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## Suppression of lung adenocarcinoma through menin and polycomb gene-mediated repression of growth factor pleiotrophin

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### Abstract

Menin up-regulates transcription of cell cycle inhibitors to suppress endocrine tumors, but it is poorly understood how menin suppresses non-endocrine tumors such as lung cancer. Here, we show that menin inhibits proliferation of human lung cancer cells and growth of lung cancer in mice. The menin-mediated tumor suppression requires repression of growth factor pleiotrophin (PTN), which binds to its cell surface receptor, anaplastic lymphoma kinase (ALK) that is activated in certain lung adenocarcinomas. Menin represses *PTN* transcription and PTN-induced proliferation of human lung cancer cells, and menin expression is substantially reduced in primary human lung adenocarcinomas. Notably, menin binds the *PTN* locus and enhances Polycomb gene EZH2-mediated histone H3 lysine 27 trimethylation (H3K27m3), a negative mark for gene transcription but does not affect histone H3K4 methylation that is usually up-regulated by menin in endocrine cells. Together, our findings indicate that menin suppresses lung cancer partly through increasing polycomb gene-mediated H3K27 methylation and repressing *PTN* transcription, unraveling a novel, epigenetically regulated PTN-ALK signaling pathway in suppressing lung cancer.

### Keywords

lung adenocarcinoma; histone H3 lysine 27; *Men1*; pleiotrophin; Polycomb

### Introduction

Lung cancer is a leading cause of cancer-related death over the world, and the 5-year survival rate remains poor despite aggressive chemotherapy (Jemal *et al.*, 2002). Somatic mutations in proto-oncogenes, such as K-Ras, epidermal growth factor receptor (EGFR), and

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anaplastic lymphoma kinase (ALK), are often identified in lung adenocarcinoma (Herbst *et al.*, 2008; Jemal *et al.*, 2002; Soda *et al.*, 2007). Pei *et al.* had reported that non-small cell lung cancers (NSCLC) develop in both *Men1*<sup>+/-</sup> and *p18*<sup>-/-</sup>; *Men1*<sup>+/-</sup> mice at a high penetrance (Pei *et al.*, 2007). However, little is known as to how menin suppresses development of NSCLC.

The *Men1* gene, which encodes the nuclear protein menin, is mutated in patients with an inherited tumor syndrome, multiple endocrine neoplasia type 1 (MEN1) (Chandrasekharappa *et al.*, 1997). In MEN1 endocrine tumors with a germline mutation in one of the *MEN1* alleles, the remaining wild type *MEN1* allele is often inactivated due to a somatic mutation (Loss of Heterozygosity, LOH), indicating *MEN1* as a bona fide tumor suppressor gene in endocrine tumors (Lemos and Thakker, 2008).

Though menin's primary sequence does not reveal any paralogs, multiple lines of evidence suggest that one of the major functions of menin is to regulate gene transcription (Karnik *et al.*, 2005; Milne *et al.*, 2005; Yokoyama *et al.*, 2004). Menin associates with chromatin and the nuclear matrix and exerts multiple biological functions including regulation of cell proliferation (Jin *et al.*, 2003; Schnepf *et al.*, 2006; Schnepf *et al.*, 2004). These diverse menin functions may be largely attributed to the crucial role of menin as a scaffold protein in coordinately regulating transcription of various target genes.

Menin interacts with mixed lineage leukemia proteins (MLL), histone H3 methyltransferases that catalyze histone H3 lysine 4 (H3K4) methylation with their highly conserved SET domain (Milne *et al.*, 2002). MLL is an orthologue of the *Drosophila trithorax group (TrxG)* genes (Yu *et al.*, 1998), which interact with and antagonize another group of genes, the Polycomb group (*PcG*) genes (Ringrose and Paro, 2007; Schuettengruber *et al.*, 2007). *PcG* genes form various protein complexes including Polycomb repressive complex 2 (PRC2), which contains Enhancer of Zeste homolog 2 (EZH2) and its regulatory protein SUZ12 (Ringrose and Paro, 2007; Schuettengruber *et al.*, 2007; Sparmann and van Lohuizen, 2006). EZH2 is also a chromatin-associating protein with a conserved SET domain (Cao *et al.*, 2002). However, unlike the SET domain in MLL that methylates H3K4 (Yokoyama *et al.*, 2004), the EZH2 SET domain specifically methylates H3K27 and the methylated H3K27 can be recognized by other specific binding proteins to compress chromatin structure, leading to repression of gene transcription (Cao *et al.*, 2002; Sparmann and van Lohuizen, 2006). Site-specific histone modifications are a major epigenetic mechanism for maintaining stable gene transcription or silencing in differentiated cells (Cao *et al.*, 2002; Martin and Zhang, 2007; Sparmann and van Lohuizen, 2006). Though menin promotes H3K4 methylation, which is presumably catalyzed partly by MLL at loci of the cell cycle inhibitors *p18<sup>Ink4c</sup>* (p18) and *p27<sup>Kip1</sup>* (p27) gene (Karnik *et al.*, 2005; Milne *et al.*, 2005), little is known as to whether *PcG* proteins ever participate in menin-regulated gene transcription.

Our current studies show that menin inhibits human lung cancer cells through repression of pleiotrophin (PTN) and its cell surface receptors including ALK (Stoica *et al.*, 2001). Instead of working with its known partner MLL to methylate H3K4, a positive mark, menin bound the promoter of *PTN* and recruited the Polycomb group (*PcG*) complex to the locus, resulting in H3K27 trimethylation, PTN suppression, and inhibition of proliferation of lung

cancer cells. These findings unravel a novel mean in suppressing lung cancer through epigenetically repressing the PTN and ALK via a menin-PcG complex.

## Materials and methods

### Cell culture

A549 (lung adenocarcinoma), NCI-H157 (NSCLC), and NCI-H446 (SCLC) human lung cancer cell lines were purchased from the American Type Culture Collection (ATCC, Philadelphia, PA, U.S.A.). A549 and NCI-H446 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Utah, USA) or RPMI 1640 (Hyclone), respectively, and supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone), and 1×Penicillin-Streptomycin (Pen-Strep, 100 U/ml-100 µg/ml) (Invitrogen, Carlsbad CA, USA). NCI-H157 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 1 mM Sodium Pyruvate (Hyclone), 10 mM Hepes (Hyclone) and 1×Pen-Strep. The *Men1<sup>+/+</sup>* and *Men1<sup>-/-</sup>* MEFs were described previously (Schnepp *et al.*, 2006) and cultured in DMEM supplemented with 10 % FBS, 1× Pen-Strep, 2 mM L-glutamine (Invitrogen), and 0.1 mM Non-essential amino acids (Hyclone).

### Immunohistochemistry (IHC) and immunofluorescent (IF) staining

IHC and IF detection was performed using an affinity-purified anti-menin antibody (Jin *et al.*, 2003), the specificity of the anti-menin antibody was verified in menin-null and menin-expressing cells (Suppl Fig. 1), anti-PTN (Abnova) or anti-ALK (Cell Signaling) antibodies. Nuclei were counterstained with DAPI, and the stained cells were analyzed and photographed under a confocal microscope (OLYMPUS FV1000, ×400).

### Western blotting and enzyme-linked immunosorbent assay (ELISA)

For Western blotting, cells were lysed in 20 mM Tris-HCl (pH 8.0), 5% glycerol, 138 mM NaCl, 2.7 mM KCl, 1% NP-40, 20 mM NaF, 5 mM EDTA, 1 mM sodium orthovanadate, 5µg/ml leupeptin, 1µg/ml pepstatin, and 1 mM DTT. The extracted proteins were resolved by SDS-PAGE before transfer onto PVDF membrane, followed by incubation with the anti-menin (Bethyl Laboratories, Montgomery, USA), anti-PTN (Abnova, Walnut, USA), or anti-beta-actin antibodies (Santa Cruz biotechnology, Santa Cruz, USA). ELISA for PTN was performed by using a PTN ELISA kit (Uscn Life Science & Technology, Wuhan, China), according to the manufacturer's instructions.

### Chromatin immunoprecipitation assay (ChIP)

ChIP assays were performed as described with certain modifications (Chen *et al.*, 2006). Briefly,  $1 \times 10^6$  *Men1<sup>-/-</sup>* and *Men1<sup>+/+</sup>* MEFs were treated with 1 % formaldehyde for 10 min, followed by pulsed sonication to shear cellular DNA. ChIP assays were then carried out with indicated antibodies according to the protocol of the ChIP Assay kit (Millipore Billerica, USA). Antibodies used for ChIP assays were: anti-menin (Bethyl Laboratories), anti-trimethyl-Histone H3 Lys27 (Millipore), anti-trimethylated histone H3 Lys 4 (Abcam, Cambridge, UK), anti-acetyl-Histone H3 (Millipore), anti-EZH2 (Cell Signaling, Danvers, USA), anti-SUZ12 (Cell Signaling), anti-RNA polymerase II (Millipore), or control IgG (Santa Cruz). After overnight incubation with the antibodies, the crosslinks between nuclear

proteins and genomic DNA were reversed, and the antibody pulled down DNA was purified by phenol/chloroform extraction. Quantitative PCR was performed using primers specific for menin target genes or *GAPDH*. Primer pair (PP) sequences were shown in Suppl Tab 1.

## Results

### Menin inhibits lung cancer cell proliferation through repression of PTN expression

p18, an inhibitor of cyclin-dependent kinase, suppresses lung cancer in collaboration with *Men1* in mice (Pei *et al.*, 2007). Our earlier work showed that loss of menin in MEFs increases expression of PTN 11 fold (La *et al.*, 2004). PTN is a heparin-binding growth factor that is highly expressed in certain solid cancers such as breast and lung cancer (Jager *et al.*, 2002; Perez-Pinera *et al.*, 2007a). Though it is still relatively unclear how PTN transduces its pro-proliferation signal into cells and several cell surface receptors have been proposed to bind *PTN* (Lu *et al.*, 2005; Stoica *et al.*, 2001), a prevalent model suggests that PTN binds to its cell surface receptor, protein tyrosine phosphatase receptor Z1 (PTPRZ1), inhibiting its phosphatase activity towards another oncogenic kinase, ALK (Lu *et al.*, 2005; Meng *et al.*, 2000; Powers *et al.*, 2002; Stoica *et al.*, 2001). To explore whether menin affects proliferation and PTN expression of lung cancer cells, we stably transfected A549 cells with either control or menin-expressing construct. Our results show that ectopic menin expression reduced the number of the menin cDNA-transfected cells (Fig. 1A). Ectopic menin expression was confirmed by RT-PCR and Western blotting (Fig. 1B and C). PTN expression was reduced in A549 cells with ectopic menin expression at the mRNA level (Fig. 1B, lane 2), as well as at the intracellular and secreted PTN protein level (Fig. 1C, D, respectively). No obvious apoptosis was observed in menin-transfected cells, using Annexin V staining and flow cytometry analysis (Suppl Fig. 2).

Next, we examined whether knockdown of *MEN1* (human gene encoding menin) using shRNAs affects proliferation of the cells. Control vector and constructs expressing each of the three distinct shRNAs that specifically target *MEN1* were generated and stably transfected into A549 cells. *MEN1* shRNAs 2-3 substantially reduced menin expression, but shRNA1 only mildly reduced menin expression, as shown by RT-PCR (Fig. 1E). Correlated with the levels of menin knockdown, shRNA 2-3 significantly increased cell proliferation ( $p < 0.05$ ), but control vector and menin shRNA1, which were unable to substantially reduce menin expression, did not significantly alter proliferation of A549 cells (Fig. 1F,  $p < 0.05$ ). Notably, *MEN1* knockdown also increased mRNA and the intracellular and secreted PTN (Fig. 1E, G and H), but did not affect expression of a PTN paralog, MK (Suppl Fig. 3). Menin also suppresses proliferation of two additional human lung cancer cell lines, NCI-H157 and NCI-H446 (Suppl Fig. 4B and C). We also examined the effect of *MEN1* point mutations, A242V and L22R, which were identified from inherited *MEN1* patients (Milne *et al.*, 2005), on expression of PTN and ALK, a receptor of PTN. We found that L22R and A242V point mutants lost or partially lost the ability to repress *PTN* and *ALK* expression (Fig. 1I). Together, these results indicate that menin is required for inhibiting PTN expression and proliferation of the human lung cancer cells.

To further reinforce the role of menin in repressing PTN expression, we next evaluated the impact of loss of menin on expression of PTN and PTN downstream regulator, ALK, in

*Men1*<sup>+/+</sup> and *Men1*<sup>-/-</sup> MEFs. *Men1* excision substantially increased expression of PTN and ALK (Fig. 1J and K). The role of menin in repressing PTN and ALK expression was also confirmed in a separate pair of *Men1*-expressing and *Men1*-null MEFs. Consistent with this observation, ectopic menin expression also repressed proliferation of MEFs. (Suppl Fig. 5). Together, these data indicate that menin is required for repressing PTN expression in human A549 lung cancer cells and MEFs.

### PTN expression was essential for Menin -inhibited lung cancer cell proliferation

We further examined if PTN is crucial for menin-mediated repression of proliferation of human A549 lung cancer cells. To this end, we generated three shRNAs that specifically target the *PTN* gene. Transfection of A549 cells with each of the three *PTN* shRNAs reduced the *PTN* mRNA (Fig. 3H) and the secreted PTN in culture medium (Fig. 2A), with *PTN* shRNAs 1-2 more effective. Notably, PTN knockdown by *PTN* shRNA1 significantly reduced proliferation of A549 cells ( $p < 0.05$ ), but *PTN* shRNA3, which was less effective in knocking down *PTN*, was also less potent in repressing proliferation of A549 cells (Fig. 2B), suggesting that it is *PTN* shRNA-targeted *PTN* knockdown, but not their off-targeting effect, that led to repressing proliferation of the lung cancer cells. Consistently, stably *PTN*-transfected cells (Fig. 2C) proliferated ~40% faster than the control vector cells at day 6 of culture (Fig. 2D,  $p < 0.05$ ), indicating that ectopic PTN expression is sufficient to increase proliferation of A549 cells. To further decipher the relationship between menin and PTN in controlling proliferation of A549 cells, these cells were transfected with either menin or menin and PTN, and their overexpression was confirmed by Western blotting (Fig. 2E). As expected, on day 6 of culture, menin reduced proliferation of the cells by over 30% (Fig. 2F,  $p < 0.05$ ). Notably, co-expression with PTN neutralized menin-induced repression of A549 cell proliferation (Fig. 2F). Collectively, these data demonstrate that menin-regulated PTN as a crucial target of menin in regulating proliferation of the lung cancer cells.

### Menin downregulates ALK expression partly through repressing PTN

*MEN1* knockdown upregulates ALK as well as *PTN* in A549 cells (Fig. 1E), but ectopic menin expression reduced ALK expression (Fig. 3E and G). To further dissect the potential relationship between PTN and ALK, we stably transfected A549 cells with either control vector or one of the three *PTN* shRNAs. *PTN* shRNAs 1-2, which reduced PTN expression, also decreased the *ALK* mRNA level (Fig. 3H). Conversely, *PTN* shRNA3 that failed to knock down PTN also failed to reduce the *ALK* mRNA level (Fig. 3H). Though it is possible that menin represses PTN and ALK separately, it could not be ruled out that PTN also up-regulates ALK expression. Thus, we ectopically expressed menin and/or PTN to determine their effect on the *ALK* mRNA level in A549 cells. Notably, PTN expression abrogated menin-induced reduction of the *ALK* mRNA level (Fig. 3I, lane 3), and ectopic PTN expression alone also increased ALK expression (Fig. 3I, lane 4). Together, these findings argue that PTN up-regulates ALK expression, but menin represses ALK expression indirectly through inhibiting PTN expression, downregulating two crucial components of the PTN pathway, PTN and the downstream receptor ALK. These findings highlight the importance of menin in controlling this signaling pathway.

### Menin inhibits growth of cancer xenograft derived from A549 cells

To explore whether menin affects growth of A549 cell-derived tumors in nude mice, A549 cells stably transfected with either control or menin-expressing constructs were subcutaneously transplanted into nude mice (n=10 per group). The size of the solid tumor was measured after various periods of time following transplantation. Menin expression resulted in smaller tumors in the nude mice after transplantation (Fig. 4A and B,  $p < 0.05$ ). To determine if menin and PTN affect the growth of the established tumors in nude mice, A549 cells were transplanted to nude mice. When visible tumors were formed (day 6 post-transplantation), control DNA or construct expressing either menin or PTN shRNA was injected into the tumor mass. Both ectopic menin expression and PTN knockdown significantly reduced the xenograft tumor sizes (Fig. 4C,  $p < 0.05$ , respectively) and weight (Fig. 4D,  $p < 0.05$ ) at day 8 and 10 post transplantation respectively. Both ectopic menin expression and PTN knockdown reduced PTN expression at the mRNA level (Fig. 4E) and protein level, as shown by ELISA assay (Fig. 4F). Ectopic menin expression in the tumor was confirmed by IHC staining (Fig. 4 G and H). These results indicate that menin represses, but PTN promotes, growth of xenograft of human lung adenocarcinoma in mice, highlighting the crucial role of menin and PTN in controlling growth of the tumors *in vivo*.

### Menin expression is reduced in certain primary human lung cancer

We have shown a crucial role of menin in repressing a human lung cancer cell line and MEFs, and we wonder if the menin protein level is altered in patients' primary lung cancers. Thus, we examined 39 adenocarcinoma samples and 9 squamous cell carcinomas, all with adjacent normal tissues. Sections from paraffin-embedded samples were stained with affinity-purified anti-menin antibody for IHC staining. Menin was easily detectable in the nucleus of the normal alveolar epithelial cells (Fig. 5A Right and C). In contrast, in certain tumors, staining for menin was much weaker or undetectable, as compared to that in the adjacent normal epithelial cells (Fig. 5A, Left). In the same section from a lung cancer sample, in normal bronchiolo epithelial cells abundant menin was detected in the nucleus (Fig. 5C), but was barely visible in the nucleus of the cancer cells (Fig. 5B). It is also obvious that the nucleus of the cancer cells tended to be larger than the normal epithelial cells.

Menin expression in tumors, as compared to that in the adjacent normal epithelial cells, was markedly reduced or not detectable in 9 out of 39 adenocarcinomas, accounting for 23 % of the tumors we examined (Suppl Table 2). On the other hand, in 9 squamous cell cancers, we failed to detect an obvious change of menin expression between the tumor cells and the adjacent normal lung tissues. Among 10 cases with loss of menin expression in lung cancers, 4 cases showed lymph node metastasis (40%) (Suppl. Table 2). In contrast, only 5 cases had lymph node metastasis out of 29 cases that still express menin in the tumors (17%), correlated with a reduction of lymph node metastasis in menin-expressing adenocarcinoma. While the number of cases that were analyzed is moderate, our findings suggest that menin expression was markedly reduced in 23% of lung adenocarcinomas, which was correlated with increased lymph node metastasis.



### Menin binds to the PTN locus and affects the H3K27 trimethylation(H3K27m3)

To determine how menin regulates *PTN* expression, we performed ChIP assays with *Men1*<sup>-/-</sup> and *Men1*<sup>+/+</sup> MEFs, using an anti-menin antibody. We designed two primers used for ChIP assays at *PTN* promoter loci (Fig. 6A). ChIP assays showed that menin bound to the *PTN* locus (PP1) in the *Men1*<sup>+/+</sup> cells but not in *Men1*<sup>-/-</sup> cells (Fig. 6B, lanes 3 and 6). These results provide the first direct evidence that menin regulates *PTN* expression by binding to the *PTN* locus. To elucidate how menin represses *PTN* expression, we turned our attention to the impact of menin on histone modification of *PTN* promoter locus. We first detected whether menin affects histone H3 lysine 4 trimethylation (H3K4m3) or acetyl-histone H3, which are correlated with positive gene transcription induced by menin and MLL in leukemia cells (Chen *et al.*, 2006; Yan *et al.*, 2006) at the *PTN* locus in *Men1* knockout MEF cells, but we failed to observe an impact of *Men1* excision on the level of H3K4m3 (Fig. 6C) or acetyl-histone H3 (Suppl Fig. 6A) at the *PTN* locus. We also failed to detect histone deacetylase 1 (HDAC1) at the *PTN* locus (Suppl Fig. 6B). On the other hand, histone H3 lysine 27 trimethylation (H3K27m3), which is catalyzed by EZH2 from transcription-repressing Polycomb group (*PcG*) genes, can be recognized by some *PcG* proteins to compress chromatin structure, leading to repression of gene transcription (Cao *et al.*, 2002). We thus further determined if menin affects H3K27 trimethylation (H3K27m3). Notably, H3K27m3 was reduced in *Men1* null cells at the *PTN* locus, but not at the *GAPDH* locus (Fig. 6C, and D lanes 3 and 6). Together, these findings suggest that menin represses *PTN* transcription at least in part through H3K27m3 at the *PTN* locus.

To assess the scope of *PcG* proteins involved in menin-dependent repression of *PTN*, we examined the impact of *Men1* excision on another *PcG* protein that associates with the H3K27-methylating complex, SUZ12, using ChIP assay. We found that SUZ12 bound the *PTN* locus in MEFs (Fig. 6E, Lane 3). Notably, loss of menin abrogated SUZ12 binding to the *PTN* locus (Fig. 6E, Lane 6), while the SUZ12 and EZH2 protein level in cells was not affected by menin excision (Supp Fig. 6C). These findings suggest that part of menin's role is to recruit the *PcG* complex including EZH2 and SUZ12 to the *PTN* locus to methylate H3K27 and then silence *PTN* expression. In addition, loss of menin enhanced detection of RNA pol II at the *PTN* locus (Fig. 6F, lane 6). EZH2 knockdown reduced the EZH2 mRNA and protein levels (Fig. 6G-H) but increased the *PTN* mRNA level (Fig. 6G) and secreted *PTN* for ~30% (Fig. 6I). As EZH2-mediated H3K27 represses gene transcription, these findings demonstrate that menin cross-talks with EZH2 and SUZ12 to enhance H3K27m3 at the *PTN* locus to actively repress *PTN* transcription. Together, our findings are consistent with a model that menin represses *PTN* transcription in part by functionally interacting with the *PcG* proteins in H3K27m3 as shown in (Fig. 6H). These findings have unraveled a previously unrecognized connection between menin and Polycomb group proteins in H3K27 methylation mediated repression of gene transcription and cell proliferation.

### Discussion

Although much has been learnt as to how active mutations in proto-oncogenes such as K-Ras and EGFR facilitate the development of lung adenocarcinoma (Herbst *et al.*, 2008; Jemal *et al.*, 2002; Soda *et al.*, 2007), little is known about how mutations in tumor





PTN signaling and tumor promotion, an attractive model suggests that PTN enhances cell proliferation and cell migration by binding to its cell surface receptor PTPRZ1 and inhibits its phosphatase activity toward its coreceptor, ALK (Perez-Pinera *et al.*, 2007b). As a result, the phosphorylation of ALK and hence the ALK activity is increased (Perez-Pinera *et al.*, 2007b). Interestingly, ALK has been reported to fuse with other partner proteins in ~7% of human lung adenocarcinoma cases, and the mutated ALK has enhanced kinase and transforming ability (Soda *et al.*, 2007). Therefore, it is likely that menin normally helps maintain the repressed status of the PTN and ALK transcription, but downregulation of the *MEN1* gene in lung cancer cells leads to enhanced expression of PTN and ALK. These findings suggest a previously unrecognized pathway in controlling proliferation of lung cancer, i.e, the menin, PTN, and ALK pathway, therefore implying that targeting the PTN-ALK pathway may prove effective in treating lung adenocarcinomas that express a low level of menin. In addition, our findings may also help subdivide the lung adenocarcinoma with menin-positive and menin-negative cancers for diagnosis, and this diagnosis may help in choosing the therapy by effectively treating the menin-negative cancers with the ALK kinase inhibitors.

In summary, we have unraveled a novel mechanism whereby menin represses gene transcription by regulating the PcG gene-mediated repression of gene transcription. Unlike the classic role of menin in enhancing MLL-mediated H3K4 methylation and transcription of p18 and p27 (Karnik *et al.*, 2005; Milne *et al.*, 2005), menin potently inhibits transcription of PTN by recruiting PcG proteins such as SUZ12 and enhancing trimethylation of H3K27. These findings have expanded the role of menin-mediated suppression of endocrine tumors to suppression of human lung cancer. These results not only highlight menin as a potential diagnostic marker to classify a subtype of human lung adenocarcinoma with negative menin expression, but also unravel the PTN-ALK pathway as a potential target for treating the menin-negative lung adenocarcinoma with the ALK inhibitor.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

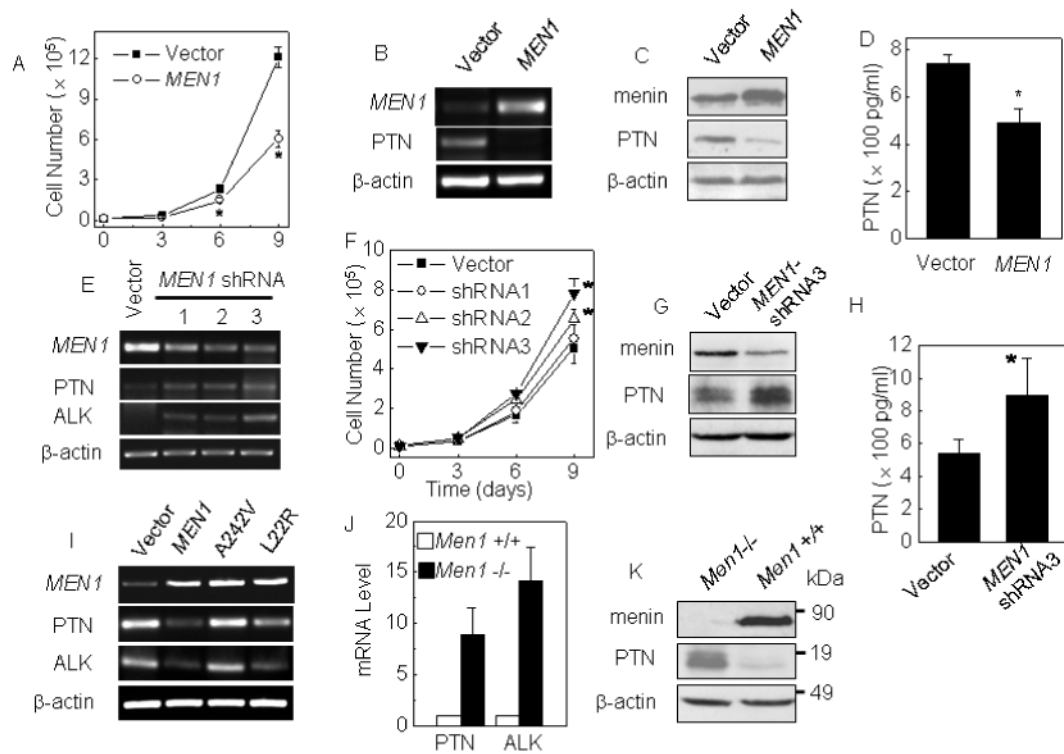
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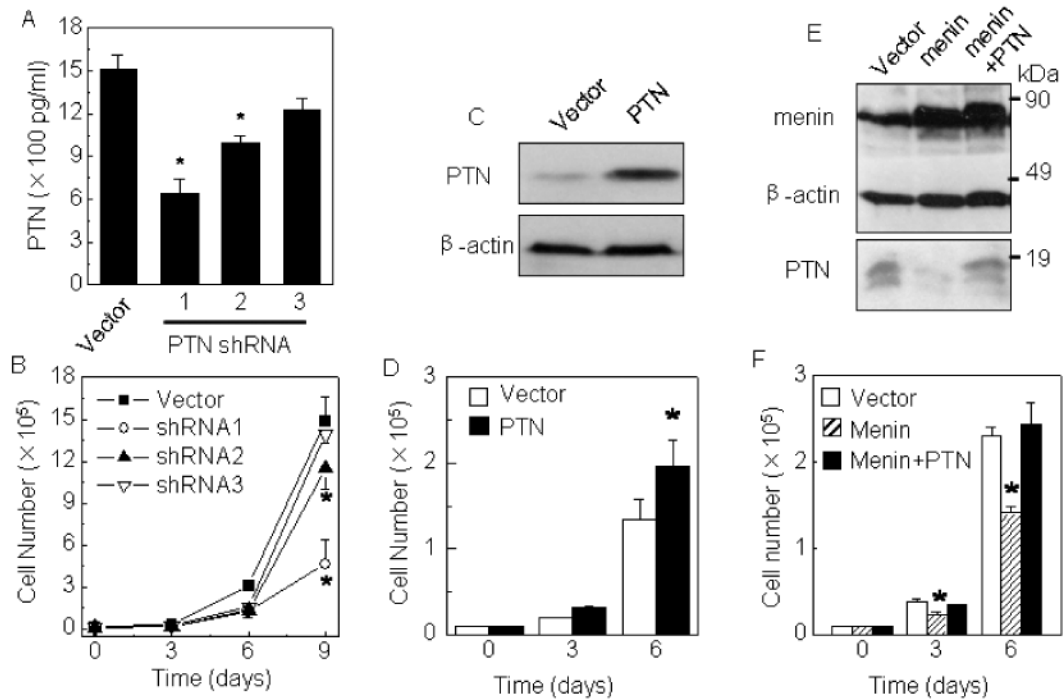
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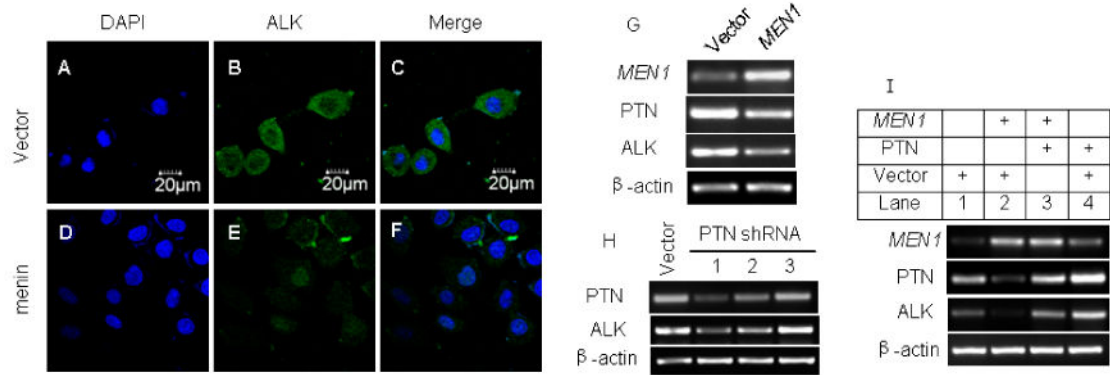


**Figure 1.**

Menin inhibits proliferation of lung cancer cells and PTN expression. **(A)** The growth curves of A549 cells transfected with either pMX-puro or pMX-menin. A549 cells were transfected with either empty vector or vector expressing the *MEN1* gene and selected by puromycin. **(B-D)** Down-regulated PTN expression was determined by RT-PCR, Western blot and ELISA, respectively. A549 cells transfected with a vector expressing shRNA against either Luc or menin. **(E)** *MEN1*, PTN and ALK mRNA level were detected by RT-PCR. **(F)** The growth curves of A549 cells with *MEN1* knockdown. **(G-H)** Impact of menin silencing on PTN expression is determined by Western blotting and ELISA, respectively. **(I)** A549 cells were transfected with empty vector, wild-type menin, point mutation A242V or L22R, and selected by puromycin. The *MEN1*, PTN and ALK expression were detected by RT-PCR. **(J)** Increased PTN and ALK mRNA levels in *Men1*<sup>-/-</sup> MEFs were detected by real-time qRT-PCR. **(K)** Western blotting detection the impact of menin silencing or expression on PTN expression. \*  $p < 0.05$  vs. control group.

**Figure 2.**

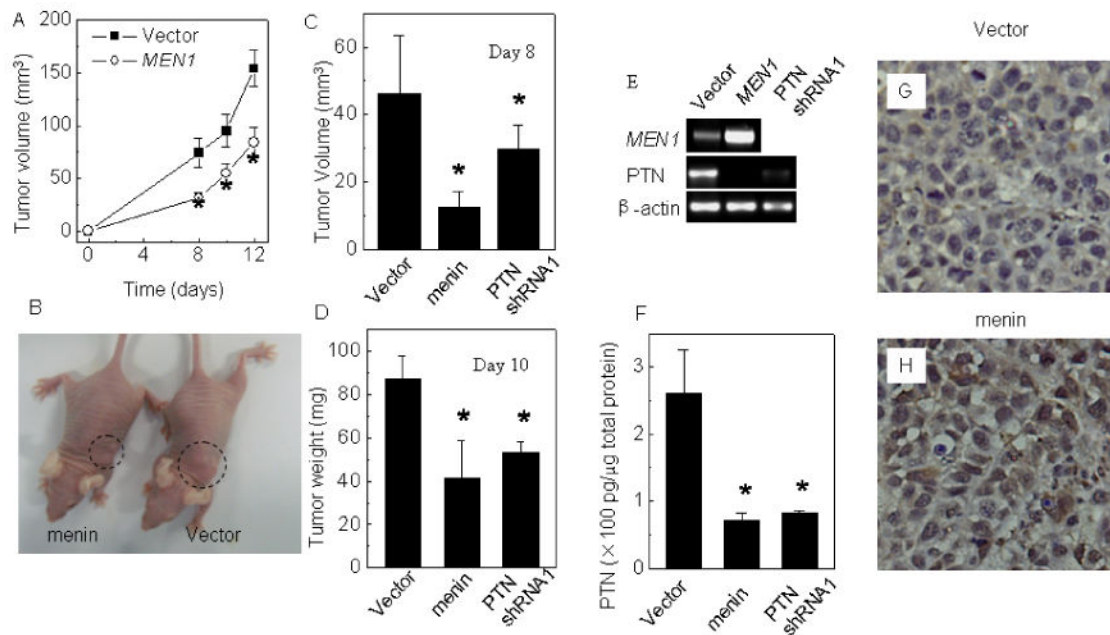
Menin inhibits A549 lung cancer cell proliferation partly through down-regulating PTN expression. **(A)** A549 cells were stably transfected with either empty vector or vector expressing one of three distinct PTN shRNAs. The efficiency of PTN knockdown was determined by measuring the secreted PTN by ELISA. **(B)** The cells generated above were evaluated for their proliferation at indicated on days of culture. **(C)** Growth curve of A549 cells stably transfected with either empty vector (pcDNA3.1) or pcDNA3.1-PTN. **(D)** A549 cells were stably transfected with either vector or vector expressing either menin or PTN, or cotransfected with menin and PTN-expressing constructs. **(E,F)** The over-expression of menin and its effect on down-regulation on PTN. \*  $p < 0.05$  vs. control group.



**Figure 3.**

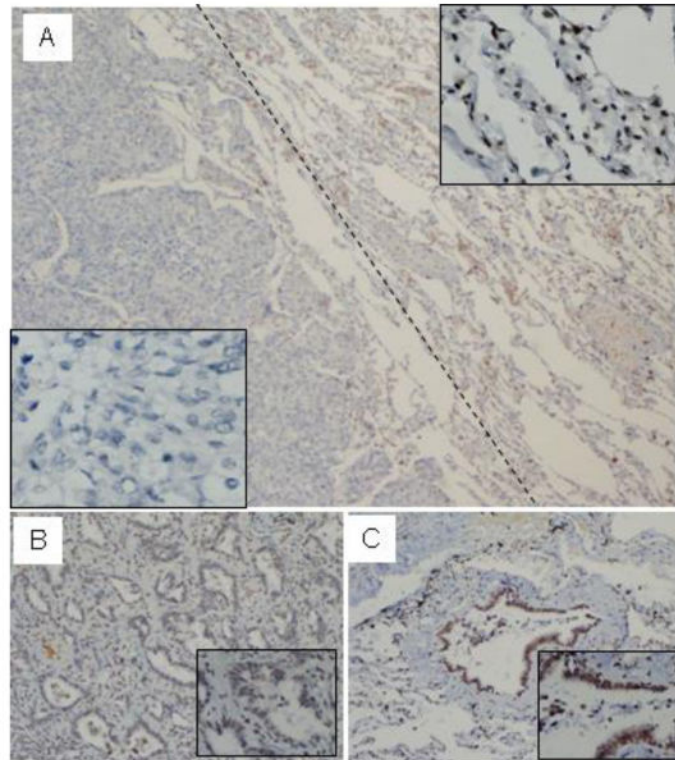
Menin indirectly down-regulates ALK expression by repressing PTN expression. A549 cells were stably transfected with pMX-puro or pMX-menin. (A-F) IF detection of ALK (green), DAPI (blue), and merge in the A549 cells. (G) Over-expression of menin and down-regulation of PTN and ALK expression were detected by RT-PCR. (H) A549 cells stably transfected with either control vector or vector expressing one of three distinct PTN shRNAs were analyzed by RT-PCR for mRNA level. (I) A549 cells were stably transfected with either control vector (30 $\mu$ g) or construct expressing either menin (*MEN1* 15 $\mu$ g+ vector 15 $\mu$ g) or PTN (PTN 15 $\mu$ g+ vector 15 $\mu$ g) or co-transfected with menin and PTN-expressing constructs (*MEN1* 15 $\mu$ g+ PTN 15 $\mu$ g). The resulting cells were processed to determine the menin, PTN and ALK mRNA level by RT-PCR.



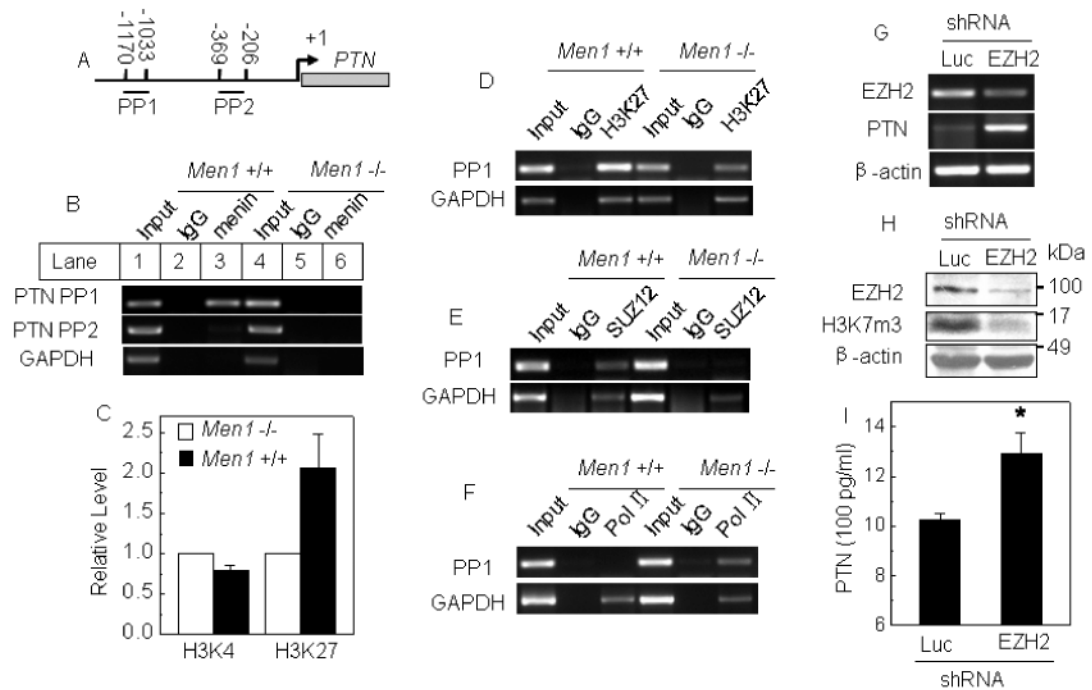


**Figure 4.**

Menin over-expression significantly inhibits growth of A549 cell-derived tumor and PTN expression *in vivo*. (A) Menin-expressing vector or empty vector were stably transfected into A549 cells and injected subcutaneously into nude mice, and tumor formation was examined day 8-post transplantation. The tumor growth rate of recipient mice (n=10 per group) is shown. (B) A representative pair of nude mice transplanted with either menin-expressing or vector control A549 cells (day 12). (C) A549 cells were transplanted to nude mice and when visible tumors were formed (day 6 post-transplantation), control vector or vector expressing menin cDNA or PTN shRNA1, which were treated with PEI, were injected into the tumor mass. The tumor volume in mice was measured on day 8 after transplantation. (D) The recipient mice were sacrificed on day 10 post transplantation; the weight of tumors was measured. (E) Both ectopic menin expression and PTN knockdown reduced PTN expression at the mRNA level, as determined by qRT-PCR. (F) PTN protein from menin cDNA or PTN shRNA-transfected tumors was detected by ELISA assay. (G, H) Immunohistochemical staining of the above various tumors with antibodies against menin. Original magnification, ×400. \*  $p < 0.05$  vs. control group.



**Figure 5.** Menin expression is reduced in certain primary human lung cancer cells. Sections from paraffin-embedded lung cancer samples were stained with affinity-purified anti-menin antibody for immunohistochemistry staining. **(A)** Menin was easily detectable in the nucleus of the normal alveolar (Right) and in certain tumors (Left), where staining for menin was not detectable or markedly reduced (40 $\times$ ). **(B)** The menin staining was markedly reduced in lung adenocarcinoma, but in normal bronchiolo epithelial cells, the staining for menin was easily detectable, **(C)**, 20 $\times$ ).

**Figure 6.**

Menin binds the *PTN* promoter and increases H3K27 trimethylation. **(A)** A schematic representation of the *PTN* gene loci and amplicons used for ChIP assays. **(B-F)** ChIP assays using one of the antibodies against menin, H3K4m3, H3K27m3, SUZ12, RNA pol II, and control IgG in *Men1*<sup>-/-</sup> and *Men1*<sup>+/+</sup> MEF cells. PCR was carried out using primers for each amplicon. **(G)** A549 cells were stably transfected with vector expressing a shRNA against either Luc or EZH2. EZH2 knockdown and *PTN* expression were determined by RT-PCR. **(H-I)** EZH2 knockdown and total H3K27m3 level were detected by Western blot, and the effect of EZH2 knockdown on secreted *PTN* protein was determined by ELISA.