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Research Article

Do differences in the endometrial transcriptome between uterine horns ipsilateral and contralateral to the corpus luteum influence conceptus growth to day 14 in cattle?†

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Abstract

Embryo transfer to the uterine horn contralateral to the ovary containing the corpus luteum (CL) negatively impacts pregnancy establishment in cattle. Our aim was to compare the transcriptome and ability of the ipsilateral and contralateral uterine horns to support preimplantation conceptus survival and growth to day 14. In experiment 1, endometrial samples from both horns were collected from synchronized heifers slaughtered on day 5, 7, 13, or 16 post-estrus ($n = 5$ per time) and subjected to RNA sequencing. In experiment 2, 10 day 7 in vitro produced blastocysts were transferred into the uterine horn ipsilateral ($n = 9$) or contralateral to the CL ($n = 8$) or into both horns (i.e., bilateral, $n = 9$) of synchronized recipient heifers. Reproductive tracts were recovered at slaughter on day 14, and the number and dimensions of recovered conceptuses were recorded for each horn. A total of 217, 54, 14, and 18 differentially expressed genes (>2-fold change, FDR $P < 0.05$) were detected between ipsilateral and contralateral horns on days 5, 7, 13, and 16, respectively, with signaling pathways regulating pluripotency of stem cells, ErbB signaling pathway, and mTOR signaling pathway amongst the top canonical pathways. Site of embryo transfer did not affect recovery rate (48.0%, 168/350) or length of conceptuses (mean ± SE 2.85 ± 0.27 mm). Although differences in gene expression exist between the endometrium of uterine horns ipsilateral and contralateral to the CL in cattle, they do not impact conceptus survival or length between day 7 and 14.

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Summary Sentence

Differences in endometrial gene expression exist between the ipsilateral and contralateral uterine horns in cattle, but conceptus growth to day 14 is not different between the horns.

Key words: transcriptome profiling, endometrium, RNA-sequencing, conceptus, cattle.

Introduction

Pregnancy loss is a major cause of reproductive failure in cattle. Despite a high fertilization rate (approximately 90%), only 30% or less of high-producing lactating cows calve after a single artificial insemination [1]. Much of this embryonic loss occurs before maternal recognition of pregnancy, encompassing the period between fertilization and day 16 after insemination [2], and is greatest from fertilization to day 7 in high-producing dairy cows [3]. During the preimplantation period, the blastocyst undergoes significant morphological changes, developing sequentially from a spherical to ovoid then tubular and finally filamentous-shaped structure that primarily involves growth of the trophectoderm cells [4]. The critical process of blastocyst elongation is initiated between days 12 and 14, with formation of a filamentous type conceptus by day 16. The uterine endometrium plays a key role in driving the elongation process via secretion and production of histotroph into the uterine luminal fluid (ULF) (see [5–7]). Spatiotemporal alterations in the endometrial transcriptome and histotroph composition are hypothesized to establish uterine receptivity to implantation and, in turn, are pivotal to the success of pregnancy [8]. These modifications are primarily regulated by progesterone (P4) and then influenced by interferon-tau (IFNT) from the elongating conceptus beginning on day 16 [5, 9, 10].

Elevated concentrations of circulating P4 in the period immediately after conception have been associated with advanced conceptus elongation [11–13], increased IFNT production [14], and higher pregnancy rates in cattle and sheep [15–17]. Differences in endometrial tissue P4 concentrations between different regions of the same uterine horn and between the horns ipsilateral and contralateral to the ovary bearing the corpus luteum (CL) during the luteal phase have been reported [18–22], indicating a local effect of the CL on P4 concentrations in the bovine endometrium. Progesterone-associated spatiotemporal changes in the transcriptome of the endometrium [23–25] and protein composition of the ULF [26–29] have been reported in cattle. Several studies have described a local counter-current transfer system through which steroid hormones may be transported from the ovarian vein to the ovarian artery, which is connected to a branch of the uterine artery by a prominent anastomosis in cattle [30, 31] supplying the oviduct and uterine horn (see [32] for review). This system may control the dynamics of the regional expression of P4-stimulated genes generating different molecular microenvironments in the bovine uterus throughout the estrous cycle.

Embryo transfer studies established that the incidence of embryo loss is higher following transfer to the uterine horn contralateral to the ovary containing the CL compared to transfer to the ipsilateral horn [33–35] (see Supplemental Table S1). Whether these differences are manifest in conceptus growth and elongation in the critical window preceding maternal recognition of pregnancy is unknown. Knowledge of differences in gene expression and ULF composition between the uterine horns during the estrous cycle could further enhance our understanding of uterine receptivity and the process of conceptus elongation, key events for the maternal recognition of pregnancy, and, in turn, successful pregnancy establishment. We hypothesized that differences in the endometrial transcriptome between the ipsilateral and contralateral horns throughout the cycle exist, and those differences would be correlated with differences in conceptus elongation after embryo transfer. Specifically, the objectives here were to compare the ipsilateral and contralateral uterine horns in terms of (i) global endometrial gene expression during the luteal phase using RNA-sequencing (RNA-Seq) technology and (ii) the ability to support conceptus growth and elongation.

Materials and methods

All experimental procedures involving animals were approved by the Animal Research Ethics Committee of University College of Dublin and licensed by the Health Products Regulatory Authority, Ireland, in accordance with Statutory Instrument No. 543 of 2012 (under Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes).

Experiment 1

Animal model

The experimental model used has been previously described by Carter et al. [11] and Forde et al. [24]. Briefly, the estrous cycles of cross-bred beef heifers were synchronized using an 8-day intravaginal P4 device (PRID®E, 1.55 g P4, Ceva Santé Animale) with a 2 ml intra muscular injection of a synthetic equivalent of natural gonadotrophin releasing hormone (Ovarelin®, Ceva Santé Animale, equivalent to 100 μg Gonadorelin) administered on the day of PRID insertion. One day prior to PRID removal, all heifers received a 5-ml intramuscular injection of a natural prostaglandin F2 alpha (Enzaprost®, Ceva Santé Animale, equivalent to 25 mg dinoprost) to induce luteolysis. Only those heifers observed in standing estrus (day 0) were used. For tissue collection, heifers were slaughtered at one of four time points during the luteal phase (day 5, 7, 13 and 16 post-estrus). In pregnant animals, these days correspond to key stages of early embryo development: (i) day 5 = 16-cell/early morula-stage embryo (n = 5); (ii) day 7 = blastocyst stage (n = 5); (iii) day 13 = ovoid conceptus at the initiation of conceptus elongation (n = 5); and (iv) day 16 = filamentous conceptus at the time of maternal recognition of pregnancy (n = 5). Blood samples were taken from the jugular vein for subsequent P4 measurement from all heifers twice daily from day 0 to day 7 after estrus, and once daily thereafter until slaughter. The P4 data were reported by Carter et al. [11].

Tissue collection

The experimental design including details of tissue collection and RNA-Seq analysis is shown in Figure 1A. On each day, the reproductive tract of each heifer was recovered and placed on ice within 30 min of slaughter. The uterine horn ipsilateral (IPSI) and contralateral (CONTRA) to the CL was flushed with 20 ml of PBS containing 5% fetal calf serum (FCS). After flushing, both uterine horns were opened longitudinally and strips of intercaruncular endometrium (approximately 300 mg) were carefully removed from the cranial third of each uterine horn and immediately immersed in 1:5 w/v of RNAlater

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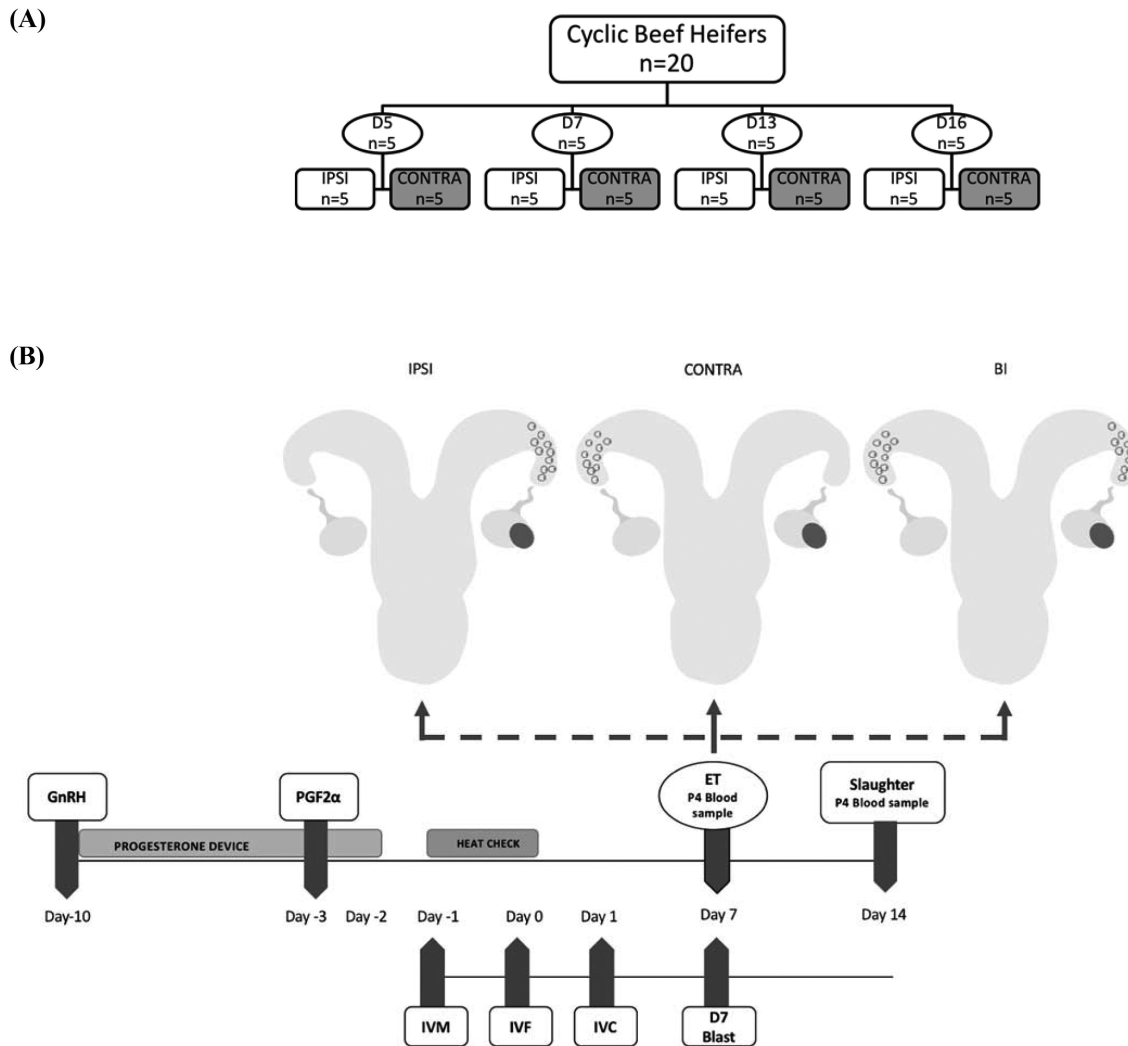

Figure 1. (A) Experimental design for tissue collection and RNA-Seq analysis. Only heifers observed in standing estrus (day 0) were used. Heifers were assigned to slaughter on either day (D) 5, 7, 13, or 16 of the estrous cycle (n = 5 per day) and endometrium samples from ipsilateral (Ipsi) and contralateral (Contra) horns of each animal were recovered. (**B**) Experimental design for the generation of day 14 embryos. GnRH = gonadotrophin releasing hormone; PGF2α = prostaglandin F2α; ET = embryo transfer; P4 = progesterone; IVM = in vitro maturation; IVF = in vitro fertilization; IVC = in vitro culture; blast = blastocyst. Fresh day 7 (n = 10) in vitro produced blastocysts were transferred to either (i) both horns of the same synchronized heifer (bilateral transfer, BI; n = 9 heifers) or to either (ii) the ipsilateral (IPSI; n = 9 heifers) or (iii) the contralateral (CONTRA; n = 8 heifers) horn of a synchronized heifer (unilateral transfer).

(Sigma). Endometrial samples were then transported on ice back to the laboratory and stored at 4°C for 24 h. Excess RNAlater was removed and samples were placed into new tubes and stored at –80°C until they were analyzed.

RNA extraction and RNA-sequencing

Total RNA extraction was carried out as described previously by Forde et al. [24]. Briefly, total RNA was extracted from approximately 100 mg of endometrial homogenate using Trizol reagent (Invitrogen, Carlsbad, CA) per the manufacturer's instructions. On-column DNase digestion and RNA clean-up was performed using the Qiagen mini kit (Qiagen). The quantity and quality of RNA were determined using the Nano Drop 1000 (Thermo Fisher Scientific) and the Agilent Bioanalyzer (Agilent Technologies), respectively. Only samples with an RNA integrity number > 8.0 were used for RNA-Seq analysis. RNA concentration was determined by quantitative high-sensitivity RNA analysis on the Fragment Analyzer instrument (DNF-472; Advanced Analytical Technologies, Inc.). RNA library preparation and sequencing was conducted by the University of Missouri DNA Core facility as described previously [36].

Data analysis

Raw sequences (fastq) were subjected to quality control (QC) using windowed adaptive quality trimming approach implemented in fqtrim (https://ccb.jhu.edu/software/fqtrim/). The quality reads were then mapped to the bovine reference genome UMD3.1 using Hisat2 mapper (https://ccb.jhu.edu/software/hisat2/), which is a fast and sensitive alignment program for next-generation sequencing data [37]. Read counts mapping to each gene were determined from the mapping data using FeatureCounts [38]. Differential expression analysis between sample groups was performed by robustly fitting the expression data to a generalized linear model using edgeRrobust [39]. Functional prediction of genes was assessed by mapping the

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genes to KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. Significant impact of differentially expressed genes (DEGs) on signaling pathways was assessed by analysis of Pathway Regulation Score (PRS) as described previously [40]. This topology-based pathway analysis was conducted using ToPAseq package [41]. The gene ontology (GO) analysis was performed using PANTHER (pantherdb.org). All statistical procedures were performed in R.

Experiment 2

Animal model

The experimental design is illustrated in Figure 1B. Thirty cross-bred beef heifers (Charolais or Limousin cross), 23.7 ± 2.6 months old and 612.6 ± 26.1 kg (mean ± SD), were used. All animals were housed indoors on slats for the duration of the experiment (August-September 2015) and were fed a diet consisting of grass silage and maize silage supplemented with a standard beef ration. All heifers were ultrasound scanned immediately before initiation of the synchronization protocol using a portable ultrasound machine (Easi-Scan™; BCF Technology Ltd) fitted with a 4.5 to 8.5-MHz linear array transducer in order to examine the reproductive tract. Only those animals free of reproductive diseases detectable by ultrasonography were used. Recipient heifers were scanned immediately before embryo transfer for CL assessment (size and side). Estrous cycles were synchronized as described in experiment 1. Only those heifers observed in standing estrus (day 0) were used as embryo recipients 7 days later. Fresh day 7 (n = 10) in vitro produced blastocysts were pooled before being loaded into straws for transcervical transfer into one of three uterine sites: (i) both horns (bilateral transfer, BI; n = 9 heifers); (ii) the ipsilateral horn (IPSI; n = 9 heifers); or (iii) the contralateral horn (CONTRA; n = 8 heifers). In all cases, the embryos were transferred as far forward (cranially) into each uterine horn without causing any trauma.

Blood samples from the coccygeal vessels were collected for P4 measurement on the day of embryo transfer (day 7) and the day of slaughter (day 14). Reproductive tracts were recovered, transported to the laboratory within 2 h of slaughter, and gently dissected in order to completely isolate each uterine horn. Each horn was independently flushed with PBS containing 5% FCS (Sigma). The number, dimensions (length and width), and site (ipsilateral or contralateral horn) of recovered embryos were recorded; in addition, each CL was dissected from the ovarian tissue, measured, and weighed.

In vitro embryo production

Blastocysts were produced in vitro as described previously [42]. Briefly, immature cumulus-oocyte complexes (COCs) were obtained from ovaries of heifers and cows slaughtered at a local abattoir. The COCs were washed and matured for 24 h in groups of 50 in 500 μL TCM-199 supplemented with 10% (vol/vol) FCS and 10 ng/mL epidermal growth factor (Sigma) at 39°C under an atmosphere of 5% CO_2 in air with maximum humidity. Matured COCs were fertilized using sperm at a concentration of 1×10^6 spermatozoa/mL. Semen from the same bull was used throughout. Gametes were co-incubated at 39°C in an atmosphere of 5% CO_2 in air with maximum humidity. At approximately 20 h post insemination, presumptive zygotes were denuded by gentle vortexing and cultured in synthetic oviduct fluid droplets (25 μL; 25 embryos per droplet). Blastocysts for transfer were removed from culture on day 7 (IVF = day 0).

Progesterone assays

After collection, blood samples were refrigerated (4°C) for 12 to 24 h before being centrifuged at 1500 × g at 4°C for 20 min. Serum was separated and stored at –20°C until analysis of P4 concentration by solid-phase radioimmunoassay using a PROG-RIA-CT Kit (DIAsource ImmunoAssays S.A.) according to the manufacturer's instructions. The sensitivity of the assay was 0.05 ng/mL. The interassay coefficients of variation for QC sample were 3.89% (low), 6.51% (medium), and 5.33% (high). The intra-assay coefficients of variation were 9.73% (low), 9.51% (medium), and 8.65% (high).

Statistical analysis

Data were checked for normality and homogeneity of variance by histograms, qqplots, and formal statistical tests as part of the UNIVARIATE procedure of SAS (version 9.1.3; SAS Institute, Cary, NC, USA). Data that were not normally distributed were transformed by raising the variable to the power of lambda. The appropriate lambda value was obtained by conducting a Box–Cox transformation analysis using the TRANSREG procedure of SAS. The transformed data were used to calculate P values. The corresponding least squares means and standard error of the nontransformed data are presented in the results for clarity. Conceptus related data and progesterone concentrations (on day 7 and day 14, respectively) were analyzed using mixed model ANOVA (PROC MIXED of SAS). The model had experimental treatment (IPSI, CONTRA or BI) as a fixed effect, and heifer within treatment was included as a random effect. Differences between treatments were determined by F tests using type III sums of squares. The PDIFF command incorporating the Tukey test was applied to evaluate pairwise comparisons between treatment means. Values were considered to be statistically significantly different when $P < 0.05$ and considered a tendency when $P < 0.10$.

RESULTS

Experiment 1

Overall transcriptional changes in the endometrium of the ipsilateral and contralateral uterine horns

RNA sequencing of IPSI and CONTRA endometrial samples generated on average 51 million reads per sample. Raw read counts, the number of reads obtained after QC, and mapping rates to reference genome of individual samples are listed in Supplemental Table S2. On average, 49 million reads were obtained after performing QC, and they were used for mapping to the bovine reference genome (approximately 95.9% of mapping rate per sample). The read counts were obtained for each protein coding gene, and they were subjected to a principal component analysis for global survey of transcriptome variation (Figure 2A).

The IPSI and CONTRA had distinct patterns of gene expression on each day (i.e., day 5, 7, 13, and 16) with day 5 exhibiting the most variation and day 16 being least variable (Figure 2). This was mirrored in the numbers of DEGs identified between IPSI and CONTRA on day 5 (n = 217), day 7 (n = 54), day 13 (n = 14), and day 16 (n = 18 DEGs) (Table 1). At Day 5, the transcriptome of the CONTRA horn was similar between heifers whereas that of the IPSI horn was more variable (Figure 2A). At Day 7, the difference in gene expression between the two horns diminished, though differences were still obvious (Figure 2A). In contrast at Days 13 and 16, there was little or no distinction in the endometrial transcriptome of the IPSI and CONTRA horns.

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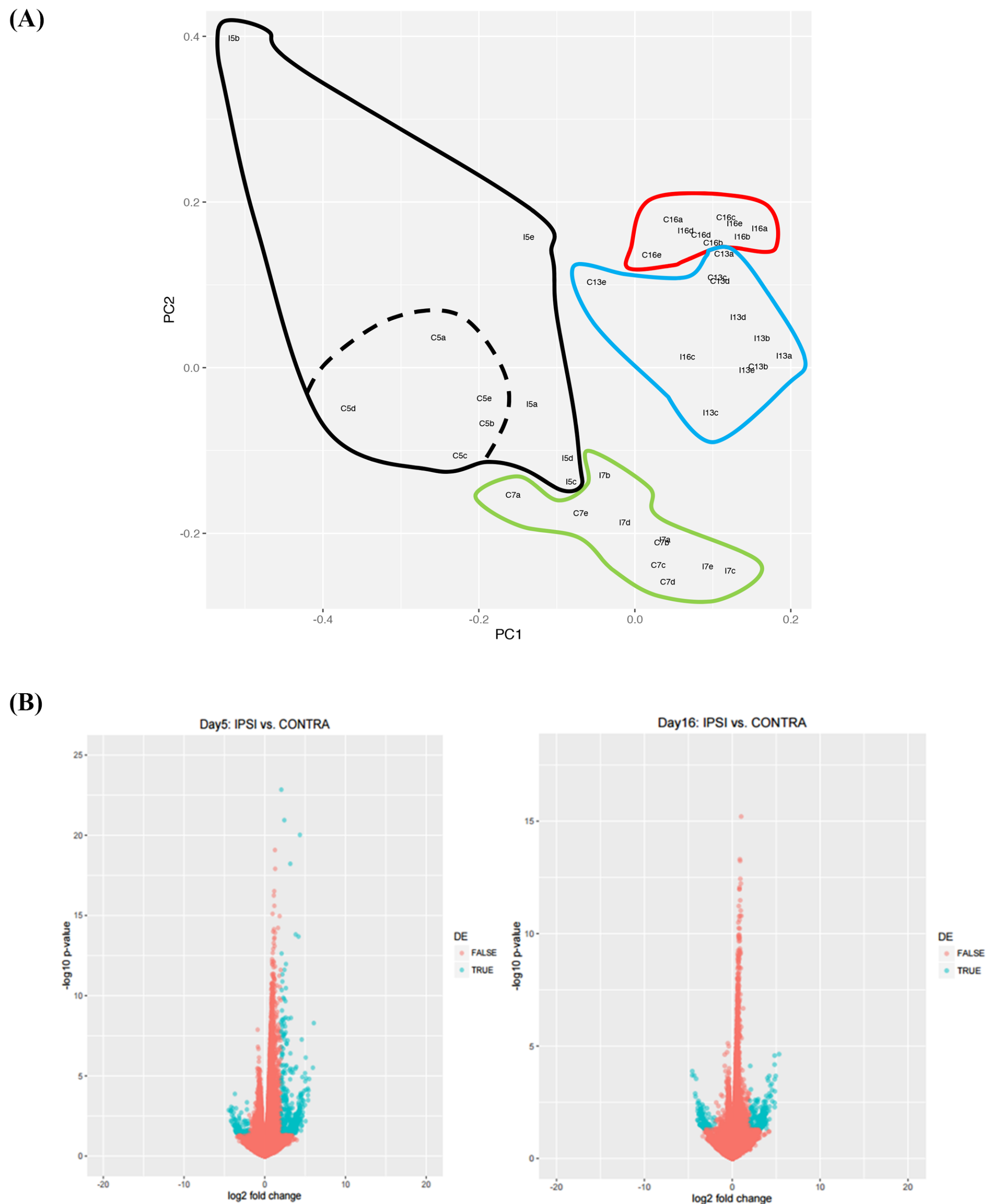

Figure 2. **(A)** Principal component analysis of endometrial gene expression variation between uterine horns on different days of estrous cycle. The plot shows that IPSI (I) and CONTRA (C) have distinct gene expressions on each day (day 5, black line; day 7 green line; day 13 blue line; day 16 red line), indicating the source of greatest variation in the overall transcriptional profile is time. Further, IPSI exhibit significantly greater variation in gene expression (solid black line) compared to CONTRA (dotted black line) on day 5. **(B)** Volcano plots of gene expression changes between uterine horns (IPSI and CONTRA) on day 5 (left) and day 16 (right) of the estrous cycle. Genes in red show nonsignificant changes in expression and those in blue are differentially expressed (DE) genes that $\geq$2 log fold changes in expression with false discovery rates $\geq$0.05.

Differentially expressed genes between IPSI vs. CONTRA

To identify genes that were significantly differentially expressed between IPSI and CONTRA, edgeR-robust analysis was conducted with read count data for samples of the two groups at each given day of the estrous cycle. The number of genes showing statistically significant changes in expression levels (based on FDR < 0.05) are listed in Table 1. Across all four time points, among the DEG, more genes (~10-fold) had higher expression in IPSI relative to CONTRA. The most up- and down-regulated DEGs at each day (>2-fold change, FDR $P < 0.05$) are shown in Table 2 and Supplemental Table S3A–D.

To understand the functional implication of DEGs between IPSI and CONTRA, we performed GO analysis (Supplemental Table S4a and b). One of the most enriched ontologies was protein deubiquitination, which was the top over-represented function among DEGs at days 5, 7, and 13. At day 16, microtubule anchoring was the most over-represented function among the significant DEGs.

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Table 1. Number of significantly differentially expressed genes (increased vs decreased expression) identified from RNA-seq data analysis.

	FDR $P \leq 0.05$		FDR $P \leq 0.05$ and FC ≥ 2	
	IPSI vs CONTRA		IPSI vs CONTRA	
Day	Increased	Decreased	Increased	Decreased
5	1964	624	201	16
7	1000	98	44	10
13	536	44	13	1
16	640	43	14	4
Ipsilateral endometrium				
	FDR $P \leq 0.05$		FDR $P \leq 0.05$ and FC ≥ 2	
Day	Increased	Decreased	Increased	Decreased
5 vs 7	2675	2134	414	296
7 vs 13	2464	2958	387	633
13 vs 16	227	318	44	71
Contralateral endometrium				
	FDR $P \leq 0.05$		FDR $P \leq 0.05$ and FC ≥ 2	
Day	Increased	Decreased	Increased	Decreased
5 vs 7	1763	1584	292	213
7 vs 13	2574	2982	476	657
13 vs 16	279	563	56	114

FDR: false discovery rate; FC: fold change in expression.

Temporal changes in the endometrial transcriptome between IPSI and CONTRA

The main driver of gene expression changes in the endometrium was time (i.e., day). More genes were differentially expressed between days (e.g., day 5 vs day 7 vs day 13 vs day16) than between IPSI vs CONTRA on a given day. Comparison of gene expression between day 5 vs day 7, day 7 vs day 13, and day 13 vs day 16 indicated that the largest temporal alteration in the transcriptome coincided with the largest time interval (day 7 vs day 13) in both uterine horns (Figure 3A). Lists of top 30 up- and downregulated DEGs between day 5 vs day 7, day 7 vs day 13, and day 13 vs day 16 in IPSI and CONTRA horns are provided in Supplemental Tables S5 and S6, respectively.

To elucidate the temporal changes in endometrial gene expression specific to each uterine horn, we compared the list of DEGs in IPSI and CONTRA within time interval (i.e., DEGs in IPSI between day 5 and day 7 were compared with DEGs in CONTRA for the same time interval, etc.). During the early luteal phase (day 5 vs day 7), there were 431 (46%) and 226 (24.1%) DEGs unique in IPSI and CONTRA, respectively (Figure 3B), while 279 (29.8%) were shared in both uterine horns. In contrast, despite the higher number of DEGs between day 7 and day 13, almost 50% of them (719) were common to both horns, while 301 (21%) and 414 (28.9%) were unique to IPSI and CONTRA, respectively (Figure 3B). During the late luteal phase, there were 48 (22%) and 103 (47.2%) DEGs in IPSI and CONTRA, respectively, with 67 (30.7%) in both uterine horns (Figure 3B).

Signaling pathway impact analysis

In an attempt to gain insight into the underlying biology of DEGs between uterine horns throughout the luteal phase of the estrous cycle, a topology-based pathway analysis approach was used. The top five canonical pathways associated with DEGs between IPSI and CONTRA were involved in signaling pathways regulating pluripotency of stem cells (73/138), progesterone-mediated oocyte maturation (55/89), endometrial cancer (31/51), ErbB signaling pathway (50/87), and mTOR signaling pathway (36/61) (Supplemental Table S7). Signaling pathway impact analysis revealed that signaling pathways regulating pluripotency of stem cells showed the highest pathway regulation score (nPRS) when IPSI was compared to CONTRA irrespective of day (Figure 4). Specifically, on day 5 the eight most significantly (q value < 0.05) dysregulated pathways between IPSI and CONTRA were (from greatest to least nPRS, Supplemental Table S8) pluripotency of stem cells (29.35), mTOR (7.24), serotonergic synapse (5.47), melanoma (5.41), long-term depression (4.45), prostate cancer (3.97), glioma (2.20), and endometrial cancer (2.07). On day 7 they were prostate cancer (11.50), pluripotency of stem cells (9.79), insulin resistance (9.38), fatty acid degradation (9.18), melanoma (8.64), RIG-I-like receptor (5.67), mTOR (5.64), and adipocytokin (5.05). On day 13 only pluripotency of stem cells (4.67) was dysregulated. Finally, on day 16 the top three dysregulated pathways were pluripotency of stem cells (14.15), thyroid cancer (9.17), and mTOR (7.22).

To identify those pathways significantly (q value < 0.05) that were also dysregulated across the cycle in each uterine horn, pathway analysis was carried out for the DEGs between day 5 vs day 7, day 7 vs day 13, and day 13 vs day 16 (Supplemental Table S8). The pathways most dysregulated in IPSI between days 5 and 7, 7 and 13, and 13 and 16 were mainly involved in metabolism (carbohydrates, lipid, and amino acid metabolism), organismal system (immune system), and, human diseases and metabolism (carbohydrates), respectively. In contrast, the pathways most dysregulated in CONTRA were associated with cellular processes and organismal system (immune system) in the early luteal phase, with metabolism (amino acid metabolism carbohydrates, and lipid) between day 7 vs day 13, and with metabolism (carbohydrates) in the late luteal phase.

Experiment 2

Conceptuses were recovered from all heifers in the BI group (9/9) and 8/9 and 7/8 in the IPSI and CONTRA groups, respectively. Recovery rate of conceptuses on day 14 (conceptuses recovered as a proportion of embryos transferred) was similar in all three groups (BI = 45.0%; IPSI = 53.3%; CONTRA = 48.7%; $P > 0.05$) (Table 3). Amongst

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Table 2. Gene ID, Entrez ID, gene name, logarithm of fold change (log FC), and *P* values of differentially expressed genes with the highest fold difference in the endometrium of cyclic heifers on days 5,7,13, and 16.

Gene ID	Entrez ID	Gene Name	log FC	*P* value
Day 5				
LOC100297621	100297621	dystrophin	6.073	5.1E-09
ZNF589	789895	zinc finger protein 589	5.963	3.1E-06
PAQR9	522374	progestin and adipoQ receptor family member IX	5.505	1.6E-05
LOC104968948	104968948	uncharacterized LOC104968948	5.420	8.0E-05
EDA2R	100301324	ectodysplasin A2 receptor	5.375	3.3E-04
LOC100848945	100848945		5.348	1.6E-04
LOC104972015	104972015		5.340	6.2E-05
MUC19	100140959	mucin 19, oligomeric	5.118	1.4E-05
LOC104969569	104969569		5.101	7.4E-05
LOC104971153	104971153		5.073	7.2E-07
LOC104971020	104971020		5.016	2.2E-05
LOC104975350	104975350		4.993	6.8E-06
LOC104971219	104971219		4.986	6.6E-04
LOC100297222	100297222	cytochrome b-c1 complex subunit 7	4.865	8.7E-05
BOSTAUV1R416	100139634	vomeronasal 1 receptor bosTauV1R416	4.820	3.7E-04
UPK3A	100336102	uroplakin 3A	−2.210	1.2E-03
TRNAE-UUC-87			−2.916	1.9E-03
PADI3	508160	peptidyl arginine deiminase, type III	−3.153	4.3E-03
CPNE6	508059	copine VI (neuronal)	−3.185	3.1E-03
LOC785133	785133		−3.547	1.0E-03
HIST1H3J	540148	histone cluster 1, H3j	−3.698	1.3E-04
CD163L1	338056	CD163 molecule-like 1	−3.859	3.6E-03
LYPD2	790087	LY6/PLAUR domain containing 2	−3.861	1.9E-03
MAG	540576	myelin associated glycoprotein	−3.954	1.0E-03
LOC104973034	104973034		−4.075	2.7E-03
LOC104971717	104971717		−4.163	8.6E-04
LRRIQ4	784301	leucine-rich repeats and IQ motif containing 4	−4.170	1.6E-03
LOC104973719	104973719		−4.176	1.4E-03
LOC104974917	104974917	uncharacterized LOC104974917	−4.268	1.5E-03
PSMB11	538873	proteasome (prosome, macropain) subunit, beta type, 11	−4.472	1.4E-03
Day 7				
LOC101905162	101905162	uncharacterized LOC101905162	5.011	1.0E-03
HOXD13	100336775	homeobox D13	4.954	3.5E-04
LOC104968876	104968876	uncharacterized LOC104968876	4.899	2.2E-05
LOC101907562	101907562	GATA zinc finger domain-containing protein 14-like	4.803	1.6E-04
LOC104972242	104972242		4.598	8.1E-04
LOC104975944	104975944		4.440	1.7E-03
LOC104969603	104969603		4.415	6.2E-04
LOC104975892	104975892	uncharacterized LOC104975892	4.395	4.8E-04
LOC104973355	104973355		4.367	1.1E-03
LOC104970245	104970245		4.330	7.6E-04
GRIFIN	528362	galectin-related inter-fiber protein	−3.849	1.1E-03
LOC505183	505183	histone H2B type 1	−3.904	1.7E-03
CLDN6	511318	claudin 6	−4.070	9.3E-04
PCDH8	513346	protocadherin 8	−4.071	1.7E-03
LOC104974902	104974902	uncharacterized LOC104974902	−4.078	1.5E-03
LOC101905008	101905008	uncharacterized LOC101905008	−4.171	5.0E-04
LOC104973274	104973274		−4.246	9.4E-04
LOC101902082	101902082		−4.632	7.3E-04
C18H19orf81	100140272	chromosome 18 open reading frame, human C19orf81	−4.813	5.2E-05
DLK1	281117	delta-like 1 homolog (Drosophila)	−4.901	1.4E-03
Day 13				
PCDHB1	504206	protocadherin beta 1	4.830	1.8E-04
LOC104975807	104975807		4.823	2.7E-04
LOC104971550	104971550	AMP deaminase 1-like	4.429	5.8E-04
IFNK	100138192	interferon, kappa	4.401	3.9E-04
LOC104969747	104969747		4.106	5.6E-04
GPR97	529961		4.077	5.0E-04
LOC101905569	101905569		3.931	4.9E-04
LOC101907661	101907661	uncharacterized LOC101907661	3.868	7.1E-04
ZNF589	789895		3.407	5.2E-04
POU5F2	104968986	POU domain class 5, transcription factor 2	2.755	1.3E-05

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Table 2. Continued

Gene ID	Entrez ID	Gene Name	log FC	*P* value
TRIM72	528804	tripartite motif containing 72, E3 ubiquitin protein ligase	−4.474	8.7E-04
Day 16				
LOC100296102	100296102	T-cell receptor alpha chain V region 2B4	5.331	2.3E-05
C11H2orf61	100335365	chromosome 11 open reading frame, human C2orf61	4.952	2.1E-04
LOC104973290	104973290		4.852	1.0E-03
LOC104974072	104974072		4.833	3.0E-04
LOC530990	530990	olfactory receptor 52Z1	4.825	2.7E-05
LOC104975364	104975364		4.822	6.7E-05
ERBB4	522419	erb-b2 receptor tyrosine kinase 4	4.547	9.1E-04
LOC104971979	104971979	uncharacterized LOC104971979	4.485	4.8E-04
LOC104973272	104973272		4.342	5.8E-04
LOC104968931	104968931	uncharacterized LOC104968931	4.219	2.1E-04
AARD	616184	alanine and arginine rich domain containing protein	−4.106	2.9E-04
CHRNB3	521702	cholinergic receptor, nicotinic beta 3	−4.333	2.0E-04
JSRP1	509378	junctional sarcoplasmic reticulum protein 1	−4.547	1.3E-04
LOC104973970	104973970	uncharacterized LOC104973970	−4.550	2.0E-04

For full list of genes see Supplemental Table S3.

Table 3. Conceptus recovery data on day 14 after the transfer of day 7 IVP blastocysts to the uterine horn(s) ipsilateral (group I) or contralateral to the CL (group C) or to both horns (bilateral) of synchronized recipients (10 embryos transferred per horn per recipient).

		No of embryos transferred			No of embryos recovered (recovery rate %)		
	No of animals	IPSI	CONTRA	Total	IPSI	CONTRA	Total
Bilateral	9	90	90	180	41 (45.5%)	40 (44.4%)	81 (45%)
Unilateral:							
I	9	90	0	90	46 (51.1%)	2[a] (4.2%)	48 (53.3%)
C	8	0	80	80	7[a] (17.9%)	32 (40%)	39 (48.7%)

[a]Embryos which had migrated from the horn into which they were transferred to the opposite horn, from where they were collected.

the embryos transferred in the IPSI group, two conceptuses from two different heifers (22.2% of recipients; 2/9) were recovered from the contralateral horn (4.2% of recovered embryos; 2/48) while seven migrated embryos (7/39; 17.9%) from four different heifers (50% of the recipients; 4/8) were collected from the ipsilateral horn in the CONTRA group.

One heifer from the CONTRA group was removed from further analysis as an outlier as all of the conceptuses recovered from her were excessively long (n = 10; mean length >30 mm, which is longer than the longest conceptuses recovered amongst the other 158 conceptuses from all heifers). Such an outlier may have arisen due to the time of transfer relative to ovulation as differences in the timing of the early rise in progesterone can significantly advance conceptus elongation [12, 13].

In terms of morphology, 83.5% (132/158) of all conceptus recovered were ovoid with 13.3% (21/158) tubular and 3.2% (5/158) being filamentous. The number of conceptuses and range in conceptus lengths recovered from each group are shown in Figure 5 and on a per heifer basis in Supplemental Figure S1. Day 14 conceptus length was not different following BI transfer (2.27 ± 0.49 mm) compared to unilateral transfers (3.03 ± 0.42 mm, $P = 0.24$). Overall, transfer to the ipsilateral uterine horn resulted in similar conceptus length and width (2.24 ± 0.35 mm and 1.09 ± 0.07 mm, respectively; n = 89) to transfer to the contralateral horn (2.91 ± 0.38 and 1.01 ± 0.07 mm, respectively; n = 69; $P > 0.10$). No differences were found in either P4 concentrations on day 7 and day 14 or in the CL weight on day 14 in recipients heifers into which embryos were transferred.

Discussion

Spatiotemporal changes in the endometrial transcriptome [23, 24, 43] and histotroph composition [27–29] are hypothesized to be essential for establishment of uterine receptivity to implantation and, in turn, are pivotal to the success of pregnancy in cattle. Main findings of the present studies were that: (1) day of the estrous cycle contributed to the greatest variation in the endometrial transcriptome; (2) there were many more altered genes between the uterine horns ipsilateral and contralateral to the CL in the early (day 5 and 7) as compared to late (day 13 and 16) luteal phase; (3) signaling pathways regulating pluripotency of stem cells were highly dysregulated when both uterine horns were compared, regardless of the day of luteal phase; and (4) the differences in endometrial gene expression between the uterine horns ipsilateral and contralateral to the CL were not associated with a reduced ability of the uterus to support conceptus survival or development to day 14 after embryo transfer on day 7.

In line with previous studies conducted in our laboratory examining endometrial gene expression during the estrous cycle (either pregnant or cyclic heifers, with high, normal, or low P4 concentrations) [24, 44, 45], the factor that contributed most to altered endometrial gene expression was the day of the cycle. Moreover, and also in agreement with those previous studies, the comparison of endometrial tissue samples collected within a short interval (day 5 vs day 7 and day 13 vs day 16), regardless of uterine horn, resulted in a smaller number of DEGs compared to samples collected from

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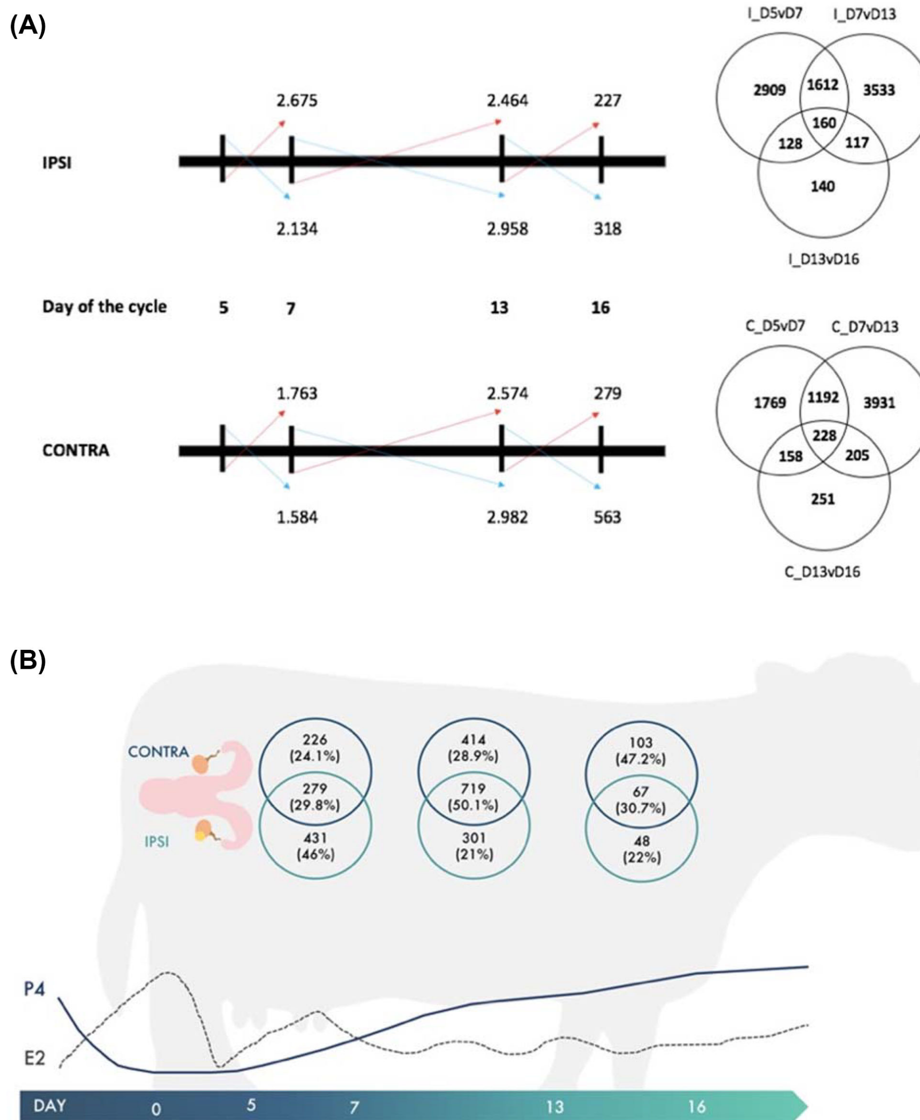

Figure 3. (**A**) Temporal changes in endometrial gene expression (FDR $\leq$ 0.05) in the uterine horns ipsilateral (IPSI) and contralateral (CONTRA) to the corpus luteum. The numbers of upregulated and downregulated genes between days 5 vs 7, 7 vs 13, and 13 vs 16 in each horn are shown. Venn diagrams illustrate the numbers of genes that were specific to each time interval and genes that were common to two or all three time intervals. (**B**) Temporal changes in endometrial gene expression (FDR $\leq$ 0.05; FC $\geq$ 2) specific to each uterine horn and in common between both horns throughout the estrous cycle. IPSI = ipsilateral uterine horn; CONTRA = contralateral uterine horn; P4 = progesterone; E2 = estradiol.

more distant days (day 7 vs day 13), highlighting the importance of the temporal changes that occur during the estrous cycle.

Comparison of the endometrial transcriptome between the uterine horns ipsilateral and contralateral to the ovary bearing the CL on a given day of the luteal phase revealed the greatest number of DEGs on day 5 and then a progressive decrease throughout the luteal phase. Moreover, the ipsilateral horn exhibited the highest variation in gene expression compared to the contralateral horn on any given day. These changes may be regulated by P4 and dependent on its concentration in the endometrium and are consistent with the finding that endometrial tissue P4 concentrations were higher in the cranial part of the ipsilateral horn than in the contralateral horn during the luteal phase [20]. In addition, endometrial P4 concentrations in both uterine horns tended to be highest at the beginning of the luteal phase and then gradually decreased throughout the cycle [20]. This is in agreement with other studies that reported differences in P4 concentrations between diverse regions of the reproductive tract in cattle [18, 19, 21, 22].

We are aware of reports that periovulatory endocrine events drive the endometrial tissue toward the receptive state during early diestrus (e.g. [8, 46]). In that model, authors concluded that during diestrus the bovine endometrial transcriptional profile was regulated by the periovulatory endocrine milieu. However, it is not clear if the differences in the endometrial transcriptome were related to estrogen or P4 or a combination of both. It is possible that the elevation in estradiol associated with first wave dominant follicle could interact with the effect of P4. However, given that the differences in gene expression between both horns declined from day 5 onwards, despite a second and probably third dominant follicle, the evidence for a strong effect of estradiol at the times we studied is weak.

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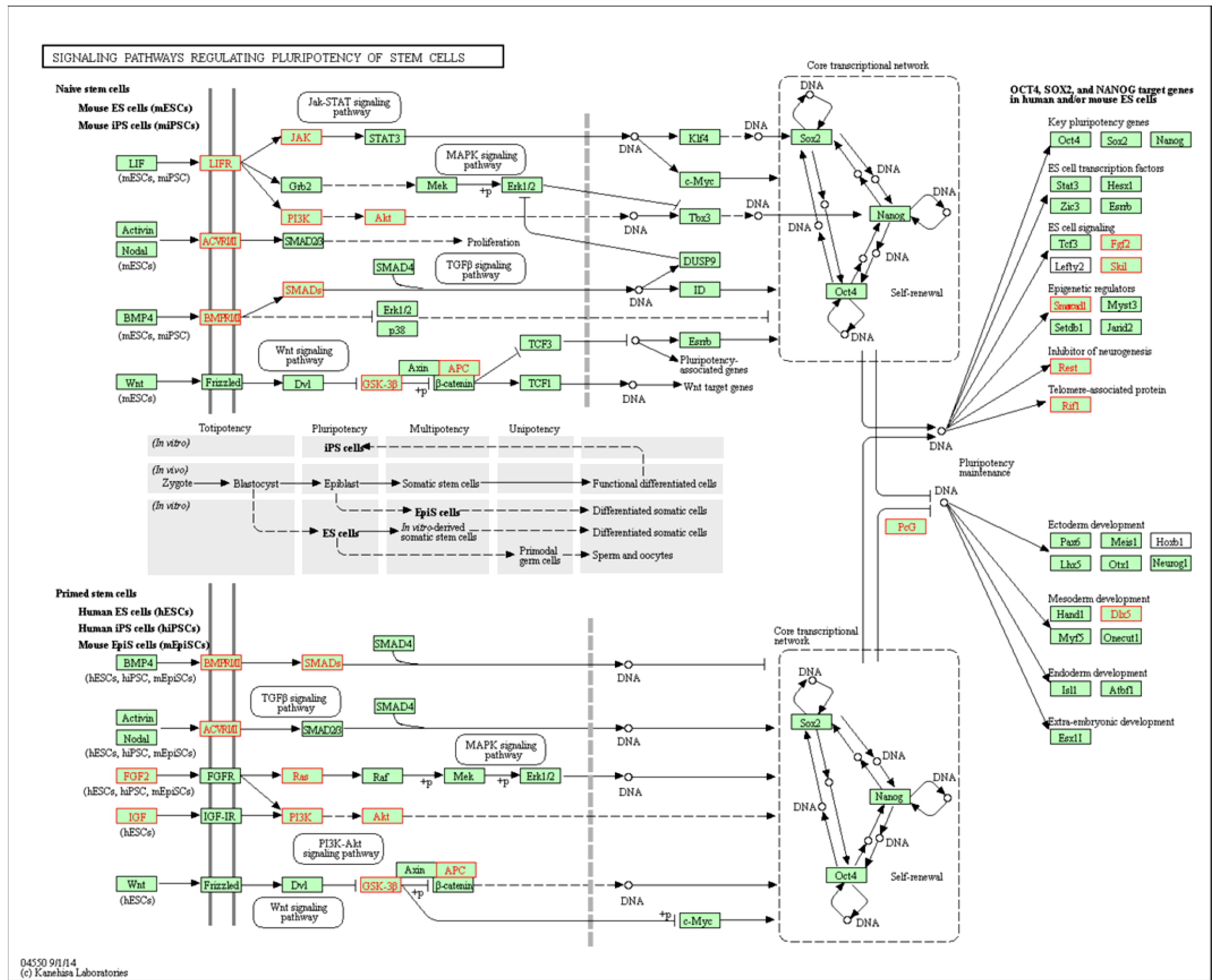

Figure 4. An example of a pathway enriched by differentially expressed genes between IPSI and CONTRA. Genes shown in red are differentially expressed.

In the present study, *PAQR9*, a receptor that belongs to the class II progestin and adipoQ receptor (PAQR) family, was one of the genes with the largest fold differences between uterine horns on day 5. This gene is associated with regulation of rapid P4 signaling in the nervous system [47]. Therefore, in our study, its expression may be altered in the presence of greater P4 concentrations in the ipsilateral horn on day 5. Consistent with our results, previous studies have also reported that other genes associated with steroid concentrations such as nuclear progesterone receptor (*PGR*), progesterone receptor membrane component 1 (*PGRMC1*) and 2 (*PGRMC2*), and estrogen receptor 1 (*ESR1*) and 2 (*ESR1*) were not differentially expressed between the ipsilateral and contralateral uterine horns in cattle [20, 48] or in sheep [49]. Additionally, no differences in the expression of *PGR*, *ESR1,* or *ESR2* were detected between bovine oviduct epithelial cells derived from oviducts ipsilateral and contralateral to the CL [50]. In contrast, others [23] found greater abundance of transcripts for *PGR* and oxytocin receptor (*OXTR*) in the horn contralateral to the CL and greater *PGRMC1* expression was reported during the early luteal phase in the cranial part of the ipsilateral horn compared with the contralateral horn [20]. Consistent with this finding, while we did not observe a difference in *PGRMC1* expression between horns on a given day, we observed an increase in *PGRMC1* expression in the ipsilateral horn compared to the contralateral horn between day 5 and day 7. Steroid hormones such as P4 and other molecules (i.e., growth factors, transcription factors, or micro RNAs) may be transported from the ipsilateral ovary to the endometrium by a local counter-current transfer system [30, 31]. In addition, the blood flow toward the uterus increases during the periestrous period in cattle [51]. This may explain greater differences in the endometrial transcriptome between the uterine horns during the early luteal phase, and especially the distinct pattern in the ipsilateral horn on day 5. Dynamic and regional changes in the bovine uterus throughout the estrous cycle may be controlled by this system, generating different molecular microenvironments and/or gradients that could be essential to the successful establishment of pregnancy. Therefore, results confirm previous speculation based on comparison of gene expression [50, 52] between ipsilateral and contralateral oviducts, and gene expression [43] and protein secretions [28, 29] between ipsilateral and contralateral endometrial tissue, that differences in the transcriptome pattern between bovine uterine horns exist during the luteal phase of the estrous cycle.

The top five canonical pathways associated with those genes altered between the uterine horns during diestrous were involved in signaling pathways regulating pluripotency of stem cells, P4-mediated

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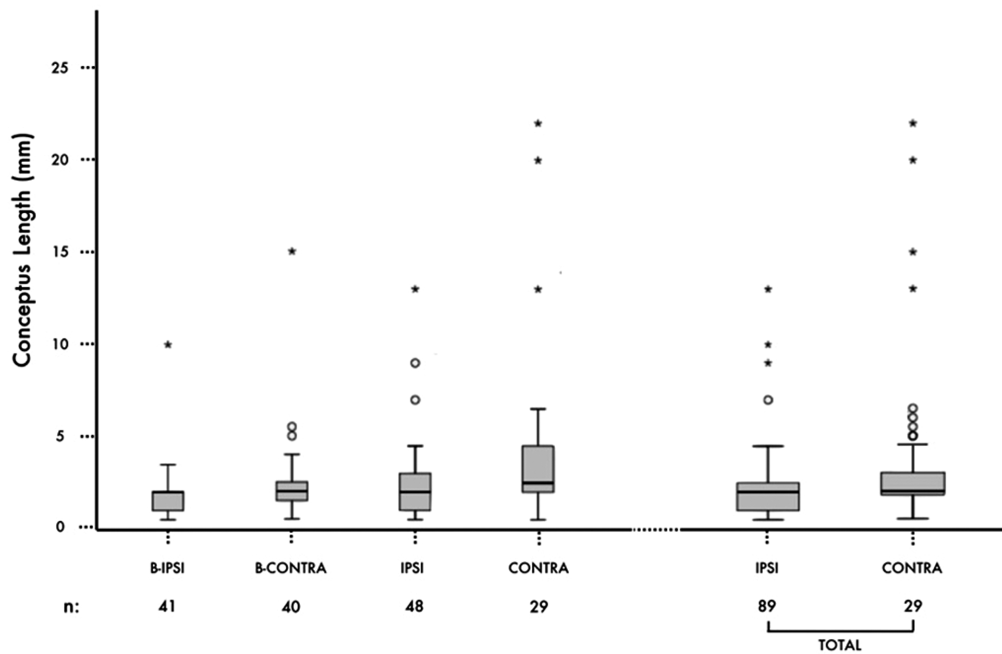

Figure 5. Box plots showing the variation in conceptus length recovered from uterine horns on day 14 of pregnancy. Each box plot represents the length (mm) of the recovered conceptuses in each group. B-IPSI: embryos recovered from the ipsilateral uterine horn to the ovary bearing the corpus luteum after the bilateral transfer of 10 embryos to each uterine horn (n = 41); B-CONTRA: embryos recovered from the contralateral uterine horn after the bilateral transfer of 10 embryos to each uterine horn (n = 40); IPSI: embryos recovered from the ipsilateral uterine horn after the unilateral transfer of 10 embryos to this horn (n = 48); and CONTRA: embryos recovered from the contralateral uterine horn after the unilateral transfer of 10 embryos to this horn (n = 29). The two box plots on the right represent the length of all recovered conceptuses in the study from either the ipsilateral (IPSI; n = 89) or contralateral (CONTRA; n = 69) uterine horns. Note that circles and asterisks represent the outliers and extreme outliers (more than three times the height of the boxes), respectively.

oocyte maturation, endometrial cancer, ErbB signaling pathway, and mTOR signaling pathway. Moreover, the impact of DEGs on signaling pathways assessed by pathway analysis revealed that signaling pathways regulating pluripotency of stem cells showed the highest dysregulation score when IPSI was compared to CONTRA, irrespective of day. These signaling systems are complex processes of signal transduction, and may play a crucial role in the coordination of the reproductive events associated with embryo–maternal interaction that leads to successful pregnancy establishment. WNT signaling is a critical pathway that regulates embryo development [53] and embryo–uterine communication and placentation [54, 55]. The inactivation of Wnt/beta-catenin signaling pathway in endometrial cancer pathways, altered in this study, promotes the downregulation of target genes involved in proliferation, cell adhesion, and inhibition of apoptosis (reviewed by [56]), and also prevents implantation [57]. Akt/PI3K (phosphatidylinositol 3-kinase) is another important signaling pathway involved in endometrial cancer and its activation occurs through binding ligands such as cytokines, growth factors or insulin (reviewed by [56]). Akt, also known as protein kinase B, promotes protein synthesis, glucose metabolism, and cell proliferation [56]. A key downstream target of PI3K is mTOR [58]. Growth factors such as insulin-like growth factor 1 (IGF-1), upregulated in the present study, and insulin block the tuberous sclerosis complex 2 (TSC2), which in turn inhibits mTOR protein [56], consistent with the downregulation of the gene in the ipsilateral horn in our study. mTOR signaling pathway positively regulates cell metabolism, growth, proliferation, and survival [59]. Moreover, mTOR mRNA and protein increased in pregnant mice, with a peak on day 5 and a reduction thereafter [60], suggesting that mTOR participates in the process of embryo implantation in rodents. It has also been reported that activation of the Akt/PI3K/mTOR signaling pathway enhances pregnancy outcome in rats [61]. Overactivation of mTOR signaling, however, leads to endometrial hyperplasia in women and mice [62]. Taken together, these studies suggest that differences in mTOR in the endometrium may play an important role during early pregnancy, especially during implantation, although its role remains unclear. ErbB signaling was significantly dysregulated in the present study. This pathway plays a vital role in early pregnancy, predominantly via the epidermal growth factor receptor (EGFR/ERBB1), which was upregulated in the ipsilateral horn compared to the contralateral in the present study. Egfr ablation in mice had no apparent defects during the preimplantation period [63]; however, there was a failure of implantation and decidualization. Despite ruminants having a relatively noninvasive type of placentation, the endometrial stroma undergoes structural changes and angiogenesis [64, 65]. Although samples were collected only from intercaruncular endometrium in this study, it has been reported that some of these changes during implantation were similar in both caruncular and intercaruncular uterine epithelium and stromal endometrium [66]. All these findings suggest that defects in uterine receptivity or embryo/uterine signaling can cause implantation failure or early pregnancy loss.

Signaling pathways regulating pluripotency of stem cells were significantly dysregulated when comparing ipsilateral to the contralateral uterine horn during the entire luteal phase, but it was at the beginning of diestrous (day 5), the day on which the embryo enters the uterus, when most changes occurred. Stem cells are capable of self-renewal or of producing multiple cell linages [67]. Adult stem cells have been found in many organs and tissues, residing in niches [68]. Uterine endometrium exhibits high regenerative potential as demonstrated by its adaptation to changes that occur during

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gestation, the post-partum period, and the early estrous cycle. The recent identification of mesenchymal stem cells in the bovine [69–71] and ovine endometrium [72] suggests that these cells play a key role in maintaining cyclicity and endometrial function. In agreement with our results, greater expression of pluripotent markers in the ipsilateral than in the contralateral horn have been reported [71], suggesting ovarian hormones influence these characteristics. Furthermore, the same authors revealed a decrease in pluripotent transcription factor expression in older compared with younger cows [71]. Recently, a progressive decrease in stem cell markers in endometrium from healthy cows compared to those exhibiting subclinical or clinical endometritis has been reported [69], while those markers were more highly expressed in cows with adenomyosis [73]. Taken together, these findings showing that a decrease in the expression of stem cell markers, or in the actual number of stem cells, is related to factors affecting pregnancy rate (uterine horn, cow age, and uterine health status) strongly support the hypothesis that uterine stem cells play a key role in many physiological and pathological reproductive events and highlight the utility of our model to study these events.

The findings that differences in endometrial gene expression exist between the uterine horns ipsilateral and contralateral to the CL during the luteal phase were not associated with reduced ability of the uterus to support conceptus survival and growth (in terms of conceptus length) on day 14. Data from embryo transfer studies indicate that transfer to the uterine horn contralateral to the ovary containing the CL significantly increases embryonic loss [33–35, 74]. We suggest three plausible reasons for a lack of difference in conceptus length: (i) the day chosen for conceptus recovery was not the most appropriate to detect slight differences in embryo development in terms of conceptus length (due to the small size of the recovered conceptuses); (ii) no significant differences in P4 concentrations at the site of embryo transfer; or (iii) differences in pregnancy rate between ipsilateral and contralateral embryo transfer are due to an increase in embryo losses after day 14. Day 14 was chosen because we have previously shown differences in conceptus length on this day in control and progesterone-manipulated heifers [45, 75]. In addition, in a previous study in beef heifers the majority of the embryonic loss occurred before day 14 [76]. Furthermore, previous personal experience has shown that in the case of multiple transfers of embryos as carried out here, separating rapidly elongating conceptuses after day 14 can be extremely difficult. We acknowledge that the use of multiple embryo transfer is associated with some issues related to interpretation and cannot rule out that the presence of up to 10 times the normal mass of conceptus tissue in the endometrium altered the uterine environment in a way that overcame deficiencies in the contralateral horn that may have compromised pregnancy if only one embryo was transferred.

In routine commercial embryo transfer, the embryo is deposited as cranial as possible in the horn ipsilateral to the CL. Depending on the difficulties encountered with an individual animal during this procedure, however, the embryo may be placed at any site less cranial in the ipsilateral horn without a detrimental effect on pregnancy rate [77]. For example, Newcomb et al. [78] reported that fetal survival at day 42 was similar when embryos were transferred into the base of the ipsilateral horn, base of the contralateral, or tip of the contralateral horn, but all were lower than when the embryos were transferred into the tip of the ipsilateral horn. This is consistent with the lack of differences in either P4 concentration or in *PGR*, *PGRMC1*, or *PGRMC2* mRNA expression in the middle part of both uterine horns [20]. In the same study, endometrial tissue P4 concentrations in the cranial portion were higher in the ipsilateral than the contralateral horn. It is possible, therefore, that conceptuses assessed in the current study developed until day 14 of the estrous cycle under an environment of similar P4 concentrations regardless the CL site.

One could hypothesize that the lower pregnancy rate observed after embryo transfer to the contralateral horn is due to the inability of a conceptus in the contralateral horn to maintain the CL unless it migrates to the ipsilateral uterine horn; if this was the case one would expect most loss to occur before or at the time of maternal recognition of pregnancy (day 16). However, Christie et al. [33] indicated that embryonic death after contralateral embryo transfers occurred after day 24. In that study, although animal numbers were low, a reduction in pregnancy rate was observed between day 24–26 (14/20 pregnant heifers) and day 42 (13/40 pregnant heifers). In a separate study, the loss of single embryo from day 27 to 107 of gestation was less in the ipsilateral horn (2 out of 14) compared with the contralateral (12 out 14) [79]. When multiple embryos (n = 10) were transferred into the contralateral horn (unilateral embryo transfer), a greater occurrence of embryo migration was observed. Transuterine migration is a rare phenomenon in cattle [80, 81] and seems to be influenced by the number of embryos present. In agreement with our results, several studies have reported an increase in the incidence of migration when more than one embryo was initially located in the same uterine horn [81–83]. Moreover, our results further support the observation that migration rate is higher if embryos are transferred exclusively to the contralateral horn [82]. Although embryo migration is postulated to be a crucial approach for assuring adequate space in multiple pregnancy [84], the physiological mechanisms involved are unknown.

In conclusion, significant differences in endometrial gene expression exist between the uterine horns ipsilateral and contralateral to the CL in cattle. Dynamic and regional changes in the bovine uterus throughout the estrous cycle may be controlled by a countercurrent transfer system that generates different molecular microenvironments and/or gradients that could be essential to the successful establishment of pregnancy. Under the conditions of this study, we did not see evidence that these changes translate into a difference in conceptus survival and growth to day 14. However, knowledge generated here regarding the most significant dysregulated signaling pathways supports the utility of the ipsilateral vs contralateral model for understanding embryonic losses in the periimplantation and/or placentation period.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplemental Figure S1. Box plot showing the variation in conceptus length on day 14 of pregnancy for each recipient. Each box plot represents the length (mm) of the recovered conceptuses from each individual recipient, identified with the acronym of the group in which the heifer was enrolled and a number (e.g. B-IPSI 1). The box plot on the right represents the length of all recovered conceptuses (n = 158) from all recipients yielding embryos (n = 23). B-IPSI: embryos recovered from the ipsilateral uterine horn to the ovary bearing the corpus luteum after the bilateral transfer of 10 embryos to each uterine horn; B-CONTRA: embryos recovered from the contralateral uterine horn after the bilateral transfer of 10 embryos to each uterine horn; IPSI: embryos recovered from the ipsilateral uterine horn after the unilateral transfer of 10 embryos to this uterine horn; CONTRA: embryos recovered from the contralateral uterine horn

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after the unilateral transfer of 10 embryos to this uterine horn. Note that points and asterisks represent the outliers and extreme outliers (more than three times the height of the boxes), respectively.

Supplemental Table S1. Review table regarding pregnancy outcomes after the embryo transfer to the ipsilateral, contralateral or bilateral uterine horn bearing the corpus luteum in cattle.

Supplemental Table S2. Raw read counts (read quality), number of reads obtained after quality control (reads mapped) and mapping rates to reference genome of individual samples.

Supplemental Table S7. Top five canonical pathways associated with differentially expressed genes (DEGs) between IPSI and CONTRA.

Supplemental Table S8. Significant dysregulation of signaling pathways of differentially expressed genes. nPRS is the score for dysregulation of the pathway. The q value, representing the corrected significance levels, for each of the listed pathways was $q < 0.05$.

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