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Culture media and format alter cellular composition and barrier integrity of porcine colonoid-derived monolayers

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ABSTRACT

Intestinal organoid technology has revolutionized our approach to *in vitro* cell culture due in part to their three-dimensional structures being more like the native tissue from which they were derived with respect to cellular composition and architecture. For this reason, organoids are becoming the new gold standard for undertaking intestinal epithelial cell research. Unfortunately, their otherwise advantageous three-dimensional geometry prevents easy access to the apical epithelium, which is a major limitation when studying interactions between dietary or microbial components and host tissues. To overcome this problem, we developed porcine colonoid-derived monolayers cultured on both permeable Transwell inserts and tissue culture treated polystyrene plates. We found that seeding density and culture format altered the expression of genes encoding markers of specific cell types (stem cells, colonocytes, goblets, and enteroendocrine cells), and barrier maturation (tight junctions). Additionally, we found that changes to the formulation of the culture medium altered the cellular composition of colonoids and of monolayers derived from them, resulting in cultures with an increasingly differentiated phenotype that was similar to that of their tissue of origin.

SUMMARY

In vitro models of the intestine are used to study the complex *in vivo* intestinal processes in a simplified context. As such, these models need to be representative of their tissue of origin. Here, we demonstrate that porcine colonoids and colonoid-derived monolayers that have comparable stem cells and differentiated cell types to those of the native tissue can be developed but are influenced by cell seeding density, culture format, and medium formulation.

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Barrier integrity; colonoid-derived monolayers; differentiation; medium formulation; Porcine colonoids; stem cells

Introduction

Until recently, immortalized intestinal cells were relied upon to study epithelial cell function *in vitro*. Although such models have been extensively used and have provided a wealth of information, they have significant limitations. Typically, these cell lines have been derived from metastatic tumors or have been immortalized using viral vectors and so have limited resemblance to normal epithelium[1], undergo genotypic alterations[2,3], and often have different expressions of key pathways compared to non-immortalized cells[2]. Primary cell cultures isolated directly from tissue are an alternative, but they have limited lifespans in culture and quickly enter replicative senescence or death[1,4,5].

More recent advances in intestinal epithelial cell culture have been centered on three-dimensional (3D) organoid cultures. Indeed, seminal work undertaken in the late 2000's[6–8] identified leucine rich repeat-containing G protein-coupled receptor (Lgr5+) intestinal adult stem cells (ISCs) that could be isolated, cultured, and propagated as organotypic cultures. These cells, when cultured in a suitable matrix and supplied with biochemical factors that mimic those found *in vivo*, form 3D structures known as organoids and are capable of self-renewal and differentiation[6–8]. Unlike immortalized cell lines that are predominantly derived from one cell type (e.g., Caco−2 as an absorptive enterocyte), intestinal organoids offer an increasingly complex model due to the presence of multiple types of cell representative of the epithelial lineage including ISCs, absorptive enterocytes, goblet and enteroendocrine cells and in some models Paneth cells.

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In addition to their more complex cellular composition, intestinal organoids are structurally more like the native tissue from which they are derived, with defined crypt and villi regions. ISCs predominate in the crypt regions while increased numbers of differentiated absorptive and secretory cells (goblet and enteroendocrine cells) can be found in villi regions. Although the abundance of the different cell types in organoids can be manipulated with suitable culture media[9–11], it is important to maintain the ISC population in organoid cultures because only stem cells have regenerative capabilities[10,12]. Culture of organoids in differentiation medium encourages increased abundance of differentiated cell types (enterocyte, goblet, enteroendocrine, and Paneth) and a decrease in *Lgr5* expression, and is typically achieved through the modulation of the Wnt and Notch pathways because these are known pathways that control differentiation of ISCs[11].

Organoid technology offers numerous possibilities to study basic and translational biology and is rapidly becoming the standard for investigating ISC biology and epithelial cell physiology[1,13]. Their 3D geometry is, however, a major limitation when studying interactions between microbial components, external antigens, or dietary factors and the host. In culture, intestinal organoids begin as simple, spherical structures, but over time become multilobed structures formed by a monolayer of cells around a central lumen-like void[12]. The apical region of the epithelium is sealed within the organoid structure. Direct access is therefore impeded[1] unless organoids are mechanically disrupted and incubated with a factor of interest (microbe, virus, etc.) prior to the organoid reforming[14,15] or using technically challenging methods such as microinjection of the factor directly into the lumen[16–18]. Unfortunately, microinjection is highly variable between organoids due to their different sizes, shapes, luminal volumes, and monolayer widths[19]. As an alternative, short-term cultures of two-dimensional (2D) polarized monolayers have been derived from organoids[20–24]. Differing methods using organoid fragments produced by mechanical shearing or single cell suspensions after enzymatic dissociation of organoids have been employed to develop monolayers on either tissue culture (TC) treated plates or Transwell inserts[1]. Those cultured on permeable Transwell inserts offer increased research versatility and allow for studies investigating barrier function[1,25,26], transport[25,27], or the differential responses to apical or basolateral stimulants[28].

To date, most studies involving intestinal organoid-derived monolayers have been centered around those developed from small intestinal enteroid cultures[25–27,29–43]. Although monolayers have been generated from organoids derived from the large intestine of mice, human, and rabbit[44–48], there are no studies that have used porcine colonoids as a starting material for such monolayers. Porcine intestinal models are used as an alternative to humans because pigs have greater morphological and functional similarities than those of mice and have previously been used as a model to study intestinal development, disease, and nutrition[49]. In this study, we optimized a method to develop porcine colonoid-derived monolayers cultured on both permeable Transwell inserts and TC treated polystyrene plates and compared their cellular composition to each other, and to the parental colonoids from which they were derived. Additionally, we determined that the culture medium formulation altered the cellular composition of colonoids and colonoid-derived monolayers, resulting in increasingly differentiated phenotypes with decreased ISCs that was more representative to that of their tissue of origin.

Materials and methods

Porcine colonoid culture

Using the method described previously,[12] colonoid cultures were generated from colon tissue from three healthy male pigs (Hampshire x (Landcare x Large white)) approximately 12 weeks of age euthanized for reasons unrelated to this project and under ethics approval obtained from the Animal Ethics Committee, Massey University, Palmerston North, New Zealand (Protocol #19/83). We have previously shown that the plating efficiency of colonoid establishment can vary between animals[12]. Thus, only colonoids developed from one of the animals were used.

Colonoids were maintained in 50+/+ medium consisting of a conditioned media (CM) generated

from L-WRN cells (American Type Culture Collection (ATCC), Manassas, VA, #CRL−3276) producing the growth factors Wnt−3A, R-spondin 3, and Noggin as described in detail by Miyoshi *et al*[50] combined at a 1:1 ratio with basal media (BM). BM consisted of Advanced DMEM/F12 (ADF – Gibco, Life Technologies, Auckland, NZ), Glutamax (2 mM, Gibco, Life Technologies), and HEPES (10 mM, Life Technologies)). This was supplemented with: primocin (0.1 mg/mL – Invivogen, San Diego, CA, USA), gastrin (15 nM), nicotinamide (10 mM), N-acetyl-cysteine (1.25 mM), human recombinant epidermal growth factor (hEGF – 50 ng/mL), p38 Mitogen-activated protein kinase (Mapk) inhibitor (SB202190–10 µM), and transforming growth factor beta (TGF-β) type receptor inhibitor (A83–01–600 nM) as final concentrations and all purchased from Sigma Aldrich (Auckland, NZ). During the initial 24 h of culture after isolation and passaging of colonoids, the RHO/Rho-associated Coiled-coil Kinase (ROCK) inhibitor (Y−27632 – 10 µM, Sigma Aldrich) was included in the culture medium. Additional medium changes without RHO/ROCK inhibitor were made every 48 h.

Passaging of colonoids

Colonoids were passaged every 5–7 days at a 1:5 ratio through mechanical disruption and re-seeded into 24 well plates in fresh Cultrex droplets (Cultrex Pathclear reduced growth factor basement membrane matrix; R&D Systems, In Vitro Technologies Ltd). Briefly, medium was removed from each well, and 500 µL of ice-cold Phosphate Buffered Saline (PBS, pH 7.4, Gibco, Life Technologies) was added to depolymerize the Cultrex matrix. Colonoids were repeatedly passed through a 25-gauge needle attached to a syringe to ensure they had been disrupted[12]. After centrifugation (100 × g for 4 min at 4°C), the Cultrex/PBS solution was removed and colonoid fragments suspended in ice-cold 50+/+ medium and combined at a 1:1 ratio with cold, liquid Cultrex. 30 µL was added per well of a 24-well plate and the plate inverted and placed in the incubator to allow the matrix to polymerize for 30 min, before the addition of 500 µL of 50+/+ medium containing RHO/ROCK inhibitor (10 µM) to each well. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Colonoid-derived 2D monolayers

The method to generate 2D monolayers from organoids has been described in part elsewhere[26] and was used with minor modifications. Briefly, after mechanical disruption, centrifugation, and removal of Cultrex/PBS solution (as described for passaging), the fragmented colonoids were incubated in TrypLE Express Dissociation Medium (Gibco, Life Technologies) for 10 min at 37°C after which they were repeatedly passed through a syringe with a 20-gauge needle attached until a single cell suspension was achieved. The cell suspension was combined at a ratio of 1:4 with BM supplemented with 20% Fetal Bovine Serum (FBS: Moregate Biotech, QLD, Australia) and centrifuged at 200 × g for 4 min at 4°C. The resultant cell pellet was suspended in 50 +/+ medium and cells counted using the Countess Automated Cell Counter (Life Technologies). Cells were seeded at low (7.6×10^4 cells/cm^2), medium (1.5×10^5 cells/cm^2), or high (2.3×10^5 cells/cm^2) cell densities onto 6.5 mm (0.33 cm^2 surface area), 0.4 µm^2 pore size, PET Transwell inserts (Corning, Lindfield, Sydney, Australia) and directly into wells of 24-well TC plates (Corning). The optimum density was determined to be 1.5×10^5 cells per cm^2. Prior to seeding, each insert was pre-coated with 100 µL of a 1:30 Cultrex:BM mix and incubated at 37°C for 1 h[45], after which the liquid was removed and the plates air-dried for an additional 10 min[26]. Monolayer formation was evaluated by measuring trans-epithelial electrical resistance (TEER; see below), bright field microscopy (Eclipse TS100 inverted microscope, Nikon, Japan) at 10× magnification with image capture (TrueChrome Metrics Camera, Tuscen Photonics Ltd., Fujian, China), and analysis of the presence and distribution of the tight junction protein zonula occludens 1 (Zo1) and the structural protein cytokeratin−18, a specific marker of epithelial cells[51] via confocal microscopy.

Sub-culture of epithelial monolayers

Monolayers cultured in TC plates were sub-cultured when they reached 80% confluence. Cells were detached with TrypLE Express and re-seeded

in 50+/+ medium into new 24-well TC plates at a density of 1.5×10^5 cells per cm^2. The cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere, with medium changes initially after 24 h with additional changes every 48 h. These cultures were passaged up to three times. Material from three replicate wells from each passage were lysed using 300 µL TRI Reagent (Life Technologies) and samples stored at −80°C until RNA isolation.

Trans epithelial electrical resistance of monolayers

For TEER measurements, the resistance across each monolayer was measured using an EndOhm culture cup connected to an EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL, USA). Measurements were made overtime as indicated in the results section. The cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere with medium changes every 48 h after the initial 24 h. TEER was calculated by subtracting the resistance of Cultrex coated inserts (no cells) from the resistance measured across inserts with cell monolayers and expressed per unit of surface area. The percentage change in TEER was calculated as ((TEER/initial TEER) x 100) − 100, where initial TEER values were recorded on day three post-seeding and before treatment with alternative medium formulations, while TEER represents values obtained on day six post-seeding (72 h post-treatment with alternative medium formulations). At completion, monolayers were processed for immunofluorescence or RNA extraction.

Alternative medium compositions

In addition to 50+/+ medium, other medium formulations were used. 50+/- medium comprised of CM combined at a 1:1 ratio with BM, to which gastrin (15 nM), nicotinamide (10 mM), N-acetylcysteine (1.25 mM) and EGF (50 ng/mL) were added but the inhibitors of p38 Mapk and TGF-β activity were excluded. EGF and both inhibitors were excluded from 50-/- medium. Lower percentages of CM (5%) were obtained by further dilution of CM with BM[24]. Thus, mediums containing 50% CM are prefixed with 50 while those containing only 5% CM are prefixed with 5. Mediums designated as +/+ include both EGF and inhibitors to p38 Mapk and TGF-β activity, those designated as +/- include EGF but exclude p38 Mapk and TGF-β inhibitors, and mediums designated as -/- mediums exclude both EGF and inhibitors (p38 Mapk and TGF-β). Colonoids and monolayers were cultured for 72 h post-seeding in 50+/+ medium before being transferred to culture with alternative medium formulations for an additional 72 h. At the end of the test period, colonoids and monolayers were either processed for immunofluorescence or RNA extraction.

Immunofluorescence staining

Colonoid-derived monolayers cultured on inserts were processed as described previously[52]. Briefly, media was removed from the basal and apical compartments, and cultures washed twice with warm PBS. Cells were fixed with 4% formaldehyde for 30 min at room temperature (RT), then washed once with PBS. Monolayers were permeabilized with 0.1% Triton X−100 (Sigma Aldrich) in PBS for 30 min at RT, washed again with warm PBS and blocked with 10% Bovine Serum Albumin (BSA) in PBS for 2 h at RT. Cells were again washed with warm PBS and incubated overnight at 4°C with diluted primary antibodies Zo1, Millipore; MABT11; research resource identifier (RRID): AB_10616098; 1:1000, and cytokeratin−18, (Sigma-Aldrich; C8541; RRID: AB_476885; 1:500). After rinsing three times with PBS, monolayers were incubated with appropriate secondary antibody either Alexa Fluor488/Goat Anti-Mouse IgG (Abcam ab150113, RRID: AB_2576208; 1:500) or Alexa Fluor647/Goat Anti-Rat IgG (Abcam ab150159; RRID: AB_2566823; 1:500) in the dark for 60 min. After the final washes (three times with PBS) membrane filters were cut from their supports and mounted on slides using ProLong Gold Antifade (Life Technologies, P36934, RRID: SCR_015961), and covered with coverslips. Monolayers on slides were visualized using a FluoView confocal laser scanning microscope (FV10i), from which images were taken under 60× magnification and captured using the FV10-ASW (version 3.1b) software (Olympus, Tokyo, Japan) and exported as TIFF images.

Medium was removed from colonoids seeded and cultured on 13-mm round coverslips and domes washed twice with warm PBS. Colonoids were fixed with 4% paraformaldehyde/PBS solution at room temperature (RT) for 20 min and washed again with warm PBS. Colonoids were permeabilized with 0.2% Triton X−100 (Sigma Aldrich) in PBS for 30 min at RT, re-washed with warm PBS and incubated with the following antibodies: sex-determining region Y (SRY)-box 9 (Sox9) conjugated to Alexa Fluor488 (Abcam ab196450; RRID: AB_2665383; 1:200[53]), mucin 2 (Muc2 - Santa Cruz; sc−515032; RRID: AB_2815005; 1:100[54]) conjugated to RPE-Cy7 (#LNK111PECY7, Bio-Rad, Auckland, NZ), and Chromogranin-A (Cga – Immunostar; #20086; RRID: AB_572226; 1:200[38,55]) conjugated to R-Phycoerythrin (R-PE - #LNK021RPE, Bio-Rad, Auckland, NZ). Colonoids were again rinsed three times with PBS, mounted using ProLong Gold Antifade (Life Technologies, P36934, RRID:SCR_015961), and coverslips sealed with clear nail varnish. Images were taken with an inverted I× 83inverted microscope equipped with a FV1200 confocal head (Olympus) using a 20×, N. A. 0.75 objective. Images were acquired using the FV10-ASW software (v4.2b, Olympus).

RNA extraction and Quantitative Real Time PCR (qPCR)

Total RNA was extracted, reverse transcribed and analyzed by qPCR using published methods[12]. Briefly, domes containing colonoids from six replicate wells were combined, depolymerized with ice-cold PBS, and centrifuged at 100 × g for 4 min at 4°C. After removal of the Cultrex/PBS solution, colonoids were lysed with TRI reagent (Life Technologies). Monolayer cultures were washed twice with PBS to remove residual medium and lysed with TRI reagent. For monolayers cultured on inserts, six replicate samples were combined, and for those cultured on TC plates three replicate samples were combined. This was due to the difference in culture surface area of inserts (0.33 cm^2) and TC plates (1.9 cm^2). All samples were stored at −80°C until RNA isolation.

Total RNA was extracted using the Direct-zol RNA purification kit (Zymo Research, Irvine, CA, USA) and treated with DNase I to remove genomic DNA. RNA quantity and quality for all samples were determined by optical density (OD 260/280) measurements using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Auckland, NZ). RNA integrity was assessed using an Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA), and all samples had an RNA integrity number (RIN) above 8.0. An RNA High-Capacity RNA-to-cDNA kit (Applied Biosystems #4387406, Foster City, CA, USA) was used to reverse transcribe RNA (275 ng). The resulting cDNA samples were stored at −20°C until required.

qPCR was conducted using 1 µL of cDNA (diluted 3:4 with nuclease-free water – Life Technologies) as template (excluding the no template controls where 1 µL of nuclease-free water was used) in a 10 µL reaction. The PowerUp™ SYBR™ Green Master Mix Lo-ROX Kit (Applied Biosystems, #A25780) was used in a MicroAmp Fast Optical 96-well Reaction Plate (0.1 mL) covered with MicroAmp Clear Adhesive Film (Applied Biosystems). The following thermal protocol was used on a QuantStudio3 real-time PCR system (Applied Biosystems): 2 min at 50°C, 10 min at 95°C followed by 40 cycles of two-step PCR denaturation at 95°C for 15 s and annealing extension at 60°C for 60 s. The genes of interest and specific primer pairs (Table 1) for each target were designed based on published sequences and were synthesized by Integrated DNA Technologies (IDT – Coralville, IA, USA). The data were normalized to the geometric mean of the stably expressed reference genes *Tbp* and *Rpl4* and examined for expression-level changes using the comparative delta-delta-cycle threshold (ΔΔCt) method[59]. Data are reported as average fold change ± SD to respective control.

Statistical analysis

SigmaPlot version 14.0. and GraphPad Prism version 9 were used for statistical analysis. Data were initially evaluated for normality with the Shapiro-Wilk test, and for equal variance using the Brown-Forsythe test. As indicated in the figure legends, data were analyzed using either the non-parametric Mann-Whitney U test or the Kruskal-Wallis test followed by Tukey's or Dunn's test for multiple comparisons. Alternatively, a one-way analysis of variance (ANOVA) followed by the

Table 1. List of primers used for qPCR.

	Primer sequences (5′ to 3′)		
Gene	Forward	Reverse	Reference
Tbp	aac agt tca gta gtt atg agc cag a	aga tgt tct caa acg ctt cg	56
Rpl4	caa gag taa cta caa cct tc	gaa ctc tac gat gaa tct tc	56
Lgr5	cct tgg ccc tga aca aaa ta	att tct ttc cca ggg agt gg	57
Sox9	cgg ttc gag caa gaa taa gc	gta atc cgg gtg gtc ctt ct	57
Sglt1	gca gct gtc ttc cta ctt gc	gca aac tcg gta atc ata cgg	57
Cga	gac cag act gag ttg aag	gcg tct tca tcg ttc tt	12
Muc2	cat cca ctc caa cat ctc	cgg gac aca ctt ctt ac	12
Ca2	ctc ttg ctg gca ctt ac	gta gct ctg cag cat att	12
Ocln	tcc tgg gtg tga tgg tgt tc	cgt aga gtc cag tca ccg ca	58
Zo1	aag ccc taa gtt caa tca caa tct	atc aaa ctc agg agg cgg c	58
Cldn1	aga aga tgc gga tgg ctg tc	ccc aga agg cag aga gaa gc	58

Abbreviations: *Ca2*, carbonic anhydrase 2; *Cga*, chromogranin A; *Cldn*, claudin; *Lgr5*, leucine-rich repeat-containing G protein-coupled receptor 5; *Muc2*, mucin 2; *Ocln*, occludin; *Rpl4*, ribosomal protein L4; *Sox9*, sex-determining region Y-box 9; *Sglt1*, sodium-glucose transporter 1; *Tbp*, tata box protein; *Zo1*, zonula occludens 1.

Holm-Sidak or Dunnett's multiple comparison test was used. Data are presented as mean ± SD, and statistical significance is indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$.

Results

Establishment of colonoid-derived epithelial monolayers

We evaluated the influence of cell seeding density on the formation of intestinal epithelial monolayers derived from porcine colonoid cultures. As anticipated, cell seeding density impacted monolayer formation. Microscopic analysis using bright field imaging demonstrated that, although there was a viable monolayer present on day two post-seeding for all seeding densities used, monolayers were not confluent and visible gaps between clumps of cells were evident (Figure 1a–c). Conversely, by day six confluent monolayers had formed regardless of the initial cell seeding density used (Figure 1d–f), although monolayers generated using medium density (1.5×10^5 cells/cm^2) appeared to result in more tightly packed cells (Figure 1e).

Immunofluorescence in conjunction with confocal microscopy confirmed that cells of colonoid-derived monolayers were indeed of epithelial origin as evidenced by staining with the structural protein cytokeratin−18, a specific marker for epithelial cells (Figure 1g). Additionally, the tight-junction protein Zo1 was found to be expressed along the cellular borders (Figure 1h).

Cell seeding density also resulted in significant differences in resistance across monolayers. Seeding at low density (7.6×10^4 cells/cm^2) resulted in lower TEER from day three to six, but conversely TEER was significantly ($p < 0.05$) higher at day nine as compared to both medium and high density (1.5×10^5 and 2.3×10^5 cells/cm^2, respectively - Figure 2). From day 10 to day 14 monolayers seeded at low density had significantly ($p < 0.05$) higher TEER as compared to monolayers seeded at high density (Figure 2). There was minimal difference between the TEER of monolayers seeded at medium or high density at any time point, except on day six when TEER was shown to be significantly ($p < 0.05$) higher for monolayers seeded at medium density (Figure 2). Indeed, by day six post-seeding, monolayers produced from medium density had attained maximal TEER values of 2780 ± 140 Ω.cm^2. By comparison, monolayers derived from high density attained maximal TEER values of 2270 ± 120 Ω.cm^2 on day seven post-seeding, while those seeded at low density only attained maximal TEER values of 2450 ± 330 Ω.cm^2 on day nine post-seeding.

Cell seeding density of colonoid-derived epithelial monolayers induces differential gene expression, but sub-culture does not

Markers for ISCs (*Lgr5* and *Sox9*), differentiated cell types (*Cga*, *Muc2, Sglt1*, and *Ca2*) and barrier maturation (*Ocln*, *Zo1*, and *Cldn*) were differentially expressed in colonoid-derived monolayers initiated with different cell seeding densities, as

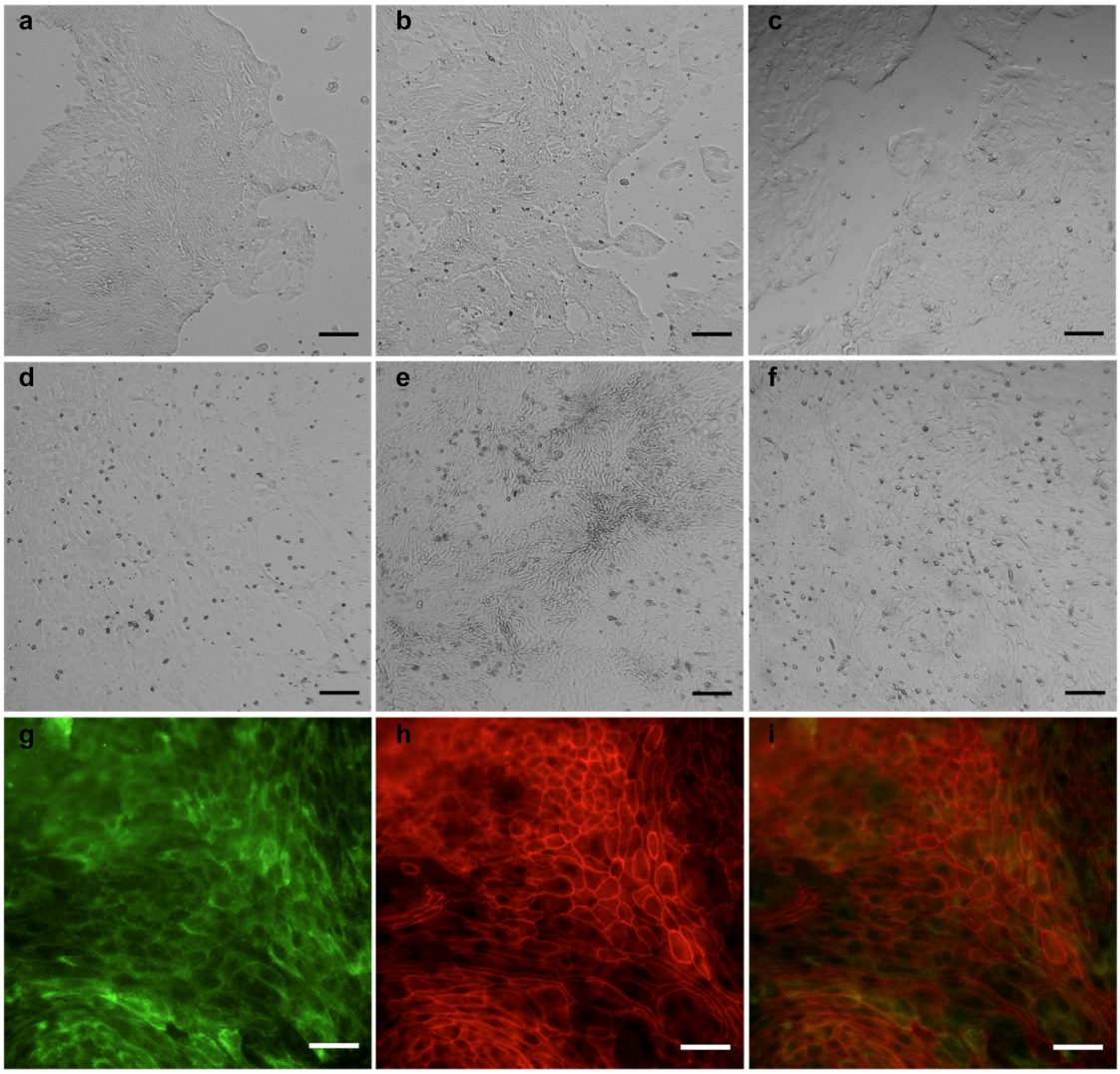

Figure 1. Microscopic imaging and immunohistochemistry of colonoid-derived monolayers. Cells were seeded at low (a, d), medium (b, e, and g-i), and high (c, f) (7.6×10^4, 1.5×10^5 and 2.3×10^5 cells/cm^2, respectively) densities and cultured for two (a-c) or six (d-i) days with 50+/+ medium. Cultures were imaged using an inverted microscope at 10× magnification (a-f) or confocal microscope (g-i). Immunoreactivity for the epithelial-specific structural protein cytokeratin – 18 (G-green) and tight-junction protein zonula occludens 1 (Zo1; H-red), and merged image of both cytokeratin – 18 and Zo1 (I). Scale bars = 50 µm.

estimated from mRNA levels. Relative to monolayers generated from a low seeding density and cultured on inserts, expression of *Lgr5* was increased by 3.0 ± 0.4-fold ($p < 0.05$) in inserts developed from medium seeding density but reduced by −2.8 ± 1.2-fold in inserts seeded at high density (Figure 3a). Expression of *Cga* and *Muc2* was decreased significantly ($p < 0.05$) in inserts seeded at medium and high density compared to inserts seeded at low density (Figure 3a). Conversely, expression of *Sglt1*, *Ca2*, *Ocln*, *Zo1*, and *Cldn* was increased in insert monolayers seeded at medium and high density (Figure 3a). Levels of *Sox9* were similar in all insert monolayers irrespective of the seeding density used (Figure 3a).

Relative to inserts seeded at medium density, those seeded at high density had decreased *Lgr5* and *Cga* expression by −8.3 ± 1.2-fold ($p < 0.001$) and −2.9 ± 1.2-fold ($p < 0.05$), respectively, but a significant increase in expression of *Sglt1* by 2.4 ± 0.5-fold ($p < 0.05$, Figure 3b).

Like monolayers cultured on inserts, those cultured in TC plates at medium and high density had significantly ($p < 0.05$) lower expression of *Muc2* compared to those seeded at low density in TC plates (Figure 3c). Expression of *Cga*

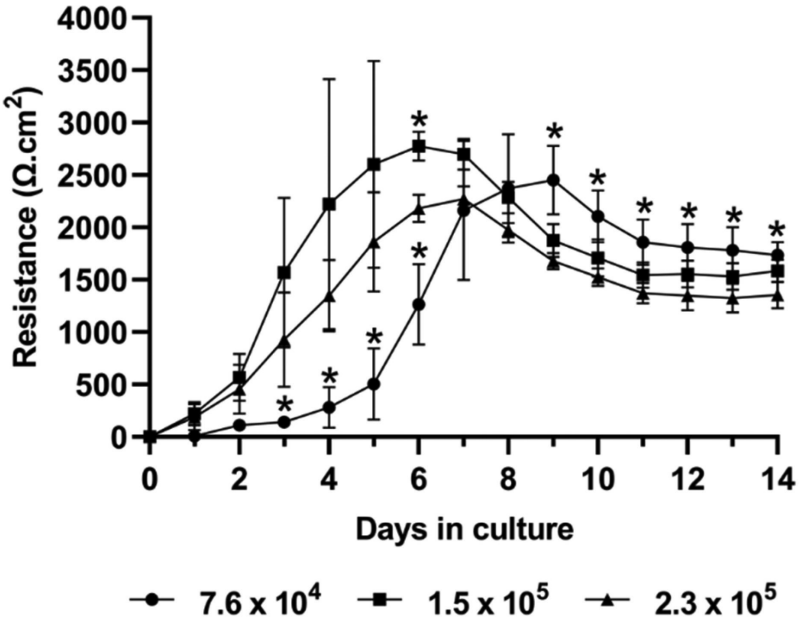

Figure 2. Influence of seeding density of colonoid-derived monolayer resistance. Cells were seeded at low, medium, and high (7.6×10^4, 1.5×10^5 and $2.3 \times 10^5/cm^2$, respectively) densities and cultured in 50+/+ medium. Resistance, presented as ohms (Ω).cm^2. Data are presented as the mean ± SD of data pooled from three independent experiments ($n = 3$). Data at the same time point were analyzed using the Kruskal-Wallis test with Tukey post-hoc test for multiple comparisons. Statistical significance is indicated as *$p < 0.05$.

was also reduced by −3.1 ± 0.7-fold ($p < 0.05$) in monolayers seeded at high density but was similar in monolayers seeded at medium density relative to those seeded at low density. Conversely, seeding at medium density significantly increased the expression of *Sglt1* and *Ca2* by 4.7 ± 0.2-fold ($p < 0.05$), and 2.7 ± 0.5-fold ($p < 0.05$), respectively, compared to TC monolayers seeded at low density (Figure 3c). Monolayers seeded at high density expressed *Ca2* at levels comparable to those seeded at low density. Expression of the stem cell markers *Lgr5* and *Sox9* was unchanged in TC monolayers irrespective of seeding density, as were *Ocln*, *Zo1*, and *Cldn* (Figure 3c). Only *Sglt1* was differentially expressed in monolayers seeded at high density relative to those seeded at medium density on TC plates, with a significant decrease in expression of −2.4 ± 0.4-fold ($p < 0.05$, Figure 3d).

In addition to comparing the effect of seeding density on differential gene expression within the same format (insert or TC plate), we also examined the effect of the same seeding density between the different formats. At low density, *Lgr5*, *Cga*, and *Ca2* expression was significantly ($p < 0.05$) decreased in monolayers cultured on TC plates compared to insert monolayers (Figure 4a). Conversely, the expression of *Sox9*, *Sglt1*, *Ocln*, *Zo1*, and *Cldn* was increased by 6.7 ± 2.8-fold ($p < 0.05$), 65.0 ± 26.5-fold ($p < 0.05$), 5.2 ± 0.5-fold, 25.9 ± 4.0-fold ($p < 0.05$), and 56.5 ± 3.0-fold ($p < 0.05$), respectively (Figure 4a). Expression of *Muc2* was comparable between formats. At medium density, *Lgr5* and *Ca2* expressions were decreased by −40.1 ± 8.7-fold ($p < 0.05$) and −2.5 ± 0.5-fold ($p < 0.05$), respectively, in monolayers cultured on TC plates compared to insert monolayers (Figure 4b). Similar to what was observed for monolayers seeded at low density, those seeded at medium density also had significant ($p < 0.05$) increases in the expression of *Sox9*, *Sglt1*, *Zo1*, and *Cldn*. In addition, expression of *Cga* and *Muc2* was increased, by 9.0 ± 1.6-fold ($p < 0.05$) and 6.3 ± 0.8-fold ($p < 0.05$), respectively (Figure 4b). Expression of *Ocln* was comparable between formats. Expression of *Lgr5* and *Ca2* was also decreased in monolayers seeded at high density and cultured on TC plates, compared to insert monolayers seeded at the same density, by −9.3 ± 2.7-fold ($p < 0.05$) and −9.4 ± 5.0-fold ($p < 0.05$), respectively (Figure 4c).

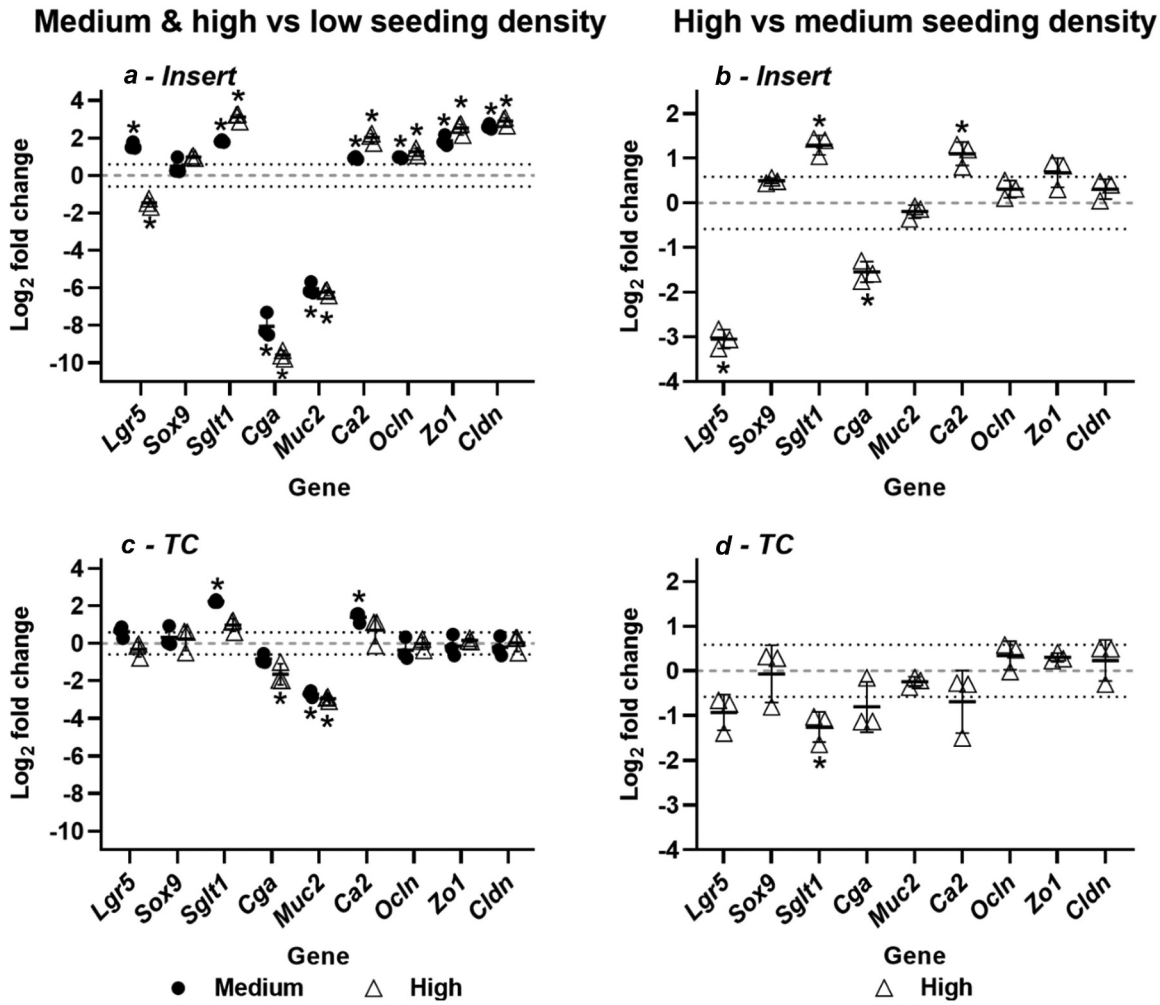

Figure 3. Differential gene expression in colonoid-derived monolayers seeded at different cell density. The relative expression of markers of stem cells (*Lgr5* and *Sox9*), differentiated colonocytes (*Sglt1*and *Ca2*), enteroendocrine and goblet cells (*Cga* and *Muc2*, respectively), and barrier maturation (*Ocln*, *Zo1*, and *Cldn*) in colonoid-derived monolayers seeded at medium (1.5×10^5 cells per cm^2) and high (2.3×10^5 cells per cm^2) densities relative to those seeded at low density (7.6×10^4 cells per cm^2) into inserts (a) or tissue culture (TC) plates (c) or at high density relative to those seeded at medium density into inserts (b) or TC plates (d) ($n = 3$ independent experiments). Data are presented as $\log_2$-fold change normalized to respective monolayers seeded at low cell seeding density (a and c) or medium density (b and d) where the mean is shown, and whiskers represent SD. Data was analyzed using Mann–Whitney U test. Values below – 0.585 and above 0.585 (corresponding to 1.5-fold change) are indicated by the dashed lines and have statistical significance ($p < 0.05$) denoted by*. *Ca2*, carbonic anhydrase 2; *Cga*, chromogranin A; *Cldn*, claudin; *Lgr5*, leucine-rich repeat-containing G protein-coupled receptor; *Muc2*, mucin 2; *Ocln*, occludin; *Sox9*, sex-determining region Y-box 9; *Sglt1*, sodium-glucose transporter 1; *Zo1*, zonula occludens 1.

Finally, the expression levels of all other genes investigated (except *Cldn* where levels were comparable) were increased (by 4.0 ± 1.5-fold ($p < 0.05$), 15.4 ± 3.3-fold ($p < 0.05$), 6.0 ± 0.5-fold ($p < 0.05$), 15.8 ± 6.6-fold ($p < 0.05$), 5.0 ± 0.4-fold ($p < 0.05$), and 7.9 ± 2.2-fold ($p < 0.05$) for *Sox9*, *Sglt1*, *Muc2, Cga*, *Zo1*, and *Cldn*, respectively) in TC monolayers (Figure 4c).

Sub-culture or passaging of cell monolayers seeded at medium density in TC plates were shown to maintain their regenerative capability. Indeed, when TC monolayers were enzymatically dissociated to single cells and re-seeded at medium density (1.5×10^5 cell per cm^2) into new wells, confluent monolayers were obtained six days post-seeding and was consistent throughout the passages. This observation shows that proliferative capacity was maintained. Sub-culture of the monolayers for three successive passages was shown to have no significant changes in the expression levels of any gene of interest (Figure 5).

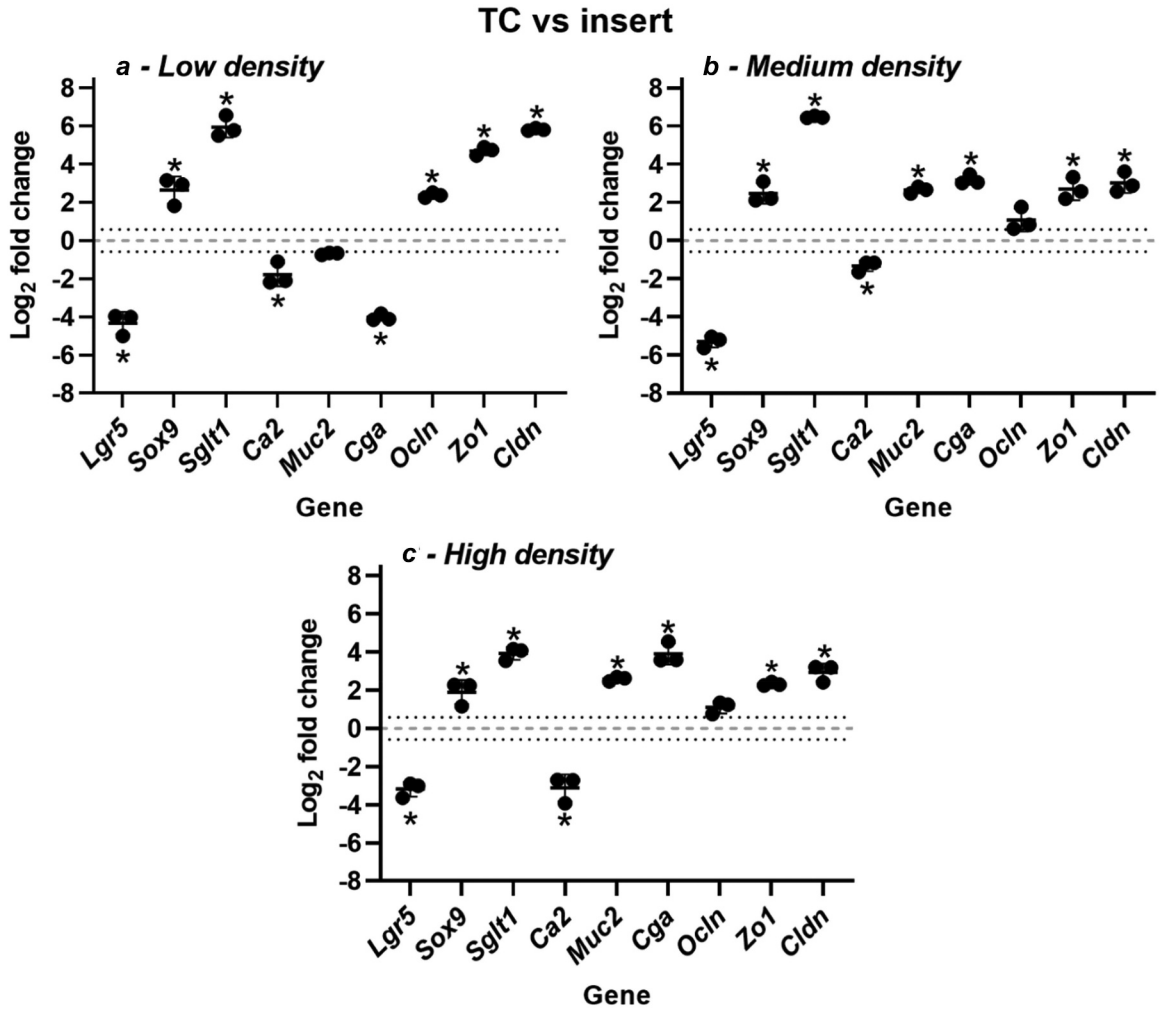

Figure 4. Differential gene expression in colonoid-derived epithelial monolayers seeded into TC treated plates at low, medium, and high cell density relative to inserts. The relative expression of mRNA transcripts of markers of stem cells (Leucine-rich repeat-containing G protein-coupled receptor 5 (*Lgr5*) and Sex-determining region Y-box 9 (*Sox9*)), colonocytes (Sodium-glucose transporter 1 (*Sglt1*) and Carbonic anhydrase (*Ca2*)) enteroendocrine cells (Chromogranin a (*Cga*)) goblet cells (Mucin 2 (*Muc2*)), and barrier maturation (Occludin (*Ocln*), Zonula occludens 1 (*Zo1*), and Claudin (*Cldn*)) in monolayers seeded at low (a), medium (b), and high (c) (7.6×10^4, 1.5×10^5 and 2.3×10^5 cells per cm^2, respectively) densities in TC plates relative to those seeded at the comparable densities into inserts ($n = 3$ independent experiments). Data represent log_2-fold change in TC monolayers normalized to insert monolayers at comparable cell seeding densities where the mean is shown, and whiskers represent SD. Data was analyzed using Mann–Whitney U test. Values below − 0.585 and above 0.585 (corresponding to 1.5-fold change) are indicated by the dashed lines and have statistical significance ($p < 0.05$) denoted by *.

Changes to medium formulation decreases stem cell mRNA expression and increases secretory cell lineage markers

We have previously shown that porcine colonoids cultured in 50+/+ medium are predominantly immature, undifferentiated structures with increased expression of the stem cell markers *Lgr5* and *Sox9* and decreased expression of markers of secretary cell lineages (*Cga* and *Muc2*) relative to the tissue from which they were derived[12]. Thus, in this study, we examined if alternative medium formulations that excluded the inhibitors (p38 MAPK and TGF-β) or EGF, or a dilution of CM, increased differentiation of colonoids and colonoid-derived monolayers cultured on permeable Transwell inserts and TC treated plates.

Relative to parental colonoid cultures grown with 50+/+ medium, expression of all genes investigated was comparable in colonoids when cultured with any medium containing 50% CM regardless of the inclusion or exclusion of inhibitors or EGF (Figure 6a).

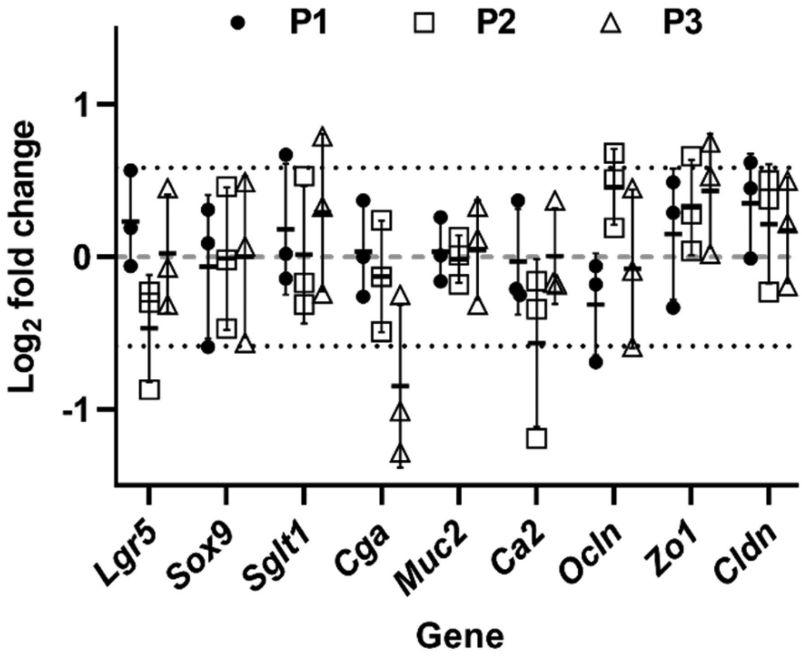

Figure 5. Differential gene expression in sub-cultured, colonoid-derived epithelial monolayers. The relative expression of mRNA transcripts of markers of stem cell (*Lgr5* and *Sox9*), specific, differentiated colonocytes (*Sglt1* and *Ca2*), enteroendocrine and goblet cells (*Cga* and *Muc2*, respectively), and barrier maturation (*Ocln*, *Zo1*, and *Cldn*) in sub-cultured colonoid-derived monolayers at passage 1, 2 and 3, relative to passage 0 monolayers as determined from qPCR. Data are presented as $\log_2$-fold change normalized to P0 monolayers and shown as scatter dot plots. The mean is shown, and whiskers represent SD from three independent experiments ($n = 3$). Data was analyzed using one-way ANOVA with Dunnett's multiple comparison test. Values below − 0.585 and above 0.585 (corresponding to 1.5-fold change) are indicated by the dashed lines and have statistical significance ($p < 0.05$) denoted by*. *Ca2*, carbonic anhydrase 2; *Cga*, chromogranin A; *Cldn*, claudin; *Lgr5*, leucine-rich repeat-containing G protein-coupled receptor; *Muc2*, mucin 2; *Ocln*, occludin; *Sox9*, sex-determining region Y-box 9; *Sglt1*, sodium-glucose transporter 1; *Zo1*, zonula occludens 1.

Levels of *Lgr5* were significantly ($p < 0.05$) reduced in colonoids when cultured with media containing 5% CM. Similarly, *Sox9* expression was also lower in colonoids cultured with 5+/- (−3.4-fold ±0.3, $p < 0.05$) and 5-/- (−4.0-fold ±0.4, $p < 0.05$) media (Figure 6a). Expression of *Sglt1* was unchanged in colonoids cultured with all media used, relative to those cultured with 50+/+ medium (Figure 6a). Reducing the concentration of CM to 5% had a significant impact on the expression of *Ca2* in colonoid cultures resulting in 32.2 ± 12.6-fold ($p < 0.05$), 30.0 ± 4.2-fold ($p < 0.05$), and 41.9 ± 8.1-fold ($p < 0.05$) increases in *Ca2* expression when cultured with 5+/+, 5+/- and 5-/- media, respectively (Figure 6a). Additionally, *Muc2* expression was increased by 24.6 ± 5.6-fold ($p < 0.05$), 12.6 ± 1.8-fold ($p < 0.05$), and 7.1 ± 0.9-fold ($p < 0.05$) when cultured with 5+/+, 5+/-, and 5-/- media, respectively. Expression of *Cga* was unchanged in colonoids cultured with any medium except those cultured with 5+/+ medium, where expression was increased significantly by 5.7 ± 1.8-fold ($p < 0.05$, Figure 6a).

Similar to what was observed for colonoids, medium containing 50% CM had a limited effect on the expression of any gene investigated in insert monolayers except that of *Ca2*, where expression was significantly decreased by −2.4 ± 0.3-fold ($p < 0.05$, Figure 6b). When cultured with any media containing 5% CM, *Lgr5* expression was decreased significantly ($p < 0.05$) compared to those cultured with 50+/+ medium, but the expression of *Sox9*, *Sglt1*, and *Cga* was unchanged when cultured with any of the media used (Figure 6b). Exclusion of both EGF and inhibitors from the media (5-/-) resulted in a significant −2.5 ± 0.6-fold ($p < 0.05$) decrease in *Ca2* expression in insert monolayers. Conversely, *Muc2* expression was increased significantly by 16.8 ± 1.7-fold ($p < 0.05$), 12.1 ± 1.0-fold ($p < 0.05$), and 3.1 ± 0.1-fold ($p < 0.05$) in insert monolayers when cultured with 5+/+, 5+/-, and 5-/- media, respectively (Figure 6b).

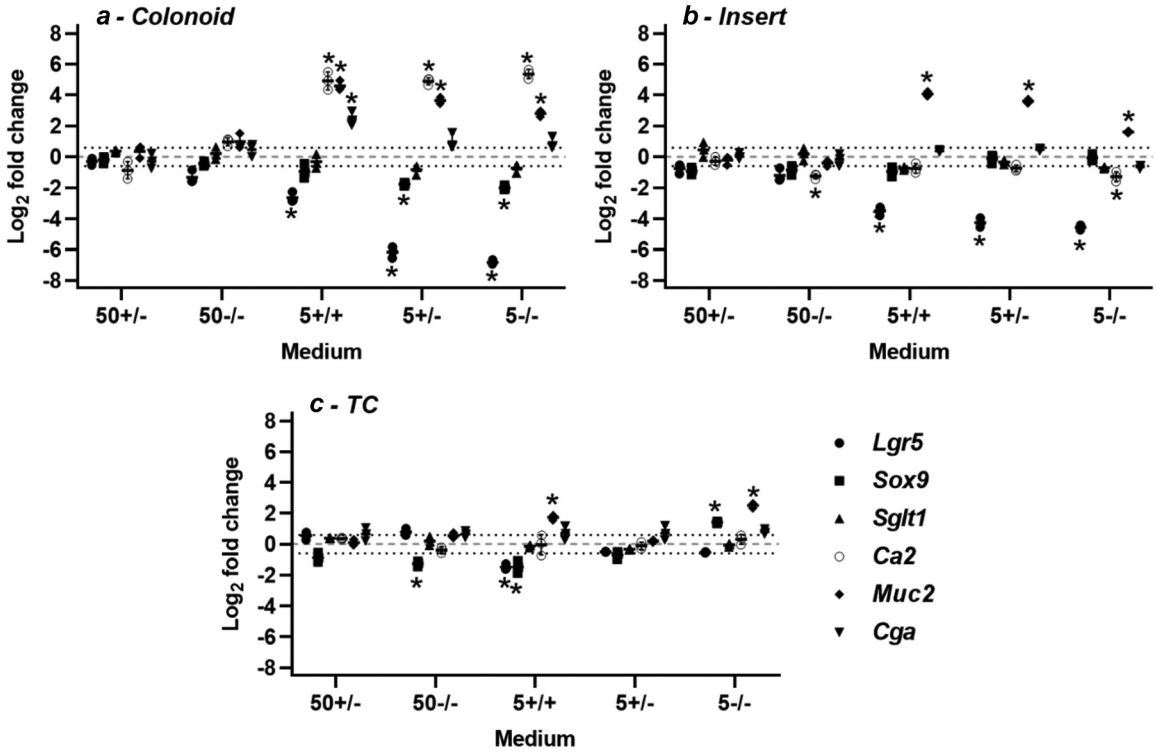

Figure 6. Differential gene expression in porcine colonoids and colonoid-derived monolayers cultured with different medium formulations. The relative expression of the stem cell markers (*Lgr5* and *Sox9*), colonocyte markers (*Sglt1* and *Ca2*), and secretory cell lineage markers (*Muc2* and *Cga*) in colonoids (a), and colonoid-derived monolayers cultured on either Transwell inserts (b), or tissue culture (TC) treated plates (c) with alternative medium formulations relative to the respective cultures grown with 50+/+ medium as determined from qPCR ($n = 3$ independent experiments). Initially all cultures were grown with 50+/+ medium for 72 h post-seeding, and then cultured with alternative media for an additional 72 h. Data are presented as $\log_2$-fold change normalized to respective cultures grown with 50+/+ medium and are shown as scatter dot plots. The mean is shown, and whiskers represent SD from three independent experiments ($n = 3$). Data was analyzed using one-way ANOVA with Dunnett's multiple comparisons test. Values below −0.585 and above 0.585 (corresponding to 1.5-fold change) are indicated by the dashed lines and have statistical significance ($p < 0.05$) denoted by*. Mediums used: 50+/+ medium (50% conditioned media (CM) + 50% basal media (BM) supplemented with epidermal growth factor (EGF) and inhibitors (p38 Mapk and TGF-β)); 50+/- (50% CM + 50% BM, +EGF – inhibitors; 50-/- (50% CM + 50% BM, -EGF – inhibitors); 5+/+ (5% CM + 95% BM, +EGF +inhibitors); 5+/- (5% CM + 95% BM, +EGF -inhibitors); and 5-/- (5% CM + 95% BM, -EGF -inhibitors). *Ca2*, carbonic anhydrase 2; *Cga*, chromogranin A; *Lgr5*, leucine-rich repeat-containing G protein-coupled receptor 5; *Muc2*, mucin 2; *Sox9*, sex-determining region Y-box 9; *Sglt1*, sodium-glucose transporter 1.

Only the expression of *Sox9* was altered in TC monolayers when cultured with media containing 50% CM relative to those cultured in 50+/+ medium (Figure 6c). Indeed, *Sox9* expression was decreased by −2.4 ± 0.3-fold ($p < 0.05$). Expression of *Lgr5* was decreased by −2.8 ± 0.4-fold ($p < 0.05$) in TC monolayers when cultured with 5+/+ medium (Figure 6c). *Sox9* expression was also decreased in TC monolayers cultured with 5+/+ media, by −2.9 ± 0.8-fold ($p < 0.05$), while those cultured with 5-/- medium had a 2.7 ± 0.2-fold ($p < 0.05$) increase in expression compared to TC monolayers cultured with 50+/+ medium. Similar to what we observed for insert monolayers, those cultured on TC plates had unchanged expression of *Sglt1*, *Ca2*, and *Cga* when cultured with any of the media formulations used (Figure 6c). *Muc2* expression increased significantly in TC monolayers when cultured with 5+/+ and 5-/- media, by 3.4 ± 0.4-fold ($p < 0.05$) and 5.7 ± 0.6-fold ($p < 0.05$), respectively (Figure 6c).

Immunofluorescence staining was used to show that ISC and secretory cell lineages were present in colonoids cultured with alternative media compositions (Figure 7). There was homogeneous distribution of Sox9 throughout colonoids cultured in any medium, although there appeared to be reduced staining of colonoids cultured with 50+/- medium (Figure 7b). Goblet cells stained with Muc2 and enteroendocrine cells stained with Cga were also identified in colonoids cultured with any

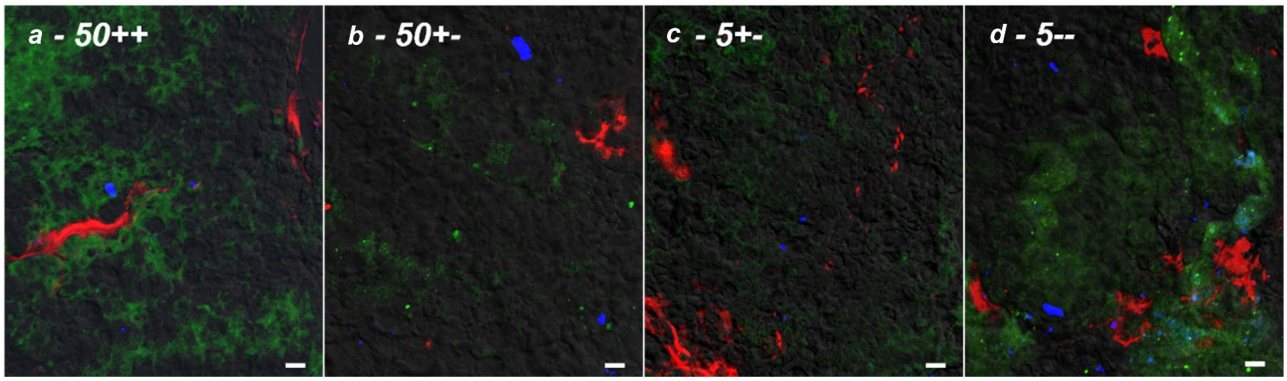

Figure 7. Immunofluorescent staining of porcine colonoids identifies stem and secretory cell lineages. the existence of stem cells and differentiated secretory cell lineages were confirmed in colonoids when cultured with alternative media formulations. Colonoids were cultured with 50+/+ medium for 72 hpost-seeding and then cultured with the same (a) 50+/+ medium or alternative media (b) 50+/-, (c) 5+/-, and (d) 5-/- for an additional 72 h. Sox9 (green) was used to identify stem cells while mucin 2 (Muc2 - red) and chromogranin-A (Cga – blue) was used to identify secretory goblet and enteroendocrine cells, respectively. Scale bar = 10 µm. Mediums used: 50+/+ medium (50% conditioned media (CM) + 50% basal media (BM) supplemented with epidermal growth factor (EGF) and inhibitors (p38 Mapk and TGF-β)); 50+/- (50% CM + 50% BM, +EGF – inhibitors; 5+/- (5% CM + 95% BM, +EGF -inhibitors); and 5-/- (5% CM + 95% BM, -EGF -inhibitors).

medium, but colonoids cultured with 5+/- and 5-/- media had more extensive staining for Muc2 (Figures 7c,d, respectively).

Culture format also influences cellular composition of colonoid-derived monolayers

When cultured in the same medium (50+/+), and relative to the parental colonoids, the expression of *Lgr5* was decreased by −2.5 ± 0.3-fold ($p < 0.001$), and −19.0 ± 10.3-fold ($p < 0.001$), respectively in insert and TC monolayers (Table 2a). Expression of *Sglt1* was increased significantly by 1.8 ± 0.2-fold ($p < 0.05$), and 5.7 ± 0.7-fold ($p < 0.001$), respectively in insert and TC monolayers, and *Ca2* expression was increased by 4.6 ± 0.1-fold ($p < 0.001$) in insert monolayers relative to colonoids (Table 2a).

Expression of *Lgr5* was decreased significantly ($p < 0.001$) in both insert and TC monolayer cultures relative to colonoids when cultured with 50 +/- medium (Table 2a). *Sox9* expression was also decreased by −3.5 ± 0.6-fold ($p < 0.05$) in insert monolayers but was unchanged in TC monolayers. Expression of *Sglt1*, *Ca2*, and *Cga* was increased significantly by 5.7 ± 0.1-fold ($p < 0.001$), 1.9 ± 0.1-fold ($p < 0.05$), and 3.0 ± 0.9-fold ($p < 0.001$), respectively in TC monolayers, and *Ca2* expression was increased by 7.0 ± 1.3-fold ($p < 0.001$) in insert monolayers relative to colonoids. Expression of *Muc2* was unchanged in both monolayer formats, relative to colonoids when cultured in 50+/- medium (Table 2a).

When cultured with 50-/- media, expression of *Sox9* and *Muc2* was significantly decreased by −2.8 ± 0.6-fold ($p < 0.001$) and −2.3 ± 0.3-fold ($p < 0.001$), respectively in insert monolayers, and *Lgr5* and *Ca2* expression was decreased by −2.3 ± 0.6-fold ($p < 0.05$) and −3.3 ± 0.4-fold ($p < 0.05$), respectively, in TC monolayers relative to colonoids cultured with the same media (Table 2a). Conversely, *Sglt1* expression was significantly increased in TC monolayers by 5.6 ± 1.1-fold ($p < 0.001$) relative to colonoids (Table 2a).

Monolayers cultured in TC plates had increased expression of *Sox9* (2.7 ± 0.3-fold, $p < 0.001$) and *Sglt1* (3.2 ± 0.4-fold, $p < 0.001$) but decreased expression of *Lgr5* (−7.7 ± 4.2-fold, $p < 0.001$) and *Ca2* (−5.9 ± 1.0-fold, $p < 0.001$), compared to insert monolayers when cultured in 50+/+ medium (Table 2b). Culturing with 50+/- medium decreased expression of *Lgr5* and *Ca2* by −2.9 ± 0.5-fold ($p < 0.001$) and −3.8 ± 0.2-fold ($p < 0.001$), respectively, but increased expression of *Sox9* (2.9 ± 0.6-fold, $p < 0.001$) and *Sglt1* (3.0 ± 0.1-fold, $p < 0.001$) in TC monolayers compared to insert monolayers cultured with the same media (Table 2b). *Sglt1* expression was also significantly increased in TC monolayers by 3.2 ± 0.7-fold ($p <$ 0.001) relative to insert monolayers when cultured with 50-/- medium (Table 2b). Additionally, the expression of *Sox9* was increased by 2.0 ± 0.2-fold (*p*

Table 2. Differential expression in porcine colonoid-derived monolayers cultured on different surfaces with mediums containing 50% conditioned media. The relative expression of mRNA transcripts of markers of stem cells (*Lgr5* and *Sox9*), differentiated colonocytes (*Sglt1* and *Ca2*), and secretory enteroendocrine and goblet cells (*Cga* and *Muc2*, respectively) in colonoid-derived monolayers cultured on Transwell inserts or tissue culture (TC) treated plates with alternative medium formulations as determined from qPCR. A) Fold change in insert and TC monolayers normalized to colonoids cultured with respective media, and B) fold change in TC monolayers normalized to insert monolayers cultured with respective media. Data represent the mean and SD from three independent experiments ($n = 3$). Data was analyzed using Mann–Whitney U test. Values below and above 1.5-fold change that have statistical significance are denoted by $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Media used: 50+/+ medium (50% conditioned media (CM) +50% basal media (BM) supplemented with epidermal growth factor (EGF) and inhibitors (p38 Mapk and TGF-β)); 50+/- medium (50% CM, + 50% BM, +EGF, -inhibitors); and 50-/- medium (50% CM, +50% BM, -EGF, -inhibitors. Abbreviations: *Ca2*, carbonic anhydrase 2; *Cga*, chromogranin A; *Lgr5*, leucine-rich repeat-containing G protein-coupled receptor 5; *Muc2*, mucin 2; *Sox9*, sex-determining region Y-box 9; *Sglt1*, sodium-glucose transporter 1.

A) Relative to colonoid

	Medium and culture format					
	50+/+		50+/-		50-/-	
Gene	Insert	TC	Insert	TC	Insert	TC
Lgr5	−2.5 ± 0.3 ***	−19.0 ± 10.3 ***	−3.4 ± 0.7 ***	−9.7 ± 1.7 ***	−2.3 ± 0.6 ***	−4.0 ± 0.6 ***
Sox9	−2.2 ± 0.6 **	1.3 ± 0.2	−3.5 ± 0.6 ***	−0.6 ± 1.4	−2.8 ± 0.6 ***	−1.4 ± 0.2
Sglt1	1.8 ± 0.2 *	5.7 ± 0.7 ***	2.0 ± 0.7 *	5.7 ± 0.1 ***	1.8 ± 0.5	5.6 ± 1.1 ***
Ca2	4.6 ± 0.1 ***	−1.3 ± 0.2	7.0 ± 1.3 ***	1.9 ± 0.1 *	0.3 ± 1.3	−3.3 ± 0.4 ***
Muc2	0.5 ± 1.3	0.3 ± 1.3	−1.3 ± 0.3	−1.2 ± 0.9	−2.3 ± 0.3	1.4 ± 0.2
Cga	0.8 ± 1.7	1.6 ± 0.4	1.7 ± 0.2	3.0 ± 0.9 ***	−0.5 ± 1.4 ***	1.8 ± 0.3

B) Relative to insert monolayers

	Medium and culture format		
Gene	50+/+ TC	50+/- TC	50-/- TC
Lgr5	-7.7 ± 4.2 ***	-2.9 ± 0..5 ***	-1.8 ± 0.3
Sox9	2.7 ± 0.3 ***	2.9 ± 0.6 ***	2.0 ± 0.2 **
Sglt1	3.2 ± 0.4 ***	3.0 ± 0.1 ***	3.2 ± 0.6 ***
Ca2	−5.9 ± 1.0 ***	−3.8 ± 0.2 ***	−3.2 ± 0.4 ***
Muc2	−1.2 ± 0.1	0.4 ± 1.3	1.7 ± 0.2
Cga	0.5 ± 1.5	0.6 ± 2.1	1.5 ± 0.3

< 0.01) but conversely, *Ca2* expression was decreased by −3.2 ± 0.4-fold ($p < 0.001$, Table 2b).

Similar results were obtained when media containing only 5% CM was used. Indeed, the expression of *Lgr5* and *Ca2* was significantly ($p < 0.001$) decreased in both insert and TC monolayers relative to colonoids cultured with 5+/+ medium (Table 3a). *Muc2* expression was also decreased in TC monolayers cultured in the same medium by −3.1 ± 0.1-fold ($p < 0.001$), but conversely, expression of *Sglt1* was increased significantly by 6.4 ± 0.4-fold ($p < 0.001$) (Table 3a). Expression of *Lgr5* was also increased in TC monolayers cultured with 5+/- and 5-/- media relative to colonoids by 3.0 ± 0.1-fold ($p < 0.001$) and 4.7 ± 0.1-fold ($p < 0.001$), respectively (Table 3a), but only insert monolayers cultured with 5-/- medium had a significant ($p < 0.001$) 9.0 ± 0.6-fold increase in the expression of *Lgr5* relative to colonoids (Table 3a). Although the expression of *Ca2* was increased by 10.2 ± 1.6-fold ($p < 0.05$) in TC monolayers cultured with 5+/- medium (Table 3a), its expression was decreased significantly ($p < 0.05$) by −43.7 ± 9.8-fold when cultured with 5-/- medium (Table 3a) relative to colonoids. Expression of *Ca2* was also decreased by −11.0 ± 1.6-fold ($p < 0.001$) and −22.5 ± 5.0-fold ($p < 0.001$) in insert monolayers cultured with 5+/- and 5-/-medium, respectively, relative to colonoids (Table 3a). Expression of *Sox9*, *Muc2*, and *Cga* in insert and TC monolayers cultured with 5+/- medium was comparable to colonoids cultured with the same medium (Table 3a). Conversely, expression of

Table 3. Differential expression in porcine colonoid-derived monolayers cultured on different surfaces with mediums containing 5% conditioned media. The relative expression of mRNA transcripts of markers of stem cells (*Lgr5* and *Sox9*), differentiated colonocytes (*Sglt1* and *Ca2*), and secretory enteroendocrine and goblet cells (*Cga* and *Muc2*, respectively) in colonoid-derived monolayers cultured on Transwell inserts or tissue culture (TC) treated plates with alternative medium formulations as determined from qPCR. A) Fold change in insert and TC monolayers normalized to colonoids cultured with respective media, and B) fold change in TC monolayers normalized to insert monolayers cultured with respective media. Data represent the mean and SD from three independent experiments ($n = 3$). Data was analyzed using Mann–Whitney U test. Values below and above 1.5-fold change that have statistical significance are denoted by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Media used: 5+/+ medium (5% conditioned media (CM) +95% basal media (BM) supplemented with epidermal growth factor (EGF) and inhibitors (p38 Mapk and TGF-β)); 5+/- medium (5% CM, +95% BM, +EGF, -inhibitors); and 5-/- medium (5% CM, +95% BM, -EGF, -inhibitors. Abbreviations: *Ca2*, carbonic anhydrase 2; *Cga*, chromogranin A; *Lgr5*, leucine-rich repeat-containing G protein-coupled receptor 5; *Muc2*, mucin 2; *Sox9*, sex-determining region Y-box 9; *Sglt1*, sodium-glucose transporter 1.

A) Relative to colonoid

	Medium and culture format					
	5+/+		5+/-		5-/-	
Gene	Insert	TC	Insert	TC	Insert	TC
Lgr5	−4.5 ± 0.9 ***	−7.7 ± 1.0 ***	1.6 ± 0.3	3.0 ± 0.1 ***	2.0 ± 0.2 *	4.7 ± 0.1 ***
Sox9	−2.1 ± 0.5 **	−1.3 ± 0.2	1.5 ± 0.3	1.6 ± 0.3	1.9 ± 0.3 *	13.9 ± 0.7 ***
Sglt1	1.3 ± 0.1	6.4 ± 0.4 ***	2.5 ± 0.2 ***	1.6 ± 0.1	1.8 ± 0.1 *	9.0 ± 0.6 ***
Ca2	−11.3 ± 2.4 ***	−42.7 ± 19.7 ***	−11.0 ± 1.6 ***	10.2 ± 1.6 ***	−22.5 ± 5.0 ***	−43.7 ± 9.8 ***
Muc2	−3.1 ± 0.1 ***	−2.2 ± 0.7	−0.4 ± 1.2	1.5 ± 0.5	−2.1 ± 0.2 **	1.5 ± 0.2
Cga	−1.3 ± 0.1	−7.4 ± 0.8 ***	1.1 ± 0.1	−1.6 ± 0.1	−2.0 ± 0.1 **	−1.3 ± 0.1

B) Relative to insert monolayers

	Medium and culture format		
Gene	5+/+ TC	5+/- TC	5-/- TC
Lgr5	-1.7 ± 0.2	1.9 ± 0.2 *	2.4 ± 0.3 ***
Sox9	1.9 ± 0.5	1.8 ± 0.4	7.5 ± 0.8 ***
Sglt1	4.8 ± 0.3 ***	3.1 ± 0.2 ***	4.9 ± 0.6 ***
Ca2	−3.8 ± 1.8 ***	−3.8 ± 0.6 ***	−2.0 ± 0.5
Muc2	−5.8 ± 0.7 ***	−12.2 ± 0.8 ***	1.6 ± 0.3
Cga	1.5 ± 0.4	1.4 ± 0.4	3.1 ± 0.4 ***

both *Sox9* and *Sglt1* was increased in TC monolayers cultured in 5-/-medium by 13.9 ± 0.8-fold ($p < 0.001$) and 7.6 ± 0.4-fold ($p < 0.001$), respectively, relative to colonoids (Table 3a).

Relative to insert monolayers, *Muc2* expression was decreased in TC monolayers cultured with 5+/+ and 5± medium by −5.8 ± 0.7-fold ($p < 0.001$) and −12.2 ± 0.8-fold ($p < 0.001$), respectively (Table 3b). *Ca2* expression was also decreased significantly in TC monolayers relative to insert monolayers when cultured in the same media (Table 3a). Conversely, the expression of *Sglt1* was increased by 4.8 ± 0.3-fold ($p < 0.001$), 3.1 ± 0.2-fold ($p < 0.001$), and 4.9 ± 0.6-fold ($p < 0.001$) in TC monolayers cultured with 5+/+, 5±, and 5-/- medium, respectively, relative to insert monolayers (Table 3b). Expression of *Lgr5* was also significantly ($p < 0.05$) increased in TC monolayers cultured with 5± and 5-/- media relative to insert monolayers, and expression of both *Sox9* and *Cga* was increased by 7.5 ± 0.8-fold ($p < 0.001$) and 3.1 ±

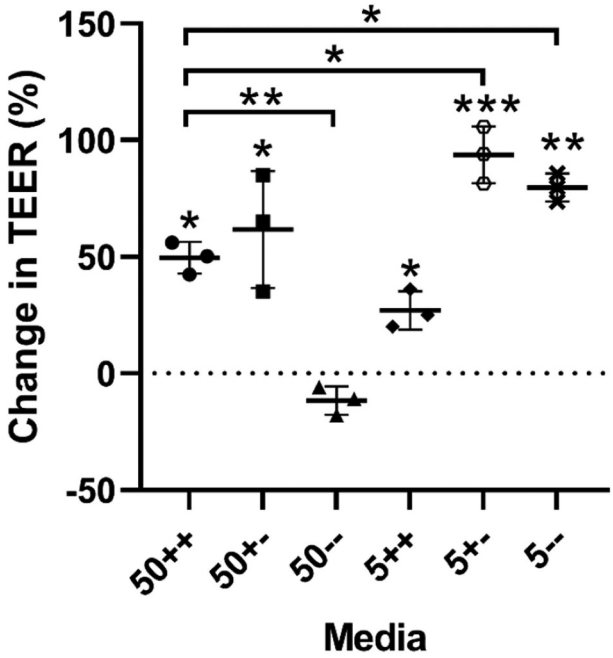

Figure 8. Influence of media composition on resistance of colonoid-derived monolayers. A single cell suspension from porcine colonoids was seeded at medium density (1.5×10^5 per cm^2) onto Transwell inserts and cultured with 50+/+ medium. After 72 h, resistance for each insert was recorded (initial TEER value) and respective inserts replenished with alternative media formulations as indicated in the graph. Inserts were cultured for an additional 72 h, and resistance for each insert recorded again. Data are presented as the percentage change in TEER compared to initial TEER for each insert. Data were analyzed using two-tailed Welch's t test. The mean is shown, and whiskers represent SD from three independent experiments ($n = 3$). Inserts cultured with alternative media were also compared to those cultured with 50+/+ media and data analyzed using one-way ANOVA with Holm-Sidak's multiple comparison test. Statistical significance is indicated as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. Media formulations: 50+/+ medium (50% conditioned media (CM) + 50% basal media (BM) supplemented with epidermal growth factor (EGF) and inhibitors (p38 Mapk and TGF-β)); 50+/- (50% CM + 50% BM, +EGF – inhibitors; 50-/- (50% CM + 50% BM, -EGF – inhibitors); 5+/+ (5% CM + 95% BM, +EGF +inhibitors); 5+/- (5% CM + 95% BM, +EGF -inhibitors); and 5-/- (5% CM + 95% BM, -EGF -inhibitors).

0.4-fold ($p < 0.001$), respectively, in TC monolayers cultured with 5-/- medium relative to inserts (Table 3b).

Culture media formulation alters barrier integrity of colonoid-derived monolayers

We examined the effect alternative media had on barrier integrity, as measured by TEER, of colonoid-derived epithelial monolayers cultured on Transwell inserts. To ensure reproducibility of results across independent experiments, TEER was measured for all inserts after the initial 72 h culture period with 50+/+ medium. Only inserts that had TEER values of 1400–1800 $\Omega.cm^2$ were used to examine the effect of alternative media on barrier integrity. Compared to initial TEER (1650 ± 140 $\Omega.cm^2$), there was a significant increase (49% ± 7, $p < 0.05$) in TEER for inserts cultured with 50+/+ media over the 72-h period (Figure 8). This increase was expected as it had been noted during the initial characterization experiments. Significant increases were also observed for inserts cultured with 50+/- (62% ± 25, $p < 0.05$), 5+/+ (27% ± 8, $p < 0.05$), 5+/- (94% ± 12, $p < 0.001$), and 5-/- (79% ± 6, $p < 0.01$) media (Figure 8). Relative to inserts cultured with 50+/+ medium, the change in TEER was significantly smaller for inserts cultured with 50-/- medium (−40% ± 5, $p < 0.01$) but greater for inserts cultured with 5+/- and 5-/- media by 31% ± 4 ($p < 0.05$) and 25% ± 13 ($p < 0.05$), respectively (Figure 8).

It has been reported previously that changes to barrier integrity can be linked to alterations in the expression of *Ocln*, *Cldn*, and *Zo1*[60–62]. Therefore, the expression of these genes was examined. Expression of *Ocln* and *Zo1* was significantly ($p <$ 0.05) increased in all insert monolayers cultured

Table 4. Differential expression in colonoid-derived insert monolayers cultured with different medium formulations. The relative expression of mRNA transcripts of stem cells (*Lgr5* and *Sox9*), differentiated colonocytes (*Sglt1* and *Ca2*), secretory enteroendocrine and goblet cells (*Cga* and *Muc2*, respectively), and barrier maturation (*Ocln*, *Zo1*, and *Cldn*) in colonoid-derived monolayers cultured on Transwell inserts with alternative medium formulations. Data are presented as fold change normalized to insert monolayers cultured with 50+/+ medium and represent the mean and SD from three independent experiments ($n = 3$). Data was analyzed using two-tailed Student's t-test with Welch's correction. Values below and above 1.5-fold change that have statistical significance are denoted by *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Mediums used: 50+/+ medium (50% conditioned media (CM) + 50% basal media (BM) supplemented with epidermal growth factor (EGF) and inhibitors (p38 Mapk and TGF-β)); 50+/- (50% CM + 50% BM, +EGF – inhibitors; 50-/- (50% CM + 50% BM, -EGF – inhibitors); 5+/+ (5% CM + 95% BM, +EGF +inhibitors); 5+/- (5% CM + 95% BM, +EGF -inhibitors); and 5-/- (5% CM + 95% BM, -EGF -inhibitors). Abbreviations: *Ca2*, carbonic anhydrase 2; *Cga*, chromogranin A; *Cldn*, claudin; *Lgr5*, leucine-rich repeat-containing G protein-coupled receptor; *Muc2*, mucin 2; *Ocln*, occludin; *Sox9*, sex-determining region Y-box 9; *Sglt1*, sodium-glucose transporter 1, and *Zo1*, zonula occludens 1.

Gene	Medium				
	50+/-	50-/-	5+/+	5+/-	5-/-
Lgr5	-1.7 ± 0.4	-2.4 ± 0.9	-11.4 ± 2.2 ***	-19.4 ± 4.0 ***	-23.7 ± 2.6 ***
Sox9	-1.9 ± 0.5	-1.8 ± 0.4	-1.9 ± 0.4	1.1 ± 0.2	1.1 ± 0.3
Sglt1	1.4 ± 0.4	1.2 ± 0.3	-1.7 ± 0.2	-1.3 ± 0.1	-1.6 ± 0.2
Ca2	-1.2 ± 0.2	-2.4 ± 0.3 ***	-1.7 ± 0.4	-1.7 ± 0.2	-2.5 ± 0.6 ***
Muc2	1.2 ± 0.2	1.3 ± 0.2	16.8 ± 1.7 ***	12.1 ± 1.0 ***	3.1 ± 0.5 ***
Cga	1.1 ± 0.1	1.2 ±0.2	1.3 ± 0.1	1.4 ± 0.2	1.6 ± 0.3
Ocln	1.9 ± 0.2 *	2.0 ± 0.3 **	5.2 ± 0.7 ***	2.7 ± 0.5 ***	4.0 ± 0.8 ***
Zo1	3.0 ± 0.5 ***	2.1 ± 0.3 **	8.8 ± 1.1 ***	4.1 ± 0.3 ***	7.5 ± 0.4 ***
Cldn	1.2 ± 0.1	1.3 ± 0.3	3.6 ± 0.8 ***	3.0 ± 0.5 ***	2.7 ± 0.3 ***

Downregulation						Upregulation			
>20	>10	>5	>3	>1.5	0	>1.5	>3	>5	>10

with any media investigated, relative to those cultured with 50+/+ medium (Table 4). *Cldn* expression was unchanged when cultured with media containing 50% CM but was differentially expressed in inserts cultured with media containing 5% CM (Table 4). Indeed, *Cldn* expression was increased by 3.6 ± 0.8-fold ($p < 0.001$), 3.0 ± 0.5-fold ($p < 0.001$), and 2.7 ± 0.3-fold ($p < 0.001$) when cultured with 5+/+, 5+/-, and 5-/- medium, respectively (Table 4).

Previous reports have also indicated that cellular composition can impact barrier integrity of insert monolayers[63,64]. For this reason, we examined the expression of *Lgr5*, *Sox9*, *Sglt1*, *Ca2*, *Cga*, and *Muc2*. Relative to inserts cultured with 50+/+ medium, expression of *Sox9*, *Sglt1*, and *Cga* was unchanged regardless of the media used (Table 4). *Lgr5* expression was significantly ($p < 0.05$) lower for all inserts when cultured with media containing 5% CM (Table 4), whereas *Ca2* expression was lower for inserts cultured with 50-/- and 5-/- media by −2.4 ± 0.3-fold ($p < 0.001$) and −2.5 ± 0.6-fold ($p < 0.001$), respectively (Table 4). Conversely, expression of *Muc2* was increased by 16.8 ± 1.7-fold ($p < 0.001$), 12.1 ± 1.0-fold ($p < 0.001$), and 3.1 ± 0.1-fold ($p < 0.001$) when cultured with 5+/+, 5+/-, and 5-/- media, respectively, but expression was unchanged when cultured in media containing 50% CM (Table 4).

Comparisons with native tissue

Finally, we compared the expression of genes coding for markers of specific cell types, differentiation, and barrier maturation in colonoids and colonoid-derived monolayers cultured on insert and TC plates with the different media, to expression in an epithelial fraction isolated from colon

Table 5. Differential expression in porcine colonoids and colonoid-derived monolayers cultured with different medium formulations relative to colon tissue. The relative expression of mRNA transcripts of markers of stem cells (*Lgr5* and *Sox9*), differentiated colonocytes (*Sglt1* and *Ca2*), secretory cell lineages (*Cga* – enteroendocrine cell, and *Muc2*—goblet cell) and barrier maturation (*Ocln*, *Zo1*, and *Cldn*) in colonoids and colonoid-derived monolayers cultured on Transwell inserts and tissue culture (TC) treated plates with alternative medium formulations as determined from qPCR. Data are presented as fold change in colonoids, insert, or TC monolayers normalized to an epithelial fraction from colon tissue. Data represents the mean and SD from three independent experiments ($n = 3$). Data was analyzed using Mann–Whitney U test. Values below and above 1.5-fold change that have statistical significance are denoted by *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Mediums used: 50+/+ medium (50% conditioned media (CM) + 50% basal media (BM) supplemented with epidermal growth factor (EGF) and inhibitors (p38 Mapk and TGF-β)); 50+/- (50% CM + 50% BM, +EGF – inhibitors; 50-/- (50% CM + 50% BM, -EGF – inhibitors); 5+/+ (5% CM + 95% BM, +EGF +inhibitors); 5± (5% CM + 95% BM, +EGF -inhibitors); and 5-/- (5% CM + 95% BM, -EGF -inhibitors). Abbreviations: *Ca2*, carbonic anhydrase 2; *Cga*, chromogranin A; *Cldn*, claudin; *Lgr5*, leucine-rich repeat-containing G protein-coupled receptor; *Muc2*, mucin 2; *Ocln*, occludin; *Sox9*, sex-determining region Y-box 9; *Sglt1*, sodium-glucose transporter 1; *Zo1*, zonula occludens 1.

Medium	Culture	Gene								
		Lgr5	*Sox9*	*Sglt1*	*Ca2*	*Cga*	*Muc2*	*Ocln*	*Zo1*	*Cldn*
Colonoid	50+/+	11.8 ± 1.6 ***	5.9 ± 0.6 ***	-0.5 ± 1.3	-1.5 ± 0.2	-5.9 ± 0.2 ***	-7.2 ± 1.4 ***	-2.7 ± 0.6 ***	-3.3 ± 0.5 ***	3.3 ± 0.6 ***
	50+/-	9.6 ± 1.4 ***	5.2 ± 0.7 ***	1.2 ± 0.1	-2.9 ± 1.5	-7.3 ± 2.3 ***	-5.7 ± 1.6 ***	-3.5 ± 0.8 ***	-2.7 ± 0.8 ***	4.8 ± 1.2 ***
	50-/-	4.8 ± 1.5 ***	4.3 ± 0.5 ***	-0.3 ± 1.5	1.3 ± 0.2	-3.7 ± 1.1 ***	-4.3 ±1.3 ***	2.6 ± 0.5 ***	-2.3 ± 0.2 **	5.9 ± 1.3 ***
	5+/+	1.9 ± 0.5	3.2 ± 1.0 ***	-0.8 ± 1.6	21.0 ± 8.4 ***	-0.4 ± 1.5	3.5 ± 0.8 ***	2.2 ± 0.5 *	3.1 ± 1.0 ***	21.9 ± 7.9 ***
	5+/-	-6.3 ± 1.6 ***	1.7 ± 0.1	-2.0 ± 0.6	19.7 ± 2.9 ***	-3.1 ± 1.0 ***	1.8 ± 0.2 *	1.6 ± 0.2	2.0 ± 0.5	19.8 ± 10.1 ***
	5-/-	-9.8 ± 1.0 ***	1.5 ± 0.2	-1.9 ± 0.4	27.4 ± 5.6 ***	-3.2 ± 0.8 ***	0.3 ± 1.3	1.3 ± 0.3	1.8 ± 0.4	18.5 ± 4.4 ***
Insert	50+/+	4.8 ± 0.5 ***	2.9 ± 1.0 ***	1.6 ± 1.0	3.0 ± 0.2 ***	-4.5 ± 1.7 ***	-6.3 ± 1.0 ***	-3.6 ± 0.8 ***	-5.0 ± 0.9 ***	5.0 ± 1.2 ***
	50+/-	2.9 ± 0.6 ***	1.5 ± 0.2	2.3 ± 0.8	2.5 ± 0.5 ***	-4.2 ± 0.5 ***	-7.3 ± 1.4 ***	-1.9 ± 0.3 *	-1.7 ± 0.4	5.8 ± 0.6 ***
	50-/-	2.1 ± 0.7 **	1.6 ± 0.3	1.9 ± 0.5	1.3 ± 0.1	-4.9 ±1.4 ***	-8.2 ± 1.1 ***	-1.8 ± 0.3 *	-2.4 ± 0.4 ***	5.7 ± 2.3 ***
	5+/+	-2.2 ± 0.6 **	1.5 ± 0.3	-0.4 ± 1.2	1.8 ± 0.4	-3.3 ± 0.3 ***	2.7 ± 0.3 ***	1.5 ± 0.2	1.8 ± 0.2 *	17.6 ± 4.2 ***
	5+/-	-4.1 ± 0.8 ***	2.6 ± 0.4 ***	1.3 ± 0.1	1.8 ± 0.3	-3.1 ± 0.2 ***	2.0 ± 0.2 *	-1.4 ± 0.3	1.2 ± 0.1	15.1 ± 2.3 ***
	5-/-	-5.0 ± 0.5 ***	2.7 ±0.4 ***	1.0 ± 0.3	0.6 ± 1.4	-6.7 ± 0.6 ***	-2.0 ± 0.1 **	1.1 ± 0.1	1.5 ± 0.3	13.2 ± 1.7 ***
TC	50+/+	-1.0 ± 1.9	7.6 ± 1.0 ***	5.1 ± 0.6 ***	-2.0 ± 0.3 **	-3.9 ± 1.1 ***	-7.2 ± 0.7 ***	-0.5 ± 1.5	0.5 ± 1.3	33.8 ± 2.5 ***
	50+/-	0.3 ± 1.3	4.3 ± 0.9 ***	6.6 ± 0.1 ***	-1.5 ± 0.1	-2.5 ± 0.7 ***	-6.7 ± 0.9 ***	1.1 ± 03	1.3 ± 0.2	26.9 ± 2.4 ***
	50-/-	1.2 ± 0.2	3.2 ± 0.5 ***	5.9 ± 1.2 ***	-2.6 ± 1.2	-2.4 ± 0.3 ***	-5.0 ± 0.5 ***	-1.1 ± 0.3	1.4 ± 0.2	25.3 ± 2.0 ***
	5+/+	-4.1 ± 0.5 ***	2.8 ± 1.0 ***	4.6 ± 0.5 ***	-2.2 ± 0.7	-2.4 ± 0.9	-2.1 ± 0.7	1.3 ± 0.3	1.9 ± 0.4	29.5 ± 4.4 ***
	5+/-	-2.1 ± 0.1 **	4.7 ± 0.8 ***	4.0 ± 0.4 ***	-2.1 ± 0.6	-2.5 ± 0.6 ***	-6.3 ± 0.3 ***	1.5 ± 0.2	1.4 ± 0.2	30.3 ± 1.4 ***
	5-/-	-2.1 ± 0.4 **	5.1 ± 0.3 ***	4.9 ± 0.4 ***	-1.6 ± 0.4	-2.2 ± 0.3 **	-1.3 ± 0.3	1.3 ± 0.3	2.1 ± 0.6	25.0 ± 2.5 ***

Downregulation				Upregulation				
>5	>3	>1.5	0	>1.5	>3	>5	>10	>20

tissue that was not used for the generation of colonoids.

When any cell model was cultured with media containing 50% CM *Lgr5* expression was significantly ($p < 0.05$) increased except in monolayers cultured on TC plates, where it was comparable to that of an epithelial fraction from colon tissue (Table 5). Reducing the concentration of CM to 5% significantly ($p < 0.05$) decreased *Lgr5* expression in all cell models except colonoids cultured with 5+/+ medium, where expression was unchanged (Table 5). The expression of *Sox9* was

increased significantly ($p < 0.05$) for all cell cultures except colonoids cultured with 5+/- and 5-/- media, and insert monolayers cultured with 50+/-, 50-/-, and 5+/+ media, where expression was similar to that of an epithelial fraction from colon tissue (Table 5).

Expression of *Sglt1* was unchanged in colonoids and insert monolayers but was significantly increased ($p < 0.05$) in TC monolayers when cultured with any media formulation relative to that of colon tissue (Table 5). Expression of *Ca2* was also unchanged in colonoids when cultured with any media containing 50% CM but was increased significantly by 21.0 ± 8.4-fold ($p < 0.001$), 19.7 ± 2.9-fold ($p < 0.001$), and 27.4 ± 5.6-fold ($p < 0.001$) when cultured with 5+/+, 5+/-, and 5-/- media, respectively (Table 5). Insert monolayers cultured with 50+/+ and 50+/- media formulations also had increased expression of *Ca2* by 3.0 ± 0.2-fold ($p < 0.001$), and 2.5 ± 0.5-fold ($p < 0.001$), respectively (Table 5). Expression of *Ca2* in TC monolayers was comparable to that of an epithelial fraction from colon tissue except TC monolayers cultured with 50+/+ media where expression was decreased by −2.0 ± 0.3-fold ($p < 0.01$, Table 5).

Cga expression was decreased significantly for all cultures except colonoids and TC monolayers cultured with 5+/+ media, where expression was comparable to that of an epithelial fraction from colon tissue (Table 5). *Muc2* expression was decreased significantly ($p < 0.001$) for all cell cultures when cultured with media containing 50% CM (Table 5). Additionally, insert monolayers cultured with 5-/- media had a significant −2.0 ± 0.2-fold ($p < 0.01$) decrease in *Muc2* expression, while in TC monolayers cultured with 5+/+ and 5+/- media, *Muc2* expression was decreased by −2.1 ± 0.5-fold ($p < 0.01$) and −6.3 ± 0.3-fold ($p < 0.001$), respectively (Table 5). Conversely, *Muc2* expression was increased by 3.5 ± 0.8-fold ($p < 0.001$) and 2.7 ± 0.3-fold ($p < 0.001$) in colonoids and insert monolayers, respectively, when cultured with 5+/+ medium, and by 1.8 ± 0.2-fold ($p < 0.05$) and 2.0 ± 0.2-fold ($p < 0.05$) in colonoid and insert monolayers, respectively, when cultured with 5+/- media (Table 5). Only colonoids and TC monolayers cultured with 5-/- media had comparable expression of *Muc2* to the epithelial fraction from colon tissue (Table 5).

Expression of both *Ocln* and *Zo1* was significantly ($p < 0.05$) decreased in colonoids and insert monolayers when cultured with media containing 50% CM, except insert monolayers cultured with 50+/- media where expression of *Zo1* was comparable to that of an epithelial fraction from colon (Table 5). Conversely, expression of *Ocln* was increased by 2.2 ± 0.5-fold ($p < 0.05$) in colonoids cultured with 5+/+ media, and *Zo1* expression was increased by 3.1 ± 1.0-fold ($p < 0.001$) and 1.8 ± 0.2-fold ($p < 0.001$) in colonoids and insert monolayers, respectively, when cultured with 5+/+ media (Table 5). Expression of both *Ocln* and *Zo1* was unchanged in TC monolayers regardless of the media used, but conversely, expression of *Cldn* was significantly ($p < 0.001$) increased in colonoids and colonoid-derived monolayers when cultured with any media formulation relative to that of an epithelial fraction from colon (Table 5).

Discussion

Intestinal organoid technology provides a complex epithelial cell culture model compared to traditional immortalized cell lines. However, their 3D geometry, where the apical membrane is trapped within the center of this structure, has the potential to limit their uses. In contrast, monolayers are easier to use and are known to increase experimental throughput. To date, colonoid-derived monolayers have been characterized for human[48] and mouse[45] but, to our knowledge, not for pig.

Here, we developed colonoid-derived monolayers and determined that cell seeding density had a limited impact on surface coverage after six days of culture, but barrier integrity (as measured by TEER) of insert monolayers was affected. Barrier integrity is an important function of any tissue or cell culture and in the intestine is crucial for regulating the entry of essential ions, nutrients, and water, while restricting the entry of luminal bacterial toxins and pathogens[64–66]. Important components that contribute to barrier integrity are junctional proteins such as Ocln, Cldn, and Zo1[12,66]. TEER is used as an indirect indicator of tight junction formation and is a fast and efficient way of measuring epithelial monolayer integrity[67]. In this study, we determined that medium density was optimal for seeding colonoid-derived

monolayers that produced confluent monolayers with consistently high TEER (1400–1800 Ω.cm^2) within six days post-seeding. Regardless of the seeding density used, the TEER values for porcine colonoid-derived monolayers in this study were comparable to those obtained previously for mouse proximal colonoid-derived monolayers[20] but were considerably greater than those for porcine and bovine enteroid-derived monolayers[25,26,40].

Cell seeding density resulted in differential gene expression in colonoid-derived monolayers, but this was also different between those cultured on inserts or TC plates. In this study, it was shown that inserts seeded at medium or high density, relative to those seeded at low density, had increased colonocyte differentiation and barrier maturation as evidenced by the increased expression of the colonocyte markers *Sglt1* and *Ca2* and the tight junction markers *Ocln*, *Cldn*, and *Zo1*.

Of interest in this study was the differential expression of the stem cell marker *Lgr5* in insert monolayers, where expression was higher in those seeded at medium density relative to those seeded at both low and high density. From microscopic analysis, it was determined that monolayers seeded at medium density had areas of densely packed cells that were not evident in monolayers seeded at low or high density. It is possible that these densely packed cell clusters observed at medium density contain an increased number of *Lgr5* expressing stem cells similar to that observed previously for murine[23] and human[68] organoid-derived monolayers, although this was not investigated further in this study.

Expression of the stem cell marker *Lgr5* was decreased in all TC monolayers compared to insert monolayers, irrespective of the seeding density used, while expression of the secretory cell lineage markers *Muc2* and *Cga* was increased in monolayers seeded at medium and high density on TC plates. Similarly, monolayers seeded at medium density and cultured on TC plates had increased colonocyte differentiation relative to those seeded at low density.

Interestingly, relative to their respective monolayers seeded at medium density, those seeded at high density had increased *Sglt1* expression when cultured on inserts, but decreased *Sglt1* expression when cultured as TC monolayers. However, TC monolayers consistently had increased *Sglt1*expression relative to insert monolayers seeded at the same cell density. These results indicate that cell seeding density may not be responsible for the differential expression of *Sglt1* but instead may relate more to the surface on which the monolayers were cultured. This interpretation is in accordance with previous reports that have shown that although seeding density has a limited impact on the expression of *Sglt1*[69], it can influence many biological processes including cellular differentiation[70], cell fate decisions[71,72], and global gene expression[73,74]. Indeed, microarray data obtained from Caco−2 cells determined that 163 genes were differentially expressed in cell populations seeded at low density compared to those seeded at high density, even though they originated from the same cell clone[75]. Additionally, microenvironmental factors, such as the extracellular matrix in which cells are cultured, can impact the migration, proliferation, and differentiation of intestinal cells[76,77], and in a recent study undertaken by Criss *et al.*[73] it was identified that 4,695 genes were differentially expressed between human enteroid-derived (duodenal) monolayers cultured on TC plates and inserts[73].

Overall in this study, we determined that although seeding density resulted in differential expression, seeding at medium density was optimal because confluent monolayers with high TEER were formed within a short period of time (6 days post-seeding), stem cell populations could be maintained in both insert and TC monolayers but were lost in inserts seeded at high density, and seeding at medium density would require less cells as compared to high density seeding.

Alterations to medium formulations also resulted in differential gene expression profiles of colonoids and colonoid-derived monolayers cultured on inserts or TC plates compared to each other but also relative to native tissue. The maintenance medium for porcine colonoids used here contained the essential factors Wnt, noggin, R-spondin, and EGF as well as inhibitors to p38 Mapk and TGF-β activity (termed 50+/+ medium). This medium has been shown to maintain porcine colonoids in an undifferentiated state relative to

their tissue of origin[12]. Similarly in this study, colonoid-derived monolayers cultured on inserts and with the same medium (50+/+) had increased expression of the stem cell markers *Lgr5* and *Sox9* but decreased expression of the secretory cell lineage markers *Muc2* and *Cga*, indicating that these cultures were also predominantly undifferentiated with increased stem cell populations relative to an epithelial fraction from porcine colon tissue. Indeed, when cultured with any media containing 50% CM with or without EGF or inhibitors, secretory cell populations were decreased for all cultures irrespective of the culture format (colonoid or monolayer), while *Sglt1* expressing cell populations were increased in TC monolayers, and *Lgr5* stem cell populations were increased for colonoids and insert monolayers but were unchanged for TC monolayers.

Although the specific reasons why there was a difference in *Lgr5* stem cell expression between the different culture formats was not investigated in this study, we suggest that when cells are seeded directly on to the surface of TC plates, the medium, and as a consequence any growth factor or signaling molecule present in the medium, such as Wnt or R-spondin, is prohibited from direct interaction with the basal membrane of the cells. In contrast, when cultured as colonoids or monolayers on permeable inserts, both apical and basal membranes are exposed. It should be noted that *Lgr5* is a Wnt target gene, and its receptor serves a critical function in ISC regulation by binding the Wnt agonist R-spondin, to amplify the local Wnt signal-[78]. Typically, in the *in vivo* situation, Wnt ligands are secreted from either the underlying mesenchymal cells or from epithelial cells, while noggin, an inhibitor of the bone morphogenetic protein (BMP) signaling pathway, is mainly expressed in mesenchymal cells beneath the crypt[79,80]. This results in the formation of a gradient of Wnt and BMP activity along the crypt-villus axis, with Wnt activity increasing from the bottom to the upper part of the crypt and villi and the reverse for BMP activity[79,81]. Wnt signaling is crucial for the maintenance of non-differentiated stem cells in the crypt, while BMP inhibits self-renewal and promotes differentiation[81]. However, because the culture medium does not have direct access to the basal membrane of TC monolayers, and because they do not have crypt-villi structures, the Wnt-noggin gradient no longer exists even though both components were included in the culture media used in this study.

Interestingly, reducing the concentration of CM to 5%, and thus reducing the concentration of the essential factors Wnt, noggin, and R-spondin in the media, decreased *Lgr5* stem cell populations for all cultures except colonoids and insert monolayers cultured with 5+/+ medium. When cultured in 5 +/+ medium, colonoids had a mixed cell phenotype as indicated by the comparable expression of the stem cell and colonocyte markers (*Lgr5* and *Sglt1*, respectively), increased expression of the goblet cell marker *Muc2*, and increased expression of the colonocyte differentiation marker *Ca2*. Indeed, *Ca2* expression was increased when colonoids were cultured with any medium containing 5% CM even though *Sglt1* expression was unchanged. Interestingly, TC monolayers had increased *Sglt1* expression, but unchanged *Ca2* expression when cultured with medium containing 5% CM indicating that the combination of culture medium and culture format is important for colonocyte differentiation. It could be suggested that increased colonocyte differentiation occurs in colonoids because they are three-dimensional, multi-lobed structures with crypt-villi domains, and colonocytes may localize to more luminal regions of the villi, similar to that observed *in vivo*[82], but when cultured as monolayers the crypt-villi structure is lost, thus reducing colonocyte differentiation.

Inclusion of EGF and inhibitors to the 5% CM (5 +/+) was essential for comparable expression levels of *Cga* in colonoids and TC monolayers, while inclusion of EGF alone was required to increase *Muc2* expression of colonoid and insert monolayers relative to colon tissue. Indeed, of all the cell culture formats and media formulations investigated, only TC monolayers cultured with 5+/+ medium had expression of both *Cga* and *Muc2* comparable to that of an epithelial fraction from colon. These results were unexpected; it was anticipated that the inclusion of the inhibitor to p38 mitogen-activated protein kinase (p38 MAPK) activity in the culture medium would decrease differentiation of goblet and enteroendocrine cells similar to that reported previously[10,83]. However, this increase only occurred when 5+/+ medium, and not 50+/+

medium, was used. Although the reason why 5+/+ and 50+/+ mediums should have a different effect on cultures was not investigated in this study, it could be suggested that a reduction of the essential factors Wnt, noggin, and R-spondin in the 5% medium may have been a contributing factor.

An increase in *Cldn* expression was observed for all cultures regardless of the format or medium, relative to that of an epithelial fraction from colon tissue. However, the increase in *Cldn* expression was much greater in colonoids and insert monolayers when cultured with 5% medium than when cultured with 50% medium. Interestingly, there was also a difference in the cellular composition of these cultures. For example, colonoids and insert monolayers cultured with medium containing 50% CM had comparable *Sglt1* expression, but increased *Lgr5* expression, and decreased *Muc2* expression, indicating these cultures had equivalent colonocyte populations relative to colon tissue, but increased stem cell, and decreased goblet cell populations. Conversely, those cultured with medium containing 5% CM had decreased stem cell and increased goblet cell populations.

The observation, in this study, that *Cldn* was more highly expressed in cultures with increased goblet cell populations, is in accordance with that of Pearce *et al.*[84] who reported that mouse enteroids with an increased goblet cell population had increased *Cldn−1* expression than those with increased stem cell populations. Of all the cultures investigated, *Cldn* and *Sglt1*expression was most highly expressed in TC monolayers irrespective of the medium used. In addition, *Sox9* was also increased in all TC monolayers. *Sox9* is a transcription factor that interacts with the Wnt signaling pathway and has a crucial function as a regulator of tissue homeostasis and regeneration in the intestine[85,86].

Previous studies suggest that in the intestinal epithelium low *Sox9* levels are linked with actively proliferating ISCs or rapidly dividing transit amplifying cells through Wnt/β-catenin signaling, but high levels of *Sox9* supress this process and aid in the selection of different cell types, such as enteroendocrine cells or those with reserve ISC function[87–89]. *Sox9* has also been shown to transcriptionally repress caudal-type homeobox transcription factor 2 (*CDX2*) and *Muc2* genes in colon-derived epithelial cells[90]. In addition, *CDX2* and β-catenin signaling have been implicated in the regulation of intestinal claudin genes[91]. Overexpression of *CDX2* in the colon epithelial cell line SW480 results in increased *Cldn−1* expression, while downregulation of β-catenin signaling decreased *Cldn−1* expression[91–93]. Although the expression of *CDX2* was not examined in this study, the increase in *Cldn*, *Sglt1*, and *Sox9* expression suggests that an interplay between the expression of these genes exists and warrants further investigation.

Conclusions

This study showed that cell seeding density, culture format, and medium formulation influence cellular composition, differentiation, and barrier maturation of porcine colonoids and colonoid-derived monolayers. Both cell seeding density and medium formulation impacted barrier integrity of insert monolayers, while seeding density, culture format, and medium formulation induced differential expression of genes associated with specific cell lineages in colonoids and monolayers derived from them. Indeed, a reduction in the essential factors Wnt, noggin, and R-spondin, or the exclusion of EGF or inhibitors to p38 MAPK or TGF-β activity in the culture medium, decreased the abundance of stem cells and increased differentiated cell types to levels comparable to that of the tissue of origin, especially those of the secretory goblet cell lineage, in colonoids and colonoid-derived monolayers.

However, a limitation of this study was that insert, and TC monolayers were always derived from colonoids. An alternative approach would be to develop colonocyte monolayers from colonoids, expose them to alternative medium formulations and subsequently passage and reseed as new monolayers. This approach would not only allow for comparisons to be made to colonoids and an epithelial fraction from colon tissue, but also to the colonoid-derived monolayers. It is possible that these alternative monolayers may be more similar to that of native tissue than the already developed models and consequently, may provide increased confidence in using such cultures as representative models of the *in vivo* situation.

Abbreviations

ADF, Advanced DMEM/F12; Anova, analysis of variance; ATCC, American Type Culture Collection; BM, Basal medium; BSA, bovine serum albumin; CA2, carbonic

anhydrase 2; CM conditioned media; CGA, chromogranin A; CLDN, claudin; DMSO, dimethyl sulfoxide; EDTA, ethylene diamine tetra acetic acid; FBS, fetal bovine serum; IDT, Integrated DNA Technologies; Ins, insert; ISC, intestinal stem cell; LGR5, Leucine-rich repeat-containing G protein-coupled receptor 5; MAPK, p38 Mitogen-activated protein kinase; MUC2, mucin 2; OCLN, occludin; PBS, Phosphate-buffered saline; Quantitative Real-Time PCR (qPCR); RIN, RNA integrity number; ROCK, Rho-associated Coiled-coil Kinase; RRID, research resource identifier, RPL4, Ribosomal protein L4; RT, room temperature; SGLT1, sodium-glucose transporter 1; SOX9, Sex-determining region Y (SRY) – box 9; TBP, Tata box protein; TC, tissue culture; TEER, trans epithelial electrical resistance, TGFβ, transforming growth factor beta; ZO1, zonula occludens 1.

Author contributions

Alicia M. Barnett conceived and designed the experiments; Alicia M. Barnett performed the experiments and analyzed the data; Alicia M. Barnett evaluated the literature; Alicia M. Barnett, Jane A Mullaney, Nicole C. Roy, and Warren C. McNabb have contributed to the interpretation of results and the writing of the manuscript. Nicole C. Roy and Warren C. McNabb sourced the funding for the research.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Institutional review board statement

The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of Massey University, Palmerston North, New Zealand (protocol code #19/83).

Data availability statement

The datasets and raw data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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