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

Aideen P. Killeen,1,2 Michael G. Diskin,3 Dermot G. Morris,3 David A. Kenny,1 and Sinéad M. Waters,1*

Authors’ Affiliations:
1Teagasc, Animal and Bioscience Research Department, Animal and Grassland Research and Innovation Centre, Grange, County Meath, Ireland; 2School of Agriculture, Food Science and Veterinary Medicine, College of Life Sciences, University College Dublin, Belfield, Dublin 4, Ireland; and 3Teagasc, Animal and Bioscience Research Department, Animal and Grassland Research and Innovation Centre, Mellows Campus, Athenry, County Galway, Ireland.

Authors’ Contributions:
AK extracted and purified the RNA from endometrial tissue, prepared it for microarray analysis, performed qPCR validation and prepared the main manuscript under the direction of SW who conceptualised the study. MGD and DK generated the animal model. MGD and SW collected endometrial tissue. MGD, DM, SW and DK collected and analysed all performance and physiological data. DM and AK performed bioinformatic analysis of microarray data. AK and SW finalized the manuscript with all other authors making valuable contributions. All authors read and approved the final manuscript.

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Corresponding Author: Dr. Sinéad M. Waters

Address: Teagasc, Animal and Bioscience Research Department
Dunsany, Co. Meath, Ireland.

Email: Sinead.Waters@teagasc.ie
ABSTRACT

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 oestrous 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 high or low fertility heifers at a later stage of the oestrous cycle; day 14 (D14). A second objective was to compare these expression profiles with those from mid-luteal HF and LF endometrium. Using the same animal model as employed in the previous study, HF and LF animals were slaughtered on D14, endometrial tissue harvested and global gene expression analysis carried out using the Affymetrix® Bovine GeneChip. Microarray analysis detected 430 differentially expressed genes (DEG) between HF and LF animals. Ingenuity Pathway Analysis (IPA), 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.

Keywords: Bovine fertility, endometrium, gene expression, microarray
INTRODUCTION

In heifers and moderate yielding dairy cows, it is estimated that early embryo mortality is approximately 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 two weeks after insemination (26).

Data from our group has 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 have 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 oestrous 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 day 7 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 sub-fertility 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 and low fertility heifers (54). In addition, Salilew-Wondim et al. (85) described changes in endometrial molecular signatures between receptive and non-receptive heifers on day 7 and day 14 of the oestrous cycle. Furthermore, Minten et al. (71) described possible innate genetic variation between fertile and sub-fertile/infertile heifers with altered endometrial expression signatures and moderate genetic associations. The challenge remains to elucidate the causes of sub-fertility. 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 high and low fertility heifers, using the fertility model as described by Killeen et al. (54), on day 14 of the oestrous cycle. In addition, genes and pathways differentially enriched between high and low fertility endometrium during mid- and late- luteal phases of the oestrous cycle (day 7 and day 14) 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, oestrous cycles of reproductively normal nulliparous crossbred beef heifers (Bos taurus n=120) were synchronized using two intramuscular administrations of 500μg of the prostaglandin F$_{2α}$ analogue (PG), cloprostenol (Estrumate®, Schering-Plough Ltd., 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 hrs 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 using a two-injection PG-regimen, inseminated and pregnancy scanned as described by Killeen et al. (54).

For the purpose of establishing an accurate high-low fertility 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 three months later, estrous cycles of animals were synchronized in preparation for endometrial harvesting on day 14 (Figure 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. Mid-uterine intercaruncular endometrial cross-sections approximately 4 sq cm, and weighing 2.5g, 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 24h 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 the day of slaughter CL diameter for each heifer was determined using vernier calipers.

**Blood sampling.** Heifers were blood sampled via jugular venipuncture for subsequent measurement of progesterone at 0900 and 2100h commencing 24h after PG for a cycle length. All blood samples were collected into 10-ml ethylenediamine tetraacetic acid (EDTA) heparinized vacutainers (Becton Dickson Vacutainer Systems, Plymouth, UK). Samples were held in iced water until centrifuged at 1500 x 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 using the Coat-a-Count assay procedure (Coat-a-Count Diagnostic Products Corporation, Los Angeles, CA, USA) with each sample tested in duplicate. The inter-assay 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 Ltd., Dublin, Ireland). Tissue samples were homogenized in 3 ml of TRIzol reagent and chloroform, and subsequently precipitated using isopropanol (Sigma-Aldrich Ireland Ltd., Dublin, Ireland). RNA samples were
stored at -80°C. Samples of RNA, 20 µg, were purified and treated for contaminating genomic DNA using RNeasy 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 using automated capillary gel electrophoresis on a Bioanalyzer 2100 with RNA 6000 Nano Lab-chips according to manufacturer’s instructions (Agilent Technologies Ireland, Dublin, Ireland). Absorbance ratios (28S/18S) and RNA integrity values recorded for all RNA samples extracted post clean-up ranged between 1.8 and 2.0, and 8.5 and 9.8, respectively.

*Microarray hybridization and analysis.* Gene expression was determined 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 LTD., Northern Ireland, according to the manufacturer’s instructions.

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 using a number of in-built quality control methods implemented in the Bioconductor affycoretools and associated packages to identify problems if they existed with array hybridization, RNA degradation, and data normalization. Microarray data were preprocessed using the mmgMOS normalization method (51) using the default settings and differential expression (DE) was calculated using the *puma*DE method both implemented 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” using 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 “bovinedaiplusv6cdf” chip definition file (CDF). This 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 Affymetrix CDF (version 26) and the custom Brainarray (version 11.0.1) CDF. This re-mapped annotation includes mapping to all RefSeq (mature RNA protein coding transcripts and validated complete coding sequences in GenBank). Annotations were also supplemented by interrogating the Ensembl Bos 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, the microarray data were further analyzed using Ingenuity Pathway Analysis (v. 8.8, Ingenuity Systems, Mountain View, CA; http://www.ingenuity.com), a web-based software application that enables identification of over-represented 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 down-regulated. 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 analysis 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 knowledge base that could potentially be included in networks.

RT-qPCR analysis. The microarray results were validated by carrying out RT-qPCR on 21 target genes. Candidate genes were chosen based on the following criteria; those that were top ranking in our microarray DEG list, genes with known functional importance in uterine mediated sub-fertility which were either up- or down-regulated, or genes which were not differentially expressed between the two treatment groups. Top ranking genes were genes showing large differential expression 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, first strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit according to manufacturer’s instructions (Applied Biosciences, Ireland). Purified total RNA (1μg) was reverse transcribed using random hexamers. The converted cDNA was quantified by absorbance at 260 nm, diluted to 50ng/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), 18s 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 2.5μM) 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 programme (84) and oligos were aligned by Basic Local Alignment Search Tool (BLASTN) on the National Centre for Biotechnology Information (NCBI) web page, to verify their identity and homology to the bovine genome (http://www.ncbi.nlm.nih.gov/BLAST/). All oligonucleotides were commercially synthesized as highly purified salt-free products by Sigma Aldrich Ireland Ltd. Primers were first tested using end point PCR to optimize amplification conditions. All amplified PCR products generated in this study were purified using the PCR purification kit (Roche, Basel, Switzerland) and sequenced (Macrogen; Nucleics Pty Ltd, Bendigo, Australia) 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 4 criteria in order of decreasing importance: i) a clear distinct melt curve absent of any additional peak(s) caused by non-specific binding, ii) a curve within the temperature range 75-85°C, iii) the primer concentration producing the lowest threshold cycle number ($C_t$) and lastly, iv) replication amongst $C_t$ values and melting temperatures ($T_m$). Subsequently, efficiencies of chosen primer concentrations were determined over a 5-fold 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 $r^2$ and amplification efficiency ($E$) values for RT-qPCR were calculated from linear regression analysis of log (input cDNA) versus $C_t$ plot. The slope for each set of standards was used to determine $E = 10^{(-1/slope)} - 1$. Slopes, amplification efficiencies and $r^2$ 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, (10ng/μl ), 10 μl Fast SYBR® Green Master Mix (Applied Biosystems, Ireland), 1 μl forward and reverse primers and 8 μl 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 60 s, followed by amplicon dissociation (95°C for 15 s, 60°C for 60 s, 95°C for 15 s and 60°C for 15 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/slope), with the slope of the linear curve of cycle threshold (Ct) values plotted against the log dilution (46). Only primers with PCR efficiencies between 90% and 110% were used. The software package GenEx 5.2.1.3 (MultiD Analyses AB, Gothenburg, Sweden) was used for efficiency correction of the raw Ct 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 Ct. Gene expression results were calculated and fold changes in gene expression between LF relative to HF heifers determined using the delta delta cycle threshold (2^-ΔΔCT) method (63).

Statistical analysis. All data were analyzed using the Statistical Analysis Systems software package (SAS Inst. Inc., Cary, NC) version 9.1. Data from RT-qPCR studies were tested for adherence to normality using PROC UNIVARIATE (SAS, 2003). Non-normal 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 difference 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
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 corpora lutea were tested for adherence to normality using PROC UNIVARIATE (SAS, 2003). CL 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.

For the analysis of progesterone profiles individual profiles were normalized relative to day of estrus (Day 0). The effect of fertility group “HF” versus “LF” was established using 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 A.I. (54). In addition, CL diameters were 22.82 ± 5.16 mm and 20.50 ± 3.51 (P>0.10) for HF and LF heifers, respectively. There were no significant differences in CL diameter between fertility groups (P > 0.10). 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; Mean +SD).

Progesterone profiles. There was no effect of fertility group x 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 high and low fertility groups (HF 5.96 ±0.41 ng ml\(^{-1}\); LF 5.65 ± 0.48 ng ml\(^{-1}\)).
Microarray differential gene expression. A total of 430 genes were found to be differentially expressed in HF versus LF animals. Of these, 156 were up-regulated and 274 down-regulated 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 4-fold down-regulated. The microarray data have been deposited in NCBI’s Gene Expression Omnibus (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 using IPA and 208 function/pathway/list eligible. Among the mapped DEG, 185 were up-regulated (Supplementary Table S1) and 75 down-regulated (Supplementary Table S2).

Biological functions. Biological categories with the largest ratio of up-regulated genes included small molecule biochemistry, lipid metabolism, and molecular transport. Categories with the largest ratios of down-regulated genes included cellular development, skeletal and muscular system development and function and reproductive system disease. Of the top 20 most significantly over-represented biological categories, gastrointestinal disease, and skeletal and muscular system development and function had the greatest ratio of up- to down-regulated genes (Figure 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, granzyme B signaling, cytotoxic T lymphocyte-mediated apoptosis of
target cells and tumoricidal function of hepatic natural killer cells (Table 4). Canonical metabolic pathways over-represented within the microarray dataset included oxidative phosphorylation, citrate cycle and arachidonic acid metabolism. There were twice as many canonical metabolic pathways (n=12) over-represented as there were canonical signaling pathways (n=6). The metabolic pathway, nicotinate and nicotinamide metabolism had the greatest ratio of up- to down-regulated genes. Overall the majority of DEG representing either canonical signaling or metabolic pathways were up-regulated (Table 4).

Networks. Using IPA a total of 24 networks were identified, 14 of which had 12 to 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 Figures 3, 4 and 5.

RT-qPCR analysis. Twenty one genes were validated 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 fifteen of the twenty one genes validated (Table 6).

Day 14 vs. Day 7. In total, seven genes were common among day 7 and day 14 microarray DEG datasets. These included four genes with known ontology, *PCCB*, *SLC25A24*, *DAP* and *COL4A4*, and three expressed sequence tags, Bt.20823, Bt.21197 and Bt.92317. *PCCB* exhibited up-regulation in LF heifers on both days of the estrous cycle (1.35 and 1.14 fold increased on D14 and D7,
respectively). However, remaining genes with known ontology exhibited contrasting differential regulation across D14 and D7, respectively: $COL4A4$, 1.90 and $-2.49$ fold; $DAP$, 1.17 and $-1.13$ fold; and $SLC25A24$, $-1.22$ and 1.22 fold. Genes $COL4A4$ and $PCCB$ were validated using RT-qPCR 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, mid-luteal 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, novel pathways and genes exhibiting changes in their expression between HF and LF heifers during this late-luteal period were identified. Furthermore, while only seven genes were common between d7 and d14, many of the same pathways were 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 $PLCB3$; cell morphology and development $COL4A3$, $COL4A4$ 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 over-represented biological pathway (with 33 DEG). Genes involved in eicosanoid synthesis; fatty acid desaturase 1 ($FADS1$), endothelin 1 ($EDN1$), low density lipoprotein receptor ($LDLR$) were up-regulated in the low fertility 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 well-known eicosanoid, $PGF_{2\alpha}$, of which endometrial
expressed \textit{EDN1} and \textit{LDLR} also induce synthesis (30, 42, 99). It was postulated that increased transcript levels of these genes, exhibited in the low fertility heifers, may subsequently result in an increased production of PGF$_{2\alpha}$, a known abortifacient (1). Interestingly, prostaglandin-endoperoxide synthase 2 (\textit{PTGS2}) which increases synthesis of PGF$_{2\alpha}$ (37), was up-regulated 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 oestrous cycle (90). Differential expression of these genes between HF and LF heifers may signify premature initiation of luteolysis, which is known to significantly impact in-utero embryo development. However, it is important to recognize \textit{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 to provide a nourishing histotroph for the developing embryo (7). Interestingly, LF animals had an up-regulation of genes \textit{EDN1}, \textit{ITGA6} and \textit{DBI}, all of which have demonstrated involvement in progesterone production in other tissues (32, 38, 39) and \textit{LDLR}, involved in the production of the steroid precursor to progesterone, was up-regulated 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 PGF$_{2\alpha}$ accompanies luteolysis (90). As neither of these metabolites were 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 sub-fertile animals (90). Additional genes
involved in the metabolism of fatty acid and synthesis of their subsequent bi-products, including ALB
(19), PCCB (57), PPARG (88) and LYN (15), were also up-regulated 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 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 to 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. High fertility heifers had increased \textit{PLCB3} expression potentially influencing endometrial receptivity. Furthermore, gene expression of calcium exchange enhancing molecule \textit{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 \textit{SLC22A18}, \textit{SLC25A1}, \textit{SLC25A3}, \textit{SLC25A11}, \textit{SLC25A28} and \textit{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 \textit{SLC2A1} was down-regulated in the endometrium of sub-fertile 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 sub-fertile strains. Furthermore, work by Salilew-Wondim et al. (85) and our laboratory (54) resulted in the discovery of 19 and 5 SLC differentially expressed genes between receptive/HF and non-receptive/LF heifers on day 7 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). Phosphate transporter \textit{SLC25A3}, which is necessary for oxidative phosphorylation of ADP to ATP (76), previously found in bovine heart and liver mitochondria (4) was up-regulated in LF heifers. In addition, mitochondrial citrate transporter \textit{SLC25A1}, which was up-regulated in LF animals, is essential for cytosolic fatty acid and sterol biosynthesis. Intermediates oxaloacetate and acetyl CoA produced from exported citrate are the substrates required for this process (52). \textit{SLC27A4} up-regulated in low conception rate animals, 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 pro-inflammatory disposition negatively influences embryo mortality (85, 100), and suggests a maternal rejection reaction to the embryo.

Transcript abundance of arachidonate 5-lipoxygenase, *ALOX5*, was increased in LF compared to 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 *PTGS*2, was simultaneously up-regulated in LF animals. *PTGS*2 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 which 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 high- and low-fertility strains stimulating pro-inflammatory cytokines and preventing the attraction of immune tolerance promoting uterine natural killer cells in sub-fertile animals, thereby potentially hindering embryo tolerance and attachment in these animals (17, 28).

Chemokine ligand 28 (*CCL28*) was up-regulated 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) also showed that non-receptive 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 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 up-regulated in LF heifers. Interestingly, an abundance of collagen genes, COL4A3, COL4A4, COL11A1 and COLQ, were up-regulated in LF heifers. In addition, decreased expression of type IV collagens was observed in decidua of spontaneously aborting women (3, 49).

Genes ITGA6 and CD82 were both up-regulated in LF heifers. These genes act antagonistically to each other; the former integrin alpha 6, a cellular adhesion molecule, promoting cellular morphogenesis by acting as a receptor for collagen/laminin and the latter suppressing this activity (43). Expression of this receptor is consistent throughout the menstrual cycle (60), and increased during the window of implantation (59). Moreover and in agreement with our results, ITGA6 was differentially expressed between receptive and non-receptive heifers during the luteal phase of the estrous cycle (85). It is therefore suggested that ITGA6 is involved in all three processes: cycle transition, pregnancy and fertility, however its mode of action is yet to be elucidated. Expression of the gene bone morphogenetic protein receptor 2 (BMPR2) was different between LF and HF heifers. It is well established that BMPR2 is involved in endometrial stromal cell decidualisation during the early stages of pregnancy, therefore facilitating implantation (89).

Small Molecule Biochemistry. Genes affecting formation of eicosanoid; ALOX5, CYP4F2 and PTGS2, 6-keto prostaglandin F1 alpha and eicosapentaenoic acid; PTGS2, prostanoid; PTGS2, and
guanine; PNP were collectively up-regulated in LF animals. Eicosanoids, eicosapentaenoic acid and prostanoids play numerous known roles in bovine reproductive tract function (110).

Increased uptake of fat in the endometrium of poor reproductive performing animals, whether from uterine lumen or membrane phospholipids, suggests an increased demand for energy substrate and (or) hormonal precursors. : LDLR (67), PLIN2 (21), PPARG (102) and SLC27A4 (95), involved in promoting lipid uptake were up-regulated in LF heifers. Increased availability of cholesterol or phospholipids as a result of increased lipid uptake, would benefit the production of prostaglandins, thromboxanes and leukotrienes (66). Uterine production of PGF₂α during early gestation possibly contributes to early embryonic mortality (91). Also, increased uptake of fat could be detrimental to embryo development, depleting histotroph lipid levels (92). However, such theories would have to be validated by the analysis of oviductal fluid fatty acid profiles and fatty acid uptake measurements.

Day 14 vs. Day 7. A small number of genes (n=7) were commonly differentially expressed between HF and LF animals on day 7 and day 14 of the estrous cycle. Similarly, Salilew-Wondim et al. (85) found only 2 genes common (GJA1 and SCARA5) among day 7 and day 14 gene expression profiles between receptive and non-receptive heifers. DEGs expressed in both day 7 and day 14 studies included: PCCB, SLC25A24, DAP and COL4A4. Of interest, PCCB; the gene encoding propionyl-CoA carboxylase beta polypeptide, is contained within the blepharophimosis-ptosis-epicanthus inversus syndrome (BPES) locus in humans. There are two types of this congenital defect: BPES I & II. Female infertility differentiates the former from the latter (80). While there were few DEGs common between day 7 and day 14, the pathway analysis software tool IPA identified a number of similarly enriched pathways from both datasets. These pathways included lipid metabolism, tissue morphology and development, molecular transport and inflammation. Furthermore, interactions of genes within these pathways were similar for fertility groups on both days with LF animals experiencing a pro-inflammatory disposition, with altered 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. Important biological pathways involved in endometrial physiology and endometrial mediated embryo survival were identified, which could be incorporated into future studies examining proteomic and metabolomic regulation of uterine mediated fertility. In addition, the expression analysis provides invaluable data on key DEGs which may be included in future SNP discovery analyses, providing potential markers for fertility which could be incorporated into future genomic selection breeding programs.

ACKNOWLEDGEMENTS

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

GRANTS

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DISCLOSURES

The authors have declared that no competing interests exist.
REFERENCES


FIGURE LEGENDS

Figure 1 Timeline detailing major events in experimental period for high/low fertility model.

Figure 2 Classification of DEG according to top 20 molecular and cellular functions, most significantly affected by endometrial related sub-fertility, using IPA.

Figure 3 Network #1; Cell Morphology, Cellular Development, Behaviour. The network is displayed graphically as nodes (genes). The node color intensity indicates the expression of genes; with red representing up-regulation and green, down-regulation in LF versus HF endometrium. The fold value is indicated under each node.

Figure 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 up-regulation and green, down-regulation in LF versus HF endometrium. The fold value is indicated under each node.

Figure 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 up-regulation and green, down-regulation in LF versus HF endometrium. The fold value is indicated under each node.
Blood sampling for progesterone

Day relative to estrus (Day 0)

Rest period

A.I.

Scan

PG1 PG2

28

Rest period

6 weeks

A.I.

Scan

PG1 PG2

28

Rest period

6 weeks

A.I.

Scan

PG1 PG2

0 28

Rest period

3 months

Slaughter

Rest period

........

Blood sampling for progesterone
**TABLES**

*Table 1* Bovine specific oligonucleotide forward and reverse primer sequences (5'-3') and PCR product length.

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<th>Gene Name</th>
<th>Sequence</th>
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<th>Amplicon Size</th>
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| **ALB**   | F: 5'-TCCCTTCGTGAAACCTATGG -3'  
R: 5'-AGGGAGGTCTGGGCTATCAT -3' | NM_180992.2 | 166 |
| **ARG2**  | F: 5'-CCCAGACCTTTGGTGTGATCT -3'  
R: 5'-AGGAAAATCCTGGGAGCTGT -3' | BC133643.1 | 121 |
| **BMPR2** | F: 5'-ACAAATTCAGTGGGCCAGAC -3'  
R: 5'-TTTGCAGCCTGTGTGAAGTC -3' | XM_002685492.1 | 181 |
| **CCL28** | F: 5'-ACTTGGCTGCTGTACATCTTT -3'  
R: 5'-CGATGTGCCCCCTTACTGTT -3' | EF654535.1 | 165 |
| **COL4A3**| F: 5'-GAGTACCCCGGTGTGAAAGGA -3'  
R: 5'-CGCGAAAAACATACCACCT -3' | NM_001166529.1 | 179 |
| **COL4A4**| F: 5'-AAAGGTGACATGGGTGAAGC -3'  
R: 5'-GGAAGCCACTGAGGTATCCA -3' | XM_002685639.1 | 107 |
**CORO1A**  
F: 5'-TCCGATGGAAGATCCTGAC-3'  
R: 5'-ACTGCTCGTGTCCAGTTCCT-3'  
BT025463.1 171

**CRYAB**  
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R: 5'-GGGATGAAAGTATGGCCGAGA-3'  
AF029793.2 146

**EGLN3**  
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**FADS1**  
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R: 5'-TCTTAGCTCACCAGCCACCT-3'  
XM_002699285.1 141

**GTF2A2**  
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R: 5'-GAACCTGAAGGCATTTTGGA-3'  
BC109831.1 163

**ITGA6**  
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R: 5'-AGCCAGGGTTTCTCCCAT-3'  
NM_001109981.1 119

**LDLR**  
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R: 5'-AGGGAACCAGAACGGAACT-3'  
NM_001166530.1 169

**LYN**  
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R: 5'-CGTGTGAAAGTCCAGATGCA-3'  
AB562971.1 185

**NSMAF**  
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ALB, albumin; ARG2, arginase type II; BMPR2, 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
Table 2 Efficiency variables for individual QPCR genes

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Inflammatory Disease

AGER, ALB, ALOX5, ARG2, ATP4A, C6, CCL28, COL11A1, COL4A3, CRYAB, DHODH, ENTPD1, GCNT3, HLA-A, HLA-DQB1, LDLR, LTF, LYN, MUT, NR3C1, ODC1, PPARG, PRDX1, PTGS2, SDC1, STX2

Immune Cell Trafficking

AGER, ALB, ALOX5, C6, CCL28, COL4A3, CORO1A, DMBT1, DPYSL2, G6PC, ITGA6, LDLR, LTF, LYN, MC4R, PLAUR, PLCB3, PPARG, PTGS2, SDC1, SPHK2

Reproductive System Disease

ABCA5, AGER, ALOX5, AMD1, ARG2, ASPH, BMPR2, BRAF, CD82, CLSTN1, EDN1, LIG1, LRP8, LTF, NR3C1, ODC1, PLAUR, PPARG, PSAT1, PTGS2, SLC25A1
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<td><strong>ENDOG, DFFB, DFFA</strong></td>
<td>12.5</td>
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<td>Reelin Signaling in Neurons</td>
<td><strong>ARHGEF4, ITGA6, LYN, LRP8, ARHGEF3</strong></td>
<td>6.1</td>
<td>0.0209</td>
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<tr>
<td>LXR/RXR Activation</td>
<td><strong>LDLR, ABCG4, ARG2, PTGS2</strong></td>
<td>4.3</td>
<td>0.0457</td>
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<tr>
<td><strong>Canonical Metabolic</strong></td>
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<td></td>
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<tr>
<td>Oxidative Phosphorylation</td>
<td><strong>ATP6V0B, NDUFC2, NDUFA6, COX5B, ATP5L, NDUFS2, NDUFB6, ATP4A, SDHC, NDUFA2</strong></td>
<td>6.06</td>
<td>0.0003</td>
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<tr>
<td>Citrate Cycle</td>
<td><strong>SUCLG1, IDH2, SDHC, MDH1, OGDH</strong></td>
<td>8.62</td>
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<td>Arachidonic Acid Metabolism</td>
<td><strong>CYP4F2, CYP2C9, CYP4F3, Cyp2c44, PTGS2, ALOX5</strong></td>
<td>2.75</td>
<td>0.0089</td>
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<tr>
<td>Glycine, Serine and Threonine Metabolism</td>
<td><strong>PSAT1, GCSH, PIPOX, CHKA, PLCB3</strong></td>
<td>3.33</td>
<td>0.0129</td>
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<tr>
<td>Ubiquinone Biosynthesis</td>
<td><strong>NDUFC2, NDUFA6, NDUFS2, NDUFB6, NDUFA2</strong></td>
<td>4.2</td>
<td>0.0129</td>
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<tr>
<td>Methionine Metabolism</td>
<td><strong>TRDMT1, AMD1, DNMT3A</strong></td>
<td>3.75</td>
<td>0.0158</td>
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<tr>
<td>Metabolism</td>
<td>Genes (Bold: down-regulated)</td>
<td>( \Delta \text{FC} )</td>
<td>( q )-value</td>
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<tr>
<td>----------------------------------------------</td>
<td>-------------------------------</td>
<td>-----------------</td>
<td>----------</td>
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<tr>
<td>Pyrimidine Metabolism</td>
<td>\textit{NT5C, POLR3F, DPYS1, ENTPD1, POLR2J, PNP, DHODH}</td>
<td>3.03</td>
<td>0.0224</td>
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<td>Nicotinate and Nicotinamide Metabolism</td>
<td>\textit{NT5C, BRAF, PNP, DFFB, G6PC}</td>
<td>3.68</td>
<td>0.0263</td>
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<td>Urea Cycle and Metabolism of Amino Groups</td>
<td>\textit{GLUD1, ARG2, ODC1}</td>
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<td>Propanoate Metabolism</td>
<td>\textit{SUCLG1, PCCB, MUT, LDHA}</td>
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<td>Glyoxylate and Dicarboxylate Metabolism</td>
<td>\textit{MTHFD2, MDH1}</td>
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<td>Galactose Metabolism</td>
<td>\textit{AKR7A2, G6PC, PFKL}</td>
<td>2.61</td>
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*Bold = genes down-regulated*
Table 5 Networks generated by IPA central to endometrial related sub-fertility

<table>
<thead>
<tr>
<th>Network ID</th>
<th>Top Functions</th>
<th>Molecules in Network</th>
<th>Score</th>
<th>Focus Molecules</th>
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<tbody>
<tr>
<td>1</td>
<td>Cell Morphology, Cellular Development, Behaviour</td>
<td><em>AGER, ALB, ATP2A2, BACE1, CD82, CHP, COL4A3, Collagen type IV, CORO1A, DENR, DFFA, DFFB, DMBT1, DRAM1, ENTPD1, GFPT2, Growth hormone, HISTONE, Immunoglobulin, Integrin alpha 3 beta 1, ITGA6, Laminin, Ldh, LDHA, LRG1, MIR124 (human), MSL1, MYST1, NFkB (complex), NFkB (family), PRDX1, RTN1, STX2, SYNPO, UBE2B</em></td>
<td>43</td>
<td>25</td>
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<tr>
<td>2</td>
<td>Gene Expression, Genetic Disorder, Nephrosis</td>
<td><em>ABLIM1, BTF3L4, CA12, Calmodulin, Calpain, Cbp/p300, CELSR2, Ck2, Collagen(s), DPYSL2, ERK, F Actin, Focal adhesion kinase, GJB1, Gpcr, GPR65, GPR68, GPR137, GTF2A2, GTF2E2, Holo RNA polymerase II, IQCB1, KCNN2, LIG1, Mapk, MC4R, MYO1B, Pkc(s), PLC, PLCB3, POLR2J, PSAT1, RNA polymerase II, SDHC, SPHK2</em></td>
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<td>20</td>
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<tr>
<td>3</td>
<td>3 Organismal Functions, Carbohydrate Metabolism, Lipid Metabolism</td>
<td>AKR7A2, ALOX5, AP3M1, ARG2, BRAF, Calcineurin protein(s), CaMKII, Caveolin, CHKA, CLASRP, Collagen Alpha1, Creb, EDN1, ERK1/2, Integrin alpha V beta 3, LDL, LDLR, LRP, LRP8, Lyn, Mek, NADPH oxidase, Nos, OIP5, Pdgf (complex), PDGF BB, PLAUR, PLIN2, PPARG, PTGS2, RASD1, Rock, SLC25A11, SMPD3, SOS2</td>
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<td>4</td>
<td>4 Cellular Growth and Proliferation, Lipid Metabolism, Small Molecule Biochemistry</td>
<td>14-3-3, Ap1, CCNF, CDC25C, CHAF1A, CYP2C9, CYP4F2, DBI, DOCK10, EGLN3, Estrogen Receptor, FADS1, Fgf, G6PC, GLUD1, hCG, HIST3H3, Histone h3, Histone h4, HPSE, Insulin, LTF, MTCH2, N-cor, Nfat (family), ODC1, PCCB, PFKL, PI3K (complex), PLA2G16, Rxr, SDC1, T3-TR-RXR, THRSP, Vegf</td>
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<tr>
<td>5</td>
<td>5 Ophthalmic Disease, Cell Morphology, Cellular Development</td>
<td>Akt, ARHGEF4, ASGR2, ATP1B3, BMPR2, C6, Caspase, COX5B, CRYAB, DAP, E2f, ENDOG, FSH, HBXIP, HCRT, Hsp70, Hsp90, IgG, IL1, Interferon alpha, Jnk, KCNJ16, Lh, NR3C1, NSF, NSMAF, P38 MAPK, PDCL3, Pka, PSMG2, Ras, Tgf beta, TMEM126A, Tnf, Trypsin</td>
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<td>6</td>
<td>6 Endocrine System Development and Function, Organ Development, Gene Expression</td>
<td>AMBP, AMD1, ARHGEF3, ARHGEF4, ASGR1, CIB2, COL11A1, COL4A3, COLQ, COTL1, CR1, GARS, GCNT3, GFPT2, HNF1A, HNF1B, MTHFD2, MUT, MYO9B, NETO2, NGFR, NME1, PARP3, PCBD1, PCSK6, PDX1, PKN1, PLXNA4, RHOA, RTP3, SAA1, TCF19, TGFB1, TNF, ZNF23</td>
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<td>7</td>
<td>Lipid Metabolism, Small Molecule Biochemistry, Nucleic Acid Metabolism</td>
<td>ACOT1, Acot1, ACOT2, ACOT4, Acot5, ACOT7, ANKZF1, C19orf44, C20orf72, CYP4F2, CYP4F3, DLST, GDAP2, HNF4A, OGDH, ONECUT1, palmitoyl-CoA hydrolase, PERP, PLSCR1, PPT1, PRRG2, PTPN11, SLC22A18, SLC25A1, SLC27A1, SREBF1, SUCLG1, TMEM216, TMIGD1, TP53, tretinoin, TRMT6, VCL</td>
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<td>8</td>
<td>Lipid Metabolism, Small Molecule Biochemistry, Cancer</td>
<td>ABTB1, AGT, APBB1, APBB2, beta-estradiol, C1QBP, CLSTN1, CS, CSTB, CTSH, Cyp2c, Cyp2c44, CYP2J2, CYP4B1, CYP4F2, ECD, EGFR, EWSR1, GFER, GJB1, HNF1B, HNF4A, IFI30, IFT122, KCNN2, MAB21L1, MDH1, PNP, POLR3F, RASL11B, RNF183, RTCD1, SAA1, SEMA3C, ZNF281</td>
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<td>16</td>
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<tr>
<td>9</td>
<td>Gene Expression, Dermatological Diseases and Conditions, Genetic Disorder</td>
<td>AR, ATF7IP, ATP6V0B, BRF1, C1orf51, CARMI, CREB1, DTNB1, FAM49B, GTF2H3, GTF3C4, HLA-A, HLA-DQB1, HPS6, Nc2, NFYB, Pias4, PNRC1, POLR3C, POLR3F, PPARGC1A, RAB11A, RFX5, RNF6, RNF14, SLC25A28, SPATA24, SRSF4, SRSF6, STBD1, TBP, VIPAR, VPS39, VPS33B, WDR89</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td>10</td>
<td>Carbohydrate Metabolism, Small Molecule Biochemistry, Cancer</td>
<td>ASPH, ATP4A, CCRN4L, CDC43, CUL1, DHODH, DPP3, ELOVL6, FBXO4, FBXO31, FBXO33, FBXW9, FBXW11, HHIP, HTT, IL4, ILVBL, LOC100129193, LRRC8C, NFKBIA, norepinephrine, NUPL1, OSBPL10, PC, PCTP, Pias4, PLIN2, PLIN4, PPARG, SKP1, SLAIN1, SLC25A3, SPARR1A, XRCC6, ZC3H18</td>
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<td>11</td>
<td>DNA Replication, Recombination, and Repair, Gene Expression, Cell-To-Cell Signalling and Interaction</td>
<td>\textit{ACTA1, CACNA1S, CISD1, CPNE2, DNA (cytosine-5-)methyltransferase, DNMT3A, EEF2, EEF1A1, EGLN1, EPPK1, GFAP, GRB2, Gsk3, HELLS, IGF1, IL5, IL3RA, IL5RA, MAK16, METTL13, MIR29B (human), MYC, MYCBP2, MYO1B, PFK, PIPOX, PLP1, PSAT1, S100A6, SCPEP1, SRI, SYNCRIPT, TRDML1, WDR44, ZNF217}</td>
<td>18</td>
<td>13</td>
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<tr>
<td>12</td>
<td>Cellular Development, Embryonic Development, Cellular Assembly and Organization</td>
<td>\textit{ACTA1, ALB, ASB8, ASFB1A, CBWD2, CLCA2, DYRK1A, FGF2, FRG1, GFAP, Hat, HMGB1L1, HRAS, Ifi202b, KLF3, MAPKAPK2, MRPL12, MRPL39, PAN2, PAN3, POUSF1, PQLC3, RFWD2, RPL37, Serpina3k (includes others), SNRPN, TCEB1, TEAD2, TEAD4, Tenascin, TEP1, TP53, VGLLI1, ZCCHC8, ZFP57}</td>
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<td>13</td>
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<td>13</td>
<td>Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry</td>
<td>\textit{22(R)-hydroxycholesterol, ABCG4, ABCG5, ABCG8, ACAT1, Aco1, APRT, ascorbic acid, cholesterol, DBI, FOS, FURIN, GCSH, HLA-DQB1, IDH1, IDH2, IKBKG, ITGAX, KBTBD10, KCNK1, Keratin, LAMP2, NFAT (complex), NMI, PGP, PIAS4, PKP2, PRKDC, PRKG2, progesterone, PTS, STAT4, TCP11, TFPI, TSPAN5}</td>
<td>16</td>
<td>12</td>
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<tr>
<td>14</td>
<td>Genetic Disorder, Metabolic Disease, Cardiovascular Disease</td>
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<td>----</td>
<td>----------------------------------------------------------</td>
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<td><strong>ATP5L, CCL28, CNGA1, CNGB1, cyclic AMP, DLG4, GLP2R, GPR65, HSPB6, IFNB1, MAPK1, MARCH9, MT-ND2, MT-ND3, MT-ND5, MT-ND4L, NADH dehydrogenase, NADH2 dehydrogenase, NADH2 dehydrogenase (ubiquinone), NDUFA2, NDUFA6, NDUFA9, NDUFAF1, NDUFB6, NDUFC2, NDUFS2, NDUFS4, NDUFS5, NDUFS6, NDUFS7, NDUFS8, palmitic acid, PNMA2, SLC27A4, TNKS2</strong></td>
<td>16</td>
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Table 6 QPCR validation results

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<tr>
<th>Gene</th>
<th>Microarray FC</th>
<th>Microarray P-Value</th>
<th>QPCR FC</th>
<th>QPCR P-Value</th>
<th>Correlation R</th>
<th>Correlation P-Value</th>
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<td>ALB</td>
<td>-6.73</td>
<td>0.000</td>
<td>-4.35</td>
<td>0.214</td>
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<td>ARG2</td>
<td>2.17</td>
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<td>1.97</td>
<td>0.049</td>
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<td>BMPR2</td>
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<td>1.11</td>
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<td>CCL28</td>
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<td>2.42</td>
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<tr>
<td>COL4A3</td>
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