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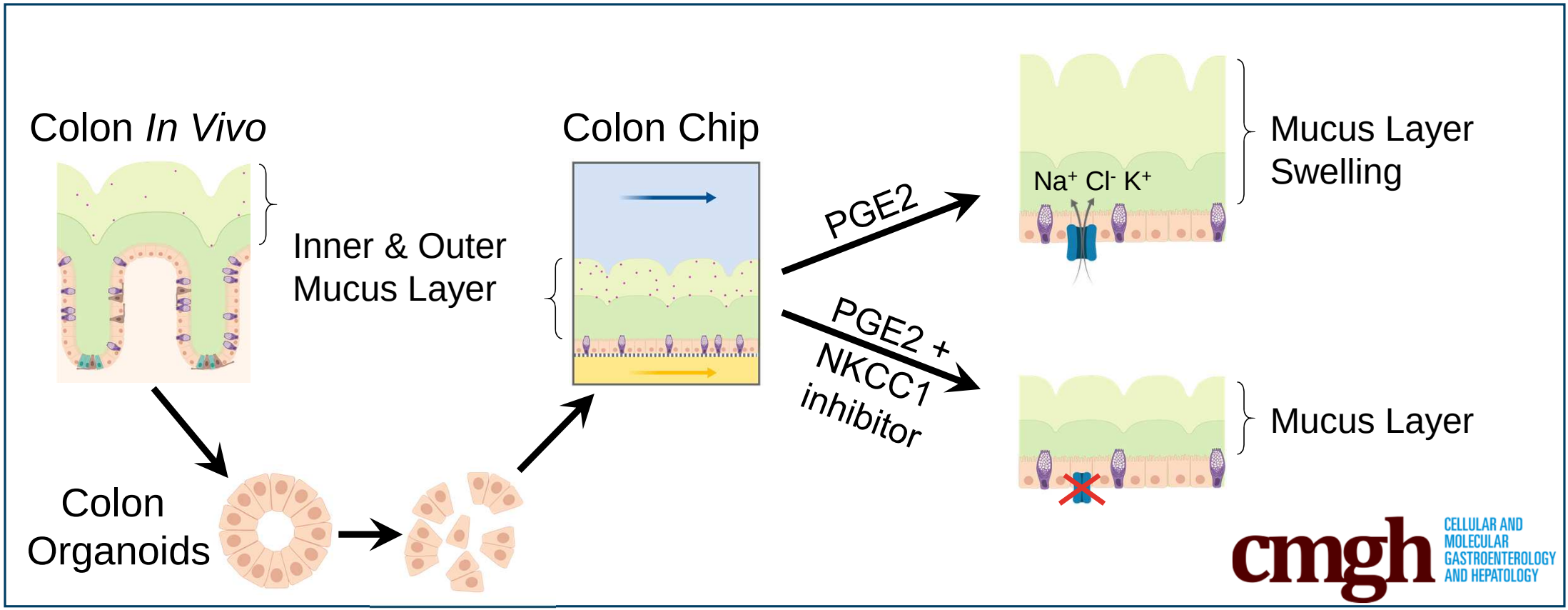

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SHORT TITLE: Probing intestinal mucus physiology with a colon-on-a-chip

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DISCLOSURES

D.E.I. is a founder and holds equity in Emulate, Inc., and chairs its scientific advisory board. A.S.-P., A.T., M.K. and D.E.I. are co-inventors on related patent applications.

SYNOPSIS

An *in vitro* method is described for studying colonic mucus physiology by integrating primary human colonic epithelial cells in a microfluidic organ-on-a-chip device. The Colon Chip produces a mucus layer with thickness and bilayered microstructure similar to the human colon.

AUTHOR CONTRIBUTIONS

A.S.-P. designed, executed and analyzed all experiments, with input and supervision from D.E.I., D.B.C., R.P.-B, and O.L.; D.B.C. helped with flow cytometry; D.B.C., A.T. and V.F. assisted with data interpretation; C.A.R. and D.T.B. provided advice on establishment of organoid cultures and interpretation of data ; T.C.F. assisted with fluorescence and transmitted light microscopy; T. D. helped with performance of chip experiments and analysis of bead data; C.F. performed the alcian blue assay; V.F. sectioned the TWs; A.D.S. helped with chip experiments and organoid cultures, and performed the qRT-PCR; S.J.-F. and J.W. helped with scanning electron microscopy; M.K. and A.T. helped with developing the Colon Chip method; D.B.C., A.T., M.K., helped with generation of human organoid cultures from resections. E.S.

generated the human organoid cultures from biopsies; A.S.-P. and D.E.I. wrote the manuscript with input from all co-authors.

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ABBREVIATIONS

AGR2: anterior gradient 2, protein disulphide isomerase family member

ALI: Air liquid interface

Colon Chip: Colon-on-a-Chip

CFTR: Cystic fibrosis transmembrane conductance regulator

CLCA1: chloride channel accessory 1

DF: Dark Field

EHEC: enterohemorrhagic E. coli

FCGBP: Fc-γ binding protein

HBSS: Hank's balanced salt solution

KCNQ: KV7 voltage-gated potassium channel

KLF4: Kruppel Like Factor 4

KLK1: Kallikrein 1

MEP1B: Meprin A Subunit Beta

MUC2: Mucin 2

Organ Chip: Organ-on-a-Chip

PDMS: Polydimethylsiloxane

PGE2: Prostaglandin E2

RETNLB: resistin-like molecule β

SPDEF: SAM pointed domain ETS factor

TFF3: Trefoil Factor 3

TW: Transwell

UC: Ulcerative Colitis

ZO1: ZONULA OCCULDENS 1

WORD COUNT

4206 words main text.

ABSTRACT

Background & Aims: The mucus layer in the human colon protects against commensal bacteria and pathogens, and defects in its unique bilayered structure contribute to intestinal disorders, such as ulcerative colitis. However, our understanding of colon physiology is limited by the lack of *in vitro* models that replicate human colonic mucus layer structure and function. Here, we investigated if combining organ-on-a-chip and organoid technologies can be leveraged to develop a human-relevant *in vitro* model of colon mucus physiology.

Methods: A human colon-on-a-chip (Colon Chip) microfluidic device lined by primary patient-derived colonic epithelial cells was used to recapitulate mucus bilayer formation, and to visualize mucus accumulation in living cultures non-invasively.

Results: The Colon Chip supports spontaneous goblet cell differentiation and accumulation of a mucus bilayer with impenetrable and penetrable layers, and a thickness similar to that observed in human colon, while maintaining a subpopulation of proliferative epithelial cells. Live imaging of the mucus layer formation on-chip revealed that stimulation of the colonic epithelium with prostaglandin E2, which is elevated during inflammation, causes rapid mucus volume expansion via an NKCC1 ion channel-dependent increase in its hydration state, but no increase in *de novo* mucus secretion.

Conclusion: This study is the first to demonstrate production of colonic mucus with a physiologically relevant bilayer structure *in vitro*, which can be analyzed in real-time non-invasively. The Colon Chip may offer a new preclinical tool to analyze the role of mucus in human intestinal homeostasis as well as diseases, such as ulcerative colitis and cancer.

Keywords: Organ chip; intestine; organoid; microfluidic; goblet cell; inflammatory bowel disease.

The mucus layer in the human colon normally protects the intestinal epithelial cells against enormous numbers of luminal commensal bacteria and potential pathogens present in the gut lumen of healthy individuals [1–5]. The human colonic mucus layer has a unique bilayer structure as it is composed of an inner layer that is normally impermeable to bacteria and a permeable outer layer [1,2,6]. The integrity of the inner layer is most crucial in preventing direct contact of the bacteria with the colonic epithelium and associated chronic inflammation [1–4]. In addition, changes of mucus layer homeostasis can indirectly influence intestinal barrier function[6]. Increased direct contact between these bacteria and the colonic epithelium can lead to gut barrier dysfunction and bacterial penetration through the epithelial tissue boundary. This can trigger injury and inflammation, for example, the mucus layer becomes penetrable to bacteria in dextran sodium sulfate-treated mice that develop colitis long before infiltration of immune cells is observed [4,5,7]. Importantly, the inner colonic mucus layer in patients with ulcerative colitis (UC), a common form of chronic inflammatory bowel disease affecting the colon [8], also has been shown to allow bacterial penetration [5,6]. Prostaglandin E2 (PGE2) is elevated during intestinal inflammation, as in patients with UC [9], where it plays an essential role in wound healing [9–11]. Recently, PGE2 also has been shown to be a direct mediator of fluid secretion using intestinal epithelial organoids [12]. This function is thought to be mainly mediated through activation of ion channels leading to ion flux-driven water flux into the lumen [12]. In the past, short term PGE2 treatment also has been reported to induce mucus secretion in cAMP-dependent manner mediated through activation of its receptor EP4 in murine intestinal loop studies and mouse proximal colon explants [13–15]. But the effect of PGE2 on the colonic mucus layer height and properties have been controversial and are not fully understood [13–16].

Unfortunately, it is impossible to study intestinal mucus physiology and changes in its behavior over time within the lumen of the living human colon to address these types of questions. Mucus physiology can be studied *in vivo* in animal models (e.g., using intestinal loop

studies) [13,14]; however, these methods are highly invasive, technically challenging, and often only low resolution imaging is possible [17]. More importantly, there are species-specific differences in mucus layer thickness and microstructure [1,2,6], and thus, there has been a search for new methods that could advance investigation in this area [6,17].

Common challenges using *in vitro* cell culture models to investigate intestinal mucus physiology include the use of cancer-derived epithelial cells, such as Caco-2 and HT29-MTX cells, which results in secretion of the gastric mucin MUC5AC, but not typical intestinal MUC2 [17,18]. Primary human intestinal organoids can produce mucus, but because it is entrapped in the central lumen of the organoid, it is difficult to investigate its physiology [17]. A few studies have demonstrated accumulation of mucins on the surface of primary human ileal or rectal organoid fragments cultured on Transwell (TW) inserts, but these cultures only accumulate thin (< 36 µm thick) mucus layers.[19,20] While a recent study reported obtaining a mucus layer of ~300 µm thickness by culturing human colonic epithelial cells on TWs under an air liquid interface (ALI)[21], this is still less than half of the thickness of the human colonic mucus layer (~600 µm)[6], and the inner mucus layer formed *in vitro* could be easily removed from the cell surface which is not the case *in vivo* [25]. Importantly, neither these cultures nor any other experimental *in vitro* method can reproduce the physiologically important bilayer structure that is seen in human colonic mucus [17]. Thus, most studies on mucus biology rely on the use of short-lived *ex vivo* mouse or human tissue explants [25]. While most of our current understanding of the colon mucus layer originated from studies of tissue explants [4,6,15,25,26], they have significant limitations including the need for repeated access to clinical biopsy specimens. In addition, clinical samples can only be used once, they cannot be used for long-term (> 1 day) cultures, and because the cells cannot be expanded *in vitro*, it is difficult to replicate results using the same donor [17].

Human organ-on-a-chip (Organ Chip) microfluidic culture technology has been used to create a human Small Intestine Chip [27,28] and Colon Chip [24], which are respectively lined by

primary human organoid-derived duodenal (Small Intestine Chip) or colonic intestinal epithelial cells (Colon Chip). These models present an alternative approach to confront this challenge. Organ Chips offer many advantages over the intestinal organoids from which they are derived, including the ability to continuously collect samples from both the luminal and abluminal compartments; the Small Intestine Chip also exhibits greater transcriptomic similarity to *in vivo* small intestine compared to organoids [28]. In the present study, we set out to explore whether Organ Chip technology could be used to develop a method to recapitulate human colonic mucus layer formation *in vitro.*

RESULTS

Reconstitution of a polarized colonic epithelium

Primary colonic epithelial cells isolated from human colon resections and endoscopic biopsies from the sigmoid and ascending colon (**Figure 1A, Table 1**) were first grown as colon organoids (**Figure 1B**), which were then fragmented and cultured on top of a porous extracellular matrix-coated membrane with 7 μm diameter pores in the top channel of a 2-channel microfluidic Organ Chip device composed of optically clear poly-dimethyl siloxane (PDMS), as previously described [24,28] (**Figure 1C,D**). Hank's balanced salt solution (HBSS) and stem cell expansion medium were continuously perfused (60 ul h^{-1}) through the top (epithelial) and bottom channels, respectively. When cultured under these dynamic flow conditions, the human colonic epithelial cells formed a confluent flat monolayer by day 3 that developed into a dense undulating epithelial sheet lined by columnar colonic cells by day 7 (**Figure 2A-C**). The timeline of experiments is based on days after monolayer formation as there were small variations in the time course of differentiation (1-2 days) in different experiments and with different donors. The colonic epithelium retained this dense morphology for at least 2 weeks in culture **(Figure 2A-C)** and covered the entire channel uniformly (**Figure 2D**).

The polarized structure of the colonic epithelium is crucial for intestinal barrier function, as well as for uptake and transport of ions and water in the colon[29]. Immunofluorescence confocal microscopy confirmed that the colonic cells formed a polarized columnar epithelium in the Colon Chip at day 7 that closely resembled that seen in human colon *in vivo*, as evidenced by restriction of nuclei to the basal cytoplasm and appearance of ZO-1-containing tight junctions and an F-actin-rich brush border along the apical surface of the epithelium (**Figure 3A**), as well as basolateral localization of E-cadherin-containing adherens junctions (**Figure 3B**). Analysis of barrier function measured with the small fluorescent tracer, Cascade blue (550 Da), confirmed that the colonic epithelium formed a tight intestinal barrier beginning at 4 days of culture on-chip, which was maintained over 2 weeks (**Figure 3C**), as previously observed in a human Small Intestine Chip [28]. Importantly, under these culture conditions, the human colonic epithelium also retained a subpopulation of proliferating cells during the entire 2 weeks of culture (**Figure 3D**).

Goblet cell differentiation

The presence of goblet cells in the colonic epithelium is a critical requirement for any study of mucus physiology as these are the specialized intestinal cells that produce and secrete mucin 2 (MUC2), which is a major component of intestinal mucus [30]. MUC2 polymers are densely packed in large secretory vesicles in goblet cells, which give the cells their typical "goblet" shape [30]. As expected based on past work that showed stem cell expansion medium drives the proliferation of stem cells in organoid cultures [22], we found that our organoids, and TW cultures created using cells isolated from these organoids, formed few, if any, goblet cells at 1 week of culture when cultured in this medium (**Figure 4A**), and similar results were obtained even when TW cultures were maintained for 14 days (**Figure 4B**). Past work has shown that it is necessary to switch from stem cell expansion medium to a differentiation medium in order to induce goblet cell differentiation in primary human intestinal cells[19,20,22,23]. Surprisingly, however, when the same organoid-derived colonic epithelial cells were cultured in the same stem cell

expansion medium under microfluidic conditions in the Colon Chip, high levels of goblet cell differentiation were observed, as indicated by appearance of MUC2+ epithelial cells that were similar in morphology and number to those seen in histological sections of human colon (**Figure 4A**). These MUC2+ cells could be detected as early as 3 days of culture and they expanded greatly in number by 1 to 2 weeks covering larger areas of the epithelium (**Figure 4B**). Flow cytometric analysis of live epithelial cell populations harvested from colon organoids, TWs, Colon Chips, and human colonic tissue confirmed that there was little goblet cell differentiation in the TW and organoid cultures, whereas ~15% of the epithelial cells differentiated into goblet cell in the Colon Chip, which is similar to the percentage of goblet cells we measured in human colon tissue samples (~10-30% depending on the donor; **Figure 4C-E**).

Importantly, despite supporting spontaneous goblet cell differentiation, the Colon Chip cultures were simultaneously able to maintain a proliferative cell subpopulation at levels similar to those present in the organoid and TW cultures (**Figure 4F**). While past work has shown that some goblet cell differentiation can be induced in TW cultures and organoids by replacing the stem cell expansion medium with differentiation medium [19,22,35], these cultures cease proliferating and are short-lived. The MUC2+ cells in the Colon Chip were also positive for goblet cell marker Trefoil Factor 3 (TFF3)[30] (**Figure 5A**), accumulated MUC2-containing mucus granules apically (**Figure 5B**), and displayed a surface morphology distinct from microvilli-covered enterocytes when analyzed by scanning electron microscopy (**Figure 5C**), which are similar to features observed in human goblet cells *in vivo* [30]. Furthermore, over the course of the 7 day cultures, there were significant increases in expression of intestinal epithelial cell genes encoding the SAM pointed domain ETS factor (SPDEF) and Kruppel Like Factor 4 (KLF4) transcription factors that control goblet cell maturation in the human colon[31,32], as well as genes encoding multiple proteins present within mucus granules of human colonic goblet cells[33,34], including Fc-γ binding protein (FCGBP), chloride channel accessory 1 (CLCA1),

resistin-like molecule β (RETNLB), Meprin A Subunit Beta (MEP1B), Kallikrein 1 (KLK1) and anterior gradient 2 protein disulphide isomerase family member (AGR2), when compared to the cells when they were cultured as organoids (**Figure 5D**). Thus, the Colon Chip effectively provided an environment for goblet cell differentiation under conditions in which proliferative cells also remain present as occurs in human colon *in vivo*, while other culture models do not.

Analysis of intestinal mucus accumulation and bilayer structure in living cultures

Given the spontaneous differentiation of large numbers of goblet cells in the Colon Chip that produce MUC2, which is the main mucin in colonic mucus, we next investigated if a physiologically relevant mucus bilayer forms on-chip. The existence of a mucus layer within the lumen of the apical epithelial channel was suggested by the appearance of increasing opacity of the Colon Chip over time when viewed from above by light microscopy (**Figure 2A,D**). Importantly, because the microfluidic Organ Chip is optically clear and has defined linear channel geometries (**Figure 1D**), we were able to develop a method to visualize live cultures in cross section across the entire channel. This was accomplished by slicing ~ 2 mm of the PDMS material away from the sides of the upper and lower channels, then turning the device 90° on its side and analyzing it using Dark Field (DF) microscopy (**Figure 6A,B**). Using this method, we detected the formation of a high light scattering layer above the cells that was well-developed by 1 week and when we analyzed the same chips over time, it continued to increase in height over the following week (**Figure 7A,B**). This layer stained positively for MUC2 (**Figure 7C**) and TFF3 (**Figure 7D**) and resided on top of the apical F-actin-rich brush border of the epithelium (**Figure 7C,D**), confirming that this material that accumulated over time in the Colon Chip is indeed a thick mucus layer. Fixation of intestine tissue with paraformaldehyde has been shown to produce a compression artifact (thinning of the layer) and disrupt the inner layer [17,36]; thus, an important advantage of this method is that it enables the *in situ* study of mucus development in its native state over an extended period of time in living cultures.

One of the unique properties of human colonic mucus is its bilayer structure, which is characterized by an inner layer that is impenetrable by bacteria and an outer penetrable mucus layer that is normally populated by commensal microbes. This feature of the mucus layer has been previously probed in tissue explants utilizing fluorescent 1 µm diameter microbeads to mimic bacteria [25]. When we flowed similar fluorescent microbeads through the lumen of the epithelial channel and allowed them to settle, we observed a bead-free region approximately 200 µm in height above the apical membrane of the epithelial cells at days 7 and 14 (**Figure 8A,B**), which is similar to the thickness of the dense impenetrable inner mucus layer observed in human colon tissue explants using this method [25]. The spread of the beads trapped in the mucus above the impenetrable layer corresponded to the penetrable outer mucus layer (**Figure 8A,B**). When we quantified the total height of the mucus layer (both inner and outer layers) formed on chip, we found that it reached ~570 µm by day 14 (**Figure 8B**). Scanning electron microscopic analysis of a cross-section of the Colon Chip also revealed the presence of a fibrous network within the thick mucus layer in tight contact with the upper surface of the epithelium (**Figure 8C**), which again is similar to what has been observed in tissue explants analyzed using lectin staining [37]. Thus, the microfluidic human Colon Chip is the first *in vitro* method to support spontaneous accumulation of a mucus layer in tight apposition to the apical surface of a cultured human colonic epithelium that replicates the thickness and unique bilayer properties displayed by human colonic mucus *in vivo*.

Modeling the response of colonic epithelium to PGE2 on-chip

Prostaglandin E2 (PGE2) has been reported to be elevated in patients with ulcerative colitis, and it appears to contribute to healing of intestinal ulcers by increasing cell proliferation and altering mucus physiology [9–11,13–15]. To explore if the human Colon Chip method can be used to study this response *in vitro*, we perfused PGE2 (1.4 nM) through the basal channel from day 2 to 8. This 6-day exposure to PGE2 resulted in a 4.5-fold increase in proliferating cells

compared to control chips, as measured by EdU incorporation (**Figure 9A**), as well as a 1.6-fold increase in MUC2+ cells (**Figure 9B**). The rise in EdU incorporation is consistent with reports that PGE2 promotes cell proliferation and is essential for wound healing [9–11].

In these studies, we noticed that the PGE2-treated chips developed blockage of the apical channel preventing fluid flow, suggesting that there also might be increased mucus production. This was surprising because the PGE2-treated Colon Chips appeared to contain less light-obscuring material compared to control Chips when imaged from above by bright field microscopy (**Figure 9C**), which stands in direct contrast to the increased opacity associated with the mucus layer development we observed in our earlier studies. Past animal and tissue explant studies exploring the effect of PGE2 on mucus production have produced contradictory results. Short term PGE2 treatment has been reported to increase colonic mucus accumulation in murine intestinal loop studies and mouse proximal colon explants [13–15], but no increase in mucus secretion could be detected in response to PGE2 treatment in isolated human colonic crypts [16] or distal colon mouse tissue explants[15].

Thus, we set out to evaluate the short-term effect of PGE2 on mucus layer height using the human Colon Chip. Short term (4 h) treatment of the Colon Chip with PGE2 resulted in an increase in mucus height by 321.8 ± 44.3 µm, which is equivalent to a ~2-fold increase compared to control chips (**Figure 9D,E**). Interestingly, however, there did not appear to be an associated increase in the total amount of mucins when quantified using an alcian blue-binding assay (**Figure 9F**). This result suggests that the increase in height we detected was not due to secretion of more mucin materials, but rather to swelling of the pre-existing mucus.

While PGE2 has a multitude of functions in intestinal physiology, it plays an important role in the regulation of fluid secretion via ion transport [12]. Because mucus production has been shown to depend on fluid secretion in mouse small intestine [38,39], we tested if this property of PGE2 was responsible for its ability to rapidly increase mucus height. PGE2-induced fluid

secretion can be blocked by inhibiting ion channels [12], and so we pretreated the human Colon Chip with a combination of 3 different ion channel inhibitors, CFTRinh-172, XE-991, and bumetanide, which are chemical inhibitors of the cystic fibrosis transmembrane conductance regulator (CFTR), K_V7 (KCNQ) voltage-gated potassium channel, and NKCC1 Na-K-Cl cotransporter, respectively, before infusing PGE2. The combination of the 3 different ion channel inhibitors led to a significant reduction in the PGE2-induced increase in mucus height confirming the importance of ion transport in this hydration response (**Figure 10A,B**).

To analyze the effects of each ion channel inhibitor individually, different Colon Chips were pre-treated with each of these ion channel inhibitors before exposing them to PGE2. Suppression of the CFTR and Kv7 K+ channel activity had no significant effect, however, inhibition of the basolateral NKCC1 Na-K-Cl cotransporter with bumetanide significantly reduced PGE2-induced mucus layer height on-chip (**Figure 10A,B**). None of these channel inhibitors altered total mucin content (**Figure 10C**). Thus, the increase in mucus height we observed upon short term exposure to PGE2 is largely due to ion and fluid secretion-induced swelling of pre-existing mucus. The reduction of mucus swelling by inhibition of NKCC1 but not CFTR or Kv7 K+, could be due to the fact that NKCC1 is a co-transporter of multiple different ions (Na^+, 2 Cl^-, K^+), whereas both other ion channels transport only single ions (Cl^- or K^+ respectively). This could indicate that PGE2-induced swelling of the mucus layer involves multiple different ions rather than just one single ion type.

As the structural integrity of the colonic mucus layer is an essential part of the intestinal barrier, local changes in mucin organization or concentration due to swelling could affect structural properties of the mucus as well. Notably, the inner impenetrable mucus layer was preserved during PGE2-induced swelling of the mucus layer (**Figure 11A,B**). Furthermore, when we exposed the mucus layers of control and PGE2-treated Colon Chips to increasing flow velocities, we found that the mucus layers in both chips exhibited similar bending angles, and

hence, similar material responses to shear stress (**Figure 11C, Supplementary Video 1**). This suggests that despite changes in hydration state of the mucus layer, its gross structural integrity was maintained.

DISCUSSION

Although the structure and function of the human colonic mucus bilayer is highly relevant for intestinal pathophysiology, previous investigation of its properties could only be carried in short-term (< 1 day) *ex vivo* tissue explants. While a recent study using colonic epithelial cells cultured under ALI in TWs demonstrated mucus layer accumulation[21], the mucus layer was thinner and more readily removed than observed *in vivo*, and this required use of a differentiation medium that depleted stem cells[19,20,22,23], which limits its ability to perform long term experiments or recovery studies. The key point, however, is that none of the past studies using human colonic epithelial cells cultured in TWs, organoids, or any other *in vitro* model[17] resulted in production of a thick mucus layer with a normal bilayer structure similar to that seen *in vivo*. In contrast, in the present study, we showed that a microfluidic 2-channel human Colon Chip enables long-term culture of primary human colonic epithelial cells under dynamic flow conditions. Moreover, this system supports the spontaneous differentiation of large numbers of highly differentiated, mucus-producing goblet cells at similar levels to those observed in human colon *in vivo*, while still maintaining a healthy subpopulation of proliferative cells. Importantly, under these culture conditions, the human colonic epithelial cells produced a mucus bilayer containing an impenetrable layer closely apposed to the apical surface of the epithelium, directly overlaid by a penetrable mucus layer, with a total thickness of 500-600 μm, which is similar to that seen in living human colon [25]. Thus, this is the first culture method to recapitulate the development of a thick human colonic mucus layer with its unique bilayer structure.

Previous work has shown that germ-free mice do not have an inner mucus layer and weeks of bacterial colonization are required for an inner mucus layer to form[40]. In contrast, in this human Colon Chip, the inner mucus layer is established without any bacteria being present on Chip. As the epithelial cells cultured on the Colon Chip come from patients who had been in contact with a complex microbiome prior to their isolation as organoids, it is possible that they were influenced by this experience (e.g., epigenetically) and no longer require their continued presence to form an inner mucus layer. However, as the inner mucus layer in germ-free mice is penetrable to microbeads[40], whereas the Colon Chip is not nor is the inner layer in human colon, this could be related to species-specific differences between human and mouse.

Another novel feature of the human Colon Chip method is that the optical clarity of the microfluidic device allows live non-invasive visual analysis of mucus accumulation and physiology over time in culture. The dynamic changes in mucus layer thickness induced *in vivo* by the inflammatory mediator, PGE2, could be replicated, quantified, and analyzed on-chip. This revealed that rapid changes in mucus layer height after short term exposure to PGE2 are primarily mediated by altering the hydration state of pre-existing mucus via ion secretion through NKCC1, and not due to additional mucus secretion. However, suppression of NKCC1 did not fully abolish the effect of PGE2, indicating that other ion channels may be involved in the PGE2-induced mucus swelling. Although analysis of the pathways that mediate these processes is beyond the scope of this study, changes in cAMP-mediated HCO_3^- secretion[39,41] or in PGE2-mediated glycosylation of mucins [42] could be involved. Thus, to further dissect the mechanism of water-mediated mucus swelling in response to PGE2, future studies could be carried out in which each type of ion is removed individually from the culture medium, as done previously[12,39], and the role of cAMP induction could be explored as well.

Furthermore, our data indicate that colonic mucus may be able to undergo significant expansion without losing barrier-function or structural stability, highlighting the remarkable

characteristics of this physiologically important structure. These findings demonstrate the usefulness of the Colon Chip as an *in vitro* tool for evaluation of mucus structure and function, which could advance our understanding of mucus physiology in disease contexts. Considering recent advances in bacterial co-cultures in intestinal microfluidic models [24,43–45], this microfluidic Colon Chip lined by patient-derived colonic epithelial cells may also facilitate development of new therapeutics or probiotics that modulate the mucus barrier, as well as provide a novel testbed for personalized medicine.

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MATERIALS AND METHODS

Isolation of human colon epithelial cells

Human colon epithelium was isolated from colon resections or endoscopic tissue biopsies (**Table 1**). Full thickness pieces of the human colon were obtained anonymously from healthy regions of colonic resection specimens processed in the Department of Pathology at Massachusetts General Hospital under an existing Institutional Review Board approved protocol (#2015P001859). Specimens were restricted to those with (non-neoplastic) disorders, and regions collected for isolation were determined to be healthy based on gross examination. Endoscopic biopsies were collected from macroscopically grossly unaffected regions of the colon of de-identified pediatric and young adult patients undergoing endoscopy for abdominal complaints. Informed consent and developmentally-appropriate assent were obtained at Boston Children's Hospital from the donors' guardian and the donor, respectively. All methods were

carried out in accordance with the Institutional Review Board of Boston Children's Hospital (Protocol number IRB-P00000529) approval.

For the isolation of colonic crypts, colon resections were processed by removing the colon epithelium with lamina propria, and then the epithelial layer or the entire biopsy was digested with 2 mg ml^{-1} collagenase I (Thermo Fisher Scientific, 17100-017) supplemented with 10 μM Y-27632 (Y0503, Sigma-Aldrich) for 40 min at 37 ℃ with intermittent agitation, as described[28,46]. Colon organoids were grown embedded in growth factor-reduced Matrigel (354230, Lot 7317015, Corning) and stem cell expansion medium supplemented with 10 μM Y-27632 [19,28]. Stem cell expansion medium is composed of advanced DMEM F12 (12634-010, Thermo Fisher Scientific) supplemented with: L-WRN (Wnt3a, R-spondin, noggin) conditioned medium (65% vol vol^{-1}) (produced by the CRL-3276 cell line, ATCC), 1x GlutaMAX (35050-061, Thermo Fisher Scientific), 10mM HEPES (15630-106, Thermo Fisher Scientific), recombinant murine epidermal growth factor (50 ng ml^{-1}) (315-09, Peprotech), 1x N2 supplement (17502-048, Thermo Fisher Scientific), 1x B27 supplement (17504-044, Thermo Fisher Scientific), 10 nM human [Leu15]-gastrin I (G9145, Sigma-Aldrich), 1 mM n-acetyl cysteine (A5099, Sigma-Aldrich), 10 mM nicotinamide (N0636, Sigma-Aldrich), 10 μM SB202190 (S7067, Sigma-Aldrich), 500 nM A83-01 (2939, Tocris), and primocin (100 μg ml^{-1}) (ant-pm-1, InvivoGen).

Colon Chip Cultures

The Colon Chip uses the exact same chip device design as our previously described Small Intestine Chip[24,27,28], but the origin of the organoids is intestinal region-specific. The Organ Chip devices are composed of poly-dimethylsiloxane (PDMS) and containing two parallel microchannels (apical channel 1000 x 1000 μm and basal channel 1000 x 200 μm; width x height) separated by a 50 μm thick PDMS porous membrane (7 μm pore diameter, 40 μm spacing) were purchased from Emulate Inc. (CHIP-S1 Stretchable Chip; RE00001024 Basic Research Kit, Emulate Inc.). After activation of the channel surfaces with 0.5 mg ml^{-1} sulfo-

SANPAH solution (A35395, Thermo Fisher Scientific), the inner surfaces of both channels and the porous PDMS membrane were coated with 200 µg ml^{-1} rat tail collagen type I (354236, Corning) and 1 % Matrigel (354230, Lot 7317015, Corning) in Dulbecco's phosphate-buffered saline (DPBS), as previously described[24,28]. Colon organoids were then isolated from Matrigel by incubating in cell recovery buffer (354253, BD) for 40 min on ice and the spun down at 400g for 5 minutes at 4°C. The colonic organoids were fragmented by incubating them in TrypLE™ Express Enzyme (12605010, Thermo Fisher Scientific) diluted in DPBS 1:1 (vol:vol) supplemented with 10 µM Y-27632 (2 mL/ well of a 24 well plate) for 1 min 45 seconds in a 37°C water bath. After adding the same volume of stem cell expansion medium with 10 µM Y-27632, the organoids fragments were spun down at 400g for 5 min at 4°C and then resuspended at 6 x 10^6 cells ml^{-1}. The colonic organoid fragments were seeded on the ECM-coated membrane in the apical channel of the Colon Chip (6 x 10^5 cells cm^{-2}) in stem cell expansion medium supplemented with 10 µM Y-27632 while filling the basal channel with the same medium, and the chips were incubated overnight at 37°C under 5% CO_2 to promote cell adhesion. The following day both channels were washed once with stem cell expansion medium, and then the chips were inserted into Pod™ portable modules (RE00001024 Basic Research Kit, Emulate Inc.) and placed within a ZOË™ culture instrument (Emulate Inc.), where they were perfused (60 µl h^{-1}) with stem cell expansion medium in the basal channel and HBSS with calcium and magnesium (21-023-cv, Corning) supplemented with 100 µg ml^{-1} primocin (ant-pm-1, InvivoGen) in the apical channel. The timeline of experiments is stated as days after monolayer formation because there was a small amount of variability (1-2 days) in terms of the time required for monolayer formation between different experiments and donors. Supplementary Table 1 lists all donors used in these studies.

Transwell insert cultures

Transwell® culture inserts (TWs; 6.5 mm) with 0.4 µm pore polyester membrane (3470, Corning) were coated as described above for the chip, and seeded with colon organoids fragments at the same density (6 x 10^5 cells cm^{-2}) on the top side of the TWs in stem cell expansion medium supplemented with 10 µM Y-27632, and the same medium was added to the bottom chamber. As with the chips, the TWs were incubated overnight at 37°C under 5% CO_2, and the following day, the TW was washed once with stem cell expansion medium before adding 1 ml stem cell expansion medium on the basal side and 250 µl of HBSS with calcium and magnesium supplemented with 100 µg ml^{-1} primocin to the apical side; medium was changed every 2 days thereafter.

Organoid cultures

Colon organoid fragments were resuspended in growth factor-reduced Matrigel at 1 x 10^6 cells ml^{-1} and plated in 24 or 48 well plates (50 or 10 µl drops/well, respectively), and covered with stem cell expansion medium supplemented with 10 µM Y-27632 (500 µl or 200 µl/ well, respectively). Stem cell expansion medium was changed every 2 days thereafter.

Immunofluorescent microscopy

Colon Chip were fixed with 200 µl of 2% paraformaldehyde (PFA) (Electron Microscopy Sciences, 15730), 25mM HEPES (Thermo Fisher Scientific, 15630-080) in DPBS (Gibco, 14190-144) with 200 µl filter tips at 4℃ on a rocker overnight. TWs and organoids were fixed at room temperature for 15 min. Chips were either stained directly or sectioned at 250 µm with a vibratome (VT1000S, Leica). Fresh frozen 7 µm *in vivo* tissue sections were fixed with 2% PFA for 12 min at 4℃. All samples were blocked and per meabilized using 0.1% Triton X-100 (X100, Sigma), 5% BSA (A2153, Sigma) in DPBS for 1 h at room temperature. Samples were then stained overnight at 4℃ in 2% BSA in DPBS with pri mary antibodies: anti-MUC2 (H-9, sc-515106, Santa Cruz, 1:100), anti-E-Cadherin (HECD-1, ab1416, abcam, 1:100), anti-ZO-1

(ZO1-1A12, 33-9100, abcam, 1:200), anti-TFF3 (EPR3974, ab108599, abcam, 1:200). The next day, after 3 washes of PBS, samples were then stained overnight at 4℃ in 2% BSA in DPBS containing secondary antibodies and phalloidin: Goat anti-mouse-IgG1 Alexa Fluor 647 (A-21240, Invitrogen, 1:100), goat anti-mouse IgG2b Alexa Fluor 555 (A-21147, Invitrogen, 1:500), Phalloidin Alexa Fluor 488 (A12379, Invitrogen, 1:200), Phalloidin Alexa Fluor 647 (A22287, Invitrogen, 1:200). The next day, after 3 DPBS washes, staining with Hoechst 33342 (H3570, Life Technologies, 1:2,000) for 30 min was performed.

Images were taken using a Leica SP5 laser scanning confocal immunofluorescence microscope with a 680-1080nm multiphoton pulsed IR laser Chameleon Vision 2 with pre compensation and Non-Descanned Detectors, a 470-670nm nm white light laser, 488 nm argon laser and coupled to HyD detectors. Acquired images were analyzed using IMARIS software (Bitplane).

Flow Cytometry

For assessment of proliferating cells, culture medium (stem cell expansion medium basally, HBSS apically) containing 10 µM EdU (Click-iT™ Plus EdU Alexa Fluor™ 350 Flow Cytometry Assay Kit, C10645, Invitrogen) was perfused through the apical and basal channels of the chip for 18h prior to cell harvest. Cells were isolated from the Colon Chips and TWs by incubation in 1mg ml^{-1} collagenase IV in TrypLE supplemented with 10 µM Y-27632 (100 µL/ channel in the chips; 100 µL above and 500 µL below the membrane in TWs) for 1h at 37℃. Detached cell fragments were incubated for an additional 45 min at 37℃ up to 1h until a single cell suspension was obtained. Epithelial cells were isolated from colonic organoids as described above except that the organoids, after extraction from Matrigel, were incubated in 200 µl enzyme solution for 45 min up to 1h at 37℃. Cells were also isolated from human colon resections by dissecting the tissue as described above. Colonic crypt isolation and digestion into single cells was performed as previously described[47]. In short, minced tissue was incubated in

8mM EDTA in DPBS (14190-144, Gibco) while slowly rotated for 75 min at 4℃, followed by vigorous shaking of the sample to enrich for dissociated colonic crypts. To obtain a single cell suspension, colonic crypts were incubated in Disaggregation Medium (Advanced DMEM/F12, 1x GlutaMAX, 10 mM HEPES, 1x N-2, 1x B-27, 10 mM nicotinamide, 1 mM N-acetyl-L-cysteine, 10 µM Y-27632 , 2U ml^{-1} dispase (17105041, Gibco), 200 KU DNAse I ml^{-1} (D5025, Sigma) and incubated for 30 min at 37℃ with occasional agitation. All harvested cells were centrifuged, resuspended in flow staining buffer composed of 1% FBS (Gibco, 10082-147), 25mM HEPES (15630-080, Thermo Fisher Scientific), 1mM EDTA (15575-020, Thermo Fisher Scientific), and 0.05% sodium azide (BDH7465-2, VWR) in DPBS (14190-144, Gibco). Surface staining was performed in 100 ul staining buffer for 30 min, followed by fixation in 2% PFA (panel 1 and 3) for 15 min or overnight fixation with eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (00-5523-00, Invitrogen) (panel 2). After fixation, EdU staining was performed following manufacturer's instructions (Click-iT™ Plus EdU Alexa Fluor™ 350 Flow Cytometry Assay Kit, C10645, Invitrogen), followed by intracellular staining in 1x saponin (Click-iT™ Plus EdU Alexa Fluor™ 350 Flow Cytometry Assay Kit, C10645, Invitrogen).

Panel 1 (2% PFA fixation): anti-MUC2 (H-9, sc-515106, Santa Cruz, 1:100), anti-mouse-IgG2b-546 (A-21143, Invitrogen, dilution 1:100), Click-iT™ Plus EdU Alexa Fluor™ 350 Flow Cytometry Assay Kit (C10645, Invitrogen). Panel 2 (Foxp3): anti-Ki67-APC (Ki-67, 350514, BioLegend, dilution 1:20), Click-iT™ Plus EdU Alexa Fluor™ 350 Flow Cytometry Assay Kit (C10645, Invitrogen). Panel 3 (fresh *in vivo* tissue): anti-CD45-Brilliant Violet 570 (HI30 clone, 304034, BioLegend, dilution 1:50), anti-CD235a-Pacific Blue (HI264 clone, 349108, BioLegend, dilution 1:40), anti-CD11b-Brilliant Violet 570 (ICRF44, 301325, BioLegend, dilution 1:40), anti-CD31-421 (WM59, 303124, BioLegend, dilution 1:20), EpCAM-PE/Cy7 (CO17-1A, 369815, BioLegend, dilution 1:20), anti-MUC2 (H-9, sc-515106, Santa Cruz, 1:100), anti-mouse-IgG2b Alexa Fluor 546 (A-21143, Invitrogen, dilution 1:100). All panels included 20 nM Syto16 (S7578,

Thermo Fisher Scientific, dilution: 1:500), Zombie NIR dye (423106, BioLegend, dilution: 1:500), Fc Block (BioLegend, 422302, dilution 1:20). Stained cells were analyzed using the LSRFortessa (BD Biosciences). Results were analyzed using FlowJo V10 software (Flowjo, LLC).

RNA isolation, reverse transcription and qRT-PCR

The cells of colonic organoids, d3 chips and d7 Colon Chips were harvested with RLT buffer from the RNeasy Mini Kit (74106, Qiagen). RNA was extracted using the RNeasy Mini Kit (74106, Qiagen) followed by cDNA synthesis with SuperScript VILO cDNA Synthesis Kit (1754250, Invitrogen). RT-PCR was performed using TaqMan Fast Advanced Master Mix (4444965, Applied Biosystems), TaqMan Gene Expression Assays (Thermo Fisher Scientific; Hs02786624_g1 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Hs00358836_m1 for Kruppel Like Factor 4 (KLF4), Hs00171942_m1 for SAM pointed domain ETS factor (SPDEF), Hs00356521_m1 for anterior gradient 2, protein disulphide isomerase family member (AGR2), Hs00395669_m1 for resistin-like molecule β (RETNLB), Hs00175398_m1 for Fc-γ binding protein (FCGBP), Hs01086545_m1 for Kallikrein 1 (KLK1), Hs00983260_m1 for Meprin A Subunit Beta (MEP1B), Hs00976287_m1 for chloride channel accessory 1 (CLCA1)), and run on a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems). All results were normalized relative to GAPDH expression and d0 organoid respective gene expression.

Permeability

Cascade Blue™ hydrazide Trilithium Salt (550 Da) (C3239, Invitrogen) at 50 µg ml^{-1} in HBSS was added to the top epithelial channel of the Colon Chip to assess barrier permeability. The concentration of dye that diffused through the membrane into basal channel was measured in the effluent, and apparent paracellular permeability (Papp) was calculated as previously described[28].

Light microscopy

The top view Colon Chip images were acquired using a differential interface contrast (DIC) or phase contrast microscopy (Zeiss Axio Observer Z1). Frozen sections were obtained during different stages of the isolation of colon epithelial cells, stained with H&E and imaged.

Side view imaging of mucus accumulation on-chip

To image mucus accumulation in living cultures on-chip, ~2 mm of PDMS were cut away from each side of the chip parallel to the channels using a razor blade secured in a press. The chips were rotated onto one side on a glass slide coated with Glycerine-Solution (11513872, Leica) and the top side was covered with glycerin and a cover glass. The images were acquired with an inverted microscope (Zeiss Axio Observer Z1) using a 2.5x objective (0.06 NA, 441010-9901, Zeiss) and condenser (0.35 NA, 424241-0000-000, Zeiss) with phase ring 2 used for DF imaging. Fluorescent images were acquired using an X-cite LED light source (Excelitas Technologies). Mucus height and area were analyzed in side view images of the Colon Chip using Fiji software.

PGE2 treatment

For long-term PGE2 studies, the basal channel of the Colon Chips was perfused with stem cell expansion medium 1.4 nM PGE2 (P5640, Sigma) for 6 days, starting at day 2. To quantify cell proliferation, 10 µM EdU (C10645, Invitrogen) was perfused through both channels of the chip for 18 hours prior to enzymatically detaching cells on day 8 and carry out flow cytometric analysis. Short term treatment with PGE2 and ion channel inhibitors was performed at 7 days after monolayer formation. In short, Colon Chips were perfused with medium (stem cell expansion medium basally, HBSS apically) containing 50 µM CFTRinh-172 (S7139, Selleckchem), 20 µM XE-991 Dihydrochloride (20010, tocris), 100 µM Bumetanide (S1287, Selleckchem), or combination of the 3 inhibitors at 60 µl h^{-1} for 4h, followed by side view imaging to determine the baseline height of the mucus layer prior to PGE2 treatment . After

baseline side view imaging, the chips were then switched to co-treatment with 1.4 nM PGE2 (basal channel) and the respective ion channel inhibitors (apically and basally). After 4h of co-treatment with inhibitors and PGE2, side view imaging was performed to determine the swelling of the mucus layer.

Scanning Electron Microscopy

To visualize the epithelium and mucus layer on-chip, SEM analysis was carried out using Colon Chips that had a top channel that was not irreversibly bonded to the membrane, which allowed the device to be dismantled manually as describe previously[48]. Cells were fixed with 4% PFA (157–4, Electron Microscopy Sciences) and 2.5% glutaraldehyde (G7776; Sigma) in DPBS and incubated in 0.5% osmium tetroxide (19152, Electron Microscopy Sciences) in 0.1 M sodium cacodylate buffer (pH 7.4) before serial dehydration in ethanol. Samples were then dried using a critical point drier and imaged using field emission SEM (Hitachi S-4700).

For cross sectional SEM images, Colon Chips were fixed with 2.5% Glutaraldehyde in 0.1 M Phosphate Buffer (P5244, Sigma) overnight and washed with water before being flash-frozen in liquid nitrogen. Frozen chips were sectioned into 5 mm cross sections on dry ice, lyophilized, mounted on aluminum pin mounts with conductive carbon tape, sputter-coated with gold, and examined with a Tescan Vega3 GMU scanning electron microscope.

Analysis of inner and outer mucus layer

Cells in the Colon Chips were stained for live cells by perfusing both channel for 30 min with medium (stem cell expansion medium basally, HBSS apically) containing 10 µM Calcein AM (C3100MP, Invitrogen) (200 µl h^{-1}); the medium perfused through the apical channel also contained 1 µm FluoSpheres™ Carboxylate-Modified Microspheres (70 µl ml^{-1}; F13083, Invitrogen). Colon Chips were incubated under static conditions for 40 min to allow the fluorescent beads to settle, and then z-stack images of 2-3 areas of each chip were collected using a Leica SP5 confocal microscope. Calcein AM was imaged with a 488 nm Argon laser,

and beads were visualized using the multiphoton laser at 1000 nm. Confocal z-stacks were reconstructed and analyzed using IMARIS software (Bitplane). To determine the thicknesses of the inner and outer mucus layers, a Gaussian distribution was fit to the data using Matlab and height of the outer layer was determined using the middle 90% of the Gaussian distribution of the beads. The inner layer was set as the distance between the apical cell surface and the lower bound of the outer layer.

Shear stress deformation assay

Increasing flow rates were applied to the Colon Chips using a Fusion Touch Syringe Pump. Side view images were acquired by transmitted light microscopy and movies were generated during flow and stop cycles of the pump at 1.6 ml h^{-1}, 6 ml h^{-1}, 10 ml h^{-1}. Images were then analyzed using Fiji software by tracing movement of mucus strains while flow was applied compared to the final position after flow was stopped. Angle between flow and no flow was calculated (great angle equals great deformation).

Drawings

All drawings were created with BioRender.

Alcian blue mucin assay

Mucus was loosened from the apical surfaces of the colon chips by reducing disulfide bonds with 250 mM Tris (2-carboxyethyl) phosphine (C4706, Sigma-Aldrich). After 1h incubation, the mucus layer was mechanically separated by washing the apical channel with phosphate buffered saline (PBS). Samples were frozen, lyophilized overnight and reconstituted in PBS. Total mucus amount was determined using an alcian blue colorimetric assay adapted from Hall et al (1980)[49]. Briefly, a standard curve was created using serial dilutions of submaxillary gland mucin (Sigma) ranging from 0 μg ml^{-1} to 500 μg mL^{-1}. Samples were diluted into the linear range of the curve using PBS. Samples and standards were equilibrated with filtered Richard Allan Scientific™ alcian blue (88043, Thermo Fisher Scientific) for 2h. The

resulting precipitant was separated by centrifugation at 1870 g for 30 min. This was followed by a series of wash/spin cycles at 1870 g in a resuspension buffer composed of 40% ethanol, 0.1 M acetic acid and 25 mM magnesium chloride. The mucin pellets were then dissociated with a 10% SDS solution (71736, Sigma) and absorbance was measured with a microplate reader (Synergy HT, BioTek) at 620 nm. Mucin concentration values for samples were interpolated from a linear fit of the standard curve.

Statistical Analysis

All graphs are depicted as mean ± standard error of the mean (SEM) and significant differences between two groups were determined using two-tailed unpaired Student's t-test. To determine significant differences between 3 groups or more, one-way ANOVA with Tukey's multiple comparisons test was used. With 3 groups or more and 2 independent variables, 2ways ANOVA with Tukey's multiple comparisons was used to determine statistical significance. Prism 7 (GraphPad Software) was used for statistical analysis.

Data Availability

The authors declare that the data supporting the findings of this study are available within the article and its supplementary information files. All authors had access to the study data and had reviewed and approved the final manuscript.

FIGURE LEGENDS

Figure 1: Patient-derived human Colon Chip. (**A**) H&E stained histological sections sections (6 µm) of human colon *in vivo*. Colon tissue with intact epithelium, lamina propria, muscularis mucosa and submucosa (left, bar, 100 µm). Dissociated epithelium including lamina propria (middle, bar, 100 µm). Smear of a 2-day colon organoid in matrigel culture (right, bar, 20 µm). The arrows are marking mitotically active epithelial cells. (**B**) Primary human colonic epithelium in Matrigel cultures. Colonic crypts embedded in Matrigel after isolation from human colon resection (left). Shortening of crypt structure embedded in Matrigel one day after isolation (middle). Closed colonic crypts (colon organoids) in Matrigel cultures 7 days after isolation (right) (bars, 200 µm). (**C**) Phase contrast images of the preparation of primary human colonic epithelial cells for seeding on the Chip starting with colon organoids that are fragmented before seeding on chip (bars, 200 µm). (**D**) Photograph (left) and schematic cross-sectional view (right) of the primary human Colon Chip microfluidic device with the colonic epithelium cultured in the top channel on the upper surface of the porous membrane that separates it from the lower channel.

Figure 2: Epithelium development in the Colon Chip after 3, 7, and 14 days of culture after monolayer formation. (**A**) Phase contrast microscopic images of colonic pithelial cells cultured on the Colon Chip (bars, 200 µm). (**B**) DIC images of the colonic epithelium (viewed from above; bars, 100 µm). (**C**) 3D confocal microscopic re-construction of z-stack images of the epithelial cells based on F-actin staining (bars, 100 µm, images are representative of 2 independent experiments). (**D**) DIC images of the full length of the channel of the Colon Chip (bars, 1000 µm; images are representative of 2 independent experiments).

Figure 3: Formation of a polarized colonic epithelium within the Colon Chip. (**A**) Immunofluorescence confocal microscopic images of histological cross sections of the Colon Chip (left) compared with human colon *in vivo* (right) showing a polarized epithelium with tight

junctions labeled with ZO1 (green) and brush border stained for F-actin (gray) restricted to the apical regions, and Hoechst-stained nuclei (blue) localized at the cell base (bars, 50 µm; white dashed line, top of the porous PDMS membrane in the Colon Chip, images are representative of 3 independent experiments). (**B**) Cross-sectional, immunofluorescence confocal microscopic images of the Colon Chip (left) compared with human colon *in vivo* (right) showing a polarized epithelium with basolateral adherens junctions labeled with E-Cadherin (green) and brush border stained for F-actin (gray) restricted to the apical regions, and Hoechst-stained nuclei (blue) localized at the cell base (bars, 50 µm; images are representative of 3 independent experiments). (**C**) Intestinal barrier function of the colonic epithelium measured over 14 days of culture on-chip by quantifying the apparent permeability (P_{app}) of Cascade blue (550 Da) (n=3-11 chips; **** p<*0.0001* compared to day 2). (**D**) Quantification of cell growth by measuring EdU incorporation over 18 h using flow cytometry (n=6-8 chips, 2 independent experiments compiled; *p<0.05 compared to d3 and d7). All data represent mean ± SEM.

Figure 4: Spontaneous goblet cell differentiation in the Colon Chip. (**A**) Cross-sectional, confocal immunofluorescence microscopic images of epithelial cells in a colon organoid (Org) (bar, 20 µm), Transwell culture (TW), Colon Chip (Chip), and human colon tissue section (*In Vivo*) (all bars, 50 µm) stained for E-Cadherin (green) and MUC2 (magenta); cultures shown are 7 days after monolayer formation (images are representative of 2 independent experiments). (**B**) 3D reconstructions of confocal immunofluorescence microscopic z-stack images of epithelial cells in TW cultures and Colon Chips at day 3, 7 and 14 after monolayer formation showing epithelium stained for E-Cadherin (green) and MUC2 (magenta) (bars, 100 µm, images are representative of 2 independent experiments). (**C**) Contour plots of flow cytometric quantification of MUC2+ cells in colon organoid (Org), TW culture (TW) and Colon Chip (Chip) at day 7 of culture versus cells isolated from human colon tissue (*In Vivo*). (**D**) A graph showing the results of the full cytometric analysis at days 3, 7, and 14 (gray area

indicates approximate range of *in vivo* values; n=3-9 devices, 2 experiments compiled; **** $p<0.0001$ compared to TW and Org). (**E**) Flow cytometric quantification of MUC2+ cells isolated from human colon tissue (n=2 donors, 2-3 samples per donor). (**F**) Flow cytometric quantification of the percentage of Ki67-labeled proliferating cells in the Colon Chip (Chip), TWs and colon organoids (Org) on day 3, 7 and 14 by flow cytometry (n=3-8 devices, 2 independent experiments compiled; * $p<0.05$, ** $p<0.01$). All data represented as mean ± SEM.

Figure 5: Differentiation of mature goblet cells in the Colon Chip. (**A**) Cross-sectional, immunofluorescence confocal microscopic images of the Colon Chip stained for goblet cell markers TFF3 (green) and MUC2 (magenta), and for the brush border marker F-actin (yellow) (bar, 50 µm; images are representative of 2 independent experiments; white dashed line, top of the porous membrane in the Colon Chip). (**B**) Higher magnification view of an immunofluorescence confocal micrograph showing cross section of the epithelium within the Colon Chip containing goblet cells stained for MUC2 (magenta), E-cadherin (green), and F-actin (gray) (bar, 20 µm, image is representative of 2 independent experiments). (**C**) Scanning electron micrograph of the apical surface of a goblet cell within the epithelium cultured in the Colon Chip surrounded by enterocytes covered with apical microvilli (bar, 5 µm). (**D**) Graphs showing the fold change in the levels of transcripts encoding SAM pointed domain ETS factor (SPDEF), Kruppel like Factor 4 (KLF4), Fc-g binding protein (FCGBP), chloride channel accessory 1 (CLCA1), resistin-like molecule β (RETNLB), Meprin A Subunit Beta (MEP1B), Kallikrein 1 (KLK1) and anterior gradient 2, protein disulphide isomerase family member (AGR2) in colonic epithelial cells cultured in the Colon Chip at day 3 or 7 (d3 or d7) relative to their levels in cells contained within colonic organoids before seeding on chip on day 0 (d0) (d0=1.0). (*$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to d0). All data represent mean ± SEM.

Figure 6: Method for non-invasive visualization of mucus layer height on-chip using Dark Field microscopy. (**A**) Schematic of how the chip is prepared for side view

imaging. (**B**) Whole chip side view images of live Colon Chips at day 3, 7 and 14 by dark field (DF) microscopy showing cells and the overlying mucus layer over time.

Figure 7: Development of MUC2+ mucus layer on Colon Chip over time. (**A**) Dark Field (DF) microscopic image of the side of an optically clear Colon Chip showing the entire length of the device with horizontal microfluidic upper and lower channels along its length, as well as upper inlet, outlet and vacuum port; a side vacuum channel is also apparent in this view. Note that mucus can be detected in the bottom half of the apical channel above the epithelium, which is cultured on the horizontal membrane that separates the upper and lower channels (dashed square corresponds to regions shown in **B-D**). (**B**) DF images of the same living Colon Chip at days 3, 7 and 14 after monolayer formation showing a reflective mucus layer overlying the epithelium that progressively increases in thickness over time above the darker cell region (dashed line, porous membrane; bar, 200 µm, images are representative of 3 independent experiments). (**C**) Higher magnification images of a region similar to the area shown in the dashed square in (**A**) within a Colon Chip fixed at day 7, demonstrating the presence of a thick mucus layer visualized by DF microscopy and MUC2 staining (MUC2) overlying the F-actin-rich brush border of the colonic epithelium (F-actin) (dashed line, porous membrane; bars, 200 µm, images are representative of 2 independent experiments). (**D**) Side view images of Colon Chip fixed at day 7, demonstrating the presence of a thick mucus layer visualized by DF microscopy and TFF3 staining (TFF3) overlying the F-actin-rich brush border of the colonic epithelium (F-actin) (dashed line, porous membrane; bar, 200 µm, images are representative of 2 independent experiments).

Figure 8: Formation of a mucus bilayer in the human Colon Chip. (**A**) A pseudo-color 3D reconstruction of representative z-stack confocal images of the Colon Chip perfused with 1 µm fluorescent beads (magenta) 3, 7 or 14 days after monolayer formation; the cells were live stained with Calcein AM (light green) (images are representative of 2 independent

experiments). (**B**) Quantification of the thickness of the inner (impenetrable) and outer (penetrable) mucus layers base on the distribution of the beads (n = 3 chips, 3 independent regions per chip; all values significantly different between all days, * p<0.05 for inner and outer layer; similar results were obtained in 2 independent experiments). (**C**) Scanning electron micrograph of a cross section of the epithelium within a Colon Chip demonstrating the presence of a filamentous network within the mucus layer in direct contact with the apical surface of the cells (bracket indicates the PDMS membrane; bar, 50 µm, image is representative of 2 independent experiments). All data represent mean ± SEM.

Figure 9: PGE2-induced mucus layer swelling on-chip. (**A**) Graph shows quantification in EdU incorporation measured over 18 hours in epithelial cell monolayers within Colon Chips after 6 days of PGE2 treatment compared to untreated controls (Con) detected using flow cytometry (n = 3 chips per treatment). Graphs showing changes in EdU incorporation measured over 18 hours (**B**) and MUC2+ cells (**C**) in epithelial monolayers within Colon Chips after 6 days of PGE2 treatment relative to untreated controls (control value set to 1) detected using flow cytometry (n = 5 chips for **A**; n = 5-6 chips for **B**; 2 independent experiments compiled). (**C**) Bright field images from above of Colon Chips that were untreated or treated with PGE2 for 6 days (bars, 200 µm). (**D**) DF side view images of a Colon Chip with epithelial cells and mucus layer before and after PGE2 treatment for 4 hours (bars, 200 µm, images are representative of 5 independent experiments). (**E**) Change in mucus height after 4h PGE2 treatment over the mucus height before treatment in the same chips (n=3-6, similar results were obtained in 5 independent experiments). (**F**) Total mucin amount per chip with and without 4h PGE2 (n=2-3) (not statistically significant by t-test). All data represent mean ± SEM.

Figure 10: Modulation of PGE2-induced changes in mucus layer thickness by ion channel inhibition. (**A**) DF side view images of a Colon Chip with epithelial cells and mucus layer before (No PEG2) and after PGE2 treatment for 4 hours without (PGE2 Control) or with

CFTRinh-172 (50 µM), XE-991 dihydrochloride (20 µM), bumetanide (100 µM) or a combination of all three ion channel inhibitors (bar, 200 µm, images are representative of 2 independent experiments). (**B**) Quantification of changes in mucus layer height based on the DF images shown in **A** and total mucin production measured using an alcian blue assay (**C**) induced by PGE2 in the absence (PGE2 control) or presence of CFTRinh-172 (50 µM), XE-991 dihydrochloride (20 µM), bumetanide (100 µM) or a combination of all three ion channel inhibitors (data are presented relative to control chips treated with PGE2 alone (n = 2-6, 2 independent experiments compiled). (** p< 0.01 vs. control). All data represent mean ± SEM.

Figure 11: Inner mucus layer is not affected by PGE2. (**A**) Side view combined DF and fluorescent image of a Colon Chip with a mucus layer labeled with 1 µm fluorescent beads (magenta) without (Con) and with PGE2 treatment for 4 hours (PGE2 4h) with the dashed square indicating a region of the chips represented in the higher magnification images in **B** (bars, 1000 µm, images are representative of 2 independent experiments). (**B**) Side view images of Colon Chip with or without 4 h PGE2 treatment with 1 µm fluorescent beads (magenta) overlaid on DF images (bars, 200 µm, images are representative of 2 independent experiments). (**C**) Response of the mucus layer to shear forces measured in angle over increasing velocity, with a higher angle being equivalent to stronger bending of the mucus layer (each data point represents 1 chip, n=3 per treatment).

Table 1: Donors used in experiments.

Supplementary Video 1: Shear force assay. Side view images compiled to movies at 6 ml h-1 and 10 ml h-1 flow rate without (No PGE2) or with 4h of PGE2 treatment (4h PGE2).

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Organoid ID	Colon Region	Goblet cells (# of experiments)	Mucus layer (# of experiments)
C07	Sigmoid	Yes (2)	Yes (1)
C08	Sigmoid	Yes (>5)	Yes (>5)
C09	Sigmoid	Yes (1)	Yes (2)
H447	Ascending	Yes (1)	Yes (1)
H480	Ascending	Yes (1)	Yes (1)

Table 1: Donors used in experiments.

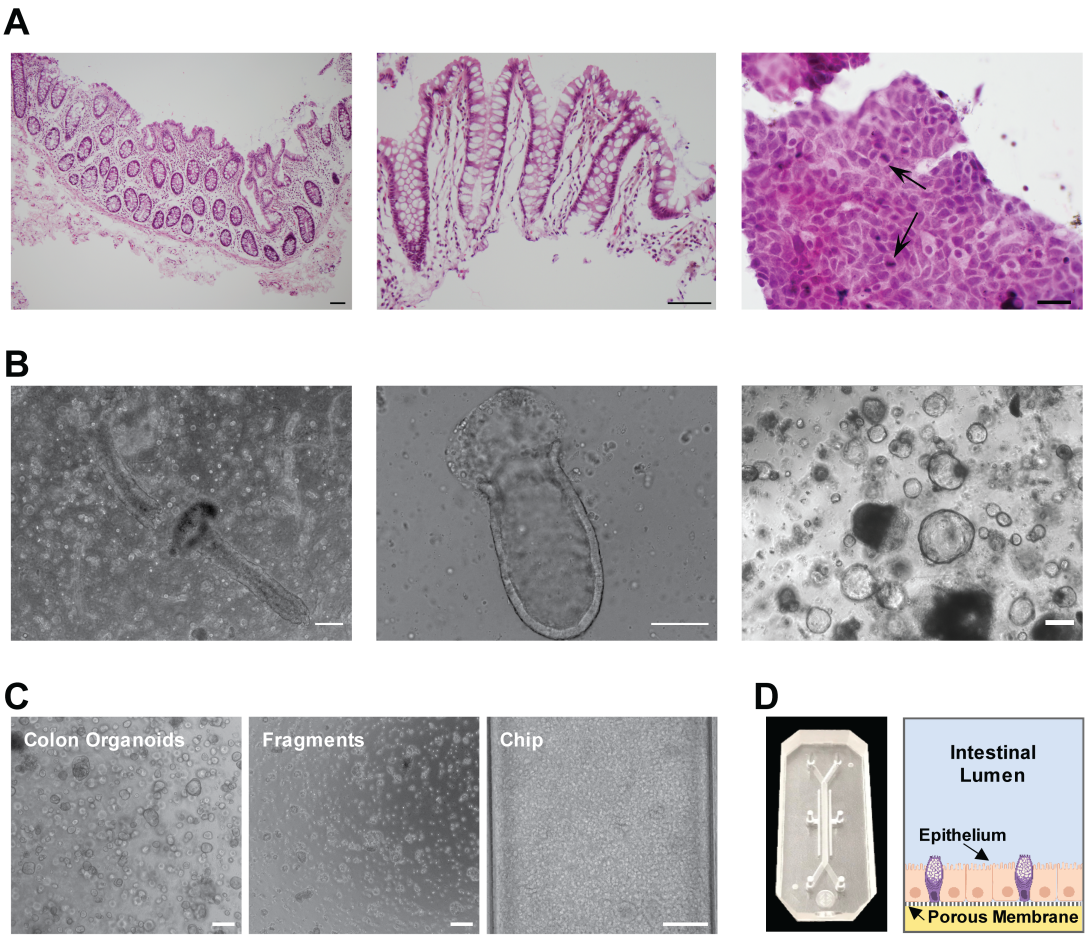

A
B
C
Colon Organoids
Fragments
Chip
D
Intestinal Lumen
Epithelium
Porous Membrane

Figure 1

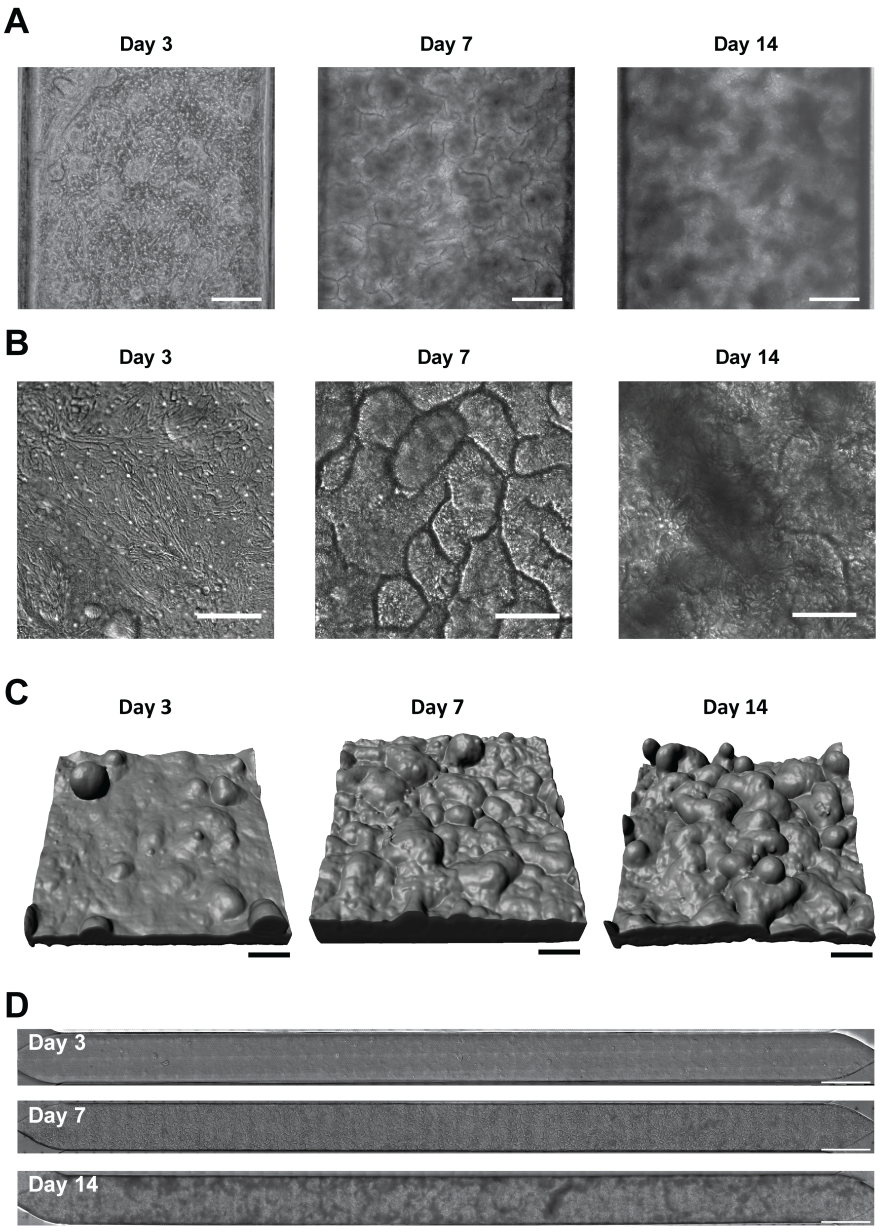

A
Day 3
Day 7
Day 14
B
Day 3
Day 7
Day 14
C
Day 3
Day 7
Day 14
D
Day 3
Day 7
Day 14

Figure 2

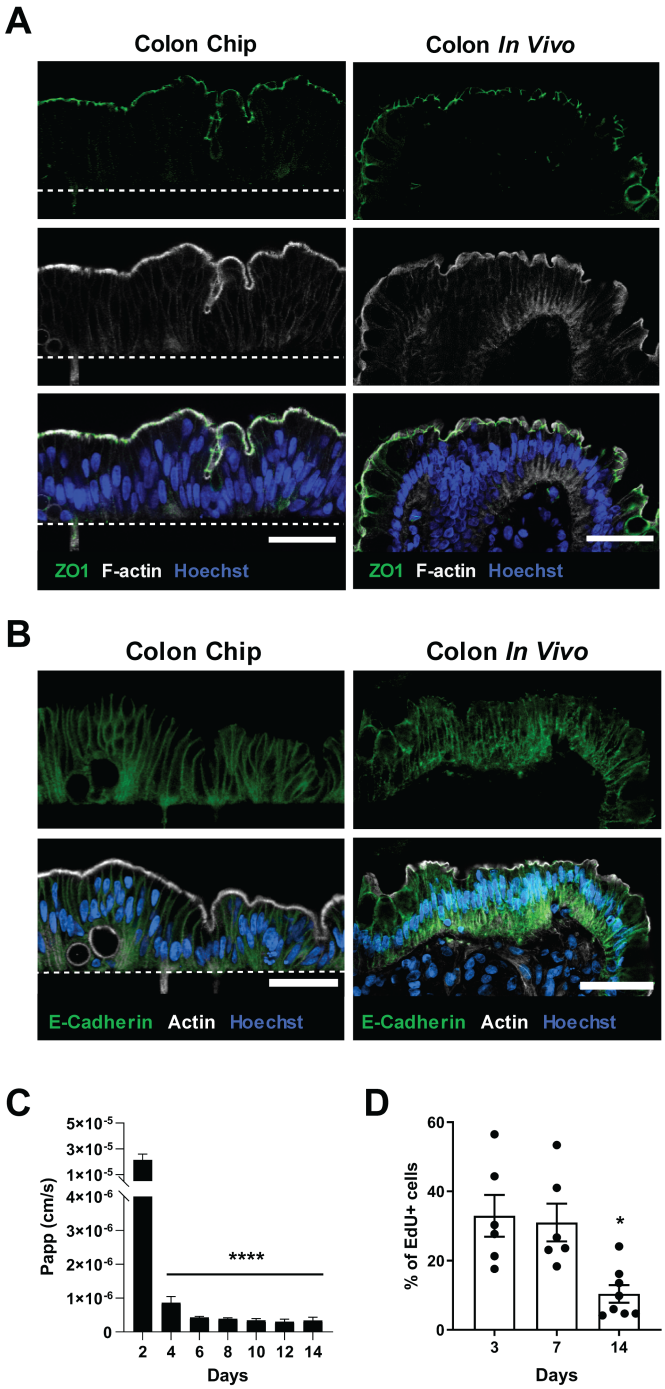

A
Colon Chip
Colon *In Vivo*
ZO1 F-actin Hoechst
ZO1 F-actin Hoechst
B
Colon Chip
Colon *In Vivo*
E-Cadherin Actin Hoechst
E-Cadherin Actin Hoechst
C
Papp (cm/s)
5×10-5
3×10-5
1×10-5
4×10-6
3×10-6
2×10-6
1×10-6
0
****
2 4 6 8 10 12 14
Days
D
% of EdU+ cells
60
40
20
0
*
3 7 14
Days

Figure 3

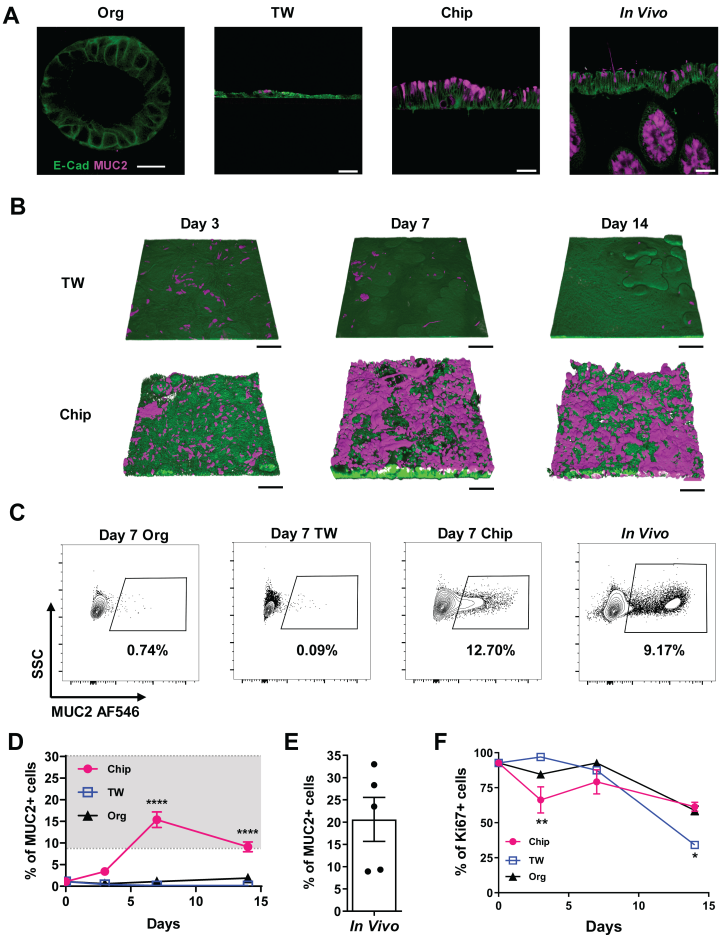
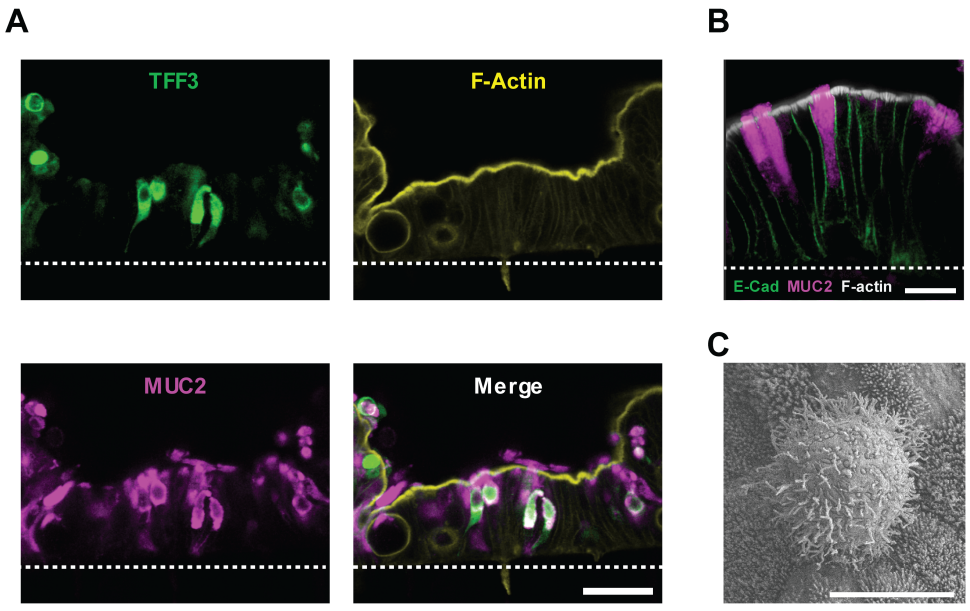

A
Org
TW
Chip
In Vivo
E-Cad MUC2
B
Day 3
Day 7
Day 14
TW
Chip
C
Day 7 Org
Day 7 TW
Day 7 Chip
In Vivo
0.74%
0.09%
12.70%
9.17%
SSC
MUC2 AF546
D
% of MUC2+ cells
Chip
TW
Org
****
****
Days
E
% of MUC2+ cells
In Vivo
F
% of Ki67+ cells
**
*
Chip
TW
Org
Days

Figure 4


A
TFF3
F-Actin
MUC2
Merge
B
E-Cad MUC2 F-actin
C

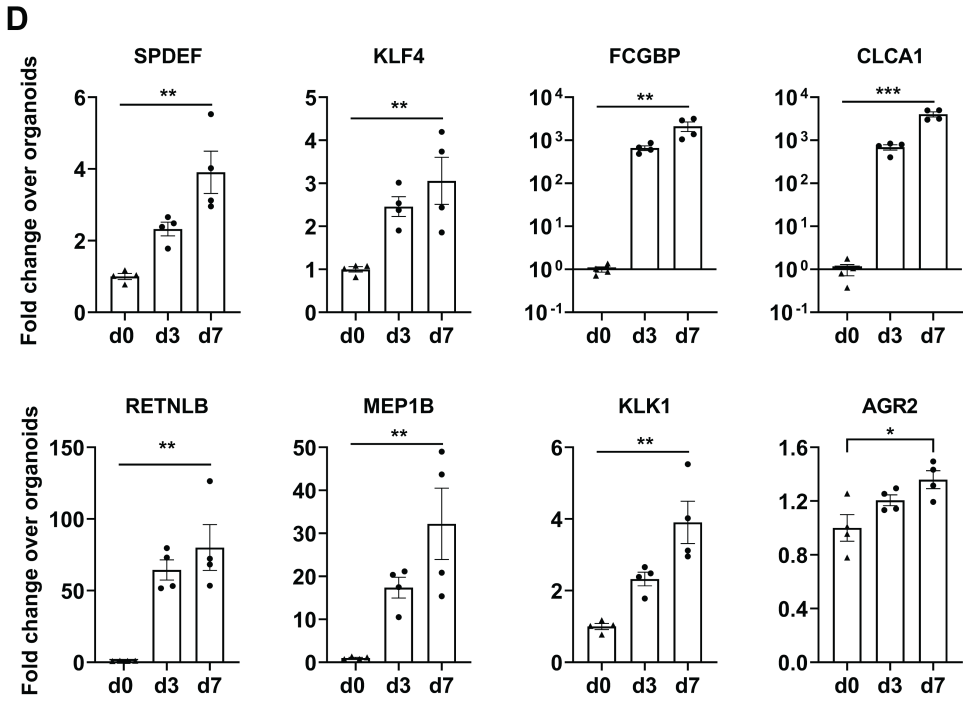
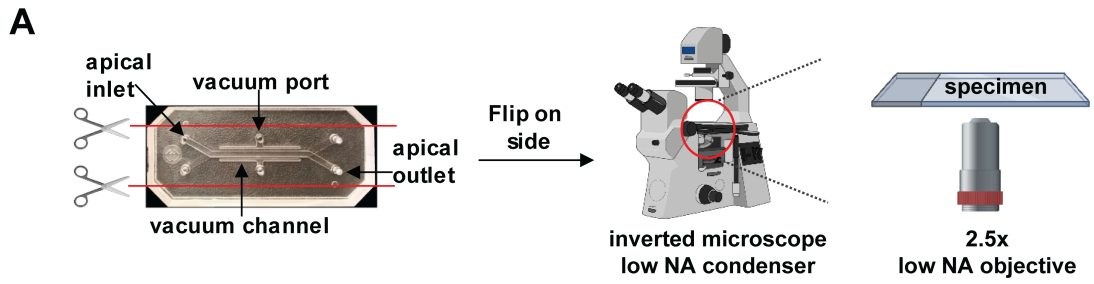

D
SPDEF
KLF4
FCGBP
CLCA1
RETNLB
MEP1B
KLK1
AGR2
Fold change over organoids
d0
d3
d7

Figure 5


A
apical inlet
vacuum port
apical outlet
vacuum channel
Flip on side
inverted microscope
low NA condenser
specimen
2.5x
low NA objective

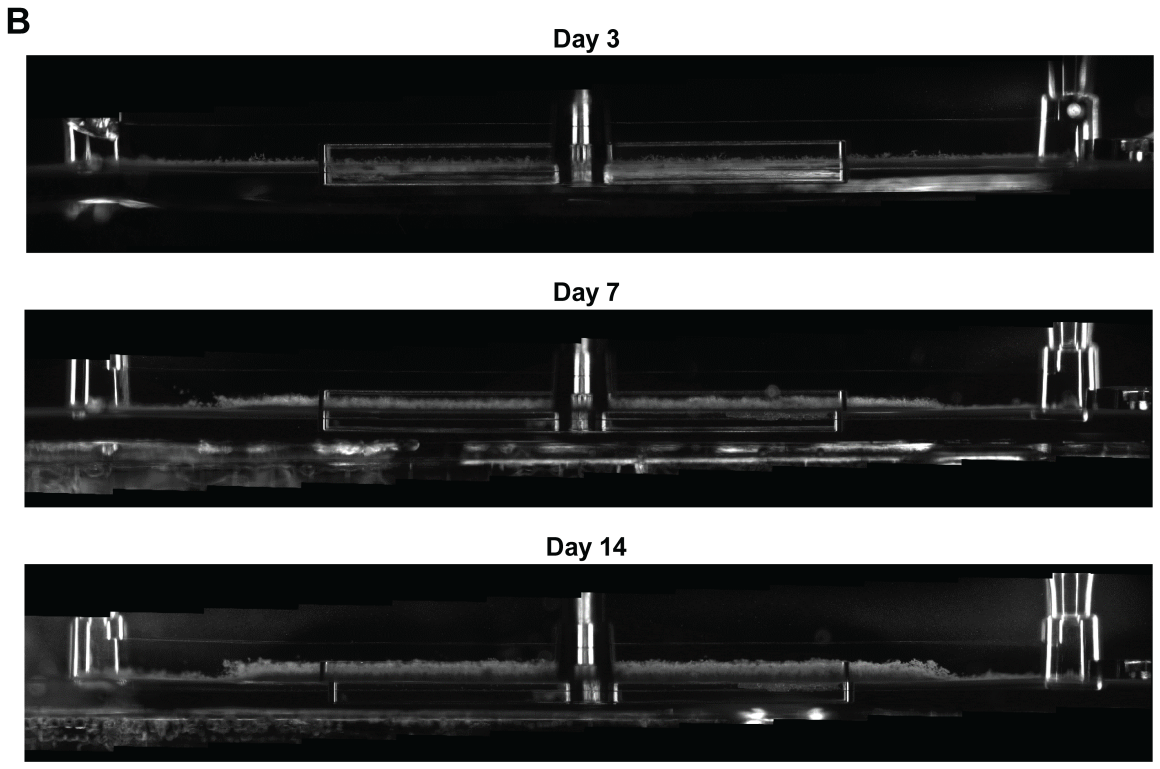

B
Day 3
Day 7
Day 14

Figure 6

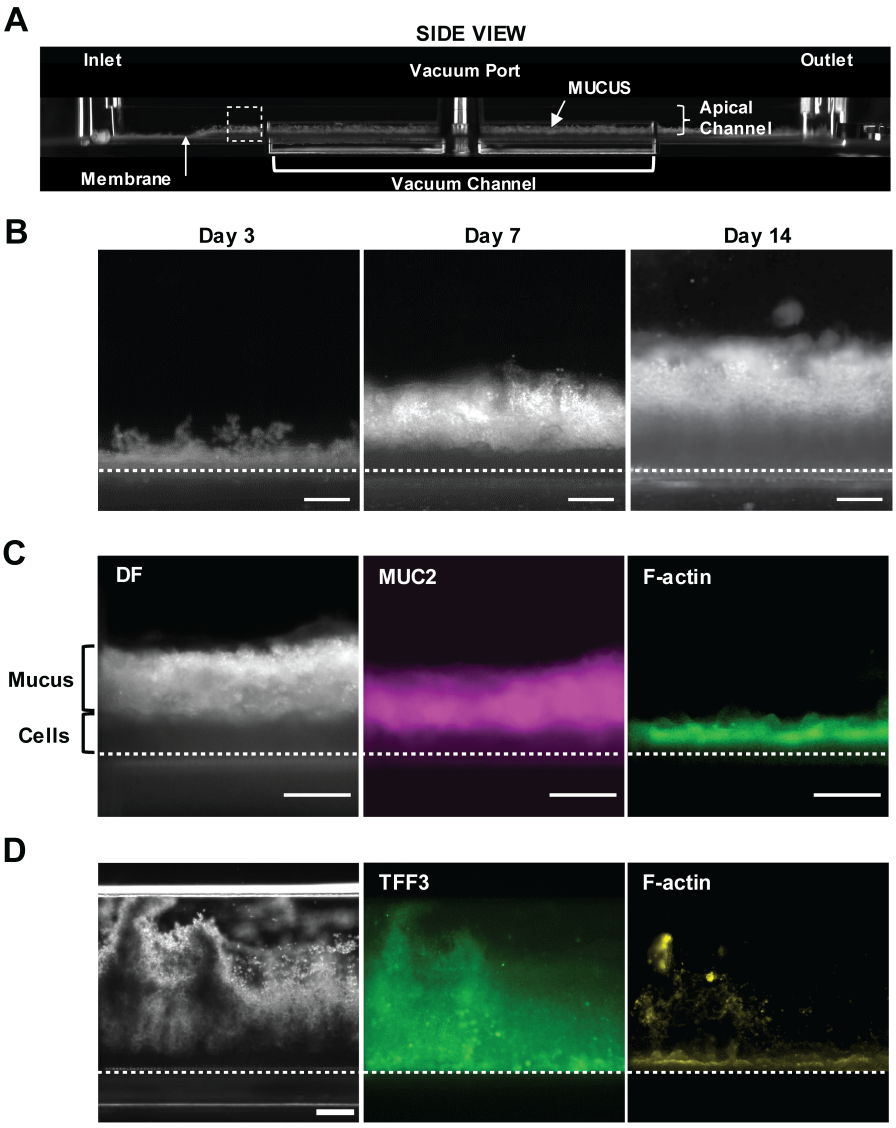

A
SIDE VIEW
Inlet
Vacuum Port
Outlet
MUCUS
Apical Channel
Membrane
Vacuum Channel
B
Day 3
Day 7
Day 14
C
DF
MUC2
F-actin
Mucus
Cells
D
TFF3
F-actin

Figure 7

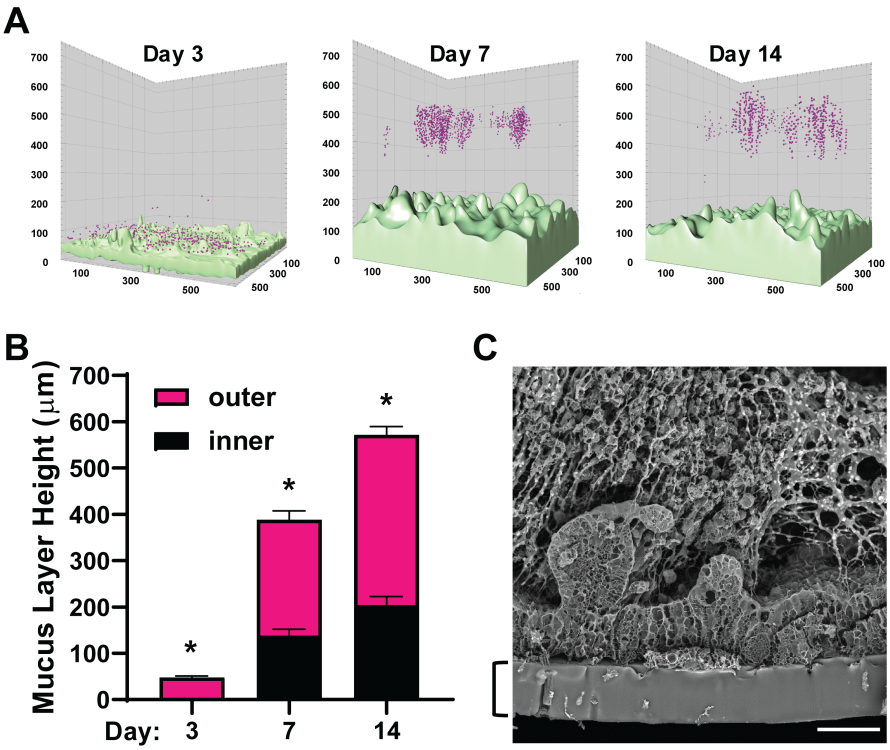

A
Day 3
Day 7
Day 14
B
Mucus Layer Height (μm)
outer
inner
Day: 3 7 14
C

Figure 8

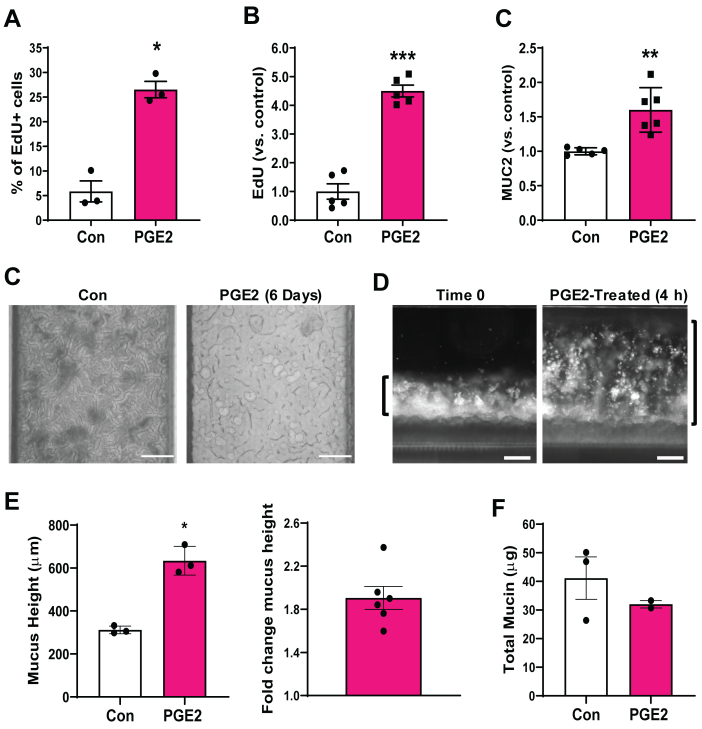

A
% of EdU+ cells
Con
PGE2
B
EdU (vs. control)
C
MUC2 (vs. control)
C
Con
PGE2 (6 Days)
D
Time 0
PGE2-Treated (4 h)
E
Mucus Height (μm)
Fold change mucus height
F
Total Mucin (μg)

Figure 9

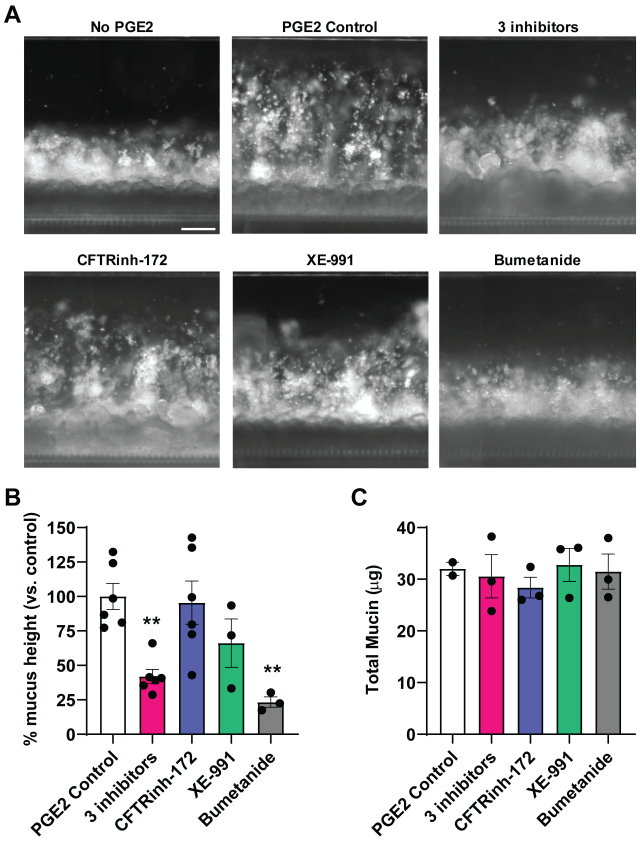

A
No PGE2
PGE2 Control
3 inhibitors
CFTRinh-172
XE-991
Bumetanide
B
% mucus height (vs. control)
150
125
100
75
50
25
0
**
**
PGE2 Control
3 inhibitors
CFTRinh-172
XE-991
Bumetanide
C
Total Mucin (µg)
40
30
20
10
0
PGE2 Control
3 inhibitors
CFTRinh-172
XE-991
Bumetanide

Figure 10

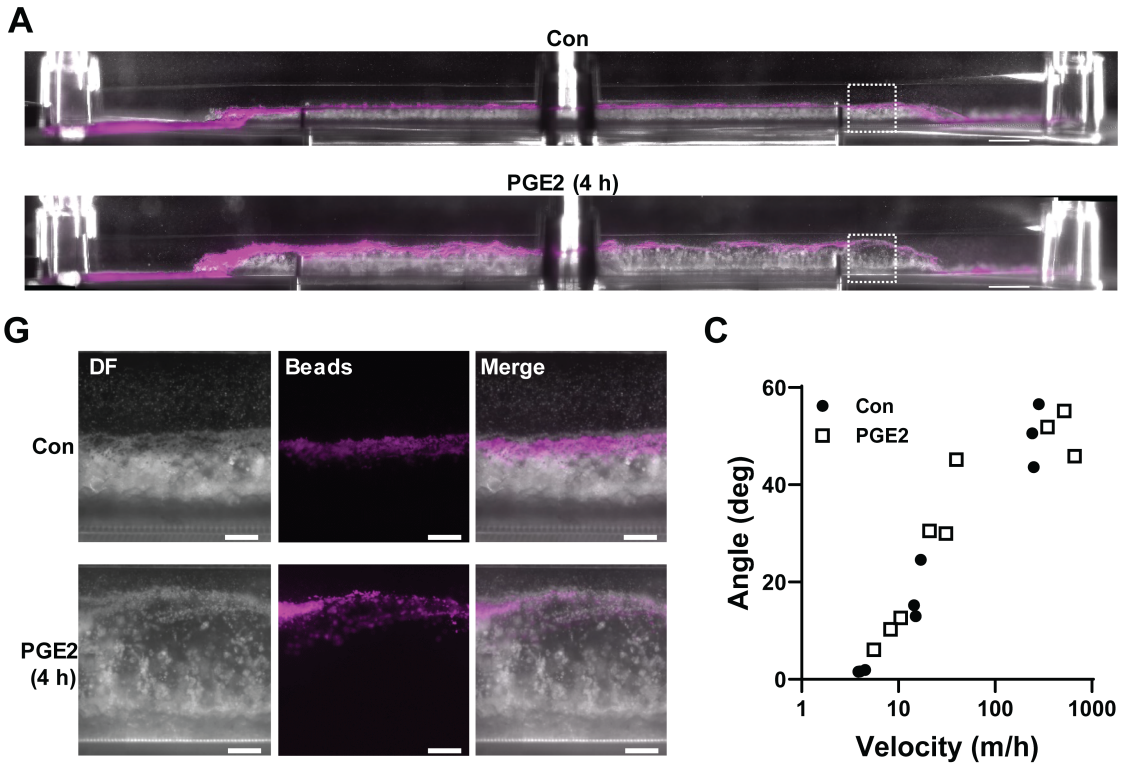

A
Con
PGE2 (4 h)
G
DF
Beads
Merge
Con
PGE2
(4 h)
C
Con
PGE2
Angle (deg)
Velocity (m/h)
0
20
40
60
1
10
100
1000

Figure 11