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PHACTR4 regulates proliferation, migration and invasion of human hepatocellular carcinoma by inhibiting IL-6/Stat3 pathway

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Abstract. – OBJECTIVE: Phosphatase and actin regulator 4 (PHACTR4) is one member of the largely uncharacterized PHACTR family of protein phosphatase 1 (PP1)-and actin-binding proteins. PHACTR4 is significantly deleted or mutant in many tumor subtypes, such as breast, colorectal, lung, neural, ovarian, and renal cancers. However, the role of PHACTR4 in human hepatocellular carcinoma (HCC) is completely unknown.

MATERIALS AND METHODS: Ten paired HCC tissues and adjacent non-cancerous tissues were used to detect the expression PHACTR4. Real-time PCR was used to detect the mRNA level of PHACTR4 in clinic samples. The protein level of PHACTR4 was determined by Western blot. Retrovirus-based gene transduction was used to generate Flag-tagged PHACTR4 HepG2 stable cell line. BrdU assay was used to determine the cell growth of HepG2 cells. The cell cycle distribution was detected by flow cytometry assay. *In vitro* scratch wounding and Matrigel invasion assays were used to test the migration and invasion ability of HepG2 cells.

RESULTS: The expression of PHACTR4 was noticeably decreased in clinical HCC tissues, compared to the non-tumoral tissues. Overexpression of PHACTR4 inhibited HCC cells proliferation, colony formation, migration and invasion, and resulted in significant cycle arrest. PHACTR4 attenuated both constitutive and IL-6-induced phosphorylation of signal transducer and activator of transcription 3 (Stat3), and inhibited Stat3 downstream genes expression.

CONCLUSIONS: Overall, our results suggest that PHACTR4 is a tumor suppressor in HCC by inhibiting IL-6/ Stat3 pathway.

Key Words:
PHACTR4, IL-6, Stat3, Tumor suppressor.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common and the third most lethal cancer, causing about 700,000 deaths per year at the worldwide[1,2]. Therefore, it's critical to understand its molecular mechanisms which might help to identify novel targets for clinic treatment of HCC.

Phosphatase and actin regulator 4 (PHACTR4) belong to a PHACTR family which contains four members, PHACTR1, 2, 3 and 4. PHACTR1 was firstly identified as a PP1a-interacting protein by a yeast two-hybrid screen and confirmed by coimmunoprecipitation (Co-IP) assay[3]. Database analysis revealed the presence of several human proteins similar to the rat PHACTR protein that arbitrarily designated as PHACTR 2-4. All four members were found to coimmunoprecipitate with actin and PP1[3]. A mutation in mouse PHACTR 4 that specifically disrupts PP1 binding, leads to an inhibitory phosphorylation of PP1, abnormal E2F activity and markedly increased proliferation, causing exencephaly and retinal coloboma[4]. The PHACTR4-PP1 interaction also antagonizes β1 integrin signaling in focal adhesions of enteric neural crest cells (ENCCs)[5,6]. Disruption the PHACTR4-PP1 interaction leads to increased β1 integrin signaling, loss of collective and directional migration. Loss of the PHACTR4 gene in mice causes an embryonic gastrointestinal defect due to colon hypoganglionosis[7,8].

Recently, the role of PHACTR4 in cancer development has been identified[9]. PHACTR4 is significantly deleted and mutant in many tumor

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subtypes. Cancer cell lines with reduced PHACTR4 expression exhibit tumor suppressor hypersensitivity upon PHACTR4 complementation[9]. However, the knowledge of the mechanisms underlying PHACTR4 is still incomplete. In the present study, we show that PHACTR4 as a tumor suppressor in human hepatocellular carcinoma (HCC) by inhibiting IL-6 /Stat3 pathway.

Materials and Methods

Human Tissue Samples

Ten paired HCC tissues and adjacent non-cancerous tissues were collected from routine therapeutic surgery at our Department. All samples are obtained with informed consent and this study is approved by the hospital Institutional Review Board.

Cell Culture

HCC cell line HepG2 cells and HEK293T cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CAS, Shanghai, China). All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Shanghai, China) supplemented with 10% fetal bovine serum (Gibco, Shanghai, China), 100 IU/ml penicillin and 100 mg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Quantitative Real-time PCR and shRNA

The Total RNA from tissues and cells were extracted by TRIzol reagent, and reverse transcriptions were performed by Takara RNA PCR kit (Takara, Dalin, China) according to the manufacturer' s instructions. To quantify the transcripts of the genes, quantitative real-time PCR was performed by using a SYBR Green Premix Ex Taq (Takara, Dalin, China) on an Applied Biosystems 7300 Real-time PCR System (Thermo Fisher, Carlsbad, CA, USA). PHACTR4 and beta-actin cDNAs were amplified by the primers: AAGTGAAGGGCAAGCAAAGG (forward) and GGCAGGGACCATATGAGTGA (reverse) for PHACTR4, and CATCCTCACCCTGAAGTACCC (forward) and AGCCTGGATAGCAACGTACATG (reverse) for beta-actin cDNA. Retroviral shRNAs were cloned into plko.1-puro vector. The following shRNAs were used in this study: Control: TTAATCAGAGACTTCAGGCGGT; Phactr4 sh1: TTGATAACAATGTACCACTGTT and Phactr4 sh2: TTCTTTAA-CATGGCATCGCTTG.

Colony Formation Assay

A base layer consisting of 0.5 ml culture media (0.6% agar) covered each 35 mm culture dish. HepG2 HCC cells stable expressing of control or Flag-PHACTR4 were then subcultured by layering 5000 cells in 0.5 ml culture media (0.3% agar) over each base layer. After 14 days incubation, cultures containing uniformly distributed single cell suspensions were considered positive colony.

BrdU Assays

A cell proliferation enzyme-linked immunosorbent assay (Beyotime, Shanghai, China) was used to analyze the incorporation of BrdU during DNA synthesis following the manufacturer's protocols. Absorbance was measured at 450 nm in the Spectra Max 190 ELISA reader (Molecular Devices, Sunnyvale, CA, USA). All experiments were performed in triplicate.

Migration Assay

For *in vitro* scratch wounding assays, the monolayer of HepG2 HCC cells stable expressing of control or Flag-PHACTR4 were cultured in 6 well plates and then physically wounded by a 200 μl yellow pipette tip. The area devoid of cells was imaged immediately and 36 h later. The Zeiss AxioVision microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) was used to visualize and image cultured cells. The T scratch software was used to acquire the imaging data. The ratio of the covered area was compared with freshly wounded samples.

Invasion Assay

The invasion ability of HepG2 cells with stable expression of Flag-PHACTR4 was determined by Matrigel invasion assays. Cells were cultured in 24-well BD invasion chambers at a concentration of 2×10^5 cells per well without serum and immediately placed onto the upper compartment of the plates. The lower compartment was added with medium plus 10% serum. Cells in chambers were incubated for 36 h, and cells on the lower surface of the membrane were stained with crystal violet and fixed in 4% paraformaldehyde. Invasion ability was expressed as percentages of control.

Cell Cycle Distribution

HepG2 cells stable expressing of control or Flag-PHACTR4 were collected, rinsed and fixed overnight in 75% cold ethanol. Then, cells were

treated with Tris-HCl buffer supplemented with RNase A and stained with propidium iodide (BD Biosciences, San Diego, CA, USA). Cell cycle distribution was determined by flow cytometry (Becton Dickinson, San Jose, CA, USA). All data were analyzed by ModFit software.

Western Blot

Clinic tissues or cultured cells were harvested and lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 6.8, 100 mM 2-mercaptoethanol, 2% SDS and 10% glycerol). After centrifugation at 15,000× g for 15 min at 4°C, proteins in the supernatants were quantified and separated by 12% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). Western blot assay was performed using the following antibodies: anti-PHACTR4, Stat3, p-Stat3 HA, Bim1, USP7, cyclin D1, Mcl1 (Santa Cruz, CA, USA) and Flag M2 antibodies (Sigma-Aldrich, St. Louis, MO, USA). Protein levels were normalized to total GAPDH, using a rabbit anti-GAPDH antibody (Santa Cruz, CA, USA).

Immunoprecipitation (IP)

Co-IP was performed on lysates from HepG2 cells transfected with indicated plasmids. Cells were lysed in RIPA buffer and supernatants were clarified, and precipitated with Flag-M2 agarose beads for overnight at 4°C. Beads with immune complexes were washed with RIPA buffer and boiled in 2x SDS loading buffer. For endogenous IP, For endogenous IP, HepG2 cells with stable expression of Flag-PHACTR4 were lysed in RIPA buffer. The supernatants were clarified, and precipitated with Flag-M2 agarose beads overnight at 4°C. Beads with immune complexes were washed with RIPA buffer and boiled in 2x SDS loading buffer.

Statistical Analysis

The data shown represent the mean ± standard (SD) of three independent experiments. Significance was analyzed using Student's *t*-test (*$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$)

Results

PHACTR4 was Noticeably Decreased in Human Primary HCC Surgical Specimens

To investigate the role of PHACTR4 in HCC, we firstly checked the mRNA expression level of PHACTR4 in ten HCC tissues and the matched adjacent non-cancerous tissues. Our quantitative RT-PCR showed that tissues from primary human HCC biopsies exhibited significantly lower expression rates of PHACTR4 mRNA compared to adjacent non-tumor tissues (Figure 1A). In general, lower level of mRNA leads to decreased expression of its encoded protein. To verify this concept in the case of PHACTR4, the lysate from HCC tissue samples were subjected to Western blot analysis by using anti-PHACTR4 antibody. As depicted in Figure 1B, the expression of PHACTR4 protein was significantly decreased in

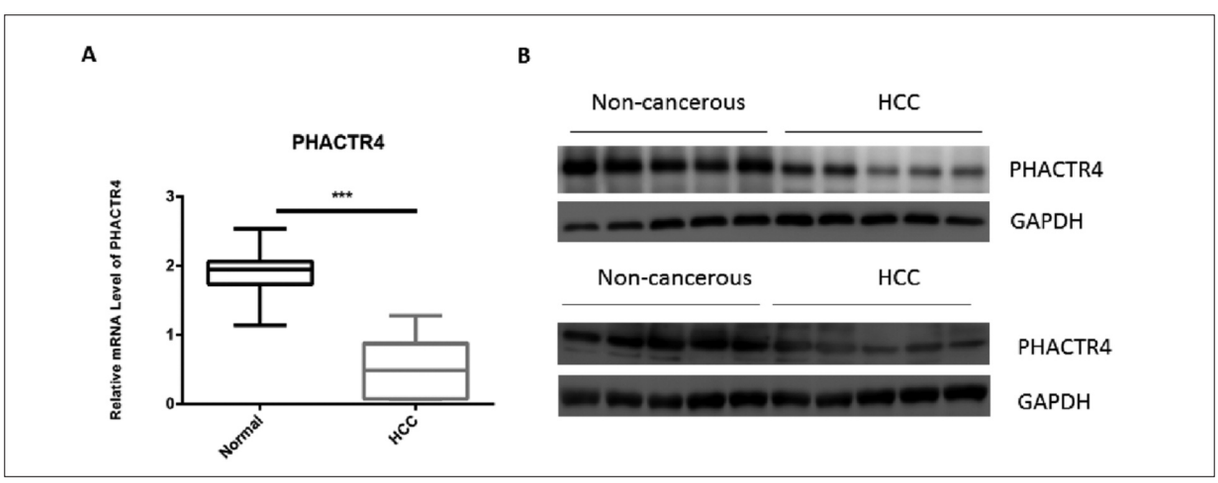

Figure 1. The mRNA and protein levels of PHACTR4 were noticeably decreased in human primary HCC surgical specimens. ***A,*** Relative mRNA expression of PHACTR4 was lower in 10 HCC tissues than in matched adjacent non-cancerous tissues ($p < 0.01$). ***B,*** Representative result of expression of PHACTR4 protein in 10 paired HCC tissues and matched adjacent non-cancerous tissues.

10 specimens of human primary HCC tissue compared to adjacent non-tumor hepatic tissues. These data suggested that PHACTR4 was down-regulated in HCC samples.

PHACTR4 Inhibited HCC Cells Proliferation and Colony Formation

To explore the potential consequences of gain-of-function in the tumorigenic phenotypes of PHACTR4 in HCC cells, a series of experiments were performed in HepG2 cells stable expression of exogenous PHACTR4. The Flag-tagged PHACTR4 expression was verified by Western blot with the anti-Flag antibody (Figure 2A). We found that HepG2 cells overexpression of PHACTR4 (PHACTR4-HepG2) showed reduced growth capacity when compared with control HepG2 cells (Figure 2B). We further confirmed this phenomenon by BrdU assay (Figure 2C). In agreement with this, the colony-forming ability was also decreased in PHACTR4-HepG2 cells (Figure 2D). To investigate the potential mechanisms underlying the anti-proliferative effect of HACTR4 in HCC cells, the cell cycle distribution was performed in PHACTR4-HepG2 cells or control HepG2 cells by flow cytometric analysis. We found that overexpression of PHACTR4 led to cell cycle arrest at G0/G1 phase and reduce the percentages of cells at S phase (Figure 2E). Taken together, these data suggested that PHACTR4 inhibited HCC cells proliferation via cell cycle arrest.

PHACTR4 Suppressed Migration and Invasion of HCC Cells in Vitro

To test whether PHACTR4 has a role in HCC cells migration and invasion, we used *in vitro* scratch wounding and matrigel invasion assays. Both assays demonstrated that the migration and invasive capability of HepG2 cells overexpression of PHACTR4 were significantly reduced when compared with control cells (Figure 3A, 3B), suggesting that PHACTR4 suppressed migration and invasion of HCC cells *in vitro*.

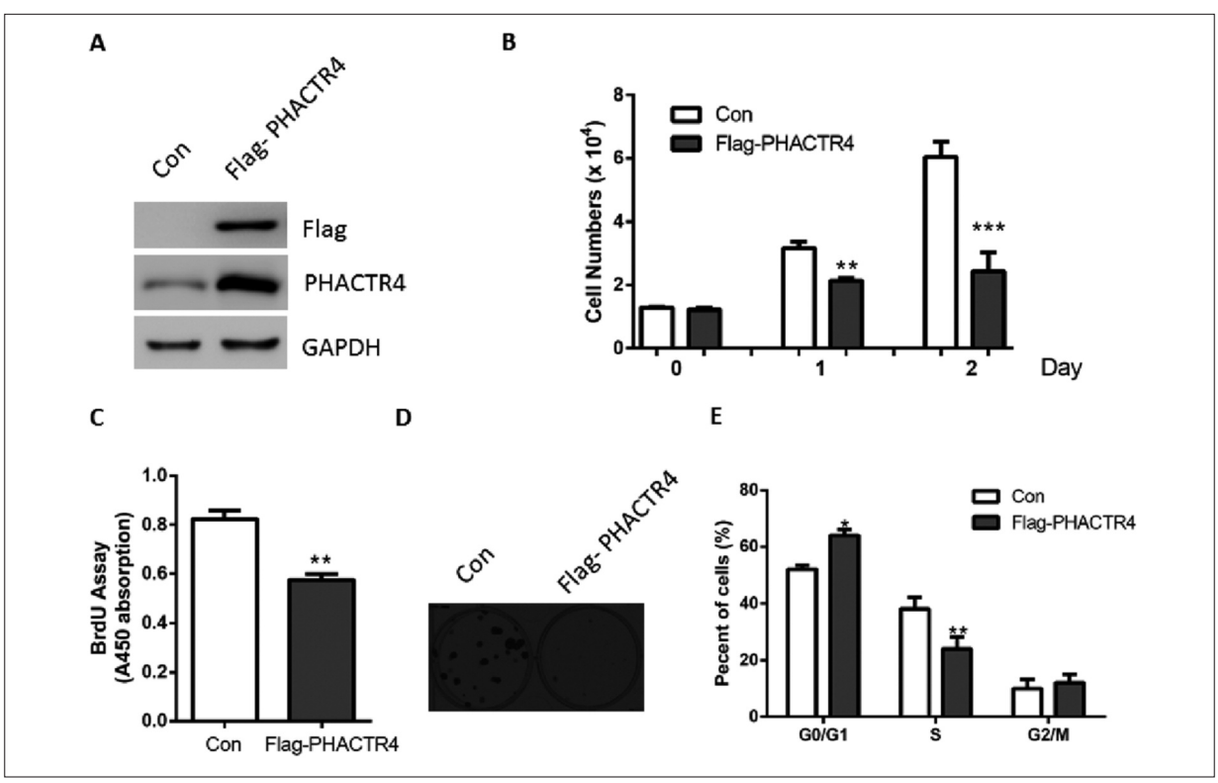

Figure 2. PHACTR4 inhibited HCC cells proliferation and colony formation. ***A,*** The protein level of exogenous PHACTR4 was analyzed by Western Blot with both Flag and PHACTR4 antibodies in HepG2 cells stably expression Flag-PHACTR4 or control vector (Con). ***B,*** The growth of HepG2 cells stably expression Flag-PHACTR4 or control vector (Con). ***C,*** The proliferative potential (Bromodeoxyuridine: BrdU) assay was determined in HepG2 cells stably expression Flag-PHACTR4 or control vector (Con). ***D,*** The colony formation was performed in HepG2 cells stably expression Flag-PHACTR4 or control vector (Con). ***E,*** The cell cycle distribution of HepG2 cells stably expression Flag-PHACTR4 or control vector (Con) was analyzed by flow cytometry.

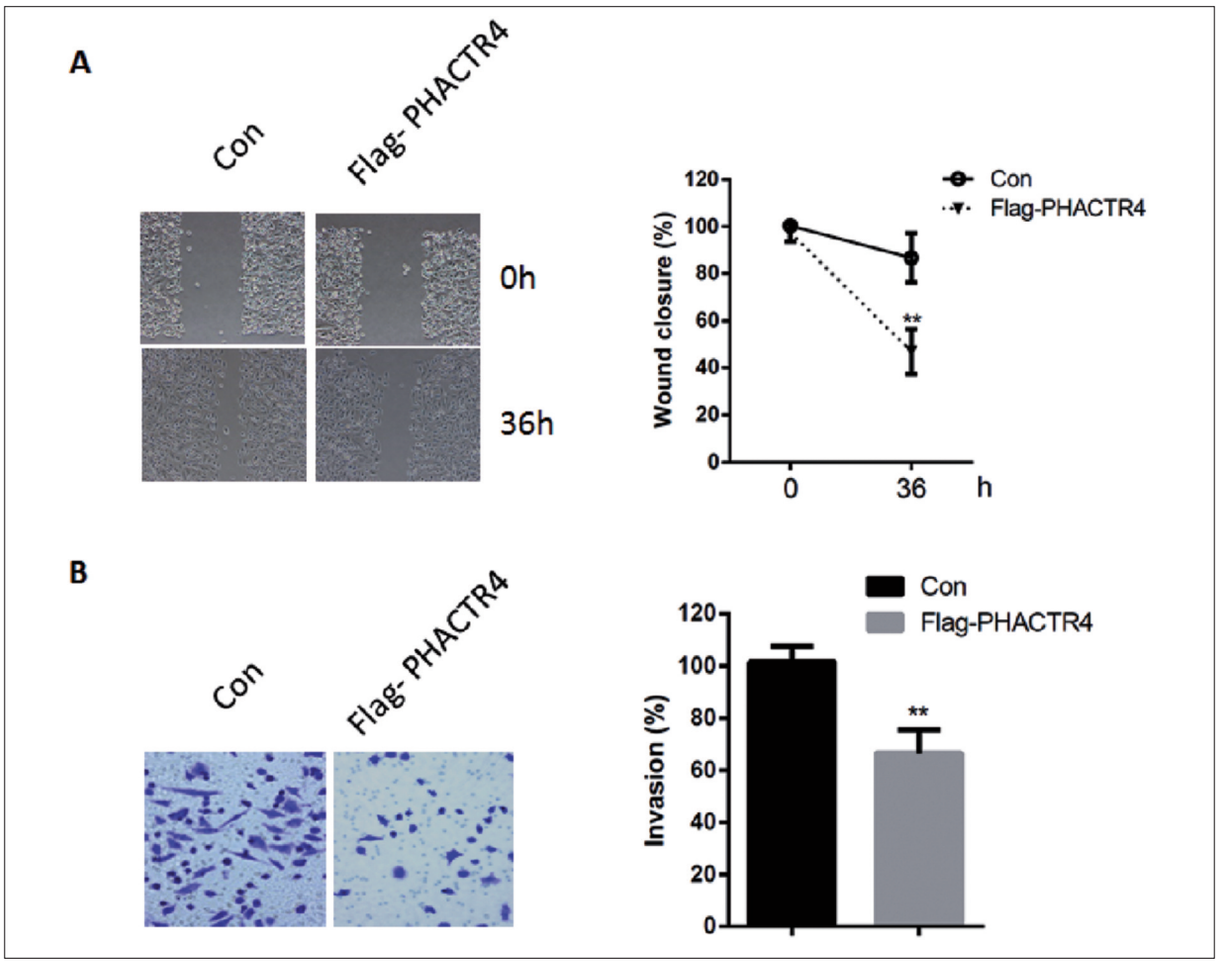

Figure 3. PHACTR4 suppressed migration and invasion of HCC cells *in vitro*. ***A,*** Representative images and quantification data of HepG2 cells stably expression Flag-PHACTR4 or control vector (Con) at 0 and 36 h after wound scratch in wound-healing assay. ***B,*** Cell invasion ability was analyzed by transwell chamber assay. The representative images and quantification data of crystal violet stained HepG2 cells stably expression Flag-PHACTR4 or control vector (Con).

PHACTR4 Inhibited the Stat3 Signaling Pathway in HCC Cells

To investigate the underline mechanisms of HACTR4 in the regulation of HCC development, we test whether HACTR4 could regulate signaling pathways that known to be involved in HCC development. We found that the Stat3 phosphorylation was impaired in PHACTR4 overexpression cells, whereas activated in PHACTR4-depleted cells (Figure 4A-B). Moreover, we also observed IL-6-induced Stat3 phosphorylation was also blocked in PHACTR4 overexpression cells (Figure 4C). These data indicated that PHACTR4 attenuated both constitutive and IL-6-induced phosphorylation of Stat3.

PHACTR4 Interacted with Stat3

The previous studies indicate that HACTR4 exhibits its biological function through protein-protein interaction[3]. To understand the detailed mechanism of HACTR4 in the regulation of Stat3 activation, we asked whether HACTR4 interacts with Stat3. To test this possibility, Flag-HACTR4 and HA-Stat3 were co-expressed into 293T cells and the interaction between HACTR4 and Stat3 were detected by IP-Western blot assay. As depicted in Figure 5A, HA-Stat3 was detected in Flag-HACTR4 immunoprecipitate. Immunoprecipitation of lysates of Flag-HACTR4-overexpressing HepG2 followed by immunoblotting revealed a physical association between tagged-HACTR4 and endogenous

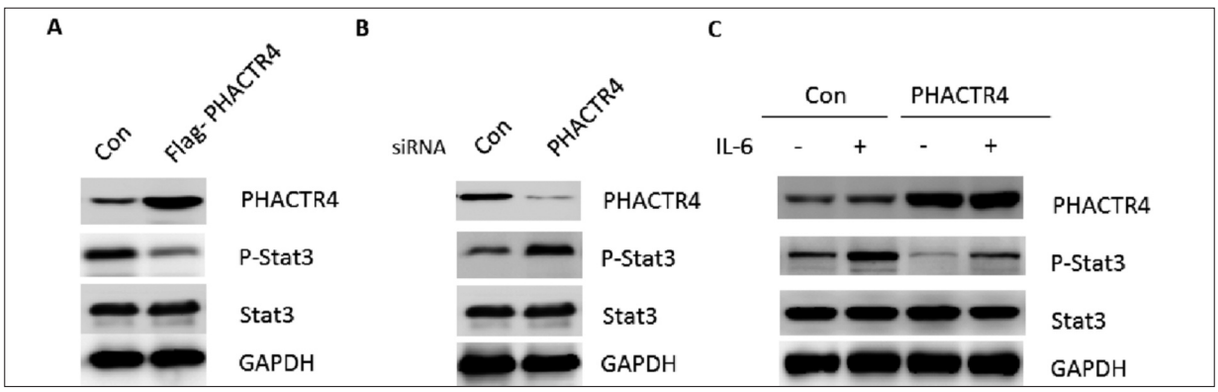

Figure 4. PHACTR4 inhibited the Stat3 signaling pathway in HCC cells. ***A,*** HepG2 cells stably expression Flag-PHACTR4 or control vector were harvested, Western blot was performed by using indicated antibodies. ***B,*** HepG2 cells were transient transfected with non-target siRNAs or siRNAs against PHACTR for 36h. After that, cells were harvested and subjected to Western blot with indicated antibodies. ***C,*** Western blot was performed for HepG2 cells stably expression Flag-PHACTR4 or control vector treated with or without IL-6.

Stat3 (Figure 5B). These findings suggested that PHACTR4 interacted with Stat3 to repress Jak2/Stat3 pathway.

PHACTR4 Repressed the Stat3 Downstream Genes Expression

To investigate whether the relation between HACTR4 and Stat3 was physiologically relevant, we detect the expression of several Stat3 downstream genes, including cyclin D1, Mcl1, Bmi-1 and USP7[10,11]. We found that overexpression of HACTR4 induced the expression of USP7, but repressed the expression of cyclin D1, Mcl1, Bmi-1 (Figure 6A). In the contrast, silencing the expression of HACTR4 achieved the opposite effects (Figure 6B), suggesting that PHACTR4 could repress the Stat3 downstream genes expression.

Discussion

Our results strongly support the notion that PHACTR4 function as a tumor suppressor in the progression of HCC. Firstly, both the mRNA and protein expression levels of PHACTR4

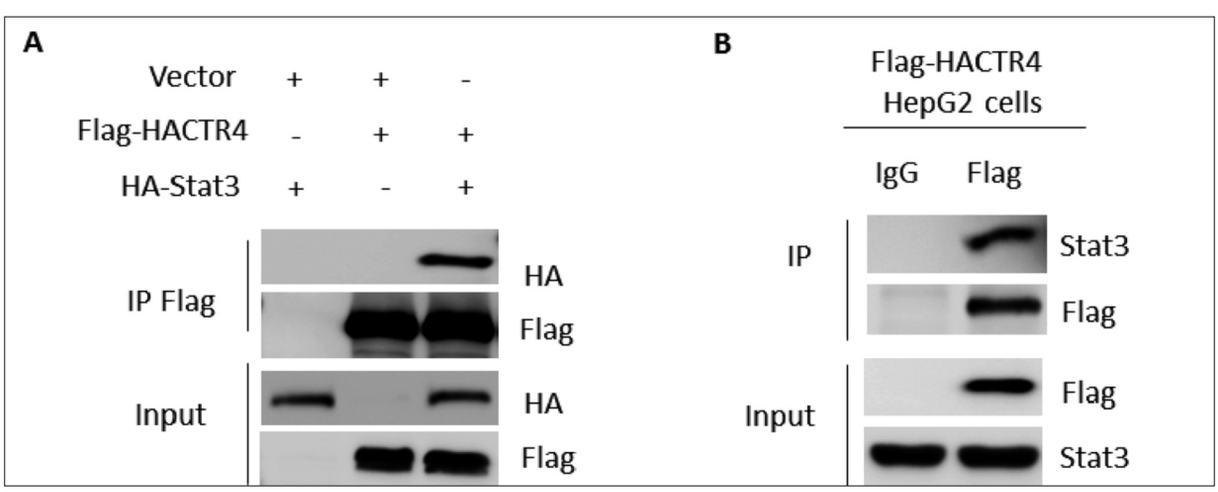

Figure 5. PHACTR4 interacted with Stat3. ***A,*** 293T cells were co-expressed with Flag-HACTR4 and HA-Stat3 for 36h, cells were harvested and co-immunoprecipitation was performed by using Flag M2 beads. The precipitates were immunoblotted by HA and Flag antibodies, respectively. ***B,*** HepG2 cells stably expression Flag-PHACTR4 were harvested, lysed and precipitated with mouse lgG or Flag-M2 antibodies overnight. Protein G agarose was then added. The precipitates were immunoblotted for Flag and Stat3.

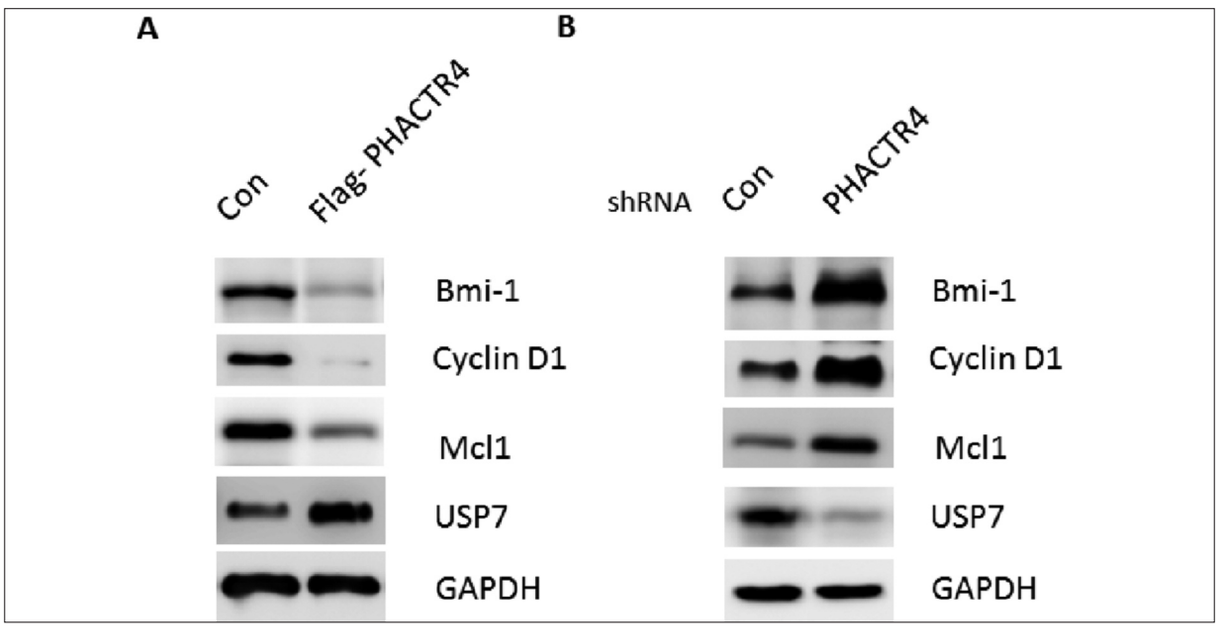

Figure 6. PHACTR4 repressed the Stat3 downstream genes expression. ***A,*** HepG2 cells stably expression Flag-PHACTR4 or control vector were harvested, Western blot was performed by using indicated antibodies. ***B,*** HepG2 cells were transient transfected with non-target siRNAs or siRNAs against PHACTR for 36h. After that, cells were harvested and subjected to Western blot with indicated antibodies.

were significantly decreased in HCC clinic samples, compared with the adjacent nontumorous liver tissues. Next, ectopic expression of PHACTR4 by retrovirus-base gene expression inhibited proliferation, colony formation, migration and invasion in HCC cells, and resulted in significant cycle arrest at G1 phase. However, *in vivo* studies using PHACTR4 knockout mice or cancer models are still needed to further clarify the exact roles of PHACTR4 in HCC development. Furthermore, PHACTR4 has been reported to be significantly deleted or mutant in many tumor subtypes, such as breast, colorectal, lung, neural, ovarian, and renal cancers[9]. Notably, a recent study demonstrates that in squamous cell carcinoma (SCC), PHACTR4 is a target of miR21, a cancer-associated miRNAs and the expression of which correlates with poor prognosis in human SCCs and enhances tumor progression in xenografts[12]. Whether the deletion or mutation or epigenetic regulation of PHACTR4 occurred in HCC tissues remain to be determined in the future studies.

At the molecular level, our results suggested that PHACTR4 is a negative regulator of the IL-6/Stat3 signaling pathway. Several investigations demonstrate that PHACTR proteins exhibit their function through directly protein-protein interaction with other key signaling factors[13]. Here, we found a physical association between tagged-HACTR4 and endogenous Stat3 and then HACTR4 inhibited both constitutive and IL-6-induced phosphorylation of Stat3. Due to the existence of negative feedback mechanisms, IL-6-induced STAT3 activation is tightly controlled in normal physiology conditions[14]. However, aberrant IL-6 and its down-stream STAT3 signaling in cancer cells have emerged as a major mechanism for cancer initiation and development[15,16]. Moreover, STAT3 signaling selectively induces and maintains a procarcinogenic inflammatory microenvironment, which is crucial for inflammatory cell-mediated transformation and tumor progression, that makes STAT3 a promising target to redirect inflammation for cancer therapy[17,18].

Conclusions

Our work provided an important insight into the function of PHACTR4/Stat3 signaling pathway in the development and HCC progression. Given the promising roles of the Stat3 inhibitors in the treatment of HCC, our results may bring some advances in better drug design, pharmacokinetics and pharmacogenetics in the future.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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