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REGULAR ARTICLE

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Factors affecting proliferation and dedifferentiation of primary bovine oviduct epithelial cells in vitro

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Abstract The oviduct is the physiological site for key events in reproduction, such as capacitation of spermatozoa, fertilization and early embryonic development. Interactions between oviduct epithelial cells and gametes or embryos cannot sufficiently be studied in vivo. Therefore, model systems are needed which mimic in vivo conditions most closely. In this study we optimised the method for isolating bovine oviduct cells and compared different cell support materials as well as two culture systems (perfusion vs static culture) for their ability to maintain characteristic morphological and functional features of oviduct cells. Out of nine different cell support materials tested, cellulose nitrate (0.45 µm pore size) was the most suitable to maintain cells in a manner similar to freshly isolated oviduct epithelial cells. Comparing static vs perfusion culture by electron microscopy, morphological differences of the cells were insignificant in the first days of culture, while they became more evident after 8 days. The cells in the static system lost typical characteristics such as columnar shape, cilia and secretory protrusions, while these features were still present in perfusion culture. In addition, intense ciliogenesis and cytoplasmic organelles for protein synthesis were found under perfusion conditions. These findings were underlined by differences in expression of the oviduct-specific oestrus-associated glycoprotein 85–97 kDa (GP 85–97) gene as revealed by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). The RNA levels of this specific gene were significantly higher in perfusion compared to the static culture system. Our data show clear advantages of perfusion vs static culture for primary bovine oviduct epithelial cells.

Key words Oviduct epithelium · Perfusion culture · Cell support · Permeable membranes · Reverse transcription-polymerase chain reaction · Bovine

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Introduction

The oviduct is an important organ in mammalian reproduction. Crucial processes, including oocyte maturation, sperm capacitation, fertilization and early embryonic development occur in the oviduct (Hunter 1988; Leese 1988; Gandolfi et al. 1989a; Ellington 1991). Oviducts from sheep (Eyestone et al. 1987) and rabbits (Ellington et al. 1990; Ectors et al. 1993) have been used as a temporary in vivo culture system for in vitro produced (IVP) bovine embryos to overcome the eight-cell developmental block (Bavister 1988). Bovine oviduct cell monolayers have been employed as an in vitro co-culture system for IVP bovine embryos (Thibodeaux et al. 1992; Xu et al. 1992; Myers et al. 1994); however, the rates of embryonic development are still lower than in vivo. This phenomenon may be due to dedifferentiation processes, i.e. loss of morphological and functional properties of oviduct cells occurring under standard culture conditions (Joshi 1988; Hishinuma et al. 1989; Walter 1995). A number of attempts have been made to maintain oviduct epithelial cells in their differentiated polarised shape and function. These include culture of cells on matrigel-coated dishes (Joshi 1995) or on collagen filter inserts (Cox and Leese 1997), use of serum-free media (Van Langendonckt et al. 1995) or hormonal stimulation (Witkowska 1979). However, all

these experiments were performed under static culture conditions. Typical features of bovine oviduct epithelial cells, such as columnar shape, cilia and specific secretory activity could not be retained sufficiently in all cases. Therefore, we employed a new perfusion culture system (Minucells and Minutissue perfusion culture system; Minuth et al. 1992; for review, see Minuth et al. 1998), providing a continuous supply of fresh medium and in addition the removal of secreted metabolites. This system has been successfully used for the culture of differentiated renal collecting duct epithelial cells (Minuth et al. 1992; Aigner et al. 1994) and stomach mucosa cells (Kloth et al. 1997). It also allows the use of various cell support materials, satisfying the specific requirements of different cells (Minuth et al. 1994). A considerable influence of different support materials on the morphology and functionality has been documented for cultured epithelial (Patrone et al. 1992) or endothelial cells (Villars et al. 1996). To study the mammalian oviduct with regard to secretory activity, influence of hormones and growth factors and to investigate mechanisms of embryo-maternal communication, a cell culture system is needed resembling the oviduct epithelium in vivo most closely. One important factor related to these aspects is the expression of an oviduct-specific oestrus-associated glycoprotein, which has been identified in cattle (M_r 85–97 kDa; Malayer et al. 1988, Boice et al. 1990; Sendai et al. 1994) and many other species (sheep: Sutton et al. 1984; Gandolfi et al. 1989b; Murray 1992; goat: Abe et al. 1995a; pig: Buhi et al. 1993; human: Rapisarda et al. 1993). The studies on regulation and function of GP 85–97 have been mainly done on in vivo collected oviduct fluid (Wegner and Killian 1991; King and Killian 1994; McNutt et al. 1992; Staros and Killian 1998) or conditioned media of short-term (6–48 h) tissue culture (Wegner and Killian 1992; Boice et al. 1990), while prolonged in vitro studies are hampered by reduced synthesis and secretion of GP 85–97 (Nancarrow and Hill 1994). In this study, we optimised the technique for isolating bovine oviduct epithelial cells, studied effects of different cell supports and culture systems (static vs perfusion culture) on cell proliferation and differentiation, and finally quantified the expression of GP 85–97 mRNA to compare both culture systems.

Materials and methods

Tissues

At a local slaughterhouse oviducts from cows were collected in the luteal phase for experiments on cell isolation and morphological characterisation, while oviducts in oestrus were used for GP 85–97 expression studies. The complete oviducts were trimmed free of adhering fat and surrounding tissue and ligated at each end. They were rinsed with Dulbecco's phosphate-buffered salt solution (PBS) containing 100 U/ml penicillin (Seromed, Berlin, Germany) and 100 µg/ml streptomycin (Seromed), disinfected with 70% ethanol and transported in PBS plus antibiotics on ice to the laboratory within 1 h.

Isolation of bovine oviduct cells

The oviducts were washed in PBS without Ca^{++} and Mg^{++} ($PBS^{(-)}$) and the infundibulum and the isthmus-uterus junction were removed. The mucosal cells were isolated using different methods (nine replicates per method).

a) Rinsing (Reed et al. 1996): the oviducts were rinsed with 12 ml Medium 199 (Life Technologies, Eggenstein, Germany), supplemented with 10% heat-inactivated fetal calf serum (FCS) (Boehringer/Mannheim, Mannheim, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (=culture medium). The cells recovered from three oviducts were pooled and collected in a centrifuge tube (Greiner, Solingen, Germany).
b) Squeezing (Eyestone and First 1989): the oviducts were stripped with a closed forceps and the cells obtained per single oviduct were collected in 2.5 ml culture medium.
c) Scraping (Witkowska 1979): each oviduct was opened longitudinally. The cells were recovered by scraping the mucosa with a sterile glass slide and were collected in 2.5 ml culture medium.
d) Enzymatic digestion (Thibodeaux et al. 1991): the oviducts were first flushed and then filled with 0.25% trypsin (Difco, Detroit, MI), 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma, Deisenhofen, Germany) solution, ligated at each end and incubated for 45 min at 37°C in $PBS^{(-)}$. Cells were recovered by flushing with 10 ml culture medium and subsequently by scraping the oviduct as described in method 3.

To evaluate the proportion of viable cells after recovery, an aliquot of the cell suspension was incubated with trypan blue solution (0.1%, Sigma) at 37°C for 4 min. After centrifugation (200×*g*, 3 min), the cell pellet was resuspended in $PBS^{(-)}$ and the proportion of stained cells was assessed in a Neubauer chamber. The proportion of epithelial cells recovered by the different methods was determined on days 2, 6 and 8 after culturing the cells on glass sheets by indirect immunofluorescence using a monoclonal antibody against cytokeratins (clone no. K8.13, Sigma). Briefly, the cells were fixed in methanol:acetone (1:1) at –20°C for 5 min and incubated with the primary antibody [diluted 1:80 in $PBS^{(-)}$ containing 1% bovine serum albumin (BSA; Sigma)] for 2 h at room temperature. Then the cells were treated with the secondary fluorescein-coupled antibody (Sigma) (diluted 1:256 in $PBS^{(-)}$+1% BSA) for 2 h at room temperature. Embryonic mouse fibroblasts were used as negative control. Cell nuclei were stained with propidium iodide as described below, and the cells were embedded in antifade medium (Vectashield, Vector Laboratories, Burlingame, CA) and analysed with a confocal laser scanning microscope (Zeiss, Munich, Germany). Additionally, the morphology of bovine oviduct cells was assessed daily by phase-contrast microscopy (Leica, Wetzlar, Germany).

Primary culture of oviduct epithelial cells on different cell support materials

For primary culture, the cell suspension obtained by scraping was washed 3 times in culture medium by centrifugation (170×*g*, 5 min). The cell pellet was resuspended in 2 ml 0.25% trypsin/0.02% EDTA solution and incubated for 12 min at 37°C. The cell number was determined and about 1×10^6 cells/ml were plated onto round cell support sheets (13 mm diameter) placed in a 28-cm^2 Petri dish (Nunc, Wiesbaden, Germany) containing 4.5 ml culture medium. The following cell support materials were used: Thermanox (Nunc), glass (Menzel, Hannover, Germany), cellulose nitrate (pore size 0.2 µm and 0.45 µm; Sartorius, Göttingen, Germany), gauze, Nucleopore (both Reichelt Chemie, Heidelberg, Germany), nylon, and polycarbonate (black and white; all from Poretics-Biotech and Trade, St. Leon-Rot, Germany). The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air and the medium was renewed every 48 h. On the transparent materials Thermanox and glass, the development of the primary cultures was assessed daily by phase-contrast microscopy. After 2 days the attachment rate on all substrates was evaluated by staining with propidium iodide (Sigma). The cells attached on the support sheets were fixed in 70% ice-cold ethanol

for 10 min at –20°C. After rinsing with PBS$^{(-)}$, the cells were stained with 200 µl propidium iodide solution (4 µg/ml) for 45 min at room temperature in the dark. The cell nuclei were assessed by UV light microscopy (Axiovert 135, Zeiss) at a magnification of ×200. To calculate the attachment rate, five different areas on two sheets per material were evaluated in three replicates.

The three support materials with the highest attachment rates were selected and their ability to sustain epithelial cell development was determined after 8 days in the perfusion culture system by scanning electron microscopy. Therefore, after 2 days pre-culture under standard static conditions, six sheets with adherent cells were washed in PBS$^{(-)}$, fixed in a Minusheet span ring (Minucells and Minutissue, Bad Abbach, Germany), transferred into the perfusion culture container (Minucells and Minutissue) and placed on a warming plate (39°C; Bachofer, Reutlingen, Germany) outside the incubator. Bovine oviduct cells were cultured for an additional 6 days in Medium 199 with 20 mM hydroxyethylpiperazine ethanesulphonic acid (HEPES) (ICN, Eschwege, Germany) supplemented with 10% FCS and antibiotics with a medium flow rate of 1 ml/h, achieved by using a peristaltic pump (Ismatec, Zürich, Switzerland). To avoid possible position effects (Reischl et al. 1998), the cell sheets at positions 2 and 3 were always removed from the perfusion container and delivered from the span ring for evaluation of morphological features by scanning electron microscopy. In each of three independent experiments two sheets per material (Thermanox, glass and cellulose nitrate 0.45 µm) and culture system (static vs perfusion) were examined. The cells on the support sheets were fixed with 2.1% glutaraldehyde (Serva, Heidelberg, Germany) and dehydrated in a graded series of ethanol (10%, 20%–90% and absolute) prior to critical-point drying over CO_2 (CDP 020, BAL-TEC, Walluf, Germany). Subsequently, the sheets were coated with a 12-nm layer of gold/palladium in a sputter coater (SCD 040, BAL-TEC) and the cell morphology on the whole sheet was assessed using a digital scanning microscope (DSM, Zeiss) and photos of randomly selected areas were taken.

Morphological evaluation of oviduct cells cultured in the perfusion vs static system

To compare oviduct cell culture under standard static and perfusion conditions, cells obtained and pooled from three different oviducts were cultured on cellulose nitrate (0.45 µm) in a Petri dish or in the perfusion chamber, respectively (n=3). To study effects on cell morphology sheets were prepared for scanning electron microscopy after pre-culture and 4, 6 and 8 days of subsequent culture in the respective system. The sampling of areas investigated by SEM was random. A pre-selection of areas by light microscopy was impossible since cellulose nitrate is non-transparent.

Additionally, we examined cultured oviduct cells by transmission electron microscopy (n=2). Two cell sheets were recovered randomly from the static and from positions 2 and 3 of the perfusion culture system after pre-culture and 4, 6 and 8 days. Cells were fixed in Karnovsky's solution (Karnovsky 1965), treated with 1% osmium tetroxide (Plano, Wetzlar)/1.5% potassium ferrocyanide (Sigma), dehydrated in a graded series of ethanol (50%, 70%, 90% and absolute, 30 min each) and embedded in Epon (Polysciences, Warrington, UK). Semithin sections of 1 µm were cut and stained with Richardson's solution (Richardson et al. 1960). Ultrathin sections with silver interference were cut with a diamond knife (Plano), placed on 200-mesh copper grids, stained with uranyl acetate and lead citrate (Plano) according to Reynolds (1963) and examined under a transmission electron microscope (Zeiss 902). From each cell support sheet, 20 grids were prepared, 200-mesh grids were used and 50 fields were examined. Photos were taken from representative regions of the different cultures.

Semi-quantitative RT-PCR analysis of GP 85–97 mRNA expression

To evaluate the effects of the two different culture systems on expression of GP 85–97, total RNA of freshly isolated oviduct cells collected in the follicular phase of oestrus cycle, pre-cultured and cultured up to 6 and 8 days in static or perfusion system, respectively, was analysed by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). The cells were incubated with trypsin and mechanically removed from the support using a cell scraper (Greiner) and centrifuged (1100×g, 3 min). The pellet was stored in a 1.5-ml Eppendorf tube at –80°C until RNA extraction. Total RNA was extracted using the RNeasy total RNA extraction kit (Qiagen, Hilden, Germany). RNA eluted with 25 µl sterile water was incubated with 10 U RNase-free DNase (Boehringer, Mannheim) to avoid DNA contamination. After quantification by photospectroscopy at 260 nm, 0.5 µg RNA was used for cDNA synthesis. The reaction mixture containing 3,4 µM oligo-(dT 17)-primer, 1 mM each of dATP, dCTP, dGTP and dTTP (MBI Fermentas, Vilnius, Lithuania), 5×–reaction buffer (50 mM TRIS-HCl, 50 mM KCl, 4 mM $MgCl_2$, 10 mM dithiothreitol) and 13 U moloney murine leukemia virus M-MuLV reverse transcriptase (MBI Fermentas) in a volume of 20 µl was incubated at 37°C for 60 min. The reaction was terminated by heating at 95°C for 10 min, cooled to 4°C and stored at –20°C until use. Semi-quantitative PCR was performed according to a previously described modified protocol (Wong et al. 1994). Two-microlitre aliquots of cDNA were amplified with 1 U Taq DNA polymerase (PAN Systems GmbH, Nürnberg, Germany) in a final volume of 20 µl containing 10×–reaction buffer (16 mM $(NH_4)_2SO_4$, 50 mM TRIS-HCl and 0.01% Tween-20), 1.5 mM $MgCl_2$, 0.1 mM of each dNTP and 0.1 µM of each specific primer. The mixture was overlaid with mineral oil (Sigma) to prevent evaporation, and amplified in a PCR cycler (Uno II Thermocycler, Biometra, Göttingen, Germany). Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 64°C for 50 s and extension at 72°C for 2 min. The secondary primer pair (0.1 µM) for bovine β-actin was added after 10 cycles during a holding step at 80°C for 2 min followed by 24 cycles performed as mentioned above. The horizontal electrophoresis was carried out on a 2% agarose gel (Gibco) containing 0.6 µg/ml ethidium bromide (Sigma) in TRIS acetate (0.04 M)-EDTA (0.001 M) buffer. The resulting DNA fragments were visualised with UV light (320 nm), documented by an Eagle eye documentation system (Stratagene, Heidelberg, Germany) and semi-quantified by digital imaging (ImageQuaNT, Molecular Dynamics GmbH, Krefeld, Germany).

PCR primers

The bovine β-actin primers represented the following sequences: (1) 5′ GCGTGACATCAAGGAGAAGC 3′ for sense primer and (2) 5′ TGGAAGGTGGACAGGGAGGC 3′ for antisense primer, amplifying an expected fragment length of 432 bp as described previously by Degen et al. (1983).

The GP 85–97 primers were designed according to the cDNA sequence published by Sendai et al. (1994), amplifying a 856-bp fragment of the middle of the molecule. The sequence of the sense primer was 5′ TGAGGTTCACCACGATGCTGT 3′ and 5′ AGTTGAGCTGAACTCATCATT 3′ for the antisense primer.

Statistical analysis

Differences in cell yield and attachment rates were tested for significance using the Mann-Whitney U-test. Data obtained by the RT-PCR analysis were statistically evaluated using the Wilcoxon test for paired samples. P<0.05 was considered significant.

Results

Effects of the different techniques for cell recovery

Total cell yield and the overall cell viability for the different isolation techniques are summarised in Table 1. The average number of cells recovered per oviduct by

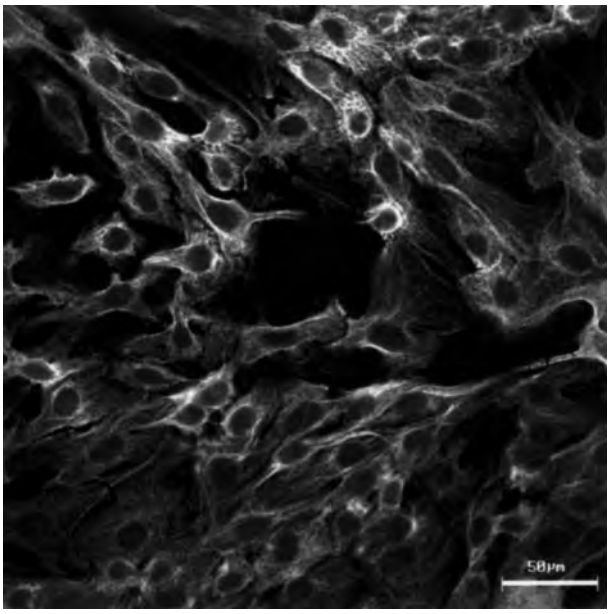


Fig. 1 Confocal laser scanning image of the immunohistochemical evaluation of cytokeratin in bovine oviduct epithelial cells after 8 days on glass under static culture conditions. *Bar* 50 µm

Table 1 Comparison of different techniques for the isolation of bovine oviduct epithelial cells

Isolation technique (n=9)	Mean cell yield per oviduct ($\times 10^6$) (±SE)	Mean proportion of viable cells (%) (±SE)	Mean yield of viable cells ($\times 10^6$) (±SE)
a) Rinsing	0.8^{a} (±0.25)	95^{a} (±0.02)	0.8^{a} (±0.24)
b) Squeezing	2.2^{b} (±0.63)	84^{c} (±0.04)	1.8^{b} (±0.51)
c) Scraping	3.1^{c} (±0.75)	84^{c} (±0.03)	2.7^{d} (±0.65)
d) Enzymatic	2.4^{b} (±0.69)	84^{c} (±0.03)	2.0^{bd} (±0.59)

Within each column, means marked by different superscripts are significantly different (Mann-Whitney U-test: *a:b, a:d, b:c, b:d:* $P<0.05$; *a:c:* $P<0.001$)

scraping (c) was significantly ($P<0.05$) higher than those obtained by squeezing (b) or enzymatic digestion (d). The rinsing technique (a) yielded the lowest amount of cells ($P<0.001$) but the greatest proportion of viable cells (95% on average). Cell viability was significantly ($P<0.001$) lower with all other procedures. Taking these findings together, the greatest number of viable cells per oviduct was obtained using the scraping technique ($P<0.05$ as compared to the rinsing and squeezing techniques; $P=0.06$ as compared to enzymatic digestion). Immunocytochemistry for cytokeratin filaments detected more than 95% epithelial cells for all recovery methods on days 2, 6 (data not shown) and 8 of in vitro culture (Fig. 1).

In primary culture, epithelial cells recovered by techniques b, c and d showed similar characteristics. The fresh cell suspensions showed ciliated and non-ciliated cells. Isolated cell clusters of about 20–100 cells attached to the culture sheet surface began to proliferate and formed small colonies within the first 48 h after seeding. The

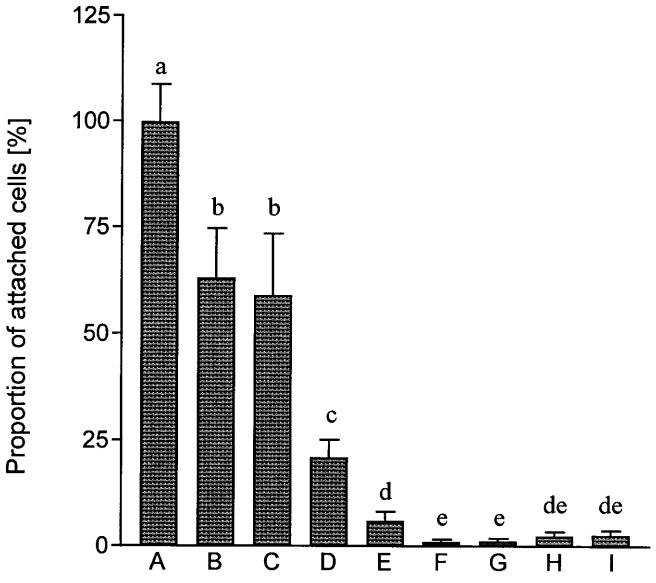


Fig. 2 Cell attachment rates on different support materials: *A* Thermanox, *B* glass, *C* cellulose nitrate 0.45 µm, *D* cellulose nitrate 0.2 µm, *E* nylon, *F* gauze, *G* nucleopore, *H* polycarbonate black, *I* polycarbonate white. Attachment rates on the different support materials are shown as a proportion of the mean number of cells attached on Thermanox. For three independent experiments (n=3), the mean number of cells on Thermanox (423, 2033 and 445/mm^2, respectively) was defined as 100%. The deviation of the two cell support sheets evaluated per experiment as described in "Materials and methods" was transformed to percentage deviation from the mean and used to calculate the standard error for Thermanox. The *bars* and *error bars* for the other materials represent means and standard errors of relative attachment rates. Means were compared using the Mann-Whitney U-test. Significant differences are indicated by different superscripts: *a:b, b:c, c:d, c:e, d:e:* $P<0.05$; *a:c, a:d, a:e, b:d, b:e:* $P<0.001$

monolayer reached confluence between days 4 and 5 and formed domes at day 7. Non-attached cells organised spherical floating aggregates, without losing their beating cilia up to 10 days. The cells isolated by rinsing (a) formed only small cell clusters and the monolayer became confluent as early as days 8–10 and created domes after day 12. There were no obvious differences in cell morphology between the isolation techniques. Ciliated cells could be commonly detected up to days 4 or 5 after seeding, but were rarely seen later in the culture period.

Effects of different cell support materials

The attachment rate of oviduct epithelial cells on nine different support materials was evaluated by propidium iodide staining (Fig. 2). It was highest on Thermanox, followed by glass and cellulose nitrate (pore size 0.45 µm), and was significantly ($P<0.05$) lower on all other support materials tested. The three most suitable materials were then tested for their ability to support oviduct epithelial cell growth under perfusion culture conditions. The morphology of the cells evaluated by scanning electron microscopy after 8 days of culture showed clear differences between the different materials. Cells grown on

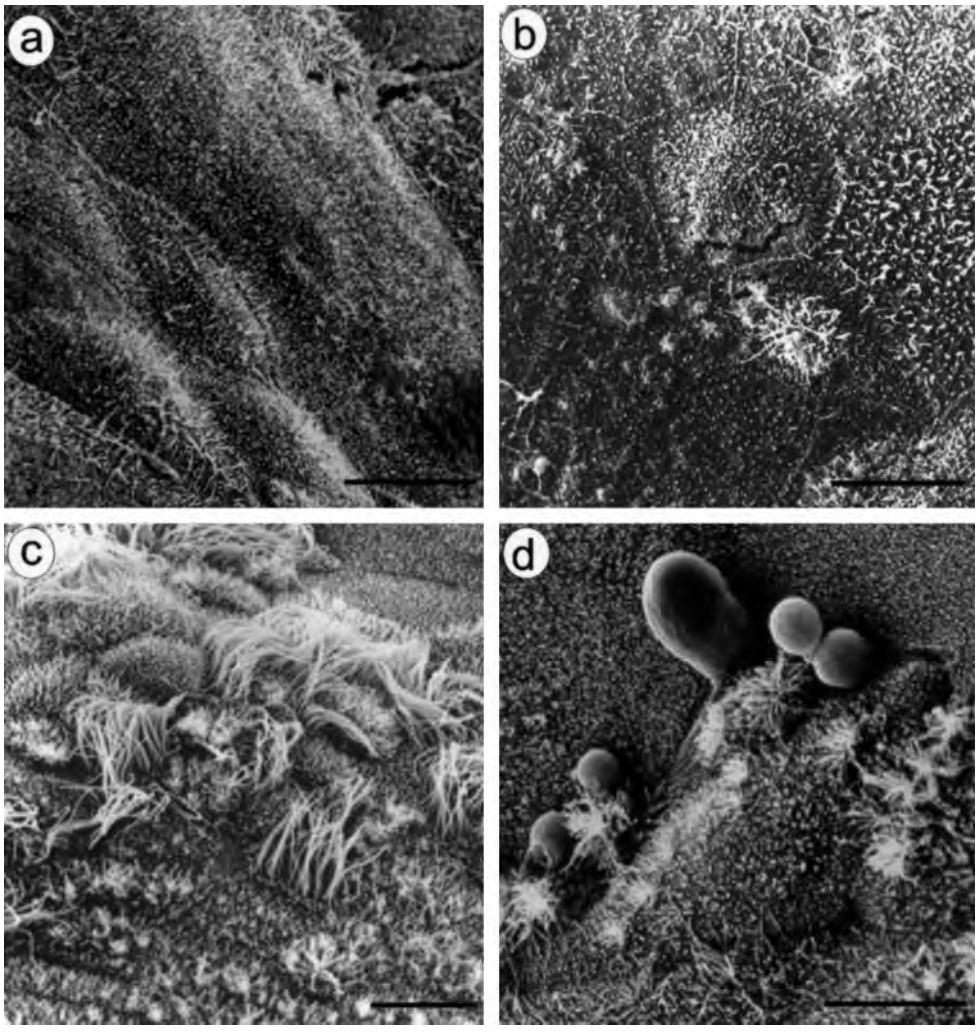


Fig. 3a–d Representative scanning electron microscopy images of bovine oviduct cell monolayers after 8 days culture in the perfusion system on different support materials. **a** Cells cultured on glass showing only microvilli; **b** cells grown on Thermanox with only short microvilli; **c** cells cultured on cellulose nitrate (pore size 0.45 µm) presenting microvilli and cilia; **d** cells grown on cellulose nitrate forming bulbous protrusions and covered by cilia. *Bars* 10 µm **(a–d)**

glass or Thermanox had formed confluent monolayers, whereas on cellulose nitrate they had only reached 70% confluence. On glass (Fig. 3a) or Thermanox (Fig. 3b) cells were flat and spindle-shaped and showed only rare cilia and no bulbous protrusions. By contrast, on cellulose nitrate the complete oviduct cell monolayer consisted of polygonal, dome-shaped cells closely connected, was covered by a dense layer of microvilli, while discrete areas with numerous cilia (Fig. 3c) were frequently seen. In addition, some secretory cells showed bulbous protrusions (Fig. 3d), characteristic of secretory activity.

Comparison of static vs perfusion culture system with respect to cell morphology

Scanning electron microscopy revealed only few differences in morphology between the static and the perfusion culture system in the first 6 days of culture. The cells grown on cellulose nitrate in both culture systems were polygonal, covered with microvilli and several cilia, and secretory cells showed some bulbous protrusions. At day 8 the differences became more obvious. While only a dense layer of microvilli and very few cilia were obvious on the surface of the cells cultured in the static culture system (Fig. 4a), cilia and bulbous protrusions were still present in the perfusion culture system (Fig. 4b–d) in distinct small regions. Beside areas with typical long cilia there were also cells present which were covered by cilia of different sizes (Fig. 4c), implicating ciliogenesis.

Transmission electron microscopy revealed ciliary and secretory epithelial cells which appeared morphologically intact or displayed only minor structural changes right after isolation by scraping (Fig. 5a). In the secretory cells the characteristic concentric lamellar granules were well maintained (Fig. 5b). Between neighbouring epithelial cells, junctional complexes appeared fully preserved (Fig. 5c). In a small number of cells some structural alterations such as dilation of the endoplasmic reticulum and mitochondrial damage occurred.

After 2 days pre-culture under static conditions the epithelial height was markedly reduced. The number of cilia was diminished and the cells showed only few small microvilli on their apical surface. The supranu-

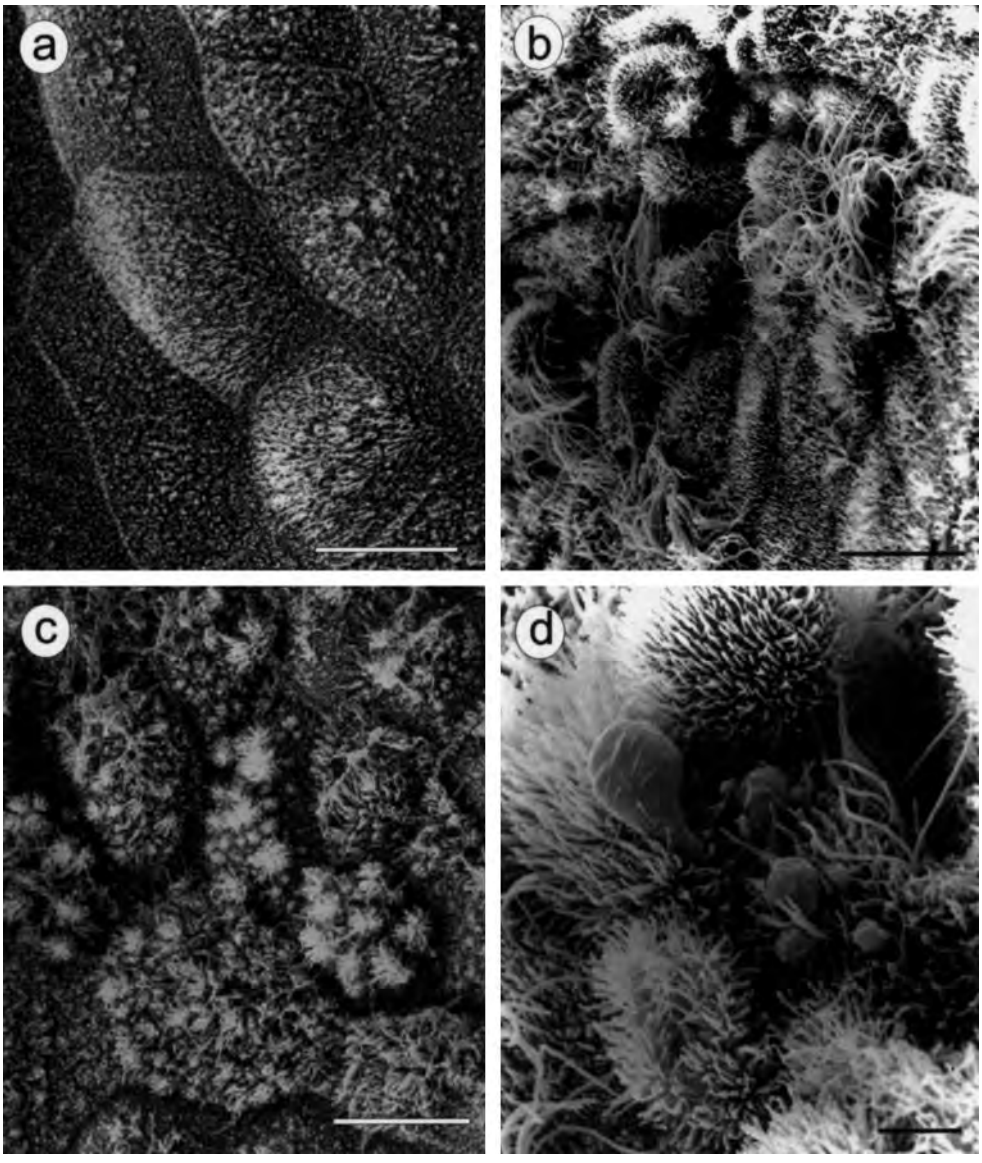


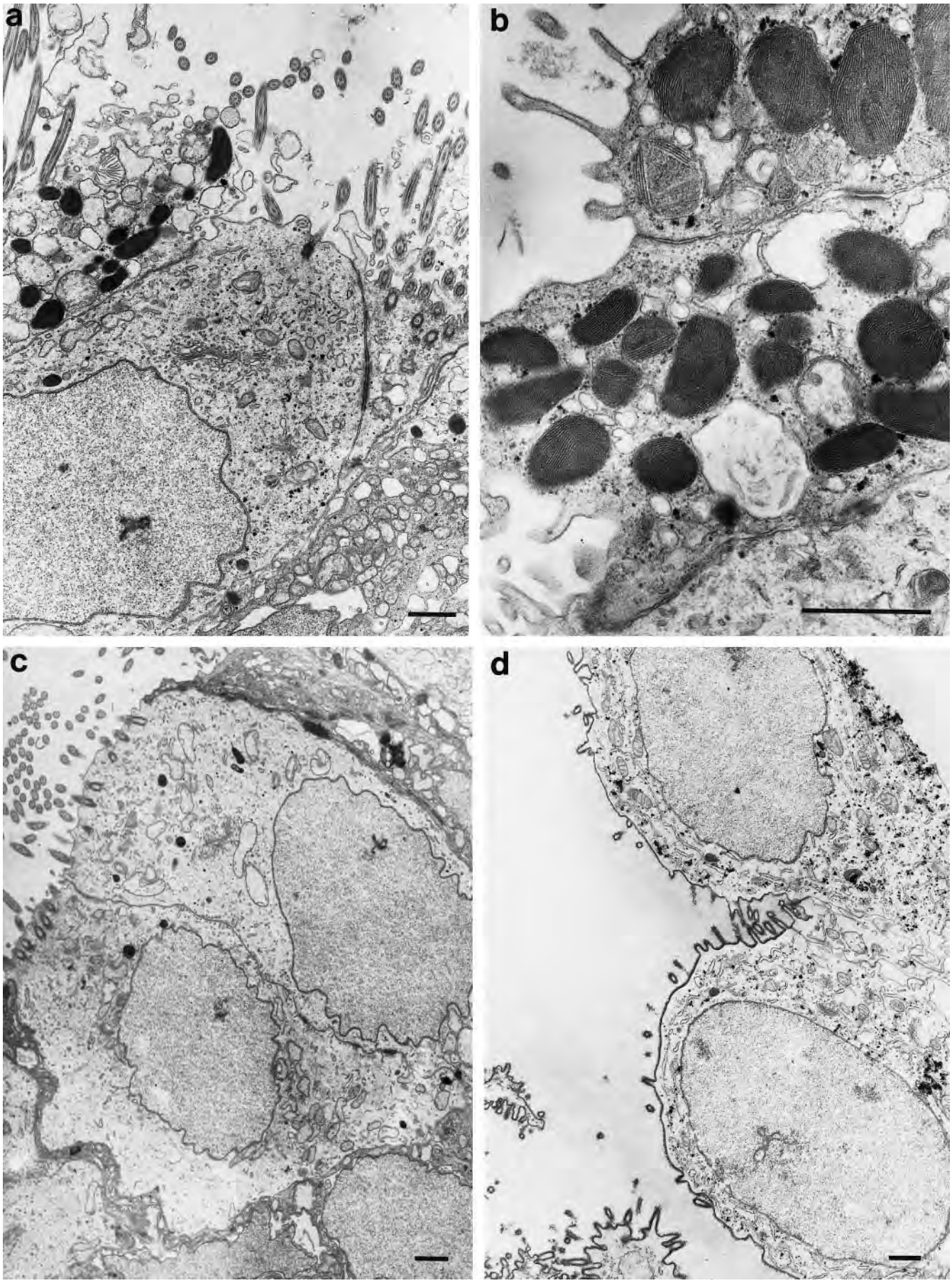
Fig. 4 Typical scanning electron microscopy images of bovine oviduct cell monolayers on cellulose nitrate after 8 days culture in **a** a static culture system. Cells showed microvilli and dense cell junctions. **b–d** A perfusion culture system; cell monolayer with microvilli and cilia; **c** cells with growing cilia of different length; **d** cells forming bulbous protrusions. *Bars* 10 µm **(a–c)**, 4 µm **(d)**

clear area was small and most of the cellular cytoplasm was occupied by the large ovoid nucleus. The amount of perinuclear glycogen had considerably increased (Fig. 5d).

On day 4 the epithelial cells cultured under both conditions did not display distinct morphological changes compared to their appearance in pre-culture. The nuclei still occupied a significant part of the cytoplasm, the supranuclear area was small and the apical surface was mainly invested with microvilli, which appeared more pronounced in cells cultured in the perfusion chamber. Only few cilia-bearing cells were found in both culture systems, which in some cases contained glycogen in their basal rootlets (Fig. 6a,b). On their basal aspect the epithelial cells extended with well-developed lobular processes into the cellulose nitrate support. At day 6 in both systems many cells displayed aggregations of centrioles in the cytoplasm, indicating the onset of intense ciliogenesis, which, however, was more obvious in perfusion culture (Fig. 6c,d).

At day 8 cells cultured in Petri dishes frequently displayed wide intercellular spaces. On their apical surface usually irregular microvilli occurred and occasionally cilia were also seen. In many cells large accumulations of glycogen were located around the nucleus (Fig. 7a). Oviductal cells cultured under perfusion conditions showed a more regular arrangement and cellular height

Fig. 5a–d Characteristic transmission electron microscopy images of bovine oviduct cells after isolation by scraping. **a** Oviduct epithelial cells with characteristic cilia and secretory granules; **b** secretory oviduct epithelial cells with concentric lamellar secretory granules, junctional complexes and dilated rough endoplasmic reticulum (rER); **c** ciliated oviduct epithelial cells showing cilia, junctional complexes and Golgi apparatus; **d** after 2 days pre-culture. Flat cells with a dominant nucleus, a few short microvilli and beginning accumulation of glycogen. *Bars* 1 µm **(a–d)**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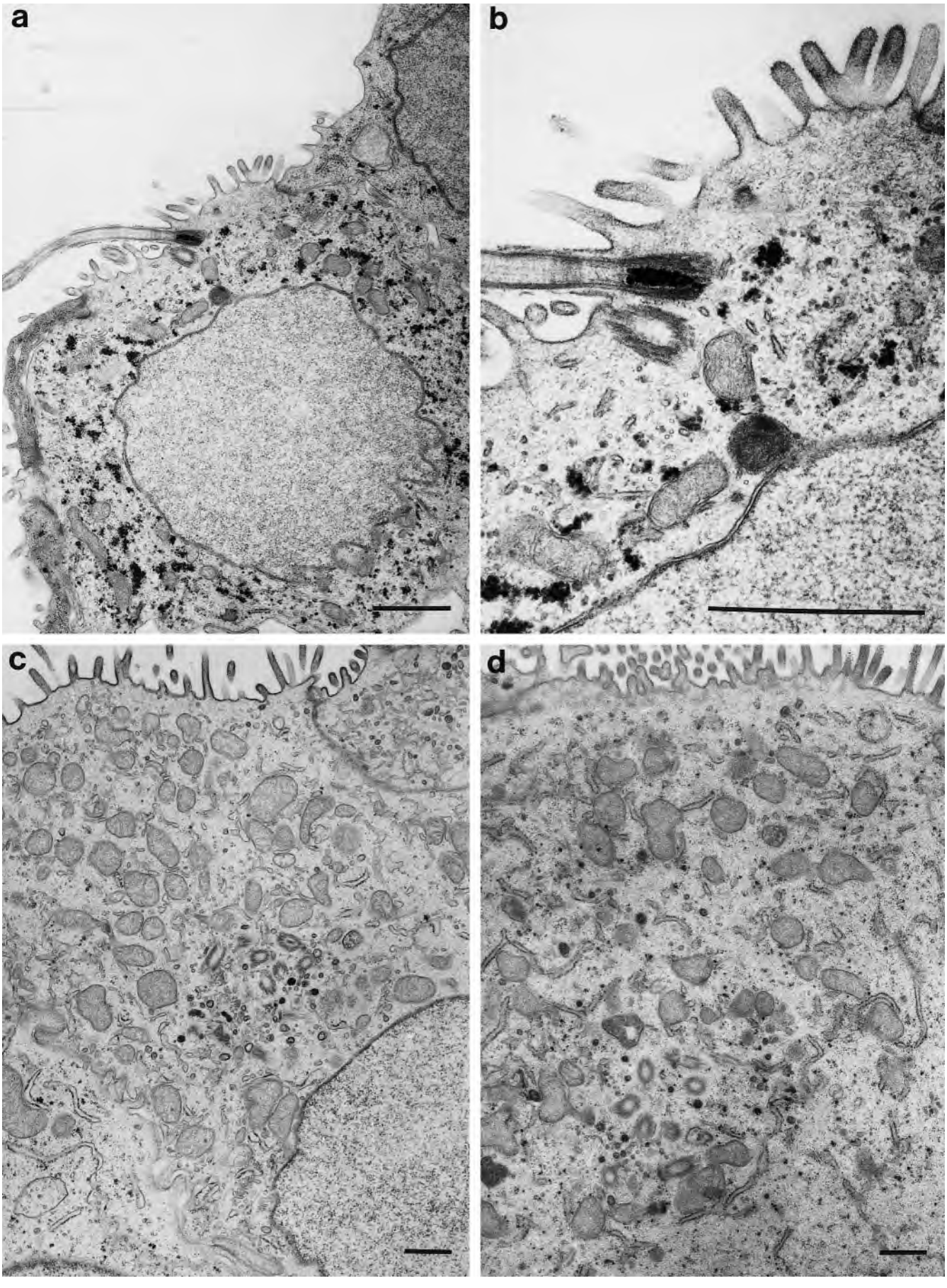
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had increased (Fig. 7b) due to an enlargement of the supranuclear cytoplasm containing numerous mitochondria, well-developed rough endoplasmic reticulum, Golgi cisternae and a few secretory granules with typical multilamellar appearance. Intercellular spaces were usually less dilated and neighbouring apical plasma membranes were sealed by small but distinct junctional complexes (Fig. 7b,c).

Gene expression of oviduct cells in perfusion vs static culture system

The mRNA expression of oestrus-associated glycoprotein 85–97 kDa in the different culture systems in respect to the culture period is shown in Fig. 8. The RNA levels in cultured cells were significantly reduced ($P<0.05$) compared to freshly isolated as well as pre-cultured cells throughout the subsequent culture period. Comparing both systems at days 6 and 8, the expression of GP 85–97 was significantly ($P<0.05$) higher in perfusion than in static culture.

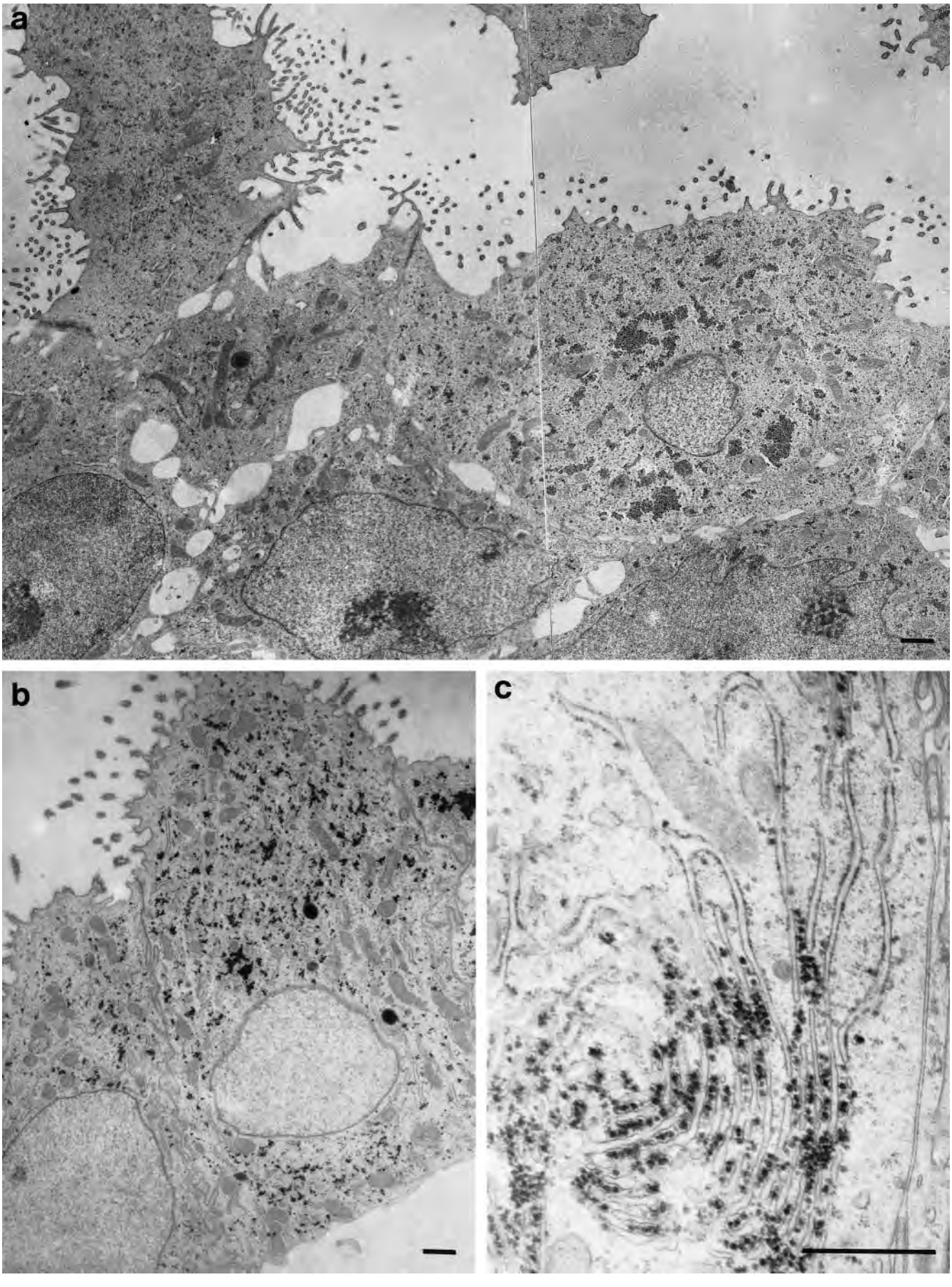
Discussion

In this study we tried to establish a suitable method for isolating viable bovine oviduct epithelial cells and to set up a culture system maintaining these cells in a manner close to the in vivo situation. Until now only few studies have compared different isolation techniques for bovine oviduct cells (Walter 1995; Abe and Hoshi 1997). Here we tested four common isolation techniques with respect to their practicability, cell yield and cellular characteristics. The mechanical techniques (rinsing, squeezing and scraping) require less time and material, are easy to perform and minimise cell damage. The highest yield of viable cells obtained by scraping enables experiments to be carried out on a confluent monolayer of oviduct epithelial cells within 5 days. The rinsing technique represents the less harmful isolation method, but the amount of harvested cells is not sufficient for cell culture experiments. Enzymatic digestion gives a high yield of oviduct cells, but is rather time consuming. Compared to our results, Thibodeaux et al. (1991) recovered more cells per oviduct using this technique, but cell viability tended to be reduced (68–87% vs 84% in our study), probably due to a harsher treatment of the cells. In our study more than 95% of the isolated cells were of epithelial origin as shown by immunostaining for cytokeratins. This proportion is in line with findings of other studies (Thibodeaux et al. 1991; van Langendonckt et al. 1995; Walter 1995). In respect to cell viability and cell yield the scraping technique is the most suitable method for isolation of bovine oviduct epithelial cells. The proportion of epithelial cells did not change during in vitro culture in the different systems as evaluated by cytokeratin staining at days 2, 6 and 8.

In addition, we compared permeable and non-permeable cell support materials first with respect to the attachment rate. The adhesion of oviduct cells to the cell support is complicated by moving cilia. This necessitates the 2 days pre-culture period, during which the cells attach to the support material, but are already subjected to culture-dependent alterations. In our study attachment rates were highest on two non-permeable materials, Thermanox and glass, and one permeable membrane, cellulose nitrate. It has previously been demonstrated that the cell support material has a great effect on the differentiation status of cultured epithelial cells (Minuth et al. 1994; Reischl et al. 1997). Various polar epithelial cell types, for example alveolar epithelial cells (Kim et al. 1992), epididymal epithelial cells (Klinefelter 1992) or Madin-Darby canine kidney cells (Cook et al. 1989), maintain more of their morphological features when cultured on permeable membranes. Cox and Leese (1997) demonstrated that confluent bovine oviduct epithelial cells cultured on a permeable membrane form a firm barrier and facilitate selective transport of medium components through the cell layer. The permeable membranes allow medium contact at the apical as well as at the basal side and may maintain cells in a more polar structure. In our experiments bovine oviduct cells stayed much more differentiated on porous cellulose nitrate, resembling the oviduct epithelium in vivo more closely than cells grown on non-permeable materials. These findings are in line with results of Gómez and Uría (1997), who achieved higher blastocyst rates in medium conditioned by bovine oviduct cells grown on a permeable membrane than in plastic petri dishes.

In further experiments we used cellulose nitrate to compare effects of static vs perfusion culture on the differentiation status of bovine oviduct epithelial cells. There were clear morphological differences evaluated by scanning and transmission electron microscopy, revealing a beneficial effect of the perfusion system. The polarity of the epithelial cells as well as oviduct-specific features such as cilia and bulbous protrusions resembling a sort of apocrine secretory activity (Nayak and Ellington 1977; Hollis et al. 1984) could be maintained in circumscribed areas for a prolonged period in perfusion culture, whereas these characteristics were almost lost during the static culture. To evaluate the physiological activity of the cells, we studied the mRNA expression of the oviduct-specific GP 85–97. This glycoprotein probably plays a role in sperm capacitation and fertilization (King et al 1994; Abe et al. 1995b; Nancarrow and Hill 1995). The mRNA expression of GP 85–97 was significantly higher in cells cultured in the perfusion system

Fig. 6a–d Representative transmission electron microscopy images of bovine oviduct cells. **a,b** After 4 days culture under static conditions. Cells with microvilli and cilia containing glycogen in their basal rootlets and also glycogen accumulation in the cytoplasm. **c,d** After 6 days culture with aggregations of centrioles in the apical part of the cytoplasm. The oviduct cells show abundant mitochondria, rER and microvilli on their surface. **c** In the perfusion system. **d** In the static system. *Bars* 1 µm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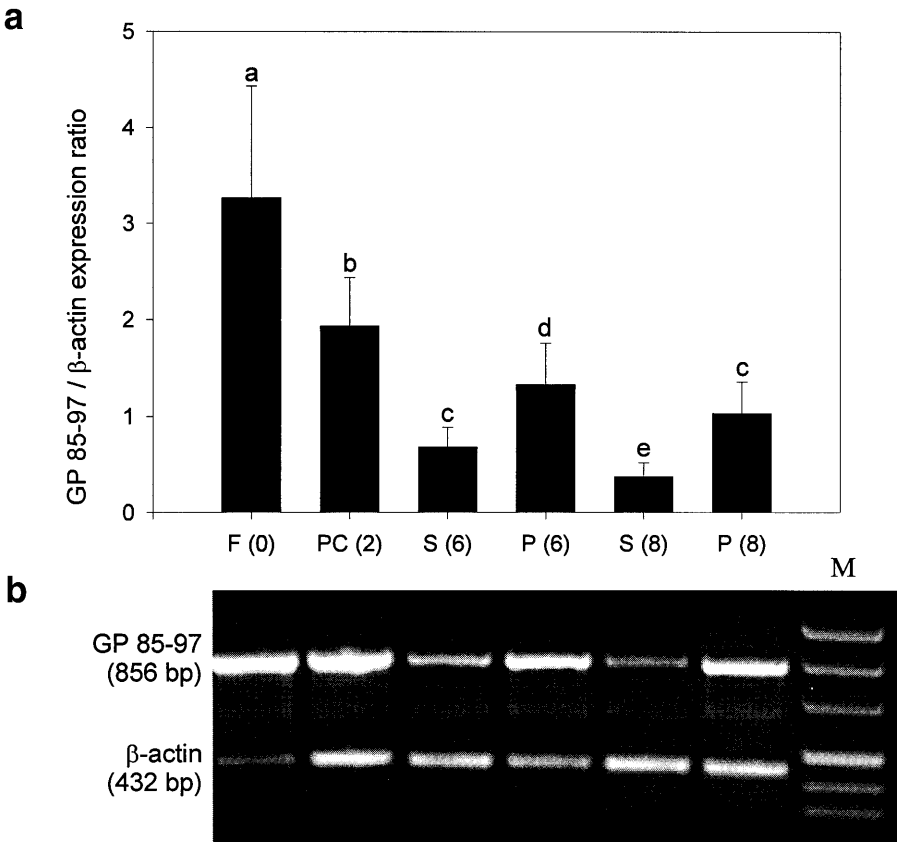

Fig. 8a, b Semi-quantitative RT-PCR analysis of oviduct-specific glycoprotein 85–97 kDa mRNA expression. **a** GP 85–97 kDa/β-actin expression ratio in the perfusion (*P*) vs static (*S*) culture system during culture period (*F(0)* freshly isolated cells, *PC(2)* cells after pre-culture, *S(6)*, *S(8)* cells cultured under static conditions for 6 and 8 days, respectively, *P(6)*, *P(8)* cells cultured under perfusion conditions for 6 and 8 days, respectively). The figure shows means and standard errors from six independent experiments (n=6). Means were compared using the Wilcoxon test for paired samples. Means marked by different superscripts are significantly different: *a:b, a:c, a:d, b:c, b:d, b:e:* $P<0.05$; *a:e:* $P<0.001$). **b** RT-PCR products from bovine oviduct epithelial cells for GP 85–97 kDa and β-actin from a representative experiment (*M* marker)

compared to those cultured in Petri dishes. These differences suggest a better maintenance of morphological and physiological features of oviduct epithelial cells cultured in the perfusion system. Dedifferentiation processes of oviduct epithelial cells in static culture, which were shown by scanning electron microscopy and transmission electron microscopy, were associated with a down-regulation of GP 85–97 expression. Future experiments using immunohistochemistry should confirm these changes on the protein level. Nevertheless, the proportion of epithelial cells as determined by cytokeratin staining did not change during prolonged culture in the static or perfusion systems. The positive effects of perfusion culture are most likely due to the medium flow resulting in a continuous supply of fresh medium and removal of toxic metabolites. Another factor may be the reduced exposure time of medium components to incubator temperatures, which may prevent labile components in the medium from degeneration (Thompson 1996). Finally, mechanical factors, i.e. vertical vs horizontal position of the cell supports, or medium flow vs static medium, may also play a role.

In conclusion, these data show the advantages of the perfusion over the static system for primary culture of bovine oviduct epithelial cells. Therefore, our culture system provides an important model for the study of oviduct physiology, specifically interactions of oviduct cells with gametes and embryos.

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Fig. 7a–c Transmission electron microscopy images of bovine oviduct cells. **a** After 8 days culture in the static system. Cells show intercellular spaces and large accumulations of glycogen in the cytoplasm. **b** After 8 days culture in the perfusion system with increased cell height, tight cell connections with junctional complexes, secretory granules and numerous microvilli on the surface. **c** Abundant ribosomes, rER, Golgi apparatus and mitochondria in the cytoplasm of a cell cultured 8 days in the perfusion system. *Bars* 1 μm **(a–c)**

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