Pancreatic neuroendocrine tumors (PanNETs) are complicated and often deadly neoplasms. A recent increased understanding of their molecular biology has contributed to expanded treatment options. DNA sequencing of samples derived from patients with PanNETs and rare genetic syndromes such as multiple endocrine neoplasia type 1 (MEN1) and Von Hippel–Lindau (VHL) syndrome reveals the involvement of MEN1, DAXX/ATRX, and the mammalian target of rapamycin (mTOR) pathways in PanNET tumorigenesis. Gene knock-out/knock-in studies indicate that inactivation of factors including MEN1 and abnormal PI3K/mTOR signaling uncouples endocrine cell cycle progression from the control of environmental cues such as glucose, leading to islet cell overgrowth. In addition, accumulating evidence suggests that further impairment of endothelial–endocrine cell interactions contributes to tumor invasion and metastasis. Recent phase III clinical trials have shown that therapeutic interventions, such as sunitinib and everolimus, targeting those signal transduction pathways improve disease-free survival rates. Yet, cure in the setting of advanced disease remains elusive. Further advances in our understanding of the molecular mechanisms of PanNETs and improved preclinical models will assist in developing personalized therapy utilizing novel drugs to provide prolonged control or even cure the disease.

Pancreatic neuroendocrine tumors (PanNETs) arise from the endocrine cells of the pancreas, also known as the islets of Langerhans. The full spectrum of disease ranges from early-stage benign hyperplasia or adenoma and localized well-differentiated neuroendocrine tumors (NETs) to more advanced metastatic or poorly differentiated neuroendocrine carcinomas (NEC) (Figure 1, A and B) (1). PanNETs can be broadly classified into functional (hormone producing) and nonfunctional tumors. Uncontrolled proliferation of islet cells increases cell numbers, and further genetic and/or microenvironmental insults induce de-differentiation of the endocrine cells. Although these tumor cells may retain their ability to release one or more hormones, such as insulin, gastrin, and glucagon, which can cause symptoms, more often the cancer cells do not cause hormone, but malignancy-related symptoms. Nonfunctional tumors account for 85% of PanNETs (2,3) and have a statistically significantly worse prognosis (4) compared to functioning PanNETs, wherein a specific syndrome from hormone oversecretion is seen. More recently, defined grading systems have helped classify these rare tumors into well-differentiated, moderately differentiated, and poorly differentiated NETs. Poorly differentiated, nonfunctional PanNETs may be further classified as small cell or large cell histological type (5).

NETs are believed to arise from various endocrine cells whose normal function is to serve at the neuroendocrine interface. For example, NETs can arise at any point in the gastrointestinal tract and bronchopulmonary system (6). Small intestinal NETs were first distinguished from other tumors in the early 20th century. They were named carcinoid tumors because their slow growth was considered to be “cancer-like” rather than truly cancerous (7). The term “gastrointestinal carcinoid” should not be used, nor should “gastroenteropancreatic neuroendocrine tumors” be used to describe PanNETs, as PanNETs are clearly a distinct biological disease with unique genetic anomalies and thus should be studied independent of NETs from other sites.

PanNETs comprise 1.3% to 2.8% of new pancreatic malignancies each year (Figure 1C) (1,2,8,9). Despite the relatively low incidence of PanNET diagnoses, the prevalence of PanNETs has been estimated to be 9.9% of pancreatic malignancies (8). PanNETs prototypically have a more indolent clinical course than do pancreatic ductal adenocarcinomas, which arise from the exocrine pancreas and comprise the majority of pancreatic cancer diagnoses (10,11). Remarkably, one of every five incidentally diagnosed pancreatic tumors is a PanNET (12), and up to 40% of all PanNETs are incidentally diagnosed (13), highlighting the number of clinically asymptomatic cases. In fact, the prevalence of PanNETs found in autopsy studies has been shown to be as high as 10%, suggesting that there may be a statistically significant number of undiagnosed PanNETs in the general population (14). The approach to treatment is management of hormonal hypersecretion and surgical resection when feasible. Unfortunately, asymptomatic PanNETs are often detected at advanced stages when complete surgical removal is not possible.
The incidence of PanNETs has been statistically significantly increasing, whereas overall survival has remained relatively unchanged over the past several decades (2,4,9). The trend of increasing incidence may be explained by multiple factors: 1) advances in imaging, which have allowed for increased detection of incidental PanNETs, or “incidentalomas” (13); 2) an aging population, as the incidence of PanNETs increases with age and peaks in the eighth decade (4); and 3) increased awareness, as the death of high-profile individuals from this disease has garnered much media attention (15).

Epidemiological studies support the conclusion that the incidence of PanNETs is increasing, and the number of asymptomatic cases and incidental diagnoses is high. Thus, PanNETs may not be as rare as they are often perceived to be (16), but rather are becoming more common. The five-year survival rate has not statistically significantly changed in more than 40 years, according to the report “Cancer Facts and Figures 2012” released by the American Cancer Society. This may change, as effective therapies were lacking until very recently when two targeted therapies received Food and Drug Administration (FDA) approval in just the last year. This delay in development of therapies highlights the urgent need for better understanding of the molecular biology and validated preclinical models of PanNETs as well as the need for increased funding to support PanNET research. The goal of the present review is to summarize current advances in signal transduction pathways underlying PanNET tumorigenesis and strategies to impact these molecular alterations.

**Cell Signaling in Pancreatic Islet Cells**

**Structure**

The main function of pancreatic islets is to modulate blood glucose levels through either release of insulin from β cells promoting cellular use of glucose or release of glucagon from α cells stimulating glucose storage. To serve their hormone secretory function, islet cells are surrounded by an endothelial capillary network. Both α and β cells do not make but rely on endothelial cells for the extracellular matrix.

**Proliferation**

Endocrine cells constantly monitor and respond to the drop or raise of glucose levels by secreting insulin or glucagon. Under certain physiological or pathophysiological conditions such as pregnancy and obesity, increased body masses constantly demand more hormones, which stimulate endocrine cell proliferation through Menin-p27/p18 control of G1 to S phase cell cycle progression (Figure 2) (17). On the other hand, damage to the cells and/or DNA blocks the cell cycle progression via upregulation of p53 (18).

Endocrine cells attach to the vascular endothelium through integrin α3β1 or α6β1 and endothelial laminin α5β1/γ (19,20).
This interaction stimulates growth/survival/migration signaling and focal adhesion kinase (FAK)/Src-related activation of Rho, Rac/PAK, RAS, and nuclear factor kB (NFkB) (Figure 2). Newly generated endocrine cells release vascular endothelial growth factor (VEGF), which binds to VEGFR and attracts endothelial cells (20). Endocrine–integrin and endothelial–laminin interactions promote islet cell survival and growth.

The endocrine cell numbers and glucose hormone levels are precisely balanced through multisignal control of cell cycle progression. Dysfunctional pathways due to genetic mutations and/or microenvironment changes can result in abnormal islet cell growth and development of PanNETs.

Genetic Factors Associated With PanNETs
An acute increase in the glucose level triggers the release of insulin from β cells, whereas a chronic glucose elevation such as pregnancy stimulates β-cell proliferation to match the increased hormone demands. Genetic mutations that mimic glucose stimulation can promote endocrine cell proliferation analogous to the physiological changes in pregnancy, resulting in the development of PanNETs. Indeed, mutations on MEN1, a gene that controls G1/S progression, are common among PanNET patients (Table 1) (21,22). Furthermore, gene mutations in DNA damage control genes such as DAXX are also linked to PanNETs (21). Uncontrolled proliferation increases islet cell numbers and hormone release, which contributes to functional PanNETs. As tumors grow, other genetic alterations can occur and promote de-differentiation, invasion, and migration. For example, ATRX and mTOR mutations are associated with PanNETs (Table 1) (21).

Impairment of G1/S Progression Control
PI3K Signaling
A single PI3K mutation was found in 1.5% of sporadic PanNETs (1/68), and this patient also had a mutation in PTEN, an inhibitory
PTEN has been reported to be mutated in 7.4% of sporadic PanNETs, and 100% of tumors with PTEN mutations also had a mutation in either MEN1 or DAXX (21). Loss of PTEN expression has been correlated with advanced PanNET stage (23). Another inhibitory gene of PI3K pathways, tuberous sclerosis complex (TSC) (24), was found to be mutated in 8.8% of sporadic PanNETs. The validity of this observation is supported by the report of PanNETs arising in patients with tuberous sclerosis in the literature, albeit uncommonly (25,26). Microarray expression profiling of PanNETs showed downregulation of PTEN and TSC2 in the majority of primary PanNETs (27). In addition, lower expression was associated with poorer disease-free and overall survival (27). Those observations demonstrated that hyperactivation of PI3K/Akt may contribute to PanNET development and behavior (Figure 3A).

**mTOR Pathways**

mTOR regulates cell survival, proliferation, and motility. As a member of the PI3K family, mTOR transmits upstream stimuli in the PanNETs, pancreatic neuroendocrine tumors (PanNETs).

Table 1. Comparison of commonly mutated genes in PanNETs and PDACs*

<table>
<thead>
<tr>
<th>Genes</th>
<th>PDACs</th>
<th>PanNETs</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRAS</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>TP53</td>
<td>85%</td>
<td>3%</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>25%</td>
<td>0%</td>
</tr>
<tr>
<td>TGFBR1/SMAD3/SMAD4</td>
<td>38%</td>
<td>0%</td>
</tr>
<tr>
<td>MEN1</td>
<td>0%</td>
<td>44%</td>
</tr>
<tr>
<td>DAXX/ATRX</td>
<td>0%</td>
<td>43%</td>
</tr>
<tr>
<td>mTOR</td>
<td>0.8%</td>
<td>15%</td>
</tr>
</tbody>
</table>

* PanNETs = pancreatic neuroendocrine tumors; PDACs, pancreatic ductal adenocarcinomas.

Thus, disabling the attachment requirement needed for cancer cells to invade and migrate. ATRX mutation-modulated chromatin modification may play a role in the abnormal activation of FAK/Src and mTOR pathways in PanNET. Underlines indicate the mutated genes or activated protein. EC = endothelial cell; FAK = focal adhesion kinase; HBA1 = hemoglobin-α; JNK = c-Jun N-terminal kinase; MEN1 = multiple endocrine neoplasia type 1; mTOR = mammalian target of rapamycin; NO = nitric oxide; PAK = p21-activated kinase; PI3K/Akt = phosphoinositide-3-kinase/protein kinase B.
including insulin and IGF1/2 and also senses cell nutrient, energy, and redox statuses (33,34). Abnormal activation of mTOR is common in patients with PanNETs (27,35). In a Rip-Tag2 transgenic mouse model of PanNET, dual targeting of the epidermal growth factor receptor pathway (erlotinib) and mTOR (rapamycin) resulted in prolonged survival compared to monotherapy and ablation of resistance/relapse seen with rapamycin alone (36).

**MEN1 Gene**

The *MEN1* gene encodes for the protein Menin. Individuals with *MEN1* mutations develop multiple endocrine neoplasia type 1 and are predisposed to a triad of NETs of the pancreas, pituitary, and parathyroid glands. More than 1300 mutations in *MEN1* have been identified to date, with the majority of mutations resulting in loss of function of *MEN1* (37). Cases of familial inheritance have long been documented, and the genetic component of this syndrome has always been suspected as *MEN1* is inherited in an autosomal dominant fashion (38), but only recently has *MEN1* been identified as the most common mutation in sporadic cases of PanNETs. Consistent with previous reports that 40% of PanNETs display abnormally low nuclear staining of menin (39), the mutation rate of *MEN1* in sporadic PanNETs was observed to be 44% (21). The finding that *MEN1* was the most commonly observed mutation in sporadic cases implicates it as a key player in the development/progression/initiation of PanNETs. Because absence of menin correlates with tumor development, menin is thought to act as a tumor suppressor gene.

Homozygous deletion of the *MEN1* gene is embryonic lethal. In accordance with its ubiquitous expression profile, this finding implicates menin in normal embryological development (40). Mice with heterozygous deletion of the *MEN1* gene develop multiple endocrine tumors, reminiscent of the human syndrome, but do so with prolonged latency (41). Conditional mutation of *MEN1* in pancreatic β cells results in the development of insulin-producing PanNETs with full penetrance (42,43). These tumors also recapitulate the early progression of PanNETs from normal islets to islet cell hyperplasia/dysplasia to frank islet adenomas. Interestingly, poorly differentiated/metastatic NECs were not observed in these mice, suggesting that additional mutational events are needed for the formation of high-grade NEC. The high rate of *MEN1* mutation in sporadic PanNETs, the substantial rate of PanNETs occurring in the *MEN1* familial syndrome, and the recapitulation of human disease in transgenic and conditional mouse models all provide strong evidence that mutation of *MEN1* is a proximal event that initiates PanNET tumorigenesis.

**PI3K Regulation of Menin Expression by Foxo1**

High glucose levels activate PI3K/Akt, which leads to the phosphorylation of the transcription factor Foxo1 (44). Phosphorylated Foxo1 has decreased binding affinity to the *MEN1* promoter, resulting in inhibition of Menin expression and enhanced proliferation. Overexpression of the *MEN1* gene suppressed the malignant phenotype of cancer cells through several pathways, including FAK signaling (45).

Menin is critical to normal pancreatic β-cell homeostasis and glucose sensing. In pregnancy, decreased Menin has been shown to mediate physiological maternal islet cell expansion, and overexpression of Menin prevents islet cell growth during pregnancy, resulting in gestational diabetes (46). Similarly, Menin has been directly implicated in the mitogenic response of β cells to glucose, and overexpression of Menin abrogated β-cell proliferation (44). These findings support the observation that impaired fasting glucose, decreased insulin sensitivity, and hyperinsulinemia occur more frequently in patients with *MEN1* mutations (47).

**Menin Modulation of p27/p18 and G1/S Progression**

Menin promotes the expression of cell cycle inhibitors. Menin appears to be ubiquitously expressed and has been shown to directly regulate proliferation in normal pancreatic islet cells (44,46,48,49). The tumor suppressor Menin is part of the mixed lineage leukemia (MLL) histone methyl transferase complex. Menin has been shown to bind directly to MLL (50) and is required for MLL-mediated H3K4 methyltransferase activity (48,51). The tumor suppressor activity of Menin is due to inhibition of proliferation, as menin has been shown to maintain promoter activity of CDKN2C and CDKN1B through H3K4 methylation (48,52). In addition, that deletion of *MEN1* acutely enhances β-cell proliferation in vivo, accompanied by decreased CDKN2C and CDKN1B in pancreatic islets within days of *MEN1* excision. Increased CDK2 activity and facilitated entry of cells from G1 to S phase of cell cycle were also observed with *MEN1* deletion in vitro (49) (Figure 3A). MLL insufficiency in pancreatic islets resulted in decreased CDKN2C and CDKN1B expression, suggesting that Menin and MLL cooperate to regulate cell cycle activity (53).

**DAXX Modulation of p53 and Cell Cycle**

The adapter protein DAXX forms an MDM2-DAXX-USP7 complex and regulates the ubiquitination activity of the RING-finger E3 ligase MDM2. DNA damage induces DAXX de-association with MDM2/USP7. This increases MDM2 autoubiquitination and reduces MDM2/USP7. This increases MDM2 autoubiquitination and facilitates entry of cells from G1 to S phase of cell cycle. Increased CDK2 activity and facilitated entry of cells from G1 to S phase of cell cycle were also observed with *MEN1* deletion in vitro (49) (Figure 3A). MLL insufficiency in pancreatic islets resulted in decreased CDKN2C and CDKN1B expression, suggesting that Menin and MLL cooperate to regulate cell cycle activity (53).

**Endothelial-Independent Islet Cell Survival in PanNETs**

Endocrine–endothelial cell interactions are essential for hormone release into the circulation. Loss of the islet cell attachment to the endothelium impairs their function and survival (55). Gene mutations that promote endocrine cell survival independent of endothelial anchorage contribute to PanNET tumorigenesis.

**DAXX and ATRX**

These proteins function together as a dimer and are implicated in chromatin stabilization (36). DAXX and ATRX have been shown to specifically associate to areas of telomeric chromatin (57). DAXX/ATRX loss is correlated with alternative lengthening of telomeres, a telomerase-independent mechanism of telomere lengthening. DAXX and ATRX mutations in PanNETs have been confirmed to occur in a mutually exclusive manner, providing further evidence support that DAXX/ATRX-mediated senescence is a mechanism for PanNET tumorigenesis.
of their cooperation, such that loss of either protein may initiate PanNET tumorigenesis. The genetic, biological, and prognostic implications of DAXX/ATRX mutational activation/suppression have been nicely reviewed recently (59).

VHL Tumor Suppressor

This is the causative gene in VHL syndrome. Patients with germline mutations in VHL develop multiple tumors including PanNETs (60). Although only some VHL patients develop PanNETs, the finding that VHL is functionally inactive in some PanNETs indicates the importance of the VHL tumor suppressor in a subset of PanNETs (61). In fact, molecular profiling of VHL PanNETs in comparison to sporadic PanNETs proves that VHL PanNETs have specific genetic alterations, most striking are those related to angiogenesis and hypoxia-inducible factor (HIF) signaling (62). Thus, VHL driven PanNETs likely represent a subset of PanNETs with unique molecular features that distinguish it from MEN1 DAXX/ATRX–driven tumors.

RET Proto-oncogene

The proto-oncogene RET can activate RAS/extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/AKT, p38 mitogen-activated protein kinase (MAPK), and c-Jun N-terminal kinase (JNK) signaling pathways. These signaling pathways are activated via binding of adaptor proteins to intracellular tyrosine residues of RET phosphorylated by its own kinase activity. RET is profoundly involved in the development of several human neuroendocrine diseases. The constitutive activation of RET by somatic rearrangement with other partner genes or germline mutations causes a considerable population of human papillary thyroid carcinomas or MEN type 2A and 2B, respectively, whereas the dysfunction of RET by germline missense and/or nonsense mutations causes Hirschsprung disease (63). Genomic profiling studies of PanNETs demonstrate gains on chromosomes 5, 7, 10, 12, 14, 17, and 20 in PanNETs. Candidate genes for targeted therapy in regions of chromosomal gains in PanNETs include RET (64).

RAS, Raf, MEK, ERK Signaling in PanNETs

K-Ras mutations are virtually nonexistent in PanNETs (21). However, inducible overexpression of Raf1 in a PanNET cell line decreased neuroendocrine marker expression and secretory granules (65). These findings were found to be mediated by FAK, as overexpressed Raf1 also increased phosphorylation of FAK at the site of tyrosine 407 (Y407), the negative regulator of FAK kinase activity (66). This was associated with decreases in cell adhesion and migration (67).

Although there are a paucity of PanNET cell lines available for study, BON1 is a permanent cell line derived from a lymph node metastasis of a human NET of the pancreas (68). The cells are able to produce tumors in nude mice and to express functional receptors for acetylcholine, 5-HT, isoproterenol, gastrin, and somatostatin. Suppression of Raf1 in BON 1 cells induced FAK activation as well as increased invasion and aggressiveness, whereas MEK inhibition had no effect on migration. Moreover, PanNET cells are known to have low levels of Raf1 activation (65). Raf deficiency may contribute to invasion and migration in late-stage PanNETs via FAK activation (Figure 3B). These data provide a stark contrast to the biology of pancreatic ductal adenocarcinomas, where activation of the RAS-Raf-MEK-ERK pathway promotes these tumors, and inhibition of Raf1 exhibits antitumor activity (69). However, it is unclear what the role of Raf in PanNETs is, due to the rarity of RAS-RAF-MEK-ERK activation. Thus, it is unlikely that the RAS-RAF-MEK-ERK pathway is implicated in PanNET proliferation/survival due to lack of KRAS mutations (21). Raf signaling may be involved in the metastatic process through alternative pathways that are associated with FAK activation (67).

ATRX has been proposed as a global regulator of transcription via remodeling chromatin. ATRXX belongs to a subgroup of the helicase superfamily contributing to DNA repair and transcription regulation (70). For example, ATRXX has been reported to down-regulate α-globin (hemoglobin-α, HBA1) (71), which is expressed in arterial endothelial cells and enriched myoendothelial junctions (72). HBA1 modulates nitric oxide (NO) bioactivity in small arteries via NO synthase/cytochrome b5 reductase 3 (72). Genetic deletion attenuates HBA1-promoted NO bioactivity (72). NO is known to induce PI3K/Akt/Src-related survival of insulin-producing cells (73). ATRXX mutation may decrease NO-modulated PI3K/Src activity and survival through downregulation of HBA1 expression in the endothelial surrounding islet cells. Dysfunctional of pancreatic endothelium may promote islet cell growth. In addition, inactivation of DAXX or ATRX is associated with alternative lengthening of telomeres in PanNETs (58). Preventing telomere shortening can lead to unlimited cell cycling of malignant islet cells. DAXX/ATRX mutation/deficiency may mimic endothelial laminin and endocrine–integrin interaction–triggered survival signaling, which bypasses the endothelial anchorage stimuli (Figure 3B). On the other hand, it is possible that DAXXX/ATRX mutations may be associated with FAK/Src activation, contributing to Rho/Rac/PAK-mediated survival, invasion, and migration. The association between DAXXX/ ATRXX mutation and FAK/Src activation in PanNETs remains to be addressed as this may be one of the key mechanisms underlying the deadly metastatic transformation of late-stage PanNETs.

Notch Signaling

Notch has been identified as a tumor suppressor in various malignancies. Whereas the downstream elements of Notch signaling pathway are conserved in gastroenteropancreatic NETs and PanNET cell lines (74), BON cells lack Notch, and overexpression of Notch in an inducible model resulted in antiproliferative effects and suppression of neuroendocrine markers and secretory granules (75). The histone deacetylase inhibitor valproic acid has also been shown to have antitumor effects in PanNET cells by upregulating Notch (76), and a recent phase 2 study of VPA in patients with low-grade PanNETs resulted in 1 of 7 partial responses, 4 of 7 stable disease, and validation of Notch upregulation in all patients (76,77). Whether Notch activation in PanNET cells is a specific or nonspecific effect of histone deacetylase inhibitors remains to be determined.

Genetics of PanNET vs Pancreatic Neuroendocrine Carcinoma (PNEC)

Although MEN1 and DAXX/ATRX mutations are the most common mutations in well- to moderately differentiated PanNETs (87%), patients with these mutations tend to have a better prognosis—approximately double the median survival—than those without these mutations (21). The protective effect of these mutations
are particularly striking in patients with metastatic PanNETs, which have mutations in both MEN1 and DAXX/ATRX, as the median survival in these patients was nearly triple that of the wild type for both MEN1 and DAXX/ATRX (21). Although the study from Jiao and colleagues (21) excluded poorly differentiated PNECs, a recent study compared genetic aberrations and immunohistochemical features of low- and intermediate-grade PanNETs vs small and large cell–type PNECs. Interestingly, PNECs seemed to represent a genetically distinct biology in comparison to PanNETs: Aberrant p53 and Rb staining occurred exclusively in PNECs at a rate of 95% and 74%, respectively. Interestingly, aberrant p16 staining was not only exclusive to PNECs, but also occurred in a mutually exclusive manner with respect to Rb such that all PNECs had disruption of the p16/Rb pathway (78). Conversely, DAXX/ATRX mutations were exclusive to PanNET tumors and occurred mutually exclusive to each other and at a rate of 45%, in accordance with previous data (21). BCL-2 overexpression was more a common feature of PNECs (74%) vs PanNETs (18%). In addition, aberrant staining of p53, Rb/p16, and BCL2 were each statistically significantly correlated with increased mitotic rate and Ki67 labeling. K-Ras mutations and SMAD4/DPC4 staining aberrations, features of pancreatic ductal adenocarcinomas, were exclusively, albeit uncommonly found in PNECs.

PI3K regulates glucose uptake through its interactions with insulin receptor substrates. Oncogene PIK3CA and tumor suppressor PTEN mutations are implicated in insensitivity of tumors to insulin and IGF-1. PI3K activation and PTEN downregulation have been found in most PanNETs (23,27). Those observations have promoted therapeutic inhibition of PI3K signaling to treat PanNETs.

Angiogenesis and Hypoxia Signaling in PanNETs

Most PanNETs (low and high grade) exhibit a high degree of vascularization (79–81), a characteristic that reflects both the underlying organ physiology and molecular evolution of the disease. Pancreatic islets predominantly consist of endocrine cells and endothelial cells (82). It is well recognized that a considerable degree of cross-talk exists between these two cell populations (83). Endocrine cells in the pancreas exhibit high levels of proangiogenic molecules, including members of the vascular endothelial growth factor (VEGF) family (84). Experimental studies have demonstrated that VEGF-A is not only critical for inducing islet microvasculature but is also required for maintenance of islet function (glucose regulation through insulin secretion) (84–86). Morphometric studies have revealed that the islets are supplied by a capillary network that is markedly denser than capillaries found in exocrine tissue (87). The volume of blood flowing through the islets is also statistically significantly (five-fold) greater than exocrine pancreatic tissue (88).

Although VEGF is considered to be the principal driver of the angiogenic switch in PanNETs (84), studies have also demonstrated activation of platelet-derived growth factor (PDGF), c-kit, and mTOR pathways as biologically significant players in PanNET angiogenesis (27,89). In tissue specimens, increased expression of VEGFR2/3, PDGF, and c-kit have been observed (89,90). An interesting phenomenon specific to PanNETs is that low-grade benign–appearing tumors exhibit higher microvessel density than high-grade tumors. Furthermore, in contrast to most epithelial tumors and carcinomas, higher microvessel density is associated with a survival benefit and better prognosis in PanNET (91).

Despite this paradox, high-grade PanNETs show activation of key regulatory pathways involved in hypoxic signaling and angiogenesis.

The HIF-1 pathway is a critical regulator of more than 100 genes involved in angiogenesis including VEGF, PDGF, angiopoietin (Ang-1 and 2), delta-like ligand (DLL4), and matrix metalloproteinases (92,93). HIF-1 transcriptional machinery is directly regulated by a family of prolyl hydroxylase domain proteins (PHDs) that mediate the molecular response to changes in tissue oxygenation. Under normoxic conditions, proline residues in the inducible subunit, HIF-1α, are hydroxylated by the PHDs (94,95) followed by ubiquitination and proteasomal degradation. Under hypoxic conditions, the functional activity of PHDs is inhibited, leading to stabilization and transcriptional activation of HIF-1α.

Studies have previously examined expression of angiogenesis and HIF-related molecules (80). In the study by Couvelard et al. (80), immunostaining sections from 45 patients with PanNET revealed that well-differentiated tumors had high cytoplasmic VEGF and HIF-1α expression whereas poorly differentiated carcinomas were associated with nuclear HIF-1α expression. Presence of HIF-1α nuclear expression was associated with the presence of necrosis, large tumor size, low microvascular density and shorter survival. Cytosplasmic and nuclear expression of PHDs has also been reported in PanNETs (96). Higher cytoplasmic and nuclear expression of the three PHD isoforms was observed in aggressive PanNETs with lymph node metastases. Tissue microarray analysis of 109 patients has shown that high nuclear expression of PHD1 and PHD3 correlates with poorer survival. In the same study, tumors with high cytoplasmic expression of PHD showed lower microvascular density, implicating PHDs in the regulation of angiogenesis in PanNET. Induction of Ang-2, a ligand of the endothelial tyrosine kinase, has also been demonstrated in PanNETs. Serum levels of Ang-2 are elevated in patients with NETs, and overexpression of Ang-2 has been shown to stimulate neoangiogenesis and enhanced lymphatic metastasis in xenografts (97). Circulating Ang-2 levels correlated with the presence of metastatic disease in patients with NETs, highlighting its potential utility as an adverse prognostic marker in PanNET.

Double-Hit Model of PanNET Tumorigenesis

Jiao and co-workers’ data show that all PTEN-mutated tumors have the second mutation of MEN1 and/or DAXX, implying that aberrant multiple pathways may be involved in the development of PanNET (21). It is possible that mutations of the checkpoint genes such as PTEN in PI3K/mTOR signaling and p53 contribute to constant cell progression, leading to overgrowth/hypertrophy that is escaped from the controls mediated by environmental cues such as nutrients/glucose levels and DNA damage (Figure 4). Increased numbers of islet cells can elevate insulin levels and induce oxygen and nutrient deficiency as the tumor grows larger. This stressful microenvironment can promote further mutations such as ATRX and mTOR genes. The second hit may uncouple the cell–cell interaction control of endocrine cell proliferation, likely through constant activation of FAK/Src or inside-outside–associated signaling.
Therapeutic Interventions Through Interference With PanNET Signaling Pathways

Targeting Angiogenesis in PanNET

Given the highly vascularized nature of PanNET, several preclinical and clinical studies have explored the potential of inhibiting angiogenesis with encouraging results. In 2011, randomized phase III studies in PanNET led to practice-changing outcomes and clinical approval of two new agents, the multitargeted tyrosine kinase inhibitor sunitinib and the mTOR inhibitor everolimus. Additional phase I and phase II trials of the antiangiogenic agent bevacizumab are currently under way.

Sunitinib

Sunitinib is a multitargeted protein tyrosine kinase inhibitor that targets VEGFR1, 2, and 3, PDGFRα and β, Ckit, flt-3, and the RET proto-oncogene. The drug was approved by the FDA in 2006 for the treatment of renal cell carcinomas and imatinib-resistant gastrointestinal stromal tumors. After preclinical studies indicated that sunitinib increased survival rates in animal models, phase II and III clinical trials were performed (98,99). Sunitinib caused tumor shrinkage and prolonged progression-free survival from 5.5 to 11.4 months in patients with advanced PanNETs (98,99). In 2011, the FDA approved sunitinib for the treatment of progressive, well-differentiated PanNETs in patients with unresectable locally advanced or metastatic tumors (100). The success of this trial provided proof that targeting distinctive tumor-related signaling pathways could improve clinical outcomes and should be the future approach to the development of therapeutic interventions for PanNETs.

Everolimus

mTOR-related mutations have been found in 15% of PanNET cases (21,59). Preclinical trials indicated that everolimus, an mTOR inhibitor, increased survival (36). Results from a phase II clinical trial (RAD001 in Advanced Neuroendocrine Tumors [RADIANT-1]) indicated that chromogranin A and neuron-specific enolase responses could be used as prognostic markers in patients with advanced PanNETs (101). In phase III clinical trials (RADIANT-3 and RADIANT-2), tumor-progression-free survival in everolimus-treated patients (11 months) was longer than control patients (4.6 months) (Figure 5) (102,103). In 2011, everolimus was approved by the FDA for the treatment of progressive or metastatic PanNETs not surgically removable. Successful control of PanNET progression in everolimus-treated patients supports the notion that mTOR signaling plays a critical role in PanNET tumorigenesis.

Both sunitinib and everolimus have a potent effect on inhibition of angiogenesis, which may cause adaptive resistance, a phenomenon often seen in antiangiogenesis-based therapies (104–106). The marked enthusiasm during the early clinical development of antiangiogenic agents has now been transformed into skepticism and concern over the safety of these agents. Although it was initially believed that antiangiogenic therapies would not induce resistance, several preclinical studies have demonstrated that tumors indeed develop adaptive resistance to antiangiogenic treatment. An increasing body of evidence also suggests that antiangiogenic agents alter the biology and the natural progression of tumors in an unfavorable manner (73,74).

This may explain the fact that sunitinib/everolimus extend survival but do not cure PanNETs. Sunitinib induces vascular

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Figure 4. A double-hit model of pancreatic neuroendocrine tumors. The first hit: Mutation of the checkpoint genes such as multiple endocrine neoplasia type 1 and p53 causes constant cell cycle progression even under conditions of low glucose levels and DNA damage. Increased numbers of islet cells can elevate insulin levels and induce a stressful microenvironment due to oxygen and nutrient deficiency. The second hit: ATRX and mTOR gene mutation can cause cell growth in the absence of cell-cell interaction through constant activation of focal adhesion kinase/Src or inside-outside-associated signaling.
pruning and intratumoral hypoxia in RIP-Tag2 mice and implanted PANC-1 cells (107). In the same model, it has also been shown that sunitinib reduces tumor growth but increases tumor invasion and metastasis, which was associated with c-met activation (107). Concurrent inhibition of VEGF and c-met overcomes this resistance and results in decreased invasion and metastasis (107).

**Menin Agonist**

*MEN1* mutations are linked to 44% of sporadic PanNETs, and Menin deficiency promotes endocrine cell proliferation. Menin activation is expected to suppress islet tumor proliferation. However, studies on screening compounds to mimic menin effects are very limited. Investigations searching for Menin agonists may lead to more effective therapies for PanNETs.

**RET Inhibitors**

*RET* is localized to chromosome 10, and genomic profiling studies have demonstrated gains of chromosome 10 in PanNETs. There are a large number of ATP-competitive RET kinase inhibitors being evaluated in preclinical studies (108). Some of these drugs have been shown to inhibit RET both in cell-free assays and in cells, thus causing proliferation arrest in oncogenic RET-transfected cells and in papillary thyroid carcinoma (PTC) cells expressing the *RET/PTC1* oncogene, but not in RET-negative control cells. In addition, RET-mediated signaling was inhibited through the ERK and JNK pathways (109). Specific RET inhibitors are actively being studied in thyroid malignancy. At present, there are no clinical trials of specific RET inhibitors in PanNETs except for those involving sunitinib, which inhibits RET in addition to several other tyrosine kinases.

**Discussion**

**Targeted Therapies**

The NET task force of the National Cancer Institute steering committee recently published the clinical and translational needs...
of this unique patient population. Rationally designed novel multitargeted combination strategies are necessary to address the treatment needs of patients with PanNETs. Advances in our knowledge of PanNET signal transduction pathways will enable screening of compounds that specifically target tumor-associated signaling. Targeting angiogenesis (sunitinib) and the PI3K/mTOR signal transduction (everolimus) delays progression-free and overall survival; however, much work remains to be done as we do not as yet have second-line therapies for patients once they progress on these regimens. The optimal sequence and combinations of these and other novel compounds need further study.

Personalized Treatments

Although progression-free survival has been demonstrated to be improved, objective response rates for everolimus and sunitinib in the treatment of advanced PanNETs are less than 10%. Preclinical studies suggest enhanced antitumor effects with combined mTOR and VEGF-targeted therapy. Data from a multicenter phase II trial of the mTOR inhibitor temsirolimus and the VEGF-A monoclonal antibody bevacizumab in well- or moderately differentiated PanNETs demonstrate a confirmed partial response in 11 of the first 25 evaluable patients. Twenty of 25 patients were progression free at six months. The combination of temsirolimus/bevacizumab was thought to have substantial activity in a multicenter phase II trial with responses well in excess of single targeted agents in PanNETs (110).

Due to an increased understanding of the molecular abnormalities in PanNETs, the treatment approach could be individualized based on the expected prognosis. Even though pancreatic NECs appear to be genetically and behaviorally distinct from well-differentiated PanNET cells, the fact that a minority of PanNET patients (those without MEN1 or DAXX/ATRX mutations) have an unfavorable prognosis strongly suggests that PanNETs without MEN1 or DAXX/ATRX mutations represent a biologically important subgroup of PanNETs with increased malignant potential and ability to progress to poorly differentiated NEC.

Similarly, mTOR and VEGF/VHL mutations are less common in PanNETs but carry a poorer prognosis. On the other hand, MEN1 mutations are correlated with DAXX/ATRX mutations in a majority of PanNETs (approximately 87%), and the majority of PanNETs are low to intermediate grade (approximately 89%), which is linked to a more favorable prognosis. Furthermore, some PanNET patients (approximately 15%) carry the mutations of genes in the mTOR pathways such as TCS2, PTEN, and PIK3CA. Thus, everolimus and sunitinib may demonstrate greater efficacy, response rates, and overall survival benefits if patients are first screened for mutations targeted by these drugs. Exploration of risks/benefits of identifying mutations for every PanNET patient can help design targeted therapies. For example, everolimus for mTOR inhibition would be likely to benefit patients with TCS, PTEN, and/or PIK3CA mutations.

Summary

Preclinical and clinical studies on the molecular biology of PanNETs indicate unique PanNET signaling events that are different from those in pancreatic ductal adenocarcinomas (MEN1/DAXX/ATRX/mTOR mutations vs K-RAS/TP53/CDKN2A/PTGFR/SMAD mutations, respectively) and other NETs. To serve their main function of hormone release and blood glucose modulation, endocrine cell cycle progression is delicately controlled by PI3K/mTOR/Menin/p27/p18 cascades. Genetic mutations along this signaling chain that attenuate glucose-sensing mechanisms can result in abnormal proliferation of islet cells in the development of PanNETs.

Molecular biological studies suggest that endocrine–endothelial contacts are mediated in part through laminin–integrin interactions. This acts as a mechanism to eliminate/control islet cells that are not close to the endothelium for hormone release to the circulation. Enlarged tumor sizes due to uncontrolled cell proliferation/growth can cause nutrient and oxygen deficiency in the center of the tumor. Cell survival under these stressful conditions can promote gene mutations that assist endocrine cells to escape their endothelial attachment requirement, likely leading to further tumor growth, invasion, and migration. DAXX, ATRX, and mTOR mutations in PanNETs can contribute to tumor progression, invasion, and metastasis.

Defining specific signal transduction pathways has promoted the design and development of new drugs for the treatment of aggressive PanNETs. For example, sunitinib inhibition of PI3K and everolimus blockade of mTOR activity cause tumor shrinkage and increase disease-specific progression-free survival. Rational combinations of multitargeted therapies will likely further extend survival or even cure PanNETs through inhibition of diverse pathways and prevent angiogenesis associated with adaptive resistance.

References


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