Research Article

Characterization and Optimization of Variability in a Human Colonic Epithelium Culture Model

Colleen M. Pike*, Bailey Zwarycz*, Bryan E. McQueen†, Mariana Castillo†, Catherine Barron†, Jeremy M. Morowitz†, James A. Levi†, Dhiral Phadke‡, Michele Balik-Meisner‡, Deepak Mav‡, Ruchir Shah‡, Danielle L. Cunningham Glasspoole†, Ron Laetham†, William Thelin†, Maureen K. Bunger† and Elizabeth M. Boazak†

*Authors contributed equally

†Altis Biosystems, Inc. Durham, NC, USA; ‡Sciome, Durham, NC, USA

Abstract

Animal models have historically been poor preclinical predictors of gastrointestinal (GI) directed therapeutic efficacy and drug-induced GI toxicity. Human stem and primary cell-derived culture systems are a major focus of efforts to create biologically relevant models that enhance preclinical predictive value of intestinal efficacy and toxicity. The inherent variability in stem-cell-based cultures makes development of useful models a challenge; the stochastic nature of stem-cell differentiation interferes with the ability to build and validate reproducible assays that query drug responses and pharmacokinetics. In this study, we aimed to characterize and reduce sources of variability in a complex stem cell-derived intestinal epithelium model, termed RepliGut® Planar, across cells from multiple human donors, cell lots, and passage numbers. Assessment criteria included barrier formation and integrity, gene expression, and cytokine responses. Gene expression and culture metric analyses revealed that controlling cell passage number reduces variability and maximizes physiological relevance of the model. In a case study where passage number was optimized, distinct cytokine responses were observed among four human donors, indicating that biological variability can be detected in cell cultures originating from diverse human sources. These findings highlight key considerations for designing assays that can be applied to additional primary-cell derived systems, as well as establish utility of the RepliGut® Planar platform for robust development of human-predictive drug-response assays.

Plain language summary

Animal models are frequently used as tools for studying gastrointestinal (GI) disease, but they poorly replicate the complexities of the human gut limiting the clinical translation of new therapeutics in development. Human stem cell derived models can better recapitulate human GI physiology, but the inherent dynamic nature of stem cells introduces variability in culture performance. We identified sources of variability in the primary stem-cell derived RepliGut® Planar model to develop robust and reliable assays that can improve preclinical therapeutic development for GI disease. Analysis of barrier formation, gene expression, and cytokine responses demonstrated that controlling cell passage number reduces variability and maximizes physiological relevance of the model. These findings highlight key assay design considerations that can be applied to additional primary-cell derived systems. Availability of reliable and physiologically relevant cell-based models can reduce animal testing, improve research accuracy, and make new treatments more relevant and effective for patients.

1 Introduction

Gastrointestinal diseases encompass a diverse range of complex and multifaceted conditions that pose significant challenges for both clinical management and preclinical drug discovery. As the understanding of the intricate mechanisms underlying these diseases evolves, there arises an increasing need for advanced research models that can recapitulate the complex microenvironment
of the gastrointestinal tract. Animal models have been pivotal in advancing our understanding of gastrointestinal diseases, yet the limited human relevance of animal models is a significant concern, as differences in gastrointestinal anatomy, physiology, and immune responses may hinder the direct translation of findings to human conditions (Olson et al., 2000; Monticello et al., 2017). In particular, the limitations of traditional in vivo models and the ethical considerations associated with human studies underscore the crucial role of in vitro models in advancing our understanding of gastrointestinal diseases.

In vitro models offer multiple advantages over animal models by providing controlled and reproducible environments that closely mimic the complexities of the human gastrointestinal (GI) tract. The intricate interplay of diverse cell types, tissue architecture, and dynamic physiological conditions within the gut necessitates models that enable systematic investigation under carefully regulated parameters. These models allow for the precise manipulation of specific cell types, genetic factors, and environmental conditions, facilitating the elucidation of complex disease processes such as inflammation, epithelial barrier dysfunction, and dysbiosis (Creff et al., 2021). Moreover, cell-based models provide a platform for high-throughput screening of potential drug candidates and personalized medicine approaches, thereby accelerating the discovery and development of novel therapeutics for digestive diseases (Wang et al., 2022).

Several in vitro models have been employed to study intestinal diseases, each with their unique advantages and disadvantages. Organoids, three-dimensional structures derived from primary tissues or pluripotent stem cells, offer a remarkable representation of intestinal architecture and function that recapitulate the cellular diversity and physiological features of the intestine, making them highly relevant for disease modeling (Sato et al., 2009; Gracz and Magness, 2014; Dutton et al., 2019; Yoo and Donowitz, 2019). However, their complexity can be a drawback, as maintaining and manipulating organoids can be technically challenging, and the variability between batches may impact reproducibility. They also have limited ability to model epithelial barrier integrity, owing to their spherical shape, fully-interior lumen and low throughput (Wang et al., 2017; Dutton et al., 2019; Franco et al., 2021). Microfluidic systems, such as gut-on-a-chip devices, aim to overcome some limitations by simulating the dynamic microenvironment of the GI tract, offering precise control over fluid flow, nutrient gradients, and cell interactions (Marrero et al., 2021). Like organoids, these systems can be technically demanding to set up, and the sheer complexity of recreating the dynamic aspects of the intestine may introduce challenges in interpretation. Cell lines, such as the commonly used Caco-2 derived from human colon adenocarcinoma, are widely used for drug permeability studies. Their simplicity and ease of use make them advantageous for high-throughput screening. However, the monolayer structure does not fully capture the diverse cell types and complexity found in the native intestine (Sambuy et al., 2005; Lennermäki, 2007; Press and Grandi, 2008; Sun et al., 2008; Larregue and Benet, 2013; VanDussen et al., 2015)). Additionally, these cell lines may undergo genetic changes over time, potentially impacting their relevance to the disease.

Within the past several decades, there has been a notable rise in the availability and utilization of commercially available in vitro models, reflecting advancements in technology and the growing demand for more physiologically relevant systems for studying GI conditions. Micro-engineered organ-on-a-chip platforms, as developed by Emulate, Inc. are valuable for studying dynamics between multiple cell types such as epithelium and endothelium (Apostolou et al., 2021). OrganoReady® (Mimetas) is a microfluidic gut-on-a-chip technology comprised of Caco-2 or colon organoids grown in 3D tubules, specialized for disease modeling and drug transport studies (Beaurivage et al., 2019). EpiIntestinal™ (MatTek), a small intestinal model comprised of epithelial cells, fibroblasts, and endothelial cells derived from normal human intestinal cells, more accurately predicts diarrhea incidence of chemotherapies (Peters et al., 2019) than Caco-2 models. While these commercially available models are undoubtedly contributing to the unravelling of the complexities of gastrointestinal diseases and accelerating drug discovery, they come with several caveats, as these systems do not implement primary cells, are low throughput, and can be technically challenging, requiring specialized microfluidics technology.

To overcome the limitations of current in vitro models, the RepliGut® Planar model was developed which is comprised of cultured colonic epithelial stem cells developed into a differentiated epithelium on a 2-dimensional (2D) scaffold in conventional Transwell® inserts (Wang et al., 2017). The model uses stem cells derived from colonic crypts that are isolated from clinically normal intestine of post-mortem transplant grade donors. Cells are plated onto a proprietary membrane-supported biomimetic hydrogel that enables differentiation and polarization into an epithelial monolayer. During polarization, the cells establish an asymmetrical distribution of apical and basolateral membranes. The RepliGut® Planar model mimics the in vivo intestine by displaying the full repertoire of proliferative (stem and transit-amplifying progenitor) and differentiated (enterocyte, goblet, and enteroendocrine) cell types, forming an impermeable barrier with high transepithelial electrical resistance (TEER), and expressing key transporters and metabolic enzymes at the gene, protein, and functional levels. The 2D planar geometry of this platform allows access to both the luminal/apical and basolateral surfaces of the polarized epithelium, mimicking interactions between the epithelium and orally administered drugs present in the intestinal lumen and intravenously administered systemic drugs, respectively.

Stem cell-derived cultures, though promising for modeling intestinal diseases, are accompanied by inherent variability that poses challenges in maintaining consistency and reproducibility (Grossmann et al., 2003). The differentiation of stem cells into specific cell types of the intestine involves a complex interplay of signaling pathways and environmental cues, leading to variability in the resulting cultures (Grossmann et al., 2003; Sato et al., 2009; Ahmad et al., 2014). Variations in differentiation efficiency, cell maturation, and tissue organization can arise, impacting the functional characteristics of the derived intestinal tissues. Standardized and reproducible methods are needed in the generation of stem cell-derived intestinal tissues to ensure consistency across experiments. Addressing this inherent variability is crucial for enhancing the reliability of findings and advancing the utility of stem cell-derived cultures as robust models for studying intestinal diseases (Mohammadi et al., 2021).
The objective of this study was to identify factors that contribute to variability in the RepliGut® Planar primary stem cell-derived intestinal culture system to develop robust and reliable assays that can advance our understanding of intestinal physiology and applications in GI disease. Specifically, we assessed the impact of cell passage number, lot-to-lot variability, and donor-to-donor variability by examining intestinal barrier formation and maintenance through transepithelial electrical resistance (TEER), conducting gene expression analysis, and evaluating cytokine responses. We identified cell passage number as a significant source of variability affecting barrier maintenance and gene expression profiles. Evaluation of four human donors showed limited variability between donors when assessing TEER kinetics, but variations were evident in responses to the proinflammatory cytokines tumor necrosis factor-alpha (TNFα) and interferon-gamma (IFNγ). These variations were observed in TEER kinetics, half-maximal inhibitory concentrations (IC50), lactate dehydrogenase (LDH) activity and chemokine release. Our findings highlight that when sources of variability are controlled, donor-specific differences in response to inflammatory stimuli persist, which is reflective of inter-individual biologic variability. Clinically validated TNFα- and IFNγ antagonists, adalimumab and tocilizumab, mitigated cytokine-induced barrier disruption and cytotoxicity, demonstrating the utility of the RepliGut® Planar as a tool for screening inflammatory mediators. Together, these results identify key considerations for designing robust assays that can be applied to additional primary-cell derived systems, enhancing the reliability and translational potential of stem cell-derived models in applications such as drug discovery and disease modeling.

2 Materials and methods

2.1 Cell culture

Human intestinal tissue was obtained post-mortem via an established Organ Procurement Organization following consent of family under strict ethical guidelines established by the Organ Procurement Transplantation Network (OPTN)1. All donors tested negative for HIV I/II, Hepatitis B (HBcAB, HBsAG), and Hepatitis C (HCV) and were free of known intestinal diseases. Intestinal crypts were isolated from human transverse colon, expanded under sub-confluent conditions, and cryopreserved essentially as described (Grossmann et al., 1998; Wang et al., 2017). Cell lots were defined as a single vialing and freeze-down process of cells pooled following expansion. A vial of frozen crypts were expanded for a single passage to generate sufficient cell numbers for an individual RepliGut experiment. For each experiment, vials were rapidly thawed in a 37°C water bath directly and seeded on a 12- or 96-well Transwell® plate (Corning 3460 or 3392) coated with a propriety hydrogel at a density of 5-8 x 10^4 cells/cm² in RepliGut® Growth Medium (RGM, Altis Biosystems, Durham, NC) containing Fetal Bovine Serum (FBS, R&D Systems, S11150H). 12-well Transwell® plates were used in gene expression analyses to collect a sufficient amount of cellular material and cell imaging experiments, whereas all other assays were performed in 96-well Transwell® plates for higher throughput analyses. Once cells reached confluence, media was changed to RepliGut® Maturation Medium (RMM, Altis Biosystems, Durham, NC) containing FBS to promote cellular differentiation and polarization. Future studies aim to replace FBS in cell culture media to reduce use of animal-derived products. Media volumes were 1000 µl/2000 µl apical/basal for 12-well plates and 100 µl/200 µl apical/basal for 96-well plates. Media was changed every 48 hours except for the switch to RMM which was based on confluence without regard to timing of the previous media change. Cells were visually monitored daily using an Entry Level Research Grade Inverted Microscope (Fisher Scientific, Entry Level Research Grade Inverted Microscope).

2.2 Cell viability

Cryopreserved cell vials were thawed, centrifuged, and resuspended in 1 ml 1X PBS. Cell solutions were aliquoted into four separate microcentrifuge tubes to serve as technical replicates. Cells were mechanically dissociated with a 28G needle and diluted 1:1 in trypan blue (Invitrogen). Viability was then measured using a Countess™ II automated cell counter (ThermoFisher).

2.3 Transepithelial electrical resistance (TEER)

Barrier integrity of cell monolayers was assessed via TEER using an Epithelial Volt/Ohm Meter (World Precision Instruments, EVOM2 or EVOM3) and STX100C96 electrode for 96-well cultures or STX2 electrode for 12-well cultures. TEER was measured daily during experiments. Percent change in TEER of cytokine-treated samples (TNFα or IFNγ) from vehicle was calculated using the following equation: \(-((\text{Average TEER}_{\text{Vehicle}} - \text{TEER}_{\text{Vehicle}})/\text{Average TEER}_{\text{Vehicle}})\times100\). IC50 were calculated using three-parameter nonlinear regression in Prism software (GraphPad Software, La Jolla, CA). Curve fitting was performed for all experimental runs, with an R-squared threshold of 0.6 applied as the minimum acceptable value and bounds on the 95% confidence interval of the computed IC50 within the range of doses tested. Runs that failed to achieve these quality metrics were deemed unsuccessful, primarily due to an inadequate dose range for a reliable curve fit and were consequently excluded from the reported TEER-based dose response metrics.

2.4 Cell fixation & staining

For 5-Ethynyl-2′-deoxyuridine (EdU) staining, cells were pulsed with 10 µM EdU 24 hours prior to fixation with 4% paraformaldehyde. EdU incorporation was detected according to the manufacturer’s protocol using Click-iT™ EdU Alexa 488 kit (Thermo Fisher, C10337). Alkaline Phosphatase (ALP) staining was performed on live cells using VECTOR Red ALP substrate.

1 https://optn.transplant.hrsa.gov/
kit (Vector Laboratories Cat#SK-5100) with a 30-minute incubation, according to the manufacturer’s instructions. The primary antibodies used in this study were as follows: Chromogranin A (CHGA, Abcam Cat#ab151560), Mucin 2 (MUC2, Santa Cruz Cat#sb-515032), Zonula Occludins-1 (ZO-1, Proteintech Cat#66452-1-lg), or E-cadherin (Proteintech Cat#20874-1-AP). Secondary antibodies were as follows: Alexa Fluor™ 594 Goat Anti-Rabbit antibody (Jackson ImmunoResearch, Cat#111-585-003), Alexa Fluor™ 488 Goat Anti-Mouse antibody (Jackson ImmunoResearch, Cat#111-545-003), or Alexa Fluor™ 647 Goat Anti-Mouse antibody (Invitrogen, A21236). For immunocytochemistry staining, cells were fixed in cold 100% methanol (ZO-1 and E-Cadherin) or in 4% paraformaldehyde (MUC-2 and CHGA) for 30 minutes. Fixed cells were permeabilized using 0.5% Triton X-100 (Promega, Cat#H5142). Primary antibodies were added at 1:250 dilution in 1X Animal-Free Blocking Solution (Cell Signaling Technology, 15019-L) to the apical side of each Transwell® for an overnight incubation at 4°C. Secondary antibodies and Hoechst 33342 Stain (Invitrogen, Cat#H3570) were diluted 1:1,000 in 1X Animal-Free Blocking Solution and incubated on the apical side of each Transwell® for 1 hour.

2.5 Imaging
EdU images were acquired with the 10x objective lens using the ImageXpress® Nano Automated Imaging System with MetaXpress Software version 6.5.4.532 (Molecular Devices). The post-laser offset and exposure time were adjusted to acquire a focus point that was comparable across the plate. Images of cell lineage and tight junction staining were acquired on an Olympus IX2-UCB microscope (Olympus, Shinjuku City, Tokyo, Japan).

2.6 Histology
Hematoxylin and Eosin (H&E) and Alcian Blue/Periodic Acid Schiff (AB/PAS) staining were performed on sections from 12-well RepliGut® Planar Donor 5 culture fixed in 4% paraformaldehyde on day 3 in RMM, following the same fixation methods as listed above. After confirming all tested Donors displayed similar TEER kinetics and culture lifespan duration, Donor 5 was chosen as a representative sample for H&E staining. Fixed tissues were processed on a Leica ASP 6025 tissue processor, embedded in paraffin (Leica Paraplast), and sectioned at 5 µm thickness. Tissue sections were baked at 60°C for 60 minutes, deparaffinized in xylene, hydrated with graded ethanols, and stained with H&E or AB/PAS using a Leica Autostainer XL. For H&E, slides were stained with Hematoxylin (Richard-Allen Scientific, 7211) for 2 minutes and Eosin -Y (Richard-Allen Scientific, 7111) for 1 minute. Clarifier 2 (7402) and Bluing (7111) solutions from Richard-Allen Scientific were used to differentiate the reaction. For AB/PAS, the slides were stained with Alcian Blue (Anatech, LTD, 867) for 10 minutes, immersed in Periodic Acid (ThermoFisher Scientific, A223-100) for 5 minutes, rinsed in water, then transferred to Schiff reagent (Fisher Scientific, SS32-500) for 30 minutes followed by a Sulfurous rinse for 1 minute, and washed in running non-DI water for 10 minutes. Histology images were captured using the 40x objective on a Leica DMi8 microscope with an Amscope 18MP Aptina Color CMOS camera and AmScope software (Version 4.11.18421).

2.7 Gene expression
For gene expression profiling, proliferative cells were collected at 3 days post-plating in RGM media and differentiated cells were collected 3 days after switching cultures to RMM media. At the time of collection, cells were rinsed once with 1X phosphate-buffered saline (PBS) and then collected in 500 µl per Transwell® of RNA Lysis Buffer from the Ambion RNAqueous kit (Invitrogen AM1912). The Ambion RNAqueous kit was used to isolate RNA based on manufacturer’s protocol. RNA concentration was quantified via Qubit. Reverse transcription was performed using the iScript™ cDNA Synthesis Kit (BioRad, 1708891). Gene expression analysis using the Biomark HD qPCR System and Dynamic Array IFCs for Gene Expression (Fluidigm) was performed at the Advanced Analytics Core Facility at the University of North Carolina at Chapel Hill School of Medicine. Taqman® probes were purchased from ThermoFisher Scientific. Assay IDs for all analyzed genes are found in Table S1. Gene expression analysis was performed using qPCR and included 91 genes corresponding to known identity and functional markers of intestinal epithelium, including enterocyte, secretary, metabolism, and inflammatory signaling related genes (Tab. S1). Relative gene expression (2-ΔΔCT ) for each gene was calculated by comparing the sample to the average proliferative cells value from that donor.

In the cell passage number analysis, CT values were converted to ΔCT using 18S as the housekeeping gene. All subsequent analysis included the 91 remaining genes (i.e., all genes except 18S). Principal component analysis, hierarchical cluster analysis, and inter-replicate correlation analysis were performed to assess sample quality and agreement between replicates in each passage. Corresponding quality control (QC) plots were generated and used to determine outlier samples and assess similarity between each passage group. Heatmaps of ΔCT values for the set of 91 genes (including housekeeping genes GAPDH and ACTB) were generated to confirm the findings from the QC analysis. Outliers were removed, and all analysis and plotting were re-performed to assess the final set of samples. Differential expression analysis was performed using a generalized linear model (GLM) with the passage number as a factor. This analysis used linear regression to compare all the passages. Foldchange, p-value, and adjusted p-value (Benjamini and Hochberg, 1995) were calculated. Differentially expressed genes (DEGs) were identified for the comparison of each higher passage (5, 10, or 15) with passage 2. To be considered a DEG, a gene had to meet the following criteria: Absolute foldchange ≥ 1.5 and Adjusted p-value ≤ 0.05.

https://doi.org/10.14573/altex.2309221s1
2.8 Proinflammatory cytokine treatments

Data from 42 experiments were evaluated to compare TNFα responses across multiple cell lots. In each experiment, cells were treated with increasing concentrations of TNFα (R&D Systems, 210-TA) in the apical and basal compartments on day 2 in RMM. TEER was measured at 24 and 48 hours after the start of treatment. IC50s were calculated using three-parameter nonlinear regression in Prism software (GraphPad Software, La Jolla, CA).

To compare cytokine responses across multiple donors, a single experiment was performed across four donors comparing TEER, LDH activity, and cytokine release (IL-8 and CXCL-11) in response to increasing concentrations of TNFα or IFNγ (0.1, 0.3, 1, 3, 10, 30, 100, and 300 ng/ml; PeproTech, 300-02). Clinical inhibitors adalimumab (Selleck Chemicals A2010) or tofacitinib (Selleck Chemicals S2789) were added with or without simultaneous treatment of TNFα or IFNγ, respectively. Cells were treated on day 2 in RMM and TEER was measured at 24 and 48 hours after the start of treatment. Supernatants were collected at the end of the experiment for LDH activity and ELISA experiments.

2.9 LDH cytotoxicity assay

Cytokine-induced cytotoxicity was measured using the CyQUANT™ LDH Cytotoxicity Assay (Invitrogen, C20300). Supernatant was collected from both apical and basal compartments at 48 hours post treatment and pooled in a representative ratio of total media (25 µL apical and 50 µL basal). LDH activity was determined on the combined media following the manufacturer’s protocol. Absorbance was measured at 490 nm and 680 nm using a BioTek Synergy H1 plate reader. The absorbance of the media blank was subtracted from each sample, followed by subtracting the absorbance at 680 nm (background) from the absorbance at 490 nm. Lysed cells served as a maximum LDH release control.

\[ \% \text{Cytotoxicity} = \left( \frac{(490 \text{ nm}-680 \text{ nm}) \text{ of sample}}{(490 \text{ nm}-680 \text{ nm}) \text{ of max LDH}} \right) \times 100 \]

Dose response curves were generated using three parameter nonlinear regression. R-squared values comparing TEER to cytotoxicity were determined using nonlinear best fit line.

2.10 Enzyme-linked immunosorbent assays (ELISAs)

Interleukin 8 (IL-8) and C-X-C motif chemokine 11 (CXCL11) ELISAs were performed using basal supernatants collected 48 hours after cytokine treatments using IL-8 Human ELISA Kit (ThermoFisher, Cat# KHC0081) and Human CXCL11/I-TAC Quantikine ELISA Kit (R&D Systems, Cat# DCX110), respectively, following the manufacturer’s protocol. Absorbance at 450 nm was measured using a BioTek Synergy H1 plate reader. Background absorbance was subtracted from all data points, including standards, samples, and controls, prior to plotting. A standard curve was generated using a sigmoidal four parameter algorithm on BioTek software from which concentrations of detected proteins were calculated.

2.11 Statistical analyses

All statistical tests were performed in GraphPad Prism 9 (GraphPad, CA, USA). Statistical significance was set at a P value of <0.05 for all analyses. Kruskal-Wallis test was used to identify significant differences in TEER metrics between cell passage numbers. One-way ANOVA with Tukey’s multiple comparison test was used to test for significance between cytokine treatments with and without adalimumab and tofacitinib.

3 Results

3.1 Sequential proliferation and differentiation over the RepliGut® Planar culture lifespan

Transverse colon intestinal epithelial cells were derived from post-mortem transplant grade donors and do not have a disease status. Demographics of the donors used in this study are shown in Table 1. A schematic of the RepliGut® Planar model with the time-course of development and representative TEER is shown in Figure 1. Crypt-resident intestinal proliferating cells were first plated at sub-confluence onto hydrogel-coated transwell membrane inserts. Barrier formation and maintenance throughout the culture duration are assessed via TEER (Figure 1A) (Gunasekara et al., 2018). Cells are grown to confluence using RepliGut® Growth Medium (RGM) formulated to promote cell proliferation. Once cells reach confluence (4-6 days), RGM is removed and replaced with a maturation media (RMM) to promote cellular polarization and differentiation into post-mitotic lineages (Figure 1A) (Gracz and Magness, 2014). Confluence is initially observed via brightfield microscopy and then confirmed by an increase in TEER above 250 Ω·cm², the TEER threshold that indicates impermeable barrier formation. TEER continues to increase for 2 days followed by a 3–5-day plateau phase (Figure 1B). The TEER plateau begins on the day when the average TEER of the 96-well plate meets or exceeds 75% of peak TEER and continues until TEER drops below 60% of peak TEER and/or the coefficient of variation (% CV, defined as the ratio of the standard deviation to the mean) between wells exceeds 25%. The TEER plateau represents the timeframe that the cells are fully differentiated with intact tight junctions. A drop in TEER indicates the end of the culture lifespan. Like in vivo physiology, intestinal epithelium in cell culture has a finite lifespan once fully differentiated, lasting 3-10 days after differentiation begins (Snippert et al., 2010).
A unique characteristic of the RepliGut® Planar model is the ability to induce the transition from a proliferative cell state to a differentiated confluent epithelium sequentially over the course of 10-12 days in culture. To characterize the lifespan of the RepliGut® Planar model, markers of cell proliferation and differentiation at multiple time points in culture were assessed via microscopy and gene expression. Cells were pulsed with EdU for 24 hours to assess the proliferative capacity on days 1 through 8 of culture (Figure 2A) with a change from RGM to RMM on day 4 corresponding to observed confluence. Representative micrographs show positive EdU staining on days 2-4 in culture (Figure 2A). On days 5 and 8, EdU staining was not detected, demonstrating that cells lost their proliferative capacity after the addition of RMM on day 4. Qualitative assessment of differentiated cell markers revealed the presence of absorptive and secretory cell lineages, as seen through positive staining of ALP, MUC2, and CHGA (Figure 2B). E-cadherin and ZO-1 staining of differentiated cells also indicated the formation of tight junctions between days 4-6 in culture (Figure 2B).

To confirm that cells had polarized after the addition of RMM, we evaluated hematoxylin and eosin (H&E) staining of histological sections of cells derived from one human donor (Donor 5) on day 3 in RMM. H&E staining revealed a polarized columnar epithelium (Figure 2C). We further assessed the presence of mucopolysaccharides via AB/PAS staining. Positive staining was localized to the apical surface, indicating the presence of mucus-producing cells with correct directional secretion (Figure 2C), further confirming the differentiated and polarized cell physiology.

To support these morphological observations, we assessed expression of genes associated with cellular proliferation and differentiation from Donor 5 on Day 3 post-plating (proliferative phase), and Day 3 in RMM (differentiated phase) corresponding to the days of highest and lowest EdU incorporation. Relative to proliferative cells, differentiated cells had lower expression of genes associated with proliferation (MKI67, LGR5 and SOX9), and higher expression of genes associated with differentiation (SI, ALPI, ANPEP and FAPB6) (Figure 2D). All differences in gene expression between proliferative cells were significant except for SI (t-test with Welch’s correction, p<0.05). Taken together, these data indicate that the RepliGut® Planar model is comprised of cells with transient proliferative capacity that can differentiate into multiple cell lineages, mimicking the cellular morphology of human intestinal epithelial cells (Gracz and Magness, 2014; Bhatt et al., 2018; Rees et al., 2020).

### 3.2 Cell passage number is a source of variability in the RepliGut® Planar model

To determine in cell passage number contributed to variability in the RepliGut® Planar model, gene expression profiles were compared between passage 2, 5, 10 and 15-derived models. Genes were analyzed relative to passage 2, the earliest feasible plating of the RepliGut® model. Principal component analysis, hierarchical cluster analysis, and inter-replicate correlation analysis of ΔCt values revealed that passage 15 samples separated from the rest of the passages (Figure 3A, 3B). Differential expression analysis performed using a generalized linear model identified 29 DEGs between p15 vs p2, 2 DEGs between p10 vs p2, and no DEGs between p5 vs p2 (Fig 3C).

---

**Fig. 1: Transverse colon RepliGut® Planar Model**

(A) Cross section schematic of the RepliGut® Planar Transverse Colon model comprised of multiple epithelial cell lineages. Cells derived from transverse colon stem and progenitor cell populations are seeded on a Transwell® cassette coated with a biomimetic scaffold and provided with differentiation cues post-confluence. (B) Representative TEER curve of RepliGut® Planar Transverse Colon over culture time course. The relative timeframe of each key measurement, time to confluence, time to plateau, and plateau length, that are used to characterize barrier formation and integrity of the cell monolayer are denoted. Data are represented as mean ± SD; n=3 technical replicates.

**Tab. 1: Donor characteristics**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Race</th>
<th>Height (in)</th>
<th>Weight (lbs)</th>
<th>BMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23</td>
<td>Male</td>
<td>Caucasian</td>
<td>75</td>
<td>182.1</td>
<td>22.8</td>
</tr>
<tr>
<td>4</td>
<td>51</td>
<td>Female</td>
<td>Caucasian</td>
<td>59</td>
<td>158.4</td>
<td>32.0</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>Male</td>
<td>African American</td>
<td>70</td>
<td>162.0</td>
<td>22.7</td>
</tr>
<tr>
<td>6</td>
<td>51</td>
<td>Male</td>
<td>African American</td>
<td>70</td>
<td>219.0</td>
<td>31.3</td>
</tr>
</tbody>
</table>

---

**ALTEX, accepted manuscript**

published April 18, 2024

doi:10.14573/altex.2309221
Fig. 2: Phase specific model characteristics

(A) Representative images of EdU incorporation over time. (B) Representative images of fully differentiated cells stained for cell proteins ALP (red), MUC2 (green), and CHGA (red), DAPI (blue) and tight junction proteins ZO-1 (green) and E-cadherin (red). (C) Representative histology image of monolayer cross sections in the differentiated phase stained for H&E (top) and for AB/PAS (bottom). (D) Gene expression of proliferative and differentiated cell genes in cells in proliferative and differentiation phases. 2ΔΔCt values were calculated, normalized to a 18S housekeeping gene and reported relative to proliferative phase cells. *p< 0.05, t-test with Welch’s correction. Data are represented as mean ± SD; n=3 biological replicates.

We next investigated whether gene expression patterns among different cell passage numbers related to functional and morphological differences during differentiation. Barrier integrity was assessed daily using TEER until day 8 in RMM from p5, p10, and p15-derived models during maturation phase. Since it was demonstrated that p5 exhibits relatively few differences in gene expression compared to p2, the analysis did not incorporate p2 cells. TEER curves from four independent runs were generated and averaged for each cell passage to observe time-driven variability in barrier formation and integrity over the duration of the RepliGut® Planar culture (Figure 3D, Figure S1A-C). No significant differences were observed in time to confluence (p>0.05, Kruskal-Wallis test; Figure S1A). P15 cells reached significantly lower peak TEER than p10 cells (p=0.04, Kruskal-Wallis test; Figure 3D). By day 2 in RMM, p5 cells reached a peak TEER of $1826 \pm 366 \Omega \cdot \text{cm}^2$ and p10 cells reached a peak TEER of $1949 \pm 169 \Omega \cdot \text{cm}^2$, whereas p15 cells only reached a peak TEER of $1032 \pm 324 \Omega \cdot \text{cm}^2$ by day 4 in RMM (Figure S1B). Relative to p15 cells, p10 and p5 cells had a longer TEER plateau length with a significant difference between p10 and p15 (p=0.0381, Kruskal-Wallis test; Figure S1C). The median plateau lengths were 4.5, 5, and 3 days for p5, p10 and p15 cells, respectively (Figure S1C).
Fig. 3: Cell passage number is a source of variability
(A) Hierarchical cluster analysis heatmap of the 91 tested genes (by ΔCt value) of passage 2, 5, 10 and 15 cells. (B) Principal component analysis of the different cell passage numbers. In (A) & (B), each data point represents a single biological replicate. Three replicates were collected across 2 (passage 2) or 3 (passes 5-15) different independent experiments; experiments comprised a single run of one cell lot and one or two runs of a second cell lot. (C) Number of differentially expressed genes of each cell passage number relative to passage 2 cells. (D) TEER of the different cell passage numbers. Data are represented as mean ± SD of ≥ 3 technical replicates per experiment from 4 experiments per passage (2 experiments for each of 2 cell lots).

3.4 Barrier formation and maintenance metrics are consistent across donors
Having determined that p10 cells resulted in cultures with similar barrier formation kinetics and gene expression patterns to p2 cultures, we moved forward to characterize donor-donor, lot-lot, and run-run variability in p10 cultures from 18 independent cell production lots across 4 donors (n = 5, 3, 9, and 1 cell lots for Donors 1, 4, 5, and 6, respectively). Donors 2 and 3 have yet to be characterized and were excluded from analysis. Post-thaw viability of ≥ 75% (87 ± 6%) was observed for all cell lots generated (Figure 4A), with no significant differences between Donors (Kruskal-Wallis test). Time to achieve culture confluence (Figure 4B) ranged from 2 to 9 days, with medians of 4, 4, 4, and 5 days for Donors 1, 4, 5, and 6, respectively. No significant differences were observed between donors in time to confluence (Kruskal-Wallis test).

Identifying an appropriate and reproducible assay window is essential to the utility of in vitro cell culture models. We sought to identify this assay window by characterizing the variability and kinetics of barrier formation and maintenance via TEER. TEER was measured across multiple days and multiple experiments using the 18 lots across four Donors described above, representing a total of 51 experiments. To allow comparison across lots and experiments, maximum TEER within each experiment was determined and all other TEER values were normalized to the within-run peak TEER. Out of the 51 experiments included in this analysis, only four runs did not reach 75% of peak TEER by Day 2 in RMM. Using a within run acceptable variability cutoff of CV<25%, only two experiments (Figure 4D) exceeded the acceptable level on Day 2 in RMM (Donor 4, one of which carried on to Day 3), and only one experiment exceeded the acceptable level on Day 5 in RMM (Donor 1). Together, this data supports predictable culture behavior with lowest experimental variability between Days 3 and 5 in maturation media, providing a clear 72-hour assay window during TEER plateau that enables meaningful assessment of barrier function using TEER. Figure 4D also includes within-plate variability data for TEER at Day 0 in RMM. Many cultures at this timepoint still demonstrated some degree
One data point from these data suggest an inherent variability in TEER. We suspect this to correspond to some instances of spontaneous differentiation; as cultures mature, within-plate variability in TEER diminishes.

The cytokine signaling molecule TNFα plays a significant role in the etiology of inflammatory bowel diseases. In addition to cytotoxicity, this proinflammatory cytokine promotes epithelial cell-chemokine release to recruit and activate immune cells involved with tissue damage and repair (Dwinell et al., 2001; Kucharzik et al., 2005; Treede et al., 2009; Sonnier et al., 2010; Friedrich et al., 2019). To investigate whether donors have a variable response to TNFα, we analyzed TEER reduction at 24 and 48 hours post-TNFα treatment in multiple cell lots from all four donors. At 24 hours post-treatment, Donors 1 and 4 had greater reductions in TEER compared to Donors 5 and 6, which increased to comparable levels by 48 hours (Figure 4E). At 24 hours post-treatment, significant differences were detected between Donors 1 and 5, Donors 1 and 6, and Donors 4 and 5 (Two-way ANOVA with Tukey’s multiple comparisons test). At 48 hours post-treatment, significant differences were detected between Donors 1 and 5, and Donors 4 and 5 (Two-way ANOVA with Tukey’s multiple comparisons test). Data shown is from 43 of 51 experiments represented in Figure 4 C & D; not all experiments included TNFα dosing. Additionally, 9 data points (six from Donor 1 and two from Donor 4) were removed from the 48-hour data sets due to a smaller percent change in TEER observed as compared to the same wells at 24 hours, a phenomenon believed to result from excess cell debris following cell death. One data point from Donor 5 was removed from all TNFα analyses due to a lack of TEER response at any dose tested, believed to result from a technical error.

To further characterize donor-to-donor variability, the 48-hour IC_{50} for percent change in TEER were compiled (Figure 4F). The IC_{50}s and 95% confidence intervals for Donors 1, 4, 5, and 6 were 7 [6, 8], 10 [8, 12], 25 [24, 27] and 11 [9, 13] ng/mL, respectively. The 95% confidence intervals on the IC_{50} reflect low variability; only for a single donor, 5, did the range of all observed IC_{50}s exceed one log. A significant difference in IC_{50} was only observed between Donors 1 and 5 (Kruskal-Wallis, p=0.0062). Together, these data suggest an inherent variability in biological sensitivity to proinflammatory insult that increases over time.
3.5 Human donors exhibit varying responses to proinflammatory cytokines

Having identified culture conditions where baseline lot to lot and experimental variability are minimized across donors, we tested functional responses to the pro-inflammatory cytokines TNFα and IFNγ that are commonly involved in manifestation of IBD (Andreou et al., 2020; Gareb et al., 2020). All donors elicited a dose-dependent response characterized by TEER reduction and LDH increase to both TNFα and IFNγ treatments with concomitant associated increase in IL-8 or CXCL11 release, respectively (Figure 5A-C, Figure S2).

IC₅₀ values for TEER reduction in response to TNFα were comparable between donors (14.68-32.8 ng/mL), demonstrating low donor-to-donor variability in sensitivity to TNFα, though the magnitude in TEER reduction was greater for Donor 1 (Figure 5A, left panel). After 48 hours of treatment with the two highest doses of TNFα, increased TEER relative to the maximum response in Donor 1 was observed, consistent with excessive cells debris that clogs the pores of the membrane. Due to increased interference from cell debris, we excluded two dose points from TEER IC₅₀ calculations (shown as annotated points in Figure 5A, left panel). In contrast, sensitivity to IFNγ was variable across Donors, with Donor 6 observed to have the highest sensitivity to IFNγ (IC₅₀ 0.12 ng/mL), compared to the other three Donors. The magnitude of response to IFNγ varied by 2.5-fold across the four Donors with Donor 1 showing the smallest reduction in TEER (-48.52±5.64%) and Donor 4 exhibiting the greatest reduction in TEER (-99.18±0.122%) (Figure 5A, right panel).

All four donors displayed a dose dependent increase in cytotoxicity in response to TNFα (Figure 5B, left panel). Similar to TEER kinetics, the magnitude of cytotoxicity in response to each cytokine was donor-dependent and correlated with barrier disruption across all four donors (R² > 0.7) (Figure S3). Cytotoxicity responses to IFNγ were not as robust as TNFα responses (Figure 5B, right panel). In line with maximum response to TNFα mediated barrier disruption, Donor 1 elicited the highest levels
of IL-8 release compared to Donors 4, 5 and 6 (Figure 5C). The concentration of CXCL11, a JAK/STAT regulated cytokine, increased similarly in cells treated with IFNγ in all four donors (Figure 5C).

To demonstrate that clinically validated TNFα- and IFNγ antagonists can protect against cytokine-induced barrier disruption and cytotoxicity, we co-treated cells from a single donor, Donor 5, with canonical marketed inhibitors of each cytokine, adalimumab or tofacitinib, respectively (Al-Bawardy et al., 2021; Antunes et al., 2021; Cai et al., 2021). For this study, doses of TNFα and IFNγ were used that corresponded to the maximum response in barrier disruption and cytotoxicity in RepliGut® Planar. Both FDA-approved clinical inhibitors effectively preserved barrier integrity (Figure 5D) and prevented cellular cytotoxicity (Figure 5E) induced by TNFα or IFNγ. These data demonstrate the utility of the RepliGut® Planar as a tool to explore drug pharmacology associated with TNFα and IFNγ pathways in IBD.

4 Discussion

In this work, we aimed to identify sources of variability in the human primary stem cell-derived platform, RepliGut® Planar, to improve in vitro modeling of GI function and disease. Intestinal stem cell models offer unique opportunities to investigate the intricate dynamics of the GI epithelium under controlled conditions. They provide a platform for exploring the molecular, cellular, and physiological aspects of GI diseases, which can be challenging to study using animal models. A major drawback of stem cell models is the inherent variability they possess due to the dynamic nature of stem cell differentiation and the influence of diverse culture conditions. Assessing sources of variability in stem cell-derived models is essential to ensure the reliability and reproducibility of experimental outcomes.

Cell passage number has been linked to variability in many immortal cell lines including Caco-2 cell culture, influencing gene expression, protein production and overall cell function (Sambuy et al., 2005). Somatic stem cells, like those found in the intestinal crypt, may possess a limited capacity for self-renewal and proper differentiation which could be accelerated in a cell culture setting. (Snippert et al., 2010; Liu and Rando, 2011). Analysis of four independent runs with cells from p5, p10 or p15 demonstrated that passage number does not limit the formation of a functional barrier, as assessed via the time to confluence (Fig. S1A), although peak TEER values and plateau length were significantly lower for p15 cells than p10 (Fig. S1B and 1C). p5 and p10 cells reached peak TEER on day 2 in RMM while p15 reached peak TEER on day 4 in RMM (Figure 3D). The plateau lengths for p5, p10, and p15 averaged 4.5±0.57, 4.75±0.50, and 3.0±0.80 days, respectively. Interestingly, these plateau lengths coincide with the expected 3-5-day life span of intestinal epithelial cells that is observed in vivo (Reynolds et al., 2014; Rees et al., 2020). However, p15 cells had a shorter assay window of 1.5 days and an almost 50% reduction in peak TEER compared to p5 and p10 cells which limits their dynamic range and use for assay development. This analysis demonstrated that while passage number does alter the magnitude of peak TEER and overall shape of the TEER profile, it does not meaningfully affect the ability of cells to form or maintain a confluent monolayer with tight junctions.

Gene expression analysis indicated p15 cells are transcriptomically distinct from p5 and p10 cells (Figure 2). Principal component analysis and hierarchical cluster analysis revealed p15 cells clustered separately from the other cell passages, suggesting that of the tested cell passages, p10 is the highest passage that resembles the native cells (p2). Together, these data suggest a fundamental change to the stem-cell identity that translates into functional differences of the epithelial monolayer and temporal behavior of the model. To avoid the possibility of stem cell identity drift affecting model performance, cells beyond passage 10 were not used in subsequent analyses.

Combined with functional evaluation of barrier formation and maintenance, the RepliGut® Planar culture timeline can be separated into three phases (time to confluence, time to TEER plateau, and plateau length). These phases were used to characterize the impact of passage number, donor and cell lot variation on overall performance of the model. Such evaluations enabled development of a robust assay within the TEER plateau phase of the RepliGut® Planar that exhibited broad dynamic range for detecting barrier disruption and release of inflammatory chemokines, thus modeling key mechanistic events in inflammatory bowel diseases. We observed that variability in TEER measurements increased with culture time, suggesting that the best opportunity to obtain meaningful assay responses are within earlier time windows following change to RMM. In this regard, variation in TEER was lowest (CV < 25%) between donors and lots during days 2-5 of the differentiation phase, which encompasses the TEER plateau phase of the RepliGut® Planar model time-course (Figure 4). Therefore, this phase was identified as the optimal window to detect meaningful and reproducible acute responses to proinflammatory cytokines.

Disruptions in barrier function have been implicated in the pathogenesis of several intestinal disorders, including inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis. Cell-based models of IBD are in high demand to better understand disease pathology and improve design of therapeutic interventions. TNFα and IFNγ are major drivers of inflammation and promising targets for drug development. The RepliGut® Planar platform displayed time-, donor- and dose-dependent responses to these proinflammatory cytokines, observed via decreased TEER, increased cytotoxicity, and increased cytokine release (Figure 6). Further, successful blockade of cytokine-induced epithelial damage was achieved using the FDA-approved clinical therapies adalimumab (neutralizing TNFα antibody) and tofacitinib (STAT inhibitor). Together, these data highlight the ability to use RepliGut® as a drug discovery tool for anti-inflammatory drugs targeting these pathways. Incorporating multiple human donors in in vitro models poses a significant challenge due to the inherent variability in genetic backgrounds, immune responses, and microbiomes among individuals. Human intestinal tissues obtained from different individuals may exhibit distinct molecular profiles, cellular compositions, and responses to stimuli, making it challenging to generalize findings across diverse populations. Surprisingly, donor-to-donor variation was not as significant as expected in terms of barrier formation.
and maintenance in RepliGut® Planar. This observation aligns with a prior study that reported low variability among 3D organoids derived from multiple human donors (Mohammadi et al., 2021). A possible explanation for the similarity observed between donors is that RepliGut® Planar does not contain an intestinal microbiome or immune cells, both of which are significant variables among humans that can influence clinical outcomes (Parlesak, 2004; Trujillo-de Santiago et al., 2018; Khan et al., 2019; Biagini et al., 2023). Developing strategies to model host-microbe interactions in RepliGut® Planar is a promising next step to enhance the clinical relevance and translatability of this platform.

Donor-donor differences were most evident in functional responses to proinflammatory cytokines, which coincides with a previous report using Emulate’s Human Colon Intestine-Chip (Apostolou et al., 2021). Notably, the magnitude and kinetics of VCAM-1 and IL-6 secretion, and permeability in response to IFNγ showed significant differences across three human donors. While these donor dependent responses may reflect individual biological differences, a larger sample size would be necessary to gain mechanistic insight. Genetic or epigenetic factors are speculated to account for the observed donor dependent differences, as polymorphisms of TNFα receptors and downstream cell signaling genes have been implicated in IBD susceptibility (Sashio, 2002; Bank et al., 2014). Leveraging advances in techniques such as single-cell RNA sequencing would address the contribution of genetic factors towards donor-to-donor variability and heterogeneity in human intestinal tissues in vitro.

Incorporating multiple human donors in intestinal disease in vitro models is crucial for enhancing the clinical relevance of research outcomes. It allows for a more comprehensive understanding of disease mechanisms, patient-specific responses to treatments, and the identification of personalized therapeutic strategies. This combination of personal response to proinflammatory cytokines coupled with the ability to control and alter the associated microbiome is a powerful concept for future development of in vitro models of inflammatory bowel disease. Future work aims to incorporate additional human donors in our intestinal disease models, allowing for a more comprehensive understanding of disease mechanisms and patient-specific responses to treatments.

5 Conclusion

This study underscores the importance of assessing variability in stem cell-derived models to ensure the reliability, reproducibility, and translatability of experimental findings. Understanding and mitigating variability not only enhances the robustness of research outcomes but also paves the way for more accurate disease modeling, drug discovery, and personalized medicine approaches, thereby accelerating progress in biomedical research and improving patient care.

References


Bank, S. et al. (2014). Polymorphisms in the Inflammatory Pathway Genes TLR2, TLR4, TLR9, L96F, NFKB1A, NFKB1, TNFA, TNFRSF1A, IL6R, IL10, IL23R, PTPN22, and PPARG Are Associated with Susceptibility of Inflammatory Bowel Disease in a Danish Cohort, PLoS ONE. Edited by M.M. Heimesaat, 9(6), p. 98815. doi: 10.1371/journal.pone.0098815


Sashio, H. et al. (2002). Polymorphisms of the TNF gene and the TNF receptor superfamily member 1B gene are associated with susceptibility to ulcerative colitis and Crohn’s disease, respectively, (53), pp. 1020–1027. doi:10.1007/s00251-001-0423-7


Data availability
All data supporting results and conclusions are contained within the article or supplementary material.

Conflict of interest
CMP, BZ, MKB, BEM, MC, CB, JMM, JAL, DCG, RL, WT, MKB and EMB are current or previous employees of Altis Biosystems, Inc. DP, MBM, DM, RS are employees of Sciome. RepliGut® Planar is developed and marketed by Altis Biosystems.

Acknowledgements
The authors thank Leah Huntress, Jacob Coyne, Erin Dancy, Lauren Boone, Earnest Taylor, Reganne Lorichon, and Vassili Kouprianov for their technical contributions to this work. We thank Mia Evangelista in the Pathology Services Core (PSC) for expert technical assistance with Histopathology. The PSC is supported in part by an NCI Center Core Support Grant (P30CA016086). We thank Gabrielle Cannon at the CGIBD Advanced Analytics Core for her expertise with BioMark. The CGIBD Advanced Analytics Core is supported in part by National Institute of Diabetes and Digestive and Kidney Diseases Grant P30 DK034987. This research was also funded by National Center for Advancing Translational Sciences Grant#1 R43 TR004230 and National Institute of Diabetes and Digestive and Kidney Diseases Grant# 1 R43 DK130708.