INTESTINAL EPITHELIAL *PTPN2* MODULATES THE NUMBER OF TUFT AND GOBLET CELLS IN MICE

By

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A capstone project submitted for Graduation with University Honors

May 22, 2026

University Honors

University of California, Riverside

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ABSTRACT

Inflammatory Bowel Disease (IBD) is characterized by inflammation along the gastrointestinal tract that causes diarrhea, cramping, weight loss, and fatigue. IBD has been associated with the loss of function of the Protein Tyrosine Phosphatase non-receptor Type 2 (*Ptpn2*) gene. Our group has reported that whole-body *Ptpn2*-knockout (KO) mice have decreased numbers and functionality of specialized small intestinal epithelial cells called Paneth cells, increased gut barrier permeability, and a dysregulated gut microbiome, all of which are factors in IBD development. Intestinal epithelial cells (IECs) directly maintain gut microbiome homeostasis, control gut permeability, create the mucus layer, and absorb nutrients, thus *Ptpn2* deletion could directly influence multiple IEC functions. Moreover, whole-body *Ptpn2* deletion causes a hyperactive immune response in mice, which significantly compromises the differentiation and function of IECs. Here, we investigated whether intrinsic epithelial *Ptpn2* loss affects the function and differentiation of IEC subtypes in vitro and in vivo. Methods: Enteroids were generated from small intestinal crypts isolated from *Ptpn2*-wildtype (WT) and global *Ptpn2*-KO mice (BALB/c). In addition, tamoxifen-inducible epithelial-specific *Ptpn2*-KO mice ($Ptpn2^{\Delta IEC}$) and *Ptpn2* controls ($Ptpn2^{fl/fl}$; C57Bl/6J) had IECs isolated for total RNA isolation followed by qPCR analysis of gene targets associated with function and differentiation of IEC subtypes. Results: In undifferentiated enteroids, the expression of IEC subtype-associated genes *Lyz1*, *Defa5*, and *Defa6* (Paneth cells), *Muc2* and *Tff3* (goblet cells), c-*Maf* (enterocytes), and *Sucnr1* and *Pou2f3* (tuft cells), showed no significant difference in *Ptpn2*-WT versus KO enteroids. In addition, there was no change in the expression of the same targets in ileal IECs of $Ptpn2^{\Delta IEC}$ versus $Ptpn2^{fl/fl}$ mice (n>5), except for Paneth cell markers that were all downregulated as reported by the McCole lab in a previous publication. However, immunostaining for *Dclk1*, a tuft

cell marker, revealed higher numbers of tuft cells in the ileum of *Ptpn2*$^{\Delta IEC}$ vs. *Ptpn2*$^{fl/fl}$ mice (n=6), while alcian blue staining revealed elevated number of goblet cells in the colon of *Ptpn2*$^{\Delta IEC}$ vs. controls (n=8). Conclusion: Epithelial *Ptpn2* deletion does not impact the expression of IEC markers in vitro, but it does increase the number of tuft and goblet cells in vivo. This implies a requirement for non-epithelial cell types in epithelial *Ptpn2* regulation of IEC differentiation.

ACKNOWLEDGEMENTS

I would like to extend my deepest gratitude to my faculty-mentors, Dr. Declan F. McCole and Dr. Vinicius Canale for their continued support and indispensable knowledge throughout this project. To Dr. Declan F. McCole, thank you for the opportunity to join your lab and for holding high academic and research standards, which were imperative for the successful completion of this capstone project. To Dr. Vinicius Canale, thank you for the wide expanse of knowledge you have passed on, for the introduction into the biomedical research field, and for your patience throughout the process. I attribute much of my academic and professional development to my mentors' guidance and teaching. I also wish to thank Dr. Begoña Echeverria and the entire University Honors team for their support and for providing the framework and resources necessary to complete this capstone.

The text of this thesis, in part, is a reprint of the material as it appears in *Journal of Clinical Investigation – 2021; and Cellular and Molecular Gastroenterology and Hepatology - 2023*. The co-authors Vinicius Canale and Declan F. McCole listed in those publications directed and supervised the research that forms the basis for this thesis.

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1. INTRODUCTION

1.1. Overview

Inflammatory Bowel Disease (IBD) is characterized by chronic inflammation along the gastrointestinal tract (GI), with contributing factors associated with environmental factors, dysregulation of the gut microbiome, and genetic susceptibility.[1,2] It affects approximately 2.39 million people in the United States alone.[3] Some of the symptoms include diarrhea, cramping, weight loss, and fatigue. The two main types of IBD are ulcerative colitis and Crohn's disease. Ulcerative colitis is characterized by small ulcers restricted to the descending colonic mucosa.[4] In Crohn's Disease inflammation may occur anywhere on the GI tract from the mouth to the anus and affects all layers of the intestinal wall.[5]

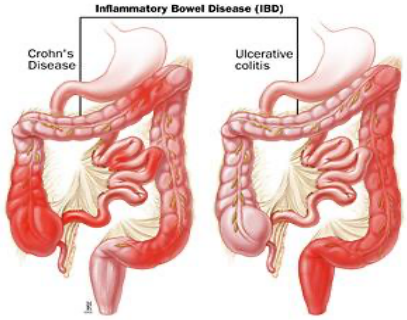

Image adapted from autoimmunesociety.org

The GI tract is a complex system made up of many organs, including the mouth, esophagus, stomach, small intestine, large intestine, and other accessory organs. The esophagus transfers food from mouth to stomach and prevents reflux of stomach acid.[6] Food is digested by enzymes in the highly acidic environment of the stomach, to create a paste called chyme, that will continue into the duodenum of the small intestine.[6] The small intestine has three morphologically similar but functionally distinct segments: the duodenum, jejunum, and ileum. The epithelium of the small intestine has characteristic protrusions called a villus, which increases the surface area, optimizing nutrient absorption.

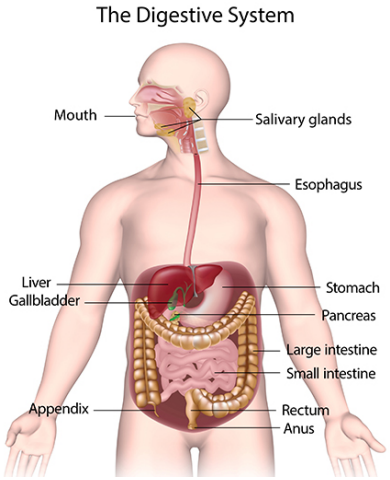

Image adapted from https://www.niddk.nih.gov/health-information/digestive-diseases/digestive-system-how-it-works

The segments of the large intestine include the cecum, colon (ascending, transverse, descending, and sigmoid), rectum, and anus. The purpose of the large intestine is to absorb water and electrolytes, secrete mucus, and move feces.[6] Many of these functions originate from the anatomy and barriers of the GI tract.

The tissue morphology of the intestines includes four layers: the mucosa, submucosa, muscularis propria, and serosa.[7] These layers aid in absorption and secretion, contain blood and lymphatic tissue to support the GI tract, control muscular contraction to push waste contents forward, and secrete fluid to lubricate the intestinal lumen to facilitate digestion or flush out potential pathogens, respectively.[8]

1.2. Intestinal Epithelial Barrier

Herein, the focus of this study lies in the intestinal epithelial barrier, which is composed of the mucus layer, intestinal epithelial cell (IEC) lining (the epithelium), and immune cells of the lamina propria.[2] The epithelium, which acts as a barrier between the lumen and external environment, generates ion solute concentration gradients, absorbs nutrients, prevents pathogens from entering the lumen, and communicates with the immune system.[9] The epithelium is composed of many IEC subtypes that have specialized functions to generate physical and chemical barriers, preventing invasion of the mucosa by the intestinal microbiota and external pathogens.[10] Some of these IECs include Paneth cells, goblet cells, and tuft cells. Enterocytes encompass an absorptive lineage of IECs.[11] The precursors to IECs are intestinal stem cells, which differentiate into transit-amplifying cells. As transit-amplifying cells move up the crypt, they differentiate into IECs.[12] Eventually, the IECs reach either the tip of the villus or luminal surface, depending on whether they are present in the small or large intestine, and shed off into the lumen.[13]

The architecture of the small intestine is made up of small invaginations called crypts, and finger-like protrusions called villi.[13] Villi maximize the amount of nutrient absorption by increasing surface area and are only present in the small intestine where nutrient absorption occurs.[13] The large intestine only has crypts, which aid in water and electrolyte absorption.[13] The makeup of IECs also varies between the small and large intestine. Enterocytes are 70% of the IEC population in the ileum, versus only 14% of the population in the large intestine.[13] In addition, 5% of the IECs in the small intestine are goblet cells, while the IECs in the large intestine are 20% goblet cells.[13]

1.2.1. Enterocytes

Enterocytes are the most abundant IECs in the small intestine. They make up an absorptive IEC lineage, with their function differing based on their location in the small intestine.[14] The role of enterocytes is the uptake of ions, water, sugar, amino acids, lipids, vitamins, and reabsorption of unconjugated bile salts.[11] These cells also aid in immune responses, with an ability to uptake and process luminal antigens via fluid-phase endocytosis.[11] Apical surfaces of enterocytes point towards the lumen and have tightly packed microvilli that increase the surface area available for nutrient absorption.[15] The microvilli also have negatively charged glycoproteins on their tip, forming a border called the glycocalyx.[11] Antigens must pass the glycocalyx to enter the enterocyte, and changes in the microvilli alters enterocyte function.[11] The gene markers for enterocytes are *cMAF* for function, and *Hes1* for differentiation.

1.2.2. Goblet Cells

Goblet cells are IECs that secrete mucin proteins, forming the physical barrier known as the mucus layer, overlaying the mucosa.[16] These IECs have a goblet-like shape, with the nucleus in the basal region and mucin secreted from the apical surface.[17] Moving from the small intestine

to colon, the number of goblet cells increases.[16] This is due to the increasing thickness of the mucus layer as the microbial contents also increase within the colon. Secreted mucin proteins include gel-forming (MUC2 and MUC6) or transmembrane (MUC3, MUC12, MUC13, MUC15, and MUC17 in the small intestine; MUC4, MUC20, and MUC21 in the large intestine).[13] Gel-forming MUC2 is considered the main mucin protein in relation to goblet cells.[16] The gene markers of goblet cell function are *Muc2* and *Tff3*, and *Klf4* for goblet cell differentiation.

1.2.3. Tuft Cells

Tuft cells are rare intestinal epithelial cells that have a chemosensory role in the intestinal epithelium.[18] Chemoreception in tuft cells is theorized due to the physical connection between tuft cells and enteric nerve terminals that are efferent in nature.[18] In addition, α-gustducin and other components in the taste transduction pathway were found in tuft cells, further supporting tuft cells' role in chemoreception.[19] Tuft cells are also dependent on the transcription factor *Pou2f3*, which is required for the differentiation of taste receptor and chemosensory cells.[18] Anatomically, tuft cells have long microvilli at the luminal surface, vesicles in the apical cytoplasm, and a tubulovesicular system down to the basal portion.[18] They are distributed along the crypt-villus axis.[18] The gene marker for tuft cells is *Sucnr1*.

1.3. Enteroids

Enteroids provide an *ex vivo* method of studying intestinal epithelial cell biology. Derived from the small intestine, enteroids are also called "mini-intestines". They are primary cell cultures from intestinal crypt-based stem cells.[20] Enteroids are not transformed and therefore reflect *in vivo* epithelial cell diversity.[21] In addition, the structure of enteroids resembles that seen *in vivo*, with villus-like epithelium present.[20] The use of enteroids is popular in the setting of

gastrointestinal research, as this model allows for studying of human intestinal physiology, developmental biology, microbial replication, and epithelial cell responses.[22]

1.4. Inflammatory Bowel Disease

IBD is associated with environmental changes, genetic susceptibility, and dysregulated microbiome, all leading to disruption of the intestinal epithelium.[1,23] The hygiene hypothesis encompasses environmental changes, explaining a correlation between increased sterilization in living environments with increased incidence of chronic inflammatory disorders.[24] Genetic susceptibility encompasses >240 gene loci associated with IBD, these genes involved in immunodeficiencies, T-cell function, cytokine production, and mycobacterial diseases.[23,25] This includes the major gene of interest in the McCole lab, protein tyrosine phosphatase non-receptor type 2 (*Ptpn2*). Increased immune responses to microbial antigens can occur from a dysregulated microbiome.[23] These factors contribute to increased permeability of the epithelial lining, leading to intestinal fluid loss and an influx of luminal bacteria into the lamina propria.[1] The subsequent inflammatory response leads to the symptoms seen in IBD.[1] Therefore, IECs have a substantial role in maintaining gut homeostasis.

Single nucleotide polymorphisms found in the *Ptpn2* gene locus, have been associated with several autoimmune chronic inflammatory diseases, including IBD, diabetes type I, and celiac disease.[26] *Ptpn2* encodes T-cell protein tyrosine phosphatase (TCPTP), which dephosphorylates substrates of the Janus activated kinase (JAK) and signal transducer and activator of transcription (STAT) protein families.[27,28] Our group and others have previously demonstrated that loss of *Ptpn2* is associated with increased intestinal barrier permeability, decreased Paneth cell viability and Paneth cell-specific antimicrobial peptide expression, changes in the tight junctions of the epithelial cells, modification of IEC-macrophage communication, and

shifts in the intestinal microbiota which allows for increased expansion of pathobiont bacteria associated with clinical IBD.[2,28-31]

1.5. Study Proposal

The physical and chemical barriers are paramount to the gut's defense against pathogens. IECs are connected by tight junctions, desmosomes, and adherens junctions to form the physical barrier.[13] A primary factor of the physical barrier is the mucus layer, created from mucin proteins secreted by goblet cells.[13] This layer traps bacteria and prevents direct interactions between luminal contents, and the epithelium and host immune system.[33] There is a double mucus layer in the colon compared to a single layer in the small intestine and this is believed to be due to the need for increased protection from the increased microbial load within the colon.[13] The inner layer is impermeable to bacteria, but bacteria can live within the outer layer.[16] In the small intestine, the single, thinner mucus layer is permeable to bacteria, which are kept away by the chemical barrier.[16] The chemical barrier is a defense system made up of secreted digestive acids, digestive enzymes, mucopolysaccharides, glycoproteins, and glycolipids.[15] It acts to protect tissues and fight against pathogens.[15] This barrier also includes the secretion of antimicrobial peptides (AMPs) by Paneth cells.[13] Paneth cells are localized to the small intestine and found at the base of crypts.[2] Secreted AMPs by Paneth cells include α-defensins (cryptdins in mice), lysozyme, and phospholipase A2 group IIA.[2] Our group has previously reported loss of *Ptpn*-2 with decreased Paneth cell viability and AMP production, which may contribute to intestinal inflammation and previously reported disruption of bacterial populations, referred to as dysbiosis.[2,32]

However, it is still unclear how loss of *Ptpn2* directly affects IECs ability to maintain gut microbiome homeostasis, create the mucus layer, and the impact on the differentiation of the IEC

subtypes. Therefore, my research aims to determine if intrinsic epithelial *Ptpn2* loss impairs the function and differentiation of IEC subtypes *in vitro* and *in vivo*.

2. METHODOLOGY

2.1. Mice

Animal procedures were performed following institutional guidelines and approved by the University of California, Riverside Institutional Animal Care and Use Committee. The mice were held in a specific-pathogen-free facility with unrestricted access to food and water. Tamoxifen-inducible epithelial-specific *Ptpn2*-KO mice (*Ptpn2*$^{\Delta IEC}$) were produced as previously described.[29] In addition, all mice experiments including handling, tissue harvest, and husbandry maintenance were performed by graduate students and/or personnel properly trained in Dr. Declan McCole's laboratory.

Inducible tissue-specific Ptpn2-KO mice

In short, a mouse line with critical *Ptpn2* exons flanked by LoxP sites (*Ptpn2*$^{tm1a[EUCOMM]Wtsi}$), was crossed with a transgenic mouse line expressing the Cre recombinase enzyme under the villin-1 promoter (Tg[Vil-cre/ERT2]23Syr). Recombination induction was achieved by tamoxifen injection administered intraperitoneally (50 mg/kg body weight) for 5 consecutive days when mice were between 6-8 weeks of age, generating *Ptpn2*$^{fl/fl}$ control mice and *Ptpn2*$^{\Delta IEC}$ mice.[2,29] (Marchelletta, R. R., et al.; 2Canale, Vinicius, et al.). Tissue harvest was performed 40 days after recombination induction, when mice were approximately 12 weeks old.

Global Ptpn2-KO mice

Mice (BALB/c) with constitutive *Ptpn2* loss were obtained from Dr. Michel Tremblay (McGill University). Harvesting of tissues occurred when mice reached 21 days old because global *Ptpn2*-KO mice die between 4 and 5 weeks of age due to systemic inflammation.[2,34]

2.2. Enteroid Models

Enteroids were generated from small intestinal crypts isolated from *Ptpn2*-wildtype (WT) and global *Ptpn2*-KO mice (BALB/c) as previously described.[2] Total RNA of undifferentiated enteroids was harvested as described below. In addition, *Ptpn2*$^{\Delta IEC}$ and *Ptpn2* controls had IECs isolated for total RNA isolation followed by qPCR analysis of gene targets associated with IEC subtype's function and differentiation.

2.3. RNA Isolation and qPCR

Quantitative polymerase chain reaction (qPCR) was used to determine whether the target gene associated with an IEC is expressed within a sample. *GAPDH* was used as the reference gene. Total RNA was extracted from enteroids using the RNeasy Mini Kit (cat. Nos. 74104 and 74106; Qiagen, Hilden, Germany) according to the manufacturer's instructions. We used a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA) to determine the RNA concentration. Complementary DNA (cDNA) was then made using qScript® cDNA SuperMix (Quantabio, Beverly, MA, USA) following the manufacturer's protocol. Real-time qPCR was performed with iQ SYBR Green Supermix (1708882; Bio-Rad, Hercules, CA, USA) on a C1000 Thermal cycler (Bio-Rad, Hercules, CA, USA) with a CFX96 Real-Time PCR system. Each PCR target was assayed in triplicate. Data were collected and analyzed using the Bio-Rad CFX Manager 3.1 software according to the manufacturer's guidelines.[35]

2.4. Immunohistochemistry

Distal ileal and colonic segments were excised and fixed in 4% paraformaldehyde overnight at 4ºC. Tissues then were rinsed with phosphate-buffered saline (PBS) and dehydrated with increasing concentrations of ethanol washes using Shandon Excelsior ES Tissue Processor (Thermo Fisher Scientific, Kalamazoo, MI). Paraffin embedding was performed using Histoplast

LP (Richard-Allan Scientific, Kalamazoo, MI) in a Tissue-Tek station set (Miles Scientific, Naperville, IL). Paraffin blocks were sectioned in a rotary microtome (RM2235; Leica, Nussloch, Germany) 5-mm wide and placed on a charged slide (Fisher Scientific, Pittsburg, PA). Slides were deparaffinized, rehydrated, and stained with H&E. Similarly, goblet cells were visualized using the periodic acid–Schiff kit (procedure no. 395; Sigma) per the manufacturer's guidelines. Slides were visualized and images were acquired with a Leica microscope (model DM5500B) coupled with a DFC450C camera (Leica). Heat-induced antigen retrieval was performed for 20 minutes at approximately 96ºC, with appropriate buffer depending on the primary antibody/epitope. In short, endogenous peroxide was quenched with 3% hydrogen peroxide in PBS for 10 minutes. Nonspecific antigens were blocked with blocking buffer (2% normal donkey serum, 1% bovine serum albumin, 0.1% Triton-X (Thermo Fisher Scientific Cat No. BP151), 0.05% Tween-20 (Thermo Fisher Scientific Cat No. BP337), and 0.05% sodium azide (Research Products Internation Cat No. S24080-250) in PBS) for 30 minutes at room temperature. We used biotin-conjugated *Tff3* as the primary antibody, which was incubated for 1 hour at room temperature in PBS with 5% normal donkey serum. Streptavidin HRP was used as the secondary antibody. Detection was performed using the biotin–streptavidin detection system. For immunohistochemistry, horseradish peroxidase signal was developed by 3,30 diaminobenzidine tetra hydrochloride incubation according to the manufacturer's protocol, and incubation time was optimized for each protein target (#8059; Cell Signaling Technology, Beverly, MA). Sections were counterstained with hematoxylin. Slides were mounted with Permount (Electron Microscopy Sciences Cat No. 17986-01) and visualized on a Leica microscope (model DM5500B with a DFC450C camera). Intestinal cross-sections were used for morphometric measurements. Only well-oriented intestinal structures (i.e., crypt and villi) with

bottom-to-top axis visibility were used in the analysis. Goblet cells were counted within the crypts and differentiated by the presence of strongly positive or strongly negative staining for *Tff3*. The ratio of strongly positive or strongly negative *Tff3* goblet cells/total goblet cells per crypt was calculated and averaged to represent a single mouse.

2.5. Statistical Analysis

Results of the qPCR were analyzed by the ΔΔCT method and graphs display geometric means ± SD of the geometric mean in a log10 scale. We used the 2-tailed Student *t* test when comparing 2 groups. Analysis of variance (ANOVA) with Tukey's post-hoc test was used when analyzing three experimental groups. The critical significance level was α=05. Data are expressed as means ± SD for *n* independent observations per group unless stated otherwise. Outliers were identified using robust regression and outlier removal (ROUT) method, with Q set at 1% and excluded when appropriate.

3. RESULTS

3.1. Optimization and Validation of qPCR Primer Sets

To ensure maximum accuracy, we validated PCR primers for our intended targets as shown in Table 1. PCR confirmed specificity of each primer target and allowed us to define the optimal annealing temperature. As an example, amplification of *GAPDH* was consistent and the melt curve gave us an annealing temperature of 55°C (Figure 1A). PCR of *Lysozyme 1* also showed consistent amplification curves and an annealing temperature of 63°C (Figure 1B). PCR optimization was completed for all primers. Subsequent gel electrophoresis validated the correct primer was expressed while no amplification of undesired targets was detected. We used 2% agarose gel ran in TAE buffer to determine the number of nucleotides of the primer match the expected product. *GAPDH* is 162 nucleotides long, which is consistent with the bands present in

the gel (Figure 1C). Gel electrophoresis was performed for all primers to ensure the correct product was expressed.

Target Name	**Primer Sequence**	**Amplicon Size (nucleotides)**	**Optimal Annealing Temperature (Tm °C)**
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	5'-GTT TGT GAT GGG TGT GAA CCA CG-3'	162	55
Kruppel-like factor 4 (*Klf4*)	5'-ACA CAG GCG AGA AAC CTT ACC-3'	78	60
Mucin 2 (*Muc2*)	5'-TCC TGA CCA AGA GCG AAC AC-3'	182	64
Trefoil factor 3 (*Tff3*)	5'-GAT TAC GTT GGC CTG TCT CCA A-3'	100	60
Cellular Musculoaponeurotic Fibrosarcoma (*cMAF*)	5'-GCA TGC TGG ACA TGT ATG GT-3'	241	60
Hairy and Enhancer of Split-1 (*Hes1*)	5'-CAC CGG ACA AAC CAA AGA CG-3'	149	62
Atonal homolog 1 (*Atoh1*)	5'-GAA GGG TGG GGT TGT AGT GG-3'	75	56
Lysozyme 1 (*Lyz1*)	5'-TGG CTG ACT GGG TGT GTT TA-3'	166	63
Defensin alpha 5 (*Defa5*)	5'-CCC CCA GCC ATG AAG ACA TT-3'	171	60
Defensin alpha 6 (*Defa6*)	5'-TGT AGA GCA AGA GGC TGC AA-3'	86	62

Succinate receptor 1 (*Sucnr1*)	5'-CCG ACA GCA GAA TGG CAC AGA-3'	146	62
POU class 2 homeobox 3 (*Pou2f3*)	5'-GGA TGG TGA ATC TGG AGC CCA T-3'	115	60

Table 1: Depicts the primer sequence, amplicon size in nucleotides, and the optimal annealing temperature for all primer targets used in our qPCR.

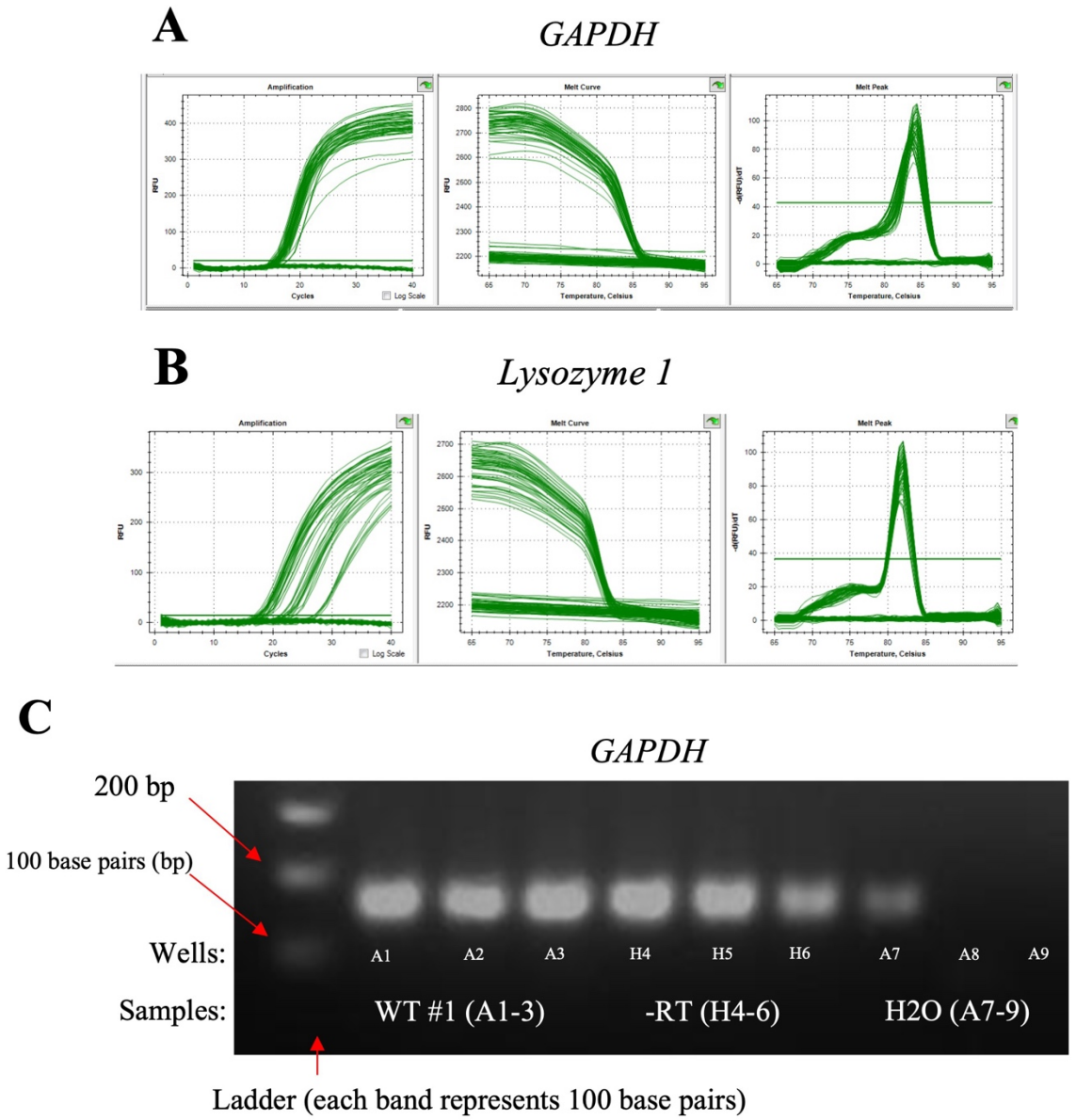

Figure 1. Primer set optimization was performed to ensure specificity and optimum annealing temperature. (A) PCR shows consistent amplification, melt curve, and melt peak for *GAPDH* and (B) *Lysozyme 1*. (C) Gel electrophoresis of GAPDH, which confirmed the correct target was expressed given *GAPDH* is 162 nucleotides long. No other targets were amplified with the primer set used.

3.2. Number of *Tff3*$^{+}$ Goblet Cells is Decreased in the Colon of *Ptpn2*-KO Mice

Our group previously sought to determine if constitutive *Ptpn2* loss compromises the function and/or numbers of IEC subtypes that restrict gut dysbiosis.[2] As stated before, the function and

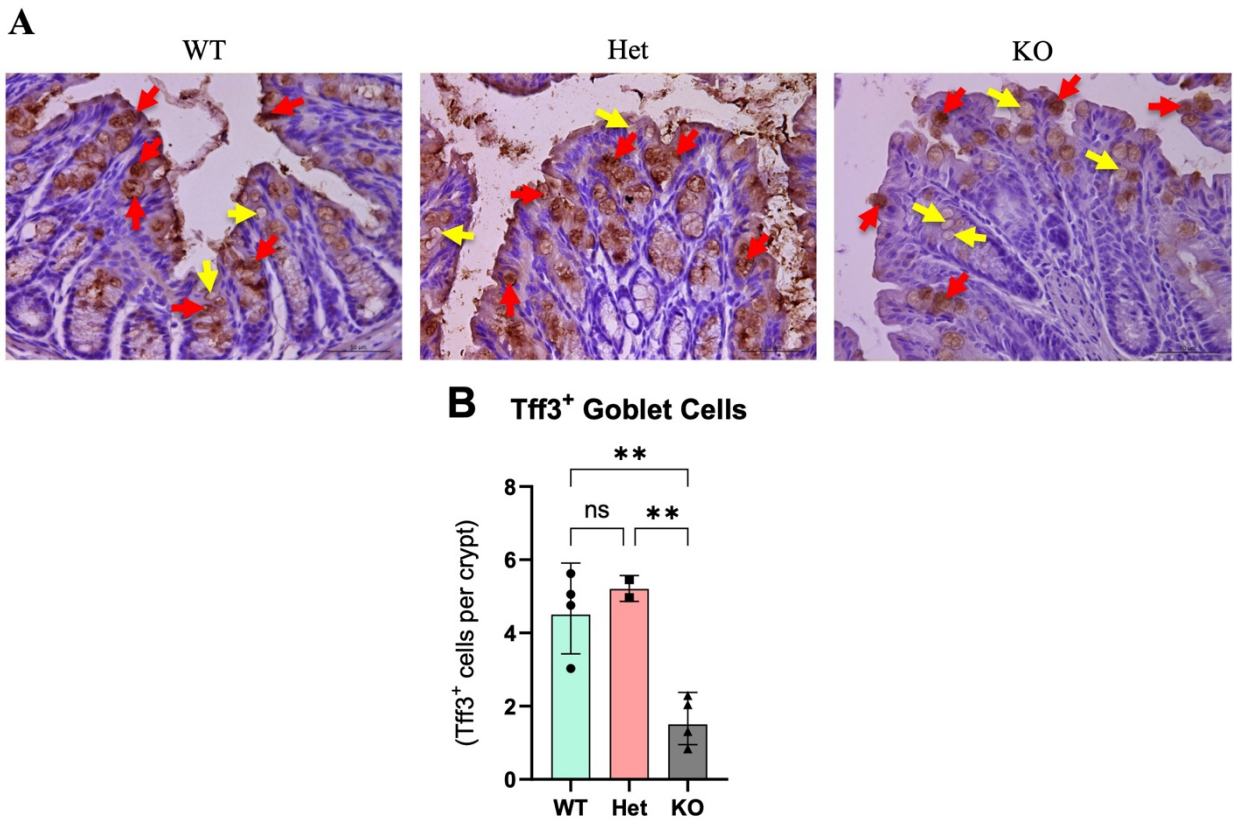

Figure 2. Identification of *Tff3*$^{+}$ colonic goblet cells through immunohistochemistry. (A) Goblet cells were either strongly stained for antimicrobial peptide and goblet cell marker *Tff3* (red arrows) or seemingly *Tff3*-negative (yellow arrows). (B) Quantification of goblet cells strongly positive for *Tff3* staining in the distal colon crypts. Bars show mean ± SD. One-way ANOVA and Tukey's post hoc.

number of ileal Paneth cells, critical orchestraters of the gut microbiome, were compromised in *Ptpn2*-KO mice. Furthermore, unpublished data revealed that constitutive *Ptpn2* loss resulted in a decreased number of ileal and cecal goblet cells when compared with *Ptpn2*-WT mice. Given that goblet cells are fundamental in the innate immune responses of the host, primarily by generating the protective mucus layer, we sought to determine the impact of *Ptpn2* function upon the function of this IEC subtype.

Here, to investigate how constitutive *Ptpn2* loss affects the function of goblet cells, we performed immunostaining for *Tff3* (Figure 2A), an antimicrobial peptide secreted by intestinal goblet cells. Goblet cells stained for *Tff3* were considered strongly positive if they were consistently dark brown in color (red arrows). Goblet cells negative for *Tff3* were not brown in

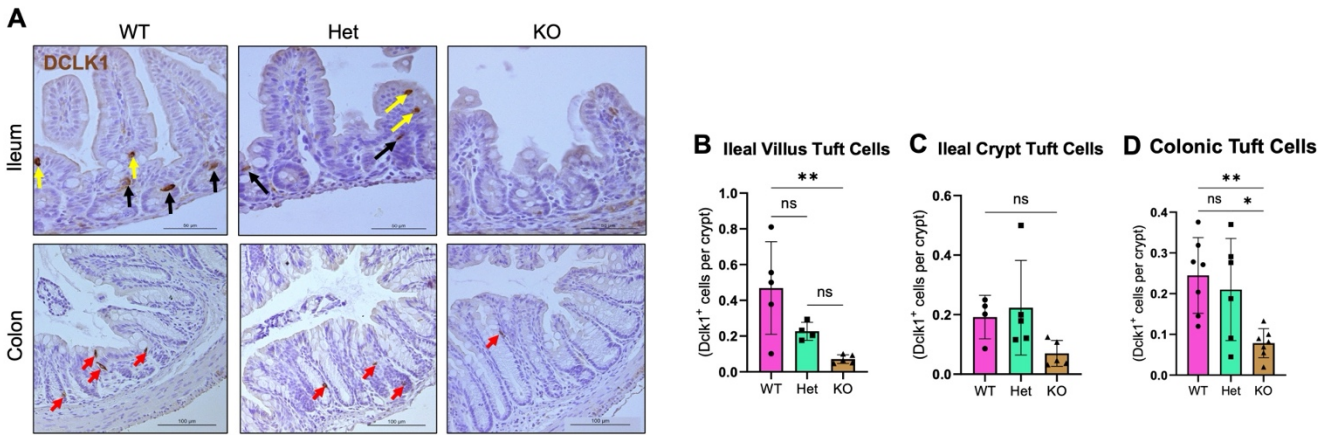

Figure 3A-D. Number of *Dclk1*$^{+}$ Tuft Cells is Reduced in the Ileum and Distal Colon of Ptpn2-KO Mice.
(A) Immunohistochemistry analysis for the Tuft cell marker *Dclk1*. Red arrows indicate IECs positively stained for *Dclk1*. Yellow arrows indicate IECs present in the ileal villi positively stained for *Dclk1*. Black arrows depict IECs within the ileal crypts positively stained for *Dclk1*. (B) Quantification of Tuft cells present in the ileal villi; (C) Ileal Crypts; and (D) Colonic Crypts. Columns show mean ± standard deviation. One-way ANOVA and Tukey's post-hoc test. (*) = $p<0.05$; (**) = $p<0.01$.

color or displayed little and diffuse staining in the cytoplasm (yellow arrows). Figure 2B shows a reduction in the number of *Tff3*$^{+}$ colonic goblet cells counted in colonic crypts between *Ptpn2*-KO versus *Ptpn2*-WT and *Ptpn2*-Het mice.

3.3. Loss of Constitutive *Ptpn2* Decreases the Number of Tuft Cells in the Ileal Villus and Colonic Crypts

Since the major IEC secretory cells, Paneth and goblet cells, were found in diminished numbers in the gut epithelium of *Ptpn2*-KO mice, we next wanted to examine the number of tuft cells in the small and large intestine of the same mice. The abundance of tuft cells in the gut is reported to be much smaller compared to goblet cells, representing only around 0.4% of all IEC subtypes in the mouse, which could further suggest an intrinsic role for *Ptpn2* in the differentiation of intestinal epithelial secretory cell subtypes.[18] In figure 3A we saw a significant decrease in the number of tuft cells present in the ileal villi of *Ptpn2*-KO mice compared to *Ptpn2*-WT mice by immunohistochemistry. In figure 3B, the counting of *Dclk1*$^{+}$ tuft cells revealed a significant difference in the number of these cells in the ileal villus of *Ptpn2*-WT versus *Ptpn2*-Het, and versus *Ptpn2*-KO mice. However, we did not observe differences in the abundance of tuft cells in the ileal crypts of the same mice (Figure 3B). On the other hand, the

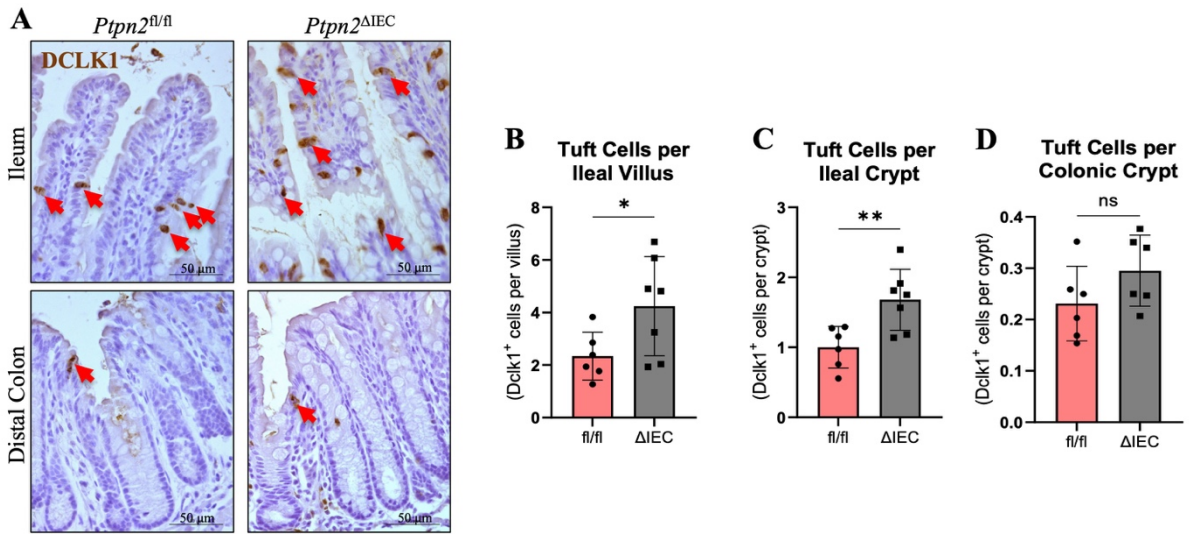

Figure 4A-D. Immunostaining for *Dckl1*, a tuft cell marker, showed increased numbers in ileal villus and in ileal crypts between *Ptpn2*$^{\Delta IEC}$ vs. *Ptpn2*$^{fl/fl}$ mice (n=6-7). However, there was no significant change in the number of tuft cells in the colon of *Ptpn2*$^{\Delta IEC}$ vs. *Ptpn2*$^{fl/fl}$ mice (n=6-7). Student t-test with alpha set at 0.05.

number of tuft cells in colonic crypts of *Ptpn2*-KO versus *Ptpn2*-WT and *Ptpn2*-Het mice was decreased (Figure 3D).

3.4. Loss of Epithelial *Ptpn2* Increases the Number of Ileal Tuft Cells and Colonic Goblet Cells in Mice

We previously showed that constitutive loss of *Ptpn2* results in a decreased number of ileal villus and colonic tuft cells. However, mice harboring constitutive loss of the *Ptpn2* gene develop systemic inflammation around day 12 post-birth, succumbing between 3-5 weeks of age as previously reported.[29,34] Since the inflammatory status of the intestine could be driving the phenotypical changes observed here, we sought to determine whether *Ptpn2* function is intrinsically important in the differentiation of intestinal epithelial secretory cells. As such, we investigated the abundance of intestinal tuft and goblet cells using an inducible epithelial-specific *Ptpn2* deletion mouse model. Figure 4A-D shows immunostaining for *Dclk1*, a tuft cell marker, displaying increased numbers in ileal villus and ileal crypts of *Ptpn2*$^{\Delta IEC}$ (epithelial specific *Ptpn2*-KO) vs. *Ptpn2*$^{fl/fl}$ (control) mice. However, there was no significant change in the number of tuft cells in the colonic crypts of our mice.

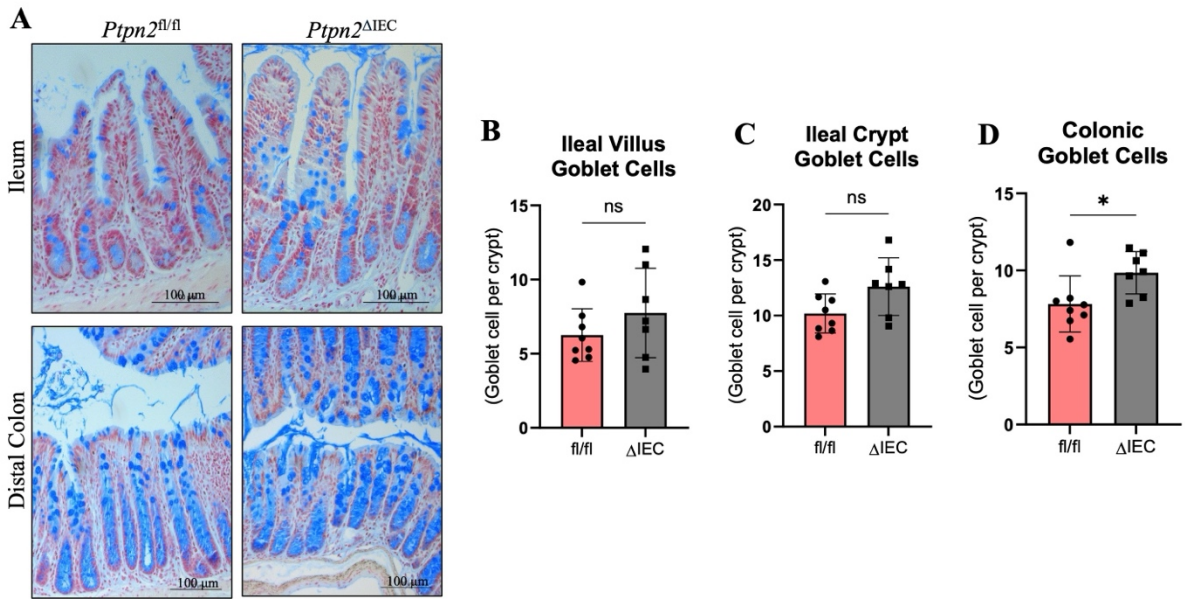

Figure 5A-D. Alcian blue staining allowed for identification of goblet cells in the ileum and colon of *Ptpn2*$^{\Delta IEC}$ vs. *Ptpn2*$^{fl/fl}$ mice. Though there was no significant change in the number of goblet cells in the ileum, both in the crypts and villus, but there was a significant increase in the number of goblet cells in the colon of *Ptpn2*$^{\Delta IEC}$ mice vs. control (n=6). Student t-test with alpha set at 0.05.

On the other hand, by using Alcian blue staining for identification of goblet cells in the ileum and colon of the same mouse line, we observed no significant change in the number of goblet cells in the crypts and villi of the ileum but there was a significant increase in in the number of goblet cells in the colon of *Ptpn2*$^{\Delta IEC}$ (epithelial specific *Ptpn2*-KO) vs. *Ptpn2*$^{fl/fl}$ (control) mice (Figure 5A-D).

3.5. Loss of Epithelial *Ptpn2* Does Not Alter Ileal Epithelial Expression of Secretory Cell Markers in Mice

To further evaluate whether *Ptpn2* is important in the function and differentiation of IEC subtypes, we isolated RNA from enriched-ileal IECs from tamoxifen-inducible epithelial-specific *Ptpn2*-KO (*Ptpn2*$^{\Delta IEC}$) and *Ptpn2*-WT (*Ptpn2*$^{fl/fl}$) mice. Our data indicated that loss of epithelial *Ptpn2* did not change ileal epithelial expression of secretory cell markers or differentiation factors. The *Kruppel-like factor 4* (*Klf4*; goblet cell differentiation factor), and the goblet cell markers, *Mucin 2* (*Muc2*), and *Trefoil factor 3* (*Tff3*), were unchanged (Figure 6A-C).

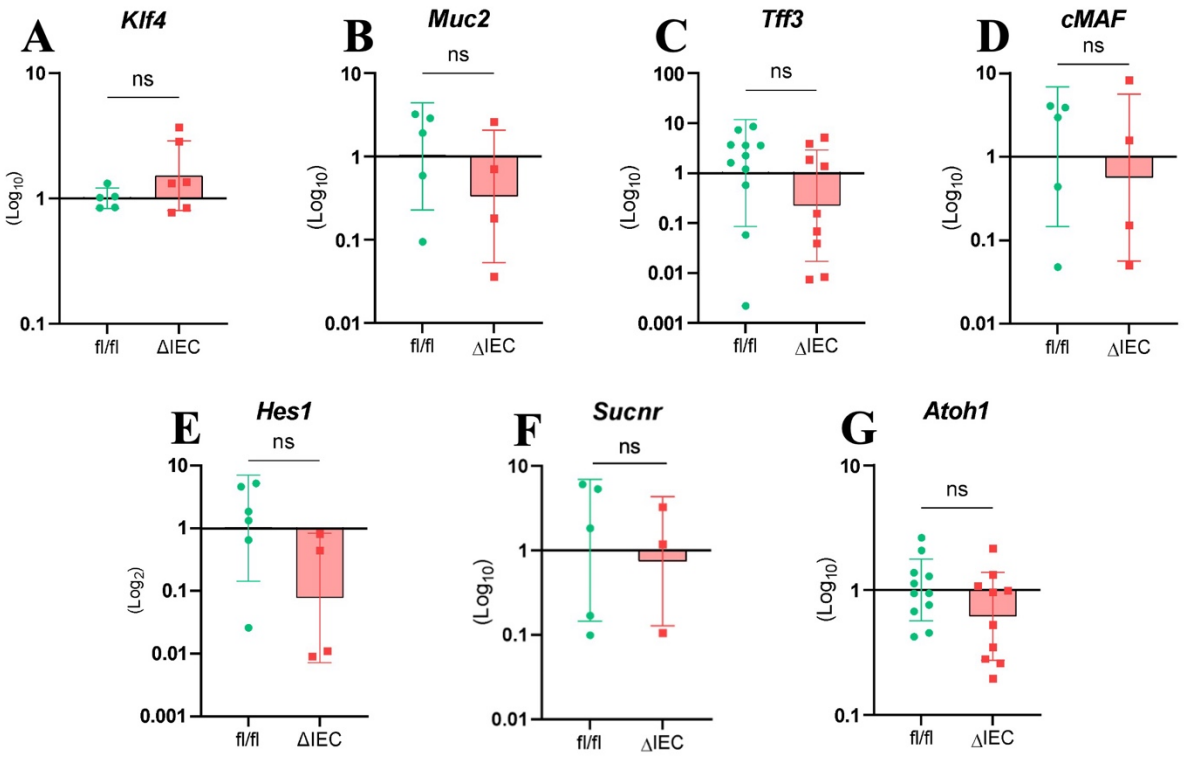

Figure 6A-G. Ileal IECs from tamoxifen-inducible epithelial-specific *Ptpn2*-KO (*Ptpn2*$^{\Delta IEC}$) and *Ptpn2*-WT (*Ptpn2*$^{fl/fl}$) mice were collected for total RNA isolation. Gene expression of targets for individual IEC subtypes was analyzed by qPCR. Figure 6A (*Klf4*), 6B (*Muc2*), and 6C (*Tff3*) show genes associated with goblet cell function and differentiation. Figure 6D (*cMAF*) and figure 6E (*Hes1*) show genes associated with enterocyte function and differentiation. Figure 6F (*Sucnr*) shows a gene associated with tuft cell function, and figure 6G (*Atoh1*) shows a gene associated with secretory cell (goblet, Paneth, and tuft cells) differentiation.

Expression of *cMAF* and *Hes1,* a marker of enterocytes, and a differentiation factor for absorptive IECs, respectively, were unchanged as well (Figure 6D-E). Expression of the Succinate receptor gene *Sucnr,* which is associated with tuft cell function, was unaltered (Figure 6F). Finally, the differentiation factor for intestinal secretory cells *Atoh1*, including goblet, tuft, and Paneth cells, was unchanged as well (Figure 6G) when compared with the control group.

3.6. Epithelial *Ptpn2* Deletion Does Not Affect Expression of IEC Subtype Markers in an Enteroid Model

Next, we wanted to further investigate whether *Ptpn2* is intrinsically important in the abundance and differentiation of IEC subtypes. Therefore, we used enteroid models which allowed for isolation of IECs as they were the only cells present in the model. Our data revealed

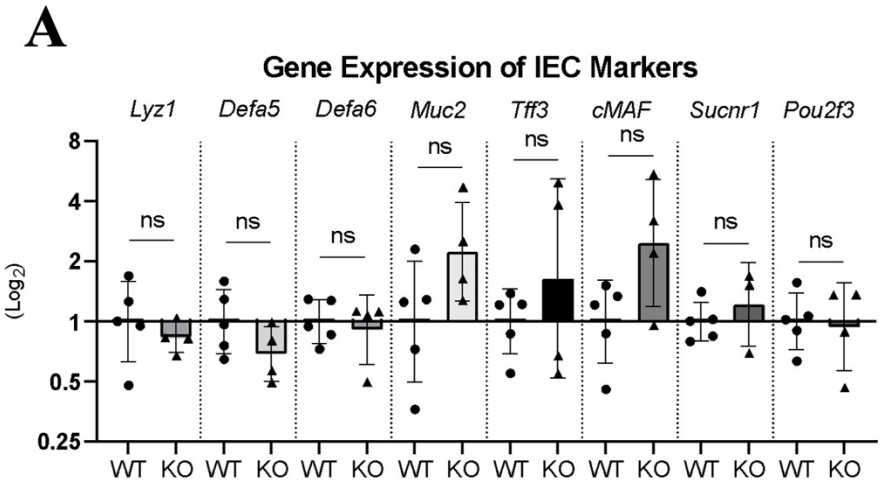

Figure 7. (A) Undifferentiated enteroids were grown in ENR media for 3-4 passages until growth and enteroid numbers were comparable between Ptpn2-KO and Ptpn2-WT genotypes. RNA was isolated from enteroids right before passing (~5-7 days). Gene expression of targets for IEC subtypes was analyzed by qPCR. The expression of IEC subtype-associated genes Lyz1, Defa5, and Defa6 (Paneth cells), Muc2 and Tff3 (goblet cells), cMAF (enterocytes), and Sucnr1 and Pou2f3 (tuft cells), showed no significant difference in Ptpn2-WT versus -KO enteroids. n=5; student t-test with alpha set at 0.05.

that epithelial *Ptpn2* deletion did not affect expression of IEC subtype markers in enteroids (*ex vivo*). These IEC subtype-associated genes included *Lysozyme 1*, *Defensin alpha 5*, and *Defensin alpha 6* for Paneth cells, *Mucin 2* and *Trefoil factor 3* for goblet cells, *cMAF* for enterocytes, and *Succinate receptor 1* and *Pou2f3* for tuft cells, all of which showed no significant difference in their expression between *Ptpn2*-WT and *Ptpn2*-KO enteroids (Figure 7A).

4. DISCUSSION

The loss of *Ptpn2* increases intestinal barrier permeability, induces changes in the tight junctions of the epithelial cells, modifies IEC-macrophage communication, decreases Paneth cell and Paneth cell-specific antimicrobial peptide viability, and causes shifts in the intestinal microbiota which allows for increased expansion of pathobiont bacteria associated with clinical IBD, as previously shown by our group and others.[2,28-32]

Additional unpublished data from our group showed constitutive *PTPN2* loss induced an increase in IEC proliferation in the large intestine, an increase in immune cell-derived TNF/IFN-y and IL-22, a decrease in the expression of differentiation factors associated with secretory cell

lineages, and a decrease in ileal and cecal goblet cell numbers (Canale, Vinicius, McCole, Declan F., *unpublished*). IEC proliferation in the proximal and distal colon was analyzed via staining for proliferation marker Ki-67 (green) and cell nucleus marker DAPI (blue).[2] Therefore, *PTPN2* loss affecting IEC differentiation was examined because data showed increased IEC proliferation, but no change in IEC death (Canale, Vinicius, McCole, Declan F., *unpublished*). In addition, potential functional changes in IEC differentiation are suggested since these mice also showed gut dysbiosis with increased abundance of the pathobiont mouse adherent and invasive *E. coli* (mAIEC).

In this study, we identified that the number of goblet cells, tuft cells, and goblet cell antimicrobial peptide production were decreased in mice with constitutive *Ptpn2* loss, suggesting an impaired innate immune response in these mice. It is known that control of immune responses is important in preventing tissue damage.[36] *PTPN2*, which is ubiquitously expressed, plays an important role in immune responses.[37] Previous research showed systemic changes due to excessive inflammation arising from hyperactive T cells and *PTPN2*-deficient dendritic cells (DCs) leading to elevated circulating and tissue levels of inflammatory cytokines such as interferon-gamma (IFN-γ). Moreover, *Ptpn2*-deficient macrophages were shown to disrupt the intestinal epithelial barrier.[38,39] This confirms intense inflammation follows constitutive loss of *Ptpn2* due to loss of *Ptpn2* inhibition of immune cell activations.

By using a mouse model with epithelial-specific *Ptpn2* loss, it allowed us to examine how IECs function in the presence of normal innate and adaptive immune responses. Surprisingly, we identified a significant increase in numbers of intestinal tuft and goblet cells when compared with $Ptpn2^{fl/fl}$ mice. Given that the $Ptpn2^{\Delta IEC}$ mice do not display severe systemic inflammation, this could contribute to a different phenotype regarding the abundance of

IEC subtypes (Canale, Vinicius, McCole, Declan F., *unpublished*). However, the increase in the number of these cells was not accompanied by elevated expression of IEC differentiation factors and cell markers analyzed by qPCR using enriched-IECs from these mice. While we were not able to identify a mechanism by which this alteration occurred, nonetheless our findings clearly show that a lack of intestinal *Ptpn2* expression leads to increased numbers of intestinal epithelial secretory cells *in vivo.* To assert, whether *Ptpn2* expression is intrinsically important regarding the differentiation and expression of IEC markers, we used enteroid models which are composed of only IECs growing *in vitro*, where contributions from the tissue surroundings, such as fibroblasts, blood stream, and immune cells were absent. In other words, they show the intrinsic relationship between epithelial loss of *Ptpn2* and IEC function and differentiation. Under these conditions, we observed no changes in the expression of IEC markers and differentiation factors, indicating that a non-epithelial factor(s) is necessary to induce the skewed number of IEC subtypes observed *in vivo*.

Future steps will include challenging the enteroids with inflammatory mediators such as cytokines to evaluate how IECs without *Ptpn2* act under inflammatory conditions. In doing so, we can determine whether inflammatory signals can induce a different response regarding IEC differentiation in epithelial-specific *Ptpn2* deficient mice.

5. CONCLUSION

In conclusion, constitutive *PTPN2* loss induces proliferation of colonic IECs while impairing expression and abundance of critical IEC subtypes, thereby compromising intestinal barrier defenses (Canale, Vinicius, McCole, Declan F., *unpublished*) In addition, epithelial-specific *Ptpn2* loss increases the number of ileal villus tuft cells and colonic goblet cells. While the mechanisms involved in this phenotype must be further investigated, the lack of effect observed

in our enteroid models indicates that a non-epithelial factor is necessary to modulate the number of IEC subtypes observed *in vivo*. As such, we plan to investigate how our enteroid models react with inflammatory markers in order to evaluate the mechanisms of how IEC subtypes act under inflammation on a background of epithelial-specific *Ptpn2* loss. We anticipate that this will reveal novel insights into how epithelial PTPN2 regulates differentiation and function of intestinal epithelial subtypes that play essential roles in gut homeostasis and host-microbe interactions.

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