Received: 24 August 2021 | Revised: 26 January 2022 | Accepted: 30 January 2022

DOI: 10.1111/cas.15301

Cancer Science WILEY

ORIGINAL ARTICLE

The link between menin and pleiotrophin in the tumor biology of pancreatic neuroendocrine neoplasms

Liping He[1,2] | Steeve Boulant[3] | Megan Stanifer[3] | Cuncai Guo[3] | Anna Nießen[1] | Mingyi Chen[1,4] | Klaus Felix[1] | Frank Bergmann[5] | Oliver Strobel[1] | Simon Schimmack[1]

[1]Department of General, Visceral and Transplantation Surgery, Heidelberg University Hospital, Heidelberg, Germany

[2]Department of Medical Oncology, The First Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, China

[3]Center for Integrative Infectious Disease Research, Heidelberg University, Heidelberg, Germany

[4]Department of Breast Surgery, The First Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, China

[5]Institute of Pathology, Heidelberg University, Heidelberg, Germany

Correspondence
Simon Schimmack, General, Visceral and Transplantation Surgery, Heidelberg University Hospital, 69120 Heidelberg, In Neuenheimer Feld 420, Heidelberg, Germany.
Email: Simon.Schimmack@med.uni-heidelberg.de

Funding information
Liping He has been supported by the Chinese Scholarship Council. Simon Schimmack has been supported by the Eva Luise und Horst Köhler Stiftung and by the Heidelberger Stiftung Chirurgic.

Abstract

MEN1, which encodes menin protein, is the most frequently mutated gene in pancreatic neuroendocrine neoplasms (pNEN). Pleiotrophin (PTN) has been reported as a downstream factor of menin that promotes metastasis in different tumor entities. In this study, the effect of menin and its link to PTN were assessed using features of pNEN cells and the outcome of patients with pNEN. The expression levels of menin and PTN in tissues from patients with pNEN were examined using qRT-PCR and western blot and compared with their metastasis status. Functional assays, including transwell migration/invasion and scratch wound-healing assays, were performed on specifically designed CRISPR/Cas9-mediated *MEN1*-knockout (MEN1-KO) pNEN cell lines (BON1$^{MEN1\text{-}KO}$ and QGP1$^{MEN1\text{-}KO}$) to study the metastasis of pNEN. Among 30 patients with menin-negative pNEN, 21 revealed a strong protein expression of PTN. This combination was associated with metastasis and shorter disease-free survival. Accordingly, in BON1$^{MEN1\text{-}KO}$ and QGP1$^{MEN1\text{-}KO}$ cells, PTN protein expression was positively associated with enhanced cell migration and invasion, which could be

Abbreviations: BMBH, Biomaterial Bank Heidelberg; BON1^{Con}, Non-specific single guide ribonucleic acid generated CRISPR/Cas9 BON1 cell line; BON1$^{MEN1\text{-}KO}$, *MEN1*-knockout CRISPR/Cas9 BON1 cell line; BON1$^{MEN1\text{-}KO+si_PTN}$, *MEN1*-knockout CRISPR/Cas9 BON1 cell line treated with pleiotrophin-small interfering ribonucleic acid; BON1$_{si_neg}$, BON1 treated with negative control-small interfering ribonucleic acid; BON1^{si_PTN}, BON1 treated with pleiotrophin-small interfering ribonucleic acid; Cas9, Clustered regularly interspaced short palindromic repeats-associated nuclease 9; CRISPR, Clustered regularly interspaced short palindromic repeats; CRP, C-reactive protein; DFS, disease-free survival; G, grading; JCRB, Japanese Cancer Research Resources Bank; L, lymphatic vessel invasion; M, distant metastasis; MAP2, microtubule-associated protein 2; MDM2, mouse double minute 2; MEN1, multiple endocrine neoplasia 1; MEN1-KO, *MEN1*-knockout CRISPR/Cas9 pNEN cell lines; MET, pancreatic neuroendocrine neoplasms with lymph node metastasis or/and liver metastasis positive; MGMT, O6-methylguanine deoxyribonucleic acid methyltransferase; N, lymph node metastasis; Non-MET, pancreatic neuroendocrine neoplasms without lymph node metastasis or/and liver metastasis positive; OS, overall survival; pM, liver metastasis; pN, lymph node metastasis; pNEN, pancreatic neuroendocrine neoplasms; pNEN^{m-}, pancreatic neuroendocrine neoplasms with negative protein expression of menin; pNEN^{m-p-}, pancreatic neuroendocrine neoplasms with negative protein expression of menin and pleiotrophin; pNEN^{m-p+}, pancreatic neuroendocrine neoplasms with negative protein expression of menin but positive pleiotrophin expression; pNET, pancreatic neuroendocrine tumors; PTN, pleiotrophin; QGP1^{Con}, Non-specific single guide ribonucleic acid generated CRISPR/Cas9 QGP1 cell line; QGP1$^{MEN1\text{-}KO}$, *MEN1*-knockout CRISPR/Cas9 QGP1 cell line; QGP1^{si_neg}, QGP1 treated with negative control-small interfering ribonucleic acid; QGP1^{si_PTN}, QGP1 treated with pleiotrophin-small interfering ribonucleic acid; qRT-PCR, quantitative real-time PCR; R, resection margin; RPTP, receptor-like protein tyrosine phosphatase; sgRNA, single guide RNA; V, invasion to vein; WHO, World Health Organization.

reversed using PTN silencing. PTN is a predicting factor of metastatic behavior of menin-deficient-pNEN. *In vitro*, menin is able to both promote and suppress the metastasis of pNEN by regulating PTN expression depending on the tumoral origin of pNEN cells.

KEYWORDS
CRISPR/Cas9, *MEN1*, metastasis, pancreatic neuroendocrine neoplasms, pleiotrophin

1 | INTRODUCTION

Pancreatic neuroendocrine neoplasms (pNEN) are a heterogeneous group of malignancies arising from different cell types within the pancreas.[1] According to the most recent WHO classification,[2] pNEN are categorized into well differentiated pancreatic neuroendocrine tumors (pNET Grade G1, G2 and G3) as well as poorly differentiated pancreatic neuroendocrine carcinoma (pNEC G3). The grading depends on the proliferation rate, which is measured in Ki67-positive cells within 10 high-powered fields (G1: <3%, G2: 3%–20%, G3: >20%) and is the strongest prognostic factor.[3] Further effective prognostic tools are the TNM classification,[4] presenting a strong correlation of size and metastasis with unfavorable prognosis, metastasis in lymph nodes[5]/distant metastasis,[6] as well as markers such as preoperative dysglycemia (blood glucose ≥140 mg% and/or HbA_{1c} ≥6.5%)[7] or peroperative CRP (>5 mg/L).[8] Furthermore, pNENs in combination with lymph node[9] or distant metastasis[6,10] are associated with worse prognosis.

Pancreatic neuroendocrine neoplasms can be inherited (as in MEN1 syndrome) or developed due to germline mutations within the *MEN1* gene that is located on 11q13.[11] *MEN1* translates into the protein menin, which is known to be a tumor suppressor in the development of different endocrine tumors.[12,13] However, the vast majority (60%–90%) of pNEN arise sporadically.[14–17] Frequently occurring genetic alterations in pNEN are LOH of *MEN1*(50%)[18] and/or *MEN1* mutations (40%).[19] *MEN1* mutations often include nonsense and missense mutations as well as in-frame deletions distributed across the gene locus.[20,21] Those alterations often result in menin protein degradation and inactivation revealing that menin may play an important role in tumorigenesis and tumor progression of all pNEN. However, the mechanism of how menin inactivation initiates tumorigenesis of pNEN is not well understood.

The heparin-binding growth factor pleiotrophin (PTN) is a secreted 19-kDa regulatory peptide with angiogenic properties that is considered as a proto-oncogene and is overexpressed in various malignancies such as breast, prostate, colon, and skin cancer.[22–25] Menin has been shown to repress PTN expression in non–small-cell lung cancer[26] and malignant melanoma.[26] As there is currently no literature on the role of PTN in pNEN, this study aimed to evaluate the link between menin and PTN in pNEN. For this purpose, CRISPR/Cas9 *MEN1*-knockout BON1 and QGP1 cells were developed and subsequent functional alternations were analyzed. The association between menin and PTN was further examined in pNEN tissue.

2 | MATERIALS AND METHODS

2.1 | Clinical samples

For this study, frozen pNEN tumor tissues ($n = 67$) were used for western blot and qRT-PCR analysis. All pNEN tumor tissues were provided from the Pancobank of the European Pancreas Center (EPZ/Department of Surgery, University Hospital Heidelberg; Ethical Approval Vote No. S-708/2019), which is part of the BMBH. An experienced pancreas pathologist (FB) performed the histological examination for paraffin-embedded and H&E-stained pancreatic tissue. Only tumor samples containing more than 90% tumor tissue were used in this study.

2.2 | Origin and culture of BON1 and QGP1 cells

The human pNEN cell line BON1, which was derived from a lymph node metastasized site of pNEN,[27] was given as a gift from Dr. M. Kidd, Yale University School of Medicine and had already been authenticated using short tandem repeat (STR) analysis. The human pNEN cell line QGP1 (pNEN primary) was purchased from the JCRB (Japan). The adherent monolayer BON1 and QGP1 cells were cultured as described previously.[28] Whole exome sequencing of both cell lines has shown significant genetic differences.[29,30]

2.3 | Generation of *MEN1*-knockout pNEN cell lines with CRISPR/Cas9 editing system

Single guide RNA (sgRNA) sequences for CRISPR/Cas9 were designed by CRISPOR (http://crispor.tefor.net/). Three insert oligonucleotides were positioned at the second exon of *MEN1*: Human *MEN1* sgRNA 1: 5′-CACCGGCTGCGCTCCATCGACGACG-3′,

Human *MEN1* sgRNA 2: 5′-CCAGGCGCACCACGTCGTCGACAAA-3′ and

Human *MEN1* sgRNA 3: 5′-CGTCGACGGCGCCTCGGATCTCAAA-3′.

The fourth insert oligonucleotide was positioned at the fourth exon of *MEN1*: Human *MEN1* sgRNA 4: 5′-CACCGCATGCGCTGTGACCGCAAGA-3′. A negative control sgRNA 5′-CACCGGTATTACTGATATTGGTGGG-3′, was selected without the overlapping sequence of the *MEN1* gene. Complementary oligonucleotides were ligated

into the lentiCRISPRv2 plasmid no. 52961 (Addgene) as described previously by Ran et al.[31] BON1 and QGP1 were infected with *MEN1* lentiCRISPRv2 lentivirus or the negative control lentivirus according to a standard protocol.[32] Transfected cells were cultured including 10 μg/ml puromycin (InvioGen).

2.4 | Sanger sequencing

Sanger sequencing was used to confirm the synthesized sgRNA-lentiCRISPRv2 vector with the U6 primer before infection of pNEN cells and the *MEN1*-knockout in CRISPR/Cas9 cell lines using the primer (AAATTGGACAGCTCCGGTGT) (Invitrogen) as previously described.[33]

2.5 | Quantitative real-time PCR (qRT-PCR)

The mRNA of frozen tissues was extracted and reverse transcribed using the RNeasy mini kit (Qiagen) and the 1st Strand cDNA Synthesis Kit (AMV) (Roche) according to the manufacturer's instructions. The primers used are shown as follows (Qiagen): Human *MEN1* forward primer 5′-ATCACAGGCACCAAA TTGGACAGC-3′; Human *MEN1* reverse primer 5′-AACACTACC CAGGCATGATCCTCA-3′; Human *PTN* forward primer 5′-TGAAGA CCCAGAGATGTAAGAT-3′; Human *PTN* reverse primer 5′-TCTTCT GGCATTCGGCATTG-3′; Human *GAPDH* forward primer 5′-GTC TCCTCTGACTTCAACAGCG-3′; and Human *GAPDH* reverse primer 5′-ACCACCCTGTTGCTGTAGCCAA-3′.

qRT-PCR analysis was performed as previously described.[28] GAPDH was used as an internal reference for normalization and the relative mRNA expression of each gene was analyzed using the ΔΔCT method as recommended.[34]

2.6 | Protein extraction and western blot analysis

Protein was extracted by RIPA buffer supplemented with complete protease inhibitors (Roche) from frozen pNEN tissue specimen (60–80 mg) or from pNEN cell pellets. Protein expression was assessed as previously described[35] using the primary antibodies Monoclonal Rabbit anti-Human-Menin (Abcam, ab92443, dilution 1:200), Monoclonal Mouse anti-Human-Pleiotrophin (Antibodies-online, ABIN 562528, dilution 1:50, Nordrhein-Westfalen, Germany) and Monoclonal Rabbit anti-Human-Beta-actin (Abcam, dilution 1:200). The secondary antibodies were HRP Goat Anti-Rabbit (IgG) and HRP Goat Anti-Mouse (IgG) (Abcam). Protein expression was quantified using ImageJ (NIH, US). Negative protein expression of patient tissue was defined as a protein expression level <5% measured by grayscale analysis in ImageJ.

2.7 | Small interfering RNA (siRNA) transfection

To investigate the impact of PTN on pNEN, the PTN translation was specifically suppressed by introduction of siRNA. The sequences of siRNA to reduce PTN expression were as follows: PTN siRNA1: 5′-GGACUGGAGCUGAGUGCAtt-3′ (Ambion) and PTN siRNA2: 5′-GGAGCUGAGUGCAAGCAAAtt-3′ (Ambion). Negative control siRNA was purchased from Ambion

Cells were plated on 6-well plates (Falcon, Colorado, USA) at a density of 500 000 cells per well, supplementing with 0.24 nM per well of siRNAs using HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's suggestions. To confirm the downregulation of each protein, the transfected cells were collected for the western blot analysis 48 h after the transfection.

2.8 | Scratch wound-healing and migration/invasion assay

Scratch wound-healing assay was used to assess the cell migration ability. A 10-μl pipette tip (Eppendorf) was used to perform a vertical scratch in each well of 6-well plates (Falcon, Colorado, USA) which was filled with more than 90% confluency monolayer cells. After removing non-adherent cells, the cell-free area was measured. At ×100 magnification, photographic documentation of cell-free areas was taken every 24 h. The wound-healing rate was calculated using the formula: wound-healing rate (%) = [(width at 0 h − width at 120 h)/(width at 0 h)] × 100.

The migration and invasion potential were assessed by transwell migration/invasion assay as described previously.[28] All experiments were repeated three times.

2.9 | Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). Relative expression and number of cells were recorded as mean ±SD. *n* describes the number of replicates. The OS time of patients with pNEN was defined as the time from resection to either death or last follow-up. Survival curves were plotted using the Kaplan–Meier method. The difference between the Kaplan–Meier curves was tested for significance applying the log-rank test. For experiments involving MTT assay, transwell migration assay, transwell invasion assay, and scratch wound-healing assay, data from a minimum of three independent experiments were used for statistical analysis. Results from three independent experiments were averaged prior to statistical analysis. A two-tailed, unpaired Student *t*-test or two-way ANOVA were used to analyze data and determine significant differences. All the analyses were considered statistically significant at a $p < 0.05$ level and *p*-values are indicated with asterisks (*$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$).

3 | RESULTS

3.1 | Downregulation of relative *MEN1* mRNA expression in metastasized patients with pNEN

Relative mRNA expression levels of *MEN1* and *PTN* were assessed using qRT-PCR in 40 random G1 and G2 pNEN tissues. All samples were divided into two groups regarding their N (lymph node metastasis) and M (distant metastasis) stages. In total, 22 samples were either N and/or M positive (N+ and/or M+) (MET group) whereas 18 samples showed no metastasis (Non-MET group) during their entire follow-up of 71.86 ± 45.85 months (mean ±SD). As shown in Figure 1A, relative mRNA expression of *MEN1* was significantly downregulated in the MET group compared with the Non-MET group ($p < 0.05$).

3.2 | PTN protein expression in menin-negative pNEN

As *MEN1* mRNA was downregulated in metastasized pNEN, tumors with negative protein expression of menin (pNEN^{m-}, no detectable protein expression on grayscale analysis using ImageJ software <5%) were analyzed further ($n = 30$). In all 30 pNEN^{m-}, PTN expression was examined. As shown in Table 1 and Figure 1B–D, 21 of 30 pNEN^{m-} were PTN positive (pNEN^{m-p+} group), whereas 9 of 30 pNEN^{m-} had no detectable PTN protein expression (pNEN^{m-p-} group). Whereas invasion to vein (V), resection margin (R) as well as grading (G) did not differ between the groups, pNEN^{m-p+} patients showed a significantly higher rate of lymph node metastasis (pN), liver metastasis (pM), lymphatic vessel invasion (L), infiltration into

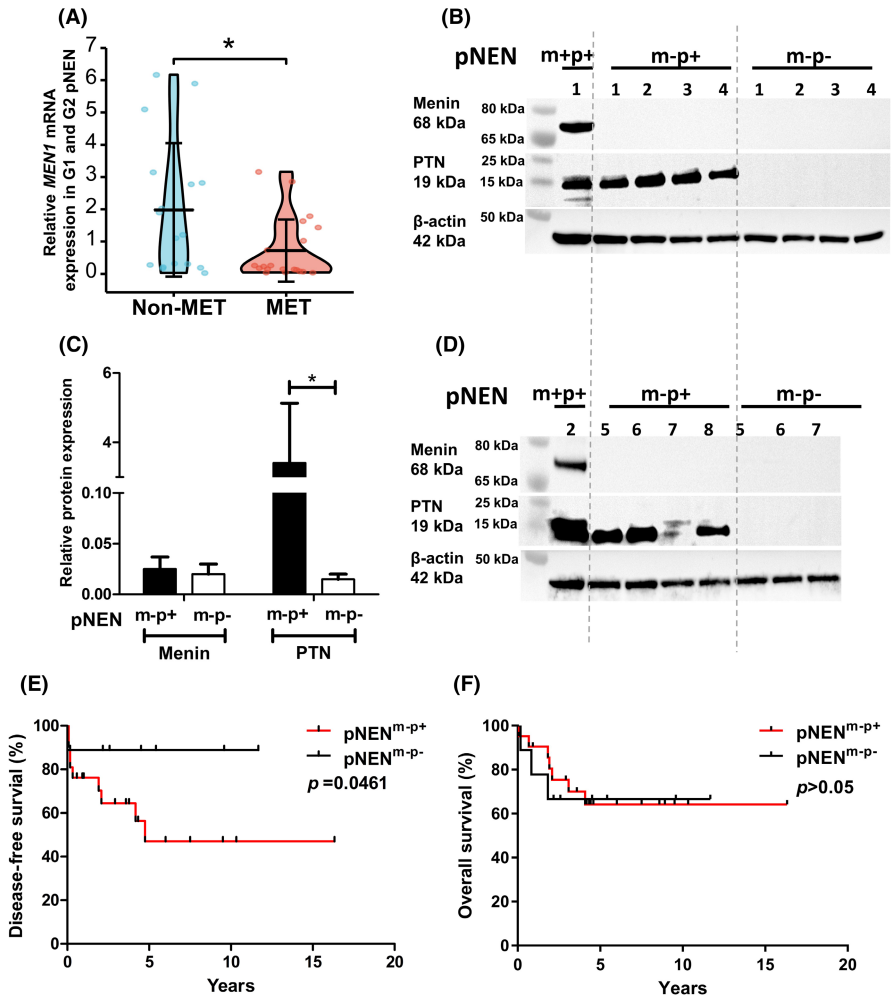

FIGURE 1 Expression and consequence of menin and PTN expression in patients with pNEN. (A) Relative mRNA expression of *MEN1* is significantly lower in the Non-MET group ($n = 22$) than in the MET group ($n = 18$) ($p < 0.05$). (B–D) Representative western blots (ImageJ software analysis below) of menin and PTN protein expression in pNEN^{m-} tissues. Two samples from the pNEN^{m+p+} group, eight samples from the pNEN^{m-p+} group and seven samples from the pNEN^{m-p-} group are displayed. (E) Kaplan–Meier analysis of disease-free survival rate shows a significantly longer DFS in pNEN^{m-p-} patients ($n = 9$, 5-year DFS = 88.89%, median DFS = 48.3 months) compared with pNEN^{m-p+} patients ($n = 21$, 5-year DFS = 57.14%, median DFS = 46.2 months) (Log-rank, $p < 0.05$). (F) Kaplan–Meier analysis of OS rate shows no significant difference between pNEN^{m-p+} patients ($n = 21$, 5-year OS = 61.9%) and pNEN^{m-p-} patients ($n = 9$, 5-year OS = 66.7%) (Log-rank, $p > 0.05$). MET: patients with pNEN with either lymph node metastasis or/and distant metastases. Non-MET: patients with pNEN with no metastasis. pNEN^{m+p+}: patients with positive menin and PTN protein expression. pNEN^{m-p+}: patients with no menin but positive PTN protein expression. pNEN^{m-p-}: patients with neither menin nor PTN protein expression

TABLE 1 The correlation between PTN and clinicopathological characteristics of pNEN^{m-} patients

	pNEN^{m-}				
	pNEN^{m-p+}(n = 21)		pNEN^{m-p-}(n = 9)		
	n	Percentage (%)	n	Percentage (%)	p-value
pN: Lymph node metastasis					
Negative	6	28.57	8	88.89	**0.0043****
Positive	15	71.43	1	11.11	
pM (HEP): Liver metastasis					
Negative	17	80.95	9	100	**<0.0001*****
Positive	4	19.05	0	0	
V: Invasion to vein					
Negative	9	42.86	7	77.78	0.1184
Positive	12	57.14	2	22.22	
L: Invasion to lymphatic vessels					
Negative	10	47.62	8	88.89	**0.0492***
Positive	11	52.38	1	11.11	
Infiltrate in adjacent tissues					
Negative	5	23.81	6	66.67	**0.0419***
Positive	16	76.19	3	33.33	
R: Resection margins					
Negative (0)	10	47.62	7	77.78	0.2293
Positive (1/2)	11	52.38	2	22.22	
Recurrence					
No	12	57.14	9	88.89	**0.0289***
Yes	9	42.86	0	11.11	
Grade					
G1	4	19.05	4	44.44	0.3498
G2	13	61.90	4	44.44	
G3	4	19.05	1	11.12	
Gender					
Male	12	57.14	5	55.56	0.9898
Female	9	42.86	4	44.44	
Age: median (min-max)	68.6 (40–96)			59.4 (26–80)	

Abbreviations: Metastatic neoplasms, pN+ or/and pM+; p−, no PTN expression; p+, PTN positive expression; pNEN^{m-}, No menin patients with pNEN. Bold values are highlight significance.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

adjacent tissues, and recurrences compared with pNEN^{m-p-} patients (Table 1; $p < 0.05$), suggesting that PTN plays an important role in the development of metastasis and aggressiveness in pNEN. Consistently, pNEN^{m-p+} patients demonstrated a significantly worse 5-year disease-free survival (n = 21, 5-year DFS = 57.1%) compared with pNEN^{m-p-} patients (n = 9, 5-year DFS = 88.9%) ($p < 0.05$; Figure 1E). However, OS did not differ between pNEN^{m-p+} patients (n = 21, 5-year OS = 61.9%) and pNEN^{m-p-} patients (n = 9, 5-year OS = 66.7%) (Figure 1F). Taken together, the combination of negative menin and positive PTN protein expression might be a potential predictive factor for metastatic behavior and represented by decreased disease-free survival of patients with pNEN.

3.3 | Establishing CPISPR/Cas9 mediated-*MEN1* knockout pNEN cell lines

Given the clinical relevance of menin deficiency in pNEN, menin protein expression was analyzed in two pNEN cell lines BON1 and QGP1 and compared with HEK293T[36–39] and HeLa cells[40] that are known to express high menin protein levels (please refer to Figure 2A). Having proven significant menin expression, *MEN1*-knockout CRISPR/Cas9 pNEN cell lines (MEN1-KO) were established for further investigations. The absence of menin and *MEN1* in pNEN cells and CRISPR/Cas9 negative control cells was confirmed at the protein level using western blot analysis and at the

gene level using Sanger sequencing analysis. The final cell lines BON1$^{MEN1\text{-}KO}$ and QGP1$^{MEN1\text{-}KO}$ were transfected using the same synthesized sgRNA4-lentiCRISPRv2 vector, the sgRNA sequence of which was 5′-CACCGCATGCGCTGTGACCGCAAGA-3′ and located at position 806–825 of the *MEN1* gene (NM_000244.3) (Figure 2B). Sanger sequencing was used to verify the correct insertion of the sgRNA into the plasmid before pNEN cell lines were transfected (Figure 2C). Both BON1$^{MEN1\text{-}KO}$ and QGP1$^{MEN1\text{-}KO}$ showed a complete and stable absence of menin at the protein level (Figure 2D–G). At the gene level, the Sanger sequencing results for MEN1-KO cell lines further verified the complete and stable fragment deletion of the *MEN1* gene (NM_000244.3) at ~780 bp to 910 bp in BON1$^{MEN1\text{-}KO}$ cells and from 763 bp onwards in QGP1$^{MEN1\text{-}KO}$ cells (Figure 2H,I). At the protein level, PTN was increased in BON1$^{MEN1\text{-}KO}$ but decreased in QGP1$^{MEN1\text{-}KO}$ (Figure 2D–G), showing a similarity to the increased PTN expression in pNEN^{m-p+} and decreased PTN in pNEN^{m-p-} patients (please refer to Figure 1B–D), suggesting the use of BON1$^{MEN1\text{-}KO}$ and QGP1$^{MEN1\text{-}KO}$ as a representative model for pNEN^{m-p+} and pNEN^{m-p-} patients, respectively.

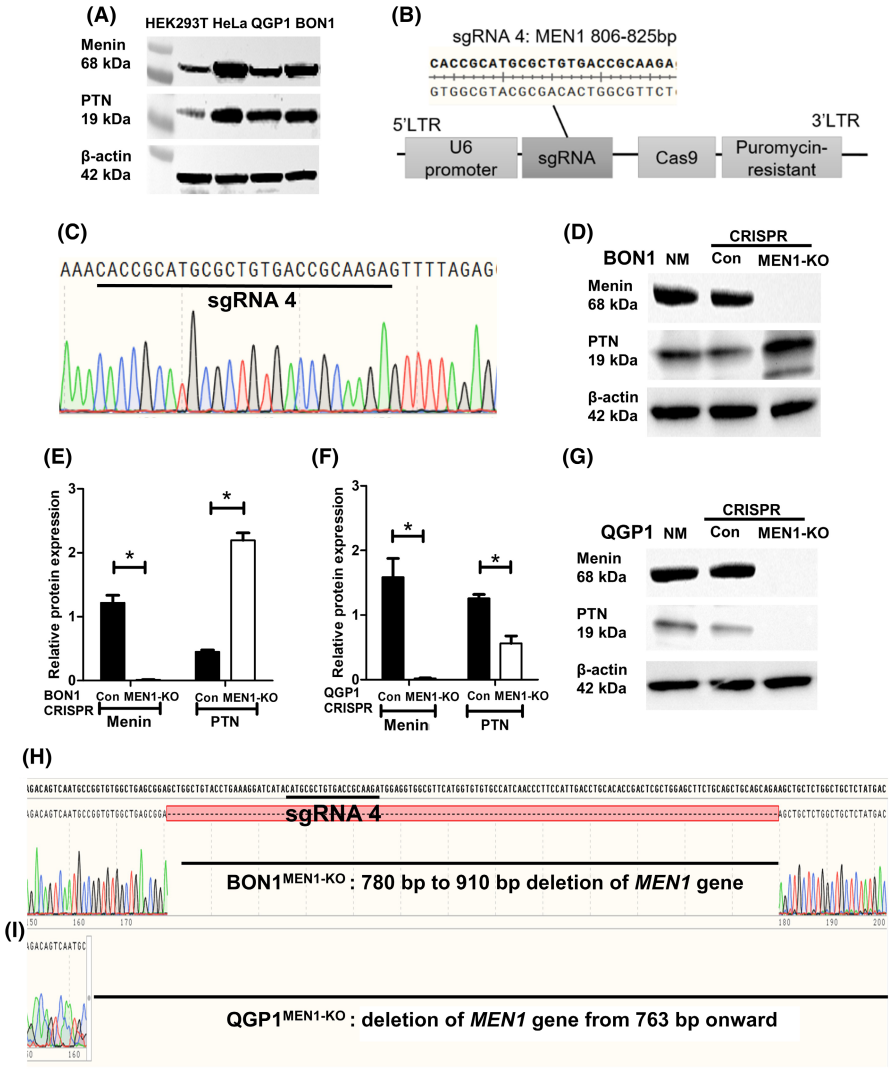

FIGURE 2 Design of CRISPR/Cas9 *MEN1*-knockout in BON1 and QGP1 cells. (A) HEK293T cells[40] and HeLa cells[36–39] are used as a menin-positive control. All lines HEK293T, HeLa, QGP1 and BON1 showed high menin and PTN protein expression. (B) Components of the lentiCRISPRv2 plasmid. The lentiCRISPRv2 plasmid includes a U6 promoter, sgRNA, Cas9, and puromycin-resistance component. The primer for the U6 promoter is used for Sanger sequencing to confirm that the candidate sgRNA is inserted into lentiCRISPRv2. sgRNA is an artificial complementary sequence of ~20 bp, which is designed according to the mRNA sequence of *MEN1* and helping the lentiCRISPRv2 plasmid to locate at the specific area of the *MEN1* gene. The puromycin-resistance component allows selection of plasmid-infected cells after puromycin treatment. (C) Sanger sequencing confirms the correctly inserted sgRNA. (D–G) Western blot of both BON1$^{MEN1\text{-}KO}$ and QGP1$^{MEN1\text{-}KO}$ cell lines demonstrates no detectable menin protein expression in comparison with their CRISPR control (BON1^{Con}/QGP1^{Con}) cell lines, respectively ($p < 0.05$). PTN protein expression increased in BON1$^{MEN1\text{-}KO}$ but is reduced in QGP1$^{MEN1\text{-}KO}$ compared with their CRISPR control group ($p < 0.05$). NM: normal untreated pNEN cells. (H) Sanger sequencing of BON1$^{MEN1\text{-}KO}$ cell line shows a 780 bp to 910 bp deletion in the *MEN1* gene. (The sgRNA targeted the 806–825 bp region of the *MEN1* mRNA gene.) (I) Sanger sequencing of QGP1$^{MEN1\text{-}KO}$ cell line shows complete *MEN1* mRNA deletion from 763 bp onward. (The sgRNA targeted the 806-825 bp region of the *MEN1* mRNA gene.)

3.4 | Impact of menin absence in metastasis of BON1 and QGP1 cells

To determine whether the lack of menin was associated with migration and invasion of pNEN cells, transwell migration and scratch wound-healing assays were performed in both BON1$^{MEN1\text{-}KO}$ and QGP1$^{MEN1\text{-}KO}$. In transwell migration and invasion assays, cell migration and invasion were markedly accelerated in BON1$^{MEN1\text{-}KO}$ ($p < 0.01$; Figure 3A,B), but significantly weakened in QGP1$^{MEN1\text{-}KO}$ ($p < 0.01$; Figure 3C,D). The scratch wound-healing assay further confirmed that the absence of menin significantly promoted the migration abilities of BON1 ($p < 0.001$; Figure 3E,F), but statistically suppressed cell migration of QGP1 ($p < 0.01$; Figure 3G,H). Taken together, these data suggested that knockout of *MEN1*, accelerated cell migration and invasion in the more aggressive BON1 cells, but suppressed the abilities of migration and invasion of QGP1 cells, reflecting the different regulation of menin on metastasis of different pNEN entities.

3.5 | Downregulation of PTN on invasion and migration of pNEN cell lines

Having seen the importance of PTN in the clinical outcome of pNEN (please refer to Figure 1), inhibition of this protein was investigated using *PTN* siRNA transfection in all the normal untreated pNEN cells and BON1$^{MEN1\text{-}KO}$ cells (si_PTN1 and si_PTN2; $p < 0.05$; Figures 4A–D and 5A,B). Loss of PTN alone (using siRNA/lipofectamine) had no impact on BON1 and QGP1 cell proliferation (data not shown).

In transwell migration and invasion assays, the decreased PTN expression led to a significant reduction of cell invasion and migration in both BON1 and QGP1 ($p < 0.05$; Figure 4E,F). Similarly, the scratch wound-healing assay further confirmed that a lack of PTN alone suppressed the cell migration of both BON1 and QGP1 ($p < 0.001$; Figure 4G–J), indicating that PTN promoted cell migration and invasion of pNEN cells.

Moreover, the significant activated cell migration and invasion by lack of menin in BON1$^{MEN1\text{-}KO}$ (BON1^{Con} and BON1$^{MEN1\text{-}KO}$, $p < 0.01$; Figure 5C,D), was markedly decreased after PTN siRNA silencing (BON1$^{MEN1\text{-}KO}$ and BON1$^{MEN1\text{-}KO+si_PTN}$; $p < 0.001$; Figure 5C,D), suggesting that menin impacted pNEN cell migration and invasion through regulating PTN expression. Furthermore, the scratch wound-healing assay confirmed that the increased cell migration of BON1$^{MEN1\text{-}KO}$ was abrogated by *PTN* siRNA ($p < 0.001$; Figure 5E,F), revealing that the absence of menin resulted in a more aggressive tumor biology mainly through an increase in PTN.

4 | DISCUSSION

As *MEN1* is the most frequently mutated gene in sporadic pNEN,[19] this study examined the loss of *MEN1* coding protein menin and its

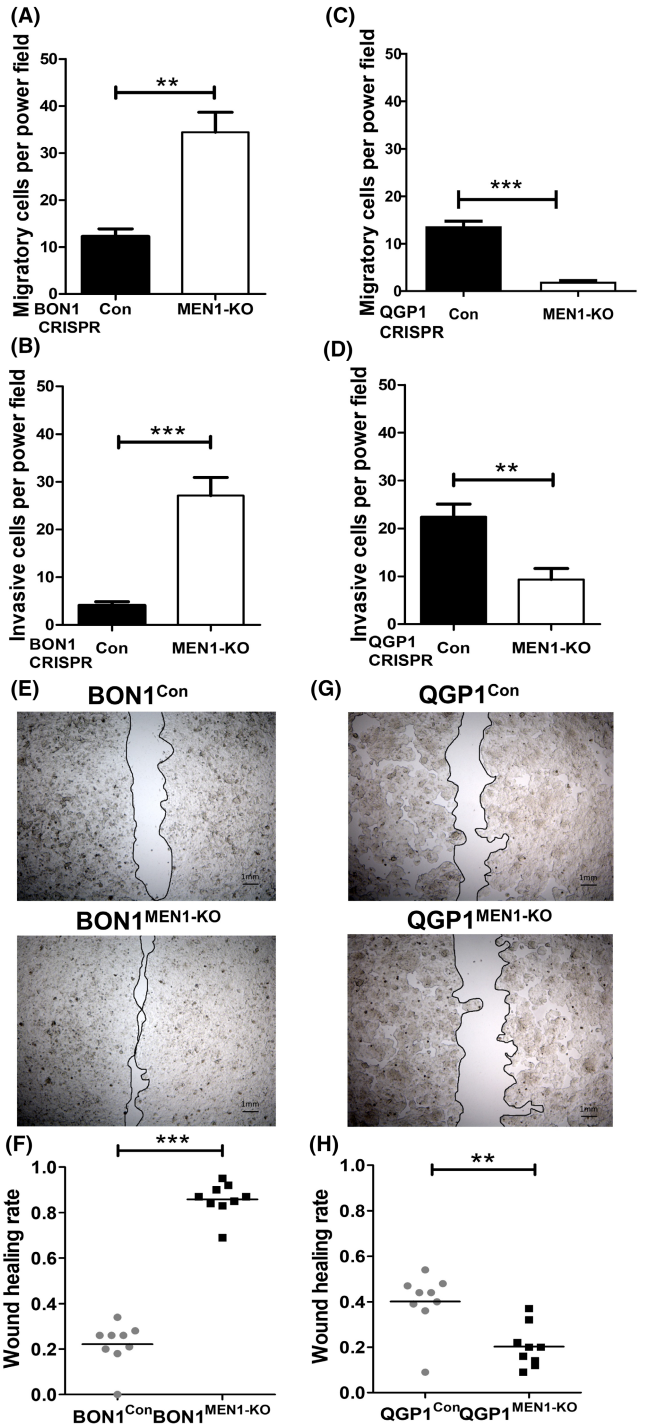

FIGURE 3 Different impact of menin knockout on migration and invasion between BON1 and QGP1 cells. (A, B) Transwell migration/invasion assays reveal more migratory and invasive cells in BON1$^{MEN1\text{-}KO}$ in comparison with BON1^{Con} cells ($p < 0.01$). (C, D) In contrast, both the numbers of migratory and invasive cells are statistically reduced in QGP1$^{MEN1\text{-}KO}$ compared with the QGP1^{Con} group ($p < 0.01$). (E, F) Scratch wound-healing assay demonstrates a significantly increased rate for wound healing in BON1$^{MEN1\text{-}KO}$ compared with BON1^{Con} ($p < 0.001$). (G, H) In contrast, QGP1$^{MEN1\text{-}KO}$ has a reduced wound-healing rate in comparison with QGP1^{Con} ($p < 0.01$)

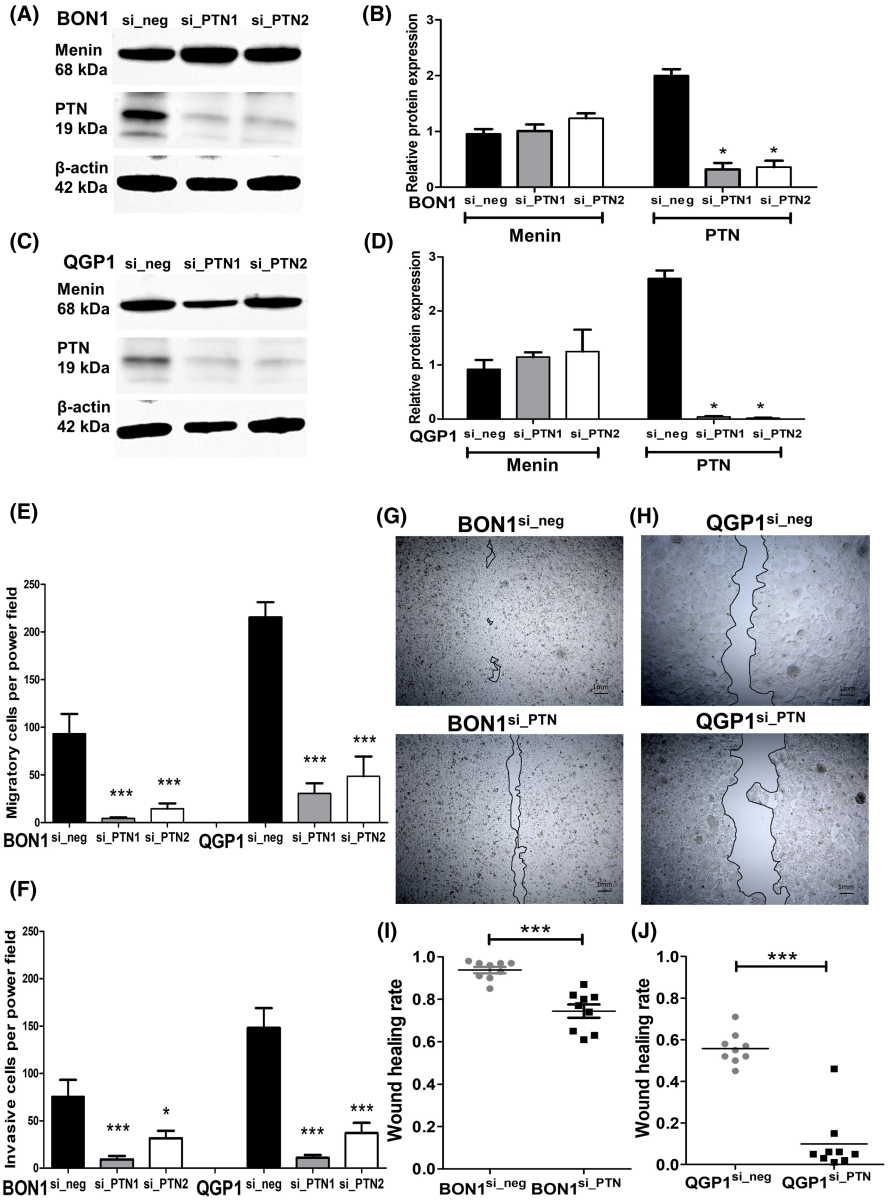

FIGURE 4 Impact of PTN siRNA silencing on migration and invasion of pNEN cells. (A–D) Western blot analysis shows normal menin, but reduced PTN, protein expression after *PTN* siRNA treatment of BON1 and QGP1 cells ($p < 0.05$). (E, F) Transwell migration/invasion assays demonstrate that cell migration and invasion of both BON1 and QGP1 are significantly reduced after *PTN* siRNA treatment (si_PTN1, si_PTN2) ($p < 0.001$). (G–J) Scratch wound-healing assays display that cell migration of both BON1 and QGP1 are suppressed after *PTN* siRNA ($BON1^{si\_PTN}$/$QGP1^{si\_PTN}$) treatment compared with their negative control siRNA ($BON1^{si\_neg}$/$QGP1^{si\_neg}$) ($p < 0.001$)

consequences, particularly on its potential downstream gene *PTN*, both in tissues from patients with pNEN and cell lines.

The relative mRNA expression of *MEN1* was decreased in metastasized patients with pNEN, and, in patients with menin-negative pNEN, PTN expression was increased and associated with metastasis and worse disease-free survival. Similar changes were also observed in BON1 cells, where the absence of menin ($BON1^{MEN1\text{-}KO}$) led to an increase in PTN expression and subsequently to an increase in cell migration and invasion. Importantly, silencing of *PTN* reversed the observed effects and therefore confirmed the downstream effect of menin on cell metastasis through PTN. Importantly, no significant difference between $pNEN^{m-p+}$ and $pNEN^{m-p-}$ patients was seen in regard to grading, which was classified using the Ki67 index, a proliferation marker, reflecting that PTN enabled the pNEN cells to migrate rather than to proliferate. This was confirmed by our *in vitro* studies that showed no differences in the proliferation assay. As resection margin (R) is an indicator for local tumor advancement or surgical quality, no association with PTN expression was expected. The combination of the absence of menin and PTN overexpression may, therefore, have the potential to predicting the metastasized clinical behavior of pNEN tumors. We here confirm one previous study showing that the absence of menin was related to a more advanced tumor stage and an increase in lymph node metastasis in gastroenteropancreatic neuroendocrine neoplasms.[41]

Whereas a recent study characterized CRISPR/Cas9-mediated *MEN1* knockout BON1 cells regarding its morphology, hormone secretion and proliferation,[42] this study mainly focused on their migration and invasion capability depending on menin and PTN expression and, for the first time, established a stable CRISPR/Cas9-mediated *MEN1*-knockout QGP1 cell line.

Most previous studies on tumor biology of pNEN have focused on the role of menin in pNEN cell growth,[43–45] but its effect on metastasis of pNEN is mainly unknown.[46] This study demonstrated that the absence of menin activated the migration and invasion abilities

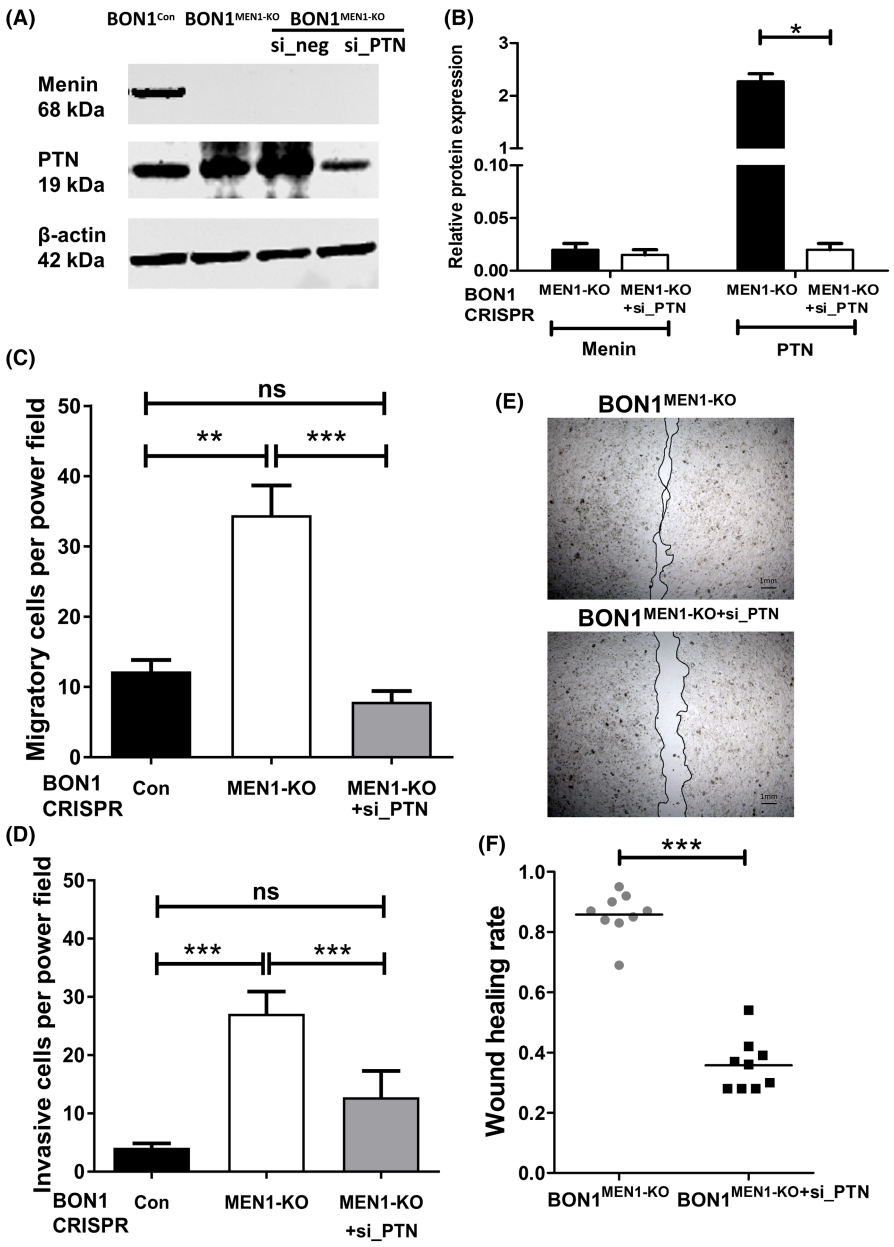

FIGURE 5 Impact of PTN siRNA silencing on BON1$^{MEN1\text{-}KO}$ cell migration and invasion. (A, B) Western blot analysis confirms the downregulation of PTN protein expression also in BON1$^{MEN1\text{-}KO}$ by *PTN* siRNA treatment ($p < 0.05$). (C, D) Both transwell migration assay and transwell invasion assay show that lack of menin results in a significant enhanced cell migration and invasion of BON1$^{MEN1\text{-}KO}$ compared with BON^{Con} ($p < 0.01$). However, this enhanced cell migration and invasion is statistically suppressed after using *PTN* siRNA (BON1$^{MEN1\text{-}KO+si_PTN}$; $p < 0.001$). (E, F) Scratch wound-healing assay reveals that *PTN* siRNA treatment on menin-deficient BON1 cells (BON1$^{MEN1\text{-}KO+si_PTN}$) alleviated the cell migration that resulted from menin deficiency ($p < 0.001$)

of BON1 cells by increasing PTN expression. Similar results in previous studies on different cancer entities have demonstrated that PTN was increased after reduction of menin and led to cell migration through different regulatory factors such as integrin $\alpha_v\beta$[47] in non–small-cell lung cancer, polycomb gene Enhancer of Zeste homolog 2,[48] and histone H3 lysine 27 trimethylation[48] in lung adenocarcinoma, and protein tyrosine phosphatase (RPTP) β/ζ in melanoma.[26]

In contrast with the metastasizing behavior of pNEN^{m-p+} patients, pNEN^{m-p-} patients showed a favorable disease-free survival. Together with the decreased PTN protein expression in both pNEN^{m-p-} patients and QGP1$^{MEN1\text{-}KO}$ cells, the biological pattern of those pNEN^{m-p-} could also be imitated through QGP1$^{MEN1\text{-}KO}$. After loss of menin, QGP1$^{MEN1\text{-}KO}$ cell migration and invasion were significantly attenuated together with a decrease in PTN expression. This study shows, for the first time, differences in the effect of menin on tumor biology both in patients with pNEN and in cell lines, and explains why loss/mutation of the tumor suppressor menin/*MEN1* has been observed previously, leading to contradicting difference in pNEN survival. Reasons for those different effects in BON1 and QGP1 cells are most likely to be different genomic mutations[29] that cause different PTN responses to *MEN1* mutation/absence of menin. This can be translated into patient outcome. Whereas Ohki et al.[18] reported that the LOH of *MEN1* had no correlation with patient survival, several other studies have shown that *MEN1* mutations led to a better[19,49,50] or to a worse survival for patients with pNEN.[51–53] The alternations of PTN expression in the absence of *MEN1*, on the most commonly used pNEN cell lines BON1 and QGP1, as well as in their abilities to metastasize, can be explained by the different response of PTN to *MEN1* knockout. The different response might influence their microenvironments to activate different histone modifications.[54] Moreover, menin was also reported to regulate the downstream factors by different histone

modifications in different environments.[54] Menin can both promote its downstream factors by catalyzing H3K4me3[55] and, also in contrast, suppress its downstream factor by enhancing H3K27me3.[48,56] In other words, menin can impair tumor progression according to its regulation of downstream factors by different modifications.[57–60] Additionally, previous studies have shown variable clinical outcome influenced by menin and its related co-factors. For example, in breast cancer, menin has been described both as a tumor promoter and a tumor suppressor, depending on the expression of estrogen receptors.[57] Similarly, menin resulted in opposite consequences in female patients with MEN1 syndrome and pNEN with or without estrogen exposure,[59] as well as in resistant prostate cancer with or without androgen receptor.[58] Moreover, PTN was associated with the expression of several proteins or enzymes[61–63] that are displayed as different expression patterns in BON1 and QGP1,[63–65] such as MGMT,[64] MAP2,[63] and MDM.[65] Therefore, the molecular factors that are abundant in QGP1 but rare in BON1 cells, may interact with menin to regulate PTN expression and metastasis in patients with pNEN and in cell lines.

In summary, in menin-negative pNEN, increased PTN expression was associated with a more aggressive behavior and poor clinical outcome. In this study, for the first time, we demonstrated that the combination of menin and PTN may serve as a novel prognostic factor and as a potential therapeutic target in patients with pNEN. In addition, we speculate on the reversible direction effects of menin in primary tumor/metastasized cell types by bringing the differences in the malignant features of BON1 and QGP1 cells into perspective. However, studies involving more patient samples and the generation of available drugs targeting PTN are needed to further elucidate the presented results.

ACKNOWLEDGEMENT

None.

DISCLOSURE

No potential conflict of interest relevant to this article was reported.

AUTHOR CONTRIBUTION

Liping He and Simon Schimmack conceived the presented idea and planned the experiments. Simon Schimmack provided laboratory space and material and supervised the experiments and analyses. Liping He carried out the experiments. Steeve Boulant, Megan Stanifer, and Cuncai Guo designed and provided the initial model of the CRISPR/Cas9 cell lines. Liping He wrote the manuscript with the support of Anna Nießen, Klaus Felix, Oliver Strobel, and Simon Schimmack. Mingyi Chen performed the calculations. Frank Bergmann performed and analyzed the tissue H&E staining of the patient samples. All authors discussed the results and revised the final manuscript.

ORCID

Simon Schimmack https://orcid.org/0000-0003-0722-7286

REFERENCES

1. Schimmack S, Svejda B, Lawrence B, Kidd M, Modlin IM. The diversity and commonalities of gastroenteropancreatic neuroendocrine tumors. *Langenbecks Arch Surg*. 2011;396(3):273-298.
2. Lloyd RV, Osamura RY, Klöppel G, Rosai J, eds. *WHO classification of tumours of endocrine organs*. Vol 10. 4th ed. WHO Classification of Tumours; 2017.
3. Fischer L, Bergmann F, Schimmack S, et al. Outcome of surgery for pancreatic neuroendocrine neoplasms. *Br J Surg*. 2014;101(11):1405-1412.
4. Rindi G, Falconi M, Klersy C, et al. TNM staging of neoplasms of the endocrine pancreas: results from a large international cohort study. *J Natl Cancer Inst*. 2012;104(10):764-777.
5. Tanaka M, Heckler M, Mihaljevic AL, et al. Systematic review and metaanalysis of lymph node metastases of resected pancreatic neuroendocrine tumors. *Ann Surg Oncol*. 2020;28(3):1614-1624.
6. Panzuto F, Merola E, Rinzivillo M, et al. Advanced digestive neuroendocrine tumors: metastatic pattern is an independent factor affecting clinical outcome. *Pancreas*. 2014;43(2):212-218.
7. Sandini M, Strobel O, Hank T, et al. Pre-operative dysglycemia is associated with decreased survival in patients with pancreatic neuroendocrine neoplasms. *Surgery*. 2020;167(3):575-580.
8. Primavesi F, Andreasi V, Hoogwater FJH, et al. A preoperative clinical risk score including C-reactive protein predicts histological tumor characteristics and patient survival after surgery for sporadic non-functional pancreatic neuroendocrine neoplasms: an international multicenter cohort study. *Cancers (Basel)*. 2020;12(5):1235.
9. Tanaka M, Heckler M, Mihaljevic AL, et al. Systematic review and metaanalysis of lymph node metastases of resected pancreatic neuroendocrine tumors. *Ann Surg Oncol*. 2021;28(3):1614-1624.
10. Yu YJ, Li YW, Shi Y, Zhang Z, Zheng MY, Zhang SW. Clinical and pathological characteristics and prognosis of 132 cases of rectal neuroendocrine tumors. *World J Gastrointest Oncol*. 2020;12(8):893-902.
11. Calender A. Molecular genetics of neuroendocrine tumors. *Digestion*. 2000;62(Suppl 1):3-18.
12. Agarwal SK, Kester MB, Debelenko LV, et al. Germline mutations of the MEN1 gene in familial multiple endocrine neoplasia type 1 and related states. *Hum Mol Genet*. 1997;6(7):1169-1175.
13. Biondi CA, Gartside MG, Waring P, et al. Conditional inactivation of the MEN1 gene leads to pancreatic and pituitary tumorigenesis but does not affect normal development of these tissues. *Mol Cell Biol*. 2004;24(8):3125-3131.
14. Cloyd JM, Poultsides GA. Non-functional neuroendocrine tumors of the pancreas: advances in diagnosis and management. *World J Gastroenterol*. 2015;21(32):9512-9525.
15. Halfdanarson TR, Rubin J, Farnell MB, Grant CS, Petersen GM. Pancreatic endocrine neoplasms: epidemiology and prognosis of pancreatic endocrine tumors. *Endocr Relat Cancer*. 2008;15(2):409-427.
16. Metz DC, Jensen RT. Gastrointestinal neuroendocrine tumors: pancreatic endocrine tumors. *Gastroenterology*. 2008;135(5):1469-1492.
17. Zhang WH, Wang WQ, Han X, et al. Infiltrating pattern and prognostic value of tertiary lymphoid structures in resected nonfunctional pancreatic neuroendocrine tumors. *J Immunother Cancer*. 2020;8(2):e001188.
18. Ohki R, Saito K, Chen Y, et al. PHLDA3 is a novel tumor suppressor of pancreatic neuroendocrine tumors. *Proc Natl Acad Sci USA*. 2014;111(23):E2404-E2413.
19. Jiao Y, Shi C, Edil BH, et al. DAXX/ATRX, MEN1, and mTOR pathway genes are frequently altered in pancreatic neuroendocrine tumors. *Science*. 2011;331(6021):1199-1203.

20. Falchetti A. Genetics of multiple endocrine neoplasia type 1 syndrome: what's new and what's old. *F1000Res*. 2017;6:73.
21. Concolino P, Costella A, Capoluongo E. Multiple endocrine neoplasia type 1 (MEN1): an update of 208 new germline variants reported in the last nine years. *Cancer Genet*. 2016;209(1–2):36-41.
22. Wellstein A, Fang WJ, Khatri A, et al. A heparin-binding growth factor secreted from breast cancer cells homologous to a developmentally regulated cytokine. *J Biol Chem*. 1992;267(4):2582-2587.
23. Chauhan AK, Li YS, Deuel TF. Pleiotrophin transforms NIH 3T3 cells and induces tumors in nude mice. *Proc Natl Acad Sci USA*. 1993;90(2):679-682.
24. Bermek O, Diamantopoulou Z, Polykratis A, et al. A basic peptide derived from the HARP C-terminus inhibits anchorage-independent growth of DU145 prostate cancer cells. *Exp Cell Res*. 2007;313(19):4041-4050.
25. Perez-Pinera P, Chang Y, Deuel TF. Pleiotrophin, a multifunctional tumor promoter through induction of tumor angiogenesis, remodeling of the tumor microenvironment, and activation of stromal fibroblasts. *Cell Cycle*. 2007;6(23):2877-2883.
26. Gao SB, Feng ZJ, Xu B, et al. Menin represses malignant phenotypes of melanoma through regulating multiple pathways. *J Cell Mol Med*. 2011;15(11):2353-2363.
27. Evers BM, Ishizuka J, Townsend CM Jr, Thompson JC. The human carcinoid cell line, BON. A model system for the study of carcinoid tumors. *Ann N Y Acad Sci*. 1994;733:393-406.
28. Schimmack S, Yang Y, Felix K, et al. C-reactive protein (CRP) promotes malignant properties in pancreatic neuroendocrine neoplasms. *Endocr Connect*. 2019;8(7):1007-1019.
29. Vandamme T, Peeters M, Dogan F, et al. Whole-exome characterization of pancreatic neuroendocrine tumor cell lines BON-1 and QGP-1. *J Mol Endocrinol*. 2015;54(2):137-147.
30. Vandamme T, Beyens M, Peeters M, Van Camp G, de Beeck KO. Next generation exome sequencing of pancreatic neuroendocrine tumor cell lines BON-1 and QGP-1 reveals different lineages. *Cancer Genet*. 2015;208(10):523.
31. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc*. 2013;8(11):2281-2308.
32. Shalem O, Sanjana NE, Zhang F. High-throughput functional genomics using CRISPR-Cas9. *Nat Rev Genet*. 2015;16(5):299-311.
33. Del Castillo V-H, van der Weyden L, Nsengimana J, et al. Comparative genomics reveals that loss of lunatic fringe (LFNG) promotes melanoma metastasis. *Mol Oncol*. 2018;12(2):239-255.
34. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*. 2001;25(4):402-408.
35. Schimmack S, Lawrence B, Svejda B, et al. The clinical implications and biologic relevance of neurofilament expression in gastroenteropancreatic neuroendocrine neoplasms. *Cancer*. 2012;118(10):2763-2775.
36. Ren F, Xu HW, Hu Y, et al. Expression and subcellular localization of menin in human cancer cells. *Exp Ther Med*. 2012;3(6):1087-1091.
37. Scacheri PC, Rozenblatt-Rosen O, Caplen NJ, et al. Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. *Proc Natl Acad Sci USA*. 2004;101(7):1892-1897.
38. Wautot V, Khodaei S, Frappart L, et al. Expression analysis of endogenous menin, the product of the multiple endocrine neoplasia type 1 gene, in cell lines and human tissues. *Int J Cancer*. 2000;85(6):877-881.
39. Srivatsan ES, Bengtsson U, Manickam P, et al. Interstitial deletion of 11q13 sequences in HeLa cells. *Genes Chromosomes Cancer*. 2000;29(2):157-165.
40. Yu D, Nagamura Y, Shimazu S, et al. Caspase 8 and menin expressions are not correlated in human parathyroid tumors. *Endocr J*. 2010;57(9):825-832.
41. Kim HS, Lee HS, Nam KH, Choi J, Kim WH. p27 loss is associated with poor prognosis in gastroenteropancreatic neuroendocrine tumors. *Cancer Res Treat*. 2014;46(4):383-392.
42. Monazzam A, Li SC, Wargelius H, et al. Generation and characterization of CRISPR/Cas9-mediated MEN1 knockout BON1 cells: a human pancreatic neuroendocrine cell line. *Sci Rep*. 2020;10(1):14572.
43. Jiang X, Cao Y, Li F, et al. Targeting beta-catenin signaling for therapeutic intervention in MEN1-deficient pancreatic neuroendocrine tumours. *Nat Commun*. 2014;5:5809.
44. Cheng P, Wang YF, Li G, et al. Interplay between menin and Dnmt1 reversibly regulates pancreatic cancer cell growth downstream of the Hedgehog signaling pathway. *Cancer Lett*. 2016;370(1):136-144.
45. Capodanno Y, Chen Y, Schrader J, et al. Cross-talk among MEN1, p53 and Notch regulates the proliferation of pancreatic neuroendocrine tumor cells by modulating INSM1 expression and subcellular localization. *Neoplasia*. 2021;23(9):979-992.
46. Razmara M, Monazzam A, Skogseid B. Reduced menin expression impairs rapamycin effects as evidenced by an increase in mTORC2 signaling and cell migration. *Cell Commun Signal*. 2018;16(1):64.
47. Feng ZJ, Gao SB, Wu Y, Xu XF, Hua X, Jin GH. Lung cancer cell migration is regulated via repressing growth factor PTN/RPTP beta/zeta signaling by menin. *Oncogene*. 2010;29(39):5416-5426.
48. Gao SB, Feng ZJ, Xu B, et al. Suppression of lung adenocarcinoma through menin and polycomb gene-mediated repression of growth factor pleiotrophin. *Oncogene*. 2009;28(46):4095-4104.
49. Park JK, Paik WH, Lee K, Ryu JK, Lee SH, Kim YT. DAXX/ATRX and MEN1 genes are strong prognostic markers in pancreatic neuroendocrine tumors. *Oncotarget*. 2017;8(30):49796-49806.
50. Chou WC, Lin PH, Yeh YC, et al. Genes involved in angiogenesis and mTOR pathways are frequently mutated in Asian patients with pancreatic neuroendocrine tumors. *Int J Biol Sci*. 2016;12(12):1523-1532.
51. Chan CS, Laddha SV, Lewis PW, et al. ATRX, DAXX or MEN1 mutant pancreatic neuroendocrine tumors are a distinct alpha-cell signature subgroup. *Nat Commun*. 2018;9(1):4158.
52. Lopez-Egido JR, Wang Y, Gronberg M, et al. Differentially regulated genes in MEN1-transfected BON cells using RT-differential display and oligonucleotide microarrays. *Anticancer Res*. 2009;29(6):1859-1866.
53. Stalberg P, Grimfjard P, Santesson M, et al. Transfection of the multiple endocrine neoplasia type 1 gene to a human endocrine pancreatic tumor cell line inhibits cell growth and affects expression of JunD, delta-like protein 1/preadipocyte factor-1, proliferating cell nuclear antigen, and QM/Jif-1. *J Clin Endocrinol Metab*. 2004;89(5):2326-2337.
54. Iyer S, Agarwal SK. Epigenetic regulation in the tumorigenesis of MEN1-associated endocrine cell types. *J Mol Endocrinol*. 2018;61(1):R13-R24.
55. Chen Y, Jones KL, Anastassiadis K, et al. Distinct pathways affected by menin versus MLL1/MLL2 in MLL-rearranged acute myeloid leukemia. *Exp Hematol*. 2019;69:37-42.
56. Funato K, Major T, Lewis PW, Allis CD, Tabar V. Use of human embryonic stem cells to model pediatric gliomas with H3.3K27M histone mutation. *Science*. 2014;346(6216):1529-1533.
57. Dreijerink KMA, Groner AC, Vos ESM, et al. Enhancer-mediated oncogenic function of the menin tumor suppressor in breast cancer. *Cell Rep*. 2017;18(10):2359-2372.
58. Malik R, Khan AP, Asangani IA, et al. Targeting the MLL complex in castration-resistant prostate cancer. *Nat Med*. 2015;21(4):344-352.

59. Qiu W, Christakis I, Stewart AA, et al. Is estrogen exposure a protective factor for pancreatic neuroendocrine tumours in female patients with multiple endocrine neoplasia syndrome type 1? *Clin Endocrinol (Oxf)*. 2017;86(6):791-797.
60. Cierpicki T, Grembecka J. Challenges and opportunities in targeting the menin-MLL interaction. *Future Med Chem*. 2014;6(4):447-462.
61. Jin L, Gao F, Zhang L, et al. Pleiotropin enhances the osteo/dentinogenic differentiation potential of dental pulp stem cells. *Connect Tissue Res*. 2021;62(5):495-507.
62. Tsai CK, Huang LC, Wu YP, Kan IY, Hueng DY. SNAP reverses temozolomide resistance in human glioblastoma multiforme cells through down-regulation of MGMT. *FASEB J*. 2019;33(12):14171-14184.
63. Asai H, Morita S, Miyata S. Effect of pleiotrophin on glutamate-induced neurotoxicity in cultured hippocampal neurons. *Cell Biochem Funct*. 2011;29(8):660-665.
64. Hijioka S, Sakuma K, Aoki M, et al. Clinical and *in vitro* studies of the correlation between MGMT and the effect of streptozocin in pancreatic NET. *Cancer Chemother Pharmacol*. 2019;83(1):43-52.
65. Hu W, Feng Z, Modica I, et al. Gene amplifications in well-differentiated pancreatic neuroendocrine tumors inactivate the p53 pathway. *Genes Cancer*. 2010;1(4):360-368.

How to cite this article: He L, Boulant S, Stanifer M, et al. The link between menin and pleiotrophin in the tumor biology of pancreatic neuroendocrine neoplasms. *Cancer Sci*. 2022;113:1575–1586. doi:10.1111/cas.15301