Assessing donor-to-donor variability in human intestinal organoid cultures

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SUMMARY

Donor-to-donor variability in primary human organoid cultures has not been well characterized. As these cultures contain multiple cell types, there is greater concern that variability could lead to increased noise. In this work we investigated donor-to-donor variability in human gut adult stem cell (ASC) organoids. We examined intestinal developmental pathways during culture differentiation in ileum- and colon-derived cultures established from multiple donors, showing that differentiation patterns were consistent among cultures. This finding indicates that donor-to-donor variability in this system remains at a manageable level. Intestinal metabolic activity was evaluated by targeted analysis of central carbon metabolites and by analyzing hormone production patterns. Both experiments demonstrated similar metabolic functions among donors. Importantly, this activity reflected intestinal biology, indicating that these ASC organoid cultures are appropriate for studying metabolic processes. This work establishes a framework for generating high-confidence data using human primary cultures through thorough characterization of variability.

INTRODUCTION

Historically, intestinal biology has been studied using immortalized or transformed cell lines. While these cell lines play a useful role, primary cell-culture models provide a more physiologically relevant system to address unanswered questions and more accurately model in vivo responses. Groundbreaking work in 2009 described modern organotypic cultures that are derived from adult mouse intestinal stem cells, are self-organizing, and contain nearly a complete repertoire of functional (terminally differentiated) cells as found in the originating tissue (Sato et al., 2009). A system derived from human intestinal stem cell was described subsequently (Sato et al., 2011a). These adult stem cell (ASC) organoid cultures enable a vast swath of in vitro studies in cells derived from a healthy host, in contrast to previous culture systems that relied on immortalized or transformed cells.

Gut ASC organoid cultures containing differentiated cells recapitulate in vivo intestinal cell composition. Absorptive and secretory cell types, which carry out the function of the intestine, are present in ASC organoid cultures. Enteroendocrine cells produce and secrete neurotransmitters and incretin hormones (Gribble and Reimann, 2019) that play major roles in gut peristalsis and vasodilation as well as pancreatic insulin secretion and satiety. Two other cell types (tuft cells [Nevo et al., 2019] and microfold cells [Dillon and Lo, 2019]) occur in vivo, but differentiation toward these lineages has to be induced by the addition of cytokines in ASC organoid cultures.

As ASC organoids contain both stem and differentiated cells, they have been deployed in a range of studies including fundamental pathway analysis efforts (Date and Sato, 2015; Dubey et al., 2020; Lindemans et al., 2015), establishing disease models (Bartfeld, 2016; Schutgens and Clevers, 2020; van der Vaart and Clevers, 2020), validating drug activity and efficacy, and genetic screening efforts (Michels et al., 2020; Ringel et al., 2020). Despite wide deployment, to date variations among donors in differentiation patterns and, therefore, functional readouts have not been characterized in human ASC intestinal organoid cultures. To address this knowledge gap, we established cultures from ileum and colon of six donors and examined donor-to-donor variability for multiple parameters. We show a high degree of correlation among donors, indicating that differentiation patterns are consistent. Additionally, small differences among donors in fundamental intestinal functions (such as metabolic activity and hormone secretion) did not hinder interpretation of results. Taken together, these data highlight the importance
Figure 1. Consistent differentiation of ileum- and colon-derived ASC organoids

(A) Schematic for time courses. Cultures were maintained for 10 days in total, switching to differentiation medium on day 3. Samples were collected on days 2, 4, 7, and 10. Day-2 cultures, maintained only in proliferation medium, were used as a baseline for gene expression in subsequent analysis.

(B) Stem/proliferative, secretory, and absorptive cell markers used to assess differentiation.

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of thoroughly characterizing human ASC organoid cultures with a diverse set of assays. Heterogeneity is an inherent and appropriate feature of human ASC organoid cultures, but we show that variability could be managed to yield robust and interpretable datasets.

RESULTS

Assessing developmental gene expression patterns

Gut ASC organoid cultures were established by isolating crypts from human intestinal tissue as previously described (Miyoshi and Stappenbeck, 2013; Sato et al., 2011a). Cultures were derived from ileum and/or colon of donated cadaver tissues from six adult donors of diverse ages and ethnicities (Table S1). Both ileum and colon cultures were established from all donors except donors 4 (ileum only) and 5 (colon only). Median age and body mass index were 31.5 (range: 18–47) years and 27 (range: 19.7–32.9) kg/m², respectively (summarized in Table S1).

These ASC cultures were grown in proliferation medium for 2 days and switched to two types of differentiation medium on day 3. Certain growth factors were removed (ENR medium [Sato et al., 2011a]) or reduced (5% L-WRN medium [Miyoshi and Stappenbeck, 2013]) to induce differentiation of cultures. Because of both technical and potential impact on signaling pathways, both media formulations were included (for details see supplemental experimental procedures).

Samples were collected at days 2, 4, 7, and 10 after the initiation of ASC organoid cultures (Figure 1A). Multiple time courses (2–3 per culture spanning several passages) were carried out for each culture to assess inter- and intra-donor variability. Furthermore, isolated crypts that were used to initiate cultures were included for the comparison of ASC organoid cultures with original tissue. Overall, 26 time-course assays were carried out, generating 556 RNA samples, leading to 13,344 qPCR observations.

Differentiation status of cultures was assessed using qRT-PCR, focusing on expression of developmental genes (Figure 1B; markers/probes are listed in Table S2). We designed a qPCR panel containing markers of stem/proliferative, secretory, and absorptive cells (Figure 1B). MKI67, LGR5, ASCL2, and AXIN2 were used as markers for proliferative and stem cells (Barker et al., 2007; Bullwinkel et al., 2006; Jho et al., 2002; Li et al., 2016; van der Flier et al., 2009).

Consistent developmental pathway gene expression patterns among donors

To assess the differentiation potential of cultures, we compared later time points (more differentiated) with an early time point (more stem/proliferative). Transcript levels of the markers were examined, comparing days 4, 7, and 10 with day 2 and visualized on a volcano plot. This analysis showed that stem/proliferative cell markers were downregulated over time whereas secretory and absorptive cell markers were upregulated (Figure 1C). Importantly, ileum- and colon-derived cultures behaved similarly. This developmental pattern was shared among all cultures analyzed, indicating that differentiation potential was similar for all cultures.

The proliferation marker MKI67 was downregulated as cultures differentiated. Additionally, previously described adult intestinal stem cell markers LGR5, ASCL2, and AXIN2 followed a similar pattern (Figure 2A). This pattern points to a shift to a mitotically arrested state over time, which is indicative of enrichment for terminally differentiated cells. ASCL2 gene expression was challenging to detect in all samples. Starting transcript levels were relatively low (day-2 Cq value 32.0 ± 2.46 for ASCL2 versus 25.9 ± 1.52 for LGR5, 23.6 ± 1.50 for AXIN2, and 21.8 ± 1.63 for MKI67; Figure S1A), making reliable detection difficult as gene expression was downregulated over time. Therefore, this marker was excluded from subsequent analysis. The overall expression pattern for stem and proliferative cell markers was similar for ileum and colon cultures (Figure S1A). Comparing ASC organoids and original tissue, expression

ATOH1 and HES1 were included as markers of secretory and absorptive progenitors, respectively (Gehart and Clevers, 2019). Additionally, markers for predominant functional secretory cell types were included: CHGA for enteroendocrine cells (Engelstoft et al., 2015), MUC2 and TFF3 for goblet cells (Pelaseyed et al., 2014), and LYZ, DEFA5, and DEFA6 for Paneth cells (Gassler, 2017). Several enterocyte markers were analyzed: CYP3A4, PGP, BCRP, SLC15A1, and SLC10A2 (International Transporter et al., 2010; Kaminsky and Zhang, 2003) were included as metabolic and absorptive markers and KRT20, PDL1, BTLN3, and BTLN8 were included as structural and intercellular signaling molecules (Di Marco Barros et al., 2016; Moll et al., 1990).
of stem and proliferative markers was similar (Figures S1A and S1D), although LGR5 expression was lower in ASC organoid cultures (average values in ileum: 0.0197 in ASC organoids, 0.302 in tissues; average values in colon: 0.0543 in ASC organoids, 0.167 in tissues).

Goblet and enteroendocrine cell markers MUC2, TFF3, and CHGA increased in expression as cultures developed, indicating the presence of these cells in well-differentiated cultures (Figure 2B). Expression of goblet cell markers was similar when comparing ileum and colon cultures (Figure S1B). Additionally, the expression pattern in ASC organoids and human tissue was similar (Figures S1B and S1C). The increase in expression for Paneth cell markers LYZ, DEFA5, and DEFA6 was not dramatic. Appropriately, Paneth cell markers were detected at a lower level in colon cultures than in ileum cultures (Figure S1B), consistent with the presence of Paneth cells in the small intestine but not colon (Clevers and Bevins, 2013). Tissue expression of Paneth cell markers was as expected: higher expression of all markers was observed in ileum (Figure S1E). Importantly, expression of DEFA5 and DEFA6 was higher in tissue than in ASC organoids. This result suggests that media conditions used were not conducive to the robust formation of Paneth cells, similar to previous results (Mead et al., 2018).

To assess the development of enterocytes, we determined the expression of several markers. Structural and signaling molecules KRT20, PDL1, BTNL3, and BTNL8 increased expression over time (Figure 2C) and did not display distinct ileum-colon expression patterns (Figure S1C). Additionally, we quantified the expression of molecules important for metabolism and transport. Expression of CYP3A4 was examined, as cytochrome P450 enzymes are important for intestinal metabolism. Expression of CYP3A4 increased dramatically over time in all cultures (Figure 2C). CYP3A4 expression was higher in ileum cultures than in colon cultures (Figure S1C), reflecting the metabolic function of the small intestine, as has been observed previously (McKinnon et al., 1995). Additionally, the expression of ATP-binding cassette family transporters PGP and BCRP was upregulated over time (Figure 2C), showing little ileum-colon expression difference (Figure S1C). Similarly, the expression of solute transporters SLC15A1 and SLC10A2 was upregulated considerably over time (Figure 2C). The expression of both solute transporters was higher in ileum cultures (Figure S1C), once again emphasizing the importance of the small intestine as the major site of nutrient transport. Interestingly, while expression levels for enterocyte markers were broadly similar in ASC organoids (Figure S1C) and original tissue (Figure S1F), ileum-colon polarity was lost for some transcripts. This was most apparent in transporters where PGP, BCRP, and SLC15A1 were more highly expressed in ileum in original tissue but not in ASC organoids, whereas SLC10A2 maintained its pattern of higher expression in ileum-derived ASC organoids.

Quantifying inter- and intra-donor variability during epithelial cell differentiation
We determined the extent of variability among cultures using principal component and correlation analysis. Gene expression data generated by qPCR for developmental pathway analysis for both ASC organoids and original human tissue (discussed in Figures 1 and 2) were used. All data points were analyzed on the basis of six features: tissue (ileum versus colon), donors, day of RNA harvest, medium type, culture passage number, and replicates. Principal component analysis (PCA) showed that samples collected on each day of time-course experiments clustered together (Figures 3A and 3B). Importantly, culture development and differentiation could be traced along principal component 1 (PC1), which captures most variation in data. Day-2 samples (highly proliferative/homogeneous stem cultures) clustered away from day 7 and day 10 (highly differentiated/heterogeneous cultures) samples. Day-4 samples, which represent an intermediate state between proliferative/stem and differentiated cultures, fell between day-2 and day-7/10 samples along PC1. Additionally, day-7 and day-10 samples clustered closely, indicating that little further differentiation occurs after day 7. This analysis clearly showed that differentiated cultures were transcriptionally distinct from proliferative cultures. Importantly, original tissue samples clustered with day-2 ASC organoid samples (Figures 3A and 3B). Original tissue samples consisted of isolated crypts, where proliferative cells are located in the intestinal gland, making this observation biologically appropriate.

Variability among donors was examined next. On PCA plots, little clustering was observed when ileum and colon samples were colored according to originating donor (Figures 3C and 3D), suggesting that differences among donors is not a major driver of variability. Next, we sought to quantify the extent of similarity among ASC organoid cultures. To quantify similarity among donors, we employed Pearson correlation analysis. When comparing gene expression
Figure 3. Quantifying variability among donors
Principal component analysis of both ileum and colon ASC organoid cultures (circles) and isolated human crypts (squares). Samples are marked by day of harvest (A and B) or donor identity (C and D). Percent variance for each principal component (PC) is reported in parentheses. Each symbol represents a separate RNA sample analyzed by qPCR. Correlation matrices comparing gene expression patterns in differentiated cultures (day 7) are presented in (E) and (F).
Expression of inflammatory immune receptors is developmentally controlled

To evaluate the potential for modeling inflammatory diseases in ASC organoid cultures, we quantified the expression of immune receptors that initiate proinflammatory signaling pathways in proliferative and differentiated ASC organoid cultures. We focused on receptors for interferons (IFNs) and tumor necrosis factor (TNF), as they have been implicated as important drivers of inflammatory gut indications (Andreou et al., 2020; Delgado and Brunner, 2019; Neurath, 2019). The expression of type I (IFNAR1 and IFNAR2), type II (IFNGR1 and IFNGR2), and type III (IFNLK1 and IL10R2) IFN receptors as well as two TNF receptors (TNFR1 and TNFR2) was quantified. Interestingly, receptor expression was higher in differentiated cultures than in proliferative cultures (Figure 4A). This pattern was consistent among the three donors analyzed, suggesting that this phenomenon is generalizable. This pattern held true for type I (Figure 4B), type II (Figure 4C), and type III (Figure 4D) IFN receptors as well as TNF receptors (Figure 4E). These findings highlight the importance of thorough characterization of immune receptor expression prior to challenging cells with cytokines or pathogens.

IFN receptors did not show a statistically significant ileum-colon culture expression bias (Figures S3A–S3C). TNF receptors appeared to be more highly expressed in ileum cultures (Figure S3D). TNFR1 expression was indeed significantly higher in ileum cultures (p = 0.0008), whereas the difference for TNFR2 expression was not statistically significant (p = 0.0997). Robust and reproducible expression of IFN and TNF receptors indicate that this ASC organoid system is suitable for modeling intestinal inflammatory disease in vitro.

Markers of functional intestinal cells are detected at protein level

The presence and fractions of functional cell types in ASC organoid cultures were determined using immunofluorescence microscopy (Figure 5). Differentiated cultures (day 7) were fixed and markers of Paneth (lysozyme), goblet (mucin 2), and enteroendocrine (chromogranin A) cells were detected. Structural proteins actin and E-cadherin were detected as markers of apical microvilli and cell junctions, respectively. This analysis showed that ASC organoids formed polarized structures with apical surfaces facing the lumen, as expected. Additionally, uniform E-cadherin staining was detected, indicating the formation of tight barrier surfaces. Finally, lysozyme, mucin 2, and chromogranin A were detected in cultures, confirming the presence of secretory cells in day-7 cultures (Figure 5A). Importantly, bright-field imaging confirmed the presence of a healthy epithelium as cultures differentiated (Figure S4). Next, the fraction of each cell type was quantified in both ileum- and colon-derived differentiated ASC organoids from several (n = 3) donors (Figure 5B). Goblet cell marker mucin 2 and enteroendocrine cell marker chromogranin A were detected at relatively high rates, whereas Paneth cell marker lysozyme occurred at much lower rates. These data indicate that while culture conditions are conducive to the formation of goblet and enteroendocrine cells, additional pathway modulators would have to be included in the culture media to increase the proportions of Paneth cells. Importantly, the staining patterns and cell proportions were consistent among the three donors examined, indicating that differentiation patterns among ASC organoid cultures are uniform.

Ileum- and colon-derived ASC organoids secrete serotonin, GLP1, and PYY

The intestine is an important endocrine tissue, and gut hormones play key roles in satiety, glucose homeostasis, and nutrient absorption (Ahlman and Nilsson, 2001; Martin et al., 2019; Worthington et al., 2018). However, cell culture systems to study gut hormone production and secretion in vitro have not been readily available. Thus, we quantified hormone secretion by differentiated ileum and colon ASC organoids (day 7 post seeding, stimulated as described in experimental procedures) derived from three donors. As enteroendocrine cell markers were robustly detected (Figures 2 and 5), we decided to assess their function by measuring levels of secreted serotonin, PYY, and GLP1. The secretion of all three hormones was detected for all donors (Figure 6). Serotonin secretion was
consistently detected more highly in colon cultures than in ileum cultures (Figure 6A). Compared with untreated cultures, serotonin secretion increased by 1.1-fold (ileum) or 7.5-fold (colon). GLP1 secretion was robustly detected in all cultures as well (Figure 6B). While both ileum and colon cultures secreted GLP1, the magnitude of secretion varied. Ileum cultures displayed an average 7.4-fold induction over untreated cultures while colon cultures displayed a 22.7-fold increase. Interestingly, the basal levels of GLP1 were lower in colon cultures than in ileum cultures. Finally,
secretion of PYY was detected in all cultures (Figure 6C). While the basal and stimulated levels of PYY were higher in colon than in ileum, the magnitude of induction upon stimulation was similar (5.7-fold for both ileum and colon cultures). This is consistent with higher colon PYY expression levels in vivo (Zhou et al., 2006). Importantly, all three donors analyzed in this experiment behaved similarly, suggesting that enteroendocrine cell function is consistently observed in ASC organoid cultures. Taken together, these data demonstrate that ASC organoid cultures faithfully recapitulate physiological conditions for hormone secretion.

Central carbon metabolism changes during ASC organoid differentiation

Central carbon metabolism is a complex enzyme-mediated network that converts sugars into precursors for metabolism. Targeted metabolomics analysis of the ileum- and colon-derived ASC organoids assessed the metabolic differences of differentiated ASC organoids compared with stem-cell-enriched cultures. Using triple quadrupole liquid chromatography-mass spectrometry, 108 central carbon metabolites were profiled at three time points (2, 4, and 7 days after initial plating) for ileum- and colon-derived ASC organoids from five and four donors, respectively. For each tissue type, metabolite concentrations were compared between donors at each time point. PCA results (Figures 7A and 7B) indicated minimal metabolic donor-to-donor variability as donors for a particular time point tended to cluster together. This robust clustering pattern indicated that the metabolic state of cultures is consistent. Clear differences in metabolism were evident between each day, as indicated by separate clusters for each time point. These metabolic trends were consistent in both ileum- and colon-derived ASC organoids.

To better understand the changes in central carbon metabolism during ASC organoid development, we visualized significantly different metabolites (p < 0.05, one-way ANOVA) by volcano plot (Figures 7C and 7D). L-citrulline, creatine, riboflavin, and L-glutathione (oxidized) were
among the list of significantly differential metabolites between days 2 and 7. Enrichment analysis (Figures 7E and 7F) revealed that amino acid metabolism—specifically glycine/serine/threonine pathway and arginine/proline pathway—was affected by differentiation to mature ileum- and colon-derived ASC organoids.

The change in abundance of several of these metabolites is consistent with metabolism in these tissues in the human body. For example, the relative abundance of L-citrulline increased nearly 10-fold in ileum-derived ASC organoids from day 2 to day 7. This result is consistent with previous studies, which have demonstrated a role for citrulline in intestinal function and that absorption of citrulline occurs in the middle to lower ileum (Fragkos and Forbes, 2018; Vadgama and Evered, 1992). Riboflavin, which significantly increased from day 2 to day 7 in both colon- and ileum-derived ASC organoids, has previously been shown to be absorbed in the small intestine (Feder et al., 1991; Hegazy and Schwenk, 1983). Flavin adenine dinucleotide, which is in the same metabolic pathway as riboflavin, also increased in both ASC organoid types. Taken together, our data suggest that the metabolic profiles detected in these ASC organoid cultures are consistent with what is known about their physiological functions. Nevertheless, to more precisely determine the ability of these ASC organoids to recapitulate tissue metabolism, additional experiments are warranted.

DISCUSSION

The advent of three-dimensional ASC organoid models allows for in vitro modeling of human tissue, providing a more physiologically relevant system than traditional tissue culture. One concern about these systems is the level of variability that may be observed from culture to culture. Here we have described the characterization of ileum and colon ASC organoids derived from various donors. We have demonstrated similar levels of epithelial cell differentiation among donors regardless of medium used for differentiation or passage number of the culture. Critically, donor-to-donor variability was minimal.

The ASC organoid cultures examined here differentiate on a time scale that matches the human gut epithelium. Within a few days differentiation is complete, as indicated by the overlap between gene expression signatures at days 7 and 10 (Figure 3). Importantly, only markers of enterocytes, goblet, Paneth, and enteroendocrine cells could be detected (Figures 1, 2, and 5); rarer cell types such as tuft and microfold cell markers were not detected (data not shown). This finding is consistent with previous work showing that differentiation toward rare cell-type lineages requires the addition of specific cytokines (Boonekamp et al., 2020).

Curiously, expression of Paneth cell markers was observed in colon cultures (Figure S1). While Paneth cell marker expression levels were higher in ileum cultures, colon culture expression was not absent. This observation could be explained by the complex nature of marker expression. Recent single-cell analysis publications (Parikh et al., 2019; Smillie et al., 2019; Wang et al., 2020) have highlighted the fact that gradients of marker expression exist in each cell type. Additionally, Paneth-like cells have been identified in the human colon (Wang et al., 2020), indicating that Paneth cell marker expression may represent this cell type.
Overall, Paneth cell differentiation was not very efficient in our organoid cultures (Figures 2, S1, and 5). It is known that both Wnt and Notch signaling pathways control Paneth cell differentiation (Sato et al., 2011b). The differentiation media conditions used in this study did not include Wnt and Notch modulators, which likely explains the modest increase in Paneth cell markers. While Paneth cells are observed in low quantities (Figure 5), increased representation of this cell type would be enhanced by adding exogenous Wnt agonists and Notch antagonists to the media, as previously described (Mead et al., 2018; Yin et al., 2014).

We show that differentiation is remarkably consistent among cultures. Appropriately, gene expression patterns are fairly uniform at early time points (denoted by a tight cluster colored in yellow: Figures 3A and 3B), reflecting homogeneity of stem/proliferative cells in the intestine. As cultures differentiate over time, gene expression patterns become less uniform (pink and blue symbols in Figures 3A and 3B), which is indicative of expected cell-type heterogeneity in terminally differentiated intestinal cells. This pattern is reminiscent of the “epigenetic landscape” described by Waddington (Goldberg et al., 2007). Our data nicely depict the widening of cell-fate trajectories as cultures differentiate to generate functional cells.

Our gene expression analysis highlighted region-specific expression patterns for certain transcripts. These included Paneth cell markers \( \text{LYZ} \), \( \text{DEFA5} \), and \( \text{DEFA6} \) and enterocyte markers \( \text{CYP3A4} \), \( \text{PGP} \), \( \text{BCRP} \), \( \text{SLC15A1} \), and \( \text{SLC10A2} \).
induce serotonin secretion (Sugisawa et al., 2020). Intestinal ASC organoids provide a robust ex vivo platform to identify modulators of endocrine cell function. These modulators will be important for studying disease processes as well as basic intestinal function.

We observed differential production of central carbon metabolites at various time points of culture differentiation. As the cultures differentiated, there was an increase in L-citrulline, creatine, and riboflavin. This represents the metabolites being utilized by the cells present in this heterogeneous population. While this targeted approach yields interesting reproducible information about central carbon metabolism, future studies could take an untargeted approach to find out whether additional metabolites can be detected in a reproducible manner. The results could indicate the ratio of a particular cell population or unique metabolic pathways that play important roles in the intestine.

In this work, we have characterized variability in primary human intestinal ASC organoids and have demonstrated that donor-to-donor variation is minimal. Additionally, we have shown that the cell-culture system recapitulates key features of the originating tissue. Identifying and characterizing the cell state for primary cell cultures is important for developing robust applications. Without thorough characterization, assay results will be difficult to interpret. Brought together, thorough characterization of cell state and functional cell information lead to successful application of this primary cell platform to ask fundamental biological questions and to advance therapeutic discovery.

**EXPERIMENTAL PROCEDURES**

For complete details, see supplemental experimental procedures.

**ASC organoid culture growth and differentiation**

Intestinal organoid cultures were established as previously described (Miyoshi and Steffenbeck, 2013; Sato et al., 2011a). Cultures were maintained in proliferation medium (50% L-WRN conditioned medium + Y27632 + SB431542) for routine passaging. To induce differentiation, we changed the culture medium to either a serum-containing medium (5% L-WRN conditioned medium with no Y27632 or SB431542) or a defined medium (base medium containing EGF, Noggin, and R-spondin) 3 days after organoids were plated.

**Transcriptional and immunofluorescence microscopy analysis**

At indicated time points, cells were harvested for RNA purification and qPCR analysis. For immunofluorescence analysis, organoids were removed from Matrigel using cell-recovery solution (Corning) and fixed using 4% paraformaldehyde. After permeabilization and blocking, organoids were incubated overnight in antibodies targeting differentiated cell markers, followed by secondary antibody staining. Stained organoids were mounted onto slides and imaged using confocal microscopy.
Principal component and correlation analysis

PCA was carried out using R. The results were visualized using GraphPad Prism. Pearson correlation analysis was performed using R, including only samples from the day-7 time point. The correlation matrix was visualized using GraphPad Prism.

Hormone secretion assay

Hormone secretion was induced in differentiated organoids (7 days post plating). In brief, secretion was induced by adding forskolin, 3-isobutylmethylxanthine (IBMX), and glucose to culture medium and analyzing supernatants after overnight incubation using kits to detect secreted PYY, GLP1, and serotonin.

Metabolomics analysis

Metabolites in organoids cultured for 2, 4, or 7 days were analyzed by mass spectrometry. Organoids were removed from Matrigel using Cell Recovery Solution (Corning) and extracted with acetonitrile/methanol/water. After homogenization, organoids were extracted for 10 min at ~20°C and centrifuged. Supernatants were dried under nitrogen and dissolved in water/methanol for mass spectrometry analysis.

SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

Conceptualization, S.M. and L.A.L.; methodology, S.M., T.P.W., and C.H.W.; software and formal analysis, C.H.W.; investigation, S.M., C.M.-P., C.W.W., T.P.W., and L.A.L.; data curation, S.M., T.P.W., C.H.W., T.R.S., and L.A.L.; writing – original draft, S.M.; writing – review & editing, all authors; visualization, T.P.W., C.H.W., T.R.S., and L.A.L.; project administration, S.M. and L.A.L.

CONFLICTS OF INTEREST

All authors were employees of Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc., Kenilworth, NJ, USA, at the time this work was performed.

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