METABOLIC AND MECHANICAL COUPLING OF ENTERIC NERVOUS SYSTEM IN REGENERATING COLON

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By
İlke Sarı
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We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Bahar Değirmenci Uzun (Advisor)

Serkan İsmail Göktuna

Mesut Muyan

Approved for the Graduate School of Engineering and Science:

Orhan Arıkan

Director of the Graduate School

ABSTRACT

Metabolic and Mechanical Coupling of Enteric Nervous System in Regenerating Colon

İlke Sarı

M.Sc. in Molecular Biology and Genetics

Advisor: Bahar Değirmenci Uzun

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Resolution of inflammation in different tissues still remains unclear. Different cell types coordinate to ameliorate inflammatory regions. In colon, enteric nervous system (ENS) is one of cell types that responds to inflammation with its heterogeneous cell types and locations throughout different layers. However, reaction of enteric glia and neuron to regeneration mechanistically and metabolically is still unknown. In this study, we found that neurons are hyperactivated upon inflammation and produce lipid droplets; enteric glia uptake these lipids to protect neurons from lipid induced toxicity. Lipid particles are stored in enteric glia. In addition to metabolic response, there is also metabolic response of ENS to regeneration which is resolved by spatial transcriptomics. This method enabled us to identify transcripts in their exact positions on the tissue; allowed us to determine transcript proximities to each other in a reorganizing tissue. We found that PTN-PTPRZ1 axis is a regulator of cell inclination of ENS cells in muscularis externa towards the submucosa. Co-expression of Ptn-Ptprz1 was increased in the regenerative area of the colon. Moreover, to determine role of ENS in tissue reorientation in vitro, we achieved to produce ENS harboring complex assembloids. Enteric progenitor cells derived neuron and glia cells from muscularis externa were able to migrate mucosa. This study underscores diverse aspects of ENS cells to aid regeneration process upon regeneration in colon.

Keywords: colon, ENS, regeneration, DSS, lipid, migration

ÖZET

Enterik Sinir Sisteminin Yenilenen Kolondaki Metabolik ve Mekanik Birlikteliği

İlke Sarı

Moleküler Biyoloji ve Genetik, Yüksek Lisans

Tez Danışmanı: Bahar Değirmenci Uzun

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İnflamasyonun çözümlenme aşaması farklı dokularda hala tam olarak anlaşılamamıştır. İnflamasyondan etkilenen bölgelerde inflamasyonun çözümlemesinde farklı hücre tipleri görev almaktadır. Kalın bağırsak için inflamasyona cevap veren hücre topluluklarından biri olan enterik sinir sistemi, farklı hücre tiplerinden oluşup kalın bağırsak katmanlarının farklı konumlarında yer almaktadır. Ancak, enterik glia ve nöronların inflamasyon sonrası doku yenilenmesindeki etkileşimi hala bilinmemektedir. Bu çalışmadaki bulgularımız, inflamasyon üzerine fazla stimüle olan nöronların lipit damlacıkları oluşturduğu, enterik glianın ise nöronları yağ partikülleri birikmesi sonucu oluşabilecek toksisiteden korumak için bu damlacıkları hücre içine aldığı yönünde olmuştur. Yağ partükülleri enterik gliada depolanmaktadır. Enterik sinir sisteminin gösterdiği metabolik tepkinin yanı sıra mekânsal transkriptomik analiziyle gösterdiğimiz üzere, enterik sinir sistemi doku yenilenmesine mekanik olarak da cevap vermektedir. Bu analiz bize transkriptlerin dokudaki yerlerini tam olarak belirlememize ve hasar sonrası kendini yenilemekte olan dokudaki transkriptlerin mekânsal yakınlıklarını analiz etmemize olanak sağlamıştır. Bu analiz sonucu PTN-PTPRZ1 reseptör ligand yolağının enterik sinir sitemi hücrelerinin kas tabakasından mukoza altına yöneliminde rolünün olduğunu gözlemledik. Kalın bağırsağın rejenerasyon gösteren bölümlerinde PTN-PTPRZ1 proteinlerinin ortak ifadesinin arttığını gözlemledik. Bunun yanında, enterik sinir sisteminin doku yenilenmesinde, hücrelerin organizasyonuna olan etkilerini göstermek için, içinde enterik sinir sistemini de barındıran kompleks kalın bağırsak assembloid yapıları oluşturduk. Bu yapılarda enterik sinir sisteminin öncü hücrelerinden farklılaşan nöron ve glialar kas tabakasından mukoza tabakasına hareket edebilmektedir. Bu çalışma, enterik sinir

sisteminin kalın bağırsaktaki rejenerasyona yardımcı çeşitli katkılarının altını çizmektedir.

Anahtar Kelimeler: kalın bağırsak, enterik sinir sistemi, rejenerasyon, DSS, lipit, hücre hareketi

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DEDICATION

To my beloved Family,

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List of Abbreviations

ACOT7	Acyl-CoA Thioesterase 7
Acta2	Actin Alpha 2
APOE	Apolipoprotein E
CD	Crohn's Disease
CNS	Central Nervous System
DSS	Dextran Sulfate Sodium
ENCC	Enteric Neural Crest Cells
ENS	Enteric Nervous System
FABP4	Fatty Acid Binding Protein 4
GDNF	Glial Cell Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
GSNO	S-Nitrosoglutathione
HSCR	Hirschspung's Disease
HuC-D	ELAV-Like RNA Binding Protein 1
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IHC	Immunohistochemistry
IL-18	Interleukin-18
LMMP	Longitudal Muscle and Myenteric Plexus
MUC2	Mucin 2
Pdgfa	Platelet Derived Growth Factor Alpha
Pdgfra	Platelet Derived Growth Factor Receptor Alpha

PEMN Putative Excitatory Motor Neurons

PIMN Putative Inhibitory Motor Neurons

PIN Putative Interneurons

PSN Putative Sensory Neurons

PSVN Putative Secondary/Vasodilator Neurons

Ptn Pleiotrophin

Ptprz1 Protein Tyrosine Phosphatase Receptor Type Z1

ROS Reactive Oxygen Species

Rspo1 R-Spondin 1

S100b S100 Calcium-binding Protein B

SCFA Short-Chain Fatty Acid

SOX10 SRY-Box Transcription Factor 10

SOX9 SRY-Box Transcription Factor 9

TGFB Transforming Growth Factor Beta

TNBS Trinitrobenzene Sulfonic Acid

Tubb3 Tubulin Beta 3 Class III

UC Ulcerative Colitis

1. INTRODUCTION

1.1 Physiology and Functions of Colon

Colon is one of the crucial organs in the body that have a role in the digestive tract. It is located between the small intestine and anus; while cecum connects the colon to the small intestine, rectum bridges the colon with anus where the digestive tract ends (Azzouz & Sharma, 2024). It is anatomically divided into four parts: ascending colon, transverse colon, descending colon and sigmoid colon (Azzouz & Sharma, 2024). It has diverse functions; one of them is water and electrolyte absorption. As unabsorbed water and indigestible food particles move through the colon, water is absorbed, and the remnants are transformed into stool (Avetisyan et al., 2015). This process is required for maintaining the water balance of the body and avoiding dehydration. The colon also plays a key role in waste elimination. Peristaltic movements with the enteric nervous system propel the formed feces through the colon to the rectum, where they are eventually expelled from the body through anus. Contents from the small intestine also contain different bacteria types along with water and food (McCracken & Lorenz, 2001). Colon resident beneficial bacteria aid in digesting certain foods and maintain immune health of the body. There resident bacteria ferment undigested carbohydrates into short-chain fatty acids (SCFAs) such as acetate and butyrate; which are useful for colonic cell types for energy requirements (Ney et al., 2023).

1.2 Anatomical Layers of the Colon

Due to its specialized functions, the colon's wall is composed of several distinct layers, each contributing to the overall operation of the organ (Rao & Wang, 2010). Mucosa, submucosa, muscularis externa and serosa compose the layers of the colon.

1.2.1 Mucosa

The innermost layer is mucosa, which is closest to the lumen of the colon (Figure 1). Contents of lumen are in direct contact with the mucosa layer. It is composed of epithelial layer, lamina propria which is a connective layer and muscularis mucosae that is a thin muscle layer (Ogobuiro et al., 2024). Because most of the luminal content is absorbed in the small intestine, colon doesn’t have villus to increase its surface area.

Therefore, it has only crypt structures that are embedded deep into the mucosa layer. These crypt structures are composed of epithelial cells that have a role on absorption of water and electrolytes, mucus secreting goblet cells and hormone secreting enteroendocrine cells. In the lower layer of epithelium, there is lamina propria that sustain structural integrity of epithelia with blood vessels, lymphatic vessels, mesenchymal cells, enteric nervous system cells and immune cells. Muscularis mucosae is the smooth muscle layer that surrounds crypt bottoms and lamina propria for enhanced movement of the colon.

1.2.2 Submucosa

Second layer of the colon wall is the submucosa layer composed of connective tissue (Rao & Wang, 2010) (Figure 1). It contains blood vessels, lymphatic vessels, a great amount of glia and a neuronal network named submucosal plexus. This is one of the two plexuses of the enteric nervous system in the colon. Because its position in the colon near the mucosa layer, it coordinates secretion of hormones, regulate inflammation and immune system cells.

1.2.3 Muscularis Externa

The third layer of the colon is called the muscularis externa (Rao & Wang, 2010) (Figure 1), as it consists of two smooth muscle layers: the inner circular muscle layer and the outer longitudinal muscle layer. In the inner circular muscle layer, the muscles are positioned around the lumen in a circular arrangement. This layer contains dense smooth muscle cells that contract to compress the lumen, aiding in the mixing of its contents. Between circular and longitudinal muscles, there is a second plexus of the enteric nervous system called myenteric plexus. This integrated neuron and glia cell network control motility of the colon. The longitudinal muscle layer consists of muscle fibers aligned parallel to the length of the colon, enabling the peristaltic movements necessary for waste elimination.

1.2.4 Serosa

The last and fourth layer of the colon is the serosa layer (Rao & Wang, 2010). This layer consists of mesothelial cells. It is a connective tissue as a thin membrane that allows the colon to move as an organ through other organs. It is not very deeply

researched; however it is known that it keeps muscle cells together throughout the colon.

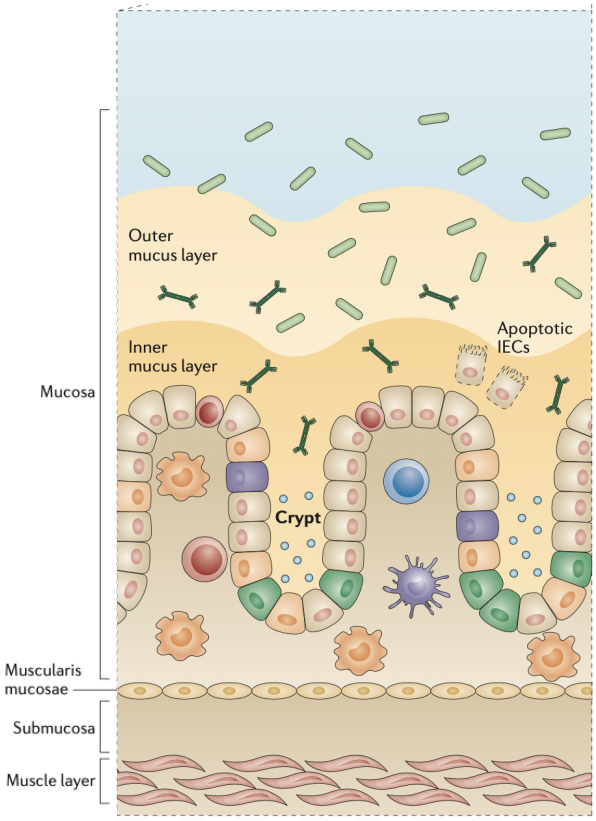


Figure 1. Different layers of the colon. Mucosa, submucosa and muscle layer (muscularis externa) are located from the inner to outer layer of the colon. (Mowat & Agace, 2014)

1.3 Colonic Cell Types

Colon has very diverse specialized cells that contribute to homeostasis of the organ. These cells cooperate and communicate to sustain the homeostasis of the colon. Because they are positioned as layers along the colon wall, every cell type has special tasks such as absorption, secretion, structural integrity or movement.

1.3.1 Epithelial Cells

Epithelial cells on the tip of the mucosa layer are named as enterocytes that absorb water and electrolyte from the lumen (Figure 2). Positioned at the luminal surface, enterocytes are the first to encounter its contents, making them vulnerable to injury from various sources, including pathogens (Rath & Haller, 2022). Despite their close

proximity and tight junctions, factors like mucus layer thinning and weakened tight junctions can allow pathogens to infiltrate the lamina propria (Kobayashi et al., 2020). This infiltration triggers an immune response by activating macrophages and antigen-presenting cells, making enterocytes not only key players in absorption but also in the initial defense against pathogens.

Colon has a mucus layer on top of the epithelial cells to protect cells from the lumen environment. One of the cell type colon has mucin-producing goblet cells (Figure 2). They are able to produce MUC2 protein which generates a polymeric sheet to provide a sustainable environment for microbiota (Cornick et al., 2019; Cornick et al., 2015). Leukotrienes, interferon-g, IL-9 and IL-13 control the mucus production in the colon (Klose et al., 2013). Also, MUC2 can be stored in goblet cells and can be over-secreted in case of pathogen infiltration.

Another epithelial cell type that is required for homeostasis of colon is enteroendocrine cells (Figure 2). These cells are known to produce hormones within the gastrointestinal tract to regulate several aspects of colon roles (Posovszky et al., 2017). Hormones produced by these cells can act locally or affect other cells such as neurons. In colon, they can affect peristaltic movement to colonic emptying and transit by affecting enteric nervous system, release of digestive enzymes or regulate barrier function by enterochromaffin cells (Furness et al., 2013). Peptides that produced from these cells are 5-HT which acts in secretion, sensation and appetite; somatostatin that is inhibitory hormone for digestive endocrine and exocrine function, stimulates colonic peristalsis; PYY which slows down gastric emptying and intestinal movement and promotes growth of goblet cells; GLP1 which enhances insulin secretion following nutrient intake and delays gastric emptying; GLP-2 and glicentin which both stimulates mucosal proliferation (Gunawardene et al., 2011).

The most fragile cell type in the colon resides at the deepest level of the mucosa in the crypt base: the colonic stem cells. These cells are well-protected by the immune system, epithelial cells, and the mucus layer (Figure 2). Colonic stem cells are responsible for the renewal of epithelial cells and can be identified by the Lgr5 marker (Barker et al., 2014). They are maintained by the Wnt signaling pathway, which

preserves their stemness and ensures the replenishment of epithelium (Choi & Augenlicht, 2024).

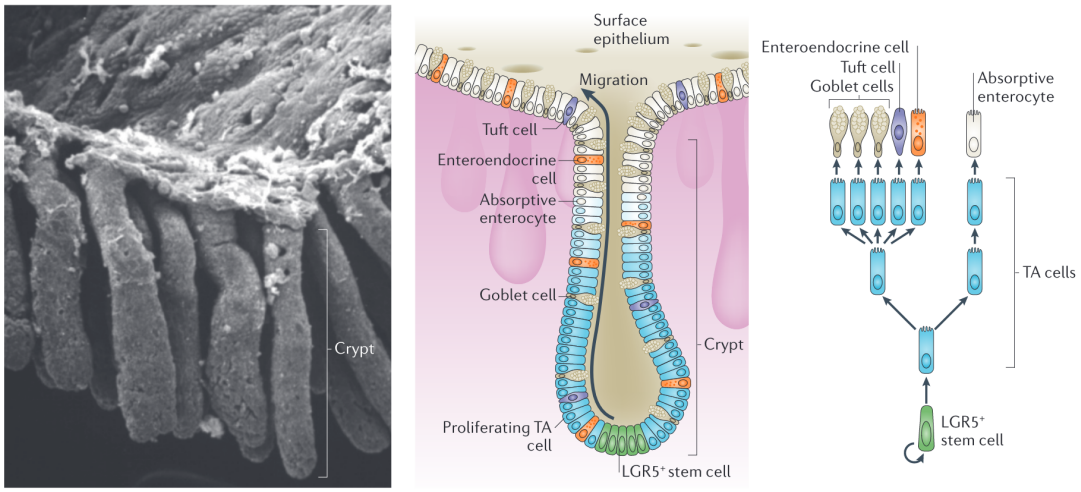


Figure 2. Different cell types of colon and their positions in crypt. Stem cells, goblet cell, enteroendocrine cells, enterocytes and tuft cell are epithelial cells of the colon. (Barker et al., 2014)

1.3.2 Immune Cells

Immune cells are the one of the prominent cell types in the colon because the colon is the most microbiome rich organ in the body. They can be found in mucosa, lamina propria, submucosa and also in the muscle layer. Because of the thick mucus layer, microbiome can sit on the mucosa layer, which is controlled by different immune mediators (Klose et al., 2013) . Innate immune cells such as macrophages, dendritic cells, innate lymphoid cells and neutrophils have an active role in keeping homeostasis in the colon. Macrophages phagocytes dead cells and produces cytokines to regulate inflammation such as mucus production in mucosa and lamina propria (Viola et al., 2023). Moreover, there is muscularis macrophages in muscularis externa region that are in close proximity with the enteric nervous system (Matteoli et al., 2014). These cells regulate the colonic peristalsis by producing BMP2 that is received by enteric neurons (Muller et al., 2014). Besides, because of their phagocytic nature, they can envelop the apoptotic neurons to clearance for homeostasis (Kulkarni et al., 2017). Adaptive immune system cells, T cells and B cells, can also be found in the colon excessively. T-cells are located in the epithelial layer and lamina propria. In the epithelial layer, immediate response can be regulated by nearby antigens from

enterocytes thanks to their position (Ma et al., 2019). B cells can produce neutralizing antibodies for pathogens that invade the epithelial layer in lamina propria (Spencer & Bemark, 2023).

1.3.3. Stromal Cells

Stromal cells consist of fibroblasts, myofibroblasts, endothelial cells and pericytes. They are essential for maintaining the homeostasis of the colon, with fibroblasts providing structural support, endothelial cells regulating the microenvironment, pericytes ensuring adequate blood flow and nutrient stability, and myofibroblasts contributing to contractile functions.

Fibroblasts are the main cell type of stromal cells in the colon. They are positioned in lamina propria, submucosa and muscularis externa. Fibroblasts in lamina propria extend through crypt length and submucosal fibroblasts sit underneath crypts. They can be identified with Pdgfra, Vimentin and Col1a1 expression (Greicius et al., 2018). According to single-cell RNA sequencing results, Pdgfra expression in crypt-tip and crypt-bottom cells were differed; while crypt-tip cells have more Pdgfra expression, crypt-bottom cells were low Pdgfra expression (Brügger et al., 2020). Crypt bottom fibroblasts were expressing high Wnt2, Wnt3, Rspo3 and Grem1 for support maintenance of stem cell identity because they are located near stem cells (Brügger et al., 2020). On the contrary, crypt-tip fibroblasts were expressing more differentiation-regulated genes such as Bmp2, Bmp3, Bmp4, Bmp5, Bmp7, Wnt5a and Dkk3 because crypt-tip has more differentiated epithelial cells (Brügger et al., 2020). Moreover, Gli1+ fibroblasts are Wnt secreting cells that are indispensable for colon stem cell viability, because colon doesn't have Paneth cells which express Wnt ligands (Degirmenci et al., 2018). Gli1+ cells also express Acta2, Vimentin and Pdgfra fibroblast markers (Degirmenci et al., 2018). Gli1+ cells that upregulate CD90 expression named as CD90+ cells also support stem cell activity by producing class 3 semaphorins (Karpus et al., 2019). Among Gli1, Foxl1 is also an important protein that is a marker for fibroblasts that provide stem cell maintenance by supplying Wnt signaling (Shoshkes-Carmel et al., 2018). CD34 is also a marker for fibroblast cells which produce Wnt2b, Grem1 and Rspo1 that support stem cells (Stzepourginski et al., 2017).

Other prominent stromal cell type is myofibroblasts, which called as activated fibroblasts that are arranged as sheath by adherent junctions (Takatsuna et al., 2016). They primarily undertake the contraction role of the colon, by the assistance of the enteric nervous system primarily. They are located on lamina propria, submucosa and muscularis externa (Takatsuna et al., 2016). Among myofibroblasts, there are endothelial cells that line blood vessels and lymphatic vessels, and act as a barrier between vessel and other cell types. They regulate immune cell regulation, nutrient and oxygen passage (Petrova & Koh, 2017; Wiggins et al., 2023). Because of their close proximity with vessels, they modulate blood flow with immune cell response. During inflammation or regeneration of epithelia, endothelial cells and pericytes govern the formation of new blood and lymphatic vessels (Vetrano et al., 2010).

1.4. Enteric Nervous System

Enteric nervous system consists of neurons, glia and supporting cells that construct complex network. They are embedded into every layer of colon to support surrounding cells for digestion and defense for pathogens. ENS is referred to as 'second brain ' because of its independent actions from the central nervous system. Their positions and molecular characteristics play a crucial role about their regulatory missions.

1.4.1 Origin of Enteric Nervous System

In literature, ENS is known as 'second brain'. However, it is the first developing neuronal system in development instead of the brain. ENS precursor cells are originally derived from vagal neural crest cells (Burke et al., 1995) and sacral neural crest cells (Burns et al., 2000). After 4 weeks of gestation in humans and day 9 in mouse embryonic day, vagal neural crest cells migrate to the foregut (Newgreen & Young, 2002). After they complete their migration, they are named as enteric neural crest cells (ENCC) which are also pluripotent. ENCCs both migrate and differentiate along the gut. To preserve their proliferative state, they express c-Ret, Sox10 and p75 (Durbec et al., 1996; Southard-Smith et al., 1998). With time lapse imaging and GFP tagging of cells, migration patterns of ENCCs were deciphered in the gut (Young et al., 2004). While some of the ENCCs migrated as a bulk, ENCCs that are supposed to migrate colon displayed a single cell migration and differentiation (Druckenbrod &

Epstein, 2005). Migration of ENCCs are also coupled with proliferation. Proliferative behavior of ENCCs at the wavefront is the main reason for the migratory process (Simpson et al., 2007).

1.4.2 Differentiation of Enteric Nervous System

Migratory ENCCs express pan-neural marker HuC-D (Young et al., 2005). Downregulation of ENCCs pluripotency marker expression of Sox10 causes differentiation into enteric neural cells. Their high c-Ret activity still remains in differentiating neurons. Moreover, low expression of c-Ret and high expression of Sox10 leads to enteric glia differentiation. B-FABP expression is also increased due to enteric glia differentiation (Young et al., 2003). Glial marker S100b is not expressed in ENCC precursor cells until embryonic day 14. Interestingly, some of the terminally differentiated neuron and glia markers such as neuropeptide Y and S100b respectively can be observed in some of the ENCC progenitors (Pham, et al., 1991; Young et al., 2005). Fully differentiated glial cells express distinctly GFAP marker.

Glial cell derived neurotrophic factor (GDNF) is a ligand for TGFB signaling, an important regulator for neurons because of its migratory signaling activity. It is important for migration of precursor cells to different bowel parts. Sánchez et al. found that GDNF lacking mice is devoid of enteric neurons. Migration of enteric neurons dependent to GDNF but not differentiation or survival were affected by its expression (Sánchez et al., 1996). Moreover, lack of GNDF neurothrophic devoid leads to aganglionosis in the colon which leads to neuropathies such as Hirschuprung disease (Mwizerwa et al., 2011).

1.4.3 Structural and Molecular Organization of the Enteric Nervous System

ENS is composed of enteric neurons and enteric glia that surround different layers of colon. While ENS cells can be found in mucosa and in the muscle area as a single cell, they specialize into two complex networks called myenteric plexus and submucosal plexus (Figure 3). Plexuses contain networks of ganglia that are connected to each other for exerting their effect on the tissue as a whole. One ganglion incorporates neurons and glia in very close proximity.

Submucosal plexus is located in the submucosa and surrounded by Pdgfra+ cells, myofibroblasts and endothelial cells. Myenteric plexus is located between circular muscle and longitudinal muscle layers (Figure 3). While submucosal ganglia is smaller than myenteric plexus in rodents, their size in higher mammals are comparable to each other (Furness et al., 2007; Sharkey & Mawe, 2023).

ENS is thought to have 200 millions of neurons and at least 4 times more glial cells (Michel et al., 2022). Considering the multitude of cells, functions of the enteric neurons and glia affect different cellular mechanisms in the colon. Myenteric plexus regulates colon movement by enabling contraction in myofibroblasts in muscle region (Sanders et al., 2010; Smith & Koh, 2017), while submucosal plexus controls epithelial functions such as absorption, secretion and pathogen defense (Furness et al., 2014; Vanner & Macnaughton, 2004).

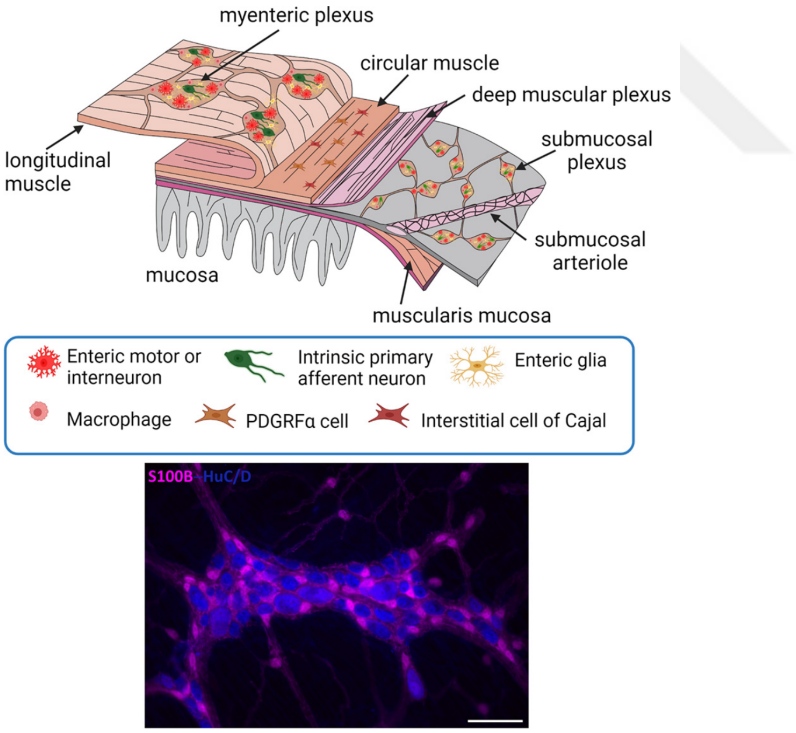


Figure 3. Enteric nervous system in different layers of colon. Submucosal plexus is found in submucosa while myenteric plexus is in muscularis externa. All the layers of colon include ENS cells to regulate homeostasis. (Sharkey & Mawe, 2023)

1.4.4 Enteric Neurons

Enteric neurons are found throughout every layer of the colon (Figure 4), but their rarity and fragility make their characterization challenging. They constitute less than 1% of the total cell population in the mouse colon. Due to these difficulties, data from studies often depend on the effectiveness of researchers in isolating neurons from tissue. As a result, there is still no consensus on the specific types of neuronal cells present in the colon.

1.4.4.1 Enteric Neuron Cell Types and Subsets

Enteric neurons are first detected and described by A.S. Dogiel which dedicates his surname to types of neuron (Dharshika & Gulbransen, 2023). Although there are many types of neurons, there is only 2 types of neurons in mouse GI tract, Dogiel type 1 and Dogiel type 2 (Furness et al., 2007; Qu et al., 2008). Dogiel type 1 neurons has elongated flat shapes that have only one axon with many dendrites. Dogiel type 2 neurons have larger cell bodies and multiple axons (Furness et al., 2007; Qu et al., 2008). While type 2 neurons communicate with mucosa and between ganglia thank to its long axons, type 1 neurons communicate nearby ganglia within plexuses and muscle only (Dharshika & Gulbransen, 2023).

For the molecular side, recent studies revealed 21 neuron subsets in colon with nuclei sorting that mainly divide neurons into 2 subsets; cholinergic and nitrergic (Drokhlyansky et al., 2020). There are 5 subsets under ‘excitatory motor neurons ’ (PEMN) that express ChAT and Tac1, 7 subsets under ‘inhibitory motor neurons ’ (PIMN) express Nos1, 3 subsets under ‘Interneurons ’(PIN) express Penk, 4 subsets under ‘sensory neurons ’(PSN) express CGPR, and 2 subsets under ‘secretory/vasodilator neurons ’(PSVN) express Glp2r (Drokhlyansky et al., 2020). Each neuronal group undertakes different roles for homeostasis. PEMNs regulate contraction of muscle cells together with PIMNs to achieve relaxation. PINs are interneurons that enable the communication between neurons. Moreover, PSN cells recognize insults or chemical cues from the epithelia and relay the signal to other cell types to respond. In the epithelium, secretory cells receive signals from PSVN cells to perform their functions (Drokhlyansky et al., 2020). Other study reveals 12 enteric

neuronal types in small intestine; excitatory and inhibitory motor neurons, interneurons and intrinsic primary afferent neurons (Morarach et al., 2021). Results show that overall clusters are similar between the small intestine and colon.

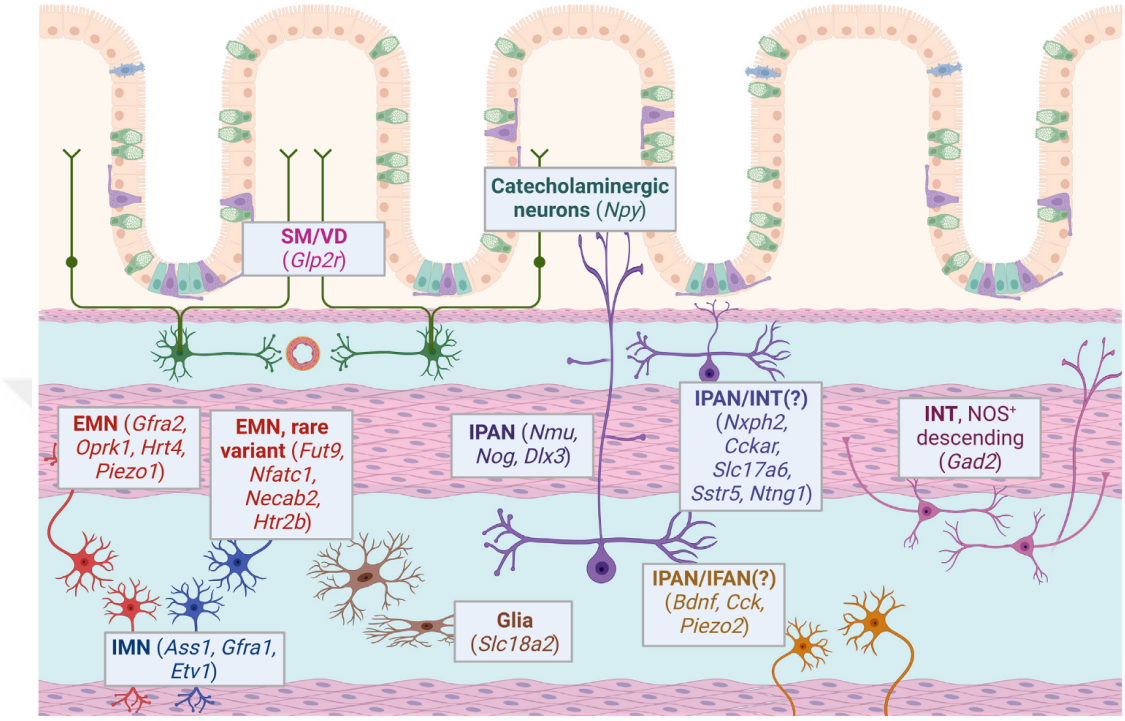


Figure 4. Types of enteric neurons in the colon. Markers of all enteric neuron subtypes can be seen from the figure. (Dharshika & Gulbransen, 2023)

1.4.4.2 Functions of Enteric Neuron

The most investigated area is how ENS regulates barrier integrity of the colon upon inflammation. Like in Figure 5, because it is known that neurons can project their endings to mucosa from submucosa and myenteric plexus, they can regulate immune function of the epithelial region (Jarret et al., 2020). Study found that neurons can release neurotransmitters and cytokines such as IL-18. Upon pathogen invasion, neurons express IL-18 to epithelial cells for protection (Jarret et al., 2020). Goblet cells receive neuronal cues and start to produce antimicrobial protein. With co-staining, inhibitory motor neurons are responsible from IL-18 production, not ChAT positive cells (Jarret et al., 2020). In another study, they found that enteric neurons that are

PIMN and PSN are in close proximity with the regulatory T cell is the mucosa (Yan et al., 2021). They secrete IL-6 in response to pathogens which leads to regulatory T cell population differentiation (Yan et al., 2021).

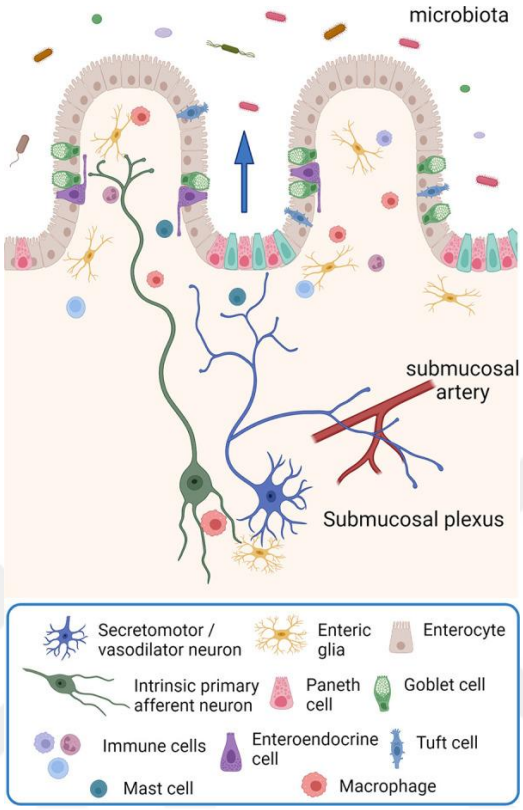


Figure 5. Enteric neurons can project their axon terminals toward mucosa and interact with epithelial cells. (Sharkey & Mawe, 2023)

1.4.5 Enteric Glia

Enteric glia is a peripheral glia population that is the most prevalent cell type in the enteric nervous system. Enteric glia regulates homeostasis in colon with its dynamic nature (Boesmans et al., 2015). They can be found in mucosa, submucosa and muscularis externa regions.Because of their location and their nature, many cell types can interact with enteric glia such as enteric neurons, fibroblasts, immune cells and

epithelial cells. Response to host defense in insult to epithelia, and safeguarding the neuronal cells are some of the enteric glial roles in colon (Laranjeira et al., 2011).

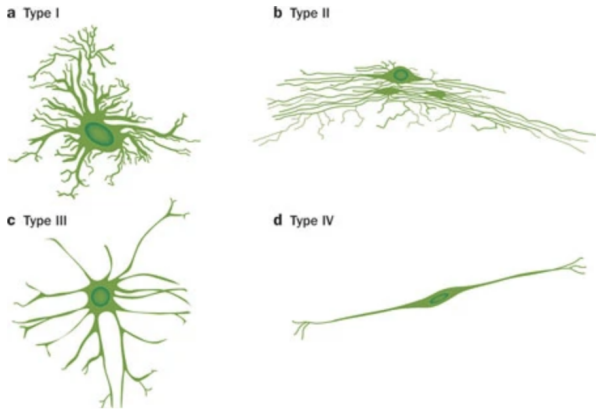
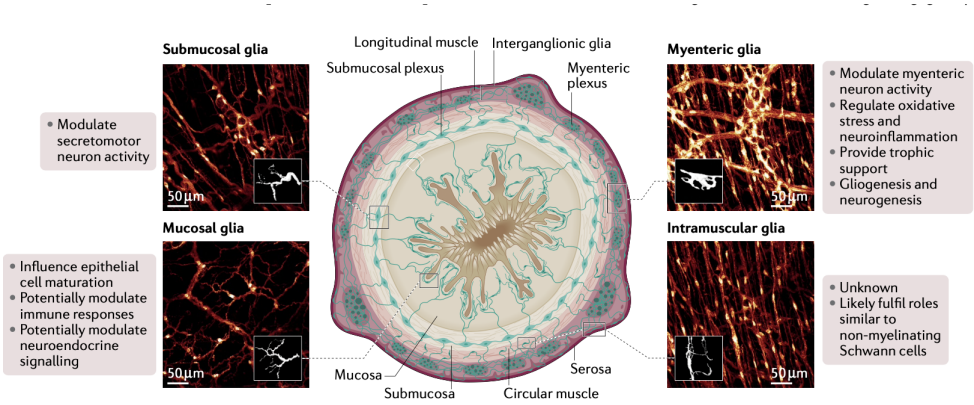


Figure 6. Types of enteric glia in colon. (Gulbransen & Sharkey, 2012)

1.4.5.1 Enteric Glial Cell Types and Subsets

Enteric glia can be classified as intraganglionic and interganglionic according to their spatial arrangement in the colon (Figure 6). Glial cells can be found in mucosa, lamina propria, in between muscle cells and in ganglia of both submucosal and myenteric plexuses. Moreover, classification of glial cells depends on their morphogenic characteristics; divided into type 1, type 2, type 3 and type 4 cells (Seguella & Gulbransen, 2021) (Figure 7). Type 1 glial cells are found in intraganglionic regions and divided into myenteric type 1 glia and submucosal type 1 glia (Seguella & Gulbransen, 2021). While myenteric type 1 glia has short branches that surround neurons in ganglia, submucosal type 1 glia does not have short branches. They regulate neuronal activity inside ganglia, protecting from neuroinflammation. Type 2 glial cells are named as interganglionic glia that extend through different myenteric ganglia to signal between them and modulate neurons across ganglia (Seguella & Gulbransen, 2021). Type 3 glial cells are divided into two depending on the location of the cell; mucosal type 3 cells are long processed cells extending through crypt length (Seguella & Gulbransen, 2021). They can influence epithelial cell maturation and modulate immune responses. Enteric plexus type 3 cells located in plexuses and run along with nerve fibers; their role is unknown. Type 4 cells are intramuscular cells that are non-

fibrous long cells that tract nerve fibers along muscle; their exact aim is also unknown



(Seguella & Gulbransen, 2021).

Figure 7. Cross section of colon shows enteric glia cell populations and their properties. (Seguella & Gulbransen, 2021)

Origin of enteric glia and neuron comes from enteric neural crest cells in embryonic development (Nagy & Goldstein, 2017). Glial cells can be detected with S100b, Sox10, GFAP and Plp1 expression in colon (Ferri et al., 1982; Hoff et al., 2008; Jessen & Mirsky, 1980). They retain their prenatal markers after differentiation such as Sox10, p75 and Nestin (Joseph et al., 2011; Laranjeira et al., 2011). Therefore, their molecular identity is heterogeneous on the data that is produced from adult ganglia. Colon glia is grouped into 3 types, named as Glia1, Glia2 and Glia3 (Drokhlyansky et al., 2020). In one study, while Glia1 group is enriched for Gfra2 receptor; Glia2 is for Slc18a2 and Glia3 group is enriched for Ntsr1 (Drokhlyansky et al., 2020). In another study, glia cells divide into 7 different subsets (Zeisel et al., 2018). Interestingly, one of the subtype is determined as proliferating which overlaps with the dynamic nature of enteric glia (Zeisel et al., 2018). This can also prove the progenitor markers inherited from enteric crest cells. Because enteric glia and neuron are derived from enteric neural crest cells, enteric glial cells are found to still retain their multipotent identity in themselves. They can support neurogenesis when tissue homeostasis is threatened with inflammation, irradiation and ablation in CNS (Joseph et al., 2011). Also, it is shown that glia can convert to neuron in vitro (Laranjeira et al., 2011).

1.4.5.2 Functions of Enteric Glia

Enteric glial cells have a role in epithelial cell homeostasis, immune cell response and neuroprotection. Bottom of the intestinal crypts reside stem cells that have a very specific niche for its maintenance. One of the niche factor Wnt is supplied by enteric glia located at the bottom of the crypt (Baghdadi et al., 2022). These glial cells are GFAP positive and they observed that GFAP+ glial cells were increased upon inflammation to support regeneration of epithelial cells (Baghdadi et al., 2022). On the contrary, some studies propose the negative effects of glial cells exacerbates inflammation in intestinal barrier (Grubišić & Gulbransen, 2022). When there is acute inflammation in the intestine, enteric glial A2BR receptor leads to decrease in Csf3, Cxcl1 and Cxcl10 that alleviate inflammation (Grubišić et al., 2022). On the contrary, they overexpress IL-6 that cause persistent barrier impairment. Also A2BR receptor activity on glia leads to overexpression of Cdln1 protein that causes barrier dysfunction (Grubišić et al., 2022b).

1.4.6 Protective Role of Glia on Neuron in CNS and ENS

In the central nervous system (CNS), enteric glia support enteric neurons in various ways such as maintaining homeostasis by providing glucose for energy needs, structural integrity and protecting from detrimental metabolic products (Bittner et al., 2010). One of the main roles of glia is supplying glucose for neurons. Glial cells preferentially metabolize glucose with glycolytic pathway, resulting in lactate released into extracellular space (Itoh et al., 2003). Neurons use lactate as one of the primary substrate for energy production.

1.4.6.1 Fat Metabolism in Brain and Colon

In the scope of this thesis, CNS glia also gatekeep for reactive oxygen species (ROS) and lipid droplets to protect neuron. Because neurons are very vulnerable to ROS (Wilson et al., 1997), it is shown by multiple groups that astrocytes in the brain neutralize ROS, nitric oxide or other type of toxic substance to the brain (Lucius & Sievers, 1996; Tanaka et al., 1999). Another group demonstrated that high ROS activity leads to lipid droplet production in neurons (Liu et al., 2017). This lipid droplets are shuttled to glia with APOE4 to protect neurons. Similar principle is also

proved by another group that neuronal lipid products were uptaken by glia and neutralized by mitochondrial oxidation in glia (Ioannou et al., 2019). For another important lipid metabolism regulators, Acyl-CoA Thioesterase 7 (ACOT7) is proposed for hydrolysis of long-chain acyl-CoA to maintain homeostatic conditions in neurons of the brain (Ellis et al., 2013). ACOT7 knockout mice displayed seizure like behavior; that indicates ACOT7 metabolism input on disease progression (Bowman et. al., 2019). Moreover, Gpr37l1 protein is G-protein coupled receptor that expressed exclusively on neuronal and glial cells on CNS which recently discovered for its neuroprotective role (Meyer et al., 2013). Prosaposin is a ligand of Gpr37l1 that binding of ligand activates cAMP-PKA pathway, leading to protection from oxidative stress in neurons (Liu et al., 2018). Moreover, it is found associated with primary cilium in brain; Gpr37l1 in Bergmann glial cell specifically interact with Shh signaling component Patch1 and precisely regulate the cerebellum development (Marazziti et al., 2013).

There is not as prevalent research in ENS metabolism as brain metabolism because of the rarity of cell type in colon. However, Abdo et al. found that enteric glia protects neurons by recruiting reduced glutathione in the cell environment (Abdo et al., 2010). Moreover, glial glutathione was found to be necessary for culture neurons (Brown & Gulbransen, 2018). Gpr37l1 protein found in brain Bergmann glial cells also found in gastrointestinal tract which specifically present in myenteric plexus (Sarkar et al., 2021). Therefore, it can also be an important regulator for neuroprotective protein in ENS.

1.4.7 Enteric Nervous System Diseases

As the commander of the gut, ENS is involved in many gastrointestinal diseases. In inflammatory bowel diseases (IBD), however, structural abnormalities such as altered myenteric plexus morphology, nerve hypertrophy and increases in numbers of neural and glial cells are also observed (Akbar et al., 2010). In Hirschspung's disease (HSCR) lack of enteric nervous system cells in the more distal parts of the intestine and functional obstruction in the gastrointestinal tract is observed due to the disruption of ENS development (Heuckeroth et al., 2018). Ret, which is one of the most important genes in ENCC development and migration, is mutated; resulting in aganglionic sites in the colon (Angrist et al., 1995). Moreover, another ENCC progenitor protein Sox10

mutation is found in human HSCR that further proves the developmental importance of ENCCs for homeostasis of colon (Herbarth et al., 1998; Sham et al., 2001). Irritable bowel disease (IBS) patients present gastrointestinal motility problems that are attributed to colorectal reflexes (Manabe et al., 2010; Ng et al., 2005). In addition, visceral hypersensitivity due to dysfunctioning sensory enteric neurons has also been reported (Hughes et al., 2013; Mertz et al., 1995).

Although it is an autonomous network, ENS is still connected to the rest of the body through extrinsic innervation (cholinergic and sympathetic) pathways (Uesaka et al., 2016). As a result, ENS is also affected by neurodegenerative diseases like Alzheimer's disease and Parkinson's disease. In Alzheimer's disease, amyloid-β accumulation in enteric neurons is observed which leads to decrease of the neuronal tissue and enhanced inflammation (Semar et al., 2013). In Parkinson's disease, it is hypothesized that the degeneration starts at the ENS and spreads from there (Braak et al., 2006). Although this hypothesis has not been fully understood, a supporting study found that pathogenic forms of α-synuclein, that forms the Parkinson's disease-typical Lewy bodies, occurs naturally in appendix and this could be the start of the spread (Killinger et al., 2018).

1.5 Organoid and Assembloid Culture Systems

Stem cells are self-renewal cells that maintain tissue homeostasis by differentiating into different cell types. In the colon, Lgr5 positive stem cells reside in the bottom of the crypt structure which is surrendered by niche cells such as fibroblast to sustain stem cell homeostasis (Barker et al., 2007). To recapitulate this environment to study the fundamental basis of organ biology and mechanism of diseases, organoid technology is developed. Organoids are self-organizing 3D tissue-like structures that mimic structural, functional and biological characteristics of the intended organ in a 3D matrix called Matrigel (Sato et al., 2009). First organoid system is developed from adult intestinal stem cells; it can be derived from adult tissue stem cells, induced pluripotent stem cells and cancer cells (Hofer & Lutolf, 2021; Sato et al., 2009). They can recapitulate the tissue architecture and cell-cell contact more than conventional 2D cell culture system. Moreover, they provide more application in personalized medicine by patient-derived organoids, drug testing for initial readouts and can be used for cell therapy (Al Shihabi et al., 2022; Kim et al., 2019). However, even with its

applications, it lacks physical contact with niche components and does not recapitulate the true environment of cell diversity of tissue such as lack of fibroblasts, vessels and immune components. Therefore, new technologies are emerging rapidly; assembloids. Assembloids are assembly of different cell types or multiple regional organoids together in a 3D environment (Birey et al., 2017). They recapitulate more accurate forms of tissue by cell-cell interactions, spatial location of each cell type and functional connectivity of tissues. The most advanced models are achieved in neural assembloids. Scientists have advanced the creation of brain-like assembloids by co-culturing organoids from various neuron types or different brain regions, enhancing the complexity of these models (Birey et al., 2022; Miura et al., 2020). In particular, human induced pluripotent stem cells (iPSCs) have been employed to generate cortical and spinal spheroids, which were then merged to construct assembloids that replicate specific regions of the brain (Andersen et al., 2020). These assembloids can be used for further applications such as drug testing on a tissue type to observe effects on the brain or vice-versa.

1.6 Spatial Transciptomics

Spatial transcriptomics is a new tool that enables quantification of genes in tissue without losing their spatial location in the tissue. It was developed by Ståhl et al. in 2016 and it is developing among its accuracy and precision since. Unlike traditional transcriptomics, which provides a bulk analysis of gene expression from a sample, spatial transcriptomics preserves the spatial context of the cells, enabling the study of how gene expression varies across different regions of a tissue (Canozo et al., 2022) . It can uncover how different cell types interact, how tissues develop, and how disease processes, such as cancer or neurodegeneration, affect specific regions of an organ (Parigi et al., 2022) .

1.7 Mouse Disease Models

Ulcerative colitis (UC) and Crohn's disease (CD) are two major types of inflammatory bowel diseases (Wilks et al., 1859). Ulcerative colitis is diagnosed by inflammation in the mucosa of colon (Rubin et al., 2019; Ungaro et al., 2017). Disrupted epithelial layer and unregulated immune response results in disease phenotype. CD is more

comprehensive in terms of its effecting sites in the colon (Hibi & Ogata, 2006). It impacts the all layers of the colon; mucosa, submucosa, muscularis externa and serosa. Fibrosis, inflammation, fistula and stricture in the colon are results of CD (Kobayashi et al., 2020).

Exact disease development of UC is still undetermined. However, it is known that shortened mucus layer in mucosa and decreased amount of immune cell diversity are the markers of UC disease (Kobayashi et al., 2020). Because of the reduced mucin production in epithelial goblet cells, mucus layer that protect epithelial lining become vulnerable to pathogens on the lumen. Lost integrity in the epithelia results in an immune system reaction in lamina propria (Kobayashi et al., 2020). Reduced number of cell types in immune cells cause inadequate defense for breach in epithelia; leads to chronic inflammation on colon. Immune cell types also produce short chain fatty acids to maintain homeostasis, their absence is also an indicator for UC diagnosis (Kobayashi et al., 2020).

1.7.1 Colitis Mouse Model

IBD diseases can be modeled in a mouse model for studying the mechanism of disease by dextran sulfate sodium (DSS), oxazolone and trinitrobenzene sulfonic acid (TNBS) (Wirtz et al., 2017). DSS is widely used as a chemical inducer of colitis due to its ease of use, low cost, and efficiency. Unlike oxazolone and TNBS, which are small molecules, DSS consists of sulphated polysaccharides with varying molecular weights, leading to an inflammation type that more closely resembles colitis in the colon (Yang & Merlin, 2024). DSS works by interacting with medium-chain fatty acids in the lumen to form vesicles (Laroui et al., 2012). These vesicles are taken up by epithelial cells, disrupting their integrity and triggering an inflammatory response (Laroui et al., 2012).

2. OBJECTIVES AND AIMS

In this thesis, our aim was the characterization of enteric nervous system response to recovery upon inflammation in the colon. It is known that mucosal structure of the colon is destroyed upon insult and various types of cells work in collaboration to alleviate damage in the tissue. ENS cells are the one of the first responders to inflammation. However, mechanisms of enteric glia and enteric neuron response to regeneration were not understood. Thus, we used DSS induced colitis mouse model to mimic inflammation and regeneration in colon and performed IHC experiments. According to our observations of inclined ENS cells from muscularis externa to submucosa, we hypothesized that enteric neurons can be protected from inflammation by fat metabolism and ENS cell inclination can be mediated by migratory pathway found in the central nervous system. Also, this inclination from muscularis externa to submucosa can be a clue for ENS cells 'regulatory role on tissue architecture.

To evaluate our hypotheses, our aims were to:

1. Identify the response of ENS cells to recovery of tissue with IHC experiments. This aim proposed to compare structural and ENS cell population changes in the mucosal and muscular externa layers.
2. Evaluate whether enteric neurons are protected from inflammatory damage through lipid transfer mechanism. Therefore, we employed a sandwich co-culture method from primary cells of glia and neurons. Neurons were labeled with C12 lipid dye.
3. Spot the ligand-receptor pair that mediates inclination of ENS cells towards submucosa by using spatial transcriptomics re-analysis. Mechanical pathways of ENS by spotting genes onto tissue provide us cell-cell proximity in tissue recovery.
4. Determine whether the observed inclination of ENS cells from the muscularis externa to the submucosa plays a regulatory role in the structural organization by 3D assembloid system in vitro.

3. MATERIAL AND METHODS

3.1 Animal Housing and Treatment

With the approval of the Animal Experiments Center Ethics Committee at Bilkent University, all mice (C57BL/6 and FVB/N) used in this study were bred, housed, and handled at the Bilkent University Animal Facility. Throughout the experiments, the mice were checked for their immune and general health status and were found to be healthy. The study involved male mice that were 8-12 weeks old.

3.2 Colitis Mouse Model

Acute inflammation was triggered by adding 2.5% dextran sulfate sodium (DSS, MP Biomedicals, CA, USA, 02160110) to the drinking water of the animals, which they had access to DSS freely for five days. These mice were sacrificed at the end of this period and referred to as 'no recovery' mice. For other groups, at the end of five-days DSS treatment, the water was switched back to drinking water for one and two days. These mice were sacrificed at the end of the one and two-day periods and referred to as ‘1 day recovery’ and ‘2 day recovery’. Control group was provided with drinking water for 5-7 days. All mice were fed with a normal chow diet (Optima, Bolu, Turkey).

3.3 Tissue Isolation and Preparation for Immunohistochemistry

The mice were euthanized with isoflurane and colon was dissected from the cecum to anus regions, flushed with ice-cold 1X PBS to remove fecal content, and divided into proximal and distal sections, each cut into 2 cm pieces. The colon pieces were washed twice with 1X PBS for 5 minutes each. For paraffin embedding, the tissues were fixed with 4% PFA overnight at 4°C, then washed and dehydrated with 30% EtOH/PBS and 50% EtOH/ddH2O for 1 hour at room temperature. The tissues were then placed in 70% EtOH before being embedded in paraffin cassettes. Leica Automatic Benchtop Tissue Processor was used for further alcohol dehydration. After dehydration and waxing procedure on tissue processor, Leica Paraffin Embedding Workstation was used to embed tissues to paraffin. After embedding, the tissues were left to solidify for 1 hour at +4°C and then kept at room temperature for an extended period. For frozen

embedding, the tissues were fixed with 4% PFA on ice, washed with PBS, incubated with 30% sucrose (Fisher Chemicals, NH, USA, 10634932) at 4℃ overnight, embedded in Tissue-Tek O.C.T (Sakura Finetek, Tokyo, Japan, 4583), and stored at -80℃.

3.4 Tissue Immunohistochemistry

For paraffin embedded tissues, paraffin blocks were sectioned for 5 um thickness and placed onto Superfrost Plus slides (Mensel Glaser). Slides were kept on a stretching table at 38℃ for at least 16 hours. Dewaxing of section was achieved with two xylol incubations for 7.5 minutes each, rehydrated with gradually decreasing ethanol concentrations as 100%, 96%, 80%, 70% and 50%, twice for each percentage for 4 minutes at room temperature (RT). 1X PBS washes are done three times for 5 minutes. Antibody retrieval with citrate or tris buffer in pressured steam was applied for 35 minutes for selected antibodies according to their working efficiency. Slides were taken out from the steamer and let it cool for 20 minutes, washed with 1X PBS two times for 5 minutes, excess PBS was removed onto tissue paper. 1 hour incubation of blocking buffer with 10% donkey serum, 10% Triton-x100 (0.3% diluted in 1X PBS), 10% 10X PBS in double distilled water at RT is applied. Primary antibody solution is prepared with respective dilutions in the blocking buffer, incubated overnight in a humidified chamber at 4 ℃. Slides washed with 1X PBS three times for 5 minutes at RT, incubated in secondary antibody solution (1:400) that contains respective antibody diluted in blocking buffer for 1 hour at RT, washed two times with 1x PBS, two times with PBST; 1x PBS containing 10% Triton-x100 (0.3% diluted in 1X PBS) for 5 minutes each. Nucleus stained with DAPI (1:1500) in PBST and incubated for 10 minutes at RT, washed 2 times with PBST and 2 times with 1x PBS for 5 minutes each. For frozen blocks, 5-15 um of tissue were sectioned onto Superfrost Plus slides and left on stretching table at 38 ℃ for 30 minutes. After slides were left on RT for 30 minutes, 10 minutes of incubation in respective fixative is applied and washed with 1x PBS three times for 5 minutes. Incubated with blocking buffer for 1 hour in RT and primary antibody solution at 4 ℃ overnight. Same secondary antibody protocol was applied. Slides were mounted with anti-face fluorescence mounting medium (Abcam), covered with coverslip, left at +4 ℃ until analysis. Images were taken with Leica Confocal SP8 microscope and LASX software by 20X, 40X and 63X objectives.

ImageJ was used for quantification of fluorescence intensity and GraphPad Prism software used for analysis of images.

3.5 Poly-D-Lysine and Laminin Coating of Coverslips

Round coverslips (18 mm) were merged into 100% ethanol for 15 minutes and let for dry in the cell culture hood for 15 minutes. Coverslips were placed into 12-well plate with sterile forceps. If coverslips were used for lipid transfer assay, dental wax which is sterilized with 100% ethanol and UV treatment cut into small pieces and 3 of them put onto coverslip for providing gap between glia and neuronal sandwich culture system. 100 µg/mL Poly-D-Lysine (PDL) solution, diluted with sterile water, was applied to coverslips to cover only the glass. The coverslips were left to sit for at least 1 hour at room temperature for quick coating. For long coating, the PDL solution can be incubated on the coverslips with the cell culture plate securely sealed with parafilm and maintained at +4°C overnight. Then, PDL was removed without disturbing the glass surface and allowed to airdrop completely under the culture hood. 10 ug/ml laminin diluted with sterile PBS was put onto coverslips containing dried PDL, only covering the surface of the glass. Coated coverslips were incubated at 37 C for 2 hours. Laminin solution was removed from coverslip without scratching the surface. Wells were gently washed with 1X PBS three times. Respective media were added to each well and stored at 37 C until cell seeding procedure.

3.6 ENS Isolation for Lipid Transfer

One FVB/N mouse was sacrificed for glia isolation, and another FVB/N mouse was sacrificed for neuron isolation. Colon was dissected into ice-cold HBSS. Longitudinal muscle and myenteric plexus (LMMP) layers was peeled (Smith et al., 2013). LMMP was put into ice-cold HBSS immediately. After this point, glia and neuron isolation protocols proceed separately. For glia isolation, LMMP was cut into tiny pieces to 5 mL Corning Cell Recovery Solution, incubated on rotating platform for 30 minutes at +4°C. Solution with LMMP pieces was further dissociated by trituration 20 times with 5 mL serological pipette coated with 0.1% BSA/PBS. For recovery of LMMP pieces, solution was filtered through 100 µm strainer and washed with 2 mL of 1X PBS. Discard supernatant. Tissue pieces on top of the strainer were transferred with clean

forceps to 1.5 mL digestion medium made up of HBSS with 10 mM HEPES, 50 µg/mL Liberase TM, 100 U/mL DNase 1 in 2 mL eppendorf and incubated on rotating platform for 30 minutes at 37°C. Enzymatic activity was stopped with complete media and solution was filtered through a 70 µm strainer immediately. Supernatant was transferred to 15 ml tube and completed to 5 ml with complete media, centrifuged in +4°C at 600xg for 5 minutes. Supernatant was discarded carefully and pellet was resuspended with 1X PBS for wash, centrifuged in +4°C. At 600xg for 5 minutes. Pellet was resuspended with 1 mL of complete media and cell number was determined. 150.000 cell/well were seeded on dental wax attached coverslips to 12-well plate. For neuron isolation, LMMP was cut into tiny pieces to digestion solution consists of HBSS with 10 mM HEPES, 100 µg/mL Liberase TM, 100 U/mL DNase 1 in 2 mL eppendorf and incubated on rotating platform for 30 minutes at 37°C. Then, tissue pieces were triturated 10 times and filtered through 100 µm strainer to recover supernatant. To stop enzymatic activity, complete media was added and centrifuged at 600xg for 5 minutes at 4°C. Pellet was washed once with complete media with the same centrifuge adjustments. Pellet was resuspended with 1 ml of complete media and cell number was determined. 150.000 cell/well were seeded on coverslips in a 12-well plate. Cells were maintained in complete media made up of DMEM/F12 with L-Glu, 15 mM HEPES, 100 U/mL Penicillin/Streptomycin and 10% FBS for 2 days. Then, cells were differentiated to glia with new medium consists of DMEM/F12 with L-Glu, 15 mM HEPES, 100 U/mL Penicillin/Streptomycin, 0.2% N2, 10 µg/mL GDNF. Neurons were differentiated with NeuroBasal Plus medium (NB+), 2% B27 Plus and 0.5 mM GlutaMAX I Supplement. Half of the media was renewed every 2 days. Glia and neuron cells were used after 7 days after initial seeding for lipid transfer protocol. Immunohistochemistry was applied to characterize and assign purity of cell types. Coverslips protected from light were washed with PBS two times for 5 minutes and fixed with 3% PFA for 15 minutes at room temperature (RT). After two PBS washes, cells were blocked and permeabilized with a blocking buffer made up of PBS, 2% BSA and 0.2% Triton-X100 for 1 hour while gently shaking at RT. After blocking, primary antibody incubation which was prepared in blocking buffer was done for 1 hour while gently shaking at RT. Coverslips were washed with wash buffer which consists of PBS with 0.1% Triton-X100 three times for 15 minutes each while gently shaking. Secondary antibody incubation was prepared in blocking buffer and incubated for 1 hour while shaking at RT. Secondary antibody was washed same as

the primary antibody. Nuclei were stained with DAPI prepared in PBS for 20 minutes while gently shaking. Coverslips were washed for 5 minutes with PBS and mounted with Anti-Fade Flourescence Mounting medium onto slide. Slides were imaged immediately after mounting to minimize loss of signal. All images were taken with Leica Confocal SP8 microscope and LASX software by 63X objective. ImageJ was used for quantification of fluorescence intensity and GraphPad Prism software used for analysis of images.

3.7 Lipid Transfer

After 5 days in culture, glial cells in the treatment group were treated with 10 ng/mL of IL-1β, TNF-α, and IFN-γ 48 hours prior to lipid transfer procedure. Also, neuron cells were treated with 2,5 mM final concentration of C12 for lipid labeling 18 hours prior to lipid transfer by changing half of the media (1 mL). In 7 day of culture, which means after 18 hours of C12 labeling, neurons were washed with pre-warmed DPBS 2 times and incubated with fresh NB+ medium for an hour at 37 ℃. Neuron and glia wells were washed with pre-warmed DPBS two times. Pre-warmed HBSS-calcium chloride medium, composed of Ca/Mg-free HBSS without phenol red, 20 mM HEPES, and 2 M calcium chloride, was added to the glial well. The coverslip containing neurons was inverted and placed onto the glial coverslip, and stabilized by pressing onto dental wax. This method was called sandwich culture and incubated at 37 ℃ for 4 hours. After 4 hours, neuronal coverslip was lifted carefully and placed into its previous well. Fat transfer can be directly imaged by staining nuclei with DAPI to minimize loss of C12 intensity. Coverslips protected from light were washed with PBS two times for 5 minutes and fixed with 3% PFA for 15 minutes at room temperature. After two PBS washes, nuclei were stained with DAPI prepared in PBS for 20 minutes. Coverslips were washed for 5 minutes with PBS and mounted with Anti-Fade Flourescence Mounting medium onto slide. Slides were imaged immediately after mounting to minimize loss of signal. All images were taken with Leica Confocal SP8 microscope and LASX software by 63X objective. ImageJ was used for quantification of fluorescence intensity and GraphPad Prism software used for analysis of images.

3.8 ENS Isolation for Assembloid Culture

2 of control FVB/N mice was sacrificed. Colon was dissected into ice-cold 1X PBS. Longitudinal muscle and myenteric plexus (LMMP) layers was peeled according to Smith et. Al., 2013. LMMP was put into ice-cold PBS immediately. LMMP was cut to 2-4 mm^2 pieces in 2 mL eppendorf containing digestion solution which contains 50 U/mL penicillin-streptomycin and 0.05 mg/mL Liberase TM in HBSS, placed on rotator for 30 minutes at 37℃. After incubation, eppendorf was shaken mildly and triturated with P1000 for 15 times after tip was wetted. Solution was spinned quickly to recover LMMP pieces from supernatant. Supernatant was taken and put onto different 1.5 mL eppendorf and FBS was added immediately to stop enzymatic activity. LMMP pieces in pellet was digested further with 0.05% Trypsin-EDTA solution at 37℃ for 10 minutes while rotating. Solution with LMMP pieces was triturated for 15 times and FBS was added immediately. Both supernatants were filtered through 70 µm strainer to a falcon and washed with Advanced DMEM/F12 at 150xg for 5 minutes. Cells were counted with NovoCyte Flow Cytometer and seeded as 150.000 cells/well in 12 well-plate. Cells were maintained in glia media made up of Advanced DMEM/F12, 100 U/mL Penicillin/Streptomycin, 10% FBS, 20 µg/mL of Gentamicin, 1x Glutamax I Supplement and 10 µg/mL of GDNF for 2-3 days. Then, cells were differentiated to neurons with NeuroBasal Plus medium, 2% B27 Plus and 0.5 mM GlutaMAX I Supplement. Half of the media was renewed every 2 days. Glia and neuron cells were used after 7-8 days after initial seeding for assembloid protocol.

3.9 Mouse Colon Crypt Isolation for Assembloid Culture

1 of control FVB/N mouse colon was isolated and flushed with ice-cold 1X PBS. Colon was opened longitudinally and washed with 1X PBS three times. Then, colon was cut into 1 cm pieces into PBS with 10 mM EDTA and 0.5 mM DTT. Cells were dissociated with EDTA/DTT solution in the rotator at 37℃ for 20 minutes. Solution changed to ice-cold 1X PBS and tissue pieces were shaken vigorously for 30 seconds. Supernatant was taken and centrifuged for 400xg for 5 minutes at 4℃. Remaining tissue pieces were used for mesenchymal cell isolation. Crypts were counted and seeded 150 crypt/dome into 10 µl matrigel as 3 drops into one well of 24-well plate. Matrigel dome was polymerized in 37℃ for 10 minutes and supplied with organoid

growth medium made from Advanced DMEM/F12 containing 10 mM HEPES, 2 mM GlutaMAX I Supplement, 100 U/mL penicillin-streptomycin, 1.25 mM N-acetyl-L-cysteine, 1x B-27, 1x N2, 50 ng/mL mouse EGF, 100 ng/mL human noggin, 0.5 µg/mL mouse R-spondin, 0.328 nM surrogate Wnt, 10 µM Y-27632, 0.5 µM A-8301 and 3 µM CHIR-99021. Media was changed every 2 days. Organoids were used after one passage.

3.10 Mesenchymal Cell Isolation for Assembloid Culture

Remaining tissue fragments from crypt isolation protocol were further used for mesenchymal cell isolation. Tissue fragments were washed with ice-cold PBS by vigorously shaking 30 seconds each to get rid of all epithelial remaining until clear supernatant was obtained. Fragments were cut into tiny pieces and merged with digestion medium which contains Ca-Mg free HBSS, 100 µg/mL Liberase TM and 400U/mL DNase I. After 20 minutes in rotator at 37 C, fragments were triturated with 10 mL serological pipette and supernatant was collected. To stop enzymatic activity, 10% FBS were put into supernatant. Fresh digestion medium was supplied to remaining tissue fragments for further digestion. After 20 minutes in the same conditions, fragments were triturated and supernatant was collected. After FBS addition, cells were filtered through 70 µm strainer and centrifuged at 400xg for 5 minutes at 4 C. Pellet was reconstituted with stromal growth medium made up of Advanced DMEM/F12, 10% FBS, 100 U/mL penicillin-streptomycin and 10 µM Y-27632; seeded into 6 well-plate. Media was changed every 2-3 days.

3.11 Assembloid Culture

3-4 days old organoids were collected from matrigel with Cell Recovery solution (Corning) and incubated in it for 30 minutes to completely depolymerize matrigel and washed once with ice-cold DBPS, centrifuged at 100xg for 1 minute. Organoids were reconstituted with Matrigel as 20 organoids in 0,5 µl. Mesenchymal cells in 6-well plates were washed once with Advanced DMEM/F12 and detached from plate with 1 mL of 0.05% Trypsin-EDTA solution (ThermoFisher Scientific) for 15 minutes at 37 ℃. When cells were detached, trituration was applied for 5 times for each well to ensure proper detachment. Cells were collected and washed once with Advanced

DMEM/F12 by centrifuging at 400xg for 5 min at 4 ℃. Cell number was determined and resuspended in Matrigel (1.5 x 10^5 cells for 1,75 µl Matrigel). Neuron and glial cells (ENS) were washed once with Advanced DMEM/F12 and detached from the coverslip with 500 µl of 0.05% Trypsin-EDTA solution (ThermoFisher Scientific) for 10-15 minutes at 37 ℃. Upon detachment, cells underwent five rounds of trituration per well to ensure complete detachment. The cells were then collected and washed once with Advanced DMEM/F12 by centrifugation at 400xg for 5 minutes at 4℃. The cell count was determined, and the cells were resuspended in Matrigel at a concentration of 10^5 cells per 1,75 µl Matrigel. 70% alcohol and UV-sterilized Parafilm sheet was placed into 100 mm petri-dish and PBS droplets were put around Parafilm sheet to sustain humidity. 1:1 ratio of ENS and mesenchymal cells were mixed and 2 µl of the mixture were placed onto Parafilm sheet with P2 pipette. Immediately, 0,5 µl of organoid with 1,5 µl of mixture were mixed and put onto the center of the previous droplet. As result, one of the 4 µl of assembloid was produced. Assembloids in petri-dish were solidified in 37 ℃ for 30 minutes. Using very fine forceps, each assembloid was carefully transferred from the Parafilm sheet into pre-warmed assembloid growth media in a 12-well plate. Assembloids were cultured at 170 rpm on an orbital shaker in cell culture incubator. Assembloid growth media consist from Advanced DMEM/F12 containing 10 mM HEPES, 2 mM GlutaMAX I Supplement, 100 U/mL penicillin-streptomycin, 1.25 mM N-acetyl-L-cysteine, 1x B-27, 1x N2, 50 ng/mL mouse EGF and 0.5 µM A-8301. Media was changed every 2 days.

3.12 Assembloid Immunohistochemistry

Assembloids were cultured for 8 days and washed once with 1x PBS and fixed with 4% PFA overnight at +4 ℃. Next day, assembloids were washed with 1x PBS for three times 5 minutes each. To avoid sticking into pipette tip, 0.1% BSA/PBS incubation was applied for 30 minutes at RT. Assembloids washed once with 1x PBS and placed into a plastic mold with a cut pipet tip. All liquids were removed from assembloids and 1% agarose was merged with assembloids. Agarose mold with assembloid was taken out from plastic mold and tissue paraffination protocol was applied manually.

For frozen embedding, After assembloids were put into plastic mold, embedded into Tissue-Tek O.C.T (Sakura Finetek, Tokyo, Japan, 4583) and stored at -20. Tissue protocols were carried out the same as for sectioning and immunohistochemistry for assembloids.

3.13 Spatial Transcriptomic Analysis

Spatial transcriptomic data from Day 0 and Day 14 (Parigi et al., 2022) were analyzed using SpaceRanger from 10x Genomics. The spaceranger count pipeline was utilized for the analysis of both datasets and parameters were not changed from original. The resulting .cloupe files were used for visualization and further analysis in the Loupe Browser from 10x Genomics. Alignment of tissue image with spots were done by Loupe Browser internal function with .cloupe file. Scale values for all features were used as Log2 in Loupe Browser.

4. RESULTS

4.1 Enteric Neuron and Glia Increases in DSS

4.1.1 Colitis Mouse Model

We used DSS induced colitis model to mimic inflammation. Therefore, we used 8- to 12-week-old male mice, treating them with a 2.5% DSS concentration in their drinking water for 5 days (Figure 8). At the end of fifth day, the mice were sacrificed, and their colons were either embedded in paraffin or frozen for immunohistochemistry analysis, forming the ‘no recovery ’group. On the same day, we also isolated the colons from a control group of mice that drink only drinking water for comparison to assess the effects of DSS treatment. For the recovery group, we treated mice with DSS for 5 days. End of the fifth day, we began giving them drinking water for the following one or two days. At the end of the six and seven days, the mice were sacrificed, and their colons were collected for analysis.

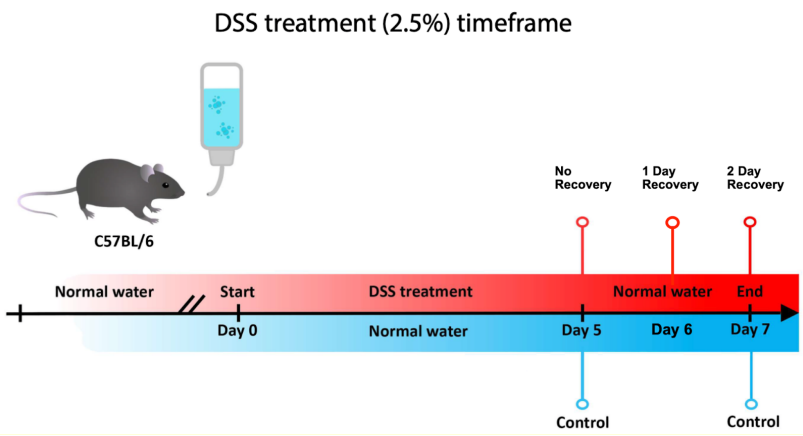


Figure 8. DSS mouse model timeline. Timepoints represent the sacrifice time of each group.

First, we assessed the success of the DSS treatment by bloody stool and weight loss. Then, Ki67 and Sox9 antibody immunohistochemistry were used foor analysis (Figure 9A,B,C). Ki67 is a marker of proliferative cells found in colonic crypts, while Sox9 is associated with stemness in the colon. In the no recovery group, we observed lack of proliferative cells and stem cells (Figure 9B, E, G). This result shows that DSS

treatment had a strong impact on colonic crypts. In recovery, proliferation and stemness markers reappear as a sign of crypt formation and regeneration (Figure 9C, F, G). Another indication of DSS damage is disrupted epithelia. Our DSS model clearly demonstrates its efficacy in disrupting the colonic epithelium, as evidenced by the absence of proliferative cells and stem cells after 5 days of administration.

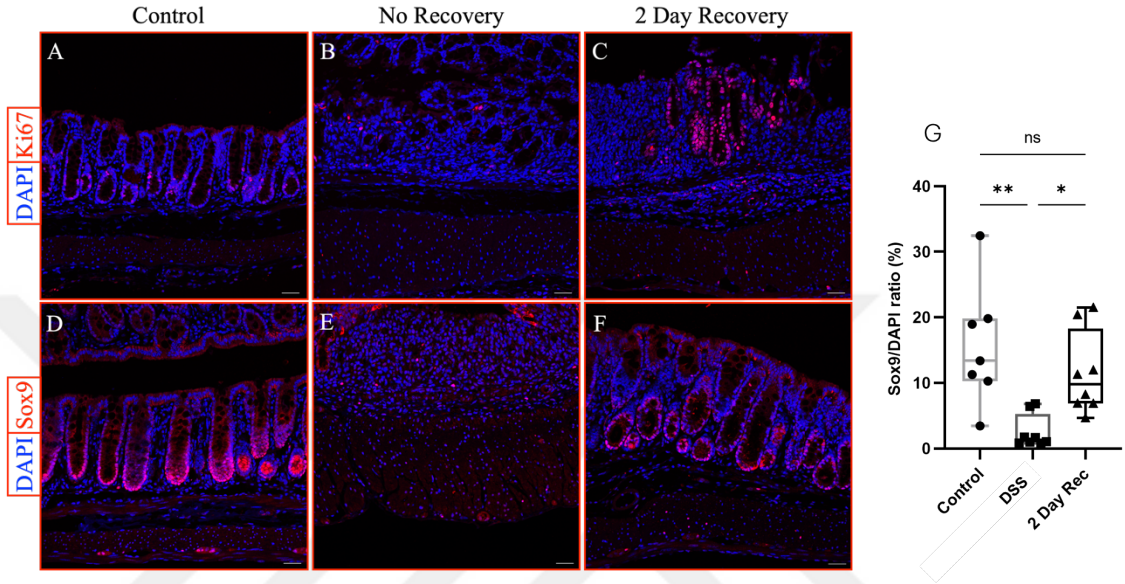


Figure 9. Proliferative cells upon DSS treatment and recovery. Ki67 and Sox9 is a proliferative cell marker shown in red, n=2. Scale bar represents 50 µm.

4.2 Enteric Nervous System Characterization

First, we characterized all cell types in the enteric nervous system. We used Tubb3 staining to identify enteric neurons, which we found in all layers of the colon (Figure 10). In the control tissue, we observed fibrous neurons in mucosa, while other types of neurons resided in the muscle layer. Additionally, we were able to locate two plexuses: the submucosal plexus and the myenteric plexus. Neurons within these plexuses formed specialized networks among themselves. Damage in mucosa leads to disruption of continuous fibrous neurons in Figure 10B; instead they appear dispersed. Muscle layer was thickened with inflammation and neurons were increased in this area (Figure 10D). In particular, neurons in the muscle area changed their orientation towards mucosa. This trend was sustained in recovery tissue (Figure 10C). With recovery, neurons in the muscle area inclined towards mucosa to restore colon epithelial function.

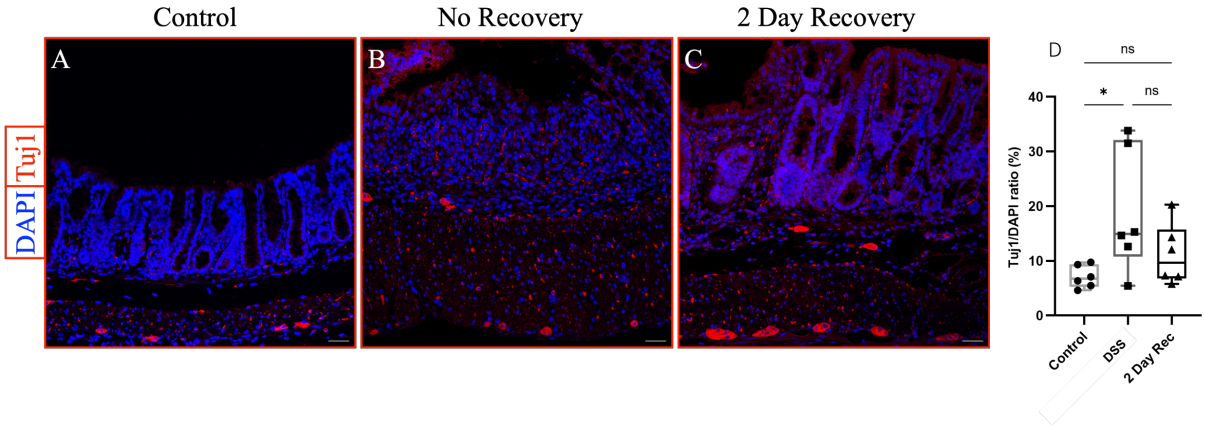


Figure 10. Tubb3 is a panmarker of enteric neurons. Tubb3 (Tuj1) in red staining was increased with DSS treatment. n=2, scale bar represents 50 µm.

After neuronal cells, glial cells were also characterized with S100b and GFAP antibodies by staining. S100b is calcium binding protein that can be localized into nucleus and cytosol of glial cells. Glial fibrillary acidic protein (GFAP) is found in ENS and marks the enteric glial cells. In Figure 11A, we observed that S100b marks glial cells in the mucosal, submucosal, and muscle areas. In the no recovery tissue, S100b was significantly upregulated in the muscle area, accompanied by an enlargement of the muscle (Figure 11B). With recovery, the expression profile returns to control levels. GFAP marker is more specialized to myenteric plexus glia population. In Figure 11D, E, F, while its expression is confined to plexus specific glia, it's expression overlaps with mesenchymal marker Vimentin in myenteric plexus.

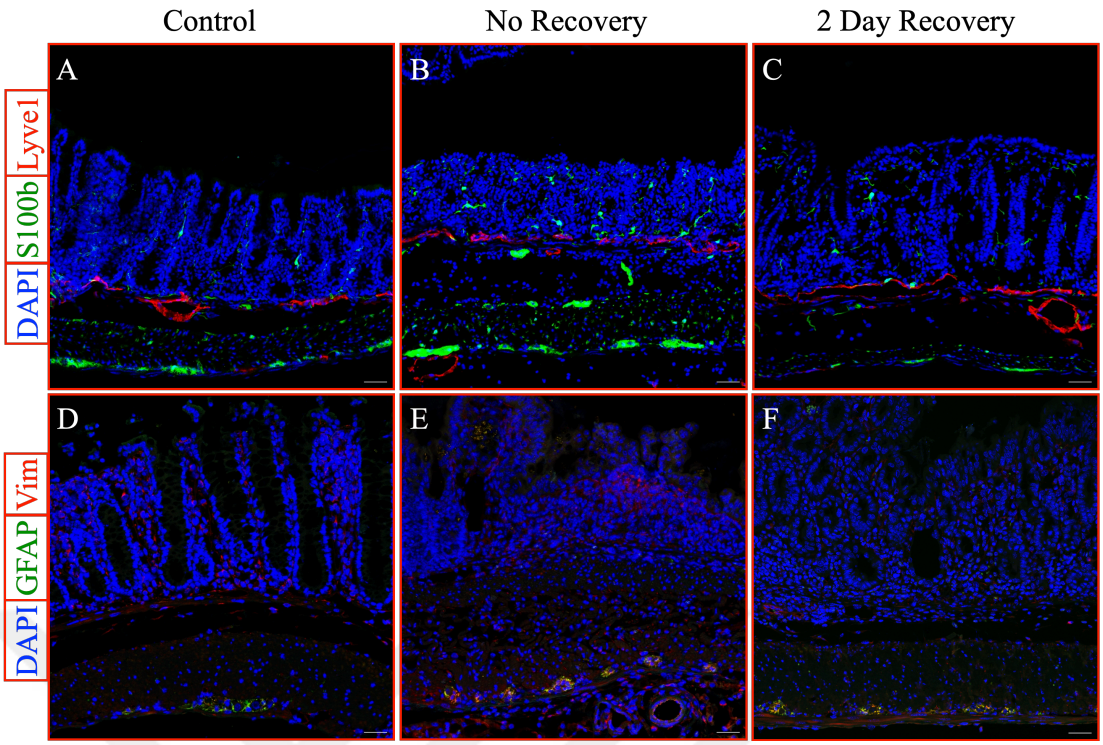


Figure 11. Enteric glia is present throughout layer of colon. S100b (green) and GFAP (green) are enteric glia markers. Lyve1 is endothelial cell marker that is found mostly in submucosal region. n=2. Scale bar represents 50 µm.

We also aimed to characterize mesenchymal cells in the colon, which reside very closely with the enteric nervous system (ENS). Therefore, we stained for CD34 and Pdgfra markers. As shown in Figure 12, we found that CD34+ mesenchymal cells were mostly located in the submucosal and mucosal regions (Figure 12), with no significant change in their expression profile. On the other hand, Pdgfra+ mesenchymal cells were found in every layer of the colon (Figure 14). Interestingly, there was a population of Pdgfra+ cells surrounding enteric ganglia in both submucosal and myenteric plexus. We thought that these cells may play important role in homeostasis of the ENS.

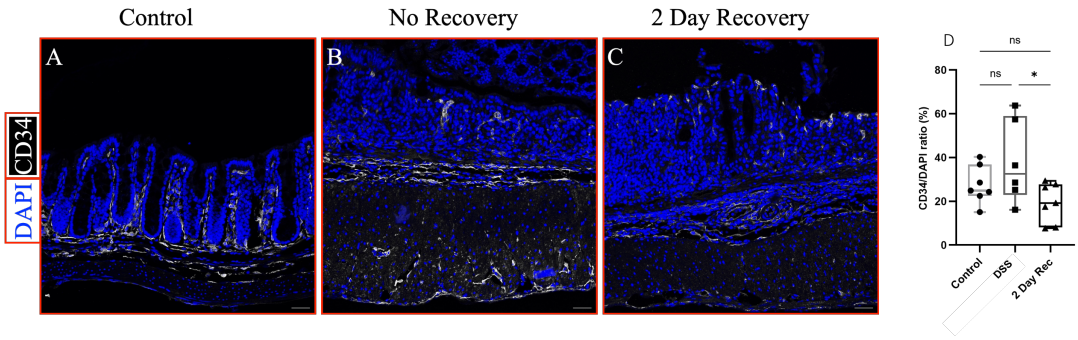


Figure 12. CD34+ mesenchymal cells are observed mostly on the dss mucosa and submucosa layers. There is no significant expression pattern change by DSS administration and recovery; except they can be observed in muscle region in DSS. n=2. Scale bar represents 50 µm.

4.3 Acot7 is a New Marker for Enteric Nervous System

We further analyzed enteric neurons using various known markers, such as HuC-D and Phox2b. HuC-D protein is a pan-neuronal marker in the central and peripheral nervous system while Phox2b protein is expressed mostly in enteric neurons. In Figure 13, we observed that ENS cells in the myenteric plexus were positive for both HuC-D and Phox2b markers. While all Phox2b-positive cells were also positive for HuC-D, some neurons were only positive for HuC-D marker. Interneurons in muscle or mucosa did not give expression in their nucleus for both markers. In the lights of these findings, we aimed to find a new marker for ENS. Therefore, previous works of re-analyzing single-cell RNA sequencing data of DSS animal model to investigate differentially regulated genes were observed. One of the prominent genes in the analysis was found to be Acyl-CoA Thioesterase 7 (ACOT7) which regulates fatty acid metabolism by hydrolysis of long-chain acyl-CoA to free fatty acids and free CoA. Therefore, we hypothesized ACOT7 to be a marker gene of ENS cells.

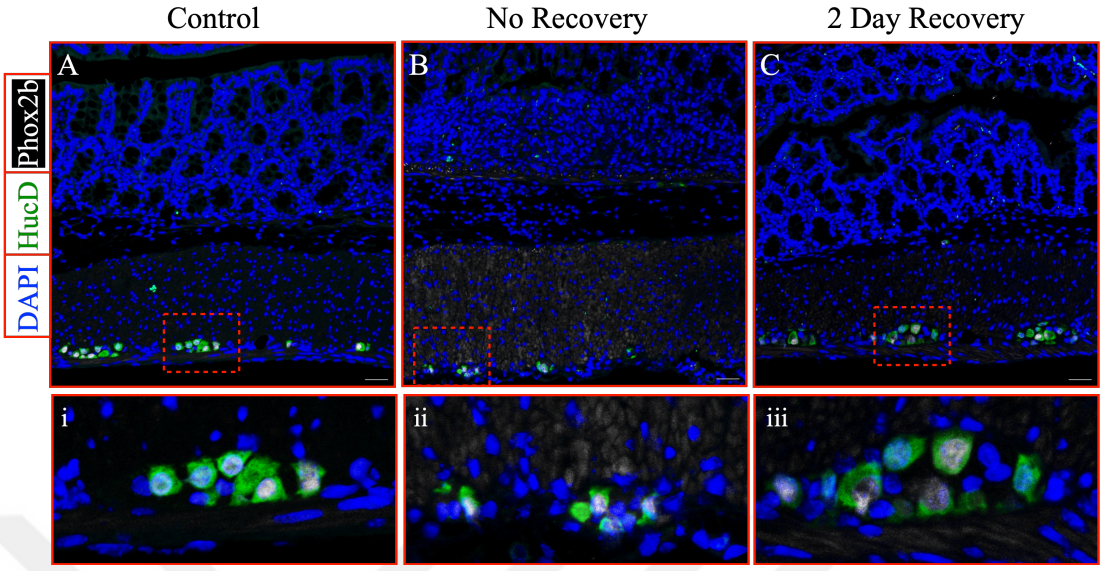


Figure 13. HuC-D and Phox2b positive neurons are located in myenteric plexus region. While Tubb3 stains every neuron in colon, HuC-D and Phox2b marks only ganglionic neurons. n=3. Scale bar represents 50 µm.

We applied IHC analysis against ACOT7 protein in DSS model and found out that it marks specifically ENS cell types (Figure 14) and Tuft cells in all three conditions. Because Tuft cells are thought to be in a close relationship with ENS, specificity of our new marker also includes epithelial cell type. To further prove our hypothesis, we applied IHC analysis with known enteric neuron marker HuC-D and Pdgfra+ mesenchymal cell marker (Figure 14). In the control sample, we detected expression of ACOT7 in both enteric glia and neuron cell types, specifically in circular and longitudinal muscle. We observe both nucleus but mostly cytosolic specific binding of ACOT7.

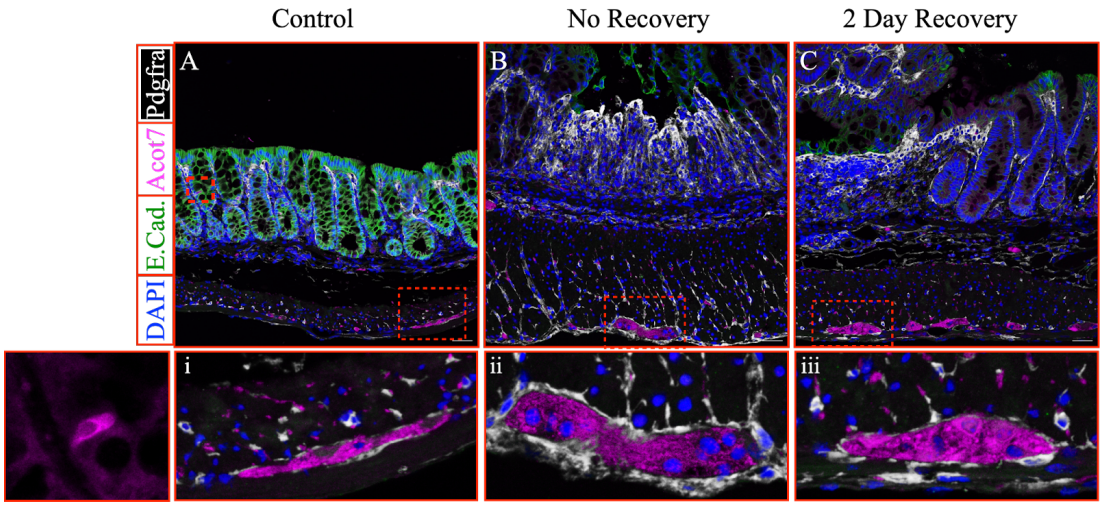


Figure 14. Acot7 is a new marker for enteric ganglia and is surrounded by Pdgfra+ cells. Acot7 specifically binds to ENS cells in myenteric plexus while also stains some of the muscle ENS cells. Also, Pdgfra+ cell surrounding indicates myenteric ganglia specific staning. n=3. Scale bar represents 50 µm.

Moreover, ACOT7 expression in myenteric ganglia is closely enclosed with Pdgfra+ cells as an indicator of ganglia (Figure 14). It also gives an expression in the tip of epithelia, which resembles Tuft cell phenotype. We also observed the same type of expression after DSS treatment. In no recovery and recovery sample, ACOT7 was found in myenteric ganglia. As expected, we observed that its expression was more obvious in interneurons in the muscle that was inclined towards mucosa. However, in the computational analysis of the IHC data, we did not find any significant change in ACOT7 expression between the control, no recovery, and recovery samples. To further support our hypothesis, we aimed to demonstrate β-catenin expression in the ENS, as it is expressed by neural crest cells. We performed IHC analysis for β-catenin and ACOT7, which revealed overlapping expressions in the myenteric ganglia (Figure 15). Therefore, we can confidently state that ACOT7 is a new marker for ENS cells residing in the muscle area.

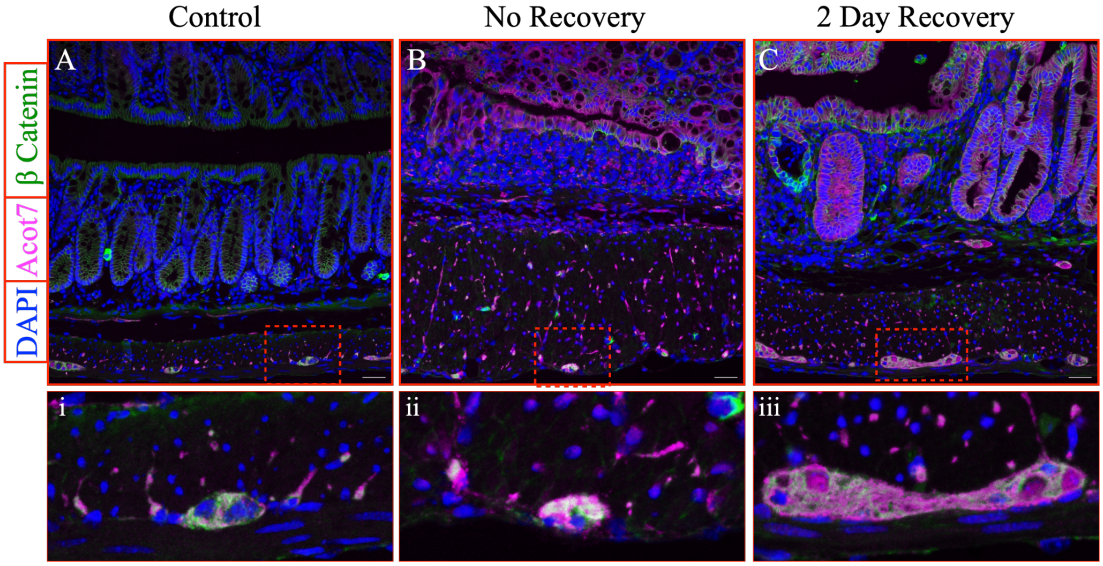


Figure 15. Acot7 cells are overlapping with β-catenin expressing cells. n=2. Scale bar represents 50 µm.

4.4 ENS and Pdgfra+ Cells Are Working Together To Improve Mucosa

In Figure 16, we observed an increase in the expression of S100b+ glial cells in response to inflammation in both the no recovery and recovery samples. Interestingly, when we examined glial cells alongside Pdgfra+ cells, we found that both S100b+ glial cells and Pdgfra+ cells re-oriented themselves towards the mucosa (Figure 16). Therefore, we found that the both cell types support and assist each other in responding to damage in the mucosal layer of the colon caused by inflammation; as they are observed in close proximity to each other in the no recovery and 2 day recovery samples. Moreover, in Figure 14, ACOT7+ cells in the muscle exhibited spatially proximal expression with Pdgfra+ cells. Because we found that ENS cells have this type of tendency to alter their direction to epithelia when there is inflammation or recovery, there was more evident expression of ACOT7 in the muscle area in DSS and recovery samples. These findings further support that ACOT7 is expressed in ENS cell types and can be suggested as a new ENS marker. Therefore, we aimed to investigate how ENS cells contribute to the recovery of the epithelium. We approached this question by examining the mechanism of how these cells support epithelial recovery both metabolically and mechanically.

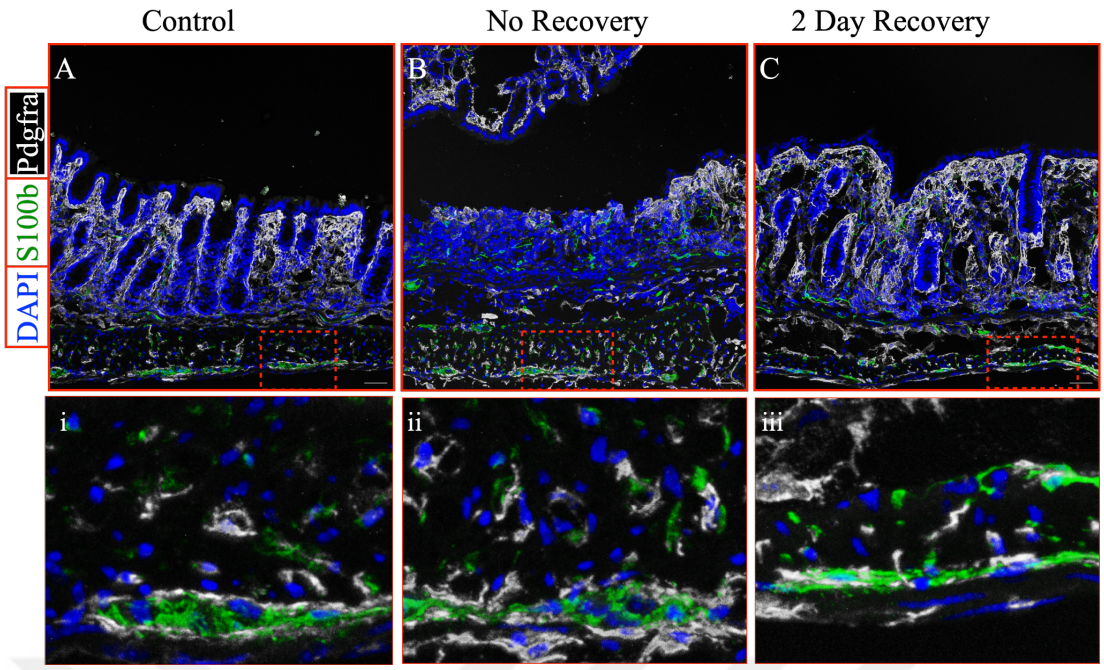


Figure 16. S100b+ and Pdgfra+ cell are located in close proximity in muscle. Besides, Pdgfra+ cells surrounding myenteric ganglia, there are Pdgfra+ cells in the muscle that reside near glia cells during DSS. n=3. Scale bar represents 50 µm.

4.5 Enteric Glia Protects Hyperactivated Neurons From Lipid Induced Toxicity

As we observed in previous figures, enteric neurons were increased and hyperactivated in the no recovery and 2 day recovery samples. It is known that hyperactivated neurons in other tissues, such as the brain, are not favorable for cell health. During hyperactivation, cells produce lipid droplets, leading to lipid toxicity in neurons and eventually resulting in neurodegeneration. Therefore, we examined lipid metabolism proteins in the ENS using IHC staining. FABP4 is an important regulatory protein for lipid metabolism in the colon. It is known for its role in fatty acid uptake from surrounding cells, transport of lipids throughout cell compartments and further breakdown of lipids. In Figure 17, we observed that FABP4 was localized in both the mucosal and muscle layers as anticipated. In the control sample, FABP4 expression exhibited a pattern reminiscent of lymphatic or blood vessels, extending towards the crypt tips. Additionally, near the crypt base, round staining was observed around vessel-like structures. We found small round stainings at all levels of the muscle layer, indicating a denser expression of FABP4 in the muscle. In the DSS sample, the expression of FABP4 in the muscle decreased with inflammation, evident by smaller and more dispersed round staining. On the second day of recovery, its expression increased in response to hyperactivation and the recovery of the epithelium. However,

we did not observe obvious staining in the myenteric plexus ENS cells in control, we observed FABP4 expression in no recovery and mostly 2 day recovery timepoints.

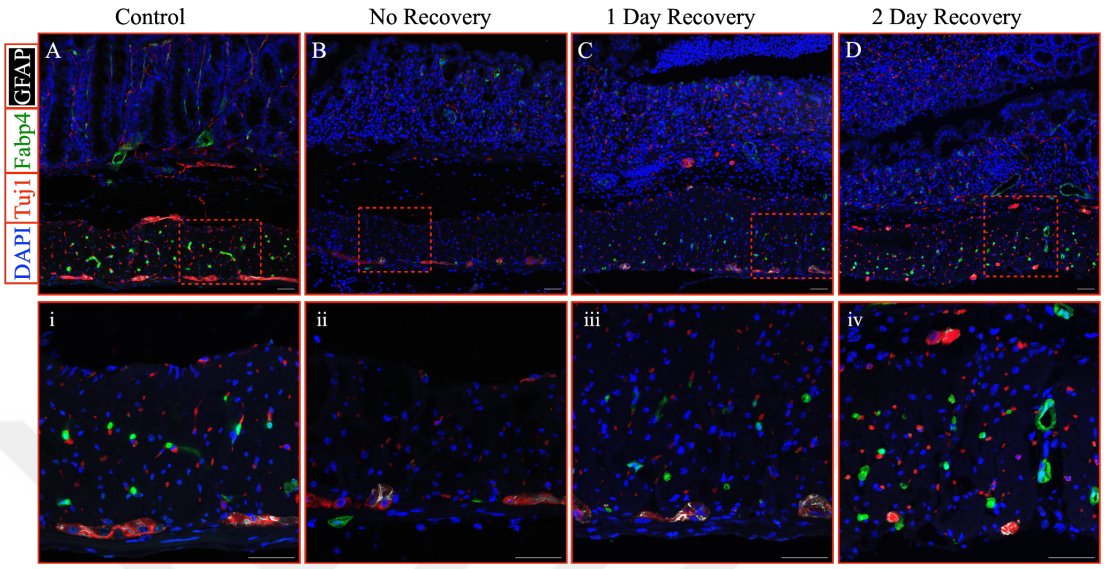


Figure 17. Lipid metabolism protein Fabp4 is detected in muscle of colon. n=3, scale bar represents 50 µm.

As a second important protein for lipid metabolism, we choose APOE protein to investigate. It is responsible for lipid transport through cells and different organs in the body. It is integrated into lipoprotein particles present in the brain. Through IHC analysis with ENS markers, we found that APOE is located in both enteric glia and neurons (Figure 18). In the control tissue, APOE was mostly found in cells at the tip of the crypt, such as enterocytes and cells in the mucosa, as well as in myenteric glia, myenteric neurons, and the muscle area. With DSS treatment, its expression increased in the myenteric ganglia. It significantly overlaps enteric glia cells in myenteric ganglia within 2 days of recovery.

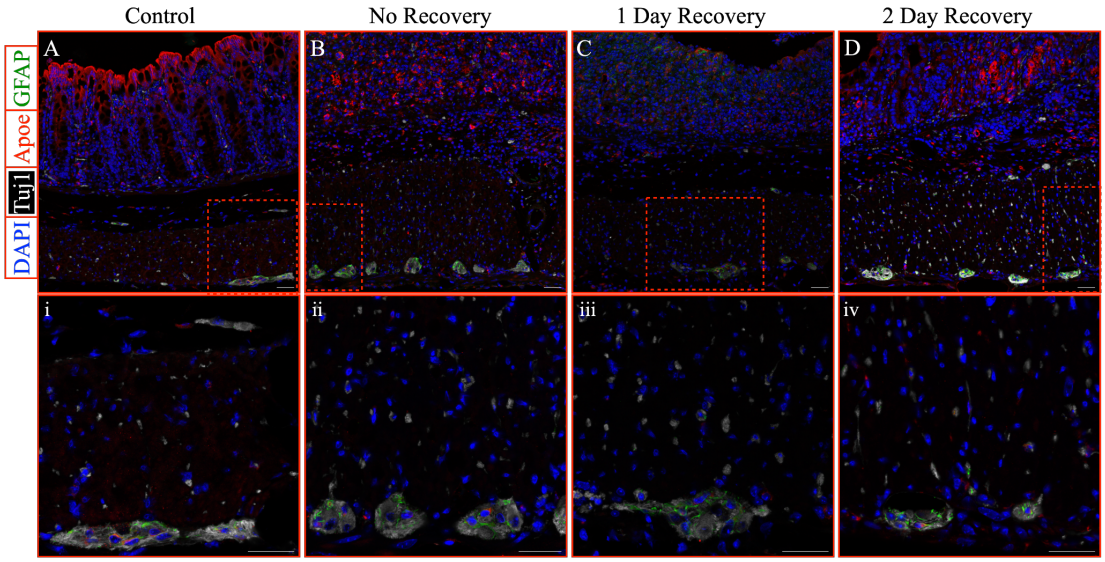


Figure 18. APOE is detected in myenteric plexus ganglia. It is found in lipid carrier lipoparticles detected in all model timepoints. n=3. Scale bar represents 50 µm.

Given that neurons have limited lipid metabolism, another cell type must assist in managing the consequences of hyperactivation, such as lipid accumulation. Therefore, we hypothesized that during inflammation, neurons transfer their lipids to glial cells to maintain their cellular health. To validate this hypothesis, we isolated primary ENS cells from control mice and differentiated them into enteric glial and neuronal cells (Figure 19A, B). Observations of upward migration and lipid accumulation in the myenteric ganglia and muscle cells led us to isolate ENS cells specifically from the serosal and muscle layers. These cells exhibited a high inclination to differentiate into both glial and neuronal lineages when cultured in specific media. We subsequently optimized the differentiation media to achieve a cell population with at least 95% purity for both neuronal and glial cell types.

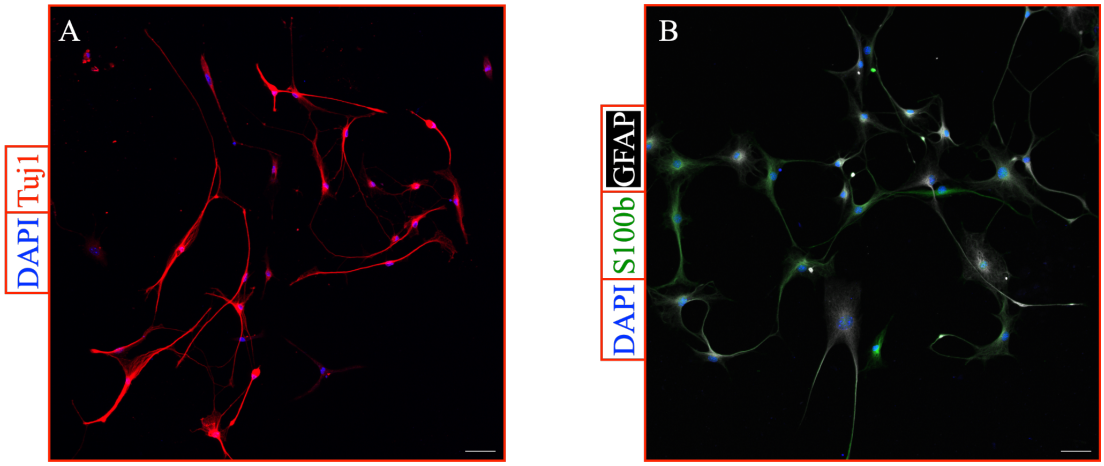


Figure 19. Enteric neuron (red) and enteric glia (green, white) cells in vitro from primary mouse cells. ENS precursor cells from colon were isolated and differentiated into neuron (A) and glia (B). n=2. Scale bar represents 50 µm.

Then, we applied lipid transfer assay to examine whether there is fat exchange between two cell types. To achieve our purpose, we designed a method that each cell type was grown on the specially coated glass coverslip (Figure 20). Because we hypothesized that hyperactive neurons pass their toxic lipids to glia, we only labeled neuronal lipids with BODIPY 558/568 C12 fluorescence fatty acid label for 18 hours. Then, neuron coverslip was turned upside down onto glia coverslip and spaced with sterilized dental wax (Figure 20). To achieve hyperactivated neurons, coculture was incubated in CaCl media for 4 hours. After incubation, coverslips were separated and analyzed for lipid transfer. In Figure 21A, we observed that neuronal lipids were successfully labeled with C12. Round staining of lipid particles can be observed in the cytoplasm of neurons. They were found both in the body and axon site of neurons. Regarding our hypothesis, we discovered C12 positive lipids in glia coverslip that were transferred from enteric neurons (Figure 21B). In Figure 21B, lipids from the labeled neurons were uptaken by glia to retain in their cell body.

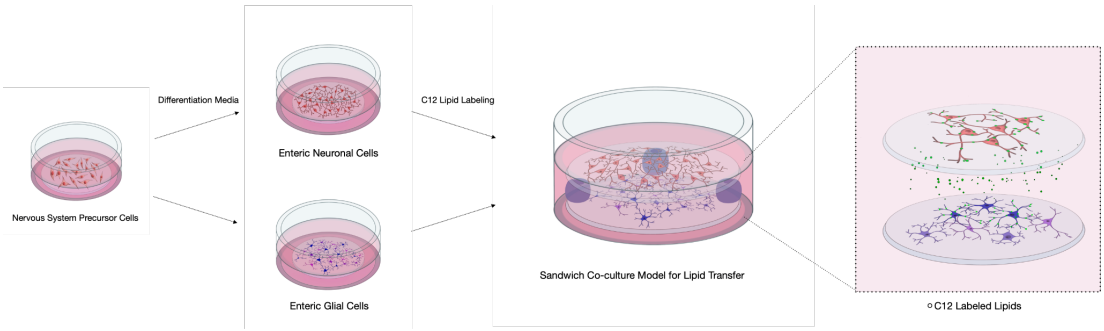


Figure 20. Sandwich method for lipid transfer.

To investigate whether inflammation has an effect on lipid transfer in ENS, we treated enteric glia with inflammatory cytokines; IL-1B, TNFa and IFNg (Figure 22). Before 48 hours of lipid transfer, we changed half of the medium with cytokine medium and repeated the lipid transfer procedure. After 4 hours of transfer, we analyzed the C12 presence in neuron and inflamed glia. There was no change in overall lipid transfer amount between control and inflamed glia (data not shown). However, in Figure 22B, we observed that C12 in inflamed glia started to disperse around, which we suggested as processing of lipid particles to protect neurons as a result of inflammation. These results confirm that enteric glia acts as a protective hub for neuron cells during hyper activation in the regenerative stage of colon.

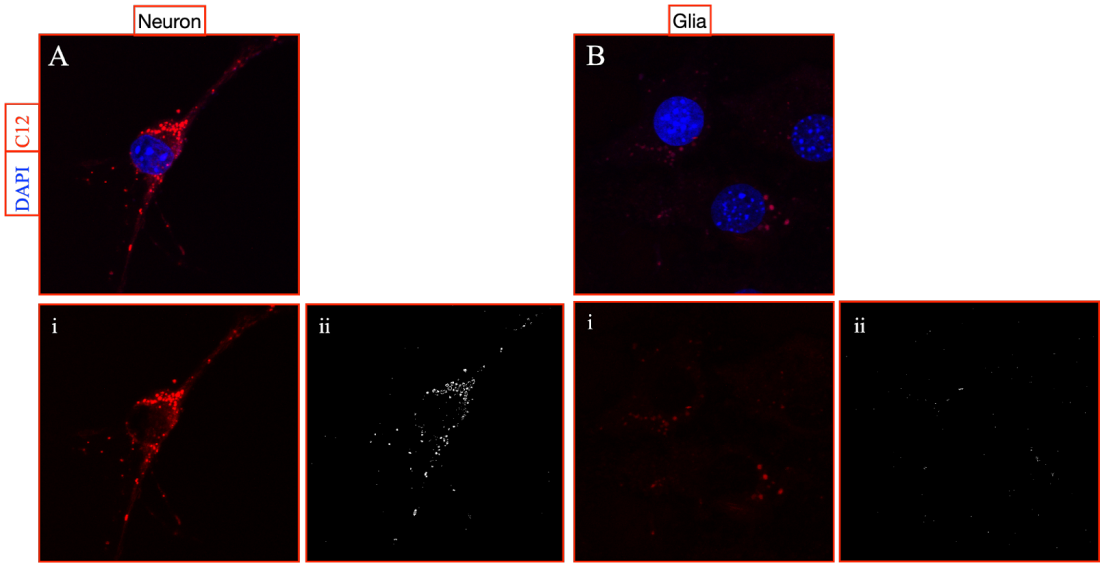


Figure 21. Lipids in enteric neurons are transferred to enteric glia. Lipids in enteric neurons are detected by C12 (red) after transfer. Neuron (A) is incubated with C12 lipid dye; after co-culture we observe neurons lipid particles in the glia (B). n=2, Scale bar represents 50 µm.

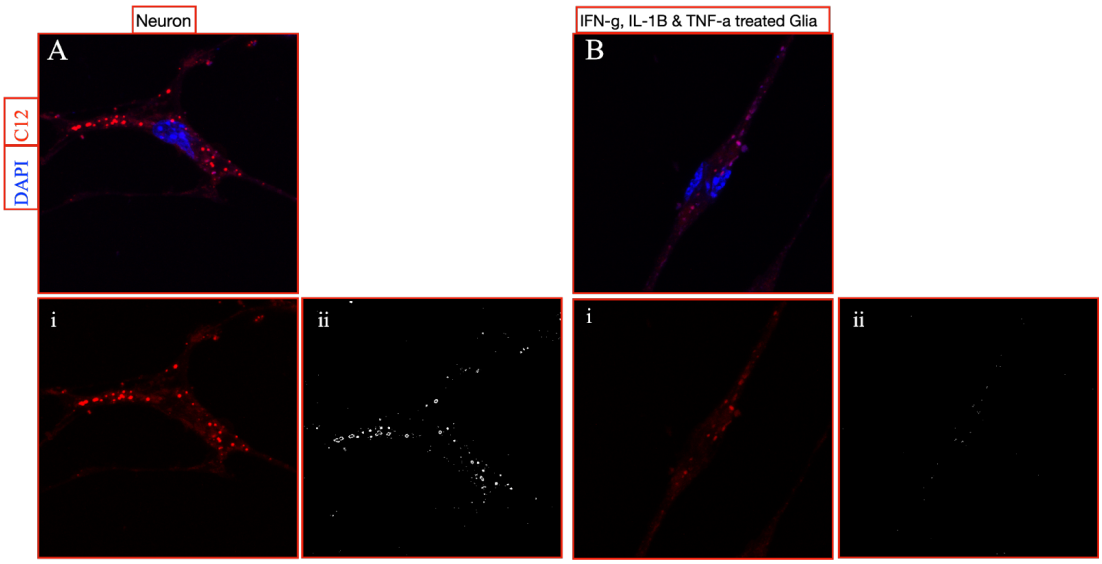


Figure 22. Lipids in inflamed enteric glia are detected by C12 (red) after transfer. Lipids in enteric neurons are detected by C12 (red) after transfer. Neuron (A) is incubated with C12 lipid dye; after co-culture we observe neurons lipid particles in the inflamed glia (B). n=2, Scale bar represents 50 µm.

4.6 Spatial Transcriptomics Reveal Mechanic Perspective of ENS Cell Inclination

After investigating the metabolic response, we wanted to examine the mechanical reaction of the ENS as inclination of cells in muscularis externa to submucosa in recovery. To do this, we utilized single-cell RNA sequencing data analysis and spatial transcriptomics. The single-cell analysis was based on mice treated with 3 cycles of 2.5% DSS for 7 days, followed by 7 days of sterile water for recovery (Jasso et al., 2022). Upon analysis using single-cell sequencing from previous work, we identified that the genes PTN, PTPRZ1, L1CAM, NCAM1, FGF2, MCAM, and PDGFA were expressed in a subset of glial cells. Notably, PTPRZ1 and L1CAM were specifically expressed only in glia (Figure 23).

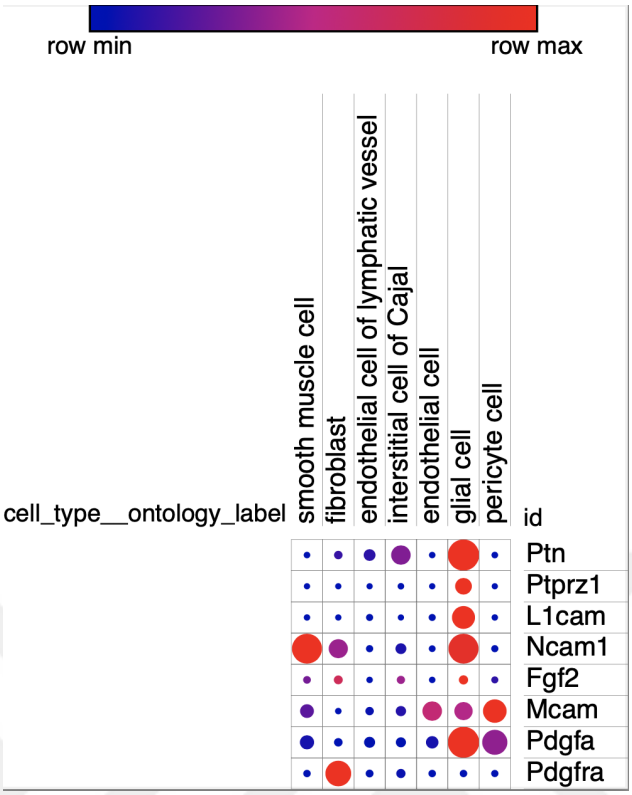


Figure 23. Dot plot for single cell RNA-seq of colon stroma dataset.

PTPRZ1 is a tyrosine phosphatase receptor typically found in the central nervous system. Although its role has not been fully deciphered, it is thought to be involved in developmental processes. One of the prominent ligands of PTPRZ1 is PTN (pleiotrophin), a gene involved in cell migration, survival, and growth. PTN is mainly expressed in enteric glia, as well as in fibroblasts and interstitial cells of Cajal (Figure 24A). Our single-cell RNA sequencing analysis revealed that PTN expression was upregulated following DSS treatment. Additionally, L1CAM, a cell adhesion molecule, plays a role in neural development and migration. Based on these findings, we hypothesized that the PTN-PTPRZ1 axis could be a significant gene pair in explaining the mechanical response of the ENS to inflammation.

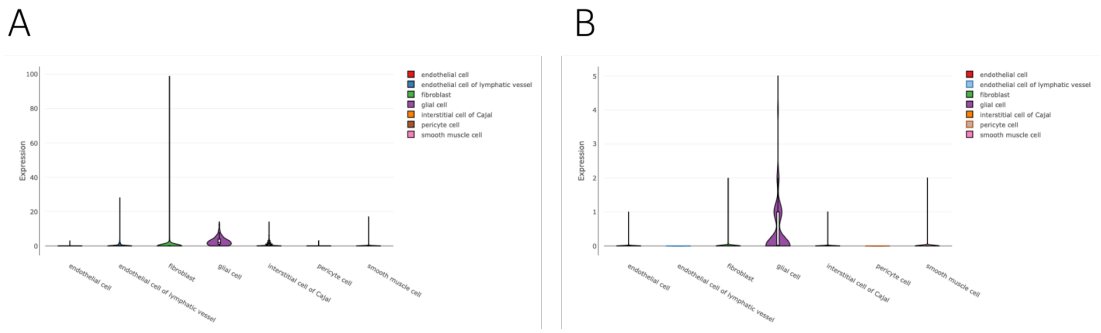


Figure 24. Violin plot of gene expression in different cell types. Ptn (A) and Ptprz1 (B) is both expressed in enteric glial cells.

We conducted IHC analysis for PTN in our animal model and confirmed its localization in enteric glia and fibroblast cells (Figure 25). Unfortunately, we were unable to get the PTPRZ1 antibody to work in the IHC experiment. However, we specifically observed PTN expression in the myenteric ganglia of control, DSS, and recovery samples. To further confirm its presence, we performed triple staining with the glial marker GFAP and the mesenchymal marker PDGFRA. PTN expression overlapped with these markers, which confirms its location in enteric glia and mesenchyme, suggesting it could be a ligand involved in cell migration during inflammation.

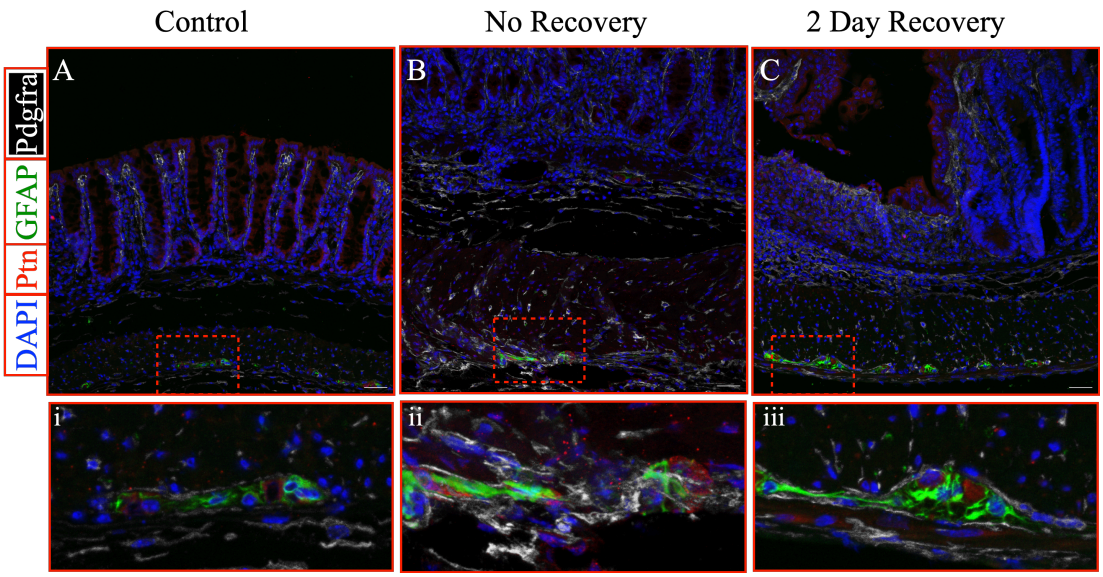


Figure 25. Ptn is expressed in the myenteric plexus ENS cells. IHC staining with Ptn (red), Pdgfra (white) and GFAP (green) indicates that Ptn is increased in DSS. n=2. Scale bar represents 50 µm.

After reanalyzing single-cell RNA sequencing, we sought to further validate the PTN-PTPRZ1 axis as a cell migration pathway involved in the ENS response to inflammation using spatial transcriptomics. Spatial transcriptomics is a method that reveals gene expression while preserving its spatial location within the tissue. We utilized spatial transcriptomics data from Parigi et al., which includes Day 0 (control) and Day 14 (recovery) samples of the colon. After reanalyzing the spatial data, we used the Loupe Browser to visualize and further investigate the results. We were able to map the expression of PTN and PTPRZ1 to the muscle area where the myenteric ganglia reside (Figure 26). Comparing the Day 0 and Day 14 samples inside red linings which highlight long crypts in control sample and regenerating areas on day14 sample by article, we observed that PTN expression increased, while PTPRZ1 expression remained unchanged. To explore this further, we conducted a co-expression analysis of PTN and PTPRZ1, coding PTPRZ1 in blue and PTN in yellow, with co-expression indicated in green. In the Day 0 analysis, we observed several green spots, suggesting that the PTN-PTPRZ1 pair is found in close proximity within the control tissue. In the Day 14 analysis, we observed a significantly higher occurrence of green spots specifically in the myenteric plexus layer (Figure 27). This data indicates that the PTN-PTPRZ1 axis plays a crucial role in cell migration during the recovery of the colon from inflammation.

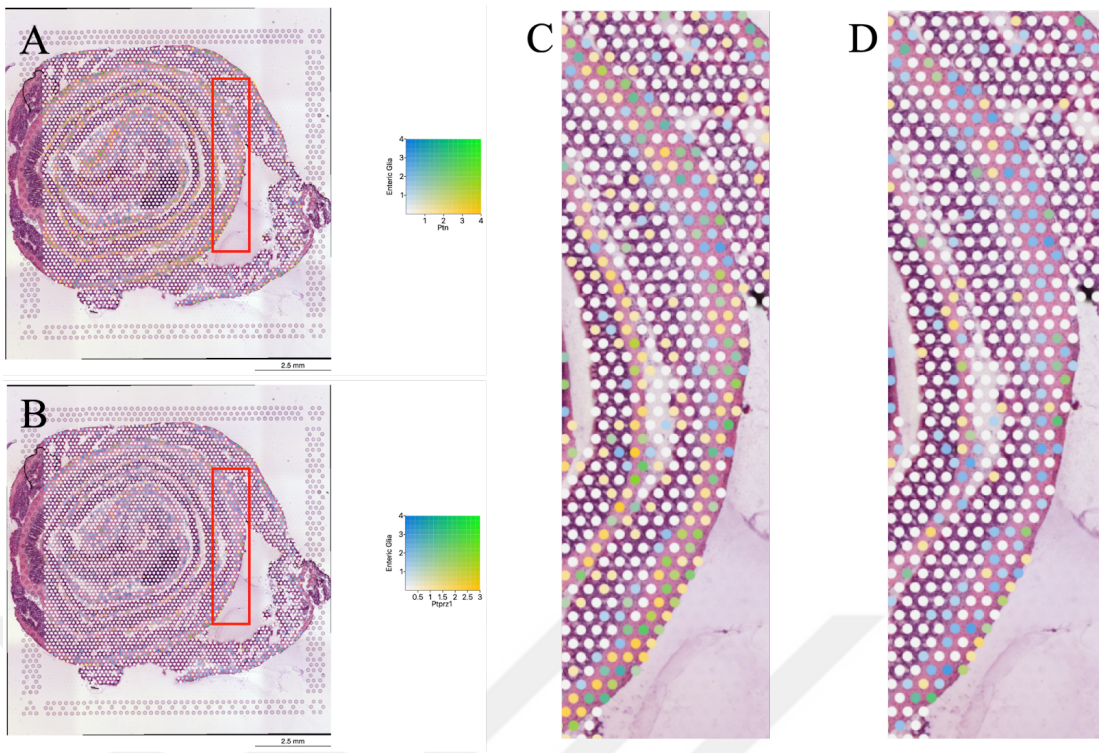


Figure 26. Ptn-Ptprz1 genes spotted onto Day14 colon sample with spatial transcriptomics. Ptn (A) and Ptprz1 (B) expressed near muscle area. Spots in green shows that they are expressed in enteric glia. Red lining defines regenerative area. C for Ptn and D for Ptprz1 are close-up images of red linings.

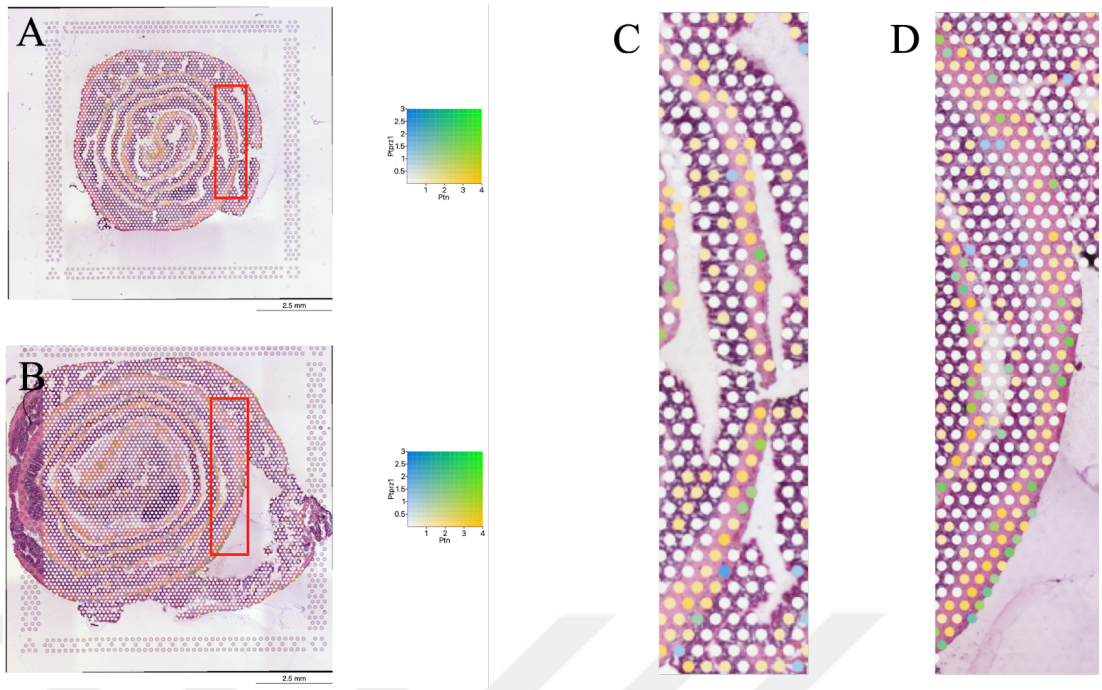


Figure 27. Ptn-Ptprz1 co-expresssion analysis spotted onto Day0 and Day14 colon samples with spatial transcriptomics. Day0 (A) green spots are more than Day14 (B) in muscle area. Red lining defines long crypt region (A) and regenerative area (B). C for Day0 and D for Day14 are close-up images of red linings.

We also identified other genes important for ENS migration, including L1CAM, MCAM, FGF2, NCAM1, PDGFA, and PDGFRA. All of these genes were localized to the muscle layer, which is our area of interest. Between the time points, we observed an increase in L1CAM, FGF2, and NCAM1 expression during recovery, while MCAM expression decreased (Appendix). These findings support our hypothesis that the ENS migrates along the mucosa to aid in the recovery of the epithelium. Moreover, we conducted a co-expression analysis of the PDGFA-PDGFRA axis, a known cell migration pathway. As expected, we observed co-expression in the control sample. We also detected co-expression on Day 14, but we could not conclude any significant change in their expression levels or spot positions (Figure 28).

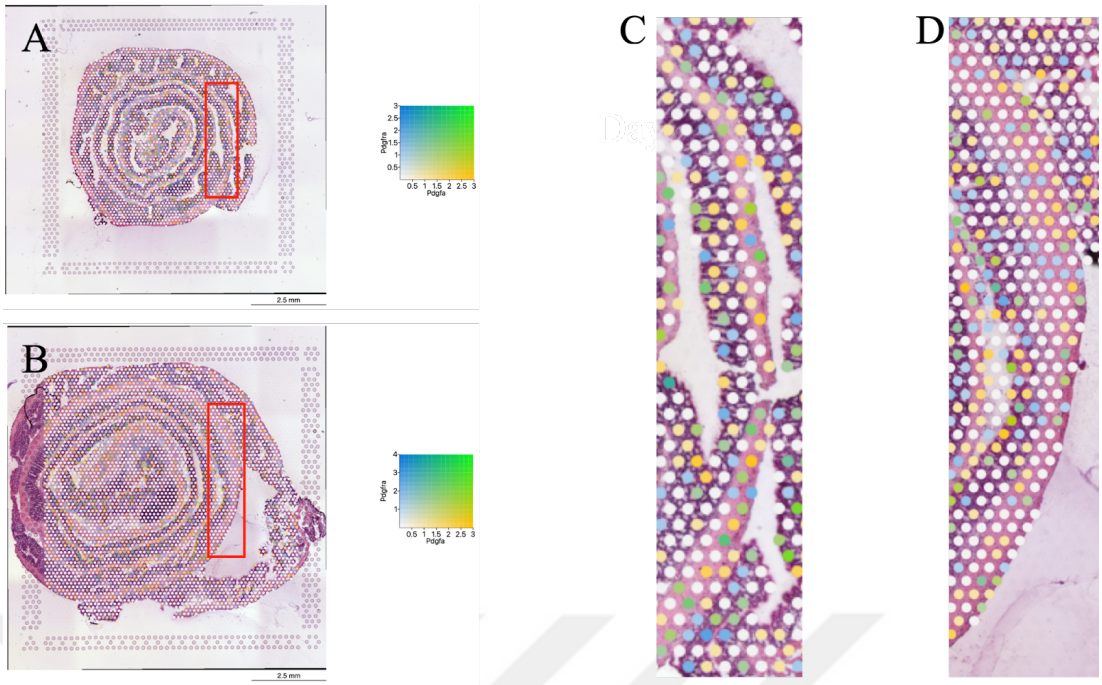


Figure 28. Pdgfa-Pdgfra co-expression analysis spotted onto Day0 and Day14 colon samples with spatial transcriptomics. Red lining defines long crypt region (A) and regenerative area (B). C for Day0 and D for Day14 are close-up images of red linings.

4.7 Neuroprotective Gpr37l1 is Detected Around Myenteric Plexus

In our reanalysis of single-cell RNA sequencing data, one glial-specific gene, GPR37L1, stood out. GPR37L1 is known for its neuroprotective and glia protective properties and is primarily found in the brain. Additionally, it has been associated with primary cilia specificity. We aimed to analyze GPR37L1 in the ENS to determine whether it has similar implications. First, we performed IHC experiments to localize primary cilia in the ENS using the ARL13B antibody. In the control sample (Figure 29), we observed primary cilia in both enteric neurons and enteric glia within the myenteric ganglia, as evidenced by overlap with neuronal HuC-D staining. Additionally, primary cilia were found in PDGFRA+ cells in the muscle. This pattern was consistent in both DSS-treated and recovery samples.

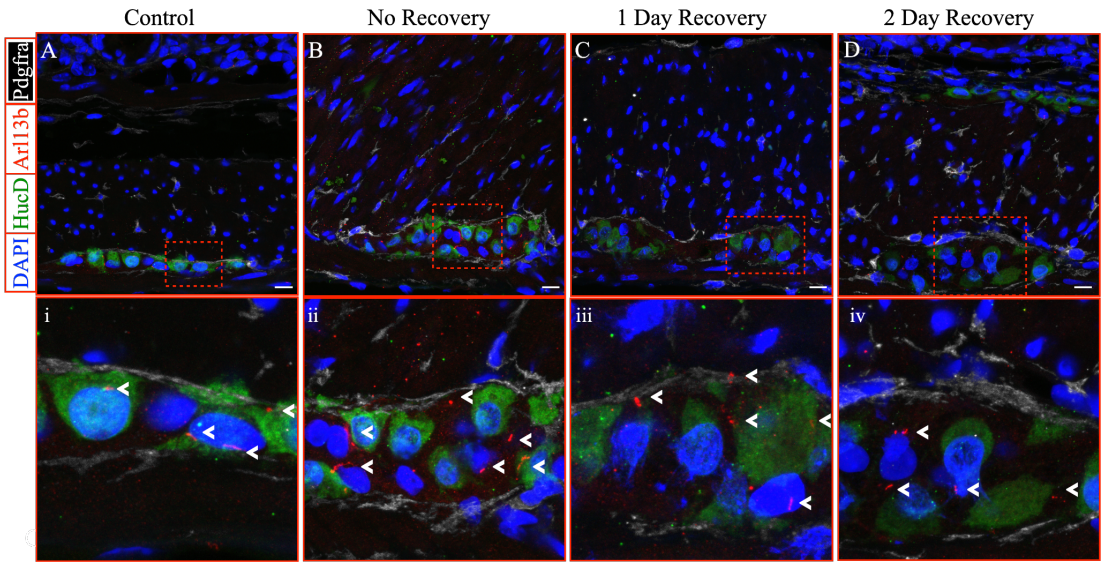


Figure 29. Primary cilia is expressed in the myenteric plexus ENS cells. IHC staining with Arl13b (red), Pdgfra (white) and HuC-D (green) indicates primary cilia presence in enteric glia and neurons. n=2. Scale bar represents 50 µm.

After identifying the presence of primary cilia in the ENS, we conducted IHC experiments using a GPR37L1 antibody in our animal model. We found that GPR37L1 did not overlap with ARL13B expression, indicating that GPR37L1 is not present in the primary cilia of the ENS. However, GPR37L1 exhibited a specific localization pattern, primarily situated on the outer sides of the myenteric ganglia. We hypothesized that GPR37L1 might colocalize with PDGFRA+ cells in the outer layer of the ganglia (Figure 30), but they were distinctly separate, regardless of whether the tissue was from control, DSS-treated, or recovery samples. We can conclude that GPR37L1 is present in the ENS with a specialized pattern in the myenteric ganglia. Interestingly, GPR37L1 can also be detected in the epithelium during epithelial damage. Its role in the ENS and epithelial response to damage require further investigation.

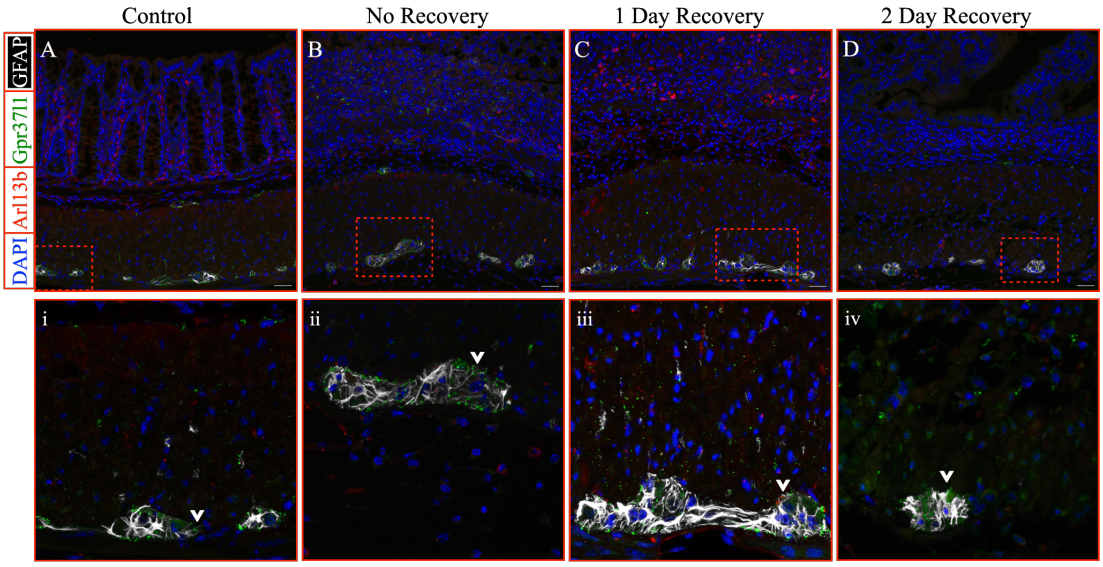


Figure 30. Gpr37l1 is expressed in the myenteric plexus. IHC staining with Arl13b (red), GFAP (white) and Gpr37l1 (green) shows that Gpr37l1 do not overlap with Arl13b expression. n=2. Scale bar represents 50 µm.

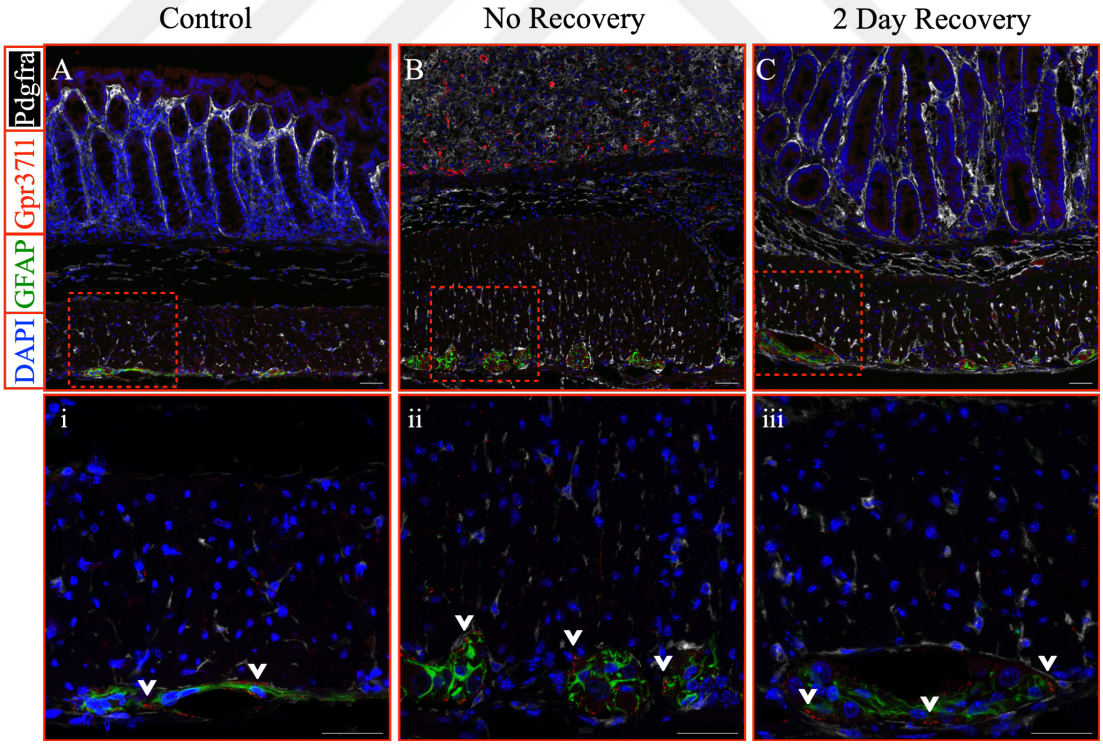


Figure 31. Gpr37l1 is expressed in the myenteric plexus. IHC staining with Gpr37l1 (red), Pdgfra (white) and GFAP(green) shows that Gpr37l1 do not overlap with Pdgfra expression. n=2. Scale bar represents 50 µm.

4.8 Complex ENS Harboring Colon Assembloids

After uncovering the mechanical response of the ENS and proposing a mechanism for it, we sought to demonstrate this phenomenon in an in vitro cell culture model. Traditional 2D cell cultures lack the complexity required to accurately model tissue-specific interactions. In contrast, 3D culture systems offer more physiologically relevant conditions, including enhanced cell-cell and cell-matrix interactions, and allow for greater heterogeneity in cell types. Therefore, we chose to use colon organoids to test our hypothesis (Figure 32).

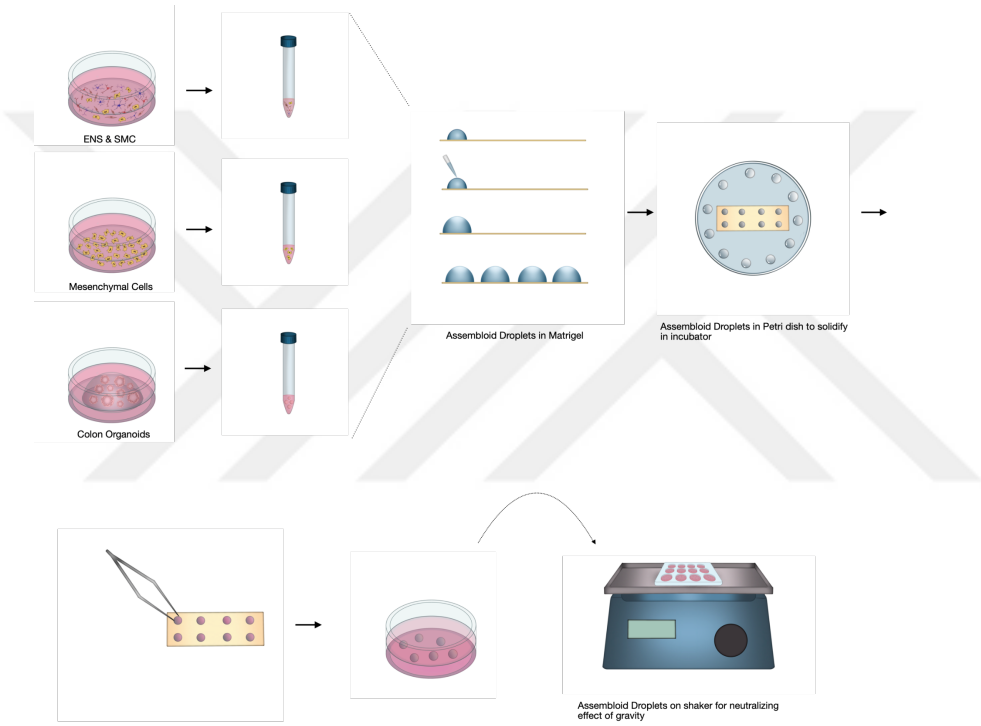


Figure 32. 3D Assembloid culture method

We hypothesized that by adding primary mesenchymal cells and primary ENS cells into the organoid culture, the colon model would better replicate the in vivo environment due to the ENS's role as an organizer. This approach should provide a more accurate model for studying the interactions and mechanisms involved for future studies. As a result, we successfully produced ENS harboring complex organoids that better recapitulate colon physiology (Figure 33). In Figure 33, we did IHC experiment to detect three components of our experiment; E. Cadherin for epithelial organoid, Tuj1 for ENS, Pdgfra for mesenchymal cells. We observed that Pdgfra+ mesenchymal and ENS cells were surrounded epithelial organoids like in the tissue. While Pdgfra+

cells at the bottom recapitulate serosa, Tuj+ cells at the bottom recapitulate ENS in the muscle, like a plexus. As we go up in the assembloid structure, epithelia was formed crypt structures that can be observed by E. Cadherin staining. Like a normal tissue crypt, mesenchymal cells can be seen in along the crypts; near epithelial cells.

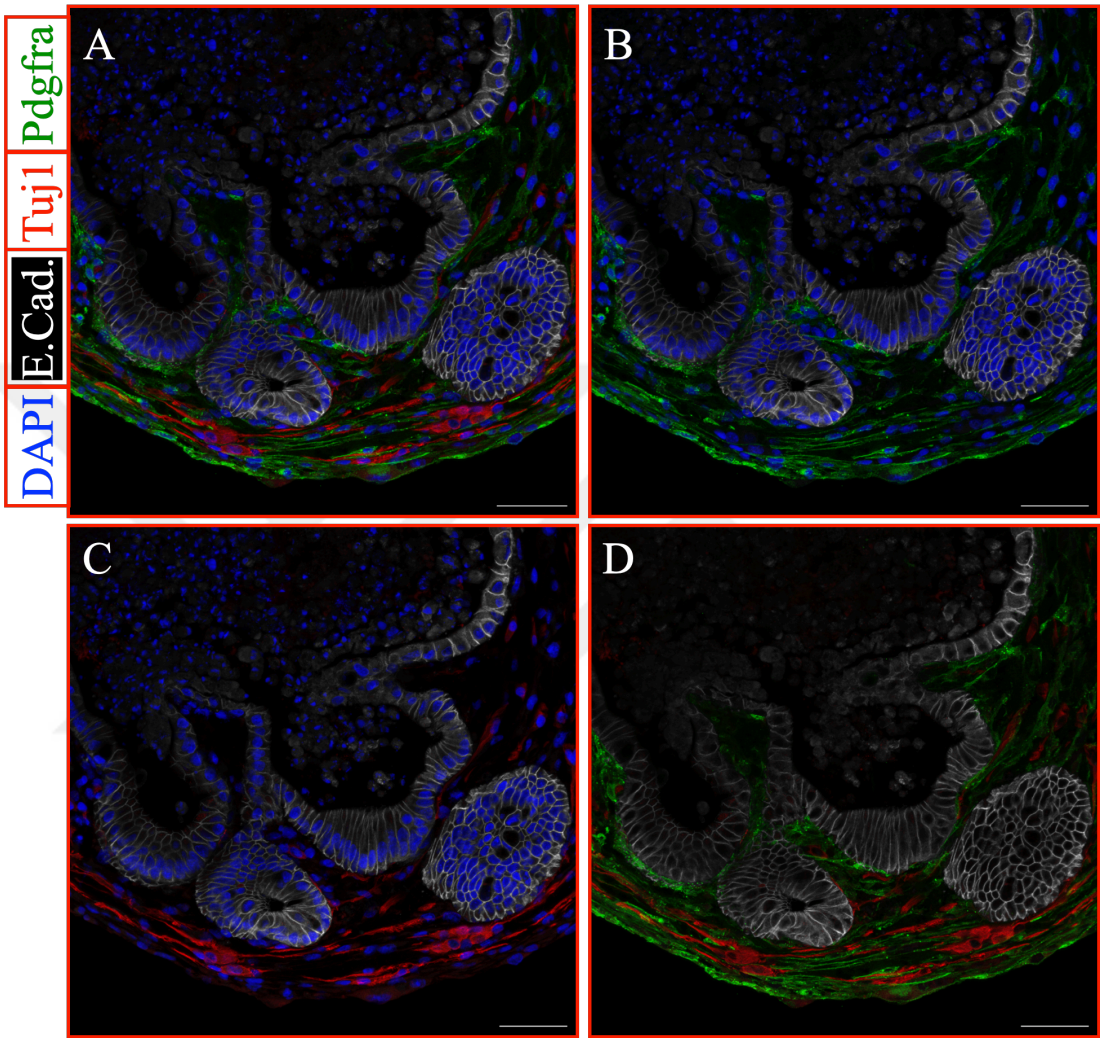


Figure 33. Complex enteric nervous system harboring colon assembloids. IHC staining with Tubb3 (red), E. Cadherin (white) and Pdgfra (green) shows crypt structures in the assembloid. n=3. Scale bar represents 50 µm.

To confirm that our epithelial cells were proliferating and healthy, we performed immunohistochemistry (IHC) with the proliferative marker Ki67 (Figure 34). We observed Ki67-positive cells at the base of the crypts, co-localized with the epithelial marker E-cadherin. This indicates that our organoids possess proliferating cells that contribute to the production of differentiated cells along the crypt, maintaining homeostasis within the assembloid.

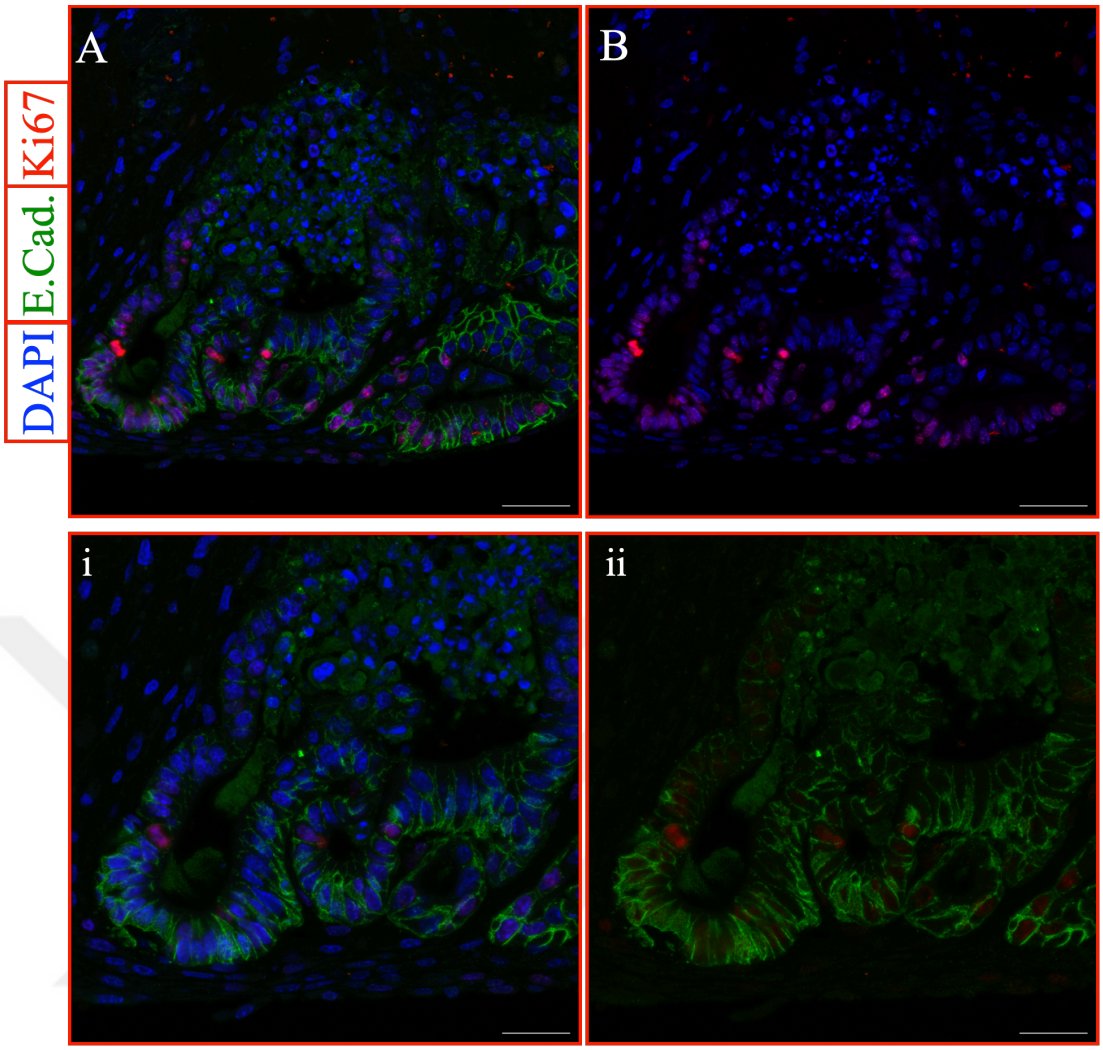


Figure 34. Colon assembloid has proliferative crypts. IHC staining with Ki67 (red) and E. Cadherin (green) indicates newly forming cells along crypt. n=3. Scale bar represents 50 µm.

To observe ENS cells more specifically, we stained assembloids with glial cell marker S100B, neuron marker Tuj1 and mesenchymal marker Pdgfra. . While we observed a similar structural organization in the muscle layer, we also detected glial cells in the mucosa, lining the crypts alongside the mesenchyme (Figure 35). This result suggests that ENS cells collaborate with mesenchymal cells to shape the epithelium. Observing this dynamic cell interaction, we investigated whether these cells were proliferating while organizing the epithelium. IHC staining with Ki67, Tuj1, and Pdgfra revealed that some Pdgfra-positive mesenchymal cells in the muscle region were also Ki67-positive (Figure 36).

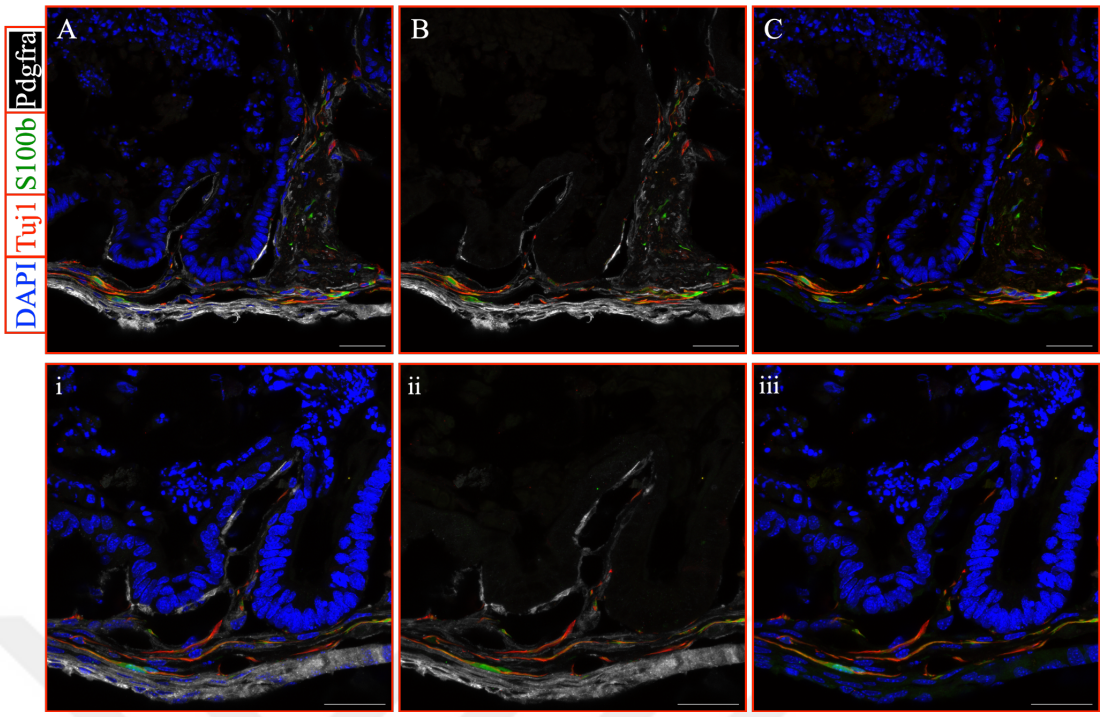


Figure 35. Assembloid compartmentalizes as muscle and mucosa layers. IHC staining with Tuj1 (red), S100b (green) and Pdgfra (white) indicates structurally defined positions of cells. ENS and mesenchymal cells migrate towards mucosa. n=2. Scale bar represents 50 µm.

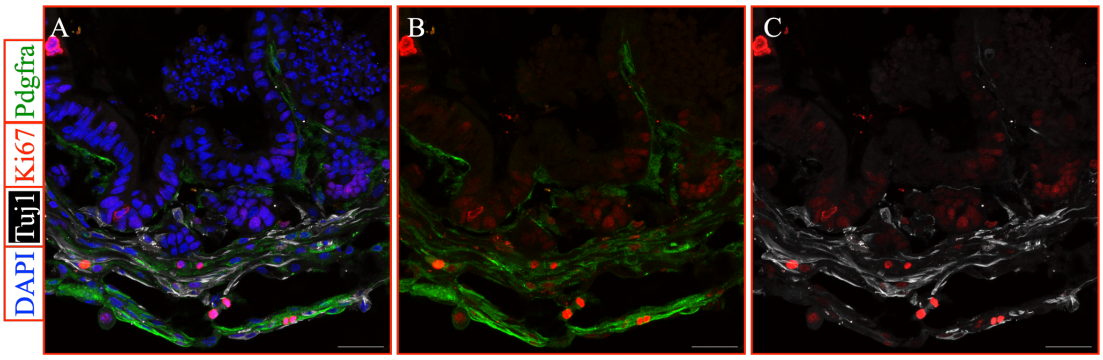


Figure 36. Crypts and some of mesenchymal cells are proliferating in assembloid culture. IHC staining with Ki67 (red), Pdgfra (green) and Tuj1 (white) indicates mesenchymal cells are proliferating. n=2. Scale bar represents 50 µm.

We also examined other neuronal markers, such as HuC-D and PHOX2B (Figure 37). We found PHOX2B-positive cells near the crypt, which were also HuC-D-positive. However, HuC-D-positive neurons in the muscle layer did not display PHOX2B positivity, indicating the presence of different neuronal cell types within our assembloid model. Given our discovery of ACOT7 as a new marker for ENS, we tested its expression by staining with the neuronal marker Tuj1 (Figure 38). We observed co-staining of ACOT7 with the neuronal marker in the outer region of the assembloid. Tuj1+ neurons in mucosa region were not positive for ACOT7, which also observed in tissue. This finding confirms that ACOT7 is present in both tissue and in vitro models, effectively identifying neurons.

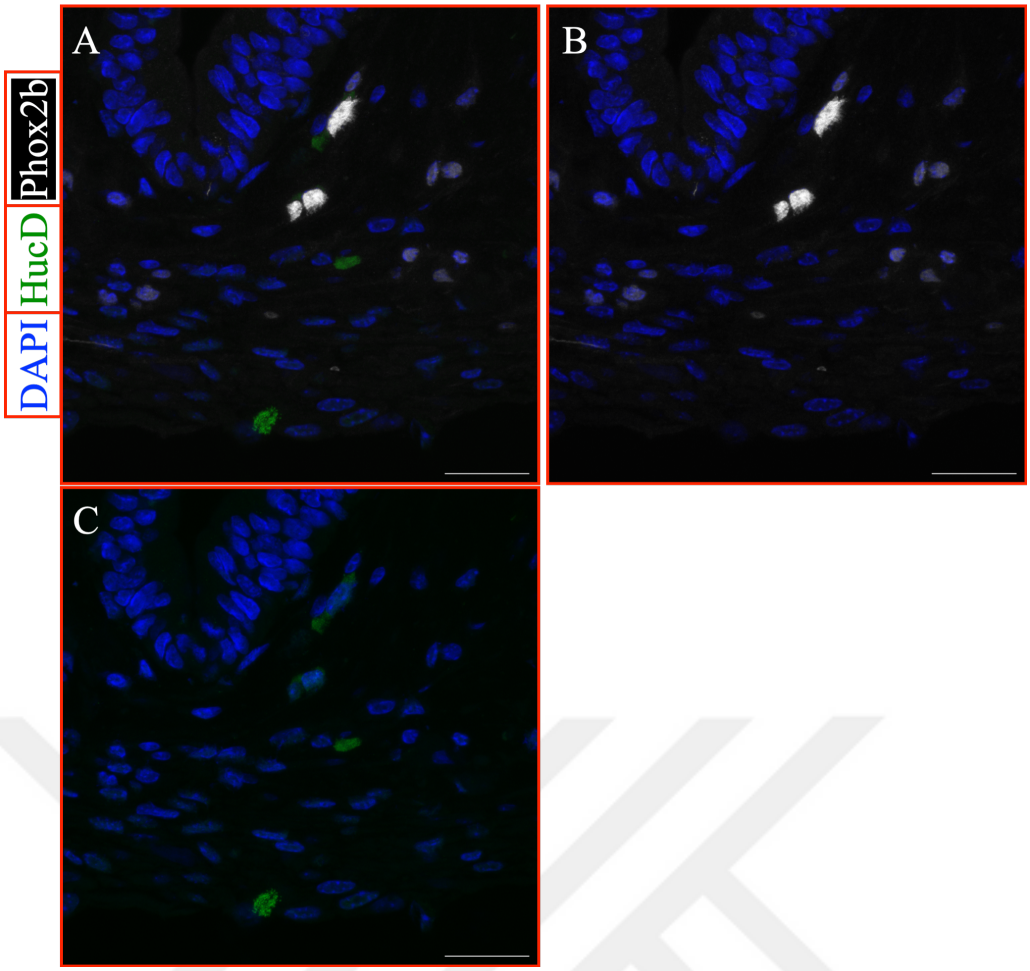


Figure 37. Enteric neurons in assembloids differentiate upon near crypt base. IHC staining with HuC-D (green) and Phox2b (white) indicates different cell populations in assembloids. n=2. Scale bar represents 50 µm.

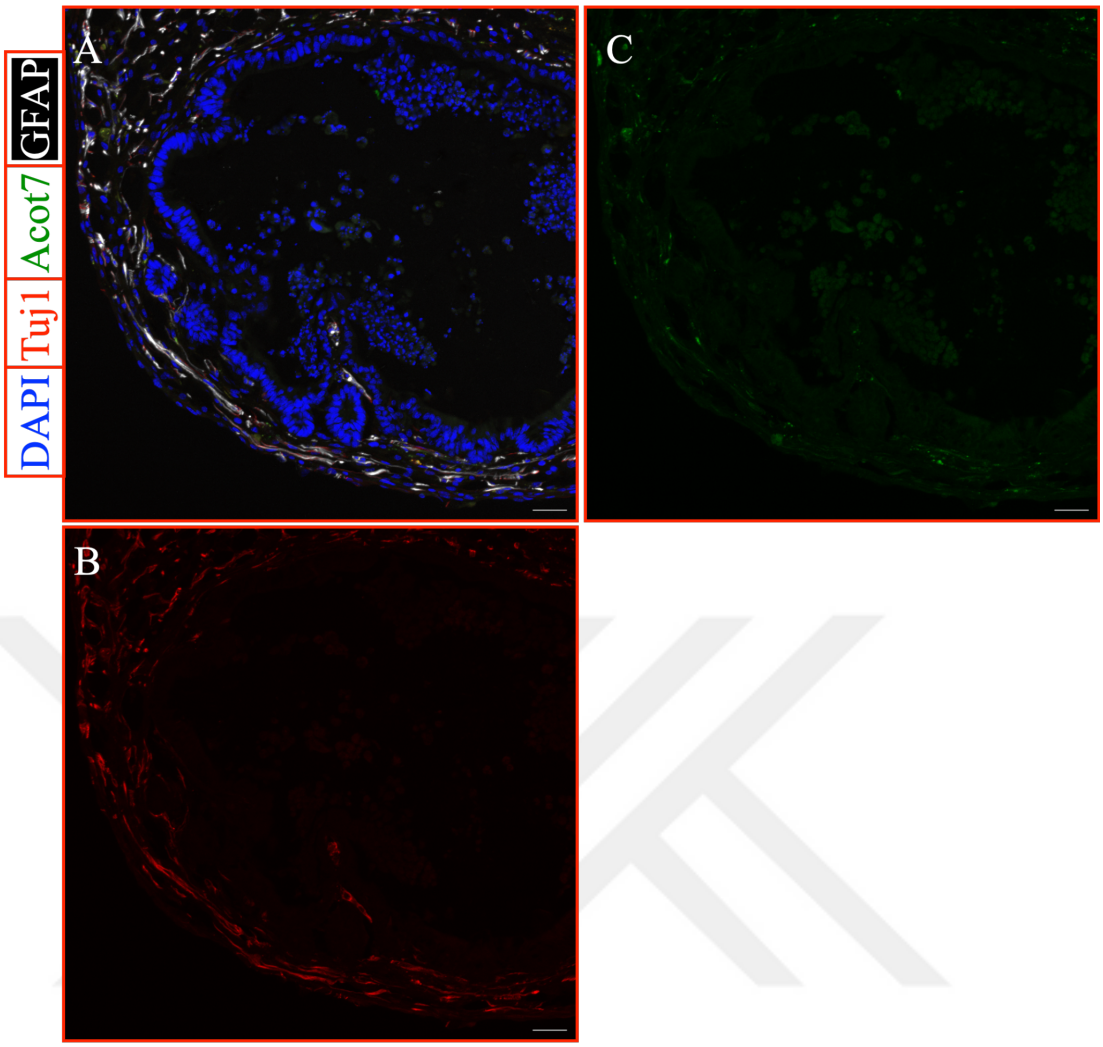


Figure 38. New enteric neuron marker Acot7 is detected in assembloid. IHC staining with Acot7 (green) and GFAP (white) indicates different cell populations in assembloids. n=1. Scale bar represents 50 μm.

To explore other components of colon cell heterogeneity, such as smooth muscle cells, secretory cells, and endothelial cells, we performed IHC staining with A-SMA (smooth muscle cells), MUC2 (secretory cells), and LYVE1 (endothelial cells) antibodies (Figure 39, 40). As shown in the figure, we successfully identified smooth muscle cells in both the muscle and mucosal layers. We also observed goblet cells in

the epithelium, responsible for mucus production, and endothelial cells near the epithelium, exhibiting a round morphology consistent with normal tissue structure.

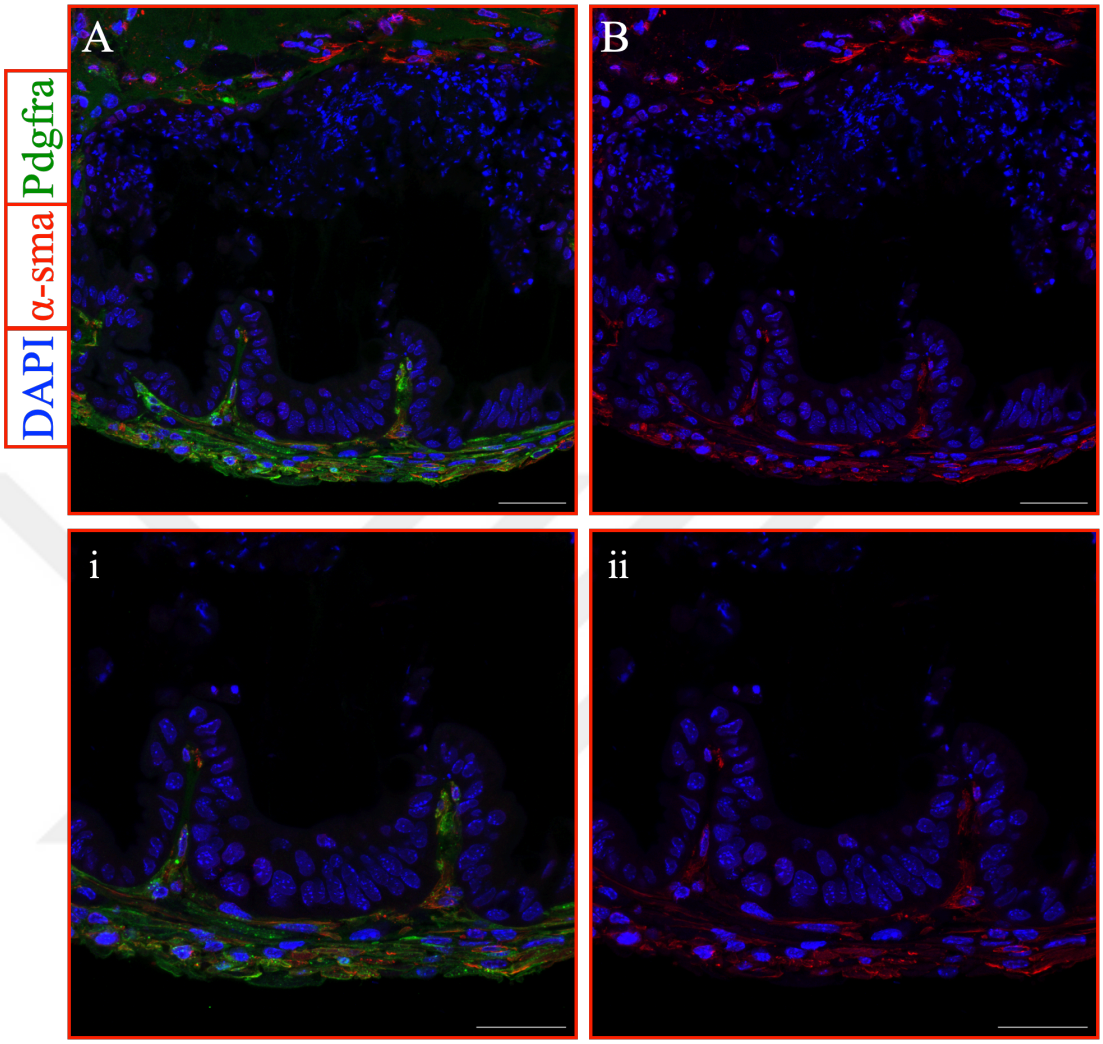


Figure 39. a-SMA positive smooth muscle cells are found in assembloid. IHC staining with Pdgfra (green) and a-sma (red) indicates smooth muscle cell presence in assembloids. n=1. Scale bar represents 50 µm.

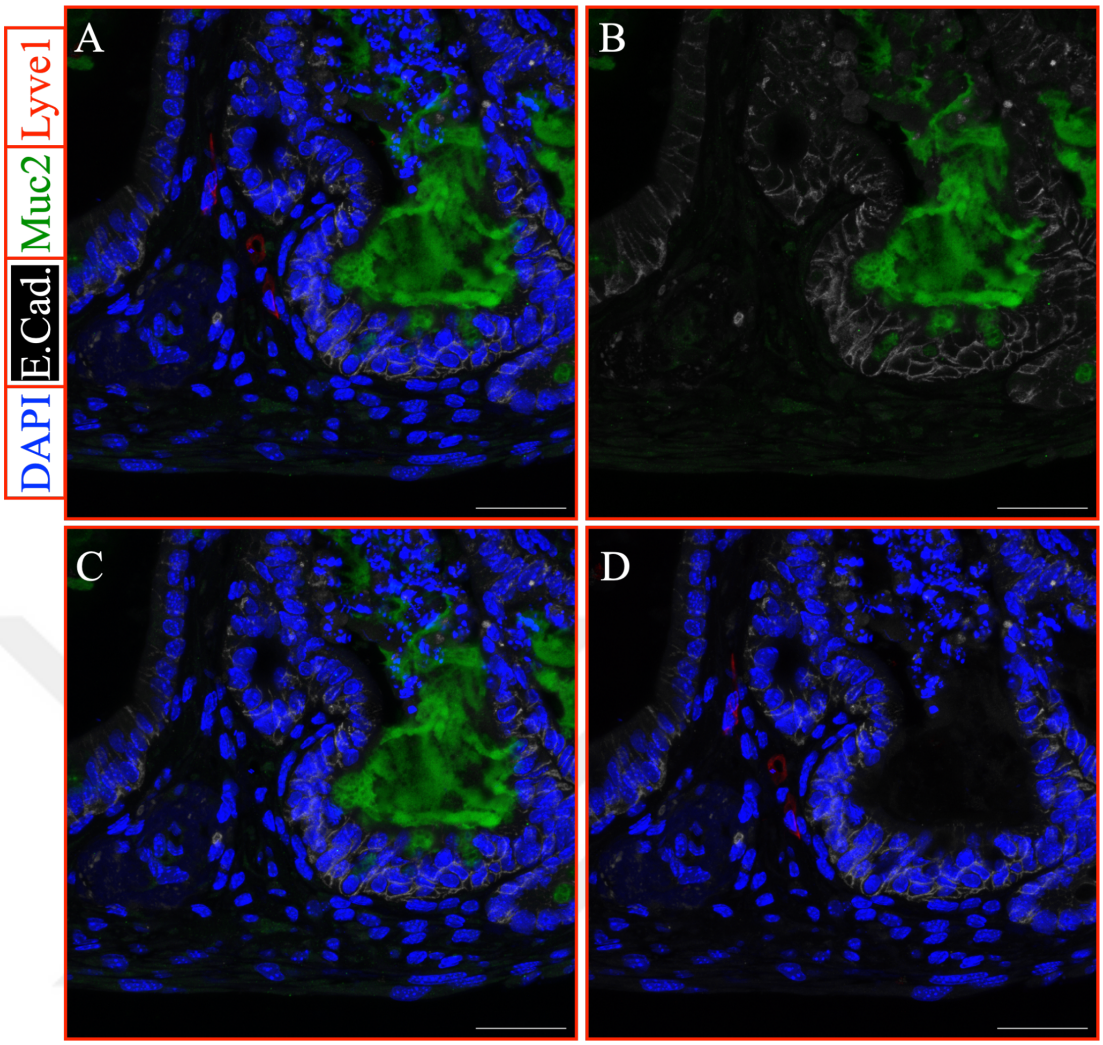


Figure 40. Goblet cells in assembloid produce mucin into the lumen. Lyve1+ cells are found near the crypt base. IHC staining with Muc2 (green), Lyve1 (red) and E. Cadherin (white) indicates different cell populations in assembloids. n=2. Scale bar represents 50 µm.

Given our observation of lipid transfer between neurons and glia, we sought to locate fat metabolism-related genes, APOE and FABP4, in our in vitro culture. We conducted IHC staining with the neuronal marker Tuj1 and the glial marker GFAP (Figures 41, 42). In Figure 42, we found that APOE was present in Tuj1+ cells, similar to what we observed in tissue. Additionally, we detected APOE+ cells in the outer layer of the assembloids, though we could not identify these cells within the scope of this work. For FABP4 staining (Figure 41), we observed positive cells in the outer layer of the assembloids, resembling the muscle layer. These cells also resembled lymphatic cells based on their similarity to Lyve1 staining observed in tissue. However, we were

unable to co-stain Lyve1 with FABP4 because both antibodies were of rabbit origin. Collectively, these results indicate that our colon assembloids closely resemble the tissue cell diversity and environment.

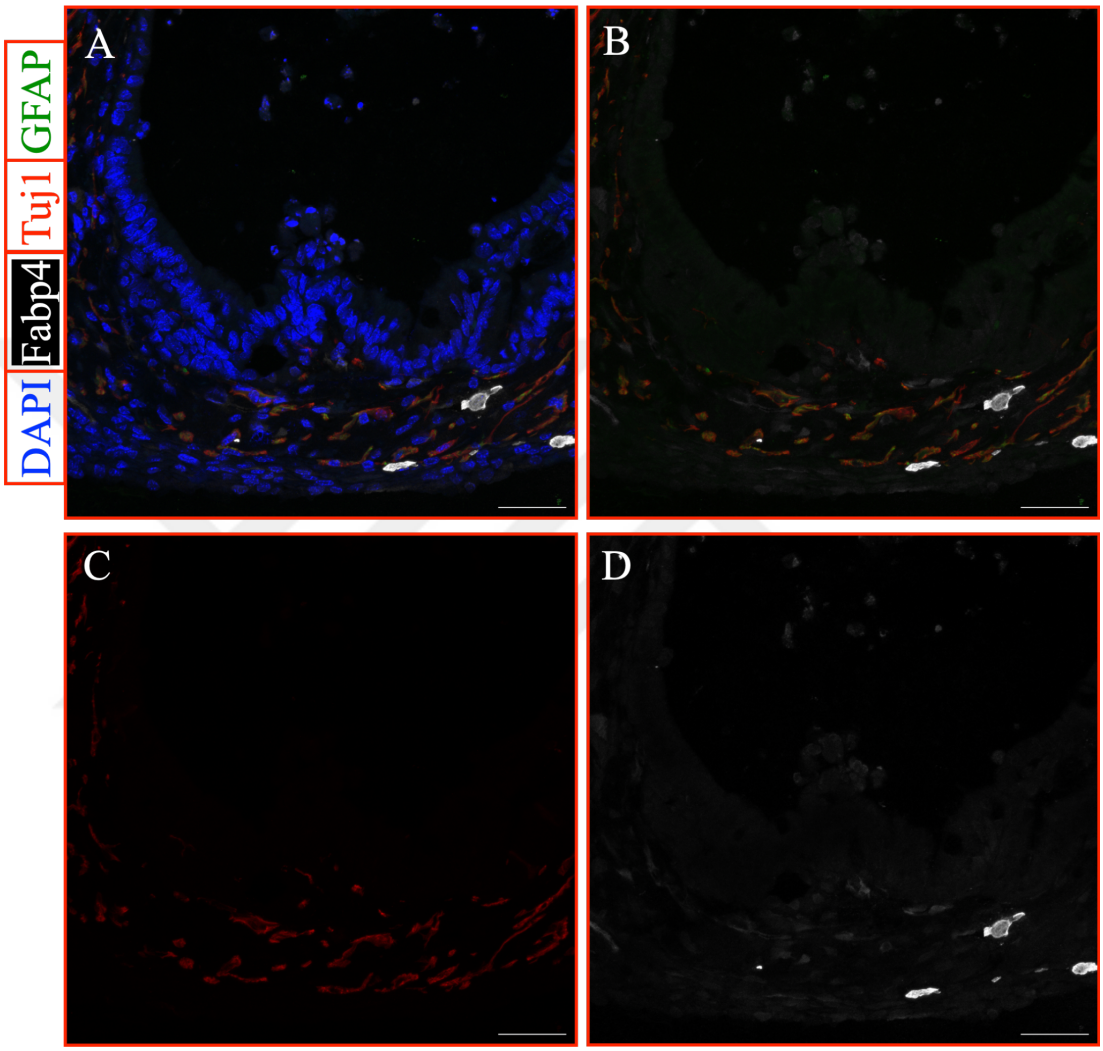


Figure 41. Muscle cells in assembloid express Fabp4. IHC staining with GFAP (green), Tuj1 (red) and Fabp4 (white) indicates different cell population in muscle express lipid metabolism protein. n=1. Scale bar represents 50 µm.

After highlighting the mechanistic role of the ENS in inflammation through the Ptn-Ptprz1 axis, we next investigated the presence of these proteins in our assembloid model. This is particularly relevant because the ENS plays a crucial role in shaping the assembloid to form the colonic structure. By examining whether Ptn and Ptprz1 are

expressed in the assembloids, we aimed to determine if the ENS contributes to the structural organization within this in vitro system, similar to its role in vivo. After IHC staining with Ptn-Ptprz1 with neuronal marker Tuj1 and the glial marker GFAP (Figures 43, 44), we found that neuronal and glial cells were positive for Ptprz1. However, the expression in assembloids was not as prominent as in vivo, presenting only as small dot-like shapes. Because it is a ligand, it is hard to detect in an in vitro system. We can say that ENS support mesenchyme and epithelium maintenance and regulate structure of colon with Ptn-Ptprz1 expression.

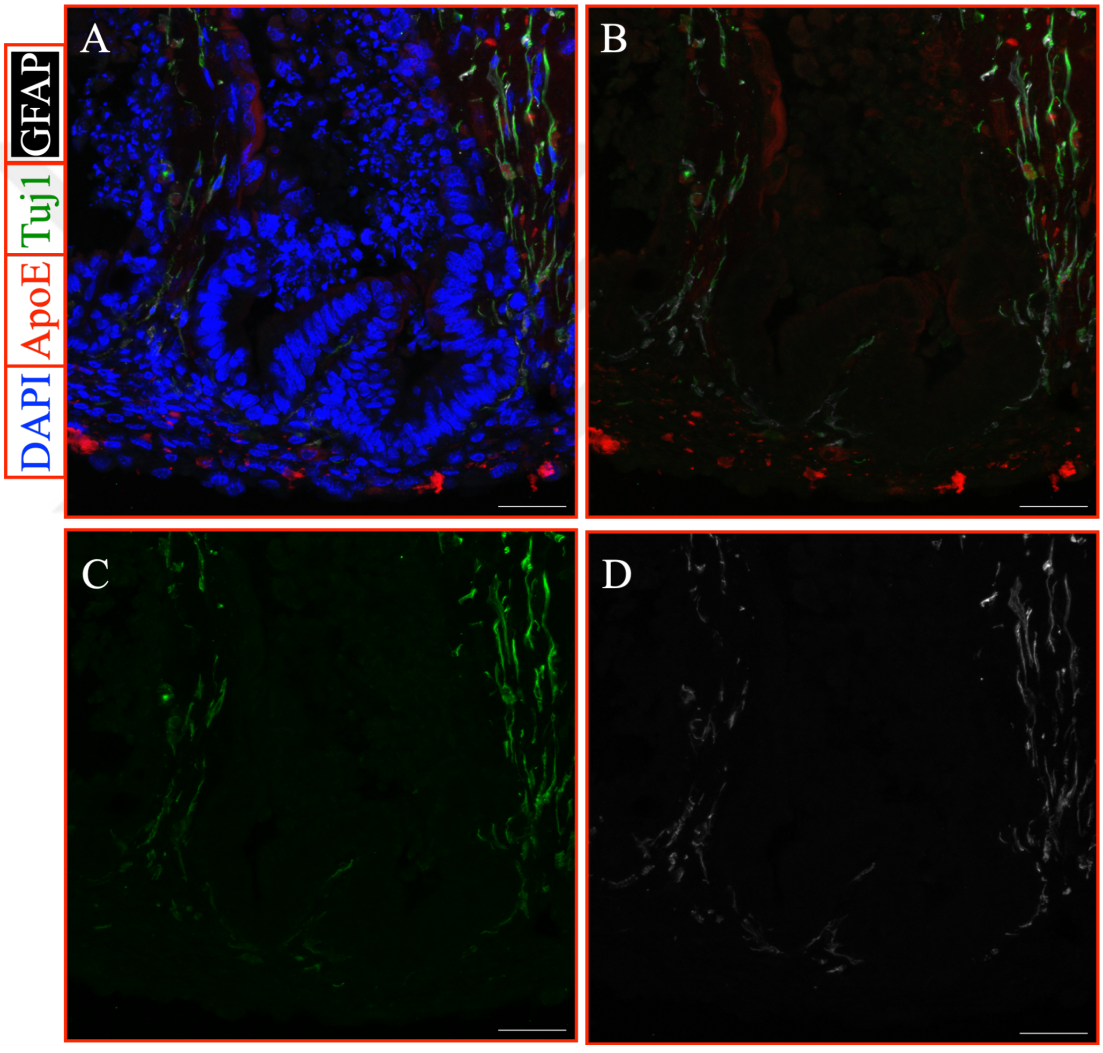


Figure 42. ENS cells in assembloid express ApoE. IHC staining with Tuj1 (green), ApoE (red) and GFAP (white) indicates ENS cells in muscle express lipoprotein. n=1. Scale bar represents 50 µm.

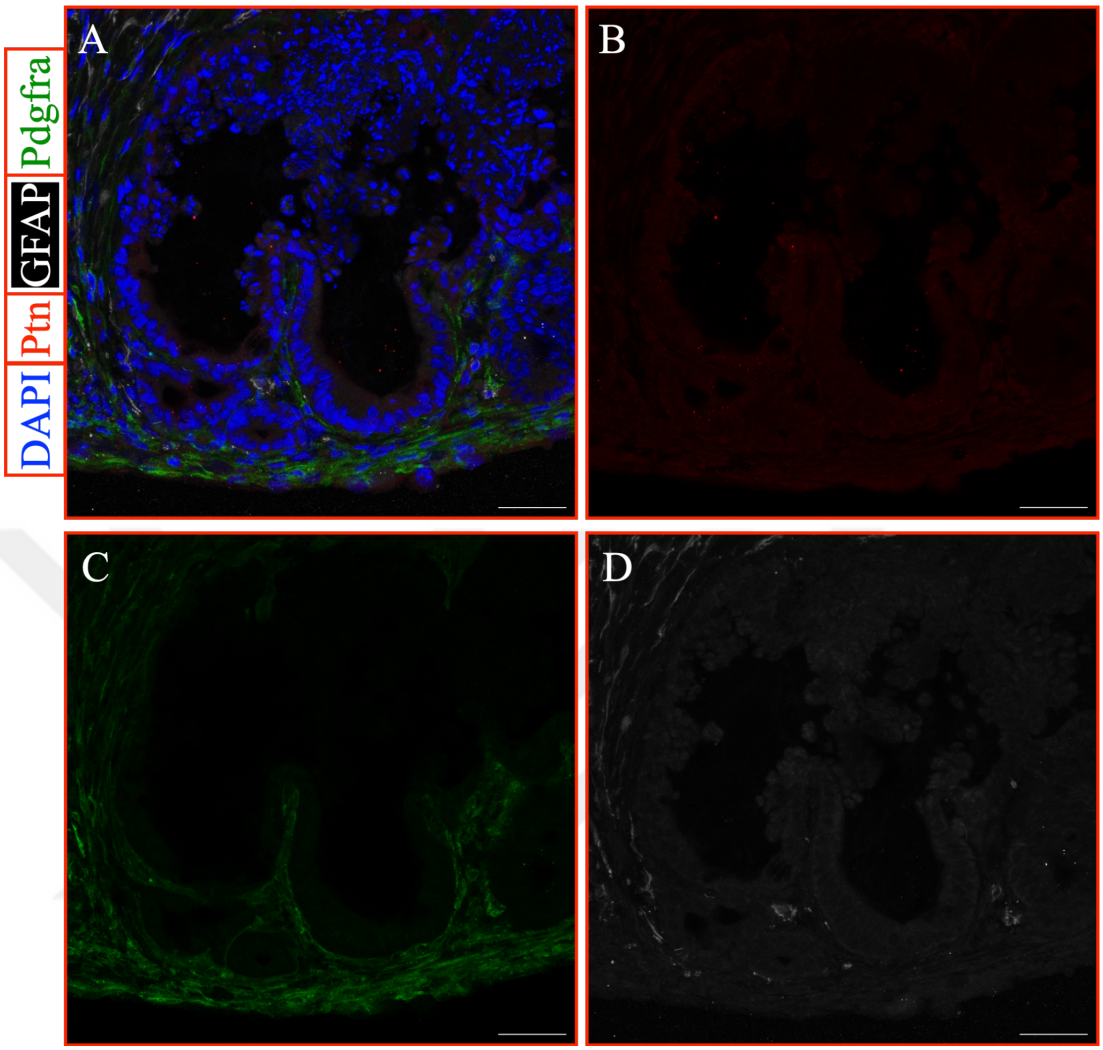


Figure 43. ENS and epitheial cells in assembloid express Ptn. IHC staining with Pdgfra (green), Ptn (red) and GFAP (white) indicates ENS cells and epithelial cells express Ptn. n=1. Scale bar represents 50 μm.

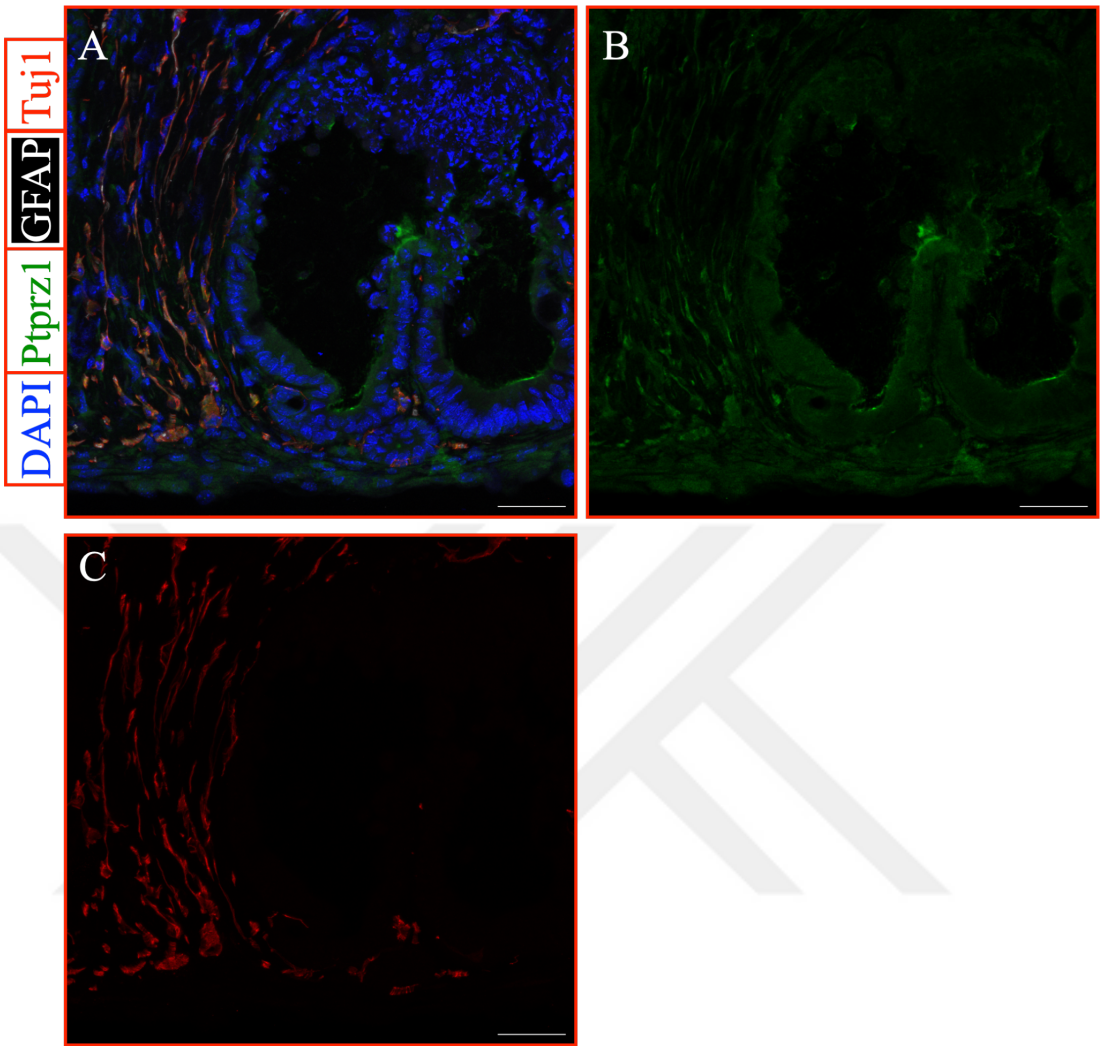


Figure 44. ENS cells in assembloid express Ptprz1. IHC staining with Ptprz1 (green), Tuj1 (red) and GFAP (white) indicates ENS cells express Ptprz1 which produce yellow staining. n=1. Scale bar represents 50 µm.

5. DISCUSSION

In this study, we aimed to reveal roles of the enteric nervous system upon inflammation and regeneration in the colon. Tissue homeostasis is one of the most specialized systems in terms of different cell type coordination. Therefore, it is still unclear what exact mechanisms are used in each unique cell type to restore normal conditions in a tissue. There are researches about epithelial cells and immune cells focusing on communication between two cell types upon inflammation (Mahapatroet al., 2021); indicating that systematic work on two cell types produce more meaningful results on how cells aid regeneration. There are several cell types in colon that are involved in the regeneration process after inflammation such as mesenchymal cells, immune cells and endothelial cells.

We were focused on ENS cells and Pdgfra+ mesenchymal cells that play crucial role on colon functions. These cells can be found in every layer of the colon; also types of cells change according to their placement in the layers. Therefore, every type in different layer exerts diverse functions such as ENS cells in lamina propria communicate with epithelial cells to sense pathogenic invasion (Jarret et al., 2020). ENS cells in the muscle layer participate in muscle contraction for propulsion of food from the body (Smith & Koh, 2017). Secretion of neurotransmitters from enteric neurons that activate smooth muscle contraction leads to peristaltic movement (Smith & Koh, 2017). Among ENS cells, mesenchymal cells also secrete Wnt, Rspo3 and other stem cell maintenance factors to support epithelial cell regeneration. To investigate inflammation and regeneration of the colon in the mouse model, we used DSS administration which mimics ulcerative colitis (UC). Like UC patient physiological conditions, mouse showed signs of inflammation such as diarrhea, bloody stool and loss of body weight. When we applied IHC analysis to colon with no recovery and recovery animals, we observed that proliferative cell marker Ki67 and stemness marker Sox9 are decreased in no recovery samples after 5 days of DSS administration. Because DSS is a sulphated polysaccharide that interacts with bacterial derived medium-chain fatty acid in the lumen, they form vesicles with fatty acids which can be uptaken by the epithelial lining of the colon. Therefore, inflammatory-inducer chemical uptaken by epithelial cells cause disruption of epithelial lining that also harms stem cells at the crypt bottom. In recovery samples, which we allowed

animals to drink normal water after five days of DSS administration showed signs of renewal of crypts by staining of Sox9 in the crypt bottom region. Also, proliferation of stem cells were observed with Ki67 marker in the regeneration areas.

After we verify colitis model efficiency, we characterized ENS cells with Tubb3, S100b, GFAP, HucD and Phox2b markers with immunohistochemistry. Enteric glia and enteric neurons can be find in all layers of colon; mucosa, submucosa and muscularis externa. There are submucosal plexus and myenteric plexus that contain ganglia of ENS cells; which regulate muscle contraction, nutrition sensing, immune regulation and hormone secretion. In no recovery and recovery samples, we found out that enteric neuronal hyperactivation and glial increase. Enteric glial cells are important regulators of neurons in ENS. However, in IBDs, there are controversial findings about the function of glial cells in inflammation. Aubé et al. states that glial ablation in the small intestine causes VIP and SP positive neurons to decrease; while ChAT positive neurons were unchanged in submucosal plexus. On the contrary, myenteric plexus cell population is changed by decrease in ChAT neurons while VIP and SP neurons remain the same in number (Aubé et al., 2006). These results indicate that absence of glial cells leads to disruption in neuronal homeostasis which negatively affects intestinal motility and permeability (Aubé et al., 2006). Another finding states enteric glial ablation leads to mucosal barrier dysfunction by scarce of enteric glia expressed protein S-Nitrosoglutathione (GSNO) (Savidge et al., 2007). Elimination of GSNO leads to decrease in epithelial junction proteins; perijunctional F-actin and tight-junction associated protein zonula occludens-1 (ZO1) which leads to epithelial barrier loosening (Savidge et al., 2007). However, one study states that glial marker protein S100b leads to signaling cascade that attenuates inflammatory response (Costa et al., 2021). Upon Clostridioides difficile (C. difficile) infection (CDI) to intestine, S100b amount in the fecal content was increased (Costa et al., 2021) . S100b inhibition in CDI leads to decrease in inflammatory cytokines such as IL-1B, IL-18, TNF-a; neutrophil recruitment and degree of intestinal damage (Costa et al., 2021). Another article states that enteric glia activation leads to continuous inflammation in the intestine. By inhibiting enteric glial activity with palmitoylethanolamide (PEA), colitis like phenotype in the mice decreased.

It is known that neuronal hyperactivation leads to lethal phenotype neurons in the brain (Bachis et al., 2008). Besides hyperactivated neurons and increased glia in DSS and recovery, we observed upward motion from myenteric externa neuron, glia and Pdgfra+ cells toward submucosa. We hypothesized that this motion is regulated for recovery of epithelial cells and ENS cells in mucosa. While we hypothesized that Pdgfra+ cells regulate inclination towards mucosa by giving structural support and cue for inclination, glial cells regulate homeostasis of colon in hyperactivation.

Neuronal activity is supported by glial cells in the central nervous system which glial cells provide ATP and metabolic substrates to maintain neuronal homeostasis (Bélangeret al., 2011; Bélanger & Magistretti, 2009). One of the important metabolic regulator is lipid droplets that regulate lipid transport, storage and cellular stress (Ralhanet al., 2021). It is known that glial cells in the brain uptake lipid droplets from enteric neurons during hyperactivation (Ioannou et al., 2019). Therefore, we were curious about how enteric neurons sustain homeostasis in DSS and regeneration. Firstly, we demonstrate important fat metabolism gene FABP4 in the colon and ApoE in the enteric nervous system. ApoE is detected in enteric neurons and glia which can be interpreted as ENS cells lipoparticles. Particularly, we observed ApoE expression in myenteric plexus neurons and glia in all mouse model timepoints. Therefore, we hypothesized that ENS cells operate the same metabolic system as the brain; lipid droplet uptake of hyperactivated enteric neurons to glia.

To prove our hypothesis, we isolated enteric neural precursor cells from mouse colon; only from serosa and muscularis externa. Reason for this is we observed our ENS cells migrate from muscularis externa to submucosa region. We treated enteric neurons with calcium chloride to achieve hyperactivation. After labeling neuronal lipids with C12, we observed lipid transfer from neuron to glia. Enteric glial cells uptake the lipids to safeguard neurons from lipid induced toxicity in the colon. To mimic inflammatory conditions in DSS, we incubated glial cells with IL-1B, TNF-a and IFN-gamma for 48 hours. Inflammatory glial cells also have taken lipids from enteric neuronal cells. We did not detect any difference in C12 signal intensity or amount in both neurons and glial cells. However, we observed more dispersed C12 signals in inflamed glial cells; which can indicate beta oxidation of lipids. Therefore, we can propose a mechanism for how enteric glial cells protect neuronal hyperactivation in DSS induced recovery

by remaining in close proximity through submucosal inclination. This protection role of glia is also demonstrated by IHC experiment on the tissue. ACOT7 signals found in ENS cells that protect from long chain acetyl-CoA. Moreover, we located Gpr37l1 protein around the myenteric plexus but not on the primary cilia of the ENS; which need further explanation.

For the mechanical perspective of cell inclination, we used single cell RNA-seq data visualization platform to visualize previous findings of our lab (Guadalupe et al., 2022) and spatial transcriptomics re-analysis (Parigi et al., 2022). Upon previous analyses, we chose Ptn, Ptprz1, Ncam1, L1cam, Mcam, Fgf2, Pdgfa and Pdgfra genes as candidate genes for ENS and mesenchymal cells in recovery. We found that Ptn is found in glia, mesenchymal stem cells, interstitial cells of Cajal, and fibroblast 3a cell types. It is most prevalent in glial cell types. Ptprz1 and L1cam are found in glial cell types only. Ncam1 and Mcam are expressed in glia and interstitial cells of Cajal; but Mcam also expressed in pericytes. Fgf2 is expressed in glia, mesenchymal stem cells and fibroblast 3a cells. Apart from these genes, we also find Pdgfa-Pdgfra expression important for mechanical support for inclination of cells. While Pdgfa is expressed in glial and pericyte cells, Pdgfra receptor is expressed in mesenchymal stem cells and fibroblast 3a subtype. We chose the Ptn-Ptprz1 pair to explain the tendency of myenteric plexus cells to orient themselves to submucosa. Ptn is a pleiotrophin that has a role in cell migration, cell adhesion, proliferation and cytoskeletal stability (Diamantopoulouet al., 2012; Lu et al., 2005). Main receptor of Ptn is found as Ptprz1; which is regulated negatively by Ptn. Ptprz1 is a tyrosine phosphatase receptor that actively removes phosphate groups from tyrosine residue (Baldauf et al., 2015; Kuboyamaet al., 2016). Ptn binding to Ptprz1 leads to dimerization of the active D1 domain of Ptprz1, which inhibits phosphatase activity (McClain et al., 2012). Accumulation of phosphate groups in the cell regulates downstream signaling cascades such as β-catenin , SRC kinase, and ALK signaling (Li et al., 2023; Meng et al., 2000). Specifically, full length form of Ptn cannot bind to Ptprz1; however, short form of Ptn can bind Ptprz1 and activate MAPK and PI3K pathways which leads to cell migration (Polykratis et al., 2005; Xia et al., 2019). Therefore, we suggest the Ptn-Ptprz1 axis as a migratory mechanism for ENS cells in recovery of inflammation.

Spatial transcriptomics is a new technology that integrates gene expression with spatial location of gene on a tissue (Cheng et al., 2023). It preserves location of transcript on cells with a barcode (Tian et al., 2023). Each spatial dot includes 5-10 cells inside. Therefore, its resolution compared to single cell sequencing is low. We cannot get single cell information from spatial transcriptomics; however, it is very advantageous to observe large scale tissues of transcripts all together. Therefore, we used spatial transcriptomics data of colitis model in mouse to reanalyze with spaceranger and visualize results with Loupe Browser from 10x Genomics (Parigi et al., 2022). We used our candidate genes from single cell RNA seq and spotted them onto Day0 and Day14 tissues. We observed that Ptn and Ptprz1 spotted onto mostly muscle areas of the colon. We define our glia cells with S100b, GFAP, Plp1 and Sox10; Tubb3, Evalv4 and Phox2b markers were used for neuronal cells. When we applied co-expression analysis, we observed that Ptn and Ptprz1 positive spots were also positive for enteric glia and enteric neuron in Day 0 and Day14. Other candidate genes also spotted onto Day0 and Day14 tissue samples. After we confirmed the expression of Ptn-Ptprz1 separately in ENS cells, we applied co-expression analysis for Ptn-Ptprz1 itself. We observed co-expression spots on Day0 in green. Moreover, we realized that co-expression spots were increased in muscle Day14 analysis. These results suggest that the Ptn-Ptprz1 axis plays an active role in resolution of DSS in day14. However, we cannot certainly claim the single cell expression increase in these genes with spatial transcriptomics because of its coverage. Therefore, in depth analysis other than spatial transcriptomics should be applied. For example, multiplexed error-robust fluorescence in situ hybridization (MERFISH) is a RNA-based technology that labels individual RNA transcripts that can provide number and location of transcripts in an individual cell. In literature, a recently published mouse DSS dataset with MERFISH reveals that there are multiple inflammation-recruited cell types in the inflammation and recovery area (Cadinu et al., 2024). They discovered multiple inflammation-associated fibroblast that only emerge in inflammation spatially near the damage site (Cadinu et al., 2024) . Moreover, inflammation-associated polarized neuron and glia cell types were discovered (Cadinu et al., 2024). We can state that our findings in both IHC analysis and spatial transcriptomics, which is inflammatory ENS and mesenchymal cells in muscle align themselves to inflammatory mucosa, coincide with findings of recent literature.

After we showed the mechanistic side of ENS cells' inclination to mucosa in regeneration upon inflammation insult with in silico analysis, we also wanted to confirm this phenomena in vitro model. For epithelial cells, we isolated colon crypts from mice and obtained colon organoids. Moreover, we isolated primary enteric neural precursor cells and mesenchymal cells from serosa and muscularis externa of the colon. After primary cells were ready for downstream application and after first passage of organoids, 'assembloids 'were obtained by kinetic co-culture system. On the contrary of traditional stationary 3D systems, we cultured matrigel droplets on swirling media, 170 RPM, to obtain more tissue-like structure in an in-vitro system. This method was also used in another study with colon organoids with only mesenchymal cells (Lin et al., 2023). Our assembloid culture include ENS cells to contribute colon physiology among mesenchymal cells compared to Lin et. al. (2023). More physiologically relevant crypt structures can be found in our assembloid system; such as neuron and glia cells migration towards mucosa along with mesenchymal cells because in the colon, there are mucosal ENS and mesenchymal cells that contribute immensely to homeostasis by sensing lumen content and organizing overall structural integrity. Therefore, we can confidently say that our assembloids achieve more structural accuracy. Moreover, our assembloids achieved to differentiate different cells types; mucin producing goblet cells, LYVE1 positive endothelial cells and a-SMA positive smooth muscle cells. While epithelial crypts were proliferative and Ki67 positive; MUC2 positive cells were producing mucus into the lumen of assembloids. Smooth muscle cells on the outer layer provided structural support along with mesenchymal cells; endothelial cells were positioned near crypt structure as observed in normal colon tissue. Interestingly, we discovered a proliferative population of mesenchymal cells on the outer region of assembloid which needs further investigation. Additionally, we identified various neuronal cell types that were either Phox2b positive, HuC-D positive, or positive for both markers. However, not all neuronal cells expressed these markers. This phenomenon can be explained by the fact that Phox2b and HuC-D-positive cells were exclusively found in ganglionic structures within the tissue. Further investigation is needed to clarify the varying expression of neuronal markers. Moreover, our novel enteric ganglia marker, Acot7, was also detected in the assembloid culture, suggesting that ENS cells within the assembloid may begin forming ganglion-like structures. We also aimed to identify fat metabolism markers ApoE and Fabp4 in the assembloids. Fabp4 exhibited a signal pattern identical

to that observed in tissue immunohistochemistry, further confirming the similarity of our assembloids to normal tissue. On the other hand, ApoE was found in ENS cells, which could be further explored in the context of a fat transfer model. To test our hypothesis regarding the role of the Ptn-Ptprz1 axis as a modulator of cell migration, we performed IHC on the assembloids. While Ptn expression was low and sparse, Ptprz1 was clearly expressed in ENS cells. This suggests that the migration of ENS cells toward the mucosal region in the assembloid may be mediated by the Ptn-Ptprz1 axis.

Among colon assembloids, there are also advanced assembloids developed for other tissues, such as the brain. The brain is a complex organ composed of various regions, making it challenging to fully replicate using organoid technology alone. While organoids can mimic broad tissue structures, they typically consist of only one cell type. To address this, researchers have co-cultured organoids from different neuron type organoids or different brain region organoids to create more sophisticated brain-like assembloids (Birey et al., 2022; Miura et al., 2020) . Human iPSCs have been used to generate human cortical and spinal spheroids, which were then fused to create brain region-specific assembloids (Andersen et al., 2020). These two-region assembloids demonstrated that neurons from each region migrate towards the other, mimicking in vivo brain connectivity (Andersen et al., 2020). Additionally, a third spheroid, a human skeletal muscle spheroid, was incorporated to form a three-part assembloid (Andersen et al., 2020). Stimulation of cortical neurons in this system activated the skeletal muscle, highlighting the potential of assembloid culture systems to replicate human functional circuits effectively. There are also ongoing investigations into improving assembloid technologies through biotechnology to achieve more tissue-like structures. Hydrogels and magnetized bioprinters are utilized to control the spatial arrangement of organoids, aiming to produce more homogeneous and structurally controlled assembloids (Roth et al., 2023)

6. CONCLUSION AND FUTURE DIRECTIONS

In this thesis, we investigated how regeneration in the colon upon inflammation was regulated by the enteric nervous system. We observed that enteric glia and enteric neurons among Pdgfra+ cells in muscularis externa region inclined towards submucosal plexus in regenerative state. We found that neurons in inflammation and regeneration are hyperactivated. Therefore, with isolated primary enteric precursor cells from colon and differentiation to intended cellular identities, we mimicked hyperactivity in neurons with calcium chloride; and co-cultured them with enteric glia for fat transfer assay. We observed massive lipid droplets with C12 signals on neuronal cells that proves lipid droplet accumulation in excessive activation. We demonstrated lipid droplet transfer from neuron to enteric glia. Enteric glia safeguards neuronal cells from hyperactivation by uptake of excess lipids to store or use as a metabolic substrate. We showed inflammatory cytokine treated glia dispersed lipid particle signals which can be an indication of usage of lipids. Among this metabolic response of enteric glia and neurons, we also pinpoint mechanical regulation of ENS cell inclination with spatial transcriptomics. We pinpoint Ptn-Ptprz1 interaction in homeostasis and observed increased interaction in the regenerative state. Moreover, we proved the mechanical role of ENS by utilizing assembloid culture in vitro. We developed ENS harboring complex assembloids to observe ENS role on physiology of colon more precisely. We observed the migration of ENS cells to the mucosal region of the assembloid, demonstrating that ENS cells from the myenteric plexus can indeed migrate to the mucosa.

For future perspectives, our main aim is characterizing the fat metabolism of glia in the culture. We can implement fatty acid oxidation assay on both neurons and glia. Also, we can locate their position in a cell with mitochondrial markers such as Tomm20 to comment on their later fate.

Another perspective for the future of our work, we can produce colon glia specific Ptprz1 knockout animal model to observe regenerative capacity of colon upon inflammation. Among Ptprz1, we can also treat animals with recombinant Ptn antibody to inhibit Ptn-Ptprz1 interaction in the colon; therefore, with DSS administration, we can observe its effects in regeneration. Also, we can use assembloid culture to observe

Ptn-Ptprz1 role by using glia ENS from the Ptprz1 knockout animal. Another perspective can be MERFISH analysis of inflammation and regeneration samples with Ptn and Ptprz1. We would be able to locate their location and quantification in the tissue.

Overall, novel findings of this thesis were hyperactivation of enteric neurons upon inflammation, generation of lipid particles in enteric neurons upon hyperactivation, lipid transfer from neuron to enteric glia to guard neuronal cell homeostasis, identification of Ptn-Ptptz1 axis for inclination of ENS cells in regeneration of colon and lastly ENS cell containing assembloid culture system that mimic colon more accurately for better in-vitro model.

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APPENDIX

Appendix I: Primary and secondary antibodies used in IHC (continued in next page).

REAGENT or RESOURCE	HOST-CLONALITY	SOURCE	Catalog Number	Dilution
Anti-ACOT7 Antibody	Rabbit Polyclonal	Sigma	HPA025735	1/100
Anti-Arl13b Antibody	Rabbit Polyclonal	ProteinTech	17711-1-AP	1/100
Recombinant Anti-CD34 Antibody	Rabbit Monoclonal	Abcam	ab81289	1/200
Purified Mouse Anti-E-Cadherin Antibody	Mouse Monoclonal	BD Biosciences	610182	1:50
Recombinant Anti-Ki67 Antibody [SP6]	Rabbit Monoclonal	Abcam	ab16667	1/100
LYVE1 Monoclonal Antibody (ALY7)	Rat Monoclonal	Thermo Fisher Scientific	14-0443-82	1/200
HuC/HuD Monoclonal Antibody (16A11)	Mouse Monoclonal	Invitrogen	A-21271	1/100
Mouse PDGFR alpha Antibody	Goat Polyclonal	R&D Systems	AF1062	1/75
Human/Mouse PHOX2B Antibody	Goat Polyclonal	R&D Systems	AF4940	1/100
Recombinant Anti-S100 beta antibody [EP1576Y]	Rabbit Monoclonal	Abcam	ab52642	1/100
Anti-Sox9 Antibody	Rabbit Polyclonal	Millipore	ab5535	1/100

Purified anti-Tubulin β-3 (TUBB3) Antibody	Rabbit Polyclonal	BioLegend	PRB-435P	1/100
Purified Mouse Anti-β-Catenin	Mouse Monoclonal	BD Biosciences	610154	1/100
GFAP Monoclonal Antibody (2.2B10)	Rat Monoclonal	Invitrogen	13-0300	1/75
Anti-beta III Tubulin antibody [2G10]	Mouse Monoclonal	Abcam	78078	1/100
Anti Vimentin EA	Rabbit Polyclonal	ThermoFisher	PA527231	1/100
Anti-Fabp4	Rabbit Polyclonal	abcam	ab13979	1/100
ApoE	Rabbit Monoclonal	Abcam	ab183597; RRID	1/100
Gpr37l1	Rabbit Polyclonal	Invitrogen	PA5-106838	1/100
PTN Polyclonal antibody 150 uL	Rabbit Polyclonal	Proteintech	27117-1-AP	1/100
Ptprz1 antibody	Rabbit Polyclonal	Bioss	bs-11327	1/100
Anti-Muc Antibody	Rabbit Polyclonal	Abcam (UK)	ab97386	1/100
Anti-acetylated tubulin	Mouse Polyclonal	Sigma	T7451	1/100

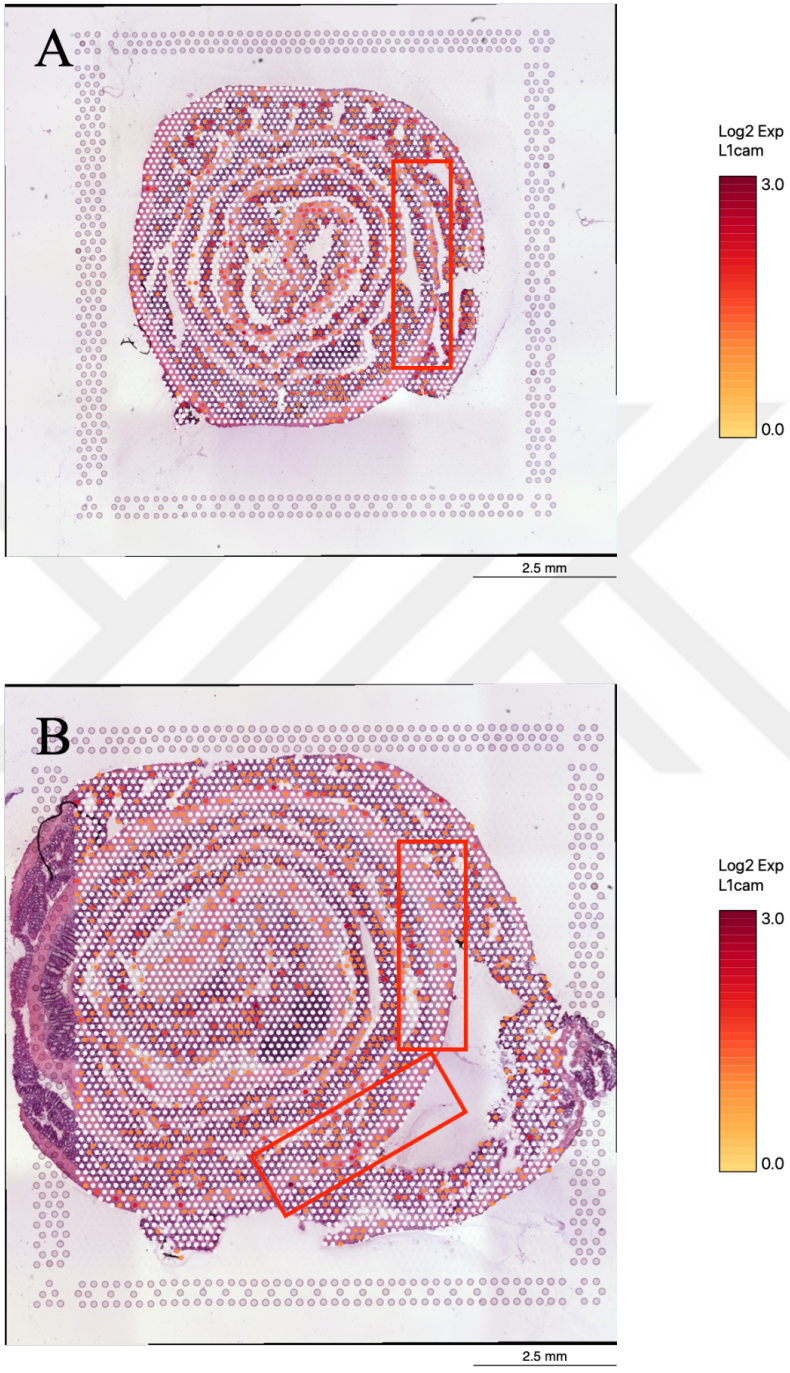
Appendix II: Reagents or resources used in the experiments (continued in next page).

REAGENT or RESOURCE	IDENTIFIER	CATALOGUE NUMBER
Mouse Chow Diet	Optima	
DSS	MP Biomedicals	02160110
Advanced D-MEM/F12 (1X)	ThermoFisher	12634028
N2 Supplement	ThermoFisher	17502048
B27 Supplement	ThermoFisher	17504044
Glutamax 1 (100X) (CE)	ThermoFisher	35050038
HBSS, w/o Ca & Mg (1X) (CE)	ThermoFisher	14170088
Antibiotic Antimycotic	ThermoFisher	15240096
DMEM, high glucose, no glutamine	ThermoFisher	11960044
FBS, HI	ThermoFisher	16140071
Penicillin Streptomycin SOL	ThermoFisher	15140148
L Glutamine (100X)	ThermoFisher	25030149
MEM NEAA (100X)	ThermoFisher	11140050
24 well round, multi dish w/lid case of 75	ThermoFisher	144530
12 well round, multi dish w/lid case of 75	ThermoFisher	150200
DPBS	ThermoFisher	14190144
HEPES Buffer 1M (CE)	ThermoFisher	15630056
REC MS EGF Biosource (TM)	ThermoFisher	PMG8041
Recombinant Murine Noggin	Peprotech	250-38
Recombinant Murine R-Spondin-1	Peprotech	315-32
WNT Surrogate-FC Fusion Protein	J-Protein Express BV	N001
N-Acetyl-L-Cysteine	Sigma	A2750
DL-Dithiothreitol	Sigma	D9779

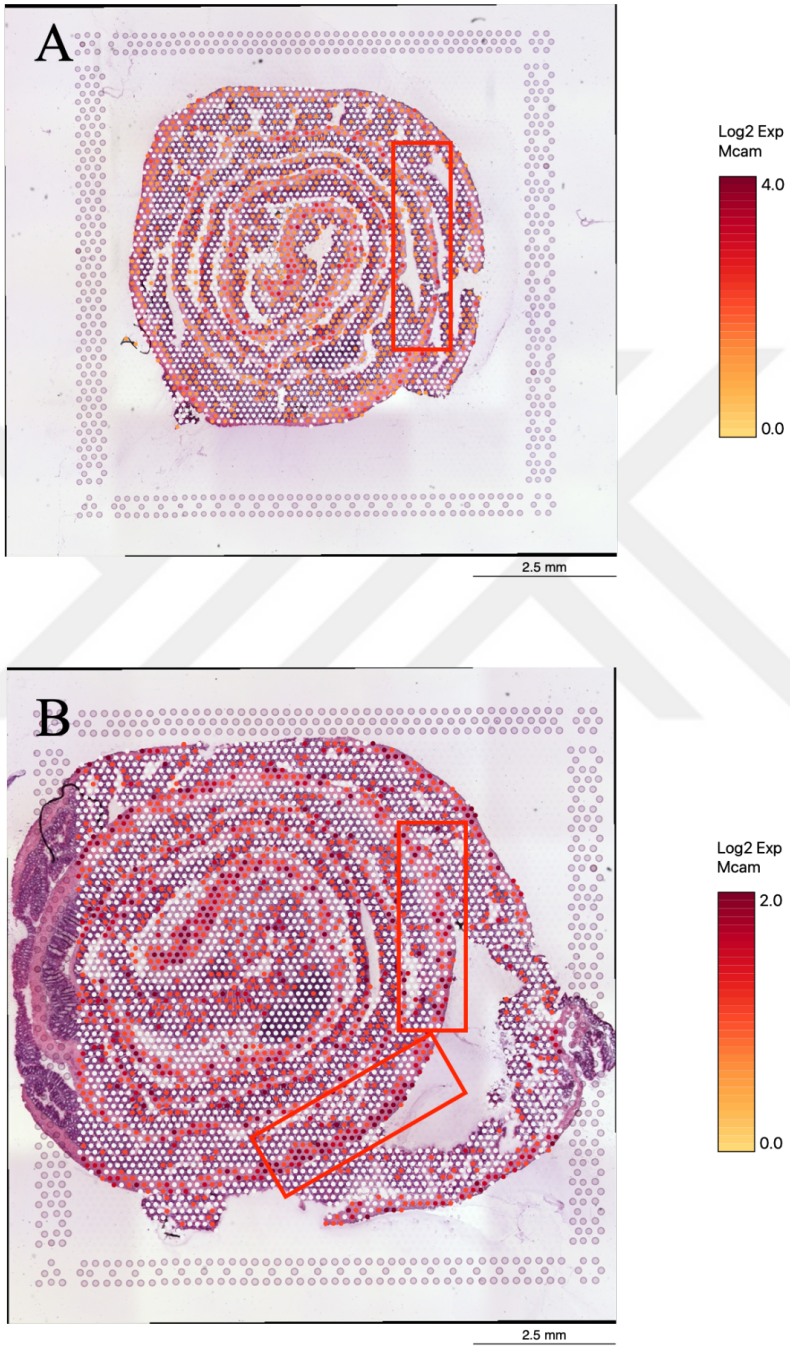
Collagenase from Clostridium histolyticum, Type XI	Sigma	C7657
Dispase II (neutral protease, grade II)	Sigma/Roche	4942078001
Bovine Serum Albumin	Sigma	A7030
Corning Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-free, LDEV-free	Life Sciences	356231
Falcon 70 um Cell Strainer, White, Sterile, Individually Packaged 50/Case	Corning	352350
Corning 25 mm Diameter Syringe Filters, 0.2 um Pore RC Membrane, Sterile, Individually Packaged	Corning	431222
Corning Matrigel Matrix for Organoid Culture, Phenol Red-free, LDEV-free, 10 mL	Corning	356255
EDTA disodium salt dihydrate	Applichem	A2937
Donkey Serum	Biowest	S2170-050
Triton x-100	Sigma	T-8787
Paraformaldehyde	Fluka	76240
Anti-Fade Fluorescence Mounting Medium	Abcam	ab104135
D(+)-Glucose	Sigma	16325
Glycerol 85%	Isolab	927.023
DAPI	Thermo Fisher Scientific	62248
Gentle Cell Disociation Reagent	Stem Cell Technologies	100-0485
Round Coverslips 18 mm	Epredia	11709875
Dental wax	BMS	1.001-S Pink Soft
Poly-D-Lysine Hydrobromide	Sigma	P6407-5mg
Laminin	Sigma	L2020

Cell Recovery Solution	Corning	354253
BODIPY 558/568 C12	Invitrogen	D3835
Liberase TM	Roche	5401119001
CHIR 99021	10 mg	Biogems
A83-01 10 mg	10 mg	tocris
Y-27632 dihydrochloride	1 mg	Sigma
Hank's Balanced Salts (1x) wo Ca & Mg wo/phenol	Capricorn	HBSS-2A
Deoxyribonuclease I from bovine pancreas	Sigma	DN25-1G
DMEM/Ham's F-12 with L-Glutamine	Capricorn Scientific	DMEM-12-A
Recombinant Murine GDNF	Peprotech	450-44
Neuronasal Plus Medium	Gibco	A35829-01
B27 Plus Supplement	Gibco	A35828-01
Recombinant Human TGF-B1	Peprotech	100-21
Recombinant Murine IFN-y	Peprotech	315-05
Recombinant Murine IL-1B	Peprotech	211-11B
Calcium chloride dihydrate	Sigma	C3306-500G
Typsin Edta Solution	Gibco	25300-054
Gentamicin	Sigma	G1397-10ML
DTT	Sigma	D9779-1G

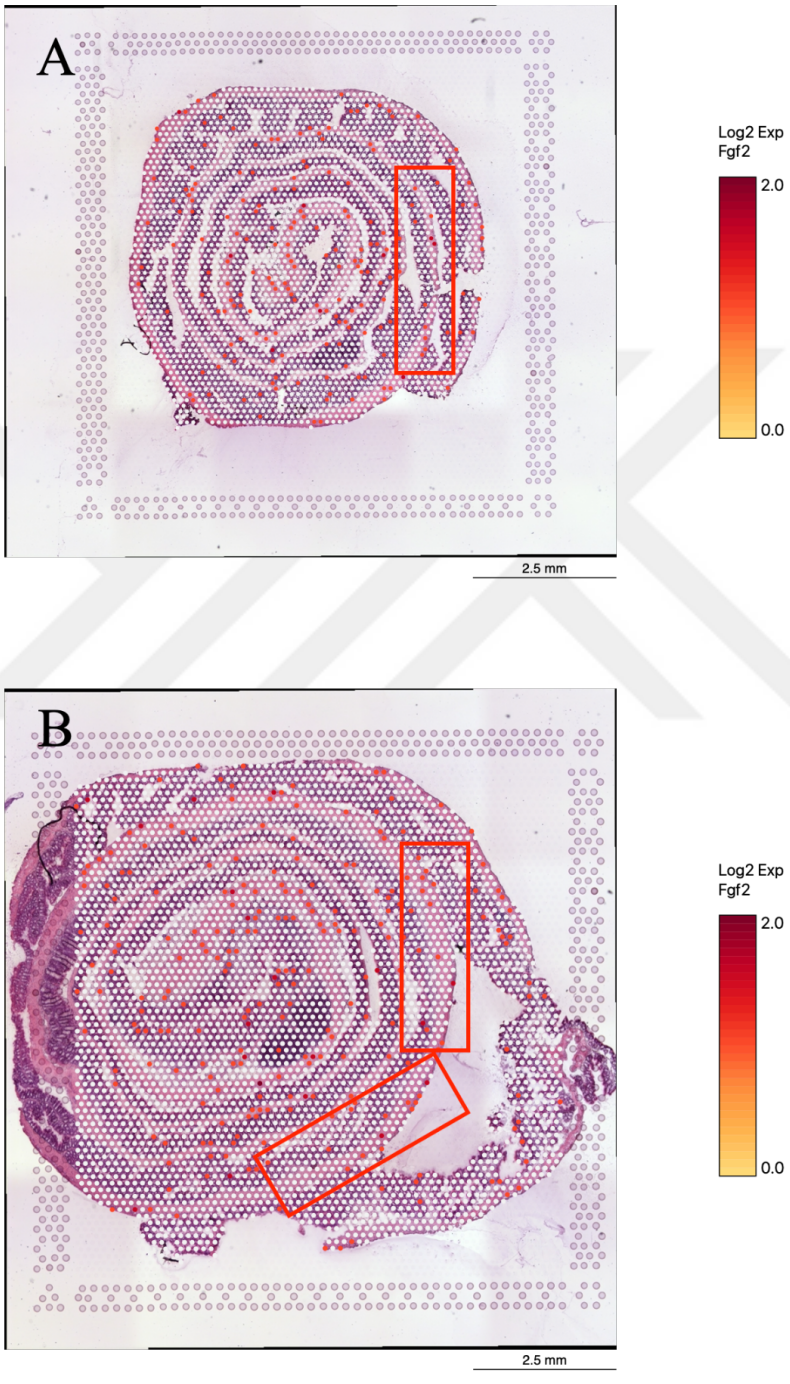
Appendix III: L1cam expression analysis spotted onto Day0 (A) and Day14 (B) colon samples with spatial transcriptomics.



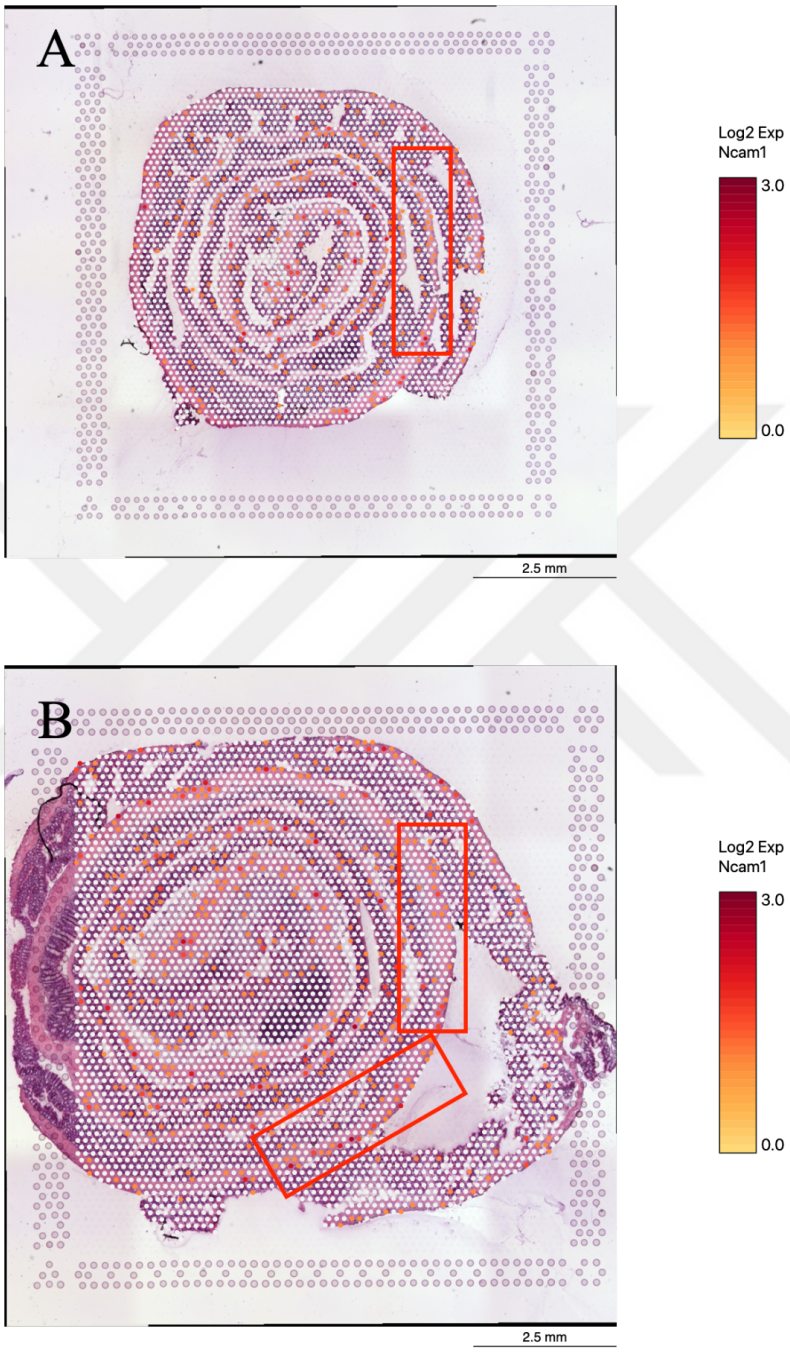
Appendix IV: Mcam expression analysis spotted onto Day0 (A) and Day14 (B) colon samples with spatial transcriptomics.



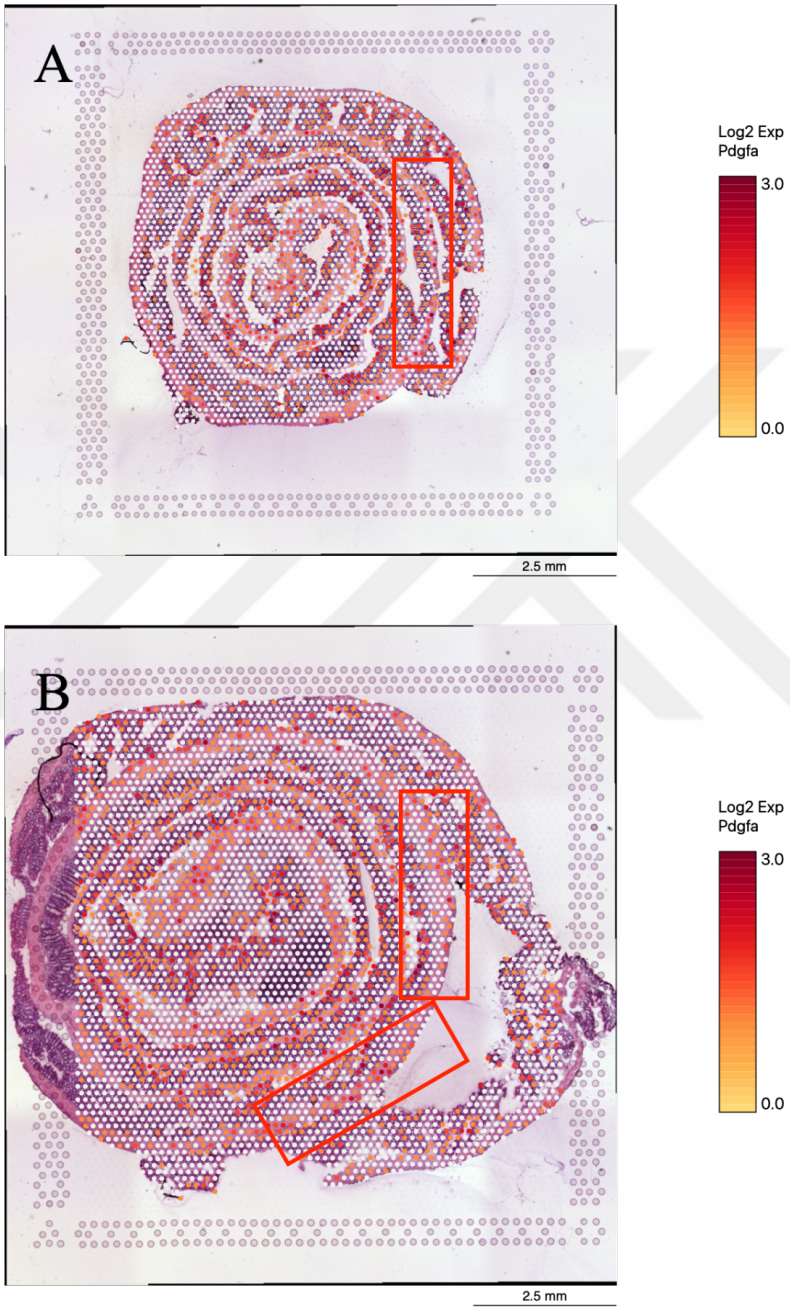
Appendix V: Fgf2 expression analysis spotted onto Day0 (A) and Day14 (B) colon samples with spatial transcriptomics.



Appendix VI: Ncam1 expression analysis spotted onto Day0 (A) and Day14 (B) colon samples with spatial transcriptomics.



Appendix VII: Pdgfa expression analysis spotted onto Day0 (A) and Day14 (B) colon samples with spatial transcriptomics.



Appendix VIII: Pdgfra expression analysis spotted onto Day0 (A) and Day14 (B) colon samples with spatial transcriptomics.

