Research article JHEP|Reports

SCYL3, as a novel binding partner and regulator of ROCK2, promotes hepatocellular carcinoma progression

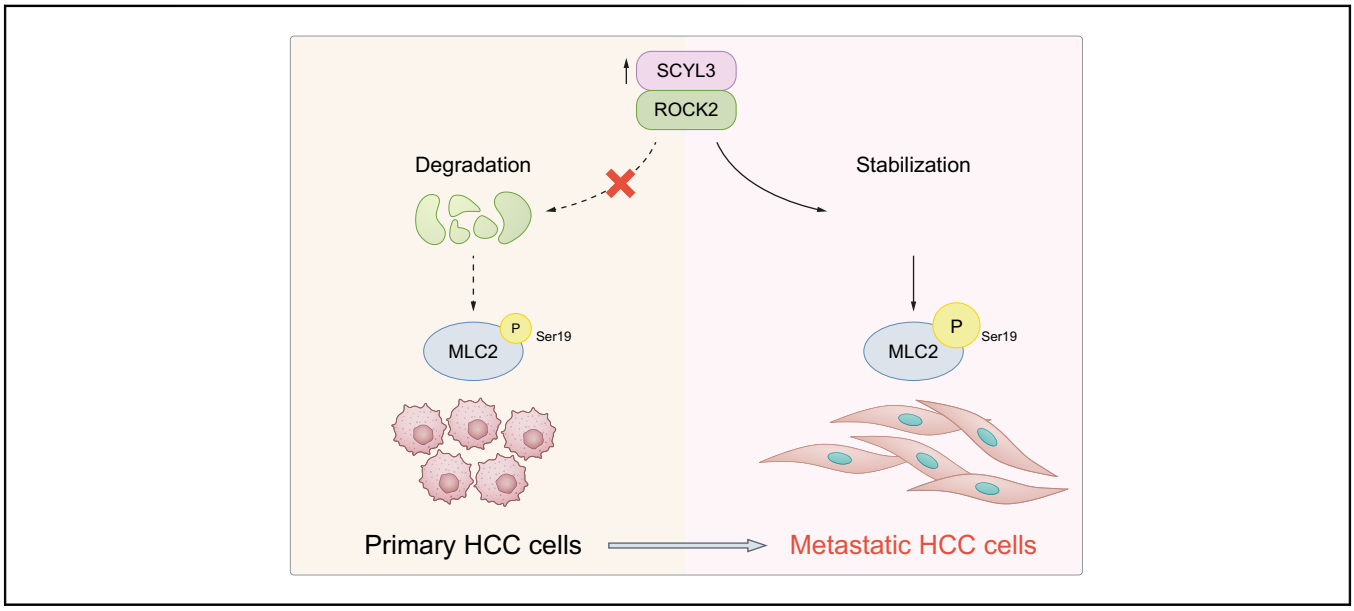
Authors

Martina Mang Leng Lei, Carmen Oi Ning Leung, Eunice Yuen Ting Lau, Rainbow Wing Hei Leung, Victor Wan San Ma, Man Tong, Yin Ying Lu, Chen Yang Huang, Qiao Hua Zhu, Irene Oi Lin Ng, Stephanie Ma, Terence Kin Wah Lee

Correspondence

terence.kw.lee@polyu.edu.hk (T.K.W. Lee).

Graphical abstract



Highlights

- SCYL3 was found to be overexpressed in HCC and was associated with metastasis and poor survival in human tumors.
- SCYL3 is critically involved in the regulation of HCC progression and metastasis.
- We identified ROCK2 as the binding partner of SCYL3.
- SCYL3 physically binds and regulates the stability and transactivating activity of ROCK2 via its C-terminal domain.

Impact and implications

SCYL3 was first reported to be a binding partner of a metastasis-related gene, ezrin. To date, the clinical relevance and functional role of SCYL3 in cancer remain uncharacterized. Herein, we uncover its crucial role in liver cancer progression. We show that it physically binds and regulates the stability and transactivating activity of ROCK2 leading to HCC tumor progression. Our data provide mechanistic insight that SCYL3-mediated ROCK2 protein stability plays a pivotal role in growth and metastasis of HCC cells. Targeting SCYL3/ROCK2 signaling cascade may be a novel therapeutic strategy for treatment of HCC patients.

https://doi.org/10.1016/j.jhepr.2022.100604

Research article

JHEP|Reports

SCYL3, as a novel binding partner and regulator of ROCK2, promotes hepatocellular carcinoma progression

Martina Mang Leng Lei,[1,†] Carmen Oi Ning Leung,[1,†] Eunice Yuen Ting Lau,[2,†] Rainbow Wing Hei Leung,[1] Victor Wan San Ma,[1] Man Tong,[3] Yin Ying Lu,[4] Chen Yang Huang,[4] Qiao Hua Zhu,[5] Irene Oi Lin Ng,[6,7] Stephanie Ma,[3,7] Terence Kin Wah Lee[1,8,9,*]

[1]*Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong;* [2]*Department of Clinical Oncology, Queen Elizabeth Hospital, Hong Kong;* [3]*School of Biomedical Sciences, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong;* [4]*Comprehensive Liver Cancer Center, The Fifth Medical Center of PLA General Hospital, Beijing, China;* [5]*Department of Interventional Radiology and Oncology, Shunde Hospital, Southern Medical University, Shunde, China;* [6]*Department of Pathology, Queen Mary Hospital, The University of Hong Kong, Hong Kong;* [7]*State Key Laboratory of Liver Research, The University of Hong Kong, Hong Kong;* [8]*Research Institute for Future Food, The Hong Kong Polytechnic University, Hong Kong;* [9]*State Key Laboratory of Chemical Biology and Drug Discovery, The Hong Kong Polytechnic University, Hong Kong*

JHEP Reports **2023.** https://doi.org/10.1016/j.jhepr.2022.100604

Background & Aims: SCY1-like pseudokinase 3 (SCYL3) was identified as a binding partner of ezrin, implicating it in metastasis. However, the clinical relevance and functional role of SCYL3 in cancer remain uncharacterized. In this study, we aimed to elucidate the role of SCYL3 in the progression of hepatocellular carcinoma (HCC).

Methods: The clinical significance of SCYL3 in HCC was evaluated in publicly available datasets and by qPCR analysis of an in-house HCC cohort. The functional significance and mechanistic consequences of SCYL3 were examined in SCYL3-knockdown/overexpressing HCC cells. *In vivo* tumor progression was evaluated in *Tp53*^{KO}/*c-Myc*^{OE} mice using the sleeping beauty transposon system. Potential downstream pathways were investigated by co-immunoprecipitation, western blotting analysis and immunofluorescence staining.

Results: SCYL3 is often overexpressed in HCC; it is preferentially expressed in metastatic human HCC tumors and is associated with worse patient survival. Suppression of SCYL3 in HCC cells attenuated cell proliferation and migration as well as *in vivo* metastasis. Intriguingly, endogenous SCYL3 overexpression increased tumor development and metastasis in *Tp53*^{KO}/*c-Myc*^{OE} mice. Mechanistic investigations revealed that SCYL3 physically binds and regulates the stability and transactivating activity of ROCK2 (Rho kinase 2) via its C-terminal domain, leading to the increased formation of actin stress fibers and focal adhesions.

Conclusions: These findings reveal that SCYL3 plays a critical role in promoting the progression of HCC and have implications for developing new therapeutic strategies to tackle metastatic HCC.

Impact and implications: SCYL3 was first reported to be a binding partner of a metastasis-related gene, ezrin. To date, the clinical relevance and functional role of SCYL3 in cancer remain uncharacterized. Herein, we uncover its crucial role in liver cancer progression. We show that it physically binds and regulates the stability and transactivating activity of ROCK2 leading to HCC tumor progression. Our data provide mechanistic insight that SCYL3-mediated ROCK2 protein stability plays a pivotal role in growth and metastasis of HCC cells. Targeting SCYL3/ROCK2 signaling cascade may be a novel therapeutic strategy for treatment of HCC patients.

Introduction

Primary liver cancer, of which the majority (~90%) of cases are hepatocellular carcinoma (HCC), is one of the deadliest malignancies, being the 6th most commonly diagnosed cancer and the 4th leading cause of cancer-related mortality in the world.[1] When patients present with early-stage HCC, they can be treated with surgical resection or liver transplantation. However, HCC is frequently complicated by the occurrence of intrahepatic and extrahepatic metastases, even after surgical resection.[2] Common sites of extrahepatic metastasis include the lung, bone, peritoneum, spleen, and lymph nodes.[3] The prognosis of HCC remains unsatisfactory, with a 5-year overall survival rate of ~70% post resection.[4] Despite recent advances in biomedical technologies, the molecular mechanisms that underlie cancer metastasis remain unclear. To improve the prognosis of individuals with HCC, an understanding of the molecular mechanisms of metastasis in HCC is urgently needed.

SCY1-like pseudokinase 3 (SCYL3) (accession number BC014662) is located on chromosome 1q24.2 and encodes a

Keywords: SCYL3; ROCK2; Hepatocellular carcinoma; Protein stability; Metastasis.
Received 12 April 2022; received in revised form 28 September 2022; accepted 1 October 2022; available online 13 October 2022

† Contributed equally

* Corresponding author. Address: Room 805, Block Y, Department of Applied Biology and Chemical Technology, Lee Shau Kee Building, The Hong Kong Polytechnic University, Hong Kong. Tel.: (852) 3400-8799, fax: (852) 2364-9932
E-mail address: terence.kw.lee@polyu.edu.hk (T.K.W. Lee).

protein of 110 kDa that has a kinase domain and four HEAT repeats. SCYL3 is also known as protein-associating with the carboxyl-terminal domain of ezrin (PACE-1) because it physically interacts with the C-terminal domain of ezrin.[5] Currently, only two reports have discussed the possible functional role of SCYL3. In one, SCYL3 was found to be preferentially expressed in the lamellipodia of highly metastatic breast cancer cells, where it colocalizes with filamentous actin, CD44 and ezrin. This observation suggests a role for SCYL3 in the regulation of cancer metastasis. However, the clinical relevance and functional role of SCYL3 in cancer remain uncharacterized. SCYL3 lacks the highly conserved histidine-arginine-aspartic acid (HRD) motif that is commonly found in kinase domains and was therefore suggested to be a pseudokinase. However, SCYL3 also demonstrated *in vitro* kinase activity,[5] suggesting that SCYL3 may bind to an active kinase rather than possessing intrinsic kinase activity itself. To characterize the physiological function of SCYL3, it is crucial to identify the direct interacting partner through which it exerts kinase activity.

In this study, we first found that SCYL3 was overexpressed in cancers of the liver, breast and colon. Specifically, SCYL3 was overexpressed in HCC at both the mRNA and protein levels and correlated with poor prognosis in individuals with HCC. SCYL3 expression was increased from non-cancerous to tumor tissues to metastatic counterparts in a stepwise manner. Using overexpression and knockdown approaches, we found that SCYL3 was critically involved in HCC tumor growth and metastasis. Interestingly, we identified ROCK2 as the novel putative binding partner of SCYL3. We found that SCYL3 stabilizes the ROCK2 protein and augments its transactivating activity, resulting in increased HCC invasiveness. Findings from our study not only provide new insights into how HCC metastasis is regulated, but also identify a new mechanism through which ROCK2 signaling is regulated by SCYL3. Targeting the SCYL3/ROCK2 signaling cascade may be a novel therapeutic strategy against HCC.

Materials and methods

Reagents

Cycloheximide (CHX) and mitomycin C were purchased from Calbiochem.

Cell lines and cell culture

Human HCC cell lines MHCC-97L and MHCC-97H (Liver Cancer Institute, Fudan University), Hep3B (ATCC), HepG2 (ATCC), Huh7 and PLC/PRF/5 (Japan Cancer Research Bank), MIHA (a gift from Dr. J.R. Chowdhury, Albert Einstein College of Medicine) and HEK293T (ATCC) were maintained in DMEM with high glucose and L-glutamine (Gibco, Invitrogen) supplemented with 10% heat-inactivated FBS (Gibco, Invitrogen), 100 mg/ml penicillin G, and 50 μg/ml streptomycin (Gibco, Invitrogen) at 37 °C in a humidified chamber containing 5% CO_2. Lentiviral infected cells were cultured in complete DMEM supplemented with 1 μg/ml puromycin. Culture medium was refreshed every 2 days. All cell lines used in this study were obtained between 2013 and 2016, regularly authenticated by morphologic observation and AuthentiFiler STR (Invitrogen) and tested for absence of mycoplasma contamination (MycoAlert, Lonza). Cells were used within 20 passages after thawing.

Human tissue specimens for mRNA expression analysis

Paired patient HCC and adjacent non-cancerous liver tissue specimens were collected at the time of surgical resection from patients at Queen Mary Hospital, Hong Kong, from 1991 to 2013. The use of human clinical specimens was approved by the Institutional Review Board (IRB) of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. Consent from patients was obtained.

HCC tissue microarray

Human primary HCCs and their metastatic counterparts, and corresponding non-tumorous liver samples in the cohort of 28 primary HCCs were obtained from patients undergoing liver resection between 1993 and 2007 at Queen Mary Hospital, Hong Kong and randomly selected for the present study. The metastatic sites of these 28 HCC cases are shown in Table S1. All specimens collected were fixed in 10% formalin for paraffin embedding. Frozen sections from tumorous and non-tumorous liver samples were cut and stained for histological examination to ensure homogenous cell population of tissues. Use of human specimens was approved by the institutional review board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. Consent from patients was obtained.

RNA extraction and quantitative PCR analysis (qPCR)

Total RNA was isolated using TRIzol reagent according to the manufacturer's protocol (Invitrogen). cDNA was synthesized using PrimeScript RT Reagent Kit (Takara) according to the manufacturer's instructions and then subjected to qPCR with BrightGreen 2 × qPCR Master Mix (Applied Biological Materials) using QuantStudio 7 Flex Real Time PCR System (Applied Biosystems) with primers specific to the sequences of genes of interest, which are provided in Table S2. Relative expression differences were calculated using $2^{-\Delta\Delta}C_T$ method with reference to β-actin. qPCRs were performed according to the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines.

Lentiviral-based transfection into HCC cells

SCYL3 short-hairpin RNA (shRNA) expression vectors were cloned into pLKO.1 vector (Addgene). The scrambled shRNA non-target control (NTC) was purchased from Sigma-Aldrich. Clone ID of the two shRNAs directed against SCYL3 are as follows: TRCN0000421240 (shSCYL3-1) and TRCN0000199443 (shSCYL3-2). Transduced cells were selected with 2 μg/ml puromycin. Table S3 lists the sequences of the shSCYL3 and NTC used. For overexpression, stable HCC cells ectopically overexpressing pReceiver-Lv105-SCYL3 were selected by 2 μg/ml puromycin.

In vivo orthotopic HCC metastasis model

Metastasis was assessed by orthotopically injecting HCC cells into the liver and then looking for metastases in the lung. Luciferase-labeled cells (NTC and shSCYL3-2) were injected into the left lobes of the livers of 6-week-old male BALB/c nude mice (n = 7/group). Six-to-eight weeks after implantation, mice were administered with 100 mg/kg D-luciferin (Gold Biotechnology) via peritoneal injection 5 min before bioluminescent imaging (Perkin-Elmer IVIS Lumina Series III Pre-clinical In Vivo Animal Imaging Systems). Livers and lungs were harvested for *ex vivo* imaging and histologic analysis.

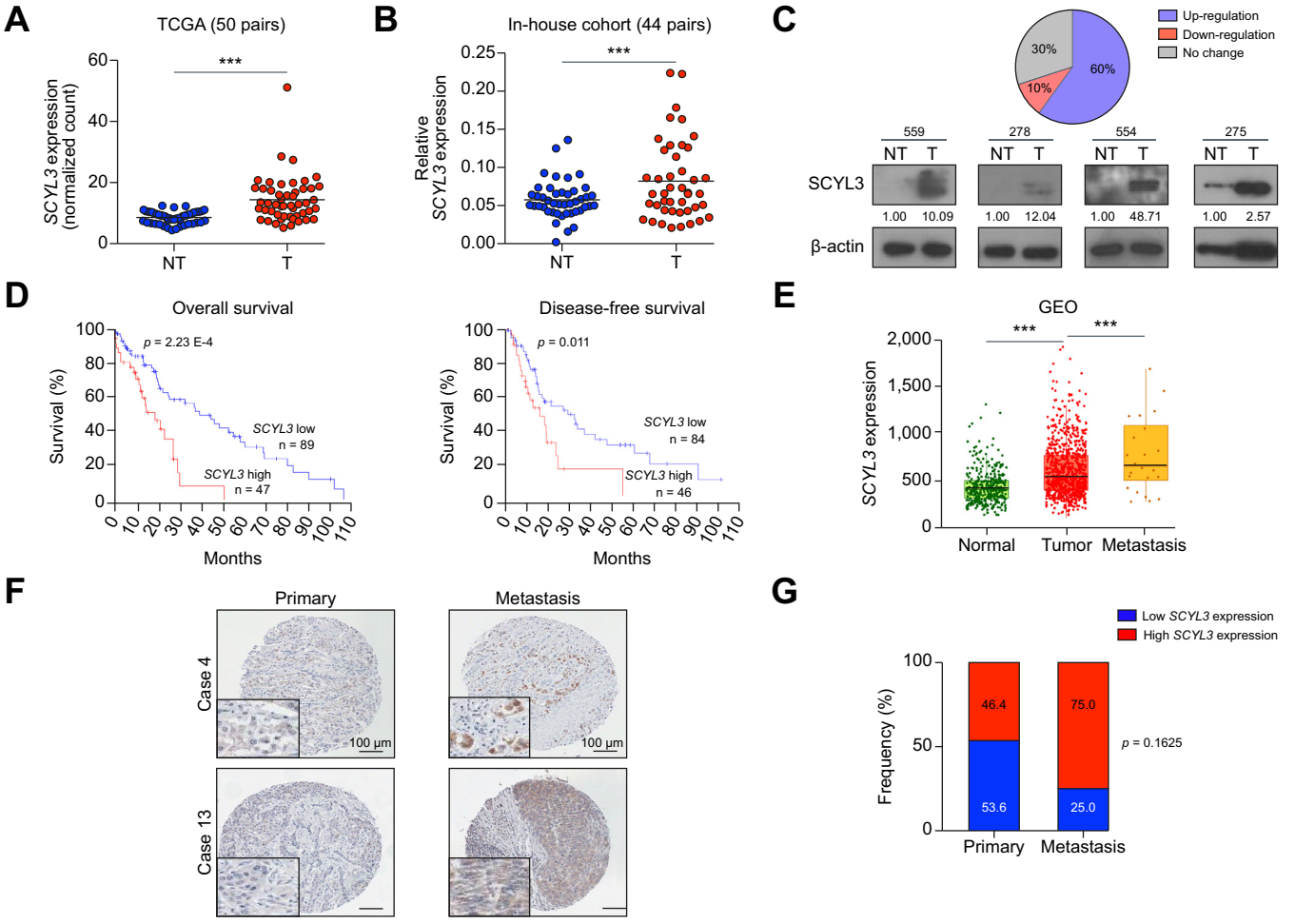


Fig. 1. SCYL3 overexpression is correlated with metastasis and poor prognosis. (A) Analysis of TCGA dataset shows upregulation of *SCYL3* mRNA expression in a cohort of 50 HCC compared to paired non-tumor samples (***p <0.001, t test). (B) qPCR analysis showed that *SCYL3* mRNA is upregulated in 44 HCC samples (***p <0.001, t test). (C) Western blotting analysis demonstrated upregulation of SCYL3 expression in 10 clinical HCC samples at the protein level. Four representative samples were shown. Chart representation of the percentage of cases with various SCYL3 expression levels. (D) In the analysis of TCGA data, the overall and disease-free survival rates of individuals with HCC and high SCYL3 overexpression were significantly lower than those of individuals with low SCYL3 expression (p = 2.23 E^{-4} and p = 0.011, respectively; log-rank test). (E) In the GEO database of HCC, a stepwise increase in the expression of *SCYL3* from the normal to primary tumor and to metastasis (***p<0.001, t test) was observed. (F) In 28 primary and matched distinct metastatic tissues, immunohistochemistry revealed significant upregulation of SCYL3 expression in metastatic HCCs compared with their primary counterparts. Two representative cases (case #4 (lung) and case #13 (colon)) were shown. Scale bar, 100 μm. (G) Strong SCYL3 expression correlated with HCC metastasis in a cohort of 28 HCC clinical samples (p = 0.1625, χ^2 test). Error bars indicate mean ± SD. GEO, Gene Expression Omnibus; HCC, hepatocellular carcinoma; NT, non-tumourous liver; T, tumourous liver; TCGA, The Cancer Genome Atlas.

Hydrodynamic tail vein *Tp53*^{KO}/*c-Myc*^{OE} HCC mouse model

Six-to-eight-week-old male wild-type C57BL/6 mice were used, and the procedure was performed as described previously. In brief, 15 μg of plasmids encoding human c-Myc tagged with luciferase (c-Myc-luc) and single-guide (sg)TP53 along with sleeping beauty (SB) transposase at a ratio of 25:1 were diluted in 2 ml saline (0.9% NaCl), filtered through a 0.22 μm filter and injected into the lateral tail vein of C57BL/6 mice in 5-7 s. The constructs used in this study showed long-term expression of genes via hydrodynamic tail vein injection (HTVI). To examine the role of SCYL3 in liver metastasis using this model, pT3-EF1a-Scyl3 (BC_043085) was generated. The mouse Scyl3 sequence was generated in pDONR^{TM}-221 vector and inserted into pT3-EF1a vector using Gateway^{TM} cloning. The pT3-EF1a-Scyl3 was also injected together with the c-Myc-luc, sgTP53 plasmids, and SB transposase (n = 8/group). The study protocol was approved by and performed in accordance with the Committee of the Use of Live Animals in Teaching and Research at the University of Hong Kong and the Hong Kong Polytechnic University (Hong Kong, P.R. China).

Statistical analysis

Statistical significance of qPCR, migration assay and invasion assay results were determined by Student's t test using Microsoft Office Excel software (Microsoft Corporation). The displayed results showed the means ± SD, and those with p values less than 0.05 were considered statistically significant (*p <0.05, **p <0.01, ***p <0.001 and ****p <0.0001). All tests are two-sided. Data points that deviate by more than 3x SD from the mean are not presented. Investigators were not blinded to the group allocation during experiments or when assessing outcomes. There is no estimate of variation within each group of data. Variance is

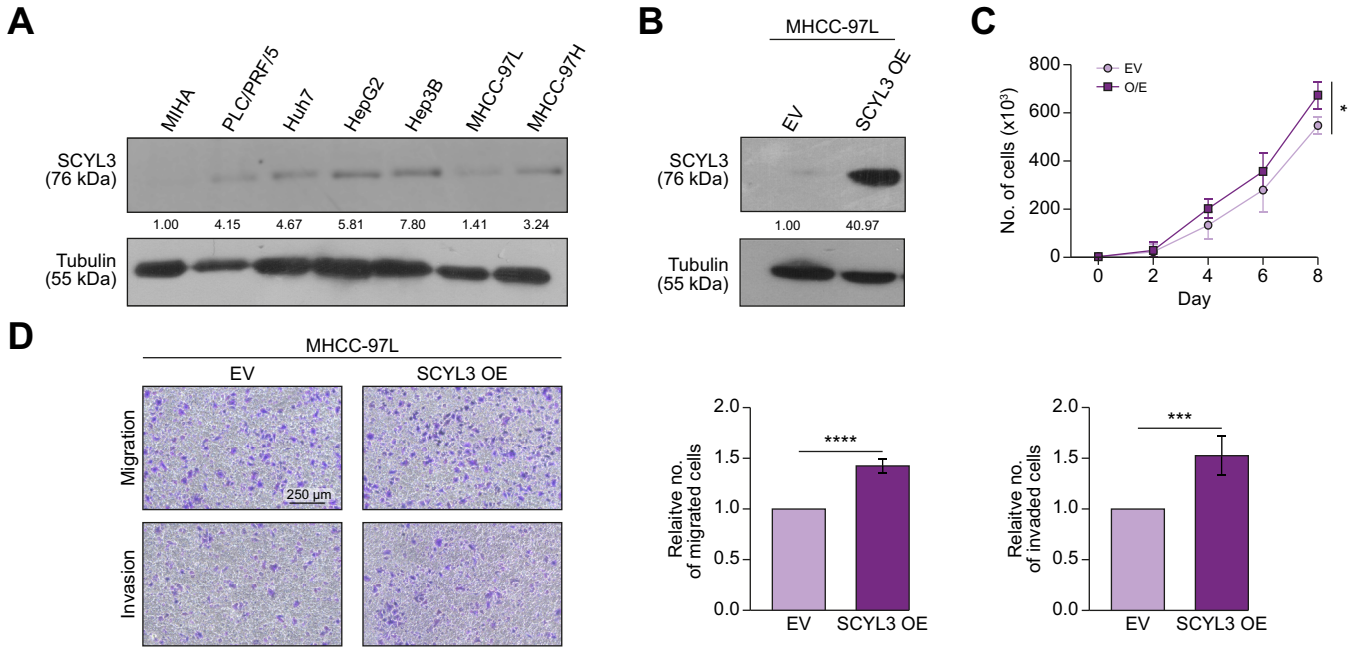


Fig. 2. SCYL3 overexpression enhances HCC migration and invasion. (A) Western blot analysis showed that MIHA, a non-tumorigenic normal liver cell line, exhibited the lowest expression of SCYL3 compared to a variety of HCC cell lines. SCYL3 was preferentially expressed in HCC cell lines, including PLC/PRF/5 and Hep3B, while MHCC-97L displayed low expression. (B) Successful stable overexpression of SCYL3 in MHCC-97 cells. (C) Using a cell counting approach, a significant increase in cell proliferation was observed in SCYL3 OE MHCC-97L cells (*p <0.05, t test). (D) Cell migration and invasion assays demonstrated that MHCC-97L SCYL3 OE cells showed an increase in migratory ability compared to control cells (***p <0.001 and ****p <0.001, t test). Error bars indicate mean ± SD. EV, empty vector; SCYL3 OE, SCYL3-overexpressing.

similar between the groups that are being statistically compared. Chi square test was employed to examine the correlation of SCYL3 with metastasis in HCC samples. Kaplan-Meier survival analysis was used to analyze overall survival and disease-free survival and a log-rank test was used to determine statistical significance; these analyses were carried out using SPSS 21 software.

For further details regarding the materials and methods used, please refer to the CTAT table and supplementary materials and methods.

Results

SCYL3 is overexpressed in HCC and correlates with metastasis

Currently, there is no information regarding the expression pattern of SCYL3 in cancers. As an initial attempt to explore whether SCYL3 expression is associated with cancer pathogenesis, we first examined SCYL3 expression in HCC in The Cancer Genome Atlas (TCGA) database of 50 individuals with HCC. Upon analysis, *SCYL3* was significantly overexpressed in HCC (p <0.001) (Fig. 1A). We further confirmed this result in a cohort of 44 clinical HCC samples using qPCR (Fig. 1B). Consistently, SCYL3 was found to be overexpressed at the protein level by western blot analysis (Fig. 1C). Patients with high *SCYL3* expression exhibited reduced overall and disease-free survival (p = 2.23 E^{-4} and 0.011, respectively; log-rank test) (Fig. 1D). In Gene Expression Omnibus (GEO) dataset of HCC, *SCYL3* was significantly upregulated in tumor specimens from non-cancerous to tumor tissues to metastatic counterparts in a stepwise manner, suggesting a metastatic role for SCYL3 in HCC (Fig. 1E). To further confirm this observation, we performed immunohistochemical analyses in 28 pairs of matched primary and metastatic HCC tissue samples. Only 13 of the 28 primary HCC samples were strong or moderately positive for SCYL3, while 15 were weak or negative. In contrast, moderate or strong SCYL3 positivity was present in 21 of 28 metastatic HCC cases, suggesting that overexpression of SCYL3 is involved in HCC metastasis (Fig. 1F,G). Next, we investigated whether there is any correlation between SCYL3 and drug resistance by analyzing 30 individuals with HCC who received prior sorafenib treatment in TCGA-LIHC dataset. Consistently, patients with high SCYL3 expression had shorter disease-free survival and progression-free survival than those with lower SCYL3 expression (p = 0.0127 and p = 0.0304, respectively), which is dependent of tumor staging (Fig. S1). These results suggest the putative role of SCYL3 in regulation of drug resistance. Using an Oncomine analysis, we found that overexpression of *SCYL3* was also observed in a variety of other neoplasms, including colon and breast cancers (Fig. S2).

Overexpression of SCYL3 promotes metastatic potential in HCC cells

To determine whether SCYL3 characterizes more tumorigenic and invasive HCC cells, we first examined its expression by western blot in a panel of liver cell lines, including the non-tumorigenic immortalized cell line MIHA and the HCC cell lines PLC/PRF/5, Huh7, HepG2, Hep3B, MHCC-97L, and MHCC-97H. SCYL3 expression was negligible in the non-tumorigenic cell line MIHA, whereas the other tested HCC cell lines demonstrated varied expression levels (Fig. 2A). Notably, elevated SCYL3 expression was also observed in the more metastatic HCC cell line MHCC-97H compared to the less metastatic cell line MHCC-97L (Fig. 2A). To further examine whether SCYL3 functionally

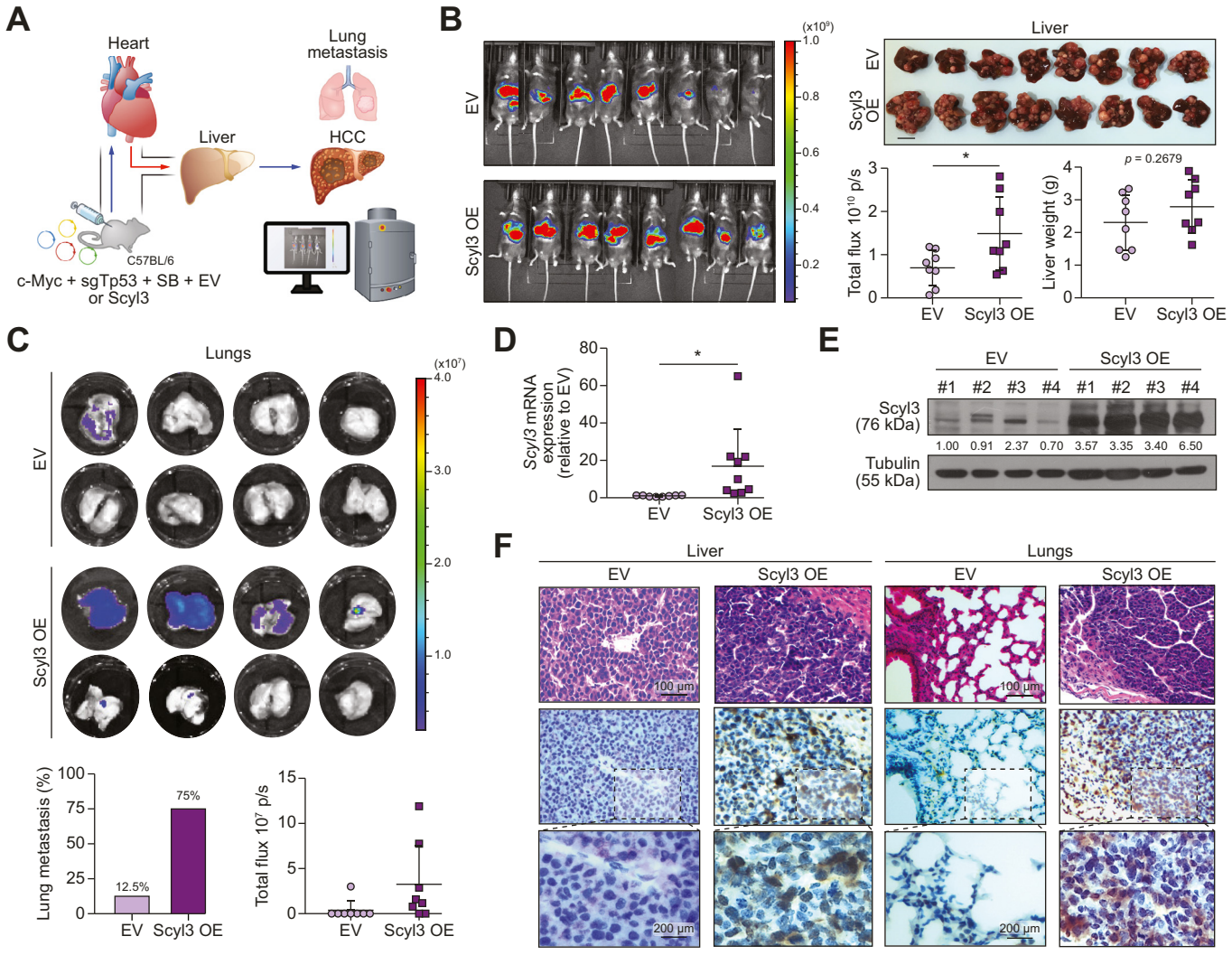


Fig. 3. Endogenous SCYL3 overexpression in the liver of an immunocompetent HCC mouse model augments tumor development and metastasis. (A) Schematic representation of the HTVI model established using C57BL/6 mice. (B) Bioluminescent images of C57BL/6 mice subjected to HTVI of EV/Scyl3, together with *Tp53*^{KO} and *c-Myc*^{OE} (n = 8 per group). Liver tumors dissected from two groups of mice (EV and Scyl3 OE). Scale bar, 1 cm. Significant increase in signal intensity in Scyl3 OE group (**p* <0.05, *t* test). Liver weight was shown as a dot plot. (C) The incidence of lung metastasis was increased in Scyl3 OE cells (1/8 *vs.* 6/8). Signal intensity of the lungs was shown as a dot plot. (D) qPCR analysis revealed enhanced Scyl3 expression in tumors injected with SCYL3 compared to those from EV in *Tp53*^{KO}/*c-Myc*^{OE} mice (**p*<0.05, *t* test). (E) Western blot analysis showed an increase in SCYL3 expression in Scyl3 OE groups when compared with control group. Four representative samples were shown. (F) H&E staining showed nodular HCC tumor and lung metastases in the Scyl3 OE group. SCYL3 was overexpressed in HCC cells in the liver and lung in the Scyl3 OE group. Scale bar, 100 μm and 200 μm. EV, empty vector; HCC, hepatocellular carcinoma; HTVI, hydrodynamic tail vein injection; Scyl3 OE, Scyl3-overexpressing.

contributes to the invasive traits of HCC cells, we performed SCYL3 overexpression experiments using a lentivirus-based approach. MHCC-97L cells were chosen for the overexpression experiment due to their relatively low endogenous SCYL3 expression levels. Efficient overexpression of SCYL3 was confirmed by western blotting (Fig. 2B). Using cell counting, overexpression of SCYL3 significantly enhanced cell proliferation (Fig. 2C). We also found that SCYL3-overexpressing (SCYL3 OE) cells exhibited a significantly enhanced ability to migrate and invade compared to control (empty vector [EV]) cells (Fig. 2D). To further confirm whether SCYL3 promotes metastatic potential *in vivo*, we induced overexpression of SCYL3 in an immunocompetent mouse model to investigate the tumor- and metastatic-promoting role of SCYL3 in HCC. We induced HCC tumors in C57BL/6 mice via HTVI using a combination of the c-Myc oncogene with sgTp53 and SB transposase with overexpression of pT3-EF1a-Scyl3 (Fig. 3A). Compared to control (EV) tumors, we found that endogenous overexpression of Scyl3 was associated with a significant increase in tumor volume, as evidenced by an increase in luciferase signals (Fig. 3B). The tumor growth fronts in the Scyl3 OE group were more invasive and irregular, whereas the tumor growth fronts in the EV group were found to be bulging, more regular, and less invasive (Fig. S3A-C). In addition, an increased number of tumor nodules was more frequently observed in the Scyl3 OE group, indicating a role for SCYL3 in intrahepatic metastasis (Fig. S3D). Strikingly, Scyl3 OE cells displayed a higher rate of lung metastasis (six out of eight mice; 75%) than control cells (one out of eight mice; 12.5%)

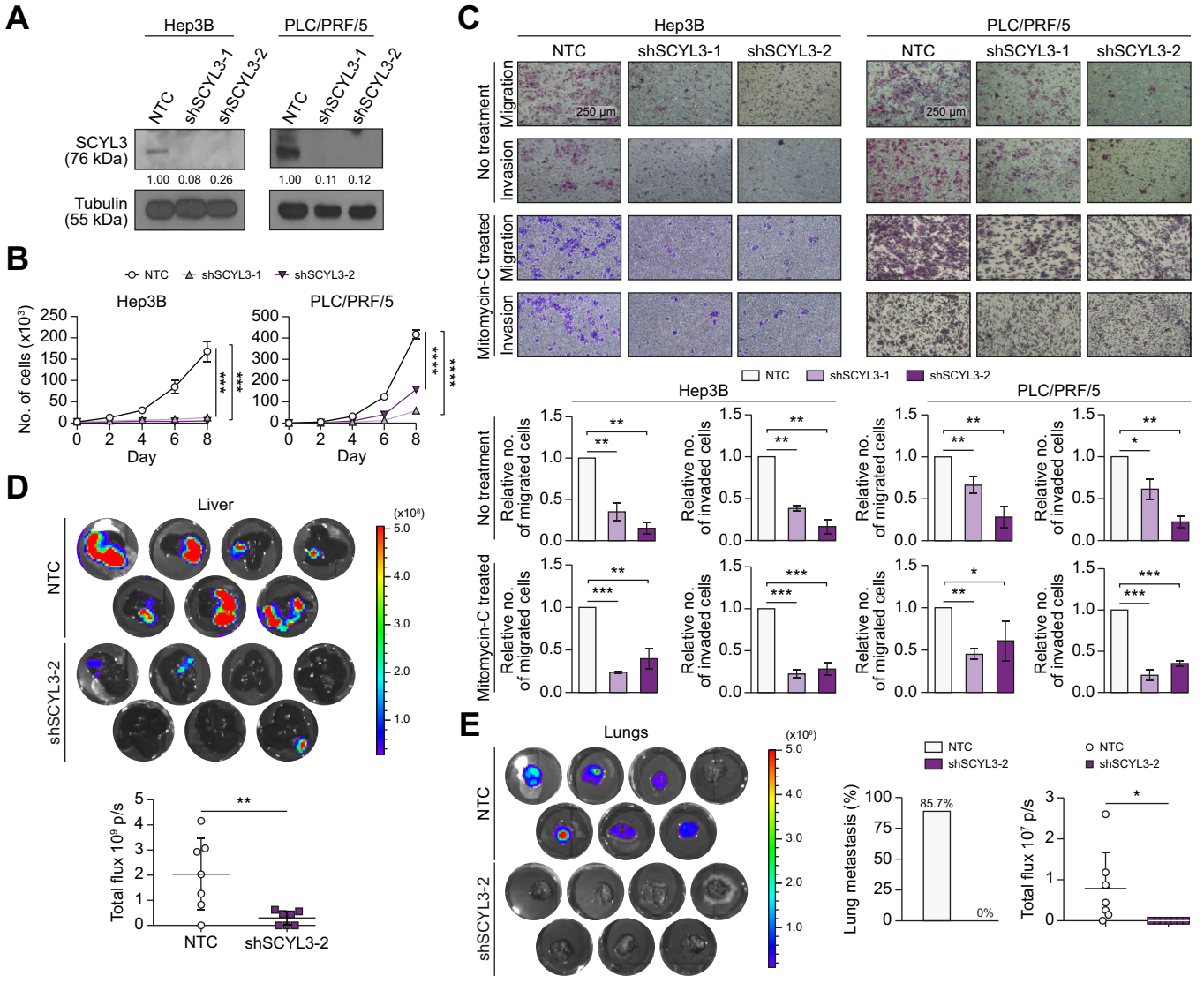


Fig. 4. Suppression of SCYL3 attenuates tumor growth and metastasis. (A) Two different shSCYL3 sequences were used (shSCYL3-1 and shSCYL3-2) to knockdown SCYL3 expression. Western blotting showed successful knockdown of SCYL3 expression in PLC/PRF/5 and Hep3B cells. (B) By direct cell counting, knockdown of SCYL3 reduced the cell proliferation rate compared to NTC (***p <0.001 and****p <0.0001, t test). (C) Cell migration and invasion assays demonstrated that the number of migratory and invading cells was significantly reduced in shSCYL3-transfected Hep3B and PLC/PRF/5 cells compared to NTC-transfected cells. shSCYL3 HCC cells treated with mitomycin C consistently showed reduced migratory and invasive abilities (*p <0.05, **p <0.01 and ***p <0.001, t test); scale bar, 250 μm. (D) Nude mice were subjected to orthotopic liver injection of NTC/shSCYL3-2 derived from PLC/PRF/5 cells and sacrificed at 8 weeks. Bioluminescence images of livers harvested from nude mice that received orthotopic injections are shown (n = 7 per group). Significant decrease in signal intensity in livers injected with shSCYL3-2 cells (**p <0.01, t test). (E) Bioluminescence imaging of lungs harvested from nude mice that received orthotopic injections are shown. Suppression of SCYL3 reduced the incidence of lung metastasis (6/7 *vs.* 0/7) *in vivo*. Significant decrease in signal intensity in shSCYL3 group (*p<0.05, t test). Error bars indicate mean ± SD. HCC, hepatocellular carcinoma; NTC, non-target control; sh, short-hairpin.

(Fig. 3C). Scyl3 was efficiently transduced into the livers of mice via HTVI, as evidenced by increased Scyl3 expression on qPCR and western blot analyses (Fig. 3D,E). Immunohistochemical analysis showed intrahepatic and lung metastases with high Scyl3 expression (Fig. 3F).

Suppression of SCYL3 reduces tumor growth and metastasis of HCC cells

To further confirm the role of SCYL3 in HCC, we also suppressed SCYL3 expression in Hep3B and PLC/PRF/5 cells, which exhibit relatively high endogenous SCYL3 expression (Fig. 2A). Successful repression of SCYL3 was confirmed by western blotting (Fig. 4A). Upon SCYL3 suppression, SCYL3 knockdown cells (shSCYL3) exhibited a decreased cell proliferation rate compared to NTC cells (Fig. 4B). Furthermore, transwell assays showed that SCYL3 knockdown reduced the migratory and invasive abilities of PLC/PRF/5 and MHCC-97L cells (Fig. 4C). Similar suppressive results were observed in shSCYL3 cells in the presence of mitomycin C, where cell proliferation was inhibited, suggesting that SCYL3-mediated migration and invasion are independent of cell proliferation (Fig. 4C). To confirm these findings, we further examined the effects of SCYL3 suppression in an *in vivo*

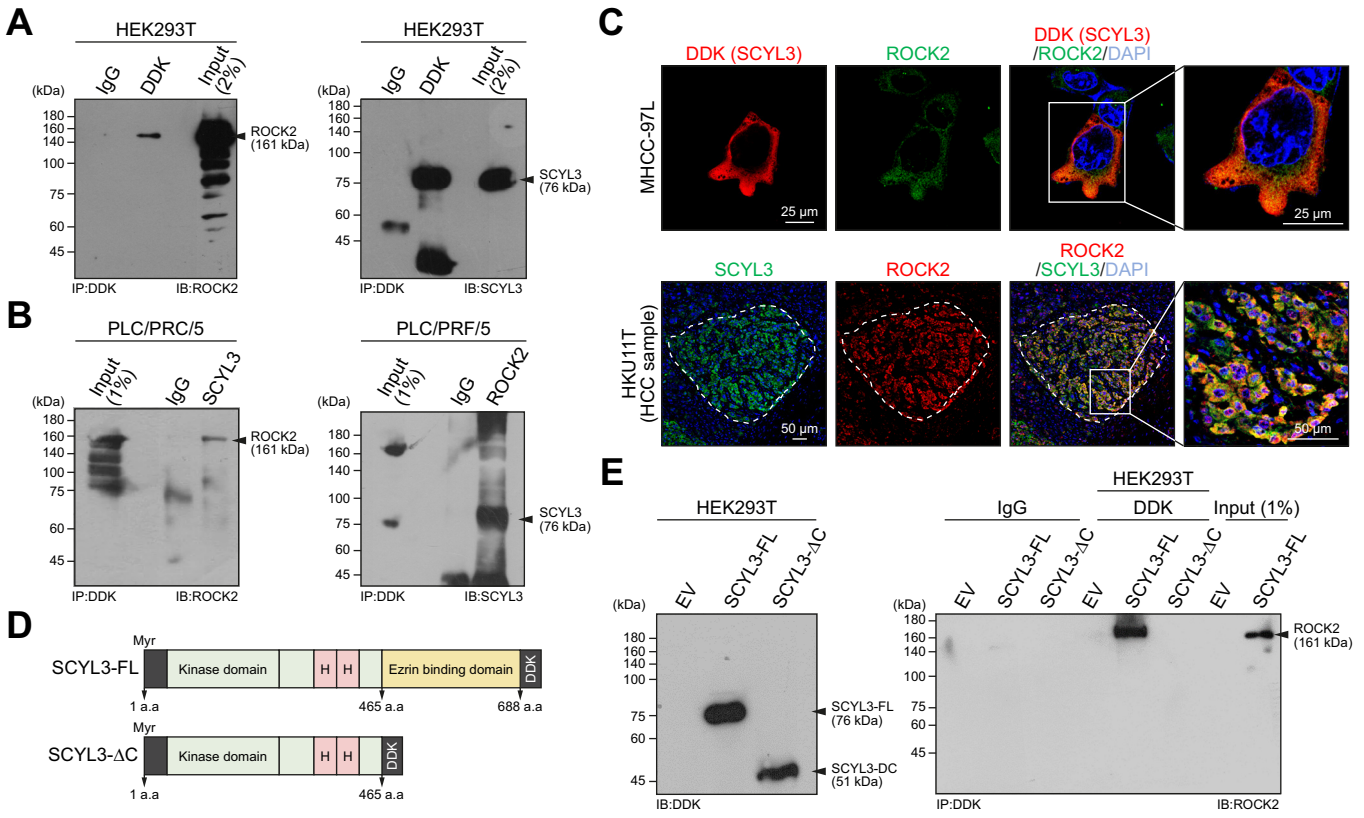


Fig. 5. SCYL3 physically binds to ROCK2 in HCC. (A) The interaction between DDK-tagged SCYL3 and ROCK2 was demonstrated by immunoprecipitation in HEK293T cells. ROCK2 is detected after pull-down of DDK-tagged SCYL3. (B) Reciprocal coimmunoprecipitation demonstrated the interaction between endogenous SCYL3 and ROCK2 in PLC/PRF/5 cells. (C) Co-localization of SCYL3 and ROCK2 was observed in MHCC-97L cells. MHCC-97L cells were transiently transfected with DDK-tagged SCYL3. SCYL3 was visualized using an anti-DDK antibody. ROCK2 expression was detected by anti-ROCK2 antibody. DDK staining (red), ROCK2 staining (green) and DAPI staining (blue); scale bar, 25 μm. In an HCC clinical tumor specimen (HKU11T), co-localization between SCYL3 and ROCK2 was clearly observed in HCC cells within tumor nodules. White dotted line represents the margin of the tumor. ROCK2 staining (red), SCYL3 staining (green) and DAPI staining (blue); scale bar, 50 μm. (D) Schematic diagram showing the domain structure of DDK-tagged SCYL3-FL and SCYL3-ΔC. (E) 293T cells were transfected with SCYL3-FL and SCYL3-ΔC as indicated. Successful transfection of these two constructs shown in HEK293T cells. Cleared cell lysates were incubated with anti-DDK to immunoprecipitate SCYL3. ROCK2 in the precipitates was detected by immunoblotting with an anti-ROCK2 antibody. Removal of the C-terminal domain (SCYL3-ΔC) resulted in complete loss of ROCK2 binding. EV, empty vector; HCC, hepatocellular carcinoma; SCYL3-ΔC, SCYL3 mutant with deletion of the C-terminal domain; SCYL3-FL, SCYL3 full length.

orthotopic HCC metastasis model in which cells were orthotopically injected into the liver to observe metastasis to the lung. SCYL3 suppression dampened the ability of PLC/PRF/5 cells not only to form tumors (3/7) in the liver but also to metastasize to the lungs (0/7) (Fig. 4D). In contrast, PLC/PRF/5 control cells displayed efficient tumor formation with an 85.7% (6/7) rate of metastasis to the lungs (Fig. 4E).

Identification of ROCK2 as a novel binding partner of SCYL3

SCYL3 was first identified as a putative binding partner for ezrin.[5] Given that SCYL3 is a pseudokinase, we hypothesized that SCYL3 performs its function through interaction with another kinase. Currently, only ROCKs and protein kinase C have been reported to induce the expression of p-Ezrin-^{Thr567}.[6,7] A gene correlation analysis of SCYL3 and ROCK2 showed a correlation score of 0.3743 between these two factors in HCC (Fig. S4). Based on this interesting finding, we examined the potential physical interaction between SCYL3 and ROCK2. We first assessed the binding between SCYL3 and ROCK2 by transfecting a DDK-tagged SCYL3 plasmid into HEK293T cells. Immunoprecipitation assays revealed a physical interaction between SCYL3 and ROCK2 (Fig. 5A). We further confirmed the endogenous interaction between SCYL3 and ROCK2 by reciprocal immunoprecipitation in PLC/PRF/5 cells (Fig. 5B). The co-localization of these two proteins was further confirmed in DDK-tagged SCYL3-transfected MHCC-97L cells and HCC clinical samples using confocal immunofluorescence microscopy (Fig. 5C). To map the region crucial for the binding of SCYL3 to ROCK2, we created a deletion mutant that lacks the C-terminal domain (SCYL3-ΔC) consisting of the ezrin-binding motif (Fig. 5D). Immunoprecipitation revealed that ROCK2 binds to the ezrin-binding domain of SCYL3 (Fig. 5E). In line with this finding, the migration- and invasion-enhancing effect of SCYL3 was abolished in SCYL3-ΔC-transfected MHCC-97L cells (Fig. S6A-B). Likewise, Scyl3-ΔC abolished the tumor growth and metastatic potential of Scyl3 in *Tp53*^{KO}/*c-Myc*^{OE} mice using the *SB* transposon system in mice (Fig. S6A-D).

SCYL3 regulates the transactivating activity of ROCK2 by sustaining its protein stability

In addition to its effect on binding affinity, we examined whether SCYL3 affects ROCK2 protein expression. In response to overexpression of SCYL3, ROCK2 expression was increased in MHCC-

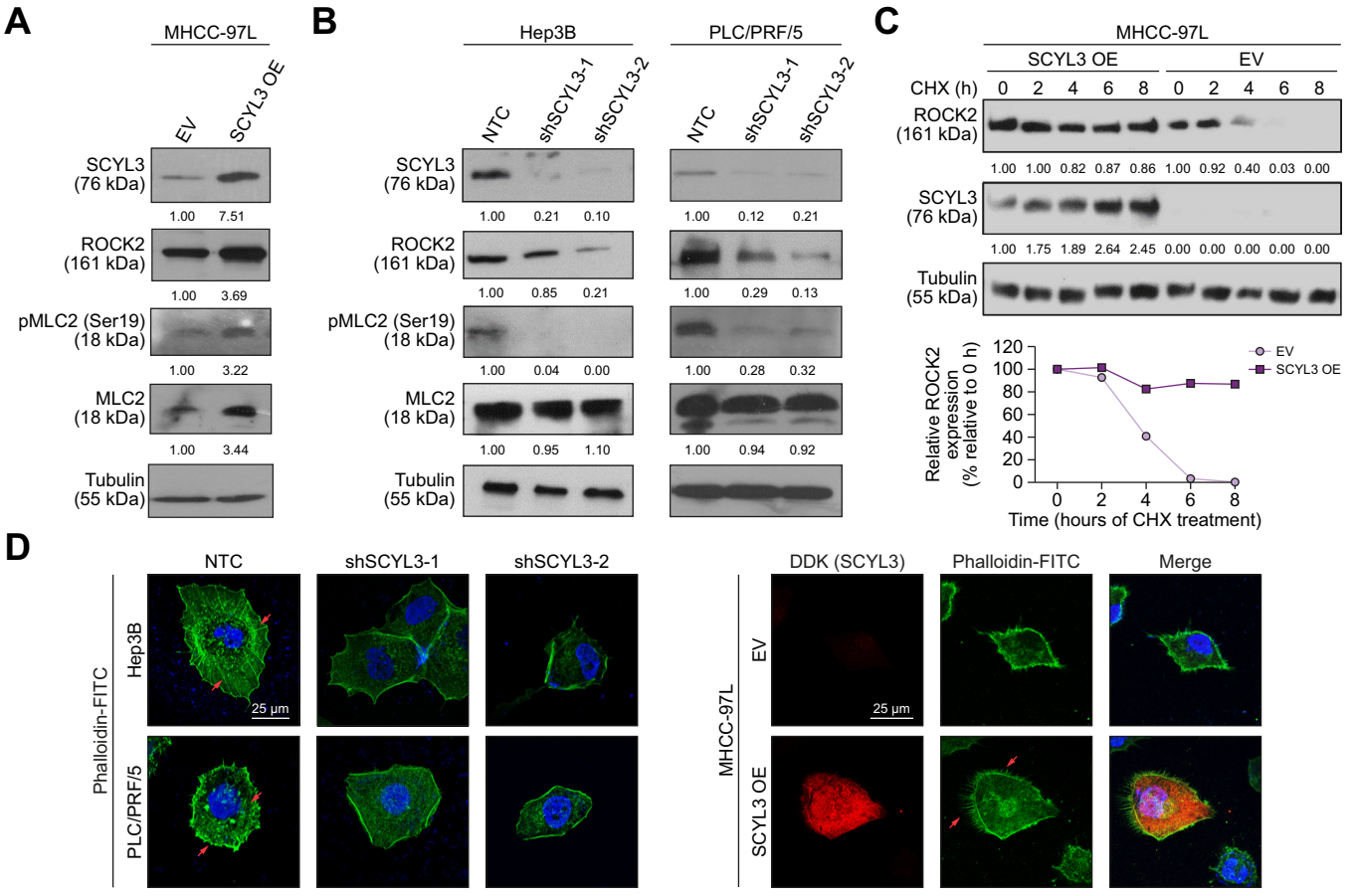


Fig. 6. SCYL3 regulates the protein stability and transactivating activity of ROCK2 in HCC cells. (A) SCYL3 overexpression led to the upregulation of ROCK2 and pMLC2 (Ser19) in MHCC-97L cells. (B) ROCK2 and pMLC2 (Ser19) protein levels were downregulated in response to SCYL3 knockdown in Hep3B and PLC/PRF/5 cells. (C) Western blot and ImageJ quantitation of the expression of SCYL3 and ROCK2 in MHCC-97L cells from 0 to 8 h with transient transfection of either EV or SCYL3 upon treatment with CHX at 100 μg/ml (D) shSCYL3 cells exhibited loss of stress fibers (polymerized actin) and actin filaments were shown by phalloidin (green), as compared to the NTC. Red arrows indicate the stress fiber formation. In contrast, SCYL3 OE exhibited an increased stress fiber network. Scale bar, 25 μm. CHX, cycloheximide; EV, empty vector; HCC, hepatocellular carcinoma; NTC, non-target control; SCYL3 OE, SCYL3-overexpressing; sh, short-hairpin.

97L cells (Fig. 6A). Conversely, the opposite effects were observed in Hep3B and PLC/PRF/5 cells treated with shSCYL3 (Fig. 6B). Consistently, HCC cells in the Scyl3 OE group showed elevated Rock2 expression in HCC tumors (Fig. S7). More specifically, we examined whether SCYL3 affects ROCK2 stability. MHCC-97L cells with or without SCYL3 overexpression were subjected to CHX treatment, a protein synthesis inhibitor, for various durations (0–8 h) prior to western blot analysis. ROCK2 degraded much slower in HCC cells overexpressing SCYL3 than in control cells (Fig. 6C). Next, we examined the effect of SCYL3 on ROCK2 kinase activity. To evaluate this potential effect, we examined phosphorylation levels of myosin light chain 2 (MLC2), a substrate of ROCK2 that is responsible for actin polymerization and depolymerization. We observed an increase in pMLC2 (Ser19) expression in SCYL3-overexpressing MHCC-97L cells. Consistently, knockdown of SCYL3 decreased the expression of pMLC2 (Ser19) (Fig. 6A,B). Finally, we examined stress fiber formation and polymerized actin in SCYL3-modified HCC cells. Using phalloidin staining, we found that stress fiber formation was suppressed in SCYL3 stable knockdown transfectants. Furthermore, we observed a significant reduction in filopodia and lamellipodia (cell protrusions) on the cell surfaces of SCYL3 knockdown cells compared to control cells. Conversely, the opposite effects were observed in SCYL3 OE cells (Fig. 6D).

Discussion

Currently, there are two reports demonstrating the role of SCYL3 in cancers. First, SCYL3 was identified as the novel putative binding partner of ezrin, regulating cell adhesion and migration of breast cancer cells.[5] Recently, SCYL3 was found to fuse with NTRK1 in colorectal cancer, which provides a novel therapeutic basis for entrectinib treatment in this subset of patients.[8] In this study, we reported for the first time that SCYL3 is overexpressed in multiple neoplasms, including liver, colon and breast cancers. Specifically, SCYL3 was overexpressed at both mRNA and protein levels, suggesting the transcriptional activation of *SCYL3* in HCC. This result is consistent with the observation that MIHA, the non-tumorigenic cell line, expressed negligible levels of SCYL3, whereas the other tested HCC cell lines demonstrated varied expression levels. Chromosome gain at 1q is one of the most frequently detected alterations in HCC (58–78%) and has been suggested to be an early genomic event in the process of HCC development.[9,10] Since SCYL3 is located on chromosome 1q24.2,

an amplified region in HCC, it is possible that SCYL3 is overexpressed due to copy number changes. Based on TCGA data, ~10% of individuals with HCC displayed amplification of *SCYL3*. On chromosome 1q24.1-24.2, Jia *et al.* identified the *MPZL1* gene to be frequently amplified and to play a crucial role in metastasis in HCC.[11] Echoing this finding, *SCYL3* expression was increased from normal to tumor tissues to metastatic counterparts in a stepwise manner. Consistently, *SCYL3* expression is more abundant in metastatic HCC than in primary tumors. Furthermore, overexpression of *SCYL3* is significantly correlated with poor patient survival.

Using lentiviral-based overexpression and knockdown approaches, we consistently found that SCYL3 regulates the migratory and invasive abilities of HCC cells and that such effects are independent of cell proliferation. Using an orthotopic HCC nude mouse model, we found that shSCYL3 cells exhibited decreased tumor growth as well as reduced ability to induce lung metastasis. Conversely, endogenous overexpression of Scyl3, via HTVI, promoted tumor development and lung metastasis in an immunocompetent mouse model. These results showed that SCYL3 is critical for regulating HCC growth and tumor progression. *SCYL3* encodes a protein with a kinase domain and four HEAT repeats. Although SCYL3 also contains a kinase domain, it lacks the highly conserved HRD motif that is commonly found in kinase domains and has therefore been suggested to be a pseudokinase. However, *SCYL3* constructs also demonstrated *in vitro* kinase activity,[5] which was eliminated with stringent purification methods. This suggests that SCYL3 may bind to an active kinase instead of possessing intrinsic kinase activity itself. To characterize the physiological function of SCYL3, an understanding of the kinase activity of this protein is crucial. We found for the first time that SCYL3 was associated with ROCK2, an active kinase that is overexpressed in HCC.[12] ROCK2 regulates HCC invasion and metastasis via a number of signaling pathways, including MMP2 ubiquitination,[13] β-catenin signaling[14] and MKP1 signaling.[15] Specifically, we found that ROCK2 binds to the C-terminal region of SCYL3. Interestingly, we found that altered SCYL3 expression in HCC resulted in changes in ROCK2 expression, which led us to hypothesize that SCYL3 regulates the protein stability of ROCK2. To test this hypothesis, we examined the effect of SCYL3 on ROCK2 protein stability by administering CHX, a protein synthesis inhibitor. We found that ROCK2 was degraded at a slower rate in SCYL3 OE HCC cells than in control cells. Clinically, co-localization of SCYL3 and ROCK2 was also found in HCC specimens. In addition to the effect of SCYL3 on ROCK2 protein stability, we examined its potential transactivating activity. We found that pMLC2 (Ser19), a direct substrate of ROCK2,[16] was consistently altered upon overexpression and repression of SCYL3 in HCC cells.

Many studies have reported the roles of the Rho/ROCK pathway in the regulation of cytoskeletal events. The Rho/ROCK pathway is critical for cell movement, including the coordination of actomyosin contraction,[17] tail retraction,[18] and the formation of stress fibers in fibroblasts.[19] Our data support a role for ROCK2 as a key regulator of MLC2 phosphorylation and thereby actomyosin contraction. We demonstrated that repression of SCYL3 in both Hep3B cells and PLC/PRF/5 cells significantly suppressed the formation of stress fibers, filopodia, and lamellipodia, while overexpression of SCYL3 exerted the opposite effects. ROCK2 was found to play a critical role in the formation of stress fibers and lamellipodia.[20] Consistently, alterations in SCYL3 led to changes in these features, indicating the regulatory role of SCYL3 on ROCK2. Besides, SMARCA4 was recently reported to be a master regulator of a number of migration-related genes in HCC.[21] In addition, chemerin, a known chemoattractant, was found to negatively regulate the migration and metastasis of HCC cells via PTEN-CMKLR1 interaction.[22] Our data provide mechanistic insight by showing the significance of the interplay between SCYL3 and ROCK2 in regulating the migration and invasion of HCC cells.

In conclusion, we found that SCYL3-mediated ROCK2 protein stability may play a role in the growth and metastasis of HCC cells (Fig. S8). Therefore, targeting the SCYL3/ROCK2 signaling pathway may be a potential therapeutic strategy for the treatment of individuals with HCC.

Abbreviations

CHX, cycloheximide; EV, empty vector; GEO, Gene Expression Omnibus; HCC, hepatocellular carcinoma; HRD motif, histidine-arginine-aspartic acid motif; HTVI, hydrodynamic tail vein injection; MLC2, myosin light chain 2; NTC, non-target control; OE, overexpression; qPCR, quantitative PCR; ROCK2, Rho kinase 2; SB, sleeping beauty; SCYL3, SCY1-like pseudokinase 3; SCYL3-ΔC, SCYL3 mutant with deletion of the C-terminal domain; SCYL3 OE, SCYL3-overexpressing; sg, single-guide; sh, short-hairpin; TCGA, The Cancer Genome Atlas.

Financial support

This study was supported by the National Natural Science Foundation of China (81871935), and Research Impact Fund (R5050-18F & R7022-20).

Conflicts of interests

The authors declare no conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

M.M.L., C.O.L., E.Y.L., and T.K.L. designed the experiment. M.M.L., C.O.L., E.Y.L., R.W.L., and V.W.M. performed the experiment. M.M.L., C.O.L., E.Y.L., M.T., S.M., and T.K.L. analyzed the data, M.M.L., C.O.L., E.Y.L., and T.K.L. wrote the paper. Y.Y.L., C.Y.H., Q.H.Z., and I.O.N. provided reagents, advice and tissue samples for this study. T.K.L. supervised the study. All authors contributed to the discussion of results and manuscript corrections.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and/or its Supplementary Materials and methods. Any additional data are available from the corresponding author T.K.L. upon reasonable request.

Acknowledgements

We thank the University Research Facility in Life Sciences at The Hong Kong Polytechnic University for providing and maintaining the equipment and technical support needed for imaging work as well as the Centralized Animal Facility at The Hong Kong Polytechnic University and The Centre for Comparative Medicine Research at The University of Hong Kong for supporting our animal studies. We thank Dr. Amaia Lujambio (Icahn School of Medicine, Mount Sinai) for sharing of plasmids used for hydrodynamic tail vein injection. We also thank Department of Pathology at The University of Hong Kong for histopathology service.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2022.100604.

References

Author names in bold designate shared co-first authorship

[1] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jermal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018;68:394–424.

[2] Poon RT, Fan ST, Wong J. Risk factors, prevention and management of postoperative recurrence after resection of hepatocellular carcinoma. Ann Surg 2000;232:10–24.

[3] El-Assal ON, Yamanoi A, Soda Y, Yamaguchi M, Yu L, Nagasue N, et al. Proposal of invasiveness score to predict recurrence and survival after curative hepatic resection for hepatocellular carcinoma. Surgery 1997;122:571–577.

[4] Poon RT, Fan ST, Lo CM, Liu CL, Wong J. Long-term survival and pattern of recurrence after resection of small hepatocellular carcinoma in patients with preserved liver function: implications for a strategy of salvage transplantation. Ann Surg 2002;235:373–382.

[5] Sullivan A, Uff CR, Isacke CM, Thorne RF. PACE-1, a novel protein that interacts with the C-terminal domain of ezrin. Exp Cel Res 2003;284:224–238.

[6] Matsui T, Maeda M, Doi Y, Yonemura S, Amano M, Kaibuchi K, et al. Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. J Cel Biol 1998;140:647–657.

[7] Ren L, Hong SH, Cassavaugh J, Osborne T, Chou AJ, Kim SY, et al. The actin-cytoskeleton linker protein ezrin is regulated during osteosarcoma metastasis by PKC. Oncogene 2009;28:792–802.

[8] Milione M, Ardini E, Christiansen J, Valtorta E, Veronese S, Bosotti R, et al. Identification and characterization of a novel SCYL3-NTRK1 rearrangement in a colorectal cancer patient. Oncotarget 2017;8:55353–55360.

[9] Jia D, Wei L, Guo W, Zha R, Bao M, Chen Z, et al. Genome-wide copy number analyses identified novel cancer genes in hepatocellular carcinoma. Hepatology 2011;54:1227–1236.

[10] Chen L, Chan TH, Guan XY. Chromosome 1q21 amplification and oncogenes in hepatocellular carcinoma. Acta Pharmacol Sin 2010;31:1165–1171.

[11] Jia D, Jing Y, Zhang Z, Liu L, Ding J, Zhao F, et al. Amplification of MPZL1/PZR promotes tumor cell migration through Src-mediated phosphorylation of cortactin in hepatocellular carcinoma. Cell Res 2014;24:204–217.

[12] Wong CC, Wong CM, Tung EK, Man K, Ng IO. Rho-kinase 2 is frequently overexpressed in hepatocellular carcinoma and involved in tumor invasion. Hepatology 2009;49:1583–1594.

[13] Huang D, Du X, Yuan R, Chen L, Liu T, Wen C, et al. Rock2 promotes the invasion and metastasis of hepatocellular carcinoma by modifying MMP2 ubiquitination and degradation. Biochem Biophys Res Commun 2014;453:49–56.

[14] Li M, Zhou W, Yuan R, Chen L, Liu T, Huang D, et al. ROCK2 promotes HCC proliferation by CEBPD inhibition through phospho-GSK3beta/beta-catenin signaling. FEBS Lett 2015;589:1018–1025.

[15] Du Y, Lu S, Ge J, Long D, Wen C, Tan S, et al. ROCK2 disturbs MKP1 expression to promote invasion and metastasis in hepatocellular carcinoma. Am J Cancer Res 2020;10:884–896.

[16] Truebestein L, Elsner DJ, Fuchs E, Leonard TA. A molecular ruler regulates cytoskeletal remodelling by the Rho kinases. Nat Commun 2015;6:10029.

[17] Yoneda A, Multhaupt HA, Couchman JR. The Rho kinases I and II regulate different aspects of myosin II activity. J Cel Biol 2005;170:443–453.

[18] Worthylake RA, Lemoine S, Watson JM, Burridge K. RhoA is required for monocyte tail retraction during transendothelial migration. J Cel Biol 2001;154:141–160.

[19] Amano M, Chihara K, Kimura K, Fukata Y, Nakamura N, Matsuura Y, et al. Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. Science 1997;275:1308–1311.

[20] Shi J, Wu X, Surma M, Vemula S, Zhang L, Yang Y, et al. Distinct roles for ROCK1 and ROCK2 in the regulation of cell detachment. Cell Death Dis 2013;4(2):e483.

[21] **Choi HI**, **An GY**, **Baek M**, Yoo E, Chai JC, Lee YS, et al. BET inhibitor suppresses migration of human hepatocellular carcinoma by inhibiting SMARCA4. Sci Rep 2021;11:11799.

[22] Li JJ, Yin HK, Guan DX, Zhao JS, Feng YX, Deng YZ, et al. Chemerin suppresses hepatocellular carcinoma metastasis through CMKLR1-PTEN-Akt axis. Br J Cancer 2018;118:1337–1348.