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In vitro mimicking of estrous cycle stages based on a comprehensive porcine oviduct epithelial cell culture model

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To Yulei

For life or for death, however separated,

To our spouses we pledged our word.

We held their hands; ---

We are to grow old together with them.

— modified from James Legge (1861)

Original text from "The book of Poetry" (in Chinese)

Contents

Abbreviations

BOEC	bovine oviduct epithelial cells
C3	complement component 3
E2	17β-estradiol
ESR1	estrogen receptor 1
EU REACH	European Registration, Evaluation, Authorization and Restriction of Chemical substances
FSH	follicle-stimulating hormone
GPER	G-protein-coupled estrogen receptor 1
HSP90B1	heat shock protein 90kDa beta (Grp94), member 1
LH	luteinizing hormone
MKI67	antigen identified by monoclonal antibody Ki-67
MUC1	mucin 1
MUC16	mucin 16
OVGP1	oviductal glycoprotein 1
PGF	prostaglandin F-2 alpha
PGR	progesterone receptor
POEC	porcine oviduct epithelial cells
P4	progesterone
SOC	high grade serous ovarian carcinoma
TEER	trans-epithelial electrical resistance
UTJ	uterinetubal junction
VCL	curvilinear velocity
VSL	straight line velocity
3T3	Swiss albino embryo fibroblast cells

1. Introduction

In mammals the oviduct is the anatomical structure that links the ovary to the uterine horn. Recent studies have proposed that it is not only a passive conduit, but rather an active reproductive organ with transport and secretion functions, orchestrating actions including storage and transport of gametes, sperm capacitation, fertilization, and early embryo development [1-4].

It is reported that the morphology and functionality of the partly ciliated oviduct epithelium obviously alter during the estrous cycle. However, regulatory mechanisms of main steroid hormones - 17β-estradiol (E2) and progesterone (P4), and correlated cellular events are still not clearly elucidated. Additionally, the demand for cells in defined functional stages necessitates the in vitro mimicking of estrous cycle.

The cilia activity is suggested to play a crucial role in the transit of gametes and early embryo through the oviduct. Still there is little knowledge on the hormonal governing mechanisms and fluid flow patterns, due to technical limits of in vivo studies. Hence novel approaches to monitor ciliary actions are needed within highly differentiated and hormone responsive in vitro models.

1.1. The significance of oviduct epithelium in reproductive biology

1.1.1. Anatomy and structure of oviduct

From the anatomical perspective, the oviduct is divided into four regions: uterinetubal junction (UTJ), isthmus, ampulla and infundibulum (Fig 1). The infundibulum is the trumpet-shaped cranial end of oviduct, which picks up ovulated oocytes by slipping fimbriae over the ovarian surface. It opens into the ampulla, where the fertilization and early embryo development occur. The ampulla takes up more than half length of the tube, with the inner lumen featured by many mucosal folds and dense ciliated cells. Compared to the ampulla, the isthmus is relatively narrow and has fewer mucosal folds. It constitutes proximal one third of the tube and extends to the UTJ, which passes through the uterine wall [5].

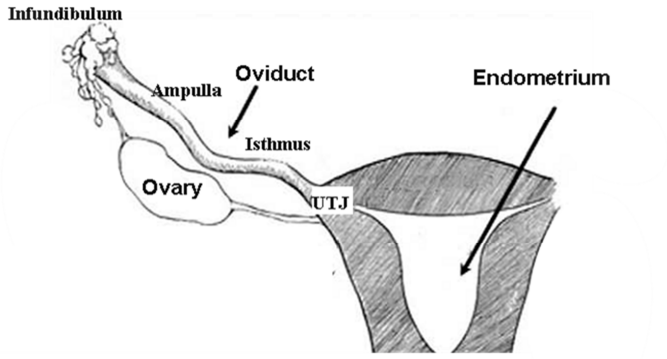

Fig 1. Scheme illustrates the anatomy of the mammalian oviduct, modified from Alexandra et al. 2006 [5].

From the longitudinal view, the oviduct is comprised of three distinct layers: serosa, muscularis and mucosa. The outer serosa layer surrounds and connects the oviduct to mesosalpinx; the muscularis lies under the serosa and is comprised of an inner circular and an outer longitudinal muscular layer. It mainly assists the transport of gametes to the fertilization site by smooth muscle activity [1]; The inner mucosa is the foremost functional site, lined by a monolayer epithelium which consists of two distinct cell populations: secretory cells and ciliated cells.

1.1.2. Oviduct epithelial cells

In the ciliated cells, motile cilia derive from the basal bodies beneath apical surface and extend towards the apical surface to approximately 10μm in length [6]. From cross section views, it is characterized by a typical 9+2 arrangement of microtubules (also called kinocilia), which causes the ciliary movement. Ciliated cells mainly regulate the tubal transport and sperm binding process. Traditionally secretory cells were considered to provide the ideal micro-milieu for fertilization and embryonic development by producing oviduct secretions [7]. However, some recent research suggested that these two types of cells might be the same cell type which adapted to different functional status [8]. To verify this deduction, further in vitro studies on the dynamic transformation between ciliated and secretory cells are needed.

The polarization of oviduct epithelium is crucial for the maintenance of reproductive functionality, such as: favoring embryo development, producing oviduct secretion and promoting spermatozoa binding [9-11]. Moreover, polarized epithelial cells form a physical barrier in the oviduct lumen, taking part in the mucosal immune system to prevent against organisms invasion in the reproductive tract [12]. The polarization and integrity of the epithelium layer relies on presence of tight junctions, which join the membranes of neighboring cells together and prevent lateral diffusion of the apical and basal surfaces [13]. The tight junctions of polarized cells could seal the paracellular spaces and thus block passage of bacterial and viral pathogens as well as selectively transport other molecules [14].

1.1.3. Roles of the oviduct in reproduction

Influence on spermatozoa. After ejaculation, small portions of spermatozoa travel through the UTJ and arrive in the caudal oviduct, where they bind by the rostral region preferably to the ciliated cells of oviduct epithelium. It has been reported that there were more sperm bound to the isthmus than to the ampulla [10]. Sperm-epithelium binding forms a sperm reservoir, which is believed to select sperm with normal acrosome morphology, control polyspermy, as well as to regulate sperm capacitation and hyperactivation [2, 15-17]. Besides, the reservoir could also synchronize the sperm release with time of ovulation [18].

Sperm-epithelium binding seems to be a process regulated by carbohydrate residues [4]. However, the molecules involved in the binding process are not well known yet. Besides, signaling events for sperm capacitation and hyperactivation need to be elucidated in reliable in vitro oviduct epithelium models.

Influence on oocyte maturation. Once ovulated, the cumulus-oocyte complex is transported by cilia beating and spontaneous muscle contractions to the ampulla.

Depending on the species, the cumulus cells expend and disaggregate within few hours in the ampulla [19]. After the expansion process, the zona pellucida is exposed to the oviductal fluid, of which the composition constantly changes according to the stage of estrous cycle [20]. The oocyte seems to be modified by the glycoprotein and sugars present in the surrounding fluid. In sow and cow, oviductal glycoprotein 1 (OVGP1), the unique oviduct secretion protein, has been proven to participate in the modification of the zona pellucida [16].

Influence on early embryo development. The embryotrophic properties of oviductal cells supporting the early embryo are emerging fields of interest. Expression alternations at the genomic and proteomic levels have been demonstrated in oviducts exposed to embryos in vivo and in vitro [21, 22]. Several studies showed that oviduct specific proteins and growth factors present in the oviduct fluid protect the embryo against adverse impacts and support embryo development [3, 23]. Therefore, biotechnicians used oviduct cultures as feeder cells or source of conditioned medium to overcome the developmental block, and boost blastocyst yield and outcomes of embryo production in vitro [24-28].

However, so far most oviductal cells applied in vitro are non-polarized, and deprived of critical characteristics of oviductal cells in vivo. This might account for the reason why co-cultures with none–reproductive tract cells, such as liver cells or kidney epithelial cells showed at least comparable positive effects on embryonic development [29, 30].

1.2. In vitro models of oviduct epithelial cells

As mentioned in the above chapter 1.1.3, to reveal mechanisms of sperm–epithelium interactions and oocyte oviductal maturation, sufficient oviduct epithelial cells in a standardized experimental environment are required. In vitro culture models of oviduct epithelium could also be employed to study the molecular composition of oviductal fluid, and to mimic estrous events in vitro.

1.2.1. Oviduct epithelial cell models for reproductive toxicity testing

Standardized oviduct epithelium model are needed to complement the test battery for reproductive toxicity testing. Due to the EU REACH policy, the use of experimental animals for toxicity safety testing is anticipated to dramatically increase [31]. Thus, the "ReProTect" project engaged on developing new strategies for in vitro reproductive toxicity testing, dividing "reproduction" process into fertility, implantation and prenatal development [32]. In the area of fertility, recent studies have shown that reproductive diseases, including ectopic pregnancy, abortion, ‘immotile cilia syndrome’, disruption of tubal development were linked with adverse effect of endocrine disruptors or toxicants on the oviduct [33-35]. Most of these studies were carried out at large scale in experimental animal models. Standardized, highly repeatable oviduct epithelial cell culture systems could serve as an alternative in the future.

1.2.2. Reported oviduct epithelial cell cultures

In vitro oviduct epithelial cells should meet criteria of oviduct epithelium in vivo as close as possible including: maintenance of two cell types; preservation of ultrastructure, such as cilia and tight junction; cellular polarity; physiologic responses to hormones. During the past decades, a variety of oviduct epithelium cultures have been developed using mainly three different approaches: conventional culture on plastic dishes, suspension culture, and culture on transwell inserts. In the dish cultures, cells have to adapt to apical nutrition supply, which is far from the physiological situation in vivo. Those oviduct epithelial cultures were normally non-polarized, with only secretory type of cells [36-38]. Rottmayer et al. (2006) reported the first suspension culture of bovine oviduct epithelial cells, which not only reflected morphological features as in vivo, but also showed hormone responsiveness [39]. Nonetheless, cells could be maintained only for a short culture period (24h) under such condition. Recently Levanon et al. (2010) reported the transwell insert method in culture of human oviduct epithelial cells, which recapitulated the morphology and ultrastructure of the epithelium for a certain period. However, since cell yield from oviductal specimen was limited, the availability of donors as well as ethical problems restricts the application of this human oviduct epithelium model.

1.2.3. Validation of oviduct epithelial cell models in vitro

Valid in vitro cell models should not only maintain features of in vivo tissues, but also require standardized protocol and optimized culture conditions. Specifically, information on culture medium, sera supplementation, culture duration, and cryopreservation of the starting cell material should be available from large-scale trials. Furthermore, for the widespread use of a cell model, especially to serve as an alternative model, pre-validation steps are needed to assess the in-lab reproducibility [40]. In terms of epithelial models, pre-validation steps might include testing endpoints such as: MTT assay, gene expression, trans-epithelial electrical resistance (TEER) measurement. For example, in the validation of human airway epithelium model, authors systematically compared culture medium, serum gradient, and effect of cryopreservation for varying culture periods, by large scale permeation and TEER testing [41].

In the field of oviduct epithelium, most studies were performed on cells isolated from low animal numbers. Important experimental parameters including different medium compositions, cryopreservation, and culture duration were mainly not considered. In sum, there is no standardized cell model of mammalian oviduct epithelium reported so far.

1.2.4. Pig as a model organism for oviduct epithelial cell cultures

Pig shares many similarities to human in respects of genetics, physiology and anatomy. Recent progress in pig genomics and proteomics further highlights it as a model organism for human biomedical studies [42]. The epithelium physiology, length of sexual cycle and anatomical features of porcine oviduct are comparable to human [43]. Porcine specimens, in contrast, are more easily accessible in large numbers.

In an earlier study, we developed our own protocol to isolate and culture porcine oviduct epithelial cells (POEC) in vitro [44]. After the systematical optimization of workflow a polarized oviduct epithelial culture was developed using the air/liquid interface method. This study was performed based on few individuals. However, to offer a tool for reproductive science as well as for toxicity testing, a highly standardized, comprehensive culture system with consistent performance at both structural and functional endpoints is upon request.

1.3. Porcine oviduct during estrous cycle

The pig is a polyestrous species with an average estrous cycle duration ranging from 18 to 24 days. The estrous cycle mainly consists of two distinct phases: luteal phase (diestrus) and follicular phase (proestrous and estrous), which are accompanied by dramatic hormonal changes during the cycle (Figure 2) [45]. Normally ovulation occurs after 24 to 48 hours of standing heat in sow, which is designated as day zero of the cycle. The ruptured follicular tissue forms corpora lutea, which consistently produce P4 and thus inhibit the level of FSH (follicle-stimulating hormone) and LH (luteinizing hormone). This period is referred to as luteal phase, which lasts approximately 10 to 14 days, and is characterized by functional corpora lutea and high plasma P4 concentration up to 35ng/ml [46]. If the sow is not pregnant at the last days of luteal phase, the uterus release PGF (prostaglandin F-2 alpha) and thus triggers luteolysis. Following the destruction of corpora lutea, the P4 level rapidly decreases whereas secretion of FSH and LH increases. These hormonal alternations stimulate the growth of follicles, which produce increasing amount of E2. This stage of estrous cycle is referred to as follicular phase, which lasts 4-7 days in sow and ends up at ovulation. The follicular phase is dominated by high plasma E2 levels of over 50pg/ml [46].

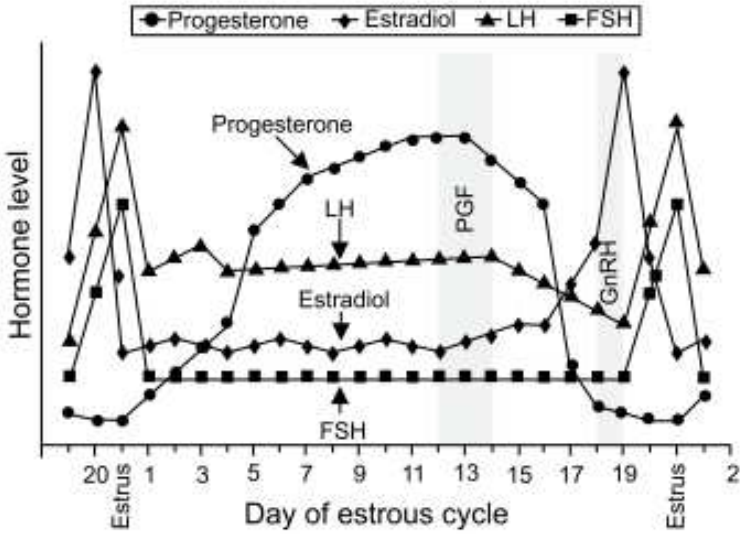

Fig 2. Plasma hormone profiles during the estrous cycle in sow, adapted from Richard et al. 1997 [47].

1.3.1. Steroid hormones and oviduct epithelial cells

Triggered by cyclic changes in circulating steroid hormones, the oviduct epithelium undergoes dramatic morphological variations in epithelial ciliogenesis and cell development. The in vivo studies by Abe et al. on porcine oviduct reported that in the luteal phase, the percentage and cellular height of ciliated cells were dramatically reduced [48, 49]. Secretory cells remain unaffected and thus apical protrusions of secretory were observed. Contrarily, in the follicular phase, ciliated cells underwent ciliogenesis and displayed more plentiful cilia. Numerous secretory granules were observed in the cytoplasm during this stage. Generally, studies on equine and bitch oviduct had similar findings, except for alternation of cellular height in both cell types [50, 51]. In primates comparable cyclic changes were reported during menstrual cycle [52, 53]. In addition to the morphological changes, mRNA and protein expression showed pronounced differences during the estrous cycle [54, 55]. All these findings suggest a role of ovarian-derived steroid hormones - especially E2 and P4 in the cyclic variations. In vitro investigations can be further demanded to reveal the regulatory mechanisms of E2 and P4 at the cellular and molecular level.

1.3.2. Do estrous stages affect sperm binding?

Although estrous stages are known to affect the oviduct epithelium, effects of cycle stages and steroid hormones on sperm binding remain a subject of debate. In rat, sperm binding was observed only in proestrus and estrus, while interaction of E2 and P4 promoted sperm adhesion [56]. In co-culture experiment with oviductal explants in equine, the number of bound sperm was also affected by the stage of estrous cycle, but not by in vitro steroid treatments [57]. Contrarily, studies in pigs and cows showed no effects of estrous cycle stages on sperm binding [58, 59].

1.3.3. Cilia activity throughout the cycle

The tubal transit is a precisely timed event, allowing sperm traveling to the distal end while transporting oocytes and early embryos in the opposite direction [60]. It is indicated that cilia beating rather than muscle contraction plays a leading role for the transport, since inhibition of muscle contractility in the oviduct did not affect the transit time of ovum, but women with ciliary dyskinesia suffer from infertility [61]. However, concerning the cilia beating pattern throughout estrous cycle, there are conflicting reports. Lyons et al. (2002) reported cilia beating frequency of human oviduct changed during menstrual cycle [62]. Consistent with this, Nakahari's group also showed similar regulation of cilia activity by steroid hormones in the guinea pig [63, 64]; however, later studies in knockout mice implied that oviductal transport was independent from cyclic stages [65]. At present there is no agreed conclusion on the cilia beating changes during the cycle. This might be due to variation of employed techniques and difficulties to sufficiently mimic the in vivo situation. Furthermore, although gametes and early embryos are moved within the oviductal fluid, the fluid flow patterns along the epithelial lumen have rarely been investigated so far.

2. Aim of the study

In conclusion, the mammalian oviduct epithelium plays a pivotal role in aspects of gamete transport, oocyte maturation, and development of preimplantation embryos. However, valid in vitro models for the oviduct epithelium are still missing. During the estrous cycle, oviduct epithelium is subjected to dynamic changes which points towards regulation by E2 and P4. Among the cyclic events, there have been conflicting reports on sperm binding and cilia activity during the estrous cycle. Besides, to date no method is available to provide information on fluid movement patterns driven by cilia.

Thus, in this study we aimed to:

1. Validate a culture model of porcine oviduct epithelial cells (POEC). Based on our previously established protocol to isolate and cultivate polarized POEC [44], we focused on developing the first standardized, comprehensive in vitro model of porcine oviduct epithelium. To offer a dossier of validation, data on culture duration, medium supplementation, and cryopreservation of primary cell material was collected from increased numbers of donor animals. Furthermore, to check the characteristic maintenance of in vivo tissue, the model was tested for morphological and functional endpoints including: degree of polarization, ultrastructure, electrophysiology, secretion of mucins, and expression of markers.

2. Mimic the estrous cycle in vitro. After validating the POEC model, we focused on mimicking the estrous cycle stages in POEC, and thus to reveal the regulatory mechanisms of E2 and P4 on cyclic events, such as: cell differentiation, ciliogenesis, and gene expression. Furthermore, the sperm-epithelium binding was investigated during mimicked estrous stages.

3. Monitor cilia beating by fluid movement in POEC. Our last objective was to develop an in vitro approach, which could be used to monitor the cilia activity, as well as to reflect movement patterns of oviduct fluid during the simulated estrous cycle.

3. Subsuming description of published work

The main experiment and discovery of this cumulative dissertation entitled

“In vitro mimicking of estrous cycle stages based on a comprehensive porcine oviduct epithelial culture model”

are in detail reported in two publications:

Publication 1:

Chen S, Einspanier R, Schoen J. Long-term culture of primary porcine oviduct epithelial cells: validation of a comprehensive in vitro model for reproductive science.

Theriogenology. 2013 Nov; 80(8):862-9. doi: 10.1016/j.theriogenology.2013.07.011

Impact factor: 2.082.

Chen S has contributed to the following parts: design and preparation, performing experiments, acquisition and analysis of data, statistical analysis, writing manuscript.

All other authors have contributed to the conception and design of study, directing experimental implementation, data interpretation, analyses discussion, edit and review of the manuscript, approval of the study.

Publication 2:

Chen S, Einspanier R, Schoen J. In vitro mimicking of estrous cycle stages in porcine oviduct epithelium cells: estradiol and progesterone regulate differentiation, gene expression and cellular function.

Biology of Reproduction. 2013 Sep 12; 89(3):54. doi: 10.1095/biolreprod.113.108829

Impact factor: 4.027.

Chen S has contributed to the following parts: design and preparation of study, performing experiments, acquisition and analysis of data, statistical analysis, writing manuscript.

All other authors have contributed to the conception and design of study, directing experimental implementation, data interpretation, analyses discussion, edit and review of the manuscript, approval of the study.

The two publications are attached as appendix at the end of this chapter.

3.1. Long-term culture of primary porcine oviduct epithelial cells: validation of a comprehensive in vitro model for reproductive science

In this work, we developed a comprehensive, standardized POEC culture system to offer an ex-vivo tool for oviduct basic research and reproductive toxicity testing. This lays the foundation for in vitro mimicking of estrous stages investigated in the second publication (chapter 3.2).

Experiments were performed on POEC isolated from 25 donor animals. After isolation from both oviducts, cells were seeded onto hanging inserts, and then cultured in air/liquid interface for either 3w or 6w (Figure 3). Remaining cells were cryopreserved in liquid nitrogen for later investigations. We systematically compared culture conditions, including culture duration, medium conditioning by 3T3 cells (Swiss albino embryo fibroblast cells), sera supplementation, and effects of cryopreservation. Cultures were examined at morphological endpoints by light microscopy, histomorphometry and electron microscopy; and functional endpoints by TEER measurement, q-PCR analysis, alcian blue staining and immunohistochemistry.

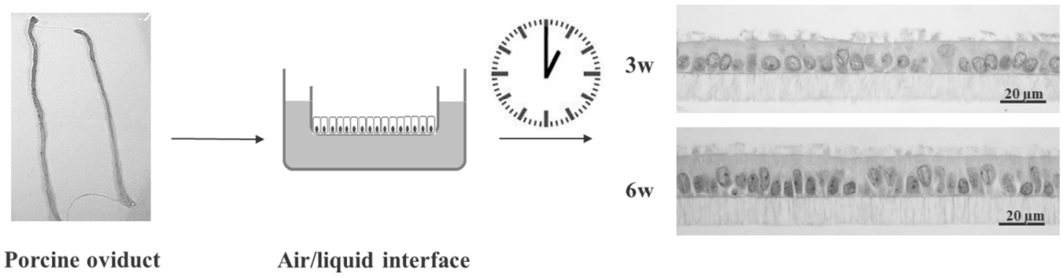

Fig 3. Schematic illustration of the cell culture workflow.

The major findings of this work are:

1. Cultures of 19 out of 20 animals in freshly isolated group, and 24 out of 25 animals in thawed group all exhibited in vivo-like phenotype such as: polarized shape, vigorous cilia, ultrastructure (protrusion, microvilli etc.), and a mixed population of ciliated cells and secretory cells.

2. Cryopreservation of POEC prior to cultivation did not affect culture quality. There is no significant difference on the mean cellular height between thawed cells and freshly isolated cells.

3. Supplementation with 3T3-enriched medium could better promote cell growth. The cellular height in conditioned culture medium (3w: 14.84 ± 0.45µm; 6w: 17.42 ± 0.54µm) were significantly higher than in non-conditioned medium (3w: 12.47 ± 0.24µm; 6w: 15.39 ± 0.53µm).

4. Cells achieved same differentiated phenotype in medium supplemented with different standard sera.

5. Although the cellular height slightly increased from 3w to 6w, the gene expression levels were stable from 3w up to 6w.
6. In our system, cells formed a tight monolayer with barrier functions, as indicated by the high and consistent TEER values.
7. Cells in our system also maintained in vivo-like functions, including mucin secretion and identical immunoprofiles to oviduct tissue.

In sum, we established a solid, highly reproducible POEC system. Under given conditions, cells recapitulated not only the cell composition, but also functionality of oviduct epithelium. This model can be used from 3 up to 6weeks, which opens up the possibility to simulate estrous cycles in vitro.

Additionally, in the process of developing POEC model, we easily and frequently observed vigorous cilia activity in our cultures. This finding inspired us to develop an in vitro method to monitor the cilia beating, which is hardly measurable in vivo (chapter 3.2).

3.2. In vitro mimicking of estrous cycle stages in porcine oviduct epithelium cells: estradiol and progesterone regulate differentiation, gene expression and cellular function

Based on the POEC model validated in chapter 3.1, we focused our study on mimicking estrous stages in vitro, and thus to investigate the regulatory mechanisms of E2 and P4 on cell differentiation, gene expression and cellular function. In this chapter, we also aimed at developing a new approach to monitor the cyclic cilia activity in vitro.

In this study, phenol red (-) Ham's F12 and a serum with low and defined steroids concentration were used for the culture medium. Before stimulation, cells were pre-cultured for either 11d (3w culture) or 31d (6w culture) following the same protocol as described in chapter 3.1. Afterwards, treatment with 35ng/ml P4 and 10pg/ml E2 for 10d resembled diestrus, while sequential treatment with 50pg/ml E2 and 0.5ng/ml P4 for 2.5d resembled estrus. Cells treated with solvent (ethanol) only served as control. Two experiments were executed in this study. In experiment 1 (N = 8 animals), we mimicked diestrus in both 3w and 6w culture; in experiment 2 (N = 6 animals), following the diestrus, estrus (10h / 2.5d) was mimicked in 3w culture. In both experiments, we performed series of analysis including histomorphometry, cell counting, electron microscopy, q-PCR analysis and TEER measurement. Furthermore, to answer whether estrous stages affect sperm binding, we investigated the sperm-epithelium interaction during the estrous simulation in experiment 2. Finally, based on the AndroVision™ software, we tracked the movement of fluorescent beads along the epithelium lining, which was driven by cilia beating (Figure 4).

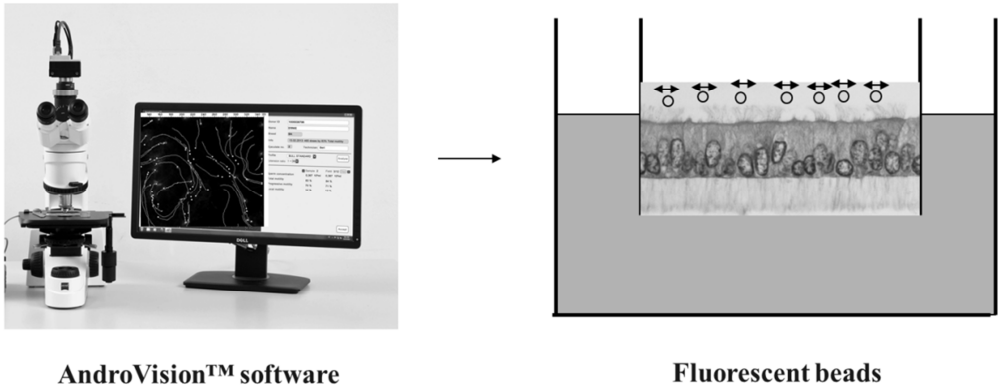

Fig. 4 Demonstration of the new approach for monitoring cilia activity based on fluorescent beads using the AndroVision™ software.

The major findings of chapter 3.2 are:

1. Simulation of estrous stages in our cell model recapitulated the cyclic changes occurring in vivo. Histology and electron microscopy showed that, simulated diestrus (high P4) led to cell regression and atrophy, while simulated estrus (high E2) led to hypertrophy and differentiation of cells.

2. E2 and P4 affect cell polarity and electrical conductivity. In experiment 1, simulated diestrus (P4-domination) caused significant decrease in cellular height (3w: to 60%; 6w: to 61%) but increase in TEER values (3w: to 127%; 6w: to 135%). In experiment 2, again similar changes in cellular height and TEER were observed in diestrus. In the followed estrus, conversely, E2-domination caused significant rise in cellular height (to 133%), but decrease in TEER values (to 84%).

3. E2 and P4 regulate ciliation and transformation of ciliated and secretory cells. There is no significant difference on the total cell number among all groups. Expression of proliferation marker MKI67 (antigen identified by monoclonal antibody Ki-67) was constant during simulation. However, in experiment 1, the proportion of secretory cells in simulated diestrus (3w: 48% of cells; 6w: 43% of cells) was significantly higher than in the control group (3w: 18% of cells; 6w: 15% of cells). In experiment 2, compared with diestrus, the percentage of secretory cells in simulated estrus was significantly decreased (3w: 33% of cells).

4. E2 and P4 caused dynamic changes in gene expression. Simulation of diestrus (high P4) led to significant decrease in expression of selected hormone receptors and oviductal markers, except for C3 (complement component 3). On the contrary, sequential simulated estrus (high E2) caused an increase in expression of most markers. Furthermore, the regulation of OVGP1, PGR (progesterone receptor) and MUC16 (mucin 16) was time-dependent.

5. There are more sperm bound in simulated estrus than in simulated diestrus. Scanning electron microscopy demonstrated that sperm mainly adhered to ciliated cells.

6. The fluid stream over epithelium layer had directional property, flowing along three typical paths. The average bead transport speed varied between different animals: with curvilinear velocity (VCL) ranging from 14.40μm/s to 56.44μm/s, straight line velocity (VSL) ranging from 10.12μm/s to 47.95μm/s. No significant difference was noticed in bead transport speed during simulation of the different groups.

To summarize, initially we mimicked the estrous events in vitro based on the validated POEC model. The regulatory mechanisms of E2 and P4 on cell differentiation were revealed in aspects of polarization, cellular conductivity, ciliogenesis and transformation of cell types. The P4-domination down regulated expression of most investigated genes, while E2-domination (or the lack of P4-domination) reversed the suppression effect.

This study states that estrous stages affect the sperm-epithelium binding, with more bound sperm observed in simulated estrus. The new approach on cilia activity demonstrated the fluid flow patterns along the epithelium lining. The frequent cilia beating drove beads traveling at high speed. However, the cilia activity was not affected by estrous stages.

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

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In Vitro Mimicking of Estrous Cycle Stages in Porcine Oviduct Epithelium Cells: Estradiol and Progesterone Regulate Differentiation, Gene Expression, and Cellular Function[1]

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ABSTRACT

Throughout the estrous cycle the oviduct epithelium undergoes dramatic morphological and functional changes. To elucidate cyclic cellular events and associated regulation mechanisms of 17beta estradiol (E2) and progesterone (P4), we mimicked estrous cycle stages in vitro using a culture system of primary porcine oviduct epithelium cells (POEC). Cells were polarized in an air/liquid interface and then treated with E2 and P4 for physiological time periods: In experiment 1, high concentration of P4 with low concentration of E2 for 10 days resembled diestrus; in experiment 2, following the previous diestrus, sequential high E2 with low P4 for 2.5 days represented estrus. Histomorphometry and electron microscopy showed cyclic changes in cellular height, cell population, and cilia density under the influence of hormone stimulation. Transepithelial electrical resistance was high in simulated diestrus but reduced in estrus. Thus, E2 and P4 affect cellular polarity, transformation of ciliated and secretory cells, as well as electrical conductivity of oviduct epithelium. Simulation of diestrus led to significant decrease in expression of hormone receptors *(PGR and ESR1)* and other epithelial markers (*MUC16, OVGP1,* and *HSP90B1*), while sequential simulated estrus caused an increase in these markers. The hormonal regulation of some marker genes was clearly time-dependent. Furthermore, POEC showed increased sperm-binding capacity in simulated estrus. In this study, we also present a novel approach based on the AndroVision software, which can be routinely utilized as a parameter for ciliary activity, and for the first time, we showed fluid movement patterns along the epithelium lining in vitro.

estrous cycle stages, fluid movement pattern, hormonal stimulation, oviduct epithelium, sperm binding

INTRODUCTION

The oviduct epithelium is mainly made up of ciliated and secretory cells, providing the micromilieu for fertilization and embryonic development as well as regulating gamete transport [1, 2]. Previous in vivo studies on sows, bitches, cows, and mares have revealed that oviduct epithelium undergoes dramatic morphological and functional changes throughout the estrous cycle [3–6]. In luteal phase, the oviduct epithelium exhibits a regressed status, characterized by substantial reduction in cellular height and marked deciliation. Conversely, cells reenter proliferative status in follicular phase, including a rise in epithelium height, a high portion of ciliated cells, and increased secretory activity. In the oviducts of primate species, similar changes have been demonstrated during the menstrual cycle [7]. Hormonal steroids, mainly 17β-estradiol (E2) and progesterone (P4), play pivotal roles in ciliogenesis and cell growth. However, the cyclic events within oviduct epithelium and associated regulation mechanisms of E2 and P4 at the cellular and molecular levels are still not clearly elucidated.

The caudal epithelium serves as a gamete reservoir and plays a significant role in sperm-epithelium interactions. It appears to lengthen the survival of sperm [8], regulate capacitation [9, 10], and synchronize sperm transit with ovulation [11]. In the past decades, conflicting reports have been published concerning the cycle-specific regulation of sperm binding. Several studies indicated estrous stages had no impact on sperm-binding patterns in bovine and porcine [12–14]. In contradiction to these findings, dependency of sperm binding on cyclic stage has been revealed in horses and rats [15, 16]. However, these studies were conducted on different models using cell vesicles, tissue explants, or animal models, which tend to create inconsistent findings.

Ciliary beating is an iconic feature regulating oviduct flow directions. Although the frequency of cilia beating has been assessed by high-speed camera-assisted video microscopy [17, 18], there is no available approach that provides information on fluid movement patterns over the luminal epithelium. Applying a technique that can be routinely used to track the liquid flow on the epithelium might bring more insight into mechanisms of cilia function.

So far, in vitro cultures of oviduct epithelial cells have been developed from several species (e.g., cows, sows, human, and monkeys) [19–23]. However, to our knowledge, there is no study on simulating estrous cycle events in these cultures, due either to the in vitro deprivation of morphological and functional characteristics [20, 23] or due to the limited culture duration [21].

Previously we established a culture method for the long-term cultivation of polarized porcine oviduct epithelium cells (POEC) [19]. In the present study, we aimed to mimic representative estrous cycle stages by periodical stimulation of the long-term cultures with E2 and P4, thus investigating the direct impact of E2 and P4 on the oviduct epithelium. In our simulation system, the morphological and ultrastructural characteristics were examined by histology and electron microscopy. Functional changes caused by the hormonal

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treatment were investigated by gene expression analysis and examination of the sperm-epithelium binding capacity. Furthermore, we describe a novel method to monitor fluid movement patterns along the epithelium lining as a parameter for cilia activity.

MATERIALS AND METHODS

Chemicals and Reagents

Reduced glutathione, ascorbic acid, E2 (E8875) and P4 (P6149) were purchased from Sigma. All the media, as well as FBS (fetal bovine serum) superior (S0615, lot No.1119X), and cell culture additives (including penicillin/streptomycin, gentamycin, and amphotericin B) were supplied by Biochrom. Phenol red free Ham F12 medium was custom synthesized by the company. PeakFlow™ Carmine flow cytometry reference beads (P14831) and Mito-Tracker Red (M-7512) were purchased from Life Technology. AndrostarPLUS was obtained from Minitube. All the other reagents were obtained from Carl Roth unless otherwise indicated.

Culture of POEC

Porcine reproductive tracts were collected from 6-mo-old gilts (hybrids, German Large White × German Landrace) in the local abattoir (Vion Lausitz GmbH, Berlin, Germany) within 15 min after slaughter. Only the oviducts of noncycling gilts were selected. Isolation of primary oviduct epithelial cells was performed following the protocol previously described by our group [19]. Subsequently, cells were seeded at 3×10^5 cells/cm^2 onto Polyethylene Terephthalate (PET) Millicell inserts (Millipore), which were placed in culture plates with the culture medium placed at the basolateral side.

Because this study focused on effects of E2 and P4, phenol red free Ham F12 and FBS superior, which has low and defined hormone levels, were applied [24]. The certificate analysis provided by the manufacturer indicated FBS superior used in this study contained 21.3 pg/ml E2 and <0.2 ng/ml P4. The composition of the culture medium was as follows: phenol red free Ham F12 (containing 10% FBS) was enriched at 2:1 (v:v) with 3T3 conditioned media and then supplemented with 1% penicillin/streptomycin, 1 μg/ml amphotericin B, 50 μg/ml gentamycin, 10 μg/ml reduced glutathione, and 10 μg/ml ascorbic acid. The description for preparation of 3T3 conditioned media was given previously [19]. Cells were maintained in a chamber at 38°C with 5% CO_2. To achieve an air/liquid interface, medium inside the inserts was suctioned off 48 h after seeding. Afterward, medium in the basolateral compartment was changed twice every week.

Steroid Stimulation

Cultures were stimulated periodically with exogenous P4 and E2 from the basolateral side. The doses of E2 and P4 were selected based on physiological plasma hormone levels reported from sows in vivo [25, 26]. The estrous cycle simulation comprised two different experiments. We present the schematic time courses of these experiments in Figure 1.

Experiment 1: determination of preculture duration and simulation of diestrus. To determine the optimal stimulation strategy, POEC from eight animals were precultured for 11 or 32 days to allow building up of cellular polarity. Thereafter, cells were treated with 35 ng/ml P4 and 10 pg/ml E2 (final concentration) for 10 days to mimic diestrus. For the control group, cells were first subjected to the same preculture for 11 or 32 days and then treated with only steroid solvent (ethanol) for 10 days. The total culture duration therefore was either 3 or 6 wk. For each animal, POEC were seeded onto four inserts to allow both histological and mRNA analysis of diestrus and the control.

Experiment 2: simulation of diestrus and estrus. Because the results of experiment 1 indicated that POEC responded similarly to steroid stimulation after 11 and 32 days of preculture, the 11 day preculture duration was selected for experiment 2. POEC from six animals were used to simulate diestrus and estrus. As in experiment 1, cells were first precultured for 11 days and then underwent diestrus simulation for 10 days. To simulate estrus, subsequently cells were treated with 50 pg/ml E2 and 0.5 ng/ml P4 (final concentration) for 2.5 days. In the control group, cells were precultured for 11 days and then treated with steroid solvent only. In this experiment for histological evaluation of diestrus, estrus, and control, POEC were seeded onto three inserts per animal.

Previous reports have indicated that hormone responsiveness of bovine oviduct epithelial cells drops after 18 h of hormonal stimulation [21]. Therefore, to detect mRNA expression profiles, cells were seeded on four additional inserts per animal and underwent stimulation as described above. Cells were harvested after 10 days of diestrus simulation and after both 10 h and 2.5 days of estrus simulation. Control cells were harvested after 12.5 days of incubation with steroid solvent only.

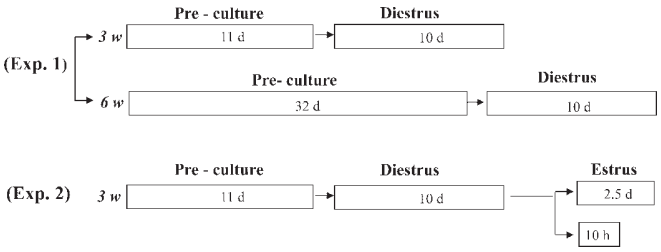

FIG. 1. Time course scheme of the simulation system for experiment 1 and experiment 2. During preculture, cells were grown in culture medium. Diestrus was simulated by medium supplementation with 35 ng/ml P4 and 10 pg/ml E2; to simulate estrus, medium was supplemented with 50 pg/ml E2 and 0.5 ng/ml P4. w, weeks; d, days; h, hours.

Transepithelial Electrical Resistance Assessment

To quantitatively assess the confluence and barrier formation of cultures, transepithelial electrical resistance (TEER) measurement was applied. The ohmic resistance was determined shortly before harvest of cultures using the EVOM^2 Epithelial Voltohmmeter (WPI). The unit area resistance value was calculated according to the manufacturer's description.

Histological Analysis and Electron Microscopy

After cultivation, membranes with cells were washed in PBS and fixed in Bouin solution. After removal from the inserts, membranes were first embedded in 2% agarose followed by postfixation in 4% formalin. After dehydration in an ascending ethanol series, the samples were embedded in paraplast. Three-micron sections were cut for haematoxylin/eosin (HE) staining, and only sections in the middle part of the membrane were collected for morphometric analysis. The height of the epithelial layer was measured by randomly taking three pictures from both ends and the middle part of the section. On each picture, the measurement was performed on five equidistant spots evenly distributed throughout the entire image. Thus, a total of 15 measurements were performed for each sample.

To monitor variation of cell population during simulation, five pictures were randomly taken for each sample. On each picture, we determined the total cell number per picture (membrane length per picture: 214 μm), as well as the number of secretory cells, which were characterized by extended protrusions. For electron microscopy, membranes were rinsed in PBS and fixed in 3% glutaraldehyde at 4°C overnight. Samples from three animals were processed in the Leibniz Institute for Zoo and Wildlife Research following standard procedures as described previously [19].

Expression of mRNA

Total RNA was extracted from POEC cultures using the mirVana miRNA Isolation Kit (Applied Biosystem). One microgram RNA was reverse transcribed by RevertAid reverse transcriptase (Fermentas) after DNase treatment. Quantitative PCR (qPCR) amplification was performed on the StepOne Plus cycler (Applied Biosystems) employing SensimixSYBR high-ROX kit (Bioline GmbH). The cycling program was: 1 cycle, 10 min at 95°C; 40 cycles, 95°C for 15 sec, corresponding annealing temperature for 20 sec, and 72°C for 30 sec. The efficiency and linearity of qPCR amplification were determined by standard curves (series dilution of purified PCR product). The geNorm software was used to normalize gene expression by computing the normalization factor based upon the geometric mean of four reference genes: actin, beta (*ACTB*); succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (*SDHA*); tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*); and 18S ribosomal RNA (*18S rRNA*). All the primer sequences and corresponding annealing temperatures are listed in Table 1.

Preparation of Sperm

All the sperm samples were provided and preprocessed by the Unit for Reproductive Medicine of Clinics, University of Veterinary Medicine Hannover (Hannover, Germany). Briefly, semen samples were collected from one

TABLE 1. List of porcine primers used for qPCR analysis.

Gene symbol	Primer sequence (5′ to 3′)	Fragment size (bp)	Tm (°C)
ACTB	Forward: CAACTGGGACGACATGGAG	234	60
	Reverse: GAGTCCATCACGATGCCAG		
C3	Forward: AACAAGGGCAAGCTGTTGAAGGTG	119	60
	Reverse: TAATAAGCCACCAGGCGGAAGGAA		
ESR1	Forward: AGGGAAGCTCCTGTTTGCTCC	234	60
	Reverse: CGGTGGATATGGTCCTTCTCT		
GPER	Forward: GTGCCCGACCTGTACTTCAT	182	58
	Reverse: AAGCTCATCCAGGTGAGGAA		
HSP90B1	Forward: TCATGAAAGCCCAAGCCTACCAGA	195	60
	Reverse: CTGACCGCAGTGTTGCTGTTTCAA		
MKI67	Forward: CTCTTGTCCCTGAATCCGCA	138	60
	Reverse: TAGCGATGGAGGTAAGTGGC		
MUC 1	Forward: AGCTGATTCTGGCCTTCCAAGACA	96	60
	Reverse: TGGTCAGGTTATAGGTGCCTGCTT		
MUC 16	Forward: AGTGGCTATGCACCCCAGAC	191	60
	Reverse: ACCAGGCAGGAGCGGAATAC		
OVGP 1	Forward: TACTTGAAGAGCTCCTGCTTGCCT	134	60
	Reverse: TCTTCCCAGAAGGCGCACATCATA		
PGR	Forward: TGAGAGCACTAGATGCCGTTGCT	198	60
	Reverse: AGAACTCGAAGTGTCGGGTTTGGT		
SDHA	Forward: CTACAAGGGGCAGGTTCTGA	141	60
	Reverse: AAGACAACGAGGTCCAGGAG		
YWHAZ	Forward: TGATGATAAGAAAGGGATTGTGG	203	60
	Reverse: GTTCAGCAATGGCTTCATCA		
18S rRNA	Forward: AATCGGTAGTAGCGACGG	276	59
	Reverse: AGAGGGACAAGTGGCGTTC		

healthy boar with proven fertility. Samples were labeled with MitoTracker Red and then preserved in the commercial extender AndrostarPLUS. Sperm were delivered overnight at 20°C to the laboratory and applied to each experiment immediately after arrival.

To eliminate diluents, 10 ml of sperm were gently added on top of 5 ml of sucrose gradient in a falcon tube, and then centrifuged at 300 × *g* for 10 min followed by another 10 min at 750 × *g*. After aspirating the supernatant, the pellet was suspended in Medium 199 and adjusted to the density of 2×10^7 sperm/ml. After that, the sperm were incubated at 38°C for 10 min before placing inside the cell inserts, and motility and viability of sperm were assessed under the light microscope.

In Vitro Sperm-Binding Test

Cells were seeded onto transparent PET inserts (Millipore), and estrous cycle simulation was performed through 3 wk of culture (n = 3 animals) as described in experiment 2. Sperm were first diluted with Medium 199 (20×10^6 sperm/ml), and then 50 µl of the suspension was added on top of each membrane with cells. After 45 min of coincubation at 38°C, sperm suspension was removed; cells were washed twice with Medium 199 to remove floating sperm. Thereafter, sperm-bearing cultures were instantly inspected under the Axiovert 35 fluorescent microscope (Carl Zeiss). In each culture, seven fields (area 0.14 mm^2) along the diametral line of membrane were captured using AxioVison (Rel.4.8) software. Afterward, cocultures were fixed in 3% glutaraldehyde for scanning electron microscopy (SEM) observation. The amount of bound sperm was counted using ImageJ software. The binding index (BI) was defined as the number of sperm bound to 1 mm^2 of cell surface.

Tracking of Apical Fluid Movement

Estrous cycle was simulated as described in experiment 2 using POEC (n = 4 animals) seeded onto transparent inserts. Fluorescent beads with a diameter of 2.5 µm were diluted 1:20 in Ham F12 medium, and then 5 µl of suspension (6×10^3 beads) was added inside the insert. After the beads settled down, five fields of view were randomly chosen in each sample (n = 5 replicates) and a 10-sec video was taken in each field. The frame rate of the video was 50 frames/sec (avA1600-50gc; Basler). These videos were analyzed by the AndroVision software (Minitube), which automatically generated the traveling paths of the beads. In parallel, curvilinear velocity (VCL) and straight line velocity (VSL) of the beads were systematically calculated at the end of each video.

Statistical Analysis

Statistical evaluations in this study were performed using SPSS Statistics 20 for Windows. The normal distribution of data was tested by the Schapiro-Wilk method. In experiment 1, comparison of cellular height, TEER, cell counts, and qPCR data were all analyzed using the paired *t*-test. In experiment 2, we used one-way repeated measures ANOVA for analysis of cellular height, TEER, cell counts, qPCR data, sperm binding, and velocity of beads. The Greenhouse-Geisser correction was applied when $\varepsilon < 0.75$, and the Huyn-Feldt correction was applied when $\varepsilon > 0.75$. If repeated measures ANOVA indicated that the overall result was significant, Fisher least significant difference (LSD) post hoc tests were conducted. To further compare sperm binding across treatments of each animal, Kruskal-Wallis test (nonparametric ANOVA) was conducted followed by Mann-Whitney *U*-test. To further compare velocity of beads across treatments of each individual animal, one-way ANOVA was performed followed by Tukey post hoc analysis. In all the experiments, $P < 0.05$ was considered as significant.

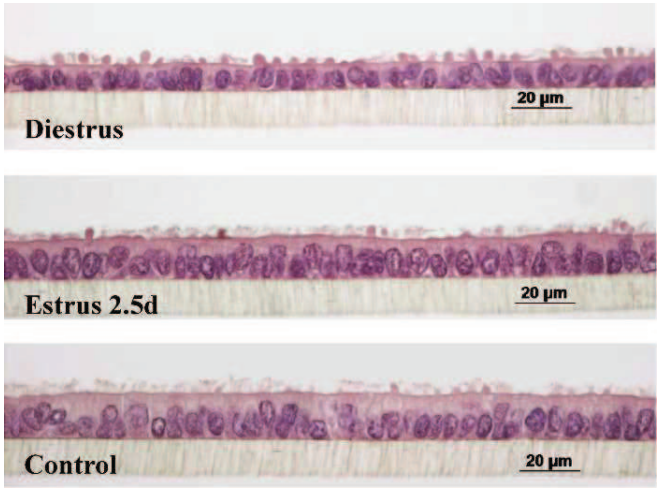

FIG. 2. Representative cross-sections of POEC cultures in different simulated cycle stages. HE staining, magnification ×400.

TABLE 2. Cellular height (μm) of POEC in different simulated cycle stages (mean ± SD) in experiment 1.*

	3 wk			6 wk		
Animal	Diestrus (D)	Control (C)	Ratio (D/C)	Diestrus (D)	Control (C)	Ratio (D/C)
A1	6.93 ± 1.72	14.13 ± 1.24	0.49	9.83 ± 0.66	17.05 ± 1.00	0.58
A2	8.85 ± 0.59	14.95 ± 0.73	0.59	11.88 ± 0.73	19.86 ± 1.08	0.60
A3	8.63 ± 1.40	14.70 ± 0.95	0.59	9.41 ± 0.78	14.48 ± 0.72	0.65
A4	9.49 ± 1.68	14.27 ± 0.63	0.67	10.37 ± 0.51	15.86 ± 1.07	0.65
A5	7.71 ± 0.44	12.67 ± 0.79	0.61	9.01 ± 0.73	16.82 ± 0.74	0.54
A6	9.01 ± 0.93	14.40 ± 0.72	0.63	12.69 ± 0.72	17.52 ± 1.60	0.72
A7	9.10 ± 1.13	14.59 ± 0.71	0.62	9.40 ± 0.83	16.95 ± 0.68	0.55
A8	8.66 ± 1.26	14.34 ± 0.72	0.60	9.71 ± 0.67	16.40 ± 1.02	0.59
Average	8.55 ± 0.78	14.25 ± 0.65	0.60	10.29 ± 1.31	16.87 ± 1.53	0.61
P value		$P < 0.001$			$P < 0.001$	

* Values represent means ± SD of 15 measurements, paired Student *t*-test.

RESULTS

Morphological Adaptions of Primary POECs in Response to Hormonal Stimuli

Histological evaluation clearly revealed morphological changes during estrous cycle simulation in POEC. In the control group, cells were polarized and maintained a mixed population of ciliated and secretory cells. In the simulated diestrus, P4-domination led to a dramatic decrease in cellular height. Cells were atrophied, appeared deciliated, and exhibited cuboidal shape. Besides, secretory cells with cytoplasmic protrusions extend the cellular boarder. Conversely, in the simulated estrus, cells regained columnar shape and showed dense cilia (Fig. 2).

In experiment 1, simulation of diestrus led to a highly significant decrease in cellular height in both 3 and 6 wk of culture (Table 2). The total cell numbers remained constant (Fig. 3). However, the proportion of secretory cells was significantly increased in simulated diestrus compared to the control in both 3 and 6 wk of culture. Morphological changes were accompanied by significant increase of cellular impedance in simulated diestrus as indicated by TEER measurement (Table 4).

In experiment 2, the same alternations in cellular height and TEER were detected in simulated diestrus (Tables 3 and 5). In the following estrous phase, subsequent treatment with high E2 and low P4 concentration caused a significant increase in cellular height. However, the cellular height in estrus was still relatively lower than in control cells (Table 3). TEER measurement showed that the cellular impedance in estrus was significantly lower than in diestrus (Table 5). There was no difference in total cell counts among the different groups ($F_{(2.0,\ 10.0)} = 0.18$, $P = 0.84$, Fig. 3C). Furthermore, qPCR analysis showed there was also no significant difference in mRNA-expression of the proliferation marker *MKI67* (antigen identified by monoclonal antibody Ki-67) among the different groups ($F_{(1.2,\ 6.2)} = 0.94$, $P = 0.39$, Fig. 3E). However, the composition of cell cultures was considerably altered by steroid stimulation. The portion of secretory cells was significantly different in all the groups ($F_{(2.0,\ 10.0)} = 130.64$, $P < 0.0001$). The highest numbers of secretory cells were found in diestrus, and the lowest in the control group (Fig. 3D).

SEM confirmed the ratio variation of ciliated and secretory cells from an aerial perspective (Fig. 4). The cilia were approximately 10 μm in length, reaching a level as found in vivo [27]. In the absence of exogenous E2 and P4, only few secretory cells extend beyond the apical boarder (Fig. 4C). In simulated diestrus, cytoplasmic protrusions of secretory cells were enlarged and dominated over the apical surface, which were interspersed with sparse straight cilia (Fig. 4D). In simulated estrus, cells became densely ciliated and exhibited swollen tips at the end of most cilia (Fig. 4E).

In spite of the predominance of secretory cells in diestrus, almost no secretory granules were found by transmission electron microscopy (TEM) in this stage (Fig. 4G). Estrus, however, was characterized by numerous electron-dense secretory granules and electron-light vacuoles in the supranuclear cytoplasm. They were well developed and large in size (Fig. 4H). A number of electron-dense granules were detected in the control cells as well (Fig. 4I). Kinocilia with typical 9+2 microtubules and mitochondria were observed in all the phases.

Functional Changes: Differential Gene Expression after Steroid Stimulation

Quantitative PCR analysis showed the dynamic changes in mRNA expression levels under steroid stimulation in both

TABLE 3. Cellular height (μm) of POEC in different simulated cycle stages (mean ± SD) in experiment 2.*

	3 wk			3 wk		
Animal	Diestrus (D)	Estrous 2.5 days (E)	Control (C)	Ratio (D/C)	Ratio (E/D)	Ratio (E/C)
A1	9.30 ± 1.11	13.31 ± 0.71	16.78 ± 1.12	0.55	1.43	0.79
A2	11.45 ± 0.97	14.82 ± 1.25	16.69 ± 1.25	0.69	1.29	0.89
A3	10.50 ± 1.42	13.67 ± 1.33	16.80 ± 1.72	0.63	1.30	0.81
A4	11.14 ± 1.22	15.27 ± 1.07	16.06 ± 1.49	0.69	1.37	0.95
A5	10.29 ± 1.04	13.72 ± 1.62	14.48 ± 0.95	0.71	1.33	0.95
A6	10.29 ± 0.70	13.00 ± 0.84	14.16 ± 0.93	0.73	1.26	0.92
Average	10.49 ± 0.75[a]	13.97 ± 0.89[b]	15.83 ± 1.20[c]	0.67	1.33	0.89
P value		$F_{(1.2,6.2)} = 75.3$, $P < 0.0001$				

* Values represent means ± SD of 15 measurements; repeated measures of ANOVA with LSD post hoc analysis.
[a,b,c] Between diestrus and control, $P < 0.001$; between diestrus and estrus, $P < 0.001$; between estrus and control, $P < 0.05$.

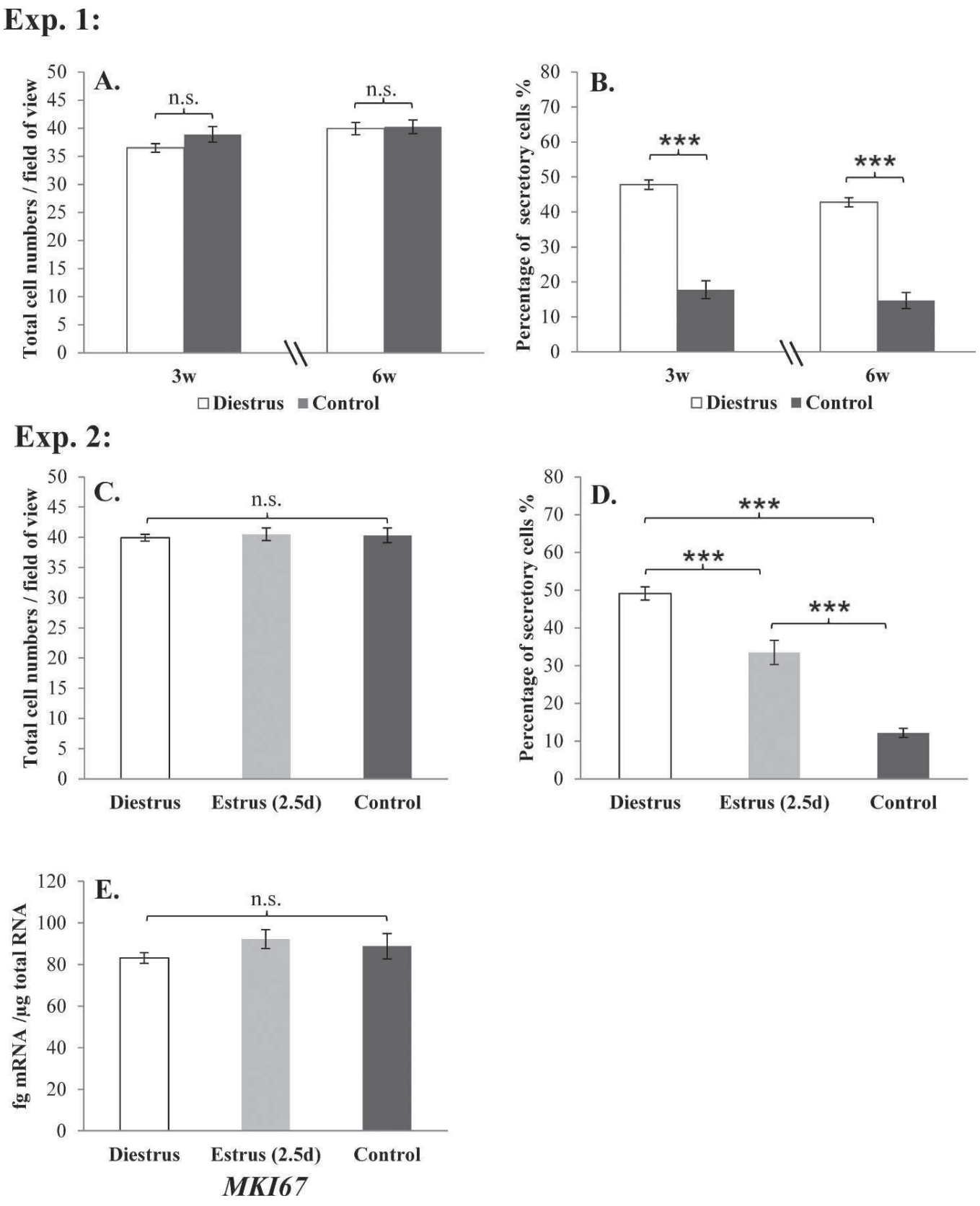

FIG. 3. Variation of cell populations during estrous cycle simulation. Experiment 1: total cell numbers/field of view remained constant (**A**) while percentage of secretory cells increased under P4 dominance (diestrus, **B**). Experiment 2: total cell number/field of view remained constant (**C**) while percentage of secretory cells increased after diestrus simulation and was gradually reduced after subsequent estrus simulation for 2.5 days (**D**); mRNA expression of MKI67 was not altered by hormonal treatment (**E**). Significance is indicated as $***P < 0.001$, while n.s. represents no statistical difference.

TABLE 4. TEER ($\Omega \times cm^2$) of POEC in different simulated cycle stages in experiment 1.*

	3 wk			6 wk		
Animal	Diestrus (D)	Control (C)	Ratio (D/C)	Diestrus	Control	Ratio (D/C)
A1	1066.89	775.17	1.38	888.03	358.71	2.48
A2	988.68	754.38	1.31	876.15	647.79	1.35
A3	1126.62	731.94	1.54	918.72	840.18	1.09
A4	920.04	830.28	1.11	956.67	770.88	1.24
A5	911.79	865.92	1.05	987.36	771.21	1.28
A6	896.61	721.38	1.24	883.08	687.72	1.28
A7	951.39	703.89	1.35	880.77	693.99	1.27
A8	973.17	770.88	1.26	923.67	767.25	1.20
Average	979.40	769.23	1.27	914.31	692.22	1.40
P value		$P = 0.001$			$P = 0.002$	

* Paired Student *t*-test.

experiments. In experiment 1, simulation of diestrus (high P4 with low E2 for 10 days) led to significant decrease in expression of most selected genes. Expression levels of progesterone receptor (*PGR*), estrogen receptor 1 (*ESR1*), the oviduct secretory glycoproteins mucin 16 (*MUC16*), and oviductal glycoprotein 1 (*OVGP1*) as well as heat shock protein 90kDa beta (Grp94), member 1 (*HSP90B1*) were highly significantly downregulated under P4 dominance. The expression of these genes were 2-fold (*MUC16*) to 26-fold (*OVGP1*) higher in the control cultures compared to simulated diestrus (Table 6). Mucin 1 (*MUC1*) and G-protein-coupled estrogen receptor 1 (*GPER*) only showed moderate downregulation of less than 50% compared to the control. In contrast, expression of complement component 3 (*C3*) was significantly increased in simulated diestrus (Table 6).

In experiment 2, when comparing the simulated diestrus with control, we detected the same regulation patterns as in experiment 1. However, the downregulation of *GPER* and *MUC1* was not significant anymore (data not shown). In the subsequent 10 h estrus simulation, expression of *PGR, OVGP1, HSP90B1,* and *ESR1* were significantly increased. When estrus was extended for a physiological duration (2.5 days), a further increase in expression of *PGR* and *OVGP1* was detected compared to the 10 h stimulation. A significant upregulation of *MUC16* was detected only after 2.5 days estrus simulation. Expression of *MUC1, GPER,* and *C3* did not differ between simulated diestrus and estrus (Fig. 5).

Effects of Steroids on Sperm-Epithelium Interaction

Estrous stages were simulated in POEC from three gilts. After 45 min of coincubation, sperm were bound to the apical surface of POEC cultures. Motility of attached sperm was over 90% across all the groups. SEM demonstrated that sperm mainly adhered by the rostral region to the ciliated cells (Fig. 6, A and B). It can be discerned that sperm also attached to microvilli of secretory cells. However, this was only rarely seen in our cultures (Fig. 6C).

The sperm-binding capacity of the epithelium cultures differed in response to the hormonal treatment. In the simulated estrus, more sperm were bound compared to other groups. In two of three animals, the number of bound sperm in estrus was significantly higher than in diestrus (Table 7).

Fluid Movement Patterns Driven by Cilia Beating

Ciliary movement during the different cyclic stages was monitored in POEC cultures (n = 4 animals). Transport of fluorescent beads driven by cilia beating could be clearly visualized using the AndroVision software (for a representative video, see Supplementary Movie S1, available online at www.biolrerpod.org). The software automatically generated the typical trajectory of the beads. Figure 7 illustrates that beads were traveling along specific routes. Hence, the fluid movement had directional properties. The average bead transport speed varied between different animals: VCL ranged from 14.40 ± 3.10 μm/sec to 56.44 ± 10.18 μm/sec and VSL from 10.12 ± 3.02 μm/sec to 47.95 ± 3.43 μm/sec (Table 8). There was no significant difference in the VCL neither across treatment of each animal or across average values of all the animals. Although in two animals the VSL across treatments are significantly different, the trends were contradictory. In sum, no apparent difference was noted in VSL among simulated estrous stages.

TABLE 5. TEER ($\Omega \times cm^2$) of POEC in different simulated cycle stages in experiment 2.*

	3 wk			3 wk		
Animal	Diestrus (D)	Estrous 2.5 days (E)	Control (C)	Ratio (D/C)	Ratio (E/D)	Ratio (E/C)
A1	832.59	729.96	671.22	1.24	0.88	1.09
A2	883.74	829.62	723.69	1.22	0.94	1.15
A3	854.37	808.83	733.59	1.16	0.95	1.10
A4	1149.39	906.51	806.85	1.42	0.79	1.12
A5	1176.45	892.98	769.89	1.53	0.76	1.16
A6	1203.84	899.25	733.59	1.64	0.75	1.23
Average	1016.73[a]	844.53[b]	739.81[c]	1.37	0.84	1.14
P value		$F_{(1.0,\ 5.2)} = 18.85$, $P = 0.007$				

* Repeated measures ANOVA with LSD post hoc tests.
[a,b,c] Between diestrus and control, $P < 0.01$; between diestrus and estrus, $P < 0.05$; between estrus and control, $P < 0.01$.

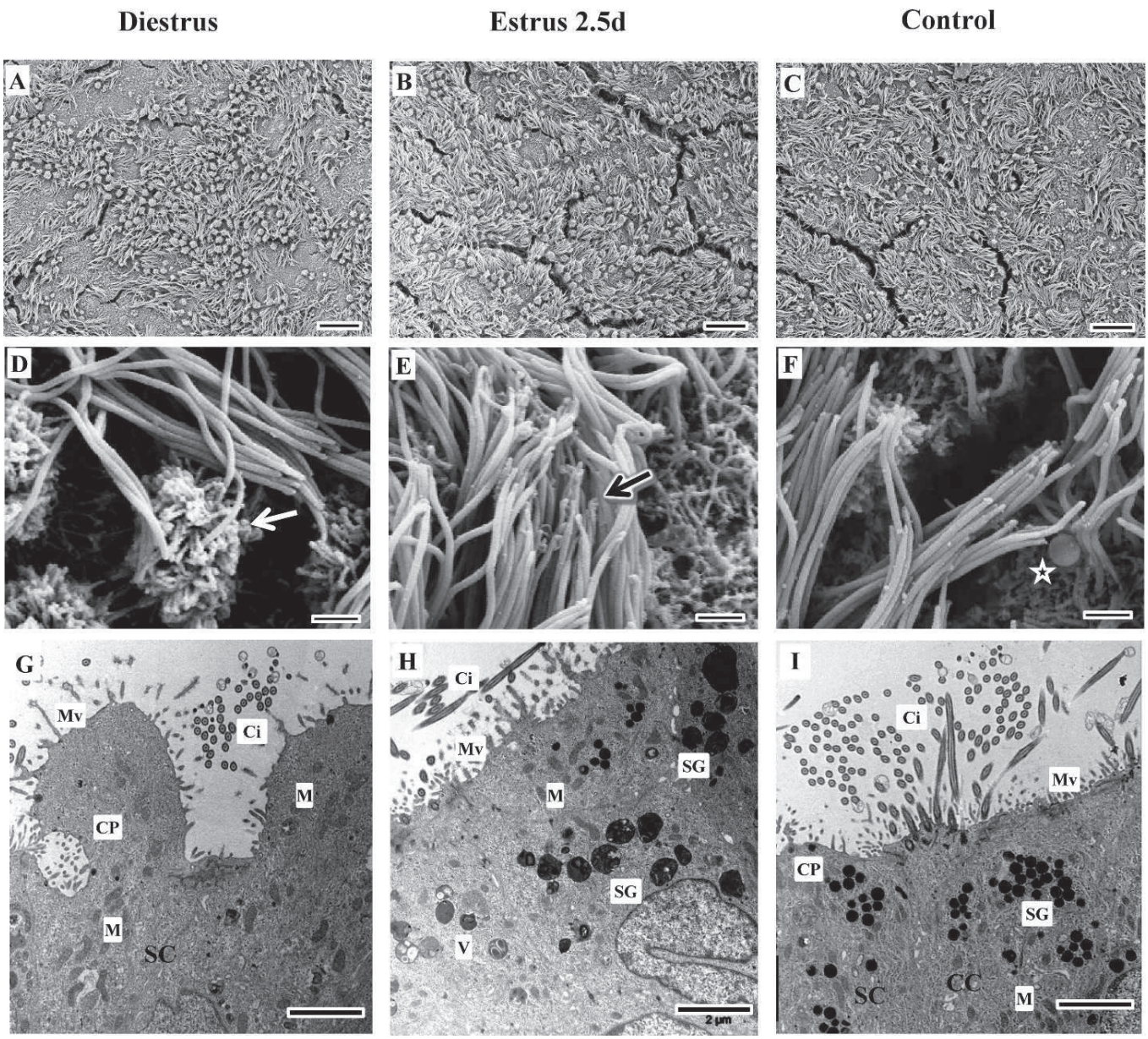

FIG. 4. Ultrastructural analysis of POEC during simulated estrous cycle in experiment 2. Scanning (**A**–**F**) and transmission (**G**–**I**) electron microscopic images of POEC. Arrowhead in **D** points to enlarged cytoplasmic protrusions of secretory cells. Arrowhead in **E** points to swollen tip of kinocilia. Star in **F** indicates secretory bubble. Bars in **A**–**C** = 20 μm and bars in **D**–**I** = 2 μm. CC, ciliated cells; SC, secretory cells; Ci, cilia; CP, cytoplasmic protrusions; M, mitochondria; Mv, microvilli; SG, electron-dense secretory granules; V, electron-light vesicles.

TABLE 6. Relative gene expression of POEC after diestrus simulation (experiment 1).*

	3 wk			6 wk		
Genes‡	Diestrus (D)	Control (C)†	*P* value	Diestrus (D)	Control (C)	*P* value
↓*PGR*	12.1 ± 2.96	100.00 ± 9.90	$P < 0.001$	32.32 ± 8.63	282.61 ± 68.55	$P < 0.001$
↓*ESR1*	35.46 ± 12.44	100.00 ± 10.19	$P < 0.001$	44.25 ± 9.36	125.47 ± 24.78	$P < 0.001$
↓*GPER*	62.91 ± 37.31	100.00 ± 14.77	$P < 0.05$	53.83 ± 10.40	84.90 ± 25.91	$P < 0.01$
↓*OVGP1*	4.22 ± 3.92	100.00 ± 19.92	$P < 0.001$	4.86 ± 3.49	130.36 ± 24.35	$P < 0.001$
↓*MUC1*	75.94 ± 22.64	100.00 ± 16.62	$P < 0.05$	59.60 ± 6.29	85.39 ± 20.81	$P < 0.01$
↓*MUC16*	48.18 ± 14.86	100.00 ± 15.7	$P < 0.001$	83.91± 12.58	159.45 ± 35.33	$P < 0.001$
↓*HSP90B1*	40.39 ± 17.02	100.00 ± 13.65	$P < 0.001$	37.82 ± 5.08	85.88 ± 15.73	$P < 0.001$
↑*C3*	154.93 ± 44.78	100.00 ± 33.33	$P < 0.05$	258.04 ± 34.58	148.15 ± 72.67	$P < 0.001$

* Expression levels are displayed relative to the 3-wk control group, and values represent mean ± SD, paired *t*-test.
† Expression level of the 3-wk control group was set at 100%.
‡ Downregulated gene indicated by ↓, and upregulated gene indicated by ↑.

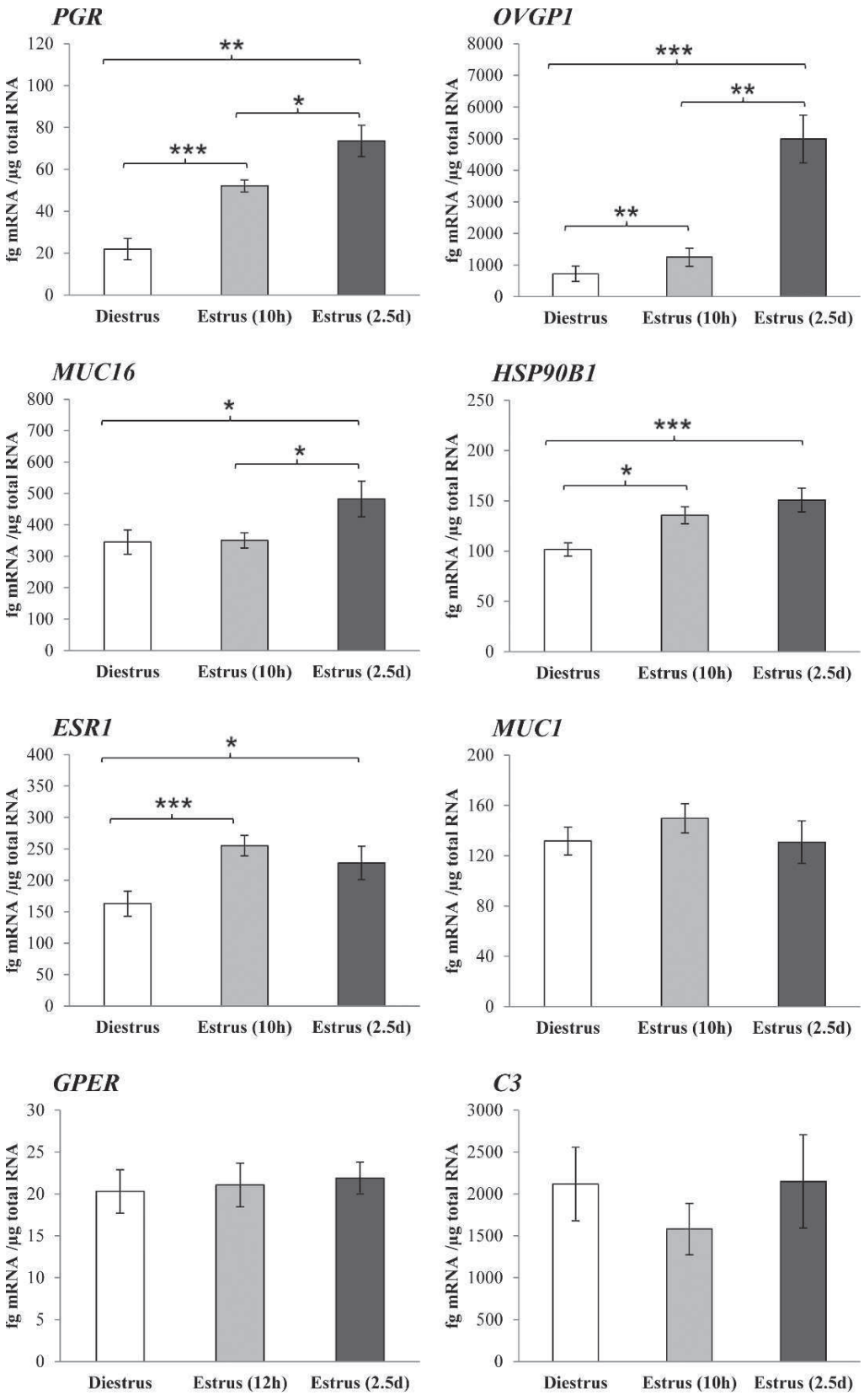

FIG. 5. Differential gene expression of marker genes after hormonal stimulation in experiment 2. Quantitative PCR analysis showed dynamic changes in mRNA expression levels under steroid stimulation. Data are analyzed by repeated measures of ANOVA followed by LSD post hoc tests. *PGR*: $F_{(1.2,\ 6.2)} = 26.80$, $P = 0.001$; *OVGP1*: $F_{(1.0,\ 5.1)} = 42.39$, $P = 0.001$; *MUC16*: $F_{(2.0,\ 10.0)} = 10.22$, $P = 0.004$; *HSP90B1*: $F_{(2.0,\ 10.0)} = 16.24$, $P < 0.001$; *ESR1*: $F_{(1.1,\ 5.5)} = 20.06$, $P = 0.005$; *MUC1*: $F_{(1.4,\ 7.2)} = 1.26$, $P = 0.32$; *GPER*: $F_{(2.0,\ 10.0)} = 0.50$, $P = 0.62$; *C3*: $F_{(1.3,\ 6.5)} = 3.75$, $P = 0.092$; Significance between two groups is indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

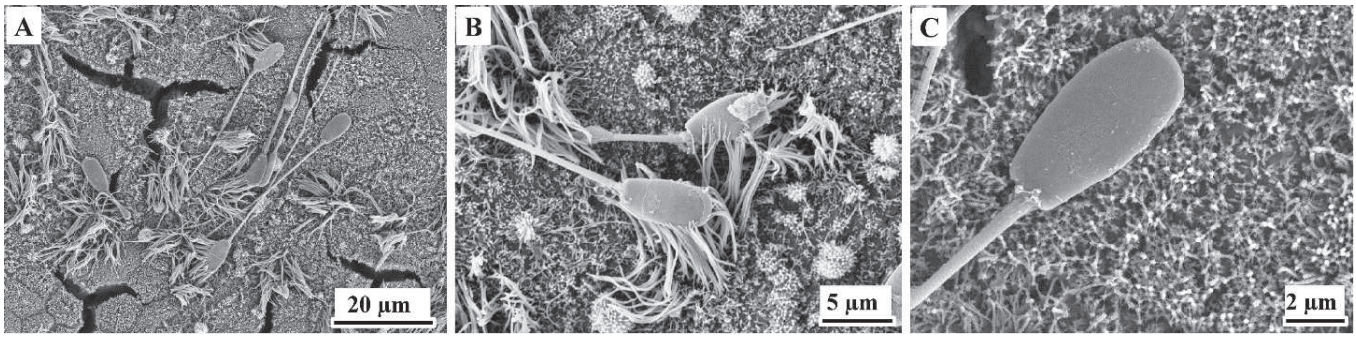

FIG. 6. Estrous cycle-dependent sperm-oviduct interaction in POEC during stimulation. **A**–**C**) SEM showing the contact between sperm and POEC. **A**) Sperm attached mainly by the rostral region. **B**) Strong binding between the cilia and the acrosomal region of sperm. **C**) Sperm attached to nonciliated cells. Bars = 20 μm (**A**), 5 μm (**B**), and 2 μm (**C**).

DISCUSSION

In this study, for the first time we mimicked estrous cycle stages on oviduct epithelium cells in vitro. This was achieved solely through administration of exogenous E2 and P4 in POEC cultures. Our simulation system has several advantages: first, the stimulation was feasible for physiological time duration; second, the system was highly reproducible; and third, it is a dynamic system, which constantly adjusted in cell population and cell morphology to steroid hormones.

Because cells were only treated by varying amount of E2 and P4, the cyclic changes revealed by histological and ultrastructural analysis provide direct evidence to the regulation effect of E2 and P4 on cellular polarization and cell composition in vitro. Total cell numbers remained constant regardless of the hormonal stimulation. Consistent with this finding, mRNA expression of *MKI67*, a marker for cell proliferation, also indicated the cell division status was unaltered during stimulation. However, the ratio of ciliated and secretory cells significantly changed in response to hormonal treatment. This suggests that the relative amount of E2 and P4 controlled the transformation between ciliated and secretory cells as well as induction of ciliogenesis in oviduct epithelium cells. Similar results were reported earlier for ovariectomized quail stimulated with different concentrations of P4 and E2 [28].

In our study, 59 out of 60 cultures developed TEER values ranging from 647 $\Omega \times cm^2$ to 1203 $\Omega \times cm^2$, which indicates the establishment of cellular polarity and barrier formation. A similar transepithelial resistance has been reported for monkey oviduct epithelium in vitro and in vivo [23]. Furthermore, we found the transcellular impedance was high in the simulated diestrus (P4-domination) and then decreased gradually in simulated estrus (E2-domination). Although in vivo impedance has rarely been investigated in oviduct, similar cyclic changes have been reported for the vagina and vulva [29, 30]. In our model, E2 and P4 showed effects on cellular electrical conductivity, which might be associated with the status of secretory activities and transcellular/paracellular transportation. TEM showed more secretory granules in estrus (E2-domination) and control (absence of E2 and P4), which might be associated with high expression of secretion-related genes (such as *OVGP1* and *MUC16)* in these two groups.

Previous studies showed that cycle stages and changing hormone levels affected gene expression in oviductal epithelium in vitro and ex vivo [21, 31, 32]. In experiment 1, we demonstrated that P4-domination downregulated the expression of most genes, including hormone receptors and secretory glycoproteins. This finding is in line with microarray results from the bovine oviduct [31]. Among all the selected genes, only *C3* showed an opposite response profile. Therefore, we hypothesize that *C3* is positively regulated by P4 in the oviduct. In experiment 2, the expression of most genes was higher in the simulated estrus compared to diestrus. At this point, it is not clear whether this upregulation is caused by the lower P4 concentration in the medium or reflects an E2 effect.

OVGP1 (MUC9) and *MUC16* are the major mucins secreted by oviduct epithelium cells [33], whereas *HSP90B1* regulates protein secretion and protein folding. The elevated expression of these genes might reflect activated secretion in simulated estrus. Increased expression of the hormone receptors *PGR* and *ESR1* in simulated estrus is again consistent with in vivo studies on bovine oviduct [31, 32].

GPER (a membrane estrogen receptor [34], which is rarely investigated in the oviduct) was slightly but significantly downregulated under P4-domination as detected in our first experiment. The study by Otto et al. [35] on *GPER* knockout mice indicated *GPER* is not essential for reproduction. In our model, *GPER* mRNA expression was regulated at least by P4, which points toward a role of *GPER* in the response of oviduct epithelium cells to hormonal stimuli. However, simulated estrus with lower levels of P4 and higher levels of E2 did not lead to significant changes in the expression levels of neither *GPER* nor *MUC1,* suggesting that even low levels of P4 (as

TABLE 7. Sperm BI during different simulated cycle stages.*

Animal	Diestrus	Estrus (2.5 days)	Control
A1	398.98 ± 42.58^{a}	754.08 ± 52.92^{b}	665.31 ± 41.72^{bc}
A2	687.76 ± 83.19	957.14 ± 130.15	803.06 ± 88.12
A3	376.53 ± 122.26^{a}	714.29 ± 140.85^{b}	401.02 ± 76.05^{ab}
Average	487.76 ± 100.21^{A}	808.50 ± 75.20^{B}	623.13 ± 117.96^{AB}
P value	$F_{(2.0,\ 4.0)} = 15.34,\ P = 0.013$		

* LSD post hoc test indicates significant difference between diestrus and estrus group ($^{A,B}P = 0.007$). For comparison across treatments of an individual animal, Kruskal–Wallis test was performed ($^{a,b,c}P < 0.05$). Values represent mean ± SEM.

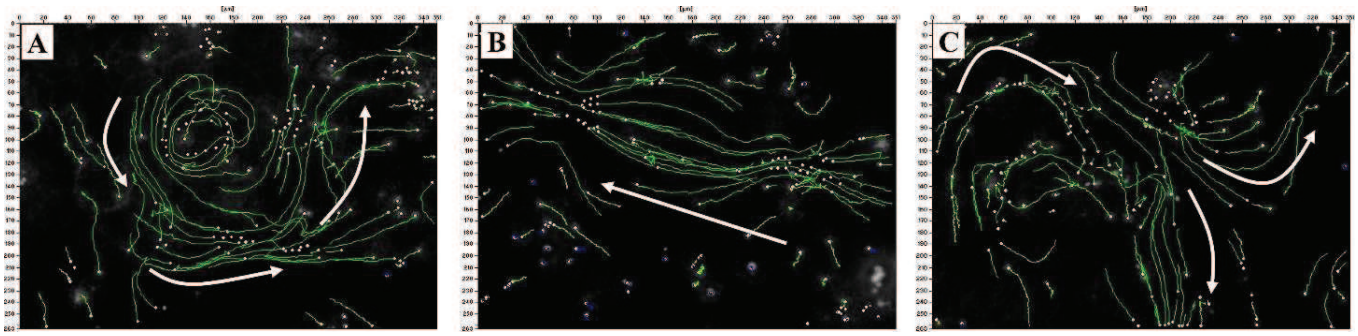

FIG. 7. Fluid movement patterns over the epithelium lining monitored by the AndroVision system tracking the transport path of fluorescent beads for 10 sec. Beads traveled along three typical paths: in circles (**A**), in straight lines (**B**), or in arcs (**C**). Direction of liquid movement was pointed out by arrows. Magnification ×200.

present in diestrus) downregulate expression of these genes and that E2 does not have a significant effect on the expression levels. When comparing 10 h of estrus simulation to 2.5 days of estrus simulation, the gene expression alterations showed different patterns: a time-dependent, long-term effect on *PGR, OVGP1,* and *MUC16* expression, but a rapid and short-term regulation of *ESR1*and *HSP90B1*.

In addition to presenting a phenocopy of the estrous cycle, we also aimed to investigate the functional changes in sperm-epithelium interaction. In our study, the binding was to both ciliated and secretory cells but preferentially to the ciliated type, which reflected previous research [15, 36]. Electron micrographs showed binding between cilia and the rostral aspect of the sperm, proving that cells in our system recapitulate the biological function of oviduct epithelium in vivo. Furthermore, our data support the notion that variation of hormone levels throughout the estrous cycle affects sperm-oviduct interaction. Previous studies in gilts demonstrated that exogenous E2 treatment increased sperm binding to oviduct explants [12]. Also in our study, more sperm bound to cells in simulated estrus. The low binding in P4-dominant group is in line with the report of Bureau et al. [37] who demonstrated that P4 pretreatment of porcine oviduct epithelial cells compromises sperm binding. In general, we propose the stage-specific binding might be a result of two mechanisms. The first is the varying numbers of ciliated cells during estrous cycle. Because most sperm were found trapped by cilia, the high degree of ciliation would probably promote the binding process in the estrous phase. The second is the observed changes in the composition and volume of oviduct secretion. It has been reported that activities of glycosidases in the oviduct fluid differ throughout the cycle [38]. The sperm-oviduct interaction is a carbohydrate-mediated process [39, 40]. Changes in glycosidase activities could act upon carbohydrate residues, thereby regulating the sperm binding.

For the first time, we adapted the AndroVision software for an epithelium cell culture model and developed a new analytic system that could be routinely used to investigate ciliary activity. In addition, we provide the first data on fluid movement patterns along the epithelium lining. Taking advantage of the cell model, the signal/noise ratio and image resolutions were substantially improved compared to other methods [18, 41]. The transport of beads proved that our culture is a dynamic system with vigorous ciliary activity. Besides, we demonstrated the directivity of the basal fluid stream guided by cilia beating. We consider that this information would facilitate studies on oviductal transport mechanisms of gametes and embryos [2]. Furthermore, this method allowed mathematical analysis of the speed of fluid movement. There were conflicting reports concerning ciliary activities throughout the cycle. One study showed changes in ciliary beating frequency (CBF) during the menstrual cycle in human [42]. Nishimura et al. [17, 18] reported that the CBF was controlled by steroid hormones during cycle in guinea pigs. However, the most recent studies revealed the specificity of ciliary beating can be attributed to anatomical positions rather than cyclic stages [41, 43]. In our study, the speed of beads transport was relatively constant in all the animals and corresponded to levels reported in murine oviduct and trachea [43]. No difference was noted in the speed of beads transport within different groups. This supports the deduction that tubal clearance of secretions takes priority over gamete transport. Nevertheless, experimental conditions such as pH values [44], vibration artifacts, and fluid volume might skew results. Therefore, further standardization of the analysis conditions is needed.

TABLE 8. Velocity parameters of beads in the apical fluid layer during different cyclic stages.*

	VCL†			VSL‡		
Animal	Diestrus	Estrus (2.5 days)	Control	Diestrus	Estrus (2.5 days)	Control
A1	26.87 ± 3.39	16.96 ± 3.68	14.40 ± 3.10	22.04 ± 2.58^{a}	11.74 ± 3.33^{ab}	10.12 ± 3.02^{b}
A2	22.68 ± 3.97	24.45 ± 3.68	22.88 ± 5.06	20.36 ± 3.47	19.04 ± 3.25	19.56 ± 4.13
A3	19.39 ± 3.31	18.24 ± 1.86	31.36 ± 5.37	13.08 ± 3.34^{a}	13.32 ± 1.98^{a}	26.51 ± 4.60^{b}
A4	52.80 ± 8.94	56.44 ± 10.18	52.60 ± 3.94	44.89 ± 8.11	46.52 ± 6.81	47.95 ± 3.43
Average	30.43 ± 3.92	29.02 ± 4.55	30.31 ± 3.85	24.71 ± 3.59	22.66 ± 3.76	25.62 ± 3.73
P value		$F_{(2.0,\ 6.0)} = 0.07$, $P = 0.93$			$F_{(2.0,\ 6.0)} = 0.29$, $P = 0.76$	

* Values represent means ± SEM. Repeated measures ANOVA to compare average values of all the groups. For comparison across treatments of an individual animal, one-way ANOVA with Tukey post hoc test was performed ($^{a,b}P < 0.05$).
† The speed (μm/sec) that beads traveled across the curved line from the beginning to the end of the analysis period.
‡ The speed (μm/sec) that beads traveled in a straight line from the beginning to the end of the analysis period.

In conclusion, for the first time we demonstrate the simulation of estrous cycle stages on mammalian oviduct epithelial cells in vitro and clearly showed the effect of E2 and P4 on differentiation, gene expression, and cellular function in oviduct epithelium. Because specific functions of oviduct epithelium are obtained under defined hormonal conditions, the in vitro simulation system will boost investigations on precisely timed local biological events occurring throughout the sexual cycle. Finally, the new approach for cilia movement analysis could be utilized to decipher basic transport mechanisms as well as human ciliary diseases, such as Kartagener syndrome, immotile cilia syndrome, ectopic pregnancy, and the influence of infections on cilia activity [2, 45].

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4. Discussion

In this study, we developed a validated, comprehensive culture model of POEC. It closely mimicked the morphology and functions of porcine oviduct epithelium in vivo, and could be maintained at a homeostatic status from 3w up to 6w. Standard culture protocol and optimal conditions are given based on large scale trials. We speculate this model will have a broad spectrum of uses in oviduct fertility studies.

Based on the validated POEC model, for the first time we demonstrated the simulation of estrous cycle on mammalian oviduct epithelial cells in vitro. Distinct regulatory functions of E2 and P4 on oviduct epithelial cells have been revealed during the simulation. Furthermore, we present a novel approach, which can be routinely utilized as a parameter for cilia activity.

4.1. A comprehensive porcine oviduct epithelial culture model

Given the broad reproductive actions taking place in the oviduct epithelium, it becomes necessary to develop in vitro models allowing the elucidation of fundamental mechanisms for these reproductive events. Hence our initial goal was to develop a comprehensive model for the ex-vivo culture of POEC.

4.1.1. A phenocopy of oviduct epithelium

Compared to 2D dish cultures reported before [36, 38], the POEC model exhibited highly morphological and functional equivalence to in vivo tissue. Histology and electron microscopy revealed that cells can well mimic in vivo architectures, by preserving natural coherence of secretory and ciliated cells, columnar shape, and cilia beating. In our model cells possessed a mean cellular height up to 17.4μm, which reflects an excellent polarization status comparable to tissue in vivo. Besides, the high TEER suggested that such cells also maintained type specific intercellular junctions. Furthermore, our model functions in a way that is observed in vivo including: secretion of acidic mucins, and expression of lineage specific markers at both mRNA and protein levels. OVGP1, MUC16 and MUC1 (mucin 1) are the major mucins secreted in the porcine oviductal fluid. The presence of these markers suggested that the POEC model retained the embryotrophic capability of native tissue.

4.1.2. Validation of the POEC model

Moreover, to our knowledge, this is the first validated model for mammalian oviduct epithelial cells. We provide information on effects of commercially available standardized sera, 3T3 medium conditioning, culture duration, and cryopreservation on cells from high numbers of donor animals. Generally the composition of serum varies among different donors and batches, which leads to high inter-laboratory variability of cell cultures. In our study, cells were tested with two commercially available standardized sera, which have continuous quality and no need for batch to batch testing. The results showed that cells achieved similar differentiated status in both standard sera. 3T3 conditioned medium would promote optimal differentiation,

as the cellular height was increased by 13 to 19%. On the other hand, for researchers who want to have clearly defined culture composition, applying conditioned medium is not mandatory, since POEC in unconditioned medium already achieved well polarized status. For the culture duration, the well differentiated phenotype together with the stable gene expression indicated that cells reached a homeostatic status from 3w up to 6w. This was also the most long lasting culture reported for primary oviduct epithelial cells, which opens the possibility to simulate complete estrous cycles. Since cells most closely present in vivo physiology after isolation, so far reported polarized epithelial cultures are mainly driven with freshly isolated cells, which are labor-intensive and inflexible in operation time [39, 66, 67]. Cryopreservation of primary isolated cells not only permits the long-term storage, but also ensures the availability of cells with continuous properties. We systematically compared freshly isolated cells and thawed cells in our POEC model. No differences were observed with respect to morphological appearance and cellular height. Thereby, researchers could freeze stocks of cells and later on perform experiments with the same cell materials. Furthermore, we quantitatively assessed POEC cultures at endpoints including cellular height, TEER, and gene expression. The results are reproducible when working with cells from 25 animals, with very low variations.

4.1.3. Application of the POEC model

We speculate this validated POEC model would serve as a useful tool for oviduct reproductive science, especially in the field of fundamental cell-mechanism research, reproductive toxicity testing, and ovarian carcinoma as explained below:

1. The biologically relevant environment offered by POEC model, may facilitate studies on germ cell maturation, tubal transport, and early embryo development, and thus improve our understanding on the underlying molecular mechanisms.

2. The model has several unique features: it is easy to handle, has a relatively long shelf-life, is adapted to commercially standard sera, cell materials (from slaughterhouse) are costless and abundant. These features suggest its potential use to reduce animal experiments, especially in the reproductive toxicity testing, where substantial numbers of animals are required for the safety testing of drugs and chemicals [32].

3. High grade serous ovarian carcinoma (SOC) is one of the most lethal ovarian cancers. New evidence suggests that it may arise from the distal oviduct and then spread to the ovary [68, 69]. Secretory cells of oviduct epithelium are the presumptive cell origin for SOC [70]. In our POEC culture, both secretory cells and ciliated cells are preserved. Thus, it might serve as an experimental model to study the transformation of secretory cells during SOC development. Exposure of cells to inflammatory mediators may simulate the carcinogenic process, thus improving our understanding in the pathogenesis of SOC.

4.2. In vitro mimicking of estrous cycle stages

In the study of chapter 3.2, we demonstrated for the first time the simulation of estrous cycle stages on mammalian oviduct epithelial cells in vitro. This is achieved

by treating cells with combinations of P4 and E2 to mimic endocrine profiles during the estrous cycle.

4.2.1. Simulation strategy

In sows, the diestrus is characterized by high plasma P4 levels for around 10d, whereas the estrus is dominated by high E2 for approximately 2.5d [71]. However, there are inconsistent reports on the concentration of serum hormones during estrous cycle in pigs (Table 1). After comparing these studies, we decided to treat cells with 35ng/ml P4 and 10pg/ml E2 for 10d to mimic main stage of diestrus, and subsequently treatment with 50pg/ml E2 and 0.5ng/ml P4 for 2.5d to mimic estrus.

We divided the simulation into two steps. In step 1, we focused on pre-test the selected hormonal concentration and stimulation strategy firstly during the simulation of diestrus. To determine optimal timing for stimulation, we performed experiments within both 3w and 6w cultures. Therefore, cells were pre-cultured for 11d or 32d respectively before 10d diestrus simulation. After step 1, the results states that POEC respond similarly to steroids stimulation within 3w and 6w cultures. This was also in agreement with our findings in our first work (chapter 3.1), that the POEC model was viable from 3w to at least 6w. Hence in step 2, we selected the 3w culture and further simulated estrus following diestrus.

Age	Sampling	Stage		References
		Diestrus	Estrus	
gilt	peripheral plasma	E2 ≤ 20pg/ml P4 up to 33.2ng/ml	E2 up to 50pg/ml P4 < 1ng/ml	Henricks et al. (1972) [46]
gilt	utero-ovarian vein plasma	neither E2 or P4 was not stated	32 ≤ E2 ≤ 69pg/ml 0.56 ≤ P4 ≤ 3.2ng/ml	Eiler et al. (1977) [72]
sow	peripheral plasma	E2 = 3.8 ± 0.4pg/ml P4 = 75 ± 7ng/ml	E2 = 33 ± 2.5pg/ml P4 = 3.6 ± 0.5ng/ml	Peralta et al. (2005) [73]
sow	Peripheral plasma	E2 around 1pg/ml P4 up to 20ng/ml	E2 up to 20.8pg/ml 0 < P4 < 1ng/ml	Noguchi et al. (2010) [74]

Table 1. Published steroid hormone concentrations during estrous cycle in pig.

4.2.2. Simulation VS stimulation

Our simulation of estrous stages based on the POEC model has distinct advantages over the hormonal stimulation in oviductal epithelial cells reported previously by other groups. Firstly, we mimicked the endocrine profiles for physiological duration. The simulation system is closely relevant to in vivo situation, and thus could better reflect co-regulatory effects of E2 and P4. In most stimulation studies, the oviduct epithelial cells were treated with either E2 or P4 alone for only very short time (less than 30h) [39, 44, 54].

Secondly, in our simulation system, the gene expression more sensitively responded to hormonal changes. Rottmayer et al. (2006) investigated the short term hormonal responsiveness of bovine oviduct epithelial cells (BOEC) in a suspension culture system. They found that E2 stimulation significantly increased PGR transcript abundance, while P4 had no effects; ESR1 (estrogen receptor 1) did not respond to either E2 or P4 treatment. Besides, transcript level of OVGP1 was significantly decreased by P4, whereas E2 had no effects. These findings were only partly consistent with in vivo studies [54, 55]. Ulbrich et al. (2003) detected the expression of hormonal receptors in bovine oviduct in vivo and in vitro [54]. In their in vitro BOEC cultures, expression of PGR and ESR1 are up-regulated by E2 stimulation while P4 showed no detectable effect, which could not fully fit their in vivo findings. In our simulation study, the expression of PGR, ESR1 and OVGP1 was significantly down regulated in simulated diestrus, but up regulated in simulated estrus. Besides, we investigated expression of other oviductal marker genes, including C3, MUC1, MUC16, GPER (G-protein-coupled estrogen receptor 1) and HSP90B1 (heat shock protein 90kDa beta (Grp94), member 1) during simulated estrous stages. Such information reflected the cyclic expression profiles of porcine oviduct epithelium in vivo, which have not been systematically investigated so far.

Thirdly, since most stimulation studies were only for short period, almost no information on morphological changes was given, except for the study by Comer et al. [8]. The author reported that administration of E2 in human oviductal epithelial cells induced a ciliated cell type, while in the absence of E2 cells adopt a secretory phenotype. In our simulation model, for the first time, we clearly demonstrated effects of E2 and P4 on cell polarization and ciliogenesis in vitro, and presented quantified data on the cellular height and portion of cell types.

Furthermore, based on our simulation system, we provided data on oviductal electrophysiology during the estrous cycle. To the best of our knowledge, so far this has been neither investigated in vitro nor in vivo. We found that the transcellular impedance significantly decreased from simulated diestrus to simulated estrus. Similar changes of electrical conductivity have been reported in other reproductive organs, the vagina and vulva [75, 76]. This suggested a decrease of epithelial monolayer integrity, as components of oviduct fluid derived from the vascular bed have been reported to increase during estrous phase [20].

4.2.3. Transformation between secretory cells and ciliated cells

It is known that the oviduct epithelium consists of two cell types: ciliated cells and secretory cells. However, there are some studies indicating that these two cell types may be in different functional status of the same cell type [8, 77]. During the simulation of estrus cycle, results showed that the total cell numbers remained constant, whereas the ratio between ciliated cells and secretory cells dramatically altered with more ciliated cells observed in simulated estrus. Meantime proliferative marker MKI67 showed stable expression over the whole period, suggesting the cell proliferation activity was not altered. Thus we infer hormonal stimulation may induce the transformation between ciliated cells and secretory cells. Similar to our findings, an immunohistochemical study in porcine oviduct epithelium in vivo concluded that, E2 may induce the proliferation of secretory cells and promote the differentiation into

ciliated cells [78]. Nevertheless, more evidence is needed to support this interesting transformation theory.

4.2.4. Estrous cycle simulation affected sperm binding

In our simulation system, we observed more bound sperm in simulated estrus than in diestrus. This is broadly in line with the in vivo study in rat and ex vivo study in equine [56, 57]. Previous reports in pigs demonstrated that P4 pre-treatment of vesicles formed from POEC interfered with sperm binding compared with E2 pre-treatment, which also confirmed our results [79]. We attribute our findings mainly to two mechanisms. Firstly, in the co-culture with sperm, the light microscopy revealed that although sperm could attach to secretory cells, most sperm were actually trapped by cilia. Thus, the high portion of ciliated cells in simulated estrus probably promoted the sperm binding process. Another potential mechanism might be the changes of oviductal secretion during the estrous cycle. It is reported that the oviduct-specific glycoproteins have the capacity to directly modulate sperm function, while glycosidases could act on sperm-binding sites present in epithelial cells [1, 80].

4.3. Novel approach for oviductal cilia activity

The AndroVision™ software is a highly efficient automated system designed by the company Minitube for computerized semen analysis. It could accurately and quickly assess the concentration, sperm integrity and motility as well as sperm viability with up to 2000 cells per field. When we looked for a method to monitor the cilia activity in our model, several features of this software appealed to us: It can provide real-time analysis of live images and video files in a broad field. The analysis time of the video could be extended up to 10sec. Moreover the software offered a detailed report on sperm movement, which includes the sperm travelling paths and several velocity parameters, inter alia, VCL, VSL and average path velocity. Lastly, the AndroVision™ is appropriate for routine application.

Hence, we planned to adopt the AndroVision™ software onto our POEC culture model. The software detects the active motion of sperm. In our model, the cilia beat vigorously and caused the movement of apical fluid. Therefore we added fluorescent beads onto the surface of the cell layer to track their movement. We then systematically compared the material, size, weight and fluorescent intensity of different commercial nanobeads, as well as the amount of liquid in the apical surface. Finally, we were able to track the travelling paths and velocity of beads in our model.

Compared to other reported methods, the image resolutions and signal/noise ratio in our method were substantially improved. Besides, driven by the motile cilia, beads travelled at a high speed up to 56.44µm/s, which was comparable to the ex vivo studies in murine oviduct and trachea [65]. The motion of beads actually reflected the fluid flow along the epithelium lining. After analyzing the trajectory of beads, we found that the basal fluid stream had directivity and fixed flow patterns. However, when we simulated estrus cycle on POEC, no difference was noted in the speed of beads transport within different groups. This result is in agreement with the study by Noreikat et al. (2012), who reported the cilia beating to be independent from cyclic stages and tubal clearance preceded gamete transport [65]. Nonetheless, experimental

conditions for this method need to be further optimized. It should be kept in mind that factors such as environmental temperature, pH value of media, and operating times might influence such experiments.

In summary, our novel fluorescent bead-based approach is easy to handle, and could be routinely used to monitor the cilia activity and apical fluid movement. It might have a broad application in basic tubal transport research, as well as in toxicity studies. Recent reports suggest that ectopic pregnancy, Kartagener syndrome, ‘immotile cilia syndrome’ in women are all related to the dysfunction of cilia activity [60]. Our model could be utilized to study the underlying pathogenesis of these diseases.

5. Summary

The oviduct epithelium is an important reproductive venue where gamete transport, sperm capacitation, oocyte maturation, and early embryo development occur. To understand the fundamental molecular mechanisms of these events, as well as to provide a tool for reproductive toxicity testing, a standardized in vitro oviduct epithelium model is on request. Although oviduct epithelium is reported to be sensitive to changes in circulating steroid hormones during estrous cycle, cyclic cellular events, especially sperm-epithelium binding and cilia activity are not well understood yet. In vitro investigations are needed to reveal principle regulatory mechanisms of hormones on cellular functions.

We firstly developed a validated, comprehensive culture model of POEC. After the systematical assessment of standard sera, 3T3 medium conditioning, culture duration, and effects of cryopreservation on cells from 25 donor animals, a dossier of validation on these parameters was provided. The results suggest that cells faithfully recapitulated the morphology, ultrastructure and functionality of oviduct epithelium in vivo. TEER measurement indicated that cells maintained tissue polarity and cell type specific tight junctions. Furthermore, the stable mucin secretion and consistent expression of functional markers revealed that cells maintained an in vivo-like, homeostatic status from 3w up to 6w.

Based on the POEC model, we could simulate estrous stages by mimicking the endocrine profiles of E2 and P4 for physiological time period. Cells exhibited distinct changes in cellular height and cilia density, which directly proved that E2-domination (estrus) induces cell polarization and ciliogenesis, but P4-domination (diestrus) suppresses these effects. Changes in composition of cell population suggested that E2 and P4 affected the transformation of ciliated cells and secretory cells. The TEER measurement revealed the cyclic changes of oviductal electrophysiology. In addition, simulated diestrus decreased expression of hormonal receptors and other oviductal markers, while subsequent estrus up-regulated these genes. Moreover, increased sperm binding was observed in simulated estrus compared to simulated diestrus.

Finally, we developed a novel approach, which could be routinely used to monitor the cilia activity. With the assistance of the commercial AndroVision™ software, we detected the typical movement patterns of apical fluid streams. Fixed flowing routes suggested the directivity of fluid stream along the epithelium lining. Driven by cilia, beads were travelling at high speed comparable to ex vivo studies. However, there was no difference in the transport speed during estrous simulation.

To summarize, our model together with the present approach to monitor cilia activity is a promising tool for basic reproductive science, as well as for reproductive toxicity testing. We present the first in vitro simulation of estrus cycle stages on mammalian oviduct epithelial cells, and revealed roles of E2 and P4 on oviduct development and functionality.

Zusammenfassung

In vitro Simulation des Sexualzyklus in einem validierten Zellkulturmodell des porcinen Eileiterepithels

Das Eileiterepithel ist ein entscheidender Ort der Reproduktion, an dem der Gametentransport, die Kapazitation, die Oozytenreifung und die frühe Entwicklung des Embryos stattfinden. Um die fundamentalen molekularen Mechanismen dieser Ereignisse zu verstehen und ein Werkzeug zur Durchführung reproduktionstoxikologischer Analysen zur Verfügung zu haben, wird ein standardisiertes in vitro Modell des Eileiterepithels benötigt. Obwohl nachgewiesen wurde, dass das Eileiterepithel sensitiv ist für Veränderungen der zirkulierenden Steroidhormone innerhalb des Sexualzyklus, ist bisher wenig über die zellulären Veränderungen, insbesondere die Bindungsfähigkeit von Spermien und die Zilienaktivität, bekannt. In vitro Untersuchungen können hier eingesetzt werden, um die regulatorischen Wirkungen der Hormone auf die zellulären Funktionen aufzudecken.

Wir haben zunächst ein umfassendes und valides in vitro Modell porciner Eileiterepithelzellen (POEC) entwickelt. Nach systematischer Untersuchung von Standardseren, einer Konditionierung des Mediums durch 3T3-Zellen, der Kulturdauer und der Auswirkung der Kryokonservierung auf die Zellen 25 verschiedener Donortiere wurde ein Dossier der Validierung dieser Parameter erstellt. Die Ergebnisse belegen eine deutliche und zuverlässige in vitro Rekapitulierung der Morphologie, der Ultrastruktur und der Funktionalität von Eileiterzellen in vivo. Messungen des transepithelialen elektrischen Widerstandes (TEER) zeigten, dass die Gewebepolarität und die Ausbildung zellartspezifischer Tight junctions erhalten sind. Zudem bestätigten die beständige Mucinsekretion und die gleichbleibende Expression verschiedener funktionaler Marker die Aufrechterhaltung eines homöostatischen Status über einen Zeitraum von drei bis sechs Wochen, der zudem dem von in vivo Eileiterepithel gleicht.

Auf Basis dieses POEC Modells wurden verschiedene Stadien des Sexualzyklus in vitro durch ein Nachstellen des endokrinen Profils von Prostaglandin E2 (E2) und Progesteron (P4) in physiologischer Dauer simuliert. Die Zellen zeigten deutliche Veränderungen in Bezug auf ihre Höhe und die Dichte ihrer ausgebildeten Zilien, so dass eine Induzierung der Zellpolarisierung und der Ziliengenese durch E2-Dominanz (Östrus) sowie eine Suppression dieser beiden Faktoren durch P4-Dominanz (Diöstrus) nachgewiesen wurde. Veränderungen in der Zusammensetzung der Zellpopulation veranschaulichten zudem, dass E2 und P4 die Transformation zilien-besetzter und sekretorischer Zellen beeinflussen. Mit Hilfe von TEER Messungen konnten die zyklischen Veränderungen in der Elektrophysiologie des Eileiterepithels aufgezeigt werden. Zudem bewirkte eine Simulation des Diöstrus eine Herunterregulierung der Expression von Hormonrezeptoren und weiteren für das Eileiterepithel spezifischen Markern, während die Expression der entsprechenden Gene im anschließenden Östrus

herauf reguliert wurde. Es wurde außerdem eine vermehrte Spermienbindung in der Simulation des Östrus im Vergleich zu der des Diöstrus nachgewiesen.

Schließlich haben wir ein neues Verfahren entwickelt, welches routinemäßig zum Monitoring der Zilienaktivität eingesetzt werden kann. Mit Hilfe der AndroVision™ Software wurden die typischen Bewegungsmuster der apikalen Flüssigkeit aufgezeichnet. Festgelegte Bewegungslinien der Flüssigkeit lassen einen gerichteten Flüssigkeitsstrom entlang der epithelialen Schicht vermuten. Durch die Zilien in Bewegung versetzte Beads zeigten eine hohe Geschwindigkeit vergleichbar zu der in ex vivo Experimenten. Es konnte kein Unterschied in der Transportgeschwindigkeit in Abhängigkeit des jeweiligen Zyklusstadiums festgestellt werden.

Zusammenfassend zeigt sich unser Eileiterepithelmodell in Kombination mit dem Verfahren zum Monitoring der Zilienaktivität als ein vielversprechendes Werkzeug für die Grundlagenforschung in der Reproduktion sowie für Analysen bezüglich der Reproduktions- und Zilientoxizität. Wir simulierten hier erstmals verschiedene Stadien des Sexualzyklus an Eileiterepithelzellen von Säugetieren und konnten die damit verbundene Funktion von E2 und P4 in Bezug auf die Entwicklung und die Funktionalität des Eileiters nachweisen.

6. Outlook

In our POEC model, cells exhibited no signs of performance decline after 6w of culture. Therefore, we hypothesize that under given circumstances the cultivation duration might be further extended. We consider that TEER measurement may serve as an additional quality indicator, which could be routinely used in long-term POEC culture. However, the proper range of TEER values and their correlation with cell morphology need to be further defined. Our first preliminary results show that cells were able to maintain differentiated status at least up to 12w.

From traditional cell lines to our 3D culture model, oviduct epithelial cells in variant differentiation statuses are available now. Performing systematic gene expression analysis of cells adapted to different conditions, would give insights into their differences at the transcriptome level. In the future data would be very helpful to reveal the underlying pathways involved in ciliogenesis, polarization and transformation of oviduct epithelial cells, as well as to identify key genes regulating these processes.

Concerning the new approach for monitoring cilia activity, further optimization of experimental conditions, such as pH value, temperature and operating procedure would improve its accuracy and consistency among labs. When successful this stable system will be used to assess toxic effects on reproductive tissue in general and cilia activity in more details.

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8. Publication list

8.1. Publications

Chen S, Einspanier R, Schoen J. Long-term culture of primary porcine oviduct epithelial cells: validation of a comprehensive in vitro model for reproductive science.
Theriogenology. 2013 Nov; 80(8):862-9.
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Cheng X *, **Chen S *,** Yu X, Zheng P, Wang H. BMP15 gene is activated during human amniotic fluid stem cell differentiation into oocyte-like cells.
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8.2. Oral presentations

Chen S, Einspanier R, Schoen J. TEER as a quality indicator for polarized porcine oviduct epithelial cells during ultra-long term cultivation.
8th PhD symposium and DRS presentation seminar. Berlin, 15.07.2013.
Berlin: mensch und buch verlag, ISBN 978-3-86387-326-4, S. 38.

Chen S, Palma Vera S, Einspanier R, Schoen J. Simulation of the estrous cycle in a long-term culture system for porcine oviductal epithelial cells (POECs).
Reproductive Biology, Biotechnology, Animal Welfare and Biodiversity Conference. Gdansk, 27.02.–01.03.2013.
Reproductive Biology, Volume 13, Supplement 2, February 2013, S. 11-12.
DOI: 10.1016/j.repbio.2013.01.143

8.3. Poster presentations

Chen S, Einspanier R, Schoen J. Long term culture of porcine oviductal epithelium cells: Optimization, characterization and validation of an in vivo-like culture system.
45th Annual Conference of Physiology and Pathology of Reproduction. Berlin, 29.2.-2.3.2012
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20. Tagung der Fachgruppe Physiologie und Biochemie der DVG. München, 16.2.-18.2.2012.

Palma Vera S, **Chen S**, Einspanier R, Schoen J. Long-term culture of primary bovine oviduct epithelial cells: Impact of insulin and EGF supplementation.
Reproductive Biology, Biotechnology, Animal Welfare and Biodiversity Conference. Gdansk 27.02.–01.03.2013
Reproductive Biology, Volume 13, Supplement 2, February 2013, S. 44.
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10. Selbständigkeitserklärung

Hiermit bestätige ich, Shuai Chen, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

Berlin, den 22.08.2013 Shuai Chen