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The E3 ubiquitin ligase SOCS-7 reverses immunosuppression via Shc1 signaling in hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is one of the most common primary liver malignancies and is the third leading cause of tumorrelated mortality worldwide. Despite advances in HCC treatment, diagnosis at the later stages, and the complex mechanisms relating to the cause and pathogenesis, results in less than 40% of HCC patients being eligible for potential therapy. Prolonged inflammation and resulting immunosuppression are major hallmarks of HCC; however, the mechanisms responsible for these processes have not been clearly elucidated. In this study, we identified SOCS-7, an inhibitor of cytokine signaling, as a novel regulator of immunosuppression in HCC. We found that SOCS-7 mediated E3 ubiquitin ligase activity on a signaling adaptor molecule, Shc1, in Huh-7 cells. Overexpression of SOCS-7 reduced the induction of immunosuppressive factors, TGF- β , Versican, and Arginase-1, and further reduced STAT3 activation. Furthermore, using an in vivo tumor model, we confirmed that SOCS-7 negatively regulates immunosuppression and inhibits tumor growth by targeting Shc1 degradation. Together, our study identified SOCS-7 as a possible therapeutic target to reverse immunosuppression in HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the primary forms of liver malignancy that affects more than 800,000 people worldwide every year¹. Depending on the stage of the disease HCC patients have a 5-year survival rate of 10-30%². Chronic viral infections with hepatitis B or C virus predispose a fraction of patients for development of HCC³. Other predispositions include alcohol consumption, diabetes, smoking, and body weight⁴. Recent treatment strategies in HCC include immunotherapy, which involves induction of the immune system to eradicate tumor cells. For example, treatment with programmed cell death-1 (PD-1) antibodies had a promising effect in a small fraction of HCC patients^{5,6}. However, there is still no broad-spectrum treatment that effectively targets HCC, which can be attributed to a poor understanding of immune deregulation and molecular pathogenesis of the disease. Hence, to identify promising therapeutic strategies, a holistic understanding of the pathways involved in promoting immunosuppression and driving HCC pathogenesis is required.

The suppressor of cytokine signaling (SOCS) family of proteins are intracellular proteins capable of regulating various signaling pathways and altering the response of cells to different cytokines^{7,8}. It consists of eight proteins including SOCS 1–7 and cytokine-inducible SH2 domain-containing protein (CIS)⁹. SOCS proteins have been shown to be associated with signal transducer and activator of transcription 3 (STAT3) hyperactivation, regulating tumorigenesis and vascular invasion¹⁰. A key member of this family, SOCS-7, alters signaling pathways by ubiquitination of targets. It negatively correlates with STAT3 activation in renal fibrosis^{11,12}. Proteasomal degradation of SOCS-7 promoted

inflammation resulting in renal failure¹³, and knockdown of SOCS-7 favored tumor growth in in vivo models of breast cancer¹⁴. A comprehensive analysis of methylation patterns of *socs* genes in HCC patients revealed hypermethylation of SOCS1 in HBV infection-related hepatocarcinogenesis but not SOCS-7. Hence, the role of SOCS-7 in HCC pathogenesis has not been described so far.

The Src homology 2 domain containing (SHC)-transforming protein 1 (Shc1) is an adaptor protein that functions in coupling growth factor receptors to intracellular signaling pathways. It has three different isoforms: p46 and p52 (activation of Ras signaling), and p66 (pro-apoptotic). The p52 isoform has been implicated in promoting breast cancer, whereas the p66 isoform has been shown to promote autophagy and disrupt mitochondrial function in B cells^{15,16}. In breast cancer, Shc1 specifically activates STAT3mediated immunosuppressive signals and inhibits STAT1 mediated immune responses to drive tumorigenesis¹⁷. Furthermore, it has been shown that inhibition of STAT3 activation or STAT3 deletion can improve immune evasion to suppress tumor progression¹⁸. In our previous experiments, it was shown that high expression of Shc1 was associated with poor survival of HCC patients, and knockdown of p66 subtype significantly reduced tumor growth, in vitro and in vivo, by inhibiting the activation of STAT3¹⁹. However, the regulation of Shc1 expression in the context of HCC is still unknown.

In this study, we identified a novel mechanism that involved SOCS-7-mediated regulation of the Shc1/STAT3 pathway in regulating tumor immunosuppression to inhibit tumorigenesis in HCC, by using both in vitro and in vivo HCC models.

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MATERIALS AND METHODS

Tissues and cell culture

Tissues was obtained from patients recruited from Zhongshan Hospital, Fudan University, Shanghai, China. Informed consent was obtained from all patients and the study was approved by, and conformed to all criteria set, by the local Ethics Committee. Human hepatoma carcinoma cell lines, Huh-7 and HepG2 cells, were purchased from Procell Life Science (Wuhan, China), and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin at 5% CO₂ at 37 °C.

Lentivirus construction and infection

The SOCS-7 gene was cloned into the lentiviral vector pENTR1A (Miaoling, Wuhan, China) and the overexpression vector pENTR1A-SOCS-7 was constructed. Transfection of the constructed plasmid into 293 T cells by Lipofectamine 3000° transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After 48 h, the culture lentiviral supernatant was collected, the virus titer was determined, and Huh-7 cells were infected with the virus to establish Huh-7 cells stably overexpressing SOCS-7. The following are the primer sequences for plasmid generation: 5'-ATGCAGGAGGCCGAGCTCCGG-3', reverse: 5'-SOCS-7 (forward: CTACGTGGAGGG TTCCACCTCTTGC-3'). Overexpression vectors used empty vectors as controls. Similarly, to silence SOCS-7 expression, shRNA expression vectors pGCSIL-GFP-shSOCS-7 (Genechem, Shanghai, China) will be constructed. It was also transfected into 293 T cells by Lipofectamine 3000° transfection reagent for stable expression. The virus infects Huh-7 cells, thereby establishing Huh-7 cells that stably silence SOCS-7, while silencing vectors used scrambled oligomer as controls. shSOCS-7 (5'-GACGUUUAUAGUAGAUAAG-3'). A scrambled oligomer (5'-UUCUUCGAACGUGUCACGUTT-3') was used as the control for shRNA experiments.

RNA extraction and qRT-PCR

Total RNA was extracted from Huh-7 cells or tumor tissues using TRIzol reagent, according to the manufacturer's instructions (Invitrogen). First strand cDNA synthesis was performed using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) and expression of *Shc1*, *SOCS-7*, and *β-actin* were evaluated using the following gene specific primers: Shc1 forward: 5'-TGGGATGAGGAG-GAGGAAGA-3', reverse: 5'-CAATGTAGCTCCCAAGTGGC-3'; SOCS-7 forward: 5'-TCAAATCCCCCCAGGTGGCTT-3', reverse 5'-GCTGGGCTTCCTTTAG AGAC-3'; TGF-β forward: 5'-GCCTGAGTGGCTGTCTTTG-3', reverse: 5'-CTGTATTCC GTCTCCT TGGTTC-3'; Veriscan forward: 5'-TCGTTTTGAGAACCAGCAGGAG-3'; reverse: 5'-CGTGAGTCGGCTTCCATTTGGAGCGATCAG-3'; Arginase-1 forward: 5'-CTGAGAGCTCCAAGC-CAAA GT-3', reverse: 5'-CGTACAGGTCCTT-3'; Gene expression was calculated as $2^{-\Delta\DeltaCCt}$ relative to β-actin.

Immunoprecipitation

For immunoprecipitation using magnetic beads, the Pierce[™] MS-Compatible Magnetic IP Kit (Pierce, Waltham, MA, USA) was used according to the manufacturer's protocol. Briefly, the cells were harvested and washed with ice-cold phosphate-buffered saline (PBS), and lysed directly in lysis buffer containing 25 mM Tris-HCI (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, and 5% glycerol with freshly added protease inhibitors (Sigma-Aldrich, St. Louis, MO, USA). After incubating for 30 min on ice, the lysates were spun at full-speed to remove cell debris and the clear supernatant was used with antibody-coupled magnetic beads for immunoprecipitation. After magnetic separation, the immunoprecipitated fraction was eluted and subjected to Western blot analysis.

Western blotting

To generate whole-cell lysates for analysis of protein expression, the cells were harvested and lysed in RIPA lysis buffer (Abcam, Cambridge, UK) for 30 min, on ice, by periodic vortexing. Cell lysates were centrifuged at 13,000 rpm for 10 min to separate cell debris. The supernatants were carefully transferred to a new tube and total protein estimation was performed using a BCA protein assay kit (Pierce). Twenty μg of total protein per sample was loaded onto a 10% sodium dodecyl sulfate (SDS)-acrylamide gel and subjected to electrophoresis. Separated proteins were then blotted onto a 0.45 µm nitrocellulose membrane (Abcam) and blocked in 5% defatted milk (in PBS or Tris-buffered saline) containing 0.1% Tween 20, for 2 h at room temperature. Membranes were then incubated with primary antibodies against SOCS-7 (1:200), Shc1 (1:500), STAT3 (1:500)

and p-STAT3 (1:500) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. After washing the membranes three times with PBS-T, they were incubated with the respective horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. The membranes were developed using a peroxidase substrate (Thermo Fisher Scientific, Waltham, MA, USA) and visualized by chemiluminescence. Quantification of protein expression was performed using ImageJ (National Institutes of Health, Bethesda, MD, USA) by comparing densities between the proteins of interest with that of β -actin, which was used as a loading control.

Immunohistochemistry

Liver tissue sections from HCC patients were processed by standard immunohistochemistry procedures [18] including deparaffinization, rehydration, and antigen retrieval. The sections were stained with primary antibody against SOCS-7 (1:100) for 2 h followed by HRP-conjugated secondary antibody for 1 h. Liquid DAB Plus Substrate Kit (Thermo Fisher Scientific) was used to visualize the proteins of interest. H-Score (H-SCORE = Σ (Pl × I) = (percentage of cells of weak intensity × 1) + (percentage of cells of moderate intensity × 2) + percentage of cells of strong intensity × 3)^{20,21} of the destination area of each image was quantified using Densito quant module in Quant Center 2.1 analysis software, the higher the value of H-score, the stronger the positive SOCS-7 intensity.

Protein half-life and ubiquitination assays

To determine the half-life of shc1, cycloheximide (100 µg/mL) (MedChem-Express, Monmouth, NJ, USA) was added to transfected cells and the cells were harvested after incubation for 0, 2, 4, 6 and 8 h. Harvested cells were lysed as described above and evaluated for Shc1 expression by western blotting. To evaluate the ubiquitination levels of proteins, transfected cells were treated with a proteasomal inhibitor (10 µM MG-132) (MedChemExpress) for 6 h prior to harvest. Harvested cells were analyzed for protein expression, as previously described.

TUNEL assay

Cell apoptosis was evaluated using a TUNEL assay kit (Sigma–Aldrich), according to the manufacturer's instructions. Briefly, paraffin-embedded tissue sections were fixed using 4% paraformaldehyde and counterstained with 4',6-diamidino-2-phenylindole (DAPI) for nuclear localization, without permeabilization. The cells were visualized by fluorescence microscopy and apoptotic cells were marked in both green (fragmented DNA) and blue (nuclear DNA).

Migration and scratch assays

Cell migration of Huh-7 cells was evaluated using a Transwell assay and scratch test. A total of 2.5×10^4 cells/well were seeded on 6-well plates overnight or starved on Transwell chambers (Corning, Corning, NY, USA) in serum-free medium. For the scratch assay, an even scratch wound was made using a disposable pipette, followed by washing with PBS. The cells were then incubated in supplemented medium and immediately imaged for the first time point (0 time point) and then subsequently imaged at 24 and 48 h. Cell migration was calculated by the scratch distance. For Transwell assays, Transwell chambers containing starved cells were incubated in wells containing media supplemented with bovine serum albumin (BSA) (10 g/L) as a chemoattractant for 0, 24, and 48 h at 37 °C/5% CO₂. The chambers were removed and migrated cells in the lower well were visualized after washing with PBS, fixing with methanol at 4 °C, and staining with 0.1% Crystal Violet. Images were taken from five different fields of view using a 200× objective.

In vivo HCC tumor mouse model

Six weeks old BALB/c nude mice (Shanghai Slac Laboratory Animal Co., China) housed under sterile pathogen-free conditions were used for the study. 1×10^{6} Huh-7 cells either stably expressing or not overexpressing SOCS-7 were subcutaneously xenografted into mice in the flank region, respectively, and normal Huh-7 cells were used as control. These mice were sacrificed 5 weeks after transfer, and the tumors were harvested. Tumor volume was evaluated using the formula: $\frac{1}{2}$ (length × width²). Tissue sections were prepared and stained with hematoxylin and eosin followed by Ki-67 (Abcam, 1:500) staining to assess cell proliferation. All animal experimentation was approved by Institutional Animal Care and Use Committee of the Fudan University, Shanghai, China.



Fig. 1 SOCS-7 expression is downregulated in hepatocellular carcinoma (HCC). A Representative western blot and densitometric quantification of HCC tissue samples and adjacent healthy control samples from 10 patients. B Relative expression of SOCS-7 mRNA in tumor tissues and adjacent tissues of 10 patients was detected by qRT-PCR. C Representative immunohistochemical (IHC) images of SOCS-7 expression in tumor tissues and adjacent tissues. D Relative IHC staining of SOCS-7 in paired tumor tissues and adjacent tissues samples are shown. Statistical significance was determined by student's *t* tests. Statistical significance was calculated by the Student's *t* test. *p < 0.05; **p < 0.01 and ***p < 0.001.

Statistical analysis

All data are expressed as the mean \pm standard deviation (SD). The Kaplan–Meier method was used to evaluate the survival of patients. Student's *t* test was used for sample comparison between two groups, and one-way analysis of variance (ANOVA) with Bonferroni's post-hoc test was used for sample comparison among multiple groups. All differences with p < 0.05 were considered statistically significant. All statistical analysis was performed using SPSS statistical software for Windows, version 21.0 (SPSS, Chicago, IL, USA).

RESULTS

Expression of SOCS-7 is downregulated in HCC

SOCS-7 is a regulator of various signaling pathways including the JAK/STAT pathway that controls cell proliferation¹². To analyze its role in HCC, tumor tissues along with adjacent healthy tissues were obtained from 10 patients, and SOCS-7 expression was evaluated by Western blotting. Compared to healthy control tissues, HCC samples were significantly lower in SOCS-7 protein expression (Fig. 1A). Similarly, the mRNA level of SOCS-7 was higher in adjacent tissues than in cancer tissues by qRT-PCR (Fig. 1B). However, as shown in Table 1, of the 77 patients studied, 36 exhibited low-SOCS-7 (H-score < 76.96), while 41 exhibited high-SOCS-7 (H-score ≥ 76.96). Data analysis showed that tumor differentiation was correlated with SOCS-7 expression (p =0.0365), but for other clinical data, such as age, gender, size, recurrence, death, cirrhosis, there was no correlation. Through the H-Score and histochemical analysis of SOCS-7 in adjacent tissues and tumor tissues (Figs. 1C, 1D), we found that the content of SOCS-7 in tumor tissues was significantly lower than that in adjacent tissues, which makes us think that SOCS-7 had a significant impact on the tumorigenesis of HCC. Taken together, these results indicated that SOCS-7 was downregulated in HCC and might play an important role in HCC pathogenesis.

Increased SOCS-7 expression can inhibit the growth of HCC in vitro

To further characterize the role of SOCS-7 in HCC, we quantitatively analyzed the mRNA expression of SOCS-7 in different hepatoma cell lines (Huh-7, HepG2). The Huh-7 cells showed a more significant reduction compared with normal human hepatocytes LO2 (Fig. 2A); therefore, Huh-7 cells were selected for subsequent experiments. We constructed vector-SOCS-7, an overexpression vector of SOCS-7, and shSOCS-7, a small interfering RNA that impedes the expression of SOCS-7. These plasmids were transfected into Huh-7 cells by liposome transfection. After 48 h, the protein level of SOCS-7 was detected by WB to evaluate the expression and effect of the vector plasmid, as shown in Fig. 2B. Next, we used siRNA knockdown and overexpression vectors in Huh-7 cells. The cells were transfected with the respective expression constructs; they were harvested and analyzed for cell apoptosis, proliferation, migration, and viability. MTT assay was used to detect the cell viability. After 72 h of infection, compared with the control group, the cell viability decreased when SOCS-7 was overexpressed, and increased significantly after knockout (Fig. 2C). The TUNEL assay revealed an increase in apoptosis upon SOCS-7 overexpression, when compared to siRNA depletion of SOCS-7 expression (Fig. 2D). The wound healing assay and Transwell migration assay showed that SOCS-7 overexpression reduced the proliferative and migratory capacities of cells, respectively, when compared to cells expressing shSOCS-7 after 24 h (Figs. 2E, F). In vitro experiments showed that SOCS-7 could inhibit the growth and migration ability of HCC cells.

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Table 1.	Correlation between SOCS-7 expression levels and
clinicopa	thological factors in HCC ($n = 77$).

Va	riable	Low SOCS-7 (36)	High SOCS-7 (41)	p value		
Gender						
	Male	29	36	0.5311		
	Female	7	5			
Age (years)						
	<50	11	20	0.1619		
	≥50	25	21			
Size (cm)						
	<5	14	20	0.5014		
	≥5	22	21			
Differentiation						
	Well	0	4	0.0365*		
	Moderate	29	24			
	Poor	6	13			
HBsAg						
	Positive	28	33	0.7699		
	Negative	8	8			
Cir	rhosis					
	Present	26	27	0.5472		
	Absent	10	14			
Recurrence						
	Yes	25	26	0.5767		
	No	11	15			
Death						
	Yes	19	22	0.9384		
	No	17	19			

Low-SOCS-7, H-score < 76.96; High-SOCS-7, H-score \geq 76.96. *p < 0.05. Chi-square test.

SOCS-7 inhibits tumor growth through immunoregulatory factors in vivo

To evaluate the relevance of SOCS-7 expression in tumor growth in vivo, we used six-week-old BALB/c nude mice and xenografted Huh-7 cells either overexpressing SOCS-7 or shSOCS-7 that reduced SOCS-7 expression. Five weeks later, tumors were harvested and characterized for weight and volume. As shown in Fig. 3A, B, SOCS-7 significantly reduced the tumor volume in mice, and as shown in Fig. 3C, its weight was also significantly decreased compared with the control group, while transfection of shSOCS-7 resulted in an increase in volume and weight. In order to examine cell proliferation in the tumor tissues, IHC staining of Ki67 was performed. As shown in Fig. 3D, up/down regulated SOCS-7 in these mouse tumors resulted in decreased/increased cell proliferation, as visualized by Ki67 staining. Next, we examined the protein and mRNA levels of immunosuppressive factors such as TGF-B, integrin-like molecule Versican, and Arginase-1 in tumor tissues (Figs. 3E, F). The protein and mRNA levels of TGF-β, Versican, and Arginase-1 were significantly decreased in tumor tissues from mice, xenografted with SOCS-7 overexpression cells when compared to controls. In the serum of mice overexpressing SOCS-7, the content of TGF-B was significantly decreased, whereas in the serum of mice knocking out SOCS-7, it was significantly increased (Fig. 3G). In addition, overexpression of SOCS-7 upregulated the production of INF-y, whereas knockdown of SOCS-7 significantly decreased INF-y (Fig. 3H). These results suggested that SOCS-7 may regulate tumor growth by modulating tumor immunity in vivo. Also, expression of programmed deathligand 1 (PD-L1) and beta-2- macroglobulin (B2M), which are critical factors expressed in response to inhibition or activation of immune responses, respectively, were also altered in response to SOCS-7 expression. PD-L1 was downregulated upon SOCS-7 overexpression, whereas it was upregulated in tissues from shSOCS-7-expressing mice (Fig. 3I). In contrast, B2M was upregulated upon SOCS-7 overexpression and downregulated upon knockdown (Fig. 3I). These results indicated a significant correlation between the expression of SOCS-7 and immunosuppressive factors that could evade host immune responses and promote tumorigenesis.

SOCS-7 targets Shc1 degradation by ubiquitination

The SOCS family of proteins is known to promote ubiquitination and degradation of targets²². To further characterize the mechanism behind the regulation of immunosuppression by SOCS-7, we used UbiBROWSER, a specific ubiquitin ligase target prediction software²³, and identified Shc1 as a downstream target of SOCS-7. To functionally verify this relationship, we evaluated the expression of Shc1 isoforms upon overexpression or knockdown of SOCS-7 in Huh-7 cells. Western blot analysis revealed a reduction in Shc1 isoforms p46 and p66 upon SOCS-7 overexpression, and an increase upon knockdown (Fig. 4A). In contrast, qPCR analysis revealed no significant differences in mRNA levels of shc1 expression (Fig. 4B). We next evaluated ubiguitination levels of Shc1 using an anti-ubiguitin antibody. Shc1 was highly ubiguitinated upon SOCS-7 overexpression and reduced upon its knockdown (Fig. 4C). We further analyzed Shc1 protein half-life by treating Huh-7 cells with cycloheximide for 0, 2, 4, 6 h. A gradual decrease in Shc1 protein levels was observed in Huh-7 cells from 0 to 6 h; however, compared with the control group, cells that overexpressed SOCS-7 decreased more rapidly, while cells that interfered with SOCS-7 decreased more slowly (Fig. 4D). Addition of the proteasomal inhibitor, MG-132, prevented degradation of Shc1 in the presence of SOCS-7 (Fig. 4E). Taken together, these results showed that in the cell line of HCC, Shc1 was ubiquitinated and targeted degradation by the E3 ubiquitin ligase, SOCS-7.

SOCS-7 inhibits tumorigenesis in HCC via regulation of Shc1 in vivo

To further verify whether the regulation of SOCS-7 on tumorigenesis is regulated by Shc1 in vivo, we xenografted Huh-7 cells transfected with shSOCS-7, sh-Shc1 and shSOCS-7+sh-Shc1 into 6-week-old mice to observe the effect of Shc1 on tumor growth in mice when interfering with or overexpressing SOCS-7. As shown in Fig. 5A-C, after interfering with the expression of SOCS-7, inhibiting the expression of Shc1 at the same time has a more significant effect on the growth of tumors in mice, while overexpressing Shc1 could promote the growth of tumors. In the mouse model, co-expressing SOCS-7 and Shc1, the growth of tumors also showed a slow trend due to the inhibition of SOCS-7 on Shc1. Similarly, the tumor immune microenvironment of mice in each group was also examined by tumor immunosuppressive factors TGF-β, Veriscan, Arginase-1 detection, as shown in Figs. 5D, E, we found that Shc1 inhibits the immunosuppress regulation of SOCS-7, which indicates that Shc1 participates in the regulation of tumor immune evasion by SOCS-7. Through intra-tissue western blot, we detected that SOCS-7 had a significant inhibitory effect on the phosphorylation modification of STAT3 in tumor tissues, as shown in Fig. 5F. Previous studies found that Shc1 promotes the phosphorylation of STAT3¹⁹, which indicated that SOCS-7 can regulate the phosphorylation modification of STAT3 by regulating Shc1. Overall, we have demonstrated experimentally that in hepatoma cells, SOCS-7 modifies Shc1 by ubiquitination, affecting the phosphorylation modification of intracellular STAT3, thereby regulating tumor immunosuppressive factors in the tumor microenvironment, and inhibiting tumorigenesis.



Fig. 2 Upregulated SOCS-7 inhibits hepatocellular carcinoma (HCC) growth in vitro. A Relative expression of *SOCS-7* mRNA was determined by qRT-PCR in different human HCC cell lines. **B** The constructed overexpression vector and empty vector, scrambled oligomer and small interfering RNA were transfected into Huh-7 cells. Cells were collected after 48 h and the protein level of SOCS 7 was detected by WB. **C** Cell viability measured by the MTT assay in Huh-7 cells transfected with SOCS-7 or shSOCS-7. Shown are the means \pm standard deviation (SD) from at least three independent experiments. **D** Cell apoptosis measured by the TUNEL assay. Huh-7 cells expressing a control vector, SOCS-7 overexpression vector, or shSOCS-7 were stained for fragmented (TUNEL) and nuclear DNA (4',6-diamidino-2-phenylindole) 48 h posttransfection. Cells undergoing apoptosis were double positive for both fragmented (green) and nuclear (blue) DNA signals. Representative micrographs are shown. **E** Cell migration using the scratch assay. Scratch area closure of Huh-7 cells expressing the respective lentiviral vectors were evaluated 0, 24, and 48 h post-transfection. Representative images were taken from five different fields using a 200× objective. **F** Cell invasion measured by the Transwell migration assay. Huh-7 cells were seeded on the upper chamber of a Transwell chamber and invasion was measured 48 h after transfection with the respective lentiviral construct, in the lower chamber. Representative images were taken from five different fields using a 200× objective. Statistical significance was calculated using the unpaired Student's *t* test. **p* < 0.05; ***p* < 0.01.

DISCUSSION

Regulation of cytokine-mediated signaling pathways and their contribution to the pathogenesis of HCC has not been clearly elucidated. Important pathways triggered during tumorigenesis, such as growth factor and interferon signaling, are regulated by the SOCS family of proteins. They are capable of interacting with each other to target proteins proteasomal degradation²⁴. In the context of HCC,

SOCS-1 and SOCS-3 have been shown to negatively regulate Janus kinase (JAK)/STAT signaling and suppressing tumorigenesis^{25,26}. In this study, we highlighted the clinical significance of SOCS-7 in HCC pathogenesis. We identified Shc1 as a downstream target of SOCS-7, which was ubiquitinated and targeted for degradation.

Constitutive activation of the JAK/STAT signaling pathway leads to aberrant cell growth and proliferation, as observed in several



Fig. 3 Overexpression of SOCS-7 inhibits the growth of HCC in vivo. A–**C** In vivo analysis of tumor growth. 1×10^{6} Huh-7 cells stably expressing control, SOCS-7 overexpression, or siRNA vectors were xenografted into mice, and tumor weight/volume were evaluated 5 weeks post-injection. **B** Evaluation of tumor weight. **A**, **C** Tumor volume calculated using the formula: $\frac{1}{2}$ (length × width2) and representative images of the harvested tumors are shown in comparison (n = 5 mice/group). **D** The representative images of H&E stain and IHC of Ki67 of tumor tissues are shown. **E** The expression of immunosuppressive factors TGF- β , Versican and Arginase-1 in tumor tissues was detected by WB. **F** Expression of immunosuppressive factors, TGF- β , Versican, and Arginase-1 in tumor tissues, quantified by qRT-PCR. **G** TGF- β was measured in mouse serum by the ELISA method (**H**) Detection of INF- γ released in the fluid of tumor tissues, quantified by ELISA. **I** B2M and PD-L1 protein expressions evaluated by western blots of tumor tissues. Representative western blot images and corresponding densitometric quantitation are shown. Data are represented as the means ± SD. Statistical significance was calculated using the unpaired Student's *t* test. *p < 0.05; **p < 0.01; ***p < 0.001.

leukemias and in vitro in transformed cell lines^{27,28}. SOCS-1 and SOCS-3 have been previously identified as negative regulators of this pathway. In our analysis from patient samples, we identified SOCS-7 to be significantly downregulated in HCC tissue samples. This downregulation in expression can be associated with epigenetic silencing, which includes methylation of CpG islands, as seen for SOCS-1, instability of mRNA, and/or protein expression²⁵. Aberrant cell proliferation and migration, along with increased tumor growth seen upon reduction of SOCS-7, can be explained by constitutive activation of the JAK/STAT signaling pathway.

The consequence of reduced SOCS-7 expression extends from promoting cell growth to favoring immunosuppressive pathways, including interferon signaling for tumor growth²⁹. Reduction in SOCS-7 levels promoted expression of immunosuppressive and tumor-inducing factors such as TGF- β , Versican, and Arginase-1. The role of TGF- β in promoting tumor invasion is well-described and in the context of HCC; it acts as a master regulator of immune activation and tolerance. In T cells, it upregulates expression of PD-1, and in association with its ligand PD-L1 can trigger immunosuppressive signals. A large genomic study on HCC tissues also revealed an increase in expression of PD-1, PD-L1, and other immunosuppressive markers³⁰. We also observed a decrease in PD-L1 expression upon SOCS-7 overexpression in tumor tissues. Additionally, transactivation of Versican, a proteoglycan, is also associated with increased tumor invasion and promoted progression of $HCC^{31,32}$.

Through in silico analysis of downstream ubiquitin E3 ligase targets, we identified Shc1 as a target of SOCS-7. Shc1 expression was regulated at the protein level but not at the mRNA level, by selective ubiquitination by SOCS-7. Although SOCS-7 is known to directly affect STAT3 activation by sequestering STAT3 in the cytoplasm to prevent its translocation into the nucleus³³. We observed that overexpression of Shc1, along with SOCS-7 knockdown, was capable of restoring tumor growth, indicating that the mechanism was regulated via Shc1. In previous experiments, we also found that the longest proapoptotic isomer (Shcp66) preferentially phosphorylated STAT3 at serine 727 position to promote tumorigenesis in HCC¹⁹. It also promotes autophagy in lung adenocarcinoma cells, and in B cells by affecting mitochondrial integrity^{15,34}. Hence, in addition to its effect on STAT3 activation, Shc1 can also induce autophagy in HCC to balance cellular stress³⁵. Therefore, Shc1 can reduce the progress of HCC, by affecting tumor progression through several mechanisms.

In summary, we identified a novel role for SOCS-7 in HCC pathogenesis, which reduces STAT3 activation through ubiquitination modification of Shc1, enhances the sensitivity of HCC cells

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Fig. 4 SOCS-7 degrades Shc1 by ubiquitination. A After 48 h of transfection, the cells were collected and the protein level of shc1 was detected by WB. **B** The mRNA levels of shc1 in different samples were detected by qRT-PCR. Shown are the means ± SD. **C** Representative western blot images of Shc1 immunoprecipitated and analyzed by anti-ubiquitin antibody. **D** SOCS-7, or shSOCS-7-expressing Huh-7 cells were treated with cycloheximide post-transfection for 0 h, 2 h, 4 h, 6 h, and Shc-1 expression was evaluated by western blotting. **E** At 42 h after transfection, Huh-7 cells were treated with MG132 for 6 h, and the expression of SOCS-7 and Shc1 was detected by WB. Representative western blot images are shown. Data are represented as the means ± SD.



Fig. 5 SOCS-7 mediated downregulation of immunosuppressive factors via Shc1. A Tumor weight evaluated from mice from different groups: Huh-7 cells expressing sh-Shc1, shSOCS-7, shSOCS-7+sh-Shc1, or control. **B** Quantification of tumor volume. **C** Representative images of mouse tumors evaluated 5 weeks after xenograft of Huh-7 cells. **D**, **E** The protein and mRNA expression of immunosuppressive factors, TGF- β , Versican, and Arginase-1 by qRT-PCR in cells which overexpressing Shc1 or sh-Shc1. **F** Western blot images of lysates from tissues indicating expressions of STAT3 and phosphor-STAT3. Representative western blot images are shown. Data are represented as the means \pm SD. Statistical significance was calculated using the unpaired Student's *t* test. **p* < 0.05; ***p* < 0.001; ****p* < 0.001.

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to NK cell-mediated immune attack, thereby inhibiting tumorigenesis. This newly identified mechanism identified a possible immunotherapeutic target by utilizing SOCS-7 and/or Shc1 to manipulate the balance established between favoring cell growth and promoting immunosuppression in HCC.

DATA AVAILABILITY

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

P.H. participated in the design of the study, animal model construction and the tissue collection, cultured cells and drafted the manuscript. Z.Z. and Y.C. participated in cultured cells and performed the data analysis. B.Y. assisted with the animal model construction and drafted the manuscript. J.X. conceived and participated in the design of the study, and final approval of the version to be submitted. All authors read and approved the final paper.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All animal experimentation was approved by Institutional Animal Care and Use Committee of the Fudan University, Shanghai, China. All experiments were conducted according to guidelines approved by the Zhongshan Hospital affiliated with Fudan University.

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