Molecular interactions at the bovine embryo–endometrial epithelium interface

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Abstract

In cattle, pre-implantation embryo development occurs within the confinement of the uterine lumen. Current understanding of the bi-lateral molecular interactions between embryo and endometrium that are required for a successful pregnancy is limited. We hypothesized that the nature and intensity of reciprocal embryo-endometrium interactions depend on the extent of their physical proximity. Bovine endometrial epithelial cells (bEECs) and morulae were co-cultured in juxtacrine (Contact+) or non-juxtacrine (Contact–) apposition. Co-culture with bEECs improved blastocyst rates on day 7.5, regardless of juxtaposition. Contact+ regulated transcription of 1797 endometrial genes vs only 230 in the Contact– group compared to their control (no embryos) counterparts. A subset of 50 overlapping differentially expressed genes (DEGs) defined embryo-induced effects on bEEC transcriptome irrespective of juxtaposition. Functional analysis revealed pathways associated with interferon signaling and prostanoid biosynthesis. A total of 175 genes displayed a graded expression level depending on Contact+ or Contact–. These genes were involved in interferon-related and antigen presentation pathways. Biological processes enriched exclusively in Contact+ included regulation of cell cycle and sex-steroid biosynthesis. We speculate that, *in vivo*, embryonic signals fine-tune the function of surrounding cells to ultimately maximize pregnancy success.

Reproduction (2020) **160** 887–903

Introduction

The endometrial epithelium is the point of contact between the maternal tissues and the embryo *in utero*. Tightly coordinated cellular and molecular interactions between the competent embryo and the receptive endometrium need to be established to safeguard the pregnancy in eutherian mammals. Disturbances in this complex communication result in early embryonic losses and, therefore, subfertility. This is of special importance in cattle, since up to 40% of pregnancies fail within the first three weeks of pregnancy (Diskin *et al.* 2011, 2016).

The embryo–maternal interaction involves dynamic processes precisely coordinated by both the maternal and the embryonic units. In the cow, it is expected that the developing embryo starts to interact with the endometrium as soon as day 5 post-fertilization when the zona pellucida-enclosed morula enters the uterus (Guillomot 1995). The embryo *in utero* is tightly surrounded by the endometrial luminal epithelium (Wolf *et al.* 2003); however, until day 20, there is no

anatomical union between the conceptus (embryo and associated membranes) and the endometrium. Thus, signaling molecules play an important role in establishing the first embryo–maternal communication. The actions of those molecules can be directed from the embryo toward the maternal tissue, and vice-versa, in a paracrine (i.e. molecules from one cell affect cells in its close proximity to it) or juxtacrine fashion (i.e. molecules from one cell affect cells in physical contact with it). One well-known example of paracrine signaling is the embryo-produced Interferon-tau (IFNT), which is released into the uterine lumen and acts in the endometrium to abrogate the luteolytic cascade (Bazer *et al.* 1997). Alternatively, juxtacrine signaling could be triggered by cell–cell and cell–matrix interactions, as for example mucins from the endometrium that prevent embryonic attachment (Thathiah & Carson 2002).

Embryos can thrive successfully until the blastocyst stage *in vitro*, independent of exposure to the maternal reproductive tract. However, it is clear that the maternal genital tract, including the uterus itself, exerts

considerable control over the ability of an embryo to develop. For instance, several studies have demonstrated that *in vivo* cultured embryos differ from their *in vitro* produced counterparts in terms of developmental competence, morphology, metabolism, gene expression profile, and cryotolerance (reviewed by Rizos *et al.* 2017). Moreover, exposure to the uterine environment at the post-hatching stage is a prerequisite for embryo elongation in the cow, given that elongation of bovine conceptuses does not occur *in vitro* (Brandao *et al.* 2004).

The uterus is a hormonally regulated organ. Ovarian steroids (i.e. estradiol and progesterone, from pre-ovulatory follicles and corpus luteum) exert classical endocrine control of morphological and functional changes in the endometrium that affect embryo development and pregnancy success (Carter *et al.* 2008, Forde *et al.* 2009, Mesquita *et al.* 2015). In contrast, programming of endometrial function exerted by the embryo has not been interrogated to the same extent. In that regard, the endometrium senses the embryo quality (Macklon & Brosens 2014). For example, the bovine endometrial transcriptome changes when exposed to *in vitro*- vs *in vivo*-produced embryos (Mansouri-Attia *et al.* 2009), and when exposed to standard *in vitro*-produced vs cloned embryos (Bauersachs *et al.* 2009). We have reported earlier that the *in vivo* endometrial transcriptome (Sponchiado *et al.* 2017) and uterine luminal metabolome (Sponchiado *et al.* 2019) respond locally to a pre-hatching embryo, as early as day 7 after conception. Other *in vitro* studies have shown that early embryos modulate the abundance of specific transcripts in endometrial epithelial cells (Talukder *et al.* 2017, Gómez *et al.* 2018) and endometrial explants (Passaro *et al.* 2018). Specific pathways modulated by the embryo include transcripts for interferon-stimulated genes (ISGs) and prostaglandin metabolism (Sponchiado *et al.* 2017). The question still remains whether the endometrial epithelial responses depend on a direct physical proximity between the embryo and the endometrial cells and thus whether the juxtacrine (i.e. physical contact) differs from the non-juxtacrine fashion. We hypothesized that nature and intensity of embryo-induced changes on endometrial transcriptome depend on physical contact between the embryos and the endometrium. Therefore, we aimed to interrogate the embryo–maternal interface for embryo-induced changes in the transcriptome of endometrial epithelial cells. Secondly, we aimed to investigate the impact and the specificity of endometrial cells on morula developmental capacity to the blastocyst stage. We used a co-culture system of embryos and bovine endometrial epithelial cells (bEECs) to elucidate basic cellular mechanisms involved in the early embryo–maternal interaction and their reciprocal effects.

Materials and methods

Overview of experimental model

To investigate our research hypotheses, we established a bEEC–embryo co-culture as a model to investigate the early embryo–maternal interactions *in vitro*. bEEC isolation and culture procedures are described in the *Isolation and culture of bovine endometrial epithelial cells* section. For validation purposes, and before starting the co-culture experiments, we characterized the bEECs regarding the expression of cell line-specific markers. In the first experiment, bEECs were juxtapositioned to morulae for 48 h, whereas non-juxtacrine condition was accomplished by using a transwell insert. Control groups of bEECs in the absence of embryos and embryos in the absence of bEECs were included. The importance of physical proximity between embryos and endometrial cells was investigated in terms of morula developmental capacity and bEEC transcriptome profiles. In the second experiment, conditioned media from bEECs and mice fibroblasts were used to test the specificity of the endometrial cell's supportive capacity on embryo development from the morula-to-blastocyst stage. An overview of the experimental procedures is given in Fig. 1.

Experiment 1

In Experiment 1, we aimed to compare the effects of juxtaposition vs non-juxtaposition between embryos and endometrial epithelial cells on bEEC transcriptome and morula developmental capacity. Embryos were co-cultured with bEEC monolayers from day 5.5 p.f. (post-fertilization; morulae) until day 7.5 p.f. (blastocysts). The start of exposure of the endometrial cells to embryos (i.e. day 5.5 p.f.) coincides with the embryo's arrival into the uterus *in vivo* in the cow (Bazer *et al.* 1991). In order to maximize the embryo–bEEC physical contact zone and the stimulus promoted by the early embryo, multiple embryos were co-cultured with the bEEC monolayers. In addition, by using pools of 15 embryos per well, differential effects caused by variation between individual embryos were avoided. Juxtacrine interaction was accomplished by placing 15 embryos directly on top of bEEC monolayers. Non-juxtacrine interaction was accomplished by placing 15 embryos in a 96-well cell culture insert (ClearView, IncuCyte®, USA). The insert membrane (8.0 µm pore size) prevented the direct physical contact between embryos and the endometrial monolayer. The distance between the basal plate and the insert is 1.425 mm. More technical details can be found at the supplier's homepage (<https://www.essenbioscience.com/en/products/reagents-consumables/incucyte-clearview-96-well-cell-migration-plate/>).

In vitro produced, day 5.5 morulae were selected under the stereomicroscope and transferred in groups of 15 ± 1 to a 96-well plate (ClearView, IncuCyte®) containing 90% confluent, untreated bEEC monolayers according to the treatments: (i) Control Embryos: morulae without bEECs; (ii) Contact+: morulae juxtapositioned to bEEC monolayers; (iii) Contact–: morulae placed on cell culture transwells, without contact with bEECs; or (iv) Control bEEC: bEECs in the absence of embryos. In each replicate, all experimental

Figure 1 Schematic overview of the experimental model. (A) Bovine endometrial epithelial cells (bEECs) were isolated from uteri ex vivo, pooled, and used for the primary culture. Cells were subcultured until passage 2, when bEECs were cryopreserved. Subculture 3 – bEECs were thawed as needed and assigned to co-culture experiments with embryos (Experiment 1; B), or to generate bEEC – conditioned medium (Experiment 2; C). In all replicates, aliquots containing cells originated from the same batch were applied. For means of characterization, cells from each passage were simultaneously cultured in 24-well plates for further PCR analyses. Created with BioRender (<https://biorender.com/>).

conditions were conducted in a single 96-well and received transwells. The experiment was performed in six replicates, with two to four wells of each treatment per replicate. Embryos were transferred using mouth-controlled, fine pore glass capillaries to minimize the volume of media transferred to the wells. Embryos–bEECs co-cultures (for all the experimental conditions) were conducted in 150 μ L of synthetic oviductal fluid (SOF) medium supplemented with 5% of fetal bovine serum (FBS), in humidified atmosphere of 5% CO_2 at 38.5°C. After 48 h of co-culture, embryos and bEEC monolayers were evaluated and processed as described subsequently.

Sample evaluation and processing

On day 7.5 p.f., embryo developmental stages were recorded and expressed as a proportion of total number of morulae assigned initially to the treatments. All morphological assessments were conducted using an inverted Olympus CKX41 microscope (Olympus, Belgium). Day 7.5 p.f. blastocysts were either fixed in paraformaldehyde for immunostaining or snap frozen. Embryos were fixed in buffered 4% paraformaldehyde for 30 min at room temperature, and stored in PBS–PVP 1% at 4°C. Groups of 5 embryos were washed in PBS–PVP 1%, snap frozen and stored at –80°C. bEEC monolayers were washed with pre-warmed PBS (Ca^{2+} Mg^{2+} free) to remove serum traces and detached cells, and then dispersed with pre-warmed TrypLE Express (Thermo Fisher Scientific) for 6 min at 38.5°C. The reaction was inactivated by dilution by adding PBS (Ca^{2+} Mg^{2+} free). bEECs were transferred and centrifuged at 200 g for 10 min at 24°C. Cell pellets were snap frozen and stored at –80°C for further processing.

Experiment 2

In this experiment, we aimed to identify potential non-specific embryo supportive effects elicited by bEECs on embryo development. By using conditioned medium from mice

fibroblasts, we tested whether heterologous, unspecific feeder cells could similarly support embryo development. Moreover, by using a medium conditioned by bEECs, we investigated the potential non-specific effect of a reduced O_2 tension caused by the bEEC monolayer that may have favored embryo development in experiment 1. Therefore, in vitro produced day 5.5 morulae/early blastocysts were cultured in fresh conditioned medium from bEECs or mice fibroblasts as described subsequently. Experiment was performed in five replicates, with two to five wells of each condition included in each replicate.

Conditioned medium preparation and treatments

Spent or control medium from six wells were pooled and centrifuged at 200 g for 5 min at 24°C to remove cells/debris. After 30 min of equilibration in the incubator, 150 μ L of medium were distributed in a 96-well plate (ClearView, IncuCyte®). Subsequently, groups of 15 ± 1 day 5.5 morulae were assigned to the following treatments: (i) Control Embryos: Culture medium (SOF + 5% FBS); (ii) bEECcond: bEEC-conditioned SOF + 5% FBS medium; or (iii) FIBRcon: fibroblast-conditioned SOF + 5% FBS medium. Embryos were transferred using mouth-controlled fine pore glass capillaries to minimize the volume of media transferred to the wells. Embryos were cultured in humidified atmosphere of 5% CO_2 at 38.5°C from day 5.5 until day 7.5 p.f. The medium was replaced with freshly prepared conditioned (or control) medium in 50% of the volume after 24 h. On day 7.5, embryos were visually scored according to their developmental stages using an inverted Olympus CKX41 microscope.

Isolation and culture of bovine endometrial epithelial cells (bEECs)

Chemicals used to prepare cell and embryo culture media were purchased from Life Technologies (Thermo Fisher Scientific) or from Sigma Chemical Co., unless otherwise stated.

Cell collection and primary culture

Reproductive tracts at early luteal phase (i.e. containing a corpus haemorrhagicum; Ireland *et al.* 1980) of the estrous cycle were collected from *Bos taurus* beef cows at a local slaughterhouse, inspected for the absence of reproductive abnormalities or disorders, and transported to the laboratory at 30°C. Four uteri were trimmed free of surrounding tissues and externally decontaminated with 70% ethanol. The uterine horn ipsilateral to the ovary containing the corpus haemorrhagicum was opened longitudinally at the mesometrial insertion and the endometrial luminal surface was carefully scraped with sterile glass slides. Cells collected from the four uteri were pooled in an equilibrated, pre-warmed cell culture medium. The cell culture medium consisted of DMEM/F-12 phenol red-free medium (Product number 11039-021; Gibco, Thermo Fisher Scientific) supplemented with 10% FBS (Product number F9665; Sigma-Aldrich), 2% penicillin/streptomycin, and 1% fungizone. Cells were pelleted at 200 *g* for 5 min at 24°C, and erythrocytes were subsequently lysed by incubation with a hyper-osmotic lysis buffer (1 mM EDTA disodium salt, 150 mM NH₄Cl and 100 mM NaHCO₃) for 1 min. Reaction was neutralized with culture medium and the cell suspension was recentrifuged. After washing twice, 1 × 10⁶ viable cells/flask were plated in 25 cm² culture flasks and pre-incubated in a humidified atmosphere of 5% CO₂ at 38.5°C for 1 h 30 min. The pre-incubation step aimed to remove contaminating non-epithelial cells. The supernatant (containing non-attached, epithelial cells) was transferred toward 25 cm² culture flasks and maintained in a humidified atmosphere of 5% CO₂ at 38.5°C. After the adhesion process started, culture medium was changed every 48 h.

Subculture and freezing procedures

Cells were subcultured when primary monolayers reached 90% of confluence. Cells were washed with PBS (Ca²⁺ Mg²⁺ free) and then dispersed using TrypLE Express (Thermo Fisher Scientific) for 6 min. Reaction was inactivated by the addition of culture medium. Cells were pelleted at 200 *g* for 5 min at 24°C. bEECs were seeded at a density of 2 × 10⁶ viable cells/flask into 75 cm² culture flasks and cultured as described for primary culture. Passage one monolayers at 95% of confluence were dissociated as described previously and plated at a density of 1.5 × 10⁶ viable cells/flask in 75 cm² culture flasks. bEECs were cultured as described for primary culture. Subconfluent (90%) monolayers were dispersed as described previously. The resulting cell pellet was diluted in cryopreservation medium (DMEM/F-12 phenol red-free medium supplemented with 15% FBS and 10% DMSO) at a concentration of 1 × 10⁶ viable cells/mL. Cryovials containing bEECs were placed in a freezing container (Nalgene® Cryo, Thermo Fisher Scientific), kept at −80°C overnight, and afterwards stored in liquid nitrogen. Experiments were performed using bEECs at passage three, thawed from the same batch.

bEEC cell line characterization

bEEC cultures were conducted to evaluate the presence of cytokeratin and vimentin, epithelial and mesenchymal cell

markers, respectively, and the abundance of functional key transcripts from primary culture to passage three. For this purpose, cells were seeded into 24-well plates for further trypsinization and retrieval for PCR analysis. For fixation and immunostaining analysis, cells were grown on sterile glass coverslips. Cell cultures were conducted as described previously (see 'Cell collection and primary culture and 'Subculture and freezing procedures' sections). Subconfluent bEEC monolayers grown on coverslips were washed with PBS (Ca²⁺ Mg²⁺ free), fixed in buffered 4% paraformaldehyde for 30 min at room temperature, and stored at 4 °C for further immunostaining procedures. For PCR analysis, bEECs were trypsinized for 6 min, retrieved and centrifuged at 200 *g* for 5 min at 24°C. Cell pellets were snap frozen and kept at −80°C until RNA isolation.

bEEC assignment to treatments (experiments 1 and 2)

Vials containing bEECs at passage three were thawed, resuspended in 10 mL of pre-warmed culture medium, and pelleted at 200 *g* for 5 min at 24°C. This step aimed to remove residual DMSO from the freezing medium. Cells were resuspended in pre-warmed culture medium and seeded into a 0.1% gelatin-coated 96-well ClearView Reservoir Plate (IncuCyte®; Sartorius) at a density of 1.5 × 10⁴ cells/well. bEECs were maintained in DMEM/F-12 phenol red-free medium supplemented with 10% FBS, 2% penicillin/streptomycin, and 1% fungizone, in a humidified atmosphere of 5% CO₂ at 38.5°C. The medium was changed every 48 h. Approximately 90 h after plating, monolayers at 90% confluency were assigned to treatments as described previously for Experiments 1 and 2. In Experiment 2, DMEM/F12-based culture medium of bEEC and mice fibroblast subconfluent monolayers was replaced by SOF medium supplemented with 5% of FBS. Additional wells containing only SOF + 5% FBS medium were included. After 24 h, spent or control medium were retrieved and assigned to experimental conditions.

In vitro production of bovine embryos

Bovine embryos were produced in vitro as described previously (Marei *et al.* 2019) with minor modifications. Briefly, ovaries were collected at a local slaughterhouse and transferred to our laboratory within 2–3 h of slaughter. Antral follicles with a diameter of 3–8 mm were aspirated. Immature, unexpanded cumulus-oocytes complexes (COCs) were matured in vitro in groups of 50 ± 5 COCs in four-well plates containing maturation medium (500 µL/well) for 24 h in a humidified atmosphere with 5% CO₂ at 38.5°C. Groups of 100 ± 10 matured COCs were transferred to Fert-TALP medium containing 1 × 10⁶ sperm cells/mL. For all replicates, frozen bull semen from the same ejaculate with proven in vitro fertility was used following selection of motile spermatozoa by centrifugation on a discontinuous 45–90% Percoll gradient. COCs were co-incubated with spermatozoa in four-well plates (500 µL/well) for 22 h in a humidified atmosphere of 5% CO₂ at 38.5°C. Following in vitro fertilization, denuded presumptive zygotes were cultured in groups of 25 ± 2 in 75 µL of SOF medium supplemented with 5% FBS. Embryo cultures were

carried out in 96-well plates under controlled atmosphere of 5% CO₂, 5% O₂, 90% N₂ at 38.5°C until day 5.5 p.f., when compact and non-compact morulae were selected and assigned to experimental conditions.

Immunofluorescence

bEEC

Cells were permeabilized with PBS containing 1% Triton X-100 and 0.05% Tween-20 for 30 min at room temperature. After washing, samples were blocked in PBS containing 2% w/v BSA and 0.05% Tween-20, for 45 min at room temperature. Cells were then incubated with primary antibodies overnight at 4°C. Cells were immunostained for an epithelial cell marker (anti-pan Cytokeratin mouse MAB; 1:100 in blocking solution; M3515, Dako), and for a mesenchymal cell marker (anti-Vimentin rabbit polyclonal antibody; 1:100 in blocking solution; ab45939, Abcam). Negative control samples were incubated with an equivalent mixture of normal mouse and rabbit IgGs replacing the primary antibodies. Monolayers were washed and subsequently incubated with the secondary antibodies Texas Red-labeled goat anti-mouse (1:200 in blocking solution; Life Technologies) and FITC-labeled goat anti-rabbit (1:200 in blocking solution; Novex, Life Technologies) for 1 h at 4°C. Nuclei were counter-stained with Hoechst 33342 (30 µg/mL in PBS-PVP) for 10 min at room temperature. Thereafter, coverslips were mounted on glass slides in droplets of 1% DABCO.

Embryos

Day 7.5 blastocysts ($n=91$) were differentially immunostained to determine inner cell mass (ICM) and trophoblast (TE) cell number using an immunofluorescence method adapted from Wydooghe *et al.* (2011). Briefly, fixed blastocysts were permeabilized in 0.1% Triton X-100 and 0.05% Tween-20 in PBS overnight at 4°C, and incubated in blocking solution (10% normal goat serum in 0.05% Tween-20 in PBS) for 2 h at 4°C. Blastocysts were incubated overnight at 4°C in primary antibody solution containing a 1:1 mixture of mouse anti-caudal type homeobox 2 (CDX2) ready-to-use antibody (BioGenex, San Ramon, USA) in blocking solution, as a marker for TE cells. Negative control embryos were incubated with normal mouse IgG at the same concentration as the primary antibody. Embryos were washed in PBS-PVP 1% and subsequently incubated with the secondary antibody Texas Red-labeled goat anti-mouse (1:200 in blocking solution; Life Technologies) for 4 h at 4°C. Nuclei were counter-stained in Hoechst 33342 (30 µg/mL in PBS-PVP 1%) for 10 min at room temperature. Blastocysts were mounted onto glass slides in droplets of 1% DABCO and covered with coverslips.

Cells and embryos were visualized under a fluorescence microscope IX71 (Olympus) equipped with CellSens software using the following three filters: DAPI (excitation/emission: 360–370/420–460 nm), FITC (460–490/520–540 nm) and RITC (510–550/>570 nm). Embryonic cell counting was performed by a blinded investigator.

Total RNA isolation and cDNA synthesis

bEEC (Experiment 1)

Total RNA isolation was carried out using PicoPure RNA Isolation Kit (Thermo Fisher). Briefly, bEEC samples were subjected to cell lysis in 500 µL of Extraction Buffer for 30 min at 42°C. Lysates were loaded into pre-conditioned purification columns and processed as per manufacturer's instructions. Membranes were subjected to on-column DNase (RNase-Free DNase Set; Qiagen) treatment at room temperature for 15 min according to the standard protocol. After subsequent washing, the membrane-bound RNA was eluted in 16 µL of the Elution Buffer provided. Total RNA yield and integrity were assessed using RNA 6000 Nano Lab-chips on a Bioanalyzer 2100 (Agilent Genomics). Absorbance ratios (28S/18S) and RNA integrity values ranged between 2.0–2.6 and 8.5–10.0, respectively. The isolated RNA samples were stored at –80°C until RNA sequencing.

bEEC (cell line characterization)

Total RNA was isolated using Trizol Reagent (Invitrogen, Thermo Fisher Scientific) in accordance with manufacturer's guidelines with minor modifications. Briefly, samples were lysed in 200 µL of Trizol and incubated at room temperature for 5 min. Subsequently, 128 µL of chloroform were added and samples were centrifuged at 10,000 *g* for 15 min at 4°C for phase separation. The aqueous phase was transferred toward a new microtube and 400 µL of isopropanol were added. At this step, samples were stored overnight at –80°C to maximize RNA precipitation. Samples were pelleted at 10,000 *g* for 12 min at 4°C, and washed in 400 µL of 75% ethanol, followed by centrifugation at 10,000 *g* for 8 min at 4°C. Air-dried RNA pellets were resuspended in 10 µL of DEPC water and, subsequently, incubated at 55°C for 10 min. Total RNA yield and purity (260/280 nm ratio) were evaluated by NanoDrop (Thermo Fisher Scientific) spectrophotometer analysis. RNA extracts were stored at –80°C. Total RNA (1 µg) samples were treated with DNase I (Promega) according to the standard protocol. First-strand cDNA was synthesized using random hexamers (High Capacity cDNA RT kit; Invitrogen) and by incubation at 25°C for 10 min, followed by incubation at 37°C for 2 h and reverse-transcriptase inactivation at 85°C for 5 min. The cDNA was stored at –20°C until PCR analyses.

Embryos (Experiment 1)

Pools of 15 embryos (developmental stages were distributed equally among samples) were subjected to total RNA isolation using PicoPure RNA Isolation Kit (Thermo Fisher) as described for bEECs. Membrane-bound nucleic acids were subjected to on-column DNase (RNase-Free DNase Set; Qiagen) treatment at room temperature for 15 min according to the standard protocol. Total RNA yield and purity (260/280 nm ratio) were evaluated by NanoDrop (Thermo Fisher Scientific) spectrophotometer analysis. Total RNA (50 ng) was reverse transcribed using Sensiscript RT kit (Qiagen) according to manufacturer's instructions with minor modifications. The master mix was

composed by the Sensiscript Reverse Transcriptase and the buffer RT supplied in the kit, supplemented with Recombinant RNasin RNase inhibitor (Promega), Oligo-dT (Promega) and Random (Promega) primers at a final concentration of 1 μ M and 10 μ M, respectively, and dNTP mix (Promega) at a final concentration of 0.5 mM/each dNTP. Samples were incubated in a thermocycler at 37°C for 1 h and stored at –20°C until PCR analyses.

Real-time PCR

Relative abundance of transcripts was analyzed by real-time PCR. Transcripts analyzed in bEECs included cell origin markers (*KRT18* and *VIM*), and mRNA associated with endometrial function (*ESR1*, *IFNAR1* and *PTGS2*). PCR analyses in day-7.5 embryos included a marker for TE cells (*CDX2*), interferon-tau (*IFNT2*), and a gene associated with apoptosis (*BAX*). Specific pairs of primers were designed based on the *Bos taurus* GenBank Ref-Seq mRNA. Reactions were carried out in duplicates in 96-well plates using the CFX Connect real-time PCR detection system (Bio-Rad Laboratories). PCR reactions were conducted in a final volume of 16 μ L, consisting of 8 μ L of SsoAdvanced Universal SYBR Green supermix (Bio-Rad Laboratories), 0.4 μ L of forward and reverse primers, and 4 μ L of cDNA template. Negative control reactions (DEPC-treated water replacing template cDNA) were included in every run. The program consisted of an initial denaturation step at 95°C for 15 s, followed by 40 cycles each of 30 s at 95°C, annealing at 59–61°C for 30 s, and extension at 72°C for 20 s. After a final extension step of 72°C for 5 min, melting curves were plotted by stepwise increases in the temperature from 50 to 95°C. The annealing temperature was optimized for each primer assay. Relative abundances were obtained after normalization of the target genes Cq (Crossing Point) values by the geometric mean of the reference genes Cq values according to the mathematical model described by Pfaffl (2001). *PPIA*, *GAPDH* and *ACTB* were used as reference genes for bEECs. *H2AFZ*, *GAPDH* and *RN18S1* were used as reference genes for embryos. Primers details are provided in Supplementary Table 1 (see section on supplementary materials given at the end of this article).

RNA sequencing

Fifteen bEEC samples (Experiment 1; 5/treatment) were selected for transcriptome analysis. Each sample consisted of bEECs retrieved from an individual well. Samples were selected based on specific criteria: (i) samples from the Contact+ and Contact– groups having embryos developed to similar blastocyst rates and stages; and (ii) samples of all three groups pertaining to the same replicates. Blastocyst developmental rates for each sample assigned to RNAseq analysis are given in Supplementary Table 2.

Poly-A containing mRNA molecules were purified from total RNA using oligo(dT)-attached magnetic beads and fragmented into small pieces using divalent cations under elevated temperature. First-strand cDNA was synthesized using random hexamer-primed RT, followed by a second-strand cDNA synthesis using DNA Polymerase I and RNase H. The synthesized cDNA was subjected to end-repair and 3' adenylation. Adapters were

ligated to the ends of 3' adenylated cDNA fragments. cDNA fragments with adapters from previous step were amplified by PCR. The resulting PCR products were purified with Agencourt AMPure XP Beads (Beckman Coulter, Beverly, MA, US), and dissolved in buffer EB. Double stranded PCR products were heat-denatured and circularized by the splint oligo sequence. Single stranded circular DNAs (ssCir DNA) were used for PE100 strand-specific library construction and validation on the Bioanalyzer 2100 (Agilent Genomics). The library was amplified with phi29 and DNA nanoballs (DNBs) were generated with ssCir DNA by rolling circle replication (RCR) to intensify the fluorescent signals during the sequencing process. The DNBs were loaded into the patterned nanoarray and pair-end reads of 100 bp were read on the BGISEQ-500 (Cambridge, MA, US) platform for subsequent data analysis.

The data presented in this publication have been deposited in the Gene Expression Omnibus (GEO) database, <https://www.ncbi.nlm.nih.gov/geo> and are accessible through GEO Series Access Number GSE151868.

Statistical analysis and bioinformatics

Statistical analyses were carried out using SAS 9.4 software. Discrete variables, such as blastocyst rates and the proportion of blastocysts reaching the expanded-stage or beyond were analyzed by PROC GLIMMIX using binomial distribution. Replicates were included as random effects in the model. When the effect of treatment was significant, means across treatments were compared by DIFF adjusted by Tukey–Kramer test. Continuous variables, such as gene expression data, cell counts and ICM:TE ratios were checked for normality of residues and homogeneity of variances by Shapiro–Wilk and Welch's test, respectively. Variables were transformed by log or square root when necessary to fulfill assumptions. Effects of treatments were determined using PROC MIXED by ANOVA considering Type III sums of squares. When significant, means across treatments were compared using DIFF adjusted by Tukey–Kramer test. P -value ≤ 0.05 indicates a significant difference, and $P > 0.05$ to $P \leq 0.1$ indicates a tendency. Data are presented as mean $\pm$ S.E.M., unless otherwise indicated.

RNA-sequencing data analysis was performed in Galaxy (<https://usegalaxy.org>). Quality control was performed by FastQC (version 0.71). Paired reads were mapped to the *Bos taurus* reference genome (UMD 3.1) using HISAT2 (version 2.1.0), after which the counts per gene model was performed with FeatureCounts (version 1.6.2). Expression values were normalized and global and pairwise statistics were performed using DESeq2 (version 1.18.1) using false discovery rate (FDR)-corrected P values.

Canonical pathways of the differentially expressed genes (DEGs) was performed using Core Analyses in Ingenuity® Pathway Analysis (IPA, QIAGEN bioinformatics). A cutoff of DEGs with fold-changes > 1.5 and adjusted P values ≤ 0.05 was applied. A detailed description of the IPA analysis is available on the manufacturer's homepage (<https://www.qiagenbioinformatics.com/products/features/>). Heatmaps were generated with heatmapmer software (<https://heatmapmer.ca>) applying average linkage and Spearman rank correlation method for clustering and distance measurements, respectively.

Results

Functional characteristics of cultured bovine endometrial epithelial cells

For validation purposes and before starting the co-culture experiments, we characterized the bEEC cell line regarding the expression of cell-line specific markers. Of note, during primary culture, isolated bEECs attached to the plastic surface of culture dishes and exhibited a mixture of elongated, round and spindle morphologies (data not shown). However, after the first passage, monolayers consisted of a homogeneous population of epithelial-like adherent cells, and that feature remained constant after two subsequent passages.

We compared the abundance of transcripts for cell-origin and functional markers by real-time PCR in bEECs among primary culture and three subsequent passages. Gene expression data showed no changes in the abundance of transcripts for keratin 18 (*KRT18*) among passages; however, we verified a six-fold increase ($P < 0.001$, Fig. 2A) in the abundance of vimentin (*VIM*) mRNA from primary culture to passage one, that remained constant until passage 3. Immunostaining revealed that the bEECs after the first passage co-expressed cytokeratin, a typical cytoplasmic marker for epithelial origin, together with vimentin, a marker of mesenchymal-derived cells (Fig. 2B). Immunofluorescence data also confirmed that expression of *VIM* mRNA in the cell lysates was not due to contaminating fibroblasts in our cell line but because of a dedifferentiated status of the cells. Abundance

of transcripts for estrogen receptor alpha (*ESR1*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), and interferon alpha and beta receptor subunit 1 (*IFNAR1*), however, remained similar among primary culture and the first three passages, suggesting that the endometrial cells conserved these functional characteristics (Fig. 2A).

Effect of co-culture with bEECs on embryo development

In vitro-produced day-5.5 morulae were cultured for 48 h in SOF+5% FBS medium in the absence of bEEC (Control Embryos), juxtapositioned to subconfluent bEEC monolayers (Contact+), or on transwells, without direct contact with bEECs (Contact-). Co-culture with bEECs significantly increased the proportion of morulae reaching the blastocyst stage on day-7.5, regardless of juxtaposition (Table 1). Additionally, the proportion of blastocysts that reached the expanded, hatching or hatched stages was significantly greater ($P=0.0048$) or tended ($P=0.0784$) to be greater in the Contact- and Contact+ groups, respectively, compared to the Control Embryos. Although the average counts of total cells in day 7.5 blastocysts were similar across treatments, there was an increase in the ICM/TE ratio in Contact+ blastocysts (Table 1). This difference in blastomere cell fate, however, did not impact the abundance of *CDX2* mRNA between the three culture conditions (Fig. 3). Surprisingly, Contact- embryos presented a four-fold increased abundance of transcripts for *IFNT2*

Figure 2 Functional characteristics of cultured bovine endometrial epithelial cells (bEECs). (A) Relative mRNA abundance of keratin 18 (*KRT18*), vimentin (*VIM*), estrogen receptor alpha (*ESR1*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), and interferon alpha and beta receptor subunit 1 (*IFNAR1*) in bEECs along primary culture (PC), subculture 1 (SC1), SC2 and SC3. Data are shown as arbitrary units; mean $\pm$ S.E.M. ^{a,b}Statistically significant difference in mRNA abundance ($P \leq 0.05$, $n=8$). (B) Light microscopy image of a confluent bEEC monolayer (x10 magnification). (C) Representative image of immunostaining for epithelial (pan cytokeratin, in red) and stromal (vimentin, in green) cell markers. The bEECs were positive for both markers. Nuclei are stained in blue. Scale bar = 50 µm. Negative controls were incubated with an equivalent mixture of normal mouse and rabbit IgGs instead of primary antibodies (top right inserts).

Table 1 Effects of co-culture with bovine endometrial epithelial cells (bEEC) on embryo development. On day-5.5 p.f. (post-fertilization), groups of 15 ± 1 untreated morulae were cultured in SOF-based medium in the absence of bEEC (Control Embryos), juxtacrine (Contact+) or non-juxtacrine (Contact-) apposition. Of the total oocytes (1970) assigned to *in vitro* fertilization, 640 (32.5%) morulae were assigned to treatments. On day-7.5 p.f., embryonic development was recorded. Blastocysts' total cell count and trophectoderm (TE): Inner Cells Mass (ICM) ratio were assessed by differential immunostaining.

Variables	Groups		
	Control embryos, $n=14$ wells	Contact+, $n=15$ wells	Contact-, $n=14$ wells
Day-7.5 blastocysts**	109/205 (53.2%) ^a	147/225 (65.3%) ^b	134/210 (63.8%) ^b
Expanded, hatching or hatched blastocysts***	50/109 (45.9%) ^{a,Y}	88/147 (59.9%) ^{ab,X}	88/134 (65.7%) ^b
Total cell counts (mean $\pm$ S.E.M.)	111.08 $\pm$ 6.07	125.36 $\pm$ 5.16	117.86 $\pm$ 5.75
ICM:TE ratio (mean $\pm$ S.E.M.)	0.36 $\pm$ 0.031 ^a	0.47 $\pm$ 0.027 ^b	0.36 $\pm$ 0.029 ^a

Within rows, values with different superscript letters differ significantly (a, b; $P \leq 0.05$) or tend to differ (X, Y; $P \leq 0.1$). **From cultured morulae; ***From total blastocysts.

compared to the Contact+ group. Contact- condition also increased the abundance of mRNA for *BAX*, an apoptosis-related gene, compared to Contact+ and Control Embryos groups (Fig. 3).

Specificity of bEEC-induced effects on embryo development

In the second experiment, *in vitro*-derived day-5.5 morulae were cultured in SOF+5% FBS medium (Control Embryos), or in SOF-based medium conditioned by bEEC (bEECcond), or by mice fibroblasts (FIBRcond). On day 7.5 p.f., the number of blastocysts was significantly greater in the bEECcond compared to the Control and FIBRcond conditions (Table 2). However, there was no effect of bEECcond condition on the proportion of blastocysts that reached or surpassed the expanded-stage.

Embryo-induced effects on bEEC transcriptome

RNA sequencing generated a total of approximately 14.6 million reads per sample. Reads were mapped to the

Bos taurus reference genome (assembly UMD3.1). After filtering, a total of 19,996 genes were used to identify DEGs (FDR ≤ 0.05). The data were deposited in Gene Expression Omnibus (GEO) under the accession number GSE151868. A total of 1797 (FDR ≤ 0.05 ; Supplementary Dataset 1) genes were differentially expressed in the Contact+ group compared to their Control bEEC counterparts (Fig. 4). Only 230 DEGs (Supplementary Dataset 2), however, were found between Contact- and the Control bEEC group. Notably, from these 230 DEGs, a subset of 225 genes was found to be also differentially expressed in the Contact+ vs Control bEEC comparison. As denoted in Fig. 4, the 50 overlapping DEGs between Contact+ vs Control bEEC and Contact- vs Control bEEC comparisons likely represent the embryo-induced effects on bEEC transcriptome irrespective to juxtaposition. Interestingly, out of those 225 DEGs, 175 genes were also differentially expressed in the Contact+ vs Contact- comparison, indicating that juxtaposition of embryos to the bEEC monolayer also changed the magnitude of embryo-induced effects on bEEC transcriptome.

The comparison between Contact+ vs Contact- yielded 899 (Supplementary Dataset 3) DEGs with 583 (~65%; Fig. 4) genes commonly changed in the Contact+ vs Control bEEC comparison plus 140 genes. The effect of embryo juxtaposition on endometrial transcriptome was accessed by this set of 723 DEGs that were exclusively changed when embryos were in contact with the bEEC monolayer (Fig. 4).

Embryo-induced effects on bEEC transcriptome independent of juxtaposition

These effects were assessed by the 50 overlapping DEGs retrieved in the Contact+ vs Control bEEC and Contact- vs Control bEEC comparisons. As shown in Fig. 5A, gene expression was modulated when compared to the Control group but similar between Contact+ and Contact-. The five most upregulated genes included interferon regulatory factor 9 (*IRF9*; 7.35-fold), sulfotransferase family 6B member 1 (*SULT6B1*; 6.46-fold), transmembrane protein 107 (*TMEM107*; -4.48-fold), TLH29 protein precursor-like (*ISG12(B)*; 2.68-fold) and ring finger protein 31 (*RNF31*;

Figure 3 Juxtaposition to bEECs changes the abundance of specific transcripts in day-7.5 embryos. Relative mRNA abundance of interferon-tau (*IFNT2*), caudal type homeobox 2 (*CDX2*), and BCL2 associated X, apoptosis regulator (*BAX*) on day 7.5 embryos cultured in SOF-based medium in the absence of bEEC (Control Embryos), juxtacrine (Contact+) or non-juxtacrine (Contact-) apposition. Each sample consists of a pool of 15 embryos from different culture wells of the same treatment group ($n=4-5$ /group). Data are shown as arbitrary units; mean $\pm$ S.E.M. ^{a,b}Statistically significant difference in mRNA abundance ($P \leq 0.05$).

Table 2 Specificity of bovine endometrial epithelial cells (bEEC)-driven effects on embryo development. On day 5.5 p.f. (post-fertilization), groups of 15 ± 1 untreated morulae were cultured in SOF-based medium (Control), in bEEC-conditioned medium (bEECcond), or in mice fibroblast-conditioned SOF medium (FIBRcond). Of the total oocytes (2320) assigned to *in vitro* fertilization, 821 (35.4%) day-5.5 morulae were assigned to treatments. On day 7.5 p.f., embryonic developmental rates were recorded.

Variables	Groups		
	Control, n=21 wells	bEECcond, n=22	FIBRcond, n=12
Day 7.5 blastocysts**	178/315 (56.5%) ^b	234/328 (71.3%) ^a	100/178 (56.2%) ^b
Expanded, hatching or hatched blastocysts***	104/178 (58.4%)	143/234 (61.1%)	52/100 (52.0%)

Within rows, values with different superscript letters differ significantly (a, b; $P < 0.001$).
From cultured morulae; *From total blastocysts.

1.59-fold). IPA analysis of DEGs (FDR ≤ 0.05; Cutoff fold-changes > 1.5; Fig. 5B) identified canonical pathways related to Interferon Signaling and Role of PKR in Interferon Induction and Antiviral Response. In addition, pathways related to inflammatory responses (HMGB1 (inflammation high mobility group box 1 chromatin-binding protein) Signaling and IL-23 Signaling Pathway), and antigen presentation (Dendritic Cell Maturation) were retrieved. Other relevant pathways identified in the functional analysis include Prostanoid Biosynthesis and Stearate Biosynthesis.

Figure 4 Embryo-induced effects on bEEC transcriptome. Venn diagram of RNAseq data from bovine endometrial epithelial cells (bEECs; $n=5$ /group) exposed to none (Control bEEC), in juxtaposition (Contact+) or non-juxtaposition (Contact-) to day-5.5 morulae for 48 h. Differentially expressed genes (DEGs) were selected using adjusted P values (FDR ≤ 0.05). The number of up and downregulated genes are shown in green and red, respectively. (i) Embryo-induced effects regardless of juxtaposition are represented by the overlapping 50 DEGs yielded in the Contact+ vs Control bEEC, and Contact- vs Control bEEC comparisons. (ii) The set of 175 DEGs returned by all three comparisons represents genes whose magnitude of changes depended upon juxtaposition. (iii) Specific effects of embryo juxtaposition on bEEC transcriptome are represented by the remaining 723 DEGs yielded in the Contact+ vs Contact- comparison. See Supplemental Dataset S4 for a complete list of DEGs.

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Figure 5 Embryo-induced effects on bEEC transcriptome regardless of juxtaposition. (A) Heatmap depicting the 50 differentially expressed genes (DEGs; FDR ≤ 0.05) that represent embryo-induced effects on bEECs but that were not affected by juxtaposition. Reads were normalized within rows. Samples and genes were clustered by average linkage and Spearman rank correlation distances. (B) Overview of canonical pathways returned by Ingenuity Pathway Analysis (IPA) analysis of DEGs. Contact+ vs Control bEEC fold-changes (Cutoff > 1.5) were considered in this analysis. The charts (y-axis) represent the top significantly enriched canonical pathways. The upper x-axis shows the -log of the value calculated based on Fisher's exact test. The ratio (bottom x-axis) represented by the orange points is calculated based on the numbers of genes in a given pathway divided by the number of genes pertaining to that pathway. Straight orange vertical line represents the threshold for significance (by default). Canonical pathways shown were selected based on their biological significance (a detailed list of canonical pathways and genes involved in each pathway is provided in Supplementary Dataset 5).

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A total of 723 genes had their expression modulated exclusively when embryos were juxtapositioned to bEECs (Contact+) but not by the Contact- condition, as shown in Fig. 7A. The top-5 DEGs include kinesin family member 5C (*KIF5C*), MX dynamin like GTPase 2 (*MX2*), IQ motif containing GTPase activating protein 3 (*IQGAP3*), ubiquitin-specific peptidase 25 (*USP25*) and cytoskeleton-associated protein 2 like (*CKAP2L*). IPA analysis identified enriched biological processes associated with cell cycle regulators, as for example Mitotic Roles of Polo-Like Kinase (upregulated), Cell Cycle: G2/M DNA Damage Checkpoint Regulation (downregulated), Cyclins and Cell Cycle Regulation (upregulated), and Cell Cycle Control of Chromosomal Replication (upregulated). Oxidative Phosphorylation, Estrogen-mediated S-phase Entry, and Pregnenolone Biosynthesis pathways were also retrieved by functional analysis of DEGs. An overview of canonical pathways impacted by juxtaposition between embryos and bEECs is provided in Fig. 7B.

To gain insights on the possible molecules triggering the transcriptome differences observed among the three conditions, upstream regulator analyses of DEGs (FDR ≤ 0.05 , Cutoff fold-changes > 1.5) were performed

in IPA software. A summary of upstream regulators among subsets of DEGs is provided in [Table 3](#) (for a complete list please see Supplementary Dataset 8). Potential upstream regulators based on the 50 DEGs

Figure 7 Juxtaposition changes nature of embryo-induced effects on bEEC transcriptome. (A) Heatmap depicting the top 50 differentially expressed genes (DEGs; $FDR \leq 0.05$) from a set of 723 genes whose expression was modulated exclusively when embryos were juxtapositioned to bEECs (Contact+) but not by the Contact– condition. Reads were normalized within rows. Samples and genes were clustered by average linkage and Spearman rank correlation distances. (B) Overview of canonical pathways returned by Ingenuity Pathway Analysis (IPA) analysis. Contact+ vs Contact– fold-changes (Cutoff >1.5) were considered in this analysis. The charts (y-axis) represent the top significantly enriched canonical pathways. The upper x-axis shows the $-\log$ of the value calculated based on Fisher's exact test. The ratio (bottom x-axis) represented by the orange points is calculated based on the numbers of genes in a given pathway divided by the number of genes pertaining to that pathway. Straight orange vertical line represents the threshold for significance (by default). Canonical pathways shown were selected based on their biological significance (a detailed list of canonical pathways and genes involved in each pathway is provided in Supplementary Dataset 7).

yielded in the Contact+ and Contact– vs Control bEECs confirmed type I interferon-signaling as an important upstream regulator triggering transcriptome changes in bEECs that were cultivated in the presence of embryos, regardless of juxtaposition. In addition, IL6 appears to be an important regulator of embryo-mediated effects on endometrial cells.

Upstream regulators predicted from the subset of 175 genes whose magnitude of expression was regulated between Contact+ and Contact– include Interferon alpha and the transcription regulator interferon regulatory factor 7 (IRF7). Analysis of the 723 genes differentially expressed exclusively when embryos were juxtapositioned to the bEEC monolayer retrieved zinc finger and BTB domain containing 17 (ZBTB17) and prostaglandin E receptor 2 (PTGER2) as potential upstream regulators. Intriguingly, medroxyprogesterone acetate also appeared as an upstream regulator of embryo-mediated effects of juxtaposition on bEEC transcriptome.

Discussion

The major players in pregnancy establishment are the embryo, the maternal reproductive tract and their reciprocal dialogue. Recent discoveries indicate that the endometrial transcriptome is locally changed by the presence of the embryo as early as day 7 *in vivo* in cattle (Sponchiado *et al.* 2017). Other *in vitro* studies showed that bovine embryos elicit responses in endometrial (Talukder *et al.* 2017, Gómez *et al.* 2018, Passaro *et al.* 2018), oviductal (García *et al.* 2017), luteal (Bridi *et al.* 2018) and immune (Talukder *et al.* 2018) cells. A critical question in this scenario is whether physical proximity of the embryo to the endometrium affects the magnitude of or is necessary for any changes in its transcriptome. Alternatively, embryo secretions could act on the surrounding cells in a classical paracrine manner. Using an exploratory approach based on a co-culture system, we interrogated the local embryo–endometrial interface for embryo-induced changes on endometrial cells transcriptome. Embryos were co-cultured in juxtacrine or non-juxtacrine apposition to bEECs for 48 h and the effects at the level of the embryo and the bEECs were investigated. We demonstrated that the bEEC supportive effects on embryo development do not depend on an embryo–bEECs juxtaposition. Nonetheless, our results revealed that the intensity and the characteristic of embryo-induced changes on endometrial epithelial cells transcriptome depend on a physical proximity between embryos and the bEEC monolayer.

We established an endometrial epithelial cell line in a manner that cells originating from the same pool were used in all replicates of each experiment. Even though the bEECs used here were at a low passage number

Table 3 Top upstream regulators predicted based on differentially expressed genes (DEGs; FDR ≤ 0.05 , cutoff fold-changes > 1.5) among the three conditions. Analysis was performed using IPA software.

Upstream regulator	Molecule type	Predicted activation state	Activation z-score	P-value of overlap
Embryo-induced effects on bEEC transcriptome independent of juxtaposition				
<i>IL6</i>	Cytokine	–	1.507	3.72E–05
<i>IFNA2</i>	Cytokine	–	1.927	4.87E–05
<i>IL1RN</i>	Cytokine	–	–	1.80E–04
<i>MAPK1</i>	Kinase	–	–	2.93E–04
9(S)-HODE	Chemical/Endogenous	–	–	8.61E–04
Embryo-induced effects on bEEC transcriptome dependent of juxtaposition – changes in magnitude				
<i>IRF7</i>	Transcription regulator	Activated	6.085	8.49E–61
Interferon alpha	Group	Activated	5.965	8.96E–59
<i>NKX2-3</i>	Transcription regulator	Inhibited	–5.385	3.73E–37
<i>PRL</i>	Cytokine	Activated	5.458	1.61E–35
<i>TRIM24</i>	Transcription regulator	Inhibited	–4.728	2.56E–34
Embryo-induced effects on bEEC transcriptome dependent of juxtaposition – changes in nature				
<i>ZBTB17</i>	Transcription regulator	–	–	1.63E–47
<i>PTGER2</i>	G-protein coupled receptor	Activated	5.375	2.52E–38
<i>CDKN1A</i>	Kinase	Inhibited	–3.042	1.26E–30
Medroxyprogesterone acetate	Chemical drug	Inhibited	–4.150	4.15E–19
<i>IL6</i>	Cytokine	–	0.437	2.03E–10

(i.e. three passages), they co-expressed cytokeratin and vimentin as previously reported (Wang *et al.* 2000, Zeiler *et al.* 2007, Li *et al.* 2016) via a process described as epithelial–mesenchymal transition (Kalluri & Weinberg 2009). At the third passage, the bEECs displayed a stable expression of markers of epithelial-cell origin (*KRT18*) and endometrial function (*ESR1*, *IFNAR1* and *PTGS2*), which are representative of the expected *in vivo* phenotype.

Co-culture with bEECs stimulated embryo development from morula to blastocyst stage regardless of juxtaposition. In the present study, morulae were exposed to bEEC monolayers on day 5.5 p.f. to mimic the timing of the bovine embryo's arrival into the uterus *in vivo* (Guillomot 1994). Although the specific mechanisms by which endometrial cells influence early embryo development remain to be elucidated, many studies report that embryos at early developmental stages benefit from co-culture with endometrial cells in several species, including bovine (Voelkel *et al.* 1985), human (Rubio *et al.* 2000), mice (Kauma & Matt 1995, Lai *et al.* 1996) and porcine (Allen & Wright 1984).

Given that embryos-bEECs co-cultures and embryos (alone) cultures were conducted at 20% O₂, which is suboptimal for early embryo development, there is also a possibility that the cell monolayer could reduce the O₂ tension of the embryo microenvironment in culture and, therefore, benefit the embryos in a non-specific manner (Nagao *et al.* 1994). In our second experiment, we eliminated the suggested importance of a reduced O₂ tension caused by a feeder cell monolayer on embryo development by using a conditioned medium. We showed that medium conditioned by bEECs increased the proportion of morulae reaching the blastocyst stage

compared to the Control group, similar to what was observed in the first experiment. Yet, the cell-specificity of the embryo supportive capacity of bEECs can be questioned by the fact that numerous investigations have demonstrated an improvement in development when embryos were co-cultured with non-endometrial homologous somatic cells, such as oviductal (Eyestone 1987, Thibodeaux *et al.* 1992), luteal (Maruri *et al.* 2018), or granulosa (Freeman *et al.* 1995, Malekshah *et al.* 2006) cells, or even in co-culture systems with adipose tissue-derived mesenchymal stem cells (Miranda *et al.* 2016). It has been also shown that co-culture with heterologous somatic cells, as for example BRL (buffalo rat liver) and Vero (green monkey kidney epithelial) cells, efficiently improve the development of mouse, bovine and human embryos (Ouhibi *et al.* 1990, Reed *et al.* 1996, Menck *et al.* 1997, Duszewska *et al.* 2000, Kattal *et al.* 2008). Taken together, these findings may suggest that the supportive role of bovine endometrial cells for embryo development in co-culture systems is non-specific. To test this, we challenged the specificity of the embryo supportive capacity of bEECs by using a medium conditioned by heterologous unspecific feeder cells. The mice fibroblast-conditioned medium had no effect on embryo development compared to the Control (SOF-based medium) condition. These observations suggest that bovine endometrial cells may provide a specific stimulus for continued development of early embryos in co-culture between days 5.5 and 7.5 after fertilization.

Although the beneficial role of co-culture on embryo development does not seem to be dependent on a juxtaposition with the bEEC monolayer, the embryo-bEEC contact did affect the cell commitment of the resulting

blastocysts, as seen by the increased ICM:TE ratio in the Contact+ group. In mouse, it has been shown that cell-to-cell interaction can influence the lineage specification of embryonic blastomeres (Lorthongpanich *et al.* 2012). The augmented ICM:TE ratio in Contact+ blastocysts, however, did not impact on transcript abundance of *CDX2*, a marker for trophectoderm cells. Additionally, day-7.5 blastocysts that were non-juxtapositioned to the bEEC monolayer displayed increased abundances of *IFNT2* and *BAX* mRNA compared to Contact+ embryos. It has been demonstrated that the attachment of trophectoderm to the uterine epithelium results in a decrease in IFNT expression during the implantation period in cattle (Ezashi & Imakawa 2017). Also, Sakurai *et al.* (2012) reported that co-culture with endometrial epithelial cells resulted in decreased *IFNT2* mRNA abundance in bovine trophoblast cells spheroids in vitro. The mechanisms by which the embryo-endometrium juxtaposition during the peri-hatching period could trigger different patterns in IFNT expression warrant further investigation.

By means of RNAseq, we obtained a genome-wide insight of the endometrial response to the presence of embryos. We verified a much stronger effect (1797 DEGs) elicited by the embryos when they were juxtapositioned to the bEEC monolayer. Transcriptome changes were rather limited between Contact– vs Control bEECs, with only 230 genes being differentially regulated. Analysis of the overlapping DEGs among the three comparisons allowed us to discriminate a subset of 50 genes whose expression was modulated in bEECs when exposed to embryos but independent of juxtaposition. Many of the upregulated DEGs were related to classical type I interferon-mediated pathways. In fact, functional analysis identified Interferon signaling as a major mediator to embryo-induced changes on bEEC transcriptome independent of juxtaposition. Blastocyst-induced changes in the abundance of Interferon-stimulated genes (ISGs) have been reported in the bovine endometrium in vivo (Sponchiado *et al.* 2017), in endometrial explants (Talukder *et al.* 2017, Passaro *et al.* 2018) and immune cells (Talukder *et al.* 2018) in vitro, and are likely triggered by IFNT produced by the embryo. Other studies later on pregnancy also reported major differences in the endometrial transcriptome between pregnant and cyclic cows being induced by IFNT (Bauersachs *et al.* 2006, Klein *et al.* 2006, Forde *et al.* 2011, Sánchez *et al.* 2019). There is no consensus about when the bovine embryo starts to produce IFNT. It has been shown that bovine blastocysts express and release IFNT into the culture medium (Hernandez-Ledezma *et al.* 1993). More recently, Talukder *et al.* (2018) reported the expression of IFNT at the protein level by bovine in vitro-produced morulae. Here, we demonstrated that in vitro-produced day-7.5 bovine embryos express transcripts for *IFNT2*, as well as that the bEECs used in our model express mRNA for

interferon alpha and beta receptor subunit 1 (*IFNAR1*). Although IFNT has been extensively studied based on its ability to mediate pregnancy recognition in ruminants, its potential role in embryo development prior to the pregnancy recognition window is still not completely understood. For instance, it has been shown that the deletion of *Isg15* results in 50% pregnancy loss before placentation in mice (Ashley *et al.* 2010). However, a remaining open question is whether and to which extent classical ISGs have a biological role in programming the endometrium toward a more receptive and supportive status for the development of the bovine embryo in the context of early pregnancy (Pestka 2007).

The magnitude and nature of embryo-induced changes on endometrial transcriptome were largely dependent on juxtaposition between embryos and the bEEC monolayer. Our results identified 175 DEGs that represent changes in the magnitude of effects, whereas a set of 723 DEGs represents changes in nature of signaling triggered exclusively by Contact+ condition. Differences in magnitude of embryo-induced effects on bEEC transcriptome due to a juxtaposition between the embryos and the endometrial cells might be attributed to a diffusion gradient of embryo-derived signals when embryos were placed on transwell inserts, similar to what is expected to occur in non-adjacent, paracrine signaling in vivo. The pore density (<2% of the surface area; 8.0 µm pore size) of the insert membrane might limit or at least slow down the diffusion of secreted signaling molecules between the upper (containing the embryos) and the basal (containing the bEEC monolayer) compartments of the culture well, leading to a less pronounced endometrial response to embryo-derived soluble factors, as for example IFNT. In this regard, our data showed an upregulation of ISGs in the Contact+ compared to the Contact– bEECs. Moreover, upstream regulator analysis of the 175 DEGs predicted Interferon alpha and the transcription regulator interferon regulatory factor 7 (IRF7) as potential candidates of factors depicting transcriptional changes triggered by the juxtapositioned embryos on bEECs (Table 3). In addition to Interferon-associated pathways, IPA analysis of the 175 DEGs yielded biological processes related to antigen presentation in association with the MHC system (i.e. Antigen Presentation Pathway and Allograft Rejection Signaling). The DEGs pertaining to these pathways, beta 2-microglobulin (*B2M*), histocompatibility 2, T region locus 24 (*H2T24*), major histocompatibility complex, class I, B (*HLA-B*), proteasome subunit beta 8 (*PSMB8*) and 9 (*PSMB9*), and transporter 1, ATP binding cassette subfamily B member (*TAP1*), were upregulated in the Contact+ condition. These results provide evidence for a local immune response due to juxtaposition of embryos. Of note, allograft rejection is triggered by allorecognition when the host immune system detects same-species, non-self antigens. It is tempting to speculate that the

local endometrial MHC system might play an important role on immune recognition and tolerance of the semi-allogeneic embryo within the uterine lumen (Koch & Platt 2003).

Pronounced effects on bEEC transcriptome were verified exclusively when embryos were juxtapositioned to the cell monolayer. A subset of 723 DEGs was used to assess the effects triggered by physical proximity between embryos and the bEECs. IPA analysis showed an enrichment for cell cycle biological processes. Further in vivo studies are necessary to address whether the endometrial epithelium changes its proliferation rate and/or its cell morphology in response to the local contact with the embryo/conceptus. Interestingly, the upstream regulator analysis showed medroxyprogesterone acetate as a potential candidate of factors depicting transcriptional changes between Contact+ and Contact– bEECs. Some of the 20 DEGs (*ANLN*, *ASPM*, *BIRC5*, *BRIP1*, *CCNB1*, *CCNB2*, *CDK1*, *CENPF*, *CKS2*, *CXCL5*, *DEPDC1*, *DLGAP5*, *KIF11*, *NCAPG*, *NEK2*, *NUSAP1*, *PBK*, *PGR*, *PRC1*, *SPC25*) pertaining to the mechanistic network (101 genes) have been found to be regulated by progesterone in the uterus (Shimizu *et al.* 2010, Forde *et al.* 2012, Xin *et al.* 2018). Limitations of the present work are that our model was hormone-free and did not take into account the glandular epithelium, limiting the direct physiological translatability of this finding.

Relatively little work has been done on the mechanisms underpinning a possible juxtracrine communication at the embryo–maternal interface prior to zona-hatching in monotocous mammals. It is known, however, that there is no intimate contact (i.e. tissue adhesion) between the pre-implantation, non-invasive embryo and the lining endometrial epithelium in the cow (Bazer *et al.* 1991). It is noteworthy that in this study, at the end of the co-culture period (day 7.5 p.f.), most embryos (~83%) were still enclosed in the zona pellucida. Direct cell-to-cell contact at the pre-hatching stage might be provided by membrane extensions through the zona pellucida (Allen & Wright 1984) and trophoctoderm projections (Gonzales *et al.* 1996). Furthermore, it has been described that the zona pellucida could also contribute with binding sites and local release of signaling molecules (i.e. growth factors and cytokines) toward the endometrial cells (Denker 2000, Herrler *et al.* 2002). Alternatively, the embryos can also release extracellular vesicles that have the potential to trigger endometrial transcriptome and immune responses independently of a cell-to-cell contact (Bridi *et al.* 2020).

Another possible explanation for the embryo-induced effects on Contact+ bEECs, that were not directly triggered by physical contact, is that Contact+ embryos may have a distinct capacity to secrete signaling molecules, compared to Contact– embryos. In the present study, no difference was observed between embryos derived from Contact+ vs Contact– conditions in terms of blastocyst

development on day 7.5 p.f. However, the embryo–bEECs juxtaposition did impact the resulting blastocysts' cell commitment as seen by increased ICM:TE ratio in the Contact+ group, as well as on the 4-fold greater abundance of transcripts for *IFNT2* in the Contact– embryos compared to their Contact+ counterparts. Thus, it is also reasonable to expect that the co-culture conditions affected the signaling capacity of those embryos. Although our experimental set-up does not allow us to distinguish between these possibilities, we speculate that differences on bEEC response to Contact+ vs Contact– embryos likely reflect a combination of the aforementioned mechanisms.

The present study provides new insights into the complex embryo–maternal interaction during the very early steps of pregnancy in cattle. On the embryonic side, the co-culture system applied in this study improved bovine embryonic development from morula to blastocyst stage. This support seems to be bEEC specific and does not rely on a juxtaposition between the embryo and the bEEC monolayer. Nonetheless, juxtaposition with bEECs altered day 7.5 blastocysts' cell fate and *IFNT2* transcripts abundance. We demonstrated for the first time that peri-hatching bovine embryos drastically change the endometrial epithelial cell transcriptome. Highlighted pathways modulated by the presence of embryos included Interferon signaling and prostanoid biosynthesis. Moreover, our data reveal that nature and magnitude of embryo-induced effects are largely influenced by physical proximity between endometrial cells and embryos, with emphasis to antigen presentation and MHC system-associated biological processes triggered in response to the semi-allogeneic embryos' presence. The functional consequences of this local recognition by the lining endometrial epithelium and how the embryo evades immune rejection in the bovine species, warrant further investigation.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-20-0344>.

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.

Funding

This work was supported by the Special Research Fund University of Antwerp (Joint-PhD Program, BOF-UA), and by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil (CAPES; Finance Code 88881.132730/2016-01 to M.S).

Data availability

RNA sequencing data are available under accession number GSE151868.

Author contribution statement

M S, M B and J L M R L conceived the study. M S drafted the manuscript. M S, W F A M and G T S B performed experiments and analyzed data. P E J B critically revised the manuscript. M B and J L M R L supervised the experiments. All authors edited and reviewed the manuscript.

Acknowledgements

The outstanding technical assistance of Gamete Research Centre staff and students to generate the embryos is gratefully acknowledged. The authors acknowledge Jens Slootmans for the assistance in performing RNA isolation and PCR analysis.

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Received 17 June 2020

First decision 20 July 2020

Revised Manuscript received 21 August 2020

Accepted 17 September 2020