DEVELOPMENT OF A RABBIT CAECUM ORGANOID MODEL: AN INNOVATIVE *IN VITRO* TOOL TO STUDY ABSORPTIVE AND BARRIER FUNCTIONS OF EPITHELIAL CELLS

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ABSTRACT

The intestinal epithelium plays a key role in digestion, nutrients absorption and in the gut barrier function. However, an *in vitro* model of rabbit epithelial cell is not available to study these functions. In this context, we tested three methods to grow organoids from epithelial crypts (containing stem cells) isolated from rabbit caecum (n=4). Key epithelial signaling pathways (Wnt and BMP) were modulated either by (i) pharmacological inhibitors (2Ki medium) or with mouse recombinant growth factors used at (ii) 50 % (WRN 50%) or (iii) 5% (WRN 5%). In the three growth conditions, organoids were formed by a monolayer of epithelial cells with the apical side enclosed towards the lumen. Organoids grown in 2Ki condition had a large diameter and a spherical morphology while organoids cultured in WRN 50% and WRN 5% conditions were smaller (-20.4% and -25.6% vs 2Ki, respectively, $P<0.05$) and some of them were non-spherical (11.2 and 12.7%, respectively), these features being suggestive of a higher differentiation level in WRN conditions. Indeed, organoids cultured in WRN 5% expressed significantly higher levels of genes markers of absorptive and secretory epithelial cells when compared to the 2Ki condition ($P<0.05$). This higher differentiation level was associated with an upregulation of antimicrobial peptides expression ($P<0.05$), an important component of the epithelial barrier function. In summary, we report for the first time a method to grow rabbit caecum organoids with a high epithelial differentiation level. This innovative *in vitro* model is a valuable tool to study the effects of nutrients or microorganisms on rabbit intestinal epithelium.

Key words: Organoid, caecum, epithelial barrier, stem cells, differentiation.

INTRODUCTION

Located at the surface of the intestinal mucosa, the epithelium is formed by a monolayer of cells constantly renewed from a pool of dividing stem cells located at the bottom of the crypts (Peterson and Artis, 2014; Gehart and Clevers, 2019). During their migration towards the intestinal lumen, epithelial cells differentiate into absorptive or secretory cells (e.g. mucus secreting cells or enteroendocrine cells). The intestinal epithelium plays a key role in digestion and nutrients absorption. Moreover, epithelial cells form a physical and immunological barrier against harmful luminal content (e.g. microorganisms, toxins and food antigens). Overall, homeostasis of the intestinal epithelium is a major determinant of nutrition and gut health.

In rabbits, intestinal epithelial cells can be studied *in vivo* but it requires animal killing, which raises ethical and cost issues. Moreover, no *in vitro* models (e.g. intestinal epithelial cells lines) are available in rabbits. In this context, the objective of our work was to develop a model of rabbit caecum organoids. We focused on the caecum since this digestive organ is relevant to study both nutritional (e.g. short chain fatty acids absorption) and barrier function (e.g. cross talk with the microbiota) in rabbits. Intestinal organoids are obtained by culture of epithelial crypts in a gel of extracellular matrix proteins with growth factors replicating the stem cell niche (high Wnt and low BMP pathways activation) (Hill and Spence, 2017; Almeqdadi et al., 2019). The main advantages of organoids are that they are derived from the species of interest, are constituted of all epithelial cells types (stem, absorptive and secretory cells) and are organized in 3 dimensions. Moreover, organoids can be multiplied and cryopreserved, which allows the test of a large number of experimental conditions with a very limited number of animals. Here, we report the development of a method to cultivate rabbit caecum organoids by using pharmacological inhibitors or mouse recombinant proteins.

MATERIALS AND METHODS

Animals and experimental design

Animal experiments were approved by the local ethical committee (SSA_2018_010). Caecal tissues were collected from 4 male 30 day old rabbits (line INRA 1777). Caecum epithelial crypts were isolated from the mucosa by incubation in a dissociation solution (3 mM DTT, 9 mM ETDA) for 30 min. After centrifugation, caecal crytps were counted and resuspended in matrigel (a gel of extracellular matrix proteins) before seeding in 48-wel plates (150 crypts/25 µL of matrigel/well). Organoids were cultured for 7 days in 3 different culture conditions (as indicated in table 1) based on methods published previously for other species (Miyoshi and Stappenbeck, 2013; Powell and Behnke, 2017; Li et al., 2018).

Table 1: Composition of the three growth media (2Ki, WRN 50% or WRN 5%)

	Function	2Ki	WRN 50%	WRN 5%
DMEM	Nutrients	x	x	x
Fetal bovine serum (10% v/v)	Growth factors	x	x	x
Penicillin/Streptomycin (1% v/v)	Antibiotics	x	x	x
HEPES (10 µM)	Buffer	x		
N-acetyl cysteine (500 mM)	Antioxidant	x		
LDN193189 (10 µM)	BMP inhibition	x		
SB431542 (10 µM)	BMP inhibition	x	x	x
CHIR99021 (10 µM)	Wnt activation	x		
Y27632 (10 µM)	Stem cells survival	x	x	x
L-WRN cells conditioned media[1]	Wnt activation/BMP inhibition		50% days 1-7	50% days 1-5 / 5% days 6-7

[1]Culture medium of L-WRN cells: mouse fibroblasts secreting three mouse recombinant growth factors (Wnt3a, R-spondin, Noggin).

Gene expression analysis

RNA was extracted with the kit Direct-zol RNA MiniPrep Plus (Zymo research) from a pool of 6 wells of organoids per growth condition. cDNA was prepared by retrotranscription from 1 µg RNA with the kit GoScript Reverse Transcription Mix, Random primer (Promega). Rabbit specific primers were used to quantify gene expression by Biomark microfluidic system using a 48.48 Dynamic Array IFC (Fluidigm). Expression level of the housekeeping gene *Atp5b* were used as a reference.

Confocal microscopy

Organoids were cultured in a Nunc Lab-Tek Chamber Slide system (Thermo Fisher Scientific) and fixed with 4% paraformaldehyde during 20 min. After permeabilisation with 0.5% triton X-100 during 20 min, actin was stained by incubation for 30 min with 10 µM phalloidin coupled with TRITC fluorochrome. Nuclei were stained by DAPI contained in the mounting medium. Organoids were observed with a confocal microscope (Leica TCS SP8).

Statistical analysis

Statistical analyses were performed using the R software with the packages lme4 and emmeans. A mixed model was used to analyze the effect of culture condition (fixed effect). Rabbit was used as a random effect, since the three growth conditions were tested on organoid lines prepared from each animal (n=4). Means of each group were compared pairwise with Tukey correction.

RESULTS AND DISCUSSION

We tested 3 different conditions to culture organoids from caecal crypts obtained from 4 rabbits (figure 1A). The stem cell niche (high Wnt/low BMP signaling pathways activation) was reproduced *in vitro* with pharmacological inhibitors (2Ki medium) or with mouse recombinant growth factors (Wnt3a, R-spondin and Noggin) at 50 % or 5 % as indicated in figure 1A. In the 3 culture conditions, organoids were constituted of a single layer of epithelial cell with actin enclosed within the organoids, indicating that epithelial cells were polarized towards the organoid lumen (figure 1B) (Klunder et al., 2017). The highest

number of organoids was obtained in the WRN 50 % condition (+35% compared to the 2Ki condition, p<0.01). The diameter of organoids was higher in the 2Ki condition when compared to WRN 50% and WRN 5% conditions (360.4 vs 287 and 268 µM, respectively, P<0.001). Only spherical organoids were obtained in 2Ki condition while non-spherical organoids were also observed in WRN 50% and WRN 5% conditions (11.2 and 12.7%, respectively) as shown in figure 1B. Since large spherical organoids are associated with a low differentiation level and a high proliferation level (Merker et al., 2016), our results suggested that rabbits organoids grown with pharmacological inhibitors (2Ki) were more proliferative and less differentiated than organoids cultured with mouse recombinant proteins (WRN conditions).

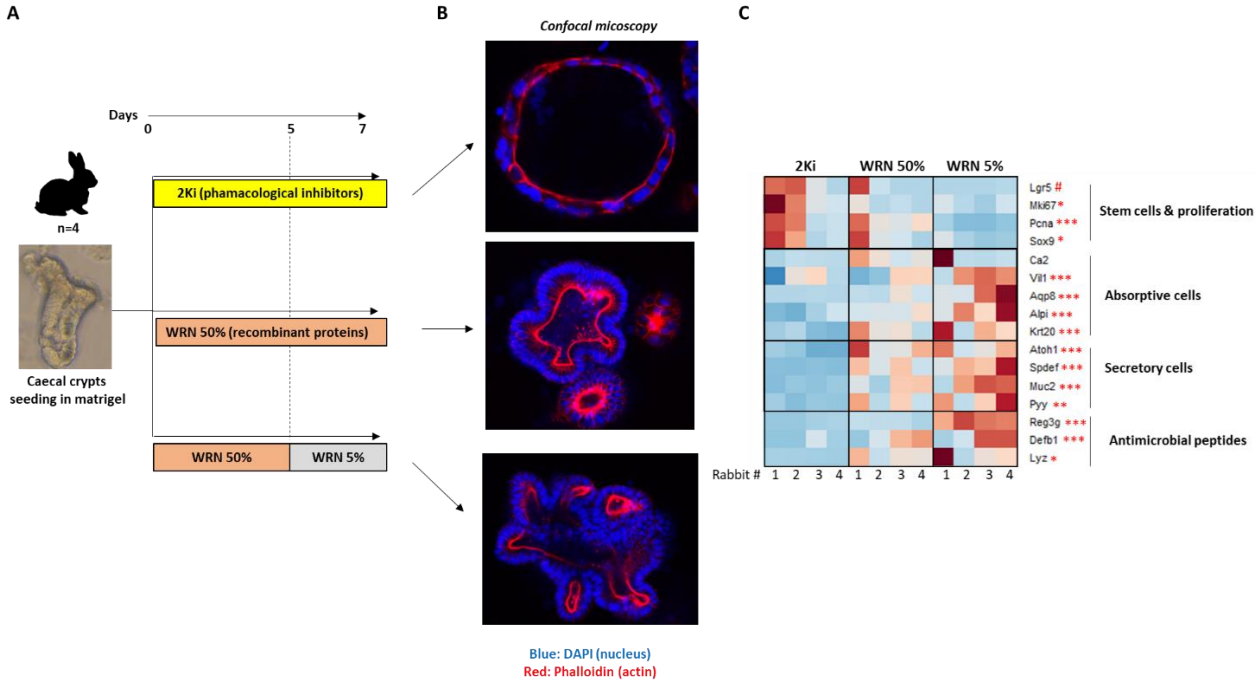

Figure 1: A- Experimental design of rabbit caecum organoid culture. B- Confocal microscopy observation of organoid after staining actin (red) and nuclei (blue). C- Heatmap representing relative expression of genes (rows) in organoids obtained from 4 rabbits and cultured in 3 conditions (columns). The color represent the Z-scores (row-scaled relative expression) from low (blue) to high (red) levels. ***: P<0.001, **: P<0.01, *:P<0.05, #: P<0.1. Pairwise comparisons are indicated in text.

To confirm these results, we analyzed the expression of genes characteristics of the different epithelial cells subtypes (figure 1C). Despite high variability between organoid lines (i.e. obtained from each animal), we observed a general trend for higher expression levels of stem cells and proliferation markers in organoids grown in the 2Ki condition compared to the WRN 5% condition. The only significant pairwise comparison was found for *Pcna* (P<0.05: WRN 5% vs 2Ki). In contrast, the highest gene expression levels of absorptive cells markers were observed in organoids grown in the WRN 5 % condition (P<0.05 WRN 5% vs 2Ki for *Vil1, Aqp8, Alpi, Krt20*). The highest gene expression level of markers of secretory cells were also observed in organoids cultured with the WRN 5% condition (P<0.05 WRN 5% vs 2Ki for *Atoh1, Spdef, Muc2, Pyy*). Altogether, gene expression profiles indicated that the highest differentiation of organoid epithelial cells were obtained with the WRN 5% condition. Moreover, organoids cultured in WRN 5% condition had the highest gene expression level of the antimicrobial peptides *Reg3g* and *Defb1* (P<0.05 WRN 5% vs 2Ki). These results suggested that a high differentiation level of organoids is associated with an increased capacity to secrete antimicrobial peptides, a key component of the epithelial barrier.

The high level of epithelial differentiation in organoids culture in the WRN 5% condition is probably related to a low activation of the Wnt signaling pathway and a high activation of BMP signaling pathway when mouse growth factors Wnt3a, R-spondin and Noggin are used at low concentrations (Gehart and Clevers, 2019). A major limitation of our organoid model is that the apical side of epithelial cells is enclosed within the organoid, which is not convenient when studying the effects of nutrients or microorganisms that reach the epithelium through the luminal side. The major perspective of our work

would be to grow organoids in 2 dimensions to have access to the apical side of epithelial cells or to reverse epithelial polarity by extracellular matrix removal, as recently shown in mouse organoids (Co et al., 2019).

CONCLUSIONS

In this work, we developed for the first time an efficient method to grow rabbit caecum organoids *in vitro*. Our results show that the utilization of mouse recombinant growth factors at low concentration allows a high differentiation level of organoids. This characteristic make them suitable to study key function of the rabbit epithelium such as nutrients absorption or its barrier role.

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