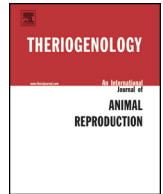




ELSEVIER

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Long-term culture of primary porcine oviduct epithelial cells: Validation of a comprehensive *in vitro* model for reproductive science

Shuai Chen, Ralf Einspanier, Jennifer Schoen*

Institute of Veterinary Biochemistry, Department of Veterinary Medicine, Freie Universitaet Berlin, Oertzenweg19b, 14163 Berlin, Germany

ARTICLE INFO

Article history:

Received 29 April 2013

Received in revised form 17 July 2013

Accepted 22 July 2013

Keywords:

Pig

Oviduct epithelium

Standardized culture system

Long-term culture

ABSTRACT

Recently, we established a protocol for the cultivation of primary porcine oviduct epithelial cells (POEC), which promoted tissue-like morphology for a prolonged culture period. The present study focuses on developing this model into a comprehensive, standardized culture system, as a candidate tool for reproductive toxicity testing and basic research. We cultivated POEC isolated from 25 animals in our culture system for both 3 and 6 weeks and systematically analyzed effects of medium conditioning, supplementation with standardized sera, and culture duration in both freshly isolated and cryopreserved cells. The differentiation status was evaluated via histomorphometry, transepithelial electrical resistance (TEER) measurement, and expression analyses. The culture system possessed high reproducibility, more than 95% of cultures achieved a fully differentiated phenotype. Cells recapitulated *in vivo*-like morphology and ultrastructure from 3 to 6 weeks. Cryopreservation of the cells prior to cultivation did not affect culture quality of POEC. Employment of conditioned medium ensured optimal promotion of POEC differentiation, and different standardized sera induced fully differentiated phenotypes. Consistent TEER establishment indicated the presence and maintenance of cell type-specific intercellular junctions. The functionality of POEC was proven by consistent mucin secretion and stable expression of selected markers over the whole culture duration. We conclude that POEC are suitable for experiments from 3 weeks up to at least 6 weeks of culture. Therefore, this culture system could be used for *in vitro* estrous cycle simulation and long-term investigation of toxic effects on oviduct epithelium.

© 2013 Elsevier Inc. All rights reserved.

1. Introduction

Standardized and well characterized *in vitro* models of the oviduct epithelium are of interest for two general reasons: 1) to understand molecular mechanisms underlying the maturation and transport of gametes, ovum fertilization, and early embryo development and 2) to provide tools that can be used in the test battery for reproductive toxicity testing especially as the use of experimental animals is anticipated to increase dramatically because of current EU risk assessment policy [1,2].

The oviduct lumen is lined by a simple columnar epithelium consisting of ciliated and secretory cells. *In vitro* models should ideally maintain morphological and functional properties of cells *in vivo*. This is especially true for epithelial cell cultures because in this cell type, cellular polarity is a prerequisite for specific cell functions [3]. Several cultivation setups so far have been employed on oviduct epithelial cultures. Previously established cell lines or primary monolayer cultures were limited to specific applications since bidimensional cultures could not support the maintenance of differentiation and specifically of ciliation [4,5]. Suspension culture of bovine oviduct epithelial cells has demonstrated *in vivo*-like morphology and hormone responsiveness, but cell-specific characteristics could be maintained only for very short periods [6,7].

* Corresponding author. Tel.: +49 (0) 30 450 539419; fax: +49 (0) 30 450 539918.

E-mail address: jennifer.schoen@fu-berlin.de (J. Schoen).

Epithelial cells derived from the human fallopian tube maintained polarity and *in vivo*-like morphology up to 28 days when grown at the air/liquid interface in transwell inserts [8]. However, the lack of healthy tissue sources and ethical issues are limitations of the human oviduct epithelium model.

With regard to anatomy and physiology, pig has close similarities to man and has thus become one of the favored model animals [9]. It has already been used as a model in studies on reproductive biology [10–12]. Therefore, we recently established a protocol for the cultivation of polarized porcine oviduct epithelial cells (POEC) [13]. Culture at the air/liquid interface using medium conditioned by mouse fibroblasts allowed maintenance of cells for extended period. Long-term culture in this model is not only possible, but a precondition for *in vivo*-like morphology and function. Only after 3 weeks (3w) of preculture, cells regained columnar shape and showed hormonal responsiveness.

The aim of the present study was to further characterize and validate this model to provide a standardized, *in vivo*-like POEC culture system as a potential tool for reproductive toxicity testing and basic science. We provide data from high animal numbers (N = 25 animals) and show the effects of culture duration, cryopreservation, medium conditioning, and supplementation with standardized sera on cell morphology during long-term culture. Primary cells cultured for 3 and 6 weeks (6w), respectively, were further compared regarding transepithelial resistance and expression of specific marker genes. We test the hypothesis that POEC cultured under the presented conditions show comparable characteristics from 3w up to 6w of culture, consequently being suitable for extended experiments as estrous cycle simulation or investigations on long-term effects of toxic substances on oviduct epithelium functions.

2. Materials and methods

2.1. Reagents and chemicals

Ham's F12 medium, cell culture antibiotics, fetal bovine serum (FBS) (S0115) and "FBS superior" (S0615) were purchased from Biochrom (Berlin, Germany). "FBS Gold" (A11-251) and Cryo Max S were supplied by PAA (Coelbe, Germany). Other reagents were purchased from Carl Roth (Karlsruhe, Germany) unless otherwise indicated.

2.2. Tissue and sample preparation

Porcine oviducts were obtained from 6-month-old fattening pigs (N = 25 animals, hybrids, German Large White × German Landrace) slaughtered on five different days in the local abattoir (Vion Lausitz GmbH, Germany).

Epithelial cells were isolated from both oviducts following the protocol previously described by our group [13]. Afterwards, part of the separated cells was immediately used for *in vitro* culture (referred to as freshly isolated cells). Remaining cells were cryopreserved in liquid nitrogen for later investigations. For cryopreservation, cells were centrifuged, resuspended in Cryo Max S, cooled down to $-80\text{ }^{\circ}\text{C}$ at the rate of $-1\text{ }^{\circ}\text{C}/\text{min}$, and then transferred to liquid nitrogen for long-term storage.

For histology, we fixed ampulla regions of five additional oviducts (N = 5 animals) in Bouin's solution within 15 minutes after slaughter.

2.3. Long-term culture of POEC

Isolated cells were suspended in growth medium and then seeded at the density of 1.0×10^5 cells/insert onto 24-well hanging inserts (PET, $0.4\text{ }\mu\text{m}$, non-transparent, Millipore, Switzerland). After seeding, cells were initially grown on membranes with 1 mL of culture medium in the basolateral side of each well and 0.2 mL medium inside the insert. To establish the air/liquid interface (access to medium from only basolateral side), medium inside the insert was removed 48 hours after seeding. Cells were maintained in a humidified atmosphere with 5% CO_2 at $38\text{ }^{\circ}\text{C}$ for 3w and 6w. Medium refreshing was performed twice per week.

In the initial seeding of freshly isolated cells, one batch of cells (N = 5 animals) failed to form a confluent cell layer because of operative errors. Therefore, we had 20 animals in total in tests with freshly isolated cells and 25 animals in tests with cryopreserved cells.

Cells were grown under different culture conditions (see the following section). The impact of these culture conditions on cell morphology was assessed by light microscopy and histomorphometry (cellular height measurement).

2.3.1. Media and FBS

2.3.1.1. Conditioned versus unconditioned medium. Ham's F12 supplemented with 10% FBS (S0115), 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, 50 $\mu\text{g}/\text{mL}$ gentamicin, 1 $\mu\text{g}/\text{mL}$ amphotericin B, 10 $\mu\text{g}/\text{mL}$ reduced glutathione (Sigma), and 10 $\mu\text{g}/\text{mL}$ ascorbic acid (Sigma) was termed non-conditioned medium (NM). To generate the 3T3-enriched medium, 3T3 Swiss albino embryo fibroblast cells (ATCC, Manassas, USA) were cultured in Ham's F12 with 10% FBS (S0115) following the protocol reported previously [13]. Afterwards, we diluted the 3T3-enriched medium in NM medium at ratio 1:2 (v/v), and termed it as conditioned culture medium (CM). FBS (S0115) was used for both NM and CM.

Freshly isolated cells were cultivated for 3w and 6w using either CM (N = 20 animals) or NM (N = 10 animals) as growth medium.

2.3.1.2. Impact of different sera supplements. To test the reproducibility of the culture system, we compared two standardized sera, FBS Superior and FBS Gold, which are assumed to have low batch-to-batch difference. The conditioned growth medium consisting of FBS Superior was termed CM-S, whereas conditioned medium containing FBS Gold was named CM-G.

Thawed cells were grown for 3w and 6w, using either CM-S (N = 10 animals), or CM-G (N = 10 animals) as growth medium.

2.3.2. Cryopreservation

To study whether cryopreservation affects the culture quality of POEC, cryopreserved cells from all 25 animals were thawed and cultured in CM for 3w and 6w.

Based on the results of these comparisons, thawed cells cultured in CM medium were defined as benchmark. All tests described in the next sections (including immunohistochemistry staining, gene expression analysis, secretion of mucosubstances and transepithelial electrical resistance [TEER] measurement) were performed using these benchmark conditions.

2.4. Histology and electron microscopy

After cultivation, cells were harvested for histological investigation. Cells and oviduct tissue were rinsed with PBS, and subsequently immersed in Bouin's solution for 2 hours. Afterwards, samples were transferred to 4% formalin, dehydrated in ascending ethanol series, and then embedded in paraplast. Sections with thickness of 3 μm were prepared. All POEC and tissue samples in this study were routinely stained with hematoxylin and eosin (HE). Additionally, Alcian blue staining pH 2.5 was performed on POEC ($N = 3$ animals) and tissue samples ($N = 3$ animals) for detection of acidic mucosubstances. After HE staining, all samples were examined for morphological criteria, such as cilia density, nuclei shape, and polarity.

2.4.1. Histomorphometry

To measure the cellular height, 3 images were captured in each section. For each image, we measured the height of 5 equidistant spots using AxioVision Rel.4.8 software (Carl Zeiss, Oberkochen, Germany). To assess oviduct epithelium height in the 5 tissue samples, areas of parallel cell growths (no tips of plicae) were located and measured following the same principles (3 pictures per section, 5 measurements per picture).

2.4.2. Immunohistochemistry staining

POEC samples ($N = 3$ animals) as well as tissue samples ($N = 3$ animals) were used for immunohistochemistry. Unspecific binding was blocked by incubation of sections with 10% normal goat serum diluted in 5% BSA for 60 minutes at 37 °C followed by incubation with primary antibodies against cytokeratin, beta-catenin, G protein-coupled estrogen receptor 1 (GPR30), and oviduct glycoprotein 1 (OVGP1) (see Table 1) at 4 °C overnight. Rabbit immunoglobulin G fraction and mouse immunoglobulin G fraction were used for negative controls. After washing 3 times in PBS, bound antibodies were visualized using the EnVision detection kit (K4065, DAKO, Denmark) following the manufacturer's instructions.

2.4.3. Electron microscopy

Samples cultured in CM for 3w and 6w were processed at the Leibniz Institute for Zoo and Wildlife Research as reported previously [13].

2.5. TEER measurement

After 3w or 6w of culture under benchmark conditions ($N = 25$ animals), TEER was measured before processing 24-well samples for histology (EVOM2 Epithelial Voltohmmeter; WPI, Sarasota, FL). To assess the ohmic resistance of the cellular layer, 200 μL of medium was added to the apical compartment of the insert and removed immediately after

the measurement. The unit area resistance value was calculated according to the manufacturer's description.

2.6. Gene expression analysis

Cryopreserved POEC from 8 animals were thawed and plated into 12-well inserts (PET, 0.4 μm , nontransparent, Millipore, Switzerland) at the density of 3.4×10^5 cells/12-well insert. After 3w and 6w of culture, total RNA was extracted using the miRNA Isolation Kit (mirVana, Applied Biosystem). Reverse transcription and quantitative polymerase chain reaction (q-PCR) amplification were carried out as previously described with minor modification [14]. Briefly, 2.5 μg total RNA from each sample was reverse transcribed into complementary DNA in a final volume of 50 μL using RevertAid reverse transcriptase (Fermentas GmbH). The q-PCR reaction consisted of 5 μL SensimixSYBR master mix and 1 μL of diluted complementary DNA (1:10 in H_2O) in a total volume of 10 μL . The cycling program was: one cycle, 10 minutes at 95 °C; 40 cycles, 95 °C for 15 seconds, at corresponding annealing temperature for 20 seconds, followed by 72 °C for 30 seconds. We used serial dilutions of extracted PCR products for preparation of standard curve, and total RNA and water as negative control. The primer sequences and their annealing temperatures are listed in Table 2. Raw PCR data were normalized using the software geNorm by calculating the normalization factor based on a panel of reference genes: actin, beta (ACTB); succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (SDHA); glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.7. Statistics

Results of cellular height measurements were analyzed by two-factor ANOVA. Significant differences in cell height achieved with different media (CM, CM-S, and CM-G) were detected by Tukey's post hoc analysis. To further compare within groups of either 3w or 6w, Student's *t*-test was performed. The significant differences within groups of certain culture periods were marked with Pentastars.

Results of TEER measurements and q-PCR analysis were both analyzed using Student's *t*-test. The software SPSS Statistics 20 for Windows was used for all statistical analyses. We considered $P < 0.05$ as statistical significance.

3. Results

The cell yield from each pair of oviducts was 0.5 to 3.0×10^7 cells, and therefore provided a stock for seeding 50 to

Table 1
Detailed information on first antibodies used for immunohistochemistry staining.

| Proteins | Manufacturer | Antibody dilution |
|--------------|------------------------|--|
| Cytokeratin | Clones AE1/AE3, Dako | 1:50 |
| Beta-catenin | ab6302, Abcam | 1:160,000 |
| GPR30 | sc-48524-R, Santa Cruz | 1:200 for cell cultures; 1:400 for tissue sections; |
| OVGP1 | ab118590, Abcam | 1:200 |

Table 2

Primer sequences and corresponding annealing temperatures for q-PCR analysis.

| Gene symbol | Primer sequence (5'–3') | Fragment size (bp) | Temperature (°C) |
|-------------|---|--------------------|------------------|
| ACTB | Forward: CAACTGGGACGACATGGAG Reverse: GAGTCCATCACGATGCCAG | 234 | 60 |
| ESR1 | Forward: AGGGAAGCTCTGTTTGTCTC Reverse: CGGTGGATATGGTCCTCTCT | 234 | 60 |
| GAPDH | Forward: ATTCCACCCACGGCAAGTTC Reverse: AAGGGGCAGAGATGATGACC | 225 | 60 |
| GPR30 | Forward: GTGCCCCGACCTGTACTTCAT Reverse: AAGTTCATCCAGGTGAGGAA | 182 | 58 |
| MUC1 | Forward: AGCTGATTCTGGCCCTCCAAGACA Reverse: TGGTCAGGTTATAGGTGCGCTGCTT | 96 | 60 |
| MUC16 | Forward: AGTGGCTATGCACCCAGAC Reverse: ACCAGGCAGGAGCGGAATAC | 191 | 60 |
| OVGP1 | Forward: TACTTGAAGAGCTCTGCTTGCCCT Reverse: TCTTCCCAGAAGGCGCACATCATA | 134 | 60 |
| PGR | Forward: TGAGAGCACTAGATGCCGTTGCT Reverse: AGAACTCGAAGTGTGGGTTGGT | 198 | 60 |
| SDHA | Forward: CTACAAGGGCAGGTTCTGA Reverse: AAGACAACGAGGTCCAGGAG | 141 | 60 |

Abbreviations: ACTB, actin, beta; ESR1, estrogen receptor α ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPR30, G protein-coupled estrogen receptor 1; MUC1, mucin 1; MUC16, mucin 16; OVGP1, oviduct glycoprotein 1; PGR, progesterone receptor; SDHA, succinate dehydrogenase complex, subunit A, flavoprotein (Fp); q-PCR, quantitative polymerase chain reaction.

300 inserts (24-well plate). Cultures of 19 of 20 animals in the freshly isolated cell group and 24 of 25 animals in the thawed cell group developed homogenous and fully differentiated phenotypes. Differences in morphology as well as in functional parameters were very low among cultures gained from different animals. Representative pictures of POEC under different culture conditions are presented in Figure 1.

3.1. Morphological investigations

3.1.1. Effect of cryopreservation

Thawed cells exhibited highly differentiated morphology identical to freshly isolated cells (Fig. 1B, C). The statistical analysis showed no significant difference in cellular height between freshly isolated and thawed cells (Fig. 2A), suggesting cryopreservation before seeding does not affect the quality of POEC cultures.

3.1.2. Impact of culture duration on POEC morphology

All freshly isolated POEC established differentiated morphology from 3w up to 6w, including columnar shape, dense cilia, and mixed population of ciliated and secretory cells (Fig. 1A, B). However, the nuclei appearance differed over culture periods: in 3w culture, the nuclei were round, located in the basal site (Fig. 1A); and in 6w culture, nuclei became elongated, localizing in mid and basal positions (Fig. 1B). Besides, statistical analysis indicated a minute but significant effect of culture duration on cellular height ($P < 0.001$ in Fig. 2A, B; $P < 0.01$ in Fig. 2C). The mean cellular height of 6w cultures was similar to the *in vivo* situation ($17.75 \pm 2.0 \mu\text{m}$, $N = 5$ animals, Fig. 1D).

After both 3w and 6w, transmission electron microscopy confirmed the presence of microvilli and kinocilia, which was characterized by the typical 9+2 arrangement of microtubules. Furthermore, a mixed population of ciliated and secretory cells as well as tissue-like polarity was maintained over the whole culture duration (Fig. 3A, B).

3.1.3. Impact of conditioned medium on the quality of POEC

Cells showed polarization and cilia structures in NM medium after 3w and 6w (Fig. 1E). However, there was a significant effect of conditioned medium on cellular height ($P < 0.001$, Fig. 2C). Cultures grown in CM (3w: $14.84 \pm 0.45 \mu\text{m}$; 6w: $17.42 \pm 0.54 \mu\text{m}$) were significantly higher than cultures grown in NM (3w: $12.47 \pm 0.24 \mu\text{m}$; 6w: $15.39 \pm 0.53 \mu\text{m}$) in both culture periods.

3.1.4. Impact of supplementation with different sera

Cells grown in medium supplemented with CM-S and CM-G both achieved fully differentiated status (Fig. 1F, G). All sera groups (CM, CM-S, and CM-G) showed comparable cellular heights in both culture periods (Fig. 2B).

3.2. Functional investigations

3.2.1. Transepithelial electrical resistance (TEER)

TEER measurement was performed in thawed POEC cultured in CM medium for 3w and 6w. The high and consistent TEER values indicated the confluence and barrier formation of cellular layer. Furthermore, statistical analysis showed a small but significant impact of culture duration on cellular electrical resistance ($P < 0.001$, Fig. 2D).

3.2.2. Secretion of mucosubstances

The secretion of acidic mucosubstances in POEC was revealed by Alcian blue pH 2.5 staining. The positive signals were mainly detected in the apical surface as well as in supranuclear cytoplasm of secretory cells. Cells displayed uniformly positive staining after both 3w and 6w, equivalent to the *in vivo* situation (Fig. 4).

3.2.3. Expression of marker genes

q-PCR analysis proved the stable expression of selected steroid hormone receptors and oviduct secretory glycoproteins in POEC cultured in CM from 3w to 6w (Fig. 5).

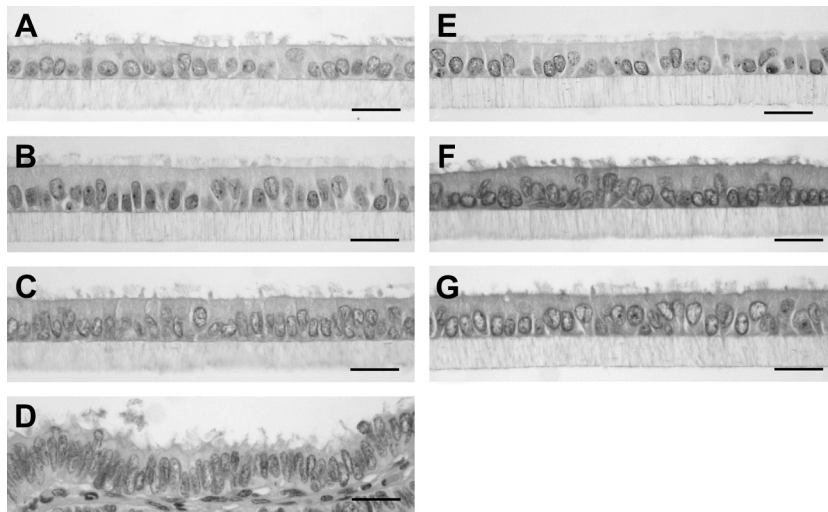


Fig. 1. Morphology of primary porcine oviduct epithelial cells (POEC) during long-term culture. Freshly isolated POEC cultured for 3 (A) and 6 weeks (B) in CM. (C) Thawed POEC cultured for 6 weeks in CM. (E) Freshly isolated POEC cultured in NM for 6 weeks. Thawed POEC cultured in CM-S (F) and CM-G (G) for 6 weeks. (D) Porcine oviduct tissue section. Hematoxylin/eosin staining; magnification $\times 400$. Scale bar = 20 μm . CM, conditioned medium; CM-G, conditioned medium containing FBS Gold; CM-S, conditioned medium containing FBS Superior; NM, nonconditioned medium.

There were no significant differences in the expression of ESR1 (estrogen receptor α), GPR30, OVGP1, MUC1 (mucin 1), and MUC16 (mucin 16) between 3w and 6w of culture. Only the transcript level of PGR (progesterone receptor) was slightly elevated in 6w cultures ($P < 0.05$).

The intense staining of cytokeratin in the cytoplasm of POEC cultures shows the purity of cultured epithelial cells (Fig. 6A, F). Beta-catenin protein was located at the joints of neighboring cells in both POEC culture and

oviduct tissue, which is a typical feature of polarized cells (Fig. 6B, G). GPR30 is an orphan receptor related to noncanonical estrogen actions [15]. It was identified in certain areas of the plasma membrane both *in vitro* and *in vivo* (Fig. 6C, H).

OVGP1, a specific marker for functional oviduct epithelial cells, was stably expressed on RNA and protein level from 3w to 6w. The protein localization of OVGP1 in POEC was comparable to the native porcine oviduct tissue (Fig. 6D, I).

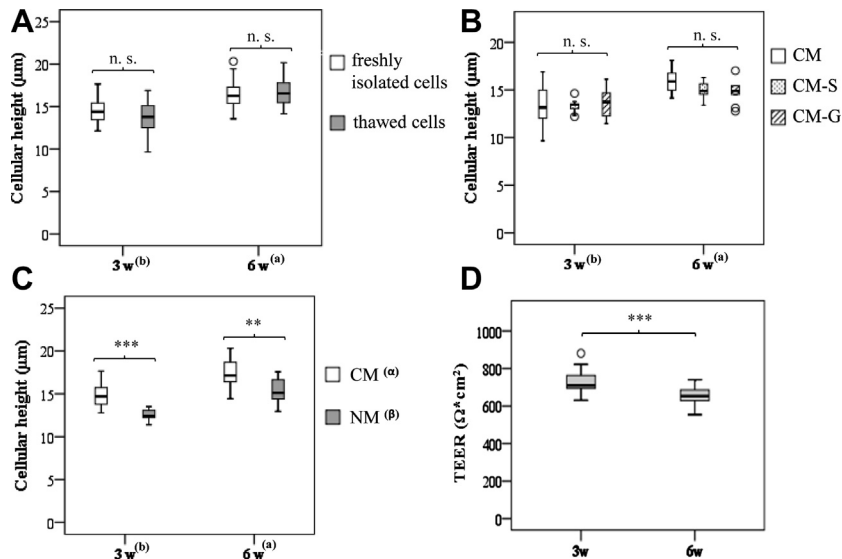


Fig. 2. Comparison of primary porcine oviduct epithelial cell (POEC) culture conditions: epithelium height and TEER measurement. (A–C) Cellular height of POEC grown under different culture conditions. Significant differences between 3 and 6 weeks' culture are indicated with an "a" and "b" following descending order ($a > b$), whereas significant differences between CM and NM are indicated with Greek letters ($\alpha > \beta$). (A) Cellular height of freshly isolated and cryopreserved POEC grown for three and six weeks ($P = 0.576$); significant effect of culture duration ($N = 19$ animals for freshly isolated cells, $N = 24$ animals for thawed cells, $P < 0.001$). (B) Cellular height of cultures grown in media supplemented with different sera ($P = 0.325$, $N = 10$ animals). (C) Cellular height of cultures grown in NM and CM ($N = 10$ animals). (D) TEER values of POEC grown in CM. Significant differences within groups of each culture period are indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. CM, conditioned medium; CM-G, conditioned medium containing FBS Gold; CM-S, conditioned medium containing FBS superior; NM, nonconditioned medium; n.s., no statistical difference; TEER, transepithelial electrical resistance.

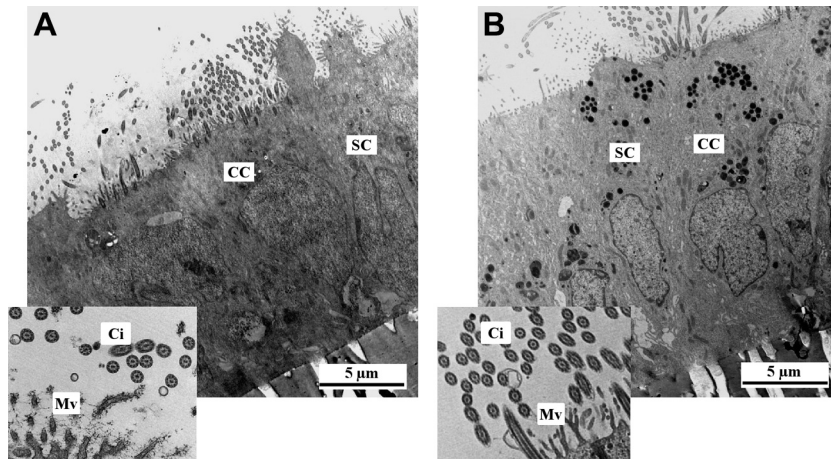


Fig. 3. Transmission electron microscopy of primary porcine oviduct epithelial cells (POEC) cultured for 3 (A) and 6 weeks (B). CC, ciliated cells; Ci, kinocilia; Mv, microvilli; SC, secretory cells.

4. Discussion

Studies introducing basic reproductive science and especially reproductive toxicology depend largely on laboratory animals [1,16]. The provision of tools suitable to investigate local mechanisms of reproduction on a cellular level as well as the demand for replacing animal experiments by *in vitro* toxicity test batteries necessitate the development of standardized *in vitro* models derived from the reproductive tract [17].

The POEC culture system validated in this study has several benefits. First, it is highly reproducible: on condition of faultless cell handling, more than 95% of cultures achieved a highly differentiated phenotype. Furthermore, it represents a long-term culture in strict sense. In our system, cells were maintained for up to 6w, which equals 10 to 15 passages of cell lines. To our knowledge, this

is the most long-lasting culture of primary oviduct epithelial cells reported so far. We deduce that the culture period could be further extended because after 6w of culture, cells exhibited no signs of performance decline. Previous studies have indicated that estrous cycle of porcine spans a period of 18 to 24 days, and the oviduct epithelium undergoes morphological and functional changes during the cycle [18,19]. Thus, our culture system provides the possibility of whole estrous cycle simulations. Above all, compared with previous reports, cells in our system are more relevant to resemble the native tissue because they achieved higher degree of polarization, coherence of population, and *in vivo*-like ultra-architecture over the whole culture duration [18,20].

The cell sources for this culture system are porcine oviducts collected from slaughterhouse, which are easily available in large quantities without consumption of experimental animals. Our dataset is gained from high animal numbers and suggests that, although cells were collected from different individual donors, the resulting long-term cultures were highly consistent under the presented conditions.

The feasibility of long-term storage is crucial for the routine application of cell cultures. So far, investigations on polarized epithelial cultures are mainly based on freshly isolated cells, which are labor-consuming and inflexible in operation time [6,8,21,22]. Therefore, we investigated the possibility of applying cryopreserved cells to our culture system. The morphological and morphometric analyses proved that thawed cells possessed the same morphological appearance and cellular height as freshly isolated cells. Because morphology reflects the functional status, we infer that these cells maintain their differentiation capacity after cryopreservation. The cell yield from each pair of oviducts is sufficiently high to perform a series of experiments. Therefore, following the present procedure, researchers can freeze stocks of cells from the same starting materials. These cells are highly homogeneous and also capable for exchange among laboratories. Furthermore, these cells can be cultured using commercially available standardized sera, proposing high

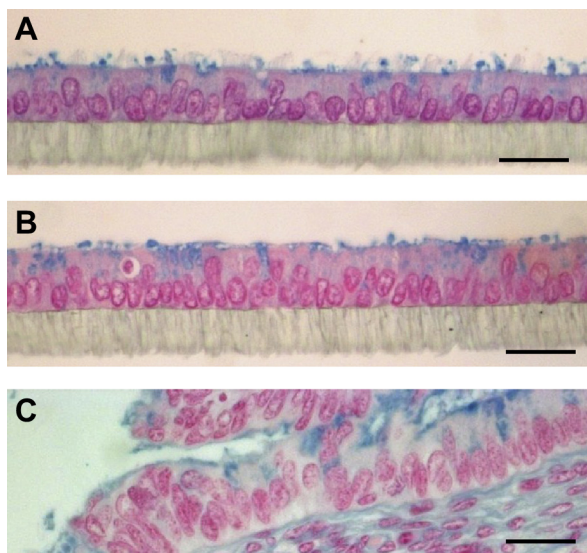


Fig. 4. Alcian blue staining in oviduct tissue (C) and primary porcine oviduct epithelial cells cultured for 3 (A) and 6 weeks (B). Scale bar = 20 μ m.

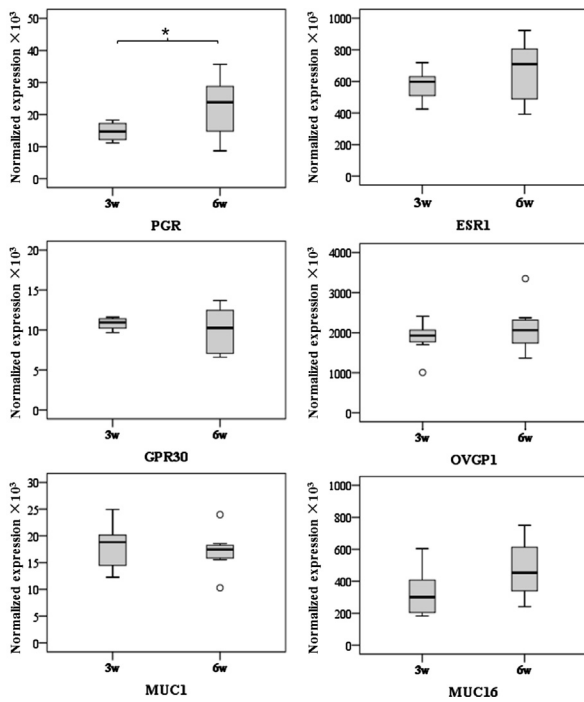


Fig. 5. Normalized gene expression in primary porcine oviduct epithelial cells (POEC) after 3 and 6 weeks of culture. Significant differences are indicated as * $P < 0.05$. $N = 8$ animals.

consistence and low interlaboratory variability of this culture system.

Applying conditioned medium to our culture system is recommended, although not mandatory. Cells cultured in

unconditioned medium also exhibit polarized status. However, the optimal promotion of polarization is achieved only with conditioned medium, which is in agreement with the findings of our previous study [13].

Epithelial cells of the oviduct are reported to synthesize oviduct specific glycoproteins into the lumen fluid, forming the micro-milieu for gametes and embryos [23]. In our study, POEC retained the capacity for continuous mucous substances secretion, which was further confirmed by the expression of mucins produced by oviduct epithelium *in vivo*, including MUC1, MUC16, and OVGP1 [24]. OVGP1 protein plays a significant role in fertilization and embryo development [25]. The stable expression of this marker over the whole culture duration indicates that our culture system retained the embryotrophic capability of native tissue, hence might serve as an ideal tool for cocultures with embryos in IVF/IVC procedures.

Recent studies showed that rather the distal oviduct than the ovarian surface epithelium is the origin for the majority of pelvic serous carcinomas [26–29]. So far there is no effective method to detect serous ovarian cancer in the early stages. Our culture system might serve as an experimental tool to determine pathogenesis of ovarian serous carcinomas and mechanisms of secretory cell transformation. Molecular and carcinogenic pathway studies based on our cell culture model could therefore facilitate the development of novel screening and prevention strategies [30].

We conclude POEC reach a functional state by 3w of culture in air/liquid interface and are usable in experiments up to at least 6w, as shown by homogenous morphology and stable expression of marker genes over this time period. We have established and hereby validated a

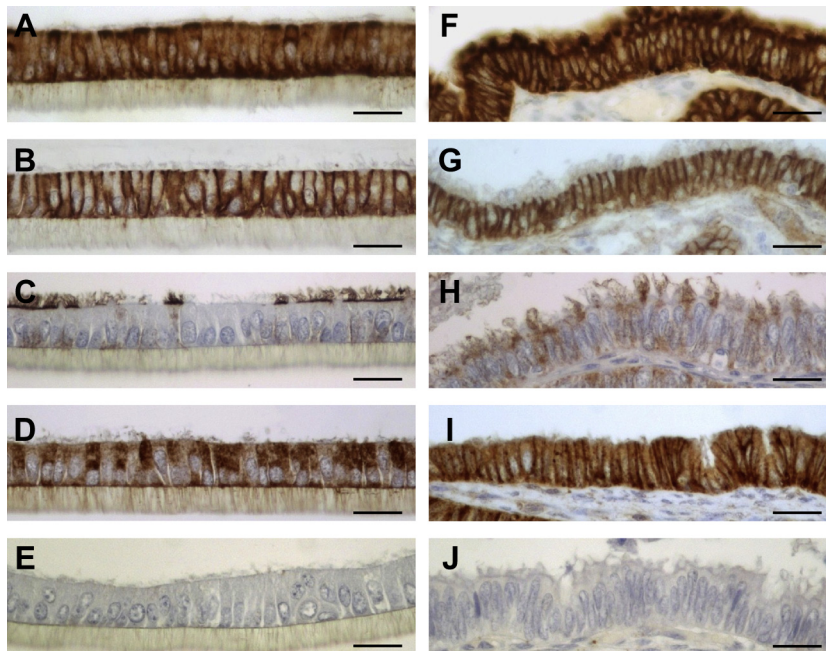


Fig. 6. Expression of cytokeratin (A and F), beta-catenin (B and G), GPR30 (C and H), and OVGP1 (D and I) in primary porcine oviduct epithelial cell (POEC) cultures and oviduct tissue. (E and J) negative control. Left column (A–E): POEC after 6 weeks of culture; right column (F–J): porcine oviduct tissue sections. Scale bar = 20 μm . GPR30, G protein-coupled estrogen receptor 1; OVGP1, oviduct glycoprotein 1.

comprehensive and highly reproducible culture system of POEC, which faithfully maintains morphological and functional properties of the oviduct epithelium *in vivo*.

Acknowledgments

The authors acknowledge Dr. Gudrun Wibbelt and Dagmar Viertel, Leibniz Institute for Zoo and Wildlife Research, Berlin, for assistance in electron microscopy. We are grateful to Lydia Mareen Koeper, Martin Schulze, and Christof Bertram, Department of Veterinary Medicine, Freie Universitaet Berlin, for their lab assistance. Special thanks to Vion Lausitz GmbH, Kasel-Golzig, Germany, for providing tissue samples. This study was supported by the Federal Institute for Risk Assessment (BfR, FK 1329-473), Germany. Shuai Chen was supported by the China Scholarship Council (CSC).

References

- [1] Spielmann H. The way forward in reproductive/developmental toxicity testing. *ATLA* 2009;37:641–56.
- [2] Coy P, Garcia-Vazquez FA, Visconti PE, Aviles M. Roles of the oviduct in mammalian fertilization. *Reproduction* 2012;144:649–60.
- [3] Adissu HA, Asem EK, Lelievre SA. Three-dimensional cell culture to model epithelia in the female reproductive system. *Reprod Sci* 2007;14:11–9.
- [4] Schoen J, Bondzio A, Topp K, Einspanier R. Establishment and characterization of an adherent pure epithelial cell line derived from the bovine oviduct. *Theriogenology* 2008;69:536–45.
- [5] Jung JG, Park TS, Kim JN, Han BK, Lee SD, Song G, et al. Characterization and application of oviductal epithelial cells *in vitro* in *Gallus domesticus*. *Biol Reprod* 2011;85:798–807.
- [6] Rottmayer R, Ulbrich SE, Kolle S, Prella K, Neumueller C, Sinowatz F, et al. A bovine oviduct epithelial cell suspension culture system suitable for studying embryo-maternal interactions: morphological and functional characterization. *Reproduction* 2006;132:637–48.
- [7] Ulbrich SE, Zitta K, Hiendleder S, Wolf E. *In vitro* systems for intercepting early embryo-maternal cross-talk in the bovine oviduct. *Theriogenology* 2010;73:802–16.
- [8] Levanon K, Ng V, Piao HY, Zhang Y, Chang MC, Roh MH, et al. Primary *ex vivo* cultures of human fallopian tube epithelium as a model for serous ovarian carcinogenesis. *Oncogene* 2010;29:1103–13.
- [9] Bendixen E, Danielsen M, Larsen K, Bendixen C. Advances in porcine genomics and proteomics—a toolbox for developing the pig as a model organism for molecular biomedical research. *Brief Funct Genom* 2010;9:208–19.
- [10] D'Cruz OJ, Erbeck D, Uckun FM. A study of the potential of the pig as a model for the vaginal irritancy of benzalkonium chloride in comparison to the nonirritant microbicide PHI-443 and the spermicide vanadocene dithiocarbamate. *Toxicol Pathol* 2005;33:465–76.
- [11] Vodicka P, Smetana Jr K, Dvorankova B, Emerick T, Xu YZ, Ourednik J, et al. The miniature pig as an animal model in biomedical research. *Ann NY Acad Sci* 2005;1049:161–71.
- [12] Mueller A, Siemer J, Renner S, Hoffmann I, Maltaris T, Binder H, et al. Perfused non-pregnant swine uteri: a model for evaluating transport mechanisms to the side bearing the dominant follicle in humans. *J Reprod Develop* 2006;52:617–24.
- [13] Miessen K, Sharbati S, Einspanier R, Schoen J. Modelling the porcine oviduct epithelium: a polarized *in vitro* system suitable for long-term cultivation. *Theriogenology* 2011;76:900–10.
- [14] Scholven J, Taras D, Sharbati S, Schon J, Gabler C, Huber O, et al. Intestinal expression of TFF and related genes during postnatal development in a piglet probiotic trial. *Cell Physiol Biochem* 2009;23:143–56.
- [15] Thomas P, Pang Y, Filardo EJ, Dong J. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology* 2005;146:624–32.
- [16] Lorenzetti S, Altieri I, Arabi S, Balduzzi D, Bechi N, Cordelli E, et al. Innovative non-animal testing strategies for reproductive toxicology: the contribution of Italian partners within the EU project ReProTect. *Ann Ist Super Sanita* 2011;47:429–44.
- [17] Brown ES, Jacobs A, Fitzpatrick S. Reproductive and developmental toxicity testing: from *in vivo* to *in vitro*. *ALTEX* 2012;29:333–9.
- [18] Abe H, Hoshi H. Regional and cyclic variations in the ultrastructural features of secretory cells in the oviductal epithelium of the Chinese Meishan pig. *Reprod Domest Anim* 2007;42:292–8.
- [19] Abe H, Hoshi H. Morphometric and ultrastructural changes in ciliated cells of the oviductal epithelium in prolific Chinese Meishan and Large White pigs during the oestrous cycle. *Reprod Domest Anim* 2008;43:66–73.
- [20] Yaniz JL, Lopez-Gatius F, Hunter RH. Scanning electron microscopic study of the functional anatomy of the porcine oviductal mucosa. *Anat Histol Embryol* 2006;35:28–34.
- [21] Rajagopal M, Tollner TL, Finkbeiner WE, Cherr GN, Widdicombe JH. Differentiated structure and function of primary cultures of monkey oviductal epithelium. *In Vitro Cell Dev Biol Anim* 2006;42:248–54.
- [22] Gualtieri R, Mollo V, Braun S, Barbatto V, Fiorentino I, Talevi R. Long-term viability and differentiation of bovine oviductal monolayers: bidimensional versus three-dimensional culture. *Theriogenology* 2012;78:1456–64.
- [23] Killian GJ. Evidence for the role of oviduct secretions in sperm function, fertilization and embryo development. *Anim Reprod Sci* 2004;82-83:141–53.
- [24] Kadam KM, D'Souza SJ, Natraj U. Identification of cellular isoform of oviduct-specific glycoprotein: role in oviduct tissue remodeling? *Cell Tissue Res* 2007;330:545–56.
- [25] Garcia ML, Peiro R, Argente MJ, Merchan M, Folch JM, Blasco A, et al. Investigation of the oviductal glycoprotein 1 (OVGP1) gene associated with embryo survival and development in the rabbit. *J Animal Sci* 2010;88:1597–602.
- [26] Reitsma W, de Bock GH, Oosterwijk JC, Bart J, Hollema H, Mourits MJ. Support of the 'fallopian tube hypothesis' in a prospective series of risk-reducing salpingo-oophorectomy specimens. *Eur J Cancer* 2013;49:132–41.
- [27] Cass I, Holschneider C, Datta N, Barbuto D, Walts AE, Karlan BY. BRCA-mutation-associated fallopian tube carcinoma: a distinct clinical phenotype? *Obstet Gynecol* 2005;106:1327–34.
- [28] Erickson BK, Conner MG, Landen Jr CN. The role of the fallopian tube in the origin of ovarian cancer. *Am J Obstet Gynecol* 2013; April 10.
- [29] Mehrad M, Ning G, Chen EY, Mehra KK, Crum CP. A pathologist's road map to benign, precancerous, and malignant intraepithelial proliferations in the fallopian tube. *Adv Anat Pathol* 2010;17:293–302.
- [30] Crum CP, McKeon FD, Xian W. The oviduct and ovarian cancer: causality, clinical implications, and "targeted prevention." *Clin Obstet Gynecol* 2012;55:24–35.