Stem Cell Research 28 (2018) 165–171

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Optimized procedures for generating an enhanced, near physiological 2D culture system from porcine intestinal organoids

B. van der Hee [a,b], L.M.P. Loonen [a], N. Taverne [a], J.J. Taverne-Thiele [a], H. Smidt [b], J.M. Wells [a,*]

[a] Host-Microbe Interactomics, Animal Sciences Group, Wageningen University, De Elst 1, Wageningen, The Netherlands
[b] Molecular Ecology, Laboratory of Microbiology, Wageningen University, Stippeneng 4, Wageningen, The Netherlands

ARTICLE INFO

Article history:
Received 24 November 2017
Received in revised form 7 February 2018
Accepted 16 February 2018
Available online 20 February 2018

Keywords:
Porcine
Organoids
Monolayer
Intestinal
Epithelium

ABSTRACT

An important practical limitation of the three-dimensional geometry of stem-cell derived intestinal organoids is that it prevents easy access to the apical epithelium for testing food components, microorganisms, bioactive and toxic compounds. To this end, we here report on a new robust method for generating confluent intestinal cell monolayers from single-cell suspensions of enzymatically-dissociated porcine organoids using modified culture conditions. With this method, cell seeding densities can be standardised, overcoming problems with methods based on mechanical dissociation of organoids. Confluent monolayers formed tight junctions with high transepithelial electrical resistance in three days and could be used in experiments for up to two weeks. Multilineage differentiation of ileal stem cells was demonstrated by immunohistochemistry and RT-qPCR of cell-specific transcripts, also unequivocally confirming the controversial existence of Paneth-like cells in the porcine small intestine. The method described here is useful to standardize primary epithelial monolayer formation from intestinal organoids and allows rapid and robust studies of intestinal physiology.

1. Introduction

Adult stem-cell derived organoids have already proven to be powerful experimental models of mammalian biology, advancing our understanding of the cellular mechanisms that drive epithelial tissue development and the role of microenvironmental factors in normal development and disease. Additionally, organoid technology enables disease phenotyping and the testing of therapeutic approaches on patient-derived cells. Adult stem cell-derived intestinal organoids are capable of self-renewal and differentiation into intestine-like structures in a 3D matrix (Sato et al., 2009; Ootani et al., 2009), which is crucial for obtaining a spatial organisation of heterogeneous tissue-specific cells resembling real-life cell lineage composition and functionality.

One practical limitation of the 3D geometry of intestinal organoids is that it prevents access to the apical epithelium for treatments with food components, microorganisms, bioactive and (toxic) compounds, unless they are small enough to pass through tight junctions or transported into cells. Although for some applications, physiological responses can be quantified in individual organoids by microscopy after microinjection of substances into the lumen (Wilson et al., 2015), this can be challenging due to heterogeneity in organoid size and problems of synchronous exposure and variability in injected volumes. Furthermore, the lumen of 3D organoids accumulates debris from cell turnover which may bind or sterically hinder interaction of injected substances with the apical membranes. To overcome these problems, methods have been reported for generating short-term cultures of polarised cell monolayers derived from mechanically dissociated organoid cultures (Moon et al., 2014; VanDussen et al., 2015; Wang et al., 2017). However, in our hands this method often leads to slower growth, increased variability between wells, and appearance of 3D organoid structures on the surface of the cell monolayer.

Cultures of polarised intestinal cell monolayers are important not only for ease of access to the apical surface but also because transepithelial permeability and responses to food and microorganisms can be measured in a more standardised way (i.e. using similar numbers of cells and trans-epithelial resistance measurements). To exploit the benefits of two-dimensional cultures, the aim of this study was to develop a robust and reproducible method for generating a confluent monolayer of porcine intestinal organoids based on enzymatic dissociation into single-cells, rather than mechanical disruption and fragment seeding. Our hypothesis was that such a method would enable the seeding density and time of culture to be standardised, thereby increasing efficiency and reducing experimental variation. Indeed, our method rapidly generates organoid-derived cell monolayers within three days after seeding, for pharmacological or transport studies. Additionally, the growth of organoid cells on a planar surface facilitated whole cell cytochemistry due to increased cellular resolution. As an example of the application of this new method we monitored cellular development

* Corresponding author.
E-mail address: jerry.wells@wur.nl (J.M. Wells).

https://doi.org/10.1016/j.scr.2018.02.013

and re-examined the controversial existence of Paneth-like cells in the porcine small intestine (Dekaney et al., 1997; Myer, 1982).

2. Materials and methods

2.1. Ileum organoid culture

Ileum organoids were generated from intestinal tissue of two 5-month-old slaughter pigs, according to the procedure described by Sato and colleagues (Sato et al., 2011a) with minor modifications (see Supplementary methods SM1.1). Porcine ileal organoids were grown in basal culture medium that was refreshed every two days (BCM: DMEM/F12 (Gibco), supplemented with 100 μg/ml primocin (Invivogen), 10 mM HEPES (HyClone), 1 × B-27 (Gibco), 1.25 mM *N*-acetylcysteine (Sigma), 50 ng/ml human epidermal growth factor (R&D systems), 15 nM gastrin, 10 mM nicotinamide, 10 μM p38 MAPK inhibitor (Sigma), 600 nM TGFβ receptor inhibitor A83-01, and conditioned media for recombinant Noggin (15% v/v), Spondin (15% v/v), and Wnt3A (30% v/v) provided by Dr Kuo and the Hubrecht Institute). Organoids were passaged at a 1:5 ratio every 5 days by mechanical dissociation and plating in fresh Matrigel matrix droplets (Basement Membrane, Growth factor reduced, REF 356231, Corning, Bedford, MA, USA).

2.2. Two-dimensional monolayers

Spherical ileum organoids were recovered from Matrigel after 5 days growth by addition of ice-cold DMEM/F12 medium, transferred into 15 ml tubes followed by centrifugation at 250 ×*g* for 5 min. The pellet of organoids was incubated in TrypLE Express dissociation medium (Gibco) for 10 min at 37 °C and dissociated by repeated pipetting to obtain a single cell suspension (see supplementary methods SM1.2 for images). Four volumes of BCM, enhanced with 20% (v/v) FBS (E-BCM) was added to the single cell suspension and centrifuged at 900 ×*g* for 5 min. Cell pellets were resuspended in E-BCM, counted manually using a Bürker chamber and seeded at 78,125 cells/cm^2 in pre-coated culture plates or Transwells. The pre-coating procedure involved incubation with 0.5% (v/v) Matrigel in F12 medium at 37 °C for 1 h after which the liquid was removed, and the plates were air-dried for 10 min. After 3 days incubation at 37 °C (5% CO_2) the cell monolayers reached confluence and were used for experiments.

2.3. Transepithelial electrical resistance (TER)

Spheroids at 5 days after passaging were recovered from Matrigel, and dissociated into single cells as described in Section 2.2. Transwell inserts were coated in the apical chamber as described in Section 2.2. Single cells were seeded at a density of 2.5 × 10^4 cells/well in 24-well transparent transwell inserts (0.33 cm^2, Falcon, BD), and incubated with 400 μl apical, and 800 μl basolateral E-BCM with or without 10 μm ROCK inhibitor (Y27632, Tocris Biotechne, United Kingdom).

For transepithelial electrical resistance (TER) measurements, six transwell inserts per group were placed in a Cellzscope apparatus (Nanoanalytics, Münster, Germany) 2 h after seeding, containing 500 μl basolateral BCM. ROCK inhibitor was removed after 24 h post-seeding, and medium was refreshed in all wells using E-BCM. TER of the forming monolayers was measured over 72 h post-seeding as previously described (Karczewski et al., 2010).

2.4. Permeability measurements of the cell monolayer

To follow epithelial permeability to small molecules, stationary TER monolayers were apically treated with 0.5 mg/ml 0.4 kDa FITC, 4 kDa or 40 kDa FITC-Dextran (Thermo-Fisher). Controls contained transwells without a monolayer. At 5, 10, 30, 60, 120 and 240 min, 50 μl of basolateral medium was measured on FITC-dextran contents using fluorescence on a Spectramax M5 (Molecular Devices, Sunnyvale, CA, USA) with 490 nm excitation and 530 nm emission.

2.5. Monolayer formation using ROCK inhibitor

To visualize the impact of seeding density and ROCK inhibitor on monolayer formation, cells were treated with or without ROCK inhibitor for 24 h (Moon et al., 2014), two-fold dilution series from 10 × 10^4 to 1.25 × 10^4 cells/well, and subsequently grown for 72 h. After 72 h, cell monolayers were fixed in 1% paraformaldehyde (PFA), and nuclei were stained (Hoechst) for 20 min. Images of cell monolayers were acquired using a high-content, high-throughput microscope (BD Pathway HT 855, BD Biosciences). Images were stitched in 4 × 5 image grids using Attovision software, and slightly adjusted for brightness and contrast to show nuclei in ImageJ. For growth videos, serial dilutions of cell suspensions were plated in pre-coated 96-well culture plates (Corning), placed in an oCelloscope (BioSense Solutions, Denmark) 2 h after seeding, and migration and proliferation was imaged every hour for 70 h.

2.6. Cell lineage identification of porcine tissue and 2D-monolayers

Confluent cell monolayers or ileal tissue were fixed with 1% PFA, Carnoy's or 4% PFA, respectively, for 1 h at room temperature. Tissues and monolayers were prepared for incubation with antibodies to identify different cell types present (see Supplementary methods SM2 for detailed methods and antibody list).

2.7. Transmission electron microscopy and RT-qPCR

A detailed description is provided in supplementary methods SM3 and 4.

3. Results

3.1. Development of a method for growing 2D organoid cultures

Growth of 3D porcine intestinal organoids has been previously described (Gonzalez et al., 2013), although a follow up study by the same authors highlighted the fact they were not long-lived and limited to about ten passages (Khalil et al., 2016). In our study, the conditions described resulted in formation of spheroids within 3–5 days and appearance of mature cryptile morphology by day 7 to 10 without restrictions of the Hayflick limit or replicative potential (Fig. 1A). In the medium of Khalil et al., stem cells may not be adequately maintained due to to absence or limiting amounts of growth factors, while our medium allowed 3D organoid cultures to be passaged for at least several months (Fig. 1A). Using our 3D culture method, stem/progenitor cells were observed at the bottom of crypts (by immunofluorescent histology), where they are normally maintained by asymmetric division. This is unaffected by long-term passage of organoids, indicating that stem cells are not lost in our 3D culture system.

Confluent monolayers were generated by disruption of 5-day old spheroids into a single cell suspension that was plated on Matrigel coated Transwells or tissue culture plates in E-BCM (Fig. 1B–C; Supplementary video S1). Although previous studies on mouse and human 2D organoid culture employed gelatine or collagen-based coatings we found Matrigel to be suitable (Wang et al., 2017; Scott et al., 2016; Moon et al., 2014; VanDussen et al., 2015). We observed that the seeding density in 96-well plates (0.33 cm^2/well) affected the capacity to generate monolayers, with an optimal density of 1.5 to 2.5 × 10^4 cells per well considering growth kinetics and efficient use of cells (Fig. 1D). At lower and higher cell densities, confluent monolayers were not obtained at three days. Although confluency was reached with higher seeding densities within 24–30 h post-seeding, this was lost after 50 h (Supplementary videos S2-S3). ROCK inhibitor was