COMMENSAL GUT BACTERIA REGULATE PANETH CELL FUNCTION PUTATIVELY VIA SIGNALING THROUGH THE SUB-EPITHELIAL TISSUE

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ABSTRACT

Crohn's Disease (CD) is an inflammatory disorder of the intestines caused by a dysregulated immune response to resident gut bacteria. Presently, most treatments for CD suppress the host immune system, which can lead to adverse side effects such as increased susceptibility to infections and malignancy. Hence, low-risk alternative treatment options are needed. Studies suggest that defects in Paneth cells (PCs) contribute to CD pathogenesis. PCs are specialized intestinal epithelial cells that regulate the gut microbiota through the secretion of antimicrobial peptides (AMPs). However, the reciprocal impact of the gut microbiota, as well as the role of the underlying intestinal stroma on PC function is still unclear. We hypothesized that microbes induce PC AMP expression through signaling from sub-epithelial stromal tissue of the intestines. To test this hypothesis, we compared the abundance of selected PC AMP mRNAs between specific pathogen free (SPF) mice that have a normal gut microbiota, and germ-free (GF) mice that have a sterile gut. AMP transcript levels were also compared in an *in vitro* system (enteroids) cultured from SPF and GF epithelial cells. The enteroid system is devoid of underlying stroma and bacteria, and thus provides a model to study the impact of these components on PC function. *In vivo*, AMP expression was significantly higher in SPF small intestine tissue than in GF tissue. However, *in vitro*, AMP expression in SPF enteroids decreased to that of GF enteroids over time. Interestingly, with the addition of sub-epithelial tissue to the enteroid system, AMP expression in SPF enteroids was restored to our *in vivo* measurements. In contrast, the addition of bacterial products (such

as LPS) without sub-epithelial tissue did not induce AMP expression. Based on these findings, we conclude that the sub-epithelial tissue is required for appropriate PC AMP expression. Given the reduction of AMP expression in GF mice, it is possible that the commensal microbiota regulate AMP expression of the PCs, through signaling from the sub-epithelial tissue. This work also highlights the limitations of the enteroid system when studying PC function since the simplified culture conditions neglect possible interactions with the underlying stroma and bacterial ligands. Hence, this limitation should be taken into consideration when using enteroids for future PC studies in CD.

ACKNOWLEDGEMENTS

This thesis would not have been possible without the guidance and support from a number of individuals. Firstly, I would like to thank my P.I., Dr. Ajay S. Gulati, for providing me the opportunity to advance my education in biology through his research lab. His enthusiasm for science has been an inspiration throughout my research experience. I would also like to thank my mentor, Alexi Schoenborn, who for almost three years provided the intense and constant initiative, guidance, and mentorship that made this thesis and my entire research experience possible. I would also like to thank Dr. Michael Shanahan and Dr. Allison Rogala for their continued support in the Gulati lab. Additionally, the flow cytometry data presented here would not have been possible without Dr. Susan Henning and Dr. Rich von Furstenberg. Along with my mentors in the lab, my friends and family provided me tremendous support throughout the completion of this thesis.

INTRODUCTION

Inflammatory bowel disease, also known as IBD, is a chronic inflammatory disease of the gastrointestinal tract. There are two major subtypes of IBD: Crohn's disease (CD) and ulcerative colitis (UC) [2]. Although these two subtypes may have overlapping symptoms, they each affect a different region of the gastrointestinal (GI) tract - ulcerative colitis is limited to the colon, while CD can affect any part of the gastrointestinal tract, though it is usually found in the distal portion of the small intestine [16]. Our lab is interested in understanding the underlying mechanisms in Crohn's disease, and therefore the focus of this project is on the small intestine.

Most CD patients develop symptoms of abdominal pain, diarrhea, and loss of appetite. Unlike many other inflammatory diseases, CD tends to emerge during adolescence or in early adulthood. Since CD is chronic, the severity of most symptoms fluctuates over time and can be accompanied with delayed puberty, decrease in bone density and eventually culminate into an overall decrease in a patient's quality of life [15]. Even though it affects nearly 1.4 million Americans, the cure for CD is yet to be discovered. Over the years, the only treatment widely used today involves suppressing the immune system of the patient [3]. However, this is not ideal, as it can result in increased adverse effects in patients such as reduced ability to fight infections and increased susceptibility to cancer.

Alternative treatment options with lower side effects can be developed by manipulating one of the key players in Crohn's Disease—Paneth cells (PCs). PCs are thought to play a role in some intestinal bowel diseases such as Crohn's disease. These

specialized cells are found throughout the small intestine and reside in the base of the crypts of Lieberkuhn in the intestine. These cells produce antimicrobial peptides (AMPs), a class of natural antibiotics made by the body that protects the host by disrupting the membranes of bacterial pathogens. In addition to regulating pathogens, AMPs also help regulate the normal, commensal bacteria that inhabit the GI tract and provide enteric homeostasis to maintain normal intestinal physiology.

In the case of CD patients, the host immune system mounts an exaggerated response to the commensal bacteria of the gut, indicating a possible role of PCs in CD. Nevertheless, the precise responsibility of these cells in CD is yet to be understood, due to the lack of *in vitro* systems to study PCs. Recently, however, an *ex vivo* system has been developed to enable the culture of intestinal crypts that contain primary intestinal epithelial cells including PCs [11]. In this system, freshly isolated intestinal crypts are obtained from mouse or human tissue and are plated in Matrigel media with growth factors under sterile conditions. These crypts form spherical organoids called enteroids, which contain all differentiated cell types of the small intestine, and contain crypts organized around a central lumen (Fig. 1) [10, 14]. Enteroids can be frozen and cultured for over eight months. Ultimately, it may be possible to study PC function through this enteroid system to clarify the role of these cells in CD and to test potential therapies that could enhance PC function in IBD patients.

Before using enteroids to study PC function, further investigation is necessary to determine if the enteroid system re-creates what is taking place *in vivo*. For example, in the mammalian intestine, resident enteric bacteria are an important factor for homeostasis and play a critical role regulating gut physiology. Some of these processes include

maintaining immune homeostasis and prevention of inflammation. However, enteroids must be grown in sterile system, devoid of microorganisms that could potentially overgrow the culture. Additionally, the enteroids contain solely the intestinal epithelium. Thus, the host intestinal stroma, such as the sub-epithelial tissue, is absent in this *in vitro* system (Fig. 2) [7]. In this study, I hypothesize that the commensal gut microbiota regulates PC function through signaling from the sub-epithelial tissue. This is important because it can then provide information regarding the role of the microbiota as well as the underlying intestinal stroma on PC function.

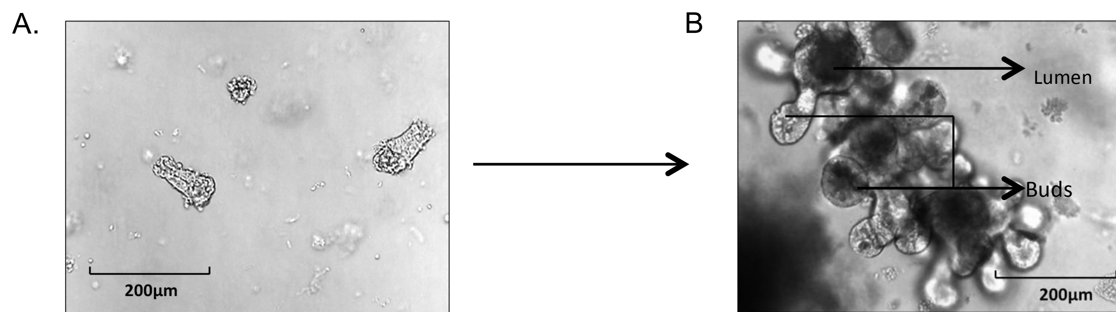


Figure 1. Isolated intestinal crypts from murine jejunum (A). In cell culture, the crypts develop buds to form enteroids (B) [14]. *Image from Stelzner et al., 2012.*

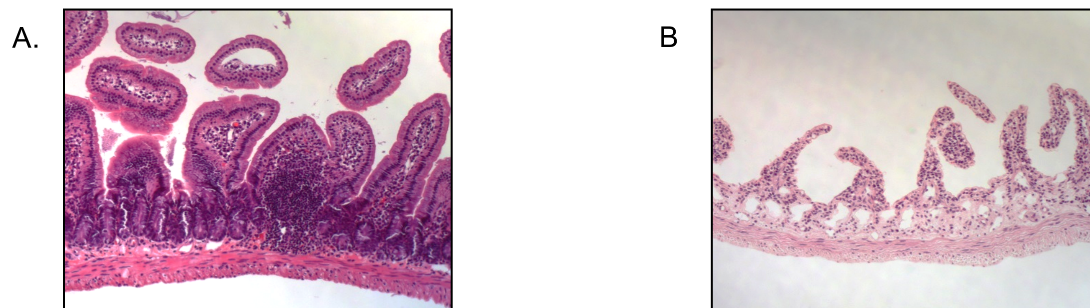


Figure 2. H&E stained jejunal section with all the tissue layers intact (A). H&E stained sub-epithelial tissue of the jejunal section (B). *Images courtesy of Susan Henning.*

METHODS

Mice

Adult, wild-type C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were housed in SPF or germ-free (GF) conditions consistent with guidelines established by the American Association for Laboratory Animal Care and Research. SPF mice were bred for multiple generations in the same animal room and sacrificed within 8-12 weeks of age. Sterilely-derived B6 mice were maintained in GF conditions at the National Gnotobiotic Rodent Resource Center at the University of North Carolina at Chapel Hill.

Histology and PC enumeration

1.5 cm sections of jejunum were fixed in 10% phosphate buffered formalin for 48 hours and were placed in paraffin longitudinally. These sections were then cut into 5 μm and stained using hematoxylin and eosin (H&E). The H&E slides were then observed under a bright field microscope for assessment. Crypts with an intact lumen were considered for enumeration. The number of PC in each location was recorded, by counting the granulated eosinophilic cells, for at least 10 crypt/villus units per section.

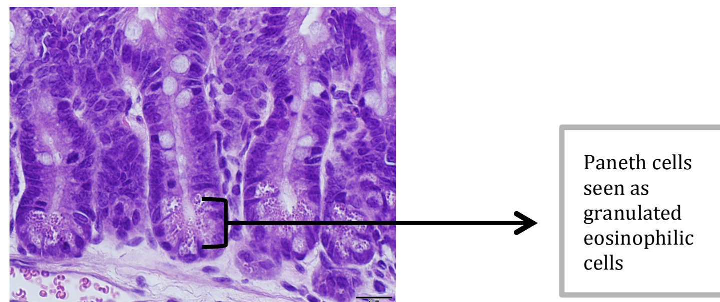


Figure 3: Histological stain of jejunal section depicting Paneth cells.

Small intestine morphology

Using Image-J software, the villus height was measured from apical tip to the base and the crypt depth was measured from the sub-mucosa to the base of the villi. For each intestinal sample, at least 10 well-oriented pictures were measured.

Flow cytometry

For each flow experiment, jejunal epithelial cells were isolated from a pool of 4 mice using EDTA/dispase digestion. The cells were fixed in 4% paraformaldehyde for 15 mins and resuspended in saponin permeabilisation buffer (Invitrogen, Carlsbad, California, USA) with Lyz-FITC (1:10 Dako, Carpenteria, California, USA) for 30 min since Lyz is an established marker for PC. Flow analysis was performed per established protocols [4].

Crypt culture

10 cm segments of jejunum from SPF and GF mice were obtained. These tissues were first flushed with 1x PBS, flayed and then cut into 1cm segments, to be placed in tubes containing 5 mL of 1x PBS on ice. They were then transferred to another tube containing 5 mL of 1x PBS and 3.5 mM EDTA (ethylenediaminetetraacetic acid) and placed in the cold room on a gentle shaking platform for 20 minutes. The tissues were then moved to fresh cold 1x PBS to be manually shaken. The solution was then filtered using a 70 µm nylon filter to enrich for crypts. The crypts were then centrifuged at 1000 RPM for 10 minutes at 4°C. The pelleted crypts were added to Matrigel membrane matrix. They were plated with 10 µl of Matrigel crypt mixture per well. The culture conditions used are listed in a table in the appendix. The culture was monitored daily, and after six days,

several wells of enteroids were pooled and isolated for RNA. The remaining wells were then passaged as below.

For sub-epithelial tissue (SET) co-culture experiments, crypts were first isolated from jejunum. The remaining tissue, devoid of crypts, was then shaken aggressively to remove the epithelium and isolate the SET. 5 cm of the SET was then minced in 200 µL phosphate buffered saline and added to the Matrigel (1.0-2.0 µl of minced SET/ 9 µl of Matrigel). Crypts (60-100) were then added to this mixture before plating.
For assessing the transcript expression level, the SET was stored in 600 µL Buffer RLT (Qiagen, Valencia, CA) with 10 µL/mL of B-ME. It was then vortexed and frozen at -80.

Passaging crypt culture

Media was removed from all the wells and then 200 uL of 1X PBS + 10 µM Y27632 solution was added to each well. The Matrigel patty was scrapped off the bottom of the plate using a P1000 tip. The contents of the well were then aspirated using a 1 mL syringe and 31g needle. The contents were next placed into a 15 mL tube and centrifuged at 1000 RPM for 10 minutes at 4°C. The pellet was then plated as per primary crypt culture.

RNA isolation

1) *Tissue:* Jejunum was placed in a tube containing RNAlater solution for RNA isolation and then stored at -80 degrees Celsius. Prior to RNA isolation, tissues were homogenized in beta-mercaptoenthanol (BME) and RLT lysis buffer. Total RNA was isolated from these samples using a Qiagen MiniKit per manufacturer’s instructions.

2) *Enteroids:* Cultures with enteroids were collected. These enteroids were stored in a solution of BME and lysis buffer RLT to inactivate intracellular RNases from degrading the RNA. Total RNA was then isolated using the Qiagen MiniKit according to the manufacturer's instructions. After total RNA has been isolated, the quality was evaluated using a spectrophotometer and then also running an RNA gel.

Quantitative RT-PCR

The expression levels of antimicrobial peptides including lysozyme (Lyz), cryptidin-related sequence-1c (Crs1c), pan C-reactive protein (Pan-crp), and angiogenin 4 (Ang4) and regenerating islet-derived protein 3 gamma (Reg3γ) were evaluated using quantitative reverse transcriptase polymerase chain reaction. The primer/probe sets for these genes were obtained from Life Technologie. The cDNA of the RNA samples was developed using SuperScript II Reverse Transcriptase. Using TaqMan Gene Expression Master Mix, the quantitative RT-PCR was performed according to the manufacturer's instructions. The expression level of β-actin was used as a baseline and as an internal control since the expression of β-actin is required in all cell types [8].

Statistical analysis

The graphical representation of qRT-PCR of the AMP expression levels as well as the PC number was done on Graph-Pad Prism 5 (GraphPad, San Diego, California, USA). All variables were found to have a normal distribution. Means were compared using Student's t test (2-tailed).

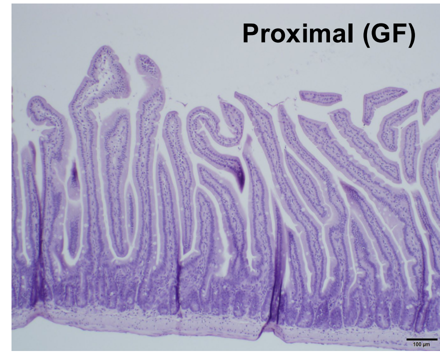
RESULTS

Jejunum morphology is impacted by the presence of microbes with variability depending on the location

Previous studies examining the role of microbiota on small intestine development have yielded conflicting data. In one study, the villus height and the crypt depth of the small intestine increased when exposed to bacteria [13]. However, another study presents a different data. Specifically, mice exposed to antibiotics were shown to have increased villus height, but decreased crypt depth [9]. We hypothesized that the variability in these data were in part due to the differential effects of the microbiota on the intestinal epithelium within varying locations of the small intestine. To test this, seven GF and seven SPF mice were utilized for histological analysis for the jejunal tissue, comparing the proximal and distal regions. Figure 4 shows the crypt depth and villus height for GF and SPF mice. Panels *A* and *B* show histology from the proximal jejunum of SPF and GF mice, while panels *E* and *F* depict histology from the distal jejunum of SPF and GF mice. Quantitative data depicting differences in villus height and crypt depth between SPF and GF mice are shown in panels *C* and *D* (proximal jejunum) and panels *G* and H (distal jejunum). Note that there is a significant difference in the villus height in both the proximal and distal regions of the jejunum when comparing SPF and GF mice. However, there is only a significant difference in crypt depth in the proximal jejunum between the SPF and GF mice.

A.

B.



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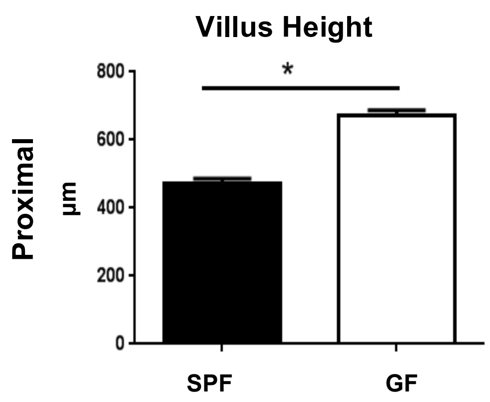

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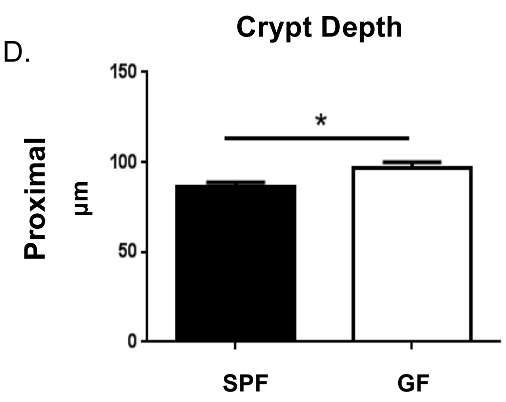

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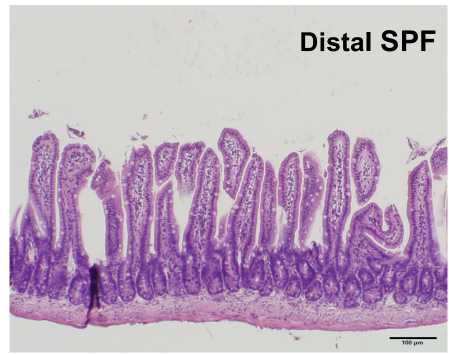

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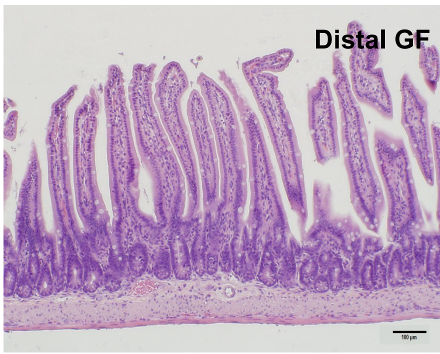


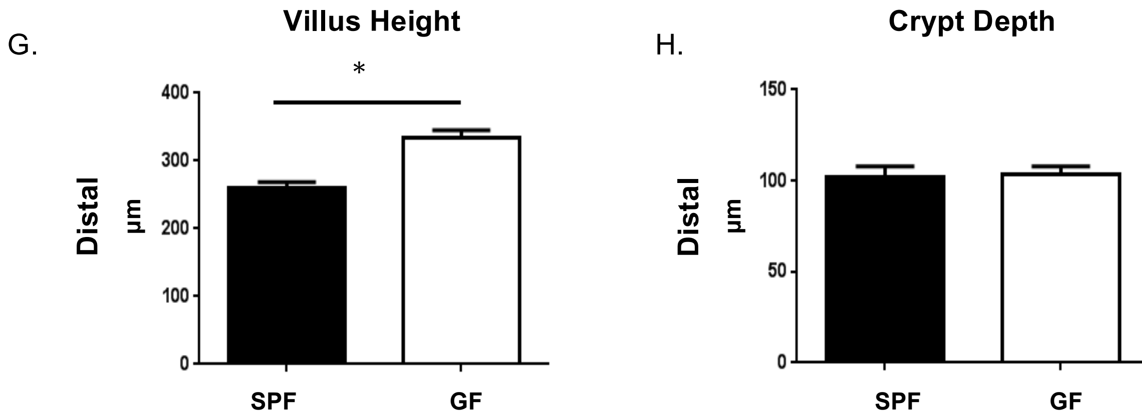


Figure 4: H&E stained images of (A,B) proximal (n=16 SPF, n=14 GF) and (E,F) distal jejunum (n=7 SPF, n=7 GF). (C, D) Proximal jejunum quantitative analysis for villus height and crypt depth. (G,H) Distal jejunum quantitative analysis of for villus height (p=0.0012) and crypt depth. *P<0.02

Paneth Cell Number is Decreased in Distal, But Not Proximal Jejunum of Germ-Free Mice

Given the clear impact of intestinal location on microbial influences in jejunal morphology, we next postulated a similar phenomenon could be relevant to PCs. To explore this further, we examined differences in PC number between GF and SPF mice in both proximal and distal regions of the jejunum. Using the same samples mentioned earlier, PC numbers were assessed by first identifying crypt-villus units. In Figure 5, images *A-D* show the stained images of proximal and distal regions of GF and SPF mice. Panels *E* and *F* of Figure 5 are the numerical representations of PC number of GF and SPF mice from proximal and distal jejunum. There was a significant decrease in the number of PCs in the distal region of the jejunum when GF and SPF mice were compared. However, no significant difference was seen in the proximal region.

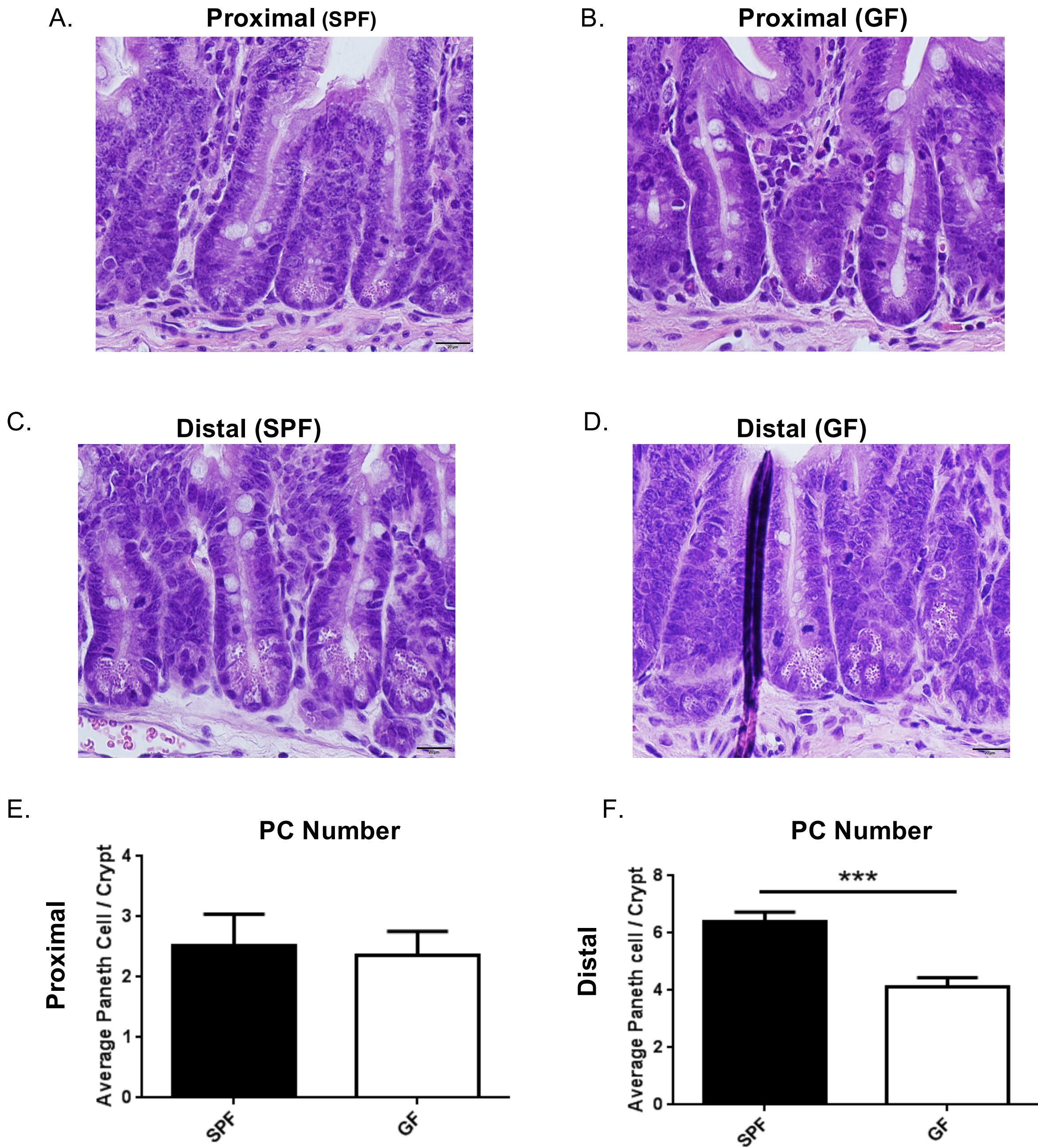
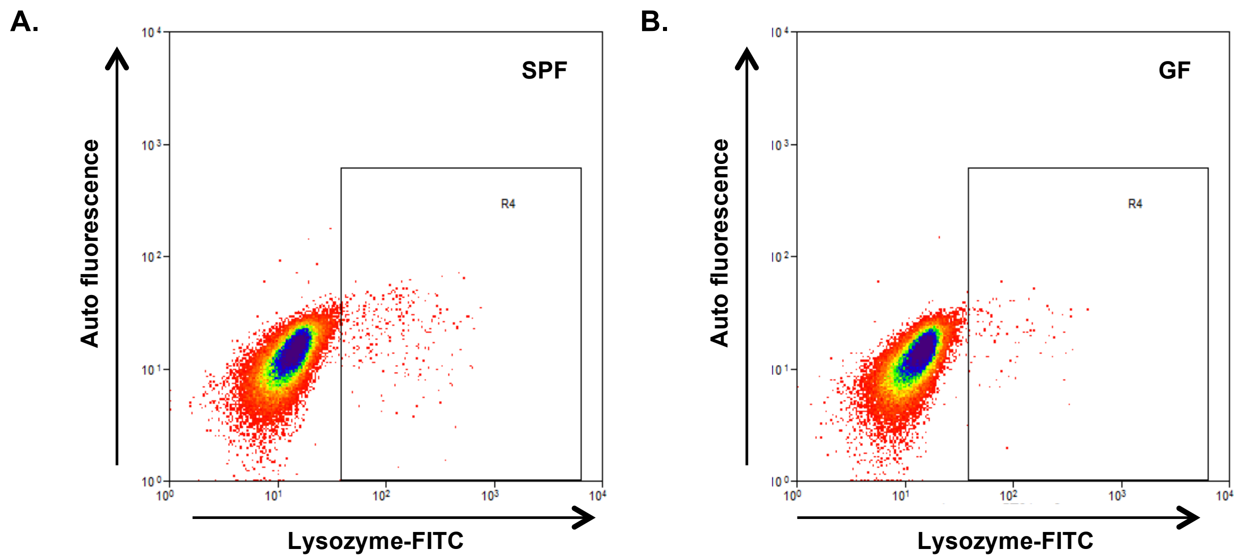


Figure 5: H&E stained images of the crypt structure of proximal jejunum of SPF (A), GF (B) as well as distal jejunum of SPF (C) and GF (D) tissues. Quantification of Paneth cell number in proximal jejunum (E) with p=0.43 and distal jejunum (F) with ***p=0.0003.

Germ-Free Mice Have Fewer Paneth Cells than SPF Mice Across the Entire Jejunum

Having demonstrated that location within the jejunum can affect relative PC numbers between SPF and GF mice, we next sought to eliminate this confounder by determining PC numbers in the entire jejunum of SPF and GF mice. To do this, we used flow cytometery of epithelial cell preparations derived from the whole jejunum. Using this approach we quantified the number of lysozyme $(Lyz)^+$ cells in the jejunum of SPF versus GF mice. Lyz is an established marker of PCs [4]. Figure 6 shows the quantification of PCs from the total jejunal tissue in GF and SPF mice. As demonstrated, GF mice have fewer jejunal PCs than their SPF counterparts.



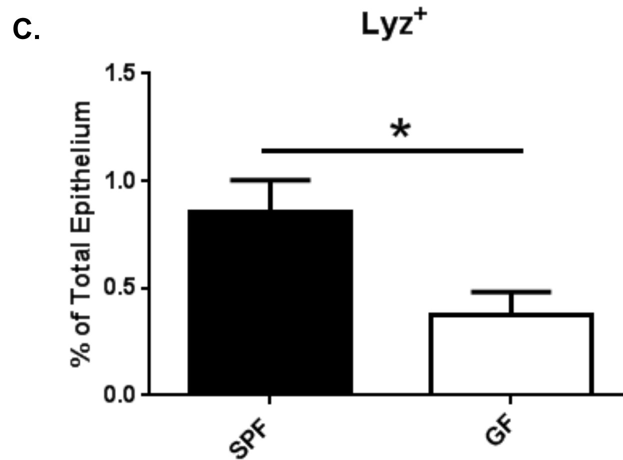


Figure 6: The percentage of PCs in the total jejunum from SPF (A) and GF (B) mice. PCs are enumerated as Lyz^+ cells. Quantification of PC number in the total epithelium (C) with $p<0.05$. The data is obtained from 5 independent experiments with n=4/ mice group.

Reg3γ Transcript Expression is Lower in GF Mice, Compared to SPF Mice

With differences in PC number established earlier, we sought to understand if the mRNA expression levels of AMPs were affected by this difference. Figure 7 represents the mRNA expression levels of major PC AMP classes from the crypts of SPF and GF murine jejunum. The data is obtained from 7 mice from both SPF and GF conditions. Only Reg3γ showed a significant difference between these two groups. The transcript expression of the other major AMPs did not show any significant differences.

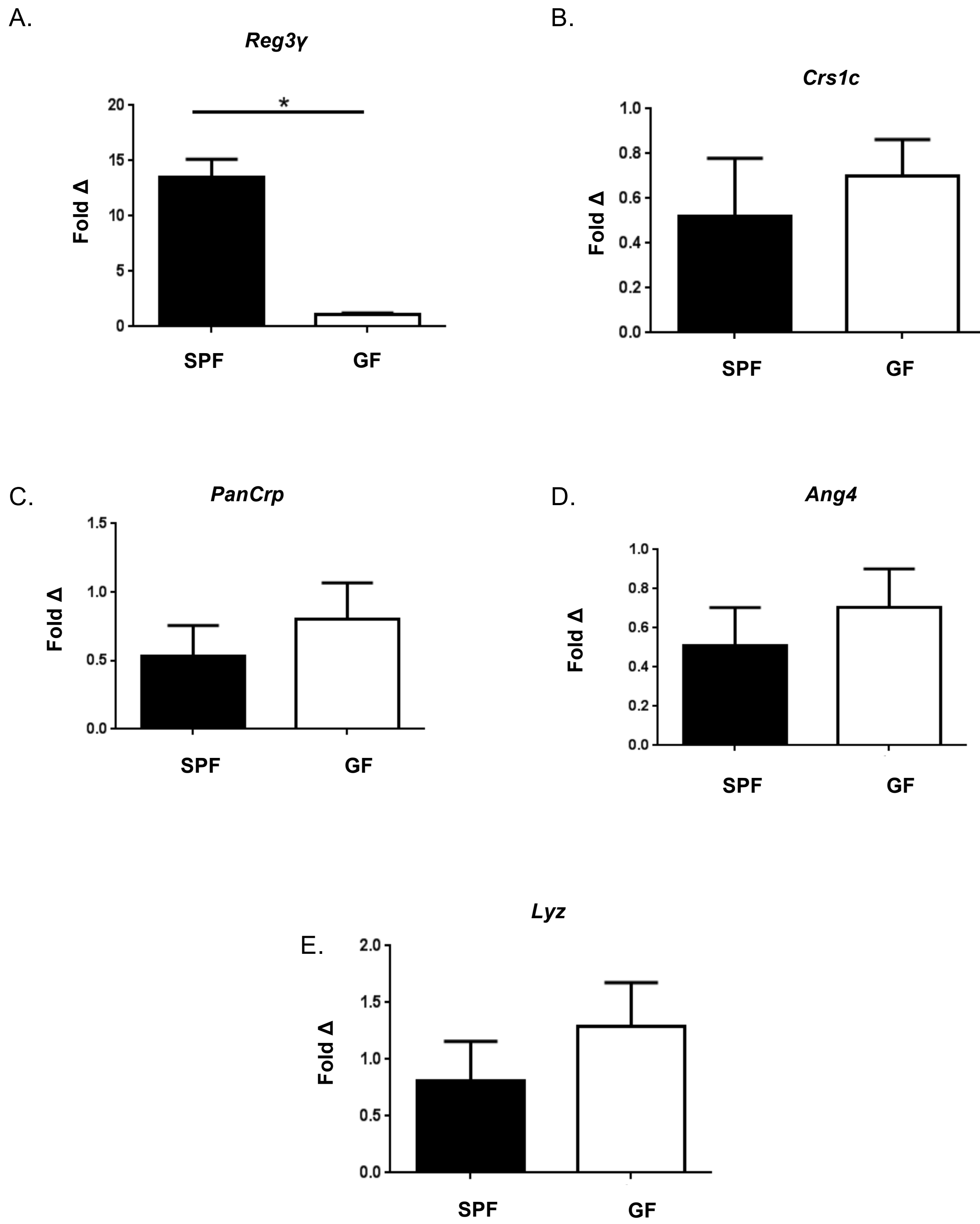


Figure 7: Paneth cell mRNA expression levels of major AMPs from crypts of GF and SPF mice (n=7 mice/group). *P=0.0006.

Reg3γ Expression is Similar in SPF- and GF-Derived Enteroids

As shown above, *in vivo,* there was a significant difference in the expression level of Reg3γ between SPF and GF mice. To study the mechanism of this differential regulation, we sought to derive an *in vitro* system that could be used to study which factors contribute to these differences. To do this, we turned to the enteroid culture system described above. In Figure 8, Reg3γ expression levels are shown in our enteroid *in vitro* system. Reg3γ transcript levels in freshly isolated crypts are shown first, expectedly reproducing our findings in Figure 7A. Reg3γ expression levels are next indicated in 6-day old enteroids and enteroids that have been passaged one or two times. Remarkably, the Reg3γ expression in SPF enteroids quickly equalizes to that of GF enteroids in this culture system, and is maintained at this level after passaging.

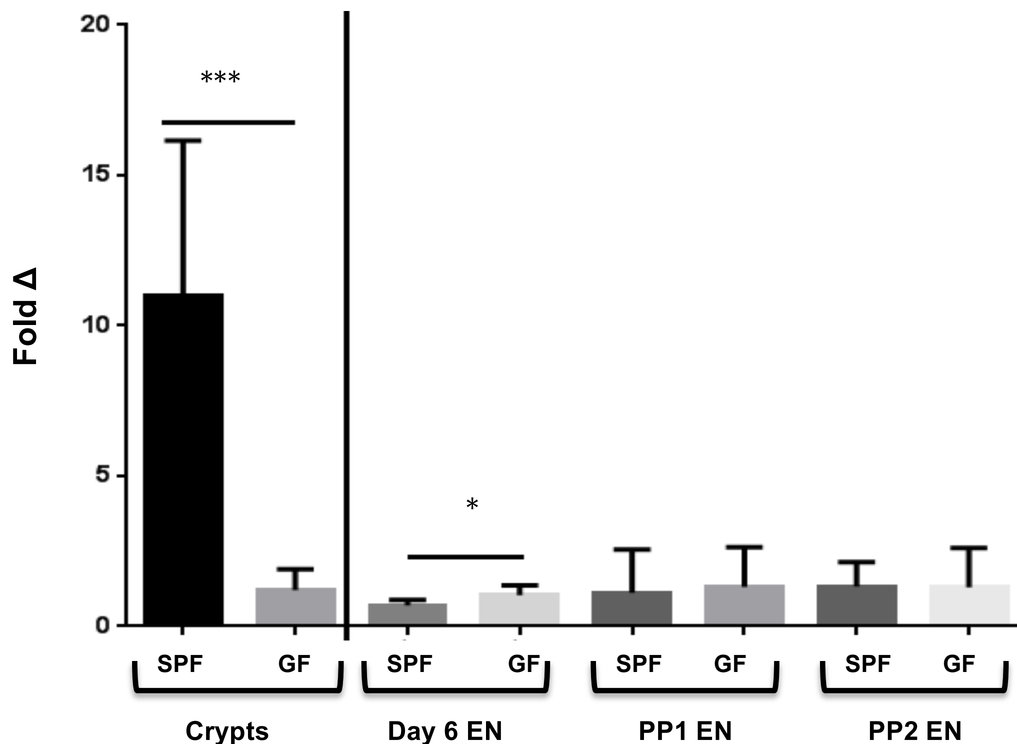


Figure 8: Reg3γ mRNA expression level in crypts, day 6 enteroids (Day 6 EN) and after passaging (PP) 1, and 2. ***P=0.0006 and *P=0.02.

Introduction of Sub-Epithelial Tissue Induces Reg3γ Expression in SPF Enteroids

As shown in Figure 8, the increased Reg3γ mRNA expression levels in SPF mice equilibrates to GF levels in enteroid culture conditions. This provides an ideal setting to understand the factors regulating the expression of this AMP. Because the enteroid culture system is sterile and devoid of bacteria and bacterial products, we first added bacterial ligands such as LPS to the cultured SPF enteroids [1]. However, the addition of these ligands did not result in the induction of Reg3γ expression. We next hypothesized that Reg3γ expression was induced by the stroma underlying the intestinal epithelium, as enteroids are cultured solely from the crypt epithelium, and are absent of any such sub-epithelial tissue. These results are shown in Figure 9. As indicated, Reg3γ mRNA is robustly expressed in SPF crypts. However, there is minimal expression in SPF sub-epithelial tissue and in SPF enteroids. Remarkably, the addition of SET to the enteroid culture saw a significant increase in the Reg3γ expression level compared with SPF enteroids alone. These findings suggest the SET plays a key role in inducing Reg3γ mRNA expression.

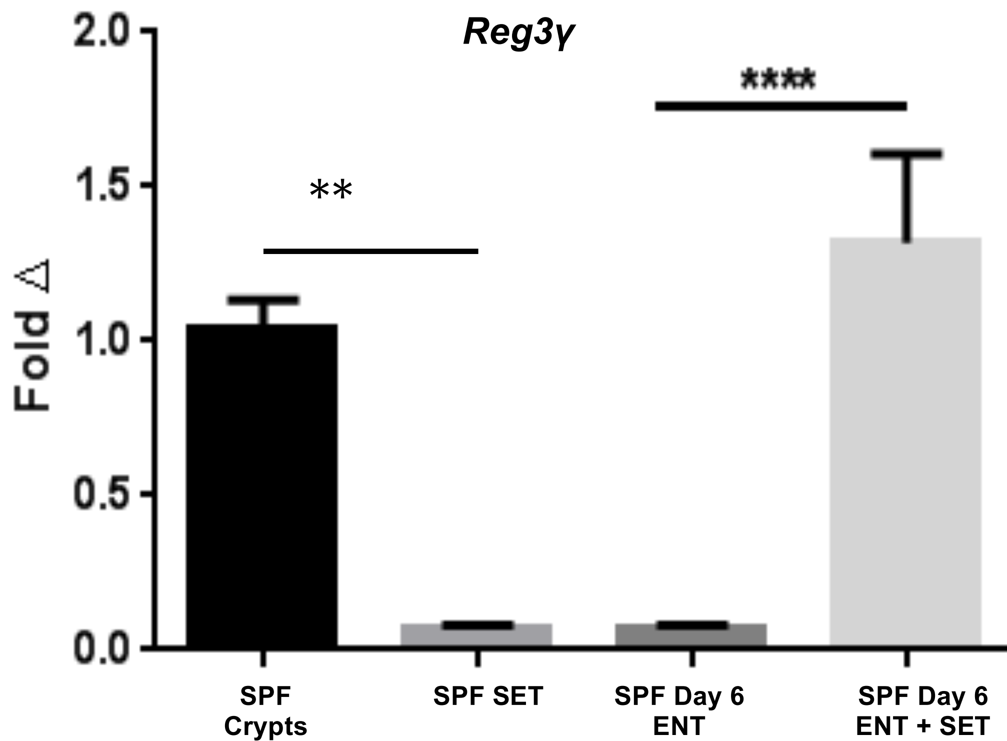


Figure 9: Jejunal mRNA expression levels of Reg3γ of crypts, sub-epithelial tissues (SET), day 6 enteroids as well as day 6 enteroids co-cultured with the sub-epithelial tissue. **P=0.002, ****P<0.0001.

DICUSSION

Previous studies examining the effects of microbiota on PCs have yielded conflicting data. For example, a study done in 1974 by Klockers showed that SPF and GF rats had similar PC numbers. However, a separate study by Satoh suggested that GF rats had lower PC number when compared to SPF rats [6,12]. We hypothesized that these conflicting results occurred because different studies examined different locations of the small intestine. This hypothesis was validated by showing differences in crypt depth and

PC numbers in SPF and GF mice of the distal jejunum, but no such differences between groups in the proximal jejunum. Having demonstrated this variability, we chose to perform subsequent experiments on the entire jejunum, thereby eliminating the confounding variable of proximal versus distal location. Using this new information, we have attempted to determine if PCs are regulated by bacteria, their underlying stroma, or interactions between both.

In this work, we reaffirm the finding that there are differences in expression of the PC AMP Reg3γ between mice raised in SPF versus GF conditions. Due to higher Reg3γ expression levels in the SPF small intestine relative to GF tissue, we can conclude that microbes induce higher transcript expression levels of Reg3γ. Intriguingly, *in vitro*, Reg3γ expression is similar between SPF and GF enteroids. This suggests that something present *in vivo*, but absent *in vitro* regulates Reg3γ expression. Possibilities for this include bacteria and/or the underlying stroma beneath PCs, which we refer to as the sub-epithelial tissue (SET).

The equalized Reg3γ levels between the SPF and GF enteroids in culture conditions, allows us to study the ability of other factors to induce expression of this AMP. The addition of bacterial ligand such as LPS did not lead to an induction of Reg3γ expression in SPF enteroids. However, the addition of SET to the culture system resulted in Reg3γ induction in the SPF enteroids. This suggests that there is signaling from the underlying stroma that regulates Reg3γ expression. This will be an important finding for investigators using this system to study PC physiology, as they must keep in mind that results may not entirely recapitulate the *in vivo* state.

The present study solely focused on the mRNA expression levels of Reg3γ. For further assessment of PC function, it will be beneficial to test and compare other components. These include assessment of Reg3γ protein levels in the same experimental conditions used above. Proteins are the end results of the gene products and hence will provide a better understanding of the molecules involved in PC function. Importantly, the expression levels of mRNA and proteins may not necessarily correlate since some messenger RNA even after transcription might not be translated [5].

In conclusion, our findings show that PC function, particularly Reg3γ expression, is regulated by the sub-epithelial tissue, putatively through signaling from the enteric microbiota. Because the microbiota can be manipulated in patients through interventions such as probiotics, antibiotics, and fecal transplantation, it possible that such therapies could be used in the future to enhance PC function in patients with Crohn's disease. Thus, based on our present findings, we can develop future studies that will assess which particular bacteria are able to influence PC function, and then develop therapies to modulate these organisms in patients with Crohn's disease.

APPENDICES

Culture Media Conditions

Reagent Name	Supplier	Cat. No	Stock Solution	Final Conc.
Advanced DMEM/ F12	Invitrogen	12634-028	_	_
Hepes, 1M	Invitrogen	15630-080	1 M	10 mM
GlutaMAX	Invitrogen	35050-061	100 X	1X
Gen/Kan	Tissue Culture Facility (Lineberger)		100 X	1X
N2 Supplement	Invitrogen	17502-048	100 X	1X
B27 Supplement	Invitrogen	17504-044	50 X	1X
Growth Factors				
hESC qualified Matrigel Membrane Matrix	Fisher		_	_
Human recombinant Noggin	Fisher	1967-NG-025	25 ug	100 ng/mL
Human recombinant R-spondin	Fisher	3474-RS-050	50 ug	500 ng/mL
Human recombinant EGF	Fisher	2028-EG-200	200 ug	50 ng/mL
Crypt Isolation Reagents				
DPBS Ca2+, Mg2+ free	Tissue Culture Facility (Lineberger)			
EDTA	Sigma – Aldrich	431788	.5M	3.5mM
Passage Reagents				
Y27632	Sigma	Y0503-1mg	1 mg	10 mM
Reagent Name	Supplier	Cat. No	Stock Solution	Final Conc.
Advanced DMEM/ F12	Invitrogen	12634-028	_	_
Hepes, 1M	Invitrogen	15630-080	1 M	10mM
GlutaMAX	Invitrogen	35050-061	100X	1X
Gen/Kan	Tissue Culture Facility		100X	1X

	(Lineberger)			
N2 Supplement	Invitrogen	17502-048	100X	1X
B27 Supplement	Invitrogen	17504-044	50X	1X
Growth Factors				
hESC qualified Matrigel Membrane Matrix	Fisher		–	–
Human recombinant Noggin	Fisher			
Human recombinant R-spondin	Fisher			
Human recombinant EGF	Fisher			
Crypt Isolation Reagents				
DPBS Ca2+, Mg2+ free	Tissue Culture Facility (Lineberger)			
EDTA	Sigma – Aldrich	431788	.5M	3.5mM

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