Bovine endometrial cells as an *in vitro* model to address early embryo maternal cross-talk

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ABSTRACT

We optimized a bovine endometrial epithelial cell (BEEC) line as a valuable research model for the study of early embryo-maternal interactions *in vitro*. Therefore, we aimed to characterize the BEEC monolayers along the primary culture and the first three passages with respect to the expression of cell-origin markers, by immunofluorescence, and abundance of functional key transcripts, by Real Time PCR. BEECs isolated from uteri (at early luteal phase) *ex vivo* were cultured and subpassed three times. Based on the markers studied, we conclude that BEEC monolayers undergo an epithelial-mesenchymal transition *in vitro* but preserve functional characteristics after few passages.

Keywords

cow; endometrium; early pregnancy; cell culture

INTRODUCTION

In cattle, up to 40% of the total embryonic losses occur in the pre-implantation period [2]. This indicates that early embryonic death is the main cause of reproductive wastage in the bovine species. These high embryonic losses are generally assumed to be a consequence of insufficient embryo-maternal cross-talk. An embryo-maternal crosstalk was discovered earlier but only few of the signals involved in the dialogue have been identified. The ability of embryonic interferon-tau to inhibit secretion of prostaglandin $F_{2\alpha}$, for example, is critical to the establishment of pregnancy in cattle [11]. A more comprehensive understanding of the important mechanisms involved in the early embryo-maternal interaction in cattle is necessary and, ultimately, would help to reduce early embryonic mortality. Modern techniques for transcriptome analysis will help to identify the signals between embryos and the maternal environment. In addition, suitable co-culturing systems are important tools to address the very local embryo-maternal interface *in vitro* [14].

Recent studies have shown a complex embryo-maternal interaction between the pre-elongated bovine embryo and oviductal [4], immune [10] and endometrial cells [9]. Embryo-induced changes are nonetheless expected to be very local in nature, due to the limited capacity of synthesis and secretion of signals by the early embryo. For instance, changes in the abundance of specific transcripts in the cranial part of the bovine pregnant uterine horn have been shown to be caused by a day-7 embryo *in vivo* [9]. The embryo-maternal interface is nonetheless difficult to be addressed in the *in vivo* environment. *In vivo* studies also require a lot of animals and are difficult to adapt. *In vitro* experiments however could be done with samples from the abattoir which is better for animal welfare and allows multiple experimental set ups. Therefore, we explored a model for the *in vitro* study of the very early embryo-maternal cross-talk by using a bovine endometrial epithelial cell (BEEC) culture. However, to effectively conduct studies on epithelial cell lines, a pure cell population is needed. Proliferating cells in monolayers tend to dedifferentiate after several days in culture with a concomitant loss of important functional characteristics [12]. Therefore, in the present study, we aimed to characterize the BEEC lineage as a valid model for the study of the embryo-maternal cross-talk.

MATERIALS and METHODS

In this study, a bovine endometrial epithelial cell culture was used. To validate this *in vitro* model, multiple parameters were compared with respect to cell-origin markers protein and gene expression among primary culture and subcultures 1 until 3. Abundances of transcripts for cellular lineage markers (*KRT18* and *VIM*), for sex-steroids hormones receptors (*ESR1)*, for prostaglandin-endoperoxide synthase 2 (*PTGS2*), and interferon alpha/beta receptor 1 (*IFNAR1*) were evaluated by Real Time PCR. Paraformaldehyde-fixed samples from the primary culture, subculture 1, 2 and 3 were immunostained for cytokeratin (epithelial cells positive) and vimentin (mesenchymal cells positive) according to the protocol described below. An overview of the different steps in the *in vitro* BEEC culture validation is given in Figure *1*.

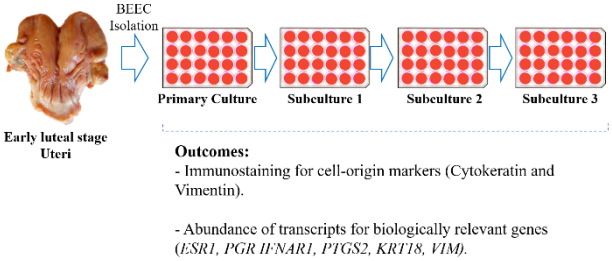

Figure 1: Experimental set-up of the validation of the in vitro BEEC culture

Bovine Endometrial Epithelial Cells isolation and Culture

Collection and primary culture.

Reproductive tracts at early luteal phase of the estrous cycle were collected from healthy, *Bos taurus* beef cows at a local slaughterhouse, inspected for absence of reproductive abnormalities or disorders, and transported to the laboratory at 30 °C. Four uteri were dissected free of surrounding tissues and externally decontaminated with 70% ethanol. The uterine horns ipsilateral to the ovary containing the corpus hemorragicus were longitudinally opened at the mesometrium insertion, and endometrial cells were mechanically isolated by slightly scraping the luminal surface with sterile glass slides. Cells from the four uteri were pooled in equilibrated, pre-warmed culture medium. The cell suspension was centrifuged at 200g for 5 min at 25 °C, the supernatant was discarded and

erythrocytes were subsequently lysed by incubation with a hyper-osmotic Lysis buffer (1 mM EDTA disodium salt, 150 mM NH_4Cl and 100 mM $NaHCO_3$) for 1 min. Next, the cell pellet was resuspended in pre-warmed culture medium. After two centrifugation-resuspension steps, 7.5 ml of cell suspension were seeded in 25 cm^2 flasks at a density of 10^6 cells/ml and pre-incubated in humidified atmosphere of 5% CO_2 at 38.5 °C for 1 h 30 min. The pre-incubation step aimed to remove the contaminating, non-epithelial cells. Subsequently, the supernatant containing non-attached, epithelial cells was transferred towards new 25 cm^2 flasks and maintained in humidified atmosphere of 5% CO_2 at 38.5 °C. After the adhesion process started, culture medium was renewed every 48 h. The cell culture medium consisted of DMEM/F-12 (Gibco, Thermo Fisher Scientific) phenol red free medium supplemented with 10% fetal bovine serum (Sigma-Aldrich), 2% Penicillin/Streptomycin, and 1% Fungizone. Flasks were daily checked for signs of contamination and to visually estimate the monolayer's confluence by inverted light microscopy.

Subculture and freezing procedures.

Cells were subcultured when primary monolayers reached 90% of confluence. Monolayers were washed once with PBS (Phosphate Buffered Saline; Ca^{2+} Mg^{2+} free) to remove residual serum. Cells were trypsinized (TrypLE Express; Gibco, Thermo Fisher Scientific) for 6 min, retrieved and centrifuged at 200g for 5 min at 25 °C. Cell count and viability were determined in a Bürker Counting Chamber (W. Schreck, Hofheim, Germany) by trypan blue exclusion test. Cells were seeded at a density of 10^6 viable cells/flask into new 75 cm^2 culture flasks. When reached 90% of confluence, BEEC monolayers were washed with PBS and trypsinized as described previously, and diluted in cryopreservation medium at a concentration of 10^6 viable cells/ml. The cryopreservation medium was based on DMEM/F-12 phenol red free medium supplemented with 15% fetal bovine serum, 10% DMSO (Invitrogen, Thermo Fisher Scientific), 2% Penicillin/Streptomycin, and 1% Fungizone. Cells were placed in a freezing container (Nalgene® Cryo, Thermo Fisher Scientific) and kept at −80 °C overnight, afterward, cryovials were stored in liquid nitrogen.

Total RNA isolation and cDNA synthesis

Total RNA was isolated using Trizol Reagent (Invitrogen, Thermo Fisher Scientific, CA, USA) in accordance with manufacturer's guidelines. Extracts containing RNA were kept at –80 °C until use. Total RNA yield and purity (260/280 nm ratio) were evaluated by NanoDrop (Thermo Fisher Scientific) spectrophotometer analysis. Samples of RNA (1 µg) were treated with DNase I (Promega) according to the standard protocol. Total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Invitrogen) according to the manufacturer's instructions. First strand cDNA was synthesized by random hexamers. Samples were incubated 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 further analyses.

Real Time PCR

Real time PCR analysis was performed to verify whether the BEEC line cultured *in vitro* recapitulates some characteristics as *in vivo* endometrial cells, at transcriptional level. The relative transcript abundance was measured by real-time PCR. The abundance of mRNA coding for the following proteins were evaluated:

- *ESR1* estrogen receptor 1
- *PGR* progesterone receptor
- *KRT18* keratin 18
- *VIM* vimentin
- *IFNAR1* interferon alpha/beta receptor 1
- *PTGS2* prostaglandin-endoperoxide synthase 2

These specific genes were selected based on their biological role in the endometrium. Transcripts for cell-origin markers *KRT18* (epithelial cells) and *VIM* (mesenchymal cells) were evaluated. *ESR1*, *PGR*, *PTGS2* and *IFNAR1* are indicatives of functional properties of endometrial cells. Specific primers were designed based on the *Bos taurus* genome.

Reactions were carried out in duplicates in 96-well plates (Bio-Rad Laboratories) sealed with Microseal B PCR plate sealing film (Bio-Rad Laboratories) using the CFX Connect Real-Time PCR detection system (Bio-Rad Laboratories B.V., Netherlands). The PCR reactions were conducted in a final volume of 16 µl, consisting of 8 µl of SsoAdvanced Universal Sybr Green supermix (Bio-Rad Laboratories), 0.4 µl of forward and reverse primers, and 4 µl 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 min, followed by 40 cycles each of 30 seconds at 95 °C, annealing at 59-61 °C for 30 seconds, and extension at 72 °C for 20 seconds. 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. *PPIA*, *GAPDH* and *ACTB* were used as reference genes. 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 [7].

Immunofluorescence

Immunofluorescence analyses were performed to evaluate the protein expression of cell-origin markers (cytokeratin and vimentin) in the BEEC monolayers. Cytokeratin is expected to be expressed in epithelial cells (along various other keratins), while vimentin is a marker for mesenchymal-derived cells [6][8].

Therefore, BEEC monolayers grown on coverslips were fixed in buffered 4% paraformaldehyde for 30 min at room temperature. Samples were permeabilized in 24-well plates containing 500 µl of 1% Triton X-100 and 0.05% Tween-20 in PBS for 30 min at room temperature. Monolayers were washed 3 times in wash solution that consisted in 0.5% BSA (Bovine Serum Albumin) and 0.05% Tween-20 in PBS. Next, samples were blocked in PBS containing 2% BSA and 0.05% Tween-20, for 45 min at room temperature, then incubated with primary antibodies overnight at 4 °C. Primary antibodies used in the present study were anti-cytokeratin mouse monoclonal antibody (M3515, Dako, CA, USA), and anti-vimentin rabbit polyclonal antibody (ab45939, Abcam, MA, USA). Negative control samples were incubated with an equivalent mixture of normal mouse and rabbit IgGs replacing the primary antibodies. Monolayers were then washed in wash solution 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 2 h at 4 °C. Nuclei were counterstained in Hoechst 33342 (30 µg/ml in PBS-PVP 1%) for 10 min at room temperature. Coverslips were mounted on glass slides in droplets of 1% DABCO. Images were captured under a fluorescence microscope with DAPI filter (excitation/emission: 360–370/420–460 nm for Hoechst stained nuclei), FITC filter (460–490/520–540 nm for vimentin), and RITC filter (510–550/>570 nm for cytokeratin-positive cells).

RESULTS

Abundance of transcripts on BEECs among passages

Real time PCR analyses showed a 6-fold increase in the abundance of *VIM* mRNA from primary culture to subculture 1, that remained constant until subculture 3; however, abundance of transcripts for *KRT18*, *ESR1*, *PTGS2*, and *IFNAR1* remained similar among primary culture and first passages, suggesting that the cells conserved these functional characteristics (Figure *3*)

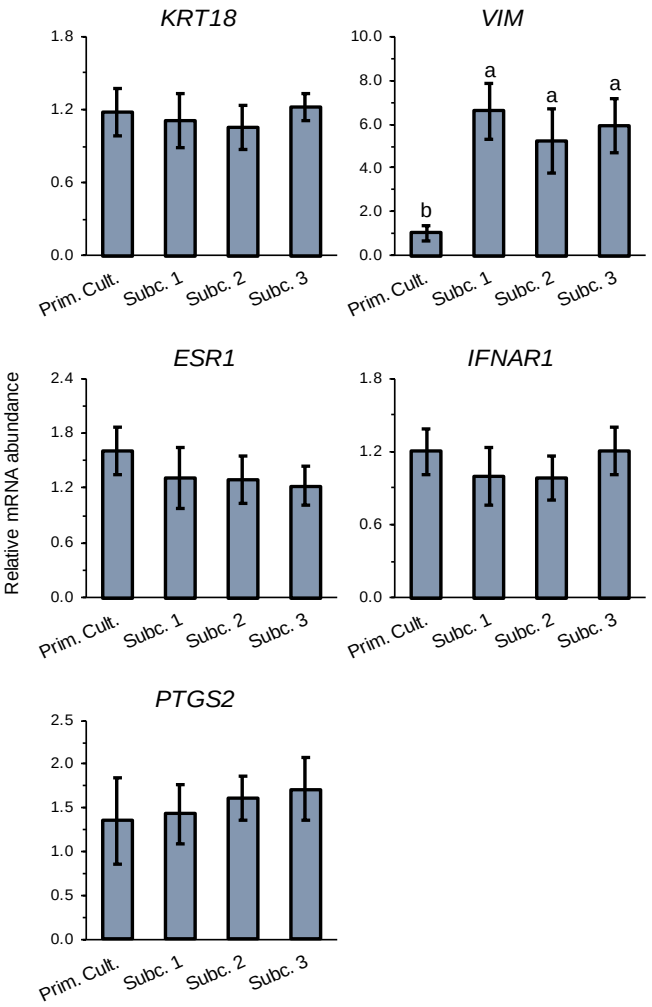

Figure 3: Relative mRNA abundance of keratin 18 (*KRT18*), vimentin (*VIM*), estrogen receptor alpha (*ESR1*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), and interferon alpha and beta receptor subunit 1 (*IFNAR1*) on bovine endometrial epithelial cells along the primary culture and the first three subcultures. Data are shown as arbitrary units; mean ± SEM. [a,b] Statistically significant difference in mRNA abundance ($P \leq 0.05$).

Cell-origin markers expression on BEECs

Immunofluorescence staining revealed that the BEECs co-express cytokeratin and vimentin after the first passage, indicating that the cells are epithelial origin but underwent an epithelial-mesenchymal transition *in vitro*, as shown in Figure *2*. Furthermore, immunolocalization of cytokeratin and vimentin protein within the same cells provides basis to conclude that presence of *VIM* mRNA verified by PCR analysis is not due to contaminating non-epithelial cells in the culture.

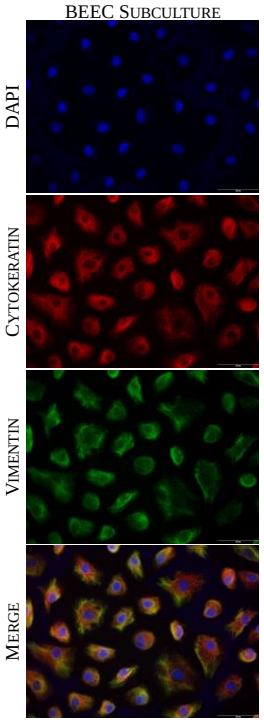

Figure 2: Immunofluorescence staining of the BEEC subculture monolayer.

The epithelial cell culture showed positive for DAPI (blue), cytokeratin (red) and vimentin (green) which indicates an epithelial-mesenchymal transition.

The bottom panel displays the merged image. The immunolocalization of cytokeratin and vimentin within the same cell indicates that the presence of vimentin, verified by PCR, is not due to contamination of non-epithelial cells. So after the first passage, cells co-expressed cytokeratin and vimentin.

CONCLUSION

Expression of cytokeratin at protein level confirms that the cells have an epithelial-origin. The increased protein expression of vimentin indicates that the epithelial cells underwent an epithelial-mesenchymal transition *in vitro* after the primary culture. The acquisition of vimentin by cytokeratin-positive epithelial cells during in vitro culture periods has already been described in literature [1][3][13][15], which has been explained by a limited epithelial to mesenchymal transition (EMT)[5]. This transition is believed to correlate with a loss of cell-to-cell contacts that result from cell disaggregation during the period of epithelial cell culture preparation [15]. In our study the significant 6-fold increase in the abundance of *VIM* mRNA from primary culture to subculture 1 might be due to the detachment protocol used in our study as TrypLE acts as a trypsin-like dissociation reagent and could select more dedifferentiated cells. However, despite their dedifferentiated status, the BEECs displayed no changes on abundance of transcripts for epithelial cell-origin (*KRT18*) and functional markers (*ESR1*, *IFNAR1* and *PTGS2*) among the first passages. In conclusion, based on the markers studied, BEEC monolayers undergo epithelial-mesenchymal transition *in vitro* but preserve functional characteristics after few passages. More studies are necessary to better establish the optimal conditions for the *in vitro* culture of epithelial cells. Taken together, these results suggest that the BEECs compose a valid model to address the very first embryo-maternal cross-talk *in vitro*.

ROLE OF THE STUDENT (MANDATORY)

Jens Slootmans was an Honours College student working under the supervision of Mariana Sponchiado. The topic was part of the PhD research of Mariana Sponchiado (supervised by Prof Jo Leroy), who designed the experimental set-up and conducted data analyses. Jen Slootmans assisted in the RNA isolation, cDNA reverse transcription, PCR analyses, and wrote the manuscript.

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