Global transcriptomic response of bovine endometrium to blastocyst-stage embryos

C Passaro¹, D Tutt², S Bagés-Arnal¹, C Maicas¹, R Laguna-Barraza³, A Gutierrez-Adán³, J A Browne¹, D Rath⁴, S K Behura⁵, T E Spencer⁵, T Fair¹ and P Lonergan¹

¹School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Ireland, ²School of Veterinary Science, The University of Queensland, Gatton, Queensland, Australia, ³Departamento de Reproducción Animal, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain, ⁴Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut, Neustadt-Mariensee, Germany and ⁵Division of Animal Sciences, University of Missouri, Columbia, Missouri, USA

Correspondence should be addressed to P Lonergan; Email: pat.lonergan@ucd.ie

Abstract

The aims of this study were (i) to investigate changes in the global transcriptome of bovine endometrial explants induced by exposure to blastocysts, (ii) to investigate if male and female blastocysts elicit a differential response in the endometrial transcriptome *in vitro* and (iii) to determine whether bovine endometrium responds to the presence of murine embryos. In Experiment 1, endometrial explants from the same uterus were cultured for 6 h with or without 20 *in vitro*-produced bovine blastocysts. In Experiment 2, endometrial explants were cultured with male or female bovine blastocysts produced *in vitro* by IVF either using sex-sorted semen or conventional unsorted semen followed by embryo sexing based on a biopsy. In Experiment 3, endometrial explants were cultured alone or in the presence of bovine blastocysts ($n=25$) or murine blastocysts ($n=25$). Following culture, explants were snap frozen and stored at -80°C until RNA extraction, qPCR or RNA-Seq. Culture with bovine blastocysts increased endometrial expression of 40 transcripts, all of which were interferon-tau induced. Culture with male or female bovine blastocysts increased transcript abundance of five classic interferon-stimulated genes (*MX1*, *MX2*, *ISG15*, *OAS1*, *RSAD2*) in explants; however, there was no difference in abundance of transcripts previously reported to be related to embryonic sex (*IFNAR1*, *IFNAR2*, *CTGF*, *ARTN*, *SLC2A1*, *SLC2A5*). Exposure to murine blastocysts did not elicit any detectable change in transcript abundance. These findings, coupled with our previous data, indicate that very local, interferon-tau-induced changes in endometrial gene expression occur in response to blastocysts; whether such changes play any role in subsequent pregnancy recognition remains to be established.

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Introduction

Following hatching of the ruminant blastocyst, communication between the elongating conceptus and the maternal endometrium is essential for maternal recognition of pregnancy, prevention of luteolysis and maintenance of progesterone secretion from the corpus luteum. Partly due to its minute size, communication between the pre-hatching, zona-enclosed embryo and the endometrium is more challenging to demonstrate and is apparently not essential for pregnancy establishment, as evidenced by the fact that pregnancies are established after embryo transfer to a uterus not previously exposed to an embryo.

There are very few, if any, global transcriptomic data sets available highlighting alterations in transcript abundance induced in the endometrium by the presence of a blastocyst(s). Indeed, many such studies carried out *in vivo*, including some of our own work (Forde *et al.* 2011) and that of others (Bauersachs *et al.* 2012), have failed to

identify changes in the endometrial transcriptome prior to Day 15/16, by which time the elongated conceptus is secreting copious amounts of IFNT.

Using qRT-PCR, we have recently shown that bovine endometrial explants respond to the presence of 8-day-old blastocysts by upregulating the expression of classical interferon-stimulated genes (ISG) (Passaro *et al.* 2018). This effect was (i) specific to the blastocyst stage – earlier stages did not induce gene expression changes, (ii) dependent on the number of blastocysts present – a minimum of five blastocysts were required to detect such changes and (iii) independent of direct contact – the effect was induced by embryos co-cultured on endometrial explants using a cell culture insert (preventing direct contact) as well as by blastocyst-conditioned medium (Passaro *et al.* 2018). Others have reported differential expression of a small number of other transcripts in the endometrium *in vivo*, induced by the presence of a single blastocyst

(Sponchiado *et al.* 2017) or in cultured endometrial cells (Talukder *et al.* 2017, Gómez *et al.* 2018a), which we failed to detect in endometrial explants using qPCR (Passaro *et al.* 2018).

A significant volume of evidence suggests that preimplantation embryo development (Bermejo-Alvarez *et al.* 2010a), metabolism (Tiffin *et al.* 1991, Gómez *et al.* 2018b) and gene expression (Bermejo-Alvarez *et al.* 2010a) are influenced by the sex of the embryo and development during this period can be further exacerbated by environmental (Hansen *et al.* 2016) and culture conditions (Larson *et al.* 2001). Male bovine embryos have been reported to develop faster (Avery *et al.* 1992) and reach the blastocyst stage more frequently (Xu *et al.* 1992) than female embryos. Indeed, bovine female *in vitro*-fertilized blastocysts display lower cell numbers and increased apoptosis than their male counterparts (Oliveira *et al.* 2010). Gómez *et al.* (2013) reported differential protein expression in Day 8 uterine fluid recovered from heifers following the transfer of Day 5 male or female embryos. More recently, Gómez *et al.* (2018a) described an embryo sex-dependent response of cultured bovine uterine epithelial (but not stromal) cells, suggesting that male and female embryos may differentially release signalling factors that induce sexually dimorphic responses in such cells.

Implantation in ruminants differs from that of rodents and primates where the conceptus enters a receptive uterus, hatches and almost immediately attaches to the uterine luminal epithelium (haemochorial placentation) (Cha & Dey 2015, Su & Fazleabas 2015). In contrast, the ruminant conceptus remains unattached for almost 3 weeks prior to implantation, during which time it rapidly elongates prior to implantation (synepitheliochorial placentation) (Lonergan & Forde 2014, Spencer *et al.* 2016). Elongation in ruminant embryos is a maternally driven process – it has not been successfully recapitulated *in vitro* (Brandão *et al.* 2004, Alexopoulos *et al.* 2005) and does not occur *in vivo* in the absence of uterine glands and associated secretions (Gray *et al.* 2002). However, whether the ruminant embryo is intrinsically programmed to elongate or whether this is exclusively driven by the maternal uterine environment is not known. Evidence for a component intrinsic to the embryo comes from the significant variation in the size of age-matched conceptuses (Betteridge *et al.* 1980), even among those recovered from the same uterus following embryo transfer (Clemente *et al.* 2009, Sánchez *et al.* 2019). Such variation in conceptus length is also evident in porcine conceptuses, despite growing in a common uterine environment, and rapid progression through this phase has been associated with conceptus competency (Blomberg *et al.* 2010).

Here, we used an endometrial explant-embryo co-culture model to elucidate the fine dialogue between the early conceptus and endometrium. Endometrial explants maintain normal cellular and extracellular

architecture and allow for communication between resident populations of endometrial cells which cannot be achieved using a 2D culture system. To extend our previous findings, the aims of this study were (i) to investigate changes in the global transcriptome of endometrial explants induced by exposure to blastocysts using RNA sequencing (RNA-Seq), (ii) to investigate if male and female blastocysts elicit a differential response in the endometrial transcriptome *in vitro* and (iii) to determine whether the bovine endometrium responds to the presence of murine embryos, which do not exhibit post-hatching elongation prior to implantation.

Materials and methods

Unless otherwise stated, all chemicals were sourced from Sigma-Aldrich.

Experiment 1: Global transcriptome analysis of endometrial response to bovine blastocysts

The aim of this experiment was to interrogate the global transcriptomic response of the bovine endometrium to the presence of blastocysts. Endometrial explants from the same uterus were cultured for 6 h with medium alone (control) or with 20 Day 8 *in vitro*-produced blastocysts using a polyester mesh to retain the embryos directly above the endometrial surface as recently described (Passaro *et al.* 2018). Five replicates (i.e. explants from five different uteri) were carried out. After incubation, explants were snap frozen and stored at -80°C until RNA extraction and RNA-Seq analysis.

Experiment 2: Endometrial response to male and female blastocysts

To investigate the transcriptomic response of the endometrium to the presence of male and female embryos, Day 8 blastocysts were produced *in vitro* by IVF either using (i) sex-sorted (X- or Y-sorted) semen or (ii) conventional unsorted semen followed by embryo sexing based on a biopsy taken on Day 6, as detailed below.

Experiment 2(a): Blastocyst production with sex-sorted semen

Bovine ovaries were collected at a local abattoir and transported to the laboratory in PBS at 35°C within 4 h. Cumulus-oocytes complexes (COCs) were aspirated from surface visible antral follicles using an 18-gauge needle attached to a 5 mL syringe. *In vitro* maturation, fertilization and culture were carried out as previously described (Rizos *et al.* 2002). Briefly, grade 1 COCs were matured for 24 h in TCM-199 supplemented with 10% fetal calf serum (FCS) and 10 ng/mL epidermal growth factor (EGF) at 38.5°C under an atmosphere of 5% CO_2 in air with maximum humidity. Fertilization (Day 0) was performed with X- or Y-sorted sperm from one of three different bulls, as previously described (Bermejo-Alvarez *et al.* 2010b). Matured COCs were inseminated with frozen-thawed,

Percoll-separated, semen added at a final concentration of 2×10^6 sperm/mL. Gametes were co-incubated at 38.5°C in an atmosphere of 5% CO₂ in air with maximum humidity. At approximately 20h post-insemination, presumptive zygotes were denuded of surrounding cumulus cells and accessory sperm and cultured in 25 µL drop of synthetic oviduct fluid (SOF) supplemented with 5% FCS until Day 8 at 38.5°C in an atmosphere of 5% CO₂, 5% O₂ with maximum humidity. On Day 8, blastocysts produced from X- and Y-sorted sperm from each bull were pooled together according to sex in groups of approximately 20 and cultured on endometrial explants for 6 h. Five replicates (i.e., separate days of ovary collection) were conducted. After incubation, explants were snap frozen and stored at -80°C until RNA extraction, reverse transcription and qPCR analysis.

Experiment 2(b): Blastocyst production and sexing by embryo biopsy

On Day 6 post fertilization, morula stage embryos were biopsied by blastomere extrusion (Tominaga & Hamada 2004) of three to eight cells for sex identification. After biopsy, embryos were returned to culture until Day 8, when those embryos that reached the blastocyst stage were cultured on an endometrial explant for 6 h in groups of 12–15 according to sex in a total of four replicates. After incubation, explants were snap frozen and stored at -80°C until RNA extraction, reverse transcription and qPCR analysis.

For Experiments 2(a-b), transcript abundance of genes previously shown by us to be induced in endometrial explants by the presence of blastocysts (*MX1*, *MX2*, *ISG15*, *OAS1*, *RSAD2*; Passaro et al. 2018) as well as genes reported by others to be altered by embryos in a sex-specific manner in cultured uterine epithelial cells (*IFNAR1*, *IFNAR2*, *CTGF*, *ARTN*, *SLC2A1*, *SLC2A5*; Gómez et al. 2018a) was investigated.

Experiment 3: Response of bovine endometrium to bovine vs murine blastocysts

In order to compare the response of bovine endometrium to embryos which intrinsically elongate (bovine) vs those that do not (murine), endometrial explants from the same uterus were cultured: (i) alone (control) or in the presence of (ii) Day 8 bovine blastocysts ($n=25$) or (iii) murine blastocysts ($n=25$). Following culture, explants were snap frozen and stored at -80°C until RNA extraction and RNA-Seq analysis. Five replicates were carried out.

To generate murine embryos, B6D2F1 female mice were superovulated by intraperitoneal injection of 7.5 IU equine chorionic gonadotrophin (Folligon 500, Intervet) followed by 7.5 IU hCG (Veterin Corion, Equinvest) 48 h later. On the night of the hCG injection, females were mated with males. Presence of a vaginal plug the next morning was designated as Day 0.5 postcoitum. Females ($n=17$) were killed by cervical dislocation on Day 2.5 and embryos were recovered by oviduct flushing. Morula stage embryos ($n=335$) were cultured in 25 µL droplets of K⁺-modified simplex optimized medium (KSOM; Millipore) under mineral oil until Day 4.

Dishes were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

Embryo sexing by PCR

In Experiment 2, PCR was used to identify embryonic sex from entire embryos following explant co-culture (Experiment 2a) and from biopsy samples (Experiment 2b), to verify the accuracy of embryo sex selection. Briefly, two primer sets were used to determine embryo sex: Y-chromosome-specific primers (*TSPY*, 5' GGTACACAAGAGCAGCGTTC forward, 5' GAGGGTGGCATAATCTGCTT reverse, 535bp amplicon) and bovine-specific satellite sequence primers (Park et al. 2001) (Sat 1.715, 5' TGGAAGCAAAGAACCCCGCT forward, 5' TCGTGAGAAACCGCACACTG reverse, 216bp amplicon). Samples were thawed, boiled at 100°C for 10min, and then held on ice. PCR reaction mix (45 µL), consisting of Dream Taq Green Master Mix (Thermo Fisher Scientific) and the primer set for *TSPY* (1 µM final concentration) was added and the tube transferred to the thermocycler. The PCR programme used an initial denaturation step at 95°C for 3 min, and then *TSPY* was pre-amplified for ten cycles of 95°C for 30s, 60°C for 45s, 72°C for 50s. After ten cycles, the tubes were held at 4°C while the primer set for satellite 1.715 (1 µM final concentration) was added, and then returned to the thermocycler for another 35 cycles on the same programme. A final extension step at 72°C for 5 min was included at the end of 35 cycles. Products were visualized on an ethidium bromide-stained 2% agarose gel under ultraviolet illumination for the positive 534bp band of *TSPY* and 216bp of the satellite sequence. Samples that exhibited both bands were assigned as male, while samples exhibiting only a satellite sequence band were assigned as female. Each PCR was carried out with three controls: negative (no template) control, female genomic DNA, male genomic DNA (Fig. 1).

Endometrial explant preparation

Bovine uteri from females estimated to be between Day 5 and 10 of the oestrous cycle based on ovarian morphology (Ireland et al. 1980) were collected at a local abattoir from post-pubertal non-pregnant heifers. Uteri were transported to the laboratory on ice and processed as described previously (Passaro et al. 2018). Briefly, the external surfaces of the uterus were washed in 70% ethanol and the uterine horn ipsilateral to the corpus luteum was opened longitudinally with sterile scissors. The exposed endometrium was washed in Dulbecco's PBS supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B. Tissue was collected from the intercaruncular areas of the endometrium from the distal part of the uterine horn (upper third) ipsilateral to the corpus luteum using a sterile 4-mm-diameter biopsy punch (Stiefel Laboratories Ltd, High Wycombe, UK). Each explant was immediately transferred to Hank's Balanced Salt Solution (HBSS) supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B, before washing twice in unsupplemented HBSS. Under sterile conditions within a class II biological safety cabinet, explants were transferred to 24-well plate, so that each well contained

Figure 1 Representative gels from embryo sexing by PCR. A single PCR using both Y-chromosome-specific primers (*TSPY*) and bovine-specific satellite sequence primers (Sat 1.715) was carried out. Lane 1: No template control. Lane 2: female gDNA. Lane 3: male gDNA. (A) From Lane 4 on: female embryos with a 216 bp specific product of Sat. (B) From lane 4 on: male embryos, a 535 bp Y-chromosome-specific product (*TSPY*) appears in the top lane.

a single explant in 1.0 mL of complete medium. Complete medium comprised Roswell Park Memorial Institute medium (RPMI 1640), supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B. The explants of intact endometrium were orientated with the

Figure 2 Representation of endometrial explant with cell culture insert used to retain *in vitro*-produced Day 8 blastocysts during co-culture.

epithelial surface uppermost and cultured in a humidified atmosphere with 5% CO₂ in air at 38.5°C. The culture medium was changed twice (every 2 h) before applying the specific treatment, according to the experimental design.

Apart from Experiment 1, in which a polyester mesh was used, cell culture inserts with a 12 µm pore size (MILLICELL-PCF 12.0 µm, Catalogue Number PIX01250, Millipore) were used during the 6 h co-culture to ensure the embryos were maintained directly above the endometrial surface (Fig. 2). A control explant (insert without embryos) was included in each replicate. To mitigate against potential variation, in each experiment, within a replicate (i.e. on a given day), each treatment was applied to explants from the same uterus, and this was replicated across multiple animals.

RNA extraction and cDNA synthesis

Total RNA was isolated using TRIzol reagent (Molecular Research Center) as per the manufacturer's instructions followed by on-column RNA clean-up using the Qiagen RNeasy mini kit (Qiagen). Briefly, 30 mg of tissue was homogenized in 1.0 mL of TRIzol using a steel bead and the Qiagen tissue lyzer (2 × 120 s at maximum speed). After homogenization, 100 µL of 1-Bromo-3-chloro-propane was added to each sample. Following centrifugation (12,000 *g*, 15 min), the upper aqueous phase was transferred directly into a RNeasy column and RNA was purified as per the manufacturer's instruction. RNA was quantified using a NanoDrop-ND1000 Spectrophotometer (Thermo Fisher Scientific).

In Experiment 2 (a-b), for each sample, cDNA was prepared from 500 ng of total RNA using the High-Capacity cDNA Reverse Transcription 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 µL and stored at –20°C for subsequent use.

RNA-Seq analysis

In Experiments 1 and 3, RNA was extracted from endometrial explants following 6-h culture. Quantity and quality of RNA were determined using the Nano Drop 1000 (Thermo Fisher Scientific) and the Agilent Bioanalyzer (Agilent Technologies), respectively. The mean RNA integrity number (RIN) ± the standard deviation was 7.4 ± 0.4 for experiment 1 and 7.6 ± 0.4 for Experiment 3. RNA library preparation and sequencing and RNA-Seq data mapping and quantification of read counts for genes were performed as described previously (Moraes *et al.* 2018). The differential gene expression analysis was performed by edgeR robust (Zhou *et al.* 2014).

Pathway and process enrichment analysis

Pathway and process enrichment analysis was carried out with using Metascape (<http://metascape.org>), an online gene annotation and analysis resource. All genes in the genome were used as the enrichment background. Terms with a *P* value <0.01, a minimum count of 3 and an enrichment factor >1.5 (the ratio between the observed counts and the counts expected by chance) are collected and grouped into clusters based on their membership similarities. More specifically, *P* values were calculated based on the accumulative hypergeometric distribution (Zar 1999), and *q* values were calculated using

the Benjamini–Hochberg procedure to account for multiple testing (Hochberg & Benjamini 1990). Kappa scores (Cohen 1960) were used as the similarity metric when performing hierarchical clustering on the enriched terms, and sub-trees with a similarity of >0.3 were considered a cluster. The most statistically significant term within a cluster is chosen to represent the cluster.

qPCR analysis of candidate genes

In Experiment 2 (a-b), qPCR was used to investigate changes in endometrial gene expression due to the presence of male or female embryos, as previously described (Passaro *et al.* 2018). Unless otherwise specified, all primers were designed using Primer-Blast software (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (Table 1). A total of eight potential reference genes (*GAPDH*, *ACTB*, *RPL18*, *PPIA*, *YWHAZ*, *RNF11*, *H3F3A*, *SDHA*) were analysed using the geNorm function within the qbase+ computer program (Biogazelle, Zwijnaarde, Belgium). For each independent sample set, geNorm was used to identify the best reference genes (Vandesompele *et al.* 2002). For Experiment 2(a), *ACTB* and *YWHAZ* were shown to be the most stably expressed (average geNorm $M \leq 0.5$), while for Experiment 2(b), *H3F3A* and *YWHAZ* were the most stably expressed. Gene expression analysis was carried out for the following genes: *MX1*, *MX2*, *ISG15*, *OAS1*, *RSAD2*, *IFNAR1*, *IFNAR2*, *CTGF*, *ARTN*, *SLC2A1*, *SLC2A5*. For Experiment 2 (a-b), one-way ANOVA was performed on the log transformed data.

Table 1 Gene abbreviation, accession number, primers sequence and reference for all genes analysed with qPCR.

Entrez gene symbol	Accession number	Primer sequence (5'–3')	Fragment size (bp)	Reference
<i>YWHAZ</i>	NM_174814.2	TGAAGCCATTGCTGAACCTG TCTCCTTGGGTATCCGATGT	114	This study
<i>H3F3A</i>	NM_001014389.2	CATGGCTCGTACAAAGCAGA ACCAGGCTGTAAACGATGAG	136	This study
<i>ACTB</i>	NM_173979.3	CAGCAGATGTGGATCAGCAAGC AACGCAGTAACAGTCCGCC	91	This study
<i>MX1</i>	NM_173940.2	CGAGCCGAGTTCTCCAAATG CAACTCTTGCCACGATACC	114	Passaro <i>et al.</i> 2018
<i>MX2</i>	NM_173941.2	ACTTTCAAGGACACAGCCAA ACCAGCTTCTCCATCCTGAA	146	Passaro <i>et al.</i> 2018
<i>ISG15</i>	NM_174366.1	CCAACCAGTGTCTGCAGAGA CCCTAGCATTCTACCGTCA	76	Passaro <i>et al.</i> 2018
<i>OAS1</i>	NM_001040606.1	CCCGGCGGACCCTACAGGAA TCCAGCCAGACCAAAGCCGC	84	Forde <i>et al.</i> 2011
<i>RSAD2</i>	NM_001045941.1	AACAGATAACCGCGCTCAAC CTTCAAACCTCCTCGTCGCTG	129	Passaro <i>et al.</i> 2018
<i>IFNAR1</i>	NM_174552.2	ACCTCCTTCCTCTGTTGACG ACATCTTCCGTTTGTTCCTCA	88	Passaro <i>et al.</i> 2018
<i>IFNAR2</i>	NM_174553.2	CTGGTCATTTGTATGGGCTCTTT GTATCCCGGGACTGTGCAATT	128	Sponchiado <i>et al.</i> 2017
<i>CTGF</i>	NM_174030.2	CACCCGGGTTACCAATGACA TTGGAGATTTGGGGGTCCG	138	This paper
<i>ARTN</i>	XM_002686516.2	AGAGAACCAGCAGTGTGAG AGGCACTTCGGCAACGAAT	89	Gómez <i>et al.</i> 2018a
<i>SLC2A1</i>	NM_174602.2	CCTGGGGCTCTTTCCTTCAG CTGGAGCCGTTAGTGTCTTG	66	This paper
<i>SLC2A5</i>	NM_001101042.2	ACCATCGGAATCCTTGTGGC GGGATCCCAGTCAATCCGAG	95	This paper

Results

Experiment 1: Global transcriptome analysis of endometrial response to bovine blastocysts

Analysis of the RNA-Seq data resulted in the identification of 63 differentially expressed transcripts (61 upregulated and 2 downregulated) in endometrial explants exposed to 20 Day 8 blastocysts for 6 h compared to control explants ($P \leq 0.001$; Table 2 and Supplementary Table 1, see section on [supplementary data](#) given at the end of this article). The most highly upregulated transcripts included *RSAD2*, *OAS1Y*, *IFI44*, *MX1*, *MX2*, *USP18* and *ISG15*. Pathway and process enrichment analysis showed that Gene Ontologies (GO) associated with specific biological processes such as defence response to virus (GO:0051607), regulation of multi-organism process (GO:0043900) and regulation of type I interferon production (GO:0032479) are significantly enriched by the differentially expressed genes. In addition, we also identified specific Reactome Gene Sets associated with Interferon Signalling (R-HSA-913531) and ISG15 antiviral mechanism (R-HSA-1169408) are also significantly enriched (Table 3).

Experiment 2: Endometrial response to male and female blastocysts

In vitro development of bovine embryos produced using sex-sorted sperm

Embryo development following IVF with X- and Y-sorted sperm from each bull is shown in Table 4. Cleavage was assessed at 48 h post insemination and blastocyst yield was recorded on Day 8. Overall, while cleavage rates were not different, IVF with X-sorted sperm resulted in lower ($P < 0.05$) blastocyst development ($8.4 \pm 2.9\%$) than insemination with Y-sorted sperm ($20.6 \pm 2.8\%$).

Sex ratio of Day 8 blastocysts

Day 8 blastocysts selected for explant culture as either male or female were predominantly of the correct sex; however, as expected, there were some inaccuracies from embryos produced with sex-sorted sperm (Table 5) and from sexing of biopsy samples (Table 6). In Experiment 2a, over the five replicates, 80.2% of the blastocysts selected as male, based on IVF with Y-sorted semen, were male and 94.1% of the blastocysts selected as female, based on IVF with Y-sorted semen, were female. In Experiment 2b, over the four replicates, 95.8% of the blastocysts selected as male, based on a biopsy taken at Day 6, were male and 76.2% of the blastocysts selected as female were female.

Response of endometrial explants to male or female blastocysts

In Experiment 2a we confirmed our previous data (Passaro *et al.* 2018) reporting an increase in transcript

abundance ($P < 0.01$) for the five classic ISG (*MX1*, *MX2*, *ISG15*, *OAS1Y*, *RSAD2*) in endometrial explants co-cultured with Day 8 blastocysts for 6 h. However, there were no differences related to embryonic sex (Fig. 3A). Similarly, in Experiment 2b, an increase ($P < 0.05$) in transcript abundance for three of the five ISG (*MX1*, *MX2*, *ISG15*) was observed following co-culture for 6 h in the presence of blastocysts; as in Experiment 2a, this was not related to embryonic sex (Fig. 3B). No difference in the relative abundance of *IFNAR1*, *IFNAR2*, *CTGF*, *ARTN*, *SLC2A1*, *SLC2A5* was observed.

Experiment 3: Response of bovine endometrium to bovine vs murine blastocysts

Representative images of bovine and murine blastocysts used for co-culture with bovine endometrial explants are shown in Fig. 4. Exposure of a bovine endometrial explants to 25 bovine blastocysts for 6 h resulted in the upregulation of 24 transcripts (Supplementary Table 2). In contrast, exposure to murine blastocysts did not elicit any detectable change in transcript abundance following RNA-Seq (Supplementary Table 2). Combining the data from Experiments 1 and 3 in terms of response of explants to bovine blastocysts ($n = 10$ replicates) revealed the differential expression of 40 transcripts (Supplementary Table 2). Among the most enriched GO terms were response to type I interferon, regulation of defence response to virus and regulation of response to cytokine stimulus (Supplementary Table 3).

Comparison of the list of differentially expressed genes in bovine endometrial explants following 6-h culture with blastocyst-stage embryos (from the combined analysis of Experiments 1 and 3 in the current study) with the common list of genes altered in endometrial explants following 6 h culture with 100 ng/mL interferon-tau (IFNT) or a Day 15 conceptus (from Sánchez *et al.* 2019) indicated that all the DEG induced in the endometrium by blastocyst-stage embryos are IFNT related (Fig. 5).

Discussion

The main findings of this study are (i) exposure of bovine endometrium to blastocyst-stage embryos results in the alteration of a number of transcripts, all of which are IFNT related; (ii) the expression of a number of candidate genes in bovine endometrium was not affected by the sex of the blastocyst and (iii) murine embryos did not elicit any detectable transcriptomic response in bovine endometrium following 6-h co-culture.

We recently demonstrated that short-term exposure of endometrial explants to bovine blastocysts (Passaro *et al.* 2018) altered the expression of classical ISGs. Here, we extended those data by examining the global transcriptome of endometrial explants exposed to blastocysts. Results of Experiment 1 revealed the

Table 2 Gene symbol, gene description, logarithm of fold change (log FC) of differentially expressed genes in bovine endometrial explants following co-culture with Day 8 blastocysts for 6 h (Experiment 1).

Gene symbol	Gene description	log FC	DE
KCND2	Potassium voltage-gated channel subfamily D member 2	5.76	UP
RSAD2	Radical S-adenosyl methionine domain containing 2	5.61	UP
MX2	MX dynamin-like GTPase 2	5.53	UP
IFI44	Interferon-induced protein 44	5.39	UP
LOC100139670	Interferon-induced protein with tetratricopeptide repeats 1	5.01	UP
IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	4.86	UP
CLEC4F	C-type lectin domain family 4 member F	4.41	UP
IFI44L	Interferon-induced protein 44 like	4.34	UP
ISG15	ISG15 ubiquitin-like modifier	4.34	UP
OAS1X	2'-5'-Oligoadenylate synthetase 40/46 kDa	3.95	UP
OAS2	2'-5'-Oligoadenylate synthetase 2	3.80	UP
USP18	Ubiquitin specific peptidase 18	3.77	UP
LOC112441507	Bone marrow stromal antigen 2-like	3.74	UP
SAMD9	Sterile alpha motif domain containing 9	3.69	UP
LOC100298356	Bone marrow stromal antigen 2	3.68	UP
MX1	MX dynamin like GTPase 1	3.59	UP
OAS1Y	2'-5'-Oligoadenylate synthetase 40/46 kDa	3.57	UP
OAS1Z	2'-5'-Oligoadenylate synthetase 40/46 kDa	3.53	UP
IFIT2	Interferon-induced protein with tetratricopeptide repeats 2	3.50	UP
KRT75	Keratin 75	3.46	UP
UBA7	Ubiquitin-like modifier activating enzyme 7	3.46	UP
LOC507055	Guanylate-binding protein 4	3.19	UP
SLFN11	Schlafen family member 11	3.03	UP
ZBP1	Z-DNA binding protein 1	2.97	UP
LOC509283	E3 ubiquitin protein ligase RNF213	2.90	UP
PARP14	Poly(ADP-ribose) polymerase family member 14	2.77	UP
EPSTI1	Epithelial stromal interaction 1	2.77	UP
CMPK2	Cytidine/uridine monophosphate kinase 2	2.65	UP
DDX58	DExD/H-box helicase 58	2.61	UP
CCDC194	Coiled-coil domain containing 194	2.53	UP
IFIH1	Interferon-induced with helicase C domain 1	2.52	UP
HERC5	HECT and RLD domain containing E3 ubiquitin protein ligase 5	2.45	UP
RTP4	Receptor transporter protein 4	2.45	UP
ZNFX1	Zinc finger NFX1-type containing 1	2.44	UP
BATF2	Basic leucine zipper ATF-like transcription factor 2	2.42	UP
LOC618737	Bone marrow stromal antigen 2	2.40	UP
LOC511531	Guanylate-binding protein 1	2.37	UP
EIF2AK2	Eukaryotic translation initiation factor 2 alpha kinase 2	2.27	UP
HERC6	HECT and RLD domain containing E3 ubiquitin protein ligase family member 6	2.22	UP
PLAC8	Placenta-specific 8	2.15	UP
IRF9	Interferon regulatory factor 9	2.04	UP
IFI27	Putative ISG12(a) protein	1.99	UP
DTX3L	Deltex E3 ubiquitin ligase 3L	1.98	UP
CGAS	Cyclic GMP-AMP synthase	1.90	UP
TIFA	TRAF-interacting protein with forkhead-associated domain	1.85	UP
DHX58	DExH-box helicase 58	1.80	UP
LOC100336669	Guanylate-binding protein 4	1.71	UP
LOC512869	Ring finger protein 213-like	1.67	UP
IRF7	Interferon regulatory factor 7	1.66	UP
XAF1	XIAP-associated factor 1	1.66	UP
PML	Promyelocytic leukaemia	1.60	UP
PARP9	Poly(ADP-ribose) polymerase family member 9	1.59	UP
LOC616948	Tripartite motif-containing protein 5-like	1.59	UP
IFIT5	Interferon-induced protein with tetratricopeptide repeats 5	1.57	UP
SP110	SP110 nuclear body protein	1.56	UP
TRIM5	Tripartite motif-containing 5	1.55	UP
PNPT1	Polyribonucleotide nucleotidyltransferase 1	1.44	UP
PARP12	Poly(ADP-ribose) polymerase family member 12	1.37	UP
PARP10	Poly(ADP-ribose) polymerase family member 10	1.30	UP
LOC512486	Interferon-induced guanylate-binding protein 1	1.27	UP
IFI16	Interferon gamma inducible protein 16	1.15	UP
LOC788077	Histone H3	-5.70	DOWN
GFY	Golgi-associated olfactory signalling regulator	-5.71	DOWN

Table 3 Gene ontology (GO) analysis of differentially expressed genes in bovine endometrial explants following co-culture with Day 8 blastocysts for 6 h (Experiment 1).

GO	Category	Description	Count	%	Log10(P)
GO:0051607	GO Biological Processes	Defence response to virus	26	50.98	-38.24
R-HSA-913531	Reactome Gene Sets	Interferon signalling	21	41.18	-29.77
GO:0043900	GO Biological Processes	Regulation of multi-organism process	16	31.37	-16.09
R-HSA-1169408	Reactome Gene Sets	ISG15 antiviral mechanism	9	17.65	-13.3
GO:0032479	GO Biological Processes	Regulation of type I interferon production	10	19.61	-13.25
GO:0060333	GO Biological Processes	Interferon-gamma-mediated signalling pathway	8	15.69	-10.58
GO:0050688	GO Biological Processes	Regulation of defence response to virus	7	13.72	-9.76
GO:0060760	GO Biological Processes	Positive regulation of response to cytokine stimulus	6	11.76	-8.72
GO:0035455	GO Biological Processes	Response to interferon-alpha	4	7.84	-6.96
R-HSA-197264	Reactome Gene Sets	Nicotinamide salvaging	3	5.88	-4.98
GO:0000209	GO Biological Processes	Protein polyubiquitination	6	11.76	-4.68
GO:0045824	GO Biological Processes	Negative regulation of innate immune response	3	5.88	-3.81
GO:2000779	GO Biological Processes	Regulation of double-strand break repair	3	5.88	-3.36
GO:0050707	GO Biological Processes	Regulation of cytokine secretion	4	7.84	-3.21
GO:0030099	GO Biological Processes	Myeloid cell differentiation	5	9.8	-2.68
GO:0002244	GO Biological Processes	Haematopoietic progenitor cell differentiation	3	5.88	-2.66
GO:0051260	GO Biological Processes	Protein homo-oligomerisation	4	7.84	-2.22
GO:0061025	GO Biological Processes	Membrane fusion	3	5.88	-2.02

Count refers to the number of genes in the given ontology term. % is the percentage of all the genes that are found in the given ontology term. Log10(P) is the *P* value in log base 10.

differential expression of 63 transcripts. Among the most upregulated were *RSAD2*, *MX2*, *ISG15* and *OAS2*. Interestingly, two transcripts (*Histone H3*, Golgi-associated olfactory signalling regulator, *GFY*) were downregulated. *Histone H3* expression levels have been used as a marker of endometrial proliferation in macaque and human endometrium (Brenner *et al.* 2003), while, to our knowledge, *GFY* has not been previously reported as differentially expressed in endometrium. Combining the data from Experiments 1 and 3 (to give a total of ten replicates per group) confirmed the upregulation of 40 transcripts in blastocyst-exposed endometrial explants compared to the control; neither *Histone H3* nor *GFY* was in this list. Comparison of this list of DEG with the common list of genes altered in endometrial explants following culture with 100 ng/mL IFNT or a Day 15 conceptus (from Sánchez *et al.* 2019) indicated that all

the DEG induced in the endometrium by blastocyst-stage embryos were IFNT stimulated. It is important to note that it was not a forgone conclusion that all the transcripts altered by a blastocyst-stage embryo would be IFNT related. Two recent studies from our group (Mathew *et al.* 2019, Sánchez *et al.* 2019) indicate that by Day 15, while the majority of altered endometrial transcripts are IFNT related, a significant number of transcripts are altered in an IFNT-independent manner. Thus, it was, we believe, not unreasonable to hypothesise that some of the changes induced at Day 8 might also be independent of IFNT.

Conceptus-derived IFNT is the primary maternal recognition of pregnancy signal in cattle (reviewed by Bazer & Thatcher 2017, Forde & Lonergan 2017) that inhibits upregulation of oxytocin receptors in the endometrial epithelia of the uterus, thereby preventing the production of luteolytic prostaglandin F₂ alpha (PGF₂α) pulses to maintain progesterone production by the corpus luteum (Hansen *et al.* 2017). In the endometrium, IFNT induces or upregulates a large number of classical ISG and regulates the expression of many other genes in a cell-specific manner that are likely important for conceptus elongation, implantation and establishment of pregnancy (Spencer *et al.* 2008). Bovine embryos begin to express IFNT upon blastocyst formation (Farin *et al.* 1990), although there is considerable variation between individual embryos in

Table 4 Cleavage rate and blastocyst yield following insemination of *in vitro*-matured bovine oocytes with X- or Y-sorted spermatozoa (Experiment 2a).

	No. oocytes	% Cleaved	% Blastocyst
Bull 1			
X sorted	439	50.2 ± 5.8	7.6 ± 3.9
Y sorted	415	58.6 ± 5.8	17.2 ± 3.9
Bull 2			
X sorted	308	75.0 ± 6.6	10.5 ± 4.4
Y sorted	394	70.4 ± 5.8	18.5 ± 3.9
Bull 3			
X sorted	397	51.6 ± 5.8	7.2 ± 3.9
Y sorted	385	68.8 ± 5.8	26.3 ± 3.9
Total			
X sorted	1144	58.9 ± 4.3	8.4 ± 2.9*
Y sorted	1194	65.9 ± 4.2	20.6 ± 2.8†

Five replicates per bull. Values are mean ± S.E.M.

*†Values with different superscripts within the same column differ significantly (*P* < 0.05).

Table 5 Verification of blastocyst sex following IVF with X- or Y-sorted sperm and subsequent blastocyst co-culture with bovine endometrial explants for 6 h (Experiment 2a, 5 replicates).

	No. of blastocysts sexed	% Male (n)	% Female (n)
X sorted	85	5.9 (5)	94.1 (80)
Y sorted	86	80.2 (69)	19.8 (17)

Table 6 Verification of blastocyst sex following embryo biopsy at Day 6, culture until Day 8 and subsequent blastocyst co-culture with bovine endometrial explants for 6 h (Experiment 2b, 4 replicates).

No. embryos biopsied on Day 6	% (n) blastocysts on Day 8	No. males based on biopsy sexing	% (n) males based on blastocyst sexing	No. females based on biopsy sexing	% (n) females based on blastocyst sexing
355	45.1 (160)	48	95.8 (46)	63	76.2 (48)

the amount they produce (Hernandez-Ledezma *et al.* 1992). This variation has been related to the origin of the embryo (Stojkovic *et al.* 1999), the age at which blastocyst formation occurs (Kubisch *et al.* 1998, 2001a), the group size in which culture takes place (Larson & Kubisch 1999), medium composition (Stojkovic *et al.* 1995, Wrenzycki *et al.* 1999, Kubisch *et al.* 2001b, Rizos *et al.* 2003) and the sex of the embryo (Larson *et al.* 2001). In contrast, later during preimplantation development, IFNT may not be the only conceptus-derived product which acts on the endometrium. For example, Mamo *et al.* (2012) described transcripts for conceptus ligands that potentially interact with corresponding receptors on the endometrium during the critical window of maternal recognition of pregnancy. Analysis of the protein content of Day 16 conceptus-conditioned culture medium with uterine luminal fluid (ULF) from cyclic and pregnant heifers on Day 16 identified 30 proteins unique to ULF from pregnant heifers which were also secreted by the conceptus *in vitro* (Forde *et al.* 2015). Lastly, comparison of transcripts altered in endometrial explants by the presence of a conceptus compared with those induced by 100 ng/mL IFNT revealed the altered expression of >100 IFNT-independent transcripts in endometrium (Mathew *et al.* 2019, Sánchez *et al.* 2019). Later during the preimplantation phase (Days 18–20), the transcriptomic response of the endometrium to pregnancy is quite specific to the type of conceptus present (AI-derived, IVF-derived, cloned) (Bauersachs *et al.* 2009, Mansouri-Attia *et al.* 2009), suggesting the endometrium is a biosensor of the developmental competency or origin of the embryo (Sandra *et al.* 2011).

The aim of Experiment 2 was to investigate sexual dimorphic alterations in the bovine endometrial transcriptome following *in vitro* exposure to Day 8 male or female blastocysts. The interest in sexual dimorphism is based on the knowledge that one-third of the transcripts present at the blastocyst stage in cattle are regulated by the sex of the embryo (Bermejo-Alvarez *et al.* 2010a). Consistent with our previous study (Passaro *et al.* 2018) and data from Experiment 1, in Experiment 2a, we observed an increase in transcript abundance of five ISG (*MX1*, *MX2*, *ISG15*, *OAS1*, *RSAD2*) in explants co-cultured with male or female blastocysts produced with sex-sorted semen; however, expression was not affected by embryonic sex. Similarly, in Experiment 2b, while transcript abundance for three of the five ISG (*MX1*, *MX2*, *ISG15*) was significantly upregulated by the presence of male or female blastocysts, there were no differences due to embryonic sex. These data are consistent with our previous study in which we failed to detect conceptus-sex-related alterations in the endometrial transcriptome *in vivo* at the initiation of implantation on Day 19 (Forde *et al.* 2016). In contrast, Gómez *et al.* (2013) reported differential protein expression in Day 8 uterine fluid recovered from heifers following the transfer of 60 Day 5 male or female embryos. More recently, Gómez *et al.* (2018a) described an embryo sex-dependent response of cultured bovine uterine epithelial (but not stromal) cells; single male embryos induced downregulation of *SLC2A1*, *SLC2A5*, *CTGF*, *ARTN*, *IFNAR1* and *IFNAR2*. While these transcripts were detectable in our dataset, they were not differentially expressed. It is important to note that these authors used cultured epithelial cells rather than

Figure 3 Quantitative real-time PCR analysis of five ISGs candidate genes (*MX1*, *MX2*, *OAS1*, *ISG15*, *RSAD2*) in bovine endometrial explants following 6-h co-culture with male or female blastocysts produced with sex-sorted semen (A) or by sexed by biopsy prior to culture (B) (Experiment 2). Relative expression values are shown (mean ± 95% confidence interval) for endometrial response to medium alone (Control, white bars); female blastocysts (black bars); male blastocysts (grey bars). Differences in gene expression between treatments ($P < 0.01$) are indicated by different superscript letters (a, b).

Figure 4 Representative images of bovine (left) and murine (right) blastocysts used for co-culture with bovine endometrial explants (Experiment 3).

intact endometrium as used here, and a much longer co-culture period (48vs 6h) which may partly explain the different observations.

Conceptus elongation is a maternally driven process dependent on secretions of the endometrium *in vivo*; it does not occur *in vitro* or *in vivo* in the absence of uterine glands (Gray *et al.* 2002). Development of bovine embryos to the blastocyst stage is supported by the reproductive tracts of other species following embryo transfer (sheep: Eyestone *et al.* 1987, Enright *et al.* 2000) or culture *ex vivo* (Mouse: Rizos *et al.* 2010). Indeed, bovine conceptuses will elongate in the ovine uterus (Rexroad & Powell 1999, Black *et al.* 2010). However, to what extent elongation is intrinsic to the embryo or whether the endometrium responds differently to embryos destined to elongate (e.g., ruminant, porcine) compared to those that do not elongate (e.g., rodent,

primate, equine) is unknown. As a first step to address this intriguing question we tested the hypothesis that murine embryos would elicit a different response from bovine endometrium than bovine embryos. Exposure to murine blastocysts did not induce any detectable changes in transcript abundance in bovine endometrial explants. While this observation is somewhat surprising, it is consistent with our previous observation that the presence of oocytes or embryos up to the morula stage did not induce changes in the expression of candidate ISG (Passaro *et al.* 2018).

In conclusion, these findings, coupled with our previous data, indicate that very local, IFNT-induced, changes in endometrial gene expression occur in response to blastocysts; whether such changes play any role in subsequent pregnancy recognition remains to be established. We believe the described blastocyst-

Figure 5 Comparison of list of differentially expressed genes in bovine endometrial explants following 6-h culture with blastocyst stage embryos (right, from the combined analysis of Experiments 1 and 3 in the current study; in yellow) with the common list of transcripts altered in endometrial explants following 6 h culture with 100 ng/mL interferon-tau (IFNT) or a Day 15 long or short conceptus (left, from Sánchez *et al.* 2019; in blue). Data indicate that all the DEG induced in the endometrium by a blastocyst are IFNT related (in contrast to Day 15 when a significant number of IFNT-independent genes are induced (Sánchez *et al.* 2019)).

induced alterations in transcript abundance are novel (not shown previously) and solid (based on ten replicates – five from each of two independent experiments in the manuscript). Further validation of these transcripts comes from the fact that they were also detected in an independent study using Day 15 conceptuses (Sánchez *et al.* 2019). Future studies we may in the future do some proteomic work on these or similar tissues in a much larger study, looking at the expression of one or two proteins here would be somewhat gratuitous and would not add much in our view.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-19-0064>.

Declaration of interest

Trudee Fair is a member of 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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