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Original Article

Grb2-associated binder-2 gene promotes migration of non-small cell lung cancer cells via Akt signaling pathway

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Abstract: Early stages of non-small cell lung cancer (NSCLC) can be successfully treated by surgical resection of the tumor, but there is still no effective treatment once it is progressed to metastatic phases. Investigation of NSCLC cancer cell migration, metastasis and development of strategies to block this process is essential to improve the disease prognosis. In the present study, we found that GRB2-associated-binding protein 2 (Gab2) is involved in the migration of NSCLC cells and demonstrated that Gab2 disruption impairs NSCLC cells migration. The requirement of Gab2 in the migration of NSCLC was further confirmed by gene silencing in vitro. In corresponding to this result, over-expression of Gab2 significantly promoted the migratory of NSCLC cells. Finally, we found that Gab2 promotes NSCLC migration through the protein kinase B (Akt) signaling pathway and up-regulation the activity of matrix metallopeptidase (MMP)-2/9. To conclude, our findings suggest a novel mechanism underlying the migration of NSCLC cells which might serve as a new intervention target for the treatment of NSCLC.

Keywords: NSCLC, migration, Gab2, Akt

Introduction

Lung cancer is the leading cause of cancer death worldwide and more than 80% of lung cancers are non-small cell lung carcinoma (NSCLC) [1]. This cancer has a very poor prognosis, principally because it is most commonly diagnosed during its later stages, and it is often characterized by aggressive local invasion, early metastasis and poor response to chemotherapy [2]. Cancer metastasis is a series of biologic events that can be envisioned a succession of cell biological changes, including cancer cells separating from original tumor, local invasion through surrounding tissues, intravasation into and transferring through the blood stream, arresting in the parenchyma of distant tissues, formation of small nodules (micro-metastasis), and finally, growth of micrometastatic lesions into macroscopic tumors [3]. Although a variety of metastasis-promoting genes have been recently identified to be related to the metastasis of NSCLC, the molecular mechanisms governing this metastasis process are still not completely understood and the treatment efficiency of metastatic NSCLC has not been significantly improved [4, 5]. Future, continue to identify the key molecules that control tumor deteriorate and development of novel treatments that can block or inhibit migration and/or metastasis is important for improving the prognosis of NSCLC.

The Grb2-associated binder (Gab) proteins including mammalian Gab1, Gab2 and Gab3, comprise a family of scaffolding or docking adaptor proteins. Gab proteins are recruited to activated receptors by direct or indirect mechanisms, mostly indirectly via Gab2 [6]. Recent studies provide evidence that Gab2 plays a critical role in human cancer. Gab2 cooperates with receptor tyrosine kinases and promotes an

invasive phenotype in breast tumorigenesis. Gab2$^{-/-}$ breast cancer cells exhibited decreased migration and impaired extracellular signal-regulated kinases (Erk) activation, suggesting its role in promoting mammary tumor metastasis [7]. In addition to breast tumorigenesis, Gab2 confers an invasive phenotype and drives progression of primary ovarian cancer and enhances their metastatic capability via activation of the protein kinase B (Akt) pathway [8]. Activation of Akt has been linked to mitogenesis, differentiation, survival, migration, invasion, and actin cytoskeletal reorganization. The Akt pathway is a major regulator of matrix metalloproteinases (MMPs) activity, which has been found to correlate with the metastatic potential of tumor cells [9]. Collectively, these results suggest that endogenous Gab2 regulates cell metastasis in a variety of cancers. In contrast, many of the functions of Gab2 in NSCLC progression, migration, and metastasis remain unclear.

In the present work, we reported the identification of a novel role of Gab2 whose disruption impaired the migration of NSCLC cell lines H1975 and H1299. By silencing and over-expression of Gab2, we further confirmed the role of Gab2 in the migration of NSCLC cells in vitro. Further investigation demonstrated that Akt signaling pathway is involved in Gab2-promoted NSCLC cells migration.

Materials and methods

Cell culture and reagents

Non-small cell lung cancer cell lines NCI-H1975, H1299, H1650, H358, H522, H650 cells, and human bronchial normal epithelial cells BEAS-2B were purchased from Cell Resource Center, Shanghai Institutes for Biological Sciences. Cells were cultured in DMEM or 1640 with 10% FBS (Gibco, Invitrogen, USA), and 1% Penicillin/Streptomycin mix (Gibco, Invitrogen, USA) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Specific inhibitor for Akt (GSK690693) was obtained from Selleck (USA). Lipofectamine™ 2000 was purchased from Invitrogen (USA).

Plasmids and DNA constructs

The pDisrup 8 retroviral vector was constructed as described previously, except a ribozyme sequence was incorporated into the vector [10]. Cells infected with pDisrup 8 retroviral vector and were selected with blasticidin S.HCl at 25 μg/ml (Invitrogen, USA). The portion of the endogenous gene that was fused with the blasticidin gene was amplified by the 3' rapid amplification of cDNA ends (3'-RACE) technique [10]. The short small interfering RNA (siRNA) was constructed with a sequence specifically targeted to human Gab2 gene: (5'-AAA CGC UGG UUU AUA CUG CGG-3'). Target and scrambled control oligonucleotides duplexes were cloned into pSilencer4.1-CMV vector (Ambion, USA) according to the manufacturer's instructions. The Gab2 colony was cloned into the sites of EcoRI and XhoI of pIRES2-EGFP vector (Clontech, USA) with gene specific primers. The primer used was as follows: Gab2 (5'-GTGAGAACGATGAGAAATA-3' and 5'-GATGCAGGCCTGACCTTTA-3'). A constitutively active mutant D2Akt (T308D/S473D) plasmid was a gift from Peter Vogt (Addgene plasmid # 49192) [11]. Transient transfection was performed using the Lipofectamine RNAi MAX reagent (Invitrogen) and following the manufacturer's instructions.

Wound healing assay

Briefly, H1975 and H1299 cells were seeded in 60 mm dishes and cultured at 37°C overnight to produce a confluent monolayer. After starvation in serum-free medium for 24 hours, a wound was created by scratching the monolayer with a 200 μl sterile pipette tip. The wounded monolayer was then washed three to remove cell debris and incubated with fresh medium. The area of cell-free scratch was photographed at 0 h and 48 h after scratching respectively. The wound healing effect was determined by measuring the percentage of the remaining cell-free area compared with the area of the initial wound [12].

Migration assay

Migration of cells was determined by BD Transwell Migration Chamber (BD Biosciences, USA) assay in vitro according to the manufacturer's instructions. In brief, 1×10^5 cells with 500 μl in serum-free medium were added into the upper chamber and 750 μl of cells conditioned medium was added into the lower chamber. After incubation in humidified tissue culture incubator, 37°C, 5% CO_2 atmosphere for 24 h, the non-migration cells in the upper surface of the membrane were removed by "scrubbing" with cotton tipped swab and the cells migrating to the lower surface of the membrane

were fixed and stained with 0.5% crystal violet for 30 minutes. Cell counting was then carried out by photographing the membrane through the microscope. Five random fields under microscope were taken and migration cells number were quantified [13].

Western blot

After washing with PBS (3.2 mM Na_2HPO_4, 0.5 mM KH2PO4, 1.3 mM KCl and 140 mM NaCl, pH 7.4) twice, cells were extracted with cold lysis buffer (20 mM Tris, 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 mM PMSF, and Roche complete protease inhibitors) and centrifuged at 15,000 g for 15 min at 4°C. Protein concentration of the supernatants was determined with Bradford assay (Bio-Rad, USA). 30 μg of samples was separated by electrophoresis on 10% SDS-PAGE and transferred to Polyvinylidene Fluoride Membrane (Millipore, USA). After blocking with 5% skimmed milk for 1 h, membranes were incubated with different specific primary antibodies in either 5% skimmed milk or 5% bovine serum albumin (BSA) (anti-Gab2, FAK, phospho-FAK^{Tyr397}, STAT3, phospho-STAT3^{Ser727}, Akt, phospho-Akt^{Ser473}, Src, phospho-Src^{Tyr416}, GSK3β and phospho-GSK-3β^{Ser9} from Cell Signaling Technology). After washing with TBST for 30 min, the membranes were further incubated with corresponding HRP-conjugated secondary antibodies and developed with Pierce's West Pico Chemiluminescence Substrate (Millipore, USA). All results were obtained from 3 independent experiments [14].

Real-time PCR

Total RNA was isolated using TRIzol according to the manufacturer's instructions (Invitrogen, USA) and the concentration of total RNA was detected by spectrophotometry at OD260. Reverse transcription (RT) was carried out using superscript III reverse transcriptase (Invitrogen, USA) as described in the manufacturer's manual. The real-time PCR was performed on ABI Prism 7500 Sequence detection system (Applied Biosystems, CA) with the KAPA SYBR® qPCR Kit (KAPA Biosystems, USA) according to the manufacturer's instructions. The primers used was as follow: Gab2 (Forward: 5'-CTG AGA CTG ATA ACG AGG AT-3', Reverse: 5'-GAG GTG TTT CTG CTT GAC-3'), β-actin (Forward: 5'-GCT CTT TTC CAG CCT TCCTT-3', Reverse: 5'-TGA TCC ACA TCT GCT GGAAG-3'). The target mRNA level of control cells normalized to the level of β-actin mRNA, was defined as 1. Results were obtained from three independent experiments.

Matrix metalloproteins (MMPs) activity assay

The activity of MMP-9 and MMP-2 were determined by QuickZyme MMPs activity assay (QucikZyme BioSciences) according to the manufacturer's instructions [15]. Briefly, after transfection, cells were washed with fresh medium and replaced with serum-free medium. After additional 24 h, the medium was collected and centrifuged at 10000 g for 10 min. Respective supernatant was added to the 96-well strip coated with MMP-9 antibody or MMP-2 antibody and incubated at 4°C overnight. After washing with wash buffer for 3 times, 50 μl assay buffer was added into the well, followed by adding 50 μl detection reagent. After incubation at 37°C for 1 h, OD405 was measured with Microplate Reader (Bio-Tek).

Statistical analysis

For quantitative PCR analyses, results were obtained from triplicate experiments on all the samples and data from all trials were averaged. Numerical results were analyzed using independent mean T-test and expressed in mean ± standard error (SE). Statistical analysis was performed using post hoc testing using Bonferroni's method. Differences were considered statistically significant at $p < 0.05$.

Result

Identification of a novel role of Gab2 in NSCLC cells migration

The NSCLC cell line H1975 is a widely used model to study the metastasis of NSCLC for its high metastatic potential [16]. To identify the key genes involved in H1975 cell mobility and migration, we transfected H1975 cells with pDisrup 8 vector to randomly produce insertions into the genomic DNA, followed by selection with blasticidin to obtain mutated H1975 cell clones. Cellular motility and migration are key hallmarks that distinguish benign from malignant tumors, enabling cells to cross tissue boundaries, disseminate in blood and lymph

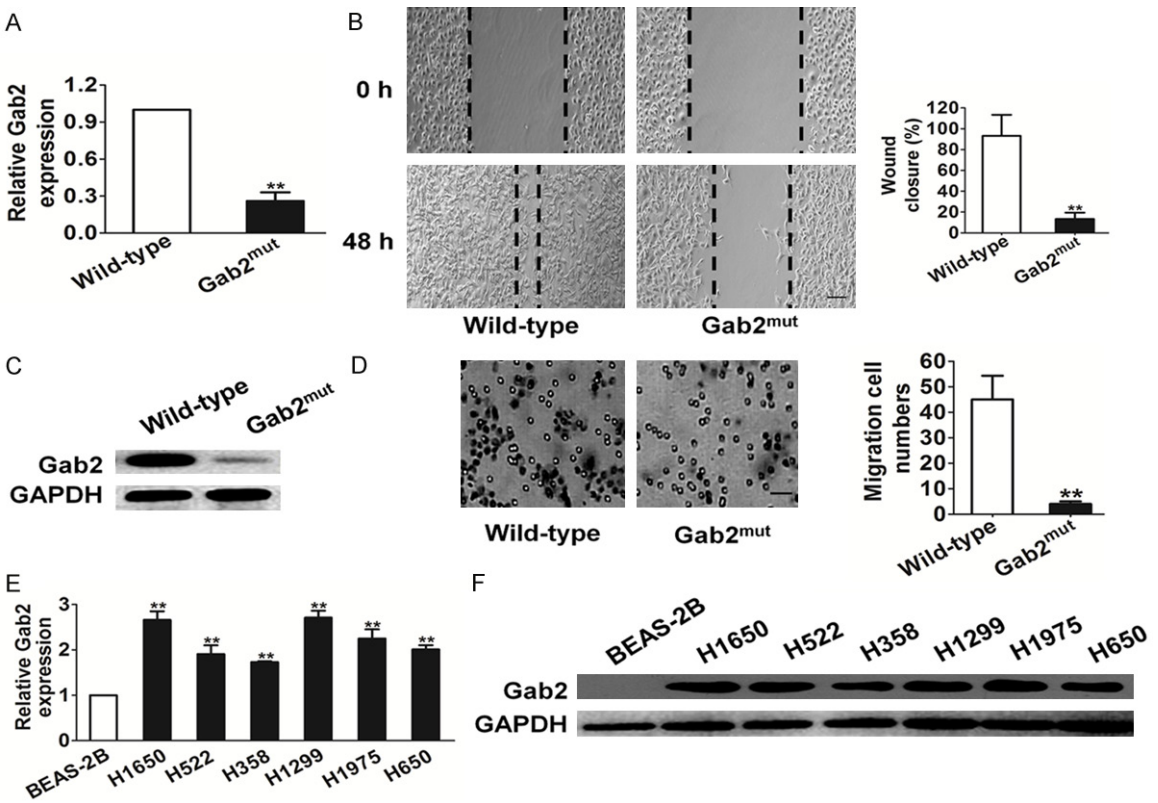

Figure 1. Identification of a novel role of Gab2 in the migration of NSCLC H1975 cells. Gab2 expression in Gab2^{mut} cells was analyzed by real-time PCR (A) and Western blotting (B). (C) Wound healing of control and Gab2^{mut} cells was performed and representative pictures of the wound distance were taken at each time point as indicated. Scale bars: 50 μm. (D) The cell motility was evaluated by transwell assay. Representative pictures were taken after staining with crystal violet. Scale bars: 50 μm. Data are collected from three independent experiments and are average ± S.E. values. $^{**}P < 0.01$, compared to wild type cells. (E) Quantification of Gab2 mRNA levels by qRT-PCR analysis. All NSCLC cells have significant up-regulation of Gab2 mRNA compared with that in the BEAS-2B (Bars are represented as the mean ± S.E, n=3, $^{**}P < 0.01$ versus BEAS-2B). (F) Immunoblotting analysis of Gab2 protein in the NSCLC cell lines and BEAS-2B cell. Gab2 protein expressions are up-regulated in all metastatic NSCLC cell lines examined compared with that in the BEAS-2B.

and establish metastases at distant sites. The mobility and migration ability of the selected mutated cell clones was then determined by wound healing and Transwell migration assay. Finally, cell clones with increased or decreased migration potential were further analyzed by the RT-PCR and 3'-RACE technique to identify the genes disrupted by pDisrup 8 vector. With this strategy, several candidate genes were identified, including a gene named Gab2 and this candidate was designated as Gab2^{mut} which exhibited decreased migration potential.

To verify whether the gene identified by this method was indeed disrupted in H1975 cells, real-time PCR and western blot were carried out to determine Gab2 gene expression. As shown in **Figure 1A** and **1B**, the expression of Gab2 was greatly reduced in Gab2^{mut} cell clone compared to wild-type cells. To determine if loss function of Gab2 affects H1975 migration, we performed wound healing and Transwell assay to evaluate the cell motility. As shown in **Figure 1C**, 48 h after scratching, the area of wound recovered by the migration of Gab2^{mut} H1975 cells was not significant and only less than half of that for control ones. Consistently, there were less Gab2^{mut} cells that migrated across the membrane of the Transwell chamber compared to the wide type cells (**Figure 1D**). In summary, disruption of Gab2 led to reduced H1975 cells mobility and significantly impaired the migration of NSCLC cell H1975.

To investigate the mRNA expression of Gab2, we performed quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis in non-small cell lung cancer cell lines

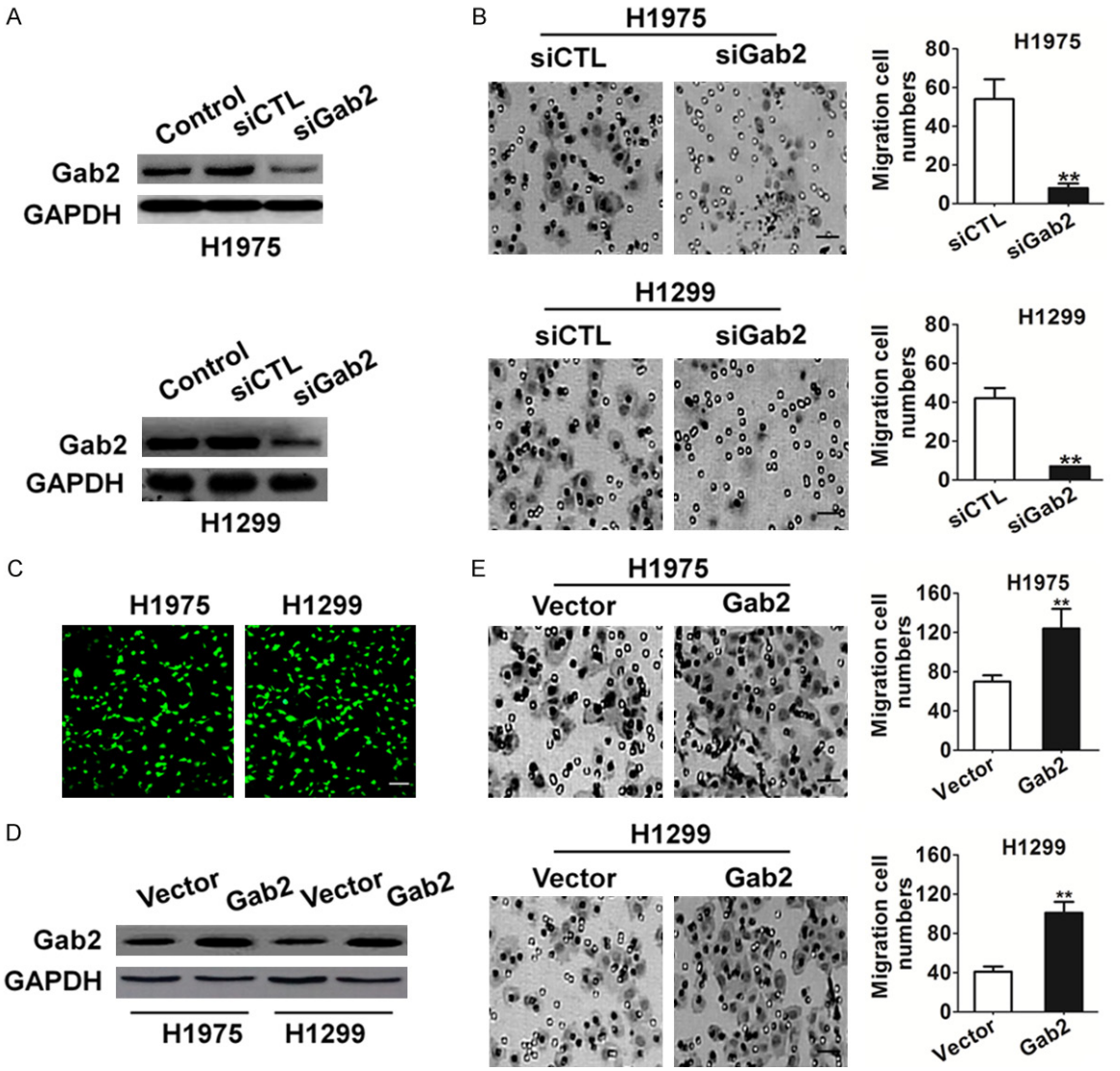

Figure 2. Gab2 silencing decreases the migration of H1975 and H1299 cells. A. Western blot shows that the Gab2 was elevated in control cells, siGab2 and siCTL transfected cells. GAPDH was used as a loading control. B. Both siGab2 and siCTL transfected cells were evaluated by transwell assay. Data are collected from three independent experiments and are average ± S.E. values. $^{**}P < 0.01$, compared to control cells. C. Gab2 was cloned into pIRES2-EGFP vector and transfected into H1975 and H1299 cells. The cells transfected with an empty vector were used as control. D. The transfection efficiency was evaluated by the expression of green fluorescence protein (GFP) and the expression of Gab2 was determined western blot with Gab2 antibody. Scale bars: 100 μm. GAPDH was used as a loading control. E. The cell motility was evaluated by transwell assay and the number of migration cells was quantified. Scale bars: 50 μm. Data are from three independent experiments and are average ± S.E. values. $^{*}P < 0.01$, compared to control cells.

(H1650, H1299, H358, H522, H1975, H650) and human bronchial normal epithelial cells BEAS-2B. Gab2 mRNA was up-regulated in all metastatic NSCLC cell lines compared with that in BEAS-2B (**Figure 1E**). We also performed western blot analysis to investigate the Gab2 protein expression status in NSCLC cells and BEAS-2B. A significant increase in Gab2 protein expression was seen in all NSCLC cell lines compared with BEAS-2B (**Figure 1F**). These analyses indicated that both transcription and translational products of Gab2 were highly expressed in metastatic NSCLC cell lines.

Confirmation of the role of Gab2 in NSCLC cells migration by gene silencing and over-expression

To ascertain Gab2 was indeed responsible for migration in NSCLC cells, we investigated wh-

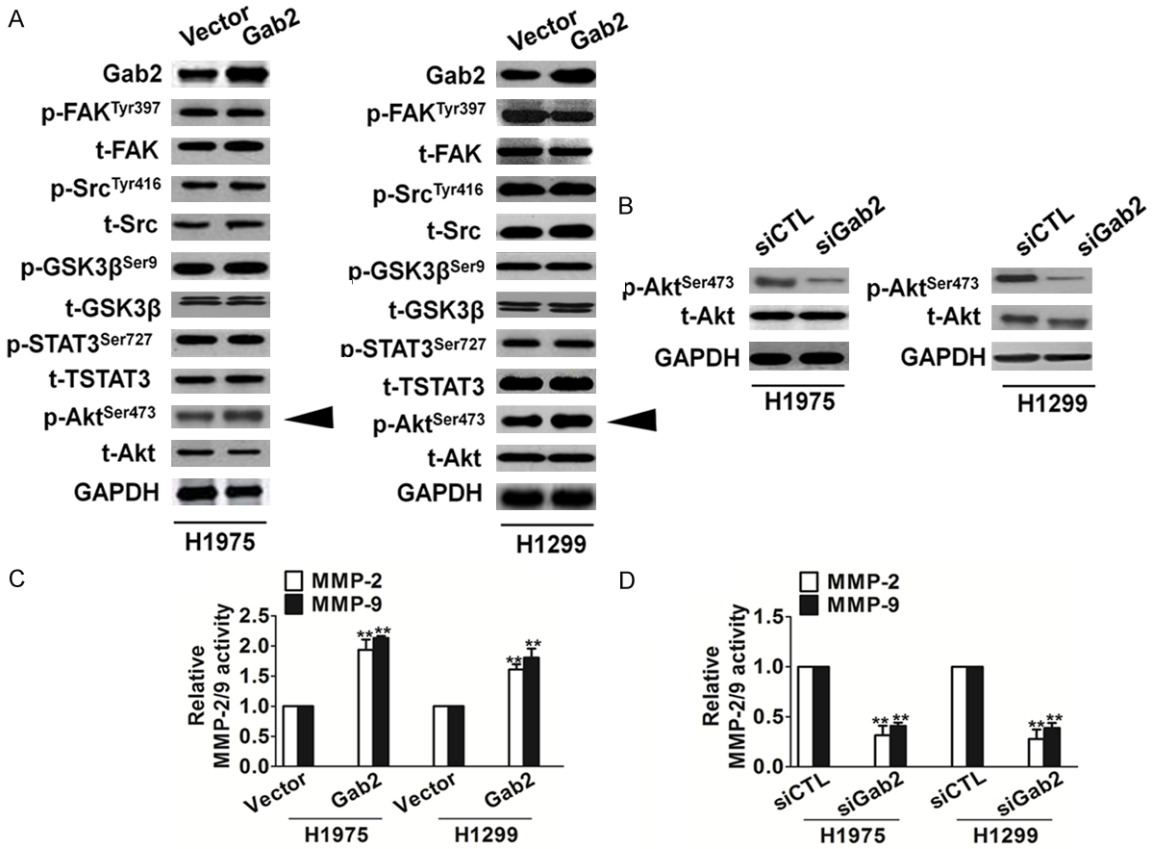

Figure 3. Effect of Akt and MMP avtivity in Gab2 gene silencing and over-expression NSCLC cells. A. Western blot results shown that the phosphorylation of Akt was elevated in cells transfected with pIRES-EGFP-Gab2 or vector. B. The phosphorylation of Akt was perturbed in cells transfected with Gab2 siRNA plasmid. C. Quantification of MMP-2/9 activity in H1975 and H1299 cells transfected with pIRES-EGFP-Gab2. Bars are represented as the mean ± S.E. n=3, $^{**}P < 0.01$ versus vector cells. D. Quantification of MMP-2/9 activity in siGab2 transfected H1975 and H1299 cells. Bars are represented as the mean ± S.E. n=3, $^{**}P < 0.01$ versus siCTL cells.

ether reduced H1975 and H1299 cells migration could be reproduced by gene silencing with siRNA. To perform this experiment, we silenced the expression of Gab2 with a siRNA-incorporated plasmid. As shown in **Figure 2A**, the expression of Gab2 in H1975 and H1299 transfected with siRNA plasmid (siGab2) was significantly decreased compared with the cells transfected with scrambled siRNA (siCTL). Then, the transfected cells were subjected to Transwell assay to evaluate their migratory potential. Transwell assay results showed that both the number of H1975 and H1299 cells transfected with siGab2 moved across the membrane was fewer than the siCTL cells (**Figure 2B**).

To further confirm the role of Gab2 in NSCLC cells migration, we cloned Gab2 into pIRES-EGFP vector and transfected it into H1975 and H1299 cells. The transfection efficiency was confirmed by the expression of green fluorescence protein (GFP) (**Figure 2C**). We also confirmed the over-expression of Gab2 by western blot with Gab2 antibody (**Figure 2D**). The migration of Gab2 over-expressed H1975 and H1299 cells was then examined by migration assay. As shown in **Figure 2E**, there were more Gab2 over-expressed cells migrated across the membrane compared with cells transfected vector. Taken together, these results indicated that silencing of Gab2 could reproduce the effect of Gab2 disruption by pDisrup plasmid and drastically reduced NSCLC cells motility.

Gab2 silencing suppressed Akt signaling and MMPs activity

To determine the signaling pathways which are involved in Gab2-mediated tumor cell migration, multiple potential signaling pathways related to migration of cancer cells were screened. As shown in **Figure 3A**, only the basal level of Akt activation was found to be significantly up-

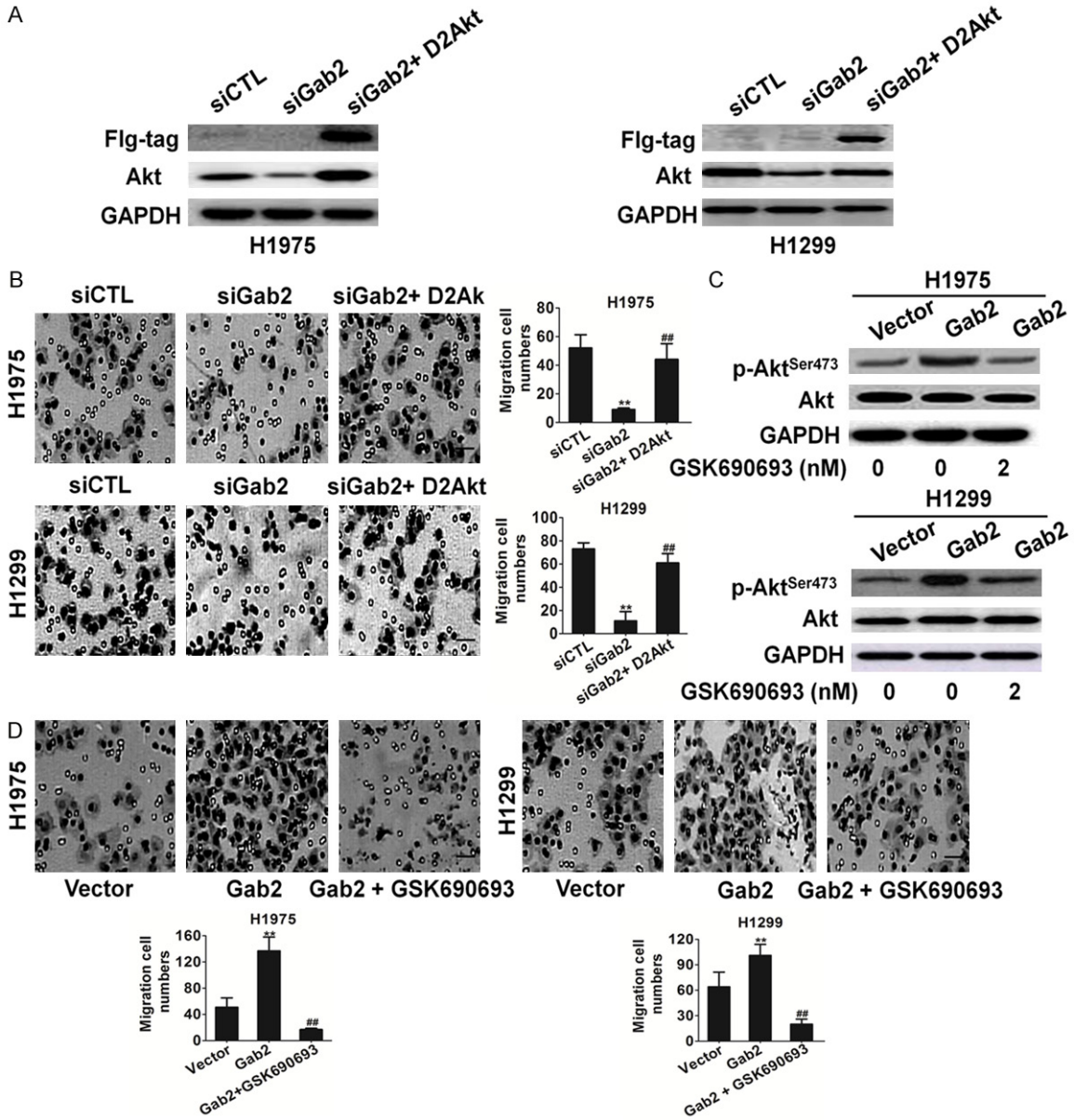

Figure 4. Gab2 facilities the migration of H1975 and H1299 cells via Akt pathway. A. Expression of D2Akt was confirmed by western blot with antibody against Flag-tag and Akt, GAPDH was used as loading control. B. Transwell assay was performed to determine the motility of cells co-transfected with Gab2 silencing plasmid and D2Akt plasmid. Columns are data collected from three independent experiments. Representative pictures were taken after staining with crystal violet. Scale bar represents 50 μm. Data are from three independent experiments and are average ± S.E. values. $^{**}P < 0.01$, compared to siCTL cells; $^{\#\#}P < 0.01$, compared to siGab2 cells. C. In the presence of GSK690693 (2 nM), H1975 and H1299 cells were incubated for 1 h, protein extracts were analyzed by western blot with antibodies against phosphorylated Akt (Ser473) or Akt. D. In the presence of GSK690693 (2 nM), transwell migration assay was conducted to evaluate the cell motility after transfection. Scale bars: 50 μm. Data are from three independent experiments and are average ± S.E. values. $^{**}P < 0.01$, compared to transfected vector cells; $^{\#\#}P < 0.01$, compared to transfected Gab2 cells.

regulated in cells over-expressing Gab2. In contrast, no obvious difference could be observed for many other signaling pathways, such as focal adhesion kinase (FAK), Src, GSK3β and signal transducer and activator of transcription 3 (STAT3). Consistently, when Gab2 was silenced by siRNA in H1975 and H1299 cells, Akt activation was also down regulated in cells (**Figure 3B**).

Taking into account that MMPs such as MMP-2 and MMP-9 can be involved in the development

of several human malignancies, as degradation of collagen IV in basement membrane and extracellular matrix facilitates tumor progression, including invasion, metastasis, and angiogenesis, we analyzed their activity. Quantification of MMP-2 and MMP-9 activities using a fluorogenic assay showed a significantly increase in extracellular MMP-2 and MMP-9 activity in Gab2 over-expressed H1975 and H1299 cells compared to vector cells (**Figure 3C**). Consistently, both MMP-2 and MMP-9 activities were decreased in Gab2 siRNA H1975 and H1299 cells (**Figure 3D**). In combination, these results strongly suggest that Gab2 facilitates the activation of Akt signaling pathway and MMPs activity in NSCLC cells.

Akt was involved in Gab2-mediated H1975 and H1299 cells migration

To confirm the role of Akt in Gab2-mediated cell migration, constitutively active form of Akt (D2Akt) was introduced into Gab2-silenced cells. The expression of D2Akt was confirmed by western blot with anti-Flag and anti-Akt antibody (**Figure 4A**). The migration of cells was then examined by migration assay. As expected, active Akt largely restored the impaired migration in Gab2-silenced H1975 and H1299 cells (**Figure 4B**) and MMPs activity (Figure S1). Furthermore, GSK690693, an Akt specific inhibitor was also employed to dissect the role of Akt signaling in cell migration [17]. As shown in **Figure 4C**, Gab2-mediated phosphorylation of Akt was completely blocked by GSK690693. As a result, Gab2-promoted migration and MMPs activity (Figure S2) in H1975 and H1299 cells were also abolished by GSK690693, as shown by the transwell migration assay (**Figure 4D**). To conclude, these data indicate that Akt signaling is involved in Gab2 promoted migration of NSCLC cells.

Discussion

Malignant non-small cell lung cancer (NSCLC) is the lung cancer with the highest risk of death for its highly metastatic potential. However, there is currently no effective treatment for metastatic NSCLC partly due to the complicated mechanism underlying its metastasis. In the present study, we identified a novel role for Gab2 in the migration of NSCLC cells. We found that Gab2 promotes the migration of tumor cells in vitro through the Akt signaling pathway. Our results may provide a new target for intervention in the NSCLC treatment and may improve the future treatment of NSCLC.

Gab2, a member of the family of Gab scaffolding adaptors, is frequently over-expression in human primary tumors and transmits, amplifies the signals from receptor tyrosine kinases [18]. Great deals of research demonstrate that Gab2 is over-expressed in ovarian, non-small cell lung cancer, gastric cancer and acute myeloid leukemia, and Gab2 is an oncogenic protein [19]. Recently studies shown Gab2 contributes to the invasive and metastatic properties of adenocarcinomas by mediating the epithelial to mesenchymal transition (EMT), the mechanism by which polarized epithelial cells acquire mesenchymal cell properties with an enhanced potential for migration, and modulating both adhesive and anti-adhesive properties of tumor cells. In addition, Gab2 in association with other molecules such as β-catenin, NF-kB p65 or epidermal growth factor receptor (EGFR), regulates transcription of several genes responsible for progression and invasiveness of cancer [20, 21]. So far, no investigation has been carried out on the involvement of Gab2 in non-small cell lung cancer.

In this work, we firstly reported a novel role for Gab2 in the migration of NSCLC cells. We found that the migration of tumor cells was significantly inhibited, when the Gab2 gene was disrupted by insertional mutagenesis. Further investigation with gene silencing of Gab2 showed that the migration of NSCLC cells was significantly decreased as revealed by the Transwell assay. In contrast, the over-expression of Gab2 in H1975 and H1299 NSCLC cells greatly enhanced the migration. All these data presented that Gab2 is involved in the migration of NSCLC cells. The underlying molecular mechanism for Gab2-regulated NSCLC cells migration is identified to be related to Akt signaling pathway. It has been demonstrated that phosphorylated Akt activates specific target genes, including those that encode c-Myc, cyclin D1, and MMP-2/9 [20]. Our results showed that over-expression of Gab2 in H1975 and H1299 non-small cell lung cancer cells induce up-regulation of Akt phosphorylation as well as MMP-2/9 activities. Furthermore, Gab2 silencing leads to reduced Akt phosphorylation and MMP-2/9 activities. Furthermore, restored Akt activity by an active form Akt plasmid could rescue the impaired migration of tumor cells and MMP-2/9 activity induced by Gab2 silencing. In the pres-

ence of GSK690693, Gab2 over-expression promoted migration and up-regulated MMPs level was significantly inhibited as revealed by the Transwell migration assay and MMP activity assay.

In the further work, it is worthwhile to elucidate the precise roles of Gab2 in regulating the Akt signaling thus mediating the migration in NSCLC. All these data presented that Gab2 is involved in the migration of NSCLC cancer cell H1975 and H1299. Due to the important roles of Gab2 in the metastasis of non-small cell lung cancer cells, it may serve as an attractive target for molecular targeting cancer therapy.

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Disclosure of conflict of interest

None.

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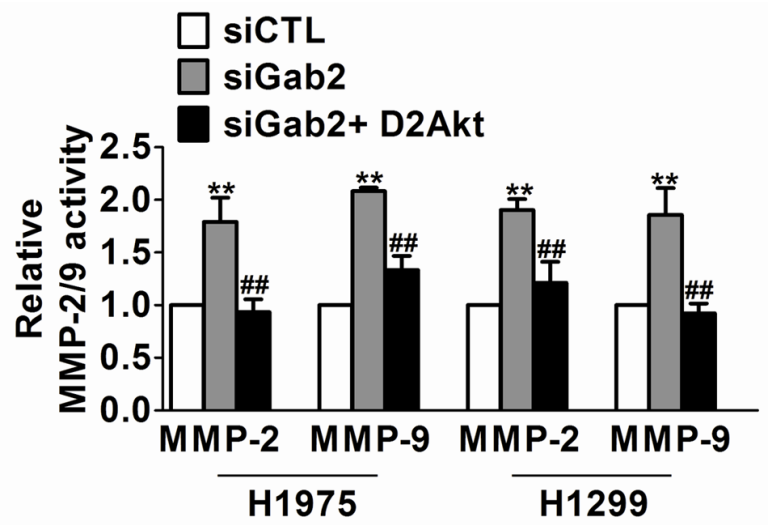

Figure S1. Restored Akt rescued MMP-2/9 activities in H1975 and H1299 cells induced by Gab2 silencing. Quantification of MMP-2/9 activities in cells transfected with Akt plasmid and siGab2 plasmid. Bars are represented as the mean ± S.E. n=3, $**P < 0.01$, compared to siCTL cells; $##P < 0.01$, compared to siGab2 cells.

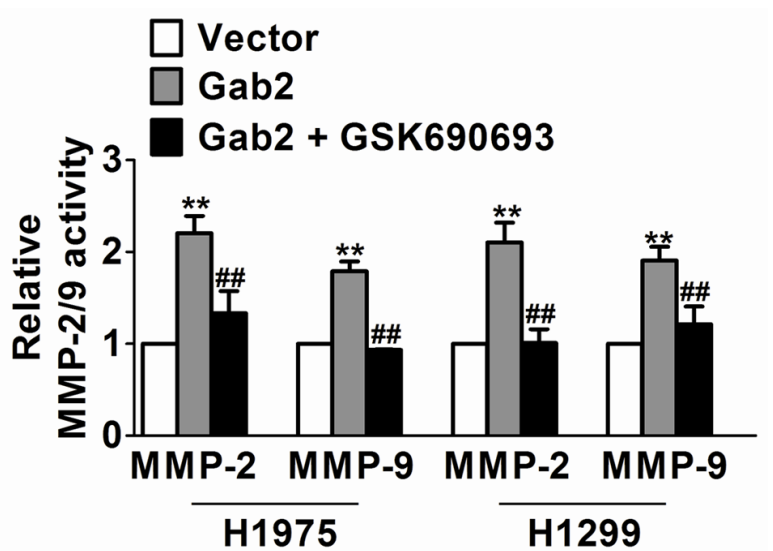

Figure S2. MMP-2/9 activities up-regulated by Gab2 over-expression was inhibited by Akt inhibitor GSK690693. In the presence of GSK690693 (2 nM), quantification of MMP-2/9 activities in H1975 and H1299 cells transfected with pIRES-EGFP-Gab2. Bars are represented as the mean ± S.E. n=3, $**P < 0.01$, compared to transfected vector cells; $##P < 0.01$, compared to transfected Gab2 cells.