Uterine influences on conceptus development in fertility-classified animals

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A major unresolved issue is how the uterus influences infertility and subfertility in cattle. Serial embryo transfer was previously used to classify heifers as high-fertile (HF), subfertile (SF), or infertile (IF). To assess pregnancy loss, two in vivo-produced embryos were transferred into HF, SF, and IF heifers on day 7, and pregnancy outcome was assessed on day 17. Pregnancy rate was substantially higher in HF (71%) and SF (90%) than IF (20%) heifers. Elongating conceptuses were about twofold longer in HF than SF heifers. Transcriptional profiling detected relatively few differences in the endometrium of nonpregnant HF, SF, and IF heifers. In contrast, there was a substantial difference in the transcriptome response of the endometrium to pregnancy between HF and SF heifers. Considerable deficiencies in pregnancy-dependent biological pathways associated with extracellular matrix structure and organization as well as cell adhesion were found in the endometrium of SF animals. Distinct gene expression differences were also observed in conceptuses from HF and SF animals, with many of the genes decreased in SF conceptuses known to be embryonic lethal in mice due to defects in embryo and/or placental development. Analyses of biological pathways, key players, and ligand–receptor interactions based on transcriptome data divulged substantial evidence for dysregulation of conceptus–endometrial interactions in SF animals. These results support the ideas that the uterus impacts conceptus survival and programs conceptus development, and ripple effects of dysregulated conceptus–endometrial interactions elicit loss of the postelongation conceptus in SF cattle during the implantation period of pregnancy.

endometrium | conceptus | fertility | gene expression | pregnancy

Infertility and subfertility are important and pervasive problems in agricultural animals and humans (1, 2). In ruminants, embryo mortality is a major factor affecting fertility and thus production and economic efficiency (3, 4). Pregnancy loss in cattle ranges from 30 to 56%, with the majority of losses occurring in the first month of pregnancy (3, 5, 6). Infertility and subfertility also impact the success of embryo transfer in cattle and humans (7, 8). In cattle, mean survival rate to calving following transfer of in vivo-derived embryos from superovulated donors ranges from 31 to 60%, and in vitro-produced embryo survival rate is lower (5, 9). Failure of the embryo to survive and establish pregnancy is due to paternal, maternal, and embryonic factors (10–12). Many of the pregnancy losses observed in natural or assisted pregnancies may be attributed to maternal factors, such as an inability of the uterus to support conceptus growth and implantation (13, 14).

After fertilization (day 0), the zona pellucida-enclosed bovine embryo enters the uterus at the morula stage by day 5 and develops into a blastocyst. The spherical blastocyst hatches from the zona pellucida on days 7 to 10 and continues to grow, changing from spherical to ovoid in shape between days 12 and 14, after which it can be termed a conceptus (embryo and associated extraembryonic membranes) (15). The conceptus undergoes elongation involving exponential growth from about 2 mm in length on day 13, 60 mm by day 16, and 20 cm or more by day 19 (16). After days 16 to 17, the time of maternal recognition of pregnancy (when the embryo signals to the mother to prevent regression of the corpus luteum), the conceptus begins the processes of implantation and placentation that involve apposition, attachment, and adhesion of the trophectoderm to the endometrial luminal epithelium and onset of trophoblast giant binucleate cell differentiation (17). Blastocyst growth into an elongated conceptus has not been achieved in vitro and requires transfer into the uterus (18), as the endometrium secretes or transports a myriad of factors critical for conceptus growth and elongation (14, 19). Dynamic changes in the endometrial transcriptome occur between days 7 and 13 that are primarily regulated by progesterone in both nonpregnant and pregnant cattle (14, 19–21). Those changes in the endometrium presumably establish a uterine environment conducive to blastocyst survival and conceptus growth into an elongated, filamentous-type conceptus and subsequently implantation and placentation. Conceptus elongation is required for production of IFN tau (IFNT) (22), which is the pregnancy recognition signal that acts on the endometrium to sustain continued production of progesterone by the ovary and regulates genes implicated in conceptus growth, implantation, and placentation (23, 24). Inadequate elongation of the conceptus presumably results in lower production of IFNT, inability to maintain the corpus luteum, and thus pregnancy loss (24–26). Although much information is known about embryo development into a blastocyst from in vitro systems (27), the essential endometrial genes and

Significance

Successful pregnancy establishment requires synchronous interactions of the conceptus with the endometrium of the uterus. This study of pregnancy outcome after assisted reproduction in fertility-classified cattle determined how the uterine environment impacts and programs conceptus survival and development. The study found that ripple effects of dysregulated conceptus–endometrial interactions elicit postelongation pregnancy loss in subfertile animals during the implantation period. This research enhances our understanding of the mechanisms that lead to pregnancy loss in both natural and assisted reproduction and has wide implications for improving pregnancy success in domestic animals and humans.

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biological pathways important for conceptus survival, growth, and implantation remain largely unknown (28, 29).

One of the major impediments to understanding maternal influences on pregnancy loss has been a lack of animals with defined high and low rates of early pregnancy loss. McMillan and Donnison (30) summarized a unique approach for experimentally identifying high and low fertility in dairy heifers based on serial transfer of in vitro-produced embryos. Their approach identified animals with high (76%) and low (11%) aggregate pregnancy rates, and a failure in the mechanism involved in conceptus elongation and thus maternal recognition of pregnancy was suggested to be the cause of early pregnancy loss in the low-fertility–classified heifers (30, 31). We recently used a similar approach to identify beef heifers with intrinsic differences in pregnancy loss (32). Serial transfer of a single in vitro-produced embryo was used to classify animals as high-fertile (HF), subfertile (SF), or infertile (IF) based on day 28 pregnancy rate. In these heifers, no difference in pregnancy rates was observed on day 14 after transfer of a single in vivo-produced embryo on day 7 postestrus (32). Thus, the observed difference in uterine competence for pregnancy in fertility-classified heifers is hypothesized to manifest during maternal recognition of pregnancy and implantation. The present study tested this hypothesis and revealed that embryo survival to day 17 is compromised in IF heifers, and that asynchronous conceptus–endometrial interactions in SF animals lead to postelongation embryonic loss by day 28 during the implantation period of pregnancy.

Results

Experiment Overview. This experiment utilized heifers that were previously fertility-classified as HF (100% pregnancy rate), SF (25 to 33% pregnancy rate), or IF (0% pregnancy rate) using serial transfer (n = 3 to 4 rounds) of a single in vitro-produced embryo (grade 1) on day 7 followed by pregnancy determination on day 28 (32). In the present study, those same fertility-classified heifers (HF, n = 21; SF, n = 14; IF, n = 6) were synchronized to estrus (day 0) and received two in vivo-produced embryos on day 7. All of the embryos were generated from superovulated donor cows using a single sire and were cryopreserved for direct thaw transfer. Each heifer received two grade 1 or 2 embryos from the same donor and developmental stages (compact morula and blastocyst). On day 17 (10 d postembryo transfer), the entire female reproductive tract was obtained, and the uterine lumen was flushed to recover the conceptus. If a conceptus was not observed in the uterine flush, the heifer was considered nonpregnant. As illustrated in Fig. 1*A*, the proportion of heifers from which one or two conceptuses were recovered was greater for HF (P = 0.02) and SF (P = 0.03) heifers compared with IF heifers but was not different (P = 0.91) between HF and SF heifers. Accordingly, pregnancy rate was higher (P < 0.05) in HF (71%) and SF (90%) than IF (20%) heifers but not different (P > 0.05) between HF and SF heifers (Fig. 1*B*). The morphology of the conceptuses recovered from HF and SF heifers was similar and ranged from tubular to elongated and filamentous (Fig. 1*C*). In contrast, a single small spherical hatched blastocyst (<0.1 cm) was

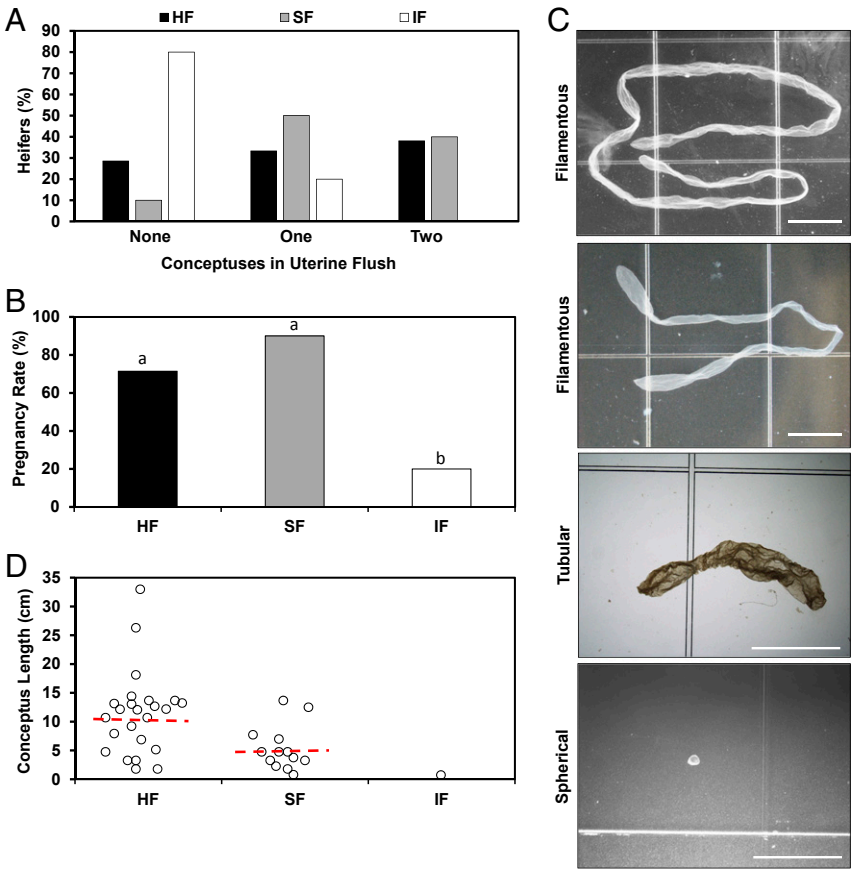

Fig. 1. Day 17 pregnancy outcome in fertility-classified heifers. HF, SF, and IF heifers were synchronized and received two in vivo-produced embryos on day 7 postestrus. The reproductive tract was acquired on day 17 (10 d postembryo transfer) and flushed to recover the conceptus(es). If present, conceptus morphology and length were determined using a stereomicroscope. (*A*) Percentage of heifers with an identifiable conceptus in the uterine flush. (*B*) Column graph showing pregnancy rate (*y* axis) of the fertility-classified heifers (*x* axis). Bars with different superscripts (a or b) indicate difference (P < 0.05) in pregnancy rate. (*C*) Representative conceptus morphology recovered from heifers. Note a single spherical embryo of less than 1.5 mm was recovered in one IF heifer. (Scale bars, 1 cm.) (*D*) Conceptus length was longer (P < 0.05) from HF compared with SF heifers. Individual conceptus length is plotted with the mean length denoted by the dashed red lines.

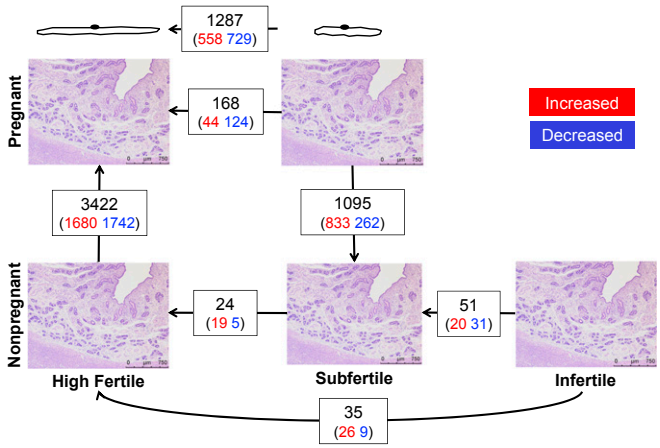

Fig. 2. Summary of endometrium and conceptus transcriptome analyses. Differentially expressed genes (FDR, $P < 0.05$) were determined by edgeR robust analysis, and the numbers of increased and decreased transcripts are indicated in red and blue, respectively, for each comparison. (Scale bars, 1 cm.)

recovered from the uterus of only one IF heifer. Overall, the conceptus was longer ($P < 0.01$) in HF (mean 10.6 cm; range 1.2 to 32.2 cm) than SF (mean 4.7 cm; range 1.5 to 13.5 cm) heifers (Fig. 1*D*).

There were no differences in circulating progesterone concentrations on day 17 between pregnant and nonpregnant heifers ($P = 0.55$) or among pregnant HF, SF, or IF heifers ($P = 0.23$) (*SI Appendix*, Fig. S1 *A* and *B*). Similarly, there was no correlation of conceptus size and circulating progesterone concentrations ($R = -0.08$; $P = 0.80$) (*SI Appendix*, Fig. S1*C*).

Dysregulation of the Endometrial Transcriptome in Response to Pregnancy. Transcriptome analysis was performed using total RNA isolated from the day 17 endometria of nonpregnant and pregnant fertility-classified heifers. Sequencing of the 25 RNA-seq (sequencing) libraries ($n = 5$ per group) generated 27.2 to 42.8 million quality reads that mapped at an ~97% rate to the *Bos taurus* reference genome (assembly UMD3.1). EdgeR robust analysis was used to identify differentially expressed genes (DEGs) [false discovery rate (FDR), $P < 0.05$] in the endometria, and a gene was defined as expressed if it presented >1 fragment per kilobase of transcript per million mapped reads (FPKM). Similar to our previous study of endometrial biopsies from these fertility-classified animals on day 14 (32), there were relatively few genes that were different (FDR, $P < 0.05$) in the endometrium of the nonpregnant HF, SF, and IF animals (Figs. 2 and 3*A* and Datasets S1–S3). Functional analysis of those DEGs did not identify any significantly enriched gene ontology (GO) terms or biological pathways. Several of the DEGs in HF and SF compared with IF endometrium encode proteins involved in immune responses or immunoglobulins (Fig. 3*A*). In SF compared with IF endometria, some of the 43 DEGs encode secreted proteins (GRP, IGFBP2, LYZ1, SERPINB7), intracellular enzymes (PLA2G2F), and transporters for water (AQP5) or amino acids (SLC2A3).

Comparison of the endometrial transcriptome of pregnant HF with pregnant SF animals detected 168 DEGs (Fig. 2 and Dataset S4). Of note, none of the DEGs were classical IFN-stimulated genes (ISGs; i.e., *IFIT1*, *IFIT2*, *ISG15*, *MX2*, *RSAD2*, *OAS2*) that are induced or considerably up-regulated by IFNT from the elongating conceptus (24) (Fig. 3*B*). The 168 DEGs were enriched for GO molecular function (extracellular matrix or ECM structural constituent), biological process (ECM organization, cell adhesion, collagen catabolic process, locomotion, cellular response to endogenous stimulus), and cellular component (ECM, extracellular space, membrane region) terms (*SI Appendix*, Table S1). Pathway analysis found enrichment in ECM organization and collagen formation. Genes increased in pregnant HF compared with SF endometrium included a water transporter (*AQP8*), lipid transporter (*FABP3*), and secreted protease (*MEP1B*). Genes decreased in HF compared with SF endometrium include cell signaling (*CAMK1G*, *IRS4*), ECM constituents, and cell-adhesion molecules (Dataset S4).

Endometrial responses to pregnancy were assessed by comparing the transcriptome of nonpregnant with pregnant animals (Figs. 2 and 3*B* and Datasets S5 and S6). This analysis found 3,422 DEGs (1,680 increased, 1,742 decreased) in HF heifers and 1,095 DEGs (833 increased, 262 decreased) in SF animals, with 848 genes commonly responsive to pregnancy in HF and SF animals (Fig. 3*B*). Thus, ~20% of total expressed genes respond to pregnancy in the endometrium of HF animals but only ~6% of genes in the endometrium of SF animals. Of note, the number of up- and down-regulated genes specific to the endometrium of HF animals was 6- and 20-fold greater, respectively, than in SF animals. The lower number of DEGs in SF animals was not due to higher levels of variation in the endometrial transcriptome, as gene expression was most variable in the endometrium of

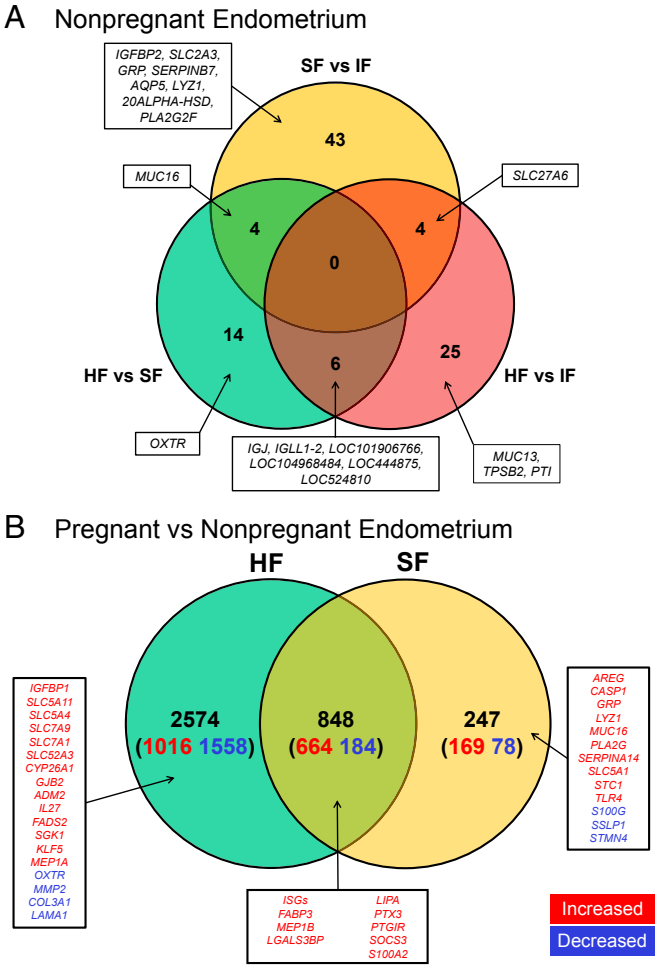

Fig. 3. Transcriptome analysis of endometrium from nonpregnant and pregnant day 17 heifers. Total RNA was extracted from five nonpregnant HF, SF, and IF heifers and five pregnant HF and SF heifers. Normalized and $\log_2$-transformed read count data and differentially expressed genes (FDR, $P < 0.05$) were determined by edgeR robust analysis. (*A*) Venn diagram showing the number of unique or common transcripts between the endometrium of nonpregnant HF, SF, and IF heifers. (*B*) Venn diagram showing the number of unique or common pregnancy-regulated transcripts in the endometrium of HF and SF heifers. For this analysis, DEGs were determined in the endometrium of nonpregnant and pregnant HF or SF heifers and used to determine the number of unique or common DEGs.

pregnant HF compared with SF animals (*SI Appendix*, Fig. S2). An impact analysis of the DEGs was performed based on pathway topology (33). This analysis found that specific signaling pathways were either negatively or positively impacted in response to pregnancy in HF and SF heifers (*SI Appendix*, Table S2). Of note, the ECM–receptor interaction pathway was negatively impacted by pregnancy only in HF animals. These findings support the idea that the endometrial response to pregnancy, namely the conceptus, is considerably altered in SF animals.

Pathways Impacted by Pregnancy. Many of the 664 genes commonly responsive to pregnancy in HF and SF animals were classical type I ISGs stimulated by IFNT (*MX2*, *ISG15*, *RSAD2*, *BST2*, *IFIT1*, *IFIT2*, *IDO1*, *ISG20*, *OAS2*, *IFIT3*, etc.) or established progesterone- and conceptus-responsive genes (e.g., *DKK1*, *FABP3*, *MEP1B*, *LGALS3BP*) (34) (Fig. 3*B* and Datasets S5 and S6). Functional analyses of those 664 up-regulated genes found enrichment for many GO terms, including molecular function (chemokine receptor binding, chemokine activity, peptidase activity), biological process (innate immune response, defense response, response to cytokine, response to type I IFN, response to virus), and cellular component (other organism, extracellular space, proteasome core complex) (*SI Appendix*, Table S3). As expected, pathways associated with the 664 commonly up-regulated genes included IFN signaling, IFNA/B signaling, cytokine signaling in the immune system, IFNG signaling, ISG15 antiviral mechanism, complement pathway, and Toll-like receptor signaling (*SI Appendix*, Tables S1 and S3), as they are linked to IFNT actions within the endometrium. Analysis of the 184 commonly down-regulated genes found they were enriched for many GO terms, including molecular function (ECM structural constituent, integrin binding, cell-adhesion binding, glycosaminoglycan binding), biological process (ECM organization, collage catabolic process, cell adhesion), and cellular component (proteinaceous ECM, ECM, collagen, basement membrane, extracellular space) (*SI Appendix*, Tables S1 and S4). Pathways associated with the 184 commonly down-regulated genes included ECM organization, collagen biosynthesis and -modifying enzymes, collagen formation, ECM–receptor interaction, ECM degradation, integrin signaling pathway, and focal adhesion (*SI Appendix*, Tables S2 and S4).

The representation of enriched GO terms and pathways was substantially different between HF and SF heifers with respect to genes responsive to pregnancy (Fig. 3*B*, *SI Appendix*, Tables S1, S2, and S5–S7, and Datasets S5 and S6). The 1,016 uniquely up-regulated genes in the endometrium of pregnant HF compared with nonpregnant HF animals were enriched for GO terms including molecular function (ubiquitin-like protein ligase binding, enzyme binding, RNA binding), biological process (carboxylic acid metabolic process, cellular catabolic process, innate immune response), and cellular component (mitochondrion, proteasome core complex) (*SI Appendix*, Tables S1 and S5). The 1,558 uniquely down-regulated genes in the endometrium of HF pregnant compared with nonpregnant HF animals were enriched for GO terms including molecular function (protein complex binding, ECM structural constituent, cell-adhesion molecule binding), biological process (ECM organization, vasculature development, biological adhesion, cell adhesion), and cellular component (proteinaceous ECM, ECM, cell junction, cell–cell junction, focal adhesion) (*SI Appendix*, Tables S1 and S6). Pathways associated with the 1,558 down-regulated genes in pregnant HF endometrium included ECM, ECM organization, collagen formation, structural components of basement membranes, focal adhesion, ECM–receptor interaction, and integrin signaling pathway (*SI Appendix*, Tables S2 and S6).

As provided in *SI Appendix*, Tables S1 and S7, GO analysis of the 169 genes uniquely up-regulated in the endometrium of SF heifers in response to pregnancy revealed enrichment in GO terms including molecular functions (amide transmembrane transporter activity, peptidase regulator activity) and cellular component (cell surface, extracellular space, plasma membrane). No biological pathways were enriched in those genes. The 78 genes uniquely down-regulated in the endometrium of SF heifers revealed no enrichment in GO terms or pathways. Collectively, these findings strongly support the idea that the endometrial response to pregnancy, the conceptus, is considerably altered in SF animals.

Alterations in the Transcriptome of HF and SF Conceptuses. Transcriptional profiling was conducted using individual conceptuses from HF (n = 17) and SF heifers (n = 10) (Fig. 2). RNA sequencing generated 36.5 to 65.5 million quality reads with an ~92% mapping rate to the *B. taurus* reference genome. EdgeR robust analysis identified 1,287 DEGs (558 increased, 729 decreased) in HF compared with SF conceptuses (*SI Appendix*, Fig. S4 and Dataset S7). To ensure that the observed differences were not related to the size of the conceptuses (35, 36), conceptuses from only HF heifers were categorized as long (range 9.8 to 32.2 cm; n = 10) or short (range 1.2 to 6.9 cm; n = 7). Subsequent edgeR robust analysis found only 18 DEGs (7 increased, 11 decreased) between the long and short conceptuses from HF heifers (Dataset S8). Thus, the differences in the transcriptome between HF and SF conceptuses are not due to their length (35, 36) but rather the fertility phenotype of the uterus.

Functional annotation of genes between HF and SF conceptuses identified many biological processes enriched in DEGs, including growth and regulation of the actin cytoskeleton in the DEGs increased in HF conceptuses (*SI Appendix*, Tables S8 and S9). GO analysis of the 558 genes increased in HF conceptuses found enrichment for molecular function (signal transducer activity, transcription factor activity, transcriptional activator activity) and biological process (embryo development, embryonic morphogenesis, animal organ morphogenesis, epithelium development, receptor signaling, tissue morphogenesis, morphogenesis of an epithelium) but no cellular component terms (*SI Appendix*, Tables S8 and S9). The 558 genes increased in HF conceptuses were enriched for pathways including integrin-mediated cell adhesion, signaling by FGFR, signaling by NGF, focal adhesion, FGF signaling, estrogen signaling, ErbB1 signaling, insulin signaling, and MAPK signaling. GO analysis of the 729 genes increased in SF conceptuses found enrichment for molecular function (molecular function regulator, receptor binding, sialyltransferase activity, heparin binding), biological process (organ development, cellular lipid metabolic process, cell adhesion, lipid metabolic process), and cellular component (extracellular space, ECM, apical part of cell, proteinaceous ECM) terms (*SI Appendix*, Tables S8 and S10). Pathways for the 729 up-regulated genes in SF conceptuses were enriched for ECM and ECM-associated proteins.

To illuminate possible mechanisms impacting the loss of conceptuses in the SF animals, the Mouse Genome Database (37) was queried to determine whether genes down-regulated in SF compared with HF conceptuses are associated with embryonic lethality. As summarized in *SI Appendix*, Table S11, many of the genes decreased in SF conceptuses have knockout database annotation terms corresponding to "embryonic lethality during organogenesis, complete penetrance," "embryonic lethality during organogenesis," "abnormal vascular development," "abnormal embryonic tissue morphology," "abnormal prenatal growth/weight/body size," "abnormal vitelline vasculature morphology," and "lethality throughout fetal growth and development, complete penetrance."

Network Analyses of DEGs in HF and SF Conceptuses. Mutual information-based network analyses were conducted by implementing a model-based cluster approach. Using the Bayesian inference criterion as model selection (38), we identified nine gene expression clusters in the conceptus transcriptome and predicted gene networks of those clusters from DEGs discovered in conceptus of HF compared with SF animals. Using the network centrality method, we predicted top key players within each cluster that may play a central role in gene expression networks in HF and SF conceptuses (*SI Appendix*, Table S12). This analysis revealed many key players, including critical enzymes (FADS1, PTGS2),

transcription factors (EFHD2, IRX4, ZBBTB7B), as well as several secreted proteins (TKDP1, TKDP4, SSLP1).

Conceptus–Endometrial Signaling. Conceptus–endometrial interactions during elongation primarily involve secreted factors from the trophectoderm and endometrial epithelium (14, 39). The GO extracellular space term (GO:0005615) was used to determine gene products secreted from the endometrium or conceptus into the uterine lumen based on DEGs in the endometrium (pregnant vs. nonpregnant) and conceptus from HF and SF animals (*SI Appendix*, Table S13). This analysis revealed substantial differences in genes encoding secreted proteins. For instance, expression of 206 genes was increased and of 296 was decreased in the endometrium by pregnancy in HF animals, but only 36 genes were increased and 5 were decreased by pregnancy in SF animals. Further, 79 genes were increased in HF conceptuses and 173 were increased in SF conceptuses.

A network of ligand receptor-mediated multicellular signaling from the FANTOM5 database of human cells (40) was used to determine ligands and receptors expressed by the endometrium and conceptus from HF and SF animals (Datasets S9 and S10) and is represented in Fig. 4. Tanglegram plots of ligand-receptor expression (*SI Appendix*, Fig. S3) found that SF heifers have relatively higher variation in branch lengths (0 through 300,000) of receptor–ligand clusters for conceptuses than those of HF animals (0 through 250,000). Thus, ligand-receptor expression in SF conceptuses is more discordant in their correspondence with endometrium ligand receptors compared with HF animals, which was measured from the distance measure (Ward's method) of gene expression variation of receptors compared with ligands between the endometrium and conceptus in SF and HF groups separately. As illustrated in Fig. 4 and *SI Appendix*, Fig. S3, some receptors are expressed with higher expression of ligands in both the conceptus and endometrium, but in the majority of cases there is a negative regulation of receptor ligands both within and between the endometrium and conceptus (*SI Appendix*, Table S14). However, there was a relative lack of receptor–ligand interactions between the conceptus and endometrium in SF compared with HF animals. Further, ligands and receptors were not as reciprocal in the SF compared with HF animals, and more ligands in the endometrium were uniquely increased or decreased by pregnancy in HF compared with SF animals. The same result was found for receptors in the endometrium. Differential expression of ligands and receptors was also observed in the conceptus from HF and SF

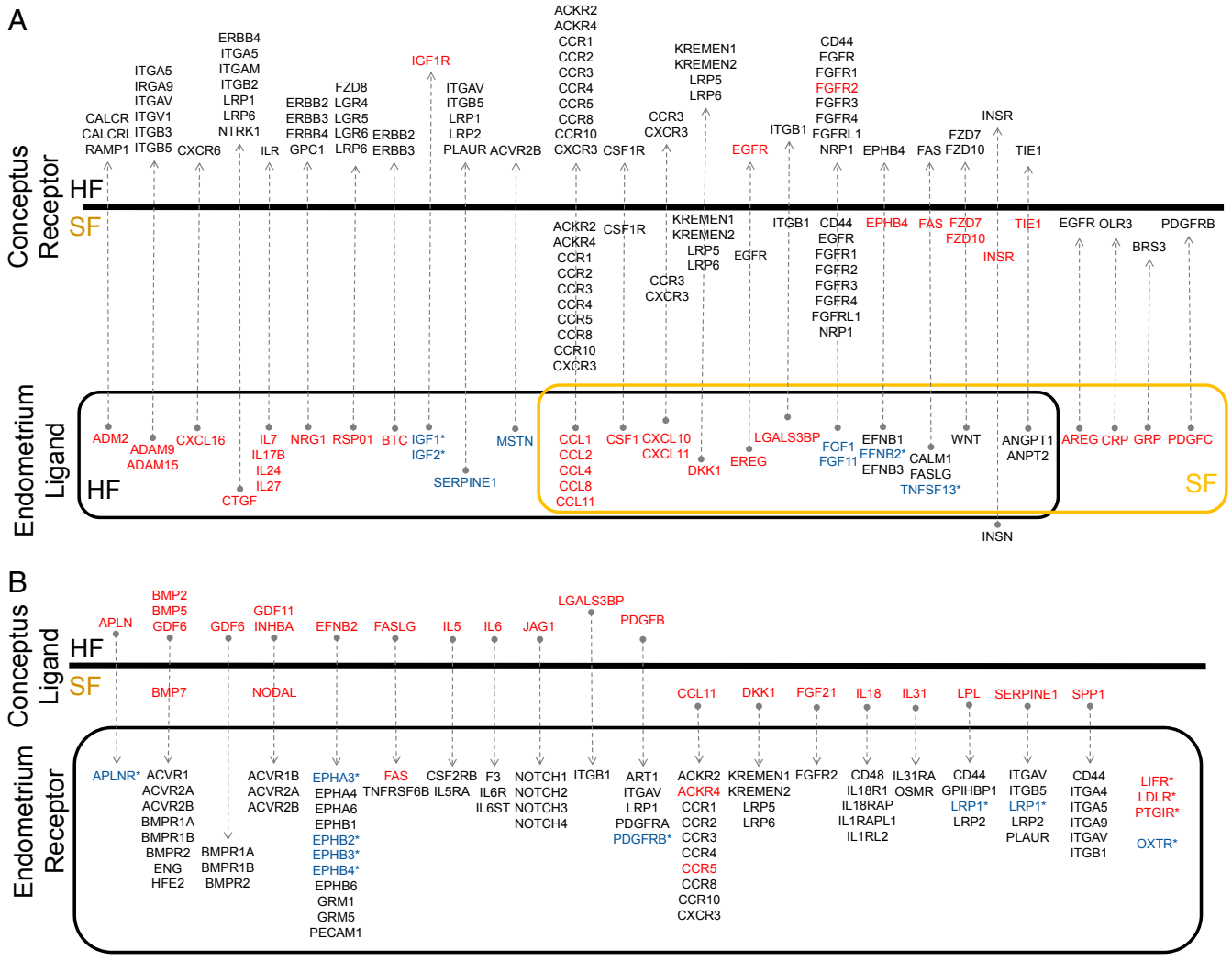

Fig. 4. Receptor–ligand interactions between the conceptus and endometrium at day 17 of pregnancy in HF and SF heifers. (*A*) Diagram of select ligands from the endometrium and receptors in the conceptus. The endometrium ligands (*Bottom*) are shown as either commonly differentially expressed in both the HF and SF endometrium (overlapping box) or exclusively to pregnant HF (black box) or SF (orange box) endometrium. (*B*) Diagram of select ligands from the conceptus and receptors in the endometrium. Genes shown in red have higher expression levels than those in black, and those in blue have lower expression than those in black.

animals. The alterations in ligands and receptors in the endometrium and conceptus would impact paracrine and autocrine signaling for conceptus growth and endometrial receptivity.

Based on protein age estimates, protein ligands are generally evolutionarily younger than the cognate receptors (40). The ages of proteins of *B. taurus* were assessed from ProteinHistorian analysis (41) that predicts age from evolutionary conservation of proteins. Next, a contingency test of up-regulated versus down-regulated genes in day 17 HF and SF endometria was conducted by differentiating them based on the "age" of the encoded protein (*SI Appendix*, Fig. S5). This analysis revealed that the transcriptome response of HF and SF pregnant heifer endometrium is associated with protein age. In HF pregnant heifers, the endometrium transcriptome did not display age differences in encoded proteins, but age of proteins was different (Pearson chi square 3.37, $P = 0.04$) in SF animals. The genes up-regulated in the SF endometrium are biased ($P = 0.0064$) toward younger age. Collectively, these analyses of ligands and receptors further support the idea that the endometrium and conceptus are asynchronous in SF animals.

Discussion

Our previous study of the fertility-classified heifers used here found no difference in pregnancy rate or conceptus development on day 14 (7 d postembryo transfer) (32). It has long been postulated that pregnancy loss in heifers and nonlactating cows was primarily due to defects in conceptus survival and elongation between conception or embryo transfer (day 7) and day 16 (5, 42). In the present study, conceptus survival was substantially compromised in IF heifers on day 17 (10 d postembryo transfer) but not in SF compared with HF animals. Given that SF animals have substantially increased embryo mortality by day 28, these findings suggest that embryonic loss in heifers after embryo transfer occurs primarily between days 17 and 28 during the implantation period of pregnancy. True infertility occurs in only 5% or so of heifers, and subfertility is more pervasive and costly in beef and dairy animals (43–45). Although pregnancy rates and embryo survival were not different on day 17 in SF and HF animals receiving two embryos on day 7, the conceptus was twofold longer in HF than SF animals on day 17 in the present study. The variability in recovered conceptus size and morphology was striking (Fig. 1), but is consistent with many other studies of beef and dairy heifers and cows (5, 16, 35, 36, 46, 47). In the present study, embryonic loss in the SF heifers had to occur between days 17 and 28, which encompasses the implantation period of pregnancy (48–50). Recent data from dairy heifers and cows as well as somatic cell nuclear transfer pregnancies support the hypothesis that embryonic loss is most prevalent during this period (3, 51–53). However, little is known of the biological pathways and gene networks regulating implantation and placentation in cattle (49, 52, 54). Progesterone action via the endometrium is critical for conceptus development and elongation (14, 24), and increased postovulatory progesterone concentrations are associated with increased conceptus growth on days 13 and 14 (55, 56). In agreement with our recent findings (32, 57), differences in levels of circulating progesterone concentrations were not associated with pregnancy outcomes. This reinforces our hypothesis that mechanisms other than circulating progesterone concentrations are associated with pregnancy loss in the fertility-classified SF and IF heifers.

A prevailing theory is that smaller conceptuses producing less IFNT are inferior in their ability to establish pregnancy compared with longer conceptuses that produce more IFNT (58). This theory has been difficult to test, given the difficulties in retrieving elongating conceptuses and transferring them into a recipient uterus without damage (16). Despite differences in the size of HF and SF conceptuses, the response of the endometrium to the conceptus was not different with respect to induction of classical ISGs by IFNT, such as *ISG15*, *MX2*, and so forth, which is not unexpected given that very little IFNT is needed to maximally stimulate expression of ISGs in cells and tissues (59). In fact, the absolute amount of IFNT that must be produced by the conceptus to signal pregnancy recognition has never been established (34). As the pregnancy recognition signal, IFNT acts in a paracrine manner on the endometrial luminal epithelium to regulate expression of the *oxytocin receptor* (*OXTR*) gene, as OXTRs are key for endometrial generation of luteolytic pulses of prostaglandin F2 alpha (34, 60). In cattle, *OXTR* expression increases in the endometrial luminal epithelium after day 14 in nonpregnant but not pregnant cattle (61). In the present study, endometrial *OXTR* mRNA levels were not different among nonpregnant HF, SF, and IF heifers and, as expected, were lower in the endometrium of pregnant (mean 2.5 FPKM) compared with nonpregnant (mean 19.5 FPKM) HF heifers. In SF heifers, *OXTR* mRNA levels were not different in the endometrium of pregnant (mean 3.8 FPKM) and nonpregnant (mean 2.1 FPKM) animals. Although *OXTR* expression was clearly different in SF heifers on day 17 postestrus, no consistent difference in estrous cycle length was observed in our previous study of these SF and IF heifers (32).

Transcriptional profiling of the endometrium was conducted to begin understanding the biology of pregnancy loss in IF and SF animals. Minimal differences were observed in the nonpregnant endometrial transcriptome among IF, SF, and HF heifers. Similarly, minimal gene expression differences were found in endometrial biopsies from these same fertility-classified heifers on day 14 (7 d postembryo transfer) (32). Few differences in the endometrium were also detected in endometria from fertility-classified heifers on day 13 postestrus (57) or day 14 postestrus (62) and also endometrial biopsies from lactating dairy cows on day 13 postestrus (63). The lack of conserved differences among these studies could be attributed to a myriad of factors, including how fertility was classified, breed effects, and endometrial sampling technique. Nonetheless, RNA-seq analysis detects some genes increased in HF or SF compared with IF endometrium that encode factors involved in reproductive tract defense against pathogens and immune system modulation (*CTLA4*, *IFI47*, *IGJ*, *IGM*, *MUC13*). In other mammals, the embryo induces expression of molecules in the endometrium that function to suppress the immune response and/or promote tolerance to the embryo (52); however, relatively little is known about the immunology of pregnancy in cattle (64). In summary, analyses of the nonpregnant endometrium transcriptome did not provide significant insights into the biological pathways underlying embryo mortality in IF and SF animals. Similar to studies of humans with fertility problems (65), the gene expression signature in nonpregnant endometrium may not be useful to predict fertility phenotype and pregnancy outcome. Further studies on the genome (57) or uterine secrotome of IF animals may provide insight into the failure of conceptus survival and growth in those animals (66, 67).

Of the 44 genes decreased in pregnant SF compared with HF heifers, several of them encode secreted factors (FABP3, MEP1B) and transporters (SLCO4C1, SLC7A1) implicated in conceptus implantation in ruminants. MEP1B is a zinc metalloendopeptidase that is expressed by and secreted from epithelial cells. In the bovine uterus, it is induced by progesterone in the endometrial glands and implicated in elongation of the conceptus (55). FABP3 is involved in the uptake, intracellular metabolism, and transport of long-chain fatty acids and up-regulated by progesterone in the endometrial luminal epithelium between days 13 and 16 in cattle (21). Long-chain fatty acids are important for cell growth and production of eicosanoids, which are important for conceptus elongation in ruminants (68, 69). SLCO4C1 is an organic ion transporter that is involved in the membrane transport of eicosanoids. Analysis of the conceptus transcriptome data identified PTGS2 as a key player in the present study, and PTGS2 is critical for conceptus elongation and pregnancy establishment in sheep (70) and a predictor of embryo viability and pregnancy success in cattle (71). SLC7A1 is an arginine transporter up-regulated in the luminal epithelium of the ovine uterus during early pregnancy, and arginine stimulates trophectoderm cell proliferation, migration, and IFNT production

in vitro (72). Functional analysis of the 44 genes found enrichment for biological processes involved in the inflammatory response (MEP1B, S100A12, C2, IL17RC, CFI, IDO1, NUPR1, TFRC). Controlled inflammation is important for the establishment of pregnancy in other mammals, although the immunological underpinnings of pregnancy are not well-understood in ruminants (64, 73).

Transcriptome analyses uncovered the remarkable finding that the endometrial response to the conceptus was extensively altered in SF animals with an approximate threefold disparity in DEGs. Comparison of the nonpregnant and pregnant endometrium revealed 2,574 unique DEGs in HF animals and 247 unique DEGs in SF animals. Further, 848 DEGs commonly responded to pregnancy, including classical ISGs up-regulated by IFNT (CLEC4F, MX2, ISG15, RSAD2, IFIT1, OAS2, etc.) as well as a number of known progesterone-induced and conceptus-stimulated genes (FABP3, MEP1B, LGALS3BP, SOCS3, S100A2). Thus, the endometrium of SF animals appears to respond appropriately to progesterone and IFNT, which are the major established regulators of endometrial gene expression and function to date in ruminants (24, 34). Comparisons of the endometrium from pregnant HF and pregnant SF heifers as well as the differential response of the endometrium of HF and SF heifers to pregnancy revealed substantial alterations in genes regulating ECM structure and organization as well as cell adhesion, cell-matrix adhesion, and cell movement. Collectively, the results support the idea that ECM remodeling is a normal response of the endometrium to the elongating and implanting conceptus, but this remodeling is substantially diminished in SF animals. Indeed, genes enriched in GO terms for cell adhesion, ECM, basement membrane, and vasculature development were down-regulated in pregnant compared with cyclic cows (74, 75). Differences in ECM remodeling were found in comparisons of the transcriptomes of chorionic, endometrial, or placental tissues from natural compared with embryo transfer or somatic cell nuclear transfer-derived pregnancies that are predominantly lost during implantation and placentation during the first few months (52). Implantation and placentation involve remodeling of the endometrial and chorionic ECM (49, 76, 77). Endometrial and fetal tissues undergo major tissue remodeling during this period to aid the proliferation, differentiation, and migration of binucleate cells, which are regulated by locally produced matrix metalloproteinases and tissue inhibitor of matrix metalloproteinase 1 (78–81). The organization of the ECM is a complex process that involves interactions between the ECM components and extracellular proteoglycans that undoubtedly impact growth and development of the placentomes. The mechanisms of pregnancy loss in SF heifers appear to be associated with the process of ECM remodeling and impaired conceptus–endometrial interactions. ECM and adhesion proteins increased in pregnant SF endometrium are normally decreased in HF endometrium by pregnancy. Trophectoderm attachment and adhesion to the endometrial luminal epithelium is a fundamental event in implantation and pregnancy establishment in mammals (82) and thus likely disrupted in SF animals. Excessive ECM in SF animals could possibly inhibit the embryonic adhesion to endometrial tissue (83).

Many of the 729 genes down-regulated in SF conceptuses are enriched for GO biological processes that include embryo development, embryo morphogenesis, animal organ morphogenesis, epithelium development, and morphogenesis of an epithelium, among others, and are associated with embryonic lethality or abnormal implantation in mice. Several genes down-regulated in the SF conceptus (GCM1, BMP2, FGFR2) are postimplantation embryonic lethal in null mice due to defects in placental development (*SI Appendix*, Table S11). Of note, GCM1 is a critical transcription factor that regulates placental development and trophoblast differentiation in mice and humans (84). Further, genes down-regulated in the SF conceptuses were enriched for GO molecular functions involving transcription factor activity and transcriptional activator activity; however, key transcription factors regulating conceptus elongation and placental development are not known in cattle (49). A number of biological processes were also down-regulated in the SF conceptus, including integrin-mediated cell adhesion, focal adhesion, FGF and FGF signaling, and MAPK signaling, which are all important pathways for conceptus growth and implantation (85). Alterations in WNTs and DKK1, a WNT inhibitor, were observed in the SF conceptus, and canonical and noncanonical WNT signaling pathways are conserved regulators of conceptus–endometrial interactions in mammals and implicated in conceptus elongation and implantation in sheep and cattle (86, 87). FGFR2 was decreased in the SF conceptus, and FGF2 from the endometrium activates FGFR2 in the elongating bovine conceptus and increases trophectoderm proliferation and IFNT production (88). Factors involved in cell migration and elongation (GJA5, TSPAN1, GJB5, ITGB2, PECAM1) were also decreased in the SF conceptus. Thus, the developmental program of conceptus growth and differentiation is likely compromised in SF animals and leads to postelongation embryonic mortality due to defects in extraembryonic tissues (placenta, allantois) and/or the embryo itself.

In addition, 558 genes up-regulated in SF conceptuses are enriched for GO molecular function (molecular function regulator, receptor binding, heparin binding) and cellular component (extracellular space, extracellular matrix). Altered secretion of proteins that are ligands for receptors expressed on the conceptus and/or endometrium could lead to defects in conceptus development and implantation (52). This supposition is supported by in silico determination of secreted factors and ligands and their differences in the endometrium and conceptus of pregnant HF and SF animals. Indeed, the endometrium is an early biosensor, and distinct endometrial responses are elicited by embryos produced by artificial means (52, 89, 90). Results of the present study suggest that other factors besides IFNT are important mediators of conceptus–endometrial interactions during early pregnancy. Bioinformatics analysis identified several secreted proteins (TKDP1, TKDP4, SSLP1) as key players in conceptus gene expression networks. The trophoblast Kunitz domain proteins (TKDPs) are a multigene family that are predominantly expressed in the trophoblast cells of the bovine placenta during early pregnancy (91). Other potentially important signaling molecules from the conceptus include BMP2, BMP5, GDF6, and GDF11, which activate signaling pathways important for endometrial function and implantation in other mammals (92, 93).

Collectively, the transcriptional profiling results strongly support the idea that conceptus–endometrial interactions are dysregulated in SF animals and underlie the observed pregnancy loss by day 28. This hypothesis is strongly supported by studies in cattle, mice, and humans finding that dysregulated interactions between the conceptus and uterus have adverse ripple effects resulting in pregnancy loss, miscarriage, or preeclampsia (94, 95). Implantation requires carefully orchestrated interactions between the receptive endometrium and elongating conceptus, and studies of somatic cell nuclear transfer-derived pregnancies in cattle clearly support the idea that altered conceptus–endometrial interactions are a cause of postelongation pregnancy loss (52, 90, 96). Given that only minimal differences were observed in the conceptus transcriptome when short and long HF conceptuses were compared, it is likely that the differences in the transcriptome of HF and SF conceptuses arise initially from influences of the endometrium.

Pregnancy loss during the first trimester of gestation of pregnancies established by embryo transfer has been hypothesized to involve failures or delays in conceptus elongation and/or embryonic development resulting in loss of pregnancy by day 30 (3). The present studies were conducted with beef heifers that were fertility-classified by embryo transfer and whose pregnancy rates were predicated on innate uterine competence to support pregnancy establishment. The defects in conceptus survival and elongation observed on day 17 in the IF heifers support the idea that an incompetent uterus is present in 5% or so of cattle, but

did not illuminate why the IF uterus fails to support pregnancy. Subfertility is a more prevalent issue in cattle, and studies here found that conceptus survival was not compromised in SF heifers, indicating that pregnancy loss occurs between days 17 and 28 in SF animals during the implantation period. Findings from the transcriptome analyses clearly support the idea that conceptus–endometrial interactions are dysregulated in the SF animals. Thus, our studies strongly support the adverse ripple effect hypothesis that aberrant communication between the endometrium and conceptus disrupts normal implantation and placentation processes, leading to pregnancy loss and later pregnancy complications (94). Based on the experimental approach of fertility classification here, we propose that the alterations in the SF conceptus transcriptome result from inappropriate responsiveness of the endometrium to the conceptus in SF animals. The alterations in endometrial and conceptus gene expression are likely caused, in part, by alterations in DNA methylation (97–101). Subsequent pregnancy loss occurs due to the ripple effects of dysregulated conceptus–endometrial interactions due to insufficient (*i*) conceptus attachment and adhesion to the endometrium; (*ii*) placental development as a consequence of inadequate endometrial remodeling or defects in allantois growth and development; and/or (*iii*) embryogenesis. In summary, the studies here provide an important foundation to understand implantation and early placentation-phase pregnancy loss and develop genetic and physiological approaches to improve the outcome of natural and assisted pregnancies.

Materials and Methods

Animals. All animal procedures were conducted in accordance with the Guide for the Care and Use of Agriculture Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committees of the USDA-ARS Fort Keogh Livestock and Range Research Laboratory and the University of Missouri. In our recent work (32), 269 beef heifers were classified based on fertility using serial embryo transfer to select animals with intrinsic differences in pregnancy loss. In each of the four rounds, a single in vitro-produced high-quality embryo was transferred into heifers on day 7 postestrus and pregnancy was determined on days 28 and 42 by ultrasound and then terminated. Heifers were classified based on pregnancy success as HF (100%), SF (25 to 33%), or IF (0%). In the present experiment, 41 heifers that had been previously (32) classified for fertility (HF, $n = 21$; SF, $n = 14$; IF, $n = 6$) were used.

Embryos. In vivo-produced embryos were generated at the USDA-ARS Fort Keogh Livestock and Range Research Station using seven Angus donor cows using previously described methods (32) and a total amount of FSH equivalent to 320 mg NIH-FSH-P1 (Folltropin-V; Vetoquinol). Donors cows were inseminated 12 and 24 h after onset of estrus (day 0 and 0.5) with semen from a single high-fertile Holstein sire, and were flushed on day 7 after breeding for embryo recovery. Recovered embryos were classified by stage of development and graded based on morphological appearance (102). Two quality grade 1 or 2 embryos (a compact morula and a blastocyst) were loaded into a 0.25-cc polyvinyl straw containing ViGro Freeze Plus medium (Bioniche Animal Health) and frozen in a programmable embryo freezer using standard techniques for direct transfer (103).

Embryo Transfer and Sample Collection. Estrous cycles of fertility-classified heifers were synchronized with an injection of PGF2α followed in 3 d by an injection of GnRH concurrent with the insertion of a controlled intravaginal drug releasing (CIDR) device. Six days later, CIDRs were removed and an injection of PGF2α was administered. Concomitant with CIDR removal, estrus detection patches (Estrotect; Rockway) were applied on the tail head of each heifer to aid in visual detection of estrus. Heifers were observed for signs of estrus three times a day, beginning at 24 h after CIDR removal. On day 7 postestrus, each heifer received two in vivo-produced embryos placed in the uterine horn ipsilateral to the ovary containing a corpus luteum using standard nonsurgical techniques by a single technician. Ultrasonography was used to identify the side and presence of the corpus luteum before embryo transfer. Only heifers that exhibited estrus signs after CIDR removal and PGF2α injection and that had a corpus luteum on day 7 postestrus received embryos. For collection of nonpregnant animals, heifers were synchronized to estrus (day 0) but did not receive embryos on day 7.

The female reproductive tract was recovered on day 17 postestrus, and their uteri were flushed with 20 mL of filtered sterile PBS (pH 7.2). If present, the state of conceptus development was assessed using a Nikon SMZ1000 stereomicroscope (Nikon Instruments) fitted with a Nikon DS-Fi1 digital camera. Conceptus size was determined using a ruler. The volume of the uterine flush was measured, and the flush was clarified by centrifugation (3,000 × *g* at 4 °C for 15 min). The supernatant was carefully removed with a pipette, mixed, aliquoted, and stored at −80 °C until analyzed. The endometrium was physically dissected from the remainder uterine horn using curved scissors. Endometrial samples as well as conceptuses were frozen in liquid nitrogen and stored at −80 °C for subsequent RNA extraction.

Circulating Progesterone Concentrations. For determination of circulating progesterone concentrations on the day of slaughter (day 17 postestrus), blood samples were collected from the median coccygeal vein or artery into evacuated tubes containing K3 EDTA (Becton Dickinson Vacutainer Systems). Blood tubes were then centrifuged at 1,200 × *g* for 20 min at 4 °C, and plasma was collected and stored at −20 °C until an RIA was performed. Plasma concentrations of progesterone were determined in duplicate 100-μL aliquots of sample using manufacturer (MP Biomedicals) reagents and recommendations for the liquid–liquid phase double-antibody precipitation assay (07-170105; MP Biomedicals). Intraassay coefficient of variation was 2%.

Statistical Analysis. Statistical analyses were conducted using SAS (SAS Institute). Statistical significance was defined as $P \leq 0.05$. Binomial data representing whether or not a conceptus was recovered on the day 17 uterine flush (yes, at least one conceptus was recovered; no, no conceptus was recovered) were analyzed by logistic regression with Firth's bias correction using the LOGISTIC procedure. The proportion of heifers that had at least one conceptus recovered on the day 17 flush within each fertility classification was determined using the FREQ procedure. The effect of fertility classification on the number of conceptuses recovered was analyzed by logistic regression using the GLIMMIX procedure with a multinomial distribution. Posttest comparisons were conducted using the contrast statement. The effect of fertility classification on conceptus length was estimated in a Poisson regression repeated measurements model using the GENMOD procedure to account for the effect of more than one conceptus being recovered per heifer. The means of conceptus length were estimated using the MEANS procedure. Continuous variables were assessed for normality using the UNIVARIATE procedure. The effect of pregnancy status (pregnant vs. nonpregnant) and fertility classification on plasma progesterone concentrations was determined by ANOVA using the GLM procedure. Posttest comparisons were conducted using the LSMEANS statement with the Fisher's protected LSD option. Only heifers with only one conceptus recovered were used in the analysis to investigate the correlation of plasma progesterone concentration and conceptus size. Pearson's correlations were determined using the CORR procedure.

RNA Sequencing. Total RNA from day 17 endometrium samples of 25 heifers, HF nonpregnant (n = 5), HF pregnant ($n = 5$), SF nonpregnant (n = 5), SF pregnant ($n = 5$), and IF nonpregnant ($n = 5$), was extracted using Isol-RNA Lysis Reagent (5 Prime). Briefly, frozen endometrium samples were disrupted and homogenized in Isol-RNA Lysis Reagent with the use of a homogenizer (VDI 25; VWR International), and total RNA was purified following the manufacturer's instructions. Total RNA from 27 conceptuses (HF, $n = 17$; SF, $n = 10$) was extracted using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen).

To eliminate DNA contamination, total RNA from endometrium and conceptuses was treated with DNase I during RNA purification using the RNase-Free DNase Set (Qiagen). RNA concentration was determined by quantitative high-sensitivity RNA analysis on the Fragment Analyzer instrument (DNF-472; Advanced Analytical Technologies). RNA library preparation and sequencing were conducted by the University of Missouri DNA Core Facility. Libraries were constructed following the manufacturer's protocol with reagents supplied in Illumina's TruSeq Stranded mRNA Sample Prep Kit. Briefly, the polyadenylated mRNA was purified from total RNA and fragmented. Double-stranded cDNA was generated from the fragmented RNA, and the index-containing adapters were ligated. The final construct of each purified library was evaluated using the Fragment Analyzer instrument, quantified with the Qubit fluorimeter using the Quant-iT HS dsDNA Reagent Kit (Invitrogen), and diluted according to Illumina's standard sequencing protocol for sequencing on an Illumina NextSeq 500 sequencer. All of the raw data and processed data from this study have been submitted to the Gene Expression Omnibus for public access (accession no. GSE107891).

Alignment of Sequences and Analysis of Differential Gene Expression. The raw sequences (fastq) were subjected to adapter removal and quality trimming using fqtrim (https://ccb.jhu.edu/software/fqtrim/). The quality reads were then mapped to the bovine reference genome UMD3.1 using Hisat2 mapper (https://ccb.jhu.edu/software/hisat2/), which is a fast and sensitive alignment program of next-generation sequencing data (104). To produce a global gene annotation file for comprehensive quantification of gene expressions across the samples, the sorted binary alignment maps of sequence reads from each sample were assembled to generate transcriptomes that were then merged, along with the gene annotations from the reference genome assembly. The transcript assembly step was accomplished using the software StringTie (https://ccb.jhu.edu/software/stringtie/), which is considered a highly efficient assembler of RNA-seq mapping data (105). The merged global transcriptome was then used in StringTie to quantify the transcript abundance of each sample. Differential expression analysis between sample groups was performed by robustly fitting the expression data to a weighted generalized linear model using edgeR robust (106).

GO, Pathway, and Network Analysis. Additional analyses determined whether DEGs were significantly enriched in specific pathways and expression networks. First, a model-based cluster analysis of differential gene expressions was performed using the Bayesian information criterion to predict whether DEGs are expressed in clusters and hence may establish expression networks. After predicting clusters, network analysis was performed based on mutual information of expression changes of genes using the R package minet (107). Key genes within each network were predicted from centrality scores of DEGs within the networks using approaches of "key player analysis," a method used in analyzing social networks (107). Functional prediction of genes corresponding to those expression modules was assessed by mapping the genes to Kyoto Encyclopedia of Genes and Genomes pathways and annotating gene ontology using DAVID (https://david.ncifcrf.gov). Significant impacts of DEGs on signaling pathways were determined using a pathway perturbation algorithm called "SPIA" (33). This topology-based pathway analysis was conducted in R using the ToPASeq package (108). Functional annotation analysis was also conducted for DEGs using the ToppGene Suite for gene list enrichment analysis (109).

Ligand-Receptor Analysis. Determination of ligands and their receptors was conducted using curated ligand–receptor pairs in the FANTOM5 database for human protein-coding genes (40). Tanglegram plots of ligand-receptor expression in the endometrium and conceptus were generated by dendextend (110) based on hierarchical clustering of ligand and corresponding receptor expression levels separately for the endometrium and conceptus. The cluster branch and nodes were color-coded, and black lines were used to show the correspondence between the same pair of ligand and receptor in the endometrium and conceptus. The hierarchical clustering was performed based on distance measured by Ward's method from expression data.

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