



Growth factors of stem cell niche extend the life-span of precision-cut intestinal slices in culture: A proof-of-concept study

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ABSTRACT

Precision-cut intestinal slices (PCIS) is an *ex vivo* culture technique that found its applications in toxicology, drug transport and drug metabolism testing, as well as in fibrosis research. The main limiting factor of PCIS as experimental model is the relatively short viability of tissue slices. Here, we describe a strategy for extending the life-span of PCIS during culture using medium that is routinely used for growing intestinal organoids. Mouse and rat PCIS cultured in standard medium progressively showed low ATP/protein content and severe tissue degradation, indicating loss of tissue viability. In turn, organoid medium, containing epithelial growth factor (EGF), Noggin and R-spondin, maintained significantly higher ATP/protein levels and better preserved intestinal architecture of mouse PCIS at 96 h. In contrast, organoid medium that additionally contained Wnt, had a clear positive effect on the ATP content of rat PCIS during 24 h of culture, but not on slice histomorphology. Our proof-of-concept study provides early evidence that employing organoid medium for PCIS culture improved tissue viability during extended incubation. Enabling lasting PCIS cultures will greatly widen their range of applications in predicting long-term intestinal toxicity of xenobiotics and elucidating their mechanism of action, among others.

1. Introduction

The shortage of reliable translational models constrains basic research of human (patho)physiology and hampers the process of drug development. Therefore, it is important to establish robust experimental models with high clinical relevance. Since *in vivo* studies often involve large numbers of animals, attention has shifted towards validation of alternative models that can replace or reduce animal experiments, while retaining the complexity of *in vivo* systems. Precision-cut intestinal slices (PCIS) is a unique and versatile *ex vivo* model that has already been successfully used to study drug metabolism (de Kanter et al., 2005; Van De Kerkhof et al., 2008), xenobiotic interactions and drug transport (Niu et al., 2013; Possidente et al., 2011; Vickers and Fisher, 2005). Recently, the application of PCIS was extended to study fibrosis and the efficacy of antifibrotic compounds (Iswandana et al., 2016; Westra et al., 2013).

The major advantage of PCIS is that, compared to widely used cell

cultures, each slice replicates the physiologic milieu, thereby ensuring that all intestinal cell types are present in their original tissue-matrix environment (de Graaf et al., 2007). However, the use of PCIS is limited by the relatively short viability of the slices: PCIS prepared from mouse tissue can be cultured up to 48 h, whereas rat PCIS remain viable only for a few hours (Li et al., 2016; Pham et al., 2015). Overcoming this limitation will pave the way for long-term drug toxicity studies and make it possible to evaluate the kinetics of slowly metabolized compounds in the intestine. Furthermore, long-term culture of PCIS aids fibrosis research, as extended incubation induces a progressive fibrogenic response, mimicking the chronic characteristics of human disease.

One of the factors that can affect the viability of PCIS during incubation is the composition of the culture medium. Previously, it was demonstrated that optimized culture conditions significantly improved the longevity of precision-cut liver slices (Starokozhko et al., 2017; Starokozhko et al., 2015). Therefore, the aim of this study was to modify the PCIS culture system by adapting the composition of culture

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Table 1
Composition of the tested culture media.

Ingredient	Supplier	Cat ^c	Final conc	WME	ADF	ADF-ENR	ADF-WENR
GlutaMax	Gibco	35,050	2 mM	+ ^b	+	+	+
D-Glucose ^a	Sigma	G8769	25 mM	+	+	+	+
EGF	Gibco	PMG8041	50 ng/mL			+	+
Noggin ^c	—	—	10% (v/v)			+	+
R-spondin1 ^c	—	—	10% (v/v)			+	+
Wnt3a ^c	—	—	50% (v/v)			+	+
N2	Gibco	17,502	1 ×			+	+
B27	Gibco	17,504	1 ×			+	+
HEPES	Gibco	15,630	10 mM			+	+
Gentamycin	Gibco	15,750	50 µg/mL	+	+	+	+
Amphotericin B	Gibco	15,290	2.5 µg/mL	+	+	+	+

WME, William's medium E; ADF, advanced DMEM/F-12; EGF, epithelial growth factor.

^a The final concentration of the D-glucose accounts for the amount of D-glucose that is already formulated in WME and ADF. For example, WME contains 11 mM D-glucose, therefore, we added D-glucose to the total concentration of 25 mM.

^b WME was purchased as supplemented with GlutaMax (cat # 32551).

^c Noggin, R-spondin1 and Wnt3a were home-made conditioned media.

medium used to produce mouse small intestine organoids and evaluate its impact on the viability of PCIS. Sato et al. established an *in vitro* culture system that is capable of growing three-dimensional (3D) self-organizing intestinal organoids (“mini-guts”) from a single intestinal stem cell and keeping them in culture for over 1.5 years (Sato et al., 2009). Resident “self-renewing” stem cells are at the core of maintenance and repair of adult tissues (Barker et al., 2010). These cells, settled at the base of intestinal crypts, give rise to all the intestinal epithelial cell lineages, and therefore, maintain intestinal physiology. A complex molecular signaling network controls intestinal morphogenesis by tightly regulating stem cell function. To be able to form and sustain an epithelial organoid from a single stem cell *in vitro*, the culture conditions must resemble the intestinal crypt microenvironment *in vivo* by including endogenous key stem cell niche signals (Sato et al., 2011b). Therefore, culture medium for mouse small intestinal organoids is supplemented with several growth factors: 1) R-spondin ligand, a Wnt agonist needed to maintain stem cell population; 2) Noggin, a bone morphogenetic protein (BMP) inhibitor, which allows for stem cell expansion; and 3) epithelial growth factor (EGF), to promote cell proliferation. Culture medium for mouse colonic organoids and human small intestine organoids additionally contains Wnt ligand, required for crypt proliferation (Sato and Clevers, 2015). Under these culture conditions, the obtained ever-expanding intestinal organoids display all hallmarks of intestinal epithelium in terms of architecture, cell type composition and self-renewal dynamics (Sato and Clevers, 2013). We hypothesized that by using the generic organoid medium (containing R-spondin, Noggin, EGF and Wnt) we can extend the life-span of PCIS in culture. To this end, we performed a short proof-of-concept study with PCIS prepared from mouse and rat jejunum. To our knowledge, the current study is the first of its kind, in which organoid medium is used to improve the viability of PCIS prepared from excised rodent intestinal tissue in culture.

2. Methods

2.1. Animals

Adult, 8–10 weeks old, male C57BL/6 mice (Centrale Dienst Proefdieren, University Medical Center Groningen, Groningen, The Netherlands) and adult, 12–14 weeks old, male Wistar rats (Envigo, The Netherlands) were housed under temperature- and humidity-controlled conditions with a 12 h light/dark cycle and free access to water and food. The experiments were approved by the Animal Ethical Committee of the University of Groningen (CCD number AVD105002017884). The study complies with the ARRIVE guidelines and was carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

2.2. Preparation of precision-cut intestinal slices (PCIS)

The intestine was harvested in a terminal procedure performed under isoflurane/O₂ anesthesia (Pharmachemie BV, Haarlem, the Netherlands) and stored in ice-cold Krebs-Henseleit buffer (KHB) supplemented with 25 mM D-glucose (Merck, Darmstadt, Germany), 25 mM NaHCO₃ (Merck) 10 mM HEPES (MP Biomedicals, Aurora, OH), saturated with carbogen (95% O₂/5% CO₂), pH 7.4. Excised murine and rat jejunum was then handled as previously described (de Graaf et al., 2010; Pham et al., 2015). Briefly, the jejunum was cleaned by flushing KHB through the lumen and subsequently divided into 2 cm segments. These segments were filled with 3% (w/v) agarose (Sigma-Aldrich, Saint Louis, USA) in 0.9% NaCl at 37 °C and embedded in an agarose core-embedding unit. Intestinal slices with wet weight of 1–2 mg (mouse) or 3–4 mg (rat) (after removal of agarose) were prepared using a Krumdieck tissue slicer and collected in ice-cold KHB.

2.3. Culture of PCIS and medium modifications

Mouse PCIS (mPCIS) were cultured in 24-well plates (one slice per well) filled with 0.5 mL culture medium up to 96 h, while rat PCIS (rPCIS) were cultured in 12-well plates filled with 1.3 mL medium up to 24 h. All slices were incubated at 37 °C in an 80% O₂/5% CO₂ atmosphere while gently shaken at 90 rpm. The medium was refreshed every 24 h. Along with the standard culture medium, William's medium E (WME, Gibco™, Life Technologies, Bleiswijk, The Netherlands, cat # 32551), we used advanced DMEM/F-12 (ADF, Gibco™, Life Technologies, cat # 12634) and two ADF-based types of media. Table 1 shows the composition of media used for PCIS culture. The names ADF-ENR and ADF-WENR stand for the growth factors that they contain (W: Wnt3a, E: EGF, N: Noggin, R: R-spondin1).

2.3.1. Preparation of R-spondin1 conditioned medium

We established a culture of 293 T-HA-Rspol-Fc cell line (kindly provided by Prof. Calvin Kuo, Stanford University) as described previously (Drost et al., 2016). In short, 293 T-HA-Rspol-Fc cells were cultured at 37 °C in selection growth medium – DMEM (Life Technologies, cat # 31966) supplemented with 10% (v/v) FBS (Sigma-Aldrich, Saint Louis, USA, cat # F7524), penicillin/streptomycin (Life Technologies, cat # 15140) and 300 µg/mL zeocin (Life Technologies, cat # R25001) – in 175 cm² flask until confluency (3–4 days). The confluent flask was passaged into 6 × 175 cm² flasks and cells were grown in basal growth medium (DMEM with 10% (v/v) FBS, and penicillin/streptomycin) at 37 °C. Once the cells reached 80% confluency (in 3–4 days), the basal growth medium was replaced by conditioning medium – ADF supplemented with GlutaMax, HEPES and penicillin/

Table 2
Histological score system.

		Grade 0	Grade 1	Grade 2
Epithelium	Necrosis	No necrosis	Moderate	Massive
	Shape	Cubic cells	30–60% cells are flat	Cells are flat (> 80%)
Villi		Preserved	Partial loss (30–60%)	Complete loss (> 80%)
Crypts		Preserved	Partial loss (30–60%)	Complete loss (> 80%)
Stroma	Necrosis	No necrosis	Moderate	Massive

streptomycin. After one week at 37 °C, the medium was collected, centrifuged at 200 xg for 5 min at 4 °C and passed through a 0.22-µm filter to remove cells and debris. The passage number did not exceed 15. The R-spondin1 conditioned medium was stored at –20 °C until further use.

2.3.2. Preparation of noggin conditioned medium

Similarly, a culture of HEK293-mNoggin-Fc cells (kindly provided by Prof. Hans Clevers, Utrecht University) was started in 175 cm² flask in selection growth medium – DMEM supplemented with 10% (v/v) FBS, penicillin/streptomycin and 500 µg/mL geneticin (Life Technologies, cat # 10131035). The confluent flask was passaged to 6 × 175 cm² flasks with DMEM +10% (v/v) FBS + penicillin/streptomycin without geneticin and cultured for 4–5 days at 37 °C. Once the cells reached confluency, the growth medium was replaced by conditioning medium – ADF supplemented with GlutaMax, HEPES and penicillin/streptomycin. After one week of incubation, the Noggin conditioned medium was collected, centrifuged at 200 xg for 5 min at 4 °C, passed through a 0.22-µm filter and stored at –20 °C until further use.

2.3.3. Preparation of Wnt3a conditioned medium and its activity check

We started a culture of L-Wnt3a cells (kindly provided by Prof. Hans Clevers) in a 75 cm² flask in selection growth medium – DMEM supplemented with 10% (v/v) FBS, penicillin/streptomycin and 125 µg/mL zeocin – at 37 °C (Fuji et al., 2015). Once the cells reached confluency (3–4 days), the flask was split into 6 × 75 cm² flasks with added basal growth medium (DMEM +10% (v/v) FBS + penicillin/streptomycin without zeocin). The cells were incubated for additional 2–3 days until they reached confluency, then each flask was passaged to 5 × 75 cm² flasks. After one week of incubation at 37 °C, the conditioned medium was collected, centrifuged at 200 xg for 5 min at 4 °C and filtered through a 0.22-µm filter. The passage number did not exceed 12. The Wnt3a conditioned medium was stored at –20 °C until further use. The activity of Wnt3a protein in the collected medium was measured using the TOP/FOP reporter system, which consists of a minimal promoter linked to either a wild type (TOP-Flash) or mutated T cell factor (TCF)-binding sites (FOP-Flash), driving expression of luciferase (Korinek et al., 1997). Briefly, HEK293T cells were plated at a density of 3 × 10⁴ cells/well in a 24-well plate in DMEM +10% (v/v) FBS + penicillin/streptomycin. After 24 h, cells were transfected with either TOP-Flash or FOP-Flash reporter constructs and a renilla luciferase plasmid (Promega, Leiden, The Netherlands) to control for transfection efficiency. Transfections were performed with polyethylenimine (PEI 25 K, Polysciences, Germany, cat # 23966). Cells were cultured for another 24 h, then stimulated with test batches of Wnt3a conditioned medium (50% Wnt3a conditioned medium was diluted in DMEM +10% (vol/vol) FBS + penicillin/streptomycin) in triplicates. Cells were harvested 24 h after stimulation using 1 × passive lysis buffer (PLB, Promega), and luciferase activity was measured with Dual Luciferase Reporter Assay kit (Promega, cat # E1910) according to the user's manual. Luciferase activity was normalized to control renilla activity. Only batches of Wnt3a conditioned medium with TOP/FOP ratio > 15 were used in culture medium for PCIS.

2.4. Viability

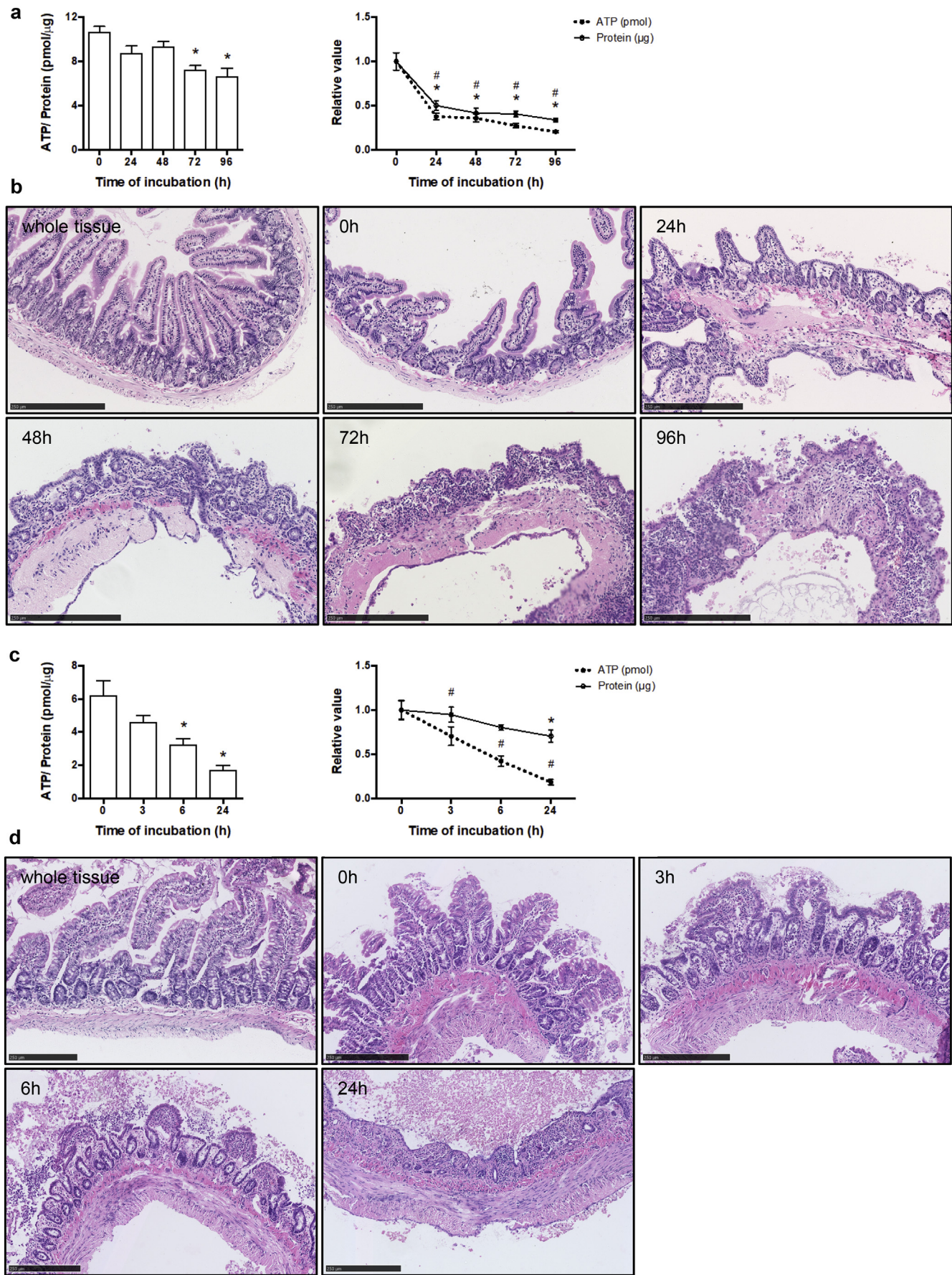
Viability of the slices after culturing was assessed by measuring the adenosine triphosphate (ATP) content (de Graaf et al., 2010). In short, after each incubation time point, three replicate slices for each experimental group were transferred to a sonication solution (containing 70% ethanol and 2 mM EDTA), snap-frozen and stored at –80 °C until further use. Samples were then thawed, homogenized and centrifuged at 10000 xg for 5 min at 4 °C. The supernatant was diluted 10 times with 0.1 M Tris HCl buffer containing 2 mM EDTA (pH 7.8), and the ATP content was determined by using the ATP bioluminescence kit (Roche diagnostics, Mannheim, Germany) according to the manufacturer's protocol with a standard ATP calibration curve. The pellet of the homogenized samples was dissolved in 200 µL of 5 M NaOH at 37 °C and diluted with 800 µL mQ water, then the protein content was determined by the Lowry assay (Bio-Rad DC Protein Assay, Veenendaal, The Netherlands). The ATP values (pmol) were normalized to the total protein content (µg) of each slice. Values are displayed as absolute ATP/Protein values or as relative values compared to the corresponding control.

2.5. Histology

All experimental groups were subjected to a morphological evaluation. PCIS were fixed in 4% buffered formalin for 24 h at 4 °C, dehydrated and then embedded in paraffin and sectioned (4 µm). Culture-induced tissue damage was assessed by staining with hematoxylin and eosin (H&E) according to the standard histological procedure. Stained tissue sections were scanned using a Nanozoomer Digital Pathology Scanner (NDP Scan U10074–01, Hamamatsu Photonics K.K., Japan). We inspected the development of tissue injury in PCIS by assigning the scores to the images based on the criteria shown in Table 2. The histological score system (adapted from Park et al. (1990)) and Roskott et al. (2010), with modifications) evaluates the integrity of four mucosal segments of PCIS – epithelium, villi, crypts and stroma – on a scale from 0 to 2 (with possibility for intermediate scores). A total score of 10 indicates severe tissue damage. E.B, J.B and C.B independently performed the blind scoring. Data are presented as the mean ± standard deviation (SD) of the three total scores.

2.6. Statistical analysis

The aforementioned analyses were performed using three replicate slices from the same animal (technical replicates) and repeated with at least four animals (biological replicates). The results are expressed as mean ± standard error of mean (SEM). We used GraphPad Prism 6.0 (GraphPad Software Inc., CA, USA) to carry out the statistical analysis. Treatment groups were compared by one-way ANOVA followed by Dunnett's multiple comparisons test. A *p*-value of < 0.05 was considered statistically significant. Correlation between ATP/protein content and the histological scores was determined using nonparametric Spearman correlation coefficient.



(caption on next page)

Fig. 1. Extended culture of mouse and rat PCIS in standard medium. (a) Viability of mouse PCIS during 96 h culture in William's medium E (WME) was measured by the ATP and protein content and expressed as normalized ATP/protein values (pmol/ μ g) or relative to 0 h values. (b) Histomorphology of mouse whole-tissue intestine and PCIS at 0, 24, 48, 72 and 96 h, as evaluated by staining with hematoxylin-eosin, scale bar 250 μ m. (c) Viability of rat PCIS during 24 h culture in WME was measured by the ATP and protein content and expressed as normalized ATP/protein values (pmol/ μ g) or relative to 0 h values. (d) Histomorphology of rat whole-tissue intestine and PCIS at 0, 3, 6 and 24 h, as evaluated by staining with hematoxylin-eosin, scale bar 250 μ m. Data are expressed as mean $\pm$ SEM, $n = 8-10$. (*) denotes statistical differences in absolute ATP/protein values (pmol/ μ g) and differences in relative ATP values compared to 0 h, while (#) denotes statistical differences in relative protein values; $p < .05$.

3. Results

3.1. Mouse and rat PCIS cultured in standard culture medium

We hypothesized that organoid medium containing growth factors of stem cell niche would improve viability of PCIS, therefore, as a first step, we described the culture of mouse and rat PCIS in standard medium - William's medium E (WME). In this study, we used two parameters - ATP content and morphological appearance - to evaluate the viability of intestinal slices. Fig. 1 illustrates the viability of mouse and rat PCIS during culture in the standard medium. Mouse PCIS were cultured in WME for up to 96 h, while rat PCIS were cultured for up to 24 h. Directly after slicing, the ATP/protein content of mPCIS was 10.58 ± 0.56 pmol/ μ g, and it gradually decreased during incubation, reaching a significant decrease of 32% and 38% at 72 h and 96 h, respectively (Fig. 1a). However, we did observe a rapid loss of both ATP and protein content in mPCIS already at 24 h that persisted throughout incubation.

Morphological evaluation (Fig. 1b) demonstrated that the normal intestinal architecture of mPCIS was well preserved after the slicing procedure (0 h). It was previously shown that the decline in ATP/protein content of intestinal slices correlates with the degree of tissue damage during culture in WME (Pham et al., 2015). Indeed, upon prolonged incubation, mPCIS showed progressive destruction of the normal intestinal architecture. Among numerous histopathological changes, we observed clear alterations in the surface epithelial cell layer associated with necrosis and loss of cubic cell shape, as well as alterations in mucosal architecture, which is generally defined by the villous morphology and crypt appearance. Culturing of mPCIS in WME led to progressing necrosis, characterized by the presence of dark colored and irregular shaped nuclei, and flattening or even severe loss of the epithelial cells. Furthermore, while intact mouse jejunum has long villi with a villous-to-crypt length ratio 3:1 to 5:1 (Erben et al., 2014), mPCIS already at 24 h displayed signs of edema, broadening and blunting of the villi, with a complete loss of villous structures at 72-96 h. Similarly, the crypt architecture became distorted over the course of incubation: irregularly shaped crypts, dilated crypts and crypts that did not run parallel were clearly present at 48 h. These changes extended to a mucosa completely devoid of crypts by 72-96 h. Of note, we observed the loss of goblet cells (cells that secrete mucus) from the epithelial cell layer already at 24 h, which can be interpreted as an indirect sign of intestinal inflammation (Heazlewood et al., 2008; Van Der Sluis et al., 2006). Taken together, along with the low ATP/protein content, severe tissue degradation present in mPCIS at 96 h in WME argued for their non-viable state, indicating that standard medium is not suitable for long-term PCIS culture.

In rPCIS, the ATP/protein content was 6.19 ± 0.92 pmol/ μ g directly after slicing (Fig. 1c). In line with previously reported results (Pham et al., 2015), we observed a rapid decline in ATP/protein values in rPCIS, with significant 48% reduction already at 6 h. By 24 h, the decrease in ATP/protein content in rPCIS reached 73%. Interestingly, the loss of ATP exceeded that of protein: the significant decrease in ATP occurred already at 3 h, while change in protein content was significant only at 24 h. Similar to mPCIS, rPCIS had a well preserved epithelial and mucosal structure directly after slicing, and upon incubation in WME rPCIS underwent a sequence of morphological changes that resulted in an immense destruction of tissue integrity (Fig. 1d). After 3 h

of culture the first signs of epithelial damage, edema, shortening of the villi and the presence of irregularly shaped or dilated crypts appeared. These histopathological changes worsened over the course of incubation, and at 24 h, rPCIS showed severe epithelial cell degeneration and complete loss of villous structures. Although crypts were not completely lost at 24 h, their structure was greatly distorted. Of note, goblet cells were present only up to 3 h, and not later during incubation when damage of the epithelial cell layer became more pronounced.

3.2. Mouse PCIS cultured in organoid media

To test our hypothesis, we cultured mPCIS in two types of organoid media up to 96 h and compared their viability with mPCIS cultured in WME. Both tested organoid media were based on advanced Dulbecco Modified Eagle Medium F-12 (ADF) and supplemented either with epithelial growth factor (EGF), Noggin and R-spondin1 (ADF-ENR) or, additionally, with Wnt3a (ADF-WENR). The complete list of added ingredients is shown in Table 1.

ATP and protein content measurements demonstrated that there was no difference in effects of ADF-ENR, ADF-WENR and WME on mPCIS at 48 h (Fig. 2a); however, ADF-ENR, but not ADF-WENR, was able to maintain significantly higher ATP/protein levels at 72 h (fold change 1.79 ± 0.24 , $P = .0133$) and 96 h (fold change 1.75 ± 0.15 , $P = .0055$) compared to WME. Interestingly, the observed effect of ADF-ENR was mainly associated with less dramatic loss of ATP content of mPCIS during culture. The gradual decrease in protein content was similar in all mPCIS, regardless of the type of culture medium. Of note, as a control for organoid media, ADF without added growth factors had no positive effect on maintaining ATP/protein content of mPCIS during prolonged culture compared to WME (Supplementary Fig. S1a).

The evaluation of morphological integrity of mPCIS cultured in different media was carried out with the aid of a histological score system that was designed to detect epithelial changes as well as changes in overall mucosal architecture (Table 2). Thus, we methodically inspected cultured mPCIS according to the defined in the scoring system criteria by evaluating histomorphology from H&E stained sections. Since we observed pronounced epithelial and mucosal changes in PCIS during culture, our scoring system included the following criteria: (1) severity of epithelial cell necrosis, (2) loss of cubic shape, (3) preservation of villous and (4) crypt structures and (5) the extent of stromal cells necrosis. A low sum histological score indicates a good preservation of epithelial and mucosal architecture, while high score (maximum sum score of 10) reflects severe tissue damage and decreased viability. As shown in Fig. 2b and c, the differences in culture-induced histopathological changes at 48 h were minimal in mPCIS cultured in WME, ADF-ENR or ADF-WENR. Upon prolonged incubation, the impact of the tested media on the preservation of tissue architecture became more apparent. The mPCIS cultured in ADF-ENR showed superior histomorphology at 72 h and, especially, 96 h. This observation was supported by a slight decrease in total histological score for the ADF-ENR experimental group at 72 h ($P = .0777$), and significantly lower score at 96 h ($P = .0008$), as compared to WME (Fig. 2c). The beneficial effects of ADF-ENR were mostly manifested by diminished necrosis of epithelial and stromal cells, as well as by prevention of crypt loss from the mucosa (Supplementary Fig. S2a). In line with the ATP data, ADF-WENR showed no improvement in maintaining normal intestinal architecture in mPCIS during long-term culture, as compared to

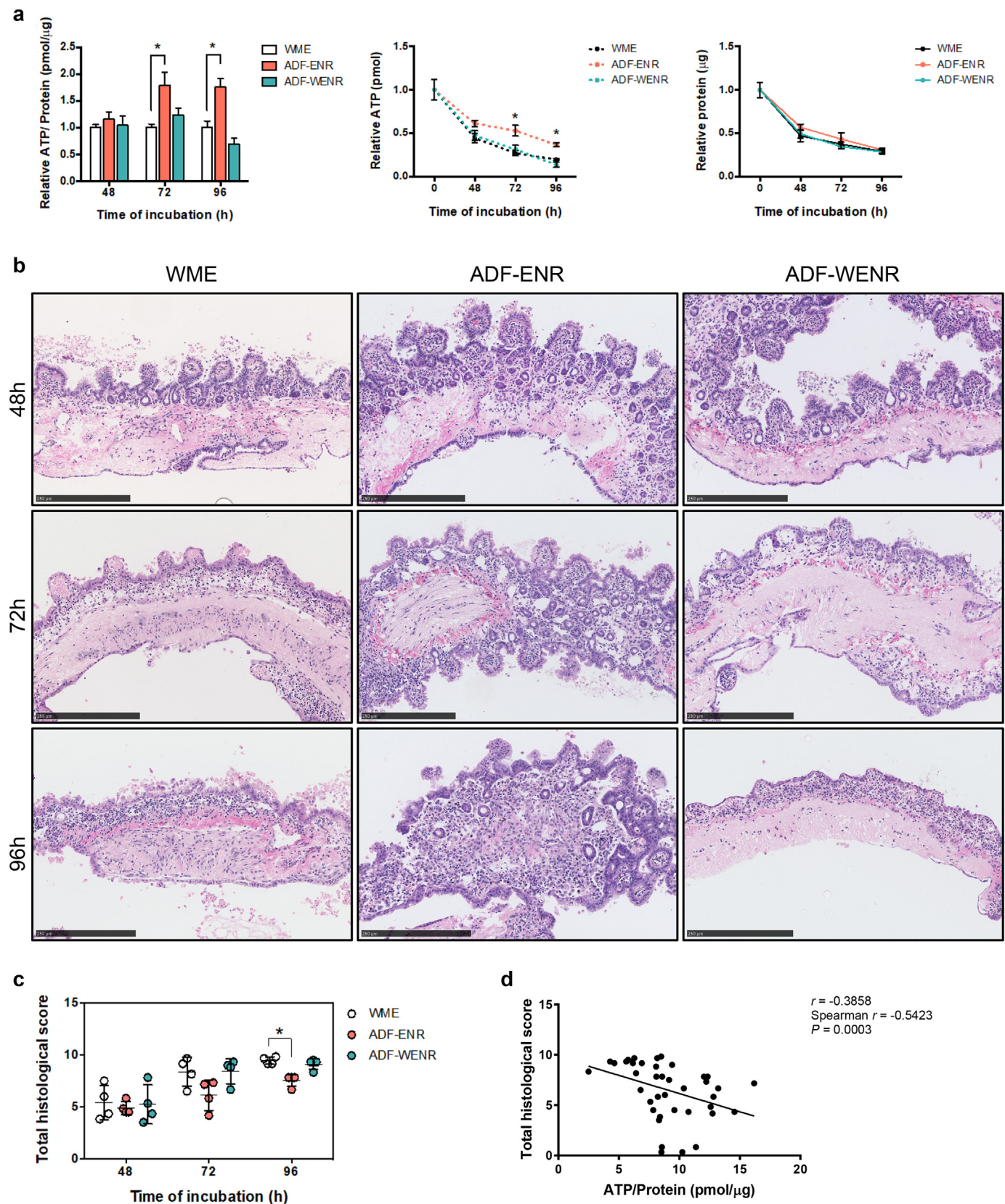


Fig. 2. Extended culture of mouse PCIS in organoid medium ADF-ENR and ADF-WENR. (a) Viability of mouse PCIS cultured in WME, ADF-ENR or ADF-WENR for 48, 72 and 96 h was measured by the ATP and protein content and expressed as values relative to WME (standard medium) or 0 h PCIS. (b) Representative images of PCIS sections stained with hematoxylin-eosin, scale bar 250 μm. (c) Histological scores of mouse PCIS after culture in tested media for 48, 72 and 96 h. (d) Correlation between ATP/protein values and histological scores. Data are expressed as mean ± SEM, $n = 4$, $p < .05$ and (*) denotes statistical differences between experimental groups within same time point.

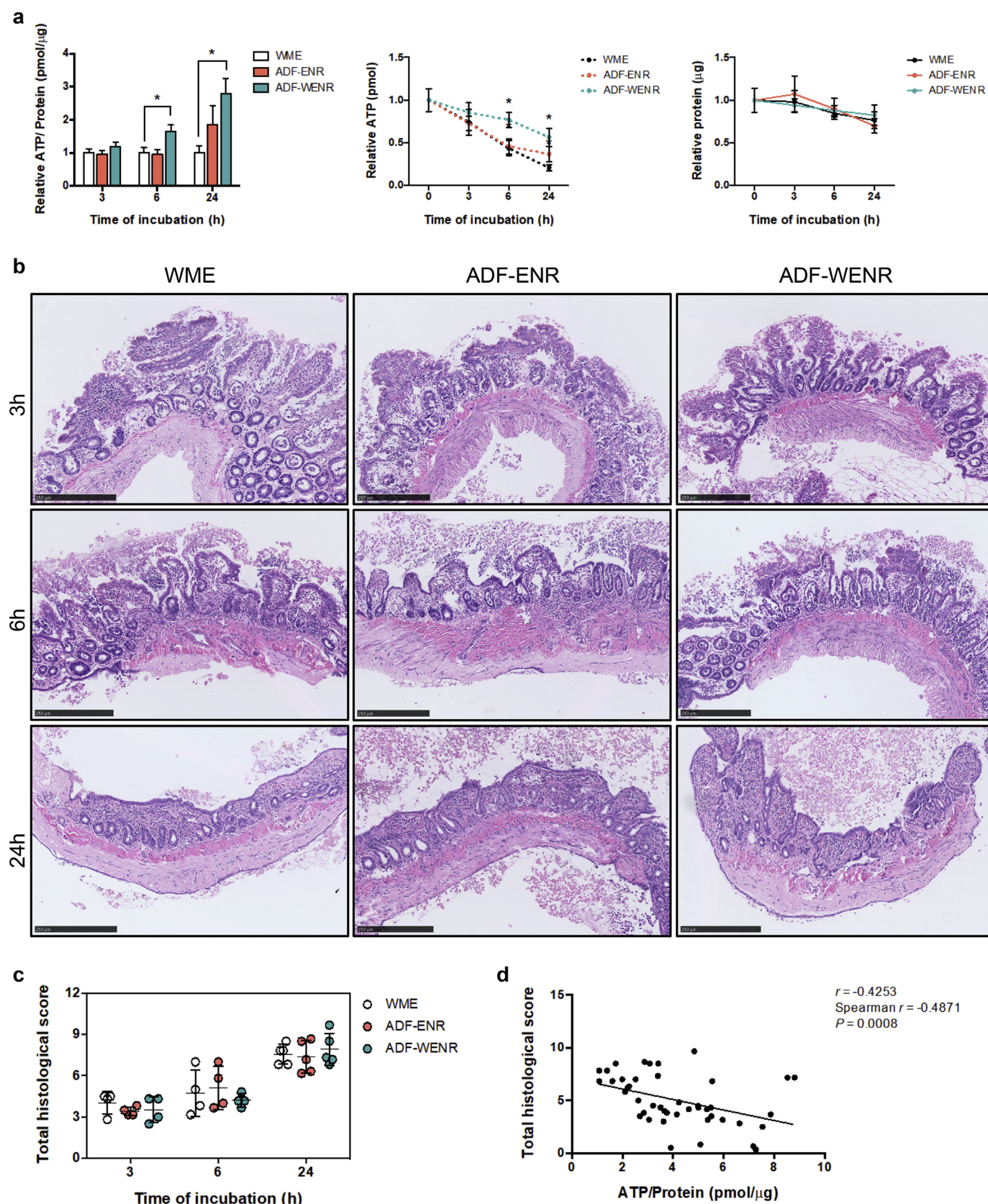


Fig. 3. Extended culture of rat PCIS in organoid medium ADF-ENR and ADF-WENR. (a) Viability of rat PCIS cultured in WME, ADF-ENR or ADF-WENR for 3, 6 and 24 h was measured by the ATP and protein content and expressed as values relative to WME (standard medium) or 0 h PCIS. (b) Representative images of PCIS sections stained with hematoxylin-eosin, scale bar 250 μm. (c) Histological scores of rat PCIS after culture in tested media for 3, 6 and 24 h. (d) Correlation between ATP/protein and histological score. Data are expressed as mean ± SEM, n = 4–6. $p < .05$ and (*) denotes statistical differences between experimental groups within same time point.

WME. Furthermore, we found a significant correlation between the ATP/protein content and total histological scores in mPCIS (Spearman $r = -0.5423$, $P = .0003$) (Fig. 2d).

3.3. Rat PCIS cultured in organoid media

We used the same two types of organoid media to culture rPCIS up to 24 h. In contrast to mPCIS, ADF-WENR, and not ADF-ENR, significantly improved viability of rPCIS, as reflected by increased ATP/protein levels at 6 h (fold change 1.64 ± 0.20 , $P = .0350$) and 24 h (2.78 ± 0.47 , $P = .0244$) (Fig. 3a). ADF-WENR mitigated ATP decline in rPCIS, especially at 6 h and 24 h, compared to ADF-ENR and WME, while it did not impact the loss of protein content during culture. As in mPCIS, ADF without growth factors had no impact on long-term viability of rPCIS (Supplementary Fig. S1b).

Despite changes in ATP content, we were unable to detect beneficial effects of the tested media on rPCIS histopathology (Fig. 3b). At 24 h, all slices, regardless of the culture media, presented disrupted epithelial and mucosal architecture, including signs of necrosis, epithelial cell loss, loss of villous structures and distorted crypts. These observations were confirmed by histological scoring, as there were no differences in the total scores between the experimental groups at any time point (Fig. 3c, Supplementary Fig. S2b). However, similar to mPCIS, there was a moderate correlation between the ATP/protein data and total histological scores in rPCIS (Spearman $r = -0.4871$, $P = .0008$) (Fig. 3d).

4. Discussion

The PCIS model is a valuable preclinical tool due to a wide range of applications that include studies of drug toxicity, metabolism and drug-drug interactions (de Kanter et al., 2002; Groothuis et al., 2013; Khan et al., 2011), as well as studies investigating the mechanism of intestinal fibrosis and the testing of antifibrotic compounds (Iswandana et al., 2016; Pham et al., 2015). However, most studies have been performed with short term PCIS cultures, often restricted to 5–48 h, due to the decline in tissue viability. In this proof-of-concept study, we tested whether culture medium routinely used to grow intestinal organoids could maintain viability of mouse and rat PCIS during extended culture. We assessed PCIS viability by two parameters – ATP/protein content and histomorphology. Our results confirm that current culture system, which relies on Williams' medium E (WME), is capable to maintain viability of mPCIS for 48 h and rPCIS for no longer than 6 h. Beyond these time points, slices suffer from severe loss of structural integrity and/or ATP content. We demonstrated that modified culture conditions, i.e. the organoid medium ADF-ENR, markedly improved the viability of mPCIS during prolonged incubation up to 96 h. In addition, ADF-WENR had a clear positive effect on the ATP content of rPCIS at 24 h, but not on slice morphology.

The decline of ATP and protein content during culture is a common phenomenon in tissue slices prepared not only from small intestine, but also from kidney (Stribos et al., 2017; Stribos et al., 2016) and liver (Catania et al., 2003; Lerche-Langrand and Toutain, 2000; Westra et al., 2016). The rate of this decline seems to differ between species. Our study found that rat intestinal slices showed a more rapid decrease in ATP content as compared to mouse. These findings are in accordance with the work of Pham et al. (Pham et al., 2015), who additionally reported that the rate of ATP loss is similar in human and mouse PCIS. In contrast, conventionally used WME maintained the viability of rat liver slices (Starokozhko et al., 2015) more efficiently than that of human slices (Starokozhko et al., 2017). These observations point towards organ- and species-specific needs of tissue slices that should be accounted for when improving the current PCIS culture system. The same holds true for the organoid model, since murine and human organoids from various organs require tissue-specific modifications of culture conditions (i.e. growth factors provided in the organoid

medium) that reflect the individual niche conditions for the resident stem cell populations and their progeny (Fatehullah et al., 2016; Kretzschmar and Clevers, 2016).

In line with our findings, previous studies showed that a decrease in ATP was accompanied by substantial morphological changes. Upon prolonged incubation in WME, intestinal slices of mouse, rat and human origin showed signs of epithelial damage and loss of mucosal architecture (Pham et al., 2015), whereas human liver slices increased in thickness and had large necrotic regions (Starokozhko et al., 2017). Furthermore, ATP content correlated with the state of morphological integrity of intestinal slices (Pham et al., 2015) and liver slices cultured under standard conditions (Westra et al., 2016), indicating that ATP levels generally reflect tissue viability. The correlation analysis in our study further confirms this association.

Culture of mouse PCIS in ADF-ENR proved to maintain tissue viability longer than culture in standard conditions: slices showed higher level of ATP production at 72 h and 96 h, and better retained epithelial and mucosal integrity. On the other hand, ADF-ENR had no impact on the steady decline in protein content during culture. The observed decrease in protein content is likely due to the detachment of outer cells following the mechanical stress inflicted by slicing. In addition, induction of autophagy might also contribute to the observed protein loss, as was argued in the study with liver slices (Starokozhko et al., 2015). Moreover, it has been shown that stress signals lead to autophagy in small intestinal epithelium and, in particular, Paneth cells (Stappenbeck et al., 2011).

As expected, addition of Wnt3a to the culture medium had no positive effect on mPCIS. As described in the study of Sato et al. (2009) culture medium for mouse small intestine organoids does not contain exogenous Wnt ligand, yet epithelial differentiation still occurs. In the intestine, Paneth cells are a major source of Wnt ligands, including Wnt3a (Gregorieff et al., 2005; Van Es et al., 2005), which are essential for controlling the stemness and the formation of Paneth cells. Thus, Wnt is abundantly present at the base of intestinal crypts. In contrast, proliferating transit-amplifying (TA) cells, which are mechanically pushed away from the crypt base by newly formed TA cells, experience a rapid decline in Wnt signaling (Clevers and Bevens, 2013). This phenomenon is known as “Wnt gradient”: high levels of Wnt drive Paneth cell differentiation, while terminally differentiated intestinal cell types – enterocytes, goblet and enteroendocrine cells – maintain their differentiated state only when Wnt signaling is absent (Crosnier et al., 2006; Farin et al., 2016). Indeed, the addition of Wnt3a to mouse small intestine organoid culture interfered with intestinal differentiation (due to the disrupted Wnt gradient) and resulted in the formation of organoids that largely consisted of undifferentiated progenitors (Sato et al., 2011b).

On the other hand, addition of Wnt3a to the culture medium was a key to establishing murine colonic organoids (Kretzschmar and Clevers, 2016; Sato et al., 2011b). In contrast to the small intestine, colon epithelium is devoid of Wnt expression (Farin et al., 2012); therefore, colon crypt cultures strictly depend on exogenous Wnt. Later, it was shown that supplementation with Wnt3a is also required for the long-term organoid cultures from primary adult human small intestinal or colonic epithelial tissue (Jung et al., 2011; Sato et al., 2011a). In our study, rat PCIS seem to benefit from the presence of Wnt in the culture medium, therefore, we speculate that rat small intestine epithelial cells produce insufficient amounts of Wnt ligands to sustain stem cells. Alternatively, Paneth cells in rat intestinal crypts might be more susceptible to injury than in mouse, resulting in weak Wnt signaling in rat PCIS. For instance, it has been shown that rat Paneth cells contain xanthine oxidase that is actively involved in the production of reactive oxygen species (ROS) (Morita et al., 2001), contributing to the rapid tissue damage in rat intestine.

It is known that Paneth cells are not the only source of Wnt *in vivo*: subepithelial mesenchyme also produces Wnt ligands, along with other signaling molecules and cytokines regulating epithelial differentiation

(Kabiri et al., 2014). Since epithelial organoids lack the mesenchymal component, their cultures rely on Paneth cell function and growth factor supply *in vitro* (Farin et al., 2012). Hou et al. (2018) described short- and long-term cultured organoid units containing epithelial and mesenchymal cells derived from mouse and human intestine, and showed that their culture did not require exogenous EGF, R-spondin1, Noggin or Wnt3a. Similarly, PCIS retain epithelial-mesenchymal cell interactions; however, the loss of viability suggests that the standard culture conditions of PCIS might be inadequate for preserving physiological functions of epithelium and/or mesenchyme in the long-term cultures, prohibiting self-sustained stem cell niche environment in slices. Therefore, growth factors added to the PCIS culture medium serve as an alternative for the lost intestinal crypt niche signaling.

As this was a preliminary proof-of-concept study, there were several limitations. First, the scoring system that we employed for the histomorphological evaluation of PCIS did not account for the loss of goblet cells (commonly associated with ulcerative colitis and other intestinal disorders (Kim and Ho, 2010)), which might serve as a sign of inflammation (Heazlewood et al., 2008; Van Der Sluis et al., 2006). Second, the effects of organoid medium on PCIS were measured by only two parameters without cell-specific observations. In order to overcome these issues, a larger study is currently being conducted, including the identification of key organoid medium ingredients for the long-term culture of PCIS and evaluation of the impact of modified medium on various intestinal cell types.

Taken together, our study provides early evidence that modifying the PCIS culture system by using generic organoid medium improved tissue viability during extended incubation. Furthermore, our results emphasize the importance of medium selection for intestinal slices originating from different species. The strategy of using the knowledge that led to the development of ever-expanding “mini-guts” for extending the life-span of PCIS cultures brings us one step closer to the superior *in vitro* model – human intestinal slices that recapitulate the original intestinal architecture with an indefinite regenerative capacity.

Author contributions

E.B., M.B. and P.O. designed the study; E.B., and J.B. carried out experiments and analyzed the data; E.B., J.B. and C.B. performed blind histomorphological evaluation; E.B. wrote the manuscript with critical review from H.A.M.M., I.A.M.d.G., M.B. and P.O. All of the authors approved the final version of the manuscript for publication.

Disclosure

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tiv.2019.05.024>.

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