

TRAF4 Regulates Migration, Invasion, and Epithelial–Mesenchymal Transition via PI3K/AKT Signaling in Hepatocellular Carcinoma

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Overexpression of the tumor necrosis factor receptor-associated factor 4 (TRAF4) has been detected in many cancer types and is considered to foster tumor progression. However, the role of TRAF4 in hepatocellular carcinoma (HCC) remains elusive. In this study, we found that TRAF4 was highly expressed in HCC cell lines and HCC tissues compared with normal liver cell lines and adjacent noncancerous tissues. TRAF4 overexpression in HCC tissues was correlated with tumor quantity and vascular invasion. In vitro studies showed that TRAF4 was associated with HCC cell migration and invasion. An in vivo study verified that TRAF4 overexpression facilitated metastasis in nude mice. In addition, overexpressed TRAF4 promoted the phosphorylation of Akt and induced Slug overexpression, leading to downregulated E-cadherin and upregulated vimentin, while silencing TRAF4 moderated the phosphorylation of Akt and repressed the expression of Slug, which resulted in upregulated E-cadherin and downregulated vimentin. These effects were inverted after pretreatment of the PI3K/Akt inhibitor LY294002 or overexpression of constitutively active Akt1. Our study demonstrated that TRAF4 was involved in promoting HCC cell migration and invasion. The process was induced by the EMT through activation of the PI3K/Akt signaling pathway.

Key words: Tumor necrosis factor receptor-associated factor 4 (TRAF4); Hepatocellular carcinoma (HCC); Epithelial–mesenchymal transition (EMT); PI3K/Akt signaling pathway

INTRODUCTION

Hepatocellular carcinoma (HCC), accounting for 90% of all primary liver neoplasias, is the fifth most common cancer and the third leading cause of cancer-related deaths throughout the world^{1–3}. The characteristic of high recurrence after surgery and the limitation of surgical management for patients with recurrent HCC or advanced HCC lead to a dismal survival outcome^{4,5}. Molecular therapies precisely targeting signaling pathways that participate in HCC development, progression, and metastasis are therefore promising prospects in the improvement of prognosis.

The tumor necrosis factor receptor-associated factor (TRAF) family, composed of seven members (TRAF1–7), serves as a signal transducer through binding tumor necrosis factor receptors (TNFRs) and interleukin-1/Toll-like receptors (IL-1R/TLRs)^{6,7}. A distinct structural feature of TRAF proteins is the presence of a carboxy-terminal

TRAF domain, which consists of an N-terminal coiled-coil region (N-TRAF) and a C-terminal β -sandwich (C-TRAF)⁸. Their main function is associated with diverse biological processes including adaptive and innate immunity, stress responses, inflammation, and bone metabolism, and they play an important role in regulating cell survival, proliferation, and differentiation^{6,9}.

As a unique member of the TRAF family, TRAF4 is the only one containing a nuclear localization signal (NLS) and possessing three CART domains (cystein-rich domain associated with RING and TRAF domain)¹⁰. Moreover, the three residues R, Y, and S, which are involved in the recognition of the cytoplasmic TRAF member interacting motif (TIM) of the TNFR, are replaced, respectively, by S, F, and F in TRAF4, leading to its reduction in interaction with most members of the TNFR family⁷. A functional distinction between TRAF4 and other TRAF

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proteins is that TRAF4 does not seem to play a role in the immune system, as immunological defects have not been detected in mice lacking TRAF4^{11,12}. TRAF4 is crucial for early embryogenesis and central nervous system myelin homeostasis^{7,13}. Upregulated TRAF4 can be observed during development. TRAF4 deficiency is embryonically lethal or, if not lethal, causes severe developmental defects in the trachea, skeleton, and nervous system^{11,12}.

TRAF4, as the first member of the TRAF family to be found in human carcinoma, was initially identified in breast cancer from a differential screen between metastatic lymph node and benign fibroadenoma biopsies¹⁴. Thereafter, TRAF4 overexpression was detected in 43% of 623 patients with 20 different tumor types through immunohistochemistry (IHC) analysis¹⁵. Recently, several studies explored the function of TRAF4 in breast cancer, lung cancer, and oral squamous cell carcinoma (OSCC), which demonstrated the participation of TRAF4 in carcinogenesis^{16–18}. Furthermore, cytoplasmic TRAF4 expression in breast cancer patients was significantly linked to a poor prognosis¹⁹. However, the role of TRAF4 in HCC remains poorly elusive.

In this study, we found that elevated expression of TRAF4 can be observed in HCC cell lines and HCC tissues. TRAF4 overexpression was associated with multiple tumor numbers and vascular invasion. In *in vitro* and *in vivo* studies we demonstrated that TRAF4-dependent cell migration and invasion occurred in HCC, which was at least partially relevant to the epithelial–mesenchymal transition (EMT), and was regulated through the PI3K/Akt signaling pathway.

MATERIALS AND METHODS

Tissue Samples

Sixty pairs of HCC and corresponding adjacent normal liver tissues were randomly collected with written consent between October 2014 and December 2015 at the Department of Hepatobiliary Surgery, the Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University. All sample collections were conducted after the approval of the ethics committee of the Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University according to the 1975 Declaration of Helsinki.

Cell Lines and Cell Cultures

Six human HCC cell lines including HepG2, Huh7, SMMC-7721, Sk-hep1, MHCC97H, and Hep3B, the hepatic stellate cell line, and the immortalized normal human liver cell line L02 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, P.R. China). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum

(FBS; Biological Industries, Israel) at 37°C with 5% CO₂. Specific inhibitor LY294002 (Cell Signaling Technology, Danvers, MA, USA) soluble in dimethyl sulfoxide (DMSO; MP Biomedicals, Solon, OH, USA) at a concentration of 50 µM was used for 30 min to inhibit PI3K/Akt activation.

Lentivirus Construction and Infection

TRAF4 overexpression, TRAF4 knockdown, and Akt1 overexpression lentivirus plasmids and their negative control lentivirus were purchased from GenePharma Company (Shanghai, P.R. China). The knockdown sequences are 5'-TTCTCCGAACGTGTCACGT-3' (NC-shRNA) and 5'-GTATGGCCTAGATGTTTCATA-3' (TRAF4 shRNA). The lentivirus was transfected into HepG2, and Sk-Hep1 cells with an optimal multiplicity of infection (MOI) of 30 TU/ml together with 5 µg/ml polybrene. At 36 h after infection, infected cells were selected with 3 µg/ml puromycin. Transfection efficiency was confirmed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blotting.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated using TRIzol reagent (TaKaRa, Japan) according to the manufacturer's instructions. Real-time PCR was performed on a LightCycler 480 Real-Time PCR system (Roche, Indianapolis, IN, USA) using SYBR Premix Ex Taq (TaKaRa). Primers of TRAF4 and GAPDH are as follows: TRAF4, 5'-CATCCAGAGCCACCAGTACC-3' (forward) and 5'-TTGATGGGCAGAGCACC-3' (reverse); GAPDH, 5'-AAGAAGGTGGTGAAGCAGG-3' (forward) and 5'-GTCAAA GGTGGAGGAGTGG-3' (reverse); primers of E-cadherin, vimentin, and Slug were used as previously described^{20,21}. The relative level of mRNA was normalized to GAPDH. Triplicate RNA samples were used for each experiment, and three independent experiments with statistical analysis were performed.

Western Blotting

Cells cultured in six-well plates in an exponentially growing phase were washed three times with ice-cold PBS before being lysed in 0.1 ml of RIPA buffer (Beyotime, P.R. China) containing protease inhibitor cocktail and phosphatase inhibitors (Beyotime). After incubation on ice for 30 min, cells were centrifuged at 4°C for 20 min at 14,000×g, and supernatants were extracted. Protein concentration was quantified with a BCA protein Assay Kit (ComWin Biotech, Beijing, P.R. China). Extractions were boiled with sample loading buffer (Beyotime). The extractions containing 30 ng of protein samples were loaded onto 10% or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and

electrophoresed. After being separated, the proteins were transferred to 0.45 μm of polyvinylidene fluoride (PDVF) membranes (Millipore, Boston, MA, USA). The membranes were blocked with 5% skimmed milk in Tween/Tris-buffered salt solution [20 mmol/L Tris (pH 7.0), 0.15 mol/L NaCl, and 0.1% Tween 20] for 60 min at room temperature and subsequently incubated overnight at 4°C with primary antibodies directly against TRAF4 (dilution 1:1,000; Abcam, Cambridge, MA, USA); E-cadherin, vimentin, Slug, p-Akt (Ser473), pan-Akt, β -actin, and GAPDH (dilution 1:1,000; Cell Signaling Technology). The PVDF membranes were washed three times for 5 min each with 15 ml of Tween/Tris-buffered salt solution and then incubated with specific horseradish peroxidase-conjugated secondary antibodies (dilution 1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 60 min at room temperature. The membranes were washed again, as mentioned previously, followed by the detection of specific antibody-antigen complexes using Immobilon Western Chemiluminescent HRP Substrate (Millipore). GAPDH protein served as an internal loading control. Intensity of bands was evaluated by the ImageJ software, version 1.49e (National institutes of Health, Bethesda, MD, USA), and each experiment was repeated three times with statistical analysis.

Histology and Immunohistochemistry

Paraformaldehyde-fixed paraffin-embedded sections (4 μm thick) was used for histology and IHC. Sections were deparaffinized twice using xylene for 20 min each, then rehydrated with a graded series of washes in ethanol, and finally rinsed in PBS. Sections were stained with hematoxylin and eosin (H&E) (Beyotime) for mouse lung structure. For IHC, rehydrated sections were treated in TE (10 mM Tris/1 mM EDTA, pH 9.0) at sub-boiling temperature for 20 min and cooled at room temperature and subsequently incubated with 3% hydrogen peroxide for 10 min at room temperature. After washing twice for 5 min each, sections were blocked with 200 μl of normal goat serum (ZSGB-BIO, Beijing, P.R. China) for 1 h at 37°C, and then incubated with 200 μl of TRAF4 antibody (Novus, Littleton, CO, USA) at a dilution of 1:1,000 overnight at 4°C. Afterward, tissue sections were washed with PBS three times and incubated with a biotinylated secondary antibody and peroxide-conjugated streptavidin working solution for 30 min each, and then stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (ZSGB-BIO). The staining results were blindly assessed by two researchers according to the score criteria described previously²⁰.

Cell Migration and Invasion Assays

For cell migration assay, 1×10^5 cells were seeded per Transwell insert (Corning, Corning, NY, USA) filled with

200 μl of nonserum medium. Afterward, inserts were dipped in 800 μl of medium and incubated at 37°C with 5% CO₂ for 24 h. Finally, cells were fixed in 4% paraformaldehyde for 30 min and stained with 1% crystal violet. Cells on the upper side of the insert were removed with a cotton swab. Six microscopic fields were captured using a digital camera per insert, and cell numbers were counted using the ImageJ software, version 1.49e (National institutes of Health). Each experiment with duplicate inserts was repeated three times with statistical analysis.

For invasion assay, 100 μl of Matrigel mixture (20 μl of Matrigel diluted in 80 μl of ice-cold nonserum medium; BD Biosciences, San Jose, CA, USA) was placed into the Transwell chambers and then incubated at 37°C with 5% CO₂ for 6 h. After the solidification of Matrigel, residual DMEM was extracted, and 1×10^5 cells suspended in 200 μl of serum-free DMEM were placed into the Transwell chambers. The same procedures were followed as with the migration assay. Incubation at 37°C with 5% CO₂ in this assay was for 36 h.

In Vivo Tumor Metastasis Assay

Male BALB/c-nude mice (6 weeks old) purchased from the Medical Science Experimentation Center of Sun Yat-sen University (Guangzhou, P.R. China) were used for animal studies. The animal study was approved by the Institutional Animal Care and Use Committee of Sun Yat-Sen University, and all animals were cared for according to institutional guidelines. Cells were suspended in PBS at a concentration of 1×10^7 cell/ml. Cell suspension (100 μl) was injected into tail veins of mice, and after 30 days of inoculation, the mice were sacrificed to detect the efficiency of metastasis.

Statistical Analysis

All continuous data were expressed as mean \pm standard deviation (SD), and differences between groups were analyzed using two-sided Student's *t*-test or one-way analysis of variance. Categorical variables were expressed as absolute numbers and compared between groups using the chi-square test. A value of $p < 0.05$ was considered as statistically significant. Statistical analyses were performed using the SPSS Statistics version 21.0 (IBM SPSS).

RESULTS

TRAF4 Is Overexpressed in HCC Cell Lines and HCC Tissues

We first verified TRAF4 expression in HCC cell lines. TRAF4 expression in an immortalized nontumorigenic hepatocyte cell line L02, hepatic stellate cell line (HSC), and HCC cell lines (HepG2, Huh7, Sk-hep1, Hep3B, SMMC-7721, and MHCC-97H) were analyzed using qRT-PCR and Western blotting. Results showed that at

the mRNA and protein levels, TRAF4 expression was prominently upregulated in HCC cell lines compared with that in a nontumorigenic hepatocyte cell line LO2 and HSC ($p < 0.05$) (Fig. 1A and B).

To further confirm whether TRAF4 expression is associated with human HCC, we detected the TRAF4

expression in 60 pairs of HCC tissues and corresponding tumor-adjacent samples (at least 1.5 cm away from the tumor) using IHC. Results showed that TRAF4 expression was evidently higher in HCC samples than that in noncancerous adjacent tissues. In addition, the expression of TRAF4 was significantly higher in HCC with

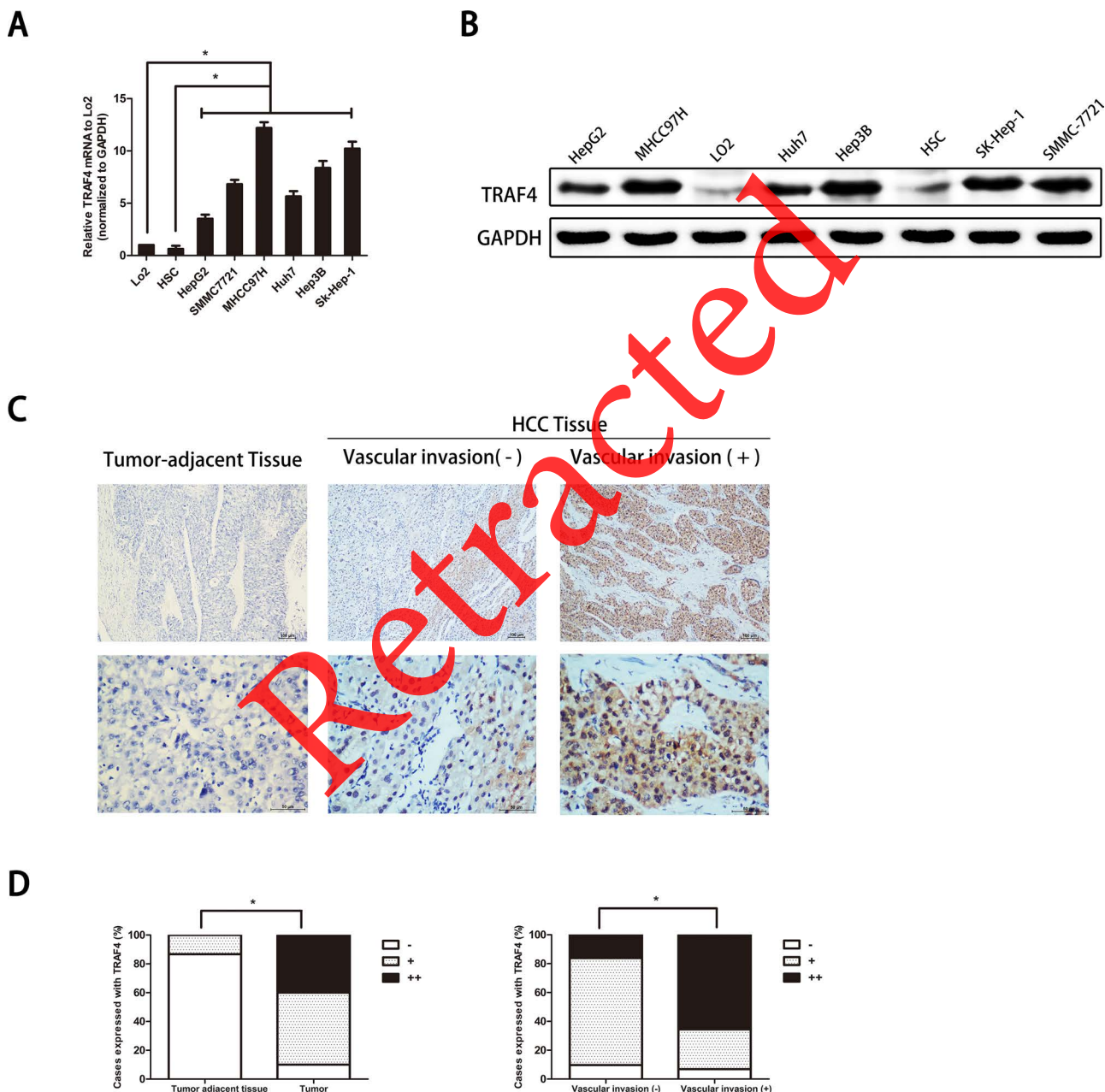


Figure 1. Tumor necrosis factor receptor-associated factor 4 (TRAF4) is overexpressed in hepatocellular carcinoma (HCC) cell lines and HCC tissues. (A) TRAF4 is overexpressed in HCC cell lines at the mRNA level. Data are presented as the mean \pm standard deviation (SD); $n = 9$. $*p < 0.05$. (B) TRAF4 is highly expressed in HCC cells at the protein level. (C) Representative immunohistochemistry images show that TRAF4 is more highly expressed in HCC tissues than in adjacent tumor tissues. Stronger stain intensity and higher stain density exist in HCC tissues with vascular invasion than in those without vascular invasion. (D) Quantitative charts of TRAF4 expression in tumor-adjacent tissues and HCC tissues are shown. The expression of TRAF4 is correlated with the incidence of HCC and vascular invasion. Data are presented as the percentage. $*p < 0.05$.

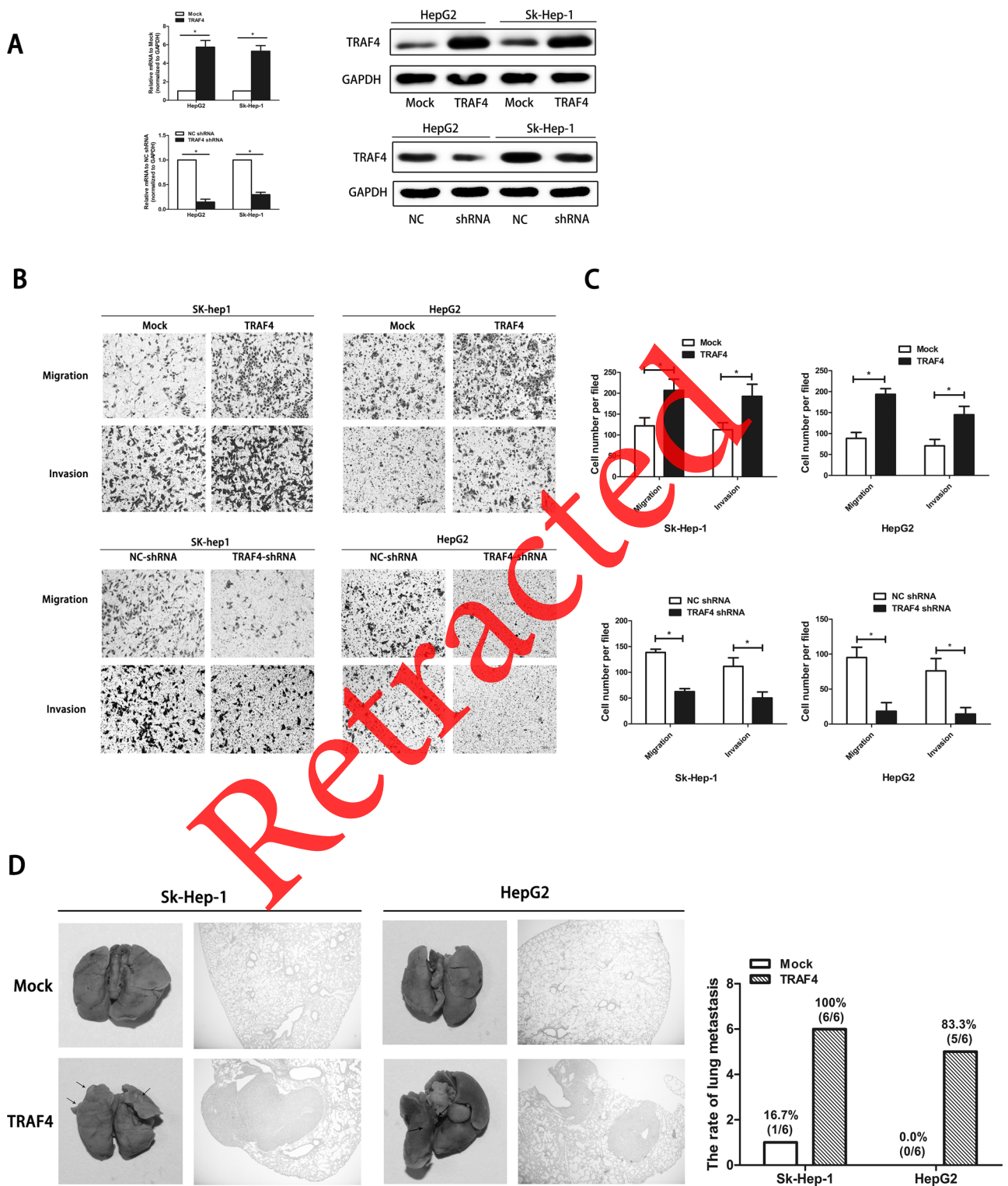
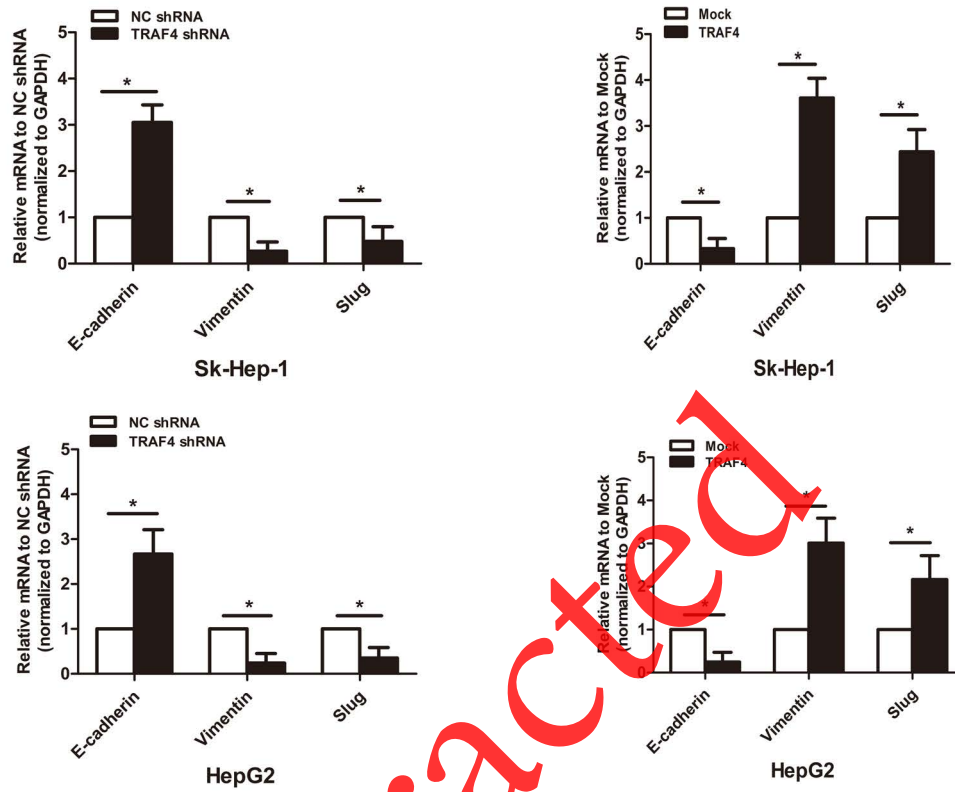
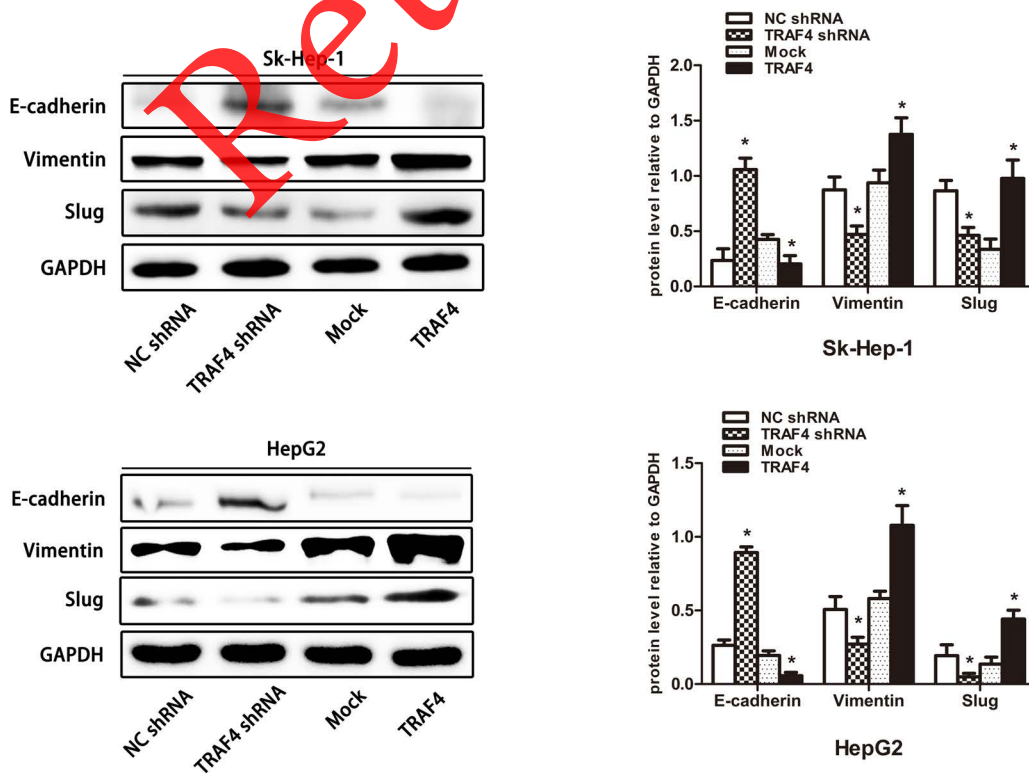


Figure 2. TRAF4 regulates HCC cell mobility. (A) The upregulation and downregulation of TRAF4 in HepG2 cells and Sk-Hep-1 cells are confirmed through qRT-PCR and Western blotting. Data are presented as the mean \pm SD; $n=9$. $*p<0.05$. (B) Cell mobility is tested using a Transwell assay. Representative views shows that upregulated TRAF4 promotes cell migration and invasion, while downregulated TRAF4 attenuates these effects. (C) Quantitation of cell migration and invasion. Data are presented as the mean \pm SD; $n=6$. $*p<0.05$. (D) TRAF4 overexpression promotes HCC metastasis in vivo. Representative views of lung tissues from each group and pulmonary metastasis rates are shown. Data are presented as the number.

A



B



vascular invasion than those without vascular invasion (Fig. 1C and D). Furthermore, to identify the clinical role of TRAF4, we investigated the correlations of TRAF4 expression with tumorous pathological characteristics including age, gender, HbsAg status, liver cirrhosis, AFP level, tumor size, tumor numbers, tumor capsule formation, vascular invasion, and TNM stage. In this study, an IHC stain score of less than 4 points, namely (–) and (+), was considered to be a low TRAF4 expression. A total score above 6 was regarded as a high TRAF4 expression. In 60 cases of tumor tissues, 24 cases expressed a high level of TRAF4 and 36 cases expressed a low level of TRAF4. Interestingly, we noted that in tissues with a high expression of TRAF4, 29.2% were associated with multiple tumors, while only 8.3% were in tissues with a low expression of TRAF4 (7/24 vs. 3/36, $p=0.034$). We also found that 79.2% of the cases in the high TRAF4 expression group were concomitant with vascular invasion compared to 27.8% of the cases in the low TRAF4 expression group (19/24 vs. 10/36, $p=0.000095$). No relationship existed between TRAF4 expression and other tumorous pathological characteristics. These results suggested that TRAF4 expression might play a critical role in HCC. High expression of TRAF4 might be associated with an invasive potential.

TRAF4 Regulates HCC Cell Migration and Invasion

To determine whether TRAF4 regulates cell mobility in HCC, we generated TRAF4 overexpression and TRAF4 knockdown of HepG2 cells and Sk-hep1 cells (HepG2-TRAF4, Sk-hep1-TRAF4, and TRAF4 shRNA) as well as their negative controls (Mock; NC-shRNA). Expression of TRAF4 in these cells was identified at both mRNA and protein levels (Fig. 2A).

Transwell assay with and without Matrigel was performed to detect migrant and invasive abilities in vitro. Results showed that both HepG2-TRAF4 and Sk-hep1-TRAF4 had significantly larger populations of migrant and invasive cells compared to their negative controls. In contrast, for cells with downregulated TRAF4, both migrant and invasive abilities were reduced (Fig. 2B and C). To further verify the function of TRAF4 in vivo, HepG2-TRAF4, Sk-hep1-TRAF4, and their corresponding control cells were injected into nude mice through the tail vein. Upregulated TRAF4 significantly promoted tumor metastasis compared with the control group (Fig. 2D). Pulmonary metastases rarely occurred in the TRAF4-Mock group compared to the TRAF4 group (Sk-Hep-1: 1:6 vs. 6:6;

HepG2: 0:6 vs. 5:6). Taken together, the results revealed that TRAF4 regulated HCC cell mobility and participated in HCC metastasis in vitro and in vivo.

TRAF4 Regulates EMT in Liver Cancer Cells

Considering that EMT plays a pivotal role in cancer cell migration and invasion²², to investigate whether TRAF4 regulates the EMT in liver cancer cells, we detected the expression level of E-cadherin, vimentin, and the EMT transcription factor Slug using qRT-PCR and Western blotting. In our study, at both the mRNA and protein levels, the changes in Slug expression was after TRAF4 expression. Upregulated epithelial cell marker E-cadherin and downregulated mesenchymal cell marker vimentin were observed in TRAF4 knockdown cell lines. TRAF4 overexpression significantly decreased the epithelial cell marker and the increased mesenchymal cell marker (Fig. 3A and B). Our results indicated that TRAF4 regulates the EMT in HCC.

Akt Activation Is Involved in TRAF4-Conducted EMT and Cell Mobility

It has been reported that TRAF4 is a critical molecule for the activation of Akt through ubiquitination in lung cancer¹⁶. The function of the pivotal cell survival kinase Akt to regulate EMT has been widely reported²³. Therefore, we investigated whether TRAF4-induced EMT is associated with Akt activation. We first found that TRAF4 overexpression unexpectedly promoted the phosphorylation of Akt and that silencing TRAF4 mitigated the phosphorylation of Akt both in Sk-Hep-1 cells and HepG2 cells (Fig. 4A). These results suggested that TRAF4 participated in the activation of Akt signaling in HCC. Afterward, we generated constitutively activated Akt1 (CA-Akt1) into silencing TRAF4 cells and treated TRAF4 overexpression cells with the PI3K/Akt inhibitor LY294002 to further explore the relationship between TRAF4-associated Akt activation and EMT. Our results showed that, in knockdown of TRAF4 cells, constitutive Akt activation could reverse the expression of Slug, leading to a decreased expression of E-cadherin and an enhanced expression of vimentin (Fig. 4B). Furthermore, HCC cells lacking TRAF4 with Akt activation showed stronger migrant and invasive abilities when compared with those without Akt activation (Fig. 5A). Similar results were also observed in TRAF4 overexpression cells. We treated TRAF4 overexpression cells with LY294002 to inhibit PI3K/Akt activation. Inhibited PI3K/Akt activation

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Figure 3. TRAF4 regulates the epithelial–mesenchymal transition (EMT) in HCC cells. (A) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) shows changes in EMT marker expression following stable overexpression or knockdown of TRAF4. (B) EMT marker expression is regulated at the protein level following the expression of TRAF4. Data are presented as the mean \pm SD; $n=9$. * $p<0.05$.

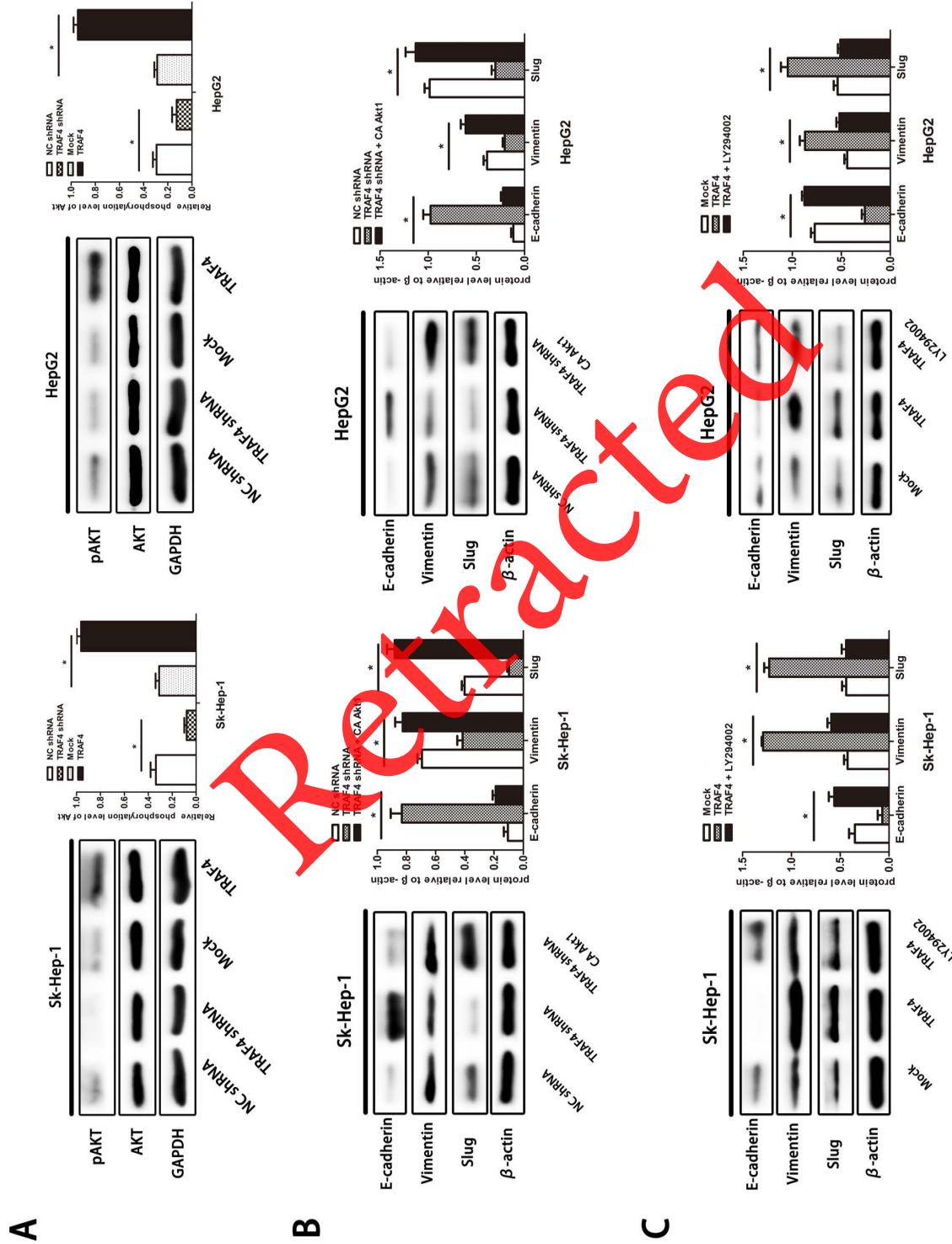


Figure 4. TRAF4 induces the EMT via PI3K/Akt signaling activation. (A) TRAF4 knockdown mitigates the phosphorylation of Akt, while forced TRAF4 expression enhances the phosphorylation of Akt. (B) Constitutive activation of Akt1 rescues TRAF4 knockdown-imposed EMT in Sk-Hep-1 and Hep2 cells. (C) PI3K/Akt inhibitor LY294002 restricts the TRAF4-induced EMT in Sk-Hep-1 and Hep2 cells. Data are presented as the mean \pm SD; $n = 3$. * $p < 0.05$.

impeded TRAF4-induced EMT in HCC cells (Fig. 4C). In addition, TRAF4-induced cell mobility was blocked without PI3K/Akt activation (Fig. 5B). These results suggested that TRAF4-regulated EMT and cell mobility were required for PI3K/Akt activation in HCC.

DISCUSSION

Since its first identification in human breast cancer in 1995, TRAF4 has been considered to be an oncogene because of its overexpression in many cancer types and its involvement in carcinogenesis via engaging in several signal pathways^{10,15,18}. For instance, TRAF4 is overexpressed in lung cancer and is required for the activation of Akt signaling via ubiquitination. TRAF4 attenuation in lung cancer blunts malignant phenotype and interferes with glucose metabolism by downregulating Glut1 and HK2¹⁶. TRAF4 plays a role in regulating cell growth, migration, and invasion by activation of the Wnt/ β -catenin pathway in OSCC and colon cancer^{17,24}. TRAF4 binds PRMT5 and is involved in the NF- κ B pathway by promoting breast cancer cell growth and proliferation²⁵. TRAF4 also promotes TGF- β receptor signaling to drive metastasis and binds TRAF2 enhancing activation of the p70s6k/S6 signaling pathway to promote the function of TRAF4 on cell proliferation in breast cancer^{18,19}. However, few studies have explored the role of TRAF4 in HCC.

To the best of our knowledge, this is the first study to investigate the function of TRAF4 in HCC. Our results revealed that upregulated TRAF4 is a frequent event in human HCC cell lines and tissues. Interestingly, tumors with a higher invasive tendency such as multiple tumor quantity and/or vascular invasion showed more highly expressed TRAF4. We established stable TRAF4 overexpression cell lines and TRAF4 knockdown cell lines and unexpectedly found that TRAF4 regulates HCC cell migration and invasion *in vitro* and *in vivo*. Previous studies have identified that TRAF4 facilitates cell migration physiologically and pathologically. In the immune system, TRAF4 promotes immune cell migration without participation in the development and function²⁶. Impaired neural tube closure and tracheal ring disruption in TRAF4-deficient mice are relevant to abnormal cell migration^{11,12}. Increasing evidence has demonstrated that TRAF4-dependent cell mobility exists in many cancer types including breast cancer, OSCC, and colon cancer^{17,18,24}. In this study, our results verified that TRAF4-dependent migration occurs in HCC and suggest that TRAF4 is a crucial potential molecule fostering HCC progression and driving metastasis in liver cancer.

The EMT is a continuous multistep biological process related to the invasion and metastasis of tumors²⁷. During EMT, tumor cells reduce cell adhesion, cut through the basement membrane, migrate into blood circulation, and finally colonize at distant sites²⁸. One of the

distinguishing features of EMT is the decreased expression of E-cadherin, which, as the cell-cell junction protein, has been considered to be a suppressor of tumor dissemination, including the dissemination of liver cancer cells^{20,29-31}. EMT-related transcription factors such as Snail and Slug bind to the E-box area, which serves as the upstream promoter of E-cadherin and then represses the expression of E-cadherin, triggering the EMT³². Our study revealed a positive correlation between TRAF4 and Slug. TRAF4 knockdown cells with low migration and invasion exhibited a lower expression of Slug, leading to the upregulation of E-cadherin and downregulation of vimentin. Overexpressed TRAF4 with elevated cell mobility prominently facilitated the EMT through the elevation of Slug, which resulted in an attenuated expression of E-cadherin and enhanced level of vimentin. These findings indicate that TRAF4-dependent cell mobility, at least partially, is associated with the transcription factor Slug that triggers the transition between epithelial and mesenchymal phenotypes in HCC.

The PI3K/Akt signaling pathway is well known in regulating cellular and physiological processes, including cell proliferation, cell cycle, differentiation, survival, apoptosis, metabolism, and migration. Additionally, activation of PI3K/Akt signaling plays an important role in maintaining the biological features of malignant cells, including the EMT²³. For instance, sustained Akt activation was associated with low E-cadherin and directly elevated the expression of Snail, which could be reversed by the Akt inhibitor³³. Twist, as a transcription factor with a strong ability to induce the EMT, has a significant effect on Akt activation³⁴. Moreover, PI3K/Akt signaling can interact with several signaling pathways including the TGF- β signaling pathway, the NF- κ B signaling pathway, and the Wnt/ β -catenin signaling pathway to induce the EMT²³. In our study, upregulated TRAF4 facilitates the phosphorylation of Akt, whereas downregulated TRAF4 leads to the opposite events, which was in line with previous studies. In lung cancer, TRAF4 was demonstrated to be indispensable for Akt activation through ubiquitination¹⁶. Zhang et al. found that TRAF4 promoted Akt activation and gave evidence of a direct interaction between Akt and TRAF4 in breast cancer³⁵. However, the mechanisms by which TRAF4 activates Akt signaling and whether TRAF4 interacts with Akt in HCC require further study.

Our study also found that TRAF4-induced EMT, cell migration, and cell invasion are dependent on Akt phosphorylation. Sustained Akt activation in TRAF4-deficient cells regained Slug expression and switched the epithelial phenotype to a mesenchymal phenotype, acquiring original cell migration and invasive abilities. For TRAF4 overexpression cells, using LY294002 to inhibit PI3K/Akt signaling can impede the TRAF4-induced upregulation of Slug, leading to enhanced expression of E-cadherin and

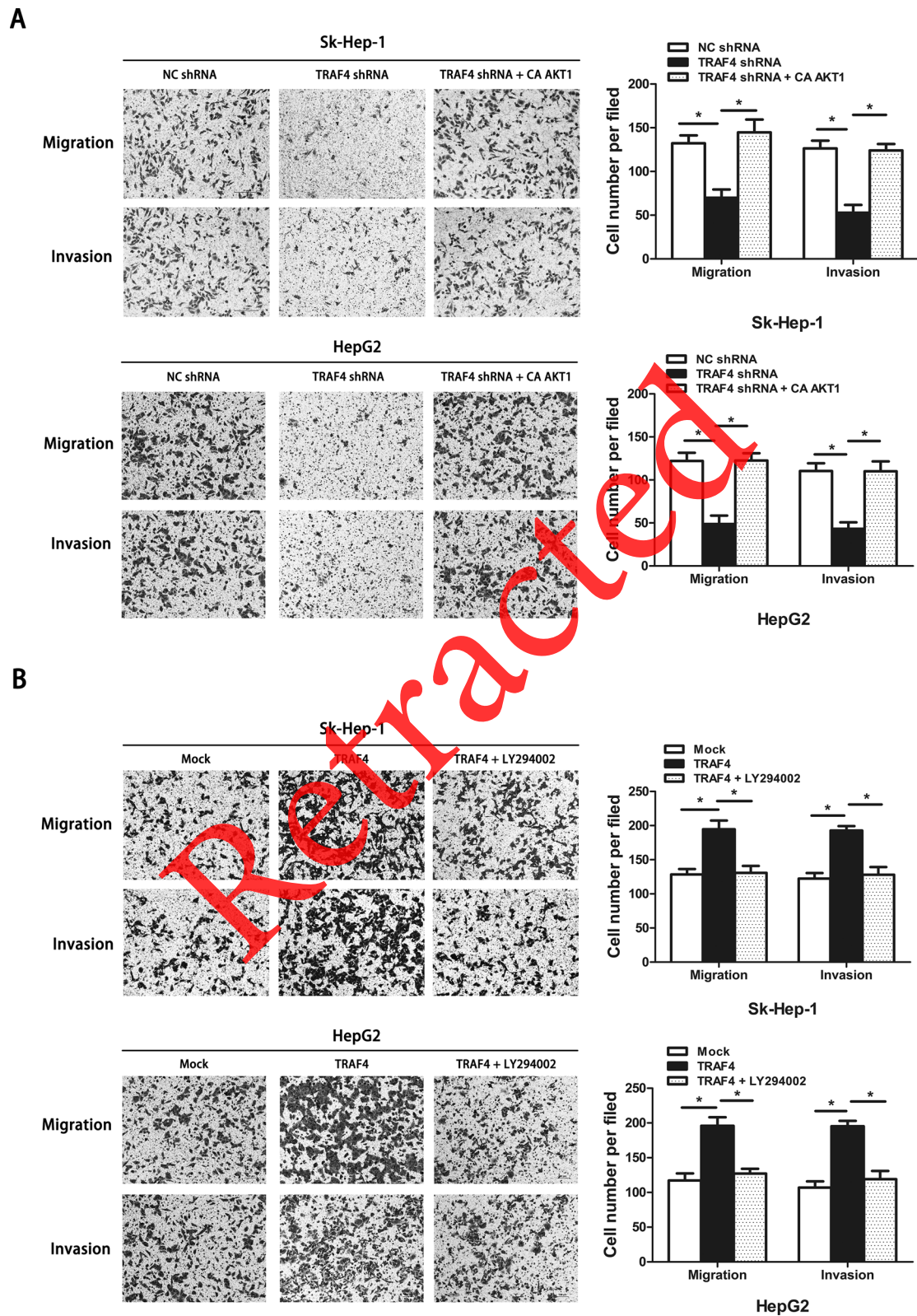


Figure 5. Regulatory effect of PI3K/Akt signaling pathways on TRAF4-induced cell mobility. (A) Constitutive activation of Akt1 recovered cell migration and invasion impaired by TRAF4 downregulation ($p < 0.05$). (B) PI3K/Akt inhibitor LY294002 hampered the TRAF4-induced cell migration and invasion ($p < 0.05$). Data are presented as the mean \pm SD; $n = 6$. $*p < 0.05$. All experiments were repeated independently three times; significant difference between groups: $*p < 0.05$.

a decreased expression of vimentin. TRAF4-dependent cell migration and invasion can also be moderated. These effects were similar to a study in melanoma showing that blockage of PI3K/Akt signaling impeded SPARC-induced Slug upregulation and cell migration while constitutively activating Akt rescue of Slug expression and the migratory ability of SPARC knockdown cells³¹. Although the underlying mechanisms and the correlation of TRAF4, Akt activation, and Slug expression in HCC remain inexplicit, this study revealed the fact that TRAF4 activated the PI3K/Akt signaling pathway, which contributed to Slug expression, leading to EMT, migration, and invasion in HCC.

In summary, our study identified that TRAF4 plays an oncogenic role in HCC by facilitating cell mobility. TRAF4 is a novel regulator of EMT through activating the PI3K/Akt signaling pathway. This study contributes to the clarification of the oncogenic function of TRAF4 in HCC and indicates that TRAF4 may serve as a potential therapeutic target for reducing HCC invasion and metastasis.

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REFERENCES

1. Maluccio M, Covey A. Recent progress in understanding, diagnosing, and treating hepatocellular carcinoma. *CA Cancer J Clin.* 2012;62(6):394–9.
2. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003;362(9399):1907–17.
3. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin.* 2013;63(1):11–30.
4. Carr BI. Hepatocellular carcinoma: Current management and future trends. *Gastroenterology* 2004;127(5 Suppl 1):S218–24.
5. Zhu AX. Systemic therapy of advanced hepatocellular carcinoma: How hopeful should we be? *Oncologist* 2006; 11(7):790–800.
6. Chung JY, Park YC, Ye H, Wu H. All TRAFs are not created equal: Common and distinct molecular mechanisms of TRAF-mediated signal transduction. *J Cell Sci.* 2002;115(Pt 4):679–88.
7. Kedinger V, Rio MC. TRAF4, the unique family member. *Adv Exp Med Biol.* 2007;597:60–71.
8. Ha H, Han D, Choi Y. TRAF-mediated TNFR-family signaling. *Curr Protoc Immunol.* 2009;Chapter 11:Unit11.9D.
9. Wajant H, Henkler F, Scheurich P. The TNF-receptor-associated factor family: Scaffold molecules for cytokine receptors, kinases and their regulators. *Cell Signal.* 2001; 13(6):389–400.
10. Regnier CH, Tomasetto C, Moog-Lutz C, Chenard MP, Wendling C, Basset P, Rio MC. Presence of a new conserved domain in CART1, a novel member of the tumor necrosis factor receptor-associated protein family, which is expressed in breast carcinoma. *J Biol Chem.* 1995;270(43): 25715–21.
11. Regnier CH, Masson R, Kedinger V, Textoris J, Stoll I, Chenard MP, Dierich A, Tomasetto C, Rio MC. Impaired neural tube closure, axial skeleton malformations, and tracheal ring disruption in TRAF4-deficient mice. *Proc Natl Acad Sci USA* 2002;99(8):5585–90.
12. Shiels H, Li X, Schumacker PT, Maltepe E, Padrid PA, Sperling A, Thompson CB, Lindsten T. TRAF4 deficiency leads to tracheal malformation with resulting alterations in air flow to the lungs. *Am J Pathol.* 2000;157(2):679–88.
13. Blaise S, Kneib M, Rousseau A, Gambino F, Chenard MP, Messadeq N, Muckenstrum M, Alpy F, Tomasetto C, Humeau Y, Rio MC. In vivo evidence that TRAF4 is required for central nervous system myelin homeostasis. *PLoS One* 2012;7(2):e30917.
14. Tomasetto C, Regnier C, Moog-Lutz C, Mattei MG, Chenard MP, Lidereau R, Basset P, Rio MC. Identification of four novel human genes amplified and overexpressed in breast carcinoma and localized to the q11-q21.3 region of chromosome 17. *Genomics* 1995;28(3):367–76.
15. Camilleri-Bonet S, Cremer I, Marmey B, Comperat E, Viguie F, Audouin J, Rio MC, Fridman WH, Sautes-Fridman C, Regnier CH. TRAF4 overexpression is a common characteristic of human carcinomas. *Oncogene* 2007;26(1):142–7.
16. Li W, Peng C, Lee MJ, Lim D, Zhu F, Fu Y, Yang G, Sheng Y, Xiao L, Dong X, Ma W, Bode AM, Cao Y, Dong Z. TRAF4 is a critical molecule for Akt activation in lung cancer. *Cancer Res.* 2013;73(23):6938–50.
17. Yang J, Wei D, Wang W, Baohong S. TRAF4 enhances oral squamous cell carcinoma cell growth, invasion and migration by Wnt- β -catenin signaling pathway. *Int J Clin Exp Pathol.* 2015;8:11837–46.
18. Zhang L, Zhou F, Garcia de Vinuesa A, de Kruijf EM, Mesker WE, Hui L, Drabsch Y, Li Y, Bauer A, Rousseau A, Sheppard KA, Mickanin C, Kuppen PJ, Lu CX, Ten Dijke P. TRAF4 promotes TGF- β receptor signaling and drives breast cancer metastasis. *Mol Cell* 2013;51(5):559–72.
19. Ren H-Y, Wang J, Yang F, Zhang X-L, Wang A-L, Sun L-L, Diao K-X, Wang E-H, Mi X-Y. Cytoplasmic TRAF4 contributes to the activation of p70s6k signaling pathway in breast cancer. *Oncotarget* 2015;6(6):2080–96.
20. Lin Z, Li W, Zhang H, Wu W, Peng Y, Zeng Y, Wan Y, Wang J, Ouyang N. CCL18/PITPNM3 enhances migration, invasion, and EMT through the NF- κ B signaling pathway in hepatocellular carcinoma. *Tumour Biol.* 2016;37(3):3461–8.
21. Zhao J, Klausen C, Qiu X, Cheng JC, Chang HM, Leung PC. Betacellulin induces Slug-mediated down-regulation of E-cadherin and cell migration in ovarian cancer cells. *Oncotarget* 2016;7(20):28881–90.
22. Heerboth S, Housman G, Leary M, Longacre M, Byler S, Lapinska K, Willbanks A, Sarkar S. EMT and tumor metastasis. *Clin Transl Med.* 2015;4:6.
23. Xu W, Yang Z, Lu N. A new role for the PI3K/Akt signaling pathway in the epithelial-mesenchymal transition. *Cell Adh Migr.* 2015;9(4):317–24.
24. Yang K, Wang F, Han J-J. TRAF4 promotes the growth and invasion of colon cancer through the Wnt- β -catenin pathway. *Int J Clin Exp Pathol.* 2015;8(2):1419–26.
25. Yang F, Wang J, Ren HY, Jin J, Wang AL, Sun LL, Diao KX, Wang EH, Mi XY. Proliferative role of TRAF4 in breast cancer by upregulating PRMT5 nuclear expression. *Tumour Biol.* 2015;36(8):5901–11.
26. Cherfils-Vicini J, Vingert B, Varin A, Tartour E, Fridman WH, Sautes-Fridman C, Regnier CH, Cremer I. Characterization of immune functions in TRAF4-deficient mice. *Immunology* 2008;124(4):562–74.
27. Talbot LJ, Bhattacharya SD, Kuo PC. Epithelial-mesenchymal transition, the tumor microenvironment,

- and metastatic behavior of epithelial malignancies. *Int J Biochem Mol Biol.* 2012;3(2):117–36.
28. Iwatsuki M, Mimori K, Yokobori T, Ishi H, Beppu T, Nakamori S, Baba H, Mori M. Epithelial-mesenchymal transition in cancer development and its clinical significance. *Cancer Sci.* 2010;101(2):293–9.
 29. Ding W, You H, Dang H, LeBlanc F, Galicia V, Lu SC, Stiles B, Rountree CB. Epithelial-to-mesenchymal transition of murine liver tumor cells promotes invasion. *Hepatology* 2010;52(3):945–53.
 30. Zhou ZJ, Dai Z, Zhou SL, Hu ZQ, Chen Q, Zhao YM, Shi YH, Gao Q, Wu WZ, Qiu SJ, Zhou J, Fan J. HNRNPAB induces epithelial-mesenchymal transition and promotes metastasis of hepatocellular carcinoma by transcriptionally activating SNAIL. *Cancer Res.* 2014;74(10):2750–62.
 31. Fenouille N, Tichet M, Dufies M, Pottier A, Mogha A, Soo JK, Rocchi S, Mallavialle A, Galibert MD, Khammari A, Lacour JP, Ballotti R, Deckert M, Tartare-Deckert S. The epithelial-mesenchymal transition (EMT) regulatory factor SLUG (SNAI2) is a downstream target of SPARC and AKT in promoting melanoma cell invasion. *PLoS One* 2012; 7(7):e40378.
 32. Prokop JW, Liu Y, Milsted A, Peng H, Rauscher FJ 3rd. A method for in silico identification of SNAIL/SLUG DNA binding potentials to the E-box sequence using molecular dynamics and evolutionary conserved amino acids. *J Mol Model.* 2013;19(9):3463–9.
 33. Silva BS, Yamamoto FP, Pontes FS, Cury SE, Fonseca FP, Pontes HA, Pinto-Junior DD. TWIST and p-Akt immunoprecipitation in normal oral epithelium, oral dysplasia and in oral squamous cell carcinoma. *Med Oral Patol Oral Cir Bucal.* 2012;17(1):e29–34.
 34. Way TD, Huang JT, Chou CH, Huang CH, Yang MH, Ho CT. Emodin represses TWIST1-induced epithelial-mesenchymal transitions in head and neck squamous cell carcinoma cells by inhibiting the beta-catenin and Akt pathways. *Eur J Cancer* 2014;50(2):366–78.
 35. Zhang J, Li X, Yang W, Jiang X, Li N. TRAF4 promotes tumorigenesis of breast cancer through activation of Akt. *Oncol Rep.* 2014;32(3):1312–8.

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