Rho Guanine Nucleotide Exchange Factor 5 Increases Lung cancer Cell Tumorigenesis via MMP-2 and CyclinD1 Upregulation

Ping He, Wei Wu, Kang Yang, Deli Tan, Meng Tang, Hongxiang Liu, Tao Wu, Shixin Zhang, Haidong Wang*

Cardiothoracic Surgery Department, Southwest Hospital, Third Military Medical University, Chongqing, China

E-mail Addresses for all authors:

Ping He: yhp0130@sina.com
Wei Wu: wuweiyahoo@sohu.com
Kang Yang: kang_yangsw@126.com
Deli Tan: tdli1029@163.com
Meng Tang: 1205469172@qq.com
Hongxiang Liu: Niumowang2000@sina.com
Tao Wu: Wutao1983.boy@163.com
Shixin Zhang: zhangshixin_2002@163.com
Haidong Wang: WangHDSci@163.com

*Corresponding author:

Haidong Wang
Cardiothoracic Surgery Department,
Southwest Hospital, Third Military Medical University, Chongqing 400038, China
Tel: +86-13883233689
Fax: +86-21-64085875
E-mail: WangHDSci@163.com

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**Key words:** Lung adenocarcinoma; ARHGEF5; MMP2; Tumorigenesis; Cell
Abstract

To elucidate the role of Rho guanine nucleotide exchange factor 5 (ARHGEF5) in tumorigenesis of lung adenocarcinoma cells. ARHGEF5 protein levels were assessed in 91 human lung adenocarcinoma specimens, and A549 and NCI-H1650 cells, by immunohistochemistry and Western blotting. In addition, ARHGEF5 mRNA expression was evaluated by quantitative reverse transcriptase-PCR (qRT-PCR). Furthermore, ARHGEF5 long- and short- isoform co-expression was detected by immunofluorescence. Finally, flow cytometry, CCK8 and wound-healing assays, cell invasion, migration and adhesion, and xenografts were used to evaluate the biological significance of ARHGEF5. ARHGEF5 was significantly increased in lung adenocarcinoma tissues and cell lines. Interestingly, ARHGEF5 levels were significantly associated with tumor grade and pathologic stage but not age, gender, T stage, or lymph node metastasis status. ARHGEF5 knockdown by RNAi resulted in dramatically reduced proliferation, adhesion, invasion, and migratory capability of A549 and NCI-H1650 cells. Likewise, protein levels of p-Src, p-Akt, and NF-κB were significantly decreased after ARHGEF5 knockdown. In parallel, increased S-phase population and MMP2/CyclinD1 expression were observed in the cancer cells, which were not apoptotic. In addition, ARHGEF5 knockdown A549 and NCI-H1650 cells injected subcutaneously and intravenously into nude mice exhibited decreased xenograft volume and overtly reduced metastasis. Conversely, ARHGEF5 overexpression in A549 and NCI-H1650 cells increased their tumorigenicity in vitro. ARHGEF5 acts as a proto-oncogene in human lung adenocarcinoma cell
tumorigenesis.
Introduction

Non-small cell lung cancer (NSCLC), caused by adenocarcinoma cells, is the most common type of lung cancer; its overall 5-year survival rate has remained at 15% for the past two decades. (1) This high mortality is attributed to early metastasis. (2) As cancer cells become metastatic and endothelial cells angiogenic, they develop altered affinity and avidity for their extracellular matrix (ECM). (3) The degradation of basement membranes and stromal ECM are crucial in tumor invasion and metastasis. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases, responsible for ECM degradation. (4) Interestingly, overexpression of ECM-degrading enzymes was observed in metastasis. (5) Indeed, expression of MMP-2 and MMP-9 is associated with high metastasis potential in several human carcinomas. (6) In addition, MMP2/9 upregulation in NSCLC is significantly associated with poor clinical improvement of patients. (7, 8) MMPs are primarily regulated at the transcriptional level through activation protein-1 (AP-1) or nuclear factor-kappaB (NF-κB). (9) Rho guanine nucleotide exchange factor 5 (ARHGEF5) belongs to the Dbl family of guanine nucleotide exchange factors (GEFs) of Rho GTPases. (10) ARHGEF5 has two isoforms encoded by a single mRNA. The short isoform consists of the 519 C-terminal amino acids of ARHGEF5 and is known as Transforming Immortalized Mammary (TIM); TIM mRNA expression is increased in lung carcinoma. (11) TIM is considered a potent RhoA activator in vivo, regulating the RhoA-mediated stress fiber reorganization; in addition, TIM was identified as a potential (11) was shown to play a role in breast tumor progression. (12) The full-length isoform, encompassing the entire ARHGEF5 gene with 1597 amino acids, has been rarely studied. Kuroiwa et al (13) demonstrated that ARHGEF5 enhances thick stress fiber formation by activating RhoA, and plays a key role in Src-induced podosome formation by linking the Src and
PI3K pathways. Interestingly, the Src–ARHGEF5–PI3K complex was detected in highly metastatic colon adenocarcinoma LuM1 cells,(14) but not in modestly metastatic NM11 cells. In addition, ARHGEF5 upregulation was observed in LuM1 cells in contrast to NM11 cells. We have previously demonstrated that co-expression of ARHGEF and src results in shorter patient survival time.(15) These intriguing examples suggest a potential correlation between the ARHGEF5 complex formation and invasive or metastatic ability of cancer cells. Therefore, we inferred that activated PI3K in the ARHGEF5 complex might induce Akt related pathways, such as AP-1 and NF-κB pathways, leading to promotion of tumor metastasis by MMPs or cyclin D1.(16)

The biological relevance of ARHGEF5 is not fully understood. Therefore, we aimed herein to elucidate the biological effect of ARHGEF5 in lung adenocarcinoma. We found a high expression level of total ARHGEF5 protein in most lung adenocarcinoma samples. Interestingly, down-regulation of both short/long ARHGEF5 isoforms inhibited the proliferation and migration potential of cancer cells in vitro and in vivo. These findings suggest a proto-oncogenic role for ARHGEF5 in human lung adenocarcinoma cell tumorigenesis.

Materials and Methods

Tissue specimens, cell lines and animals

Ninety-one tumor tissues were obtained from lung adenocarcinoma patients who underwent surgery from January 2004 to July 2010 at Southwest Hospital of Third Military Medical University. Tissue microarrays and immunohistochemistry (IHC) scoring were performed as previously described.(15) This study was carried out in accordance with the principles of the Helsinki Declaration and approved by the Ethics
Committee of the Third Military Medical University. Written informed consent was provided by each patient.

The human lung adenocarcinoma cell lines A549 and NCI-H1650 were obtained from the American Type Culture Collection (ATCC, USA) in June 2014. A549 cell line was isolated from a 58-year-old male Caucasian and NCI-H1650 cell line was isolated from a metastatic site of pleural effusion of 27-year-old male Caucasian white who suffered from a stage 3B adenocarcinoma of the lung, and maintained in RPMI 1640 or DMEM (Trace, Australia) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C, in a humid environment containing 5% CO2.

Six-week-old female nude mice were purchased from the Experimental Animal Center of Third Military Medical University. Animal care was provided in accordance with the Guidelines for the Care and Use of Laboratory Animals, and all experiments were approved by the Ethics Committee of the Third Military Medical University. Animals were allowed free access to food and water.

Quantitative reverse transcriptase-PCR

Total RNA was extracted from frozen tissue specimens and cultured cells with Trizol (Roche, Germany) following the manufacturer’s instructions. Relative ARHGEF5 mRNA levels were determined by reverse transcriptase (RT)-PCR on a Thermo PCR System (Thermo Fisher, USA). The expression of target genes was normalized to β-actin and assessed by the 2^{-ΔΔCt} method. The following primers were used:

ARHGEF5-F (5’-GGGGGAAAAGTGTGAAATGAAGC-3’), ARHGEF5-F (5’-CGGGGCTTGTAGGCTCGA-3’); MMP2-F (5’-GCGGCGGTCACAGCTACTTC-3’), MMP2-R (5’-TCCGGGTCTTCTAGTTCTCC-3’); Cyclin D1-F
(5’-AGCGCTGTTTTTGTTGTGTGTG-3’), Cyclin D1-R
(5’-CACGCAGCCTCCCAAACAC-3’); β-actin-F
(5’-CGGGGCTTGTAGGCTCGA-3’), β-actin-F
(5’-ACATCTGCTGGAAGGTGGAC-3’).

**Immunoblotting**

Tumor tissues or cells were lysed in lysis buffer containing 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% NP40, 0.5% sodium deoxycholate, and Protease Inhibitor Cocktail Set III (Calbiochem, Germany). Proteins were separated by SDS-PAGE and electro-transferred onto PVDF membranes. After blocking, membranes were incubated with rabbit polyclonal anti-human ARHGEF5 and β-actin antibodies (Abcam, USA), and anti-p-src (R&D, USA), p-Akt, MMP2, NF-κB p65, and CyclinD1 (Santa Cruz, USA). The signals were detected using the enhanced chemiluminescence kit (GE Healthcare Bio-sciences, USA).

**Confocal laser scanning microscopy**

Immunofluorescent staining was performed to localize the expression of ARHGEF5 and Src (R&D, USA). A549 and H1650 cells cultured on glass coverslips were fixed and successively incubated with primary antibodies against human ARHGEF5 (Abcam) overnight at 4°C, and myc (Santa Cruz) or Cy5 (Beyotime, China) conjugated secondary antibodies, at 37°C for 1 hour. Finally, cells were counterstained with Hoechst 33342 and analyzed by confocal microscopy.

**Immunohistochemistry (IHC)**

For immunohistochemistry, anti-ARHGEF5 or Src antibodies were added for 1 hour at 37°C and 4°C overnight. The reactions were developed by EnVision (DAKO, USA); signals were visualized using the diaminobenzidine solution (DAKO, USA) and light counterstaining with hematoxylin. Human pancreas tissues were chosen as
positive controls. The slides were assessed by 2 blinded histopathologists.

Construction of stable ARHGEF5 over-expression and knock-down cell lines

ARHGEF5 was stably over-expressed and knocked down in cells according to methods described previously.(17) ARHGEF5-specific RNAi targeting GATTAAGGTACCGTTGCA (positive-sense strand, 5’-GAUUAAGGUCACCGUGCA-3’; antisense strand, 3’-CUAAUUCCAGUGGCAACGU-5’) was designed. Full-length ARHGEF5 cDNA (5.5 kbp) and RNAi expression vectors were constructed using pMagic 7.1 (Sunbio, China) and transfected into A549 and NCI-H1650 cells (cDNA and RNAi, respectively). Empty virus was transfected into cells in negative control groups (Mock for cDNA and RNAi). The intact A549 or H1650 were considered parental cells (Par). The transfected cells were seeded into a 24-well plate at 1.67×10^5 cells/well, incubated overnight, and infected with lentivirus at a multiplicity of infection (MOI) of 20 and 40, respectively, with polybrene at 5µg/ml. Cells were cultured under selection with 2µg/ml puromycin. Stable expression or knockdown in cells was verified by Western blotting after 3 weeks of growth. As controls, cells were transfected with empty pMagic 7.1 (Par) to generate shControl.

Cell Counting Kit-8 (CCK8) assay

Cell viability was performed using Dojindo Cell Counting Kit-8 (Kumamoto, Japan) according to manufacturer’s instructions. After cell treatment with triptolide at various concentrations for 24, 48, and 72 h, 10 µl tetrazolium substrate was added for 1 h at 37 °C and optical density (OD) was measured at 450 nm on a Fluorescence Spectrophotometer (F-2500, HITACHI, Japan). Cell inhibitory rate was calculated as [1-(OD experiment – OD blank)/(OD control - OD blank)] x 100%.

Cell cycle analysis using flow cytometry
Cancer cells were harvested and fixed in 75% ethanol at 4°C for 24 hours. After incubation with RNase at 37°C, cells were stained with propidium iodide (PI) for 15 minutes and analyzed on a FACScan flow cytometer (BD Biosciences, USA).

**Cell invasion, migration and adhesion assays**

For invasion assays, cells were plated onto matrigel coated chambers. The number of invading or migrated cells was counted in 5 distinct areas at ×400 magnification. Four wells were set per cell line.

Migration assays included wound healing and trans-well chamber assays. For wound healing experiments, A549 and H1650 cells were seeded into 6-well dishes. At 80 - 90% confluence, a sterilized pipette tip was used for wound generation across the monolayer. Cells migrated into the wounded area or protruded from the wound border were visualized and imaged under inverted microscopy. Nine areas were randomly selected in each well at 100× magnification and three wells per cell group were analyzed. For trans-well chamber migration assay, A549 and H1650 cells that migrated into the lower chambers were counted.

For adhesion assays, A549 or H1650 cells were plated at 4×10^4/mL onto fibronectin coated culture dishes, and the attached cells were harvested and quantified. Cell invasion and migration were evaluated in transwells: 1×10^4 cells/well were added in upper chambers and pure fetal calf serum was placed in lower chambers as chemoattractants. After incubation (12h, 37°C) the cells remaining in upper chambers were removed and those that migrated to lower chambers fixed and stained with hematoxylin.

**Xenografts generation**

Nude mice were acclimated for a week and randomly divided into 2 groups to receive ARHGEF5 knock-down (RNAi group) or control (Par group and Mock group) A549
or H1650 cells, injected subcutaneously in the groin (5x10^5 in 0.1 ml serum-free DMEM medium, 5 mice per group) or lateral tail veins (1x10^7 in 0.2 ml serum-free DMEM medium, 10 mice per group). Animals were sacrificed after 4 weeks. Tumor size, defined as 3.14 × length × width × depth)/6 (mm^3), was measured with calipers. Incidence of lung metastasis in nude mice and regional lymph nodes was assessed by an experienced pathologist. IHC was performed for ARHGEF5 detection. Tumor tissues were stained using hematoxylin and eosin (H&E).

**Statistical analysis**

SPSS 19.0 (SPSS Inc., Chicago, IL, USA) was used to perform statistical analyses. The Chi-square and likelihood ratio tests were used to assess the significance of differences recorded in various clinical parameters. The chi-square test was used to assess the association between clinical features and the expression of biological factors. Data were obtained from 3 to 5 independent experiments. P<0.05 was considered statistically significant.

**Results**

**ARHGEF5 is expressed in most human lung adenocarcinoma tissues and cell lines**

To determine the clinical significance of ARHGEF5, patients with ARHGEF5-negative and -positive tumors were compared with respect to demographic (age and gender) and prognostic (tumor size [cm] and stage [I–III]) variables using the chi-square test. For the 91 patients analyzed, the mean age was 58.76±11.94, and 56.04% were males. Of the patients whose staging data were available, resected lesions measured 3.1±0.93 cm. The ARHGEF5 protein was detected mainly in the cytoplasm of tumor cells near the basement membrane (Fig. 1A) and less frequently in adjacent normal tissues (data not shown). A classification by
pathologic staging yielded 35 (38.46%) of stage I, 20 (21.98%) of II, and 36 (39.56%) of III (Table S1). Interestingly, ARHGEF5 over-expression was correlated with tumor grade, pathologic stage I/II/III, with $p=0.026$ and $p=0.044$, respectively. However, patient gender, age, and lymph node metastasis were not correlated with ARHGEF5 expression (Table 1). These findings demonstrated the over-expression in ARHGEF5 in tumor tissues.

We further assessed the expression of ARHGEF5 isoforms in lung adenocarcinoma cells A549 (primary tumor) and H1650 (isolated from metastatic sites). Short length ARHGEF5 isoform expression level was higher in H1650 than in A549. The opposite was obtained for ARHGEF5 long isoform (Fig. 1B). Confocal microscopy revealed that ARHGEF5 long and short isoforms were co-expressed in the cytoplasm of both cell lines (Fig. 1C).

**ARHGEF5 induces cell proliferation but not apoptosis**

As shown in Fig. 2A, 2B, ARHGEF5 was over-expressed in cDNA (overexpression) group and down-regulated in RNAi (knockdown) group compared with Par or Mock group in both A549 and H1650 cells. Cell proliferation was inhibited in the RNAi group and increased after ARHGEF5 over-expression (Fig. 2C, 2D). In agreement, ARHGEF5 over-expression significantly increased cell percentage of S-phase and ARHGEF5 knock-down decreased this parameter, with approximately 25.27 (Par), 21.15 (Mock), 37.66 (cDNA), and 8.73 (RNAi) % in A549 cells, and 27.55, 23.84, 35.66 and 17.55 % for Par, Mock, cDNA, and RNAi groups, respectively, in H1650 cells. Apoptotic cells were scarce (Fig. 2E, 2F).

**ARHGEF5 activates cancer cell adhesion, invasion and migration**

Next, we assessed the effect of ARHGEF5 regulation on cancer cell adhesion. ARHGEF5 knock-down cells (RNAi) exhibited significantly lower invasion and
migration capability compared with controls (Par and Mock cells) at 60 minutes (Fig. 3A-F). In adhesion assays, cells with reduced ARHGEF5 levels (RNAi) showed lower cell adhesion capability (Fig. 3G,3H).

**ARHGEF5 overexpression or silencing results in regulation of signaling effectors**

Next, we determined whether the expression of MMPs, key mediators in ECM degradation and cell migration into surrounding tissues, was regulated by ARHGEF5. We found that ARHGEF5 knock-down by RNAi resulted in the reduction of MMP2 levels, while ARHGEF5 over-expression increased the levels of MMP2. Similar results were obtained for Cyclin D1, an important protein affecting cell cycle and proliferation. Moreover, p-Src, p-Akt and NF-κB presented the same trend in A549 and H1650 cell lines (Fig. 4).

**ARHGEF5 knock down inhibited tumor growth and metastasis in vivo**

To analyze the effect of ARHGEF5 on tumorigenic potential of A549 and H1650, cells transfected with ARHGEF5 RNAi were implanted orthotopically into nude mice. Fig. 5A shows tumor volumes in these mice: cells transfected with ARHGEF5 RNAi significantly reduced tumor growth. Fig. 5B shows H&E staining images of xenograft tumors. IHC assays showed that xenografts from ARHGEF5 knock-down cells exhibited less ARHGEF5 expression (Fig. 5C). Moreover, the incidence of lung metastasis in nude mice subcutaneously injected ARHGEF5 knock-down (RNAi) A549 or H1650 cells was significantly lower than that of Par cells (Table S2).

**Discussion**

ARHGEF5 plays an important role in cancer. However, little is known about its expression pattern in NSCLC and its role in tumor invasion, metastasis, and other aspects of cancer progression. Herein, we demonstrated the elevated ARHGEF5
protein levels in lung adenocarcinoma tissues and cell lines. To our knowledge, this is
the first report showing that ARHGEF5 may contribute to lung adenocarcinoma cell
tumorigenesis by promoting the expression of migration-related MMP2 and
proliferation-related cyclinD1, with subsequent downstream changes in biological
behavior. We further demonstrated that regulation of ARHGEF5 modulates the
p-Akt/NF-κB/MMP2/cyclinD1 pathway, suggesting the p-src/ ARHGEF5/ PI3K/ Akt/
NF-κB/ MMP2/ cyclinD1 axis to be, at least in part, an oncogenic route by which
ARHGEF5 contributes to lung adenocarcinoma tumorigenesis.

In human lung cancer, multiple studies have revealed involvement of the deregulation
of GEFs,(18) such as Tiam1, ECT2, and Vav1-3.(19-21) In agreement, we
demonstrated that ARHGEF5 was highly expressed in lung adenocarcinoma tissues
and cultured cells. In addition, we showed that ARHGEF5 levels were highly
correlated with tumor grade and pathologic stage. Likewise, high Src kinase
activity(22) has been observed in many cancer types, including lung cancer.(23, 24)
Src may stimulate tumorigenesis in NSCLC in a variety of ways, e.g. impacting the
signal transducer and activator of transcription 3 (STAT-3) and focal adhesion kinase
(FAK)-related pathways, both of which are involved in tumor survival.(25, 26) Indeed,
Src activates the VEGF pathway via STAT-3(27) in response to hypoxia in human
lung adenocarcinoma cells, thus increasing blood supply to the oxygen-starved
tumors.(28)

Multiple genetic abnormalities have been observed in NSCLC: activation of the
MAPK pathway, EGFR, and KRAS mutations.(29) These changes may directly affect
lung cancer etiology or indirectly impact the regulation of the cancer-related signaling
network.(30) As a potential oncogene, the role of ARHGEF5 in the signaling network
of cancer is unclear.
Interestingly, ARHGEF5 is able to bind Src and PI3K upon phosphorylation to form a complex in Src-induced podosome formation. (13) Our previous report demonstrated a direct interaction between ARHGEF5 and Src. (15) Herein, we found that long (170 kDa) and short (60 kDa) ARHGEF5 isoforms were co-expressed in lung adenocarcinoma cells A549 and H1650.

TIM is a potent activator of RhoA that also exhibits activity toward Rac1 and Cdc42 in vivo; it also potentiates the serum response factor (SRF)- and AP-1- regulated transcriptional activities, and activates the SAPK/JNK signaling pathway. (11) Moreover, ARHGEF5 is the first RhoA GEF found to be directly regulated by Gi proteins. (31)

Phosphoinositide 3-kinase (PI3K) plays a role in cell proliferation and anti-apoptosis, both of which are crucial to cancer development. (32) AKT is one of the most important downstream targets of PI3K that transmits oncogenic signals and mediates a variety of cellular responses. (33) In present study, we demonstrated that ARHGEF5 regulation modulated p-Akt, which is upstream to NF-κB. Therefore, depletion of ARHGEF5 resulted in decreased activation of NF-κB as shown above. However, the mechanisms underlying ARHGEF5 effect on NF-κB remain unclear. Of note, constitutive activation of NF-κB is significantly correlated with tumor metastasis in various cancers. (34, 35) In addition, stromal cell-derived factor-1 enhanced motility through an ERK and NF-κB dependent pathway in human lung cancer cells. (36)

Compared to control cells, ARHGEF5 overexpression and knockdown cells resulted in, respectively, increase and decrease of proliferation, indicating that ARHGEF5 promotes the proliferative capacity of these cells. It is known that NF-κB binds to cyclin D1 promoter. (26) Since decrease in cellular proliferation could result from increased apoptosis, we examined whether ARHGEF5 depletion alters cell survival in
lung adenocarcinoma cells. Interestingly, ARHGEF5 silencing did not affect apoptosis in A549 and H1650 cells, indicating that ARHGEF5 in lung adenocarcinoma participates in cellular proliferation but not cell survival pathways. Taken together, these data suggest that ARHGEF5 induces proliferation of lung adenocarcinoma cells, likely through cyclin D1 regulation.

Enzymatic degradation of ECM is a crucial step in cancer invasion and metastasis. MMPs play a key role in carcinogenesis. Multiple reports have confirmed that expression of MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) is strongly linked to tumor metastasis in various human cancers and critical for the invasive and metastatic potential of lung carcinoma cells. In the lung, MMP-2 and MMP-9 are produced by a variety of cells. Pardo et al. (1996) reported high gene expression of MMP-2 in type II alveolar epithelial cells (e.g. A549 cells). In addition, MMP-2 and MMP-9 have been associated with increased tumor spread and poor prognosis in lung cancer. Furthermore, previous studies have shown a significant prognostic value for MMP-2 in NSCLC. Interestingly, expression and activation of MMP-2 and MMP-9 are complexly controlled by upstream signaling pathways, such as mitogen-activated protein kinases, integrin, and discoidin domain receptor 2 (DDR2). The transcription of MMP-2 and MMP-9 genes is regulated by upstream regulatory sequences, including NF-κB, AP-1, and Ets-1 binding sites. As shown above, knock-down of ARHGEF5 resulted in decreased levels of MMP2. MMP-9 plays an important role in tumor cell invasion, metastasis and angiogenesis, but no relationship was found between ARHGEF5 and MMP9. It should be noted that MMP expression is modulated by activation of AKT, MAPK, and NF-κB.

In conclusion, ARHGEF5 is expressed at high levels in most human lung adenocarcinoma, and its over-expression increased proliferation and invasion of
cancer cells in vitro, reflecting its action on growth and metastasis of xenograft tumors in vivo. In addition, the present work suggests a plausible mechanism where Src-ARHGEF5-PI3K activates the p-Akt pathway and increases NF-κB levels, leading to MMP2 and CyclinD1 up-regulation. Finally, our findings provide further insight into the PI3K/Akt signaling pathway and NF-κB transcriptional activity in A549 and H1650 cancer cells. Overall, ARHGEF5 may promote lung adenocarcinoma cells and therefore constitutes a candidate molecular target for cancer therapy.

Acknowledgments

None.
References


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Figure legends

Figure 1. ARHGEF5 is expressed in most human lung adenocarcinoma tissues and lung adenocarcinoma cell lines. A, representative ARHGEF5 immunohistochemical staining images of primary human lung adenocarcinoma tissues. ARHGEF5 positive cells appear brown in the cytoplasm. The slides were counterstained with hematoxylin. Magnitude, Top: ×50; Bottom: ×200. B, protein levels of ARHGEF5 isoforms were determined by immunoblot in lung adenocarcinoma A549 and H1650 cell lines. Top: proteins in total cell lysates were resolved by SDS-PAGE. β-actin was blotted as the loading control. Bottom: protein expression levels were analyzed by densitometry using the Image J software. Each bar represents mean±standard error (SE) of three independent experiments. C, lung adenocarcinoma A549 and H1650 cells were cultured on cover-slips and stained with anti-h-ARHGEF5 (short isoform, 60 kDa) and anti-h-ARHGEF5 (long isoform, 170 kDa) antibodies. Nuclei were revealed by Hoechst 33342. Top: A549 cell lines. Bottom: H1650 cell lines. Scale bars represent 50µm.

Figure 2. Proliferative effect of ARHGEF5 knockdown (RNAi) and over-expression (cDNA) in A549 and H1650 cells. A-B, cell protein levels of ARHGEF5 isoforms were determined by immunoblot in A549 and H1650 cell lines transfected with ARHGEF5 knockdown (RNAi) or over-expression (cDNA) plasmids. Top: proteins in cellular lysates were resolved by SDS-PAGE. Bottom: relative intensity (A, A549; B, H1650). β-actin was used as an internal control. (C, A549; D, H1650; * P < 0.05 versus Par). E-F, Top: cell cycle stage and cellular DNA diploidy were assessed using PI staining and flow cytometry (E, A549; F, H1650).
Figure 3. Invasion, migration and adhesion of A549 and H1650 cells after knockdown (RNAi) or over-expression (cDNA) of ARHGEF5. A-B, cells were plated onto matrigel coated chambers and after 60 minutes, the number of invasion cells was counted in 5 distinct areas at ×400 magnification. Cells that migrated from matrigel coated culture dishes to the lower chambers are shown: A, A549; B, H1650 (*P<0.05, **P<0.01 versus Par). C-D, A549 and H1650 cells were seeded into 6-well dishes and wound healing assays were performed. A sterilized one-milliliter pipette tip was used for wound generation across the cell monolayer. Migration of cells into the wound was then observed at different time points. Magnitude, ×100. Cells that migrated into the wound are shown: C, A549; D, H1650 (*P<0.05, **P<0.01 versus Par). E-F, cell migration was determined using a transwell assay. Cells that migrated into the lower chambers were counted and a migration graph was plotted for different cell transfectants. G-H, cells were plated onto fibronectin coated plates and adherent cells were counted (E, A549; F, H1650; *P<0.05, **P<0.01 versus Par).

Figure 4. ARHGEF5 regulates the expression of signaling proteins. A-B, mRNA levels of ARHGEF5, MMP2 and CyclinD1 in Par, Mock, RNAi and cDNA groups, were determined by RT-PCR. Relative expression levels: A, A549; B, H1650 (*P<0.05, **P<0.01 versus Par). C-D, protein levels in ARHGEF5, MMP2, CyclinD1, phosphorylate-Src, phosphorylate-Akt and NF-κB in Par, Mock, RNAi and cDNA groups were determined using immunoblot assays. Relative band intensities: C, A549; D, H1650 (*P<0.05, **P<0.01 versus Par). β-actin was used an internal control.

Figure 5. ARHGEF5 modulates tumor growth in vivo. A, Par, Mock and RNAi groups for A549 and H1650 cells were injected subcutaneously into nude mouse groin
(5x10^5 in 0.1 ml serum-free DMEM) or lateral tail veins (1x10^7 in 0.2 ml serum-free DMEM), and the animals were sacrificed after 4 weeks. The volumes of xenograft tumors were calculated and growth curves plotted. Top: A549; Bottom: H1650. ** P<0.01 versus Par group. B, xenograft tumors were cut into slices and H&E staining was performed (Magnitude, ×50). Arrow: lung metastasis tumor. Left: orthotopic xenograft tumors derived from the A549 group. Right: orthotopic xenograft tumors derived from the H1650 group. C, immunohistochemical staining for ARHGEF5 in xenograft tumors (Magnitude, ×200).
Fig 1

A

No staining  Strong staining  Moderate staining  Weak staining

B

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<thead>
<tr>
<th></th>
<th>A549</th>
<th>H1650</th>
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<tbody>
<tr>
<td>Long isoform</td>
<td></td>
<td>170 KDa</td>
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<tr>
<td>Short isoform</td>
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<td>60 KDa</td>
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<td>β-actin</td>
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C

Relative Intensity (a.u.)

H1650

<table>
<thead>
<tr>
<th>A549</th>
<th>H1650</th>
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<tr>
<td>anti-h-ARHGEF5 (60KDa)</td>
<td>anti-h-ARHGEF5 (170KDa)</td>
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**Figure 4**

**(A)** Western Blot Analysis of ARHGEF5, MMP2, CyclinD1, and β-actin in Par, Mock, RNAi, Mock, and cDNA conditions.

**(B)** Relative intensity graph showing the expression levels of ARHGEF5, MMP2, and CyclinD1. The bars are labeled with Par, Mock, RNAi, Mock, and cDNA, with error bars indicating standard deviation.

**(C)** Western Blots for p-src, p-Akt, NF-κB, MMP2, and β-actin under Par, Mock, RNAi, Mock, and cDNA conditions.

**(D)** Western Blots for p-src, p-Akt, NF-κB, MMP2, CyclinD1, and β-actin under Par, Mock, RNAi, Mock, and cDNA conditions, with asterisks indicating statistical significance.
Molecular Cancer Therapeutics

Rho Guanine Nucleotide Exchange Factor 5 Increases Lung cancer Cell Tumorigenesis via MMP-2 and CyclinD1 Upregulation

Ping He, Wei Wu, Kang Yang, et al.

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