Menin encoded by the multiple endocrine neoplasia type 1 (MEN1) gene is associated with chromatin and the nuclear matrix and exerts multiple biological functions including regulation of cell proliferation and adhesion. Men1 mutations increase the likelihood of lung cancer development in mice. Menin expression is reduced in certain human non-small cell lung cancer cells, and reduction of menin is closely correlated with increased lung cancer metastasis to lymph nodes. However, it is poorly understood whether menin affects migration of lung cancer cells. In this study, we show that menin-regulated A549 lung cancer cell migration, which was mediated by growth factor pleiotrophin (PTN) and its cell surface receptor, protein tyrosine phosphatase beta/zeta (RPTP $\beta/\zeta$). Ectopic menin expression significantly repressed PTN transcription, but indirectly inhibited RPTP $\beta/\zeta$ expression through repressing PTN expression. Further studies revealed that menin-regulated cell migration through PTN/RPTP $\beta/\zeta$, in conjunction with integrin $\alpha$, $\beta$, focal adhesion kinase, phosphatidylinositol 3-kinase and phosphorylated extracellular signal regulated kinase 1/2. These findings provide mechanistic insights into the molecular basis for menin/PTN-mediated regulation of A549 lung cancer cell migration.

**Keywords:** lung cancer; migration; Men1; pleiotrophin; RPTP $\beta/\zeta$

**Introduction**

Menin encoded by the multiple endocrine neoplasia type 1 (MEN1) gene is a nuclear protein, which is mutated in patients with an inherited tumor syndrome, MEN1 (Chandrasekharappa et al., 1997). In 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). As menin does not show an obvious homology to any known protein motifs, it has been challenging to elucidate how menin acts as a tumor suppressor.

Recently, multiple lines of evidence suggest that menin is associated with chromatin and nuclear matrix and exerts multiple biological functions including regulation of cell proliferation, apoptosis and DNA repair (Gao et al., 2008). These diverse menin functions may be largely attributed to the crucial role of menin as a scaffold protein, which can alter histone tail modifications and epigenetic status of its target genes to control their transcription (Wu and Hua, 2008). For instance, menin represses proliferation of pancreatic islet cells and mouse embryonic fibroblasts (MEFs) partially through upregulating transcription of p18$^{ink4c}$ (p18) and p27$^{kip1}$ (p27), potent inhibitors of cyclin-dependent kinases (Schnepf et al., 2006). In this regard, menin interacts with mixed lineage leukemia proteins (Karnik et al., 2005; Milne et al., 2005), histone H3 methyltransferases that catalyze histone H3 lysine 4 methylation (H3K4me3) with their highly conserved SET domain (Milne et al., 2002). Menin and mixed lineage leukemia bind the p18 and p27 gene loci, enhancing H3K4me3 at these loci, to promote their transcription in endocrine cells, leading to repression of cell proliferation (Karnik et al., 2005; Milne et al., 2005).

Nonetheless, it is still poorly understood whether menin influences development of non-endocrine tumors, such as lung cancer. Lung carcinoids occur sporadically and are also detectable infrequently in patients with MEN1 syndrome (Debelenko et al., 1997, 2000; Petzmann et al., 2001). Non-small cell lung cancers develop in both Men1$^{+/+}$ and p18$^{-/-}$;Men1$^{+/+}$ mice, but with a much higher penetrance in mice with the latter genotype (Pei et al., 2007). Further study indicated that p18$^{-/-}$ or Men1$^{+/+}$ increased pRb phosphorylation at cyclin-dependent kinase 4/6 site Ser608, and more obviously, increased pRb was detected in tumor cells from p18$^{-/-}$;Men1$^{+/+}$ mice (Pei et al., 2007). These observations suggest a role for menin in suppressing lung carcinogenesis through regulating cell proliferation. Consistent with this notion, we recently found that menin inhibits proliferation of several human lung cancer cell lines and growth of lung cancer xenograft (Gao et al., 2009). Consistent with a role of menin in suppressing development of lung cancer, in 23% of...
primary human lung adenocarcinomas, menin expression is significantly reduced. Moreover, a reduction in menin expression is also closely correlated with lymph node metastasis of the cancer (Gao et al., 2009). As cancer metastasis involves cell migration and invasion, these findings raise the possibility that menin may affect cell migration.

Our previous work suggests that menin-mediated suppression of lung cancer is partly through polycomb gene (PcG)-dependent repression of pleiotrophin (PTN) expression. PTN is a heparin-binding growth factor that is highly expressed in certain solid cancers, such as in breast and lung cancers (Jager et al., 2002; Perez-Pinera et al., 2007). PTN activates its cell surface receptors, regulating multiple functions including cell adhesion, cell migration, cell proliferation and cytoskeletal stability (Lu et al., 2005; Pariser et al., 2005; Duces et al., 2008). These studies raise the possibility that downregulated menin expression facilitates the development of lung cancer not only by repressing lung cancer cell proliferation, but also by altering lung cancer cell migration and metastasis. However, the precise mechanisms whereby menin regulates cell migration are not well understood. We have previously shown that menin interacts with a scaffold protein, IQ motif containing GTPase activating protein 1, which links the cytoskeleton to cell adhesion and migration in endocrine cells (Yan et al., 2009). However, it remains poorly understood as to how menin influences migration of non-endocrine tumors, such as lung cancer cells. In this study, we show that menin regulates A549 lung cancer cell migration through downregulating expression of PTN and its cell surface receptor, protein tyrosine phosphatase beta/zeta (RPTP b/ζ) (Perez-Pinera et al., 2007). PTN-RPTP b/ζ expression and activation of focal adhesion kinase (FAK) and phosphoinositide 3-kinase (PI3K) were repressed by menin, and menin-repressed cell migration involved inhibition of PI3K-extracellular signal regulated kinase (ERK)1/2 signaling, which is crucial for cell migration. Together, these results suggest a novel menin–PTN pathway that controls cell migration in A549 cells.

Results

Menin inhibits cell migration and represses PTN/RPTP b/ζ expression

Previously, we showed that menin expression was reduced in lung adenocarcinomas, and menin reduction was highly correlated with increased lymph node metastasis (Gao et al., 2009). As cancer metastasis usually involves enhanced cell migration, we sought to determine whether one of the functions of menin is involved in controlling cell migration. To this end, we performed a modified transwell chamber assay to evaluate the effect of loss of menin on migration of Men1+/+ and Men1−/− MEFs. The results indicate that Men1 ablation significantly increased cell migration (Figure 1a, P<0.05; Supplementary Figure 1a). Next, we evaluated the effect of loss of menin on PTN and RPTP b/ζ expression in Men1+/+ and Men1−/− MEFs. Men1 excision (Supplementary Figure 1c) increased mRNA expression of RPTP b/ζ (6.5-fold), as detected by quantitative reverse transcriptase–PCR (Figure 1d) and the downregulated PTN mRNA expression (eightfold) by menin has been reported early (Gao et al., 2009). Similar results were also obtained by detection of PTN and RPTP b/ζ using regular reverse transcriptase–PCR (Supplementary Figure 1d). Increased PTN and RPTP b/ζ protein levels were detected in asynchronous Men1 null MEFs, as compared with the Men1-expressing cells, using western blotting (Figure 1e).

We further determined PTN and RPTP b/ζ expression in the Men1+/+ and Men1−/− MEFs in an exponential growth phase, using immunofluorescence staining. As illustrated in Supplementary Figure 2, the Men1-null cells failed to show menin staining (Supplementary Figures 2b and j), but showed an obvious cytoplasmic staining for PTN and RPTP b/ζ (Supplementary Figures 2c and k). Conversely, menin-expressing MEFs showed a clear staining for nuclear menin (Supplementary Figures 2f and n), but a reduction of PTN and RPTP b/ζ staining (Supplementary Figures 2g and o). Together, these results indicate a pivotal role for menin in downregulating both PTN and its receptor, RPTP b/ζ expression. To further confirm these results, we generated three independent pairs of MEFs to analyze the intrinsic biological effect of menin on cell phenotype and target gene expression. Similar results from one of the three pairs of MEFs were presented in Supplementary Figures 3a and b.

Menin regulates cell migration partly through PTN and RPTP b/ζ signaling

Next, we examined whether menin regulates PTN and RPTP b/ζ expression in A549 adenocarcinoma cells.
Our previous work has shown that ectopic menin expression reduces PTN expression (Gao et al., 2009). A549 cells were stably transfected with either control vector or a construct-expressing MEN1 (pMX-menin). Ectopic menin expression was confirmed by western blotting, and as expected, menin overexpression reduced the protein level of RPTPβ/ζ (Figure 2a). Further, MEN1 knockdown by shRNA also increased the protein level of RPTPβ/ζ in A549 cells (Supplementary Figure 4). We also examined the effect of MEN1 point mutations, A242V and L22R, which were identified from inherited MEN1 patients (Pannett and Thakker, 1999) on RPTPβ/ζ expression; we found that A242V and L22R lost or partially lost the ability to repress RPTPβ/ζ expression (Figure 2b). Correlated with this result, both wild-type menin and L22R mutant overexpression significantly reduced A549 cell migration (P<0.05, respectively), however, A242V was unable to reduce RPTPβ/ζ expression and did not significantly repress cell migration (Figure 2c). Next, we used an alternative approach, the scratch wound assay, to compare the motility of mock versus MEN1-overexpressed A549 cells. The extent of wound closure in control cells within 4 days of wounding was much higher than that in ectopically MEN1-expressed A549 cells (Figure 2d). The dramatic difference between these two cells reinforces the notion that menin normally represses cell migration. Next, another pair of stable menin overexpressed A549 cell lines was established by using PLNCX2 retrovirus system, and similar results on the role of menin in regulating PTN/RPTPβ/ζ expression and cell migration were shown in Supplementary Figures 5a and b. In addition, another human nonsmall cell lung cancer cell line NCI-H157 was used to confirm the above results. It is noteworthy that ectopic menin expression not only inhibited PTN/RPTPβ/ζ expression, but also repressed NCI-H157 cell migration (Supplementary Figures 5c and d). We further tested whether PTN/RPTPβ/ζ is required for menin-repressed A549 cell migration. Three distinct RPTPβ/ζ shRNAs were transfected into A549 cells, and RPTPβ/ζ shRNA3 reduced the RPTPβ/ζ protein level effectively (Figure 2e). It is noteworthy that RPTPβ/ζ knockdown by shRNA3 reduced migration of A549 cells (Figure 2f). Furthermore, PTN knockdown effectively reduced intracellular PTN expression (Figure 2g), concomitant with reduced A549 cell migration (Figure 2h). Together, these data indicate that menin inhibits A549 cell migration at least partly through repressing expression of PTN and RPTPβ/ζ.
Menin indirectly regulates RPTP β/ζ expression through PTN

As menin represses PTN transcription partly through PcG-mediated H3K27me3 at the PTN locus (Gao et al., 2009), we determined if menin also bound to the RPTP β/ζ locus using chromatin immunoprecipitation (IP) assay. However, we failed to detect menin binding to the RPTP β/ζ promoter region (data not shown). This may suggest that menin regulates RPTP β/ζ transcription through an indirect mechanism. PTN is a natural ligand for RPTP β/ζ, a phosphatase of phosphorylated tyrosine, and PTN signaling suppresses the phosphatase activity of RPTP β/ζ in reducing steady-state of tyrosine phosphorylation of downstream signaling molecules (Perez-Pinera et al., 2007). Although it is possible that menin represses PTN and RPTP β/ζ separately, we cannot rule out that PTN positively regulates RPTP β/ζ expression.

To further dissect the potential relationship between menin, PTN and RPTP β/ζ, we stably transfected A549 cells with PTN shRNA, and the resulting cells were analyzed for the mRNA and protein levels of PTN and RPTP β/ζ. Interestingly, PTN knockdown diminished the RPTP β/ζ mRNA and protein levels (Figures 3a and b). In a complementary approach, we ectopically expressed menin and/or PTN to determine their effect on the RPTP β/ζ mRNA level. As shown in Figure 3c, menin overexpression reduced both the PTN and RPTP β/ζ mRNA levels (lane 2); notably, ectopic PTN expression abrogated menin-induced reduction of the...
Menin regulates cell migration through PTN signaling

Z-J Feng et al

RPTP β/ζ mRNA level (lane 3). Consistent with this observation, menin overexpression significantly reduced A549 cell migration (Figure 3d, P < 0.05), but PTN abrogated menin-induced reduction of cell migration (Figure 3d). Furthermore, addition of recombinant human PTN to culture medium upregulated the RPTP β/ζ protein level, as shown by western blotting (Figure 3e), as well as A549 cell migration (Figure 3f). Together, these findings strongly suggest that PTN upregulates RPTP β/ζ expression, whereas menin represses RPTP β/ζ expression indirectly through inhibiting PTN expression.

PTN increases interaction between RPTP β/ζ and integrins

Integrin αvβ3 is important for cell signaling, survival and migration particularly during angiogenesis and tumorogenesis (del Pozo et al., 2004). However, the role of integrins for the migration of lung cancer cells has not been analyzed extensively. Integrin αvβ3 is a potential receptor for PTN and is required for PTN-induced endothelial cell migration through RPTP β/ζ (Mikelis et al., 2009). Thus, PTN/RPTP β/ζ signaling may affect lung cancer cell migration in conjunction with integrins. Our results indicate that RPTP β/ζ expression was stimulated by PTN in A549 cells (Figure 3e). It is noteworthy that immunofluorescence staining results indicate that PTN substantially increased expression of both RPTP β/ζ and integrin β3 and colocalization of RPTP β/ζ and integrin β3 in the A549 cells (Figure 4a). This result prompted us to analyze whether αv/β3 associates with RPTP β/ζ. We performed co-IP from extracts of A549 lung cancer cells and found that endogenous integrin αv and β3 were co-immunoprecipitated with endogenous RPTP β/ζ (Figure 4b). As a control, IgG failed to pull down αv and β3 (Figure 4b). The interaction between αv and RPTP β/ζ was further confirmed by IP of endogenous RPTP β/ζ with αv antibody (Figure 4c). In addition, interaction between αv and β3 was also detected in A549 cells (Figures 4c and d). We further examined the effect of menin on αv and β3 expression in A549 cells and in Men1+/− and Men1−/− MEFs. Western blotting revealed that menin overexpression did not affect expression of either αv or β3 in A549 cells (Supplementary Figure 6a) and similar results were obtained in Men1 null MEFs (Supplementary Figure 6b), suggesting that PTN transiently increases the integrin expression and their colocalization with RPTP β/ζ. Overall, the results above indicate that PTN stimulation increases expression of both RPTP β/ζ and integrins, as well as their interaction.

Menin inhibits activation of FAK, PI3K and ERK1/2, which were crucial for cell migration

RPTP β/ζ is an important regulator in the reciprocal control of the steady-state tyrosine phosphorylation levels of β-catenin by tyrosine kinases and phosphatases (Meng et al., 2000). β-Catenin acts as a key factor in the E-cadherin-mediated cell–cell adhesion (Nelson and Nusse, 2004). To elucidate cell signaling underlying menin/PTN-regulated cell migration, we tested if the menin-regulated cell migration requires β-catenin signaling. However, no substantial difference in expression of β-catenin or its Tyr 142 phosphorylation was detected in both A549 and MEFs (Supplementary Figures 6a and b), raising the possibility that other key factors link menin/PTN signaling to cell migration. FAK, a protein tyrosine kinase that is recruited at an early stage to focal

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**Figure 3** MEN1 indirectly regulates RPTP β/ζ expression through PTN in A549 cells. (a, b) A549 cells were transfected with PTN shRNA, and the resulting cells were processed to determine PTN and RPTP β/ζ mRNA and protein levels using reverse transcriptase (RT)-PCR and western blotting, respectively. (c) A549 cells were transfected with either control vector (30 μg) or construct expressing either MEN1 (MEN1 15 μg + vector 15 μg), or co-transfected with MEN1 and PTN-expressing constructs (MEN1 15 μg + PTN 15 μg). The resulting cells were processed to determine menin, PTN and RPTP β/ζ mRNA levels by RT-PCR. (d) The resulting cells were monitored for their migration, N = 3, *P < 0.05 vs control. (e) recombinant human PTN (rhPTN) (100 ng/ml) increased RPTP β/ζ expression and migration of A549 cells.

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**Oncogene**
adhesions, mediates many downstream adhesion responses, including activation of the p85-subunit of PI3K (Baillat et al., 2008). To examine whether menin affects FAK, we evaluated the effect of loss of menin on expression of FAK in Men1+/+ and Men1−/− MEFs. Men1 excision did not affect the total amount of FAK, but substantially increased the level of its Tyr 397-phosphorylated form (Figure 5a). We further determined the effect of menin on PI3K and ERK1/2, other downstream effectors of FAK (Baillat et al., 2008), in the MEFs. The results indicate that Men1 excision increased expression and consequently, phosphorylation (Tyr458) of PI3K, as well as ERK1/2 phosphorylation (Thr202/Tyr204) (Figure 5a). Indeed, similar results on menin’s role in reducing the phosphorylated forms of FAK and ERK1/2 were obtained in menin over-expressed A549 cells (Figure 5b). Consistently, the menin-null MEFs showed obvious cytoplasmic staining for pERK1/2, while menin-expressing MEFs showed much weaker staining for pERK1/2 (Supplementary Figure 7). Interestingly, the Cdc42-GTPase and Rac1-GTPase activity were transiently induced by PTN at 10–30 min (Figure 5d), similar to the induction of RPTPβζ and integrins by PTN (Figure 4a). As menin inhibited expression of PTN, as well as phosphorylation of FAK and ERK1/2, but did not affect Cdc42 and Rac1 activities, it is likely that menin-mediated repression of PTN leads to relatively long-lasting repression of the FAK-ERK1/2, but only transient repression of the Rho family of the GTPases in regulating cell migration.

Menin inhibits FAK-ERK activation through PTN/RPTPβζ and partly represses migration through inhibiting ERK1/2 phosphorylation

It is unclear how menin regulates FAK activation. We examined whether RPTPβζ interacts with FAK in A549 cells, but failed to observe interaction between them using co-IP. It has been reported that integrin–FAK interaction may serve as a downstream effector of PTN (Mikelis et al., 2009), and integrin αζ/β3 directly interacts with RPTP α or RPTP βζ, which is essential for integrin β3 activation (von Wichert et al., 2003; Mikelis et al., 2009). FAK is itself regulated by various
mechanisms, including tyrosine phosphorylation, serine/threonine phosphorylation and protein–protein interactions (McLean et al., 2005). Therefore, we tested if downregulation of FAK phosphorylation is mediated by menin through downregulating PTN/RPTP β/ζ expression. To this end, serum-starved A549 cells were stimulated by addition of recombinant human PTN and allowed to progress for various periods of time before analysis. The results indicate that phosphorylation of FAK was obviously increased after 15 min of treatment with PTN, reached a peak between 30 and 60 min after the treatment, but went down after 120 min (Figure 6a). In contrast, phosphorylation of ERK1/2 was abruptly increased 15 min after exposure to PTN, but rapidly reduced 30 min after treatment with PTN (Figure 6a). Consistently, knockdown of PTN or RPTP β/ζ reduced basal level of phosphorylation of FAK and ERK1/2 (Figure 6b). These results suggest that PTN can increase phosphorylation (or activation) of both FAK and ERK1/2; however, as the kinetics of phosphorylation of ERK1/2 and FAK is distinct, they might be activated by the PTN pathway in a distinct manner. Activated FAK has an essential role for maintenance of several cell phenotypes, including cell migration through PI3K/mitogen-activated protein kinase signaling (McLean et al., 2005). We thus examined if PI3K and ERK1/2 were involved in menin-regulated cell migration. A549 cells were treated with LY294002 or U0126, respectively, inhibitors for PI3K and MEK1/2, respectively. The PI3K inhibitor reduced phosphorylation of ERK1/2, but not the total amount of ERK1/2 in A549 cells (Supplementary Figure 8), and decreased A549 cell migration in a dose-dependent manner (Figure 6c). In addition, we collected six cases with menin downregulated primary lung adenocarcinomas, and found that the levels of RPTP β/ζ and pERK1/2 were high, as shown by immunohistochemistry in two of six cases (Supplementary Figure 9). These results suggest that menin-regulated PTN-ERK1/2 pathway could be involved in development of certain lung adenocarcinoma. Together, our results suggest that menin inhibits FAK and ERK1/2 phosphorylation partly through PTN/RPTP β/ζ expression, and ERK1/2 activation may have a role for menin-mediated repression of lung cancer cell migration.

Discussion

Cell migration is a crucial step of cancer metastasis, a major cause of cancer-related death (Molloy and van ’t Veer, 2008). Many oncogenes and tumor suppressor genes are involved in regulating tumor cell migration, invasion and metastasis (Klein, 2008). For example, PTEN is well known for its role in regulating tumor growth, invasion, and metastasis, and it is capable of restricting growth and survival by antagonizing the activity of PI3K (Tamura et al., 1999; Chen et al., 2009a). MEN1, a bona fide tumor suppressor gene in endocrine tumors, exerts multiple biological functions including regulation of cell proliferation (Gao et al., 2008). Until recently, little has been known regarding the function of menin in controlling cancer cell migration.

We found that menin expression in tumors, as compared with the adjacent normal epithelial cells, was markedly reduced or not detectable in 10 out of 45 adenocarcinoma samples, and reduction of menin
expression was correlated with an increase in lymph node metastasis (Gao et al., 2009). Further investigation has revealed that menin represses migration of lung cancer cells, at least partly through inhibiting expression of PTN/RPTP $\beta_\gamma$, a growth factor and its cell surface receptor that are involved in controlling cell proliferation, migration and apoptosis (Perez-Pinera et al., 2007). However, unlike menin-mediated epigenetic repression of PTN transcription through H3K27me3, menin represses RPTP $\beta_\gamma$ expression possibly indirectly through inhibiting PTN expression. It remains unclear how PTN normally upregulates expression of its receptor, RPTP $\beta_\gamma$. As PTN has several candidate receptors including anaplastic lymphoma kinase and integrin $\alpha_v\beta_3$ (Stoica et al., 2002; Mikelis et al., 2009), one possibility is that PTN activates these receptors and thus indirectly upregulates expression of RPTP $\beta_\gamma$. Consistent with these scenarios, our previously finding that menin can directly and indirectly downregulate at least two crucial components of the PTN pathway, both PTN and a downstream receptor, anaplastic lymphoma kinase (Gao et al., 2009), respectively, highlights the importance of menin in controlling this signaling pathway.

Integrin signaling is a major pathway in regulating cell adhesion to extra-cellular matrices. Interaction between integrins and their substrates regulates various cellular functions associated with tumor development and metastatic progression, including cell adhesion, migration and invasion (Tamura et al., 1999; Chen et al., 2009b). Integrin-mediated cell adhesion not only initiates signals directly, but also modulates signaling downstream of growth factor receptors; the integrin-FAK signaling pathway enables RhoA to activate its effectors in cell migration (Palazzo et al., 2004). It has been reported that PTN activates signal transduction through RPTP $\beta_\gamma$ or integrin ($\alpha_v\beta_3$) and migration of endothelial cells (Mikelis et al., 2009). In particular, PTN induces migration of the cells through activating FAK, PI3K and ERK (Polykratis et al., 2005). Our results show that RPTP $\beta_\gamma$ interacted with integrin $\alpha_v$ and $\beta_3$ in A549 cells, and that menin not only inhibits expression of PTN, but also represses the activation (phosphorylation) of FAK, PI3K and ERK1/2. The FAK signaling complex acts to recruit and/or phosphorylate a number of signaling proteins and is involved in cell adhesion and the motile and invasive phenotype in lung cancer cells (Liu et al., 2008). FAK is upregulated in non-small cell lung cancers (Carelli et al., 2006), and fibronectin-mediated activation of FAK leads to lung cancer metastasis through ERK or PI3K/Akt pathway (Meng et al., 2009). Furthermore, inhibition of MEK1/2 using PD98059 also reduced cell migration and invasion (Meng et al., 2009). In agreement with these observations, our results indicate that A549 lung cancer cell migration was significantly reduced by PI3K and MEK1/2 inhibitors, respectively.
Menin regulates cell migration through PTN signaling
Z-J Feng et al

Materials and methods

Cell culture and gene transfection
MEF cell line generation and culture were previously described (Schnepp et al., 2006). A549 and NCI-H157 cell culture and polyethylenimine-mediated plasmid transfection were performed, as previously described (Gao et al., 2009). A549 cells were treated with or without recombinant human PTN (R&D, Minneapolis, MN, USA), PI3K inhibitor (LY294002) (Cell Signaling, Danvers, MA, USA), and MEK1/2 inhibitor (U0126) (Cell Signaling).

Western blotting
The cells were lysed in radioimmunoprecipitation assay buffer (Jin et al., 2007), and the extracted proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis before transfer onto PVDF membrane, and each of the antibodies was incubated against one of the following proteins: menin (Bethyl, Montgomery, AL, USA), PTN (Abnova, Walnut, CA, USA), RPTP β/ζ (BD, San Jose, CA, USA), FAK (Santa Cruz, Santa Cruz, CA, USA), Tyr397 phospho-FAK (Invitrogen, Carlsbad, CA, USA), ERK1/2 (Cell Signaling), Thr202/Tyr204 phospho-ERK1/2 (Cell Signaling), integrin αv (Millipore, Billerica, MA, USA), integrin β3 (Santa Cruz), PI3K p85 (Cell Signaling), Tyr458 phospho-PI3K (Cell Signaling), β-catenin (Cell Signaling), Tyr142 phospho-β-catenin (Abcam, Cambridge, MA, USA) and β-actin (Santa Cruz). Membranes were further washed and incubated with an anti-rabbit or anti-mouse secondary antibody (Pierce, Rockford, IL, USA). Detection of immunoreactive bands was performed using an ECL detection kit (Pierce), according to the manufacturer’s instructions. Equal protein loading was indicated by Ponceau-S staining of blotted membranes.

Statistical analysis
Values were presented as the mean ± s.d. Statistical comparisons between groups were conducted using the Student’s t-test. For scratch wound assay, subconfluent cells were scraped using sterilizing 10 μl pipette tips, washed with phosphate-buffered saline and cultured in normal medium. The cells were observed under a Nikon Eclipse TE 300 inverted microscope (Nikon Corporation, Tokyo, Japan), and images were captured daily.

Conflict of interest
The authors declare no conflict of interest.

Acknowledgements
This work is supported by an NFSC grant (No. 30701003, GH Jin) and a National Cancer Institute grant (R01CA113962, XH). We appreciate the valuable comments from other members of our laboratories.

(5424) Hence, menin may inhibit A549 cell migration partly by repressing PTN and its receptor RPTP β/ζ expression, and ectopic menin expression reduced the active and phosphorylated forms of FAK, PI3K and ERK1/2, but did not affect β-catenin expression and nuclear localization. However, the precise mechanism for downregulation of FAK, PI3K and ERK1/2 by menin remains to be analyzed.

Collectively, our findings unravel a previously unrecognized pathway in controlling A549 lung cancer cell migration, that is, the menin-PTN pathway. This mechanism is quite distinct from menin-regulated intercellular adhesion of pancreatic β-cells, in which menin interacts with the IQ motif containing GTPase activating protein 1, a scaffold protein, and reduces GTP-Rac1 interaction with IQ motif containing GTPase activating protein 1 (Schnepp et al., 2006). Although PTN increases β-catenin phosphorylation and nuclear translocation in lung epithelial cells (Weng et al., 2009), we failed to observe an effect of PTN on β-catenin phosphorylation in A549 lung cancer cells (Supplementary Figure 6a). It is likely that the cancer cells respond distinctly to PTN stimulation. These findings suggest multiple roles for menin in regulating adhesion, migration and signal transduction in distinct types of cells.

Our results have provided new insight into menin-regulated PTN/RPTP β/ζ signal transduction in controlling A549 lung cancer cell migration. These studies have unraveled the crucial mechanisms whereby menin represses migration at least partly through suppressing the PTN/RPTP β/ζ in repressing lung cancer migration. As menin actively represses PTN-induced lung cancer cell migration and loss of menin expression is closely correlated with enhanced lung cancer metastasis to lymph nodes, the menin-PTN pathway in regulating cell migration and metastasis may serve as a target for therapy against lung adenocarcinoma.
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Z.-J. Feng et al.

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