Location relative to the corpus luteum affects bovine endometrial response to a conceptus

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Abstract

In cattle, embryo transfer into the uterine horn contralateral to the corpus luteum results in a higher incidence of pregnancy loss compared to transfer into the ipsilateral horn. We have previously reported temporal changes in the endometrial transcriptome during the estrous cycle which differ between uterine horns. The objective of this study was to compare the transcriptomic response of endometrium from the ipsilateral and contralateral horns to an elongating conceptus. Cross-bred beef heifers ($n=16$) were synchronized and either used to generate day 14 conceptuses following the transfer of *in vitro*-produced blastocysts or to obtain day 14 endometrial explants. Conceptuses were recovered on day 14 by post-mortem uterine flushing, placed individually on top of explants collected from the ipsilateral (IPSI-D14) or the contralateral (CONTRA-D14) uterine horn of cyclic heifers, and co-cultured for 6 h. The response to a conceptus was markedly different between uterine horns, with 61 and 239 differentially expressed genes (DEGs; false discovery rate <0.05) in the ipsilateral and contralateral horns, respectively, compared to their controls. Direct comparison between IPSI-D1 and CONTRA-D14 revealed 32 DEGs, including *CXCL11*, *CXCL10*, *IFIT2*, *RSAD2* and *SAMD9*. Gene Ontology analysis of these 32 genes revealed ten enriched biological processes, mainly related to immune response and response to an external stimulus. These data indicate that the endometrial response to the presence of a conceptus varies between uterine horns in the same uterus and may contribute to the higher incidence of pregnancy loss following embryo transfer to the contralateral horn.

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Introduction

Uterine receptivity in ruminants is established through spatial and temporal changes of the endometrial transcriptome and subsequent uterine luminal fluid (ULF or histotroph) composition (Lonergan & Forde 2014). Those changes are mainly regulated by progesterone (P4) from the corpus luteum (CL) and conceptus-derived interferon tau (IFNT), which blocks the endometrial luteolytic mechanism (Forde & Lonergan 2012, Brooks *et al.* 2014).

The role of P4 in uterine receptivity is unequivocal (Forde *et al.* 2009, Lonergan *et al.* 2016, Spencer *et al.* 2016). Low circulating P4 concentrations in the first week after ovulation are associated with under-developed conceptuses (Forde *et al.* 2012) with an altered transcriptomic signature (Barnwell *et al.* 2016) and a lower likelihood of establishing pregnancy (Diskin *et al.* 2006, Wiltbank *et al.* 2014). On the other hand, elevated concentrations of circulating P4 in the period immediately after conception have been associated with

advanced conceptus elongation (Carter *et al.* 2008), increased IFNT production (Mann & Lamming 2001) and, in some cases, greater pregnancy rates in cattle and sheep (Ashworth *et al.* 1989, Stronge *et al.* 2005, McNeill *et al.* 2006).

Endometrial tissue P4 concentrations are higher in the uterine horn ipsilateral to the CL than in the contralateral horn (Weems *et al.* 1988, Takahashi *et al.* 2016), especially during the early luteal phase (days 5 and 6) (Takahashi *et al.* 2016). Those differences have been associated with an increased incidence of embryo loss following transfer to the contralateral horn compared to transfer to the ipsilateral horn (Christie *et al.* 1979, Del Campo *et al.* 1983, Izaiké *et al.* 1988), most of which occurred between days 24–42 (Christie *et al.* 1979).

The bovine endometrium responds differently to cloned vs IVF- or AI-derived conceptuses (Bauersachs *et al.* 2009, Mansouri-Attia *et al.* 2009), as well as to conceptuses produced *in vivo* or *in vitro* (Mathew *et al.* 2018) or age-matched conceptuses of different lengths (Sánchez *et al.* 2019). In a previous study from our

group, we reported differential gene expression in the endometrium between the ipsilateral and contralateral horns on days 5, 7, 13 and 16 of the estrous cycle (Sánchez *et al.* 2018), indicating that the environment in both horns is potentially different. However, whether the endometrium from the uterine horn ipsilateral or contralateral to the CL responds differently to an elongating conceptus is not known. Therefore, the objective of the present study was to compare the local response of the ipsilateral and contralateral endometrium to a day 14 conceptus. We hypothesized that the endometrium from the ipsilateral and contralateral uterine horns would respond differently to the presence of a conceptus, which might partly explain the reported differences in embryo loss. In order to disentangle the effect of a potentially compromised conceptus (e.g. a conceptus developed in the contralateral horn) from the effect of a compromised endometrium, conceptuses were generated under 'normal' conditions (in the horn ipsilateral to the CL) and, following recovery, were placed on top of explants from either the ipsilateral or contralateral horn of cyclic heifers.

Materials and methods

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

In vitro embryo production

Immature cumulus-oocyte complexes (COCs) were obtained by aspirating follicles (3–8 mm) from the ovaries of heifers and cows collected at a local abattoir. *In vitro* maturation, fertilization and culture were carried out as previously described (Rizos *et al.* 2002). Briefly, only grade 1 and 2 COCs were selected, washed and matured for 24 h in 500 µL TCM-199 supplemented with 10% (vol/vol) fetal calf serum (FCS) and 10 ng/mL EGF in groups of 50 COCs at 38.8°C under an atmosphere of 5% CO₂ in air with maximum humidity. Matured COCs were washed and transferred in the same groups into fertilization medium (Tyrode medium with 25 mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg/mL fatty acid-free BSA and 10 µg/mL heparin-sodium salt (184 U/mg heparin; Calbiochem)) and inseminated with frozen-thawed Percoll-selected bull sperm (GE Healthcare Biosciences) at a concentration of 1×10^6 sperm/mL. Gametes were co-incubated at 38.8°C in an atmosphere of 5% CO₂ in air with maximum humidity. Approximately 20 h after insemination, presumptive zygotes were denuded of surrounding cumulus cells and accessory sperm by gentle vortexing, washed and transferred into 25 µL droplets of culture medium (synthetic oviduct fluid supplemented with 5% FCS) under mineral oil in

groups of 25 embryos per droplet. Embryos were cultured until day 7 (day 0 = day of *in vitro* fertilization) at 38.8°C under an atmosphere of 5% CO₂, 5% O₂ and 90% N₂ with maximum humidity.

Animal model

The experimental design is illustrated in Fig. 1A. Cross-bred beef heifers ($n=16$) were randomly allocated to one of the following groups: embryo recipients ($n=10$) used to generate elongated conceptuses or endometrial donors ($n=6$) used to generate endometrial explants. The reproductive tract of all heifers was examined for normality before the experiment using a portable ultrasound machine (Easi-Scan™; BCF Technology Ltd, Dundalk, Ireland). Heifers were synchronized as previously described by Sánchez *et al.* (2018) using an 8-day P4-releasing intravaginal device (PRID®E, 1.55 g P4, Ceva Santé Animale, Libourne, France). Briefly, on the day of the PRID insertion, all animals were given 2 mL of a synthetic equivalent of natural gonadotrophin releasing hormone (GnRH; Ovarelin®, Ceva Santé Animale, equivalent to 100 µg Gonadorelin) by i.m. injection. One day prior to PRID removal, all heifers received a 5 mL i.m. injection of a natural prostaglandin F2 alpha (PGF2α; Enzaprost®, Ceva Santé Animale, equivalent to 25 mg Dinoprost) to induce luteolysis. Only those heifers observed in standing estrus ($n=14$) were used. Day 0 was assigned as the day of the expected ovulation, which occurs approximately 28 h after the start of standing estrus (Walker *et al.* 1996, Valenza *et al.* 2012, Randi *et al.* 2018). Recipient heifers ($n=9$) were scanned immediately before embryo transfer for assessment of CL size and location. On day 7, *in vitro*-produced blastocysts ($n=15$ per recipient) were transferred into the ipsilateral horn. All heifers were slaughtered on day 14 to recover elongating conceptuses or endometrial tissue for explant culture. Reproductive tracts were recovered, transported to the laboratory on ice (for explants) or at ambient temperature in a polystyrene box (for conceptuses) within 2 h of slaughter. The uterine horns of the recipient heifers were independently flushed with PBS containing 5% FCS. The number and dimensions (length and width) of recovered conceptuses were recorded. The endometrium of the endometrial donor heifers ($n=5$) was processed for explants as described.

Endometrial explant procedure

Intercaruncular endometrial explants were prepared as described by Borges *et al.* (2012). Briefly, once at the laboratory, reproductive tracts ($n=5$) were removed from the ice, trimmed of connective tissue and both horns were isolated. Holding the dissected end closed, the outside of each uterine horn was washed with PBS containing 1% Antibiotic/Antimycotic (ABAM; Gibco, Thermo Fisher Scientific). Then, the horns were opened longitudinally along the anti-mesometrial side and the endometrium was washed with Hank's Balanced Salt Solution (HBSS; Gibco, Thermo Fisher Scientific) supplemented with 1% ABAM. Uterine explants ($n=4$ per heifer, i.e., two from the ipsilateral and two from the contralateral uterine horn; see Fig. 1) were removed using an 8 mm biopsy punch (Kruuse, Langeskov, Denmark) from the cranial third of each horn

Figure 1 Experimental design: (A) synchronization protocol, *in vitro* embryo production and embryo transfer for the generation of day 14 conceptuses. IVM=*in vitro* maturation; IVF=*in vitro* fertilization; IVC=*in vitro* culture; (B) endometrial explants ($n=20$) were recovered from the uterus of donor's heifers ($n=5$). On the right, a summary of the experimental groups with number of replicates for each and a summary of DEGs between groups. Increased and decreased transcripts are indicated in red and blue, respectively. *One of the explants did not have sufficient RNA quality for sequencing and was therefore removed from further analysis.

and placed into a 35-mm dish with HBSS with 1% ABAM. The myometrium was carefully removed with a scalpel, and the explants were washed again in HBSS with 1% ABAM. Afterwards, they were transferred into the culture wells in 1 mL of RPMI medium (Gibco, Thermo Fisher Scientific) with 1% ABAM and incubated in 5% CO₂ and air at 38.8°C for 1 to 4 h before use. At this point, the medium of all the explants was completely replaced with fresh medium. A single day 14 conceptus was placed on top of the explants from the uterine horn ipsilateral (IPSI-D14; $n=5$) or contralateral (CONTRA-D14; $n=5$) to the CL, with the help of an insert with a 12 μ m pore size (Millipore). We have previously demonstrated that direct contact between the embryo and the endometrium was not necessary to detect a response of the endometrium (Passaro *et al.* 2018). As a control, explants from the ipsilateral (IPSI-CTRL; $n=5$) or contralateral (CONTRA-CTRL; $n=5$) uterine horn were incubated with medium only.

The experimental groups and replicates are summarized in Fig. 1. After 6 h of culture, the inserts with the conceptuses were removed and all explants were snap frozen and stored at -80°C for further analysis.

RNA extraction

Total RNA extraction and purification was carried out as described by Passaro *et al.* (2018). Briefly, ~ 50 mg of endometrial tissue was homogenized in 1.0 mL of TRIzol (Molecular Research Center, Cincinnati, OH, USA) using a steel bead and the Qiagen tissue lyzer (2 $\times$ 120 s at maximum speed). On-column RNA purification was performed using the Qiagen RNeasy mini kit (Qiagen) as per the manufacturer's instructions. RNA was quantified using a NanoDrop-ND1000 Spectrophotometer (Thermo Fisher Scientific) and all samples were shown to have 260/280 nm ratio greater than 1.8. RNA

integrity number (RIN) was determined using the RNA 6000 chip on the Agilent 2100 Bioanalyzer (Agilent Technologies) and the average RIN $\pm$ s.d. was 6.62 ± 0.3 .

RNA sequencing analysis

RNA library preparation and sequencing were conducted by the University of Missouri DNA Core Facility as previously described (Moraes *et al.* 2018). Briefly, libraries were constructed following the manufacturer's protocol with reagents supplied in Illumina's TruSeq Stranded mRNA Sample Prep Kit. The polyadenylated mRNA was purified from total RNA and fragmented. Double-stranded cDNA was generated from the fragmented RNA and the index-containing adapters were ligated. The final construct of each purified library was evaluated using the Fragment Analyzer instrument, quantified with the Qubit fluorimeter using the Quant-iT HS dsDNA Reagent Kit (Invitrogen) and diluted according to Illumina's standard sequencing protocol for sequencing on an Illumina NextSeq 500 sequencer. All raw and processed data from this study have been submitted to the Gene Expression Omnibus for public access.

Alignment of sequences and analysis of differential gene expression

The raw sequences (fastq) were subjected to adapter removal and quality trimming using Trimmomatic (Bolger *et al.* 2014). The quality reads were then mapped to the bovine reference genome ARS-UCD1.2 using Hisat2 mapper which is a fast and sensitive alignment program of next-generation sequencing data (Kim *et al.* 2015). The sorted binary alignment maps along with the NCBI gene annotation of the ARS-UCD1.2 assembly were subjected to FeatureCounts (Liao *et al.* 2014) to quantify read counts of genes for each sample. Differential expression

analysis between sample groups was performed by robustly fitting the expression data to a weighted generalized linear model using edgeR robust (Zhou *et al.* 2014). Gene ontology (GO) was determined using DAVID (<https://david.ncicrf.gov>).

RNA sequencing validation using quantitative real-time PCR

We selected nine genes to validate the RNA sequencing (RNA-seq) results with quantitative real-time PCR (qRT-PCR): interferon-induced very large GTPase 1-like (*LOC783920*), C-X-C motif chemokine ligand 11 (*CXCL11*), C-X-C motif chemokine ligand 10 (*CXCL10*), interferon-induced protein with tetratricopeptide repeats (IFIT-2) (*IFIT2*), sterile alpha motif domain containing 9 (*SAMD9*), chemokine (C-C motif) ligand 8 (*CCL8*), cystatin E/M (*CST6*), radical S-adenosyl methionine domain containing 2 (*RSAD2*) and 2',5'-oligoadenylate synthetase 1, 40/46 kDa (*OAS1Y*). Genes for qRT-PCR were selected based on three criteria: being differentially expressed between at least two groups, biological significance (all interferon stimulated genes, ISGs) and fold change. Primer sequences were obtained from the literature or were designed based on Gen-Bank Ref Seq mRNA sequences using the National Center for Biotechnology Information tool, Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). The protocol followed for the cDNA preparation and qRT-PCR has been previously described by Passaro *et al.* (2018). For each sample, cDNA was prepared from 1000 ng of total RNA using the High Capacity cDNA RT Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The purified cDNA was then diluted in RNase- and DNase-free water up to a volume of 300 μ L. Duplicate qRT-PCR assays were performed in a total volume of 20 μ L, containing 10 μ L FastStart Universal SYBR Green Master (Roche Diagnostics Ltd.), 1.2 μ L forward and reverse primer mix (300 nM final concentration), 2.6 μ L

Table 1 Gene symbol, accession number, primers sequence and amplicon size for all genes analyzed by quantitative real-time PCR.

Gene symbol	Accession number	Primer sequence (5'–3')	Amplicon size	Reference
<i>LOC783920</i>	XM_015474717.2	GGATCGGCATTCTGACTGT ACTTCTCCGAGCATCTCTTGG	112	Primer-Blast
<i>CXCL11</i>	NM_001113173.1	AAGCATGAGTGTGAAGGGCA ACCGTCCGCCTTTGAACATA	92	Primer-Blast
<i>CXCL10</i>	NM_001046551.2	CTGCCACGTGTGCGAGATTA TGCCTCTTCCGTGTTCCGAG	145	Primer-Blast
<i>IFIT2</i>	XM_002698356.5	GAGAAGTGCACGGCAATCAT TCCCTTACCAAGTCCAGGT	99	Primer-Blast
<i>SAMD9</i>	NM_001205781.2	TTGAATGCCTCCTACACCGA CAAAGCAGCAGGGCATGAAG	102	Primer-Blast
<i>CCL8</i>	NM_174007.1	GCTGGACAGCTACACGAGAAT CATCCCTGTCCGCTTTGGTC	80	Primer-Blast
<i>CST6</i>	NM_001012764.3	TGGCTGGAGACCATGTAGAC TTCTTCCAGGGAACCAAGG	103	Primer-Blast
<i>RSAD2</i>	NM_001045941.1	AACAGATAACCGCGCTCAAC CTTCAAACCTCCTCGTCGCTG	129	(Passaro <i>et al.</i> 2018)
<i>OAS1</i>	NM_001040606.1	CCCGGCGGACCCTACAGGAA TCCAGCCAGACCAAGCCGC	84	(Forde <i>et al.</i> 2011)
<i>RNF11</i>	NM_001077953.1	TCCGGGAGTGTGTGATCTGTATGAT GCAGGAGGGGCACGTGAAGG	131	(Passaro <i>et al.</i> 2018)
<i>YWHAZ</i>	NM_174814.2	TGAAGCCATTGCTGAACCTTG TCTCCTTGGGTATCCGATGT	114	(Passaro <i>et al.</i> 2018)

nuclease-free water and 5 µL cDNA template on the ABI Prism 7500 Fast Sequence Detection System (Life Technologies, Thermo Fisher Scientific) and the appropriate positive and negative controls were included. Thermo-cycling conditions were as follows: 95°C for 10 min for one cycle, followed by 95°C for 15 s and 60°C for 1 min for 40 cycles. A dissociation curve was also added to ensure specificity of amplification. The presence of a single sharp peak in the melt curve analysis confirmed the specificity of all targets. geNorm was used to identify the most stable (average geNorm $M \leq 0.5$) and optimal number of reference genes (Vandesompele et al. 2002). The reference genes selected were ring finger protein 11 (*RNF11*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*), which were subsequently used to normalize the gene expression data. A standard curve was included for each gene of interest to confirm whether primer efficiencies were between 85% and 110%. The threshold cycle (Ct) for each sample was automatically calculated using the default settings within the SDS software (SDS 1.4, ABI). Ct values were imported into the qbase+ analysis package (Biogazelle, Zwijnaarde, Belgium). Data were normalized using the geometric mean of the reference genes as identified by geNorm. Relative expression values were automatically calculated by the software using a modified version of delta-delta Ct method ($\Delta\Delta C_q$; also known as $\Delta\Delta CT$) (Hellemans et al. 2008). One-way ANOVA was performed on the log transformed data.

Results

Conceptus recovery

Day 14 conceptuses were recovered from seven out of the nine (77.7%) recipient heifers. The recovery rate (proportion of conceptuses recovered relative to the number transferred) was 51.8% (70/135). The mean length of the recovered conceptuses was 11.32 ± 1.34 mm (mean $\pm$ S.E.M.). Only intact filamentous conceptuses with a length between 10 and 20 mm were individually co-cultured on top of the endometrial explants ($n=10$ conceptuses, mean length 14.58 ± 0.73 mm).

RNA sequencing and gene ontology analysis

One of the explants in the CONTRA-D14 group was of insufficient quality for RNA-seq and was therefore removed from further analysis. The RNA-seq of the remaining uterine explants ($n=19$) generated an average of 39.3 million reads per sample. The average mapping rate per sample was 96.2%. The number of differentially expressed genes (DEGs) and the status of expression (up- or down-regulated) is summarized in Fig. 1B. There were no differences between the transcriptome of the IPSI-CTRL and CONTRA-CTRL endometrial explants. However, the response of the ipsilateral and contralateral endometrium to a day 14 conceptus was remarkably different. Analysis of the RNA-seq data revealed 61 DEGs (all upregulated, based on false discovery rate (FDR) < 0.05 , Supplementary Table 1, see section on supplementary materials given at the end of this article) between IPSI-D14 and IPSI-CTRL and 239 DEGs (220 upregulated, 19 downregulated; Supplementary Table 2) between CONTRA-D14 and CONTRA-CTRL. Further, the comparison of these sets of DEGs revealed that 54 transcripts were upregulated in both experimental groups, while 7 and 185 were unique to IPSI-D14 and CONTRA-D14, respectively (Fig. 2).

The majority of the top upregulated genes by a D14 conceptus that were shared between groups were typical ISGs (16 out of the top 20, see Table 2). These included: *RSAD2*, MX dynamin like GTPase 2 (*MX2*), interferon-induced protein with tetratricopeptide repeats 1 (*LOC100139670*), *IFIT3*, C-type lectin domain family 4 member F (*CLEC4F*), interferon-induced protein 44 (*IFI44*), ISG15 ubiquitin-like modifier (*ISG15*), *IFIT2*, 2',5'-oligoadenylate synthetase 1, 40/46 kDa (*OAS1Z*) and *CCL8*.

GO analysis of the 61 DEGs between IPSI-D14 and IPSI-CTRL identified 39 enriched biological processes (BP) ($P \leq 0.05$; Supplementary Table 3). The top five BP include: (1.) response to interferon-alpha (IFNA), (2.) cytoplasmic pattern recognition receptor signaling

Figure 2 Venn diagram (Venny 2.1.0 BioinfoGP) showing the number of unique or common differentially expressed genes (DEGs) between endometrial explants of uterine horns ipsilateral and contralateral to the corpus luteum following exposure to a day 14 conceptus (IPSI-D14 and CONTRA-D14). DEGs were previously determined in each group relative to the respective controls. Increased and decreased transcripts are indicated in red and blue, respectively.

Table 2 Top 20 genes upregulated in both ipsilateral and contralateral endometrium exposed to a day 14 conceptus (IPSI-D14 and CONTRA-D14, respectively).

Gene symbol	Gene description	Log FC IPSI-D14	Log FC CONTRA-D14	FDR IPSI-D14 vs CONTRA-D14
<i>RSAD2</i>	radical S-adenosyl methionine domain containing 2	5.63	8.03	<0.05
<i>MX2</i>	MX dynamin like GTPase 2	5.84	7.81	0.148
<i>LOC100139670</i>	interferon-induced protein with tetratricopeptide repeats 1	5.30	6.90	<0.05
<i>IFIT3</i>	interferon-induced protein with tetratricopeptide repeats 3	5.20	6.36	0.227
<i>CLEC4F</i>	C-type lectin domain family 4 member F	6.72	4.30	0.906
<i>IFI44</i>	interferon-induced protein 44	5.00	5.81	0.142
<i>ISG15</i>	ISG15 ubiquitin-like modifier	4.39	5.66	0.286
<i>IFIT2</i>	interferon-induced protein with tetratricopeptide repeats 2	3.97	5.56	<0.05
<i>OAS1Z</i>	2',5'-oligoadenylate synthetase 1, 40/46 kDa	3.69	5.06	0.051
<i>LOC100298356</i>	bone marrow stromal antigen 2	3.56	5.19	0.185
<i>CCL8</i>	chemokine (C-C motif) ligand 8	3.37	5.36	<0.05
<i>OAS1X</i>	2',5'-oligoadenylate synthetase 1, 40/46 kDa	3.42	5.00	0.467
<i>LOC112441507</i>	bone marrow stromal antigen 2-like	3.42	4.97	0.071
<i>IFI44L</i>	interferon-induced protein 44 like	3.81	4.41	0.521
<i>USP18</i>	ubiquitin specific peptidase 18	3.28	4.88	<0.05
<i>LOC781710</i>	guanylate-binding protein 2	2.95	4.73	0.400
<i>LOC507055</i>	guanylate-binding protein 4	2.73	4.86	<0.05
<i>CCDC194</i>	coiled-coil domain containing 194	3.04	4.55	0.489
<i>UBA7</i>	ubiquitin like modifier activating enzyme 7	3.00	4.39	0.228
<i>OAS2</i>	2'-5'-oligoadenylate synthetase 2	3.19	4.19	0.286

Logarithm of fold change (Log FC) relative to its respective controls. False discovery rate (FDR) from the direct comparison between IPSI-D14 vs CONTRA-D14; FDR < 0.05 means they are differentially expressed. For full list of DEGs, see Supplementary Tables 1, 2 and 5.

pathway, (3.) negative regulation of viral genome replication, (4.) positive regulation of response to cytokine stimulus and (5.) negative regulation of viral life cycle. For the contralateral uterine horn, the GO analysis of the 239 DEGs between the CONTRA-D14 and CONTRA-CTRL group identified 87 enriched BP (Supplementary Table 4), including: (1.) interferon-gamma (IFNG)-mediated signaling pathway, (2.) response to type I interferons (IFNs), (3.) regulation of IFNA production, (4.) negative regulation of viral genome replication and (5.) positive regulation of IFNA production.

The direct comparison of RNA-seq data from IPSI-D14 vs CONTRA-D14 identified 32 DEGs (25 upregulated, 7 downregulated; Supplementary Table 5). The ten most upregulated genes (greatest to least) in CONTRA-D14 relative to IPSI-D14 included: *LOC783920*, *CXCL11*, *CXCL10*, *IFIT2*, *RSAD2*, *SAMD9*, guanylate-binding protein 4 (*LOC507055*), CD274 molecule (*CD274*), cytidine/UMP kinase 2 (*CMPK2*) and *LOC100139670*. The seven down-regulated genes (from most down-regulated to least) were: lipopolysaccharide binding protein (*LBP*), *AQP3*, *CST6*, SEM1 26S Proteasome Complex Subunit (*SEM1*), S100 calcium binding protein A2 (*S100A2*), microfibril associated protein 2 (*MFAP2*) and plexin A3 (*PLXNA3*).

GO analysis of the 32 DEGs between IPSI-D14 and CONTRA-D14 identified ten enriched BP (Supplementary Table 6): (1.) positive regulation of cAMP-mediated signaling, (2.) regulation of innate immune response, (3.) defense response to other organism, (4.) positive regulation of cytokine production, (5.) immune effector process, (6.) response to other organism, (7.) response to

external biotic stimulus, (8.) response to biotic stimulus, (9.) regulation of immune system process and (10.) response to external stimulus.

qRT-PCR validation of RNA-seq results

Using qRT-PCR, nine genes of interest (*LOC783920*, *CXCL11*, *CXCL10*, *IFIT2*, *SAMD9*, *CCL8*, *CST6*, *RSAD2* and *OAS1Y*) were validated in all groups (four comparisons for each gene). Thirty of the 36 comparisons (83.3%) were in agreement between RNA-seq and qRT-PCR, that is, one gene was found to be differentially expressed, or not, between two groups in both analyses. Although qRT-PCR failed to detect a significant difference among six of the comparisons that were found to be differentially expressed in the RNA-seq, the trend (e.g. upregulated in CONTRA-D14 compared to CONTRA-CTRL) was always consistent (Fig. 3 and Table 3).

Discussion

In order to understand the lower pregnancy rates observed following embryo transfer to the contralateral uterine horn, this study aimed to compare the local response of bovine endometrium ipsilateral and contralateral to the CL to an elongating conceptus. In order to disentangle the effect of a potentially compromised embryo/conceptus from the effect of a compromised endometrium, conceptuses were generated under 'normal' conditions (in the horn ipsilateral to the CL) and placed on top of endometrial explants from either the ipsilateral or contralateral horn. Major findings

Figure 3 Comparison between quantitative real-time PCR (qRT-PCR) and RNA sequencing (RNA-seq) data for nine selected genes. Note that for 4 of the 9 genes (A to D), all comparisons are in agreement; while the qRT-PCR failed to detect a significant difference in transcript abundance in some comparisons from the other 5 genes (E to I) the trend was always consistent.

from this study are: (1.) although no differences in gene expression were detected between IPSI-CTRL and CONTRA-CTRL (i.e. non-pregnant) endometrium, the response of the endometrium to a day 14 conceptus was distinct in each uterine horn; (2.) interestingly, more genes were differentially expressed in the contralateral than in the ipsilateral endometrium after exposure to a conceptus and (3.) many of the biological processes enriched in the DEGs between CONTRA-D14 and IPSI-D14 are associated with immune response and

response to stimuli. These data extend the results of [Sánchez et al. \(2018\)](#) who described temporal changes in the transcriptome of the endometrium ipsilateral and contralateral to the CL by describing differential response of the endometrium in both uterine horns to an elongating conceptus.

In terms of the relevancy of the model, while it is true that, in commercial practice, most single embryo transfer is done into the ipsilateral horn, there are occasions when it may be done deliberately into the

Table 3 Summary of RNA sequencing (RNA-seq) results validation through quantitative real-time PCR (qRT-PCR).

Comparisons	RNA-seq	qRT-PCR	Log FC RNA-seq
<i>RSAD2</i>			
IPSI-CTRL vs IPSI-D14	YES	YES	5.63
IPSI-CTRL vs CONTRA-CTRL	NO	NO	
IPSI-D14 vs CONTRA-D14	YES	NO	1.98
CONTRA-CTRL vs CONTRA-D14	YES	YES	8.03
<i>OAS1Y</i>			
IPSI-CTRL vs IPSI-D14	YES	YES	2.87
IPSI-CTRL vs CONTRA-CTRL	NO	NO	
IPSI-D14 vs CONTRA-D14	NO	NO	
CONTRA-CTRL vs CONTRA-D14	YES	YES	3.82
<i>CXCL10</i>			
IPSI-CTRL vs IPSI-D14	NO	NO	
IPSI-CTRL vs CONTRA-CTRL	NO	NO	
IPSI-D14 vs CONTRA-D14	YES	YES	2.16
CONTRA-CTRL vs CONTRA-D14	YES	YES	3.75
<i>IFIT2</i>			
IPSI-CTRL vs IPSI-D14	YES	YES	3.97
IPSI-CTRL vs CONTRA-CTRL	NO	NO	
IPSI-D14 vs CONTRA-D14	YES	NO	2.04
CONTRA-CTRL vs CONTRA-D14	YES	YES	5.56
<i>LOC783920</i>			
IPSI-CTRL vs IPSI-D14	NO	NO	
IPSI-CTRL vs CONTRA-CTRL	NO	NO	
IPSI-D14 vs CONTRA-D14	YES	NO	2.42
CONTRA-CTRL vs CONTRA-D14	YES	YES	3.84
<i>SAMD9</i>			
IPSI-CTRL vs IPSI-D14	YES	NO	2.87
IPSI-CTRL vs CONTRA-CTRL	NO	NO	
IPSI-D14 vs CONTRA-D14	YES	NO	1.81
CONTRA-CTRL vs CONTRA-D14	YES	YES	4.39
<i>CST6</i>			
IPSI-CTRL vs IPSI-D14	NO	NO	
IPSI-CTRL vs CONTRA-CTRL	NO	NO	
IPSI-D14 vs CONTRA-D14	YES	NO	-1.84
CONTRA-CTRL vs CONTRA-D14	NO	NO	
<i>CXCL11</i>			
IPSI-CTRL vs IPSI-D14	NO	NO	
IPSI-CTRL vs CONTRA-CTRL	NO	NO	
IPSI-D14 vs CONTRA-D14	YES	YES	2.21
CONTRA-CTRL vs CONTRA-D14	YES	YES	3.51
<i>CCL8</i>			
IPSI-CTRL vs IPSI-D14	YES	YES	3.37
IPSI-CTRL vs CONTRA-CTRL	NO	NO	
IPSI-D14 vs CONTRA-D14	YES	YES	1.67
CONTRA-CTRL vs CONTRA-D14	YES	YES	5.36

'YES' or 'NO' refers whether the gene expression was found significantly different for each of the comparisons or not. 'Log FC RNA-seq' refers to the logarithm of fold change (Log FC) of the second group relative to the first (i.e. IPSI-D14 relative to IPSI-CTRL for the first comparison) for the RNA-seq data.

contralateral horn or when an embryo can find itself in that horn. One such scenario is in the beef industry where, in order to induce twins to increase efficiency, an embryo may be transferred to the contralateral horn of a female which had been bred by AI at oestrus 7 days earlier (or two embryos may be bilaterally transferred). (Sreenan *et al.* 1981, Sreenan & Diskin 1989, Guerra-Martinez *et al.* 1990, Dahlen *et al.* 2012). Coupled with

poor profitability of the sector and increasing focus on its carbon footprint, the future sustainability of beef cow herds in some countries, including Ireland, is under pressure. Twinning, along with improvements in genetic gain through use of selected sires, offers the potential to increase output, profitability and reduce greenhouse gas emissions due to increased output and a dilution in overhead costs per animal. Furthermore, results from the long-term twinning project at the US Meat Animal Research Center (USMARC), Clay Center, Nebraska, where the frequency of fraternal twin births increased from 3.1% to >50% per year in about 20 years through selection, indicate that dams producing twins weaned 70.8% more calves than dams with a single birth (Echternkamp & Gregory 2002).

A second scenario in which the embryo may end up in the contralateral horn is due to transuterine migration. The incidence of transuterine migration is low in cattle following fertilization *in vivo* by natural mating or AI (about 10% based on studies by Rowson *et al.* 1971, Scanlon 1972). When two ovulations occurred in the same ovary and two fetuses were present, 20% of cows had evidence of transuterine migration (Scanlon 1972). Importantly, however, transuterine migration is significantly higher following embryo transfer, particularly when more than one embryo is transferred. Several studies have reported an increase in the incidence of migration when more than one embryo was initially located in the same uterine horn (Scanlon 1972, McMillan & Peterson 1995, 1999). In the study by McMillan and Peterson (1995), following the uni-lateral transfer of two embryos, more than half of all twin pregnancies were bilateral (23/38, 61%). Thirdly, although perhaps less relevant, but nonetheless noteworthy is the fact that, nowadays, a high proportion of double ovulations occur in high-producing dairy cows. In one study, the incidence of double ovulation was nearly three-fold greater for high (20.2%) vs low (6.9%) milk producing cows (Fricke & Wiltbank 1999). Clearly, if ovulations occur bilaterally, then each horn is essentially ipsilateral to a CL; however, where they occur unilaterally, in a small number of cases one embryo may end up in the contralateral horn. Surprisingly, there are very few data available describing the proportion of such cows that go on to deliver twins. Garcia-Ispuerto and López-Gatius (2019) examined the incidence and timing of abortion in Holstein Friesian dairy cows with advanced twin pregnancies in north-eastern Spain. From 1194 twin pregnancies at around Day 60 (522 bilateral and 672 unilateral), abortion was recorded in 278 (23.3%) cows before Day 260 of pregnancy: 7 (1.3%) in bilateral and 271 (40.3%) in unilateral twin pregnancies.

Another point to consider is the possibility that the response of an endometrial explant from the uterine horn contralateral to CL to a conceptus which developed in the uterine horn ipsilateral to CL may not be same as that to a conceptus developed in the contralateral horn.

While this is a possibility, as mentioned previously, the design used here allows the disentangle of the effect of a potentially compromised embryo from the effect of a compromised endometrium. There is very little evidence to indicate that there is any meaningful response from the endometrium to the presence of a conceptus before Day 7 (the day on which the embryos were transferred in this study). Many studies from our own group (Forde *et al.* 2011) as well as others (Bauersachs *et al.* 2012) have demonstrated that no transcriptomic response is detectable from the endometrium prior to Day 15/16. This is entirely consistent with what occurs in commercial embryo transfer practice, where embryos, typically on Day 7, are transferred into the uterus of a synchronised recipient which has had no pre-exposure to an embryo or products derived therefrom.

In two recent studies from our group, we have clearly demonstrated that, using an explant model such as that described in this study, it is possible to detect subtle embryo-induced changes in the endometrial transcriptome (Passaro *et al.* 2018, 2019). This was only possible by placing multiple blastocysts on top of explants and by being able to analyse the tissue that was directly below the embryos. Interesting, all of the genes altered in the endometrium were IFNT-related, as conformed by comparison with the response of endometrial tissue to stimulation with recombinant IFNT or Day 15 conceptuses (Sánchez *et al.* 2019).

In a previous study from our group (Sánchez *et al.* 2018), we compared the transcriptome of the endometrium ipsilateral and contralateral to the CL in cyclic heifers at four different time points: day 5, 7, 13 and 16. The number of DEGs was highest on day 5 (217 DEGs) and progressively decreased with time (54, 14 and 18 DEGs, respectively) (Sánchez *et al.* 2018). Consistent with these results, in the present study, we did not detect any differences between the non-pregnant ipsilateral and contralateral (IPSI-CTRL vs CONTRA-CTRL) endometrial transcriptome on day 14. The slight difference between the two studies (14 DEGs on day 13 in Sánchez *et al.* 2018 and none on day 14 in the present study) could be due to the difference in sample type; in the first case, samples were intercaruncular endometrial strips collected and snap frozen immediately after slaughter, while in the current study, samples were endometrial explants collected after slaughter and incubated for 7 to 10 h before being snap frozen.

Despite the lack of differences in the endometrial transcriptome between the IPSI-CTRL and CONTRA-CTRL samples, the response of the endometrium to a day 14 conceptus was substantially different between uterine horns. The number of altered genes due to the presence of a conceptus was almost four-fold higher in the CONTRA-D14 compared to IPSI-D14 (239 vs 61 DEGs, respectively). This observation is consistent with the study of Moraes *et al.* (2018), where relatively few differences were detected in the endometrial

transcriptome of non-pregnant high-fertile, subfertile and infertile heifers; however, the response of the endometrium from high-fertile and subfertile animals to pregnancy was remarkably different (3422 vs 1095 DEGs, respectively).

Endometrial response to a day 14 conceptus in the IPSILATERAL uterine horn

Most of the DEGs in the ipsilateral horn exposed to a conceptus were also differentially expressed in the contralateral endometrium (54 out of 61). Only seven genes were uniquely upregulated in the ipsilateral horn (Fig. 2): adipogenesis regulatory factor (*ADIRF*), aminomethyltransferase (*AMT*), amylase, alpha 2B (pancreatic) (*AMY2B*), growth arrest and DNA damage inducible gamma (*GADD45G*), GDNF family receptor alpha 4 (*GFR4*), interleukin 6 (*IL6*) and solute carrier family 26 member 10 (*SLC26A10*).

AMT gene expression has been previously reported as being more abundant in endometrial explants from pregnant than cyclic and/or uterine gland knockout (UGKO) sheep on day 14 (Gray 2005). *AMT* is part of the glycine cleavage system (Okamura-Ikeda *et al.* 2010), which produces 5,10-methylenetetrahydrofolate as a donor of one-carbon units, which is essential for the synthesis of DNA during cell proliferation (Fleming & Copp 1998). Glycine is one of the most abundant amino acids in the ULF of cyclic and pregnant heifers (Groebner *et al.* 2011, Forde *et al.* 2014b) and sheep (Gao *et al.* 2009). On day 15, pregnant heifers had higher levels of glycine than cyclic heifers (Groebner *et al.* 2011), which could explain a higher *AMT* gene expression in IPSI-D14 compared to IPSI-CTRL. This gene could be important for the support of conceptus growth and development due to its role in DNA synthesis.

SLC26A10 belongs to a family of anion transporters expressed in many epithelia (Ohana *et al.* 2009). Members of the solute carrier family 26 (SLC26) are responsible for regulating the ULF pH, secreting bicarbonate into the lumen in exchange for the luminal chlorine, which is in turn is secreted via the cystic fibrosis transmembrane conductance regulator (CFTR) (Xie *et al.* 2018). Interestingly, *CFTR* is one of the top ten down-regulated genes in CONTRA-D14 relative to its control. The acid-base homeostasis in the reproductive tract is critically important for reproduction (Xie *et al.* 2018). The different expression of genes related to pH balance could reflect differences in the ULF pH between the uterine horns, making the contralateral a less favorable environment for embryo development and implantation.

In agreement with our results, *IL6* was found to be upregulated in a previous study from our group in which endometrial explants were exposed to a day 15 conceptus compared with the controls and explants treated with IFNT (Mathew *et al.* 2018). *IL6* encodes a cytokine that has both pro-inflammatory and anti-

inflammatory activities and is a major player in the regulation of the acute phase response and immune response (Heinrich *et al.* 1990, Scheller *et al.* 2011). One of the functions of IL6 is coordinating the balance of T cell phenotypes (Prins *et al.* 2012). T-helper (Th) cells are required for almost all adaptive immune responses and can differentiate into several distinct subtypes, including Th1, Th2, Th17 and T regulatory (Tregs) cells (Oliveira *et al.* 2013). Th1 and Th17 play a central role in the induction of inflammation and are involved in the allograft rejection process (Alberts *et al.* 2002, Saini *et al.* 2011). In contrast, Th2 and Treg are crucial for the induction and the maintenance of allograft tolerance (Saini *et al.* 2011). IL6 is a very important factor in determining Th17/Treg balance, promoting the generation of Th17 and inhibiting Treg differentiation (reviewed by Kimura & Kishimoto 2010).

Pregnancy requires a delicate balance between pro-inflammatory and anti-inflammatory molecules to maintain maternal immune system integrity while preventing rejection of the embryo (Walker *et al.* 2010, Mansouri-Attia *et al.* 2012). In humans, both IL6 deficiency and IL6 excess have been linked with reproductive impairment (Prins *et al.* 2012). *Il6* null mutant mice exhibit normal estrous cycles, ovulation, fertilization and embryo implantation, but frequent occurrence of fetal resorption in mid-gestation (Robertson 2010). Although the mechanisms underlying fetal loss in the absence of IL6 are not defined (Prins *et al.* 2012), the fact that *IL6* is exclusively upregulated in the IPSI-D14 and not in the CONTRA-D14 may suggest an imbalance in the T cell population that could lead to a loss of pregnancy in a more advance stage. This notion would be consistent with the observations from Christie *et al.* (1979) that a great number of developing embryos died between days 24–42 when transfers were done into the contralateral horn.

Regarding the GO analysis of these 61 DEGs between IPSI-D14 and IPSI-CTRL, only 2 of the 39 enriched BP were exclusive to the ipsilateral endometrium: response to IFNA (GO:0035455) and cytoplasmic pattern recognition receptor (PRRs) signaling pathway (GO:0002753). Interestingly, those were the ones with highest fold enrichment (over 100). Both BP are somewhat related to the immune response. In the first case, IFNA is an important antiviral cytokine quite similar to IFNT (~50% identity) and with similar biological activities (Green *et al.* 2005, Bauersachs *et al.* 2012). It is secreted mainly by plasmacytoid dendritic cells and serves to alert cells of both innate and adaptive immunity to the threat of infections (reviewed by Huber & Farrar 2011). In addition, it ensures the proper differentiation of Th1 cells by restricting the development of alternative subsets like Th2 and Th17 (Huber & Farrar 2011). In the second case, toll-like receptors (TLRs), a type of PRRs, are one of the main regulators of innate immunity and are key regulators in immunologic events during stages of normal pregnancy such as implantation (reviewed by

Amirchaghmaghi *et al.* 2013). These two BP uniquely enriched in the ipsilateral endometrium suggest that the immune response to a conceptus is different between uterine horns.

Overall, analysis of the unique DEG and BP in the ipsilateral uterine horn suggests that a favorable environment includes support of conceptus growth and development (*AMT*), an appropriate regulation of ULF homeostasis (*SLC26A10* - *CFTR*) and a balanced immune response (*IL6*, GO:0035455, GO:0002753).

Endometrial response to a day 14 conceptus in the CONTRALATERAL uterine horn

Comparison between CONTRA-D14 and its control revealed 239 DEGs, 185 of which were differentially expressed uniquely in the contralateral uterine horn. Of those 185 genes, *Schlafen* Family Member 11 (*SLFN11*), *CXCL10*, *CXCL11* and T cell immunoglobulin and mucin domain containing 4 (*TIMD4*) were among the top ten upregulated. The major function of chemokines, such as *CXCL10* and *CXCL11*, is to recruit immune cells including leukocytes, lymphocytes and macrophages to inflammatory regions (Janeway & Medzhitov 2002). Furthermore, it has been hypothesized that endometrial chemokines may regulate conceptus adhesion to the uterine epithelium due to the expression of chemokine receptors not only in the endometrium but also in trophoblast cells from mice, goats and sheep (Dominguez *et al.* 2003, Nagaoka *et al.* 2003, Imakawa *et al.* 2006). Chemokines *CXCL10* and *CXCL11* were expressed during the peri-implantation period (from day 14 to 20) in ovine endometrium; expression of both factors increased as implantation advanced, with maximum expression on day 17 for *CXCL11* and on day 20 for *CXCL10* (Imakawa *et al.* 2006).

TIMD4 serves as the natural ligand for T cell immunoglobulin and mucin domain (TIM)-1, another protein of the TIM family, and this interaction is necessary for T cell proliferation (Umetsu *et al.* 2005). The TIM family is involved in modulating host immune responses likely by altering the Th1/Th2 balance (Jones 2002). *SLFN11* is an inhibitor of DNA replication that promotes cell death in response to DNA damage (Zoppoli *et al.* 2012). In mammals, preparation of the uterus for implantation includes endometrial tissue remodeling, including cell death (Tassell *et al.* 2000, Liu *et al.* 2005, Okano *et al.* 2007). However, the expression profile of cell death-related genes in the bovine pregnant uterus still remains unverified (Suzuki *et al.* 2018).

SLFN11, *CXCL10*, *CXCL11* and *TIMD4* were also found upregulated in endometrial explants from the ipsilateral uterine horn exposed to IFNT, a long, or a short day 15 conceptus (Sánchez *et al.* 2019). The same genes were upregulated on days 15, 17 and 18 in pregnant heifers compared to the respective non-pregnant controls (Bauersachs *et al.* 2012, Moraes *et al.* 2018) but not on

days 12 and 13 (Forde *et al.* 2011, Bauersachs *et al.* 2012). The expression of these genes does not seem to be related with fertility since they were not differentially expressed between high-fertile and subfertile pregnant heifers (Moraes *et al.* 2018). Present results in the ipsilateral uterine horn combined with results from the aforementioned studies suggest that day 14 might be too early for those genes to be upregulated. Furthermore, differential expression of 185 genes only in the contralateral uterine horn and not in the ipsilateral may indicate a dysfunctional response to a conceptus which could be a consequence of an inadequate P4 priming, especially during the first days after ovulation (Takahashi *et al.* 2016).

Among the top ten downregulated genes from the 185 unique to the contralateral uterine horn were chromosome 15 open reading frame, human C11orf34 (*C15H11orf34*), *AQP3* and *CFTR*. *C15H11orf34*, also known as Placenta Expressed Transcript 1 (*PLET1*), has been reported to mark distinct populations of progenitor cells in several tissues and to provide essential cues to direct trophoblast stem cell differentiation (Murray *et al.* 2016). Forde *et al.* (2009) examined how elevated P4 from day 3 to day 7, which has been associated with increased pregnancy success in cattle, affected the endometrial transcriptome. In this study, *C15H11orf34* was found to be upregulated on day 16 of pregnancy in the endometrium of pregnant heifers with high compared with normal P4 concentrations. Moreover, in the study of Moraes *et al.* (2018), it was also upregulated on day 17 of pregnancy in high-fertile and subfertile heifers compared to its respective controls and expression was significantly higher in pregnant high-fertile compared to pregnant subfertile animals.

One of the functions of *AQP3* and *CFTR* is water transport. In the pig, *AQP1* and *AQP5* may play an important role in maintaining local fluid balance within the uterus and embryo-maternal communication during implantation (Skowronska *et al.* 2016). Hayashi *et al.* (2017) found that *AQP2* and *AQP5* were upregulated in the ipsilateral horn endometrium of non-repeat breeder compared to repeat breeder cows. In agreement with our results, *CFTR* was one of the top ten down-regulated genes in the contralateral horn endometrium of repeat breeder cows (Hayashi *et al.* 2017). As mentioned earlier, *CFTR* is also involved in the maintenance of the ULF pH. Defects in the regulation of these genes may lead to disturbance of the fluid microenvironment resulting in fertility problems.

Regarding the GO analysis of the 239 DEGs between CONTRA-D14 and CONTRA-CTRL, 50 of the 87 enriched BP were exclusive to the contralateral endometrium (Supplementary dataset 5). Among those with a highest enrichment fold were many related with response to and production of type I IFNs (alpha and beta) and type II IFN, IFNG. Type I IFNs are primarily induced by viral infection but can also be secreted

in response to a variety of biological stresses, such as inflammation (Huber & Farrar 2011). Some of the functions of type I IFNs are to modulate innate immune responses in a balanced manner and to activate the adaptive immune system (reviewed by Ivashkiv & Donlin 2014). Although IFNA is similar to IFNT and has similar biological activity (Green *et al.* 2005, Bauersachs *et al.* 2012), administration of IFNA to heifers on day 13 after insemination decreased pregnancy rates by day 42 (Barros *et al.* 1992).

IFNG plays important roles in diverse cellular processes, including activating innate and adaptive immune responses, inhibiting cell proliferation and inducing apoptosis (reviewed by Murphy *et al.* 2009). High circulating levels of IFNG have been associated with pregnancy loss in humans (Mueller-Eckhardt *et al.* 1994, Jenkins *et al.* 2000), mice (Haddad *et al.* 1997) and pigs (Tayade *et al.* 2006). In pigs, IFNG elevation was strongly implicated in peri-implantation losses (Tayade *et al.* 2006, Murphy *et al.* 2009). Therefore, an excess production of both IFNA and IFNG, as might be the case in the contralateral uterine horn, could be detrimental for embryo development and/or successful pregnancy establishment.

Direct comparison IPSILATERAL vs CONTRALATERAL

The direct comparison between the IPSI-D14 and CONTRA-D14 revealed 32 DEGs (25 upregulated, 7 downregulated), in contrast with the 54 genes that were commonly upregulated when compared with their respective controls (Fig. 2). This apparent discrepancy likely arises due to the variation of expression within each group which will have an effect on the multiple correction of raw *P* values to determine the FDR, based on which a gene is declared significant or not (Sánchez *et al.* 2019).

At least 22 of the 25 upregulated genes in CONTRA-D14 relative to IPSI-D14 are ISGs (Supplementary Table 3), which further demonstrates that the endometrium from the ipsilateral and contralateral uterine horns respond differently to IFNs. Type I IFNs can lead to immune cell recruitment and activation (Makris *et al.* 2017); therefore, an exacerbated response to type I IFNs, as observed in the contralateral uterine horn in our study, could increase the risk of tissue rejection and pregnancy loss (Ribeiro *et al.* 2016).

S100A2, *CST6* and *LBP* were three of the seven downregulated genes in CONTRA-D14 relative to IPSI-D14 (i.e. were upregulated in IPSI-D14 relative to CONTRA-D14). In agreement with our results, *S100A2* and *LBP* were upregulated on day 15 of pregnancy compared to day 15 cyclic heifers (Bauersachs *et al.* 2012). *S100A2* is part of the S100 protein family which are involved in virtually all normal and pathological cell functions including cell proliferation, Ca²⁺ homeostasis, inflammatory and immune responses (Carafoli 2002, Berridge *et al.* 2003, Leclerc & Heizmann 2011, Fleming

et al. 2012). LBP encodes a protein that binds bacterial endotoxin (Schumann *et al.* 1996). Increased expression of antimicrobial genes in pregnant animals may confer innate immune protection against potential bacterial infection during a time of local immune suppression, as occurs during pregnancy (Walker *et al.* 2010). Forde *et al.* (2014a) found that CST6 was one of the most abundant proteins in the ULF during the pregnancy recognition period and hypothesized that it might be involved in driving conceptus elongation during the pre-implantation period of pregnancy in cattle. Expression of cystatins and cathepsins in the uterine endometrium has been suggested to play important roles in endometrial remodeling during the reproductive cycle and pregnancy (Salamonsen 1999).

The most enriched BP in the GO analysis of the 32 DEGs between CONTRA-D14 and IPSI-D14 was positive regulation of Adenosine 3',5'-cyclic monophosphate (cAMP)-mediated signaling with over 100-fold-enrichment (GO:0043950). cAMP is a nucleotide that acts as a key second messenger in numerous signal transduction pathways, converting and amplifying extracellular signals (first messengers) (Yan *et al.* 2016). The enrichment of this and five other BP associated to response to stimulus might point to a different capacity of the endometrium of the contralateral and ipsilateral uterine horns to respond to extracellular signals produced by the conceptus.

The other four BP are related to immune response and cytokine production. Deregulation of the immune response may be partly responsible for the large number of pregnancy losses that occur during the implantation time (Walker *et al.* 2010). In human, exaggerated maternal immune response to the fetus has been suggested as one of the underlying causes of a proportion of idiopathic recurrent pregnancy loss (Babbage *et al.* 2001, Saini *et al.* 2011).

The large difference in the number of DEGs between the endometrium ipsilateral and contralateral to the CL in response to a day 14 conceptus may be related to the differences in P4 concentrations during the first few days after ovulation (Takahashi *et al.* 2016), since P4 is one of the major regulators of the uterine receptivity through changes induced in the endometrium transcriptome (Spencer & Bazer 1995, Forde & Lonergan 2012, Brooks *et al.* 2014). In cattle, unlike twin- or litter-bearing species, the presence of conceptus tissue is not expected in the contralateral uterine horn until day 18–19, when the filamentous conceptus occupies both horns. Therefore, the precocious presence of a conceptus on day 14 may provoke an exaggerated response, particularly immune-related, that may cause problems later during the implantation period and lead to a pregnancy loss. Furthermore, differential expression of genes related to homeostasis suggest that the environment in the contralateral uterine horn may not be favorable for embryo development.

Conclusion

In conclusion, we report here the differential conceptus-induced transcriptomic response of bovine endometrium ipsilateral and contralateral to the CL. The response of the endometrium to a day 14 conceptus was distinct in each uterine horn with many of the biological processes enriched in the DEGs between CONTRA-D14 and IPSI-D14 being associated with immune response and response to stimuli. Knowledge generated in this and other such studies contribute to our knowledge of maternal embryo communication during this critical period and our understanding of the factors affecting embryonic loss.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-19-0464>.

Declaration of interest

Trudee Fair is on the editorial board of *Reproduction*. Trudee Fair was not involved in the review or editorial process for this paper, on which she is listed as an author. The other authors have nothing to disclose.

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Author contribution statement

P L, J M S and T F conceived the study. S B A, J M S, B F F, M M and P L performed the experiment. S B A performed the lab work and analyzed the data. S K B and T E S performed the RNA sequencing and analyzed the data. S B A and P L wrote the manuscript. All authors discussed the results and contributed to the manuscript.

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