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HNRNPC downregulation inhibits IL-6/STAT3-mediated HCC metastasis by decreasing HIF1A expression

Danfei Liu[1] | Xiangyuan Luo[1] | Meng Xie[1] | Tongyue Zhang[1] | Xiaoping Chen[2] | Bixiang Zhang[2] | Mengyu Sun[1] | Yijun Wang[1] | Yangyang Feng[1] | Xiaoyu Ji[1] | Yiwei Li[3] | Bifeng Liu[3] | Wenjie Huang[2] | Limin Xia[1]

[1]Department of Gastroenterology, Hubei Key Laboratory of Hepato-Pancreato-Biliary Diseases, Institute of Liver and Gastrointestinal Diseases, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei Province, China

[2]Hubei Key Laboratory of Hepato-Pancreato-Biliary Diseases, Hepatic Surgery Center, Clinical Medicine Research Center for Hepatic Surgery of Hubei Province, Key Laboratory of Organ Transplantation, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Ministry of Education and Ministry of Public Health, Wuhan, Hubei, China

[3]The Key Laboratory for Biomedical Photonics of MOE at Wuhan National Laboratory for Optoelectronics-Hubei Bioinformatics and Molecular Imaging Key Laboratory, Systems Biology Theme, Department of Biomedical Engineering, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, China

Correspondence
Limin Xia and Wenjie Huang, Department of Gastroenterology, Institute of Liver and Gastrointestinal Diseases, Hubei Key Laboratory of Hepato-Pancreato-Biliary Diseases, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China.
Email: xialimin@tjh.tjmu.edu.cn and huangwenjie@tjh.tjmu.edu.cn

Abstract

RNA-binding protein (RBP) dysregulation is functionally linked to several human diseases, including neurological disorders, cardiovascular disease, and cancer. Heterogeneous nuclear ribonucleoproteins (hnRNPs) are a diverse family of RBPs involved in nucleic acid metabolism. A growing body of studies has shown that the dysregulated hnRNPs play important roles in tumorigenesis. Here, we found that heterogeneous nuclear ribonucleoprotein C (C1/C2) (HNRNPC) had good performance in distinguishing between hepatocellular carcinoma (HCC) and normal liver tissues through bioinformatics analysis. Further investigation revealed that HNRNPC was significantly correlated with multiple malignant characteristics of HCC, including tumor size, microvascular invasion, tumor differentiation, and TNM stage. Patients with HCC with positive HNRNPC expression exhibited decreased overall survival and increased recurrence rate. HNRNPC downregulation inhibited HCC invasion and metastasis. The decreased expression of hypoxia inducible factor 1 subunit alpha (HIF1A) was identified as the molecular mechanism underlying HNRNPC downregulation-inhibited HCC metastasis by RNA sequencing. Mechanistically, HNRNPC downregulation decreased HIF1A expression by destabilizing HIF1A mRNA. HIF1A overexpression rescued the decrease in invasiveness and metastasis of HCC induced by HNRNPC downregulation. Additionally, interleukin (IL)-6/STAT3 signaling upregulated HNRNPC expression in HCC cells, and knockdown of HNRNPC significantly inhibited IL-6/STAT3-enhanced HCC metastasis. Furthermore, anti-IL-6 antibody siltuximab significantly inhibited IL-6-mediated HCC metastasis. In summary, our research revealed the clinical value, functional role, and molecular mechanism of HNRNPC in HCC and showed the potential of HNRNPC as a biomarker for diagnosis, prognosis, and further therapeutic targets for HCC.

Abbreviations: ARE, AU-rich element; HCC, hepatocellular carcinoma; HIF1A, hypoxia inducible factor 1 subunit alpha; HNRNPC, heterogeneous nuclear ribonucleoprotein C; hnRNPs, heterogeneous nuclear ribonucleoproteins; IHC, immunohistochemistry; IL-6, interleukin 6; RBP, RNA-binding protein; STAT3, signal transducer and activator of transcription 3; STAT3C, constitutively active STAT3.

Danfei Liu, Xiangyuan Luo, and Meng Xie contributed equally to this work.

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KEYWORDS
heterogeneous nuclear ribonucleoprotein C, hypoxia inducible factor 1 subunit alpha, interleukin 6, signal transducer and activator of transcription 3, siltuximab

1 | INTRODUCTION

Hepatocellular carcinoma is the sixth leading cause of cancer, and its mortality rate ranks third among cancer-related deaths.[1] With advances in surgery and chemotherapy, the overall survival of patients with HCC has been greatly improved. However, this survival benefit only occurs +in patients without metastasis.[2] The treatment effect and prognosis of patients with metastatic HCC remain poor. Therefore, there is a sense of urgency to further investigate the mechanism of HCC metastasis and identify metastasis-related molecules for HCC, which may provide potential therapeutic targets.

RNA-binding proteins are key regulatory components for cellular homeostasis by controlling RNA abundances and functions at the post-transcriptional level.[3] RBP dysregulation is related to several human diseases, including cardiovascular disease,[4] neurological disorder,[5] and cancer.[6] Numerous RBPs have been identified as dysregulated and associated with poor prognosis from the analyses of 1225 clinical HCC samples,[7] indicating their essentialness for HCC biology and severity. HNRNPC, an RNA-binding protein belonging to the hnRNP family, controls multiple aspects of RNA metabolism, including alternative splicing,[8] mRNA stabilization,[9] and translation.[10] Emerging evidence suggests that HNRNPC plays an important part in various types of cancer. Silencing of HNRNPC inhibits the metastatic potential of glioblastoma by increasing PDCD4 protein stability.[11] Repression of HNRNPC arrests tumorigenesis in breast cancer by activating a type I interferon response.[12] Although the involvement of HNRNPC in HCC proliferation and migration has been reported,[13] little information is known about the underlying mechanism. Further studies are needed to determine the molecular mechanism underlying the function of HNRNPC in HCC.

HIF1A is one driver of tumor progression by controlling cell proliferation, metabolism, angiogenesis, and invasion.[14] The expression of HIF1A is regulated through synthesis and degradation at translational or post-translational levels. Beyond that, transcription factors, RBPs, and microRNAs are involved in the regulation of HIF1A expression at transcriptional and post-transcriptional levels.[15] The regulation of HIF1A mRNA stability is one of the mechanisms influencing HIF1A expression due to the presence of multiple AREs in the 3′UTR of HIF1A mRNA. For example, zinc-finger RBP ZFP36L1 downregulates HIF1A expression by decreasing HIF1A mRNA stability in bladder cancer,[16] whereas latent membrane protein 1 (LMP1) upregulates HIF1A expression by enhancing HIF1A mRNA stability in nasopharyngeal carcinoma cells.[17]

Chronic inflammation in the tumor microenvironment is a driving force for cancer development.[18] Notably, HCC arises almost exclusively in the chronic inflammatory environment.[19] IL-6, a multifunctional inflammatory cytokine, functions by binding to its specific receptor IL-6R. The resultant IL-6/IL-6R complex induces the signal transducing receptor glycoprotein 130 (GP130) homodimerization, resulting in the activation of several signaling pathways, including the MAPK, the PI3K, and, most prominently, the STAT3 pathway.[20] Aberrant hyperactivation of IL-6/STAT3 signaling is frequently observed during tumor progression, and this hyperactivation is closely related to poor prognosis.[21,22] In HCC, IL-6/STAT3 signaling promotes HCC development by controlling cell proliferation, angiogenesis, invasiveness, and stemness.[23] In recent years, IL-6-targeted approaches have been approved for the treatment of multiple diseases, such as rheumatic diseases, Castleman disease, and cytokine release syndrome.[24] Currently, targeting IL-6 as a promising therapeutic strategy is being extended to cancer.[25] However, the effect of targeting IL-6 in HCC treatment is still unclear.

In this study, we demonstrated that HNRNPC expression was correlated with malignant characteristics and poor prognosis of HCC. HNRNPC downregulation inhibited IL-6/STAT3-mediated HCC metastasis by decreasing HIF1A expression. Siltuximab, the anti-IL-6 antibody, significantly inhibited IL-6-induced HCC metastasis.

2 | MATERIALS AND METHODS

2.1 | Patients and follow-up

This study was approved by the Ethics Committee of Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology. All HCC samples were collected on condition of informed consent. This study enrolled 286 and 180 adult patients with HCC who underwent surgical resection at the Tongji Hospital of Tongji Medical College between 2003 and 2005 and between 2006 and 2008, respectively. The clinicopathologic characteristics have been shown in Table 1. Patients underwent a regular check-up every 2–3 months in the first 2 years and every 3–6 months thereafter. The examinations of serum alpha-fetoprotein (AFP) level and abdominal ultrasonography were used for the evaluation of tumor recurrence. Computed tomography and/or MRI examinations were performed every 3–6 months, along with chest radiographic examination. Follow-up data were summarized at the ends of December 2013 (Cohort I) and December 2016 (Cohort II). The primary endpoints were the overall survival and probability of recurrence. The period from surgery to recurrence was defined as the recurrence time. The period between surgery and death or last follow-up was defined as the overall survival time.

In addition, 15 normal liver tissues were obtained from individuals with accidental death. In total, 75 with HCC who had undergone surgical resection at the Tongji Hospital of Tongji Medical College

TABLE 1 Correlation between HNRNPC expression and clinicopathological characteristics in two independent cohorts of human HCC tissues

Clinicopathological variables	Cohort I: Tumor HNRNPC expression Negative (*n* = 134)	Cohort I: Tumor HNRNPC expression Positive (*n* = 152)	Cohort I *p*-value	Cohort II: Tumor HNRNPC expression Negative (*n* = 81)	Cohort II: Tumor HNRNPC expression Positive (*n* = 99)	Cohort II *p*-value
Age	51.33 (9.00)	53.72 (8.629)	0.516	52.22 (11.08)	50.16 (9.656)	0.381
Sex						
Female	18	29	0.206	15	17	0.846
Male	116	123		66	82	
Serum AFP						
≤20 ng/ml	48	47	0.45	21	21	0.483
>20 ng/ml	86	105		60	78	
Virus infection						
HBV	99	105	0.268	61	72	0.379
HCV	9	20		10	7	
HBV + HCV	6	9		2	5	
None	20	18		8	15	
Cirrhosis						
Absent	41	56	0.317	23	28	1.000
Present	93	96		58	71	
Child–Pugh score						
Class A	112	139	0.048*	65	72	0.293
Class B	22	13		16	27	
Tumor number						
Single	74	65	0.044*	40	35	0.069
Multiple	60	87		41	64	
Maximal tumor size						
≤5 cm	88	80	0.030*	45	35	0.010*
>5 cm	46	72		36	64	
Tumor encapsulation						
Absent	35	52	0.157	25	57	0.001*
Present	99	100		56	42	
Microvascular invasion						
Absent	84	74	0.023*	61	40	<0.001*
Present	50	78		20	59	
Tumor differentiation						
I–II	101	93	0.011*	61	55	0.008*
III–IV	33	59		20	44	
TNM stage						
I–II	113	99	<0.001*	75	68	<0.001*
III	21	53		6	31	

*$p<0.05$.

between 2017 and 2019 were divided into the following four groups according to the presence or absence of metastasis and recurrence records at the time of initial diagnosis: (1) "no-metastasis" group; (2) "metastasis" group; (3) "no-recurrence" group; and (4) "recurrence" group.

2.2 | RNA sequencing

RNA library preparation, high-throughput sequencing, and data analysis were conducted by Jiayin Biotechnology Co., Ltd. Briefly, total RNA was extracted from triplicate of MHCC97H-shcontrol and

MHCC97H-shHNRNPC cells using the TRIzol reagent (Invitrogen). Here, 3 μg of RNA per sample were used as input material. RNA-seq libraries were prepared using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB) and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina). After cluster generation, the library preparations were sequenced using the Illumina Novaseq 6000 platform, and 150-bp paired-end reads were generated. The differentially expressed genes were filtered using the DEseq2 algorithm under the criteria ($p<0.05$ and a fold change >2). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted to determine the biological implications of these differentially expressed genes. RNA-seq data were deposited in the Gene Expression Omnibus depository (GSE180789).

2.3 | Plasmid construction

Plasmids were constructed following standard procedures described in our previous study.[26] The *HNRNPC* promoter sequence (−1984/+158) was obtained from human genomic DNA using PCR. This sequence is located at the position of the transcriptional start site (−1984 to +158) in the 5′-flanking region of the human *HNRNPC* gene. The vector was constructed by incorporating forward and reverse primers at the 5′- and 3′-ends of the *Kpn*I and *Xho*I sites, respectively. The PCR product was cloned into the *Kpn*I and *Xho*I sites of the pGL3-basic vector (Promega). The 5′-flanking deletion constructs of *HNRNPC* promoter, (−1898/+158) HNRNPC, (−1395/+158) HNRNPC, (−631/+158) HNRNPC, (−263/+158) HNRNPC, (−135/+158) HNRNPC, (−24/+158) HNRNPC, were similarly generated using the (−1984/+158) HNRNPC construct as the template. The STAT3 binding sites on the *HNRNPC* promoter were mutated using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene). The constructs were confirmed by DNA sequencing. Similarly, for the *HIF1A* 3′UTR truncation constructs, (2800–2962)-WT1, (2971–3133)-WT2, and (3345–3534)-WT3 were generated with forward and reverse primers incorporating *Bam*HI and *Xho*I sites at the 5′- and 3′-ends, respectively. The primers are listed in Table S3.

2.4 | ChIP assay

In total, 1×10^7 cells were cross-linked with 1% formaldehyde at room temperature for 10 min and quenched by the addition of glycine for 10 min. Next, cells were lysed in 300 μl of ChIP lysis buffer (1 mM PMSF, 1% SDS, 10 mM EDTA, and 50 mM Tris pH 8.1). DNA fragments were prepared by sonication. The recovered supernatant was then incubated for 2 h with ChIP grade antibody anti-STAT3 or isotype control IgG in the presence of protein G-Sepharose beads and herring sperm DNA (Sigma-Aldrich), followed by antibody denaturation with 1% SDS in lysis buffer. Precipitated DNA was extracted from the beads by immersing in 1.1 M $NaHCO_3$ solution and 1% SDS solution at 65°C for 6 h. The DNA was then purified using a PCR Purification Kit (Qiagen).

HCC and normal liver tissues ($n=6$) were first cut into small pieces (≤1 mm^3). Then, DNase I (20 mg/ml; Sigma-Aldrich) and collagenase (1.5 mg/ml; Sigma-Aldrich) were added for digestion. The 1× red cell lysis solution was used for red blood cells; 1% formaldehyde was used to crosslink dissociated cells for 10 min. Sonication was used to fragment DNA from cell lysate. The fragmented DNA was immunoprecipitated when incubating with ChIP grade antibody anti-STAT3 or isotype control IgG at 4°C. Then, specific primers were designed (provided in Table S3) and RT-PCR was performed for amplification of the corresponding binding fragments on the promoters.

2.5 | Statistical analysis

Data were presented as mean ± SD and were analyzed using Student's *t*-test for quantitative variables or Fisher's exact test for categorical variables using SPSS21.0 software. A *p*-value <0.05 was considered as statistically significant.

Additional materials and methods are provided in Appendix S1.

3 | RESULTS

3.1 | Identification of *HNRNPC* as an important gene closely associated with HCC progression

To screen key HCC-related RBPs, we intersected 474 RBPs predicting HCC identified from 1225 clinical HCC samples[7] with 5985 prognostic genes in HCC from The Cancer Genome Atlas (TCGA) Clinical Data Resource (TCGA-CDR)[27] (Appendix S1), yielding 221 overlapping genes (Figure 1A). The hnRNPs are a large family of RBPs with crucial roles in cancer and metastasis,[28,29] and seven members were present in these overlapping genes, including HNRNPA0, HNRNPA2B1, HNRNPC, HNRNPR, PCBP2, PTBP1, and SYNCRIP (Figure 1A). With the best capacity in differentiating between normal liver and HCC tissues shown by receiver operating characteristic (ROC) curves, HNRNPC was selected for further study (Figure 1B). In addition, HNRNPC was significantly associated with poor prognosis, high tumor grade, and vascular invasion of patients with HCC (Figure 1C–E). Given the lack of metastatic samples in the public database, we next detected HNRNPC expression in 15 normal liver samples and 70 HCC samples and their paired nontumor tissues. Recurrent or metastatic patients exhibited higher HNRNPC levels compared with nonrecurrent or nonmetastatic ones (Figure 1F). Additionally, immunohistochemical (IHC) and qPCR analyses revealed that HNRNPC expression was the highest in metastatic HCC tissues, followed by primary HCC tissues, and finally adjacent nontumor tissues (Figure 1G). We further detected HNRNPC expression in two HCC cohorts (Figure 1H) and found that patients who were HNRNPC positive exhibited decreased overall survival and

increased recurrence rates (Figure 1I). The high HNRNPC expression in HCC tissues was positively correlated with increased tumor size, microvascular invasion, poor tumor differentiation, and advanced TNM stage (Table 1). Univariate and multivariate analyses determined HNRNPC as an independent prognostic factor for overall survival and recurrence rates (Tables S1 and S2). Taken together, HNRNPC was a potential biomarker for HCC.

3.2 | HNRNPC downregulation inhibits invasiveness and metastasis of HCC

Expression profiles of HNRNPC on human HCC cell lines showed that the levels of HNRNPC was the highest in highly metastatic MHCC97H cells (Figure 2A and Figure S1A). MHCC97H cells were chosen to investigate the effect of HNRNPC downregulation on HCC cell migration and invasion. Given the potential off-target effect, three different shRNAs targeting HNRNPC were transfected into MHCC97H cells (Figure 2B). All of them significantly inhibited the migration and invasion of MHCC97H cells (Figure 2C). Furthermore, we investigate the effects of knockdown of HNRNPC in MHCC97H cells on the proliferation, survival, and apoptosis in vitro by colony formation assays, CCK-8 assays, and flow cytometry. The results showed that HNRNPC knockdown significantly inhibited the proliferation and survival of MHCC97H cells (Figure S1B,C), and significantly promoted the apoptosis of MHCC97H cells (Figure S1D). We selected MHCC97H cells transfected with shHNRNPC-3 lentivirus for the further in vivo experiments. An HCC orthotopic implantation model was constructed by transplanting MHCC97H-shHNRNPC cells into the livers of nude mice. We found that HNRNPC downregulation slowed tumor growth, decreased lung metastasis, and prolonged overall survival time (Figure 2D–I).

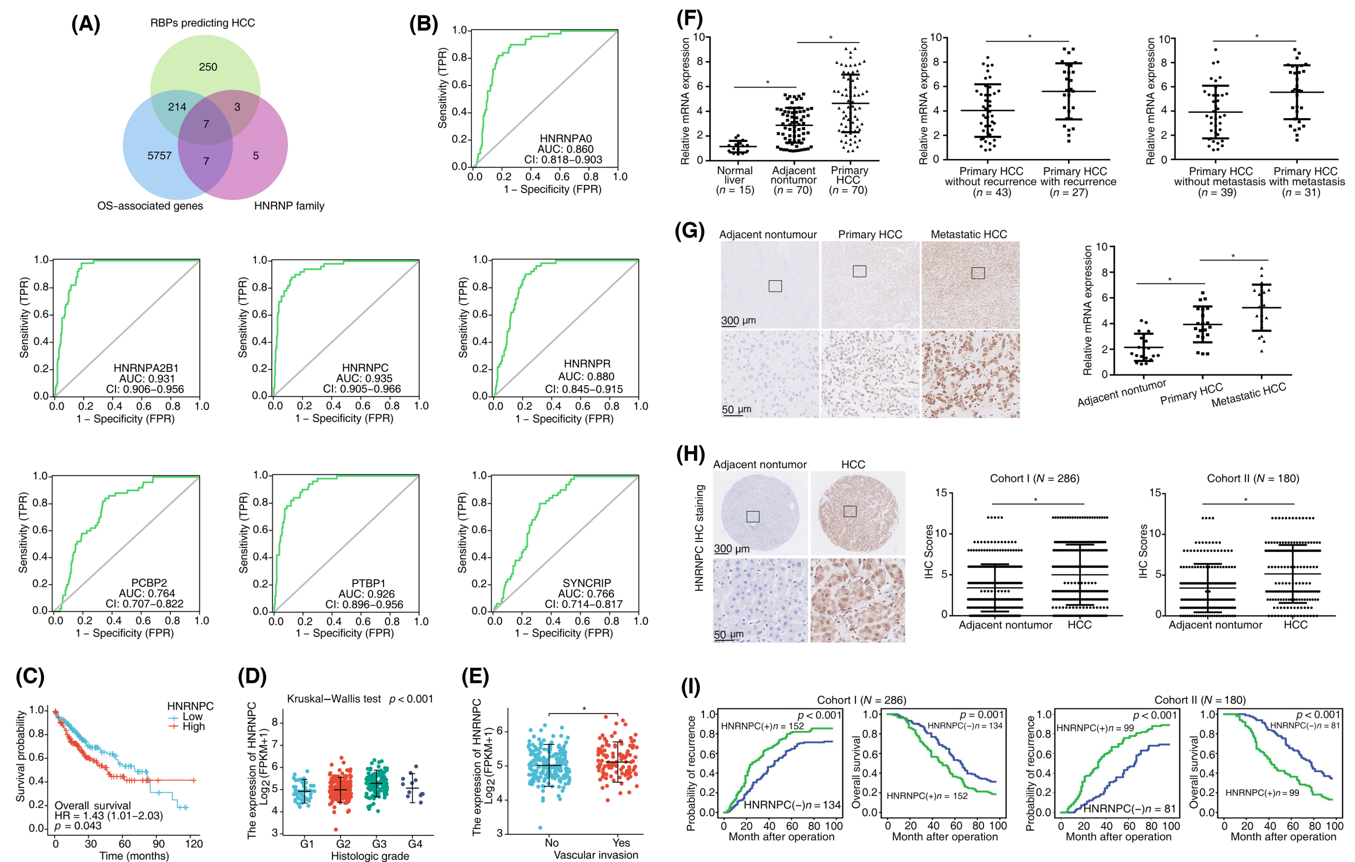

FIGURE 1 Identification of heterogeneous nuclear ribonucleoprotein C (HNRNPC) as an important gene closely associated with HCC progression. (A) Venn diagram showing the candidate HNRNP members by intersecting 474 RBPs predicting HCC with 5985 genes that are significantly associated with overall survival of patients with HCC. (B) ROC curves of seven candidate HNRNP members showed their capacities in differentiating between normal and HCC tissues. (C–E) The association of HNRNPC expression with overall survival, histologic grade, and vascular invasion in patients with HCC was analyzed from TCGA Clinical Data Resource. (F) Relative mRNA levels of HNRNPC in 15 normal liver tissues and 70 paired HCC tissues and adjacent nontumor tissues (left), in HCC samples from patients with HCC with recurrence ($n = 27$) or without recurrence ($n = 43$) (middle), and in HCC samples from patients with HCC with metastasis ($n = 31$) or without metastasis ($n = 39$) (right). (G) IHC and qPCR for HNRNPC expression in adjacent nontumor tissues, primary HCC tissues, and metastatic HCC tissues ($n = 20$). (H) Left: representative IHC staining for HNRNPC in tissue microarrays. Right: IHC scores of HNRNPC in two HCC cohorts. (I) The prognostic value of HNRNPC was analyzed in two HCC cohorts by Kaplan–Meier tests.

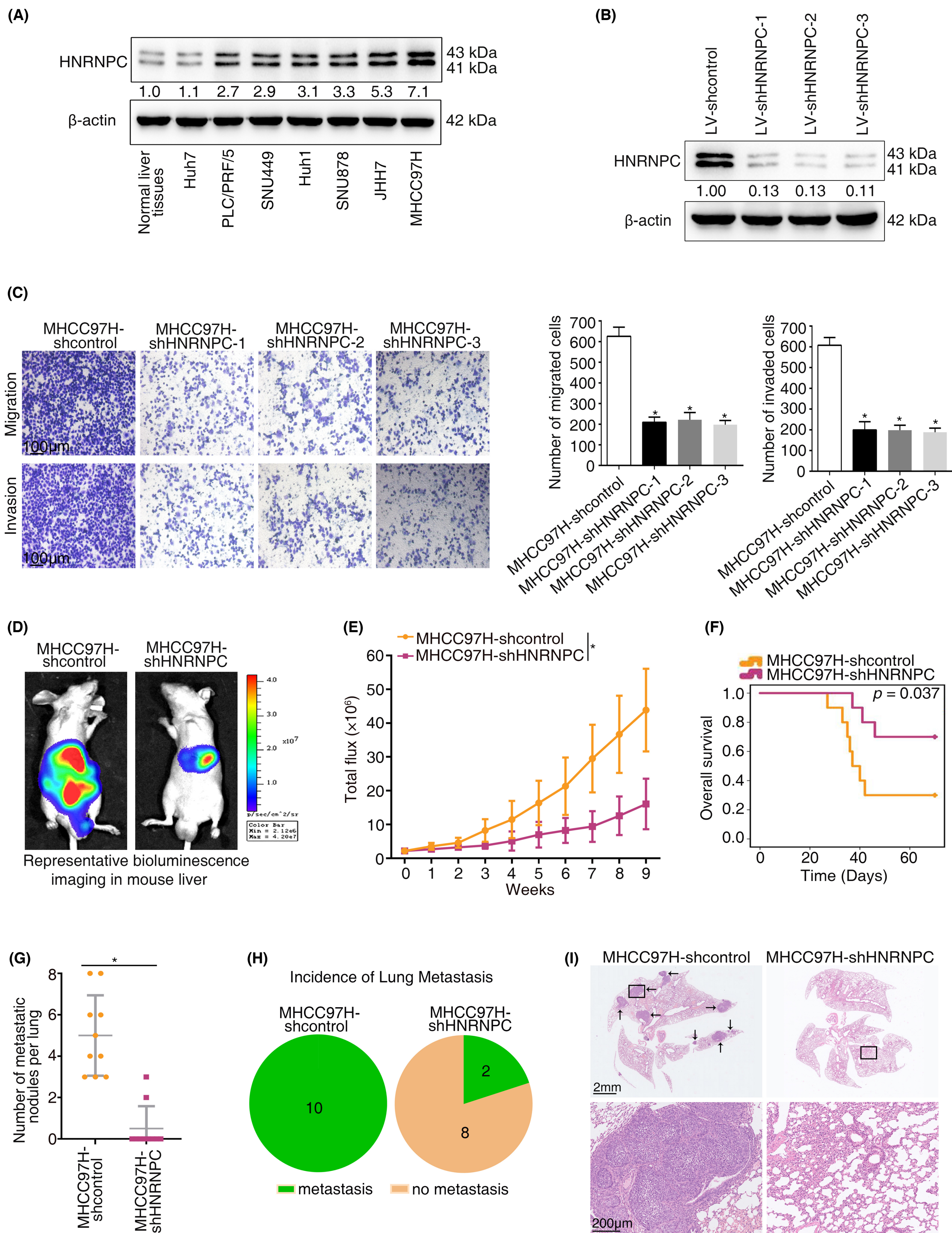
(A)
HNRNPC
43 kDa
41 kDa
1.0 1.1 2.7 2.9 3.1 3.3 5.3 7.1
β-actin
42 kDa
Normal liver tissues
Huh7
PLC/PRF/5
SNU449
Huh1
SNU878
JHH7
MHCC97H
(B)
LV-shcontrol
LV-shHNRNPC-1
LV-shHNRNPC-2
LV-shHNRNPC-3
HNRNPC
43 kDa
41 kDa
1.00 0.13 0.13 0.11
β-actin
42 kDa
(C)
MHCC97H-shcontrol
MHCC97H-shHNRNPC-1
MHCC97H-shHNRNPC-2
MHCC97H-shHNRNPC-3
Migration
Invasion
100μm
Number of migrated cells
Number of invaded cells
(D)
MHCC97H-shcontrol
MHCC97H-shHNRNPC
Representative bioluminescence imaging in mouse liver
(E)
MHCC97H-shcontrol
MHCC97H-shHNRNPC
Total flux (×10⁶)
Weeks
(F)
MHCC97H-shcontrol
MHCC97H-shHNRNPC
p = 0.037
Overall survival
Time (Days)
(G)
Number of metastatic nodules per lung
MHCC97H-shcontrol
MHCC97H-shHNRNPC
(H)
Incidence of Lung Metastasis
MHCC97H-shcontrol
MHCC97H-shHNRNPC
10
2
8
metastasis
no metastasis
(I)
MHCC97H-shcontrol
MHCC97H-shHNRNPC
2mm
200μm

FIGURE 2 Heterogeneous nuclear ribonucleoprotein C (HNRNPC) downregulation inhibits invasiveness and metastasis of HCC. (A) Expression profiles of HNRNPC in different human HCC cell lines by western blotting. (B) Western blotting for HNRNPC expression after three different shRNAs targeting HNRNPC were transfected into MHCC97H cells. (C) Transwell assays for the effect of HNRNPC knockdown on the migration and invasion of MHCC97H cells. (D–I) In vivo metastatic assay. Representative bioluminescence imaging (BLI) (D), the bioluminescence signals (E), the overall survival of the nude mice (F), the number of metastatic nodules (G), the incidence of lung metastasis (H), and the H&E staining of the lungs (I) in the different groups following orthotopic implantation for 9 weeks. *$p < 0.05$.

3.3 | HNRNPC downregulation inhibits HCC metastasis by decreasing HIF1A expression

We next investigated the mechanism underlying HNRNPC downregulation and inhibited HCC metastasis. As HNRNPC is an RBP that affects RNA metabolism, we knocked down HNRNPC in MHCC97H cells and then conducted RNA sequencing (RNA-seq). In total, 183 downregulated genes and 241 upregulated genes (fold change ≥2 and $p < 0.05$) were identified following HNRNPC knockdown (Appendix S2). Both GO and KEGG analyses identified that the "HIF-1 signaling pathway" was the significantly downregulated pathway in the HNRNPC-knockdown group (Figure 3A). Additionally, HIF1A was one of the most significantly downregulated genes (Figure 3B). The bioinformatics prediction software ENCORI (http://rna.sysu.edu.cn/encori/rbpClipRNA.php) showed that HNRNPC was positively correlated with HIF1A (Figure 3C). RT-PCR and western blot assays further confirmed that HNRNPC downregulation decreased HIF1A expression (Figure 3D). Therefore, we posited that the decreased expression of HIF1A might be the molecular mechanism underlying HNRNPC downregulation-inhibited HCC metastasis.

RIP was performed to investigate the interaction between HNRNPC and HIF1A. HIF1A was significantly enriched in the HNRNPC antibody group (Figure 3E), indicating that HNRNPC protein was physically associated with HIF1A mRNA. The regulation of HNRNPC on mRNA stability prompted us to explore whether HNRNPC changed the transcript abundance of HIF1A by influencing its stability. To test this idea, actinomycin D, an RNA synthesis inhibitor, was used to block de novo mRNA synthesis. The half-life of HIF1A mRNA was markedly decreased in the HNRNPC-knockdown group (Figure 3F), indicating that HNRNPC downregulation decreased HIF1A expression by destabilizing HIF1A mRNA. The 3′UTR of HIF1A contains multiple AREs that are responsible for mRNA stability (Figure S2). To determine the regions required for HNRNPC-mediated HIF1A mRNA stability, we constructed three truncations of HIF1A 3′UTR, including WT1, WT2, and WT3 (Figure 3G). The luciferase activities of the reporters carrying WT2 and WT3 significantly decreased upon HNRNPC knockdown (Figure 3H). This decrease in luciferase activities could be restored by the site-directed mutation of WT2 and WT3 (Figure 3I). These results indicated that AREs in WT2 and WT3 regions of the HIF1A 3′UTR were essential for the regulation of HIF1A mRNA stability by HNRNPC.

To determine the involvement of HIF1A in HNRNPC-mediated HCC metastasis, we upregulated HIF1A expression in HNRNPC-silencing MHCC97H cells with lentiviral infection (Figure 4A). HIF1A upregulation rescued the decrease in cell migration and invasion caused by HNRNPC knockdown (Figure 4B). In addition, HIF1A upregulation abolished the suppressive effect of HNRNPC knockdown on the proliferation and survival of MHCC97H cells (Figure S3A,B), and weakened the promoting effect of HNRNPC knockdown on the apoptosis of MHCC97H cells (Figure S3C). In vivo experiments showed that HIF1A overexpression accelerated tumor growth, increased lung metastasis, and shortened overall survival time in the HNRNPC-silencing group (Figure 4C–H). Taken together, HNRNPC downregulation inhibited HCC metastasis by decreasing HIF1A expression.

3.4 | IL-6/STAT3 signaling mediates upregulation of HNRNPC expression

To identify the mechanism by which HNRNPC was upregulated in HCC, the ENCODE TFBS ChIP-seq data were analyzed. Several transcription factors that may regulate HNRNPC transcription were identified (Figure 5A). STAT3 is a known negative regulator of type I interferon-mediated antiviral responses.[30,31] Interestingly, type I interferon signaling was the most significantly upregulated pathway in the HNRNPC-knockdown group (Figure 5B). We wondered about the relationship between STAT3 and HNRNPC. Sequence analysis revealed several STAT3 binding sites on the *HNRNPC* promoter (Figure S4). Additionally, a positive expression correlation was found between STAT3 and HNRNPC by analyzing TCGA data (http://gepia.cancer-pku.cn/) (Figure 5C). Therefore, we hypothesized that the expression of HNRNPC may be controlled by STAT3. The overexpression of STAT3C significantly upregulated HNRNPC expression and enhanced the *HNRNPC* promoter activity (Figure 5D). To determine the region responsible for the binding of STAT3 and the *HNRNPC* promoter, luciferase reporters containing truncations and mutations of the *HNRNPC* promoter were cotransfected with STAT3C or control plasmids. We found that the region deletion between −135 to −24 bp and the binding site 1 mutation significantly decreased luciferase reporter activity (Figure 5E). Moreover, ChIP-PCR results showed significant enrichment of STAT3 binding site 1 on *HNRNPC* promoter by using a STAT3 antibody in HCC cells and HCC tissues (Figure 5F and Figure S5A).

IL-6 is the best known activator of STAT3.[32] It plays a crucial role in liver inflammation and cancer through sustaining STAT3 activation.[33] To identify whether IL-6 could regulate HNRNPC, we treated HCC cells with gradient concentrations of IL-6. IL-6 significantly upregulated HNRNPC expression, and this effect was dose dependent (Figure 5G). Additionally, IL-6 significantly enhanced the promoter activity of *HNRNPC* (Figure 5H). The potential binding site indispensable for IL-6-enhanced promoter activity of *HNRNPC* was further identified by serial truncation and site-directed mutagenesis (Figure 5I). ChIP-PCR results showed that the STAT3 binding site

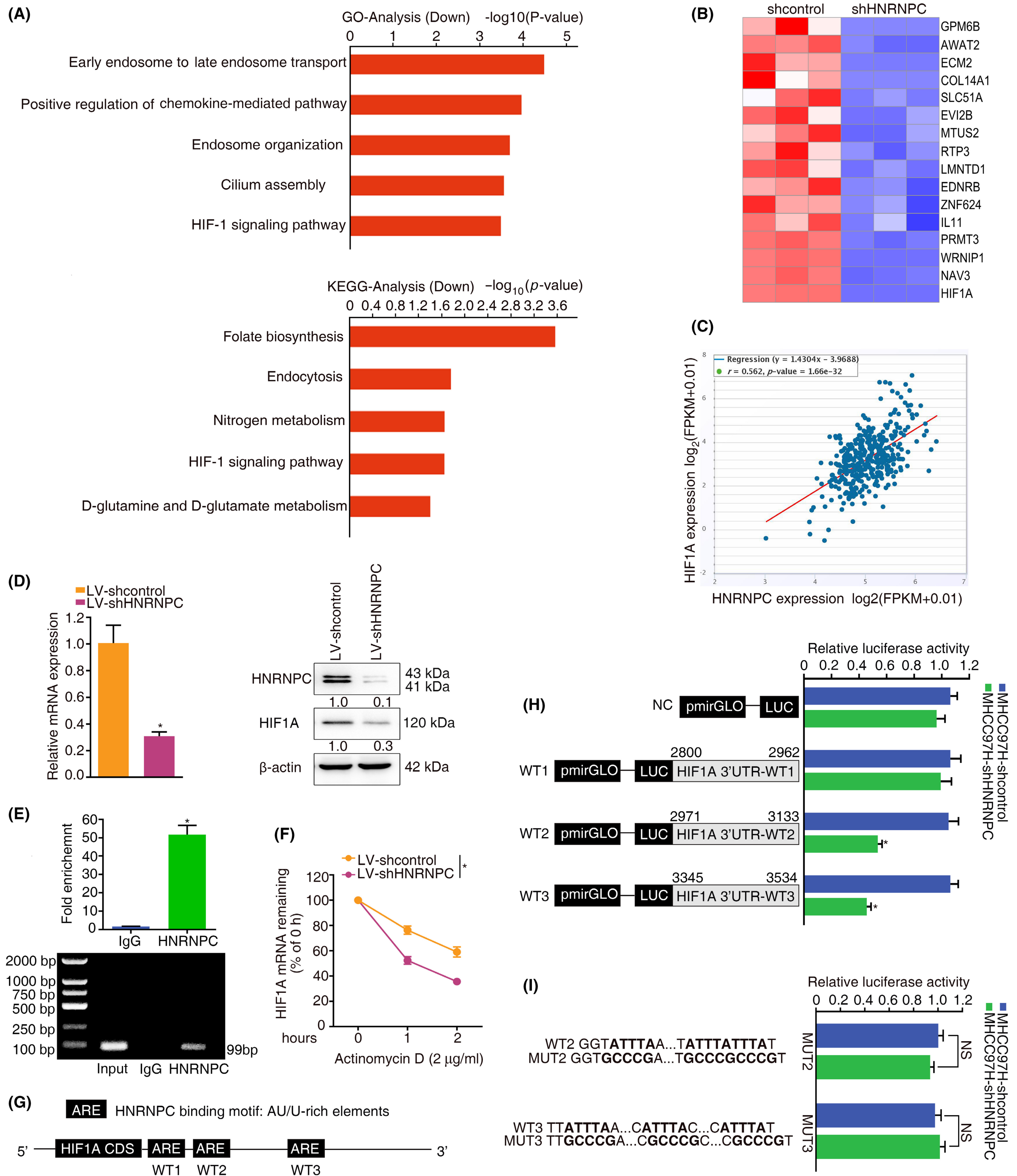

FIGURE 3 Heterogeneous nuclear ribonucleoprotein C (HNRNPC) downregulation decreases HIF1A expression by destabilizing HIF1A mRNA. (A) Top five GO terms in biological process and top five enriched pathways in KEGG analysis among differentially downregulated genes following HNRNPC knockdown. (B) Most significantly downregulated genes (fold change ≥4, $p<0.05$) following HNRNPC knockdown are presented in a heatmap. (C) Correlation analysis between HNRNPC and HIF1A expression in HCC using the ENCORI website. (D) qPCR and western blotting for HIF1A expression following HNRNPC knockdown. (E) The interaction between HNRNPC and HIF1A was determined by RIP. RNA enrichment was measured by RT-PCR. (F) The half-life of HIF1A mRNA was examined by qPCR in MHCC97H cells after HNRNPC knockdown and actinomycin D treatment (2 μg/ml) for the indicated times. (G) Schematic of various regions in the 3′UTR of HIF1A mRNA. (H, I) The relative luciferase activities were measured in MHCC97H cells carrying different regions and mutated sequences of the HIF1A 3′UTR after HNRNPC knockdown. *$p<0.05$.

1 on the *HNRNPC* promoter was significantly enriched by using a STAT3 antibody after IL-6 treatment (Figure 5J and Figure S5B). To determine the necessity of STAT3 for IL-6-mediated HNRNPC expression, we knocked down STAT3 in IL-6-treated Huh7 cells. STAT3 knockdown significantly suppressed IL-6-enhanced *HNRNPC* promoter activity (Figure 5K) and reduced IL-6-upregulated HNRNPC expression (Figure 5L). These results indicated that IL-6/STAT3 signaling mediated the upregulation of HNRNPC expression.

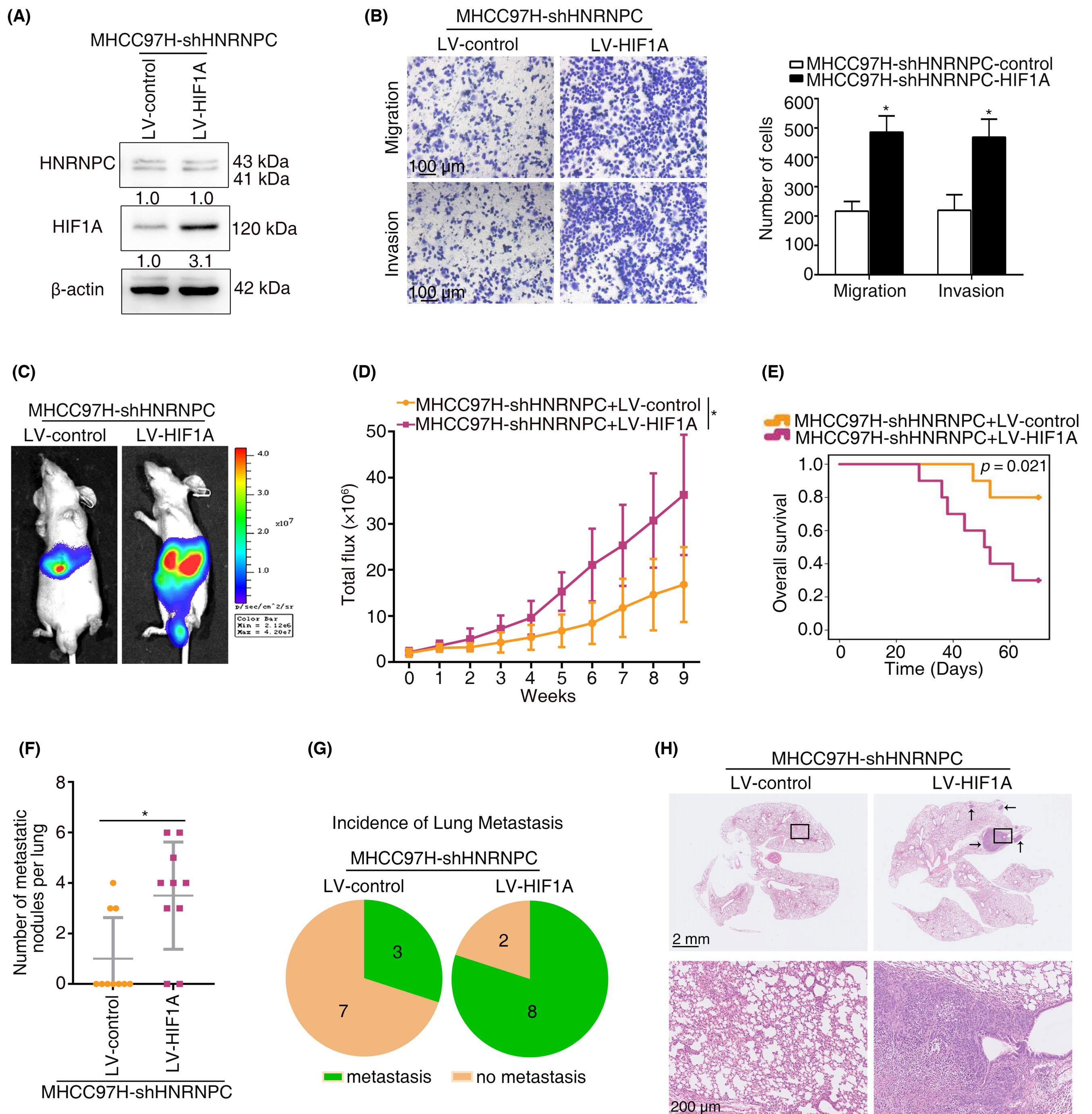

FIGURE 4 Heterogeneous nuclear ribonucleoprotein C (HNRNPC) downregulation inhibits HCC metastasis by decreasing HIF1A expression. (A) Western blotting for HNRNPC and HIF1A expression in MHCC97H cells transfected with HNRNPC-silencing lentivirus and HIF1A-overexpressing lentivirus. (B) Transwell assays for the effect of HIF1A overexpression on the migration and invasion of HNRNPC-silencing MHCC97H cells. (C–H) In vivo metastatic assay. Representative bioluminescence imaging (BLI) (C), the bioluminescence signals (D), the overall survival of the nude mice (E), the number of metastatic nodules (F), the incidence of lung metastasis (G), and the H&E staining of the lungs (H) in the different groups following orthotopic implantation for 9 weeks. *$p < 0.05$.

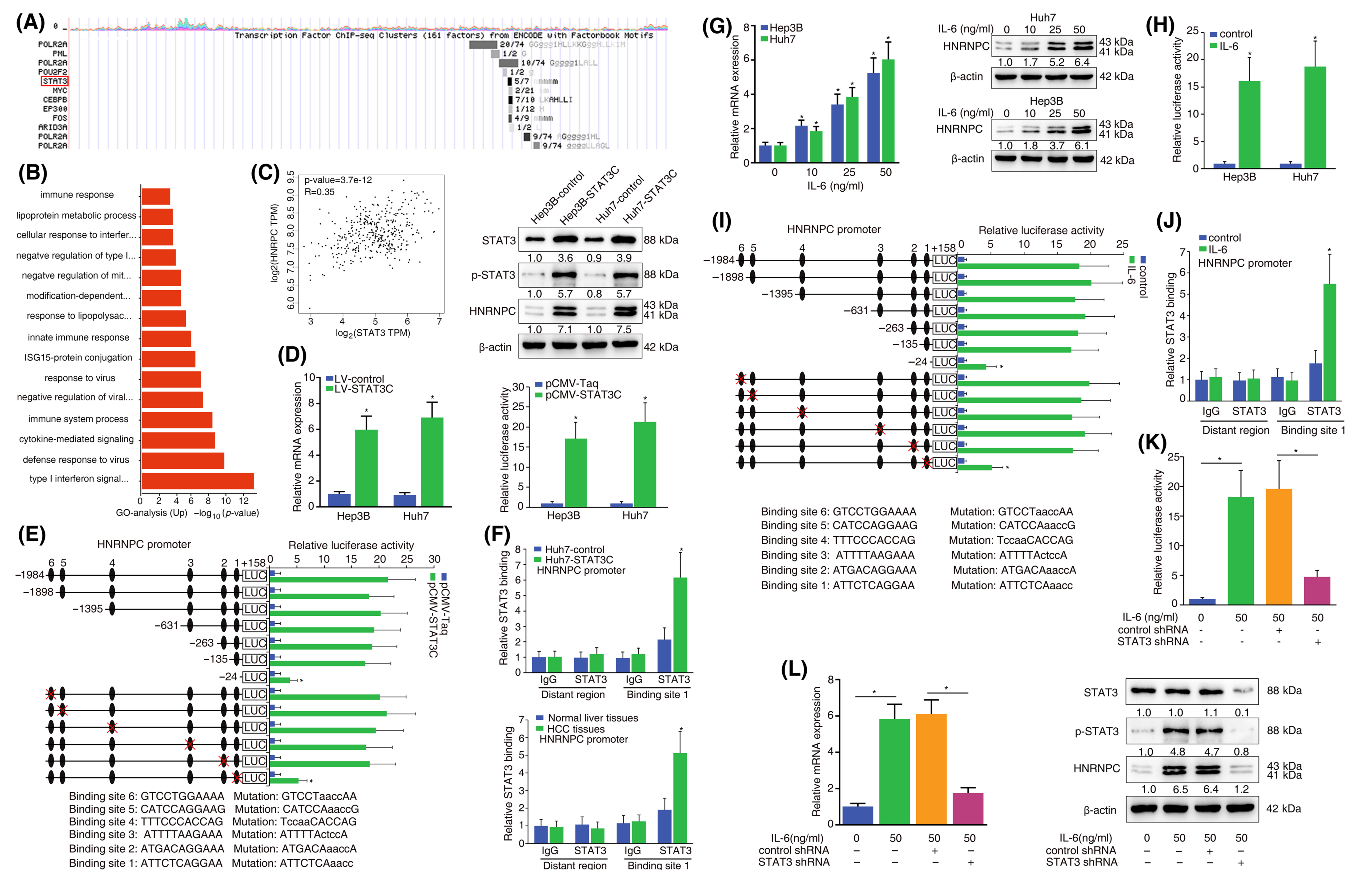

FIGURE 5 IL-6/STAT3 signaling upregulates heterogeneous nuclear ribonucleoprotein C (HNRNPC) expression. (A) Potential transcription factors in the *HNRNPC* promoter in the UCSC Genome Browser (assembly >hg38, DNA range: chr14:21,271,442–21,269,143; strand, –). (B) Top 15 GO terms in biological process among differentially upregulated genes following HNRNPC knockdown. (C) Expression correlation between STAT3 and HNRNPC was analyzed using the Gene Expression Profiling Interactive Analysis (GEPIA) website. (D) The regulation of STAT3 on HNRNPC expression was determined by qPCR, western blotting, and luciferase reporter assay. (E) Luciferase activity was measured in Huh7 cells after cotransfection with truncations and mutations of the *HNRNPC* promoter and STAT3C plasmid. (F) Direct recruitment of STAT3 to the *HNRNPC* promoter was determined by a ChIP assay in HCC cells and HCC specimens. (G) Regulation of IL-6 on HNRNPC expression was determined by qPCR and western blotting. (H) Luciferase activity was measured in HCC cells treated with or without IL-6 (50 ng/ml) for 24 h. (I) Luciferase activity was measured in Huh7 cells after transfection with truncations and mutations of the *HNRNPC* promoter and treatment with IL-6. (J) Direct recruitment of STAT3 to the *HNRNPC* promoter was determined by ChIP assay in IL-6-treated Huh7 cells. (K) Luciferase activity was measured in HCC cells after IL-6 treatment and STAT3 knockdown. (L) Regulation of STAT3 on IL-6-induced HNRNPC expression was determined by qPCR and western blotting. *$p < 0.05$.

3.5 | HNRNPC is essential for IL-6/STAT3 signaling-mediated HCC metastasis

To test if HNRNPC is necessary for STAT3-mediated HCC metastasis, HNRNPC was knocked down in STAT3C-overexpressing Huh7 cells (Figure 6A). HNRNPC knockdown inhibited STAT3C-enhanced cell migration and invasion (Figure 6B), slowed tumor growth, decreased lung metastasis, and prolonged overall survival time in the STAT3C-overexpressing group (Figure 6C–H). Similarly, we silenced HNRNPC in IL-6-overexpressing Huh7 cells (Figure 6I). HNRNPC inhibition attenuated IL-6-enhanced cell migration and invasion (Figure 6J), slowed tumor growth, decreased lung metastasis, and prolonged overall survival time in the IL-6-overexpressing group (Figure 6K–P). These data suggested that HNRNPC was essential for IL-6/STAT3-mediated HCC invasion and metastasis.

3.6 | Siltuximab significantly attenuates IL-6-enhanced HCC metastasis

Siltuximab is a chimeric mouse–human antibody targeting IL-6 with promising results in clinical trials alone or in combination with other agents for the treatment of multiple human malignancies.[34] To determine the effect of siltuximab in HCC metastasis, HCC cells were exposed to siltuximab (100 μg/ml) for 24 h. Siltuximab treatment markedly inhibited STAT3 activation and HNRNPC expression (Figure 7A), as well as attenuated IL-6-enhanced migration and invasion capacities of HCC cells (Figure 7B). Moreover, siltuximab weakened the promoting effect of IL-6 overexpression on the proliferation and survival of Huh7 cells (Figure S6A,B), and abolished the suppressive effect of IL-6 overexpression on the apoptosis of Huh7 cells (Figure S6C). Ulteriorly, nude mice were administered

i.p. with siltuximab (20 mg/kg) twice weekly for 8 weeks (Figure 7C). Siltuximab treatment significantly slowed tumor growth, decreased lung metastasis, and prolonged overall survival time in the IL-6-overexpressing group (Figure 7D–I). These findings suggested that siltuximab significantly attenuated IL-6-induced HCC metastasis.

4 | DISCUSSION

Metastasis remains the major obstacle to the successful treatment of HCC. Therefore, more attention needs to be paid to the field of HCC metastasis for novel therapies. RBPs could interact with thousands of RNAs, and perturbations in RBP-RNA networks activity leads to cancer development.[35] hnRNPs are a large family of RBPs that is altered in many types of cancer and plays important roles in cancer metastasis. HNRNPLL suppresses colorectal cancer metastasis through the regulation of CD44v6.[36] HNRNPF induces epithelial–mesenchymal transition (EMT) in bladder cancer by regulating Snail1 expression.[37] HNRNPC was identified as one of 474 RBPs used to classify tumor and nontumor in HCC.[7] The good capacity of HNRNPC in differentiating between normal liver and HCC tissues in TCGA-LIHC datasets are presented by ROC curve, showing the potential of HNRNPC as a biomarker for HCC diagnosis. Beyond that, we found that HNRNPC expression was significantly associated with malignant characteristics and poor prognosis of HCC in a 286-patient cohort and a 180-patient cohort, highlighting the potential of HNRNPC as a biomarker for HCC prognosis. In vitro and in vivo metastatic assays suggested that HNRNPC downregulation inhibited HCC invasion and metastasis, revealing the potential of HNRNPC as a further therapeutic target for HCC.

To determine the molecular mechanism underlying HNRNPC downregulation-inhibited HCC metastasis, RNA-seq was conducted to identify the difference in transcript expression following HNRNPC knockdown. HIF1A and “HIF-1 signaling pathway” were identified as

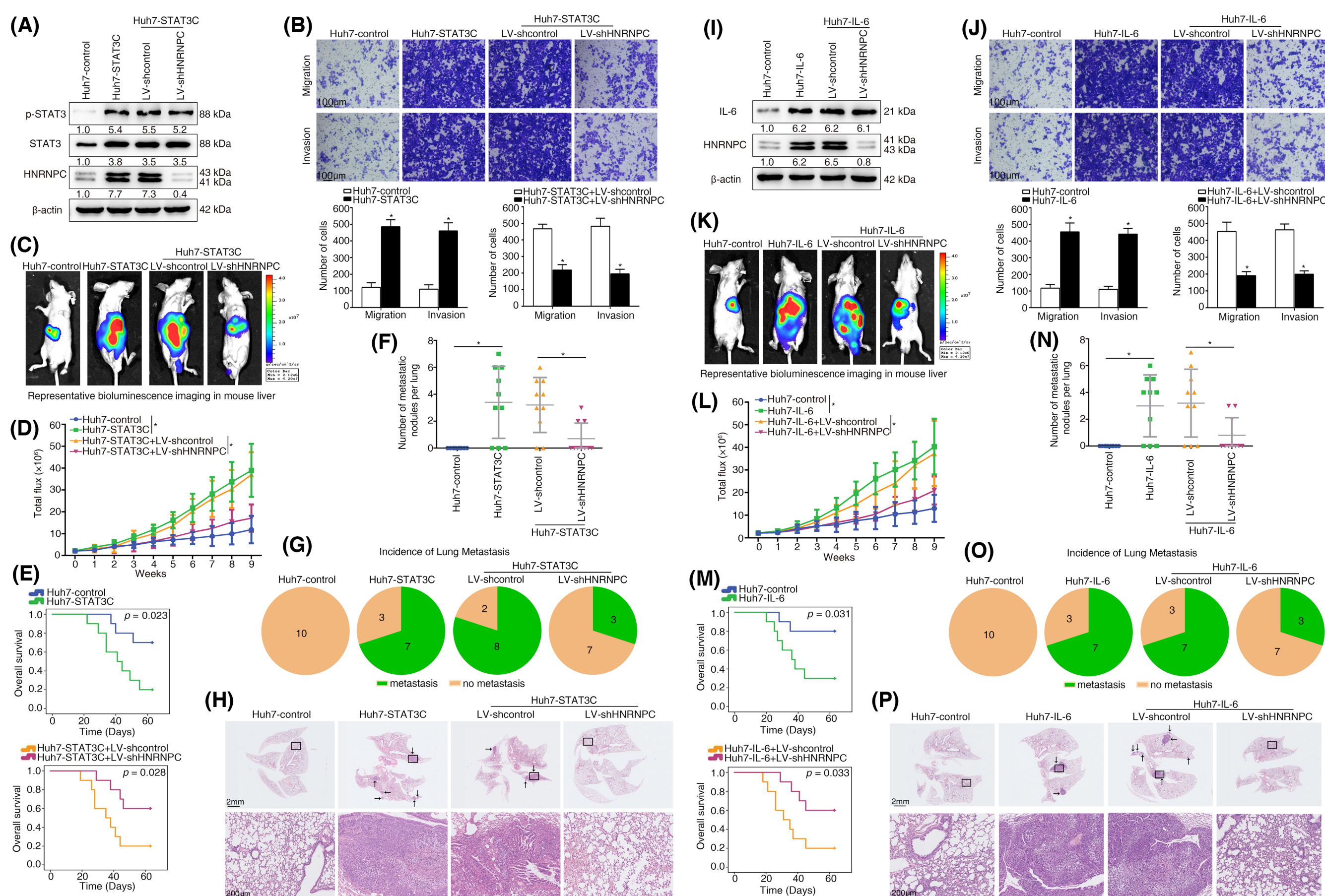

FIGURE 6 Heterogeneous nuclear ribonucleoprotein C (HNRNPC) is essential for IL-6/STAT3-mediated HCC metastasis. (A) Western blotting for STAT3, p-STAT3, and HNRNPC expression in lentivirus-transfected Huh7 cells. (B) Transwell assays for the effect of HNRNPC on STAT3C-mediated cell migration and invasion. (C–H) In vivo metastatic assay. Representative bioluminescence imaging (BLI) (C), the bioluminescence signals (D), the overall survival of nude mice (E), the number of metastatic nodules (F), the incidence of lung metastasis (G), and H&E staining of lungs (H) in the different groups following orthotopic implantation for 9 weeks. (I) Western blotting for IL-6 and HNRNPC expression in lentivirus-transfected Huh7 cells. (J) Transwell assays for the effect of HNRNPC on IL-6-mediated cell migration and invasion. (K–P) In vivo metastatic assay. Representative BLI (K), the bioluminescence signals (L), the overall survival of nude mice (M), the number of metastatic nodules (N), the incidence of lung metastasis (O), and H&E staining of the lungs (P) in the different groups following orthotopic implantation for 9 weeks. *$p < 0.05$.

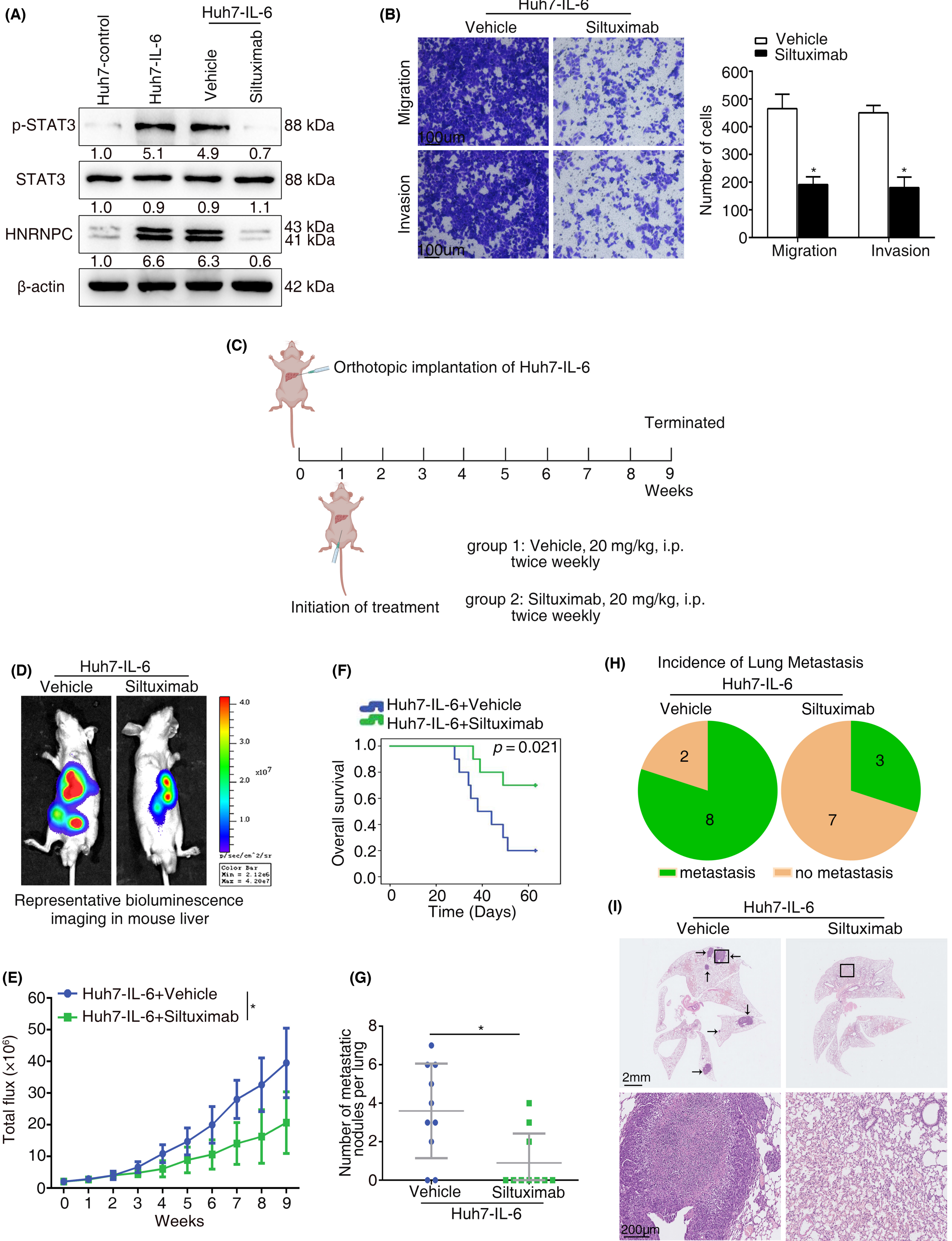
(A)
Huh7-IL-6
Huh7-control
Huh7-IL-6
Vehicle
Siltuximab
p-STAT3
88 kDa
1.0 5.1 4.9 0.7
STAT3
88 kDa
1.0 0.9 0.9 1.1
HNRNPC
43 kDa
41 kDa
1.0 6.6 6.3 0.6
β-actin
42 kDa
(B)
Huh7-IL-6
Vehicle
Siltuximab
Migration
Invasion
100um
Vehicle
Siltuximab
Number of cells
Migration
Invasion
(C)
Orthotopic implantation of Huh7-IL-6
Terminated
Weeks
Initiation of treatment
group 1: Vehicle, 20 mg/kg, i.p. twice weekly
group 2: Siltuximab, 20 mg/kg, i.p. twice weekly
(D)
Huh7-IL-6
Vehicle
Siltuximab
Representative bioluminescence imaging in mouse liver
(E)
Huh7-IL-6+Vehicle
Huh7-IL-6+Siltuximab
Total flux (×10^6)
Weeks
(F)
Huh7-IL-6+Vehicle
Huh7-IL-6+Siltuximab
p = 0.021
Overall survival
Time (Days)
(G)
Number of metastatic nodules per lung
Vehicle
Siltuximab
Huh7-IL-6
(H)
Incidence of Lung Metastasis
Huh7-IL-6
Vehicle
Siltuximab
metastasis
no metastasis
(I)
Huh7-IL-6
Vehicle
Siltuximab
2mm
200μm

FIGURE 7 Siltuximab significantly attenuates IL-6-enhanced HCC metastasis. (A) Western blotting for p-STAT3 and heterogeneous nuclear ribonucleoprotein C expression in Huh7 cells after lentiviral transfection and siltuximab treatment (100 μg/ml, 24 h). (B) Transwell assays for the effect of siltuximab on IL-6-mediated cell migration and invasion. (C) Schematic diagram showing siltuximab administration in vivo. Nude mice were injected i.p. with siltuximab (20 mg/kg) twice weekly for 8 weeks. (D–I) In vivo metastatic assay. Representative bioluminescence imaging (BLI) (D), the bioluminescence signals (E), the overall survival of the nude mice (F), the number of metastatic nodules (G), the incidence of lung metastasis (H), and H&E staining of lungs (I) in the different groups following orthotopic implantation for 9 weeks. $*p < 0.05$.

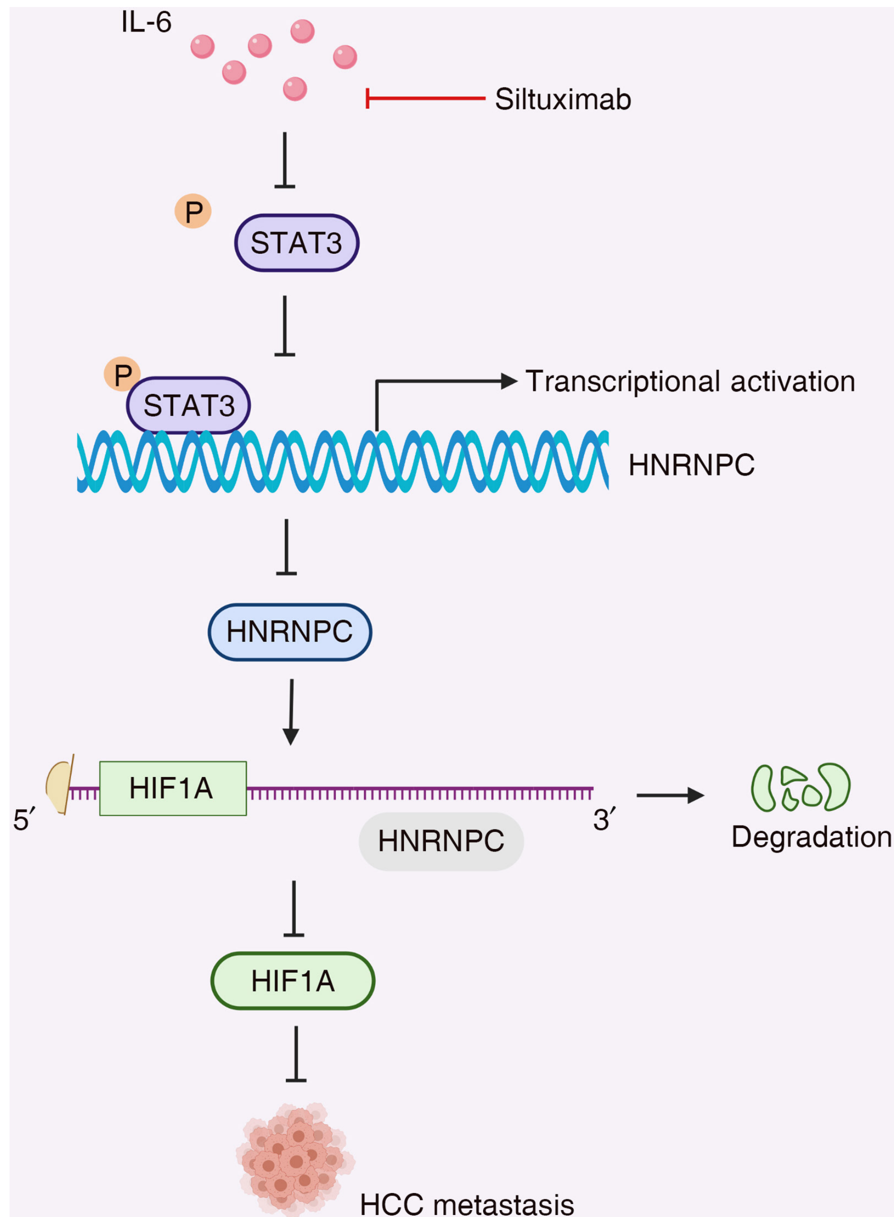

FIGURE 8 Mechanism scheme of siltuximab in HCC metastasis. Siltuximab blocks IL-6/STAT3-mediated transcriptional activation of heterogeneous nuclear ribonucleoprotein C (HNRNPC), and then faint HNRNPC is incapable of binding to HIF1A mRNA, which makes HIF1A mRNA unstable and degraded, therefore inhibiting HCC metastasis.

one of the most significantly downregulated genes and pathways, indicating the decrease in HIF1A expression and the subsequent inhibition of HIF-1 signaling might be the molecular mechanism underlying HNRNPC downregulation inhibited HCC metastasis. HNRNPC is an important regulator in mRNA stability. For example, the decrease in HNRNPC expression accelerates the instability of ANLN-210 mRNA in head and neck squamous cell carcinoma.[38] Our study revealed that HNRNPC downregulation decreased HIF1A expression by destabilizing HIF1A mRNA. AREs in the 3′UTR of mRNAs are the most common determinants of mRNA stability.[39] It has been reported that USP52 depletion destabilizes HIF1A mRNA through AREs in HIF1A 3′UTR.[40] In our study, AREs in WT2 and WT3 regions of HIF1A 3′UTR were essential for the regulation of HIF1A mRNA stability by HNRNPC.

In addition to the significant downregulation of the HIF-1 signaling pathway, HNRNPC knockdown contributed to the significant upregulation of type I interferon signaling, which is consistent with previous finding that HNRNPC deficiency could activate the type I interferon response in breast cancer.[41] Type I interferon plays an important inhibitory effect in HCC metastasis.[42] Activated type I interferon responses might be another molecular mechanism for the suppression of HCC metastasis induced by HNRNPC downregulation. Given the negative regulation of STAT3 on type I interferon response and its potential binding to the *HNRNPC* promoter, we speculated that STAT3 may be the upstream regulator of HNRNPC. The regulation of STAT3 on HNRNPC expression was confirmed by luciferase reporter assay and ChIP assay. IL-6, the best known activator of STAT3, was also determined to regulate HNRNPC expression. HNRNPC was critical for the function of IL-6/STAT3 in HCC. IL-6/STAT3-mediated HCC metastasis was significantly inhibited by HNRNPC downregulation.

IL-6 is a pleiotropic cytokine with roles in cancer metastasis, including HCC.[43] Targeting IL-6 is a promising strategy to inhibit tumor progression and improve the efficacy of cancer therapy. For example, IL-6 blockade suppresses the development and progression of lung cancer[44] and enhances the responsiveness to chemotherapy in gastric cancer.[45] Recently, the implication of IL-6 in the invasiveness and chemoresistance of ovarian cancer has been discussed.[46] Siltuximab, a chimeric mouse–human antibody targeting IL-6, was approved by the United States Food and Drug Administration (US FDA) to treat multicentric Castleman disease in 2014. In addition, siltuximab could be used for various other disorders in which IL-6 overproduction plays a crucial part.[47] The preclinical and clinical studies have demonstrated the antitumor efficacy of siltuximab against ovarian cancer[48] and lung cancer.[49] However, its effect in HCC is yet to be testified. Our study revealed that siltuximab treatment significantly impeded HCC metastasis by inhibiting IL-6-induced STAT3 activation and HNRNPC expression, indicating that siltuximab had therapeutic effects in HCC, at least in animals. However, the issue that whether siltuximab could be applied to patients with HCC needs to be determined by clinical trials.

In conclusion, HNRNPC is closely associated with the malignant characteristics of HCC and independently predicts the prognosis of patients with HCC. Siltuximab blocks IL-6/STAT3-mediated transcriptional activation of HNRNPC, subsequent downregulated HNRNPC leads to the instability of HIF1A mRNA and the decrease in HIF1A expression, therefore inhibiting HCC metastasis (Figure 8). The suppression of HNRNPC or the use of siltuximab could serve as a therapeutic strategy for IL-6-induced HCC metastasis.

AUTHOR CONTRIBUTIONS

Danfei Liu performed the experiments and wrote the paper. Xiangyuan Luo and Meng Xie gave assistance in immunohistochemistry staining and animal experiments. Xiaoping Chen and Bixiang Zhang provided HCC samples. Tongyue Zhang, Mengyu Sun, Yijun Wang, Yangyang Feng, and Xiaoyu Ji collected tissues samples. Yiwei Li and Bifeng Liu gave advice in bioinformatics analysis. Danfei Liu, Limin Xia, and Wenjie Huang were responsible for the conception and design of experiments. All authors have read and approved the final manuscript.

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DISCLOSURE

The authors have no conflict of interest.

DATA AVAILABILITY STATEMENT

All supporting data are included in the article and its additional files.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: This study was approved by the Ethics Committee of Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology. Informed Consent: All HCC samples were collected on condition of informed consent. Registry and the Registration No. of the study/trial: N/A. Animal Studies: All animal experiments in this study were performed under the institutional ethical guidelines approved by the Animal Ethics Committee of Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology.

ORCID

Limin Xia https://orcid.org/0000-0002-6327-6034

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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