A bovine oviduct epithelial cell suspension culture system suitable for studying embryo–maternal interactions: morphological and functional characterization

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Abstract

We established a short-term (24 h) culture system for bovine oviduct epithelial cells (BOECs), obtained on day 3.5 of the estrous cycle and evaluated the cells with respect to morphological criteria, marker gene expression, and hormone responsiveness. BOEC sheets were isolated mechanically from the ampulla with similar yields from oviducts ipsi- and contralateral to the ovulation site (57.9 ± 4.6 and $56.4 \pm 8.0 \times 10^6$ cells). BOECs showed >95% purity and cells cultured for 24 h maintained morphological characteristics present *in vivo*, as determined by light microscopy, scanning electron microscopy, and transmission electron microscopy. Both secretory cells with numerous secretory granules and ciliated cells with long, well-developed, and vigorously beating kinocilia were visible. Quantitative real-time PCR failed to detect significant differences in transcript levels between ipsi- and contralateral BOECs for the majority of marker genes (estrogen receptors α and β (*ESR1* and *ESR2*), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMGCR*), oviductal glycoprotein 1 (*OVGP1*), progesterone receptor (*PGR*), and tumor rejection antigen 1 (*TRA1*)) throughout the 24 h culture period. However, the combined data of all time points for glutathione peroxidase 4 (*GPX4*), a gene previously shown to be expressed at higher levels in the ipsilateral oviduct *in vivo*, also indicated significantly different mRNA levels *in vitro*. The expression of marker genes remained stable after 6 h cell culture, indicating only a short adaptation period. Western blot analysis confirmed *ESR1* and *PGR* protein expression throughout the culture period. In agreement with cyclic differences *in vivo*, estradiol-17 β stimulation increased *PGR* transcript abundance in BOECs. Our novel culture system provides functional BOECs in sufficient quantities for holistic transcriptome and proteome studies, e.g. for deciphering early embryo–maternal communication.

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Introduction

The oviduct plays a pivotal role in mammalian reproduction, providing an optimal environment for oocyte maturation, sperm capacitation, fertilization, and transport of gametes and embryos (Ellington 1991, Hunter 2003). The oviduct epithelium consists of ciliated and secretory cells. Kinocilia are actively involved in the transport of oocytes, sperm, and early embryos, while secretory products are essential for providing optimized

microenvironments (Abe & Hoshi 1997, Leese *et al.* 2001). The relative proportions of ciliated and secretory cells in the ruminant oviduct and their morphology change markedly during the estrous cycle. Ciliated cells increase in the late luteal phase in the infundibulum and ampulla, with less pronounced differences towards the caudal ampulla. Cell height in goat oviduct epithelium was reported to decrease in the luteal phase, which was more pronounced in the ciliated cells of the infundibulum and ampulla (Abe *et al.* 1999, Yaniz *et al.* 2000). Consistent

with morphological changes, transcriptome studies identified extensive estrous cycle-dependent changes in gene expression patterns in bovine oviduct epithelial cells (BOECs). Most genes upregulated in the diestrous/luteal phase are involved in regulation of transcription and cell proliferation, while the majority of genes upregulated in the estrous/follicular phase are involved in protein secretion and modification (Bauersachs *et al.* 2003).

Cultured oviduct epithelial cells have been employed to study the effects of ovarian and environmental steroids (Wijayagunawardane *et al.* 1999), sperm–epithelial interactions (Baillie *et al.* 1997), and the formation and composition of oviductal fluids (Cox & Leese 1997, Mahmood *et al.* 2002). Beneficial effects of oviduct epithelial cells on co-cultured embryos have been used to overcome the developmental block at the 8–16 cell stage in ruminants, and to improve the pregnancy rates and outcomes with embryos generated *in vitro* (Gandolfi & Moor 1987, Galli *et al.* 2003). Studies performed in hamster and rat showed accelerated transport of embryos as compared with unfertilized oocytes in the oviduct, a phenomenon that was not mediated systemically, but locally, indicating that oviduct cells are not only signaling, but also responding to embryos (Ortiz *et al.* 1986, 1989). Such findings are consistent with *in vivo* data obtained in mouse, where the presence of embryos affected gene expression of oviduct cells (Lee *et al.* 2002). Systematic studies addressing very early embryo–maternal interactions in bovine have now become feasible with a battery of functional genomics technologies (Hiendleder *et al.* 2005). However, holistic transcriptome and proteome analyses, as well as specific experiments targeting embryo–maternal interactions in the oviduct, require sufficient numbers of well-defined cells and embryos in a standardized experimental environment. This is often difficult or impossible to achieve in bovine *in vivo*. Furthermore, the functional knockdown of genes by RNA interference is possible in somatic cells and embryos *in vitro*. Thus, an appropriate oviduct cell-culture system would greatly aid the analysis of early embryonic and maternal signaling (Wolf *et al.* 2003).

BOECs have been isolated by enzymatic, mechanical, or mixed enzymatic/mechanical procedures (Abe & Hoshi 1997, Reischl *et al.* 1999), but cell yield per oviduct has only been assessed for procedures involving trypsinization, and were found to be rather limited (Thibodeaux *et al.* 1991). BOECs have been cultured as monolayers or in suspension. Proliferating cells grown in monolayers dedifferentiate with a concomitant loss of important morphological characteristics. These include reduction of cell height, loss of cilia, and loss of secretory granules and bulbous protrusions (Thibodeaux *et al.* 1992, Walter 1995). Perfusion culture of BOECs grown on cellulose-nitrate sheets sustained morphology, including ciliated cells in circumscribed regions, more faithfully, but nevertheless, the cells showed signs of

dedifferentiation (Reischl *et al.* 1999). In a direct comparison between BOECs grown in monolayer or in suspension culture, only suspended cells maintained cilia and secretory activity (Walter 1995). Semi-quantitative expression analysis of *OVGP1*, encoding the oviduct-specific glycoprotein 1, was used for quality assessment of cultured BOECs (Reischl *et al.* 1999). We have developed a short-term 24 h culture system for BOECs isolated on day 3.5 of the estrous cycle as a tool for studying embryo–maternal interactions in the oviduct. Comprehensive light microscopic, scanning electron microscopic, and transmission electron microscopic examinations were employed to validate functional morphology and ultrastructural integrity of cultured cells. In addition, cell function was assessed at the molecular level by quantitative mRNA expression studies of marker genes during culture and after hormone stimulation, and by demonstrating the presence and physiological regulation of steroid hormone receptors.

Materials and Methods

Animals

Synchronized Simmental heifers ($n=11$) aged 16–23 months were slaughtered on day 3.5 after the onset of standing heat. Follicle development and ovulation were monitored by ultrasound examinations every 6 h, starting 52 h after application of 500 µg Cloprostenol (Estrumate, Essex Pharma, Munich, Germany). Additionally, blood samples were taken every 6 h, starting 24 h after Cloprostenol application, to confirm estrous cycle stage by measuring serum concentrations of estradiol-17β (E2; Meyer *et al.* 1990), progesterone (P4; Prakash *et al.* 1987), and luteinizing hormone (Schams & Karg 1969). All experiments with animals were carried out with the permission from the responsible animal welfare authorities, the Regierung von Oberbayern.

Preparation of BOECs, cell culture, and hormone stimulation of cultured cells

After slaughter, removal of the skin, and opening of the abdominal cavity, the reproductive tract was recovered. Both oviducts were trimmed free from connective tissue, ligated, dissected, and washed in PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (PBS–), and dipped in 70% ethanol before being transported on ice to the laboratory in PBS– supplemented with 2% penicillin–streptomycin solution (Sigma). Oviducts were dipped again in ethanol and washed in PBS– before removing the ligature in a laminar flow hood. The ampulla was divided into three segments and gently squeezed in a stripping motion with forceps to obtain BOECs. Cells appeared as a yellowish paste that was collected in culture medium TCM-199 (Invitrogen) supplemented with 2% estrous cow serum (ECS) and 0.25 mg/ml gentamicin (Sigma). The cell

suspension was pipetted 15 times with a 1000 µl filter tip (Eppendorf, Hamburg, Germany) before being passed twice through a 30 gauge syringe needle. After three steps of washing, each followed by 25-min sedimentation in culture medium in the cell culture incubator, an aliquot of the cell suspension was further disaggregated for cell counting by passing it 15 times through a 30 gauge needle. Cell viability at seeding was analyzed by Trypan blue staining (Sigma). BOECs were cultured in the central eight wells of a 24-well culture plate (Nunc, Roskilde, Denmark) with 10^6 cells each in 800 µl TCM-199 and 0.25 mg/ml gentamicin supplemented with 2% ECS (experiment 1, three animals) or cow serum collected on day 3.5 (CS 3.5) of the estrous cycle (experiment 2, four animals). Hormone assays showed that ECS contained 8.16 pg/ml E2 and <0.05 ng/ml P4, and CS 3.5 contained 1.81 pg/ml E2 and 0.07 ng/ml P4. Hormone responsiveness of BOECs (experiment 3) was investigated in cells cultured with CS 3.5. After 6 h pre-culture without hormone supplementation, E2 or P4 (Sigma) was added at a concentration of 10 pg/ml or 10 ng/ml culture medium respectively, and samples were taken after 6 h (four animals) and 18 h (three animals) treatment. Cells treated with hormone carrier solution alone served as controls. Culture took place at 38 °C in a humidified atmosphere with 5% CO₂ in air. The time span from slaughter of heifers to seeding of cells was approximately 6 h.

Immunocytochemistry

For immunocytochemical examinations, cells were grown as monolayer on coverslips for 5–6 days, washed in PBS – and fixed in a mixture of methanol and acetone (7:3, v/v) at –20 °C for 10 min. After washing, one coverslip of each was covered with anti-vimentin antibody (clone V9, V-6630; Sigma), anti-cytokeratin antibody (cytokeratin 8.13, C-6909; Sigma), or PBS – supplemented with 0.1% (w/v) BSA (Sigma) as a control, and incubated at 37 °C in a humidified atmosphere for 1 h. After washing in PBS –, the coverslips were coated with FITC-labeled secondary antibody (F-2883; Sigma) and again incubated for 1 h. Incubation with propidium iodide (Sigma) was performed for 10 min in the dark at room temperature. Coverslips were evaluated using a Zeiss AxioCam on a Zeiss Axiovert 200 microscope with UV light (Zeiss, Jena, Germany).

Cytokeratin antibody was chosen according to the previous data concerning expression in oviduct epithelial cells during different stages of the estrous cycle (Perez-Martinez *et al.* 2001). Cytokeratin 8.13 detects cytokeratins 1, 5, 6, 7, 8, 10, 11, and 18. In metestrus, *in vivo* cytokeratins 5, 6, 7, 8, and 18 are staining reported to stain positive, whereas vimentin staining shows <10% strongly positive epithelial cells (Perez-Martinez *et al.* 2001).

Scanning electron microscopy (SEM)

BOECs obtained *ex vivo*, i.e. immediately after slaughter, and cultured BOECs were washed twice in Soerensen buffer (pH 7.4; 1:5 solution of 0.07 M KH₂PO₄ and 0.07 M Na₂HPO₄–2H₂O). Specimens were fixed in 1% glutaraldehyde in Soerensen buffer at 4 °C for 24 h. After washing in Soerensen buffer, the cells were dehydrated in an ascending series of acetone (10, 20, 30, 40, 50, and 60%: twice, 5 min each; 70, 80, and 90%: 1 h each; 100%: 12 h). BOECs were dried in a Union Point Dryer CPD 030 (Bal-Tec, Walluf, Germany) using liquid CO₂ as the transitional fluid. After drying, specimens were coated with 12 nm gold–palladium by a Union SCD 040 sputtering device (Bal-Tec). SEM observations were made with the Zeiss scanning electron microscope DSM 950 using magnifications of 200×–10 000×.

Transmission electron microscopy (TEM)

BOECs were washed twice in cacodylate buffer (0.2 M sodium cacodylate, pH 7.2). After fixation in Karnovsky's fluid (2.5% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate buffer) for 24 h, the epithelial cells were post-fixed in 1% OsO₄ and 1.5% KFe(CN)₆. Specimens were then transferred to a drop of 20% BSA in cacodylate buffer. By adding 25% glutaraldehyde, the BSA was polymerized to a pellet containing the cells. The pellet was dehydrated in a graded series of ethanol and embedded in Epon (Polysciences, Eppenheim, Germany). Ultrathin sections (50 nm) were mounted on grids, post-stained with osmium tetroxide and examined using a Zeiss electron microscope with magnifications from 3000× to 25 000×.

Total RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was extracted using Trizol reagent according to the manufacturer's instructions (Invitrogen). RNA samples were quantified with a spectrophotometer at 260 nm and then stored at –80 °C. The purity of RNA was assessed by the 260/280 nm ratio and integrity was confirmed by gel electrophoresis. One microgram of each RNA sample was reverse transcribed in a total volume of 60 µl (5× buffer; (Promega), 10 mM dNTPs (Roche), 50 µM hexamers (Gibco BRL), using 200 U MMLUV Point Mutant H-RT enzyme (Promega)). Primers were synthesized (MWG-Biotech, Ebersberg, Germany) to amplify specific fragments of bovine transcripts as described in Table 1. All amplified fragments were sequenced once to verify PCR-products (MWG-Biotech). During quantitative real-time PCR (qPCR), the specific melting point of amplified products carried out by the LightCycler standard PCR protocol served as verification of the product identity. In each PCR, 1 µl cDNA was used

Table 1 Primer sequences and real-time PCR parameters used for the quantification of gene expression.

Gene	Direction	Primer sequence	Fragment size (bp)	Annealing temperature (°C)	Fluorescence acquisition point (°C)
18S rRNA	Forward	5'-AAGTCTTTGGGTCCGGG-3'	365	60	82
	Reverse	5'-GGACATCTAAGGGCATCACA-3'			
ESR1	Forward	5'-AGGGAAGCTCCTATTGCTCC-3'	234	63	86
	Reverse	5'-CGGTGGATGTGGTCCTCTCT-3'			
ESR2	Forward	5'-GCTTCGTGGAGCTCAGCCTG-3'	262	64	81
	Reverse	5'-AGGATCATGGCCTTGACACAGA-3'			
PGR	Forward	5'-GAGAGCTCATCAAGGCAATTGG-3'	227	64	81
	Reverse	5'-CACCATCCCTGCCAATATCTTG-3'			
GPX4	Forward	5'-GAGAAACCTAAAGACCTTGGC-3'	276	60	82
	Reverse	5'-TTGACTGGAAGAATGGGGAC-3'			
TRA1	Forward	5'-TGGCAGAGACCATCGAAAG-3'	120	60	77
	Reverse	5'-GGTAACTCCCTTCAGCAG-3'			
OVGP1	Forward	5'-TGTCCACGTTTCCAACCG-3'	856	60	85
	Reverse	5'-GGAGGGCGATCACTGAACG-3'			
HMGCR	Forward	5'-GCATAGGAGGCTACAACGCC-3'	241	63	80
	Reverse	5'-CCTTGAACACCTAGCATCTGC-3'			

to amplify a specific target gene. PCRs using the LightCycler DNA Master SYBR Green I protocol (Roche Diagnostics, Mannheim, Germany) were performed as described previously (Ulbrich *et al.* 2004). Annealing temperatures (AT) and fluorescence acquisition (FA) points for quantification within the fourth step of the amplification segment are shown in Table 1. In each PCR, 17 ng/ μ l cDNA were introduced and amplified in a 10 μ l reaction mixture (3 mM MgCl₂, 0.4 μ M primer forward and reverse each, 1 $\times$ Light-Cycler DNA Master SYBR Green I, Roche). The cycle number required to achieve a definite SYBR Green fluorescence signal was calculated by the second derivative maximum method (crossing point, CP; Light-Cycler software version 3.5.28). The CP is correlated inversely with the logarithm of the initial template concentration. As negative controls, water instead of cDNA was used. All RT-qPCR data were standardized to 18S rRNA.

Western blot analysis

Western blot analysis was performed as described previously (Ulbrich *et al.* 2003). Briefly, approximately 10⁶ BOECs were repeatedly frozen in liquid nitrogen with lysis buffer containing proteinase inhibitor. Protein samples (36 μ g/lane) were separated on a 4–12% Bis-Tris Gel (NuPage; Invitrogen) in MOPS running buffer and transferred onto nitrocellulose membranes. The membranes were blocked overnight with 1% dried milk in TBS containing 0.1% Tween-20. These were incubated with the MAB against ESR1 (2-185, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or PGR (Clone 10A9, Coulter Immunotech, Marseille, France) in TBS-0.1% Tween-20–1% dried milk powder for 75 min at room temperature. The dilutions used were 1:200 (ESR1) and 1:50 (PGR) respectively. After washing, the

membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (DAKO, Hamburg, Germany) at a dilution 1:2000 in TBS-0.1% Tween-20–1% dried milk powder for 45 min at room temperature. After washing in TBS-0.1% Tween-20, and TBS alone, the membrane was incubated with ECL reagent detection solution (Amersham) for 3 min in the dark. Finally, an X-ray film was exposed to the membrane to visualize protein expression. Protein extract from bovine endometrium (30 ng/lane) was used as a positive control as described previously (Schams *et al.* 2003).

Statistical analysis

ANOVA, calculation of least squares means (LSM) and standard errors of means (S.E.M.), was performed with the general linear model procedure as implemented in SPSS 12.0G for Windows, version 12.0.1 (SPSS GmbH Software, Munich, Germany). Student's *t*-test with Bonferroni correction for multiple testing was used in comparisons of marker gene expression differences throughout the culture period. Paired *t*-test was employed in comparisons of marker gene expression between ipsi- and contralateral BOECs and in comparisons between hormone-stimulated BOECs and controls. Differences were considered significant at $P < 0.05$. Graphs were plotted with Graph Pad Prism, version 3.00 for Windows (GraphPad Software, San Diego, CA, USA). Quantitative PCR CP-data are presented as mean $\pm$ S.E.M. subtracted from the arbitrary value 50 (Δ CP) to transform CP values into proportional values reflecting decreases and increases in transcript abundance. The combined data set consists of all data presented in the graph irrespective of different sampling times.

Results

Characterization of isolated cells and morphology of cultured BOECs

The number of isolated cells ranged from 30.7×10^6 to 87.0×10^6 cells/ampulla. Mean cell yield was very similar for ipsi- and contralateral oviducts with $57.9 \pm 4.6 \times 10^6$ and $56.4 \pm 8.0 \times 10^6$ cells/ampulla respectively. Trypan blue staining was positive in the majority of singularized cells, while cells organized in aggregates were viable at 0.5 h and after 24 h in culture (Fig. 1a and b). As cells were not singularized, no cell counting demonstrating percentages of viable cells was performed at this stage. To perform further characterization, the cells were therefore cultured for 5–6 days to finally settle as monolayers. These cells were from the same cell populations as the ones that were used for the short-term culture. Immunocytochemical examinations showed >95% cells staining positive with anti-cytokeratin antibody, thus confirming the epithelial character of the cultured BOECs population. Less than 5% of the cells grown on a coverslip stained positive for the fibroblast marker vimentin (Fig. 1c and d).

Light microscopy showed vigorously beating cilia on all aggregate surfaces throughout the 24 h culture period. The mechanically obtained flat cell sheets changed into worm-like structures during the first 6 h culture. This morphology was maintained throughout culture, but the apical surface of individual cells in the aggregates attained a slightly more rounded shape after 24 h (Fig. 2a and b). All aggregates remained in rapid and constant motion due to vigorous ciliary beating throughout the culture period. During 48 h culture, no cell attachment occurred.

SEM revealed ciliated and secretory cells in their natural coherence. BOECs after 0.5 and 24 h in culture

appeared very similar to BOECs in *ex vivo* tissue samples obtained on day 3.5 of the estrous cycle (Fig. 2c–f). TEM confirmed ciliated and secretory cells in the cultured aggregates. BOEC characteristics, such as numerous kinocilia with their basal bodies, numerous secretory granules, and mitochondria were visible at seeding and after 24 h in culture. The maintenance of cell polarity was confirmed by the presence of cilia and microvilli on the apical cell surface and of tight junctions on the lateral cell surfaces (Fig. 3a–f).

Gene expression of BOECs during culture

Quantitative PCR did not detect significant differences in the expression of marker genes *ESR1* (estrogen receptor α), *ESR2* (estrogen receptor β), *GPX4* (glutathione peroxidase 4), *HMGCR* (3-hydroxy-3-methylglutaryl coenzyme A reductase), *OVGP1* (oviductal glycoprotein 1), *PGR* (progesterone receptor), and *TRA1* (tumor rejection antigen 1) between BOECs derived from oviducts ipsi- and contralateral to the ovulation site at different time points during the 24 h culture period (Fig. 4). However, ipsi- and contralateral BOECs differed significantly ($P < 0.05$) in *ESR2* expression at 0.5 h after seeding. Moreover, *GPX4* expression was significantly different between ipsi- and contralateral BOECs ($P < 0.01$) in the combined analysis of data obtained at all sampling time points (Fig. 4).

The combined statistical analysis of marker gene expression in ipsi- and contralateral BOEC at 0.5, 6, 12, and 24 h revealed stable expression of *HMGCR* and *OVGP1* over all time points. The expression of *ESR2*, *GPX4*, and *PGR* was stable at 6 h in culture and thereafter, *TRA1* was upregulated at 6 h in culture ($P < 0.001$),

Figure 1 Evaluation of cell viability and purity. (a) Trypan blue staining at 0.5 h in culture: viable cells (Trypan blue negative) were only present in aggregates in contrast with singularized cells (Trypan blue positive). (b) Trypan blue positive cells were not found later in culture. (c) Immunocytochemical examination using anti-cytokeratin antibodies. (d) Immunocytochemical examination using anti-vimentin antibodies. The cells used in the short-term culture formed a monolayer after 5–6 days culture clearly demonstrating >95% cytokeratin positive cells and <5% vimentin positive cells. Nuclei are stained using propidium iodide. Bar=100 μm .

Figure 2 Light and scanning electron microscopic (SEM) images of BOECs. (a) Cells at 0.5 h in culture. (b) Cells after 24 h in culture. BOECs were organized in aggregates and showed intense ciliary beat throughout culture period. Bar = 100 μ m. (c) SEM images of ciliated and secretory BOECs in oviduct tissue *ex vivo*. ((d) and (e)) BOEC aggregates at 0.5 h in culture. (f) BOECs after 24 h in culture. Bar = 10 μ m.

returned to the 0.5 h expression level after 12 h in culture, and was stable at 24 h. *ESR1*, in contrast, was down-regulated at 6 h in culture ($P < 0.05$), returned to the 0.5 h expression level after 12 h in culture, and was stable at 24 h (Fig. 4). Supplementation of TCM-199 with 2% CS 3.5, instead of 2% ECS did not change gene expression patterns, with the exception that downregulation of *ESR1* was no longer significant ($P = 0.094$; Fig. 5).

Expression of steroid hormone receptors and *OVGP1* in cultured BOECs after stimulation with steroid hormones

Analysis of *ESR1* and *PGR* in cultured BOECs by Western immunoblot showed stable expression of both steroid

hormone receptors throughout the culture period. *PGR* showed predominantly isoform A (Fig. 6). Transcript levels of *ESR1*, *ESR2*, *PGR*, and *OVGP1* were quantified by qPCR to assess hormone responsiveness of cultured BOECs. Stimulation of cells with 10 pg/ml E2 for 6 h resulted in a significant increase of *PGR* transcript ($P < 0.05$). After 18 h E2 stimulation, the difference in *PGR* expression between hormone stimulated and control cells was no longer significant. However, combined data from both sampling time points again showed a significant E2 effect ($P < 0.05$) on *PGR* expression (Fig. 7). The decreased transcript abundance of *OVGP1* was significant ($P < 0.05$) after 18 h P4 stimulation. This downregulation was also significant in the combined data ($P < 0.05$; Fig. 7).

Figure 3 Transmission electron microscopy of BOECs. ((a) and (b)) BOECs in oviduct tissue *ex vivo*. ((c) and (d)) BOECs at 0.5 h in culture. ((e) and (f)) BOECs after 24 h in culture. Cilia, mitochondria, and basal bodies were visible throughout culture period. B, basal bodies; C, cilia; L, lymphocyte; M, mitochondria; N, nucleus; S, secretory vacuoles. Bar = 2 μ m.

Discussion

The aim of this study was to establish a BOECs isolation procedure and culture system suitable for holistic and specific analyses of early embryo–maternal interactions on day 3.5 of the estrous cycle. Previous studies have isolated BOECs from animals in a wide range of different estrous cycle stages. Most studies used oviducts obtained from slaughtered cows at the abattoir, where estrous cycle stage was either unknown (Walter 1995, Lim *et al.* 1997, Gualtieri & Talevi 2000) or estimated based on ovarian morphology (Abe & Hoshi 1997, Cox & Leese 1997, Sun *et al.* 1997, Ellington *et al.* 1998, Pegoraro *et al.* 1998, Boquest & Summers 1999, Reischl *et al.* 1999, Bosch *et al.* 2001, Kubisch *et al.* 2001), sometimes complemented by inspection of the cervix (Rief *et al.*

2002) and measuring luteal progesterone levels (Wijayanawardane *et al.* 1996). A single study used estrous synchronized animals, estrous behavior, plasma progesterone concentrations, and ovarian morphology at slaughter, to assign BOECs to four defined cycle stages (Thibodeaux *et al.* 1991). The pronounced estrous cycle-dependent changes in BOECs morphology (Yaniz *et al.* 2000) and gene expression (Einspanier *et al.* 1999, Gabler *et al.* 2003, Bauersachs *et al.* 2004), clearly show that a precise definition of estrous cycle stage is essential for obtaining functional cells for specific applications. Proliferating cells in monolayers after several days in culture are, therefore, less likely to mimic the oviduct environment *in vivo* than BOECs in short-term suspension culture. This is evident from various degrees of dedifferentiation and loss of morphological hallmarks

Figure 4 RT-qPCR data for marker genes obtained from BOECs cultured in TCM-199 supplemented with 2% ECS. Transcript abundance was determined at 0.5, 6, 12, and 24 h after seeding. Data for BOECs isolated from ipsi- or contralateral oviducts are shown separately for the different time points and as combined data regarding all time points. One ΔCP unit represents a twofold increase in mRNA. BOECs isolated from three individuals were analyzed. Different superscript letters indicate significant differences between sampling time points for the combined ipsi- and contralateral data. *ESR1*, *GPX4*, and *PGR*: $P < 0.05$; *ESR2* and *TRA1*: $P < 0.001$. Significant differences between ipsi- and contralateral cells (*ESR2* and *GPX4*) are indicated by asterisks. * $P < 0.05$, † $P < 0.01$.

such as cilia, as observed in previous monolayer studies (Thibodeaux *et al.* 1991, Walter 1995).

The number of cells isolated from individual ampullae using purely mechanical means ranged from 30.7×10^6 to 87.0×10^6 , with $57.51 \pm 3.88 \times 10^6$ cells on average. This is a tenfold higher yield than previously reported cell yields after enzymatic or combined mechanical/enzymatic treatment (Thibodeaux *et al.* 1991, Reischl *et al.* 1999), and is consistent with our observation that a considerable proportion of the cells is lost by enzymatic (e.g. trypsin) treatment. Moreover, in our hands, this effect appeared to be estrous cycle dependent, i.e. was much more pronounced for oviducts in early luteal phase as compared with luteal phase (data not shown). Considering the morphological differences between oviduct epithelial cell types, this could also indicate that enzymatic treatment during cell preparation might adversely affect the faithful representation of BOEC populations *in vitro*. The purity (>95%) of mechanically isolated BOEC in the

present study was comparable to cells obtained by combined mechanical/enzymatic treatment (Walter 1995, Abe & Hoshi 1997, Reischl *et al.* 1999).

Consistent with the previously reported of BOECs phenotype in suspension culture (Harvey *et al.* 1995, De Pauw *et al.* 2002), the examination of gross BOEC morphology by light microscopy showed that all cell aggregates were in constant motion due to vigorously beating cilia. Electron microscopic analysis confirmed the presence of numerous cells with intact cilia adjacent to secretory cells during the 24 h culture period. Cell aggregates exhibited a surface morphology highly similar to oviduct epithelium *in vivo*. In contrast, data reported for BOECs cultured in monolayer on glass or Thermanox showed a reduction in cell height and loss of cilia. In the same study, cells grown on permeable cellulose-nitrate sheets maintained morphology more faithfully, especially in perfusion culture (Reischl *et al.* 1999). It has been speculated that the ciliation process is

Figure 5 RT-qPCR data for marker genes obtained from BOECs cultured in TCM-199 supplemented with 2% CS 3.5. Transcript abundance was determined at 0.5, 6, and 24 h after seeding. Cells were isolated from ipsilateral oviducts of four animals. One Δ CP unit represents a doubling of mRNA. Different superscripts indicate significant differences ($P < 0.05$) between sampling time points.

Figure 6 Western blot analysis of ESR1 and PGR isoforms in BOECs during culture. Lane 1, control (bovine endometrium); lanes 2–4, BOECs from animal 1, cultured for 0.5, 12, and 24 h respectively; lane 5, BOECs from animal 2, cultured for 24 h. (a) Blot for ESR1. (b) Blot for PGR isoforms A–C. BOECs express predominantly PGR-isoform A.

the endpoint of differentiation and cannot be induced in an *in vitro* system (Thibodeaux *et al.* 1991). Cultured cells of the present study maintained ultrastructural characteristics, including cell organelles, such as basal bodies, secretory vacuoles, mitochondria, and polarity throughout culture. This is also in line with a previous report on BOECs in suspension culture (Walter 1995). Therefore, a short-term culture system, which is ready for use on the same day that the cells are recovered from the oviduct, has significant advantages over monolayer systems that need several days to grow to confluence.

Quantitative examination of gene expression is an additional valuable tool to assess the functional integrity of cells in culture. Oviduct specific glycoprotein 1 (OVGP1) is synthesized in BOECs and known to support embryonic development *in vivo* and *in vitro* (Nancarrow & Hill 1994). Semi-quantitative qPCR analysis of OVGP1 mRNA levels in BOECs has revealed significant differences between freshly isolated cells and cells cultured as monolayers on different supports (Reischl *et al.* 1999). In the present study, qPCR failed to detect significant differences in OVGP1 expression throughout the 24 h culture period as compared with freshly isolated cells, indicating the maintenance of physiological competence. Recent *in vivo* studies have revealed additional marker genes for BOECs function (Bauersachs *et al.* 2003, 2004, Ulbrich *et al.* 2003). This includes genes known to be differentially regulated in ipsi- and contralateral oviduct (GPX4, HMGCR) or in different stages of the estrous cycle (TRA1), as well as steroid hormone receptors (ESR1, ESR2, and PGR). Comparison of gene expression between ipsi- and contralateral BOECs at individual sampling time points during the culture period failed to detect significant differences in GPX4 and HMGCR transcript levels but showed a significant difference in ESR2 mRNA at 0.5 h in culture. However, the combined analysis of all time points revealed a significantly higher transcript abundance ($P < 0.01$) for glutathione peroxidase 4 (GPX4) in the ipsilateral oviduct. This is consistent with the

Figure 7 Quantitative PCR data for marker genes obtained from BOECs after stimulation with steroid hormones. Cells were precultured for 6 h, hormone stimulated and sampled after 6 and 18 h treatment. Data are shown separately for the two sampling time points and as combined data. Cells from four and three animals respectively were analyzed after 6- and 18-h stimulation. (a) Transcript abundance in controls and in cells stimulated with 10 pg/ml E2. (b) Transcript abundance in controls and in cells stimulated with 10 ng/ml P4. Significant differences are indicated. * $P < 0.05$.

situation *in vivo* (Bauersachs *et al.* 2003). *GPX4* transcripts in the bovine oviduct were found to be almost restricted to the luminal epithelium, with highest levels in the isthmus proximal to the dominant follicle during the follicular stage, and in the post-ovulatory period (Lapointe *et al.* 2005).

Analysis of all seven marker genes at 0.5, 6, 12, and 24 h in culture showed that the gene expression was

stable after 6 h, indicating only a short adaptation period. Thus, the presented BOECs suspension culture system is suitable for co-culture experiments with embryos as early as 6–12 h after seeding.

BOEC gene expression profiles show pronounced differences between estrous and diestrous (Bauersachs *et al.* 2004), and hormone stimulation is expected to affect gene expression in cultured BOECs. We confirmed

the presence of ESR1 and PGR in cultured cells at different time points by Western blot. BOECs isolated from the ampulla expressed only PGR isoform A, which is known to predominate in the early luteal phase (Ulbrich *et al.* 2003). This is consistent with previous studies, which failed to detect PGR isoform C in the bovine oviduct and observed isoform B in isthmus rather than ampulla (Ulbrich *et al.* 2003). Supplementation with E2, significantly increased PGR transcript abundance ($P < 0.05$) in BOECs after 6 h stimulation, but P4 showed no effect. ESR1 and ESR2 did not respond to E2 or P4 treatment. Gene expression of BOECs *in vivo* varies markedly during the estrous cycle, i.e. is steroid hormone dependent (Bauersachs *et al.* 2004). Our data are in agreement with data obtained from BOECs *ex vivo* that showed upregulation of PGR, but stable ESR1 and ESR2 expression in the follicular phase (Ulbrich *et al.* 2003). The increase in PGR mRNA may have been caused by increased PGR gene expression or post-transcriptional stabilization of PGR mRNA (Petersen *et al.* 1989). Previous reports demonstrated an estradiol-dependent OVGP1 expression (Briton-Jones *et al.* 2004), but other reports held LH/hCG responsible for the upregulation during estrous (Sun *et al.* 1997, Briton-Jones *et al.* 2003). The regulation of OVGP and the steroid concentrations causing OVGP1 effects differ between species. The bovine specific physiological hormone doses used in our study did not provoke an E2 response of OVGP1 *in vitro*, which is consistent with previous data (Sun *et al.* 1997). This might be different for physiological and/or higher than normal hormone levels in other species, as demonstrated in human (Briton-Jones *et al.* 2004). In our study, transcript levels of OVGP1 decreased after P4 stimulation, which is in agreement with low transcript abundance during the luteal phase of the estrous cycle (Boice *et al.* 1990) and confirms other *in vitro* data (Umezue *et al.* 2003).

In conclusion, the presented short-term culture system supplies well-defined and functional BOECs in numbers sufficient for functional genomics and proteomics studies of early embryo–maternal interactions in co-culture and other experiments.

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