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Cancer Research

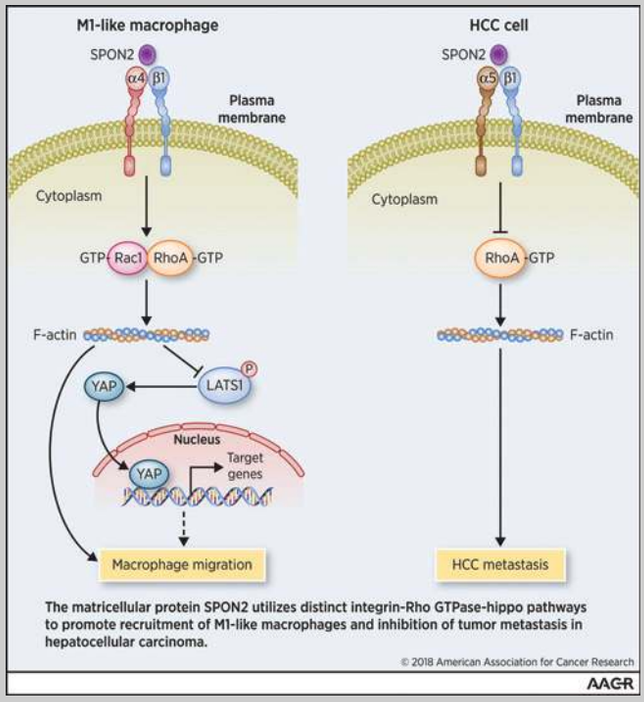
SPON2 Promotes M1-like Macrophage Recruitment and Inhibits Hepatocellular Carcinoma Metastasis by Distinct Integrin–Rho GTPase–Hippo Pathways

Yan-Li Zhang[1], Qing Li[1], Xiao-Mei Yang[1], Fang Fang[1], Jun Li[1], Ya-Hui Wang[1], Qin Yang[1], Lei Zhu[1], Hui-Zhen Nie[1], Xue-Li Zhang[1], Ming-Xuan Feng[2], Shu-Heng Jiang[1], Guang-Ang Tian[1], Li-Peng Hu[1], Ho-Young Lee[3], Su-Jae Lee[4], Qiang Xia[2], and Zhi-Gang Zhang[1]

Abstract

Tumor-associated macrophages (TAM) represent key regulators of the complex interplay between cancer and the immune microenvironment. Matricellular protein SPON2 is essential for recruiting lymphocytes and initiating immune responses. Recent studies have shown that SPON2 has complicated roles in cell migration and tumor progression. Here we report that, in the tumor microenvironment of hepatocellular carcinoma (HCC), SPON2 not only promotes infiltration of M1-like macrophages but also inhibits tumor metastasis. SPON2-α4β1 integrin signaling activated RhoA and Rac1, increased F-actin reorganization, and promoted M1-like macrophage recruitment. F-Actin accumulation also activated the Hippo pathway by suppressing LATS1 phosphorylation, promoting YAP nuclear translocation, and initiating downstream gene expression. However, SPON2-α5β1 integrin signaling inactivated RhoA and prevented F-actin assembly, thereby inhibiting HCC cell migration; the Hippo pathway was not noticeably involved in SPON2-mediated HCC cell migration. In HCC patients, SPON2 levels correlated positively with prognosis. Overall, our findings provide evidence that SPON2 is a critical factor in mediating the immune response against tumor cell growth and migration in HCC.

Significance: Matricellular protein SPON2 acts as an HCC suppressor and utilizes distinct signaling events to perform dual functions in HCC microenvironment.



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Introduction

Abundant macrophage infiltration is a common feature of tumors (1, 2). M1 or M2 subtype represents tumor suppressive or tumor-supportive macrophages, respectively. Specific tumor microenvironmental signals further determine the polarization and functions of macrophages (3). For many solid tumor types, a high density of cells expressing macrophage-associated markers is generally associated with a poor clinical outcome (4). However, conflicting data exist for lung, stomach, prostate, and bone malignancies, where both positive and negative outcome associations with increased macrophage density have been detected

[1]State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, P.R. China. [2]Department of Liver Surgery, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, P.R. China. [3]College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul, Republic of Korea. [4]Department of Life Science, Research Institute for Nature Sciences, Hanyang University, Seoul, Republic of Korea.

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Y.–L. Zhang, Q. Li, and X.-M. Yang contributed equally to this article.

Corresponding Author: Zhi-Gang Zhang, State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, P.R. China. Phone: 8621-3420-6763; Fax: 8621-3420-6022; E-mail: zzhang@shsci.org

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(5, 6). These discrepancies are likely due to the type/stage of the cancer evaluated, or to the subtype of macrophages analysis performed (7).

Tumor-associated macrophages (TAM) represent key regulators of the complex interplay between the immune system and cancer. Therapeutics impacting macrophage presence and/or bioactivity have shown promise in preclinical models and are now being evaluated in the clinic. Macrophages can be induced to phagocytose tumor cells by blocking the interaction between signal regulatory protein alpha (SIRPα) and CD47, and this therapeutic strategy is currently the subject of multiple clinical trials in cancer (8). In addition, programmed cell death protein 1 (PD-1) and programmed cell death ligand 1 (PD-L1) may serve as a new regulatory "checkpoint" for macrophages, given that macrophages express PD-1 in the tumor microenvironment (9). Because the C-C chemokine ligand 2 (CCL2) and colony-stimulating factor 1 (CSF-1) play critical roles in recruiting macrophages to neoplastic tissue, interest in therapeutics that target these ligands and/or their corresponding receptors to ablate the protumorigenic properties of macrophages has grown (10). These therapeutic approaches enabled improved outcomes in various preclinical models, but other targets can serve a complementary role.

Moreover, it should be considered that macrophages also participate in antitumor responses (11). Preclinical studies have revealed minimal macrophage proliferation and shorter half-lives for TAMs compared with resident macrophages in the counterpart homeostatic tissues (12). Therefore, recruitment of TAMs, especially TAMs of the M1 subtype with phagocytic activity, to the tumor microenvironment is necessary to sustain TAM numbers and is essential for antitumor immunity in human cancers. The molecules involved in this process will be new targets for immunotherapy.

Hepatocellular carcinoma (HCC) has a high mortality rate and develops as a consequence of chronic liver inflammation (13). The multistep hepatocarcinogenesis is accompanied with progressive changes in the liver microenvironment (14). No drug is effective as the first-line treatment for patients with advanced HCC, which represents 40%–70% of the whole HCC population (15). Thus, identifying and targeting critical pathways that improve therapeutic efficacy by bolstering antitumor immune responses holds great promise for improving HCC outcomes and impacting long-term patient survival.

The extracellular matrix (ECM) is the molecular basis of the interaction between cancer cells and the surrounding microenvironment. SPON2, also known as Mindin and DIL-1, is a member of the Mindin F-Spondin family of evolutionarily conserved, secreted ECM proteins (16). SPON2 is composed of an N-terminal F-spondin domain, which binds to integrin receptors, and a C-terminal thrombospondin type 1 repeat domain, which binds to bacterial lipopolysaccharide (17). Recent studies have shown that SPON2 is essential for the initiation of immune responses and represents a unique a pattern recognition molecule for microbial pathogens (18). Remarkably, SPON2 also functions as an integrin ligand for inflammatory cell recruitment and T-cell priming (19, 20). The binding of bacteria by SPON2 promotes phagocytosis of the bacterium and stimulates the production of proinflammatory cytokines by the macrophage (21). Furthermore, a number of clinical studies have suggested that SPON2 might be a new serum and histologic diagnostic biomarker for malignant tumors as the levels of SPON2 in patients are higher than those in healthy individuals (22–24). However, the molecular events underlying SPON2-mediated malignancies remain undefined, thus limiting the development of novel anticancer-targeted therapies. Here, we examined the potential role of SPON2 in HCC progression and focused on the potential contribution of SPON2 to the recruitment of macrophages and inhibition of tumor metastasis.

Materials and Methods

Cell culture

Cell lines HuH7, Hep3B, SMMC-7721 (TCHu13), MHCC-LM3, MHCC-97L, and MHCC-97H have been described previously (25). SNU-423, SNU-449, HepG2, THLE-2, and THP-1 were purchased from ATCC and cultured in the indicated medium according to the protocols. All cell lines underwent verification in January 2017 by Shanghai Cancer Institute and regular testing (every 4 months) to ensure lack of contamination with the Mycoplasma. The number of passages between thawing cell lines and their use in the described experiments was 2–30. THP-1 cells were stimulated with 200 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 24 hours to induce differentiation as described (26).

Cell migration and invasion assays

For the migration assay, 2×10^4 cells in 200 μL of the medium with blocking antibodies were seeded into the top chambers of Transwells (Millipore). The invasion assay was performed with Matrigel-coated filters (BD Biosciences). Culture medium containing 5% FBS and rSPON2 was added to the bottom chamber. Cells were incubated at 37°C and allowed to migrate for 24 hours or invade through the Matrigel for 48 hours. The blocking antibodies used in this study were purchased as follows: integrin α4 (9C10; Biolegend), integrin α5 (5H10-27; Biolegend), integrin αM (MCA711; Biosource), integrin αX (3.9; Biolegend), β1 (HMβ1-1; Biolegend), β2 (MA-1806; Endogen), and CCL5 (21418; R&D Systems). The experiments were performed in quintuplicate and repeated twice.

Immunofluorescence staining

Assays for tissue staining were performed as described previously (27). We cultured the cells in 12-well chambers (Ibidi) and cells were incubated with primary antibodies against SPON2 (HPA043890; Sigma-Aldrich), integrin α4 (ab22858; Abcam), integrin α5 (ab6131; Abcam), and YAP (4912; Cell Signaling Technology). The nuclei were counterstained for 2 minutes with DAPI (Sigma-Aldrich). F-Actin bundles were stained with FITC-phalloidin. Images were acquired by confocal microscopy (LSM 510, METALaser Scanning Microscope, Zeiss).

Western blotting

Proteins were separated by SDS-PAGE under reducing conditions, followed by blocking in PBST containing 1% BSA. The primary antibodies used included the following: SPON2 (AF2609; R&D Systems), anti-HA antibody (05-904; Merck Millipore), LATS1 (A300-479A; Bethyl Laboratories), p-LATS1 Tyr1079 (8654; Cell Signaling Technology), YAP (4912; Cell Signaling Technology), p-YAP Ser127 (13008; Cell Signaling Technology), α4 (ab81280; Abcam), α5 (ab150361; Abcam), and α-Tubulin (T6199; Sigma-Aldrich). After incubating with the anti-mouse IRDye 680 (LI-COR) and anti-rabbit IRDye 800 (LI-COR) secondary antibodies for 1 hour at room temperature, the bands were visualized using an Odyssey infrared imaging system (LI-COR). Quantification was performed the using ImageJ software.

Animal studies

Mice were housed and used according to protocols approved by the East China Normal University Animal Care Commission. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the NIH. A total of 2×10^6 MHCC-LM3 or SMMC-7721 cells was suspended in 20 μL serum-free DMEM/Matrigel (1:1) for each BALB/c (nu/nu) mouse. Through a 1-cm transverse incision in the upper abdomen under anesthesia, each mouse (6 per group, 6-week-old males) was orthotopically inoculated in the left hepatic lobe with a microsyringe. After 6 weeks, the mice were sacrificed, and their livers were dissected, fixed with phosphate-buffered neutral formalin and prepared for standard histologic examinations.

Dynabead immunoprecipitation

Immunoprecipitation was performed as described previously (28). The protein G Dynabeads (Invitrogen) were precleaned and incubated with the anti-HA antibody (05-904; Merck Millipore), anti-α4 antibody (provided by Prof. Jian-Feng Chen, Shanghai Institute of Biochemistry and Cell Biology, Shanghai, P.R. China), anti-α5 antibody (ab6131; Abcam), or normal mouse IgG (Sigma-Aldrich).

Clinical samples

All tissue samples were collected in the Department of Liver Surgery, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University. Fresh samples including tumor tissues and CNL tissues were obtained from HCC patients during tumor resection. A total of 202 HCC samples were collected from 2004 to 2010 and constructed into tissue microarrays. The median age was 50 years (range 17–73 years). Most of these patients were HBV-positive (187/202). The follow-up time ended in December 2012, and the median follow-up period was 33 months (range 2–90 months). All tissue samples were obtained with informed consent, and all procedures were performed in accordance with the China Ethical Review Committee.

GTPase pull-down and G-LISA activation assays

Pull-down assays were conducted as reported previously (29). Primary antibodies used in the assays as follows: RhoA (2117; Cell Signaling Technology), Rac1 (2320346; Merck Millipore), or Cdc42 (2466; Cell Signaling Technology). Activation of RhoA and Rac1 were measured using G-LISA activation assay kits (Cytoskeleton) according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using SPSS 16.0 for windows (IBM). Data were presented as the mean ± SD or ± SEM from at least three samples or experiments per data point. Student *t* test or one-way ANOVA was used for comparisons between groups. Correlation of SPON2 expression and TAM density in patients with HCC was evaluated by Pearson test. Cumulative survival time was calculated by the Kaplan–Meier method and analyzed by the log-rank test. $P < 0.05$ was considered statistically significant.

A more detailed description of the experimental procedures and reagents used in this study can be found in the Supplementary Materials and Methods.

Results

SPON2 is significantly overexpressed in the clinical samples and closely correlate with vascular invasion and patient prognosis in HCC

We first investigated SPON2 expression in HCC by analyzing The Cancer Genome Atlas (TCGA) database. The data showed that the mRNA levels of *SPON2* in HCC tissues were higher than that in the corresponding noncancerous liver (CNL) tissues (Supplementary Fig. S1A). Similar results were obtained in four independent HCC microarray datasets from the GEO database (Supplementary Fig. S1B). We further confirmed that the *SPON2* expression level was significantly higher in the HCC tissues than in the paired CNL tissues (Supplementary Fig. S1C). IHC analysis revealed stronger SPON2 staining in the HCC tissues than in the CNL tissues (Supplementary Fig. S1D). The SPON2 protein was overexpressed in 52% of the HCC patients (Supplementary Fig. S1E). In addition, the SPON2 expression levels were higher in most of the tested HCC cell lines, with the exceptions of SMMC-7721 and HepG2, compared with immortalized human liver THLE-2 cells (Supplementary Fig. S2A and S2B).

To further investigate the clinical significance of SPON2 in HCC, we analyzed the SPON2 expression status relative to various pathologic parameters in 202 HCC patients. The results indicated that SPON2 expression in HCC tissues was closely associated with gamma-glutamyltransferase, vascular invasion, and TNM stage (Supplementary Table S1). Interestingly, we found that the patients with high SPON2 expression had better overall survival (Fig. 1A) and relapse-free survival (Fig. 1B) than patients with low SPON2 expression.

SPON2 positively correlates with the M1-like macrophage density in HCC

Previous reports have shown that SPON2 is especially critical for inflammatory cell recruitment (21). TAMs represented up to 40% of all patrolling and infiltrating lymphocytes in HCC according to The Cancer Immunome Atlas (30). We wondered whether there was any potential link between SPON2 expression and macrophage infiltration. M1-specific markers (HLADR and CCR7) and M2-specific marker (Fizz1) were used to distinguish TAMs in human HCC tissue microarrays. IHC staining showed that the HCC tissues with higher SPON2 levels contained more $HLADR^+$ and $CCR7^+$ macrophages, whereas the HCC tissues with lower SPON2 levels had fewer $HLADR^+$ and $CCR7^+$ macrophages (Fig. 1C). We also found positive correlations between SPON2 expression levels and $HLADR^+$ and $CCR7^+$ macrophages (Fig. 1D). However, there was no significant correlation between SPON2 and $Fizz1^+$ macrophages (Fig. 1E). These data suggest a positive correlation between the SPON2 levels and M1-like TAM density in human HCC.

SPON2 promotes macrophage-like cell migration while inhibiting HCC cell migration *in vitro*

To validate whether SPON2 functioned as a potent chemoattractant, we performed a series of migration and invasion assays to examine the capacity of SPON2 to attract macrophages/monocytes *in vitro*. SPON2-silenced and SPON2-overexpressing stable cell lines that were generated with transductions of lentivirus carrying the *SPON2*-shRNA (shSPON2) or *SPON2* gene (lenti-SPON2), respectively, were established in

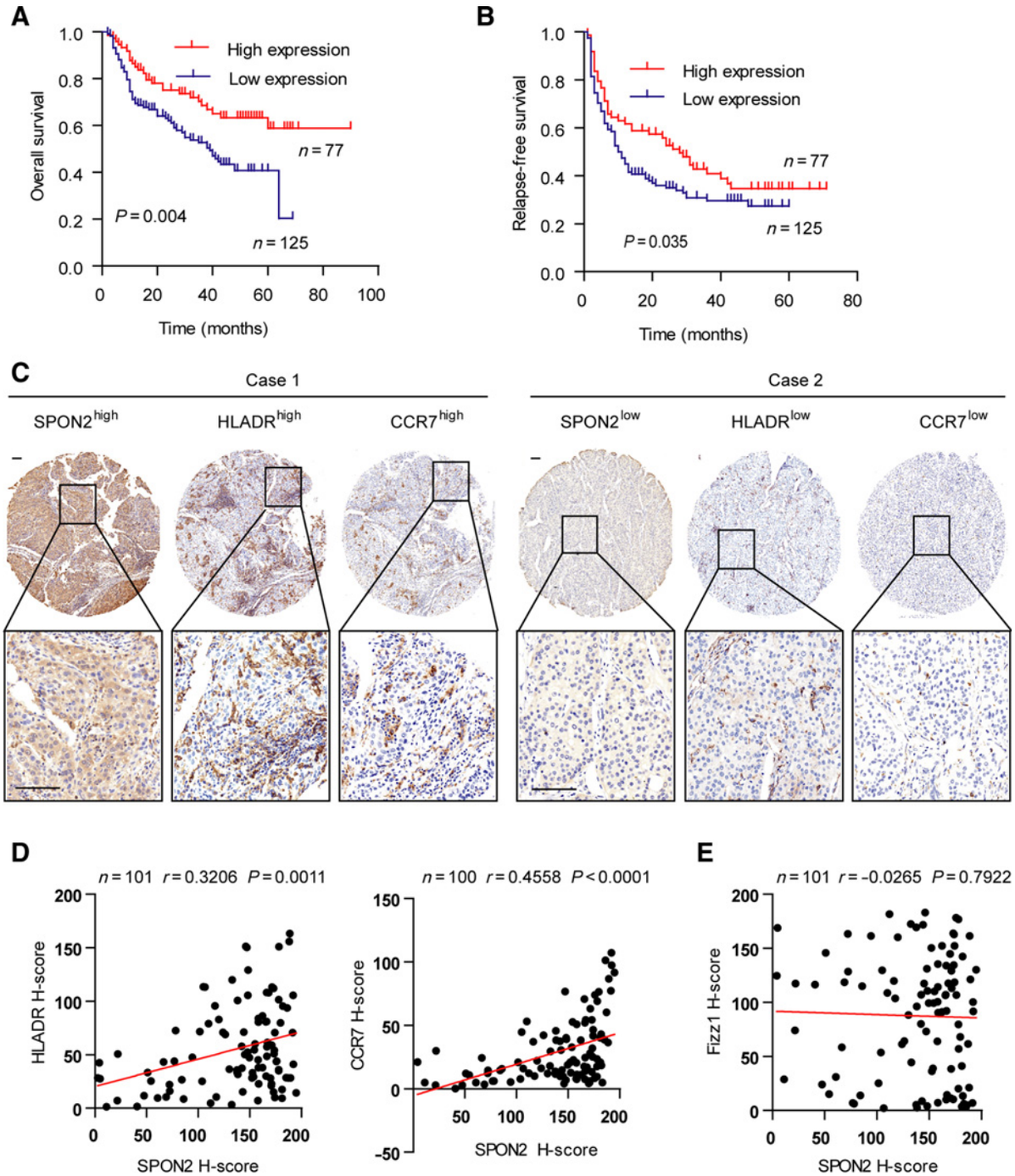

Figure 1.

Positive correlation of SPON2 expression with patient prognosis and M1-like TAM infiltration. **A** and **B,** Kaplan–Meier analysis of overall and relapse-free survival for the expression of SPON2. **C,** Representative images of IHC staining show that HCC Case 1 with higher SPON2 levels contains more HLADR$^+$ and CCR7$^+$ TAMs, and Case 2 with lower SPON2 levels has less HLADR$^+$ and CCR7$^+$ TAMs. Insets in the IHC stains are enlarged and are shown below each picture. Scale bar, 100 μm. **D,** Correlation analysis between SPON2 and HLADR$^+$ and CCR7$^+$ macrophages in HCC tissue microarray slides. **E,** Correlation analysis between SPON2 and Fizz1$^+$ macrophages in HCC tissue microarray slides. Two-tailed Pearson test was used ($n = 104$, except a few samples that detached from the slides during the staining process and that were not included in statistics).

four HCC cell lines; control cells were transfected with a scramble shRNA (shNC) or mock vector (lenti-vector; Supplementary Fig. S2C–S2E).

We then used the THP-1 cell line, which could be primed from monocytes to become macrophage-like cells, for the Transwell assays. Conditioned media (CM) from SPON2-silenced MHCC-

LM3 and MHCC-97H cells dramatically reduced migration of the PMA-primed macrophage-like THP-1 cells compared with the matched control cells (Fig. 2A). Likewise, CM from SPON2-overexpressing SMMC-7721 and HepG2 cells significantly increased migration of the THP-1–derived macrophage-like cells (Fig. 2A). Because SPON2 is an ECM protein, we further confirmed the supportive role of SPON2 in primed THP1 cell migration and invasion using a recombinant SPON2 (rSPON2) protein (Supplementary Fig. S2F). THP-1 macrophage migration and invasion toward rSPON2 were significantly enhanced in a dose-dependent manner (Fig. 2B). Furthermore, the same phenomena were also observed in human peripheral blood mononuclear cells (hMC; Fig. 2C), indicating that SPON2 displayed strong capacity to attract macrophages/monocytes.

Previous reports have shown that SPON2 is associated with the migration and invasion of several types of HCC cells (31). Transwell assays confirmed that silencing of SPON2 significantly increased the migration of both MHCC-LM3 and MHCC-97H cells, whereas overexpression of SPON2 dramatically decreased the migration of both SMMC-7721 and HepG2 cells (Fig. 2D). We further confirmed the inhibitory effect of SPON2 on HCC cell movement with the rSPON2 protein. The results showed that rSPON2 significantly inhibited migration and invasion of SMMC-7721 and HepG2 cells in a dose-dependent manner (Fig. 2E and F).

Collectively, these data demonstrate that SPON2 has a strong capacity to attract macrophages but prevents HCC cell migration and invasion.

SPON2 suppresses HCC metastasis and recruits M1-like macrophages *in vivo*

We further evaluated the effect of SPON2 silencing and overexpressing on HCC progression in xenografts. The number of intrahepatic metastatic nodules was much higher in the mice inoculated with the shSPON2/MHCC-LM3 cells than mice transplanted with the shNC/MHCC-LM3 cells (Fig. 3A and B). While mice transplanted with the lenti-SPON2/SMMC-7721 cells showed fewer intrahepatic metastatic nodules than that with lenti-vector/SMMC-7721 cells (Fig. 3A and B).

To further confirm the correlation between SPON2 and M1-like macrophages in HCC, immunofluorescence staining of TAM markers in xenografts was performed. Fraction of $HLADR^+$ cells were markedly reduced in the tumors derived from shSPON2/MHCC-LM3 (Fig. 3C). While fraction of $HLADR^+$ cells were significantly increased in the tumors derived from lenti-SPON2/SMMC-7721 cells (Fig. 3D). Consistently, silencing SPON2 led to dramatically decreased $CCR7^+$ M1 fraction (Fig. 3E), whereas overexpressing SPON2 resulted in noticeably increased $CCR7^+$ M1 fraction (Fig. 3F), confirming a positive correlation between the SPON2 levels and M1-like TAM infiltration.

SPON2-silenced metastatic liver nodules displayed an increase in the fraction of $Fizz1^+$ M2 compared with the control group (Supplementary Fig. S3A). Moreover, SPON2-overexpressed metastatic liver nodules exhibited a decrease in the fraction of $Fizz1^+$ M2 compared with the control group (Supplementary Fig. S3B).

Collectively, these data suggest that SPON2 has an inhibitory effect on HCC metastasis, which is consistent with previous reports, and that the infiltrated TAMs in the SPON2-abundant regions are maintained as M1-like subtype macrophages, which may secrete tumor-suppressive factors to prevent HCC progression.

Distinct integrin receptors mediate diverse functions of SPON2 on the motility of macrophage-like cells and HCC cells

We further uncovered the underlying mechanism of SPON2-modulated movement of macrophages and HCC cells. Previous studies have revealed that SPON2 serves as a ligand for integrins α4, α5, αM, β1, and β2 (19, 20). Given the uniform expression of α4, α5, αM, αX, β1, and β2 receptors in macrophages (Supplementary Fig. S4A), specific anti-integrin mAbs were used to block SPON2-induced recruitment of macrophages. Among the six antibodies, anti-α4 and anti-β1 significantly inhibited SPON2-induced migration of primed THP-1 cells, whereas the other mAbs displayed no effect (Fig. 4A). The combination of anti-α4 and anti-β1 synergistically inhibited SPON2-induced migration of primed THP-1 cells (Fig. 4A). To further confirm that integrin α4β1 was involved in SPON2-induced macrophage recruitment, integrin α4 was silenced with siRNA (Supplementary Fig. S4B). Both the migration and invasion assays showed that the promotive effects of SPON2 on primed THP-1 cell movement were almost completely abolished by ITGA4 silencing (Fig. 4B). Taken together, these data indicate that SPON2-induced migration of macrophages is mainly mediated by the integrin α4β1 receptors.

We further explored which subtype of integrin receptor was responsible for SPON2-modulated HCC cell migration. The results showed that blocking integrin α5 and β1 abrogated the inhibitory effects of SPON2 on HCC cell migration (Fig. 4C). Simultaneous α5 and β1 blockade synergistically reversed SPON2-medated migration of HCC cells (Fig. 4C). Furthermore, using siRNA against ITGA5, we confirmed that the inhibitory effects of SPON2 on HCC cell migration and invasion were mainly mediated through integrin α5β1 (Fig. 4D; Supplementary Fig. S4C).

We next determined whether SPON2 could directly interact with integrin α4 in macrophages and with integrin α5 in HCC cells. Using a coimmunoprecipitation approach, SPON2 or α4 was readily detected in primed THP-1 cell lysates that were immunoprecipitated with the anti-α4 or anti-SPON2 antibody (Fig. 4E). In MHCC-LM3 cells, SPON2 was coimmunoprecipitated with α5 as one complex and vice versa (Fig. 4F).

Consistently, coimmunofluorescence staining showed that the endogenous SPON2 and integrin α4 were colocalized in primed THP-1 cells, forming a ring-like pattern on cell surfaces (Supplementary Fig. S5A). Likewise, endogenous SPON2 and integrin α5 were spatially colocalized in MHCC-LM3 cells, with the highest fluorescent signals surrounding the cell membranes (Supplementary Fig. S5B).

To validate whether SPON2 and the integrin receptors temporally and spatially existed in one complex, the Duolink *in situ* PLA was performed. On the primed THP-1 cell membrane, PLA spots were detected in samples labeled with the anti-SPON2 and anti-α4 antibodies, but not in samples treated with corresponding control IgG (Fig. 4G). Similarly, fluorescent PLA foci were visualized on MHCC-LM3 cell surface in the presence of the SPON2 and integrin α5 antibodies (Fig. 4H).

These results indicate that SPON2 promotes macrophage-like cell and inhibits HCC cell movement through interactions with the two distinct integrin receptors.

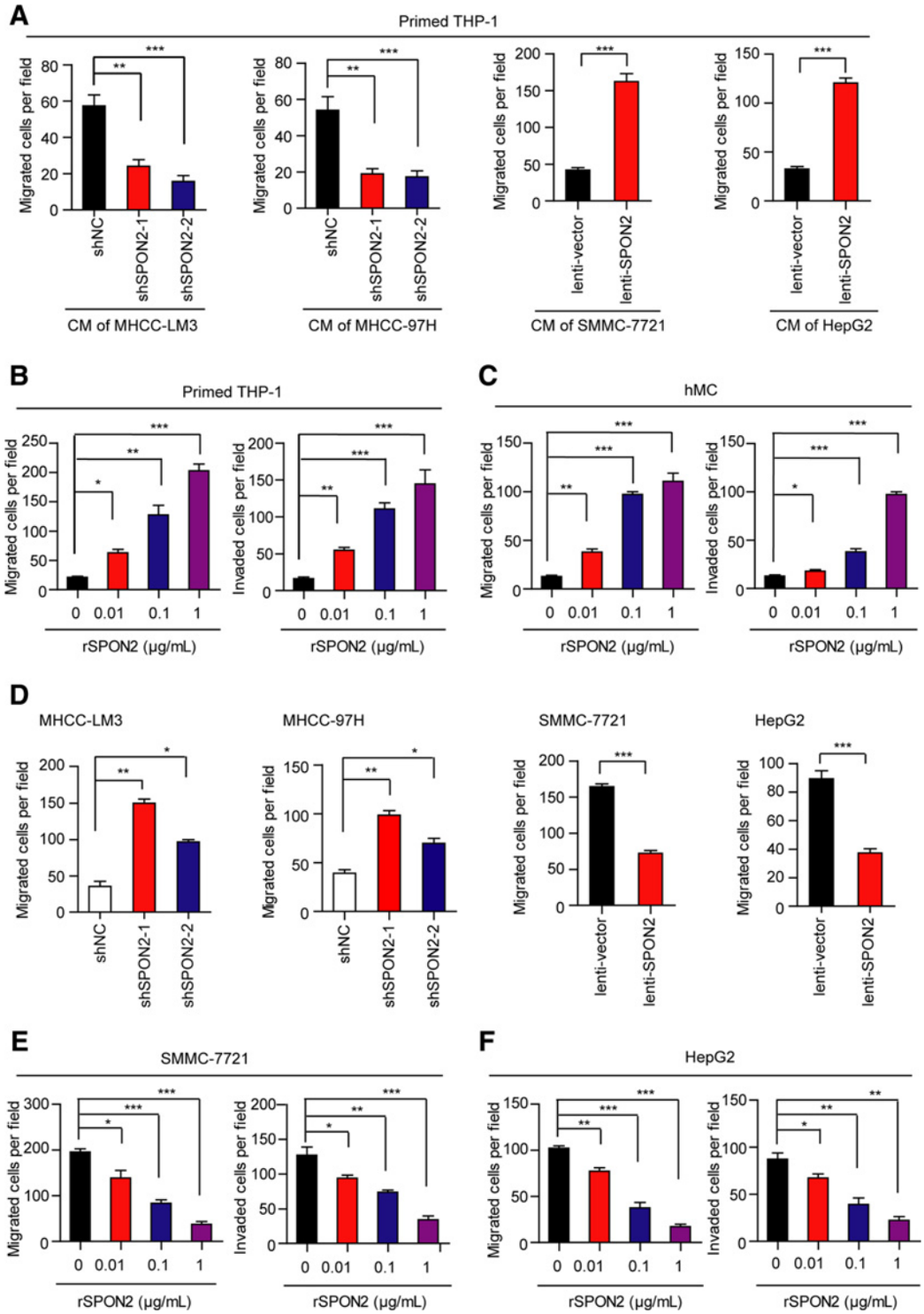

Figure 2.

SPON2 promotes macrophage-like cell migration but inhibits HCC cell migration *in vitro*. **A,** Quantification of migrated macrophage-like THP-1 cells toward CM from shSPON2/MHCC-LM3, shSPON2/MHCC-97H, lenti-SPON2/SMMC-7721, or lenti-SPON2/HepG2 cells in Transwell assays. **B,** Quantification of migrated and invaded THP-1 cells treated with 0.01, 0.1, or 1 μg/mL rSPON2 in Transwell assays. **C,** Quantification of migrated and invaded human mononuclear cells (hMC) treated with 0.01, 0.1, or 1 μg/mL rSPON2 in Transwell assays. **D,** Quantification of migrated HCC cells with SPON2 silencing or SPON2 overexpressing in Transwell assays. **E,** Quantification of migrated and invaded SMMC-7721 cells treated with 0.01, 0.1 or 1 μg/mL rSPON2 in Transwell assays. **F,** Quantification of migrated and invaded HepG2 cells treated with 0.01, 0.1 or 1 μg/mL rSPON2 in Transwell assays. Quantification of migrated and invaded cells was performed for six randomly selected fields. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ ($n = 6$ fields; two-tailed unpaired t test).

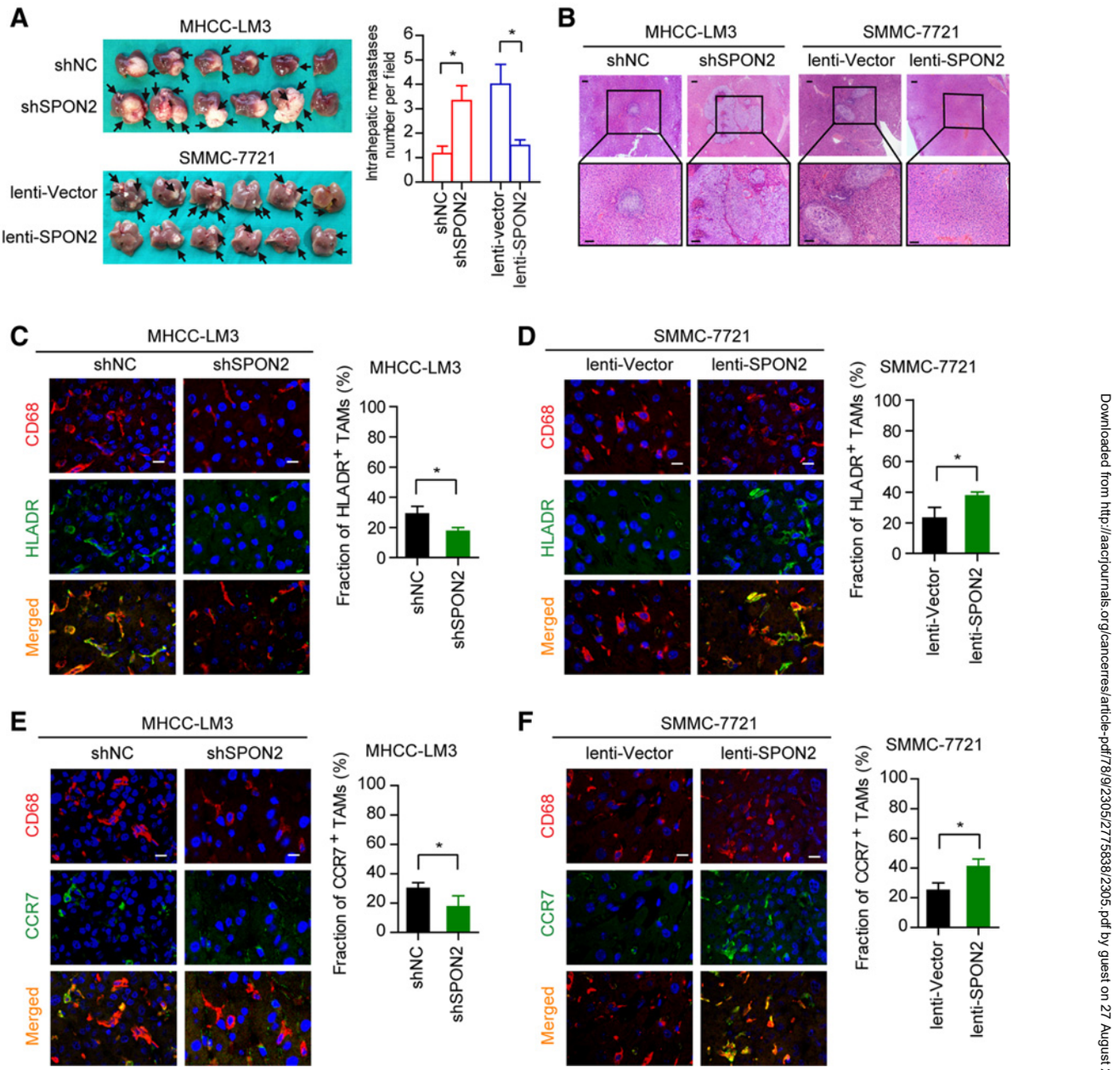

Figure 3.

SPON2 suppresses xenograft tumor intrahepatic metastasis and enhances M1-like TAM infiltration. **A,** Representative images of intrahepatic metastases in shNC/MHCC-LM3-inoculated and lenti-SPON2/SMCC–7721–inoculated mice are shown. Black arrows, metastatic nodules. **B,** Representative images of hematoxylin and eosin stains of liver tissues from mice that were orthotopically inoculated with SPON2-silenced MHCC-LM3 or SPON2-overexpressing SMCC-7721 cells. Scale bars, 100 μm. **C** and **E,** Immunofluorescence staining of the total macrophage marker, CD68 (red), and the M1 TAM markers, HLADR (green) and CCR7 (green), in MHCC-LM3–derived tumors expressing shNC or shSPON2. Nuclei were counterstained with DAPI (blue). Scale bar, 10 μm. **D** and **F,** Immunofluorescence staining of the total macrophage marker, CD68 (red), and the M1 TAM markers, HLADR (green) and CCR7 (green), in SMCC-7721-derived tumors expressing lenti-vector or lenti-SPON2. Nuclei were counterstained with DAPI (blue). Scale bar, 10 μm. The $HLADR^+$ and $CCR7^+$ TAM fraction was determined by the percentage of $HLADR^+$ and $CCR7^+$ TAMs within the $CD68^+$ TAM populations of the shSPON2 or lenti-SPON2 xenografts. Nonspecific staining was ruled out. *, $P < 0.05$ ($n = 6$ tumors; two-tailed unpaired t test).

SPON2 activates RhoA/Rac1-Hippo signaling in macrophage-like cells and suppresses RhoA signaling in HCC cells

Small Rho guanosine triphosphatases (Rho GTPases) are key molecules in integrin signaling (32). In addition, the activity of Rho GTPases regulates the assembly of actin stress fibers and cell locomotion (33). Thus, we examined the effects of SPON2 on the activities of RhoA, Rac1, and Cdc42 in both macrophages and HCC cells. Using a Rho GTPases pull-down assay, we observed

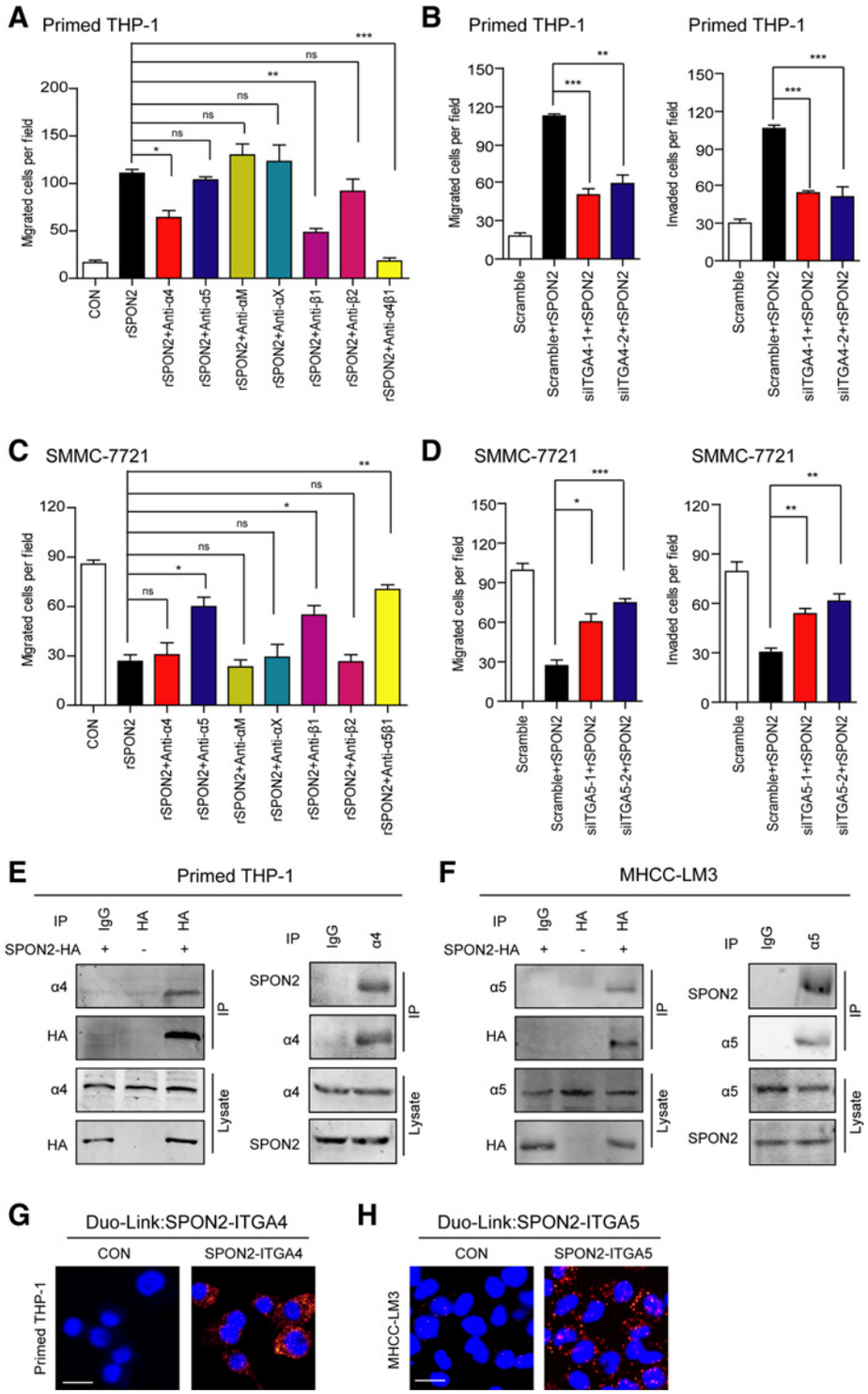

Figure 4.
SPON2 mediates migration of macrophage-like cells and HCC cells through different integrin receptors. **A,** Transwell migration assay of PMA-primed THP-1 cells toward rSPON2 in the presence of blocking antibodies to α4, α5, αM, αX, β1, and β2. **B,** Densitometric analysis of migrated and invaded THP-1 cells transfected with scramble siRNA or ITGA4 siRNA. **C,** Transwell migration assay of SMMC-7721 cells towards rSPON2 in the presence of blocking antibodies to α4, α5, αM, αX, β1, and β2. **D,** Densitometric analysis of migrated and invaded SMMC-7721 cells transfected with scramble siRNA or ITGA5 siRNA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, no significance ($n = 6$ fields; two-tailed unpaired t test). **E,** Co-IP of SPON2 with integrin α4 in primed THP-1 cells. **F,** Coimmunoprecipitation of SPON2 with integrin α5 in MHCC-LM3 cells. **G,** The interaction between SPON2 and integrin α4 on primed THP-1 cell membranes was detected by *in situ* PLA (red dots). **H,** The interaction between SPON2 and integrin α5 on MHCC-LM3 cell surfaces was detected by *in situ* PLA (red dots). Nuclei were counterstained with DAPI (blue). Scale bar, 20 μm.

that the rSPON2 protein significantly enhanced the activity of RhoA and Rac1 but not Cdc42 in macrophages (Fig. 5A). We then used another approach, the G-LISA activation assay, to measure the activities of RhoA and Rac1; the G-LISA analysis also demonstrated that the activities of RhoA and Rac1 in the primed THP-1 cells were noticeably increased by rSPON2 (Fig. 5B). Moreover, 3D spheroid immunofluorescence showed that the stress fiber-like actin level in the rSPON2-treated THP-1 cells was higher than in the vehicle-treated cells (Fig. 5C), indicating that SPON2 could regulate cytoskeleton assembly.

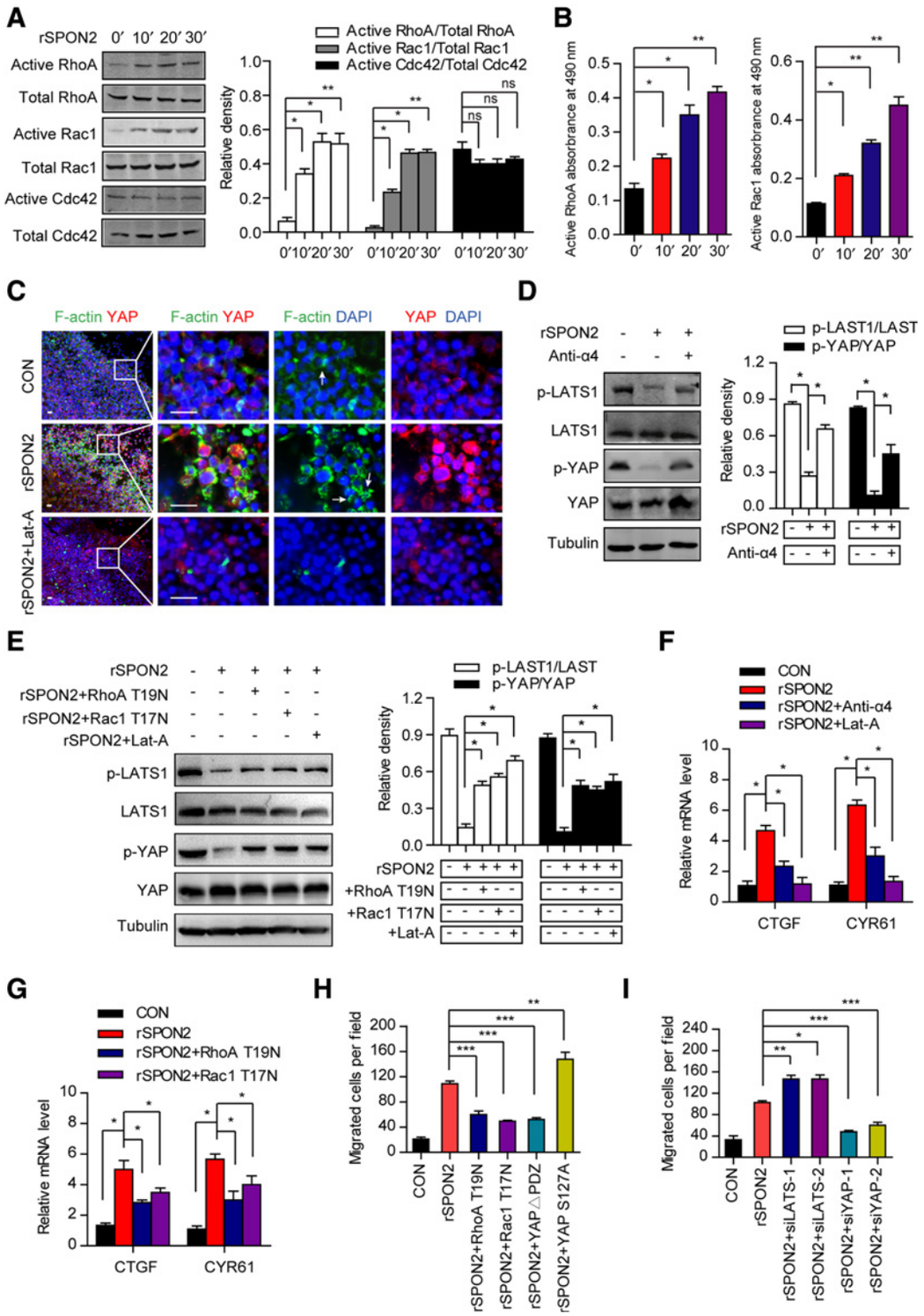

Figure 5.

SPON2 stimulates migration of macrophage-like cells through α4 integrin-RhoA/Rac1-Hippo signaling. **A,** Pull-down assays for the active RhoA, Rac1, and Cdc42 in primed THP-1 cells treated with rSPON2 at different time points. **B,** The G-LISA activation assays showed that both active RhoA and active Rac1 were enhanced in the primed THP-1 cells treated with rSPON2. **C,** Immunofluorescence of F-actin (green) and YAP (red) in primed THP-1 cells embedded in 3D Matrigel spheroids. Nuclei were counterstained with DAPI (blue). Arrows, F-actin aggregates. Scale bar, 20 μm. **D** and **E,** The phosphorylation levels of LATS1 and YAP in the primed THP-1 cells. Tubulin was used as the loading control. **F** and **G,** Expression of YAP-regulated genes, CTGF and CYR61. **H** and **I,** Transwell migration assay of PMA-primed THP-1 cells. $n = 6$ fields. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ns, no significance (two-tailed unpaired t test).

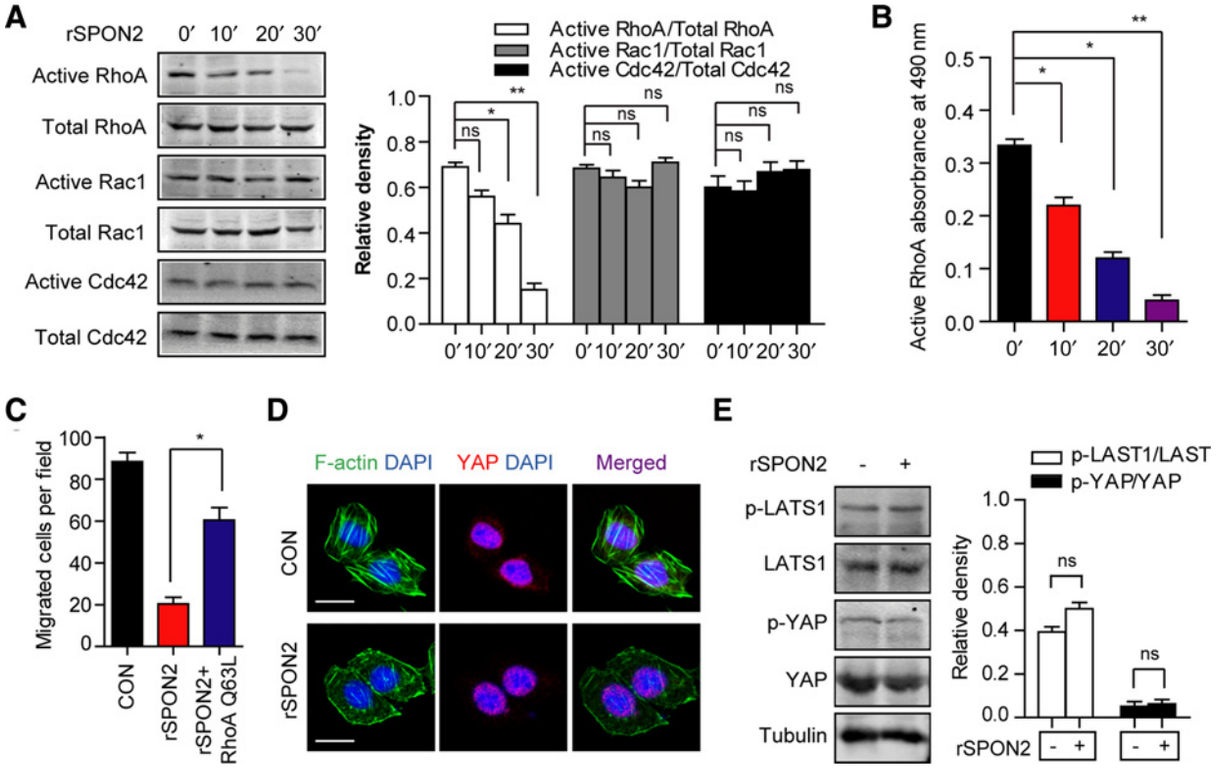

Figure 6.

SPON2 suppresses migration of HCC cells through α5 integrin-RhoA signaling. **A,** Pull-down assays for the active RhoA, Rac1, and Cdc42 in SMMC-7721 cells treated with rSPON2 at different time points. **B,** G-LISA activation assay showed that active RhoA was decreased in SMMC-7721 cells treated with rSPON2. **C,** Decreased migration of rSPON2-induced SMMC-7721 cells was reversed in SMMC-7721 cells expressing the RhoA Q63L mutant. $n = 6$ fields. **D,** Immunofluorescence of F-actin (green) and YAP (red) in SMMC-7721 cells. Nuclei were counterstained with DAPI (blue). Scale bar, 20 μm. **E,** The phosphorylation levels of LATS1 and YAP in SMMC-7721 cells. Tubulin was used as the loading control. *, $P < 0.05$; **, $P < 0.01$, ns, no significance (two-tailed unpaired t test).

Recently, the Hippo pathway was reported to be modulated by integrin-mediated changes in the actin cytoskeleton and Rho GTPase activity (34, 35). Therefore, we further investigated the effects of SPON2 on the Hippo pathway. The results showed that rSPON2 significantly increased YAP nuclear localization in primed THP-1 cells, while inhibition of F-actin with Lat-A reversed YAP nuclear localization (Fig. 5C). And SPON2-mediated YAP nuclear localization was enhanced by Hippo signaling restriction (siLATS1) and YAP nuclear localization mutant (YAP S127A; Supplementary Fig. S6A–S6C). However, both siYAP and YAP cytoplasmic localization mutant (YAPΔPDZ) could reverse SPON2-mediated YAP nuclear localization (Supplementary Fig. S6B–S6D). In addition, rSPON2-mediated suppression of LATS1 and YAP phosphorylation was partially restored by anti-α4 (Fig. 5D). Similar results were also obtained by Lat-A, dominant negative mutants of RhoA T19N or Rac1 T17N (Fig. 5E; Supplementary Fig. S7A and S7B).

With the rSPON2 treatment, YAP translocated to nucleus and subsequently initiated downstream expression of genes, such as CTGF and CYR61 (Fig. 5F). The rSPON2-mediated YAP downstream gene expression was abated by anti-α4 or Lat-A (Fig. 5F). RhoA T19N and Rac1 T17N also inhibited rSPON2-mediated YAP transcriptional activity (Fig. 5G). Moreover, SPON2-mediated macrophage recruitment was obviously reversed by siYAP, RhoA T19N, Rac1 T17N, or YAPΔPDZ (Fig. 5H and I). However, both siLATS1 and YAP S127A strengthened SPON2-mediated macrophage migration (Fig. 5H and I). These results indicate that SPON2-induced macrophage migration requires persistent activation of a cytoskeleton-regulated pathway, which may cooperate with the inactivation of the Hippo pathway.

We further investigated the effects of SPON2 on the activities of the Rho GTPases in HCC cells. The pull-down assay showed that SPON2 significantly reduced the activity of RhoA but had no effect on the activity of Rac1 or Cdc42 (Fig. 6A). The G-LISA activation assay further confirmed these results (Fig. 6B). Furthermore, the constitutively activated mutant of RhoA (RhoA Q63L) restored the SMMC-7721 migration that was inhibited by rSPON2 (Fig. 6C; Supplementary Fig. S7C). Compared with the control cells, the SMMC-7721 cells treated with rSPON2 showed fewer stress fiber actin bundles (Fig. 6D). However, no obvious differences were observed in the nuclear translocation of YAP (Fig. 6D). The Western blotting analysis showed that SPON2 had no effect on LATS1 and YAP phosphorylation (Fig. 6E). Taken together, by interacting with α5β1 integrin receptor, SPON2-induced alterations, which include reduced RhoA activity and decreased F-actin accumulation, likely contribute to the inhibitory effects of SPON2 on HCC cell migration.

Discussion

Previous studies have reported that members of the integrin family mediate leukocyte adhesion and migration by interacting

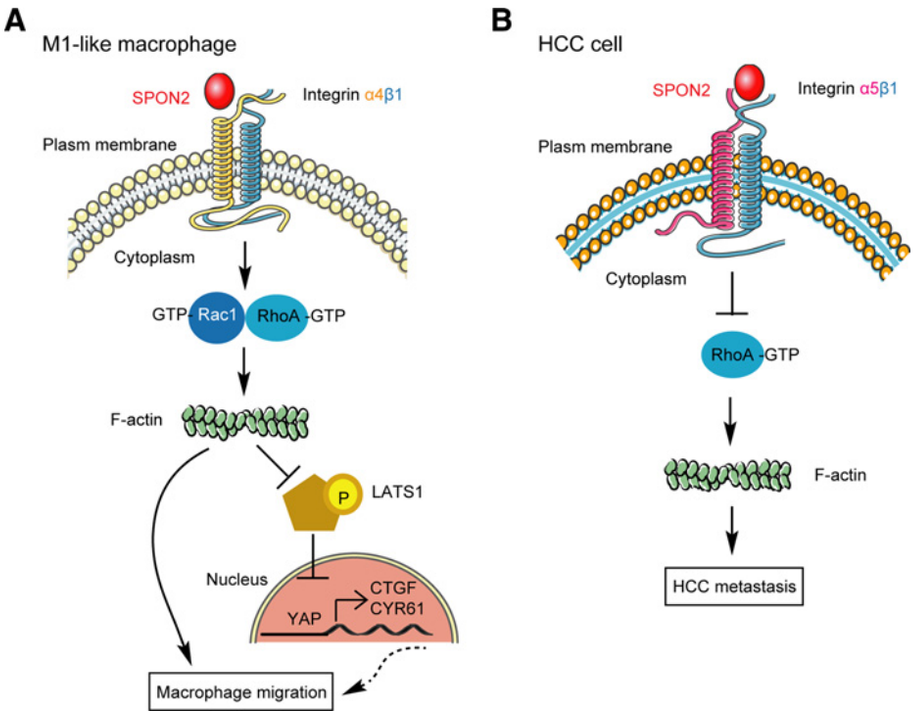

Figure 7.
A schematic presentation illustrating SPON2-mediated recruitment of M1-like macrophages and suppression of HCC metastasis through distinct integrin-Rho GTPase-Hippo pathways. **A,** Illustration of the SPON2-regulated specific integrin signaling in M1-like macrophage. SPON2 interactions with integrin α4β1 receptors activate RhoA and Rac1, resulting in more stress fiber-like actin bundles. F-Actin accumulation not only promotes M1-like macrophage migration but also inhibits the Hippo pathway by restricting phospho-LATS1, promoting YAP nuclear translocation, initiating YAP-dependent gene expression, and ultimately accelerating M1-like macrophage infiltration. **B,** Illustration of the SPON2-regulated specific integrin signaling in HCC cell. SPON2 interactions with integrin α5β1 receptors suppress activation of RhoA, disrupt F-actin assembly, and eventually inhibit HCC cell migration and tumor metastasis. The solid lines with arrows and blunted ends refer to positive and inhibitory actions, respectively. The dotted lines with arrows indicate less well-characterized pathways.

with endothelial cells and ECM proteins (36, 37). Several ECM proteins, including laminin, thrombospondin, and fibronectin, play important roles in the recruitment of monocytes and neutrophils to inflamed sites (38). In this study, we demonstrated that the ECM protein SPON2 and its integrin receptors α4β1 play critical roles in the recruitment of M1-like subtype TAMs in HCC microenvironment. It has been reported that SPON2 directly binds to bacterial and viral pathogens to initiate innate immune responses, and functions as an opsonin for macrophage phagocytosis (21). SPON2 deficiency significantly suppressed inflammatory cell infiltration, cytokine, and chemokine production (39). Chronic infection with HBV or HCV is a major cause of HCC (13). Upregulated SPON2 can function as a potentially strong chemoattractant for monocytes and macrophages to inhibit the hepatitis virus and clear infected hepatocytes. Therefore, the accumulation of macrophages in the SPON2-abundant regions of HCC is expected to be composed of M1-like macrophages, which exhibit antitumor immune responses.

It is well-known that chemokines are involved in recruitments of monocytes/macrophages in cancer (40, 41). Indeed, chemokine ligand 5 (CCL5) production was increased in HCC cells treated with rSPON2 comparing with controls (Supplementary Fig. S8A). However, inhibition of CCL5 slightly blocked rSPON2-mediated macrophage migration (Supplementary Fig. S8B). Therefore, SPON2 mainly functions as a potent chemoattractant and interacts with α4β1 integrin receptor in macrophages to attract macrophages.

TAMs can not only provide tumorigenic signals during chronic inflammation, but also clear premalignant senescent hepatocytes to prevent HCC (42). Therapeutic targeting of hepatic macrophages might be able to prevent HCC in patients with chronic liver diseases or improve current therapies in established HCC (10, 43). However, although these results are encouraging, any macrophage-directed HCC therapies would have to take into account for the heterogeneous functions of hepatic macrophages during chronic inflammation, fibrosis, and cancer progression.

Cell morphology is an important factor in regulating the Hippo pathway. It has been suggested that stress fibers consisting of F-actin, which act upstream of LATS, regulate YAP through Hippo signaling (44). Recent studies have indicated that the integrin–Gα13–RhoA–YAP pathway regulates JNK signaling and down-regulates proinflammatory gene expression, thereby affecting monocyte attachment and infiltration (34, 45). Our data are the first to support a model in which SPON2 functions through α4β1 integrin receptors to activate RhoA and Rac1, increase stress fiber assembly, and eventually drive M1-like macrophages to the tumor microenvironment. F-Actin accumulation in response to SPON2 suppresses LATS1 phosphorylation, triggers YAP translocation into the nucleus, and ultimately initiates YAP-dependent transcription (Fig. 7A). However, how YAP regulates macrophage movement is not fully understood. In the presence of SPON2, expression of YAP-regulated growth-promoting genes might promote JNK signaling and upregulate proinflammatory cytokine expression, thereby enhancing macrophage attachment and infiltration. Out study is the first to reveal that the Hippo pathway is involved in integrin-mediated macrophage recruitment.

Notably, SPON2-mediated movement of macrophages and HCC cells requires distinct integrin receptors and downstream signaling events. SPON2-integrin α5β1 signaling plays a critical role in suppressing RhoA activity, disrupting F-actin assembly, and consequently inhibiting migration and invasion of HCC cells (Fig. 7B). However, nuclear accumulation of YAP did not change in the presence of SPON2, indicating that the Hippo pathway was most likely not involved in SPON2-mediated migration of HCC cells. Recent studies have revealed that the regulation of YAP and transcriptional coactivator with PDZ-binding motif (TAZ) can be

disrupted. Hyperactivation of these two proteins is widespread in cancer (46). Future studies are necessary to determine why the SPON2–integrin α5β1–RhoA signaling restricts F-actin assembly, but does not affect YAP activation.

Analysis of TCGA database indicated that ITGA5 was significantly overexpressed in HCC (Supplementary Fig. S9), which may be the reason that SPON2 preferentially interacts with integrin α5 over α4 in HCC cells. However, why SPON2 attracts M1-like macrophages in HCC through α4 instead of α5 remains unknown. Integrin α4 has been shown to initiate lymphocyte attachment and rolling under physiologic flow (36). This result is consistent with RhoA and Rac1 functioning downstream of α4β1 integrins to promote macrophage migration. In addition, it is expected that α4β1 and α5β1 integrins have different roles and require different signaling events in tumor microenvironment (47, 48).

Because of its elevated level, SPON2 has already been established as a prognostic biomarker of colorectal cancer (24), and has been investigated as a serum and histologic diagnostic biomarker for ovarian cancer alike (22). Conflicting results regarding the effects of SPON2 on the migratory and invasive abilities of tumor cells have been reported by multiple studies. Schmid and colleagues reported that *ITGA5*, as a transcriptional target gene of MACC1, induces cell motility, drives colorectal cancer metastasis, and can serve as an important biomarker for predicting a poor colorectal cancer prognosis (49). Conversely, thyroid hormone–regulated SPON2 has an inhibitory effect on HCC cell migration and invasion (31). Nevertheless, our data reveal that SPON2 not only suppresses HCC metastasis but also facilitates M1-like macrophage recruitment to the tumor microenvironment to prevent HCC progression. Immunohistostaining indicates that SPON2 is significantly upregulated in HCC patients and predicts good survival. Further investigations will be required to determine whether elevated SPON2 can serve as a novel diagnostic and prognostic biomarker for patients with HCC. An improved understanding of the cellular and molecular mechanisms whereby SPON2 promotes M1-like macrophage recruitment and restricts hepatocarcinogenesis will provide new strategies for therapeutic approaches to HCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y.-L. Zhang, X.-M. Yang, F. Fang, X.-L. Zhang, Q. Xia, Z.-G. Zhang

Development of methodology: Y.-L. Zhang, Q. Li, F. Fang, X.-L. Zhang, M.-X. Feng, G.-A. Tian, Q. Xia, Z.-G. Zhang

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.-L. Zhang, Q. Li, F. Fang, J. Li, Q. Yang, L. Zhu, H.-Z. Nie, M.-X. Feng, S.-H. Jiang, G.-A. Tian, L.-P. Hu, Z.-G. Zhang

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.-L. Zhang, Q. Li, F. Fang, Q. Yang, L. Zhu, H.-Z. Nie, M.-X. Feng, S.-H. Jiang, L.-P. Hu, Z.-G. Zhang

Writing, review, and/or revision of the manuscript: Y.-L. Zhang, Q. Li, X.-M. Yang, F. Fang, Y.-H. Wang, Q. Yang, L. Zhu, S.-H. Jiang, G.-A. Tian, L.-P. Hu, H.-Y. Lee, S.-J. Lee, Q. Xia, Z.-G. Zhang

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X.-M. Yang, Y.-H. Wang, M.-X. Feng, S.-H. Jiang, Q. Xia, Z.-G. Zhang

Study supervision: X.-M. Yang, M.-X. Feng, G.-A. Tian, Q. Xia, Z.-G. Zhang

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