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ORIGINAL ARTICLE

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

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Menin upregulates 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 Enhancer of Zeste homolog 2 (EZH2)-mediated histone H3 lysine 27 trimethylation (H3K27m3), a negative mark for gene transcription but does not affect histone H3K4 methylation that is usually upregulated 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.
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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, and anaplastic lymphoma kinase (ALK), are often identified in lung adenocarcinoma (Jemal *et al*., 2002; Soda *et al*., 2007; Herbst *et al*., 2008). Pei *et al*. (2007) had reported that non-small cell lung cancers (NSCLC) develop in both $Men1^{+/-}$ and $p18^{-/-}$; $Men1^{+/-}$ mice at a high penetrance. 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 because of a somatic mutation (loss of heterozygocity), indicating *MEN1* as a bona fide tumor suppressor gene in endocrine tumors (Lemos and Thakker, 2008).

Though menin's primary sequence does not show any paralogs, multiple lines of evidence suggest that one of the major functions of menin is to regulate gene transcription (Yokoyama *et al*., 2004; Karnik *et al*., 2005; Milne *et al*., 2005). Menin associates with chromatin and the nuclear matrix and exerts multiple biological functions including regulation of cell proliferation (Jin *et al*., 2003; Schnepp *et al*., 2004, 2006). 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* 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, which contains Enhancer of Zeste homolog 2 (EZH2) and its regulatory protein SUZ12 (Sparmann and van Lohuizen, 2006; Ringrose and Paro, 2007; Schuettengruber *et al*., 2007). 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

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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; Sparmann and van Lohuizen, 2006; Martin and Zhang, 2007). Though menin promotes H3K4 methylation, which is presumably catalyzed partly by MLL at loci of the cell-cycle inhibitors $p18^{Ink4c}$ (p18) and $p27^{Kip1}$ (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 binds the promoter of *PTN* and recruits the 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 through a menin–PcG complex.

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* (Stoica *et al*., 2001; Lu *et al*., 2005), a prevalent model suggests that PTN binds to its cell surface receptor, protein tyrosine phosphatase receptor Z1, inhibiting its phosphatase activity toward another oncogenic kinase, ALK (Meng *et al*., 2000; Stoica *et al*., 2001; Powers *et al*., 2002; Lu *et al*., 2005). 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 (Figure 1a). Ectopic menin expression was confirmed by RT–PCR and western blotting (Figures 1b and c). PTN expression was reduced in A549 cells with ectopic menin expression at the mRNA level (Figure 1b, lane 2), as well as at the intracellular and secreted PTN protein level (Figures 1c and d, respectively). No obvious apoptosis was observed in menin-transfected cells, using Annexin V staining and flow cytometry analysis (Supplementary Figure 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 (Figure 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 (Figure 1f; $P<0.05$). Notably, *MEN1* knockdown also increased mRNA and the intracellular and secreted PTN (Figures 1e, g, and h), but did not affect expression of a PTN paralog, MK (Supplementary Figure 3). Menin also suppresses proliferation of two additional human lung cancer cell lines, NCI-H157 and NCI-H446 (Supplementary Figures 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 (Figure 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 $Men1^{+/+}$ and $Men1^{-/-}$ MEFs. *Men1* excision substantially increased expression of PTN and ALK (Figures 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 (Supplementary Figure 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 whether 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 (Figure 3h) and the secreted PTN in culture medium (Figure 2a), with *PTN* shRNAs 1–2 be 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 (Figure 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.

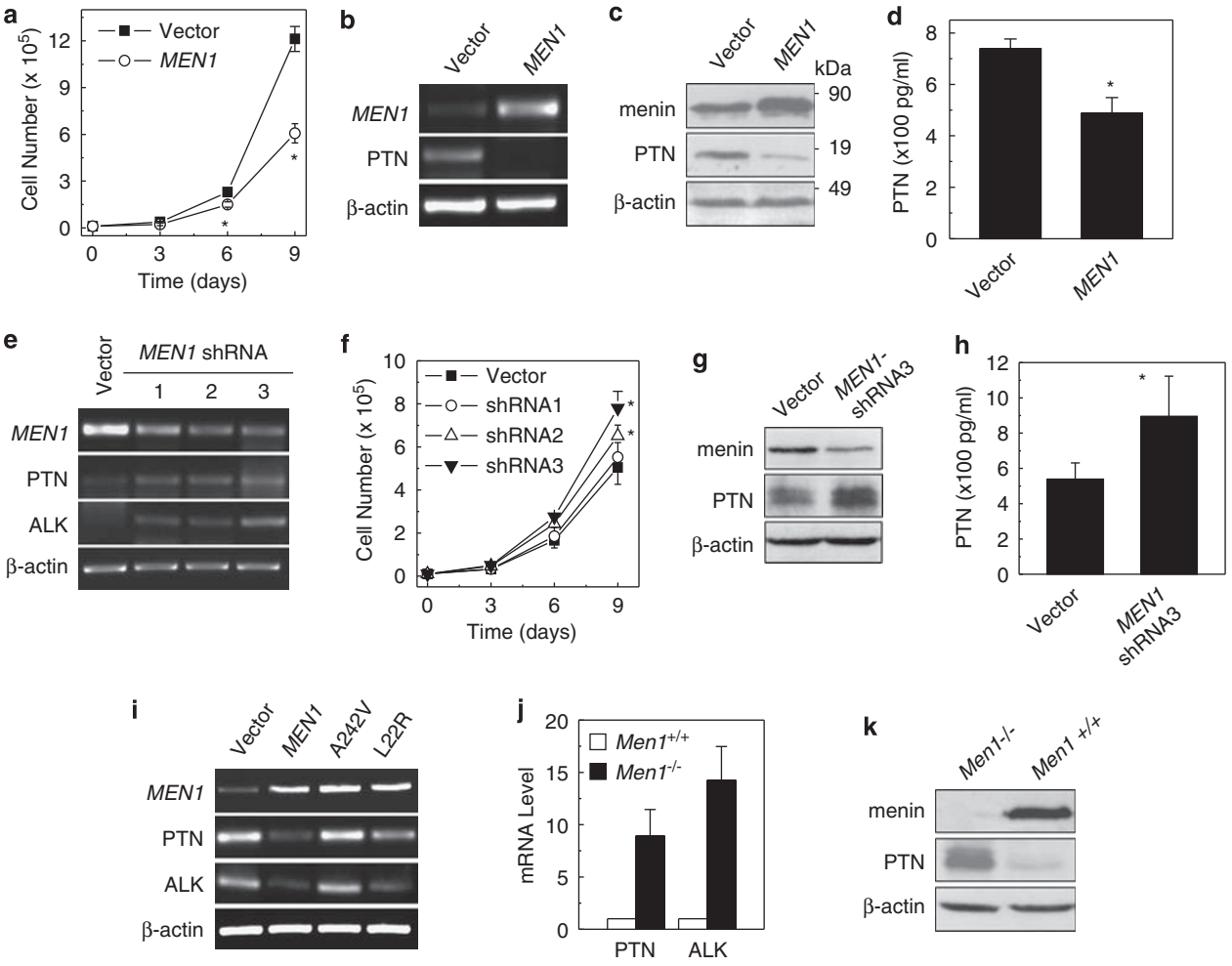

Figure 1 Menin inhibits proliferation of lung cancer cells and PTN expression. (**a**) The growth curves of A549 cells transfected with either pMX-puro or pMX-menin. A549 cells were transfected with either empty vector or vector expressing the *MEN1* gene and selected by puromycin. (**b**–**d**) Downregulated 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*$^{-/-}$ 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.

Consistently, stably PTN-transfected cells (Figure 2c) proliferated ~40% faster than the control vector cells at day 6 of culture (Figure 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 (Figure 2e). As expected, on day 6 of culture, menin reduced proliferation of the cells by over 30% (Figure 2f; $P<0.05$). Notably, co-expression with PTN neutralized menin-induced repression of A549 cell proliferation (Figure 2f). Collectively, these data show 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 upregulated ALK as well as *PTN* in A549 cells (Figure 1e), but ectopic menin expression reduced ALK expression (Figures 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 (Figure 3h). Conversely, *PTN* shRNA3 that failed to knock down PTN also failed to reduce the *ALK* mRNA level (Figure 3h). Though it is possible that menin represses PTN and ALK separately, it could not be ruled out that PTN also upregulates ALK expression. Thus, we ectopically expressed menin and/or PTN to determine their effect

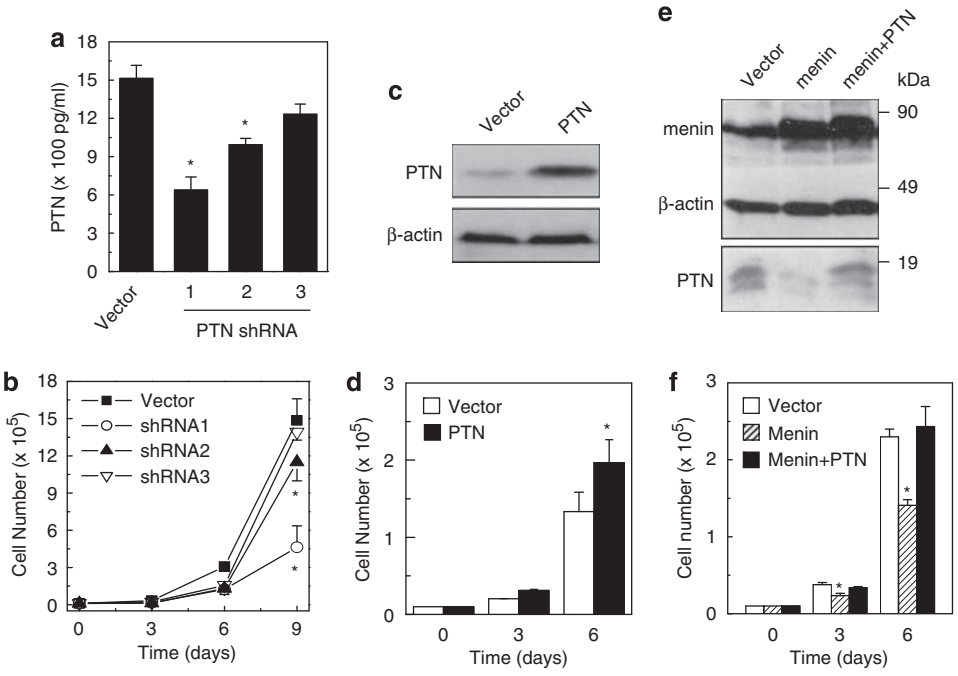

Figure 2 Menin inhibits A549 lung cancer cell proliferation partly through downregulating PTN expression. (**a**) A549 cells were stably transfected with either empty vector or vector expressing one of three distinct PTN shRNAs. The efficiency of PTN knockdown was determined by measuring the secreted 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 co-transfected with menin and PTN-expressing constructs. (**e**, **f**) The overexpression of menin and its effect on downregulation on PTN. $*P<0.05$ vs control group.

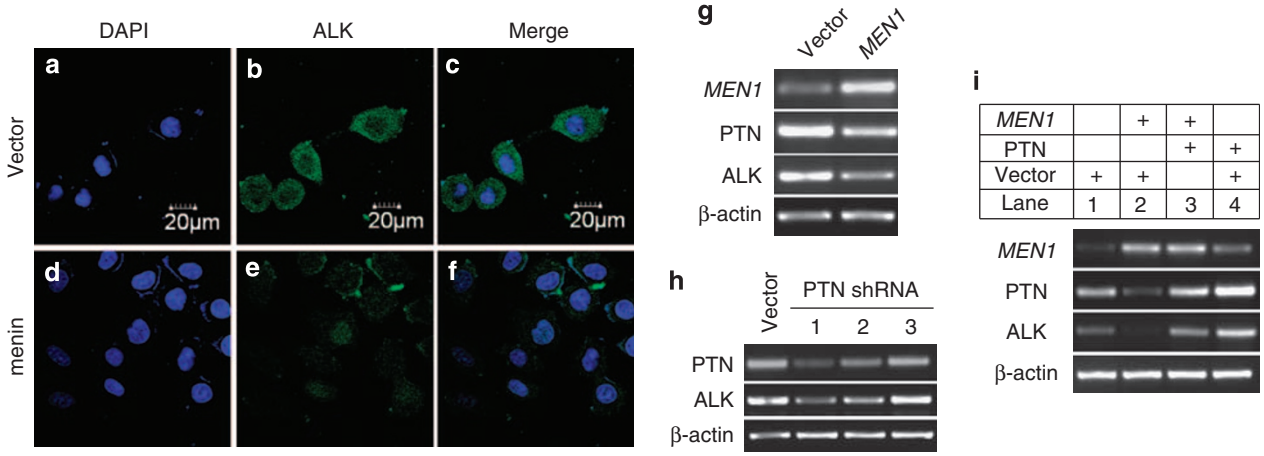

Figure 3 Menin indirectly downregulates ALK expression by repressing PTN expression. A549 cells were stably transfected with pMX-puro or pMX-menin. (**a**–**f**) Immunofluorescent detection of ALK (green), DAPI (blue), and merge in the A549 cells. (**g**) Overexpression of menin and downregulation of 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 μg) or construct expressing either menin (*MEN1* 15 μg + vector 15 μg) or PTN (PTN 15 μg + vector 15 μg) or co-transfected with menin and PTN-expressing constructs (*MEN1* 15 μg + PTN 15 μg). The resulting cells were processed to determine the menin, PTN, and ALK mRNA level by RT–PCR.

on the *ALK* mRNA level in A549 cells. Notably, PTN expression abrogated menin-induced reduction in the *ALK* mRNA level (Figure 3i, lane 3), and ectopic PTN expression alone also increased ALK expression (Figure 3i, lane 4). Together, these findings argue that PTN upregulates 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 that were subcutaneously transplanted into nude mice ($n=10$ per group). The size of the solid tumor was measured after various periods of time after transplantation. Menin expression resulted in smaller tumors in the nude mice after transplantation (Figures 4a and b; $P<0.05$). To determine whether 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 (Figure 4c; $P<0.05$, respectively) and weight (Figure 4d; $P<0.05$) at days 8 and 10 post transplantation, respectively. Both ectopic menin expression and PTN knockdown reduced PTN expression at the mRNA level (Figure 4e) and protein level, as shown by enzyme-linked immunosorbent assay (ELISA) (Figure 4f). Ectopic menin expression in the tumor was confirmed by immunohistochemistry staining (Figures 4g 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 whether 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 immunohistochemistry staining. Menin was easily detectable in the nucleus of the normal alveolar epithelial cells (Figures 5a right and c). In contrast, in certain tumors, staining for menin was

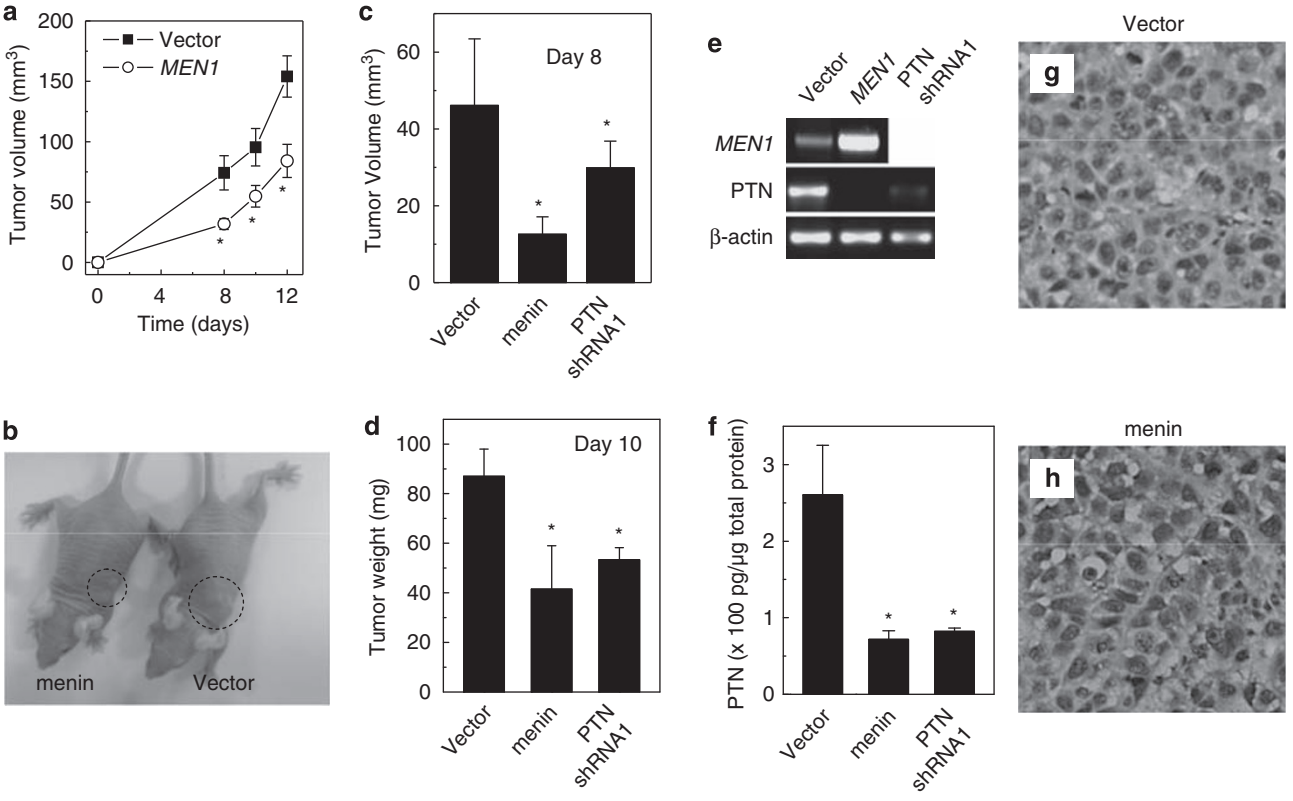

Figure 4 Menin overexpression significantly inhibits growth of A549 cell-derived tumor and PTN expression *in vivo*. (**a**) Menin-expressing vector or empty vector were stably transfected into A549 cells and injected subcutaneously into nude mice, and tumor formation was examined on day 8 post transplantation. 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 killed 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, $\times 400$. $^*P<0.05$ vs control group.

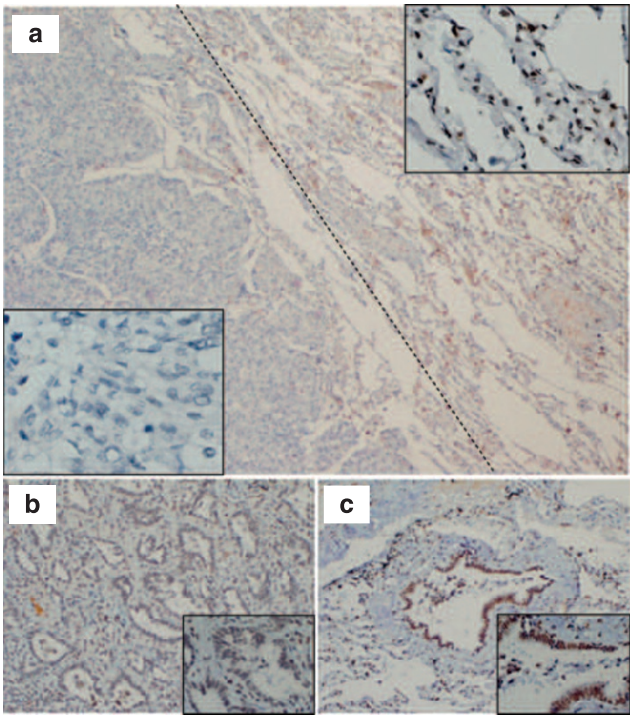

Figure 5 Menin expression is reduced in certain primary human lung cancer cells. Sections from paraffin-embedded lung cancer samples were stained with affinity-purified anti-menin antibody for immunohistochemistry staining. (**a**) Menin was easily detectable in the nucleus of the normal alveolar (right) and in certain tumors (left), where staining for menin was not detectable or markedly reduced (40 ×). (**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 ×).

much weaker or undetectable, as compared with that in the adjacent normal epithelial cells (Figure 5a, left). In the same section from a lung cancer sample, in normal bronchiolo epithelial cells abundant menin was detected in the nucleus (Figure 5c), but was barely visible in the nucleus of the cancer cells (Figure 5b). It is also obvious that the nucleus of the cancer cells tended to be larger than that in the normal epithelial cells.

Menin expression in tumors, as compared with 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 (Supplementary Table 2). On the other hand, in nine 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%) (Supplementary 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 in lymph node metastasis in menin-expressing adenocarcinoma. Although 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 chromatin immunoprecipitation (ChIP) assays with *Men1*$^{-/-}$ and *Men1*$^{+/+}$ MEFs, using an anti-menin antibody. We designed two primers used for ChIP assays at *PTN* promoter loci (Figure 6a). ChIP assays showed that menin bounds to the *PTN* locus (PP1) in the *Men1*$^{+/+}$ cells but not in *Men1*$^{-/-}$ cells (Figure 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 (Figure 6c) or acetyl–histone H3 (Supplementary Figure 6A) at the *PTN* locus. We also failed to detect histone deacetylase 1 at the *PTN* locus (Supplementary Figure 6B). On the other hand, histone H3 lysine 27 trimethylation (H3K27m3), which is catalyzed by EZH2 from transcription-repressing *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 whether menin affects H3K27 trimethylation (H3K4m3). Notably, H3K27m3 was reduced in *Men1*-null cells at the *PTN* locus, but not at the *GAPDH* locus (Figures 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 bounds the *PTN* locus in MEFs (Figure 6e, lane 3). Notably, loss of menin abrogated SUZ12 binding to the *PTN* locus (Figure 6e, lane 6), whereas the SUZ12 and EZH2 protein level in cells was not affected by menin excision (Supplementary Figure 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 (Figure 6f, lane 6). EZH2 knockdown reduced the EZH2 mRNA and protein levels (Figures 6g and h) but increased the *PTN* mRNA level (Figure 6g) and secreted PTN for ~30% (Figure 6i). As EZH2-mediated H3K27 represses gene transcription, these findings show that menin crosstalks 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

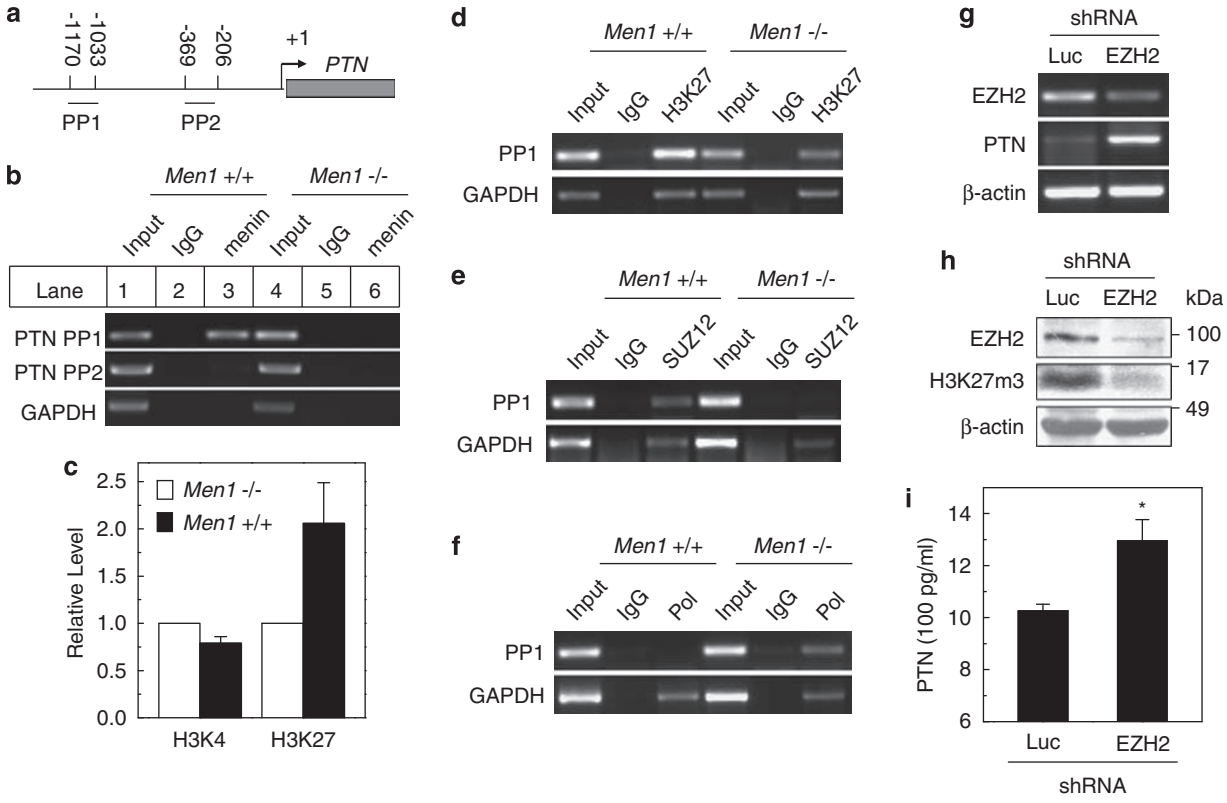

Figure 6 Menin binds the *PTN* promoter and increases H3K27 trimethylation. (**a**) A schematic representation of the *PTN* gene loci and amplicons used for ChIP assays. (**b**–**f**) ChIP assays using one of the antibodies against menin, H3K4m3, H3K27m3, SUZ12, RNA pol II, and control IgG in *Men1*$^{-/-}$ and *Men1*$^{+/+}$ MEF cells. PCR was carried out using primers for each amplicon. (**g**) A549 cells were stably transfected with vector expressing an 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, $*P<0.05$.

functionally interacting with the PcG proteins in H3K27m3 as shown in Figure 6h. These findings have unraveled a previously unrecognized connection between menin and PcG 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 epidermal growth factor receptor facilitate the development of lung adenocarcinoma (Jemal *et al*., 2002; Soda *et al*., 2007; Herbst *et al*., 2008), little is known about how mutations in tumor suppressor menin affect development of lung cancer (Pei *et al*., 2007). Menin suppresses endocrine tumors partly through enhancing MLL-mediated H3K4 methylation and upregulation of p18 and p27 transcription (Karnik *et al*., 2005), but it is poorly understood how menin suppresses other types of tumors. Our findings demonstrate that menin potently represses proliferation of the lung cancer cells and growth of the cancer cell-derived tumors at least partly through epigenetically repressing *PTN* transcription through PcG gene-mediated H3K27 methylation. This mechanism is quite distinct from menin suppression of endocrine tumors through upregulating a positive histone mark, H3K4 methylation (Karnik *et al*., 2005). PTN, a heparin-binding growth factor that binds its cell surface receptors protein tyrosine phosphatase receptor Z1 and ALK, is a major effector of menin in repressing human lung adenocarcinoma cells. Our results have linked the tumor suppressing function of menin to the PTN–ALK pathway that is known to be active in certain lung adenocarcinomas (Jager *et al*., 2002). This link is mediated partly through regulating EZH2-mediated H3K27 and gene silencing.

The relationship between menin and repression of PTN is disrupted in certain lung adenocarcinoma cells, as menin expression is abrogated or substantially diminished in certain primary lung cancer cells. This conclusion is supported by several lines of evidence. First, menin potently inhibited proliferation of several lung cancer cell lines and PTN expression. Second, ectopic PTN expression increased cell proliferation, whereas PTN knockdown reduced proliferation of the cancer cells as well as growth of lung cancer xenograft. Third, ectopic expression of PTN abrogated the menin-mediated inhibition of the cancer cells. Fourth, menin expression was markedly reduced in 23% of

primary human lung adenocarcinomas. PTN possesses a pleiotrophic role in regulating neurite growth, mitogenesis, cell migration, and angiogenesis (Choudhuri *et al*., 1997). PTN is highly expressed in a number of cancers including lung cancer, and PTN concentrations in serum were over 10-fold higher in lung cancer patients as compared with the control group (Jager *et al*., 2002). Moreover, *Men1* mutation coupled with mutation in the *p18* gene leads to the development of NSCLC in certain mice (Pei *et al*., 2007). Thus, our findings are consistent with the notion that menin-mediated repression of PTN contributes to repression of growth of lung cancer cells and in particular, lung adenocarcinoma cells.

Menin seems to preferentially suppress development of a subtype of NSCLC, lung adenocarcinoma, because reduction in menin expression was detected in more primary lung adenocarinomas but not in squamous cell cancers (Supplementary Table 2). As the epidermal growth factor receptor and K-Ras pathway is often excessively upregulated in adenocarcinomas but not in squamous cell cancers (Herbst *et al*., 2008), it is possible that one of menin's functions is to repress the Ras pathway, which could in part be upregulated by PTN or epidermal growth factor receptor pathways. From the cultured A549 cells and human lung adenocarcinoma cell-derived xenograft, we have observed tight menin-mediated repression of PTN and the crucial role of PTN in proliferation of cancer cells. These results strongly suggest that the menin and PTN pathway has a crucial function in suppressing lung adenocarcinoma. Further work still remains to elucidate how menin preferentially suppresses lung adenocarcinoma.

Our findings have uncovered the menin, PTN, and ALK pathway in controlling proliferation of lung cancer cells, and this pathway may serve as a target for therapy against lung adenocarcinoma. Though still lacking of a well-established and unified model to account for PTN signaling and tumor promotion, an attractive model suggests that PTN enhances cell proliferation and cell migration by binding to its cell surface receptor protein tyrosine phosphatase receptor Z1 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 $\sim$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 that is 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.

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, USA). A549 and NCI-H446 cells were cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) or RPMI 1640 (Hyclone), respectively, and supplemented with 10% (v/v) fetal bovine serum (Hyclone), and 1 × Penicillin–Streptomycin (100 U/ml–100 μg/ml) (Invitrogen, Carlsbad CA, USA). NCI-H157 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate (Hyclone), 10 mM Hepes (Hyclone), and 1 × Penicillin–Streptomycin. The $Men1^{+/+}$ and $Men1^{-/-}$ MEFs were described earlier (Schnepp *et al*., 2006) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1 × Penicillin–Streptomycin, 2 mM L-glutamine (Invitrogen), and 0.1 mM non-essential amino acids (Hyclone).

Immunohistochemistry and immunofluorescent staining

Immunohistochemistry and immunofluorescent detection were 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 (Supplementary Figure 1), anti-PTN (Abnova, Walnut, CA, USA) or anti-ALK (Cell Signaling, Danvers, MA, USA) antibodies. Nuclei were counterstained with DAPI, and the stained cells were analyzed and photographed under a confocal microscope (OLYMPUS FV1000, × 400).

Western blotting and 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, 5 μg/ml leupeptin, 1 μg/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, TX, USA), anti-PTN (Abnova), or anti-β-actin antibodies (Santa Cruz

biotechnology, Santa Cruz, CA, USA). ELISA for PTN was performed by using a PTN ELISA kit (Uscn Life Science & Technology, Wuhan, China), according to the manufacturer's instructions.

ChIP assay

ChIP assays were performed as described with certain modifications (Chen *et al*., 2006). Briefly, 1×10^6 *Men1*$^{-/-}$ and *Men1*$^{+/+}$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, MA, 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), 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 sequences were shown in Supplementary Table 1.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)