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The Role of Short-Chain Fatty Acids on Intestinal Epithelial Cell Migration

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The Role of Short-Chain Fatty Acids on Intestinal Epithelial Cell Migration

by

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Dedication

This dissertation is dedicated to my wife and children. Without their love and support this long journey through medicine would not be possible. I would also like to dedicate this dissertation to my parents and three sisters, who have been by my side every day to give me advice, comfort, and love. Lastly, to my friends who have been through this arduous journey of the MD/PhD program with me, especially during Hurricane Harvey and the Covid-19 Pandemic.

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The Role of Short-Chain Fatty Acids on Intestinal Epithelial Cell Migration

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Abstract

Inflammatory Bowel Disease (IBD) is a chronic remitting and relapsing autoimmune disorder of the gastrointestinal tract that affects millions of people worldwide with incidence and prevalence on the rise. Increases in the rates of IBD have been partly attributed to shifts toward a western diet, which emphasizes calorie dense, nutrient poor foods high in saturated fats, sugars, and artificial sweeteners. Importantly, dietary fiber intake is low in the United States and abroad. Dietary fiber is fermented into short-chain fatty acids (SCFAs) by the gut microbiota and is vital to proper gut function. Previous studies have found that diets lacking fiber, SCFAs, or receptors for SCFAs predispose both animals and humans to IBD and colorectal cancer. However, in order to utilize SCFAs for clinical management of IBD, we must further understand their functions and mechanisms of action. This work describes the effects of SCFAs, specifically propionate, on intestinal epithelial cell migration and wound healing. Intestinal epithelial cell migration is critical for epithelial restitution, the first phase in wound healing in the

intestine. The studies in this dissertation demonstrated the effectiveness of SCFAs to trigger epithelial cell migration independent of proliferation up the crypt-villus axis and reduce ulceration in mice in a model of colitis. Notably, the effects of SCFAs on epithelial migration in a histone deacetylase inhibition (HDACi) and GPR43 dependent manner allows for further targeting of these pathways to circumvent some of the current pitfalls of SCFAs, mainly their ability to inhibit epithelial proliferation, which could be detrimental to ulcer healing.

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List of Abbreviations

AC (alternating current)
AFM (atomic force microscopy)
AMP (antimicrobial peptide)
AMPK (5' AMP-activated protein kinase)
ARP (actin Related Protein)
BrdU (5-bromo-2'-deoxyuridine)
cAMP (cyclic AMP)
CD (Crohn's Disease)
CDC42 (cell division control protein 42 homolog)
CFSE (carboxyfluorescein diacetate succinimidyl ester)
CRC (colorectal cancer)
DAPI (4′,6-diamidino-2-phenylindole)
DSS (dextran sulfate sodium)
FAK (focal adhesion kinase)
GERD (gastroesophageal reflux disease)
GI (gastrointestinal)
GPR (G-couple protein receptor)
HDAC (histone deacetylase)
HDACi (histone deacetylase inhibitor)
IBD (inflammatory bowel disease)
IBS (irritable bowel syndrome)
IEC (intestinal epithelial cell)
KO (knockout)
MEK (mitogen activated-protein kinase)
MSD (mean squared displacement)
MSIE (Mouse small intestinal epithelial cells)
mTOR (mammalian target of rapamycin)
NFKβ (nuclear factor kappa-light-chain-enhancer of activated B cells)
RAC1 (ras-related C3 botulinum toxin substrate 1)
SCFAs (short-chain fatty acids)
SMCT (sodium coupled monocarboxylate transport)
STAT3 (signal transducer and activator of transcription 3).
TJ (tight junction)
UC (ulcerative colitis)

WAVE (WASP-family verprolin-homologous protein)

Chapter 1-SCFAs and Intestinal Homeostasis[1]

Introduction

Ulcerative colitis (UC) and Crohn's disease (CD), collectively known as inflammatory bowel disease (IBD), have emerged as a significant health challenge in the 21st century. IBD affects millions of people in the United States, Europe, and Asia with incidence and prevalence increasing worldwide.[1] The role of microbiota in the pathogenesis of IBD is well established. However, the components of the microbiota which are responsible for these effects remain largely unknown. Studies have identified a crucial role for gut microbiota metabolites in modulating intestinal homeostasis and immunity, with dietary fibers and their bacterial fermentation products, short chain fatty acids (SCFAs), playing an essential part.[2,3] Of particular interest are the SCFAs acetate, propionate, and butyrate, which collectively account for >95% of the SCFA population.[4] Their importance to intestinal health cannot be overstated, as SCFAs have been linked to protection against IBD, allergic asthma, and diabetes.[5–8] In this review, we will highlight SCFAs regulation of barrier protection and their role in the pathogenesis of IBD.

Formation of SCFAs

The intestines harbor trillions of bacteria that have developed both a mutualistic and symbiotic relationship with their host. The intestinal microbiome plays pertinent roles in maintaining homeostasis, and alterations in the microbiome are associated with chronic inflammatory conditions including IBD, diabetes, obesity, and allergic asthma[6,8,9]. Among various microbiota metabolites important in regulation of host physiology and health,

[1] This work has been previously published in: **Bilotta AJ**, Cong Y. Gut microbiota metabolite regulation of host defenses at mucosal surfaces: implication in precision medicine. *Precis Clin Med*. 2019;2(2):110-119. doi:10.1093/pcmedi/pbz008

SCFAs (including acetate, propionate, and butyrate) are derived from the bacterial fermentation of dietary fibers, such as inulin, which escape absorption in the small intestines and enter into the colon[4] (**Figure 1.1**). Acetate is the most abundantly produced SCFA, followed by propionate and butyrate in a 3:1:1 molar ratio, respectively.[9] SCFA formation is dictated by both the type of bacteria and type of dietary fiber present in the colon. For example, most bacteria produce acetate which can be derived from acetyl-CoA or alternatively via formate, hydrogen, and carbon dioxide, via the Wood-Ljungdahl pathway.[10] While many bacteria can produce acetate, propionate production occurs most commonly in the phyla of Bacteroidetes via the succinate pathway, which requires hexoses and pentoses.[10,11] Propionate can also be produced by species such as Veilonella using lactate through the acrylate pathway or through the propanediol pathway found in *Roseburia* and *Ruminococcususes*, which utilize fucose and rhamnose.[10,11] Conversely, butyrate is primarily produced through the condensation of a thiol group of coenzyme A with the carboxy group of acetyl-CoA, resulting in butyrl-CoA, which can then ultimately be converted to butyrate.[10,12] There are many butyrate producing species, with the Firmicutes phyla being the primary producer in the human colon.[13]

Transport of SCFAs

SCFAs, particularly butyrate, provide colonic cells with 80% of their daily energy supply and thus appreciable quantities are not found in the hepatic vein.[14] This is in contrast to acetate and propionate, which are primarily taken up by colonocytes and transported into the portal vein for metabolism in peripheral tissues such as muscle.[4] SCFA absorption occurs by three mechanisms: passive diffusion, electroneutral, or electrogenic uptake[15] (**Figure 1.1**). The charge of a SCFA determines if its uptake occurs via passive diffusion

or carrier mediated. For example, passive diffusion of SCFAs is primarily seen when the SCFA is in the protonated form; this is a major mechanism of SCFA transport at physiological pH.[16] In contrast, SCFAs in anion form are dependent on carrier-mediated uptake, which can occur through four primary transporters. Monocarboxylate transporter 1 (MCT1) and MCT4 are electroneutral transporters, which rely on hydrogen[16] in contrast to sodium coupled monocarboxylate transport 1 (SMCT1) and SMCT2, which rely on sodium and are electrogenic and electroneutral transporters, respectively.[16]

SCFAs Mechanisms of Action

The effects of SCFAs in the intestines and elsewhere are derived from their ability to stimulate three G-couple-protein receptors (GPRs), GPR41, GPR43, and GPR109a, as well as their ability to act as histone deacetylase inhibitors (HDACi) (**Figure 1.1**). GPR41 is coupled to the pertussis toxin-sensitive $G_{i/o}$ family, which regulates cyclic AMP (cAMP) production. GPR41 has its highest affinity for propionate>butyrate>>acetate.[17] GPR41 is found in appreciable levels on peripheral blood monocytes (PBMC), dendritic cells (DC), and polymorphonuclear neutrophils (PMN), as well as in the spleen, lymph nodes, bone marrow, lung, small intestine, and adipose tissue.[17] Conversely, GPR43 expression is more restricted, as it is located mainly in the intestines and specific immune populations such as PMN, PBMC, monocytes, and lymphocytes.[17] GPR43 has a dual coupling to both pertussis toxin-sensitive $G_{i/o}$ as well as to the pertussis toxin–insensitive G_q. GPR43 primarily signals through $G_{i/o}$, except in the intestine, where GPR43 via its G_q coupling promotes glucagon-like peptide 1 (GLP-1) secretion.[17,18,19,20] GPR43 has affinity for all SCFAs with propionate>acetate≥ butyrate.[17,18] Unlike GPR41 or GPR43, GPR109a engages only butyrate, while also being the endogenous receptor for niacin.[21,22] GPR109a, similar to

GPR41, is coupled to the pertussis toxin-sensitive $G_{i/o}$.[21] GPR109A is expressed in the intestines, macrophages, monocytes, PMNs, DC, adipocytes, and Langerhans cells.[10,23,24] Lastly, SCFAs can act as potent HDACi with butyrate>propionate>>acetate.[25] HDACi play a role in gene modulation, protein stability, and pathway activation. With regards to gene modulation, histone acetylation allows enhanced access for transcriptional machinery to gene promoters by relaxing the chromatin structure. Thus, histone acetyltransferases (HATs) via acetylation allow for more open and accessible chromatin, whereas HDACs remove acetylation, leading to closed chromatin and gene repression. Additionally, through their HDACi action, SCFAs also play a role in modulating protein stability and activation via acetylation, such as seen with the modulation of p53 activity.[26]

SCFAs Regulation of Mucous

SCFAs are able to stimulate mucous production, which plays a critical role in the gut by creating a barrier to pathogens and toxins. The impact of SCFAs on mucous production was demonstrated by Finnie et al[27] who showed that butyrate increased colonic mucous glycoprotein (mucin) when incubated with epithelial biopsy specimens from colonic resection samples. SCFA regulation of mucin (MUC) gene expression was shown by Hatayama et al[28] who found that butyrate stimulated expression of MUC2, the primary mucin which comprises the colonic mucous layer, in the human goblet-like colon cells LS174T. This induction of MUC was dependent on mitogen-activated protein kinase kinase (MEK) signaling as the MEK inhibitor U0126 completely abrogated butyrate's effect on MUC2 protein expression. Later, this finding was extended by Paassen et al[29] who found that butyrate, acetate, and propionate stimulated MUC2 via binding of the butyrate responsive region by AP1. The difference in findings in the regulation of MUC2 expression

at the RNA and protein level suggest a role for butyrate as both a transcriptional and translational regulator, most likely by acting via HDACi and through GPR41 or GPR43. This is supported by the findings of Paassen et al[29] that propionate, which has high affinity for GPR41 and GPR43, stimulated greater MUC2 expression than butyrate at every concentration except 1mM. Further exploration of MUC2 regulation is of importance as MUC2 KO mice spontaneously develop colitis.[30] Beyond colitis, mucin serves an important role in protection from pathogens which was demonstrated by Jung et al[31] who showed that butyrate increases MUC 3, 4, and 12 expression while also increasing lactobacillus adherence and decreasing *E. coli* adherence *in vitro*. Thus, the role of SCFAs in modulating mucin synthesis serves as an important mechanism by which the host can allow for the colonization of beneficial bacteria, which may outcompete pathogenic bacteria and prevent inflammation and infection. Thus, a deeper understanding of the role of mucin may lead to the development of probiotics that would allow for alteration of the microbiome through colonization and expansion while also protecting from gastrointestinal infection and inflammation.

SCFAs Regulation of Antimicrobial Peptides

In addition to mucous, SCFAs stimulate antimicrobial peptides (AMPs), which are critical for innate defenses against pathogens and serve as a first line of defense for the underlying epithelial layer. In this regard, Hase et al[32] demonstrated that the human cathelicidin LL-37 was expressed constitutively in the colon, specifically in cells at the surface and in the upper crypts. This effect was independent of the commensal bacteria, as human fetal colon transplanted onto the backs of severe combined immunodeficiency (SCID) mice under sterile conditions demonstrated similar LL-37 expression as human colon *in vivo*.

Additionally, butyrate increased levels of LL-37 in Caco-2 and HT-29 cells. The mechanism underlying the stimulation of LL-37 by butyrate was uncovered by Schauber et al[33] who showed that LL-37 expression was dependent on butyrate activation of MEK in the human colon cancer cell line SW620. The potential implications of LL-37 in host protection was unraveled by Raqib et al[34] who demonstrated that butyrate upregulates the expression of CAP-18, the rabbit homologue to LL-37, and that this upregulation was critical for protection against shigella infection, as pretreatment of rabbits with butyrate prior to shigella infection led to decreased severity of infection. This is an important finding because it suggests that prevention and treatment of gastrointestinal bacterial infections could be done through dietary intervention. However, the contribution of AMPs in the protection against specific pathogens like shigella must be further examined as SCFAs stimulate mucous production, and dietary deficiencies in fiber have been shown to increase mucous degrading bacteria and susceptibility to pathogens.[35]

Aside from cathelicidin, Zeng et al[36] found that in IPEC-J2 cells (a porcine-derived colon cell line) acetate, propionate, butyrate, as well as phenyl derivatives of butyrate, increased β-defensin 2 and β-defensin 3 expression. This finding was further elucidated by Xiong et al[37] who found that butyrate could stimulate the *in vivo* expression of β-defensin 2 and β-defensin 3 in the colon and ileum of pigs, which ultimately lead to protection against severe infection when pigs were challenged with E. *coli*. This effect was found to be through HDACi, as treatment of 3d4/2 cells (immortalized porcine alveolar macrophages) led to increased expression of several AMPs including β-defensin 2 and β-defensin 3. Thus, this finding suggests an important role of macrophages in AMP production in response to SCFAs, while also confirming the work of Raqib et al[34], demonstrating the potential

feasibility of diet modification in the protection from gastrointestinal infection. Lastly, our group recently uncovered that SCFAs via GPR43 regulate the expression of REGIIIγ and β-defensin 1, 3, and 4. [38] This mechanism was dependent on SCFA induction of STAT3 and mTOR activation, as both inhibition of STAT3 and mTOR chemically or with siRNA knockdown, abrogated the effects of SCFAs on AMP production.

SCFAs Regulation of the Epithelial Layer

SCFAs regulate the daily turnover of the epithelial lining and stem cell proliferation. In recent years, reports on the effects of SCFAs, specifically butyrate, on the epithelium have been conflicting. This conflicting data gave rise to the butyrate paradox, which describes differential responses of cells to butyrate when treated *in vitro* and *in vivo*.[39] This paradox was elegantly unraveled by Donohoe et al,[40] who showed that cell metabolism, i.e. the Warburg effect, dictated the impact of butyrate on epithelial cells. This report demonstrated that tumor cells do not preferentially metabolize butyrate, leading to the intracellular accumulation of butyrate which blocks proliferation and promotes differentiation and apoptosis. However, in normal colonocytes or in tumor cells in which the Warburg effect is blocked, butyrate metabolism could promote the proliferation of colonocytes by acting as a carbon donor for acetyl-CoA and histone acetylation. This model proposes that lower doses of butyrate at the bottom of the crypt drive HAT and proliferation, whereas high doses at the top of the crypt lead to HDACi, apoptosis, and sloughing of cells into the lumen. This model was further verified by Kaiko et al[41], who showed that butyrate inhibited proliferation in cryptless animals and around areas of ulceration where the stem cell compartment would be exposed to the high luminal butyrate concentration. Thus, this study suggests that crypts, as well as colonocytes, are critical in metabolizing butyrate and

creating a butyrate gradient, which permits HAT activity at the base of the crypt. Additionally, the findings of both these articles support the long-term health effects of a high fiber diet in protecting against the development of colorectal cancer. [42]

SCFAs Regulation of Tight Junctions

Tight junctions (TJs) are complex protein-protein associations between individual cells that maintain the epithelium's selective permeability. Several studies have focused on both indirect effects of SCFAs on TJs via modulation of cytokines, as well as the direct effects of SCFAs on epithelial cell TJs. In terms of cytokines, Heller et al[43] showed that treatment of HT-29 cells with IL-13, a highly upregulated cytokine in UC patients, increased cell permeability, while also promoting the expression of the pore forming claudin-2. More recently, Wang et al[44] showed that IL-10 KO mice have decreased ZO-1 and occludin expression and that mixed feedings of IL-10 KO mice with a diet supplemented with acetate, propionate, and butyrate could increase occludin and ZO1 expression. However, whether or not this effect was through direct actions of SCFAs on the epithelium, or through modulation of effectors such as TNFα was not investigated. This is important, as IL-10 and SCFAs are important modulators of several inflammatory cytokines such as IFNγ and TNFα, which have well-characterized roles in modulating TJ permeability.[45] Additionally, Zheng et al[46] found that in the human colon cancer cell lines T84 and Caco-2, butyrate upregulated IL-10RA via a STAT3 and HDACi dependent pathway which led to an increase in transepithelial electrical resistance (TEER). However, KO of IL-10RA in T84 abrogated the effects of butyrate on increasing TEER, which appeared to be through the ability of IL-10RA to downregulate the pore forming claudin-2. Furthermore, Chen et al[47] recently found that butyrate protected mice from increased epithelial permeability in a

GPR109a dependent manner in a model of TNBS colitis. This effect was dependent on macrophage GPR109a suppression of LPS-induced phosphorylation of AKT in macrophages and was demonstrated using a transwell system where RAW246.7 macrophages were co-cultured with Caco-2 cells and pretreated with LPS in the presence or absence of butyrate. Thus, this finding exemplifies the important role macrophages play in modulating epithelial integrity through proinflammatory regulation.

SCFAs also have direct effects on epithelial cells in modulating TJ formation. For example, Feng et al[48] found that butyrate increased claudin-3, occludin, and ZO1 expression in a GPR109a dependent manner in piglets and Caco-2 cells. The effect on claudin-3 was abrogated with GPR109a knockdown (KD) in Caco-2 cells. Additionally, Cheng et al[49] found that NLR family CARD domain-containing 3 (NLRC3) KO mice have increased epithelial permeability. Treatment with butyrate increased NLRC3 expression and overexpression of NLRC3 increased TEER, implicating a role for butyrate in NLR3 induction of TJs, possibly through upregulating ZO1.

Finally, metabolism is an important driver of TJ formation. In this regard, Zhang et al[50] showed that in kidney cells, activation of 5' AMP-activated protein kinase (AMPK) lead to increased endogenous Ca^{2+} levels, which drove TJ formation. Additionally, Kelly et al[51] showed that hypoxia inducible factor 1 (HIF-1α) expression is critical for SCFA regulation of intercellular permeability. Interestingly, AMPK activation has been shown to stabilize HIF-1α and prevent the switch to glycolysis, the Warburg effect, implicating an important role for butyrate in modulating glycolysis.[52] Finally, Peng et al demonstrated

that butyrate, a known activator of AMPK, modulates TJ formation through regulation of AMPK.[53] Thus, it appears that by regulating energy status via AMPK in several tissues, butyrate may have a universal role in driving TJ formation.

SCFAs and Immune Regulation

The immune cells, which reside intraepithelially and in the lamina propria of the intestines play a vital role in regulation of host homeostasis to microbiota, with accumulating evidence suggesting that SCFAs are the key regulator of this process (**Figure 1.2**).

SCFAs Regulation of Neutrophils

SCFAs have been shown to regulate neutrophil functions. In this regard, Vinolo et al[54] demonstrated the differential effects of SCFAs on neutrophil killing. This was examined via the isolation of rat peritoneal neutrophils, in which butyrate inhibited the phagocytosis and killing of C. *albicans*, while also decreasing reactive oxygen species (ROS) production in neutrophils. This is in contrast to propionate, which had no effect on phagocytosis, killing, or ROS production; similarly, acetate only moderately increased ROS production. Vinolo et al[55] later uncovered that butyrate and propionate treatment of neutrophils diminished TNFα, cytokine- induced neutrophil chemoattractant-2 (CINC-2αβ), and nitric oxide (NO) production in LPS treated neutrophils. This downregulation of inflammatory cytokines was found to be HDACi dependent and cyclooxygenase (COX) independent. This data by Vinolo et al points toward a major role of HDACi in modulating neutrophil function, given the potency of butyrate as compared to other SCFAs. More interestingly, it implicates butyrate as a key player in priming neutrophils in the gut, possibly to protect against invading pathogens. With this data, it would be of great interest to further examine

the role of systemic butyrate, possibly through the use of tributyrin, the rapidly absorbed prodrug form of butyric acid.[56]

SCFAs modulation of chemotaxis was uncovered by Sina et al[57] who examined chemotaxis of neutrophils under acute and chronic inflammation in wild-type (WT) and GPR43 KO mice. In the study, it was shown that GPR43 KO mice had decreased neutrophil influx into the colon upon both acute and chronic inflammation. Using transwell assays, they found that SCFAs activate neutrophil migration, and that this migration was abrogated in GPR43 KO neutrophils. However, under non-inflammatory conditions, GPR43 KO neutrophils *in vivo* did not demonstrate any alterations in chemotaxis. Most interestingly though is that GPR43 KO aggravated acute DSS colitis but was protective in chronic colitis. This begs the question as to the differential regulation of GPR43 and its importance under non-inflammatory versus inflammatory reactions, and in acute versus chronic inflammation. Given that neutrophilic infiltrate is a hallmark of ulcerative colitis, it would be of interest to investigate whether a GPR43 antagonist would be beneficial in modulating chronic colitis and colitis-associated cancer.

The work from Vieira et al[58] further demonstrated the role of SCFAs and GPR43 in neutrophil chemotaxis. Using a mouse model of gout where monosodium urate (MSU) crystals are injected into the capsule of the knee, treatment of mice with acetate led to increased neutrophil influx and elevated IL-1β. However, in GPR43 KO, the effects of acetate were abrogated, which led to decreased PMN influx and IL-1β. However, despite the increased neutrophils and elevated IL-1β, later work by Vieira et al[59] showed that

although neutrophils and IL-1β were elevated within 6 hours following MSU deposition, treatment with SCFAs led to quicker resolution of inflammation. Thus, this finding of GPR43 dependent resolution of neutrophil inflammation in the acute setting supports the work by Sina et al[57] who showed that GPR43 KO mice are more susceptible to severe inflammation and death in the acute DSS model.

Aside from GPR43, Chen et al[60] also found that dimethyl fumarate (DMF) and its metabolite monomethyl fumarate (MMF) decreased neutrophil chemotaxis into the spinal column in a model of experimental autoimmune encephalomyelitis (EAE). This effect was dependent on GPR109a expression on neutrophils, as GPR109a KO abrogated the effects of DMF and appears to be modulated by decreased neutrophil adhesion to endothelial cells. Thus, it appears that SCFAs via GPR43 and GPR109a are key regulators of neutrophil chemotaxis and implicate the potential systemic use of SCFAs to treat inflammatory conditions.

SCFAs Regulation of T Lymphocytes

SCFAs modulate the differentiation of Th1, Th17, and T regulatory (Treg) cells, as well as their function. The role of SCFAs in Treg induction was demonstrated by Arpaia al[61] who showed that butyrate could drive CNS1 dependent differentiation of extrathymic Tregs. This was further confirmed by Furusawa et al[62], who showed that luminal concentrations of SCFAs correlated with the number of Tregs present in the colon. Recently, Haghikia et al[63] demonstrated that SCFAs as compared to long-chain fatty acids, were protective in the preventative setting, but not the treatment setting, in experimental EAE. This mechanism occurred via SCFA induction of Tregs, which was demonstrated by adoptive transfer of

Tregs from propionate treated or non-treated mice into recipient mice with simultaneous induction of EAE. Additionally, Schwarz et al showed that butyrate induction of Tregs was protective against contact hypersensitivity reactions in the skin, similar to their role in colitis and EAE.[64] These data support that SCFAs may be an important environmental factor that could dictate the onset of inflammatory diseases, however, the ability of SCFAs to modulate inflammation following disease onset is less convincing. The lack of SCFA protection post-inflammation onset may be due to their differential effects on other T cell populations as well as their concentration. For example, Salkowska et al[65] found in human Jukrat T-cells that butyrate decreased RORγt expression in naïve CD4 T cells under Th17 polarizing conditions, but promoted RORγt and IL-17A expression if butyrate was added to differentiated Th17 cells. Furthermore, Park et al[66] found administration of super physiological doses of SCFAs led to the development of T cell mediated ureteritis, which progressed to kidney hydronephrosis. These data offer interesting perspectives on the role of SCFAs on inflammation by the fact that they demonstrated that SCFAs may not be a beneficial treatment for acute inflammation, and that dosing of SCFAs could be critical in determining its therapeutic potentials. Additionally, Asarat et al[67] found that PBMCs cultured in the presence of LPS and SCFAs had decreased Th17 cells, increased Treg cells, and decreased IL-6, with butyrate being the most potent inducer of Tregs. Furthermore, Zhang et al[68] demonstrated that butyrate administration increases peripheral Treg induction, while increasing IL-10 and IL-12 and decreasing IL-17 and IL-23 expression. Recently our group showed that SCFAs induce IL-10 production in Th1 effector cells in a GPR43 dependent manner mediated by Blimp-1.[69] The importance of IL-10 production in Th1 was further verified by showing that the SCFA treated microbiota specific Th1 cells

induced less severe colitis as compared to untreated Th1 cells when transferred into RAG KO mice. However, administration of an anti-IL-10R antibody abrogated the effect of SCFA treated Th1 cells. Our groups finding was further extended by Luu et al[70] who demonstrated that another SCFA, pentanoate, effectively inhibited IL-17 production in Th17 cells and increased IL-10 production, with IL-10 induction being regulated by glucose oxidation in T cells.

SCFAs Regulation of Macrophages

SCFAs play several roles in modulation of macrophage activation, recruitment, and anti-microbial responses. The role of SCFAs in the activation of macrophages was shown by Lukasova et al[71] who demonstrated the importance of GPR109a in modulating M1 macrophage differentiation by downregulating M1 macrophage markers CD68 and arginase 2. Additionally, GPR109a activation decreased IFNγ induction of monocyte chemotactic factor 1α (MCP-1α) as well as macrophage recruitment following peritoneal MCP-1α injection. This anti-inflammatory effect of SCFA receptors was extended by Nakajima et al[72] who showed that WT mice are thinner and have higher insulin sensitivity than GPR43 KO mice. To demonstrate this, it was shown that M2 macrophages isolated from WT, but not GPR43 KO mice had elevated levels of TNFα. In this context, elevated levels of TNFα expression by M2 macrophages are associated with adipocyte tissue remodeling and decreased fat accumulation. Furthermore, Chang et al[73] demonstrated that butyrate via HDACi leads to the downregulation of LPS-induced proinflammatory release from macrophages, specifically affecting IL-6. Most recently, Schulthess et al[74], using single cell RNA-seq analysis, identified that butyrate induced an anti-microbial signature

characterized by the expression of S100A8, S100A9, S10012, LYZ, and FCN1, which was driven by inhibition of HDAC3. Thus, these data implicate SCFAs as major modulators of basal levels of inflammation driven by macrophages, and also exemplifies their potentially protective effect against pathogens through the promotion of anti-microbial responses at epithelial surfaces.

SCFAs Regulation of Dendritic Cells

SCFA regulation of DCs is critical in the induction of tolerance. In this regard, a report by Tan et al[75] demonstrated the importance of GPR43 and GPR109a in the development of tolerance to food antigens. Here, the lack of GPR43 or GPR109a in mice fed a high fiber diet led to a reduction of $CD103^+$ DCs and Aldh1a2 expression [the retinaldehyde dehydrogenase-2 (RALDH2) enzyme is encoded by Aldh1a2]. RALDH2 is responsible for vitamin A metabolism to retinoic acid (RA), which is critical for the induction of Tregs by $CD103^+$ DCs.[76,77] Supporting this evidence, it was shown that GPR43 KO and GPR109a KO mice had impaired Treg responses in the mesenteric lymph nodes (MLN), increased serum IgE, and heightened clinical anaphylaxis scores when challenged with antigen. A later report by Goverse et al[78] showed that SCFAs and a high fiber diet were able to induce vitamin A metabolism in epithelial cells and $CD103^+$ dendritic cells and this was correlated with increased Foxp3 expression in T cells. The ability of SCFAs to induce vitamin A metabolism via Aldh1a1 expression in intestinal epithelial cells (IEC) was dependent on HDAC1 inhibition as demonstrated by increased expression of Aldh1a when IEC were treated with MS344, an HDACi targeting HDAC1. With this data, it would be of interest to examine the selective inhibition of HDAC1 in the prevention and treatment of colitis, as Treg induction has been shown to be important for protection against colitis.[62]

Recently, our group demonstrated that DCs play an important role in the induction of IgA production in the gut following stimulation with SCFAs.[79] Here, we showed that GPR43 KO mice had decreased levels of IgA compared to WT mice and that feeding WT mice but not GPR43 KO mice with acetate led to induction of intestinal IgA. This effect of acetate was shown to be independent of T cells, as TCRβδ KO mice, which have B cells but lack T cells, also had elevated IgA production in response to SCFAs. *In vitro*, acetate induced RA signaling in DCs, which drove increased IgA production from B cells.

SCFAs and Inflammatory Bowel Disease

Harig et al[80], who successfully treated a small cohort of patients with diversion colitis via rectal irrigation, first showed the relevance of SCFAs as a potential therapeutic. This finding was later extended by Scheppach et al[81] who was able to successfully treated patients with ulcerative colitis with a regiment of butyrate. The basis for using SCFAs as a treatment is exemplified by the findings of Treem et al[82], who showed that children with UC and CD have decreased fecal SCFAs, and Frank et al[83], who uncovered that patients with IBD often have a decrease in Firmicutes and Bacteroidetes, which are noted for their production of butyrate and propionate. However, despite these findings, the role of SCFAs for the treatment of colitis remains controversial. For example, Furusawa et al[62] in a preventative model of colitis, showed that the treatment of mice with butyrate post transfer of $CD4^{+}CD45RB^{hi}$ T cells prevented the onset of colitis. Additionally, Maslowski et al[84] showed that acetate could reduce the severity of acute and chronic colitis in a GPR43 dependent manner, which was abrogated in GPR43 KO mice. GPR43

KO mice were more susceptible to both acute and chronic DSS colitis, with neutrophils also showing enhanced migration into the peritoneum following injection of heat inactivated *S. aureus*. The findings of Maslowski et al[84] differ from those of Sina et al[57], who showed that GPR43 KO mice had less severe colitis in the chronic DSS model. However, due to the differences in DSS protocols, it is difficult to perform a direct comparison. Thus, further evaluation across several models of colitis should be explored. Consistent with the importance of SCFAs in colitis prevention, Singh et al[23] demonstrated the importance of GPR109a in colitis development, with GPR109a KO mice developing lethal colitis in the acute model, while also having increased risk of colorectal cancer development in the azoxymethane (AOM) DSS model. The findings by Singh et al[23] in the AOM/DSS model supports the work performed by Kaiko et al[41] who proposed that butyrate might play a critical role in the prevention of cancer development by preventing the proliferation of stem cells while exposed to higher luminal concentrations of butyrate. Additionally, while SCFAs may play an important role in the prevention of inflammation, Chang et al[73] demonstrated that in a treatment model where butyrate supplementation began the day prior to DSS colitis onset rather than 5-7 days prior, butyrate was no better than control in terms of colitis severity in the acute DSS colitis model.[73] The reason for SCFAs effect in prevention rather than treatment of colitis may be offered by the findings from Kaiko et al[41], who found that butyrate inhibition of stem cell expansion led to increased ulcer size in the acute model of DSS. Thus, the beneficial effects of butyrate on inflammation may be partially counteracted by this delay in repair to ulcerated tissue. To circumvent this issue, in the future it may be beneficial to begin investigating compounds

that target individual GPRs or HDACs in IBD, which allow for the anti-inflammatory properties of SCFAs while avoiding possible alterations in epithelial barrier function.

Concluding Remarks

Given the importance of SCFAs in barrier protection and regulation of inflammation, dietary supplementation of SCFAs or modulation of diet to increase dietary fiber intake is an attractive option for potentially reversing the increase we see today in chronic inflammatory diseases. This could be beneficial in the preventative setting, where SCFAs have been linked to lower risk of chronic inflammatory diseases and colorectal cancer.[8,42,85] However, while SCFAs have a clear role in the regulation of host immunity, it is unclear whether SCFAs represent a feasible treatment following the onset of chronic inflammatory conditions. This is further supported by the conflicting clinical data which to date have failed to show conclusive evidence for the use of SCFAs in the acute setting. Nevertheless, further work is needed in this area for the purposes of precision medicine if we hope to one day treat IBD patients with chemical agonists or antagonists of GPRs or HDACs.

Figures

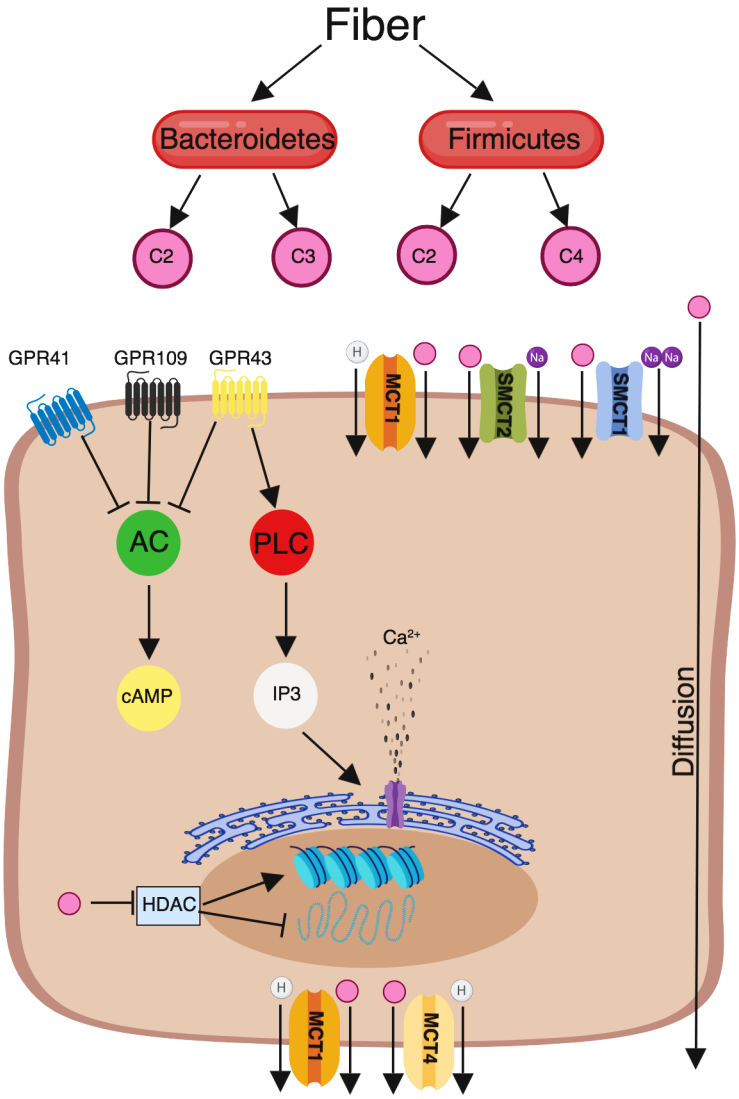

Figure 1.1: Formation, transport, and mechanisms of action of SCFAs.

Fiber is fermented by bacteria in the colon which release SCFAs acetate (C2), propionate (C3), and butyrate (C4). Uptake is via passive diffusion, electroneutral, or electrogenic uptake. SCFAs engage three receptors, GPR41, GPR43, and GPR109a, which are linked to Gi/o and/or Gq. Gi/o modulates adenylate cyclase (AC) and cAMP production, whereas Gq activates phospholipase C (PLC), leading to inositol triphosphate (IP3) formation, and modulation of calcium release from the endoplasmic reticulum. SCFAs

also act as HDACi, which promotes the open form of chromatin. *Figure created with BioRender*.

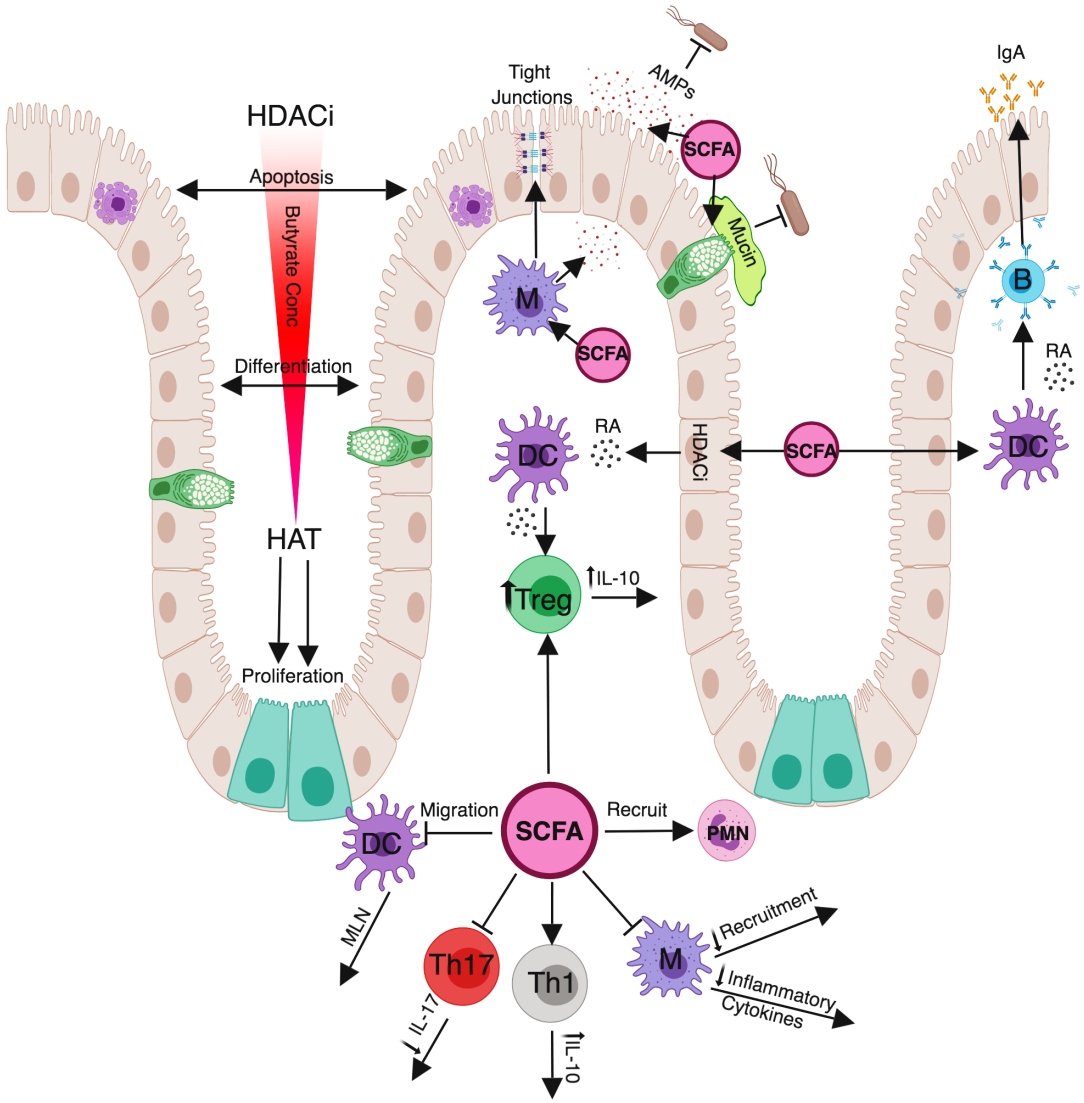

Figure 1.2: SCFA Modulation of Barrier Defenses.

A butyrate concentration gradient is established by the epithelium, where high concentrations of butyrate stimulate HDACi, leading to differentiation or apoptosis, and low concentration stimulate acetylation via HATs and stem cell proliferation. SCFA modulate tight junction formation directly and indirectly through influencing cytokine production from macrophages (M). SCFAs also stimulate antimicrobial peptide (AMP) production from the epithelium and macrophages. SCFAs stimulate several classes of

mucins (MUC) including MUC2, the primary colonic mucin. SCFAs regulate dendritic cell (DC) function by promoting retinoic acid signaling (RA) which promotes IgA secretion from B cells (B) and promoting T regulatory cells (Tregs), while also inhibiting DC migration to the draining mesenteric lymph nodes (MLN). SCFA act on T cells to promote IL-10 production from Tregs and Th1 cells, while modulating Th17 differentiation and IL-17 production. SCFAs recruit neutrophils (PMN) to the gut. SCFAs modulate macrophage function, where they inhibit their recruitment to the gut, while decreasing pro-inflammatory production. *Figure created with BioRender*.

Chapter 2-Migration and Techniques to Study Cell Movement

Mechanics of Cell Migration

The intestinal epithelial tract is constantly renewing every 3-5 days.[86] This process is energetically expensive and relies on coordination between proliferation in the crypts and apoptosis at the villus apex. However, migration is also critical in epithelial turnover driven largely by the role of several major proteins in the class of Rho GTPases activated by upstream signaling from trefoil factors, IL-22, TGFβ, and other cytokines.[87–92] Cell migration is a complex process that depends on continuous remodeling of cellular junctions, interactions with underlying substrates, as well as cytoskeleton rearrangement. This process begins with signaling through focal adhesions which include proteins such a integrins linked to focal adhesion kinase (FAK) which is a major protein that regulates the turnover of focal adhesion complexes.[93] Phosphorylation of FAK at Tyr-397 leads to its activation, which sets off a series of downstream events, which include the three major Rho GTPase family members RhoA, CDC42, and RAC1. This action is mediated by the ability of FAK to modulate guanine exchange factors (GEFs) which are responsible for Rho GTPase activation.[93] Two major Rho GTPases, CDC42 and RAC1 are important for assembly of cell extensions including lamellipodia and filopodia. For a migrating cell, extension of a hood like structure (lamellipodia) is driven by coordination of RAC1 and CDC42 with downstream proteins such as actin related proteins 2/3 (ARP 2/3) and WAVE, which promotes actin branching, extension, and turnover, allowing progressive growth of the lamellipodia.[93–95] Meanwhile, at the site of assembly where the nascent lamellipodia or filopodia interacts with the underlying substrate, new focal adhesions develop driven by

the interaction of alpha-beta integrins with the basement membrane.[93,94,96] Finally, at the rear of the cell, adhesion complexes are broken and remodeled, driving activation of RhoA and Rho-associated protein kinase (ROCK), leading to the recruitment of myosin light chain, which leads to contraction along actin-myosin stress fibers.[93,94] This brings the rear of the cell forward toward the nucleus and completes one round of extension and retraction of the cell. The importance of cell migration, especially FAK, RhoA, CDC42, and RAC1 cannot be overstated. These pathways are well characterized and mutations in either CDC42, RAC1, ARP, FAK or RhoA leads to serious diseases and malformations including variable growth dysregulation, facial dimorphisms, neurodevelopmental ,immunological, and hematological abnormalities.[93,97] These pathways form the core of cell movement under both homeostatic and disease states .

Techniques to Study Migration Past and Present

Migration assays have evolved over time to allow for complete dissection of cell movement. Historically, these assays were typically carried out on uncoated plastic plates with cell lines most commonly derived from epithelial or fibroblast cells. Following confluency of the monolayers, cells were scratched with a pipette tip and treated with or without serum for a given length of time and the difference in the denuded area at baseline and at some time point (usually hours to days after scratching) was calculated.[98] This difference in area between the two points is known as migration distance. Although simplistic in its approach, the traditional scratch assay missed major cellular events that could allow scientist to elucidate this dynamic process of cell migration. In response, new techniques were developed to assess the properties of cell migration, one of which is live cell imaging.[99] Live cell imaging is performed using the traditional scratch assay, or cells

seeded into a 3-dimensional matrix, and movement is tracked by imaging repeatedly over the course of hours to days at small time intervals (usually 5-15 minutes). These serial images are then stitched to allow for the analysis of cell motion. With this technique, cell speed, and certain parameters such as mean squared displacement, which examines the displacement of total area of a cell over time and directionality are able to be calculated. However, directionality may be misleading because it is influenced by speed and thus does not allow us to directly measure persistence.[100] Similarly, MSD may be used to calculate alpha, which is a measure of persistence, but this method is not suitable over long periods of time.[100] To overcome these limitations, Gorelik deduced a formula which allows us to investigate cell speed and cell persistence independently by focusing on the cosign of the vectors of cell movement between each point, which eliminates the influence of cell speed on directionality.[101] This additional information is vital for the dissection of underlying molecular pathways via other means such as actin dynamics, which regulate cell adhesion and spreading.

Techniques to Study Actin Dynamics

There exist several ways *in vitro* to study the dynamics of cell movement. Starting with cell adhesions, the cell attachment assay has classically been used to understand the way cells interact with underlying substrates.[102] This assay involves plating cells onto surfaces that are coated with either polystyrene, collagen, matrigel, polylysine or various mixtures of substrates. By altering the substrate and/or its stiffness, one is able to determine the types of integrins that are mediating attachment. Conversely, by including inhibitors or activators in the assay, one is able to deduce which pathways are likely to be involved in the process of attachment.[102] To quantify the degree of attachment, cells may either be stained with

crystal violet and imaged via brightfield microscopy or stained with DAPI which allows for more convenient automatic quantification with immunofluorescence microscopy.[102] However, although informative, this assay does not take into account adhesive strength, which can allow for further understanding of the types of interactions that are occurring as well as the strength of those interactions. In order to probe this phenomenon, a spinning disk method known as hydrodynamic shear may be utilized.[103] Due to the nature of adhesive bonds between integrins and underlying matrix mixtures, the spinning disc method allows for force to be applied to a group of cells equally and thus the determination of the average shear force needed to remove those cells from the disk.[104] The data obtained from this assay is then used to calculate the number of bonds formed between each cell and the underlying substrate. This assay is practical for use in the lab, however, there is a subset of other methods which allow for more precise measurement of cell detachment including cell adhesion force microscopy, which relies on atomic force microscopy (AFM) paired with laser beam deflection to measure force applied to individual cells. [105] Additionally, AFM itself can be used to explore the cell surface during focal adhesion assembly, but investigation of this is often slow as only a single cell at a time may be visualized and measured.[104]

One convenience of the cell attachment assay is that it is often paired with the cell spreading assay allowing one to study both attachment and cell spreading simultaneously. Cell spreading assays rely on plating cells onto a surface coated with various substrates. After a certain amount of time (often 30-90 minutes) cells are then stained with either crystal violet and imaged via brightfield, or stained with DAPI and phalloidin, which allows for visualization of the perimeter of the actin network.[106] These cells can then be imaged via

immunofluorescence microscopy and their cell size is quantified either manually or automatically using software. The cell size after a given length of time is indicative of how fast a cell is able to spread, which gives information on both the ability to develop focal adhesion complexes as well as polymerize actin. However, because several processes are affected during this assay, one is not able to deduce whether or not spreading is caused by increased actin polymerization or increased focal adhesion development. To deduce this information, cell spreading assays are often paired with actin polymerization assays. Actin polymerization assays utilize fluorescent pyrene conjugated to G-actin.[107] Cell lysates are mixed with pyrene conjugated actin, which fluoresces brighter when conversion into F-actin occurs. The amount of polymerization can be followed over time to determine a rate of polymerization. Additionally, actin depolymerization can also be calculated by following the breakdown for styrene conjugated F-actin, which results in a loss of fluorescence.[107] Lastly, another common technique to follow cell spreading which does not rely on fluorescence is electric cell-substrate impedance sensing.[108] In this technique, the alternating current (AC) impedance is between the cell surface and the underlying substrate.[108] This is possible by depositing gold-filmed electrodes onto the substrate.[108] The benefit of this method is it allows for time course data on both cell attachment and spreading to be obtained, rather than relying on static time points with conventional spreading assays. This also allows for less user bias, better data replication, and a more high-throughput method for investigating the roles of different inhibitors and activators on cell spreading.[108]

Techniques to Study Migration *In Vivo* In the Intestinal Tract

Studying intestinal epithelial cell migration has been particularly difficult and relies on the tracking of BrdU labeled cells. The rationale for using this technique was largely due to the fact that most people believe that migration is a passive process in the gut driven by cell division.[109] Thus, to measure cell migration, BrdU was used to label cells at various timepoints, and similar to the traditional scratch assay, the area displaced between two time points was assumed to be equivalent to migration distance. Countless papers have relied on this technique to study migration in recent decades.[90,110] However, discounting the role of cell proliferation on migration may lead to erroneous results. Recently, it was shown that cell migration in the gut is not passive, but rather an active process that is driven by continuous actin turnover.[111] In this model, cell division drives cell migration in the crypt due to increased pressure from the base of the crypt, but outside of the crypt, pressure on neighboring cells from cell division is low, and actin turnover is the primary driver of cell migration.[111] Thus, to measure cell migration in the gut without confounding the results due to the impact of cell division, we can measure cell migration by using the S phase inhibitor hydroxyurea to temporarily halt cell proliferation, which allows for a more accurate measurement of cell migration along the crypt villus axis.[111]

Chapter 3-Propionate Enhances Cell Speed and Persistence to Promote Intestinal Epithelial Turnover and Repair [2]

Abstract

Background and Aims: Gut bacteria-derived short-chain fatty acids (SCFAs) play crucial roles in the maintenance of intestinal homeostasis. However, how SCFAs regulate epithelial turnover and tissue repair remain incompletely understood. In this study, we investigated how the SCFA propionate regulates cell migration to promote epithelial renewal and repair.

Methods: Mouse small intestinal epithelial cells (MSIE) and human Caco-2 cells were used to determine the effects of SCFAs on gene expression, proliferation, migration, and cell spreading *in vitro*. Video microscopy and single cell tracking were used to assess cell migration kinetically. 5-bromo-2'-deoxyuridine (BrdU) and hydroxyurea were used to assess the effects of SCFAs on migration *in vivo*. Lastly, an acute colitis model using dextran sulfate sodium (DSS) was used to examine the effects of SCFAs *in vivo*.

Results: Using video microscopy and single cell tracking, we found that propionate promoted intestinal epithelial cell migration by enhancing cell spreading and polarization, which led to increases in both cell speed and persistence. This novel function of propionate was dependent on inhibition of class I histone deacetylases (HDAC) and GPR43 and required signal transducer and activator of transcription 3 (STAT3). Furthermore, using 5-bromo-2'-deoxyuridine (BrdU) and hydroxyurea *in vivo*, we found that propionate

[2] This work has been previously published in: **Bilotta AJ**, Ma C, Yang W, Yu Y, Yu Y, Zhao X, Zhou Z, Yao S, Dann SM, Cong Y. Propionate Enhances Cell Speed and Persistence to Promote Intestinal Epithelial Turnover and Repair. *Cell Mol Gastroenterol Hepatol*. 2020;(December):1-22. doi:10.1016/j.jcmgh.2020.11.011

enhanced cell migration up the crypt-villus axis under homeostatic conditions, while also protecting against ulcer formation in experimental colitis.

Conclusion: Our results demonstrate a mechanism by which propionate stimulates cell migration in an HDAC inhibition, GPR43, and STAT3 dependent manner, and suggest that propionate plays an important role in epithelial migration independent of proliferation.

Background and Aims

The gut epithelium, which separates the host from the external environment and forms the first line of defense to enteric infection, is comprised of a single layer of epithelial cells which renew themselves constantly, turning over every 3-5 days [112 113]. In order to maintain homeostasis, the epithelial layer depends on a host of growth signals and energy sources to proliferate, differentiate, and migrate. This begins with stem cells in the crypt base dividing every 2-3 hours driven by the secretion of growth factors such as Wnt from underlying mesenchymal fibroblast [114]. Following division, stem cells give rise to transient amplifying cells, which then differentiate in response to both host and microbial factors including short chain fatty acids (SCFAs) [115]. These cells migrate toward the villus axis where they are extruded into the gut lumen. It has been shown that gut microbiota plays a crucial role in epithelial maturation and turnover, however, the mechanisms remain poorly understood.

Intestinal epithelial restitution is a complex process that is important for tissue regeneration in inflammatory bowel disease (IBD) [87]. It has been shown that during the first several hours following injury, cells from adjacent crypts migrate into the wound bed to form a temporary barrier to protect the underlying stem cells from exposure to luminal contents [116–118]. This process is independent of cellular proliferation as seen in other organs such as the skin. Several signals have been described to play a role in epithelial restitution including prostaglandins, transforming growth factor beta (TGFβ), trefoil factors, and gut microbiota-derived short chain-fatty acids such as propionate [88,91,119,120]. However, the mechanisms behind how these signals contribute to the restitution remain unclear.

SCFAs are bacterial metabolites from the fermentation of dietary fibers by microbes in the gut lumen [11,12,15]. There are five short chain fatty acids, with acetate, propionate, and butyrate making up the large majority of SCFAs in the gut [4]. SCFAs are vital for intestinal homeostasis, where they affect both the epithelial cells as wells as immune cells in the underlying lamina propria [2,121]. SCFAs are known to enhance mucus production by promoting goblet cell differentiation and mucus production [27–29]. Previously, we and several other groups have shown that SCFAs are important for stimulating intestinal epithelial cell (IEC) antimicrobial peptide production to protect against enteric infection [122]. It has been demonstrated that a SCFA gradient exists in the gut, with high levels of SCFAs exerting an apoptotic effect on cells via inhibition of histone-deacetylases, and low concentration allowing for histone-acetyltransferase activity and increased stem cell proliferation [40]. Furthermore, it has been shown that SCFAs such as propionate can enhance epithelial restitution *in vitro*, and promote epithelial turnover by promoting proliferation in the crypt [86,120]. Previously, it was thought that epithelial migration that occurs during epithelial turnover was a passive process as a result of the force generated from dividing cells in the crypt [109]. However, recent data suggest that actin polymerization, but not proliferation, is the major driving force for cell migration on the villus [111].

Propionate, a major SCFA present at high levels in the gut lumen, has been shown to contribute to intestinal homeostasis through promoting epithelial proliferation and migration [4,39,40,120]. However, how propionate drives epithelial cell proliferation and migration, and the mechanisms involved are still largely unclear. In the current study, we utilized video microscopy to track individual cells to analyze cell speed and persistence

using the classic scratch assay (wound healing model) and DiPer *in vitro* [98,101]. Additionally, we used BrdU and hydroxyurea to investigate the effects of propionate on IEC migration *in vivo* [111]. We report here that propionate increases both cell speed and persistence through inhibition of HDAC and activation of GPR43 and STAT3 to promote epithelial cell migration and wound closure, which enhances epithelial turnover and protection against ulcer formation.

Materials and Methods

Animals and Models

C57BL/6 WT mice were purchased from the Jackson Laboratory. All mice were housed in the specific pathogen-free animal facility in the Animal Resource Center at UTMB. All described animal experiments were performed in accordance with protocols reviewed and approved by the Institutional Animal Care and Use Committees of the University of Texas Medical Branch (UTMB).

For DSS model of colitis, WT mice were fed water +/- 200 mM propionate ad libitum for 7 days. Mice were then fed water containing 1.9% DSS (Cat# DS1004, Gojira FC) w/v +/- 200 mM propionate for 7 days. A 3-day washout was performed where mice were fed water +/- 200 mM propionate. Mouse weights were monitored daily.

For epithelial migration *in vivo*, mice were fed water ad libitum containing an antibiotic cocktail containing 1g/L of metronidazole (Acros Organics, Cat# 443-48-1), ampicillin (Fisher Scientific, Cat# BP1760-5), kanamycin (Fisher Scientific, Cat# BP906-5), and 0.5g/L of vancomycin (Acros Organics, Cat# 1404-93-9) for 10 days. Mice were then treated with water containing the antibiotic cocktail +/- 200 mM propionate for an additional 21 days. Mice were intraperitoneally injected with 100mg/kg of BrdU (Abcam, Cat# ab142567). Half of the mice were sacrificed 36 hours post-BrdU injection. The remaining mice were injected with 50 mg/kg of the S phase Inhibitor hydroxyurea (Acros Organics, Cat# 127-07-1) to inhibit crypt proliferation, and sacrificed 12 hours post hydroxyurea injection.

Reagents

Recombinant murine gamma interferon (IFN-γ) (Cat# 575306) was purchased from BioLegend. Culture medium RPMI 1640 (Cat# SH30027.01), penicillin/streptomycin

(Cat# SV30010), were purchased from GE healthcare. ITS (Cat# 354350,) and Matrigel (Cat# 356231) were purchased from Corning. Dulbecco's Modified Eagle's Medium (DMEM) (Cat# 30-2002) was purchased for ATCC. Advanced Dulbecco's Modified Eagle's Medium/F12 (Advanced DMEM) was purchase for Gibco. Recombinant mouse EGF (Cat# 2028-EG), human WNT-3A (Cat# 5036-WN), mouse Noggin (Cat# 1967-NG), mouse R-Spondin (Cat# 3474-RS) were purchased from R&D Systems. The ROCK inhibitor Y-27632 dihydrochloride (Cat# 1254) was purchased from TOCRIS. STAT3 inhibitor HJC0152 was synthesized by Dr. Jia Zhou's laboratory at the University of Texas Medical Branch at Galveston following their reported procedures [123]. STAT3 Inhibitor Stattic (Cat# S7024), PAK1 inhibitor IPA3 (Cat# S7093), HDACI TMP195 (Cat# S8502), Valproate (Cat# S1168), RGFP966 (Cat# S7229), and 1-Naphthohydroxamic Acid were purchased from Selleckchem. MEK Inhibitor U0126 (Cat# U120) butyrate (Cat# 303410), acetate (Cat# S5636), propionate (Cat# P1880), TSA (Cat# t8552), sodium dichloroacetate (Cat# 347795), 6-Diazo-5-oxo-L-norleucine (DON) (Cat# D2141), Bz-423 (Cat# SML1944), metformin hydrochloride (Cat# PHR1084), 2-deoxy-D-glucose (2-DG) (Cat# D6134) and etomoxir (Cat# E1905) were purchased from Sigma Aldrich. Primary antibody against BrdU (Cat# ab6326) and Texas red goat anti-rat secondary (Cat# T-6392) were purchased from Abcam. Primary antibody against golgin 97 (Cat# A-21270), Texas red goat anti-mouse secondary (Cat# T-6390), phalloidin-488 (Cat# A12379), phalloidin-Texas Red (Cat# T7471), Hoechst 33342 (Cat# 62249) and Prolong Diamond Antifade Mount containing DAPI (Cat# P36970) were purchased from ThermoFisher. The following ELISA assay kits were purchased from BioLegend: mouse IFN-γ, IL-17, TNF, IL-6, and human IL-10 antibodies (Cat#: 431414, 430804, 432504, 430904, 431304, and

430604). For flow cytometry, Ki67 was purchased from BioLegend. Anti-MFGE8 monoclonal antibody (Cat# D199-3) was purchased from MBL International Corporation.

Knockout of STAT3 using CRISPR

Knockout of STAT3 was performed as previously described.[124] LentiCRISPR vector (plasmid no. 52961; Addgene, Cambridge, MA) was used to knockdown STAT3 in MSIE cells. The design and cloning of the target guide RNA (gRNA) sequences were performed using the Zhang laboratory's protocol (http://www.genome-engineering.org) [125]. Briefly, the suitable target sites for the gRNA sequence against STAT3 were established using the CRISPR design tool software (http://crispr.mit.edu). Cas9 target sequences for the indicated genes were designed in http://www.genome-engineering.org. Then, gRNA (Integrated DNA Technologies) were synthesized and subcloned into the lentiCRISPR-v2 vector. The newly constructed lentiCRISPR plasmids were then transfected into MSIE cells. Following antibiotic positive selection, transfected cells were established as a stable cell line. STAT3 Forward: *5'ACCGCGATTACCTGCACTCGCTTC3'*, Reverse:*5' AAACGAAGCGAGTGCAGGTAATCGC3'*.

Epithelial Cell Culture

Mouse small intestinal epithelial cells (MSIE) were cultured in RPMI 1640 medium supplemented with 5 U/ml murine gamma interferon (IFN-γ), 5% fetal bovine serum (FBS), ITS (5 μg/mL insulin, 5 μg /mL transferrin and 5 ng/mL selenous acid) and 100 U/mL penicillin/streptomycin at the permissive temperature of 33 °C. Before treatment with SCFAs, cells were starved in RPMI 1640 medium with 0.5% or indicated FBS for 16 hours at 37 °C. Caco-2 and IEC-18 cells were cultured in DMEM supplemented with 10%

FBS and 100 U/mL penicillin/streptomycin at 37 °C and 5% CO2. Before treatment with SCFAs, Caco-2 and IEC-18 cells were starved overnight in medium with 1% FBS at 37 °C.

Enteroid Culture

Enteroids were generated as previously described.[122] Briefly. The jejunum was dissected from the mouse, minced, and rocked for 30 minutes at 4°C with 2mM EDTA. The tissue was then treated with PBS containing 43.3 mM sucrose and 54.9 mM sorbitol and rocked for 2 minutes. Supernatant was filtered through a 70um cell strainers. The pellet containing detached crypts was resuspended in a 50% matrigel plug and overlayed with LWRN conditioned media and cultured at 37 °C as previously described.[126] For the first two days of culture, cells were additionally supplemented with 10uM of the ROCK inhibitor Y-27632. After two days, the media was changed to LWRN media without Y-27632. On day 3-4, spheroids were gently dissociated and passaged using TrypLE. For treatment of cells for RNA analysis or Seahorse XF Cell Mitro Stress Test, cells were washed twice with Advanced DMEM F12 containing 4mM glutamine, 10mM HEPES, and 100 U/mL penicillin/streptomycin. Treatment media was composed of Advanced DMEM F12 with 4mM glutamine, 10mM HEPES, 10 ng/mL WNT3A, 71 ng/mL Noggin, 75 ng/mL RSPO, and 50 ng/mL EGF as described previously.[127]

Epithelial Migration Assay

MSIE, IEC-18, and Caco-2 were seeded in either 24 or 96-well plates and allowed to attach for 24 hours. Following attachment, cells were placed into serum starve media overnight. For MSIE and IEC-18, a scratch was made in the cell monolayer using a ruler and 200uL

pipette tip. For Caco-2 cells, a scratch was made in the cell monolayer using a 200 μL pipette tip attached to a vacuum source. Cells were washed once in their respective media before the addition of treatments. Control cells were treated with equivalent amounts of PBS or DMSO. The cells were then placed onto the microscope stage of a Nikon Eclipse TI located inside of an Okolab cage incubator a and imaged at 10× at 37 °C and 5% CO2. For some experiments, cells were placed into the Biotek Cytation 5 (Biotek Instruments, Winooski, VT USA), and imaged at 4x or 10x at 37 °C and 5% CO2. For single time point experiments, wound closure was calculated as the $\frac{Original\ Area\ of\ Wound - Final\ Area\ of\ Wound}{\frac{Length\ of\ Wound}{2}}$. The widths of the wounds were measured using the MRI Wound Healing Tool macro for FIJI software (NIH) (**http://dev.mri.cnrs.fr/projects/imagejmacros/wiki/Wound_Healing_Tool**) and manually checked for accuracy. When quantification could not be done manually, the Fiji line tool was used to measure the area of the wound. For some experiments, cells were stained for F-actin using phalloidin-488 (Invitrogen, Cat# A12379) to assess adhesion of the monolayers. For time-lapse experiments, cells were imaged continuously every 15 minutes until monolayer closure occurred. Images were exported and stacked to create time-lapse videos. Single cell tracking was performed by tracking the centroid position of 15-20 cells per sample that moved the furthest using Fiji [128]. Data was uploaded and analyzed via DiPer for autocorrelation (cell persistence), cell speed, mean-squared displacement, and plots at origin [101]. Alpha values were calculated by taking the $\frac{\log(MSD\ slope)}{\log(time).}$ at each time point and averaged together over the course of the entire experiment.

Epithelial Cell Spreading

96-well plates were coated with 60 μL of a (1:50) dilution of matrigel in ice-cold DPBS for 1 hour at 37 °C and 5% CO2. Plates were washed and blocked with 0.1% bovine serum albumin (BSA) for 30 minutes. MSIE cells were subjected to an epithelial migration assay with or without 5mM propionate. 16 hours into the epithelial migration assay, cells were harvested using TrypLE Express (ThermoFisher, Cat# 12604021), counted, resuspended into fresh reduced serum media, and plated at 5,000 cells per well onto matrigel coated plates for 30, 60, or 90 minutes. Cells were washed to remove cells that did not adhere and fixed in 4% paraformaldehyde for 10 minutes at room temperature. Following fixation, cells were permeabilized with 0.1% triton-x100 in PBS for 10 minutes, blocked for 60 minutes using 10% goat serum and 22.5 mg/mL glycine in PBST, followed by overnight incubation in phalloidin-Texas Red-X (1:40) and Hoechst 33342 (1:1000). Cells were washed with PBST and imaged using the Biotek Cytation 5. For cell counts, the entire well was image automatically, and all the cells from each frame were calculated using Gen5.3 (Biotek Instruments, Winooski, VT USA) and added together to get the total number of cells per well. For cell spreading, a 5x5 grid which encompassed approximately 75% of the well was imaged automatically at 10×. Cell size was quantified automatically by Gen5.3 by calculating the total area of phalloidin staining. Images were checked manually for accuracy. To calculate cell spreading, the average cell size and SEM were determined for each sample. For publication images, cells that represented the average cell size at their respective time-points were imaged at 40×.

Epithelial Polarization

MSIE were seeded in 96-well plates and allowed to attach for 24 hours. Following attachment, cells were placed into serum starve media overnight. Following starvation, cells were treated with or without propionate in serum reduced media for 16 hours. After 16 hours, a scratch was made in the monolayer using a ruler and a 200 μL pipette tip and placed and place back into the incubator at 37 °C and 5% CO2 for two hours. After two hours, cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature. Following fixation, cells were permeabilized with 0.1% triton-x100 in PBS for 10 minutes, blocked for 60 minutes using 10% goat serum and 22.5 mg/mL glycine in PBST followed by overnight incubation with golgin-97 (1:100). The following day, cells were washed with PBST and incubated in goat anti-mouse Texas Red secondary (1:500) for two hours at room temperature. Cells were then washed in PBST and incubated overnight in phalloidin-488 (1:40) and Hoechst 33342 (1:1000). Cells were washed with PBST and imaged using the Biotek Cytation 5 at 40x. For imaging, 2 spots were randomly chosen on each side of the wounded monolayer (4 spots total) for each sample. Cells at the leading edge were then quantified for the extent of polarization. Cells were considered polarized if the majority of the golgi was located between the nucleus and the developing lamellipodia and facing the wounded gap. Between 35 and 55 cells were analyzed per sample and the percent of cells polarized was calculated as $\frac{\#\ of\ cells\ polarized}{total\ number\ of\ cells}$.

Flow Cytometry Analysis

For analysis of proliferation, MSIE cells were plated in a 24 well plate. Following serum starvation, cells were treated with CFSE and treated for 24 hours with SCFAs. Cells were then fixed with 1% paraformaldehyde. For Ki67 staining, serum starved MSIE cells were

treated for 24 hours with SCFAs. Cells were fixed and permeabilized followed by fluorochrome-conjugated anti-mouse Ki67 (1:100). Cells were then fixed in 1% paraformaldehyde. Quantification was performed with a LSRII/Fortessa and FACSDiva software (Becton Dickinson, Mountain View, CA, USA). Data was analyzed with FlowJo.

BrdU Staining

5 μm formalin-fixed ileal tissue was deparaffinized with CitriSolv (Thermofisher, Cat# 04-355-121) and rehydrated using a series of ethanol washes. Antigen-retrieval was performed with sodium citrate (pH 6.8) at 100°C for 20 minutes and then allowed to cool for 20 minutes at room temperature. Tissue was permeabilized for 10 minutes at room temperature using 0.2% triton-x100 in PBST. Slides were blocked in 10% goat serum, 22.5 mg/mL glycine in PBST for 1 hour at room temperature. Slides were incubated overnight in rat anti-BrdU (1:200) in blocking solution at 4°C. Slides were washed 3x with PBST before proceeding with incubation with rabbit anti-rat 588 in PBST for 2 hours. Slides were washed 3 times in PBST, dried, and mounted using Prolong Diamond Antifade Mount containing DAPI. Images were acquired at 20x with a Biotek Cytation 5. The BrdU front was measured using Gen 5.3 [128]. To do this, the line tool was used to measure the distance from the base of the crypt to the BrdU front. 15-30 villi were quantified per sample to obtain a BrdU front average and SEM.

ELISAs

ELISAs were performed according to the manufacturer's protocols for IL-6, IFN, TNFα, and IL17A using culture supernatant derived from organ cultures. High Affinity 96 well plates were coated with capture antibody at a (1:200) dilution and incubated overnight at

4°C. After blocking, supernatants were incubated for with detection antibody at a (1:200) followed by Streptavidin conjugated to horseradish peroxidase at (1:1000) prior to the addition of TMB substrate. Absorbance was measured using a plate reader.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted with TRIzol reagent (Life Technologies; Carlsbad, CA), quantified via nanodrop, and used for cDNA synthesis. Quantitative real-time PCR was performed by using SYBR Green Gene Expression Assays (Bio-Rad, Hercules, CA, USA). GAPDH was used as the endogenous reference gene. The relative gene by normalizing to GAPDH. GAPDH Forward: *5'TCAACAGCAACTCCCACTCTTCCA3'*, Reverse: *5'ACCCTGTTGTAGCCGTATTCA3*; TFGβ Forward:*5'TGACGTCACTGGAGTTGTACGG3'*, Reverse: *5'GGTTCATGTCATGGATGGTGC3'*; Mfge8 Forward: *5'CGCACAGGATCGTCAAT3'*, Reverse: *5'CGCAGAAGGTTCACCTGGAT3'*; Pak1 Forward: *5'GTGTCTGAGACCCCAGCAGTA3'*, Reverse: *5'GTGGTTCAATCACAGATCGTGT3'*.

Cell metabolism measurement

MSIE cells were seeded at 8,000 cells per well into a 96-well Seahorse plate. Following serum starvation, cells were treated for 8 hours with propionate and subjected to the Seahorse XF Cell Mito Stress Test (Agilent, Cat# 103708-100) to determine OCR and ECAR. For enteroid monolayers, spheroids grown in LWRN were dissociated and plated at 350 crypt pieces per well onto a 96-well Seahorse plated coated with 30uL of 10% matrigel (1:10 dilution into DPBS). Cells were overlayed with LWRN conditioned media for 2 days supplemented with Y-27632. After two days, cells were treated in treatment

media as described above with or without propionate. Following 8 hours of treatment, cells were subjected to the Seahorse XF Cell Mito Stress Test (Agilent, Cat# 103708-100) to determined OCR and ECAR.

Histopathological Assessment

At necropsy, the colon was swiss-rolled and fixed in 10% neutral buffered formalin for 24 hours. Tissue was then paraffin embedded and 5 μm sections were prepared and stained with H&E. The severity of the disease was calculated based upon epithelial architecture and inflammatory. A score between 0-4 was given for each category and summed together to give a total pathology score between 0-8. Additionally, the total number of ulcers were quantified per sample. All slides were read by a blinded pathologist.

Statistical Analysis

All results were presented as mean or mean ± SEM. Student t test was used to test for differences between the means of two groups. One-Way ANOVA was used to assess the differences between the means of three or more groups. Post-hoc pairwise comparisons were assessed if more than two groups were present in an experiment with adjustment for multiple comparisons using the Tukey method. When data was compiled from multiple experiments, Two-Way ANOVA assessing the treatment and experiment effects were conducted. We also conducted a post-hoc test of the statistical interaction assessing if the treatment effect differed across the experiments, but this was not significant ($p>0.05$). We reported our Two-Way ANOVA results without the statistical interaction. We also conducted pairwise comparisons between the means of the treatment and control groups at pre-specified time points adjusting for multiple comparisons with the Tukey method. For experiments with multiple time points comparing two treatments, multiple T test were

performed with each row analyzed individually without assuming a consistent SD, corrected for multiple comparisons using the Holm-Sidak method. All the statistical analysis was performed using Prism 8. Significance was set a priori at $p<0.05$.

Results

Propionate promotes the migration of IECs

To determine whether propionate stimulates epithelial migration, we used a classic *in vitro* wound healing model to monitor the movement of cells into a denuded area [98]. Mouse small intestinal epithelial cells (MSIE), an immortalized non-transformed epithelial cell line that retains properties of primary IECs [129], were used. MSIE cells treated with propionate enhanced cell movement into the denuded area over 20 hours (**Figure 3.1 A-B**). Acetate and butyrate also functioned similarly in promoting cell movement into the denuded area (**Figure 3.1 A-B**), which is consistent with previous reports [120]. To determine if proliferation was a driving factor behind the enhanced wound closure, MSIE cells were treated with acetate, propionate, and butyrate for 24 hours and stained for Ki67 to measure the proliferating cells. Cells treated with SCFAs did not affect proliferation as evidenced by no difference in Ki67 expression between SCFAs treated cells and untreated cells (**Figure 3.1 C-D**). To confirm the Ki67 data, MSIE cells were labeled with the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CSFSE) and treated for 24 hours. No differences were seen between control and SCFA treated cells, with most cells undergoing one division within 24 hours, which is similar to the average doubling time of MSIE cells **(Figure 3.1 E-F).** These data indicated that the ability for SCFA treated cells to fill the denuded area is due to cell migration but not proliferation. Next, to ensure that performing the migration experiments in serum-reduced media was not the reason for the difference between control and SCFA treated cells, we performed a migration experiment using propionate under various concentrations of FBS. Although cells at baseline migrated faster with higher concentrations of FBS, propionate was able to significantly enhance migration over control cells under each scenario (**Figure 3.1 G**). Finally, to ensure that SCFA

stimulates IEC migration occurred in different species, IEC18, a non-transformed rat intestinal epithelial cell line, and Caco2, a human colorectal cell line, were treated with or without propionate. Propionate treatment of both IEC18 and Caco2 enhanced cell migration (**Figure 3.2 A-D**). These data suggest that SCFAs promotion of IEC migration is conserved across several species.

Propionate promotes IEC spreading and polarization

Epithelial migration requires continuous actin remodeling to allow for extension and retraction of cell lamellipodia, filopodia, and adhesions [130,131]. This process is critical for epithelial turnover and wound healing [111,132]. To investigate whether propionate promotes actin remodeling, we pretreated MSIE cells for 16 hours with or without propionate. After 16 hours, cells were gently dissociated and re-plated onto matrigel coated plates for 30, 60, or 90 minutes, followed by actin staining and quantification for attachment and cell spreading. No differences were observed in cell attachment (**Figure 3.3 A-B**). However, there was a significant increase in cell size in MSIE cells treated with propionate, with many cells spreading rapidly by 60 minutes post plating (**Figure 3.3 C-D**). To investigate if propionate could enhance cell polarization, which is important for cell directionality [133], we pretreated MSIE cells with or without propionate for 16 hours before wounding the monolayers with a scratch. Two hours post scratch, cells were stained for golgin-97 to visualize the Golgi complex, which relocates between the nucleus and leading edge of polarized cells, and is indicative of cell polarization [133]. Propionate was able to increase the number of cells polarized along the wound margins, with many cells positioning the Golgi between the nucleus and forming lamellipodia as compared to controls in which Golgi staining was more often equally distributed perinuclear with smaller lamellipodia (**Figure**

3.3 E-F). Additionally, propionate treatment upregulated the expression of P21-associated kinase 1 (Pak1), a downstream target of CDC42, important for cell spreading and lamellipodia dynamics in MSIE cells [134], and milkfat-globule EGF 8 (Mfge8) in MSIE, which has also been shown to play a role in cell spreading with cells taking on a type II migration phenotype [110] (**Figure 3.4 A**). This finding was also extended to butyrate in MSIE cells **(Figure 3.4 B)**. Propionate treatment also upregulated MFGE8 but not PAK1 expression in enteroids (**Figure 3.4 C**). To determine whether propionate induces cell spreading and polarization through PAK1 and MFGE8, we included either anti-MFGE8 or the PAK1 inhibitor IPA-3 in scratch assays. However, neither neutralization of MFGE8 nor inhibition of PAK1 was able to decrease propionate-induced cell migration (**Figure 3.4 D-E**). Together, these data suggest that propionate could increase cell migration by promoting cell spreading and Golgi positioning independent of PAK1 and MFGE8.

Propionate promotes IEC speed and persistence

Appropriate cell migration is critical to the development and homeostasis of tissues [135]. It has been shown that there is a universal coupling between cell speed and cell persistence, and that faster cells move in straighter lines [136]. However, cell persistence can also be affected by the physical constraints of neighboring cells during sheet migration, which constantly remodel their junctions to uniformly migrate together [137]. To investigate whether SCFA treatment affects cell speed and persistence, we performed video microscopy. MSIE cells were treated with or without propionate and cell movement was recorded every 15 minutes for up 24 hours (**Movie S3.1**). Treatment with propionate enhanced cell migration (**Figure 3.5 A-B**), which was seen from approximately 8-12 hours post treatment of propionate (**Figure 3.5 A**). Cell motion was analyzed by tracking the

centroid position of cells that moved the furthest during the assay. Plot at origins revealed that cells treated with propionate moved further and straighter than non-treated cells (**Figure 3.5 C**). Analysis via DiPER showed a significant increase in mean-squared displacement (MSD), which has been shown to take into account both cell speed and persistence, when treated with propionate (**Figure 3.5 D**). To determine cell persistence, we calculated alpha values, indicative of more persistent movement toward the wounded area, by taking the log slope of the MSD curve. Alpha values range from 1 to 2, with 1 being completely random motion, and 2 indicating non-random directed motion. Propionate treatment increased alpha values (**Figure 3.5 E**). To confirm this finding, we also calculated cell autocorrelation, which measures persistence independent of speed by using only the angles of the vector tangential to the cells trajectory [100]. This calculation revealed increased cell persistence in response to propionate treatment (**Figure 3.5 F**). Lastly, we calculated cell speed by averaging the instantaneous speed of cells every 15 minutes. This revealed that propionate treatment significantly increased cell speed (**Figure 3.5 G**). Taken together, this data indicates that by promoting cell spreading and polarization, propionate treatment increases both cell speed and persistence, which are critical for enhanced cell movement.

HDAC inhibition and GPR43 mediate the effects of propionate on IEC migration

It has been shown that SCFAs function through binding their receptors GPR41, GPR43, and GPR109, and through inhibition of histone-deacetylase (HDACi) [17,25,138–140]. HDAC inhibitors (HDACi) are global regulators of gene transcription due to their ability to module histones [26]. HDACi have been shown to stimulate cell migration through a TGFβ-dependent pathway and enhance wound healing *in vivo* [141]. Additionally, GPR43 is known

to promote neutrophil migration into the gut during inflammation [57]. To determine whether HDAC inhibition and GPR stimulation mediate propionate induction of IEC migration, we treated MSIE cells with the global HDACi trichostatin A to mimic the HDAC inhibitory function of propionate, as well as ligands for GPR41 and GPR43, the receptors for propionate. We found that trichostatin A was able to significantly enhance MSIE cell migration, with GPR43 agonist also playing a role and GPR41 having an inhibitory effect (**Figure 3.6 A-B**). HDACs are divided into 3 classes, type I, IIA, and IIB. Next, we investigated the specificity of propionate-mediated HDACi in MSIE cells. To determine which HDACs were important for MSIE migration, we performed an HDACi screen with various inhibitors specific for different classes of HDAC, including TMP195a (a class IIa inhibitor), SBHA (a HDAC1 and HDAC3 inhibitor), 1-napthohydroxamic acid (a HDAC8, HDAC1, HDAC6 inhibitor), RGFP966 (a HDAC3 inhibitor), and valproate (an inhibitor of Class I HDAC and proteasomal degradation of HDAC2) [74]. We found that only valproate was able to recapitulate the effects seen with propionate (**Figure 3.6 C**). Next, we investigated the role of TGFβ in this process, as HDACi have been shown to promote TGFβ expression and TGFβ dependent cell migration [91,141]. We confirmed that propionate increased TGFβ expression in MSIE cells (**Figure 3.6 D**) and enteroids (**Figure 3.6 E**) as previously reported [142]. We then performed a scratch assay with propionate in the presence of a TGFβ neutralizing antibody. However, TGFβ neutralization had little effect on migrating MSIE cells treated with propionate (**Figure 3.6 F**).

To determine whether valproate could enhance both cell speed and persistence in a similar manner to propionate, we performed video microscopy with MSIE cells and analyzed cell

motion in response to valproate treatment (**Movie S3.2**). Valproate treatment was able to enhance cell migration similar to propionate treatment (**Figure 3.7 A and B**). However, valproate-treated cells did not close the wound gap as fast as propionate treated cells with a difference occurring with propionate-treated cells between 8-12 hours versus 12-14 hours with valproate (**Figure 3.7 A**). Plot at origins showed that propionate and valproate increased the distance and straightness of cell migration (**Figure 3.7 C**). Plotting MSD showed that both propionate and valproate increased displacement but to different extents (**Figure 3.7 D**). Analysis of cell persistence by calculating alpha values revealed that both propionate and valproate increased cell persistence, albeit to different degrees (**Figure 3.7 E**), whereas autocorrelation found that both valproate and propionate enhanced cell persistence to a similar extent (**Figure 3.7 F**). Furthermore, both treatments with propionate and valproate enhanced cell speed (**Figure 3.7 G**). Together, this data indicates that class I HDACi can recapitulate many of the effects of propionate-induced IEC migration, and that this mechanism is independent of TGFβ.

STAT3 is critical for propionate induction of cell persistence

SCFAs are known to act as an energy source for intestinal epithelial cells [140]. Additionally, SCFAs affect cell metabolism of epithelial cells by promoting oxidative phosphorylation [41,143]. To investigate whether propionate affects IEC metabolism, MSIE cells or enteroids monolayers were treated with or without propionate for 8 hours and subjected to an extracellular flux Seahorse analyzer to measure the oxygen consumption rate (OCR), which is primarily attributed to mitochondrial oxidation, and the extracellular acidification rate (ECAR) that represents glycolysis. There were no significant differences in oxygen consumption or extracellular acidification rate, indicating that propionate at this early time

point did not affect cell metabolism (**Figure 3.8 A-D**). To verify this result, we performed scratch assays in the presence of various metabolic inhibitors for glycolysis and mitochondrial oxidation, including etomoxir (a fatty acid oxidation inhibitor), 6-Diazo-5-oxo-L-norleucine (DON, a glutamine inhibitor), Oligomycin (a complex 5 inhibitor), metformin (a TCA inhibitor), and 2-deoxy glucose (a glycolysis inhibitor). All metabolic inhibitors tested had no effect on propionate-induced migration (**Figure 3.8 E-F**). We have previously shown that SCFAs in a GPR43 dependent manner promoted activation of STAT3, mammalian target of rapamycin (mTOR), and mitogen activated-protein kinase (MEK) [122]. Because GPR43 agonist increased cell migration of MSIE (**Figure 3.6 B**), we investigated whether any of these pathways had an effect on propionate-induced IEC migration. We performed a screen using U0126 (a MEK inhibitor), HJC0152 and Stattic (STAT3 inhibitors), and rapamycin (a mTOR inhibitor) with or without propionate. We found that both Stattic and HJC0152, but not other inhibitors, attenuated propionates ability to induce migration (**Figure 3.8 G**). To examine the effects of STAT3 on propionate-induced migration, we performed video microscopy with WT and STAT3 KO MSIE cells as we previously described [144], in the presence or absence of propionate (**Movie S3.3**). Interestingly, deficiency in STAT3 had no effect on total migration distance in response to propionate treatment (**Figure 3.9 A-B**). However, further analysis revealed major differences between WT and STAT3 KO MSIE cells. Plots at origins revealed that although propionate treated STAT3 KO cells moved a similar distance as propionate treated WT cells, the movement was more random (**Figure 3.9 C**). During migration experiments, the leader cells at the front edge of the wound margins in STAT3 KO cells tended to separate from the rest of the monolayer (**Figure 3.9 A**). To confirm this finding,

we performed phalloidin staining for F-actin. Propionate treatment promoted sheet migration in WT cells, but often led to dissociation of the epithelial sheet in STAT3 KO cells (**Figure 3.9 D**). Plotting MSD confirmed similarities in the total migration distance, as there was no difference between propionate treated WT and STAT3 KO cells (**Figure 3.9 E**). However, calculating alpha values indicated large differences in cell persistence between propionate treated WT and STAT3 KO cells (**Figure 3.8 H**), which was further confirmed by autocorrelation (**Figure 3.9 F**). Furthermore, although propionate significantly increased cell speed over WT cells, propionate treatment further enhanced STAT3 KO cell speed (**Figure 3.9 G**). Collectively, this data indicated that STAT3 is critical for propionate-induced directional persistence, but is dispensable for the enhanced speed seen with propionate treatment. This is important as it has been shown that conditional STAT3 KO in IEC leads to severe experimental colitis and aberrant wound healing [145].

Propionate stimulates migration of IECs in vivo

It has been shown that SCFAs are pertinent for epithelial turnover [86]. However, until recently, it was not known that epithelial cells actively migrate up the villus independent of crypt proliferation [109]. To quantitatively assess whether propionate could stimulate migration of cells up the crypt villus axis *in vivo*, we employed a protocol as previously reported using hydroxyurea [111]. WT mice were fed a cocktail of antibiotics including vancomycin, metronidazole, ampicillin, and kanamycin for 10 days as we previously described [79]. After ten days, mice were continuedly fed the antibiotic cocktail with or with 200 mM propionate for an additional 21 days. All groups of mice were intraperitoneally injected with 100 mg/kg of BrdU. Thirty-six hours post injection, some of the mice were

sacrificed and used for baseline measurements of epithelial migration. The remaining mice received an injection of 50 mg/kg hydroxyurea to halt crypt proliferation and sacrificed 12 hours later (**Figure 3.10 A**). Thirty-six and forty-eight hour time points were used based on data from a previous report which found that proliferation in the crypts affects villus migration the least when cells were at the midway point of the villus [111]. To further protect against the effect of proliferation, low dose hydroxyurea was also used. We found that the BrdU front in control and propionate treated mice moved a similar distance 36 hours post injection (**Figure 3.10 B-C**). However, by 48 hours post BrdU injection, the BrdU front in propionate treated mice was significantly increased compared to control mice, with propionate treated cells moving an average of 73.6 μm (95% CI 49.61-97.71 μm) compared to 29.3 μm (95% CI 3.32-55.28 μm) in controls (**Figure 3.10 B-C**). Taken together, these data indicate that propionate stimulates the migration of IECs along the villus independent of proliferation.

Propionate reduces ulceration in experimental colitis

Finally, we examined if propionate could protect mice against the development of colitis in experimental colitis upon dextran sulfate sodium (DSS) insult (**Figure 3.11 A**). WT mice were pretreated for 7 days with or without 200mM propionate in their drinking water. Then mice were given DSS containing water with or without 200mM propionate for 7 days before being switched to regular drinking water with or without propionate for an additional 3 days. Mice were monitored daily for weight changes. Mice were sacrificed 10 days post administration of DSS. There were no differences in weight loss between either groups **(Figure 3.11 B)**. However, propionate treated mice had a significant reduction in ulcer development in the colon (**Figure 3.11 C and D**). There were no differences in inflammatory cytokine production, including TNFα, IL-17, IFNγ, and IL-6, between

control and propionate treated mice (**Figure 3.11 E**). These results indicate that propionate mainly affects intestinal epithelial cell migration and wound healing but not inflammatory responses.

Discussion

SCFAs are the primary gut bacterial products of fermentation of soluble fibers contributed from foods such as fruits, vegetables, and grains, which are known to be protective against colorectal cancer and potentially beneficial for patients with inflammatory disorders such as IBD [42,80,81,146]. However, the mechanisms by which they protect the intestinal epithelium are still being unraveled. In this study, we found that propionate and other SCFAs stimulated IEC migration that was independent of cell proliferation. The propionate-enhanced IEC migration was collective rather than single cell in nature, which is pertinent for processes such as epithelial turnover and wound healing, where neighboring junctions with other cells remain intact [135,137]. Importantly, propionate drives intestinal epithelial migration through regulating cell speed and persistence in a HDAC inhibition, GPR43, and STAT3 dependent manner.

It has been shown that migrating cells require three processes for effective migration including attachment, polarization, and actin polymerization. In the gut, actin polymerization was shown to be a major driving factor for cell migration on the villus [111]. We found that propionate upregulated genes involved with actin reorganization such as Pak1 and Mfge8 [94,110,147–149]. However, although propionate drives cell spreading and polarization without affecting attachment, this process was independent of both Pak1 and MFGE8 despite these proteins being linked to this process. These differences suggest that each of these genes is dispensable for this function possibly via the redundant actions of downstream proteins. Using video microscopy, we found that propionate enhanced both cell speed and persistence, most likely due to its ability to increase cell spreading and

enhance polarization which is pertinent for both cell speed and directionality. Our findings, thus, support the argument that both cell speed and persistence are linked for cell migration [136].

Propionate exerts its actions on cells via G-coupled protein receptors and by acting as an inhibitor for HDAC [17,138]. Here we found that HDAC inhibition and GPR43 mediated propionate-induced cell speed and persistence. This indicates that modulation of gene transcription is most likely pertinent to propionate-induced migration, although we were not able to identify a single gene that contributed to this process. We also found that inhibition of Class I HDACs via valproate could recapitulate the effects of propionate on cell migration. Valproate has been shown to be beneficial in ameliorating colitis in experimental colitis, which raises the possibility that valproate is stimulating epithelial migration to allow for recovery in these mice [150]. Additionally, our finding is in line with a previous report, which found that the HDAC inhibition could stimulate the migration of intestinal epithelial cells [141]. However, in our model, TGFβ was not responsible for the induction of migration. This is most likely due to the duration of the migration experiment. Epithelial restitution is a fast process, occurring within 24-48 hours. Thus we chose to assay over a shorter time period, whereas the previous report chose to assess migration over 6 days, where TGFβ secretion into the supernatant would most likely have a major impact on migration as is well known [91].

SCFAs are known to alter the metabolism of IEC driving them toward oxidative phosphorylation and β-oxidation [14,41,140]. In our study, we found that propionate was unable

to change the metabolic activity in IECs in the early stages. We assessed metabolic activity 8 hours post treatment with propionate, whereas other groups in previous reports have examined this effect at 24 hours with SCFAs such as butyrate [41,143]. Thus, the difference in incubation, SCFA used, and dosage could be a significant factor affecting the results. However, this time point was used due to the significant changes in cell migration between non-treated and propionate at the 8-12 hour time frame. Additionally, the use of etomixir, a β-oxidation inhibitor, alongside propionate, further confirmed that propionate was not being metabolized at this crucial time frame, but rather exerting its functions via alteration of gene expression. However, our data does not exclude a possible metabolic regulation of IECs for SCFAs at later stages of tissue repair.

SCFAs have been shown to activate many enzymes including MEK, mTOR, and STAT3 to exert their functions [122]. We found that inhibition of STAT3, but not MEK and mTOR, could affect propionate-induced cell persistence. This is consistent with a previous report, which found that STAT3 was important for cell persistence in fibroblast, with STAT3 KO cells unable to effectively polarize and move collectively [151]. Furthermore, we found that STAT3 KO IEC dissociated from one another and moved individually instead of as a collective sheet. Thus, although propionate-treated STAT3 KO IEC moved as far as propionate-treated WT IEC, their migration was disorganized. This is critical as collective migration is required for epithelium tissue organization and wound healing. This is in line with a previous report that Vil^{Cre} $Stat3^{fl/fl}$ mice, in which STAT3 is deficient specifically in IEC, have defective wound healing with acute DSS injury leading to severe disease and aberrant wound healing [145].

Given that propionate was able to induce cell migration *in vitro* via promoting cell spreading and polarization, we assessed its ability to promote migration *in vivo*. To eliminate the effect of existing bacteria produced propionate in the intestinal lumen, we treated mice with an antibiotic cocktail. BrdU time points of 36 and 48 hours post injection were used to minimize the effect of cell proliferation on migration as found in a previous report [111]. Additionally, to further reduce the proliferative effect, we injected mice with hydroxyurea, an S phase inhibitor. We found that propionate induced migration of cells on the villus. This finding suggest that propionate is a major regulator of epithelial turnover, enhancing both cell proliferation and cell migration, which would allow the coordinated renewal of the intestinal epithelium. Of note, we did not find that propionate significantly enhanced proliferation at the 36 hour time point as previously reported [86]. However, our finding does not contradict theirs, as they assess proliferation at a 48 hour time point and used a cocktail of SCFAs, rather than a single SCFA propionate as in our study. Based on our findings that propionate induces IEC migration, and previous results indicating that HDAC inhibition ameliorated colitis [141,150], we expected that propionate would protect against colitis. Indeed, our results demonstrated that propionate was able to reduce ulceration of the epithelium.

In summary, we demonstrate that propionate promotes epithelial migration to drive epithelial turnover and repair, which depends on HDAC inhibition of class I HDACs, GPR43, and activation of STAT3, allowing for collective cell migration. This suggest that at the base of the crypt, where HDAC inhibitory activity is low, SCFAs are able to promote

stem cell proliferation [40]. However, as SCFA concentrations rise as cells move out of the crypt, HDAC inhibition enhances cell migration by promoting cell spreading to coordinate the movement of cells out of the crypt with active migration of cells up the villus and eventual cell extrusion into the lumen. Our findings thus suggest a novel mechanism for how SCFAs contribute to intestinal homeostasis.

Figures

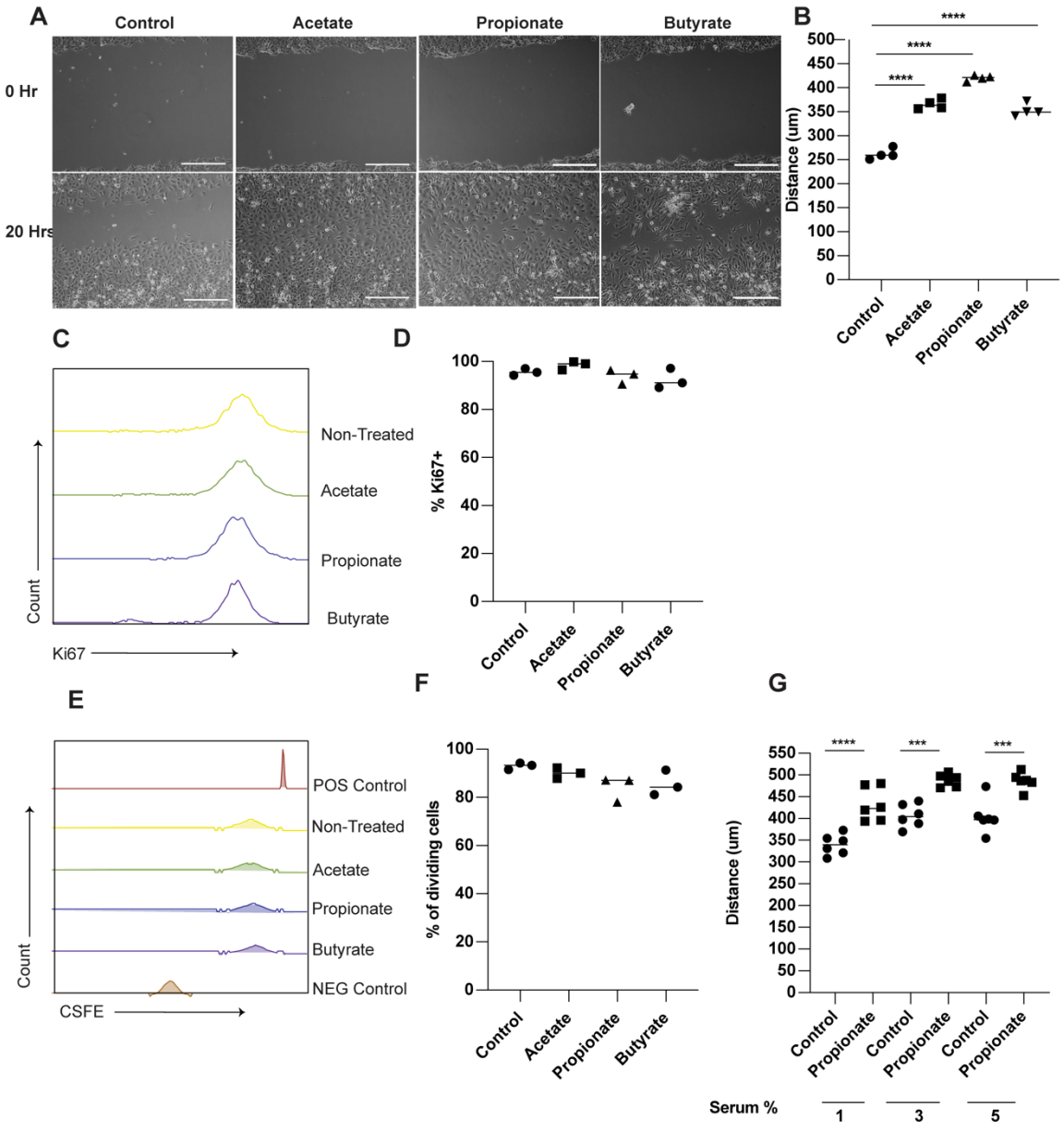

Figure 3.1: Propionate promotes the migration of intestinal epithelial cells.

(A-B) MSIE cells were wounded and treated with SCFAs. (A) Representative phase contrast images of MSIE cells, scale bars 300 μm. (B) Quantification of average migration distance of n=3 samples per treatment. (C-D) MSIE cells were treated with

SCFAs, stained for Ki67, and analyzed via flow cytometry. (C) Representative graphs and (D) quantification of Ki67 from n=3 samples per treatment. (E-F) MSIE cells were labeled with CSFE, treated with SCFAs, and analyzed via flow cytometry. (E) Representative graphs and (F) quantification of proliferation from n=3 samples per treatment. (G) MSIE cells were wounded and treated with propionate in increasing concentrations of FBS. Quantification of average migration with n=6 samples per treatment. *P<.05, ***P<.001 ****P<.0001 by one-way ANOVA with Tukey's post-test.

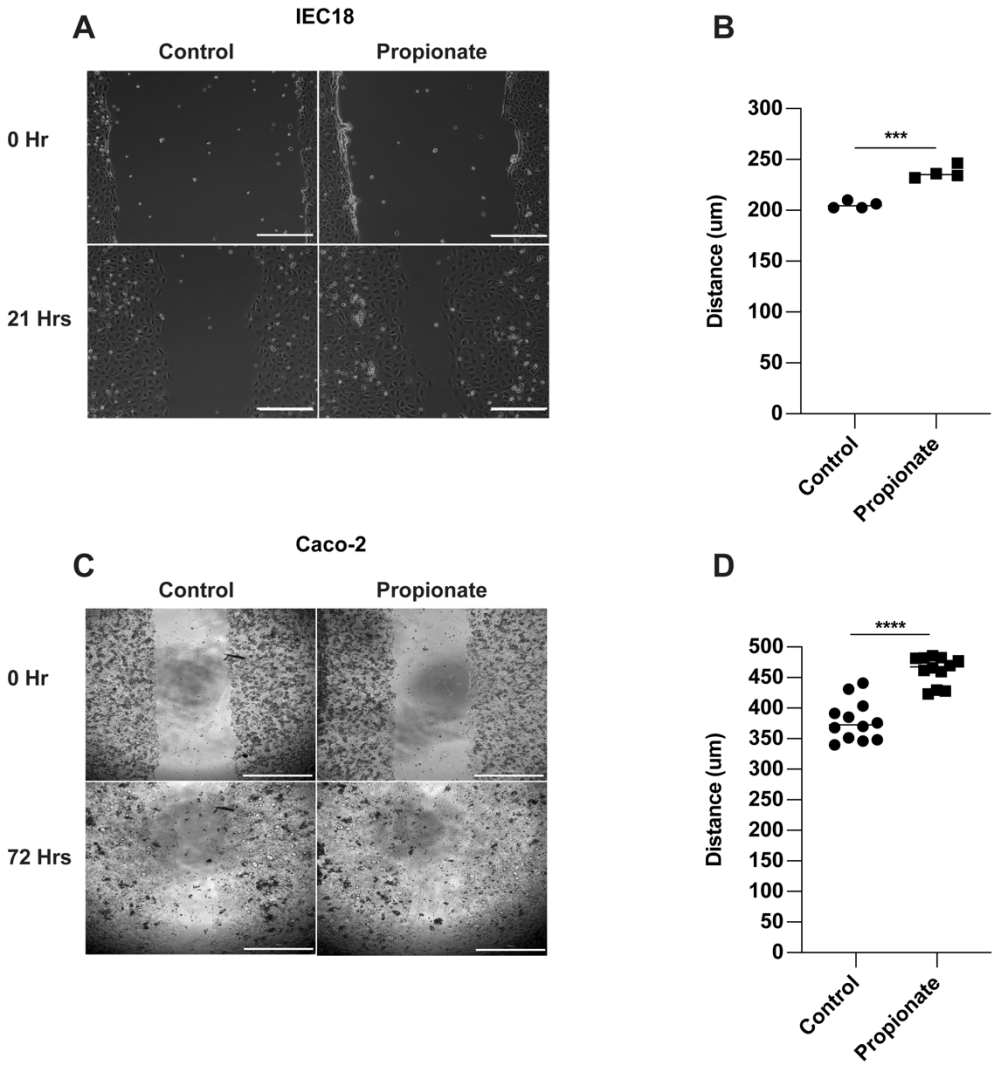

Figure 3.2: Propionate promotes the migration of rat and human epithelial cells. (A-B) IEC-18 cells were wounded and treated with propionate. (A) Representative phase contrast images, scale bars 300 μm. (B) Quantification of average migration distance with n=4 samples per treatment. (C-D) Caco-2 cells were wounded and treated with propionate. (D) Representative phase contrast images, scale bars 1000 μm. (D)

Quantification of average migration distance with n=12 samples per treatment group. ***P<.001, ****P<.0001 by two tailed student's T-test.

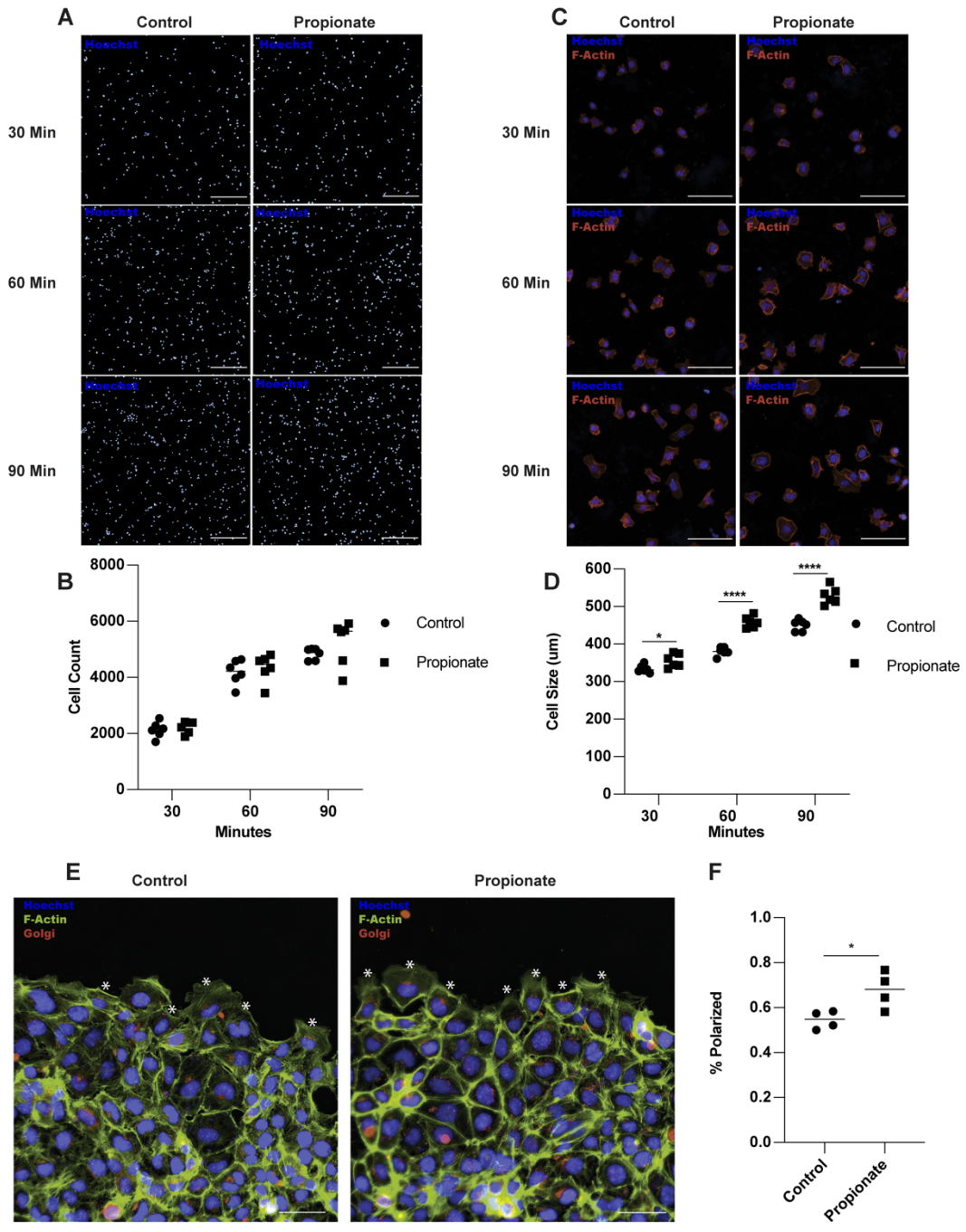

Figure 3.3: Propionate promotes IEC spreading and polarization.

(A-D) MSIE were pretreated with propionate for 16 hours and then re-plated onto matrigel coated plates for 30, 60, or 90 minutes to assess attachment and spreading. (A)

Representative images of cells stained for Hoechst (blue), scale bars 300 μm. (B) Quantification of cell attachment with n=6 samples per treatment. (C) Representative images of the cells stained for phalloidin (red) or Hoechst (blue), scale bars, 20 μm. (D) Quantification of average cell area per sample with n=6 samples per treatment. (E-F) MSIE were pretreated with propionate for 16 hours, wounded, and stained for phalloidin (green), golgin-97 (red), and Hoechst (blue) two hours post wounding. (E) Representative immunofluorescent images with (*) indicating polarized cells, scale bars 100 μm. (F) Quantification of cell polarization with n=4 samples per treatment. *P<.05, **P<.01, ***P<.001, ****P<.0001 by one-way ANOVA with Tukey's post-test for groups of three or more and by student's T-test for groups of two.

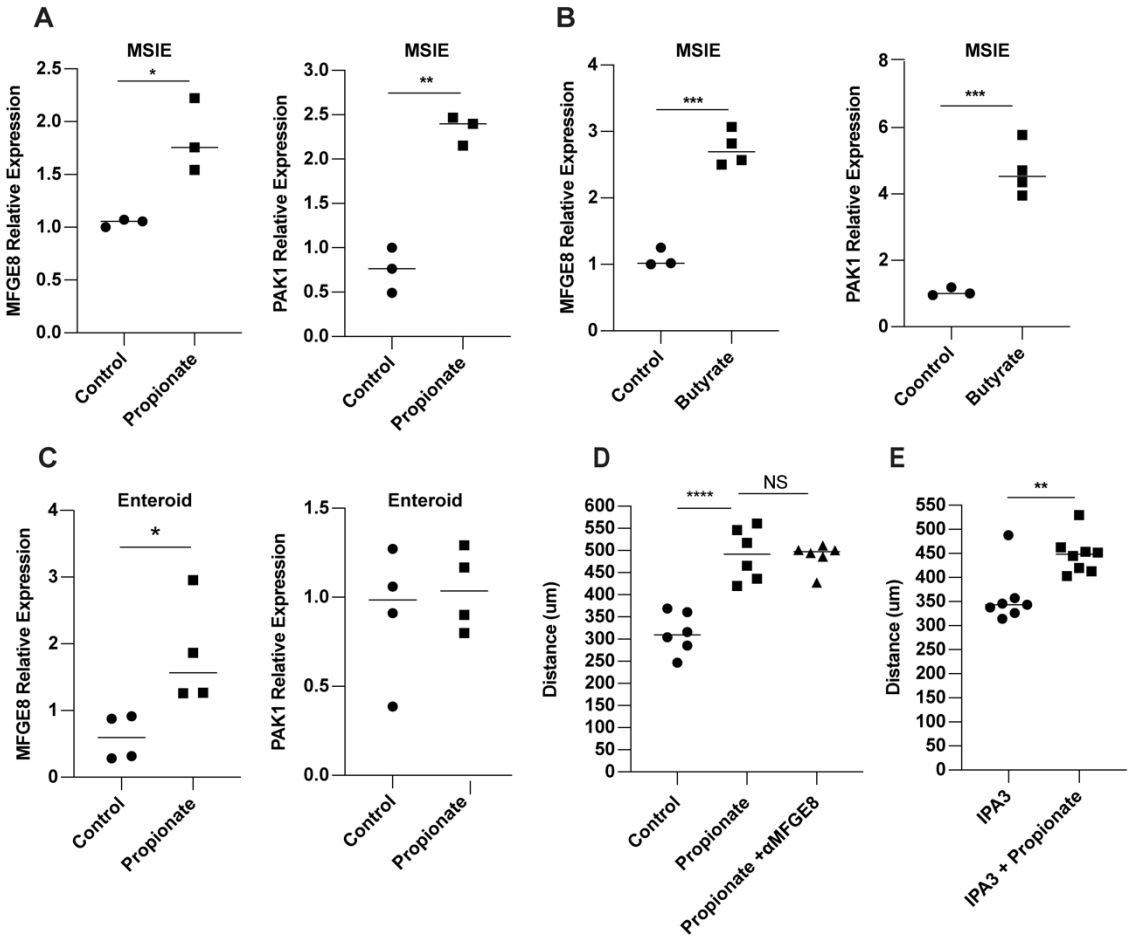

Figure 3.4: Propionate induced migration is independent of MFGE8 and PAK1.

(A-B) MSIE cells were treated for 24 hours with propionate or butyrate and analyzed for mRNA expression of MFGE8 and PAK1. (C) Jejunal enteroids were treated for 24 hours with propionate and analyzed for mRNA expression of MFGE8 and PAK1. Relative expression of MFGE8 and PAK1 with n = 3 to 4 samples per treatment group. (D-E) MSIE cells wounded and treated with propionate with or without inhibitors. (D) Quantification of average migration distance of cells treated with MFGE8 neutralizing antibody with n=7 samples per treatment. (E) Quantification of average migration distance of cells treated with the PAK1 inhibitor IPA3 with n=7 samples per treatment.

**P<.01, ***P<.001, ****P<.0001 by one-way ANOVA with Tukey's post-test for groups of three or more and by Student's T test for groups of two.

Movie S3.1: Propionate promotes IEC cell speed and persistence.

MSIE cells were wounded and treated with the SCFA propionate and video microscopy was performed with images taken every 15 minutes. Representative phase contrast images of MSIE cells taken every 15 minutes and stacked into a movie. Scale bars 300 μm.

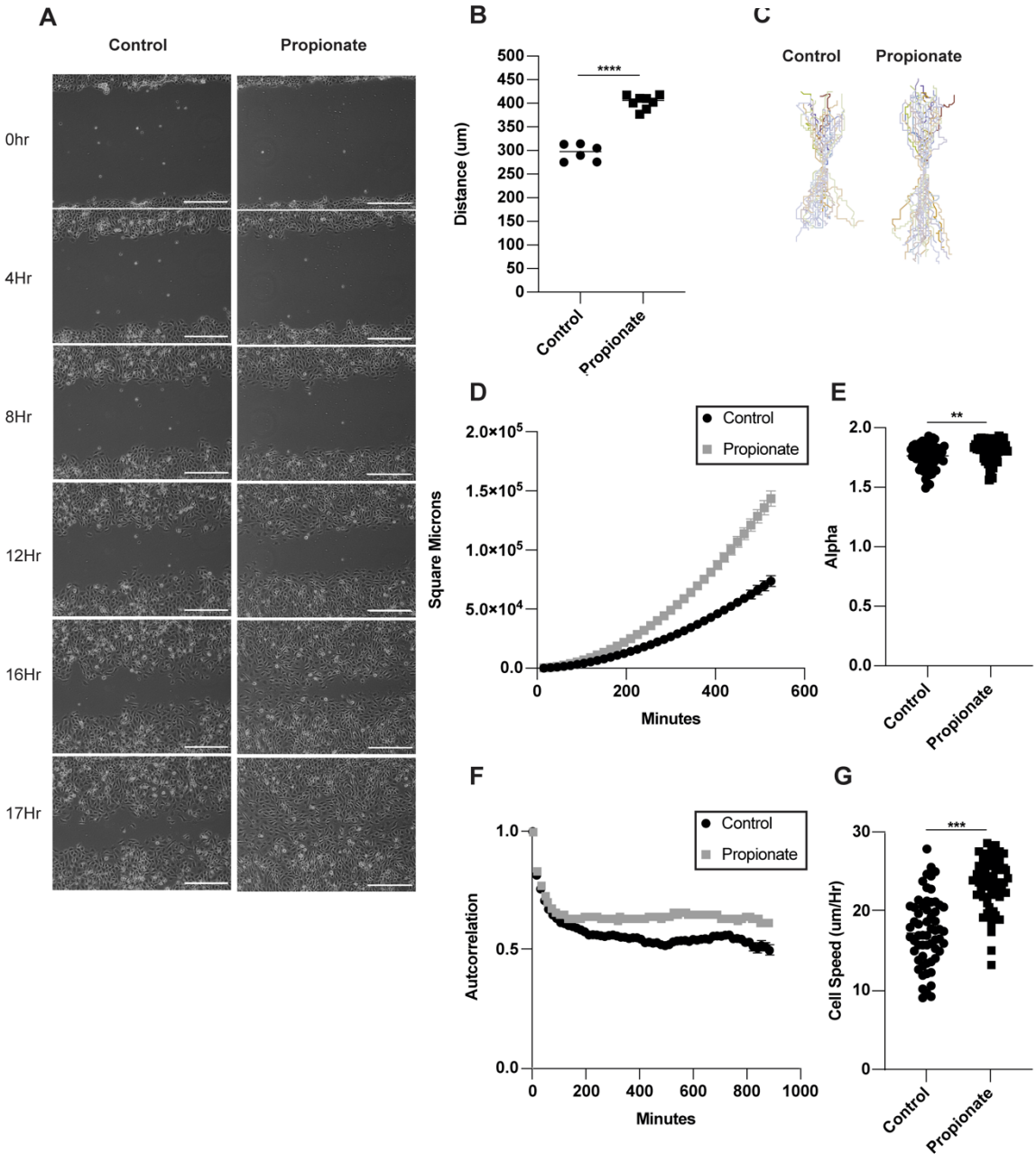

Figure 3.5: Propionate promotes IEC speed and persistence.

(A-G) MSIE cells were wounded and video microscopy was performed. Videos were analyzed by tracking the centroid position of 15-20 cells per sample that moved the furthest during the assay. (A) Representative phase contrast images of MSIE cells at 0, 4, 8, 12, 16, and 17 hours. Scale bars, 300 μm. (B) Quantification of average migration

distance with n=5-6 samples per treatment. (C) Plot at origin graphs of n=58 cells from n=3 samples per treatment. (D) Mean Squared Displacement (MSD) of n=58 cells from n=3 samples per treatment. (E) Alpha values of n=58 cells from n=3 samples per treatment. (F) Autocorrelation of cells over time representative of n=58 cells from n=3 samples per treatment. (G) Average cell speed per hour of n=58 cell from n=3 samples per treatment. **P<.01, ***P<.001, ****P<.0001 by one-way ANOVA with Tukey's post-test for groups of three or more and by Student's T test for groups of two.

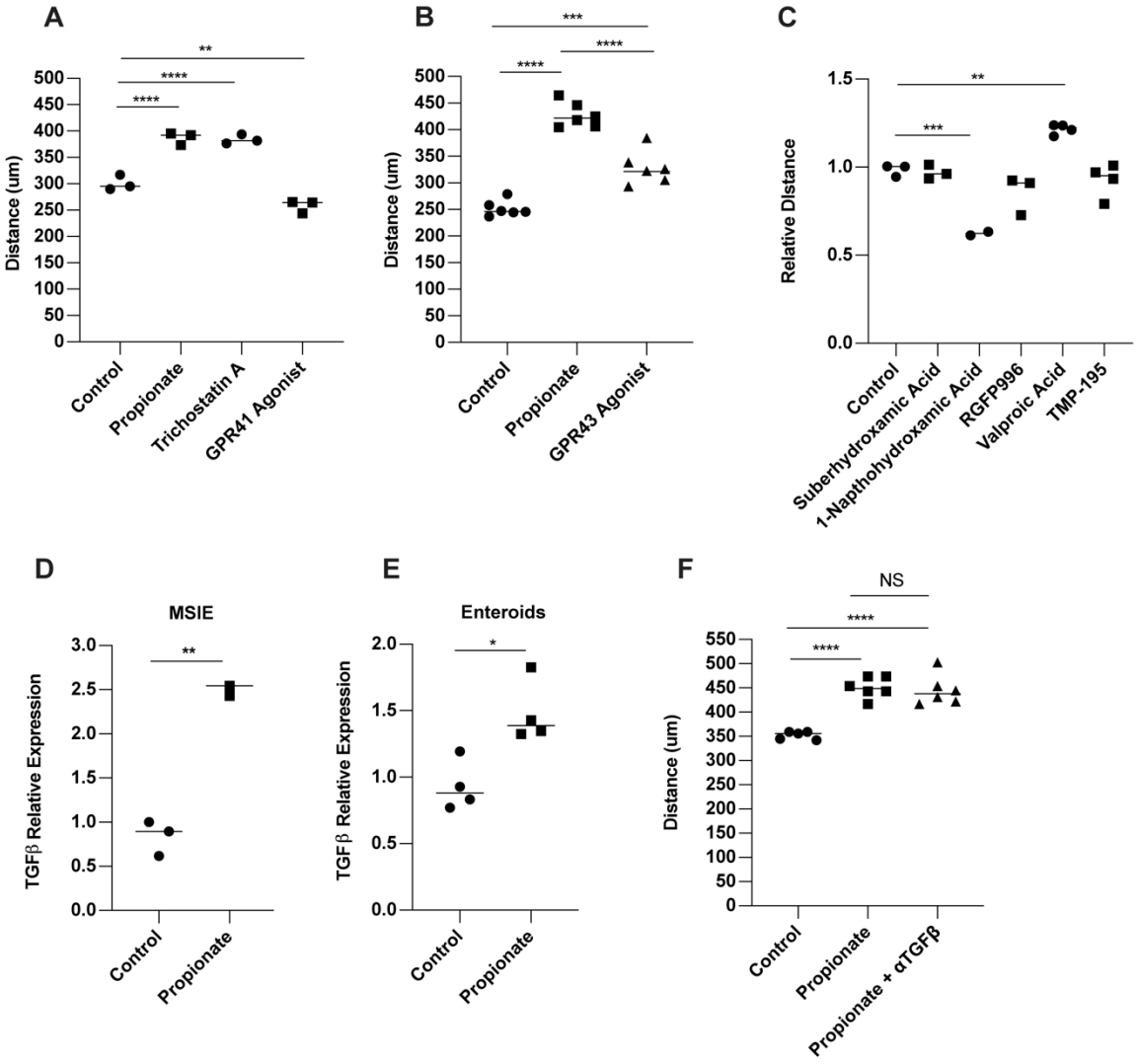

Figure 3.6: GPR43 and inhibition of Class I HDAC mediate propionate promotion of IEC migration.

(A-C and E) MSIE cells were wounded, then cultured with propionate, HDAC inhibitors, or anti-TGFβ antibody. Quantification of average migration distance with (A) propionate, trichostatin A, and GPR41 agonist with n=3 per treatment, (B) propionate and GPR43 agonist with n=6 per treatment, (C) HDAC inhibitors with n=2-3 per treatment. (D) MSIE cells or (E) jejunal enteroids were treated for 24 hours with propionate and

analyzed for mRNA expression. Relative expression of TGFβ with n=3 samples per treatment group. (F) Quantification of average migration distance with propionate with or without anti-TGFβ antibody. *P<.05, **P<.01, ***P<.001, ****P<.0001 by one-way ANOVA with Tukey's post-test for groups of three or more and by Student's T test for groups of two.

Movie S3.2: Inhibition of HDAC mediates the effects of propionate on IEC migration.

MSIE cells were wounded and treated with the SCFA propionate or valproate and video microscopy was performed with images taken every 15 minutes. Representative phase contrast images of MSIE cells taken every 15 minutes and stacked into a movie. Scale bars 300 μm.

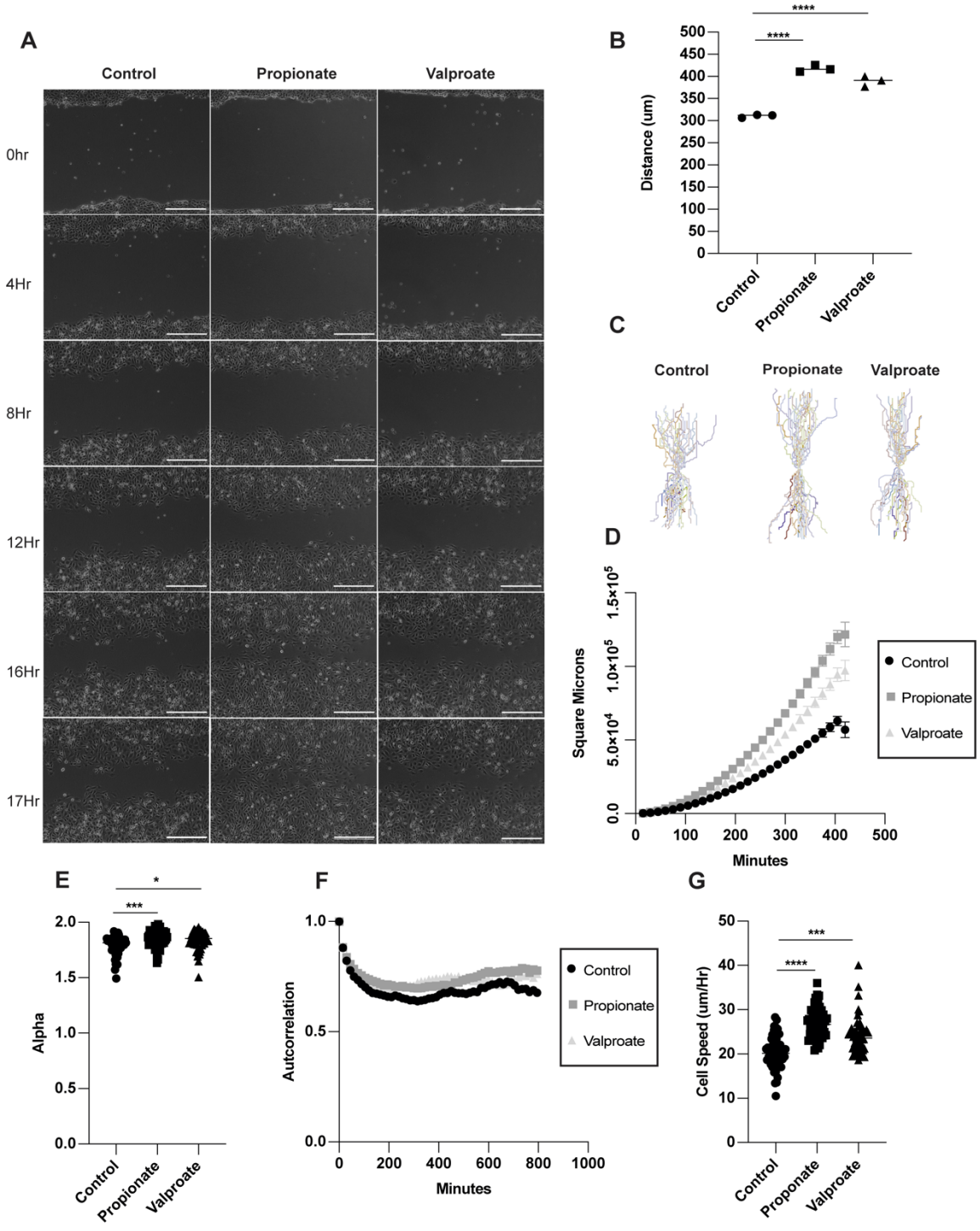

Figure 3.9: STAT3 is critical for propionate induction of cell persistence.

WT and STAT3 KO MSIE cells were wounded and video microscopy was performed. Videos were analyzed by tracking the centroid position of 15-20 cells per sample that moved the furthest during the assay. (A) Representative phase contrast images of MSIE cells at 0, 4, 8, 12, 16, and 17 hours. Scale bars, 300 μm. (B) Quantification of average migration distance with n = 4 samples per treatment. (C) Plot at origin graphs of n=45, 48, and 47 cells for control, propionate, or STAT3 KO treated samples from n=3 samples per treatment. (D) MSIE cells were wounded and phalloidin (green) and Hoechst (blue) staining was performed 16 hours post wounding, scale bars 100um. (E) Mean Squared Displacement (MSD) of n=45, 48, and 47 cells for control, propionate, or STAT3 KO treated samples from n=3 samples per treatment. (F) Autocorrelation of cells over time representative of n=45, 48, and 47 cells for control, propionate, or STAT3 KO treated samples from n=3 samples per treatment. (G) Average cell speed per hour of n=45, 48, and 47 cells for control, propionate, or STAT3 KO treated samples from n=3 samples per treatment. **P<.01, ***P<.001, ****P<.0001 by one-way ANOVA with Tukey's post-test for groups of three or more and by Student's T test for groups of two.

Movie S3.3: STAT3 is critical for propionate induction of cell persistence.

Puromycin control and STAT3 KO cells were wounded and treated with the SCFA propionate and video microscopy was performed with images taken every 15 minutes. Representative phase contrast images of MSIE cells taken every 15 minutes and stacked into a movie. Scale bars 300 μm.

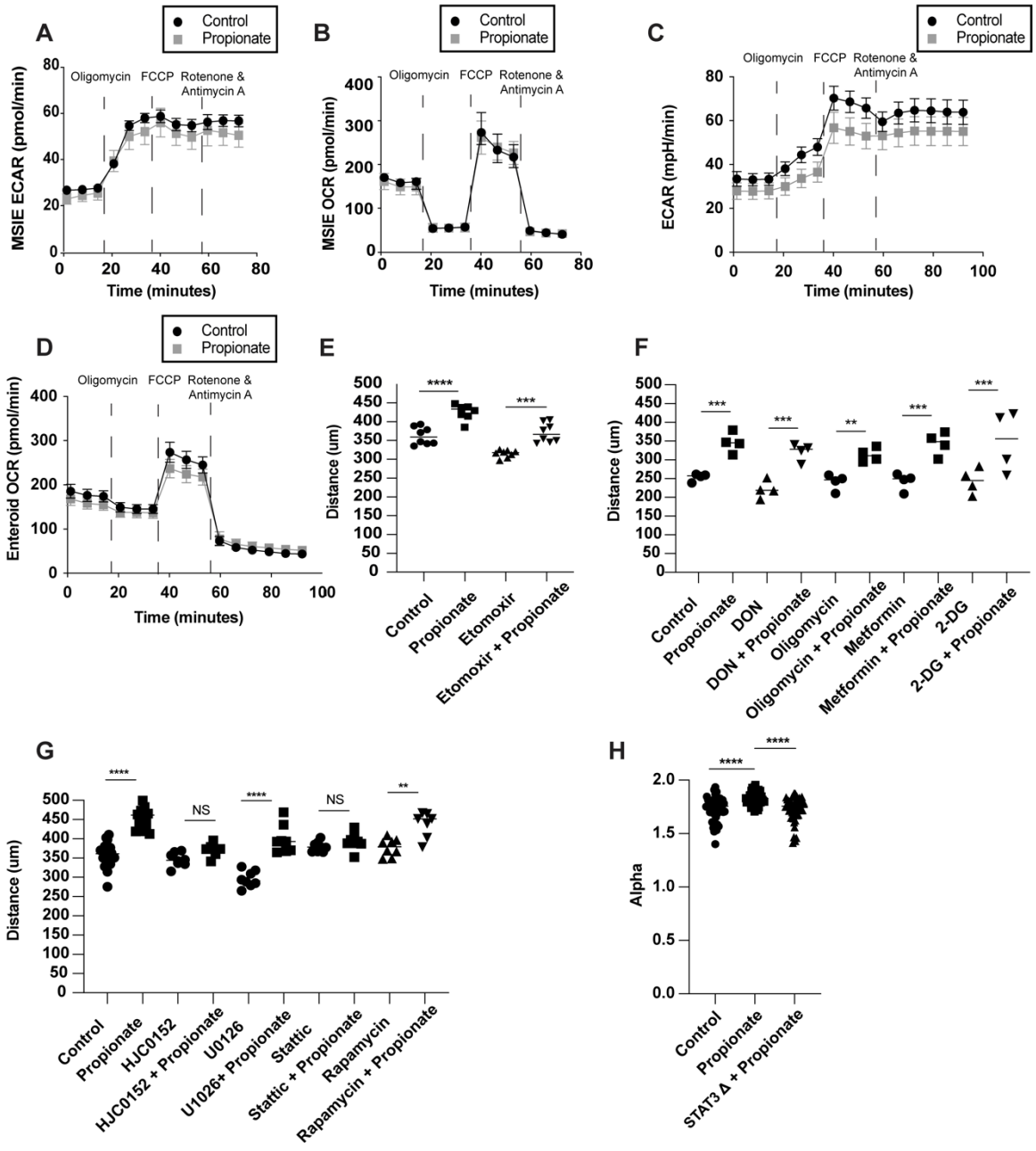

Figure 3.8: Propionate induced migration is independent of metabolism and dependent on STAT3.

(A-B) MSIE cells or (C-D) jejunal enteroids monolayers were treated for 8 hours with propionate followed by a mito-stress test. (A,C) Extracellular acidification rate (ECAR) and (B,D) Oxygen consumption rate (OCR) over time with n=3 samples per treatment for

(A,B) and n=4-6 samples per treatment for (C,D). MSIE cells were wounded and treated with propionate with or without etomoxir (E) or 6-Diazo-5-oxo-L-norleucine (DON), oligomycin, Metformin, 2-deoxyglucose (2-DG) (F). Quantification of average migration distance with n=3-8 samples per treatment group. (G) Quantification of average migration distance of MSIE cells treated with propionate with or without HJC0152, U0126, Stattic, or Rapamycin with n=3 or more samples per treatment. (H) Alpha values of n=45, 48, and 47 cells for control, propionate, or STAT3 KO treated samples from n=3 samples per treatment. *P<.05, **P<.01, ***P<.001, ****P<.0001 by one-way ANOVA with Tukey's post-test for groups of three or more and by Student's T test for groups of two.

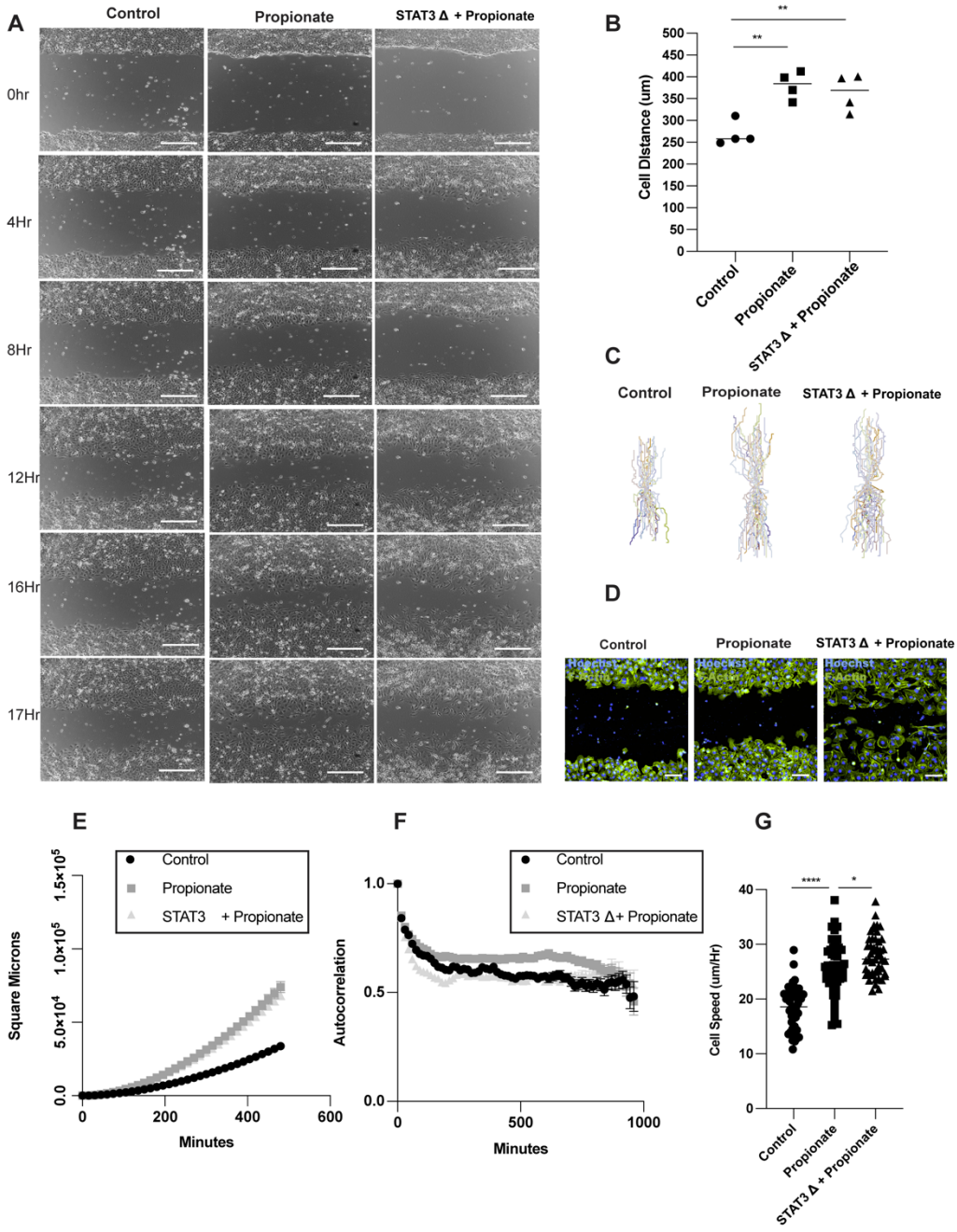

Figure 3.9: STAT3 is critical for propionate induction of cell persistence.

WT and STAT3 KO MSIE cells were wounded and video microscopy was performed. Videos were analyzed by tracking the centroid position of 15-20 cells per sample that moved the furthest during the assay. (A) Representative phase contrast images of MSIE cells at 0, 4, 8, 12, 16, and 17 hours. Scale bars, 300 μm. (B) Quantification of average

migration distance with n = 4 samples per treatment. (C) Plot at origin graphs of n=45, 48, and 47 cells for control, propionate, or STAT3 KO treated samples from n=3 samples per treatment. (D) MSIE cells were wounded and phalloidin (green) and Hoechst (blue) staining was performed 16 hours post wounding, scale bars 100um. (E) Mean Squared Displacement (MSD) of n=45, 48, and 47 cells for control, propionate, or STAT3 KO treated samples from n=3 samples per treatment. (F) Autocorrelation of cells over time representative of n=45, 48, and 47 cells for control, propionate, or STAT3 KO treated samples from n=3 samples per treatment. (G) Average cell speed per hour of n=45, 48, and 47 cells for control, propionate, or STAT3 KO treated samples from n=3 samples per treatment. **P<.01, ***P<.001, ****P<.0001 by one-way ANOVA with Tukey's post-test for groups of three or more and by Student's T test for groups of two.

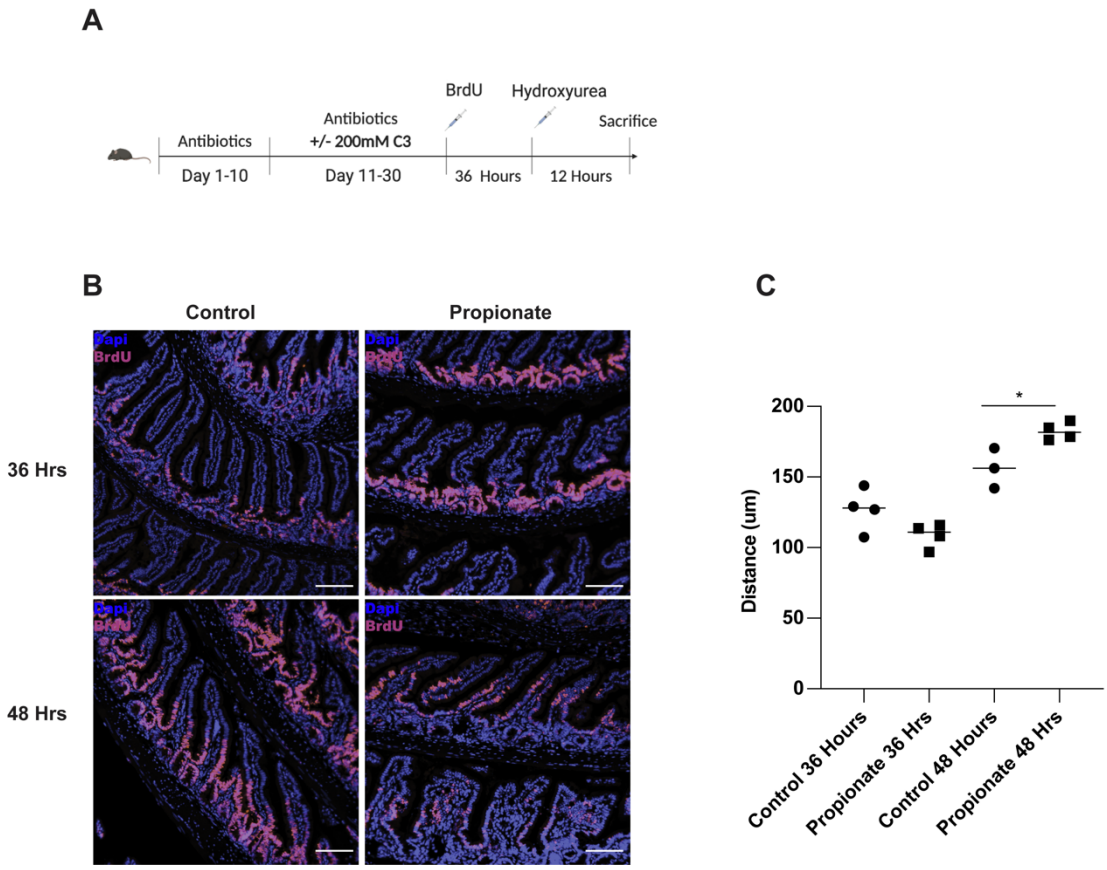

Figure 3.10: Propionate stimulates IEC migration in vivo.

(A) Schematic of measuring epithelial migration *in vivo*. (B) Representative images of BrdU (red/pink) and DAPI (blue) in the ileum of control and propionate treated mice at 36 and 48 hours post BrdU injection, scale bars 100 μm. (C) Quantification of BrdU height from the base of the crypt to the uppermost positive BrdU cell with n=3-4 samples per group. *P<.01 by Two-way ANOVA with Tukey post-test.

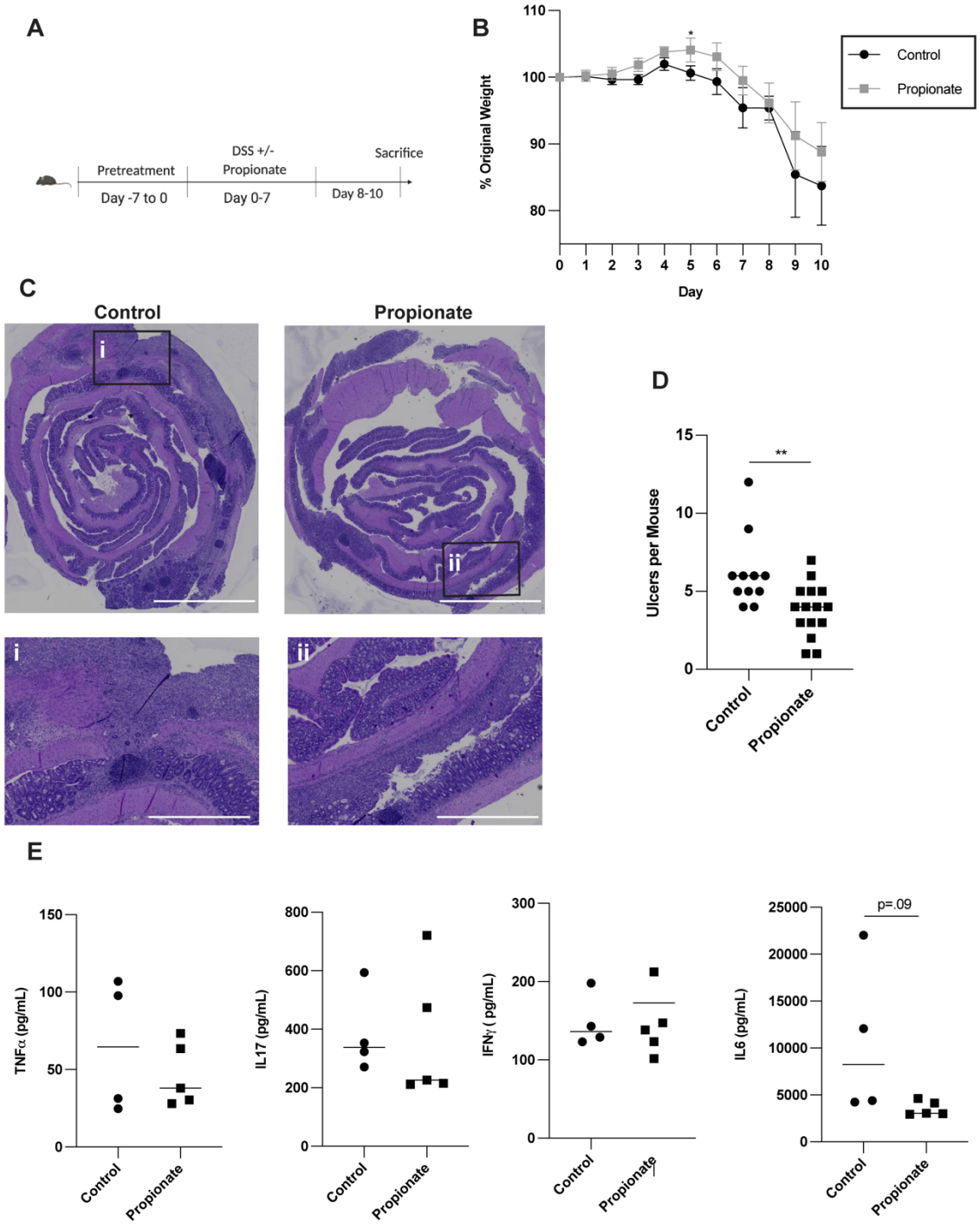

Figure 3.11: Propionate reduces ulceration in experimental colitis.

(A) Schematic of DSS colitis model. (B) % of original weight with n=4-5 mice per treatment. (C) Representative H&E images of control and propionate treated mice. Scale

bars, 4000 μm for whole mount images and 1000 μm for zoomed regions. (D) Total ulcers per mouse for n=12 control and n=15 propionate treated mice pooled from 3 independent experiments. (E) Colonic organ culture for inflammatory cytokines with n=4-5 samples per treatment. (D) *P <.05, **P<.01 by Two-way ANOVA with Tukey post-test and (B) by multiple T test corrected for multiple comparisons using the Holm-Sidak method.

Chapter 4-Implications for Using Short-Chain Fatty Acids in Human Disease

Chronic Diseases in the US

The number of individuals diagnosed with a chronic disease on a yearly basis is quickly increasing in the United States and around the world. Six in ten adults in the US have at least one chronic condition, and nearly 40% of individuals have two or more chronic conditions.[152,153] These include diseases such as obesity, diabetes, heart disease, cancers, and digestive diseases. In terms of digestive diseases, more than 70 million Americans have reported to have at least one of the following: IBD, diarrhea, celiac sprue, constipation, irritable bowel syndrome (IBS), and gastroesophageal reflux (GERD) amongst others.[153,154] With these diseases on the rise, scientist worldwide strive to understand these alarming trends. In the recent decades, there has been a major focus on diet and environmental factors and how they drive these disease processes.[2,155]

Diet and Disease

The diet in the United States and around the globe has been shifting away from nutrient dense foods such as fruits and vegetables, toward highly processed foods which are calorie rich and nutrient poor.[2,156,157] These foods include ingredients such as artificial sweeteners, high fructose corn syrup, and trans-fatty acids. On the surface, diets rich in highly processed foods increases the risk of diabetes and obesity. However, one key nutrient, dietary fiber, is often absent in these calorie dense foods, which has major implications for gut health. Dietary fiber comes in many forms, with some of the most prevalent being fructans, inulins, and oligosaccharides.[158] These compounds are found in highest concentrations in fruits, green vegetables, and beans. Dietary fiber, also known are roughage, comes in two forms, soluble and insoluble. Insoluble fiber acts as a bulking agent

in the GI tract, increasing stool weight and caliber. Soluble fiber is consumed by the vast number of microbes that inhabit the gut and are fermented leading to the production of several compounds, one of which is SCFAs.[159,160]

Short-Chain Fatty Acids and IBD

SCFAs, including acetate, propionate, and butyrate, are the primary SCFAs produced in the intestines and are responsible for 95% of SCFA production in the gut.[4] SCFAs are vital for human health, with increased SCFA productions being associated with lower risk of chronic diseases such as allergic asthma, IBD, diabetes and cancer. [3,42,84] SCFAs function in several ways in the intestine, affecting both epithelial cells and the immune cells in the underlying lamina propria. So, it is crucial to understand how these compounds affect intestinal health and if their absence leads to increased risk for development of IBD. In the context of IBD, nearly all functions of SCFAs are seen as vital. First, the ability of SCFAs to promote mucus production is important for limiting access of luminal antigens to both the underlying epithelial cells as well as the immune cell populations residing in the lamina propria.[27,35,161,162] Thus, decreased dietary fiber intake, and as a consequence decreased SCFA production, may lead to a thinning of the mucus layer, enhancing susceptibility to bacterial and antigen invasion.[30] Furthermore, a lack of dietary fiber, which is essential for bacterial population health, may lead to an increase in the number of mucus degrading bacteria, where bacteria break down the mucus layer for use as energy when dietary fiber is in short supply.[163] Another mechanism for how a lack of dietary fiber may enhance susceptibility to colitis has to do with its ability to affect both IgA production as well as AMP production.[122,164] Both AMP and IgA are secreted into the inner dense mucus layer of the intestine. Here, both antibodies and AMPs can neutralize any incoming virus, parasite,

or bacteria. With reduced production of either AMPs or antibodies, there is an increased risk of invasion of the epithelial layer, leading to heightened inflammation. Furthermore, the ability of SCFAs to promote TJ formation via upregulation of both ZO1 and Occludin is another mechanism by which SCFAs can protect from luminal antigens crossing into the lamina propria.[53,165,166] Lastly, SCFAs play a major role in modulating cell differentiation in the epithelial layer, ensuring appropriate stem cell proliferation, goblet cell and enterocyte differentiation. Thus, the importance of dietary fiber and SCFA production in the intestine cannot be overstated. Collectively, any perturbations of this system may lead to a leaky epithelial barrier and low levels of baseline inflammation, increasing the risk for both IBD and cancer.

Current Treatments in IBD

Many treatments are used to combat IBD, including mesalamines, anti-TNF agents such as Remicade, immunosuppressants such as methotrexate, and new classes of biologics such as $\alpha 4\beta 7$ blockers, also known as Entyvio. [167,168] All of these medications are effective at some level in helping to induce clinical and endoscopic remission. Despite their efficacy, most patients will experience periods of relapse, with up to 20% of patients progressing to surgical management, although rates of resection are gradually declining.[169,170] Additionally, not everyone who undergoes surgical resection will achieve remission, and the risk of cancer in these patients is generally higher than the population average.[171] These clinical outcomes in patients despite the availability of several diverse classes of medication, warrants further research and development to manage these life altering diseases. To date, many trials are underway for new classes of medications including new anti-TNFs. For example, AVX-470 is a unique anti-TNF in that it is taken

orally and is not systemically available.[167] This advancement would allow for IBD management without some of the side effects of traditional anti-TNFs, which cause systemic immune suppression. Furthermore, research is ongoing for the use of SCFAs as potential treatments.

Short-Chain Fatty Acids as a Treatment in Human Disease

The available data on the use of SCFAs for therapeutic use in IBD is limited and there has yet to be a large scale randomized trial. There have been several small scale clinical studies on the effects of SCFAs or prebiotic treatment and their effects on colitis, but the results are mixed. For example, some of the earliest studies showed that in a small cohort of patients with left sided colitis or diversion colitis, SCFA enemas helped to resolve inflammation and restore clinic and endoscopic remission.[80,81] Furthermore, it was shown in cases of refractory ulcerative proctosigmoiditis that butyrate enemas led to at least a partial response as demonstrated by lower disease activity scores on follow-up colonoscopies.[172] More recently, in a small randomized trial, it was shown that 30mL of sodium butyrate at a concentration of 600mmol/L twice a day for 30 days decreased mucosal atrophy and improved endoscopic scores as compared to controls who received saline.[172] Additionally, another group is working with derivatives of SCFAs, such as propionyl-L-carnitine, which is derived from propionate. In a randomized control trial of 120 patients, patients receiving at least 1 gram of propionyl-L-carnitine daily induced endoscopic remission in 55% of patients with mild to moderate ulcerative colitis compared to 35% in the placebo.[173] The way in which SCFAs are promoting remission in patients versus placebo may be due to the ability of SCFAs to downregulate the production of TNF-α as well as inhibit the expression of inflammatory cytokines following stimulation with

lipopolysaccharide via modulation of NFK-β. [174] If so, this mechanism is similar to how mesalamines, the first-line medication for IBD, function. [175] Additionally, SCFAs, specifically butyrate, may function by upregulating enzymes important for guarding against oxidative stress, as butyrate enemas have been shown to decrease both inflammatory and oxidative stress markers in humans. [176] Although several small-scale studies show promise, some have found that SCFAs have limited or no effect on disease activity. For example, a study using both oral mesalamine with or without oral sodium butyrate tablets in a group of 25 patients found no significant difference in UC disease activity index between patients treated with mesalamine versus mesalamine and butyrate tablets.[177] Additional studies have looked at the effect of butyrate enemas for the treatment of left sided colitis but found no difference between butyrate enemas and saline enemas three or six weeks post initiation of treatment. [172]

Pitfalls of Clinical Studies on Short-Chain Fatty Acids in IBD

Several deficiencies exist in our current understanding of the role of SCFAs in disease that may impact past, present, and future studies on the role of SCFAs in IBD. First and foremost, existing clinical studies on the role of SCFAs are small in scale, often involving less than 20-30 patients, thus any effect of SCFAs on disease activity may be masked by the relatively small scale of study. Additionally, the type of butyrate and the amount of butyrate used are different between each study. The lack of standardization of therapy makes it difficult to evaluate the results of several studies over time. The formulation of SCFAs in these studies may be vital do to the normal physiology of the intestinal tract. For example, all intestinal epithelial cells have the capability of using SCFAs, especially butyrate, for a source of energy via beta-oxidation.[140] This means that oral formulations of

SCFAs may not be appropriate, as delivering an accurate and adequate dose to the colon of individuals with disease may be difficult. Additionally, the pH of the intestinal lumen varies from person to person, ranging anywhere between 5.1-7.5.[178] Lower pH values are often found in the small bowel, with a gradual rise in pH at the ileum, before becoming more acidic in the caecum/right colon. This pH gradually rises again toward 6.6-7.5 in the left colon and rectum.[178] However, in patients with active disease, these pH values tend to trend toward more acidic levels. To complicate this further, luminal pH and surface pH of the colonic mucosa tend to differ.[178] The pH of the lumen and surface of the mucosa are vital to future use of SCFAs in IBD. This is because SCFAs are acids, with pKa values of approximately 5. With wide-ranging pH values of the colonic lumen, this indicates that each patient being treated with SCFAs may have more or less availability of SCFAs in both tablet and enema form depending on their gut pH. Lastly, the effects of SCFAs on colonic homeostasis are dose dependent. In mice, it has been well demonstrated that at high doses, SCFAs, especially butyrate, increases the rate of apoptosis, whereas at lower rates, stimulation of AMPs and mucins may occur.[38,161,179] Thus standardization of SCFA dosage, pH, and formulations are vital for furthering our understanding of SCFAs for potential therapeutic use.

Histone Deacetylase Inhibitors as a Treatment for IBD

SCFAs in their physiological form have several shortcomings for their use in IBD as mentioned above. Additionally, some effects of SCFAs may not be beneficial for patients in an acute flare. For example, butyrate may inhibit or promote stem cell proliferation depending on its concentration. This was shown elegantly by Kaiko *et al*, who showed that colonocytes above the stem cell compartment lower the concentration of SCFAs at the base

of the crypt, leading to histone acetylation.[40] However, removal of these colonocytes, for example during ulceration, halts stem cell proliferation which impedes fast resolution of ulceration. [41] This effect is due to the limited ability of stem cells to metabolize high levels of butyrate, which leads to butyrate accumulation and cell senescence.[41] However, it is possible to avoid some of these detrimental effects by targeting certain pathways induced by SCFAs. For example, in our study we show that in an HDACi dependent manner, SCFAs can stimulate cell migration, which is beneficial for wound healing in the gut, where epithelial migration to form a temporary covering of the wound bed is the first step to resolving the underlying ulceration.[87,118,180] Additional studies have also highlighted these potential effects. For example, it was shown that the treatment of mice with HDACi both prevents the onset of colitis in a DSS model of colitis, as well as promotes the resolution of colitis when given post DSS.[141,150] These effects may be through HDACi ability to modulate tight junction formation, proliferation, and migration[141,150,181] Lastly, many HDACs have been shown to have increased expression or enhanced activity in IBD patients leading to the hypoacetylation of many gene promoters known to function in the anti-inflammatory response. [141,182] Some of the functions of HDACi in the anti-inflammatory response include enhancement of Foxp3 expression leading to increased Treg suppressive function and the ability to downregulate IL12/IL23 secretion from dendritic cells and macrophages.[183–185] The functions of HDACi in colitis have been well-established in mouse models and with several HDACi such as valproate currently clinically available, the investigation of HDACi use in IBD is warranted. To date, no human clinical trials have been carried out to examine the function of HDACi such as valproate in IBD. However, some groups have treated biopsies from patients with IBD to investigate a potential use for

HDACi in human disease. For example, one group found that in patients with active inflammation, there was a reduction in H3K27ac$^+$ cells as compared to inactive IBD controls and an increased level of pro-inflammatory cytokine production in *ex vivo* biopsy cultures. [186] Following treatment with valproate, H3K27ac$^+$ levels increased in the biopsies from patients with active inflammation and expression of IL-6, IL-10, IL-1 and IL-23 decreased as compared to control samples.[186] This new data is the most promising to date for the potential use of HDACi in IBD.

Concluding Remarks

IBD is a chronic remitting and relapsing autoimmune disorder of the gastrointestinal tract that effects millions worldwide with rates on the rise.[187,188] Current therapies are not sufficient to maintain remission in a large portion of patients leading to potential long-term complications such as surgical resection and colorectal cancer.[169,171] Thus more research into therapeutics is warranted. SCFAs have been identified as a potential target for therapeutic development; however, our understanding of how SCFAs work in a concentration dependent manner is still lacking. Thus, this work describes a novel function, epithelial cell migration, in response to SCFA treatment that may be beneficial for future investigations into the use of SCFAs in human disease. More importantly, this work details a mechanism for how SCFAs can stimulate epithelial cell migration in an HDACi and GPR43 dependent manner. The identification of these pathways are of utmost importance as targeting specific pathways relevant to SCFA function may be better suited for use as potential treatment in humans. This would allow us to avoid some of the effects of SCFAs on cell senescence and apoptosis, which could be detrimental to early stages of wound healing in the gut. Finally, the identification of HDACi in epithelial cell migration, the first

step in epithelial restitution, allows us to circumvent some of the problems with the development, delivery, and epithelial cell metabolism of SCFAs, as many of these problems have already been solved for HDACi such as valproate, which has been in use clinically for over 60 years.

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Vita

Anthony James Bilotta II was born on October 7th, 1991 to Anthony James Bilotta and Mary Beth Bilotta in Houston, TX. When he was young, Anthony moved with his family to Leominster, MA where he spent most of his childhood and adolescence. Anthony attended and graduated from Worcester Academy, a boarding school, in Worcester, MA. During his junior year in high school, Anthony met his eventual wife, Nicole, who he would marry in 2016. Anthony attended Vanderbilt University where he discovered his passion for science and began his research in colorectal cancer and colitis in the laboratory of Dr. Christopher Williams. Anthony Graduated from Vanderbilt University with a degree in Molecular and Cellular Biology in 2014.

Following graduation, Anthony moved with his wife, Nicole, to Galveston, TX where he entered into the MD/PhD Combined Degree Program in the Summer of 2015. Following his first two years of medical school, Anthony joined the laboratory of Dr. Yingzi Cong to study the effects of short-chain fatty acids and long-chain fatty acids on intestinal epithelial cell homeostasis and disease. Under his supervision, Anthony has published two first author papers and is currently working on his third. Additionally, he has coauthored nine papers, with three more currently under review. Anthony was also the recipient of a NIH NIDDK F30 as well as a handful of other awards including the Edith and Robert Zinn Presidential Scholarship, Brian C. and Stephanie M. Sauer Endowed Scholarship, Dennis William Bowman Scholarship in Biomedical Research, Mardelle Susman Scientific Writing Award, American Association of Immunologist (AAI) Trainee Poster Award, James W. McLaughlin Travel Awardee, UTMB Student Government

Association Leadership Scholarship, and the Harry B. and Marie S. Kelso Endowed Blocker Scholarship for Biomedical Sciences. Anthony was selected for a presentation at Digestive Disease Week (DDW) 2020. During his PhD, Anthony and Nicole had two beautiful girls, Lilah and Fiona.

After graduating with his PhD, Anthony will rejoin his medical school class to complete his last two years of training. Following graduation from the MD/PhD program, Anthony will enter into residency and then fellowship, where he hopes to train in gastroenterology.

Publication List

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