From the Institute of Animal Breeding
Veterinary Faculty of the
Ludwig-Maximilian-University Munich
Chair of Molecular Animal Breeding and Biotechnology
Univ.-Prof. Dr. Eckhard Wolf

Supervisor: PD Dr. Stefan Hiendleder

A short-term suspension culture system for bovine oviduct epithelial cells suitable for the study of embryo-maternal communication processes

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Regine Rottmayer
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Dekan: Univ.-Prof. Dr. E. P. Märtlbauer

Referent: Univ.-Prof. Dr. E. Wolf

Korreferent: Univ.-Prof. Dr. Dr. F. Sinowatz

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Abbreviations

°C	degree Celsius
µl	microliter
µm	micrometer
AngII	angiotensin II
ANOVA	analysis of variance
ANP	atrionatriuretic peptide
AT	annealing temperature
BDMA	benzyldimethylamine
BOEC	bovine oviduct epithelial cells
BRL	buffalo rat liver
BSA	bovine serum albumin
cDNA	complementary DNA
CO_2	carbon dioxide
CP	crossing point
CS 3.5	cow serum obtained on Day 3.5 of the estrous cycle
DDSA	dodecenylsuccincanhydride
DMEM/F12	Dulbecco´s modified Eagle´s Medium / Hams F12
DNA	deoxyribonucleic acid
e.g.	for example
E2	estradiol-17β
ECS	estrous cow serum
ESR1	estrogen receptor α
ESR2	estrogen receptor β
et al.	and others
ET-1	endothelin-1
FGF	fibroblast growth factor
Fig.	Figure
FITC	fluorescein isothiocyanate
G	gauge
g	gravity
GH	growth hormone
GPX4	B. taurus non-selenium glutathione phospholipid hydroperoxide peroxidase

h	hour(s)
H_2O	water
HA	hyaluronan
HMGCR	H. sapiens 3-hydroxy-3-methylglutaryl-coenzyme A reductase
IFNT	interferon tau
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IgG	immunoglobulin G
IL	interleukin
INOS	inducible nitric oxide synthase
IVC	*in vitro* culture
IVF	*in vitro* fertilization
IVP	*in vitro* production
kDA	kilodalton
l	liter
LH	luteinizing hormone
LSM	least squares means
M	molar
MB2	Menezo´s B2 medium
min	minute(s)
ml	milliliter
mm	millimeter
MPM	modified Parker medium
mRNA	messenger RNA
mV	millivolt
nm	nanometer
NMA	nadicmethylanhydride
O_2	oxygen
OF	oviduct fluid
OVGP1	oviduct-specific glycoprotein
P4	progesterone
PAF	platelet-activating factor
PAF-AH	platelet-activating factor acetylhydrolase
PAF-R	platelet-activating factor receptor

PBS P/S	phosphate-buffered saline with penicillin/streptomycin
PBS	phosphate-buffered saline
PBS–	phosphate-buffered saline without calcium and magnesium
PDGF	platelet-derived growth factor
Pen/Strep	penicillin/streptomycin
PGE2	prostaglandin E2
PGF2α	prostaglandin F2α
PGR	progesterone receptor
RHAMM-IHABP	receptor for HA-mediated motility/intracellular HA-binding protein
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-qPCR	real-time quantitative polymerase chain reaction
SEM	scanning electron microscopy
TCM-199	tissue culture medium 199
TEM	transmission electron microscopy
TGFβ2	transforming growth factor β2
TNFα	tumor necrosis factor α
TRA1	tumor rejection antigen 1
v/v	volume per volume
VEGF	vascular endothelial growth factor

1 Introduction

One of the most important problems in bovine reproduction and breeding is, also in economic matters, early embryonic death (Wolf et al. 2003).

The oviduct provides the optimal environment for gamete maturation, fertilization and early embryonic development. As it is the first organ in contact with the early embryo, the search for factors influencing fertility and signals exchanged between the embryo and the maternal environment should start here.

Early pregnancy events are difficult to study *in vivo*, as the differentiation between successful and unsuccessful artificial insemination at this stage is difficult and experiments are expensive. An adequate cell culture model suitable for co-culture experiments with bovine embryos would ease these studies and is therefore needed to further explore physiological changes occurring during the first days of pregnancy.

Oviduct cell culture systems were mainly used to improve culture conditions for *in vitro* produced (IVP) embryos. It was shown that co-culture of IVP-embryos with oviduct epithelial cells was beneficial due to embryotrophic effects of secretions of cultured cells as well as due to the removal of embryotoxic substances such as ammonia from the culture medium. It was shown that bovine embryos receive signals from cells from the reproductive tract, but similar signals, sent by the embryo and affecting cells of the maternal organism, were shown only in the mouse model.

The aim of this study was to establish and evaluate a culture system for BOEC suitable for studying early processes of embryo-maternal communication in the oviduct. A short-term culture system, allowing co-culture experiments for bovine embryos for 12 to 24 hours and maintaining physiological cell functions during this time would be eligible. A very important factor to consider was the cell yield for further sample processing.

Current methods for functional genome reseach, such as holistic transcriptome or proteome studies well defines biological material in sufficient quantities. This requirement could not be fulfilled by the established culture systems for BOEC. As an essential part of the DFG research group "Mechanisms of embryo-maternal communication" (DFG 478/1,2), this project created the basis for co-culture experiments of BOEC and the corresponding embryonic stages to systematically analyse early embryo-maternal interactions

2 Literature

2.1 Morphological characteristics of the bovine oviduct

2.1.1 Anatomy of the bovine oviduct

The bovine oviduct, also referred to as bovine fallopian tube, is a pairwise tubular structure, about 20 to 28 cm in length, embedded in a serosa fold, the *mesosalpinx*, a peritoneal protuberance. The oviduct provides a connection between ovary and uterine horn of either side passable for gametes and embryos. It consists of five morphological and functional distinguishable parts, the infundibulum, the ampulla, the ampullary-isthmic junction, the isthmus and the utero-tubal junction. The **infundibulum** as the most proximal part is funnel-shaped and shows fringes, *fimbriae*, sometimes adhering to the ovary (*Fimbriae ovaricae*) as an anchorage (Nickel et al. 1987). It takes actively part in oocyte uptake after ovulation. The **ampulla**, representing two thirds of the oviduct length, is the wider part of the tube, where oocyte maturation and fertilization take place. It is connected to the narrower part, the isthmus, by the **ampullary-isthmic junction**, which shows characteristics of both parts. The convoluted **isthmus** with its narrow lumen is known to act as a sperm reservoir as described below, together with the **utero-tubal junction** that marks the distal end of the oviduct (Yaniz et al. 2000).

In cross-section, the oviduct consists of different tissue layers: on its outer surface, turned towards the peritoneal cave, it is covered by a serosa layer, *Tunica serosa*, followed by the *Tela subserosa*, which contains muscle fibers and various blood vessels (Hees and Sinowatz 1986). The muscle layer, *Tunica muscularis*, is differentially developed dependent on the oviduct segment. In the infundibulum and ampulla, it is markedly thinner than in the isthmus. However, fibers are arranged in circular and longitudinal direction as well as spirally / helically (Liebich 1990). Their peristaltic contractions assist at transporting oviductal contents towards the uterus, especially regarding ovum transport and oviductal clearance. The *Tunica mucosa* as the inner tissue layer is composed of the *Lamina propria mucosae* which is represented by fibrous and cellular connective tissue building the framework for mucosal folds (Ellington 1991), and the *Tunica mucosa*, the epithelial cell layer (Liebich 1990).

2.1.2 Epithelium of the bovine oviduct

The epithelial cell layer covering the luminal surface of the bovine oviduct consists mainly of two different cell types, ciliated and secretory cells. Cilia are special protuberances found on the apical cell membrane, about 10 µm in length. Fixed beneath the apical cell membrane on a basal body, their inner structure shows 9 double microtubules encircling 2 microtubules ("9x2+2-structure"), in contrast to microvilli which consist of actin filaments and which are only 1-2 µm in length (Hees and Sinowatz 1986). For successful transportation processes, ciliary movement is synchronized via the arrangement of ciliary rootlets, the anchor of cilia structures in the apical cell region, and leads to a directed flow of fluids (Abe and Hoshi 1997, Hagiwara et al. 1997). Secretory cells show numerous microvilli on their apical side and are known to secrete products, e.g. the embryotrophic oviduct-specific glycoprotein, by exocytosis, especially in the first few days of the estrous cycle and of early pregnancy (Ellington 1991, Murray 1997, Menezo and Guerin 1997). Proportions of those cell types are reported to change during the estrous cycle in different species. In ruminants, studies show marked changes particularly in proximal parts of the oviduct. The mean percentage of ciliated cells significantly decreases in infundibulum and ampulla during the luteal phase compared to the follicular phase (Yaniz et al. 2000, Walter and Miller 1996). Furthermore, the height of ciliated cells was reported to decrease in the infundibulum and ampulla in the luteal phase, whereas in more distal parts of the oviduct the height of non-ciliated cell populations was diminished (Abe et al. 1999). In another study it was suggested that the cell population remains unchanged but cell morphology alters in the sense of development and degradation of cilia due to cyclic changes affecting the hormonal environment (Suuroia et al. 2002).

There are two more, but rare cell types of the oviduct epithelium described, narrow cells in the epithelial layer which are suggested to be secretory cells after secretion, probably in preparation of their removal towards the lumen out of the epithelial assembly, and rounded basal cells which were long time thought to be new developing epithelial cells (Liebich 1990, Hees and Sinowatz 1986). Though, the absence of mitotic figures as well as the lack of cytokeratin and S-100 expression in these cells strongly suggest their lymphocyte character (Walter and Miller 1996), affirmed by the demonstration of CD3 expression, a T-lymphocyte cell marker, in all basal cells in human oviduct epithelium (Comer et al. 1998).

2.2 Physiological functions of the bovine oviduct

2.2.1 Gamete maturation, sperm capacitation and fertilization

Crucial reproductive events take place in the oviduct which appears to be an extremely specialized organ although multifaceted to fulfill its many functions. The infundibulum picks up the oocyte during ovulation in an actively moving manner. Cilia on its epithelial covering support the oocyte in finding the right direction towards the oviductal lumen, as observed in hamster (Talbot et al. 2003). In the ampulla, oocyte maturation and fertilization take place (Hees and Sinowatz 1986, Ellington 1991, Talbot et al. 2003).

Ascending sperm after mating or artificial insemination are known to remain in the isthmus, especially the distal part of the isthmus, establishing adhesive contact to epithelial cells. This phenomenon was reported in different species such as cattle (Hunter and Wilmut 1984, Lefebvre et al. 1995), sheep (Hunter et al. 1980), hamster (DeMott et al. 1995), horse (Troedsson et al. 1998), pig (Rodriguez-Martinez et al. 2005) and human (Mastroianni, Jr. 1999), and therefore seems to be a highly conserved mechanism in reproductive biology. Sperm survival until ovulation is ensured by a prolonged motility, viability and fertility due to the interactions with oviduct epithelial cells (Talevi and Gualtieri 2004). Sperm release due to a change in affinity of spermatozoa towards epithelial cells may be caused by heparin-like glycosaminoglycans that are physiological components of the bovine oviduct fluid, whose concentrations and capacitating activities change, being maximal at estrous (Bosch et al. 2001, Talevi and Gualtieri 2004). Oviduct adhesion processes do not only conduce to sperm storage in the oviduct until ovulation, but also assist in selecting a higher quality sperm subpopulation (Ellington et al. 1999, Gualtieri and Talevi 2000, Gualtieri and Talevi 2003). It was shown that sperm selected through adhesion to the fallopian tube have a strong advantage both in the ability to bind to the zona pellucida and to fertilize the oocyte (Talevi and Gualtieri 2004).

After fertilization in the ampulla, the developing embryo passes through the oviduct, supported by ciliary beat and muscular contraction of the oviduct, until reaching the uterus at about the 16-cell-stage (Ellington 1991).

2.2.2 Oviduct fluid

The main role of providing the optimal environment for the various events taking place in the oviductal lumen is represented by the production of oviduct fluid (OF), containing ions, nutrients, proteins, hormones, growth factors, carbohydrates, lipids and amino acids. OF composition has been analyzed in different species, including cattle, rabbit and human (Leese et al. 2001). It is generated by transsudation into the oviduct lumen as well as by secretion of substances synthesized in epithelial cells (Ellington 1991, Nancarrow and Hill 1994, Menezo and Guerin 1997).

Changes to cell morphology as described above indicate changes in secretion activity that also may contribute to a variation in OF production. The produced OF amount is dependent on the stage of estrous cycle: cows produce 0.2 ml OF per day in diestrous and 2.0 ml in estrous (Roberts et al. 1975). In ewes, the produced amount could be experimentally influenced by hormones, effecting an increase by estradiol-17β and a decrease by progesterone supplementation (Leese et al. 2001).

Amino acid concentrations in OF were analyzed in different species, showing that glycin and alanin are representing the highest proportions, with taurine and hypotaurine playing an important role in oviduct function. Glycin shows a protective effect on preimplantation mouse embryos against the detrimental influence of inorganic ions (Leese et al. 2001). Taurine and hypotaurine are major constituents of OF and are important in supporting the viability of gametes and preimplantation embryos (Leese et al. 2001).

Glucose levels in bovine oviducts are extremely low in comparison with serum concentrations suggesting that this is important for successful sperm capacitation and embryonic development in bovine. Bovine embryos during pre-elongation development gain their energy by oxidative phosphorylation, i.e. the oxidation of pyruvate and amino acids. Only in the stage of compaction and blastulation they switch to glycolysis. This means in early stages, there is no need of high glucose amounts in their environment, in contrast, high glucose levels may be deleterious (Takahashi and First 1992, Kim et al. 1993, Thompson 2000).

Oviduct-specific glycoprotein (OVGP1) is well-documented and appears in OF of many species in a highly conserved manner. It is known to interact as well with oocytes as with sperm (Buhi 2002). Its quantity is highest in the periovulatory phase. Synthesis and secretion are thought to be related to estrogen stimulation (Buhi 2002, Briton-Jones et al. 2004, Killian 2004) or to stimulation by luteinizing hormone (LH; Sun et al. 1997). Its binding to the zona pellucida of oocytes and early embryos points towards a role in early development (Buhi 2002). Also, interactions with spermatozoa are reported leading to increased rates of

fertilization and embryonic development (Killian 2004). Despite the enhancement of fertilization, sperm capacitation and penetration, it was suggested that OVGP1 stabilizes the microenvironment immediately surrounding gametes and embryo, preventing dispersal of essential nutrients and ions, particularly during ciliary beating or muscular contraction, by increasing the viscosity of luminal fluid (Hunter 1994). Interestingly, OVGP1 is not necessarily inalienable *in vitro*, as oocytes, spermatozoa and embryos are able to survive without co-cultured oviduct epithelial cells or other type somatic cells. The more, null mutation mice showed regular fertility characteristics, proving that at least in mice, OVGP1 is not essential for the process of *in vivo* fertilization (Araki et al. 2003). These results suggest OVGP1 is not the "universal oviduct elixir" but a variety of factors acting together is responsible for successful fertilization and embryonic development (Killian 2004).

2.2.3 Transport mechanisms in the oviduct

All reproductive processes described are dependent on transportation mechanisms. The oocyte passes through the proximal parts of the oviduct to the site of fertilization, the distal part of the ampulla, spermatozoa have to migrate to this site arriving from the uterus, and after successful fertilization the early embryo passes the isthmus towards the uterus again. Ciliary beat and muscular contractions support these migration processes.

Ciliary currents in mammalian oviducts of different species were examined by Gaddum-Rosse and Blandau (1976). In cows, only transportation processes towards the uterus could be observed, which were independent of the stage of estrous cylce and particularly striking in the isthmus. Differences between species were reported, as in pigs and rabbits in some parts of the isthmus pro-ovarian ciliary currents were observable. In guinea pigs and rats, ciliary current was only detected in the ampulla or merely in its proximal part, but not in the isthmus, due to the presence of very few cilia. In humans and pig-tailed macaques, the results were similar to those described in cows (Gaddum-Rosse and Blandau 1976).

Another important mechanism for transportation processes, taking place in the oviduct, are contractions of the surrounding muscle layer, *Tunica muscularis*. The role of myofibroblasts in the oviduct was elucidated, suggesting that a network of these contractile cells in the oviductal mucosa might influence condition and volume of the mucosal folds and might therefore be indirectly involved in tubal transport mechanisms. The activity or presence of myofibroblasts changes during the estrous cycle and might be influenced by steroid hormones (Walter 1998).

Wijayagunawardane et al. studied processes related to oviduct contractions. In cell culture experiments, the ovarian steroids estrogen and progesterone and prostaglandins E2 (PGE2) and F2α (PGF2α), and oxytocin and endothelin-1 (ET-1) were shown to synergistically control oviduct contraction. Particularly, combination of luteinizing hormone (LH) and high estrogen (E2) with low progesterone (P4) levels in the medium, mimicking the hormonal situation around ovulation *in vivo*, led to stimulation of the production of contraction-related substances such as prostaglandins and endothelin-1 by bovine oviduct epithelial cells. Prostaglandin stimulates smooth muscle cells and the ciliary activity of epithelial cells in the bovine oviduct and therefore facilitates ovum or zygote transport through the oviduct. This results in adequate conditions for embryo transport during the periovulatory period (Wijayagunawardane et al. 1998, Wijayagunawardane et al. 1999). Furthermore, the role of vasoactive peptides angiotensin II (AngII) and atrial natriuretic protein (ANP) was examined by a microdialytic oviduct system. The existence of a functional endothelin-angiotensin-ANP-system in the bovine oviduct was suggested to be possibly involved in regulatory processes of oviduct contractions during the periovulatory period (Wijayagunawardane et al. 2001a). Interestingly, oxytocin released from the newly-formed corpus luteum blocked these mechanisms, pointing towards a slow-down of transport speed in the oviduct (Wijayagunawardane et al. 2001b). Another member of this complex regulatory network is tumor necrosis factor alpha (TNFα), known as an important mediator of cell-to-cell communication. In *in vitro* experiments it stimulated secretion of PGs, ET-1, and AngII in oviducts obtained in follicular and periovulatory stage (Wijayagunawardane et al. 2003). The TNFα system was suggested to further optimize the release of contraction-related substances and to modulate local contraction processes (Wijayagunawardane et al. 2003). In addition, embryos at 2- to 4-cell stage express TNFα, so that the secretions by the embryo may further act locally to enhance the production of PGs, ET-1 and AngII in the oviduct, which may result in an active oviductal contraction in the microenvironment surrounding the embryo (Wijayagunawardane and Miyamoto 2004). Vascular endothelial growth factor (VEGF) is a potent angiogenic and permeability enhancing factor. Its role in periovulatory stage, when it shows highest oviductal activity, was examined recently. VEGF directly stimulates synthesis and release of PGs and ET-1 in the oviduct and is stimulated itself by LH. Due to a negative feedback regulatory mechanism, VEGF shows a very temporary activity pattern, even more under LH influence (Wijayagunawardane et al. 2005). These very complex interactions between different regulatory systems in the bovine oviduct resulting in ideal conditions for

embryo transport to ensure arrival in the uterus just at the right time for further embryonic development and establishment of pregnancy (Wijayagunawardane et al. 2005).

2.3 Embryo-maternal communication

2.3.1 Embryo-maternal communication in the oviduct

For establishment of a successful pregnancy, there must be a signal exchange between the embryo and the maternal organism. In ruminants, the best known signal sent by the embryo essential for pregnancy-recognition is interferon τ (IFNT). It is secreted by the trophoblast from Day 10 up to Day 21-25 with maximal production on Days 14 to 16 (Spencer and Bazer 2004). As up to 40 % of embryonic losses occur between Day 8 and 17 of pregnancy (Humblot 2001, Thatcher et al. 2001), there may be disturbances in these first days of pregnancies also possibly independent of IFNT. The oviduct provides the first environment for the developing embryo, therefore the search for embryo-maternal communication signals must start here (Wolf et al. 2003).

A study in hamsters by Ortiz et al. (1986) showed that fertilized oocytes were transported towards the uterus faster than their unfertilized counterparts. This counted for fertilization by mating as well as by *in vitro* procedures. Astonishingly, in hamsters with fertilized oocytes transferred into one oviduct and unfertilized oocytes into the other, fertilized ova appeared earlier in the uterus. Thus, the suggestion of a maternal answer to the presence of embryos at that early stage is reasonable (Ortiz et al. 1986). This phenomenon appears to be mediated locally, not systemically. Changes in the zona pellucida occuring either by fertilization or by aging of the unfertilized oocytes may account for a change in elasticity or adhesiveness in the oviduct. Also, secretion of a chemical signal is a possible explanation of this phenomenon (Ortiz et al. 1986). However, the same research group did not find identical oviductal reactions to embryo presence in rats. Here, the transport rate was dependent on the stage of the transferred embryos as more advanced stages were transported faster than earlier stages. In rats, the differential transport rate of unfertilized *versus* fertilized oocytes seems to become relevant no earlier than in the uterus (Ortiz et al. 1989, Villalon et al. 1982). A self-supporting mechnism of embryos was also observed in the mare, where Day 5 morulae secrete prostaglandine E2 (PGE2) thereby stimulating their transport towards the uterus (Weber et al. 1991, Freeman et al. 1992). In accordance to hamster studies, platelet-activating factor (PAF)

secretion by human embryos was examined as well as the expression of platelet-activating factor receptor (PAF-R) and platelet-activating factor acteylhydrolase (PAF-AH) in the oviduct. The results point towards a key role of embryo-derived PAF and its receptor and catabolizing enzyme in the fallopian tube in early interactions between an embryo and the maternal organism (Velasquez et al. 2001). A recent study using RT-qPCR technique proved changes in gene expression patterns in the mouse oviduct depending on the presence or absence of embryos passing through the organ *in vivo* (Lee et al. 2002a).
In bovine, systems involved in very early embryo-maternal communication may include the growth hormone, the IGF-system and other growth factors, the hyaluronic acid system and PAF (Wolf et al. 2003).

2.3.2 Growth hormone

Growth hormone (GH) is an important modulator in many aspects of female reproduction. Obligatory for growth and development, it is involved in processes of sexual differentiation and pubertal maturation, gonadal steroidogenesis, gametogenesis and ovulation and has additional functions in pregnancy and lactation. It directly acts endocrine itself or via induction of hepatic or local insulin-like growth factor I (IGF1) production. Synthesized in gonadal, placental and mammary tissues, it may also act in an autocrine or paracrine manner (Hull and Harvey 2001).
Bovine embryos were shown to synthesize GH and its receptor in a stage-specific manner. *In vitro*-derived embryos cultured in medium supplemented with GH showed characteristics very similar to *in vivo*-derived embryos, with respect to morphological features such as increased cell numbers in the inner cell mass and trophectoderm and decreased apoptosis rates. Also, metabolic characteristics such as glycogen elimination and increased exocytosis of lipids were shown (Koelle et al. 2001, Koelle et al. 2002). Compatibly, bovine oviduct epithelial cells express the GH receptor (Hull and Harvey 2001) enabling them to react with embryo-produced GH.

2.3.3 IGF-system

Insulin and insulin-like growth factors (IGFs) are a group of structurally related polypeptides that regulate growth of many cell types. The growth-promoting activity of these polypeptides is mediated by binding to receptors on the cell membrane and IGF transport and function are modulated by interactions with at least six insulin-like growth factor binding proteins

(IGFBPs), which are present in many extracellular fluids and in early embryos (Winger et al. 1997, Pushpakumara et al. 2002). In the bovine oviduct, the IGF system was studied *in vivo*, revealing different expression patterns of IGF types and their receptors and binding proteins. Locally or systemically produced IGFs, regulated by IGFBPs, may act directly on the embryo or via modulation of oviduct secretions and muscular activity to positively influence the success of early embryonic development (Pushpakumara et al. 2002).

2.3.4 Other growth factors

Growth factors are hormone-like polypeptides and proteins, acting predominantly paracrine and autocrine by promoting mitogenic activity in local tissue proliferation and remodeling, e.g. transformation of the ovarian follicle into a corpus luteum (Hafez and Hafez 2000). Fibroblast growth factor (FGF) is a polypeptide with angiogenic as well as mitogenic properties and fulfills diverse functions in the female reproductive tract. It stimulates proliferation of various cell types required with blastocyst implantation and embryonic development (Hafez and Hafez 2000). Vascular endothelial growth factor (VEGF) is connected with physiological alteration processes in the female reproductive organs, accompanied by changes of blood flow. It takes part in regulation of follicular development, steroidogenesis, corpus luteum formation and maintenance, endometrial vascular growth and permeability and fetal development and placental function. As it has been detected in the oviduct in human and bovine, a possible role in the regulation of early reproductive events in the oviduct has been discussed (Hafez and Hafez 2000, Wijayagunawardane et al. 2005). FGF and VEGF are known to be secreted in the bovine oviduct in different amounts depending on the stage of estrous cycle. FGF-1 is increased at ovulation whereas FGF-2 is increased after ovulation. VEGF increases before ovulation, therefore a role in creating an optimal environment for physiological processes in the oviduct such as gamete maturation, fertilization and early embryo development is possible (Wijayagunawardane et al. 2005). *In vitro*, FGF-2 promotes development of bovine embryos beyond the 8-cell block (Larson et al. 1992) and VEGF enhances *in vitro* maturation of oocytes and accelerates development of early embryos (Gabler et al. 1999, Luo et al. 2002a, Luo et al. 2002b). A possible VEGF function in regulation of embryo transport was discussed recently (Wijayagunawardane et al. 2005).

Similarly, another growth factor, tumor necrosis factor alpha (TNFα), was reported to possibly lead to an embryo-induced modulation of muscle contractions in the oviduct, giving

a real hint to very early embryo-maternal communication processes (Wijayagunawardane and Miyamoto 2004).
Beneficial effects of platelet-derived growth factor (PDGF) on embryonic development *in vitro* were shown, providing the possibility of another factor in embryo-maternal crosstalk (Larson et al. 1992, Thibodeaux et al. 1993).

2.3.5 Hyaluronic acid system

Hyaluronic acid (HA), a glucosaminoglycan and component of the extracellular matrix, regulates cellular events such as signaling, gene expression, proliferation, differentiation, adhesion, migration and morphogenesis. HA is a normal component of mammalian follicular, oviduct and uterine fluids (Lee, Ax 1984). Granulosa and expanding cumulus cells produce large amounts of HA, the secretion by embryos starts on Day 2 and is stage-dependent (Stojkovic et al. 2002, Stojkovic et al. 2003). The system is regulated by the binding of HA to proteins and surface and intracellular receptors. Positive effects of HA on cell numbers and apoptosis rates in blastocysts were described as well as the regulative effect on the receptor for HA-mediated motility/intracellular HA-binding protein (RHAMM/IHABP) expression, a phenomenon probably related to the polarization and differentiation processes finally resulting in formation of trophectoderm and inner cell mass (Stojkovic et al. 2003). A marked increase takes place during elongation of the embryo and establishment of embryo-maternal interactions (Stojkovic et al. 2003). The influence of HA-receptor RHAMM on ciliary beat in the bovine oviduct epithelium was discussed (Ulbrich et al. 2004), confirming a function of HA as a signal in early embryo-maternal communication.

2.3.6 Platelet-activating factor

Platelet-activating factor (PAF) is a potent phospholipid mediator produced by several cell types: neutrophils, macrophages, endothelial cells, and preimplantation embryos. PAF induces a wide range of physiologic and pharmacologic responses involving reproductive processes, platelet aggregation, anaphylaxis, and vascular permeability. PAF, produced by sperm, enhances sperm motility and *in vitro* fertilization during co-incubation of sperm and egg (Hafez and Hafez 2000).
Platelet-activating factor is described in hamsters and humans as a promising candidate for an early signal of embryo-maternal communication in the oviduct (Velasquez et al. 1995, Velasquez et al. 2001).

In sheep IVP-embryos, PAF is produced under different culture conditions (Battye et al. 1991). In human embryos, PAF secretion has been shown as well (O'Neill et al. 1990). Interestingly, in different species expression of platelet-activating factor receptor (PAF-R) in the oviduct followed a compatible pattern, PAF-R increasing at stages when the embryo passes the oviduct (Yang et al. 1992, Lash, Legge 2001, Velasquez et al. 2001). PAF was described to increase intracellular Ca^{2+} level by Ca^{2+} influx from the extracellular space (Tiemann et al. 1996, Downing et al. 2002) and therefore it might be another factor involved in the regulation of embryo transport towards the uterus (Hermoso et al. 2001).

2.4 Somatic cells in culture

2.4.1 Cell culture techniques

Cell culture techniques are helpful to examine biological processes that are not easy to study *in vivo*. Concerning the oviduct, it is a useful tool as this organ cannot easily be reached for taking biopsy material. Examinations of the oviduct are always connected with surgical procedures implying the disturbance of the physiological environment compared to real *in vivo* conditions. In contrast, under culture conditions behavior of cells can be studied free of systemic variations that might arise in the animal both during normal homeostasis and stress of an experiment. For culture, cells are taken from oviducts of slaughtered animals or oviducts are obtained during surgery and cultured in a nutrient mixture for the following days, sometimes being passaged for multiplying cell numbers. The aim is to gain differentiated cell material, maintaining the physiological cell characteristics comparable to their characteristics *in vivo* and therefore obtaining a reliable *in vitro* model system. The evaluated characteristics might include cell morphology and ultrastructural features, the maintenance of tissue structures such as connections between neighbouring cells and cell polarity, enzyme activities and antigenic markers (Freshney 2000).

Obtained cells may be singularized for consistant seeding using enzyme solutions e.g. containing collagenase or trypsin or they may stay in a state of natural coherence to their neighbouring cells (Freshney 2000). Cells may adhere to the culture dish or to a substrate as support (e.g. different types of plastic, glass, nylon membranes, cellulosenitrate) and grow to confluence (Reischl et al. 1999) or they may stay suspended in culture medium without adhering to the bottom of the culture dish as shown below.

When cells are singularized before seeding there is a high probability for a uniform cell population and distribution of different cell types at seeding. However, the possibility exists that enzymatic actions may cause damage to cells or their surfaces or to surfaces of special cell populations and therefore proportions may be altered in culture not anymore representing the distribution like *in vivo* (Freshney 2000). Additionally, certain cell types may adhere easier to the support than others, causing loss of representative proportions (Fortier et al. 1988). To obtain a monolayer, the seeded cells have to divide, but a connection between mitosis in culture and cell dedifferentiation is strongly suggested (Walter 1995). Cell characteristics may be lost in accordance with the celerity of monolayer growth.

2.4.2 Organ or organotypic culture

Cell functions in tissue of a multicellular organism depend on the interactions among cells. Using explants, small tissue pieces potentially containing different cell types in their natural arrangement, tissue architecture and connections between the integrated cells are maintained (Fig. 1). Organ cultures tend to retain the differentiated properties of the tissue and histological characteristics. However, as differentiated cells in organ culture do not grow, there may be a reduced amount of cells for sampling for modern biotechnological assays such as array hybridization techniques. Exact quantification of cells in explants is difficult and as each experiment requires new explants, reproducibility may be less than using established cell lines. In a organotypic culture, a recombination of two or more separately cultured and established cell lines, e.g. in a three-dimensional structure, is possible (Fig. 2; Freshney 2000).

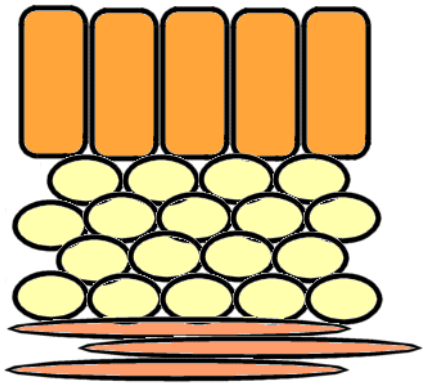

Fig. 1 Schematic depiction of an explant in organ culture. A small piece of tissue containing different cell types in their natural coherence is cultured.

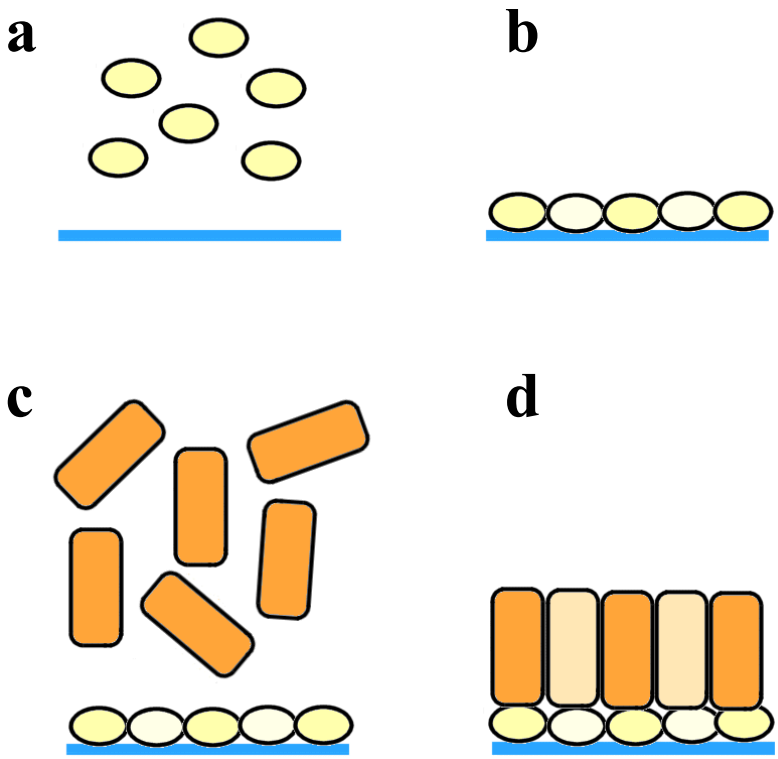

Fig. 2 Schematic depiction of an organotypic culture system. One cell type, e.g. stromal cells, is seeded (a) and forms a monolayer (b). A second cell type, e.g. epithelial cells, is seeded on top of the first monolayer (c) and grows itself to a monolayer (d). Thus, tissue architecture as found in vivo is reconstructed.

2.4.3 Primary explant culture

A small tissue fragment (up to 1 mm^3) is placed on a glass- or plastic-liquid-interface, where after attachment migration is promoted in the plane of the solid substrate forming a monolayer (Fig. 3; Freshney 2000). An advantage of this technique is the lack of enzymatic procedures for cell singularization which could damage cells. However, when cells divide and grow to a monolayer, there is the strong possibility of dedifferentiation processes (Gualtieri and Talevi 2000).

Explants may also remain in suspension, not adhering to the culture dish, especially in short-term culture systems (Fig. 4). These cells remain in their natural coherence to each other and may maintain specific cell-to-cell interactions (Walter 1995, Freshney 2000). Polarity and cell morphology do not underlie dedifferentiation processes in such a fast rate as described above. In suspension, no mitoses will occur (Walter 1995), meaning that dedifferentiation is prevented, but also that cell numbers remain constant, so that initial cell numbers at seeding have to be clearly higher to gain comparabale yields for sampling.

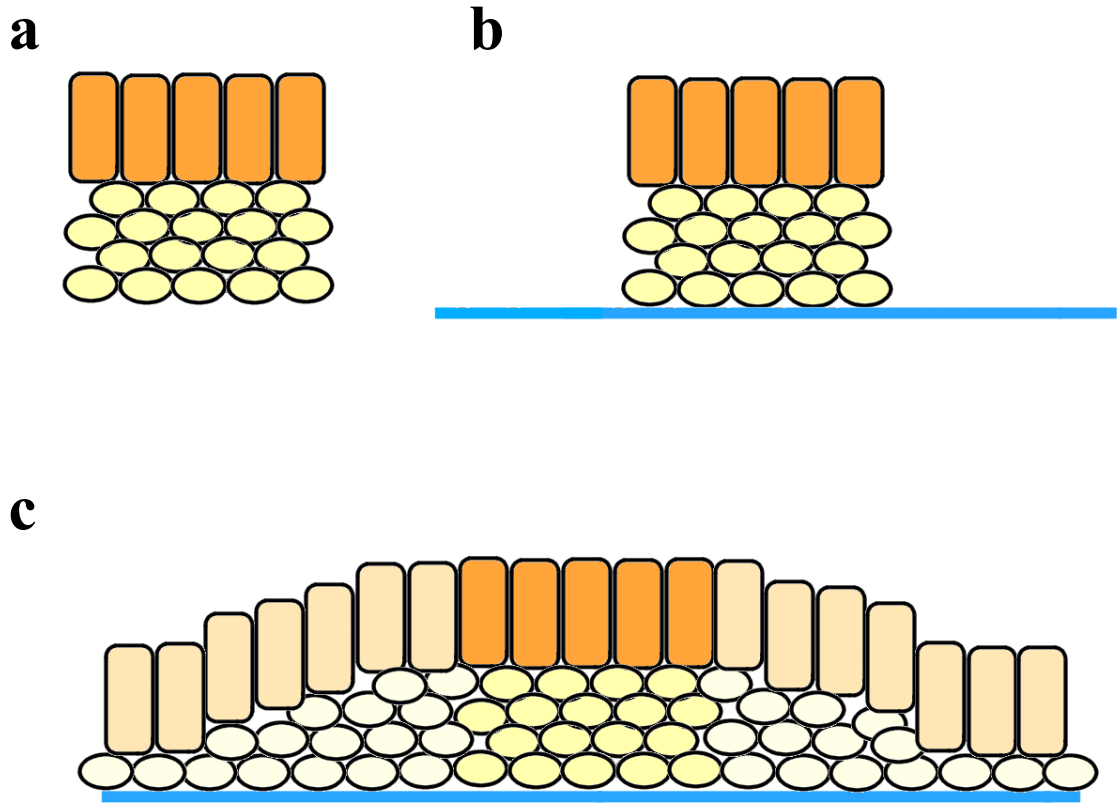

Fig. 3 Schematic depiction of a primary explant culture with outgrowth. Small pieces of tissue (a) are cultured and attach to the culture dish (b). After adhesion, cells begin to divide and form a monolayer (c). Explants may also consist of only one cell type.

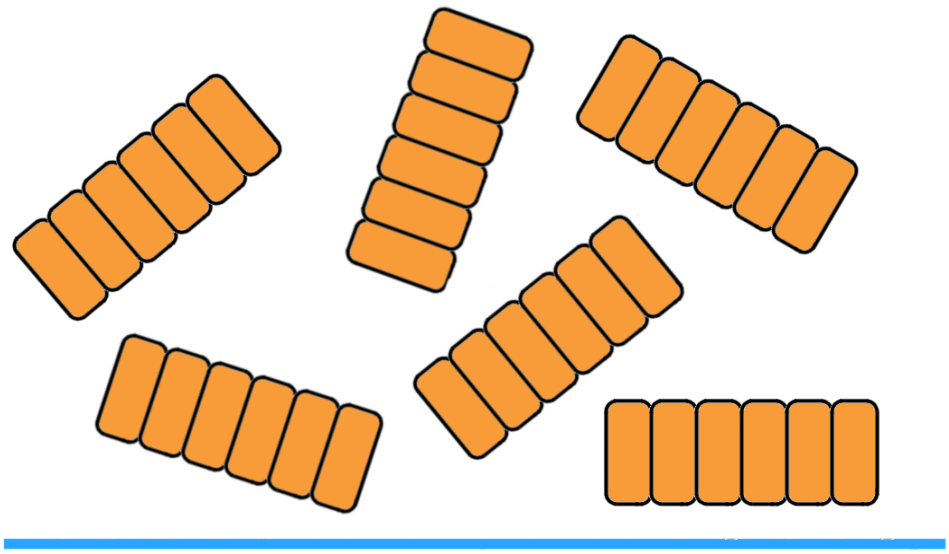

Fig. 4 Schematic depiction of a suspended primary explant culture. Explants do not attach to the culture dish and cells in explants do not further divide.

2.4.4 Cell culture

Tissue, or outgrowth from the primary explant, is dispersed into a cell suspension which may then be cultured after attachment of cells as an adherent monolayer on a solid substrate (Fig. 5) or, non-attached, as a cell suspension in the culture medium (Fig. 6; Freshney 2000).

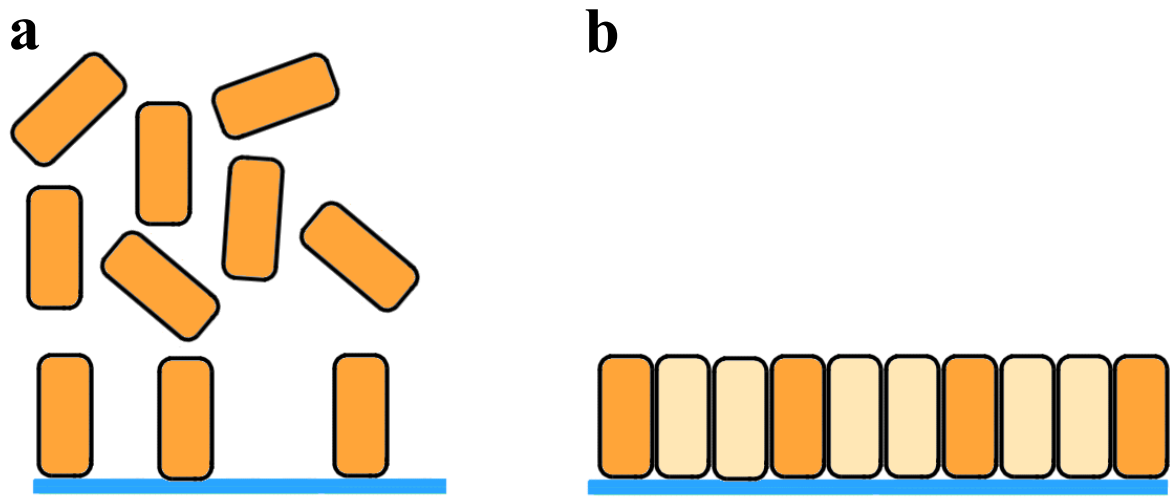

Fig. 5 Schematic depiction of a monolayer cell culture system. Singularized cells are seeded, some of the cells attach to the culture dish (a) and begin dividing, resulting in a confluent monolayer (b).

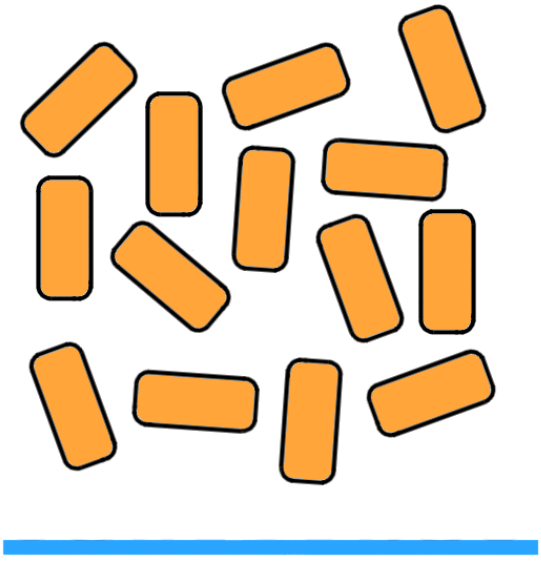

Fig. 6 Schematic depiction of a suspension cell culture system. Singularized cells are seeded and they do not adhere to the culture dish, due to their own movement or due to stirring of the culture medium.

2.5 Bovine oviduct epithelial cells in culture

2.5.1 Different cell preparation methods for BOEC

In former studies using cultured BOEC, cells were mostly obtained from cows slaughtered at the slaughterhouse. This seems to be problematic as a considerable proportion of cows are slaughtered for fertility reasons. Different studies showed that 20 to 40 % of slaughtered cows were culled for fertility reasons (Grunert 2003). Hence, there is a high probability of working with affected cell material not showing physiological or healthy conditions.

Mostly, the stage of estrous cycle has been estimated by ovarian morphology according to criteria described by Ireland et al (1979). However, no consistent pattern appears recognizable. For co-culture experiments with spermatozoa, BOEC obtained from oviducts in the luteal phase (Boquest and Summers 1999, Boquest et al. 1999) were used as well as cells from follicular phase (Bosch et al. 2001, Ellington et al. 1998). Experiments on LH function employed BOEC derived from late follicular phase oviducts, what is reasonable with respect to the physiological conditions in the oviduct at the time when LH is present (Mishra et al. 2003, Sun et al. 1997). For co-culture with embryos, cells from estrous (Rief et al. 2002) or short after ovulation (Pegoraro et al. 1998) have been used, whereas Gandolfi et al. (1987) used cells derived from the oviducts of donor animals after superovulation and embryo flushing, ensuring an analogy between BOEC and embryos in co-culture experiments (Gandolfi and Moor 1987).

For the most part, BOEC were obtained by enzymatic procedures using trypsin and pancreatin together (Cox and Leese 1997, McNutt-Scott, Harris 1998), trypsin alone (Thibodeaux et al. 1991, Winger et al. 1997, Reischl et al. 1999) or collagenase (Sun et al. 1997, Bosch et al. 2001, Mishra et al. 2003) in different concentrations. Cells were singularized by enzymatic treatment and seeded to attach to the culture dish and grow to a confluent monolayer. However, as the oviduct epithelium consists of two different cell types, ciliated and secretory ones, there is the possibility that the two cell populations may show different adhesion behavior. While ciliated cells are in constant motion, the non-moving secretory cells seem to adhere easier and faster. This could cause an overproportional quantity of secretory cells under culture conditions. The same phenomenon may be caused by specific damage of ciliated cells by enzymatic treatment due to their bigger cell surface. Thus, in some studies and particularly in recent years, cells were obtained by mechanical procedures remaining in cell aggregates which were mostly cultured non-adhesively in a suspension culture system

(Boquest and Summers 1999, De Pauw et al. 2002, Gualtieri and Talevi 2000, Kamishita et al. 1999, Lim et al. 1997, Pegoraro et al. 1998, Ulbrich et al. 2003, Yadav et al. 1998).
Obtained cell numbers after using the standard trypsin method were reported in two studies: Between 0.8 and 3.1 x 10^6 cells per oviduct were gained, depending on different preparation methods (Thibodeaux et al. 1991, Reischl et al. 1999). Considering the use of modern biotechnology techniques such as construction of subtracted cDNA libraries, these cell numbers are not sufficient for analyses. Thus, a protocoll yielding higher cell numbers is needed.

2.5.2 Co-culture experiments with embryos

Most experiments using cultured BOEC aimed at providing an optimized *in vitro* environment for embryo culture as BOEC were shown to support IVP-embryos to overcome the developmental block occurring in the 8-16 cell stage in ruminant embryos (Gandolfi et al. 1989, Ellington et al. 1990). Positive effects on embryos by co-culture with BOEC were caused by secretion of embryotrophic substances such as growth factors (Nancarrow and Hill 1994, Vanroose et al. 2001) as well as by modulating the surrounding environmental conditions, i.e. medium. BOEC in co-culture were reported to lower the oxygen level in the culture medium, thereby preventing the formation of deleterious radicals (Thompson 2000, Vanroose et al. 2001), as well as by removing embryotoxic substances, such as ammonia (Nancarrow and Hill 1994) from the medium and lowering glucose and ion levels that might have detrimental effects on embryos (Vanroose et al. 2001). However, these effects do not seem to be limited to oviduct cells from the same species but co-culture does also work with embryos and heterologous cells obtained from different species such as rabbit, mouse, bovine or human:

- **Bovine** embryos and **rabbit** oviduct epithelial cells (Carney et al. 1990, Ellington et al. 1990)
- **Mouse** embryos and **human** oviduct epithelial cells (Ouhibi et al. 1990 Takeuchi et al. 1992, Liu et al. 1995, Xu et al. 2000, Lee et al. 2001, Xu et al. 2001, Xu et al. 2003)
- **Human** embryos and **bovine** oviduct epithelial cells (Wiemer et al. 1993, Feng et al. 1996)
- **Goat** embryos and **bovine** oviduct epithelial cells (Yadav et al. 1998)

Additionally, even co-culture with cumulus cells or with non-reproductive tract cells such as BRL or Vero cells shows positive effects on embryonic development *in vitro* (Goto et al. 1988, Goto et al. 1992, Goto et al. 1994, Rehman et al. 1994, Menck et al. 1997, Krisher et al. 1998, Pegoraro et al. 1998). This may be due to the fact that not one or only few soluble factors are responsible for successful development but an ensemble of different signals and factors acts cooperatively (Killian 2004). This hypothesis may be affirmed by the perception that no direct contact between BOEC and embryos is required to achieve positive effects (Lim et al. 1997, Eyestone and First 1989, McCaffrey et al. 1991).

However, in recent years, successful IVF- and IVC-protocols without the need of co-culture with somatic cells or cell-conditioned media were established (Vanroose et al. 2001). This appears to be reasonable, as standardized culture conditions are better controllable, because cells, especially in primary culture, may always be a risk of contaminating cultures with pathogens as long as no well characterized and established cell lines are used (Pegoraro et al. 1998). The use of co-culture systems for commercial embryo production seems impractical in terms of difficult standardization (Vanroose et al. 2001).

However, none of the described co-culture studies examined the impact of a present embryo on BOEC, whereas the other direction was investigated quite intensively aiming to gather information about how to obtain optimal developmental rates of commercial *in vitro* embryo production in different species. As studies in mouse detected an alteration in gene expression patterns in oviduct cells caused by the presence of embryos (Lee et al. 2002b), this new direction of research employing co-culture systems may be considered more intensively now also *in vitro* and in other species such as bovine, giving the opportunity to learn more about the physiology of successful reproduction.

2.5.3 Studies on oviduct function

Additionally to the improvement of IVP-conditions, there are *in vitro* studies aiming at elucidation of physiological processes in the oviduct such as formation of oviduct fluid or function of pathways involved in physiological and pathological processes. Many studies address interactions between oviduct epithelium and spermatozoa. Sperm binding to the epithelial cells was observed *in vitro* (Boilard et al. 2002, Boquest and Summers 1999, Boquest et al. 1999, Gualtieri and Talevi 2000, Hunter 2003, Talevi and Gualtieri 2004), gaining the insight that this process is repeatable (Gualtieri and Talevi 2000) and that a kind of sperm selection may take place (Ellington et al. 1999, De Pauw et al. 2002, Talevi and Gualtieri 2004).

Effects of factors that might play a role in early pregnancy were studied as well by *in vitro* means. The role of regulative and embryotrophic mechanisms important in early pregnancies could be verified in cell culture experiments. The impact of PAF (Downing et al. 2002), the AngII system (Mahmood et al. 2002, Wijayagunawardane et al. 2001a) and LIF secretion (Reinhart et al. 1998) on the formation of the oviductal microenvironment was studied to gain knowledge of possible regulative mechanisms that could be positively affected also in the living organism.

Cell culture was further used for studying physiological processes and their regulatory mechanisms e.g. in a mouse model embryotrophic function of complement C3b was proven (Lee et al. 2002b). In human oviduct epithelial cells *in vitro*, Lyons et al. (2002) showed that endometriotic peritoneal fluid leads to a decrease in ciliary beat frequency in oviduct tissue, which may lead to poor fertility in endometriosis patients (Lyons et al. 2002). This phenomenon would be very difficult to study *in vivo*, even by modern techniques such as endoscopy, but the cell culture results provide valuable aspects of endometriosis pathogenetic mechanisms (Lyons et al. 2002). The differential effects of natural estradiol-17β, in contrast to environmental estrogens (phyto- and xenoestrogens), has also been shown in oviduct cell culture experiments. Interestingly, it was detected that environmental estrogens may act as endocrine disruptors and may have deleterious effects on the reproductive system by adversely influencing biology and physiology of the oviduct compared with the effects of natural estradiol-17β (Reinhart et al. 2003).

Also *in vitro*, a new role of LH was found to be up-regulation of oviduct-specific glycoprotein (OVGP1) by increasing transcript stability (Sun et al. 1997) probably in preparation for the passing embryo that is positively influenced by OVGP1. This phenomenon was further proven by co-culture experiments in which LH supplementation positively affected embryonic development due to an increase of OVGP1 (Mishra et al. 2003).

Several studies examined the formation of OF (Leese et al. 2001) and regulatory influences on secretion processes. Protein patterns were compared *in vivo versus in vitro* using different culture systems (Woldesenbet and Newton 2003) and regulatory effects on single proteins were examined (Eberhardt et al. 1999).

2.5.4 Quality assessment of BOEC in monolayer or suspension culture

To gain reliable results qualified for interpretation of physiological events by cell culture techniques, cells should maintain their physiological characteristics as good as possible. In many studies special examinations were undertaken to ensure these requirements.

Morphological criteria were mostly observed, especially the presence of cilia has been examined. However, in monolayer studies the loss of cilia after cell attachment and growth to confluence has been described (Thibodeaux et al. 1992, Walter 1995, Reischl et al. 1999), whereas in cell aggregates remaining in suspension, cilia were still detected after 10 days in culture (Walter 1995). This means an important advantage of the suspension culture system, as a striking characteristic of the bovine oviduct epithelium *in vivo* is the existence of ciliated and secretory cell types.

Ultrastructural features of BOEC in monolayer or suspension were examined via electron microscopy (Walter 1995, Reischl et al. 1999, Gualtieri and Talevi 2000). Monolayers showed numerous microvilli with a glycocalyx on their apical side, but no cilia were detectable. Cells were connected to their neighboring cells by interdigitating cytoplasmic projections, outfoldings and by tight and adhering junctions. Numerous mitochondria, golgi apparatus and rough endoplasmatic reticulum were present.

Suspended cells contained in aggregates showed polarized BOEC in a differentiated state, representing both ciliated and secretory cells. Cilia were localized on the outer aggregate surface. However, intercellular spaces appeared wide although terminal bars and finger-like projections between neighboring cells were developed (Walter 1995).

In both culture systems, glycogen accumulation were detected, probably a sign of abundant glucose in the culture medium (Walter 1995, Reischl et al. 1999).

An important indicator for the purity of the cultured epithelial cell population was immunocytochemistry staining against cytokeratin as a marker for cells of epithelial origin and against vimentin to detect fibroblast contamination (Abe and Hoshi 1997, Boquest and Summers 1999, Comer et al. 1998, Cox and Leese 1997, Ellington et al. 1998, Reischl et al. 1999, Walter 1995, Mishra et al. 2003). If the epithelial fraction was > 95 %, this was a sign for good purity of the culture. Cell viability at the time of seeding or during culture was reviewed via trypan blue exclusion. Values of 90 % viable cells at seeding were reported regularly and presented a criterium for adequate cell isolation techniques (Cox and Leese 1997, Reischl et al. 1999, Mishra et al. 2003, Ulbrich et al. 2003).

Also, results of embryonic development in co-culture studies were used to prove BOEC functionality (Ando et al. 2000, Okada et al. 2005, Yadav et al. 1998, Gandolfi and Moor 1987, Ellington et al. 1990).

In recent studies, expression of physiologically important embryotrophic factors such as oviduct-specific glycoprotein (OVGP1; Reischl et al. 1999, Briton-Jones et al. 2002, Briton-Jones et al. 2004), endothelin-1 (ET-1; Reinhart et al. 2003), members of the insulin-like growth factor (IGF) system (Winger et al. 1997, Xia et al. 1996), or leukaemia inhibitory factor (LIF), vascular endothelial growth factor (VEGF), epithelial growth factor (EGF), IGF1, tumor growth factor β 2 (TGFβ2), and interleukin-4 (IL4) (Okada et al. 2005) was applied to obtain information about the differentiation state of cultured cells. Gene expression studies provide an interesting tool particularly for characterization of newly established cell lines. However, to use these methods properly, it is very important to achieve sufficient information about cell behavior *in vivo*. Studies concerning gene expression in the bovine oviduct were conducted recently referring to differential expression patterns in ipsi- and contralateral bovine oviducts (Bauersachs et al. 2003) and during the estrous cycle. This basic research is essential for the interpretation of *in vitro* findings (Bauersachs et al. 2004).

In some studies, polarity of cells in a monolayer has not only been determined by ultrastructural studies but also by measuring the transepithelial electric potential difference. Confluent BOEC monolayers were able to retain a potential of 1.00 ± 0.34mV or 3.3 ± 0.2 mV, respectively, and allowed examinations on secretory and absorption activities (Cox and Leese 1997, Downing et al. 2002).

A study in goat concerning protein expression of cultured oviductal cells revealed similar patterns in organ culture and in monolayer culture (Woldesenbet and Newton 2003). In bovine, in both culture systems, no significant difference could be observed, except a lesser IGF-2 release from monolayers compared to vesicles (Winger et al. 1997, Xia et al. 1996).

For optimal maintenance of cell characteristics, different materials were used as a support to maintain cell polarity *in vitro* (Cox and Leese 1997, Reischl et al. 1999). In a comparison of different support materials such as plastic, glass, nylon membranes and cellulosenitrate membranes, cellulosenitrate showed a positive influence on cell differentiation (Reischl et al. 1999), yet increasable by perfusion culture conditions, providing a constant supply of fresh medium. This effect was shown regarding ultrastructural cell features and an increase in OVGP1 expression in cells cultured on cellulosenitrate under perfusion conditions (Reischl et al. 1999).

Though, even considering such efforts in creating an optimized environment for cultured cells *in vitro*, cells in aggregates, non-attaching to the culture dish, seemed to maintain morphological and ultrastructural characteristics best.

3 Material and methods

3.1 Equipment and expendable items

3.1.1 Cell preparation

Suture material:	Supramid®, metric 4, Serag-Wiesner, Naila
Petri dishes:	35x10 mm, NunclonTM, Roskilde, Danmark
	60x15 mm, Cellstar, Greiner, Solingen
Sterile bench:	Lamin Air®, HB 2472, Heraeus, Munich
Glassware:	Schott, Brand, Wertheim
Syringes:	Estra®, 1 / 5 / 10 / 20 ml, CODAN Medical ApS, Rodby, Danmark
Canules:	Sterican®, 23G / 30G, Braun, Melsungen
Pipettes:	2 / 10 / 20 / 100 / 200 / 1000 µl, Eppendorf, Hamburg
Pipette tips:	Eppendorf, Hamburg
Pipette filter tips:	Eppendorf, Hamburg
Neubauer Chamber:	Brand, Wertheim
Sterile filters:	Minisart, 0.2 µm, Sartorius, Goettingen
Centrifuge tubes:	15 ml, Greiner 50 ml, Falcon®, Becton-Dickinson

3.1.2 Cell culture

Incubator:	Type B 5060, Heraeus
Microscopes:	Leica DMIL, Leica, Wetzlar Axiovert 135 with UV lamp, Zeiss, Goettingen
Documentation:	Axiocam, Zeiss, Goettingen AxioVision 4.0, Zeiss, Goettingen
24-well-multidish:	Nunc, Nunclon, Roskilde, Danmark
Glass coverslips:	∅ 12 mm, Marienfeld, Lauda-Koenigshofen
Reaction tubes:	0.5 ml / 1.5 ml, Eppendorf, Hamburg

3.1.3 Histological analyses

Union Point Dryer CPD 030 , Bal-Tec, Walluf

Union SCD 040 sputtering device, Bal-Tec, Walluf

Zeiss scanning electron microscope DSM 950, Zeiss, Goettingen

3.1.4 Molecular analyses

Spectrophotometer:	Gene Quant II, RNA/DNA calculator, Pharmacia Biotech, Cambridge
Thermomixer:	Type 5436, Eppendorf, Hamburg
Vortexer:	M S1 minishaker, IKA, Staufen
Centrifuges:	Type 5415 D, Eppendorf, Hamburg
	Type 5417 R, Eppendorf, Hamburg
	Biofuge pico, Heraeus, Hanau

3.2 Used media and stock solutions

3.2.1 Cell preparation

PBS (Phosphate-buffered saline)

8.00 g	NaCl	(Sigma, Deisenhofen)
0.20 g	KCl	(Sigma)
1.15 g	Na_2HPO_4 x 2 H_2O	(Fluka, Neu-Ulm)
0.20 g	KH_2PO_4	(Merck, Darmstadt)
0.10 g	$CaCl_2$	(Sigma)
0.10 g	$MgCl_2$ + 6 H_2O	(Sigma)
1.0 l	Milli-Q water	

sterile filtered, stored at room temperature

PBS− (Phosphate-buffered saline without calcium and magnesium)

8.00 g	NaCl	
0.20 g	KCl	
2.14 g	Na2HPO4 x 7 H2O	(Merck)
0.20 g	KH2PO4	
1.0 l	Milli-Q water	

sterile filtered, stored at room temperature

PBS P/S (Phosphate-buffered saline with 2 % Penicillin/Streptomycin

98 ml	PBS−
2 ml	Pen/Strep stock solution

freshly prepared before use

Pen/Strep stock solution

0.65 g	Penicillin	(Seromed, Berlin)
1.33 g	Streptomycin	(Seromed)
100 ml	Milli-Q water	

(contains 100 U/ml penicillin and 100 µg/ml active streptomycin)

sterile filtered, stored at -20°C

3.2.2 Cell culture

TCM-199

TCM-199 25 mM HEPES with Earle´s Salts with L-Glutamine without L-Amino-Acids, Invitrogen, Karlsruhe

Culture medium

97.5 ml	TCM-199
2 ml	serum (estrous cow serum or cow serum obtained on Day 3.5)
0.5 ml	gentamicin stock solution

Gentamicin stock solution

50.0 mg	gentamicin	(Sigma)
1.0 ml	NaCl-solution (0.9 %)	(Sigma)

Estrous cow serum (ECS)

Blood samples from cows in standing heat were obtained and centrifuged twice at 1,320 x*g* for 20 min to obtain serum. Serum was pooled from four different animals and inactivated in the water bath at 56°C for 30 min. Serum stock was stored at -20°C.

Cow serum obtained on Day 3.5 of the estrous cycle (CS 3.5)

Blood samples from cows on Day 3.5 of the estrous cycle were obtained and centrifuged twice at 1,320 x*g* for 20 min to obtain serum. Serum was pooled from four different animals and inactivated in the water bath at 56°C for 30 min. Serum stock was stored at -20°C.

Estradiol-17β, Sigma

Progesterone water soluble, Sigma

Trypan blue solution 0.4 %, Sigma

Bovine Serum Albumin (BSA), Sigma

Monoclonal Anti-Vimentin, Clone V9, Sigma

Monoclonal Anti-Cytokeratin 8.13, Clone 8.13, Sigma

Anti-Mouse IgG (whole molecule) FITC conjugate, Sigma

Vectashield® antifade solution, Vector Laboratories, Burlingame

Propidium iodide solution, 40µg / ml, Sigma

3.2.3 Electron microscopy

Glutaraldehyd 2.1 %

25 ml	glutaraldehyde 25 %	(Serva, Heidelberg)
275 ml	Soerensen-Buffer	

Soerensen-Buffer

Solution A:

9.078 g	KH_2PO_4	(Sigma)
1 l	Milli-Q water	

Solution B:

11.876 g	Na_2HPO_4 x 2 H_2O	(Sigma)
1 l	Milli-Q water	

Work solution:

80.8 ml Solution B,
ad 100 ml Solution A
pH 7.4, stored at 4°C

Lead citrate solution

16 ml	Aqua dest	
3 ml	natrium citrate	(Sigma)
2 ml	lead citrate	(Sigma)
4 ml	NaOH (1M)	(Sigma)
ad 100 ml	Aqua dest	

Epon

16 ml	Poly/Bed	(Polysciences, Waorington, USA)
10 ml	DDSA	(Polysciences)
9 ml	NMA	(Polysciences)
0.3 - 0.5 ml	BDMA	(Polysciences)

Karnovsky solution

Stock Solution

10 g	paraformaldehyde	(Sigma)
100 ml	Aqua dest	
6 drops	NaOH (1M)	

Work solution:

4 ml	Karnovsky Stock solution	
6 ml	glutaraldehyde 8 %	(Polysciences)
10 ml	natriumcacodylat-buffer (0.2M)	

Na-cacodylat-Buffer (0.2M)

Solution A:

8.56 g	$Na(CH3)_2AsO_2 \times 3H_2O$	(Polysciences)
200 ml	Aqua dest	

Work solution:

50 ml	Solution A	
4.2 ml	0.2M HCl	(Sigma)
ad 100 ml	Aqua dest	

3.2.4 RNA extraction

Trizol® reagent, Invitrogen, Karlsruhe

Isopropanol, Roth, Karlsruhe

Chloroform, Roth, Karlsruhe

Ethanol, Roth, Karlsruhe

3.3 Animals

With respect to the manifold changes in cell proportions in the bovine oviduct epithelium during the estrous cycle, it seems important to use organs of animals at a well defined stage of the estrous cycle. We chose to work with cells obtained on Day 3.5 of the estrous cycle because at this point of time, the embryo is located in the oviduct and if there are signals to be detected, it should be around this time.

Synchronized Simmental heifers aged 16 - 23 months were slaughtered on Day 3.5 after the onset of standing heat. Follicle development and ovulation were monitored by ultrasound examinations every 6 h starting 52 h after application of 500 µg Cloprostenol (Estrumate®, Essex Pharma, Munich, Germany). Additionally, blood samples were taken every 6 h starting 24 h after Cloprostenol application to confirm estrous cycle stage by measuring serum concentrations of estradiol-17β, progesterone and luteinizing hormone (LH). All experiments with animals were carried out with permission from the responsible animal welfare authorities at the Regierung von Oberbayern.

3.4 Culture of bovine oviduct epithelial cells obtained on Day 3.5 of the estrous cycle

3.4.1 Oviduct preparation

After slaughter, removal of the skin and opening of the abdominal cavity, the reproductive tract was recovered. Both oviducts were trimmed free from connective tissue, ligated, dissected, washed in PBS without Ca^{2+}/Mg^{2+} (PBS–), and dipped in 70 % ethanol before being transported on ice to the laboratory in PBS– supplemented with 2 % penicillin-streptomycin. Oviducts were dipped again in ethanol and washed in PBS– before removing the ligature in a laminar flow hood.

3.4.2 Preparation of cells

The oviduct ampulla was divided into three segments and gently squeezed in a stripping motion with forceps to obtain BOEC. Cells appeared as a yellowish paste that was collected in 5 ml culture medium TCM-199 supplemented with 2 % estrous cow serum (ECS) and 0.25 mg/ml gentamicin. The cell suspension was pipetted 15 times with a 1000 µl filter tip before being passed twice through a 23G syringe needle. The suspension was stored for 30 min in the incubator to allow fibroblast cells to selectively adhere to the culture dish. After transferring the supernatant to an 15 ml tube and three steps of washing with fresh culture medium, each followed by 25 min sedimentation in culture medium in the incubator, cells were seeded into the central eight wells of a 24-well culture plate in a concentration of 10^6 cells per well in 800 µl culture medium and cultured as described below.
The time span from the slaughter of heifers to the seeding of cells was approximately 6 h.
Before seeding, an aliquot of the three times washed cell suspension was further disaggregated for cell counting by passing it 15 times through a 30G needle. After this procedure, BOEC were mostly singularized. Cell counting was performed using a Neubauer improved chamber.

3.4.3 Culture conditions and experiments

BOEC were cultured with 10^6 cells each in 800 µl TCM-199 and 0.25 mg/ml gentamicin at 38°C in a humidified atmosphere with 5 % CO_2 in air. Serum supplementation during the culture period was 2 % ECS (experiment 1) or 2 % cow serum collected on Day 3.5 of the estrous cycle (CS 3.5, experiment 2). Hormone assays showed that ECS contained 8.16 pg/ml estradiol-17β (E2) and < 0.05 ng/ml progesterone (P4), CS 3.5 contained 1.81 pg/ml E2 and 0.07 ng/ml P4.
In experiment 1, ipsi- and contralateral oviducts were used and compared to each other. In experiments 2 and 3, only oviducts ipsilateral to the ovulation site were obtained (Fig. 7).

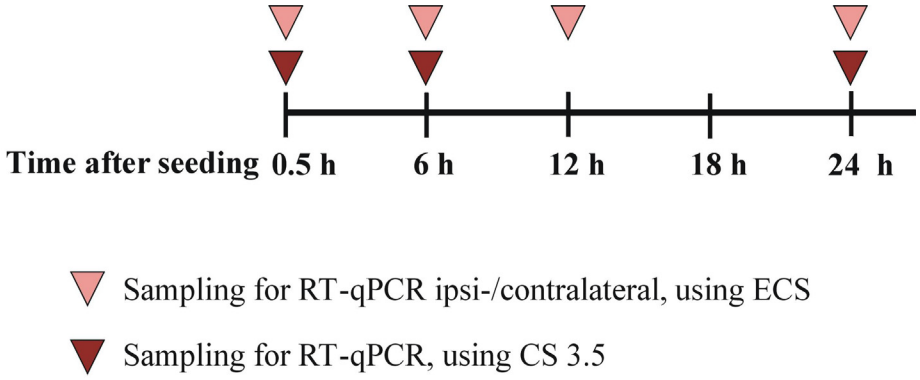

Fig. 7 Sampling schedule for quantitative PCR examinations during the course of culture (experiment 1 and experiment 2). In experiment 1, BOEC derived from ipsi- and kontralateral oviducts were cultured in medium supplemented with 2 % ECS and examined at different time points. In experiment 2, BOEC from ipsilateral oviducts were cultured in medium with 2 % CS 3.5 and transcript abundance of marker genes was compared to that of ipsilateral oviduct cells from experiment 1.

To evaluate conservation of physiological cell functions, BOEC responsibility to steroid hormone supplementation was examined (experiment 3). After 6 h of pre-culture of 10^6 BOEC in 750 µl standard cell culture medium, supplemented with 2 % CS 3.5, 50 µl culture medium containing estradiol-17β (E2) or progesterone (P4) were added in a concentration to achieve an hormone addition of 10 pg/ml E2 or 10 ng/ml P4. Cells treated with hormone carrier solution only served as controls. After 6 and 18 h stimulation, samples were taken for quantitative PCR (Fig. 8).

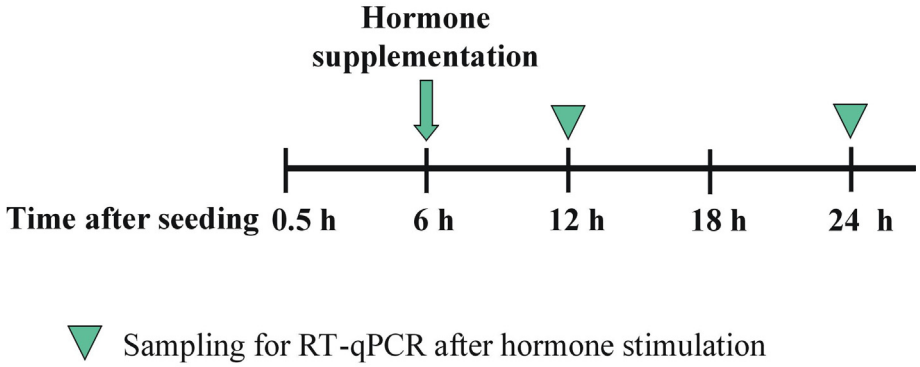

Fig. 8 Sampling schedule for quantitative PCR after supplementation with 10 pg/ml estradiol-17β or 10 ng/ml progesterone (experiment 3). After a adaptation period of 6 hours, steroids were added to the culture medium and samples for RT-qPCR examinations were taken after 6 h and 18 h of stimulation.

3.5 Morphological examinations

3.5.1 Trypan blue staining

Cell viability at seeding and after 24 h in culture was assessed by trypan blue. While cells with an intact membrane inhibit permeation of the dye, destroyed or damaged cells stain positive by trypan blue.
A 50 µl aliquot of the final cell suspension was washed twice with PBS- and centrifuged at 500 x*g*. The cell pellet was suspended in 50 µl PBS- and 25 µl 0.4 % trypan blue solution. After one minute, cell staining was examined in a Neubauer improved chamber.

3.5.2 Immunocytochemistry

Immunocytochemistry was performed on BOEC grown as a monolayer on a glass coverslip. Therefore, glass coverslips were equilibrated in culture medium at least for 4 h before seeding. Cells were seeded as described above, but were incubated for 3 days to allow cell aggregates to adhere to the support. Medium change took place on the third day and on the fifth day. Cells grown as monolayers on the coverslips were examined after six days in culture. The coverslips were washed three times in PBS– and fixed and precooled in a mixture of methanol and acetone (7:3 v/v) at –20°C for 10 min. After washing, one glass coverslip each was covered with anti-vimentin antibody, anti-cytokeratin antibody, or PBS– supplemented with 0.1 % bovine serum albumin (BSA) as a control and incubated for 1 h at 37°C in a humidified atmosphere. After washing three times in PBS–, the coverslips were coated with FITC-labeled secondary antibody and again incubated for 1 h. Incubation with propidium iodide was performed for 10 min in the dark at room temperature to stain cell nuclei. The coverslips were evaluated using a Zeiss AxioCam on a Zeiss Axiovert 200 microscope with UV light at 546 nm.

3.5.3 Light microscopy

Cell morphology was assessed at seeding and after 6 h, 12 h and 24 h in culture (Fig. 9). For documentation, digital pictures were taken and processed with Axiovision® 4.0.

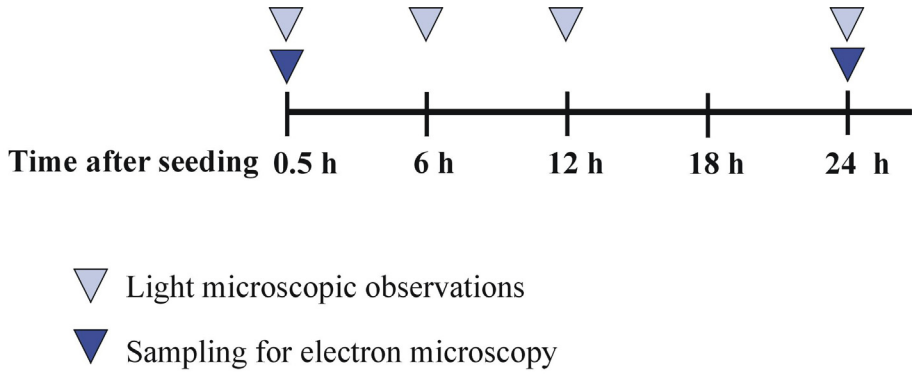

Fig. 9 Time schedule for light and electron microscopic examinations.

3.5.4 Scanning electron microscopy

BOEC obtained *ex vivo* and cultured BOEC after centrifugation at 500 x*g* were washed twice in Soerensen buffer pH 7.4 (1:5 solution of 0.07 M KH_2PO_4 and 0.07 M Na_2HPO_4-$2H_20$). Specimens were fixed in 1 % glutaraldehyde in Soerensen buffer at 4°C for 24 h. After washing in Soerensen buffer, cells were dehydrated in an ascending series of acetone (10 %, 20 %, 30 %, 40 %, 50 %, 60 %: twice, five minutes each; 70 %, 80 % and 90 %: 1 h each; 100 %:12 h). BOEC were dried in a Union Point Dryer CPD 030 using liquid CO_2 as the transitional fluid. After drying, specimens were coated with 12 nm gold-palladium by a Union SCD 040 sputtering device. SEM observations were made with the Zeiss scanning electron microscope DSM 950 using magnifications of 200× - 10,000×.

3.5.5 Transmission electron microscopy

BOEC were washed twice in cacodylate buffer (0.2 M sodium cacodylate, pH 7.2). After fixation in Karnovsky's fluid (2.5 % glutaraldehyde and 2 % formaldehyde in 0.1 M cacodylate buffer) for 24 h, epithelial cells were postfixed in 1 % OsO_4 and 1.5 % $KFe(CN)_6$. Specimens were then transferred to a drop of 20 % BSA in cacodylate buffer. By adding 25 % glutaraldehyde, BSA was polymerized to a pellet containing the cells. The pellet was dehydrated in a graded series of ethanol and embedded in Epon. In order to assess the cellular compartments of the oviductal cells, semithin sections (1 µm) were cut and stained with methylene blue. Ultrathin sections (50 nm) were mounted on grids, post-stained with osmium tetroxide and examined using a Zeiss electron microscope with magnifications from 3,000× to 25,000×.

3.6 Quantitative Real-time PCR examinations

3.6.1 Total RNA extraction

Total RNA was extracted using Trizol® reagent according to the manufacturer's instructions
Frozen samples were thawed on ice for approximately 15 min. One sample consisted of material from 2 x 10^6 cells. 500 µl Trizol® were added and samples were left at room temperature for 5 min. 0.1 ml chloroform were added and the tubes were vigorously shaken for 15 sec. After 3 min at room temperature, samples were centrifuged at 11,900 x*g* for 15 min. Afterwards, the developed watery phase, containing RNA, was carefully transferred into separate tubes with 250 µl isopropanol. These new tubes were shaken and left at room temperature for 10 min. Samples were centrifuged again for 20 min at 4°C at 11,900 x*g*. The resulting supernatant was removed and 1 ml 75 % ethanol was added. A short vortex showed a white flake to assure the presence of RNA. The RNA pellet was centrifuged at 7,500 x*g* for 10 min and ethanol was carefully removed. The tubes were left open in a sterile work bench for 10 min allowing the RNA pellet to dry. Then, 20 µl autoclaved H_2O were added and the samples were left on ice for 30 min before shaking slightly in thermomixer at 55°C for 10 min.
1 µl of the solution was used to measure RNA yield with a spectrophotometer at 260 nm and to determine RNA purity by the 260/280 nm ratio. Each sample was stored at -80°C.

3.6.2 Reverse transcription quantitative PCR (RT-qPCR)

RT-qPCR examinations were performed by Dr. Susanne Ulbrich from the Department of Physiology at the TU Munich-Weihenstephan.

RNA integrity was assured by gel electrophoresis. One µg of each RNA sample was reverse transcribed in a total volume of 60 µl [5x Buffer (Promega, Madison, USA), 10 mM dNTPs (Roche, Mannheim, Germany), 50 µM hexamers (Gibco BRL, Grand Island, USA), using 200U MMLUV Point Mutant H-RT enzyme (Promega, Madison, USA)].

Gene-specific primers were synthesized (MWG-Biotech, Ebersberg, Germany) to amplify specific fragments of bovine transcripts as described in Table 1. All amplified fragments were sequenced once to verify PCR-products (MWG-Biotech, Ebersberg, Germany). During RT-qPCR, the specific melting point of amplified products carried out by the LightCycler® standard PCR protocol served as verification of the product identity. For each RT-qPCR reaction, 1 µl of cDNA was used to amplify a specific target gene. PCR reactions using the LightCycler® DNA Master SYBR Green I protocol (Roche, Mannheim, Germany) were performed as described previously (Ulbrich et al. 2004). Annealing temperatures (AT) and fluorescence acquisition (FA) points for quantification within the fourth step of the amplification segment are shown in Table 1. In each PCR reaction 17 ng/µl cDNA were introduced and amplified in a 10 µL reaction mixture (3 mM $MgCl_2$, 0.4 µM primer forward and reverse each, 1× Light Cycler® DNA Master SYBR Green I, Roche, Mannheim, Germany). Real-time PCR reactions using the LightCycler® DNA Master SYBR Green I protocol (Roche Diagnostics, Mannheim, Germany) were performed as described previously (Ulbrich et al. 2004). The cycle number required to achieve a definite SYBR Green fluorescence signal was calculated by the second derivative maximum method (crossing point CP) (LightCycler software version 3.5.28). The CP is correlated inversely with the logarithm of the initial template concentration. As negative controls, water instead of cDNA was used. All samples for the candidate genes were standardized to the internal control *18S* rRNA.

Gene	Direction	Primer sequence	Fragment size (bp)	Annealing Temperature (°C)	Fluorescence Acquisition Point (°C)
18S rRNA	Forward	5`-AAGTCTTTGGGTTCCGGG-3`	365	60	82
	Reverse	5`-GGACATCTAAGGGCATCACA-3`			
ESR1	Forward	5`-AGGGAAGCTCCTATTTGCTCC -3`	234	63	86
	Reverse	5`-CGGTGGATGTGGTCCTTCTCT-3`			
ESR2	Forward	5`-GCTTCGTGGAGCTCAGCCTG-3`	262	64	81
	Reverse	5`-AGGATCATGGCCTTGACACAGA-3`			
PGR	Forward	5`-GAGAGCTCATCAAGGCAATTGG-3`	227	64	81
	Reverse	5`-CACCATCCCTGCCAATATCTTG-3`			
GPX4	Forward	5`-GAGAAACCTAAAGACCTTGGC-3`	276	60	82
	Reverse	5`-TTGACTGGAAGAATGGGGAC-3`			
TRA1	Forward	5`-TGGCAGAGACCATCGAAAG-3`	120	60	77
	Reverse	5`-GGTAACTTCCCCTTCAGCAG-3`			
OVGP1	Forward	5`-TGTCCACGTTTTCCAACCG-3`	856	60	85
	Reverse	5`-GGAGGGCGATCACTGAACTG-3`			
HMGCR	Forward	5`-GCATAGGAGGCTACAACGCC-3`	241	63	80
	Reverse	5`-CCTTGAACACCTAGCATCTGC-3`			
INOS	Forward	5`-ACCATCCAGCTGACGGGAGAT-3`	216	61	86
	Reverse	5`-TGGCAGGGTCCCCTGTGATG-3`			

Table 1 Primer details and parameters of RT-qPCR.

Gen	Gen name	Functions
ESR1	Estrogen receptor alpha	Steroid receptor, Mediation of estrogen actions / Regulation of gene expression, predominant subtype in reproductive organs (Pelletier et al. 2000)
ESR2	Estrogen receptor beta	Steroid receptor, Mediation of estrogen actions predominantly in ovary, mammary gland and embryo (Kuiper et al. 1997, Wang et al. 2000, Saji et al. 2000, Kowalski et al. 2002)
GPX4	B. taurus non-selenium glutathione phospholipid hydroperoxide peroxidase	Importance for male reproductional processes and fertility (Beckett et al. 2005), Differential gene expression in the ipsi- versus the contralateral oviduct in vivo (Bauersachs et al. 2003), Functions in antioxidative defense (Lapointe et al. 2005)
HMGCR	H. sapiens 3-hydroxy-3-methylglutaryl-coenzyme A reductase	Role in steroid / cholesterol synthesis (Ohashi et al. 2003, Goldstein et al. 1990) Up-regulation in the oviduct situated ipsilateral to the ovulation site *in vivo* (Bauersachs et al. 2004)
INOS	Inducible nitric oxide synthase	Modulation of the oviductal environment, regulation of contractility and immune response in the bovine oviduct (Ulbrich 2005).
OVGP1	Oviduct-specific glycoprotein 1	Interactions with gametes and the embryo in the oviduct (Buhi 2002); Highest gene expression around ovulation (Bauersachs et al. 2003), Used in former studies to evaluate BOEC quality in culture (Reischl et al. 1999, Briton-Jones et al. 2002)
PGR	Progesterone receptor	Steroid receptor, Mediation of progesterone actions (Ulbrich et al. 2003)
TRA1	Tumor rejection antigen 1	Chaperone and tumor marker (Watanabe et al. 2001), Differential gene expression in bovine oviducts in vivo (Bauersachs et al. 2003)

Table 2 Examined candidate genes and their known functions in reproduction.

3.7 Western blot analysis

Western blot analysis was performed at the Physiology Weihenstephan, Technical University Munich, as described previously (Ulbrich et al. 2003). Approximately 10^6 BOEC were repeatedly frozen in liquid nitrogen with lysis buffer containing proteinase inhibitor. Protein samples (36 µg per lane) were separated on a 4-12 % Bis-Tris Gel (NuPage, Invitrogen, California, US) in MOPS running buffer and transferred onto nitrocellulose membranes. The membranes were blocked with 1% dried milk in TBS containing 0.1 % Tween-20 over night. These were incubated with the monoclonal antibody against ESR1 (2-185, Santa Cruz Biotechnology, California, US) or PGR (Clone 10A9, Coulter Immunotech, Marseille, France) in TBS-0.1 % Tween-20 and 1 % dried milk for 75 min at room temperature. The dilutions used were 1:200 (ESR1) and 1:50 (PGR), respectively. After washing, membranes were incubated with anti-mouse horseradish peroxidase-conjugated IgG secondary antibody (DAKO, Hamburg, Germany) at a dilution of 1:2,000 in TBS-0.1 % Tween-20 and 1 % dried milk for 45 min at room temperature. After washing in TBS-0.1 % Tween-20 and TBS alone the membrane was incubated with enhanced chemiluminescence reagent detection solution (Amersham, Buckinghamshire, UK) for 3 min in the dark. Finally, an X-ray film was exposed to the membrane to visualize protein expression. Protein extract from bovine endometrium (30 ng per lane) was used as a positive control as described (Schams et al. 2003).

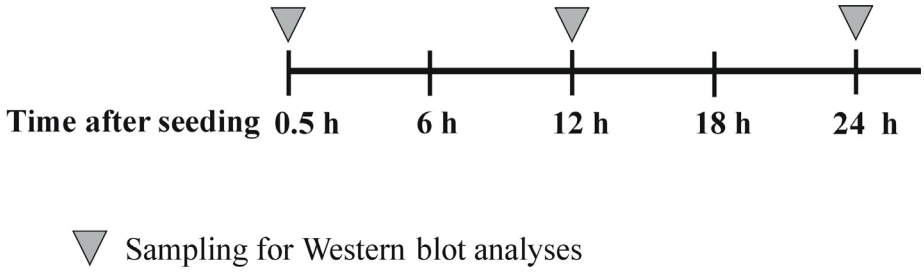

Fig. 10 Sampling schedule for Western Blot examinations to demonstrate the existence of steroid receptors ESR1 and PGR at seeding and after 12 and 24 h in culture.

3.8 Statistical analysis

Analysis of variance (ANOVA), calculation of least squares means (LSM) and standard errors of means (SEM) were performed with the general linear model procedure as implemented in SPSS 12.0G for Windows, version 12.0.1 (SPSS GmbH Software, Munich, Germany). Student´s *t*-test with Bonferroni correction for multiple testing was used in comparisons of marker gene expression differences throughout the culture period. Paired *t*-test was employed in comparisons of marker gene expression between ipsi- and contralateral BOEC and in comparisons between hormone-stimulated BOEC and controls. Differences were considered significant at $P < 0.05$. Graphs were plotted with Graph Pad Prism, version 3.00 for Windows (GraphPad Software, San Diego, California USA). RT-qPCR CP-data are presented as means ± SEM subtracted from the arbitrary value 50 (ΔCP) to transform CP values into proportional values reflecting decreases and increases in transcript abundance.

4 Results

4.1 Cell yield, cell viability and purity of the epithelial cell culture

The number of isolated cells ranged from 30.7 × 10^6 to 87.0 × 10^6 cells per ampulla. Mean cell yield was very similar for ipsi- and contralateral oviducts with 57.9 ± 4.6 × 10^6 and 56.4 ± 8.0 × 10^6 cells per ampulla, respectively.

Trypan blue staining was positive in the majority of singularized cells, while cells organized in aggregates were viable at 0.5 h and after 24 h in culture (Fig. 11a, b).

Immunocytochemical examinations showed > 95 % cells staining positive with anti-cytokeratin antibody, thus confirming the epithelial character of the cultured BOEC population. Less than 5 % cells grown on a glass coverslip stained positive for the fibroblast marker anti-vimentin (Fig. 11c, d).

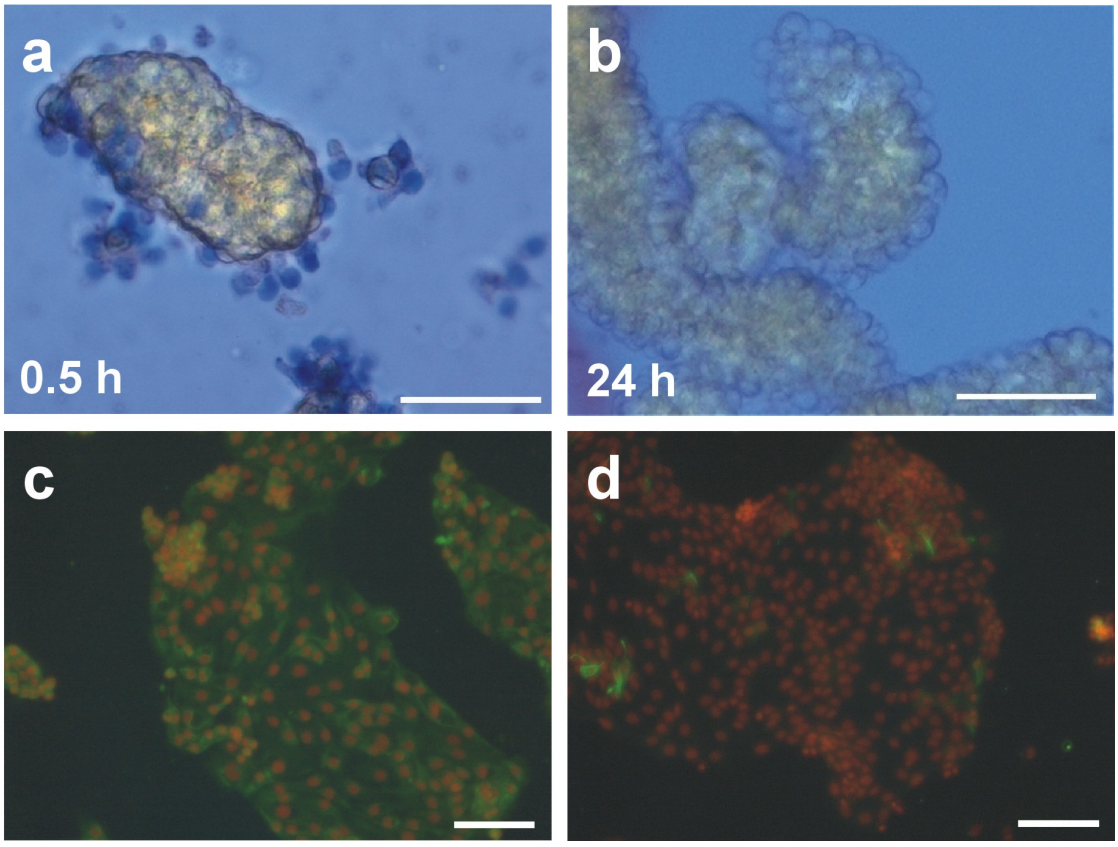

Fig. 11 Evaluation of cell viability and purity. Viable trypan blue negative cells were only present in aggregates and singularized cells stained trypan blue positive at 0.5 h in culture (a). Trypan blue positive cells were not found later in culture (b). Immunocytochemical examination using anti-cytokeratin antibodies indicate a high purity of the epithelial cell culture (c). Immunocytochemical examination using anti-vimentin antibodies shows < 5 % fibroblast contamination (d). ***Bars* 100 µm.**

4.2 Cell morphology and ultrastructure

Light microscopy showed BOEC contained in aggregates with vigorously beating cilia on all aggregate surfaces throughout the 24 h culture period. Mechanically obtained flat cell sheets changed into worm-like structures during the first 6 h of culture. This morphology was maintained throughout culture but the apical surface of individual cells in the aggregates attained a slightly more rounded shape after 24 h (Fig. 12a, b). All aggregates remained in rapid and constant motion due to vigorous ciliary beating throughout the culture period.

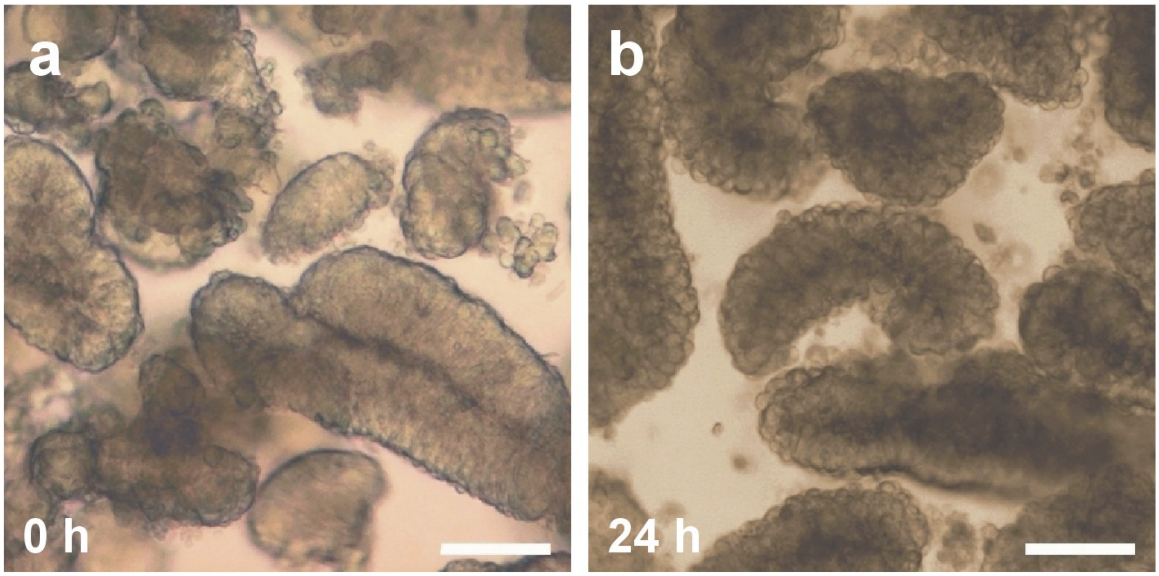

Fig. 12 Light microscopic images of BOEC. Cells at 0.5 h (a) and after 24 h (b) in culture. BOEC were organized in aggregates and showed intense ciliary beat throughout the culture period. *Bars* 100 µm

Scanning electron microscopy revealed ciliated and secretory cells in their natural coherence. Cilia were long and well-developed. BOEC after 0.5 h and 24 h in culture appeared identical to BOEC in *ex vivo* tissue samples obtained on Day 3.5 of the estrous cycle (Fig. 13a-d).

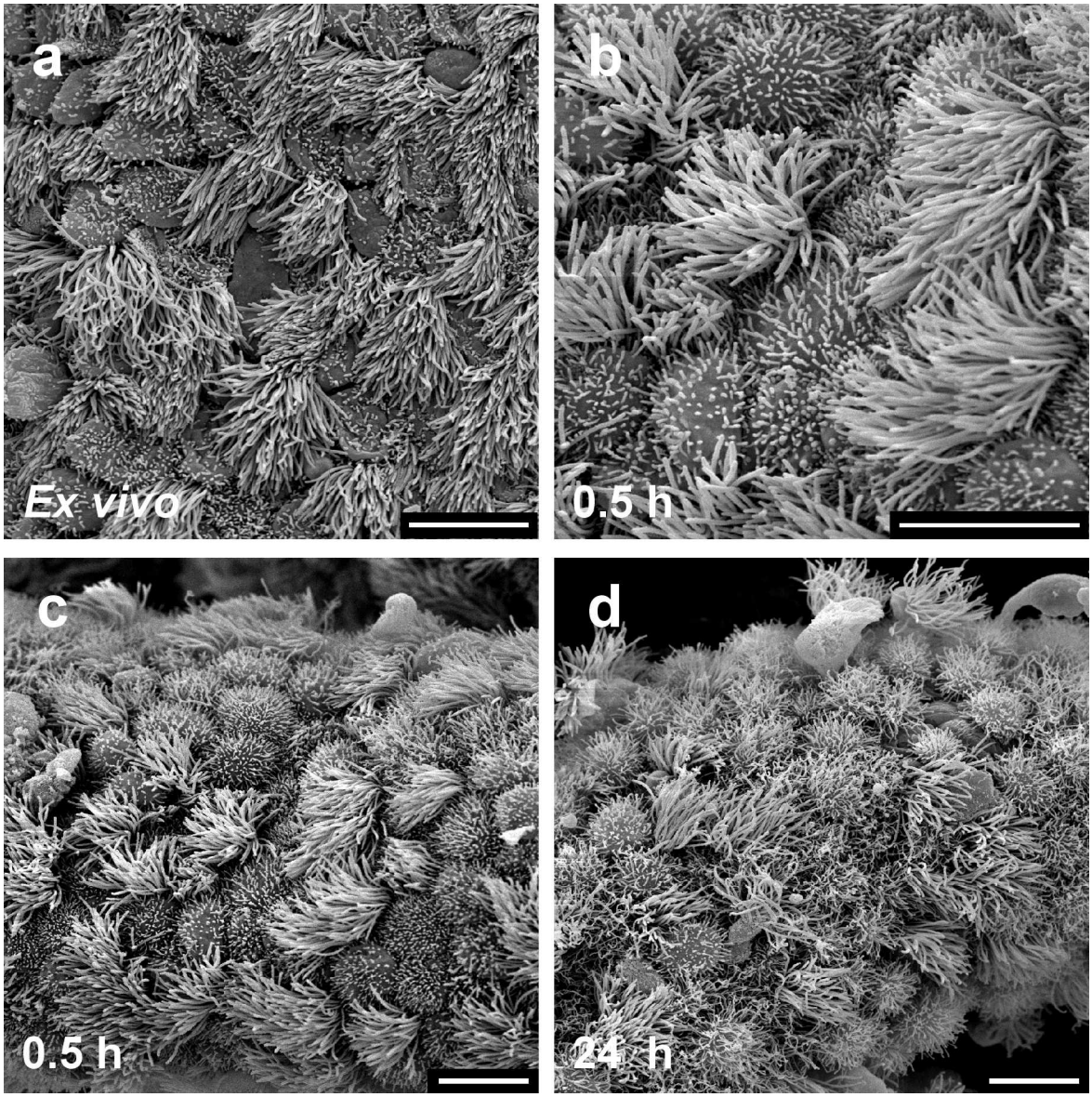

Fig. 13 Scanning electron microscopic (SEM) images of ciliated and secretory BOEC in oviduct tissue ex vivo (a) and BOEC in aggregates after 0.5 h in culture (b, c). Cells after 24 h in culture (d). Cultured BOEC reveal a morphology very similar to that ex vivo. *Bars* 10 µm.

Transmission electron microscopy confirmed the presence of ciliated and secretory cells in the cultured aggregates. BOEC characteristics such as numerous kinocilia with their basal bodies, numerous secretory granules, and mitochondria were visible at seeding and after 24 h in culture. The maintenance of cell polarity was confirmed by the presence of cilia and microvilli on the apical cell surface and tight junctions on the lateral cell surfaces (Fig. 14a-f).

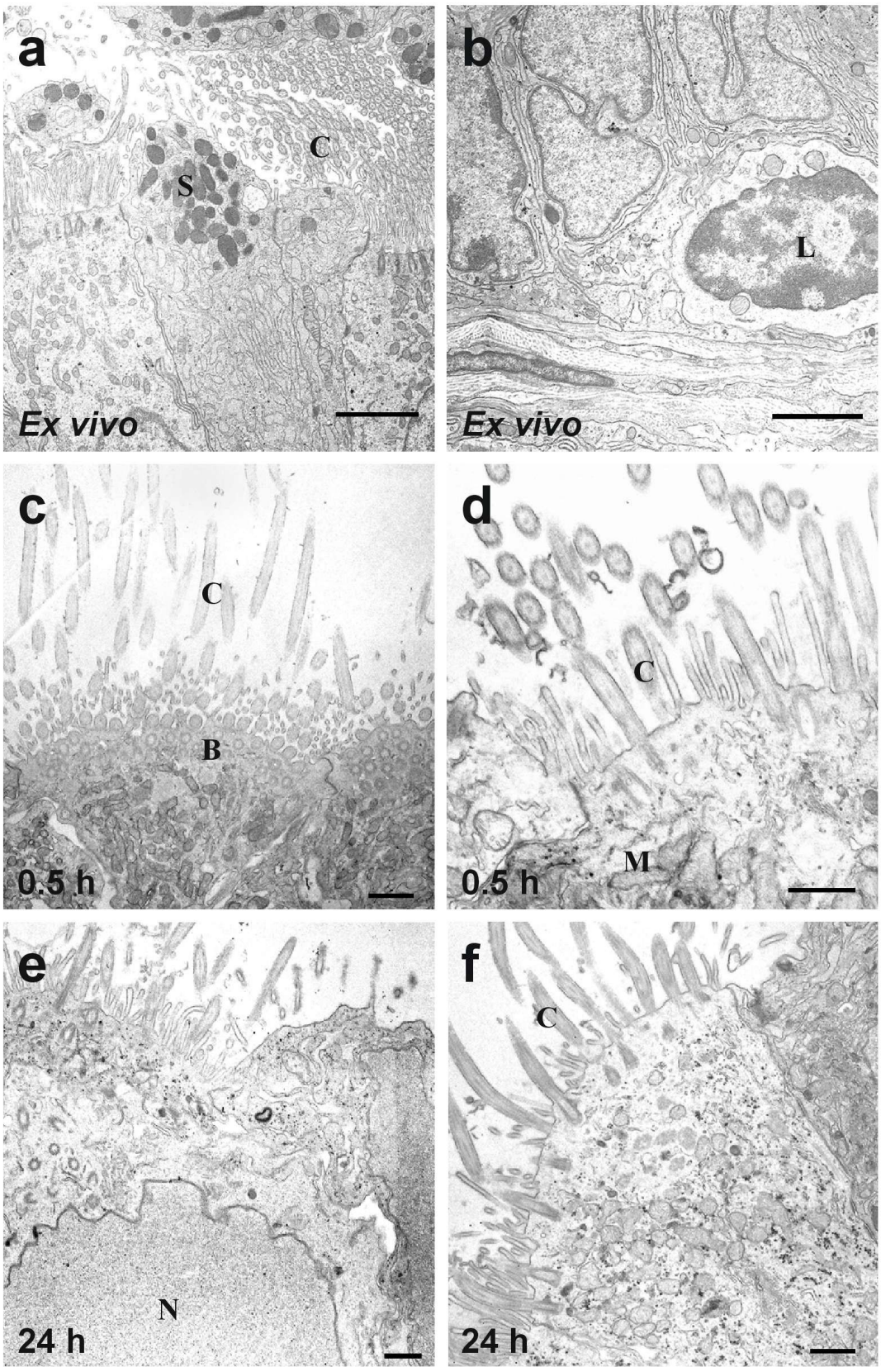

Fig. 14 Transmission electron microscopy of BOEC. BOEC in oviduct tissue ex vivo (a, b) and BOEC at 0.5 h in culture (c, d). BOEC after 24 h in culture (e, f). Cilia, mitochondria and basal bodies were visible throughout culture period. B = basal bodies, C = cilia, L = lymphocyte, M = mitochondria, N = nucleus, S = secretory vacuoles. *Bars* 2 µm.

Semithin section of BOEC aggregates after 24 h in culture confirmed the epithelial cell character and showed that a single layer of BOEC is forming the aggregate and that there are no cells contained between the epithelial layers. On the aggregate surface, many cilia are visible. Darker stained cells are secretory BOEC, brighter stained cells bear cilia (Fig. 15).

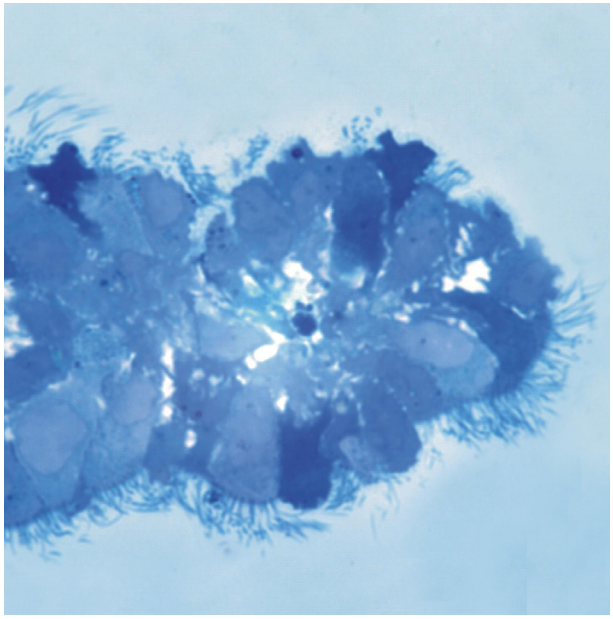

Fig. 15 Semi thin section of bovine oviduct epithelial cells (BOEC) organized in a suspended aggregate. Ciliated and secretory cells are visible. BOEC are arranged in a tight epithelial layer. Length of cilia approximately 8 µm.

4.3 RT-qPCR

4.3.1 RNA isolation and RNA yield

Cultured BOEC isolated from the ampullae of ipsilateral oviducts yielded significantly higher amounts of RNA per culture well with 10^6 seeded cells than BOEC isolated from the corresponding contralateral segment (2.73 ± 0.98 µg, n = 24 *versus* 2.31 ± 0.14, n = 12; $P < 0.05$). Sampling time (0.5, 6, 12, and 24 h) had no effect on RNA yield ($P = 0.974$).

4.3.2 Gene expression patterns during the course of culture

Quantitative RT-PCR failed to detect significant differences in the expression of marker genes *ESR1*, *ESR2*, *GPX4*, *HMGCR*, *OVGP1*, *PGR*, and *TRA1* (Table 2) between ipsi- and contralateral BOEC at different time points during the 24 h culture period (Fig. 16). However, *ESR2* expression of ipsi- and contralateral BOEC differed significantly ($P < 0.05$) at 0.5 h after seeding. Moreover, differences in *GPX4* expression between ipsi- and contralateral BOEC were significantly different ($P < 0.01$) in the combined analysis of all sampling time points (Fig. 16).

The combined statistical analysis of marker gene expression in ipsi- and contralateral BOEC at 0.5, 6, 12, and 24 h in culture revealed stable expression of *HMGCR* and *OVGP1* over all time points. The expression of *ESR2*, *GPX4* and *PGR* was stable at 6 h in culture and thereafter. *TRA1* was up-regulated at 6 h in culture ($P < 0.001$), returned to expression level at 0.5 h after 12 h in culture, and was stable at 24 h. *ESR1*, in contrast, was down-regulated at 6 h in culture ($P < 0.05$), returned to expression level at 0.5 h after 12 h in culture, and was stable at 24 h (Fig. 16).

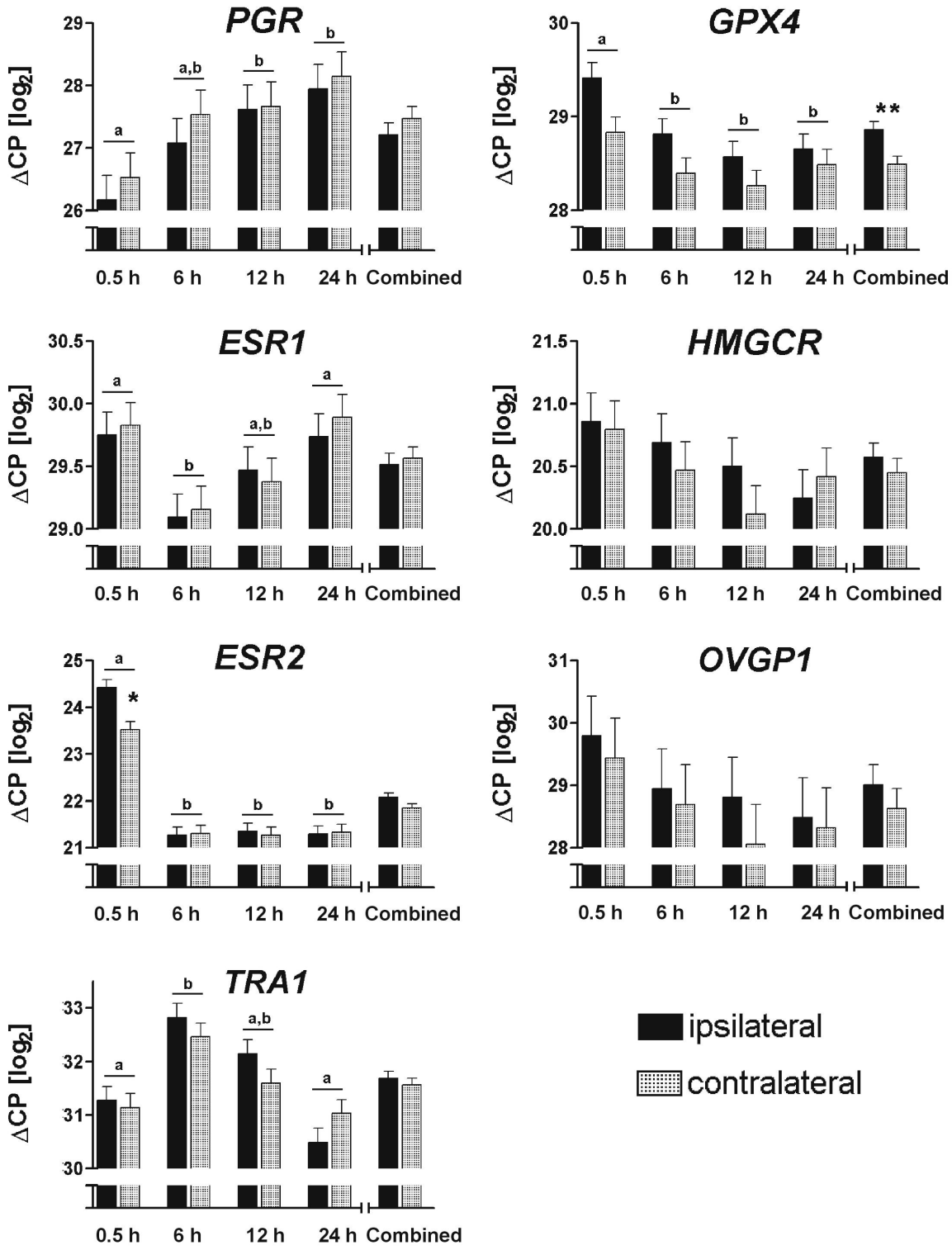

Fig. 16 RT-qPCR data for marker genes obtained from BOEC cultured in TCM-199 supplemented with 2% ECS. Transcript abundance was determined at 0.5, 6, 12, and 24 h after seeding. Data for BOEC isolated from ipsi- or contralateral oviducts are shown separately for the different time points and as combined data for all time points. One ΔCP unit represents a two-fold increase in mRNA. BOEC isolated from 3 individuals were analyzed. Different superscript letters indicate significant differences between sampling time points for the combined ipsi- and contralateral data. ESR1, GPX4, PGR: $P < 0.05$; ESR2, TRA1: $P < 0.001$. Significant differences between ipsi- and contralateral cells are indicated by asterisks. *: $P < 0.05$, **: $P < 0.01$.

4.3.3 Influence of the supplementation with different serum

Supplementation of TCM-199 with 2 % CS 3.5 instead of 2 % ECS did not change gene expression patterns, with the exception that down-regulation of *ESR1* was no longer significant ($P = 0.094$) (Fig. 17).

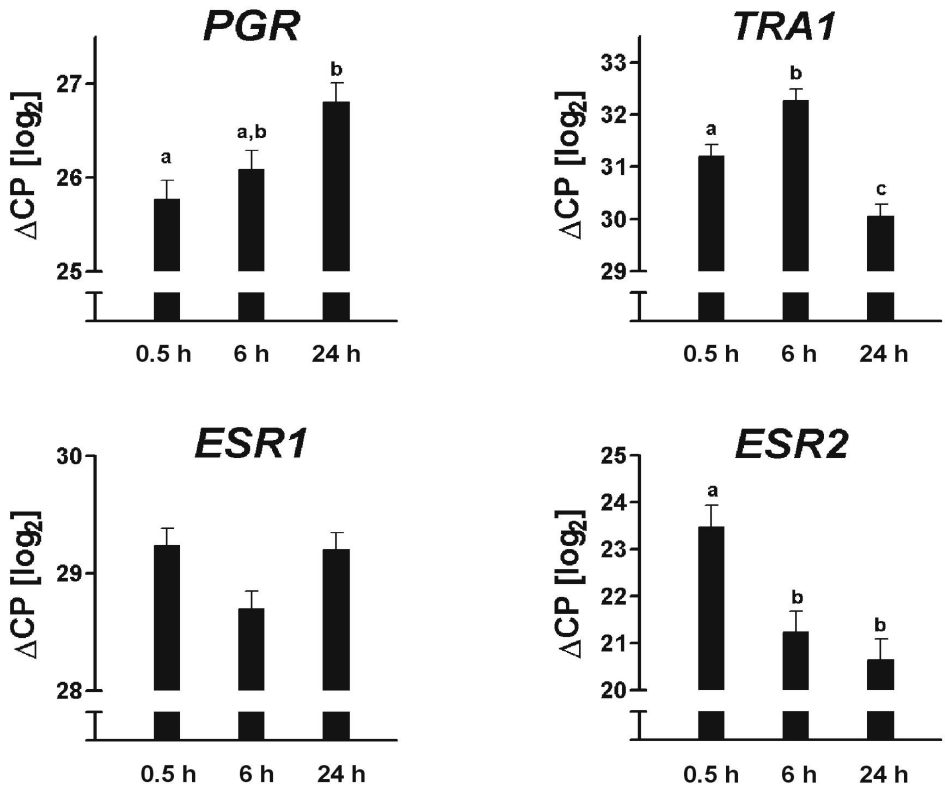

Fig. 17 RT-qPCR data for marker genes obtained from BOEC cultured in TCM-199 supplemented with 2 % CS 3.5. Transcript abundance was determined at 0.5, 6, and 24 h after seeding. Cells were isolated from ipsilateral oviducts of 4 animals. One ΔCP unit represents a two-fold difference in mRNA. Different superscripts indicate significant differences ($P < 0.05$) between sampling time points.

4.3.4 Gene expression of cultured BOEC after stimulation with steroid hormones

Transcript levels of *ESR1*, *ESR2*, and *PGR* were quantified by RT-qPCR to assess hormone responsiveness of cultured BOEC. Stimulation of cells with additional 10 pg/ml estradiol-17β (E2) for 6 h resulted in a significant increase of *PGR* transcript ($P < 0.05$). After 18 h of E2 stimulation, the difference in *PGR* expression between hormone stimulated and control cells was no longer significant. However, combined data from both sampling time points again showed a significant E2 effect ($P < 0.05$) on *PGR* expression. A significant increase in INOS transcript level after stimulation with P4 was observed in the combined data set (Fig. 18).

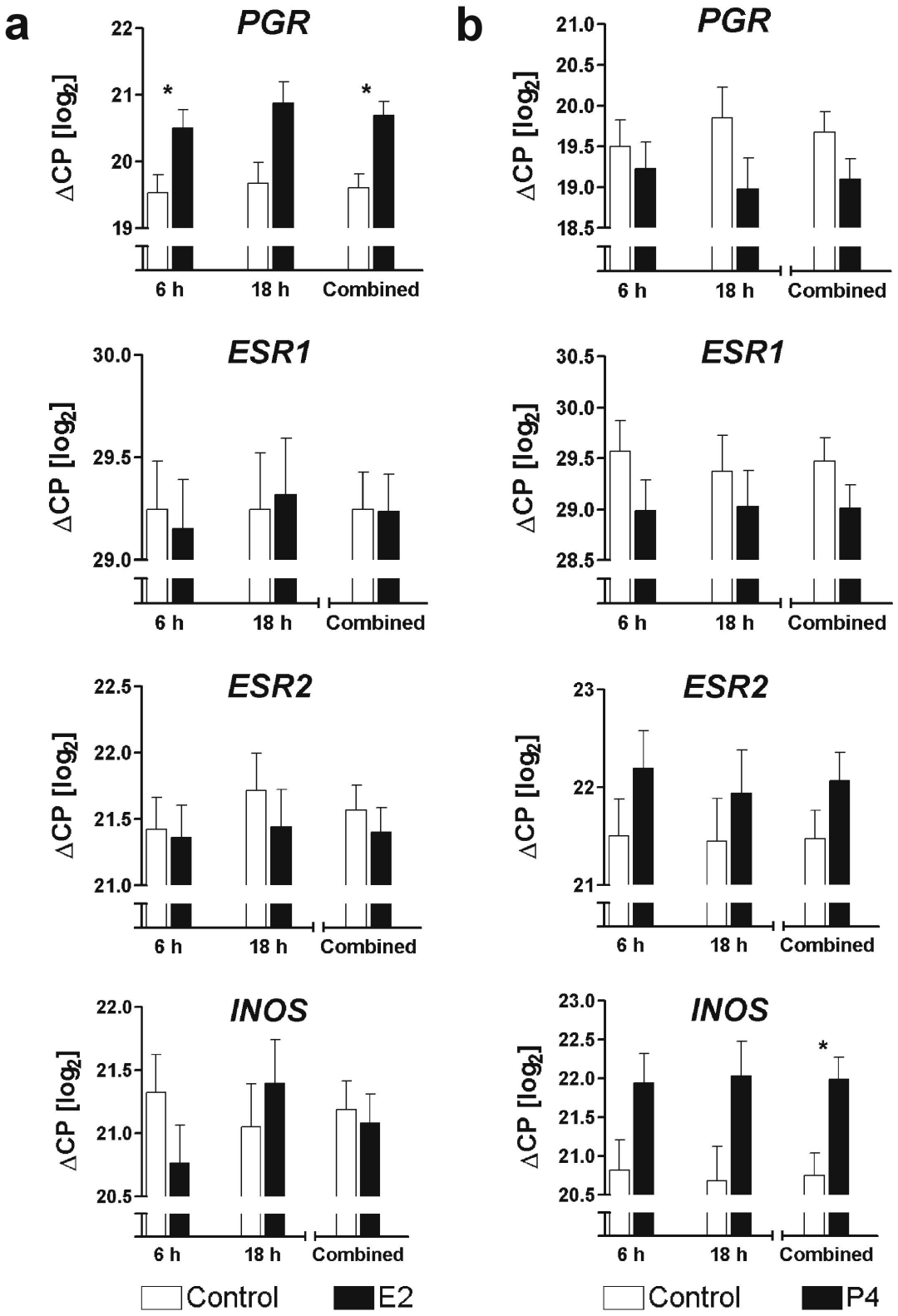

Fig. 18 RT-qPCR data for marker genes obtained from BOEC after stimulation with steroid hormones. Cells were precultured for 6 h, hormone stimulated and sampled after 6 and 18 h treatment. Data are shown separately for the two sampling time points and as combined data. Cells from 4 and 3 animals, respectively, were analyzed after 6 and 18 h stimulation. a Transcript abundance in controls and in cells stimulated with 10 pg /mL estradiol-17β (E2). b Transcript abundance in controls and in cells stimulated with 10 ng/mL progesterone (P4). Significant differences are indicated. *: $P < 0.05$.

4.4 Western blot analysis

Analysis of ESR1 and PGR in cultured BOEC by Western immunoblot showed stable expression of both steroid receptors throughout the culture period. PGR showed predominantly isoform A (Fig. 19).

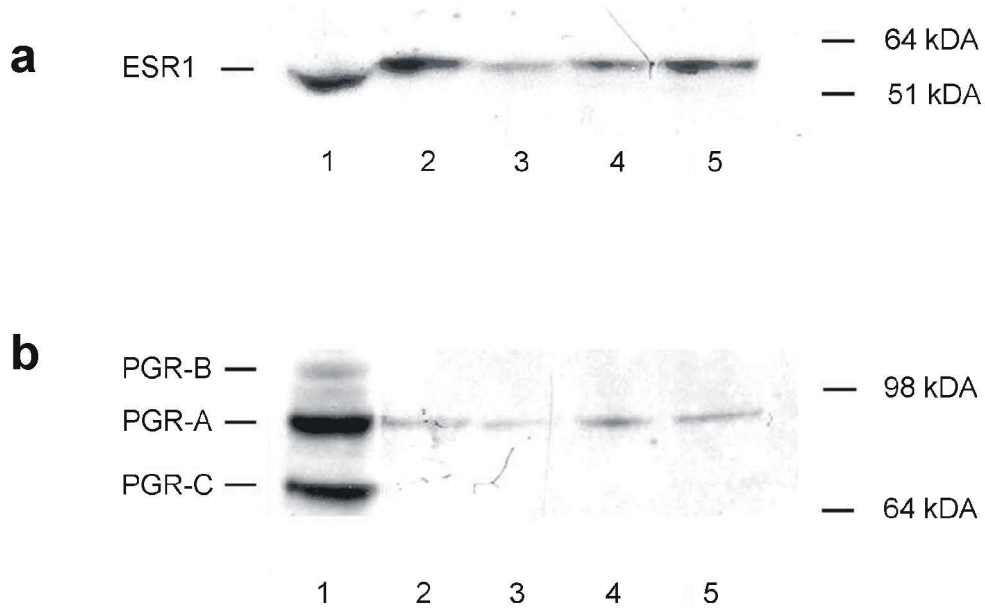

Fig. 19 Western blot analysis of ESR1 and PGR isoforms in BOEC during culture. Lane 1: control (bovine endometrium), lanes 2-4: BOEC from animal 1, cultured for 0.5, 12 and 24 h, respectively; lane 5: BOEC from animal 2, cultured for 24 h. a Blot for ESR1. b Blot for PGR isoforms A-C. BOEC express predominantly PR-isoform A.

5 Discussion

5.1 Stage of the estrous cycle

As there are marked morphological cyclic changes in the oviduct epithelium during the estrous cycle (Abe 1996, Yaniz et al. 2000) with pronounced changes in gene expression patterns (Einspanier et al. 1999, Gabler et al. 2003, Ulbrich et al. 2003, Bauersachs et al. 2004, Ulbrich et al. 2004), it seems is of critical importance to work with defined cells to gain insights in early pregnancy events. Cultured cells maintaining their special characteristics under culture conditions may keep cycle-specific features relevant for physiological processes concerning embryo-maternal interactions. Day 3.5 of the estrous cycle was chosen with respect to the physiological presence of the embryo in the oviduct at this timepoint. Thus, synchronous BOEC are provided for co-culture studies with embryos. Furthermore, working with cells from a well-defined stage of the estrous cycle is a requirement for standardized experimental conditions required for holistic transcriptome and proteome studies (Hiendleder et al. 2005). In most previous studies, bovine oviducts obtained from the slaughterhouse were used (Gandolfi and Moor 1987, Ellington et al. 1990, Sun et al. 1997, Boquest and Summers 1999, Bosch et al. 2001, Rief et al. 2002, Mishra et al. 2003, Pegoraro et al. 1998) to obtain material for cell culture experiments. As there is no control over the cyclic activity during the last months or of microbial contamination at the time of sampling, these culture systems cannot be standardized.

5.2 Cells derived from oviducts ipsi- or contralateral to the ovulation site

The oviduct location, ipsilateral or contralateral to the ovulation site, could affect cell behavior. Respective studies were recently undertaken to reveal differential gene expression between epithelial cells derived from the ipsi- or contralateral oviduct *in vivo* (Bauersachs et al. 2003). In our study, no differential gene expression between ipsi- and contralateral cell origin was observed under culture conditions. This is likely due to the complex counter-current blood flow mechanisms described in the female reproductive tract, e.g. between ovary and oviduct or uterus (McCracken et al. 1984, Stefanczyk-Krzymowska and Krzymowski 2002, Einer-Jensen and Hunter 2005). Under culture conditions, cell reactions are free of

these systemic steroidal regulations. To gain further insight into local hormonal conditions in the bovine reproductive tract, concentrations of estradiol-17β, progesterone, prostaglandin E, prostaglandin F2α, oxytocin, and endothelin-1 were measured in oviduct tissue, but these data did not represent the distribution in epithelial cells alone (Wijayagunawardane et al. 1998).

5.3 Cell preparation and culture conditions

In former studies, BOEC were often isolated by enzymatic treatments (Thibodeaux et al. 1991, Cox and Leese 1997, Sun et al. 1997, McNutt-Scott 1998, Reischl et al. 1999, Winger et al. 1997, Bosch et al. 2001, Mishra et al. 2003). In recent years, researchers favored cell material isolated via mechanical treatment (Lim et al. 1997, Pegoraro et al. 1998, Yadav et al. 1998, Boquest and Summers 1999, Kamishita et al. 1999, Gualtieri, Talevi 2000, De Pauw et al. 2002, Ulbrich et al. 2003). This appears to be a more promising approach as enzymatic solutions may cause damage to cells with fragile structures on their surface, e.g. cilia (Freshney 2000). A comparison between cell numbers obtained from bovine oviducts reported after enzymatic treatment (Thibodeaux et al. 1991, Reischl et al. 1999) and cell numbers obtained by mechanical processing only, as in the present study, shows that the dimension of the 10-fold difference is unlikely to be related to the person preparing cells or to variations in the cell counting procedure. Rather, it is a clear indication of cell loss by enzymatic treatments. Unfortunately, the available data about obtained cell numbers per oviduct using different cell isolation protocols are very limited.

Different media were used for BOEC cultures such as DMEM/F12, MB2, RPMI-1640, TCM-199, CMRL1066 (Abe, Hoshi 1997). More recently, TCM-199 was frequently in use (Lim et al. 1997; Sun et al. 1997, Winger et al. 1997, Yadav et al. 1998, Boquest and Summers 1999, Hwang et al. 2000, Kamishita et al. 1999, De Pauw et al. 2002, Gualtieri and Talevi 2000, Nandi et al. 2003, Ulbrich et al. 2003). For planned co-culture experiments with the described culture system, TCM-199 bears the advantage that IVP embryos can be cultured in modified Parker medium (MPM) with a composition very similar to TCM-199. Therefore, embryos might be less disturbed by the change from embryo culture medium to cell culture medium.

The typical trypan blue staining pattern in cell aggregates shows that our cell preparation method is suitable for BOEC to be cultured in aggregates, but not for cell singularization as it would be achieved by enzymatic digestion. Therefore, the use of enzymes with lower digestive activities than trypsin should be considered where cell singularization is required for further culture and studies.

Immunocytochemistry is a useful way to determine cell characteristics and to distinguish between epithelial cells and fibroblast contamination. In the present study, attached and proliferating cells were evaluated after 6 days in culture. Cells were stained after 6 days in culture. Fibroblast contamination in attached cell populations was very low, and the actual contamination of BOEC in aggregates is likely to be even lower, since fibroblasts can be expected to adhere easier to the support than their epithelial counterparts which are contained in vigorously moving aggregates. Therefore, fibroblasts can be expected to grow faster in monolayer than epithelial cells, which would lead to a higher fibroblast proportion. In endometrium epithelium cultures, cells after singularization are pre-incubated for 18 hours (Fortier et al. 1988) so that fibroblasts attach to the culture dish. The medium is then changed and selective attachment thus used to obtain a epithelial cell culture with higher purity. Histological studies using *in situ* staining methods on fixed BOEC aggregates could be endorsed to confirm our results, but did not seem obligatory as ultrastructural observations did not reveal an indication of the presence of fibroblast cells contained in BOEC cell aggregates after 24 h in culture. In an independent study, cell types contained in BOEC aggregates were examined and no fibroblasts were detected (Lefebvre et al. 1995).

5.4 Morphological findings

Light and electron microscopic examinations showed the presence of numerous cells with actively beating cilia on their apical membrane during the 24 h culture period. In monolayer studies, BOEC regularly lost their cilia (Thibodeaux et al. 1991, Walter 1995, Reischl et al. 1999). Cells retaining this important morphological feature are a major advantage of the developed short-term suspension culture system. Furthermore, conservation of cell polarity, an important charcteristic of epithelial cells, was demonstrated.

In other research groups, positive aspects of BOEC cultured in aggregates were observed concerning cell morphology, ultrastructure (Lefebvre et al. 1995, Walter 1995), and BOEC interaction with sperm. Sperm binding to BOEC aggregates was reported to be heavily concentrated on ciliated cells (Lefebvre et al. 1995), therefore it seems reasonable to use co-culture systems providing cilia to gain reliable results. In another study, spermatozoa bound preferably to cell aggregates as compared to monolayers (Baillie et al. 1997). Dedifferentiation of cells outgrowing from explants or aggregates while forming a monolayer has been described. Higher numbers of ciliated cells were found on the surface of attaching aggregates as compared with the adjacent outgrowing cells (Gualtieri and Talevi 2000). *In*

vitro, IGF2 release from vesicles was significantly higher than observed for monolayer cultures, showing a slight advantage of aggregate culture, although this was the only significant difference with respect to IGFs und IGFBPs analyzed in both culture systems (Winger et al. 1997).

Another clear advantage of using cells in their natural coherence is the faster availability for experiments. Cells may be used as soon as 6 to 12 h after seeding. The fact that singularized cells have to attach and establish new cell-to-cell contacts raises the question whether these structures are similar and functionally comparable to those observed *in vivo* or whether cells are already dedifferentiated when ready for use.

Thus, BOEC cultured in non-attached aggregates offer advantages for co-culture experiments because of their availability and well conserved morphology.

5.5 Gene expression patterns during the culture period

Quantitative PCR studies are a tool suitable to evaluate cell behavior *in vitro*. Changes in gene expression levels mirror dedifferentiation processes prior to the perception of ultrastructural alterations. Comparisons between gene expression patterns *in vitro* and *in vivo* allow a very specific characterization of the differentiation state of cultured cells.

Until now, gene expression patterns during BOEC culture were not routinely moitored, but semi-quantitative examinations on *OVGP1* expression in cultured cells has previously been used as an indicator of cell quality. *OVGP1* production is assumed to be a basic feature of oviduct epithelial cells (Reischl et al. 1999, Briton-Jones et al. 2002, Briton-Jones et al. 2004).

In this study, seven candidate genes were used to confirm stable culture conditions during the whole culture period. Genes under examination in addition to the steroid receptors *ERa*, *ERβ* and *PR* (Ulbrich et al. 2003, Bauersachs et al. 2004) and *GPX4*, which was recently shown to be differentially expressed in the ipsi- *versus* the contralateral oviduct *in vivo* (Bauersachs et al. 2003, Lapointe et al. 2005). In our study, *GPX4* was the only gene maintaining a tendency for an ipsi-contralateral difference in expression level under culture conditions. This suggests a long-term activation of *GPX4* in vivo.

OVGP1, oviduct specific glycoprotein 1, is a glycoprotein supporting embryonic development *in vivo* and *in vitro* (Leese et al. 2001). *OVGP1* mRNA levels appear to be highest around ovulation and decrease slightly on Day 3.5 in the ipsilateral oviduct (Bauersachs et al. 2003). It was previously used to evaluate BOEC quality in culture (Reischl et al. 1999, Briton-Jones et al. 2002). Monolayer cultures showed a significant decrease in *OVGP1* expression from

cell seeding to monolayer achievement. The presented suspension culture system, in contrast, did not show significant changes during the 24 h culture period. Therefore, the beneficial effects of OVGP1 on embryonic development can be expected to be maintained as well. This provides further evidence for the high quality of BOEC cultured in aggregates. Clearly, the maintenance of the expression level is connected to the short culture period, but as aggregated cells are utilizable after a short time, this is a definite advantage of the suspension culture system.

TRA1, B. taurus tumor rejection antigen 1, formerly known as 94-kDa glucose-regulated protein (*GRP94*), is differentially expressed in ipsi- *versus* contralateral bovine oviducts *in vivo*. It acts as a chaperone and tumor marker, whereas its expression increases under conditions that interfere with normal glycosylation and folding or assembly of proteins (Watanabe et al. 2001).The up-regulation after 6 h in culture and the prolonged adaptation period could thus be a response to change in environmental conditions. After 12 h in culture, the transcript level returned to the level at seeding.

Taken together, these findings indicate that gene expression is stable after 6 h in culture for the majority of genes examined. For stable culture conditions, an adaptation period of 6 to 12 h after seeding is therefore recommended until further treatment, e.g. addition of embryos for co-culture experiments.

The absence of differences in the gene expression patterns of cultured BOEC derived from ipsi- versus contralateral oviducts confirms the presence of local regulatory mechanisms *in vivo* such as a counter-current blood flow between ovary and oviduct on either side of the female genital tract (Einer-Jensen and Hunter 2005). These results lead to the assumption that both oviducts may be used for cell culture experiments. On the other hand, additional genes have to be examined because the increased RNA amount in cells derived from the ipsilateral oviduct might indicate responses of other genes not chosen as candidates in the present experiments.

5.6 Steroid responsiveness of cultured BOEC

Bovine oviduct epithelial cell gene expression profiles show pronounced differences between estrous and diestrous (Bauersachs et al. 2004) and hormone stimulation is expected to affect gene expression in cultured BOEC. The presence of ESR1 and PGR protein in cultured cells at different time points was confirmed by Western blot analysis. BOEC isolated from the ampulla expressed only PGR isoform A, which is known to predominate in the early luteal

phase (Ulbrich et al. 2003). This is consistent with previous studies, which failed to detect PGR isoform C in the bovine oviduct and observed isoform B in isthmus rather than ampulla (Ulbrich et al. 2003).

Supplementation with E2 significantly increased *PGR* transcript abundance in BOEC after 6 h stimulation, P4 showed no effect. *ESR1* and *ESR2* did not show a response to E2 or P4 treatment 6 and 18 hours after supplementation. Gene expression of BOEC *in vivo* varies markedly during the estrous cycle, i.e., it is steroid hormone dependent (Bauersachs et al. 2004). Our data are in agreement with data obtained from BOEC *ex vivo* that showed up-regulation of *PGR*, but stable *ESR1* and *ESR2* expression in the estradiol-17β-dominant follicular phase (Ulbrich et al. 2003). The increase in *PGR* mRNA may have been caused by increased PGR transcript level or posttranscriptional stabilization of *PGR* mRNA (Petersen et al. 1989).

In the combined data, a significant increase as a respond to P4 treatment was observed. *INOS*, inducible nitric oxide synthase, is known as a regulator of the oviductal environment and plays a role in regulating contractility and immune response in the bovine oviduct (Ulbrich et al. 2005). In vivo, it shows cycle dependent changes in expression level only in the isthmus, especially an up-regulation on Day 3.5, when P4 begins to increase, *versus* Day 0 and Day 12 (Ulbrich et al. 2005).

5.7 Conclusion

According to the comprehensive morphological and functional characterization, BOEC cultured as suspended cell aggregates over a 24 h time period are a very promising tool for co-culture studies with embryos for obtaining samples for holistic studies on embryo-maternal communication.

As there is rising evidence that the presence of embryos in the oviduct causes changes in the maternal organism, the oviduct may not just be routinely prepared for the possible presence of embryos but could give a specific maternal response to the presence of early embryonic stages. Further research will help to elucidate the role of the oviduct in reproduction and, as demanded by Conway-Myers (1998) "to recognize the potential of the fallopian tube as more than a highway for oocytes to transverse in their pathway to the uterus".

6 Summary

A short-term suspension culture system for bovine oviduct epithelial cells suitable for the study of embryo-maternal communication processes

A short-term (24 h) culture system for bovine oviduct epithelial cells (BOEC) suitable for co-culture experiments with embryos was established and evaluated. BOEC were obtained on Day 3.5 of the estrous cycle and processed by mechanical means only to obtain cell aggregates. Cell yields were 10-fold higher as described in former studies employing enzymatic treatment to achieve a single cell suspension for seeding to obtain a BOEC monolayer.

Light microscopic examinations showed vigorously beating cilia on the apical side of the BOEC in aggregates and a rapid and constant motion of cell aggregates due to active ciliary beat. Scanning electron microscopy and transmission electron microscopy confirmed ultrastructural characteristics of BOEC at seeding and after 24 h in culture very similar to the situation *in vivo*. Both secretory cells with numerous secretory granules and ciliated cells with long, well-developed kinocilia were visible. The purity of the epithelial cell culture was > 95 %, as assessed by immunocytochemical methods.

For further characterization of cultured BOEC, gene expression patterns were examined after different time spans in culture. Cultured BOEC isolated from ampullae ipsilateral to the ovulation site yielded significantly higher amounts of RNA than their contralateral counterparts (2.73 ± 0.98 *versus* 2.31 ± 0.14 µg per 10^6 cells). However, quantitative PCR did not detect significant differences in transcript levels between ipsi- and contralateral BOEC for the majority of marker genes (*ESR1*, *ESR2*, *HMGCR*, *OVGP1*, *PGR*, *TRA1*) throughout the 24 h culture period. The analysis of combined data obtained from different sampling time points during the culture period revealed an effect only for *GPX4* (*B. taurus* non-selenium glutathione phospholipid hydroperoxide peroxidase), a gene known to be differentially expressed *in vivo.*. Marker gene expression of five genes remained stable after 6h of cell culture, indicating only a short adaptation period of cultured cells. The use of two different sera (estrous cow serum *versus* cow serum obtained on Day 3.5 of the estrous cycle) in a concentration of 2 % did not affect gene expression patterns.

Western blot analysis confirmed ESR1 (estrogen receptor α) and PGR (progesterone receptor) protein expression throughout the culture period. In agreement with cyclic differences *in vivo*,

stimulation with 10 pg/ml estradiol-17β increased *PGR* transcript abundance in BOEC significantly. A response to the stimulation with 10 ng/ml progesterone was shown as *INOS* (inducible nitric oxide synthase) gene expression increased significantly after steroid treatment.

Thus, the developed culture system provides functional BOEC with an unchanged morphology and maintained functionality as compared with cells *in vivo*. The system is rapidly available for use in co-culture experiments with bovine embryos and provides cultured cells in sufficient quantities for holistic transcriptome and proteome studies, thereby helping to decipher early embryo-maternal communication.

7 Zusammenfassung

Ein Kurzzeit-Suspensions-Kultursystem für Oviduktepithelzellen des Rindes zur Untersuchung von Mechanismen der frühen embryo-maternalen Kommunikation

Es wurde ein Kurzzeit-Kultursystem (Kulturdauer 24 h) für Oviduktepithelzellen des Rindes (BOEC), welches sich für Ko-Kulturversuche mit Rinderembryonen eignet, entwickelt und validiert. BOEC wurden von Tieren an Tag 3,5 des Zyklus unter Anwendung lediglich mechanischer Methoden gewonnen und befanden sich in Zellaggregaten, in welchen die natürlichen Zell-Zell-Verbindungen erhalten blieben. Die Zellausbeute war 10-mal höher als in früheren Studien, in welchen die Zellen unter Enzymeinwirkung vereinzelt worden waren, bevor sie zum Monolayer auswuchsen.

Im Lichtmikroskop konnte man kräftig schlagende Zilien auf den apikalen Zelloberflächen beobachten. Die Zellaggregate befanden sich während der gesamten Kulturdauer in rotierender Bewegung aufgrund des aktiven Zilienschlages an der Außenseite der Aggregate. Untersuchungen im Raster- sowie Transmissionselektronenmikroskop bestätigten die sehr gut erhaltene Ultrastruktur der BOEC zum Zeitpunkt der Aussaat und nach 24 h in Kultur. Sowohl sekretorische Zellen mit zahlreichen sekretorischen Granula als auch zilientragende Zellen mit langen, gut entwickelten Zilien waren sichtbar. Die Reinheit der Epithelzellkultur, bestimmt mittels Immunzytochemie, betrug > 95 %.

Zur weiteren Charakterisierung der kultivierten BOEC wurden Genexpressionsmuster der Zellen nach verschieden langer Kulturdauer untersucht. Kultivierte Zellen, die ursprünglich aus Eileiterampullen ipsilateral zur Ovulationsstelle gewonnen worden waren, zeigten signifikant höhere RNA-Erträge als die kontralateral gewonnenen BOEC (2.73 ± 0.98 *versus* 2.31 ± 0.14 µg pro 10^6 Zellen). Quantitative PCR-Untersuchungen der eingesetzten Kandidatengene (*ESR1*, *ESR2*, *HMGCR*, *OVGP1*, *PGR*, *TRA1*) ließen jedoch während der 24-stündigen Kulturdauer keinen Unterschied im Expressionsmuster ipsilateral bzw. kontralateral gewonnener Zellen erkennen. Lediglich für *GPX4*, ein *in vivo* differentiell exprimiertes Gen, ergab die statistische Auswertung über die gesamte Kulturdauer hinweg einen signifikanten Effekt. Die Expression von fünf Kandidatengenen war nach 6 h in Kultur stabil, dies bedeutet eine nur kurze Adaptationszeit an die Kulturbedingungen, nach welcher konstante Bedingungen in der Zellkultur herrschen. Die Verwendung zweier unterschiedlicher Seren (Serum östrischer Kühe *versus* Serum von Kühen an Tag 3,5 des Zyklus) in einer

Konzentration von 2 % im Kulturmedium rief keine signifikante Veränderung der Genexpressionsmuster hervor und bewies die Stabilität der Kulturbedingungen.

Das Vorhandensein der Steroidrezeptoren ESR1 (Estrogenrezeptor α) und PGR (Progesteronrezeptor) wurde mittels Western blot-Verfahren nachgewiesen. Die Hormonresponsivität der kultivierten Zellen wurde anhand der Zugabe von 10 pg/ml Estradiol-17β oder von 10 ng/ml Progesteron überprüft. Wie es der physiologischen Situation in vivo entspricht, wurde die Expression von *PGR* durch Estradiol-17β gesteigert. Die Genexpression von *INOS* (induzierbare Stickstoffoxidsynthase) konnte durch Progesteronzugabe signifikant gesteigert werden.

Das entwickelte System ermöglicht es, morphologisch intakte und funktionell erhaltene BOEC zu kultivieren und innerhalb kürzester Zeit für nachfolgende Ko-Kulturexperimente mit Rinderembryonen zur Verfügung zu stellen. Die Gewinnung ausreichender Mengen an Zellmaterial für unfassende Transkriptom- und Proteom-Untersuchungen ist gewährleistet, so dass das Kultursystem ein wertvolles Instrument zur Untersuchung der frühen embryo-maternalen Kommunikation darstellt.

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Curriculum vitae

Name	Regine Elisabeth Rottmayer
Date of Birth	13.05.1977
Place of Birth	Munich
Family status	married

Education

09/1983 - 07/1985	Primary school Uffenheim
09/1985 - 07/1987	Primary school Anzing
09/1987 - 06/1996	Grammar school "Franz-Marc-Gymnasium Markt Schwaben"
11/1996 - 08/2002	Studies of Veterinary Medicine, Ludwig-Maximilian-University Munich, State examination
since 01/2003	Department for Molecular Animal Breeding and Biotechnology, Ludwig-Maximilian-University Munich, Chair: Prof. Dr. E. Wolf
since 04/2003	Dissertation: " A short-term suspension culture system for bovine oviduct epithelial cells suitable for the study of embryo-maternal communication processes "