A 3-D cell culture system to study epithelia functions using microcarriers

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Abstract In vitro cell culture models used to study epithelia and epithelial diseases would benefit from the recognition that organs and tissues function in a three-dimensional (3D) environment. This context is necessary for the development of cultures that more realistically resemble in vivo tissues/organs. Our aim was to establish and characterize biologically meaningful 3D models of epithelium. We engineered 3D epithelia cultures using a kidney epithelia cell line (MDCK) and spherical polymer scaffolds. These kidney epithelia were characterized by live microscopy, immunohistochemistry and transmission electron microscopy. Strikingly, the epithelial cells displayed increased physiological relevance; they were extensively polarized and developed a more

differentiated phenotype. Using such a growth system allows for direct transmission and fluorescence imaging with few restrictions using wide-field, confocal and Light Sheet Fluorescence Microscopy. We also assessed the wider relevance of this 3D culturing technique with several epithelial cell lines. Finally, we established that these 3D micro-tissues can be used for infection as well as biochemical assays and to study important cellular processes such as epithelial mesenchymal transmission. This new biomimetic model could provide a broadly applicable 3D culture system to study epithelia and epithelia related disorders.

Keywords Three-dimensional cell culture · Live cell imaging · Epithelial systems · Cytodex 3 · EMT

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Introduction

Epithelial tissue constitutes the largest tissue group in humans and its study can reveal much about the body

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and its related disorders. The most frequently used in vitro model of epithelia is the two-dimensional (2D) monolayer. 2D cell culture is usually defined as the practice of growing mammalian cells on flat plastic surfaces such as petri dishes (Abbott 2003). 2D cultures of epithelial cells have been implemented for several decades to evaluate the nature of these cells (Nauli et al. 2003), their associated pathological conditions (Förster 2008; Tanos and Rodriguez-Boulan 2008) and host pathogen relationships (Robbins et al. 1999). While this system has provided important insights into the biology of epithelial cells, it is not without restrictions. 2D epithelial monolayers lack complex physiological relevance, with many of the morphological and functional characteristics inherent to the cell's in vivo differentiated state lost; such as cell polarization, tissue specific protein secretion and a well-developed microvilli brush border (Page et al. 2012). Epithelial cells cultured in a three-dimensional (3D) microenvironment can partially regain their in vivo phenotype and thus provide more predictive and accurate data (Asthana and Kisaalita 2012). 3D cell culture attempts to simulate in vivo conditions by providing cells with additional microenvironment factors. 3D systems generally utilize at least one of the following factors: biomaterials with complex chemical and biochemical characteristics (e.g. Collagen), a substrate with physiologically relevant stiffness and porosity, a 3D geometry where cells interact with each other and the substrate in all three dimensions (Griffith and Swartz 2006). There are many 3D epithelial cell culture techniques currently available; however, issues associated with these techniques make them unsuitable for use as routine tissue culture models and for imaging assays.

One of the first steps towards the third dimension in cell culture was the development of permeable membrane filter supports (Misfeldt et al. 1976; Cereijido et al. 1978). Cells that are grown on such supports exhibit characteristic features of polarized epithelial cells seen in vivo (Vega-Salas et al. 1987; von Bonsdorff et al. 1985). However, imaging of this type of support is difficult as the translucent membrane filter generates high levels of light scattering and needs to be dissected, breaking the epithelial barrier prior to observation. Alternative techniques utilize the epithelial cell's ability to form polarized cysts when

encapsulated in extracellular matrix materials (O'Brien et al. 2002) and to form aggregates known as spheroids under specific conditions (Messner et al. 2013). However, cysts are closed structures with an internalized lumen that do not allow access to the apical membrane for manipulation (infection, transfection and microinjection), and spheroids are disorganized cell aggregates that lack the structure necessary to mimic healthy in vivo tissue (Vörsmann et al. 2013).

In 1967, van Wezel described the use of small particles (0.2 mm) known as microcarriers, for the growth of anchorage-dependent cells (van Wezel 1967). Microcarriers are small spheres, either porous or non-porous, with diameters in the range of 100–500 µm, made of several biocompatible materials (Kadouri 1994). Microcarriers have been used with epithelial cells mainly for vaccine production (Sun et al. 2004). However, a limited number of studies pointed out their relevant use in infection research (Carterson et al. 2005), viral glycoprotein transport (Mayer et al. 1996), patch-clamp experiments (Poronik et al. 1988) and biochemical assays (Mayer et al. 1996). With these studies in mind, we chose to comprehensively investigate whether microcarriers are an efficient 3D epithelial tissue model suitable for microscopic analysis and assays.

In this paper we describe a static cell culture system for microcarriers that avoids microcarrier aggregation and produces single epithelial organoids. It uses commercially available Cytodex 3 to establish micro-tissues using multiple epithelial cell types (lung, liver, Kidney and colon) and is quick, easy and affordable. We illustrate that our model is compatible with most major imaging modalities, including transmission, live time lapse, confocal, light sheet and electron microscopy. Using these techniques we observed that MDCK microcarriers have a highly polarized morphology with distinct basolateral/apical domains, a dense microvilli network at the apical membrane and well developed cellular junctions. These features suggest that the MDCK microcarriers are functional. Finally, we established that this model allows the investigation of epithelial processes and diseases such as Epithelial Mesenchymal Transition (EMT), membrane trafficking and infections (demonstrated using the obligate intracellular parasite *Toxoplasma gondii*).

Materials and methods

Cell lines

MDCK-I (NBL-2, CCL-34), A549 and Caco-2 cell stocks were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). MDCK-II (ECACC, Porton Down, Salisbury, U.K., #62107) was a kind gift from Dr. Carsten Schultz. HepG2 and Huh7 were a kind gift from Dr Markus Meißner. Stock cultures of cells were grown in Modified Eagle's Medium (MEM) (A549 cells were grown in Ham's F-12 K medium) with 10 % (v/v) Fetal Calf Serum (FCS) (Caco-2 cells with 20 % FCS), penicillin–streptomycin (100 U/ml and 100 µg/ml, respectively) and 2 mM glutamine at 37 °C and 5 % CO₂. Cells were sub-cultured twice per week following standard trypsinization protocols.

Primary antibodies and reagents

All materials were supplied by Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise stated. GM130 (BD Biosciences, Oxford, UK), Sarg1, Hoechst 33342 (stock 1 µg/ml), Phalloidin-Alexa488 (Molecular Probes, Loughborough, Leicestershire, UK) and Concanavalin A (stock 1 mg/ml).

Cell culture on microcarriers

Cytodex 3 beads (GE Healthcare, Uppsala, Sweden) were rehydrated and stored in Phosphate Buffered Saline (PBS) with 10 % penicillin/streptomycin. 80 % Confluent MDCK-II cells were detached with 0.5 % trypsin and 2 mM EDTA. For cell culture on Cytodex 3 microcarriers, approximately 10 swollen Cytodex 3 beads/cm² were seeded with 1×10^3 cells/cm², resulting in a ratio of 100 cells per bead, only 5–10 cells attached to the bead and expanded. Microcarrier culture was conducted using appropriate medium in 24-well plates coated with a repellent layer (Gel-riteTM). The Cytodex 3 beads were confluent 4 days after seeding and unattached cells were removed by filtering through a 70 or 100 µm cell strainer (BD Biosciences, Erembodegem, Belgium). The remaining beads were transferred into a coated well with fresh medium, and the beads were left to differentiate for up to 6 weeks. Once established the epithelial Cytodex 3 culture did not require the use of trypsin to pass the cells. Simple addition of new Cytodex 3 led to cell

transfer from established Cytodex 3 to the newly added microcarriers. This can improve culture by limiting cell passage trauma due to regular trypsinization.

Immunofluorescence staining and fluorescence imaging

Cells were rinsed twice with pre-warmed PBS and then fixed for 10 min with 3 % paraformaldehyde, washed with 0.1 M PBS/glycin and permeabilized with 0.1 % PBS/Triton for 20 min. After extensive washing with PBS, cells were blocked for 30 min with 10 % PBS/FCS and then incubated in primary antibody GM130 (1:400) at room temperature for 30 min. After washing, cells were incubated with Cy3 labeled goat anti-mouse secondary antibody (1:200) for 30 min at room temperature and then washed. Cells were then incubated for 30 min in Phalloidin Alexa-488 (1:40) or (1:10) for 15 min, and subsequently with Hoechst 33342 (1:1000) for 5 min. Samples were kept in PBS after washing and observed with a Leica SP5 confocal microscope or on our custom built Light Sheet Fluorescence Microscope (LSFM). Images were analyzed using ImageJ and FIJI.

Sample preparation for LSFM

The sample was embedded in a cylinder of a 0.5–2 % (w/v) type VII low melting agarose gel. A glass capillary with an inner diameter of 1.1 mm (or a syringe with an inner diameter of 4.7 mm) held the agarose gel rod. The sample was inserted from above into a PBS filled chamber on the custom built LSFM. The capillary or syringe could be translated along three axes and rotated around its center.

Sample preparation for transmission electron microscopy

The Epithelial Cytodex were fixed with 2.5 % glutaraldehyde in Cacodylate (CaCo) buffer. Samples were washed with CaCo buffer, and kept cold on ice during incubation with 2 % OsO₄ in CaCo buffer for 40 min. The sample was rinsed with water, before uranyl acetate (0.5 % in water, 30 min) was added to increase the contrast. The fixed epithelial Cytodex were embedded in Epon and left to polymerize at 60 °C (2–2.5 days). After polymerization, samples were trimmed only around the epithelial Cytodex of interest. Ultra-thin (50–60 nm) sections were made [on average

58 ± 21 (SD), serial sections covered the complete epithelial Cytodex and was transferred to copper-palladium slot grids (2_1 mm, Plano, Wetzlar, Germany) freshly coated with Formvar. The sections were post stained with uranyl acetate and lead citrate. Images were taken using a BioTwin CM120 electron microscope (FEI Company, Eindhoven, Netherlands).

EMT Cytodex motility assay

Confluent MDCK-II Cytodex 3 beads were transferred into an 8 well Labtek dish (Nunc, Schwerte, Germany) and cultivated for 2–3 days under standard conditions. After ~ 24 h (or at specific time points) the beads attached and cells started to grow on the bottom surface of the dish. Time lapse experiments were performed on a Delta Vision Microscope (20 $\times$, frame rate: 1/20 min). Images were analyzed using ImageJ and FIJI.

Toxoplasma infection assay

100 cells per well (384 well plate) were seeded the day before infection and ~ 10 confluent beads were transferred into another well. For Infection 1×10^5 GFP-Toxoplasma/per well were added and incubated overnight. After washing the cells with PBS, they were fixed with 3 % paraformaldehyde/0.06 % glutaraldehyde in PBS, washed once with PBS and blocked with 3 % Bovine Serum Albumin/PBS. To stain parasites remaining on the cell surface an anti-Sarg1 antibody (mouse) was used (1:400) and incubated for 1 h at room temperature followed by several washing steps with PBS and further incubation with an Alexa-596 anti-mouse secondary antibody for 1 h. Cells were washed with PBS and nuclei were stained with Hoechst 33342 (1:1000) for 5 min. After a final washing step with PBS cells were covered with 40 μ l PBS and stored at 4 °C. Cells grown as a monolayer were imaged on an Axiovert200 Microscope (40 $\times$) and cells on Cytodex 3 beads were embedded in 1.5 % agarose/PBS and imaged using LSMF (40 $\times$).

Results

Growth of MDCK kidney epithelial cells on polymer scaffolds

We tested several microcarrier types for their ability to grow an epithelial polarized monolayer (Fig. S1 and

Supplementary Material). Cytodex 3 was found to be the optimal choice (porous Sephadex beads coated with denatured collagen, 175–300 microns in diameter). They allow quick and stable monolayer establishment (with cells adhering better on the cross-linked collagen layer) and are compatible with most microscopic techniques.

MDCK kidney epithelia cells were cultivated statically on the beads in 24-well plates. In normal tissue culture dishes, cells preferentially grow on the bottom of the dish then spread on the beads (Fig. 1a and d). In order to increase the numbers of cells on the microcarriers, we coated the bottom of the dish with a number of repellent layers (PLL-PEG, agarose, agarose-EDTA or GelriteTM). GelriteTM was found to be the most efficient coating due to its effectiveness at promoting cellular adherence on the microcarriers (Fig. 1b and e), ease of use and relatively low cost (see Supplementary Material). Finally, as already observed during microcarrier based cell growth in microgravity conditions, beads tend to aggregate and can form up to cm size aggregates that cannot be easily manipulated. Under our conditions, beads also formed aggregates as they clustered in the center of the dish. We developed a simple damping system made out of a rubber pad that can be placed under the cell culture dish to reduce the vibrations inherent to cell culture incubators. Our final set-up produces single beads covered by a cell monolayer (Fig. 1c, f and Electronic Supplementary Material, Movie S1 and Movie S2). This technical improvement allows growth of microcarriers without spinner flasks or shaking incubators, thus limiting cell trauma. We tested several epithelial cell lines for their ability to grow on microcarriers and observed that all of them could generate single epithelial beads: HepG2 (hepatocellular carcinoma), Caco-2 (colon carcinoma), A549 (lung epithelial cell line) as well as Huh7 (hepatoma cells) (Fig. S2 and Supplementary Materials).

Epithelial microcarriers display a highly developed reversed polarity

Epithelia cells in tissues are characterized by a polarized phenotype towards the free apical surface where epithelium functions occur: secretion, absorption and excretion, as well as infection from parasites or bacteria. In order to prove that static epithelial microcarriers can be used to engineer epithelia, we performed immunofluorescence and electron microscopy to investigate their polarity. Cells grown on

Fig. 1 Development of the epithelia cell culture microcarrier model. **a** The initial attempt to simply add microcarriers to a growing cell monolayer leads to reduced cell invasion and polarization as cells prefer the 2D substrate over the bead surface (**d**). **b** The use of a repellent layer (GelriteTM) forces

MDCK-II cells to grow on the bead surface but microcarrier aggregates (**e**) form due to incubator vibrations. **c** A damping system placed underneath the dish allows the formation of single epithelial microcarriers (**f**). Scale bars **d** 50 μ m, **e** 50 μ m, **f** 20 μ m

Cytodex 3 beads displayed a polarized phenotype and had a columnar morphology (Fig. 2a). They had a reversed polarity to that of *in vivo* epithelial tissue, the apical membrane of the cells facing the medium environment as opposed to forming an internalized lumen facing the bead. The Golgi apparatus as well as the centrosome was found on the apical side (Fig. 2b, c). The presence of microvilli and cell junctions on the apical side ensure the tightness of the epithelia and the control of permeability (Fig. 2c, d). We performed confocal microscopy analysis of cell polarity using the Golgi apparatus and the actin network as markers (Fig. 2e–g). As shown on a single optical slice, the Golgi apparatus was localized above the nucleus towards the apical side and just below the largest part

of the actin network that supported the microvilli (Fig. 2f, g). To confirm the functionality of the polarized epithelia, we performed a transport assay using a thermo-sensitive adenovirus GFP tagged VSVG from the vesicular stomatitis virus (Presley et al. 1997). VSVG can be followed along the secretory pathway and has been reported to traffic through the Golgi apparatus to the basolateral and lateral membranes of MDCK cells (Farr et al. 2009). Upon release, we observed the accumulation of the GFP signal within the Golgi apparatus region and the appearance of a strong signal at the basolateral region of the polarized epithelia close to the microcarrier surface (see Fig. S3 and Supplementary Materials as well as Movies S6 and 7).

Fig. 2 The epithelia cells on microcarriers are polarized. **a** As observed by electron microscopy, MDCK-II cells on the bead (bottom) are elongated along their polarization axis as previously shown (von Bonsdorff et al. 1985), with the basal membrane facing the bead surface and the apical side facing the culture medium. **b** Magnification of frame in Fig. 2a. The Golgi apparatus is asymmetrically positioned in between the nucleus and the apical membrane as well as the centrosome (c).

d Desmosomes can be observed close to the apical side and seal the epithelial monolayer. **e** Confocal microscopy, the cell monolayer is polarized as the Golgi apparatus (GM130, red) is positioned in between the cell nucleus (Hoechst, blue) and the plasma membrane (actin, green). **f** A single optical slice through the center of the microcarrier clearly displaying the polarization of the epithelia **g** Magnification of frame in Fig. 2f. Scale bars **a** 5 μm , **b** 1 μm , **c** and **d** 200 nm, **e** and **f** 20 μm , **g** 5 μm

Epithelial beads can be easily imaged

To investigate the limitations of our microcarrier based epithelia cell culture model we imaged MDCK beads using several microscopic modalities. First we imaged them using a simple widefield microscope (Fig. 3a–f). We could easily measure the cell perimeter and cell surface (Fig. 3a), as well as followed cell division (Fig. 3b–f and Electronic Supplementary Material, Movie S3), which required confocal microscopy when using porous membrane growth conditions.

Due to their transparency, the beads can be imaged on an upright as well as an inverted confocal microscope; this allows direct imaging of the beads

using water lenses, limiting diffraction effects. We imaged by confocal microscopy a single bead (Fig. 3g–i). In order to be able to fully image the epithelial beads we used LSFM as its multiview capability, rotatory stage and imaging system (CCD) is much faster compared to confocal microscopy (Huisken et al. 2004). A single bead can be imaged fully in less than 10 min using four channels, while it takes up to 3 h for a half bead stack to be acquired in three channels on a confocal microscope. Improved resolution can also be achieved through the use of the multiview mode (4 views) (Fig. 4). This necessitates a longer acquisition time (40 min) but allows for multiview image fusion and total bead imaging as well as removal of artefacts.

Fig. 3 The epithelia microcarriers are suitable for imaging. **a** MDCK-II cells on microcarriers can be imaged at high magnification ($\times 40$) on a widefield microscope (E-cadherin, green; Nucleus, red) Scale bar 5 μm **b–f** Selected frames of the time lapse analysis of cell division on an epithelial bead (**b** 0 min, **c** 20 min, **d** 40 min, **e** 1 h, **f** 4 h). Arrows indicate the tracked cell and its daughter cells. Scale bar 15 μm . **g** Projection of a confocal imaging stack of the cell surface (Concanavalin A, red) and the nucleus (Hoechst, green) of a week old half bead. A

dividing cell bulging out of the monolayer can be seen on the top right. Scale bar 20 μm . **h** Projection of a confocal imaging stack of the nucleus (Hoechst, grey) of a 2 week old half bead, the position of the nuclei illustrate that the cells are interlocking with each other, forming a tight epithelial barrier. Scale bar 20 μm . **i** Projection of a confocal imaging stack of the nucleus (Hoechst, blue), the Golgi apparatus (GM130, red) and actin network (Phalloidin, green) of a 2 week old half bead

Studying EMT using epithelial beads

EMTs have been described primarily during embryonic development as well as in pathological situations such as tumor progression. During EMT, cells down-regulate epithelial-specific proteins and induce the expression of mesenchymal proteins, losing the adherent and tight junctions that keep them in contact with their neighbors; breaking through the basal membrane and migrating over long distances owing to profound changes in their cytoskeleton architecture. We tested the possibility to investigate such a crucial process using our microcarrier system. We positioned a single epithelial bead on a glass coverslip and followed its behavior over time (Fig. 5a–d and Movie S4). The

polarized cells on the bead attached to the glass surface and started to spread. This expansion was a linear process (Fig. 5e). By confocal microscopy we could image the EMT process (Fig. 5f and Electronic Supplementary Material, Movie S5). Cells at the center were elongated and showed an increased actin staining similar to a migrating cell phenotype (Fig. 5f). In order to confirm this initial EMT observation we performed a simple EMT experiment. We assumed that cells growing on beads were mesenchymal in nature at the early growth stage and began to polarize and acquire their full epithelial potential over a 2 week culture period. We assumed that the EMT abilities of epithelial beads should decrease over time. Therefore, we repeated our EMT assay using epithelial beads of

Fig. 4 Multiple-view imaging of a Cytodex microcarrier using single plane illumination Microscopy (SPIM). MDCK-II cells that stably express an E-cadherin EGFP construct were grown on a Cytodex 3 microcarrier for 2 weeks prior to imaging with the SPIM. The nuclei were stained with Draq5 (Biostatus), the cell borders and, thus, the location of the cells' outer plasma membrane are highlighted by the E-cadherin EGFP expression. The Cytodex bead was fixed in paraformaldehyde and then

embedded in 1 % agarose in a capillary (inner diameter 1.1 mm). It was imaged using a $\times 20$ objective (Zeiss Achromplan; NA 0.5) along four angles (0° , 90° , 180° , and 270°). Each 3D stack of images consisted of 1033 images (z spacing, $0.32 \mu\text{m}$). The complete multi-view super set of images represented 4132 images (i.e., 1.1 GB). (Reprinted with permission from Reynaud et al. 2008)

different ages (3, 10, 14 and 17 days) (Fig. 5g). It was observed that EMT ability decreased as the epithelial beads aged and gained epithelial characteristics.

Studying an infection process using epithelial beads

Epithelia are semi permeable barriers. They perform selective permeability tasks, absorption and secretion, but are also the first defense barrier of our bodies against bacteria and parasites. To address whether our epithelial beads can be used for infection assays we used the apicomplexan parasite *Toxoplasma gondii* as

a model system and analyzed host cell invasion, intracellular replication and egress of polarized epithelial MDCK-II. Polarized filter grown MDCK monolayers are an established model for studying *T. gondii* transmigration (Taylor et al. 2006). To test the feasibility of using our epithelial beads, we infected polarized MDCK-II cells with *T. gondii* constitutively expressing GFP and found effective invasion and growth of the parasite (Fig. 6). Intracellular *T. gondii* actively modulated the host cell, including its cytoskeleton (Laliberte and Carruthers 2008). The ease of performing microscopic characterizations allowed us to follow the infection over time and to

Fig. 5 Epithelial beads EMT characteristics. **a–d** Selected frames of a time lapse analysis of an EMT assay using an MDCK-II epithelial bead (Deltavision, widefield, 20X, NA: 0.4, frame rate: 1/h) (**a** 0, **b** 6 h, **c** 12 h, **d** 18 h) Scale bars 150 μm . **e** The graph represents the relation between the area covered by the mesenchymal cells versus time. Area was measured with ImageJ. **f** Projection of a confocal imaging stack of the nucleus

(Hoechst, blue), the Golgi apparatus (GM130, red) and actin network (Phalloidin, green) of a bead and its EMT process extended below. This projection only used the first five slices of the stack close to the coverslip for better visualization of the EMT process. **g** Graph representing the relationship between the area covered by the mesenchymal cells and the age of the beads

analyze the behavior of host cells. We found that during infection we did not observe a significant depolarization of the host cell, indicating that the parasite was not modulating the host cell in terms of polarization or its differentiation status. We also compared invasion efficiency and growth of the parasite between 2D cell culture and epithelial beads and did not find significant differences (data not shown).

In conclusion the epithelial microcarrier system, allows analysis of the infection rate, growth and possible host cell modifications of polarized epithelial cells.

Discussion

Cytodex 3 microcarriers promote epithelial differentiation

Cytodex 3 microcarriers have a number of properties which promote the development of a well differentiated physiologically relevant epithelial organoid. (1) They

are coated with a layer of denatured type I collagen that is functionally similar to the basal lamella in epithelial tissue. Cells recognize and bind protein ligands present in the collagen network using transmembrane receptors such as integrins. The cells then build up a network of adherence proteins around these points of interaction with the substrate, which is followed by adhesion to other cells. This ensures tightly associated and well organized epithelia. (2) The collagen can sequester soluble substances such as growth factors present in the medium or released by cells and concentrate their effect locally on the tissue, as well as bind other extracellular matrix proteins produced by the attached cells (Griffith and Swartz 2006). (3) The cross linked dextran core of Cytodex 3 is porous and promotes the creation of nutrient and signaling gradients which contributes to the polarization of the epithelia. This mechanism is similar in principle to porous membrane filters. (4) A sphere (cyst) is one of the basic architectural units of in vivo epithelial tissue (O'Brien et al. 2002), culturing cells in the geometry of a sphere causes a distinct arrangement

Fig. 6 Epithelial Cytodex *Toxoplasma gondii* infection over-time. Selected 3D stacks from a time lapse analysis of an infection assay using an MDCK-II epithelial bead (SPIM, 20X, NA: 0.4, stack acquisition rate: 1/h). SPIM imaging stack projections of the nucleus (Hoechst), *T. gondii* apparatus (GFP),

nucleus (blue)/parasite (green) merge and transmission channel at 24, 72 and 96 hours are shown. Localized parasitic infection of host cells (24h) is followed by spreading of the parasite to neighboring cells (72h), and eventually leads to cell death and disruption of the epithelia (96h). Scale bars 20 μ m

of the cytoskeleton and changes the intrinsic strain experienced by the tissue. This results in a more physiologically relevant tissue organization. (5) A free floating collagen based substrate as opposed to collagen attached to a dish can stimulate the differentiation of epithelial cells (Wozniak and Keely 2005). Cells are able to apply force to and remodel the substrate, whereas attached substrates resist these forces.

We believe these characteristics support the development of an efficient and predictive 3D epithelial tissue model.

Epithelial microcarriers: a predictive and efficient 3D model

In this study we described a new technique for culturing epithelial cells on Cytodex 3 microcarriers

which allows for comprehensive imaging with few limitations and the investigation of epithelial functions. The epithelial cells grown on such microcarriers displayed a similar polarized morphology and physiological attributes as cells conventionally grown on permeable membranes. Growing polarized epithelial cells on microcarriers is a long established practice (Nilsson 1988); however, to date investigation of the polarized cells functionality has been limited to the application of biochemical assays (Ming et al. 2009) or electron microscopy (Malhi et al. 2002). In this paper we took the unique approach of investigating the cells functionality from a cell biology perspective, using several light microscopy techniques in conjunction with sub-cellular markers to characterize the cells differentiated morphology.

Cells formed a confluent polarized monolayer, developed apically located cell junctions, microvilli and cilia, in addition to having asymmetrically distributed intracellular structures (Golgi apparatus, centrosome). A major limitation of other polarized epithelial cell models (filters, cysts, spheroids and embryos) is their imaging. The porous membrane filter needs to be excised prior to imaging, leading to destruction of the permeable barrier and shearing of the cell monolayer. Cysts and embryos are usually distorted to improve imaging (coverslip pressure, mounting media) and can be difficult to mount due to their size and shape and presence of extracellular matrices. Additionally it is often impossible to apply drugs or agents to the apical membrane, as the lumen of a cyst is internal. The epithelia microcarriers overcome these problems. The system uses commercially available microcarriers (Cytodex 3) at a lower cost than porous membranes or extracellular matrices. Additionally, due to their size, the amount of antibody needed for immunofluorescence staining is lower (30 µl incubation volume). Reagents can be applied easily to the apical side of the epithelia, which we demonstrated using the parasitic organism *T. gondii*. The excreted components could also be analyzed by collection of the Cytodex well supernatant. Importantly this simple method can be used to grow various types of epithelial cells of different origins (kidney, lung, colon and liver).

Highly compatible with microscopic analysis

The epithelia beads can be easily mounted for use with most microscopes, requiring minimal manipulation other than direct transfer to a microscope grade glass coverslip. Microcarriers are also ideal for follow up experiments as no damaging or permanent mounting medium is required to image and they are resilient to shear stresses. We demonstrated this using widefield, confocal and LSMF time lapse imaging. LSMF technology (Reynaud et al. 2008; Huiskens et al. 2004) has allowed us to rapidly image the entire epithelia at once, giving us access to larger amounts of cells and opening up the possibility to investigate the behavior of a single controlled and defined epithelia.

Studying epithelial functions using microcarriers

The importance of a model system is not only its price or robustness but also its ability to answer fundamental

questions. EMT is an important stage during developmental processes (gastrulation) and pathological progression [metastasis (Acloque et al. 2009)]. So far investigations have been mainly restricted to 2D cell cultures and animal model systems (Radisky et al. 2005; Zhou et al. 2004). 2D cell culture provides a limited representation of a 3D process like EMT and animal models are expensive to maintain, can require extensive experimentation periods (e.g. mutant generation) and are difficult to image due to their size and heterogeneity. We were able to study the dynamics of the EMT process using epithelia beads, and found that cells that were allowed to differentiate for longer periods had a delayed EMT initiation (Fig. 5). This technique can be extended to studying cell architecture changes in real time (Fig. 5). In future cytodex EMT studies we plan on investigating the expression of structural proteins associated with epithelial (E-cadherin) and mesenchymal (vimentin) phenotypes. Interestingly, we observed that the migration process does not empty the bead but seems to form an “Epithelial Cell Reservoir” that continuously releases cells through an EMT process at the points of interaction between the bead and the coverslips. This characteristic is similar to a so-called “stem cell niche”, where stem cells with limited division properties generate “dividing cells” that can repopulate or differentiate in a tissue repair context (Imitola et al. 2004). Immortalized epithelial cells induced to enter EMT have been shown to express stem cell markers and possess other properties of stem cells (Mani et al. 2008).

We believe a natural extension of these studies would be to conduct EMT blocking drug trials using epithelial microcarriers. Attempts at testing EMT blockers using 3D cultures have already shown that a more physiologically relevant microenvironment results in alterations to the levels of active compound required for inhibiting EMT (Aref et al. 2013).

Epithelium is the ultimate barrier between the outside world and our body. Often, this single layer of epithelial cells must prevent the entry of bacteria, parasites and exogenous antigens while allowing absorption of essential nutrients. Additionally it must also initiate effective and appropriate immune responses when pathogens are present. Many parasite-host studies have to be performed on flat cell monolayers or on animals. There are a very limited number of models allowing full control of a complete

epithelial tissue. Microcarriers provide a bridge for parasitic studies between in vivo models and cell monolayers. The established and stable number of cells per Cytodex, developed epithelial structure and imaging compatibility made them highly suitable for parasite-host studies. The possibility to grow various types of epithelia could promote a higher standard 3D cell culture model, using specific parasites and specific target epithelia. Furthermore, the capacity to keep epithelial Cytodex in culture for over a month without trypsinization results in a more differentiated physiologically realistic model.

Conclusion

We have developed an open static microcarrier culture system that produces individual epithelial micro-tissues with a reversed polarity displaying high levels of physiological relevance. It allows for easy observation and manipulation of samples at all stages of growth, is simple and robust, cost-effective and can be easily tailored to specific needs. Microcarriers represent a versatile model to study epithelial functions and events such as EMT, secretion, absorption and infections using highly defined epithelial micro-tissues that possess a 3D physiologically relevant geometry.

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Compliance with ethical standards

This study did not involve human participants and/or animals.

Conflict of interest The authors declare no conflict of interest.

References

- Abbott A (2003) Cell culture: biology’s new dimension. *Nature* 424:870–872
- Acloque H, Adams MS, Fishwick K et al (2009) Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. *J Clin Invest* 119:1438
- Aref AR, Huang RY, Yu W et al (2013) Screening therapeutic EMT blocking agents in a three-dimensional microenvironment. *Integr Biol* 5:381–389
- Asthana A, Kisaalita WS (2012) Biophysical microenvironment and 3D culture physiological relevance. *Drug Discov Today* 18:533–540
- Carterson A, Höner zu Bentrup K, Ott CM et al (2005) A549 lung epithelial cells grown as three-dimensional aggregates: alternative tissue culture model for *Pseudomonas aeruginosa* pathogenesis. *Infect Immun* 73:1129–1140
- Cereijido M, Robbins ES, Dolan WJ et al (1978) Polarized monolayers formed by epithelial cells on a permeable and translucent support. *J Cell Biol* 77:853–880
- Farr GA, Hull M, Mellman I et al (2009) Membrane proteins follow multiple pathways to the basolateral cell surface in polarized epithelial cells. *J Cell Biol* 186:269–282
- Förster C (2008) Tight junctions and the modulation of barrier function in disease. *Histochem Cell Biol* 130:55–70
- Griffith LG, Swartz MA (2006) Capturing complex 3D tissue physiology in vitro. *Nat Rev Mol Cell Biol* 7:211–224
- Huiskens J, Swoger J, Del Bene F et al (2004) Optical sectioning deep inside live embryos by selective plane illumination microscopy. *Science* 305:1007–1009
- Imitola J, Raddassi K, Park KI et al (2004) Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1 α /CXC chemokine receptor 4 pathway. *Proc Natl Acad Sci USA* 101:18117–18122
- Kadouri A (1994) Cultivation of anchorage-dependent mammalian cells and production of various metabolites. *Colloids Surf B* 2:265–272
- Laliberte J, Carruthers VB (2008) Host cell manipulation by the human pathogen *Toxoplasma gondii*. *Cell Mol Life Sci* 65:1900–1915
- Malhi H, Irani AN, Gagandeep S et al (2002) Isolation of human progenitor liver epithelial cells with extensive replication capacity and differentiation into mature hepatocytes. *J Cell Sci* 115:2679–2688
- Mani SA, Guo W, Liao MJ et al (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 133:704–715
- Mayer A, Ivanov IE, Gravotta D et al (1996) Cell-free reconstitution of the transport of viral glycoproteins from the TGN to the basolateral plasma membrane of MDCK cells. *J Cell Sci* 109:1667–1676
- Messner S, Agarkova I, Moritz W et al (2013) Multi-cell type human liver microtissues for hepatotoxicity testing. *Arch Toxicol* 87(1):209–213
- Ming M, Li X, Fan X et al (2009) Retinal pigment epithelial cells secrete neurotrophic factors and synthesize dopamine: possible contribution to therapeutic effects of RPE cell transplantation in Parkinson’s disease. *J Transl Med* 7:53
- Misfeldt DS, Hamamoto ST, Pitelka DR (1976) Transepithelial transport in cell culture. *Proc Natl Acad Sci* 73:1212–1216
- Nauli SM, Alenghat FJ, Luo Y et al (2003) Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells. *Nat Genet* 33:129–137
- Nilsson K (1988) Microcarrier cell culture. *Biotechnol Eng Rev* 6:404–439
- O’Brien LE, Zegers MMP, Mostov KE (2002) Building epithelial architecture: insights from three-dimensional culture models. *Nat Rev Mol Cell Biol* 3:531–537
- Page H, Flood P, Reynaud EG (2012) Three-dimensional tissue cultures: current trends and beyond. *Cell Tissue Res* 352(1):123–131
- Poronnik P, Cook D, Young J (1988) The use of Cytodex microcarrier beads in patch-clamp studies on cultured epithelial cells. *Pflügers Arch* 413:90–92

- Presley JF, Cole NB, Schroer TA et al (1997) ER-to-Golgi transport visualized in living cells. *Nature* 389:81–85
- Radisky DC, Levy DD, Littlepage LE et al (2005) Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. *Nature* 436:123–127
- Reynaud EG, Krzic U, Greger K et al (2008) Light sheet-based fluorescence microscopy: more dimensions, more photons, and less photodamage. *HFSP J* 2:266–275
- Robbins JR, Barth AI, Marquis H et al (1999) *Listeria monocytogenes* exploits normal host cell processes to spread from cell to cell. *J Cell Biol* 146:1333–1350
- Sun MB, Jiang YJ, Li WD et al (2004) A novel process for production of hepatitis A virus in Vero cells grown on microcarriers in bioreactor. *World J Gastroenterol* 10:2571–2573
- Tanos B, Rodriguez-Boulan E (2008) The epithelial polarity program: machineries involved and their hijacking by cancer. *Oncogene* 27:6939–6957
- Taylor S, Barragan A, Su C et al (2006) A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen *Toxoplasma gondii*. *Science* 314:1776–1780
- van Wezel A (1967) Growth of cell-strains and primary cells on micro-carriers in homogeneous culture. *Nature* 216:64–65
- Vega-Salas DE, Salas PJ, Gundersen D et al (1987) Formation of the apical pole of epithelial (Madin–Darby canine kidney) cells: polarity of an apical protein is independent of tight junctions while segregation of a basolateral marker requires cell-cell interactions. *J Cell Biol* 104:905–916
- von Bonsdorff CH, Fuller SD, Simons K (1985) Apical and basolateral endocytosis in Madin–Darby canine kidney (MDCK) cells grown on nitrocellulose filters. *EMBO J* 4:2781
- Vörsmann H, Groeber F, Walles H et al (2013) Development of a human three-dimensional organotypic skin-melanoma spheroid model for in vitro drug testing. *Cell Death Dis* 4:e719
- Wozniak MA, Keely PJ (2005) Use of three-dimensional collagen gels to study mechanotransduction in T47D breast epithelial cells. *Biol Proced Online* 7:144–161
- Zhou BP, Deng J, Xia W et al (2004) Dual regulation of Snail by GSK-3 beta-mediated phosphorylation in control of epithelial-mesenchymal transition. *Nat Cell Biol* 6:931–940