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The role of tumor suppressor menin in *IL-6* regulation in mouse islet tumor cells

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ABSTRACT

Menin is a gene product of multiple endocrine neoplasia type1 (*Men1*), an inherited familial cancer syndrome characterized by tumors of endocrine tissues. To gain insight about how menin performs an endocrine cell-specific tumor suppressor function, we investigated the possibility that menin was integrated in a cancer-associated inflammatory pathway in a cell type-specific manner. Here, we showed that the expression of IL-6, a proinflammatory cytokine, was specifically elevated in mouse islet tumor cells upon depletion of menin and *Men*$^{-/-}$ MEF cells, but not in hepatocellular carcinoma cells. Histone H3 lysine (K) 9 methylation, but not H3 K27 or K4 methylation, was involved in menin-dependent IL-6 regulation. Menin occupied the IL-6 promoter and recruited SUV39H1 to induce H3 K9 methylation. Our findings provide a molecular insight that menin-dependent induction of H3 K9 methylation in the cancer-associated interleukin gene might be linked to preventing endocrine-specific tumorigenesis.

1. Introduction

Tumor-associated inflammation has been recognized as an important hallmark of cancer [1]. The role of inflammation in tumor promotion has been extensively studied, and recent evidence supports its function in tumor recurrence and metastasis [2,3]. Most inflammatory signals affect tumorigenesis by activating signal transducer and activator of transcription 3 (STAT3) [2,4,5], of which persistent activation in malignant cells stimulates proliferation, survival, angiogenesis, and invasion. Interleukin-6 (IL-6) and leukemia inhibitory factor (LIF), two STAT3 activating cytokines, were found to be produced robustly upon KRas activation during pancreatic tumorigenesis, and ablation of IL-6 in KRas^{G12D} mice demonstrated that IL-6 is one of the key STAT3 activators during PADC initiation and progression [5,6].

Menin was initially identified as a product of the multiple endocrine neoplasia type 1 (*Men1*) tumor suppressor gene [7,8], and its loss of function due to various mutations along the gene is associated with tumors of parathyroid, enteropancreatic neuroendocrine tissue, and anterior pituitary [9,10]. Overexpression of menin in Ras-transformed cells results in decreased proliferation and tumor growth, supporting the idea that menin serves as a tumor suppressor protein [11]. Until now, menin has been reported to interact with a broad spectrum of more than 20 proteins [9]. Importantly, menin functions as a transcription factor by tethering various chromatin remodeling factors [12]. For example, it represses transcription by counteracting the activity of JunD in a histone deacetylase (HDAC)-dependent manner [13] or epigenetically suppresses Hedgehog signaling through histone arginine methylation [14]. Menin suppresses lung adenocarcinoma by repressing the gene transcription of growth factor pleiotrophin (PTN), where the Polycomb gene Enhancer of Zeste homolog 2 (EZH2)-mediated H3 lysine (K) 27 trimethylation is required [15]. Furthermore, menin directly interact with Suppressor of variegation 3–9 homolog 1 (SUV39H1) to repress homeobox gene transcription, such as *Gastrulation Brain Homeobox* 2 (*GBX2*), through H3 K9 methylation [16]. Menin also up-regulates certain cyclin-dependent kinase inhibitor genes and developmental regulatory genes through interaction with mixed lineage leukemia complexes (MLL1 and MLL2) which have histone H3 K4 methylation (HMT) activities [17–20]. Interestingly, contrary to its well-known role as a tumor suppressor, menin functions as a critical oncogenic partner for MLL fusion proteins in blood cells and mediates transcriptional activation of target genes such as *Hoxa9* by employing H3 K4 methylation, an essential step in aggressive human acute leukemia [21–23]. Also, hepatocellular carcinogenesis is in part promoted by menin as it plays an essential role in the activation of an oncogenic protein Yap1 (Yes-associated protein) [24,25].

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In this study, to understand how menin acts as a tumor suppressor specifically in the endocrine lineage, we investigated the role of menin in transcriptional regulation of target genes involved in tumor-associated inflammation and found that menin in pancreatic insulinoma cell lines significantly represses the expression of *IL-6* which is a major effector molecule with multiple functions in cell proliferation and anti-apoptosis [2,26,27].

2. Materials and methods

2.1. Cell culture

Mouse pancreatic islet tumor cells (TGP61 from ATCC) were cultivated in 50% DMEM (Dulbecco's modified Eagles' medium)/50% F12 K medium (Mediatech) supplemented with 10% fetal bovine serum and L-Glutamine/penicillin/streptomycin. Pancreatic beta cells (MIN6 from ATCC) were cultivated in DMEM supplemented with 15% fetal bovine serum and 50 units/ml penicillin/streptomycin (HyClone). HeLa (Human cervical cancer cells from ATCC) cells and HepG2 (Human liver hepatocellular cells from ATCC) were cultivated in DMEM or MEM (Eagles' minimum essential medium) supplemented with 10% fetal bovine serum and 50 units/ml of penicillin/streptomycin. *Men1*$^{+/+}$ MEF (Mouse embryonic fibroblasts) and *Men1*$^{-/-}$ MEF were described [16].

2.2. siRNAs and quantitative real-time polymerase chain reaction (qRT-PCR)

For RNA interference assays, cells were transfected with a control siRNA (AccuTarget™ Negative control siRNA, Bioneer) or siRNAs against *Men1* (ST Pharm), *SUV39H1* (ST Pharm), *G9a* (ST Pharm), and *Ezh2* (ST Pharm) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Total RNA was isolated using RNeasy kit (Qiagen), and cDNA synthesis was performed with the Reverse Transcription System (Fermentas). Quantitative real-time PCR was performed with specific primers using KAPA™ SYBRFAST qPCR KIT (KAPA BIOSYSTEMS) and CFX96™ real-time PCR detector (Bio-Rad). Relative levels of mRNA were normalized to the values of *GAPDH* mRNA for each reaction.

2.3. Western blot analysis

Cells were washed once with cold PBS (without calcium) and lysed in lysis buffer [20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.5% NP-40, and inhibitors of proteases and phosphatases]. After a10 min incubation on ice, the cell lysate was centrifuged (13,000 rpm at 4 °C) for 15 min. The extracted proteins were resolved by SDS–PAGE, transferred onto nitrocellulose membranes, and immunoblotted with anti-menin (Bethyl Laboratories), anti-GAPDH (Santa Cruz Biotechnology), or anti-Actin (Abcam) antibodies.

2.4. IL-6 enzyme-linked immunosorbent assay (ELISA)

An IL-6 ELISA was performed to quantify the secreted IL-6 present in the cell culture medium using BD optEIA mouse IL-6 ELISA kit (BD Biosciences). Briefly, cell culture medium was centrifuged to remove cellular debris, and 100 μl of cleared cell medium was used in the IL-6 ELISA following the manufacturer's instructions. The IL-6 ELISA was read using a 450-nm wavelength. A standard curve was generated with known quantities of IL-6.

2.5. Chromatin immunoprecipitation (ChIP)

Cells were cross-linked with 1% formaldehyde at 37 °C for 15 min. The crosslinking was quenched by addition of 0.125 M glycine for 5 min. Cells were washed twice with PBS and the nuclei were isolated with Lysis buffer A [10 mM Tris HCl (pH 7.5), 10 mM KCl, 5 mM $MgCl_2$, 0.5% NP40]. The nuclei were resuspended in SDS lysis buffer [50 mM Tris HCl (pH 7.9), 10 mM EDTA, 0.5% SDS] and sonicated at 0 °C to produce DNA fragments ranging from 200 to 1000 bp using the Bioruptor (Cosmo Bio). After centrifugation, antibodies and A/G beads were added to the chromatin samples and incubated overnight at 4 °C with rotation. After washing and de-crosslinking, the DNA was purified using a PCR purification kit (Qiagen). Relative amount of immuno-precipitated (IP) DNA was represented as the percentage of input DNA (IP DNA/input). All primer sequences are provided in Supplementary Materials.

3. Results

3.1. Men1 knockdown affects the mRNA levels of IL-6 in a cell type-specific manner

To understand the functional difference of menin in various cellular backgrounds and the functional significance that is related to tumorigenic or anti-tumorigenic activity, we sought to examine menin's molecular activity in different cell types. Previously, we reported that menin represses the transcription of *GBX2* via mediating H3 K9 tri-methylation [16]. One of the downstream targets of *GBX2* is *IL-6*, which plays a critical role by stimulating the inflammatory autocrine/paracrine IL-6 signaling loop through the activation of the STAT3 pathway to confer tumorigenic properties [28]. As inflammation can lead to the development of cancer, we wondered whether menin directly affects the level of IL-6 in endocrine cells. Two different mouse insulinoma cell lines, MIN6 and TGP61, were treated with siRNAs targeting *Men1*. Both are epithelial-like cell lines derived from a pancreatic tumor with islet cell morphology. The knock down of menin was confirmed by immunoblot analysis and the quantitative RT-PCR (Fig. 1). Interestingly, the mRNA level of *IL-6* was increased when menin was depleted in both cell lines (Fig. 1A and B). In contrast, there were no obvious effects on *IL-6* mRNA levels in HeLa cervical cancer cells (Fig. 1C). Whereas, *Men1* siRNA significantly reduced *IL-6* mRNA expression in HepG2 liver carcinoma cells (Fig. 1D), which is consistent with the previous report that menin is associated with inflammation pathways with elevated level of *IL-6* [24]. These results suggest that the integration of menin in inflammatory pathways through IL-6 signaling might be pivotal in determining its role in tumorigenesis with different cellular backgrounds.

3.2. Exogenous expression of menin rescued the IL-6 level in mouse insulinoma cell

To confirm the potential role of menin in *IL-6* regulation and to exclude the possibility of off-target effects of *Men1* siRNAs, we synthesized two more siRNAs carrying different target sequences within the *Men1* gene. All three siRNAs successfully reduced the expression of *Men1* to a different extent and resulted in the increase of *IL-6* levels (Fig. 2A). Although the effect of siRNAs on *IL-6* induction varies, at least more than a twofold increase was observed repeatedly in each sample by treatment with different siRNAs. To further validate the significance of the *IL-6* phenotype, we performed a rescue experiment employing a DNA construct driving the expression of human *Men1* gene. The human *Men1* gene contains a two or four nucleotide sequence difference within the respective regions targeted by each siRNA (Fig. 2 B, top panel), which was sufficient for human *Men1* to escape from the mouse RNA interference. Cells were treated with the mixture of siRNAs, *Men1*–1, -2, and -3. It was highly selective and efficiently reduced the endogenous menin, while add-back expression of human *Men1* successfully recovered the level of menin (Fig. 2B, bottom panel).

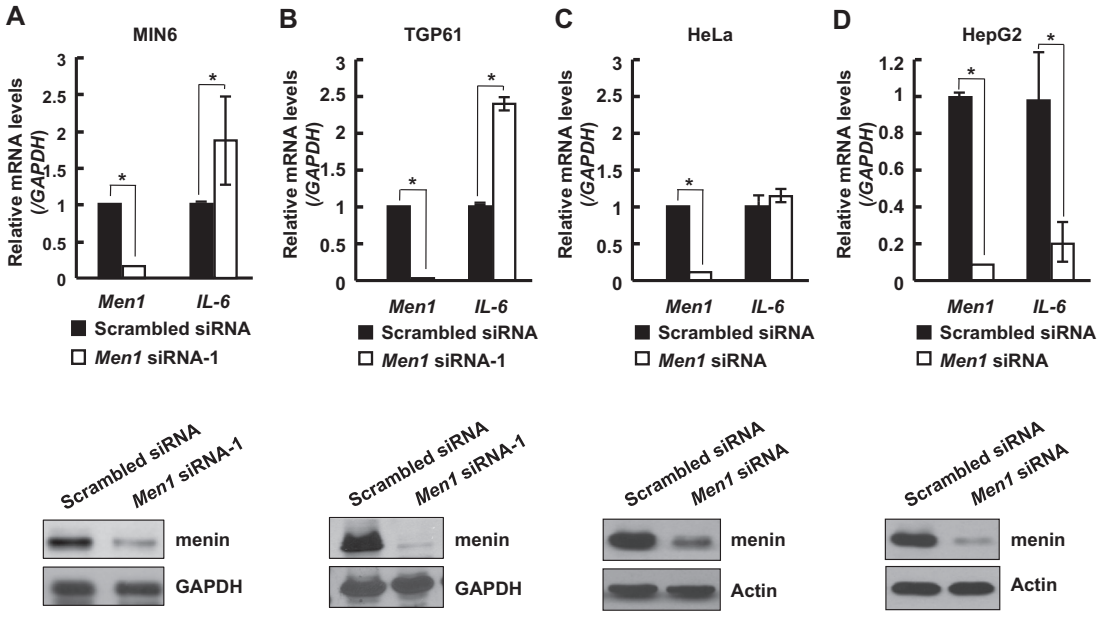

Fig. 1. Knockdown of *Men1* increased *IL-6* level in mouse insulinoma cell lines. Expression of *IL-6* was measured using qRT-PCR in MIN6 (A), TGP61 (B), HeLa (C), and HepG2 (D). Cells were treated with scrambled siRNAs as a control or siRNAs specifically targeting *Men1* for 48 h and total RNA was isolated to detect the steady state level of *IL-6* by qRT-PCR. The efficiency of knockdown of *Men1* was confirmed by qRT-PCR and Western blotting (bottom). Each mRNA level was normalized for *GAPDH* and presented as a relative value. Error bars represent S.D., $n = 3$ ($^*p$-value < 0.05).

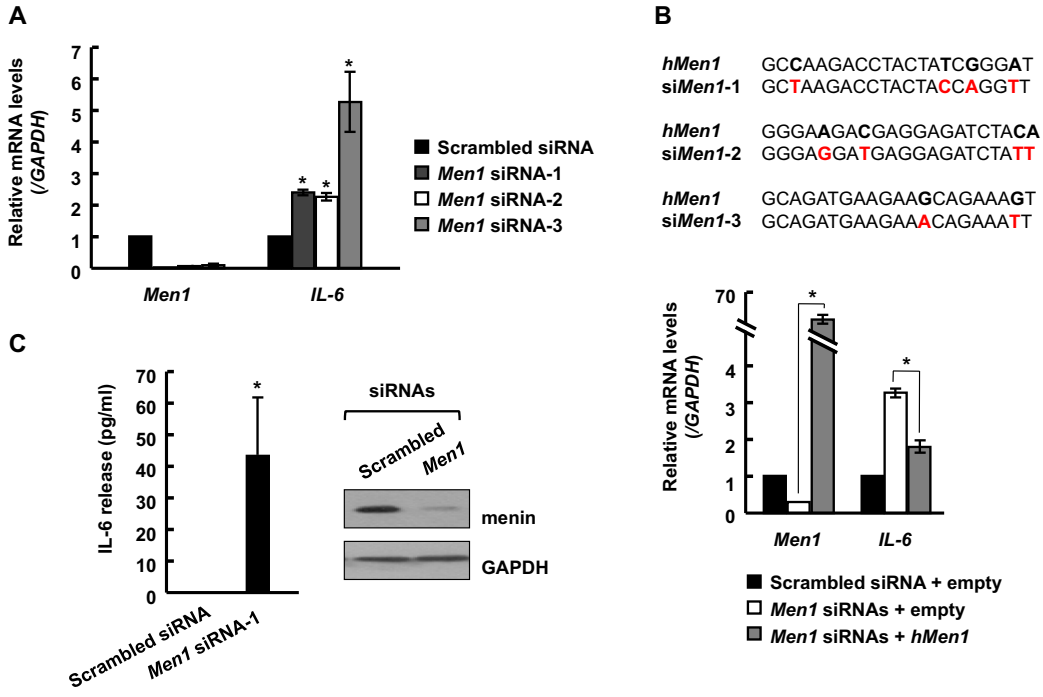

Fig. 2. Rescue of *Men1* gene decreased the *IL-6* level in pancreatic islet tumor cells. (A) Three independent siRNAs targeting different sequences within the *Men1* gene were tested in TGP61. Expression levels of *Men1* and *IL-6* were measured using qRT-PCR after treatment of each of the siRNA as indicated. (B) Rescue experiments were performed by co-transfection of mixture of *Men1* siRNAs with a vector control or the construct expressing human menin (*hMen1*). Total RNA was prepared for analysis of mRNA by qRT-PCR. The primers used for cDNA amplification to detect mRNA levels of menin can recognize both mouse and human *Men1* transcript. Each mRNA level was normalized for *GAPDH* and presented as a relative value. (C) The effect of *Men1* knockdown on IL-6 production was assayed by ELISA. TGP61 cells treated with scrambled or *Men1* siRNAs were harvested and tested using an ELISA with the culture supernatants (left panel), and then the cells were lysed and tested for *Men1* knockdown (right panel). Error bars represent S.D., $n = 3$. Significance of differences was evaluated ($^*p$-value < 0.05).

Importantly, our data demonstrated that the exogenous *hMen1* construct significantly suppressed *IL-6* levels and overrode the effect of siRNAs, suggesting that IL-6 expression is directly coupled to the presence of menin in TGP61 cells (Fig. 2B, bottom panel).

Next, to investigate further whether the *IL-6* mRNA levels targeted by menin are accompanied by changes in IL-6 protein secretion, ELISA detection of IL-6 with the cell culture supernatants was performed. As expected, IL-6 secretion was elevated by *Men1*

knockdown in TGP61 pancreatic islet cells (Fig. 2C, left panel). Immunoblotting performed with the cellular lysates validated the efficient depletion of menin (Fig. 2C, right panel). Together, these results indicate that the expression of *IL-6* is down-regulated by menin in rodent islet tumor cells.

3.3. H3 K9 histone methyltransferases are critical for the menin-dependent regulation of IL-6

We and others have proposed that menin might act as a scaffold or adaptor protein to interact with various chromatin remodeling proteins to control gene transcription [16,29,30]. As menin is known to tether mSin3A/HDAC complex to the target genes to repress their expression, we decided to examine the relative contribution of menin-dependent HDAC activity to *IL-6* regulation. We treated TGP61 cells with HDAC inhibitor, TSA. When genes are under the control of HDACs, their transcription is de-repressed by TSA in a dose-dependent manner. As shown in Fig. 3A, *IL-6* levels were greatly increased by the treatment of *Men1* siRNAs, however, its induction was not dependent on TSA. These results indicated that menin contributed to *IL-6* regulation independently of the HDAC activity.

Menin also has an ability to interact with SUV39H1 and G9a, thereby influencing the H3 K9 methylation states of target genes [16,31–34]. Any role of SUV39H1 or G9a in *IL-6* expression was investigated using RNA interference-mediated knock down of those HMTs in TGP61 cells. *SUV39H1* siRNAs efficiently reduced its target, but did not interfere with the level of menin. Notably, depletion of SUV39H1 significantly increased the level of *IL-6* mRNA (Fig. 3B). By analogy, RNA interference-mediated knock down of G9a showed that the *IL-6* level was up-regulated by G9a depletion, indicating that SUV39H1 and G9a might be critical for the down-regulation of *IL-6* (Fig. 3C). In contrast to H3 K9 HMTs, the expression of the *IL-6* gene was barely affected by Ezh2 (H3 K27 HMT) depletion (Fig. 3D). The relationship of menin and SUV39H1 in *IL-6* expression was further analyzed (Fig. 3E and F). Notably, the individual depletion of *Men1* or *SUV39H1* significantly increased the levels of *IL-6* mRNA to different extents, while the simultaneous depletion of both proteins did not have an additive effect on *IL-6* levels (Fig. 3G), indicating that menin and SUV39H1 function in the same pathway in a cross-dependent manner. Taken together, our data demonstrate that SUV39H1 and G9a, but not HDACs or Ezh2, are important for the repression of *IL-6,* implying that methylation of H3 K9 is likely to have a role.

3.4. Menin and SUV39H1 lead cooperatively to down regulation of IL-6 by targeting H3 K9 trimethylation at the IL-6 promoter in endocrine cells

To provide further evidence, a ChIP assay was performed to measure the recruitment of menin and SUV39H1 around the *IL-6* gene. We carried out ChIP with anti-menin and anti-SUV39H1

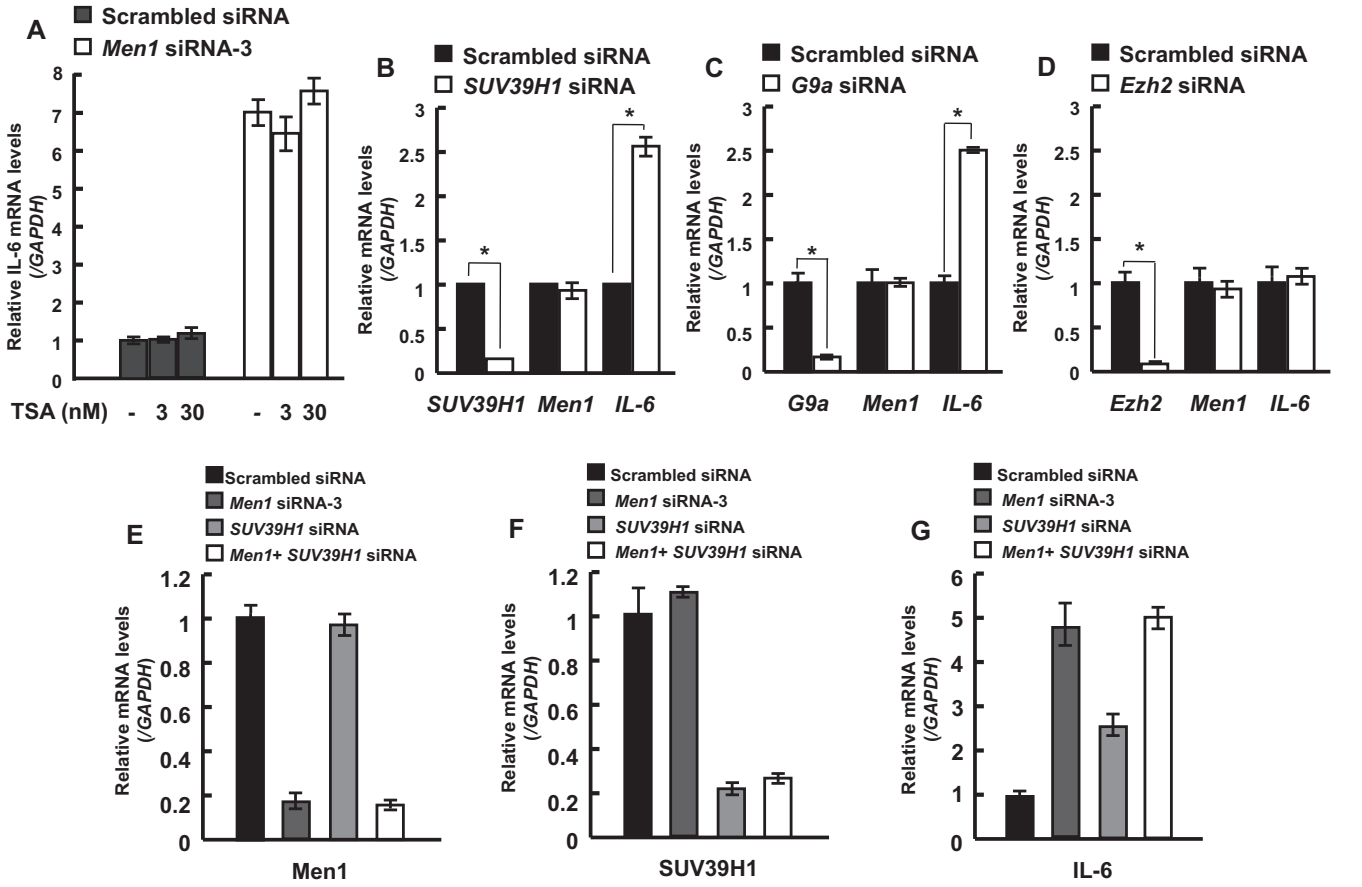

Fig. 3. *IL-6* regulation is mediated by menin-dependent coupling of SUV39H1. (A) TGP61 cells were treated with siRNAs targeting *Men1* for 48 h. Cells were then treated with TSA for 24 h with increasing doses as indicated. Total RNA was prepared to perform qRT-PCR. TSA-dependency was shown as the fold difference between samples by considering expression levels obtained from each TGP61 cell without TSA treatment as 1. (B) TGP61 cells were treated with siRNA targeting *SUV39H1* and total RNA was isolated to detect the steady state level of *IL-6* mRNA by qRT-PCR. (C and D) TGP61 cells were treated with siRNA for knocking down *G9a* or *Ezh2*. The mRNA levels were normalized to the level of *GAPDH* and shown as relative values. (E and F) Expression of *Men1* and *SUV39H1* were measured using qRT-PCR in TGP61 cells. (G) Simultaneous knockdown of menin and SUV39H1 did not show an addictive effect on the mRNA levels of *IL-6*. The PCR values were normalized to *GAPDH* and presented as relative values. Error bars represent S.D., $n = 3$ (*p value < 0.05).

antibodies once cells treated with control or *Men1* siRNA (Fig. 4A and B) and used three distinct pairs of primers (pp) to amplify the promoter proximal and distal regions (pp1 and pp2, respectively) or gene body (pp3) of *IL-6* gene for analysis (Supplementary Fig. S1A). The *Men1* knock-down was confirmed in TGP61 cells during the ChIP assay (Supplementary Fig. S1B). Our results show that menin was specifically recruited to the *IL-6* promoter proximal regulatory region (pp2) in control cells, but not in *Men1* siRNA treated cells (Fig. 4A). Relative occupancy along the gene indicates that menin might be important for transcriptional initiation of *IL-6*. Interestingly, SUV39H1 was also enriched in the promoter proximal region (Fig. 4B) and importantly, its recruitment was abolished by the depletion of menin, indicating that menin is responsible for recruitment of SUV39H1. To gain insight into the mechanism by which menin and SUV39H1 regulate *IL-6* transcription, we next analyzed the covalent modification states of H3 in the *IL-6* promoter by ChIP. The H3 K9 trimethylation (H3K9me3) was reduced in cells treated with *Men1* siRNA, specifically in the promoter proximal region (Fig. 4C, pp2), whereas the level of H3 K9 acetylation (H3K9ac) was increased in the same region (Fig. 4D). However, H3 K4 trimethylation (H3K4me3) was relatively unchanged, suggesting MLL-dependent H3 K4 methylation was not the target of menin in this region (Supplementary Fig. S2). A ChIP assay within *IL-6* region revealed that menin contributes to the formation of a repressive chromatin environment, which is in part via SUV39H1. Confirming this again, a ChIP analysis with $Men1^{+/+}$ or $Men1^{-/-}$ MEF cells also showed that $Men1^{-/-}$ MEF cells had increased *IL-6* level (Fig. 4E) and menin and SUV39H1 were recruited to the *IL-6* promoter (pp2) (Fig. 4F) to contribute to *IL-6* repression with concomitant regulation of H3K9me3 (Fig. 4G), while H3 K9 acetylation or H3 K4 methylation was negligibly altered (Supplementary Fig. S3). Taken together, these results indicate that SUV39H1 and menin function together at the protein level and contribute significantly to the down-regulation of *IL-6*, most likely through the methylation of H3 K9 on the *IL-6* promoter.

4. Discussion

Menin is peculiar in the sense that its function as a tumor suppressor is endocrine tissue-specific. The underlying mechanisms of menin's different effect on specific organs are not clear, but they likely target gene expression profiles and various distinct signaling pathways in a context-dependent or cell type-specific manner [35].

We have previously reported that menin has an ability to mediate methylation of H3 K9 through integration of SUV39H1 for *GBX2* gene repression in MEF cells [16]. Gbx2 is a homeotic transcription factor associated with pancreatic differentiation and is consistently overexpressed in cancer cells such as human prostate cancer [28,36]. Interestingly, one of the downstream targets of Gbx2 is *IL-6* [28]. In this study, we showed that menin specifically downregulates IL-6 in pancreatic insulinoma islet cells and MEF cells, but not in breast or hepatocellular cancer cells. It binds to *IL-6* within the promoter proximal region and employs SUV39H1 and possibly G9a to induce H3 K9 methylation, which is a striking contrast to the reports that menin mediates the expression of Yap1 and inflammation pathways through MLL-dependent H3 K4 methylation to promote liver tumorigenesis [24].

Inflammation is frequently associated with an increased risk of cancer, promoting initiation, development, and progression of the disease, during which a variety of cytokines and chemokines play a role [2,37]. In this sense, it is noteworthy that menin targets IL-6, a pro-inflammatory cytokine. IL-6 has been shown to be overexpressed in human pancreatic cancer cells and its expression levels correlate with its tumorigenic and metastatic potential

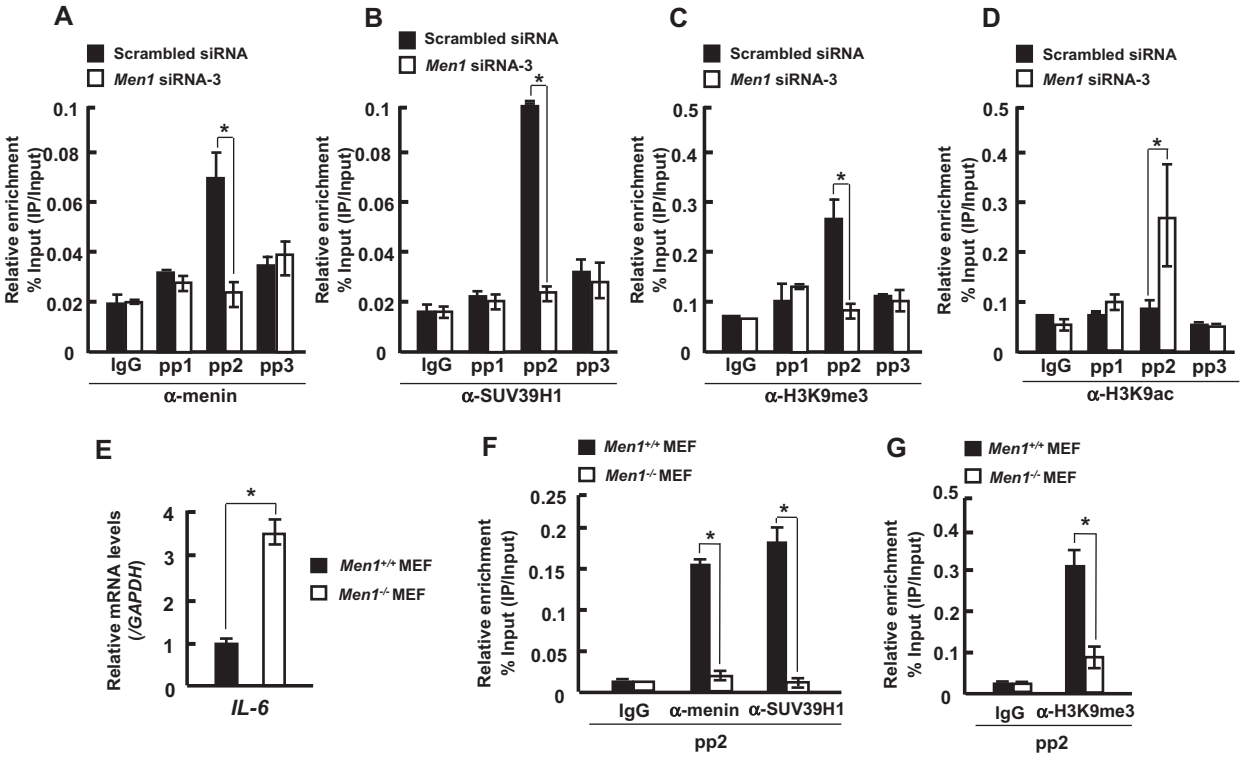

Fig. 4. Menin recruits SUV39H1 to promote trimethylation of H3 K9 around the *IL-6* promoter in pancreatic islet cells. (A and B) Relative occupancy and recruitment of menin and SUV39H1 along the *IL-6* promoter locus was analyzed by ChIP. Chromatin solution was obtained and subjected to ChIP using anti-menin, anti-SUV39H1 antibodies, or normal rabbit IgG as a control. (C and D) ChIP was performed using anti-H3 K9 trimethylation (H3K9me3) or H3 K9 acetylation (H3K9ac) antibodies. The levels of H3K9me3 and H3K9ac in *IL-6* promoter were analyzed as described in Section 2. (E) The expression level of *IL-6* was measured by qRT-PCR in $Men1^{+/+}$ or $Men1^{-/-}$ MEF cells. (F and G) Recruitment of menin, SUV39H1, and the level of H3K9me3 at the promoter proximal region (pp2) were analyzed by ChIP assay. Error bars represent S.D., $n = 2–3$ (*p-value < 0.05).

[38,39]. IL-6 secreted by microenvironmental compartments such as myeloid cells, and the ones aberrantly expressed from the pancreatic lesion itself, can influence tumor development in an autocrine/paracrine fashion, through abnormal activation of the growth signaling pathway [40]. Importantly, our observation supports menin as a bona fide tumor suppressor that could thwart inflammation and tumor growth in the endocrine lineage. Since SUV39H1 and G9a were also shown to repress *IL-6* in this study, we propose the hypothetical model in which menin functions as a tumor suppressor by regulating histone methylation states at the promoters of specific cytokine genes that govern inflammation and tumorigenesis in this particular cell type. We suggest that targeting menin, epigenetic modifications of histone, and pro-inflammatory cytokines such as IL-6 (and the downstream signaling pathway) might have novel therapeutic potential.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.113.

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