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Lung cancer cell migration is regulated via repressing growth factor PTN/RPTP β/ζ signaling by menin

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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 β/ζ). Ectopic menin expression significantly repressed PTN transcription, but indirectly inhibited RPTP β/ζ expression through repressing PTN expression. Further studies revealed that menin-regulated cell migration through PTN/RPTP β/ζ, in conjunction with integrin $\alpha_v\beta_3$, 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.
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Keywords: lung cancer; migration; *Men1*; pleiotrophin; RPTP β/ζ

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 (Schnepp *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 Ser^{608}, 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

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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 β/ζ) (Perez-Pinera *et al*., 2007). PTN-RPTP β/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 β/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 examined whether *MEN1* knockdown using short hairpin RNAs (shRNAs) affects migration of A549 lung adenocarcinoma cells. Control vector and three distinct shRNAs that specifically target human *MEN1* were stably transfected into A549 cells. The resulting cells were analyzed for knockdown of endogenous *MEN1* using both quantitative reverse transcriptase–PCR and western blotting. *MEN1* shRNAs 2–3 substantially reduced expression of *MEN1* mRNA and protein, but shRNA1 failed to knockdown *MEN1* expression (Figure 1b; Supplementary Figure 1b). Interestingly, correlated with the levels of *MEN1* knockdown by shRNAs, shRNA 2–3 significantly increased A549 cell migration ($P<0.05$, respectively), but shRNA1 that was unable to reduce *MEN1* expression failed to do so (Figure 1c). Together, these results suggest that menin normally restricts cell migration.

Our previous work has shown that menin suppresses lung cancer cell proliferation partly through epigenetically repressing transcription of growth factor PTN (Gao *et al*., 2009). PTN is highly expressed in certain solid cancers (Jager *et al*., 2002; Perez-Pinera *et al*., 2007), binds to cell surface receptors, such as RPTP β/ζ, and has a crucial role in cell proliferation, adhesion and migration (Lu *et al*., 2005; Pariser *et al*., 2005; Duces *et al*., 2008). To examine whether menin represses lung cancer cell migration partly through affecting RPTP β/ζ expression, we evaluated the effect of loss of menin on RPTP β/ζ expression in $Men1^{+/+}$ and $Men1^{-/-}$ MEFs. *Men1* excision (Supplementary Figure 1c) increased mRNA expression of RPTP β/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 β/ζ using regular reverse transcriptase–PCR (Supplementary Figure 1d). Increased PTN and RPTP β/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 β/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 β/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 β/ζ staining (Supplementary Figures 2g and o). Together, these results indicate a pivotal role for menin in downregulating both PTN and its receptor, RPTP β/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 β/ζ signaling

Next, we examined whether menin regulates PTN and RPTP β/ζ expression in A549 adenocarcinoma cells.

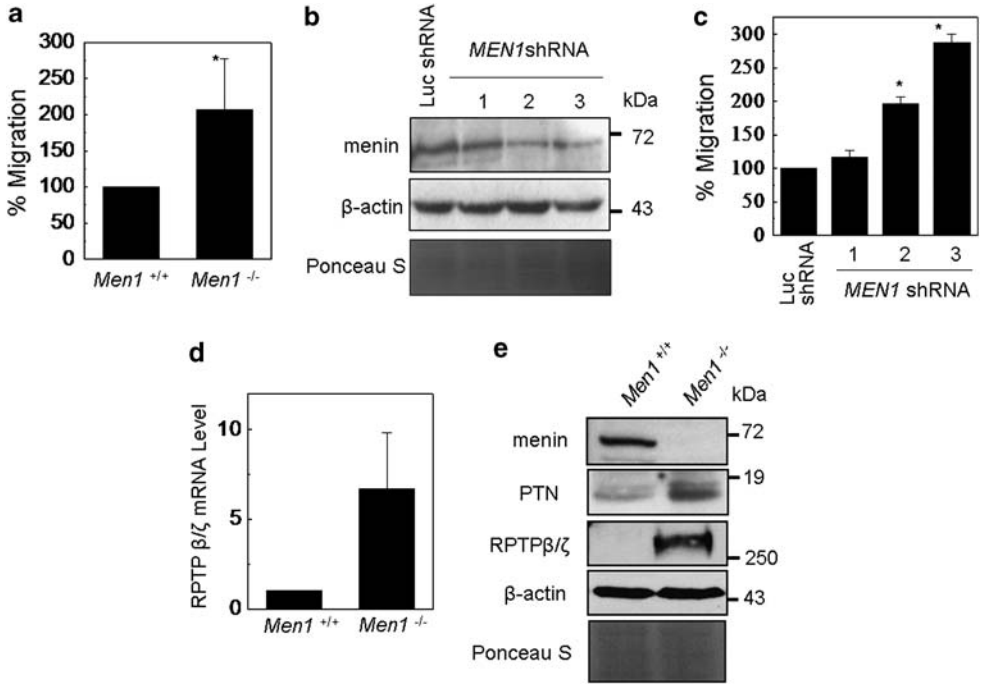

Figure 1 Menin inhibits cell migration and represses PTN/RPTP β/ζ expression. (**a**) *Men1*$^{+/+}$ and *Men1*$^{-/-}$ MEFs were added to the upper-filter of a transwell, and 24 h later, the migrated cells were stained with crystal violet, and cell number was counted under a microscope. (**b**) A549 cells were transfected with either vector-expressing shRNAs against Luc or one of the three shRNAs against *MEN1* and selected by G418. The efficiency of menin silencing was determined by western blotting. Equal sample loading was confirmed by Ponceau S staining of the Western blot membrane. (**c**) The selected A549 cells were added to the upper-filter, and cell migration was determined. (**d**) Increased RPTP β/ζ mRNA levels in *Men1*$^{-/-}$ MEFs were detected by real-time quantitative reverse transcriptase (qRT)–PCR. The amount of RPTP β/ζ mRNA was determined by normalizing their mRNA quantity with the control β-actin mRNA level, and mean values and s.d. were calculated from triplicates of a representative experiment. (**e**) The efficiency of menin ablation and the effect of *Men1* expression on PTN (18 kDa) and RPTP β/ζ (≈250 kDa) expression were determined by western blotting, and β-actin was used as a loading control. Equal sample loading was also confirmed by Ponceau S staining of the western blot membrane. Results in all cases are mean ± s.d. percentage change in number of migrating cells vs corresponding untreated cells (default = 100) and calculated from triplicates of an independent experiment, *$P<0.05$ vs control.

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 RPTP β/ζ (Figure 2a). Further, *MEN1* knockdown by shRNA also increased the protein level of RPTP β/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 RPTP β/ζ expression; we found that A242V and L22R lost or partially lost the ability to repress RPTP β/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 RPTP β/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/RPTP β/ζ expression and cell migration were shown in Supplementary Figures 5a and b. In addition, another human non-small 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/RPTP β/ζ expression, but also repressed NCI-H157 cell migration (Supplementary Figures 5c and d). We further tested whether PTN/RPTP β/ζ is required for menin-repressed A549 cell migration. Three distinct RPTP β/ζ shRNAs were transfected into A549 cells, and RPTP β/ζ shRNA3 reduced the RPTP β/ζ protein level effectively (Figure 2e). It is noteworthy that RPTP β/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 RPTP β/ζ.

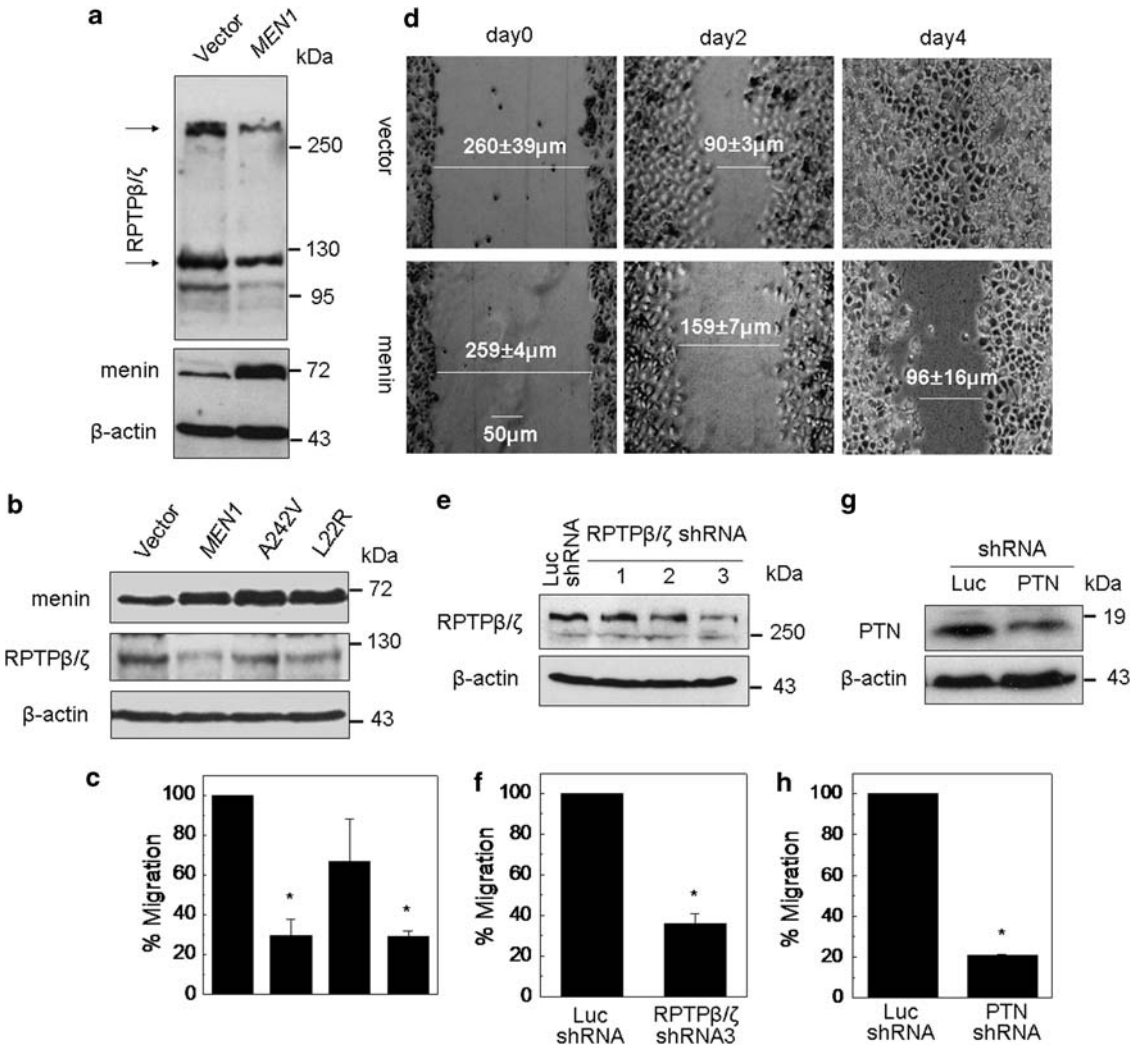

Figure 2 Menin inhibits A549 cell migration partly through repressing PTN and RPTP β/ζ expression. (**a**) A549 cells were transfected with *MEN1* and selected by puromycin. Expression of menin and downregulated RPTP β/ζ (≈250 and 130 kDa) were determined by western blotting. (**b**) A549 cells were transfected with an empty vector, wild-type *MEN1*, point mutation A242V or L22R. Menin and RPTP β/ζ expression in the various cell lines above were analyzed by western blotting. (**c**) The various A549 cell lines were used to determine cell migration. (**d**) Menin overexpression inhibited closure of artificial wounds made in confluent cellular monolayers. (**e**) A549 cells were stably transfected with either Luc shRNA or vector expressing one of three distinct RPTP β/ζ shRNAs, and the efficiency of RPTP β/ζ knockdown was determined by western blot. (**f**) A549 cells transfected with RPTP β/ζ shRNA3 were used to determine cell migration. (**g**) A549 cells were transfected with PTN shRNA, and the efficiency of PTN knockdown was determined by western blotting. (**h**) The cell migration of the above A549 cells was determined by three independent experiments, $*P<0.05$.

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

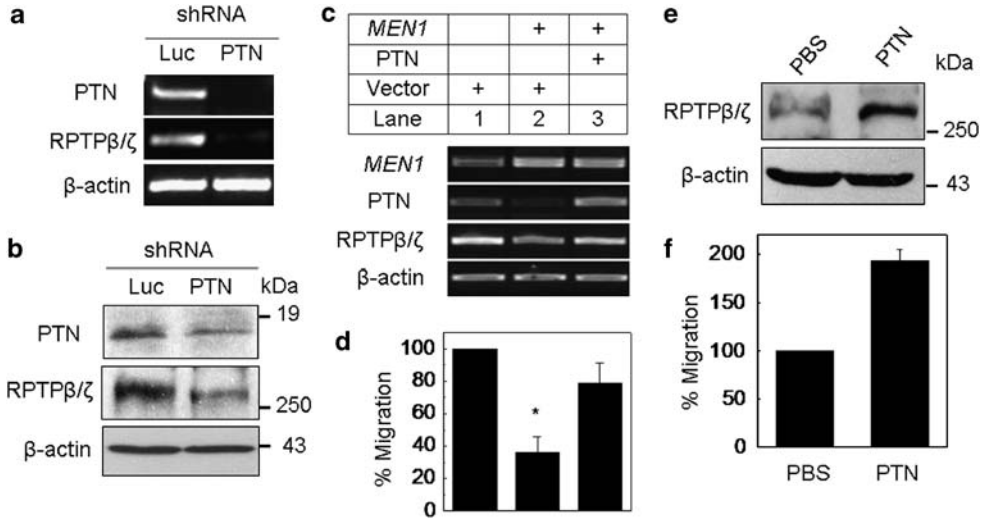

Figure 3 *MEN1* indirectly regulates RPTP β/ζ expression through PTN in A549 cells. (**a**, **b**) A549 cells were transfected with PTN shRNA, and the resulting cells were processed to determine PTN and RPTP β/ζ mRNA and protein levels using reverse transcriptase (RT)–PCR and western blotting, respectively. (**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**, **f**) recombinant human PTN (rhPTN) (100 ng/ml) increased RPTP β/ζ expression and migration of A549 cells.

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 $\alpha_v\beta_3$ is important for cell signaling, survival and migration particularly during angiogenesis and tumorigenesis (del Pozo *et al*., 2004). However, the role of integrins for the migration of lung cancer cells has not been analyzed extensively. Integrin $\alpha_v\beta_3$ 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 β_3 in the A549 cells (Figure 4a). This result prompted us to analyze whether α_v/β_3 associates with RPTP β/ζ. We performed co-IP from extracts of A549 lung cancer cells and found that endogenous integrin α_v and β_3 were co-immunoprecipitated with endogenous RPTP β/ζ (Figure 4b). As a control, IgG failed to pull down α_v and β_3 (Figure 4b). The interaction between α_v and RPTP β/ζ was further confirmed by IP of endogenous RPTP β/ζ with α_v antibody (Figure 4c). In addition, interaction between α_v and β_3 was also detected in A549 cells (Figures 4c and d). We further examined the effect of menin on α_v and β_3 expression in A549 cells and in *Men1*$^{+/+}$ and *Men1*$^{-/-}$ MEFs. Western blotting revealed that menin overexpression did not affect expression of either α_v or β_3 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

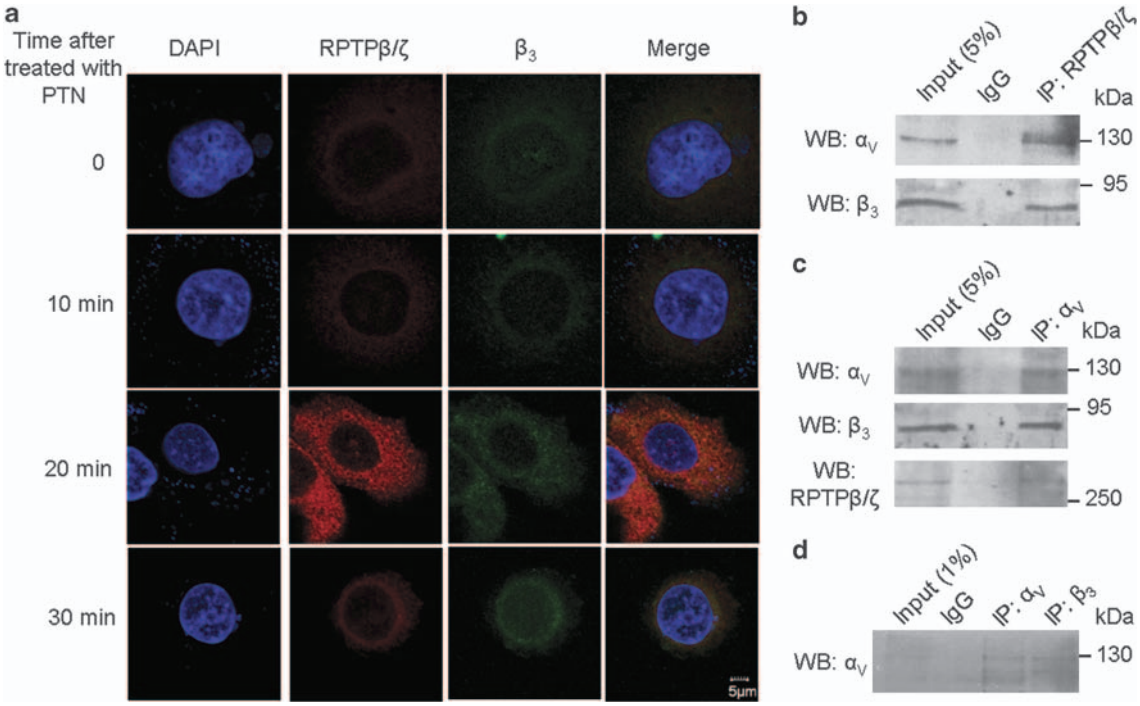

Figure 4 PTN increases interaction of RPTP β/ζ and integrins. (**a**) Serum-starved A549 cells were treated with 100 ng/ml recombinant human PTN (rhPTN) and harvested at various time points. RPTP β/ζ (red), β_3 (green) and 4,6-diamidino-2-phenylindole (DAPI) (blue) were detected by IF, × 1600-fold. (**b**–**d**) A549 cell lysates were IP for RPTP β/ζ, α_v and β_3, and immunoprecipitates were analyzed by western blotting for the presence of RPTP β/ζ, α_v, β_3.

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 6c). Collectively, these results suggest that FAK signaling may link menin/PTN to cell migration partly through regulating PI3K and ERK1/2 phosphorylation.

Cell morphology and migration are known to be regulated by members of the Rho family of small GTPases, including Rho, Rac1 and Cdc42 (Hall, 1998). Rac1 and Cdc42 are activated by ligation of integrins, whereas Rho activation relies on either integrins, syndecan-4 or additional cell-surface receptors (Hood and Cheresh, 2002). Hence, we further examined if menin-regulated PTN expression controls cell migration partly through Rho family signaling. Ectopic menin expression did not alter the amount of either activated forms (GTP-bound) or the total amount of Rho, Rac1 and Cdc42 in A549 cells (Figure 5c). Similar results were obtained in *Men1*-null or *Men1*-expressing MEFs (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 RPTP β/ζ 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/RPTP β/ζ and partly represses migration through inhibiting ERK1/2 phosphorylation

It is unclear how menin regulates FAK activation. We examined whether RPTP β/ζ 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 α_v/β_3 directly interacts with RPTP α or RPTP β/ζ, which is essential for integrin β_3 activation (von Wichert *et al*., 2003; Mikelis *et al*., 2009). FAK is itself regulated by various

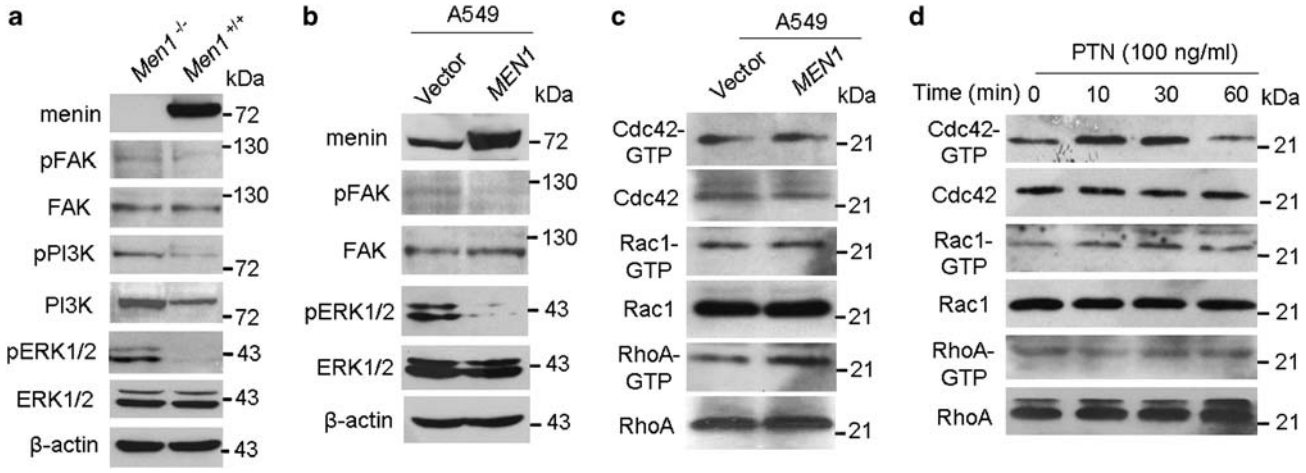

Figure 5 Menin inhibits activation of FAK, PI3K and ERK1/2. (**a**) The efficiency of menin ablation and its effect on upregulation of pFAK, PI3K (85 kDa), pPI3K and pERK1/2 were determined by western blotting. (**b**) The effect of ectopic menin expression on FAK (125 kDa), pFAK, ERK1/2 (42 and 44 kDa) and pERK1/2 in A549 cells were determined using western blotting. (**c**) PAK1-PBD agarose and Rhotekin RBD agarose were used to isolate GTP-Cdc42, GTP-Rac1 and GTP-RhoA from whole cell lysates from menin-overexpressing A549 cells. The Cdc42-GTP, Rac1-GTP and RhoA-GTP were detected using western blotting and normalized by the total input protein. (**d**) Serum-starved A549 cells were treated with 100 ng/ml recombinant human PTN (rhPTN) and harvested at various time points. The activation of Cdc42, Rac1 and RhoA were detected by western blotting and normalized by the total input protein.

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). As expected, the migration of A549 cells treated with the MEK1/2 inhibitor was also reduced 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

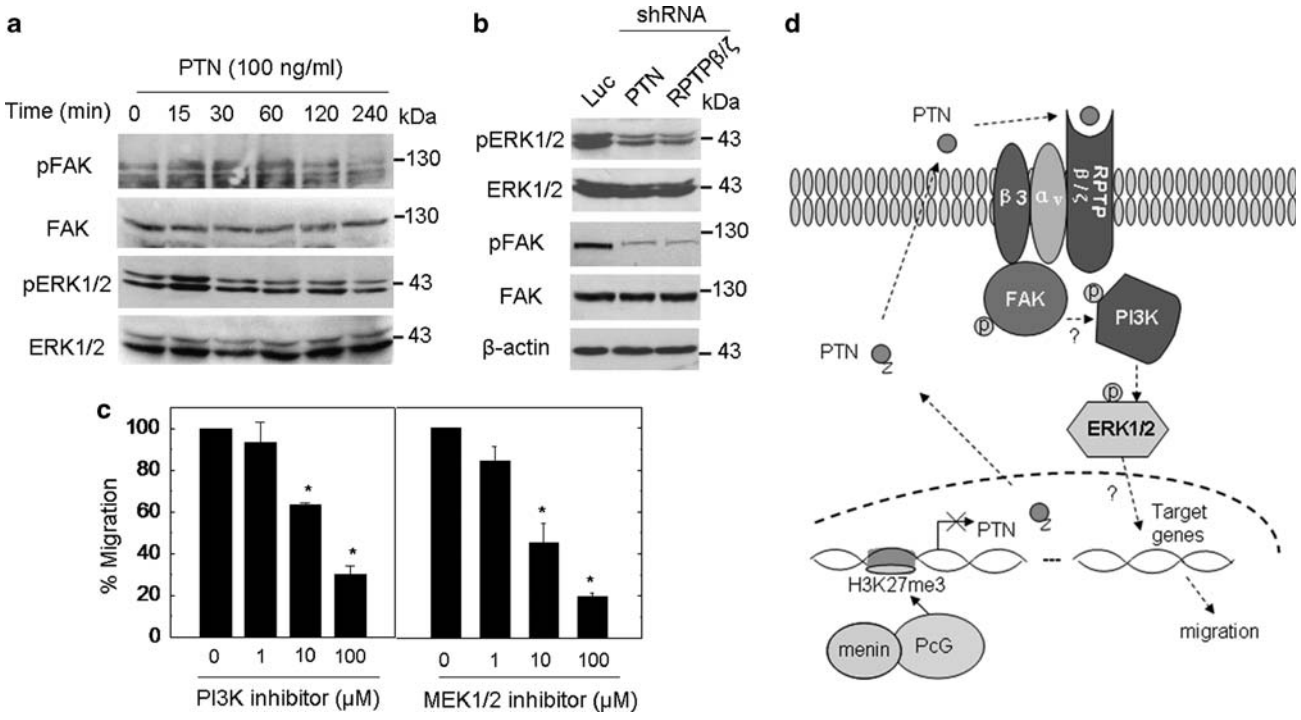

Figure 6 Menin regulates FAK and ERK1/2 activation through PTN/RPTP β/ζ and represses cell migration partly through ERK1/2. (**a**) Serum-starved A549 cells were treated with 100 ng/ml recombinant human PTN (rhPTN) and harvested at various time points. The activation of FAK and ERK1/2 were determined with western blotting. (**b**) A549 cells were transfected with PTN or RPTP β/ζ shRNA, pFAK and pERK1/2 were examined with western blotting. (**c**) Migration of A549 cells treated with LY294002 or U0126 were significantly reduced in a dose-dependent manner. $N=3$, $^*P<0.05$. (**d**) A model for regulation of cell migration by menin through growth factor PTN/RPTP β/ζ.

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 β/ζ, 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 β/ζ expression possibly indirectly through inhibiting PTN expression. It remains unclear how PTN normally upregulates expression of its receptor, RPTP β/ζ. 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 β/ζ. 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 β/ζ 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 β/ζ interacted with integrin α_v and β_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

(Figure 6c). 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, but increases E-cadherin/β-catenin interaction with IQ motif containing GTPase activating protein 1 (Yan *et al*., 2009). Consistent with these findings, PTN is abundantly expressed in fetal lung epithelial cells of rats and enhances cell proliferation (Weng *et al*., 2009). 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.

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 α_v (Millipore, Billerica, MA, USA), integrin β_3 (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.

IP assay

IP assays were performed essentially, as previously described (Yan *et al*., 2009). Briefly, the harvested cells were lysed and incubated with a primary antibody or normal rabbit IgG (Millipore). Protein A or protein G-agarose beads (Santa Cruz) were added. Bound proteins were collected by centrifugation, washed and analyzed by western blotting.

Cell migration assay and scratch wounding assay

Migration assays were performed, as previously described (Mikelis *et al*., 2009), in 24-well transwell plates (Millipore) using uncoated polycarbonate membranes with 8 μm pores. Serum-starved cells were harvested and re-suspended at a concentration of 1×10^4 cells per 0.2 ml in serum-free medium containing 10% bovine serum albumin, and added to the upper chamber. At 24 h after incubation, non-migrated cells were scraped off the upper side of the filter, and filters were stained with 0.1% crystal violet. The number of migrated cells on the reverse side of the membrane was quantified by average cell counts from six random fields in each well, under a microscope at $\times 100$ magnifications (Supplementary Figure 1a). Each condition was assayed in triplicate wells, and the resulting data were subjected to statistical analysis 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.

Statistical analysis

Values were presented as the mean ± s.d. Statistical comparisons between groups were conducted using the Student's *t*-test, and $P<0.05$ was considered statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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