Menin Is Required for Optimal Processing of the MicroRNA let-7a*

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Multiple endocrine neoplasia type I (MEN1) is an inherited syndrome that includes susceptibility to pancreatic islet hyperplasia. This syndrome results from mutations in the MEN1 gene, which encodes menin protein. Menin interacts with several transcription factors, including JunD, and inhibits their activities. However, the precise mechanism by which menin suppresses gene expression is not well understood. Here, we show that menin interacts with arsenite-resistant protein 2 (ARS2), a component of the nuclear RNA CAP-binding complex that is crucial for biogenesis of certain miRNAs including let-7a. The levels of primary-let-7a (pri-let-7a) are not affected by menin; however, the levels of mature let-7a are substantially decreased upon Men1 excision. Let-7a targets, including Insr and Irs2, pro-proliferative genes that are crucial for insulin-mediated signaling, are up-regulated in Men1-excised cells. Inhibition of let-7a using anti-miRNA in wild type cells is sufficient to enhance the expression of insulin receptor substrate 2 (IRS2) to levels observed in Men1-excised cells. Depletion of menin does not affect the expression of Drosha and CBP80, but substantially impairs the processing of pri-miRNA to pre-miRNA. Ars2 knockdown decreased let-7a processing in menin-expressing cells but had little impact on let-7a levels in menin-excised cells. As IRS2 is known to mediate insulin signaling and insulin/mitogen-induced cell proliferation, these findings collectively unravel a novel mechanism whereby menin suppresses cell proliferation, at least partly by promoting the processing of certain miRNAs, including let-7a, leading to suppression of Irs2 expression and insulin signaling.

Significance: Understanding how menin represses beta cell proliferation will aid toward improving therapies targeting endocrine tumors and metabolic diseases including diabetes.

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**Menin** promotes the processing of certain miRNAs, including let-7a, whose target Irs2 plays an important role in insulin signaling and beta cell proliferation. Moreover, menin suppresses cell proliferation, at least partly by miRNA-mediated suppression of cell proliferation. These findings collectively unravel a novel mechanism of menin-mediated suppression of cell proliferation.

Multiple endocrine neoplasia type I (MEN1) is an inherited syndrome, with development of neoplasia in several endocrine organs including pancreatic islets (1–4). The gene mutated in this syndrome, MEN1, encodes a nuclear protein of 610 amino acids, menin (5, 6). Menin interacts with multiple proteins and is involved in a variety of cellular processes including gene transcription, cell proliferation, apoptosis, and genome stability (7–12), but the precise mechanisms involving menin-mediated suppression of cell proliferation remain to be elucidated. The recently solved crystal structure of menin reveals that it contains a deep pocket that binds short MLL1 or JunD peptides in the same manner, but has opposite effects on gene transcription (13), supporting the capacity of menin to act as either a context-dependent transcription activator or repressor.

MicroRNAs (miRNAs) are short (20–24 nucleotides), endogenous non-protein-coding RNA molecules that negatively regulate gene expression post-transcription. MiRNAs either bind to their target gene mRNA and promote their degradation and/or inhibit protein translation (14, 15). The primary transcripts of miRNA genes (pri-miRNA) are processed in the nucleus by the Microprocessor complex consisting of DGCR8 and Drosha to ~70-nucleotide hairpin structures called pre-miRNAs (16–18). Pre-miRNAs are exported out of the nucleus and processed to ~22-nucleotide double stranded miRNA duplexes by Dicer (19–21). The miRNA duplexes are unwound, and the mature miRNA is incorporated into the RNA-induced silencing complex containing the Argonaute protein (22, 23). MiRNAs are involved in diverse biological processes during development and disease (24, 25) including type 2 diabetes (26–28). Aberrant expression of miRNAs has also been linked to several types of cancer (29, 30). miRNA-mediated regulation of pancreatic islet cell proliferation and insulin secretion has previously been demonstrated (31–34). Conditional deletion of Dicer1 early in pancreas development results in dramatic reduction of insulin-producing beta cells.
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EXPERIMENTAL PROCEDURES

Plasmids and Cell Culture—Retroviral plasmids expressing FLAG-tagged menin have been described previously (36). Lentiviral constructs expressing Ars2 were obtained from Open Biosystems. Lentiviral packaging plasmids, pMD2G and pAX2G, were purchased from Addgene. MEFs and HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and 1% Pen/Strep.

Western Blotting—Cells were lysed in radioimmunoprecipitation assay buffer (Sigma) on ice for 10 min and sonicated to shear the genomic DNA. Protein concentrations were determined by BCA assay, and the samples were subjected to SDS-PAGE. The proteins were transferred to a PVDF membrane. Antibodies used were anti-menin (Bethyl, A300-105A), anti-Irs2 (Cell Signaling, antibody 4502), anti-RAS (Millipore, catalog 05-516), anti-Ars2 (37), anti-Drosha (Cell Signaling, antibody 3364), anti-CBP80 (Bethyl, A301-793A) and anti-β-actin (Sigma, A5441).

RNA Extraction and Quantitative Real-time PCR—Total RNA was extracted from cultured cells with TRIzol and an RNeasy extraction kit from Qiagen. One microgram of RNA was transcribed into cDNA using SuperScript III RT from Invitrogen, and real-time PCR was performed on the 7500 Fast Real Time PCR machine from Applied Biosystems. Either Gapdh or Hprt1 mRNA levels were used as endogenous controls, and analysis was done using the relative quantification method according to instructions from ABI.

Isolation of miRNA and cDNA Synthesis—miRNA was isolated using the mirVana miRNA isolation kit from Invitrogen. Briefly, cells were lysed in lysis/binding solution, and RNA was extracted with acid-phenol:chloroform. One-third volume of ethanol was added to the aqueous phase and passed through a filter cartridge. Another two-thirds volume of ethanol was added to the filtrate, mixed, and passed through a second filter cartridge. Finally, the mRNA was eluted with 100 μl of elution solution at 95 °C. cDNA was synthesized using the NCoded miRNA First-Strand cDNA Synthesis kit from Invitrogen. Briefly, a poly(A) tail was added to the mRNA samples using poly(A) polymerase, followed by incubation at 37 °C for 15 min. First strand cDNA was synthesized from the polyadenylated miRNA by reverse transcription using the supplied Universal RT Primer Superscript III RT/RNaseOUT enzyme mix and incubated at 50 °C for 50 min.

Primary miRNA Processing Assay—To generate pri-let-7a, PCR was performed using primers flanking the pre-let-7a hairpin of the mouse locus. Forward and reverse primers added ssRNA flanks as well as a 5′-XhoI and 3′-XbaI site. The resulting PCR products were digested and cloned into pcDNA3.1. Pri-let-7a template for in vitro transcription was generated from the pri-miRNA-pcDNA3 construct described above. PCR was employed to add on a 5′ T7 promoter (5′-TCG TAA TAC GAC TCA CTA TAG GGA TAT CCA TCA CAC TGG CGG CC-3′) and (5′- GCT GAT CAG CGA GCT GTA GCG GC-3′). Radiolabeled pri-let-7a was generated using the T7 High Yield RNA Synthesis kit (New England Biolabs) in the presence of [32P]UTP according to the manufacturer’s instructions. Cell lysates were prepared from MEFs in radioimmunoprecipitation assay buffer followed by sonication and centrifugation. A reaction mixture containing 15 μl of cell lysate, 6.4 mM MgCl2, and radiolabeled pri-let-7a transcript was incubated at 37 °C for 90 min. RNA was extracted using TRIzol and resolved on a 6% urea-polyacrylamide gel, and the bands were detected by autoradiography.

Statistical Analyses—Statistical analysis was performed using GraphPad Prism (v 5.0, GraphPad Software). The data are presented as the mean ± S.D. A two-tailed Student’s t test was used for measuring statistical differences.

RESULTS

Menin Interacts with ARS2, a Component of the Nuclear RNA Cap-binding Complex Important for miRNA Biogenesis—Menin acts as a scaffold protein coordinating the function of different proteins (38), but the precise mechanisms by which it represses gene transcription and cell proliferation are not fully understood. We sought to identify novel menin-interacting partners to provide further insight into menin-mediated regulation of cell proliferation. To this end, ectopically expressed menin was purified by affinity chromatography using FLAG M2 beads, and the menin-interacting proteins were excised for characterization by mass spectrometry. Analysis of the purified proteins showed that ARS2 was among the major eluted proteins (Fig. 1A). ARS2 is a component of the nuclear RNA cap-binding complex that stabilizes the primary miRNA (pri-miRNA) transcript for processing by the Microprocessor complex consisting of Drosophila and DGC8R8, and thus plays an essential role in miRNA-mediated silencing (37, 39). To confirm ARS2 as a menin-interacting partner, we performed co-immunoprecipitation in cell lysates from HEK293 cells.
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Menin Enhances the Processing of pri-let-7a to pre-let-7a—Because menin does not affect the transcript level of pri-let-7a (Fig. 3A), and ARS2 has been shown to be involved in the processing of pri-miRNA to pre-miRNA, we determined whether menin affects the processing of pri-let-7a to pre-let-7a biochemically. We performed a miRNA-processing assay using [32P]-radiolabeled pri-let-7a as the substrate and cell lysates from either menin-expressing or menin-null cells. Lysates from menin-null cells contained diminished processing activity compared with lysates from control WT cells, which generated a cleavage product of ~94 nucleotides (Fig. 4). This result indicates that menin plays a role in the biogenesis of let-7a by enhancing the processing of pri-let-7a to pre-let-7a.

FIGURE 1. Menin interacts with ARS2. A, silver staining of menin-containing fractions after affinity purification using anti-FLAG M2 beads in HEK293 cells ectopically expressing menin. Visible bands were excised for identification by mass spectroscopy. Asterisk indicates ARS2 peptide fragments identified by mass spectroscopy; MDAAV, LTPLSVR, NINGITGHK, RWGVTDFR, VALSEPQPER, SKYHPDEVGK, FTVNTQELQK, VLDDLILYLR, DLADPPVDVDF, VRNINGITGHK, VALSEPQPER, ISHGVLEWOK, ESLSFAAQMGR, EVAFFN-NFLTDAK, TFEKETTLPSVR, LGSIAEIDLGVPPPVMK, MLDAAVIEKMGITENDLR, AIVEYREDLAPDDVF, ILEQEEEEEQAGKPGEPSK, and ILEQEEEEEQAGKPGEP-SKK. B, nuclear extract from HEK293 cells ectopically expressing menin were immunoprecipitated (IP) with anti-menin antibody, and immunoblotted for ARS2. Asterisk indicates a nonspecific band.

FIGURE 2. Mature let-7a miRNA levels are increased in menin-expressing cells. A, Men1fl/fl and Men1fl/fl;CreER MEFs were treated with 4-hydroxytamoxifen (4-OHT), and excision of menin was determined by Western blotting. B and C, qRT-PCR showing the levels of let-7a (B) and miR-155 (C) in Men1fl/fl and Men1fl/fl;CreER MEFs treated with 4-OHT. D, qRT-PCR showing let-7a levels in BON cells ectopically expressing either vector or wild type (WT) menin; inset, Western blotting showing levels of ectopic menin expression. Error bars indicate ± S.D.

To determine whether the decrease in the processing of pri-let-7a in Men1-excised cells results from decreased levels of components of the pri-miRNA processing machinery, we examined the levels of Drosha and CBP80. Our results show that the protein levels of Drosha, an RNase III enzyme that executes the initial step of miRNA processing in the nucleus along with DGCR8 (17), and CBP80, a nuclear cap-binding protein (37), are comparable in both menin-excised and wild type cells (Fig. 5). This indicates that the role of menin in the processing of pri-let-7a does not occur via regulation of the expression levels of the Microprocessor complex, but rather by enhancing the stability of the pri-let-7a transcript.
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Functional Interaction between Menin and ARS2 Is Crucial for Processing of let-7a—To elucidate the causal relationship between the interaction of menin and ARS2 in miRNA processing, we knocked down Ars2 expression in both WT and menin-null cells. A significant reduction in the levels of ARS2 protein was observed in both cell types using three independent clones of shRNA targeting Ars2 (Fig. 6A). qRT-PCR analysis indicated a 40% decrease in let-7a levels in WT cells for all three shRNAs examined (Fig. 6B). On the contrary, Ars2 knockdown did not affect let-7a levels in Men1-excised cells (Fig. 6B). These findings indicate that the impact of ARS2 on miRNA processing depends on the presence of menin, strongly suggesting that menin and ARS2 interact functionally to control processing of let-7a.

IRS2 Levels Are Enhanced upon Men1 Excision—Using prediction algorithms, Insr and Irs2 were previously identified as let-7 targets based on sequence complementarity between the miRNA and the 3' UTR of target genes (41). Inhibition of let-7a using anti-miRs prevented down-regulation of IRS2 expression in the liver of mice on a high fat diet, confirming IRS2 as a let-7a target (41). Importantly, IRS2 plays a central role in peripheral insulin signaling and pancreatic beta cell proliferation, and disruption of IRS2 causes type 2 diabetes in mice (42). We have shown previously that excision of the Men1 gene ameliorated pre-existing glucose intolerance and increased both glucose-stimulated insulin release and circulating insulin levels in mouse models for diabetes (43). We therefore examined whether a decrease in let-7a levels upon Men1 excision leads to increased IRS2 expression. Our results show that upon Men1 excision, Irs2 expression is increased ~2-fold at the mRNA level (Fig. 7A), with substantial increase at the protein level (Fig. 7B). This is consistent with the role of miRNAs in regulating gene expression by degradation of target gene mRNA and/or inhibiting translation (14, 15). Furthermore, Men1 excision increased levels of insulin receptor, Insr, another known target of let-7a that phosphorylates and activates IRS2 (41) (data not shown). In agreement with this finding, ectopic expression of menin in mouse βHC9 cells, insulin-producing cells derived from pancreatic islets with beta cell hyperplasia, decreases Irs2 mRNA and protein levels (Fig. 7C). Additionally, decreased levels of IRS2 protein levels were observed in BON cells ectopically expressing menin (Fig. 7D). These findings suggest that the elevated level of mature let-7a in menin-expressing cells targets the Irs2 transcript for degradation, and excision of Men1 subsequently relieves this degradation, resulting in enhanced expression of IRS2.

Anti-miR-induced Knockdown of let-7a Restores the Levels of IRS2—To determine whether menin-mediated biogenesis of let-7a plays a key role in menin-induced repression of Irs2 expression, we examined the effect of let-7a inhibition on Irs2 levels. To this end, cells were transfected with either let-7a or control anti-miR, and the levels of IRS2 were quantitated 5 days after transfection. A significant increase in the protein levels of IRS2 was observed in menin-expressing MEFs transfected with let-7a anti-miR compared with control anti-miR-treated cells (Fig. 8, second panel, lane 2 versus 1). As positive controls, the protein level of RAS, a known target of the let-7 microRNA family (44), was also up-regulated in menin-expressing MEFs treated with let-7a anti-miRNA compared with control anti-miR-treated cells, as expected (Fig. 8, third panel, lane 2 versus 1). These results clearly demonstrate that the decreased levels of IRS2 in menin-expressing MEFs compared with Men1-excised cells can be, at least partly, attributed to the presence of elevated levels of let-7a. To examine whether the impact of the let-7a anti-miR is menin/let-7a-specific, we treated menin-null cells with let-7a anti-miR. Our results indicate that the level of IRS2 in Men1-excised cells was not affected by let-7a anti-miR treatment (Fig. 8, lane 4 versus 3), possibly due, in part, to the
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FIGURE 7. IRS2 levels are reduced in menin-expressing cells. A, Men1+/+ and Men1+/−CreER MEFs were treated with 4-hydroxytamoxifen, and the mRNA levels of Irs2 were determined by qRT-PCR. B, Immunoblotting for IRS2 in cell lysates from Men1+/+ and Men1+/−CreER MEFs is shown. Ponceau S staining is included as a loading control. C, retrovirus expressing either control or menin was stably transduced into JHC9, and the mRNA levels of Irs2 were determined by qRT-PCR; inset, Western blotting showing protein levels of IRS2. D, cell lysates from BON cells ectopically expressing either vector or menin were immunoblotted for menin and IRS2. Ponceau S staining is included as a loading control. Error bars indicate ± S.D.

FIGURE 8. Anti-miR-induced repression of let-7a expression results in increased levels of IRS2. Men1+/+ and Men1+/− MEFs were harvested 5 days after transfection with either control (−) or let-7a (+) anti-miR, and the protein levels of IRS2 and RAS were determined by Western blotting. Immunoblotting for actin is included as a loading control.

relatively low levels of let-7a present in the menin-null cells, as expected. Similarly, the protein levels of RAS were unchanged after let-7a anti-miR treatment in Men1-excised cells compared with control anti-miR-treated cells. Collectively, these results indicate that the difference in IRS2 protein levels in menin-null and menin-expressing cells is largely attributed to menin-regulated production of mature let-7a, which degrades Irs2 mRNA and inhibits protein translation.

DISCUSSION

Here, we identify the role of menin in the biogenesis of certain miRNAs including let-7a and miR-155. We show that menin interacts with ARS2, a protein previously known to contribute toward stability and delivery of capped pri-miRNA transcripts to the Microprocessor complex containing Drosha and DGC8 (37). Excision of Men1 results in decreased levels of several miRNAs including let-7a and miR-155 (Fig. 2). Analysis of the transcript levels indicated that the levels of pri-miRNA were not altered by menin (Fig. 3). Additionally, radiolabeled processing assay indicates that menin plays a role in the processing of pri-miRNA to pre-miRNA (Fig. 4). The recently solved crystal structure of menin (13) suggests that it acts as a scaffold protein and regulates gene expression by interacting with distinct partners including transcription factors JunD (7, 13), NF-kB (45), PPARγ (46), and various histone modification enzymes including MLL (8, 13, 47, 48), PRMT5 (12), EZH2 (49) and HDACs (50). Furthermore, it has previously been shown that menin binds to the promoter of mir-26-a and induces its expression, resulting in decreased SMAD1 levels during osteoblastic differentiation of human adipose tissue-derived stem cells (51). Ars2 knockdown resulted in significant decrease of let-7a levels in menin-expressing cells whereas it had little effect in menin-null cells. This indicates that the functional interaction between menin and ARS2, in addition to the miRNA processing machinery, is essential for proper processing of the let-7a miRNA (Fig. 6). Additionally, this is the first report detailing a post-transcriptional role for menin in regulating gene expression and cell proliferation via regulation of miRNA processing.

The role of miRNAs in regulating pancreatic beta cell proliferation and insulin secretion has been demonstrated extensively (27, 28, 31–33). We show here that menin plays a role in the processing of let-7a, a member of the let-7 family of miRNAs that regulates glucose metabolism in multiple organs (41). Furthermore, global knockdown of let-7a in mice prevents and treats impaired glucose tolerance in diet-induced obese mice, partly by increasing the levels of IRS2 and INSR (41). Similarly, we observed decreased levels of let-7a in Men1-excised cells with significant increase in IRS2 expression (Fig. 7), and inhibition of let-7a using specific anti-miR resulted in derepression of IRS2 (Fig. 8) suggesting that the increased levels of Irs2 upon Men1 excision can be attributed, in part, to reduced let-7a levels. It has previously been reported that IRS2 is markedly overexpressed in rat insulinoma cells compared with rat primary islet β cells (52). Furthermore, the importance of IRS2 in insulin signaling has been demonstrated using transgenic knock-out mouse models where Irs2−/− mice show a marked decrease in beta cell mass and develop type 2 diabetes (53). It is thus conceivable that elevated serum insulin and decreased blood glucose levels in Men1-null transgenic mice (54) can be attributed partly to decreased let-7a with subsequent increase in IRS2 and increased pancreatic beta cell mass. As a corollary, it can also be conceived that disruption of the menin-let-7a-IRS2 axis contributes, at least in part, to tumorigenesis caused by menin mutations in MEN1 syndrome. Moreover, it is likely that menin-mediated processing of miRNAs such as let-7a also contributes partly to the suppression of endocrine tumors.

A recent report described insulin-mediated down-regulation of menin and localization in the cytoplasm in a time-dependent manner via the human insulin receptor (55). It is conceivable that activation of the insulin signaling pathway by insulin leads to reduction in menin levels particularly in the nucleus, consequently resulting in decreased processing of pri-let-7a and increased Irs2 expression. However, in the cell lines used for our
study, we did not observe any decrease in global menin levels or increased localization in the cytoplasm upon treatment with insulin for either 15 min or 24 h (data not shown). However, increased p-AKT levels were detected in cells treated with insulin (data not shown). It is possible that the effect of insulin on menin expression was not observed in our cells because of the lack of ectopic expression of human insulin receptor.

In addition to let-7a, we observed that menin plays a role in the biogenesis of miR-155 with reduced levels upon Men1 excision (Fig. 2C). A previous screen for miRNAs aberrantly regulated in pancreatic neuroendocrine tumors identified significant down-regulation of miR-155 expression in pancreatic endocrine tumors compared with normal pancreas (34). As >40% of pancreatic endocrine tumors harbor somatic mutations in the Men1 gene (56), it is possible that the decreased levels of miR-155 observed in pancreatic endocrine tumors can be partly attributed to the dysregulation of miR-155 biogenesis upon Men1 mutation.

In conclusion, we have uncovered a novel mechanism by which menin regulates gene expression post-transcriptionally via regulating biogenesis of miRNAs including let-7a and miR-155, and provide a rationale for possible use of anti-miR-based therapy for alleviating symptoms associated with MEN1 syndrome and metabolic diseases including diabetes.

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