Gene expression and metabolic response of bovine oviduct epithelial cells to the early embryo

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Abstract

During its journey through the oviduct, the bovine embryo may induce transcriptomic and metabolic responses, via direct or indirect contact, from bovine oviduct epithelial cells (BOECs). An *in vitro* model using polyester mesh was established, allowing the study of the local contact during 48h between a BOEC monolayer and early embryos (2- or 8-cell stage) or their respective conditioned media (CM). The transcriptomic response of BOEC to early embryos was assessed by analyzing the transcript abundance of *SMAD6*, *TGDF1*, *ROCK1*, *ROCK2*, *SOCS3*, *PRLP* and *AGR3* selected from previous *in vivo* studies and *GPX4*, *NFE2L2*, *SCN9A*, *EPSTI1* and *IGFBP3* selected from *in vitro* studies. Moreover, metabolic analyses were performed on the media obtained from the co-culture. Results revealed that presence of early embryos or their CM altered the BOEC expression of *NFE2L2*, *GPX4*, *SMAD6*, *IGFBP3*, *ROCK2* and *SCN9A*. However, the response of BOEC to two-cell embryos or their CM was different from that observed to eight-cell embryos or their CM. Analysis of energy substrates and amino acids revealed that BOEC metabolism was not affected by the presence of early embryos or by their CM. Interestingly, embryo metabolism before embryo genome activation (EGA) seems to be independent of exogenous sources of energy. In conclusion, this study confirms that early embryos affect BOEC transcriptome and BOEC response was embryo stage specific. Moreover, embryo affects BOEC via a direct contact or via its secretions. However transcriptomic response of BOEC to the embryo did not manifest as an observable metabolic response.

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Introduction

During its 4-day journey through the oviduct (Hackett *et al.* 1993), the bovine embryo undergoes important morphological and transcriptional events such as the first mitotic cell divisions, the timing of which is reflective of developmental competence (Lonergan *et al.* 1999) and embryonic genome activation (EGA) involving a switch from dependence on transcripts stored in the oocyte to *de novo* transcription (Memili & First 2000). There is convincing evidence demonstrating a positive influence of the oviduct on embryo development in terms of embryo cryotolerance, gene expression and developmental competence (Enright *et al.* 2000, Lazzari *et al.* 2002, Rizos *et al.* 2002, Lonergan *et al.* 2003). However, the embryo effect on the oviduct is less studied. In mice, the presence of embryos in the oviduct increased the expression of specific genes such as thymosin beta-4, ribosomal protein L41 and nonmuscle myosin light

chain 3 (Lee *et al.* 2002). In pigs, Almiñana *et al.* (2012) reported that the presence of embryos reduced the expression of genes related with immune function. Also, transforming growth factor- α , transforming growth factor- β -binding protein 2 and atrial natriuretic factor receptor-like were upregulated in the presence of four-cell porcine embryos (Chang *et al.* 2000).

It is likely that the effect of any putative embryo-derived signals on the cells of the oviduct would be magnified in litter-bearing species due to the presence of multiple embryos in the oviduct. That notion is supported by a previous study from our group in cattle, a mono-ovulatory species, which failed to detect a transcriptomic response of the oviduct to the presence of a single embryo, while a response was observed after the transfer of multiple embryos (Maillo *et al.* 2015). In the horse, the presence of a single embryo altered the oviduct transcriptome (Smits *et al.* 2016), when epithelial cells were collected locally from the ampullary–isthmic

junction. Combined, these findings suggest that the presence of a single embryo in the oviduct may induce a very local effect on oviduct transcriptome which is difficult to detect. Furthermore, *in vivo* studies remain challenging, due to the relative inaccessibility of the oviduct. BOECs have been cultured *in vitro* in many systems, as monolayers, in perfusion chambers, in suspension, in polarized or in 3D systems (reviewed by Maillo *et al.* 2016). Recent *in vitro* culture strategies have mimicked the morphological and functional changes occurring in the oviduct epithelium using an air–liquid interface (Chen *et al.* 2017, 2018) or using the state-of-the-art oviduct-on-a-chip platform (Ferraz *et al.* 2018). However, considering the complexity of studies aiming to understand the regulatory mechanisms controlling embryo–maternal communication, the simple BOEC *in vitro*, cultured as a monolayer might provide a good starting point to study embryo signals. For instance, Schmaltz-Panneau *et al.* (2014) identified differentially expressed genes related to the immune system and interferon signaling by co-culturing early embryos for 8 days on a 13-day-old BOEC monolayer (Schmaltz-Panneau *et al.* 2014). Also, in a recent study carried out by our group using the BOEC monolayer model, we reported that bovine embryo–oviduct interaction *in vitro* reveals an early cross-talk mediated by bone morphogenetic protein (BMP) signaling (García *et al.* 2017).

In order to test the hypothesis that the early embryo induces transcriptional and metabolic changes in BOEC, we established an *in vitro* co-culture system allowing both local and temporal contact between early bovine embryos and oviduct isthmus epithelial cells, where development occurs *in vivo*. The objectives were (i) to assess the gene expression response of BOEC to early embryos and to test whether it is due to a contact-dependent signal or the result of BOEC interaction with embryo secretions and (ii) to examine the metabolic changes of BOEC in the presence or absence of early embryos.

Materials and methods

Chemicals

All reagents were purchased from Sigma Chemical Química S.A Company unless otherwise stated.

Bovine oviduct epithelial cell isolation and *in vitro* culture

Bovine oviducts at the early luteal phase (corresponding to days 3–5 of the estrous cycle) were obtained from slaughtered heifers and selected based on ovarian morphology, according to Ireland *et al.* (1980). Oviduct epithelial cell isolation was performed as described previously (García *et al.* 2017). Cells were cultured at a final concentration of 2×10^6 cells/mL with TCM 199 supplemented with 10% fetal calf serum (FCS), 2.5% gentamycin and 1% amphotericin, in four-well plates at 38.5°C

Figure 1 (A) Immunofluorescence analysis of BOEC monolayers showing positive staining for the epithelial markers, cadherin and cytokeatin and negative staining for the fibroblast marker, vimentin. Cell nuclei were counterstained with Hoechst (blue). (B) Representative image of the polyester meshes employed to establish a direct and indirect contact area between the embryos and the BOEC monolayer. A guide mesh of 41×41 openings, covering an area of 121 mm^2 , containing delimited marked areas of 50 openings ($(7 \times 7) + 1$) was placed outside underneath each well of a four-well dish and was used as a guide to localize the co-culture area. Another mesh square of the same size, fitting exactly into the well, was introduced in a way that the external and internal meshes overlapped during the co-culture.

in an atmosphere of 5% CO_2 , 20% O_2 and saturated humidity until confluence (6–7 days). Half of the media was renewed every 48 h. Twenty-four hours before starting the co-culture with embryos or their CM, the cell culture media was replaced with Synthetic Oviduct Fluid supplemented with amino acids (SOFaa) + 5% FCS (see ‘Experimental design’ section for more details and Fig. 2).

Immunocytochemistry

The epithelial nature of the cultured cells was confirmed by immunocytochemical analysis using antibodies anti-bovine-pancadherin (C1821), anti-bovine-pancytokeatin (C2931) and anti-bovine-vimentin (V2258), as previously described (Lopera-Vásquez *et al.* 2016) (Fig. 1A).

In vitro production of embryos and their CM

Immature cumulus oocyte complexes recovered by aspirating follicles (2–8 mm in diameter) from ovaries of heifers slaughtered at a local abattoir were submitted to *in vitro* maturation and fertilization, as described previously (García *et al.* 2017). Approximately 18–22 h after insemination, presumptive zygotes were completely denuded of cumulus cells by vortexing and groups of 50 were initially cultured in 500 μL of SOFaa supplemented with 5% of FCS in a four-well dish under an atmosphere of 5% CO_2 , 5% O_2 and 90% N_2 at 38.5°C. Embryos at the two- and eight-cell stage were selected at 31 and 52 h post insemination (hpi), respectively, and then randomly cultured either in groups of 50 for 48 h to produce embryo CM or co-culture with BOEC (see Experimental design, Fig. 2).

Figure 2 Experimental design. (A) Replicates of BOEC ($n=4$), embryo CM ($n=4$) and embryo ($n=4$) production were carried out in a synchronized way. BOEC were cultured until confluence for 6 days. Half of the media was renewed every 48 h, and 24 h before starting the co-culture, it was replaced with SOFaa + 5% FCS. Embryos at the two- and eight-cell stages were generated by IVF (0h) and selected at 31 or 52 h post insemination (hpi) respectively to be cultured during 48 h to either generate fresh CM or co-cultured with BOEC. The following experimental groups were established: (1) BOEC directly beneath to 50 embryos at two- or eight-cell stage (BOEC*); (2) BOEC in the same well as two- or eight-cell embryos but not in direct contact (BOEC^{CM}); (3) BOEC cultured with CM produced from 50 embryos at two- or eight-cell stage (BOEC^{CM}); (4) BOEC cultured without embryos (BOEC); (5) embryos at two- or eight-cell stage cultured alone (EMB) and (6) embryo/BOEC-free media as control (BLANK MEDIA). After 48 h of co-culture, BOEC were recovered from the marked area from groups 1, 2, 3 and 4 to perform gene expression analysis (B), while the media obtained from groups 1, 3, 4, 5 and 6 were collected to assess metabolic analysis of energy substrate and amino acid composition (C).

Mesh preparation and co-culture condition

In order to limit the area of contact between the embryos and the BOEC monolayer, a nontoxic woven polyester mesh (Sefar Petex; Sefar, Bury, Lancashire, UK) was used as described previously (García *et al.* 2017). Grids with size of 41×41 openings covering an area of 121 mm^2 , fitting perfectly inside the well of a Nunc 4-well dish, were used. First, a template grid containing delimited marked areas of 50 openings ($(7 \times 7) + 1$) was placed outside underneath each well of a four-well dish (Fig. 1B). A second mesh square of the same size was washed once with 70% ethanol and three times with PBS followed by SOFaa media, and then it was introduced into the well, so that the external and internal meshes overlapped (Fig. 1B). During the co-culture, 50 embryos (at the two- or eight-cell stage) were placed inside the BOEC well, over the central marked area. After 48 h of co-culture, BOEC were collected from the marked area directly beneath the embryos and as well as from other areas of the well that were not in direct contact with the embryos (Fig. 1B) by mechanical scraping with a micropipette tip and snap frozen in liquid nitrogen and maintained at -80°C until gene expression analysis.

RNA isolation and reverse transcription

mRNA from each experimental group of BOEC was extracted from four biological replicates using Dynabeads mRNA Direct Extraction Kit (DynaL Biotech, Oslo, Norway), following the manufacturer's instructions with minor modifications as

described previously (Bermejo-Alvarez *et al.* 2008). After 10 min of incubation in lysis buffer with Dynabeads, poly(A) RNA attached to the Dynabeads was extracted with a magnet and washed twice in washing buffer A and washing buffer B. RNA was eluted with Tris-HCl. Immediately after extraction, the reverse transcription reaction was performed as recommended by the manufacturer (Epicentre Technologies Corp, Madison, WI, USA). Briefly, oligo-dT ($0.2 \mu\text{M}$) and random primers ($0.5 \mu\text{M}$) were added to the RNA and were then heated for 5 min at 70°C to denature the secondary RNA structure. Next, the tubes were incubated at 25°C for 10 min to promote the annealing of random primers. Then, the RNA was reverse-transcribed for 60 min at 37°C in a final volume of $40 \mu\text{L}$ containing 0.375 mM dNTPs (Biotools, Madrid, Spain), 6.25 U RNasin RNase inhibitor (Promega), $10 \times$ MMLV-RT buffer with 8 mM dithiothreitol and 5 U MMLV high-performance reverse transcriptase (Epicentre Technologies Corp), followed by incubation at 85°C for 5 min to inactivate the RT enzyme.

Gene expression analysis by quantitative real-time polymerase chain reaction

The mRNA expression levels of the selected genes were determined by real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) using specific primers designed with Primer-BLAST software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) to span exon-exon boundaries when possible (Table 1). Primers were previously validated for adequate primer efficiency and specificity of their PCR

Table 1 Set of primers used for qRT-PCR assays.

Gene	Primer sequence (5'–3')*		Fragment size (bp)	Gene bank accession
	Forward	Reverse		
<i>H2AFZ</i>	AGGACGACTAGCCATGGACGTGTG	CCACCACCAGCAATTGTAGCCTTG	209	NM_174809
<i>ACTG1</i>	GAGAAGCTCTGCTACGTCTG	CCAGACAGACCCGTGTTGG	255	NM_001033618.1
<i>GPX4</i>	TGTGGTGAAGCGGTATGGTC	TATCCCACAAGGCAGCCAG	266	NM_174770.4
<i>NFE2L2</i>	GCTCAGCATGATGGACTTGGAG	GGGAATGTCTCTGCCAAAAGC	390	NM_001011678.2
<i>ROCK1</i>	ACGTGACCTAGTGCCTTGTG	CCTCAGTGTGCTTTTGTGCC	164	XM_002697789.5
<i>ROCK2</i>	CTTGGCTGCTCAACTGGAGA	TGCTCTTGGGCTTCCCTTCAG	276	NM_174452.2
<i>AGR3</i>	TGTCACACTCAGTTCTGGTCC	GTCATCTCCCCACCCTCTTGA	119	NM_001191502.1
<i>SMAD6</i>	GGAGAAATTCGCTCCAAGTGC	CCCTGCCTTTAAAACCCAAGC	242	NM_001206145.1
<i>TDGF1</i>	ATGGTGAGAGACGGGCTGCTAG	GCCCTTGCTCTCATACAGCTTC	201	NM_001080358.1
<i>SCN9A</i>	GTTGATAACCCTGTGCCTGGA	CTTCAAAAGCCAGAGCACCAC	250	XM_005202453.3
<i>SOCS3</i>	GCGAGAAGATCCCTCTGGTG	CTAAAGCGGGCATCGTACT	167	NM_174466.2
<i>IGFBP3</i>	GAGTCCAAGCGTGAGACAGAA	GCGGCACTGCTTTTCTTGTA	150	NM_174556.1
<i>PRELP</i>	CAGCATCGAGAAAATCAATGGGA	AGCACATCATGAGGTCCAGC	158	NM_174434.3
<i>EPSTI1</i>	AAACGACAGCAACAGGAGGAA	CCTTGGAGTCGGTCCAGAAA	89	XM_002691775.5

*All the primers were designed with NCBI Primer-BLAST online tool of the National Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

products was confirmed by electrophoresis on a 2% agarose gel. All target genes showed efficiencies between 95 and 100% and correlation coefficients close to 1.0.

All mRNA transcripts were quantified in duplicate. Each RT-qPCR reaction was performed in a final volume of 20 µL, containing 0.25 mM of forward and reverse primers, 10 µL of GoTaq RT-qPCR Master Mix (Promega) and 2 µL of each cDNA sample derived from BOEC (≈60 ng/µL) using a Rotorgene 6000 Real-Time Cyclor (Corbett Research, Sydney, Australia) and SYBR Green as double-stranded DNA-specific fluorescent dye.

Relative expression levels were quantified by the comparative cycle threshold ($\Delta\Delta CT$) method (Schmittgen & Livak 2008). Values were normalized using two housekeeping genes (*H2AFZ* and *ACTG1*). Fluorescence was acquired in each cycle to determine the threshold cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. According to the comparative CT method, the ΔCT value was determined by subtracting the mean CT value of the two housekeeping genes for each sample from each gene CT value of the sample. Calculation of $\Delta\Delta CT$ involved using the highest sample ΔCT value (i.e. the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$ (Livak & Schmittgen 2001).

Metabolic analysis

Glucose lactate and pyruvate consumption

Concentrations of glucose, lactate and pyruvate in spent medium following culture of BOEC in the presence or absence of embryos were measured using fluorometric assays based on those described by Gardner & Leese (1990), but with modifications (Guerif *et al.* 2013). All values were expressed as mM and were relative to a blank control to calculate consumption rate.

Amino acid profiling

The analysis of 18 amino acids in spent medium was performed using high-performance liquid chromatography (HPLC) as previously described (Houghton *et al.* 2002). All values were expressed in µM and were relativized to blank control to calculate consumption rate.

Experimental design

Experiment 1: Direct and indirect effects of the embryo on the BOEC gene expression

An *in vitro* co-culture system was established to allow a localized and temporal contact between BOEC and early bovine embryos. Isthmic epithelial cells obtained from a pool of three post-ovulatory stage oviducts on each day (day=replicate, four replicates) were cultured until confluence/monolayer for 6 days. Based on our previous studies (Barrera *et al.* 2017, García *et al.* 2017), where the highest proportions of two- and eight-cell embryos were observed at 31 and 52 hpi respectively, in this experiment embryos, at the two- and eight-cell stages were generated by IVF and removed from culture at 31 or 52 hpi respectively to be cultured during 48 h to either generate fresh CM (four replicates) or co-cultured with BOEC (four replicates) (Fig. 2A). BOECs were cultured for 48 h as follows: (1) directly beneath and in direct contact with 50 embryos at the two- or eight-cell stage (BOEC⁺); (2) in the same well as 50 embryos at the two- or eight-cell stage but not in direct contact with the embryos (BOEC⁻); (3) with CM from embryos at the two- or eight-cell stage (BOEC^{CM}) and (4) without embryos or CM (control, BOEC) (Fig. 2B). Following 48 h of culture, BOECs were collected from the marked area from all groups by mechanical scraping to perform RT-qPCR. The mRNA abundance of genes that had previously been shown to be affected by the presence of embryos from an *in vivo* study (Maillo *et al.* 2015: *SMAD6*, *TDGF1*, *ROCK1*, *ROCK2*, *SOCS3*, *PRELP*, *AGR3*, *NFE2L2*) and from *in vitro* studies (Schmaltz-Panneau *et al.* 2014, 2015: *GPX4*, *SCN9A*, *EPSTI1*, *IGFBP3*) were analyzed as described earlier.

Experiment 2: Effect of early embryo on BOEC metabolism

After 48 h of co-culture, the media obtained from the previous experimental groups (BOEC⁺, BOEC^{CM}, BOEC⁻) as well as the spent media obtained from embryos cultured alone (EMB) were collected to assess the metabolic changes that may result from interaction of BOEC with the embryos or with their CM, by analyzing the energy substrate and amino acid composition. As blank control, embryo/BOEC-free media was treated in the same manner as the experimental groups (Fig. 2C).

Statistical analysis

Statistical analysis was performed with the SigmaStat software package (Jandel Scientific, San Rafael, CA, USA). The Kolmogorov–Smirnov test was applied to assess data normality. Differences in embryo development were analyzed using chi-square. Differences in the relative mRNA abundance levels and metabolic results were analyzed using one-way ANOVA followed by Holm–Sidak *post hoc* test, when applicable, to determine the statistical differences between the experimental groups. When normality test failed Kruskal–Wallis, a non-parametric test was conducted. Values were considered significantly different when the *P* value was lower than 0.05.

Results

Effect of BOEC co-culture on early embryo development in vitro

Cleavage rates at 31 hpi (two-cell=774/1177) and 52 hpi (eight-cell=652/1197) were $65.5 \pm 2.5\%$ and $54.0 \pm 7.4\%$ respectively. After 48 h of co-culture, no differences were found in the percentage of two-cell embryos developed to eight-cell stage between embryos co-cultured with ($57.0 \pm 1.4\%$) or without BOEC ($65.0 \pm 2.6\%$). Similarly, the percentage of 8-cell embryos developed to 16-cell stage was not different between both groups ($51.0 \pm 4.2\%$ vs $57.7 \pm 1.9\%$ for embryos cultured with or without BOEC respectively).

Experiment 1: Direct and indirect effects of the embryo on the BOEC gene expression

The presence of 50 two-cell embryos, in direct contact, or their CM decreased the relative abundance of *GPX4*, *ROCK2* and *SCN9A* in BOEC in comparison with the control group ($P < 0.05$). Abundance of *SMAD6* was decreased in the presence of embryos, irrespective of direct contact, but was not altered following exposure to CM. The only difference between cells cultured in direct or indirect contact with embryos was observed for *NFE2L2* gene, which decreased in cells directly cultured with embryo or their CM in comparison with the culture in indirect contact or control groups. Culture of BOEC with CM increased expression of *IGFBP3* ($P < 0.05$) compared with the control, but no difference was observed with the rest of groups (Fig. 3A). The remaining genes were unaffected by treatment ($P > 0.05$) (Fig. 3A).

Co-culture in the presence of eight-cell embryos or their CM revealed a different expression pattern of many of the analyzed genes to that observed with two-cell embryos. *PRELP* was decreased ($P < 0.05$) in the direct or indirect presence of eight-cell embryos or their CM in comparison with the control group. Also, BOEC directly exposed to eight-cell embryos or their CM exhibited decreased expression of *SOC33* and increased expression of *SCN9A* in comparison with BOEC cultured in indirect contact with embryos and with the control ($P < 0.05$). *GPX4* was only decreased in BOEC cultured directly with embryos in comparison with the other groups. Furthermore, the expression level of *NFE2L2* was increased ($P < 0.05$) in cells cultured with CM compared to the control but no differences were observed with the rest of groups. Also, abundance of *ROCK1* and *ROCK2* was increased ($P < 0.05$) in cells cultured with CM in comparison to the control or to the direct culture with embryos, but it was similar to the indirect contact group. *SMAD6* is the only gene that maintained the pattern of expression between BOEC co-cultured with either

Figure 3 Relative mRNA abundance of genes assessed in BOEC cultured with two-cell (A) or eight-cell (B) stage embryos. BOEC⁺: BOEC directly beneath to embryos. BOEC⁻: BOEC in the same well as embryos but not in direct contact. BOEC^{CM}: BOEC cultured with embryo CM. BOEC: cultured without embryos or CM. Bars represent the relative abundance of the transcripts analyzed and normalized to *H2AFZ* and *ACTG1* as housekeeping genes. Results are expressed as means $\pm$ S.E.M. Different superscripts indicate significant differences ($P < 0.05$) between groups. Data were obtained from four replicates of independent BOEC samples per experimental group.

two- or eight-cell embryos, where it decreased in BOEC cultured in direct or indirect contact with embryos in comparison with the rest of groups ($P < 0.05$) (Fig. 3B). The remaining genes did not exhibit an alteration of their expression due to treatment ($P > 0.05$) (Fig. 3B).

Experiment 2: Effect of early embryo on BOEC metabolism

Glucose, lactate and pyruvate consumption

Glucose, lactate and pyruvate consumption by BOEC was not affected by the presence of two-cell or eight-cell embryos (Fig. 4A and B). Indeed, a similar consumption of glucose and pyruvate by BOEC was observed whether they were cultured independently with or without embryos or CM. However, BOEC consumption of those substrates was higher than embryo consumption ($P < 0.001$ for two-cell embryos; $P < 0.005$ for eight-cell embryos). In contrast, lactate production was elevated in BOEC cultured with CM from two-cell embryos and in media from two-cell embryos cultured alone in comparison with the other groups ($P < 0.001$) (Fig. 4A). Lactate consumption was also higher in BOEC cultured alone or with eight-cell embryos and in media from eight-cell embryos cultured alone in comparison with BOEC cultured with CM ($P < 0.005$) (Fig. 4B). Regarding embryo metabolism, two-cell embryos cultured without BOEC did not reveal a consumption of energy substrates when they were compared to the blank control (data of blank media were not included in the graphs).

Amino acid profiling

The results of HPLC revealed that among the 18 amino acids analyzed, the concentration of ten was affected in BOEC co-cultured with two-cell embryos and 12 in the BOEC co-cultured with eight-cell embryos. These amino acids were significantly highly depleted in BOEC cultured alone or with embryos or with CM compared with both stage embryos cultured alone, except for alanine having the opposite pattern (Fig. 5A and B) ($P < 0.005$). Similar to alanine, serine had the same tendency of accumulation, but significant differences were observed only for eight-cell embryos in BOEC cultured alone or with embryos or CM compared with eight-cell embryos cultured alone ($P < 0.005$) (Fig. 5B). Regarding embryo metabolism, two-cell embryos cultured without BOEC did not reveal a consumption of amino acids substrates when they were compared to the blank control (data of blank media were not included in the graphs).

Discussion

We have previously reported that oviduct transcriptome changes produced by the presence of an embryo may be very local in nature (Maillo *et al.* 2015). The present study aimed to validate these data using an *in vitro* model

Figure 4 Energy substrate analysis (glucose, lactate and pyruvate) in BOEC cultured with two-cell (A) or eight-cell (B) stage embryos. Depletion/production data are expressed relative to a control blank media. Values are expressed as means $\pm$ S.E.M. BOEC⁺, BOEC co-cultured with embryos; BOEC^{CM}, BOEC cultured with embryo CM; BOEC, BOEC cultured neither with embryos nor with their CM; EMB, embryos cultured alone. Different superscripts indicate significant differences between groups (A, $P < 0.001$); (B, $P < 0.005$). All the groups included under the brackets are statistically similar between each other, but they are different to the other group(s).

which offered the double benefit of immobilizing the embryo on the surface of the BOEC monolayer during co-culture, enabling the analysis of the local interaction between BOEC and embryos. In this model, we chose to use 50 embryos as we wanted to amplify any putative signal, also this number corresponds to the number of embryos transferred in the study of Maillo *et al.* (2015).

The response of BOEC to two- and eight-cell embryos or their CM was evaluated at gene expression and metabolic levels. The overall transcriptional profile of a panel of candidate genes between BOEC co-cultured with two-cell embryos or with their CM was different from that observed after co-culture with eight-cell embryos or their CM. In cattle, the major activation of transcription occurs during the 8- to 16-cell stage (reviewed by Graf *et al.* 2014). These events perhaps are accompanied by specific embryo signaling. Evidence is emerging of a dynamic mutual paracrine communication between both the embryonic and the maternal environments, through extracellular vesicles (EVs), at the early stages of preimplantation embryo development (Saadeldin *et al.* 2015, Almiñana *et al.* 2017).

Figure 5 Amino acid metabolism in BOEC co-cultured with two-cell (A) or eight-cell (B) stage embryos. Depletion/production data are expressed relative to a control blank media. Values are expressed as means $\pm$ S.E.M. BOEC⁺, BOEC co-cultured with embryos; BOEC^{CM}, BOEC cultured with embryo CM; BOEC, BOEC cultured neither with embryos nor with their CM; EMB, embryos cultured without BOEC. Different superscripts indicate differences between groups ($P < 0.005$). All the groups included under the brackets are statistically similar between each other, but they are different to the other group(s).

For example, the secretome of *in vitro*-cultured human embryos contains EVs that are taken up by the maternal side (Giacomini *et al.* 2017). In support of the existence of stage-specific effects of the embryo on the reproductive tract, data from Passaro *et al.* (2018) have shown that bovine endometrial explants respond to the presence of 8-day-old blastocysts and not to earlier stages (oocytes, two-cell, Day 5 morula).

Serum has been associated with reduced blastocyst quality, although it is still used for embryo culture (e.g., 5–10%) (reviewed by Rizos *et al.* 2017). We have reported that 10% FCS accelerates blastocyst development, with more blastocysts appearing at Day 6 after IVF than when it is absent and that this accelerated development was associated with poorer survival following cryopreservation (Rizos *et al.* 2003). However, others working in commercial IVF/ET in Brazil routinely use low concentrations (e.g., 2.5%) of FCS and report no difference in survival following cryopreservation or conception rates after transfer (Sanches *et al.* 2016). Also, BOEC cultures typically require the presence of serum for normal development, cell attachment, growth and proliferation (Brunner *et al.* 2010). Thus, FCS was supplemented to all experimental groups.

Results indicate that BOEC mRNA abundance was affected directly by embryos or by their secretions, depending on the transcript analyzed, which indicates the complexity of the mechanisms implicated in embryo–maternal communication. The oviductal mRNA abundance of *SMAD6* decreased in the presence of both two- and eight-cell embryos, consistent with our

previous data indicating the relevance of *SMAD6* as a component of the BMP signaling pathway (García *et al.* 2017).

SOCS3, *PRELP* and *SCN9A* expression was affected in BOEC cultured in direct contact with eight-cell embryos and their CM. The fact that eight-cell embryos decreased the expression of *SOCS3* and *PRELP*, implicated in immune defense, supports the idea that embryo may avoid the maternal immune response by decreasing inflammation (Maillo *et al.* 2015). This agrees with data obtained from the comparison of ipsilateral oviducts from pregnant and cyclic mares, showing that the embryo modifies the expression of immune response-related genes with marked upregulation of interferon-associated genes (Smits *et al.* 2016). This is also consistent with recent data indicating that BOEC stimulates embryos to produce IFNT, which then acts on immune cells to promote an anti-inflammatory response in the oviduct (Talukder *et al.* 2018). Expression of *SCN9A* was upregulated in BOEC co-cultured with eight-cell embryos or their CM. This is consistent with the study of Schmaltz-Panneau *et al.* (2014) who hypothesized that there is neurotrophin signaling by the embryo at the time of blastocyst formation (Schmaltz-Panneau *et al.* 2014). Furthermore, *SCN9A* mediates the voltage-dependent sodium ion permeability of excitable cell membrane. (Chan *et al.* 2012). Taking this information into account, our results indicate that during its passage through the oviduct, the early embryo might affect the epithelial ion channels modulating the luminal fluid volume and composition according to its needs.

The *IGFBP3* transcript was also increased in BOEC cultured with CM of two-cell embryos, a finding consistent with previous reports of an increase of *IGFBP3* in BOEC co-cultured for 8 days with 20 early embryos (Schmaltz-Panneau *et al.* 2014). *IGFBP3* belongs to a family of six binding proteins that bind insulin-like growth factor 1 and 2 (IGF-1 and -2). It was demonstrated that the bovine oviduct expresses *IGFBP-3* in a region-specific manner in the isthmus epithelium. This pattern of distribution might help to create an IGF-1 gradient along the oviduct as the embryo travels through it (Pushpakumara *et al.* 2002).

Recently, changes in the oviduct microenvironment in its metabolite levels related to the stage of the cycle and the proximity of ovulation have been reported (Lamy *et al.* 2018). However, whether the embryo can elicit similar responses is largely unknown. Consequently, we sought to evaluate the effect of the early embryo on the metabolism of energy substrates and amino acids of BOEC cultured *in vitro*. Results indicate that BOEC metabolism was not affected by the presence of early embryos (two-cell or eight-cell stage) or by their CM. Furthermore, results indicate that BOEC metabolism is different to that of embryos, suggesting that BOEC and embryos have metabolic requirements that may be independently satisfied *in vitro* by the culture media. BOEC consumed glucose, lactate and pyruvate as well as amino acids, except for serine and alanine, which were produced by cells. It has previously been proposed that BOEC altered the composition of culture medium in a manner consistent with embryo development; indeed, BOEC-depleted glucose concentrations while there was an accumulation of lactate and pyruvate, two key metabolites required for successful development of the early embryo indicating that BOEC might be attempting to create an environment favorable for embryo development (Edwards *et al.* 1997). However, it is important to note that in their experiment, Edwards *et al.* (1997) performed the co-culture in SOFaa media supplemented with high levels of carbohydrates, mimicking those present in TCM-199. In contrast, our study used SOFaa that was free of carbohydrates, following the composition previously described (Holm *et al.* 1999) which may explain the discrepancies in results.

Interestingly, embryo metabolism before EGA seems to be independent of the environment. The results indicated no consumption of energy substrate or amino acids by two- to eight-cell embryos. However, this was not the case for embryos at the 8- to 16-cell stage which displayed an active metabolism. The lack of metabolism by two- to eight-cell embryos may suggest that the embryo has sufficient endogenous energy sources, most likely fat but possibly protein, to support its early development (Sturmey *et al.* 2009, Leese 2015).

The amino acid analysis revealed the production of serine and alanine by BOEC independent of the presence of embryos. The release of alanine amino acid in the extracellular medium is witness of ammonium detoxification mediated by pyruvate metabolism (reviewed by de Souza *et al.* 2015). Furthermore, alanine is involved in the regulation of intracellular pH and may have a role in protecting the embryo from osmotic stress (Van Winkle 2001), and it has shown to promote bovine preimplantation development *in vitro* (Moore & Bondioli 1993). Thus, BOEC could be predisposed to increase alanine concentration in the media to sustain embryo needs.

In conclusion, using an *in vitro* approach allowing a local and temporal interaction between the embryo and oviduct epithelial cells, this study confirms that the early embryo affects the gene expression of BOEC. The effect was embryo stage specific and resulted from a direct contact with BOEC or from embryo secretions released into the media. Under our experimental conditions the early embryo did not affect BOEC metabolism indicating that during its short passage through the oviduct, it might elicit changes at signaling level necessary for its development without inducing metabolic changes.

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.

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