

Molecular genetic studies of pancreatic neuroendocrine tumors reveal new therapeutic approaches

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SYNOPSIS (99 words - 100 word limit)

Pancreatic neuroendocrine tumors (PNETs) can arise sporadically or as part of familial syndromes. Genetic studies of hereditary syndromes and whole exome sequencing analysis of sporadic PNETs have revealed the roles of some genes involved in PNET tumorigenesis. The most commonly mutated gene in PNETs is the multiple endocrine neoplasia type 1 (*MEN1*) gene, whose encoded protein menin, has roles in transcriptional regulation, genome stability, DNA repair, protein degradation, cell motility and adhesion, microRNA biogenesis, cell division, cell cycle control and epigenetic regulation. Therapies targeting epigenetic regulation and *MEN1* gene replacement have been reported to be effective in pre-clinical models.

KEY POINTS (up to 5)

- Pancreatic Neuroendocrine Tumors (PNETs) can occur as sporadic neoplasms or as part of hereditary syndromes such as multiple endocrine neoplasia type 1 (MEN1).
- MEN1, which is an autosomal dominant disorder, is due to loss-of-function mutations of the tumour suppressor *MEN1* gene that encodes menin.
- Approximately 40% of non-familial (i.e. sporadic) PNETs have *MEN1* mutations, with subsequent loss of menin which acts as a tumour suppressor.
- Menin is a scaffold protein with roles in transcriptional regulation, genome stability, DNA repair, protein degradation, cell motility and adhesion, microRNA (miRNA) biogenesis, cell division, cell cycle control, epigenetic regulation and Wnt signaling.
- Emerging therapies targeting the functional roles of menin with *Men1* gene replacement therapy, epigenetic modulators and antagonists of Wnt-signaling may prove useful for future treatment of PNETs.

INTRODUCTION

Pancreatic neuroendocrine tumors (PNETs), which account for 1-2% of all pancreatic neoplasms, are pathologically heterogeneous and consist of epithelial cells with phenotypic and ultrastructural neuroendocrine differentiation^{1,2}. PNETs may be classified according to their proliferation, which is assessed by the Ki-67 index or mitotic count, with: well-differentiated PNETs grade 1 (G1) having a Ki-67 index of <3% or mitotic count of <2 mitoses/10 high-power fields (HPF); well-differentiated grade 2 (G2) PNETs having a Ki-67 index of 3-20% or 2-20 mitoses/10 HPF; and well-differentiated grade 3 (G3) PNETs or poorly differentiated pancreatic neuroendocrine carcinomas (PNECs) having a Ki-67 index of >20% and >20 mitoses/10 HPF^{1,3,4}. The incidence of PNETs, also known as pancreatic islet cell tumors, has doubled between 1973 and 2004 from 0.16 to 0.33 per 100,000 individuals, and this may in part be due to improvements in diagnostic imaging and detection, and autopsy studies have shown that as many as 0.8%-10% of individuals have PNETs^{2,5-7}. PNETs may secrete hormones or vasoactive substances, such as gastrin, insulin, glucagon or vasoactive intestinal peptide (VIP), and be associated with clinical symptoms due to the hormonal over production, or they may be non-secreting (i.e. non-functional) and clinically present often with locally advanced or metastatic disease⁸.

Treatments for primary PNETs include surgery, while nonresectable PNETs and metastases are treated with biotherapies that include somatostatin analogues, inhibitors of receptors and monoclonal antibodies, chemotherapy and radionuclide therapy⁹. The median survival time for patients with PNETs is ~3.6 years¹⁰, while prognosis for patients whose PNETs have metastasised is poor with a survival of 1-3 years^{5,6,11,12}. However, the molecular pathology of well-differentiated PNETs is not sufficiently well understood for prediction of: the aggressiveness of individual tumors that would reliably identify patients who would benefit from early therapy; or indolent disease for which the risk to benefit ratio of treatment may not be advantageous for patients¹³. Thus, improved understanding of the molecular basis of PNETs as well as better treatments are required. Recent preclinical studies have identified new cellular pathways and therapeutic targets for treating PNETs, and these include epigenetic modification, the β -catenin/Wnt pathway, hedgehog signaling, somatostatin receptors and *MEN1* gene replacement therapy. This chapter will review these advances in the molecular genetics of PNETs that have stemmed from studies of hereditary syndromes associated with PNETs, as well as sporadic PNETs, and the cellular pathways that these studies have identified for therapeutic approaches.

PNETS IN HEREDITARY SYNDROMES

PNETs most frequently (~90%) arise as a non-familial isolated endocrinopathy (i.e. sporadically), but they can also occur as part of a complex familial syndrome that includes multiple endocrine neoplasia type 1 (MEN1), von Hippel Lindau disease (VHL), neurofibromatosis type 1 (NF1) and the tuberous sclerosis (TS) complex^{1,2,9}. Indeed, PNETs have been reported to occur in 30-80% of MEN1 patients, >15% of VHL patients, <10% of NF1 patients and <1% of patients with TS⁹. These hereditary tumor syndromes will be briefly reviewed, as they provide molecular insights in PNET development .

MULTIPLE ENDOCRINE NEOPLASIA TYPE 1 (MEN1)

The most common hereditary syndrome associated with PNETs is MEN1, which is an autosomal dominant disorder characterised by the combined occurrence of two or more tumors involving the parathyroids, pancreatic islets, anterior pituitary and adrenals^{14,15}. In MEN1, PNETs are typically multiple whilst in sporadic cases tumors are generally solitary masses. Patients with MEN1 syndrome

have germline mutations in the *MEN1* tumor suppressor gene, which is located on chromosome 11q13 and encodes a 610 amino acid protein, menin. *MEN1* germline mutations are found in >90% of *MEN1* patients and comprise whole or partial gene deletions, frameshift deletions or insertions, in-frame deletions or insertions, and splice site, missense, and nonsense mutations, which result in a functional deficiency of menin^{16,17}. *MEN1* tumors have somatic mutations as well as germline mutations consistent with the Knudson two-hit hypothesis for the role of tumor suppressor genes in oncogenesis, and in the majority (>90%) of *MEN1* tumors the somatic abnormality is loss of heterozygosity (LOH), with the remaining 10% having intragenic deletions or point mutations¹⁷⁻¹⁹.

Menin is a ubiquitously expressed protein that functions as a nuclear scaffold protein that interacts with >40 interacting proteins (Figure 1), thereby enabling it to have multiple roles in pathways of cellular proliferation by influencing transcriptional regulation, genome stability, DNA repair, protein degradation, cell motility and adhesion, microRNA (miRNA) biogenesis, cell division, cell cycle control and epigenetic regulation^{17,20-25}. For example, menin inhibits: wingless integration 1 (Wnt) signaling by transferring β -catenin from the nucleus, which reduces cell proliferation^{21,26}; the activity of JunD proto-oncogene (JunD) by blocking its phosphorylation and therefore potentially subsequent interaction with co-activators^{22,27}, causing JunD to prevent rather than promote cell growth²⁸; and Hedgehog pathway signaling, which influences several functions including tumorigenesis, by recruitment of a protein arginine methyltransferase (PRMT5), which inactivates the Hedgehog pathway promoter growth arrest specific 1 (*Gas1*) gene²⁹ (Figure 2). In addition, menin interacts with the mixed lineage leukaemia protein 1 (MLL1) histone methyltransferase complex to methylate histone H3 (Lys4), causing chromatin modification and increased transcriptional activity of genes including those encoding the cyclin dependent kinase inhibitors p27 and p18, which are involved in cell cycle regulation^{30,31} (Figure 2). Menin also promotes the cytostatic effects of transforming growth factor-beta (TGF- β) by interaction with the small body size mothers against decapentaplegic homolog (Smad) pathway³²⁻³⁴ (Figure 2), as well as interacting with nuclear factor kappa B subunit (NF- κ B) proteins to modulate NF- κ B transactivation³⁵. Furthermore, menin can prevent the interaction between the GTPase Kirsten rat Sarcoma viral oncogene homolog (K-RAS) and sons of sevenless (SOS) that is essential for K-RAS activation³⁶. Moreover, in murine pancreatic β -cells menin has been shown to activate opposing K-RAS pathways that comprise a proliferative pathway, likely via regulation of

mitogen activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) phosphorylation, and an anti-proliferative pathway via ras association domain family member 1 (RASSF1A)³⁷. Thus, menin is considered to interact with K-RAS to block the MAPK/ERK pathway, thereby inhibiting proliferation, and menin loss removes this inhibition and leads to increased cell proliferation³⁷. Menin also acts as a suppressor of ERK-dependent phosphorylation of target proteins³⁸ (Figure 2). In addition, somatostatin receptor (SSTR) modulation of proliferation may also occur through K-RAS signaling, thereby highlighting the importance of K-RAS signaling in PNETs, and indicating that menin may play a possible role in SSTR downstream signaling³⁹ (Figure 2). Furthermore menin is an inhibitor of the phosphatidylinositol-3-kinase (PI3K)/viral oncogene of Akt8 retrovirus (Akt) (also known as protein kinase B (PKB)/mammalian target of rapamycin (mTOR) (PI3K/Akt/mTOR)) signaling pathway, by binding to Akt and preventing its translocation to the plasma membrane⁴⁰ (Figure 2). The mTOR pathway regulates cell proliferation, cell metabolism, survival, motility and autophagy⁴¹.

VON HIPPEL LINDAU (VHL)

PNETs develop in patients with VHL disease, which is an autosomal dominant disorder with an incidence of 1 in 36000 individuals⁴². The *VHL* gene is located on chromosome 3p25 and encodes a protein, von Hippel-Lindau disease tumor suppressor (pVHL), involved in the oxygen-sensing pathway that regulates hypoxia-inducible factors (HIFs)⁴³. Truncation of the pVHL prevents the ubiquitination of HIF transcription factors leading to expression of target genes favouring angiogenesis (Figure 2). Individuals with VHL disease typically inherit a germline *VHL* mutation and those cells that acquire a somatic mutation (i.e. the “second hit”) of the wild-type allele develop tumors. *VHL* germline mutations, which are detected in virtually all VHL patients^{44,45}, comprise missense, frameshift, nonsense, in-frame deletions/insertions, large/complete deletions and splice site abnormalities with mutations located in exon 3 being associated with an increased risk of malignant PNETs⁴⁵⁻⁴⁷. Mouse models of conditional inactivation of *Vhl* in specific pancreatic cell populations have revealed that a lack of pVHL in pancreatic progenitor cells results in significant postnatal death, and the few surviving mice then develop islet hyperplasia and microcystic adenomas, which are also found to occur in 35-70% of VHL patients⁴⁸.

NEUROFIBROMATOSIS TYPE 1 (NF1)

Patients with NF1, which is an autosomal dominant disorder, may develop PNETs. The *NF1* tumor suppressor gene is located on chromosome 17q11.2 and encodes the cytoplasmic protein neurofibromin that controls cellular proliferation by inactivating the ras signal transduction pathway⁴⁹ (Figure 2). Loss of function *NF1* mutations lead to increased activity of the MAPK and PI3K/Akt/mTOR pathways⁵⁰, and the PNETs that develop have a great potential for malignancy^{51,52}.

TUBEROUS SCLEROSIS (TS)

Patients with TS may develop PNETs such as gastrinomas, insulinomas and non-functioning tumors. TS is an autosomal dominant disease caused by mutations in tuberous sclerosis 1 (*TSC1*) located on chromosome 9q34 or *TSC2* located on chromosome 16p13.3, which encode the hamartin and tuberin proteins, respectively. Mutations in *TSC1* and *TSC2* can activate the mTOR pathway to increase cell proliferation^{52,53} (Figure 2).

SPORADIC PNETS

The majority of sporadic PNETs are associated with somatic mutations, although some patients with sporadic PNETs may have germline mutations.

SOMATIC MUTATIONS IN SPORADIC PNETS

The commonest mutated gene in sporadic PNETs is the *MEN1* gene. Thus, 40% of sporadic PNETs have somatic *MEN1* mutations, indicating that *MEN1* mutations are major drivers in the development of all PNETs (Table 1)^{13,54}. In addition, whole exome and whole genome sequencing studies have identified the involvement of other genes and cellular pathways in chromatin modification, telomere length maintenance, growth control, DNA damage and cell metabolism as well as alterations in gene copy number, chromosomal rearrangements, gene fusions, telomere integrity and miRNAs^{13,54}.

Mutations in Genes Involved in Chromatin Remodelling

Approximately 25-45% of sporadic PNETs have somatic mutations in *MEN1* with loss of heterozygosity seen at the *MEN1* locus in 30-70% of cases, including those without somatic *MEN1* mutations^{13,54-56}; and ~45% have inactivating somatic mutations of either the death-domain-associated

protein (*DAXX*) or α -thalassemia/mental retardation syndrome X-linked (*ATRX*) genes that are associated with loss of protein expression, detected by immunohistochemical analysis of PNETs⁵⁷⁻⁶¹. Mutations in both *MEN1* and either *ATRX* or *DAXX* occur in 18-25% of PNETs, whereas *ATRX* and *DAXX* mutations do not occur concurrently in the same tumor^{13,54}, and this is consistent with the functional roles of *DAXX* and *ATRX* encoded proteins being within the same pathway⁶². Indeed, the *DAXX* and *ATRX* proteins, which are involved in apoptosis and chromatin remodelling^{13,54,63}, are critical for the maintenance of stable telomere length. Thus, both *DAXX* and *ATRX* proteins, which form a H3.3 histone chaperone complex, are required to enable incorporation of histone H3.3 at telomeres. *ATRX* is also required for suppressing the expression of the long non-coding RNA (lncRNA) telomeric repeat-containing RNA (TERRA), which maintains telomeric structure by regulating telomerase activity and heterochromatin formation⁶⁴⁻⁶⁸. Thus, *DAXX* and *ATRX* are involved in controlling telomere length, and the loss of *DAXX* or *ATRX* function due to mutations leads to an increase in telomere length in tumors with *DAXX/ATRX* mutations¹³, and these tumors are referred to as having the alternative lengthening of telomeres (ALT) phenotype (Figure 2)^{58,59}. Reactivation of telomerase leads to resistance to senescence in tumor cells with *ATRX* mutations, and tumors lacking telomerase activity display telomerase independent ALT^{69,70}. Patients that have PNETs with mutations in *MEN1*, *DAXX/ATRX* or a combination of both *MEN1* and *DAXX/ATRX* are reported to have a better prognosis when compared to patients who had PNETs that lacked these mutations⁵⁴. Somatic mutations of the set domain containing 2 (*SETD2*) gene, have also been detected in ~20% of sporadic PNETs (Table 1)^{13,71}. Inactivating mutations of *SETD2*, which is involved in chromatin remodelling, have previously been linked with clear cell renal carcinoma⁷². *MEN1*, *DAXX* and *ATRX* are tightly related to chromatin remodelling, thereby suggesting that PNETs are tightly regulated by epigenetic mechanisms, which are important drivers of tumorigenesis. Other epigenetic alterations found in PNETs include hypermethylation of the promoter region of *RASSF1* and cyclin dependent kinase inhibitor 2A (*CDKN2A*)⁷³⁻⁷⁵. In addition, insulin like growth factor 2 (*IGF2*) has a unique hypermethylation signature in insulinomas, and this is of interest in relation to the reported epigenetic changes in insulin like growth factor and insulinoma⁷⁶. Finally, the pleckstrin homology-like domain family A member 3 (*PHLDA3*) genomic locus, which is a p53-regulated repressor of Akt, has been reported to have LOH and promoter methylation at a high frequency, thereby indicating that *PHLDA3* is a likely tumor suppressor in PNETs⁷⁷.

The occurrence of *MEN1* and *DAXX/ATRX* mutations in sporadic non-MEN1 insulinomas is reported to be 3-8% and 3%, respectively^{78,79}. However mutations and differential expression of epigenetic modifying genes and their targets are reported to occur more frequently^{78,79}. Thus, the somatic Trp372Arg gain of function mutation of the Ying Yang 1 (YY1) transcription factor, which regulates mitochondrial function and insulin/insulin-like growth factor signaling and is a target for mTORC1^{80,81}, has been reported to occur in 15-30% of sporadic insulinomas^{78,79}. The Trp372Arg mutation increased transcriptional activity of YY1 that resulted in greater transcription of target genes isocitrate dehydrogenase 3 (NAD(+)) alpha (*IDH3A*) and uncoupling protein 2 (*UCP2*)⁷⁸. Other recurrently mutated genes detected in insulinomas include H3 histone family 3A (*H3F3A*), lysine-specific demethylase 6A (*KDM6A*) and ATR (ataxia telangiectasia and Rad3-related protein) serine/threonine kinase (*ATR*), which are reported in 8%, 8% and 8% of insulinomas, respectively⁷⁹.

Mutations Affecting the mTOR Signaling Pathway

Approximately 15% of sporadic PNETs harbor somatic mutations of genes in the mTOR cell signaling pathway, and these include mutations of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), phosphatase and tensin homolog (*PTEN*), *TSC1* and *TSC2*, and DEP (Dishevelled, Egl-10 and Pleckstrin) domain containing protein 5 (*DEPDC5*)^{13,54} (Table 1, Figure 2). Mutations in *PTEN*, *TSC2* and *DEPDC5*, which are mutually exclusive, are typically inactivating mutations and 75% of sporadic PNETs have decreased expression of PTEN and TSC2⁸², whereas the *PIK3CA* mutation, which involves a hotspot for activation of the kinase domain, has been reported to be oncogenic^{62,83}. Moreover, gastroenteropancreatic NETs with *TSC2*, *KRAS* and tumor protein p53 (*TP53*) mutations are reported more likely to be associated with disease progression and reduced survival in patients, and these possible aggressive alterations were observed in 6% of patients with PNETs <2cm and limited to the pancreas (defined as stage 1 disease by the European Neuroendocrine Tumor Society Tumor Node Metastasis (ENET TNM) classification and combined ENET/American Joint Commission on Cancer (AJCC))⁸⁴, thereby identifying a potential group of patients that may benefit from early surgery or systemic therapy⁸⁵. Gene fusion events involving the ewings sarcoma breakpoint region 1 (*EWSR1*) gene, that can activate mTOR signaling, have also been putatively identified in PNETs¹³.

Copy Number Changes

Copy number changes, which may reflect chromosomal instability comprising loss and gain have been reported in PNETs, and these alterations may accumulate during tumor growth and progression with multiple chromosomal abnormalities being associated with a worse prognosis^{1,86}. Recurrent regions of chromosomal loss involve the known NET tumor suppressors *MEN1*, *CDKN2A*, EYA (eyes absent homolog) transcriptional coactivator and phosphatase 1 (*EYA1*), Scm (sex comb on midleg) like with four Mbt (malignant brain tumor) domains 1 (*SFMBT1*), and RAB GTPase activating protein 1 like (*RABGAP1L*) which are located on chromosomes 11q13.1, 9q21.3, 8q13.3, 3p21.1 and 1q25.1, respectively¹³. Recurrently amplified regions include persephein (*PSPN*) on chromosome 19p13.3 and Unc-51 (C.elegans) like autophagy activating kinase 1 (*ULK1*) on chromosome 12q24.33, which are involved in RET signaling and mTOR-regulated autophagy, respectively¹³.

MiRNAs

MiRNAs *miR-103* and *miR-155* are up- and down-regulated in PNETs and pancreatic acinar cancers when compared to normal tissue respectively⁸⁷. *MIR-204* expression in insulinomas is reported to correlate with insulin expression, and mir-21, which represses *PTEN*, is reported to be associated with an increased proliferation index in insulinomas and their hepatic metastases⁸⁷. The miRNA profiles of insulinomas and non-functioning PNETs are reported to be indistinguishable, although overexpression of *miR-204* and *miR-211*, which are closely related, have been reported to be restricted to insulinomas^{73,86,88}.

Pancreatic Neuroendocrine Carcinomas (PNECs)

G3 PNETs or PNECs have a distinct molecular signature from G1 and G2 PNETs, in having frequent mutations of the *TP53* and retinoblastoma 1 (*RB1*) genes which are very rare in well-differentiated PNETs^{13,89}. The prevalence of *TP53* aberrations is also significantly greater in liver metastases than in primary PNETs⁸⁵. There are also significant differences in the distribution of variants in the rearranged during transfection (*RET*), Harvey rat sarcoma viral oncoprotein (*HRAS*), *MEN1*, *DAXX* and *ATRX* genes, with *MEN1* variants being more common in early stage disease and *DAXX* and *ATRX* being more frequent in advanced disease⁸⁵.

GERMLINE MUTATIONS IN PATIENTS WITH SPORADIC PNETS

Germline mutations of the *MEN1*, *CDKN1B* and *VHL* genes have been reported to occur in ~6%, ~1%, and ~1% of patients with sporadic PNETs, which also had LOH involving these genes in the tumors¹³ (Table 1). The identification of germline *MEN1* mutations in patients with apparently sporadic PNETs is important, as it indicates that such patients and their children are at greater risk of developing MEN1 associated tumors. This has implications for the clinical management and surveillance of MEN1-associated tumors in the patients and for genetic screening and tumor surveillance in their children and also possibly their first degree relatives. In addition, germline alterations in DNA repair genes have also been reported to result in characteristic mutational signatures in tumour genomes. Thus, inactivating germline mutations of the base-excision repair gene Mut Y homolog (*MUTYH*), which occurred with somatic LOH in PNETs of ~5% of patients¹³ (Table 1), had a mutational signature of G:C>T:A transversions throughout the tumor genome. Germline biallelic inactivation of *MUTYH* has been also reported to cause autosomal recessive *MUTYH*-associated colorectal polyposis syndrome, which is also associated with somatic G:C>T:A transversions involving the *APC* gene, that is the driver of colorectal polyps^{13,90}. This suggests that *MUTYH* deficiency likely plays a role in the development of PNETs and that poly adenosine diphosphate (ADP) ribose polymerase (PARP) inhibitors, which are directed at defective DNA-damage repair mechanisms, may represent potential treatments that would require further assessments. In addition, ~1% and ~4% of patients with sporadic PNETs have been observed to have germline mutations of breast cancer 2 (*BRCA2*), and checkpoint kinase 2 (*CHEK2*) that were associated with reduced kinase activity, respectively¹³ (Table 1).

CURRENT THERAPEUTIC APPROACHES FOR PNETS

Surgical resection with regional lymph node dissection offers the only potentially curative treatment for well-differentiated PNETs (G1 and G2)⁹. For advanced PNETs and metastatic disease medical therapies (e.g. biotherapies, which target tumor-specific receptors and intracellular pathways, and chemotherapies, which generally target cell division) and radiological treatments (e.g. peptide receptor radionuclide therapy (PRRT), radiofrequency ablation, transarterial embolization, and selective internal radiation therapy) are available⁹. Medical chemotherapy is usually reserved for

patients with PNET metastases, a high tumor burden, high proliferative index (e.g. Ki-67 index >5%), rapid tumor progression or symptoms not controlled by biotherapy⁹. Chemotherapy for such PNET disease usually comprises combined cytotoxic regimes (e.g. Streptozocin with fluorouracil or doxorubicin⁹¹; or temozolamide with capecitabine⁹²) and tumor response rates of 70% have been reported⁹². Medical biotherapies for PNETs, which can be: hormonal and based on somatostatin (a peptide hormone that inhibits release of other hormones, cell proliferation and angiogenesis⁹³); or targeted to tumor-specific molecular changes (e.g. in receptors and signaling pathways) that help tumors to grow and spread, include mTOR signaling inhibitors, receptor tyrosine kinase (RTK) inhibitors, and antibodies targeting vascular endothelial growth factor (VEGF) or its receptor (VEGFR)⁹ (Figure 2). The recent advances in medical biotherapies will be briefly reviewed, prior to discussing emerging therapies.

SOMATOSTATIN ANALOGUES

Somatostatin analogues (e.g. octreotide and lanreotide) have been used to control excessive hormone secretion and for their potential anti-proliferative effects in patients with low-grade (Ki-67 index <5%) PNETs that express somatostatin receptors (SSTRs), which are G-protein-coupled receptors (GPCRs)⁹⁴⁻⁹⁷. There are 5 SSTRs (SSTR₁₋₅) and each has specific functions with: SSTR₂ and SSTR₅ predominantly mediating inhibition of hormone secretion; SSTR₁, SSTR₂, SSTR₄ and SSTR₅ mediating cell cycle arrest and anti-proliferative actions; and SSTR₃ mediating apoptosis^{98,99}. Moreover, the aberrantly truncated variant of SSTR₅ (sst5TMD4) is overexpressed in gastroenteropancreatic NETs (GEPNETs) where it is associated with increased aggressiveness¹⁰⁰. In addition, sst5TMD4 disrupts the normal function of SSTR₂ and decreases the response of different tumor cells and tissues in response to somatostatin analogues^{101,102}. These findings suggest that identification of sst5TMD4 may be a useful biomarker to predict responses to somatostatin analogues and the aggressiveness of PNETs^{86,100}. PNETs may express all 5 subtypes, although ~80% of PNETs will predominantly express SSTR₂, for which octreotide and lanreotide have high affinities (Figure 2)⁹⁴, such that treatment with: lanreotide resulted in a ~50% reduction in the risk of disease progression and a prolonged progression-free survival (PFS) (median in lanreotide treated group not reached versus median in placebo treated group of 18 months, p<0.001) in non-MEN1 patients with treatment naive well-differentiated G1 and G2 non-functioning GEPNETs^{96,103,104}, and octreotide, resulted in

tumor response in 10%, stable disease in 80%, and progression of disease in 10% of MEN1 patients with duodeno-pancreatic NETs who were treated, over 12-15 months¹⁰⁵. In addition PRRT using a somatostatin analogue (e.g. octreotide, octreotate, dototate, and dota-toc) labelled with a β -emitting nuclide in the form of either ¹⁷⁷Lutetium or ⁹⁰Yttrium has been reported to be effective for treating NETs. Thus, treatment with: ¹⁷⁷Lutetium- or ⁹⁰Yttrium-octreotide in patients with gastro-intestinal NETs and metastatic NETs¹⁰⁶⁻¹¹¹, has been reported to result in objective response rates of ~20-60%^{106,108-110}, progression free survival (PFS) of 20-34 months^{108,110}, and an overall survival of 53 months¹⁰⁸; combining ¹⁷⁷Lutetium and ⁹⁰Yttrium nuclides has also been reported to increase survival in patients with PNETs¹¹¹; and the combination of ¹⁷⁷Lu-octreotate, capecitabine and temozolomide, in a phase1/2 study, has been reported to result in complete or partial response in >50% of the patients with advanced NETs (including PNETs)¹⁰⁷.

mTOR INHIBITORS

mTOR activation is associated with poor prognosis and there is a significant correlation between high proliferation index (Ki-67) values and expression of mTOR, PIK3CA and eukaryotic translation initiation factor 4E binding protein 1 (p-4EBP1)^{86,112}. Moreover, somatic mutations of genes associated with the mTOR pathway^{13,54} are found in ~15% of PNETs, and the mTOR inhibitor everolimus, which suppresses multiprotein complexes to inhibit downstream signaling (Figure 2), has been reported to increase PFS from ~6 to 11 months in patients with advanced NETs, including PNETs¹¹³⁻¹¹⁶. However, a companion diagnostic test that would help to identify patients who could benefit from treatment with mTOR inhibitors are not available², although preclinical data have suggested that aberrations of *PI3KCA* and *PTEN*, and high pAKT levels, may predict sensitivity to rapamycin in NET cell lines and rapalogs in NET patients¹¹⁷. However, protein and/or mRNA analysis might be a better indicator of mTOR pathway activation than mutational status, as it would avoid errors due to epigenetic changes that affect protein expression⁸⁶. Loss of AKT repression, may also occur in patients with *PHLDA3* LOH or methylation, and thus such patients may also potentially respond well to everolimus⁷⁷.

RTK INHIBITORS

PNETs are highly vascular and frequently express VEGFRs^{118,119}, and RTK inhibitors (e.g. sunitinib, which targets VEGFRs and platelet-derived growth factor receptors (PDGFRs) (Figure 2)) have been reported to increase PFS from ~5.5 to 11.4 months, in patients with PNETs¹²⁰. Pazopanib, another RTK inhibitor, has also been reported, in a phase 2 trial, to result in response and disease control rates of ~20% and >75%, respectively of non-MEN1 patients with metastatic GEPNETs¹²¹. However, therapeutic resistance to RTK inhibitors may arise through induction of hypoxic stress and upregulation of transcription factors controlling expression of pro-angiogenic molecules, or reduced lysosomal stability leading to autophagy^{122,123}, and companion diagnostic tests that would help to identify such resistance remain to be developed.

EMERGING THERAPIES FOR PNETS

MEN1 mutations, which occur in ~40% of sporadic PNETs and almost all MEN1 PNETs, represent the major drivers for PNET development, and therapies based on an increased understanding of menin, together with that of receptors and signaling pathways in PNETs are helping to develop new therapies. Assessment of the efficacy of these therapies have been facilitated by cellular and *in vivo* models which include conventional and conditional *Men1* knockout mouse models that develop MEN1-associated tumors, including PNETs¹²⁴⁻¹³³, and some of these pre-clinical advances will be discussed.

MEN1 GENE REPLACEMENT THERAPY

Menin acts as a tumor suppressor, and as such its loss due to *MEN1* mutations leads to tumor development and growth. Thus, *MEN1* gene replacement therapy should be able to suppress tumor proliferation, and indeed *Men1* gene replacement therapy into pituitary NETs that developed in conventional knockout mice lacking one allele of *Men1* (*Men1*^{+/-}), using a recombinant non-replicating adenoviral serotype 5 vector (rAd5), containing *Men1* cDNA under the control of a cytomegalovirus promoter (rAd5-MEN1), resulted in increased menin expression with decreased proliferation of the pituitary NETs. The *Men1* gene therapy did not induce an immune T-cell response or increased apoptosis¹³⁴. These findings, established proof of principle for the efficacy of *Men1* gene replacement therapy. In addition use of a hybrid adeno-associated virus and phage (AAVP) vector displaying

biologically active octreotide on the viral surface for ligand-directed delivery of the pro-apoptotic tumor necrosis factor (*TNF*) transgene to PNETs developing in a pancreatic specific *Men1* knockout mouse model, has also been reported to reduce tumor size, and improve survival of the mutant mice¹³⁵. These results suggest that systemic, ligand-directed transgene treatment of PNETs could potentially evolve as a novel and effective treatment.

EPIGENETIC MODULATORS

Menin interacts with a number of histone modifying proteins including histone methyltransferase (MLL1 and PRMT5) and deacetylase complexes (mSin3A-histone deacetylase)¹⁷ (Figure 1), and the use of epigenetic modulators, which represent a novel class of anti-cancer drugs¹³⁶ to treat PNETs has been employed in *in vitro* and *in vivo* studies¹³⁷. These studies utilised JQ1, an inhibitor of the bromo and extra terminal domain (BET) family of proteins that bind to acetylated histone residues to promote gene transcription¹³⁷. *In vitro* studies revealed that JQ1 decreased proliferation and increased apoptosis of a PNET cell line and *in vivo* studies, using a pancreatic β -cell specific conditional *Men1* knockout mouse model revealed that JQ1 decreased proliferation and increased apoptosis of PNETs¹³⁷. CP103, another BET inhibitor, has also been reported to reduce PNET proliferation in a human PNET cell line xenograft mouse model¹³⁸. Thus, epigenetic modulators, e.g. via BET inhibition, may offer potential therapies for PNETs lacking *MEN1* expression.

WNT SIGNALING MODULATORS

Menin inhibits Wnt signaling, as it promotes phosphorylation of β -catenin and its transfer from the nucleus via nuclear-cytoplasmic shuttling, which reduces cell proliferation^{21,26}. Moreover, the conditional knockout of β -catenin in PNETs of mice with pancreatic β -cell conditional knockout of menin, decreased the number and size of PNETs, as well as increasing survival, while use of a β -catenin antagonist (PKF115-584) decreased PNET cell proliferation in β -cell menin knockout mice¹³⁹. Thus, Wnt-signaling modulators may provide a novel approach for treatment of MEN1-deficient PNETs.

RAS SIGNALING

Aberrent activation of Ras signaling can occur in NETs due to mutations in Ras or Ras regulatory proteins like NF1, or downstream effectors such as Raf/MEK/ERK. Aberrent activation of Ras depends on absolute dependency on protein kinase C delta (PKC δ)-mediated survival pathways^{140,141}. This sensitizes cells to apoptosis induced by suppression of PKC δ activity which is not toxic to cells with normal Ras activity. The PNET cell line - BON-1, is sensitive to PKC δ inhibition induced by shRNA knockdown or diverse small molecule inhibitors such as Rottlerin¹⁴², and such molecules may prove to be useful for treating PNETs which have activated Ras signaling.

ANTI-ANGIOGENIC COMPOUNDS

Most anti-angiogenic compounds block the activity of the cytokine VEGF, which promotes the growth and survival of blood vessels. Treatment with bevacizumab, which is an anti-VEGF monoclonal antibody, in combination with chemotherapy, has been reported to delay the progression of moderately well-differentiated and advanced gastro-intestinal NETs, which included metastatic well-differentiated, PNETs¹⁴³⁻¹⁴⁶. However, inhibiting VEGF signaling has been reported to be accompanied by increased invasiveness and metastasis of cancers possibly due to the upregulation of proangiogenic factors which include fibroblast growth factors (FGFs) and therefore targeting FGFs in addition to VEGF and PDGF may improve the efficacy of treatment¹⁴⁷⁻¹⁴⁹. Furthermore, the combination of sunitinib, or an anti-VEGF antibody, with inhibitors (e.g. PF-04217903 or PF-0241066 (crizotinib)) of hepatocyte growth factor receptor (HGFR or c-Met), which promotes cell proliferation, invasion and metastasis, prevented the increased tumor aggressivity without impairment to the activity of restricting tumor growth¹⁵⁰. Thus, these studies suggest that the increase in invasion and metastasis that are promoted by selective inhibition of VEGF signaling, may be reduced by combining the treatment with a c-met inhibitor. In addition, inhibition of nitric oxide (NO) synthase may have a role, since the use of L-arginine methyl ester (L-NAME), a NO synthase inhibitor, in *ex vivo* treatment of PNETs from conventional *Men1*^{+/-} knockout mice, caused impaired blood perfusion and increased constriction of the tumour supplying arterioles¹⁵¹.

USE OF SOMATOSTATIN ANALOGUES FOR PNET CHEMOPREVENTION

Somatostatin analogues may have a potential role in chemoprevention of MEN1-associated NETs as they have been shown to have antiproliferative, and antisecretory effects on NETs^{103,104,152,153}. Thus, treatment with pasireotide, which is a multiple-receptor-targeted somatostatin analogue that acts via SSTR_{1,2,3} and SSTR₅⁹⁴, decreased proliferation and increased apoptosis of PNETs in conventional *Men1*^{+/-} and pancreatic specific conditional *Men1* knockout mutant mice, as well as increasing survival of the conventional *Men1*^{+/-} mutant knockout mice^{154,155}. In addition to these anti-proliferative and pro-apoptotic effects, pasireotide was also found to inhibit the development of PNETs in the *Men1*^{+/-} mutant mice¹⁵⁴. These findings, which indicate that pasireotide-treatment resulted in fewer *Men1*^{+/-} mice with PNETs who also had fewer PNETs per pancreas, when compared to control phosphate buffered saline (PBS)-treated *Men1*^{+/-} mice, are consistent with a lower development of new NETs, in pasireotide treated *Men1*^{+/-} mice¹⁵⁴. These findings suggest that somatostatin analogues may have a chemopreventative role in the treatment of MEN1-associated PNETs in patients, and two studies in MEN1 patients have reported such anti-proliferative actions of somatostatin analogues. In one study octreotide was given to MEN1 patients to treat duodeno-pancreatic NETs, and retrospective analysis revealed that 10% of PNETs had tumour response, and that 80% had stable disease¹⁰⁵; and in another study, octreotide was given prospectively to 8 MEN1 patients with GEPNETs, and found to be safe and decrease gastro-intestinal hormone secretion, and to be associated with stable PNET disease¹⁵⁶.

SUMMARY

MEN1 mutations are major drivers for development of familial and sporadic forms of PNETs. Additional genetic studies of PNETs have further improved our understanding of the molecular alterations and cellular pathways associated with these tumours and these include the roles of genes involved in: chromatin remodelling and epigenetic regulation; mTOR signaling; K-RAS signaling; β -catenin/Wnt signaling; and SSTR signaling. This has led to the development of medical biotherapies, which can inhibit SSTR, mTOR or RTK signaling. In addition recent pre-clinical studies have also demonstrated the efficacy of: *MEN1* gene replacement therapy; epigenetic modulators; Wnt pathway targeting β -catenin antagonists; and multi-somatostatin receptor targeted analogues in treating

PNETs. Clinical evaluation of such emerging treatments may help to provide new therapies for improving outcomes and life expectancies in PNET patients.

FIGURE LEGENDS

Figure 1. Menin interacting proteins. More than 40 different proteins have been reported to interact with menin, which is a multifunctional protein with roles in: transcriptional regulation as a co-repressor or co-activator (via interactions with chromatin modifying proteins, transcription factors and transcription initiation or elongation proteins); DNA repair associated with response to DNA damage; cell signaling; cytoskeletal structure; cell division; cell adhesion; or cell motility^{17,20}. DAXX = death domain associated protein; HDACs = histone deacetylases; mSIN3A = SIN3 transcription regulator family member A; LEDGF = lens epithelium-derived growth factor; MLL = mixed lineage leukemia; PRMT5 = protein arginine methyltransferase 5; SUV39H1 = suppressor of variegation 3-9 homolog 1; DNMT1 = DNA methyltransferase 1; FBP1 = fructose biphosphatase 1; FOXA2 = forkhead box A2; MNX1 = motor neuron and pancreas homeobox 1; JUND = JunD proto-oncogene; c-MYB = MYB (myeloblastosis) proto-oncogene; c-MYC = MYC (myelocytomatosis) proto-oncogene; NFκB = nuclear factor kappa B; AR = androgen receptor; ERα = estrogen receptor alpha; LXRα = liver X receptor alpha; PPARα = peroxisome proliferator activated receptor alpha; RXR = retinoic X receptor; VDR = vitamin D receptor; PEM = PEM homeobox; RUNX2 = runt related transcription factor 2; SMAD = small body size mothers against decapentaplegic homolog; SIRT = Sirtuin 1; SON = SON DNA binding protein; TCF = transcription factor; SKIP = SKI interacting protein; ARS2 = arsenite-resistance protein 2; CHIP = carboxy terminus of hsp70-interacting protein; ASK = apoptosis signal regulating kinase; CHES1 = checkpoint suppressor 1; FANCD2 = fanconi anemia complementation group D2; RPA2 = replication protein 2; GFAP = glial fibrillary acidic protein; IQGAP1 = IQ motif containing GTPase activating protein 1; NMMHC-IIA = non-muscle myosin heavy chain IIA; AKT1 = viral oncogene of Akt8 retrovirus (AKT) serine/threonine kinase 1; FOXO1 = forkhead box O1; NM23β = NME/NM23 nucleoside diphosphate kinase 2; GRB2 = growth factor receptor bound protein 2; RAS = KRAS (Kirsten rat sarcoma virus) proto-oncogene; SOS1 = son of sevenless homolog 1

Figure 2. Major pathways associated with PNET tumor progression. In the nucleus menin interacts with: the transcription factor JunD to prevent its phosphorylation and activation of target genes such as gastrin (*GAST*); β -catenin mediating its export from the nucleus and inhibiting Wnt signaling; PRMT5 to inactivate the Hedgehog pathway promoter *Gas1*; and MLL1 and MLL2, and Smad3, which is a TGF- β signaling component, to promote transcription of target genes that reduce cellular proliferation. DAXX and ATRX are responsible for telomere maintenance with genetic mutations leading to the Altered Lengthening of Telomere (ALT) phenotype, while MUTYH, CHEK2 and BRCA2 are involved in DNA repair. In the cytoplasm menin inhibits: the mTOR pathway which regulates cellular proliferation and autophagy by binding to Akt, which are part of RTK and SSTR signaling pathways; and K-RAS induced proliferation by both inhibition of ERK dependent phosphorylation of target proteins and prevention of the SOS/K-RAS interaction. PTEN, TSC1, TSC2 and DEPDC5 are also involved in regulating the mTOR signaling pathway, while NF1 regulates RAS signaling via MEK/ERK and mTOR pathways by regulating the conversion of inactive RasGDP to active RasGTP. VHL is part of a multiprotein complex responsible for ubiquitination and degradation of the α subunits of HIF1 and 2, and thus *VHL* mutations can lead to stabilisation of HIFs increasing angiogenesis through increased expression of hypoxia-inducible target genes. Targeted therapies (white text in ovals with solid outlines) include mTOR inhibitors such as everolimus; Wnt pathway inhibitors such as β -catenin antagonists; epigenetic modulators; *MEN1* gene therapy; RTK inhibitors such as sunitinib; and somatostatin analogues such as octreotide and lanreotide or PRRT. VEGFR = vascular endothelial growth factor receptor; SSTRs = somatostatin receptors; SOS = son of sevenless; RAS = KRAS proto-oncogene; Raf = B-Raf proto-oncogene; MEK = mitogen-activated protein kinase/ERK kinase 1; ERK = extracellular signal-regulated kinase; RASSF1A = ras association domain family member 1; HIF- α = hypoxia inducible factor alpha; VHL = von hippel lindau; B-cat = β -catenin; PI3K = phosphatidylinositol-4,5-bisphosphate 3 kinase; AKT = AKT serine/threonine kinase 1; mTOR = mechanistic target of rapamycin kinase; PTEN = phosphatase and tensin homolog; TSC1/2 = tuberous sclerosis 1/2; DEPDC5 = DEP domain containing protein 5; SMAD = small body size mothers against decapentaplegic homolog; JUND = JunD proto-oncogene; MLL = mixed lineage leukemia; CDKN1B/2C = cyclin dependent kinase inhibitor 1B/2C; PRMT5 = protein arginine methyltransferase 5; GAS1 = growth arrest specific 1; DAXX = death domain associated protein; ATRX = alpha thalassemia/mental retardation syndrome X-Linked; MUTYH = MutY homolog; CHEK2

= checkpoint kinase 2; BRCA2 = breast cancer 2; Wnt = wingless integration 1; RTK = receptor tyrosine kinase; PRRT = peptide receptor radionuclide therapy.

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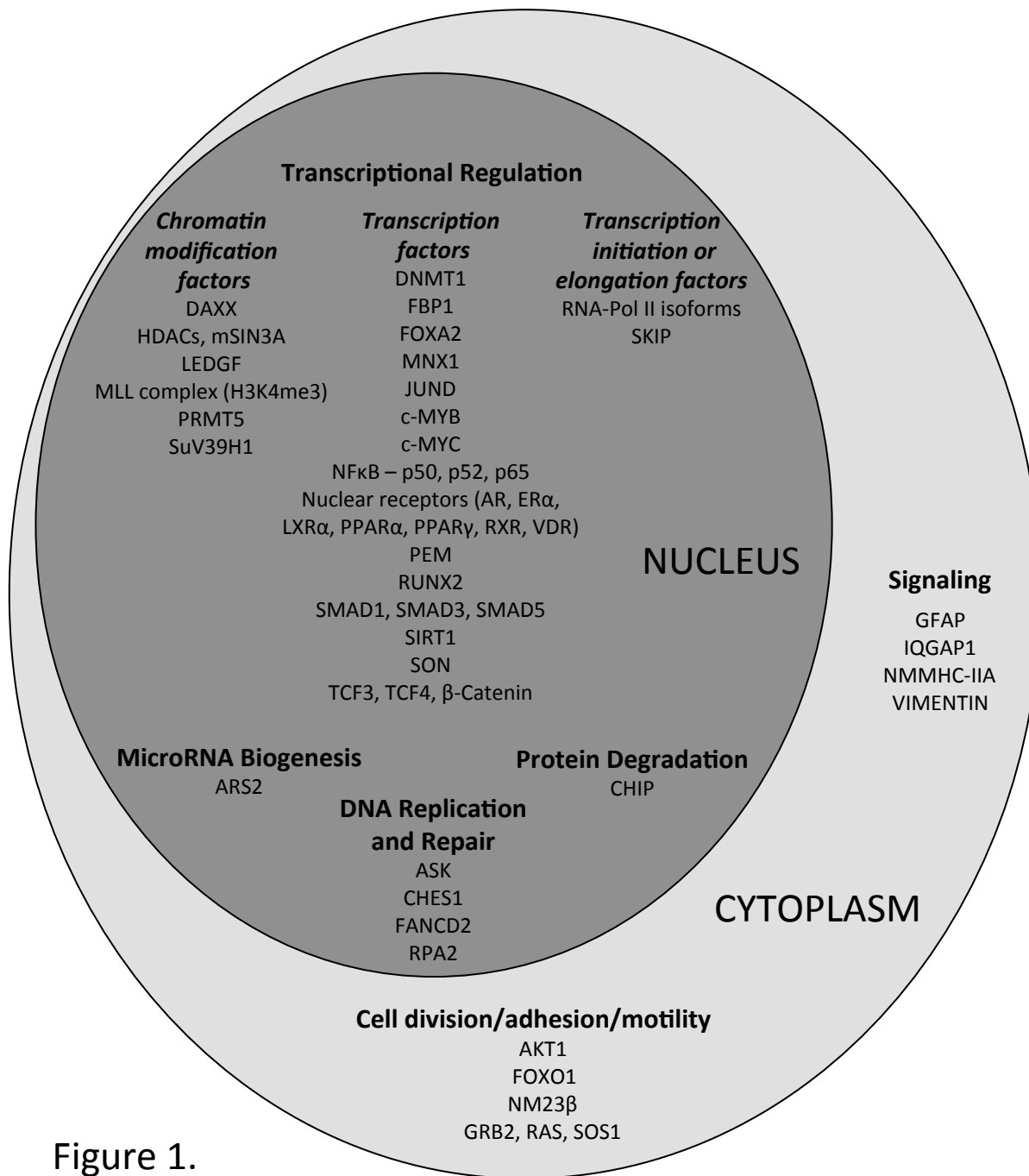


Figure 1.

Figure 2.

