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Tripartite motif 16 inhibits epithelial–mesenchymal transition and metastasis by down-regulating sonic hedgehog pathway in non-small cell lung cancer cells

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ABSTRACT

The present study was to examine the effect of Tripartite motif 16 (TRIM16) on epithelial–mesenchymal transition (EMT) and metastasis in non-small cell lung cancer (NSCLC) cells, and its clinical significance in NSCLC. The correlation of TRIM16 expression and clinical features of NSCLC was analyzed in paraffin-embedded archived normal lung tissues and NSCLC tissues by immunohistochemical analysis. The effect of TRIM16 on EMT and metastasis was examined both in vitro and in vivo. The expression of TRIM16 was markedly decreased in NSCLC and correlated with tumor metastasis. Upregulation of TRIM16 significantly inhibited EMT and metastasis of NSCLC cells. In contrast, silencing TRIM16 expression significantly promoted the EMT and metastasis of NSCLC cells both in vitro and in vivo. Moreover, we demonstrated that downregulation of TRIM16 activated the sonic hedgehog pathway, and that inhibition of the sonic hedgehog pathway by cyclopamine abrogated the effect of TRIM16-downregulation induced EMT and metastasis on NSCLC cells. Our results suggest that TRIM16 is a potential pharmacologic target for the treatment of NSCLC and promotion TRIM16 expression might represent a novel strategy to NSCLC metastasis.

1. Introduction

Lung cancer is the most common cause of cancer deaths worldwide, ~1.2 million deaths people die from it per year [1]. It is the leading type of new cancer cases leading to death. Non-small cell lung carcinoma (NSCLC) is the most frequent subtype, ~85% of all cases [1]. Most of NSCLC patients have locally advanced or distant metastatic disease from onset of symptoms. It is strongly associated with poor prognosis and has a 5-year survival rate of <10 and 5% in male and female patients, respectively [1,2]. While NSCLC usually reveals better responsiveness to chemotherapy and radiation, NSCLC is relatively insensitive to both therapeutic modalities [2–4]. Hence, identification of novel targets for more effective anti-NSCLC strategies with minimal toxicity is urgent.

The TRIM16, also called the estrogen-responsive B box protein, which is a member of the RING-B box-coiled-coil/TRIM protein family [5]. It is associated with many different kinds of cancers [6–8]. Previous studies have identified TRIM16 as a DNA binding protein with histone acetyltransferase activity, which is necessary for the retinoic acid receptor β2 transcriptional response in cancer cells [9]. Overexpressed TRIM16 reduced neuroblastoma cell growth, enhanced retinoidinduced differentiation, and decreased tumourigenicity in vivo [6]. Recently studies revealed that TRIM16 acts as a tumor suppressor, promoting neuritic differentiation, cell migration, and replication through interactions with cytoplasmic vimentin and nuclear E2F1 in neuroblastoma cells [6,10]. Therefore, TRIM16 may act as a tumor suppressor, but whether TRIM16 has a function in NSCLC development, EMT, and metastasis remains unknown.

This study showed that TRIM16 expression was decreased in NSCLC tissues and NSCLC cell lines. Upregulation of TRIM16 significantly inhibited EMT and metastasis of NSCLC cells. In contrast, silencing TRIM16 expression significantly promoted the EMT and metastasis of NSCLC cells both in vitro and in vivo. In addition, we also found that TRIM16 had a function in NSCLC cell EMT and invasion could be partly associated with sonic hedgehog

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pathway. These results indicate the pivotal function of TRIM16 in NSCLC cell EMT and invasion.

2. Material and methods

2.1. Chemicals and antibodies

Lipofectamine 2000 transfection and TRIZOL LS Reagents were purchased from Invitrogen (Grand Island, NY, USA). Antibodies against TRIM16, Shh, Smo, Ptc, and Gli-1 were purchased from Abcam (Cambridge, MA, USA). E-cadherin, N-cadherin, vimentin, and β-actin antibodies were from Cell Signaling technology (Danvers, MA, USA). Anti-α-catenin antibody was from BD (Franklin Lakes, NJ, USA). Cyclopamine was from Sigma (Sigma–Aldrich, St. Louis, MO, USA). Unless otherwise noted, all other chemicals were from Sigma.

2.2. Patients and specimens

Paraffin-embedded, archived tissue samples were obtained from 51 normal lung tissues and 121 patients diagnosed with NSCLC between 2009 and 2013 at the Department of Pathology, First Affiliated Hospital, Xi'an Jiaotong University. The histologic characterization and clinicopathologic staging of the samples were determined according to the WHO criteria and current International Union against Cancer TNM (tumor-node-metastasis) Classification. Detailed clinical information of all patients is summarized in Supplementary Table 1.

2.3. Histological and immunohistochemical analysis

The normal human lung tissues and human lung tumor tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight and subsequently embedded in paraffin wax. Sections cut at a thickness of 4 μm were stained with hematoxylin and eosin for histological analysis. Immunohistochemical analysis was performed for different markers in these arrays as described previously. The proportion of stained cells was semiquantitatively determined following published protocols.

2.4. Cell culture

NSCLC cell lines (ATCC, Manassas, VA, USA) were cultured under the following conditions: A549, H1299, H460, A427 and H1650 cell lines were cultured using 10% fetal bovine serum (Cat#10099-141, Invitrogen, Carlsbad, CA) in RPMI-1640 (Cat#C11875, Invitrogen). BEAS2B cell line was cultured using 10% fetal bovine serum (Invitrogen) in Dulbecco's modified Eagle medium (Cat#C11965, Invitrogen). Cell culture was according to manufacturer's protocol. All the cell lines were grown at 37 °C in a 5% CO_2/95% air atmosphere and were revived every 3–4 months.

2.5. Establishment of TRIM16 stable expression and knockdown cell lines

Retroviral construct containing human pBabe-TRIM16 cDNA, and pSuper.retro.puro with shRNA against human TRIM16 were prepared as described previously [11]. The generation of retrovirus supernatants and transfection of lung carcinoma cells were conducted as described previously [12]. The expression of TRIM16 was confirmed by qRT-PCR and Western blotting analysis.

2.6. Cell invasion and motility assay

Invasion of cells was measured in Matrigel (BD, Franklin Lakes, NJ, USA) -coated Transwell inserts (6.5 mm, Costar, Manassas, VA, USA) containing polycarbonate filters with 8-μm pores as detailed previously [13,14]. The inserts were coated with 50 μl of 1 mg/ml Matrigel matrix according to the manufacturer's recommendations. 2×10^5 cells in 200 μl of serum-free medium were plated in the upper chamber, whereas 600 μl of medium with 10% fatal bovine serum were added to lower well. After 24 h incubation, cells that migrated to the lower surface of the membrane were fixed and stained. For each membrane, five random fields were counted at ×10 magnification. Motility assays were similar to Matrigel invasion assay except that the Transwell insert was not coated with Matrigel.

2.7. Confocal immunofluorescence microscopy

Cell lines were plated on culture slides (Costar, Manassas, VA, USA). After 24 h, the cells were rinsed with PBS and fixed with 4% paraformaldehyde, and cell membrane was permeabilized using 0.5% Triton X-100. These cells were then blocked for 30 min in 10% BSA and then incubated with primary antibodies overnight at 4 °C. After three washes in PBS, the slides were incubated for 1 h in the dark with FITC-conjugated secondary antibodies (Invitrogen, Grand Island, NY, USA). After three further washes, the slides were stained with DAPI for 5 min to visualize the nuclei, and examined using a Carl Zeiss confocal imaging system (LSM 780) (Carl Zeiss, Jena, Germany).

2.8. Western blotting

Cells were lysed in lysis buffer and total protein contents were determined by the Bradford method. 30 μg of lysis were separated by reducing SDS-PAGE and probed with specific antibodies. Blots were washed and probed with respective secondary peroxidase-conjugated antibodies, and the bands visualized by chemoluminescence (Amersham Biosciences).

2.9. qRT-PCR

Total RNA was extracted using Trizol reagent and cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen). qRT-PCR and data collection were performed with an ABI PRISM 7900HT sequence detection system. The primers used for the amplification of the indicated genes are available upon request.

2.10. Gene expression profiling

Total RNA quality and quantity were determined using Agilent 2100 Bioanalyzer and NanoDrop ND-1000. Affymetrix HU U133 plus 2.0 arrays were used according to manufacturer's protocol. The data were initially normalized by robust multiarray average (RMA) normalization algorithms in expression console software (Affymetrix). Significantly altered genes between TRIM16 knockdown and its control cells were considered by scatter plots and the genes up- and down-regulated ≥5-fold. Clustering analysis was done using gene list by Gene Cluster v3.0 software, and heat maps were visualized using Java TreeView v1.1.4r3 software. Gene set enrichment analysis was carried out using ConceptGen (http://conceptgen.ncibi.org). Gene sets were either obtained from the ConceptGen or from published gene signatures.

2.11. In vivo tumor metastasis

Nude mice were purchased from the Shanghai Slac Laboratory Animal Co. Ltd and maintained in microisolator cages. All animals were used in accordance with institutional guidelines and the current experiments were approved by the Use Committee for Animal Care. For metastasis assays, cells were resuspended in PBS at a concentration of 1×10^7 cells ml^{-1}. Cell suspension (0.1 ml) was injected into tail veins of nude mice. All of the mice were killed by CO_2 60 days after inoculation.

2.12. Statistical analysis

The results were analyzed using SPSS 18.0 software (Chicago, IL, USA). Each experiment was repeated a minimum of three times. A two-tailed t-test was used to determine statistical significance. The results were presented as the means ± S.D. *P*-values < 0.05 were considered to be statistically significant.

3. Results

3.1. Expression of TRIM16 was downregulated in NSCLC cancer tissues

To test whether TRIM16 may function as a tumor suppressor in NCSLC, we first compared the expression levels of TRIM16 in 121 NCSLC and 51 normal lung tissue samples using IHC. TRIM16 protein levels were found to be reduced in the tumor lesions compared with the normal lung tissue lesions in most of the samples (Fig. 1A), suggesting a possible role for TRIM16 in the development or progression of NCSLC. We then analyzed TRIM16 expression in NCSLCs without or with distant metastasis; we found that TRIM16 expression was significantly correlated with distant metastasis in NCSLC tissues (Fig. 1B).

3.2. Establishment of stable TRIM16 transfectants in lung cancer cells

We measured the TRIM16 expression levels in five lung cancer cell lines (A549, H1299, A427, and H1650) and one normal lung cell line, BEAS2B, by western blot and qRT-PCR. The results show that levels of TRIM16 were decreased in all the tumor cell lines compared with the BEAS2B cell (Fig. 2A, Supplemental figure 1A). We used A549 cells to establish a stable cell line that constitutively overexpressed the TRIM16 protein with the aim of revealing the role that TRIM16 expression has in the development or progression of lung cancer. We also used shRNA to generate a stable TRIM16 knockdown in the H1650 lung cancer cell line. The transfection efficiency was confirmed using western blotting and qRT-PCR analyses. As shown in Fig. 2B and Supplemental figure 1B, the A549 cells that had been transfected with the TRIM16 expression plasmid displayed significantly increased TRIM16 expression at both the mRNA and protein levels compared with the vector cell lines. In addition, the H1650 cells that had been transfected with the TRIM16 shRNA plasmid displayed significantly decreased TRIM16 expression at both the mRNA and protein levels compared with the control cells (Fig. 2B and Supplemental figure 1C).

3.3. TRIM16 regulates the transition between epithelial and mesenchymal phenotypes in lung cancer cells

To investigate whether TRIM16 positively regulates cell migration and invasion, we first observed the morphological changes and found thatA549-TRIM16 cells reverted to an epithelial phenotype as compared to their respective control cells (Fig. 2C). This observation was further confirmed by expression analyses of epithelial and mesenchymal markers. We showed that TRIM16 overexpression increased the levels of epithelial markers (E-cadherin and α-catenin) and decreased the levels of mesenchymal markers (N-cadherin and vimentin) in A549 cells (Fig. 2C and D). Conversely, H1650-shTRIM16 cells exhibited fibroblastic morphology (Fig. 2E). Consistent with this, silencing TRIM16 decreased levels of epithelial markers, and increased levels of mesenchymal markers (Fig. 2E and F). Taken together, these findings suggest that TRIM16 plays an important role in regulating EMT of lung cancer cells.

3.4. TRIM16 inhibited migratory and invasive capacities of lung cancer cells in vitro and inhibited metastasis in vivo

We next assessed whether TRIM16 could affect the ability of lung cancer cells to migrate and invade using a Boyden's chamber assay. TRIM16 overexpression inhibited both migration and invasion in A549 cells (Fig. 3A). In addition, TRIM16 knockdown in H1650 cells significantly promoted cell migration and invasion (Fig. 3B). These results indicated that TRIM16 significantly inhibited the invasion and migration of lung cancer cells.

We then investigated the functional relevance of JARID1B for metastasis in vivo. A549-TRIM16, H1650-shTRIM16 and their corresponding control cells were injected into nude mice through the tail vein. TRIM16 overexpression not only significantly decreased the number of mice with liver distant metastasis (Supplemental figure 2), but also dramatically decreased the number of metastatic tumors in liver of each mouse (Fig. 3C). Silencing TRIM16 in H1650 cells promoted metastatic behavior, both in terms of the number of mice with distant metastasis (Supplemental figure 2) and the number of metastatic tumors in the liver of each mouse (Fig. 3D). Therefore, the in vivo results further demonstrate the critical role of TRIM16 in lung cancer metastasis.

3.5. TRIM16 inhibited lung cancer metastasis via inactivation of the sonic hedgehog pathway

To better understand the mechanisms by which TRIM16 engaged in lung cancer development and progression, we performed gene expression profiling on H1650-shTRIM16 and its control cells. Microarray analyses identified a list of genes significantly differentially expressed after TRIM16 silencing including upregulation of sonic hedgehog pathway (Fig. 4A). Furthermore, gene set enrichment analysis indicated that proliferation, neoplasm metastasis and invasion, cell movement and motility, and sonic hedgehog pathway related gene signatures_ENREF_28 were significantly changed in TRIM16 silencing cells (Fig. 4B), supporting the idea that TRIM16 regulates lung cancer EMT, invasion and metastasis. These data also led us to hypothesize that TRIM16 exerts these functions possibly via sonic hedgehog pathway. To test this, we first determined whether sonic hedgehog pathway is a downstream target of TRIM16 in NSCLC cells. Expression of sonic hedgehog pathway in the cells with altered TRIM16 expression was further evaluated by qRT-PCR and Western blotting. A549-TRIM16 cells exhibited greatly decreased Shh, Smo, Ptc, and Gli-1 both PTEN mRNA and protein levels (Supplemental figure 3A, Fig. 4C), whereas silencing TRIM16 in H1650 cells dramatically increased theirs mRNA and protein levels (Supplemental figure 3B, Fig. 4D). To test whether the sonic hedgehog pathway was involved in the anti-EMT and anti-metastatic function of TRIM16, we pretreated the A549 cells with sonic hedgehog pathway inhibitor, cyclopamine. As shown in Fig. 4E and F, treatment with the sonic hedgehog pathway inhibitor, cyclopamine, drastically weakened the abilities of these TRIM16-shRNA-transduced cells to induce migration, invasion and

EMT markers changes. The results confirmed that the sonic hedgehog pathway was involved in TRIM16-mediated EMT and metastasis in lung cancer.

4. Discussion

Despite the decline in lung cancer mortality, a number of lung cancer patients develop metastatic tumors even after surgical removal of the primary tumors. Therefore, metastasis continues to be the main obstacle to the effective treatment of lung cancer, and there is an urgent need to identify novel molecular factors that lead to the invasiveness and metastasis of lung cancer [1]. Mounting evidence shows that in epithelial cancers, including NSCLC, induction of epithelial–mesenchymal transition (EMT) is a major event that provides mobility to cancer cells in order to generate metastases [15]. EMT is characterized by the loss of epithelial characteristics and acquisition of a mesenchymal phenotype, which confers the ability for cancer cells to invade adjacent tissue and migrate to distant sites, where these cancer cells proliferate to generate new tumors [16]. Hence, clarifying the regulation of proliferation and EMT will greatly benefit our understanding of NSCLC metastasis. In the present manuscript, we identified TRIM16 as a candidate target gene for the inhibition of lung cancer EMT and metastasis.

The tripartite motif or TRIM, family of proteins were originally described by its multi-domain design of three structurally distinct motifs, the RING finger zinc-binding domain, a B-box zinc-binding domain and the coiled-coil domain [17]. Presently, there are approximately 70 TRIM proteins which are structurally similar, yet involved in a diverse range of cellular processes including cell proliferation and differentiation, oncogenesis, apoptosis and retroviral replication [18]. TRIM16 is also known as the estrogen-responsive B-box protein due to its original discovery as an estrogen responsive protein in human mammary epithelial cells [19]. Estrogens and keratinocyte growth factor regulate mRNA expression levels of TRIM16 in human mammary epithelial cells and keratinocytes, respectively. Increased expression of TRIM16 induced the differentiation of keratinocytes [10].

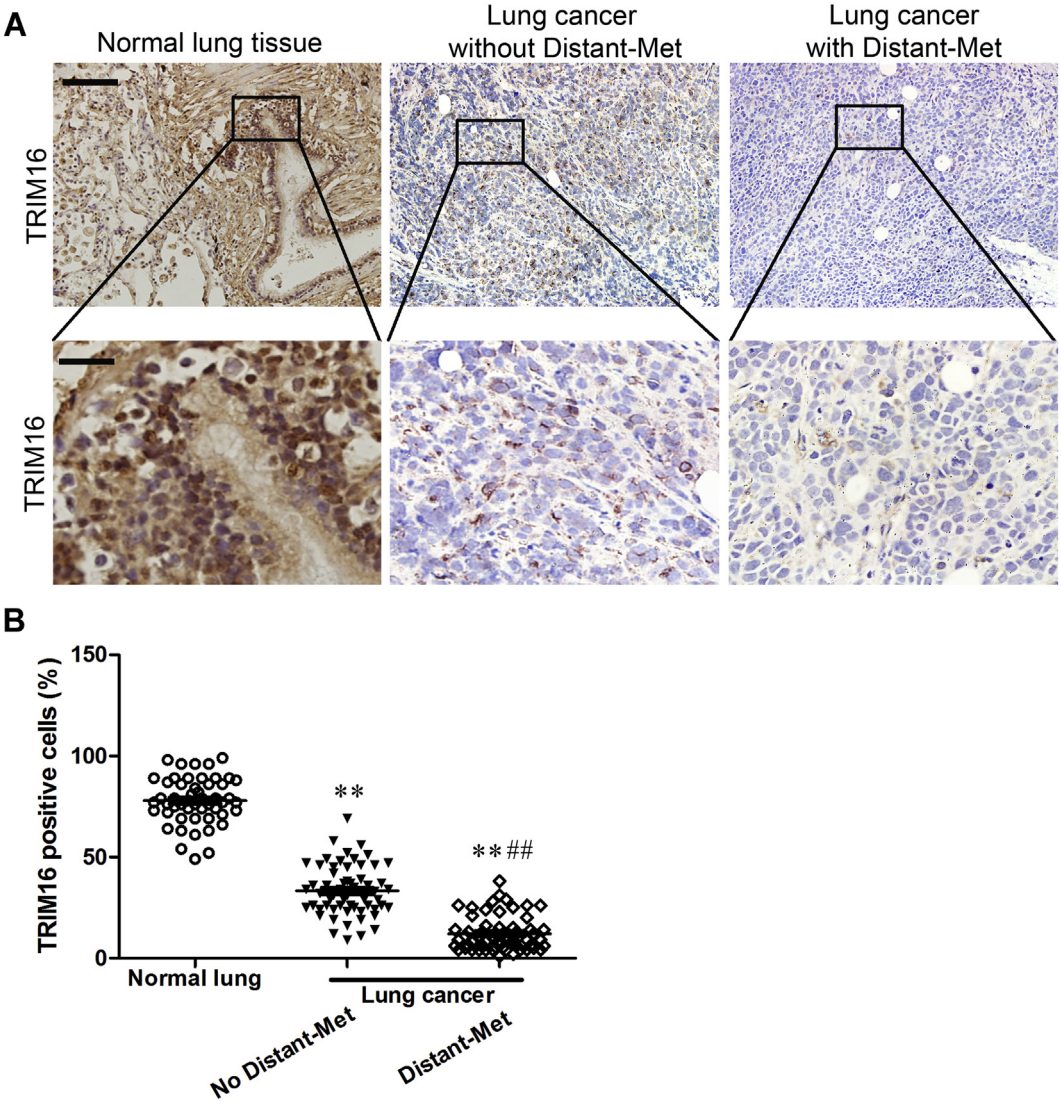


Fig. 1. Expression of TRIM16 was downregulated in NSCLC cancer tissues. A, TRIM16 protein expression was analyzed by immunohistochemical analysis in 121 NSCLC tissues and 51 normal lung tissues and the representative results were shown. B, semiquantification of TRIM16 expression in normal lung tissues, and primary NSCLC tissues without or with distant metastasis. **, compared with normal lung tissues $P < 0.01$; ##, compared with not metastasis tissues $P < 0.01$ is based on the Student t test. Error bars, SD. Scale bars, 50 μm (top) and 20 μm (bottom) in A.

TRIM16 has been shown to suppress tumor progression through regulatory pathways involved in growth inhibition, migration, differentiation and apoptosis. Recently research has demonstrated that TRIM16 can heterodimerize with other TRIM proteins and has E3 ubiquitin ligase activity [17]. All of these data strongly support a role for TRIM16 as a tumor suppressor gene. However, the exact mechanisms of TRIM16 involvement in lung cancer remain unclear.

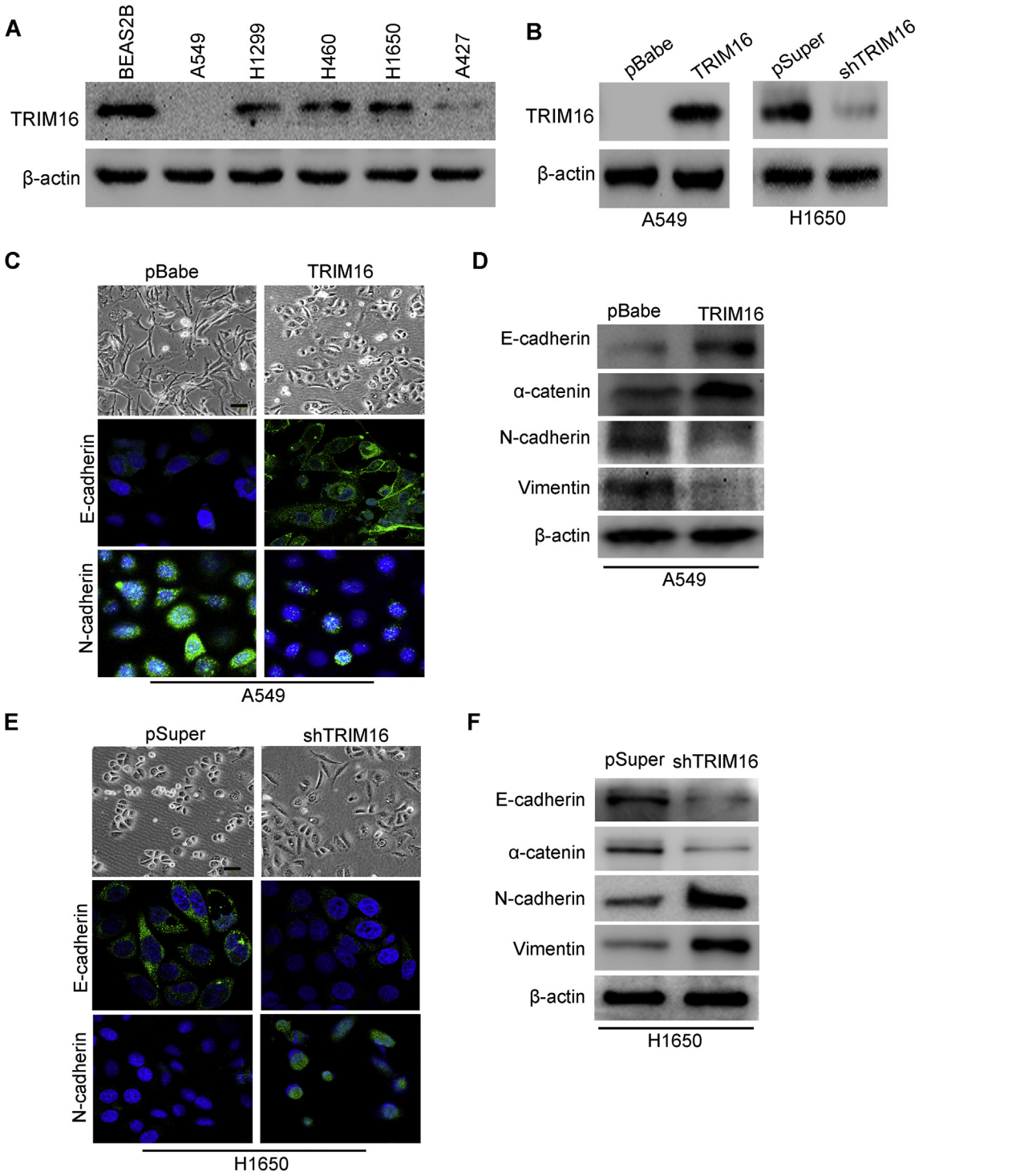
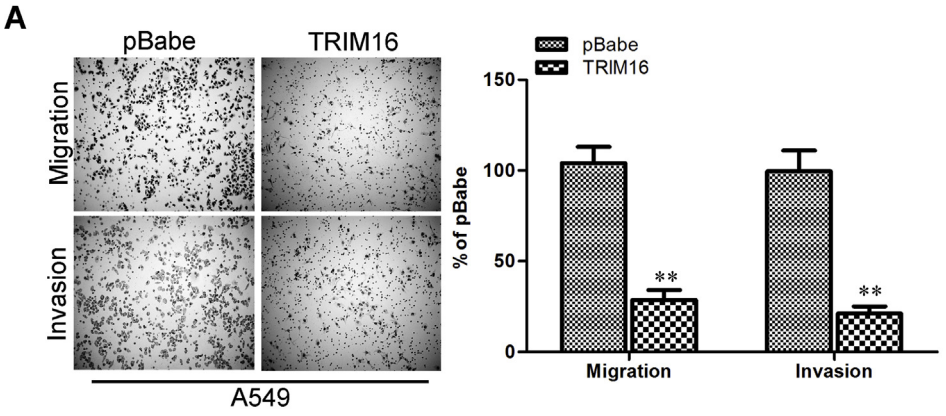


Fig. 2. TRIM16 regulates the transition between epithelial and mesenchymal phenotypes in lung cancer cells. A, expression level of TRIM16 was measured by Western blotting in BEAS2B, A549, H1299, H460, H1650, and H427 cell lines. B, The transfection efficiency of TRIM16 was analyzed by measuring protein levels by western blotting. C, representative phase-contrast images of A549 cells showed TRIM16 overexpression-modulated morphologic changes, and expression of epithelial and mesenchymal marker was analyzed by immunofluorescence stains in A549-TRIM16 and its control cells. D, expression of epithelial and mesenchymal marker was analyzed by Western blotting in A549-TRIM16 and its control cells. E, representative phase-contrast images of H1650 cells showed TRIM16 knockdown-modulated morphologic changes, and expression of epithelial and mesenchymal marker was analyzed by immunofluorescence stains in H1650-shTRIM16 and its control cells. F, expression of epithelial and mesenchymal marker was analyzed by Western blotting in H1650-shTRIM16 and its control cells.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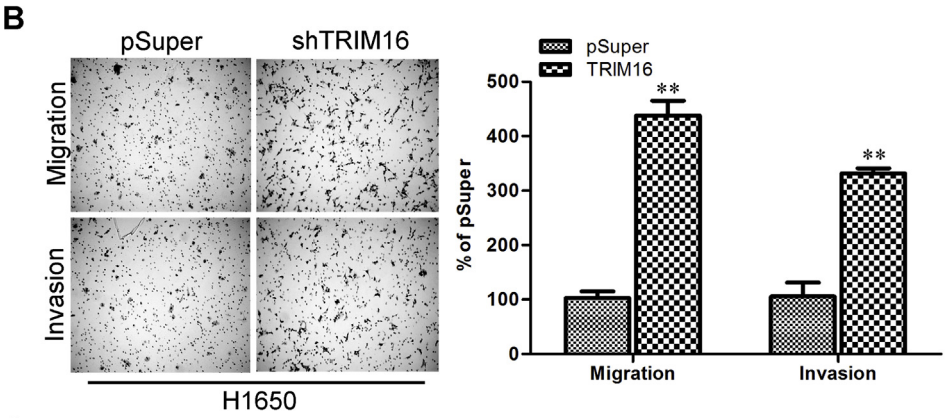
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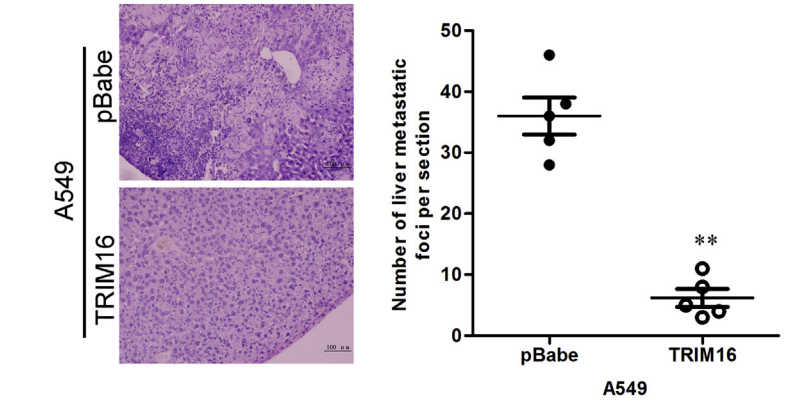
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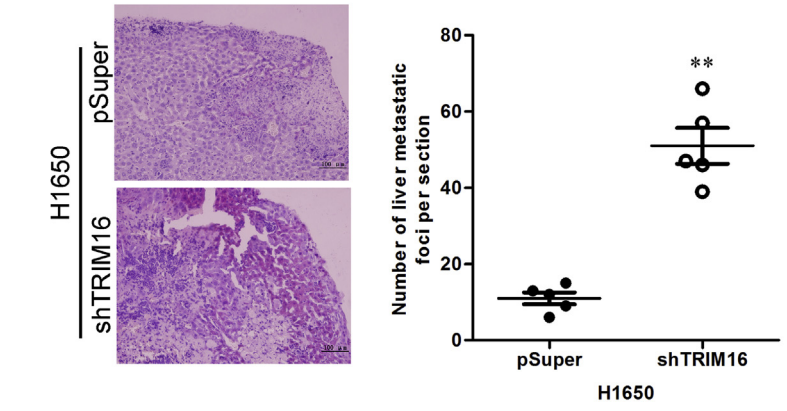
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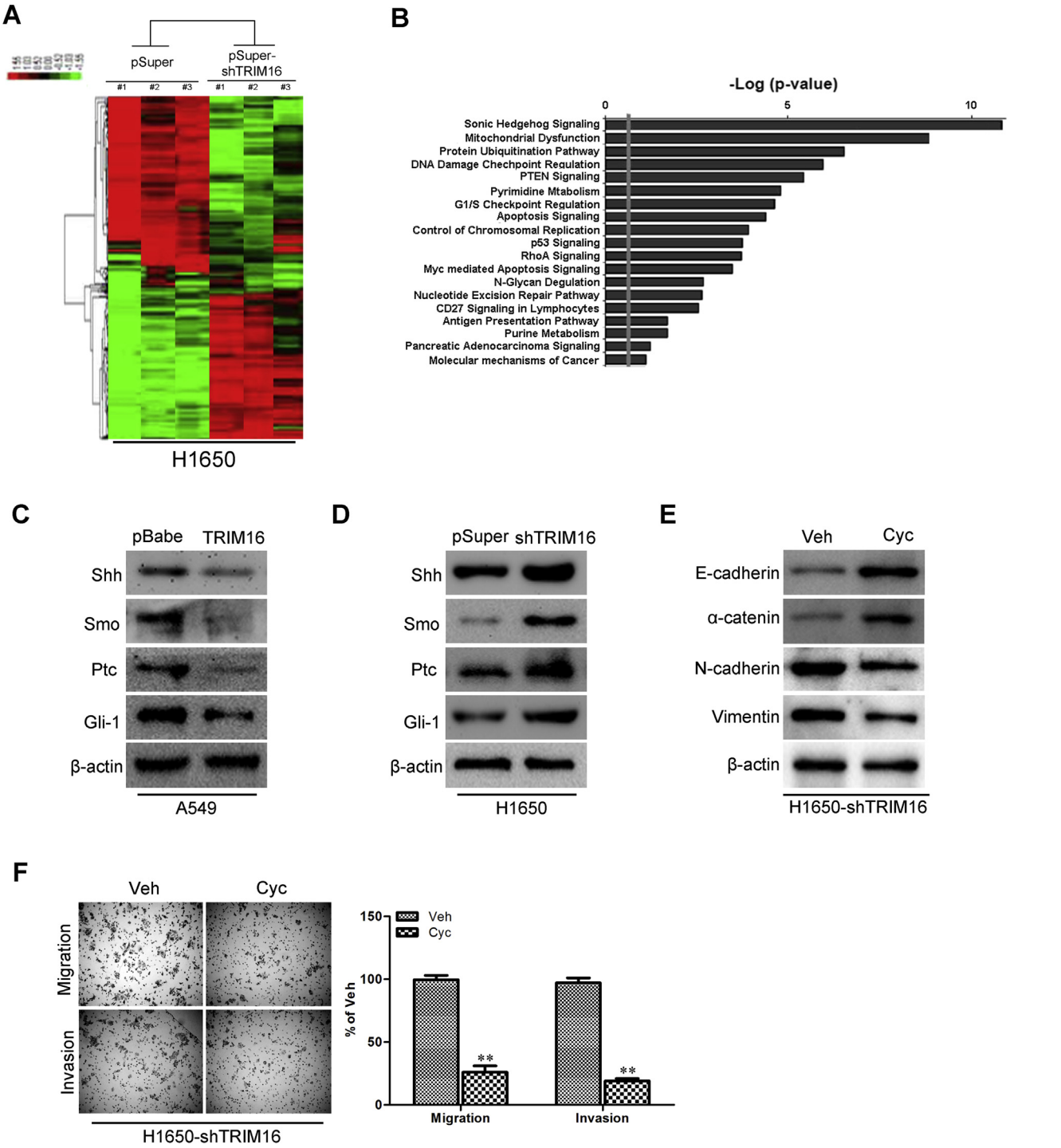

Fig. 4. TRIM16 inhibited lung cancer metastasis via inactivation of the sonic hedgehog pathway. A, supervised hierarchical clustering of the genes differentially expressed after TRIM16 knockdown in H1650 cells. B, gene set enrichment analysis was carried out using ConceptGen. C and D, protein levels of Shh, Smo, Ptc, and Gli-1 were measured in lung cancer cells with TRIM16 overexpression (C) or silencing (D) by Western blot assay. E, protein levels of E-cadherin, α-catenin, N-cadherin, and Vimentin were measured in H1650-shTRIM16 cells treated without or with cyclopamine (10 μM) by Western blot assay. F, H1650-shTRIM16 cells treated without or with cyclopamine (10 μM) were subjected to Transwell migration (top), and Matrigel invasion assays (bottom), quantification of migrated cells through the membrane and invaded cells through Matrigel of each cell line are shown as proportions of their vector controls. **, $P < 0.01$ is based on the Student t test. All results are from three independent experiments. Error bars, SD.

Fig. 3. TRIM16 inhibited migratory and invasive capacities of lung cancer cells in vitro and inhibited metastasis in vivo. A, A549-TRIM16 and its control vector cells were subjected to Transwell migration (top), and Matrigel invasion (bottom) assays, quantification of migrated cells through the membrane and invaded cells through Matrigel of each cell line are shown as proportions of their vector controls. B, H1650-shTRIM16 and its control vector cells were subjected to Transwell migration (top), and Matrigel invasion assays (bottom), quantification of migrated cells through the membrane and invaded cells through Matrigel of each cell line are shown as proportions of their vector controls. C, the numbers of metastatic foci per section in liver of individual mouse with injection of A549-TRIM16 or its control cells. D, the numbers of metastatic foci per section in liver of individual mouse with injection of H1650-shTRIM16 or its control cells. n = 5 for tail vein injection. **, $P < 0.01$ is based on the Student t test. All results are from three independent experiments. Error bars, SD.

Tumor suppressor genes can inhibit tumor invasive and metastatic potential. Previous studies have reported that the expression levels of tumor suppressor genes, such as p53, p21 and PTEN, were decreased in tumors compared with normal tissues [20]. To confirm the tumor suppressor function of TRIM16, we first examined the levels of TRIM16 in lung cancer samples and normal lung tissue samples using IHC. We found that TRIM16 was significantly reduced in lung cancers, which suggested that TRIM16 was a candidate tumor suppressor gene in lung cancer. To further explore the role of TRIM16 in lung cancer, we transfected lung cancer cells either to ectopically express TRIM16 or to inhibit its expression using RNA interference. Knockdown of TRIM16 in vitro significantly enhanced the migration and invasion of lung cancer cells and induced the EMT formation, while overexpression of TRIM16 inhibited cell mobility and EMT. Our in vivo experiments also demonstrated that TRIM16 markedly inhibited metastasis to the liver. These data further supported the tumor suppressor role of TRIM16 in lung cancer.

The activated sonic hedgehog pathway has been demonstrated to have an essential role in lung cancer cell EMT, motility and invasion [21]. Higher expression of sonic hedgehog pathway molecules were observed in lung cancer tissues compared with normal samples, and higher levels of sonic hedgehog pathway molecules were associated with both an increased risk of lymph node metastases and poor prognoses [21]. Sonic hedgehog pathway breakdown the extracellular matrix, contributing to cancer metastasis [21]. Therefore, we investigated whether TRIM16 suppressed EMT and metastasis in lung cancer via inactivation of the sonic hedgehog pathway. Our results indicated that the levels of Shh, Smo, Ptc, and Gli-1 were significantly decreased in TRIM16-overexpressing cells, and Shh, Smo, Ptc, and Gli-1 were upregulated in TRIM16 knockdown cells. When we pretreated the TRIM16 knockdown cells with a sonic hedgehog pathway inhibitor, the increased migration and invasion ability of TRIM16 knockdown cells was inhibited. All of these data revealed that TRIM16 suppresses the migration and invasion activities of lung cancer cells via activation of the sonic hedgehog pathway. However, how TRIM16 might regulate the sonic hedgehog pathway is still unknown. So in our following studies, we will reveal the detailed mechanism about how TRIM16 might regulate sonic hedgehog pathway in lung cancers.

In conclusion, we found that TRIM16 expression was generally lower in lung cancer lesions compared with normal lung tissues. Our in vitro and in vivo data demonstrate that TRIM16 has a vital function in inhibiting cell mobility, which is at least partially controlled by the sonic hedgehog pathway. Thus, we propose that the candidate tumor suppressor gene TRIM16, together with the sonic hedgehog pathway, maybe an effective novel therapeutic target in the management of lung cancer.

Competing interests

The authors have no competing interests to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.144.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.144.

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