502. Organoids as a research tool in animal breeding and nutrition

L.M.G. Verschuren[1*], D. Schokker[2], S. Verstringe[3], L. Kruijt[2], A.A.C. de Wit[2], E.G.T. van der Valk[2], S.K. Kar[2] and E.D. Ellen[2]

[1]Topigs Norsvin Research Center B.V., Schoenaker 6, 6641 SZ, Beuningen, the Netherlands; [2]Wageningen University & Research, P.O. Box 338, 6700 AH Wageningen, the Netherlands; [3]Nutrition Sciences N.V., Booiebos 5, 9031, Drongen, Belgium; lisanne.verschuren@topigsnorsvin.com

Abstract

In vitro miniaturized and simplified three-dimensional versions of an organ, commonly known as organoids, are an important innovative key technology in human and, more recently, animal research. So far, the *in vitro* organoid system has been applied on a small number of animals in a research setting. However, to apply the *in vitro* organoid system on a larger scale for industrial purposes, a high throughput system needs to be developed. The major aim of this research is to pave the way for a high throughput *in vitro* organoid system of the pig intestinal tract to be used in animal breeding, nutrition, and health. We developed high throughput tools to generate organoids from pig intestines on a large scale. We also looked at different applications addressing intestinal functionality, which allow to accurately phenotype animals for complex traits. Organoid research enables animal breeding companies to switch more quickly towards less animal testing.

Introduction

Developments in cell biology show that it is possible to grow and maintain three-dimensional (3D) organ-like structures in culture for a virtually indefinite time. These structures, called organoids, contain multiple cell types, resemble the *in vivo* tissue, and recapitulate key function(s) of the organ. It has been demonstrated that organoids can be dissociated and seeded onto Matrigel- or collagen-coated Transwell membranes to form two-dimensional (2D) epithelial monolayers (Foulke-Abel *et al.*, 2014; Van der Hee *et al.*, 2020). These 2D cultures allow the study of interactions with apically applied exogenous stimuli, such as pathogens or compounds, i.e. feed ingredients (Kar *et al.*, 2020), nutrients, drugs, and chemicals. In previous studies, the transport and reaction on different agents has been investigated using 2D monolayers (Van der Hee *et al.*, 2020). Hence, the 2D organoid structure could potentially be used to test differences in responses to various exogenous stimuli between organoids derived from different animals. Organoids can then be used as a phenotyping tool in animal breeding. So far, porcine intestinal organoids have been used to study host-pathogen interaction in relation to feed efficiency on a small number of pigs (Ellen *et al.*, 2018). However, to apply the *in vitro* organoid system on a larger scale for industrial purposes, like phenotyping of animals, a high throughput system needs to be developed. The major aim of this research was to pave the way for high throughput *in vitro* organoid systems of the pig intestinal tract to use as a key technology in animal breeding, nutrition, and health. To develop an *in vitro* high throughput organoid system, several steps within the organoids system need to be optimized: (1) collection of tissue samples; (2) production of organoids; (3) storing of the tissue and organoids; and (4) testing. Furthermore, the resemblance between the *in vitro* system and the *in vivo* system needs to be validated.

Materials & methods

Collection of tissue samples. Tissue samples have been collected from the small intestine (jejunum and ileum) and large intestine (colon). These locations have been selected because the jejunum and ileum are important for nutritional research, whereas the colon could be beneficial in future research to collect tissue samples without sacrificing the animals. Tissue samples have been collected from the slaughterhouse. So far, tissue samples have been collected from 26 three-way crossbred (Synthetic boar × (Landrace × Large

R.F. Veerkamp and Y. de Haas (eds)

DOI: 10.3920/978-90-8686-940-4_502, © L.M.G. Verschuren *et al.* 2022

White)) pigs raised at research farm 'Laverdonk' of Agrifirm Innovation Center (Heeswijk-Dinther, the Netherlands).

Establishing a cryo-stock for pig organoids. Adult stem cells (ASCs) from the collected tissue have been used to derive 3D organoids. In our study, intestinal crypts have been used. Under appropriate conditions using different growth factors and Matrigel, ASCs can grow, and their daughter cells can differentiate in multiple cell types, resulting in 3D structures (Kar *et al.*, 2021). We can produce large units of 'mother' organoids from a large number of animals to use as a phenotyping tool in animal breeding. We can also produce a large number of 'daughter' organoids to perform multiple tests on tissue from the same animal, for example for multiple phenotypes per pig or as a screening tool in animal nutrition. To validate the resemblance between the organoids and the respective tissue, gene expression measurements using RNA-Sequencing technology has been used as a read-out parameter. To trim low-quality data, we used Trim Galore version 0.6.6 (Krueger, 2018) and Cutadapt version 1.16 (Martin, 2016) with default settings except for length 35 (default 20) and stringency 6 (default 1). This resulted in paired-end reads if both reads were ≥35 bp. These trimmed reads were aligned against the pig reference genome (Ensembl *Sus scrofa* 11.1.103, (Howe *et al.*, 2021) using STAR version 2.7.3a with settings at default (Dobin *et al.*, 2013). Gene expression was quantified using RSEM version 1.3.0 (Li and Dewey, 2011) with default settings, except for the strand specific protocol, which was set to 0 to derive all upstream reads from the reverse strand. RSEM expected counts and Transcripts per Million (TPM) values were quantified. Principal component analyses have been performed to compare the tissue and organoids.

Optimization of the 2D monolayer Transwell system. Single cells, obtained after dissociation of 3D intestinal 'daughter' organoids, were seeded on Transwells to obtain 2D monolayers for further testing. We determined the optimal conditions to obtain stable and reproducible trans-epithelial electrical resistance (TER) values in 2D organoids, and to confirm the integrity and permeability of the monolayer. For this, the cellZscope (NanoAnalytics GmbH, Münster Germany) was used to measure the TER every half an hour to investigate the optimal Matrigel concentration, cell density, and timestamp.

Results

Gene expression from organoids act as a proxy for the respective tissue. We successfully produced organoids from different pigs and different locations. By measuring gene expression of both the tissue of interest and its derived organoids, we established if a relationship exists. Here, we targeted the colon as an example. When focusing on the moderate and highly expressed genes, i.e. transcripts per million above 100, we observed an overlap of 1,539 genes (75%) between tissue and organoids, 242 genes (12%) unique for tissue, and 276 genes (13%) unique for organoids. This shows that many genes are expressed in both the *in vivo* and *in vitro* situation. To assess the functionality of these genes we subsequently performed a Gene Ontology (GO) enrichment analysis for the genes in 'unique tissue', 'overlap', and 'unique organoids'. As we were interested in the biological processes that could be affected, we solely focused on the GO biological processes. Furthermore, we focused on the top 10 biological processes based on the false discovery rate (FDR<0.01, Table 1).

2D Transwell system. Figure 1 shows preliminary results of the TER measurements. So far, the optimal Matrigel concentration we observed is 1% with a cell density of 10^5 cells. This resulted in a window-of-opportunity to perform functional studies between approximately 80-90 hours after seeding, because the TER values were above 1000 Ωcm^2.

Table 1. Top 10 significantly enriched[1] Gene Ontology biological processes.

Ranking	'Unique tissue'	'Overlap'	'Unique organoid'
1	Immune response	Organonitrogen compound biosynthetic process	Small molecule metabolic process
2	Complement activation	Cellular amide metabolic process	Localization
3	Humoral immune response	Organonitrogen compound metabolic process	Macromolecule localization
4	RNA splicing	Peptide metabolic process	Organic substance transport
5	Iron ion transmembrane transport	Amide biosynthetic process	Actin filament organization
6	Immune system process	Translation	Cellular localization
7	RNA splicing, via transesterification reactions	Peptide biosynthetic process	Oxidation-reduction process
8	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	Generation of precursor metabolites and energy	Protein localization
9	MRNA splicing, via spliceosome	Oxidation-reduction process	Actin filament depolymerization
10	Tricarboxylic acid cycle	Protein metabolic process	Protein transport

[1] False discovery rate <0.01.

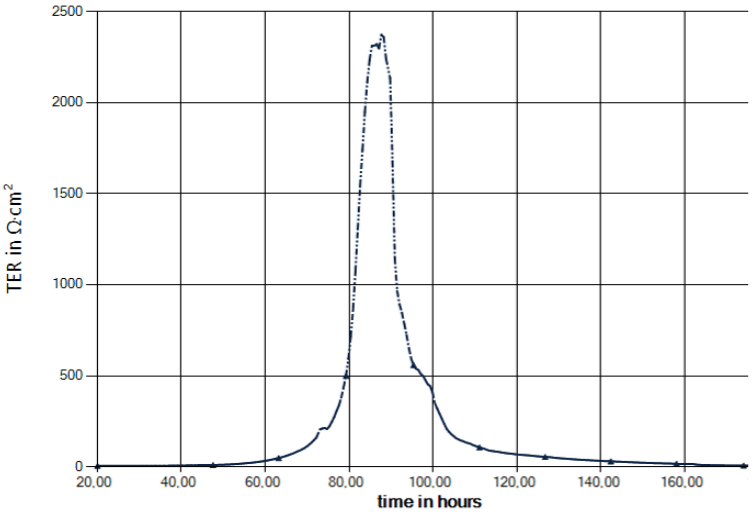

Figure 1. Trans-epithelial electrical resistance (TER) measurements with 1% Matrigel and 10^5 cells.

Discussion

Organoids have been identified as a key *in vitro* tool to reduce animal testing and to define new phenotypes for animal breeding. For pigs, especially for the intestinal tract, this *in vitro* system has been optimized. Results from the gene expression study of the colon indicate that organoids resemble the *in vivo* tissue to a large extend, which is in line with results for jejunum organoids (Van der Hee *et al.*, 2020). The main difference between tissue and organoids is the lack of immune cells in organoids, which our study showed by the genes from 'Unique tissue', indicating multiple processes involved in immunity. The GO terms of 'Unique organoid' genes showed more processes such as localization and actin organization, which was expected because the organoids grew in an artificial environment, i.e. Matrigel. The genes from 'Overlap' were by far the greatest number, i.e. 1,539 genes among the comparison. These 'Overlap' genes were more involved in generic biological processes, i.e. metabolic processes. As we aim to use the organoids as phenotyping and screening tool, similarity in metabolic processes between organoids and tissue is highly

important. Hence, the organoids are likely suitable for comparing responses of animals to exogenous stimuli on the organoids when this is related to metabolic processes, but not when the aim is to test the immune response.

Preliminary results of the 2D system show that a Matrigel concentration of 1% with 10^5 cells resulted in the most optimal TER values. For transport studies, TER values of >750 Ωcm^2 results in low variability between replicates (Van der Hee *et al.*, 2020). Future work will focus on extending the window-of-opportunity when the TER values are above a certain threshold, e.g. 1000 Ωcm^2. The latter will benefit the application for investigating differences between organoids derived from individual animals.

Currently, a high-throughput *in vitro* screening system is under development that will enable breeding and nutrition companies to test and phenotype pig intestinal organoids on a larger scale. Furthermore, simultaneously a biobank will be developed, including (intestinal) organoids and tissue from several pigs, for future new research questions.

References

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, *et al.* (2013) Bioinformatics 29(1): 15-21. https://doi.org/10.1093/bioinformatics/bts635

Ellen, E. D., N. Taverne, J. J. Taverne-Thiele, O. Madsen, H. Woelders, *et al.* (2018) Proc. of the 69th EAAP, Dubrovnik, Croatia.

Foulke-Abel, J., J. In, O. Kovbasnjuk, N. C. Zachos, K. Ettayebi, S. E. Blutt, *et al.* (2014) Exp Biol Med (Maywood) 239: 1124-1134. https://doi.org/10.1177/1535370214529398

Howe, K. L., Achuthan, P., Allen, J., Allen, J., Alvarez-Jarreta, *et al.* (2021). Nucleic acids res 49(D1): D884-D891. https://doi.org/10.1093/nar/gkaa942

Kar, S. K., B. van der Hee, L. M. P. Loonen, N. Taverne, J. J. Taverne-Thiele, *et al.* (2020) J. Anim. Sci 11: 51. https://doi.org/10.1186/s40104-020-00443-4

Kar, S. K., J.M. Wells, E.D. Ellen, M.F.W. te Pas, O. Madsen, *et al.* (2021) Vet. Res. 52: 43. https://doi.org/10.1186/s13567-021-00904-2

Krueger, F. Trim Galore! Available at: http://www.bioinformatics.babraham.ac.uk/projects/trim_galore

Li, B. and Dewey, C. N. (2011) BMC bioinform 12(1): 1-16. https://doi.org/10.1186/1471-2105-12-323

Martin, M. (2011). EMBnet. journal 17: 10-12. https://doi.org/10.14806/ej.17.1.200

Van der Hee, B., O. Madsen, J. Vervoort, H. Smidt, and J. M. Wells. (2020) Front. Cell Dev. Biol. 8. https://doi.org/10.3389/fcell.2020.00375