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Establishment of *in vitro* cultures of porcine reproductive epithelia - oviduct and cervix uteri -

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1. Introduction

1.1. The porcine female reproductive tract

The porcine female reproductive tract consists of paired ovaries and fallopian tubes, a uterus bicornis with a cervix uteri, a vagina and vulva (see figure 1.1).

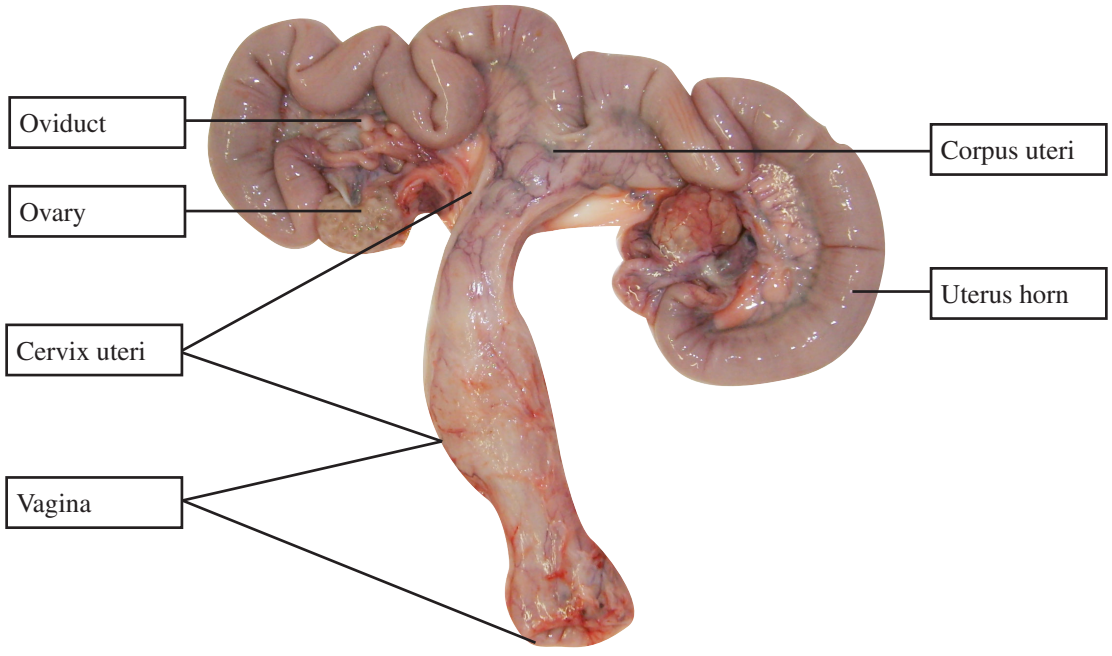

Figure 1.1.: Female reproductive tract of a prepubertal pig.

Each section hosts its own important properties to enable successful reproduction.

The ovaries synthesize steroid hormones to regulate cyclic events like the preparation of a possible pregnancy and provide fertile oocytes.
In the oviduct, essential processes such as maturation and transport of gamete and embryo (Blandau et al., 1975; Suarez and Pacey, 2005), sperm-epithelium interaction (Rodriguez-Martinez, 2007), selection of sperm, fertilization of the ovum (for review see Brüssow et al. (2008)), and early embryo-maternal communication (Hill, 2001) take place.
In the uterus, sperm capacitate and are passively transported by streams of uterine secrets towards the oviducts. Pregnancy recognition is also located in the uterus. Here, the embryonic and the following fetal development is facilitated by implantation and development of a fetal and maternal placenta (Bazer et al., 2010).
The cervix uteri maintains the physiological and healthy intrauterine milieu (Quayle, 2002; Timmons et al., 2010; Ochiel et al., 2008), takes part in sperm-epithelium interaction and signal reception from the seminal plasma (Song et al., 2010; Robertson, 2007). Furthermore, it provides in cooperation with the vagina a physical and microbicidal barrier to protect the proximal reproductive tract. The cervix, the vagina and the vulva form the soft birth canal.

1.1.1. Epithelial cells

Epithelial cells cover the inner and outer surfaces of the body as an interface between the organism's connective tissue and the environment, and form a barrier between biologically different compartments (Liebich, 2004). Epithelia can be divided in epithelial types due to cellular form (squamous, isoprismatic or columnar) and the numbers of cell layers (single-layer, stratified or pseudostratified, figure 1.2). Tight intercellular connections between neighbouring cells are characteristic for epithelial cells. Next to barrier-functions, the cells are involved in resorption, transport of ions, macromolecules and water, or secretion depending on tissue properties. The epithelium is clearly separated from the underlying tissue by the basement membrane,

and is supplied with nutrients from basolateral by means of diffusion. An important feature of differentiated epithelial cells is their polarity. The apical or luminal compartment of the cellular membrane is functionally and structurally different from the basolateral side. The two domains of the cellular plasma membrane are separated by tight junctions encircling the apex of the cell and sealing neighbouring cells to each other (figure 1.3).

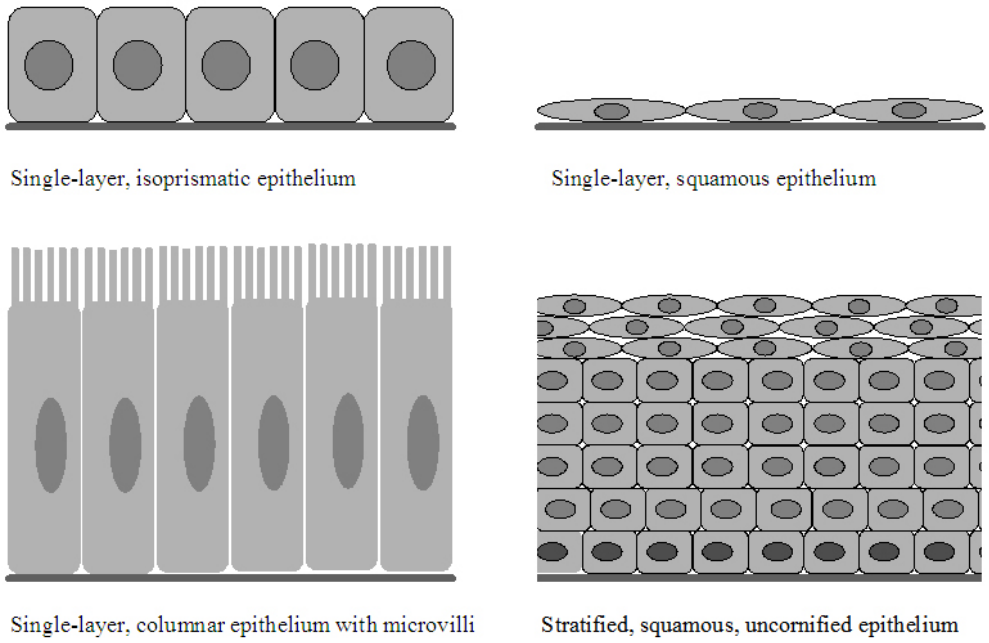

Figure 1.2.: Epithelial tissue, modified from Liebich (2004).

1.1.2. The porcine oestrus cycle

The cyclic activity of the pig is non-seasonal and polyoestrus with a mean cycle length of 21 days. The mammalian cycle in general can be divided into three stages: prooestrus (day 18-21), oestrus (day 1-2) and interoestrus or dioestrus (day 3-17). During the early interoestrus, a large number of follicles start to grow in the ovaries. During the prooestrus, only selected dominant follicles develop further, while all other follicles become atretic. Here, in parallel to the follicular growth, the peak of estradiol concentration is reached. During oestrus the level of estradiol falls already.

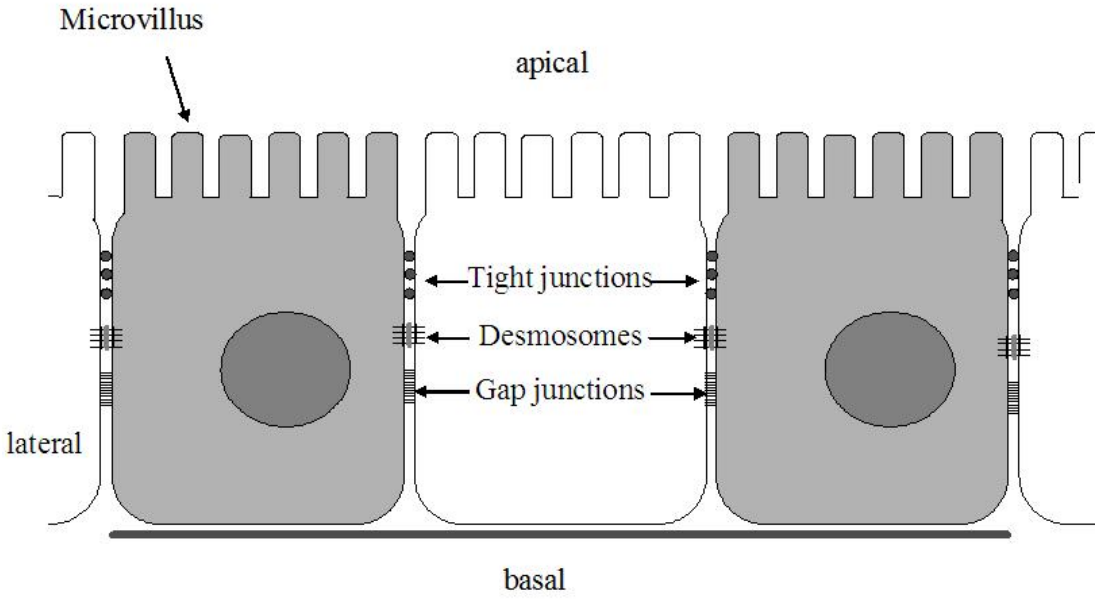

Figure 1.3.: Polarized, epithelial cells. Modified from Simons and Fuller (1985).

In the early oestrus, as a positive feed-back to estradiol, a LH[1]-peak occurs and fosters the further ripening of the dominant follicles until ovulation. The corpus luteum develops from the follicular walls as a result of the luteotropic effect of LH. The follicular granulosa cells in the corpus luteum produce progesterone. After ovulation at the end of the oestrus, the FSH[2]-peak occurs and stimulates growth of new follicles for the next cycle. The level of progesterone increases until it reaches a plateau from day 8 to day 14. As a negative feed back, progesterone suppresses gonadotropin secretion from the anterior pituitary gland. After that, in parallel to the regression of the corpus luteum, the concentration of progesterone decreases to the lowest level and a new cycle can begin (Schnurrbusch and Hühn, 1994; Bostedt, 1995; Schnurrbusch and Vogler, 2005).

[1]LH: Luteinizing Hormone
[2]FSH: Follicle Stimulating Hormone

1.1.3. Epithelia of the porcine reproductive tract

The histological structure of the porcine tubular reproductive tract is classically built up of the tunica mucosa containing the epithelium mucosae and the lamina propria mucosae, the tunica muscularis with its stratum circulare and longitudinale, and the tunica serosa.

The reproductive epithelia undergo cyclic changes due to the hormonal regulation as described before (section 1.1.2).

The oviductal epithelium consists of two cell types: secretory and ciliated cells that cover the connective tissue as a columnar single-layer (Abe and Hoshi, 2007, 2008). During prooestrus and oestrus with high estradiol levels, the height of the oviductal epithelial cells is decreased (Patek, 1974). The secretory cells secrete the oviductal fluid containing proteins and glycoproteins (Oliphant, 1986). Their activity is at its maximum at this stage of the cycle (Patek, 1974; Verhage et al., 1979). Ciliated cells show a high level of ciliation (Verhage et al., 1979; Abe and Oikawa, 1992) and the cilial beat frequency is increased during oestrus (Lyons, 2002). During interoestrus, a deciliation of 10% of the ciliated cells and a decreased secretory activity was detected by Verhage et al. (1979).

The epithelial layers of the endometrium change from simple isoprismatic or low columnar in the late interoestrus and prooestrus to a high columnar or pseudostratified epithelium in oestrus and mid interoestrus (Kaeoket et al., 2001). Temporarily, epithelial cells show kinocilia (Liebich, 2004; Leiser et al., 1988). The epithelium forms tubular uterine glands by invaginations. During the proliferative phase of the uterus under estradiol dominance, the tubular uterine glands are prolonged and the number of epithelial cells is increased (Liebich, 2004). The amount of secretory vesicles is highest in dioestrus (Kaeoket et al., 2001) which indicates a high secretory activity under progesterone influence. The uterine glands are shortened, tortuous and covered with columnar epithelial cells at this stage of the cycle (Liebich, 2004). There are only few studies dealing with the investigation of the porcine cervical

epithelium (Krebs, 2007). These studies do not come to uniform results concerning the epithelial structure. In a PhD-thesis, Krebs (2007) compared these studies and investigated cyclic or age-dependent alterations in the cervix uteri. The cervical epithelium type of the sow changes from cranial to caudal, as it is a columnar single-layer epithelium on the inner, uterine side and a stratified epithelium with two to four, (Libal, 1990) or up to ten (Krebs, 2007) on the outer, vaginal side. Only Libal (1990) could show microvilli and cilia on the apical side of the epithelial cells throughout the cycle. Libal (1990) detected epithelial alterations correlated with cyclic changes of hormonal levels. In the interoestrus, she could show a mixture of epithelial types from a purely stratified to a stratified epithelium covered with a columnar single-layer in the caudal part of the cervix whereas in the oestrus, she could only find stratified epithelium. Krebs (2007) could not show any morphological alteration correlating with the cycle. The cervix uteri does not contain any glandular structure, so mucus is probably secreted into the cervical lumen through holocrine secretion (Libal, 1990; Kleinen, 2006; Krebs, 2007).
The vagina is covered by a stratified epithelium that is keratinized depending on cycle stage (Liebich, 2004).

1.2. Cell culture

1.2.1. History and terminology

In 1885, for the first time, cells could be maintained alive outside the animal body for several days by Wilhelm Roux (Alberts, 2002). A great development of *in vitro* research began. The first human immortal cell line (HeLa), derived from a cervical adenocarcinoma of Henrietta Lacks, was established by George Otto Gey in 1952 (Alberts, 2002). HeLa has become a widely used and important cell line for *in vitro* studies.

Since the middle of the 20th century, *in vitro* systems based on cell culture help to investigate molecular biology of single cells and tissues, and find application in a range of scientific fields like immunology, pharmacology, toxicology and tissue engineering (Freshney, 2005a).
In 1990, the Tissue Culture Association Terminology Committee officially published the following definitions concerning cell and tissue culture (Schaeffer, 1990):

Primary Culture *A culture started from cells, tissues or organs taken directly from organisms. A primary culture may be regarded as such until it is successfully subcultured for the first time. It then becomes a "cell line".*

Cell Line *A cell line arises from a primary culture at the time of the first successful subculture. The term cell line implies that cultures from it consist of numerous lineages of cells originally present in the primary culture. The terms finite or continuous are used as prefixes if the status of the culture is known.* [...]

Passaging *The transfer or transplantation of cells, with or without dilution, from one culture vessel to another. It is understood that any time cells are transferred from one vessel to another, a certain portion of the cells may be lost and, therefore, dilution of cells, whether deliberate or not, may occur. This term is synonymous with the term "subculture".*

1.2.2. Epithelial cell culture systems in reproductive research

The molecular pathways in the morphologically and functionally very different tissues of the reproductive tract are still not completely explored. An increasing subfertility and infertility in human as well as fertility problems in livestock like dairy cattle (Walsh et al., 2011) makes the investigation of reproductive epithelia essential. Cell culture systems derived from reproductive epithelia are necessary tools to

understand potential causes for the decrease of fertility and to generate knowledge about the molecular pathways within the individual tissues.

The maintenance of basoapical polarity of epithelial cells *in vitro* is a necessary prerequisite to control survival and proliferation as well as pivotal functions of the cells (Adissu et al., 2007).

As an example, non-polarized human oviductal epithelium in culture did not show an *in vivo*-like response to estradiol stimulation concerning oviductal protein expression, whereas the polarized cell culture followed the expected principles (Briton-Jones et al., 2004). In addition, Baghaban Eslami Nejad et al. (2005) could show an improved development of ovine *in vitro* embryos when co-cultured with polarized endometrial cells in comparison to a co-culture with non-polarized cells.

To study human reproductive biology, the investigation of cell cultures derived from human material would be ideal. Polarized cell cultures of the human reproductive tract are already established and find application in a variety of scientific questions (see table 1.1).

However, the limited availability of healthy human tissues, the ethical tenability respectively, and the need for reproductive research in veterinary medicine necessitates model systems derived from other species. In scientific approaches investigating the mammalian reproductive physiology and pathology, cell cultures of some parts of the reproductive tract are already established using the pig as favoured model species. Table 1.2 gives an overview of such systems derived from porcine tissues, assuming the pig as model species.

Oviductal epithelium Concerning the oviduct, the oestrus cycle has not been simulated *in vitro* yet, since existing *in vivo*-like cultures were only usable for short-term cultivation. Investigation of specific expression patterns in different cycle stages will reveal fascinating regulation mechanisms that can be further explored and modulated *in vitro*, and will provide valuable information about new targets to support fertility or enable contraception. Next to that, the regulation of ciliary beat fre-

Tissue	Cells	Culture	Polarity	References
oviduct	primary	growth on filters	morphology, detection of polarity markers	Levanon et al. (2010)
endometrium	primary	growth on filters coated with ECM[a]	morphology	Classen-Linke et al. (1997)
endometrium	cell line	growth on filters coated with ECM	morphology	Park et al. (2003)
cervix uteri and vagina	passage 1	growth on filters	morphology	Ayehunie et al. (2006)
cervix uteri	primary	growth on filters	TEER[b]	MacDonald et al. (2007)
vagina	cell line	multicellular spheroids [c]	morphology	Hjelm et al. (2010)

Table 1.1.: Published polarized epithelial cell culture systems derived from the human reproductive tract. The single cell cultures are introduced mentioning the original tissue, the used cell types (primary, once passaged or cell lines) and method of culture. Most of the cells are grown on permeable filters either uncoated or coated with ECM. Polarity was shown either by morphological criteria in cross-sections, measurement of TEER or by detection of markers for polarity

[a]ECM: Extracellular matrix, usually a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells. Used as coating of cell culture plates for cellular differentiation Mazzoleni et al. (2009).

[b]TEER: Transepithelial electrical resistance, a sign for a confluent and dense epithelium

[c]Multicellular spheroid: Form of organotypic culture. Epithelial cell aggregates to reconstitute tissue-like organisation (Mazzoleni et al., 2009). Cells on the outer surface show a differentiated, polarized morphology.

Tissue	Cells	Culture	Polarity	References
oviduct	primary	growth on cell culture plate	morphology (SEM[a])	Areekijseree and Vejaratpimol (2006)
endometrium	primary	growth on filters[b]	morphology	Guseva et al. (2003)
endometrium	passage 1	growth on filters	TEER[c]	Deachapunya and O'Grady (1998)
endometrium	primary	growth on cell culture plate coated with ECM[d]	morphology (SEM)	Zhang and Davis (2000)
endometrium	primary	growth on filters coated with ECM	TEER, distinct secretory profiles	Bowen et al. (1996)
cervix uteri	primary	filter	morphology	Guseva et al. (2003)
vagina	-	-	-	-

Table 1.2.: Pubblished polarized epithelial cell culture systems derived from the porcine reproductive tract. The single cell cultures are introduced mentioning the original tissue, the used cell types (primary, once passaged or cell lines) and method of culture. Most of the cells are grown on permeable filters or cell culture plates either uncoated or coated with ECM. Polarity was shown either by morphological criteria in cross-sections or via SEM, measurement of TEER. In one study, polarity was proved showing different secretion profiles on the basolateral and apical side.

[a]SEM, Scanning electron microscopy. Visualization of sufaces

[b]Culture of cells in layers on top of porous membranes/surfaces supports polarization of epithelial cells.

[c]TEER: Transepithelial electrical resistance, a sign for a confluent and dense epithelium

[d]ECM: Extracellular matrix, usually a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells. Used as coating of cell culture plates for cellular differentiation Mazzoleni et al. (2009).

quency is also not yet completely understood. Even the significance of the cilial activity for successful reproduction is not clear (Lyons, 2002). A new approach for cilia movement analysis could be utilized to decipher basic transport mechanisms as well as human ciliary diseases, such as Kartagener syndrome, 'immotile cilia syndrome', ectopic pregnancy and the influence of infections (Lyons, 2006). It is already known that substances from cigarette smoke (Jin et al., 1998; Genbacev-Krtolica, 2005; Talbot and Riveles, 2005) or other substances (Buckley and Stewart, 1983; Liao et al., 2011) have modulating effects on cilial movement.
In general, establishing and validation of alternative assay methods to evaluate local toxic effects and genotoxicity related to REACH [3] make progress. For the toxicological end point "reproduction", there are no *in vitro* methods approved yet (Lilienblum, 2008; Spielmann, 2009). Such test systems ideally consist of a set of single tests to display the complex course of reproduction. During the process of developing new strategies for safety studies in reproduction by the project "ReProTec" of the ECVAM [4] the oviduct was discarded. As mentioned before, essential steps during the reproductive cycle take place in the oviduct. Therefore, the development of reliable cell cultures of the oviductal epithelium is necessary to complete the set of toxicological end point protocols concerning reproduction on the one hand, and, on the other hand, to elucidate the important physiological actions within the oviduct.

Cervical epithelium Cell cultures of cervical epithelium are already applied for serial testing of cytotoxic effects of diverse substances like local contraceptives and antiviral or antibiotic agents (Elmore et al., 2000; Ayehunie et al., 2006). Providing a cost-effective and validated alternative to human tissue would help to perform standardized screenings. For microbial studies, primary cell cultures derived from the porcine reproductive tract served as model to investigate infection mechanisms of chlamydia in human. The pig seems to be the ideal model in comparison to

[3]REACH: Registration, Evaluation, Authorization and Restriction of Chemicals
[4]ECVAM: European Centre for the Validation of Alternative Methods

commonly used laboratory animals (Guseva et al., 2003). After delivery, the maternal cervical epithelium is involved in the remodelling of the reproductive tract (Timmons et al., 2010). Regulating mechanisms are still not completely known and also need to be investigated *in vitro*. Sperm-binding structures are expressed in the porcine cervical epithelium (Song et al., 2010). It can be concluded, that already in the cervix a sperm-epithelium contact occurs. Next to that, signals from the seminal fluid are recepted in the cervical epithelium and lead to immunomodulating effects (Robertson, 2007).

Each part of the reproductive tract differs in type and function of epithelia (from single-layered to stratified epithelium, secretory and ciliated cells). One main goal of differentiated culture systems is to study tissues under simplified conditions (Adissu et al., 2007). Cell cultures of these epithelia only achieve reliable results, when differentiated, meaning the maintenance of tissue specific morphology and function. Thus, reliable *in vitro* model systems of the oviductal and cervical epithelium that resemble the *in vivo* situation are needed, but their establishment is demanding and appears difficult to be realized.

1.2.3. Demands on the epithelial in vitro model systems of oviduct and cervix uteri

During the process to establish *in vivo*-like epithelial cell culture systems derived from the porcine oviduct and cervix uteri, the following objectives were set (see table 1.3):

Maintenance of the in vivo phenotype Since both cell cultures are derived from epithelial tissues, the purity of the culture has to be investigated by keratin detection, a common method to verify epithelial origin. The expression of beta catenin, as a part of the intercellular junctional complexes, and a columnar shaped morphology (oviductal epithelium) will prove a polarization of the cultured cells. As ectocervical

epithelium is stratified *in vivo*, the objective of this culture was to support a comparable multi-layered growth.
Tissue-specific functions have to be maintained. So, in the oviductal culture, the native composition of cell types, both secretory and ciliated cells with actively beating kinocilia, have to remain intact. The oviduct known as a hormone-responsive tissue should react to hormonal stimulation comparable to the native response. One main characteristic of cervical tissue is the production of mucus, which has to be detected in cell culture.

Optimized culture conditions For cell culture, there are plenty of different basic culture media commercially available. Each medium has its own specific composition of ingredients. There are also media preparations especially for specific tissues like airway epithelium or placental tissue available. It is imaginable that the nutritive demands of each cell type - that are mostly unknown - vary between cells derived from different tissues or species. Therefore, the evaluation of the cellular behaviour in the different growth media is necessary for every single cell type to provide an optimal and hopefully tissue-specific culture environment. Cell transport, isolation, growth media composition and culture conditions have great influence on cellular development during culture. Therefore, the culture protocols have to be optimized from the very beginning in order to create *in vivo* like culture systems for the respective cell types. To ensure practicability for the use of the models in a great number of replicates, the protocols need to be uncomplicated and reproducible.
The metabolism in the reproductive tract is controlled by different steroid hormones during the cycle. Due to the unknown effects of the interactions between such hormones, an *in vitro* model of reproductive tissue ideally includes the possibility to be kept up without any disadvantageous effect over a longer period of time. This enables a modelling of cyclic hormonal changes and the analysis of their effects on cellular basis.

Availability of the native tissue Unlike healthy human tissue, slaughterhouse-by products from pigs are continuously available and cost-effective at the same time. Advantageously, animals for slaughter normally are healthy and of the same age and race. This makes such tissues interesting as basic material for standardizable cell cultures.

Demands and aims	importance
maintenance of the *in vivo* function	1
maintenance of the *in vivo* morphology	1
ease of use	2
possibility of long-term cultivation	2
easy and continuous availability of the native tissue	3
low or no costs in tissue collection	3

Table 1.3.: Demands on and aims for our *in vitro* systems of the reproductive epithelia
(importance: 1 = essential, 2 = very important, 3 = important).

1.3. Scientific question

Easily accessible, differentiated *in vitro* systems of the cervical as well as the oviductal epithelium are not yet available to elucidate the molecular mechanisms important for successful reproduction in mammalians. Aim of this project is the establishment of useful cell culture systems derived from porcine tissues representing *in vivo* - like behaviour to investigate essential actions in reproductive biology and toxicology avoiding animal experiments.

Initially, cell isolation protocols has to be developed and optimized. Culture media and supplements are investigated concerning optimal cellular growth in culture. An

important claim is to reproducibly generate differentiated phenotypes of these cells. Thus, different culture conditions are evaluated using histology, immunohistochemistry and real time RT-PCR regarding support of a tissue specific phenotype.

2. Paper I:

2.1. Miessen, K., Sharbati, S., Einspanier, R. and Schoen, J. (2011). Modelling the porcine oviduct epithelium: a polarized in vitro system suitable for long-term cultivation. Theriogenology, 76(5):900-910

online available:
http://dx.doi.org/10.1016/j.theriogenology.2011.04.021

3. Paper II:

3.1. Miessen, K., Einspanier, R. and Schoen, J. (2012). Establishment and characterization of a differentiated epithelial cell culture model derived from the porcine cervix uteri. BMC Veterinar Research, 8:31

online available:
http://dx.doi.org/10.1186/1746-6148-8-31

Miessen et al. BMC Veterinary Research 2012, 8:31
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BMC Veterinary Research

METHODOLOGY ARTICLE Open Access

Establishment and characterization of a differentiated epithelial cell culture model derived from the porcine cervix uteri

Katrin Miessen, Ralf Einspanier and Jennifer Schoen*

Abstract

Background: Cervical uterine epithelial cells maintain a physiological and pathogen-free milieu in the female mammalian reproductive tract and are involved in sperm-epithelium interaction. Easily accessible, differentiated model systems of the cervical epithelium are not yet available to elucidate the underlying molecular mechanisms within these highly specialized cells. Therefore, the aim of the study was to establish a cell culture of the porcine cervical epithelium representing *in vivo*-like properties of the tissue.

Results: We tested different isolation methods and culture conditions and validated purity of the cultured cells by immunohistochemistry against keratins. We could reproducibly culture pure epithelial cells from cervical tissue explants. Based on a morphology score and the WST-1 Proliferation Assay, we optimized the growth medium composition. Primary porcine cervical cells performed best in conditioned Ham's F-12, containing 10% FCS, EGF and insulin. After cultivation in an air-liquid interface for three weeks, the cells showed a discontinuously multilayered phenotype. Finally, differentiation was validated via immunohistochemistry against beta catenin. Mucopolysaccharide production could be shown via alcian blue staining.

Conclusions: We provide the first suitable protocol to establish a differentiated porcine epithelial model of the cervix uteri, based on easily accessible cells using slaughterhouse material.

Background

The uterine cervical epithelium protects the upper reproductive tract from insults providing a physical barrier, secretions containing bactericidal and virucidal agents and a pathogen-dependent direct immunomodulation [1-3]. During estrous, it takes part in direct sperm-epithelium interaction [4] as well as in the signal reception from seminal fluid [5].

To elucidate cell type-specific actions of hormones and cytokines, signal transduction pathways, cell-cell interactions, and gene expression in these highly specialized cells, model systems resembling the original tissue need to be developed.

Cervical cell cultures of a variety of species are already applied in various fields of science. They serve as *in vitro* systems for basic research [6], in oncological and microbiological studies [7-9] as well as for assessment of product-and pharmaco-toxicity [10,11].

The cells used in these studies are mainly derived from human ectocervical tissue, which *in vivo* is covered by a polarized, multilayered epithelium. However, the cells (primary, immortalized or transformed) are cultured as monolayers and therefore lost these tissue specific characteristics. Maintenance of multilayered growth and polarity is pivotal for the *in vivo*-like functionality of the ectocervical epithelium *in vitro*, as apical polarity forms physical paracellular and functional barriers based on cell-cell contacts [12-15].

Cell culture models used in basic research as well as in toxicology ideally should meet two requirements at the same time: to a) be easily and continuously available and b) resemble the *in vivo* properties of the specific cell type. Therefore, we investigated, if porcine material from the slaughterhouse could provide to establish a suitable and differentiated cell culture model of the uterine cervical epithelium. Pigs for slaughter are usually healthy and

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roughly of the same age. During the last decades the pig also became one of the favoured models for humans, since anatomy, physiology and genetics are highly comparable [16]. The mean length of estrous cycle and hormone profiles as well as cervical mucus production also resemble the human characteristics [7,17].

In order to provide a practical tool to analyze the complex pathways within the cervical epithelium, the aim of this study was to establish an accessible model of the porcine ectocervical epithelium based on tissue derived from slaughterhouse. Cell isolation and culture conditions were optimized in order to support proliferation and differentiation *in vitro*. The culture was characterized by specific markers to describe the cell type, state of differentiation and functionality in comparison to the native tissue.

Results

Cell transport and isolation

Transport conditions from the slaughterhouse to the laboratory (transportation time approximately 2 h) turned out to be a crucial factor for cell viability. Tissue that was transported in growth medium at room temperature showed best survival of the epithelial cells.

Pure epithelial cervical cells could reproducibly be isolated only by outgrowth from tissue explants (Figure 1) as other previously described cell isolation methods did not lead to a pure and viable primary cell population in our hands.

Primary cells can be passaged and cryopreserved using standard protocols.

After passaging the cervical cells up to five times, the cultured cells were still viable and showed some epithelial characteristics (keratin staining). However, the epithelial morphology was completely lost in passage 5 (Figure 2). Already in passage one morphological alterations can be found in some parts of the monolayer (Figure 3).

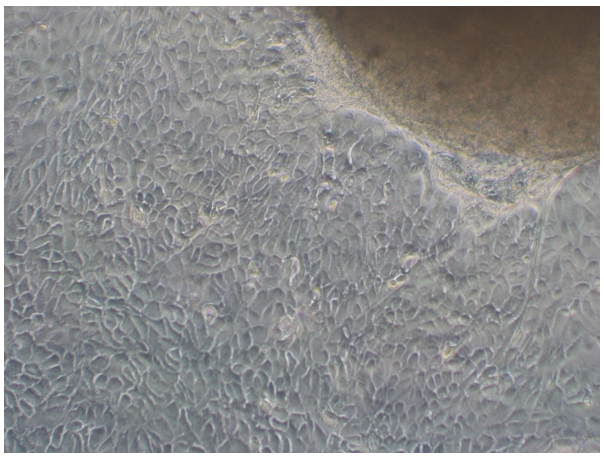

Figure 1 Tissue explant from ectocervical tissue, cultured in Ham's F-12 containing 10% FCS for five days. Epithelial cells grow out of the tissue and attach to the cell culture dish.

Initial media testing

Growth media were evaluated using a simple morphology score. The growth medium Ham's F-12 containing 10% FCS, antibiotics, antimycotics and antioxidants gained the highest score of 180 points concerning proliferation, the absence of vacuoles and epitheloid phenotype of the grown-out primary cells and cells in passage one (Table 1).

Test of media conditioning and media additives by WST-1 assay

Conditioning of the growth medium and the addition of media supplements were investigated using cervical cells of passage one. Cells cultured in conditioned Ham's F-12 showed an increase in mitochondrial turnover of WST-1 of 12% after 48 h and 44% after 72 h relative to the unconditioned growth medium. Supplementation of EGF and porcine insulin to the conditioned Ham's F-12 led to a two-fold higher mitochondrial activity after 48 h and 72 h compared to the conditioned growth medium without any hormone substitution (Figure 4). Other additives or other additive combinations led to a less pronounced increase (EGF, EGF + hydrocortisone, EGF + hydrocortisone + insulin) or stagnation (insulin, hydrocortisone, insulin + hydrocortisone) of mitochondrial activity.

Differentiated, multilayered cell culture of cervical cells

Primary cervical cells and cells of passage one were cultured on Millicell inserts either with access to growth medium from both basolateral and apical or in air-liquid interface with access to growth medium only from basolateral. We analyzed the respective setups histologically after three weeks considering *in vivo*-like morphology.

A differentiated phenotype was established when culturing primary cells in an air-liquid interface (without medium in the upper compartment) for three weeks. The cells showed a reproducible discontinuously stratified phenotype with up to six layers. When cultured conventionally with access to growth medium from both basolateral and apical the epithelial cells were mainly columnar and also showed partially multilayered growth. However, height of the multilayer was increased by growth in air liquid interface (conventional growth: up to 4 layers, air-liquid interface: up to six layers). After passaging, the epithelial cells grew as single-layer consisting of non-polarized cells. Passaged cells showed multilayered growth only in very few areas of the membranes (Figure 5).

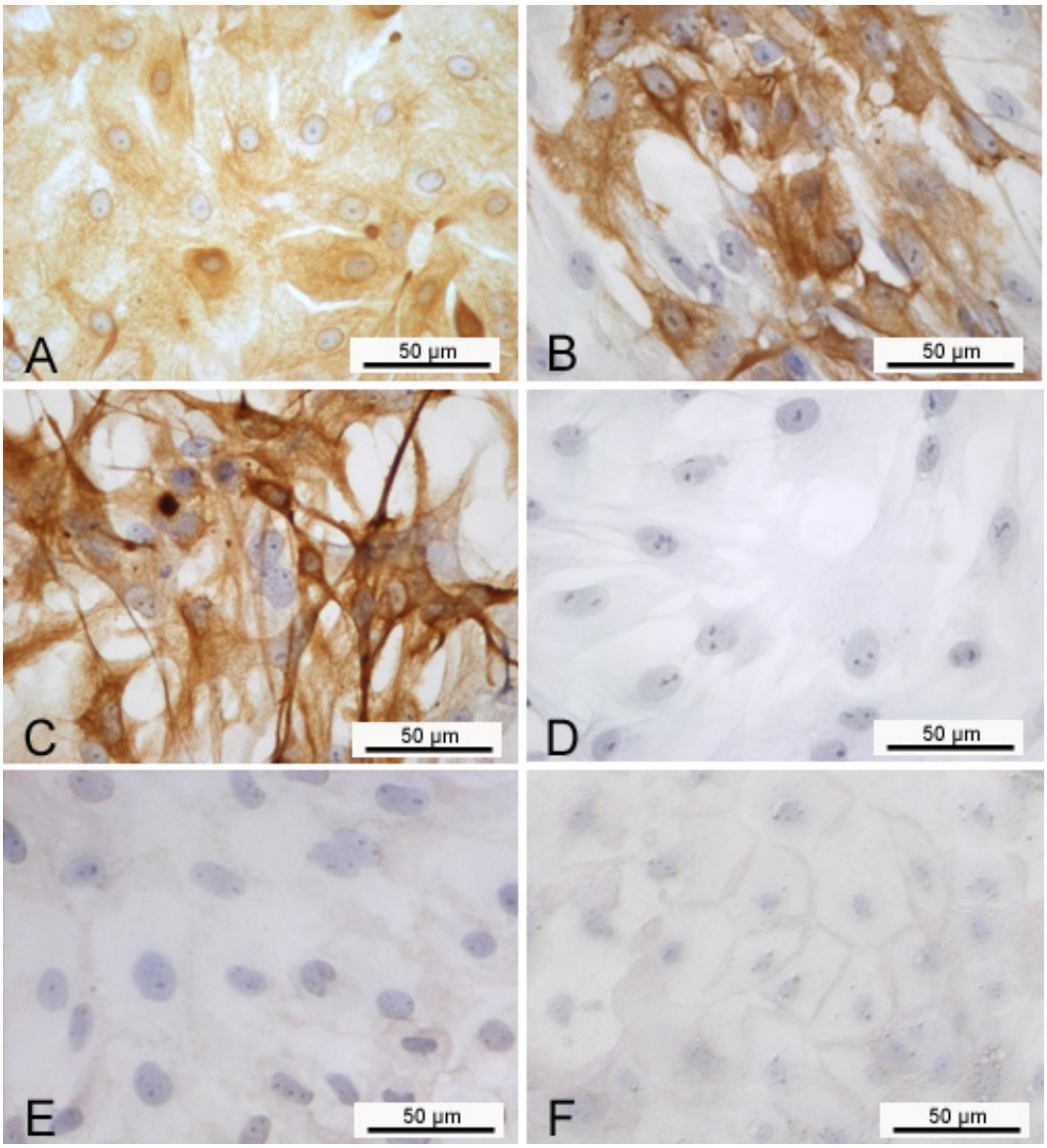

Figure 2 Immunocytochemical detection of keratins and beta catenin in porcine cervical epithelial cells. Keratins were detected in A) primary cells, B) passage one, C) passage 5; D) negative control. Beta catenin is shown in E) primary cells and F) passage one. Counterstain: hemalum.

Characterization of the in vivo-like cell culture

Purity of the epithelial cell culture and differentiation was shown by immunohistochemistry using antibodies against keratin, as epithelial marker, and beta catenin, as part of adherens junctions. Cervical epithelial cells cultured on membranes in air-liquid interface for three weeks showed keratin-expression in the cytoplasm comparable to the ex vivo-epithelium (Figure 6A/B). Likewise, beta catenin could be visualized near the cellular membrane on cell culture and tissue sections (Figure 6C/D). In the tissue samples beta catenin expression is detectable in all layers of the porcine cervical epithelium, but the intensity of the immunostaining is lower in the superficial cell layer. This difference could not be detected in the *in vitro*-model.

To show cervix specific function mucopolysaccharides were visualised. The multilayered cell cultures showed a blue signal in the apical layers after alcian blue staining comparable to the *in vivo* situation (Figure 6G/H).

Discussion

Epithelial cells of different tissues or species diverge substantially in their culture requirements. Thus, *in vivo*-like culture of epithelial cells necessitates optimization of the entire culturing process including transport, isolation, medium composition and culture conditions.

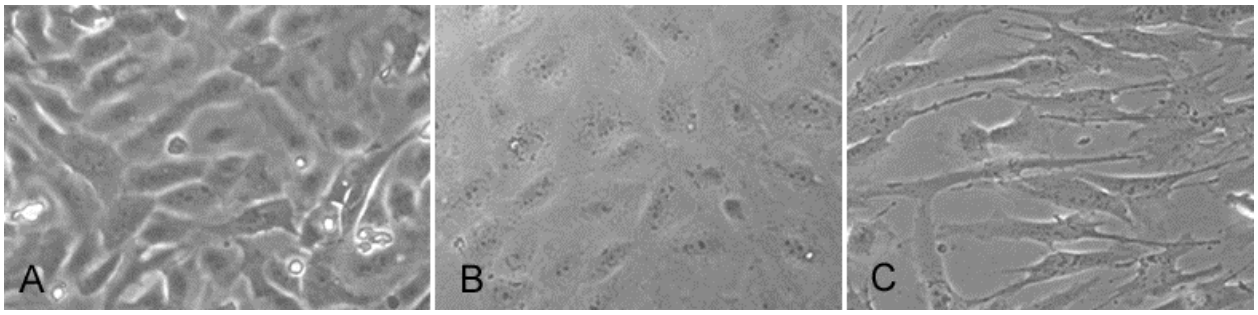

Figure 3 Epithelial cells of the cervix uteri, conventionally cultured for 10 days in cell culture plates using Ham's F-12, containing FCS, EGF and insulin as growth medium. A) primary cells, B and C) passage one. The passaged cells show an inhomogeneous morphology.

In the present study we established a new protocol for a differentiated cell culture system of the porcine cervical epithelium, based on easily accessible slaughterhouse material. The morphology and tested functional markers of our culture system are comparable to the native tissue as shown by histology, immunohistochemistry and alcian blue staining. The use of fibroblast-conditioned medium supported proliferation of cervical epithelial monolayers suggesting that stromal growth factors or cytokines released into the medium are required for cell growth in these epithelia. The supplementation of the conditioned medium with EGF further optimized proliferation and mitochondrial activity of the cervical epithelial cells. Insulin intensified the proliferative effect of EGF, as cellular metabolic effects of EGF (next to synthesis of DNA, RNA and proteins) also include stimulation of glucose metabolism [18]. Providing an air-liquid interface to the primary cells promoted the formation of multilayers. In contrast to reports on cervical epithelial cells from other species [13,19] passaging the primary cells, however, caused disadvantageous effects. Already in passage one cells showed a decreased potential to form multilayers and extended passaging led to the loss of epitheloid cell character.

Cervical epithelial cells play a relevant role in nowadays research as cell cultures derived from this tissue are frequently used as *in vitro* models. The level of differentiation, however, varies widely between different culturing systems.

Cervical epithelial cells cultured as monolayers on solid support dedifferentiate [20] and are more susceptible to certain toxic agents than ex vivo tissue cultures or animal models [10,21]. Therefore, a fully differentiated three-dimensional *in vitro* system of the human cervicovaginal epithelium was recently established for toxicological studies [13,22]. This homologous system is surely ideal for human research, but the general availability and the resulting small number of possible biological replicates are limiting factors for its usability. Advanced cell culture technologies like the rotating wall vessel bioreactor showed to provide differentiated vaginal epithelial cells from immortalized cells [23]. However, special equipment is needed to conduct this technique.

Table 1 Morphology scores of cervical epithelial cells grown in different growth media

Medium		Score
Ham's F-12	+10% FCS	180
DMEM/Ham's F-12 (1:1)	+10% FCS	170
Medium199	+10% FCS	165
DMEM	+10% FCS	165
McCoy's 5A	+10% FCS	140

The highest score achievable was 240 points per growth medium (8 points in three categories, 30 animals in total; primary cells after five days of culture: n = 15 animals; cells in passage one after two days of culture: n = 15 animals)

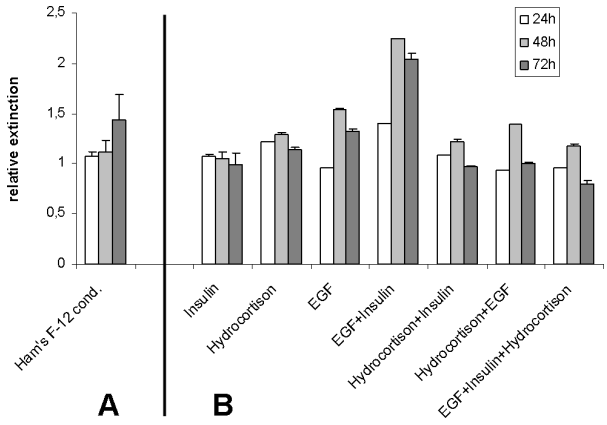

Figure 4 Cervical epithelial cells of three animals were cultured for 24 h, 48 h and 72 h in different growth medium compositions. Viability and proliferation were measured in triplicates using the Roche WST-1 Proliferation Assay. Extinctions of the media were measured at 450 nm after 30 min incubation with the WST-1 reagent. A) Ham's F-12 relative to the unconditioned medium, and B) conditioned Ham's F-12 with different additives relative to the basic conditioned Ham's F-12.

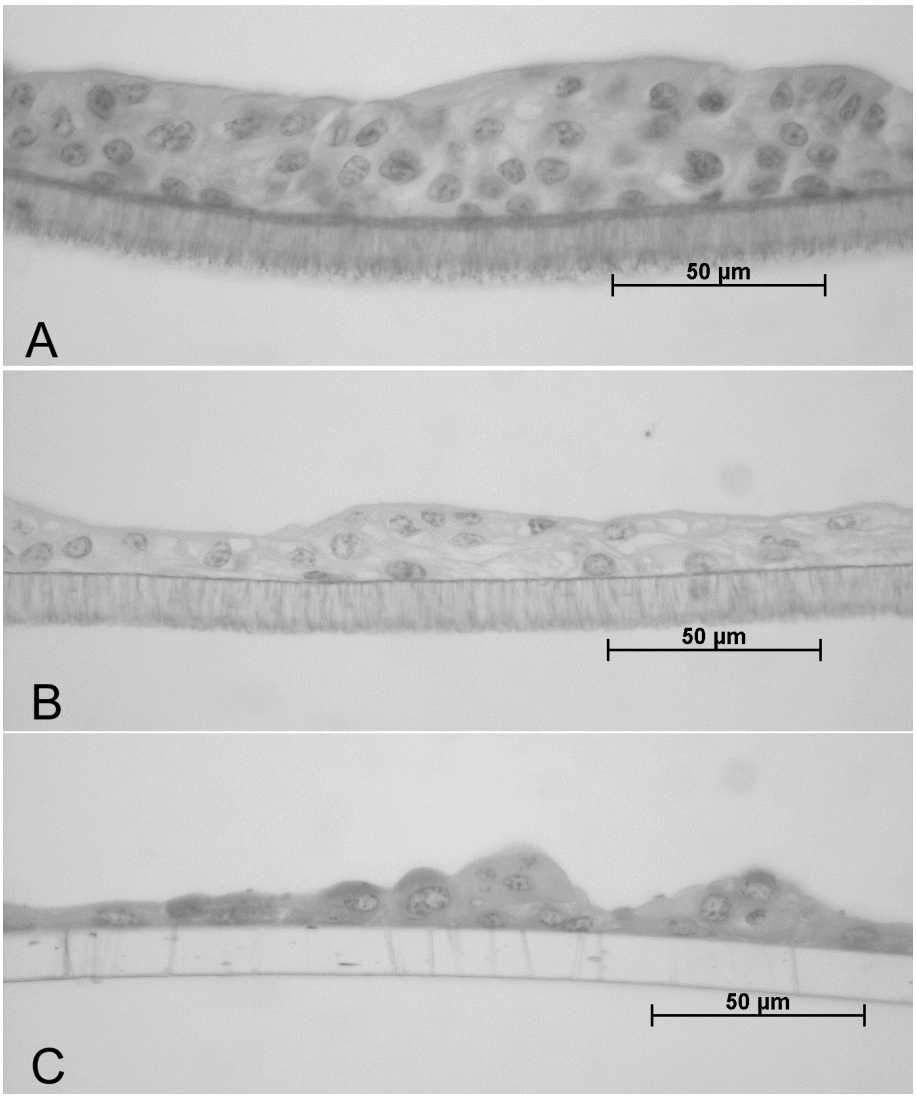

Figure 5 Ectocervical epithelial cells cultured on hanging membranes with conditioned Ham's F-12 containing 10% FCS, EGF and insulin for three weeks. A) Primary cells cultured in air-liquid interface (with access to growth medium only from basolateral), B) primary cells with access to growth medium from both basolateral and apical side, and C) epithelial cells of passage one cultured in air-liquid interface.

The porcine cells used for our model of the cervical epithelium are unlimitedly accessible and maintenance as well as handling of the cells can be carried out in a standard cell culture lab. The differentiated cell culture resembles physiological properties of the native tissue and can, therefore, be called *in vivo*-like model.

However, with regard to the different scientific questions addressable with this model system (pathogen-dependent immunomodulation, sperm-epithelium interaction, signal reception etc.) we did not carry out a functional characterization of the cell culture. It is now essential to describe and validate its specific functional properties by focusing directly on the particular processes under investigation.

Conclusions

We developed an *in vitro* system modelling the mammalian cervical epithelium that is cost-effective and easily available as it is based on slaughterhouse by-products. It is a practical tool in veterinary science and might also promote human reproductive research.

Methods

Overview

We tested different methods to isolate epithelial cells from porcine cervical tissue. Standard growth media and a range of additive-compositions were investigated regarding their influence on proliferation and morphology. The cultured cells were characterized by histology,

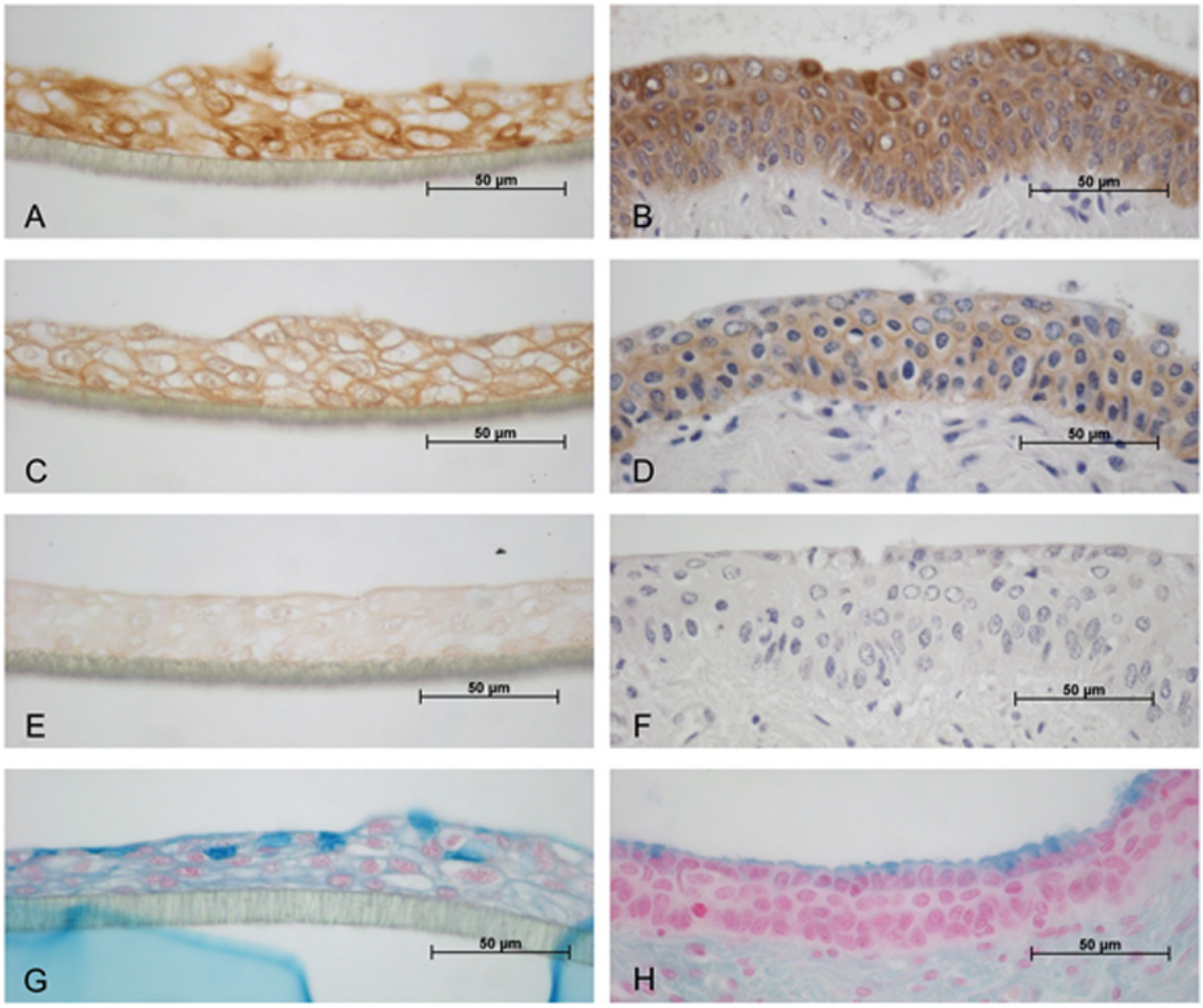

Figure 6 Detection of keratins (A, B; E and F: negative control), beta catenin (C, D) and mucopolysaccharides (G, H) in porcine cervical epithelial cells. Left column (A, C, E, G): primary cervical epithelial cells cultured in air-liquid interface for three weeks; Right column (B, D, F, H): ectocervical tissue sections.

immunocyto-and-histochemistry. Studying differentiation, we cultured tissue explants and cells from passage one on hanging membranes either with access to medium from both basolateral and apical side or in an air-liquid interface with access to medium only from the basolateral side.

Material

Media, antibiotics and serum for cell culture were supplied by Biochrom AG, Berlin, Germany. All other chemicals were obtained from Sigma-Aldrich, St. Louis, USA, unless otherwise indicated.

Tissue preparation and transport conditions

Porcine uterine cervices were collected in a local slaughterhouse from healthy, 6-month-old, pre-pubertal animals, approximately 15 min after death. Ovaries of all animals only showed small follicles of the same size as a sign for cyclic inactivity. The organs were washed immediately with Dulbecco's PBS.

Conditions of transport were investigated concerning the influence on cell viability after isolation. Cervical tissues were transported to the laboratory either dry or in growth medium containing 10% FCS, fetal calf serum (on ice or at room temperature).

After optimization of transport conditions the cervical tissues were transported in basic growth medium (Medium 199, or after intial media testing: Ham's F-12) containing 10% FCS at room temperature within 2 hours.

For histology, tissues of four animals were transversally cut in 5 mm thick pieces and fixed in chilled Bouin's solution on-site.

Isolation of cervical epithelial cells

To isolate pure epithelial cells from porcine cervical tissue, we applied different previously published enzymatic digestion methods [7,13,24] as well as a modified protocol for cellular outgrowth from primary tissue explants [25]. Briefly, cervices were opened longitudinally and rinsed with Dulbecco's PBS. Tissue pieces of the ectocervical mucosa (approximate length 1 cm, width 2 mm and height 1 mm) were cut with scissors. 10 pieces were placed (mucosal side up) in a 25 cm^2 culture flask with 2 mL of growth medium. After initial media testing, the growth medium consisted of conditioned Ham's F-12, 10% FCS, 10 ng/mL murine EGF (epidermal growth factor), 1 μg/mL porcine insulin, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL gentamycin, 1 μg/mL amphotericin B, 10 μg/mL reduced glutathione and 10 μg/mL ascorbic acid as antioxidants. After 48 and 96 h, culture medium was changed. After five days the tissue explants were removed from the culture vessel.

If necessary for further experiments, the cells were passaged (3 mL of Accutase, 20 min incubation at 37°C) after five days of culture.

Cells were cryopreserved using a standard protocol. 5 × 10^4 cells were resuspended in 500 μL CryoMaxx (PAA Laboratories GmbH, Pasching, Austria) and cooled down to -80°C (1°C/min) before storage in liquid nitrogen.

Immunocytochemistry

To proof cell type and purity of the collected cells we applied immunocytochemistry against pan-keratins. Differentiation was shown using an antibody against beta catenin, as part of adhearens junctions. For primary antibodies and protocol details see Table 2.

Either tissue explants or 1 × 10^5 cervical cells in passage one were grown on Superfrost Plus® slides (Gerhard Menzel, Glasbearbeitungswerk GmbH & Co. KG, Braunschweig, Germany) until confluence. They were rinsed with Dulbecco's PBS, fixed in ice-cold acetone for 10 min and washed three times in Dulbecco's PBS. Blocking of unspecific binding sites was not necessary. The primary antibody was incubated for 1 h at room temperature (pan-keratins) or at 4°C overnight (beta catenin). Mouse immunoglobulin G fraction or normal rabbit serum (DakoCytomation, Glostrup, Denmark) served as negative control. After three washing steps in PBS-T 0.01%, the secondary antibody (Goat Anti-Rabbit or Anti-Mouse Poly-HRP, Thermo Scientific, Rockford, USA) was incubated for 30 min at room temperature. After three more washing steps, diaminobenzidin (DAB-/Metal Concentrate 10×, Thermo Scientific) was used as chromogen.

Initial media testing

To evaluate different media for further use as basic growth medium, we applied a morphology score.

Cervical epithelial cells of 30 animals were cultured (in conventional cell culture plates) as described above in five different growth media containing 10% FCS as well as antibiotic, antimycotic and antioxidant reagents (Table 3). Cell morphology of primary (five days after explantation) and cells in passage one (two days after passaging) was assessed by light microscopy. The morphology was scored concerning proliferation, presence of vacuoles and epitheloid phenotype (Table 4). The highest achievable score of each medium was 240 points (8 points in three categories, 30 animals in total; primary cells after five days of culture: n = 15 animals; cells in passage one after two days of culture: n = 15 animals).

Preparation of conditioned medium

The growth medium with the best results in the initial media testing was further modified. Conditioned growth medium (mimicking stromal-epithelial interaction) was prepared referring to standard protocols [19]. In short, 5 × 10^5 3T3 Swiss albino embryo fibroblast cells (ATCC, Manassas, USA) were grown in a 75 cm^2 cell culture flask in 20 mL Ham's F-12, 10% FCS. After 48 h (subconfluence), fresh growth medium was incubated for 24 h on the fibroblasts. The sterile filtered supernatant was stored at -20°C. To prepare conditioned growth medium, one part of the supernatant was diluted in two parts of unconditioned medium.

Test of media conditioning and media additives by WST-1 assay

Influence on cell viability and proliferation of different growth media (for media composition details see Table 3)

Table 2 Antibodies and protocol details for immunohistochemistry (IHC) and immunocytochemistry (ICC) procedures

	name	manufacturer	dilution	blocking solution
IHC	Beta Catenin, ab6302	Abcam plc, Cambridge, Uk	1:160.000 in PBS	no block
	Anti human cytokeratin, clones AE1/AE3	DakoCytomation, Glostrup, Denmark	2.4 μg/mL in PBS	Casein, Candor Bioscience, Münster, Germany
ICC	Beta Catenin, ab6302	Abcam plc, Cambridge, Uk	1:2.000 in PBS	no block
	Anti human cytokeratin, clones AE1/AE3	DakoCytomation, Glostrup, Denmark	1 μg/mL in PBS	no block

Table 3 Media composition in the different steps of cell culture optimization

Initial media testing	*Basic media*	*Supplementation*
	DMEM +10% FCS	-
	DMEM/Ham's F-12 (1:1) +10% FCS	-
	Ham's F-12 +10% FCS	-
	McCoys-5A +10% FCS	-
	Medium 199 +10% FCS	-
Media conditioning and additives	*Basic media*	*Supplementation*
	Ham's F-12 +10% FCS	-
	Ham's F-12 +10% FCS, cond.	-
	Ham's F-12 +10% FCS, cond.	+ EGF
	Ham's F-12 +10% FCS, cond.	+ insulin
	Ham's F-12 +10% FCS, cond.	+ hydrocortisone
	Ham's F-12 +10% FCS, cond.	+ EGF, insulin
	Ham's F-12 +10% FCS, cond.	+ insulin, hydrocortisone
	Ham's F-12 +10% FCS, cond.	+ EGF, hydrocortisone
	Ham's F-12 +10% FCS, cond.	+ EGF, insulin, hydrocortisone
Differentiated cell culture	*Lower compartement*	*Upper compartment*
	Ham's F-12 +10%FCS, cond., EGF, insulin	a) Ham's F-12 +10%FCS, cond., EGF, insulin
		b) no medium (air-liquid interface)

DMEM Dulbecco's MEM medium, *FCS* fetal calf serum, *cond.* conditioned. Concentration of the supplements: 10 ng/mL murine EGF, 1 μg/mL porcine insulin, 0.5 μg/mL hydrocortisone. All growth media were supplemented with 100 U/mL penicillin/100 μg/mL streptomycin, 50 μg/mL gentamycin, 1 μg/mL amphotericin B, 10 μg/mL reduced glutathione and 10 μg/mL ascorbic acid.

was monitored using WST-1 Proliferation Assay (Roche Deutschland Holding GmbH, Grenzach-Wyhlen, Germany) according to the manufacturer's guide. In short, 1×10^4 cervical cells of passage one were seeded in 96-well culture dishes. 24 h, 48 h and 72 h after seeding, cells were incubated for 30 min with the reagent and WST-1 turnover was measured photometrically at wavelength 450 nm and a reference wavelength of 630 nm. All measurements were conducted for three animals as triplicates.

Differentiated cell culture

Cells were grown on permeable membranes (6-well and 24-well Millicell hanging inserts, PET membrane, Millipore, Temocura, Canada) hanging in tissue culture plates and creating two compartments, a basolateral and an apical one. We applied different cultivation setups: access to complete growth medium from either sides or air-liquid interface for nutrition exclusively from the basolateral side.

1×10^5 cervical epithelial cells (passage one) were seeded in 24-well inserts containing 200 μL growth medium with 1 mL medium in the well. To culture non-passaged cells on membranes, four pieces of cervical mucosa were placed in a 6-well insert containing 500 μL growth medium with 2 mL medium in the well. The culture plates were incubated at 37°C, 5% CO2 in a humid chamber. After 48 and 96 h growth medium was changed. After five days of culture, the mucosal pieces were carefully stripped off the membrane. To achieve an air-liquid interface, growth medium was completely removed from the insert. After three weeks of culture, the membranes were carefully drawn off the plastic inserts and further processed for histology.

Preparing ex vivo tissue and cell culture membranes for histology

For immunohistochemistry, the cervical tissue or cells on membranes (embedded in 1.2% agarose to ensure a

Table 4 Morphology score criteria applied in the initial media testing

score	Proliferation (P0)	Proliferation (P1)	Vacuole formation	Phenotype
3	Continuous margin of cells around explants	> 90% confluence	no vacuoles	-
2	Discontinuous margin of cells around explants	70%-90% confluence	< 10% cells vacuolated	epitheloid phenotype
1	single cells and small clusters of cells around explants	< 70% confluence	> 10% cells vacuolated	-
0	not assessable, all or many cells dead	not assessable, all or many cells dead	not assessable, all or many cells dead	fibroblastoid phenotype

P0 primary cells, *P1* cells in passage one

cutting ancle of 90°) were fixed in Bouin's solution at 4° C for at least 24 h. After washing in 4% buffered formalin, the samples were dehydrated and embedded in paraffin. Sections of approximately 5 μm were cut, mounted on Superfrost Plus® slides and dried at 37°C overnight.

Hemalum/eosin staining to identify cellular morphology and alcian blue staining at pH 2.5 to visualize mucus production was performed for both tissue and membrane sections.

Immunohistochemistry

To proof the differentiation state of the epithelial cells cultured on membranes, we applied immunohistochemistry using antibodies against keratins and beta catenin (for primary antibodies and protocol details see Table 2).

After deparaffination and rehydratation, sections were washed in Dulbecco's PBS. Antigens were demasked by boiling slides for 2 min and cooling down for 20 min in citrate buffer, pH6. Endogenous peroxidase was blocked in 3% H_2O_2 in methanol (twice, 15 min). Sections were incubated with blocking solution at 37°C in a humid chamber for 1 h. Sections were incubated with primary antibodies at 4°C in humid chamber over night. Mouse immunoglobulin G fraction or normal rabbit serum respectively (DakoCytomation) served as negative control. After washing in PBS-T 0.01% (3 times, 15 min), slides were incubated with goat anti-mouse or anti-rabbit secondary antibody (Thermo scientific) conjugated with horseradish-peroxidase for 30 min. Peroxidase was visualized using diaminobenzidin.

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Authors' contributions

KM participated in study design, carried out tissue collection, cell culture experiments, histological sample procedures, immunohistochemistry and other histological studies, and drafted the manuscript; RE participated in study design and supervision, and revised the manuscript; JS conceived of the study, coordinated and supervised the study performance, and drafted and revised the manuscript. All authors read and approved the final manuscript.

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4. Discussion

Since the molecular mechanisms leading to successful reproduction in mammalians are not yet completely understood, model systems helping to elucidate these processes are needed (Schön et al., 2011). Aim of this project was to establish easily accessible cell culture systems of the porcine oviductal and cervical epithelium maintaining physiological morphology and function of the native tissues.

Demands and aims	**oviduct**	**cervix uteri**
maintenance of the *in vivo* function	+	+/-
maintenance of the *in vivo* morphology	+	+/-
possibility of long-term cultivation	+	+
ease of use	+	-
easy and continuous availability of the native tissue	+	+
low or no costs in tissue collection	+	+

Table 4.1.: Demands on and aims for our *in vitro* systems of the reproductive epithelia of porcine oviduct and cervix uteri. Which criteria could be fulfilled successfully?
(+: successful, +/-: partly successful, -: finally unsuccessful.)

Oviductal cell culture For the porcine oviductal cell culture, the possibility of a long-term culture is sophisticated. Here, its comparability to the *ex vivo* tissue could

be shown in morphology and cellular composition as well as in selected function like polarization characteristics, cilial beating and a physiological response to steroid hormone stimulation. As shown, a culture is easy to handle and can conveniently be applied in various approaches. The establishment including the fulfilling of the previously set aims was completely successful (table 4.1).

Currently, Areekijseree and Vejaratpimol (2006) established a short-term porcine oviductal *in vitro* system based on cultured epithelial cells. In comparison to the presented culture system in our study, their culture showed *in vivo*-like morphology with two cell types, secretory and ciliated cells, but *in vivo*-like function was not investigated. Further on, the cells were cultured in conventional culture dishes not allowing access to both apical and basolateral side of the epithelial cells. Such cells were cultured maximally for 48 h in that study, so, information about the possibility of long-term cultivation is not available. With experiences from the here presented study, long-term cultivation under such culture conditions will not support a differentiated phenotype of the oviductal cells. Since isolation procedure and source of porcine tissue are the same, ease of use, tissue availability and cost-effectiveness are comparable to that of our presented culture system.

Differentiated cell cultures have also been established derived from human oviducts. Levanon et al. (2010) reported on a well-characterized *in vitro* system of the human oviduct that sustained many important *in vivo*-like properties in morphology and function. Since the cells were also cultured on permeable membranes in air-liquid interface, a long-term cultivation for up to 28 days as well as access to both sides of the polarized epithelium was possible. Here, a side-specific secretion could be detected for oviduct specific proteins. Although a responsiveness to sex hormones was not investigated, it is highly likely that the *in vitro* system maintained particular physiological properties. Compared to the porcine oviductal epithelial culture developed and used in our study, the human model can also be called *in vivo*-like, but, as disadvantage, the availability of fresh and healthy human tissue limits the wide use of such systems in biomedical science. In general, the detailed investiga-

tion of the culture by Levanon et al. (2010) should be seen as a quality standard for establishment of cell culture models (table 4.2).
The here presented *in vitro* system provides the possibility to investigate effects on oviductal cilia without the necessity for labortory animal use since a cilial beating was detected over an extended period of time up to six weeks.

Demands and aims	**Areekijseree and Vejaratpimol (2006)**	**Levanon et al. (2010)**
	porcine tissue	human tissue
maintenance of the *in vivo* function	not tested	+
maintenance of the *in vivo* morphology	+	+
possibility of long-term cultivation	not tested	+
ease of use	+	+
easy and continuous availability of the native tissue	+	-
low or no costs in tissue collection	+	-

Table 4.2.: Evaluation of previously established *in vitro* systems of the oviduct. (+: successful, -: unsuccessful)

Cervical cell culture The development of a protocol for a porcine cervical cell culture was only partly successful (table 4.1). Although outgrowth of tissue explants (Freshney, 2005b) was identified as the only feasible method to gain pure epithelial cells in our hands (Mießen et al., 2009), the protocol needs experience in handling of primary cervical cells and had to be further optimized. The success of outgrowth depends on the volume of growth medium in the culture dish and the size of tissue explants. An adequate volume of medium provides an optimal nutrition of the

tissue and minimizes the risk for the tissue of floating and losing contact to the culture surface. An optimal size of the primary explants facilitates an adhesion on the culture surface and also reduces the rate of floatation. Epithelial cells grow out radially from the tissue explants. Therefore, cells on the culture surface are irregular regarding the time point of outgrowth and the distribution on the culture surface. This could be an explanation for the discontinuity of the multilayer in the culture on membranes in air-liquid interface. In order to generate a regular distribution and growth on the culture surface, cervical epithelial cells were subcultured. To gain an adequate cell number enabling a vital culture of the first passage, the outgrowth had to last for an extended period of time. A culture of the cells for longer than five days led to strongly attached cells on the culture dish surface. Trypsin (an enzyme conventionally used for subculture) in various concentrations or Accutase led to a cellular detachment after extended incubation periods. Such long incubation periods caused fatal damage to most of the cells. Only a few cells remained viable. Passaging the cells with Accutase was more effective and led to a higher number of viable cells compared to trypsin treatment. A subculture of the cells after five days was a compromise of an adequate cell number and an acceptable incubation time without remarkable loss of vitality. Unfortunately, passaged cells grew partly as monolayer with areas of an irregular multilayer (Mießen et al., 2010, 2011). Damages caused by the subculture enzymes can not be excluded as they are known to destroy cellular parts on the outer membrane (Freshney, 2005a). Therefore, the culture of primary cells, meaning an outgrowth directly onto the membrane, is to be preferred as it provides an *in vivo*-like multilayered morphology of the cervical cell culture. Our primary culture in air-liquid interface expressed markers of functions comparable to the *in vivo* situation like beta catenin as a marker for differentiation, and production of mucus . In conclusion, the porcine cervical cell culture maintains partly *in vivo* morphology and function, and can be held in long-term culture, but the yet established protocol is complicated, needs experienced experimentators, and is, therefore, not easy to use (table 4.1).

Guseva et al. (2003) established a primary polarized epithelial cell culture derived from the cervix uteri of mature gilts for the purpose of Chlamydia-infection studies. A polarized monolayer could be shown by transmission electron microscopy culturing the cells on permeable support. Since there was no information given about the origin of the cervical cells from either endo- or ectocervix, a comparability to the native tissue could not be clearly demonstrated. In addition, for the infection study, cells were grown on conventional culture dishes. The culture support has a great influence on cellular phenotype. Therefore, a polarized cell morphology is not obligatorily expressed under such culture conditions. Furthermore, specific cervical functions or a possible long-term cultivation were not investigated. Consequently, although complicated in handling, the here presented model of the porcine cervical epithelium has many advantages compared to the cell culture of Guseva et al. (2003) referring to the *in vivo*-like morphology and function.

Cultures of cervical epithelium have also been established using human tissue. MacDonald et al. (2007) cultured human cervical cells on permeable ECM-coated membranes for up to 20 days. ECM is known to trigger polarization of epithelial cells (Mazzoleni et al., 2009). Polarization was detected measuring transepithelial resistance to evaluate the presence of tight junctional cell-cell contacts, but specific morphology or function remained unclear. In contrast to that, the morphological and functional investigation of the differentiated model of human vaginal-ectocervical epithelium for toxicological studies by Ayehunie et al. (2006) is very detailed. The cells were cultured in passage one, a status that led to unsatisfying results in our study. Though, Ayehunie et al. (2006) established a multilayer comparable to the *ex vivo* tissue of vagina and ectocervix after culture in an air-liquid interface for 11 days. In addition to the highly differentiated morphology, an *in vivo*-like distribution of glycogen could be shown. Compared to the present study, the availability of human tissue is a limiting factor for the applicability of both culture systems. And also the fact that the model system by Ayehunie et al. (2006) is derived of a mixture of vaginal and ectocervical cells, which are morphologically not to distinguish,

but presumably possess different properties, is highly questionable. Comparing the previously established cervical *in vitro* models to the here presented porcine cell culture, the model system of Ayehunie et al. (2006) is the only higher developed and characterized model of the cervix uteri (table 4.3).

Demands and aims	**Guseva et al. (2003)**	**MacDonald et al. (2007)**	**Ayehunie et al. (2006)**
	porcine tissue	human tissue	human tissue
maintenance of the *in vivo* function	not tested	not tested	+/-
maintenance of the *in vivo* morphology	unclear	not tested	+
possibility of long-term cultivation	not tested	+	+
ease of use	+	+	+
easy and continuous availability of the native tissue	+	-	-
low or no costs in tissue collection	+	-	-

Table 4.3.: Evaluation of previously established *in vitro* systems of the cervix uteri. (+: successful, +/-: partly successfull , -: unsuccessfull)

In veterinary medicine, such model systems will also be useful for toxicological studies, e.g. optimization of sperm dilutors. The evaluation of ingredients could be tested for irritant effects on the cervical tissue getting in contact with such substances at artificial inseminations. Cervical epithelial cells are regulated by steroid hormones that alter epithelial permeability, microbicidal activity, cytokine/chemokine secre-

tion as well as immune cell recruitment and activation to ensure maternal and fetal protection (Ochiel et al., 2008). These local immune modulating effects and also mucus production as a part of the protective properties of the cervix can be investigated more detailed *in vitro* (Ochiel et al., 2008).

4.1. Discussion of methods

4.1.1. Model systems

Models simplify complex mechanisms and help to understand detailed processes. In comparison to *in vivo* experiments, *in vitro* approaches can be performed in a short period of time, show little biological variability and are cost-effective. Impact of single events on the process of interest can be investigated isolated from environmental influences. Model systems help to elucidate and to predict effects and effectors. They are ideal to investigate isolated mechanisms. To obtain reliable results, the models have to be well defined and characterized. Thus, before the performance of an experiment based on a model system, the individual model has to be checked for its differentiated properties to solve the particular scientific question (Adissu et al., 2007; Mazzoleni et al., 2009; Einspanier et al., 2011).

4.1.2. Cell culture

In vitro systems based on cell culture are ethically less problematic since animal use in research is replaced or reduced (RRR approach[1]). Culture systems can be established using almost all tissues and species, depending on availability and practicability, and, therefore, can serve as model systems for the respective subject of interest. In many studies, cell culture is in use for evaluation of cytotoxicity for various cell and tissue types (Elmore et al., 2000). Environmental conditions like pH,

[1]RRR: Replacement, Refinement, Reduction. Search for alternate methods to avoid animal use for research

temperature as well as the concentration of nutritions and hormones in the growth media can be easily controlled and varied. Cells can mostly be preserved in liquid nitrogen, and hence, experiments can be performed or be repeated using the same material. They enable standardized and reproducible studies in a high number of biological and technical replicates. Studies using cell culture are cost-effective at this point, since reagents can be saved due to the direct application onto the cells and the reduced volumes compared to animal experiments.

On the other hand, to enable growth of cells necessary distinct requirements have to be investigated, optimized and ensured. Cell culture equipment to ensure defined conditions is expensive. Furthermore, the characteristic morphology of the individual cell type is mostly difficult to recreate and can possibly be atypical in the culture. This is due to a possibly unwanted cell type selection during the isolation process or by means of dedifferentiation.

Physiological epithelial function (secretion, transport and absorption) mainly depends on cellular polarity maintained by an ability to establish tight junctional complexes and functionally distinct membrane domains (distinction into apical and basolateral) (Sabatini et al., 1983; Simons and Fuller, 1985).

In conventional cell cultures, epithelial cells are cultured on solid artificial supports like plastic culture dishes or coverslips. In these systems, one surface of the epithelial cells is forced to be in contact with the support, while the other is in contact with growth medium. Whereas *in vivo*, epithelial cells get their nutrition through diffusion from the bloodstream, meaning from their basolateral side (Zegers et al., 2003). In an adherent monolayer, supply of nutrition is located at the apical surface of the cells. Growth of epithelial cells on porous filters supports polarization of the cells by simulating the natural nutrition from basolateral (Rodriguez-Boulan and Powell, 1992; Simons and Fuller, 1985). In many studies, clear morphological alterations as well as functional differences between polarized and non-polarized cell cultures of reproductive epithelia could be shown. Simplified conditions (e.g., growing cells on plastic culture dishes as a non-polarized monolayer) do not sustain tissue-specific

architecture, biochemical metabolism and cell-cell communication as the complex *in vivo* environment is not restored (Mazzoleni et al., 2009).
Since reproductive epithelial cells are highly differentiated in morphology and function, this status is necessary to maintain in *in vitro* systems.
In general, such systems do not completely reflect the real complex interactions between different cell types and the surrounding tissues *in vivo*. This is due to the fact that in cell culture, individual cell types are investigated isolated from their native environment. During culture procedure a selection of particular cell types can occur. Despite this or precisely for this reason, *in vitro* systems provide the possibility to study direct effects on the respective cell type, to modify culture conditions and to examine a possible cellular reaction excluding any influence of other organs and of the circulatory and immune system (Sultan and Haagsmann, 2001).
Conclusively, only cell cultures expressing a phenotype comparable to the *in vivo* tissue will enable the collection of valid data (Bhadriraju and Chen, 2002). Therefore, establishment of a reliable cell culture system includes an optimization of culture conditions and a detailed investigation of morphology and functions. Thus, the cells in the applied system have to be characterized and their validity has to be determined using specific methods for the particular scientific question.

4.1.3. Cell lines versus primary cells

Both primary cells and cell lines are used for standard cell culture experiments. For individual scientific questions, the appropriate method should be applied, but distinct advantages and limitations have to be considered. Using cell lines, regardless to continuity or finity, the cells have to be passaged. Subculturing opens many possibilities. The specific cell type can be propagated and the number of available cells can be increased. A continuous cell line can be subcultured repeatedly, reliably recovered from cryopreservation and maintains many of the characteristics of its cell type or tissue of origin (Wistuba et al., 1998; Ross et al., 2000). Furthermore, the

culture's homogeneity is promoted.

But, especially referring to finite cell lines, treatment with enzymes during subculture procedure causes damage to the cells and cellular surface (Freshney, 2005a). Some cells in the original tissue-specific cell type composition are more adapted to such culture conditions than others. For this reason, a selection of single cell types is inevitable (Freshney, 2005a). It is known from previously established epithelial cell cultures of the oviduct, that secretory cells were lost during culture procedures (Schoen et al., 2008). The risk of overgrowth by other unspecified or stromal cells is also increased in early cell lines (Freshney, 2005a). Phenotypic instability often results in dedifferentiation, adaptation or selective growth and might leads to invalid results. As we see in the cervical cell culture in this study, subculturing led to a loss of differentiation properties and alters cellular characteristics.

Also freezing the cells for storage may have negative influence on cellular function (Bank and Mazur, 1973). Next to microbial contamination, a major problem of reliability of cell cultures based on cell lines are cross-contaminations with other cell lines. Capes-Davis et al. (2010) report a worldwide contamination of specific cell lines with HeLa-cells up to 29%. A strict separation of cell lines during hands-on work has to be ensured. Since continuous cell lines are mainly derived from cancer tissue or acquire a cancer-like phenotype during establishment, the application in experiments investigating physiological molecular mechanisms is questionable.

Primary cells are less altered than cell lines and the original cell type composition is mainly preserved as well as morphological and functional properties. For that reason, a primary cell culture is more comparable to the native tissue. However, replicates of primary cell cultures can be inhomogeneous, especially when the native material varies in age, body condition or oestrus cycle stage. To minimize such uncertainties concerning reproducibility, the native material has to be selected to be uniform (Schoen et al., 2008).

4.1.4. Cell isolation of cervical epithelial cells

To gain cervical epithelial cells several isolation methods for epithelial from the reproductive tract or from cervical epithelium have been tested (Stanley, 2005; Guseva et al., 2003; Ayehunie et al., 2006). However, standard protocols did not lead to a viable epithelial cell culture. Since most of the protocols were established for human cells, it is likely that cells derived from other species may need their own optimized protocol, even if the cells are derived from the same tissues. Therefore, specifically adjusted isolation methods have to be tested and validated for the respective cell type before successfully establishing a new cell culture.

4.1.5. Evaluation of growth media

For an initial media evaluation, we applied a simple morphology score. Microscopically, we could evaluate the epitheloid phenotype, which definitely altered to a more fibroblast-like morphology after passaging. Establishing the oviductal cell culture, we could refer to a number of easily accessible tissue specific characteristics like maintenance of cilial beating. Additionally, the proportion of cells showing vacuoles as sign of cellular senescence helped to find an optimal composition of growth media. Other markers like proliferative activity by reaching confluence or subconfluence during a defined period of time could be included.

The ability and speed of proliferation is essential for cells in culture. Cells that do not proliferate at least at the beginning of culture can not be maintained. Many validation procedures in cell culture are based on proliferation assessment. Such methods like measurements of impedance or mitochondrial activity are easy to apply and deliver useful information about cells and culture conditions.

To assess proliferation of cells, measurement of the mitochondrial activity is a widely used method. Most applications are based on photometrically detectable changes of extinction due to a mitochondrial redox reaction of a colour-reagent. The main idea of this test is that the higher the cell density in the culture well, the more substance

is metabolized by mitochondria. There are possible sources of error due to the simple redox reaction. Most of the reagent will surely be converted by mitochondrial redox enzymes. Unfortunately, other cytoplasmic enzymes are also involved in the turnover. The second interference factor can be irregular activity of the mitochondrial enzymes, since cells which are in an adaptative phase towards new influences (new medium or additives, other substances, etc) can show a higher metabolic activity in general, and therefore, alter the redox reaction and finally the reliability.
The mitochondrial turnover of WST-1[2] does not only give information on proliferation but also includes metabolic activity. In summary, the use of proliferation profiles as parameter for optimization can be seen critically. Proliferative activity also depends on the cell type. Fibroblasts as well as some transformed cells or cells of cancer-origin grow faster than epithelial cells (Freshney, 2005a). In case of cell cultures that are impure (a mixture of fibroblasts and epithelial cells directly after isolation, for example), a measurement of proliferation will not give uniform and valuable results, especially, if proportions of cell types vary in the different replicates. Another problem is the process of dedifferentiation. As shown in the publication Miessen et al. (2012), morphology of epithelial cells altered during the passages. These cells showed modified growth characteristics. So, even a pure epithelial primary cell culture must be controlled regarding regularity before any proliferation assay can be applied.
Application of such tests is limited regarding a differentiated cell culture. In the oviductal cell culture, we could show that (re-)differentiation was established after three weeks of culture without an increase of cell number. The WST-test would probably not detect any differences between morphologically completely different cell cultures after one week and three weeks. Although the xCelligence technology (Roche) would presumably detect such differences due to polarization and a higher impedance, a culture in an air-liquid interface promoting differentiation in both the oviductal and cervical cell culture is not yet possible in the xCelligence

[2]WST-1: water soluble tetrazolium.

system. Therefore, a combination of proliferation measurement with an assessment of morphology and function is necessary for an optimal monitoring of living cell quality.

4.1.6. Specific growth conditions

Standard cell culture conditions do not sustain maintenance or development of morphological and functional polarity of epithelial cells and rapidly lead to an undifferentiated phenotype (Hadley et al., 1987; Schatz et al., 1990). Culture on permeable membranes includes the advantage to simulate *in vivo*-like nutrition properties of epithelial cells adjusted to basal laminae. Growth medium from basolateral, comparable to supply of nutrient from the bloodstream, sustains cellular maintenance of differentiation or redifferentiation of the cells. This could clearly be shown either by the oviductal and the cervical cell culture in an air-liquid interface. In comparison to the conventionally grown cells on plastic surfaces, culture on membranes includes problems in being visualized microscopically due to the often opaque or not completely translucent membranes or the polarized and, therefore, higher or multilayered growth of the cells. Next to that, histological processing of the membranes revealed problems and had to be optimized. The membranes had to be embedded in agarose before the dehydratation process to protect the cells and to ensure an exact cutting angle of 90° during sectioning of the paraffin-embedded samples.

4.1.7. The pig as a model in research for human reproduction

Due to the industrial pork production, porcine material for cell culture is easily available. Biological variations between the different individuals concerning age, cycle stage (mostly pre-pubertal animals) and breed (hybrids of German Edelschwein, German Landrace and Pietrain) are reduced. Since anatomy of the organs, as well as physiological and pathophysiological responses have been found to be very similar to those of humans (Hannon et al., 1989; Larsen and Rolin, 2004; Lunney, 2007), the

pig is a more valid model for human research than rodent species and, therefore, an important alternative as non-rodent animal species (Nunoya et al., 2007). On the molecular level, there is a very high sequence and chromosome structure homology with humans (Lunney, 2007). Main topics using the pig as model are heart physiology, reproductive functions, transplantation of organs, skin physiology, brain, gut physiology and nutrition, as biomechanical models, for tissue engineering, respiratory functions, infectious disease models (Vodicka et al., 2005; Lunney, 2007), and investigation of inflammatory processes (Sullivan et al., 2001; D'Cruz et al., 2005). Focussing on reproductive biology, the non-seasonal porcine and human hormone cycles are quite similar and, therefore, hormonal regulation mechanisms seem to follow the same principles. Maturation of oocytes in the oviduct is comparable between the two species (Vodicka et al., 2005), although ovulation characteristics differ with multiple ovulations in the pig and a single ovulation per cycle in humans. Although deposition of sperm in the reproductive tract is not similar (vaginal deposition in humans (Suarez and Pacey, 2005) and cervical deposition in pigs (Hunter, 1981)), and the anatomy of the uterus in not comparable (uterus simplex in humans and uterus bicornis in pigs), the porcine uterus has been used to investigate sperm transport and its regulation in the uterus (Mueller et al., 2006) which seems to be identical between the two species (Suarez and Pacey, 2005). Studies investigating microbiotic infection mechanisms in the reproductive tract utilized porcine cells successfully (Guseva et al., 2003). Nevertheless, the use of porcine tissue derived from slaughter house material from pre-pubertal swine for reproduction studies remain questionable. Are the processes in the reproductive tissues already comparable to those of adult animals? Is it possible to simulate cycle dependent actions in such pre-pubertal tissues? In previous studies, tissue only from mature gilts was used to establish polarized cell cultures to maintain physiological functions of reproductive epithelia in culture (Bowen et al., 1996; Guseva et al., 2003). However, in the presented oviductal cell culture, a physiological hormone responsiveness could be shown although the native tissue was derived from immature gilts. Sex hormone

receptors could be detected. Therefore, detailed investigations for characterization and validation of the established primary cultures will follow.

5. Outlook

We have established an innovative protocol to culture porcine cervical epithelial cells expressing a differentiated morphology. Additionally, the cell culture shows physiological properties comparable to the native tissue. In order to use the *in vivo*-like cell culture as model system for specific scientific questions regarding pathogen-dependent immunomodulation, sperm-epithelial interaction, signal reception etc., it has to be further validated and, in particular, be functionally characterized.

The newly established oviductal cell culture is very promising to serve as tool in future research. Since we have shown a gradual (re-)differentiation process over three weeks, this system will be useful to study differentiation mechanisms in the porcine oviduct. Meanwhile, morphology and morphometry changes have been validated over a prolonged period of time, in comparison of different biological replicates, and before and after cryopreservation. Recent studies from our research group proved a high reproducibility of this differentiated long-term culture system with a possibility of cryopreservation prior to cultivation (Chen et al., 2013b). Furthermore, previously observed donor-specific differences in response to hormonal stimuli could be adjusted by hormonal synchronisation of the cells. In response to hormonal treatment, the model system recapitulates oestrus cycle events such as changes in morphology, ultrastructure, and functional features like trans-epithelial electrical resistance, sperm binding capacity and gene expression (Chen et al., 2013a)

Culture in dual chamber-systems reveals new opportunities to study side-specific effects of substances like hormones or pharmaceuticals. Effects of such substances applied either from basolateral or apical can now be studied easily. This is especially

important in hormonal regulation of oviductal physiology. Here, after ovulation, the epithelium gets in contact with steroid hormones also from the apical side. Estradiol in the follicular fluid has surely an influence on oviductal functions at this stage of the porcine oestrus cycle. In the first publication (Miessen et al., 2011b) physiological responses to hormonal stimuli from basolateral could be detected. A closer investigation of the currently irregular expression patterns of oviduct specific proteins on RNA[1] level due to probable donor- or replicate-specific influence will follow. Preliminary, unpublished experiments substantiate that application of estradiol to either the apical or the basolateral compartment of the oviductal cell culture leads to different responses in maturation of miRNA[2] that is involved in regulation of gene expression in the reproductive tract (Miessen et al., 2011a).

Since the oviductal cells still showed a cilial activity after six weeks of culture this oviduct specific property has been further investigated regarding enhancement or reduction of the cilial beating. Fluid movement patterns along the epithelium *in vitro* could be presented for the first time applying AndroVision™ Software (Minitüb, Tiefenbach, Germany) that tracks the path of kinetic beads (Chen et al., 2013a).

In conclusion, the conveniently available cervical cell culture appears to be a useful tool to investigate cellular and molecular mechanisms concerning mammalian reproduction and shall further be optimized and finally characterized.

The *in vivo*-like oviductal epithelial culture turns out to be a brilliant system to further elucidate detailed questions concerning regulation of the cyclic events, cell differentiation, sperm-oviduct interaction, the importance of cilial movement and toxicological influence of chemicals on reproduction.

[1] RNA: ribonucleic acid

[2] miRNA: microRNA

6. Summary

Establishment of in vitro cultures of porcine reproductive epithelia - oviduct and cervix uteri, Katrin Mießen

The molecular pathways in the morphologically and functionally different tissue of the reproductive tract of mammals are still incompletely elucidated. For exploring the processes leading to successful reproduction, differentiated *in vitro* systems modelling the mammalian reproductive tract are needed.

The pig serves as a valuable resource for adequate material for several reasons. Since anatomy of the organs, as well as physiological and pathophysiological responses have been found to be very similar to those in humans, the pig is a more valid model for human research than rodent species and, therefore, an important alternative as a non-rodent animal species. This is especially true for the early processes in reproduction. Furthermore, due to the industrial porc production, porcine material for cell culture is easily available (slaughterhouse). Biological variations between the different individuals concerning age, cycle stage (mostly pre-pubertal animals) and breed (hybrids of German Edelschwein, German Landrace and Pietrain) are reduced. This leads to a low biological variation.

In the present studies, culture conditions for primary porcine epithelial cells derived from the oviduct and the cervix uteri were optimized with regard to morphological differentiation and usability for extended cultivation periods. To evaluate different

growth media for the primary cells, we used morphological criteria as well as real-time impedance measurement. After an initial media testing, the cells were grown on hanging membranes and the culture settings (conventionally cultured, serum gradient over the membrane (for the oviductal cell culture only) and air-liquid interface) were assessed by histology and electron microscopy (only oviductal cells).

For the oviductal cell culture, we proved long-term expression of an oviduct specific marker (oviductal glycoprotein 1) and showed a specific hormone responsiveness of the culture system by means of quantitative reverse transcription-PCR (qPCR). Differentiated epithelial cells could reproducibly be cultured up to six weeks in an air-liquid interface. After three weeks of culturing, the cells were clearly polarized and exhibited motile cilia. The model maintains physiological properties such as morphological features (mixed cell population of ciliated and secretory cells, apical cell-cell contacts typical for columnar epithelial cells) and oviduct-specific markers showing hormone responsiveness.

We could reproducibly culture pure epithelial cells out-growing from fresh tissue explants of the porcine cervix uteri. The optimised growth medium was conditioned Ham's F-12, containing 10 % FCS[1], EGF[2] and insulin. When growing cells in an air-liquid interface for three weeks, the cells showed a multilayered phenotype. The cells were of epithelial origin and showed beta catenin expression as well as production of mucopolysaccharides.

Finally, polarized in vitro-systems of the porcine cervical and oviductal epithelium preserving detailed features of the native tissue were successfully established.

Especially the oviductal cell culture model is very promising and will further promote subsequent research projects.

[1]FCS: Fetal Calf Serum

[2]EGF: Epidermal Growth Factor

7. Zusammenfassung

Etablierung porciner in vitro-Zellkulturen aus Eileiter und Gebärmutterhals, Katrin Mießen

Die molekularen Vorgänge innerhalb der morphologisch und funktionell sehr unterschiedlichen Gewebe des weiblichen Reproduktionstraktes des Säugetieres sind bisher nicht komplett aufgeklärt. *In vitro*-Systeme, die die physiologischen und pathophysiologischen Gegebenheiten widerspiegeln, sind notwendig, um die Prozesse zu verstehen, die zu einer erfolgreichen Reproduktion führen.

Als Ressource für adäquates Zellmaterial bietet sich das Schwein aus mehreren Gründen an. Es ist eines der favorisierten Modelle für den menschlichen Organismus, da das Schwein dem Menschen, im Vergleich zu den für *in vivo*-Versuche häufig eingesetzten Nagetieren, unter vielen anatomischen und physiologischen Gesichtspunkten ähnlicher ist. Dies trifft insbesondere auf die frühen Prozesse des Reproduktionsgeschehens zu. Zudem ist Gewebe aus dem porcinen Reproduktionstrakt als Nebenprodukte der Fleischproduktion (Schlachthof) leicht verfügbar. Darüber hinaus zeigen die Donor-Tiere bedingt durch die industrialisierte Schweinezucht wenig individuelle Unterschiede betreffend z.B. des Zyklusstandes (präpubertäre Tiere) und der Rasse (fast ausschließlich Hybride aus Deutschem Edelschwein, Landrasse und Pietrain). Dies reduziert die biologische Schwankungsbreite entsprechend.

In der vorliegenden Doktorarbeit wurden Isolierungsmethoden und Kulturbedingungen für Epithelzellen aus Eileiter und Gebärmutterhals unter dem Gesichtspunkt

der morphologischen Differenzierung und einer möglichen Langzeitkultivierung untersucht und optimiert.

Um die verschiedenen Wachstumsbedingungen der Epithelzellkulturen zu bewerten wurden sowohl morphologische Kriterien als auch Echtzeit-Impedanzmessungen herangezogen. Nach einer anfänglichen Untersuchung von Wachstumsmedien wurden die Epithelzellen unter unterschiedlichen Kulturbedingungen auf hängenden, permeablen Membranen kultiviert. Die Zellkulturen der einzelnen Kulturbedingungen (konventionell, mit einem Serumgradienten über die Membran (nur für die Eileiterzellkultur) oder in einem Air-Liquid-Interface mit Kontakt zum Medium ausschließlich von der basolateralen Zellseite) wurden histologisch und elektronenmikroskopisch (nur Eileiterzellkultur) ausgewertet.

Rein epitheliale Zellen aus dem Gebärmutterhals konnten über ein Auswachsen aus Gewebeexplantaten gewonnen werden. Als optimiertes Wachstumsmedium diente konditioniertes Ham's F-12, mit 10 % FCS, EGF und Insulin. Eine dreiwöchige Kultur der Zellen im Air-Liquid-Interface führte zum Wachstum eines mehrschichtigen Cervixepithels. Hier konnten sowohl beta-Catenin als Differenzierungsmarker sowie eine Mukusproduktion nachgewiesen werden.

Differenzierte Epithelzellen des Eileiters in ihrer natürlichen Zusammensetzung (Zilien tragende und sekretorische Zellen) konnten reproduzierbar über sechs Wochen im Air-Liquid-Interface gehalten werden. Schon nach einer Kulturdauer von drei Wochen polarisierten die Zellen deutlich und zeigten motile Kinozilien. Die Expression eines Eileiter-spezifischen Markers (oviductal glycoprotein 1) wurde nachgewiesen und eine spezifische Antwort auf Hormonstimulation konnte mittels quantitativer Polymerasekettenreaktion (qPCR) gezeigt werden.

In den hier etablierten in vitro-Systemen der Epithelien von Eileiter und Gebärmutterhals konnten mit denen im natürlichem Gewebe vergleichbare Verhältnisse erhalten beziehungsweise eingestellt werden.

Das in der Dissertation vorgestellte Oviduktzellkultursystem lässt neue Impulse auf nachfolgende Forschungsprojekte erwarten.

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A. Appendix

A.1. Nachdruckgenehmigungen

Die hier gezeigte Kopie der Veröffentlichung *Miessen, K., Sharbati, S., Einspanier, R. and Schoen, J. (2011). Modelling the porcine oviduct epithelium: a polarized in vitro system suitable for long-term cultivation.* (Miessen et al., 2011b) darf als Druckversion genutzt werden.

Bei der Veröffentlichung *Miessen, K., Einspanier, R. and Schoen, J. (2012). Establishment and characterization of a differentiated epithelial cell culture model derived from the porcine cervix uteri.* (Miessen et al., 2012) handelt es sich um ein Open Access-Dokument und darf in der vorliegenden Doktorarbeit sowohl in gedruckter als auch online-Form verwendet werden.

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A.3. Selbstständigkeitserklärung

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

Güstrow, den Unterschrift