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Modeling early embryo-maternal interactions *in vitro*

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Abstract

Environmental conditions experienced during early embryonic development influence growth, metabolism, and gene expression of the embryo as well as the epigenetic profile of the offspring. The environment of the early embryo consists of the luminal fluid within the oviduct and uterus and the epithelial cells composing this fluid. Whether the embryo is able to shape its own microenvironment by interacting with the epithelial lining of the oviduct/uterus and which factors potentially interfere with or regulate these interactions remains to be elucidated. As early embryonic signals and the respective maternal responses are subtle and local events, it is challenging to study them *in vivo*. Therefore, adequate *in vitro*-models optimally mimicking the contact zone between the maternal reproductive tract and the early embryo are needed to a) elucidate basic mechanisms involved in early embryonic development and b) reduce the number of experimental animals used for such studies. Functional epithelial cells are generally defined by a polarized distribution of organelles and proteins. Proper polarization is tightly connected with physiological cell behavior and *in vivo*-like reactivity of the epithelium. Therefore, this review summarizes current strategies for *in vitro* preservation of epithelial cell polarity. It presents recent advances in 3D culture of female reproductive tract epithelia and embryo-epithelial co-cultures. A special emphasis is set on compartmentalized culture systems, powerful tools for studying early embryo-maternal interactions *in vitro*. In such systems, cultured epithelial cells are manipulable from their basolateral as well as their apical cell pole, allowing concomitant application of embryonic as well as maternal effectors from the appropriate cellular compartment.

Keywords: embryo-maternal interactions, endometrium, oviduct, three dimensional cell culture models.

Introduction

Early embryos of eutherian mammals reside within the oviduct (species-specific up to the 4-cell, 8-cell, morula or blastocyst stage) before they transition into the uterus. It is now well established that the environmental conditions experienced during early embryonic development (zygote to blastocyst stage) influence growth, metabolism, and gene expression of the embryo as well as the epigenetic profile of the offspring (recently reviewed by Fazeli and Holt, 2016; Rizos *et al*., 2017).

So far, clear evidence is given for a mutual, reciprocal interaction between the female reproductive tract (FRT) and the developing conceptus during maternal recognition of pregnancy and implantation. However, the biological relevance of earlier interactions of the embryo with the upper FRT is still a matter of debate.

Studies in litter bearing species like pigs and mice show that the oviduct responds to preimplantation embryos, long before the embryonic signal for maternal recognition of pregnancy (Lee *et al*., 2002; Alminana *et al*., 2012; Li *et al*., 2015). In the monovulatory cow, where only one single embryo resides in the oviduct, this could not be confirmed *in vivo* at the transcriptomic level (Maillo *et al*., 2015), but first hints exist for an early embryonic influence on the oviduct fluid proteome in this species (Maillo *et al*., 2016). There is clear *in vivo* evidence for early embryo-maternal communication in another monovulatory species, the horse, where embryonic prostaglandin E2 causes relaxation of the oviductal isthmus and allows selective transport of the embryo to the uterus (Weber *et al*., 1991; Freeman *et al*., 1992). Differential transcriptomic profiles between pregnant and non-pregnant oviducts also suggest an impact of one single early embryo on the innate immune response in the equine FRT (Smits *et al*., 2016).

However, whether the embryo is actually able to shape its own microenvironment by interacting with the epithelial lining of the oviduct/uterus and which factors potentially interfere with or regulate this fine-tuned interactome (Fazeli, 2011) remains to be elucidated.

As early embryo-maternal interactions are presumably subtle and local events, they are challenging to study *in vivo* (not only, but especially in monovulatory species), both from a technical and an ethical point of view. Biological variation, very limited numbers of possible replicates and the unfavorable signal-to-noise ratio might hamper the success of such *in vivo* studies.

Therefore, adequate *in vitro*-models optimally mimicking the contact zone between the maternal reproductive tract and the early embryo could help to elucidate basic mechanisms involved in early embryonic development and programming. At the same time such models reduce the number of experimental animals needed for basic research and might proof useful also for reproductive toxicity testing (Simintiras and Sturmey, 2017).

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The environment of the early embryo

The environment of the early embryo consists of the luminal fluid within the FRT and the epithelial cells composing this fluid.

It is suggested that the oviduct and uterine luminal fluids represent the optimal milieu for the early embryo. Its basic composition has been analyzed with respect to protein composition, ion content and energy substrates using *in situ* and *ex vivo* techniques (reviewed in Leese *et al*., 2008). Differences in the fluid composition have been documented regarding the estrous cycle stage and sampling region. However, the mechanisms of oviductal and uterine fluid formation and its regulation are far from being understood.

The epithelial cells assembling the luminal surface of the oviduct and uterus compose this fluid in accordance to the developmental needs of the embryo (Absalon-Medina *et al*., 2014) and directly get in contact with the early embryo.

This contact zone is a simple epithelium which not only nourishes the early embryo but also provides protection from or clearance of unfavorable molecules and (in case of the oviduct) transport of the embryo towards the uterus.

Epithelia of the FRT: developmental origin and structural hallmarks

Oviduct and uterus both derive from a pair of Mullerian ducts (MDs), which consists of three elements: an inner epithelium layer, surrounding mesenchyme, and the external Mullerian coelomic epithelium. During MD development, the opening cranial end forms into the oviduct, and the caudal ends of left and right MD fuse and give rise to the uterus. Epithelia in the oviduct and uterus both develop from the MD epithelium, while the stromal compartment of uterus evolves from the MD-surrounding mesenchyme (Kurita, 2011).

Epithelia are generally defined by a polarized distribution of organelles and proteins within each cell. The paracellular space between adjacent epithelial cells is sealed by cell-cell junctions at the apical part of the lateral plasma membrane. Especially tight junctions are essential for epithelial polarity and functionality as they form both a paracellular barrier (regulating selective paracellular permeability) as well as a barrier within the membrane which restricts the exchange of membrane components between apical and basolateral cell surface domains. In recent years it became evident that beside their role as simple diffusion barriers, tight junctions are cellular signaling platforms which are regulated by diverse physiological and pathological stimuli (reviewed in (Zihni *et al*., 2016). Loss of cellular polarity, in turn, is a pathological condition frequently seen in cancer development, which alters specific cell functions and responsiveness to external signaling events (Ellenbroek *et al*., 2012).

The basal part of the cell membrane of an epithelial cell is attached to the basement membrane, which separates the epithelium from the underlying connective tissue. With this basolateral cell pole, it takes in systemic effector molecules and nutrients as well as molecules secreted by the connective tissue. In contrast, the contact to gametes or embryos takes place on the apical surface of the cell, which is morphologically and functionally different from its basolateral counterpart in terms of membrane properties and abundance of receptor molecules.

Therefore, proper polarization of the epithelial cells in the FRT is tightly connected with their physiological behavior and *in vivo*-like reactivity towards systemic maternal as well as embryonic stimuli.

Modelling the contact zone: oviductal and uterine epithelial cells *in vitro*

Maintenance of epithelial polarity during culture is an important prerequisite for *in vitro* investigations concerning the fine-tuned interactions possibly taking place between the early embryo and the maternal organism.

Under standard culture conditions (2D, adherent on cell culture plastic ware), which are most frequently used to explore embryo-maternal interactions *in vitro*, epithelial cells (primary or cell lines) from the FRT attach to the plastic surface and are submerged in medium. Even if cells build cell-cell contacts and a certain level of cellular polarization under such conditions, they receive nutrition from the apical pole. This leads to rapid adaptation processes within the cells and to marked changes in their morphological and functional integrity (Fig. 1A). Polarization, expression of marker genes and ciliation get lost (Danesh *et al*., 2016). A well-known example for the loss of marker gene expression during *in vitro* culture is the oviductal glycoprotein 1 (OVGP1), one of the most abundant glycoproteins in the oviduct of most mammals (Coy *et al*., 2008). Under 2D culture conditions, OVGP1 is promptly down-regulated and cannot be triggered by ovarian steroids anymore (Briton-Jones *et al*., 2002, 2004; Schoen *et al*., 2008; Danesh *et al*., 2016).

Cell culture conditions preserving epithelial cell polarity in vitro

Suspension culture

Suspension culture (Fig. 1B) is frequently used for primary oviduct epithelial cells (OEC), especially for analysis of sperm binding in mammalian species (De Pauw *et al*., 2002; Waberski *et al*., 2005; Henry *et al*., 2015). Cells are obtained by squeezing out or scraping off the oviduct epithelium, and later maintained in suspension culture dishes as cell clusters (also termed explants) with their cilia directed outwards. Suspension culture is a particularly useful tool for short-term experiments as it preserves oviduct specific characteristics for approximately 12 h before first signs of de-differentiation are observed (Rottmayer *et al*., 2006).

3D Organoids

Epithelial cells cultured in extracellular matrix (ECM) substitutes can form three dimensional structures with a lumen (Fig. 1C). An organoid culture system from clonal cells was established from the human oviduct, which showed fully *in vivo*-like epithelial differentiation including OVGP1 expression (Kessler *et al*., 2015). Likewise, three dimensional human glandular endometrial cultures were recently developed, leading to highly differentiated, hormone-responsive organoids, which secreted uterine marker proteins like glycodelin and osteopontin (Turco *et al*., 2017). In these models stemness was maintained within the cell population and therefore allowed long-term culture. Differentiation is induced via exogenous factors. Gland-like endometrial spheroids have also been used to study human endometrium-trophoblast interaction and trophoblast invasion (Buck *et al*., 2015). However, manipulation of the organoid lumen, e.g. applying an embryo or embryonic effector molecules on the apical cell pole, is not possible. Yet such organoid cultures enable propagation of differentiated FRT epithelia *in vitro* (e.g. from one individual). Organoid cultures represent self-organizing 3D systems which are genetically stable and contain progenitor and/or stem cells as well as differentiated cells. Therefore, they can be used as a substitute for freshly isolated primary cells in other culture systems.

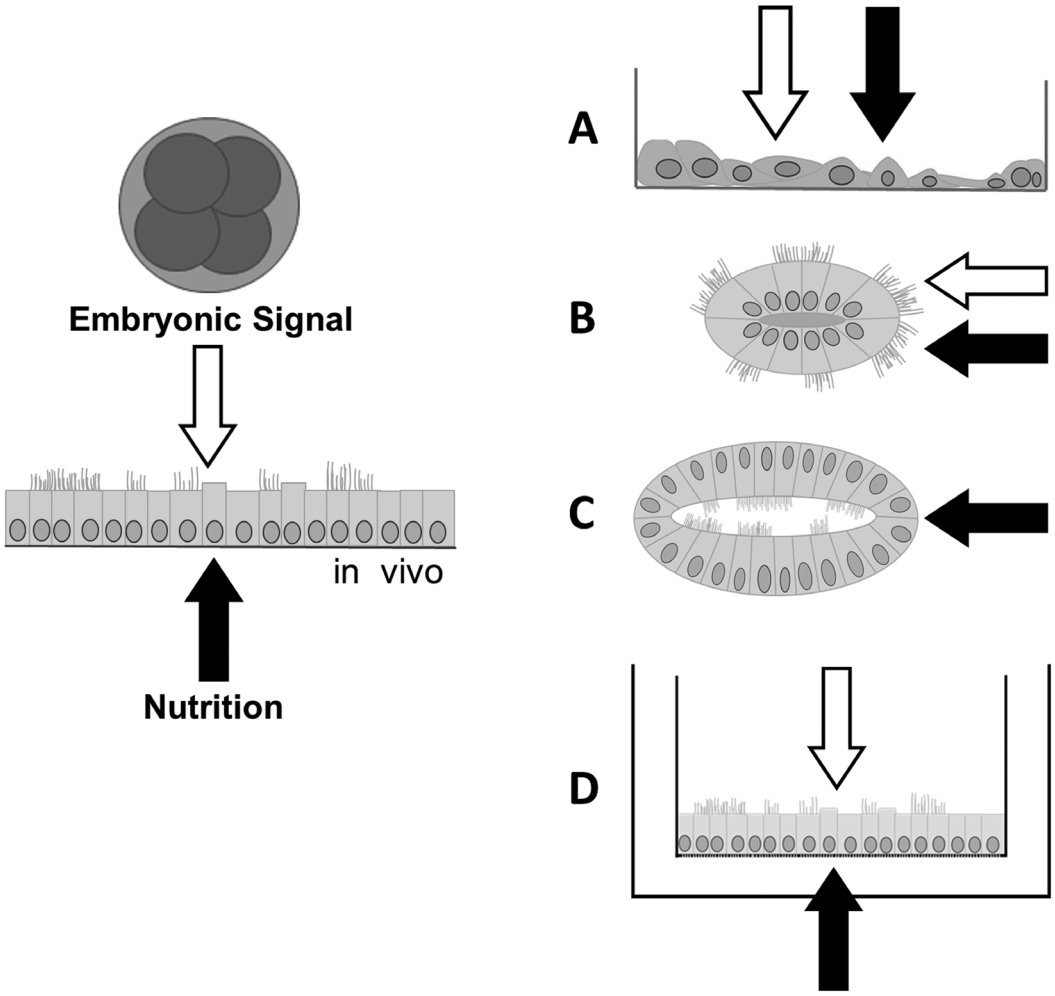

Figure 1. Schematic illustration of possible reception routes for embryonic signals and nutrition in different culture systems of epithelial cells. (A) Standard 2D adherent submerged culture; embryonic signals and nutrition from apical side. (B) Epithelial sphere in suspension culture; signals and nutrition from outside of the sphere (apical epithelial cell pole). (C) Epithelial 3D organoid; nutrition is provided from outside the organoid; embryonic signal is not applicable. (D) Compartmentalized culture system; nutrition from the basolateral side, embryonic signal could be given from the apical side.

Compartmentalized culture systems

In compartmentalized culture systems, cells are grown in inserts on either porous membranes or scaffolds (Fig. 1D). Placing the insert into the cell culture medium mimics the *in vivo* nutrient supply from the basolateral side of the cell, and thereby supports epithelial differentiation and polarization. The compartmentalized system can be used to grow cells submerged (liquid-liquid interface) with either the same or two different media in the basolateral and apical compartment (e.g. creating a serum gradient over the

membrane). Porous filter supports can also be used to grow FRT epithelia at the air-liquid interface (ALI), i.e. with no medium in the apical compartment. The ALI method is primarily known from differentiated long-term cultures of skin and airway epithelia. When applied to FRT epithelia, ALI culture systems engendered excellent long-term differentiation of oviduct (Levanon *et al*., 2010; Miessen *et al*., 2011; Gualtieri *et al*., 2012) and uterine epithelial cells (Munson *et al*., 1990; Classen-Linke *et al*., 1997). In general, as known for airway epithelia, ALI culture seems to better support differentiation of FRT epithelia than submerged conditions. Furthermore, ALI models allow acquisition and analysis of the luminal fluid created by the epithelia. Initial analysis of oviductal fluid surrogates obtained from ALI cultures showed apparent similarities to oviductal fluid *in vivo* (Simintiras *et al*., 2016; Chen *et al*., 2017). This makes the ALI approach a promising tool to investigate effectors regulating or modifying the environment of the early embryo.

In compartmentalized culture systems, cell polarization can be further enhanced by coating the membrane or scaffold with ECM components like collagens (Munson *et al*., 1990; Chen *et al*., 2017). Especially collagen IV, a structural protein present in the apical *lamina densa* of the basement membrane, could facilitate the initial attachment rate of isolated cells during seeding and therefore prevent dedifferentiation due to excessive proliferation (Aumailley and Timpl, 1986). Also conditioning the basolateral medium with homo- or heterologous fibroblasts enhances the structural differentiation of the cultured epithelial cells (Munson *et al*., 1990; Ostrowski and Nettesheim, 1995; Miessen *et al*., 2011).

3D models combining epithelial and stromal cells

Stromal cells regulate cell fate, morphology, and function of epithelia through epithelial-mesenchymal interactions (Cunha *et al*., 1985; Kurita *et al*., 2001). Interactions between epithelial and stromal cells are evident in the oviduct (Umezu and Tomooka, 2004) as well as in the uterus, where they have been extensively studied in human models for decidualization and embryo invasion (reviewed in Weimar *et al*., 2013). Stromal cells seem to translate systemic signals within the maternal organism (e.g. from steroid hormones) and to modulate their effect on the epithelial lining (Pierro *et al*., 2001; Qi *et al*., 2012).

Stromal cells can be co-cultured with epithelial cells in compartmentalized culture systems with or without ECM resembling scaffolds (e.g. hydrogel, agarose or matrigel; Fig. 2). While 3D models of the oviductal or luminal endometrial epithelium with the underlying stroma can relatively easily be constructed (Arnold *et al*., 2001; Simintiras *et al*., 2016; Fig. 2A, B), modeling the endometrium with both its glandular and luminal epithelium in 3D is a more complex endeavor. In contrast to the luminal epithelium, the glandular endometrial epithelium finds its niche within the interstitial tissue (Fig. 2C). To our knowledge, there is only one report documenting the capability of uterine epithelial cells to form both the endometrial luminal epithelium as well as glands *in vitro*. Primary epithelial endometrium cells were co-cultured with stromal cells seeded in fibrin-agarose and gland formation occurred spontaneously (Wang *et al*., 2012).

More recent epithelial-stromal co-culture models are based on *ex vivo* ECM scaffolds as the de-cellularized endometrium (Olalekan *et al*., 2017) or novel artificial scaffold structures (MacKintosh *et al*., 2015) which provide a more *in vivo*-like 3D environment for the stromal cells.

Perfused culture systems and microfluidic devices

The FRT epithelium of most mammalian species undergoes dramatic morphological and functional changes throughout the estrous cycle. In the luteal phase (progesterone dominance), the epithelium exhibits a regressed status; conversely, cells re-enter proliferative status in the follicular phase (estradiol dominance), including a rise in epithelium height and increased secretory activity.

However, the exact and time resolved impact of hormones (as well as many other dynamic systemic maternal cues) on luminal fluid formation and epithelium responsiveness to embryonic signals are not elucidated yet. Even if different stages of the estrus cycle can be mimicked in compartmentalized models of the FRT epithelium (Chen *et al*., 2013), these systems are still static. Devices which allow constant perfusion of the cell culture vessel therefore provide much better options for modeling the dynamic changes induced by maternal cues and to elucidate their effects on the embryonic environment and the embryo itself. Perfusion approaches were already used to model FRT epithelia, and were proven to enhance structural differentiation (Reischl *et al*., 1999). Lately, new dynamic culture systems, which enable long-term maintenance of differentiated and hormone responsive epithelia, have been established. An organ-on-a-chip model of the bovine oviduct (suitable for live cell imaging) recapitulates the oviduct epithelium over extended culture periods (up to six weeks). In co-culture experiments, epithelial interactions with sperm and oocytes as well as fertilization events were observed (Ferraz *et al*., 2017). Beyond that, organ modules of the murine ovary, fallopian tube, uterus, cervix and liver, with a sustained circulating flow between all tissues, were recently coupled in a multiple unit microfluidic platform. This system simulated not only the female reproductive tract, but also the endocrine loops between different organs (Xiao *et al*., 2017). In the era of 3D (bio-)printing, these approaches surely represent the next generation of *in vitro* models for studying early embryo-maternal interactions.

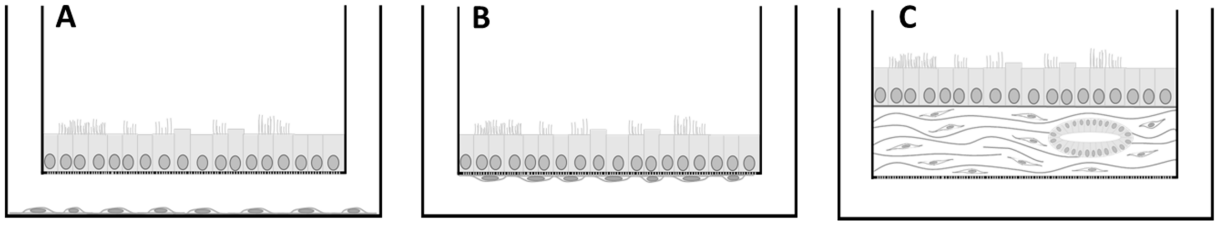

Figure 2. Schematic illustration of strategies for co-culturing stromal cells with FRT epithelial cells in compartmentalized culture systems. (A, B) Stromal and epithelial cells grown in separate compartments. (A) Stromal cells seeded in the basal culture dish. No direct epithelial-stromal cell contact; (B) Stromal cells seeded on the basal side of the insert directly beneath epithelial cells; (C) Stromal and epithelial cells within the same compartment. Stromal cells embedded in ECM support, which may permit the formation of glandular structures.

Co-culture of embryos with epithelia of the FRT

FRT epithelial cells cultured under standard 2D submerged conditions were widely used as feeder layers to improve IVP outcomes and showed a positive effect on *in vitro* embryo quality (Locatelli *et al*., 2005; Cordova *et al*., 2012, 2014; Schmaltz-Panneau *et al*., 2015). In terms of their embryo supporting capacity, however, no difference was detected between oviduct and uterine epithelial cells and oviduct stromal cells (Goff and Smith, 1998). Feeder origin also had no influence on bovine embryonic development and transcriptome when bovine oviduct epithelial cells were compared with a primate kidney cell line as feeder layer (Carvalho *et al*., 2017). In recent years, co-cultures have also been applied to examine early embryo-maternal dialogue. In several studies bovine embryos were co-cultured with OEC to mimic the maternal environment *in vitro.* These experiments demonstrated bi-directional responses at the transcriptional level from both the maternal and embryo side (Schmaltz-Panneau *et al*., 2014; Garcia *et al*., 2017). However, OEC were subjected to multiple embryos and/or prolonged co-incubation, which does not ideally imitate the *in vivo* situation in the monovulatory cow.

Taking advantage of the ALI approach (see paragraph compartmentalized culture systems), co-culture experiments for the first time demonstrated embryo development on OEC up to the blastocyst stage without supplementation of any embryo culture medium (Chen *et al*., 2017). However, more *in vivo*-like mRNA expression of bovine embryos could not be proven as a result of co-culture (van der Weijden *et al*., 2017). We deduce that to enhance embryo quality in co-culture including a dynamic hormonal stimulation procedure might be necessary to better mimic the *in vivo* oviductal environment.

Conclusion

Depending on the researcher's specific scientific question, different culture models are available to reconstruct the upper FRT *in vitro*, either for the short or long term. In compartmentalized culture systems, epithelia are manipulable from their basolateral as well as apical surface, allowing co-culture of embryos/zygotes on the apical and concomitant application of maternal effectors to the basolateral compartment. This makes them powerful tools for studying early embryo-maternal interactions. Stromal cells and ECM components can be incorporated, which is of special interest for modeling the endometrium *in vitro*. Considering that the oviduct and uterus are highly dynamic, hormone responsive organs, perfused culture systems or microfluidic devices allow a more *in vivo*-like recapitulation of the early embryonic environment. The recent advances achieved in these model systems provide the basis for deciphering the possibly fine-tuned interactions between the single early embryo and the maternal organism as well as their effects on offspring development and health.

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