REPRODUCTION
RESEARCH

Blastocyst-induced changes in the bovine endometrial transcriptome

C Passaro[1], D Tutt[2], D J Mathew[3], J M Sanchez[1], J A Browne[1], G B Boe-Hansen[2], T Fair[1] and P Lonergan[1]

[1]*School of Agriculture and Food Science, University College Dublin, Belfield, Dublin, Ireland,* [2]*School of Veterinary Science, The University of Queensland, Gatton, Queensland, Australia and* [3]*Division of Animal and Nutritional Sciences, West Virginia University, Morgantown, West Virginia, USA*

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

Abstract

The objectives of this study were (i) to determine whether blastocyst-induced responses in endometrial explants were detectable after 6- or 24-h co-culture *in vitro*; (ii) to test if direct contact is required between embryos and the endometrial surface in order to stimulate endometrial gene expression; (iii) to establish the number of blastocysts required to elicit a detectable endometrial response; (iv) to investigate if upregulation of five interferon-stimulated genes (ISGs) in the endometrium was specific to the blastocyst stage and (v) to test if alterations in endometrial gene expression can be induced by blastocyst-conditioned medium. Exposure of endometrial explants to Day 8 blastocysts *in vitro* for 6 or 24 h induced the expression of ISGs (*MX1*, *MX2*, *OAS1*, *ISG15*, *RSAD2*); expression of *IFNAR1*, *IFNAR2*, *NFKB1*, *IL1B*, *STAT1*, *LGALS3BP*, *LGALS9*, *HPGD*, *PTGES*, *ITGB1*, *AKR1C4*, *AMD1* and *AQP4* was not affected. Culture of explants in the presence of more than five blastocysts was sufficient to induce the effect, with maximum expression of ISGs occurring in the presence of 20 blastocysts. This effect was exclusive to blastocyst stage embryos; oocytes, 2-cell embryos or Day 5 morulae did not alter the relative abundance of any of the transcripts examined. Direct contact between blastocysts and the endometrial surface was not required in order to alter the abundance of these transcripts and blastocyst-conditioned medium alone was sufficient to stimulate a response. Results support the notion that local embryo–maternal interaction may occur as early as Day 8 of pregnancy in cattle.

Reproduction (2018) **156** 219–229

Introduction

In cattle, the embryo descends into the uterus from the oviduct between Day 4 and 5 after insemination, at approximately the 16-cell stage (Hackett *et al.* 1993). By Day 7, the embryo develops to a blastocyst, characterized by the differentiation of two cell types: the inner cell mass, which will give rise to the fetus, and the trophectoderm, which will form the placenta later in development. By Day 8–9, the blastocyst hatches from the zona pellucida and develops sequentially into an ovoid (Day 13), and then tubular structure (Day 14), which subsequently begins to elongate to form a filamentous conceptus (Degrelle *et al.* 2005), occupying the entire length of the ipsilateral horn by Day 19, when implantation is initiated.

Up to the blastocyst stage, development is independent of uterine signaling, as illustrated by the relative ease with which blastocysts can be produced *in vitro*. However, after hatching from the zona pellucida, the bovine embryo becomes entirely dependent on uterine secretions for its further development. This is demonstrated by the fact that blastocysts fail to undergo elongation *in vitro* (Alexopoulos *et al.* 2005), while they will do so if transferred to synchronized recipients (Clemente *et al.* 2009). The importance of uterine luminal fluid is further demonstrated by studies in which ablation of uterine glands in ewes resulted in a failure of blastocysts to elongate after transfer (Gray *et al.* 2002).

Pregnancy recognition in cattle is initiated around Day 15–16, both at the physiological and transcriptomic level. Prior to that time, strong evidence of embryo/pregnancy-induced changes in the endometrial transcriptome is lacking (Forde *et al.* 2011, 2014, Bauersachs *et al.* 2012); in those two studies, differences in the transcriptome between pregnant and cyclic animals were not detected prior to Day 15–16, by which time the conceptus is secreting copious amounts of interferon-tau (IFNT), the pregnancy recognition signal (Forde & Lonergan 2017). Nonetheless, the first week of development is critical as evidenced by the fact that, at least in high-producing dairy cows, about 50% of embryos are no longer viable by Day 6–7 (Sartori *et al.* 2010). Whether communication between the embryo

ISSN 1470–1626 (paper) 1741–7899 (online)
https://doi.org/10.1530/REP-18-0188
Online version via www.reproduction-online.org

and endometrium at this stage is important remains to be demonstrated convincingly. There is unequivocal evidence that when development occurs *in vivo*, blastocyst quality is improved in terms of ultrastructure (Rizos *et al*. 2002*a*), gene expression profiles (Lonergan *et al*. 2003*a*,*b*), cryotolerance (Rizos *et al*. 2002*b*) and pregnancy rate after transfer (Hasler *et al*. 1995) compared to when blastocysts are produced *in vitro*. However, evidence of a reciprocal effect of a single embryo on the cells of the oviduct and/or uterus is more difficult to detect.

Several groups, including our own, have reported the use of multiple embryo transfer to study early embryo development and maternal communication (Clemente *et al*. 2011, Ledgard *et al*. 2012, Spencer *et al*. 2013, O'Hara *et al*. 2014, Gómez & Muñoz 2015). Through amplification, such an approach may facilitate the identification of molecular changes that would otherwise be difficult to observe under physiological conditions when a single embryo is present. For example, transfer of large numbers of embryos to the oviducts of recipient heifers resulted in detectable alterations in the transcriptome of the epithelial cells, which were not detectable in the presence of a single embryo (Maillo *et al*. 2015). Furthermore, while we did not detect differences in the proteome of uterine lumen fluid in pregnant and cyclic heifers at Day 7 (Passaro *et al*. 2016), differences have been reported following transfer of large numbers of embryos (up to 60) to recipients from Day 5 to Day 8 (Muñoz *et al*. 2012).

In vitro studies have demonstrated that preimplantation embryos secrete a variety of biochemical messengers, embryotropins, which act in an autocrine manner to promote embryonic development (reviewed by Wydooghe *et al*. 2015). For many of these factors, expression of corresponding receptors in the uterus has been identified, the activation of which could lead to cellular and tissue responses in regions that are in close physical contact with the embryo. Others have reported that the early bovine embryo (from Day 5 to Day 9) induces an anti-inflammatory response in uterine epithelial cells and immune cells *in vitro* (Talukder *et al*. 2017) and that uterine flushings from Day 7 superovulated and inseminated cows stimulated expression of interferon-stimulated genes (ISGs) and immune-related genes in peripheral blood mononuclear cells (Rashid *et al*. 2018). Therefore, if factors secreted by the pre-elongating embryo enhance changes in the transcriptome and in the proteome of the endometrium, those changes are most likely to be local in nature and may not be detectable using crude methods of sample collection (Fazeli & Holt 2016). Thus, there is a need to adopt alternative approaches to detect such local embryo-induced changes in the endometrium during the very early stages of pregnancy.

Most recently, local embryo-induced alterations in the endometrial transcriptome from spatially defined regions in response to the presence of a Day 7 bovine embryo were reported (Sponchiado *et al*. 2017). In that study, the presence of an embryo altered the abundance of 12 transcripts in the cranial part of the uterine horn ipsilateral to the corpus luteum, including classical ISGs (*ISG15*, *MX1*, *MX2*, *OAS1Y*), genes involved in prostaglandin biosynthesis (*PTGES*, *HPGD*, *AKR1L4*), water channels (*AQP4*) and a solute transporter (*SLC1A4*); however, the extent of change was relatively minor in nature ranging from 1.35- to 2-fold. Based on this, we hypothesized that the blastocyst induces local changes in the endometrial transcriptome through the production of interferon-tau and potentially other diffusible factors. The specific objectives of this study were (i) to determine whether embryo-induced responses in uterine endometrial explants were detectable after 6- or 24-h co-culture *in vitro*; (ii) to test if direct contact is required between embryos and the endometrial surface in order to stimulate endometrial gene expression; (iii) to establish the optimum number of blastocysts required to elicit a detectable endometrial response; (iv) to investigate if upregulation of candidate ISGs in the endometrium in response to exposure to Day 8 embryos *in vitro* was specific to the blastocyst stage and (v) and to test if alteration in endometrial gene expression can be induced by blastocyst-conditioned medium.

Materials and methods

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

In vitro *embryo production*

Bovine ovaries were collected at a local abattoir and transported to the laboratory in ~37°C saline (0.9% w/v) within 4 h. Cumulus-oocytes complexes (COCs) were aspirated from surface visible antral follicles using a 19-gauge needle attached to a 20 mL syringe. *In vitro* maturation, fertilization and culture were carried out using standard procedures. Briefly, COCs were matured for 18–22 h in maturation medium consisting of TCM 199 Earle's-buffered, 0.02 IU/mL FSH, 0.02 IU/mL LH, 100 IU/mL penicillin, 100 µg/mL streptomycin, 10% (v/v) FCS and 0.045 mM L-glutamine and 0.2 mM sodium pyruvate, under oil and incubated at 38.5°C in an atmosphere of 5% CO_2 in air with maximum humidity. Matured COCs were inseminated with a pool of frozen–thawed sperm from three highly fertile bulls. Motile sperm were separated using a swim-up procedure by placing 180 µL thawed semen under 1 mL of Sperm TALP (Parrish *et al*. 1985) and incubating at 38.5°C. After 90-min incubation, 800 µL of the upper fraction was removed and centrifuged for 20 min at 800 ***g*** to pellet the sperm. The pellet was resuspended in 100 µL Sperm TALP to sperm concentration, which was adjusted to give a final concentration of 1×10^6 sperm/mL. Fertilization (=Day 0)

was carried out in 24-well dishes, each well with a working volume of 500 µL. Fertilization medium was a modified TALP (Ball *et al.* 1983), consisting of 1.8 U/mL heparin, 200 mM caffeine and 6 mg/mL fatty acid-free BSA. Gametes were co-incubated at 38.5°C in an atmosphere of 5% CO_2 in air with maximum humidity. At approximately 22 h post insemination, presumptive zygotes were denuded of surrounding cumulus cells and accessory sperm and cultured in groups of 40 in 1 mL wells of modified KSOM with 0.5X non-essential amino acids, 3 mg/mL fatty acid-free BSA, 100 units/mL penicillin and 100 µg/mL streptomycin, under oil and cultured at 38.5°C in an atmosphere of 5% CO_2 in air with maximum humidity until Day 8.

Endometrial explant procedure

Bovine uteri from females estimated to be between Day 5 and 10 of the estrous cycle were collected at a local abattoir from post-pubertal non-pregnant heifers. Staging of the tracts was based on the study of Ireland *et al.* (1980). Uteri were kept on ice until further processing in the laboratory and processed as described by Borges *et al.* (2012). 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 phosphate-buffered saline solution (D-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 Wycome, 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 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 epithelial surface uppermost and cultured in a humidified atmosphere with 5% CO_2 in air at 38.5°C. The culture medium was changed twice (every 2 hours) before applying the specific treatment, according to the experimental design. In each of the five experiments, each treatment was applied to explants from the same uterus, and this was replicated across multiple animals.

Experiment 1: embryo-induced responses in uterine endometrial explants after 6- or 24-h co-culture

The aim of this experiment was to determine whether embryo-induced responses in uterine endometrial explants were detectable after 6 or 24 h co-culture *in vitro*. Twenty zona-enclosed *in vitro*-produced Day 8 blastocysts were cultured within a polyester mesh (Matoba *et al.* 2010) for 6 h or 24 h on an endometrial explant ($n = 8$ replicates) in 1 mL RPMI medium (Fig. 1). Control explants were cultured with the mesh but in the absence of embryos. In the absence of a mesh, a proportion of embryos tend to roll off the explant (possible due to the movement involved in placing the culture dish in the incubator and/or barely perceptible vibrations in the incubator). After incubation, explants were snap frozen and stored at −80°C until RNA extraction, reverse transcription and quantitative PCR analysis. The 18 transcripts analyzed are shown in Table 1.

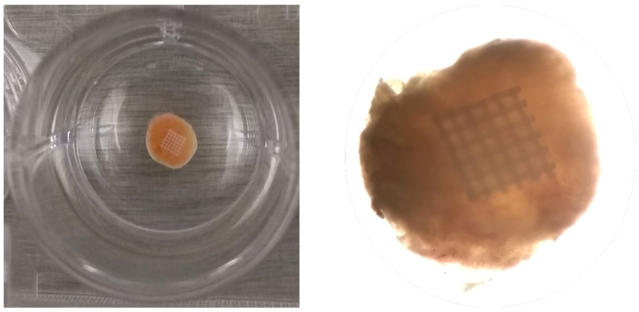

Figure 1 Endometrial explant with polyester mesh used to retain *in vitro*-produced Day 8 blastocysts during culture.

Experiment 2: differences in the transcriptomic response of endometrial explants when cultured with or without direct contact with Day 8 blastocysts

The aim of this experiment was to test if direct contact is required between the embryo and the endometrial surface in order to stimulate endometrial gene expression. While the mesh used in Experiment 1 works well, it is somewhat laborious to use and, in some cases, it too can fall off the explant. Endometrial explants, collected from the same uterus, were cultured for 6 h with medium alone (Control) or with 20 Day 8 blastocysts using a cell culture insert with a 12 µM pore size (Millipore) (preventing direct contact) or a polyester mesh (direct contact) to retain the embryos directly above the endometrial surface (five replicates in total) (Fig. 2). After incubation, explants were snap frozen and stored at −80°C until RNA extraction, reverse transcription and quantitative PCR analysis.

Experiment 3: effect of exposure to 0, 1, 5, 10 or 20 blastocysts on endometrial explant gene expression

The aim of this experiment was to test the sensitivity of endometrial explants to the presence of increasing numbers of blastocysts in terms of expression of ISGs. Endometrial explants, collected from the same uterus, were cultured for 6 h with medium alone (Control), 1, 5, 10 or 20 Day 8 blastocysts, three replicates in total. After incubation, explants were snap frozen and stored at −80°C until RNA extraction, reverse transcription and quantitative PCR analysis.

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

Entrez gene symbol	Accession number	Primer sequence (5′–3′)	Fragment size (bp)	Reference
YWHAZ	NM_174814.2	TGAAGCCATTGCTGAACTTG TCTCCTTGGGTATCCGATGT	114	This study
RNF11	NM_001077953.1	TCCGGGAGTGTGTGATCTGTATGAT GCAGGAGGGGCACGTGAAGG	131	This study
SDHA	NM_174178.2	ACTTCACCGTTGATGGCAATAA CGCAGAAATCGCATCTGAAA	59	This study
PPIA	NM_178320.2	CATACAGGTCCTGGCATCTTGTCC CACGTGCTTGCCATCCAACC	108	This study
RPL19	NM_001040516.1	GAAAGGCAGGCATATGGGTA TCATCCTCCTCATCCAGGTT	86	This study
MX1	NM_173940.2	CGAGCCGAGTTCTCCAAATG CAACTCTCTGCCACGATACC	114	This study
MX2	NM_173941.2	ACTTTCAAGGACACAGCCAA ACCAGCTTCTCCATCCTGAA	146	This study
OAS1Y	NM_001040606.1	CCCGGCGGACCCTACAGGAA TCCAGCCAGACCAAAGCCGC	84	Forde *et al.* (2011)
ISG15	NM_174366.1	CCAACCAGTGTCTGCAGAGA CCCTAGCATTCTCACCGTCA	76	This study
RSAD2	NM_001045941.1	AACAGATAACCGCGCTCAAC CTTCAAACTCCTCGTCGCTG	129	This study
IFNAR1	NM_174552.2	ACCTCCTTCCTCTGTTGACG ACATCTTTCCGTTTGTTCCTCA	88	This study
IFNAR2	NM_174553.2	CTGGTCATTTGTATGGGCTCTTT GTATCCCGGGACTGTCGAATT	128	Sponchiado *et al.* (2017)
NFKB1	NM_001076409.1	ATACTGAACAATGCCTTCCGG CACGTCAATGGCCTCAGTGTAG	135	This study
IL1B	NM_174093.1	ACCTGAACCCATCAACGAAATG TAGGGTCATCAGCCTCAAATAACA	74	This study
STAT1	XM_005202573.3	GCATTAGTCAGGGCCCAAATTGTTACAG GCCAGATACAGGAAGCTTTGCAC	139	This study
ITGB1	NM_174368.3	TCAGACTTCCGAATTGGGTTTG AAATGGGCTCGTGCAGTTCT	118	Sponchiado *et al.* (2017)
LGALS9	NM_001015570.3	TCAGCTTCCAGCCTCCAGGG TCCAGGGGCGCTGTGTATGGT	86	This study
LGALS3BP	XM_015458977.1	CAACTGCAGACACGACAAGG AGGGATTTCGCCAGATAGGT	88	This study
HPGD	NM_001034419.2	TGATCAGTGGAACCTACCTGG TGAGATTAGCAGCCATCGC	183	Oliveira *et al.* (2017)
PTGES	NM_174443.2	GCTGCGGAAGAAGGCTTTTGCC GGGCTCTGAGGCAGCGTTCC	101	Oliveira *et al.* (2017)
AKR1C4	NM_181027.2	TCCTGTCCTGGGATTTGGAACCTT ATCGGCAATCTTGCTTCGAATGGC	166	Oliveira *et al.* (2017)
AMD1	NM_173990.2	TGCTGGAGGTTTGGTTCTC TCAAAAGTATGTCCCACTCGG	96	Ramos *et al.* (2014)
AQP4	NM_181003.3	GTGTCTGTTGCAGTGAGAT CAAAGGGACCTGGGATTTAG	157	Sponchiado *et al.* (2017)

Experiment 4: transcriptomic response of endometrial explants to different stages of early embryo development

The aim of this experiment was to investigate if the transcriptomic response of the endometrium was dependent on the developmental stage of the embryo and was not simply a general inflammatory response. For this purpose, endometrial explants, collected from the same uterus, were cultured for 6 h with medium alone (Control), matured denuded oocytes, 2-cell embryos, Day 5 morulae or Day 8 blastocysts ($n = 20$ for each developmental stage and five replicates in total). Cell culture inserts with a 12 µM pore size were used during the 6 h culture to ensure the embryos were directly above the endometrial surface. After incubation, explants were snap frozen and stored at −80°C until RNA extraction, reverse transcription and quantitative PCR analysis.

Experiment 5: transcriptomic response of endometrial explants to blastocyst-conditioned medium

To extend the results of Experiment 2, the aim of this experiment was to test the effect of diffusible factors present in blastocyst-conditioned medium (BCM) on endometrial gene expression. For this purpose, endometrial explants, collected from the same uterus, were cultured for 6 h in culture medium (Control) or in BCM (four replicates in total). BCM was obtained by culturing

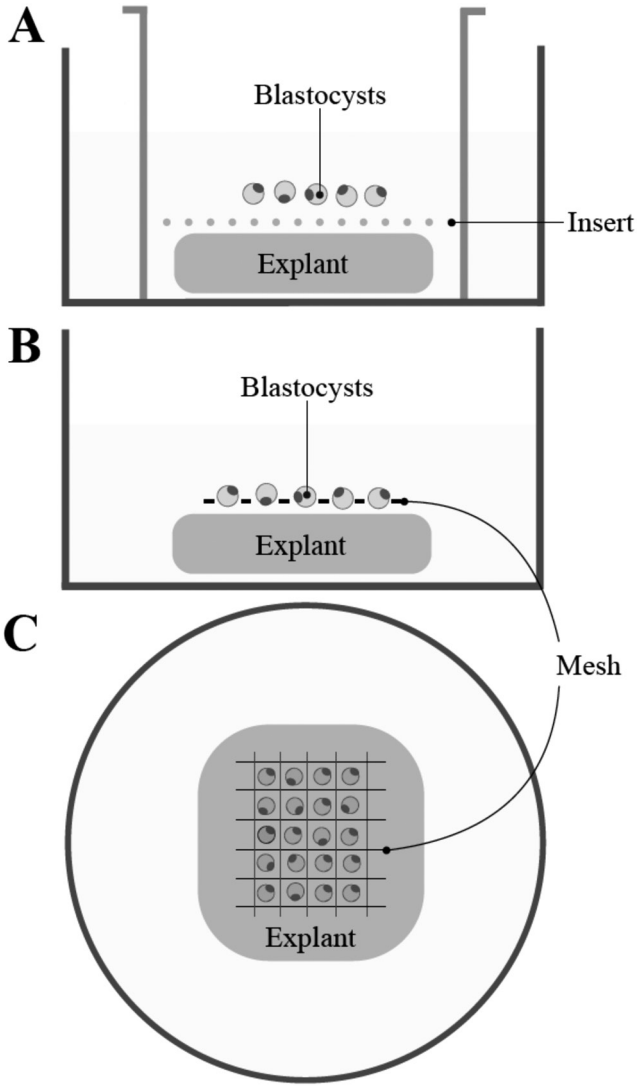

Figure 2 Representation of (A) endometrial explant with cell culture insert and (B and C) endometrial explant with polyester mesh, used to retain *in vitro*-produced Day 8 blastocysts during culture.

100 Day 7 blastocysts in 1 mL culture medium (RPMI) for 24 h. After 24 h, the blastocysts were removed from the RPMI and the medium was used to culture the endometrial explant for 6 h. Control medium was incubated in parallel during the 24-h conditioning period and was then used for explant culture for a further 6 h. After incubation, explants were snap frozen and stored at −80°C until RNA extraction, reverse transcription and quantitative PCR analysis.

RNA extraction and cDNA synthesis

Total RNA was isolated using TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA) 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 an RNeasy column, and RNA was purified as per the manufacturer's instruction. RNA was quantified using a NanoDrop-ND1000 Spectrophotometer (Thermo Fisher Scientific Inc.), and all samples were shown to a have 260/280 nm ratio greater than 1.8. 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 analysis.

Quantitative real-time PCR analysis

Quantitative real-time PCR (qPCR) was used to investigate changes in endometrial gene expression due to treatment. Unless otherwise specified, all primers were designed using Primer-Blast software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). Duplicate qPCR assays were performed in a total volume of 20 µL, containing 10 µL FastStart Universal SYBR Green Master (Roche Diagnostics Ltd.), 1.2 µL forward and reverse primer mix (300 nM final concentration), 2.6 µL nuclease-free water and 5 µL cDNA template on the ABI Prism 7500 Fast Sequence Detection System (Life Technologies), 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. A total of eight potential reference genes (*GAPDH*, *ACTB*, *RPL18*, *PPIA*, *YWHAZ*, *RNF11*, *H3F3A*, *SDHA*) were analyzed using the geNorm function with the qbase+ package (Biogazelle, Zwijnaarde, Belgium). For each independent sample set, geNorm was used to identify the best reference genes (Vandesompele *et al.* 2002). The reference genes selected were Experiment 1: *YWHAZ* and *RNF11*; Experiment 2: *RPL19* and *RNF11*; Experiment 3: *RNF11* and *SDHA*; Experiment 4: *PPIA* and *SDHA*; Experiment 5: *RPL19* and *YWHAZ*. They were shown to be the most stably expressed (average geNorm $M \leq 0.5$) and were subsequently used to normalize the gene expression data for each experiment. A standard curve was included for each gene of interest as well as for the reference genes to confirm primer efficiencies for all targets were between 90% and 110%. The threshold cycle (C_t) for each sample was automatically calculated using the default settings within the SDS software (SDS 1.4, ABI). C_t values were imported into the qbase+ analysis package. 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 C_t method ($\Delta\Delta C_q$; also known as $\Delta\Delta C_T$) (Hellemans *et al.* 2007). For Experiments 1, 2, 3 and 4 one-way ANOVA was performed on the log transformed data. For Experiment 5, as the data were not normally distributed, the Mann–Whitney test was performed.

Results

Experiment 1: embryo-induced responses in uterine endometrial explants after 6- or 24-h co-culture

Culture of endometrial explants with *in vitro*-produced Day 8 blastocysts for 6 h and 24 h increased ($P<0.01$) the relative abundance of five ISGs (*MX1*, *MX2*, *OASY1*, *ISG15*, *RSAD2*) (Fig. 3). Expression of *IFNAR1*, *IFNAR2*, *NFKB1*, *IL1B*, *STAT1*, *LGALS3BP*, *LGALS9*, *HPGD*, *PTGES*, *ITGB1*, *AKR1C4*, *AMD* and *AQP4* was not affected. There was no effect of duration of co-culture on transcript relative abundance; therefore, in all subsequent experiments, a co-culture of 6 h was used.

Experiment 2: differences in transcriptomic response of endometrial explant when cultured with or without direct contact with Day 8 blastocysts

No differences in gene expression for the five candidate ISGs were observed when endometrial explants were cultured for 6 h in direct contact or without direct contact with Day 8 blastocysts (Fig. 4). The abundance of all five transcripts was increased ($P<0.05$) in the presence of blastocysts, irrespective of direct or indirect contact. In agreement with Experiment 1, abundance of *IFNAR2*, *NFKB1*, *STAT1*, *HPGD* and *PTGES* was not affected by the presence of embryos. Based on the outcome, we used the cell culture inserts in all subsequent experiments.

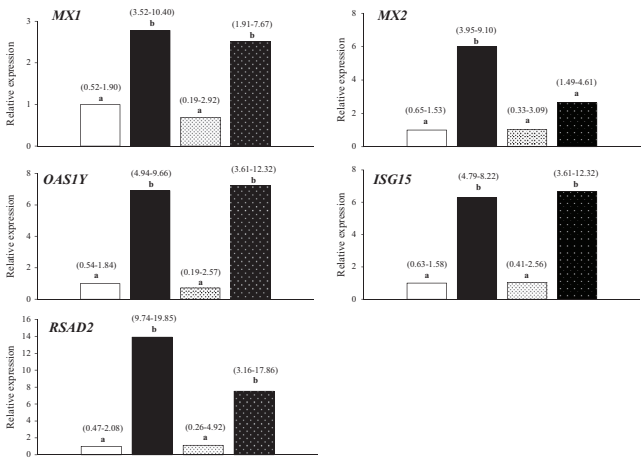

Figure 3 Quantitative real-time PCR analysis of five interferon-stimulated genes (*MX1*, *MX2*, *OAS1Y*, *ISG15*, *RSAD2*). Relative expression values are shown (mean ± 95% CI) for endometrial response to medium alone at 6 h (Control 6 h, white bars); to Day 8 blastocysts at 6 h (treatment 6 h, black bars); to medium alone at 24 h (Control 24 h, white bars black stipples); to Day 8 blastocysts at 24 h (treatment 24 h, black bars white stipples). Significant differences in gene expression between treatments are indicated by different superscript letters (a, b) when $P<0.01$.

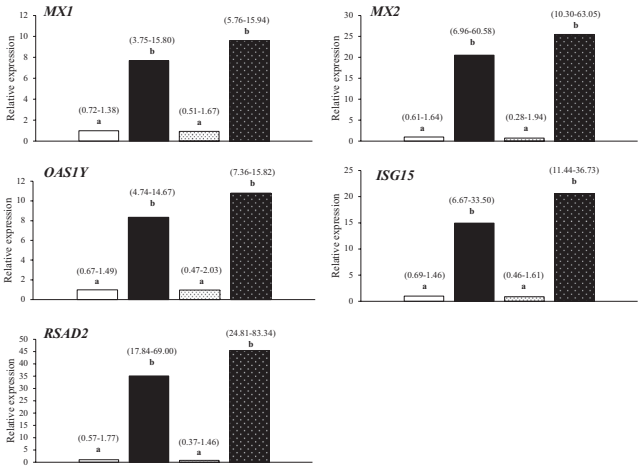

Figure 4 Quantitative real-time PCR analysis of five interferon-stimulated genes (*MX1*, *MX2*, *OAS1Y*, *ISG15*, *RSAD2*). Relative expression values are shown (mean ± 95% CI) for endometrial response to culture medium plus cell culture insert (Control Insert, white bars); Day 8 blastocysts within cell culture insert (Treatment Insert, black bars); culture medium plus polyester mesh (Control Mesh, white bars black stipples); Day 8 blastocysts within polyester mesh (Treatment Mesh, black bars white stipples). Significant differences ($P<0.01$) in gene expression between treatments are indicated by different superscript letters (a, b).

Experiment 3: effect of exposure to 0, 1, 5, 10 or 20 blastocysts on endometrial explant gene expression

Culture of endometrial explants in the presence of five or more blastocysts increased ($P<0.05$) the relative abundance of the five candidate ISGs with maximum alteration in abundance observed following exposure to 20 blastocysts (Fig. 5). Therefore, all subsequent experiments were conducted with 20 blastocysts.

Experiment 4: transcriptomic response of endometrial explants to different stages of early embryo development

Culture of endometrial explants with Day 8 blastocysts for 6 h caused an increase ($P<0.01$) in the transcript abundance of the five candidate ISGs. In contrast, exposure of explants to oocytes, 2-cell embryos or Day 5 morulae did not alter the relative abundance of the tested transcripts (Fig. 6).

Experiment 5: transcriptomic response of endometrial explants to BCM

The relative abundance of the five candidate ISGs was increased ($P<0.05$) when endometrial explants were cultured for 6 h in BCM compared to the control (Fig. 7).

Discussion

We have previously reported a lack of difference in the endometrial transcriptome (Forde *et al.* 2011) and uterine lumen fluid proteome (Passaro *et al.* 2016)

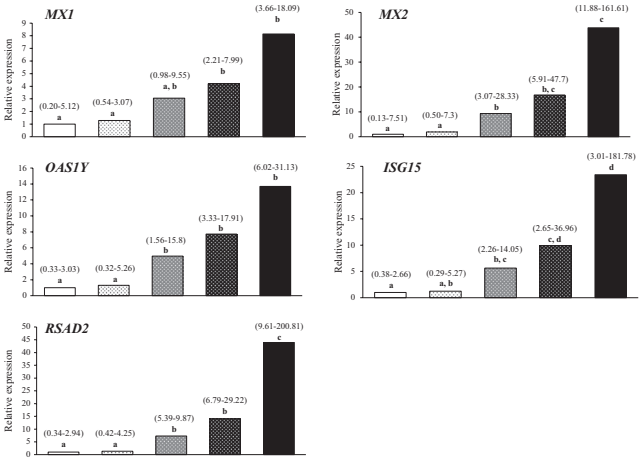

Figure 5 Quantitative real-time PCR analysis of five interferon-stimulated genes (*MX1*, *MX2*, *OAS1Y*, *ISG15*, *RSAD2*). Relative expression values are shown (mean ± 95% confidence interval) for endometrial response to medium alone (Control, white bars); 1 Day 8 blastocyst (white bars black stipples); 5 Day 8 blastocysts (light gray bars white stipples); 10 Day 8 blastocysts (black bars white stipples); 20 Day 8 blastocysts (black bars). Significant differences ($P < 0.01$) in gene expression between treatments are indicated by different superscript letters (a, b).

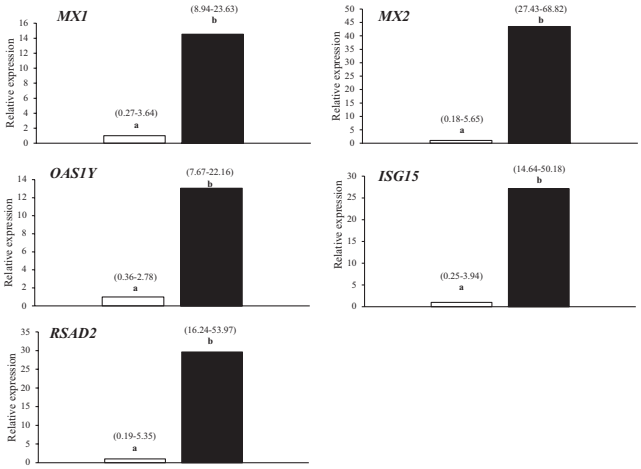

Figure 7 Quantitative real-time PCR analysis of five interferon-stimulated genes (*MX1*, *MX2*, *OAS1Y*, *ISG15*, *RSAD2*). Relative expression values are shown (mean ± 95% confidence interval) for endometrial response to RPMI culture medium (Control, white bars); blastocyst-conditioned medium (BCM, black bars). Significant differences ($P < 0.05$) in gene expression between treatments are indicated by different superscript letters (a, b).

of pregnant and non-pregnant heifers on Day 7 post estrus. Here, we used an *ex vivo* model of intact bovine endometrium to amplify any potential embryo-derived signals to investigate potential local effects of blastocyst stage embryos on endometrial gene expression. We extended those observations by investigating how many blastocysts were required to induce the effect, whether this altered gene expression was specific to

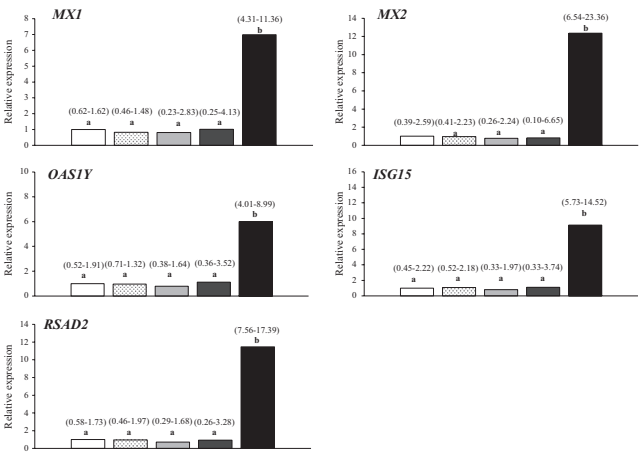

Figure 6 Quantitative real-time PCR analysis of five interferon-stimulated genes (*MX1*, *MX2*, *OAS1Y*, *ISG15*, *RSAD2*). Relative expression values are shown (mean ± 95% confidence interval) for endometrial response to medium alone (Control, white bars); Oocytes (white bars black stipples); 2-cell embryos (light gray bars); Day 5 embryos (dark gray bars); Day 8 blastocysts (black bars). Significant differences ($P < 0.01$) in gene expression between treatments are indicated by different superscript letters (a, b).

the blastocyst stage, whether direct contact between embryos and the endometrial surface was necessary to induce the endometrial response and whether the same effect can be observed in the presence of BCM. The main findings of this study are (1) the ability to detect a response of the endometrium to the embryo is dependent on the number of embryos present, (2) the response of the endometrium to the early embryo is stage specific, (3) direct contact between the embryo and the endometrium is not required to induce expression of candidate ISGs and (4) diffusible factors present in BCM alter the expression of ISGs in the endometrium.

The fact that blastocysts can be produced routinely *in vitro* in the absence of contact with the reproductive tract and subsequently establish a pregnancy after transfer to a recipient supports the notion that exposure of the reproductive tract to the early embryo, or vice versa, is not required for pregnancy. Consistent with these observations, previous global gene expression studies from our group (Forde *et al.* 2011) and others (Bauersachs *et al.* 2012) failed to detect differences in gene expression between pregnant and non-pregnant heifers prior to Day 15–16. However, recent *in vitro* studies (Talukder *et al.* 2017, Rashid *et al.* 2018) and one *in vivo* study (Sponchiado *et al.* 2017) have provided limited evidence of embryo-induced altered gene expression in the cells of the endometrium. Consistent with the latter study, in Experiment 1, exposure of endometrial explants to *in vitro*-produced Day 8 blastocysts for 6 h or 24 h caused a significant upregulation of five ISGs (*MX1*, *MX2*, *OASY1*, *ISG15*, *RSAD2*). Expression of *IFNAR1*, *IFNAR2*, *NFKB1*, *IL1B*, *STAT1*, *LGALS3BP*, *LGALS9*, *HPGD*, *PTGES*, *ITGB1*, *AKR1C4*, *AMD1* and

AQP4, which have been reported to be altered by the early embryo in other studies (Sponchiado *et al.* 2017, Talukder *et al.* 2017, Gómez *et al.* 2018) was not affected. Sponchiado *et al.* (2017) investigated changes in the transcriptome of spatially defined regions in the endometrium in response to Day 7 embryo *in vivo*, reporting that the abundance of 12 transcripts, including *ISG15*, *MX1*, *MX2* and *OAS1Y*, was modulated but only in the endometrial region in proximity with the embryo. Talukder *et al.* (2017) investigated the effect of the early developing embryo on the immune-related gene profile in bovine uterine epithelial cells (BUEC) *in vitro*. They found that the embryo modulated BUEC gene expression, inducing *ISG15*, *OASY1*, *MX2*, *STAT1*, *IFNAR1*, *IFNAR2*, *PTGES* and *PGE2* and with the suppression of *NFKB2*, *NFKBIA TNFA* and *IL1B*. We did not detect any embryo-induced alteration in the expression of *IFNAR2*, *STAT1*, *NFKB1*, *HPGD* and *PTGES* under the conditions of our study. The reasons for this discrepancy are unclear but may be due to the different systems employed in both studies (monolayer of epithelial cells vs intact endometrial explant).

Results of Experiment 3 demonstrated that more than five blastocysts were required to detect a significant increase in ISG expression, with maximum alteration in expression seen with 20 blastocysts; therefore, 20 blastocysts were used in all subsequent experiments. In Experiment 4, endometrial explants were exposed to mature oocytes or embryos at different stages of early development (two-cell embryos; Day 5 morulae; Day 8 blastocysts). Upregulation of the five candidate ISGs was only observed following exposure to blastocysts, suggesting that the response of the endometrium is most likely related to blastocyst-secreted IFNT and not simply a general inflammatory response. IFNT is the primary agent responsible for maternal recognition of pregnancy in cattle (reviewed by Bazer & Thatcher 2017, Forde & Lonergan 2017) involving suppression of the endometrial luteolytic mechanism to maintain progesterone production by the corpus luteum via upregulation of oxytocin receptors in the endometrial epithelia of the uterus, thereby preventing the production of luteolytic prostaglandin F2 alpha (PGF2α) pulses. In the endometrium, IFNT induces or upregulates a large number of classical ISGs and regulates expression of many other genes in a cell-specific manner that are likely important for conceptus elongation, implantation and establishment of pregnancy (Hansen *et al.* 2017).

Bovine embryos begin to express IFNT as the blastocyst forms (Farin *et al.* 1990), although there is considerable variability between individual embryos in the amount they produce (Hernandez-Ledezma *et al.* 1992), which may be related to the origin of the embryo (Stojkovic *et al.* 1999), the age at which blastocyst formation occurs (Kubisch *et al.* 1998, 2001*a*), the group size in which culture takes place (Larson & Kubisch 1999), the medium composition (Stojkovic *et al.* 1995, Wrenzycki *et al.* 1999, Kubisch *et al.* 2001*b*, Rizos *et al.* 2003) or to the sex of the embryo (Larson *et al.* 2001). In a previous study from our group (Rizos *et al.* 2003), we observed a significantly higher level of expression of IFNT mRNA in blastocysts produced in the absence of serum, which was correlated with higher cryotolerance, consistent with the notion that mRNA levels for this transcript are higher in good-quality embryos. In agreement, Wrenzycki *et al.* (1999) reported increased levels of IFNT mRNA in hatched blastocysts produced in the presence of polyvinyl alcohol, compared with those in which serum was present. In contrast to these observations, transcripts levels for IFNT have been reported to be significantly higher in *in vitro*-cultured embryos compared with those derived *in vivo* (Wrenzycki *et al.* 2001, Lonergan *et al.* 2003*a*,*b*). It may be that the temporal expression of IFNT transcripts is a better indicator of embryo quality than the absolute expression at a particular stage because the latter is known to vary widely (Hernandez-Ledezma *et al.* 1992). Indeed, Kubisch *et al.* (1998) observed a negative relationship between early IFNT production and developmental competence.

In Experiment 2, endometrial explants were cultured with or without direct contact with Day 8 blastocysts for 6 h. We observed that direct contact was not required in order to enhance the transcription of the candidate ISGs. Similarly, we found medium conditioned by blastocyst culture was sufficient to enhance the transcription of those genes in the endometrial explants (Experiment 5). This is supported by previous work in which the biological activity of IFNT was detected in BCM, where blastocysts were produced *in vitro* or *in* vivo (Kubisch *et al.* 1998, 2001*a*,*b*, Kimura *et al.* 2004).

Clearly, IFNT does not act on the endometrium in a vacuum and there are likely other conceptus-derived factors within the uterine environment that could influence endometrial gene expression. For example, the Day 13 bovine conceptus secretes prostaglandins that act locally in a paracrine manner to alter gene expression in the endometrium prior to pregnancy recognition in cattle (Spencer *et al.* 2013). Furthermore, comparison of the protein content of the uterine luminal fluid from cyclic and pregnant heifers on Day 16 and Day 16 conceptus-conditioned culture medium revealed 30 proteins produced by the conceptus, which contribute to the composition of uterine lumen fluid around pregnancy recognition (Forde *et al.* 2015). Lastly, using an explant culture model similar to that described in the current study in the presence of IFNT or a conceptus, we have recently demonstrated that endometrial gene expression is altered by the conceptus in both an IFNT-dependent and -independent manner (Mathew *et al.* 2017, Sánchez *et al.* 2016).

In conclusion, results from the present study contribute to the concept that the early embryo is capable of communicating with the reproductive tract. The effect on the endometrial transcriptome is dependent on the

stage of embryo development and is due to diffusible substances, most likely IFNT, but potentially other factors also, secreted by the blastocyst. Direct contact is not required between embryos and the endometrial surface in order to upregulate ISGs and BCM alone is sufficient to stimulate a response. Failure to detect embryo/pregnancy-induced alterations in gene expression in the endometrium *in vivo* prior to conceptus elongation in cattle in several studies is likely due to the small size of the embryo, which may elicit a very local effect on the endometrium that is undetectable by transcriptome analysis of a relatively large endometrial sample. The functional significance of such induced changes remains to be fully elucidated given that it is possible to transfer embryos from Day 7 onward to a uterus that has not previously been exposed to an embryo and achieve normal pregnancy rates.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. Trudee Fair is a member of the Editorial Board of Reproduction.

Funding

This work was supported by the Science Foundation Ireland (13/IA/1983).

Acknowledgments

The authors thank Dr Nana Satake (The University of Queensland, Australia) who assisted in sample collection. They also thank Dr Lisa Kidd (The University of Queensland, Australia) for contributing resources and Dr Constantine Simintiras (University College Dublin, Ireland) for helping with figures.

References

Alexopoulos NI, Vajta G, Maddox-Hyttel P, French AJ & Trounson AO 2005 Stereomicroscopic and histological examination of bovine embryos following extended in vitro culture. *Reproduction, Fertility and Development* **17** 799–808. (https://doi.org/10.1071/RD04104)

Ball GD, Leibfried ML, Lenz RW, Ax RL, Bavister BD & First NL 1983 Factors affecting successful in vitro fertilization of bovine follicular oocytes. *Biology of Reproduction* **28** 717–725. (https://doi.org/10.1095/biolreprod28.3.717)

Bauersachs S, Ulbrich SE, Reichenbach H-D, Reichenbach M, Büttner M, Meyer HHD, Spencer TE, Minten M, Sax G, Winter G *et al.* 2012 Comparison of the effects of early pregnancy with human interferon, alpha 2 (IFNA2), on gene expression in bovine endometrium. *Biology of Reproduction* **86** 46. (https://doi.org/10.1095/biolreprod.111.094771)

Bazer FW & Thatcher WW 2017 Chronicling the discovery of interferon tau. *Reproduction* **154** F11–F20. (https://doi.org/10.1530/REP-17-0257)

Borges ÁM, Healey GD & Sheldon IM 2012 Explants of intact endometrium to model bovine innate immunity and inflammation ex vivo. *American Journal of Reproductive Immunology* **67** 526–539. (https://doi.org/10.1111/j.1600-0897.2012.01106.x)

Clemente M, de La Fuente J, Fair T, Al Naib A, Gutierrez-Adan A, Roche JF, Rizos D & Lonergan P 2009 Progesterone and conceptus elongation in cattle: a direct effect on the embryo or an indirect effect via the endometrium? *Reproduction* **138** 507–517. (https://doi.org/10.1530/REP-09-0152)

Clemente M, Lopez-Vidriero I, O'Gaora P, Mehta JP, Forde N, Gutierrez-Adan A, Lonergan P & Rizos D 2011 Transcriptome changes at the initiation of elongation in the bovine conceptus. *Biology of Reproduction* **85** 285–295. (https://doi.org/10.1095/biolreprod.111.091587)

Degrelle SA, Campion E, Cabau C, Piumi F, Reinaud P, Richard C, Renard J-P & Hue I 2005 Molecular evidence for a critical period in mural trophoblast development in bovine blastocysts. *Developmental Biology* **288** 448–460. (https://doi.org/10.1016/j.ydbio.2005.09.043)

Farin CE, Imakawa K, Hansen TR, McDonnell JJ, Murphy CN, Farin PW & Roberts RM 1990 Expression of trophoblastic interferon genes in sheep and cattle. *Biology of Reproduction* **43** 210–218. (https://doi.org/10.1095/biolreprod43.2.210)

Fazeli A & Holt WV 2016 Cross talk during the periconception period. *Theriogenology* **86** 438–442. (https://doi.org/10.1016/j.theriogenology.2016.04.059)

Forde N & Lonergan P 2017 Interferon-tau and fertility in ruminants. *Reproduction* **154** F33–F43. (https://doi.org/10.1530/REP-17-0432)

Forde N, Carter F, Spencer TE, Bazer FW, Sandra O, Mansouri-Attia N, Okumu LA, McGettigan PA, Mehta JP, McBride R *et al.* 2011 Conceptus-induced changes in the endometrial transcriptome: how soon does the cow know she is pregnant? *Biology of Reproduction* **85** 144–156. (https://doi.org/10.1095/biolreprod.110.090019)

Forde N, Simintiras CA, Sturmey R, Mamo S, Kelly AK, Spencer TE, Bazer FW & Lonergan P 2014 Amino acids in the uterine luminal fluid reflects the temporal changes in transporter expression in the endometrium and conceptus during early pregnancy in cattle. *PLoS ONE* **9** e100010. (https://doi.org/10.1371/journal.pone.0100010)

Forde N, Bazer FW, Spencer TE & Lonergan P 2015 'Conceptualizing' the endometrium: identification of conceptus-derived proteins during early pregnancy in cattle. *Biology of Reproduction* **92** 156. (https://doi.org/10.1095/biolreprod.115.129296)

Gómez E & Muñoz M 2015 Multiple-embryo transfer for studying very early maternal-embryo interactions in cattle. *Reproduction* **150** R35–R43. (https://doi.org/10.1530/REP-14-0465)

Gómez E, Sánchez-Calabuig MJ, Martin D, Carrocera S, Murillo A, Correia-Alvarez E, Herrero P, Canela N, Gutiérrez-Adán A, Ulbrich S *et al.* 2018 In vitro cultured bovine endometrial cells recognize embryonic sex. *Theriogenology* **108** 176–184. (https://doi.org/10.1016/j.theriogenology.2017.11.038)

Gray CA, Burghardt RC, Johnson GA, Bazer FW & Spencer TE 2002 Evidence that absence of endometrial gland secretions in uterine gland knockout ewes compromises conceptus survival and elongation. *Reproduction* **124** 289–300. (https://doi.org/10.1530/rep.0.1240289)

Hackett AJ, Durnford R, Mapletoft RJ & Marcus GJ 1993 Location and status of embryos in the genital tract of superovulated cows 4 to 6 days after insemination. *Theriogenology* **40** 1147–1153. (https://doi.org/10.1016/0093-691X(93)90285-D)

Hansen TR, Sinedino LDP & Spencer TE 2017 Paracrine and endocrine actions of interferon tau (IFNT). *Reproduction* **154** F45–F59. (https://doi.org/10.1530/REP-17-0315)

Hasler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, Neely B, Shuey LS, Stokes JE & Trimmer SA 1995 Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology* **43** 141–152. (https://doi.org/10.1016/0093-691X(94)00020-U)

Hellemans J, Mortier G, De Paepe A, Speleman F & Vandesompele J 2007 qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biology* **8** R19. (https://doi.org/10.1186/gb-2007-8-2-r19)

Hernandez-Ledezma JJ, Sikes JD, Murphy CN, Watson AJ, Schultz GA & Roberts RM 1992 Expression of bovine trophoblast interferon in conceptuses derived by in vitro techniques. *Biology of Reproduction* **47** 374–380. (https://doi.org/10.1095/biolreprod47.3.374)

Ireland JJ, Murphee RL & Coulson PB 1980 Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. *Journal of Dairy Science* **63** 155–160. (https://doi.org/10.3168/jds.S0022-0302(80)82901-8)

Kimura K, Spate LD, Green MP, Murphy CN, Seidel GE & Roberts RM 2004 Sexual dimorphism in interferon-tau production by in vivo-derived bovine embryos. *Molecular Reproduction and Development* **67** 193–199. (https://doi.org/10.1002/mrd.10389)

Kubisch HM, Larson MA & Roberts RM 1998 Relationship between age of blastocyst formation and interferon-tau secretion by in vitro-derived bovine embryos. *Molecular Reproduction and Development* **49** 254–260. (https://doi.org/10.1002/(SICI)1098-2795(199803)49:3<254::AID-MRD5>3.0.CO;2-N)

Kubisch HM, Larson MA & Kiesling DO 2001*a* Control of interferon-tau secretion by in vitro-derived bovine blastocysts during extended culture and outgrowth formation. *Molecular Reproduction and Development* **58** 390–397. (https://doi.org/10.1002/1098-2795(20010401)58:4<390::AID-MRD6>3.0.CO;2-V)

Kubisch HM, Larson MA, Ealy AD, Murphy CN & Roberts RM 2001*b* Genetic and environmental determinants of interferon-tau secretion by in vivo- and in vitro-derived bovine blastocysts. *Animal Reproduction Science* **66** 1–13. (https://doi.org/10.1016/S0378-4320(01)00086-0)

Larson MA & Kubisch HM 1999 The effects of group size on development and interferon-tau secretion by in-vitro fertilized and cultured bovine blastocysts. *Human Reproduction* **14** 2075–2079. (https://doi.org/10.1093/humrep/14.8.2075)

Larson MA, Kimura K, Kubisch HM & Roberts RM 2001 Sexual dimorphism among bovine embryos in their ability to make the transition to expanded blastocyst and in the expression of the signaling molecule IFN-tau. *PNAS* **98** 9677–9682. (https://doi.org/10.1073/pnas.171305398)

Ledgard AM, Berg MC, McMillan WH, Smolenski G & Peterson AJ 2012 Effect of asynchronous transfer on bovine embryonic development and relationship with early cycle uterine proteome profiles. *Reproduction, Fertility and Development* **24** 962–972. (https://doi.org/10.1071/RD11225)

Lonergan P, Rizos D, Gutierrez-Adán A, Moreira PM, Pintado B, de la Fuente J & Boland MP 2003*a* Temporal divergence in the pattern of messenger RNA expression in bovine embryos cultured from the zygote to blastocyst stage in vitro or in vivo. *Biology of Reproduction* **69** 1424–1431. (https://doi.org/10.1095/biolreprod.103.018168)

Lonergan P, Rizos D, Kanka J, Nemcova L, Mbaye AM, Kingston M, Wade M, Duffy P & Boland MP 2003*b* Temporal sensitivity of bovine embryos to culture environment after fertilization and the implications for blastocyst quality. *Reproduction* **126** 337–346. (https://doi.org/10.1530/rep.0.1260337)

Maillo V, Gaora PÓ, Forde N, Besenfelder U, Havlicek V, Burns GW, Spencer TE, Gutierrez-Adan A, Lonergan P & Rizos D 2015 Oviduct-embryo interactions in cattle: two-way traffic or a one-way street? *Biology of Reproduction* **92** 144. (https://doi.org/10.1095/biolreprod.115.127969)

Mathew DJ, Sánchez JM, Passaro C, Charpigny G, Behura S, Spencer TE & Lonergan P 2017 Interferon tau (IFNT)-dependent and -independent effects of the bovine conceptus on the endometrium during early pregnancy. In *Proceedings of the 50th Annual Meeting of the Society for the Study of Reproduction*, Washington D.C., 13–16 July 2017, Abstract P87.

Matoba S, Fair T & Lonergan P 2010 Maturation, fertilisation and culture of bovine oocytes and embryos in an individually identifiable manner: a tool for studying oocyte developmental competence. *Reproduction, Fertility and Development* **22** 839–851. (https://doi.org/10.1071/RD09277)

Muñoz M, Corrales FJ, Caamaño JN, Díez C, Trigal B, Mora MI, Martín D, Carrocera S & Gómez E 2012 Proteome of the early embryo-maternal dialogue in the cattle uterus. *Journal of Proteome Research* **11** 751–766. (https://doi.org/10.1021/pr200969a)

O'Hara L, Forde N, Carter F, Rizos D, Maillo V, Ealy AD, Kelly AK, Rodriguez P, Isaka N, Evans ACO *et al.* 2014 Paradoxical effect of supplementary progesterone between Day 3 and Day 7 on corpus luteum function and conceptus development in cattle. *Reproduction, Fertility and Development* **26** 328–336. (https://doi.org/10.1071/RD12370)

Oliveira ML, D'Alexandri FL, Pugliesi G, Van Hoeck V, Mesquita FS, Membrive CMB, Negrão JA, Wheelock CE & Binelli M 2017 Peri-ovulatory endocrine regulation of the prostanoid pathways in the bovine uterus at early dioestrus. *Reproduction, Fertility and Development* **29** 544–556. (https://doi.org/10.1071/RD15269)

Parrish JJ, Susko-Parrish JL & First NL 1985 Effect of heparin and chondroitin sulfate on the acrosome reaction and fertility of bovine sperm in vitro. *Theriogenology* **24** 537–549. (https://doi.org/10.1016/0093-691X(85)90060-3)

Passaro C, Forde N, Spencer TE & Lonergan P 2016 73 Proteomic analysis of uterine luminal fluid on Day 7 of pregnancy in cattle. *Reproduction, Fertility and Development* **28** 166–166. (https://doi.org/10.1071/RDv28n2Ab73)

Ramos R dos S, Mesquita FS, D'Alexandri FL, Gonella-Diaza AM, Papa P de C & Binelli M 2014 Regulation of the polyamine metabolic pathway in the endometrium of cows during early diestrus. *Molecular Reproduction and Development* **81** 584–594. (https://doi.org/10.1002/mrd.22323)

Rashid MB, Talukder AK, Kusama K, Haneda S, Takedomi T, Yoshino H, Moriyasu S, Matsui M, Shimada M, Imakawa K & Miyamoto A 2018 Evidence that interferon-tau secreted from Day-7 embryo in vivo generates anti-inflammatory immune response in the bovine uterus. *Biochemical and Biophysical Research Communications* **500** 879–884. (https://doi.org/10.1016/j.bbrc.2018.04.178)

Rizos D, Fair T, Papadopoulos S, Boland MP & Lonergan P 2002*a* Developmental, qualitative, and ultrastructural differences between ovine and bovine embryos produced in vivo or in vitro. *Molecular Reproduction and Development* **62** 320–327. (https://doi.org/10.1002/mrd.10138)

Rizos D, Ward F, Duffy P, Boland MP & Lonergan P 2002*b* Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. *Molecular Reproduction and Development* **61** 234–248. (https://doi.org/10.1002/mrd.1153)

Rizos D, Gutiérrez-Adán A, Pérez-Garnelo S, De La Fuente J, Boland MP & Lonergan P 2003 Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression. *Biology of Reproduction* **68** 236–243. (https://doi.org/10.1095/biolreprod.102.007799)

Sánchez JM, Mathew DJ, Passaro C, Charpigny G, Behura S, Butler ST, Spencer, TE & Lonergan P 2016 Effect of conceptus size on embryo-maternal communication during early pregnancy in cattle. In *Proceedings of the 33rd Annual Scientific Meeting of the European Embryo Transfer Association (AETE)*, Bath, United Kingdom, September 8th and 9th, 2017. Animal Reproduction 14 979 Abstract A335E.

Sartori R, Bastos MR & Wiltbank MC 2010 Factors affecting fertilisation and early embryo quality in single- and superovulated dairy cattle. *Reproduction, Fertility, and Development* **22** 151–158. (https://doi.org/10.1071/RD09221)

Spencer TE, Forde N, Dorniak P, Hansen TR, Romero JJ & Lonergan P 2013 Conceptus-derived prostaglandins regulate gene expression in the endometrium prior to pregnancy recognition in ruminants. *Reproduction* **146** 377–387. (https://doi.org/10.1530/REP-13-0165)

Sponchiado M, Gomes NS, Fontes PK, Martins T, Del Collado M, Pastore A de A, Pugliesi G, Nogueira MFG & Binelli M 2017 Pre-hatching embryo-dependent and -independent programming of endometrial function in cattle. *PLoS ONE* **12** e0175954. (https://doi.org/10.1371/journal.pone.0175954)

Stojkovic M, Wolf E, Büttner M, Berg U, Charpigny G, Schmitt A & Brem G 1995 Secretion of biologically active interferon tau by in vitro-derived bovine trophoblastic tissue. *Biology of Reproduction* **53** 1500–1507. (https://doi.org/10.1095/biolreprod53.6.1500)

Stojkovic M, Büttner M, Zakhartchenko V, Riedl J, Reichenbach HD, Wenigerkind H, Brem G & Wolf E 1999 Secretion of interferon-tau by bovine embryos in long-term culture: comparison of in vivo derived, in vitro produced, nuclear transfer and demi-embryos. *Animal Reproduction Science* **55** 151–162. (https://doi.org/10.1016/S0378-4320(99)00015-9)

Talukder AK, Yousef MS, Rashid MB, Awai K, Acosta TJ, Shimizu T, Okuda K, Shimada M, Imakawa K & Miyamoto A 2017 Bovine embryo induces an anti-inflammatory response in uterine epithelial cells and immune cells in vitro: possible involvement of interferon tau as an intermediator. *Journal of Reproduction and Development* **63** 425–434. (https://doi.org/10.1262/jrd.2017-056)

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A & Speleman F 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3** Research0034. (https://doi.org/10.1186/gb-2002-3-7-research0034)

Wrenzycki C, Herrmann D, Carnwath JW & Niemann H 1999 Alterations in the relative abundance of gene transcripts in preimplantation bovine embryos cultured in medium supplemented with either serum or PVA. *Molecular Reproduction and Development* **53** 8–18. (https://doi.org/10.1002/(SICI)1098-2795(199905)53:1<8::AID-MRD2>3.0.CO;2-K)

Wrenzycki C, Herrmann D, Keskintepe L, Martins A, Sirisathien S, Brackett B & Niemann H 2001 Effects of culture system and protein supplementation on mRNA expression in pre-implantation bovine embryos. *Human Reproduction* **16** 893–901. (https://doi.org/10.1093/humrep/16.5.893)

Wydooghe E, Vandaele L, Heras S, De Sutter P, Deforce D, Peelman L, De Schauwer C & Van Soom A 2015 Autocrine embryotropins revisited: how do embryos communicate with each other in vitro when cultured in groups? *Biological Reviews of the Cambridge Philosophical Society* **92** 505–520. (https://doi.org/10.1111/brv.12241)

Received: 13 April 2018
First decision 9 May 2018
Revised manuscript received 22 May 2018
Accepted 12 June 2018