Mutation of Tumor Suppressor Gene Men1 Acutely Enhances Proliferation of Pancreatic Islet Cells

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Abstract

Multiple endocrine neoplasia type 1 (MEN1), an inherited tumor syndrome affecting endocrine organs including pancreatic islets, results from mutation of the tumor suppressor gene Men1 that encodes protein menin. Although menin is known to be involved in regulating cell proliferation in vitro, it is not clear how menin regulates cell cycle and whether mutation of Men1 acutely promotes pancreatic islet cell proliferation in vivo. Here we show that excision of the floxed Men1 in mouse embryonic fibroblasts (MEF) accelerates G0/G1 to S phase entry. This accelerated S-phase entry is accompanied by increased cyclin-dependent kinase 2 (CDK2) activity as well as decreased expression of CDK inhibitors p18*kip1 and p21*cip1. Moreover, Men1 excision results in decreased expression of p18*kip1 and p21*cip1 in the pancreas. Furthermore, complementation of menin-null cells with wild-type menin represses S-phase entry. To extend the role of menin in repressing cell cycle in cultured cells to in vivo pancreatic islets, we generated a system in which floxed Men1 alleles can be excised in a temporally controllable manner. As early as 7 days following Men1 excision, pancreatic islet cells display increased proliferation, leading to detectable enlargement of pancreatic islets 14 days after Men1 excision. These observations are consistent with the notion that an acute effect of Men1 mutation is accelerated S-phase entry and enhanced cell proliferation in pancreatic islets. Together, these results suggest a molecular mechanism whereby menin suppresses MEN1 tumorigenesis at least partly through repression of G0/G1 to S transition. (Cancer Res 2006; 66(11): 5707-15)

Introduction

Multiple endocrine neoplasia type 1 (MEN1) is a dominantly inherited tumor syndrome that results from the mutation of the tumor suppressor gene Men1, which encodes menin (1, 2). Menin interacts with multiple proteins that play critical roles in the regulation of cell proliferation, including JunD (3), Smad 3 (4), and activator of S-phase kinase (5). Activator of S-phase kinase is the crucial regulatory factor for protein kinase cdc7 that is required for initiation of DNA replication (6, 7) and menin functionally represses the activity of activator of S-phase kinase (5). In addition, menin interacts with a protein complex containing the mixed lineage leukemia protein (8, 9) and up-regulates transcription of various target genes, including the cyclin-dependent kinase (CDK) inhibitors p27*kip1 and p18*kip1, in transformed fibroblasts (10) and insulinoma cells (11). Whereas these observations provide a potential mechanistic link between menin and cell cycle regulation, a direct link between menin function and cell cycle progression has not been established. An obstacle to answering this question has been the lack of synchronizable cells in which Men1 can be conditionally inactivated in vitro so that the effect of Men1 deletion on the cell cycle progression can be examined.

Mouse models have greatly increased our understanding of molecular pathology of the MEN1 syndrome. Tumors derived from mice heterozygous for Men1 display loss of heterozygosity (12, 13), confirming the role of menin as a bona fide tumor suppressor. Tumors arise in the parathyroid (14), pituitary (15), and pancreatic islet cells (15–17) from the mice in which Men1 is conditionally inactivated in these respective organs, establishing an important role for menin in suppressing tumor development in endocrine organs. However, because the excision of Men1 is not under temporal control in these mice, it is challenging to study the acute effects of deletion of Men1 on proliferation of pancreatic islet cells. Thus, although tumor cells in insulinomas of the mice display enhanced cell proliferation as shown by 5-bromo-2-deoxyuridine-5-triphosphate (BrdUrd) uptake (17), it is difficult to determine how soon after Men1 deletion increased islet cell proliferation occurs. If increased islet proliferation is an acute consequence of Men1 deletion, then this would suggest that loss of menin-mediated repression of cell proliferation is at least in part responsible for the early events of MEN1 tumorigenesis. A mouse model in which Men1 can be deleted in a temporally controllable manner will help to address this question. Answering this question is important for identifying the pathways through which menin controls tumorigenesis in endocrine organs and for understanding how to potentially manipulate these pathways for therapeutic intervention.

In the current studies, mouse embryonic fibroblasts (MEF) in which Men1 could be conditionally deleted were generated. Unlike viral oncogene-immortalized Men1−/− MEFs (18), these MEFs could be synchronized to determine the effect of menin on cell cycle progression. In addition, we established a model system in which the floxed Men1 locus could be excised in a temporally controlled fashion so that the acute effect of in vivo Men1 excision on cell proliferation could be examined. These technical advances allowed us to establish a crucial role for menin in repressing cell cycle entry into S phase in vitro as well as in suppressing proliferation of normal pancreatic islet cells in vivo.

Materials and Methods

Mouse breeding, genotyping, and excision of the floxed Men1 locus. All animal studies were approved by University Laboratory Animal
Resources, the University of Pennsylvania committee on animal care, and were carried out in accordance with the mandated standards. Mεntελειακον mice (designated Mεntελειακον; mixed FVB/129Sv background) were kindly provided by Dr. Francis Collins (National Institute for Human Genome, Research; ref. 17). The pan-active human UBC9 promoter-driven Cre-ERT2 (19, 20) was introduced into murine fertilized eggs to generate Cre-ER transgenic mice1 using the method of lentivirus transgenesis (21). Breeding was carried out by crossing Mεntελειακον and Cre-ER mice. Mεntελειακον-Cre-ER mice were genotyped by PCR using the following primers: P1, 5′-ctccacatgcctctttcagc-3′; P2, 5′-aaatcagctggagagctaacc-3′; and P3, 5′-ggacaggtggggat-3′. The primers for genotyping Cre-ERT2 were 5′-tacacacaaatttgctcatgtagc-3′ and 5′-ttctaatggaggaacctggt-3′. Mεntελειακον-Cre-ER and Mεntελειακον-Cre-ER mice at 12 weeks of age were first fed with tamoxifen (Sigma, St. Louis, MO) at a dose of 200 mg/kg body weight/d for 2 consecutive days, followed by a day off and then for a second 2 consecutive days at the same dose. After 7, 14, and 30 days, the mice were sacrificed for analysis. In total, 22 mice (11 male and 11 female) were analyzed, with the male and female mice randomly distributed between the two groups.

**Immunofluorescent staining of pancreatic sections.** Mεntελειακον-Cre and control Mεntελειακον-Cre-ER mice (both tamoxifen-fed) were injected ip. with 50 mg BrdUrd (Sigma)/kg body weight 2 hours before sacrifice and dissection. Pancreata were isolated and processed for H&E staining and three separate sections from each mouse were stained to quantify area of islets using Metamorph software (Molecular Devices Corporation, Sunnyvale, CA). For immunofluorescent staining, a rabbit antimenin antibody (800, ref. 18) and a sheep anti-BrdUrd antibody (2284, Abcam, Inc., Cambridge, United Kingdom) were used in combination with FITC-conjugated antirabbit IgG and TRITC-conjugated antishheep IgG secondary antibodies, together with 4′,6-diamidino-2-phenylindole (DAPI; 10 μg/mL). Images were captured using a Nikon eclipse E800 fluorescence microscope equipped with a CCD digital camera and the BrdUrd-positive cells among the total DAPI-stained cells per islet were quantified. To color BrdUrd with insulin or glucagon in islet cells, the following antibodies were used: monoclonal rat anti-BrdUrd (B7-HI, Accurate Chemical & Scientific Corp., Westbury, NY), Cy2-conjugated antirat IgG, guinea pig anti-insulin, monoclonal rat anti-BrdUrd (BU1/75, Accurate Chemical & Scientific Corp., Westbury, NY), Cy2-conjugated antirat IgG

**RT-PCR and real-time TaqMan PCR.** Total RNA was extracted from cell lines and pancreata using the RNeasy Mini Kit (Qiagen, Valencia, CA). One-step RT-PCR was done with RNA derived from pancreata using the Titan One Tube RT-PCR System (Roche, Indianapolis, IN) following the instructions of the manufacturer. Real-time TaqMan PCR quantification of gene expression was done with RNA derived from cultured cell lines using TaqMan probes for p18Ink4c (Applied Biosystems, Foster City, CA; Mm00483243_m1), p27kip1 (Mm00438167_g1), and GAPDH as an internal control (Mm99999915_g1). Analysis was done using the relative quantification method according to instructions from the ABI.

**Plasmid construction and production of recombinant viruses.** Plasmids for generating recombinant retroviruses were constructed by inserting PCR-amplified human menin cDNA into the BamHI/NotI site of the retroviral vector pMX-puro to generate pMX-menin. The production of recombinant adenoviruses and retroviruses was as previously described (22). For complementation with wild-type menin, Mεntελειακον-Cre-ER cells were seeded on day 0, infected with various retroviruses [including green fluorescent protein (GFP)—expressing retroviruses as a control for infection efficiency] on day 1, and switched to fresh media on day 2 before selection with 2 μg/mL puromycin on day 4.

**Generation of MEF cell lines and fluorescence-activated cell sorting analysis.** MEFs from Mεntελειακον embryos were isolated on embryonic day 14 (E14) and were immortalized using the 3T6 protocol (23). Briefly, 8 × 10⁵ MEFs were plated on a 60-mm plate and passaged every 3 days. After 30 to 35 passages, immortalized cells emerged. After immortalization, the cells were infected with adenoviruses expressing either GFP (Ad-GFP) or Cre recombinase (Ad-Cre), generating one control cell line (designated Mεntελειακον-Cre) and two menin-null cell lines (designated Mεntελειακον-Cre-1 and Mεntελειακον-Cre-2). After two to three passages of Mεntελειακον excision, the cells were seeded in MEF medium (22) at a density of 1.5 × 10⁵ per 100-mm dish on day 0 for cell cycle analysis. On day 1, cells were switched to medium containing only 0.1% FBS. On day 5, normal MEF medium containing nocodazole (Sigma; 200 ng/mL) was added to cells, releasing them from arrest in G0/G1. At various time points after release, cells were pulsed with 10 μmol/L BrdUrd for 2 hours immediately before harvest and fixation. Cell pellets were processed for double staining with an anti-BrdUrd antibody (PharMingen, San Jose, CA) and propidium iodide (10 μg/mL in PBS; Sigma), followed by analysis on a FACS Calibur (Becton Dickinson, Franklin Lakes, NJ). Gating was done to focus on the G1, S, and G2/M populations.

**Antibodies and Western blotting.** Whole-cell lysates were prepared with EMB lysis buffer (0.1% NP40, 160 mmol/L NaCl, 50 mmol/L HEPES (pH 7.4), 5 mmol/L EDTA (pH 8.0), 1 mmol/L DTT, 0.2 mmol/L phenylmethylsulfonyl fluoride; ref. 24) supplemented with protease inhibitor cocktail set (Calbiochem, San Diego, CA) and subjected to Western blotting analysis as previously described (22). The primary antibodies used were rabbit antitenin (BL-342, Bethyl Lab, Montgomery, TX), goat antianti-insulin (C-11, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-p27kip1 (BD Transduction, San Jose, CA), rabbit anti-p21 (N-20), rabbit anti-p21 (C-19), and rabbit anti-p16 (M-156; Santa Cruz Biotechnology) or control rabbit IgG. Immunoprecipitates were incubated with 2 μg histone H1 (Upstate Biotech, Norcross, GA) and 5 μCi [γ-32P]ATP for 30 minutes before SDS-PAGE separation, as previously described (25), and subjected to phosphoimaging analysis and quantification. Total histone substrate was visualized by Coomassie blue staining.

**Statistical analysis and quantification.** Microsoft Excel and GraphPad Prism software were used to prepare graphs and for statistical analyses. When appropriate, the Student’s t test was used to determine significance of results.

**Results**

**Ablation of Mεntελειακον in vitro increases cell proliferation and transition from G0/G1 to S phase.** We immortalized MEFs from mouse embryos with the floxed Mεntελειακον (17) using the 3T6 protocol (23), and then infected the cells with recombinant adenoviruses expressing either GFP (Ad-GFP) or Cre (Ad-Cre) that could excise the floxed Mεntελειακον from the genome. The cell lysates from the infected cells were subjected to Western blotting analysis. Ad-Cre (lanes 2 and 3), but not Ad-GFP (lane 1), abrogated expression of menin (Fig. 1A). Mεntελειακον excision was also confirmed by genotyping (Fig. 1B) because Mεntελειακον excision yielded a PCR fragment of the increased size. The Mεntελειακον-Cre and the Mεntελειακον-Cre-2 cells, two independent pools of the MEFs infected by Ad-Cre, proliferated more quickly than the menin-expressing Mεntελειακον cells (2.7 and 2.9 × 10⁵ cells/10⁵ cells; P < 0.03), Mεntελειακον versus Mεntελειακον-Cre-1; P < 0.02, Mεntελειακον-Cre-1 versus Mεntελειακον-Cre-2; Fig. 1C). To further confirm this difference in cell proliferation in vitro, we excised Mεntελειακον from one additional independent clone and similar results were obtained.

We next determined whether menin inhibits cell cycle progression and, if it does, at what phase it inhibits cell cycle progression. Serum-starved menin-null or menin-expressing cells were stimulated by addition of serum and allowed to progress for various periods of time up to 24 hours. Cells were harvested at various time points after release and processed for staining with anti-BrdUrd.

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1. E. Brown, unpublished data.
2. R. Schnepf, data not shown.
Figure 1. Ablation of Men1 in MEFs results in increased cell proliferation in vitro. A, Cre-mediated excision of the Men1 flanked by the lox P sites abrogates menin protein expression. Men1<sup>fl</sup> cells were either infected with adenovirus Ad-GFP (Men1<sup>fl</sup> cell line) or Ad-Cre (Men1<sup>fl/A</sup> and Men1<sup>fl/2</sup>, two independent pools of the infected cells) before detection of menin and with control actin 5 days after infection. B, excision of Men1 in the Men1<sup>fl/A</sup> and Men1<sup>fl/2</sup> cells was confirmed by genotyping using primers P2 and P3 (lane 1) or P1 and P3 (lanes 2 and 3) as described in Materials and Methods. C, deletion of Men1 in MEFs increases cell proliferation. Men1<sup>fl/A</sup> and Men1<sup>fl/2</sup> cells were seeded in triplicate on day 0 and counted using a hemocytometer on day 4. Data were derived from the mean of triplicate cultures. Representative of three independent experiments.

Ablation of Men1 increases CDK2 activity, but decreases p18<sup>ink4c</sup> and p27<sup>kip1</sup> RNA and protein levels. The cell cycle is positively regulated by various CDKs and CDK2 plays a crucial role in controlling G<sub>0</sub>/G<sub>1</sub> to S transition (26). Thus, we determined whether menin inhibits CDK2 activity. Lysates from Men1<sup>fl</sup> cells precipitated kinase activity was detected using histone H1 as a substrate and whether menin inhibits CDK2 activity. Lysates from Men1<sup>fl</sup> cells precipitated kinase activity was detected using histone H1 as a substrate. Twenty hours after release from serum starvation, only 12.2% of the Men1<sup>fl</sup> cells progressed from G<sub>0</sub>/G<sub>1</sub> to S phase (Fig. 2A, top left) as compared with that of asynchronous cells (55%; data not shown). Similarly, Men1<sup>fl/A</sup> cells were primarily distributed in G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>-M phases, with only 7.5% cells in S phase (Fig. 2A, bottom left). Twelve hours after release from serum starvation, only 12.2% of the Men1<sup>fl</sup> cells progressed from G<sub>0</sub>/G<sub>1</sub> to S phase (Fig. 2A, top middle). In contrast, 32% of Men1<sup>fl/A</sup> cells entered S phase (Fig. 2A, bottom middle). At 24 hours of release, 41.5% of Men1<sup>fl</sup> cells reached S phase (top right) whereas only 28% of the Men1<sup>fl/A</sup> cells were in S phase, as they had already passed their peak at S phase (18 hours) and progressed to G<sub>2</sub>-M (Fig. 2A, bottom right). The detailed kinetics of cell cycle progression for both Men1<sup>fl</sup> cells and Men1<sup>fl/A</sup> cells are shown in Fig. 2B. These results show that loss of menin expression accelerates progression from G<sub>0</sub>/G<sub>1</sub> to S phase.

Although menin-dependent transcription of p27<sup>kip1</sup> and p18<sup>ink4c</sup> was recently reported (10), this is the first time that menin was shown to suppress cell cycle progression, repress CDK2 activity, and up-regulate p27<sup>kip1</sup> and p18<sup>ink4c</sup> in a well-controlled system. These data suggest that menin regulates CDK2, at least in part, by regulating p27<sup>kip1</sup> and p18<sup>ink4c</sup>. The detailed kinetics of cell cycle progression for both Men1<sup>fl</sup> cells and Men1<sup>fl/A</sup> cells are shown in Fig. 2B. These results show that loss of menin expression accelerates progression from G<sub>0</sub>/G<sub>1</sub> to S phase.

Complementation of Men1<sup>A/A</sup> cells with wild-type menin inhibits cell proliferation and G<sub>0</sub>/G<sub>1</sub> to S phase progression and restores p18<sup>ink4c</sup> and p27<sup>kip1</sup> protein and RNA levels. If Men1 excision leads to enhanced cell proliferation and G<sub>0</sub>/G<sub>1</sub> to S phase transition, complementation of menin-null cells with menin should suppress cell proliferation and G<sub>0</sub>/G<sub>1</sub> to S phase progression. Thus, we infected Men1<sup>A/A</sup> cells with control vector retroviruses or retroviruses encoding wild-type menin, and the resulting cells were monitored for cell growth, expression of p27<sup>kip1</sup> and p18<sup>ink4c</sup>, and G<sub>0</sub>/G<sub>1</sub> to S phase progression. As expected, expression of both p18<sup>ink4c</sup> and p27<sup>kip1</sup> was higher in Men1<sup>A/A</sup> cells than in Men1<sup>A/A</sup> cells. In addition, the mRNA levels of p27<sup>kip1</sup> and p18<sup>ink4c</sup> in Men1<sup>A/A</sup> cells were 2.5-fold (P < 0.02) and 3.5-fold (P < 0.0001) higher, respectively, than in Men1<sup>A/A</sup> cells (Fig. 3C). Although mening-dependent transcription of p27<sup>kip1</sup> and p18<sup>ink4c</sup> was recently reported (10), this is the first time that menin was shown to suppress cell cycle progression, repress CDK2 activity, and up-regulate p27<sup>kip1</sup> and p18<sup>ink4c</sup> in a well-controlled system. These data suggest that menin regulates CDK2, at least in part, by regulating p27<sup>kip1</sup> and p18<sup>ink4c</sup>.
Notably, 12 hours after release, 33% of vector-complemented cells were in S phase, as compared with 16% in menin-complemented cells (Fig. 4D). Twenty-four hours after release, vector-complemented cells progressed out of the peak of S phase (31%) whereas a greater percentage of menin-complemented cells remained in S phase (36%; Fig. 4D). A more detailed cell cycle profile at multiple time points after G0/1 release further supports the role of menin in slowing down G0 to S phase transition (Fig. 4E).

Figure 2. Ablation of Men1 in MEFs accelerates cell cycle progression from G0/G1 to S phase. A, serum-starved Men1<sup>−/−</sup> and Men1<sup>−/+</sup> cells were stimulated with the addition of serum and harvested 0, 6, 12, 18, and 24 hours after release. The cells were pulsed with BrdUrd, harvested, and processed for analysis by flow cytometry. B, detailed kinetics of cell cycle change in G0, G1, S, and G2-M phases in Men1<sup>−/−</sup> and Men1<sup>−/+</sup> cell lines. Duplicate cultures were examined for each time point. Representative of two independent experiments.

Figure 3. Ablation of Men1 results in increased CDK2 activity and decreased levels of p27<sup>Kip1</sup> and p18<sup>Ink4c</sup> protein and RNA. A, ablation of Men1 in MEFs increases CDK2 activity. As in Fig. 2A, serum-starved Men1<sup>−/−</sup> and Men1<sup>−/+</sup> cells were released from serum starvation and harvested at the indicated time points, and then subjected for Western blotting with the indicated antibodies. B, excision of Men1 in MEFs decreases p18<sup>Ink4c</sup> and p27<sup>Kip1</sup> protein levels. The indicated cells were released from serum starvation and harvested at the indicated time points, and then subjected for Western blotting with the indicated antibodies. C, ablation of Men1 decreases p18<sup>Ink4c</sup> and p27<sup>Kip1</sup> RNA levels. Real-time TaqMan PCR analysis was carried out using TaqMan probes for p18<sup>Ink4c</sup>, p27<sup>Kip1</sup>, and GAPDH. D, the p27<sup>Kip1</sup> and p18<sup>Ink4c</sup> mRNA levels decrease in Men1<sup>−/−</sup>Cre-ER mice 1 month after tamoxifen treatment as shown by RT-PCR. Representative samples of four mice for each of the genotypes.
Men1 excision in pancreatic islets acutely results in increased islet cell proliferation and size. The above studies in cultured cells showed a crucial role for menin in controlling S-phase entry. However, it is still unclear whether this role of menin also applies to in vivo endocrine cells such as pancreatic islet cells, in which a germ-line mutation in only one Men1 allele predisposes the patient to the development of insulinomas (28). In addition, because it takes ~6 months for mice carrying a Men1 mutation to develop insulinomas, which have a high proliferation index (17), an important unresolved question is whether time-controlled Men1 excision can quickly lead to enhanced proliferation of pancreatic islet cells. To address these questions, we bred mice with the Men1 locus flanked by lox P sites (Men1<sup>fl/fl</sup>, previously Men1<sup>AV/AV</sup>; ref. 17) with mice (Men1<sup>fl/+</sup>) expressing Cre-ER (estrogen receptor) driven by a pan-active UBC9 promoter, to generate mice with the Men1<sup>fl/fl</sup>; Cre-ER genotype. Cre-ER expressed from a transgene can be activated by tamoxifen, resulting in excision of genes flanked by lox P sites (29). Both control mice (Men1<sup>fl/+</sup>) expressing Cre-ER and the Men1<sup>fl/fl</sup>; Cre-ER mice were fed with tamoxifen, and then pancreata were harvested to determine excision of the conditional Men1 locus. Tamoxifen effectively induced Men1 excision in the pancreata of the Men1<sup>fl/fl</sup>; Cre-ER mice (Fig. 5A and B, lane 2), but not in Men1<sup>fl/+</sup>; Cre-ER mice (Fig. 5B, lane 1). Conversely, in the absence of tamoxifen, the floxed Men1 remained intact in the pancreata of Men1<sup>fl/fl</sup>; Cre-ER mice, indicating no leakiness in excision of the Men1 locus in the absence of tamoxifen (Fig. 5B, lane 3). Given the effective control of Men1 excision, further experiments were done using Men1<sup>fl/fl</sup>; Cre-ER and Men1<sup>fl/+</sup>; Cre-ER mice to control for any nonspecific effects of tamoxifen treatment.
multiple BrdUrd-positive cells (Fig. 5G, H, and J). Quantification of the BrdUrd-positive cells from islets of multiple mice indicates that ∼0.4% of islet cells were BrdUrd positive in Men1+/+;Cre-ER mice, but notably 2.0% of cells were BrdUrd positive in Men1l/l;Cre-ER mice (Fig. 5H and K; P < 0.008). To determine whether the BrdUrd-positive cells are either insulin-secreting β-cells or glucagon-secreting α-cells, pancreatic sections were costained with the anti-insulin antibody or the anti-glucagon antibody. In the islet from tamoxifen-fed Men1l/l;Cre-ER mice, there were two BrdUrd-positive cells, both costained with the anti-insulin antibody (red in the nucleus, Fig. 5N). Conversely, BrdUrd-positive cells were not costained with the anti-glucagon antibody (Fig. 5O). Together, these results indicate that Men1 excision leads to increased proliferation of islet cells well before the development of insulinomas. These results further support the in vitro results that menin represses G$_0$/G$_1$ progression or S-phase entry of cultured cells (Fig. 2).

To extend our in vitro findings about the role of menin in up-regulating p27$^{kip1}$ and p18$^{ink4c}$ to the in vivo organ such as pancreatic islets, we also determined whether loss of Men1 affects p27$^{kip1}$ and p18$^{ink4c}$ expression in the murine pancreata. Pancreata were harvested from Men1+/+;Cre-ER and Men1l/l;Cre-ER mice that were fed with tamoxifen. Quantification of various mRNAs from the pancreata by RT-PCR shows that Men1 expression was detectable in Men1+/+;Cre-ER control mice (Fig. 3D, top, lane 1) but greatly reduced in Men1l/l;Cre-ER mice (lanes 2 and 3). Similarly, expression of p27$^{kip1}$ and p18$^{ink4c}$ was also markedly decreased in Men1l/l;Cre-ER mice, as compared with the control mice, whereas expression of control GAPDH was comparable between Men1+/+;Cre-ER and Men1l/l;Cre-ER mice (Fig. 3D). These data show that menin may also regulate p27$^{kip1}$ and p18$^{ink4c}$ levels in vivo.

Enhanced islet cell proliferation after Men1 excision may affect the size of the islets after certain period of enhanced proliferation.

Figure 5. Excision of floxed Men1 results in increased islet cell proliferation. A, a schema for the floxed Men1 locus (Men1$, previously Men1$^{D/N}$, ref. 17) and the excised product (Men1$^{D/D}$). One of the two floxed Men1 alleles is depicted. In the absence of tamoxifen, primers 2 and 3 amplify fragment 1 (F1); following tamoxifen treatment and excision of the floxed Men1 allele, primers 1 and 3 amplify fragment 2 (F2). B, inducible and effective excision of the floxed Men1 locus. Mice 12 weeks of age were fed with tamoxifen and genotyped as described in Materials and Methods. C to J, one month after tamoxifen treatment, Men1$^{D/D}$;Cre-ER control mice and Men1$^{D/D}$;Cre-ER mice were i.p. injected with BrdUrd 2 hours before harvesting pancreata for immunofluorescent staining. Pancreatic sections were stained with antimenin and anti-BrdUrd antibodies to determine menin expression and the proliferative index of cells. DAPI staining was used to visualize nuclei. Images were acquired using 20× objective lens. Merged image of (F) and (J) correlates expression of menin and the uptake of BrdUrd. The islet is circled by a dashed line. K, quantification of BrdUrd-positive islet cells from three tamoxifen-treated Men1$^{+/+}$;Cre-ER (control) mice and four Men1l/l;Cre-ER mice. L to O, BrdUrd-positive cells express insulin. Pancreatic sections from tamoxifen-fed (1 month after feeding) Men1$^{+/+}$;Cre-ER mice (L) and Men1l/l;Cre-ER mice (N) were costained with the anti-BrdUrd antibody (red) and the anti-insulin antibody (green). M and O, pancreatic sections were costained with the anti-BrdUrd antibody (red) and the anti-glucagon (green) antibody as indicated. Images were captured using 20× objective lens.
Notably, the size of islets from Men1<sup>+/c; Cre-ER</sup> mice was, on average, larger than that of the control mice 1 month after tamoxifen treatment (Fig. 6A). The mean of the area of the islets from the Men1<sup>+/c; Cre-ER</sup> mice was ~3.5-fold larger than that from the control mice (Fig. 6B, 0.50 versus 1.73, P < 0.0001). Collectively, these results indicate that deletion of Men1, within a month, leads to enhanced cell proliferation and enlargement of pancreatic islets, a tissue commonly affected in MEN1 syndrome.

To further determine how soon after Men1 deletion BrdUrd uptake increases in islet cells, we further examined pancreata at 7 and 14 days following tamoxifen treatment. At 7 days, ~0.2% of islet cells in Men1<sup>+/c; Cre-ER</sup> mice were BrdUrd positive in comparison with 0.6% of islet cells in Men1<sup>+/d; Cre-ER</sup> mice (Fig. 7A, P < 0.005), indicating a significant increase in islet cell proliferation 7 days after Men1 excision. At 14 days, 0.2% of islet cells from control mice were BrdUrd positive as compared with 1.4% of islet cells in Men1<sup>+/d; Cre-ER</sup> mice (Fig. 7A, P < 0.4 × 10<sup>-5</sup>). The mean islet area was not significantly different between Men1<sup>+/c; Cre-ER</sup> and Men1<sup>+/d; Cre-ER</sup> mice on day 7. However, on day 14, the mean of the area of the islets from the Men1<sup>+/d; Cre-ER</sup> mice was ~1.5-fold larger than that from the control mice (Fig. 7B, 0.84 versus 1.22, P < 0.005). These results strongly suggest that deletion of Men1 acutely results in increased cell proliferation, which may accelerate the accumulation of islet cells, resulting in islet enlargement and hyperplasia 14 days after Men1 excision.

**Discussion**

To understand how Men1 mutations result in MEN1 syndrome, it is important to elucidate how menin regulates cell cycle progression, proliferation of pancreatic islet cells, and the acute effect of Men1 deletion on islet cell proliferation. In this study, we initially examined the role of menin in regulating cell cycle progression in vitro and then extended the study to the proliferation of pancreatic islet cells in vivo. Using MEFs with homozygous conditional Men1 alleles, we showed that Men1 excision accelerated S-phase entry for 4 to 5 hours, the first direct evidence linking menin to inhibition of cell cycle progression. Accelerated S-phase entry in Men1-excised cells was accompanied by decrease of p18<sup> Ink4c </sup>and p27<sup> Kip1 </sup>in protein levels (Fig. 3B). This is consistent with a recent report that expression of p18<sup> Ink4c </sup>and p27<sup> Kip1 </sup>can either directly or indirectly inhibit CDK2, and p27<sup> Kip1 </sup>is decreased in mouse MEN1 insulinomas as well as an insulinoma cell line expressing low level of menin.

CDK2 plays an important role in G1 to S transition (26) and p18<sup> Ink4c </sup>and p27<sup> Kip1 </sup>can either directly or indirectly inhibit CDK2 (27). Consistent with this, we found that Men1 excision led to elevated CDK2 activity, concomitant with down-regulation p18<sup> Ink4c </sup>and p27<sup> Kip1 </sup>and earlier entry to S phase in MEFs. To our knowledge, this is the first time that loss of menin expression is simultaneously linked to down-regulation of p18<sup> Ink4c </sup>and p27<sup> Kip1 </sup>, increased CDK2 activity, and accelerated S-phase entry. Although it is attractive to hypothesize that increased CDK2 activity in menin-null cells contributes to the accelerated S-phase entry, the increased CDK2 activity may not directly cause the accelerated S-phase entry. Our findings that menin-mediated expression of p18<sup> Ink4c </sup>and p27<sup> Kip1 </sup>correlates with the delayed entry to S phase are also in agreement with the report that double mutation of p16<sup> Ink6c </sup>and p27<sup> Kip1 </sup>enhances transition from G<sub>0</sub> to S phase in muscle cells (30).

However, our findings do no necessarily mean that p18<sup> Ink4c </sup>and p27<sup> Kip1 </sup>are the major effectors of menin-mediated inhibition of G<sub>0</sub> to S phase transition. It is possible that the potential effect of Men1 excision on cell cycle withdrawal and maintenance of quiescence

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**Figure 6.** Excision of the floxed Men1 results in enlargement of pancreatic islets. A, enlargement of pancreatic islets after excision of Men1. H&E staining of pancreatic sections prepared from tamoxifen-fed Men1<sup>+/c; Cre-ER</sup> and Men1<sup>+/d; Cre-ER</sup> mice 1 month after tamoxifen treatment. Images were acquired using 20× objective lens. I, islets. B, quantification of the size of islets derived from three Men1<sup>+/c; Cre-ER</sup> and four Men1<sup>+/d; Cre-ER</sup> mice as described in Materials and Methods. Circles, value of the area for a single islet in arbitrary units. Line, mean of areas of all the measured islets.

**Figure 7.** Excision of Men1 acutely accelerates islet cell proliferation. A, 7 and 14 days after tamoxifen treatment, pancreata from mice (four Men1<sup>+/c; Cre-ER</sup> and three Men1<sup>+/d; Cre-ER</sup> mice for day 7; four Men1<sup>+/c; Cre-ER</sup> and four Men1<sup>+/d; Cre-ER</sup> mice for day 14) were processed for BrdUrd staining as described in Fig. 6C to J. B, quantification of pancreatic islets from mice (four Men1<sup>+/c; Cre-ER</sup> and three Men1<sup>+/d; Cre-ER</sup> mice for day 7; four Men1<sup>+/c; Cre-ER</sup> and four Men1<sup>+/d; Cre-ER</sup> mice for day 14) was done as described in Fig. 6B.
also in part contributes to the quicker transition to S phase. In addition, we previously also showed that menin interacted with and functionally inhibited activator of S-phase kinase, an essential component of protein kinase complex cdcl7/activator of S-phase kinase that is required for S-phase entry (7). Thus, the menin and activator of S-phase kinase interaction may also contribute to repression of entry to S-phase.

To extend our findings on the role of menin in repressing cell proliferation and S-phase entry to pancreatic islets in vivo, we determined whether deletion of Men1 quickly results in increased islet cell proliferation. Within 7 days of Men1 excision, pancreatic islet cells displayed increased BrdUrd uptake, a reliable indicator of entry into S phase. Consistent with the crucial role of menin in keeping proliferation of islet cells in check, the mean islet size gradually increased from day 14 to day 30 after Men1 excision. These results establish for the first time that loss of menin expression acutely results in enhanced cell proliferation in islet cells, a tissue commonly affected in the MEN1 patient. Thus, these results indicate that menin normally represses proliferation of islet cells, and an acute and early effect of the Men1 mutation is enhanced proliferation of islet cells including β-cells. However, these results do not exclude the possibility that secondary effects, such as defects in genome instability and apoptosis, following Men1 mutation, also contribute to MEN1 tumorigenesis.

An important related observation is that excision of Men1 accelerated proliferation of islet cells, but not the adjacent exocrine cells, indicating a tissue-specific role for menin in regulating proliferation of the endocrine pancreata. The floxed Men1 was effectively excised in the pancreas including both the exocrine and endocrine cells, based on analysis of the genomic DNA for the floxed Men1 (Fig. 5B). The floxed Men1 locus was also effectively excised from other tissues such as bone marrow and the mouse tail (data not shown). This is consistent with the expected broad expression of the Cre-ER transgene. These results are also in accordance with a recent study showing that inactivation of Men1 in the liver, a tissue not affected in MEN1 syndrome, does not result in tumorigenesis (31). Thus, menin may play an especially critical role in suppressing cell proliferation in endocrine organs. It is not clear how menin specifically represses the proliferation of islet cells but not the adjacent exocrine cells.

The current studies lead to a model in which menin normally regulates the levels of p18Ink4c and p27Kip1 to repress CDK2 activity and limit islet cell proliferation. Accordingly, mutation of Men1 results in increased islet cell proliferation. Men1 excision quickly results in increased pancreatic islet cell proliferation, which may help to initiate development of islet hyperplasia. The enhanced proliferation of pancreatic islet cells, perhaps in combination with decreased apoptosis and genome stability, may further accelerate the rate of secondary genetic and/or epigenetic alterations, leading to the development of insulinomas. However, the current studies do not rule out that additional menin-related regulators, such as JunD (3), cyclin D1 (32), and activator of S-phase kinase (5), are also involved in the regulation of islet cell proliferation. In conclusion, these studies show that menin plays an essential role in the tissue-specific suppression of pancreatic islet cell proliferation and in inhibition of the G0/G1-S transition. Furthermore, these findings suggest the possibility that targeting the CDK2 axis may prove useful in treating MEN1 islet tumors, and set the stage to further investigate precisely how menin specifically up-regulates the transcription of the p18Ink4c and p27Kip1 genes.

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