Deep into the niche: deciphering local endoderm-microenvironment interactions in development, homeostasis and disease of pancreas and intestine

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Subtitle: The microenvironmental contribution to *in vivo* and *in vitro* pancreatic and intestinal development

Abbreviations: ECM, extracellular matrix; hPSC, human pluripotent stem cell; IBD, inflammatory bowel diseases; PaSC, pancreatic stellate cell; PPs, pancreatic progenitors; SM, splanchnic mesoderm; vSMCs, vascular smooth muscle cells; single cell RNA-sequencing, scRNA-seq.

Summary:

Unraveling molecular and functional heterogeneity of niche cells within the developing endoderm could resolve mechanisms of tissue formation and maturation. Here, we discuss current unknowns in molecular mechanisms underlying key developmental events in pancreatic islet and intestinal epithelial formation. Recent breakthroughs in single-cell and spatial transcriptomics, paralleled with functional studies *in vitro*, reveal that specialized mesenchymal subtypes drive the formation and maturation of pancreatic endocrine cells and islets via local interactions with epithelium, neurons and microvessels. Analogous to this, distinct intestinal niche cells regulate both epithelial development and homeostasis throughout life. We propose how this knowledge can be used to progress research in the human context using pluripotent stem cell-derived multilineage organoids. Overall, understanding the interactions between the multitude of microenvironmental cells and how they drive tissue development and function could help us make more therapeutically relevant *in vitro* models.

Introduction

Epithelial cells throughout the endoderm carry out pivotal functions to regulate digestion and metabolism. In the pancreas, epithelial cells are broadly divided into two major cellular compartments: the endocrine and exocrine compartments. The proper function and development of pancreatic endocrine cells is essential, as tightly regulated and coordinated action of hormones from five distinct endocrine cell types is pivotal for maintaining blood glucose homeostasis. The two most abundant pancreatic endocrine cell types are α- and β-cells, which produce and secrete glucagon and insulin, respectively. These two endocrine hormones' actions are counterregulatory and the postprandial increase in glucose concentration in the blood serum stimulates insulin release from β-cells allowing uptake of glucose by peripheral tissues, while glucagon prevents the decrease in glucose levels by promoting glycogen breakdowns in the liver. Developing adjacently to the pancreas, diverse epithelial cells lining the lumen of the intestine are constantly sloughing off and excreted as waste with new cells continuously generated. The development and regeneration of the epithelial lumen, with epithelial cells maintaining proper proportion, function, and localization, is essential for digestive and metabolic health. In divots in the intestinal epithelial lining called crypts, intestinal stem cells reside while supportive cells regulate the self-renewal and differentiation of cells.[1]

While epithelial cells drive essential functions throughout the pancreas and gastrointestinal tract, these cells cannot develop into fully functional cells without the contribution of a melange of abundant non-epithelial cell types. Very early fate decisions rely on a combination of intrinsic and extrinsic signals, many of which have been well defined. For example, extrinsic signals from notochord, aorta, and adjacent developing organs drive initial pancreatic specification, formation, and growth.[2] The intrinsic transcription factors driving endodermal lineage development have been comprehensively discussed elsewhere.[3–6] Later in development, as cells differentiate and begin to mature into physiologically competent adult cell types, extrinsic signals from the niche are abundant; however, much work is yet to be done to identify, define, and mechanistically understand the spatial, cellular, and molecular interplay of cell types and signals that maintain epithelial diversity and function.

Gaining an understanding of the cellular and molecular niche has therapeutic and technological potential. Pancreatic diseases are a high burden to society of which most common within the exocrine part are cancer, pancreatitis; and within endocrine – diabetes (537 million people affected in 2021).[7] Untreated diabetes leads to multiorgan failure and eventually death, and in 2021 approximately 6.7 million people worldwide died due to diabetes.[7] Diabetes results from prolonged inability to sustain blood glucose homeostasis due to β-cell dysfunction often combined with impaired ability of peripheral tissues to respond to insulin (type II diabetes), or β-cell loss (type I diabetes). In parallel, a large majority of pervasive intestinal diseases are caused by dysfunction of cells or signals in the microenvironment. Inflammatory bowel diseases (IBD), including Crohn's and Ulcerative Colitis, are characterized by inflammation of the intestinal epithelium, affecting 6 million people globally and steadily rising in incidence each year.[8] It is not conclusively known, however, what the initiating factor or cell type is in

many of these pancreatic and intestinal diseases, nor is it known how cells in the microenvironment shift states to perpetuate pathology. Thus, understanding how neighboring cells communicate with one another and change over time may provide new knowledge regarding disease intervention. Beyond this, studying dynamics of the niche *in vivo* guides the generation of complex cell types and multicellular tissue models *in vitro*. This can be accomplished through mimicking the complex *in vivo* processes in a dish, either through the addition of small molecules and growth factors normally provided by cells in the microenvironment, or through the differentiation of multiple cell types in tandem. New cellular technologies, such as intestinal organoids, allow for modeling of human organ development, cellular crosstalk, and different stages of pathogenesis. In addition, providing signals exogenously allows for the production of pure and more physiologically competent β-cell cultures to be used for drug screening and potentially regenerative therapies for diabetic patients. These major therapeutic and technological breakthroughs are entirely dependent upon understanding the cellular and molecular diversity of the niche.

In this review, we examine the progress made in recent years to understand these microenvironmental cells and signals, specifically focusing on niche contributions to the development, maturation, and regeneration of cells in the pancreas and intestine. These advances are critical not only for our biological understanding of normal tissue function, but also to apply towards the generation of therapeutically relevant *in vitro* systems. Recent work defining the niche-epithelium interactions through single-cell methods has provided a deeper understanding of development and has paved the way towards the generation of glucose responsive beta cells, intestinal organoids or “mini-guts”, and even mid-gestation stage mouse embryos by rebuilding the cellular or molecular microenvironment in a dish.[6,9–11] Finally, we will discuss the remaining open questions regarding the local microenvironmental actions as a driver of pancreatic and intestinal development and examine how we can use this knowledge to advance *in vitro* models for digestive health.

A shifting microenvironment guides pancreatic endocrine cell formation and function.

The pancreas, an endoderm derivative, initially develops at the duodenal loop of the primitive gut tube around E8.5 in mice.[3] By E9.5-E10.0, expanding multipotent pancreatic progenitors (PPs) form epithelial buds at ventral and dorsal sides of the gut tube. Around E11.5, PPs within the bud interior acquire apico-basal polarity and organize into microlumens that by E12.5 connect into a plexus structure. Starting from E13.5 in mice, in a process known as the secondary transition, the expanded plexus core undergoes plexus-to-duct remodeling by forming ramified primitive ducts, while the plexus exterior undergoes extensive branching morphogenesis within ductal ends. As a result of the secondary transition, the developing pancreas acquires a lobular structure similar to that of the adult organ. A portion of progenitors within the late plexus core undergo endocrine fate specification to give rise to all islet endocrine cell types.

The pancreas develops as an epithelial sheet surrounded by a complex, multicellular microenvironment, composed of mesenchymal, endothelial, neuronal, and immune cells, together forming the pancreatic niche. In fact, during the secondary transition, cells in the pancreatic niche greatly outnumber epithelial cells.[12] Throughout development the microenvironment provides mechanical and chemical cues that are indispensable for the initial fate commitment, organ growth and spatial organization, as well as functional maturation of endocrine and exocrine cells.[2,13–15] Initial specification and growth of the pancreas is driven and controlled by signals from adjacent tissues like the notochord and aorta (in the case of the dorsal pancreas), and cardiac mesoderm and septum transversum (in the case of ventral pancreas).[2] These processes are also supported by signals from major blood vessels.[2,16–19] At later timepoints, newly formed PPs become surrounded by proliferating mesenchyme, which is the most abundant component of the pancreatic microenvironment. Golosow and Grobstein demonstrated in the 1960s that pancreas deprived of mesenchyme cannot develop *ex vivo*.[20] More recently, the necessity of the mesenchyme for proper pancreatic development was confirmed using mouse conditional models in which pancreatic mesenchyme was specifically ablated at multiple timepoints during development.[12,21,22] Different studies showed that depending on the developmental timepoint, the mesenchyme plays a different role during pancreas formation. The early mesenchyme acts to prevent precocious differentiation of endocrine cells, maintaining PPs in a proliferative, multipotent state before initiation of epithelial branching,[23,24] while the later mesenchyme exhibits a positive effect on both exocrine and endocrine differentiation.[12,22,25,26] Recent studies, including ours, showed evidence that the pancreatic mesenchyme is molecularly heterogeneous and shifts in time, and highlighted the functions, organ-specificity and origins of mesenchyme.[27–32] However, knowledge on the origin of the pancreatic mesenchyme, as well as its development, function, and topological relationship with other cell types, remains grossly incomplete.

Pancreatic mesenchyme dynamically changes over development

Mesenchymal cells within the developing gastrointestinal and pulmonary tracts, including the pancreas, are descendants of lateral plate mesoderm and its progeny – splanchnic mesoderm (SM).[33] Between E8.5 and E9.5 in mice, SM lineages diversify along foregut concomitantly to gut tube remodeling and initiation of endodermal organ formation.[34] Developmental trajectories of SM derivatives and adjacent foregut organs are highly coordinated via signaling pathways,[34] suggesting orchestrated two-way interplay between endoderm and mesoderm derivatives. At E9.5, SM surrounds peri-pancreatic foregut as an organized bilateral layer of elongated cells marked by Nkx3-2 (also known as Bapx1).[12,35] Nkx3-2+ mesenchyme descendants remain widespread in the developing pancreas.[12,29] Interestingly, Nkx3-2 expression level is side asymmetric, higher on the left-dorsal side of pancreas, which also induces asymmetric, left-biased growth of both dorsal pancreas and pancreatic mesenchyme.[35] SM and its derivatives are a source of fibroblast growth factor 10 (Fgf10), which is essential for pancreatic epithelium growth,[36] and more Fgf10-producing mesenchyme at the left side

of dorsal pancreas might explain the leftward growth of pancreatic epithelium. Interestingly, Fgf10 produced by endothelial cells is necessary for initial pancreatic mesenchyme survival and growth,[24] underpinning the complexity of interactions and dependencies in the microenvironment of developing pancreas. Loss of Nkx3-2+ mesenchyme prevents asymmetric growth and induces intestinal fate acquisition by pancreatic progenitors via loss of hedgehog signaling inhibition, [22,35] further highlighting role of mesenchyme in early gut development.

Around E9.5-10.5, Isl1+ cells from mesothelium (also known as coelomic epithelium, the SM derivative lining gut tube epithelium), start to infiltrate the pancreatic anlage.[21,32,33] Lineage tracing by DiL staining suggests that the coelomic epithelium but not SM as a sole source of pancreatic mesenchyme at E8.5.[32] Yet it is possible that the specific region of pro-pancreatic SM was not detected in the experiments or that Nkx3-2+ mesenchyme, which also expresses Isl1,[29] is derived from coelomic epithelium. Thus, the relationship of coelomic epithelium-derived cells to Nkx3-2+ SM and underlying mesenchymal populations remains to be clarified by use of lineage tracing methods at earlier developmental stages.

Analyses of individual cell transcriptomes within developing pancreas by single-cell RNA sequencing (scRNA-Seq) revealed increasing diversity among mesenchymal populations at E12.5-E16.5.[27,28,37] The application of scRNA-Seq at E14.5 pancreas allowed the identification of ten mesenchymal clusters, including mesothelial Wt1+ cells, spleno-pancreatic Nkx2-5+ mesenchyme, pancreatic stellate cells (PaSC)/vascular smooth muscle cells (vSMCs), a large archetypal mesenchyme cluster, and multiple discrete mesenchymal clusters of unknown identity.[27–29,35] These subtypes were identified based on their transcriptomic features, but the development and functional heterogeneity remains mostly elusive with only a few of these subtypes studied so far. For example, mesothelial Wt1+ cells have a very specific localization at E12.5-E14.5, when they outline pancreas mostly as a single-cell layer.[28,35,38] Yet, how other pancreatic mesenchymal subtypes are related to Wt1+ mesothelium as well as the ancestry of these mesothelial progenitors themselves needs to be further clarified.

Mesothelial cells change transcriptomically between E12.5 and E16.5, and are likely ancestors of at least some PaSCs/vSCMs.[27,28,38] PaSCs are a pancreatic specific mesenchymal subtype localized near vasculature, ducts, and acinar cells. While part of the microenvironment themselves, PaSCs also respond dynamically to changes in their surroundings and have the ability to transition into reactive states. However, the functional consequence of PaSC reactivity is highly dependent on what inductive signals push them to activation and what other factors are present in the niche.[39] For example, in pancreatic cancer, reactive PaSCs can upregulate secretion of the chemokine CXCL12 and prevent cytotoxic T-cell migration to the tumor to increase tumor burden.[40] These cells have also been found to secrete alanine and Nrf2 to directly support tumor growth and metabolism.[41,42] This shows that even in the same disease, these cells can differentially respond to microenvironmental cues and have different consequences on pathogenesis through either direct influence on tumor cells themselves or

indirectly through interaction with immune cells. While studies have focused on understanding the heterogeneous responses PaSCs transition through in adult disease states including pancreatitis and pancreatic cancer,[43–45] the functional significance and developmental relationship of PaSCs during organogenesis and cellular maturation are largely overlooked. As these cells constantly monitor their environment and rapidly transition from “quiescence” into an activated state, it is reasonable to speculate that these cells are also highly active throughout the massive changes occurring during tissue formation and development.

By E18.5 pancreatic mesenchyme becomes more homogenous.[27,28,37] The loss of heterogeneity might indicate acquisition of cellular composition more resembling postnatal pancreas, characterized by low number of mesenchymal cells and cell types, i.e. quiescent and activated PaSCs), pericytes, telocytes, and vSMCs.[14,22,46–50] In fact, adult pancreatic mesenchymal cells are so transcriptomically akin that scRNA-Seq clustering of whole human pancreas recognizes only quiescent and activated PSCs.[47,48,50] The acquisition of adult-like mesenchymal composition also coincides in time with pancreas remodeling to adult-like morphology. Developmental relationships between recognized adult pancreatic mesenchyme cells is not clear and requires further investigation.

Pancreatic niche controls plexus-to-duct remodeling and branching morphogenesis

After initial bud growth and its enveloping by proliferating mesenchyme, the developing pancreas undergoes structural remodeling during the secondary transition (**Figure 1**). Starting from E11.5, PPs segregate into exterior unipotent tip (ancestors of acinar cells) and interior bipotent trunk (ancestors of ductal and endocrine cells) progenitor domains, with PPs retained at ductal termini.[51,52] During the secondary transition, the exterior of pancreatic plexus undergoes extensive stochastic branching morphogenesis, driven by PPs and tip progenitors at ductal termini.[51,53,54] Principles of branching morphogenesis are proposed to be universal for organs that develop branched morphology, e.g. kidney and mammary gland, and are based on both chemical and mechanical cues from the surrounding niche.[55] However, the precise signaling mechanisms that instruct specific progenitors at ductal termini to induce branching remain unknown. Similarly, termination of branching is partially explained by short-range inhibition when a termini gets into proximity of a neighboring duct,[54,56] but the inhibitory signals identity and origins remain unknown. Meanwhile, the interior of plexus remodels at E14.5-E18.5 in an asymmetric and localized fashion with microlumens pruning and forming a ductal network,[57,58] but site-specific inducers of these events have not been identified.

During the secondary transition, mesenchyme, endothelial, and neural crest cells invade the interior of branching epithelium cavities, creating local niches. Significant mesenchyme influence on pancreas development at multiple timepoints (E15.5-E18.5) was observed when Nkx3-2+ descendants were depleted by either diphtheria toxin or due to loss of pro-survival canonical Wnt signaling.[12] In these experiments, mesenchyme exclusion blocked late progenitor proliferation and branching.

Similarly, epithelial branching was ablated by the dysfunctional Hox6/Wnt5a pathway in pancreatic mesenchyme.[25] In addition to mesenchyme, progenitors of endothelial and neural lineages infiltrate pancreas during the secondary transition attracted by signals from pro-endocrine trunk regions and repelled by tip cells.[59–63] Ongoing vascularization regulates pancreas growth and branching by inhibiting tip cell growth in the trunk region.[18,19] In the above examples global mesenchyme and endothelial populations in pancreas were targeted, yet studies targeting more specific mesenchymal subpopulations are needed to uncover the functionality beyond the molecular mesenchyme heterogeneity.

Niche cells are necessary for endocrine cell specification and islet development

EPs marked by transient Ngn3 expression, form in the plexus core prior to local ductal remodeling.[6,57] EPs undergo few rounds of symmetric replication, delaminate from epithelium through partial epithelial-mesenchymal transition (EMT), and acquire specific endocrine fate.[64–66] A current model proposes that nascent islets emerge as endocrine cell clusters, called peninsulas, attached to the epithelial cord, and eventually detach from the ancestor duct remaining in its proximity.[66–68] The nascent islets initially coalesce into cord-like structures, while postnatally they undergo fission.[66,69] During islet maturation endocrine cells assemble with endothelial, neuronal and mesenchymal cells from the niche to acquire specific architecture allowing their functionality.[15] Yet, detailed mechanisms of delamination, islet formation and maturation steps as well as differences between mouse and human islet architecture have not been explained.

Some important insights into specific roles of microenvironment in delamination, islet formation and maturation steps were recently made. Ablation of mesenchymal Hox6/Wnt5a pathway *in vivo* blocks EP specification.[25] Interestingly, we recently showed that fetal pancreatic mesenchyme-derived Wnt5a promotes human β-cell derivation from hPSCs *in vitro* via non-canonical Wnt signaling.[30] Similarly, Pbx1 KO in Nkx2-5 mesenchyme impairs EP-endocrine transition in dorsal pancreas due to trunk-adjacent basement membrane disruption and Slit/Robo pathway deregulation by loss of mesenchymal Slit3.[29,70] Slit and Robo2 expression in β-cells is necessary for correct islet architecture.[71] A recent 3D imaging of intact pancreas revealed that Nkx2-5+ mesenchyme progeny tighten its contacts with epithelium between E12.5-E14.5,[72] further supporting notion that some specific mesenchymal subtypes function locally within a pro-endocrine niche. Therefore, together these studies imply that the different subtypes of pancreatic mesenchyme are dynamic, with unique temporal molecular signature, and likely have a specific function during pancreatic development.

An essential component of developing and mature islets are endothelial and neural crest lineages. Endothelial and neural crest cells approach closely endocrine cells to regulate balance between their proliferation and maturation through development, ensure proper islet architecture and precise function of islets in adult organisms.[60,62,73–75] For example, by releasing VEGF β-cells attract endothelial cells and organize themselves around them, and loss of VEGF prevents islet formation,

whereas its excess results in hypervascularization that prevents β-cells to form islets.[18,59,76,77] On the other hand, loss of neural crest cells induces excessive β-cell proliferation but blocks β-cell maturation.[60,62] In addition, pericytes which together with endothelial cells create islets vasculature, were recently shown to influence β-cell maturation, via bone morphogenetic protein (BMP)-4 pathway, and adult islet function.[49,78,79] On the other hand development and maturation of islet vasculature and innervation is also regulated by signals from developing pancreatic epithelium and islet cells, but this crosstalk is not well understood. Excitingly, analysis of our E14.5 murine pancreas scRNA-Seq data,[27] suggests that specific mesenchymal subpopulations can preferentially interact with endothelial or neural cells, making attractive hypothesis for specialized roles of these subpopulations in angio- and neurogenesis. Thus, islet development might be orchestrated by interplay between mesenchyme, endothelial, neuronal and endocrine progenitors, navigating them into each other's proximity and governing proper islet architecture.

The development and maturation of the intestine relies on cell non-autonomous signaling

While the pancreas buds out from the primitive gut tube, the tube itself is what gives rise to the gastrointestinal tract. Initially growing as a sheet, the endoderm folds around murine embryonic day 8 to form a tube of endoderm surrounded by mesoderm (called "tubulogenesis).[80] The endoderm will later give rise to the hindgut (including but not limited to the pancreas, stomach, esophagus, and upper duodenum), the midgut (including but not limited to the lower duodenum, jejenum, ileum, cecum), and hindgut (including but not limited to the distal regions of the colon). The splanchnic mesoderm, which is wrapped around the gut tube, is incredibly diverse [34] and forms both the connective tissue that holds the gut tissues together called mesentery and the cells that become the non-epithelial layers of the gut. These layers include cells in the lamina propria inside of the villi in the small intestine, the longitudinal and circular smooth muscle layers, submucosal connective tissue, blood vessels, and the outside serosa. Around murine embryonic day 10, neural crest cells migrate to and invade the gut wall in a rostrocaudal manner. These cells differentiate into enteric nervous system cells, first mixing with these primitive mesodermal tissues and staying in the outermost region.[81] In the mouse, it is not until postnatal stages that enteric nervous system cells migrate cetripetally towards the lamina propria until all layers of the gut are fully innervated. Finally, while the cell bodies remain outside of the gut, peripheral nervous system cells like extrinsic sensory neurons innervate the entire length of the intestine. With the menagerie of cell types in the intestine outlined above, there is a wealth of opportunity for cell non-autonomous signaling to affect cell fate and function of all cells.

While mesoderm and ectoderm derived cells make up the many cells outlined above in the intestine, the intestinal epithelia develop from the gut tube endoderm itself. Initially the intestinal epithelium forms as a smooth tube before mesenchyme and epithelium folding into longitudinal ridges, followed by a zigzag pattern,[82] until finally the finger-like projections called villi form, expanding the intestinal surface area approximately 100,000-fold.[83] While in humans intestinal crypts (where the intestinal stem cells and niche cells reside) develop shortly after villi in utero, murine crypts do not form until after birth.[84] An elegant sc- and spatial RNA-Seq by Zhao et al. reveals that the intestinal mesenchyme and

epithelium develop in a coordinated manner.[85] Using interactome analyses, they predict a wide range of intercellular signaling that can direct intestinal fate decisions, including signaling from mesenchymal telocytes to developing epithelial cells. They show in ex vivo experiments that hepatocyte growth factor (HGF) treatment to embryonic intestinal fragments promoted secretion of Olfm4, a secreted intestinal stem cell marker, along with morphological changes concurrent with intestinal maturation. Thus, there is an intimate relationship between intestinal niche cells and the epithelium during early development and maturation.

Spatial organization promotes the cellular diversity of the intestinal microenvironment

Epithelial cells line the intestinal lumen and are poised to interact with the deluge of intestinal contents that pass through every day ranging from food matter to microbiota. These cells have a short lifespan, turning over every 3-5 days and contributing to approximately 40% of human cellular mass lost each day.[86,87] Intestinal stem cells express LGR5 and reside in crypts nestled between finger like protrusions called villi; as new cells are made, older cells migrate upwards along the crypt-villus axis until they hit an extrusion zone and are eventually sloughed off into the lumen to be expelled as waste. Proximal-to-distal patterning along the crypt-villus axis mirrors the birth-to-death of epithelial cells. These intestinal epithelial cells sit atop a rich base of diverse niche cells that help to maintain epithelial cell integrity, regeneration, and physiology (**Figure 2**).

The decision for intestinal stem cells to either replicate themselves or give rise to one of many diverse epithelial subtypes present throughout the intestinal lining is tightly regulated and relies on many interactions and signals from different cells in the local niche. Paneth cells, a specialized epithelial cell type that huddle next to intestinal stem cells in the crypt, secrete a cocktail of signaling factors to regulate the stem cell niche. While many of these signals promote the stem cell state, including epidermal growth factor (EGF), Wnt3, and Delta-like ligands, Paneth cells can also act to decrease regeneration through release of the extracellular Wnt inhibitor Notum.[88] The balance between regeneration and senescence in the intestine can be modulated through the function of Paneth cells, with an imbalance in the aged gut contributing to the diminished cellular turnover and many age associated gastrointestinal maladies.

Among the most well studied cells in intestinal development and the intestinal niche are mesenchymal cells. The mesenchyme changes over time as the intestine develops, with elegant work recently showing the subtypes of mesenchyme present in the developing human gut via scRNA-Seq.[83] Early in development, the mesenchyme differentiates into primitive smooth muscle cells which provide trophic support through secretion of WNT and R-SPONDIN-1.[89] A separate population of mesenchyme present during early development lines the epithelium and secretes both WNT and Neuregulin-1 (NRG1), a ligand that promotes the differentiation of secretory epithelial cells.[89] NRG1 is classically studied in the nervous system, where it is released from neurons and promotes the development and repair of both neurons and glia in the central and peripheral nervous systems.[90] As the enteric nervous

system is developing concurrently with the epithelium, the consequence of NRG1 secretion from a specialized mesenchymal subtype in the developing human gut on enteric nervous system development and organization would be interesting to understand. The enteric nervous system also retains the ability to regenerate [81,91,92] it will also be important to identify the source and secretion of NRG1 in the adult gut and during epithelial and nervous system regeneration.

A specialized large and rare mesenchymal cell type called telocytes sit beneath the crypts in the intestine and secrete Wnt ligands and the BMP inhibitor Gremlin1 to promote intestinal stem cell self-renewal.[1,93] Spatial analysis of the intestinal niche reveals that subtypes of mesenchyme are organized into different sectors in a proximal-distal pattern, including specialized subtypes of telocytes nestled at the tip of the villi.[94] These tip associated telocytes provide signals such as BMP and the non-canonical WNT5A ligand to regulate epithelial cell gene expression. Thus, telocytes exhibit a spatial switch from anti-BMP and canonical WNT signaling to pro-BMP and non-canonical WNT signaling. This spatial switch in the telocyte secretome along the crypt-villi axis differentially influences epithelial cell function. These directly opposing functions in the same cell type in different regions emphasize the importance of understanding the spatial, molecular, and functional heterogeneity of cells in the microenvironment. Further, it is not yet known what other cell types and signals influence telocytes across the crypt-villi axis to contribute to their functional heterogeneity.

Diverse cell types coordinate to maintain intestinal physiology and homeostasis

Beyond development and regeneration, the gut microenvironment continues to drive mature intestinal function. The intestines move food through the body, secrete digestive enzymes, absorb nutrients and water, and defend the body against infection. This impressive range of functions is driven by a combination of specialized epithelial cells including not only enterocytes, but also hormone-secreting enteroendocrine cells, immune-modulating Paneth cells, and mucus-producing goblet cells. The intestinal epithelium is constantly renewing itself and maintaining healthy function in this setting of frequent cell turnover requires tightly regulated communication with surrounding cells. Enteric glia cells (of the enteric nervous system) contribute to this epithelial homeostasis, secreting Wnt ligands during times of regeneration to regulate intestinal stem cell proliferation.[95] Paneth cells and mesenchymal cells also secrete Wnt ligands, suggesting redundant and potentially fine-tuned signaling to ensure robust control over such an important process.[96,97] In the enteric nervous system, enteric neurons coordinate muscle contractions required to move material through the gut, and they are also able to transmit signals from the gut lumen to the brain via the vagus nerve, forming the gut-brain axis.[98] Immune cells interact with the luminal contents in the gut. While epithelial cells provide a physical barrier to protect the body against toxins and potentially harmful bacteria, resident immune cells work to recognize pathogenic antigens in the environment and defend the body against infection. Specialized gut-associated lymphoid tissue (GALT), composed of B-cells, T-cells, and M- cells, sample antigens from the lumen and communicate with nearby dendritic cells to prime the intestine for adaptive immune

response.[99] Other immune cells such as macrophages are diffusely spread through intestinal tissue and regulate the innate immune response.[100] Importantly, the immune system can differentiate between harmless commensal microbes of the healthy gut microbiome and harmful foreign microbes, a vital distinction in an environment that depends so heavily on microbial influence for proper functioning. Finally, the secretory functions of epithelial cells are also regulated by local extrinsic cues. As one example, enteric neurons stimulate enteroendocrine cells to secrete serotonin in addition to secreting it themselves, regulating gastric acid secretion, pancreatic secretions, gut motility, and more.[101] Without these extensive contributions from the microenvironmental niche, the intestine could not function properly.

Changes in the intestinal microenvironment may contribute to the initiation and progression of disease

Abnormal functioning of cells of the intestinal microenvironment can both cause gastrointestinal disease and be a reaction to disease – malfunctioning niche cells contribute to disease manifestation, and disease processes modify niche cell behavior. For instance, consider IBD, which involves chronic inflammation of the intestinal epithelium, leading to symptoms such as abdominal pain, abnormal stools, and weight loss. The disease is thought to be initiated by immune cells in the intestinal microenvironment that enact an inappropriately exaggerated response to gut microflora, leading to damage of intestinal epithelial cells, impaired barrier function, and further inflammation.[102] The function of other niche cells such as enteric glia change in IBD, which undergo a transition to a transcriptionally distinct reactive subtype with both damaging and beneficial functions.[103,104] These reactive cells can promote both continued inflammation and epithelial regeneration.[105] This bidirectional relationship between disease and microenvironment has the potential to form a positive feedback loop, leading to aggravated disease symptoms until interrupted by therapeutic interventions. This paradigm is also present in other gastrointestinal pathologies. In colon cancer, signals from the microenvironment cells including immune cells, enteric glial cells, and fibroblasts contribute to tumorigenesis,[106–109] and then continued growth of tumors interrupts normal functioning of these same microenvironmental cells, aggravating symptoms and promoting tumor progression.[110,111] In infection, immune cells initiate the inflammatory response driving a patient's symptoms, and in response to this inflammation fibroblasts and enteric glial cells activate to promote not only epithelial healing, but also scarring and further inflammation.[112,113] Across all GI diseases, better understanding the mechanistic details of microenvironmental interaction with pathogenesis will improve our ability to prevent and treat patients.

Gut organoids recapitulate multicellular tissue formation providing a system to probe human development

Understanding how the microenvironment contributes to development and physiology, including the many subtypes of cells, their spatial organization, and their changes over time, will provide knowledge that allows for the generation of cells and tissue *in vitro*. Protocols to generate complex, three-dimensional intestinal organoids from intestinal explants or pluripotent stem cells hinge upon the knowledge from *in vivo* development and utilize growth factors and small molecules that mimic the *in vivo* environment during early cell fate decisions (**Figure 3a-b**).[114,115] These major breakthroughs have opened up a new realm of science to allow for the modeling of the human gut.[116,117] However, while the complex interplay of cell types and signals during early development are relatively well defined, the cellular and molecular mechanisms dictating later stages of development and maturation have remained elusive. These organoids develop to early human fetal stages in the dish while suspended in a Matrigel dome, with mesenchyme and epithelial cells self-organizing around a simple lumen into human “mini-guts”.[117–120] When transplanted into the mouse kidney capsule, subtypes of mesenchyme begin to differentiate into smooth muscle in opposing layers (circular and longitudinal muscle) while the epithelium starts to organize into crypt-villi units around the lumen with more diverse cell types present.[117] Yet even with transplantation, which undoubtedly provides trophic support from the mouse, these organoids only mature to late fetal stages.

Recent work has focused on understanding how the microenvironment promotes further maturation and differentiation in the gut. Many studies identifying mesenchyme subtypes that change over space and time in the developing gut have revealed factors that promote the differentiation of human pluripotent stem cells into intestinal organoids that develop to later stages, including the addition of NRG1 to organoid models, which specifically promotes differentiation of intestinal stem cells into epithelial secretory cells.[89] Even targeting niche factors that are found to change over time, like the increase in Notum in the aged gut, can restore phenotypes and function in aged intestinal organoids.[88] While suspending organoids in Matrigel domes promotes their assembly and development, efforts to culture organoids in tissue-specific extracellular matrices (ECM) or designer matrices have shown that the type of ECM can also alter tissue development, with many benefits of more precise and tissue-specific ECMs.[121–124]

Beyond the addition of *in vivo* identified molecules, many efforts have concentrated on the addition of other cell types at the right proportion and right timing to guide differentiation *in vitro*. The modularity of this alternative approach can shed light onto the functional significance of different cell types during development and allow for the identification of cell type derived signals as they are identified to promote or prevent tissue development. Some examples of this in recent years are the addition of vasculature, immune cells, or enteric nervous system cells into intestinal organoid protocols.[125–127] It has even been found that the addition of mechanical cues to mimic the peristaltic activity of the functional gut promotes intestinal organoid maturation.[122] While great work has been done to show how mechanical cues influence intestinal epithelial cell development, there has been considerably less focus on other cell types in the intestine such as the enteric nervous system. A recent study has shown that smooth muscle contractions shaping enteric nervous system plexus

formation.[128,129] It will be of great importance to understand how mechanosensation affects the development and function of the enteric nervous system, as these cells are integral in regulating peristalsis. Interestingly, innervated intestinal organoids grown *in vitro* only develop to early fetal stages, with a smooth epithelial lining surrounded by mesenchymal cells and disorganized enteric nervous system cells. It is not until after transplantation to the kidney capsule that these organoids further mature, with epithelial cells forming villi-crypt units, smooth muscle cells developing and organizing into circular and longitudinal layers, and enteric nervous system plexus formation.[125] This shows that cues not provided *in vitro*, but that arise after transplantation, further potentiate intestinal maturation. As the intestine contains a menagerie of cell types that interact dynamically with one another throughout development (**Figure 2**), continuing to dissect the cellular, spatial, molecular, and temporal heterogeneity of the niche will unlock our ability to build therapeutically relevant human “mini-guts” in a dish.

Mechanosignaling regulation of pancreas development

In the pancreas, knowledge of key extrinsic signaling pathways driving development has been adapted for human β-cell *in vitro* differentiation, enabling great advancements in pancreatic cell generation. Multiple signaling pathways controlling pancreas development that are regulated by microenvironment-derived chemical cues were identified, including FGF2, RA, BMP, TGFβ, Notch, Hedgehog and canonical Wnt,[2,13] and many of these factors are incorporated into the *in vitro* hPSC-differentiation protocols .

However, microenvironment guides pancreatic development also by physical cell-cell interactions and ECM scaffold, which are harder to recapitulate *in vitro* than soluble factors.[57,130–132] The microenvironment is composed not only of adjacent cells (mesenchymal, epithelial, neuronal, endothelial and immune cells), but also of ECM fibers such as collagens, fibronectin, and laminins. The cellular and ECM compartments are separated by basement membrane, which is produced by endothelial cells and pericytes. Moreover, cells and ECM are source of multiple secreted soluble factors that influence the signaling landscape and each other. Together, these different microenvironment components provide the cells with adhesion substrates, physical support and adhesion substrates which all are critical for proper differentiation, proliferation and morphogenesis. This relationship between cells and ECM was proposed as dynamic reciprocity by Sage and Bornstein in 1982.[133] Yet we are still far for fully understand it. The development of multiple tissues, for instance the intestine as discussed above, the lung, and the mammary gland, is dramatically affected by the composition, density, and stiffness of the ECM. Cells generally interact with ECM molecules via integrins, receptors located at the cell membrane and whose primary function is to disseminate signals between the cells with ECM matrix. However, the type and quantity of integrins on given cell are specific to the cell and tissue type. Several studies have suggested the requirement of integrins and basement membrane for pancreatic branching and cell differentiation, and deletion of the β1-integrin subunit in the pancreas induce basement membrane defects and actin cytoskeleton disorganization.[131] Inhibiting of cell-ECM

interactions in pancreatic explant cultures by the tetrapeptide arginyl-glycyl-aspartyl-serine, impairs branch formation.[131] Furthermore, ECM interactions can also affect cell fate decisions in the pancreas through mechanotransduction; in both mice and in vitro, cell spreading, or the ability for cells to move away from a central region of proliferating cells, influences the expression of PDX1.[132] This spreading phenomenon is promoted through integrin a5b1, a canonical fibronectin receptor. Cell spreading or tip-trunk patterning influences the activity of the mechanoresponsive factor YAP1 via the formation of actin bundles. YAP1 is reduced during endocrine cell formation; in fact, deletion of YAP1 in pancreatic progenitors in mice leads to precocious and robust endocrinogenesis and hypoglycemia. When cells are plated onto laminin, they exhibit reduced YAP1 and increased NGN3; this shows that mechanosignaling and ECM proteins are important for pancreatic cell fate decisions.

Studies have even shown that the ECM can change in disease and in aging;[134] thus it may be important to work towards the application of age-specific and disease-specific ECM (possibly from decellularized patient tissue) in addition to developmental stage-specific and tissue-specific ECM, to use in cell culture methods and to determine the effects of ECM and ECM-derived signals on tissue function. Despite these examples, the specific mechanisms behind the complex and dynamic relationship between epithelial cells and extracellular elements are still unraveling.

Most of current protocols of hPSC differentiation towards pancreatic or intestinal lineages are based on the application of cocktail of growth factors and small molecules, which selection is based on the knowledge gained from developmental studies. However, in these protocols little attention is directed towards the application of developmental stage-specific ECM proteins. Application of different ECM substrates at various stages of *in vitro* derivation might improve the terminal differentiation, given that the expression of key integrin receptors changes over the embryogenesis. For example, during oligodendrocytes *in vitro* derivation, cells are initially grown on Matrigel to be later transferred to poly-l-lysine-coated plates for terminal differentiation.[135]

Boosting pancreatic organoid systems for research on human organ development and disease

Despite its critical role, microenvironment and its cellular components, including mesenchyme, endothelium or neural cells, are not significantly present during *in vitro* pancreatic differentiation. This is because signaling pathways leading to pancreatic endoderm are exclusive with mesenchymal lineage, development of the latter is blocked early during differentiation. However, as we and others showed, mesenchymal or endothelial cell incorporation at various stages of hPSC pancreatic differentiation facilitates the development of human pancreatic β-cells and their progenitors.[136–139] In our recent work we showed that coculture of hPSC-derived pancreatic progenitors with human stage- and organ-specific mesenchymal and endothelial cells promotes the functional human β-cell formation *in vitro*.[30] We also showed that both secreted factors and ECM enhance endocrine cell formation.

Moreover, we identified growth factors, like WNT5A and Endocan that promote human endocrine cell fate *in vitro* and are released by human fetal pancreatic stage-specific microenvironment-derived cells.

Yet, recent studies showing the molecular, temporal and likely functional diversity of pancreatic niche components inclines that there are more signals specific for key pancreatic developmental events still waiting to be uncovered and incorporated in the *in vitro* hPSCs differentiation. Noteworthy, most of our knowledge on the pancreatic niche during development is based on mouse studies and more studies based on human cells are needed.

In vitro reconstruction of such events as plexus-to-duct remodeling, branching morphogenesis or islet formation and maturation would require reconstruction of non-epithelial niche components. Gel scaffold-based hPSC systems were recently established for use in developmental research on PPs, which allowed their long-term culture, self-organization into polarized microlumens in form of cysts, and observation of delamination events (**Figure 3c**).[140,141] Although branching can be observed in mouse pancreatic organoids (pancreatoids, **Figure 3d**) derived from dissociated embryonic pancreata,[26,56,142] this has not been achieved in hPSC-derived systems. Moreover, we showed that development of endocrine lineage requires preservation of native, organotypic mesenchyme in mouse pancreatoids.[26,143] In terms of islet formation during hPSC differentiation, creation of nascent islets can be observed in form of endocrine buds loosely attached to bulk progenitor sphere,[68] but their further maturation requires the enveloping microenvironment, missing in the *in vitro* culture. Reconstruction of functional islets for cell transplantation purposes might be beneficial over pure β-cells. Indeed, this was confirmed by studies that incorporated endothelial cells or microvessels into hPSC-derived pancreatic endocrine cells, which boosted their functionality and survival.[144,145] Similarly, addition of neural crest stem cells to cadaveric human islets improved transplantation outcome in mice.[146,147] This indicates that further identification of temporal, local signals and molecular mechanisms beyond microenvironment-epithelium cross-talk is crucial for better understanding and controlling *in vitro* β-cell derivation and functional maturation.

In contrast to β-cell differentiation, multiple human 3D organoid systems, for example cerebral, lung and gastrointestinal, by default include microenvironment cells, facilitating the organoids architecture well resembling *in vivo* counterparts. [117,148–150] Often these organ-specific organoids were derived with a goal to create miniorgans for drug testing, disease modeling and developmental research. In contrast, pancreatic differentiation protocols were developed with the aim to maximize production of pure, functional β-cells or other pancreatic lineages for therapeutic and research applications (**Figure 3e**),[151–156] and not to holistically model pancreas development. To overcome this limitation and create all-hPSC derived multilineage, epithelial-mesenchymal pancreatic organoids, a separate differentiation of each lineage might be followed by their assembly at the *in vitro* differentiation step representing the pre-secondary transition stage *in vivo* (**Figure 3f**). As shown by research pointing to the importance of organ-specific mesenchyme, it is important though to develop protocols of pancreatic mesenchyme derivation. Although this has not been achieved yet, the ancestral lateral

mesoderm lineage has been already derived.[157] In such a system, other niche components - endothelial and neuronal - could be added, to better mimic the *in vivo* niche.

Conclusion

More focused research on individual mesenchyme subpopulations and their interaction with both epithelial and niche cells over time will not only promote our understanding of basic biological mechanisms (**Boxes 1-2**), but also will help us open a route towards new therapies and cellular technologies. This research could include re-analysis of existing scRNA-Seq data, loss-of function studies, lineage tracing, spatial transcriptomics and 3D imaging; not only at a static point in time, but throughout development, maturation, and disease progression to get a better picture of what is happening as cells change states or fate in response. These efforts would be facilitated by research in the human context using novel hPSC-derived 3D systems dedicated to developmental studies. The co-culturing of hPSC-derived tissues with other developmentally relevant cell types in the niche will show what specific signals drive different processes and at what exact time they take effect, helping efforts to generate mature tissues for regenerative therapy, drug screening, and the modeling of human specific processes. This would bring a new realm of science and medicine, providing for the first time access to human tissues to answer previously inaccessible questions.

Figures:

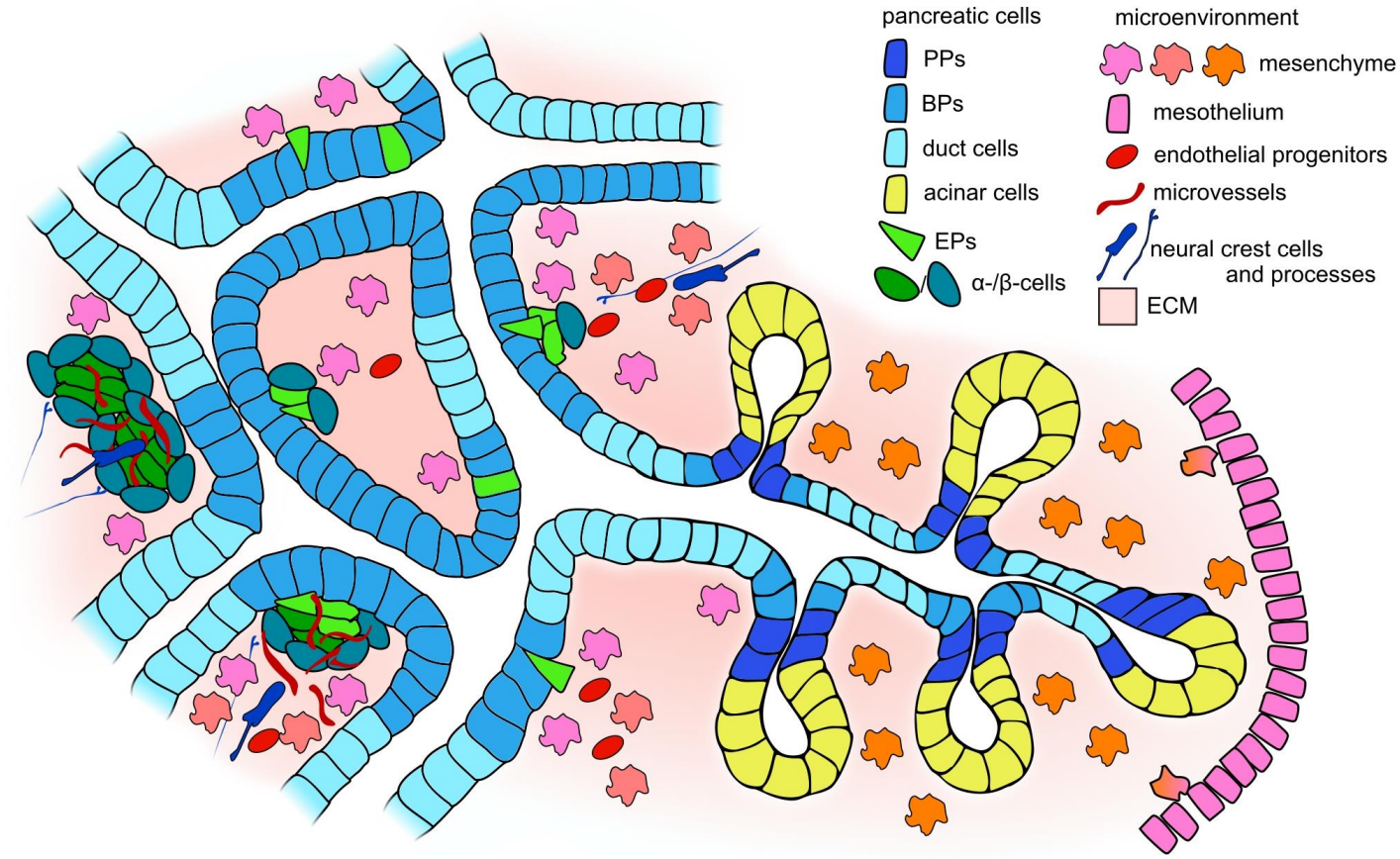

Fig. 1. Overview of dynamic niches during development of pancreatic endocrine islets. A scheme representing pancreatic trunk (left) and tip (right) compartments during secondary transition at approximately E14.5-E16.5. The plexus undergoes remodeling into the ductal network by BP divisions and duct cell specification, while PPs and unipotent acinar progenitors at ductal termini drive branching of the tip domain. Some BPs within the core of trunk domain become EPs that proliferate and delaminate to become endocrine islet cells (α- and β-cells, as well as less abundant δ-, ε- and PP-cells that are not included on the scheme). The mesenchymal cells and ECM might differentially invade and allocate within compartments and cavities, creating specific niches promoting ductal growth, endocrine specification or branching. Pro-endocrine trunk regions additionally attract endothelial followed neural crest cells, and neural processes. Together, endocrine cells with mesenchyme, endothelial and neural crest-derivatives organize into pancreatic islets, initially as epithelial-cord attached peninsulas. The nascent islets detach into surrounding mesenchyme later, coalesce with neighboring islets and further mature to become functional islets in the postnatal period. Pancreas is outlined by mesothelial cell layer, which is hypothesized to be a progenitor of at least some mesenchymal subtypes.

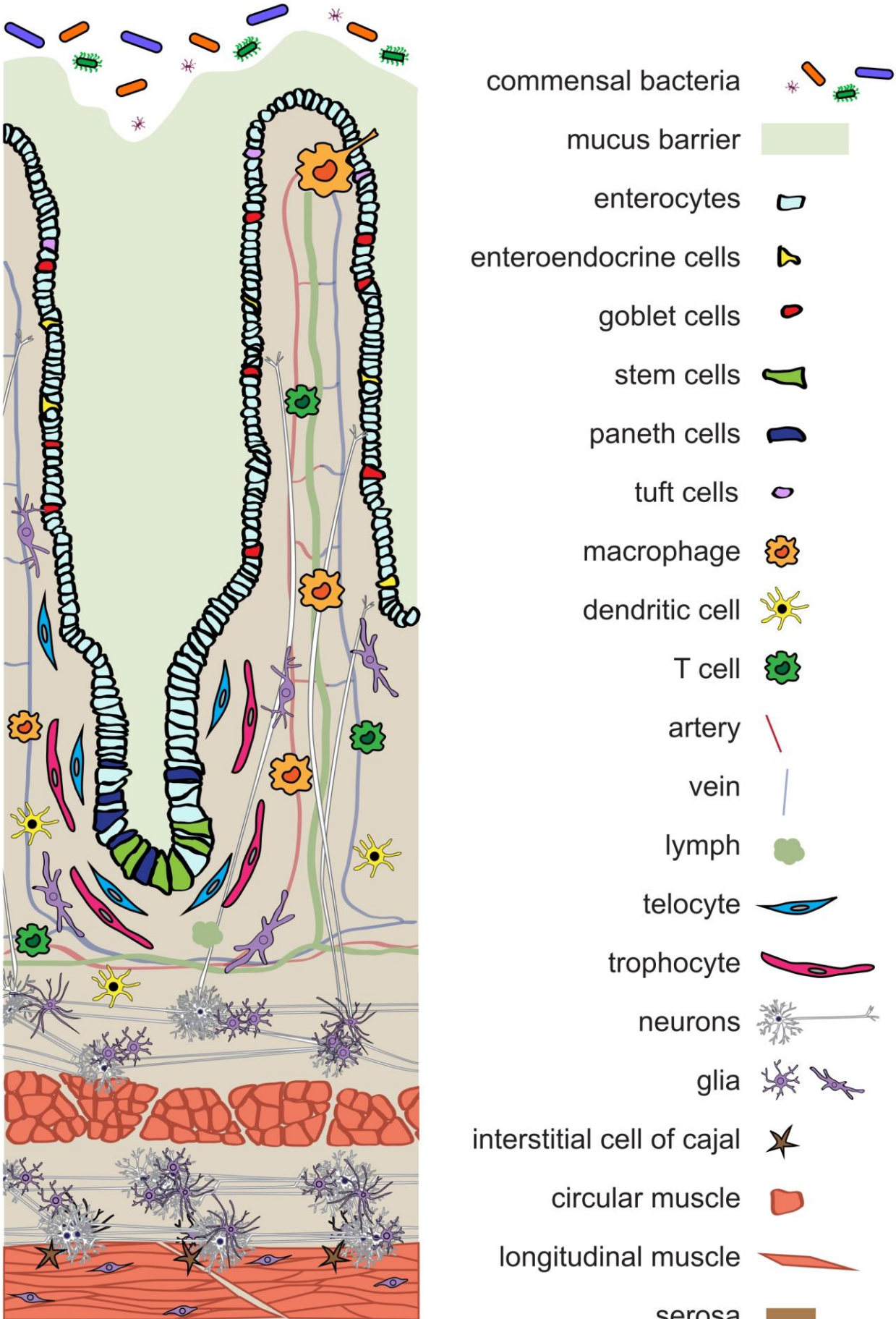

Fig. 2. The intestinal microenvironment is dynamic and composed of diverse cell types. Schematic of intestinal section, with lumen at the top and outer intestinal tissue shown at the bottom. Above the epithelium is a specialized mucus barrier produced by cells in the intestine to protect from bacterial infiltration from species residing in the lumen. The intestine is lined with diverse epithelial cells that are patterned in a specific proximal-distal pattern from crypt to villi tip, with each region promoting different epithelial fates and functions. At the base of the epithelium in the crypts, cells are surrounded by specialized mesenchyme subtypes such as a unique telocyte subtype called trophocytes that provide signals in the intestinal stem cell niche to promote proliferation and maintain cells in an undifferentiated state. Further up the villi, telocytes become specialized to secrete other factors with the opposite effect of trophocytes, albeit these are the same type of cell, they act differently depending on their spatial organization. The intestinal niche is also abundantly stocked with immune cells, including macrophages, T-cells, and dendritic cells, and heavily vascularized. The enteric nervous system (composed of different subtypes of neurons and glia) are found in every spatial compartment of the gut from the muscle layer in the myenteric plexus to the submucosa up into the lamina propria inside the villi. How these cells change throughout each spatial compartment is not yet known.

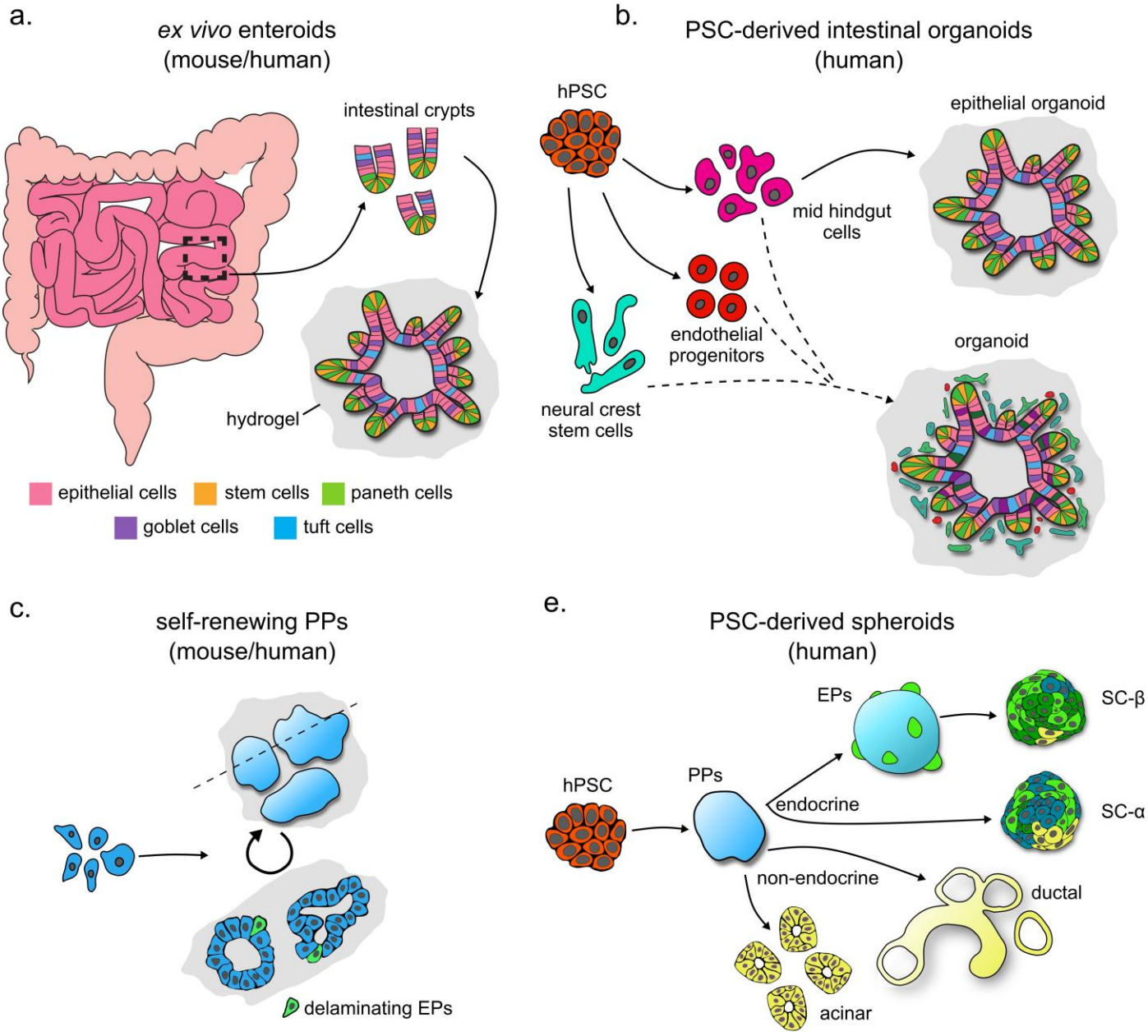

Fig. 3. Pancreatic 3D *in vitro* systems. a). Intestinal crypts can be isolated from mature human or mouse intestine. When transferred to a hydrogel matrix, these crypts grow into epithelial intestinal organoids ("enteroids"), which contain multiple epithelial cell types and form crypt and villi-like structures that recapitulate in vivo tissue organization. **b).** Human intestinal organoids can also be formed by differentiating hPSCs into mid hindgut cells that form spheroids in culture, and then transferring these spheroids into a hydrogel matrix for continued differentiation and maturation into epithelial organoids. When additional hPSC-derived microenvironmental cell precursors are added to these cultures, they integrate into the epithelial organoid and increase its epithelial cell specialization and functional relevance. **c)-f).** Multiple systems of pancreatic development have been established *in vitro* to study organ development, for cell therapy and disease modeling. **c).** Both mouse E10.5 pancreas-derived and human hPSC-derived self-renewing PPs (circulating arrow) can be long-term cultured as hydrogel-embedded spheroids (gray). These spheroids can be used for either further differentiation towards specific pancreatic lineages or for research on development, including studies in specification and delamination of EPs (dashed line denotes optical section, shown below). **d).** Pancreatic progenitors (PPs) can be obtained from dissociated E10.5 mouse pancreas. These cells can be reaggregated and propagated in hydrogel matrices (gray field), where they form a lobular structure composed mostly of non-endocrine cells. Addition of organotypic mesenchyme from the same developmental stage, and without hydrogel matrix, strongly enriches endocrine cell formation. **e).** Human PSCs are applied to derive relatively pure pancreatic cell populations, with most focus on β-cells for therapeutic applications. The differentiation process is based on mimicking subsequent *in vivo* pancreas development steps, including PP and EP stages. The process is governed by addition of soluble factors targeting also specific niche-derived signaling pathways. Yet, the niche is not present during the *in vitro* differentiation and thus these systems lack complex interactions present in the pancreatic microenvironment. Thus, the topological organization of these spheroids lacks *in vivo* complexity limiting their use in research on some aspects of pancreas development. **f).** We propose that human PSC-derived organoids assembled from PSC-derived epithelial and microenvironment cells will develop structures more faithfully reflecting *in vivo* complexity of pancreas during secondary transition, which would facilitate research on pancreas development in human context. With such systems pancreatic-microenvironment interactions can be spotted, and possibly these systems would allow creation of islet-like structures *in vitro*. All included lineages could be separately derived from hPSCs and assembled together at specific differentiation stages. Dashed lines denote steps that have not been reported yet.

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Author contributions:

All authors contributed to the writing of the manuscript. W.J.Sz and K.L. created the figures.

Data availability statement:

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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