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RASAL1 inhibits HepG2 cell growth via HIF-2α mediated gluconeogenesis

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Abstract. RAS protein activator like 1 (RASAL1) is a member of the RAS GTPase-activating protein (GAP) family, and has been identified as a tumor suppressor in various types of cancer. In the present study, it was determined that decreased levels of RASAL1 were accompanied by a higher pathological stage and larger tumor size in human liver cancer. Therefore, it was hypothesized that RASAL1 may serve an inhibitory role in liver cancer. In the present study, the following was demonstrated: i) Exogenous expression of RASAL1 may inhibit the proliferation and invasion ability of HepG2 cells; ii) overexpression of RASAL1 may downregulate HIF-2α transcription activity and HIF-2α-mediated gluconeogenesis through extracellular signal-related kinase 1/2 activation; iii) RASAL1 may reduce the xenograft tumor size in nude mice by inhibiting the expression of hypoxia-inducible factor (HIF)-2α and gluconeogenesis enzymes. These data suggest that the RASAL1/HIF-2α axis may serve an essential role in the growth of HepG2 cells, and that this signaling cascade may be a novel therapeutic target for the treatment of liver cancer.

Introduction

Liver cancer is a type of malignancy prevalent in less-developed regions, and was the fifth most common cancer in males and the ninth in females worldwide in 2012; it is also the second most common cause of cancer-associated mortality (1). Developing an optimum therapeutic strategy is one of the major aims of clinical studies at present.

It has been identified that RAS proteins are involved in a number of cellular processes, including migration, proliferation, differentiation and survival (2). RAS protein activator like 1 (RASAL1) is a member of the RAS GTPase-activating protein (GAP) family, and has been revealed as downregulated in several solid tumors (3), and also to function as a tumor suppressor gene that negatively modulates the RAS signaling pathway by catalyzing RAS inactivation (4). Previously, evidence has indicated that RASAL1 levels are correlated with liver injury and hepatic fibrosis (5,6). However, little is known about the association between RASAL1 and liver cancer.

Hypoxia-inducible factor (HIF)-1 and HIF-2 are transcription factors that serve major roles in the cellular responses to hypoxia, and have recently been considered mediators of cancer progression and targets for cancer therapy (7). HIF-1α has been identified as a positive factor for tumor growth, and increased HIF-1α activation was correlated with the development of more aggressive carcinogenic phenotypes (8,9). Not only a prognostic marker, high HIF-2α levels have also been associated with advanced stages or poor patient outcomes in several types of tumor (10), HIF-2α has also been suggested to serve an important role in the development of various diseases: HIF-2α-null embryos have exhibited vascular disorganization throughout the yolk sac and the embryo itself (11), and can perish due to adrenal insufficiency, although they may survive with adrenal catecholamine replacement therapy (12). A prior study identified that HIF-2α-mediated hypoxic signaling and hepatic insulin action may modulate glucose metabolism (13,14), and may participate in the postprandial hepatic glucagon response (15). Increased metabolic autonomy, nutrient absorbance and metabolism to support growth and proliferation has been demonstrated among diverse tumor types (16), so targeting metabolic transformation is a promising strategy for cancer therapy. Therefore, the present study focused on the glucose metabolism effect to clarify the correlation between RASAL1 and HIF-2α in liver cancer development.

In the present study, it was identified that RASAL1 was significantly downregulated in liver cancer tissues compared with the corresponding non-tumor tissues, and may serve as an independent predictor for the overall survival of patients with liver cancer. Furthermore, RASAL1 regulated cell proliferation and invasion through its inhibitory effect on HIF-2α, which may partly account for HIF-2α-mediated gluconeogenesis via the extracellular signal-related kinase (ERK)1/2 pathway, thus affecting the proliferation of liver cancer cells *in vitro* and *in vivo*. This assisted understanding of the tumor suppressive function of RASAL1. In addition, the present study

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aimed to reveal a novel regulatory mechanism of RASAL1 in the development of liver cancer, and provide a novel direction for its clinical application.

Materials and methods

Tissue collection and ethics statement. A total of 16 primary human liver cancer tissues and adjacent non-tumor tissues were collected from patients who had undergone surgery at the Linyi People's Hospital (Linyi, China) between August 2013 and October 2015. All patients had not received chemotherapy or radiotherapy prior to surgery. The study was approved by the Linyi People's Hospital Ethics Committee, and was performed in compliance with the Declaration of Helsinki Principles. Written informed consent was obtained for all patient samples. The animal experiments were performed with the approval of The Institutional Committee for Animal Research of Linyi People's Hospital and in conformity with National Guidelines for the Care and Use of Laboratory Animals (17).

Cell culture. Hepatoblastoma HepG2 cells (18) were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured at 37˚C with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal calf serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), 4.5 g/l glucose, 2 mM L-glutamine (Thermo Fisher Scientific, Inc.,), 100 U/ml penicillin and 100 µg/ml streptomycin.

Plasmids and cell transfection. For RASAL1 overexpression experiments, HepG2 cells ($5x10^6$ per reaction) were transfected with 1 µg pcDNA3.1-RASAL1 plasmid at 37˚C for 48 h, designed and synthesized by Shanghai GenePharma Co., Ltd (Shanghai, China), using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RASAL1-overexpressing HepG2 stable cell line was generated using 10 µg/ml G418 (Invitrogen; Thermo Fisher Scientific, Inc.) in the culture medium for 4 weeks, and the resulting single clones were expanded to obtain stably transfected cells. Cells transfected with an empty vector and un-transfected cells were used as controls. To detect the function role of ERK1/2 in RASAL1 mediated HIF-2α expression, 100 nM ERK1/2 inhibitor SCH772984 (S7101, Selleck Chemicals, Houston, TX, USA) was added to the medium 24 h after transfection to inhibit the activation of ERK1/2.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses. The total RNA was extracted from tissues or cultured cells with TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Briefly, tissues or cells were homogenized and RNA was isolated following phase separation with chloroform, precipitated with 80% isopropanol, washed twice with 75% ethanol, and finally re-dissolved in water. RNA concentration was determined by UV spectrophotometry (NanoDrop 2000; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). A volume of 1 µg total RNA was reverse transcribed to a final volume of 20 µl, using random primers under standard conditions with the PrimeScript RT Reagent kit and gDNA Eraser (Takara Biotechnology Co., Ltd., Dalian, China; cat. no. RR047A). Following the RT reaction, 1 µl cDNA was used for subsequent RT-qPCR reactions (SYBR Premix Ex Taq; Takara Biotechnology Co., Ltd., according to the manufacturer's protocol. Sequences of all primers are summarized in Table I. The RT-qPCR and data collection were carried out on an ABI 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction was initially denatured (95˚C for 15 sec), followed by 40 cycles of 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec, with a final melting curve analysis of the fluorescence performed between 60˚C and 95˚C with increments of 0.5˚C every 10 sec. The $2^{-\Delta\Delta Cq}$ value was calculated for every sample, finally the mRNA expression levels were indicated with $2^{-\Delta\Delta Cq}$ and normalized to GAPDH (19).

MTT assay. A 100 µl suspension of HepG2 cells was seeded into a 96-well plate following transfection for different times (0, 24, 48, 72 and 96 h), at a density of $0.5x10^5$ cells/well at 37˚C, Following this, MTT was added to each well at a final concentration of 0.5 mg/ml for 4 h, and the resulting formazan crystals were dissolved in dimethyl sulfoxide. Optical density was measured at 490 nm using a plate microreader (Tecan Austria GmbH, Grodig, Austria). The growth inhibition ratio was calculated for three independent repeats.

Transwell chamber assay. HepG2 cells that stably expressed RASAL1 and the control cells were trypsinized with 0.25% phenol red trypsin (cat no. 25200056; Thermo Fisher Scientific, Inc.), centrifuged at room temperature for 3 min at 100 x g and resuspended in serum-free DMEM. A total of $x10^5$ HepG2 cell suspension was added to the upper wells of Transwell chambers (Corning Incorporated, Corning, NY, USA) pre-coated with matrigel (to observe migration ability, upper Transwell chamber wells were not coated with Matrigel). The medium was added to the lower chamber. Subsequent to culturing at 37˚C, the cells for 48 h, the cells remaining in the upper chamber were removed with a cotton swab. The wells were washed twice with PBS and stained at room temperature for 10 min with 2 mg/ml crystal violet. The migrated/invaded cells were counted under a light microscope at magnification, x200 in at least 6 fields of view. The experiments were repeated three times.

Cell proliferation assay. To measure the effect of RASAL1 on proliferation activity, $3x10^3$ cells/well HepG2 cells were plated onto 96-well plates. Following overnight culture at 37˚C, HepG2 cells were transfected with 100 ng/well pcDNA3.1 or RASAL1 using Lipofectamine® 2000 at 37˚C, and after 24 h of incubation at 37˚C, cell proliferation was measured with a BrdU assay kit (Roche Applied Science, Penzberg, Germany) in accordance with the manufacturer's protocol. All experiments were repeated three times independently.

Western blot analysis. Liver cancer tissues and HepG2 cells were collected, lysed with radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EGTA, 1 mM EDTA) containing complete protease inhibitor (Roche Applied Science) on ice for 30 min and subjected to protein extraction. A total of 25 µg total lysate per sample was separated on 10% SDS-PAGE gels and

Table I. Primers sequences, PCR conditions and products sizes.

Gene	Sequence	Annealing Temperature, ˚C	PCR product size (bp)
HIF-1α (NM_181054)	Forward: 5'-GAACGTCGAAAAGAAAAGTCTCG-3' Reverse: 5'-CCTTATCAAGATGCGAACTCACA-3'	60	124
HIF-1β (NM_001197325)	Forward: 5'-TAGTGCCCTGGCTCGAAAAC-3' Reverse: 5'-GGTTCAAAACAGGAGTCACGG-3'	61	239
HIF-2α (NM_001430)	Forward: 5'-CGGAGGTGTTCTATGAGCTGG-3' Reverse: 5'-AGCTTGTGTGTTCGCAGGAA-3'	62	115
HIF-3α (NM_022462.4)	Forward: 5'-CCTGTGGAGTCATCTCACCG-3' Reverse: 5'-GACTTTTCCTTGCGCAGCTC-3'	60	151
RASAL1 (NM_004658)	Forward: 5'-CAGCTCCCTGAATGTTCGC-3' Reverse: 5'-TCCTCATCCAGCACGTAGAAG-3'	61	216
PEPCK (NM_001018073)	Forward: 5'-AGTAGAGAGCAAGACGGTGAT-3' Reverse: 5'-TGCTGAATGGAAGCACATACAT-3'	60	179
G6Pase (NM_000151)	Forward: 5'-CTACTACAGCAACACTTCCGTG-3' Reverse: 5'-GGTCGGCTTTATCTTTCCCTGA-3'	61	160
GAPDH (NM_001256799)	Forward: 5'-GGAGCGAGATCCCTCCAAAAT-3' Reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'	60	197

PCR, polymerase chain reaction; HIF, hypoxia-inducible factor; RASAL1, RAS protein activator like 1; PEPCK, phosphoenolpyruvate carboxy kinase.

then transferred onto nitrocellulose membranes. Specific monoclonal anti-RASAL1 (cat. no. ab170711; 1:1,000 dilution), anti-HIF-2α (cat. no. ab73895; 1:1,000 dilution) and anti-glucose 6-phosphatase (G6Pase; cat. no. ab83690; 1:1,000 dilution) primary antibodies (all Abcam, Cambridge, MA, USA), and anti-ERK1/2 (cat. no. 4695; 1:1,000 dilution), anti-phospho-ERK1/2 (cat. no. 4370; 1:1,000 dilution) and anti-phosphoenolpyruvate carboxy kinase (PEPCK; cat. no. 8565; 1:1,000 dilution) (all Cell Signaling Technology, Inc., Danvers, MA, USA) were used. An anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5,000 dilution, cat. no. 7074; Cell Signaling Technology, Inc.,) was also used. West Pico Chemiluminescent substrate kit (Pierce; Thermo Fisher Scientific, Inc.) was used as a substrate to visualize the protein bands, which were quantified using densitometry image analysis software version 3.0 (Image Master VDS; Pharmacia Biotech; GE Healthcare, Chicago, IL, USA). Normalization was performed using β-actin (cat. no. ab6276; 1:3,000 dilution, Abcam) expression.

Glucose production. A total of 48 h after HepG2 cells were transfected with pcDNA3.1 or RASAL1, cells were washed twice with PBS and then incubated at 37˚C with KRB buffer for 2 h. Following incubation, 0.5 mM pyruvate and 1 mM lactate were added to the KRB buffer and incubated at 37˚C for an additional 4 h. Glucose release was measured using a glucose LiquiColor® diagnostic kit (Stanbio Laboratories; EKF Diagnostics, Inc., Boerne, TX, USA).

Plasmid construction and luciferase assay. The entire human HIF-2α 3'-untranslated region segment was amplified by PCR using homo genomic DNA extracted from HepG2 cell as a template using Prime STAR® HS DNA Polymerase (R045Q, Takara Biotechnology Co., Ltd.). Primers were as follows: Forward: 5'-CCGCTCGAGGCCAGGCCTTCTACCTGGGCAGCACC-3', Reverse: 5'-GAATGCGGCCGCTAGGATCAGAATACTTTAATAAGATACC-3', and the thermocycling conditions were as follows: 95˚C initial denaturation for 5 min; 95˚C degeneration for 1 min, 56.1˚C annealing for 1 min, 72˚C for 1 min in 35 cycles; 72˚C extension for 10 min with *Mlu*I and *Xho*I overhangs. The PCR products were inserted into *Mlu*I and *Xho*I sites in the pGL3-Basic (Ambion; Thermo Fisher Scientific, Inc.) vector to obtain the reporter pGL3-HIF-2a construct. *Mlu*I (cat no. 1071B) and *Xho*I (cat no. 1094B) restriction enzymes were obtained from Takara Biotechnology Co., Ltd., (Dalian, China). For the luciferase reporter assays, 0.3 μg pGL3-HIF-2α plasmid, 0.3 μg *Renilla* luciferase (Ambion; Thermo Fisher Scientific, Inc.) and 0.3 μg pcDNA3.1 or RASAL1 were transfected into HepG2 cells using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) in 6-well plates at 37˚C for 48 h. *Renilla* was used as the transfection control. A total of 48 h after transfection, cells were assayed using Dual-Luciferase Reporter Assay kits (E1910, Promega Corporation, Madison, WI, USA).

Oxygen consumption rate (OCR). Measurement of the OCR was performed using a Seahorse XF96 analyzer (Seahorse Bioscience; Agilent Technologies, Inc., North Billerica, MA, USA). HepG2 cells were transfected with pcDNA3.1 or RASAL1 using Lipofectamine® 2000 at 37˚C for 48 h, and then resuspended with un-buffered medium and seeded at $1x10^5$ cells/well in XF96 plates. Cells were equilibrated in the

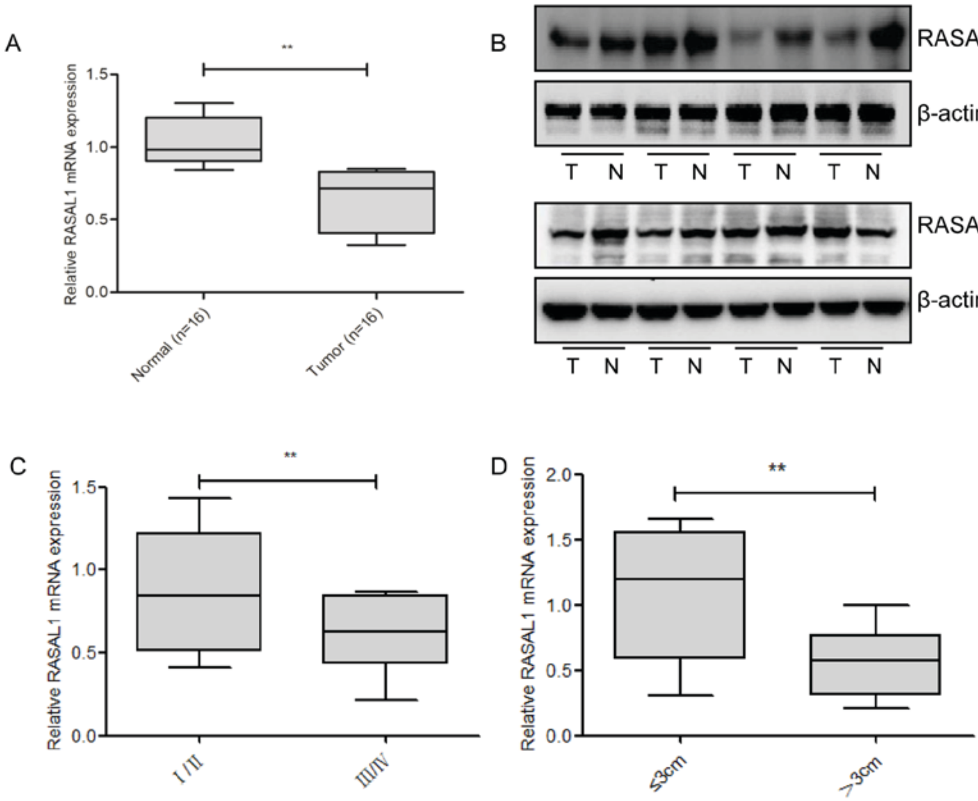

Figure 1. Analysis of RASAL1 expression in liver cancer tissues and clinical parameters. (A) RASAL1 was detected in 16 pairs of liver cancer tissues by reverse transcription-quantitative polymerase chain reaction. The levels of RASAL1 in liver cancer tissues were significantly low compared with those in non-tumorous tissues. (B) The protein levels of RASAL1 in liver cancer tissues were detected using a western blot assay. RASAL1 expression was significantly lower in patients with (C) higher pathological stages and (D) larger tumor sizes. **P<0.01. RASAL1, RAS protein activator like 1.

un-buffered medium for 45 min at 37˚C in a CO_2-free incubator, prior to being transferred to the XF96 analyzer. Basal OCR and the change in oxygen consumption were measured upon treatment with oligomycin and carbonyl cyanide p-trifluoromethoxyphenylhydrazone in succession, according to the manufacturer's protocol (cat no. 103344-100, Seahorse Bioscience; Agilent Technologies, Inc., Santa Clara, CA, USA).

In vivo tumor study. A total of 20 male nude mice (4-6 weeks; 18-20 g) were purchased from the Model Animal Research Center of Shandong University (Jinan, China). The animals were housed in a temperature- (20-26˚C) and humidity- (40-70%) controlled room with a 12:12 light: dark cycle, and provided free access to food and water. After 1week adaptive feeding, 20 mice were randomly divided into two groups: The control group was injected with control HepG2 cells and the RASAL1 group, which was injected with RASAL1-overexpressing HepG2 cells. A total of $5x10^6$ RASAL1-overexpressing HepG2 stable or control cells were injected subcutaneously into the right flank of each mouse. Tumor volumes were determined every 5 days after injection and calculated as described previously (20). Mice were sacrificed by CO_2 asphyxiation in a 1.5 l cage with 1.8 m^3/min CO_2 flow rate, approximately 5 min later, the mice died the death was confirmed by observing no spontaneous breathing for 2-3 min and no blink reflex, the final concentration of CO_2 in the cage reach approximately 80%. Tumors were dissected for RT-qPCR and western blot analysis. All animal studies were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Linyi People's Hospital and the Research Institute Animal Care and Use Committee. All protocols were approved by the Shandong Cancer Hospital and Research Institute Animal Care and Use Committee (approval number, 1040608). All surgery was performed under sodium pentobarbital anesthesia (60 mg/kg, i.p.), and all efforts were made to minimize suffering.

Statistical analysis. The results are expressed as mean ± standard error of the mean from ≥3 independent experiments. Data between the groups were analyzed using the Student's t-test or one-way analysis of variance, followed by the Bonferroni-Dunn multiple comparisons test with SPSS statistical software program (v 20.0; IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

RASAL1 is downregulated in human liver cancer tissues and is associated with poor prognosis. To detect the levels of RASAL1 expression, RT-qPCR and western blotting were used in 16 pairs of liver cancer tissues, and compared with the corresponding non-tumor tissues; it was identified that RASAL1 was significantly downregulated at the mRNA and protein level in the cancerous tissues (Fig. 1A and B). Subsequently, the associationbetweenRASAL1 expression levels and the clinical parameters of liver cancer was

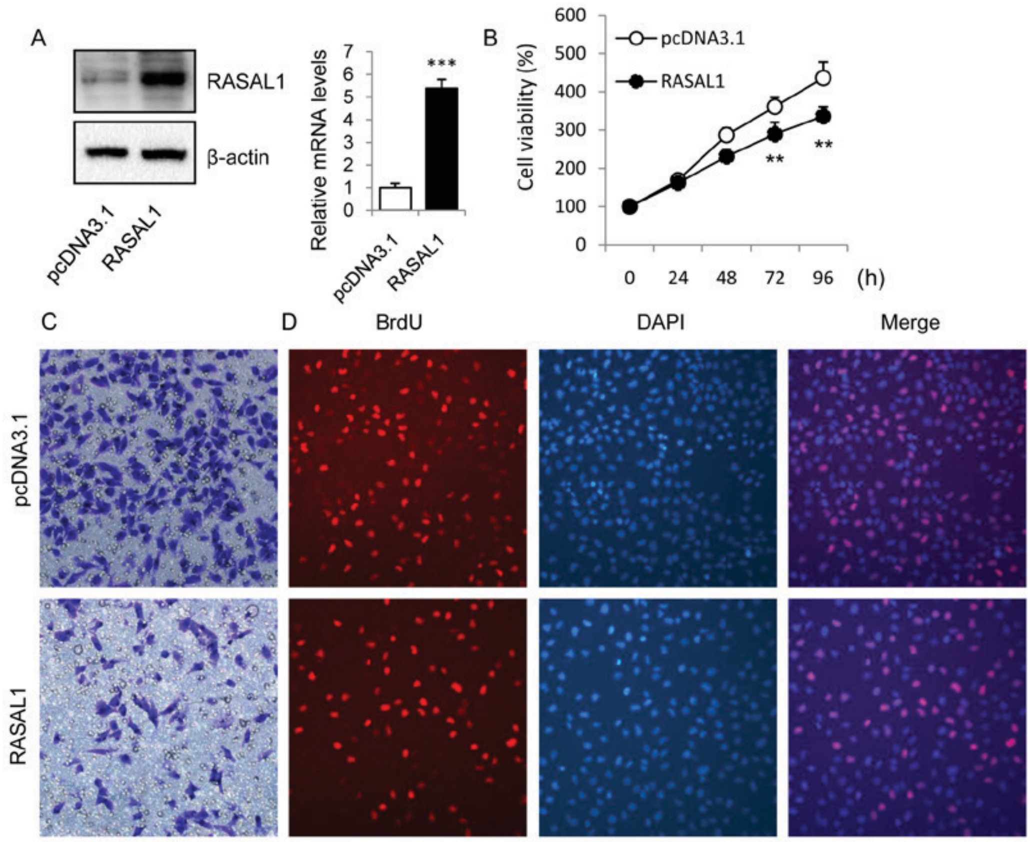

Figure 2. Overexpression of RASAL1 may inhibit the proliferation and invasion of HepG2 cells. (A) Western blot and reverse transcription-quantitative polymerase chain reaction analyses of RASAL1 protein and mRNA levels 48 h after transfection in HepG2 cells. Total lysates were prepared either from vector control orRASAL1 groups. Antibodies against RASAL1 were used in this assay. The protein loading control was performed using anti-β-actin. Bars represent the mean ± SEM. (B) The viability of HepG2 cells following transfection with pcDNA3.1 or RASAL1 for the indicated time. Bars represent the mean ± SEM. (C) Cell invasion ability was demonstrated using a Transwell assay. (D) Cell proliferation ability was detected using a BrdU assay. The results were reproduced in three independent experiments. **P<0.01, ***P<0.001 vs. pcDNA3.1 group. SEM, standard error of the mean. RASAL1, RAS protein activator like 1.

examined. As presented in Fig. 1C and D, RASAL1 downregulation was correlated with advanced pathological stage (P=0.009) and increased tumor size (P=0.008). Combined, these results suggest that the downregulation of RASAL1 may serve an important role in liver cancer development and progression.

RASAL1 inhibits proliferation and invasion in HepG2 cells. Next, the functional role of RASAL1 in the proliferation and invasion ability of HepG2 cells was explored using gain-of-function methods. It was observed that the expression of RASAL1 was significantly upregulated in HepG2 cells transiently overexpressing RASAL1 at the protein and mRNA levels, as compared with in the vector control cell line (Fig. 2A). To examine the effects of RASAL1 overexpression on cell proliferation and invasion, MTT (Fig. 2B), Transwell (Fig. 2C) and BrdU (Fig. 2D) assays were used 48 h following transfection. As demonstrated, transfection with RASAL1 resulted a significant inhibition of growth and invasion ability in the HepG2 cell line.

Overexpression of RASAL1 in HepG2 cells decreases HIF-2α expression and tumor metabolism. To validate whether the inhibition effect of overexpressed RASAL1 in HepG2 cells was mediated by its decreasing the expression of HIF proteins, the mRNA levels of HIF-1α, HIF-1β, HIF-2α and HIF-3α in RASAL1-overexpressing HepG2 cells were investigated using RT-qPCR. The results indicated that only HIF-2α was significantly decreased at the mRNA and the protein level, and that the phosphorylation of ERK1/2 was increased (Fig. 3A and B).

As previously demonstrated, the upregulation of HIF-1 and/or HIF-2 may decrease hepatic expression of the glucose transporter Glut2 and the gluconeogenic gene G6Pase, and also decrease the levels of the PEPCK rate-limiting enzyme of gluconeogenesis (21). Therefore, the protein and mRNA levels of PEPCK and G6Pase were detected by western blot analysis to determine whether RASAL1 inhibited tumor growth through the metabolism pathway. As presented in Fig. 3B and C, the two key gluconeogenic enzymes were downregulated at the protein and mRNA levels, while the inhibitor of ERK1/2, SCH772984, upregulated PEPCK and G6Pase protein and mRNA levels. These data also suggest that HIF-2α transcription activity and glucose production was suppressed in RASAL1-overexpressing HepG2 cells, but that SCH772984 weakened the inhibitory effect (Fig. 3D and E). OCRs were additionally measured *in vitro* in the presence and absence of RASAL1 in HepG2 cells, and it was identified that RASAL1 caused a significant decrease, while SCH772984 induced an increase (Fig. 3F).

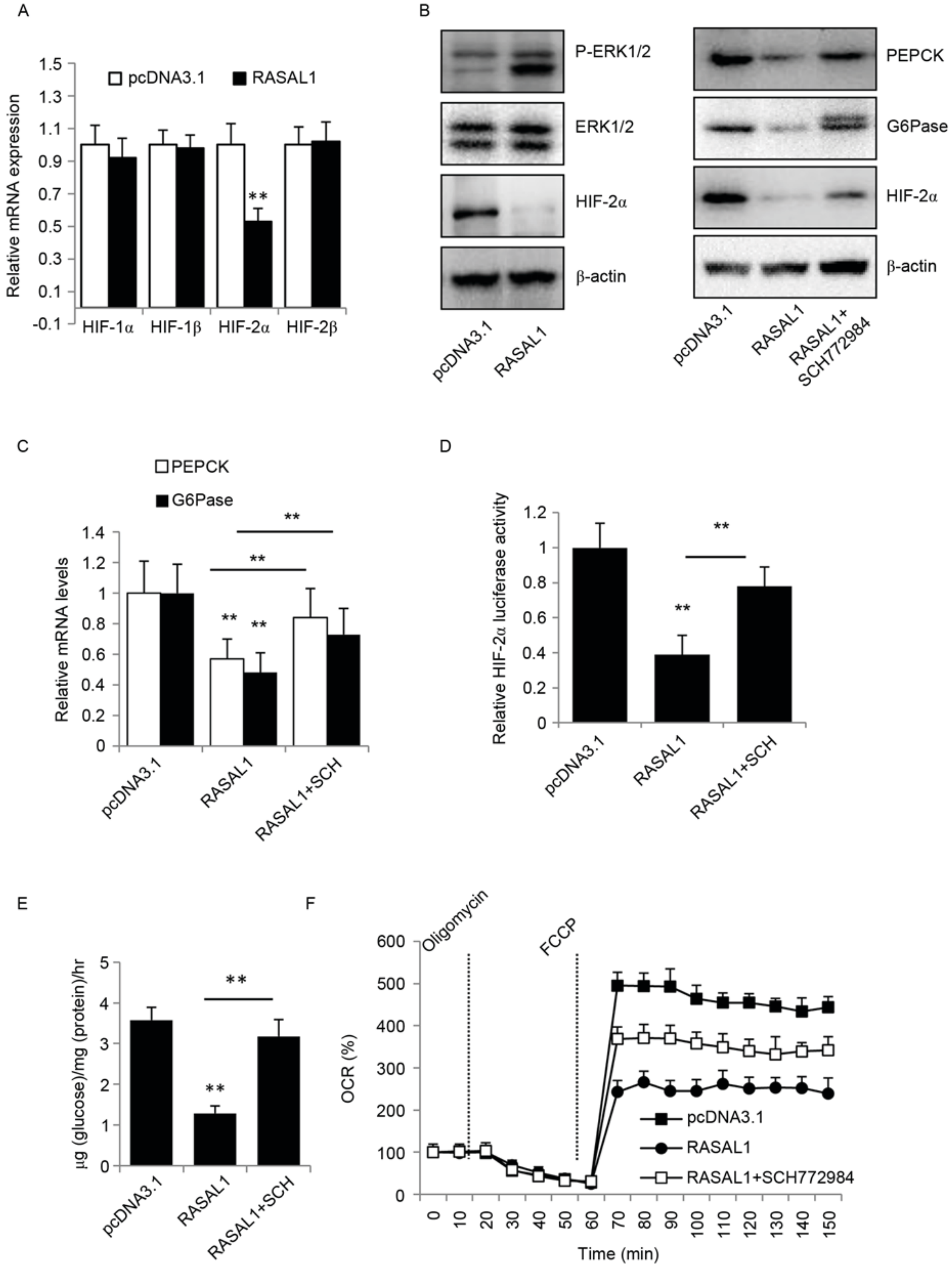

Figure 3. Overexpression of RASAL1 in HepG2 inhibits HIF-2α expression and tumor metabolism. (A) Reverse transcription-qPCR was performed to detect the expression of HIF genes in transfected cells, **P<0.01 vs. pcDNA3.1 group, and (B) western blot assays were used to detect the levels of HIF-2α, PEPCK, G6Pase and the phosphorylation of ERK1/2, with or without 1 μM SCH772984 treatment, following the transfection of RASAL1. (C) The mRNA levels of PEPCK and G6Pase were analyzed with qPCR in HepG2 cells, with or without 1 μM SCH772984 treatment, following the transfection of RASAL1, **P<0.01 vs. pcDNA3.1 or RASAL1 group. (D) Luciferase activity of the HIF-2α promoter was detected via the overexpression of RASAL1 with or without SCH772984, **P<0.01 vs. pcDNA3.1 or RASAL1 group. (E) Glucose production from HepG2 cells incubated with 9 mM lactate and 1 mM pyruvate with overexpression of RASAL1 or inhibition of ERK1/2 phosphorylation, **P<0.01 vs. pcDNA3.1 or RASAL1 group. (F) The OCR of HepG2 cells was measured 48 h after transfection with RASAL1 or treatment with SCH772984 using the Seahorse Bio analyzer. Data are normalized to basal OCR and are representative of three independent experiments. Each bar represents the mean ± standard error of the mean of three independent experiments. **P<0.01 vs. pcDNA3.1 or RASAL1 group. HIF, hypoxic-inducible factor; qPCR, quantitative polymerase chain reaction; OCR, oxygen consumption rate; p, phosphorylated; ERK, extracellular signal-regulated kinase; RASAL1, RAS protein activator like 1; SCH, SCH772984; FCCP, Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose 6-phosphatase.

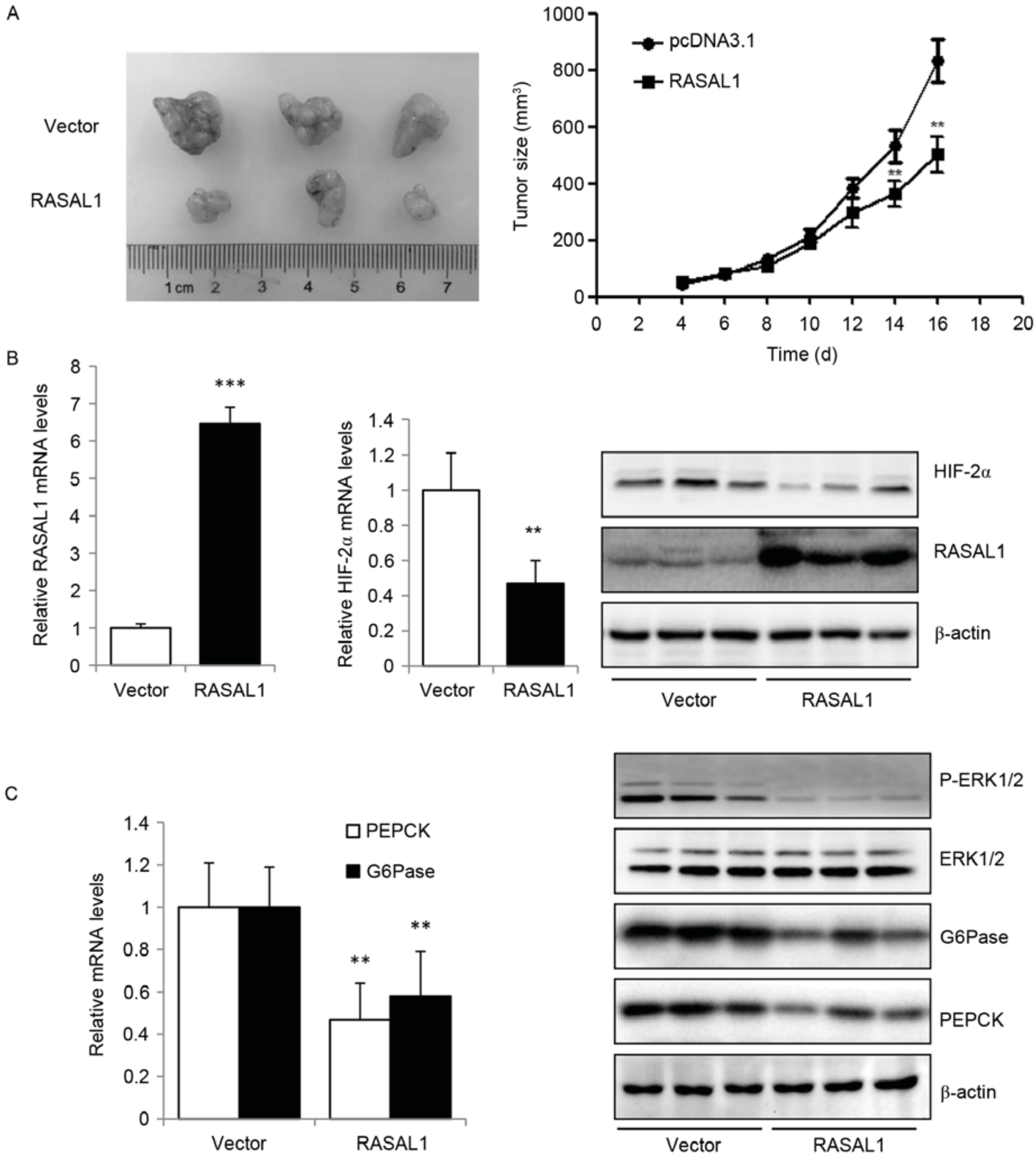

Figure 4. Upregulation of RASAL1 in HepG2 cells inhibits tumor growth through HIF-2α-mediated glucose metabolism. (A) Decreased tumor volume in nude mice four weeks following the subcutaneous injection of $5x10^6$ RASAL1-overexpressing HepG2 stable cells or control cells. Tumors sizes were calculated. (B) The mRNA and protein levels of RASAL1 and HIF-2α were detected via reverse transcription-qPCR and western blotting. (C) The PEPCK and G6Pase mRNA levels were determined by qPCR and normalized to GAPDH. PEPCK, G6Pase protein expression and the phosphorylation of ERK1/2 in xenograft tumor tissues were detected by western blot analysis. Each bar represents the mean ± standard error of the mean of three independent experiments. **P<0.01, ***P<0.001 vs. vector control. HIF, hypoxia-inducible factor; qPCR, quantitative polymerase chain reaction; p, phosphorylated; ERK, extracellular signal-regulated kinase; RASAL1, Ras protein activator like 1; PEPCK, phosphoenolpyruvate carboxy kinase; G6Pase, glucose 6-phosphatase.

Upregulation of RASAL1 in HepG2 inhibits tumor growth via HIF-2α-mediated glucose metabolism in vivo. Based on the data from the present study that the exogenous expression of RASAL1 in liver cancer cells inhibited cancer cell proliferation and invasion *in vitro*, which maybe mediated by reducing the expression of HIF-2α, the effect of RASAL1 overexpression in tumor xenografts *in vivo* was additionally explored. The results indicated that RASAL1 exhibited an inhibitory effect on tumor size compared with the vector control group (Fig. 4A). The higher expression levels of RASAL1 in tumor xenografts transfected with RASAL1-overexpressing HepG2 stable cells, as compared with those transfected with vector control cells, were validated by RT-qPCR and western blotting, are concomitant with the decreased expression of HIF-2α at the mRNA and protein levels (Fig. 4B). The key gluconeogenic enzymes PEPCK and G6Pase were downregulated at the mRNA and protein levels, and the phosphorylation of ERK1/2 was significantly increased (Fig. 4C). These *in vivo* data were concordant with the *in vitro* observations, and suggest that RASAL1 may elicit a tumor suppressive effect through the inhibition of HIF-2α expression, gluconeogenesis and oxygen consumption rate. Thus, theRASAL1/HIF-2α axis maybe a novel therapeutic target for liver cancer treatment.

Discussion

RASAL1 has been suggested to be a tumor suppressor gene in colorectal, thyroid and gastric cancer (4,22,23), through its negative modulation of the RAS signaling pathway, and also to function as an RasGAP that catalyzes RAS inactivation (24,25). Although efforts have been made, the specific molecular mechanisms of its tumor suppressor function remain unknown. Therefore, the present study focused on the tumor suppressive effects of RASAL1 in liver cancer.

In the present study, it was identified that the average levels of RASAL1 in liver cancer tissues were significantly low when compared with those in corresponding non-tumor tissues. The low RASAL1 expression levels of patients with liver cancer are associated with advanced pathological stage and larger tumor size. Consistent with previous data, RASAL1 gene expression was decreased in gastric carcinoma tissues and cell lines (26). HepG2 is a hepatoblastoma cell line that has been previously misidentified as hepatocellular carcinoma. However, it may be used to investigate the functional role of RASAL1 in the development and treatment of liver cancer (18,27). The *in vitro* experiments conducted withRASAL1-overexpressing HepG2 cells in the present study demonstrated that RASAL1 can inhibit the proliferation and invasion ability of HepG2 cells. These results indicate that RASAL1 may have a crucial role in liver cancer development and progression.

As a metabolic regulator, HIF-2α has been identified to be involved in cancer progression via a regulatory role in cancer cell metabolism (28,29). It has been demonstrated previously that the overexpression of HIF-2α in rat glioma tumors may reduce growth by increasing caspase-3-mediated tumor cell apoptosis (30). However, HIF-2α has been revealed to promote tumor growth in a renal carcinoma xenograft model, suggesting a unique role for HIF-2α in tumor growth (31). In the present study, increased expression of RASAL1 in HepG2 cells was observed at the mRNA and protein levels, as compared with the vector control cell line, 48 h after transfection, and this upregulation was concomitant with reduced expression of HIF-2α and increased phosphorylation of ERK1/2. The phosphorylation of ERK1/2 has been indicated to modulate HIF-1 or HIF-2 activity in several cell types (32). HIF-2α restored the expression of the gluconeogenic genes *Pepck* and *G6Pase*, and rescued the hypoglycemic phenotype of Vhlh mutants, supporting a role as a regulator of hepatic lipid metabolism (33). Furthermore, the present study also identified that the overexpression of RASAL1 in HepG2 cells decreases PEPCK and G6Pase mRNA and protein levels, the luciferase assay conducted in the present study indicated that RASAL1 decreased HIF-2α transcription activity, and measurement of the OCR *in vitro* in the presence and absence of RASAL1 in HepG2 cells demonstrated a significant decrease in oxygen consumption in liver cancer cell lines. Notably, inhibition of the activation of ERK1/2 with SCH772984 rescued the effect of RASAL1 downregulation on gluconeogenesis induced by HIF-2α. These data provide evidence that RASAL1 may be a critical inhibitor in liver cancer, via HIF-2α-mediated glucose metabolism.

Based on the *in vitro* data obtained in the present study, which indicated that RASAL1 may be involved in HIF-2α-mediated metabolism in liver cancer cells, the *in vivo* efficacy of the RASAL1-inhibition effect was explored. The results demonstrated that the enhanced tumor growth inhibition efficacy induced by RASAL1-overexpressing HepG2 stable cells with decreased HIF-2α, PEPCK and G6Pase mRNA and protein expression in tumor xenografts, indicates that the RASAL1/HIF-2α axis maybe a potential therapeutic target for current liver cancer therapy.

The present study demonstrated that RASAL1 may partially abrogate HIF-2α-mediated gluconeogenesis through the activation of ERK1/2. Using *in vitro* and *in vivo* bioassays, it was demonstrated, that RASAL1 is an important inhibitory factor for patients with liver cancer, and that it modulates HepG2 cell proliferation. Regulation of HIF-2α, as a component of RASAL1-mediated metabolism, participates in the occurrence and development of liver cancer. Thus, the present study may present a novel strategy for targeting with the RASAL1/HIF-2α interaction as a novel therapeutic application for patients with liver cancer.

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