
DIFFUSE HORMONAL SYSTEMS

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ABSTRACT

Neuroendocrine (NE) cells are rare epithelial cells that, in addition to having an endocrine function, express markers and peptides otherwise associated with neurons and the central nervous system. NE cells can be found as either single cells or small clusters of cells dispersed throughout the parenchymal surface epithelium of different tissues, including the lung, the intestine, and the pancreas. The observation that NE cells, which are dispersed throughout the body in different tissue sites, are often innervated and secrete bioactive compounds that can act both locally and systemically, led to the idea of the diffuse neuroendocrine system, a diffuse hormonal system composed of NE cells. NE cells perform important endocrine functions. Furthermore, NE cells are implicated in several human diseases. In particular, a group of rare tumors that presumably arise from NE cells, neuroendocrine neoplasms (NENs), have sparked a great deal of interest in NE cell biology. NENs can arise in almost all tissues but they show the highest incidence in the lung and the gastroenteropancreatic (GEP) system. In this chapter, we will outline what is currently known about NE cell differentiation and function, focusing specifically on

NE cells of the lung, pulmonary neuroendocrine cells (PNECs), and the most prominent NE cells of the GEP system: enteroendocrine cells (EECs) of the small intestine and stomach and pancreatic endocrine cells. We will also discuss the potential role of these specific NE cells in the context of tissue injury. Finally, we will provide a brief overview of NEN biology with regards to NENs arising in the lung and GEP system.

INTRODUCTION

Neuroendocrine (NE) cells are epithelial cells that, in addition to having an endocrine function, express markers and peptides otherwise associated with neurons and the central nervous system (1,2). NE cells can be defined by the presence of dense secretory granules and the expression of general NE markers including chromogranin A and synaptophysin. The first identified NE cells were the enterochromaffin (EC) cells of the small intestine, whose distinctive shape and histological properties piqued the interest of scientists during the late nineteenth and early twentieth centuries. In particular, dense secretory granules within NE cells hint to their endocrine function. These secretory granules also make NE cells reactive to chromium and silver, which makes them

easy to identify using histological staining methods. Throughout their history, NE cells in the intestine have been referred to in the literature with a variety of names recalling their distinct reactions to histological stains: clear cells (did not pick up conventional stains), chromaffin cells (reacted to chromium salts), argentaffin cells (affinity for silver stains), and Kulchitsky cells (in honor of one of the scientists who studied them) (3,4).

Following the description of NE cells in the intestine, histological studies revealed the presence of NE cells not only throughout the intestinal mucosa, but also in other epithelial tissues (1–8). NE cells can be found as either single cells or small, often innervated clusters of cells dispersed throughout the parenchymal surface epithelium of different tissues, including the small intestine, the lung, and the urogenital tract. As is the case for the cells of the pancreatic islets of Langerhans, the C cells of the thyroid, and adrenal medullary cells, NE cells can also form distinct clusters of cells within endocrine glands.

In 1938, drawing on his histological studies of NE cells in the pancreas and intestine, Friederich Feyrter proposed that NE cells comprise a diffuse neuroendocrine system. Nearly 30 years later, Anthony Pearse refined this idea of the diffuse neuroendocrine system by showing that NE cells, much like neurons, are able to metabolize amines and produce polypeptide hormones. Thus, the concept of

a diffuse neuroendocrine system that functions as a diffuse hormonal system and is composed of cells dispersed throughout the body that, through the secretion of bioactive compounds, communicate in a coordinated fashion with their surroundings and with the nervous system solidified (8,9). Famously, Pearse also suggested that NE cells are all derived from the neural crest. This hypothesis, however, was later disproved by several elegant lineage tracing experiments. With the exception of the cells of the adrenal medulla, the extra-adrenal paraganglia, and C cells of thyroid, which are indeed derived from the neural crest, different types of NE cells are derived from the epithelial progenitors of their respective tissue sites (3).

While the specific function of pancreatic islet cells, the cells of the adrenal medulla, and C cells of the thyroid, for example, have been well-established both in terms of their contribution to specific organ function and the maintenance of homeostasis; the specific functions of other NE cells are less well defined. Furthermore, the list of polypeptide hormones and neuropeptides secreted by NE cells is continuously being updated and further refined. Studies that elucidate the developmental differentiation trajectories of NE cells from different tissues have expanded the list of common NE marker genes so that it no longer includes only hormones and neuropeptides but also lineage specific transcription factors. A summary of common NE markers is provided in Table 1.

Table 1. Common NE Markers		
NE Marker	Function	Associated NE cell
ASCL1	Transcription factor	PNECs, some gastric EECs
NEUROD1	Transcription factor	GEP EECs
INSM1	Transcription factor	All NE cells
Chromogranin A (CHGA)*	Secretory protein	All NE cells
Synaptophysin (SYP)*	Synaptic vesicle glycoprotein	All NE cells
NCAM1 (a.k.a. CD56)*	Cell adhesion molecule	All NE cells
UCHL1 (a.k.a. PGP9.5)*	Deubiquitinating enzyme	All NE cells
Neuron Specific Enolase (NSE)*	Metabolic enzyme	All NE cells

* Indicates markers used in clinical diagnosis

Much of the interest in NE cell biology has been initiated by observations that have been made regarding their behavior in disease. In particular, a group of rare tumors that presumably arise from NE cells, neuroendocrine neoplasms (NENs), have sparked a great deal of interest in NE cell biology inasmuch as this might relate to the genesis and peculiar clinical behavior of some of these tumors. NENs have been observed in almost all tissues, and consist of well-differentiated neuroendocrine tumors (NETs), tumors that proliferate and progress slowly, and neuroendocrine carcinomas (NECs), poorly differentiated tumors that have a poor prognosis (10). While NENs were initially classified according to the embryological origin of their tissue site of incidence (i.e., foregut, midgut, or hindgut), they are now referred to according to their specific tissue site of origin and, in the case of tumors that elicit hormonal

syndromes, according to the primary hormone they secrete.

NENs show the highest incidence in the lung and the gastroenteropancreatic (GEP) system (Figure 1). For this reason, as outlined above, while there are many different kinds of NE cells arising in many different tissue sites, for the purposes of this chapter, we will focus on the NE cells of the lung, pulmonary neuroendocrine cells (PNECs), and the most prominent NE cells of the GEP system: enteroendocrine cells (EECs) of the small intestine and stomach, and pancreatic islet cells or pancreatic endocrine cells (pECs). These cells are key components of the body's diffuse hormonal system (Table 2).

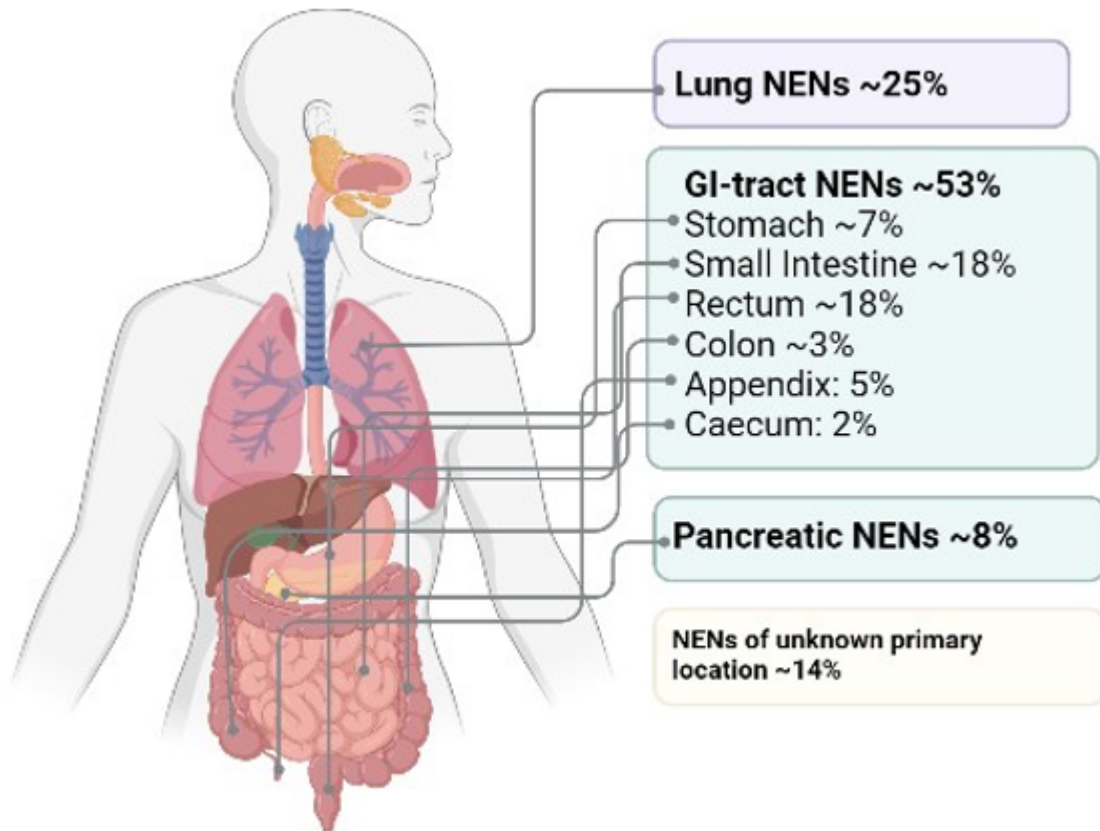


Figure 1. Occurrence of the most common types of neuroendocrine neoplasms. The occurrence of the main types of neuroendocrine tumors presented as the percentage of all NENs (315,441). GI-NENs represent the largest subgroup of NENs, followed by lung and pancreatic NENs. Subtypes not listed in this figure include NENs from the thyroid, kidney, adrenal gland, breast, prostate and skin.

Table 2. Types of NE cells and Their Tissue Site

NE cell type	Tissue site	Predominant hormone
PNEC	lung	numerous (see text)
Alpha cells	Pancreas	Glucagon
Beta cells	Pancreas	Insulin
Gamma/ PP cells	Pancreas	PPY
Delta cells	Pancreas	Somatostatin
Epsilon cells	Pancreas (during development)	Ghrelin
G cells	Stomach, duodenum, pancreas	Gastrin
D cells	Stomach, small intestine	Somatostatin
Enterochromaffin cells (EC) cells	Stomach, small intestine, colon	Serotonin (5-HT)
EC-like (ECL) cells	Stomach	Histamine
X and X/A cells	Stomach (mainly), small intestine	Ghrelin
L-I-N lineage cells	Small intestine (distal), Colon (L cells)	GLP-1, GLP-2, PYY, serotonin (L-cells), CCK, serotonin (I cells), NTS (N cells)
K cells	Small intestine (proximal)	GIP, serotonin

PULMONARY NEUROENDOCRINE CELLS (PNECS)

Pulmonary neuroendocrine cells (PNECs), the neuroendocrine cells of the lung, are prominent constituents of the diffuse neuroendocrine system. Although PNECs account for only 0.5% of the lung epithelium, their distinct morphological and histological staining properties, which are shared with the majority of NE cells, led to their prominence in early histological studies of the lung (11). First described in 1949 as “helle zellen” (‘bright cells’ in German), it was later appreciated that PNECs contain

secretory granules and that they produce and secrete bioactive compounds, including serotonin (12–14). PNECs were thus added to the growing list of NE cells that make up the diffuse neuroendocrine system.

PNECs are found both as single cells within the lung parenchyma and as small clusters, called neuroendocrine bodies (NEBs) (Figure 2A) (15). In mammalian lung tissue, NEBs are distinctly located next to airway bifurcation points of the branching airways. Solitary PNECs, on the other hand, show a more divergent pattern of localization between

species. Whereas in mice, solitary PNECs are mostly found in the trachea, in human lung tissue, they can

be found throughout the airways. A distinct feature of PNECs is their direct innervation (16).

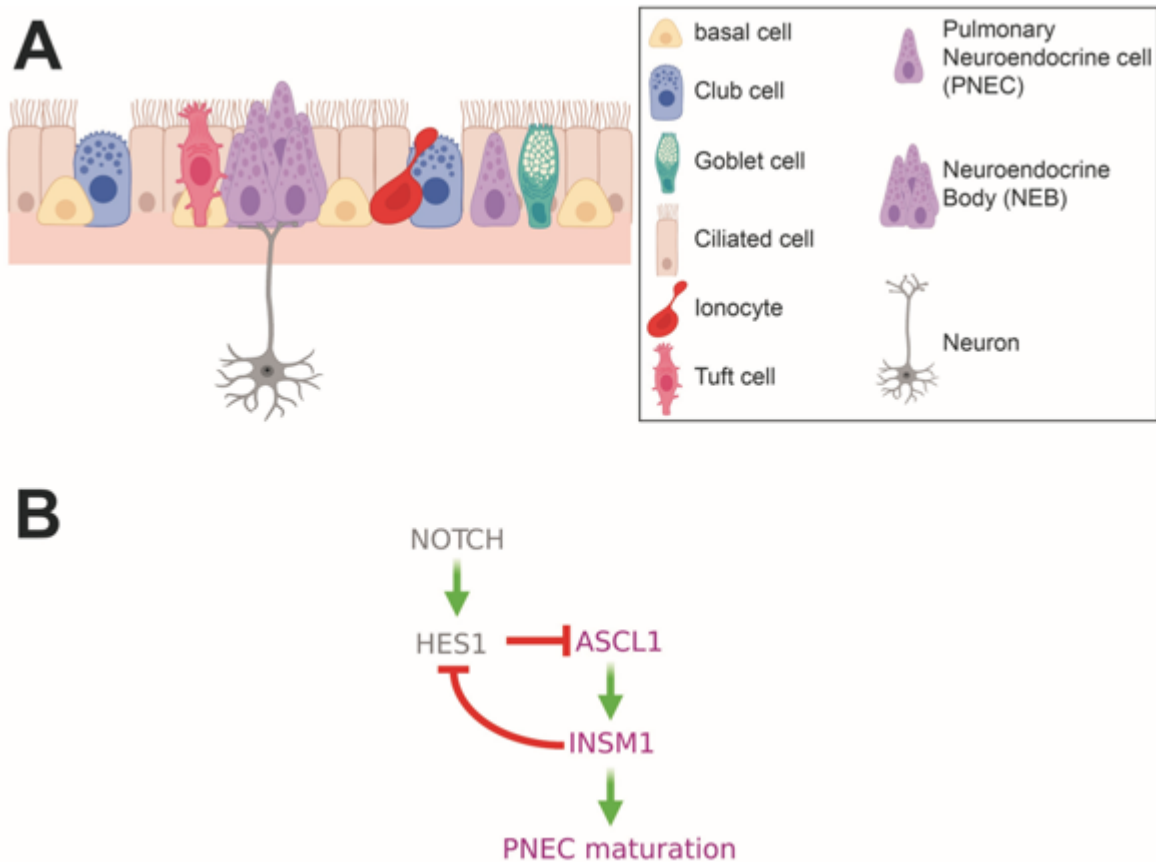


Figure 2. Pulmonary Neuroendocrine Cells (PNECs) in the upper airways. (A) Schematic depicting epithelial cell types found in the upper airways. Solitary PNECs and innervated Neuroendocrine Bodies (NEBs) are shown. (B) Diagram of signaling and transcription factor interactions that regulate PNEC differentiation.

Although the precise function of PNECs is not well-defined, their preservation across evolution -- similar cell types are found in fish gills and in all air-breathing vertebrates -- suggest an important physiological function (17–19). Furthermore, the position at airway bifurcation points of PNECs, their contact with the airway lumen, and innervation suggest a role in airway sensing. The bioactive compounds, hormones, and neuropeptides that PNECs secrete are known to affect oxygen sensing, pulmonary blood flow and bronchial

tonus, and lung immune responses. The bioactive compounds secreted by PNECs include serotonin, calcitonin, calcitonin gene-related peptide (CGRP), gastrin-releasing peptide (GRP), chromogranin A, gamma aminobutyric acid (GABA), and synaptophysin, clearly suggesting an endocrine function for these cells (20–23). A summary of the hormones and neuropeptides expressed by PNECs and other NE cells discussed in this text is included in Table 3.

Table 3. NE Cell Expressed Hormones		
Hormone	Associated NE cell types	Reported function in described NE cell types
Calcitonin	PNEC	pulmonary blood flow and bronchial tonus
Calcitonin gene-related peptide (CGRP)	PNEC	vasoregulation, bronchoprotection, immune cell recruitment
Gastrin releasing peptide (GRP)	PNEC	Regulates mucus and cytokine production upon inflammation or inflammation-associated processes
Gamma aminobutyric acid (GABA)	PNEC and pECs	Regulates mucus and cytokine production upon inflammation or inflammation-associated processes
Somatostatin (SST)	PNECs and GEP NE cells (D cells)	Inhibits secretion of insulin, glucagon, PYY, serotonin, and gastrin
Vasoactive intestinal polypeptide (VIP)	PNECs and GEP EECs	Acts as a neurotransmitter, an immune regulator, a vasodilator, and a secretagogue
Histamine	PNECs and Gastric EECs (ECL cells)	Stimulates gastric acid secretion
Ghrelin (GHRL)	PNECs, Gastric EECs (X/A cell), and pECs (Epsilon cells)	Stimulates appetite, promotes gluconeogenesis and increased gastric acid secretion
Cholecystokinin (CCK)	PNECs and GI EECs (I cells)	Stimulates gallbladder contraction, pancreatic enzyme secretion, gut motility, satiety, and inhibits acid secretion
Serotonin (5-HT)	PNECs and GI EECs (EC cells)	Regulates vasodilation and smooth muscle contraction
Secretin (SCT)	GI EECs	Stimulates the release of bicarbonate and water to neutralize gastric acid

Gastric inhibitory peptide (GIP)*	GI EECs (K cells)	Inhibits insulin secretion and to a lesser extent gastric acid secretion
Substance P (neuropeptide)	GI EECs (EC cells)	Regulates intestinal motility and mucosal permeability
Glucagon-like peptide 1 (GLP-1)	Intestinal EECs (L cells)	Stimulates insulin secretion and inhibits glucagon secretion
Neurotensin (NTS)	Intestinal EECs (N cells)	Inhibits gastric acid secretion
Glucagon (GCG)	pECs (alpha cells)	Increases blood glucose levels by stimulating glucose production and inhibiting glycogen storage by the liver
Insulin (INS)	pECs (beta cells)	Stimulates uptake of blood glucose by other tissues
Gastrin	pECs (G cells)	Stimulates gastric acid secretion
Pancreatic polypeptide (PPY)	pECs (PP/gamma cells)	Inhibits glucagon and somatostatin

* Gastric inhibitory peptide (GIP) is also known as glucose-dependent insulintropic polypeptide

PNECs in Development: Specification, Differentiation, and NEB Formation

PNECs are the first differentiated cell type to appear in the developing lung (15). The timing of PNEC differentiation in human lungs has not been comprehensively delineated but several studies have reported the appearance of PNECs in human fetal airways at 8 - 9 weeks of gestation (24). In mice, the fetal epithelial progenitor cells that give rise to mature PNECs appear for the first time at around embryonic day (E) 12.5 (25). These cells are defined by expression of the basic helix loop helix (bHLH) lineage transcription factor, ASCL1, which is required for their specification. Mice that carry null alleles of *Ascl1* do not have PNECs (26,27).

PNEC lineage specification by expression of *Ascl1* is followed by two key events that appear to happen in

parallel: the formation of NEBs and the maturation of early *Ascl1* expressing cells to fully differentiated PNECs. Using a lineage trace of *Ascl1*-positive cells in mouse embryonic lung, Kuo and Krasnow observed the first signs of PNEC clustering at around E13.5 to E14, followed by the appearance of bonafide NEBs, which contained mature PNECs at around E15.5 to E16 (25). In human fetal lungs PNEC clustering was first observed at about 9 to 10 weeks of gestation (28).

Although one might imagine that the formation of NEBs is likely achieved through proliferation of nascent PNECs, a different mechanism has been shown to be at play. Sparse lineage tracing of early fetal *Ascl1*-positive PNECs in mice using a multi-color lineage reporter showed that NEBs contained either different colored cells or a single labeled cell and multiple unlabeled cells, arguing that the PNECs in NEBs are not clonal (25). Live cell imaging of fetal

mouse lung tissue showed that NEBs are formed through the migration and subsequent aggregation of PNECs at airway branchpoints (25,29). This process appears to be regulated by Slit-Roundabout (ROBO) signaling, a pathway more classically associated with axonal guidance (30). PNECs express the ROBO receptor and the lungs of mice where the Slit-ROBO pathway has been disrupted by mutation of either the Slit ligands or the ROBO receptor itself, have fewer NEBs and more solitary PNECs than the lungs of wildtype mice (21).

Concomitant with NEB formation, the transcriptional events initiated by expression of *Ascl1* culminate in the emergence of fully differentiated, functional PNECs. In particular, expression of *Ascl1* in lung progenitors induces expression of the zinc finger transcription factor, INSM1. PNECs in mice carrying mutant alleles of *Insm1* fail to express the mature PNEC markers, CGRP and UCHL1 (ubiquitin C-terminal hydrolase L1, a.k.a. PGP9.5). While the INSM1 targets that mediate the PNEC maturation process have not been delineated, INSM1 has been shown to directly repress the bHLH transcription factor and Notch target gene, *Hes1* (Figure 2B) (31).

HES1 and other Notch signaling components play crucial roles in repressing the differentiation and specification of PNECs and, thereby, in mediating the NE versus non-NE cell fate choice. The lungs of mice in which *Hes1* has been conditionally deleted in early lung progenitors have more ASCL1-positive cells and, in particular, fewer solitary PNECs, showing instead more and larger NEBs compared to the lungs of wildtype mice (29). The increased size of NEBs in *Hes1*-deficient lungs suggests a mechanism of cell fate specification through Notch-mediated lateral inhibition whereby Notch is activated in PNEC neighboring cells through binding of Notch receptors to the Notch ligands expressed on the surface of PNECs themselves. The activated Notch signaling in

these PNEC neighboring cells induces expression of *Hes1*, which in turn represses the PNEC fate.

PNECs express the Notch ligands Dll1, Dll4, Jag1, and Jag2 shortly after their specification during lung development and the cells surrounding PNECs in NEBs express Notch receptors (32). Genetic loss of either all three Notch receptors or *Dll1* and *Dll4* Notch ligands in the developing mouse lung leads to a dramatic increase in the number and size of NEBs, phenocopying conditional deletion of *Hes1* (32,33). In cultures of human airway cells derived from induced pluripotent stem cells, inhibition of Notch signaling leads to increased numbers of PNECs (34,35).

As we will discuss in other parts of this text, two other bHLH transcription factors, NEUROG3 and NEUROD1 have been shown to play central roles in the differentiation of EECs and pancreatic islet cells. In contrast, as of yet, there is little evidence that these transcription factors are decisive for PNEC specification or differentiation. To date, Neurog3 expression has not been described in PNECs. While Neurod1 expression has been observed in some PNECs in both fetal and adult mouse lung, a limited number of studies have investigated its specific role in the PNEC lineage (27,36). *Neurod1*-null mouse lungs from mice less than 2 weeks old showed decreased numbers of solitary PNECs and more NEBs compared to lungs from age-matched wild type mice. However, this difference normalized once mutant mice were 6 weeks old (36). As will be discussed later on in this text, NEUROD1 is a marker of a subtype of the high-grade lung NEN, small cell lung cancer (SCLC), suggesting it might also play a role in normal PNEC biology.

Reactive PNEC Proliferations: PNECs in the Response to Lung Injury

Multiple studies have shown that PNEC numbers and NEB size are altered in several human disease conditions. Increased numbers of PNECs have been observed in the lungs of patients with COPD, asthma, cystic fibrosis, and some forms of pneumonia. Other pathological conditions associated with increased PNECs include sudden infant death syndrome (SIDS), bronchopulmonary dysplasia (BPD), and congenital diaphragmatic hernias (CDH) (15). Studies in animal models and PNEC culture systems provide experimental evidence that PNECs respond to environmental stimuli by both proliferation and/or secretion of bioactive compounds.

The early observation that murine PNECs proliferate in response to a common form of experimental lung injury, naphthalene administration, and that this proliferation precedes epithelial lung regeneration, led to the hypothesis that PNECs are multipotent stem cells that aid in lung regeneration (37). Indeed, lineage tracing studies of PNECs following naphthalene induced lung injury showed that a rare subpopulation of PNECs, termed NE^{stem}, can function as stem cells in this context (38). In response to naphthalene, NE^{stem} cells proliferate and sometimes migrate to the site of injury where they dedifferentiate (lose NE identity) and take on other lung cell fates. The process of dedifferentiation and reprogramming was shown to be mediated by Notch signaling, recalling the role of this pathway in PNEC fate specification, and by EZH2 (38,39). Nonetheless, NE^{stem} cells are not solely responsible for regenerating the lung after injury, as they were shown to contribute only to a small portion of the regenerated surface epithelium (38). Furthermore, genetic ablation of PNECs does not abrogate lung regeneration following naphthalene injury (23,39).

In thinking about PNECs as components of a diffuse hormonal system, two questions arise from the studies of PNECs in the context of naphthalene lung injury.

The first is, how do PNECs detect injury? Club cells, which express cytochrome P450 2F2 (Cyp2f2), metabolize naphthalene to a toxic metabolite and the accumulation of this toxic metabolite leads to cell death specifically in these cells (40). Given that PNECs proliferate at time points shortly after peak Club cell injury, it is likely that they are responding to the Club cell injury and not to the naphthalene itself. Consistent with this hypothesis, selective ablation of Club cells using genetic ablation techniques also resulted in PNEC proliferation (41). Nonetheless, injury associated signals that are specific to PNECs and their responses have not been identified.

The second question that arises is, besides functioning as stem cells, do PNECs have an endocrine or paracrine/autocrine function in the context of lung injury? Although this question has not been explored in the naphthalene injury model, evidence from other model systems and from human diseases suggest that PNECs respond to some forms of lung injury or disease through the secretion of bioactive compounds. Cigarette smoke, a common culprit of lung injury, provides a good example. Bronchoalveolar lavage (BAL) fluid from smokers has increased levels of peptides secreted by PNECs, implicating these cells in the cellular response to cigarette smoke in humans (42). The PNEC-secreted bioactive compounds associated with this response include GABA and GRP, and both of these molecules have been implicated in inflammation and inflammation-associated processes (42,43). It is likely that PNEC proliferation is also involved in the response to cigarette smoke and its primary component, nicotine. Increased PNECs have been observed in rats exposed to cigarette smoke pre- and postnatally and in rhesus monkeys exposed to nicotine prenatally (44,45).

Pointing to a critical role for PNECs in oxygen sensing in the lung, hypoxia and hypoxia-mimicking genetic

modifications have been shown to result in higher numbers of PNECs in mice, rats, rabbits, and guinea pigs (22,46–49). Shortly after showing that the distinct dense cored vesicles of PNECs carried serotonin, Lauweryns and Cokelaere went on to show that this serotonin was secreted by PNECs upon exposure to hypoxia (14,49). This finding was further refined and shown to be dependent on changes in intracellular Ca^{2+} concentrations using cultured rabbit and hamster lung slices (50,51). Serotonin release by PNECs is likely a physiologically relevant functional response to hypoxia as serotonin has been shown to induce vasoconstriction of pulmonary arteries (52).

Increased expression in PNECs of the neuropeptide, CGRP, has also been linked to hypoxia (46,53). CGRP has been implicated in promoting alveolar regeneration and in mediating immune cell responses in the lung (21,54). Importantly, results from a study by Shivaraju et al. linked the expression of CGRP by PNECs to the hypoxia-induced regenerative response of epithelial cells in the trachea. When the authors ablated PNECs and exposed mice to hypoxia, they observed a defective regenerative response that could be rescued by intranasal administration of CGRP (46).

PNECs appear to also respond to hyperoxia-induced lung injury. Patients with BPD, a chronic lung disease associated with oxygen supplementation of premature infants, have increased numbers of GRP-expressing PNECs (55). In a baboon model of BPD, some of the lung defects associated with the disease could be prevented by treatment of the animals with a GRP blocking antibody, demonstrating that GRP is directly linked to the disease phenotype (56). GRP also plays a role in the lung's response to viral pneumonia and in the fibrotic response to radiation therapy (57,58).

There is clear evidence for a close interplay between PNECs and immune cells. In particular, the effector molecules secreted by PNECs can recruit and activate

different populations of immune cells. In one of the first studies to show this, researchers developed a mouse model of CDH, a birth defect that results in pulmonary hypoplasia and pulmonary hypertension (21). CDH is associated with both a heightened immune response and increased numbers of PNECs (59). To study CDH, since point mutations in SLIT and ROBO genes are associated with the disease, Branchfield et al. generated mice with lung-specific deletions of the roundabout receptors, *Robo1* and *Robo2*. When *Robo1* and *Robo2* were deleted in the entire lung epithelium the authors noted elevated immune cell infiltration in the lung, thus mimicking one of the features of CDH. When *Robo1* and *Robo2* were deleted only in PNECs, the mice displayed the same phenotype, directly linking the defect to PNECs. Interestingly, ROBO1- and ROBO2-deficient PNECs have increased levels of CGRP and knockout of the gene encoding CGRP partly reversed the immune and lung phenotypes of mice deficient for ROBO1 and ROBO2 in the lung epithelium.

A potential immune regulatory role for PNECs is also suggested by the observation that PNEC numbers are elevated in patients with asthma and that more chromogranin A-positive PNECs are seen in guinea pigs after allergen sensitization and challenge (60). Mice deficient of PNECs due to deletion of *Ascl1* in the lung epithelium, show a dampened response to allergen challenge -- reduced goblet cell hyperplasia and reduced immune cell infiltration -- and this was tied directly to reduced levels of PNEC-derived GABA and CGRP, respectively (61).

Diseases of Primary PNEC Hyperplasia: NEHI and DIPNECH

Up to now we have highlighted instances of increased PNEC number or NEB size that appear to be consequent to or at least associated with some forms of acute or underlying lung injury. These examples are

instructive in that they point to a role for PNECs and the molecules they secrete in mediating the response to external stimuli and injury in the lung. Save for lung NENs, which will be discussed in further detail later in this text, there are two notable clinical instances of primary -- as opposed to reactive -- PNEC proliferation that have no known etiology and are not associated with common pathogenic triggers: neuroendocrine cell hyperplasia of infancy (NEHI) and diffuse idiopathic neuroendocrine hyperplasia (DIPNECH).

NEHI is a rare pediatric lung disease consisting histologically of hyperplastic GRP-positive and serotonin-positive PNECs in the distal lung epithelium of otherwise normal lung tissue. Symptoms are usually first noted between 6 to 8 months of life and include tachypnea, retractions, crackles and hypoxemia (62). In some cases, patients with NEHI show an inconspicuous, patchy pattern of inflammation or fibrosis, generally assumed to be a consequence of the increased PNEC numbers rather than its cause (63). Interestingly, despite increased PNEC numbers in the lungs of patients with NEHI, from a study on a small patient cohort (5 patients), it appeared that PNECs were not actively proliferating in the lungs of these patients as no Ki67 and GRP double positivity was observed (63). Unfortunately, treatment for patients with NEHI are currently limited to supportive oxygen supplementation and, in some cases, additional nutritional support. The majority of NEHI patients show gradual improvement of symptoms and the disease is not associated with mortality. Nonetheless, recent reports show that some patients experience abnormal lung function persisting into adulthood (62,64,65). While the etiology of this disease remains unknown, there are indications of a genetic basis for the disease. One study identified four families with multiple members diagnosed with NEHI and showing an autosomal dominant pattern of inheritance (66). Another study identified a heterozygous mutation in the *NKX2.1* gene in

members of a family with a history of childhood lung disease consistent with NEHI (67).

DIPNECH is a rare syndrome with adult onset consisting histologically of increased PNECs in the small bronchi and bronchioles and confined to the basement membrane, appearing as scattered PNECs, small nodules, or a linear proliferation of PNECs (68). These features are often seen in concomitance with what are referred to as tumorlets, PNEC proliferations that extend beyond the basement membrane but are less than 5 mm in diameter (69). Other histological features include fibrosis, chronic inflammatory cell infiltrate, and constrictive obliterative bronchiolitis. The majority of patients with DIPNECH are women and the disease is not associated with smoking or other lung diseases. Patients diagnosed with DIPNECH often present with symptoms including cough, exertional dyspnea and an obstructive or mixed obstructive/restrictive defect on pulmonary function test. A small number of patients with DIPNECH are diagnosed due to incidental findings (69).

DIPNECH was first recognized and formally defined in 1992 by Aguayo et al., who described the symptoms and histological features of 6 DIPNECH patients (42). While DIPNECH is considered a disease of primary rather than reactive PNEC proliferation, cases associated with parathyroid gland hyperplasia, acromegaly and pituitary adenoma, multiple endocrine neoplasia type I syndrome, and pulmonary adenocarcinoma have been reported (70–72). The World Health Organization (WHO) classifies DIPNECH as a preinvasive, possibly preneoplastic condition (73). Most patients with DIPNECH have multiple PNEC nodules, sometimes including both tumorlets and frank low grade lung NET (carcinoid) tumors (70). In contrast to NEHI where Ki67-positive PNECs were not observed, the PNEC proliferations in DIPNECH patients contain some Ki67-positive cells (63,74). While patients with DIPNECH most often

follow a clinical course showing stability or slowly progressing functional decline, a small subset of patients have rapidly progressive disease including progression to respiratory failure or metastatic carcinoid tumors (70,75). To date, there is no standard of care for DIPNECH and the most effective treatment strategy for patients with DIPNECH are somatostatin analogues (SSAs), which have shown effectiveness in improving symptoms of cough and dyspnea in some patients (76–78). Considering that it has been well-established that SSAs inhibit the secretion of bioactive compounds from gastrointestinal NETs, the effectiveness of SSAs in treating cough and dyspnea in patients with DIPNECH suggests these symptoms are caused by the secretion of bioactive compounds by DIPNECH PNECs (79).

Lung NENs

Lung NENs account for 20-25% of all lung cancers and for 25-30% of NENs from all tissue sites (80,81). As is the case for NENs in general, lung NENs comprise both low grade, well-differentiated NETs and high grade, poorly differentiated NECs. Lung NENs can thus be subdivided into the high-grade carcinomas, small cell lung cancer (SCLC) and large cell neuroendocrine carcinomas (LCNEC), and the low-grade tumors, atypical carcinoids (AC), classified as intermediate grade, and typical carcinoids (TC), classified as low grade.

SMALL CELL LUNG CANCER (SCLC)

The most common lung NEN, SCLC, accounts for 79% of all lung NENs and 30% of all lung cancers and is also the best studied among lung NENs (82). Consistent with its classification as NEC, SCLC is a highly aggressive tumor with a high rate of metastasis and a 10-year survival rate of only 1-2% (83). Among patients with SCLC, 97% have a history of smoking (18). While rare, patients with SCLC sometimes

experience paraneoplastic endocrine syndromes, most commonly a syndrome of inappropriate antidiuretic hormone (SIADH) and ectopic Cushing's syndrome (84). Studies of SCLC biology have been aided by a collection of tumor-derived cell lines, several patient-derived xenograft (PDX) models, and genetically engineered mouse models (GEMMs) of the disease (85–87). These preclinical model systems have allowed scientists to address questions in two key areas: the cell of origin of SCLC and molecular signatures predictive of therapeutic vulnerabilities.

Genetically, SCLC is a relatively homogeneous disease -- *RB1* and *TP53* are both almost universally lost in patient tumors (88). Conditional simultaneous genetic deletion of *Rb1* and *p53* in the mouse epithelium results in tumors that recapitulate many of the key features of the human disease at both the histological and molecular levels (87,89–91). Targeting the deletion of *Rb1* and *p53* to specific epithelial cell types in the lung provided definitive evidence that *Cgrp*-expressing PNECs are a cell of origin for tumors in this model (23,92). However, PNECs are not the only epithelial lung cell type that can be a cell of origin for mouse SCLC. A separate study showed that an, as of yet, unidentified CGRP-negative cell that is also negative for the canonical markers of two other common lung epithelial cell types gives rise to mouse SCLC lesions that are molecularly distinct from those initiated in CGRP-positive PNECs (93).

Genomic analysis of human and mouse SCLC primary tumors and cell lines has revealed commonly mutated genes and pathways, most notably loss of *PTEN*, *NOTCH*, and histone modification genes, and amplification of *MYC* family oncogenes (88,90,91). Several studies focused on metastasis in mouse SCLC have highlighted the role of *NFIB* in driving progression of some of these tumors, and data from human patients with SCLC support the clinical

relevance of these findings (94–96). These studies, in combination with preclinical testing in mouse models and cell lines have suggested some degree of patient stratification. In particular, high expression of MYC is associated with tumor sensitivity to Aurora Kinase inhibitors (97).

The standard treatment regimen for patients with SCLC is a combination therapy of a platinum agent combined with etoposide (82). Despite a clinical response to these therapies in the majority of patients, almost all patients will then experience tumor recurrence (83). The analysis of the transcriptomes of both mouse and human SCLC tumors and cell lines has identified 4 molecular subtypes of SCLC, defined by their expression (or lack of expression) of 3 lineage-specific transcription factors: ASCL1 high, NEUROD1 high, POU2F3 high, and a fourth subtype that has low expression of NE transcription factors and has been proposed to be defined by expression of YAP1. A more recent study suggests a classification in which this fourth subtype is defined by expression of immune checkpoint genes and human leukocyte antigens (98,99). It has been hypothesized that the cell of origin for the POU2F3 high subtype of SCLC might be the pulmonary tuft cell, another chemosensory cell type in the lung distinct from PNECs (100,101).

Importantly, several studies using preclinical models of SCLC suggest that these molecular classifications can be used to stratify patients according to potential therapeutic vulnerabilities (102). Adding complexity to this schema, single cell RNAseq studies of mouse SCLC and of xenografts derived from circulating tumor cells from SCLC patients suggest that different molecular subtypes might represent different stages of progression where tumors begin in an ASCL1-high state and progress towards a non-NE state and that individual tumors might comprise cells belonging to different subtypes (101,103). Several studies have also shown other forms of intratumor heterogeneity in

SCLC that have implications for patient therapy (104–106). Other new therapies suggested for SCLC include tricyclic antidepressants, therapies that target specific metabolic vulnerabilities, and therapies targeting the GNAS/ PKA/PP2A signaling axis (107–109).

LARGE CELL NEUROENDOCRINE CARCINOMA (LCNEC)

Pulmonary LCNEC is less common than SCLC, accounting for 16% of all lung NENs (82). Like SCLC, pulmonary LCNECs are highly metastatic and are associated with smoking history and with an overall 5-year survival rate ranging from 15% to 25% (110). In contrast to SCLC, however, there are relatively few preclinical models for LCNEC and we know much less about the basic biology of this disease. This might partly explain why guidelines for treating patients with LCNEC are rather rudimentary (82,111).

Pathohistological analysis of tumors from GEMMs of SCLC found that a portion of the mouse tumors in these models had a histological pattern consistent with LCNEC. While these LCNEC tumors only accounted for 10% of the tumors from the GEMM in which only *Rb1* and *p53* were conditionally deleted in the lung epithelium, they were much more prominent in the GEMM in which *Rb1*, *p53*, and *Pten* were conditionally deleted specifically in CGRP-expressing PNECs (87). A different GEMM, based on loss of *Rb1* and expression of mutant *p53* alleles, also develops both SCLC and LCNEC mouse tumors (112). These GEMMs have, thus far, not been used to explore the biology specifically of LCNEC and doing so might present some technical challenges. Recently, the first GEMM specifically for LCNEC was reported. In this model, *Rb1*, *p53*, *Pten*, and *Rbl1* were simultaneously deleted in the mouse lung epithelium, resulting in a tumor spectrum consisting primarily of LCNEC and low-grade NETs (113).

The majority of insights into LCNEC have been provided by molecular analysis of primary patient tumor samples. The most comprehensive analysis, consisting of whole exome sequencing of 60 LCNEC tumors and RNA-sequencing expression analysis of 69 LCNEC tumors, highlighted the existence of two major molecular subtypes of LCNEC (114). Type I LCNECs had a higher rate of alterations in *TP53* and *STK11/KEAP1* and an NE expression profile defined by high expression of *ASCL1* and *DLL3* and low expression of *NOTCH*. Type II LCNECs had frequent mutations in *RB1* and *TP53*, therefore resembling SCLC at the genomic level. The expression profile of type II LCNECs, however, was distinct from SCLC and instead was defined as NE low, with low expression of *ASCL1* and *DLL3* but high expression of *NOTCH*.

The description of these two molecular subtypes for LCNEC highlights a clinical conundrum relating to the treatment of patients with LCNEC: should they be treated with SCLC chemotherapy regimens or with chemotherapy regimens for non-NE non-small cell lung cancer (NSCLC) (111)? The report of an SCLC-like subtype of LCNEC (type I) and a NSCLC-like subtype of LCNEC (type II), might suggest a way to stratify patients for different chemotherapy regimens. In line with this idea, a retrospective analysis of LCNEC cases found that patients whose tumors either had wildtype *RB1* or showed expression of *RB1* protein had a better outcome when treated with a NSCLC chemotherapy regimen as opposed to a SCLC chemotherapy regimen (115).

Other therapies beyond traditional chemotherapy regimens are also being explored for patients with LCNEC. One example that relates to patient stratification according to LCNEC subtype, which was defined in part by differential patterns of *DLL3* expression, involves therapeutic strategies that use *DLL3* to target tumor cells. Given that *DLL3* is also

expressed by some SCLC tumors, this also represents a potential therapeutic opportunity in SCLC. Although a *DLL3*-antibody conjugated to the DNA-damaging pyrrolobenzodiazepine dimer toxin did not provide a survival benefit in 2 phase 3 clinical trials, other *DLL3* targeting approaches are being developed (116). In addition, several studies have uncovered potentially targetable molecular alterations in some LCNEC tumors, including activating *EGFR* mutations, *FGFR1* amplifications, activating *BRAF* mutations, *ALK* rearrangements, and mutations affecting *BDNF/TrkB* signaling (114,117–119). Given that the majority of these targetable mutations have been identified in LCNEC tumors with wildtype *RB1*, the question remains as to how best to treat patients with *RB1* mutant LCNEC (120).

LUNG NETs: TYPICAL AND ATYPICAL CARCINOIDS

Lung NETs comprise low grade typical carcinoids (TC) and intermediate grade atypical carcinoids (AC), accounting for 5% and 0.5% of all lung NENs, respectively (82). TC and AC tend to present in younger patients than LCNEC and SCLC, and the majority of patients are women and non-smokers (121). Although the majority of lung NETs are sporadic and non-functional, a small percentage of patients with Lung NETs, 5% of TC and < 2% of AC, present with paraneoplastic syndromes including those associated with adrenocorticotrophic hormone (ACTH), growth hormone releasing hormone (GHRH), histamine, and serotonin (122,123). Some of these are more commonly associated with metastatic lesions of TC or AC (123). Approximately 5% of lung NETs are associated with the familial cancer syndrome caused by germline mutations in multiple endocrine neoplasia gene type I (*MEN1*). Interestingly, 5% to 10% of lung NETs are also associated with tumor multiplicity, a feature which might suggest a connection with either an unappreciated familial predisposition syndrome or

with premalignant conditions such as DIPNECH (80,121).

The overall 10-year survival rates for stage I TC and AC are comparable ranging from 98% to 91%, respectively. In the case of stage IV tumors, 10-year survival for TC patients is 49% but is only 18% for patients with AC (124). A distinguishing feature of TC and AC is their relatively slow growth. Indeed, the pathological criteria for diagnosing carcinoids are the number of mitoses per mm² and the presence of absence of necrosis: < 2 mitoses per mm² and no necrosis for a diagnosis of TC, 2 to 10 mitoses per mm² and demonstration of necrosis for a diagnosis of AC (82). Morphologically, carcinoids typically contain small cells that show nested, rosette, and trabecular growth patterns with peripheral palisading (125).

Complete surgical resection is the most common treatment for patients with TC and AC, and for the majority of these patients' surgery is associated with a favorable survival prognosis. Unfortunately, however, a fraction of carcinoid tumors metastasizes, and tumor recurrence (even after apparent curative resection) has been reported in 1 to 6% and 14 to 29% of patients with TC and AC, respectively. Due to a highly variable time to relapse for patients with recurrence (0.2 to 12 years), the recommended follow-up period is 15 years (121,126–129). The reported incidence for lymph node metastasis for TC and AC is variable with rates ranging from 12% to 17% for patients with TC and from 35% to 64% for patients with atypical carcinoid (80,130). The incidence of distant metastases for both TC and AC is 3% and 21%, respectively (129,131).

The incidence of tumor recurrence and metastasis calls attention to a clinical need for systemic treatment options for patients with TC and AC tumors that are unresectable, as well as for the need for effective adjuvant therapy options that can be offered to patients after surgery. Unfortunately, standard

chemotherapy and radiotherapy regimens have proven to be mostly ineffective in this patient population (80). The only treatment option shown to improve progression-free survival in patients with advanced and progressive TC and AC is the mTOR inhibitor everolimus (132).

Other therapeutic options for patients with TC and AC that are not currently considered standard of care due to limited clinical trials, include somatostatin (SST) analogues and peptide receptor radionuclide therapy (PRRT), and temozolomide with or without capecitabine. Given that pulmonary carcinoids can express SST receptors, patients with these tumors can be potentially considered for palliative treatment with unlabeled or radiolabeled SST analogues.

The lack of clarity regarding standard of care for patients with unresectable, metastatic, or recurrent TC and AC, points to an unmet need for not only new and effective systemic treatment strategies for these patients, but also for clear patient stratification criteria for predicting the probability of a response of a given patient tumor to specific therapeutic options. Furthermore, given the broad range of tumor malignancy for TC and AC, biomarkers that can predict the potential for tumor progression and metastasis or recurrence are also needed. Efforts to address these clinical needs have been hindered by both difficulties in performing molecular characterization of these tumors and by a dearth of preclinical models representative of the disease. Only a handful of cell lines exist for TC and AC and only one GEMM for TC and AC has been reported (133,134).

In contrast to SCLC and LCNEC, pulmonary carcinoids have a low tumor mutational burden, have very few recurrent or characteristic mutations, and rarely contain "driver" mutations in known oncogenes. The most commonly mutated gene in pulmonary carcinoids is *MEN1*, and up to 5 to 13% of patients

with germline mutations of this gene are diagnosed with pulmonary carcinoids (135–137). The most commonly mutated class of genes in pulmonary carcinoids are chromatin remodeling genes, a category that includes *MEN1*, *PSIP1*, and *ARID1A*. Though prevalent in SCLC and LCNEC, mutations in *RB1* and *TP53* are rare in pulmonary carcinoids (135,136). Recurrent copy number alterations have also been identified in pulmonary carcinoids, including in genes that would imply targetable therapeutic vulnerabilities such as, *EGFR*, *MET*, *PDGFRB*, *AKT1/PKB*, *PIK3CA*, *FRAP1*, *RICTOR*, *KRAS*, and *SRC* (136,138).

Transcriptional and methylation analysis of primary pulmonary carcinoids has also revealed distinct subclasses of these tumors. Using multi-omics factor analysis (MOFA), Alcala et al. identified 3 molecular clusters, termed A1, A2, and B (139). While most of the tumors in clusters A1 and A2 were TC, tumors in cluster B were primarily classified as ACs. Tumors in cluster B had high expression of *ANGPTL3* and *ERBB4*, were enriched for mutations in *MEN1*, and were associated with a worse overall survival. Consistent with a worse prognosis for patients with tumors in cluster B, tumors in this cluster also showed universal downregulation of the orthopedia homeobox protein gene, *OTP*, whose expression has previously been associated with an improved prognosis in patients with pulmonary carcinoids (126). A separate study performed a similar multi-omic analysis of an independent set of pulmonary carcinoids and also identified 3 molecular subtypes that they termed LC1, LC2, and LC3 (140). The concordance between the molecular subtypes identified in these two studies was shown through integration of the two datasets, further validating the use of these molecular classifications for pulmonary carcinoids (141).

The molecular analysis of pulmonary carcinoids has provided evidence that supports the idea that a

fraction of lung NENs may actually fall into a category that lies between G2 ACs and G3 NECs in terms of malignancy. While such a category is recognized in GEP-NENs and is termed well-differentiated G3 NET, its existence has only recently been suggested for lung NENs (142). The study by Alcala et al. identified a subgroup of ACs, termed “supra-carcinoids,” that showed the morphologic characteristics of pulmonary carcinoids, but whose transcriptional profile was closer to that of LCNECs (139). In their analysis of the transcriptional profiles of a series of LCNECs and ACs, Simbolo et al. identified 3 molecular clusters, C1, enriched for LCNECs, C3 enriched for ACs, and C2, which was mixed in terms of number of ACs and LCNECs and which showed intermediate molecular features (143). Finally, an earlier study by Rektman et al. had identified 2 examples of what they referred to as “carcinoid-like” LCNEC tumors -- tumors that showed a clear carcinoid-like morphology and a molecular profile consistent with ACs (low tumor mutational burden and mutation in *MEN1*) but that had been classified as LCNEC due to a high proliferation rate (118).

The supra-carcinoids in the Alcala et al. study showed a higher expression of *MKI67* than other carcinoids in the series, supporting an idea that has been purported in the literature concerning a potential role for percent Ki67 positivity in identifying pulmonary carcinoids more likely to be associated with a poor prognosis (144–146). Typically, ACs show a Ki67 positivity rate of less than 20%. However, some tumors diagnosed as AC show rates between 20 and 50% (147,148). Likewise, as indicated by the “carcinoid-like” LCNEC tumors in the Rektman et al. study, some tumors that would otherwise be considered ACs, are diagnosed instead as LCNEC due to having a high proliferation rate (118,149). Furthermore, the comparison of proliferation rates between matched primary stage IV pulmonary carcinoids and metastases indicated an increased proliferation rate in 35% of the metastases,

suggesting increased proliferation as a feature of progression (148). This idea is further supported by the observation that Ki67 positivity was heterogeneous in the analyzed tumors with some regions of the tumors showing hot-spots of increased proliferation compared to the rest of the tumor. Beyond Ki67 positivity, a list of defining features of supra-carcinoids or borderline pulmonary carcinoids/neuroendocrine carcinomas has yet to be established.

ENDOCRINE CELLS IN THE GASTROENTEROPANCREATIC TRACT

Together, the organs connected throughout the mouth to the anus are known as the gastrointestinal (GI) tract, and when the pancreas is included, these organs are collectively referred to as the gastroenteropancreatic (GEP) tract. Throughout the GEP tract endocrine cells can be found as either solitary cells, as is the case in the GI tract, or as innervated clusters, as is the case in the pancreas.

Throughout the gastrointestinal (GI) tract the solitary endocrine cells, which have a slender, elongated shape, are referred to as enteroendocrine cells (EECs). This classification helps to distinguish them from endocrine cells of other organs e.g., lung and pancreas. Despite representing only 1% of the gut epithelial cells, the large size of the intestinal epithelium makes it the body's largest endocrine organ (150,151).

Compared to the slender EECs of the GI tract, the pancreatic endocrine cells, dispersed as clusters (known as islets of Langerhans) throughout the organ, have a more pyramidal or round-oval shaped appearance. An adult human has millions of islets, which collectively correspond to roughly 2% of the pancreatic epithelium (152,153). These islets are highly vascularized, a feature that enables pancreatic hormones to travel via the bloodstream to reach their target organs. In fact, the pancreatic hormones act both locally and systemically, eliciting responses throughout the body, consequently affecting the overall metabolic state of the organism. The sections below will provide an overview of the endocrine cells of the GEP tract as components of both the diffuse neuroendocrine system and the body's diffuse hormonal systems.

GASTRIC ENDOCRINE CELLS

The first major organ of the GI tract is the stomach, which, in humans, can be divided into 4 functionally distinct compartments. From proximal to distal; the cardia is the connective region between the esophagus and the stomach, the fundus stores undigested food and gases, the corpus is the largest compartment and performs the digestive action of the stomach, and, finally, the pylorus regulates gastric emptying (Figure 3A) (154,155).

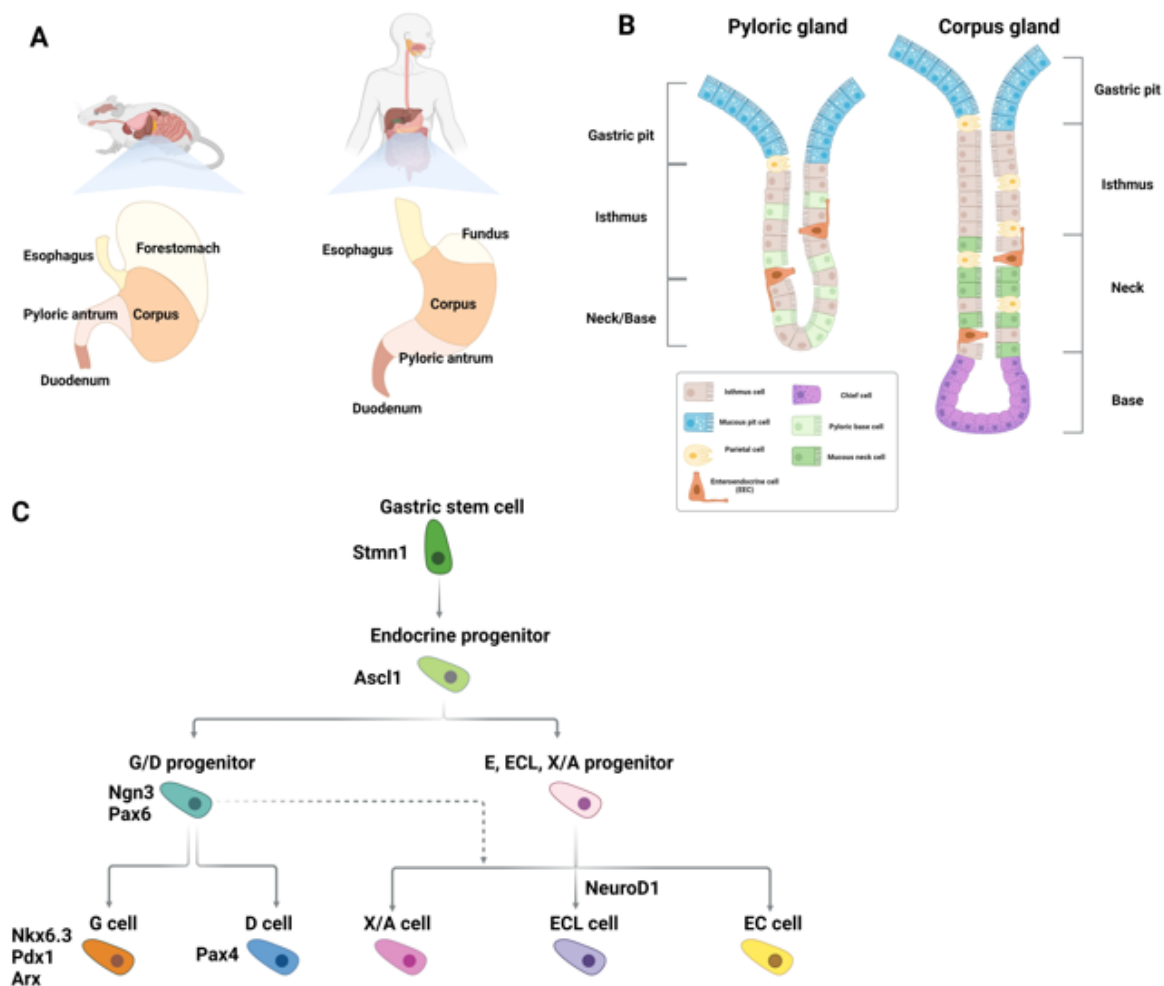


Figure 3. Gastric enteroendocrine cells. (A) Schematic showing the anatomical differences between the murine and human stomach (B) Schematic depicting epithelial cell types found in the gastric pylorus and corpus glands. Solitary gastric enteroendocrine cells are shown in orange. (C) Diagram of signaling and transcription factor interactions that regulate gastric enteroendocrine cell differentiation.

Histologically, the stomach comprises tubular-shaped mucosal invaginations containing a pit region of primarily surface mucous cells, and a gland region. The latter is further subdivided into the isthmus, neck, and base (Figure 3B). The primary differentiated cell types of the stomach are: mucus-producing pit cells, chief cells, which secrete digestive enzymes, acid-secreting parietal cells, gastric tuft cells, whose function is ill defined, and gastric EECs. These cells are continuously formed throughout life, albeit at different rates, by progenitor cells located in the

isthmus of the gland region (156,157). With the exception of the cardia, which primarily contains pit cells and scattered parietal cells, gastric EECs can be found in all of the compartments of the stomach. In the corpus and fundus EECs are located in the lower third of the glands. EECs in the pylorus are located in the neck region (158).

Gastric EECs are divided into the following 5 main subtypes defined by their predominantly expressed

hormone: G cells (gastrin), D cells (somatostatin), enterochromaffin (EC) (serotonin), EC-like (ECL) cells (histamine), and X/A cells (ghrelin) (Figure 3C) (159). While some gastric EEC subtypes overlap with those found in the intestine, comparison of duodenal EECs and gastric EECs by single cell RNA sequencing (scRNA-seq) suggested a distinct gastric EEC expression profile. These differences are most likely reflective of the tissue specific microenvironment of these EECs, the stimuli they are exposed to, and their different functions (160).

As will be outlined in subsequent parts of this text, differentiation of intestinal EECs requires expression of the master bHLH transcription factor, *NEUROG3*. While *NEUROG3* is important for gastric EECs, its role appears to be EEC subtype specific. Most gastric EECs are also dependent on *ASCL1*, the same transcription factor that initiates PNEC specification. Studies from two different groups, which independently generated *Neurog3* null mice showed that while these mice lacked D-cells and G-cells and had decreased numbers of EC cells, ECL and X/A cells were unaffected (161,162). *Ascl1* null mice displayed a similar but not identical phenotype, showing lack of D, G and EC cells and severely decreased numbers of X/A cells (163). ECL cells were not examined in *Ascl1* null mice. Thus, some gastric EECs, such as D- and G- cells are dependent on both *NEUROG3* and *ASCL1*, while others, such as X/A cells, are dependent on *ASCL1* but not *NEUROG3*. EC cells appear to be entirely dependent on *ASCL1*, and only partially dependent on *NEUROG3* (163). *Ascl1* is not expressed in intestinal EECs from the mouse. Nonetheless, scRNA-seq of human intestinal EECs identified expression of *ASCL1*, suggesting a role for this transcription factor not only in gastric EECs but also in human intestinal EECs (164).

The EEC hormone most clearly associated with gastric function is gastrin, secreted by G-cells located

in the pyloric compartment of the stomach. These cells are also found in the duodenum, but their function has been best studied in the stomach (160,164). Gastrin secreted into the bloodstream by G-cells binds to its receptor expressed by ECL cells in the corpus thereby stimulating them to secrete histamine, which, in turn, stimulates neighboring parietal cells to secrete gastric acid. Given that parietal cells themselves also express the gastrin receptor, gastrin release by G-cells can also directly stimulate parietal cells to secrete gastric acid (165–167). The other hormones produced by the gastric EECs are also secreted by other gastroenteropancreatic endocrine cells (GEP-ECs) and will be discussed in later sections.

INTESTINAL AND COLONIC ENDOCRINE CELLS

Architecture and Cell Types of the Intestine

The gut can be divided into the small and large intestine (also known as the colon). The two primary functions performed by the small intestinal epithelium are 1) to form a barrier against the continuous chemical and mechanical insults induced by the undigested food, microorganisms, and toxins present in the intestinal lumen, and 2) to absorb nutrients from ingested food (150). This latter process occurs with an exceptionally high efficiency made possible by the large surface area generated by the intestinal epithelium's folded structure. Protrusions known as villi contain differentiated non-mitotic cells, while invaginations known as crypts contain proliferative, self-renewing stem cells and their epithelial niche cells, Paneth cells. The colon lacks villi and its primary function is water absorption and movement of the stool. The continuous damage experienced by the intestinal epithelium necessitates a high cellular turnover to maintain organ function. The intestinal stem cells, marked by the expression of leucine-rich G-protein-coupled receptor 5 (LGR5), continuously

replace lost cells via rapid division which regenerates the epithelium within 4-5 days (150).

The differentiated cells that originate from the LGR5+ stem cells can be divided into two main functional categories, absorptive (enterocytes and microfold cells) and secretory (EECs, goblet, Paneth, and tuft cells) (Figure 4A). Intestinal EECs secrete hormones in response to stimuli such as nutrients from digested

food and metabolites produced by the gut microbiota (168,169). The stimuli that EECs respond to can be both mechanical and chemical, and the hormones they produce are secreted both locally and into the bloodstream, thereby allowing them to act not just locally but also systemically. Gut hormones regulate important functions such as digestion, nutrient absorption, appetite, and gastric as well as gut motility (170).

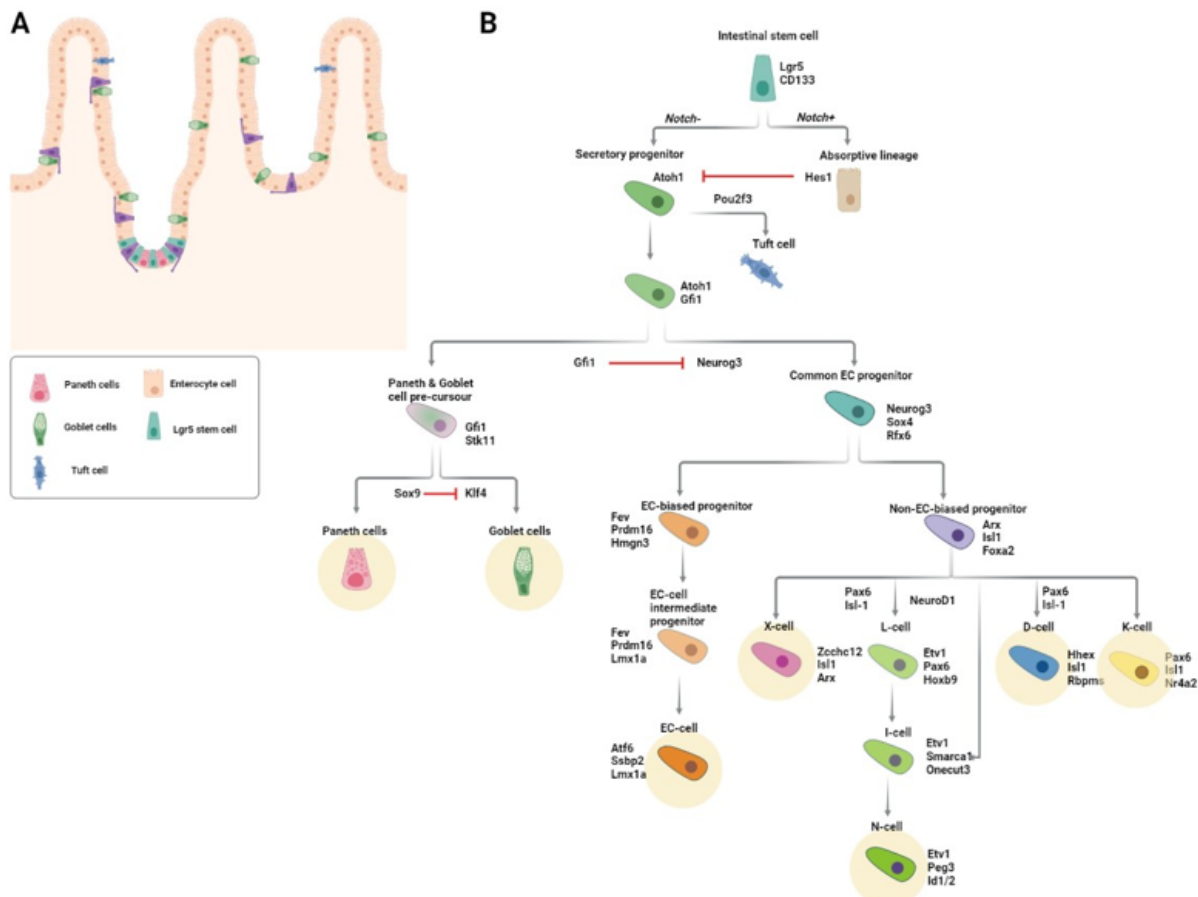


Figure 4. Intestinal enteroendocrine lineage specification. (A) Schematic of the intestinal epithelium. Solitary enteroendocrine cells (EECs) are depicted in purple. (B) Diagram of signaling and transcription factor interactions that regulate intestinal enteroendocrine cell differentiation. Differentiated EEC subtypes are highlighted with yellow circles.

Factors Regulating Commitment to the Secretory Lineage

The rapid division of LGR5-positive stem cells gives rise to progenitor cells, the majority of which differentiate as they migrate upwards along the crypt-villus axis. The following section describes the differentiation of intestinal EECs and highlights some of the essential regulatory factors and signaling pathways that direct this process.

NOTCH SIGNALING

The first step in becoming an EEC is commitment to the secretory lineage, a process that is initiated by the transcription factor, ATOH1 (Protein atonal homolog 1). At the crypt bottom, active Notch signaling prevents the differentiation of stem and progenitor cells. Cell-cell contact between Notch ligand (DII1 and DII4) expressing Paneth cells and stem/progenitor cells results in the expression of the Notch target gene, *HES1*, which in turn represses the secretory cell fate by repressing ATOH1 (171,172). Hence, commitment to the secretory lineage requires inactivation of Notch signaling.

Inactivation of Notch signaling is concomitant with loss of contact with Paneth cells. Due to the high ratio of progenitor to Paneth cells in the crypt, not all progenitors can simultaneously be in touch with a Paneth cell and this results in stochastic loss of Paneth cell-stem cell contact. Additionally, as new progenitors are continuously generated by the stem cells in the crypt, older progenitors are pushed upward along the crypt-villus axis, causing them to lose contact with the Notch ligand-presenting Paneth cells. The resultant loss of active Notch in these cells enables expression of ATOH1 and commitment to the secretory lineage (173,174). Mice lacking *Atoh1* do not have any

secretory cells (173). In contrast, mice with null alleles of *Hes1* have excessive numbers of secretory cells.

Factors Regulating Commitment to the EEC Lineage

Transient expression of *NEUROG3* commits Atoh1-expressing secretory progenitors to the EEC fate. Whereas *Neurog3* knockout mice completely lack EECs, overexpression of *Neurog3* leads to increased numbers of EECs and decreased numbers of goblet cells (162,175,176). Homozygous *NEUROG3* mutations have been identified in children with generalized malabsorption and reduced numbers of intestinal EECs (177). Downstream targets of *NEUROG3* include other transcription factors important for EEC differentiation such as *NEUROD1*, *PAX4/6*, *NKX2.2*, *INSM1*, and *PDX1* (162,178–181). *NEUROG3* is also implicated in cell cycle control: *Neurog3* expression in mouse pancreatic endocrine progenitors leads to upregulation of the cell cycle inhibitor, *Cdkn1a*, and consequent cell cycle exit (182). Consistent with the idea that cell cycle exit biases secretory progenitors to the EEC lineage, inhibition of either epidermal growth factor receptor (EGFR) or mitogen activated protein kinase (MAPK) signaling induced quiescence of intestinal stem cells in organoid culture. When this quiescence was reversed by reactivation of these pathways, the resulting organoids had an increased proportion of EECs (183).

Specification of the Different EEC Lineages

While most progenitors generated in the crypt immediately begin migrating upwards, those primed to become EECs remain in the crypt for anywhere between 48h and 60h (184). During this time, these cells become committed to one of several divergent

differentiation trajectories, each of which results in a different EEC subtype. Prior to leaving the crypt, EEC-committed progenitors have already started to express and secrete their lineage-defining hormones. The time required to produce a specific hormone varies between the different EEC lineages and this may explain why some EEC-committed progenitors remain in the crypt longer than others (184).

Altogether, intestinal EECs produce more than 20 different hormones. The earliest classification of EECs was based on immunostainings and consisted of the following 8 EEC lineages as defined by the main hormone they were found to express: enterochromaffin (EC) cells that secrete serotonin (5-hydroxytryptamine, 5-HT), I cells that secrete cholecystokinin (CCK), K cells that secrete gastric inhibitory peptide (GIP), L cells that secrete glucagon-like peptide 1 (GLP-1), X cells that secrete ghrelin (GHRL), S cells that secrete secretin (SCT), D cells that secrete somatostatin (SST), and N cells that secrete neurotensin (NTS) (185).

At the time that this classification was first proposed, it was believed that EECs belonging to a given subtype predominantly expressed the hormone that defined that EEC subtype. Thus, for example, it was believed that EC cells only predominantly expressed serotonin, and L cells only predominantly expressed GLP-1. However, new techniques for performing hormone co-stainings using multiple antibodies, fluorescent hormone reporter mice, and transcriptome-based sequencing of EECs have all led to the observation that some EECs express multiple subtype-defining hormones (186–190). The question thus arose, did these multihormonal EECs represent previously unidentified and distinct EEC subtypes? Or were they simply cells caught in a transition state along the EEC subtype differentiation trajectory? The latter would suggest that EECs are capable of hormonal plasticity and are therefore capable of transitioning from

expression of one lineage defining hormone to another.

RESOLVING LINEAGE IDENTITY AND HORMONE SWITCHING

One of the first lines of evidence that EECs might undergo hormone switching was the observation that, while serotonin-producing EC cells were rapidly labeled after a single pulse of radioactive thymidine, secretin-producing S cells were labeled much later and only after multiple injections of the isotope (191,192). Thus, it was concluded that serotonin-expressing cells but not secretin-expressing cells had the ability to self-renew and that secretin cells did not differentiate before reaching the villus. Based on this data, one could postulate that serotonin-expressing cells might become secretin-expressing cells once they reach the villus.

A lineage relationship between EECs localized in the crypt and EECs localized in the villus was first suggested in 1990 by Roth and Gordon based on an immunohistochemical study in which it was observed that cells expressing substance P (encoded by the *Tac1* gene) but not secretin were found in the crypt, cells expressing both substance P and secretin were found in the bottom of the villus, and cells expressing secretin but not substance P were found exclusively at the top of the villus. The majority of substance P-expressing and secretin-expressing cells co-expressed serotonin. The authors thus concluded that these hormones were sequentially expressed along the crypt villus axis (186). The fact that substance P-expressing cells were labeled by the thymidine analogue, BrdU, faster than secretin-producing cells further supported this idea of sequential expression of substance P and secretin by the same EEC (193). Functional evidence for dynamic hormone-switching in EECs was provided first by cell ablation studies showing that ablation of one EEC subtype led to

decreased numbers of other EEC subtypes (194). Subsequently the generation of a novel mouse Neurog3 reporter allele, Neurog3Chrono, enabled more definitive delineation of dynamic hormone-expression patterns of single EECs (184).

In the Neurog3Chrono mouse, two fluorescent reporter proteins, an unstable mNeonGreen and highly-stable tdTomato, are expressed concurrently with endogenous Neurog3. As a result, the ratio of red to green fluorescence of a given EEC provides real-time information about the age of that cell relative to when it expressed Neurog3 (184). ScRNAseq of EECs from Neurog3Chrono mice provided definitive evidence that many EECs switch the hormone they produce throughout the course of their lives and furthermore suggested a more simplified EEC subtype classification consisting of five mature EEC lineages. One of these five lineages was the one proposed by Roth and Gordon of substance P expressing cells that give rise to serotonin-expressing cells and then to secretin-expressing cells. This lineage was also confirmed independently by lineage tracing of Tac1-expressing cells in the mouse intestinal epithelium (195).

Two key observations from the Neurog3Chrono study substantiated a simplified EEC lineage classification. First, all EEC lineages except for SST-expressing D-cells began to express secretin upon entering the villus, thus rendering the S-cell lineage obsolete. Second, L-, I- and N- cells were shown to belong to a single lineage. Prior to this, observations from a mouse model of L-cell ablation had led the Schwartz lab to propose that L- and N- cells were part of the same lineage (194). The Neurog3Chrono study showed that, when located at the bottom of the crypt, L-cells secrete GLP-1 but, upon reaching the upper regions of the crypt, begin to express the I-cell defining hormone, CCK, and, finally, upon reaching the villus region, they begin to express the N-cell defining

hormone, NTS. Thus, the EEC lineages or subtypes could be reduced to the following 5 main lineages: enterochromaffin (EC) cells that secrete Serotonin, K cells that secrete GIP, X cells that secrete GHRL, D cells that secrete SST, and LIN cells that secrete GLP-1, CCK and NTS (Figure 4B) (184).

ENTEROCHROMAFFIN (EC) CELLS

EC cells are the most prevalent EEC subtype and they can be found in all regions of the intestine (196,197). They are slender, triangularly-shaped cells that can have protrusions extending towards the luminal surface of the intestine (198,199). EC cells are defined by their expression of both serotonin and tryptophan hydroxylase 1 (TPH1), an enzyme that catalyzes the rate limiting step in the biosynthesis of serotonin (189). Serotonin in EC cells is stored in pleomorphic granules and is released in response to chemical and mechanical stimuli. Although commonly associated with brain development and regulation of mood and stress, 95% of the body's serotonin is produced by the intestine where it regulates functions such as motility, fluid secretion, and vasodilation (200–202). The response of EC cells to different types of stimuli is mediated, in part, through their expression of various receptors, including the olfactory receptor, OLF558, which acts as a sensor for microbial metabolites, and the transient receptor potential A1 (TRPA1), a receptor-operated ion channel that detects dietary irritants (203). A subset of EC cells expresses the mechanosensitive channel Piezo2, which converts mechanical forces to secretion of fluids and serotonin (204).

Given the important functions exerted by serotonin, it is not surprising that EC cells are implicated in several GI pathologies (202). Consistent with the observation that most SI-NETs express serotonin, intestinal EC cells are thought to be the cell of origin for these tumors. An early hint that SI-NETs might indeed arise

from EC cells was provided by a immunohistochemical study in which serial sections of an entire ileal SI-NET tumor were stained for several EEC markers, including serotonin. The authors observed aggregates of proliferating EC cells within crypts in close proximity to the tumor, which the authors speculated were indicative of where the tumor had originated (205). A later study made the same observation in a larger cohort of SI-NET samples from eight different patients, supporting the idea that aberrant proliferation of EC cells within the crypt is associated with SI-NET formation (206).

More recently, it has been suggested that the cell of origin of SI-NETs is not necessarily a fully differentiated EC cell, but rather an EC cell that expresses not only the EC cell markers, TPH1 and CHGA, but also markers of reserve stem cells (207). As we will discuss later in this text (see, EECs can act as reserve niche and stem cells), lineage tracing of both progenitor cells and of cells expressing mature EC cell markers in the mouse intestinal epithelium has shown that EC cells can adopt a stem cell fate upon injury (208). This observation suggests a plausible scenario whereby, if an EC cell were to acquire a genomic (or other) aberration capable of driving NET genesis, it could be long lived enough to indeed give rise to a tumor. Consistent with this hypothesis, Sei et al. identified human EC cells co-expressing the EC cell marker TPH1, and markers associated with both canonical and reserve stem cells, within crypt EC cell microtumors in tissue sections from patients with familial SI-NETs (209,210).

WHAT CAUSES HORMONE SWITCHING?

Growth factor gradients change from high WNT, high EGF, high NOTCH, and low BMP, at the crypt bottom, to increasingly high BMP, low WNT, low EGF, and low NOTCH along the villus. Thus, upon leaving the crypt, EEC progenitors are exposed to increasingly different signaling environments. It was therefore attractive to

speculate that these signaling gradients could induce hormone switching in EECs.

Adult stem cell (ASC) derived mouse and human intestinal organoids lack mesenchymal cells and are therefore not exposed to growth factor gradients but instead experience a constant environment determined by the media composition (211). Consequently, the ASC-derived intestinal organoid system provides researchers with a controlled, *in vitro* setting in which the signaling environment that intestinal cells experience can be modulated. Under expansion conditions that mimic the crypt environment and are optimized to promote stem cell maintenance, the EECs in mouse small intestinal organoids display a crypt hormone profile. However, when BMP4 was added to the culture media to mimic the villus environment, the EECs in the organoids expressed Secretin suggesting they had taken on a villus-like profile (195). The molecular mechanisms governing the hormone switching from GLP-1 to CCK to NTS observed in the L-I-N lineage remain to be unraveled but are likely to similarly involve cellular signals that differ along the crypt-villus axis.

Non-Neoplastic EEC Hyperplasia

The previous sections described the formation of intestinal EECs. These cells and the hormones they secrete play a central role in regulating processes that are important for maintaining organismal function and energy homeostasis. It is therefore not surprising that EECs are implicated in a number of human disease conditions. Increased plasma level of EEC hormones and, in some cases, direct evidence of increased numbers of specific intestinal EEC subtypes have been reported in inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), lymphocytic colitis, Celiac disease, *H. pylori* infection, and *Giardia* infection (212–215). A genome-wide association study (GWAS) identified a strong association between a

single nucleotide polymorphism (SNP) in the promoter of the EEC transcription factor, *PHOX2B*, and Crohn's Disease (CD), a form of IBD (216). The mechanism leading to intestinal EEC hyperplasia in these conditions is not clear, though there is some evidence that inflammatory cytokines are involved (213). Consistent with this idea, treatment of mice with IFN γ and TNF α led to increased numbers of Chromogranin A-positive colonic EECs (217). Likewise, IL-13 has been linked to the response of EC cells to enteric parasite infection (218).

It is plausible that the observed changes in EEC numbers in these conditions are not necessarily or not only a result of the disease pathology, but are also mediators of the pathology itself. There is clear evidence for interplay between EECs, the immune system, sensory neurons, and commensal bacteria (168,219). Together with goblet cells, EECs have been shown to secrete the cytokine IL-17C in patients with IBD (220). EEC hormones have been shown to have immunomodulatory functions (219). Some EECs are directly innervated and one study showed that serotonin-expressing intestinal ECs form synapses with nerve fibers through which they can modulate nerve fiber activity (203). EECs express functional toll-like receptors (TLR) and can thereby interact with commensal bacteria by responding to the metabolites they produce (221). Furthermore, as EEC hormones are known to also act systemically, the consequences of disease associated alterations in their abundance or function are not limited to the GI tract. Most notably, GLP-1 and GIP, which amplify glucose stimulated insulin secretion, are less effective in patients with type 2 diabetes (T2D) and GLP-1 receptor agonists are currently used to treat T2D and obesity (185).

The most well-documented examples of non-neoplastic EEC hyperplasia are hyperplasias of gastric and duodenal EECs. These include: ECL cell hyperplasia and G-cell hyperplasia. ECL-cell

hyperplasia is associated with chronic excessive gastrin production, hypergastrinemia, resulting most commonly from achlorhydria due to chronic atrophic gastritis (CAG), gastrin-producing tumors in Zollinger-Ellison syndrome (ZES), and long-term proton-pump inhibitor (PPI) treatment (222). Lineage tracing of gastrin/cholecystokinin-2 receptor (CCK2R)-expressing cells in mice showed that CCK2R-expressing ECL cells in the isthmus but not the base of the stomach proliferated in response to PPI-induced hypergastrinemia (223). ECL cell hyperplasia only rarely progresses to neoplastic gastric ECL NETs. G-cell hyperplasia is most commonly observed as a secondary change associated with CAG in patients with pernicious anemia. In rare cases, it has also been observed in patients with peptic ulcer disease in conjunction with decreased numbers of a different gastric EEC, the SST producing D-cell. G-cell hyperplastic lesions do sometimes progress to G-cell NETs, gastrinomas (222).

EECs CAN ACT AS A RESERVE NICHE AND STEM CELLS

The microenvironment in which LGR5-positive intestinal stem cells reside is known as the stem cell niche and includes Paneth cells. The stem cell niche presents the stem cells with cellular signals that prevent them from differentiating and help preserve their self-renewal capacity. Loss of Paneth cells or severe injury such as irradiation, chemotherapy treatment, or surgery can cause loss of intestinal stem cells. In these situations, several different epithelial cell populations have been shown to act as reserve stem cells (224–228). The first studies indicating that EECs could act as reserve stem cells followed the fate of secretory progenitors (224–228). As this progenitor population also contained progenitor cells fated to become Paneth or goblet cells, the contribution specifically of EECs could not be explicitly determined. A later study that used lineage tracing of a population

of EEC committed secretory progenitors expressing markers of differentiated EECs showed that these cells can act as reserve stem cells, capable of both contributing to intestinal regeneration after irradiation and generating organoids in vitro (228).

Lineage tracing of mouse intestinal cells expressing either NEUROD1 or Tryptophan hydroxylase (TPH1; the rate limiting enzyme in Serotonin biosynthesis and considered an EC cell marker) showed that these cells, similar to the EEC committed secretory progenitors mentioned above, had the ability to contribute to homeostasis and to regeneration following irradiation induced injury (208). Given that the EEC-committed progenitors in the earlier study expressed *Neurod1*, and that a subset of these cells also expressed *Tph1*, it is unclear whether the Tph1 lineage-traced cells with regenerative capacity in this study were indeed mature differentiated EECs or the same multi-potent EEC committed secretory progenitors described before. More recently, it was shown that, upon genetic Paneth cell ablation, mature EECs replaced the ablated Paneth cells, serving as Notch-presenting niche cells for intestinal stem cells (229).

PANCREATIC ENDOCRINE CELLS

Located in the upper left abdomen behind the stomach, the adult pancreas, although being one anatomical entity, originates from two individual buds that arise from either side of the distal foregut endoderm during early embryonic development. As development progresses, these buds fuse to form the final glandular composite organ consisting of two compartments, one exocrine and one endocrine,

which contain functionally and morphologically distinct cell types. Acinar cells secrete digestive enzymes which are transported to the duodenum by mucin-secreting ductal cells, and together these two cell types comprise the exocrine compartment. The endocrine compartment comprises 5 different pancreatic endocrine cell (pEC) types, each of which secretes a specific hormone: alpha (glucagon), beta (insulin), gamma/ PP (pancreatic polypeptide), delta (SST) and epsilon cells (ghrelin). As is the case for EEC-derived hormones, pancreatic hormones act both locally and systemically. Indeed, some pancreatic hormones are transported throughout the body via the bloodstream and instruct other organs to, among other things, release or store glucose from the blood. Hence, the pECs play a key role in nutrient metabolism, digestion, and glucose homeostasis (230).

Development and Differentiation of Pancreatic Endocrine Cells

In contrast to the GI tract where EECs are generated throughout the life of the organism, the pECs are almost exclusively generated during the fetal development of the organ (231). This process is orchestrated by an interplay of regulatory transcription factors. Whereas some of these transcription factors are expressed constitutively, others play a transient yet essential role in mediating pEC lineage commitment and differentiation (see table 4). Murine models have made invaluable contributions to our understanding of pEC generation. In rodents, pancreatic development is divided into 3 transition phases, each outlined below (Figure 5).

Table 4. NE Cell Lineage Transcription Factors

Transcription Factor	Associated NE cell types	Role (specifically in NE cells)
ASCL1	PNECs and gastric EECs	Lineage specification (26,27,163)
NEUROG3	GEP NE cells	Lineage specification (transiently expressed) (161,162,175,176,232,233)
NEUROD1	PNECs*, Intestinal EECs (L-I-N lineage), and pECs (beta cells)	Specification of some NE cell subtypes (not defined in lung, secretin-expressing cells and I-cells in the intestine, beta cells in the pancreas) (27,36,255,407,408)
ATOH1 (a.k.a. MATH1)	Intestinal EEC precursors	Specification of the intestinal secretory cell fate (precedes EEC lineage commitment) (173)
INSM1	PNECs and GEP EECs**	NE cell maturation (31,160,180)
GFI1	PNECs and Intestinal EECs	Maturation of CGRP+ PNECs (409); lineage specification (via inhibition of EEC fate in intestinal secretory progenitors) (410,411)
PDX1	GEP EECs (G cells, some intestinal EECs, and pECs)	Patterning and specification of gastric and duodenal EECs (G cells, SST- and GIP-expressing duodenal EECs) (412,413); Maintenance of beta cell maturity (414,415)
ISL1	GEP EECs (some gastric EECs**, non EC intestinal EECs, and pECs)	Specification of non-EC intestinal EECs (416); maturation and survival of pECs in development (417,418); maintenance of adult beta cell function (419)
RFX3	pECs	pEC differentiation during development (420); differentiation and maintenance of mature beta cells in the adult (421)
RFX6	GEP EECs (gastric EECs**, some intestinal EECs, and pECs)	Differentiation of intestinal EECs (K, L, X, I, and EC cells) (184,422); pEC specification and differentiation in development (423,424); Regulation of insulin expression in beta-cells (425)

PAX4	GEP EECs (some gastric EECs, duodenal EECs, some pECs)	Differentiation of EECs in the duodenum, of EC cells and D cells in the stomach (178), and of beta and delta cells in the pancreas (426,427)
PAX6	GEP EECs (some gastric EECs, some duodenal EECs, some pECs)	Differentiation of D and G cells in the stomach, of K cells in the duodenum (178), and of alpha and epsilon cells in the pancreas (428,429)
NKX2.2	Some intestinal EECs and some pECs	Regulation of cell fate within the intestinal EEC population (promotes EC, L-I-N, and K lineages) (179) and within the pEC population (promotes beta cell, alpha cell, and PP cell lineages) (252)
NKX6.1	Some gastric EECs and some pECs	Differentiation of EC cells in the stomach** (430) and of beta cells in the pancreas (253)
NKX6.2	Some pECs	Differentiation of alpha, beta, and PP cells in the pancreas (431,432)
NKX6.3	Some gastric EECs	Differentiation of G cells in the stomach (433)
MAFA	Some pECs	Maintenance of beta cell identity in the adult pancreas (257,434)
MAFB	Some pECs	Differentiation of alpha and beta cells in the developing pancreas (246,247); Maturation of alpha and beta cells in the adult pancreas (247,256)
ARX	GEP NE cells (some gastric EECs, some intestinal EECs, and some pECs)	Differentiation of G cells in the stomach (435), of L-, I-, and K cells in the intestine (435), and of alpha cells in the developing pancreas (244,245); Maintenance of alpha cell identity in the adult pancreas (436)
FOXA2	Some intestinal EECs and some pECs	Differentiation of L cells and D cells in the intestine (437); Maintenance of adult beta cell maturation in the pancreas (438,439); Maturation of alpha cells in the developing pancreas (440)
HHEX	Some intestinal EECs and some pECs	Differentiation of and maintenance of delta cell identity in the pancreas (262) (implicated in delta cells of the intestine (184,195))

* Role in PNECs no well defined

** Role in gastric EECs implicated by expression pattern

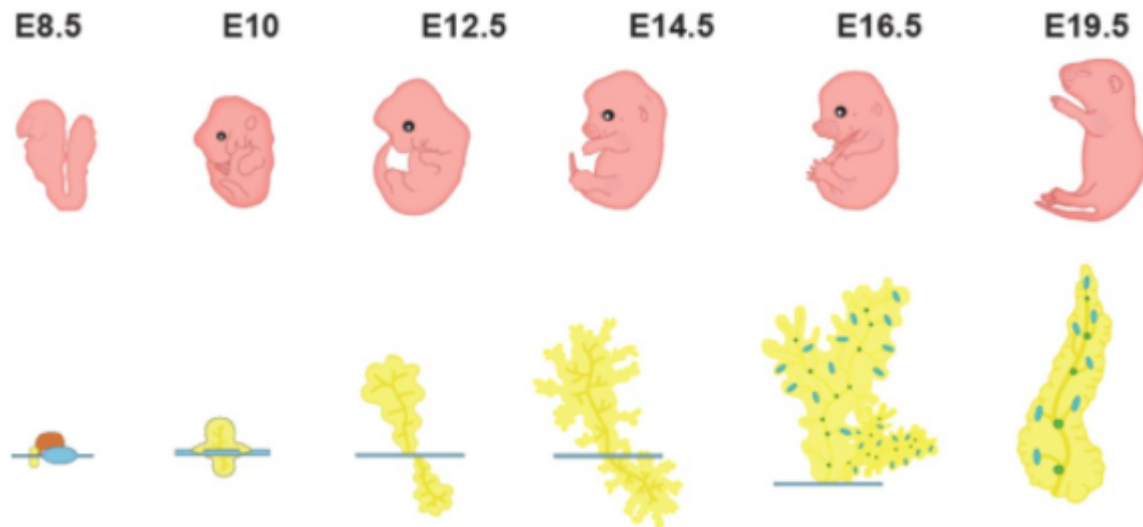


Figure 5. Rodent pancreas development. Schematic depicting the development of the murine pancreas. Islets of Langerhans containing the hormone-secreting endocrine cells are shown in blue and green.

PRIMARY TRANSITION

The first transition phase starts at around embryonic day (E) 9.0-9.5 with the formation of the pancreatic buds and ends at around E12.5 with the start of branching morphogenesis. During this period, the dorsal bud gives rise to the first pancreatic endocrine progenitor cells. As is the case for intestinal EECs, the formation of pECs depends on the expression of the bHLH transcription factor *NEUROG3*, which is transiently expressed at a relatively low level starting at ~E9.0-E11.0. Mice with targeted disruption of *Neurog3* lack pECs and die postnatally of diabetes (232). Consistent with this data in mice, human induced pluripotent stem cells (iPSCs) with null alleles of *NEUROG3* fail to differentiate into pECs (233). Following this first pulse of *Neurog3* expression, a limited number of pancreatic progenitors becomes

committed to the pEC lineage, and some of these cells begin to express glucagon (234).

SECONDARY TRANSITION

Branching morphogenesis continues throughout the first half of the second transition, (~E12.5-E15.5), during which the plexus expands and segregates into tip and trunk domains. From E16.5-E18.5 the epithelial plexus is remodeled into a ductal network with a tree-like structure. Inter- and intralobular ducts branch off from the main pancreatic duct, which connects the pancreas to the common bile duct. During this period, multipotent pancreatic progenitor cells, which make up the majority of cells at the beginning of this transition phase, become either unipotent, fated to become acinar cells at the tip, or bipotent, with the potential to form endocrine and ductal cells. These lineage commitments coincide with a second, more

pronounced wave of Neurog3 expression that peaks at around E14.5-E15.5, and then rapidly decreases at around E17.5(235). This second wave of Neurog3 expression commits progenitors to the pEC fate and primes them towards one of the five pEC types.

TERTIARY TRANSITION

During the tertiary transition, individual progenitor cells that have now become fated to the pEC fate by the second, higher pulse of Neurog3 expression delaminate from the ductal network through what is thought to be asymmetric cell division and/or epithelial to mesenchymal transition (EMT). These pECs then migrate throughout the mesenchyme until they encounter other pEC-fated cells, aggregate with the help of Neural cell adhesion molecule (NCAM), and form oval shaped proto-islets (236,237). This process is reminiscent of the way, during lung development, PNECs form NEBs. The formation of fully mature islets requires their vascularization by endothelial cells and their innervation by neurons, both processes that take place from E16.5 onwards, and during the first weeks postnatally.

POSTNATAL

At birth the majority (more than 80%) of the pECs originates from endocrine progenitors and the remainder results from proliferation of preexisting pECs within the islets.

DIFFERENCES IN PANCREATIC ENDOCRINE CELL DEVELOPMENT BETWEEN RODENTS AND HUMANS

Although studies of human pEC development are limited, they have uncovered a few main differences compared to rodents. First, there seems to be only one pulse of NEUROG3 during the formation of human pECs (compared to two in the mouse), which occurs at around 8 weeks of gestation. Second, the premature proto-islet structures described above are formed earlier in human development (at 12 weeks post conception) (238,239). Finally, the mature islet architecture differs between rodents and humans (Figure 6). Rodent islets have a central core of beta cells, which are also the most common of all the islet cells (60–80%). The remainder of the endocrine cells, alpha cells (15–20% of the cells of the islet), delta cells (<10% of islet cells), and gamma/ PP cells (<1% of cells) surround the beta cells in a circular structure known as the mantle (240). Human islets do not display the same organized structure as rodent islets. Instead, there is a salt and pepper pattern where the different endocrine cells are randomly scattered within the islets. The proportion of the different pECs also differs as human islets have proportionally less beta but more alpha cells compared to rodent islets, consisting of circa 30% alpha cells, 60% beta cells, about 10% delta cells, and rare <1% gamma/PP cells and epsilon cells (241,242).

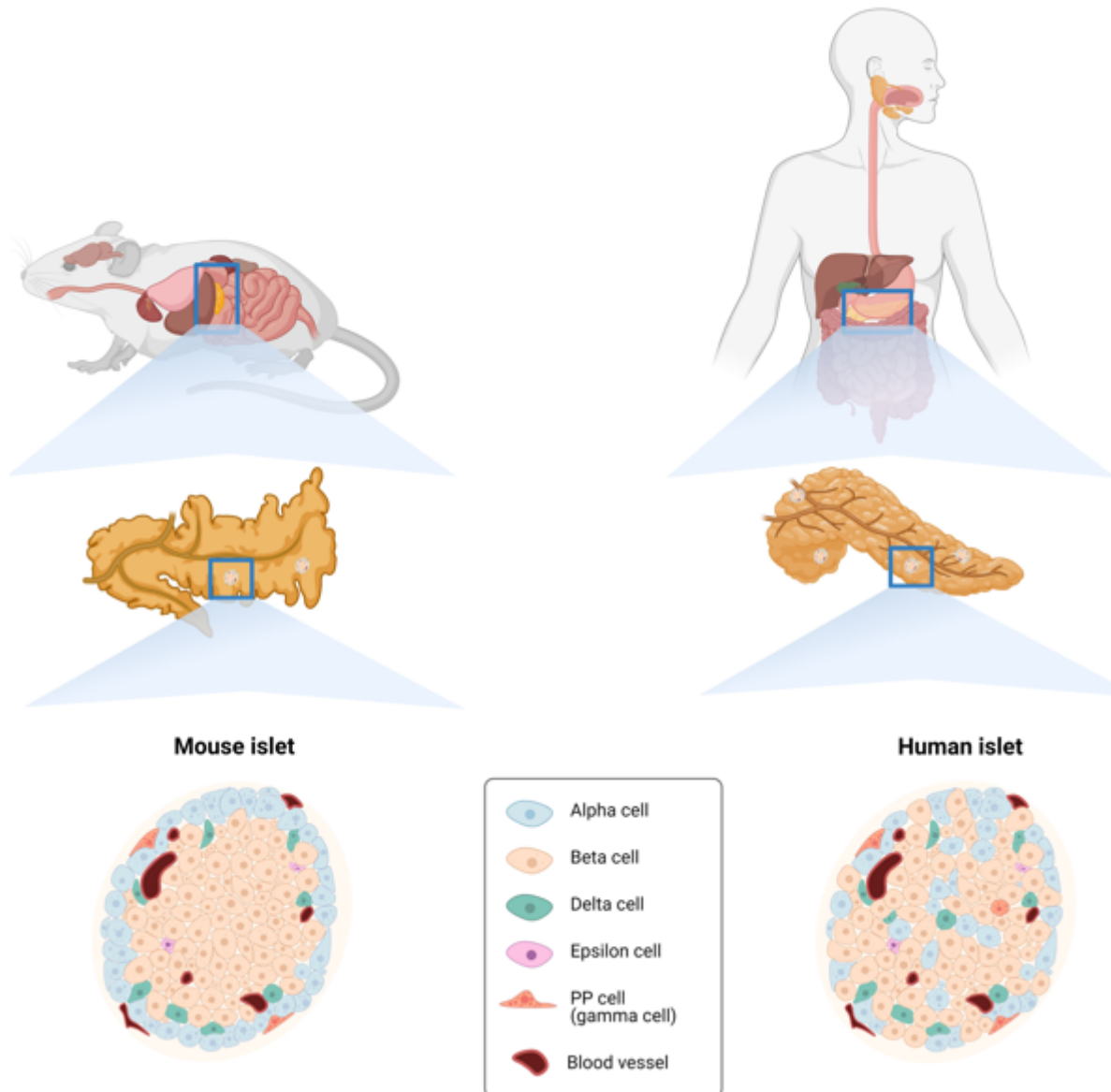


Figure 6. Differences between the murine and human pancreas. Schematic showing the anatomical differences between the murine and human pancreas in terms of both organ and islet architecture.

Pancreatic Islet Cells and Hormones – All with Their Own Function

their specific hormones and function during adult homeostasis (Figure 7).

This section describes the molecular and cellular characteristics of the different subtypes of pECs and

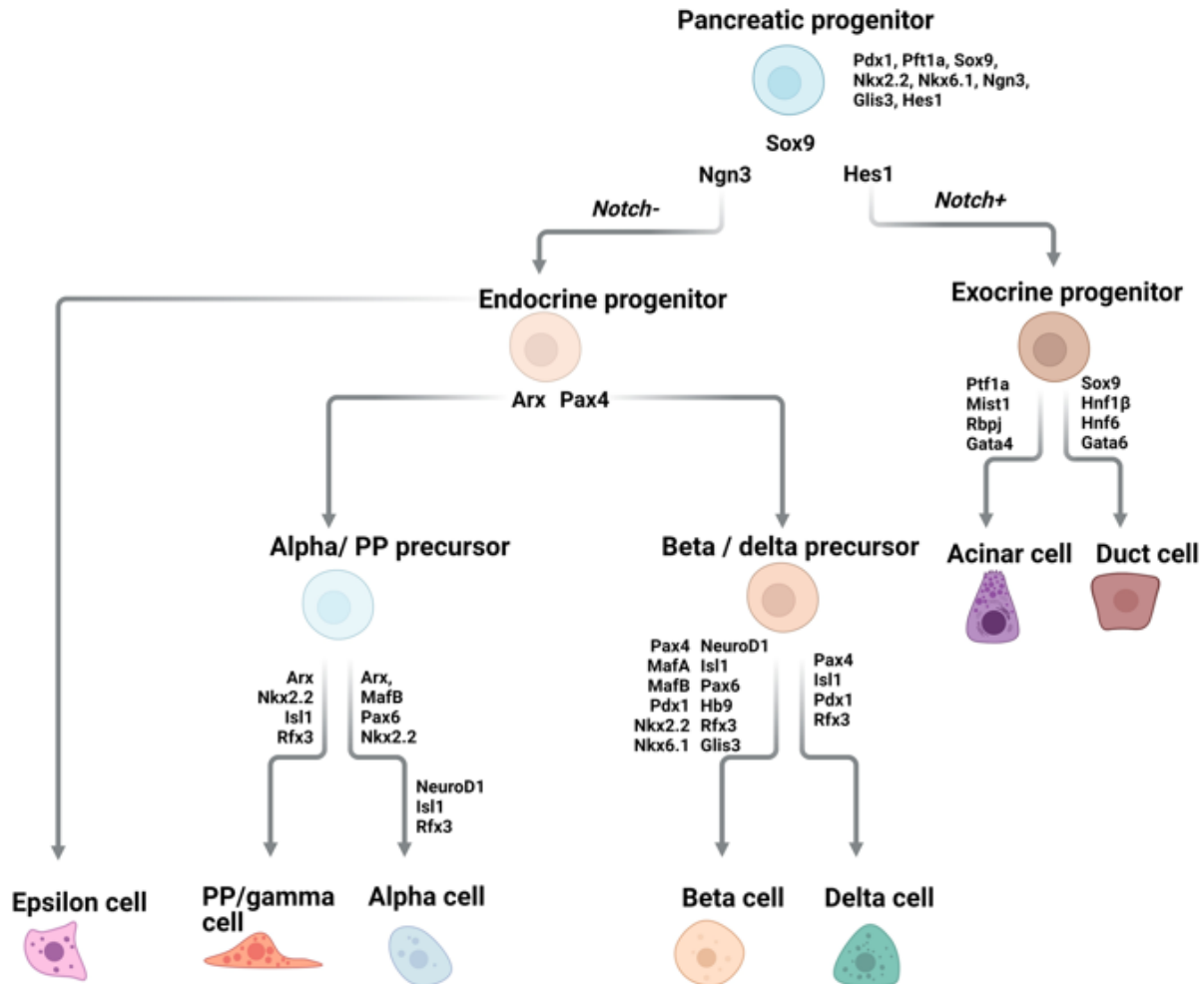


Figure 7. Pancreatic endocrine cells. Diagram of signaling and transcription factor interactions that regulate pancreatic endocrine cell differentiation.

ALPHA CELLS, GLUCAGON

Alpha cells secrete glucagon and are the second most common pEC subtype. Glucagon contributes to maintaining homeostatic blood glucose levels by stimulating glucose production and inhibiting glycogen storage by the liver. The glucagon receptor, GCGR, is widely expressed by multiple organs besides the liver, and glucagon mediates multiple other physiological processes including amino acid metabolism,

glomerular filtration by the kidney, lipolysis, and gastric motility (243).

The key factor promoting commitment of pEC progenitors to alpha cells is the transcription factor ARX. Deletion of *Arx* in Pdx1-expressing progenitors in the mouse leads to a complete loss of alpha cells, accompanied by a compensatory increase in the number of beta and delta cells, which results in the

same total numbers of pECs in mutant and wildtype mice (244). Similarly, overexpression of *Arx* in the embryonic mouse pancreas or in developing islet cells results in an increase in the number of alpha and PP cells while diminishing the number of beta and delta cells (245). While *ARX* is required for alpha cell specification, the transcription factor, *MAFB*, is required for their final maturation and hormone expression. Mice with null alleles of *MafB* show a 50% reduction in insulin and glucagon positive cells (246). Moreover, adult mice in which *MafB* has been conditionally deleted in either *Neurog3*-expressing pEC progenitors or in mature pECs had reduced numbers of glucagon-positive cells (247).

BETA CELLS, INSULIN

The most well-known and abundant cell type of the Islets of Langerhans is the beta cell. Beta cells produce insulin which lowers the blood glucose levels via direct and indirect effects on target tissues. Binding of insulin to its receptor facilitates glycolysis in liver hepatocytes and skeletal muscle cells and promotes lipogenesis in the liver and white adipose tissue. Additionally, insulin inhibits hepatic gluconeogenesis and glucagon production by alpha cells (248–251).

Some of the most important transcription factors involved in the differentiation of beta cells are *NKX2.2*, *NKX6.1*, *NEUROD1*, *MAFA*, and *MAFB*. In mice lacking the homeodomain transcription factor *NKX2.2*, beta cell precursors fail to fully mature or produce insulin and, as a consequence, the mutant mice develop hyperglycemia and die shortly after birth (252). *NKX2.6* lies downstream of *NKX2.2*, and in mice lacking *Nkx6.1* beta cell neogenesis during the second transition is blocked, and the development of fully differentiated beta cells is prevented (253). *NEUROD1* is also required for beta cell maturation (254). *Neurod1* null mice have reduced beta cell numbers and fail to develop mature islets (255).

Differentiating beta cells express both *MafA* and *MafB*, but fully mature beta cells in the mouse only express *MafA*. Conditional deletion of *MafB* in the mouse pancreas delayed beta cell maturation (247). Notably, *MAFB* is expressed in adult human beta cells and while human pluripotent stem cells with *MAFB* knockout are capable of pEC differentiation, they favor delta cell and PP cell specification at the expense of beta cells (256). *MAFA* is not essential for beta cell development but is required for insulin secretion and for maintenance of the beta cell identity (257).

DELTA CELLS, SOMATOSTATIN

Pancreatic delta cells, which make up ~5-6% of all islet cells, are responsible for the production of SST. Like insulin and glucagon, SST is a peptide hormone and cleavage of the precursor pro-hormone gives rise to two active isoforms, a long form that acts primarily in the central nervous system and a short form that acts on the organs of the GEP system. Delta cells predominantly secrete the short isoform of SST in response to a variety of stimuli including acetylcholine, glutamate, GLP-1, and urocortin3 produced by beta cells, ghrelin produced by epsilon cells, and high blood glucose levels (258). Within the islets, SST binds to one of its five different receptors on the surface of beta cells and alpha cells, thereby inhibiting the secretion of insulin and glucagon (259,260). SST also acts on cholangiocytes in the liver, inhibiting their secretion of fluids and thereby mediating bile flow (261).

The delta cell-specific transcription factor, haematopoietically expressed homeobox (*HHEX*), is required for both differentiation of delta cells during embryogenesis and maintenance of their identity in the adult. Deletion of *Hhex* in mouse pEC progenitors during development led to loss of delta cells by E16.5. Likewise, deletion of *Hhex* in adult mouse pancreatic delta cells led to a reduced number of delta cells and reduced secretion of SST (262). The phenotype of

mice lacking HHEX in the pancreas speaks to the hormonal interplay between pECs: reduced SST secretion in mutant mice led to increased hormone secretion from alpha and beta cells in response to different stimuli. Indeed, despite not being expressed in beta cells, *HHEX* has been repeatedly identified through GWAS studies as a locus conferring susceptibility to T2D (263).

EPSILON CELLS, GHRELIN

Epsilon cells, described for the first time in 2002, were the last islet cell type to be discovered. The number of pancreatic epsilon cells is highest during embryogenesis, comprising up to 10% of islet cells at mid-gestation, but their numbers then decrease such that they make up only a bit more than 1% of islet cells in the adult pancreas (264). Scattered throughout the islets, epsilon cells produce the growth hormone secretagogue ghrelin. The name of this hormone comes from the Proto-Indo-European root “ghre-”, meaning “to grow,” which seems appropriate for a hormone that was discovered in efforts to identify the ligand for the growth hormone secretagogue receptor (GHS-R) (265). Also known as the hunger hormone, ghrelin is a 28 amino acid peptide that needs to be post-translationally acetylated before it can bind to its receptor, GHS-R, and exert its function on its target cells in an expanding list of target organs (266).

Consistent with its stimulatory effect on food intake, ghrelin concentrations are highest during fasting. This function is primarily mediated by ghrelin secreted by gastric X cells. The other hallmark functions of ghrelin are stimulating fat deposition and stimulating growth hormone release from the pituitary. The list of functions attributed to ghrelin is still growing and includes regulating glucose and energy homeostasis, cardioprotection, muscle atrophy, and bone metabolism (267). Secretion of ghrelin is stimulated by glucagon and is inhibited by glucose, insulin, leptin,

and GLP-2(268–270). In the pancreas, ghrelin secreted by epsilon cells binds to the GHS-R on delta cells, thereby inducing them to secrete SST, which in turn inhibits the secretion of insulin by beta cells (271,272). There is also some evidence that ghrelin promotes the survival and proliferation of beta cells (264).

A complex interaction between NKX2.2 and NEUROD1 appears to be involved in epsilon cell specification during development (264). However, the transcription factor most closely linked specifically to epsilon cells is PAX6, which appears to inhibit epsilon cell formation. Ghrelin-expressing cells are increased in the developing pancreas of *Pax6* knock-out mice at the cost of alpha cells (273). Likewise, when *Pax6* was deleted in the adult pancreas of mice, the same increase in epsilon cell numbers was observed, concomitant with loss of beta cells, alpha cells, and delta cells (274). A later study demonstrated that, upon loss of *Pax6* expression, beta and alpha cells began to express ghrelin (275).

PP (GAMMA) CELLS, PANCREATIC POLYPEPTIDE

PP cells comprise less than 1-2% of the islets and the majority are located in the head of the pancreas. Perhaps due to their scarcity in the islets, not much is known about the genetic determinants of PP cell specification and maturation. Signals from the vagus nerve, enteric neurons, and arginine following meal intake stimulate PP cells to release pancreatic polypeptide (PP) (276). This hormone has the opposite effect of ghrelin and is referred to as the satiety hormone (277). Transgenic mice that overexpress PP show reduced weight gain and decreased fat mass, and long-acting PP analogues are being explored for the treatment of human obesity (278,279). Within the pancreatic islet, PP indirectly affects insulin secretion by inhibiting glucagon and

SST secretion via a receptor expressed on alpha and delta cells, respectively (280,281).

Other members belonging to the same peptide hormone family as PP are neuropeptide Y (NPY) and peptide YY (PYY) which are produced in the GI tract. PYY is produced by L cells in the intestine and colon and its function mimics that of PP in the pancreas (282).

Pancreatic Endocrine Cells in Injury Repair

Injury to the pancreatic epithelium in the form of surgery, inflammation or metabolic trauma disrupts homeostasis by causing extensive cell loss. Failure in injury repair can result in organ dysfunction and cause clinical symptoms.

The youthful pancreas has a high potential to regenerate damaged tissue following injury (283,284). In contrast to the GI tract, however, where this capacity to regenerate is maintained throughout life, the regenerative capacity of pECs is limited in adult tissue (285,286). Through observations made in rodent injury models, mostly looking at beta cells, it has become clear that the endocrine pancreas employs multiple strategies to regain homeostasis following injury.

REPLICATION OF PRE-EXISTING BETA CELLS

The ability of pre-existing beta cells to replicate in response to injury was first shown by the Melton lab. Using a beta-cell-specific lineage trace they found that the newly generated beta cells that arose following partial pancreatectomy originated from pre-existing ones (287). Using the same injury model, but an unbiased thymidine analogue based labelling technique, a different group came to the same conclusion (288).

NEOGENESIS OF BETA CELLS FROM A STEM/PROGENITOR CELL

An alternative hypothesis is that beta cell regeneration in the adult pancreas involves a process called neogenesis, which involves the formation of de novo beta cells derived from a stem or progenitor cell expressing Neurog3. Initial studies of neogenesis were based on *in vivo* pulse labelling with the thymidine analogue, BrdU. In one study, IFN-gamma induced beta cell depletion resulted in the appearance of budding duct/islet-like structures containing proliferative ductal cells as well as newly formed acinar and endocrine cells (289). Similar observations were made in two different models of pancreatic injury in rats, where regeneration involved marked proliferation of ductal cells, some of which expressed ductal and endocrine markers, followed by the formation of new pECs and islet structures (290,291). Researchers from the Heimberg lab found that, subsequent to pancreatitis induced by pancreatic duct ligation in mice, the region of the pancreas undergoing regeneration contained NEUROG3-positive cells, a portion of which also expressed ductal markers (292). Finally, a lineage trace of ductal cells in mice showed that, following injury, ductal, acinar, and endocrine cells all contained the lineage trace (293). Altogether, these studies suggest that, following injury, new pECs can be derived from a progenitor cell in the pancreas ductal epithelium, whose differentiation to the pEC fate is dependent on transient expression of Neurog3.

TRANS-DIFFERENTIATION OF NON-BETA CELLS INTO BETA CELLS

Finally, a landmark study from the lab of Pedro L. Herrera, provided evidence for yet another strategy employed by the pancreatic endocrine compartment to regenerate. Prior to inducing genetic ablation of adult beta cells, the authors induced a lineage trace of

glucagon-producing alpha cells. In the initial months following beta cell ablation, mice required supplementation with insulin. However, at 6 months post-ablation, mice no longer required supplemental insulin and their pancreata showed increased beta cell mass and beta cells that expressed both insulin and glucagon. At early time points following ablation, the majority of the regenerated beta cells in these mice contained the alpha cell lineage trace, arguing that the beta cells had resulted from trans-differentiation of the alpha cells (294). This provided new evidence of endocrine cell plasticity.

Pancreatic Endocrine Cell Hyperplasia

In the previous section we discussed observations relating to pEC regeneration in the context of conditions that lead to direct loss of pECs. Given defining pEC characteristics such as direct innervation and the role of pECs in mediating a number of physiological processes, it is likely that pECs also respond to stimuli produced in the context of other pathological conditions. As we have discussed in previous sections of this text, EEC and PNEC hyperplasia have each been observed in the context of inflammation or injury of their respective tissue sites. Likewise, an increase in the number pECs has been observed as a response to some pathological conditions relating to the pancreas (295,296). Moreover, focal endocrine hyperplasias have been observed incidentally in the pancreas of up to 10% of screened adults at autopsy (297).

The pEC mass is normally 1% and 3% of the total pancreatic mass in adults and infants, respectively. If this increases to more than 2% or 10% of the total pancreatic mass in adults or infants, respectively, it is defined as pEC hyperplasia (222,297,298). Some instances of pEC hyperplasia involve a general increase in the size of pancreatic islets that results in an increase in overall islet cell numbers but not in a

change in the relative frequencies of one pEC subtype versus another. However, the majority of pEC hyperplasia are associated with an increase in the number of a specific subtype of pEC, most commonly alpha and beta cells. Hyperplasia of other pEC subtypes including the rare gamma/PP cells have also been reported. Morphologically, pEC hyperplasia either appears as large islets (larger than 250 μ m in diameter) or as budding structures protruding from the ductal epithelium (297). The latter budding structures are reminiscent of the structures described above in mouse models of pEC injury and are suggestive of pEC neogenesis.

BETA CELL HYPERPLASIA

Beta cell hyperplasia is commonly observed in patients with insulin resistance and early T2D and is likely a physiological response to these conditions. Other clinical conditions in which beta cell hyperplasia is implicated include persistent hyperinsulinemic hypoglycemia of infancy (PHHI) and non-insulinoma pancreatogenous hypoglycemia syndrome (NIPHS) (299–302). These conditions are associated with dysregulated insulin secretion and hypoglycemia in infants and neonates or in adults, respectively.

The pancreas of patients with PHHI is characterized by the presence of either focal beta cell hyperplasia, resulting in a focal increase in islet size or, more commonly, of diffuse beta cell hyperplasia in which enlarged islets and small, irregularly shaped endocrine cell clusters are found throughout the pancreas (297). The percentage of beta cells present within the islets of patients with PHHI is increased such that they account for 70-90% of the islet (222). Increased proliferation of not just beta cells but also of ductal and centroacinar cells has been reported in the pancreas of patients with PHHI (303). PHHI is caused by mutations in *ABCC8* and *KCNJ11*, which encode for subunits of the ATP-sensitive potassium channel

involved in insulin secretion, as well as by mutations in genes affecting beta cell metabolism such as glucokinase (GCK), glutamate dehydrogenase (GLUD1), and short chain fatty acid hydroxyacyl dehydrogenase (SCHAD) (304).

NIPH is defined by postprandial hypoglycemia and, unlike PHHI, the genetic cause has not been clearly identified. The pancreas of patients with NIPH exhibits an increase in both number and size of the islets, and contains endocrine cells budding from the ductal epithelium (305,306). Symptoms of both PHHI and NIPH can be resolved through either partial or near total pancreatectomy.

ALPHA CELL HYPERPLASIA

Alpha cell hyperplasia (ACH) is a rare condition most commonly caused by mutations in the gene that encodes for the glucagon receptor, *GCGR* (307,308). The number of islets in patients with ACH is increased and the islets vary in size, are often larger, and contain a high proportion of alpha cells (309). Patients with ACH often, though not always, also present with hyperglucagonemia, and multiple pancreatic NETs. The multifocality of ACH and pancreatic NET lesions in these patients, and the observed presence of large islets showing signs of morphological transition from ACH to glucagonomas suggests that ACH lesions can progress to frank glucagonomas (308,309). Interestingly, these patients do not display features of glucagonoma syndrome due to their *GCGR* mutations. Mice with germline null mutations in *Gcgr*, or with liver-specific deletion of *Gcgr* also develop ACH that can progress to glucagonomas. Pharmacologic interruption of glucagon signaling in mice also leads to ACH. In these models, ACH appears to be primarily driven by alpha cell proliferation, though it is possible that transdifferentiation of other pECs or of ductal cells is also involved. It is interesting to note that the clear link between *GCGR* function and ACH implies a

signaling feedback loop that regulates both the number of glucagon-producing alpha cells and their secretion of glucagon. One of the signals that is likely to contribute to ACH is amino acids. Serum amino acid levels are increased in patients with ACH and transcriptomic analysis of mouse models of ACH have shown altered expression patterns for genes involved in amino acid catabolism and transport (310).

GAMMA/PPCELL HYPERPLASIA

Compared to alpha and beta cell hyperplasia even less is known about PP cell hyperplasia, which occurs very rarely. As with alpha and beta cell hyperplasia the number and size of the pancreatic islets in patients with PP cell hyperplasia is increased and contain a high proportion of PP cells (311–313). In 50% of the reported PP hyperplasia cases, the patients had suffered from gastrinoma or ZES. In addition, there is no correlation between PP cell number and PP serum levels. It is possible that PP hyperplasia arises as an effect of gastrinomas (297). There are also no genetic changes directly related to the onset of this condition.

GEP NEUROENDOCRINE NEOPLASMS (GEP-NENS)

GEP-NENs encompass all NENs that arise along the GEP tract and account for 55 to 70% of NENs from all tissue sites (82,314,315). GEP-NENs comprise both well-differentiated NETs and poorly differentiated NECs. In addition, some GEP-NENs occur as GEP-mixed neuroendocrine/non-neuroendocrine neoplasms (GEP-MiNEN) that can be either well- or poorly-differentiated and are characterized by their mixed morphology showing endocrine and non-endocrine features. Based on proliferation (measured by mitotic count and Ki67 index) GEP-NETs are further subdivided into low (G1), intermediate (G2), and high (G3) grade NETs (82). G3 well-differentiated NETs are associated with a poor prognosis and show

a decidedly more aggressive clinical behavior than G1 and G2 GEP-NETs. G3 well-differentiated NETs are more commonly observed in the pancreas than in other GEP tissue sites. GEP-NECs can be subdivided into small cell NEC and large cell NECs.

GEP-NECs

GEP-NECs comprise 10-20% of all NENs, with roughly 38% arising in the GI tract (colon, anus, rectum) and 23% in the pancreas (316). GEP-NETs and GEP-NECs display different mutational profiles and are therefore considered separate disease entities. Indeed, the most common site for NECs is the large bowel, whereas the most common site for NETs is the ileum (316). In addition, the observations that some GI-NECs show features resembling adenocarcinomas or squamous cell carcinomas and that up to 40% of NECs show non-endocrine features, have led some researchers to hypothesize that GEP-NECs are more closely related to non-endocrine tumor types than to high grade (G3) NETs (82,317,318).

The importance of the two genes *RB1* and *TP53* in the genesis of NECs is highlighted by the fact that these genes are commonly mutated in both lung-NECs and GEP-NECs. *TP53* mutations have been identified in 20-73% of all GEP-NECs (319–323) while *RB1* mutations have been identified in 44-86% of all GEP-NECs (322,324–326). The limited number of studies that have performed genomic analyses of GEP-NECs have also identified other genes that are commonly mutated in GEP-NECs, including *KRAS*, *SMAD4* and *APC* (318,327,328). In addition, GEP-NECs are characterized by frequent and severe chromosomal abnormalities (79).

As discussed previously, generating a GEMM of SCLC was achieved through conditional tissue-specific deletion of *Rb1* and *p53* in the mouse lung epithelium (89). In contrast, attempts to generate GEMMs of *RB1*; *TP53* mutant GEP-NECs by deletion

of *Rb1* and *p53* in targeted mouse GEP tissues have proven to be less straightforward and have given mixed results (329). Conditional deletion of *Rb1* and *p53* in renin-expressing mouse pECs led to the generation of highly aggressive, metastatic, glucagon-producing tumors (330). Given the expression of glucagon in combination with the aggressive course of the tumors developed by this GEMM, however, it is unclear whether they are a more suitable model for sporadic glucagonomas or for pancreatic NECs. In human patients, pancreatic NECs are almost exclusively non-functional, i.e., they do not secrete symptom-inducing hormones (331). In the RIP-Tag2 GEMM, instead, SV40 T-antigen is expressed in beta cells, thereby effectively abrogating the *Rb1* and *p53* pathways in these pECs. RIP-Tag2 mice primarily develop aggressive insulinomas and, to a lesser extent, poorly differentiated pancreatic NECs (332). The RIP-Tag2 model has been instrumental in delineating stepwise aspects of pancreatic NEN tumorigenesis and in identifying therapeutic strategies for these tumors in patients (329). Nonetheless, whether this GEMM can be used as a reliable model for pancreatic NECs is unclear.

In addition to GEMMs, cell lines and, more recently, GEP-NEC patient-derived tumor organoid lines have been generated (333,334). Patient-derived tumor organoids, 3D long-term cultures of tumor cells, can be expanded long-term, can be cryopreserved, and have been shown to be representative of the patient tumor tissue from which they were derived at both the genetic and phenotypic levels (335). Recently, a genetically engineered organoid model of GEP-NECs was generated by CRISPR/Cas9 mediated compound knock-out of *RB1* and *TP53* combined with overexpression of 6 transcription factors in otherwise normal colon organoids (333). Interestingly, in the absence of transcription factor overexpression, compound knock-out of *RB1* and *TP53* was not sufficient to generate GEP-NECs from these cells.

Together with genomic analyses of GEP-NECs, studies using preclinical models of GEP-NECs have been informative. Nonetheless, to date, strategies for stratifying patients in terms of the molecular characteristics of their tumors and the likely response of their tumors to specific therapies are lacking. Currently, the primary treatment strategies for GEP-NECs use platinum agents combined with etoposide, based on the relative effectiveness of this approach in treating SCLC, which is a more common and better studied NEC subtype (336–338). Overall, the response rate for GEP-NECs to first line therapy is 40–60%. Upon relapse or the tumors becoming refractory, there are no well-established second-line therapies. This is reflected in a median survival of 38 months in patients with localized disease and only 5 months in patients with metastatic disease (339). Other potential treatment strategies for patients with GEP-NECs that are currently undergoing clinical trials are the mTOR inhibitor everolimus and some forms of immunotherapy (336–338).

GEP-NETs

GEP-NETs represent 80–90% of all GEP-NENs and comprise many different tumor types. GEP-NETs are classified as functional or nonfunctional depending on whether they secrete symptom-causing hormone peptides. Functional GEP-NETs, which can arise throughout the GEP-tract, include gastrinomas and insulinomas (10). GEP-NETs are highly heterogeneous with regards to their biological behavior and clinical presentation, course, and prognosis. The most common tissue sites of primary GEP-NETs are the small intestine, the rectum, the colon, the pancreas (12.1%), and the appendix (315). In general, G1 and G2 GEP-NETs are associated with high 5-year survival rates ranging from 75 to 79% and from 62 to 74%, respectively. The 5-year survival rate for patients with G3 well-differentiation NETs, on the

other hand, shows more variability between NETs of the intestine (40%) and NETs of the pancreas (7%) (340). Notably, the recognition of the category of G3 well-differentiated pancreatic NET by the WHO in 2017 has had important implications for patients and clinicians, as it highlighted the fact that some pancreatic NETs, despite showing morphological features and differentiation more commonly associated with low-grade NETs, show aggressive behavior more similar to that of NECs (82,331).

The most prevalent sites of origin for GEP-NETs show regional differences that are likely reflective of differences in both environmental and genetic factors. GI-NETs arising from the small intestine or colon are most common in the USA, small intestinal or pancreatic NETs are more common in Europe, while in Asia gastric and rectal NETs are most prevalent (341). The most well studied GEP-NETs are pancreatic NETs and SI-NETs.

Gastric NETs (G-NETs) are relatively rare and only account for 4 to 6% of NENs (342,343). These tumors are classified into one of four categories according to their clinical characteristics. Most G-NETs are type I tumors, which are associated with CAG and arise as multiple small nodules. These tumors rarely metastasize. Type II G-NETs are very similar to type I tumors but are commonly associated with MEN1 syndrome (in conjunction with gastrin producing pancreatic NETs) or ZES. Type I and type II G-NETs arise as a consequence of excessive gastrin. Type III G-NETs are sporadic tumors that are not associated with other gastric conditions and present as large, solitary lesions. Finally, type IV tumors, G-NECs, are both the very rare and the most malignant. These tumors arise sporadically and are poorly differentiated. While type I, II, and III G-NETs are ECL cell tumors, type IV G-NECs arise from other endocrine cell types (344).

Small intestinal NETs (SI-NETs) are the most common neoplasm arising in the small intestine (345). They include NETs arising in the jejunum and ileum, with the ileum being the major site of incidence (346). A unique feature of ileal SI-NETs is that they are multifocal in 10-20% of cases, with the different tumors arising independently (347). Moreover, ileal SI-NETs have a high rate of metastasis, with >50% of ileal SI-NET patients presenting with metastases at the time of diagnosis (348). Finally, the majority of SI-NETs produce serotonin, causing many of these to induce carcinoid syndrome, the most common functional hormonal syndrome of patients with NETs (349). Clinical symptoms of carcinoid syndrome include watery diarrhea, flushing, hypotension, breathlessness, wheezing, and loss of appetite, all attributable to not just increased serum levels of serotonin but also of prostaglandins, histamine, bradykinin, and tachykinins (9,350). Carcinoid syndrome is most often observed when NETs, having metastasized to the liver, secrete their biologically active compounds directly into the systemic circulation (351). Carcinoid syndrome can cause carcinoid heart disease in which elevated serum serotonin levels cause fibrosis in the heart. Symptoms of carcinoid syndrome attributable to serotonin can be ameliorated through the administration of serotonin-inhibiting therapies (340,352).

Of note, duodenal NETs are sometimes considered separately from SI-NETs as they more closely resemble gastric and pancreatic NETs in terms of their mutational profile (353). Whereas the serotonin producing EC cells are thought to be the cell of origin of SI-NETS, duodenal NETs more commonly express gastrin and somatostatin (described in more detail later in this chapter) and are therefore thought to arise from G and D cells (354,355).

Pancreatic NETs (PanNETs) are the best studied subtype of GEP-NET and are the only subtype for

which the category of high grade G3 well-differentiated NET has been officially recognized (356). While some well-differentiated PanNETs are functional and consist of a single hormone-producing cell type, the majority of PanNETs are non-functional and contain a mixture of cells expressing markers for the different pEC types (357,358). PanNETs are thought to arise from differentiated endocrine cells of the islets of Langerhans. However, a recent study based on next generation DNA methylation analysis suggests that they may also arise from the exocrine pancreas (359).

Familial GEP-NETS

While the majority of the GEP-NETS arise sporadically, approximately 5% occur as part of a hereditary cancer predisposition syndrome (360). The most common of these syndromes are associated with the development of duodenal and PanNETs and are caused by germline mutation in one of five different genes: *MEN1*, tuberous sclerosis complex 1 (*TSC1*), tuberous sclerosis complex 2 (*TSC2*), Von Hippel–Lindau (*VHL*), and neurofibromatosis type 1 (*NF1*) (361). Of these, *MEN1* is the gene most strongly implicated in PanNETs. *MEN1* encodes for the protein menin, which acts as a scaffold for both transcription factors and chromatin-modifying enzymes (362–364). Thus, although its exact function is yet to be determined, menin has been suggested to play a role in multiple processes including DNA damage repair, cell cycle regulation, histone methylation, and mTOR pathway activity (365).

Familial SI-NETs are far rarer and have been associated with germline mutations in *CDKN1B*, inositol polyphosphate multikinase (*IPMK*), and MutY DNA glycosylase gene (*MUTYH*) (366–369). Notably, germline mutations in *CDKN1B* have been shown to be causative of the MEN4 familial cancer syndrome in which patients develop parathyroid, pituitary, and, more rarely, SI-NETs (323). Finally, there is one

example of a single consanguineous family in which several family members were affected by hypergastrinemia and consequent gastric ECL NETs. These familial G-NETs were shown to be caused by germline inactivating mutations in the gene for a proton pump expressed by parietal cells and involved in gastric secretion, *ATP4a* (370).

Genetics of Sporadic GEP-NETs

Although rare, familial cancer syndromes associated with NETs have pointed to genes and pathways that are also important for the genesis of sporadic NETs. Genetic studies have revealed that somatic inactivation by mutation, chromosomal alteration, or epigenetic silencing of genes such as *MEN1*, *TSC2*, and *VHL*, each associated with a familial NET syndrome, are found in approximately 40%, 35%, and 25% of sporadic pancreatic NETs (371,372). Familial SI-NET syndromes are rarer, and of the causative genes for these syndromes, only mutations in *CDKN1B* have also been identified in 9% of sporadic SI-NETs (373). Of note, the largest whole genome analysis of pancreatic NETs to date, uncovered germline mutations in *CDKN1B* and *MUTYH* in pancreatic NETs from patients that had no family history of the disease, therefore implicating alterations in these genes also in the genesis of pancreatic NETs and, perhaps, of GEP-NETs in general (371,372). Incidentally, germline variants in pancreatic NET samples in this cohort were also identified in checkpoint kinase 2 (*CHEK2*), and *BRCA2* (371,372).

Molecular analysis of pancreatic NETs also uncovered alterations in genes that are not implicated in familial NET syndromes. In particular, Jiao et al. identified recurrent mutations in *PTEN* and *PIK3CA* in pancreatic NETs. This study also identified recurrent mutations in the chromatin remodeling enzymes *DAXX* (death domain associated protein) and *ATRX* (α thalassemia/mental retardation syndrome X-

linked), in 25% and 18% of pancreatic NETs (374). *DAXX* and *ATRX* function together in a complex that deposits histone H3.3 at different sites including telomeres and mutations in *ATRX/DAXX* were mutually exclusive in pancreatic NETs (372). *ATRX/DAXX* mutations in pancreatic NETs were correlated with chromosomal instability and the activation of a telomerase independent telomere maintenance mechanism, alternative lengthening of telomeres (ALT) (375,376). Perhaps it is thus not entirely surprising that *ATRX/DAXX* mutations are more often found in G3 pancreatic NETs than G1/G2 pancreatic NETs and are associated with reduced patient survival (323,375). Other mutations found in G3 pancreatic NETs and associated with shorter survival times are mutations in *ARID1A* and *CDKN2a* (377,378).

Whereas several driver mutations have been identified in pancreatic NETs, identifying a clear genetic driver of SI-NETs has been more difficult. Instead, the development of sporadic SI-NETs seems to be dependent on chromosomal aberrations. Loss of chromosome (chr) 18 is observed in more than 60% of SI-NETs. While the functional importance of this chromosomal loss in SI-NETs has not been determined, one study identified allelic loss of *BCL2*, *CDH19*, *DCC*, and *SMAD4* (all on chr 18) in 44% of SI-NETs (379). Loss of chr 9 and 16 or gain of chr 4, 5, 7, 14 or 20 are also recurrently observed in SI-NETs, albeit at lower frequencies (353,380–383). Targeted mutational and copy number analysis of metastatic SI-NETs has identified recurrent mutations in *APC*, *CDKN2C*, *BRAF*, *KRAS*, *PIK3CA*, and *TP53*, though at relatively low frequencies (ranging from 4 to 10%) (328,379,384).

Although genomic studies demonstrate that SI-NETs and pancreatic NETs have distinct genomic profiles, a common pathway alteration stands out -- activation of mTOR/PI3K signaling. In pancreatic NETs the

pathway is recurrently activated by frequent loss of negative regulators of the pathway (PTEN, TSC1/2) and by recurrent activating mutations in PIK3CA. Likewise, this pathway is implicated in SI-NETs by the observation of recurrent activating mutations in KRAS, BRAF, and PIK3CA as well as, more commonly, of frequent copy number gains in components of this pathway, including SRC and mTOR itself (323). Frequent alteration of CDKN family genes also appears to be a common feature of both GEP-NET subtypes, suggesting deregulation of the cell cycle is also important for NETs. Finally, though not discussed in this text, aberrant methylation patterns are likely to contribute to GEP-NETs in general and some studies have suggested that methylation patterns can be used to stratify GEP-NETs with implications for patient prognosis (385–387).

Treatments for GEP-NETs

The main alternatives used to treat GEP-NETs for which surgical resection is not possible are hormone analogs, PRRT, the mTOR inhibitor everolimus, and for pancreatic NETs, sunitinib. Chemotherapy mainly targets proliferative cells and is therefore predominantly used on NECs and G2 pancreatic NETs.

Somatostatin analogues (SSAs) such as octreotide and lanreotide bind to the somatostatin receptors (SSTR1-5) and mimic the natural hormone's ability to exert an inhibitory function on the cell's hormone secretion. To prolong their effect, these analogues have been synthetically engineered to have an increased half-life compared to the endogenous hormone. Administration of SSAs is common in the treatment of functional GEP-NETs as they prevent the tumors from secreting excessive amounts of hormones and thus alleviate clinical symptoms (388,389). Although GEP-NETs can express all five somatostatin receptors, the majority express SSTR2.

Binding of SSAs to SSTR2 has been shown to decrease proliferation and lead to disease stabilization in GEP-NET patients (390–392). In addition, SSAs can also be coupled to radioactive isotopes and used for targeted radiological therapy. The internalization of the radioactive isotope by the cancer cells causes DNA damage and cell death (393,394).

As discussed previously, the mTOR-AKT pathway plays an important role in GEP-NETs. Consistent with this, inhibitors of this pathway such as everolimus have been shown to have a positive effect (132,395,396). Cell type-specific drugs have also been used in the treatment of pancreatic NETs and one of the oldest drugs is streptozotocin, a beta cell specific cytotoxic agent that has been used for almost four decades (397). Streptozotocin selectively enters beta cells via the glucose transporter, GLUT2, causing DNA damage resulting in cell death (398). Finally, inhibitors of receptor tyrosine kinases, vascular endothelial growth factor and its receptor have been effective in the treatment of GEP-NETs, most notably, pancreatic NETs as they are often highly vascularized (399,400).

CONCLUSIONS

When comparing neuroendocrine cells from different tissues, multiple recurrent themes can be identified. For one, their development relies on the expression of bHLH transcription factors ASCL1 and NEUROG3. Animal models have highlighted the differential roles played by these two transcription factors in the formation and differentiation of NE cells of the diffuse NE system. Whereas ASCL1 drives PNEC lineage commitment in the lung, NEUROG3 is essential for the formation of the intestinal EECs and pECs. Both ASCL1 and NEUROG3 appear to be important for the development of gastric EECs. Another interesting difference can be seen in the expression pattern of these transcription factors. Whereas NEUROG3 is

transiently expressed during the formation of GEP endocrine cells, ASCL1 is constitutively expressed in lung progenitor and mature PNECs. The mechanistic differences and biological reason behind these differential dependencies on either ASCL1 or NEUROG3 remain to be determined.

Second, NE cells of the diffuse NE system appear to be involved in the response to some forms of injury, as indicated by their increased numbers in some disease conditions. Importantly, comparing NE cells during development or homeostasis to NE cells in disease conditions has the potential to uncover aberrations in common pathways and regulatory factors that contribute to or mediate the pathological state. This kind of analysis is likely to provide candidate targets for the development of new (targeted) therapies.

Another recurrent theme in the biology of neuroendocrine cells from different tissues, is the crosstalk between NE cells and their environment. This crosstalk can be seen both in the response of NE cells to environmental stimuli and in the direct influence they can exert both locally and systemically through the bioactive compounds they secrete. Finally, the ideas of NE cell plasticity and NE cell heterogeneity are ones that have not been fully explored but might be important to their function. As an example of the former, although EECs are named after their predominantly expressed hormone, they can also be multi-hormonal and even change their hormone expression depending on their location within the tissue. While EEC hormonal switching is just starting to be discovered within the intestinal tract, it is likely that a similar degree of plasticity in hormone expression exists for other NE cell types. Indeed, some hints of plasticity between endocrine cells can also be seen in pancreatic endocrine cells, where some studies imply trans-differentiation of alpha cells to beta cells under certain conditions. Regarding NE cell heterogeneity, it is interesting to note that,

whereas the initial studies of NE cells were driven by observations about their shared features, with the advancement of single-cell sequencing technologies, the more recent era of NE cell research has highlighted a previously underappreciated heterogeneity of different tissue-specific NE cell populations (34,35,164,183,401,402). NE cell heterogeneity highlights the plasticity and dynamic nature of these cells as they respond to external stimuli and microenvironmental signals in a context-specific manner.

With regards to NENs, next generation sequencing efforts have started to characterize the mutational landscape of NENs. So far, these have been mostly focused on pancreatic and lung NENs. Similar studies of NENs originating from other organs would be of value as they could provide new insights into NEN biology. Importantly, candidates discovered in genome-wide genomic studies need to be validated to determine whether they represent drivers or passengers throughout disease progression. Additionally, their exact mechanistic function is of importance if they are to become targets for drug development. For NENs with a lower mutational burden and few recurrent mutations, epigenome, non-coding or protein level changes may play a more significant role in disease initiation and progression. These possibilities are just starting to be explored.

A significant challenge in studying NENs is the development of model systems that can be used to study both basic as well as translational NEN biology. Currently, the most commonly used models include GEMMs, cell lines, and patient derived xenografts (PDX). Although these model systems have provided invaluable knowledge about NEN biology they also have their limitations. GEMMs have been instrumental in the study of SCLC and some very specific subtypes of pancreatic NETs (RIP-Tag2 mice and Men1 mutant mice). Nonetheless, the current GEMMs for different

NENs do not cover the entire range of different tumor types encompassed by NENs. Furthermore, species differences require comparisons and/or end stage studies in human derived model systems.

Cell lines, which while being robust and relatively cheap to culture, suffer from genetic changes occurring over time and may not recapitulate the full tumor heterogeneity.

PDX models circumvent those limitations while providing analysis in an *in vivo* environment, but have a very low engraftment rate of <10% and tedious logistics (403). Organoids derived from healthy tissue provide researchers with the means to study normal NE cell differentiation via the establishment of differentiation protocols (183,195) and offer the potential to model NEN disease progression by stepwise genome engineering, as has been achieved for other tumor types (333,404–406). Moreover, *in vitro* drug screens on patient-derived tumor organoids have the potential to aid personalized treatment.

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However, to date only a handful of NEN organoid lines have been established. Of note, most of those organoid lines represent NECs, rather than NETs (333,334).

Although NEN biology is starting to be explored in more detail, much remains to be discovered. A combination of both basic and translational research will be needed to provide the biological insights necessary to significantly be able to improve or establish novel clinical treatment options for NENs.

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