ABSTRACT

CALDWELL, MADISON. Defining an Age-Dependent Relationship Between the Epithelium, Glia, and Microbiota in a Pig Model of Acute Intestinal Wound Healing (Under the direction of Dr. Amanda Ziegler and Dr. Anthony Blikslager).

Diseases that injure the intestinal epithelial barrier (IEB) cause disproportionately higher mortality in neonates compared to mature individuals. Previous work in pigs demonstrated this is due to a failure in acute repair of the neonatal intestinal epithelium. However, signals from juvenile intestine rescues acute repair in neonates. This suggests that while neonates are innately capable of acute repair, they lack proper signals from the surrounding microenvironment to initiate these repair pathways. Among the intestinal microenvironment, a subepithelial population of enteric glial cells (EGC) and the gut microbiota both mature postnatally. The work in this dissertation investigated the age-dependent relationship between the intestinal epithelium, EGC, and gut microbiota in the context of acute repair using a pig model.

Primary cultures of EGC were isolated from the colonic submucosal plexus of unweaned neonatal (2-week-old) and weaned juvenile (>6 weeks old) pigs. These were co-cultured with the IPEC-J2 intestinal epithelial cell line isolated from the jejunum of an unsuckled 2-week-old piglet and exposed to neonatal or juvenile lumen content to assess the interactions between age, gut microbiota, and EGC on acute intestinal epithelial repair during *in vitro* scratch wound healing. While juvenile EGC promoted intestinal epithelial repair regardless of treatment, neonatal EGC only promoted repair following exposure to juvenile lumen content. Moreover, juvenile lumen content increased release of the protein SERPINB2 from neonatal EGC and SERPINB2 promoted acute intestinal epithelial wound healing.

The work in this dissertation asserts that acute intestinal epithelial repair develops postnatally in concert with maturation of subepithelial EGC and the gut microbiota (Chapter 1).

It highlights the relationship between the enteric nervous system (ENS) and IEB in the context of intestinal ischemia/reperfusion as an injury model (Chapter 2). This work also describes and characterizes a novel method for the primary culture of EGC from the colonic submucosal plexus (SMP) and longitudinal muscle/myenteric plexus (LMMP) of pigs (Chapter 3). Application of this method is then applied to assess the relationship between age, EGC, and the gut microbiota during acute intestinal epithelial wound healing (Chapter 4). The results from these studies are reflected on and considered in connection with recent findings in relevant research and clinical applications to conclude (Chapter 5). Overall, findings from this dissertation suggest that EGC are immature in their ability to promote acute intestinal epithelial repair in neonates but can be modulated by factors produced by the juvenile gut microbiota thus defining an age-dependent relationship. Results from this work highlight EGC and the microbiota as potential therapeutic targets for diseases that affect the IEB in the postnatal period and perhaps beyond in life.

Defining an Age-Dependent Relationship Between the Epithelium, Glia, and Microbiota in a Pig Model of Acute Intestinal Wound Healing

by
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BIOGRAPHY

Madison Caldwell was born in Chambersburg, Pennsylvania on May 8, 1996. Madison spent her formative years in Chambersburg living with her older brother, Devon; parents Mary and Scott; and a consistent household of pets. After graduating from Chambersburg Area Senior High School in 2014, Madison pursued her undergraduate education at nearby Gettysburg College earning her Bachelor of Science in Health Sciences in 2018. During her undergraduate years, Madison completed her own pilot research study under Dr. Eric Noreen and continued to pursue her passion in research as a molecular biology technician at GlaxoSmithKline following her undergraduate education. In 2019, Madison was accepted into and started the DVM/PhD dual degree program at NC State University College of Veterinary Medicine with an interest in small animal medicine and comparative gastroenterology.

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11βPGF2α	11β-prostaglandinF2α
15dPGJ2	15-deoxy-(12,14)-prostaglandin J2
15-HETE	15-hydroxyeicosatatraenoic acid
5-HT	Serotonin
Ach	Acetylcholine
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
ChAT	Choline acetyl transferase
CNS	Central nervous system
DAPI	4',6-diamidino-2-phenylindol
DMSO	Dimethyl sulfoxide
EGC	Enteric glial cells
ENS	Enteric nervous system
FFAR2	Free fatty acid receptor 2 (or GPR43)
FFAR3	Free fatty acid receptor 3 (or GPR41)
FITC	Fluorescein isothiocyanate
GABA	γ-aminobutyric acid
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GI	Gastrointestinal
GSNO	S-nitrosoglutathione
HDAC	Histone deacetylase inhibitor

IEB	Intestinal epithelial barrier
iNOS	Inducible nitric oxide synthase
IPAN	Intrinsic sensory neuron
IPEC-J2	Intestinal porcine epithelial cells
I/R	Ischemia/reperfusion
Juv	Juvenile
LC	Lumen content
LGR5	Leucine-rich repeat-containing G-protein coupled receptor 5
LMMP	Longitudinal muscle/myenteric plexus
LPS	Lipopolysaccharide
MCT	Monocarboxylate transporter
MODS	Multiple organ dysfunction syndrome
NEC	Neonatal necrotizing enterocolitis
Neo	Neonate
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
NOD	Nucleotide-binding oligomerization domain
NPY	Neuropeptide Y
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PLP-1	Proteolipid protein 1

Pro-EGF	Pro-epidermal growth factor
PRR	Pattern recognition receptors
ROS	Reactive oxygen species
S-100β	S100 calcium-binding protein β
SCFA	Short chain fatty acid
SEM	Standard error of measurement
SIRS	Systemic inflammatory response syndrome
SMP	Submucosal plexus
SOX10	SRY-box transcription factor 10
TFF3	Trefoil factor 3
TGF-β_1	Transforming growth factor-β_1
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VIP	Vasoactive intestinal peptide
ZO	Zonula occludens

Chapter 1

An Orchestra of Maturation: The Intestinal Epithelial Barrier, Enteric Nervous System, and Gut Microbiota

1.1 Abstract

From conception to birth, the gastrointestinal tract undergoes a crucial period of growth and development that lays forth the foundational anatomical and functional requirements necessary for life. While much of this development occurs prenatally, many aspects of gastrointestinal physiology are immature at birth and continue developing during the neonatal period. Among these processes, maturation of the intestinal epithelial barrier stands as a cornerstone of vital importance. The intestinal epithelial barrier constitutes one of the largest interfaces separating the external luminal environment and internal milieu of the body serving as the frontline for defense against luminal challenges while ensuring absorption of essential nutrients, ions, and water. One of the most remarkable characteristics of this barrier is its ability to rapidly repair following injury to prevent translocation of toxic luminal contents including gut microbes into systemic circulation. While many barrier functions have been shown to mature postnatally, literature regarding the developmental timeline of barrier repair mechanisms remains scarce. This is significant as diseases of barrier breakdown in neonates such as necrotizing enterocolitis affect a devastating 2-5% of all premature infants resulting in mortality rates up to 50%.[1] Not only will a better understanding of the mechanisms underlying development of acute intestinal barrier repair mechanisms enhance our ability to treat these neonatal patients, but it also holds implications in adults as barrier dysfunction is associated with a variety of intestinal and extra-intestinal diseases. To highlight barrier repair as an age-dependent process, we will

first provide an overview of the development and postnatal maturation of the intestinal epithelial barrier including its regulation by the gut microbiota and enteric nervous system concluding with repair mechanisms and current information of this process in neonates.

1.2 Introduction

The intestinal epithelial barrier (IEB) is a semi-permeable structure that regulates interactions between the internal environment of the body and external lumen. Given that the lumen encompasses a harsh array of microbial products and dietary antigens, it is crucial to minimize translocation of these luminal components across the intestinal mucosa to prevent overwhelming immune cell activation and inflammation that can potentiate sepsis. At the same time, the intestine must also facilitate the transportation of nutrients and bidirectional flow of water and electrolytes. To coordinate this delicate balance of selective permeability, the intestinal epithelium itself establishes a multifaceted system of defenses. In addition to its inherent defense system, the IEB is modulated by microenvironmental cues. Two major microenvironmental modulators of this barrier are the subepithelial enteric nervous system (ENS) and the supra-epithelial gut microbiota. Input from these microenvironmental sources allows the IEB to dynamically adapt to changes and maintain homeostasis.

At birth, however, the IEB and its modulators are immature rendering neonates—especially those born prematurely—vulnerable to intestinal insults and breaches of defense. Such breaches to the immature barrier can result in life-threatening diseases of preterm infants including neonatal necrotizing enterocolitis (NEC). This is further compounded by the apparent lack of acute barrier repair mechanisms in neonates compared to adults. What is driving this age discrepancy in repair and how these repair mechanisms develop and mature remains unknown

and understudied. A better understanding of mechanisms underlying barrier repair development and maturation will not only advance intestinal disease treatment in newborns but may also provide novel insights for improving the myriad of intestinal and extra-intestinal diseases associated with IEB dysfunction in adults. Therefore, this review will first summarize postnatal development of the IEB and its regulation by the gut microbiota and ENS. We will then relate this knowledge to acute IEB mechanisms highlighting what is known about this process in neonates and what critical questions remain to be answered.

1.3 Intestinal Epithelial Barrier

1.3.1 Barrier Structure

The IEB is comprised of a single layer of polarized columnar-shaped cells that line the mucosa of the small intestine and colon. In the small intestine, this epithelium is organized into a pattern of invaginated crypts and finger-like villi while in the colon arranged into crypts connected by inter-crypt surface epithelium. The crypts are sites of proliferation where intestinal stem cells give rise to all intestinal epithelial lineages including enterocytes, goblet cells, Paneth cells, enteroendocrine cells, microfold cells, and tuft cells which are briefly summarized below (Table 1). While our understanding of this differentiation process has become increasingly complex and specific to each cell type, in general, intestinal stem cells characterized by LGR5 expression produce progenitors known as transit amplifying cells that divide as they migrate up the crypt differentiating into absorptive and secretory lineages that ultimately terminally differentiate into their specialized epithelial cell types.[2] This is a rapidly continuous process as most mature cells of the intestinal epithelium are reported to renew every 3-5 days shedding into the lumen through a process of controlled cell death.[3] Several key signaling pathways carefully

control this balance between intestinal stem cell proliferation and differentiation with Wnt signaling being of particular importance.[4] Signaling molecules are provided both by the intestinal epithelium itself as well as the surrounding niche which allows the intestinal epithelium to act dynamically adapting to cues from the subepithelial and luminal microenvironments to maintain homeostasis. For simplicity, we have briefly summarized the structure of the epithelial monolayer that comprises the IEB and endows it with its unique functions here. It is important to recognize, however, that growing bodies of literature devoted to individual intestinal epithelial cell lineages and the unprecedented transcriptomic resolution provided by single cell RNA sequencing have highlighted the increasing complexity and heterogeneity of the intestinal epithelium and we direct interested readers to recent reviews and atlases for a deeper understanding.[5,6]

Table 1.1. Major differentiated epithelial cell types of the intestinal barrier and their observed functions

Cell Type	Observed Functions	References
Enterocytes	Predominant cell type of the intestinal epithelium; Serve in absorption and trafficking of nutrients using microvilli on apical membrane and transporters on basolateral membrane; Able to depolarize and form lamellipodia to close wounds	3,7-9
Goblet cells	Secrete components of mucus layer including mucin2 (MUC2) and trefoil factor 3 (TFF3) to provide defense and enhance barrier function	9-11
Paneth cells	Sense and respond to bacteria by secreting antimicrobial peptides; Provide essential niche factors to support intestinal stem cells Only found in small intestinal crypts; Appear to have species-specific differences and existence in some species is controversial; Longer reported lifespan of 20-60 days	9,12-18
Enteroendocrine cells	Assortment of cells expressing mechanical, olfactory, and taste receptors; Transduce luminal signals by secreting peptide hormones; Exhibit axon-like basal processes referred to as neuropods that contain peptide-secreting vesicles and interact with enteric glia cells, enteric neurons, and vagal neurons	9,19-21
Microfold (M) cells	Initiate mucosal immune responses by transporting luminal antigens across the epithelium for presentation to underlying immune cells; Predominantly found in follicle-associated epithelium	9,22-25
Tuft cells	Chemosensory luminal sentinel cells; Regulate epithelial responses to injury; Role in microbial sensing including promotion of type 2 immune responses in helminth and protozoal infection	9,26-28

1.3.2 Barrier Mechanisms

As mentioned, the IEB relies on several lines of defense inherent to the epithelium to maintain permeability while preventing translocation of potentially damaging luminal contents. The mucins—large glycoprotein molecules secreted by goblet cells—establish a mucus layer over the epithelium that serves as the first and most luminal line of defense. Mucus is primarily

composed of the gel-forming mucins, namely MUC2, whose heavily glycosylated structures promote formation of a hydrophilic network.[10,29] In the small intestine, the mucus barrier is a single layer thick and lightly adhered to facilitate absorption. The mucus barrier of the colon is comprised of a loosely adhered outer layer that interacts with gut microbes and a firmly adhered inner layer meant to remain sterile.[10,29,30] The mucus layer functions in protection of the intestinal epithelium against harmful luminal agents by preventing pathogen adhesion and translocation while also reducing friction during peristalsis. Further, this mucus serves as an environmental niche to resident gut microbiotas who, in turn, modulate mucus composition.[31,32] The intestinal mucus layer is continuously secreted allowing for changes in mucin composition and thickness to rapidly adapt to fluctuations in internal host factors as well as external luminal ones.[33] This ongoing regulation of the mucus layer is essential for physiological barrier function as compromises to the mucus layer are associated with both intestinal diseases including inflammatory bowel disease and extra-intestinal pathologies such as diabetes.[34,35]

Directly beneath the mucus layer, the intestinal epithelial monolayer itself serves as a physical barrier and second line of defense. Adjacent epithelial cells are tightly adhered to one another by three main junctional complexes that establish structural support. Each of these complexes utilize interactions between transmembrane proteins and their intracellular counterparts to establish a connection to the cytoskeleton. The major proteins and functions of each junctional complex in the intestinal epithelium have been well-described elsewhere and are summarized in Table 2 below. While all of the junctional complexes contribute to the structural integrity of the intestinal epithelium, tight junctions are particularly essential to barrier function as the primary regulator of paracellular permeability. Paracellular permeability—movement of molecules between cells—is mediated by two distinct routes referred to as the pore and leak

pathways. Briefly put, the pore pathway allows for the passage of small molecules and is charge-selective whereas the leak pathway allows for the passage of larger solutes irrespective of charge.[36-40] In addition to paracellular permeability, molecules can also cross the intestinal epithelium via transcellular transport mediated by substrate-specific transporters expressed on the apical and basolateral cell surfaces. Altogether, these properties not only allow the intestinal epithelium to act as a cohesive physical barrier but also endow it with the selective permeability inherent to IEB function.

The third line of defense contributed by the intestinal epithelium is the secretion of biochemical factors which primarily act as antimicrobials. Paneth cells are especially established for their role in defense and known to secrete antimicrobial peptides such as α-defensins and lysozyme.[45-47] Beyond their direct antimicrobial role, Paneth cells also drive innate immune responses by secreting the inflammatory cytokines IL-17 and TNFα.[48,49] Tuft cells, though still being defined in their functions, may similarly drive innate immune responses via secretion of IL-25 which is a member of the IL-17 family of cytokines.[48] These secretory defense mechanisms highlight yet another aspect in which the intestinal epithelium acts as a barrier between the external lumen and internal milieu.

Table 1.2. Major intestinal epithelial junction complexes and their observed functions

Junction Type	Transmembrane Protein(s)	Associated Cytoplasmic Proteins	Associated Cytoskeletal Element	Observed Function	References
Tight Junction	Claudins; Occludin; Junctional adhesion molecules; Tricellulin	Zonula occludens (ZO-1, ZO-2, ZO-3); Cingulin	Actin	Seal intercellular space; Regulate paracellular permeability (gate function); Restrict intramembrane diffusion of molecules between apical and basolateral surfaces to maintain polarity (fence function)	36,41-43
Adherens Junction	E-cadherin; Nectin	Catenins (α-catenin, β-catenin, p120 catenin, plakoglobin); Vinculin; Afadin	Actin	Cell-cell adhesion; Mechanical integrity; Facilitates assembly of other junctional complexes; Maintain cell polarity	36,43
Desmosome	Desmocadherins (desmoglein, desmocollin)	Plakoglobin; Plakophilin; Desmoplakin	Intermediate filaments	Cell-cell adhesion; Mechanical integrity	36,43,44

1.4 Intestinal Barrier Regulation

Although these barrier properties are inherent to the intestinal epithelium, they are carefully regulated by the surrounding microenvironment. This includes input from the subepithelial ENS as well as the luminal gut microbiota. Together, these components of the intestinal microenvironment not only maintain homeostatic barrier function but also ensure proper barrier repair following insults. In-depth reviews on each of these individual components and their roles regarding barrier function have been published, so we briefly summarize the structure and barrier roles of each to establish their significance.

1.4.1 Enteric Nervous System

The entire gastrointestinal tract is densely innervated by a network of enteric glial cells (EGC) and neurons collectively referred to as the ENS that controls nearly all gastrointestinal functions including motility, secretion, and barrier function. In mammals, the ENS is organized into two major ganglionated plexi. The submucosal plexus (SMP) is localized to the submucosa and primarily regulates mucosal functions, and the larger myenteric plexus is found between the circular and longitudinal muscle layers and primarily controls motility.[50,51] While the cell bodies of neurons are confined to the ganglia of these plexi, EGC are located within and extend beyond ganglia to innervate every layer of the gut wall. The ENS forms a complete sensorimotor circuit comprised of intrinsic primary afferent neurons (IPANs), interneurons, and motor neurons and can act autonomously without input from the central nervous system (CNS).[51-53] Though independent, the ENS maintains a bidirectional relationship with the CNS as part of the canonized gut-brain axis. The CNS modulates gastrointestinal functions via efferent sympathetic and parasympathetic innervation but also receives an immense amount of sensory input from the ENS via the vagus nerve.[54] Enteric neurons are unmyelinated and sub-types have been classified

by morphology (Dogiel Type I and II in humans), electrophysiological properties (AH and S), neurochemical expression (acetylcholine, vasoactive intestinal peptide, nitric oxide synthase, serotonin or 5-HT, substance P, ATP, dopamine, γ-aminobutyric acid or GABA, neuropeptide Y, among others) and most recently resolved molecularly by single cell transcriptomics which is well summarized in the review by Sharkey and Mawe.[55-58] EGC are a heterogenous population of cells that work in concert with enteric neurons to regulate intestinal functions. Glia communicate bidirectionally with enteric neurons and glial cell excitation has been characterized by increases in intracellular calcium that propagates via connexin-43 hemichannels.[59] Similar to enteric neurons, EGC have been categorized by their anatomical location and morphology (Types I-IV) and are being further resolved with single cell transcriptomics identifying 3 to 7 distinct subtypes depending on the study and model.[56,60-62] Canonical markers of EGC include GFAP (glial fibrillary acidic protein), S-100β (S100 calcium-binding protein B), SOX10 (SRY-box transcription factor 10), and PLP-1 (proteolipid protein-1), though glia have been shown to be phenotypically plastic and expression of these markers is dynamic varying based on microenvironmental cues.[61] The heterogeneity and phenotypic plasticity of EGC are believed to reflect functional specializations supporting the various roles played by EGC and the entire ENS in regulating intestinal physiology. This has been well demonstrated by research highlighting the importance of both enteric neurons and glia on IEB maintenance.

As stated, a body of evidence has highlighted the regulatory role of enteric neurons on IEB function primarily through studies assessing the consequences of neuronal inhibition on permeability. Early *in vivo* studies demonstrated alterations in epithelial permeability in response to neuromediators such as neurokinin A, vasoactive intestinal peptide (VIP), and acetylcholine.[63-65] While these studies did not directly implicate the involvement of enteric neurons as opposed to

extrinsic innervation of the intestine, *ex vivo* studies elaborated on this. Hardin et al., utilized *ex vivo* Ussing chamber experiments to demonstrate that neural blockade of proximal rabbit colon via tetrodotoxin significantly decreased mucosal uptake of intact BSA as a marker of permeability.[66-68] These results were also corroborated by Cameron and Perdue in their Ussing chamber experiments on mouse jejunum showing that baseline horseradish peroxidase permeability decreased following general neural inhibition with tetrodotoxin and muscarinic cholinergic inhibition with atropine.[69] Likewise, tetrodotoxin pretreatment of porcine ileum decreased paracellular flux of the smaller macromolecule 4 kDa-FITC dextran through epithelial tight junctions and prevented increases in barrier permeability induced by corticotropin-releasing factor.[70] However, the authors suggest that these effects may have been partially mediated by off-target effects of corticotropin-releasing factor on mast cell degranulation highlighting the complexity of direct and indirect enteric neuronal involvement in regulating barrier permeability. Using a human co-culture model, Neunlist et al., showed that electrical field stimulation of the submucosal plexus preparations resulted in decreased permeability to 4 kDa-FITC dextran in two different colonic epithelial monolayers and that this decrease in permeability was abrogated by the presence tetrodotoxin.[71] They further showed that this effect on epithelial permeability could be blocked using a VIP receptor antagonist and mimicked by exposing monolayers to VIP which the authors linked to increases in expression of the tight junctional protein zonula occludens-1 (ZO-1). Interestingly, in another co-culture experiment neurons differentiated from neural crest stem-like cells of the mouse longitudinal muscle/myenteric plexus resulted in decreased transepithelial electrical resistance and increased permeability to 4 kDa-FITC dextran in Caco-2 colonic monolayers.[72] Enteric neurons also affect intestinal permeability in the context of nutrient status. Tetrodotoxin was once again used to show that neural inhibition led to increased

permeability in the jejunum of pigs that were fed but not those fasted for 48 hours, whereas the acetylcholine mimetic carbachol reduced permeability in a fasted but not fed state.[73] Additionally, Calvin et al., analyzed permeability of mouse jejunum and ileum on Ussing chambers following mucosal stimulation with a variety of nutrients as well as the bacterial cell membrane component lipopolysaccharide (LPS) while inhibiting (tetrodotoxin for neurons or connexin-43 blocker Gap26 for glia) or activating (veratridine for neurons) the ENS.[74] They found that inhibiting or activating enteric neurons or glia had no effect on baseline permeability, but tetrodotoxin did abolish previously observed decreases in permeability following fat exposure (Intralipid) and exacerbated LPS-induced increases in permeability thereby implicating enteric neuronal control of permeability in a stimulus-specific manner. Overall, these studies demonstrate that enteric neurons regulate barrier function and their inhibition generally results in decreased permeability.

EGC have also become well known for their impact on the IEB. This was first shown in a 1998 study using a *Gfap*-HSV-1 TK (herpes simplex virus type I thymidine kinase) transgenic mouse model to preferentially ablate GFAP-expressing cells exposed to the antiviral ganciclovir which resulted in epithelial monolayer disruption and ultimately a fatal fulminant jejuno-ileitis.[75] Using the same mouse model, ablation of GFAP-expressing EGC significantly delayed mucosal wound healing in one study and increased *in vivo* mucosal permeability to FITC-dextran in another.[76,77] The authors of the latter also reported that monolayers co-cultured with EGC exhibited greater transepithelial electrical resistance, decreased macromolecule permeability, and increased expression of the tight junctional proteins ZO-1 and occludin. They also identified EGC-derived s-nitrosoglutathione (GSNO) as a mediator of these barrier-promoting effects both *in vivo* and *in vitro* which has since been supported by others in various models.[77-79] However,

the reliability of using the HSV-1 TK model to ablate GFAP-expressing EGC has been called into question as a later study identified toxic off-target effects in non-glial cells contributed to the observed pathology and further showed using a diphtheria toxin model to eliminate glia expressing PLP-1 that there was no effect on barrier function.[80] As previously discussed, these contradictory findings may be explained in part due to glial cell heterogeneity with specific subsets of glia expressing different markers.[61,81] This idea was supported by a 2022 study that investigated the effects of ablating GFAP-expressing and PLP-1-expressing EGC using a diphtheria toxin model and found ablation of either subset alone had no effect on intestinal epithelial architecture. However, double ablation resulted in intestinal collapse preceded by significant increases in epithelial permeability supporting a redundant role of EGC subsets in maintaining barrier integrity.[82] Additionally, these authors showed that GFAP-expressing but not PLP-1-expressing subsets of glia regulate intestinal stem cells by expressing Wnt ligands and promoting regeneration following injury.[82] Similarly, Kovler et al., also utilized diphtheria toxin to target GFAP-, PLP-1-, and SOX10-expressing EGC and while baseline barrier function was not assessed, loss of each EGC subset tested resulted in worsened inflammation in their neonatal necrotizing enterocolitis (NEC) model, and GFAP-expressing glia specifically were lost as the disease progressed in mice, pig, and human samples.[83] They also identified brain-derived neurotrophic factor (BDNF) produced by EGC as a mediator of these effects demonstrating that exogenous BDNF was able to rescue NEC severity *in vivo* and in other injury models *in vitro*.[83] In addition to GSNO and BDNF, a number of studies have identified several other glial-derived mediators purported to influence specific IEB functions via paracrine signaling as summarized in Table 3.

Table 1.3. Notable enteric nervous system mediators and their observed effects on intestinal epithelial barrier function

Factor	Observed Effect(s)	Observed Source	Reference(s)
11β-prostaglandinF2α (11βPGF2α)	Promotion of intestinal epithelial cell spreading and wound healing via epithelial PPARγ activation	Enteric Glia	[93]
15-hydroxyeicosatetraenoic acid (15-HETE)	Promotion of intestinal epithelial cell spreading and decreased permeability	Enteric Glia	[94]
15-deoxy-(12,14)-prostaglandin J2 (15dPGJ2)	Neuroprotection and control of epithelial cell proliferation	Enteric Glia	[95,96]
Brain-derived neurotrophic factor (BDNF)	Prevention of experimentally-induced NEC by restraining epithelial TLR4 signaling	Enteric Glia	[83]
Glial cell line-derived neurotrophic factor (GDNF)	Prevention of apoptosis perhaps through an autocrine loop; Reduction in epithelial permeability and inflammation	Enteric Glia	[97-99]
Glial-derived S-nitrosoglutathione (GSNO)	Upregulation and localization of tight junctional proteins to promote barrier integrity	Enteric Glia	[77-79]
Nerve growth factor (NGF)	Mediation of gut inflammation and modulation of visceral sensitivity	Enteric Glia	[100]
Pro-epidermal growth factor (pro-EGF)	Promotion of mucosal wound healing to enhance epithelial restitution	Enteric Glia	[76]
S-100β	Stimulation of inflammatory nitric oxide production associated with a RAGE-mediated NF-kB pathway; Integrates bacterial TLR-signaling	Enteric Glia	[101,102]
TGF-β_1	Partly involved in inhibition of epithelial cell proliferation	Enteric Glia	[103]
Acetylcholine (ACh)	Anti-inflammatory effects; Mediation of intestinal barrier permeability and integrity; Potential role in intestinal stem cell niche; Regulation of intestinal motility; Stimulation of mucosal secretions	Enteric Neurons	[104-109]
Neuropeptide Y (NPY)	Increased epithelial permeability via increases in pore-forming claudin-2 in a TNF-dependent manner	Enteric Neurons	[110]

Table 1.3. (continued)

Serotonin (5-HT)	Regulation of mucosal growth and turnover; Neuroprotection; Regulation of intestinal secretion	Enteric Neurons	111-113
Vasoactive intestinal peptide (VIP)	Control of epithelial cell proliferation; Decrease in paracellular permeability; Maintenance of gut microbiota homeostasis Role in immunomodulation; Stimulation of mucosal secretions	Enteric Neurons	71,86,114-116

Beyond direct roles on barrier function, the ENS offers additional influence by regulating epithelial proliferation, controlling secretion to regulate ion and fluid flux, and via bidirectional interactions with local immune cells to regulate mucosal immunity.[84-87] Considering that many of the known ENS effects on the IEB are driven by the release of cell-derived mediators we have briefly summarized them for the purposes of this review (Table 3). While the majority of these mediators and the ENS in general promote barrier function, it is also important to consider this in the context of disease as inflammation can alter the phenotype and function of enteric neurons and glia driving further inflammation and intestinal dysfunction.[88-91] Though beyond our scope, we direct readers seeking an in-depth discussion regarding the many aspects of IEB function and intestinal physiology modulated by the ENS to recent reviews.[58,92]

1.4.2 Gut Microbiota

The gut is colonized by an estimated 100 trillion microorganisms including bacteria, viruses, fungi, and protozoa residing primarily in the intestinal lumen.[117] These microorganisms—referred to as gut microbiota—and their genomes make up the gut microbiome. Composition of the gut microbiota is dynamic and varies with age, health status, diet, drugs, and genetics; though environmental factors are believed to play a predominant role in

gut microbiota composition.[118] The gut microbiome serves a variety of functions including vitamin production, protection against pathogens, fermentation of indigestible foods, modulation of immune responses, and importantly, IEB regulation.[119] We will focus primarily on bacteria as these are the most well-studied component of the gut microbiota due to their abundance and the widely available research techniques to study them. Further, the gut boasts the greatest concentrations of bacteria with an estimated magnitude of 10^{11} bacteria/mL in the colon and 10^{3}-10^{4} in the small intestine.[120] The major phyla of bacteria comprising the gut microbiota include Bacteroidetes and Firmicutes as the dominant phyla constituting over 90% of the total community as well as Bacillota, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia.[121-124]

The intestinal epithelium recognizes and responds to structural components and metabolites produced by these microbes. Pattern recognition receptors (PRRs) including toll-like receptors (TLRs) and nucleotide-binding oligomerization domain or NOD-like receptors are expressed by the intestinal epithelium and bind to pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) from the outer cell wall of gram-negative bacteria, lipoteichoic acid from gram-positive bacteria, peptidoglycan, foreign nucleic acids, and flagellin.[125] This allows not only for immune regulation, but also modulation of the gut barrier. Much of this research has focused on the effects of various probiotic (non-pathogenic naturally occurring) bacterial species. One of the first studies demonstrating this was conducted in 2001 and found the probiotic cocktail VSL#3 decreased colonic permeability to the macromolecule mannitol in controls and rescued increased permeability observed in IL-10 deficient mice *in vivo*.[126] The authors confirmed these findings *in vitro* showing that monolayers of the human colon epithelial T84 cell line exhibited increased transepithelial electrical resistance and

decreased mannitol permeability within hours of exposure to VSL#3. They were also able to replicate these results with exposure to two out three individual bacterial strains confirming that the response to VSL#3 was specific to one or more species in the probiotic cocktail. Further, they found similar barrier enhancing results using VSL#3 conditioned media that had been filtered to remove bacteria and identified a soluble protein factor as a potential mediator. Similarly, a 2006 study showed that the probiotic EcN (Escherichia coli Nissle 1917 strain) was able to rescue decreases in barrier function in T84 monolayers via upregulation of the tight junctional protein zonula occludens-2 (ZO-2) resulting in increased transepithelial electrical resistance.[127] Importantly, this study like many others, not only demonstrates the barrier dysfunction caused by pathogenic bacteria but also highlights the ability of probiotic species that may not have barrier-enhancing effects at baseline to rescue barrier dysfunction following insults and in the context of barrier diseases such as NEC.[126,128-136] Like previously, the majority of these results demonstrate that the barrier promoting effects of probiotic bacteria are attained at least in part by altering expression of intestinal epithelial tight junctional proteins (including ZO-1, ZO-2, occludin, and claudins) resulting in decreased permeability. Accordingly, antibiotics have the opposite effect impairing IEB function and increasing permeability, though whether all of these effects are microbiota-dependent is unclear.[137-139]

Also similar to the ENS, the effects of the gut microbiota on IEB function are largely mediated by microbial release of signaling molecules and metabolites. These microbial products include indole derivates from tryptophan metabolism, polyphenol derivatives, polyamines, bile acid metabolites, and short-chain fatty acids (SCFAs). The majority of these bacterial metabolites have barrier-promoting effects primarily through their regulation of junctional proteins and paracellular permeability, though contradictory findings have been reported for bile acid

derivatives. This topic and the role of bacterial structural components was extensively reviewed in a recent publication by Ghosh and colleagues, so we will limit our discussion to SCFAs as their role in barrier function has been well-studied and summarized in Table 4 below.[140] SCFAs include a number of fatty acid compounds comprised of six or less carbon atoms that are produced as byproducts of bacterial fermentation with butyrate, acetate, and propionate being the most well-known. The greatest concentration of SCFAs is found in the colon where they enter epithelial cells via passive diffusion or through apical transporters such as monocarboxylate transporter-1 (MCT-1) and sodium-coupled monocarboxylate transporter-1 and 2 (SMCT-1 and SMCT-2), or can activate signaling pathways via the apical G-protein coupled receptors GPR41 (free fatty acid receptor 3 or FFAR3), GPR43 (free fatty acid receptor 3 or FFAR2), or GPR109a for butyrate only.[141,142] Additionally, SCFAs can be transported out of epithelial cells basolaterally via MCT-1 and MCT-4 (monocarboxylate transporter-4). The effects of SCFAs on barrier function depend on the concentration, SCFA, and intestinal epithelial cell type studied.

The majority of studies have focused on butyrate as it serves as the predominant energy source for colonocytes and because, out of the SCFAs, butyrate has the greatest ability to modulate gene expression by acting as a histone deacetylase (HDAC) inhibitor.[143,144] Butyrate improves barrier function in Caco-2 cells by increasing transepithelial electrical resistance and decreasing macromolecular permeability at low doses (2 mM) but not at higher doses (8 mM) by inducing AMP-activated protein kinase dependent assembly of the tight junction proteins ZO-1 and occludin.[145] Zheng at al., elaborated on this showing that 2 mM butyrate improved barrier function via IL-10 receptor-dependent repression of pore-forming claudin-2 in Caco-2 and T84 cells.[146] Another study in Caco-2 cells found that butyrate only enhanced transepithelial electrical resistance at 5 mM and had no effect on paracellular permeability at any dose. However, butyrate

still resulted in a decrease of pore-forming claudin-2 and 7 as well as an increase in barrier-promoting claudin-1.[147] Nevertheless, studies in other cell types have also supported an overall barrier-promoting role of butyrate on IEB function as evidenced by increased resistance and decreased permeability *in vitro*.[148] In addition to these positive effects on resistance and permeability, butyrate also promotes barrier integrity by increasing scratch wound healing at periods >36 hours in T84 as well as jejunal IPEC-J2 cells.[149,150] The former study further identified the actin-associated protein synaptopodin as a target increased by butyrate via HDAC inhibition that promotes epithelial migration after injury.[149] Results from *in vivo* studies have proved more contradictory likely due to differences between injury models. Butyrate was shown to protect barrier function following chemical-induced injuries from 2,4,6-trinitrobenzene sulfonic acid (TNBS) and DSS colitis as well as against NEC in mice but showed no protection from Compound 48/80-induced injury and can induce a developmental-dependent NEC.[151-155] In addition to these effects, butyrate also acts on the ENS. One study showed that butyrate increased the proportion of cholinergic (ChAT-positive) neurons along with colonic motility and contractility.[156] Butyrate also exhibits protective effects and was also shown to prevent colitis-induced loss of colonic neurons and increases of GFAP-positive glia in the myenteric plexus.[156,157]

As for acetate and propionate, while some studies have shown no effect on barrier function or intestinal wound healing, others have found positive results including that the combination of butyrate, acetate, and propionate increased resistance, decreased permeability, modulated tight junctions (increased ZO-1 and decreased claudin-2), and protected against injury-induced inflammasome and autophagy.[149,158] Acetate increased resistance across monolayers derived from human colonic organoids and increased colonic epithelial wound

healing compared to other SCFAs, though at a dose of 20 mM likely exceeding beneficial *in vitro* concentrations of butyrate and propionate.[159,160] Similar to acetate, recent studies have begun investigating the effects of propionate and have found barrier-promoting effects through decreases in paracellular permeability, increases in epithelial resistance, and upregulation of the tight junction protein endothelial cell-selective adhesion molecule (ESAM).[161] Propionate also enhanced epithelial cell spreading and scratch wound healing and while the authors demonstrated similar increases in wound healing following butyrate and acetate exposure, they did not examine the effects of butyrate and acetate on cell spreading and speed which would have been an interesting comparison.[162] Overall, while butyrate has historically dominated studies, the effect of SCFAs on IEB function remains an exciting area ripe for further mechanistic elucidation.

Table 1.4. Microbial short chain fatty acids and their observed effects on intestinal epithelial barrier function

Short Chain Fatty Acid	Observed Effect(s)	Major Bacterial Phyla Source(s)	Reference(s)
Butyrate	Paradoxical effect on permeability depending on dose; Promotes epithelial wound healing; Facilitates tight junction assembly	Firmicutes	145,147-150,163,164
Acetate	Strengthens tight junction integrity; Inhibits increases in permeability from DSS-induced colitis by increasing junctional protein expression	Bacteroidetes	159,160
Propionate	Upregulation of pro-barrier genes and decrease of pro-inflammatory cytokines; May promote epithelial wound healing and cell spreading	Bacteroidetes; Some Firmicutes	162,165

1.5 Postnatal Maturation of the Intestinal Barrier

While the structures of the IEB are in place at birth, research indicates that maturation of the intestinal epithelium, ENS, and gut microbiota continues postnatally coinciding with adult functionality of the barrier. For context, we will briefly summarize the developmental and early postnatal maturation processes of the intestinal epithelium, ENS, and microbiota but an exhaustive review would be beyond our scope and we direct readers to excellent previous literature covering each of these.[166-169]

1.5.1 Intestinal Epithelial Barrier Development and Maturation

The intestinal epithelium develops from the endoderm of the primitive gut tube which completes organogenesis at 8 weeks of gestational age in humans. Soon after, the epithelium undergoes a process of reorganization in a rostro-caudal direction developing from its primitive stratified epithelium into a mature simple columnar pattern with crypt-villus architecture appearing by 9-10 weeks of gestation driven by epithelial-mesenchymal interactions.[170-173] By 12 weeks, enterocytes can be identified differentiating from a population of stem-like *lgr5* low-expressing progenitor cells as shown by Fawkner-Corbett and colleagues using spatial transcriptomics.[174] This elaborated on an earlier study which identified the intermediate filament cytokeratin 20 as an early marker of absorptive enterocytes and goblet cells and was appreciable at week 8 of gestation.[175] By 17 weeks, all specialized intestinal epithelial cell types seen in adults can be seen in the human fetus, though functionally not yet mature. For example, Paneth cells are detectable at 12-13 weeks of gestation coinciding with the expression of antimicrobial defensins, but remain immature until later in development as premature infants born at 24 weeks of gestation exhibit Paneth cells in lower number and deficient in function.[176-178] Goblet cells are detectable by 8-10 weeks of gestation with numbers and expression of various mucins increasing

through gestation.[179] Through 24 weeks of gestation, crypt length continues to increase and proliferative cells become confined to the crypt base as in adults.[180-182]

Unlike the anatomical development of the fetal intestine which resembles that of adults relatively early in gestation, intestinal functionality begins to develop in mid-gestation with some aspects maturing postnatally. Intercellular tight junctions between intestinal epithelial cells have been observed as early as 10 weeks of gestation, yet barrier permeability does not reach adult levels until after birth.[183-186] This results in a double-edged sword as increased permeability is necessary to allow for passive transfer of maternal antibodies in colostrum but increases the risk of exposure to antigens that threaten the immature immune system. Postnatal environmental exposures appear to drive early life decreases in intestinal permeability as both preterm infants and those born at 34-37 weeks of gestation exhibit declines in permeability to lactulose in response to oral feedings.[184] Similarly, another study found that permeability in premature infants was not related to gestational age or birth weight with comparable levels of lactulose to mannitol ratios seen after two days of birth indicating that the postnatal intestine adapts rapidly to enteral feeding.[185] However, this decline in permeability may also be related to the food source as breast milk consumption also significantly reduces intestinal permeability of premature infants compared to formula.[187] This has been further investigated *in vitro* as addition of breast milk to the apical side of monolayers derived from human enteroids resulted in a dose-dependent increase in transepithelial electrical resistance but had no effect on FITC-dextran permeability.[188] Breast milk contains a number of hormones, growth factors, and other bioactive compounds that likely contribute to this maturation process preparing the newborn intestine for adult functionality and has been previously reviewed.[189] Early life interactions with the gut microbiota further drive IEB maturation as probiotics have been shown to accelerate barrier maturation by

decreasing permeability and upregulating expression of claudin-3.[190] Lastly, in addition to barrier function and regulation of permeability, the absorptive and secretory roles of the fetal intestine develop later in gestation from 26 weeks until birth and require the presence of amniotic trophic factors.[191,192] Overall, while the intestine is largely formed prior to birth, significant adaptations are required postnatally to establish proper functioning of the IEB and prepare the neonatal intestine for its adult roles.

1.5.2 Enteric Nervous System Development and Maturation

Both enteric neurons and glia originate from multipotent neural crest cells arising from the ectoderm during development. Early experiments using ablation and grafting techniques in avian embryos demonstrated that the majority of the ENS is colonized by vagal neural crest cells with contributions from sacral neural crest cells.[193,194] However, recent lineage tracing studies have found contradictory results regarding the contribution of sacral neural crest cells to the ENS in chickens compared to mice.[195,196] The definition of both vagal and sacral neural crest cell populations has also been questioned by more modern studies that have highlighted contributions from Schwann cell precursors.[197,198] Origin aside, once they migrate to the embryonic foregut and hindgut, these progenitors now referred to as enteric neural crest cells colonize the entire gut by week 7 of gestation.[199] Proliferation and differentiation of these progenitors is dictated by the expression of major molecular mediators. For example, SOX10 is expressed by all progenitors and necessary for cell survival and potency. However, as differentiation occurs, maintenance of SOX10 expression appears to be required for glial cell differentiation while decreased expression prepares cells for a neuronal fate and may even impact neuronal subtype.[200-204] Additional mediators such as RET and its binding partner GDNF, PHOX2B, and various other signaling pathways also shape the developing ENS and mutations in these key factors have been associated

with intestinal aganglionosis at birth characteristic of Hirschsprung's disease.[205-208] After full colonization by week 7 of gestation, evidence of myenteric plexus formation has been reported by 9 weeks of gestation with the submucosal plexus following 2-4 weeks later indicating an "outside-in" manner of development.[209] The rest of gestation is characterized by continued growth and differentiation into distinct neuronal subtypes as well as some apparent glial subtypes, though the timeline and order of subtype appearance may differ by model.[200,210-212]

Functional maturation of the ENS also develops later in gestation and continue postnatally, particularly in regard to motility. Early intestinal motility appears to be of myogenic origin with a recent study by Dershowitz and colleagues that assessed GI motility of fetal human jejunum *ex vivo* showing that tetrodotoxin had no effect on early motility patterns at 13-15 weeks post conception but did have drastic inhibitory effects on motility patterns at 18-22 gestational weeks indicating that the ENS begins to gain function at this time.[213-215] Despite this, premature infants display immature motility patterns compared to those born at term indicating continued functional maturation throughout gestation.[216] Additionally, several studies have demonstrated postnatal changes to the ENS. One of the first studies to investigate this found a postnatal increase in the percent of myenteric neurons identified as cholinergic and a concomitant increase in cholinergic-dependent neuromuscular transmission in the colon of rats.[217] Similarly, Foong et al., demonstrated changes to neuron morphology and electrophysiological properties during the early postnatal period from the duodenal myenteric plexus of mice.[218] In a following study, this group analyzed these changes later in life comparing 2-week-old mice to 6-week-old mice and found significant changes to ENS architecture in the submucosal and myenteric plexi as well as neurochemical changes in the submucosal plexus.[219] This study also highlighted a decrease in enteric neurons, at least in the myenteric plexus, during adolescence as noted in other studies in a

process referred to as “pruning” that is necessary for proper adult functionality.[219-222] As for EGC, Cossais and colleagues characterized the maturing postnatal glial network noting a progressive decrease in SOX10- and SOX10-/Ki67-positive cells and increase in GFAP and S-100β expressing cells in the myenteric plexus of rat colons.[223] In addition to these postnatal changes, proper maturation of the ENS has been closely associated with the development of the gut microbiota.

1.5.3 Gut Microbiota Development and Maturation

Though the sterility of the womb has been contested, it is generally accepted that the vast majority of microbial colonization within the newborn intestine occurs following birth during contact with maternal vaginal, gut, and skin microbial populations.[224,225] The early gut microbiota composition is influenced by a variety of factors including mode of delivery, geography, diet, and antibiotic exposure among others.[226,227] Of these factors, receipt of breast milk has been considered the leading determinant of infant gut microbiota composition, at least for term infants.[226,228]. The gut microbiota purportedly stabilizes around 2-3 years of age following cessation of breastfeeding and transition to solid foods with compositions between individuals converging towards a more diverse mature microbiota.[228-231] By adulthood, the gut microbiota is considered relatively stable within individuals under healthy conditions becoming less stable and diverse in the elderly.[232-234]

Colonization of the gut by resident microbes is also closely associated with ENS development during the postnatal period. A 2013 study noted significant differences in jejunum and ileum whole mount specimens of myenteric plexi from germ-free neonatal mice including decreased nerve densities and diminished motility.[235] Similarly, neonatal antibiotic exposure resulted in gastrointestinal dysfunction characterized by altered ENS structure and function that

persisted in juvenile mice even after antibiotic administration was discontinued.[236-238] In mature mice, antibiotic treatment also induces alterations to microbiota composition, changes to the glial network, and disruptions to normal enteric neuron number and neurochemistry.[239-242] However, the study by Vicentini and colleagues showed that these antibiotic-induced effects were reversible with spontaneous recolonization of the gut microbiota restoring normal gastrointestinal transit time and density of enteric neurons and glia in the colon and ileum.[242] The results from this study, like others, suggest that the gut microbiota mediates postnatal enteric neurogenesis.[242,243] The exact mechanism by which the gut microbiota drives these changes requires further elucidation as this study found that while LPS enhanced neuronal survival and SCFA enhanced neuronal survival and stimulated neurogenesis, neither were able to restore metrics of gut function.[242] A possible explanation may be related to serotonin (5-HT) as conventionalizing germ-free mice in adulthood was shown to restore normal enteric neuroanatomy and intestinal transit by inducing the release of 5-HT and activating the 5-HT4 receptor which has also been linked to enteric neurogenesis.[244]

Similarly, EGC and their products have also received recent attention for their interactions with the gut microbiota. EGC appear to respond to butyrate *in vivo* and *in vitro* with data suggesting butyrate exerts an antiproliferative effect on EGC which express canonical SCFA receptors.[223] A seminal study by Kabouridis expanded on this by investigating the development of mucosal EGC specifically showing that the lamina propria of mice was essentially devoid of glia at birth but increased within the early postnatal period and following weaning reaching adult levels by 4-5 weeks of age.[245] The invasion of mucosal EGC into the lamina propria was shown to require the presence of the gut microbiota as villus-crypt units from germ-free mice contained significantly less EGC, but conventionalization of mice at 4 weeks was

able to restore numbers suggesting that this process is not restricted to early postnatal life.[245] Using elegant inducible lineage-tracing experiments, this group also demonstrated that mucosal EGC appear to be continuously supplied with cells originating mainly from the myenteric plexus. This process is regulated by gut microbiota as antibiotic treatment dramatically reduced the incoming flow of mucosal EGC.[245] While this appears to be true in mice, the translation of these results in humans has been refuted. Inlender et al., corroborated that mucosal EGC are absent in mice at birth but found that they were present in human fetal tissue at 8-12 weeks of gestational age.[246] Using xenografts of human fetal gut, they also provided evidence that the establishment of mucosal EGC did not require microbial gut colonization, nor did maintenance of these glial cells require an intact myenteric plexus.[246] Lastly, this group compared single cell RNA sequencing data to assess differences between mouse and human EGC and found differentially expressed marker genes clustered into species-specific populations with one of the most obvious differences being the lack of GFAP expression by human EGC.[246] While these authors did not investigate functionality of fetal human mucosal EGC, this study does highlight the importance of model consideration. The majority of studies informing current knowledge about intestinal development and postnatal maturation have been performed in rodents whose gestation and postnatal period differ significantly from humans. For example, some aspects of intestinal morphogenesis including crypt formation occur after birth in mice but are evident in the first trimester of humans meaning the intestine of a term human infant is most comparable to a 4-week-old mouse.[181,247] Thus, while these findings provide key insights regarding development and maturation of the IEB, ENS, and gut microbiota, translation to humans requires further research.

1.6 Intestinal Epithelial Barrier Repair

One of the most remarkable and important attributes of the IEB is its acute ability to heal following a variety of injuries including ischemia/reperfusion, infectious agents, and bile acids.[248] Rapid restoration of barrier function is paramount to prevent translocation of microbes, their products, and other toxic luminal content from accessing underlying immune cells and systemic circulation. Acute restoration of the epithelial barrier has been well described in the small intestine and occurs in three distinct but overlapping phases. In the first step of acute mucosal repair, prostaglandins drive contraction of subepithelial myofibroblasts in an energy-dependent process decreasing the surface area of the wound.[248-252] After this, a process referred to as restitution occurs in which epithelial cells depolarize and assume a flattened, migratory phenotype crawling across the basement membrane to close the defect.[248,249] This requires the extension of epithelial cell membranes into lamellipodia-like protrusions in the direction of migration that interact with the basement membrane at focal adhesions which serve as mechanical linkages between the cell and its underlying matrix.[253] Dynamic remodeling of the cytoskeleton through actin-myosin interactions propels the cell forward essentially allowing the cell to pull itself across the wound bed. Intestinal epithelial cells are also unique in their ability to undergo collective sheet migration in which a single leading cell depolarizes and forms the characteristic lamellipodia-like protrusions while the remaining cells are pulled along maintaining their polarization to minimize the impact on barrier function.[254,255] Closure of the paracellular space by reassembly of cell-cell junctions marks the final phase of acute IEB repair. Considered to be the most critical step in restoring barrier function, this process is also mediated in part by prostaglandins and critically serves to reseal the paracellular space via redistribution of tight junctional proteins to the apical membrane through endosomal recycling.[251,256] Together

these three processes effectively restore the intestinal epithelial barrier within hours to prevent systemic complications and overwhelming inflammation associated with translocation of harmful lumen contents. Following acute repair to prevent immediate decompensation of barrier function, the intestine must replenish the various epithelial cell types sloughed off during injury to restore normal tissue architecture and complete functionality. Driven by proliferation and differentiation of pools of progenitors and crypt-residing stem cells, the intestinal epithelial architecture can be fully restored within days of injury marking complete repair.[257]

Given their roles in barrier regulation, it is not surprising that both the ENS and gut microbiota mediate intestinal epithelial repair as well. For example, cholinergic neurons were recently found to activate calcium signaling in the intestinal epithelium to promote healing.[109] EGC are also well-known for their ability to promote intestinal epithelial healing by releasing mediators such as pro-EGF, 11βPGF2α, and GDNF.[76,93,99] Moreover, commensal microbes promote barrier healing by stimulating intestinal epithelial generation of reactive oxygen species that phosphorylate focal adhesion kinase required for epithelial restitution.[253,258] As further evidence of their roles in barrier repair, ENS and gut microbiota alterations are associated with a variety of digestive diseases including those perpetuated by a lack of repair observed in NEC.[259-261]

1.6.1 Age-Dependence of Intestinal Epithelial Barrier Repair

Although the intestinal epithelium and barrier regulators including the ENS and gut microbiota mature after birth, the majority of functional studies have focused on barrier measures such as permeability and resistance. Few studies have assessed the impact of age and maturation on IEB wound healing. Clinically, this represents a critical area necessitating further research as younger age has been associated with worse outcomes and higher mortality in pediatric

conditions involving IEB damage such as volvulus and NEC.[262] Seeking to better understand this, our laboratory discovered an age-dependent defect in barrier repair using a translational porcine model in which we surgically induce ischemia to cause intestinal epithelial damage and recover the tissues *ex vivo* on Ussing chambers. While the jejunum of juvenile pigs recovered barrier function in terms of permeability and resistance, neonates failed to repair and neonatal enterocytes lacked the migratory phenotype characteristic of restitution.[249] Interestingly, we were able to rescue barrier repair in neonates by applying homogenized mucosa from ischemia-injured juvenile intestine.[249] This suggests that while neonates exhibit immature barrier repair mechanisms, these pathways appear to be intact but require activation from key factors present in the mucosa of older pigs. Based on the findings from Kabouridis et al., that mucosal EGC develop postnatally and their ability to promote repair via paracrine signaling, we believed these cells may play a role in age-dependent barrier repair.[245] We confirmed that EGC density is increased in the mucosa of juvenile pigs as compared to neonates and found that chemical inhibition of EGC with fluoroacetate in juvenile pigs resulted in a failure to repair mimicking the defect observed in neonates.[263] From these studies, we have begun to better understand the postnatal development of IEB repair. However, we still lack relevant mechanisms and while we have implicated EGC as a potential mediator, several additional mucosal factors may also be involved. For one, it is likely gut microbiota play a role given their influence on both the ENS and intestinal epithelium. Further, one of the greatest differences between our 2-week-old neonatal pigs and 6-week-old juveniles is weaning off milk and the transition to solid feed that occurs at 3 weeks of age, which is known to induce major maturational shifts in the gut microbiota.[228] Based on this evidence, we believe postnatal IEB repair mechanisms are driven by

a microbiota-ENS-intestinal epithelial axis. Future studies seek to investigate these relationships to better understand mechanisms underlying age-dependent IEB repair.

1.7 Conclusions

Overall, the IEB serves as a critical line of defense against the external luminal environment. In health, it orchestrates a delicate balance between nutrient absorption and protection against harmful luminal agents that hinges on its selective permeability. While the intestinal epithelium establishes several lines of defense in its role as a barrier, it is also modulated by its microenvironment allowing it to adapt as needed. The ENS and gut microbiota serve as two major microenvironmental regulators of the IEB utilizing secreted factors to mediate many of their effects. Proper development both prenatally and postnatally is required for normal IEB function. This includes not only the intestinal epithelium itself, but also the ENS and gut microbiota. Though IEB function matures after birth in terms of permeability and resistance, few studies have assessed the impact of age on other crucial roles of the barrier including its ability to repair following injury. Indeed, our laboratory has recently identified an age-dependent defect in epithelial restitution exhibited by neonatal but not juvenile pigs and have implicated EGC as potential mediators of postnatal repair. Despite these findings, definitive details and mechanisms are lacking including potential involvement of other intestinal factors such as the gut microbiota that likely play a role. Future studies will be essential to unravel the complex, age-dependent interactions between the intestinal epithelium, ENS, and microbiota that govern IEB regulation and repair following injury.

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Chapter 2

Caught in the Crossfire: Is the ENS an Innocent Victim or Active Perpetrator in Intestinal Ischemia/Reperfusion Injury?

2.1 Abstract

Intestinal ischemia/reperfusion (I/R) injury is a notoriously devastating condition complicated by a multifactorial pathophysiology and limited therapeutic interventions resulting in high rates of morbidity and mortality. If not repaired in time, intestinal I/R injury leads to fatal outcomes including systemic inflammatory response syndrome and multiple organ dysfunction syndrome due to loss of intestinal epithelial barrier integrity. As the first line of defense against injurious luminal contents, proper functioning of this barrier is essential for maintaining gut homeostasis. The enteric nervous system is closely involved in intestinal barrier function but the relationship between these two during intestinal I/R injury has not been fully elucidated. Evidence suggests that the enteric nervous system is both a victim and active player in intestinal I/R injury and as such, may be a promising therapeutic target. This review provides an overview of I/R pathophysiology and examines the relationship between the intestinal barrier and the enteric nervous system, specifically in the context of intestinal I/R injury concluding with findings from our work in a translational pig model.

2.2 Introduction

Proper blood perfusion of tissues is essential in maintaining a healthy cellular environment as this accomplishes both nutrient and oxygen delivery as well as waste removal. When ischemic conditions arise, blood flow and oxygenation of tissues drop to levels insufficient

to support cell viability. Causes of ischemia include severe systemic vascular blockages as in myocardial infarction, peripheral blockages such as intestinal infarction or volvulus, iatrogenic blockages during surgical interventions, or more complex disease states such as neonatal necrotizing enterocolitis in which ischemia appears to develop secondary to mucosal inflammation.[1] Though reperfusion of the affected tissue is necessary to ameliorate ischemia, restoration of blood flow paradoxically induces further injury due to inflammation, immune system activation, and oxidative stress referred to collectively as ischemia/reperfusion (I/R) injury.[2-6]

While I/R injury can result from a variety of pathologies, they consistently represent serious clinical burdens with high morbidity and mortality rates. All organs are susceptible to varying extents, but those of particular concern for ischemic injury include the brain, heart, kidneys, and—perhaps less intuitively—the gut. Indeed, the intestines are argued to be the most sensitive organ to I/R injury, exemplified by the high mortality rate of 50-80% for acute mesenteric ischemia that has not significantly improved in recent decades.[7-11] Such high mortality rates underscore the role played by intestinal I/R injury in the initiation of life-threatening disorders such as systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) due to intestinal barrier disruption and bacterial translocation.[3,12-15] Even after years of research, our understanding of the pathophysiology underlying intestinal I/R injury is not yet comprehensive. In many instances, clinical prognoses for intestinal diseases involving I/R remain guarded due to a lack of early diagnostic markers and therapeutic treatment options.

One major reason why intestinal I/R injury results in such devastating outcomes is the damage it causes to the intestinal epithelial barrier (IEB). The IEB serves as the primary line of

defense separating the intestinal lumen from underlying circulation and immune components. Breakdown of this barrier leads to unregulated translocation of luminal content including microbes and their products, antigens, and toxins. Intestinal barrier integrity is mediated by the local environment including the enteric nervous system (ENS). While some studies have examined the effects of intestinal I/R on the ENS following injury, few have investigated its role prior to and during intestinal I/R injury. In this review, we will summarize the pathophysiology of intestinal I/R injury and highlight the potential role of the ENS including recent evidence from our laboratory using a porcine model.

2.3 Pathophysiology of I/R injury

For context, we will provide a brief description of the mechanisms underlying I/R injury but direct interested readers to more thorough reviews.[16,17] During ischemia, decreased blood flow results in oxygen deprivation to the affected tissue. Without oxygen to maintain cellular respiration, cells deplete their ATP supplies and begin to rely on anaerobic metabolism. Consequently, cellular lactic acid accumulates, reducing the cytosolic pH. Reduced ATP leads to a cascade of detrimental effects including limited ability to remove calcium from the cell and an inability to maintain Na+/K+ ATPase activity resulting in cell swelling due to an influx of sodium, calcium, and water. [18] Additionally, breakdown of ATP leads to an accumulation of hypoxanthine as a byproduct which is normally metabolized by xanthine dehydrogenase. Under ischemic conditions, xanthine dehydrogenase is converted into xanthine oxidase due to calcium influx and proteolysis.[17,19] During reperfusion, the sudden reintroduction of oxygen is utilized by xanthine oxidase leading to the production of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide.[17,20-22] ROS activate inflammatory pathways and cause oxidative damage

to cells by reacting with proteins, DNA, and lipids. In addition to this direct damage, ROS act as chemoattractants resulting in neutrophil infiltration that further injures the reperfused tissue.[17,23]

2.4 Intestinal Barrier and the Enteric Nervous System

Intestinal I/R injury occurs from conditions that decrease blood flow without occlusion such as shock and cardio-pulmonary diseases or conditions that cause occlusion such as thrombi, emboli, and volvulus as well as more complex states induced during surgery, intestinal transplantation, trauma, and in neonatal necrotizing enterocolitis due to inflammation.[17] This ultimately causes deleterious effects by disrupting the integrity of the IEB. The IEB is a semi-permeable structure comprised from a monolayer of polarized columnar-shaped epithelial cells that line the mucosa of the small intestine and colon serving to regulate interactions between the internal environment of the body and external environment of the lumen. Adjacent intestinal epithelial cells are tightly adhered to one another by three main junctional complexes including tight junctions, adherens junctions, and desmosomes. Each of these complexes utilize interactions between transmembrane proteins and their intracellular counterparts to establish a connection to the cytoskeleton and provide structural support. This creates a physical barrier responsible for regulating permeability through two main routes. The transcellular route allows substances to move through cells using specific membrane transporters while the paracellular route allows substances to travel between neighboring intestinal epithelial cells dictated primarily by intercellular tight junctions.[24] Normally, the IEB is relatively impermeable and responsible for uptake of essential nutrients, solutes, and water while excluding noxious luminal contents such as pathogens and their products. Damage to barrier integrity leads to increases in permeability that are characteristic of several intestinal diseases and can contribute to pathologies

beyond the gut. Various factors can result in IEB damage including ischemia. Ischemia injures the IEB by decreasing blood flow and therefore oxygen delivery to the intestine. Cells at the villus tips in the small intestine and inter-crypt surface in the colon exist in a state of physiologic hypoxia due to countercurrent circulation and are more susceptible to further decreases in oxygen.[17,25-27] During ischemic events, these cells are the first to detach from their underlying basement membrane followed by progressive cell loss toward the crypt base with increasing durations of ischemia.[17,28,29] Sloughing of these epithelial cells disrupts their continuity creating defects that allow microbes and their products to cross the IEB. Thus, regardless of the cause, acute repair of the IEB is paramount to survival and has been characterized into three distinct yet overlapping steps: 1.) contraction of the lamina propria to reduce wound surface area, 2.) migration of epithelial cells across the basement membrane to cover the denuded wound, and 3.) reassembly of tight junctions to restore proper permeability.[30,31]

IEB function is modulated by its surrounding microenvironment including the ENS. The ENS is comprised of neurons and enteric glial cells (EGC) that innervate the gastrointestinal tract to regulate nearly every aspect of intestinal physiology including motility, secretion, and barrier function. In mammals, the ENS is organized into two major ganglionated plexi. The submucosal plexus (SMP) is localized to the submucosa and primarily regulates mucosal functions, and the larger myenteric plexus (LMMP) is found between the circular and longitudinal muscle layers and primarily controls motility. While the cell bodies of enteric neurons are confined to the ganglia of these plexi, EGC extend to densely innervate every layer of the gut wall. EGC play a crucial role in the regulation of IEB functions including absorption, secretion, and barrier function primarily through the release of paracrine signaling molecules. These mediators influence the IEB by regulating epithelial cell proliferation, reducing barrier permeability,

promoting tight junction integrity, and enhancing wound healing.[6,32-38] In addition to these effects, EGC also modulate inflammatory responses that regulate gut health and the IEB.[39,40] Of relevance, the EGC-derived mediator GDNF increases under hypoxic conditions and is capable of attenuating hypoxia-induced alterations in IEB function.[41] However, the same study showed that inducible nitric oxide synthase (iNOS) derived from EGC also increases under hypoxic conditions and has a negative effect on barrier function highlighting the dual effects the ENS can have on intestinal barrier function.

2.5 Intestinal I/R Injury and the Enteric Nervous System

2.5.1 The Effects of Intestinal I/R Injury on the Enteric Nervous System

Intestinal I/R injury induces profound effects on the ENS indicated by both anatomical and physiological changes. Following I/R, several studies have reported degenerative changes in neuronal histology including cell swelling, increased apoptosis, nuclear abnormalities, and altered expression of canonical markers.[42-47] While some of these changes were evident within hours of injury, others manifested and persisted weeks to months later indicating both the acute and long-term damage of intestinal I/R injury. Additionally, though most of these studies focused on the myenteric plexus, others have noticed similar morphological alterations to submucosal neurons as well.[48,49] To better understand these effects, a team lead by Furness was interested in whether some neuronal subtypes were more affected by I/R injury and found that nitric oxide synthase (NOS) inhibitory motor neurons and interneurons appeared to be selectively damaged at least compared to calretinin-expressing neurons.[43] They elaborated on this study to investigate the mechanism behind the neuronal death and implicated cytoplasmic loss of the RNA binding protein Hu and aggregated nitrosylated proteins in NOS-expressing neurons likely due to

reactions of nitric oxide and ROS during early reperfusion as contributing factors.[50] However, knocking out NOS in these neurons to reduce nitric oxide worsened outcomes from intestinal I/R injury suggesting a complicated role of these neurons having both protective and detrimental effects.[51] The Castelucci group has also contributed a number of studies regarding the effects of ischemia on specific receptor-expressing neuronal subpopulations in the ileum finding significant decreases in neurons expressing calbindin and ChAT (choline acetyl transferase) in the submucosal plexus and calretinin, calbindin, NOS, and ChAT in the myenteric plexus with calbindin most severely impacted in both plexi.[49] This group also investigated receptors expressed by a variety of neuronal subpopulations and showed significant decreases in neurons expressing the ATP-activated $P2X_2$ and P2X7 receptors.[49] Both Furness and Castelucci also examined the effects of intestinal I/R injury on EGC finding evidence of glial distortion.[52,53] Specifically, Furness identified unusual staining patterns of GFAP in myenteric EGC that appeared to be associated with loss of Hu immunoreactivity in neighboring neurons suggesting alterations to EGC may precipitate neuronal damage following I/R injury.[52] Castelucci also showed an increase in glial cell area at longer time points (1-2 weeks) after injury and found that the density of S-100β-expressing glia in the myenteric plexus increased at 24 hours, 72 hours, and 1 week following injury.[53,54] Besides these morphological changes, intestinal I/R injury results in alterations to intestinal physiology and most notably results in decreased motility and contractility.[45,51,53,55-59] Given that intestinal motility is controlled in large part by the ENS, it is likely that the observed morphological changes to enteric neurons and glia contribute to this dysmotility.

2.5.2 The Effects of the ENS on Intestinal I/R Injury

While the majority of these studies reported the effects on the ENS following injury, several studies have also investigated modulation of the ENS to improve outcomes following intestinal I/R injury. Pretreatment with a NOS inhibitor or NMDA receptor inhibitor both prevented intestinal I/R-induced delays in intestinal motility and altered myenteric neuron expression including increased vasoactive intestinal peptide (VIP) immunoreactivity.[55] Ketamine, an NMDA receptor antagonist, also protects against intestinal I/R injury and seems to require an intact ENS to do so suggesting involvement of enteric glutamate in potentiating deleterious effects of intestinal I/R injury.[60,61] Castelucci also showed that blockage of the myenteric neuronal P2X7 receptor attenuated morphological alterations in neurons and decreased contractility caused intestinal I/R injury.[62] Similar results were obtained by inhibiting the pannexin-1 channel which has an established role transporting ATP as a cell-cell signaling molecule between enteric neurons and glia.[62,63] Another study investigated the effects of inhibiting both pannexin-1 and the P2X7 receptor immediately following intestinal I/R injury. Inhibition of either pannexin-1 and the P2X7 receptor prevented neuronal loss, restored contractility, and decreased the number of EGC back to normal levels similar to the findings of others. However, when looking at specific subsets of EGC, it was found that the pannexin-1 inhibition group did not exhibit recovery of S-100β-expressing glia to the same extent of GFAP-expressing glia. Because functional metrics were still restored in the pannexin-1 inhibition group, these findings indicate that GFAP-expressing glia specifically may play a larger role in intestinal I/R injury.[64] Dexmedetomidine, a selective α2 adrenergic agonist, is also effective in alleviating intestinal I/R injury and a recent study proposed that this is due in part to its ability to prevent mitochondrial damage and apoptosis of EGC.[65-67] Lastly, a number of studies have shown that

vagal nerve stimulation immediately following intestinal I/R-injury is protective and appears to exert these effects by activating EGC.[68-71] Based on this evidence, the ENS appears to be a promising target for treating intestinal I/R injury and may provide alternate therapeutic options to limit the intestinal barrier breakdown that precipitates outcomes such as SIRS.

2.6 Perspectives from a Pig Model of Intestinal I/R Injury

While the majority of studies have focused on intestinal I/R injury in rodent models, our laboratory has focused on surgically induced ischemia in a porcine model. Pigs are more anatomically and physiologically similar to humans including their villus structure, possession of sacculations and tenia in the colon resulting in similar transit times, and colonic fermentation of an omnivorous diet.[72-74] The gut microbiota composition is similar between pigs and humans and their microbiomes share a 96% identity in functional pathways.[75] Single cell RNA sequencing studies have further highlighted transcriptomic similarities between humans and pigs.[76-79] One of these recent reports revealed highly conserved regional programs between the human and porcine ENS including response to vagal nerve stimulation.[79] These similarities are also particularly relevant for intestinal I/R injury studies as pigs and human express relatively low levels of xanthine oxidase which has been identified as a major contributor of reperfusion injury in rodent and feline studies suggesting that reperfusion injury may not play as important of a role in humans and pigs.[80,81]

From our research, we have discovered an age-dependent difference in acute IEB repair in which suckling neonatal pigs (2-weeks-old) failed to restore barrier function compared to their weaned juvenile (6-8-weeks-old) counterparts following ischemic injury.[31] Importantly, IEB repair in neonates could be rescued by application of a homogenized mucosal homogenate from

ischemia-injured juvenile intestine during *ex vivo* recovery on Ussing chambers implicating an age-dependent microenvironmental factor driving these acute repair mechanisms. This led us to investigate the effect of the ENS and EGC specifically as one of the possible microenvironmental contributors to age-dependent repair following intestinal I/R. We found that not only is the network of GFAP-positive EGC underdeveloped in neonatal pigs, but also using fluoroacetate, which EGC are preferentially sensitive to, as an inhibitor recreated the repair defect in juvenile pigs. This is particularly interesting considering the mucosal population of EGC develops postnatally driven by gut microbe colonization in rodents.[82] However, this was not shown to be the case in humans and has not been investigated in the pig highlighting an area for further research.[83] Altogether, our results thus far indicate that an intact network of EGC is required for acute barrier restoration following intestinal I/R injury in the pig and future studies hope to undercover key mechanisms driving this repair process.

2.7 Conclusions

Overall, intestinal I/R injury represents a significant clinical burden with persistently high rates of mortality. Though the pathophysiology underlying intestinal I/R injury is fairly well understood, new treatment options for these patients are still lacking. One explanation for these high rates of mortality is the destructive effect of intestinal I/R injury on the IEB which can lead to translocation of toxic luminal content precipitating overwhelming inflammatory reactions as in SIRS and MODS. Additionally, another cause of these devastating outcomes is the impact on the ENS which suffers acute and long-term impacts to both morphology and function following intestinal I/R injury. In fact, the ENS is not just a victim of intestinal I/R injury but also plays an active role. Several studies found that therapeutic interventions targeting the ENS resulted in

protection from intestinal I/R injury. Despite these findings, the exact mechanisms remain unclear and the effects are likely specific to certain subsets of enteric neurons and glia. As such, studies should consider differences between these subsets in future investigations to potentially uncover which are most relevant in the context of intestinal I/R injury. Lastly, considering that the majority of studies regarding the ENS and intestinal I/R injury have been performed in rodents, future research should investigate this relationship in a more clinically relevant and translational model such as the pig.

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Chapter 3

Protocol to Culture Enteric Glial Cells from the Submucosal and Myenteric Plexi of Neonatal and Juvenile Pig Colons

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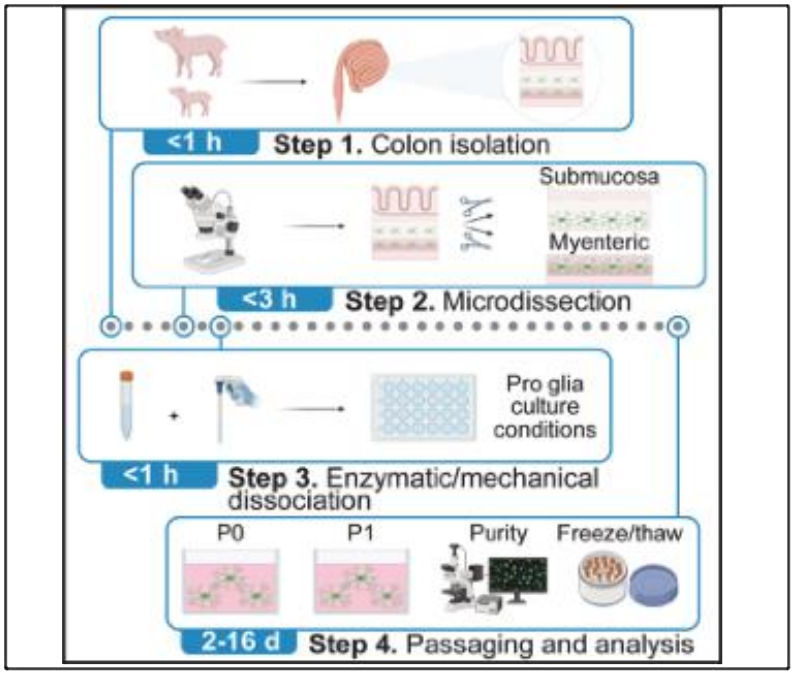
Protocol to culture enteric glial cells from the submucosal and myenteric plexi of neonatal and juvenile pig colons



Here, we present our protocol to culture enteric glial cells from the submucosal and myenteric plexus of neonatal and juvenile pig colons. We describe steps for colon isolation, microdissection, and enzymatic and mechanical dissociation. We include procedures for passaging and analyzing cell yield, freeze/thaw efficiency, and purity. This protocol allows for the generation of primary cultures of enteric glial cells from single-cell suspensions of microdissected layers of the colon wall and can be used to culture enteric glia from human colon specimens.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Enteric glial cell isolation from both the submucosal and myenteric plexi of pig colon

Establishment and maintenance of enteric glial cell primary cultures

Cell yield, passaging, freezing/thawing, and purity of primary cultures of enteric glia

Comparison of enteric glial cell primary cultures from neonatal and juvenile pigs

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Protocol

Protocol to culture enteric glial cells from the submucosal and myenteric plexi of neonatal and juvenile pig colons

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SUMMARY

Here, we present our protocol to culture enteric glial cells from the submucosal and myenteric plexus of neonatal and juvenile pig colons. We describe steps for colon isolation, microdissection, and enzymatic and mechanical dissociation. We include procedures for passaging and analyzing cell yield, freeze/thaw efficiency, and purity. This protocol allows for the generation of primary cultures of enteric glial cells from single-cell suspensions of microdissected layers of the colon wall and can be used to culture enteric glia from human colon specimens.
For complete details on the use and execution of this protocol, please refer to Ziegler et al.[1]

BEFORE YOU BEGIN

The protocol below describes the specific steps to isolate, grow, maintain, freeze, and thaw primary cultures of enteric glial cells from the colon of neonatal and juvenile pigs. Of note, we have used this protocol to establish primary cultures of enteric glial cells from the small intestine and colon of pigs at various ages as well as from human colon resections (data not shown). Our protocol differs from other published methods by combining: (1) microdissection of colonic layers to isolate both the submucosa and the longitudinal muscle/myenteric plexus, (2) single cell suspensions through enzymatic and mechanical dissociation and (3) the pig model. For other methods describing isolation and culture of enteric glial cells, please refer to previously published work in rodents[2–8] and humans.[9,10]

Institutional permissions

All live animal use for experiments should be approved in accordance with relevant institutional and national guidelines and regulations. If human samples are used, patient samples should be collected in accordance with the Institutional Review Board.

All procedures used in this work were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC).

Prepare reagents and materials

Timing: 2 h

1. At least one day prior to experiment.
   a. Prepare the dissecting buffer, dissociation medium, and plating medium as described in the "materials and equipment" section.
   b. Prepare bovine serum albumin (BSA), collagenase IA, and protease I for enzymatic dissociation.
      i. BSA is prepared at 50 mg/mL in sterile 1X PBS and stored in 400 µL aliquots at −20°C.
      ii. Collagenase IA is prepared at 5 mg/mL in sterile 1X PBS and stored in 250 µL aliquots at −20°C.
      iii. Protease I is prepared at 20 mg/mL in sterile 1X PBS and stored in 250 µL aliquots at −20°C.
   c. Prepare poly-L-lysine at 1.0 mg/mL in 2X Pierce borate buffer diluted in sterile distilled water. Store aliquots at −20°C.

Note: We suggest storing poly-L-lysine in 1 mL aliquots at −20°C. However, we recommend adjusting the volume of the aliquot to the needs of the specific experiment to prevent excessive freeze/thaw cycles.

   d. Prepare Sylgard as per manufacturer instructions and coat PYREX dissection dish.
   e. Sterilize Sylgard-coated dissection dishes in an autoclave.

⚠ CRITICAL: BSA, collagenase IA, protease I, and poly-L-lysine should be sterile filtered through a 0.2 µm filter in a biosafety cabinet before aliquoting.

Coat culture plates

Timing: 3 h

⚠ CRITICAL: Coating plates should be performed in a biosafety cabinet.

2. At least one day prior to experiments.
   a. Thaw poly-L-lysine aliquot on ice.
   b. Coat plates with poly-L-lysine in a biosafety cabinet.
      i. Add one volume of sterile distilled water to 1.0 mg/mL poly-L-lysine aliquot for a final concentration of 0.5 mg/mL.
      ii. Coat plates such that the bottom of the well is covered or following the suggested volumes in Table 1.
      iii. Allow poly-L-lysine to coat plates for at least 12 h at 20°C–22°C.

Note: For faster coating, plates can be coated for 2 h at 37°C.

   c. Rinse plates in a biosafety cabinet.
      i. Remove poly-L-lysine with sterile glass pipet and vacuum suction.

Table 1. Poly-L-lysine coating volumes based on plate size (Before you begin, Step 2bii)

Plate size	Volume of poly-L-lysine (µL)
96-well	100
24-well	300
6-well	1000

ii. Rinse the coated wells three times with sterile distilled water at the same or higher volume used for coating with poly-L-lysine.
iii. If not plating immediately, the third rinse should remain in the wells and the plates can be stored in an incubator at 37°C until needed.
iv. Remove final rinse prior to plating.

Note: Coated plates can be prepared ahead of time and stored with their final sterile distilled water rinse in an incubator at 37°C for up to 7 days.

3. On the day of the experiment.
   a. Thaw 2 aliquots each of BSA, collagenase IA, and protease I per animal on ice.
   b. Thaw N-2, G-5, and Gem21 growth supplements on ice.

Note: N-2 and Gem21 provide nutrients for neural cell growth and G-5 supports glial cell specific growth.

   i. The growth supplements can be prepared as a cocktail and added to medium with a final concentration of 1X each.
   c. Pre-warm plating medium at 37°C in a water bath.
   d. Set heated oven with rotating capabilities to 37°C.
   e. Set swinging-bucket centrifuge to 15°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Chicken anti-GFAP (1/1,000)	Abcam	Cat # ab4674
Rabbit anti-S100β (ready to use)	Dako	Cat # IR504
Mouse anti-Sox10 (1/100)	Santa Cruz	Cat # sc-365692
Mouse anti-alpha smooth muscle actin (1/500)	Abcam	Cat # ab7817
Chicken anti-PGP9.5 (1/300)	Abcam	Cat # ab72910
Alexa Fluor 488 donkey anti-mouse (1/500)	Thermo Fisher Scientific	Cat # A-21202
Alexa Fluor 568 goat anti-chicken (1/500)	Thermo Fisher Scientific	Cat # A-11041
Alexa Fluor 647 goat anti-rabbit (1/500)	Thermo Fisher Scientific	Cat # A-21245
Chemicals, peptides, and recombinant proteins		
Amphotericin B	Sigma-Aldrich	Cat # A9528
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat # A3311
Hank's balanced salt solution (HBSS)	Gibco	Cat # 14175-095
DMEM/F-12 50/50	Corning	Cat # 10-090-CV
Sodium bicarbonate	Sigma-Aldrich	Cat # S5761
L-glutamine	GeminiBio	Cat # 400-106
Fetal bovine serum (FBS)	GeminiBio	Cat # 100-106
Penicillin-Streptomycin	GeminiBio	Cat # 400-109
Gentamicin	Sigma-Aldrich	Cat # G1272
Collagenase type IA	Sigma-Aldrich	Cat # C9891
Protease type I	Sigma-Aldrich	Cat # P4630
N-2 NeuroPlex supplement	GeminiBio	Cat # 400-163
Gem21 NeuroPlex supplement	GeminiBio	Cat # 400-161
G-5 supplement	Thermo Fisher Scientific	Cat # 17503012
Poly-L-lysine hydrobromide	Sigma-Aldrich	Cat # P2636
Pierce borate buffer	Thermo Fisher Scientific	Cat # 28341
Trypsin-EDTA	GeminiBio	Cat # 400-151
DPBS	Corning	Cat # 21-031-CV
Cell culture grade water	Corning	Cat # 25-055-CM
DAPI (4',6-diamidino-2-phenylindole)	Invitrogen	Cat # D3571

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Trypan blue	Gibco	Cat # 15250061
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat # D1435
Paraformaldehyde (PFA)	Sigma-Aldrich	Cat # P6148
PBS 1X (without calcium or magnesium)	Corning	Cat # 21-040-CM
Horse serum	Gibco	Cat # 16050130
Sodium azide	Sigma-Aldrich	Cat # S2002
Triton X-100	Sigma-Aldrich	Cat # X100
Sylgard 184	Electron Microscopy Sciences	Cat # 24236-10
Software and algorithms		
Prism	Prism	http://www.graphpad.com/
ImageJ	Schneider et al.[11]	https://imagej.nih.gov/ij/download.html
Other		
Microdissection scissors, VANNAS-extra special, micro fine, 3', curved	Biomedical Research Instruments, Inc.	Cat # 11-1005
Sharp scissors, fine	Fine Science Tools	Cat # 14060-09
Curved 14 cm Mayo scissors	World Precision Instruments	Cat # 501750
Microdissection curved #7 forceps	World Precision Instruments	Cat # 504505
Microdissection straight forceps	World Precision Instruments	Cat # 504507
Dissecting microscope	Zeiss Stemi 305 stereo zoom microscope 0.8x – 4.0x K EDU	SKU # 435063-9010-100
0.20 mm Minutiens pins	Fine Science Tools	SKU # 26002-20
100 µm cell strainer	Thermo Fisher Scientific	Cat # 22-363-549
70 µm cell strainer	Thermo Fisher Scientific	Cat # 22-363-548
40 µm cell strainer	Thermo Fisher Scientific	Cat # 22-363-547
15 mL polypropylene conical tube	Thermo Fisher Scientific	Cat # 339651
50 mL polypropylene conical tube	Thermo Fisher Scientific	Cat # 339652
Hemocytometer	Hausser Scientific	Cat # 3200
2 mL cryogenic vials	Corning	Cat # 430488
Tissue culture-treated non-pyrogenic polystyrene 24-well plates	Corning	Cat # 3524
Tissue culture treated non-pyrogenic polystyrene 96-well plates	Corning	Cat # 3596
Tissue culture treated non-pyrogenic polystyrene 6-well plates	Corning	Cat # 3516
PYREX 150 × 20 mm Petri dish with cover	Corning	Cat # 3160-152
0.2 µm PES Nalgene syringe filter	Thermo Fisher Scientific	Cat # 725-2520
60 mL sterile plastic Luer lock syringe	Thermo Fisher Scientific	Cat # 14955461
Hybridization incubator	Thermo Fisher Scientific	Model # FBH10

MATERIALS AND EQUIPMENT

Dissecting buffer		
Reagent	Final concentration	Amount
Hank's Balanced Salt Solution (HBSS)	N/A	500 mL
Penicillin-Streptomycin (100X)	1.5%	7.5 mL
Gentamicin (10 mg/mL)	0.06 mg/mL	3 mL
Amphotericin B (4.4 mg/mL)	0.003 mg/mL	375 µL
Total	**N/A**	**510.875 mL**

Note: Extra buffer can be stored at 4°C for up to a month.

Dissociation medium		
Reagent	Final concentration	Amount
DMEM/F-12 50/50	N/A	47.1 mL
Sodium bicarbonate (4.5%)	0.12%	1.32 mL

(Continued on next page)

Continued		
Reagent	Final concentration	Amount
Penicillin-Streptomycin (100X)	1.5%	750 µL
L-glutamine (200 mM)	2 mM	500 µL
Gentamicin (10 mg/mL)	0.06 mg/mL	300 µL
Amphotericin B (4.4 mg/mL)	0.003 mg/mL	37.5 µL
Total	**N/A**	**50 mL**

Note: Amphotericin B should be resuspended in culture grade water.

Note: Medium can be stored at 4°C for up to one week.

Plating medium		
Reagent	Final concentration	Amount
DMEM/F-12 50/50	N/A	42.1 mL
Fetal bovine serum (FBS)	10%	5 mL
Sodium bicarbonate (4.5%)	0.12%	1.32 mL
Penicillin-Streptomycin (100X)	1.5%	750 µL
L-glutamine (200 mM)	2 mM	500 µL
Gentamicin (10 mg/mL)	0.06 mg/mL	300 µL
Amphotericin B (4.4 mg/mL)	0.003 mg/mL	37.5 µL
Total	**N/A**	**50 mL**

Note: Medium can be stored at 4°C for up to one week.

Note: It may be beneficial to prepare one aliquot of plating medium for steps performed at the bench and a separate sterile aliquot used only in a biosafety cabinet for plating.

Maintenance medium		
Reagent	Final concentration	Amount
DMEM/F-12 50/50	N/A	48.9 mL
Fetal bovine serum (FBS)	0.5%	250 µL
Penicillin-Streptomycin (100X)	0.5%	250 µL
L-glutamine (200 mM)	2 mM	500 µL
Gentamicin (10 mg/mL)	0.02 mg/mL	100 µL
Amphotericin B (4.4 mg/mL)	0.001 mg/mL	12.5 µL
Total	**N/A**	**50 mL**

Note: Medium can be stored at 4°C for up to one week.

Passaging medium		
Reagent	Final concentration	Amount
DMEM/F-12 50/50	N/A	44.1 mL
Fetal bovine serum (FBS)	10%	5 mL
Penicillin-Streptomycin (100X)	0.5%	250 µL
L-glutamine (200 mM)	2 mM	500 µL
Gentamicin (10 mg/mL)	0.02 mg/mL	100 µL
Amphotericin B (4.4 mg/mL)	0.001 mg/mL	12.5 µL
Total	**N/A**	**50 mL**

Note: Medium can be stored at 4°C for up to one week.

Blocking solution		
Reagent	Final concentration	Amount
0.1% PBS azide	95%	950 µL
Horse serum	5%	5 µL
Total	N/A	1 mL

Note: Blocking solution can be stored at 4°C for up to one month.

Permeabilization solution		
Reagent	Final concentration	Amount
0.1% PBS azide	90%	900 µL
Horse serum	5%	50 µL
10% Triton X-100	5%	50 µL
Total	N/A	1 mL

Note: Permeabilization solution can be stored at 4°C for up to one month.

Note: A 10% Triton X-100 is prepared by diluting 100 µL of Triton X-100 into 900 µL of distilled water and mixing well. 10% Triton X-100 is stable at 20°C–22°C.

⚠ CRITICAL: All non-sterile solutions added to sterile medium should be sterile filtered through a 0.2 µm filter and syringe in a biosafety cabinet.

⚠ CRITICAL: Medium should be prepared sterilely in a biosafety cabinet.

Alternatives: Here we provide a list of alternative reagents and equipment.

- Cell freezing container alternative.
  - Any cell freezing container can be used such as Cornell CoolCell LX Cell Freezing Container (Sigma Aldrich CLS432001).
- Dissecting microscope alternative.
  - Any dissecting microscope can be used such as National Optical Dual Magnification (1X/3X) Stereo Microscope (Thermo Fisher Scientific 430TBL).
- Growth supplement alternative.
  - Gem21 can be substituted one for one with B-27 (Gibco 17504044).
  - N-2 can be substituted one for one with N-2 Supplement (Gibco 17502048).
- Hemocytometer.
  - An automated cell counter can be used but is not recommended for initial plating due to heterogeneity of cell sizes and shapes in cell suspension.
- Horse serum.
  - For immunofluorescence protocols, serum from other species can be substituted one for one including donkey (Sigma-Aldrich D9663) and goat (G9023).
- Inverted fluorescence microscope alternative.
  - Any inverted fluorescence microscope with 10X and 20X objectives equipped with filters compatible with the panel of secondary antibodies can be used.
- L-glutamine alternative.
  - L-glutamine can be substituted one for one with GlutaMAX Supplement (Gibco 35050061).
- Media alternative.

- DMEM/F-12 50/50 supplemented with exogenous L-glutamine can be substituted one for one with DMEM/F-12 GlutaMAX Supplement (Gibco 10565018) without exogenous L-glutamine supplementation.
- Hybridization Incubator.
  - Any oven with rotating capabilities can be used such as Roto-Therm Incubated Tube Rotators (Thomas Scientific 1159P17).
- Primary antibodies.
  - Primary antibodies from other sources and hosts can be used. For this experiment, we used a panel of primary antibodies from 3 different host species to be able to label all 3 general enteric glial cell markers in the same well.
- Secondary antibodies.
  - Secondary antibodies from other sources and hosts can be used. For this experiment, we used a panel of secondary antibodies conjugated with 3 different fluorophores (wavelengths chosen according to the filters of fluorescence microscope) to be able to label all 3 general enteric glial cell markers in the same well.

STEP-BY-STEP METHOD DETAILS

Part 1: Isolation, plating, and maintenance of primary cultures of porcine colonic enteric glial cells

Timing: 5 h

In this section, we describe steps for establishing enteric glial cell primary cultures from pigs.

Note: This method allows for the establishment of primary cultures of enteric glial cells from both the submucosa and longitudinal muscle/myenteric plexus of spiral colons from pigs using dissection, enzymatic digestion, and mechanical dissociation. There are currently no known labeling-based methods to discriminate enteric glial cells from the submucosal and myenteric plexi due to shared cell marker expression. Therefore, to establish cultures from both plexi, microdissection of the colonic wall layers is necessary as it allows for the isolation of (1) the submucosa which encloses the submucosal plexus and (2) the longitudinal muscle which inextricably supports the myenteric plexus. Of note, based on H&E staining of dissected layers, the submucosa specimens are likely to also contain some of the muscularis mucosa and lamina propria of the mucosa. As a result of the enzymatic digestion and mechanical dissociation of whole layers (submucosa and longitudinal muscle/myenteric plexus (LMMP)), cells other than enteric glial cells will also initially be present in the cell suspension, but culture conditions were designed to selectively favor enteric glial cell growth using poly-L-lysine as a preferred substrate, the glial cell-specific growth supplement cocktail G-5, and 0.5% heat-inactivated FBS. Under these conditions, >90% of the cells present in the culture are immunoreactive for the enteric glial cell markers GFAP and S-100β. For proof of principle, we tested our technique on two age groups of pigs—neonates 2 weeks old and juveniles 6–8 weeks old—since there are notable gross differences in colon tissue thickness between the two age groups. In our hands, neonate cultures in general grew faster and to a greater amount. However, purity, measured as the percentage of cells immunoreactive for enteric glial cell markers GFAP and S-100β, did not significantly differ between the two age groups. By providing data on two age groups, we hope to provide a reference to others utilizing our technique or adapting it for use in human studies.

1. Euthanize pigs using an appropriate ethically approved method.

Note: In this study, neonates were anesthetized with isoflurane vaporized in 100% oxygen via mask inhalation and euthanized by exsanguination. Juveniles were sedated with 3 mg/kg

xylazine injected intramuscularly and euthanized via penetrating captive bolt followed by exsanguination.

2. Make a midline incision into the abdominal cavity using a #10 scalpel blade.
3. Remove a section of the spiral colon, approximately 3–5 cm in length, using Mayo scissors.

Note: For consistency in location, the central flexure can be used as a landmark.

4. Open the colon longitudinally using Mayo scissors and rinse thoroughly under cold tap water to remove all lumen contents.

Note: If transporting the colon to a secondary location for dissection, place it in cold dissecting buffer on ice in a 50 mL conical tube.

5. Place the colon in a Sylgard-coated dissection dish filled with ice cold dissection buffer.

Note: Replace the dissection buffer with fresh cold dissection buffer as needed throughout the dissection.

6. Using a dissecting microscope, orient the colon serosa up in the dissection dish.
7. Trim away fat and mesentery using sharp scissors.
8. Reorient the specimen mucosa up and pin it with minutien pins.
9. Use pins and curved microdissection forceps to stretch the spiral colon so that it is taut but not tearing.

Note: Several iterations of stretching may be needed to reach optimal tissue tension. Tissue should be re-stretched as needed throughout the dissection.

10. Trim the stretched colon to a 3 cm × 3 cm square using sharp scissors (Figure 1).
11. Remove the mucosa using curved microdissection forceps (Methods video S1).
12. After the entire mucosa has been removed, carefully separate the submucosa from the underlying circular muscle at the edge of the tissue.
13. Continue dissecting out the submucosa using microdissection scissors to cut any potential perpendicular structures connecting the submucosa to the circular muscle (Methods video S2).

Note: Remove pins as they are encountered and re-pin the exposed muscle layers to maintain tension.

14. When the submucosa has been entirely removed, pin it aside or in another sterile Sylgard-coated plate in ice-cold dissecting buffer.

Note: The submucosal tissue comprises the submucosal plexus.

15. Strip circular muscle fibers individually from one end of the tissue to the other using straight forceps (Methods video S3).

Note: The tissue remaining after stripping the circular muscle fibers mainly comprises the longitudinal muscle/myenteric plexus (LMMP).

16. Optional: a small ~3 mm × 3 mm piece of full thickness colon, mucosa, submucosa, and LMMP may each be fixed for hematoxylin and eosin histological staining to assess precision of dissection (Figure 2).

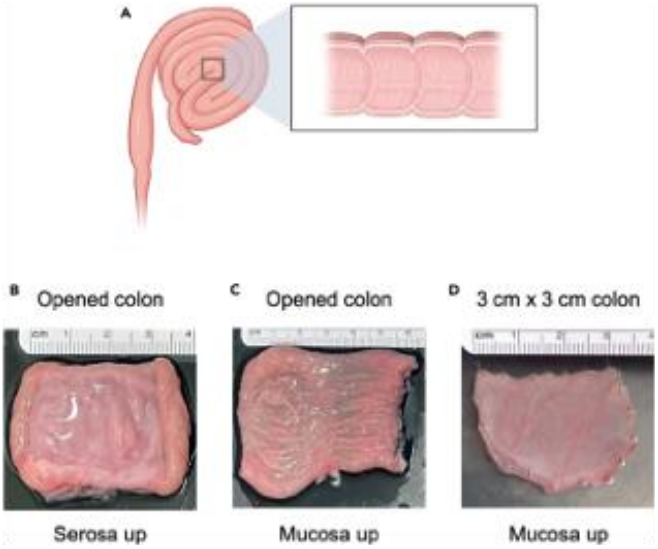

Figure 1. Schematic and gross images of pig spiral colon

Pictures demonstrate:

(A) obtaining a spiral colon specimen from the central flexure (related to Part 1, Step 3).

(B–D) (B) trimming fat and mesentery away from the serosal surface (related to Part 1, Step 7). (C) orienting the colon specimen mucosa up (related to Part 1, Step 8), and (D) cutting and stretching the colon specimen to a 3 cm × 3 cm square (related to Part 1, Step 10). Created with BioRender.com.

17. Using a dissecting microscope, use the straight forceps and microdissection scissors to cut the submucosa into 1 mm × 1 mm pieces and place these pieces in 5 mL of dissociation medium in a 15 mL conical tube on ice.
18. Repeat Step 17 with the LMMP.
19. Add one aliquot of BSA (20 mg), collagenase IA (1.25 mg), and protease I (5 mg) to each conical tube.
20. Incubate the tubes in an oven set at 37°C for 35 min while rotating.

Note: This time should be extended to 45 min for adult pigs and adult human specimens.

21. Add 10 mL of pre-warmed plating medium to each tube to stop enzymatic digestion.
22. Use a serological pipet to mechanically triturate the tissues by forcefully pipetting up and down 20 times.
23. Vortex for 30 s to further dissociate the tissues.
24. Spin at 300 × *g* at 15°C for 5 min using a swinging-bucket centrifuge.
25. Carefully remove and discard the supernatant.
26. Repeat steps 21–23.
27. Filter the mixture progressively through 100 μm, 70 μm, and 40 μm cell strainers to filter out debris and clumps.
28. Spin the filtered cell preparation at 300 × *g* at 15°C for 5 min using a swinging-bucket centrifuge.

⚠ CRITICAL: The remaining steps should be performed sterilely in a biosafety cabinet.

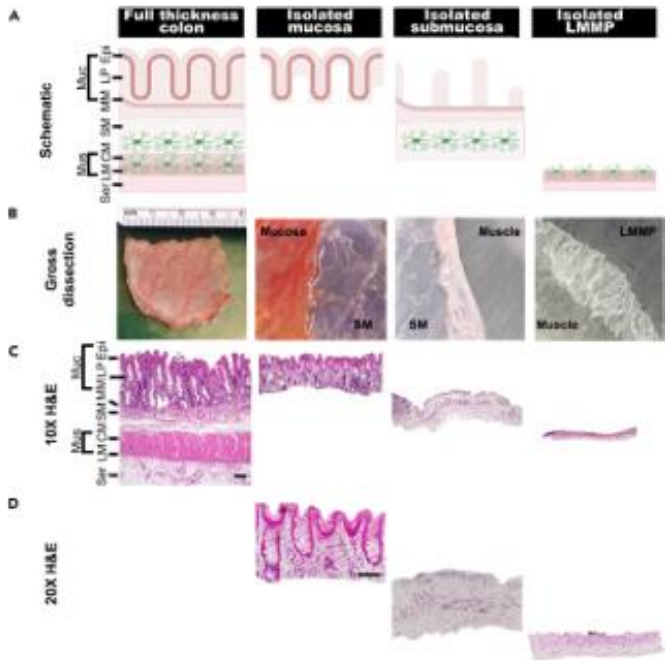

Figure 2. Microdissection of the different layers of the colon

Pictures illustrate the different steps mentioned in the protocol.

(A) Schematics illustrate the steps of isolation detailed in A: mucosa (Muc), epithelium (Epi), lamina propria (LP), muscularis mucosa (MM), submucosa (SM), circular (CM) and longitudinal (LM) layers of the muscularis (Mus), and serosa (Ser).

(B) Gross removal of the mucosa (related to Part 1, Step 11), isolation of the submucosa containing the submucosal plexus (SM) (related to Part 1, Step 12), and removal of the circular muscle to isolate the longitudinal muscle/myenteric plexus (LMMP) (related to Part 1, Step 15).

(C) H&E staining confirms precision of dissection at 10X magnification (related to Part 1, Step 16).

(D) H&E staining at 20X magnification (related to Part 1, Step 16).

Scale bar for H&E, 100 μm. Created with BioRender.com.

29. Use vacuum suction to carefully remove the supernatant.
30. Gently resuspend the pellet in ~1 mL of sterile plating medium.
31. Count the total number of live cells using trypan blue and a hemocytometer.

Note: Average cell yields at the time of plating for submucosa and LMMP cell preparations obtained from colons of neonatal and juvenile pigs are provided for reference (Table 2).

32. Plate cells on poly-L-lysine-coated plates in sterile plating medium with 1X growth supplement cocktail according to the parameters of Table 3.

Note: For this experiment, cells derived from the submucosa were plated at 400,000 cells/well in 4 poly-L-lysine coated wells of a 24-well plate and 40,000 cells/well in 8 poly-L-lysine coated wells of a 96-well plate. Cells derived from the LMMP were plated at 150,000 cells/well in 4 poly-L-lysine coated wells of a 24-well plate and 15,000 cells/well in 8 poly-L-lysine coated wells of a 96-well plate. All wells in the 24-well plate will be passaged for analysis of P1 cultures. All wells in 96-well plate will be fixed and used for P0 purity analysis. The experimental

Table 2. Average cell yield ± SEM in initial cell preparations from the submucosa and longitudinal muscle/myenteric plexus (LMMP) of 3 cm × 3 cm sections of porcine colons (related to Part 1, Step 31)

	Average cell yield (millions of cells)	
Colon wall layer	Neonate	Juvenile
Submucosa	0.9 ± 0.3[a]	2.1 ± 0.3[a,b]
LMMP	1.0 ± 0.2	0.5 ± 0.2[b]

Significant differences were identified between neonate and juvenile submucosa as well as between juvenile submucosa and juvenile LMMP yield by Two-way ANOVA Tukey's multiple comparisons test (n=4).
[a](p = 0.03)
[b](p = 0.005)

outcomes of all plates are described in Figure 3. Multiple plate sizes were utilized throughout this experiment to provide data and protocols for different plate and well sizes.

Note: A higher density of cells is plated for submucosal preparations compared to LMMP preparations to account for the respective fraction represented by enteric glia in the two preparations.

Note: Higher plating densities can be used to achieve faster growth.

33. Place the plated cells at 37°C with 5% CO_2.
34. On the following day replace the medium with fresh maintenance medium and 1X growth supplement cocktail (Figure 4).

Note: Perform the first medium change using a micropipette rather than vacuum to achieve gentler suction and prevent cell loss.

35. Replace medium with fresh maintenance medium and 1X growth supplement cocktail every other day until the desired level of confluence is reached (Figure 4).

Part 2: Analysis and passaging of primary cultures of porcine colonic enteric glial cells

Timing: 6 h

In this section, we describe steps for passaging, freezing and thawing, and evaluating purity of enteric glial cell primary cultures.

Note: To demonstrate stability of our cultures after passaging, we collected data on various parameters at passage 0 and passage 1. We first assessed the purity of our cultures using immunostaining assays targeting the enteric glial cell markers GFAP, S-100β, and SOX10 as well as PGP9.5 and α-SMA, respectively markers of neurons and myofibroblasts/smooth muscle cells, the two predominant potential contaminants (Part 2C). We also quantified the number of enteric glial cells obtained from each layer and each age group at the time of first and

Table 3. Suggested plating density for cell preparations of enteric glial cells from the submucosa and longitudinal muscle/myenteric plexus (LMMP) of porcine colons (related to Part 1, Step 32)

	96w plate	24w plate
Volume of plating medium (μL)	200	500
Submucosal cells/well	40,000	400,000
LMMP cells/well	15,000	150,000

These cultures will be referred to as passage 0 or P0.

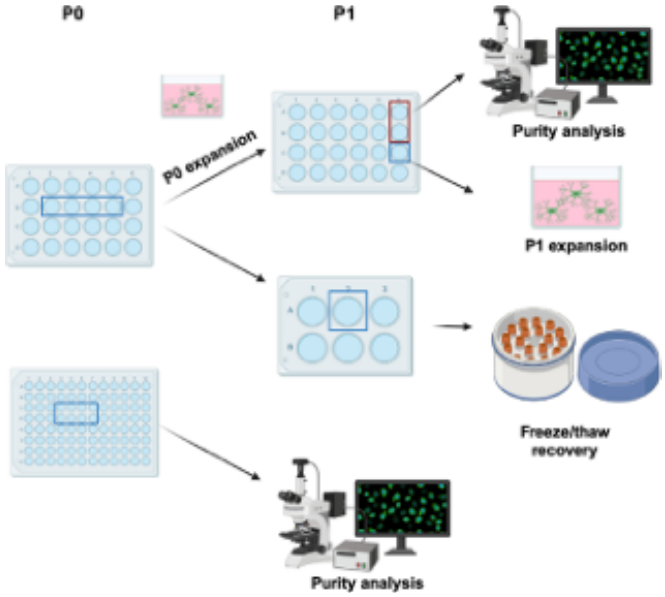

Figure 3. Schematic depicts experimental design
Created with BioRender.com.

second passages to demonstrate expansion of the glial cell network (Part 2A). We further report the average number of days between plating and passaging. To demonstrate that the cultures resist freezing and thawing cycles, we evaluated the effect of freezing on cell viability by comparing the number of live cells prior to and following freezing and thawing of cultures at passage 2 (Part 2B).

Part 2A: Passaging

36. Passage cells at 70–80% confluence but before large cell clusters have formed.

Note: The days from first to second passage can be determined by counting the days from initial plating until the first passage. Average number of days from initial plating to first passage of submucosa and LMMP cell cultures obtained from colons of neonatal and juvenile pigs in this experiment are provided for reference (Table 4).

Note: Neonate cultures generally grow faster and are ready for passaging sooner than juveniles.

⚠ CRITICAL: When enteric glial cells are extensively clustered they tend to lift off the plate and may be lost so we recommend adding and removing the warm PBS solution gently.

Note: The following passaging method uses volumes corresponding to the 24-well plates used in this experiment. Volumes should be changed accordingly for different well sizes.

37. Remove medium using a micropipette.

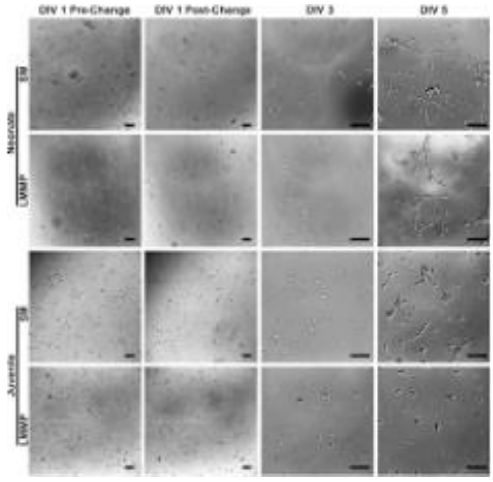

Figure 4. Establishment and expansion of primary cultures of enteric glial cells

Bright-field images of primary cultures of enteric glial cells isolated from neonatal and juvenile pig colon submucosa (SM) and longitudinal muscle/myenteric plexus (LMMP) on day in vitro 1 (DIV1) before and after medium change to illustrate changes in cellular density due to the removal of unadhered cells and debris, DIV3 to illustrate the beginning of the formation of enteric glial cell clusters, and DIV5 to illustrate the expansion of enteric glial cells and the formation of a glial network. Scale bar, 100 μm (related to Part 1, Steps 34–35).

38. Rinse each well with ~500 μL of pre-warmed sterile 1X PBS.
39. Remove the PBS using a micropipette.
40. Add 200 μL of pre-warmed trypsin-EDTA diluted 1:3 in sterile 1X PBS to each well.
41. Incubate the cells at 37°C checking every 2 min whether they have detached from the plate and if necessary, gently tapping the plate to encourage cells to detach.

⚠ CRITICAL: Thoroughly monitoring cells during trypsinization is important to prevent cell death from prolonged exposure to trypsin.

42. When the vast majority of the cells have detached, pipet up and down with a micropipette to break up any cell clusters as needed.
43. Add 600 μL of pre-warmed passaging medium to neutralize the trypsin.
44. Use a micropipette to transfer the cell solution from each well into a conical tube.

Note: A 15 mL conical tube can be used for 2–15 wells of 24 well plate(s).

Table 4. Average number of days ± SEM from initial plating until first passage of enteric glial cell primary cultures from the submucosa and longitudinal muscle/myenteric plexus (LMMP) of 3 cm × 3 cm sections of porcine colon (related to Part 2A, Step 36)

	Average numbers of days until first passage	
Colon wall layer	Neonate	Juvenile
Submucosa	5.0 ± 0.4	8.5 ± 1.6
LMMP	5.3 ± 0.3*	10 ± 1.2*

Significant differences were identified between neonate LMMP and juvenile LMMP by Two-way ANOVA Tukey's multiple comparisons test ($n = 4$).
*($p = 0.03$)

Table 5. Average cell yield ± SEM at first passage of enteric glial cell primary cultures from the submucosa and longitudinal muscle/myenteric plexus (LMMP) of 3 cm × 3 cm sections of porcine colon (related to Part 2A, Step 48)

	Average cell yield (x1,000 cells)	
Colon wall layer	Neonate	Juvenile
Submucosa	60 ± 8.5	44 ± 15
LMMP	60 ± 19	37 ± 10

No significant differences were identified between ages or plexi by Two-way ANOVA Tukey's multiple comparisons test ($n = 4$).

45. Spin the cells at 300 × *g* at 20°C–22°C for 5 min.
46. Use a vacuum to carefully remove the supernatant.
47. Gently resuspend the pellet in ~1 mL of pre-warmed passaging medium using a micropipette.
48. Count the total number of live cells using trypan blue and a hemocytometer.

Note: The cell yield can be determined by counting the live cells at passaging. Average cell yields from initial plating to first passage of submucosa and LMMP cell cultures obtained from colons of neonatal and juvenile pigs in this experiment are provided for reference (Table 5).

Note: The average fold change of cells from initial plating to first passage can be determined by dividing the number of live cells at the time of the first passage by the total number of cells initially plated. Average fold changes from initial plating to first passage of submucosa and LMMP cell cultures obtained from colons of neonatal and juvenile pigs in this experiment are provided for reference (Table 6). A decrease in cell numbers is expected as non-glial cells included in the initial plating do not adhere nor grow under the glial-specific culture conditions.

49. Plate cells on poly-L-lysine-coated plates in plating medium with 1X growth supplement cocktail according to the parameters of Table 7.

Note: For this experiment, primary cultures of enteric glial cells from the submucosa were plated at 20,000 cells/well in 3 poly-L-lysine coated wells of a 24-well plate and primary cultures of enteric glial cells from the LMMP were plated at 12,000 cells/well in 3 poly-L-lysine coated wells of a 24-well plate. Remaining cells were plated in 1 poly-L-lysine coated well of a 6-well plate for freeze/thaw experiments (Figure 3). For P1 cultures, 2 wells of the 24-well plate were fixed at confluency for purity analysis and the third well of the 24-well plate was counted at confluency to determine cell expansion. The single well in the 6-well plate was frozen at confluency to determine freeze/thaw recovery.

50. Place the plated cells at 37°C with 5% CO_2.
51. On the following day (Figure 5), use a micropipette to replace the medium with fresh maintenance medium and 1X growth supplement cocktail at the working volumes listed in Table 7.

Table 6. Average fold change ± SEM in the number of cells in primary cultures from the submucosa and longitudinal muscle/myenteric plexus (LMMP) of porcine colon between the initial plating and first passage (related to Part 2A, Step 48)

	Average fold change in cell number at the time of first passage over number of cells initially plated	
Colon wall layer	Neonate	Juvenile
Submucosa	0.15 ± 0.02	0.11 ± 0.03
LMMP	0.40 ± 0.13	0.20 ± 0.05

No significant differences were identified between ages or plexi by Two-way ANOVA Tukey's multiple comparisons test ($n = 4$).

Table 7. Suggested first passage plating density for enteric glial cell primary cultures from the submucosa and longitudinal muscle/myenteric plexus of pig colon (related to Part 2A, Steps 49 and 51)

	24w plate
Working volume of plating medium (µL)	500
Submucosal cells/well	20,000
LMMP cells/well	12,000

52. Replace medium with fresh maintenance medium and 1X growth supplement cocktail every other day until the desired level of confluence is reached.
53. When passage 1 cultures reach 70–80% confluence, repeat steps 36–48 on 1 well of the 24-well plate.

Note: We do not recommend passaging primary cultures of enteric glial cells beyond the third passage due to phenotypic changes.

Note: The days from first to second passage can be determined by counting the days from plating the first passage until the second passage. Average numbers of days from first to second passage of submucosa and LMMP cell cultures obtained from colons of neonatal and juvenile pigs in this experiment are provided for reference (Table 8).

Note: The cell yield can be determined by counting the live cells at passaging. Average cell yields from first to second passage of submucosa and LMMP cell cultures obtained from colons of neonatal and juvenile pigs in this experiment are provided for reference (Table 9).

Note: The average fold change of cells from first to second passage can be determined by dividing the number of live cells at the time of the second passage by the total number of cells plated at the first passage. Average fold changes from first to second passage of submucosa and LMMP cell cultures obtained from colons of neonatal and juvenile pigs in this experiment are provided for reference (Table 10).

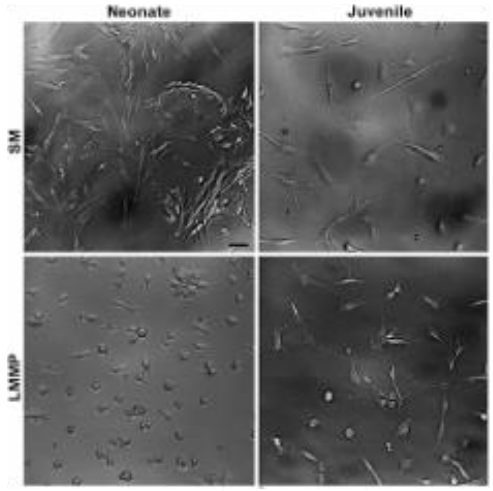

Figure 5. Enteric glial cell primary cultures from the submucosa (SM) and longitudinal muscle/myenteric plexus (LMMP) on day 1 *in vitro* following the first passage (related to Part 2A, Steps 51–52)
Scale bar, 50 µm.

Table 8. Average number of days ± SEM from first passage until second passage of enteric glial cell primary cultures from the submucosa and longitudinal muscle/myenteric plexus (LMMP) of porcine colon (related to Part 2A, Step 53)

	Average number of days from first to second passage	
Colon wall layer	Neonate	Juvenile
Submucosa	2.8 ± 0.3	4.5 ± 0.6
LMMP	2.5 ± 0.5[a]	4.8 ± 0.6[a]

Significant differences were identified between neonate LMMP and juvenile LMMP by Two-way ANOVA Tukey's multiple comparisons test ($n = 4$).
[a]($p = 0.05$)

Part 2B: Freezing and thawing

54. Passage cells for freezing at 70–80% confluence but before large cell clusters have formed.

Note: For this experiment, we plated cells at passage 1 in 1 poly-L-lysine coated well of a 6-well plate to determine freeze/thaw efficiency.

Note: The following passaging method uses volumes corresponding to the 6-well plate used in this experiment. Volumes should be changed accordingly for different well sizes.

55. Rinse each well with ~1 mL of pre-warmed sterile 1X PBS.
56. Remove the PBS using a micropipette.
57. Add 1 mL of pre-warmed trypsin-EDTA diluted 1:3 in sterile 1X PBS to each well.
58. Incubate the cells at 37°C checking every 2 min whether they have detached from the plate and if necessary, gently tapping the plate to encourage cells to detach.
59. When the vast majority of the cells have detached, pipet up and down with a micropipette to break up any cell clusters as needed.
60. Add 3 mL of pre-warmed passaging medium to neutralize the trypsin.
61. Use a micropipette to transfer the cell solution from each well into a conical tube.

Note: A 15 mL conical tube can be used for 1–2 wells of a 6-well plate.

62. Spin the cells at 300 × *g* at 20°C–22°C for 5 min.
63. Use a vacuum to carefully remove the supernatant.
64. Gently resuspend the pellet in ~1 mL of sterile passaging medium using a micropipette.
65. Count the total number of live cells using trypan blue and hemocytometer.
66. Freeze the cells in 5% DMSO supplemented with passaging medium in a cryovial.
67. Cryovials can be placed in an appropriate freezing container and stored at −80°C.
68. After at least 24 h, transfer cryovials to liquid nitrogen storage.

Table 9. Average cell yield ± SEM at second passage of enteric glial cell primary cultures from the submucosa and longitudinal muscle/myenteric plexus (LMMP) of porcine colon from 1 well of a 24-well plate (related to Part 2A, Step 53)

	Average cell yield (x1,000 cells)	
Colon wall layer	Neonate	Juvenile
Submucosa	43 ± 3.9	31 ± 2.8
LMMP	51 ± 6.6[a]	21 ± 2.0[a]

Significant differences were identified between neonate LMMP and juvenile LMMP by Two-way ANOVA Tukey's multiple comparisons test ($n = 4$).
[a]($p = 0.001$)

Table 10. Average fold change ± SEM in the number of cells in primary cultures from the submucosa and longitudinal muscle/myenteric plexus (LMMP) of porcine colon between the second passage and first passage (related to Part 2A, Step 53)

	Average fold change in cell numbers at the time of second passage over first passage	
Colon wall layer	Neonate	Juvenile
Submucosa	2.2 ± 0.2[a]	1.6 ± 0.1
LMMP	4.2 ± 0.6[a,b]	1.7 ± 0.2[b]

Significant differences were identified between neonate submucosa and neonate LMMP and between neonate LMMP and juvenile LMMP by Two-way ANOVA Tukey's multiple comparisons test ($n = 4$).
[a]($p = 0.003$)
[b]($p = 0.0005$)

69. After at least 24 h in liquid nitrogen, cells can be thawed per the following steps:
    a. If necessary, place the cryovial on dry ice for transportation.
    b. Place the cryovial in a water bath set to 37°C.
    c. Thaw the cells for ~1 min until only a small ice cube remains.
    d. Quickly add 1 mL of pre-warmed passaging medium and pipet up and down twice very gently.
    e. Transfer the cell solution to a 15 mL conical tube containing 5 mL of pre-warmed passaging medium.
    f. Spin the cells at 300 × g and at 20°C–22°C for 5 min.
    g. Use a vacuum to carefully remove all but ~500 µL of supernatant.
    h. Gently resuspend the pellet using a micropipette.
    i. Plate the cells in an appropriate size and number of poly-L-lysine coated wells in passaging medium with 1X growth supplement cocktail.

    Note: In this experiment, 1 well of a 6-well plate was used to determine freeze/thaw efficiency.

    j. Place the plates at 37°C with 5% CO_2.
    k. Repeat steps 55–65 to determine the number of live cells 24 h after thawing.

Note: To determine freeze/thaw recovery, divide the number of live cells 24 h after thawing by the number of live cells frozen. Average percent recovery following freezing and thawing of submucosa and LMMP enteric glial cell primary cultures obtained from colons of neonatal and juvenile pigs are provided for reference (Table 11).

Part 2C: Purity analysis

70. At confluence, fix all wells of the 96-well plate from Step 32 (passage 0) in ~200 µL 4% PFA and the remaining 2 wells of the 24-well plate from Step 49 (passage 1) in ~500 µL 4% PFA for 15 min at 20°C–22°C.

Table 11. Average percent recovery of cells ± SEM following freezing and thawing of enteric glial cell primary cultures from the submucosa and longitudinal muscle/myenteric plexus of porcine colon (related to Part 2B, Step 69)

	Average cell recovery (%)	
Colon wall layer	Neonate	Juvenile
Submucosa	35 ± 8.2	23 ± 11
LMMP	49 ± 7.0	23 ± 4.2

No significant differences were identified by Two-way ANOVA Tukey's multiple comparisons test ($n = 4$).

Note: Alternatively, plates can be left in 4% PFA for at least 12 h at 4°C.

Note: 4% PFA is made with 1X PBS.

71. Remove the PFA.
72. Rinse the wells three times with an equal volume of 1X PBS.

Note: The last PBS rinse can be replaced with 0.1% NaN_3 in 1X PBS for long term storage. Remove NaN_3-containing PBS solution before beginning immunofluorescence protocol.

II Pause point: Plates can be sealed with parafilm and stored at 4°C until immunofluorescence assays are performed but ensure wells do not dry out.

73. Remove 1X PBS from wells.
74. Incubate cells with 100 µL (96-well plate) or 350 µL (24-well plate) of blocking solution for 45 min at 20°C–22°C on a plate rocker.
75. Remove the blocking solution.
76. Permeabilize cells with 100 µL (96-well plate) or 350 µL (24-well plate) of permeabilization solution for 15 min at 20°C–22°C on a plate rocker.
77. Remove the permeabilization solution.
78. Prepare primary antibody solutions in permeabilization solution following dilutions suggested in key resources table.
79. Incubate cells with 100 µL (96-well plate) or 350 µL (24-well plate) of primary antibody solution for at least 12 h at 4°C.

Note: In the first well, antibodies against the glial cell markers S-100β, GFAP, and SOX10 were utilized to assess the percentage of enteric glial cells. In the second well, antibodies raised against the neuronal marker PGP9.5, the myofibroblast marker α-SMA, and the glial cell marker S-100β were utilized to quantify potential contaminants.

Note: The primary antibody solution can be incubated at 20°C–22°C for 2 h for faster staining.

80. Remove the primary antibody solution.
81. Rinse each well with 1X PBS three times for 5 min on a plate rocker.
82. Prepare secondary antibody solutions as a 1:500 dilution in permeabilization solution.
83. Incubate cells with 100 µL (96-well plate) or 350 µL (24-well plate) of secondary antibody solution for 2.5 h at 20°C–22°C on a plate rocker.
84. Remove the secondary antibody solution.
85. Repeat step 81.
86. Prepare DAPI solution as a 1:1000 dilution in permeabilization solution.
87. Incubate cells with 100 µL (96-well plate) or 350 µL (24-well plate) of DAPI solution for 15 min at 20°C–22°C on a plate rocker.
88. Repeat step 81.
89. Seal the plate with parafilm and store at 4°C protected from light until imaging.

Note: The last PBS rinse can be replaced with 0.1% NaN_3 in 1X PBS for long term storage.

Note: Fill empty wells of plate with PBS or water and seal with parafilm to prevent stained wells from drying.

90. Perform purity analysis.

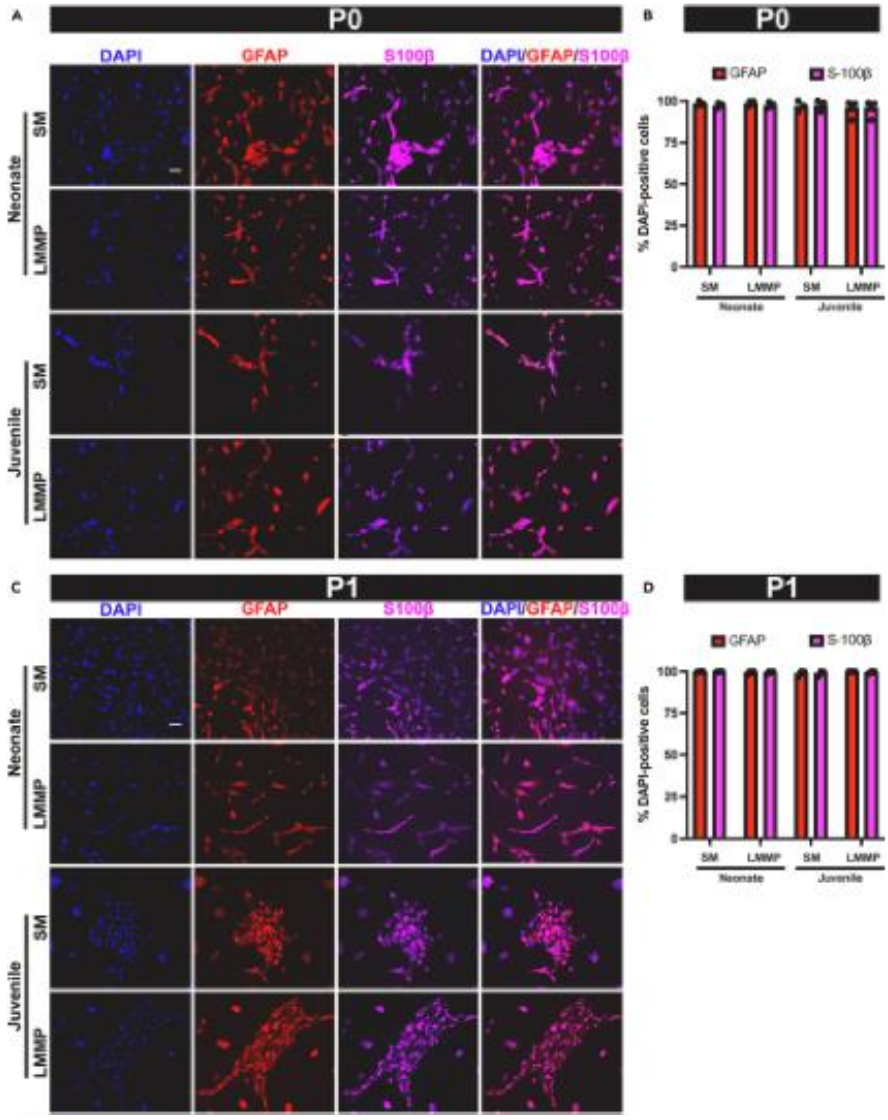

Figure 6. Expression of the enteric glial cell markers GFAP and S-100β in primary cultures isolated from the submucosa and longitudinal muscle/myenteric plexus of neonatal and juvenile pig colons

Primary cultures of enteric glial cells isolated from neonatal and juvenile pig colon submucosa (SM) and longitudinal muscle/myenteric plexus (LMMP) are >95% positive for the enteric glial markers GFAP and S-100β at passages 0 and 1 (related to Part 2C, Step 90).

(A) Representative pictures of enteric glial cell primary cultures isolated from neonatal and juvenile pig colon SM and LMMP at passage 0 stained for the enteric glial cell markers GFAP (red) and S-100β (magenta).

(B) Quantification of the average percentage of cells immunoreactive for the enteric glial cell markers GFAP and S-100β in SM and LMMP enteric glial cell primary cultures at passage 0.

Figure 6. Continued

(C) Representative pictures of enteric glial cell primary cultures isolated from neonatal and juvenile pig colon SM and LMMP at passage 1 stained for the enteric glial cell markers GFAP (red) and S-100β (magenta).

(D) Quantification of the average percentage of cells immunoreactive for the enteric glial cell markers GFAP and S-100β in SM and LMMP enteric glial cell primary cultures at passage 1.

Data are expressed as mean ± SEM. No significant differences were identified by Two-way ANOVA Tukey's multiple comparisons test (n = 3–4 independent experiments, at least 6 fields were analyzed per well). DAPI (blue) shows nuclei. Scale bar, 50 μm.

Note: In this study, at least 6 fields per well were analyzed to determine the percentage of cells that were positive for S-100β, GFAP, or SOX10 (Figures 6 and 7) and for PGP9.5, α-SMA, and S-100β (Figures 8 and 9). Images were captured using an inverted fluorescence microscope (Olympus IX81, Tokyo, Japan) with a digital camera (ORCA-flash 4.0, Hamamatsu, Japan) using a 20x objective lens (LUC Plan FLN, Olympus, Tokyo, Japan).

EXPECTED OUTCOMES

This protocol to establish and maintain primary cultures of enteric glial cells from the submucosa and longitudinal muscle/myenteric plexus of porcine colons was developed for future investigations on enteric glia biology using porcine and human specimens. To illustrate expected outcomes such as cell yield from a specific tissue size, purity of passage 0 and passage 1 cultures, culture expansion over time, and freeze/thaw recovery, we provide descriptive statistics for 3–4 independent cultures. We further compared enteric glial cells derived from neonatal and juvenile pigs and found that, from a size matched spiral colon specimen, neonatal cultures grow faster than juveniles, but purity is expected to be comparable between both age groups.

QUANTIFICATION AND STATISTICAL ANALYSIS

Prism (version 9.3.1) was used to conduct statistical analyses. For all data analyzed, a Two-way ANOVA was performed and post-hoc analysis via Tukey's multiple comparisons test. All data is presented as mean ± SEM.

LIMITATIONS

This protocol was designed to establish primary cultures of porcine colonic enteric glial cells from the submucosa and the longitudinal muscle/myenteric plexus (LMMP) isolated by microdissection. Microdissection of the intestinal layers is challenging, and for instance, it is impossible to remove all circular muscle fibers. Thus, enteric glial cells from the circular muscle layer are potentially present in the LMMP cultures. Similarly, enteric glial cells from the submucosa specimens also contain enteric glial cells from the lamina propria of the mucosa.

Phenotypic stability of the primary cultures is another potential limitation. In our hands, primary cultures of enteric glial cells appear to be phenotypically stable up until passage 4, therefore much of their use must be immediate. While freezing is an option, since recovery is not 100% upon thawing, some of the heterogeneity of the primary cultures may be lost upon freezing and thawing cycles. Similarly, it is also possible that some of the heterogeneity of the primary cultures may be lost upon passaging.

TROUBLESHOOTING

Problem 1

Clogging serological pipets during mechanical dissociation (Part 1, Step 22).

Potential solution

Cutting the pieces of submucosa and LMMP as small as possible helps prevent clogging. Serological pipets can also be tapped repeatedly against the conical tube while pipetting up and down or a larger 25 mL serological pipet can be used to help prevent clogging.

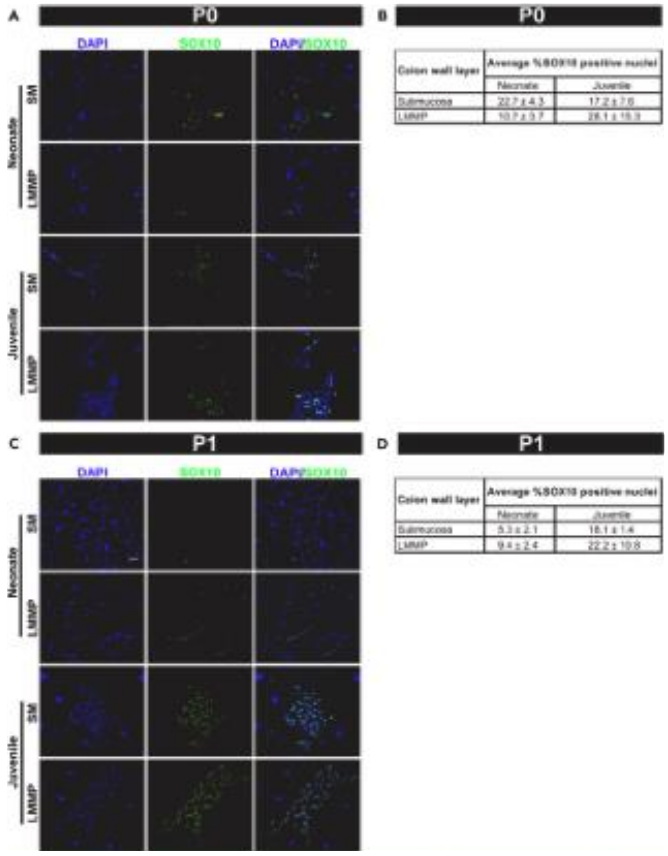

Figure 7. Expression of the enteric glial cell marker SOX10 in primary cultures isolated from the submucosa and longitudinal muscle/myenteric plexus of neonatal and juvenile pig colons

Only a fraction of enteric glial cells in primary cultures isolated from neonatal and juvenile pig colon submucosa (SM) and longitudinal muscle/myenteric plexus (LMMP) are immunoreactive for SOX10 at passage 0 and 1 (related to Part 2C, Step 90).

(A) Representative pictures of enteric glial cell primary cultures isolated from neonatal and juvenile pig colon SM and LMMP at passage 0 stained for the enteric glial cell marker SOX10 (green).

(B) Quantification of the average percentage of cells immunoreactive for the enteric glial cell marker SOX10 in SM and LMMP enteric glial cell primary cultures at passage 0.

(C) Representative pictures of enteric glial cell primary cultures isolated from neonatal and juvenile pig colon SM and LMMP at passage 1 stained for the enteric glial cell marker SOX10 (green).

(D) Quantification of the average percentage of cells immunoreactive for the enteric glial cell marker SOX10 in SM and LMMP enteric glial cell primary cultures at passage 1.

Data are expressed as mean ± SEM. No significant differences were identified by Two-way ANOVA Tukey's multiple comparisons test (n = 3–4 independent experiments, at least 6 fields were analyzed per well). DAPI (blue) shows nuclei. Scale bar, 50 μm.

Problem 2

Low initial cell yield (Part 1, Step 31).

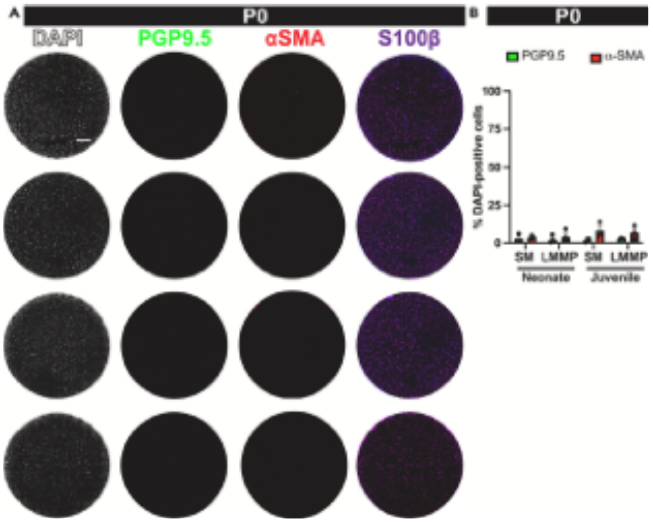

Figure 8. Purity analysis of P0 primary cultures of enteric glial cells isolated from the submucosa and longitudinal muscle/myenteric plexus of neonatal and juvenile pig colons

Enteric glial cell primary cultures isolated from neonatal and juvenile pig colon submucosa (SM) and longitudinal muscle/myenteric plexus (LMMP) comprise less than 10% of cells immunoreactive for myofibroblast or neuronal markers at passage 0 (related to Part 2C, Step 90).

(A) Representative images (whole well stitching) of enteric glial cell primary cultures isolated from neonatal and juvenile pig colon SM and LMMP at passage 0 immunolabeled for the pan-neuronal marker PGP9.5 (green), myofibroblast marker α-SMA (red), and glial cell marker S-100β (magenta).

(B) Quantification of the average percentage of cells immunoreactive for the pan-neuronal marker PGP9.5 and myofibroblast marker α-SMA in SM and LMMP enteric glial cell primary cultures at passage 0. Scale bar, 1 mm. Data are expressed as mean ± SEM. No significant differences were identified by Two-way ANOVA Tukey's multiple comparisons test ($n = 3–4$ independent experiments, at least 6 fields were analyzed per well). DAPI (white) shows nuclei. Scale bar, 1 mm.

Potential solution

Decreasing the dissection time to under 2 h total can improve tissue viability and thus, cell yield. Similarly, regularly replacing cold dissecting medium improves tissue viability and cell yield. Cutting the pieces of submucosa and LMMP as small as possible also helps improve cell yield by limiting clogging.

Problem 3

Enteric glial cell cultures growing slowly (Part 1, Step 35).

Potential solution

Sometimes decreasing the frequency of medium changes can help with low confluency, slow growing cultures.

Problem 4

Cell clusters during passaging (Part 2A, Step 42).

Potential solution

Cell clusters can be broken up by pressing the micropipette tip against the bottom of the well while homogenizing cells during trypsinization. Overdoing this may damage cells, so it should be performed only a few times until clusters are mostly dissociated into single cells.

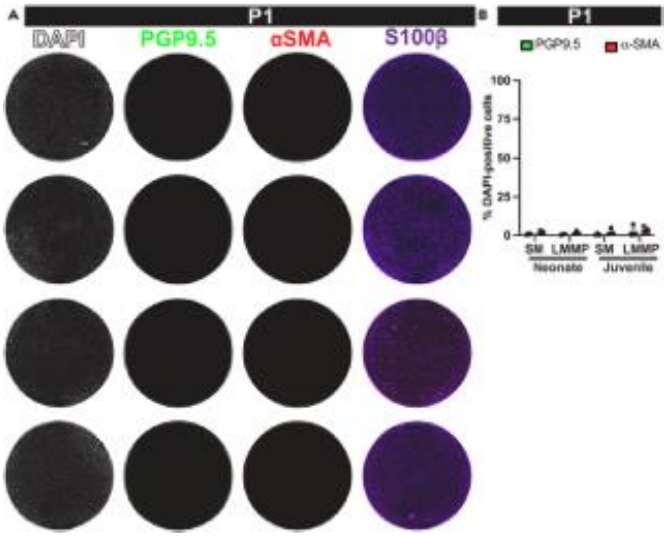

Figure 9. Purity analysis of P1 primary cultures of enteric glial cells isolated from the submucosa and longitudinal muscle/myenteric plexus of neonatal and juvenile pig colons

Enteric glial cell primary cultures isolated from neonatal and juvenile pig colon submucosa (SM) and longitudinal muscle/myenteric plexus (LMMP) comprise less than 10% of cells immunoreactive for myofibroblast or neuronal markers at passage 1 (related to Part 2C, Step 90).

(A) Representative images (whole well stitching) of enteric glial cell primary cultures isolated from neonatal and juvenile pig colon SM and LMMP at passage 1 immunolabeled for the pan-neuronal marker PGP9.5 (green), myofibroblast marker α-SMA (red), and glial cell marker S-100β (magenta).

(B) Quantification of the average percentage of cells immunoreactive for the pan-neuronal marker PGP9.5 and myofibroblast marker α-SMA in SM and LMMP enteric glial cell primary cultures at passage 1.

Data are expressed as mean ± SEM. No significant differences were identified by Two-way ANOVA Tukey's multiple comparisons test (n = 3–4 independent experiments, at least 6 fields were analyzed per well). DAPI (white) shows nuclei. Scale bar, 1 mm.

Problem 5

Low cell yield after passaging (Part 2B, Step 48).

Potential solution

Following centrifugation, it is important to aspirate supernatant off pelleted cells in a slow and smooth manner to avoid dislodging the pellet and losing cells.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Laurianne Van Landeghem (lcvanlan@ncsu.edu).

Technical contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Laurianne Van Landeghem (lcvanlan@ncsu.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103057.

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AUTHOR CONTRIBUTIONS

M.L.C. and C.A.C. performed the experiments. C.L.M. and M.T. participated in method development. J.O. provided the animals. M.L.C. wrote the manuscript. J.O., A.L.Z., A.T.B., and L.V.L. edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Chapter 4

Juvenile Lumen Content Rescues Neonatal Enteric Glial Cell Ability to Promote Intestinal Epithelial Restitution *in vitro*

4.1 Abstract

Failure to repair the intestinal epithelial barrier is associated with devastating outcomes due to the translocation of luminal contents across the epithelium and into contact with underlying immune cells and systemic circulation. We previously reported that neonatal pigs exhibit a defect in acute mucosal wound repair in which they are unable to restore barrier function due to a specific lack of epithelial restitution aligning with clinical observations that younger age increases the risk of mortality from intestinal ischemia due to impaired barrier function. However, this defect can be rescued by application of ischemia-injured juvenile mucosa suggesting a specific maturation factor is responsible. Believing this to be the subepithelial network of enteric glial cells which normally supports barrier function, we further showed that this network is underdeveloped in neonatal pigs. Another major difference between the age groups we investigated is that juvenile pigs have been weaned and on solid feed inducing changes to the gut microbiota composition while neonates remain nursing. Therefore, we hypothesized that an age-dependent relationship between the developing enteric glial network, gut microbiota, and intestinal epithelium may drive differences in acute barrier repair. We test that hypothesis in this study showing that exposure to juvenile lumen content stimulates neonatal EGC to promote intestinal epithelial wound healing *in vitro*. Furthermore, we report significant differences in lumen content composition between neonatal and juvenile pigs, examine butyrate as a potential age-dependent luminal cue stimulating neonatal EGC, and identify SERPINB2 as a candidate molecule for enhancing acute intestinal epithelial wound healing.

4.2 Introduction

The intestinal epithelial barrier is a semi-permeable structure comprised of a monolayer of cells lining the intestinal mucosa that serves critical yet paradoxical functions. On one hand, the intestinal epithelium is responsible for secretion of digestive enzymes and absorption of nutrients. While on the other, this single layer of cells must also serve as a barrier from luminal products such as antigens, toxins, and microbes and their metabolites. Defects in the intestinal epithelium must be rapidly healed to prevent translocation of these products across the barrier where they have access to underlying immune cells and systemic circulation. Acute mucosal wound healing has been well described in the small intestine as occurring in three distinct yet overlapping steps: 1.) contraction of the villus to decrease the denuded wound area, 2.) migration and spreading of epithelial cells to cover the exposed basement membrane referred to as restitution, and 3.) reassembly of tight junctions to restore molecular barrier.[1,2] Following this acute phase of healing, the intestinal epithelium proliferates and differentiates to replace cells lost to injury. Barrier restoration is essential to homeostasis and failure to rapidly repair the intestinal barrier can precipitate life-threatening conditions such as systemic inflammatory response syndrome (SIRS) characterized by overwhelming inflammation due to the translocation of luminal products.

Previous work from our laboratory found that barrier repair is an age-dependent process that develops postnatally as neonatal pigs exhibit a defect specifically in epithelial restitution. Using our surgically induced model of mesenteric ischemia to damage the intestinal epithelium, we found juvenile (6-8 weeks of age) pigs rapidly restored barrier function during *ex vivo* recovery on Ussing chambers while neonatal (2 weeks of age) pigs did not, likely due from failure to adopt an migratory epithelial cell phenotype.[2] Surprisingly, we were able to rescue this

neonatal defect in repair by applying homogenized mucosa from injured juvenile tissues. This indicates that intrinsic acute mucosal repair pathways are present in neonates but lack proper repair induction signals that are present in the more mature intestine. Considering which factors may require postnatal maturation, we speculated that the enteric nervous system and specifically, enteric glial cells (EGC) as a possible source. EGC are well-known mediators of intestinal epithelial function primarily through paracrine signaling and are known to release molecules that regulate the barrier and promote intestinal wound healing.[3-9] Importantly, a population of EGC present in the lamina propria in close proximity to the intestinal epithelium develops after birth in rodents.[10] We confirmed that the subepithelial network of EGC is underdeveloped in neonatal pigs relative to their juvenile counterparts.[11] Moreover, chemical inhibition of EGC with fluoroacetate prevented barrier restoration in juveniles recreating the defect seen in neonates.[11]

An additional understudied component of the relationship between EGC and the intestinal barrier is the presence of the gut microbiota. Similar to EGC, the commensal gut microbiota modulates barrier function primarily through microbial cell components such as lipopolysaccharide as well as the production of metabolites such as short chain fatty acids (SCFAs).[12] Microbial-produced SCFAs—most notably butyrate—promote barrier function and mucosal wound healing.[13-16] Microbial colonization of the gut also occurs primarily after birth and is required for invasion of EGC into the lamina propria in rodents.[10] The most significant shifts in the gut microbiota composition occur following weaning during the transition from a milk-based diet to solid feed after which the mature composition remains fairly stable.[17-20] Similarly, mucosal EGC expand significantly after weaning and begin to stabilize as they reach adult levels purportedly driven by changes in the gut microbiota composition.[10] A key difference between the age groups in our model is weaning of the piglets at 3 weeks of age, so we

postulated that a postnatal relationship between the intestinal epithelium, network of EGC, and gut microbiota may be driving age-dependent differences in acute mucosal repair.

In the present study, we utilized a non-contact co-culture model to study the effect of lumen content and age of EGC on intestinal epithelial wound healing *in vitro*. Monolayers of IPEC-J2 cells, which are a non-cancerous and non-transformed cell line isolated from neonatal pig jejunum, were co-cultured with EGC isolated from the submucosal plexus of neonatal or juvenile pig colons and exposed to filtered colonic lumen content from the respective age groups prior to scratch wounding. Using mass spectrometry of supernatants from these experiments, we identified several possible targets that are differentially secreted from neonatal EGC exposed to neonatal versus juvenile lumen content including an understudied serine protease inhibitor SERPINB2. We also investigated differences in lumen content composition between the two age groups to better understand the specific mechanism driving our findings. Overall, we demonstrated that juvenile lumen content is capable of rescuing the ability of neonatal EGC to promote intestinal epithelial restitution *in vitro* and identify SERPINB2 as a secreted signaling molecule that promotes this process.

4.3 Materials and Methods

Animals

All procedures were approved by North Caroline State University Institutional Animal Care and Use Committee. Commercial crossbred pigs of both sexes were sedated using xylazine (1.5 mg/kg) and ketamine (11 mg/kg) and euthanized with an overdose of pentobarbital or via captive bolt followed by exsanguination at an academic animal production facility for immediate tissue collection. Neonates were considered pigs that were approximately 2 weeks of age and not

weaned. Juveniles were considered pigs that were 6-8 weeks of age and had been weaned at 3 weeks of age.

Enteric glial cell isolation and culture

Primary cultures of EGC from neonatal and juvenile pig colons were isolated and maintained as previously described.[21] Briefly, specimens of spiral colon were collected from the central flexure, emptied of their lumen contents, and rinsed thoroughly. The submucosa enclosing the submucosal plexus was isolated by microdissection followed by enzymatic digestion with 4 mg/mL BSA (Sigma #A3311 prepared in 1X PBS and sterile-filtered), 0.25 mg/mL collagenase (Sigma #C9891 prepared in 1X PBS and sterile-filtered), and 1 mg/mL protease (Sigma #P4630 prepared in 1X PBS and sterile-filtered) in 5 mL of DMEM/F-12 50/50 (Corning #10-090-CV) with 0.12% sodium bicarbonate (Sigma #S5761), 1.5% penicillin-streptomycin (Gemini #400-109), 2 mM L-glutamine (Gemini #400-106), 0.06 mg/mL gentamicin (Sigma #G1272), and 0.003 mg/mL amphotericin B (Sigma #A9528). Tissues were incubated for 35 minutes at 37°C in a rotating incubator then inactivated with 10 mL of the same media used earlier supplemented with 10% heat-inactivated fetal bovine serum (Gemini #100-106). Tissues were then mechanically dissociated by pipetting and vortexing, centrifuged, mechanically dissociated again with fresh media, and then strained sequentially through 100-µm, 70-µm, and 40-µm cell strainers to obtain single cells. Cells were seeded in the FBS-supplemented media above with a 1X growth factor cocktail containing N2 (Thermo #17502048), G5 (Thermo #17503012), and B27 (Thermo #17504044) onto a tissue-cultured treated T25 flask coated overnight with 0.5 mg/mL poly-L-lysine (Sigma #P2636) in 0.5 M borate buffer (Thermo #28341) and rinsed 3X with sterile water prior to plating. Cultures were maintained in DMEM/F-12 50/50 medium with 0.5% heat-inactivated fetal bovine serum

(Gemini #100-106), 0.5% penicillin-streptomycin (Gemini #400-109), 2 mM L-glutamine (Gemini #400-106), 0.02 mg/mL gentamicin (Sigma #G1272), and 0.001 mg/mL amphotericin B (Sigma #A9528) supplemented with the 1X growth factor cocktail listed above to enrich for glial cells. Cells were maintained at 37°C and 5% CO_2. Primary cultures of EGC were passaged at 80% confluence using trypsin-EDTA (Fisher #302101) diluted 1:3 in sterile 1X PBS or frozen in 5% DMSO (Sigma #D1435). All cultures of EGC used in this study were from the first or second passage.

Lumen content isolation and filtration

Colonic lumen content was removed from spiral colon specimens and diluted with 1X PBS until liquified. Afterwards, lumen content was flash frozen on dry ice and stored at -80°C and a 1 mL aliquot was dried in an oven to remove all liquid content to determine dry matter concentration. Prior to use, thawed lumen content samples were centrifuged at 17,000 x g at 4°C for 45 minutes on a benchtop centrifuge, the supernatant filtered using a 0.2 µm column (Corning #8160) and centrifuged at 17,000 x g at 4°C for 30 minutes, and the filtrate of this was filtered on another 0.2 µm column centrifuged at 17,000 x g at 4°C for 5 minutes.

Wound healing assays

Wound healing assays were modified from a previously published protocol.[11] For lumen content wound healing assays, primary cultures of EGC were passaged on Day 0 and plated in 24-well tissue culture treated plates seeded at 20,000 cells/well onto wells coated overnight with 0.5 mg/mL poly-L-lysine (Sigma #P2636) in 0.5 M borate buffer (Thermo #28341) and rinsed 3X with sterile water prior to plating. On Day 1, IPEC-J2 cells were passaged using trypsin-EDTA (Corning #25-053-CI) and seeded at 40,000 cells/well onto 0.4 µm Transwell® filter inserts (Corning #3470) and maintained in DMEM/F-12 50/50 with 15 mM HEPES (Corning

#10-092-CV) supplemented with 5% heat-inactivated FBS (Gemini #100-106), 5 ng/mL insulin-transferrin/selenium supplement (Gibco #1400045), 0.12% penicillin-streptomycin (Gemini #400-109) and 5 ng/mL recombinant human EGF (Gibco #PHG0311). All cells were maintained at 37°C and 5% CO_2. On Day 2, filter inserts containing IPEC-J2 cells were transferred to wells containing primary cultures of EGC or blank wells containing only enteric glial cell culture medium with 1X growth factor cocktail. Also on Day 2, filter inserts containing IPEC-J2 cells were dosed with 0.083 mg/mL of neonatal or juvenile filtered lumen content diluted in IPEC-J2 medium. On Day 3, IPEC-J2 filters received a half dose of neonatal or juvenile filtered lumen content and were subsequently manually scratched using a disposable 200 µL micropipette tip. Wounds were imaged at 0-, 2-, 4-, 6, and 8-hours after scratch wounding using an inverted microscope (Olympus IX81, Tokyo, Japan) with a digital camera (ORCA-flash 4.0, Hamamatsu, Japan) using a ×20 objective lens (LUC Plan FLN, Olympus, Tokyo, Japan) and plate mapping software (CellSens, Olympus, Tokyo, Japan). Percent wound closure ([Area of wound at Time 0 – Area of wound at Time 8]/Area of wound at Time 0) was calculated after measuring the total wound area using ImageJ software (NIH, Bethesda, MD). This protocol was also used for butyrate, acetate, and propionate wound healing assays but these were imaged with an inverted fluorescence microscope (Keyence BZ-X810) using a 4X objective (Plan Fluorite 4X LD PH Keyence BZ-PA04. Sodium butyrate (Sigma #B5887), sodium acetate (Sigma #S5636), and sodium propionate (Sigma #P5436) were prepared following manufacturer recommendations in sterile cell culture grade water (Corning #25-055-CM) and filtered using a 0.2 µm column (Corning #8160) centrifuged at 17,000 x g at 4°C for 5 minutes. For SERPINB2 wound healing assays, the same protocol was followed with the exception of steps involving primary cultures of EGC. IPEC-J2 filter inserts were instead maintained in IPEC-J2 medium and recombinant

SERPINB2 (R&D Systems #9206-PI-025) was added to the 24-well plates containing the filter inserts. After 24 hours, IPEC-J2 monolayers were scratched and imaged with an inverted fluorescence microscope (Keyence BZ-X810) using a 4X objective (Plan Fluorite 4X LD PH Keyence BZ-PA04).

Total living cell assays

For IPEC-J2 lumen content toxicity assays, cells were seeded at 20,000 cells/well in a 48-well tissue culture-treated plate and grown to a confluent monolayer over 24 hours prior to dosing with filtered lumen content diluted 1:10, 1:20, 1:60, 1:100, 1:250, 1:500, and 1:600 in technical duplicates. Cells were maintained at 37°C and 5% CO_2. After 24 hours, IPEC-J2 cells were trypsinized with trypsin-EDTA (Corning # 25-053-CI), centrifuged at 1500 x g for 3 minutes at 20°C, and living cells were counted using trypan blue (Thermo #15250061) and a hemocytometer. The same protocol was repeated for all other IPEC-J2 cell toxicity assays except cells were seeded at 175,000 cells/well in 24-well tissue culture-treated plates. Toxicity assays for EGC were performed at the end of wound healing experiments. One well per condition was rinsed with 1X PBS, trypsinized with trypsin-EDTA (Fisher #302101), centrifuged at 300 x g for 5 minutes at 20°C, and living cells were counted using trypan blue (Thermo #15250061) and a hemocytometer.

Immunocytochemistry

Immunocytochemistry assays were performed as previously described[22]. Briefly following wound healing assays, cells were fixed in 4% paraformaldehyde in PBS (Santa Cruz #281692) overnight at 4°C. Fixed cultures were rinsed three times in 1X PBS and maintained in 0.1% sodium azide (Sigma-Aldrich #S2002) in 1X PBS. Cells were permeabilized and saturated for 30 minutes at room temperature in PBS with 0.01% Triton-X (Sigma #X100) in PBS and 4%

donkey serum (Sigma #D9663) and incubated overnight at 4°C with mouse anti-Ki67 (DAKO #M7240) diluted 1:500 in 0.01% Triton-X PBS and 4% donkey serum. Primary antibodies were removed by rinsing three times with 1X PBS. Cells were then incubated with a goat anti-mouse secondary antibody conjugated to Alexa Fluor 568 (Thermo #A11004, RRID:AB_2534072) at a 1:500 dilution in 0.01% Triton-X PBS and 4% donkey serum for 2 hours at room temperature. Cell nuclei were counterstained with DAPI (Invitrogen #D1306) at a dilution of 1:1000 in PBS for 5 minutes at room temperature. DAPI staining was rinsed three times with 1X PBS and stained cells were maintained in 0.1% sodium azide in 1X PBS. Images were captured using an inverted fluorescence microscope (Keyence BZ-X810) using a 40X objective (Plan Fluorite 40X LD PH Keyence BZ-PF40LP). Color channels were merged and recolorized in ImageJ software (NIH, Bethesda, MD) to visualize overlay images for counting. Six fields per well were counted for each condition.

Mass spectrometry

Immediately following lumen content wound healing assays, the supernatants from the basolateral compartment of the co-culture setup were collected and flash frozen on dry ice. Each culture media sample was concentrated using a 3 kD Nanosep centrifugal filter (DSC #OD003C34) at 25°C, 14,000 x g for 50 minutes. Proteins in 30 µL concentrated samples were conjugated into 100 µg magnetic beads (10 µg protein captured) (Cytiva #65152105050350) and digested on beads with 0.8 µg of a trypsin and LysC protease mixture (Promega #V5073) resuspended in 80 µL of 0.1 M ammonium bicarbonate buffer. Peptides (20 µL) were analyzed via nano LC-MS/MS. For nano-LC-MS/MS-based proteomic analysis, all measurements were conducted at the Molecular Education, Technology, and Research Innovation Center (METRIC) at NC State University. Samples were loaded as 20-µl injections and peptides were first trapped

in Acclaim™ PepMap™ 100 C18 column (3 μm particle, 75 μm x 20 mm) separated by a 120 minute linear gradient using Aurora Frontier C18 analytical column (1.7 μm, 75 μm x 600 mm) at 200 nL/minute of mobile-phase A (0.1% formic acid in 2% acetonitrile and 97.9% water) and mobile-phase B (0.1% formic acid in 80% acetonitrile) from 4–22% mobile phase B in 50 minute, followed by 22-32% mobile phase B in 21 min, 32-44% mobile phase B in 23 minutes and 44-55% mobile phase B in 10 minutes, the column was washed with 95% mobile phase B in 15 minute. The Orbitrap Eclipse (Thermo #FSN04-10000) was operated as follows: 275°C ion transfer tube temperature, 1800 V spray voltage in positive ion mode, acquisition—full scan (m/z 375–1,500) with 60,000 resolving power in MS mode, MS/MS acquisition using 45 variable isolation windows (m/z 352-418, 417.25-428.75, 428.25-439.75, 439.25-450.75, 450.25-461.75, 461.25-472.75, 472.25-483.75, 483.25-494.75, 494.25-505.75, 505.25-516.75, 516.25-527.75, 27.25-538.75, 538.25-549.75, 549.25-560.75, 560.25-571.75, 571.25-582.75, 582.25-593.75, 593.25-604.75, 604.25-616.75, 616.25-628.75, 628.25-640.75, 640.25-652.75, 652.25-664.75, 664.25-676.75, 676.25-689.75, 689.25-702.75, 702.25-715.75, 715.25-728.75, 728.25-741.75, 741.25-754.75, 754.25-767.75, 767.25-781.75, 781.25-795.75, 795.25-809.75, 809.25-827.75, 927.25-846.75, 846.25-866.75, 866.25-886.75, 886.25-906.75, 906.25-926.75, 926.25-950.75, 950.25-976.75, 976.25-1004.75, 1004.25-1110.75, 1110.25-1200.75) in data-independent acquisition (DIA) with 30,000 resolving power. The collision energy mode is set as stepped with a normalized collision energy setting of 25, 28 and 32%. Raw nano-LC-MS/MS data were processed using FragPipe (Version 21.0, University of Michigan). Searching was performed with a 20 ppm fragment mass tolerance against the Sus scrofa (Pig) proteome database (downloaded from Uniprot database, 2024, 22,830 entries) and 298 commonly used contaminant database embedded in FragPipe. The database search settings were specific for trypsin digestion.

Specified modifications included dynamic Met oxidation and static Cys carbamidomethylation, and the maximum dynamic modification per peptide is set as 3. Identifications were filtered to a strict protein false discovery rate (FDR) of 1%. Statistically different proteins were calculated based on the grouping and using T-test with permutation-based FDR adjustment for multiple testing.

16s rRNA sequencing

Sequencing of bacterial 16s rRNA was performed by the UNC Microbiome Core. For DNA isolation samples were transferred to a 2 mL tube containing 200 mg of ≤106 μm glass beads (Sigma #G4649) and 0.3 mL of Qiagen ATL buffer (Qiagen #939011), supplemented with 60 mg/mL lysozyme (Thermo #89833). The suspension was incubated at 37°C for 1 hour with occasional agitation. Bead beating was performed for 1 minute in a Qiagen TissueLyser II at 30 Hz followed by a 5-minute incubation at 95°C. The suspension was agitated for 40 minutes on a Digital Vortex Mixer at 3000 rpm. Subsequently the suspension was supplemented with 600 IU of Qiagen proteinase K (Qiagen #19157) and 0.3 mL of Qiagen AL buffer (Qiagen #19075) followed by a 55°C overnight incubation. After a brief centrifugation, supernatants were aspirated and transferred to a new tube containing 0.3 mL of ethanol. DNA was purified using a standard on-column purification method with Qiagen buffers AW1 (Qiagen #19081) and AW2 (Qiagen #19072) as washing agents and eluted in DNase free water. For validation of the DNA isolation process, a known bacterial community, ZymoBIOMICS Microbial Community Standard (Zymo #D6300), and a blank composed of only DNA isolation reagents were included in the DNA extraction process and again in the library preparation. In addition to the isolation controls, the library preparation included a library blank composed of library preparation reagents alone.

For sequencing, 12.5 ng of total DNA was amplified using universal primers targeting the V4 region of the bacterial 16S rRNA gene. Primer sequences contained overhang adapters appended to the 5' end of each primer for compatibility with the Illumina sequencing platform. The primers used were F515/R806. Master mixes contained 12.5 ng of total DNA, 0.5 μM of each primer, and 2x KAPA HiFi HotStart ReadyMix (Roche #07958927001). The thermal profile for the amplification of each sample had an initial denaturing step at 95°C for 3 minutes, followed by cycling of denaturing at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and a 30-second extension at 72°C (25 cycles), a 5-minute extension at 72°C, and a final hold at 4°C. Each 16S amplicon was purified using the AMPure XP reagent (Beckman Coulter #A63881). In the next step, each sample was amplified using a limited cycle PCR program, adding Illumina sequencing adapters and dual-index barcodes (index 1(i7) and index 2(i5)) (Illumina) to the amplicon target. The thermal profile for the amplification of each sample had an initial denaturing step at 95°C for 3 minutes, followed by a denaturing cycle of 95°C for 30 seconds, annealing at 55°C for 30 seconds and a 30-second extension at 72°C (8 cycles), a 5-minute extension at 72°C and a final hold at 4°C. The final libraries were again purified using the AMPure XP reagent (Beckman Coulter #A63881), quantified, and normalized before pooling. The DNA library pool was then denatured with NaOH, diluted with hybridization buffer, and heat-denatured before loading on the MiSeq reagent cartridge (Illumina) and the MiSeq instrument (Illumina). Automated cluster generation and paired–end sequencing with dual reads were performed according to the manufacturer's instructions

For analysis, sequencing output from the Illumina MiSeq PE250 platform was converted to fastq format and demultiplexed using Illumina Bcl2Fastq 2.20.0. The resulting paired-end reads were processed using QIIME 2 2024.5. Index and linker primer sequences were trimmed

using the QIIME 2 invocation of Cutadapt. The resulting paired-end reads were processed with DADA2 through QIIME2 including merging paired ends, quality filtering, error correction, and chimera detection. Amplicon sequencing units from DADA2 were assigned taxonomic identifiers based on the Silva 138 database using the QIIME 2 q2-feature classifier. Alpha diversity indexes Faith PD whole tree, Evenness (Shannon), and observed species were estimated using QIIME2 at a rarefaction depth of 5,000 sequences. Beta diversity estimates were calculated within QIIME2 using weighted and unweighted Unifrac distances and Bray-Curtis dissimilarity between samples at a subsampling depth of 5,000. Results were summarized, and visualized through Principal Coordinate Analysis, and significance was estimated as implemented in QIIME2. The significance of differential abundance was estimated using ANCOM as implemented in QIIME.

Short chain fatty acid extraction and quantification

After thawing, unfiltered lumen content samples were acidified with1 mL of 0.5 N HCl overnight at 4°C. The samples were then centrifuged at 17,000 x g for 15 minutes at room temperature. From the supernatant, 1 mL of sample was combined with 5 M metaphosphoric acid in a 25% solution of 2-ethylbutyric acid as an internal standard. This was centrifuged again at 17,000 x g for 15 minutes at room temperature. Short chain fatty acids were analyzed by gas liquid chromatography, GLC, (model CP-3380; Varian: Walnut Creek, CA) using a fused-silica capillary column, 30 m × 0.25 mm with 0.25-µm film thickness (Nukol; Supelco Inc., Bellefonte, PA) according to a previously published protocol.[23]

Statistical analysis

Data analysis was performed using Prism statistical software (GraphPad, La Jolla, CA). Data were reported as mean ± SEM for a given number of animals for each experiment. Results

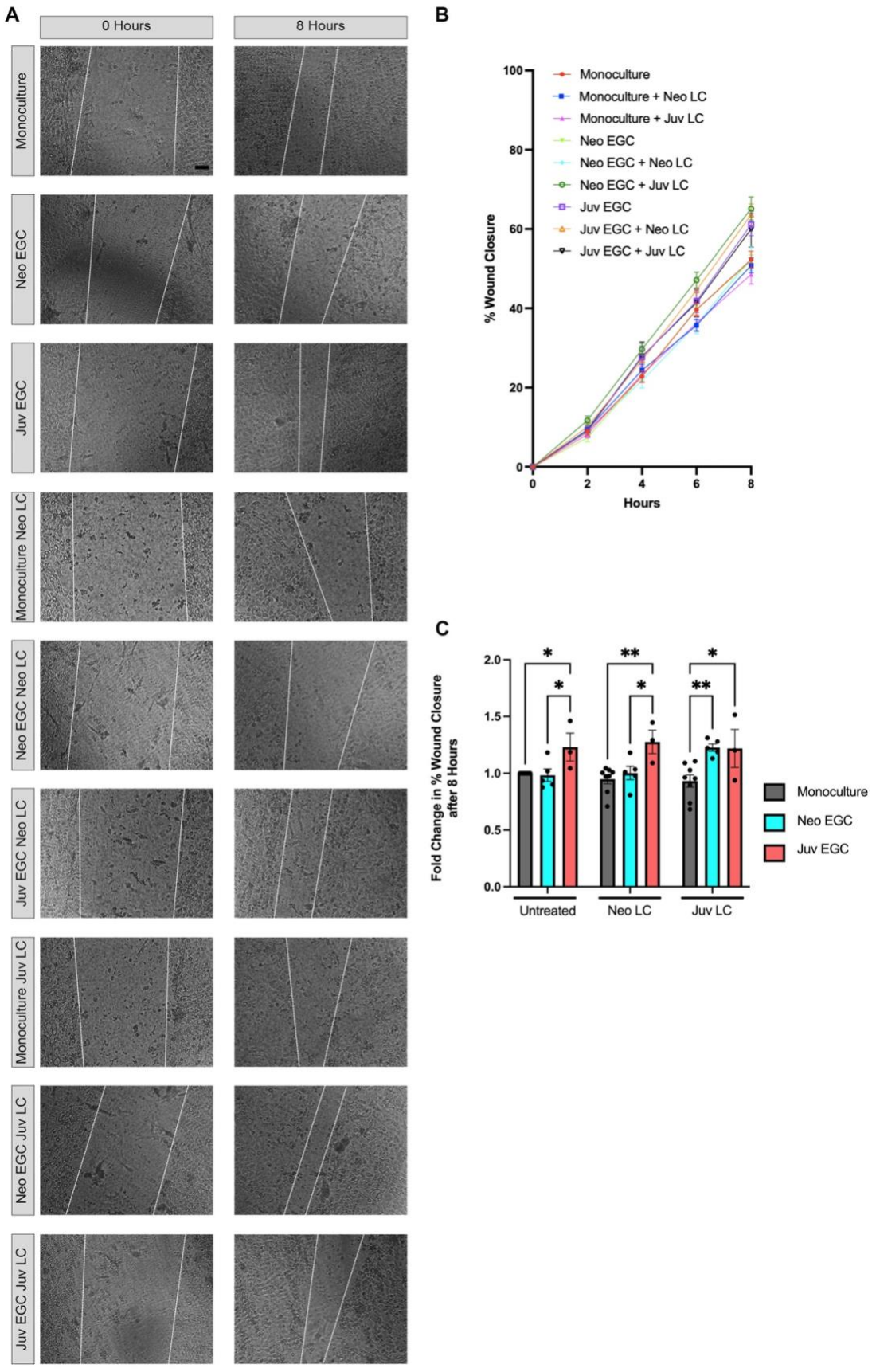
were analyzed by unpaired Mann-Whitney test for nonparametric data, one-way ANOVA, two-way ANOVA (or mixed-model on datasets with missing data points), or two-way ANOVA with repeated measures. For analyses where significance was detected by ANOVA, Tukey's multiple comparisons test was utilized for post hoc comparisons. The α-level for statistical significance was set at $P < 0.05$. For 16s rRNA sequencing, MicrobiomeAnalyst 2.0 software was used to generate figures and analyze results by performing a Welch's T-test for alpha diversity measures and pairwise PERMANOVA for beta diversity measures.[24]

4.4 Results

4.4.1 Juvenile lumen content rescues ability of neonatal EGC to promote intestinal epithelial wound healing in vitro

To determine the effect of lumen content and age of EGC on acute intestinal wound healing, we developed a non-contact co-culture scratch wound model in which IPEC-J2 monolayers were grown on 0.4 μm filters in the apical compartment and primary cultures of EGC isolated from the submucosal plexus of neonatal and juvenile pig colons were grown in the basolateral compartment. IPEC-J2 filters were co-cultured with EGC and dosed with lumen content from each respective age group 24 hours prior to the assay to allow for bidirectional paracrine signaling. Immediately before manually inflicting a scratch wound, IPEC-J2 monolayers received an additional half-dose of lumen content due to their unknown volatility (Figure 1A). We assessed percent wound closure over 8 hours finding that the greatest observable difference between the groups occurred at the 8-hour time point (Figure 1B). Controlling for differences between IPEC-J2 passages, we found that juvenile EGC significantly enhanced wound healing regardless of treatment (Figure 1C). Neonatal EGC were not capable of enhancing wound healing unless they had been exposed to juvenile lumen content (Figure 1C).

This suggests that neonatal EGC possess the innate ability to promote mucosal wound healing but lack proper signals from the surrounding microenvironment. To control for differences in lumen content dry matter between the age groups, we diluted all lumen content to the same concentration to prevent significant effects on IPEC-J2 viability (Figure S1A). We also did not identify any significant differences in the viability of our EGC between any of the conditions (Figure S1B). These results support our previous *ex vivo* findings that neonates exhibit a defect in acute intestinal epithelial wound healing but can be rescued by exposure to proper signaling molecules provided by juvenile lumen content.


A
0 Hours
8 Hours
Monoculture
Neo EGC
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Monoculture Neo LC
Neo EGC Neo LC
Juv EGC Neo LC
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Neo EGC Juv LC
Juv EGC Juv LC
B
Monoculture
Monoculture + Neo LC
Monoculture + Juv LC
Neo EGC
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Neo EGC + Juv LC
Juv EGC
Juv EGC + Neo LC
Juv EGC + Juv LC
% Wound Closure
Hours
C
Fold Change in % Wound Closure after 8 Hours
Untreated
Neo LC
Juv LC
Monoculture
Neo EGC
Juv EGC

Figure 4.1 Juvenile lumen content stimulates neonatal EGC to promote intestinal epithelial wound healing. A.) Representative phase-contrast images depict scratch wound healing over 8 hours of IPEC-J2 monolayers grown in monoculture or co-culture with primary cultures of neonatal enteric glial cells (Neo EGC) or juvenile enteric glial cells (Juv EGC) isolated from the colonic submucosal plexus of pigs and exposed to neonatal lumen content (Neo LC) or juvenile lumen content (Juv LC). B.) Time course depicts IPEC-J2 relative percent wound closure of these conditions over 8 hours. C.) Quantification of percent wound closure after 8 hours expressed as fold change over untreated monoculture controls. Data are expressed as mean ± SEM. Biological replicates n = 3-8, 2-3 technical replicates per condition. *P<0.05, **P<0.01 by two-way ANOVA with Tukey's multiple comparisons test. Scale bar = 100 μm (20X).

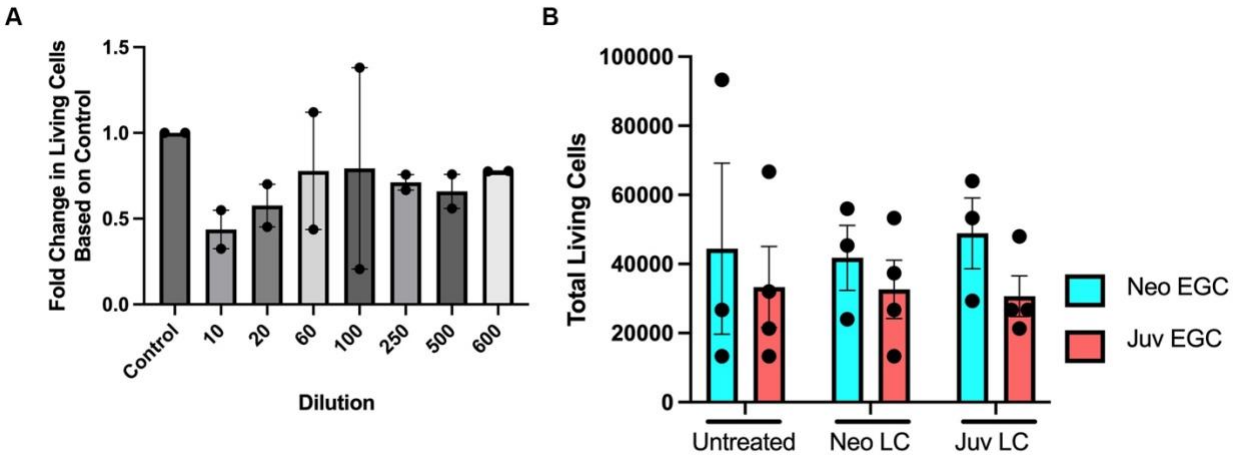

Figure 4.S1 Concentrations used of colonic lumen content from juvenile and neonatal pigs do not affect cell viability. A.) Quantification of living IPEC-J2 cells after 24 hours of exposure to lumen content expressed as fold change over untreated controls shows non-significant decreases at lower dilutions of lumen content that stabilize to less than 25% loss of viability at higher dilutions. B.) Quantification of total living enteric glial cells following scratch wound assay show no significant differences in cell number between neonatal (Neo EGC) and juvenile (Juv EGC) cultures of EGC nor significant differences in cell number based on treatment of scratch wound with neonatal lumen content (Neo LC) or juvenile lumen content (Juv LC). Data are expressed as mean ± SEM. Biological replicates n = 2, 2 technical replicates per condition for S1A. Biological replicates n = 3 for S1B. No significant differences were identified by one-way ANOVA with Tukey's multiple comparisons test for S1A or two-way ANOVA with Tukey's multiple comparisons test for S1B.

4.4.2 Juvenile lumen content specifically enables neonatal EGC to promote intestinal epithelial restitution in vitro

Considering we had previously attributed the neonatal defect in repair as a failure to adopt the migratory epithelial phenotype characteristic of restitution, we next verified that the effects we observed in our model were specific to restitution and not due to increased proliferation. To do so, we stained the injured IPEC-J2 monolayers from the prior experiments to assess the percent of cells that were immunoreactive for the proliferation marker Ki67 (Figure 2A). None of the EGC conditions significantly increased the percent of cells immunoreactive for Ki67 (Figure 2B). Interestingly, there was a significant decrease in Ki67+ epithelial cells co-cultured with the juvenile EGC control group and a trend toward decrease in the juvenile EGC co-culture group treated with juvenile lumen content (Figure 2B). These results indicate that the significant improvements in acute epithelial repair previously observed were due to increased restitution and not increased proliferation.

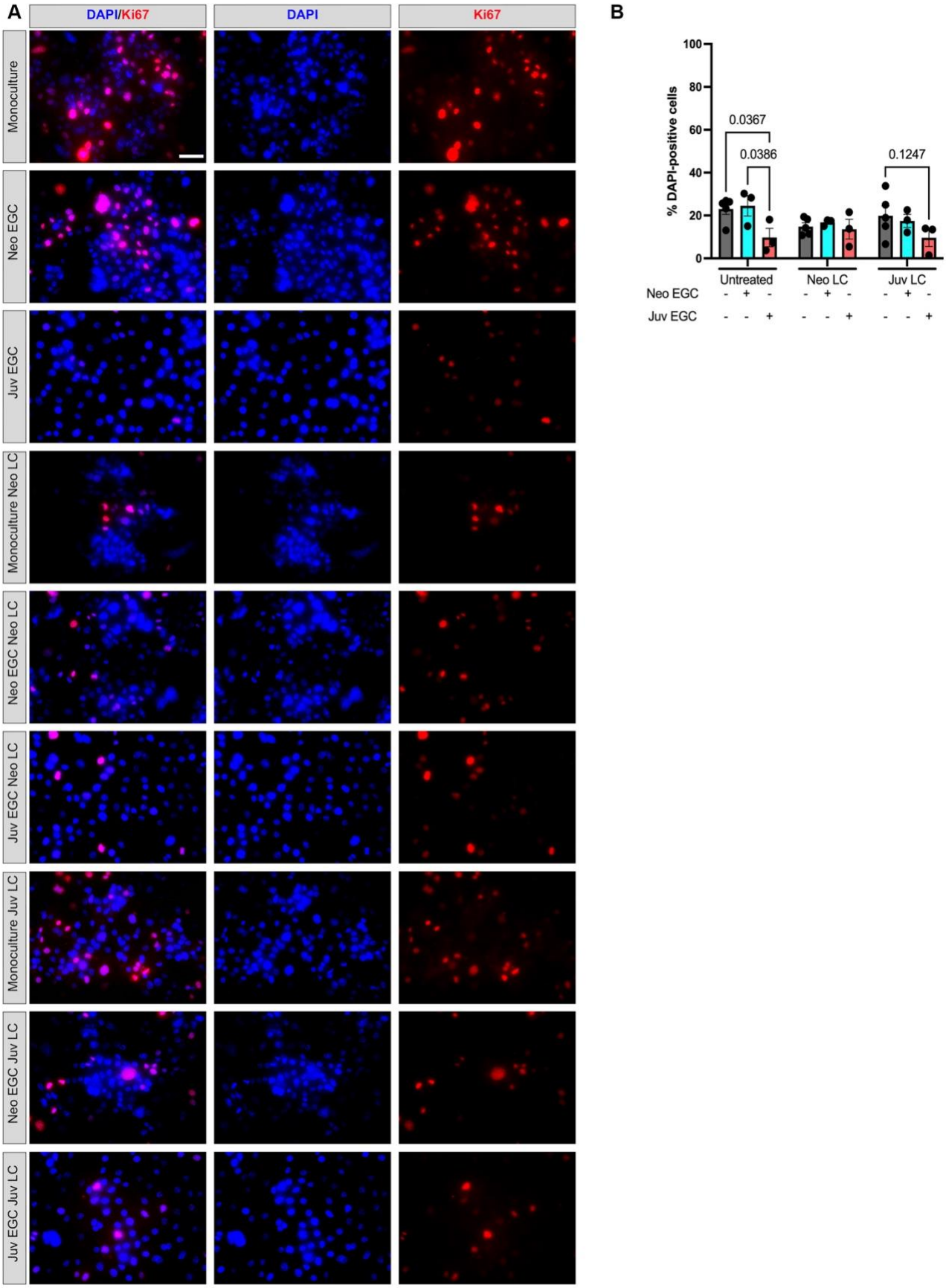
A
DAPI/Ki67
DAPI
Ki67
Monoculture
Neo EGC
Juv EGC
Monoculture Neo LC
Neo EGC Neo LC
Juv EGC Neo LC
Monoculture Juv LC
Neo EGC Juv LC
Juv EGC Juv LC
B
% DAPI-positive cells
100
80
60
40
20
0
0.0367
0.0386
0.1247
Untreated
Neo LC
Juv LC
Neo EGC
Juv EGC

Figure 4.2 Juvenile lumen content stimulates ability of neonatal EGC to enhance wound healing by promoting intestinal epithelial restitution specifically. A.) Representative immunofluorescent images of IPEC-J2 monolayers grown in monoculture or co-culture with primary cultures of neonatal enteric glial cells (Neo EGC) or juvenile enteric glial cells (Juv EGC) isolated from the colonic submucosal plexus of pigs and exposed to neonatal lumen content (Neo LC) or juvenile lumen content (Juv LC) stained for the proliferation marker Ki67 (red) 8 hours after scratch wounding. B.) Quantification of the average percent of IPEC-J2 cells immunoreactive for Ki67. Data are expressed as mean ± SEM. Biological replicates n = 3-5, 6 fields per condition. Significance assessed by two-way ANOVA with Tukey's multiple comparisons test. Scale bar = 50 μm (40X).

4.4.3 Juvenile lumen content upregulates release of SERPINB2

Considering that EGC primarily regulate wound healing through the release of paracrine factors, we questioned if exposure to juvenile lumen content results in different paracrine factors compared to neonatal lumen content leading to enhanced wound healing. To better investigate this, we performed mass spectrometry on conditioned supernatants from the basolateral chambers following scratch wounds to identify differentially expressed proteins between neonatal EGC exposed to juvenile lumen content compared to those treated with neonatal lumen content. Only one protein was significantly increased in basolateral supernatants from neonatal EGC treated with juvenile lumen content relative to those treated with neonatal lumen content and was identified as the serine protease inhibitor SERPINB2 (Figure 3A). We next replicated our co-culture model with IPEC-J2 monolayers in the apical compartment but instead exposed them to recombinant SERPINB2 protein in the basolateral compartment for 24 hours prior to inflicting the scratch wound (Figure 3B). After 8 hours, we found that the 1 ng/mL dose significantly increased the percent wound closure and fold change in wound closure relative to the vehicle (Figures 3C-D). The 10 ng/mL dose of SERPINB2 trended toward an increase in wound closure, while the 100 ng/mL dose had no effect on wound closure suggesting SERPINB2 likely has a dose-dependent effect that is seemingly unrelated to toxicity as neither of these doses significantly affected IPEC-J2 viability (Figure S2A). Overall, these results showed

that the protein SERPINB2 that was upregulated in scratch wounds of neonatal EGC co-cultured with IPEC-J2 cells exposed to juvenile lumen content is also capable of promoting intestinal epithelial restitution *in vitro*.

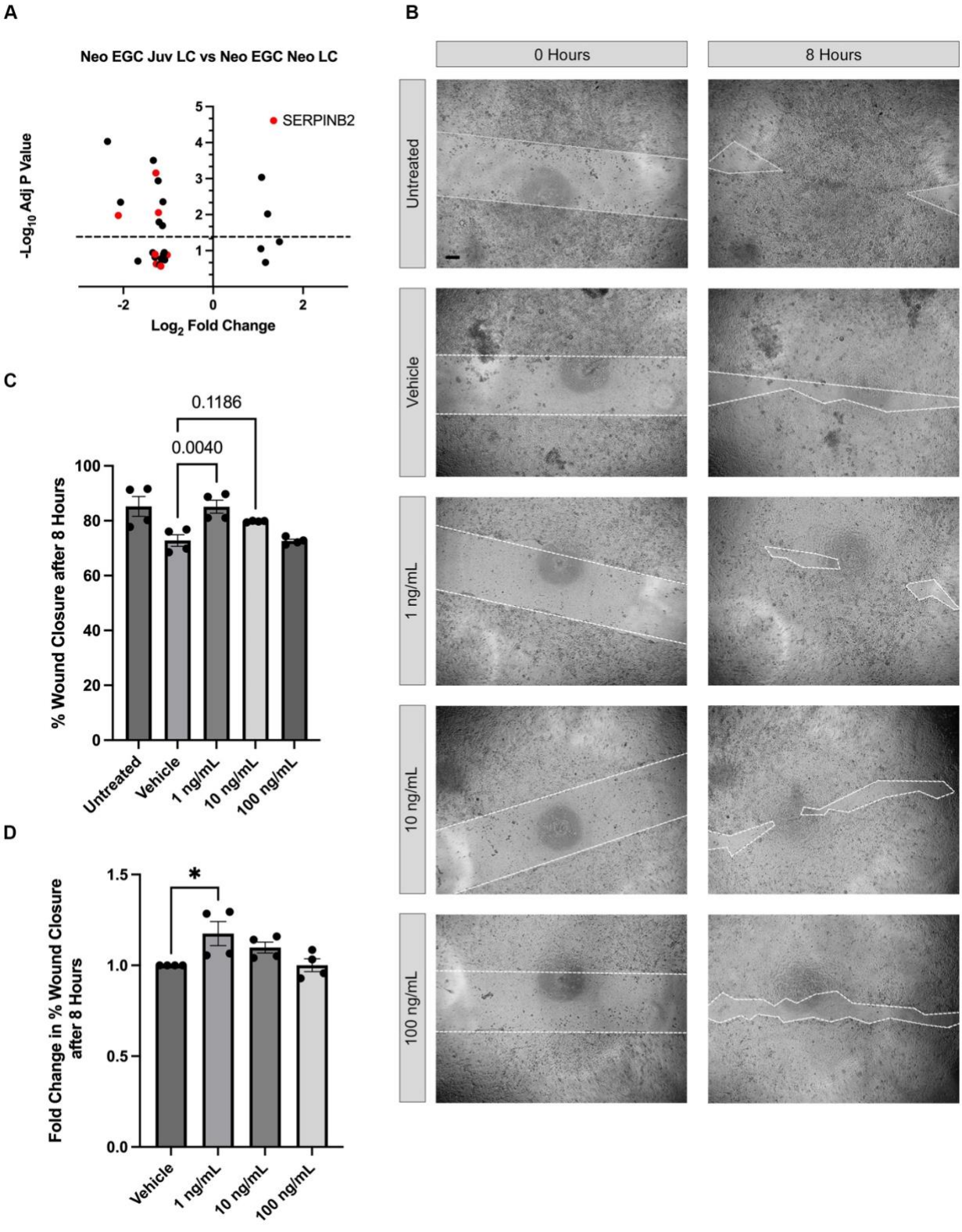
A
Neo EGC Juv LC vs Neo EGC Neo LC
SERPINB2
-Log10 Adj P Value
Log2 Fold Change
B
0 Hours
8 Hours
Untreated
Vehicle
1 ng/mL
10 ng/mL
100 ng/mL
C
0.1186
0.0040
% Wound Closure after 8 Hours
Untreated
Vehicle
1 ng/mL
10 ng/mL
100 ng/mL
D
Fold Change in % Wound Closure after 8 Hours
*
Vehicle
1 ng/mL
10 ng/mL
100 ng/mL

Figure 4.3 Juvenile lumen content upregulates release of SERPINB2 to promote intestinal epithelial restitution. A.) Volcano plot depicts differentially expressed proteins determined by mass spectrometry performed on supernatants isolated from the basolateral compartment of neonatal enteric glial cell (Neo EGC) co-cultures exposed to juvenile lumen content (Juv LC) versus neonatal lumen content (Neo LC) following scratch wound experiments. Biological replicates n = 5, 2-3 pooled technical replicates of each condition. Above dashed line indicates $P<0.05$. Red data points indicate proteins predicted to be secreted. B.) Representative phase-contrast images depict scratch wound healing over 8 hours of IPEC-J2 monolayers exposed basolaterally to 1, 10, and 100 ng/mL of SERPINB2 recombinant protein. C.) Quantification of relative percent wound closure after 8 hours identified a significant increase in wound healing in IPEC-J2 monolayers exposed to 1 ng/mL of SERPINB2 compared to the vehicle. D.) Quantification of percent wound closure after 8 hours expressed as fold change over vehicle-treated controls also identified a significant increase in wound healing in IPEC-J2 monolayers exposed to 1 ng/mL of SERPINB2 compared to the vehicle. Data are expressed as mean ± SEM. For 3B-D, biological replicates n = 4, 2-3 technical replicates per condition. *$P<0.05$ by one-way ANOVA with Tukey's multiple comparisons test. Scale bar = 200 μm (4X).

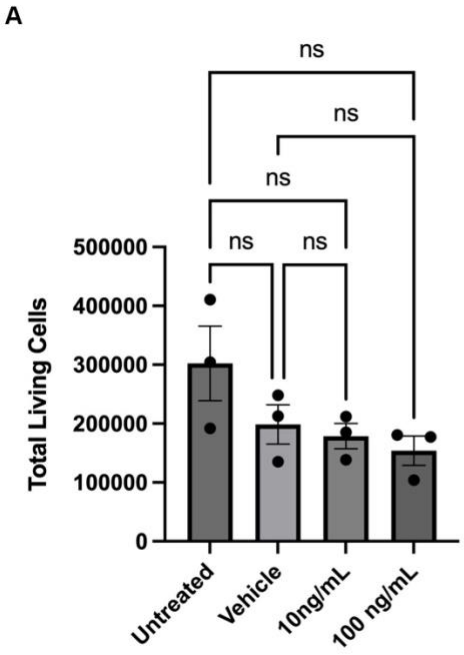

Figure 4.S2 Recombinant SERPINB2 dose does not affect cell viability. A.) Quantification of total living IPEC-J2 cells after 24 hours of exposure shows non-significant decreases in viability of cells exposed to the vehicle or SERPINB2 compared to untreated controls. Data are expressed as mean ± SEM. Biological replicates n = 3, 2 technical replicates per condition. No significant differences were identified by one-way ANOVA with Tukey's multiple comparisons test.

4.4.4 Microbial composition differs significantly between juvenile and neonatal lumen content

Juvenile and neonatal lumen content likely vary due to a number of factors including differences in diet (solid feed versus milk) and gut microbiota composition. One of the major differences between the juvenile and neonatal pigs in our study is weaning which occurs at 3 weeks of age. Given the differential responses of neonatal EGC to juvenile lumen content compared to neonatal lumen content and that this lumen content is likely heavily impacted by the microbial populations present, we postulated that a shift in gut microbiota composition may be the source of signals necessary for neonatal EGC to promote intestinal epithelial restitution. To investigate differences in gut microbiota compositions between these two age groups we performed 16s rRNA sequencing on unfiltered colonic lumen content. All measures of alpha diversity between juvenile and neonatal lumen content identified significant differences between the groups indicating a greater diversity of microbial populations in juveniles (Figure 4A). Additionally, beta diversity significantly differed between the age groups with neonatal samples clustering closely together while juveniles also clustered together but in a more widespread pattern (Figure 4B). To determine if there were any specific differences in the populations of gut microbes between neonates and juveniles, we next examined relative abundance at the genus level (4C). Several differences in relative abundance were identified between the two groups but the most striking was the genus *Mitsuokella*. *Mitsuokella* was present in all but one juvenile sample but was absent from all neonates except a very small contribution in one animal (4C). The *Mitsuokella* genus belongs to the Firmicutes phylum and is gram-negative bacteria known to produce SCFA that play a major role in intestinal physiology and interestingly, wound healing.

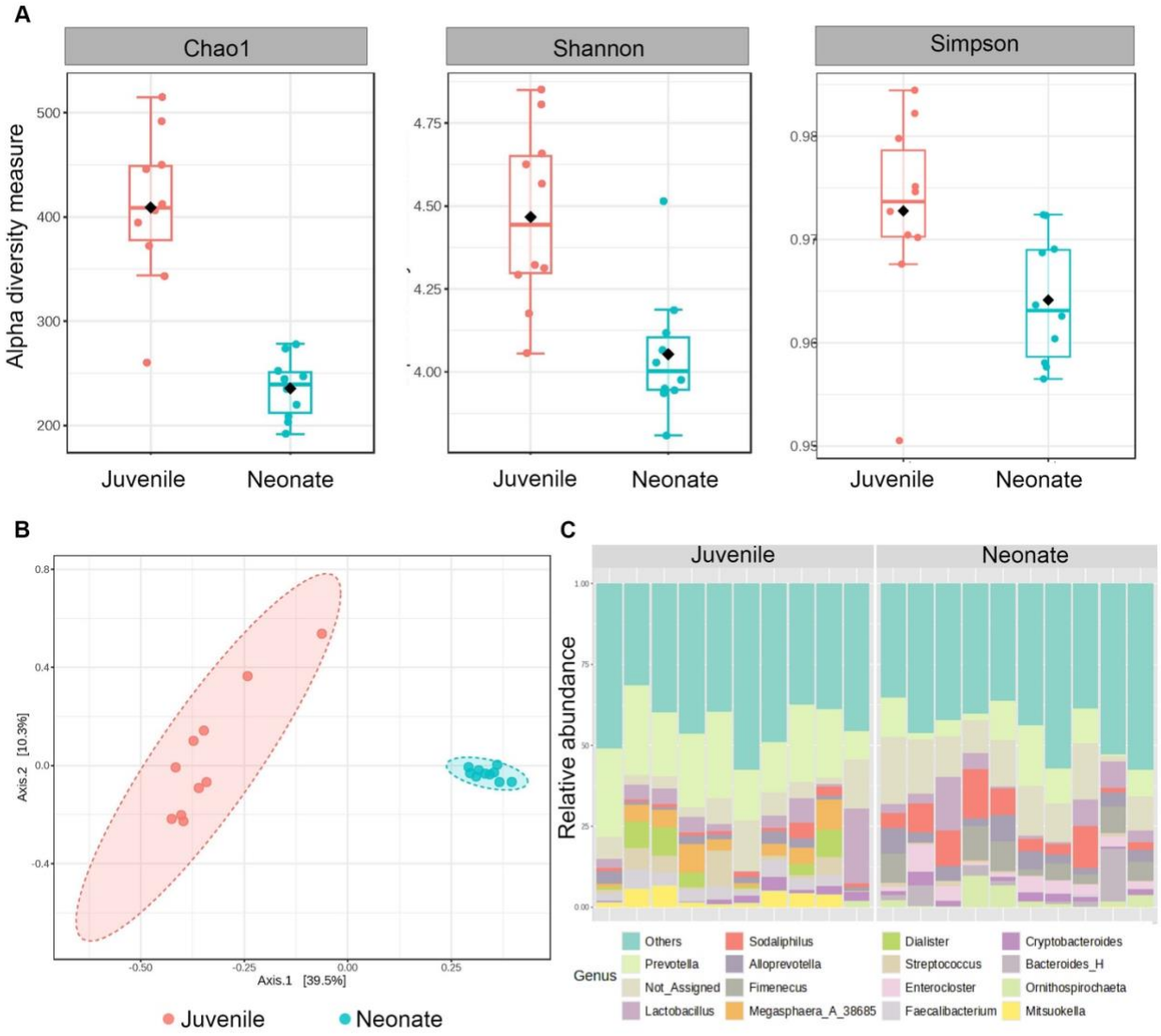

Figure 4.4 Microbial composition of juvenile pig colonic lumen content exhibits increased diversity compared to that of neonates. A.) Alpha diversity measures of Chao1 ($P<0.001$), Shannon ($P<0.001$), and Simpson ($P<0.05$) were significantly increased in juveniles as compared to neonates based on Welch's T-test. B.) Beta diversity measure based on Bray-Curtis dissimilarity matrix was significantly different between juveniles and neonates based on pairwise PERMANOVA ($P=0.001$). C.) Relative abundance of gut microbial composition at the genus level between juvenile and neonatal colonic lumen content. Biological replicates n = 10.

4.4.5 Short chain fatty acid concentration and composition differs significantly between juvenile and neonatal lumen content

Gut microbe composition differed significantly between the colons of juvenile and neonatal pigs and most notably, a major missing genus missing from neonates but present in juveniles known as *Mitsuokella* is a known producer of SCFAs. This piqued our interest into whether the observed differences in gut microbiota composition were reflected in the concentrations of SCFAs in our juvenile and neonatal lumen content. SCFAs are one of the main metabolic byproducts produced during microbial fermentation in the intestine and have been shown to influence intestinal health including the epithelial barrier, so we next quantified SCFA in our colonic lumen content. The concentration of the SCFAs acetate, propionate, butyrate, valerate, isobutyrate, and isovalerate were significantly higher in juvenile lumen content compared to that of neonates (Figure 5A-F). Based on their diet of solid feed with more indigestible fiber to support microbial fermentation and higher absolute colonic microbe numbers, it was expected that the concentration of SCFAs would be higher in juvenile pigs. To account for this, we also compared the percent of total each of our SCFAs comprised for both age groups (5A-F). Here we found that the contribution of propionate and butyrate to total SCFA concentration was higher in juveniles, while that of isobutyrate and isovalerate was lower (5B-C, 5E-F). This supports that SCFAs and particularly propionate and butyrate may be one of the signals provided to EGC necessary to promote intestinal epithelial restitution in mature animals.

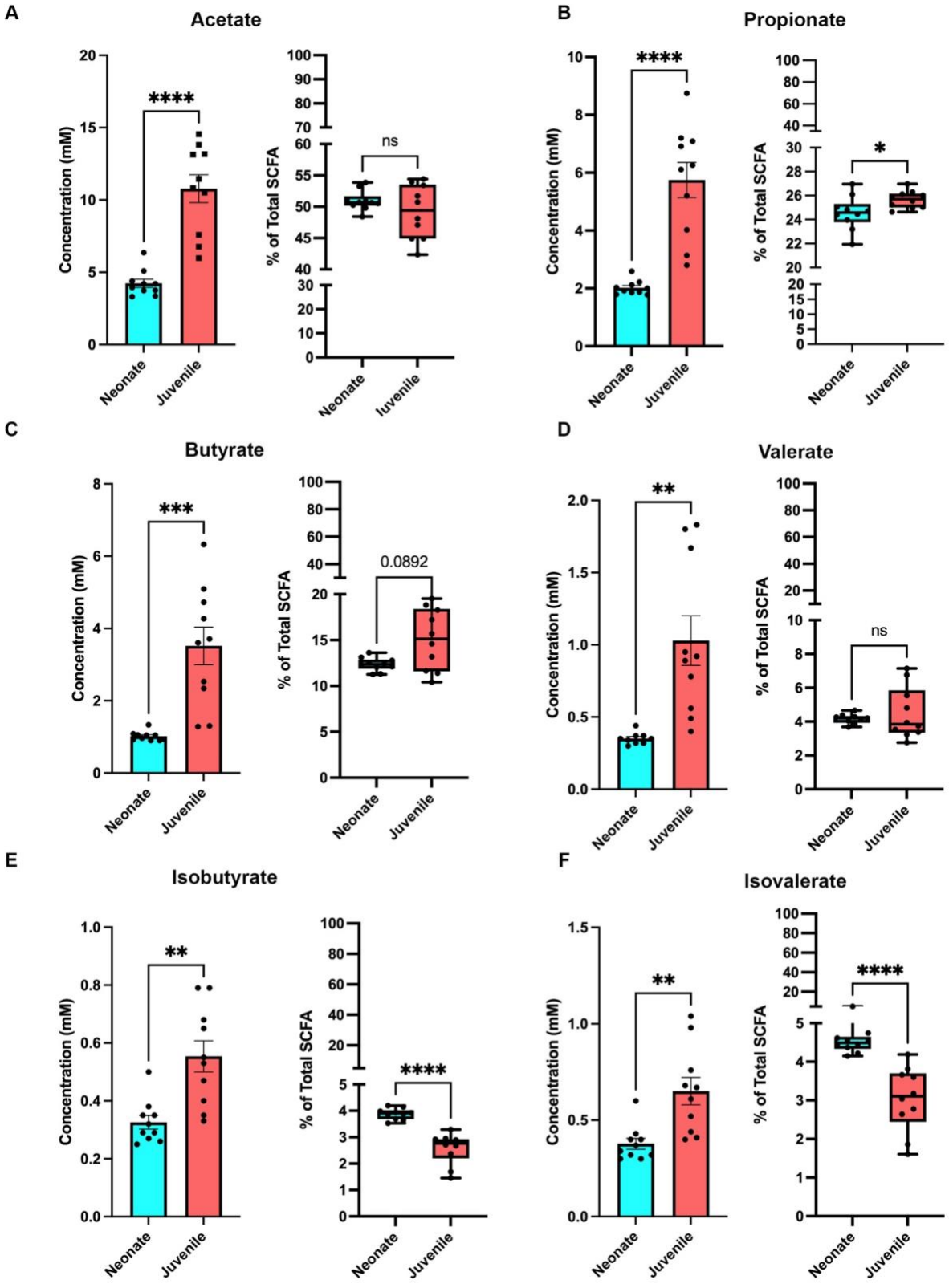
A
Acetate
Concentration (mM)
****
Neonate
Juvenile
% of Total SCFA
ns
B
Propionate
****
*
C
Butyrate
***
0.0892
D
Valerate
**
ns
E
Isobutyrate
**
****
F
Isovalerate
**
****

Figure 4.5 Short chain fatty acid (SCFA) composition differs between colonic lumen content of juvenile and neonatal pigs. A.) Absolute concentration and percent of total SCFA concentration for acetate in neonatal versus juvenile pig colonic lumen content. B.) Absolute concentration and percent of total SCFA concentration for propionate in neonatal versus juvenile pig colonic lumen content. C.) Absolute concentration and percent of total SCFA concentration for butyrate in neonatal versus juvenile pig colonic lumen content. D.) Absolute concentration and percent of total SCFA concentration for valerate in neonatal versus juvenile pig colonic lumen content. E.) Absolute concentration and percent of total SCFA concentration for isobutyrate in neonatal versus juvenile pig colonic lumen content. F.) Absolute concentration and percent of total SCFA concentration for isovalerate in neonatal versus juvenile pig colonic lumen content. Data are expressed as mean ± SEM. Biological replicates n = 10. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 by unpaired T-test for absolute concentrations and Mann-Whitney test for percent of total.

4.4.6 Butyrate recapitulates effect of juvenile lumen content on neonatal EGC ability to promote intestinal epithelial wound healing in vitro

As one of the major SCFAs and primary energy source of colonocytes, butyrate plays a variety of roles in intestinal health and barrier regulation including promotion of wound healing. Based on this and our findings that butyrate was one of the only SCFAs that was increased in absolute and relative concentration in juvenile lumen content, we postulated that butyrate may be capable of inducing neonatal EGC to promote epithelial restitution. To investigate this, we utilized our original non-contact co-culture model with IPEC-J2 monolayers and primary cultures of EGC but treated the apical compartment with neonatal (1 mM) and juvenile (5 mM) concentrations of sodium butyrate 24 hours prior to scratch wounding (Figure 6A). We found that while butyrate appeared to have an overall effect of decreasing wound healing, the juvenile concentration of 5 mM butyrate significantly enhanced the ability of neonatal EGC to promote epithelial restitution unlike the neonatal concentration of 1 mM butyrate (Figure 6B). We verified that this effect was specific to butyrate as similar findings were not identified for acetate, while propionate exhibited a trend toward increasing neonatal EGC ability to enhance epithelial restitution at the juvenile concentration (Figure 6C). We also confirmed that none of the doses

tested had significant effects on the viability of IPEC-J2 monolayers or EGC, but butyrate did trend toward a dose-dependent toxicity toward IPEC-J2 cells perhaps explaining the observed decreases in restitution at higher doses (Figure S3A-F). From these results, we concluded that juvenile concentrations of butyrate are sufficient to recapitulate the effect of juvenile lumen content on enhancing the ability of neonatal EGC to promote epithelial restitution *in vitro*.

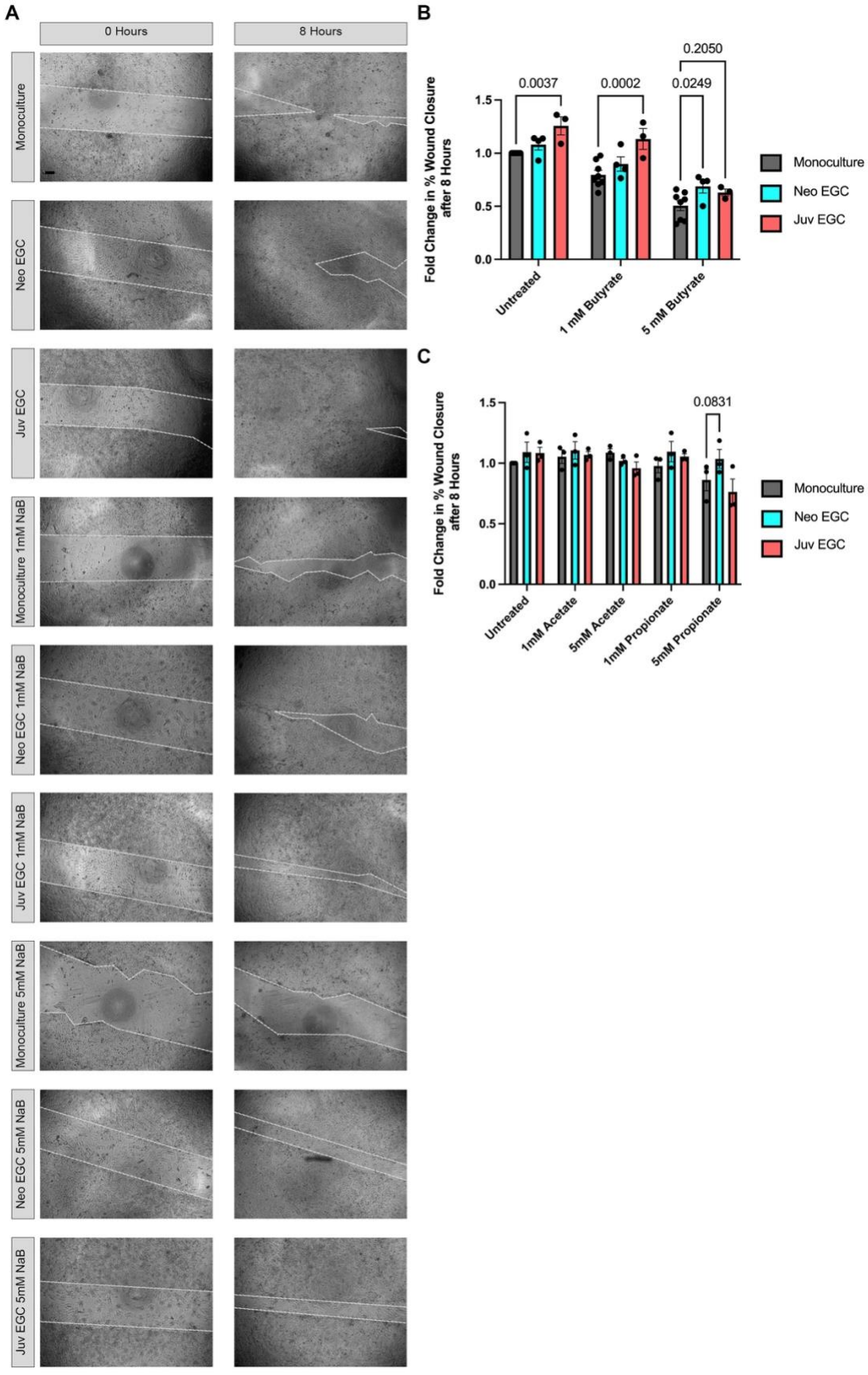
A
0 Hours
8 Hours
Monoculture
Neo EGC
Juv EGC
Monoculture 1mM NaB
Neo EGC 1mM NaB
Juv EGC 1mM NaB
Monoculture 5mM NaB
Neo EGC 5mM NaB
Juv EGC 5mM NaB
B
Fold Change in % Wound Closure after 8 Hours
0.0037
0.0002
0.0249
0.2050
1.5
1.0
0.5
0.0
Untreated
1 mM Butyrate
5 mM Butyrate
Monoculture
Neo EGC
Juv EGC
C
Fold Change in % Wound Closure after 8 Hours
0.0831
1.5
1.0
0.5
0.0
Untreated
1mM Acetate
5mM Acetate
1mM Propionate
5mM Propionate
Monoculture
Neo EGC
Juv EGC

Figure 4.6 Juvenile concentrations of butyrate enable neonatal EGC to promote intestinal epithelial wound healing *in vitro*. A.) Representative phase-contrast images depict scratch wound healing over 8 hours of IPEC-J2 monolayers grown in monoculture or co-culture with primary cultures of neonatal enteric glial cells (Neo EGC) or juvenile enteric glial cells (Juv EGC) isolated from the colonic submucosal plexus of pigs and exposed to 1 mM or 5 mM of sodium butyrate (NaB). B.) Quantification of percent wound closure after 8 hours expressed as fold change over untreated monoculture controls. C.) Quantification of percent wound closure after 8 hours expressed as fold change over untreated monoculture controls for IPEC-J2 monolayers grown in monoculture or co-culture with primary cultures of neonatal enteric glial cells (Neo EGC) or juvenile enteric glial cells (Juv EGC) isolated from the colonic submucosal plexus of pigs and exposed to 1 mM or 5 mM of sodium acetate or sodium propionate. Data are expressed as mean ± SEM. Biological replicates n = 3-8, 2-3 technical replicates per condition for 6A-B. Biological replicates n = 3, 2-3 technical replicates per condition for 6C. Significance assessed by two-way ANOVA with Dunnett's multiple comparisons test. Scale bar = 200 μm (4X).

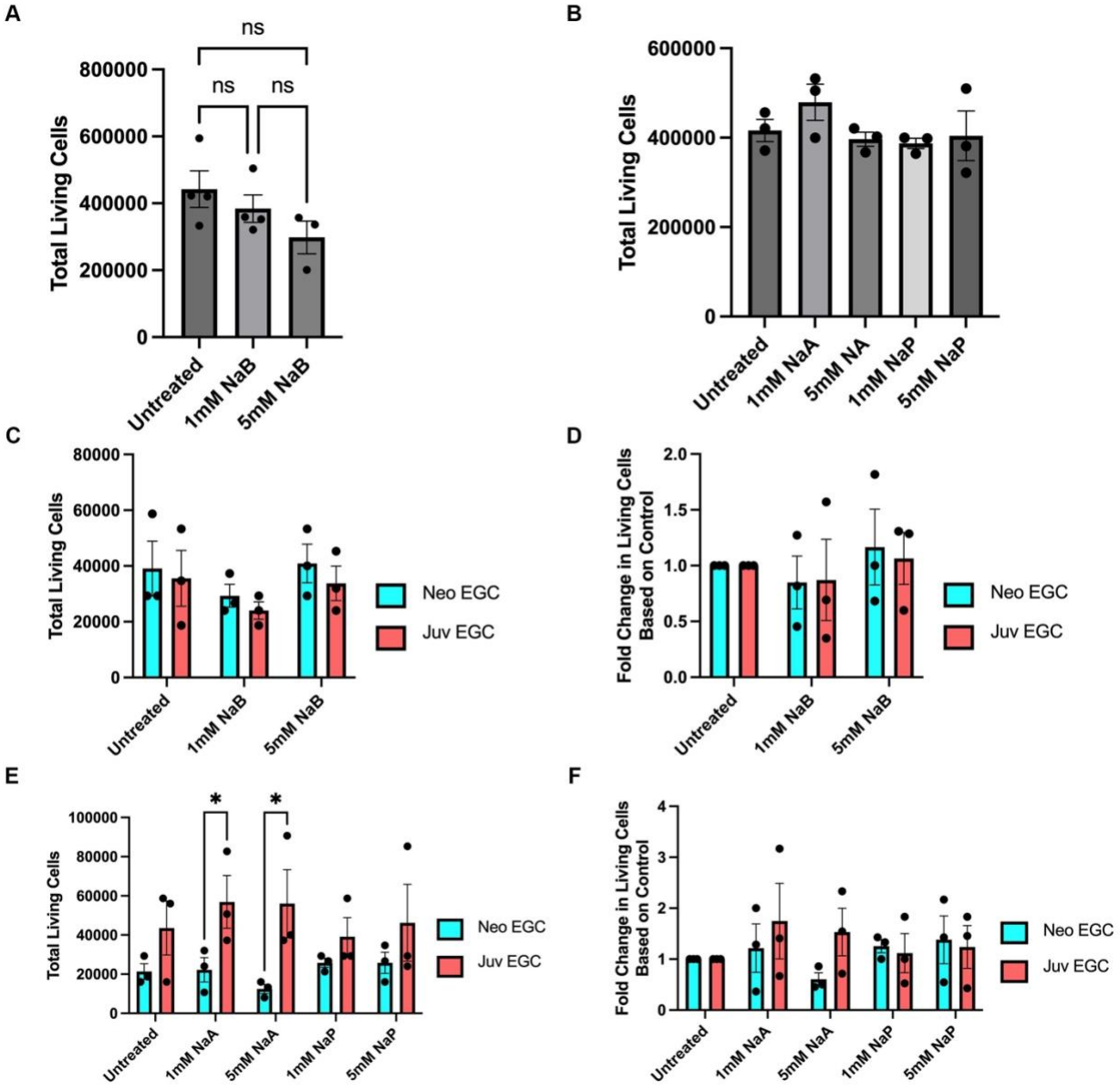

Figure 4.S3 Concentrations used of butyrate, propionate, and acetate used do not affect cell viability. A.) Quantification of living IPEC-J2 cells after 24 hours of exposure to 1 mM and 5 mM sodium butyrate (NaB) show non-significant decreases in cell viability. B.) Quantification of living IPEC-J2 cells after 24 hours of exposure to 1 mM and 5 mM sodium acetate (NaA) or 1 mM and 5 mM sodium propionate (NaP) show no significant changes in cell viability. C.) Quantification of total living enteric glial cells following scratch wound assay show no significant differences in cell number between neonatal (Neo EGC) and juvenile (Juv EGC) cultures nor significant differences in cell number based on exposure to 1 mM or 5 mM NaB. D.) Quantification of total living enteric glial cells expressed as fold change over untreated control cultures of EGC for respective age groups following scratch wound assay show no significant differences in cell number based on exposure to 1 mM or 5 mM NaB. E.) Quantification of total living enteric glial cells following scratch wound assay show no significant differences in cell number between Neo EGC and Juv EGC nor significant differences in cell number based on

exposure to 1 mM or 5 mM NaA or NaP. F.) Quantification of total living enteric glial cells expressed as fold change over untreated control cultures of EGC for respective age groups following scratch wound assay show no significant differences in in cell number based on exposure to 1 mM or 5 mM NaA or NaP. Data are expressed as mean ± SEM. Biological replicates n = 3-4, 2 technical replicates per condition for S3A-B. Biological replicates n = 3 for S3C-F. No significant differences were identified by one-way ANOVA with Tukey's multiple comparisons test for S3A-B or two-way ANOVA with Tukey's multiple comparisons test for S3C-F.

4.5 Discussion

Following surgically induced mesenteric ischemia, neonatal pigs exhibit a deficiency in acute intestinal wound healing. This is characterized by a failure of enterocytes neighboring the injury to adopt a migratory phenotype indicating that this is a specific defect in the restitution step of barrier restoration.[11,25] Juvenile pigs do not exhibit this defect and are efficiently capable of restoring barrier function during an acute recovery period indicating that acute intestinal repair mechanisms develop postnatally. While considering what other postnatal developments may influence acute intestinal barrier repair, EGC emerged as an interesting candidate. EGC, along with enteric neurons, comprise the enteric nervous system which regulates nearly all gastrointestinal functions. A population of EGC colonize the lamina propria after birth in rodents forming a network believed to regulate barrier functions given their proximity to the intestinal epithelium. This colonization process was previously shown to depend on the presence of gut microbiota which also develops primarily after birth with major shifts seen after weaning.[10,19,26]

Given this relationship, we postulated that an immature relationship between EGC, gut microbiota, and the intestinal epithelium may explain neonatal defects in acute barrier repair. Thus, we first assessed the effect of age on intestinal epithelial wound healing using an *in vitro* non-contact co-culture scratch model in which IPEC-J2 monolayers were grown with primary cultures of neonatal and juvenile EGC isolated from the colonic submucosal plexus of pigs and

exposed these monolayers to filtered colonic lumen content from neonatal or juvenile pigs. This experiment supported that juvenile EGC promote intestinal epithelial wound healing regardless of the condition while neonatal EGC lack this ability agreeing with our previous *ex vivo* findings.[2] However, exposure to juvenile lumen content stimulated neonatal EGC to promote wound healing also similar to our reports that application of homogenized injured juvenile intestine rescues neonatal barrier repair *ex vivo*.[2] Additionally, because acute intestinal epithelial repair reportedly occurs prior to proliferation, we wanted to verify that the effects we saw of lumen content age and co-culture condition were not due to increases in epithelial proliferation. Staining of IPEC-J2 monolayers after these scratch wounds for the proliferation marker Ki67 revealed that neither co-culture condition nor lumen content exposure significantly increased the percent of cells immunoreactive for Ki67. In fact, juvenile EGC significantly decreased the percentage of proliferating epithelial cells when untreated and trended toward a similar outcome in those exposed to juvenile lumen content. This further supports that any observed changes were due to increased intestinal epithelial restitution during wound healing and not increased proliferation. Interestingly, neonatal EGC had no effect on the percentage of proliferating epithelial cells which may indicate another aspect in which neonatal EGC are immature as EGC have been previously shown to inhibit intestinal epithelial proliferation highlighting an area for further research.[6,7]

Considering EGC are known for their ability to modulate intestinal epithelial function largely via the release of paracrine signaling molecules, we next assessed protein differences in the basolateral supernatants which we reasoned would primarily contain molecules released by EGC. To do so, we performed mass spectrometry to identify proteins that were differentially expressed between co-cultures of neonatal EGC exposed to neonatal versus juvenile lumen

content. We identified SERPINB2 as the only protein predicted to be secreted and significantly increased in neonatal EGC exposed to juvenile lumen content versus neonatal lumen content. SERPINB2 or plasminogen activator inhibitor-2 (PAI-2) is a serine protease inhibitor originally noted for its role in the coagulation cascade as an inhibitor of urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA) both of which convert plasminogen to its active form plasmin.[27] SERPINB2 has received the moniker the "undecided" serpin due to a lack of consensus regarding its functions including those outside of uPA and tPA inhibition as well as its method of secretion. Accordingly, SERPINB2 has been associated with an array of cell types and processes including proliferation and differentiation, inhibition of apoptosis, and senescence as well as molecular interactions such as those with retinoblastoma proteins, annexins, and vitronectin.[28-37] Clinically, SERPINB2 upregulation has been correlated with a litany of inflammatory conditions and is reportedly one of the most highly upregulated genes in inflammatory bowel disease.[38] In cancer, SERPINB2 has paradoxical effects acting to promote tumor growth in some while exhibiting tumor-suppressor qualities in others [39-42]. Relevant to our studies, some of these report that SERPINB2 increases cell motility and migration while other report that it decreases these properties.[27,39,40,42] For our purposes, we investigated the effects of varying concentrations of basolateral exposure to recombinant SERPINB2 protein on IPEC-J2 intestinal epithelial wound healing and found that our lowest dose significantly increased wound closure over 8 hours. Based on the literature and these findings, it is likely that SERPINB2 has unique functions that are dose-dependent and specific to cell type. As future studies seek to elucidate the precise mechanisms of SERPINB2 it will be important to consider the effects of these various experimental designs. To our knowledge, this is the first study to explore the effect of SERPINB2 on intestinal epithelial cell wound healing and despite its association with

inflammatory bowel disease, this may be not be due to its direct effects on the intestinal epithelium but rather upregulation of SERPINB2 in immune cells, especially macrophages.[43] Regardless, the role of SERPINB2 in the intestinal epithelium and in the context of wound healing deserves further research.

Additionally, in the present study we also verified that the observed increases in intestinal restitution induced by neonatal EGC exposed to juvenile lumen content could be attributed to differences in gut microbe composition between juveniles and neonates. We first assessed this using 16s rRNA sequencing and found that juvenile colonic lumen samples were significantly more diverse than neonates. This was expected because the neonatal pigs in this study were still suckling while the juveniles had already been weaned and exposed to solid, fiber-containing feed. In our relative abundance comparison, a single bacterial genus was present in almost all juveniles and virtually absent from neonates and was identified as *Mitsuokella* belonging to the Firmicutes phylum. *Mitsuokella* are gram-negative obligate anaerobes also found in the human intestinal tract that ferment carbohydrates into SCFAs.[44,45] In terms of disease, decreased abundance of *Mitsuokella* has been linked to irritable bowel syndrome and interestingly was one of the most significantly depleted genera in children with pediatric Crohn's disease.[46,47] This agrees with our findings and supports that *Mitsuokella* may provide key signals required for intestinal mucosal repair.

Knowing that a major producer of SCFAs was mostly absent in our neonatal lumen content samples and that SCFAs are integral to intestinal barrier function, we proceeded to quantify the SCFAs between juveniles and neonates in terms of absolute concentration and percent of total. These results showed that while juveniles exhibited significant increases in absolute concentration for each of the six SCFAs analyzed, only butyrate and propionate were

significantly increased in percent of total. This led us to consider that butyrate or propionate could be the signal present in juvenile lumen content necessary to stimulate neonatal EGC to promote intestinal epithelial repair. We chose to first focus on butyrate as it has a well-established role in intestinal homeostasis. For one, butyrate is the preferred energy source of colonocytes and been shown to support intestinal integrity by increasing resistance and decreasing permeability of the epithelial barrier.[13-15,48-51] Moreover, studies have highlighted the ability of butyrate to promote intestinal epithelial wound healing *in vitro*, alleviate intestinal inflammation, and improve outcomes from experimental neonatal necrotizing enterocolitis (NEC) and DSS-induced colitis *in vivo*.[13,16,52-57] Butyrate induces these effects by activating pathways governed by SCFA-specific G-protein coupled receptors or by entering cells where butyrate has been shown to modulate gene expression acting as a histone deacetylase (HDAC) inhibitor.[13,58] Unlike results from others, butyrate had an overall effect of decreasing wound closure in our study and seemingly decreased the ability of juvenile EGC to promote intestinal wound healing at the 5 mM dose. However, the juvenile concentration of butyrate at 5 mM was able to recapitulate the effect of juvenile lumen content by stimulating neonatal EGC to promote intestinal epithelial wound healing. Differences in study design may explain the discrepancy in effect of butyrate on wound healing. The majority of other studies have looked at wound healing over periods >36 hours only seeing significant increases in healing at later time points, whereas we analyzed an 8-hour period to focus more specifically on acute intestinal restitution.[13,16] Additionally, our results may have differed due to our use of the IPEC-J2 cell line derived from the jejunum of a neonatal pig which we chose for higher fidelity to physiology as compared to tumorigenic cell lines. We still chose to utilize EGC and lumen content from the colon because the quantity of microbiota is greatest here and we theorized this design would best indicate the

relationship between EGC and gut microbial products. Future studies focusing solely on the small intestine and colon are warranted to better clarify this relationship. Lastly, varying doses of butyrate appear to have differing effects on intestinal epithelial cells and may have accounted for some of our results. As for the other SCFAs we tested, neither acetate nor propionate had any significant effects on wound healing, though there was a trend at the 5 mM dose of propionate toward improving neonatal EGC wound healing ability. While some studies have found that acetate and propionate do not significantly improve intestinal wound healing compared to butyrate similar to us, others show that propionate and acetate produce similar increases to butyrate.[13,59,60] Again, these variations in results are likely due to the use of different cell types and concentrations of SCFAs. All three of the SCFAs tested in our study have been shown to act as HDAC inhibitors to varying extents with butyrate possessing the greatest activity followed by propionate and the least activity seen in acetate.[61] Considering this, it is possible that the effect observed in our study is the result of butyrate acting as an HDAC inhibitor to modulate gene expression in neonatal EGC which would explain why a similar, yet non-significant trend was observed for propionate. It is thus tempting to consider whether butyrate might exert its effects in our study by increasing expression of SERPINB2 in neonatal EGC via HDAC inhibition and is a focus of ongoing studies.

Overall, this study sought to elucidate mechanisms responsible for the age-dependent relationship between EGC, gut microbiota, and acute intestinal epithelial wound healing. We show that neonatal EGC can be modulated by the mature luminal microenvironment to improve intestinal epithelial wound healing and that this appears to be a maturity dependent process. Ongoing studies to understand these interactions may lead to novel life-saving interventions as neonates of younger ages have been shown to suffer worse clinical outcomes from intestinal

barrier damage in ischemia-related diseases including volvulus and NEC.[62,63] Furthermore, these results exemplify the ability to alter EGC function using external stimuli which may prove useful in the treatment of various intestinal diseases associated with dysfunctional EGC such as inflammatory bowel disease. We also identified SERPINB2 as a novel candidate for improving acute intestinal wound healing that holds therapeutic potential for other disorders associated with the intestinal barrier. This study was limited in its ability to fully characterize our juvenile and neonatal colonic lumen content which may have highlighted other molecules including dietary components responsible for the observed effects. Additionally, due to our co-culture model, we are unable to definitively say whether the lumen content acted directly on EGC to drive the observed effects or acted indirectly using the intestinal epithelium as an intermediary. Finally, we did not test whether higher concentrations of butyrate found in juvenile lumen content specifically induced increased SERPINB2 expression in neonatal EGC. Future work aims at addressing these limitations and will be essential in clarifying the mechanisms involved. Such discoveries will not only further our understanding of acute intestinal epithelial wound healing but may also help discover novel therapeutics applicable in the treatment of disorders related to intestinal barrier dysfunction.

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Chapter 5

Discussion and Future Directions: Insights into an Age-Dependent Relationship Between the Epithelium, Enteric Glial Cells, and Microbiota During Acute Intestinal Wound Healing

5.1 Clinical Relevance

Proper functioning and integrity of the intestinal barrier is essential to homeostasis and survival. Breaches in the barrier can precipitate life-threatening diseases due to the translocation of luminal content across the intestinal epithelial monolayer where these luminal agents may activate underlying immune cells or enter systemic circulation resulting in overwhelming inflammation. While the process of barrier repair has been well described as occurring in three distinct and overlapping steps, we still do not fully understand the mechanisms involved. Further, despite evidence suggesting barrier functions such as permeability mature postnatally, the development of barrier repair mechanisms has not been extensively investigated. This highlights a critical knowledge gap with real world implications as neonates of all species suffer worse outcomes from diseases that result in barrier breakdown. In dogs, mortality from canine parvovirus was reported to be 91% in puppies compared to 10% in adults.[1] Survival from porcine epidemic diarrhea virus is also age-dependent with mortality in piglets under 10 days old reaching up to 100% and dropping to 10% in pigs older than 10 days.[2] For equines, survival from surgical colic treatment was less than 10% in foals under 14 days of age compared to 45% for foals between ages 15 and 150 days.[3] Similar trends are seen in human infants with neonatal necrotizing enterocolitis (NEC) which occurs due to a complex pathophysiology involving ischemic injury to the intestinal epithelial barrier. Prematurity has been identified as one of the most important contributing factors to NEC development.[4] Despite increased preventative

measures, the mortality of NEC has not significantly improved over the past 10 years, and it remains a leading cause of death in preterm infants with mortality rates of 20-50%.[5] Additionally, current treatments for NEC do not target the source of the systemic inflammation caused by intestinal barrier damage and treatment often requires surgical removal of the affected bowel further increasing mortality rates.[6] Advancing our understanding of neonatal intestinal barrier repair is therefore critical for developing effective interventions to reduce the high mortality associated with barrier-related diseases in all species.

5.2 Summary of Findings

While trying to understand the discrepancy in outcomes from intestinal barrier damage between ages, our laboratory found that neonatal pigs exhibit a defect in epithelial restitution characterized by an apparent lack of enterocyte migration ultimately resulting in failure to restore barrier function but rescuable by exposure to injured juvenile intestinal tissue.[7] Given their role in barrier regulation, we then postulated an underdeveloped network of subepithelial enteric glial cells (EGC) in neonates may be a contributing factor.[8] This population of EGC develops postnatally and requires colonization by gut microbiota in rodents.[9] Considering that both the gut microbiota and EGC regulate barrier function, this highlights a tridirectional nexus in which all three interact with and influence one another. The exact mechanisms of these relationships are not fully understood as many studies have looked at the individual parts rather than the relationship as a whole. This is especially important in the context of development as functionality of the intestinal epithelium, EGC, and gut microbiota all involve maturation after birth and may explain the observed age-dependence of acute intestinal barrier repair, but this has not been studied. Thus, the work presented here sought to fill this knowledge gap by utilizing a

porcine model of intestinal epithelial wound healing to elucidate the age-dependent relationship between the intestinal epithelium, EGC, and the gut microbiota.

In Chapter 1, a literature review was performed to summarize functions of the intestinal epithelial barrier as well as its regulation by enteric neurons and glia comprising the enteric nervous system (ENS) and gut microbiota. We also describe the prenatal and postnatal aspects of intestinal barrier development and maturation of the intestinal epithelium itself as well as its regulators. We then propose that acute intestinal wound healing is a barrier property that, similar to permeability regulation, develops postnatally driven by maturation of the ENS and gut microbiota. This chapter provides relevant background information regarding the intestinal barrier, ENS, and gut microbiota and highlights a gap in knowledge as to when and how the intestine develops mechanisms necessary for intestinal epithelial wound healing.

The relationship between intestinal mucosal repair and the ENS is further interrogated in Chapter 2, specifically in the context of intestinal ischemia/reperfusion (I/R) injury. Ischemic injuries to the intestine are one of the major causes of barrier damage and provide a relevant clinical model to study repair as these injuries can be carefully titrated by altering the length of ischemia. This chapter summarizes the pathophysiology of intestinal I/R injury and details the observed effects on the ENS morphologically and functionally. While many studies have investigated the aftermath of intestinal I/R injury on the ENS, we also highlight evidence suggesting that the ENS may be a possible target for improving outcomes from intestinal I/R injury especially given the role of the ENS in barrier regulation. This chapter also details our use of surgically induced intestinal ischemia in a porcine model and the relevant advantages of using a pig model to study intestinal wound healing.

Chapter 3 details our novel protocol to establish primary cultures of EGC from the submucosal and myenteric plexi of both neonatal and juvenile pig colons which was essential for carrying out our future co-culture experiments. We quantify a variety of metrics regarding the cells obtained using this protocol and compare them between neonatal and juvenile pigs. Developing and analyzing outcomes of this protocol showed our ability to establish highly pure cultures based on the expression of canonical glial cell markers. Additionally, given the differences in techniques between isolating EGC from pigs compared to rodents, our protocol offers a translational alternative that can be applied to other large species including humans.

Finally, we test our hypothesis that signaling molecules present in the more mature juvenile lumen can stimulate neonatal EGC to drive intestinal epithelial wound healing in Chapter 4. In the previous chapters, we built up evidence supporting this hypothesis including: (1) neonatal pigs exhibit a defect in intestinal barrier repair rescuable by application of juvenile tissue, (2) EGC are underdeveloped in neonatal pigs and their maturation is driven by gut microbe colonization in rodents, (3) chemical inhibition of EGC in juvenile pigs recapitulates the defect in barrier repair, and (4) a major difference between our neonatal and juvenile pigs is weaning which causes drastic shifts in gut microbiota composition. Findings in this chapter support our hypothesis as exposure to juvenile, but not neonatal, lumen content resulted in increased wound healing of intestinal epithelial cells co-cultured with neonatal EGC. We also determine that these effects are specific to restitution not proliferation and in doing so, potentially detected another aspect in which neonatal EGC are immature as juvenile EGC significantly decreased intestinal epithelial proliferation in the untreated group while neonatal EGC did not. Previous studies reported that EGC in other models do inhibit intestinal epithelial proliferation, so this could be an interesting avenue for further research.[10,11] Additional findings

from Chapter 4 include the identification of SERPINB2 which was significantly increased in co-cultures of neonatal EGC and IPEC-J2 monolayers exposed to juvenile versus neonatal lumen content. At the lowest dose tested, SERPINB2 significantly improved acute intestinal wound healing and is deserving of more research as this was seemingly the first study to examine its effects in the intestinal epithelium. We then verify that the lumen content of juvenile and neonatal pigs differs significantly both in microbial composition and concentration of short chain fatty acids (SCFAs). This led us to investigate whether higher concentrations of butyrate in juvenile lumen content may be responsible for stimulating neonatal EGC to improve intestinal wound healing, which we found to be true. As the primary data chapter, this section provides key insights supporting postnatal development of acute intestinal wound healing capabilities and begins to assess the players and mechanisms involved.

Altogether, the work presented in this dissertation supports that acute intestinal wound healing mechanisms are underdeveloped at birth and this appears to be due, in part, to the network of subepithelial EGC being functional immature. However, when presented with proper signaling molecules found in the lumen content of more mature animals, these neonatal EGC are capable of promoting repair at levels similar to more mature juvenile EGC. Identification of the major signaling factors in this pathway including (1) the specific component of juvenile lumen content that stimulates neonatal EGC and (2) signaling molecules produced by these stimulated EGC that promote intestinal wound healing may lead to the development of novel therapeutics. Such therapeutics, along with mechanistic insights gained from this work, could guide the treatment of the many diseases characterized by barrier dysfunction.

5.3 Key Findings Aligned with Current Research Trends

5.3.1 Enteric glial cell plasticity

The data presented in this study support the notion that EGC are phenotypically plastic meaning their responses are dictated by microenvironmental cues. This was evident as neonatal EGC were incapable of promoting intestinal epithelial restitution when they were untreated or exposed to neonatal lumen content, but exposure to juvenile lumen content endowed neonatal EGC with the ability to enhance intestinal epithelial wound healing. Similarly, no effects were observed when neonatal EGC were treated with neonatal levels of butyrate while exposure to juvenile levels of butyrate produced a significant increase in intestinal epithelial wound healing. Boesmans previously brought attention to the plasticity of EGC showing that expression of canonical glial cell markers is highly dynamic and may be up- or downregulated depending on the environment.[12] It is, therefore, important to consider this when interpreting results especially from *in vitro* studies when EGC are cultured in a foreign environment. However, this also presents EGC as prime targets for modulation in the context of disease. Perhaps with the right signals we can influence EGC to promote desired outcomes as we did in our study. This could be especially important for reversing the "reactive" glial cell state observed in response to pro-inflammatory mediators.[13,14] Along with plasticity, EGC exhibit heterogeneity with specific populations of EGC believed to support distinct functions. This was recently supported by findings that intestinal stem cell function is mediated by only a specific subset of GFAP-expressing EGC.[15] Notably, in Chapter 3 we observed similar plasticity and heterogeneity as all EGC were expected to be immunoreactive for SOX10 but less than 50% were and those that were tended to form certain network types with surrounding cells. While we did not assess these

attributes of EGC in Chapter 4, it would be interesting to determine if a specific subset of EGC is responsible for driving intestinal wound healing in our model.

5.3.2 Altering the microbiota in disease treatment

We used filtered lumen content to recapitulate the presence of microbial signals *in vitro* and found that juvenile lumen content stimulated neonatal EGC to promote intestintal wound healing while neonatal lumen content did not. These differential effects on EGC function were due to differences in lumen content composition between neonatal and juvenile pigs confirmed by 16s rRNA sequencing and analysis of SCFAs. Such findings highlight our ability to modulate intestinal processes by altering gut microbiota composition, which has become an area of active research for digestive diseases. Indeed, investigations into optimizing gut microbiota health remain a hotspot in research and have resulted in commercial successes with the advent of prebiotic and probiotic treatments for a range of conditions, though these products have been met with conflicting reviews regarding their efficacy. On the other hand, transferring of a healthy gut microbiota during fecal microbiota transplantation (FMT) is considered generally effective in the treatment of specific diseases. For example, FMT treatment of *Clostridium difficile* infection has reported cure rates of 85-95%.[16] Animal studies have also demonstrated efficacy of FMT in the treatment of experimental NEC but this has not yet been tested in humans.[17,18] While FMT remains a promising therapy for several diseases, its use is guarded due to public perception, biosafety concerns, and reported adverse side effects. To overcome these challenges, sterile fecal filtrate transfer (FFT) offers an effective alternative approach. FFT is similar to what was performed in our study as fecal matter is processed into a sterile filtrate before transplantation to decrease the risk of transferring live pathogens. Our results supported that microbe-free filtered lumen content was sufficient to stimulate neonatal EGC to enhance intestinal epithelial wound

healing indicating that live microbes are not required. FFT studies have produced similar results showing efficacy in the treatment of *Clostridium difficile* infection as well as experimental NEC. In our studies, we sought to determine if a single component in lumen content might be able to reproduce the effects on the ability of neonatal EGC to improve intestinal epithelial wound healing and focused on butyrate. We found that treatment with butyrate at levels found in juvenile lumen content replicated our previous observations indicating that we may be able to identify key molecules present in the lumen that can drive intestinal epithelial wound healing. Identification of these key molecules could eliminate the need for full FMT or FFT and better target the specific conditions being treated. For instance, butyrate has shown efficacy in the treatment of intestinal disorders such as inflammatory bowel diseases and NEC.[19,20] Overall, the work in this dissertation further supports the use of filtered lumen content as a surrogate for the gut microbiota and highlights our ability to identify specific lumen molecules that promote intestinal health and ameliorate disease.

5.4 Future Directions

Several findings from the work presented in this dissertation warrant additional investigation. As previously mentioned, our observation that untreated juvenile EGC significantly decreased intestinal epithelial proliferation while neonatal EGC did not was an unexpected result. It would be interesting to know if this characteristic develops later in maturation or can be stimulated in neonatal EGC similar to what we saw with intestinal epithelial wound healing. We also identified SERPINB2 as the only secreted protein significantly increased in co-cultures of neonatal EGC treated with juvenile versus neonatal lumen content. In preliminary studies, 1 ng/mL of recombinant SERPINB2 significantly increased wound healing

of IPEC-J2 monolayers. The effect of SERPINB2 on cell migration is controversial in literature so it would be interesting to further study this in our model. Specifically, how SERPINB2 increases wound healing in IPEC-J2 monolayers could be assessed morphologically using electron microscopy to determine if enterocytes adopt a migratory phenotype and transcriptomically to better understand what genes and pathways might be involved. We also need to show that exposure of neonatal EGC to juvenile lumen content without the presence of IPEC-J2 monolayers results in an increase of SERPINB2 release and to determine if this is an age-dependent characteristic by assessing if more SERPINB2 is released from juvenile EGC relative to neonatal EGC. Additionally, we examined the effect of neonatal and juvenile levels of butyrate, acetate, and propionate in our co-culture model and found that only juvenile levels of butyrate stimulated neonatal EGC to promote intestinal wound healing. Considering many SCFAs are known histone deacetylase (HDAC) inhibitors with butyrate being the most potent, we are curious what genes may have been affected. Specifically, we plan to determine if SERPINB2 expression is regulated by SCFAs acting as HDAC inhibitors. Outside of the direct findings from our study, we remain curious about the effect of gut microbiota colonization on the function of EGC. In the future, this could be assessed by repeating our study with juvenile EGC from gnotobiotic pigs that contain a limited microbiota or by maintaining pigs on a milk-only diet until they reach juvenile age. Results from this would provide key insights regarding the functional maturation of EGC that are lacking in literature.

5.5 Concluding Remarks

Integrity of the epithelial barrier is a critical component of intestinal health and its ability to repair following injury is essential to survival. Mechanisms of acute intestinal barrier repair

are immature in neonates leaving them vulnerable to life-threatening outcomes if intestinal epithelial injury occurs. EGC play a major role in barrier regulation including promoting repair but are functionally immature at birth. However, this function can be stimulated by components of a more mature gut microbiota indicating the pathways necessary for acute mucosal repair in neonates are intact but require specific signals not yet present in the neonatal lumen. The studies presented are some of the first to investigate the effect of age on EGC, gut microbiota, and the intestinal epithelium in the context of acute wound healing. It is our hope that insights from these studies will incite further mechanistic understandings that drive the development of novel therapeutics. As a veterinary clinician scientist, I am especially excited about the translational nature of this work and hope it can be utilized to save the lives of neonatal patients of all species.

5.6 References

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