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DISSERTATION

Mechanisms of Intestinal Morphogenesis, Enteric Nervous System Development, Function, and Disease: Insights from Mouse, Stem Cell Models, and Human Genetics

by

Ali Kalantari

Submitted in partial satisfaction of the requirements for degree of

DOCTOR OF PHILOSOPHY

in

Developmental and Stem Cell Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Approved:

Committee Chair

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Committee Member

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by

Ali Kalantari

DEDICATION

To Rebecca Grace Cornwell, whose presence fills my days with warmth and whose spirit lights my path. To my family—Alireza Kalantari, Sholeh Tamaddon, and Yaas Kalantari— your kindness, wisdom, and unwavering support have been the heartbeat of this journey.

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The research presented in this dissertation was conducted under the supervision of Dr. Zev Gartner and Dr. Faranak Fattahi. Chapters 2 and 4 represent published, peer-reviewed work appearing in *Cell* and *Nature*, respectively. Significant portions of the work detailed in Chapters 2, 4, and 5 were performed in close collaboration with Tyler Huycke, Homa Majd, and Alireza Majd, alongside other listed co-authors. Chapter 3 is currently prepared as a manuscript for submission.

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դրանքն արարեցա՞նք զայնքն արարեցա՞նք

մահացան մահա՞նք դրանքն մահա՞նք

•• արարեցա՞նք մահացանք ինչպե՞ս

“Truth is good, is best of all that is good.

Desire is, desire for that which is best truth”

— Zarathustra, *Ashem Vohu*, *Yasna* 27.14

Mechanisms of Intestinal Morphogenesis, Enteric Nervous System Development, Function, and Disease: Insights from Mouse, Stem Cell Models, and Human Genetics

Ali Kalantari

ABSTRACT

The gastrointestinal tract is a complex organ system whose function depends on coordinated activity of epithelial, mesenchymal, and neural compartments. During embryonic development, these compartments undergo orchestrated morphogenetic events to establish the architectural and functional features of the mature intestine. This thesis integrates developmental biology, stem cell biology, and human genetics to elucidate mechanisms governing intestinal morphogenesis and enteric nervous system (ENS) development, and to identify genetic and cellular pathways underlying disorders of gut-brain interaction (DGBIs).

Chapter 1 provides an introduction to intestinal villus morphogenesis, ENS development and patterning, motor neuron specification, and the genetic susceptibility to DGBIs, establishing the context for the experimental work presented in subsequent chapters.

Chapter 2 investigates the role of extracellular matrix (ECM) adhesion and remodeling in intestinal villus morphogenesis. Using mouse explant cultures, pharmacological inhibition of matrix metalloproteinases, and single-cell RNA sequencing, this chapter demonstrates that integrin-mediated cell-ECM interactions and MMP-mediated matrix degradation are essential for PDGFR α ⁺ mesenchymal condensation and epithelial folding. These findings establish ECM remodeling as a central mechanism underlying villus formation.

Chapter 3 investigates the mechanisms of ENS patterning within the regionally specialized gut environment. Using multiplexed single-cell RNA sequencing of the developing mouse small intestine (E13.5–E18.5), this chapter demonstrates that while the epithelium and mesenchyme maintain strong anterior–posterior (A-P) graded gene expression, the ENS lacks overt intrinsic regionalization. Instead, the ENS follows a predominantly temporal maturation trajectory defined by distinct glial and neuronal differentiation states. Integrative ligand-receptor analysis identifies the PTN/MDK-PTPRZ1 signaling axis as a critical, spatially graded microenvironmental input derived from the mesenchyme. Functional perturbation of this pathway in human pluripotent stem cell-derived ENS cultures confirms that PTPRZ1 signaling fine-tunes progenitor proliferation, neurogenesis, and neurotransmitter specification. Collectively, these findings support a model in which ENS maturation relies on a uniform core program that is secondarily refined by extrinsic, region-specific mesenchymal cues.

Chapter 4 focuses on enteric motor neuron specification and functional characterization, particularly nitrergic inhibitory neurons. Using hPSC-derived enteric ganglioid models and high-throughput screening, this chapter identifies signaling pathways that promote nitrergic neuron differentiation and drugs that modulate nitrergic neuron activity.

Chapter 5 leverages genome-wide association studies using the All of Us Research Program database to identify genetic variants and candidate genes associated with DGBIs, including irritable bowel syndrome, functional diarrhea, functional dyspepsia, and functional fecal incontinence. The chapter reveals enrichment of genes involved in neuronal excitability, synaptic transmission, and ion channel regulation, supporting the hypothesis that genetic variants affecting ENS development or function contribute to DGBI pathophysiology.

Collectively, these studies demonstrate the power of integrating mouse developmental genetics, human pluripotent stem cell and *ex-vivo* models, transcriptomics, and human genetics to address fundamental questions in gut biology and disease.

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LIST OF ABBREVIATIONS

AP	Anterior-Posterior
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BMP	Bone Morphogenetic Protein
BQ	EDNRB Antagonist
c-FOS	Proto-oncogene c-Fos
cGMP	Cyclic Guanosine Monophosphate
ChAT	Choline Acetyltransferase
CMMC	Colonic Migrating Motor Complex
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DE	Differential Expression
DESeq2	Differential Expression Sequence analysis 2
DGBI	Disorder of Gut-Brain Interaction
E (+ number)	Embryonic Day (e.g., E13.5, E14.5)
ECM	Extracellular Matrix
EDN3	Endothelin-3
EDNRB	Endothelin Receptor Type B
ENCC	Enteric Neural Crest Cell
ENS	Enteric Nervous System
EPI	Epithelium/Epithelial
ESC	Embryonic Stem Cell
FACS	Fluorescence-Activated Cell Sorting
FDR	False Discovery Rate
FGF	Fibroblast Growth Factor
GABA	Gamma-Aminobutyric Acid
GAD1/GAD2	Glutamate Decarboxylase 1/2
GDNF	Glial Cell Line-Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GFRα1	GDNF Family Receptor A-1
GI	Gastrointestinal
GRN	Gene Regulatory Network
GSEA	Gene Set Enrichment Analysis
GSK3β	Glycogen Synthase Kinase 3 B

GWAS	Genome-Wide Association Study
Hh	Hedgehog
HIF1α	Hypoxia-Inducible Factor 1- α
HIO	Human Intestinal Organoid
hPSC	Human Pluripotent Stem Cell
HVG	Highly Variable Gene
IBS	Irritable Bowel Syndrome
IBS-C	IBS with Constipation
IBS-D	IBS with Diarrhea
IBS-M	IBS with Mixed Bowel Habits
IBS-U	Unclassified IBS
ICC	Interstitial Cell of Cajal
iPSC	Induced Pluripotent Stem Cell
L-NAME	N(G)-Nitro-L-Arginine Methyl Ester
LR	Ligand-Receptor
MAPK	Mitogen-Activated Protein Kinase
MDK	Midkine
MECE	Mutually Exclusive, Collectively Exhaustive
MES	Mesenchyme/Mesenchymal
MMP	Matrix Metalloproteinase
MSI	Mouse Small Intestine
MULTI-seq	Multiplexed Single-Cell RNA Sequencing
NANC	Non-Adrenergic, Non-Cholinergic
NAZ	PTPRZ1 Inhibitor (NAZ2329)
NES	Normalized Enrichment Score
NO	Nitric Oxide
nNOS	Neuronal Nitric Oxide Synthase
NOS1	Nitric Oxide Synthase 1
PC	Principal Component
PCA	Principal Component Analysis
PDGF	Platelet-Derived Growth Factor
PDGFRα	Platelet-Derived Growth Factor Receptor A
PDGFRβ	Platelet-Derived Growth Factor Receptor B
PI3K	Phosphoinositide 3-Kinase
PTN	Pleiotrophin
PTPRZ1	Protein Tyrosine Phosphatase Receptor Type Z1
QC	Quality Control

RA	Retinoic Acid
RAS	Rat Sarcoma (protein)
RET	REarranged during Transfection (receptor tyrosine kinase)
RNA	Ribonucleic Acid
RNA-seq	RNA Sequencing
scRNA-seq	Single-Cell RNA Sequencing
SLC6A4	Solute Carrier Family 6 Member 4 (Serotonin Transporter)
SLC32A1	Solute Carrier Family 32 Member 1 (GABA Transporter)
smFISH	Single-Molecule Fluorescence In Situ Hybridization
SNP	Single-Nucleotide Polymorphism
TF	Transcription Factor
TGF-β	Transforming Growth Factor B
TPH2	Tryptophan Hydroxylase 2
UCSF	University of California, San Francisco
UMAP	Uniform Manifold Approximation and Projection
UMI	Unique Molecular Identifier
VIP	Vasoactive Intestinal Peptide
vNC	Vagal Neural Crest
Wnt	Wingless-related integration site
YAP	Yes-Associated Protein

CHAPTER 1: INTRODUCTION

The Gastrointestinal Tract: An Integrated System of Epithelial, Mesenchymal, and Neural Compartments

The gastrointestinal (GI) tract represents one of the most complex and functionally diverse organ systems in the human body. Beyond its primary role in nutrient absorption, the intestine serves as a critical interface between the external environment and internal physiology, regulating immune responses, hosting trillions of commensal microorganisms, and coordinating sophisticated neuroendocrine signaling. The remarkable functional capacity of the intestine depends on the precise coordination of three major tissue compartments: the epithelium, the mesenchyme, and the enteric nervous system (ENS). During embryonic development, these compartments undergo coordinated morphogenesis to establish the architectural and functional properties that enable lifelong intestinal homeostasis. Understanding how these tissue layers communicate during development, and how developmental programs can be recapitulated in therapeutic contexts, represents a fundamental challenge in developmental biology with profound clinical implications.

Disorders affecting intestinal structure and function affect millions of individuals worldwide and represent a significant burden on healthcare systems. Congenital malformations such as Hirschsprung disease, characterized by absence of enteric neurons in the distal colon, can be life-threatening without surgical intervention (Heuckeroth RO, 2018). Disorders of gut-brain interaction (DGBIs), formerly termed functional gastrointestinal disorders, affect approximately 40% of the global population and manifest as chronic symptoms including abdominal pain,

bloating, constipation, and diarrhea in the absence of identifiable structural abnormalities (Sperber AD et al., 2021). These conditions impose substantial morbidity and economic costs, yet treatment options remain limited due to incomplete understanding of the cellular and molecular mechanisms underlying intestinal morphogenesis, neural circuit development, and disease pathogenesis.

Recent advances in stem cell biology, single-cell genomics, and tissue engineering have created unprecedented opportunities to study human intestinal development and disease. Human pluripotent stem cells (hPSCs) can be directed to differentiate into intestinal epithelial organoids, mesenchymal cells, and enteric neurons, providing scalable experimental systems that recapitulate key aspects of embryonic development. These platforms enable high-throughput interrogation of developmental mechanisms, identification of disease-associated genes, and development of cell-based regenerative therapies. This thesis leverages hPSC-derived models alongside mouse genetic approaches and single cell transcriptomics to dissect the mechanisms governing intestinal villus morphogenesis, enteric nervous system patterning, motor neuron specification, and genetic susceptibility to disorders of gut-brain interaction.

INTESTINAL VILLUS MORPHOGENESIS: EPITHELIAL-MESENCHYMAL INTERACTIONS AND ECM REMODELING

The Structural Foundation of Intestinal Function

The small intestine is characterized by finger-like projections called villi that extend into the lumen and dramatically increase the absorptive surface area. These villi, along with

invaginations called crypts, form repetitive functional units that are essential for nutrient absorption and epithelial renewal throughout life. Villus formation occurs during mid-gestation in mammals, beginning around embryonic day 14.5 (E14.5) in mice, and represents a critical morphogenetic event that establishes the fundamental architecture of the mature intestine (Wells JM & Spence JR, 2014). Prior to villus emergence, the intestinal epithelium is organized as a flat, pseudostratified layer overlying undifferentiated mesenchyme. The transformation from this smooth configuration to a highly folded villus-crypt architecture requires coordinated changes in both epithelial and mesenchymal compartments, involving cell proliferation, differentiation, migration, and mechanical forces (Figure 1.1).

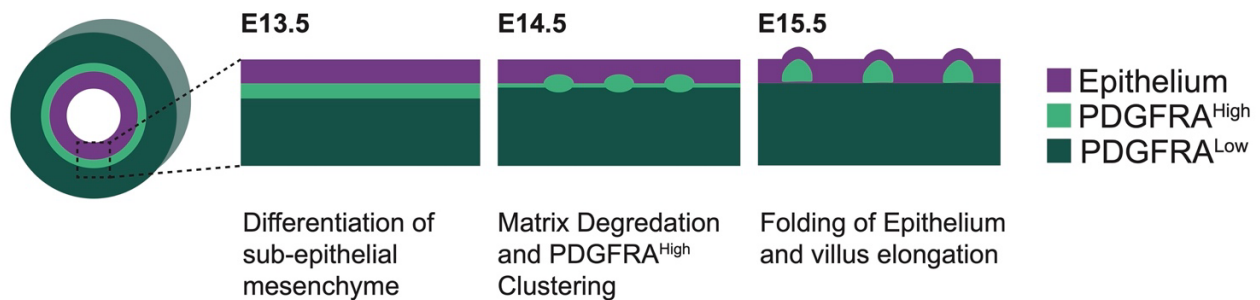


Figure 1.1: Intestinal villus morphogenesis and the role of PDGFR α ⁺ mesenchymal condensation. Schematic illustration of villus formation showing the progression from flat pseudostratified epithelium (E13.5) to the emergence of villus structures (E14.5-E15.5). PDGFR α ⁺ mesenchymal cells (green) condense beneath the epithelium (purple), generating forces that fold the overlying epithelial layer into finger-like projections. ECM remodeling and actomyosin contractility drive mesenchymal condensation and epithelial folding.

PDGFR α ⁺ Mesenchymal Condensation as a Driver of Villus Patterning

A key initiating event in villus morphogenesis is the condensation of subepithelial mesenchymal cells that express high levels of platelet-derived growth factor receptor α (PDGFR α). Studies using genetic lineage tracing and live imaging in mice have demonstrated that, beginning at

E13.5, a subset of mesenchymal cells immediately beneath the epithelium upregulates PDGFR α expression, becoming highly motile and subsequently aggregating into discrete clusters (Walton KD et al., 2012), (Huycke TR et al., 2024)). These PDGFR α high condensates form in a proximal-to-distal wave along the anterior-posterior axis of the developing small intestine and spatially correlate with sites where the overlying epithelium begins to buckle and evaginate to form nascent villi (Huycke TR et al., 2024). Genetic ablation of PDGFR α or disruption of non-muscle myosin II activity in mesenchymal cells prevents both condensate formation and epithelial folding, establishing that mesenchymal dynamics are necessary for villus initiation (Huycke TR et al., 2024).

The mechanisms by which PDGFR α high cells generate forces sufficient to deform the overlying epithelium have been the subject of intensive investigation. Recent work combining ex vivo organ culture, live imaging, and computational modeling has revealed that PDGFR α high cells undergo a process resembling liquid-phase condensation, wherein initially dispersed motile cells coalesce into compact, rounded aggregates (Huycke TR et al., 2024). This process is driven by actomyosin-dependent contractility and is accompanied by dramatic remodeling of the extracellular matrix (ECM). Inhibition of matrix metalloproteinases (MMPs), enzymes that degrade ECM proteins, blocks both mesenchymal condensation and epithelial folding, suggesting that ECM remodeling is an essential component of the morphogenetic process.

Signaling Pathways Coordinating Mesenchymal Condensation

Multiple signaling pathways have been implicated in regulating the behavior of subepithelial mesenchyme during villus formation. Hedgehog (Hh) signaling, emanating from the epithelium, is both necessary and sufficient for the formation of PDGFR α ^{high} clusters (Walton KD et al.,

2012). Pharmacological inhibition of Hh signaling prevents mesenchymal condensation, while ectopic activation of the pathway induces precocious or ectopic cluster formation. Bone morphogenetic protein (BMP) signaling, conversely, appears to antagonize villus formation; inhibition of BMP signaling enhances mesenchymal clustering (Walton KD et al., 2012). Wnt signaling in the epithelium is induced by PDGFR α^{high} mesenchyme and promotes epithelial proliferation in the regions that will become villi, creating a feed-forward loop that amplifies the initial patterning cues.

The molecular effectors downstream of these signaling pathways that control the cell behaviors underlying condensation, motility, adhesion, and contractility, are only beginning to be understood. Analysis of single-cell RNA sequencing (scRNA-seq) data from E14.5 small intestine has revealed that PDGFR α^{high} cells express high levels of genes involved in ECM interaction, including integrins (Itga2, Itga11) that bind to collagens, and genes encoding ECM components themselves, including non-fibrillar collagens (Col6a1, Col23a1) and laminin subunits. These cells also express Mmp2 and Mmp14, membrane-bound and secreted metalloproteinases that degrade fibrillar collagens. The coordinated upregulation of ECM synthesis, ECM receptors, and ECM-degrading enzymes suggests a model in which PDGFR α^{high} cells actively remodel their local microenvironment to facilitate cell movement and clustering, while simultaneously secreting new matrix components that stabilize the condensed state.

Epithelial Responses and Mechanical Coupling

The deformation of the epithelium during villus emergence is not simply a passive response to mesenchymal forces. Recent studies using atomic force microscopy, traction force microscopy, and finite element modeling have shown that the epithelium possesses elastic properties that

resist bending, and that the magnitude and spatial pattern of mesenchymal forces must overcome this resistance to generate stable folds (Huycke TR et al., 2024). The epithelium also exhibits mechanosensitive responses: application of curvature in cultured intestinal organoids activates Yes-associated protein (YAP) signaling in epithelial cells, which promotes proliferation and crypt formation. Thus, mechanical deformation generated by mesenchymal condensation may serve not only to initiate the villus structure but also to activate epithelial signaling programs that elaborate and maintain the crypt-villus axis.

Taken together, these findings reveal villus morphogenesis as a paradigm of tissue-level self-organization, wherein local cell-cell and cell-ECM interactions, coupled with long-range signaling gradients, generate repetitive three-dimensional structures with reproducible spacing and geometry. The insights gained from studying this process have implications beyond developmental biology: villus atrophy is a common feature of intestinal diseases including celiac disease, inflammatory bowel disease, and chemotherapy-induced enteropathy, and strategies to promote villus regeneration or to engineer vascularized intestinal tissue for transplantation will benefit from a mechanistic understanding of villus morphogenesis.

THE ENTERIC NERVOUS SYSTEM: DEVELOPMENT, MIGRATION, AND REGIONAL PATTERNING

The ENS as an Autonomous Neural Network

The enteric nervous system is the largest division of the peripheral nervous system, consisting of an estimated 200–600 million neurons in humans, comparable in number to the neurons of the spinal cord (Furness JB, 2012). Embedded within the wall of the gastrointestinal tract from the

esophagus to the anus, the ENS controls motility, secretion, local blood flow, and immune responses with remarkable autonomy, earning it the designation of the “second brain.” ENS neurons are organized into two major ganglionated plexuses: the myenteric (Auerbach’s) plexus, located between the longitudinal and circular muscle layers, which primarily regulates motility, and the submucosal (Meissner’s) plexus, which modulates secretion and blood flow. Enteric neurons exhibit remarkable diversity, encompassing more than 15 functional subtypes characterized by distinct morphologies, neurotransmitter profiles, synaptic connectivity patterns, and physiological roles (Hao MM & Young HM, 2009).

The ENS derives almost exclusively from vagal neural crest cells that emigrate from the caudal hindbrain at the level of somites 1–7 in mice (corresponding to the post-otic region in humans) around E8.5–E9.0 (Le Douarin NM & Teillet MA, 1973). These vagal neural crest-derived enteric neural crest cells (ENCCs) enter the foregut and migrate caudally in a rostral-to-caudal wave over several days, colonizing the full length of the gastrointestinal tract by E14.5 in mice (Anderson MH et al., 1995). During migration, ENCCs proliferate extensively; failure of either migration or proliferation results in Hirschsprung disease, a congenital disorder characterized by aganglionic segments of the distal colon. After the gut is fully colonized, ENCCs undergo coordinated differentiation into neurons and glia, with the peak of neurogenesis occurring between E12.5 and E15.5, and gliogenesis following neurogenesis consistent with in vivo developmental timelines (Pham TD et al., 1991), (Liu C & Yang J, 2022), (Laranjeira C et al., 2011), (Charrier B & Pilon N, 2017).

GDNF-RET and Endothelin Signaling in ENS Development

The molecular pathways governing ENCC survival, migration, and proliferation have been extensively studied, with the glial cell line-derived neurotrophic factor (GDNF) and its receptor

RET emerging as central regulators. GDNF is secreted by the mesenchyme of the developing gut, establishing a chemoattractant gradient that guides ENCC migration (Taraviras S et al., 1999). RET is a receptor tyrosine kinase expressed by ENCCs; upon binding GDNF (in complex with the co-receptor GFR α 1), RET activates intracellular signaling cascades including the PI3K-AKT and RAS-MAPK pathways, which promote cell survival, proliferation, and motility (Takahashi M, 2001). Loss-of-function mutations in RET are the most common genetic cause of Hirschsprung disease, accounting for approximately 50% of familial and 15–20% of sporadic cases (Amiel J & Lyonnet S, 2001).

Endothelin-3 (EDN3) and its receptor EDNRB constitute a second essential signaling axis for ENS development. EDNRB is expressed by ENCCs, and EDN3 is produced by the gut mesenchyme. Like GDNF-RET, the EDN3-EDNRB pathway promotes ENCC proliferation and migration. Mutations in either EDN3 or EDNRB cause Hirschsprung disease, and mouse models deficient in *Ednrb* (e.g., piebald lethal mice) exhibit colonic aganglionosis resembling the human disease (Baynash AG et al., 1994). Intriguingly, recent lineage-tracing studies have revealed that there are at least two distinct vagal neural crest-derived lineages contributing to the ENS, which respond differentially to GDNF and endothelin signaling, suggesting that ENS development involves more complex lineage relationships than previously appreciated (Poltavski DM et al., 2024).

Do Enteric Neurons Acquire Regional Identities?

A central question in ENS biology is whether enteric neurons, after colonizing the length of the gut tube, acquire region-specific molecular identities that reflect their position along the anterior-posterior (AP) axis. The gut epithelium and mesenchyme exhibit pronounced AP regionalization,

characterized by spatially restricted expression of Hox transcription factors and other regional markers that specify distinct organ identities (e.g., stomach, duodenum, jejunum, ileum, colon). It has been unclear whether the ENS, which differentiates within this highly patterned environment, similarly adopts regionally distinct transcriptional programs, or whether enteric neurons retain a uniform “vagal” identity throughout the gut.

Addressing this question requires spatially resolved, single-cell resolution analysis of gene expression across developmental time. Recent work using multiplexed single-cell RNA sequencing (MULTI-seq) on mouse small intestine from E13.5 to E18.5, with samples segmented along the AP axis, has provided new insights into this question. Variance partitioning analysis revealed that developmental time (i.e., maturation stage) explains the majority of transcriptional variance in ENS cells, whereas AP position contributes minimally. In contrast, epithelial and mesenchymal compartments showed strong AP-dependent variance, with clear Hox expression gradients. ENS cells, across all developmental stages and positions, predominantly expressed anterior (vagal) Hox genes and lacked the posterior Hox signatures seen in surrounding mesodermal tissues. Clustering and UMAP projection of ENS cells revealed organization primarily along a temporal differentiation trajectory, from proliferating progenitors

to immature neurons to mature neurons and glia, with minimal evidence of region-specific cell states.

These findings support a model in which the ENS retains a largely uniform intrinsic transcriptional program that is organized along a temporal axis rather than a spatial one. This uniformity is consistent with the common vagal origin of all ENS cells and suggests that positional information, so critical for patterning the gut tube itself, is not directly encoded within ENS neurons.

Microenvironmental Cues Shape ENS Maturation

Although ENS cells lack strong intrinsic regional identities, they do not develop in isolation from the surrounding gut environment (Figure 1.2). Ligand-receptor interaction analysis using CellChat on spatiotemporally resolved scRNA-seq data revealed that the mesenchyme is the dominant source of signaling inputs to the ENS throughout development. Among the most prominent signals are members of the pleiotrophin (PTN) and midkine (MDK) families, which bind to the receptor protein tyrosine phosphatase PTPRZ1 expressed by ENS progenitors and neurons. Both PTN and MDK exhibit spatially graded expression within the mesenchyme along the AP axis, and PTPRZ1 expression within ENS cells also shows spatial and temporal dynamics. Despite the absence of overt regional transcriptional identity in ENS cells, subtle differences in gene expression correlate with AP position, suggesting that localized niche signals fine-tune ENS maturation.

To test the functional role of PTN-MDK-PTPRZ1 signaling, hPSC-derived enteric ganglioids, three-dimensional aggregates of enteric neurons and glia, were treated with recombinant PTN, MDK, or a small-molecule PTPRZ1 inhibitor (NAZ2329). Bulk RNA-seq analysis revealed that

modulation of this pathway broadly affects ENS lineage specification: treatment with PTN, MDK, or NAZ2329 upregulated progenitor and proliferative gene signatures while suppressing neuronal maturation and synaptic gene programs. Neurotransmitter identity was also affected, with consistent downregulation of GABAergic markers and selective upregulation of serotonergic markers in response to PTN. These findings demonstrate that PTPRZ1 signaling serves as a molecular rheostat controlling the balance between ENS progenitor expansion and neuronal differentiation and modulates neurotransmitter specification programs.

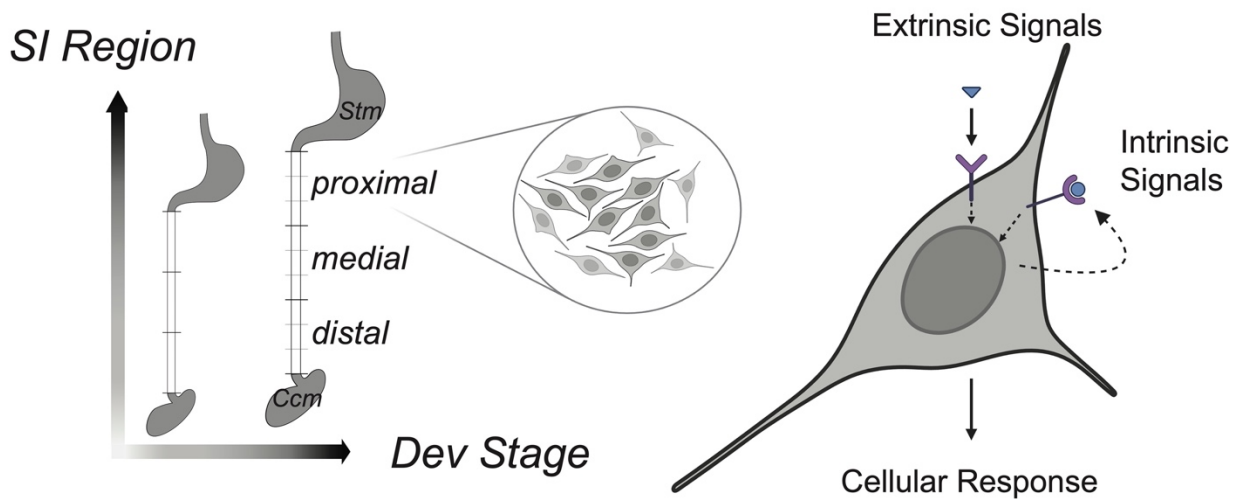


Figure 1.2: Microenvironmental PTN/MDK-PTPRZ1 signaling modulates ENS maturation and neurotransmitter specification. Schematic showing mesenchyme-derived PTN and MDK ligands (spatially graded along the A-P axis) binding to PTPRZ1 receptors on ENS progenitors and neurons. Functional validation in hPSC-derived enteric ganglioids demonstrates that modulation of PTPRZ1 signaling regulates the balance between progenitor expansion and neuronal differentiation, and influences neurotransmitter identity specification

Additional microenvironmental signals identified by ligand-receptor analysis include endothelin, Notch, and extracellular matrix components, each of which is expressed in spatially and temporally restricted patterns by the mesenchyme and epithelium. The integration of these diverse signals likely contributes to the fine-scale heterogeneity observed in ENS cell states,

even in the absence of overt regional transcriptional programming. This model, wherein ENS cells follow a uniform developmental trajectory but are secondarily modulated by localized niche cues, reconciles the observation of transcriptional uniformity with the functional specialization of ENS neurons in different gut regions.

ENTERIC MOTOR NEURONS: SPECIFICATION, NEUROTRANSMITTER IDENTITY, AND CONTROL OF GUT MOTILITY

Functional Classification of Enteric Neurons

Gastrointestinal motility is orchestrated by intrinsic reflex circuits within the ENS that operate with remarkable independence from central nervous system control. These circuits integrate sensory information from the gut lumen, including mechanical stretch, chemical composition, and pH, and coordinate appropriate motor outputs to the smooth muscle layers. Enteric motor neurons, which directly innervate the circular and longitudinal smooth muscle, can be functionally classified into two major categories: excitatory motor neurons, which promote muscle contraction, and inhibitory motor neurons, which induce muscle relaxation (Sharkey KA & Mawe GM, 2023). The coordinated activity of these two populations generates the complex patterns of gut motility, including peristaltic waves, segmentation contractions, and receptive relaxation.

Excitatory motor neurons predominantly use acetylcholine as their primary neurotransmitter, acting on muscarinic receptors (M2 and M3) on smooth muscle cells to cause depolarization and contraction (Gwynne RM & Bornstein JC, 2007). Some excitatory neurons co-release tachykinins such as substance P, which act on neurokinin receptors to further enhance contractile responses. Inhibitory motor neurons, by contrast, utilize nitric oxide (NO) as their principal neurotransmitter, along with purines (ATP, β -NAD⁺) and vasoactive intestinal peptide (VIP) (Sanders KM & Ward SM, 1992). NO diffuses into smooth muscle cells and activates soluble guanylate cyclase, leading to increased cyclic GMP levels, which cause smooth muscle

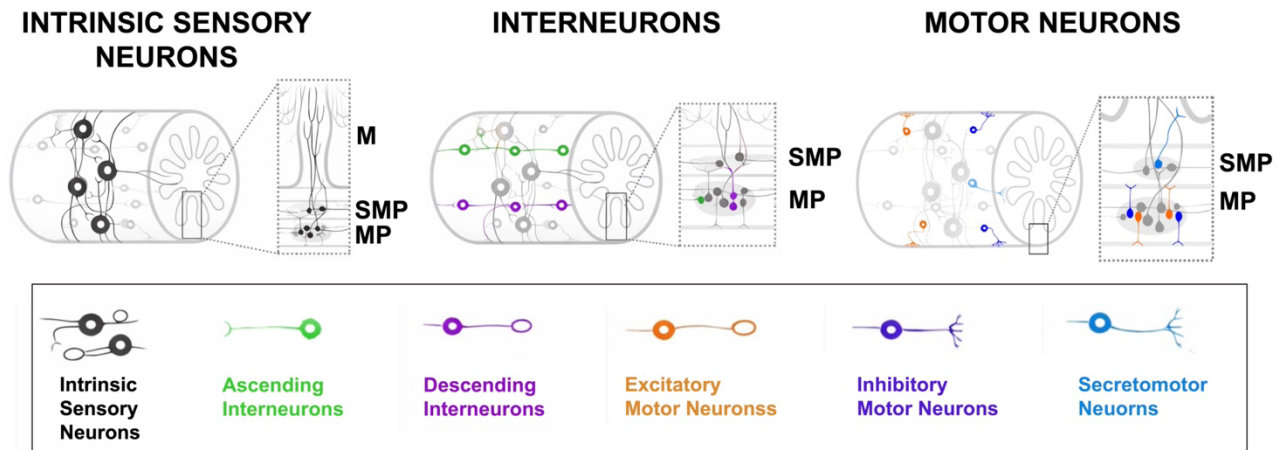


Figure 1.3: Neuronal organization of enteric circuits. Intrinsic sensory neurons form circumferential networks with extensive mucosal innervation. Myenteric plexus (MP) interneurons create longitudinal chains via ascending (oral) and descending (anal) projections. The circular and longitudinal muscle layers are innervated by excitatory and inhibitory motor neurons, whereas submucosal plexus (SMP) secretomotor neurons project to the mucosa. Adapted from Candice Fung and Pieter Vanden Berghe, “Functional circuits and signal processing in the enteric nervous system,” *Cellular and Molecular Life Sciences*, vol. 77, 2020, p. 4506.

relaxation (Figure 1.3). Inhibitory neurons are often referred to as “nitrergic” neurons due to their expression of neuronal nitric oxide synthase (nNOS, encoded by NOS1), the enzyme responsible for NO synthesis (Huang PL et al., 1993).

Nitrergic Neurons and Gastrointestinal Motility Disorders

Nitrergic inhibitory motor neurons play an essential role in coordinating gut motility by enabling sphincter relaxation, accommodation of ingested food, and propagation of peristaltic contractions. Selective loss or dysfunction of NO neurons is implicated in a range of gastrointestinal motility disorders, collectively termed enteric neuropathies. Achalasia, a disorder of the esophagus characterized by failure of the lower esophageal sphincter to relax, is associated with loss of nitrergic neurons in the myenteric plexus (Mearin F et al., 1993). Gastroparesis, a condition marked by delayed gastric emptying, is similarly linked to reduced nitrergic

innervation, particularly in diabetic patients (Watkins CC et al., 2000). Infantile hypertrophic pyloric stenosis and chronic intestinal pseudo-obstruction also involve deficits in inhibitory neurotransmission. In mouse models, genetic ablation of *Nos1* results in hypertrophic pyloric stenosis and impaired colonic motility, phenocopying aspects of human enteric neuropathy (Huang PL et al., 1993).

The vulnerability of nitrergic neurons extends beyond congenital conditions. Chemotherapy with platinum-based agents (e.g., cisplatin, oxaliplatin) causes selective degeneration of enteric neurons, with nitrergic neurons being particularly susceptible (McQuade RM et al., 2018). Up to 50% of cancer survivors experience chronic gastrointestinal symptoms, including constipation and diarrhea, long after treatment has concluded, highlighting the clinical importance of understanding and protecting nitrergic neuron function.

Developmental Specification of Neurotransmitter Identity

How enteric neurons acquire specific neurotransmitter identities during development remains incompletely understood. Unlike the central nervous system, where spatial patterning cues (e.g., dorsal-ventral and anterior-posterior gradients of morphogens) play dominant roles in specifying neuronal subtypes, the ENS appears to rely more heavily on spatiotemporal cues from the niche and cell-intrinsic transcriptional programs. Single-cell RNA sequencing of developing mouse and human ENS has revealed that neuronal diversification occurs progressively as progenitors exit the cell cycle and undergo terminal differentiation (Majd H et al., 2025). Subclustering of enteric neurons based on neurotransmitter marker expression identifies distinct populations: cholinergic neurons expressing choline acetyltransferase (ChAT); nitrergic neurons expressing NOS1; serotonergic neurons expressing tryptophan hydroxylase (TPH2) and the serotonin

transporter (SLC6A4); and GABAergic neurons expressing glutamate decarboxylase (GAD1/GAD2) and the GABA transporter (SLC32A1).

Remarkably, many of these neurotransmitter identities are established relatively late in ENS development. In mice, NOS1 expression begins around E13.5–E14.5 and increases through postnatal stages. The transcriptional regulators that control NOS1 expression in enteric neurons are beginning to be identified: recent work has shown that hypoxia-inducible factor 1 α (HIF1 α) binds to the NOS1 promoter and activates its transcription in nitrergic neurons, and that stabilizing HIF1 α can rescue nitrergic function in diabetic gastroparesis (Gao F et al., 2023). Other transcription factors implicated in enteric neuron subtype specification include Hand2, which is required for the development of noradrenergic neurons; Phox2b, essential for pan-neuronal differentiation; and Ascl1, which promotes neuronal differentiation at the expense of glial fates (Lasrado R et al., 2017), (Nagy N & Goldstein AM, 2017).

High-throughput chemical screens using hPSC-derived enteric neurons have provided new insights into the signaling pathways that regulate neurotransmitter specification. Treatment of enteric neural crest progenitors with PP121, a multi-targeted receptor tyrosine kinase inhibitor that blocks PDGFR signaling, dramatically increases the proportion of NOS1+ nitrergic neurons in differentiated cultures (Majd H et al., 2025). This effect is recapitulated by genetic knockout of PDGFRA and PDGFRB, confirming that PDGFR signaling negatively regulates nitrergic neuron specification. Conversely, addition of PDGF ligand reduces nitrergic differentiation. These findings suggest that mesenchymal-derived PDGF signals may regulate the balance between different neuronal subtypes in the developing ENS, perhaps by influencing the timing of progenitor cell cycle exit or by biasing lineage choice at critical decision points.

Functional Characterization and Therapeutic Modulation of Nitrergic Neurons

The availability of hPSC-derived enteric neurons has enabled the development of scalable functional assays to interrogate nitrergic neuron activity. Expression of the immediate early gene c-FOS, induced upon neuronal depolarization, provides a convenient readout for neuronal activity that can be measured by flow cytometry or immunofluorescence (Majd H et al., 2025). Using a NOS1GFP reporter hPSC line, in which GFP expression is driven by the endogenous NOS1 promoter, it becomes possible to specifically track nitrergic neurons and assess their responses to pharmacological stimuli.

A high-throughput screen of 582 neuromodulatory compounds identified multiple drug candidates that increase c-FOS expression in NOS1-GFP⁺ neurons, indicating enhanced nitrergic activity. These compounds converged on several protein target classes, including serotonin receptors, adrenergic receptors, acetylcholine receptors, glutamate receptors, sodium channels, and opioid receptors. A parallel screen measuring NO release directly, using a colorimetric assay that detects nitrite, the stable oxidation product of NO, identified a largely overlapping set of hits, validating the c-FOS assay as a surrogate for NO neuron function. Among the top hits, the α 2-adrenergic agonist dexmedetomidine showed particularly robust effects: treatment of isolated mouse colon segments *ex-vivo* with dexmedetomidine increased the frequency of colonic migrating motor complexes (CMMCs), rhythmic propulsive contractions mediated by the ENS, and this effect was abolished by co-treatment with L-NAME, a non-selective NOS inhibitor (Majd H et al., 2025). Furthermore, dexmedetomidine failed to relax colonic muscle from *Nos1*^{-/-} mice, demonstrating that its effects on motility are dependent on intact nitrergic signaling.

These findings illustrate the power of hPSC-derived ENS models for identifying novel therapeutic targets and drugs that modulate specific neuronal subtypes. They also highlight a potential strategy for treating enteric neuropathies: pharmacological enhancement of residual nitrergic function may partially compensate for neuronal loss, and drugs that promote nitrergic neuron differentiation or survival could be developed as disease-modifying therapies.

GENETIC ARCHITECTURE OF DISORDERS OF GUT-BRAIN INTERACTION

Clinical Burden and Classification of DGBIs

Disorders of gut-brain interaction (DGBIs), previously referred to as functional gastrointestinal disorders, represent a heterogeneous group of chronic conditions characterized by gastrointestinal symptoms, abdominal pain, bloating, altered bowel habits, that cannot be attributed to structural or biochemical abnormalities detectable by conventional diagnostic tests (Sperber AD et al., 2021). According to the Rome IV diagnostic criteria, which provide standardized definitions for 33 different DGBIs organized by anatomical region (esophageal, gastroduodenal, bowel, anorectal), the global prevalence of DGBIs is approximately 40%, making them among the most common reasons for gastroenterology consultations worldwide (Sperber AD et al., 2021)

Irritable bowel syndrome (IBS), the most extensively studied bowel DGBI, affects an estimated 10–15% of adults in Western countries and exhibits variable prevalence across populations (Sperber AD et al., 2021). IBS is subdivided into subtypes based on predominant bowel habit: IBS with constipation (IBS-C), IBS with diarrhea (IBS-D), IBS with mixed bowel habits (IBS-

M), and unclassified IBS (IBS-U). Other common DGBIs include functional dyspepsia (affecting 5–10% of the population), characterized by postprandial fullness and epigastric pain; functional constipation; and functional diarrhea. Although once dismissed as psychosomatic, DGBIs are now recognized to have a complex multifactorial etiology involving altered gut motility, visceral hypersensitivity, immune activation, gut microbiota dysbiosis, and gut-brain axis dysfunction (Enck P et al., 2016).

The economic and social burden of DGBIs is substantial. Patients with DGBIs report reduced quality of life, increased absenteeism from work and school, and higher healthcare utilization compared to the general population (Eijsbouts C et al., 2021). Work productivity loss in individuals with DGBIs is comparable to that seen in inflammatory bowel disease, underscoring the significant impact of these “functional” disorders on daily functioning. Current treatment options are largely symptomatic and include dietary modifications, pharmacological agents targeting gut motility or visceral pain, and psychological interventions. The limited efficacy of available treatments reflects our incomplete understanding of disease mechanisms.

Genetic Susceptibility to DGBIs

Family and twin studies have provided evidence for heritable components of DGBIs, with estimates suggesting that genetic factors account for 20–50% of IBS risk (Henström M & D'Amato M, 2016). However, DGBIs are complex polygenic disorders, likely involving the combined effects of multiple common genetic variants of small effect size, together with environmental risk factors (Figure 1.4). Genome-wide association studies (GWAS) have begun to identify specific genetic loci associated with IBS and related DGBIs, although progress has

been slower than for other complex diseases, in part due to challenges in defining precise disease phenotypes and recruiting large cohorts with standardized diagnostic criteria.

Recent GWAS efforts have identified several genome-wide significant loci for IBS, including variants near genes involved in ion channel function, neurotransmitter signaling, and immune regulation. Single-nucleotide polymorphisms (SNPs) associated with IBS have been found near SCN5A (encoding a voltage-gated sodium channel), KCTD8 (a potassium channel regulator), NCAM1 (a neural cell adhesion molecule), and DOCK9 (a guanine nucleotide exchange factor involved in cytoskeletal regulation) (Eijsbouts C et al., 2021). These genetic associations provide hints about biological pathways contributing to disease, but functional validation of individual variants and genes has been limited.

To extend genetic studies of DGBIs and identify candidate susceptibility genes relevant to enteric neurobiology, genome-wide association analyses were performed using the All of Us Research Program database, which includes genetic and medical record data from a large and diverse cohort of participants. GWAS was conducted for six DGBIs: irritable bowel syndrome, functional chest pain, functional diarrhea, functional dyspepsia, functional dysphagia, and functional fecal incontinence. Significant SNPs ($p < 5 \times 10^{-8}$) were identified for five of these conditions (all except functional dysphagia) in individuals of European ancestry.

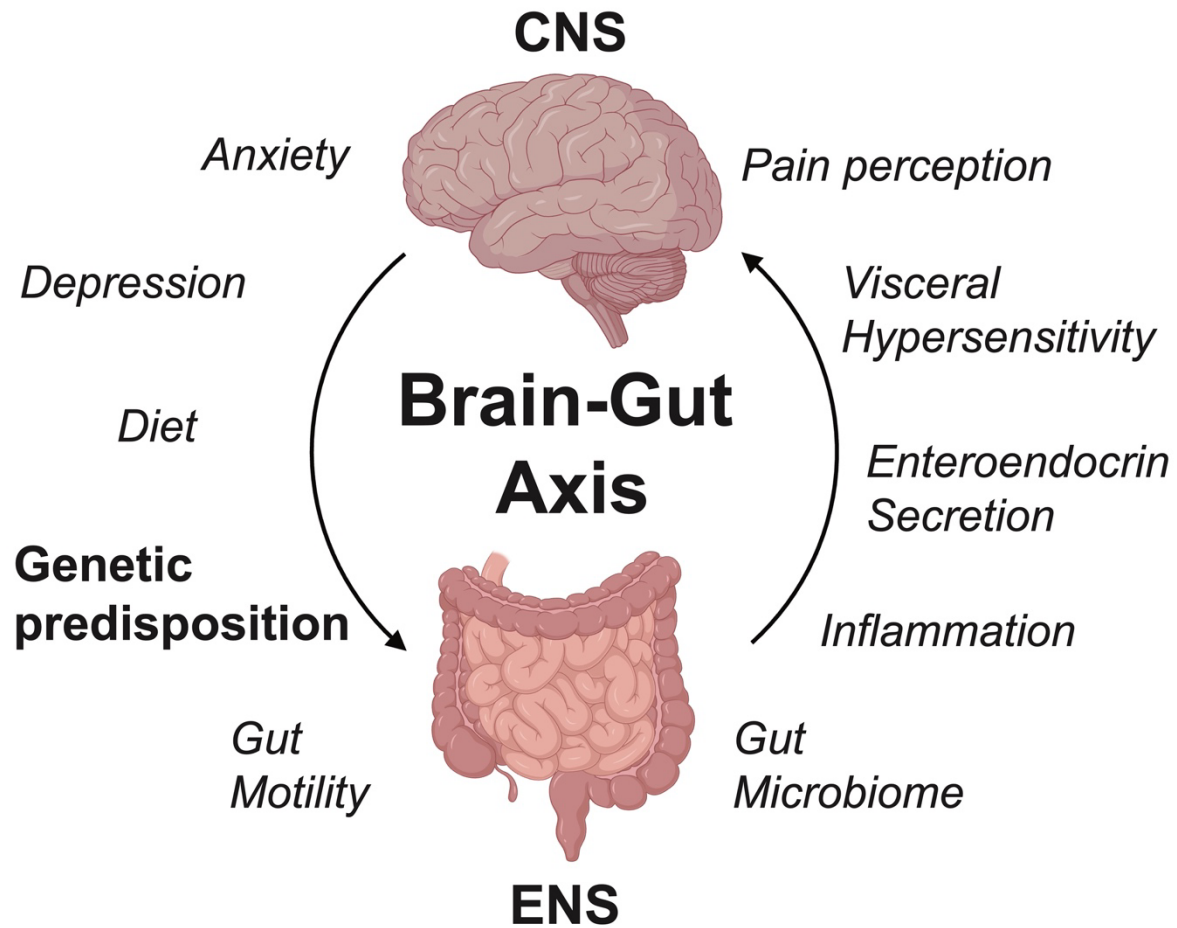


Figure 1.4: The Brain-Gut Axis and the contribution of genetic predisposition to DGBI pathophysiology. Schematic representation of the bidirectional communication between the central nervous system (CNS) and the enteric nervous system (ENS). Symptoms of DGBIs arise from dysregulation within this axis, involving altered gut motility, visceral hypersensitivity, enteroendocrine secretion, inflammation, and microbiome interactions. These physiological processes are reciprocally linked to CNS states such as anxiety and depression. Genetic predisposition (bold) acts as a fundamental modifier of this system, determining an individual's susceptibility to developing chronic gastrointestinal dysfunction in response to environmental and psychological triggers.

HUMAN PLURIPOTENT STEM CELLS AS TOOLS FOR MODELING GUT DEVELOPMENT AND DISEASE

Directed Differentiation Strategies Recapitulate Embryonic Development

Human pluripotent stem cells (hPSCs), including embryonic stem cells (ESCs) (Thomson JA et al., 1998) and induced pluripotent stem cells (iPSCs) (Takahashi K et al., 2007), possess self-renewal and pluripotency (Zakrzewski W et al., 2019), making them uniquely suited for studying human development and disease (Avior Y et al., 2016). Directed differentiation protocols recapitulate major stages of gut development by mimicking embryonic signaling events. Definitive endoderm is efficiently induced by high levels of Nodal/Activin signaling (D'Amour KA et al., 2005). The resulting FOXA2+/SOX17+ endoderm can be patterned into CDX2+ mid/hindgut progenitors by activating WNT signaling (e.g., via CHIR99021) and FGF pathways (McCracken KW et al., 2011).

Following hindgut specification, CDX2+ progenitors spontaneously aggregate into spheroids that mature into human intestinal organoids (HIOs) containing polarized epithelium and mesenchyme (McCracken KW et al., 2011). The regional identity of these organoids can be controlled by varying the duration of FGF/WNT exposure (Tsai YH et al., 2017). However, because early HIOs typically lack an enteric nervous system, complementary strategies are required to derive and incorporate enteric neural progenitors.

Enteric Nervous System Differentiation from hPSCs

Generating enteric neurons from hPSCs requires differentiation toward the vagal neural crest lineage. This is achieved by combined activation of BMP and WNT signaling together with retinoic acid (RA), which caudalizes neural crest cells toward a vagal identity (Fattahi F et al., 2016), (Barber K et al., 2019), (Majd H et al., 2025). The resulting SOX10+/FOXD3+/EDNRB+ enteric neural crest cells (ENCCs) form enteric crestospheres, which can be further differentiated with GDNF to generate enteric neurons and glia (Majd H et al., 2025).

Over 4–12 weeks, crestospheres mature into "enteric ganglioids" containing diverse neuronal subtypes (cholinergic, nitrergic, serotonergic, GABAergic) and glia that closely resemble primary human ENS profiles (Majd H et al., 2025). These ganglioids exhibit functional maturation, including electrical excitability and synaptic connectivity confirmed by calcium imaging and optogenetics (Majd H et al., 2025), making them suitable for the high-throughput screening and disease modeling applications in Chapters 4 and 5.

Advantages and Limitations of hPSC Models

hPSC models offer key advantages over traditional systems. First, they utilize human cells, circumventing interspecies differences where rodent models may obscure disease mechanisms (Majd H et al., 2025). Second, they are scalable, enabling high-throughput screening and the creation of isogenic disease models via CRISPR-Cas9 editing (Schwank G et al., 2013). Third, hPSC-derived cells can functionally engraft *in vivo*; transplantation of nitrergic neuron-enriched ganglioids into *Nos1*^{-/-} mice rescues gastrointestinal motility deficits, establishing proof-of-principle for cell therapy (Fattahi F et al., 2016), (Majd H et al., 2025).

However, limitations remain. hPSC-derived tissues often resemble fetal rather than adult stages (Tsai YH et al., 2017), and in vitro systems lack complex interactions with the microbiome, vasculature, and immune system. Despite these caveats, integrative approaches combining hPSC models with mouse *ex-vivo* and transcriptomics provide a powerful framework for dissecting ENS development and disease.

THESIS OVERVIEW AND OBJECTIVES

This thesis integrates developmental biology, stem cell biology, and human genetics to elucidate mechanisms governing intestinal morphogenesis and enteric nervous system development, and to identify genetic and cellular pathways underlying disorders of gut-brain interaction. The work encompasses four chapters, each addressing a distinct but interconnected aspect of gut development and disease.

Chapter 2 investigates the role of metalloproteinase-mediated matrix degradation in the condensation of PDGFR α ⁺ mesenchymal cells during intestinal villus morphogenesis. Using a combination of mouse explant cultures, pharmacological inhibition of matrix metalloproteinases, and single-cell RNA sequencing, this chapter defines the extracellular matrix remodeling events that enable mesenchymal cell motility and aggregation. Additionally, the chapter examines how Hedgehog signaling regulates expression of ECM-related genes in PDGFR α ⁺ cells, connecting epithelial-to-mesenchymal signaling with mechanical forces that drive villus formation. These findings establish villus morphogenesis as a self-organizing process dependent on coordinated ECM synthesis, degradation, and cell contractility, and have implications for understanding villus atrophy in disease and for engineering vascularized intestinal tissue.

Chapter 3 addresses the question of regional patterning in the enteric nervous system by using spatiotemporally resolved single-cell RNA sequencing of the developing mouse small intestine from E13.5 to E18.5. By segmenting the intestine along its anterior-posterior axis and performing multiplexed scRNA-seq (MULTI-seq), this chapter directly compares the degree of regional specialization in epithelial, mesenchymal, and ENS compartments. The data reveal that, in contrast to the strong Hox-mediated regionalization of epithelium and mesenchyme, the ENS

is organized primarily along a temporal maturation axis and retains a uniform vagal identity. However, ligand-receptor analysis identifies mesenchyme-derived PTN/MDK-PTPRZ1 signaling as a spatially graded microenvironmental cue that fine-tunes ENS progenitor proliferation, neuronal differentiation, and neurotransmitter specification. Functional validation in hPSC-derived enteric ganglioids confirms that modulation of PTPRZ1 signaling alters ENS developmental trajectories, demonstrating that extrinsic niche signals shape ENS maturation despite the absence of intrinsic regional programming.

Chapter 4 focuses on the developmental specification and functional characterization of enteric motor neurons, particularly nitrergic inhibitory neurons. Using hPSC-derived enteric ganglioid models and high-throughput screening approaches, this chapter identifies signaling pathways, including PDGFR inhibition, that promote differentiation of nitrergic neurons from enteric neural crest precursors. The chapter also describes chemical screens to identify drugs that modulate nitrergic neuron activity and enhance gastrointestinal motility *ex vivo*. Transplantation of nitrergic neuron-enriched ganglioids into *Nos1*^{-/-} mice demonstrates functional engraftment and rescue of motility deficits, establishing proof-of-concept for cell-based therapies for enteric neuropathies. This work provides a framework for understanding neurotransmitter specification in the ENS and for developing therapeutic strategies targeting specific neuronal subtypes in motility disorders.

Chapter 5 leverages genome-wide association studies using the All of Us Research Program database to identify genetic variants and candidate genes associated with disorders of gut-brain interaction, including irritable bowel syndrome, functional diarrhea, functional dyspepsia, and functional fecal incontinence. The chapter maps significant SNPs to nearby genes and annotates their known functions, revealing enrichment of genes involved in neuronal excitability, synaptic

transmission, ion channel regulation, and chromatin remodeling. Gene expression analysis in hPSC-derived enteric neural crest cells and enteric ganglioids confirms that many DGBI-associated genes are expressed during ENS development, supporting the hypothesis that genetic variants affecting ENS development or function contribute to DGBI pathophysiology. This chapter lays the groundwork for future functional studies using CRISPR-mediated perturbation and perturb-seq approaches to dissect the role of individual candidate genes in ENS biology and disease.

Collectively, these studies demonstrate the power of integrating mouse ex-vivo models, human pluripotent stem cell models, transcriptomics, and human genetics to address fundamental questions in gut biology and disease. By elucidating the mechanisms that govern intestinal morphogenesis, enteric nervous system patterning, and motor neuron specification, this work provides insights into both normal development and disease pathogenesis. The identification of genetic risk factors for DGBIs and the validation of candidate genes using hPSC models represents a step toward precision medicine approaches for gastrointestinal disorders. Finally, the development of cell-based therapies for enteric neuropathies offers hope for patients with debilitating motility disorders for which few effective treatments currently exist. The marriage of developmental biology with translational medicine illustrated in this thesis exemplifies how understanding developmental mechanisms can illuminate disease pathogenesis and inspire novel therapeutic strategies.

CHAPTER 2: EXTRACELLULAR MATRIX

REMODELING IN INTESTINAL VILLUS

MORPHOGENESIS

INTRODUCTION

Intestinal villi are finger-like projections that line the lumen of the small intestine, increasing its absorptive surface area by approximately 100-fold (Helander HF & Fändriks L, 2014), (Walton KD et al., 2016). These structures are essential for nutrient absorption and represent one of the most architecturally striking examples of tissue morphogenesis in mammalian development (Chin AM et al., 2017). Villus formation occurs during mid-gestation in mice, beginning around embryonic day 14.5 (E14.5), and involves coordinated changes in both epithelial and mesenchymal compartments (Shyer AE et al., 2013), (Walton KD et al., 2016). Prior to villus emergence, the intestinal epithelium is organized as a flat, pseudostratified layer overlying undifferentiated mesenchyme (Grosse AS et al., 2011). The transformation from this smooth configuration to a highly folded villus-crypt architecture requires coordinated cell proliferation, differentiation, migration, and the generation of mechanical forces. Understanding the cellular and molecular mechanisms that drive villus morphogenesis has profound implications for regenerative medicine, tissue engineering, and the treatment of disorders characterized by villus atrophy, including celiac disease, inflammatory bowel disease, and chemotherapy-induced enteropathy (Schumann M et al., 2017); (Fraser JS & Ciclitira PJ, 2001); (Dahlgren D et al., 2021)

A key initiating event in villus morphogenesis is the condensation of subepithelial mesenchymal cells that express high levels of platelet-derived growth factor receptor α (PDGFR α) (Walton KD et al., 2012). Studies using genetic lineage tracing and live imaging in mice have demonstrated that, beginning at E13.5, a subset of mesenchymal cells immediately beneath the epithelium upregulates PDGFR α expression, becoming highly motile and subsequently aggregating into discrete clusters (Walton KD et al., 2012), (Shyer AE et al., 2015). These PDGFR α^{high} condensates form in a proximal-to-distal wave along the anterior-posterior axis of the developing small intestine and spatially correlate with sites where the overlying epithelium begins to buckle and evaginate to form nascent villi (Walton KD et al., 2012), (Shyer AE et al., 2013). Genetic ablation of non-muscle myosin genes *Myh9* and *Myh10* in PDGFR α -expressing mesenchyme disrupts both condensate formation and epithelial folding, establishing that actomyosin-mediated contractile force in PDGFR α^{high} cells is necessary to initiate villus morphogenesis (Freddo AM et al., 2016), (Huycke TR et al., 2023).

While the requirement for mesenchymal cell motility and contractility in villus formation is well established, the role of the extracellular environment in facilitating these cellular behaviors remains poorly understood. The extracellular matrix (ECM) is a complex network of proteins and polysaccharides that provides structural support to tissues and regulates cell adhesion, migration, proliferation, and differentiation (Frantz C et al., 2010), (Hynes RO, 2009). ECM composition and architecture are dynamic during development, and ECM remodeling—through both proteolytic degradation and force-mediated reorganization—is essential for many morphogenetic processes (Bonnans C et al., 2014). However, the specific changes in ECM architecture and composition that occur during intestinal villus morphogenesis, and the molecular mechanisms that regulate these changes, have not been systematically investigated.

In this chapter, I investigate the role of ECM adhesion and remodeling in intestinal villus morphogenesis with a particular focus on PDGFR α^{high} subepithelial mesenchyme. Using single-cell gene expression analysis, I identify a PDGFR α^{high} lineage that is transcriptionally enriched for integrins, ECM components, and matrix metalloproteinases during the window of villus patterning. Through explant culture and small-molecule inhibition, I demonstrate that integrin-mediated cell–ECM interactions and matrix metalloproteinase (MMP)-mediated matrix degradation are both necessary for PDGFR α^{high} mesenchymal condensation and for the initiation of epithelial folding. I further show that the spatiotemporal expression pattern of Tgf- β signaling genes correlates with the expression of ECM-related genes in PDGFR α^{high} cells and that pharmacologic modulation of this pathway alters condensate size and villus patterning. Although the causal relationships between Tgf- β signaling, specific ECM programs, and PDGFR α^{high} cell behaviors remain to be fully defined, these findings establish ECM adhesion and remodeling as essential components of the morphogenetic machinery that drives villus formation and identify Tgf- β signaling as a candidate upstream regulator of this program.

RESULTS

Pdgfra^{High} Cells Have Transcriptional Signatures of ECM Adhesion and Remodeling During Patterning of Villi

To identify molecular programs that could drive mesenchymal condensation, I first analyzed the transcriptional states of the developing mesenchyme along the mesenchymal–subepithelial interface (MSI). Integration and annotation of scRNA-seq data from E13.5–E18.5 small intestine revealed a continuum of mesenchymal populations that included muscle/ICC progenitors, subepithelial mesenchyme, and a distinct Pdgfra^{high} subset (Figure 2.1A).

Focusing on the subepithelial mesenchyme, reclustering isolated a Pdgfra^{high} lineage that was transcriptionally distinct from neighboring Pdgfra^{low} and cycling progenitors (Figure 2.1B). Cell-cycle scoring showed that Pdgfra^{high} cells were largely post-mitotic compared with more proliferative mesenchymal populations suggesting that condensation is driven by changes in cell state rather than proliferation (Figure 2.1B, Figure 2.1C). Density plots of Pdgfra populations from E13.5 to E14.5 (villus initiation) show increased in Pdgfra^{high} cells (Figure 2.1F), temporally aligning this cell state with the onset of clustering and epithelial folding. Gene expression analysis of the Pdgfra^{high} lineage revealed robust enrichment of genes related to ECM adhesion and remodeling, including integrin-mediated cell–ECM interactions and matrix degradation (Figure 2.1E). Pdgfra^{high} cells displayed elevated expression of integrin subunits, collagens, laminins, and matrix metalloproteinases, consistent with interaction and reshaping of the local matrix environment (Figure 2.1F). This transcriptional signature suggests that the Pdgfra^{high} state is specifically adapted to facilitate the dynamic cell–matrix interactions necessary for mesenchymal condensation.

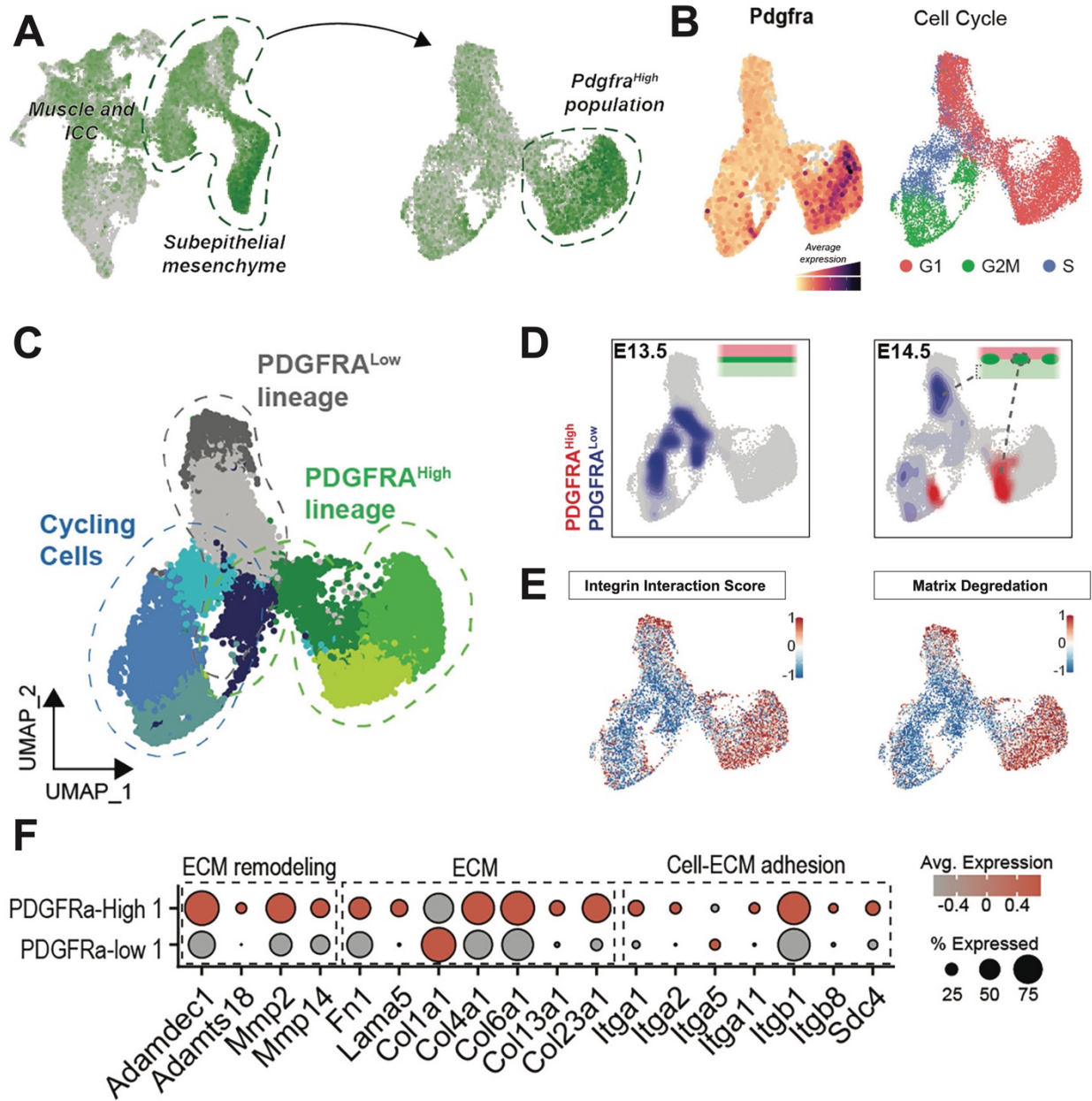


Figure 2.1: $\text{Pdgfra}^{\text{High}}$ cells have transcriptional signatures of ECM adhesion and remodeling during patterning of villi

A. UMAP visualization of integrated scRNA-seq data from developing mouse intestinal mesenchyme (E13.5–E18.5), showing the separation of Muscle/ICC progenitors from the subepithelial mesenchyme (left) and the isolation of the subepithelial subset (right).

B. Feature plots projecting Pdgfra expression (left) and assigned cell cycle phases (right) onto the subepithelial mesenchyme manifold.

C. UMAP visualization of the reclustered subepithelial mesenchyme identifying three distinct populations: a $\text{Pdgfra}^{\text{Low}}$ lineage (gray), a $\text{Pdgfra}^{\text{High}}$ lineage (green), and cycling progenitors (blue).

D. Density plots comparing the distribution of $\text{Pdgfra}^{\text{High}}$ (red) and $\text{Pdgfra}^{\text{Low}}$ (blue) populations at E13.5 versus E14.5, highlighting the expansion of the $\text{Pdgfra}^{\text{High}}$ state during villus initiation.

E. Feature plots showing enrichment of transcriptional module scores for "Integrin Interaction" and "Matrix Degradation" within the $\text{Pdgfra}^{\text{High}}$ population.

F. Dot plot showing differential expression of genes related to ECM remodeling (Mmp2 , Mmp14), ECM composition (Colla1 , Lama5), and Cell-ECM adhesion (Itga1 , Itgb1) between $\text{Pdgfra}^{\text{High}}$ and $\text{Pdgfra}^{\text{Low}}$ clusters. Dot size represents the percentage of cells expressing the gene; color intensity indicates average expression levels.

Integrin-Mediated Cell-ECM Interactions Are Required for Mesenchymal Condensation

The enrichment of ECM adhesion genes in Pdgfra^{high} cells led to the hypothesis that specific integrin heterodimers are required for condensation and villus formation. To test this, I established *ex-vivo* cultures of E13.5 small intestine, a stage when the epithelium is still flat and Pdgfra^{high} cells have not yet formed mature condensates. In control cultures, Pdgfra^{high} clusters emerged beneath the epithelium over 48 hours, coincident with the formation of stereotyped epithelial folds that resemble nascent villi (Figure 2.2A).

Pharmacologic inhibition of $\alpha 2\beta 1$ integrin using the small-molecule inhibitor TC-I 15 disrupted this process in a dose-dependent manner. Whereas control explants formed rounded, evenly spaced condensates that underpinned epithelial invaginations, TC-I 15–treated explants exhibited irregular, poorly defined mesenchymal clusters and a largely flat epithelial sheet (Figure 2.2B). Quantification of cluster morphology showed a significant reduction in circularity and an increase in cluster area with increasing doses of TC-I 15, consistent with impaired compaction and coalescence of Pdgfra^{high} cells (Figure 2.2D).

Similarly, inhibition of $\alpha 5\beta 1$ integrin using the peptide antagonist ATN-161 perturbed villus patterning. ATN-161–treated explants displayed enlarged and misshapen condensates with attenuated epithelial folds relative to controls (Figure 2.2C). Morphometric analysis again revealed decreased circularity and increased area of Pdgfra^{high} clusters (Figure 2.2E). Together, these data demonstrate that $\alpha 2\beta 1$ - and $\alpha 5\beta 1$ -mediated adhesion to the ECM is required for proper mesenchymal condensation and epithelial folding, and they functionally validate the transcriptional signatures of enhanced cell–ECM adhesion in Pdgfra^{high} mesenchyme.

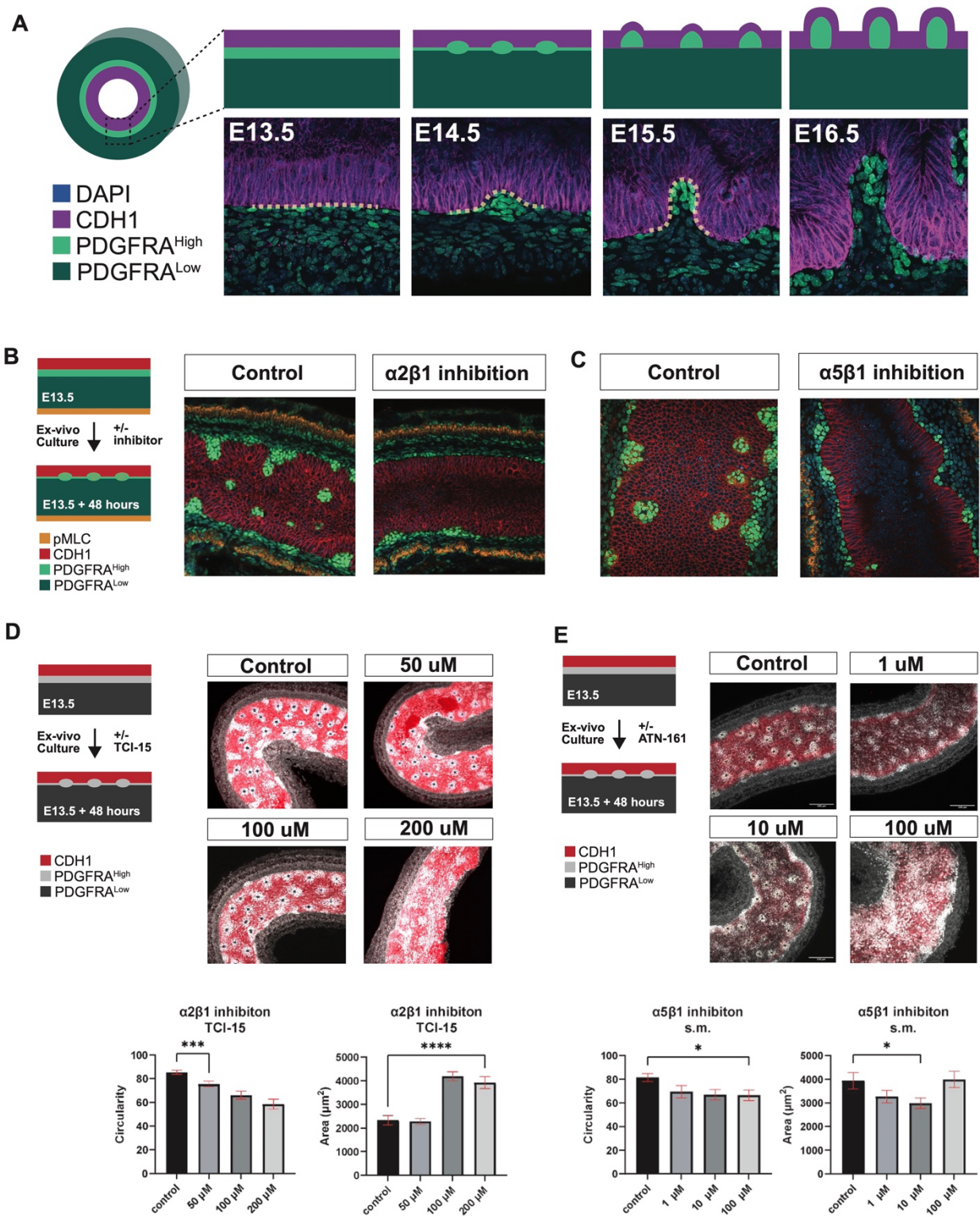


Figure 2.2: Integrin-Mediated Cell-ECM Interactions Are Required for Mesenchymal Condensation

A. Schematic illustration of the stages of villus morphogenesis. Villus patterning (E13.5–E14.5) involves the clustering of Pdgfra^{High} cells (light green) beneath the epithelium (purple) to form condensates. Villus elongation (E15.5–E16.5) follows as the epithelium folds outward.

B. Representative immunofluorescence images of E13.5 intestinal explants cultured for 48 hours with vehicle control or the $\alpha 2\beta 1$ integrin inhibitor TCI-15 ([50, 100 μ M]). Tissues are stained for E-Cadherin (Red, epithelium) and Pdgfra-GFP (Green, mesenchyme). Inhibition disrupts the formation of defined mesenchymal clusters. Scale bar: [100 μ m].

C. Representative immunofluorescence images of E13.5 explants cultured for 48 hours with vehicle control or the $\alpha 5\beta 1$ integrin inhibitor ATN-161. Staining as in (B).

D. Quantification of cluster morphology for the $\alpha 2\beta 1$ inhibition experiment in (B). Bar graphs show the Cluster Circularity (left) and Cluster Area (right). Data represent $n = 2$ explants per condition. Statistical significance determined by one-way ANOVA: * $p < 0.05$, ** $p < 0.01$.

E. Quantification of cluster morphology for the $\alpha 5\beta 1$ inhibition experiment in (C). Bar graphs show Cluster Circularity and Area.

Matrix Metalloproteinase Activity Is Necessary for Mesenchymal Condensation

Because $\text{Pdgfra}^{\text{high}}$ cells also express genes associated with matrix remodeling, I next asked whether proteolytic degradation of ECM is required for condensation. Immunofluorescent images of ECM components such as fibrillar collagen I, fibronectin, elastin, and laminin confirm their expression in subepithelial layer of the developing small intestine prior to mesenchymal condensation. (Figure 2.3A). Broad inhibition of MMP activity was achieved by treating E13.5 intestinal explants with Marimastat or GM6001 throughout a 48-hour culture period. In vehicle-treated controls, $\text{Pdgfra}^{\text{high}}$ cells organized into compact condensates beneath the epithelium, and villus-like epithelial folds were evident (Figure 2.3B). In contrast, both Marimastat- and GM6001-treated explants failed to generate discrete $\text{Pdgfra}^{\text{high}}$ clusters: subepithelial mesenchyme remained dispersed, and the epithelium largely retained a flat morphology reminiscent of the pre-patterning state.

Live imaging of Pdgfra -labeled subepithelial mesenchyme confirmed that, under broad MMP inhibition, cells remained motile but did not coalesce into stable condensates (Figure 2.3C). Instead, they exhibited transient contacts that failed to mature into long-lived aggregates, suggesting that dynamic matrix turnover is required for cells to rearrange and pack efficiently. In situ zymography using quenched fluorescent substrates demonstrated that GM6001 effectively abolished MMP activity in the subepithelial layer, whereas robust activity was observed in control sections, particularly at sites of emerging villus clusters (Figure 2.3D).

To directly test whether reducing collagen density is sufficient to promote condensation, I treated explants with exogenous collagenase. Collagenase treatment thinned and perforated the

subepithelial collagen network and led to the formation of larger, more coalesced $\text{Pdgfra}^{\text{high}}$ clusters compared with untreated controls (Figure 2.3E–F). These manipulations establish that matrix metalloproteinase–dependent degradation of fibrillar ECM, and more broadly collagen clearance, is necessary for the transition from a dispersed to a condensed mesenchymal state and for the accompanying epithelial folding.

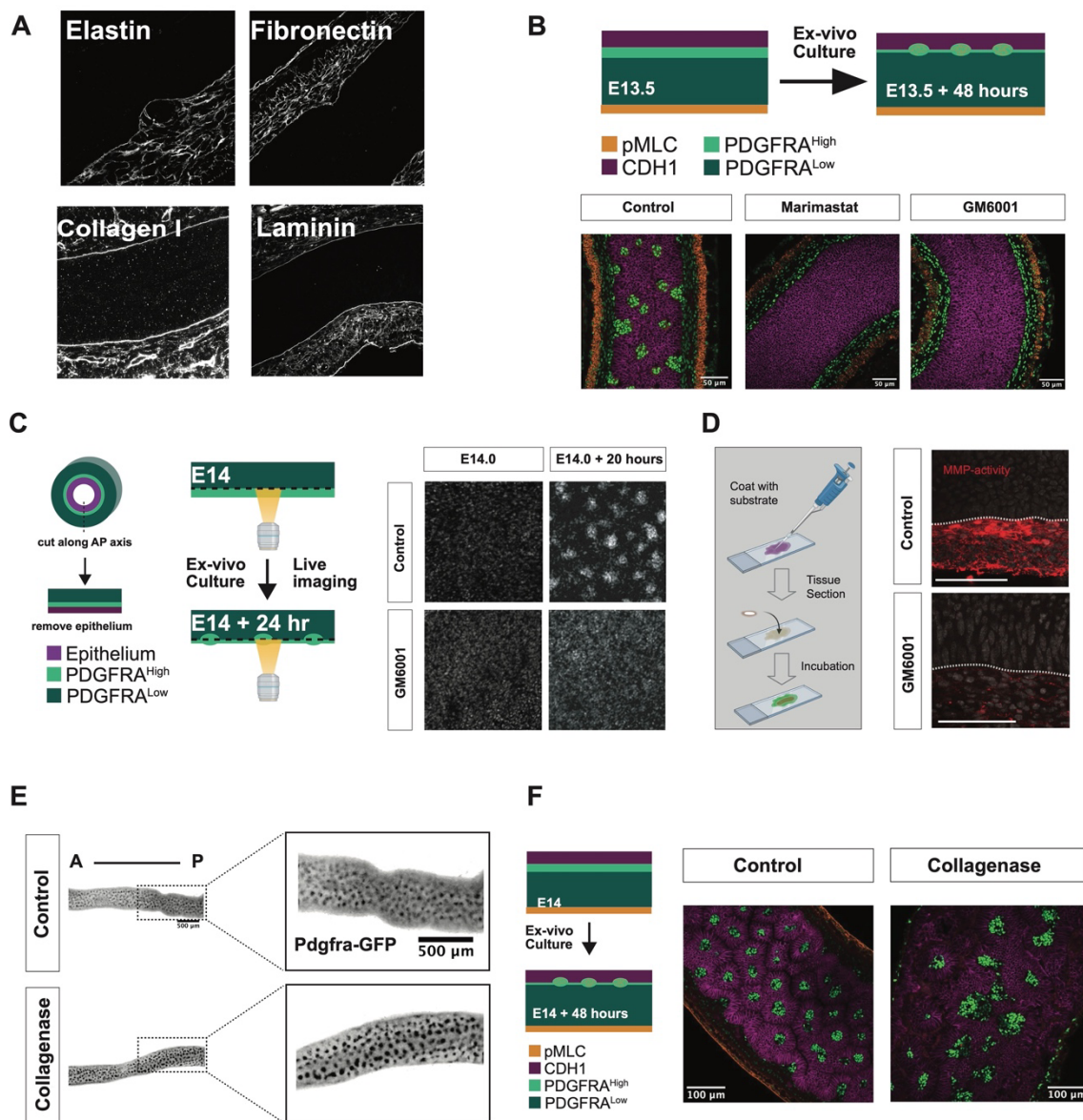


Figure 2.3: Matrix Metalloproteinase Activity Is Necessary for Mesenchymal Condensation

A. Representative immunofluorescence images of key ECM components present at E14.0 in subepithelial mesenchyme.

B. Representative immunofluorescence images of E13.5 explants cultured for 48 hours with broad-spectrum MMP inhibitors Marimastat (10 μ M) or GM6001 (10 μ M).

C. Time-lapse live imaging sequences of subepithelial mesenchyme in E14.0 explants treated with GM6001 vs Control show persistent random motion without aggregation in treated samples.

D. In situ zymography of E14.5 intestinal sections. Gelatinase activity is visualized by the unquenching of a fluorescent substrate (Red). Treatment with GM6001 (bottom) abolishes the signal, confirming specificity. Dotted line marks the epithelial-mesenchymal interface. Scale bar: [100 μ m].

E, F. Representative immunofluorescence images of E13.5 explants treated with exogenous Collagenase for 48 hours. Collagenase-treated explants compared to controls, showing enlarged condensates.

TGF- β Signaling Expression Pattern Matches ECM Genes in Pdgfra^{high} Cells and Is Required for Proper Villus Patterning

Given the strong enrichment of ECM-related genes in Pdgfra^{high} cells, I next sought to identify upstream signaling pathways that coordinate these programs. Gene expression profiling across developmental time revealed that ECM genes enriched in the Pdgfra^{high} lineage—including non-fibrillar collagens, laminins, and matrix remodeling enzymes—show a temporal increase from E13.5 through the villus patterning window that closely parallels the rise of Tgf β ligands and receptors (Figure 2.4A). Spatial (pseudotemporal) analysis along the proximal–distal axis at E15.5 further showed that segments with higher expression of these ECM genes also displayed elevated expression of Tgf- β pathway components (Figure 2.4B), suggesting a spatial coupling between Tgf- β activity and the ECM-remodeling state of Pdgfra^{high} mesenchyme.

To test whether Tgf- β signaling is functionally required for villus patterning, I cultured E13.5 intestinal explants in the presence of the TGF- β inhibitor SB431542 or exogenous TGFB1. After 48 hours, control explants exhibited regularly spaced, spherical Pdgfra^{high} condensates and well-defined villus folds (Figure 2.4C). SB431542 treatment markedly reduced the number and definition of mesenchymal clusters and resulted in a largely flattened epithelium, indicating a failure to initiate or stabilize condensation. In contrast, TGFB1 treatment produced enlarged Pdgfra^{high} clusters and exaggerated epithelial protrusions, consistent with enhanced condensation and folding.

Together, these results support a model in which Tgf- β signaling promotes an ECM-remodeling and adhesion program in Pdgfra^{high} mesenchyme. By upregulating ECM components and remodeling enzymes whose temporal and spatial expression aligns with villus patterning, Tgf β

signaling licenses subepithelial cells to both adhere to and remodel their surrounding matrix. This coordinated regulation of integrin engagement, matrix degradation, and ECM composition is essential for mesenchymal condensation and the emergence of patterned villi.

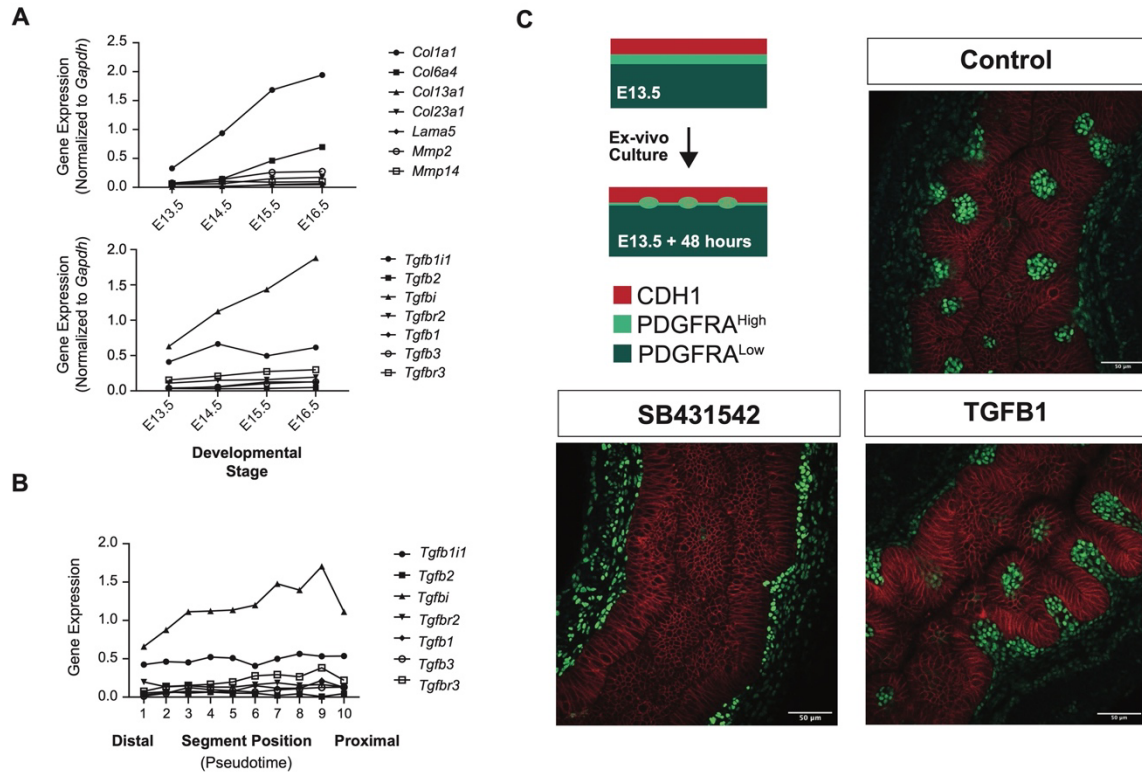


Figure 2.4: Tgf-β signaling is necessary for patterning of villi and its expression patterns match ECM genes enriched in *Pdgfra*⁺ mesenchyme.

A. Comparative temporal gene expression plots showing that the upregulation of ECM genes enriched in *Pdgfra*^{High} mesenchyme (top) parallels the expression of TGF-β ligands (*Tgfb1*, *Tgfb2*) and receptors (*Tgfb1*, *Tgfb2*) (bottom) during the patterning window (E13.5–E16.5).

B. Spatial gene expression analysis along the proximal-distal axis of the E15.5 intestine (Pseudotime). ECM remodeling genes and TGF-β pathway components show correlated expression gradients, being higher in the Proximal segments where villi are more developed.

C. Functional perturbation of TGF-β signaling in E13.5 explants cultured for 48 hours. Representative images show: Control (left), TGF-β inhibitor SB431542 (10 μM, middle), and exogenous recombinant TGF-β (100 ng/mL, right). SB431542 treatment prevents condensation and folding, while TGF-β1 activation results in enlarged, hyper-condensed clusters.

Discussion

In this chapter, I have investigated how extracellular matrix adhesion and remodeling contribute to intestinal villus morphogenesis. Using a combination of single-cell gene expression analysis, explant culture, small molecule inhibition, and imaging, I have demonstrated that a transcriptionally distinct $\text{Pdgfra}^{\text{high}}$ subepithelial mesenchymal population is enriched for ECM adhesion and remodeling genes during the villus patterning window. Functional perturbations reveal that integrin-mediated cell–ECM interactions and MMP-dependent matrix degradation are necessary for $\text{Pdgfra}^{\text{high}}$ mesenchymal condensation and epithelial folding. Finally, I provide evidence that Tgf- β signaling exhibits spatiotemporal expression patterns that mirror ECM-related gene expression in $\text{Pdgfra}^{\text{high}}$ cells and that modulation of this pathway alters condensate morphology and villus architecture. Together, these findings support a model in which ECM adhesion, proteolysis, and upstream signaling converge to control the mechanics of villus morphogenesis, while also highlighting important limitations and open questions.

The Role of Integrin Signaling in Mesenchymal Condensation

The requirement for integrin-mediated adhesion in mesenchymal condensation underscores the importance of bidirectional signaling between cells and their extracellular environment during morphogenesis. Integrins are heterodimeric transmembrane receptors that link the ECM to the intracellular cytoskeleton and activate signaling pathways that regulate cell survival, proliferation, migration, and differentiation. In this chapter, pharmacologic inhibition of $\alpha 2\beta 1$ and $\alpha 5\beta 1$ integrins in intestinal explants disrupted the formation of compact, spherical $\text{Pdgfra}^{\text{high}}$ clusters and impaired epithelial folding, as quantified by reduced cluster circularity and altered

cluster area. These results indicate that both collagen-binding ($\alpha 2\beta 1$) and fibronectin-binding ($\alpha 5\beta 1$) integrins contribute to the organization of subepithelial condensates.

Mechanistically, $\alpha 5\beta 1$ –fibronectin interactions are well positioned to regulate actomyosin-based force generation through focal adhesion kinase (FAK), Src, and Rho/ROCK pathways.

Engagement of fibronectin by $\alpha 5\beta 1$ integrin could promote myosin light chain phosphorylation and enhance contractility within $\text{Pdgfra}^{\text{high}}$ cells, establishing a positive feedback loop in which ECM adhesion strengthens cytoskeletal tension, which in turn remodels the ECM and reinforces cell clustering. Although the current experiments do not directly measure contractility (e.g., pMLC levels) under integrin inhibition, the pronounced defects in condensate compaction are consistent with impaired force transmission and highlight the need for future work using live reporters or phospho-specific staining to quantify mechanical outputs.

By contrast, $\alpha 2\beta 1$ –collagen interactions may influence condensation through partially distinct mechanisms. Collagen-binding integrins can regulate focal adhesion turnover, cell polarity, and directional migration through PI3K and other signaling pathways. The observation that $\alpha 2\beta 1$ inhibition leads to enlarged, irregular clusters suggests that collagen engagement helps stabilize cell–cell and cell–matrix contacts necessary for cluster refinement, even if overall contractility is maintained. However, the reliance on pharmacologic inhibitors introduces potential off-target effects and does not fully resolve the relative contributions of epithelial versus mesenchymal integrin pools. Conditional genetic deletion of specific integrin subunits in $\text{PDGFR}\alpha$ -expressing cells, ideally combined with quantitative force measurements, will be critical to test these mechanistic hypotheses.

The Requirement for MMP-Mediated ECM Degradation

The requirement for MMP activity in mesenchymal condensation is consistent with the broader role of matrix metalloproteinases in tissue morphogenesis and remodeling. MMPs are a family of zinc-dependent endopeptidases that degrade ECM components and regulate cell behavior by releasing ECM-bound growth factors, generating bioactive ECM fragments, and modulating cell–matrix adhesion. In this study, broad-spectrum pharmacologic inhibition of MMPs using Marimastat and GM6001 prevented Pdgfra^{high} cluster formation and villus folding, while collagenase treatment enlarged condensates, implying that controlled collagen degradation is required to permit cell rearrangements and packing.

Single-cell gene expression analysis further revealed that Pdgfra^{high} cells upregulate MMPs, including Mmp2 and Mmp14, during the villus patterning window. These proteases preferentially target fibrillar collagens and certain ECM glycoproteins but spare many non-fibrillar and transmembrane collagens. This substrate preference suggests a model in which Pdgfra^{high} cells locally degrade fibrillar ECM to loosen mechanical constraints, while maintaining or even increasing non-fibrillar matrix components that may support cell adhesion and signaling. However, because the functional experiments relied on broad MMP inhibition rather than specific genetic perturbation of Mmp2 or Mmp14, the relative contribution of individual MMPs and potential compensation among family members remain unresolved.

Moreover, the explant system does not fully capture the complex in vivo gradients of protease and inhibitor activity. Previous work has implicated TIMP3, a broad-spectrum MMP inhibitor, in restricting MMP activity to specific tissue compartments. Although scRNA-seq data suggest differential expression of TIMP family members across mesenchymal populations, direct

measurements of TIMP protein distribution and activity were not performed here. Future studies integrating spatial transcriptomics, protease activity reporters, and conditional manipulation of TIMP genes will be important to determine whether spatially confined proteolysis sharpens the boundaries between condensed and non-condensed mesenchyme and thereby contributes to villus periodicity.

Dynamic ECM Remodeling as a Mechanism of Morphogenesis

The combined integrin and MMP perturbation experiments support a view of villus morphogenesis in which ECM remodeling is not a passive consequence of condensation but an active driver of tissue shape change. By simultaneously engaging integrins and degrading specific ECM components, *Pdgfra*^{high} cells may transition the subepithelial niche from a relatively rigid, fibrillar collagen-rich environment to a more permissive matrix that allows cells to slide past one another, compact, and transmit force to the overlying epithelium. This conceptual model is consistent with work in other systems showing that local MMP activity and ECM remodeling are required for branching morphogenesis and collective cell migration.

Nevertheless, the current study primarily infers changes in ECM architecture and composition from gene expression and functional perturbations rather than from direct, quantitative measurements of matrix structure. Detailed imaging of ECM fibers (e.g., second harmonic generation, quantitative texture analysis) and mechanical measurements (e.g., local stiffness mapping) across developmental time and under perturbation conditions would be necessary to fully validate the proposed shift from fibrillar to non-fibrillar or basement-membrane-like matrices during condensation. Incorporating such biophysical measurements in future work will help bridge the gap between molecular programs and tissue-scale mechanics.

Tgf- β Signaling as an Upstream Regulator of ECM Programs

The observation that Tgf- β signaling genes exhibit temporal and spatial expression patterns that parallel ECM-related genes in Pdgfra^{high} cells suggests that Tgf- β signaling may coordinate ECM adhesion and remodeling programs during villus morphogenesis. TGF- β ligands and receptors are known regulators of ECM synthesis, MMP expression, and integrin signaling in diverse contexts, including fibrosis and tissue repair. In this chapter, pharmacologic inhibition of TGF- β receptors reduced Pdgfra^{high} condensate formation and villus folding, whereas exogenous TGF- β 1 enlarged condensates and exaggerated epithelial protrusions. These findings position Tgf- β signaling as an important modulator of condensate size and villus architecture.

However, several caveats temper the interpretation that Tgf- β is a master upstream regulator of the Pdgfra^{high} ECM program. First, the pharmacologic perturbations affect all TGF- β -responsive cell types in the explant, including epithelium and non-Pdgfra^{high} mesenchyme, making it difficult to ascribe phenotypes specifically to Pdgfra^{high} cells. Second, although transcriptomic data show correlated expression of ECM and Tgf- β pathway genes, direct evidence that Tgf- β signaling cell-autonomously induces specific integrins, collagens, or MMPs in Pdgfra^{high} cells is lacking. Third, Tgf- β signaling can promote both pro- and anti-proliferative effects depending on context, and altered cell cycle dynamics could contribute to changes in cluster size independently of ECM remodeling.

Addressing these limitations will require cell-type-specific manipulation of Tgf- β signaling (e.g., conditional deletion of Tgfbr1/2 or expression of pathway reporters in Pdgfra^{high} cells) combined with single-cell and spatial transcriptomics to identify direct transcriptional targets. It will also be important to disentangle Tgf- β from other pathways known to impact villus morphogenesis, such

as BMP, Wnt, and mechanical feedback from the epithelium, to determine whether Tgf- β acts as a primary instructive cue or as a permissive modulator of a broader morphogenetic network.

Implications for Villus Regeneration and Intestinal Disease

The mechanistic understanding of villus morphogenesis developed in this chapter has important implications for intestinal disease and tissue engineering. Villus atrophy is a common feature of many intestinal diseases, including celiac disease (in which villus atrophy results from immune-mediated destruction of the epithelium), inflammatory bowel disease (characterized by chronic inflammation and tissue damage), and chemotherapy-induced enteropathy (where anti-cancer drugs damage both epithelium and mesenchyme). Strategies to promote villus regeneration or to engineer intestinal tissue with physiological villus architecture will require a mechanistic understanding of the cues and cellular behaviors that drive villus formation. The identification of ECM remodeling—and its regulation by integrins, MMPs, and Tgf- β signaling—as an essential component of villus morphogenesis suggests that therapeutic modulation of these pathways, or direct engineering of the ECM microenvironment, may provide new avenues for promoting intestinal repair.

Furthermore, the availability of three-dimensional human intestinal organoids (HIOs) derived from human pluripotent stem cells has created new opportunities to apply these developmental insights to tissue engineering. HIOs can form epithelial and mesenchymal compartments but often lack the proper villus-crypt architecture necessary for physiological function. Findings presented in this chapter suggest that providing organoids with ECM scaffolds that mimic the compliant, dynamically remodeled matrix of the Pdgfra^{high} niche, and engineering mesenchymal compartments to express appropriate integrin and MMP repertoires under controlled Tgf- β

signaling, may enhance villus-like morphogenesis. Systematic manipulation of matrix composition, ligand presentation, and protease activity in organoid systems will be an important future direction for translating these developmental mechanisms into regenerative strategies.

Caveats and Future Directions

Several caveats of the current work warrant emphasis. First, the reliance on small molecule inhibitors for integrins, MMPs, and Tgf- β receptors raises the possibility of off-target effects and incomplete inhibition. Complementary genetic approaches—including conditional knockouts or knockdowns in PDGFR α -expressing cells and CRISPR-based perturbations in organoid models—will be necessary to confirm the specificity and sufficiency of the pathways implicated here. Second, the explant culture system, while recapitulating many aspects of in vivo development, lacks vascular, immune, and neural components that may influence ECM remodeling and villus patterning. Intravital imaging and in situ perturbations in intact embryos will be important to validate whether the dynamics observed ex vivo accurately reflect in vivo processes.

Third, the current analyses largely treat Pdgfra^{high} cells as a homogeneous population, yet single-cell data hint at further heterogeneity within this lineage. Subpopulations may differ in their ECM synthesis, proteolytic capacity, or responsiveness to Tgf- β and other signals. Future work leveraging higher-resolution clustering, lineage tracing, and spatial transcriptomics could reveal functionally distinct Pdgfra^{high} subtypes and clarify how they cooperate to build villi. Finally, integrating quantitative biophysical measurements—such as traction force microscopy, laser ablation, or computational modeling of tissue mechanics—will be essential to move from

qualitative descriptions of condensation to a predictive, quantitative framework for how ECM remodeling, cell adhesion, and contractility together generate the villus architecture.

Summary

In summary, the work presented in this chapter establishes ECM remodeling as a central mechanism underlying intestinal villus morphogenesis. A transcriptionally defined *Pdgfra*^{high} mesenchymal population engages integrin-mediated adhesion and MMP-dependent proteolysis to remodel its local microenvironment, enabling cell motility, condensation, and force transmission to the overlying epithelium. Tgf- β signaling emerges as a candidate upstream regulator that tunes ECM-related gene expression and condensate morphology, although its precise cell-type-specific and molecular roles remain to be fully elucidated. By identifying specific adhesion receptors, proteases, and signaling pathways that control villus morphogenesis, this work provides a framework for understanding the physical and molecular basis of intestinal tissue architecture and opens new avenues for therapeutic intervention in intestinal disease and for engineering intestinal tissue with appropriate villus structure.

CHAPTER 3: REGIONAL PATTERNING AND MICROENVIRONMENTAL REGULATION OF ENTERIC NERVOUS SYSTEM DEVELOPMENT

Introduction

During embryogenesis, the gut tube emerges from endodermal and mesodermal precursors and subsequently develops into a regionally patterned organ with distinct morphological and functional domains along its anterior-posterior (AP) axis (Wells JM & Spence JR, 2014). These specialized domains are shaped in part by the epithelium (endoderm-derived) and the surrounding mesenchyme (mesoderm-derived), each adopting unique transcriptional programs that drive tissue morphogenesis and organ-specific functions (Zorn AM & Wells JM, 2009).

Cellular diversity within and between these compartments generate discrete niches that influence the fate and behavior of other cell lineages during intestinal development. Notably, the small intestine harbors migrating progenitor populations derived from other anatomical locations, such as neural crest, endothelial, and myeloid cells, that integrate into the gut and contribute to its complexity (Nagy N & Goldstein AM, 2017). Although AP patterning in the epithelium and mesenchyme is well established, whether and how the ENS acquires region-specific transcriptional identities after settling into these niches remains an open question.

While the ENS transcriptional landscape remains less defined, the small intestine's functional output exhibits clear regionalization along the anterior-posterior axis. Physiologically, motility patterns shift aborally: contraction frequencies step down from the duodenum to the ileum, and

propulsive efficacy varies to accommodate the transition from rapid mixing to prolonged absorption (Huizinga JD & Lammers WJ, 2009). Structurally, recent work revealed that the ENS is organized into circumferential stripes of varying neuronal density that differ along the length of the gut, potentially scaffolding these distinct motility gradients (Hamnett R et al., 2022). Furthermore, the neighboring epithelium is strictly zoned: recent study demonstrated that the small intestinal epithelium is divided into five discrete metabolic domains, each governed by unique transcriptional programs (Zwick RK et al., 2024). Given these overt functional and epithelial boundaries within the small intestine, it is reasonable to hypothesize that the ENS possesses a parallel, regionally distinct transcriptional architecture.

The enteric nervous system is a complex and autonomous network of neurons and glia embedded within the gastrointestinal tract. It regulates motility, secretion, absorption, local blood flow, and immune responses (Furness JB, 2012). The ENS contains more neurons than the spinal cord and is organized into two major plexuses: the myenteric plexus, which regulates motility, and the submucosal plexus, which modulates secretion and vascular tone (Furness JB, 2008). These neurons form intrinsic reflex circuits that sense mechanical and chemical cues within the gut lumen and coordinate appropriate responses. Derived from vagal neural crest (vNC) progenitors, ENS development involves extensive AP migration, proliferation, and differentiation (Le Douarin NM & Teillet MA, 1973). vNC cells originate from the caudal hindbrain, migrate through the somites, enter the foregut around E9 in mice, and then colonize the entire gastrointestinal tract (Young HM et al., 1998). This AP migration imparts a temporal gradient to ENS assembly, but whether it also establishes enduring spatial specialization remains unresolved.

While early migratory and colonization events are well characterized, the later stages of ENS development remain far less understood, including the interplay between ENS progenitors and local tissue compartments, the transcriptional mechanisms of neuronal and glial diversification, and the establishment of neurotransmitter specification, axon trajectories, and circuit organization (Ganz J, 2018), (Sasselli V et al., 2013). Classic studies have shown that Enteric Neural Crest Cells (ENCCs) require pathways such as Gdnf/Ret and Edn/Ednrb for colonization and survival (Taraviras S & Pachnis V, 1999), (Baynash AG et al., 1994). However, it is unknown whether ENS cells subsequently adopt region-specific transcriptional programs that parallel the strong AP identities of surrounding epithelial and mesenchymal tissues, or whether they instead maintain a largely uniform intrinsic program that is fine-tuned by local environmental cues.

To dissect the relationship between gut regionalization and ENS development, we integrated multiplexed single-cell RNA sequencing (MULTI-seq) and functional perturbations. This combined approach allowed us to compare the spatial patterning of epithelium and mesenchyme with that of the ENS across developmental time, and to determine whether ENS cells acquire distinct regional identities or instead follow a predominantly temporal differentiation trajectory. By aligning ligand-receptor expression patterns with spatially resolved transcriptomics, we further investigated whether localized microenvironmental cues from the mesenchyme contribute to subtle transcriptional tuning in the ENS despite the absence of overt intrinsic regionalization. This framework enabled us to evaluate both the extent of AP patterning within each compartment and the mechanisms through which extrinsic signals may refine ENS maturation within the context of a strongly regionalized gut.

We find that the epithelial and mesenchymal compartments display pronounced and persistent AP patterning, including in expression of Hox genes and presence of region-specific cell types. In contrast, the ENS maintains a uniform vagal Hox identity and is primarily organized along a temporal maturation axis. Despite the absence of strong intrinsic regionalization, we show that region-specific mesenchymal ligands, including PTPRZ1 ligands PTN and MDK, correlate with subtle ENS transcriptional shifts. Functional perturbations in human pluripotent stem cell-derived ENS cultures confirm that these microenvironmental cues regulate ENS progenitor proliferation, neuronal maturation, and neurotransmitter specification. Together, these findings support a model in which the ENS retains a stable core neuroglial program that is secondarily refined by localized, region-specific niche signals within the patterned gut.

Results

The gut is strongly regionalized along the A-P axis, but the ENS is largely spatially uniform

We previously generated a spatiotemporal single-cell atlas of mouse small intestine (MSI) spanning E13.5–E18.5 where we segmented the tissue along the A-P axis at 2.5 mm spatial resolution (Figure 3.1A). These samples were multiplexed from a single gut to minimize batch effects, with multiple experiments providing data across this developmental window. To better understand the detailed transcriptional events controlling ENS patterning we refined the resolution of this data set by integrating across experimental datasets using CONCORD. The resulting unified latent space was well integrated across experimental data sets yet revealed the expected transcriptional differences across developmental time (Figure 3.1B).

To investigate the extent of patterning within each tissue compartment we applied variance partitioning using split-metadata PCA. We found that A-P position explained substantially more variance in EPI and MES than ENS, whereas developmental time contributed comparably to all (Figure 3.1C). Subsetting this atlas yielded a high-resolution mesenchymal (MES) compartment encompassing known fibroblast- and smooth-muscle derived subtypes (Figure 3.1D,E, Figure S1A). Hox expression patterns revealed AP-graded signatures in MES (Figure 3.1F). The epithelial (EPI) compartment consisted of enterocytes, goblet cells, and enteroendocrine cells (Figure 3.1G,H, Figure S1B) with AP-graded Hox expression as well (Figure 3.1I). ENS cells formed clear progenitor, neuronal, and glial identities based on canonical markers (Sox10 for progenitors; Elavl4 for neurons; S100b for glia-like progenitors), Figure 3.1J,K; Figure S1C). In

contrast to the epithelium and mesenchymal cells, Hox gene expression analysis showed that ENS retained a largely uniform vagal Hox profile with minimal regional variation (Figure 3.1L).

To investigate patterning and cellular composition across regions and stages, we quantified the proportion of each cluster across the A-P axis and developmental time. MES and EPI displayed clear A-P partitioning and sharper stratification by developmental age and region (Figure 3.1E,H; Figure S1D, E). In contrast, projecting A-P position and developmental stage onto the ENS UMAP revealed no substantial differences in regional cell composition (Figure 3.1K; Figure S1F). Quantification of ENS subtype proportions showed overall stability across A-P regions and stages, aside from modest increases in two PTN⁺/PTPRZ1⁺ clusters (clusters 1 and 9; Figure 3.1K). MES and EPI, on the other hand, exhibited both changes in cell-type proportion and clusters that emerge at specific time points (Figure 3.1E,H). Combined, these data indicate that ENS transcriptional program is shaped primarily by temporal maturation rather than A-P regionalization.

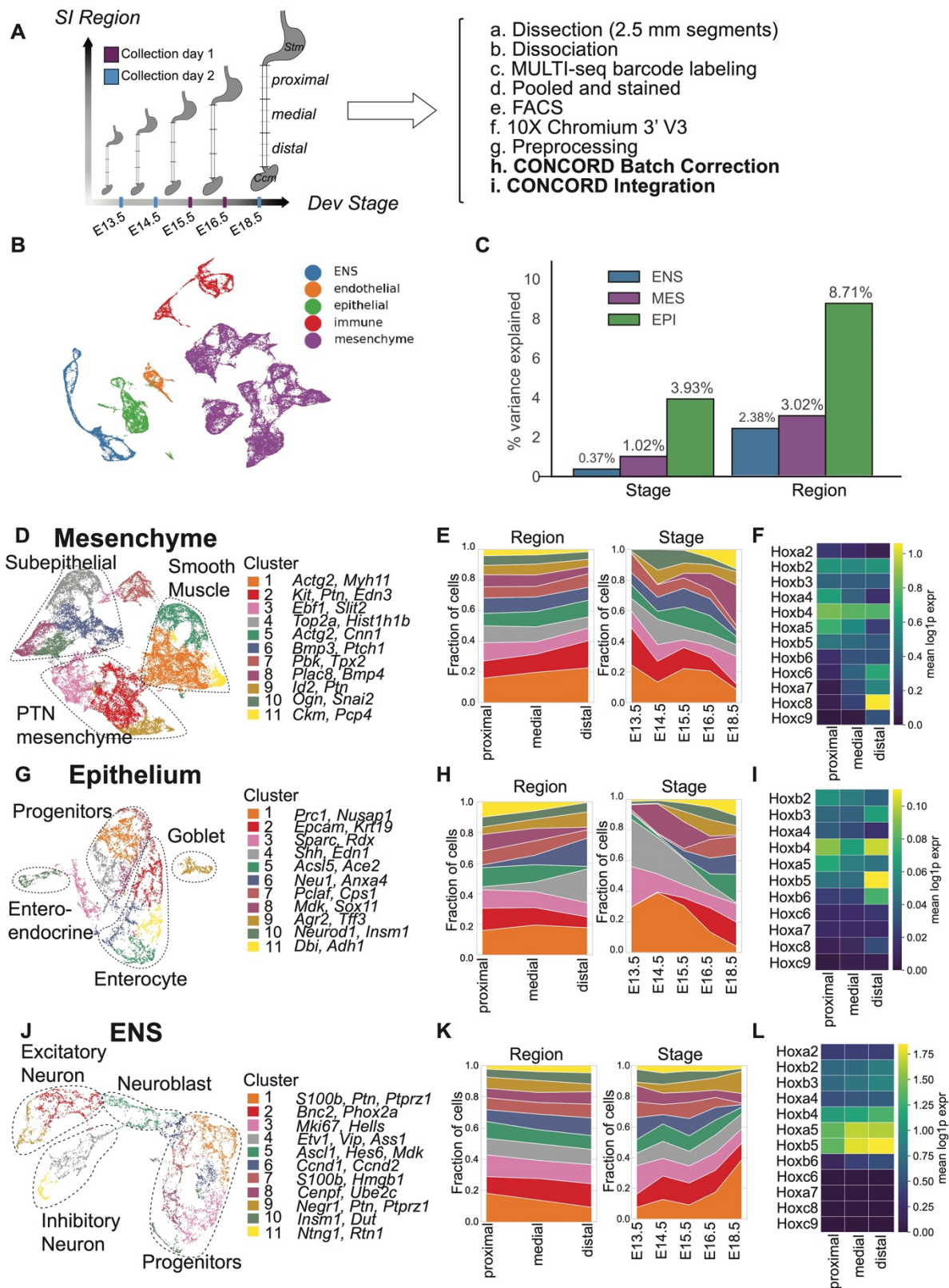


Figure 3.1. The developing gut is strongly regionalized along the A-P axis, but the ENS is largely spatially uniform.

A: Schematic of the spatiotemporal single-cell atlas of the developing mouse small intestine.

B: UMAP of all MSI cells colored by broad compartment annotation (ENS, mesenchyme, epithelium, endothelium, immune).

C: Bar plot showing the percentage of variance in gene expression explained by developmental stage and region for the ENS, mesenchyme (MES), and epithelium (EPI) compartments.

D: UMAP of the MES subset colored by leiden clusters.

E: Fraction of MES clusters across regions and developmental stages.

F: Heatmap showing the mean expression of Hox genes along the anterior-posterior (AP) axis in the MES.

G: UMAP of the EPI subset colored by leiden clusters.

H: Fraction of EPI clusters across regions and developmental stages.

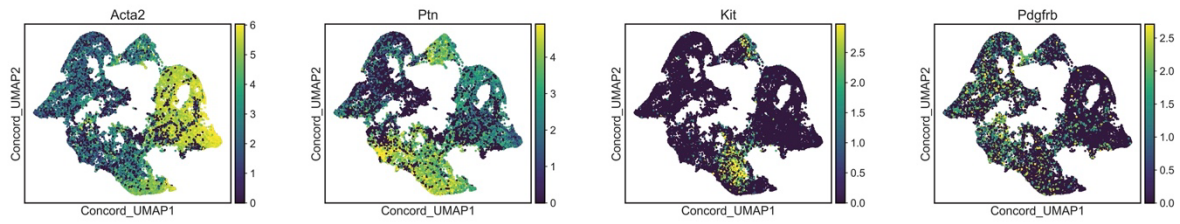
I: Heatmap showing the mean expression of Hox genes along the anterior-posterior (AP) axis in the EPI.

J: UMAP of the ENS subset colored by leiden clusters.

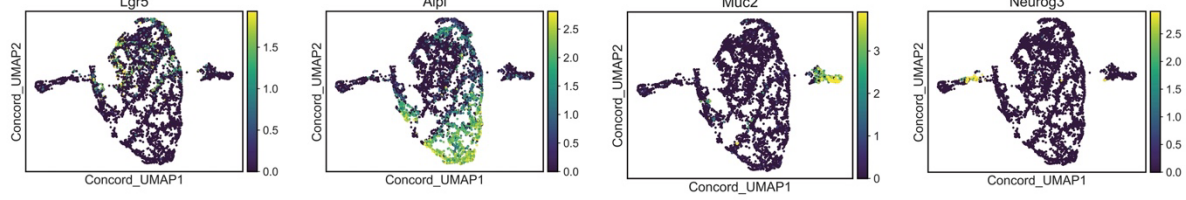
K: Fraction of ENS clusters across regions and developmental stages.

L: Heatmap showing the mean expression of Hox genes along the anterior-posterior (AP) axis in the ENS.

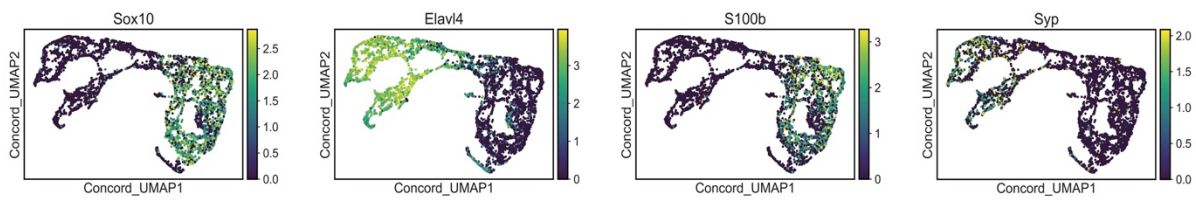
A



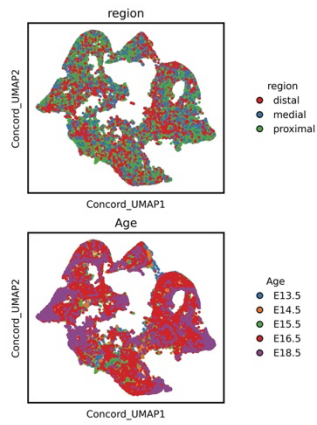
B



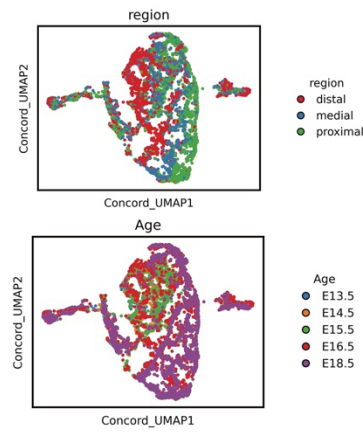
C



D



E



F

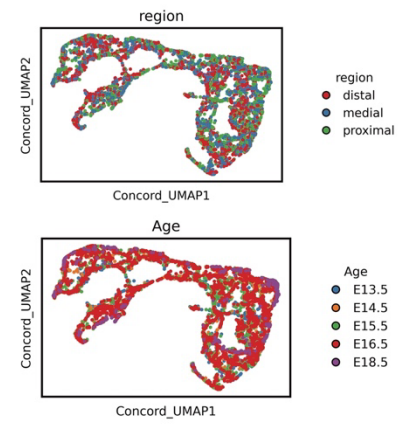


Figure S3.1. MES, EPI, and ENS cells marked by the marker genes, region, and developmental stage

A: UMAP of the expression of canonical markers in MES.

B: UMAP of the expression of canonical markers in EPI.

C: UMAP of the expression of canonical markers in ENS.

D: UMAP of the MES subset, with cells colored by MSI region and developmental stage.

E: UMAP of the EPI subset, with cells colored by MSI region and developmental stage.

F: UMAP of the ENS subset, with cells colored by MSI region and developmental stage.

Microenvironmental signals, including PTN/MDK, provide spatially structured inputs to the ENS

Given that the ENS lacks a robust intrinsic transcriptional code for A-P patterning, we hypothesized that regional specialization is instead imposed by extrinsic cues from the highly patterned gut microenvironment. To systematically identify position-dependent signals capable of instructing ENS development, we modeled intercellular communication between the ENS and its surrounding niches using CellChat (Figure 3.2A). Mesenchyme emerged as the dominant source of incoming ligand-receptor signals to ENS across all stages and regions, with ENS autocrine interactions contributing secondarily (Figure 3.2B).

Pathway-level contrasts revealed a restricted set of spatially or temporally regulated signaling families, including PTN, MDK, EDN, NOTCH, and NCAM (Figure 3.2C,D). Among these, PTN and MDK ranked consistently high across A-P regions and developmental stages, showing coherent spatial and temporal trends (Figure 3.2E-G).

To identify the transcriptional basis for these effects, we modeled ligand and receptor expression as a function of position and developmental stage (Figure 3.2H, I, Tables 7-9). At E15.5, PTN ligand exhibited strong spatial gradients within MES, EPI, and ENS (Figure 3.2J,K; Figure S2A-D), while the receptor PTPRZ1 showed one of the most pronounced spatial slopes within ENS (Figure 3.2P). Temporally, MDK decreased but PTN and PTPRZ1 expression increased within ENS, paralleling the rise of PTN-enriched subclusters (Figure 3.2J, Figure 3.1J; Figure S2E-H).

These analyses position PTN/MDK-PTPRZ1 signaling as a spatially graded, developmentally dynamic microenvironmental axis with potential activity on the ENS.

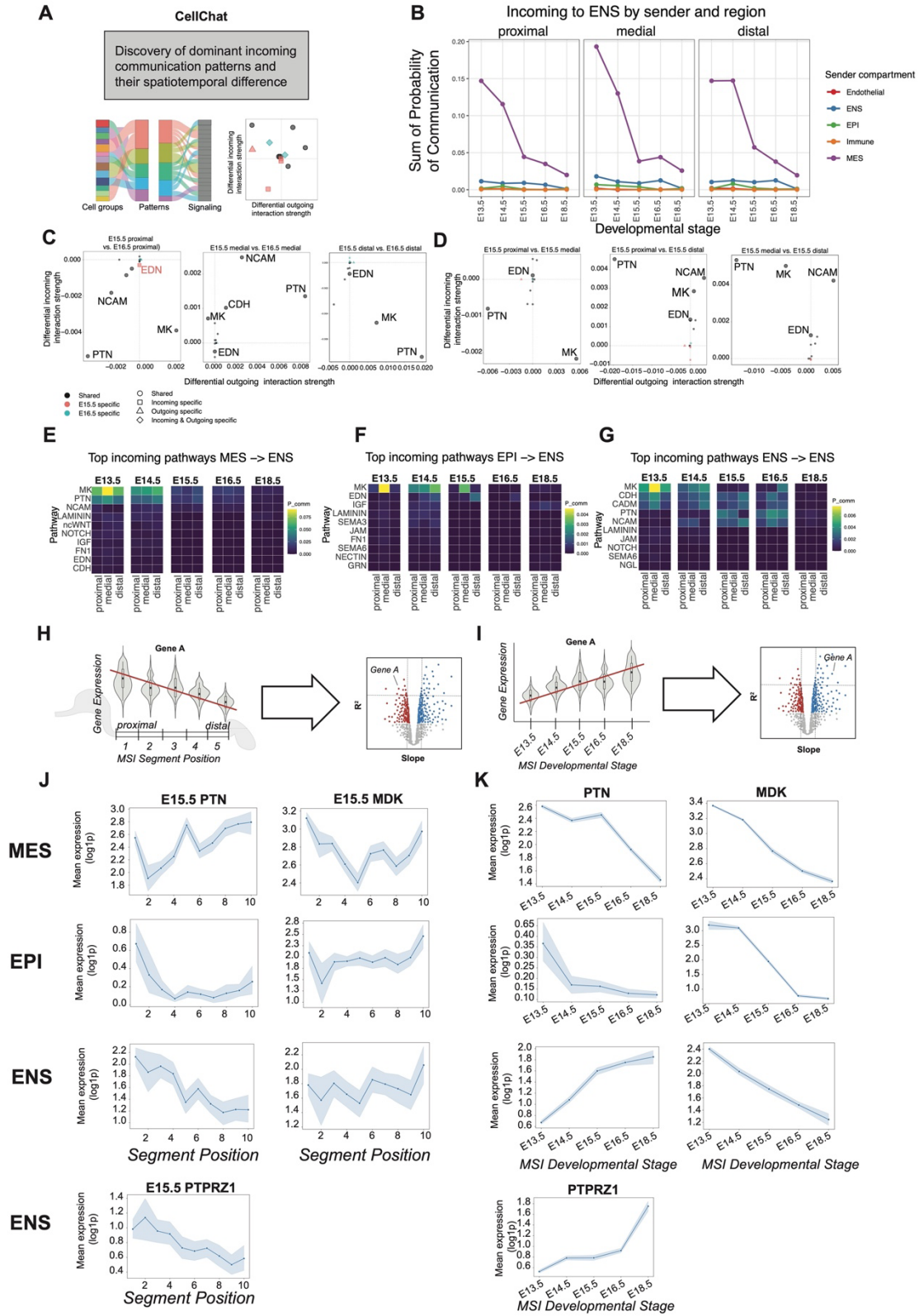


Figure 3.2. Microenvironmental signaling to ENS highlights PTN/MDK pathways with spatial and temporal structure.

A: Conceptual schematic of the intercellular communication analysis using CellChat to model ligand–receptor (LR) communication across compartments.

B: Line plot showing the net incoming signaling strength (Probability of Communication from CellChat) to the ENS from the sender compartments stratified by developmental stage and A-P region. MES is the dominant signaling source.

C: Volcano plots summarizing pathway-level contrasts along the developmental axis (E15.5 proximal vs. E16.5 proximal), highlighting pathways like PTN, MDK, EDN, NCAM.

D: Volcano plots summarizing pathway-level contrasts along the A-P axis of MSI (distal vs. proximal) and temporal axis (E15.5 proximal vs. E15.5 distal), highlighting pathways like PTN, MDK, EDN, CDH, NCAM.

E: Heatmap showing the strength of top incoming pathways from MES to the ENS across A-P regions and developmental stages. PTN and MDK have the highest probability of communication.

F: Heatmap showing the strength of top incoming pathways from EPI to the ENS across A-P regions and developmental stages. EDN and MDK signaling have the highest probability of communication.

G: Heatmap showing the strength of top incoming pathways from ENS to the ENS across A-P regions and developmental stages. PTN and MDK signaling have the highest probability of communication.

H, I: Schematics of the spatial (H) and temporal (I) regression models used to link Ligand-Receptor transcript levels to position and age.

J: Mean expression of Mdk and Ptn and their receptor Ptprz1 vs MSI segment position from the spatial regressions at E15.5.

K: Mean expression of Mdk and Ptn and their receptor Ptprz1 vs MSI segment position from the temporal regressions.

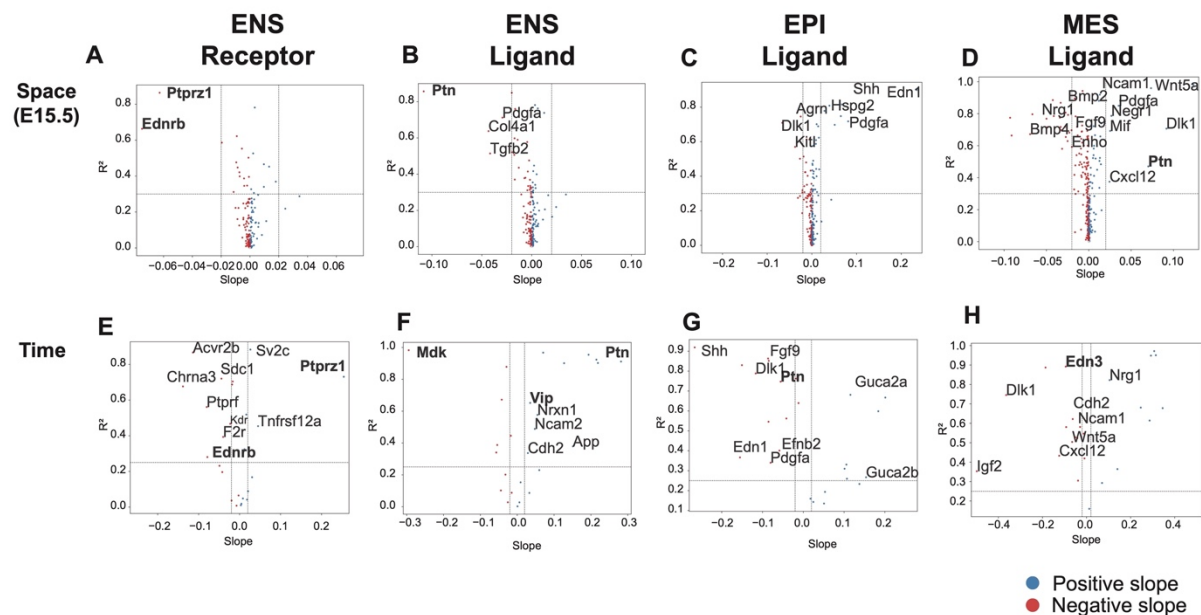


Figure S3.2. Volcano plots of the spatial and temporal regression models for ligand-receptor expression.

A-D: Volcano plots of the spatial regression models for ENS Receptors (A), ENS ligands (B), EPI ligands (C), MES ligands (D).

E-H: Volcano plots of the temporal regression models for ENS Receptors (E), ENS ligands (F), EPI ligands (G), MES ligands (H).

PTN-expressing ENS subclusters represent distinct developmental states

Given the prominence of signatures for PTN signaling in these data, we examined two ENS subclusters highly enriched for PTN/MDK–PTPRZ1 components (Figure S3): cluster 1 (Ptn⁺/Ptpz1⁺; Plp1⁺, S100b⁺) and cluster 9 (Ptn⁺/Ptpz1⁺; Elavl4⁺, Negr1⁺, Hoxb5⁺), both of which increased in abundance over developmental time (Figure 3.1J, K).

DEG analysis between cluster 1 and other progenitors (Figure 3.3A) displayed a progenitor-like transcriptional signature, including translation at synapse level (Figure 3.3B, C). Gene regulatory network analysis using SCENIC highlighted glial-biased or early progenitor regulons such as NFIX, FOS, and SOX10 (Figure 3.3D). PTN- and PTPRZ1-centered GRNs consisted of TF hubs and targets associated with progenitor maintenance, cytoskeletal remodeling, and early lineage priming (Figure 3.3E, F).

Cluster 9 (Figure 3.3G) demonstrated a mature neuronal-committed profile, with upregulation of axonogenesis, neurite extension, synaptic programs, and ion channels (Figure 3.3H, I) in comparison to other neuronal clusters. Its regulon landscape differed markedly from cluster 1, showing increased activity of neuronal maturation TFs, including HOX family members, KLF7, and ZFH2 (Figure 3.3J-L).

Together, these data reveal that PTN-enriched ENS populations encompass transcriptionally and regulatorily distinct developmental states, suggesting that PTN signaling may serve different roles in progenitor versus neuronal lineages.

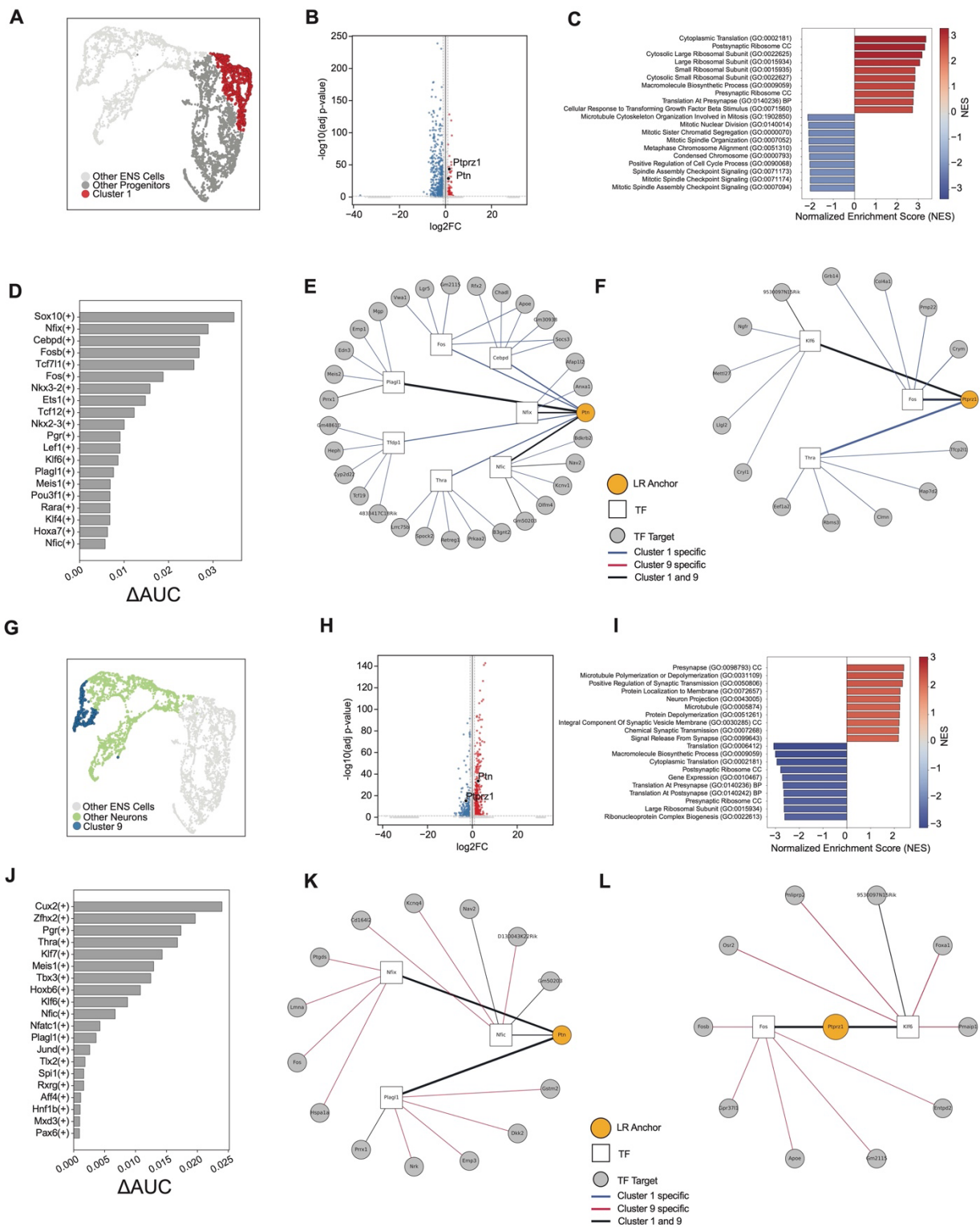


Figure 3.3. PTN-expressing ENS subclusters exhibit distinct developmental programs and gene regulatory networks.

A: UMAP of the ENS subset highlighting progenitor cluster 1 with high Ptn and Ptprz1 expression.

B: Volcano plot of DEGs between cluster 1 and other progenitors.

C: GSEA for ranked genes from B, showing enrichment of top significant terms related to translation, transcription, and TGF- β signaling.

D: The change in AUCell score of top TF regulons activities (pySCENIC AUCell) that distinguish cluster 1 amongst other progenitors. Showing high activity of regulons like Sox10, Nfix, and Fos.

E, F: Gene regulatory networks (GRNs) centered on Ptn (E) and Ptprz1 (F) in cluster 1.

G: UMAP of the ENS subset highlighting neuronal cluster 9 with high Ptn and Ptprz1 expression.

H: Volcano plot of DEGs between cluster 9 and other neuronal progenitors.

I: GSEA for ranked genes from H, showing enrichment of top significant terms related to synaptogenesis, axonogenesis, and neurotransmission.

J: The change in AUCell score of top TF regulons activities (pySCENIC AUCell) that distinguish cluster 9 amongst other neurons. Showing high activity of regulons like Hox family members, Klf7, and Zfhx2.

K, L: Gene regulatory networks (GRNs) centered on Ptn (K) and Ptprz1 (L) in cluster 9.

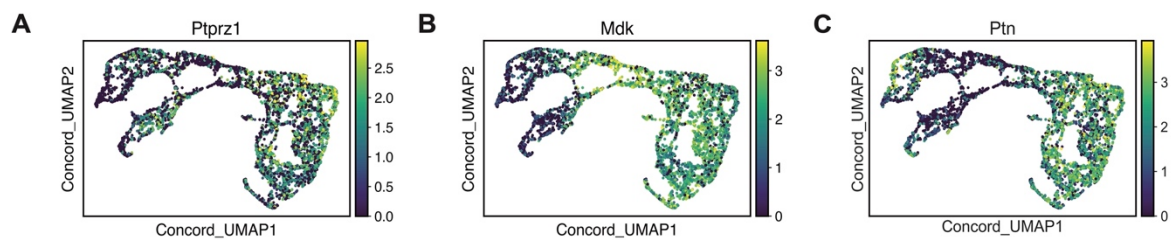


Figure S3.3. Expression of PTPRZ1 signaling components on Concord UMAP.

A, B, C: UMAP highlighting the ENS cells with expression (log₂ of counts) of Ptprz1 (A), Mdk (B), and Ptn (C).

PTN/MDK-PTPRZ1 signaling modulates neurogenesis and neurotransmitter specification in hPSC-derived ENS

To test the functional relevance of these pathways, we perturbed the PTN/MDK-PTPRZ1 pathway in hPSC-derived ENS cultures. PTPRZ1 (protein tyrosine phosphatase receptor type Z1) is a receptor-type tyrosine phosphatase that is catalytically inactivated upon ligand binding. When PTN or MDK bind to the extracellular domain of PTPRZ1, they induce receptor dimerization and allosterically inhibit its phosphatase activity. Similarly, NAZ2329 is a cell-permeable small molecule that binds to the active D1 catalytic domain of PTPRZ1 and stabilizes an open, inactive conformation of the WPD catalytic loop. Thus, all three perturbations inhibit PTPRZ1 phosphatase activity, leading to increased tyrosine phosphorylation of downstream substrates and enhanced activity of kinase signaling pathways normally suppressed by PTPRZ1, including Src, β -catenin, and MAPK cascades.

Day 30 enteric ganglioids were treated with PTN, MDK, or the PTPRZ1 inhibitor NAZ2329 followed by bulk RNA-seq (Figure 3.4A). PCA revealed condition-specific transcriptional shifts, with biological replicates clustering by treatment (Figure 3.4B). PC1 loadings represent genes involved in mitosis and PC2 reflects extracellular matrix organization (See Tables 10-13).

Across perturbations, ENS lineage programs were broadly altered (Figure 3.4C-H). PTN, MDK, and NAZ2329 downregulated neuronal and synaptic maturation modules while upregulating progenitor and proliferation signatures. GSEA confirmed negative enrichment of axon development and synapse assembly, accompanied by positive enrichment of E2F, G2/M, and mitotic pathways (Figure 3.4D).

Treatment with PTPRZ1 inhibitors also changed the expression of neurotransmitter-identity modules (Figure 3.4I, J). GABAergic identity was consistently suppressed, with PTN showing the strongest effect (reductions in GAD2, SLC32A1). PTN selectively increased serotonergic markers, including SLC18A2. Other neurotransmitter classes showed more modest or condition-specific responses.

Inhibition of PTPRZ1 receptor via PTN, MDK, and NAZ2329 increased expression of HOX genes and decreased the expression of POU transcription factors (Figure 3.4K). These perturbations also induced widespread remodeling of cytokine, neuropeptide, and receptor expression, indicating broader changes to signaling interactions (Supplementary Figure S3.4A-J; Tables 14 and 15).

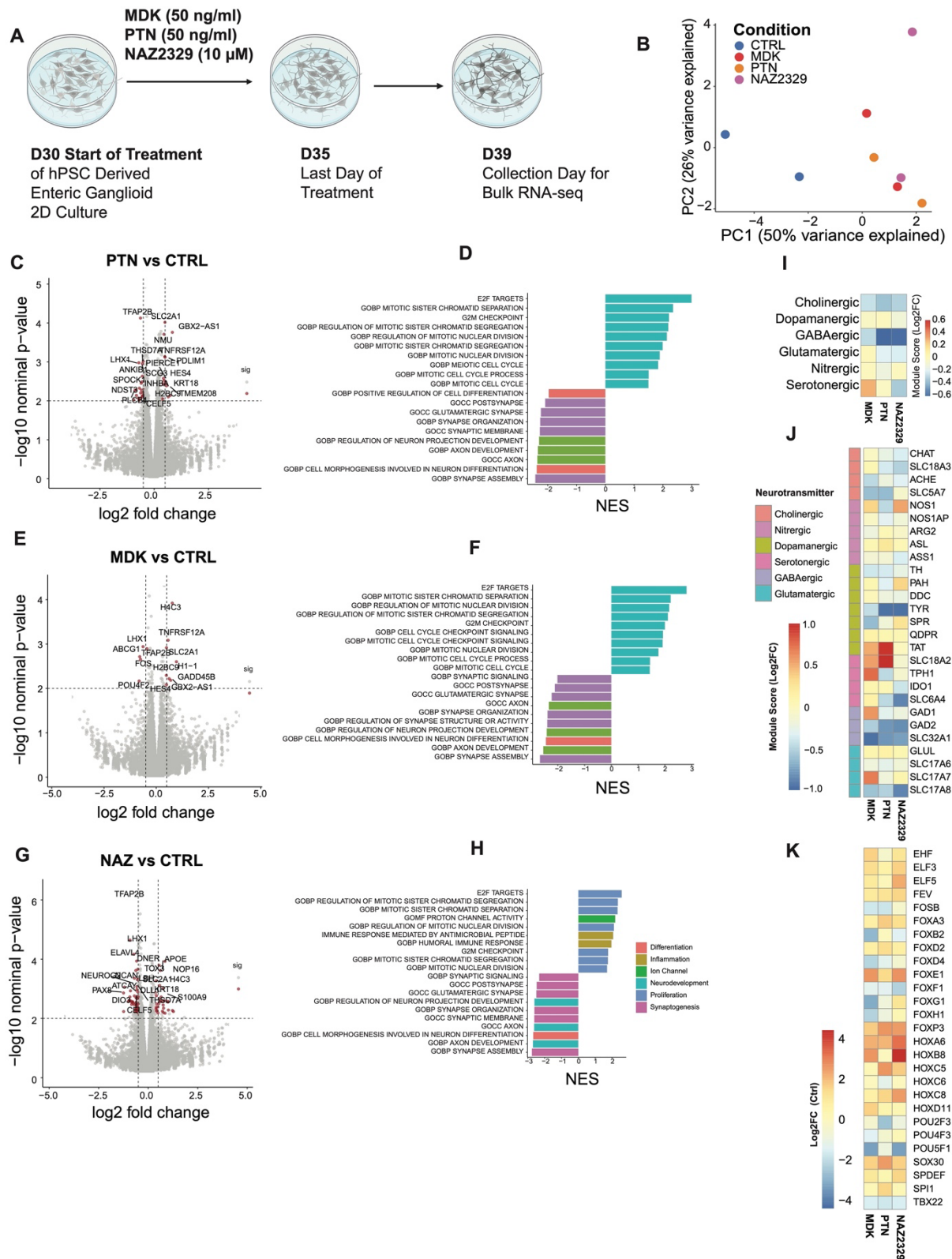


Figure 3.4. Ligand perturbations of PTN/PTPRZ1 axes reweight proliferative and synaptic maturation programs in human ENS cultures.

A: Schematic of the bulk RNA-seq perturbation experiment on hPSC-derived ENS cultures.

B: PCA of bulk RNA-seq samples, showing that samples cluster by treatment condition (CTRL, MDK, PTN, NAZ2329).

C: Volcano plot showing the DEGs of PTN relative to CTRL.

D: GSEA of ranked genes from C show positive enrichment of terms related to proliferation and negative enrichment of maturation terms.

E: Volcano plot showing the DEGs of MDK relative to CTRL.

F: GSEA of ranked genes from E show positive enrichment of terms related to proliferation and negative enrichment of maturation terms.

G: Volcano plot showing the DEGs of NAZ2329 relative to CTRL.

H: GSEA of ranked genes from G show positive enrichment of terms related to proliferation and negative enrichment of maturation terms.

I: Heatmap showing the log₂ fold-change in neurochemical module scores relative to CTRL. MDK increases neuronal serotonergic modules, while PTN and NAZ2329 downregulate GABAergic-associated modules.

J: Heatmap showing the log₂ fold-change in neurochemical genes relative to CTRL. MDK and PTN increase neuronal serotonergic modules, while PTN, MDK, and NAZ2329 downregulate GABAergic-associated genes.

K: Heatmap of log₂ fold-changes for selected TF genes. HOX and POU families had largest changes.

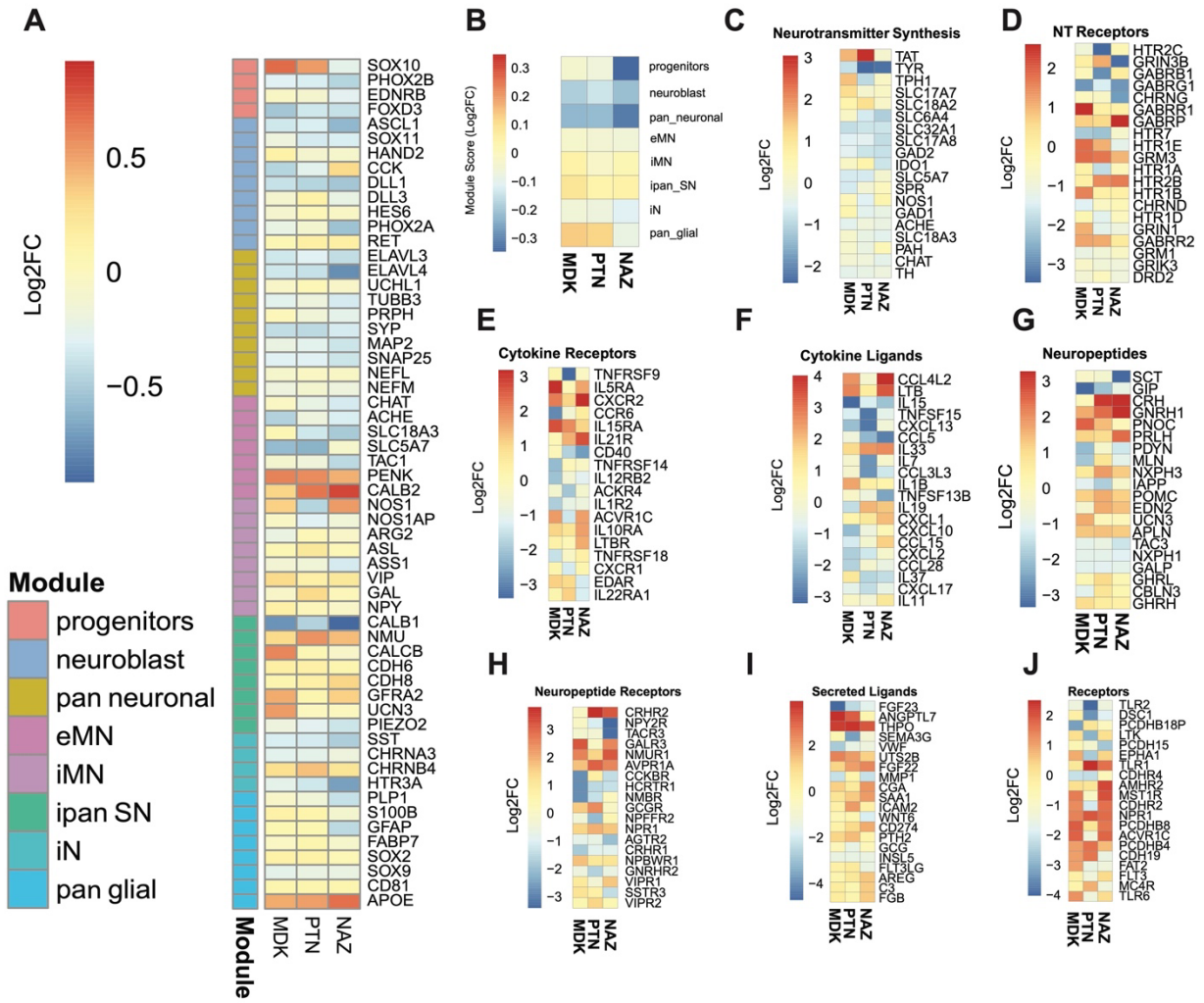


Figure S3.4. Transcriptomic consequences of PTPRZ1 inhibition via MDK, PTN, and NAZ2329.

A: Heatmap showing the $\log_2$ fold-changes in ENS state genes relative to CTRL. MDK, PTN, and NAZ2329 increase APOE, PENK, CALB2. MDK and PTN also increase SOX10 levels.

B: Heatmap showing modest $\log_2$ fold-changes in module scores relative to CTRL. MDK and PTN increase glial module, while NAZ2329 downregulates neuronal-associated modules.

C: Heatmap showing modest $\log_2$ fold-changes in neurotransmitter synthesis genes relative to CTRL.

D: Heatmap showing modest $\log_2$ fold-changes in neurotransmitter receptor genes relative to CTRL.

E: Heatmap showing modest $\log_2$ fold-changes in cytokine receptor genes relative to CTRL.

F: Heatmap showing modest $\log_2$ fold-changes in cytokine ligands relative to CTRL.

G: Heatmap showing modest $\log_2$ fold-changes in neuropeptide genes relative to CTRL.

H: Heatmap showing modest $\log_2$ fold-changes in neuropeptide receptor genes relative to CTRL.

I: Heatmap showing modest $\log_2$ fold-changes in secreted ligands relative to CTRL.

J: Heatmap showing modest $\log_2$ fold-changes in receptor genes relative to CTRL.

These data demonstrate that PTPRZ1 signaling regulates proliferative, neurogenic, and neurotransmitter-specification programs during human ENS differentiation, although the observed transcriptional shifts reflect a convolution of cell-intrinsic gene expression changes within individual cell types (e.g., progenitors, neurons, glia) and potential changes in the relative proportions of these cell types due to limitations of bulk RNA-seq.

Collectively, these results support a model in which the epithelium and mesenchyme establish strong A-P regional identities, whereas ENS cells maintain a largely uniform intrinsic program centered on temporal neuroglial maturation. Despite the absence of intrinsic ENS regionalization, local mesenchymal cues, particularly PTN/MDK family ligands, fine-tune ENS progenitor states, neuronal maturation trajectories, and neurotransmitter identity. This framework explains how ENS cells integrate into a highly regionalized organ while preserving a stable core identity and highlights localized niche signaling as a key mechanism for region-specific ENS refinement.

Discussion

The Absence of Overt Regional Transcriptional Identity in the ENS

In this study we find that gut epithelium and mesenchyme autonomously form strong AP patterning, including a prominent region-specific HOX code. In contrast, this regional patterning is not mirrored within the ENS. Instead, ENS cells retain a largely uniform intrinsic transcriptional identity anchored in vagal neural crest origin and organized predominantly along a temporal maturation axis. This contrast indicates that ENS progenitors do not adopt local positional identities in the same manner as surrounding gut tissues, despite experiencing the same spatial environment.

The absence of strong regional transcriptional identity in the ENS, despite the presence of strong regionalization in surrounding tissues, is a striking finding that contrasts with longstanding assumptions about ENS patterning. This result suggests that ENS progenitors do not adopt local positional identities in the same manner as the endoderm- and mesoderm-derived tissues through which they migrate. Instead, ENS cells appear to follow a uniform developmental trajectory dictated by their common vagal neural crest origin, with transcriptional variance driven primarily by differentiation state rather than spatial position.

Several factors may contribute to the transcriptional uniformity of the ENS. First, the ENS is derived from a single population of vagal neural crest cells, which emigrate from a restricted region of the hindbrain and colonize the entire gut over a relatively short developmental window. Unlike the mesoderm and endoderm, which are patterned by Hox codes and other positional cues during gastrulation, neural crest cells do not appear to acquire regional identities prior to migration. Second, the migration of ENCCs occurs in a rostral-to-caudal wave, such that cells at

different AP positions are exposed to developmental cues at different times. This temporal gradient may mask or override potential spatial patterning cues. Third, the ENS is highly interconnected, both within the myenteric plexus and between the myenteric and submucosal plexuses, and these interconnections may homogenize transcriptional states across regions.

Mesenchymal Niche Signals Fine-Tune ENS Development

While the ENS lacks overt AP regionalization, our analyses point to a variety of microenvironmental inputs to its development. Ligand-receptor profiling and spatial transcriptomics identify a small number of mesenchyme-derived signaling pathways, including PTN/MDK, EDN, and NOTCH, as spatially enriched and developmentally dynamic inputs to ENS cells. Among these, the PTN/MDK–PTPRZ1 signaling emerges as a prominent regulator with coherent spatial gradients and temporal increases that parallel ENS maturation. These signals correlate with subtle transcriptional tuning in ENS subpopulations, suggesting that local microenvironmental cues modulate ENS development even in the absence of strong intrinsic regionalization.

The identification of PTN/MDK-PTPRZ1 signaling as a spatially graded, temporally dynamic input to the ENS provides a molecular mechanism by which localized niche signals can fine-tune ENS development. PTN and MDK are secreted heparin-binding growth factors that have been implicated in neural stem cell maintenance, neurogenesis, and axon guidance in the central nervous system. PTPRZ1 is a receptor protein tyrosine phosphatase that is expressed by neural progenitors and immature neurons and has been shown to regulate cell proliferation, migration, and differentiation in various contexts.

The functional perturbation experiments in hPSC-derived ENS cultures provide direct evidence that PTPRZ1 signaling regulates ENS progenitor proliferation and neuronal maturation. The observation that PTN, MDK, and the PTPRZ1 inhibitor NAZ2329 all shift ENS cultures toward a more proliferative, less differentiated state is consistent with a model in which PTPRZ1 phosphatase activity promotes neuronal differentiation. In this model, inhibition of PTPRZ1 phosphatase activity—either by ligand binding (PTN/MDK) or pharmacological blockade (NAZ2329)—maintains the progenitor state by increasing kinase signaling in PTPRZ1-regulated pathways. This model is supported by previous studies showing that PTPRZ1 dephosphorylates and inactivates receptor tyrosine kinases, including PDGFR and FGFR, which are known to promote cell proliferation. By modulating the balance between progenitor expansion and neuronal differentiation, PTPRZ1 signaling may serve as a molecular rheostat that allows ENS development to be tuned in response to local tissue cues.

PTN/MDK-PTPRZ1 Signaling and Neurotransmitter Specification

Functional perturbation of PTPRZ1 signaling in human pluripotent stem cell–derived ENS cultures support this model. Perturbation of PTPRZ1 signaling alters ENS lineage trajectories by enhancing proliferative programs, suppressing synaptic and neuronal maturation modules, and shifting neurotransmitter specification, for example, reducing GABAergic identity while elevating serotonergic markers. Together, these findings suggest that PTPRZ1 signaling balances ENS progenitor expansion against neuronal differentiation, i.e., when PTPRZ1 is inhibited, the resulting enhancement of kinase signaling promotes a more proliferative, less differentiated state while simultaneously biasing neurotransmitter fate decisions toward specific identities such as serotonergic neurons. These coordinated changes across transcriptional, regulatory, and signaling layers highlight how microenvironmental cues fine-tune ENS development at multiple levels.

Importantly, these effects are observed across both mouse and human systems, underscoring the evolutionary conservation of these niche interactions.

The finding that PTPRZ1 signaling also modulates neurotransmitter specification programs is particularly intriguing. The consistent downregulation of GABAergic markers and selective upregulation of serotonergic markers in response to PTN treatment suggests that PTPRZ1 signaling influences not only the timing of neuronal differentiation, but also the acquisition of specific neurotransmitter identities. This observation has important implications for understanding the mechanisms that generate neuronal diversity in the ENS. While previous studies have identified transcription factors that regulate broad neuronal versus glial fates, the extrinsic signals that control neurotransmitter subtype specification remain poorly understood. Our findings suggest that microenvironmental cues, mediated by pathways such as PTN/MDK-PTPRZ1, play an important role in this process.

Temporal vs. Spatial Organization of the ENS

Together, our findings support a conceptual model in which ENS cells establish a uniform neuroglial core program early in development that is subsequently sculpted by localized, regionally patterned signals from the mesenchyme. This model reconciles two longstanding observations: that the gut exhibits high regional specialization while the ENS is largely transcriptionally uniform (Lasrado R et al., 2017); (Lake JI & Heuckeroth RO, 2013). While ENS cells do not encode overt AP positional identities intrinsically, they appear to integrate spatial information through microenvironmental cues that fine-tune their maturation, connectivity, and neurotransmitter specification as they differentiate within distinct gut niches.

This organizational principle has important implications for regenerative medicine. Strategies to generate region-specific intestinal epithelium or mesenchyme must carefully control morphogen exposure to establish appropriate anterior-posterior identity. In contrast, enteric neuron generation may be less constrained by positional specification, enabling production of generic enteric neurons that can adapt to their local environment after transplantation. Indeed, successful functional integration of hPSC-derived enteric neurons into host colon regardless of their in vitro differentiation conditions supports this model.

Implications for ENS Development and Therapeutic Applications

The findings presented in this chapter have several important implications for understanding ENS development and for developing therapeutic applications. First, the observation that the ENS retains a largely uniform transcriptional identity despite strong regionalization in surrounding tissues suggests that ENS cells are intrinsically programmed to follow a stereotyped developmental trajectory, but can be modulated by local microenvironmental signals. This has implications for cell-based therapies: ENS progenitors or neurons derived from hPSCs may not require region-specific patterning prior to transplantation, as they may be able to adapt to their local environment after engraftment. However, exposure to appropriate microenvironmental cues may be necessary to promote their functional maturation and integration into host circuits.

Second, the identification of PTN/MDK-PTPRZ1 signaling as a key regulator of ENS progenitor proliferation and neuronal differentiation suggests that this pathway could be targeted therapeutically to promote ENS regeneration or repair in conditions characterized by enteric neuronal loss, such as Hirschsprung disease, achalasia, or diabetic gastroparesis. Small molecules that modulate PTPRZ1 activity could be used to bias enteric neuron differentiation

toward specific subtypes in vitro, enabling production of nitrergic neuron-enriched or cholinergic neuron-enriched populations for cell therapy. Conversely, aberrant PTPRZ1 signaling during development could contribute to ENS developmental disorders such as intestinal neuronal dysplasia.

Third, the observation that neurotransmitter specification is influenced by microenvironmental signals has implications for understanding the origins of neuronal diversity in the ENS. While transcription factors clearly play important roles in specifying neuronal subtypes, our findings suggest that extrinsic signals also contribute to this process, potentially by modulating the activity or expression of these transcription factors. Future studies should investigate how PTN/MDK-PTPRZ1 signaling interacts with cell-intrinsic transcriptional programs to regulate neurotransmitter specification, and whether other microenvironmental signals similarly influence this process.

Limitations and Future Directions

Our findings lead to several intriguing questions that warrant future investigations. One is whether ENS regionalization enhances at later developmental or postnatal stages, potentially through activity-dependent maturation or epigenetic reprogramming. Another is how different signaling axes, such as PTN/MDK, EDN, NOTCH, and WNT, cooperate or act sequentially to shape ENS lineage transitions and circuit formation. Finally, the mechanisms by which epithelial and mesenchymal compartments influence ENS patterning, whether via secreted ligands, extracellular matrix components, metabolic gradients, or short-range interactions, remain incompletely understood.

The findings presented in this chapter focus on the small intestine, but the ENS colonizes the entire length of the gastrointestinal tract, including the stomach, colon, and rectum. Regional differences in ENS development and function may be more pronounced along the broader gut axis (e.g., small intestine versus colon) than within the small intestine itself. Extending the spatiotemporal scRNA-seq analysis to include the stomach and colon would provide a more complete picture of ENS regionalization and microenvironmental regulation across the entire gut. Additionally, our functional studies used bulk RNA-seq, which provides population-level information but cannot resolve cell-type-specific responses. Future studies using single-cell RNA-seq following perturbation would enable more precise identification of which ENS cell types are most sensitive to PTPRZ1 modulation.

By providing a multi-compartment, multi-species view of ENS development, our study defines how intrinsic lineage programs interface with regionally patterned gut niches to produce a mature, spatially integrated enteric nervous system. This framework not only refines our understanding of ENS development but also has implications for regenerative strategies, disease modeling, and cell-based therapies, which must account for the role of microenvironmental context in shaping ENS function.

Summary

In this chapter, I have investigated the regional patterning and microenvironmental regulation of enteric nervous system development. Using spatiotemporally resolved single-cell RNA sequencing, I have demonstrated that the epithelium and mesenchyme exhibit strong anterior-posterior regionalization, including prominent Hox expression gradients, whereas the ENS is organized primarily along a temporal maturation axis and retains a uniform vagal Hox identity. Despite the absence of intrinsic regional transcriptional identity in the ENS, ligand-receptor interaction analysis reveals that mesenchyme-derived signals, particularly PTN and MDK acting through PTPRZ1, are spatially graded along the AP axis and temporally dynamic during development.

Functional perturbation of PTPRZ1 signaling in hPSC-derived ENS cultures confirms that this pathway regulates ENS progenitor proliferation, neuronal differentiation, and neurotransmitter specification. These findings support a model in which the ENS retains a stable core neuroglial program that is secondarily refined by localized, region-specific niche signals within the patterned gut. This model reconciles the observation of transcriptional uniformity in the ENS with the functional specialization of ENS neurons in different gut regions, and has important implications for understanding ENS development, the generation of neuronal diversity, and the development of cell-based therapies for enteric neuropathies. By bridging fundamental developmental biology with translational applications, this work exemplifies how mechanistic understanding of ENS patterning can inform therapeutic strategies for gastrointestinal disease.

CHAPTER 4: DEVELOPMENTAL SPECIFICATION AND FUNCTIONAL CHARACTERIZATION OF ENTERIC MOTOR NEURONS

Introduction

Gastrointestinal motility disorders represent a significant clinical challenge, affecting millions of individuals worldwide and imposing substantial morbidity and economic burden (Sperber AD et al., 2021); (Peery AF et al., 2015). These disorders frequently arise from dysfunction or loss of specific neuronal subtypes within the enteric nervous system (ENS), particularly inhibitory nitric oxide (NO)-producing motor neurons that are essential for coordinating gut motility. Nitrergic neurons, characterized by expression of neuronal nitric oxide synthase (nNOS, encoded by NOS1), release NO to promote smooth muscle relaxation, enabling critical functions including sphincter opening, receptive accommodation, and propagation of peristaltic contractions (Parsons CL, 2006). Selective loss or dysfunction of NO neurons is implicated in severe motility disorders including achalasia, gastroparesis, hypertrophic pyloric stenosis, intestinal pseudo-obstruction, and chronic constipation (Duarsa GWK et al., 2019); (Aslanian H et al., 2005); (Pan G et al., 1993); (Hsu PI et al., 2001). Despite their clinical importance, the mechanisms governing nitrergic neuron specification during development and strategies to modulate their function therapeutically have remained incompletely understood, largely due to limited access to human enteric tissue and challenges in maintaining functional neurons in vitro.

The Critical Role of Nitrergic Neurons in Motility Disorders

Enteric motor neurons are functionally classified into two major categories: excitatory motor neurons, which promote muscle contraction primarily through acetylcholine release, and inhibitory motor neurons, which induce muscle relaxation through release of NO, purines (ATP, β -NAD⁺), and vasoactive intestinal peptide (VIP). The coordinated activity of these two populations generates the complex patterns of gut motility necessary for normal digestive function (Furness JB, 2012); (Sarna SK, 2010). Among inhibitory neurons, nitrergic neurons play a particularly essential role. NO diffuses into smooth muscle cells and activates soluble guanylate cyclase, leading to increased cyclic GMP levels and smooth muscle relaxation (Parsons CL, 2006). This inhibitory neurotransmission is critical for sphincter function, accommodation reflexes, and the descending limb of the peristaltic reflex.

The clinical importance of nitrergic neurons is underscored by their selective vulnerability in numerous gastrointestinal disorders. Achalasia, a disorder of the esophagus characterized by failure of the lower esophageal sphincter to relax, is associated with loss of nitrergic neurons in the myenteric plexus (Duarsa GWK et al., 2019); (Aslanian H et al., 2005); (Pan G et al., 1993). The progressive loss of nitrergic inhibitory innervation in the esophagus likely plays a major role in the progression of achalasia, with histological studies demonstrating decreased neurons containing nitric oxide synthase and interstitial cells of Cajal (Pan G et al., 1993). Gastroparesis, particularly in diabetic patients, correlates with reduced nitrergic innervation and impaired NO-mediated relaxation (Unknown, n.d.); (Hsu PI et al., 2001); (Watkins CC et al., 2000). Diabetic gastroparesis occurs as a result of dysfunction in the autonomic and enteric nervous systems, with chronically high blood glucose levels leading to neuronal damage resulting in abnormal myenteric neurotransmission, impaired inhibitory (nitric oxide) neuronal function, and

dysfunctional smooth muscle and pacemaker cells (Hsu PI et al., 2001); (Watkins CC et al., 2000); (Rudloff U et al., 2005).

In mouse models, genetic ablation of *Nos1* results in hypertrophic pyloric stenosis, achalasia-like phenotypes, and impaired colonic motility, phenocopying aspects of human enteric neuropathy (Alam SM et al., 1996); (Sivarao DV et al., 2001); (Huang PL et al., 1993). Studies using *Nos1*-deficient mice have revealed that nitric oxide-induced relaxation of the lower esophageal sphincter is impaired, and achalasia can be confirmed in vivo by manometry (Sivarao DV et al., 2001); (Alam SM et al., 1996). Beyond congenital and metabolic conditions, nitrergic neurons are particularly susceptible to chemotherapy-induced neurotoxicity and other forms of enteric neuropathy (Marino F et al., 2016); (Orozco LD et al., 2015).

Current Understanding of Motor Neuron Specification

How enteric neurons acquire specific neurotransmitter identities during development remains a fundamental question in ENS biology. Unlike the central nervous system, where spatial patterning cues such as dorsal-ventral and anterior-posterior morphogen gradients play dominant roles in specifying neuronal subtypes, the ENS appears to rely more heavily on temporal differentiation cues and cell-intrinsic transcriptional programs. Single-cell RNA sequencing of developing mouse and human ENS has revealed that neuronal diversification occurs progressively as progenitors exit the cell cycle and undergo terminal differentiation (Morarach K et al., 2021); (Appenzeller T & Science News Staff, 2017). Enteric neurons can be classified by their neurotransmitter profiles: cholinergic neurons expressing choline acetyltransferase (CHAT); nitrergic neurons expressing NOS1; serotonergic neurons expressing tryptophan

hydroxylase (TPH2); and GABAergic neurons expressing glutamate decarboxylase (GAD1/GAD2) (Morarach K et al., 2021).

The transcriptional regulators controlling neurotransmitter specification are beginning to be identified. Hypoxia-inducible factor 1 α (HIF1 α) binds to the NOS1 promoter and activates its transcription in nitrergic neurons, and stabilizing HIF1 α can rescue nitrergic function in diabetic gastroparesis (Musah S et al., 2018). Transcription factors including Hand2, Phox2b, Ascl1, ETV1, and BNC2 have been implicated in enteric neuron subtype specification, with ETV1 expression associated with nitrergic lineage and BNC2 with cholinergic lineage (Morarach K et al., 2021). However, the extracellular signaling pathways that regulate these transcriptional programs and control the balance between different neuronal fates have remained largely unknown.

hPSC-Derived Enteric Neurons as a Model System

Human pluripotent stem cells (hPSCs) provide a scalable experimental platform to study human ENS development and disease. Directed differentiation protocols have been established to generate vagal enteric neural crest cells (ENCCs) through sequential activation of BMP and WNT signaling combined with retinoic acid treatment, which caudalizes neural crest cells toward a vagal identity (Wang J et al., 2015); (Larmuseau MHD, 2019). These ENCCs can be cultured in suspension to form enteric crestospheres, which differentiate in the presence of GDNF to generate enteric neurons and glia that organize into three-dimensional enteric ganglioids. These ganglioid cultures recapitulate key aspects of human ENS development and contain multiple neuronal subtypes including cholinergic, nitrergic, serotonergic, and GABAergic neurons (Majd H et al., 2025); (Wang J et al., 2015).

hPSC-derived enteric neurons offer several advantages for studying NO neuron biology: they provide unlimited access to human cells, circumventing interspecies differences and limited availability of primary human tissue; they enable high-throughput screening applications that would be impractical with primary tissue; and they can be genetically manipulated using CRISPR-Cas9 genome editing to model disease-associated mutations or perturb developmental pathways (Wang J et al., 2015); (Majd H et al., 2025). Furthermore, hPSC-derived enteric neurons can be transplanted into immunocompromised mice to assess their in vivo engraftment and functional integration, providing a path toward cell-based therapies for enteric neuropathies (Wang J et al., 2015); (Kaynak Ö et al., 2024); (Dominiak-Felden G et al., 2016).

In this chapter, we leverage hPSC-derived enteric ganglioid models to address three interconnected objectives: (1) identification of drugs that modulate enteric neuron activity, particularly nitroergic and cholinergic neurons, and enhance gastrointestinal motility; (2) validation of candidate compounds in ex vivo colonic motility assays; and (3) elucidation of developmental signaling pathways that regulate NO neuron specification (Majd H et al., 2025). Through unbiased high-throughput screening approaches, we identify compounds that increase NO neuron and cholinergic neuron activity and demonstrate the pro-motility effects of selected hits in ex vivo colonic tissue assays (Majd H et al., 2025). We discover that inhibition of platelet-derived growth factor receptors (PDGFRs) promotes nitroergic neuron differentiation from enteric neural crest precursors, providing a strategy to generate NO neuron-enriched cultures for therapeutic applications (Majd H et al., 2025).

Results

High-Throughput Screening Identifies Modulators of Enteric Neuron Activity

To identify drugs that modulate enteric neuron activity and could serve as potential therapeutic agents for motility disorders, we leveraged our scalable hPSC-derived ENS culture platforms to perform high-throughput functional screens. We employed two complementary screening strategies: (1) direct measurement of NO release from 2D ENS cultures, and (2) flow cytometric quantification of neuronal activation using c-FOS expression in genetically labeled neuronal subtypes (Majd H et al., 2025).

For the NO release screen, we established a high-throughput colorimetric assay to assess NO neuron activity by measuring NO release in culture supernatants. We treated 2D ENS cultures with a library of 582 neuromodulatory compounds and quantified NO release using a commercially available detection system (Figure 4.1A) (Majd H et al., 2025). This screen identified 17 compounds that substantially enhanced NO concentration in the supernatant with z-score ≥ 2.0 (Figure 4.1B). Analysis of predicted protein targets for these hits revealed convergence on several target classes including serotonin receptors, cytochrome P450 enzymes, monoamine oxidases, adrenergic receptors, glutamate receptors, dopamine receptors, histamine receptors, COX inhibitors, and sodium channels (Figure 4.1C) (Majd H et al., 2025).

To complement the NO release assay and enable detection of neuronal activation more broadly, we performed a second high-throughput screen using c-FOS expression as a readout of neuronal activity. c-FOS is a well-established immediate-early gene marker induced by neuronal depolarization (Biedzinski S et al., 2020). To specifically label and track NO neurons, we generated a NOS1-GFP reporter hESC line using CRISPR-Cas9 knock-in to insert a GFP

cassette under control of the endogenous NOS1 promoter. Differentiation of this reporter line produced ganglioids with NO neurons co-expressing GFP and NOS1. We treated NOS1-GFP ganglioids with the neuromodulatory compound library for 1 hour and measured co-expression of c-FOS and GFP by flow cytometry to quantify NO neuron activation (Figure 4.1D) (Majd H et al., 2025). This screen identified 20 compounds that significantly increased the proportion of c-FOS⁺ cells within the NOS1-GFP⁺ population (z-score ≥ 1.5) (Figure 4.1E). Compilation and classification of predicted protein targets for these hits revealed convergence on target classes including serotonin receptors, COX inhibitors, dopamine receptors, sodium channels, adrenergic receptors, acetylcholine receptors, GABA receptors, FAAH (fatty acid amide hydrolase), histamine receptors, and vasopressin receptors (Figure 4.1F) (Majd H et al., 2025).

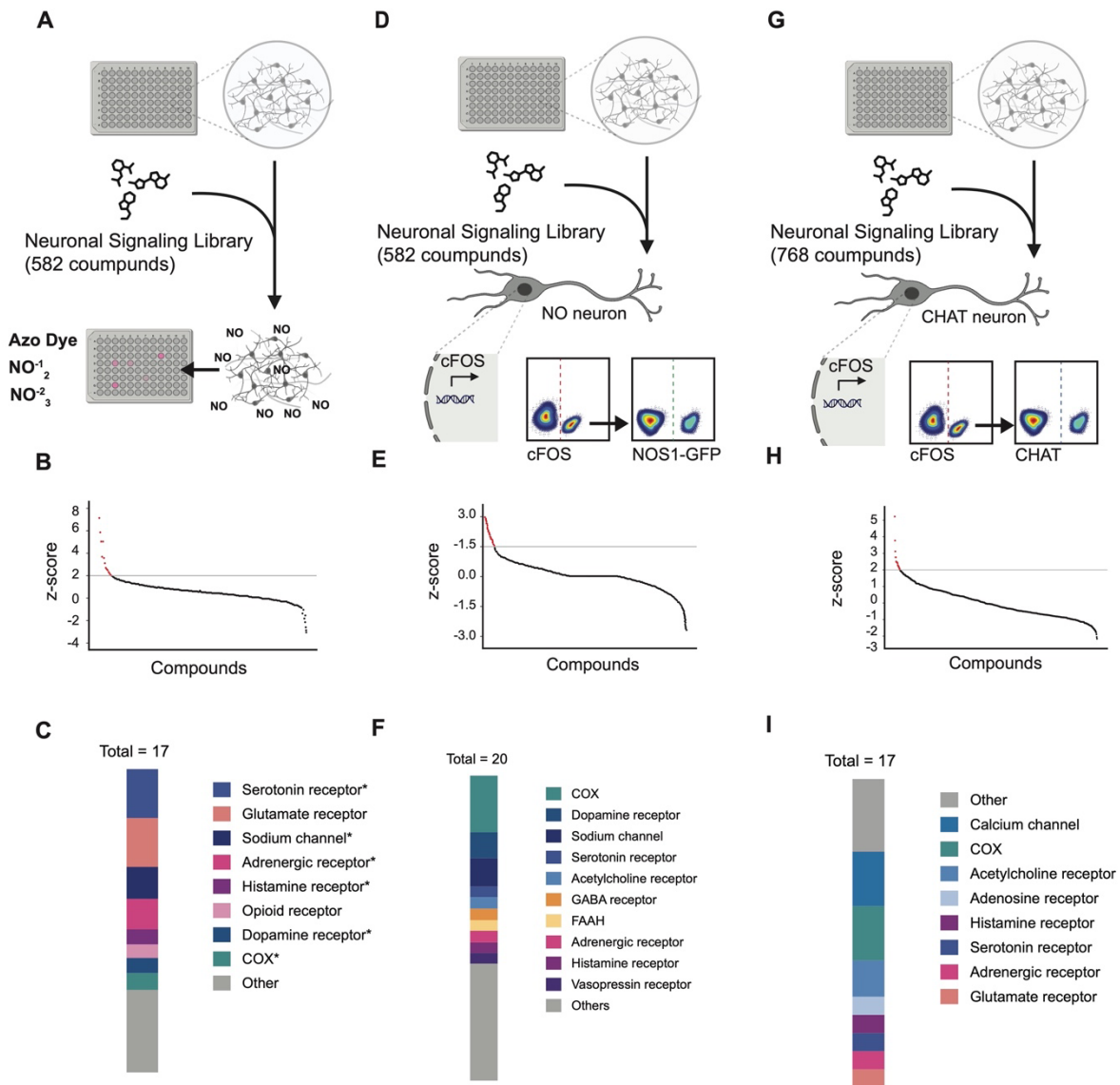


Figure 4.1: High-throughput screening identifies modulators of enteric neuron activity

A: Schematic of high-throughput colorimetric screen to identify compounds inducing NO release in 2D ENS cultures.

B: Z-score scatter plot of compounds for NO release assay ($z\text{-score} \geq 2.0$).

C: Target classes of top 17 compounds identified in the NO release screen.

D: Schematic of high-throughput flow cytometry screen to identify compounds inducing cFOS expression in NOS1-GFP+ enteric ganglioid NO neurons.

E: Z-score scatter plot of compounds for cFOS induction in NO neurons ($z\text{-score} \geq 1.5$).

F: Target classes of top 20 compounds identified in the nitrergic cFOS induction screen.

G: Schematic of flow cytometry screen for cholinergic neuron activation measuring cFOS induction in CHAT+ cells.

H: Z-score scatter plot of compounds for cFOS induction in cholinergic neurons.

I: Target classes of top 17 compounds identified in the cholinergic cFOS induction screen.

To extend our screening approach beyond nitrergic neurons, we also performed a flow cytometry-based screen to identify modulators of cholinergic neuron activity. Using a similar strategy, we generated a CHAT reporter line to label cholinergic neurons and screened for compounds that induce c-FOS expression in CHAT⁺ cells (Figure 4.1G). This screen identified 17 compounds that significantly increased cholinergic neuron activity ($z\text{-score} \geq 1.5$) (Figure 4.1H), with target classes including serotonin receptors, glutamate receptors, adrenergic receptors, histamine receptors, opioid receptors, dopamine receptors, COX inhibitors, and sodium channels (Figure 4.1I) (Majd H et al., 2025).

The substantial overlap between predicted targets across the three independent screening modalities (NO release, nitrergic c-FOS induction, and cholinergic c-FOS induction) provides robust validation. The convergence on specific target classes—particularly serotonin, adrenergic, acetylcholine, glutamate, and histamine receptors—across multiple assays suggests that these neuromodulatory pathways play central roles in regulating enteric neuron activity (Majd H et al., 2025).

Ex-Vivo Validation of Candidate Drugs on Colonic Motility

We selected a subset of hit compounds representing diverse target classes and prioritizing FDA-approved drugs for further validation in ex vivo colonic motility assays. To assess whether these compounds modulate gastrointestinal motility, we tested their effects on mouse colonic tissue using organ bath assays with video-based spatiotemporal mapping.

Paired control and drug-treated colon segments were mounted in a GI motility monitor apparatus and spatiotemporal maps were generated from video recordings to quantify colonic migrating motor complexes (CMMCs)—rhythmic propulsive contractions initiated by the ENS—and slow

waves mediated by interstitial cells of Cajal. Representative spatiotemporal maps showed changes in colonic contractions along the proximal-distal axis over 10-minute recording periods (Figure 4.2A) (Majd H et al., 2025).

Initial screening of candidate compounds measured CMMC intervals at the 75th percentile of the cumulative CMMC distribution across five consecutive 10-minute acquisitions. Multiple compounds showed effects on shortening CMMC intervals relative to untreated controls (Figure 4.2B), indicating enhanced colonic motility (Majd H et al., 2025). To determine whether these effects were mediated through NO neuron modulation, we performed more detailed sequential treatments in the presence and absence of the NOS1 inhibitor L-NAME.

For detailed validation, we performed experiments consisting of four 6-minute acquisitions with sequential treatments: buffer control, drug alone, buffer washout, and drug + L-NAME co-treatment (Figure 4.2C) (Majd H et al., 2025). Representative 26-minute spatiotemporal maps

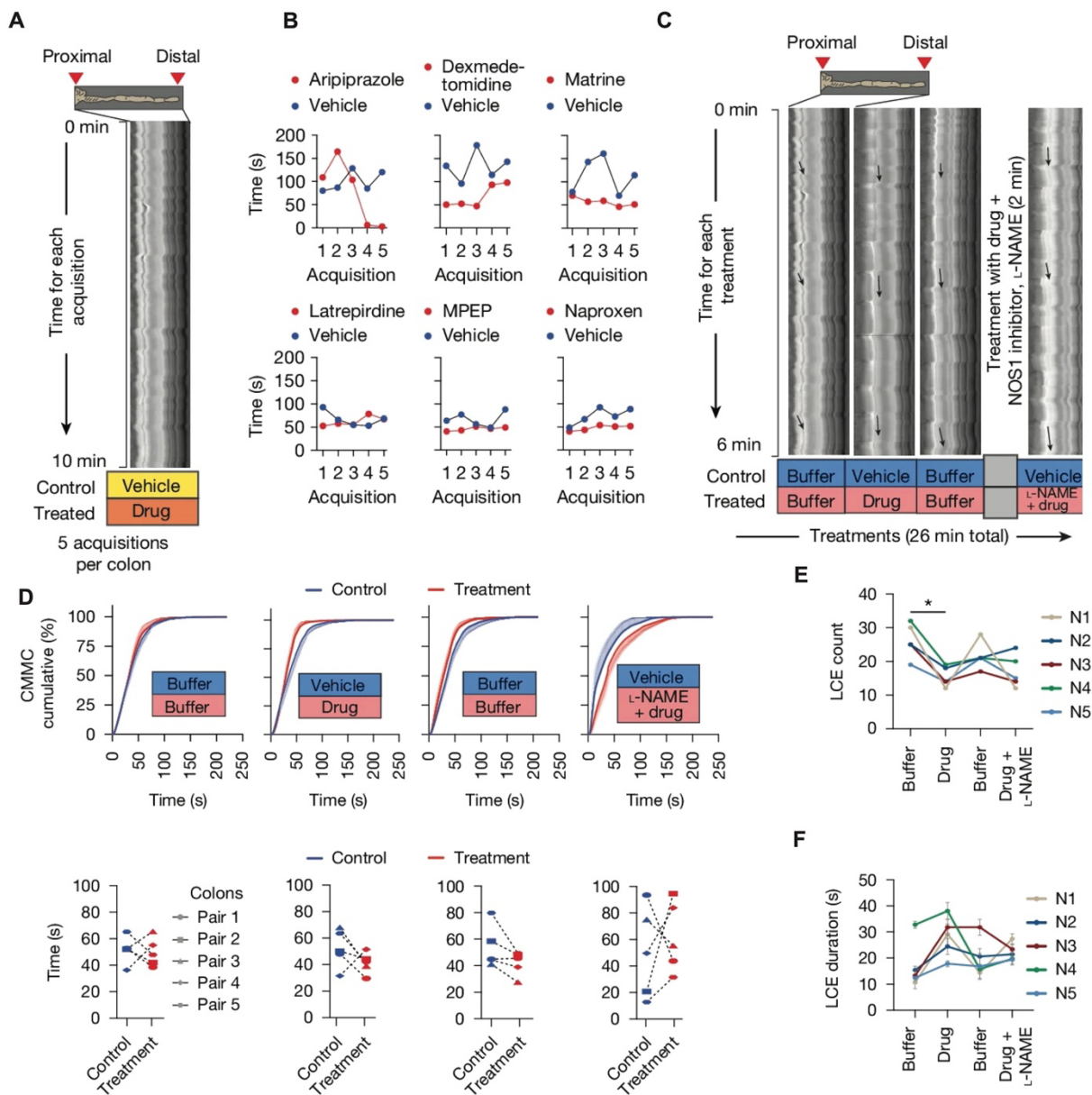


Figure 4.2: Ex vivo validation of candidate drugs on colonic motility

A: Representative spatiotemporal map of mouse colonic contractions along the proximal–distal axis over 10 min.

B: Quantification of CMMC intervals at the 75th percentile of cumulative CMMC distribution for each compound in A.

C: Schematic of sequential drug treatment and representative 26-min spatiotemporal maps; arrows indicate longitudinal contractile events (LCEs).

D: Quantification of CMMC intervals at the 75th percentile under sequential treatment conditions.

E: Total number of LCEs per 6-min segment under sequential treatment conditions.

F: Mean LCE duration under sequential treatment conditions.

showed longitudinal contractile events (LCEs), indicated by arrows, under different treatment conditions (Figure 4.2C). Quantification revealed that several compounds decreased CMMC intervals at the 75th percentile (Figure 4.2D), an effect that was abolished or reduced by L-NAME co-treatment in some cases, demonstrating dependence on intact nitrergic signaling (Majd H et al., 2025). Analysis of LCEs showed that drug treatments also affected the total number of LCEs per 6-minute segment (Figure 4.2E) and the mean LCE duration (Figure 4.2F), with these effects being reversed by drug washout and blocked by L-NAME co-treatment for compounds acting through nitrergic mechanisms (Majd H et al., 2025).

Effect of Candidate Drugs on Colonic Motility Assays

To further characterize the effects of selected hit compounds on colonic motility, we performed comprehensive analysis of both CMMCs and slow waves. Cumulative percentile diagrams were generated for CMMCs (Figure 4.3A) and slow waves (Figure 4.3B) to visualize the distribution of event intervals. We quantified the latency between slow waves at the 75th percentile for selected high-throughput screen hits (Figure 4.3C) (Majd H et al., 2025).

For individual compound validation, we analyzed three compounds that showed promising effects: aripiprazole (a dopamine receptor modulator), matrine (a sodium channel modulator), and MPEP (a glutamate receptor antagonist). For aripiprazole, cumulative percentile diagrams showed changes in CMMC intervals at the 75th percentile (Figure 4.3D), with quantification revealing modest effects on CMMC intervals (Figure 4.3E) (Majd H et al., 2025). Similarly, matrine treatment showed altered CMMC cumulative percentile distributions (Figure 4.3F) with corresponding changes in quantified CMMC intervals (Figure 4.3G) (Majd H et al., 2025).

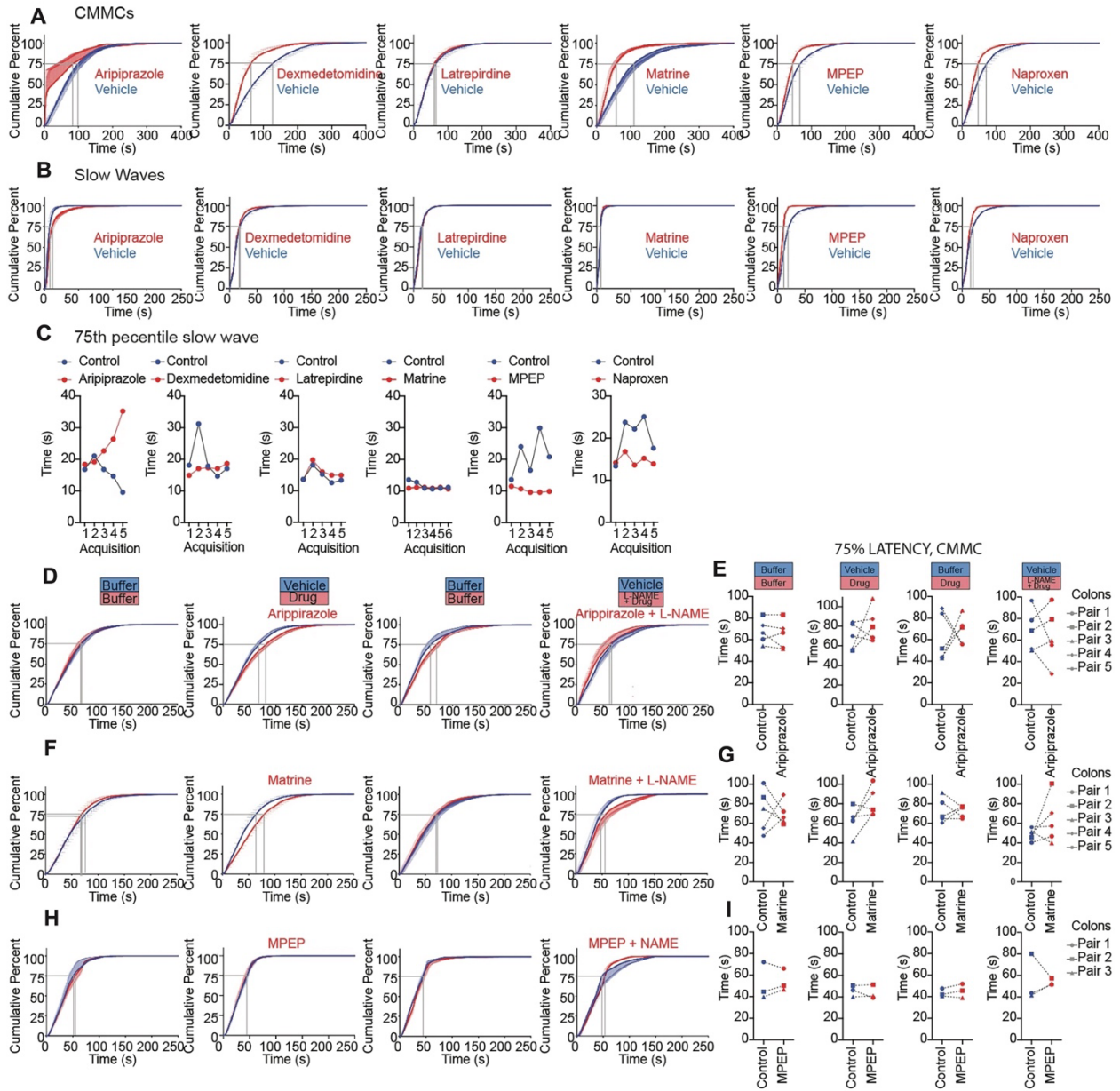


Figure 4.3: Effect of candidate drugs in ex vivo mouse colonic motility assays

- A: Diagrams of CMMC cumulative percentiles for selected hits.
- B: Diagrams of slow wave cumulative percentiles for selected hits.
- C: Quantification of latency between slow waves at 75th percentile for selected HTS hits.
- D: Diagrams of CMMC cumulative percentile at 75th percentile for aripiprazole.
- E: Quantification of CMMC intervals at 75th percentile for aripiprazole.
- F: Diagrams of CMMC cumulative percentile at 75th percentile for matrine.
- G: Quantification of CMMC intervals at 75th percentile for matrine.
- H: Diagrams of CMMC cumulative percentile at 75th percentile for MPEP.
- I: Quantification of CMMC intervals at 75th percentile for MPEP.

MPEP demonstrated comparable effects, with altered cumulative percentile diagrams (Figure 4.3H) and quantified changes in CMMC intervals (Figure 4.3I) (Majd H et al., 2025).

Effect of Candidate Drugs on Ex Vivo Mouse Colonic Slow Waves

While CMMCs represent neurally mediated contractile events, slow waves are generated by interstitial cells of Cajal and provide the electrical pacemaking activity underlying gut motility (Sanomura T et al., 2023); (Nolte E et al., 2006). To determine whether our candidate compounds selectively affect neural versus non-neural components of motility, we analyzed their effects on slow waves.

For aripiprazole, cumulative percentile diagrams of slow wave intervals at the 75th percentile (Figure 4.4A) and quantification of latency between slow waves (Figure 4.4B) showed minimal effects, suggesting that aripiprazole primarily acts through neural mechanisms rather than affecting pacemaker activity (Majd H et al., 2025). Similarly, dexmedetomidine (an α 2-adrenergic agonist) showed limited effects on slow wave cumulative percentile distributions (Figure 4.4C) and quantified slow wave latency (Figure 4.4D) (Majd H et al., 2025).

Matrine analysis revealed slow wave cumulative percentile diagrams (Figure 4.4E) and quantified latency measurements (Figure 4.4F) that were largely unchanged compared to control conditions (Majd H et al., 2025). Likewise, MPEP showed minimal alterations in slow wave cumulative percentile distributions (Figure 4.4G) and quantified slow wave latency (Figure 4.4H) (Majd H et al., 2025).

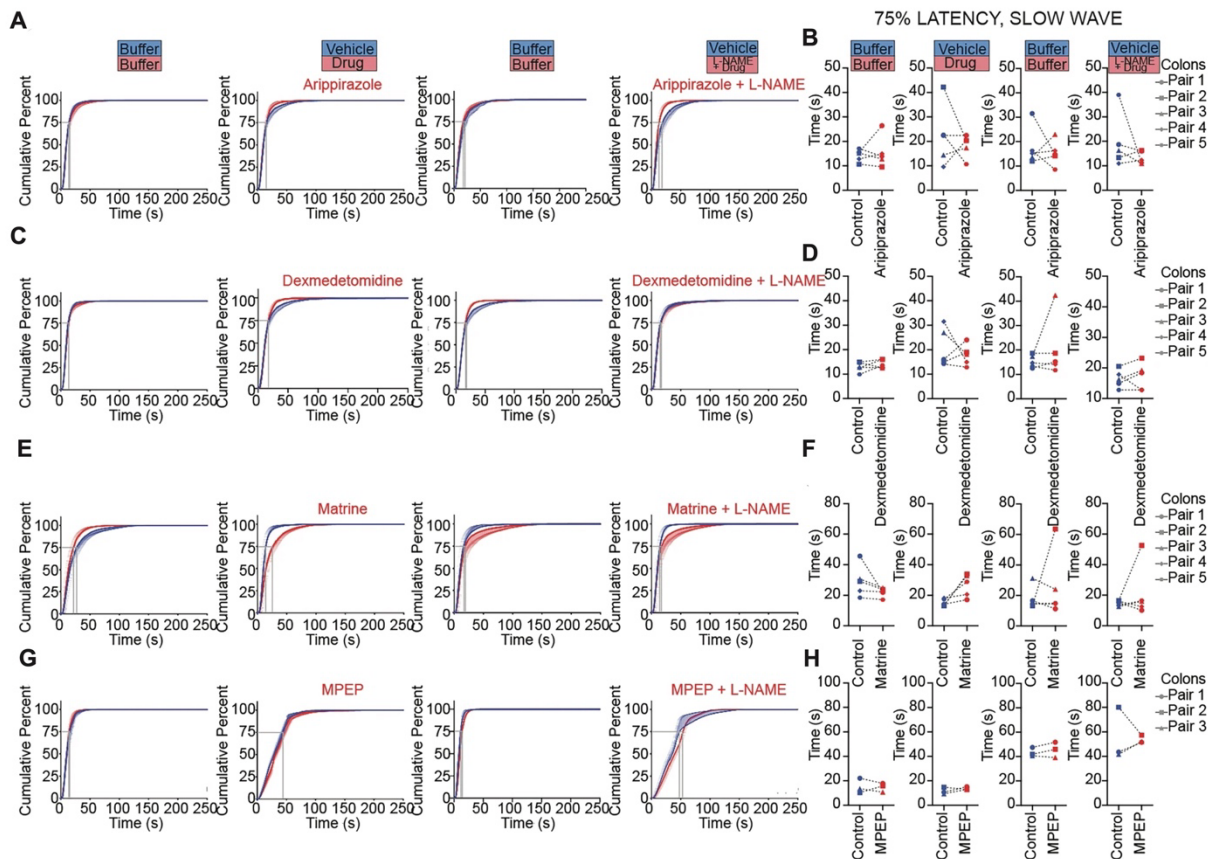


Figure 4.4: Effect of candidate drugs on ex vivo mouse colonic slow waves

A: Diagrams of slow wave cumulative percentile at 75th percentile for aripiprazole.

B: Quantification of latency between slow waves at 75th percentile for aripiprazole.

C: Diagrams of slow wave cumulative percentile at 75th percentile for dexmedetomidine.

D: Quantification of latency between slow waves at 75th percentile for dexmedetomidine.

E: Diagrams of slow wave cumulative percentile at 75th percentile for matrine.

F: Quantification of latency between slow waves at 75th percentile for matrine.

G: Diagrams of slow wave cumulative percentile at 75th percentile for MPEP.

H: Quantification of latency between slow waves at 75th percentile for MPEP.

The selective effects of these compounds on CMMCs without substantial impact on slow waves indicate that their pro-motility actions are mediated primarily through modulation of enteric neural circuits rather than direct effects on interstitial cells of Cajal or smooth muscle pacemaking activity (Majd H et al., 2025). This finding supports the conclusion that pharmacological targeting of neurotransmitter receptors expressed by enteric neurons can enhance motility through neural mechanisms.

PDGFR Inhibition Promotes Enteric NO Neuron Induction

While the screens described above identified compounds that modulate activity of differentiated enteric neurons, we sought to identify developmental signaling pathways that regulate NO neuron specification during differentiation. Understanding these mechanisms could enable generation of NO neuron-enriched cultures for transplantation and provide insights into developmental origins of nitrergic neuron deficits.

We performed a high-throughput small-molecule screen treating enteric crestospheres during the differentiation window with inhibitors targeting diverse signaling pathways, then quantifying NOS1+ neurons in resulting ganglioid cultures (Figure 4.5A) (Majd H et al., 2025). This screen identified compounds that substantially increased the proportion of NOS1+ neurons. Target prediction analysis revealed that several structurally similar top hits were predicted to interact with receptor tyrosine kinases including platelet-derived growth factor receptors (PDGFRs), vascular endothelial growth factor receptors (VEGFRs), and epidermal growth factor receptors (EGFRs/ERBBs) (Majd H et al., 2025).

We focused on PP121, a multi-targeted receptor tyrosine kinase inhibitor with known activity against PDGFRs, VEGFRs, and EGFRs. Treatment with PP121 during a critical developmental window (days 15-20 of differentiation) substantially increased the percentage of NOS1+ enteric neurons in day-40 ganglioid cultures compared to control conditions (Figure 4.5B) (Majd H et al., 2025). To determine the mechanism by which PP121 enhances NO neuron induction, we evaluated the roles of its known receptor targets.

Single-cell RNA-sequencing analysis confirmed expression of PDGFRA, PDGFRB, ERBB2, and ERBB3 in enteric crestospheres, whereas VEGFR mRNAs were not detectable. We tested the effects of receptor-specific agonists and antagonists: treatment with PDGF (PDGFR agonist) reduced NO neuron proportions, while treatment with sunitinib (PDGFR and VEGFR antagonist) increased them, implicating PDGFR signaling as the relevant pathway (Majd H et al., 2025). By contrast, treatment with NRG1 (ERBB agonist) or sapitinib (ERBB antagonist) showed no significant effect on NO neuron induction.

To definitively confirm the role of PDGFR signaling, we used CRISPR-Cas9 to knockout PDGFRA, PDGFRB, or both in enteric crestospheres and quantified NO neurons in resulting ganglioids. NO neurons were enriched in both PDGFRA and PDGFRB single-knockout cultures and to an even greater extent in double-knockout cells, confirming that PDGFR signaling negatively regulates nitrergic neuron specification (Majd H et al., 2025).

Representative immunofluorescence images of NOS1 and neuronal marker TUBB3 in control versus PP121-treated ganglioids demonstrate the substantial enrichment of NO neurons achieved through PDGFR inhibition (Figure 4.5C) (Majd H et al., 2025). Single-nucleus RNA sequencing of control and PP121-treated ganglioids confirmed that all major cell types were present in both

conditions, and that PP121 treatment enriched for specific nitroergic neuronal subtypes without generating entirely novel populations. These findings reveal a previously unknown role for PDGFR signaling in controlling the balance between nitroergic and cholinergic neuron fates during ENS development (Majd H et al., 2025).

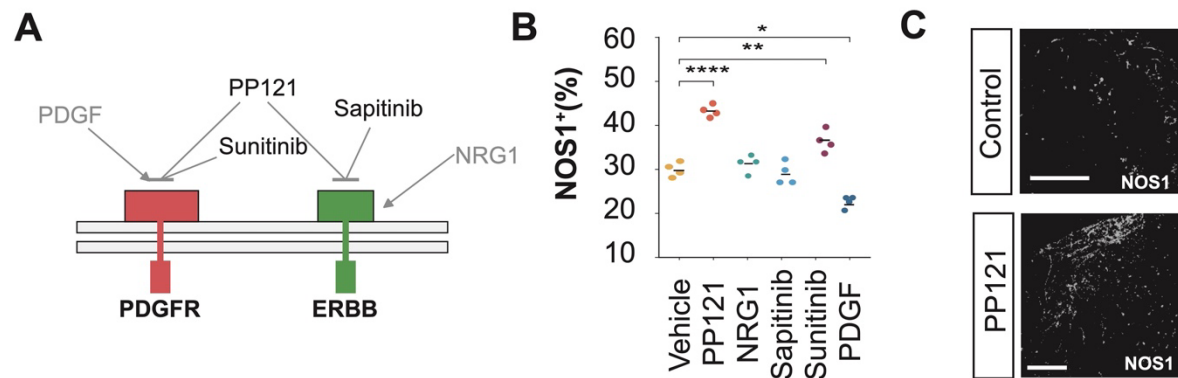


Figure 4.5: PDGFR inhibition promotes enteric NO neuron induction

A: Schematic of high-throughput pharmacological screening to identify compounds that enrich NO neurons.

B: Quantification of %NOS1+ enteric neurons in control and PP121-treated conditions.

C: Representative immunofluorescence staining of NOS1 (nitroergic) and TUBB3 (neuronal) in control and PP121-treated ganglioids.

Discussion

Drug Discovery and Therapeutic Target Identification

The high-throughput screening platform described here identified multiple neuromodulatory compounds that enhance enteric neuron activity through diverse mechanisms. The convergence on specific target classes—particularly serotonin, adrenergic, acetylcholine, glutamate, and histamine receptors—across three independent screening modalities (NO release, nitrgenic c-FOS induction, and cholinergic c-FOS induction) provides robust validation (Majd H et al., 2025).

The demonstration that several hits enhance colonic motility *ex vivo* establishes proof-of-principle that pharmacological modulation of enteric neuron activity can improve motility.

The compounds identified—including aripiprazole, dexmedetomidine, matrine, and MPEP—represent diverse target classes and mechanisms of action. Several are FDA-approved drugs currently used for other indications, suggesting potential for rapid repurposing for treating motility disorders. For example, aripiprazole is approved as an atypical antipsychotic acting primarily as a dopamine D2 receptor partial agonist, but our findings suggest it may also modulate enteric dopamine signaling to enhance motility (Majd H et al., 2025).

Dexmedetomidine, an α_2 -adrenergic agonist approved for sedation, showed effects on colonic motility that appeared to be mediated in part through nitrgenic mechanisms based on sensitivity to NOS1 inhibition, consistent with known expression of adrenergic receptors on enteric neurons (Furness JB, 2012).

The broader finding that enteric neurons express diverse neurotransmitter receptors and respond to multiple neuromodulatory signals has important physiological implications. It suggests that enteric neurons integrate diverse inputs from intrinsic enteric circuits, extrinsic autonomic

innervation, and circulating factors to fine-tune their activity (Biedzinski S et al., 2020). This pharmacological accessibility may offer therapeutic opportunities beyond direct NO pathway modulation.

An important caveat is that the ex vivo motility assays used here assessed acute effects of compounds on neural and muscular function in isolated colonic tissue, which may not fully recapitulate the complex in vivo environment including hormonal, immune, and microbiome influences. Additionally, while selectivity for CMMCs over slow waves suggests neural mechanisms, the precise cellular targets and downstream signaling pathways engaged by each compound require further investigation (Majd H et al., 2025). Some compounds may act directly on enteric neurons expressing cognate receptors, while others may act indirectly through enteric glia, immune cells, or other cell types in the gut wall.

Mechanisms of Nitrergic Motor Neuron Specification

The discovery that PDGFR signaling negatively regulates nitrergic neuron specification represents a major advance in understanding ENS development (Majd H et al., 2025). PDGF receptors are receptor tyrosine kinases that exist as two isoforms, PDGFR α and PDGFR β , which can form homodimers or heterodimers upon ligand binding (Andrae J et al., 2008); (Toftager M et al., 2013). PDGFR α signaling controls gastrulation and the development of several organs including lung, intestine, skin, testis, kidney, bones, and neuroprotective tissues (Hoch RV & Soriano P, 2003). PDGFR β signaling is recognized as an essential regulator of early hematopoiesis and blood vessel formation (Andrae J et al., 2008).

The observation that PDGFR inhibition or genetic ablation increases nitrergic neurons at the expense of cholinergic neurons suggests that PDGFR signaling acts as a fate switch during

enteric neurogenesis. The differential expression of PDGFRA/PDGFRB and the transcription factors BNC2 (cholinergic-associated) versus ETV1 (nitroergic-associated) in distinct ENCC clusters supports a model in which PDGFRs promote cholinergic fate while suppressing nitroergic fate (Majd H et al., 2025).

The mechanism by which PDGFR signaling influences neurotransmitter specification likely involves regulation of the timing of cell cycle exit or modulation of lineage-determining transcription factors. PDGF signaling is known to promote proliferation and inhibit differentiation in multiple contexts through activation of PI3K/Akt/mTOR and RAS/MAPK pathways; in enteric neural crest, it may prolong the progenitor state and bias cells toward cholinergic fate upon differentiation (Andrae J et al., 2008); (Toftager M et al., 2013).

Alternatively, PDGFR signaling may directly regulate expression of BNC2 or repress ETV1, thereby instructing cholinergic versus nitroergic identity.

Interestingly, the mesenchyme is a major source of PDGF ligands during gut development, suggesting that regional or temporal variation in mesenchymal PDGF expression could influence the balance of excitatory versus inhibitory neurons in different gut regions or developmental stages. This finding connects to the observation in Chapter 3 that mesenchyme-derived signals fine-tune ENS development, adding PDGF-PDGFR signaling to the repertoire of microenvironmental cues that shape ENS cell fate (Majd H et al., 2025). The spatially graded expression of PDGF ligands in the developing gut mesenchyme may provide positional information that fine-tunes the ratio of excitatory to inhibitory motor neurons along the anterior-posterior axis, optimizing motility patterns for regional gut function (Majd H et al., 2025).

From a translational perspective, the availability of FDA-approved PDGFR inhibitors such as imatinib, sunitinib, and others raises the intriguing possibility that pharmacological PDGFR inhibition during critical developmental windows could increase nitrergic neuron numbers in vivo (Unknown, n.d.). While systemic PDGFR inhibition would have pleiotropic effects given the broad roles of PDGF signaling in development and homeostasis, targeted delivery or time-limited exposure might be feasible. More immediately, PDGFR inhibition provides a robust protocol for generating NO neuron-enriched cultures for transplantation and disease modeling applications (Majd H et al., 2025).

Translational Applications and Future Directions

The identification of drugs that enhance enteric neuron activity and promote motility provides a foundation for developing new therapies for motility disorders. The compounds identified here could enter clinical testing for gastroparesis, chronic constipation, or other conditions characterized by reduced enteric neural function (Majd H et al., 2025). Prioritizing FDA-approved drugs with established safety profiles could accelerate translation.

The discovery that PDGFR inhibition promotes nitrergic neuron specification enables generation of NO neuron-enriched cultures that could be used for cell-based therapies (Majd H et al., 2025). Previous work has demonstrated successful transplantation of hPSC-derived enteric neural crest cells into mouse models of Hirschsprung disease, and enriching for specific neuronal subtypes could enhance therapeutic efficacy for disorders characterized by selective neuronal loss (Wang J et al., 2015); (Dominiak-Felden G et al., 2016); (Rollo BN et al., 2016). Future studies should assess the engraftment, migration, and functional integration of NO neuron-enriched ganglioids

in animal models of diabetic gastroparesis, achalasia, or other nitrergic neuropathies (Majd H et al., 2025); (Khieu M & Butler SL, 2025); (Kaynak Ö et al., 2024).

Several questions remain for future investigation. First, the precise cellular mechanisms by which the identified neuromodulatory compounds enhance motility require detailed characterization, including electrophysiological recordings from identified neuronal subtypes, calcium imaging to assess neuronal activation patterns, and analysis of neurotransmitter release (Majd H et al., 2025). Second, the transcriptional programs downstream of PDGFR signaling that control the cholinergic-nitrergic fate decision should be elucidated through single-cell multi-omics approaches. Third, in vivo validation of the pro-motility effects of candidate drugs in mouse models of motility disorders is essential before clinical translation. Fourth, the potential of NO neuron-enriched ganglioids for treating enteric neuropathies should be tested in transplantation experiments with rigorous assessment of engraftment, functional integration, and long-term safety (Majd H et al., 2025).

Limitations

Several limitations of the current study warrant discussion. First, the high-throughput screens were performed on hPSC-derived cultures that may not fully recapitulate all aspects of primary human enteric neurons. While single-cell transcriptomic analyses demonstrated high similarity between in vitro-derived and primary human neurons, functional responses to neuromodulatory compounds should be validated in primary human tissue when available (Majd H et al., 2025).

Second, the ex vivo motility assays were performed on mouse colonic tissue, and species differences in enteric neural circuits, receptor expression, and motility patterns may limit direct

translation to humans (Majd H et al., 2025). Future validation in human intestinal tissue obtained from surgical specimens or organ donors would strengthen translational potential.

Third, while PDGFR inhibition substantially enriched nitrergic neurons, the resulting cultures still contained other neuronal subtypes and non-neuronal cells (Majd H et al., 2025). Whether further purification is necessary or desirable for therapeutic applications remains to be determined. Complete purity may not be optimal given the functional interdependence of ENS cell types and the importance of neuron-glia interactions.

Fourth, the molecular mechanisms linking PDGFR signaling to transcriptional control of neurotransmitter identity remain incompletely understood. Future studies using ChIP-seq, ATAC-seq, and other genomic approaches could identify direct transcriptional targets of PDGFR signaling and elucidate how this pathway intersects with cell-intrinsic transcription factor networks.

Summary

This chapter establishes hPSC-derived enteric ganglioids as a powerful platform for identifying drugs that modulate enteric neuron activity and for elucidating developmental pathways controlling neuronal subtype specification (Majd H et al., 2025). High-throughput functional screening identified multiple neuromodulatory compounds that enhance nitrergic and cholinergic neuron activity through diverse receptor targets including serotonin, adrenergic, glutamate, histamine, and dopamine receptors. Validation in ex vivo colonic motility assays demonstrated that several hits promote colonic contractions, with selective effects on neurally mediated CMMCs rather than pacemaker-driven slow waves (Majd H et al., 2025). A developmental screen revealed that PDGFR signaling negatively regulates nitrergic neuron specification, and

pharmacological or genetic PDGFR inhibition enables generation of NO neuron-enriched cultures.

These findings advance understanding of enteric neuron development and function, identify drug candidates for treating motility disorders, and provide tools for generating enriched neuronal populations for disease modeling and regenerative medicine applications. The integration of high-throughput screening, ex vivo functional validation, and developmental pathway dissection exemplifies a comprehensive approach to translating stem cell biology into therapeutic applications (Majd H et al., 2025). Future studies should assess these compounds in animal models of motility disorders and evaluate NO neuron-enriched ganglioids for cell-based therapies targeting enteric neuropathies (Majd H et al., 2025).

CHAPTER 5: GENETIC ARCHITECTURE OF DISORDERS OF GUT-BRAIN INTERACTION

Introduction

Disorders of gut-brain interaction (DGBIs), formerly known as functional gastrointestinal disorders, are extraordinarily common conditions affecting billions of people worldwide. Characterized by recurrent gastrointestinal symptoms including abdominal pain, bloating, diarrhea, and constipation in the absence of organic structural disease or standard biochemical abnormalities, DGBIs represent a significant public health burden. The most prevalent DGBI is irritable bowel syndrome (IBS), affecting approximately 4–5% of the global population according to strict Rome IV criteria, though earlier estimates using Rome III criteria were higher (Sperber AD et al., 2021). Other major DGBI categories include functional dyspepsia, functional constipation, functional diarrhea, and abdominal pain disorders (Sperber AD et al., 2021). Despite their frequency, effective treatments remain limited, in part because the underlying biological mechanisms have remained obscure.

Historical Context and Disease Classification

For decades, functional gastrointestinal disorders were viewed primarily as psychosomatic illnesses, with symptoms attributed to psychological stress or psychiatric comorbidity rather than to identifiable biological abnormalities. This conceptualization led to a bifurcation between "organic" GI diseases (such as inflammatory bowel disease, celiac disease, or peptic ulcer disease) and "functional" disorders, with the latter receiving substantially less research attention and fewer therapeutic options. However, accumulating evidence over the past two decades has

firmly established that functional GI disorders are not psychosomatic but rather reflect genuine alterations in GI physiology, neural function, and microbiota composition (Drossman DA, 2016).

The Rome IV classification system, published in 2016, represents a paradigm shift toward understanding functional GI disorders as manifestations of disordered gut-brain interactions (Drossman DA, 2016). This framework emphasizes that symptoms result from dysregulation of the bidirectional communication between the brain, gut, and microbiota, involving altered intestinal motility, heightened visceral sensation, mucosal barrier dysfunction, immune activation, and altered neuroendocrine signaling (Drossman DA, 2016). The transition from "functional" to "disorders of gut-brain interaction" reflects this etiological perspective and acknowledges that these conditions involve identifiable, if complex, biological mechanisms.

Genetic Contributions to DGBIs

While environmental factors—including stress, diet, infections, and antibiotics—clearly play important roles in DGBI pathogenesis, family and twin studies provide compelling evidence for genetic contributions to disease susceptibility. Twin studies in IBS, for example, estimate heritability at 30–50%, indicating that genetic variation accounts for a substantial proportion of disease risk (Levy RL et al., 2001). Similarly, family studies demonstrate that first-degree relatives of IBS patients have approximately 2–3 times higher risk of developing IBS compared to the general population (Levy RL et al., 2001). These observations firmly establish that DGBIs are not purely environmental or psychological in origin but rather reflect complex interactions between genetic predisposition and environmental triggers.

However, despite growing evidence for genetic involvement, the specific genetic variants that confer risk for DGBIs have remained largely unknown until recently. Genome-wide association studies (GWAS), which systematically test associations between common genetic variants across the entire genome and phenotypic traits, have revolutionized understanding of complex disease genetics. GWAS have successfully identified hundreds of genetic variants associated with major psychiatric diseases, neurological conditions, and gastrointestinal diseases. Yet, comprehensive GWAS of DGBIs have been limited, in part because of inconsistent disease definitions and phenotypic characterization, and in part because of the proprietary nature of large electronic health record datasets.

Recent Advances in DGBI Genetics from Large-Scale Population Studies

Recent large-scale genetic studies, leveraging UK Biobank data and the All of Us Research Program, have begun to elucidate the genetic architecture of DGBIs. Combined GWAS and single-cell transcriptomics analyses have identified genetic variants and cell-type-specific mechanisms associated with multiple DGBI categories, including functional chest pain, functional dyspepsia, functional diarrhea, functional dysphagia, and functional fecal incontinence (Majd A et al., 2023). These studies have revealed several key insights: (1) DGBIs share genetic risk factors, suggesting common pathophysiological pathways; (2) identified GWAS loci implicate genes involved in immune function, neuronal signaling, and mucosal barrier integrity; (3) integration with cell-type-specific transcriptomics has nominated specific cell types and biological pathways as mediators of genetic risk; and (4) Mendelian randomization analyses have provided evidence for bidirectional causal relationships between DGBIs and brain structure, supporting the gut-brain axis hypothesis (Majd A et al., 2023). Furthermore, large-scale meta-

analyses have highlighted shared genetic pathways between IBS and anxiety disorders, reinforcing the gut-brain nature of these conditions (Eijssbouts C et al., 2021).

The All of Us Research Program and DGBI Genetics

The All of Us Research Program, a landmark initiative of the National Institutes of Health, aims to recruit one million participants for comprehensive genomic, phenotypic, and health outcome assessment (All of Us Research Program Investigators et al., 2019). Importantly, the All of Us program includes detailed phenotyping of gastrointestinal symptoms through standardized questionnaires, enabling precise phenotypic definition of DGBI cases and controls. Preliminary analyses of All of Us data have begun to characterize the genetic variants and associated biological pathways underlying DGBIs in a large, diverse population-based cohort (All of Us Research Program Investigators et al., 2019).

In this chapter, I report findings from genome-wide association study analyses of DGBI phenotypes in the All of Us Research Program. I identify genome-wide significant loci associated with specific DGBI phenotypes and with broad DGBI burden (a measure of cumulative DGBI symptom severity). I then integrate these GWAS results with single-cell transcriptomics data from the developing and adult ENS, intestinal epithelium, and immune cells to identify cell types and biological pathways mediating genetic risk. These findings provide new insights into the fundamental mechanisms underlying DGBIs and identify promising targets for therapeutic intervention and biomarker development.

Results

Phenotypic Characterization of DGBIs in the All of Us Cohort

The All of Us Research Program recruited participants through a distributed network of healthcare organizations across the United States. Participants consented to share electronic health records (EHRs), provide biological samples for genomic analysis, and complete detailed surveys covering medical history, symptoms, lifestyle factors, and health outcomes. At the time of analysis, the All of Us cohort included over 633,000 participants with available genotype and phenotype data, representing one of the most diverse biomedical datasets in history (All of Us Research Program Investigators et al., 2019).

To systematically identify DGBI cases and controls, we applied standardized Rome IV criteria to data from the All of Us baseline survey and supplementary gastrointestinal symptom questions (Drossman DA, 2016). Specifically, we classified participants with recurrent abdominal pain or distension for at least 1 day per week over the previous 3 months as having IBS or functional dyspepsia, depending on symptom location and character. Participants with recurrent loose or watery stools in the absence of prior diarrhea-related conditions were classified as having functional diarrhea. Participants reporting functional constipation were identified by the presence of straining, lumpy or hard stools, or sensation of incomplete evacuation in >25% of defecations (Sperber AD et al., 2021).

In total, we identified more than a thousand participants meeting Rome IV criteria for IBS, functional dyspepsia, functional diarrhea, functional constipation, and unspecified DGBIs. Control participants were defined as those without significant GI symptoms (reported GI

symptoms <1 day per week or absent over the previous month) and without prior diagnosis of inflammatory bowel disease, celiac disease, or other organic GI disease.

Genome-Wide Association Study of DGBI Phenotypes

GWAS was conducted for six DGBIs: irritable bowel syndrome, functional chest pain, functional diarrhea, functional dyspepsia, functional dysphagia, and functional fecal incontinence.

Significant SNPs ($p < 5 \times 10^{-8}$) were identified for five of these conditions (all except functional dysphagia) in individuals of European ancestry.

Table 1. SNPs that were found to be significantly associated with each condition.

DGBI	Leading SNP(s)	Chr.	Position(s)	p-value
Irritable bowel syndrome	rs200076067	3	37178147	1.24e-8,
	rs112128385		37324403	2.34e-8,
	rs111823385		37043269	4.20e-8,
	rs112928350		36951365	5.07e-6
Functional chest pain	rs11063527	12	656599	1.67e-8
Functional Dyspepsia	rs115574037	3	194087833	1.89e-8
Functional Diarrhea	rs145044248	1	85221148,	7.34e-9,
	rs138302836	21	37129365	2.46e-8
Functional Incontinence	rs558633201	1	34379986	9.49e-9

For irritable bowel syndrome, leading SNPs mapped to or near genes including LRRFIP2, MLH1, DCLK3, ITGA9, MYD88, and TRANK1. Several of these genes have known roles in immune signaling (MYD88, a key adaptor in Toll-like receptor signaling) and epithelial barrier function (ITGA9, an integrin). For functional chest pain, significant associations were found near B4GALNT3, WNK1, KDM5A, and ERC1. Of particular interest, WNK1 encodes a serine/threonine kinase that regulates chloride channels in sensory neurons and is critical for

controlling neuronal excitation. KDM5A is a histone demethylase involved in neuronal differentiation, and ERC1 encodes a component of the presynaptic active zone involved in neurotransmitter release.

Table 2. Protein-coding genes were found to be significantly associated with each condition.

DGBI	Genes mapped from significant SNPs
Irritable bowel syndrome	<i>LRRFIP2, MLH1, EPM2AIP1, GOLGA4, DCLK3, ITGA9, VILL, ACAA1, MYD88, OXSRI, EXOG, TRANK1, APRG1(C3orf35)</i>
Functional chest pain	<i>B4GALNT3, RAD52, WNK1, KDM5A, ERC1, CCDC77, FBXL14</i>
Functional Dyspepsia	<i>LRRC15, TMEM44, ATP13A4, GP5, CPN2, OPA1</i>
Functional Diarrhea	<i>MCOLN3, RPF1, CTBS, SSX2IP, TTLL7, DNASE2B, DOPEY2, PRKACB, RCAN1, CLIC6, RUNX1, CBR1, CBR3, CLDN14, LPAR3, GNG5, KCNE1, SET4D, SMAD13</i>
Functional Incontinence	<i>CSMD2, HMGB4, GJA4, GJB3, GJB5</i>

Functional diarrhea-associated SNPs mapped near RUNX1, a transcription factor essential for development of nociceptive and proprioceptive sensory neurons (Marmigère F & Ernfors P, 2007); DOPEY2, important for neural morphogenesis and cognitive development (Xiao Y et al., 2023); RCAN1, which regulates calcineurin signaling in neurons (Hoeffler CA et al., 2013); and CLIC6, a chloride channel that interacts with dopamine receptors (Griffon N et al., 2003). For functional fecal incontinence, significant SNPs were found near CSMD2, a synaptic transmembrane protein critical for dendritic spine formation and synaptic maturation (Gutierrez MA et al., 2019); HMGB4, a chromatin regulator of neuronal differentiation; and connexin genes (GJA4, GJB3, GJB5), which encode gap junction components essential for coordinated neural and smooth muscle activity.

Collectively, these genetic findings point to neural and synaptic dysfunction as a central feature of DGBIs, consistent with the conceptual framework of gut-brain axis pathology (Majd A et al., 2023). The identification of specific candidate genes provides an entry point for functional studies to determine how genetic variants alter protein function and contribute to disease phenotypes.

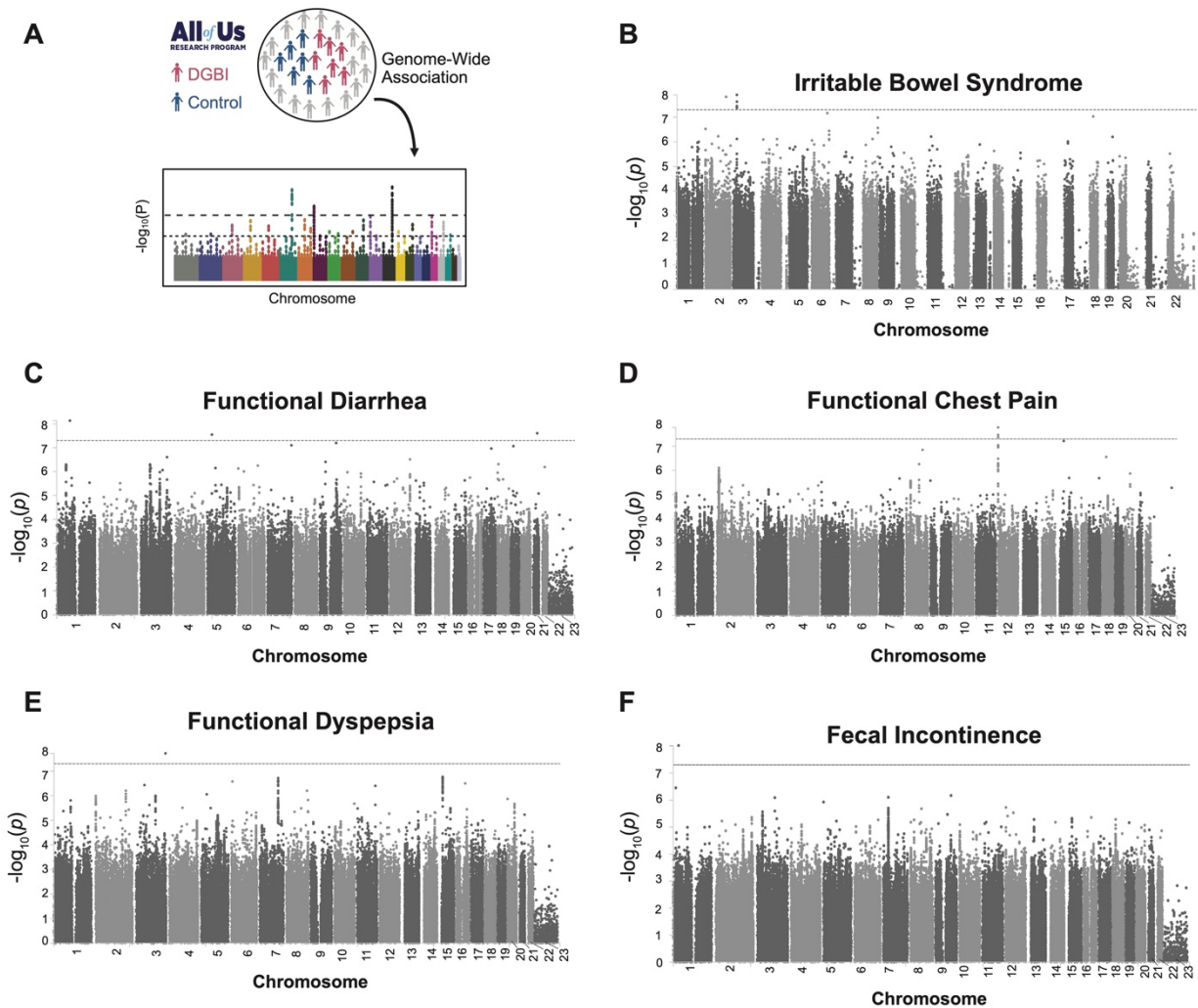


Figure 5.1: Manhattan plots showcasing significant SNPs associated with DGBIs

A: Schematic of DGBI GWAS

B: Manhattan plot of IBS

C: Manhattan plot of Functional Diarrhea

D: Manhattan plot of Functional Chest Pain

E: Manhattan plot of Functional Dyspepsia

F: Manhattan plot of Fecal Incontinence

Discussion

A critical next step is to determine whether and how DGBI-associated genes influence enteric nervous system development and function. Preliminary analysis of gene expression in MSI and hPSC-derived enteric neural crest cells revealed that a substantial fraction of DGBI-associated genes is expressed during ENS development, with expression patterns that vary across developmental time. This temporal expression pattern suggests that DGBI-associated genes may influence distinct developmental processes, from neural crest migration and proliferation to neuronal differentiation and synapse formation.

To systematically assess the functional role of DGBI candidate genes, a perturb-seq approach is planned, in which CRISPR-mediated knockdown or knockout of each gene will be performed in hPSC-derived enteric ganglioids, followed by single-cell RNA sequencing to identify gene expression changes and functional assays (calcium imaging, neurotransmitter release measurements) to assess neuronal activity. This approach will enable identification of genes that specifically affect nitrergic, cholinergic, or other neuronal subtypes, and will reveal how genetic perturbations alter ENS development and function in ways that may contribute to DGBI pathophysiology.

These studies illustrate a paradigm in which human genetics provides an unbiased entry point to disease biology, and hPSC-derived models provide a platform for functional validation and mechanistic dissection. By linking GWAS hits to cellular phenotypes in an experimentally tractable system, this approach has the potential to nominate novel therapeutic targets and to stratify patients based on genetic risk profiles.

CHAPTER 6: METHODS

Intestinal Explant Culture

E13.5 small intestines were dissected and placed on 0.4 μm pore polycarbonate transwell membrane inserts (Corning) in 24-well plates containing 500 μL of basal culture medium (DMEM/F12, Gibco) supplemented with N2 supplement (1 $\times$, Gibco), B27 supplement (1 $\times$, Gibco), GlutaMAX (2 mM, Gibco), and penicillin/streptomycin (100 U/mL). Explants were cultured at 37°C in 5% CO₂ for 48 hours. For pharmacological perturbation experiments, small molecules were added to the culture medium at the indicated concentrations: GM6001 (10 μM , Sigma-Aldrich), Marimastat (10 μM , Tocris), obtustatin (10 $\mu\text{g/mL}$, custom synthesis), TC-I 15 (10 μM , Tocris), ATN-161 (10 μM , Tocris), cyclopamine (10 μM , Sigma), and SAG (1 μM , Cayman Chemical). Vehicle controls (DMSO, 0.1% final concentration) were included for all experiments. After culture, explants were fixed in 4% PFA for 1 hour at room temperature for immunofluorescence analysis.

Immunofluorescence and Imaging

Tissue Preparation (Whole-Mount and Cryosections)

Fixed intestinal tissues (whole-mount or cryosections) were permeabilized in PBS containing 0.5% Triton X-100 (PBST) for 30 minutes at room temperature, then blocked in PBST containing 5% normal donkey serum and 1% bovine serum albumin (BSA) for 1 hour. Primary antibodies were diluted in blocking solution and incubated overnight at 4°C. Secondary

antibodies (Alexa Fluor-conjugated, Invitrogen) were diluted 1:500 in blocking solution and incubated for 2 hours at room temperature. Nuclei were counterstained with DAPI (1 µg/mL).

Antibodies

Primary antibodies used: anti-PDGFR- α (1:200, R&D Systems, AF1062), anti-phospho-myosin light chain 2 (Ser19) (1:200, Cell Signaling, 3671), anti-collagen I (1:200, Abcam, ab34710), anti-collagen VI (1:200, Abcam, ab6588), anti-laminin (1:200, Sigma, L9393), anti-MMP2 (1:100, Abcam, ab97779), anti-TUBB3/TUJ1 (1:1000, BioLegend, 801202), anti-nNOS/NOS1 (1:200, Immunostar, 24287), anti-CHAT (1:200, Millipore, AB144P), anti-SOX10 (1:200, R&D Systems, AF2864), anti-GFAP (1:500, DAKO, Z0334).

Immunofluorescence Staining for hPSC-Derived Ganglioids

For immunofluorescence staining of ganglioids, cells were initially fixed in 4% PFA in PBS (30 min, room temperature) and then blocked and permeabilized by permeabilization buffer (Foxp3/Transcription Factor Staining Buffer Set, 00-5523) for another 30 min at room temperature. After fixation and permeabilization steps, cells were incubated in primary antibody solution overnight at 4°C, and then washed three times with permeabilization buffer before their incubation with fluorophore-conjugated secondary antibodies at room temperature. Before imaging, stained cells were incubated with DAPI fluorescent nuclear stain and washed an additional three times.

Preparation of Enteric Ganglioid Frozen Sections

hPSC-derived ganglioids were collected, rinsed twice in PBS and fixed on ice in 4% PFA (SCBT sc-281692) for 3 h, followed by replacing 90% of the supernatant with PBS for storage at 4°C

for up to 6 months. Ganglioids were treated with 5% sucrose (RPI Research Products 524060) in PBS for 10 min at room temperature, followed by 10% sucrose in PBS for 2 h at room temperature and 20% sucrose at 4°C overnight. Sucrose-treated ganglioids were positioned in cryomoulds (Tissue-Tek Cryomold medium, VWR 25608-924), all 20% sucrose was removed and they were incubated in 2:1 (20% sucrose:OCT Tissue Plus O.C.T. Compound Fisher HealthCare 5484) for 2 h at room temperature before flash freezing in ethanol/dry ice. Sections of 12–20 µm were taken on a cryostat (Leica 3050S), adhered to a Superfrost Plus Micro Slide, Premium (VWR 48311-703) and dried at 42°C on a slide dryer for up to 2 h before storing at –80°C for up to a year.

Staining Enteric Ganglioid Frozen Sections

Unless otherwise specified, all steps were performed at room temperature. Ganglioid frozen sections were prepared as above and then washed three times in PBS and blocked for 1–2 h in serum (10% donkey or 10% goat with 0.5% v/v Triton X-100, VWR 0694). Slides were then incubated with primary antibody diluted in serum (10% donkey or 10% goat with 0.1% Triton X-100) at 4°C for 12–20 h. Slides were washed six times for 20 min each in PBS with 0.1% Tween-20 (Sigma P1379) and incubated for 1 h with Alexa Fluor-conjugated secondary antibodies. The diluted secondary antibody solution was removed and replaced with 1.0 µg/ml DAPI in water for 10 min. The slides were washed six times for 20 min each in PBS with 0.1% Tween-20, and coverslips were mounted with Fluoromount-G (Southern Biotech 0100-01).

Tissue Staining for Whole-Mount Colon Preparations

Colonic longitudinal muscle myenteric plexus tissues were fixed with 4% PFA (1 h on ice, Thermo Scientific, J19943-K2) and blocked and permeabilized with a buffer containing 1% BSA

and 1% Triton X-100 in PBS (45 min, room temperature). Then, tissues were incubated with primary antibody solutions in the same buffer, overnight, 4°C and were washed three times before treatment with fluorophore-conjugated secondary antibodies.

Imaging

Confocal images were acquired using a Zeiss LSM 880 confocal microscope with 20×/0.8 NA and 40×/1.4 NA oil immersion objectives. Z-stacks were acquired with 1–2 µm step size. Tile scans of whole intestinal segments were acquired using the automated stage and stitched using Zeiss ZEN software. Widefield fluorescence images were acquired using a Zeiss Axio Imager.Z2 with Apotome.2 structured illumination. Images were processed using Fiji/ImageJ. Brightness and contrast were adjusted linearly for display; all comparisons used identical imaging parameters and post-processing. For images that were stitched, we used Leica's LAS X tiling feature or the Grid/Pairwise stitching plugin for FIJI.

Macro Fluorescence Imaging

Images were taken on a Nikon AZ100M Macro laser scanning confocal instrument configured with long-working-distance low-magnification lenses. The microscope is equipped with the standard 405 nm, 488 nm, 561 nm and 640 nm laser lines and has photomultiplier tube detectors with a detection range from 400 nm to 700 nm. To reduce signal drop-off at the image edges, we used an optical zoom factor of 2.1 and increased our lateral resolution using a digital zoom factor of 1.873.

Quantification

Cell counts, fluorescence intensity measurements, and condensate morphology analyses were performed using Fiji/ImageJ and CellProfiler. For fiber alignment quantification, picrosirius red-stained sections were imaged under polarized light, and fiber orientation was quantified using the Directionality plugin in Fiji. For each experimental condition, at least 3 biological replicates and 5 imaging fields per replicate were analyzed.

Mouse embryonic small intestine scRNA-seq dataset

The single-cell RNA sequencing (scRNA-seq) dataset of embryonic mouse small intestine (MSI) analyzed in this study was generated previously and described elsewhere (Huycke TR et al., 2024). In brief, wild-type mouse embryos were collected at embryonic days (E) 13.5, 14.5, 15.5, 16.5 and 18.5. For each embryo, the small intestine was dissected from stomach to cecum in ice-cold PBS under a stereomicroscope. The MSI was then subdivided into contiguous 2.5 mm segments along the anterior-posterior (AP) axis. Each segment was assigned a segment position index (SegPos), and the total number of segments per embryo increased with developmental stage to approximate equal physical lengths. For some analyses, segments were subsequently grouped into proximal, medial, and distal regions by binning SegPos into thirds along the A-P axis. Embryo ID, SegPos, and derived region labels were retained as metadata throughout the analysis.

For each embryo, pooled segments were enzymatically and mechanically dissociated into single-cell suspensions as previously described (Haber AL et al., 2017). Briefly, tissue was incubated in a digestion buffer containing collagenase/dispase and DNase I at 37 °C with gentle agitation,

followed by trituration and filtration through a 40 μ m strainer. Live cells were enriched either by flow cytometry or density-based methods. Single-cell suspensions from multiple segments and embryos were then multiplexed using MULTI-seq lipid-modified DNA oligonucleotide barcodes following the published protocol (McGinnis CS et al., 2019). After barcode labeling, cells from multiple samples were pooled, optionally re-sorted to enrich for viable singlets, and loaded onto the 10x Genomics Chromium platform for single-cell 3' RNA-seq library preparation according to the manufacturer's instructions. Libraries were sequenced on an Illumina platform to a depth sufficient to achieve approximately tens of thousands of reads per cell.

Raw base call files were processed using Cell Ranger (10x Genomics) (Zheng GX et al., 2017) against the appropriate mouse reference genome (mm10) to generate gene-by-cell count matrices. MULTI-seq barcodes were demultiplexed as described previously (McGinnis CS et al., 2019) to assign each cell to a single sample or to identify barcode collisions and likely doublets. Our analyses used a curated AnnData object generated from this dataset, in which initial quality control and demultiplexing had been performed. Low-quality cells were excluded based on minimum UMI counts, gene complexity, and mitochondrial read fraction; thresholds were tuned per batch and matched those used in the original study. Doublets were identified with Scrublet (Wolock SL et al., 2019) and by MULTI-seq barcode conflicts and removed. Genes expressed in very few cells were excluded prior to normalization.

Normalization, batch integration, and clustering

All single-cell analyses were performed in Python using scanpy and anndata unless otherwise stated (Wolf FA et al., 2018). For each cell, raw counts were library-size normalized (for example, scaling total counts to 10,000 per cell) and log-transformed using the natural logarithm

of counts plus one ($\log_1 p$) to generate a log-normalized expression matrix. Highly variable genes (HVGs) were identified within each batch using scanpy's HVG functions, and batch-specific HVGs were merged after excluding mitochondrial and ribosomal genes from consideration. To integrate data across developmental stages and embryos, we used CONCORD to learn a shared low-dimensional latent space. Principal component analysis (PCA) was performed on the scaled HVG expression matrix, and k-nearest neighbor (kNN) graphs were constructed for each batch. CONCORD was then applied with default domain alignment hyperparameters to jointly align these graphs across batches and generate a unified latent representation for all cells. This latent embedding was stored in the AnnData object and used to compute a global kNN graph and a UMAP embedding (Becht E et al., 2018). All UMAP visualizations shown in Figures were derived from this CONCORD-based neighbor graph. Cell clustering was performed using the Leiden community detection algorithm on the integrated kNN graph (Traag VA et al., 2019). We explored a range of resolutions and selected those that yielded stable and biologically interpretable clusters. A global clustering solution was used to identify major compartments (enteric nervous system [ENS], mesenchyme [MES], epithelium [EPI], endothelium, immune), and independent clustering was then performed on compartment-specific subsets for higher resolution analyses.

Cell-type annotation and compartment subsetting

Broad cell types were annotated on the integrated atlas by inspecting canonical marker genes and, where appropriate, referencing annotations from the original study (Huycke TR et al., 2024). ENS cells were identified by expression of Sox10, Ednrb, Ret, Phox2a, Phox2b and related markers. Mesenchymal cells were defined by Pdgfra, Colla1, Vim and other mesenchymal markers; epithelial cells by Epcam and keratins; endothelial cells by Pecam1 and

Kdr; and immune cells by Ptprc and lineage-specific genes. For downstream analyses, we created separate AnnData subsets for ENS, MES and EPI compartments by filtering on these broad labels. Each subset retained CONCORD latent and UMAP coordinates, Leiden cluster identities, SegPos, region assignment, Age, embryo ID and QC covariates.

Composition across developmental stage and A-P region

To quantify how ENS, MES, and EPI subtypes are distributed across developmental stages and A-P regions, we computed cluster proportions at the embryo level. For each embryo and condition (for example, a particular stage or region), we counted the number of cells belonging to each Leiden cluster within a given compartment and divided by the total number of cells from that compartment in the same embryo and condition. These embryo-level proportions were then aggregated by taking the mean across embryos for each condition and visualized as stacked bar or area plots. To derive uncertainty estimates, we performed nonparametric bootstrap resampling of embryos within each condition and recomputed proportions to obtain 95% confidence intervals. Where formal statistical testing of compositional changes was required, generalized linear models with binomial or Dirichlet-multinomial likelihoods were fitted to embryo-level counts, with condition as the main predictor (Smillie CS et al., 2019); (Yu P & Shaw CA, 2014).

Variance partitioning

To compare the relative contributions of developmental stage and A-P region to transcriptional variance within ENS and MES, we implemented a split-metadata PCA framework. For each compartment, log-normalized HVG expression was scaled, and PCA was performed. For each principal component (PC), we fitted a linear model regressing PC score on Age, region, cell-cycle covariates and batch. From these models, we extracted partial eta-squared values for each

factor, representing the fraction of variance in that PC explained uniquely by that factor conditional on the others. We then aggregated partial eta-squared values across PCs by weighting them by the proportion of total variance explained by each PC. This yielded compartment-specific estimates of the variance attributable to stage and region, which were visualized with bootstrapped confidence intervals.

Ligand-receptor communication analysis with CellChat

Intercellular communication between compartments was inferred using CellChat (R package), focusing on signaling pathways targeting ENS (Jin S et al., 2021). Raw counts and metadata from the integrated atlas were exported to R, and CellChat objects were constructed after filtering for relevant sender and receiver groups. ENS, MES, and EPI were included as both potential senders and ENS receivers, along with endothelial and immune compartments. For each Age and region combination, we derived a communication network using the CellChatDB.mouse database of ligand-receptor pairs. Overexpressed genes and interactions were identified using CellChat's standard pipeline, communication probabilities were computed for each LR pair, and pathway-level communication probabilities were aggregated by summing over LR pairs belonging to the same signaling family. Net incoming signaling strength to ENS was defined as the sum of pathway-level communication probabilities for all interactions with ENS as the receiver, broken down by sender compartment, stage, and region. To identify pathways with spatial or temporal signatures, we compared pathway-level "information flow" metrics along the A-P axis and across development. For spatial contrasts (for example, distal vs proximal), we computed differences in information flow and assessed significance by permutation testing of condition labels (typically several thousand permutations per comparison), followed by Benjamini-Hochberg correction for multiple pathways. An analogous strategy was used for

temporal contrasts (for example, E13.5 vs E14.5). Volcano plots summarize standardized differences in information flow versus $-\log_{10}(\text{FDR})$, highlighting pathways such as PTN, MDK, EDN, NOTCH. For selected top pathways, including PTN, we also plotted pathway strength across regions and stages with bootstrapped confidence intervals.

Spatial and temporal regression of ligand and receptor expression

To link pathway-level CellChat results to underlying transcript dynamics, we modeled expression of individual ligands and receptors as functions of A-P position and developmental stage. For each gene of interest, we considered log-normalized expression in the relevant compartment (for example, EPI, MES, ENS ligands, ENS receptors) and fitted linear models at the single-cell level. Spatial models related expression to numeric segment position (SegPos) within a fixed developmental stage, including covariates for batch, cell-cycle score, total UMI counts and mitochondrial fraction. Temporal models related expression to Age across stages, with region included as a covariate alongside the same technical and biological covariates. From each model, we obtained regression slopes, coefficients of determination (R^2), and p-values for the variable of interest (SegPos or Age). P-values were adjusted across genes using Benjamini-Hochberg. Genes were visualized on volcano plots with slope on the x-axis and R^2 on the y-axis. For top candidates such as Ptn and Ptprz1, we also plotted mean expression across ordered segments or developmental stages with bootstrapped 95% confidence intervals to illustrate spatial gradients and temporal trajectories in ligand and receptor expression.

ENS subcluster definition and differential expression

Within the ENS subset, we identified subclusters with strong PTN/PTPRZ1 expression by overlaying Ptn and Ptprz1 expression on the ENS UMAP and inspecting Leiden clusters. Two

clusters were selected for detailed analysis: a Ptn⁺/Ptpz1⁺/Plp1⁺ progenitor-like cluster (cluster 1) and a Ptn⁺/Ptpz1⁺/Elavl4⁺ neuronal-committed cluster (cluster 9). Cluster 1 was compared to other ENS progenitor-like clusters, whereas cluster 9 was compared to other ENS neurons. Differential expression between each focal cluster and its corresponding reference group was performed using scanpy's rank_genes_groups function (Wolf FA et al., 2018). Unless otherwise indicated, we used the Wilcoxon rank-sum test on log₁p-normalized expression values and restricted analysis to genes expressed in at least a minimal fraction of cells in either group. For each gene, we obtained log₂ fold-changes and adjusted p-values (Benjamini–Hochberg). Genes were visualized on volcano plots with log₂ fold-change versus –log₁₀(FDR), and significantly differentially expressed genes above an effect-size threshold were labeled. These DE signatures were then used as ranked lists for downstream gene set enrichment analysis.

Gene set enrichment analysis for ENS subclusters

To interpret differential expression profiles for clusters 1 and 9, we performed gene set enrichment analysis (GSEA) using gseapy (Marron MM et al., 2022). Genes were ranked by a signed statistic derived from the DE results (for example, log₂ fold-change weighted by significance), and this ranked list was queried against MSigDB Hallmark and Gene Ontology (biological process and cellular component) gene sets. Gene set definitions were obtained via msigdb (Liberzon A et al., 2011) and harmonized with mouse gene symbols by case conversion and orthology mapping where necessary. Normalized enrichment scores (NES) and FDR-adjusted q-values were computed using permutation-based tests (Subramanian A et al., 2005). Top 10 enriched pathways with FDR < 0.05 were summarized in bar plots and highlighted key differences between progenitor-like and neuronal-committed PTN⁺/Ptpz1⁺ states, including

enrichment of mitotic, transcription, and translation programs at synapse in cluster 1 and neurite extension, synaptic pathways, and neurotransmission in cluster 9.

Regulatory network inference and PTN/PTPRZ1-centered GRNs

Transcription factor (TF) regulons and gene regulatory networks were inferred using the pySCENIC workflow applied to the ENS subset (Aibar S et al., 2017). Co-expression modules were first identified using GRNBoost2, using a curated list of mouse TFs as candidate regulators. These co-expression relationships were then filtered based on motif enrichment and cis-regulatory evidence using RcisTarget and species-specific motif databases, resulting in regulons defined as TFs and their direct target gene sets. Regulon activity for each cell was quantified with AUCell, which computes the area under the recovery curve of regulon genes in each cell's ranked expression profile, yielding regulon activity scores (AUCs). To identify regulons distinguishing clusters 1 and 9 from their respective reference groups, regulon AUCs were compared using linear models or rank-based tests with cluster identity as the primary predictor and stage and region as covariates. Regulons with significant differences after FDR correction were selected and visualized as barplot, showing patterns of regulon activity across clusters and developmental states. To construct PTN- and PTPRZ1-centered gene regulatory subnetworks, we extracted TF–target edges from the pySCENIC-inferred networks that involved PTN or PTPRZ1 either as TF targets or as TFs with strong regulon activity in PTN+/Ptprz1+ clusters. For each edge, we derived a composite weight by averaging normalized co-expression importance and normalized motif enrichment scores. We then selected TFs with high out-degree or cumulative edge weights within cluster 1 or cluster 9 and visualized these subnetworks. The edge thickness represented composite weights, highlighting distinct TF hubs and downstream programs associated with PTN/PTPRZ1 in progenitor-like versus neuronal-committed ENS cells.

hPSC maintenance and ENS differentiation

Human pluripotent stem cells (hPSCs) were maintained in a defined medium (for example, mTeSR1 or Essential 8) and passaged using non-enzymatic dissociation reagents according to standard protocols. Cells were routinely tested for mycoplasma contamination. Differentiation toward enteric neural crest and ENS-like ganglioid cultures followed a previously established protocol (Fattahi F et al., 2016), (Penaud-Budloo M et al., 2019), (Yang S et al., 2024). In brief, hPSCs were first induced toward neural crest using combinations of dual-SMAD inhibition and Wnt activation, then patterned toward a vagal ENS fate using GDNF over the course of approximately two to three weeks. Cultures were then maintained in conditions that support ENS-like neuronal and glial maturation, producing dense networks of ENS-like ganglia by around Day 30 (D30) of differentiation.

ENS small molecule perturbation

To interrogate PTN/PTPRZ1 and EDNRB signaling axes, Day-30 ENS ganglioid cultures were treated with recombinant ligands and small-molecule inhibitors. Cultures were exposed to midkine (MDK; 50 ng/mL), pleiotrophin (PTN; 50 ng/mL) or the small-molecule PTPRZ1 inhibitor NAZ2329 (NAZ; 10 μ M). In parallel, cells were treated with an EDNRB agonist (EDN3) and an EDNRB antagonist (BQ), using concentrations previously shown to modulate ENS lineage behavior. Treatments were administered in neuronal induction/basal medium from D30 to D35, with media changes at appropriate intervals. On D35, treatments were discontinued, and cultures were maintained in basal medium until Day 39 (D39) to allow a brief chase period. All conditions, including vehicle-treated controls (CTRL), were performed with three

independent biological replicates, defined as separate differentiation batches or independently seeded wells.

RNA extraction, bulk RNA-seq library preparation, and sequencing

On D39, cultures were washed with PBS and lysed directly in RNA extraction buffer. Total RNA was isolated using a column-based kit according to the manufacturer's instructions, including on-column DNase treatment to remove genomic DNA. RNA quality and integrity were assessed by TapeStation or Bioanalyzer, and concentration was measured by Qubit. Only samples with sufficient yield and acceptable RNA integrity were used for library preparation. Bulk RNA-seq libraries were prepared by Plasmidsaurus using Oxford Nanopore Technology with custom analysis and annotation. Raw sequencing reads were processed using a standard alignment and counting pipeline. Reads were adapter- and quality-trimmed, aligned to the human reference genome (GRCh38) using STAR (Dobin A et al., 2013), and assigned to genes using featureCounts with GENCODE gene annotations (Liao Y et al., 2014). This yielded a sample-by-gene count matrix that was imported into R for differential expression analysis with DESeq2.

DESeq2 analysis, PCA, and sample-level QC

Differential expression analysis of bulk RNA-seq data was carried out in R using DESeq2 (Love MI et al., 2014), with additional visualization performed using tidyverse and related packages. Raw counts and sample metadata (condition, replicate) were used to construct a DESeqDataSet, and size factors and dispersions were estimated using DESeq2's standard workflow. A design formula with condition as the main factor was used to model expression differences among CTRL, MDK, PTN, and NAZ2329. For visualization, we applied the variance-stabilizing transformation (VST) to generate homoscedastic expression values. Principal component

analysis (PCA) was performed on the VST-transformed matrix using prcomp. Samples are plotted in the PC1–PC2 plane, colored by condition, showing separation of treatment groups and clustering of replicates. To aid interpretation and assess replicate consistency, condition centroids in PC space were computed and optionally overlaid. Outlier samples, defined by large distances from their condition centroid or inconsistent clustering based on correlation metrics, were identified in a separate QC script. Where outliers were detected, they were excluded from downstream DESeq2 contrasts used in figure panels; this filtering step is documented in Supplementary Methods. Differential expression contrasts were computed between each treatment and control (MDK vs CTRL, PTN vs CTRL, NAZ2329 vs CTRL, EDN vs CTRL, BQ vs CTRL) using DESeq2's Wald test. For each gene in each contrast, DESeq2 reported log₂ fold-change, standard error, Wald statistic and adjusted p-value after Benjamini–Hochberg correction. Genes with FDR < 0.05 and |log₂ fold-change| above a nominal threshold were considered significantly differentially expressed.

Module-based summaries and GSEA for bulk RNA-seq

To connect bulk perturbation responses to ENS developmental and neurotransmitter programs identified in the mouse scRNA-seq data, we defined curated gene modules representing progenitor, neuroblast, pan-neuronal, neuronal subtype and glial states, as well as neurotransmitter classes (for example, cholinergic, nitrergic, serotonergic, GABAergic) and signaling-related genes (PTN/PTPRZ1/EDNRB and downstream TFs). Module membership was based on literature and on cluster-specific markers from the mouse ENS atlas. For each module and each DESeq2 contrast, we summarized treatment effects by computing the mean log₂ fold-change of genes within the module relative to CTRL. These module-level log₂ fold-changes were

visualized as heatmaps, with rows corresponding to modules or selected marker genes and columns corresponding to conditions. Where the goal was to compare absolute magnitudes across conditions, colors represent raw $\log_2$ fold-changes. To obtain pathway-level insights, we performed gene set enrichment analysis on the bulk RNA-seq DESeq2 results using GSEA (Subramanian A et al., 2005) and fgsea and msigdb (Liberzon A et al., 2011). For each contrast, genes were ranked by a signed statistic (for example, $\log_2$ fold-change multiplied by $-\log_{10}(\text{FDR})$, or the DESeq2 Wald statistic), and this ranking was compared against MSigDB Hallmark and GO gene sets for Homo sapiens. Normalized enrichment scores and FDR-adjusted q-values were computed by fgsea. We focused on gene sets related to cell cycle and proliferation (for example, E2F targets, G2M checkpoint, mitotic nuclear division), neurodevelopment and axon guidance, and synaptic structure and function. This analysis revealed distinct and sometimes opposing effects of PTN/MDK/PTPRZ1 versus EDNRB signaling perturbations on proliferative and synaptic maturation programs in hPSC-derived ENS cultures. Category-specific heatmaps of $\log_2$ fold-changes for neurotransmitter synthesis genes, neuropeptides, cytokines and receptors were also constructed to illustrate perturbation-induced changes in neurochemical identity and cell–cell communication components, using curated gene lists and the same DESeq2-derived $\log_2$ fold-changes.

GWAS methods for DGBIs

For the genome-wide association studies conducted using the All of Us Research Program data (All of Us Research Program Investigators et al., 2019), genetic association analyses were performed using Hail, a scalable open-source library for genomic data analysis built on Apache Spark. Hail provides efficient methods for quality control, variant annotation, and association

testing on biobank-scale datasets. Analysis workflows followed established protocols as documented in the Hail GWAS tutorial (https://hail-notebook-image.readthedocs.io/en/latest/examples/hail_GWAS_v0.0.1.html).

Quality Control and Variant Filtering

Genotype data from the All of Us Research Program were imported into Hail MatrixTables. Sample-level quality control metrics were computed using Hail's `sample_qc()` method, including call rate, heterozygosity, and relatedness estimates. Samples with call rates <0.95 or outlier heterozygosity values (>3 standard deviations from the mean) were excluded. Variant-level QC metrics were computed using Hail's `variant_qc()` method. Variants were filtered for call rate >0.95 , Hardy-Weinberg equilibrium $P > 1 \times 10^{-6}$, and minor allele frequency >0.01 following standard GWAS quality control practices.

Association Testing

For each DGBI phenotype defined according to Rome IV diagnostic criteria with extensive exclusion of overlapping conditions, logistic regression models were fit using Hail's `logistic_regression_rows()` method for case-control phenotypes. Models were adjusted for age, sex, and the first 10 genetic principal components to control for population stratification. For traits with small sample sizes or rare variants, Firth logistic regression was applied to reduce bias in effect size estimates. Association results were annotated with variant functional consequences using Hail's Variant Effect Predictor (VEP) integration, linking variants to genes and predicting their impact on protein structure. Genome-wide significant associations ($P < 5 \times 10^{-8}$) were identified and prioritized for downstream analysis.

Statistics and software

Unless otherwise indicated, all statistical tests were two-sided, and multiple comparisons were controlled using the Benjamini–Hochberg false discovery rate procedure. For single-cell analyses, effect sizes are reported as partial eta-squared for variance partitioning, regression slopes and R^2 values for spatial and temporal models, $\log_2$ fold-changes for differential expression, and normalized enrichment scores for GSEA. For bulk RNA-seq, effect sizes are reported as DESeq2 $\log_2$ fold-changes and normalized enrichment scores. Confidence intervals, where shown, represent 95% intervals derived from bootstrap resampling or model-based estimates. Single-cell analyses were implemented primarily in Python using scanpy (Wolf FA et al., 2018), anndata, numpy, pandas, scikit-learn, gseapy (Marron MM et al., 2022), pySCENIC (Aibar S et al., 2017) and custom scripts. CellChat (Jin S et al., 2021), DESeq2 (Love MI et al., 2014), msigdb (Liberzon A et al., 2011), GSEA (Subramanian A et al., 2005), tidyverse and heatmap packages (such as pheatmap or ComplexHeatmap) were used in R for ligand–receptor communication, bulk RNA-seq differential expression and pathway analyses.

CHAPTER 7: DISCUSSION

The gastrointestinal tract is a remarkably complex organ system whose function depends on the coordinated activity of multiple tissue compartments—epithelium, mesenchyme, and enteric nervous system—that undergo highly orchestrated morphogenetic events during embryonic development. This thesis has investigated fundamental mechanisms governing intestinal morphogenesis and enteric nervous system development and has identified genes underlying disorders of gut-brain interaction. By integrating mouse, human pluripotent stem cell models, transcriptomics, and human genetics, this work provides new insights into how tissues self-organize during development, how developmental programs can be recapitulated for therapeutic purposes, and how genetic variation affecting developmental processes contributes to disease susceptibility.

Integration of Findings Across Chapters

ECM Remodeling and Mesenchymal Signaling in Gut Development

Chapter 2 demonstrated that extracellular matrix remodeling, mediated by integrin-ECM interactions and matrix metalloproteinase activity, is essential for intestinal villus morphogenesis. PDGFR α -high mesenchymal cells actively remodel their local microenvironment during condensation, degrading fibrillar collagens while synthesizing non-fibrillar matrix components that facilitate cell motility and clustering. Hedgehog signaling from the epithelium promotes the formation of PDGFR α -high clusters, while downstream of Hh, TGF- β signaling may act as an upstream regulator of the ECM gene programs—including integrins, MMPs, and collagens—expressed in these subepithelial mesenchymal cells. Together,

these findings establish ECM remodeling as a central mechanism linking epithelial-mesenchymal signaling to mechanical force generation during tissue morphogenesis.

The broader principle that mesenchymal cells actively sculpt their environment to drive morphogenesis resonates across subsequent chapters. In Chapter 3, ligand-receptor analysis revealed that the mesenchyme is the dominant source of signaling inputs to the developing ENS, including matrix-associated growth factors such as pleiotrophin (PTN) and midkine (MDK). In Chapter 4, PDGFR signaling was identified as a negative regulator of nitrergic neuron specification in hPSC-derived enteric cultures. Although Chapter 4 did not directly investigate integrin or ECM gene expression in differentiating enteric neurons, the finding that inhibition of PDGFR, the same receptor that marks the subepithelial mesenchyme enriched in ECM programs in Chapter 2, promotes nitrergic fate suggests that mesenchyme-derived signals continue to influence neural development well beyond initial neural crest migration. Thus, mesenchymal signaling—whether through ECM remodeling, secreted growth factors, or receptor tyrosine kinase pathways—emerges as a conserved regulatory strategy employed across multiple tissue compartments and developmental stages in the gut.

Temporal vs. Spatial Organization of Developing Tissues

A central finding of this thesis is the contrasting organizational principles governing different tissue compartments in the developing gut. Chapter 3 revealed that the intestinal epithelium and mesenchyme exhibit strong anterior-posterior regionalization driven by spatially restricted Hox gene expression, whereas the enteric nervous system is organized primarily along a temporal maturation axis with minimal regional transcriptional identity. This dichotomy—strong spatial

patterning in mesodermal and endodermal compartments, weak spatial patterning in neural crest-derived ENS—reflects their distinct embryonic origins and developmental strategies.

The uniform vagal identity of ENS cells throughout the gut may confer functional advantages.

First, it may enable flexible adaptation to local microenvironments: rather than being locked into region-specific transcriptional programs, ENS cells can respond dynamically to spatially graded niche signals (such as PTN/MDK-PTPRZ1) to fine-tune their proliferation, differentiation, and neurotransmitter specification. Second, uniform identity may facilitate neural circuit assembly by allowing stereotyped synaptic connectivity patterns to be established independent of gut region, with functional specialization arising from local modulation of neuronal excitability and neurotransmitter release rather than from intrinsic transcriptional differences.

This organizational principle has important implications for regenerative medicine. Strategies to generate region-specific intestinal epithelium or mesenchyme must carefully control morphogen exposure to establish appropriate anterior-posterior identity. In contrast, enteric neuron generation may be less constrained by positional specification, potentially enabling production of enteric neurons that can adapt to their local environment after transplantation. Consistent with this idea, Chapter 4 demonstrated successful functional integration of hPSC-derived enteric neurons into the host colon under the differentiation conditions tested, though whether this generalizes across diverse culture protocols remains to be established.

Microenvironmental Regulation of ENS Development

The spatiotemporal dynamics of ENS transcriptional identity and the regulation of signaling cues to ENS cells have remained incompletely understood. The identification of PTN/MDK-PTPRZ1 signaling as a spatially graded microenvironmental input to the ENS (Chapter 3) addresses this

gap by providing a molecular mechanism through which localized niche signals fine-tune ENS maturation. PTN and MDK are expressed in spatially and temporally dynamic patterns within the mesenchyme, and their receptor PTPRZ1 is expressed by ENS progenitors and neurons.

Functional perturbation of this pathway in hPSC-derived ENS cultures demonstrated that PTPRZ1 modulation broadly affects the balance between progenitor expansion and neuronal maturation: treatment with PTN, MDK, or the PTPRZ1 inhibitor NAZ2329 upregulated progenitor and proliferative gene signatures while suppressing neuronal maturation and synaptic gene programs. Neurotransmitter identity was also affected, with consistent downregulation of GABAergic markers and selective modulation of serotonergic markers.

These findings establish PTPRZ1 signaling as a molecular rheostat that tunes the pace of ENS maturation and influences neurotransmitter specification. Importantly, they complement the findings of Chapter 4, where PDGFR signaling was identified as a distinct mesenchymal pathway regulating nitrergic neuron specification. Together, Chapters 3 and 4 reveal that multiple receptor tyrosine kinase and phosphatase pathways—PTPRZ1, PDGFR α/β —converge on ENS progenitors to regulate the timing and outcome of neuronal differentiation. This convergence suggests that the mesenchymal niche provides a combinatorial signaling environment in which the relative levels of different growth factors bias enteric progenitors toward specific fates. Future work should investigate how these pathways interact at the level of intracellular signal transduction and gene regulatory networks.

Neurotransmitter Specification and Functional Diversity in the ENS

The balance between excitatory and inhibitory motor neurons is critical for normal gut motility. Chapter 4 made the key finding that PDGFR signaling negatively regulates nitrergic neuron

specification: pharmacological inhibition with PP121 or genetic ablation of PDGFRA and PDGFRB dramatically increased the proportion of NOS1+ neurons in hPSC-derived enteric cultures, while addition of PDGF ligand suppressed nitrergic differentiation. This effect positions PDGFR signaling as a gatekeeper that restricts the nitrergic fate, potentially by maintaining progenitors in a proliferative state or by biasing lineage choice at critical decision points.

The high-throughput drug screen in Chapter 4 further identified multiple neuromodulatory compounds that enhance nitrergic neuron activity, converging on serotonin, adrenergic, acetylcholine, glutamate, and opioid receptor targets. Among these, the α 2-adrenergic agonist dexmedetomidine showed particularly robust effects on colonic migrating motor complexes *ex vivo*, effects that were dependent on intact NOS1 signaling. These findings demonstrate the translational utility of hPSC-derived ENS platforms for drug discovery and provide candidate compounds for further preclinical evaluation.

Taken together with the PTN/MDK-PTPRZ1 findings of Chapter 3, these results reveal that neurotransmitter specification in the ENS is regulated by multiple, converging mesenchymal signals. This multi-input model is consistent with the functional diversity observed in the mature ENS and suggests that perturbation of any single pathway may shift the balance of neuronal subtypes rather than completely ablating a specific population.

Genetic Architecture of Gut-Brain Disorders and ENS Dysfunction

Chapter 5 extended the developmental and functional studies of Chapters 2–4 to the human population level by performing genome-wide association studies of disorders of gut-brain interaction using the All of Us Research Program database. Significant genetic associations were identified for irritable bowel syndrome, functional diarrhea, functional dyspepsia, functional

chest pain, and functional fecal incontinence. Notably, DGBI-associated genes were enriched for functions in neuronal excitability, synaptic transmission, ion channel regulation, and chromatin remodeling. Expression analysis confirmed that many of these genes are expressed in hPSC-derived enteric neural crest cells and ganglioids, supporting the hypothesis that genetic variants affecting ENS development or function contribute to DGBI pathophysiology.

These genetic findings complement the developmental studies in a significant way. The identification of ENS-expressed genes at DGBI risk loci provides human genetic evidence that the pathways studied in Chapters 3 and 4—those governing neuronal specification, synaptic function, and ion channel activity—are clinically relevant. Future studies combining CRISPR-based perturbation of DGBI candidate genes in hPSC-derived ganglioids with functional readouts (calcium imaging, motility assays) will be critical for establishing causality and prioritizing therapeutic targets.

Broader Implications for Developmental Biology

Self-Organization and Emergent Pattern Formation

The villus morphogenesis program described in Chapter 2 represents a striking example of tissue-level self-organization. PDGFR α -high cells undergo condensation through a dewetting-like process that depends on cell-intrinsic contractility, ECM remodeling, and local signaling interactions rather than pre-patterned positional information. The emergent spacing and geometry of villus structures arise from physical parameters—including cell-ECM adhesion strength, matrix degradation rate, and actomyosin contractility—that can be modeled using frameworks from soft matter physics. This model contrasts with classical Turing reaction-diffusion models that invoke interacting morphogens to explain periodic patterning, and it

underscores the importance of integrating mechanical and biochemical perspectives in developmental biology.

Similarly, ENS maturation (Chapter 3) proceeds through a self-organizing temporal program that generates diverse neuronal subtypes from a common progenitor pool, with local niche signals providing modulatory inputs rather than instructive commands. This organizational strategy—a uniform intrinsic program refined by extrinsic context—may be a general principle for neural crest-derived tissues that colonize large territories during development.

Integration of Genomic and Phenotypic Diversity

Chapters 3 and 5 together illustrate how genomic approaches at different scales illuminate complementary aspects of gut biology. Single-cell RNA sequencing reveals the transcriptional architecture of individual cell states during development, enabling identification of signaling pathways and gene regulatory networks at cellular resolution. In contrast, population-level GWAS identifies common genetic variants that influence disease risk across millions of individuals. The convergence of these approaches—for example, the finding that DGBI risk genes are expressed in the same ENS cell types whose development is regulated by PTN/MDK-PTPRZ1 and PDGFR signaling—provides multi-level evidence linking developmental programs to disease susceptibility.

Clinical and Translational Impact

Cell-Based Therapies for Enteric Neuropathies

The findings of this thesis have direct implications for the development of cell-based therapies for enteric neuropathies, including Hirschsprung disease, achalasia, gastroparesis, and chronic

intestinal pseudo-obstruction. A key question for such therapies is whether hPSC-derived ENS progenitors must be patterned to adopt region-specific identities prior to transplantation. The results of Chapter 3 suggest that this may not be necessary. Because the ENS retains a largely uniform vagal transcriptional identity throughout the gut and lacks overt anterior-posterior regionalization, ENS progenitors appear to follow a stereotyped developmental trajectory that is subsequently refined by local microenvironmental cues rather than by cell-intrinsic positional programs. This implies that a single, standardized ENS differentiation protocol may be sufficient to generate progenitors capable of adapting to diverse gut regions after engraftment—a considerable practical advantage for clinical translation.

However, the same findings also highlight the importance of the host microenvironment in shaping transplanted cell fate. The mesenchymal niche provides critical signals—including PTN/MDK-PTPRZ1 and PDGF—that regulate the balance between progenitor expansion, neuronal differentiation, and neurotransmitter specification (Chapters 3 and 4). Whether the niche environment at the site of transplantation, which may be diseased or structurally disrupted, can adequately support the maturation and functional integration of engrafted ENS cells remains an open question. Strategies to co-deliver appropriate niche factors—or to pre-condition grafted cells with signals such as GDNF, PDGFR inhibitors, or PTPRZ1 modulators—may be necessary to bias differentiation toward desired neuronal subtypes (e.g., nitrergic neurons for motility disorders) and promote functional circuit integration. Additionally, the identification of PDGFR inhibition as a driver of nitrergic neuron specification (Chapter 4) provides a concrete strategy for generating nitrergic neuron-enriched populations for transplantation in conditions where inhibitory motor neuron loss is the primary pathology.

Precision Medicine for Disorders of Gut-Brain Interaction

The identification of genetic risk factors for DGBIs, as described in Chapter 5, opens new avenues for precision medicine. Genetic risk scores integrating information from multiple GWAS loci could stratify individuals by disease susceptibility, enabling earlier diagnosis, preventive interventions, or personalized treatment selection. The functional validation of DGBI candidate genes using hPSC-derived enteric ganglioids and CRISPR-mediated perturbation will be critical for nominating therapeutic targets and understanding how common genetic variation translates into altered ENS function.

Implications for Understanding and Treating Intestinal Disease

The developmental mechanisms elucidated in this thesis have relevance beyond congenital disorders. Villus atrophy, a common feature of celiac disease, tropical sprue, and chemotherapy-induced enteropathy, involves loss of the three-dimensional villus architecture established during development. Understanding how ECM remodeling drives villus morphogenesis may inspire strategies to promote villus regeneration: delivery of MMPs, integrin-activating peptides, or Hedgehog agonists could potentially stimulate mesenchymal condensation and epithelial folding in atrophic intestine.

Similarly, acquired enteric neuropathies resulting from diabetes, chemotherapy, or infection involve selective loss or dysfunction of enteric neurons. The molecular pathways identified in this thesis—PDGFR signaling, PTPRZ1 signaling, WNK1-mediated chloride homeostasis—may be dysregulated in these conditions and represent targets for neuroprotection or regeneration.

Limitations and Future Directions

While this thesis has made significant progress in understanding intestinal morphogenesis, ENS development, and DGBI genetics, several important limitations and unresolved questions remain.

Maturation and Fidelity of hPSC-Derived Models

The hPSC-derived ENS models employed in this thesis—enteric ganglioids and 2D enteric cultures—recapitulate the remarkable neuronal and glial diversity of the primary human ENS, including cholinergic, nitrergic, serotonergic, and GABAergic neuronal subtypes. Module scoring of ganglioid cell-type signatures against primary human colon single-nucleus RNA-seq data has demonstrated high transcriptional similarity between in vitro and in vivo enteric neurons (Majd et al., 2025), and the neurotransmitter complexity observed in ganglioids closely parallels that of primary tissue. Nevertheless, several important caveats must be considered when interpreting findings from these models.

First, hPSC-derived enteric neurons exhibit gene expression profiles most consistent with fetal and neonatal rather than adult ENS, a limitation shared broadly across PSC-derived organoid and neural models (Werner & Gillis, 2023). Prolonged culture partially addresses this, as ganglioids gain functional maturity—including increased c-FOS responsiveness and synaptic connectivity—over weeks in culture. However, full maturation may require cues absent from standard culture conditions, including co-culture with interstitial cells of Cajal and smooth muscle, exposure to mechanical stretch mimicking peristalsis, innervation by extrinsic autonomic neurons, and colonization with commensal microbiota. The absence of these inputs likely limits the fidelity of circuit-level properties such as rhythmic motor pattern generation.

Second, like other PSC-derived systems, enteric ganglioid cultures contain off-target cell populations. Single-cell RNA-seq of ganglioids has revealed the presence of mesenchymal and other non-neural cell types whose composition and transcriptional identity can vary between 2D and 3D culture formats (Majd et al., 2025). While these off-target populations may in some cases provide beneficial paracrine signals, they also introduce variability and complicate the interpretation of bulk assays. Strategies to reduce off-target differentiation—such as purification of SOX10⁺ enteric neural crest cells by FACS prior to ganglioid formation, or CRISPR-based lineage selection—could improve the consistency of downstream analyses.

Third, current hPSC-derived ENS models lack the architectural complexity of native tissue. In vivo, enteric neurons and glia are organized into ganglionated plexuses embedded within the muscular wall of the gut, with defined connectivity between myenteric and submucosal circuits. Ganglioids, by contrast, are three-dimensional aggregates that contain the appropriate cell types but lack this higher-order spatial organization. As a consequence, functional readouts available in ganglioids—calcium imaging, c-FOS expression, NO release—capture neuronal activity at the single-cell or population level but do not recapitulate emergent circuit behaviors such as colonic migrating motor complexes. The ex vivo validation experiments in Chapter 4, which tested drug candidates on intact mouse colon segments, were essential precisely because they provided the tissue-level architecture missing from the in vitro system.

Fourth, organoid-to-organoid heterogeneity—variability in size, cell-type composition, and differentiation state—remains a practical challenge for screening applications. Recent advances in bioprinting and guided self-organization, including the use of composite granular biomaterials (MAGIC matrices) to deliver cells at tissue-like densities with sub-100 μm spatial resolution, have demonstrated order-of-magnitude improvements in organoid uniformity and assay

sensitivity. Integrating such fabrication strategies with ENS differentiation protocols could substantially improve the reproducibility and scalability of ganglioid-based functional assays.

Finally, the question of how faithfully hPSC-derived ENS models represent region-specific ENS biology remains open. Chapter 3 demonstrated that the ENS *in vivo* follows a predominantly temporal rather than spatial transcriptional program, suggesting that hPSC-derived enteric neurons—which are generated under uniform culture conditions—may in fact capture the core developmental program of the ENS reasonably well. However, the subtle transcriptional refinements imposed by region-specific mesenchymal cues (PTN/MDK-PTPRZ1, PDGF) are absent from standard ganglioid cultures, and incorporating such signals may be necessary to model region-specific ENS phenotypes relevant to diseases that preferentially affect particular gut organ or segments.

Interspecies Differences and Translatability

Mouse models have been invaluable for dissecting developmental mechanisms, but interspecies differences in gut anatomy, physiology, and ENS function limit translatability. Enteric neuron density, neurotransmitter expression, and response to pharmacological agents differ between rodents and humans. These differences underscore the importance of validating findings in human tissue or human cell-based models, as was done in Chapters 3 and 4 with hPSC-derived ganglioids.

Functional Validation of DGBI Genetic Associations

While Chapter 5 identified multiple DGBI-associated genetic loci, establishing causality between specific variants and disease phenotypes will require extensive functional studies. Most GWAS hits are noncoding variants that likely affect gene regulation rather than protein sequence.

Identifying the causal variants, target genes, and mechanisms will require fine-mapping, chromatin conformation capture, and CRISPR-based enhancer editing in relevant cell types.

Single-Cell and Spatial Transcriptomics

While Chapter 3 employed spatiotemporally resolved single-cell RNA-seq to profile the developing ENS, emerging technologies enable even higher resolution interrogation of development. Spatial transcriptomics methods that preserve native tissue architecture while providing single-cell resolution can map gene expression patterns in intact tissues and reveal how spatial microenvironments influence cell fate.

Expansion to Other GI Disorders and Tissues

The experimental and computational approaches developed in this thesis are generalizable to other aspects of GI development and disease. Esophageal development, gastric patterning, pancreatic morphogenesis, and hepatobiliary development all involve epithelial-mesenchymal interactions, ECM remodeling, and signaling gradients analogous to those studied here.

Concluding Remarks

This thesis has elucidated fundamental mechanisms governing intestinal morphogenesis and enteric nervous system development, identified genetic variants associated with disorders of gut-brain interaction, and established proof-of-concept for cell-based therapies for enteric neuropathies. By integrating mouse developmental genetics, human pluripotent stem cell models, spatial transcriptomics, and human genetics, this work exemplifies the power of multi-scale, multi-system approaches to understanding development and disease.

The gastrointestinal tract, with its complex architecture and diverse cell types, has long been a challenging system to study. Recent advances in stem cell biology, genomics, and imaging have transformed our ability to interrogate GI development at unprecedented resolution and to model human GI disorders in vitro. The findings presented here demonstrate that developmental mechanisms—ECM remodeling, microenvironmental signaling, neurotransmitter specification—are not only fascinating from a basic science perspective but also have direct clinical relevance.

As the fields of developmental biology, stem cell biology, and genomics continue to advance, the integration of these approaches will yield increasingly powerful insights. The marriage of developmental biology and translational medicine, exemplified in this thesis, represents a path forward for addressing the fundamental questions of how organs form and how we can harness developmental principles to treat disease. The work presented here provides a foundation for future studies that will continue to illuminate the remarkable complexity of the gut and to translate developmental insights into clinical impact for the millions of individuals affected by gastrointestinal disorders.

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