Recapitulation of Pancreatic Neuroendocrine Tumors in Human Multiple Endocrine Neoplasia Type I Syndrome via Pdx1-Directed Inactivation of Men1

H.-C. Jennifer Shen,1 Mei He,1 Anthea Powell,1 Asha Adem,1 Dominique Lorang,1 Charles Heller,1 Amelia C. Grover,1 Kris Ylaya,2 Stephen M. Hewitt,2 Stephen J. Marx,1 Allen M. Spiegel,1 and Steven K. Libutti1

1Tumor Angiogenesis Section, Surgery Branch and 2Laboratory of Pathology, National Cancer Institute and 3Metabolic Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, Maryland and 4Albert Einstein College of Medicine, Bronx, New York

Abstract

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal syndrome caused by mutations in the MEN1 tumor suppressor gene. Whereas the protein product of MEN1, menin, is ubiquitously expressed, somatic loss of the remaining wild-type MEN1 allele results in tumors primarily in parathyroid, pituitary, and endocrine pancreas. To understand the endocrine specificity of the MEN1 syndrome, we evaluated biallelic loss of Men1 by inactivating Men1 in pancreatic progenitor cells using the Cre-lox system. Men1 deletion in progenitor cells that differentiate into exocrine and endocrine pancreas did not affect normal pancreas morphogenesis and development. However, mice having homozygous inactivation of the Men1 in pancreas developed endocrine tumors with no exocrine tumor manifestation, recapitulating phenotypes seen in the MEN1 patients. In the absence of menin, the endocrine pancreas showed increase in cell proliferation, vascularity, and abnormal vascular structures; such changes were lacking in exocrine pancreas. Further analysis revealed that these endocrine manifestations were associated with up-regulation in vascular endothelial growth factor expression in both human and mouse MEN1 pancreatic endocrine tumors. Together, these data suggest the presence of cell-specific factors for menin and a permissive endocrine environment for MEN1 tumorigenesis in endocrine pancreas. Based on our analysis, we propose that menin's ability to maintain cellular and microenvironment integrity might explain the endocrine-restrictive nature of the MEN1 syndrome. [Cancer Res 2009;69(5):1858–66]

Introduction

Multiple endocrine neoplasia type 1 (MEN1; OMIM 131100) is a dominant inherited syndrome caused by mutations in the MEN1 tumor suppressor gene (1, 2). Patients with a family history of the MEN1 syndrome are predisposed to develop multiple endocrine tumors, primarily affecting parathyroid, anterior pituitary, and pancreatic islets. More than 95% of MEN1 patients develop clinical manifestations of the disorder by the fifth decade (3, 4), whereas the earliest occurrence has been reported at 5 years old (5). Consistent with Knudson’s two-hit hypothesis for tumor suppressor genes (6), MEN1 monoclonal expansion is initiated when loss of heterozygosity (LOH) at 11q13 occurs in patients with inherited germ line mutations of the MEN1 gene (7–9). Additionally, somatic inactivation and LOH of the MEN1 alleles have been reported in a variety of sporadic endocrine tumors, such as parathyroid adenomas and pancreatic insulinsomas (10, 11). Mutations in the MEN1 gene seem to be inactivating, and no clear genotype-phenotype correlations have been established for mutations detected along the coding sequence of MEN1 in both familial and sporadic tumors (12).

The protein product of MEN1, menin, does not display significant homology to any known family of proteins, and it has been described predominantly as a transcriptional regulator by interacting with nuclear proteins, such as JunD, nuclear factor-κB, Smad3, and FANCD2 (13, 14). Further biochemical studies have shown that menin complexes with mixed-lineage leukemia protein to regulate gene expression via chromatin modification in mouse embryonic fibroblast cells (15), bone marrow cells (16), and human HeLa and leukemia cells (17, 18). Similar epigenetic regulation by menin has also been reported in endocrine tumors, wherein menin modulates histone methylation and expression of cyclin-dependent kinase inhibitors, p27 and p18 (19). More recently, menin has been implicated in the control of pancreatic β-cell growth during pregnancy (20). Together, these studies have broadened our knowledge of the biochemical, physiologic, and pathologic roles of menin in both endocrine and nonendocrine contexts.

Using mouse models to understand the human MEN1 syndrome has proved to be informative (21–23), perhaps due to the highly conserved genomic structures, nucleotide (89% identity), and amino acid (97% identity) sequences shared by mouse Men1 and human MEN1 genes (24, 25). Although mice deficient of both Men1 alleles die in utero at E11.5-13.5 with developmental defects in multiple organs, mice heterozygous for Men1 alleles show an increased incidence of endocrine tumors and demonstrate a similar phenotype to the human MEN1 patients (21, 22, 26). To circumvent the embryonic lethality, conditional inactivation of Men1 has further confirmed that biallelic loss of menin in endocrine tissues can lead to the development of parathyroid adenoma (27), pancreatic insulinoma (28, 29), and pituitary prolactinoma (30). These observations are reminiscent of the tumor spectrum observed in mice with heterozygous germ line deletion of Men1, as well as human MEN1 patients. Whereas the generation of mouse models has effectively mimicked the human MEN1 syndrome, the mechanisms...
leading to the endocrine-specific tumorigenicity of the MEN1 syndrome remain to be elucidated.

Based on published biochemical and genetic analyses on Men1, we postulated that tumorigenicity of the MEN1 syndrome may be explained by cell-specific cofactors for menin, because expression of menin is not restricted to endocrine tissues (25, 31), whereas most manifestations of MEN1 essentially are endocrine specific. To test this hypothesis, we chose to selectively inactivate Men1 in pancreatic progenitor cells during embryogenesis, such that Men1 alleles are deleted in exocrine and endocrine cells of the pancreas. Our aim was to determine whether complete loss of menin in cells of pancreatic lineage leads to tumorigenesis only in MEN1-affected endocrine tissues or in pancreatic exocrine tissues as well. Here, we describe that similar to MEN1 patients with neuroendocrine tumors, mice completely deficient of Men1 in both endocrine and exocrine pancreas developed only pancreatic endocrine tumors, supporting the hypothesis that cell-specific factors exist to explain the tissue-selective tumorigenicity of the MEN1 syndrome. We further provide evidence demonstrating that alterations in the endocrine microenvironment, such as up-regulation in vascular endothelial growth factor (VEGF) expression, are involved in developing pancreatic endocrine tumors resulting from the loss of menin. Together, these findings suggest that cell-specific factors are involved in MEN1 pancreatic tumorigenesis and that a permissive microenvironment is essential in this process.

Materials and Methods

Animals and genotyping. Mice carrying the Men1 alleles flanked by loxp sites (Men1 1/1 or Men1 ΔΔ/ΔΔ; ref. 29) were crossed with Pdx1-Cre transgenic mice (a kind gift from D. Melton; ref. 32) to generate Pdx1-Cre;Men1 f/+ heterozygous mice in mixed FVB;129Sv background. Pdx1-transgenic mice (a kind gift from D. Melton; ref. 32) to generate Pdx1-loxPsites(Men1f/forMen1 (Pierce) containing 1 most manifestations of MEN1 essentially are endocrine specific. To explained by cell-specific cofactors for menin, because expression

www.aacrjournals.org 1859 Cancer Res 2009; 69: (5). March 1, 2009

Histologic analysis. FITC-lectin perfusion to visualize the vasculature was performed as described (33). Briefly, mice were injected i.v. via tail vein with 50 μg of FITC-labeled lectin (Lycopersicon esculentum, Vector Laboratories), which was allowed to circulate for 3 min. Then, mice were euthanized via cervical dislocation, and their pancreas was removed. The pancreatic tissue was processed for both frozen histologic analysis by embedding tissues in Tissue-Tek optimal cryo temperature (OCT) freezing medium and for formalin-fixed paraffin embedding (FFPE). For all immunostaining experiments described below, appropriate positive and negative controls were run concurrently for all the applied antisera on the adjacent pancreas sections.

Frozen (10–20 μm) and FFPE sections (5 μm) of mouse pancreas were routinely stained with Mayer's H&E for histopathologic analysis. For immunohistochemical staining of VEGF, a goat anti-mouse VEGF (1:50, R&D Systems) or a monoclonal antibody against human VEGF (clone VG1; 1:50, Thermo Fisher Scientific) was used. Sections were counterstained in Mayer's hematoxylin, mounted, and photographed using a Zeiss microscope.

For immunostaining of FITC-lectin injected pancreas, frozen sections were briefly fixed in 4% paraformaldehyde, washed in PBS, and incubated in blocking buffer (5% normal goat serum/2.5% bovine serum albumin in PBS). Primary antibodies were diluted in 0.5% blocking buffer: guinea pig anti-swine insulin (1:500, DAKO), rabbit anti-human gastrin (1:500, Novocastra/DAKO), rabbit anti-human chromogranin A (ready to use, Invitrogen), rabbit anti-synaptophysin (1:50, Diagnostic Biosystems), sheep anti-human CDBI (1:50; R&D Systems), and monoclonal rabbit anti-human Ki67 (1:500, Thermo Fisher Scientific).

For quantitative analysis of immunofluorescent images and islet areas within the pancreas, multiple images from the same animals were analyzed using the Zeiss imaging software AxioVision (Carl Zeiss MicroImaging). Application-specific macros written by Zeiss support were applied to each image to allow unbiased quantification of the positive signals. Densitometry for each of the images were additionally evaluated and controlled for staining variability among different samples. Human neuroendocrine tumor images were "mosaic" images consisting of a quilt of four consecutive low-powered fields stitched together using the same imaging software as described (34).

VEGF ELISA. Mouse VEGF protein was measured using Quantikine mouse VEGF immunoassay according to the manufacturer's protocols (R&D Systems).

In vivo treatment study. Sunitinib was suspended in an aqueous solution of 0.5% carboxymethylcellulose and 0.25% Tween 80, as described previously (34). Age, weight, and sex-matched mice for each genotype, and Men1 1/1 and Pdx1-CreMen1 1/1, were treated with sunitinib (20 mg/kg of weight; ref. 35) or vehicle via oral gavage daily for 3 mo. Mice began treatment at an average age of 3 mo. During the treatment period, body weight for each animal was monitored for treatment toxicity and for calculating sunitinib dosing monthly. For each animal, images of all islets in each section were captured for quantitative analysis. All animal experiments were conducted in accordance with NIH-approved and AALAC-approved protocols and guidelines.

Human tissue samples. Normal pancreas and MEN1 pancreatic neuroendocrine tumors were obtained from patients under NIH IRB-approved protocols. After surgical removal, the samples were flash frozen in liquid nitrogen and stored at −80°C or formalin-fixed for paraffin embedding. Frozen pancreas were embedded in OCT compound (Tissue Tek II, Miles) on dry ice before being sectioned into 8 to 10 μm thickness for

www.aacrjournals.org 1859 Cancer Res 2009; 69: (5). March 1, 2009

Histologic analysis. FITC-lectin perfusion to visualize the vasculature was performed as described (33). Briefly, mice were injected i.v. via tail vein with 50 μg of FITC-labeled lectin (Lycopersicon esculentum, Vector Laboratories), which was allowed to circulate for 3 min. Then, mice were euthanized via cervical dislocation, and their pancreas was removed. The pancreatic tissue was processed for both frozen histologic analysis by embedding tissues in Tissue-Tek optimal cryo temperature (OCT) freezing medium and for formalin-fixed paraffin embedding (FFPE). For all immunostaining experiments described below, appropriate positive and negative controls were run concurrently for all the applied antisera on the adjacent pancreas sections.

Frozen (10–20 μm) and FFPE sections (5 μm) of mouse pancreas were routinely stained with Mayer's H&E for histopathologic analysis. For immunohistochemical staining of VEGF, a goat anti-mouse VEGF (1:50, R&D Systems) or a monoclonal antibody against human VEGF (clone VG1; 1:50, Thermo Fisher Scientific) was used. Sections were counterstained in Mayer's hematoxylin, mounted, and photographed using a Zeiss microscope.

For immunostaining of FITC-lectin injected pancreas, frozen sections were briefly fixed in 4% paraformaldehyde, washed in PBS, and incubated in blocking buffer (5% normal goat serum/2.5% bovine serum albumin in PBS). Primary antibodies were diluted in 0.5% blocking buffer: guinea pig anti-swine insulin (1:500, DAKO), rabbit anti-human gastrin (1:500, Novocastra/DAKO), rabbit anti-human chromogranin A (ready to use, Invitrogen), rabbit anti-synaptophysin (1:50, Diagnostic Biosystems), sheep anti-human CDBI (1:50; R&D Systems), and monoclonal rabbit anti-human Ki67 (1:500, Thermo Fisher Scientific).

For quantitative analysis of immunofluorescent images and islet areas within the pancreas, multiple images from the same animals were analyzed using the Zeiss imaging software AxioVision (Carl Zeiss MicroImaging). Application-specific macros written by Zeiss support were applied to each image to allow unbiased quantification of the positive signals. Densitometry for each of the images were additionally evaluated and controlled for staining variability among different samples. Human neuroendocrine tumor images were "mosaic" images consisting of a quilt of four consecutive low-powered fields stitched together using the same imaging software as described (34).

VEGF ELISA. Mouse VEGF protein was measured using Quantikine mouse VEGF immunoassay according to the manufacturer's protocols (R&D Systems).

In vivo treatment study. Sunitinib was suspended in an aqueous solution of 0.5% carboxymethylcellulose and 0.25% Tween 80, as described previously (34). Age, weight, and sex-matched mice for each genotype, and Men1 1/1 and Pdx1-CreMen1 1/1, were treated with sunitinib (20 mg/kg of weight; ref. 35) or vehicle via oral gavage daily for 3 mo. Mice began treatment at an average age of 3 mo. During the treatment period, body weight for each animal was monitored for treatment toxicity and for calculating sunitinib dosing monthly. For each animal, images of all islets in each section were captured for quantitative analysis. All animal experiments were conducted in accordance with NIH-approved and AALAC-approved protocols and guidelines.

Human tissue samples. Normal pancreas and MEN1 pancreatic neuroendocrine tumors were obtained from patients under NIH IRB-approved protocols. After surgical removal, the samples were flash frozen in liquid nitrogen and stored at −80°C or formalin-fixed for paraffin embedding. Frozen pancreas were embedded in OCT compound (Tissue Tek II, Miles) on dry ice before being sectioned into 8 to 10 μm thickness for
histologic analysis. Frozen sections from two different MEN1 patients were used for immunofluorescent staining of insulin, CD31, and chromogranin A. These two patients were operated on for tumor size and/or metastasis, and tumors were positive for general pancreatic neuroendocrine tumor markers (chromogranin A and synaptophysin). Additional pancreatic neuroendocrine tumors from four different MEN1 patients (two insulinomas and two gastrinomas) were used for immunohistochemical staining of VEGF on their FFPE tumor sections. Sections of normal and neuroendocrine tumors were blindly evaluated and identified by pathologist (S.M.H.).

Statistical analysis. Statistical analysis was performed using GraphPad InStat v.3.05, GraphPad Prism v.4.02, and Microsoft Excel. Imaging quantification data were analyzed with a Mann-Whitney nonparametric test, but percentage of islet area per pancreas section (Fig. 1C) was evaluated with two-tailed Student’s t test. Plasma insulin levels, blood glucose level, and VEGF ELISA were evaluated with one-way ANOVA with multiple comparisons testing. A P value of <0.05 was considered statistically significant.

Results
Loss of menin in pancreatic progenitor cells does not affect normal pancreas development but leads to increase in cell proliferation only in endocrine pancreas. To further understand the tissue-specific role of MEN1 in tumorigenesis, we generated a conditional knockout mouse model to evaluate the loss of Men1 in both exocrine and endocrine pancreas cells using a Cre-loxP system. Transgenic mice expressing Cre recombinase from the

---

**Figure 1.** Generation of Pdx1-Cre;Men1 f/f mice. A, deletion of the Men1 alleles in mouse pancreas. Genotyping PCR using genomic DNA isolated from pancreas exocrine (T, tissues) and endocrine cells (Is, islet) using laser-captured microdissection. Top, the status of Men1 alleles (f, flox, 430 bp; +, wild-type, 380 bp); bottom, the presence of deleted Men1 allele (del, 638 bp) only in Pdx1-Cre–positive pancreas. B, Western blot analysis of menin protein expression (76 kDa) in mouse pancreas using total pancreatic protein lysate in a 12-month-old cohort. Genotype of each mouse is indicated on top (wt, wild-type), and β-actin (43 kDa) is used as loading control. C, immunofluorescent staining for insulin (red) and Ki-67 (green) of representative control Men1 f/+ and mutant Pdx1-Cre;Men1 f/f islets of 2-month-old to 3-month-old mice. Magnification, 400×. D, quantification of proliferating cells in endocrine islets or exocrine cells of pancreas in mice at ages of 2 to 3 mo. Representative of two different animals of the same genotype; multiple images were analyzed for each animal. The absolute number of Ki-67–positive proliferating cells was counted within the endocrine and exocrine tissues for each image using the AxioVision software.
Pdx1 (pancreatic and duodenal homeobox 1) promoter allowed target gene deletion in all pancreatic endocrine and exocrine cells due to Pdx1-Cre expression in pancreatic progenitor cells (32). The Pdx1-Cre transgenic mice bred with mice whose Men1 alleles are flanked by loxP sites from exons 3 to 8 (Men1f/f; ref. 22) gave expected Mendelian frequencies for all genotypes (Supplementary Table S1). Laser capture microdissection of genomic DNA from pancreatic endocrine and exocrine cells showed the expected deletion of Men1 alleles in all pancreatic cells of Pdx1-Cre;Men1f/f animals (Fig. 1A, bottom). Furthermore, using total pancreatic lysate, we confirmed a significant reduction of menin protein expression in Pdx1-Cre;Men1f/f homozygous mice (Fig. 1B).

Together, these results showed that Pdx1-Cre–mediated loss of menin in the pancreas is not lethal to embryogenesis and does not affect normal pancreas development.

Histopathologic analysis of pancreatic tissues at early time points (2–3 months) showed normal exocrine glandular components and endocrine islets in Pdx1-Cre (n = 1), Men1 f/f (n = 5), Pdx1-Cre;Men1 f/+ (n = 10), and Pdx1-Cre;Men1 f/f (n = 6) animals (Supplementary Data A). Because Men1 deletion has been shown to lead to increased β-cell proliferation (36), we tested whether or not loss of Men1 in pancreatic progenitor cells would result in an increase in proliferation potential in both endocrine and exocrine cells of pancreas. We elected to use Ki-67 as the cell proliferation marker for all pancreatic cells and insulin as a marker for endocrine cells by immunofluorescent staining (Fig. 1C and Supplementary Data B). As expected, Pdx1-Cre;Men1 f/f mice indeed had more proliferating cells within their islets when compared with age-matched control genotype Men1 f/+ mice (Fig. 1D). However, although deletion of Men1 alleles was confirmed by PCR in exocrine cells of Pdx1-Cre;Men1 f/f pancreas (Fig. 1A), no significant difference in the number of proliferating cells was detected in nonendocrine cells outside islets (Fig. 1D).

Inactivation of Men1 in exocrine and endocrine cells of pancreas leads exclusively to endocrine tumor development in Pdx1-Cre;Men1 f/f mice. Further characterization of older Pdx1-Cre;Men1 f/f mice showed enlarged and hyperplastic islets as early as at ages of 5 to 6 months, and these mice exhibited progression to insulinoma by ages of 10 to 12 months (Fig. 2A). Gastrinoma was not observed in Pdx1-Cre;Menf f/f pancreas at ages of >12 months (n = 5, data not shown) although gastrinoma has been reported in a different mouse model of MEN1 (21). Insulinomas were filled with blood islands or lacunae.
and characterized by disorganized tumor cells with aberrant nuclei (Fig. 2A, right bottom). Yet, no histologic abnormality was observed in the exocrine tissues of Pdx1-Cre;Men1 f/f animals. Consistent with the development of hyperplastic islets and insulinomas, the Pdx1-Cre;Men1 f/f mice had elevated plasma insulin levels that were significantly different from the control genotypes (Pdx1-Cre and Men1 f/f) starting at age of 5 months and persisting throughout their lifetime (Fig. 2B; Supplementary Table S2). Notably the 16-month-old Pdx1-Cre;Men1 f/+ mice began to display elevated plasma insulin levels, suggesting the development of hyperplastic islets due to the loss of the other wild-type Men1 allele, as has been described (21–23). As expected, elevated insulin levels were accompanied by a decrease in fasting blood glucose starting at age of 10 months (Supplementary Data C). Moreover, the Pdx1-Cre;Men1 f/f mice had a shorter life span, as 50% of them died at ages of ~14 months whereas >50% of the control genotypes lived beyond 24 months (Fig. 2C).

VEGF contributes to the abnormal islet vasculature and the insulinomas developed in Pdx1-Cre;Men1 f/f mice. To investigate why complete loss of Men1 in the pancreas only resulted in an endocrine phenotype in Pdx1-Cre;Men1 f/f mice, we speculated that, in addition to cell-specific factors, pancreatic endocrine tissues provide a more permissive environment for tumor development. Because it is well-established that vascular changes and angiogenesis are critical steps during tumor progression (37, 38), we hypothesized that loss of Men1 alters islet vasculature and correlates with the insulinomas developed in Pdx1-Cre;Men1 f/f mice. To visualize pancreatic vasculature, mice were i.v. injected with FITC-lectin before euthanasia. By immunofluorescent staining with anti-insulin antibodies, we were able to distinguish endocrine islets from exocrine tissues of the pancreas (Fig. 3A). Quantitative analyses of immunofluorescent images of control and Pdx1-Cre;Men1 f/f pancreas showed a significant increase in islet vascularity in Pdx1-Cre;Men1 f/f mice as early as at ages of 3 and 10 months (Fig. 3B). In addition, the vasculature of some Pdx1-CreMen1 f/f islets exhibited structural abnormalities, as indicated by dilation of blood vessels and intense tortuosity present in both 3-month-old and 12-month-old animals (Fig. 3C). These changes in islet vascularity seemed to predate the development of endocrine tumors. At all time points analyzed, we did not detect differences in vascular density and vessel morphology in exocrine tissues between control and Pdx1-CreMen1 f/f pancreas (Supplementary Data D).

Because VEGF is a central regulator of angiogenesis, we next performed VEGF ELISA to determine if the vascular alterations in...
with sunitinib exhibited a significant decrease in islet vascularity. Indeed, elevated pancreatic VEGF protein was detected in mice as early as at age of 3 months and was significantly different between control and Pdx1-Cre;Men1 f/f homozygous mice at age of 12 months (Fig. 4A). Immunohistochemical staining using a VEGF antibody further showed that elevation of VEGF expression predominantly localized in the endocrine tissues of pancreas (Fig. 4B). To confirm that VEGF plays an important role during insulinoma progression in Pdx1-Cre;Men1 f/f mice, we elected to inhibit VEGF signaling with sunitinib, a small-molecule tyrosine kinase inhibitor that is known to inhibit all VEGF receptors (39). A cohort of sex-matched and weight-matched Men1 f/f and Pdx1-Cre;Men1 f/f mice (averaged ages, 3 months) were treated with vehicle or sunitinib via daily oral gavage for 3 months. At the end of treatment, the pancreas of these mice was subjected to immunofluorescent imaging analysis for islet cell proliferation and islet vascularity. The number of animals and images analyzed were included in Supplementary Table S3. As indicated by percentage of Ki-67–positive cells within each islet, a significant reduction in cell proliferation was observed in Pdx1-Cre;Men1 f/f animals treated with sunitinib, but not in sunitinib-treated Men1 f/f animals (Fig. 5A and B). Similarly, Pdx1-Cre;Men1f/f mice treated with sunitinib exhibited a significant decrease in islet vascularity, whereas no differences in islet vascularity was observed between vehicle-treated and sunitinib-treated Men1 f/f mice (Fig. 5C and D). We also determined pancreatic VEGF protein expression and found no differences between vehicle and treatment group for each genotype (Supplementary Data E). This result is not surprising, as sunitinib functions to inhibit VEGF signaling, but not VEGF secretion. Together, these data suggest that VEGF signaling is a critical pathway involved in tumors developed in this mouse model of MEN1.

Human MEN1 pancreatic neuroendocrine tumors displayed similar characteristics as endocrine tumors developed in mice deficient of menin in pancreas. Similar to MEN1 patients with pancreatic neuroendocrine tumors, the Pdx1-Cre;Men1 f/f homozygous mice progressively developed insulinomas due to inactivation of menin in pancreas. To evaluate if our mouse model of MEN1 recapitulates the human MEN1 syndrome, we used immunofluorescent staining to analyze human MEN1 neuroendocrine tumors for cell proliferation and vascular morphology, variables used to establish our mouse model. As shown in Fig. 6A, in the normal portion of the pancreas that was not involved in tumor, only a few proliferating cells were detected in islets in contrast to the neuroendocrine tumors containing many Ki-67–positive proliferating cells (Fig. 6A). Using a CD31 antibody to identify blood vessels, we further showed hypervascularity and dilation of vessels within the MEN1 neuroendocrine tumors from patients (Fig. 6B). Moreover, immunohistochemical analysis revealed abundant VEGF expression in all MEN1 neuroendocrine tumors tested (n = 4). Whereas positive VEGF expression was detected also in islets of the adjacent normal pancreas, VEGF expression is absent in the exocrine pancreas (Fig. 6C). The similarities shared between the Pdx1-Cre;Men1 mouse model and the human MEN1 neuroendocrine tumors supports that our mouse model represents a powerful tool for investigating the molecular mechanisms related to MEN1 tumorigenesis in patients.

Discussion

In the present study, we aimed to elucidate the tissue-specific nature of the lesions associated with the MEN1 syndrome using a mouse model wherein menin is deleted in progenitor cells that gave rise to both exocrine and endocrine pancreas during development. We revealed that pancreatic progenitor cells lacking menin were able to differentiate into normal endocrine and exocrine pancreas. However, adult endocrine cells lacking menin developed tumors, in contrast to adult exocrine cells, which remained phenotypically normal in the absence of menin. In other words, we established that loss of menin is sufficient to cause endocrine tumorigenesis but insufficient to initiate exocrine tumorigenesis in the pancreas. This novel observation supports the hypothesis that cell-specific factors must exist to explain the tissue specific tumorigenesity of the MEN1 syndrome. In addition, we showed, for the first time in literature, that up-regulation in VEGF expression is associated with MEN1 pancreatic neuroendocrine tumors developed in human and mice. Together, these findings imply that cell-specific modulators for menin and alterations in microenvironment are crucial for MEN1 tumorigenesis in the endocrine pancreas. Therefore, the tissue-restricted nature of MEN1 lesions may reside within not only the cellular context but also the surrounding microenvironment of the endocrine cells affected in MEN1 syndrome.
The major difference distinguishing our conditional murine model of MEN1 from the previously described MEN1 models of insulinomas (28–30) is that all pancreatic cells are deficient of menin in our mice. Not previously described in literature, we showed that menin is not required for pancreatic morphogenesis and differentiation during development. We further revealed that loss of menin in exocrine cells does not result in histologic and functional abnormalities in the exocrine pancreas. Notably, loss of menin led to an increased cell proliferation and tumor development in endocrine pancreas, and those phenotypes was not observed in exocrine pancreas. This is a significant observation in that deletion of Men1 in the same progenitor cells of pancreatic lineage only leads to tumor formation in cells that differentiate into pancreatic endocrine cells. Our findings were similar to a report wherein homozygous loss of menin in hepatocytes is well-tolerated without neoplasia (40). Collectively, these murine models of Men1 imply that the endocrine-restricted tumor pattern seen in the MEN1 syndrome is likely due to cell-specific or tissue-specific factors for menin, because expression of menin is ubiquitous (25, 31). Whereas the identities of endocrine cell–specific factors for menin remain a mystery, it has been speculated that the endocrine tumor bias in the MEN1 syndrome results from a requirement of menin to maintain expression of cyclin-kinase inhibitors, such as p27 and p18, in endocrine cells (19, 41, 42). Yet, it does not exclude the possibility that other protective molecules and mechanisms exist in nonendocrine tissues in the absence of menin. Therefore, our model system will serve as a valuable tool to test these hypotheses and may be used to uncover potential target genes and cofactors for menin.

Another plausible explanation for the tumorigenicity of the MEN1 syndrome might reside in the unique characteristics of the endocrine microenvironment. It has been established that endocrine pancreatic β cells require endothelial signals for differentiation and function (43) and that distinctly different extracellular proteins are detected in the basement membranes of endocrine and exocrine cells (44). Recently, it has been shown that endocrine β cells, in contrast to exocrine pancreatic cells, do not produce a basement membrane of extracellular matrix. Instead, β cells use VEGF to attract endothelial cells to form basement membranes next to them, which contain signals to promote β-cell proliferation and insulin secretion (45). In the present study, we showed that the insulinomas developed due to loss of menin are associated with an increase in VEGF expression and that blocking VEGF signaling with sunitinib was sufficient to inhibit islet cell proliferation in our Pdx1-Cre;Men1

Figure 5. Quantitative analysis of immunofluorescent images for percentage of Ki-67–positive areas within islets (A) and islet vascularity (C) in vehicle-treated or sunitinib-treated (trx) animals of control Men1 f/f and mutant Pdx1-Cre;Men1 f/f genotypes. Representative images are shown B and D. B, Ki-67–positive areas, and islets are identified using antibodies against Ki-67 (red) and insulin (cyan). Percentage of Ki-67–positive areas within islet are shown to account for differences of islet size between control Men1 f/f and mutant Pdx1-Cre;Men1 f/f genotypes. D, blood vessels are visualized via FITC-lectin injection (green), whereas pancreatic islets are identified using an anti-insulin antibody (magenta). Magnification, 100×.

Cancer Res 2009; 69: (5). March 1, 2009 1864 www.aacrjournals.org

Downloaded from cancerres.aacrjournals.org on April 20, 2017. © 2009 American Association for Cancer Research.
f/f animals. Similarly, inhibiting VEGF with a monoclonal VEGF antibody has been shown to result in a decrease in tumor volume in a pituitary mouse model of MEN1 (46). The significance of VEGF in contributing to tumors developed in our model ought to be further validated using specific VEGF inhibitors because sunitinib is also known to block other tyrosine kinase signaling pathways (39, 47). However, our data, together with the published literature, support a critical role of VEGF in vascularization and growth of MEN1-associated tumors. Analysis of other MEN1-associated endocrine tumors, such as parathyroid adenoma, might provide further evidence to underline the future potential for an anti-VEGF therapy in MEN1 endocrine neoplasms.

It is also important to note that our molecular and histologic analysis on a vascular phenotype of MEN1 pancreatic neuroendocrine tumors is the first one in literature that links VEGF in driving tumor formation after Men1 deletion. Whereas a direct functional relationship between menin and VEGF is yet to be determined, angiogenesis and VEGF have been shown to be critical in a non-MEN1 model of insulinoma (48, 49). Thus, it would be of great interest to learn whether menin loss is involved in this model system. In addition, although angiogenesis and VEGF are the focus of this study, possibilities exist that loss of menin may lead to other microenvironment changes, allowing the endocrine islets to be more permissive for tumor development.

Based on our data using both human and mouse pancreatic neuroendocrine tumors, we speculate that islet cells lacking menin use VEGF to induce endothelial cells to create a permissive islet microenvironment for the growth of naturally occurring MEN1 tumors. However, further molecular studies will be required to elucidate the functional relationship between menin and VEGF and to determine if the absence of exocrine lesions in MEN1 patients and in our Pdx1-Cre;Men1f/f mice might be due to the differences in extracellular signals generated by cells deficient for menin.

Based on our mouse model of MEN1 described here, we propose that the endocrine tissue restrictive nature of the MEN1 syndrome is due to both cellular factors for menin and the permissive endocrine microenvironment. It is, thus, possible that menin mediates tumor suppression by maintaining cellular and microenvironment integrity. Our mouse model of MEN1 serves as an ideal system to further decipher cell-specific factors for menin and to investigate the significance of microenvironment alterations in MEN1 tumorigenesis. Comparative analysis of both exocrine and endocrine tissues lacking menin will enhance our understanding of the MEN1 syndrome and provide vital clues for the presence of protective mechanisms in tissues not affected by the loss of Men1. The use of mouse models may ultimately derive important insights into the biology of the disease, which may guide toward novel therapeutics for the MEN1 patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 9/19/2008; revised 10/31/2008; accepted 12/15/2008; published OnlineFirst 02/10/2009.

Grant support: Intramural Research Program of the NIH National Cancer Institute and National Institute of Diabetes and Digestive Diseases.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. F. Collins for his insightful comments and participation during the development of this project, Drs. A. Tindle and M. Kwon for useful discussions, and Drs. S. Agarwal and S. Chandrasekharappa for helpful advice.
References
Men1-Directed Inactivation of Pdx1 Human Multiple Endocrine Neoplasia Type I Syndrome via Pdx1-Directed Inactivation of Men1

H.-C. Jennifer Shen, Mei He, Anathea Powell, et al.