Endometrial gene expression in high- and low-fertility heifers in the late luteal phase of the estrous cycle and a comparison with midluteal gene expression

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Endometrial gene expression in high- and low-fertility heifers in the late luteal phase of the estrous cycle and a comparison with midluteal gene expression. Physiol Genomics 48: 306–319, 2016. First published February 5, 2016; doi:10.1152/physiolgenomics.00042.2015.—Embryonic mortality is a major constraint to improving reproductive efficiency and profitability in livestock enterprises. We previously reported differential expression of genes with identified roles in cellular growth and proliferation, lipid metabolism, endometrial remodeling, inflammation, angiogenesis, and metabolic exchange in endometrial tissue on day 7 of the estrous cycle (D7), between heifers ranked as either high (HF) or low (LF) for fertility. The aim of the current study was to further elucidate the underlying molecular mechanisms contributing to early embryo loss by examining differential endometrial gene expression in HF or LF heifers at a later stage of the estrous cycle; day 14 (D14). A second objective was to compare these expression profiles with those from midluteal HF and LF endometrium. Using the same animal model as employed in the previous study, we slaughtered HF and LF animals on D14, harvested endometrial tissue, and carried out global gene expression analysis using the Affymetrix Bovine GeneChip. Ingenuity Pathway Analysis revealed enrichment for a host of biological pathways including lipid metabolism, molecular transport, immune response, cell morphology and development, and cell growth and proliferation. Important DEG included ALB, BMPR2, CCL28, COL4A3/4, FADS1, ITGA6, LDLR, PLCB3, PPARG, PTGS2, and SLC27A4. Furthermore, DEG expressed on both D7 and D14 included: PCCB, SLC25A24, DAP, and COL4A4. This study highlights some of the pathways and mechanisms underpinning late luteal bovine endometrial physiology and endometrial-related conception rate variance.

IN HEIFERS AND MODERATE YIELDING dairy cows, it is estimated that early embryo mortality is ~40%, with annual trends across the globe indicating further deterioration (75, 93). The peri-implantation embryogenesis (embryo development prior to implantation) period is extended in cattle compared with other mammalian species. During this stage, the healthy embryo undergoes rapid mitotic division, otherwise known as cleavage, advancing through three critical stages of embryonic development (compaction, blastocyst formation, and elongation) and is critically reliant on a variety of extrinsic factors including its extracellular uterine environment. Not surprisingly, the vulnerable in vivo embryo is subject to exterior inadequacies, and, as a result, much loss is reported in the first 2 wk after insemination (26).

Data from our group have confirmed evidence for repeatability of conception rate, reporting a repeatability estimate of 0.18 (27). In addition, it has been suggested that uterine factors contribute to these differences (69). However, studies have revealed uterine endometrial thickness or morphology has no predictive value for conception in assisted reproductive technologies (111). It is thus likely that discreet changes in the endometrial molecular signature are potentially influencing such variation.

Temporal changes in endometrial gene expression profiles have been demonstrated during various stages of the estrous cycle (54, 71). Furthermore, modifications in endometrial transcriptional signatures occur due to dietary supplementation (107), variation in progesterone levels (35, 36, 68), infection, and pregnancy (33, 106). Moreover, the effect of fertility on bovine endometrial gene expression on D7 of pregnancy has been described (8). It is thus clear that the endometrium is acutely susceptible to both local and systemic changes, likely to affect embryo growth and survival. These studies provide information on the genes, pathways, and networks regulating the provision of an optimum uterine environment and contributing to retarded or irregular embryo growth. However, it is important to examine changes inherent to subfertility excluding those produced by the presence of an embryo.

Recent data from our group revealed altered endometrial gene expression for components of various biochemical pathways in high- (F) and low-fertility (LF) heifers (54). In addition, Salilew-Wondim et al. (85) described changes in endometrial molecular signatures between receptive and non-receptive heifers on day 7 (D7) and day 14 (D14) of the estrous cycle. Furthermore, Minten et al. (71) described possible innate genetic variation between fertile and subfertile/infertile heifers with altered endometrial expression signatures and moderate genetic associations. The challenge remains to elucidate the causes of subfertility. Given the greatest increment of embryo loss occurs by day 14–16 (26), the primary objective of this study was to identify the potential molecular mechanisms contributing to embryo loss during this period by examining endometrial gene expression in HF and LF heifers, using the fertility model as described by Killeen et al. (54), on D14 of the estrous cycle. In addition, genes and pathways differentially enriched between HF and LF endometrium during mid-
late luteal phases of the estrous cycle (D7 and D14) were examined to identify any spatiotemporal biochemical pathways surrounding this period of exponential embryo loss.

**MATERIALS AND METHODS**

All experimental procedures involving heifers were licensed by the Department of Health and Children, Ireland (licence number B100/846). Protocols were in accordance with the Cruelty to Animals Act (Ireland 1876, as amended by European Communities regulations 2002 and 2005) and the European Community Directive 86/609/EC and were sanctioned by the Institutional Animal Research Ethics Committee.

**Animal model.** The animal model used was that described by Killeen et al. (54), except endometrial samples for this study were instead harvested on D14. In brief, estrous cycles of reproductively normal nulliparous crossbred beef heifers (Bos taurus n = 120) were synchronized using two intramuscular administrations of 500 μg of the prostaglandin F2α, analog (PG), cloprostenol (Estrumate; Schering-Plough, Shire Park, Welwyn Garden City, Hertfordshire, UK). Animals were visually observed for signs of estrous activity, and those observed to be in standing estrous were inseminated at 6–18 h after onset of heat. At point of insemination heifers weighed 410 ± 45 kg and had a body condition score of 4.1 ± 0.2.

Pregnancy was diagnosed 28 days after insemination using the criteria set out by Kastelic et al. (53). Following diagnosis, all pregnant heifers received PG on day 28 to induce embryo loss. Six weeks after induced embryo loss all heifers were subjected to estrous reprogramming by a two-injection PG regimen and inseminated, and pregnancy was scanned as described by Killeen et al. (54).

For the purpose of establishing an accurate HF/LF model, this schedule was followed for a further two occasions. Thus, following four inseminations, animals that established a pregnancy on all four occasions were categorized as high fertility and designated “HF” heifers, while those achieving pregnancy on only one occasion were categorized as low fertility and designated “LF” heifers. After the fourth insemination and subsequent pregnancy diagnosis, pregnant heifers were returned to estrus (estrus = day 0). Approximately 3 mo later, estrous cycles of animals were synchronized in preparation for endometrial harvesting on D14 (Fig. 1).

**Tissue sampling.** Animals from HF (n = 8) and LF groups (n = 8) were slaughtered on D14 in a licensed abattoir (KEPAK, Athleague, Co. Roscommon, Ireland). Following slaughter, the reproductive tract and ovaries were checked for gross abnormalities but none were recorded. Uterine tissues were opened longitudinally along the mesenteric border. Miduterine intercaruncular endometrial cross-sections ~4 cm², and weighing 2.5 g, were harvested from the animals by peeling from the underlying uterine myometrium from the middle third of the uterine horn ipsilateral to the corpus luteum (CL) within 20 min of slaughter. Samples were washed in sterile PBS and stored in RNA later at 4°C for 24 h before being transferred for long-term storage at −20°C. All surgical instruments used for tissue collection were sterilized and treated with RNA Zap (Ambion, Applera Ireland, Dublin, Ireland). In addition, on all day of slaughter CL diameter for each heifer was determined by vernier calipers.

**Blood sampling.** Heifers were blood sampled via jugular venipuncture for subsequent measurement of progesterone at 0900 and 2100 commencing 24 h after PG for a cycle length. All blood samples were collected into 10 ml ethylenediamine tetraacetic acid heparinized vacutainers (Becton Dickson Vacutainer Systems, Plymouth, UK). Samples were held in iced water until centrifuged at 1,500 g at 4°C for 15 min after which plasma was extracted and stored in sterile 7 ml vials at −20°C until assayed.

**Progesterone assays.** Progesterone profiles for each of the eight heifers within HF and LF groups were established. Concentration of progesterone was measured in plasma as the mean of the two samples taken on each cycle day by the Coat-a-Count assay procedure (Coat-a-Count Diagnostic Products, Los Angeles, CA) with each sample tested in duplicate. The interassay and intra-assay coefficients of variation for low, medium, and high control samples were 17.4 and 4.43%, 5.56 and 28.4%, and 4.16 and 4.94% with mean concentrations of 0.24, 2.54, and 7.21 ng/ml, respectively. The minimum detectable limit for this assay was 0.06 ng/ml.

**RNA extraction and quality analysis.** Total RNA was prepared from 100–200 mg of fragmented frozen endometrial tissue using the TRIzol reagent (Sigma-Aldrich Ireland, Dublin, Ireland). Tissue samples were homogenized in 3 ml of TRIzol reagent and chloroform and subsequently precipitated using isopropanol (Sigma-Aldrich Ireland). RNA samples were stored at −80°C. Samples of RNA, 20 μg, were
purified and treated for contaminating genomic DNA using RNasea
clean-up kits in accordance with manufacturer’s guidelines supplied
(QIAGEN, Crawley, West Sussex, UK). This protocol included an
on-column DNase treatment step. RNA quality and quantity were
assessed by automated capillary gel electrophoresis on a Bioanalyzer
2100 with RNA 6000 Nano Lab-chips according to manufacturer’s
instructions (Agilent Technologies Ireland, Dublin, Ireland). Absor-
bance ratios (28S/18S) and RNA integrity values recorded for all
RNA samples extracted postclean-up ranged between 1.8 and 2.0, and
8.5 and 9.8, respectively.

Microarray hybridization and analysis. Gene expression was de-
termined using a 24,027 probe set bovine oligonucleotide array
(Affymetrix, High Wycombe, UK), representing ~23,000 bovine transcripts based on the original mapping using Unigene build 57
(March 24, 2004). RNA from each heifer was hybridized to a separate
array. All 16 RNA samples were hybridized and scanned by ALMAC
GROUP, Northern Ireland, according to the manufacturer’s instruc-
tions. All microarray analyses including preprocessing, normalization
and statistical analysis were carried out using R (R, 2007) version 2.6
and Bioconductor (41) version 2.1 as described by Morris et al. (72).
Data were quality assessed before and after normalization by a
number of inbuilt quality control methods implemented in the Bio-
conductor affycoretools and associated packages to identify problems
if they existed with array hybridization, RNA degradation, and data
normalization. Microarray data were preprocessed by the mmgMOS
normalization method (51) using the default settings and differential
expression (DE) was calculated by the pumaDE method both imple-
mented in the Bioconductor package “puma” (77). The puma method
uses a Bayesian hierarchical model to calculate the probability of
positive likelihood ratio (PPLR). The PPLR associates probability
values of genes being differentially expressed, which is a measure of
the false positive detection of DE, to each ratio and generates lists of
genes ranked by the probability of DE. This PPLR statistic was
converted into “P-like values” by the recommended formula in the
puma method prior to subsequent analysis. DE was calculated as
LF/HF ratio.

As many of the original annotations for the Affymetrix bovine chip
are erroneous (20), the remapped annotations were determined using the
“bovinaidpluscdf” chip definition file (CDF). The annotation
is based on the CDF-Merger procedure as described by De Leeuw et al. (22), which generates a hybrid CDF based on the standard Af-
nymetrix CDF (version 26) and the custom Brainarray (version 11.0.1)
CDF. This remapped annotation includes mapping to all RefSeq
(mature RNA protein coding transcripts and validated complete cod-
ing sequences in GenBank). Annotations were also supplemented by
interrogating the Ensembl B. taurus database version 46 using the
BioMart package in Bioconductor and manual annotation where possible with recent entries in Entrez Gene.

Pathway analysis. To examine the molecular functions and genetic
networks, we further analyzed the microarray data with Ingenuity
Pathway Analysis (IPA; v. 8.8, Ingenuity Systems, Mountain View,
CA; http://www.ingenuity.com), a web-based software application
that enables identification of overrepresented biological mechanisms,
pathways, and functions most relevant to experimental datasets or
genes of interest (64, 72).

A dataset containing gene identifiers and corresponding expression
and P-like values was uploaded into IPA. Briefly, each identifier was
mapped to its corresponding gene object in the Ingenuity knowledge
base. A P-like value of $P < 0.05$ from the puma analysis was set to
identify genes whose expression was significantly up- or downregu-
lated. These genes, called “focus” genes, were overlaid onto a global
molecular network developed from information contained within the
Ingenuity knowledge base. Networks of these focus genes were then
algorithmically generated based on their connectivity. Network anal-
ysis returns a score that ranks networks according to their degree of
relevance to the network eligible molecules in the dataset. The score
takes into account the number of network eligible molecules in the
network and its size, as well as the total number of network eligible
molecules analyzed and the total number of molecules in the knowl-
dge base that could potentially be included in networks.

RT-qPCR analysis. The microarray results were validated by car-
rying out RT-qPCR on 21 target genes. Candidate genes were chosen
based on the following criteria; those that were top ranking in our
microarray differentially expressed gene (DEG) list, genes with
known functional importance in uterine mediated subfertility that
were either up- or downregulated, or that genes that were not differentially
expressed between the two treatment groups.

Top ranking genes were genes showing large DE between fertility
groups. Such large differential gene expression may reflect functional
relevance in the specific target tissue and therefore warranted further
validation.

Using the same RNA samples that were analyzed in the microarray
studies, we synthesized first-strand cDNA with the High Capacity
cDNA Reverse Transcription kit according to manufacturer’s instruc-
tions (Applied Biosciences, Ireland). Purified total RNA (1 μg) was
reverse transcribed using random hexamers. The converted cDNA
was quantified by absorbance at 260 nm, diluted to 50 ng/μl working
stocks and stored at −20°C for subsequent analyses.

Analysis of putative reference genes for RT-qPCR studies was
carried out using GeNorm version 3.5 Microsoft Excel Add in
(Microsoft, Redmond, WA) (104). The stability of the expression of
several cited reference genes, including ribosomal protein L15 (6),
18 s ribosomal RNA (81), ubiquitin (18), glyceraldehyde phosphate
dehydrogenase and β-actin (87, 103), was investigated across all
samples in this study. Similar to Coyne et al. (18), ubiquitin (at an
optimal concentration of 2.5 M) exhibited the greatest stability
during qPCR analysis of endometrial mRNA samples analyzed, with
an M value of 0.022. Based on a recommended cut-off V value of
0.15, ubiquitin was selected as a single standard reference gene for
these experiments as the use of additional reference genes did not
contribute to a more accurate normalization factor. Primers were
designed using the Primer3 software program (84), and oligos were
aligned by Basic Local Alignment Search Tool (BLASTN) on the
National Center for Biotechnology Information web page, to verify
their identity and homology to the bovine genome (http://www.ncbi.n-
lm.nih.gov/BLAST/). All oligonucleotides were commercially syn-
dthesized as highly purified salt-free products by Sigma Aldrich Ire-
land. Primers were first tested using end-point PCR to optimize
amplification conditions. All amplified PCR products generated in this
study were purified with the PCR purification kit (Roche, Basel,
Switzerland) and sequenced (Macrogen; Nucleic Pys, Bendigo, Aus-
tralia) to verify their identity. Primer sequences used in this study are
listed in Table 1.

Primer concentrations were optimized for each gene by titrating 5,
10, and 20 μM per primer. The most suitable primer concentration
was chosen based on four criteria in order of decreasing importance:
1) a clear distinct melt curve absent of any additional peak(s) caused
by nonspecific binding, 2) a curve within the temperature range
75–85°C, 3) the primer concentration producing the lowest threshold
cycle number (Ct), and lastly, 4) replication amongst Ct values and
melting temperatures. Subsequently, efficiencies of chosen primer
concentrations were determined over a fivefold dilution series,
whereby cDNA was diluted into working solutions: stock, 1:2, 1:4,
1:8, 1:16, and RT-qPCR assays carried out. This was repeated for
every gene. The r2 and amplification efficiency (E) values for RT-
qPCR were calculated from linear regression analysis of log (input
cDNA) vs. Ct plot. The slope for each set of standards was used to
determine E = 10 −1/Ct slope − 1. Slopes, amplification efficiencies, and
r2 estimates for individual genes are reported in Table 2.

RT-qPCR analysis. Each RT-qPCR reaction was carried out in a
96-well plate format with a total volume of 20 μl, containing 1 μl
cDNA, (10 ng/μl), 10 μl Fast SYBR Green Master Mix (Applied
Biosystems, Ireland), 1 μl forward and reverse primers, and 8 μl

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nuclease-free H₂O. Performance of RT-qPCR was carried out using the Applied Biosystems Fast 7500 v2.0.1 with the following cycling parameters: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 s, followed by dissociation (95°C for 15 s, 60°C for 1 s, 95°C for 15 s, and 60°C for 1 s). Dissociation curves were examined for the presence of a single PCR product. Amplification efficiencies were determined for all candidate and reference genes using the formula $E = 10^{-1/C_{T}} - 1$ (slope), with the slope of the linear curve of $C_{T}$ values plotted against the log of the initial number of copies with PCR efficiencies between 90% and 110% were used. The software package GenEx 5.2.1.3 (MultiD Analyses, Gothenburg, Sweden) was used for efficiency correction of the raw $C_{T}$ values, interplate calibration based on a calibrator sample included on all plates, averaging of replicates, normalization to the reference gene, and the calculation of quantities relative to the greatest $C_{T}$ value. Gene expression results were calculated and fold changes in gene expression between LF relative to HF heifers determined using the delta delta $C_{T}$ ($2^{ΔΔC_{T}}$) method (63).

Statistical analysis. All data were analyzed using the Statistical Analysis Systems software package (SAS Inst., Cary, NC) version 9.1. Data from RT-qPCR studies were tested for adherence to normality using PROC UNIVARIATE (SAS, 2003). Nonnormal data were subsequently transformed using the best fit function as described by PROC TRANSREG (SAS, 2003). Differences in mean values between the two groups (HF and LF) were tested using ANOVA (PROC MIXED). Animal within treatment was used as the error term. The Tukey critical difference test was used to determine statistical differences between LF and HF mean values. The CORR procedure of SAS (PROC CORR, SAS 2003) was used to determine correlations between microarray and RT-qPCR data. Pearson correlation coefficients were estimated for each individual gene across all animals ($n = 16$). A $P$ value of $P < 0.05$ was considered to be statistically significant.

Data collected from CL were tested for adherence to normality using PROC UNIVARIATE (SAS, 2003). CL differences in mean

<table>
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<th>Gene Name</th>
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<th>Accession Number</th>
<th>Amplicon Size</th>
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Table 1. Bovine-specific oligonucleotide forward and reverse primer sequences (5'-3') and PCR product length

ALB, albumin; ARG2, arginase type II; BMP2, bone morphogenetic protein receptor type II; CCL28, chemokine (C-C motif) ligand 28; COL4A3, collagen type IV alpha 3; COL4A4, collagen type IV alpha 4; CORO1A, coronin actin binding protein 1A; CRYAB, crystallin alpha B; EGLN3, egl nine homolog 3; FADS1, fatty acid desaturase 1; GTF2A2, general transcription factor IIA 2; ITGA6, integrin alpha 6; LDLR, low density lipoprotein receptor; LYN, V-yes-1 Yamaguchi sarcoma viral related oncogene homolog; NSMAF, neutral sphingomyelinase (N-SMase) activation associated factor; PCCB, propionyl CoA carboxylase beta polypeptide; PLAUR, plasminogen activator urokinase receptor; PLCB3, phosphoinositide phospholipase C-beta-3; PPARG, peroxisome proliferator-activated receptor gamma; PTGS2, prostaglandin-endoperoxide synthase 2; SLC27A4, solute carrier family 27 member 4; UBQ, ubiquitin.
values between the two groups (HF and LF) were tested using ANOVA (PROC MIXED). Animal within treatment was used as the error term.

For the analysis of progesterone profiles individual profiles were normalized relative to day of estrus (day 0). The effect of fertility group “HF” vs. “LF” was established by a repeated-measured analysis (PROC MIXED; SAS).

RESULTS

Animal model. Retrospective analysis of embryo survival rates were high, averaging 72% across the entire cohort of nulliparous heifers for the four rounds of artificial insemination (54). In addition, CL diameters were 22.82 ± 0.99 for nulliparous heifers for the four rounds of artificial insemination rates were high, averaging 72% across the entire cohort of animals. The day of slaughter weight between HF and LF animals (overall 625 ± 54). In addition, CL diameters were significantly higher (P < 0.05) for HF and LF heifers, respectively. There was also no difference in slaughter weight between HF and LF animals (overall 625 ± 15.1 kg for the HF and 619 ± 18 kg for LF; means ± SD).

Progesterone profiles. There was no effect of fertility group × day of cycle (P > 0.10) or fertility group (P > 0.10) on the concentration of progesterone. On the day of slaughter plasma concentrations did not differ between the HF and LF groups (HF 5.96 ± 0.41 ng/ml, LF 5.65 ± 0.48 ng/ml).

Microarray differential gene expression. A total of 430 genes were found to be differentially expressed in HF vs. LF animals. Of these, 156 were upregulated and 274 downregulated in the LF compared with HF heifers. Transcript abundance differences between LF and HF groups resulted in fold changes ranging from 6.7-fold up to fourfold downregulated. The microarray data have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus (GEO) (31) and are accessible through GEO Series accession number GSE50091.

Pathway analysis. Of the 430 DEG, a total of 260 genes were successfully mapped to a molecular/biological pathway and/or category, while 252 of these were network eligible with IPA and 208 function/pathway/list eligible. Among the mapped DEG, 185 were upregulated (Supplementary Table S1) and 75 downregulated (Supplementary Table S2).

Biological functions. Biological categories with the largest ratio of upregulated genes included small molecule biochemistry, lipid metabolism, and molecular transport. Categories with the largest ratios of downregulated genes included cell death, skeletal and muscular system development and function, and reproductive system disease. Of the top 20 most significantly overrepresented biological categories, gastrointestinal disease and skeletal and muscular system development and function had the greatest ratio of up- to downregulated genes (Fig. 2). Pathways with the greatest number of DEG, including their respective number of DEG, were cell death (n = 56), small molecule biochemistry (n = 52), molecular transport (n = 38), lipid metabolism (n = 33), cellular movement (n = 28), inflammatory disease (n = 26), immune cell trafficking (n = 21), reproductive system disease (n = 21) as presented in Table 3.

Canonical pathways. Canonical signaling pathway analysis uncovered genes with functions in mitochondrial dysfunction, granulocyte B signaling, cytotoxic T lymphocyte-mediated apoptosis of target cells, and tumoricidal function of hepatic natural killer cells (Table 4). Canonical metabolic pathways overrepresented within the microarray dataset included oxidative phosphorylation, citrate cycle, and arachidonic acid metabolism. There were twice as many canonical metabolic pathways (n = 12) overrepresented as there were canonical signaling pathways (n = 6). The metabolic pathway and nicotinamide and nicotinamide metabolism had the greatest ratio of up- to downregulated genes. Overall the majority of DEG representing either canonical signaling or metabolic pathways were upregulated (Table 4).

Networks. Using IPA, we identified a total of 24 networks, 14 of which had 12–25 focus molecules among DEG. The 14 top networks are listed in Table 5. Lipid metabolism and small molecule biochemistry features in five of the top 14 networks. In addition, cell morphology, gene expression, cellular development, and carbohydrate metabolism appear most frequently throughout the networks generated, and, in particular, they constitute the top three scoring networks. Illustrations of gene interactions among DEG contained within the top three scoring networks are presented in Figs. 3, 4, and 5.

RT-qPCR analysis. We validated 21 genes by real-time RT-qPCR (Table 1). There was good consistency between methodologies in direction and magnitude of differential gene expression among genes analyzed. Correlation coefficients exceeded 0.58 in 15 of the 21 genes validated (Table 6).

D14 vs. D7. In total, seven genes were common among D7 with known ontology, PCCB, SLC25A24, DAP, and COLA4A4, and three expressed sequence tags, Br.20823, Br.21197, and Br.92317. PCCB exhibited upregulation in LF heifers on both days of the estrous cycle (1.35- and 1.14-fold increased on D14 vs. D7, respectively). However, remaining genes with known ontology exhibited contrasting differential regulation across D14 and D7, respectively: COLA4A4, 1.90 and −2.49-fold; DAP, 1.17 and −1.13-fold; and SLC25A24, −2.2.2 and 1.12-fold. Genes COLA4A4 and PCCB were validated by RT-qPCR

1 The online version of this article has supplemental material.
and correlated highly significantly with expression results from the Affymetrix microarray platform ($P < 0.001$; $r = 0.78$ and 0.75, respectively).

**DISCUSSION**

Recently, our group examined endometrial gene expression in HF and LF heifers during the critical, midluteal phase of the estrous cycle (54). However, taking into consideration that early embryo loss culminates by day 14–16 (25), endometrial gene expression analyses during the late luteal phase is pertinent in establishing the full complement of molecular mechanisms preceding early embryo loss. In the current study, we identified novel pathways and genes exhibiting changes in their expression between HF and LF heifers during this late luteal period. Furthermore, while only seven genes were common between D7 and D14, many of the same pathways were

**Table 3. Biological categories from IPA analysis with the largest number of DEG**

<table>
<thead>
<tr>
<th>Biological Category</th>
<th>Gene Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell death</td>
<td>AGER, ALB, ALOX5, APBB2, ARG2, ARHGEF3, ARHGEF4, BRAF, C1QBFP, C6, CDC25C, CHKA, CLCA2, COL4A3, CRYAB, CYP2C9, DAP, DFFA, DFFB, DHODH, DNM1T3A, DRAM1, EDN1, ENDOG, G6PC, GLUD1, HBXIP, HCRT, ITGA6, LDHA, LDLR, LIG1, LTF, LYN, M4B1, NR3C1, NSMAF, ODC1, PERP, PL2A2, PLAU, PNP, PPARG, PRDX1, PSMD2, PTGS2, RASD1, RFWD2, RPL37, RTN1, SDC1, SDHC, SLC25A24, SOS2, SPHK2, UBE2B</td>
</tr>
<tr>
<td>Small molecule biochemistry</td>
<td>ABCG4, ACOT4, AGER, ALB, ALOX5, AMD1, APRT, ARG2, ASGR2, BMP2, C1QBFP, CDC25C, CHKA, COL11A1, Cyp2c44, CYP2C9, CYP4F2, CYP4F3, DBI, EDN1, ENTPD1, FADS1, G6PC, GCSD, GMPT2, GJB1, GLUD1, HCRT, HPS, ITGA6, LDHL, LTF, LYN, M4C4, NR3C1, NSF, NSMAF, NT5C, ODC1, PCCB, PFKL, PLA2G16, PLAUR, PLIN2, PNP, PPARG, PRDX1, PTGS2, SLC27A4, SMPD3, SUCLG1, THRSP</td>
</tr>
<tr>
<td>Molecular transport</td>
<td>AGER, ALB, ALOX5, APRT, ATP1B3, ATP2A2, ATP4A, ATP5L, ATP5V0B, C1QBFP, CHKA, CHP, CLCA2, CNGA1, CORO1A, EDN1, ENTPD1, G6PC, GJB1, HCRT, HC2N2, LDLR, LYN, M4C4, NR3C1, NSF, NT5C, NUPL1, ODC1, PLIN2, PNP, PPARG, PTGS2, SLC25A11, SLC25A3, SLC27A4, SLC27A4, SLC30A8, SRI, STX2, VPS33B</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>ABCG4, ACOT4, AGER, ALB, ALOX5, ASGR2, BMP2, CHKA, Cyp2c44, CYP2C9, CYP4F2, CYP4F3, DBI, EDN1, FADS1, G6PC, ITGA6, LDHL, LTF, LYN, M4C4, NR3C1, NSF, NT5C, NUPL1, ODC1, PLIN2, PNP, PPARG, PTGS2, SLC25A11, SLC25A3, SLC27A4, SLC30A8, SRI, STX2, VPS33B</td>
</tr>
<tr>
<td>Inflammatory disease</td>
<td>ABLM1, AGER, ALB, ALOX5, AMD1, BRAW, C6, CCL28, COL1A3, CORO1A, DMT1, DYSPL2, EDN1, G6PC, HPS, ITGA6, LDHL, LTF, LYN, M4C4, MTC2H2, PLAU, PLCB3, PPARG, PTGS2, SCPEP1, SDC1, SPHK2</td>
</tr>
<tr>
<td>Reproductive system disease</td>
<td>AGER, ALB, ALOX5, ARG2, ASPH, BMP2, BRAF, C6, CCL28, COL1A3, CORO1A, DMT1, DYSPL2, G6PC, ITGA6, LDHL, LTF, LYN, M4C4, PLAUR, PLCB3, PPARG, PTGS2, SDC1, STX2</td>
</tr>
</tbody>
</table>

IPA, Ingenuity Pathway Analysis; DEG, differentially expressed genes.
enriched in both mid- and late luteal endometrium (54), indicating that consequential changes in gene expression was occurring in endometrial transcriptome between these days. Significant pathways include lipid metabolism FADS1 and LDLR; molecular transport ALB, SLC27A4, and ABCG4; cell morphology and development COL4A4, COL4A5, and BMPR2; and immune response PTGS2, CORO1A, and CCL28.

Lipid metabolism. Lipid metabolism features in five of the top 14 generated networks and is the fourth most overrepresented biological pathway (with 33 DEG). Genes involved in eicosanoid synthesis [fatty acid desaturase 1 (FADS1), endothelin 1 (EDN1), low density lipoprotein receptor (LDLR)] were upregulated in the LF animals. Recently found to be expressed in bovine endometrial tissue, FADS1 codes for a rate-limiting enzyme involved in the synthesis of long chain fatty acids: arachidonic acid, eicosapentaenoic acid, and docosahexanoic acid (108). Such long chain polyunsaturated fatty acids are precursors to the well-known eicosanoids, PGF2α, of which endometrial-expressed EDN1 and LDLR also induce synthesis (30, 42, 99). It was postulated that increased transcript levels of these genes, exhibited in the LF heifers, may subsequently result in an increased production of PGF2α, a known abortifacient (1). Interestingly, prostaglandin-endoperoxide synthase 2 (PTGS2), which increases synthesis of PGF2α (37), was upregulated in LF heifers. In the context of this study, increased prostaglandin synthesis during the late luteal phase would be deleterious to the maturing CL, inducing luteolysis (2). This is consistent with the life-cycle of the CL with regression occurring in the late luteal phase of an estrous cycle (90). Differential expression of these genes between HF and LF heifers may signify premature initiation of luteolysis, which is known to significantly affect in-utero embryo development. However, it is important to recognize PTGS2 is implicated in the synthesis of a variety of prostaglandins, some of which are embryo-trophic, facilitating growth and elongation, and uterine regulating.

The relationship between progesterone concentration and embryo survival has been well examined. It has been reported that increased plasma progesterone in the luteal phase before and after insemination is associated with higher pregnancy rates (96). Furthermore, progesterone facilitates and maintains pregnancy by preparing the uterus for implantation (5) and, working in concert with a multitude of other metabolites, provides a nourishing histotroph for the developing embryo (7). Interestingly, LF animals had an upregulation of genes EDN1, ITGA6, and DBI, all of which have demonstrated involvement in progesterone production in other tissues (32, 38, 39), and LDLR, involved in the production of the steroid precursor to progesterone, was upregulated in LF animals. This may suggest endometrial progesterone metabolism in LF animals is different to HF animals, which could be deleterious to early embryo survival. Overall, decreased progesterone production and increased PGF2α accompanies luteolysis (90). As neither of these metabolites was measured in uterine fluid, it is unclear whether pathways eliciting CL regression were activated or repressed in LF heifers. Measurement of these metabolites would be instrumental in clarifying whether premature initiation of luteolysis is affecting subfertile animals (90). Additional genes involved in the metabolism of fatty acid and synthesis of their subsequent biproducts, including ALB (19), PCCB (57), PPARG (88), and LYN (15), were also upregulated in LF animals.

**Molecular transport.** Genes involved in the transportation of long chain fatty acids ALB (105), PLIN2 (21, 34), PPARG (86), and SLC27A4 (95) were differentially expressed between fertility groups. In addition, modification in expression was found in genes with roles in lipid uptake LDLR (23), NR3C1 (58), PPARG (73), and SLC27A4 (95). Alterations in lipid uptake are
Table 5. Networks generated by IPA central to endometrial related subfertility

<table>
<thead>
<tr>
<th>Network ID</th>
<th>Top Functions</th>
<th>Molecules in Network</th>
<th>Score</th>
<th>Focus Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cell morphology, cellular development, behavior</td>
<td>AGER, ALB, ATP2A2, BACE1, CD82, CHF, COL4A3, Collagen type IV, CORO1A, DEXR, DFFA, DFFB, DMBT1, DRAM1, ENTPD1, GFFT2, Growth hormone, HISTONE, Immunoglobulin, Integrin alpha 3 beta 1, ITGAX, Laminin, Ldh, LDHA, LRG1, MIR124 (human), MSL1, MYST1, NFkB (complex), NFkB (family), PRDX1, RTN1, STX2, SYNPO, UBE2B</td>
<td>43</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>gene expression, genetic disorder, nephrosis</td>
<td>ABLIM1, BTF3L4, CA12, Calmodulin, Calpain, Chpp300, CELSR2, CK2, Collagen(s), DPYSL2, ERK, F Actin, Focal adhesion kinase, GBJ1, Gnr, GPR65, GPR86, GPR137, GTF2A2, GTF2E2, Holo RNA polymerase II, IQC81, KCNN2, LIG1, Mapk, MC4R, MYO1B, Pks(s), PLC, PLCB3, POLR2J, PSAT1, RNA polymerase II, SDC1, T3-TR-RX, TRS3P, Vesf</td>
<td>32</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>organismal functions, carbohydrate metabolism, lipid metabolism</td>
<td>AKR7A2, ALOX5, AP3M1, ARG2, BRAF, Calcineurin protein(s), CadMKII, Caveolin, CHKA, CLASRP, Collagen Alpha, Creb, EDN1, ERK1/2, Integrin alpha V beta 3, LDL, LDLR, LRP, LYN, Mek, NADPH oxidase, Nos, OIP5, Pdgf (complex), PDGF BB, PLAUR, PLN2, PPARG, PTGS2, RASD1, Rock, SLC25A11, SMDP3, SOS2</td>
<td>32</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>cellular growth and proliferation, lipid metabolism, small molecule biochemistry</td>
<td>I4-3-3, Ap1, CCNCF, CDC25C, CHAF1A, CYP2C9, CYP4F2, DBI, DOCK10, EGF, G6PC, GLUD1, Hcg, HIST3H3, Histone h3, Histone h4, HPSE, Insulin, LTF, MCH2, N-or, Nfat (family), ODC1, PCCB, PFKL, PI3K (complex), PLA2G16, Rsr, SDC1, T3-TR-RX, TRS3P, Vesf</td>
<td>30</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>ophthalmic disease, cell morphology, cellular development</td>
<td>Akt, ARHGEF4, ASGR2, ATP1B3, Bmp2, Caspase, COX5B, CRYAB, Dap, E2f, ENDOG, FSH, HBxIP, Hcrt, Hsp70, Hsp90, IgG, IL1, Interferon alpha, Jnk, Kcnj16, Lh, Nsr1, Nsf, NSMAF, P38 Mapk, Pdcd1, Pka, Psma2, Ras, Tgf beta, Tmem126A, Tpr, Trypsin</td>
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<td>18</td>
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<tr>
<td>6</td>
<td>endocrine system development and function, organ development, gene expression</td>
<td>AMBP, AMD1, ARHGEF3, ARHGEF4, ASGR1, Bcl2, COL1A1, COL4A3, COLQ, COTL1, CR1, Gars, Gcnt3, GFFT2, Hnf1A, Hnf1B, Mdh2, Mthfd2, Mut, Myo9b, Neto2, Ngrf, Nme1, Parp3, Pcdh1, Pcsks6, Pdx1, Pkn1, Pnxn4, Rhoa, Rpit3, Saa1, Tcf19, Tgfb1, Tnf, Znf2f3</td>
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<td>16</td>
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<tr>
<td>7</td>
<td>lipid metabolism, small molecule biochemistry, nucleic acid metabolism</td>
<td>ACOT1, Acot1, Acot2, Acot4, Aco5, Acot7, Ankrf1, C1orf94, C20orf72, CYP4F2, CYP4F3, Dlst, Gap2, Hnf4a, Ift122, Kcn2, Mab21l1, Mdh1, Pnp, Polr3f, Rsl11b, Rnf183, Rtcdf1, Saa1, Sema3c, Znf2f1</td>
<td>23</td>
<td>16</td>
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<tr>
<td>8</td>
<td>lipid metabolism, small molecule biochemistry, cancer</td>
<td>Abtb1, Agt, Apb1, Apb2, Beta-estadiol, C1qbp, Clstn1, Cs, Cstb, Cth, Cyp2a, Cyp2f2, Cyp4b1, Cyp4f2, Ecd, Egfr, Ewsi1, Gfer, Gjbn, Hnf1b, Hnf4a, Ifi19, Ift122, Kcn2, Mab21l1, Mdh1, Pnp, Polr3f, Rsl11b, Rnf183, Rtcdf1, Saa1, Sema3c, Znf2f1</td>
<td>23</td>
<td>16</td>
</tr>
<tr>
<td>9</td>
<td>gene expression, dermatological diseases and conditions, genetic disorder</td>
<td>Ar, Atf7ip1, Atp6v0b, Brf1, C1orf51, Carm1, Crebl1, Dnbp1p, Fam49b, Gtf2h3, Gtf3c4, Hla-a, Hla-qrb1, Hps6, N-or, Nfyb, Pias4, Pnrc1, Polrc3, Polr3f, Ppargc1a, Rab11a, Raf5, Rnf6, Rnf14, Sclc25a28, Spata24, Srsf4, Srsf6, Stbd1, Tbf, Vipar, Vps39, Vps33b, Wdrl89</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td>10</td>
<td>carbohydrate metabolism, small molecule biochemistry, cancer</td>
<td>Aspan, Atph4a, Ccr4nl4, Cdc3, Cul1, Ddhod, Dpp3, Elov16, Fbx0, Fbx031, Fbx033, Fbxw9, Fbxw11, Hhip, Htt, Il4, Ilvbl, Loc100129193, Lrrc3c, Nkfb1a, Norepinephrine, Nup1l, Osbpl10, P, Pctp, Pias4, Pln2, Pln4, Pparg, Sfpk, Slain1, Sclc25a3, Spbr1a, Xrc6, Zch3h8</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td>11</td>
<td>DNA replication, recombination, and repair, gene expression, cell-to-cell signaling and interaction</td>
<td>Acta1, Cacna1a, Cisd1, Cpn2e, DNA (cytosine-5)-methyltransferase, Dnmt3a, Eef2, Eff1a1, Egl1, Eppk1, Gafp, Grb2, Gs3, Hell, Igif1, Il5, Il3ra, Il3ra, Maki6, Mettl13, Mirk29b (human), Myc, Mypb2, Myo1b, Pfk, Pipox, Plp1, Psat1, St00as6, Scepcp1, Srd, Syncre, Trmd1, Wdr4, Znf2f7</td>
<td>18</td>
<td>13</td>
</tr>
<tr>
<td>12</td>
<td>cellular development, embryonic development, cellular assembly and organization</td>
<td>Acta1, Alb, As8b, Asf1a, Bcwd2, Clca2, Dyrkia, Fgf2, Frg1, Gafap, Hat, Hmgbl1, Hras, I620b2, Klf3, Mapkapk2, Mrpl12, Mrpl39, Pan2, Pan3, Pou5f1, Pqlc3, Rwfd2, Rpl37, Serpin3k (includes others), Snrpm, Tcebl1, Tead2, Tead4, Tenasin, Tep1, Tp53, Vgl1, Zcchc8, Zfp57</td>
<td>17</td>
<td>13</td>
</tr>
</tbody>
</table>

Continued
associated with a number of metabolic disease states (10, 45). However, increased uptake and transport of fatty acid, particularly long chain fatty acid, in low reproductive capacity animals may indicate there is increased substrate for eicosanoid synthesis in such animals. Eicosanoid synthesis negatively influences embryo survival (11). For example, PLIN2 gene expression has been reported to increase during the later luteal phase of the estrous cycle and in the presence of a conceptus (34). Data from our fertility study, the first of its kind, revealed upregulated PLIN2 in LF animals compared with HF animals.

Numerous studies report electrolyte transport activities across the endometrial epithelium (12–14), thereby influencing uterine fluid composition and in turn intrauterine sperm capacitance and motility and embryo development and implantation. It has been observed that a high molar ratio of K⁺/Na⁺ contributes to an improvement in in vitro fertility in rats (103). Also, across species K⁺/Na⁺ ratios are higher in uterine than oviductal fluid (47, 48), suggesting the importance of this electrophysiological signaling pathway in overall endometrial physiology. Genes NSF (79), ATP4A (65), and ATP1B3 (62) with known involvement in K⁺ and Na⁺ transport displayed differential expression between HF and LF heifers.

Phospholipase Cβ3 (PLCB3) was found to be previously expressed in ovarian follicular (29) and uterine myometrial cells (113) and is likely to be involved in calcium signaling (70). Calcium signaling in the endometrium has been shown to influence endometrial receptivity (112), a decrease of which causes a corresponding reduction in production of receptivity factors and subsequent implantation failure. HF heifers had increased PLCB3 expression potentially influencing endometrial receptivity. Furthermore, gene expression of calcium exchange enhancing molecule GJB1 (16) was also different between HF and LF heifers, highlighting the prospect for calcium signaling in endometrial function and receptivity.

Solute carrier (SLC) genes SLC22A18, SLC25A1, SLC25A3, SLC25A11, SLC25A28, and SLC27A4 were among the most important and widely cited endometrial molecular transport molecules featuring in gene expression profiles from HF and LF animals. Walker et al. (106) observed that glucose transporter SLC2A1 was downregulated in the endometrium of subfertile cows on day 17 of pregnancy and suggested the ability of these cows to transport this basic energy source to growing embryos may be altered. In such circumstances, embryo development would be compromised, and this would contribute to increased embryo mortality rates, as observed in subfertile strains. Furthermore, work by Salilew-Wondim et al. (85) and our laboratory (54) resulted in the discovery of 19 and five SLC differentially expressed genes between receptive/HF and nonreceptive/LF heifers on D7 of the estrous cycle, respectively. Such genes are thought to play a role in transport of cellular materials across the cell membrane, the absence of which could result in defective metabolic exchange between mitochondria and cytoplasm (44), again potentially affecting the intrauterine peri-implantation embryo environment.

Differentially expressed SLC genes in the current study included drug transporters, SLC 22 family (55); mitochondrial transporters, SLC 25 family (76); and fatty acid transporters, SLC 27 family (94). Phosphatase transporter SLC25A3, which is necessary for oxidative phosphorylation of ADP to ATP (76), previously found in bovine heart and liver mitochondria (4), was upregulated in LF heifers. In addition, mitochondrial citrate transporter SLC25A1, which was upregulated in LF animals, is essential for cytosolic fatty acid and steroid biosynthesis. Intermediates oxaloacetate and acetyl CoA produced from exported citrate are the substrates required for this process (52). SLC27A4, upregulated in low conception rate ani-

**Table 5.—Continued**

<table>
<thead>
<tr>
<th>Network ID</th>
<th>Top Functions</th>
<th>Molecules in Network</th>
<th>Score</th>
<th>Focus Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>lipid metabolism, molecular transport, small molecule biochemistry</td>
<td>22(R)-hydroxycholesterol, ABCG4, ABCG5, ABCG8, ACAT1, Acat1, APRT, ascorbic acid, cholesteryl, DBI, FOS, FURIN, GCSH, HLA-DQb1, IDH1, IDH2, IKBKG, IKT, KRTBD10, KCNK1, Keratin, LAMP2, NEAT (complex), NML, PGP, Pias4, PKP2, PRKDC, PRKG2, progestosterone, PTS, STAT4, TCP11, TFF1, TSPAN5</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>14</td>
<td>genetic disorder, metabolic disease, cardiovascular disease</td>
<td>ATP5L, CCL28, CNGA1, CNGB1, cyclic AMP, DLG4, GLP2R, GPR65, HSPB6, IFNB1, MAPK1, MARCH9, MT-N2D, MT-N3D, MT-N5D, MT-NDA4L, NADH dehydrogenase, NADH2 dehydrogenase, NADH2 dehydrogenase (ubiquinone), NDUF2, NDUF6A, NDUF9, NDUF9A1, NDUF9B, NDUFC2, NDUF5, NDUF54, NDUF55, NDUF56, NDUF57, NDUF8, palmitic acid, PNMA2, SLC27A4, TNKS2</td>
<td>16</td>
<td>12</td>
</tr>
</tbody>
</table>

Fig. 3. Network 1: cell morphology, cellular development, behavior. The network is displayed graphically as nodes (genes). The node color intensity indicates the expression of genes, with red representing upregulation and green downregulation in low-fertility (LF) vs. high-fertility (HF) endometrium. The fold value is indicated under each node.
imals, is necessary for the uptake of long chain and very long chain fatty acids (95, 105). It is therefore evident that a disparity in the mRNA expression of metabolite transport proteins exists between fertility groups.

**Immune response.** Studies have demonstrated an in-utero proinflammatory disposition negatively influences embryo mortality (85, 100) and suggest a maternal rejection reaction to the embryo.

Transcript abundance of arachidonate 5-lipoxygenase, ALOX5, was increased in LF compared with HF animals. This transcript codes for an important protein involved in arachidonic acid metabolism and catalyzes the first step in leukotriene biosynthesis, critical to initiating an acute inflammatory response (42). Furthermore, prostaglandin-endoperoxide synthase 2, otherwise known as COX-2 or PTGS2, was simultaneously upregulated in LF animals. PTGS2 is also implicated in inflammation by its involvement in arachidonic acid metabolism and prostaglandin release (42, 82). Other genes with specific roles in the inflammatory response that were differentially expressed include ALB, CORO1A, LYN, and PLAUR (56, 78, 83, 109). Similarly, Walker et al. (106) found expression of chemokines CCL16, CXCL10, and CXCL11 to be altered between HF and LF strains, stimulating proinflammatory cytokines and preventing the attraction of immune tolerance, promoting uterine natural killer cells in subfertile animals, thereby potentially hindering embryo tolerance and attachment in these animals (17, 28).

Chemokine ligand 28 (CCL28) was upregulated in LF compared with HF heifers. Chemokines act on neutrophils and monocytes attracting them to sites where they are “needed” to strengthen the inflammatory response (9). The effects of endometrial cytokines on embryo implantation have been widely studied (24); however, their effects on embryo tolerance have been examined to a lesser extent. Salilew-Wondim et al. (85)

**Fig. 4.** *Network 2: gene expression, genetic disorder, nephrosis.* The network is displayed graphically as nodes (genes). The node color intensity indicates the expression of genes, with red representing upregulation and green downregulation in LF vs. HF endometrium. The fold value is indicated under each node.

**Fig. 5.** *Network 3: cellular growth and proliferation, lipid metabolism, small molecule biochemistry.* The network is displayed graphically as nodes (genes). The node color intensity indicates the expression of genes, with red representing upregulation and green downregulation in LF vs. HF endometrium. The fold value is indicated under each node.
also showed that nonreceptive heifers exhibited CCL28 up-regulation compared with receptive heifers.

**Cell morphology and development.** The provision of a structurally and functionally efficient endometrium is of utmost importance for endometrial receptivity and successful implantation. Abnormal expression of the major constituents of the extracellular matrix (ECM), collagens, matrix metalloproteinases (MMP), and tissue inhibitors of MMPs (TIMP) could be associated with difficult or negated implantation and subsequent embryo death (50). More importantly, Minten et al. (71) found that gene MEP1B, from the meprin family of ECM cleavage proteins, was more abundantly expressed in HF than SF endometrium. MMPs cause ECM degradation. They act by breaking down ECM components into collagens, fibronectin, and laminins. TIMP bind conserved zinc-binding sites on MMPs suppressing their activity (74). Furthermore, expression of TIMP1 is heightened by CD82 (61), which was upregulated in LF heifers. Interestingly, an abundance of collagen genes, COL4A3, COL4A4, COL11A1, and COLQ, were upregulated in LF heifers. In addition, decreased expression of type IV collagen was observed in decidua of spontaneously aborted endometrial extracellular remodeling and metabolite exchange, and increased fatty acid synthesis, all of which are thought to be responsible for poor conception rates in these animals.

**Conclusions**

This work provides novel data on late luteal, global endometrial gene expression signatures between HF and LF heifers. We identified important biological pathways involved in endometrial physiology and endometrial-mediated embryo survival that could be incorporated into future studies examining proteomic and metabolomic regulation of uterine-mediated embryo survival. In addition, the expression analysis provides invaluable data on key DEG that may be included in future single gene studies and functional validation of these DEGs.
nucleotide polymorphism discovery analyses, providing potential markers for fertility that could be incorporated into future genomic selection breeding programs.

ACKNOWLEDGMENTS
The authors thank all the farm and technical staff at Teagasc Athenry for expertise and diligent management and care of the heifers used in this study.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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