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Garima (Jeeya) Sharma March 27, 2024

PTGER4-dependent epigenetic regulation of intestinal epithelial subtypes during homeostasis and Crohn's Disease

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An abstract of a thesis to be submitted the Faculty of the College of Arts and Sciences of Emory University in partial fulfillment of the requirements of the degree of Bachelor of Sciences with Honors

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Abstract

Background: Crohn's Disease (CD) is a relapsing-remitting inflammatory bowel disease (IBD) that can also manifest in the rectum as perianal fistulizing CD. During this time, mucosal inflammation can disrupt epithelial cell differentiation, decreasing the abundance of fully differentiated cell types that mitigate healing. Gastrointestinal organoids lack fully differentiated cell types and thus can serve as a model to mechanistically investigate pathways that promote them, potentially giving rise to new therapeutic targets. We hypothesize that PTGER4, an epithelial receptor encoded by an IBD risk gene, is involved in epigenetic modulation that promotes expression of gene programs involved in secretory lineage formation. **Methods:** Rectal mucosal biopsy samples from Emory University/Children's Healthcare of Atlanta (CHOA) were used for crypt extraction and organoid culturing. Localization of HDAC cell type expression was determined by immunofluorescence on surgical tissue sections. Freshly isolated crypts and established organoids were used for biochemical analysis on chemically inhibited HDAC or HES1 samples, including rt-PCR and Western blotting (WB). Inhibition of PTGER4 was also investigated under these conditions. **Results:** Class I and II HDACs were broadly expressed in the epithelium, stromal, and immune compartments of the mucosa, with relatively high levels of HDAC4 in epithelial cells. We found that PTGER4 stimulation in organoids with prostaglandin E2 (PGE2) decreased histone deacetylases phosphorylation at HDAC4(S246)/5(S259)/7(S155), but not HDAC4(S632)/5(S661)/7(S486), and found that LMK-235, an inhibitor of HDACs 4/5, can increase *SPINK4* expression. This contrasted with butyrate stimulation of organoids that increased the phosphorylation of HDAC4(S246)/5(S259)/7(S155), increased levels of HDAC 4 and 5, and depleted levels of HDAC7, but also increased *SPINK4* expression. Inhibition of HDACs with butyrate or small molecule inhibitors of HDACs had variable effects on freshly isolated crypts and organoids, likely reflecting patient heterogeneity in these pathways. **Conclusion:** These results suggest that PTGER4 regulates secretory lineage pathways by activating HDAC4 and 5 in a manner opposite of the signals coming from the microbiome (i.e. butyrate), and these opposing signals, one received apically by the epithelium from the microbiome, the other basolaterally from stromal cells, likely balance one another during homeostasis to promote the appropriate proportions of epithelial subtypes, but the process is disrupted during inflammation and further complicated by patient specific responses.

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Introduction

Our overall wellbeing is intricately linked to intestinal homeostasis. The human gastro-intestinal tract is indeed a system-of-systems that includes the mouth, esophagus, stomach, small intestine, large intestine (or colon), rectum and anus that all work together to process food, extract nutrients, and expel waste. The intestinal mucosa is organized in layers. The first layer, the epithelium, is comprised of intestinal epithelial cells (IECs) that are in contact with the luminal contents of the gut. Organized into millions of crypts and small intestinal villi, IECs rest on the lamina propria, forming crypts that fold in or villi that extend outward into the lumen. Connected side-by-side, these IECs constitute an epithelial “sheet” that covers the entire surface area of the gastrointestinal tract while selectively transporting biochemicals in and out of the cells (**Figure 1**).

This selective barrier of epithelial cells protects the next layer, called the lamina propria, from external biomaterials that have been ingested. Within the lamina propria there are a variety of other cell types including immune (i.e. macrophage, B-cell, etc), endothelial, and mesenchymal cells (myo-/fibroblasts). The immune cells defend against microbes that penetrate the epithelial barrier [1], the mesenchymal stromal cells (MSC) provide various soluble cues to the epithelium for proliferation and/or differentiation signaling, and the endothelium provides blood flow in and out of the mucosa. MSCs also play a key intermediary role in regulating the behavior of the epithelium and immune cells during homeostasis and inflammation/repair [2]. This interplay and crosstalk between mucosal cell types is fundamental to maintaining wellbeing.

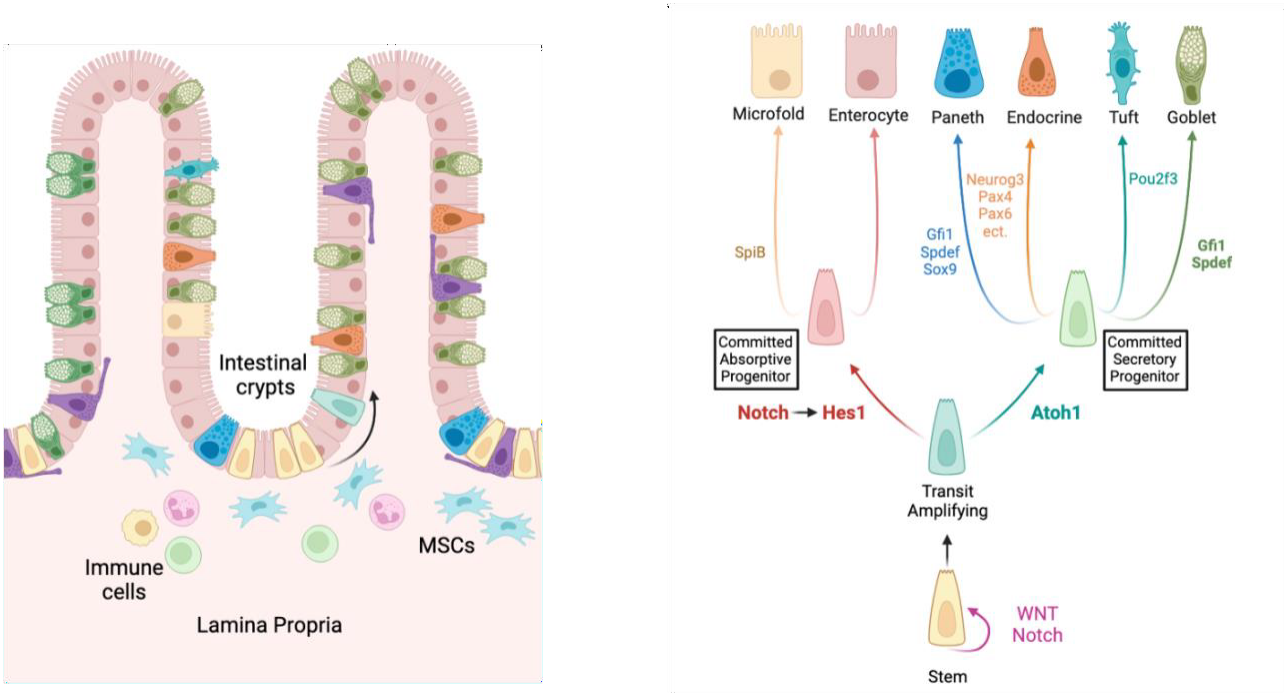

Figure 1. Intestinal crypt stem cell differentiation is mediated by signaling factors and differential gene expression.

Key Signaling Involved in Intestinal Epithelial Cell Differentiation

All the intestinal epithelial cells of the gut are replenished every 3-5 days by a rich source of leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5+) stem cells at the base of each crypt [3]. As depicted in **Figure 1**, IECs differentiate as they travel vertically along the crypt axis towards the lumen and are exposed to different mitogenic factors, cytokines, chemokines, and immunoregulatory metabolites released by a heterogeneous population of MSCs [4]. Accordingly, these epithelial cells differentiate into either absorptive or secretory cell types depending on the signaling factors they receive. Absorptive cell types, such as enterocytes, make up most of the gut lining and are responsible for nutrition uptake along the surfaces of villi in the small intestine; whereas,

secretory cell types, such as Paneth and goblet cells that are responsible for secreting antimicrobial (Paneth) or mucus that forms a dense but permeable boundary between the luminal contents and epithelium (goblet cells), make up a much smaller percentage of the epithelial subtype population [5]. Other epithelial cell subtypes critical for barrier function and homeostatic maintenance include enteroendocrine cells (EEC) that are responsible for hormone secretion, along with tuft cells that support immune function [1] (**Figure 1**). These different cell types necessitate tightly controlled signaling pathways in maintaining homeostasis.

Canonical Notch and Wnt signaling regulate highly conserved pathways that are crucial to intestinal progenitor cell proliferation and differentiation. Notch signaling has been evidenced to promote absorptive lineages while inhibiting secretory cell programming. A Notch receptor is activated when Notch ligands, such as Delta-like ligand 1 (DLL1), are expressed by neighboring cells and bind to the receptor. Subsequent proteolytic cleavage of the receptor produces the Notch intracellular domain (NICD) that forms a part of a transcriptional protein activation complex in the nucleus to promote the activation of target genes, such as *hairy and enhancer of split-1* (*HES1*) [6]. HES1 appears to inhibit the transcription of the basic helix-loop-helix transcription factor *atonal homolog 1* (*ATOH1*) gene responsible for promoting secretory cell types, by binding to *ATOH1's* promoter and acting as a transcriptional repressor [7]. Thus, Notch signaling not only promotes progenitor cell proliferation but also favors the differentiation of absorptive enterocytes.

The Wnt signaling pathway similarly promotes IEC growth and differentiation. Extracellular Wnt proteins can bind to Frizzled G-Protein Coupled Receptors (GPCRs)

and other co-receptors, such as LRP5/6 to initiate signaling that modulates the levels of β-catenin available to a cell. β-catenin phosphorylated at S552 accumulates in cells with high Wnt concentrations, localizing to the nucleus and promoting activation of Wnt target genes, including those necessary for secretory cell type generation [8,9]. As such, both independently and at their intersections, the opposing effects of Notch and Wnt signaling pathways allow for the development of different IEC phenotypes [10].

As described, expression of *HES1* by epithelial cells towards the base of the crypt in the transit amplifying zone favors an absorptive lineage through inhibition of *ATOH1* [11]. Whereas a secretory lineage is promoted by cells expressing *ATOH1,* operating through several mechanisms to propagate mature secretory IECs [12]. Of note, the genetic loci encoding a zinc-finger protein, GFI1, that promotes the formation of goblet or Paneth cells, is a target gene of ATOH1 [13]. The SAM pointed domain ETS factor (SPDEF) is another transcription factor implicated in goblet and Paneth cell differentiation [14]. Furthermore, the SOX9 transcription factor is also involved in the development of Paneth cells [15]. Studies demonstrate that the ATOH1 target gene encoding the bHLH transcription factor Neurogenin 3 (NEUROG3) initiates EEC differentiation [16]. The differentiation of these IECs is also highly influenced by interactions with other cell types in the lamina propria, including MSCs and immune cells, along with microbiota in the lumen, that release molecules such as short chain fatty acids like butyrate that are absorbed by epithelial cells. Altogether, the behaviors of these diverse, specialized cell types work collectively to maintain intestinal homeostasis. When this intricate network and collaboration begins to malfunction, disease of the gastro-intestinal tract likely occurs.

Inflammatory Bowel Diseases and Perianal Crohn's Disease

Crohn's disease (CD) is a relapsing and remitting inflammatory bowel disease (IBD) that can affect the entire gastrointestinal track and even peripheral organs, such as the eyes and skin [17,18]. Current immunotherapies for CD, such as anti-TNF monoclonal antibodies, alleviate disease symptoms but they are not curative or universally effective [19]. The cause of CD is not clear, but multiple factors, including environmental stimuli, genetic predisposition, and microbiome composition-based factors, are associated with the disease [20–22]. While CD can manifest throughout the body, it typically presents in the distal ileum, with luminal stricturing and deeply penetrating mucosal fibrosis as hallmark features of disease [23]. Perianal fistulizing CD, or perianal disease, is a severe penetrating form of CD that can manifest during rectal manifestations of CD, often with poor outcomes, leaving individuals debilitated and suffering from a diminished quality of life [24]. As such, perianal CD is marked by a loss of intestinal homeostasis, increased cellular permeability, and changes to the anatomical structures of the intestine, including the development of abscesses, skin tags, and fistulae. Unfortunately, up to 35% of CD patients form fistulas during their disease course, over half of which are perianal manifestations [25]. This aggressive phenotype of CD does not adequately respond to traditional therapies targeting the immune system and often requires surgical intervention [26]. The lack of response to therapies urges clinicians and researchers to determine root causes or consider novel therapies to ameliorate patients' disease. At the cellular level, perianal CD is marked by increased inflammation and a greater proportion of absorptive cell types in the rectum. Therefore, determining methods to study human IECs and

stimulating more the growth of more secretory cell types may lead to the discovery of healing mechanisms that may assist in treating disease.

Ongoing studies in the Kugathasan Lab show that the homeostatic proportions of IECs are disrupted by rectal inflammation during perianal disease. The mucosa in perianal disease appears to have lower than normal proportions of mature goblet cells with concurrently higher than normal proportions of absorptive lineages. The functional consequences of fewer goblet cells in each crypt may exacerbate disease by contributing to a compromised mucosal barrier, allowing for increased invasion of microbes with concomitant immune activation [27,28], or diminishes the pool of dedifferentiating cells that facilitate repair [29]. Therefore, elucidating IEC differentiation signals and mechanisms will give needed insight into potential therapeutic targets for Crohn's disease and its complex phenotypes.

PTGER4 as an Important Target in Intestinal Healing

MSCs and IECs work together to maintain the epithelial barrier by the exchange of soluble molecules like Wnt and bone morphogenetic proteins (BMPs) that promote the appropriate levels of proliferation and differentiation, giving rise to the necessary proportions of epithelial cell subtypes [30]. During injury, MSCs release prostaglandin E2 (PGE2) (made by COX-2 enzyme) that stimulates a prostaglandin EP4 receptor (PTGER4), a GPCR expressed on the epithelium, to initiate differentiation of wound associated epithelium (WAE) [31]. As a GPCR, PTGER4 belongs to the largest family of membrane proteins, each of which are composed of seven membrane bound α-helical segments. GPCRs are responsible for facilitating a wide range of cellular functions, including hormonal and neurotransmitter functions that regulate vision, olfaction, and

taste [32]. Notably, GPCR-targeting drugs have been used to treat multiple diseases including antihistamines, cardiovascular drugs, antacid drugs, and antipsychotics [33]. Their widespread use bolsters confidence in the notion of modulating PTGER4 activity or its downstream targets as a therapy. In fact, *ptger4* deficient mice, and knockouts of *ptgs2*, a COX-2 encoding gene, have impaired epithelial function and reduced populations of secretory cells, suggesting that the PGE2-PTGER4 axis is also involved in differentiation during homeostasis [34,35]. Remarkably, of the hundreds of risk loci for IBD identified through genome-wide association studies (GWAS), one of these loci maps to a small nucleotide polymorphism (SNP) at 5p13.1 that is just upstream of *PTGER4* on human chromosome 5 [36,37]. As such, defining the role of PTGER4 in regulating epithelial cell differentiation along the secretory lineage during CD has increased priority. To this end, we are reasoning that the therapeutic manipulation of intestinal epithelial-specific PTGER4 and downstream signaling will promote higher levels of goblet cells in the mucosa and stimulate healing.

Intestinal Organoids as a Model

Intestinal organoids are a proven model to study many of the properties of epithelial cells [38]. Organoids are grown from epithelial stem cells in crypts isolated from a patient's mucosal biopsy. Data from the Kugathasan Lab shows that organoids are transcriptionally distinct from mucosal epithelium and lack almost all differentiated cell types, including goblet cells, and show high levels of *HES1* expression when grown in Stem Cell Technologies Intesticult™ media (**Figures 2 and 3**). Secretory cell type markers, including *SPINK4, MUC2,* and *SPDEF*, were notably diminished and not co-

expressed in these organoids' transcriptomes (**Figure 3**). Thus, like inflamed intestinal mucosa with decreased secretory lineages, lack of these cell types in rectal organoid cultures provides the basis to dissect the missing signals needed to promote these lineages in the mucosa and give mechanistic to new therapeutic targets. The spatial-temporal microenvironment environment (crypt-axis) with MSC-derived signaling factors that influence IEC differentiation is missing in cell culture and presumably prevents these other differentiated lineages from occurring. Data produced by the Kugathasan Lab and others suggests that the desired differentiation state for a subset of these cells can be achieved with the appropriate manipulation of chemical cues in cell culture [39]. For example, we have shown that rectal organoids treated with PGE2 express higher levels of SPINK4 mRNA and protein. This result further links PGE2-PTGER4 signaling with a goblet cell phenotype and implies a potential pathway worth pursuing.

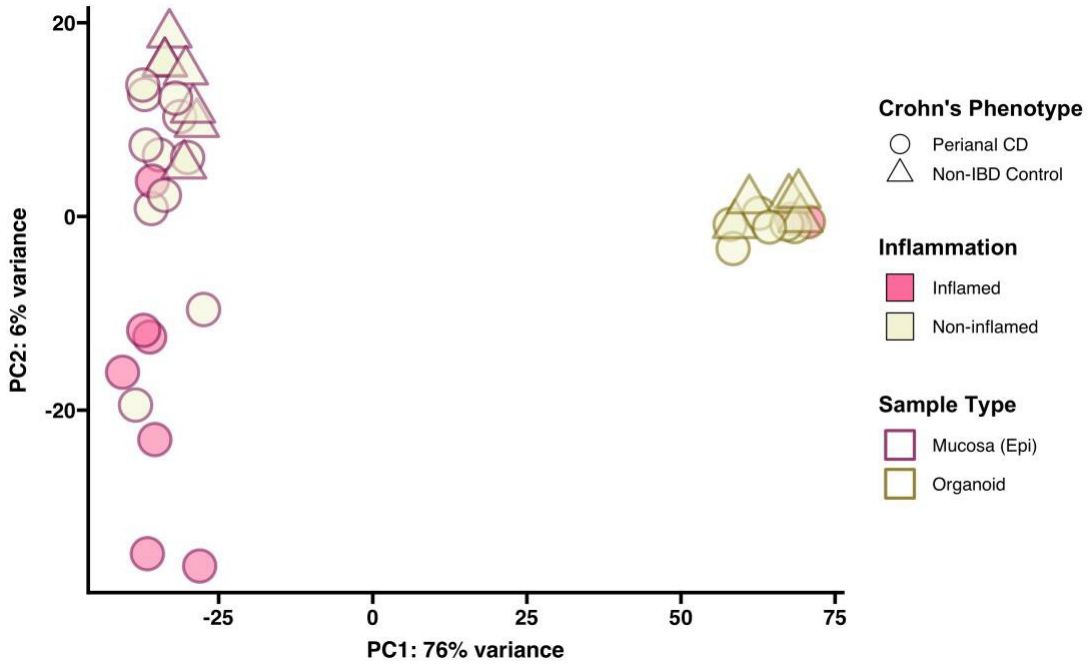

Figure 2. PCA analysis of scRNA-seq from mucosal epithelium and organoid culture.

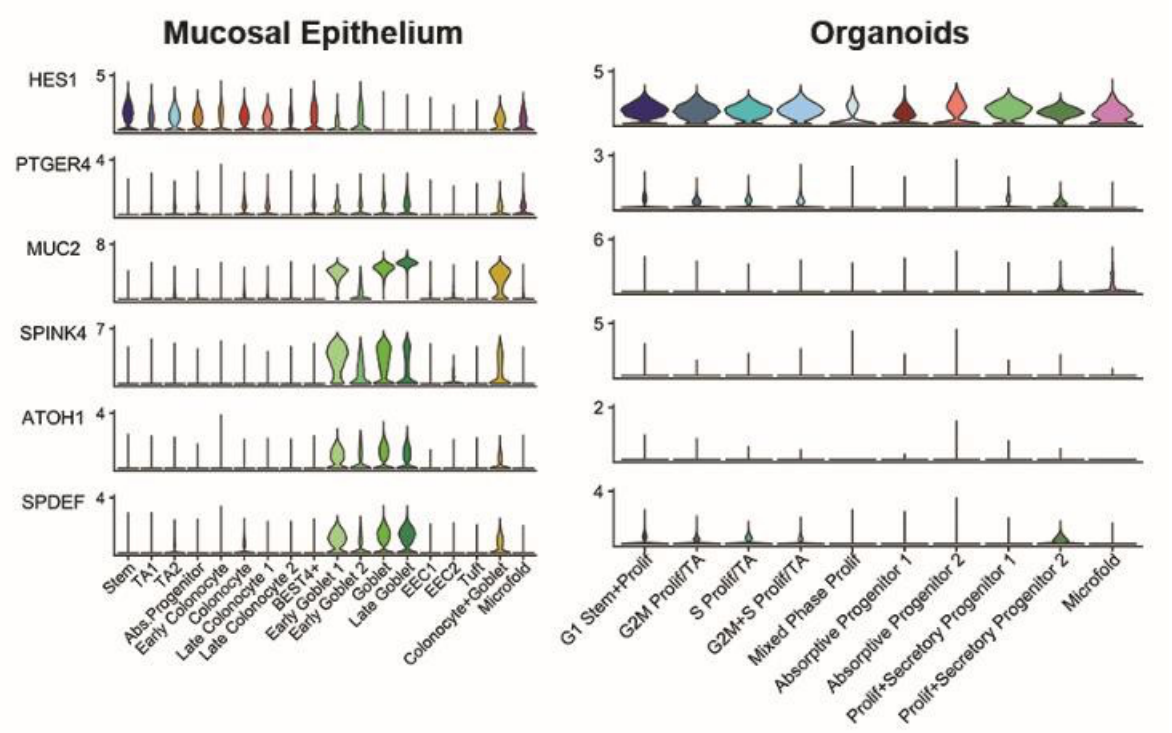

Figure 3. Analysis of scRNA-seq data derived from mucosal and organoid samples from the same patients. Violin plots highlight the presence of distinct goblet cell clusters marked by the co-expression of ATOH1, SPDEF, SPINK4, and MUC2 that are absent in the organoids.

Intestinal Epithelial Cell Differentiation and The Role of Epigenetic Proteins

We hypothesize that similar epigenetic modifications occurring in epithelial cells during mucosal inflammation are also taking place in cell culture, down regulating expression of genes involved in secretory lineage differentiation. Naturally, this idea invokes the role and regulation of histone modifying enzymes that can increase or decrease gene transcription by altering the architecture of the chromatin and impacting accessibility to gene enhancers and promoters. While the epigenetic code is still being deciphered, it is becoming increasingly clear that methylation and/or acetylation of histones can have dramatic effects on gene expression. Accordingly, histone

acetyltransferase (HATs) activity can “open” and histone deacetylases (HDAC) can “close” the chromatin through the transfer and removal of acetyl groups, respectively, on long N-terminal residues on histones. It is noteworthy that the health benefits of short chain fatty acid producing bacteria like lactobacillus have been observed with HDAC inhibition as the basis to their mechanism of action [40].

The readers, writers, and erasers of the epigenetic code appear to be a complex group, with a handful of HAT enzymes and close to 20 different isoforms of HDACs (encoded at different chromosomal loci). The enzymes can modify proteins in the cytoplasm and nucleus, and in some cases translocate between the subcellular compartments to perform their functions [41]. HDACs regulate chromatin availability by deacetylating histones, leading to chromatin condensation. The different forms of HDACs are divided into four classes based on shared homology with yeast HDACs: the Class I HDACs (HDAC1, HDAC2, HDAC3, and HDAC8), Class II HDACs (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10), Class III Sir2-like proteins (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7), and the Class IV protein HDAC11 [42].

Histone Deacetylases in the Intestine

Class I HDACs are globally localized to and function in the nucleus across cell types, as they are involved in vital cell processes, including cell proliferation, differentiation, and apoptosis. They share great homology with yeast HDAC Rpd3, maintaining a completely conserved deacetylase domain. When independently knocked out, they are lethal to mice embryos, implicating their importance in organism development. When knocked out together, however, increased IEC proliferation and

differentiation was observed in mice enteroids. Moreover, deletion of *Hdac1* and *Hdac2* in enteroids correlated with more secretory cell precursors [43].

Class II HDACs have been observed to shuttles between the nucleus and the cytoplasm, regulating genes involved in cell signaling and cytoskeleton integrity [44]. Like Class I HDACs, Class II HDACs also share significant homology with the yeast HDA1 with a common C-terminal deacetylase domain [44]. There are two subdivisions for this class. Class IIa involves HDAC4, 5, 7, and 9 while Class IIb includes HDAC6 and HDAC10. The Class IIa HDACs contain a unique N-terminal adaptor DNA site for MEF-2 binding, and they also contain 3-4 phosphorylation sites, allowing for multiple levels of precise control of their activity in association with 14-3-3 proteins [41]. Within this class, HDAC7 has been identified as being associated with an IBD risk SNP [45]. HDAC7 has been found to regulate intestinal epithelial differentiation into absorptive cell types while suppressing a secretory phenotype, making this protein an potential therapeutic target to promote secretory cells in culture [46]. The Class IIb HDACs have been understudied and are less well understood for their role in IECs. Altogether, however, regulation of this class of HDACs demonstrates much promise towards specific targeting and regulation of IEC behavior.

The Sirtuins, or Class III HDACs, are NAD^+-dependent proteins that behave primarily as transcriptional silencers and share homology with the yeast Sir2. There are seven different sirtuin proteins, demonstrating a vast range of variety and function in this class of HDACs. These sirtuins also take up various locations in the cell, existing in the nucleus, cytoplasm, and mitochondria. In regards to intestinal health, SIRT3 overexpression has been implicated in regulation of the intestinal epithelium, as

concomitant downregulation of Notch-1 signaling decreased human gastric cancer cell proliferation, and it has also been shown to promote enteric nervous system integrity and function [47,48].

The Class IV HDAC refers to HDAC11 that shares homology with yeast Hos3 and is like HDAC Class I and Class II in its functional domains. While it has been linked to immune regulation [49], HDAC11 is relatively understudied.

The vast diversity and levels of control exhibited in HDACs and their potential roles in IEC differentiation makes them a promising target for IBD therapy [50]. In fact, HDAC inhibitors have already been explored as therapeutics in other diseases, such as leukemias [51], solid tumor cancers [52], metabolic disorders [53], and autoimmune or inflammatory diseases [54]. In this study, we used surgical tissue, freshly derived intestinal crypts from patient biopsies, and established rectal organoids, to investigate the role of HDAC signaling in epithelial differentiation pathways. We show cytoplasmic expression of HDAC4 in the mucosal epithelium, along with the activity of class II HDACs4, 5 and 7 being regulated by PGE2-PTGER4 signaling that increases *SPINK4* expression. We also show that these pathways interface with signals from the microbiome to cause a range of transcriptional changes in genes related to differentiation in the epithelial cells derived from different patients.

Methods

Partially adapted from: Shanta Murthy*, Murugadas Anbazhagan*, Sushma Maddipatla*, Vasantha L Kolachala, Anne Dodd, **Garima Sharma**, Amanda Radunne, Duke Geem, Ranjit Pelia, Savannah Washburn, Yeonjoo Hwang, Tarun Koti, Sharmistha Rudra, David J. Cutler, Jason D. Matthews, Subra Kugathasan. Comparative single cell profiling of

mucosa and organoids implicates altered epithelial-mesenchymal interactions mediated by perianal fistulizing Crohn's Disease. **(Manuscript under preparation)**

Biospecimen collection and organoid culturing

Protocols were approved by Emory University Institutional Review Boards (IRB). Mucosal biopsies were obtained from patients having perianal CD, CD, or non-IBD patients lacking gastrointestinal inflammation or infection. Inflammation was determined by Simple Endoscopic Score for Crohn's Disease (SES-CD) being greater than 3 after clinical, endoscopic, and histological evaluation of the patient. Organoid cultures were established from patient biopsies as previously described [55] and maintained in Matrigel (Corning) with Intesticult Human Organoid Growth Media (Stem Cell Technologies). Experiments were performed between passage 3 and 10. De-identified surgical tissue used for immunofluorescence microscopy was obtained under an approved discarded tissue protocol that did not require consent.

Extracting organoid crypts and treating with different HDAC inhibitors

During isolation of intestinal crypts from patients' mucosal biopsies, the following inhibitors were added: LMK-235 (1 μM), HES1i (JI130) (10 μM), and HDAC pan-inhibitor butyrate (1 mM). These cultures were propagated for 2-4 days and harvested for RNA extractions and quantitative PCR or Western blot.

Treating organoid cultures with different inhibitors

During culturing of intestinal organoids, TMP195 (1 μM), a Class IIa HDAC4, HDAC5, HDAC7 and HDAC9 inhibitor; CS055/Chidamide (1 μM), a Class I HDAC1, HDAC2, HDAC3, and Class IIb HDAC10 inhibitor; LMK-235 (4 μM), an HDAC4/5 inhibitor; butyrate (1 mM), a pan-HDAC inhibitor; EP4i (10 μM), a PTGER4 inhibitor; and PGE2 (1 μM) were independently added to cultures, and organoids were collected for RNA extractions and quantitative PCR after 24 hours. Some treatments were analyzed by Western blotting.

Western blotting

Organoid lysates prepared by sonication in reducing SDS loading buffer with protease/phosphatase inhibitor cocktail (Thermo) were heat denatured and separated on Any kD mini-protein TGX precast protein gels (Bio-Rad) before transferring to nitrocellulose membranes (Bio-Rad). Primary antibodies were used at 1/1000 and the secondary antibodies (GE, donkey anti-rabbit or -mouse, HRP conjugated) at 1/5000. The following primary antibodies were used against: (HDAC 4 S246/ HDAC5 S259/ HDAC7 S155) and (HDAC 4 S632/ HDAC 5 S661/ HDAC 218 7 S486), and total HDAC 1-7, along with GAPDH, PDGFR and E-cadherin (Cell Signaling Technologies).

RNA Extraction and Quantitative PCR

Total RNA from organoids was extracted using a Qiagen micro-RNA kit and cDNA prepared with a High-Capacity RT kit (ThermoFisher). Gene expression analysis was

performed using a 7500 Fast Real-Time quantitative PCR (qPCR) system (Applied Biosystems). Taqman gene expression primers for *SPINK4, EPCAM, ATOH1, PTGER4, SPDEF*, and *ACTB* were obtained from ThermoFisher. Data were expressed using the 2^{-ddCt} method with Ct limit of 40.

Immunofluorescence

Formaldehyde-fixed, paraffin embedded (FFPE) surgical tissue sections (5 μm) from the intestine were subjected to antigen retrieval by heat before immunofluorescence. Tissue sections were blocked with 3% bovine serum albumin/PBS and then incubated with primary antibodies at 1/250 dilution: E-cadherin, or HDAC 1-7, and next with secondary antibodies (1/1000 dilution) with DAPI (1/10,000) (ThermoFisher). Images were obtained on an Olympus FV100 confocal microscope and processed minimally for brightness and contrast. During optimization of these antibodies for staining, only anti-HDAC 1, 2 and 4 proved suitable for appropriate staining and further microscopic analysis.

Results

Detection of HDACs in the intestinal epithelium in vivo and in vitro

To ensure that HDACs are present in the intestinal epithelium, we used available reagents to detect the presence of Class I (HDACs 1-3) and Class II HDACs (HDACs 4-7) in the intestinal epithelium, organoids, and MSC. Immunofluorescent imaging of patient derived intestinal tissue sections demonstrated that only antibodies against HDAC1, 2 and 4 worked for immunofluorescence. In those imaging assays, variable cellular localization of these HDACs in the epithelial cells was observed, with HDAC1 being more

nuclear, HDAC2 being more cytoplasmic, and HDAC4 being mainly cytoplasmic with some nuclear localization (**Figure 4A**). All the antibodies tested worked for Western blotting (**Figure 4B**). HDACs 1-7 were detected in lysates from mucosal biopsies, MSC, and organoid cultures via western blotting (**Figure 4B**), demonstrating their expression in multiple cell types, but most importantly their expression in the epithelium. However, due to the sequence homology across the classes of HDACs, the availability of small molecules specific to only one isoform is limited, since most of the inhibitors that will inhibit one form, will at least inhibit another in the same group or sometimes multiple isoforms or ones in other groups. LMK-235 is one of the more specific inhibitors, primarily targeting HDAC4 and to some degree HDAC5 [56].

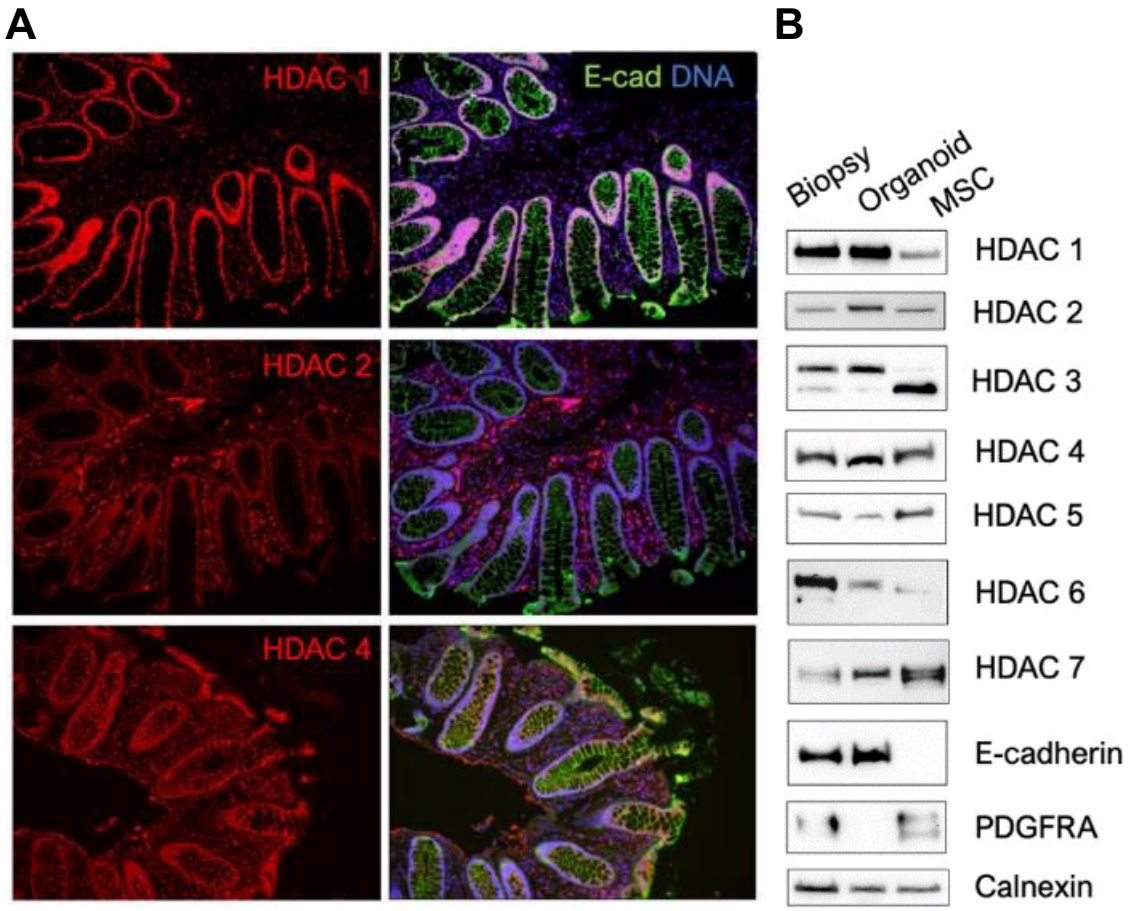

Figure 4. Class I and II HDACs are expressed in the intestinal epithelium. A) Immunofluorescence on intestinal surgical tissue showing the presence of HDAC1, 2, and 4 in intestinal crypts. B) Western blot of HDAC 1-7 total protein in intestinal biopsies, organoids, and MSCs with calnexin as an internal loading control.

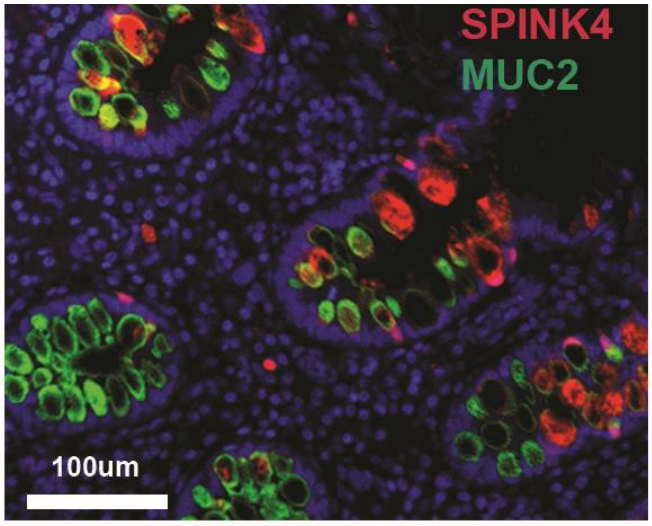

Figure 5. SPINK4 (red) expression in MUC2 positive goblet cells (green) in surgical tissue.

Preliminary data from the Kugathasan Lab and others demonstrates that *SPINK4* is a representative marker for goblet cell lineages (**Figure 5**). In addition, treatment of rectal organoids with PGE2 increases *SPINK4* expression and organoid swelling that can both be blocked by inhibiting PTGER4 during this treatment (**Figure 6**). To test whether blocking HDAC 4/5 phenocopies PGE2-PTGER4 mediated increases in *SPINK4* expression, LMK-235 was incubated with patient derived rectal organoids for 24 hrs, and consistent with our hypothesis, we found increases in *SPINK4* expression in many of the patient samples (**Figure 6**). Similarly, *ATOH1*, another marker for secretory cell types, increased upon LMK-235 treatment. Evidently, inhibition of HDAC4/5 demonstrated changes in secretory lineage gene expression that were like activating PTGER4 with PGE2, suggesting that they inhibit HDAC 4/5 activity when signaling together.

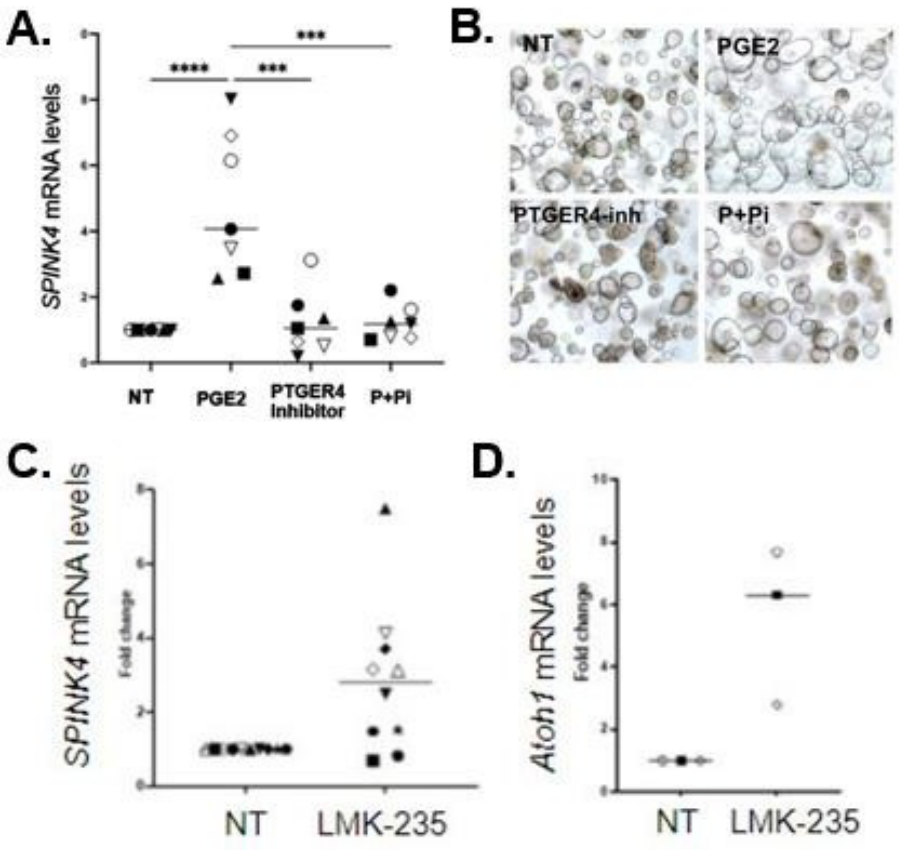

Figure 6. PGE2 and LMK-235 increases SPINK4 expression levels. A) Fold change in SPINK4 mRNA levels in patient organoid samples (n=7) as determined by RT-qPCR results normalized to B-actin. B) Images of organoids swelling upon PGE2 stimulation. C) Fold change in SPINK4 (n=10) and ATOH1 (n=3) mRNA levels are increased in some patients upon LMK-235, a selective inhibitor of HDAC4/5, treatment. RT-qPCR results normalized to B- actin housekeeping gene.

PGE2-PTGER4 axis regulates HDAC 4, 5, and 7 phosphorylation levels

To gain mechanistic insight into PTGER4 mediated changes in HDAC activity, we measured levels of HDAC phosphorylation at multiple amino acid residues within isoforms 4, 5, and 7 upon PGE2 stimulation since some of its activity is known to be regulated through phosphorylation [57]. We found that phosphorylation at HDAC4(S246)/5(S259)/7(S155), but not HDAC4(S632)/5(S661)/7(S486), decreased upon treatment with PGE2 (**Figure 7A**). Blocking PTGER4 with a small molecule inhibitor

(EP4i) mitigated decreases of HDAC 4, 5, 7 phosphorylation during PGE2 treatment (**Figure 7B**), demonstrating a link between this signaling pathway and HDAC function. Because the polyclonal phospho-specific antibodies used in these experiments are cross reactive with class IIa HDACs, it is difficult to distinguish HDAC 4 and 5 since they are the same molecular weight, but different than HDAC 7. Comparison of protein molecular weights implies that dephosphorylation is occurring on HDAC4/5 bands during PGE2 treatment. Unexpectedly, we did not observe increases in *SPINK4* expression in the LMK-235 experiments for this patients' organoids, in fact, this sample even decreased

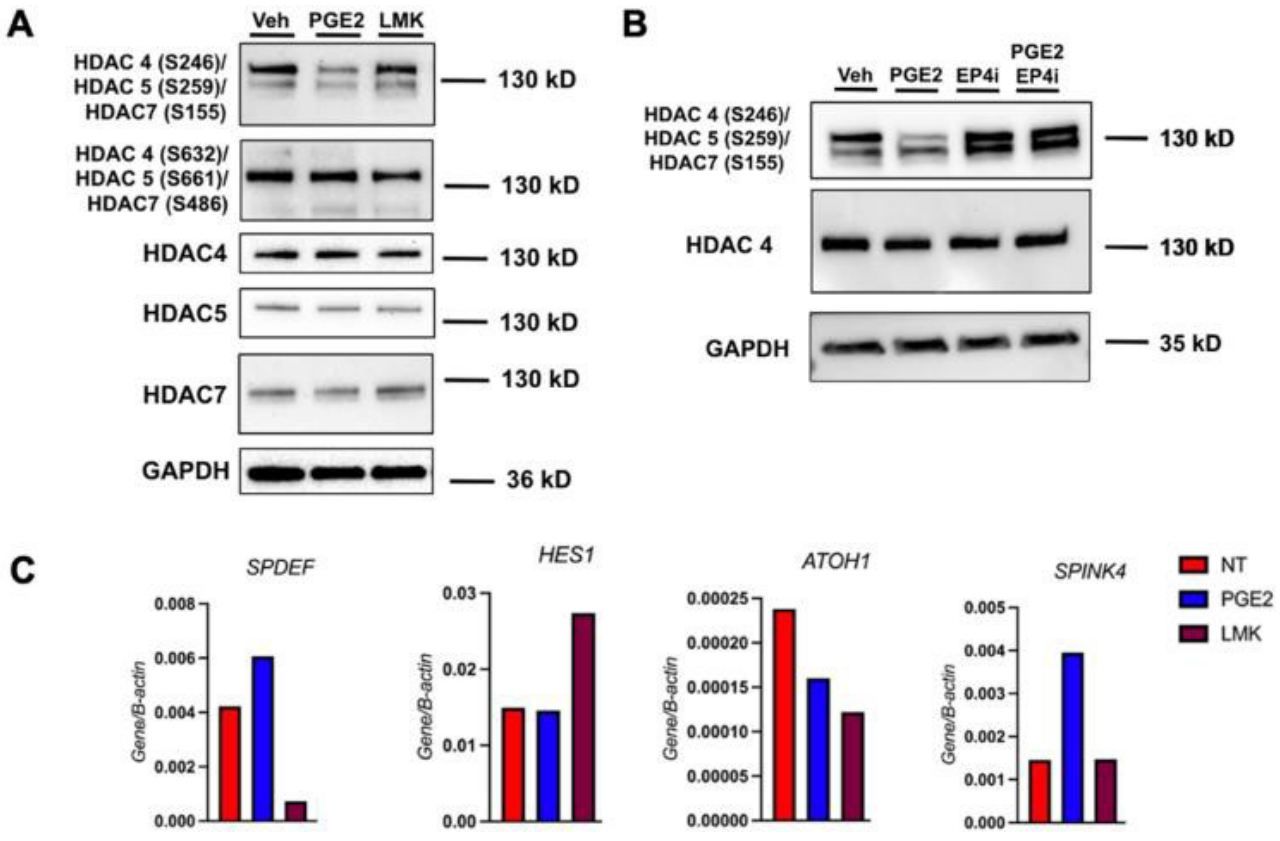

Figure 7. Histone deacetylases HDAC4(S246)/5(S259)/7(S155) phosphorylation, but not HDAC4(S632)/5(S661)/7(S486) change during PGE2 treatment through PTGER4. A) Western blot of HDAC 4,5, 7 phosphorylation sites and total proteins upon PGE2 and LMK-235 stimulation and B) PTGER4 inhibition with EP4i with GAPDH as an internal control. C) RT-qPCR measuring mRNA levels of absorptive and secretory cell marker genes normalized to B-actin gene expression upon PGE2 and LMK-234 treatment.

expression of *SPDEF* and *ATOH1* while increasing *HES1* expression under these conditions (**Figure 7C**). Surprisingly, although treatment with PGE2 increased *SPINK4* expression as seen previously for all patients tested (**Figure 6**), it decreased the expression levels of *ATOH1* (**Figure 7C**).

Pan-inhibition of HDACs changes phosphorylation and total HDAC protein levels

Taken together, with activation of HDACs having long been associated with decreased gene expression, considered with the increases in *SPINK4* expression levels concomitant with decreases in HDAC 4/5 phosphorylation levels during PGE2 treatment in our experiments, all suggested that this PTGER4 mediated decrease in HDAC phosphorylation was associated with their functional inhibition. To test this hypothesis, we used butyrate for organoid treatment, a known HDAC inhibitor produced by the microbiome. Expecting this treatment to produce similar decreases in HDAC phosphorylation like PGE2 treatment, we were surprised to find that it conferred the opposite effect by increasing phosphorylation of HDAC 4, 5, and 7 (**Figure 8A**). Remarkably, the total protein levels of HDAC 4 and 5 were increased at 24 hrs of butyrate treatment, and by 48 hrs HDAC 7 levels were substantially decreased but were back to baseline levels by 72 hrs (**Figure 8A**). Furthermore, we assessed gene expression of secretory and absorptive cell type marker genes (**Figure 8B**), finding marked decreases in the expression of some secretory marker genes, *SPDEF* and *ATOH1*, at the 24-hour time point, but an overwhelming increase in *SPINK4* expression levels by 72 hrs. Moreover, we observed an increase in the absorptive marker gene, *HES1*, during this

butyrate treatment. These results suggest that PGE2-PTGER4 signaling activates HDAC 4 and 5 by decreasing their phosphorylation.

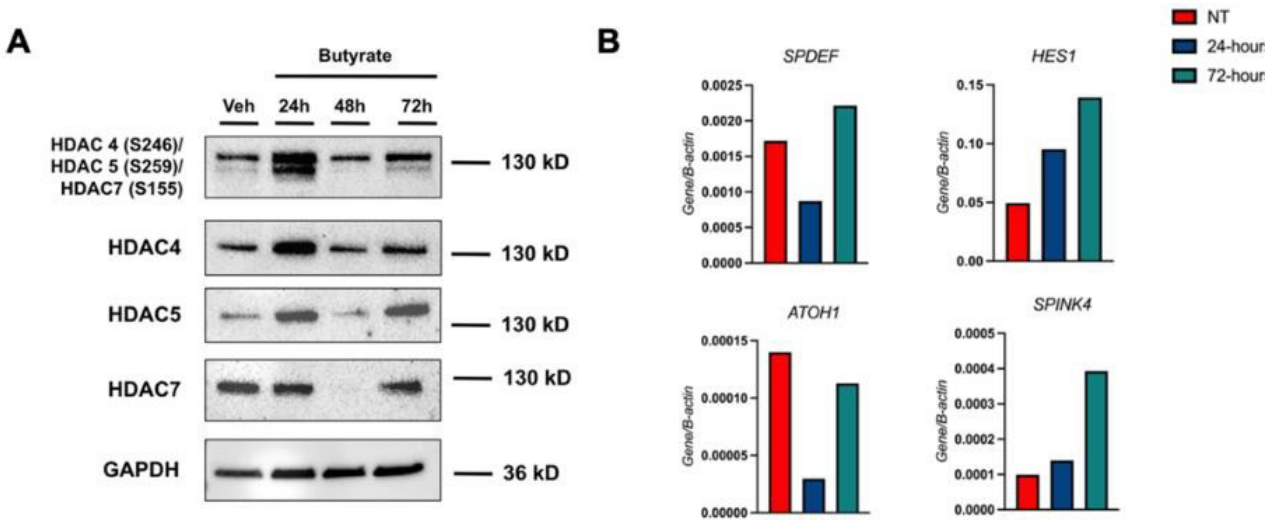

Figure 8. Inhibition of HDACs by a pan-inhibitor, sodium butyrate, increases HDAC4(S246)/5(S259)/7(S155) phosphorylation and total HDAC protein levels and influences gene expression of epithelial differentiation marker genes. A) Western blot of an HDAC4, 5, 7 phosphorylation site and total proteins with GAPDH as an internal loading control and B) RT-qPCR measuring mRNA levels of absorptive and secretory cell marker genes normalized to β- actin gene expression upon sodium butyrate (1mM) treatment for 24, 48, and 72 hours.

Stem Cell Technologies Human Organoid Differentiation Media™ is not sufficient to induce secretory cells

To determine if commercially available organoid differentiation media could induce the proliferation of secretory cells, we determined secretory and absorptive cell marker genes for organoids grown in standard organoid media versus differentiation media independently (n=2) (**Figure 9**). Results showed no increase in secretory marker genes' expression. On the contrary, a visible decrease was observed in both *SPINK4* and *SPDEF,* while *HES1* levels slightly increased. This result suggests that the commercially available differentiation media used in this experiment is not sufficient to promote

secretory lineages in culture and, thus, validates further studies exploring the manipulation of other cellular mechanisms to propagate secretory cells in culture.

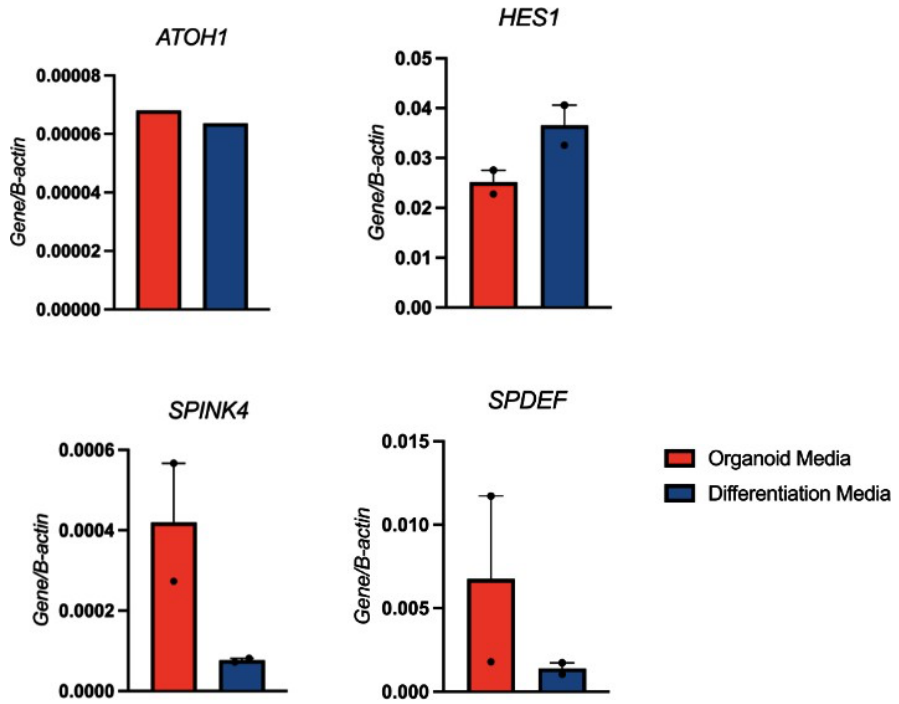

Figure 9. No significant increase in secretory cell marker genes in organoids treated with differentiation media versus standard organoid media. RT-qPCR measuring mRNA levels of absorptive and secretory cell marker genes normalized to β-actin gene.

Inhibition of HDACs by HDAC Class I and Class II inhibitors in organoids

To understand whether inhibition of different classes of HDACs would result in differentiating gene expression, HDAC Class I and HDAC Class II inhibitors were added to growing organoid cultures. Gene expression across the different cultures did not differ significantly across treatments (**Figure 10**). However, there are notable patient-specific responses that indicate the biological effects of these inhibitors on IEC gene expression. For instance, a non-inflamed control patient responded with an increase in *ATOH1*

expression upon treatment with CS055, a Class I HDAC inhibitor, with levels for *HES1, SPINK4, and SPDEF* remaining relatively stable in comparison to the no treatment control. On the contrary, another non-inflamed patient with disease exhibited undetectable levels of *ATOH1* expression upon treatment with the same drug. These contrasting results may be reflective of variable responses due to genotypic and phenotypic differences in the patients.

Inhibition of HES1 and pan-inhibition of HDACs in freshly isolated rectal crypts

We hypothesized that early blockade of HES1 with a small molecule inhibitor JI130 or HDAC inhibition with butyrate would result in increased gene expression of secretory marker genes. At the concentrations used in this study, no significant changes were observed in general for gene expression, however a notable increase in *ATOH1* and *HES1* expression levels were observed with butyrate treatment, yet no effect on *SPINK4* levels (**Figure 11**). There were, however, interesting expression signals for individual patients. For example, the organoids derived from an inflamed patient with disease expressed lower levels of secretory marker genes (**Figure 11A, C, D**) but expressed higher levels of the absorptive marker gene *HES1* (**Figure 11B**). Another non-inflamed patient showed higher levels of both *ATOH1* and *SPINK4* secretory markers. LMK-235 was also used to inhibit HDACs in these cultures; however, the resulting RNA yields were not sufficient to continue with experimental data collection, suggesting the need to adjust concentration or the necessity of HDAC4/5 early on in crypt-to-organoid establishment.

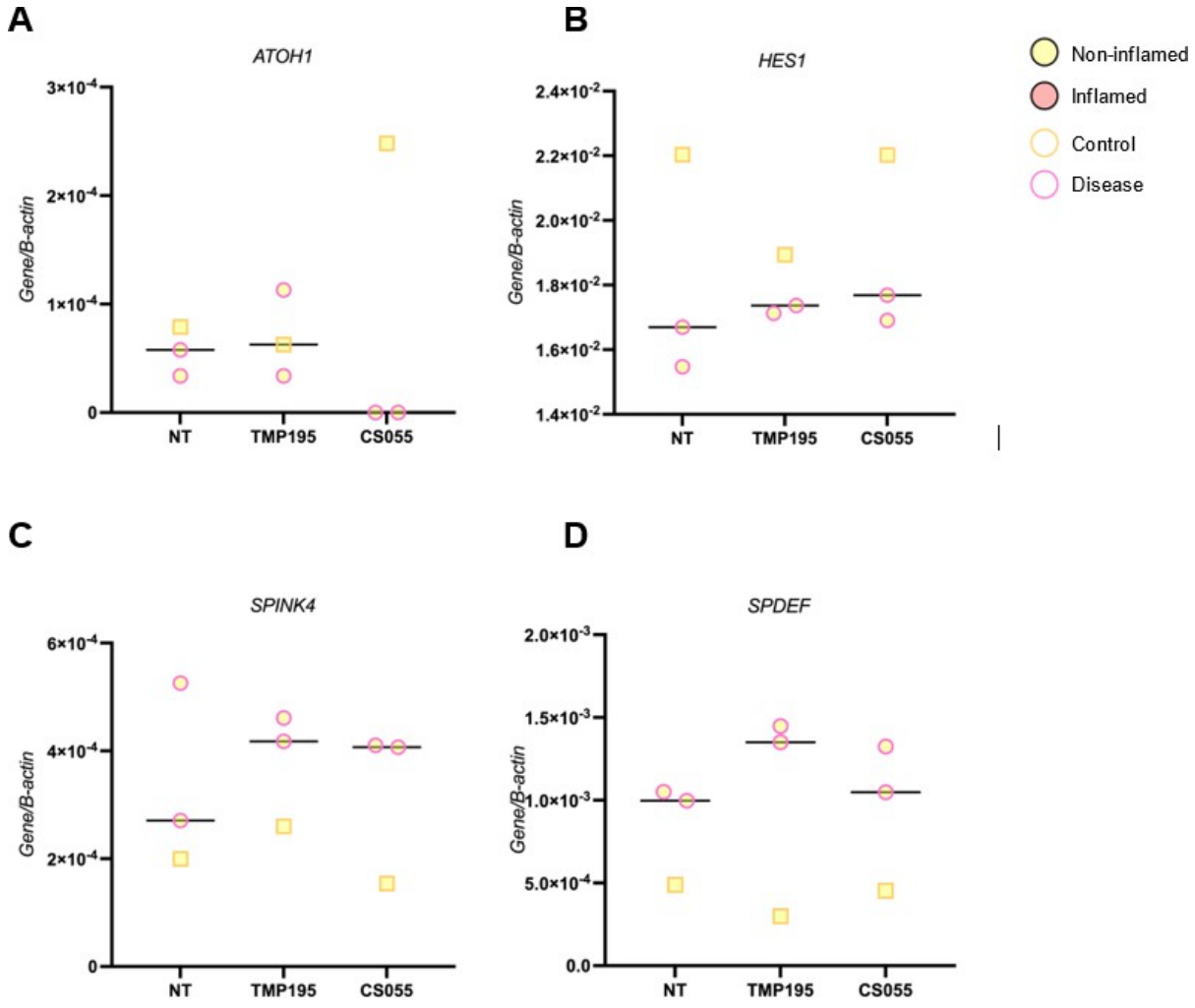

Figure 10. Inhibition of HDACs by HDAC Class I and Class II inhibitors in organoids. RT-qPCR measuring mRNA levels of absorptive and secretory cell marker genes normalized to β-actin gene. Each symbol corresponds with a different patient.

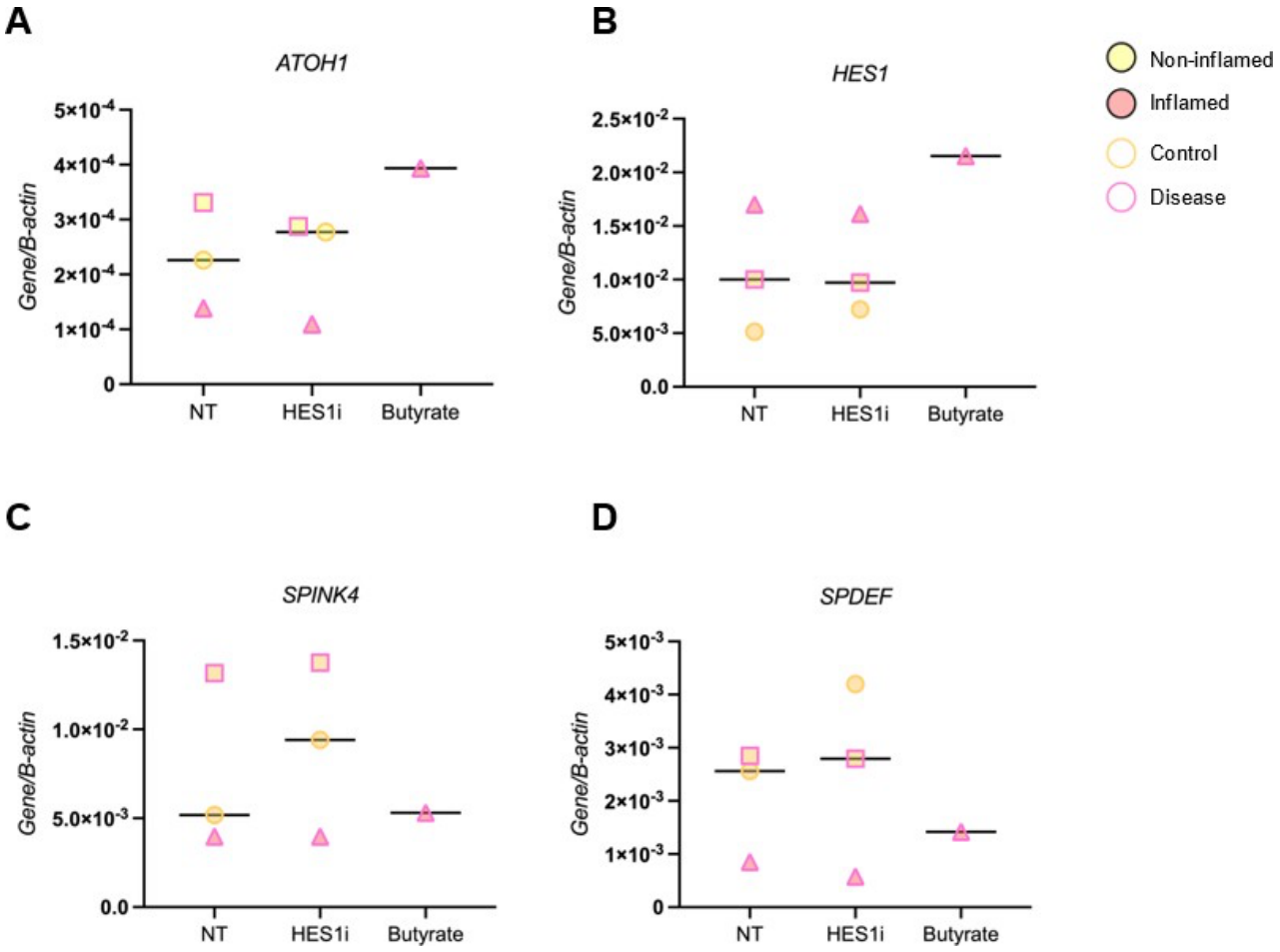

Figure 11. Inhibition of HES1 and pan-inhibition of HDACs by butyrate in organoid crypts. RT-qPCR measuring mRNA levels of absorptive and secretory cell marker genes normalized to β-actin gene. Each symbol corresponds with a different patient.

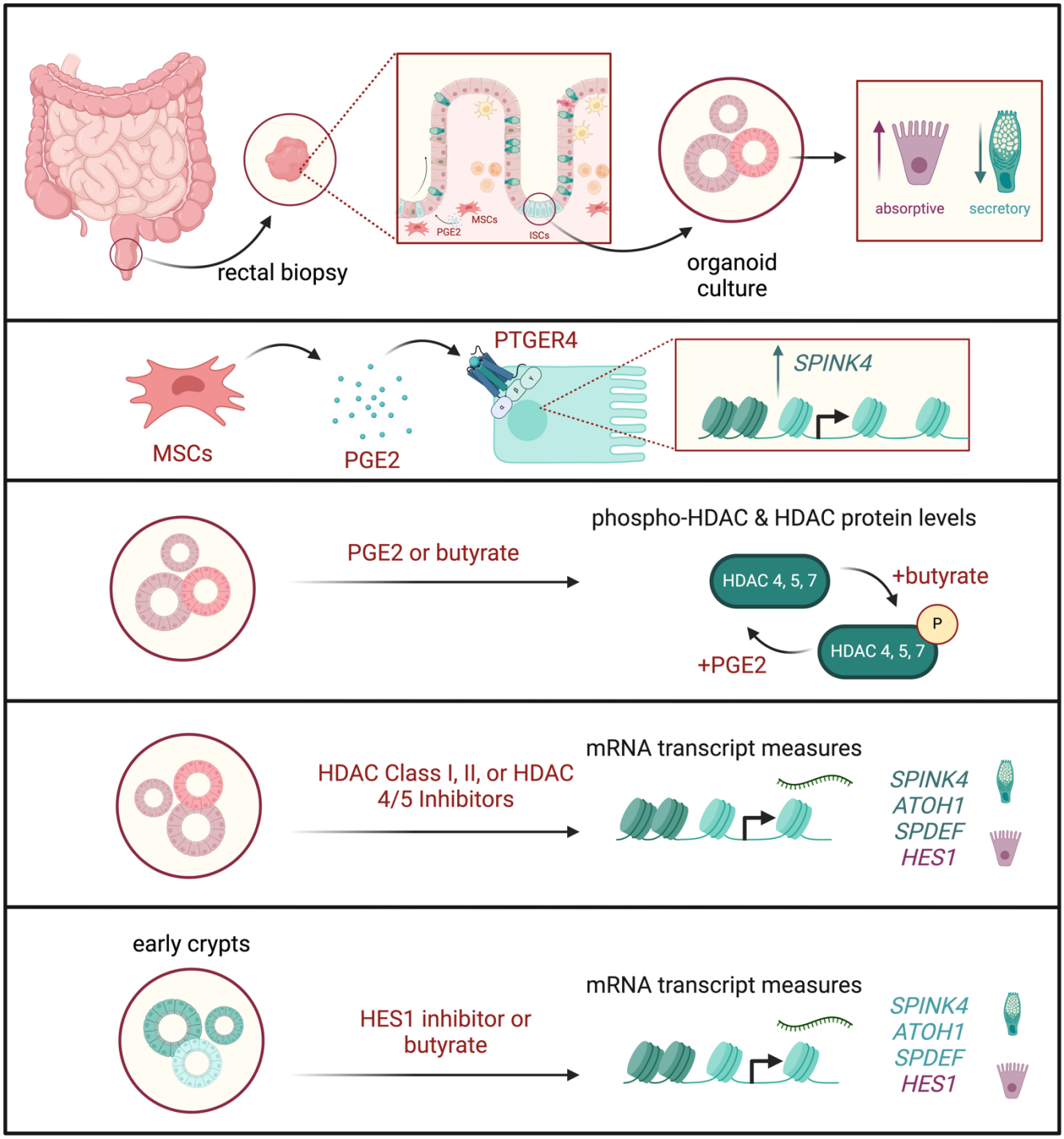

Figure 12. Graphical summary.

Discussion

Herein, we have demonstrated the expression of HDACs 1-7 in epithelial cells and some of their modulatory effects on gene expression. Given the stem-like nature of *in vitro* intestinal epithelial cell cultures, we sought to determine whether there is a connection between HDACs and the PGE2-PTGER4 signaling axis, and whether their manipulation would promote propagation of more secretory cell types in culture. Although we were not able to produce fully differentiated secretory cells in culture, we demonstrate differential effects of inhibiting HES1 and HDACs on absorptive and secretory marker gene expression along with a connection between HDAC 4, 5, and 7 activity and the PGE2-PTGER4 signaling axis that regulates *SPINK4* expression.

In the ileum, both goblet and Paneth cells exert healing effects in response to mucosal damage [29] , but the rectum lacks Paneth cells thus leaving this function to goblet cells. The decrease in secretory cell types during mucosal inflammation of perianal CD suggests that targeted therapies that promote increases in their abundance would also increase healing in those patients. The lack of differentiated cell types in organoids provided the ideal model to investigate how these pathways are stimulated and we found that like intestinal tissues, HDAC 1-7 were expressed in organoid cells thus distinguishing them as a viable target to modulate for a potential increase in secretory cell types. HDAC4 was of particular interest due to its comparatively high expression in the epithelium, however, the lack of available reagents and inhibitors targeting each of the individual HDAC isoforms without affecting the others, limited their specific study.

Collectively, results presented here suggest that PTGER4 is regulated by MSC ligands that stimulate gene expression in the epithelium, at least in part, by modulating

epigenetic enzymes and thus the state of IEC differentiation towards a secretory phenotype. On the other hand, using butyrate as a control for HDAC inhibition, we found that it converged on similar pathways as PGE2 stimulation but sometimes with opposite effects. Butyrate treatment affected the total protein levels of the Class IIa HDACs in a cyclic manner. The increase in HDAC 4/5 may be indicative of a feedback loop to produce more HDACs 4/5 in response to their inhibition by butyrate. Alternatively, the effect of butyrate on HDAC7 may represent another adaptive cellular mechanism through which HDAC7 levels stabilize after an initial decrease due to butyrate treatment. For instance, it may be possible that proteosome complexes that degrade HDAC7 are regulated by butyrate, or inhibition of proteosome activity may lead to decreased ubiquitination of HDAC4/5 and these proteins' concomitant accumulation in the cells. To this end, the activities of the proteosome or changes in ubiquitination levels of these proteins have likely been modified. Other possibilities include the global increase in gene expression, including those of HDAC-protein-coding genes, upon pan-HDAC inhibition by butyrate. In addition, we found that within this time-course of butyrate treatment, mRNA transcript levels of *SPINK4* increase markedly after 72 hours. This increase in gene expression is also the case for the other genes observed, suggesting that butyrate treatment over extended periods of time induced stronger responses in gene expression, an effect expected by a pan-HDAC inhibitor. Unexpectantly, however, we also observed that butyrate treatment increased phosphorylation of HDACs at residues HDAC4(S246)/5(S259)/7(S155), suggesting that the decreases in phosphorylation at these residues during PGE2 treatment is an enzyme-activating post-translational modification. We had originally interpreted our results as PGE2-PTGER4 signaling increasing SPINK4 expression by inactivating HDACs and thus allowing for opening of chromatin to promote gene expression. Considering the butyrate results however, it

appears that HDACs are being activated by PTGER4, suggesting that the deacetylation of non-histone proteins in the cytoplasm might be the other intermediates in regulating SPINK4 expression, and is supported by our immunofluorescence analysis of mucosal tissue sections that revealed a mostly cytoplasmic localization to HDAC4. One possible mechanism here is supported by studies showing HAT protein p300 and HDAC4 competing to modulate acetylation marks on a transcription factor GATA4 to regulate its activity in cardiomyocyte differentiation [58]. Interestingly, GATA4 has been identified to be an important factor in ileal intestinal barrier integrity [59]. Alternatively, the increase in *SPINK4* expression could be mechanistically related to HDAC activity localized to other genes or genetic elements that control *SPINK4* expression i.e. micro-RNA, enhancers, etc. Further experimentation is needed to discern the implications of dephosphorylation at these amino acid residues within class II HDACs 4, 5, and 7 and their biologic effects regarding the PGE2-PTGER4 and secretory gene signaling process. One avenue considers the phosphorylation of HDAC4 at the Ser-426 site being modulated by Calcium/calmodulin-dependent protein kinase (CaMKII/IV) to regulate its catalytic activities [60] and how the involvement of the CaMKII/IV activity might play a role in the PGE2-PTGER4 pathways. Altogether, the converging signals of the MSCs of the lamina propria and the microbial products in the lumen suggest a mechanistic tug-of-war intersecting with HDACs and other cellular proteins to mediate the fate of IECs.

The differences in patient responses and the notion of personalized medicine can be appreciated from the results of investigating the PGE2-PTGER4 signaling axis. We have shown that inhibition of HDAC 4 and 5 with LMK-235 can increase the expression levels of *SPINK4* and *ATOH1* in many patients, but not all, and the additional patient

tested in these additional studies belongs to the latter group of non-responders. In fact, we show in that patients' sample that LMK-235 resulted in a decrease in *ATOH1* and *SPINK4,* and an increase in *HES1*. Moreover, the diverse set of responses in gene expression when class I or class IIa HDACs were targeted with inhibitors CS055 and TMP195, respectively, indicates there are numerous variables affecting signaling properties of each patients' cells. Some patients' organoids increased gene expression of secretory marker genes, such as *SPINK4*, while others increased expression of absorptive marker gene *HES1* upon stimulation with these HDAC inhibitors. To explain this, we propose a model that implicates genetic and epigenetic factors regulating chromosomal architecture in vivo being maintained in cell culture and thus creating nuanced signaling reactions in common biological pathways occurring in each patients' cells.

Other HDAC inhibitor experiments we preformed involved treating freshly isolated crypts that would grow into organoids, with the idea being that early inhibition of HDAC would allow secretory gene programs to be expressed or at least maintained at their current rate in the crypts. In these experiments, it is notable that for one patient (inflamed disease), butyrate resulted in slight increases in all the genes tested, but especially *ATOH1* and *HES1*. By inhibiting all HDACs, unregulated acetylation of histones may promote global gene expression that could lead to such a result. The increase in several marker genes, including *ATOH1*, for this patient warrants further investigation into the potential utility of butyrate as an early treatment during crypt extraction and organoid establishment to promote secretory cells *in vitro*. In fact, butyrate was shown to increase expression of goblet cell marker gene *MUC2* in colon cancer cell lines, further supporting

this hypothesis [61]. Other inhibitors, such as LMK-235, did not allow for the collection of sufficient RNA to proceed with assessment. This setback can be expected as LMK-235 is a cancer therapeutic, so it is not surprising that LMK-235 exhibited anti-proliferative effects and low RNA yields on the treated IECs. We did not treat cells with Class I and Class IIa HDAC inhibitors simultaneously yet; however, due to the global involvement of Class I HDACs in processes necessary for cell survival, expectations for the proper growth of the crypts into organoids after treatment would be low.

As suggested by the results in this thesis, activation and inhibition of different HDACs resulted in variable effects in epithelial cell gene expression involving specific differentiation lineages. Other studies have also shown inconsistencies with HDAC functions *in vivo*, whereby Hdac1/2 are required for intestinal healing in a mouse DSS-induced colitis model, but the absence of Hdac2 in a mouse knockout model was shown to be protective against colitis through regulation of IEC differentiation [62]. For some patients, our results are consistent with a study using intestinal organoid models to show broad-spectrum inhibition of HDACs resulted in increased *Math1/ATOH1* and *Muc2* expression [63]. Regarding the role of HES1 in regulating the expression of these secretory genes, contrary to expectations its inhibition early on in establishing organoid cultures from freshly isolated crypts would promote secretory lineage formation, the HES1 inhibitor JI130 resulted in the increase of *HES1* expression levels, possibly another feedback loop like that observed for HDAC 4/5 protein levels during butyrate treatment. Further examination of protein levels of HES1, ATOH1, SPDEF and MUC2 would give more insight to the mechanisms of these treatments.

In conclusion, the work here has shown a link between PTGER4, encoded by an IBD risk gene, with HDAC activity and secretory gene expression. Although inhibition of HES1 or HDACs in crypts and organoids did not demonstrate generalizable changes in gene expression across patients, the results have shed new light on the complexity of the signaling downstream of PTGER4 and the control it has or does not have on gene expression, depending on the patient. After all, it is important to keep in mind that the epigenetic proteins we investigate are susceptible to environmental influences on cellular programming within an individual that are likely retained to some degree during cell culturing, thus influencing the outcome of our experiments. As such, limitations of these experiments involve the inability to control the environmental influences and heterogeneity among patient samples. The reliance of this project on patient samples and the time-associated constraints with organoid culturing limit sample sizes. Given more time for sample collection, a case-control approach might aid in the delineation of mechanistic trends in HDAC activity based on phenotypic grouping of patient samples. Nevertheless, the vast variety of HDACs in their form and function allows for a nuanced control of gene regulation once the underlying mechanisms have been determined and the appropriate isoforms can be therapeutically targeted. This is a challenging endeavor due to the homology of the HDAC isoforms but a necessary one, nonetheless. As such, exploring the function of different HDAC isoforms in their effects on IEC gene expression may result in discovery of mechanisms that can be advantageous towards propagating specific IECs in culture or *in vivo*, particularly for those patients harboring IBD risk mutations affecting *PTGER4*.

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