Effects of a novel co-culture system on development, metabolism and gene expression of bovine embryos produced *in vitro*

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An *in vitro* model using co-culture of bovine *in vitro*-produced (IVP) embryos and bovine oviduct epithelial cells (bOECs) was established to study embryo–maternal interactions in the oviductal environment. *In vitro* conditions maintaining differentiated growth of oviductal cells were determined by evaluating several media supplemented with different sera at various concentrations. Morphological features were used as indicators of physiological growth, and it became obvious that synthetic oviduct fluid (SOF) supplemented with either oestrous cow serum (OCS) or dextran-coated charcoal-treated fetal calf serum (DCC-FCS) helped to prevent dedifferentiation of bOECs (Expt 1). RT-real-time-PCR analysis revealed an increased mRNA content of the oviduct-specific glycoprotein GP 85-97 when using lower serum concentrations (2 and 5% compared with 10%; Expt 2). In subsequent experiments in which cell-free cultured controls and co-cultured embryos were compared, co-cultured embryos

showed an increased rate of cleavage ($P < 0.05$) after 3 days. Successive cell-free culture until day 8 resulted in a lower rate of blastocyst development ($P < 0.05$) and reduced ATP content ($P < 0.05$) of co-cultured versus control embryos (Expt 3). Long-term co-culture (8 days) in SOF with 5% OCS increased the expression of developmentally relevant genes (glucose transporter 1 (Glut-1) and heat shock protein (HSP 70)) in co-cultured versus control embryos (Expt 4). Higher embryonic Glut-1 mRNA expression after co-culture was obvious when using 10% DCC-FCS, but was not significant when culture medium was supplemented with 10% rather than 5% OCS (Expt 5). In conclusion, SOF with 5% OCS supports differentiated growth of bOECs. Co-culture under these conditions improves early cleavage rate, but not blastocyst development, and increases the expression of developmentally relevant genes influenced by type of serum and serum concentration.

Introduction

Early embryonic development takes place in the oviduct, where the preimplantation embryo experiences a changing environment during the reproductive cycle (Hunter, 1988; Leese, 1988; Ellington, 1991). A good *in vitro* model of this system is needed to broaden our understanding of the complex embryo–maternal interactions.

Historically, *in vitro*-cultured bovine embryos struggled to pass the so-called '8- to 16-cell developmental block', a culture-induced event during the maternal-to-zygotic transition (Wright and Bondioli, 1981; Eyestone and First, 1991). *In vitro* culture systems resembling the reproductive tract environment were improved to support preimplanta-

tion embryo development to pass this block. Synthetic oviduct fluid (SOF), a composition of ovine oviduct fluid (Tervit *et al.*, 1972), and other simple media (Chatot *et al.*, 1989; Lawitts and Biggers, 1991) were capable of supplying the embryonic requirements *in vitro*. Most of these media were supplemented with serum; however, the undefined nature and variability in serum composition directed research efforts to serum-reduced or completely serum-free 'defined' media (Thompson, 1996). Nevertheless, defined media in which proteins are replaced, for example, by polyvinyl alcohol (PVA), are less able to support embryo development and the resulting embryos are metabolically compromised (Pinyopummintr and Bavister, 1991; Krisher *et al.*, 1999).

The general lack of understanding about the developmental block *in vitro*, as well as early developmental processes and metabolism of bovine embryos, led to the advent of co-culture systems using somatic cells of the reproductive tract, typically oviductal epithelial cells (OECs) (Gandolfi and Moore, 1987; Thibodeaux *et al.*,

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1992). The beneficial effects on blastocyst development are thought to be due to embryotrophic factors provided by the epithelial cells (Gandolfi *et al.*, 1989, 1992), and also to reduction of O₂ tension (Fukui *et al.*, 1991) and glucose concentration by these cells (Bavister, 1995).

Embryo co-culture has proven to be an inappropriate system for production of embryos for commercially focused purposes, but it is a suitable model for studying embryo–maternal interactions. A deeper insight into this complex, including the signalling between both components, can be efficiently obtained by using an *in vitro* system that mimics the *in vivo* environment and most closely fulfils the requirements of both the cellular component and embryos that might be quite different. The most important prerequisite is to prevent the loss of physiological features (dedifferentiation) of the OECs, which mediate the maternal and cellular effects on embryonic development *in vivo*. This can be achieved in a number of ways including growing bovine OECs (bOECs) on permeable matrices such as cellulose nitrate (Reischl *et al.*, 1999). Furthermore, medium and serum supplementation have to be empirically determined, and differences must be evaluated by cell morphology and by studying the expression of oviduct-specific genes, such as glycoprotein GP 85-97 (Malayer *et al.*, 1988; Sendai *et al.*, 1994).

The effects of co-culture on embryo development are typically evaluated by rates of cleavage and development to the blastocyst stage, as well as by assessing metabolic activity and expression of genes that indicate developmental capacity. As ATP production is a key regulator of embryo development and reflects metabolic activity, ATP content can be used to characterize embryo viability (Thompson, 2000). Expression of the glucose transporter 1 (Glut-1) gene is frequently used to describe differences between different culture systems (Wrenzycki *et al.*, 1998, 2001). Glut-1 mediates cellular glucose incorporation into embryonic cells and is necessary for transition from the morula to blastocyst stage (Leese, 1995). Glut-1 mRNA expression of *in vivo*-derived embryos was more than double that of *in vitro*-produced embryos (Niemann and Wrenzycki, 2000). The regulation of expression of genes related to 'stress' responses (Leese *et al.*, 1998), such as heat shock protein (HSP) genes (Donati *et al.*, 1990), is of particular interest because it is known to be affected by *in vitro* culture conditions and is different between *in vivo*- and *in vitro*-derived embryos (Wrenzycki *et al.*, 1999).

The aim of the present study was to establish a co-culture system for bovine *in vitro*-produced (IVP) embryos with bOECs as a model to study embryo–maternal communication. First, the effect of common cell culture media (tissue culture medium (TCM) 199 and Ham's F10) and embryo culture media (SOF and modified Parker's medium (MPM)) on differentiated bOEC growth was compared. Different parameters of cell morphology were investigated by electron microscopy and expression of GP 85-97 was quantified by RT–real-time-PCR to define culture conditions that support cells in a state most similar to that *in vivo*. Effects of

short-term co-culture to mimic the 3 days of embryo migration through the oviduct *in vivo* (Gordon, 1994) were described by rate of embryonic development, ATP content and number of cells. Long-term co-culture for 8 days using different types and quantities of serum was evaluated by comparing blastocyst development and Glut-1 and HSP 70 mRNA expression.

Materials and Methods

Isolation and culture of bovine oviduct epithelial cells (bOECs)

Oviducts from cows at oestrus detected by a mature pre-ovulatory follicle together with oestrous mucus were collected at a local abattoir, trimmed free of surrounding tissue and ligated at the infundibulum and at the isthmus–uterus junction. The complete organ was rinsed with PBS containing 100 iu penicillin ml⁻¹ (Seromed, Berlin) and 100 µg streptomycin ml⁻¹ (Seromed), disinfected with 70% (v/v) ethanol and transported in PBS plus antibiotics on ice to the laboratory within 1 h.

The epithelial cells were isolated according to Witkowska (1979) by opening the oviduct longitudinally and scraping the mucosal epithelial layer with a sterile glass slide, and were further processed as described by Reischl *et al.* (1999). In brief, cells were collected in 2.5 ml Hepes-buffered TCM 199 (ICN, Eschwege) (TCM/H) + 10% fetal calf serum (FCS; Gibco BRL, Karlsruhe), and were pooled from three oviducts before being washed twice by centrifugation at 170 *g* for 5 min each. The cell pellet was incubated in 2 ml 0.25% (w/v) trypsin–0.02% (v/v) EDTA solution (Sigma, Deisenhofen) for 8 min at 37°C. Finally, the cells were washed in TCM/H + 10% FCS, centrifuged at 170 *g* for 5 min and counted before plating.

In vitro production of bovine embryos

Bovine embryos were produced using slight modifications to previously described methods (Stojkovic *et al.*, 1995). In brief, cumulus–oocyte complexes (COCs) were collected from ovaries obtained from an abattoir by aspirating follicles 2–8 mm in diameter and washing them twice in preincubated (39°C, 5% CO₂ in air) TCM 199 buffered with 1 mmol sodium bicarbonate l⁻¹ (TCM/B) supplemented with 10% OCS. OCS was prepared by collecting blood from cows at standing oestrus that had been hormonally synchronized and verified by determination of progesterone concentration. After centrifugation at 4800 *g* for 25 min, serum was pooled from three cows and heat-inactivated (56°C, 25 min) before aliquots were stored at –20°C. COCs with a dense cumulus layer and a dark, evenly granulated cytoplasm were selected and matured *in vitro* in TCM/B supplemented with 10% OCS and 0.01 iu ml⁻¹ of each of bFSH and bLH (Sioux Biochemicals, Sioux Center, IA) for 24 h at 39°C in 5% CO₂ in air. *In vitro* fertilization was performed for an additional 20 h in Tyrode's albumin–lactate–pyruvate medium containing 6 mg BSA ml⁻¹

(Sigma), 10 µg heparin ml⁻¹ (Sigma) and frozen-thawed semen (10⁶ spermatozoa ml⁻¹) prepared by swim-up procedure (Parrish *et al.*, 1986). Cumulus cells were removed from the presumptive zygotes (day 1) by vortexing (180 s). The embryos were washed twice in the same medium used in the subsequent culture experiment and groups of 30 embryos were either transferred on to the precultured bOECs on cellulose nitrate in one well of a four-well plate (Nunc, Roskilde) in 600 µl of the specific culture medium (co-culture) or into the same medium without bOECs (control).

Experiment 1: comparison of different media and serum sources with respect to bOEC morphology

For a 2 day pre-culture at 37°C in a humidified atmosphere of 5% CO₂ in air, 0.8 × 10⁶ cells were plated in 800 µl TCM/B with 10% FCS per well of a four-well plate on round cellulose nitrate sheets (13 mm in diameter, 0.45 µm pore size; Sartorius, Göttingen). Four different media and three different sera (4 × 3 factorial experiment) were compared. The pre-cultured cells were subsequently cultured for 6 days in either TCM/B (Gibco BRL), MPM, SOF or Ham's F10 (Sigma) as basic media, and each was supplemented with either 10% FCS, OCS or dextran-coated charcoal-treated fetal calf serum (DCC-FCS), and the medium was changed every 48 h. DCC-FCS was prepared by mixing 400 ml FCS two times with 900 ml 0.01 mol Tris-HCl l⁻¹ containing 0.25 g dextran (Pharmacia Biotech, Uppsala) and 2.25 g charcoal (Merck, Darmstadt). The solution was incubated two times for 45 min each at 45°C and centrifuged at 850 g for 20 min before filtration (Wobus *et al.*, 2001).

Morphological evaluation of oviductal cells cultured in different media and sera. After 8 days of culture the epithelial cell surface (cilia, pores, microvilli and dome shape), as well as intercellular attachment, was evaluated by scanning electron microscopy. Transmission electron microscopy was used to examine intracellular organelles (mitochondria, rough endoplasmic reticulum (rER) with cisterna, Golgi apparatus), glycogen accumulation and intercellular junctions.

For scanning electron microscopy, the cellulose nitrate sheets with bOECs were washed in PBS(-) (without Ca²⁺ and Mg²⁺), fixed with 2.1% (w/v) glutaraldehyde (Serva, Heidelberg) and dehydrated in a graded series of acetone before critical-point drying over CO₂ (CDP 020; BAL-TEC, Walluf). After fixing the samples on an aluminium holder, they were coated with a 12 nm layer of gold-palladium in a sputter coater (SCD 040; BAL-TEC) and evaluated using a digital scanning microscope (DSM; Zeiss, Jena).

For transmission electron microscopy, bOECs were rinsed in PBS(-) and sodium cacodylate buffer, fixed in Karnovsky's solution (Karnovsky, 1965) and pre-contrasted with 1% (v/v) osmium tetroxide (Plano, Wetzlar) and 1.5% (w/v) potassium ferrocyanide (Sigma). The cells were dehydrated in a graded series of ethanol and embedded in

Epon (Polysience, Warrington). Ultrathin sections (60 nm) with silver interference were placed on copper grids, stained with uranyl acetate and lead citrate (Plano), and examined under a transmission electron microscope (Zeiss 902). For each electron microscopy technique, two sheets were prepared per replicate and treatment. The media/sera comparison was repeated three times.

Experiment 2: effect of serum concentrations on bOEC morphology and expression of GP 85-97 mRNA

SOF and TCM/B were supplemented with 2, 5 or 10% FCS or OCS to study the effect of reduced serum content. bOECs were prepared and pre-cultured as in Expt 1 and cultured for an additional 6 days in the different media combinations with a change in medium every 48 h. The serum reduction experiment was performed five times. Morphological evaluation of oviduct cells was done as in Expt 1.

RT-real-time-PCR analysis of mRNA expression in bOECs. The mRNA expression of the oviduct-specific glycoprotein GP 85-97 was analysed by RT-real-time-PCR after 8 days of culture to determine the physiological status of the bOECs. Four cellulose nitrate sheets per treatment with the attached monolayer were transferred to a 1.5 ml reaction tube and vortexed in 300 µl lysis buffer (Qiagen, Hilden). After removing the sheets, the lysate containing RNA was frozen at -80°C. Total RNA was extracted according to the manufacturer's protocol of the RNeasy total RNA extraction kit (Qiagen) and incubated with 10 U RNase-free DNase (Roche Diagnostics, Mannheim) for 25 min at 37°C. RNA (40 ng) was reverse transcribed in 40 µl RT reaction mixture containing 0.01 mol dithiothreitol l⁻¹ (Gibco BRL), 1.32 µg random hexamer primers (Roche Diagnostics), 1 mmol of each dNTP l⁻¹ (MBI Fermentas, St Leon-Rot), 40 U Moloney murine leukaemia virus (M-MuLV) reverse transcriptase (Gibco BRL), 5 × reaction buffer (Gibco BRL) and RNase-free water for 1 h at 40°C. The reaction was terminated by heating at 95°C for 5 min, and was cooled on ice and stored at -20°C until use.

Quantification of mRNA was performed using an ABI PRISM® 7700 Sequence Detection System (PE Applied Biosystems, Weiterstadt) and SYBR® Green (PE Applied Biosystems) as a double-strand DNA-specific fluorescent dye. Nine RNA dilutions (1:2) from 10 ng µl⁻¹ to 0.039 ng µl⁻¹ were used to produce a standard curve to calculate the GP 85-97 expression in bOECs. The dilutions were supplemented with carrier synthetic A-oligonucleotides (Pharmacia Biotech) to a final concentration of 10 ng µl⁻¹ RNA solution and were reverse transcribed together with the bOEC RNA samples. Aliquots of the standards were primed separately for GP 85-97 (gene of interest) and GAPDH to calculate the corresponding amplification efficiencies by the ABI PRISM® software. GAPDH was used as a housekeeping gene for normalization. Regulation of this gene due to different media and various serum concentrations was evaluated in

Table 1. Expression of GAPDH mRNA in bovine oviduct epithelial cells (bOECs) cultured in different media, types of serum and serum concentrations (Expt 2)

Culture	SOF plus OCS	SOF plus FCS	TCM plus OCS	TCM plus FCS
2% Serum	4.46 ± 0.56	4.6 ± 0.78	6.23 ± 0.86	9.35 ± 1.9
5% Serum	4.25 ± 0.48	8.37 ± 1.77	8.4 ± 2.2	9.86 ± 1.94
10% Serum	9.25 ± 2.77	7.96 ± 2.05	7.84 ± 1.1	10.98 ± 1.98

SOF: synthetic oviduct fluid; FCS: fetal calf serum; TCM: tissue culture medium; OCS: oestrous cow serum.

Data are presented in RNA units (mean ± SEM).

advance by RT-PCR analysis. Although differences in GAPDH mRNA abundance were found between the experimental groups, these differences were not significant (Table 1). Furthermore, the differences in expression of the gene of interest (GP 85-97) were much greater than those of GAPDH, thereby justifying its use for normalization.

Amplification mixes (25 µl) contained 2 µl bOEC cDNA solution, 2 × SYBR®Green Master Mix, 0.25 U AmpErase uracil *N*-glycosylase (PE Applied Biosystems) and 0.3 µmol l⁻¹ of forward and reverse primers for GAPDH, or 0.4 µmol l⁻¹ of forward and 0.2 µmol l⁻¹ of reverse primer for GP 85-97, respectively. Amplification primers (bovine GP 85-97, accession no. D16639: forward primer: 5'-TGTCC-ACGTTTCCAACCG-3' (324–342); reverse primer: 5'-GG-AGGGCGATCACTGAAGT-3' (374–355); bovine GAPDH, accession no. U85042: forward primer: 5'-ACCACTTG-GCATCGTGGAG-3' (461–480); reverse primer: 5'-GGG-CCATCCACAGTCTTCTG-3' (536–517)) were designed using Primer Express® software (PE Applied Biosystems). PCR started with activation of AmpErase for 2 min at 50°C, followed by 10 min denaturation at 95°C and 40 cycles of denaturation (95°C, 15 s) and annealing/extension (60°C, 1 min). The identity of the amplified GP 85-97 cDNA was confirmed by purification and restriction enzyme digestion (*Mse*I; MBI Fermentas; 17 and 34 bp), and the specificity of the GAPDH PCR product was determined by sequencing.

Relative mRNA expression of GP 85-97 in the bOEC samples was calculated using ABI PRISM® 7700 software according to the C_T (threshold) of the samples. C_T is defined as the cycle number at which the fluorescence intensity determined after each PCR cycle exceeds a fixed base line and represents RNA arbitrary units (RAU) relative to the specific standard curve. The mRNA expression of GP 85-97 was calculated after normalization to GAPDH by dividing the corresponding RAU in each sample (RAU GP 85-97/RAU GAPDH). The RT-real-time-PCR was performed using bOECs from three repeated culture experiments and real-time-PCR analysis was repeated twice per cDNA sample.

Experiment 3: short-term co-culture of bovine IVP embryos with bOECs

For the co-culture experiment, 4 × 10⁶ cells per 5 ml were pre-cultured on six cellulose nitrate sheets in a 6 cm Petri dish for 2 days in TCM/B with 10% FCS. The denuded day 1 zygotes were cultured with this bOEC monolayer on

cellulose nitrate (co-culture) or without (control) both in SOF (supplemented with 2% (v/v) essential amino acids stock (Gibco BRL), 1% (v/v) non-essential amino acids stock (Gibco BRL), 0.025% (w/v) gentamycin (Sigma)) and in 5% OCS in a humidified atmosphere of 39°C, 5% CO₂, 5% O₂ and 90% N₂. This experiment was repeated seven times.

Evaluation of rates of development. After short-term (48 h) co-culture, the developmental potential of both embryo groups was evaluated at day 3 of development on the basis of the cleavage rate (number of cleaved embryos out of selected oocytes). Afterwards, both groups were transferred separately to a cell-free culture system of SOF containing the same ingredients but 10% OCS, and the rate of blastocyst development (number of blastocysts out of selected oocytes) was determined at day 8.

Determination of ATP content and total number of cells of day 8 blastocysts. The ATP content of short-term co-cultured and control embryos was measured at day 8 using a commercial assay based on the luciferin–luciferase reaction (bioluminescent somatic cell assay kit; Sigma) (Stojkovic *et al.*, 1999). In brief, after washing the embryos three times in ATP sample buffer, each embryo was transferred individually into a polypropylene tube (Sarstedt, Nürmbrecht) containing 50 µl ATP buffer on ice/water. After addition of 100 µl ice-cold somatic cell-releasing buffer, the reaction mix was incubated for 5 min on ice/water. One hundred microlitres of the ice-cold luciferin–luciferase assay mix prepared by adding 960 µl ATP dilution buffer to 40 µl luciferin–luciferase stock solution was added to each embryo and incubated for 5 min at room temperature in darkness. The resulting light emission was measured by a luminometer (Bioluminat Junior; Berthold, Wildbad) and the ATP content was determined by linear regression of a seven-point standard curve (0–6 pmol per tube).

After ATP determination, the embryos were washed in PBS and the zona pellucida of non-hatched embryos was removed by pronase digestion (0.5% (w/v) in PBS for 2 min). Subsequently, the embryos were transferred to 50 µl 4'-6'-diamino-2-phenyl-indole (DAPI) (Sigma; 10 µg ml⁻¹) for staining of cell nuclei and were incubated for 1 min at 37°C in darkness. Each individual embryo was transferred in a small amount of PBS on to a glass slide and the number of cells was counted using an epifluorescence microscope (Axiovert 135; Zeiss) at × 400 magnification under UV light (365 nm excitation).

Experiment 4: long-term co-culture of IVP embryos in SOF with 5% OCS

Long-term co-culture was performed for 8 days on bOECs prepared as described above (Expt 3). The culture medium SOF was supplemented with 5% OCS and half of the medium (300 μ l) was changed every 48 h to avoid the negative effects of, for example, ammonia, generated from the spontaneous degradation of amino acids during culture and amino acid metabolism, and other toxic metabolites (Gardner *et al.*, 1994). The control embryos were also cultured in SOF plus 5% OCS but without bOECs. This experiment was repeated five times. Rate of blastocyst development was determined on day 8 before RNA extraction.

RT-real-time-PCR analysis of expression of *Glut-1* and *HSP 70* mRNA in long-term co-cultured embryos. Embryos were washed three times in PBS supplemented with 0.1% (w/v) polyvinyl pyrrolidone (Sigma) and stored in pools of four embryos of the same developmental stage at -80°C in a minimum volume until processed for RT-real-time-PCR. Total embryonic RNA of four embryos was isolated as described by Prelle *et al.* (2001). In brief, 100 μ l extraction buffer (0.2 mol NaCl l^{-1} , 0.025 mol Tris l^{-1} (pH 7.2), 1 mmol EDTA l^{-1}) and 4 ng synthetic A-oligonucleotides (Pharmacia Biotech) as a carrier were added to the thawed embryos, and RNA was extracted with 75 μ l chloroform:isoamyl alcohol (24:1) and 75 μ l phenol. After centrifugation at 16 000 *g* for 10 min, the aqueous phase was mixed with 100 μ l chloroform:isoamyl alcohol and after another centrifugation at 16 000 *g* for 10 min, 2 μ l of the co-precipitant seeDNA[®] (Amersham Pharmacia, Amsterdam) and $0.1 \times$ volume of the aqueous phase of 3 mol sodium acetate l^{-1} were added. The RNA pellet was washed in ethanol and dissolved in 20 μ l RNase-free water. For the RT reaction, 10 μ l total embryonic RNA (equivalent to 2 blastocyst RNA units (BRUs)) was mixed with 10 μ l RT master mix containing 0.66 μ g random hexamer primer (Roche Diagnostics), 1 mmol of each dNTP l^{-1} (MBI Fermentas), 0.01 mol dithiothreitol l^{-1} , 20 U M-MuLV-RT and $5 \times$ RT buffer (all Gibco BRL).

Real-time PCR reaction mix (25 μ l) contained 1.75 μ l embryonic cDNA (equivalent to 0.175 BRU), $2 \times$ SYBR[®]Green master mix, 0.25 U AmpErase and 0.3 μ mol l^{-1} of each specific forward and reverse primer (bovine *Glut-1*, accession no. M60448: forward primer: 5'-AAC-CTGCTGGCCTTCGTGT-3' (468–486); reverse primer: 5'-AGCGACCCAGGATCAGCAT-3' (549–531); bovine *HSP 70*, accession no. X53827: forward primer: 5'-CCCCA-AGCTATGTCGCCTT-3' (147–165); reverse primer: 5'-GGATTCAATGCGACTTGGTTC-3' (222–202)). GAPDH was proven to be a non-regulated housekeeping gene in advance (C_T GAPDH co-culture: 28.78 ± 0.36 ; C_T GAPDH control: 28.23 ± 0.38). Four dilutions of embryonic cDNA (0.3, 0.15, 0.075 and 0.037 BRU) were primed separately for each gene of interest to create standards for calculating amplification efficiency during real-time-PCR. PCR conditions were the same as for GP 85-97, and amplification and

quantification were performed following the standard curve method described previously (Kölle *et al.*, 2001; Prelle *et al.*, 2001). The expression (in arbitrary units (AU)) obtained for co-cultured embryos was calibrated by the corresponding values obtained for cell-free cultured control embryos, which were set to 1. Specificity of the amplification products was confirmed by sequencing.

Experiment 5: long-term co-culture of IVP embryos in SOF with 10% DCC-FCS versus 10% OCS

The influence of different types of serum on differentiation of bOECs with respect to their embryotrophic potential on embryos was investigated in a 8 day co-culture using SOF with 10% OCS and 10% DCC-FCS, respectively. The control embryos for each group were cultured in the same medium without bOECs and half of the medium was changed every second day in each system. The experiment was repeated three times. Rate of development was determined at day 8 and mRNA expression of blastocysts was investigated as described for Expt 4 (C_T GAPDH with 10% OCS: 27.68 ± 0.55 (co-culture) versus 27.38 ± 0.92 (control); C_T GAPDH with 10% DCC-FCS: 30.97 ± 0.41 (co-culture) versus 29.53 ± 0.39 (control)).

Statistical analyses

Data for GP 85-97 mRNA expression of bOECs (Expt 2) were analysed using Kruskal–Wallis *h* test to evaluate the influence of basic medium (TCM and SOF) and type of serum (OCS and FCS). Subsequently, differences between various serum concentrations (2, 5 and 10%) were analysed by Mann–Whitney U test.

Rate of cleavage and ATP content after short-term co-culture (Expt 3) were analysed using one-way ANOVA, and ATP content and number of cells at different developmental stages were analysed by Mann–Whitney U test. The correlation between ATP content and number of cells was evaluated by calculating the Spearman-rho coefficient.

Rates of development in the long-term co-culture experiment using 5% OCS (Expt 4) were analysed by ANOVA. Data for embryonic mRNA expression were analysed using Student's one-sample *t* test for logarithmic factors of calibrated expression values of co-cultured embryos compared with 1 (value of corresponding control embryos).

In the final co-culture experiment (Expt 5), the influence of type of serum (OCS or DCC-FCS) on embryonic development in the two culture systems was evaluated by chi-squared analysis.

Results

Experiment 1: effect of different media and serum sources on bOEC morphology

Evaluation by electron microscopy revealed a polygonal, dome-shaped morphology of bOECs when cultured in TCM 199 or SOF similar to that observed *in vivo*. Regardless of the serum source, several pore-like membrane lesions ($< 1 \mu\text{m}$) were visible at the apical cell surface (Fig. 1a). In

Fig. 1. (a,b) Scanning electron microscope images of bovine oviduct epithelial cell (bOEC) monolayers after 2 + 6 days culture on cellulose nitrate (Expt 1). (a) Polygonal dome-shaped bOECs cultured in tissue culture medium (TCM) 199 with 10% fetal calf serum (FCS) showing pore-like

Fig. 2. Relative differences in abundance of GP 85-97 mRNA (in RNA arbitrary units, RAU) of bovine oviduct epithelial cells (bOECs) cultured in different combinations of basic media (tissue culture medium (TCM; ■), synthetic oviduct fluid (SOF; □)), serum sources (fetal calf serum (FCS), oestrous cow serum (OCS)) and concentrations (2, 5 and 10%) after normalization for GAPDH expression (Expt 2). Values are mean $\pm$ SEM. Significant differences are marked by different superscripts within basic media (^{a-c} and ^{de}, $P < 0.05$, Mann-Whitney U test). Significant differences between media ($P < 0.001$) are not shown.

contrast, bOECs cultured in MPM or Ham's F10 were flat and spindle-shaped. Tight intercellular adhesion characterized by desmosomes, the typical *in vivo* epithelial cell connections, was observed only in cells cultured in SOF with OCS (Fig. 1e). This combination also showed an increased proportion of intracellular organelles in bOECs including a widespread Golgi apparatus, high number of mitochondria and numerous dilated rER (Fig. 1f). Culture in enriched media (TCM 199 and MPM) induced accumulation of glycogen in some cells (Fig. 1c). Regardless of culture medium, DCC-FCS increased the proportion of ciliated cells (Fig. 1b), which are characteristic of OECs *in vivo*, compared with other serum sources.

Experiment 2: effect of serum concentrations on bOEC morphology and expression of GP 85-97 mRNA

Independent of basic media, bOECs cultured in the presence of OCS showed epithelial-like cell morphology, whereas in the presence of FCS, cells were more flat and loosely connected with fewer cilia. In TCM 199 the effects of different serum concentrations or sources on cell

morphology were less distinct compared with SOF, in which cells cultured in low serum concentrations contained a higher proportion of cell organelles, especially numerous mitochondria, Golgi apparatus, dilated rER and small vacuoles with secretory granules. Lower OCS concentrations also induced an increased proportion of cells carrying cilia with typical '9 + 2' microtubule arrangement (Fig. 1d).

Significant differences in expression of GP 85-97 mRNA were detected between the cells cultured in different FCS concentrations ($P < 0.05$). Independent of basic media, the abundance of GP 85-97 mRNA increased with decreasing serum content. When OCS was used, the GP 85-97 mRNA expression of bOECs was similar to that of bOECs cultured with FCS in both basic media (Fig. 2). Culture in SOF significantly increased expression of GP 85-97 mRNA compared with culture in TCM, regardless of serum source and concentration ($P < 0.001$).

Experiment 3: rates of development, ATP content and number of cells of bovine IVP embryos after short-term co-culture

Short-term co-culture with bOECs significantly ($P < 0.05$) increased the cleavage rate of embryos on day 3 compared with the cell-free control (Table 2). However, the proportion of blastocyst development by day 8 was significantly reduced ($P < 0.05$).

The ATP content of individual expanded and hatching blastocysts at day 8 was significantly lower in the co-culture group compared with the control group ($P < 0.05$) (Fig. 3a). The number of cells of these embryos was determined by DAPI staining (Fig. 3b), and no differences were found between controls and co-cultured embryos (Table 3). As a consequence, the ATP content per cell was lower in co-cultured embryos compared with controls (blastocysts: 6.29 ± 0.64 versus 6.98 ± 0.68 fmol per cell; expanded blastocysts: 6.37 ± 0.62 versus 7.07 ± 0.62 fmol per cell; hatching blastocysts: 6.65 ± 1.12 versus 6.69 ± 1.21 fmol per cell; hatched blastocysts: 6.96 ± 1.84 versus 7.26 ± 1.05 fmol per cell). A positive correlation was detected between number of cells and ATP content for both co-cultured ($r = 0.54$) and control ($r = 0.65$) embryos (Fig. 3c).

Experiment 4: effect of long-term co-culture on embryonic development and mRNA expression

After long-term co-culture in SOF with 5% OCS, there was no significant difference in rates of development between the co-cultured and control embryos at day 8

membrane lesions ($< 1 \mu\text{m}$) (arrowheads). (b) Cell grown in synthetic oviduct fluid (SOF) with 10% dextran-coated charcoal-treated (DCC)-FCS showing cilia. (c-f) Transmission electron microscope images of bOECs after 2 + 6 days cultured on cellulose nitrate. (c) Intracytoplasmic glycogen accumulation (white arrowheads) in one cell close by another without glycogen after culture in TCM with 10% FCS. (d) Cells cultured in SOF with 5% OCS displaying cilia with typical '9 + 2' microtubule arrangement (arrow), numerous organelles (white arrowheads) and rough endoplasmic reticulum with visible cisternae (black arrowheads). (e) Cells in SOF with 10% OCS with tight intercellular connections (arrowheads) like desmosomes (centre), and tight junctions (apical). (f) Cells cultured in SOF with 10% OCS displaying numerous organelles including mitochondria and distinct Golgi apparatus (arrowheads). Scale bars represent (a) $10 \mu\text{m}$, (b,c) $5 \mu\text{m}$, (d) $2 \mu\text{m}$ and (e,f) $1 \mu\text{m}$.

Table 2. Rates of development of bovine *in vitro*-produced (IVP) embryos after short-term co-culture (Expt 3)

Culture system	Number of oocytes	Cleavage rate (day 3)	Blastocysts (day 8)	Expanded blastocysts	Hatched blastocysts	Total blastocyst rate (day 8)
Co-culture (with bOECs)	872	81.2 ± 1.8 ^a	8.8 ± 1.2	4.6 ± 0.9	2.6 ± 0.3 ^a	16.1 ± 1.1 ^a
Control (without bOECs)	794	74.3 ± 2.2 ^b	12.6 ± 2.6	7.4 ± 1.1	4.9 ^b ± 0.9 ^b	25.0 ± 3.6 ^b

bOECs: bovine oviduct epithelial cells.

Data are presented as mean ± SEM of percentage of oocytes selected for IVF.

^{ab}Values with different superscripts within columns differ significantly ($P < 0.05$, ANOVA).

(Table 4). The content of Glut-1 mRNA tended to be higher in co-cultured embryos compared with controls, although this difference was not significant ($P < 0.08$; Fig. 4a). The content of HSP 70 mRNA was significantly higher ($P < 0.05$) in co-cultured expanded blastocysts than in the corresponding controls (Fig. 4b).

Experiment 5: influence of different serum sources on co-cultured embryos

Co-culture using each of 10% OCS and 10% DCC-FCS resulted in a lower rate of blastocyst development when compared with the cell-free control system ($P < 0.05$ in DCC-FCS; $P < 0.01$ in OCS). The proportion of hatched blastocysts in DCC-FCS was reduced markedly in the co-culture system as well as in the control system compared with 10% OCS ($P < 0.001$). The total rate of blastocyst development was significantly lower in DCC-FCS independent of the culture system ($P < 0.05$) (Table 5).

Relative Glut-1 mRNA content was higher in co-cultured blastocysts in SOF supplemented with 10% DCC-FCS (Fig. 5a), although this increase was not significant ($P < 0.08$), whereas 10% OCS did not show an obvious effect on the expression of both HSP 70 and Glut-1 in co-cultured embryos (Fig. 5b).

Discussion

In the present study the effects of bOEC co-culture on bovine IVP embryo development were evaluated to assess its suitability as a model for studying embryo–maternal communication. Initially, four different media were compared using porous cellulose nitrate matrices that prevent dedifferentiation of bOECs (Reischl *et al.*, 1999). These media, which were designed for somatic cell culture (TCM 199 and Ham's F10) and embryo culture media (SOF and MPM), were combined with three sera (FCS, DCC-FCS and OCS), and their effects on bOEC morphology and function *in vitro* were compared. SOF supplemented with OCS maintained polygonal morphology, typical of epithelial cells, as well as the functional status of bOECs most effectively, although this medium was initially designed and optimized for embryo culture (Tervit *et al.*, 1972; Fukui *et al.*, 1991). The DCC-FCS also efficiently supported the differentiated growth of bOECs, characterized by a high

proportion of ciliated cells even after prolonged culture. Treating FCS with dextran-coated charcoal removes various molecules, including oestradiol (reduction of 97% from 75 pg ml⁻¹ to 2.2 pg ml⁻¹) (Fortunati *et al.*, 1999), endogenous retinoids and growth factors (Wobus *et al.*, 2001) such as fibroblast growth factor (FGF) and epidermal growth factor (EGF). A reduced content of mitogenic substances is likely to prevent dedifferentiation by not inducing cell proliferation, which is usually antagonistic to cell differentiation (Buchner and Wartenberg, 1997).

A lower concentration of serum in culture medium also reduces the growth factor content; however, some beneficial effects are maintained, including binding of toxic metals, support of cell attachment and provision of protease inhibitors (Barnes and Sato, 1980). In the presence of OCS, cells retained the epithelial-like morphology and secretory activity of embryotrophic substances characterized by small contrasted vacuoles and pore-like apical membrane lesions independent of the serum concentration. In addition, the occurrence of large Golgi apparatus typical of *in vivo* cells and numerous mitochondria in bOECs cultured in SOF with OCS was indicative of high metabolic activity, especially high synthesis of proteins stored in dilated rER (Geneser, 1986; Joshi, 1995), without obvious differences between 2% and 5% OCS. This finding might be due to a positive effect of oestrogen contained in OCS, as Nayak and Ellington (1977) demonstrated an influence of balanced oestrous hormones on the morphological and functional status of bOECs.

Lower serum concentrations affected the expression of oviduct-specific GP 85-97 mRNA in bOECs, which is secreted *in vivo* at the time of ovulation stimulated by oestrogen (Gerena and Kilian, 1990; Wegener and Kilian, 1992; Staros and Kilian, 1998). Significantly higher gene expression, probably due to differentiated growth as a consequence of reduced mitotic activity, was shown when using 2% or 5% instead of 10% FCS. GP 85-97 expression has been used as a marker of bOEC functionality in a previous study (Reischl *et al.*, 1999), in which *ex vivo*-derived bOECs showed high expression, whereas in cultured cells the expression was reduced depending on time *in vitro* and culture system. In the present study, GP 85-97 mRNA abundance was not significantly influenced by the OCS concentration but expression was high when OCS was used instead of FCS, possibly due to a higher

oestrogen content in OCS. Therefore, OCS or low FCS concentrations in a co-culture system could support differentiated cell growth and subsequent embryonic development by cellular secretion of embryotrophic factors. A low content of serum might also be beneficial because secreted factors will not be masked by serum proteins.

In the present study, SOF, which is known for its beneficial effect on embryo development, was shown for the first time to support differentiated bOEC growth. This was the initial step to establish a novel co-culture system as a model to study interactions between oviductal cells and bovine preimplantation embryos. Short-term co-culture in SOF with 5% OCS simulated the oviduct passage *in vivo* and increased the cleavage rate of co-cultured embryos on day 3 (81.2% versus 74.3%), confirming results in sheep (Gandolfi and Moor, 1987) and cattle (Eyestone and First, 1989). This effect might be due in part to reduction of serum-derived glucose by differentiated bOECs (Bavister, 1995). Glucose has a negative effect on early embryonic development (Edwards *et al.*, 1997) but can be metabolized into lactate and pyruvate by co-cultured cells (Cox and Leese, 1997). As early embryonic stages can use these two substances as energy sources (Leese *et al.*, 1993), co-culture might have a beneficial effect on early cleavage rate.

However, the lower rate of blastocyst development in the short-term co-culture group on day 8 might be due to the harsh transfer on day 3 into the standard cell-free system during a sensitive phase of development (8- to 16-cell block) (Gordon, 1994). Alternatively, feeder cells could be removed and embryos could be cultured in conditioned medium, as an increased understanding of the changing requirements of early embryo development and the temporal relationship to the reproductive tract has led to the concept of 'sequential' culture by altering media components and physical conditions during the *in vitro* period (Thompson, 2000). Therefore, transfer into the cell-free culture system at day 3 might also act to select a small number of the most viable embryos that develop to blastocysts just because of these changing culture conditions. This presumptive higher viability should be proven by their tolerance to cryopreservation or by transferring co-cultured and control embryos to recipients and following their development to offspring.

Van Blerkom *et al.* (1995) demonstrated that for human

Fig. 3. Evaluation of ATP content and number of cells in bovine *in vitro*-produced (IVP) embryos (Expt 3). (a) ATP content (pmol) of various blastocyst stages of bovine IVP embryos generated in different culture systems. ■: Co-culture; □: control. Values are mean $\pm$ SEM. Asterisks indicate significant differences between co-cultured and control embryos (ANOVA, $P < 0.05$). (b) Hatched blastocyst, stained by 4'-6'-diamidino-2-phenylindole (DAPI). Scale bar represents 20 μ m. (c) Positive correlation between number of cells and ATP content (fmol $\times$ 10) in both culture systems; grey regression curve and black diamonds (◆): co-culture group, $r = 0.542$, $y = 0.5545x + 7.1472$; black regression curve and open squares (□): control group, $r = 0.646$, $y = 0.5953x + 14.343$ (Spearman-rho).

Table 3. Effect of short-term co-culture on number of cells of day 8 *in vitro*-produced (IVP) bovine embryos (Expt 3)

Culture system	Blastocysts (day 8)	Expanded blastocysts	Hatching blastocysts	Hatched blastocysts
Co-culture (with bOECs)	79.6 ± 5.9 (n = 16)	119.7 ± 7.0 (n = 19)	138.5 ± 13.9 (n = 5)	151.9 ± 14.7 (n = 3)
Control (without bOECs)	83.0 ± 6.2 (n = 25)	137.6 ± 7.1 (n = 31)	137.1 ± 16.6 (n = 7)	152.0 ± 10.8 (n = 12)

bOECs: bovine oviduct epithelial cells; n: number of embryos evaluated.
Data are mean ± SEM.

Table 4. Rates of development of bovine embryos after long-term co-culture in synthetic oviduct fluid (SOF) with 5% oestrous cow serum (OCS) (Expt 4)

Culture system	Number of oocytes	Cleavage rate (day 3)	Blastocysts (day 8)	Expanded blastocysts	Hatched blastocysts	Total rate of blastocyst development (day 8)
Co-culture (with bOECs)	685	ne	10.7 ± 1.4	6.6 ± 1.1	3.1 ± 0.7	20.4 ± 0.9
Control (without bOECs)	638	ne	9.8 ± 1.4	8.4 ± 1.5	6.6 ± 2.0	24.7 ± 2.1

bOECs: bovine oviduct epithelial cells; ne: not evaluated.
Data are presented as mean ± SEM of percentage of oocytes selected for IVF.

blastocysts, rates of development are not a sufficient indicator of embryo quality, as even morphologically intact class 1 embryos do not necessarily initiate pregnancies. Slotte *et al.* (1990) showed that metabolic defects caused low implantation rates of *in vitro*-produced embryos. As quantitative metabolic differences could serve as more relevant selection criteria, in the present study ATP content was evaluated in day 8 embryos. ATP production via oxidative phosphorylation is essential for bovine embryo development *in vitro*. However, transient inhibition of this process sometimes appears to be beneficial to embryo development (Thompson *et al.*, 2000). Therefore, the lower ATP content in co-cultured embryos might not necessarily reflect a lower quality, as reduced ATP content was found in *ex vivo* embryos compared with IVP embryos (M. Stojkovic, unpublished) and did not hinder early embryonic development (Van Blerkom *et al.*, 1995). Instead, the reduced ATP content may reflect higher energy demand of productive processes in co-cultured embryos (Leese, 1991). In the present study there was no difference in number of cells between the two groups (which would also result in a different ATP content), whereas Orsi *et al.* (2000) found fewer cells in co-cultured embryos. Nevertheless, ATP determination should be combined with measurement of ADP to evaluate the energy turnover via the ATP:ADP ratio (Slotte *et al.*, 1990).

In addition, quantification of mRNA transcripts of selected developmentally relevant genes enables studies of the effects of bOECs on embryos. The Glut-1 gene was selected after it was shown that *ex vivo* embryos had higher Glut-1 expression compared with IVP embryos (Morita *et al.*, 1994; Uechi *et al.*, 1997; Wrenzycki *et al.*, 1998).

Expression of Glut-1 was higher in co-cultured blastocysts when using 5% OCS compared with cell-free culture; this finding was in accordance with the results of Wrenzycki *et al.* (2001), who detected an influence of different culture conditions on Glut-1 expression. In the present study, the increased Glut-1 expression in co-cultured embryos was not found in later blastocyst stages, probably due to lower suitability of the oviductal feeder cells for these embryos, and therefore a sequential culture, for example a change from oviductal to uterine feeder cells, might be more appropriate.

Expression of HSP 70 might indicate suboptimal conditions, related to inadequate nutrient supply or oxidative stress, and was increased in co-cultured expanded blastocysts compared with controls, due to insufficient binding of free oxygen radicals normally mediated by bOEC co-culture (Thompson *et al.*, 1990). HSP 70 expression might also resemble a reaction to other stressors such as enhanced toxic cell metabolites in co-culture (Bavister, 1995). This effect was reduced by the replacement of half the medium every second day in our experiments. However, HSP 70 expression does not necessarily indicate reduced vitality. It could also be a marker of embryonic cell functionality, as HSP is a chaperone that supports protein folding after *de novo* synthesis (Beckmann *et al.*, 1992).

In medium supplemented with DCC-FCS, the higher expression of Glut-1 mRNA by co-cultured blastocysts indicates that the ability of this serum to maintain the differentiated status of bOECs also influences embryo development. However, higher Glut-1 mRNA expression was accompanied by a lower rate of blastocyst development and especially reduced hatching ability in DCC-FCS.

Fig. 4. RT–real-time-PCR quantification of expression of glucose transporter 1 (Glut-1) and heat shock protein 70 (HSP 70) mRNA in bovine blastocyst stages generated under different culture conditions (co-culture (■) versus control (□) in synthetic oviduct fluid (SOF) with 5% oestrous cow serum (OCS)) (Expt 4). Relative (a) Glut-1 and (b) HSP 70 mRNA abundance of co-cultured embryos after normalization for GAPDH were calibrated on expression of control embryos (set to 1). Values are mean $\pm$ SEM. Asterisk indicates differences in Glut-1 expression, although this effect was not significant ($P < 0.08$), and HSP 70 expression ($P < 0.05$) (one-sample t test).

The latter effect might be due to a lack of specific serum factors that usually hinder the so-called ‘zona hardening’ (Schroeder *et al.*, 1990) and to a reduced content of growth

Fig. 5. RT–real-time-PCR to quantify mRNA expression of glucose transporter 1 (Glut-1) and heat shock protein 70 (HSP 70) in bovine blastocysts generated in co-culture (■) versus a cell-free system (□) with different sera (Expt 5). (a) Synthetic oviduct fluid (SOF) with 10% dextran-coated charcoal-treated fetal calf serum (DCC-FCS) increased Glut-1 mRNA content in co-cultured blastocysts, although the effect was not significant ($P < 0.08$; indicated by asterisk). (b) SOF with 10% oestrous cow serum (OCS) did not cause significant differences in mRNA expression of Glut-1 and HSP 70, respectively, in different culture systems.

factors such as EGF that stimulate blastocyst development and hatching (Lonergan *et al.*, 1996). Therefore, the results of the present study indicate that DCC-FCS is not optimal in a co-culture system to study embryo–maternal communication. However, Thompson *et al.* (1998) found similar rates of

Table 5. Rates of development of bovine embryos after long-term co-culture in different sera (Expt 5)

Culture system	Number of oocytes	Cleavage rate (day 3)	Blastocysts (day 8)	Expanded blastocysts	Hatched blastocysts	Total rate of blastocyst development (day 8)
Co-culture, 10% (v/v) OCS	285	ne	27 (9.5)	13 (4.6)	21 (7.4) ^c	61 (21.4) ^g
Control, 10% (v/v) OCS	283	ne	28 (9.9)	22 (7.8)	38 (13.4) ^d	88 (31.1) ^h
Total OCS	568		55 (9.0)	35 (6.2)	59 (14.4) ^k	149 (26.2) ^m
Co-culture, 10% (v/v) DCC-FCS	297	ne	37 (12.5)	14 (4.7) ^a	2 (0.7) ^e	53 (17.8) ⁱ
Control, 10% (v/v) DCC-FCS	271	ne	36 (13.3)	27 (10.0) ^b	4 (1.5) ^f	67 (24.7) ^j
Total DCC-FCS	568		73 (12.9)	41 (7.2)	6 (1.1) ^l	120 (21.1) ⁿ

OCS: oestrous cow serum; DCC-FCS: dextran-coated charcoal-treated fetal calf serum; ne: not evaluated;

Data are presented as total numbers and means of percentage (in brackets) of the oocytes selected for IVF.

Values with different superscripts within columns differ significantly (^{ab}, ^{cd}, ^{ij} and ^{mn}, all $P < 0.05$; ^{gh}, $P < 0.01$; ^{ce}, ^{df} and ^{kl}, all $P < 0.001$, chi-squared test).

development in the presence of DCC-FCS without co-cultured cells compared with BSA-supplemented media.

The supportive influence of serum concentration on the functional status of bOECs became obvious when 5% OCS modulated the embryotrophic effect more efficiently resulting in a higher Glut-1 expression of co-cultured embryos compared with 10% OCS.

In the present study we have described a sensitive system to investigate embryo–maternal interactions, taking into account biologically relevant indicators (energy metabolism and expression of selected genes) of developmental capacity. Knowing that the requirements of embryos and oviductal cells *per se* are different, the results of this study showed that culture of bOECs in the embryo culture medium SOF with 5% OCS on cellulose nitrate matrix promotes differentiated growth and embryotrophic activity of feeder cells. Despite the lower rates of blastocyst development obtained in this system, which must be studied in more detail, for example by proving a suspected selection mechanism of more viable embryos by means of embryo transfer experiments or tolerance to cryopreservation, this co-culture system using bOECs in SOF plus OCS is a promising step forward in establishing an *in vitro* model to study embryo–maternal communication.

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