Original Article

CD44 potentiates hepatocellular carcinoma migration and extrahepatic metastases via the AKT/ERK signaling CXCR4 axis

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Background: Cell adhesion molecule cluster of differentiation 44 (CD44) plays a significant role in cancer cell local invasion, intravasation, migration, and the establishment of metastatic lesions. However, little is known about the underlying mechanism of how CD44 regulates hepatocellular carcinoma (HCC) extrahepatic metastasis (EHM).

Methods: The expression of CD44 in HCC tissues and cell lines was detected through western blot and immunohistochemistry (IHC). Through gain- and loss-of-function assays, we examined the oncogenic roles of CD44 in regulating HCC cell growth and metastasis *in vitro* and *in vivo*. To identify the potential mechanism, we employed quantitative real-time polymerase chain reaction, and western blot.

Results: In this study, CD44 was highly expressed in HCC cells and HCC-patient specimens that exhibited high malignancy potential. The overall survival (OS) was worse and the cumulative recurrence rate was higher in HCC patients with CD44 overexpression than those with low levels of CD44 expression. Our *in-vitro* and *in-vivo* experiments showed that CD44 downregulation reduced HCC cell colony formation, migration, and invasion, and HCC tumor growth and metastasis, and that the pro-metastasis effect of CD44 was mediated by the protein kinase B (AKT)/extracellular signal-regulated kinase (ERK) signaling-chemokine receptor C-X-C chemokine receptor type 4 (CXCR4) axis. The reported capacity of CD44 to induce CXCR4 expression and increase the propensity of tumors to invade and metastasize to distant organs is consistent with the aggressive clinical characteristics of HCCs.

Conclusions: CD44 could represent a future therapeutic target for EHM.

Keywords: Hepatocellular carcinoma (HCC); extrahepatic metastasis (EHM); CD44; AKT/ERK signaling; CXC chemokine receptor (CXCR)

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Introduction

Hepatocellular carcinoma (HCC), which has a 5-year survival rate <20% (1,2), is still the leading cause of cancer-related death in less developed countries (3). The high mortality rate of HCC is mainly related to its frequent recurrence or metastasis after curative therapy. Extrahepatic metastasis (EHM) is one of the most important causes of poor prognosis and may occur at the time of recurrence or in patients who present with advanced HCC (4,5). The most common site of HCC EHM is the lung, followed by lymph node, bone, adrenal gland, and peritoneum (6,7). The presence of EHM is a critical barrier to further improving the overall survival (OS) of HCC patients; however, there is a definite lack of evidence and pertinent studies on its underlying mechanism, and even fewer studies on the key molecules involved in the process.

Cluster of differentiation 44 (CD44), which is both a cell-cell and cell-stromal adhesion protein, is a plasma membrane protein that is often overexpressed in tumor cells, and is indicative of poor survival in most solid cancers. The changes and effects of CD44 on the histomorphology of HCC is few reported. But CD44 have been regarded as an essential marker in histochemistry staining of tumor biopsies because the numbers of tumor-associated macrophages (TAMs) correlated with the numbers of CD44(+) cells (8). It has been reported that CD44 is related to tumor malignancy, and indicates an unfavorable prognosis in multiple malignancies, including breast cancer (9), gastric cancer (10), and liver cancer (11). However, the role of CD44 in HCC EHM remains elusive, and understandings of its underlying mechanism need to be extended.

CXC chemokine receptors (CXCRs) are integral membrane proteins that specifically bind and respond to cytokines of the CXC chemokine family. The important role of CXCRs in liver repair and regeneration has been thoroughly analyzed (12), and some CXCRs have been found to have close relationship with tumor prognosis; for example, CXCR4 was found to be overexpressed in at least 23 different cancer types, including HCC (13), and is associated with more aggressive diseases and a poorer prognosis. To date, only limited research appears to have been conducted on the relationship between CD44 and CXCRs, and their roles in the EHM of HCC.

In this study, we found that CD44 was a potent predictor of EHM and the OS of HCC patients. Our *in-vitro* study showed that the knockdown of CD44 resulted in the significant inhibition of HCC cell migration, invasion, and colony formation. Additionally, our *in-vivo* study showed that HCC invasion and lung metastasis were significantly enhanced in the CD44 overexpression group. Mechanistically, among all of the CXCR1-7 molecules, silencing CD44 resulted in the maximum reduction of CXCR4 via the downregulation of the protein kinase B (AKT)/extracellular signal-regulated kinase (ERK) signaling pathway, which was supported by a reduced migration ability in response to stromal cell-derived factor 1α (SDF-1α). The inhibition of AKT and ERK significantly blocked the chemotactic responses of the HCC cells triggered by SDF-1α. In this study, we clarified the regulating mechanism between CD44 and CXCR4. To the best of our knowledge, we for the first time illustrated the relationship between CD44 and the CXCR4/SDF-1α axis in the EHM of HCC, which might become an important therapeutic target in the future. We present the following article in accordance with the ARRIVE and MDAR reporting checklists (available at https://atm.amegroups.com/article/view/10.21037/atm-22-2482/rc).

Methods

Patients and specimens

We enrolled 2 independent cohorts comprising a total of 338 HCC patients from a single liver cancer center (Liver Cancer Institute, Zhongshan Hospital, Fudan University) in this study. Cohort 1 comprised 76 patients, of whom 38 (the EHM group) were selected between 2002 and 2007 from patients with EHM HCC at the time of hepatic resection, or during follow-up. Both primary HCC tissues in the liver and EHM tissues were collected by surgical resection or needle aspiration. Cohort 2, our independent validation cohort, comprised 262 HCC patients, who were recruited from the same institute in the year 2006. The HCC diagnosis was according to the World Health Organization's criteria. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) (14). The study was approved by the Research Ethics Committee of the Zhongshan Hospital, Fudan University (No. 2006-653).

Informed consent was taken from all the patients.

Table 1 sets out the baseline clinicopathologic characteristics of all the patients in Cohort 2. The follow-up procedures and treatment modalities after relapse have been described in previous study (15). OS was defined as the interval between the dates of surgery and death or the last observation. Time to recurrence (TTR) was defined as the time between the first surgery and the first report of intrahepatic or distant recurrence (excluding patients who had died from non-liver cancer causes before recurrence) (16). The data for patients who had not relapsed or died were censored at the last follow-up date.

Tissue microarray (TMA) and IHC

The TMAs and immunohistochemistry (IHC) of serial TMAs were constructed as previously described (17). All the HCC cases were histologically reviewed by hematoxylin and eosin (H&E) staining. Duplicate cylinders from intratumoral and peritumoral areas (a total of 4 punches per patient) were obtained. The primary monoclonal antibody was CD44 (#37259) obtained from Cell Signaling Technology (Danvers, MA, USA). After immunostaining for 5 mins, the reaction products were visualized using a diaminobenzidine tetrahydrochloride (DAB) Detection Kit (Maixin Biotechnology, Fuzhou, China). IHC staining was assessed by 2 independent pathologists with no previous knowledge of patient characteristics. Discrepancies were resolved by consensus. Membranous CD44 expressions were interpreted according to previous study (18). The staining extent score used a scale of 0–3 that corresponded to the percentage of immunoreactive tumor cells (0%, 1–25%, 26–50%, and >50%, respectively). The staining intensity was scored as negative (score =0), weak (score =1), or strong (score =2). A score ranging from 0–6 was calculated by multiplying the staining extent score with the intensity score.

Cell culture and reagents

The HCC cell lines with stepwise metastatic potential (MHCC97H and HCCLM3) generated on the same genetic background were established at our institute (19). HepG2, Huh7, and PLC/RFP/5 were obtained from the Chinese Academy of Sciences. Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) was used to culture the cells. All the cell lines were incubated in a humidified incubator containing 5% carbon dioxide at 37 ℃. LY294002, PD98059, and SDF-1α were purchased from Cell Signaling Technology (Danvers).

Plasmids, lentivirus package, and infection

The complementary deoxyribonucleic acid (cDNA) corresponding to human CD44 was introduced into the plenti6.3-IRES2-EGFP, and the lentivirus was packaged according to the Nature Protocol (20). The CD44 overexpression cell lines were achieved by lentivirus infection and Blasticidin selection. The following 3 targets were chosen for CD44 knockdown: 5'-CCGTTGGAAACATAACCATTA-3', 5'-GGACCAATTACCATAACTATT-3', and 5'-CGCTATGTCCAGAAAGGAGAA-3'. The scramble short-hairpin ribonucleic acid (shRNA) sequence for the control was 5'-CCTAAGGTTAAGTCGCCCTCG-3'. Stable cell lines were obtained using the Addgene Protocol (21). The functional reconstruction of CD44 was achieved by introducing a new engineered shRNA-insensitive CD44 open reading frame (ORF). Specifically, the sequence of 5'-CGCTATGTCCAGAAAGGAGAA-3' was nonsense mutated to 5'-AGATACGTGCAGAAAGGAGAA-3' by site-directed mutation.

Orthotopic growth of HCC tumors in animal models

LM3-wt (HCC-LM3 without modification), LM3-LV-shNT, LM3-LV-shCD44, and LM3-LV-shCD44-RES (1×10^7) were subcutaneously inoculated into the right flanks of 6-week-old BALB/c male mice, which were purchased from Shanghai model organisms company After 3–4 weeks, non-necrotic tumor tissues were cut into 1 mm^3 pieces and orthotopically implanted into the liver. Tumor samples were then extracted for further analysis. Under a blinded condition, all the animals were randomly allocated into different groups by a technician. Animal experiments were performed under a project granted by the Animal Ethics Committee of Zhongshan Hospital, Fudan University (No. 2020632), in compliance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" for the care and use of animals. A protocol was prepared before the study without registration.

Cell proliferation, migration, and invasion assays

96-well plates (5×10^3 cells/well) were culture cells for 24, 48, or 72 h. The methods for cell growth has been referred previously (19). Cell migration was evaluated through

Table 1 Clinicopathologic features

Characteristics	Intratumoral CD44 Level					Intratumoral CXCR4 Level				
	High (n=59)		Low (n=203)		P value	High (n=56)		Low (n=206)		P value
	No. of patients	%	No. of patients	%		No. of patients	%	No. of patients	%	
Age (years)	52.92		52.08		0.609	51.95		52.35		0.807
Sex					0.815					0.707
Male	51	86.4	173	85.2		47	83.9	117	85.9	
Female	8	13.6	30	14.8		9	16.1	29	14.1	
Hepatitis B history					0.173					0.550
Yes	47	79.7	180	88.7		47	83.9	180	87.4	
No	11	18.6	22	10.8		8	14.3	25	12.1	
AFP, U/L	5,979.8		4,636.8		0.518	7,725.41		4,181.84		0.093
ALT, U/L	53.42		53.49		0.994	51.11		54.12		0.745
Liver cirrhosis					0.029					0.104
Yes	6	10.2	47	23.2		7	12.5	46	22.3	
No	53	89.8	156	76.8		49	87.5	160	77.7	
Tumor number					0.062					0.080
Single	42	71.2	167	82.3		40	71.4	169	82	
Multiple	17	28.8	36	17.7		16	28.6	37	18	
Tumor size, cm	7.29		5.93		0.043	6.673		6.119		0.418
Intrahepatic metastasis					0.062					0.080
Yes	17	28.8	36	17.7		16	28.6	37	18.0	
No	42	71.2	167	82.3		40	71.4	169	82.0	
Tumor encapsulation					0.912					0.560
Complete	28	47.5	98	48.3		25	44.6	101	49.0	
No	31	52.5	105	51.7		31	55.4	105	51.0	
Recurrence in 1 year					0.000					0.000
Yes	28	47.5	46	22.7		28	50.0	46	22.3	
No	31	52.5	157	77.3		28	50.0	160	77.7	
Microvascular invasion					0.213					0.182
Yes	28	47.5	78	38.4		27	48.2	79	38.3	
No	31	52.5	125	61.6		29	51.8	127	61.7	
TNM stage					0.1					0.019
I	21	35.6	102	50.2		21	37.5	102	49.5	
II	18	30.5	60	29.6		14	25.0	64	31.1	
III	17	28.8	37	18.2		20	35.7	34	16.5	
IV	3	5.1	4	2		1	1.8	6	2.9	

AFP, alpha-fetoprotein; ALT, alanine aminotransferase; TNM, tumor-node-metastasis.

Transwell assays (Boyden Chambers, Corning, Cambridge, MA, USA). Generally, 6×10^4 cells in serum-free DMEM were cultured on membranes (8.0-μm pores) in a 24-well plate. Lower chamber of each well was added with DMEM containing 10% FBS. 48 h later, Giemsa (Sigma Chemical Co., St Louis, MO, USA) was used to stain cells which had reached the underside of the membranes and cells were counted at 200× magnification. The cell invasion assays were performed similarly, except that 10 μL of Matrigel (BD Biosciences, Franklin Lakes, NJ) was added to each well 6 h before the cells were seeded on the membranes.

To determine the impact of CD44 with exogenous SDF-1α–induced tumor cell migration, cell migration assays were performed as mentioned before except that the lower chamber contained different concentrations of SDF-1α instead of 10% FBS.

To determine the combined inhibitory effect of hyaluronan and AMD 3100 (Plerixafor, chemokine receptor 4 inhibitor), the cells suspended in 2% tetracycline-free FBS containing medium were cultured in the upper chamber of the inserts and treated with AMD3100 (100 ng/mL). The culture medium in the lower chamber contained HA (100 ng/mL). The cells without any treatment served as a control in these experiments. After treating for 24 h, the number of migrated cells was counted as mentioned before.

Western blot analysis

The procedures used in this study have been described elsewhere (19). The primary antibodies included anti-CD44 (#37259), anti-AKT (#37259), phospho-Akt (#4060), anti-ERK (#9102), and phospho-ERK (#4370), which were obtained from Cell Signaling Technology (Danvers). CXCR4 (ab124824) was obtained from Abcam (Cambridge, UK).

Real-time polymerase chain reaction (RT-PCR) analysis

The primers used in the RT-PCR are listed in Table S1. RNA was extracted using Trizol Reagent (Takara, Japan), and the messenger RNA (mRNA) was transcripted to cDNA with m-mlv reverse-transcriptase (Promega, USA) following the user's manual. The RT-PCR analysis was conducted on ABI 9700 using a Power SYBR Green® PCR Master Mix (Life Technology, Carlsbad, CA, USA).

Statistical analysis

The χ^2 test or the Fisher exact test as appropriate were used to analyze the associations between the immunoreactive markers and clinicopathologic variables. The Pearson χ^2 test or Fisher exact test was applied in the qualitative variables comparation, and the *t* test was used to compare the quantitative variables. Survival curves of the patients were plotted employing the Kaplan-Meier method, and the log-rank test was used to compare the differences. The Cox regression model for the multivariate analysis only used factors which achieved statistical significance in the univariate analysis ($P<0.05$). All the statistical analyses were performed using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA).

Results

High expression of CD44 in high metastatic HCC cell lines and HCC tissues with EHM

We began the study with an analysis of CD44 protein level in different HCC cell lines with varying metastatic potential. Among the examined HCC cell lines, CD44 was readily detected in the Huh7, MHCC-97H, and HCCLM3 cells, and was correlated with a high-invasion capacity in the nude mice. Conversely, and absence of CD44 or very low CD44 levels were found in HCC cell lines that were relatively less metastatic (i.e., HepG2, and PLC) (see *Figure 1A*).

Based on the results of variant CD44 expression in the HCC cell lines, we next analyzed CD44 protein expression levels using TMAs based on an IHC staining study of 76 patients (15 HCC pairs with primary and lung tumor nodules, 14 HCC pairs with primary and lymph node metastatic lesions, 9 HCC pairs with primary and adrenal gland tumor sites, and 38 HCC patients without distant metastasis in the 5-year follow-up after resection). We found that patients suffering EHM had much higher expression levels of CD44 than those without EHM (non-EHM, NM) (see *Figure 1B*). Generally, CD44 was mainly expressed in the cell membrane, and was rarely expressed in the cytoplasm or nucleus (see *Figure 1C*).

In all, our findings suggested that CD44 plays a key role in the metastatic process of HCC and might be a good predictor of EMH in HCC patients.

CD44 upregulation in HCC patients is correlated with poor clinical outcomes and early recurrence

To study the correlation between CD44 expression and clinical outcomes, we analyzed CD44 expression in HCC

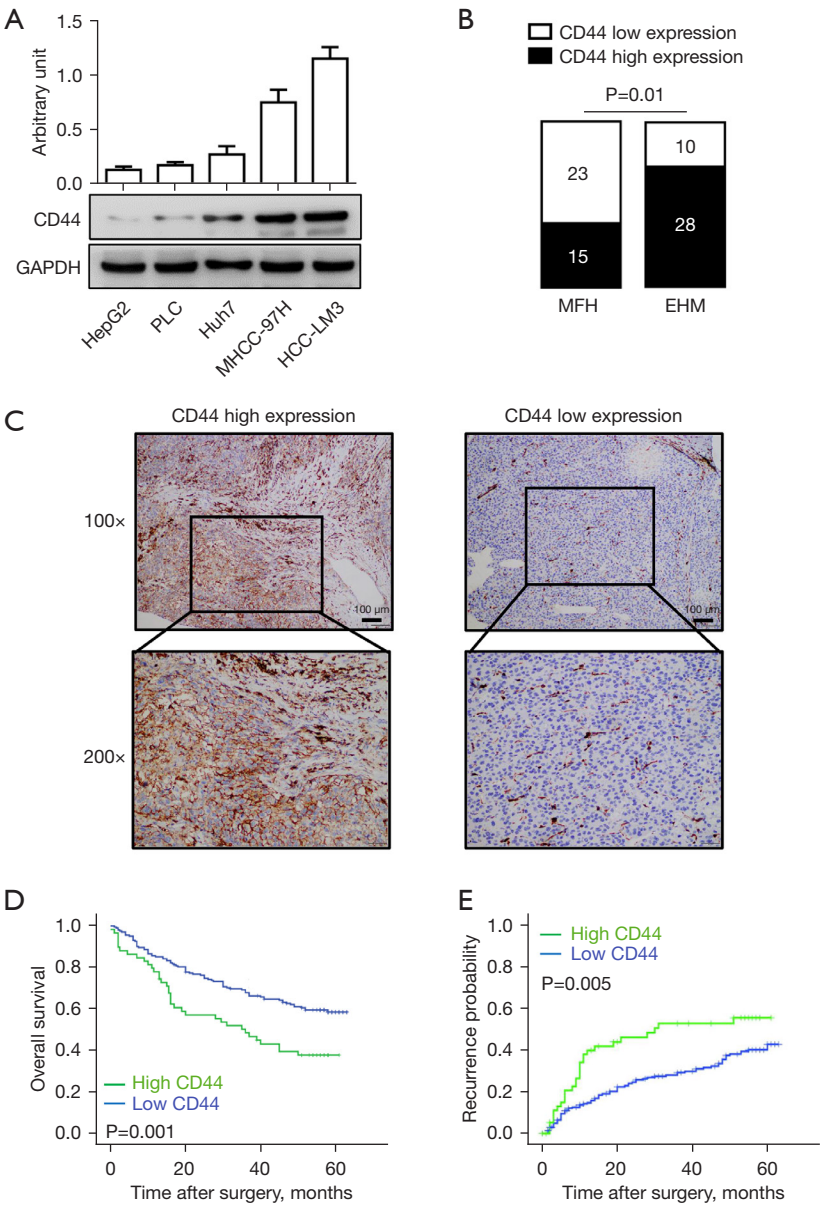

Figure 1 CD44 is highly expressed in HCC cells and HCC specimens with higher malignant potential and is correlated with poor outcomes. (A) The western blot analysis of CD44 in different HCC cell lines showed that CD44 levels increase as the malignant potential increased. (B) IHC showed that the ratio of high CD44 expression in HCC with EHM was much higher than that in non-metastatic HCC. (C) Representative immunohistochemical images of CD44 expression in HCC tissues (magnification: 100×, 200×; scale bar, 100 μm). (D) OS of HCC patients with CD44 high or low expression (P=0.001). (E) Recurrence rate of HCC patients with CD44 high or low expression (P=0.005). HCC, hepatocellular carcinoma; MFH, metastasis free hepatocellular carcinoma; EHM, extrahepatic metastasis; IHC, immunohistochemistry; OS, overall survival.

specimens derived from 262 postoperative HCC patients with available 5-year follow-up data (see *Table 1*). All the patients were allocated to CD44 low (n=203) or high (moderate and strong, n=59) groups, except for 2 patients whose specimens were deciduous during the IHC staining. The Kaplan-Meier analysis showed that HCC patients with high CD44 expression had significantly worse OS than those with low CD44 expression (median OS: 36 *vs.*

Table 2 Univariate and multivariate analysis of factors associated with OS and TTR

Features	OS				TTR			
	Univariate, P value	Multivariate			Univariate, P value	Multivariate		
		Hazard ratio	95% CI	P value		Hazard ratio	95% CI	P value
Age: <51 *vs.* ≥51 years	0.376			NA	0.403			NA
Gender: female *vs.* male	0.519			NA	0.272			NA
Hepatitis B history: yes *vs.*no	0.587			NA	0.791			NA
Liver cirrhosis: yes *vs.* no	0.385			NA	0.075			NA
AFP: <400 *vs.* ≥400 ng/mL	0.008			NA	0.850			NA
Preoperative ALT: ≤75 *vs.* >75 U/L	0.885			NA	0.778			NA
Tumor size: ≤5 *vs.* >5 cm	<0.001			NA	<0.001			NS
Tumor encapsulation: complete *vs.* none	<0.001	1.543	1.049–2.270	0.027	0.037			NA
Microvascular invasion: yes *vs.* no	<0.001			NA	0.013			NA
Intrahepatic metastasis: yes *vs.* no	<0.001			NA	0.010			NS
TNM stage: IIIa *vs.* II *vs.* I	<0.001	2.177	1.609–2.945	<0.001	<0.001	1.915	1.361–2.695	<0.001
Intratumoral features: low *vs.* high								
CD44 level	0.001			NA	0.005			NA
CXCR4 level	0.001			NA	0.009			NA
Combined CD44 and CXCR4 level	<0.001	1.197	1.024–1.399	0.024	0.004	1.300	1.092–1.546	0.003

OS, overall survival; TTR, time to recurrence; AFP, alpha-fetoprotein; ALT, alanine aminotransferase; TNM, tumor-node-metastasis.

>63 months; Log Rank (LR) =15.179; P=0.001; see *Figure 1D*). In the 5-year follow-up period, 49 of the 80 patients died (61.3%) in the CD44 high-expression group compared to 68 of 180 patients (37.8%) in the CD44 low expression group. Another feature distinguishing the CD44 high group from the CD44 low group was the former's significantly shortened TTR (median TTR: 36 *vs.* >63 months; LR =12.931; P=0.005; see *Figure 1E*).

Other factors that had significant predictive value in relation to the OS and TTR of the patients in the univariate analysis included large tumor size, the presence of intrahepatic metastasis, no encapsulation, microvascular invasion, and an advanced tumor-node-metastasis (TNM) stage. High alpha-fetoprotein (AFP) levels and liver cirrhosis were also associated with OS and disease-free survival, respectively. The significant factors in the univariate analysis were included in the multivariate Cox proportional hazards analysis (see *Table 2*). Altogether, the results of the analysis indicated that CD44 was an independent risk factor for both OS [hazard ratio (HR) =1.485; P=0.045] and TTR (HR =1.663; P=0.016).

CD44 promotes HCC cell colony formation and tumorigenicity in vivo

Tumor cell proliferation, migration, invasion, and colony formation in distant organs are the most important steps in the cascade of tumor metastasis. Considering the clinical findings and high expression of CD44 in metastatic HCC cell lines, we conjectured that CD44 might play a key role in the metastatic process of HCC. To optimize our *in-vitro* experiments, we generated a series of lentivirus-mediated CD44-specific shRNAs to silence the expression of CD44 (shCD44) in highly aggressive HCCLM3 and Huh-7 cells. The control cells were infected with the lentivirus expressing the scrambled shRNA precursor. Among the 3 shCD44 vectors, the shCD44 #2 vector, which showed the best knock down effect in endogenous CD44 expression based on western blotting validation, was chosen for the subsequent studies (see *Figure 2A*).

First, we sought to assess the effects of CD44 on HCC proliferation. Somewhat surprisingly, silencing endogenous CD44 in the HCCLM3 and Huh7 cells had very little

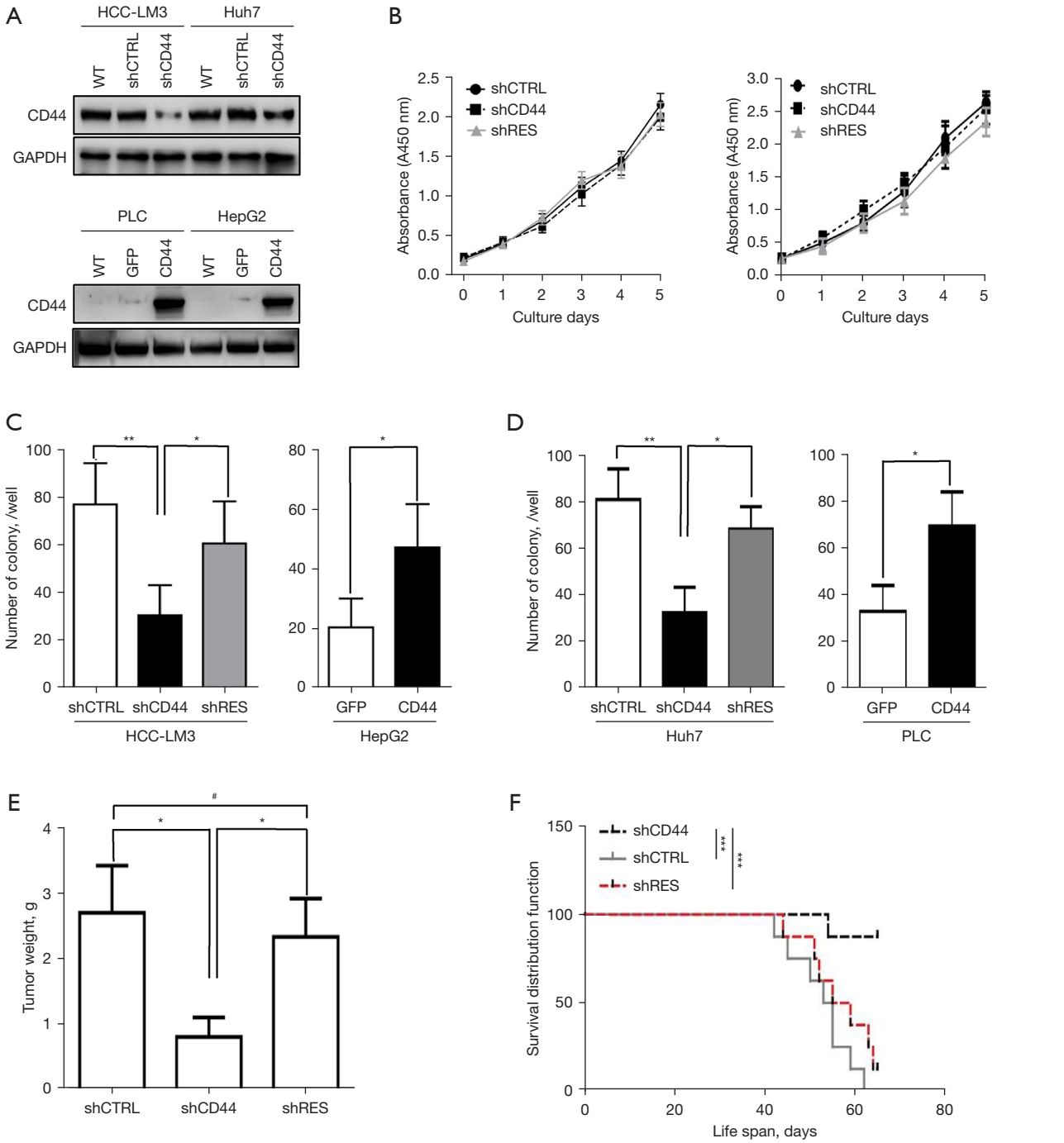

Figure 2 Downregulation of CD44 significantly decreased HCC cell colony formation and HCC progression. (A) Confirmation of the effects of CD44 knockdown using specific shRNA (shCD44 was the most effective in the interference of CD44) and the overexpression in the HCC cell lines used in the following study. (B) CCK8 assays were employed to detect the growth ability of HCC cells from days 0–5 ($P>0.05$). (C,D) Colony formation assays were employed to detect the colony formation ability of HCC cells. (E) Tumor weights of mice 8 weeks after orthotopic implantation (n=6 mice/group). (F) Survival distribution function of mice in different groups. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; #, not significant. HCC, hepatocellular carcinoma; CCK8, Cell Counting Kit-8.

effect on cell proliferation (see *Figure 2B*), which excluded the possibility that acute CD44 depletion is toxic to cells. However, the results of the colony formation assays showed that introducing shCD44 to the HCC-LM3 cells resulted in the significant inhibition of colony formation (see *Figure 2C*, left panel), and similar results were found in the Huh7 cells (see *Figure 2D*, left panel). Conversely, the overexpression of CD44 significantly increased the colony formation compared to lentiviral-green fluorescent protein (LV-GFP)-treated cells in the lower CD44 expression HCC cell lines of HepG2 and PLC (see *Figure 2C*,*2D*, right panel). To exclude the possibility of off-target effects, we reintroduced CD44 with CD44-cDNA engineered to be insensitive to shRNA (shRES) into the CD44 knockdown cells to examine whether the re-expression of CD44 rescued the migration and invasion features. We found that as a result of the reintroduction of CD44, the HCC cells' colony formation ability recovered to an almost normal level (see *Figure 2C*,*2D*).

To determine whether CD44 promotes the tumorigenicity of HCC *in vivo*, HCC-LM3-shCTRL, HCC-LM3-shCD44, and HCC-LM3-shRES were separately transplanted into the nude mice subcutaneously, and intact subcutaneous tumor tissues (1 mm × 1 mm × 1 mm) derived from the indicated cell lines (n=6 mice/group) were orthotopically impanated into the nude mice. We found that 8 weeks after orthotopic implantation, the weights of the HCC-LM3-shCD44-derived xenografts were significantly lighter than the HCC-LM3-shCTRL- and HCC-LM3-shRES derived xenografts (see *Figure 2E*). Further, compared to the mice implanted orthotopically with HCC-LM3-shCTRL, the mice implanted orthotopically with HCC-LM3-shCD44 had a significantly longer survival rate (at day 65, shCTRL *vs.* shCD44, survival rate =0% *vs.* 75%, P=0.001; see *Figure 2F*).

CD44 promotes HCC migration and invasion in vitro and in vivo

To further investigate the role of CD44 in HCC migration and invasion, we performed transwell migration and invasion assays in 3 independent experiments, and found a positive correlation between CD44 and HCC cell invasion (see *Figure 3A*) and migration (see *Figure 3B*,*3C*). Specifically, we found that shCD44 reduced the migration and invasion ability significantly in CD44 high-expression HCC cells, while CD44 overexpression in HepG2 had the opposite effects. Further, following the reintroduction of CD44, shCD44-HCC-LM3 migration and invasion almost recovered to normal levels (see *Figure 3A-3C*). Similarly, in the wound-healing migration assays, microscopic examination at 24 and 48 h revealed a significant delay in the wound closure rate of shCD44 HCC-LM3 compared to that of the control group (see *Figure 3D*,*3E*).

In our *in-vivo* experiments, 2×10^5 GFP-HepG2 or GFP-HepG2-CD44 cells were injected into nude mice (n=6) via the tail vein. After 14 days, the formation of tumor foci in the lungs was evaluated using a fluorescence stereomicroscope imaging system. All the mice injected with the GFP-HepG2-CD44 cells showed tumor formation in the lungs, which was confirmed by H&E staining. Conversely, only 2 of the 6 mice injected with the GFP-HepG2 cells showed tumor formation in the lungs (see *Figure 3F*,*3G*).

CD44 regulates the expression of CXCR4 and the response of HCC cells to SDF-1α

To determine whether CD44 affected the expression of the CXC chemokine receptors, we performed quantitative RT-PCR to detect CXCR1-7 expression with or without CD44 knockdown. We found that the expression level of CXCR4 was the highest among CXCR1-7 in MHCC-97H, and the downregulation of CD44 resulted in the maximum reduction of CXCR4 (see *Figure 4A*). The subsequent verification test showed that CD44 was positively correlated with CXCR4 protein levels in multiple HCC cell lines treated with shCD44 (see *Figure 4B*). To exclude the possibility that CD44 was the downstream molecule of CXCR4, we detected the expression of CD44 in the HCCLM3 cell line when stable CXCR4 was silenced. The results of the western blot showed that there was no difference in the expression of CD44 between the CXCR4 high and low expression groups (see *Figure 4C*). To further confirm the correlation between CD44 and CXCR4 expression in HCC cells, we treated cells with HA, an important ligand of CD44, and observed a significant increase of CXCR4 expression in a time- and dose-dependent manner (see *Figure 4D*). Thus, our results showed that CD44 plays an indispensable role in maintaining CXCR4 expression in HCC cells.

To examine the role of CXCR4 in CD44 mediating the migration of HCC cells, we detected the migration ability of the shCTRL-HCCLM3 cells and shCD44-HCC-LM3 cells in response to SDF-1α. As *Figure 4E* shows, the transwell migration assays demonstrated that the migration ability of shCD44-HCCLM3 cells in response to serial concentrations of SDF-1α (0–100 ng/mL) was at least

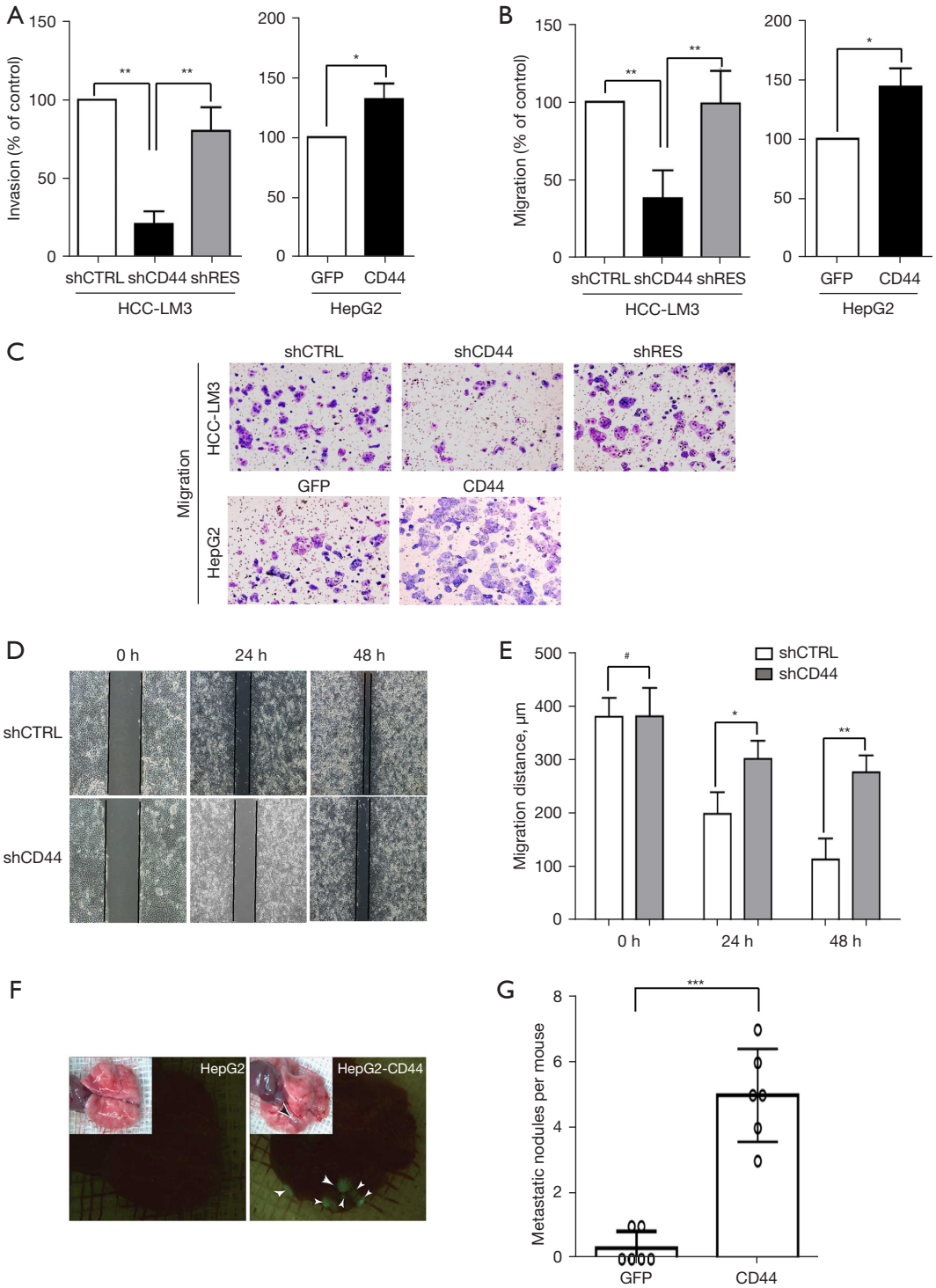

Figure 3 CD44 promoted HCC cell migration and invasion *in vitro* and *in vivo*. (A-C) Transwell assays were employed to detect the migration and invasion ability of HCC cells (observation method: optical microscope; magnification: 100×). (D,E) Wound-healing assays were employed to detect the migration ability of HCC cells, and the migration distances were also evaluated (observation method: inverse microscope; magnification: 100×). (F,G) Images of tumor foci in the lungs were evaluated using a fluorescence stereomicroscope imaging system, and metastatic nodules per mouse were counted using a fluorescence stereomicroscope (n=6 mice/group). *, P<0.05; **, P<0.01; ***, P<0.001; #, not significant. HCC, hepatocellular carcinoma.

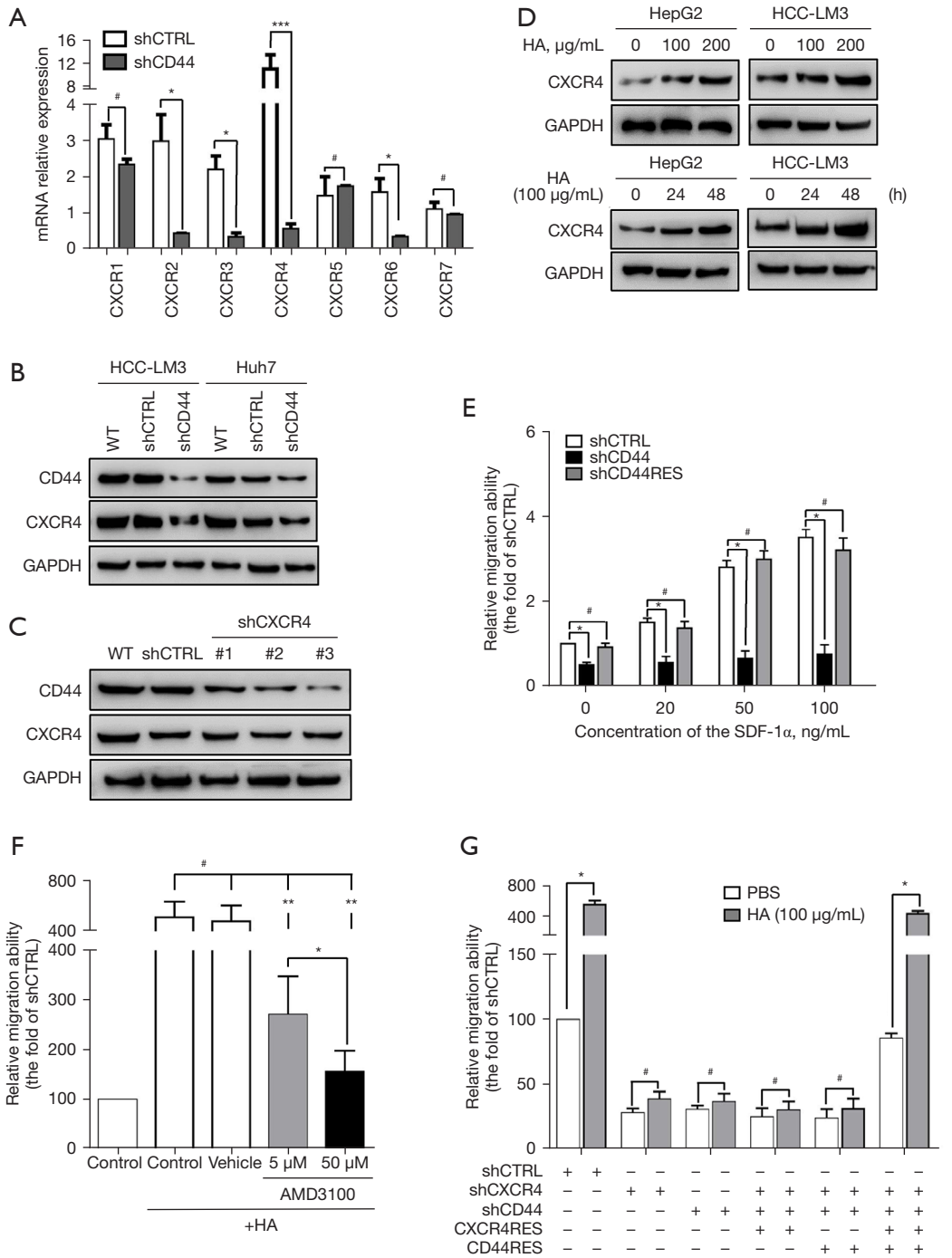

Figure 4 CD44 regulated the migration ability and response to SDF-1α through CXCR4. (A) qRT-PCR was used detect CXCR1-7 expression with or without CD44 knockdown. (B,C) Western blot was employed to detect CD44 and CXCR4 expression with CD44 or CXCR4 knockdown. (D) HCC cells were treated with HA in a time- and dose-dependent manner and western blot was used to detect CXCR4 expression. (E) Transwell migration assays were used to evaluate the migration ability of HCCLM3 cells treated by serial concentrations of SDF-1α (0–100 ng/mL). (F) CXCR4 antagonist AMD3100 and HA were used to treat HCC-LM3 cells, and the migration ability of cells was detected by transwell migration assays. (G) Transwell migration assays were used to evaluate the migration ability of HCCLM3 cells with indicated treatments. *, P<0.05; **, P<0.01; ***, P<0.001; #, not significant, +, adding agents; –, not adding agents. qPCR, real-time quantitative reverse transcription PCR; HA, hyaluronic acid; SDF-1α, stromal cell-derived factor 1α; PBS, phosphate buffer solution.

two times less than that of the shCTRL-HCCLM3 cells, and the reintroduction of exogenous CD44 to the CD44-silenced cells almost returned migration repression back to normal levels.

CXCR4 underpins the role of HA/CD44 in promoting the migration of HCC cells

To explore whether the expression of CXCR4 is necessary in sustaining the HA/CD44 role of promoting migration, a pharmacologic approach was used first with a specific CXCR4 antagonist AMD3100 (22) to attenuate CXCR4 activity. HA/CD44-promoting motility was abrogated by AMD3100 in a concentration-dependent manner (see *Figure 4F*). Next, we generated a series of lentivirus-mediated small-inhibitory RNAs to silence the expression of CXCR4 in HCC-LM3. CXCR4 knockdown significantly reduced the migration of HCC-LM3 cells, but there was no obvious change in the expression of CD44. Further, reintroducing CD44 produced a slight effect in restoring the HA stimulating migration of HCC-LM3-shCD44 when CXCR4 was simultaneously knocked down, whereas reintroduction of CD44 and CXCR4 together produced a significant effect (see *Figure 4G*).

HA-CD44–induced CXCR4 upregulation is AKT/ERK-activity dependent

The roles of the PI3K/AKT and MEK pathways in modulating tumor cells motility are well established. Thus, to better characterize the molecular mechanism by which CD44 regulates CXCR4 in HCC, we first compared AKT and ERK activation between CD44 high and low conditions. We found that the phosphorylation of AKT and ERK increased at indicated times in the condition of HA stimulation in the CD44 high-expression HCC-LM3 cells, while the HA-stimulated activation of AKT and ERK was inhibited when CD44 was downregulated (see *Figure 5A,5B*). These data indicated that CD44 plays a critical role in AKT and ERK phosphorylation during the HA-induced migration of HCC-LM3 cells.

To further confirm that the observed downregulation of CXCR4 and migration inhibition were dependent on AKT and ERK signaling, we treated the HCC-LM3 cells with AKT and ERK inhibitors and subsequently measured the expression of CXCR4 and cell migration, respectively. In relation to the cells treated with the AKT inhibitor LY294002 (5 μM), we found that the expression of CXCR4 was decreased in the HCC-LM3-shCTRL cells, while in the HCC-LM3-shCD44 cells, the expression of CXCR4 was low in the absence or presence of the LY294002 treatment synchronously (see *Figure 5C*). Similar results were found in relation to the HCC-LM3-shCTRL and HCC-LM3-shCD44 cells treated with the ERK inhibitor PD98059 (see *Figure 5D*). Further, the inhibition of AKT or ERK activation resulted in a 2–3-fold inhibition of migration in the HCC-LM3-shCTRL cells when higher concentration of LY294002 or PD98059 were used (see *Figure 5E*).

Discussion

In addition to participating in the development and maintenance of tissue architecture, cell adhesion molecules serve as cell surface receptors and are critical for capturing, integrating, and transmitting signals from the extracellular milieu to the cell interior, and thus play a key role in modulating tumor metastasis. Among a wide variety of adhesion molecules, CD44 and CXCR4 are 2 molecules that have been the subject of increasing studies in recent years. However, currently, the relationship between CD44 and CXCR4 and their combined role in HCC metastasis are largely unknown.

In this study, we confirmed that the expression of CD44 was low in non-invasive HCC cells, but high in invasive HCC cells, which suggests that CD44 plays a promoting role in HCC metastasis. In relation to the prognostic value of CD44, we found that CD44 was correlated with tumor size, AFP level, OS, and TTR. Our univariate and multivariate analyses showed that HCC patients with high levels of CD44 expression generally had worse OS and increased recurrence than those with low levels of CD44 expression. In line with the results of the clinical analysis, we showed the effects of the HA/CD44 interaction on HCC migration and metastasis in our *in-vitro* and *in-vivo* studies. Downregulating the expression of CD44 severely inhibited the migration of the HCC cells. Notably, we found that CD44 upregulated the expression of CXCR4 through the AKT and ERK signaling pathway, and the expression of CXCR4 was critical in the HA-CD44 pro-metastasis role. The combination of CD44 and CXCR4 had significant prognostic value in HCC after hepatectomy.

Our previous study (23) and other studies (24-26) have shown that CD44 is aberrantly expressed in HCC and is implicated in the metastatic process. Additionally, it is reported that miR-199a-3p can target CD44 to inhibit

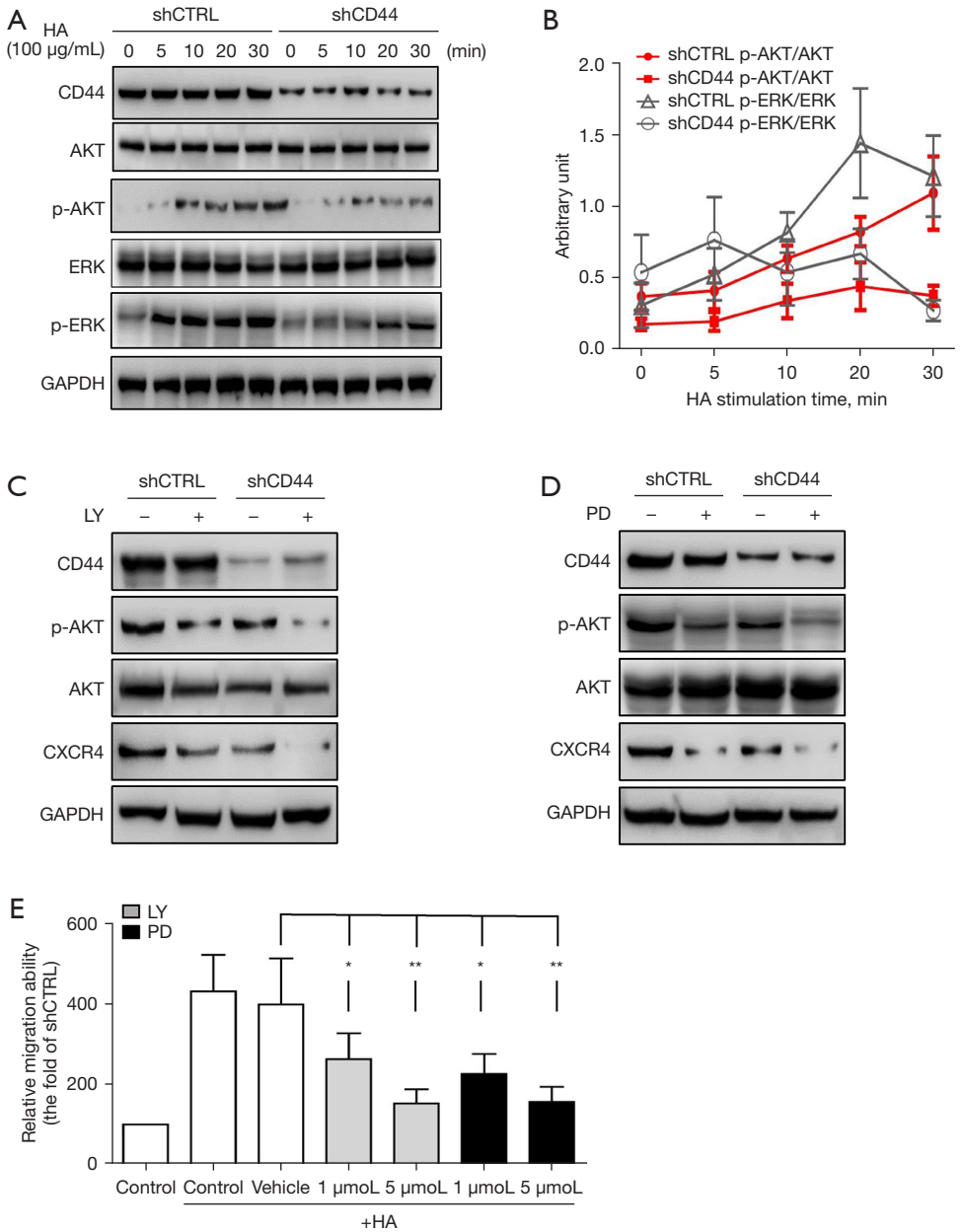

Figure 5 HA-CD44-induced CXCR4 upregulation was AKT/ERK-activity dependent. (A) CD44 knockdown significantly attenuated HA-induced AKT/ERK phosphorylation. (B) The variation tendency of pAKT/AKT and pERK/ERK are shown. (C,D) AKT inhibitor LY294002 (5 μM) or ERK inhibitor PD98059 (5 μM) were used to treat HCCLM3 cells with or without CD44 knockdown. (E) Both LY294002 and PD98059 significantly inhibited HA-induced HCC cell migration in a dose-dependent manner. *, P<0.05; **, P<0.01. HA, hyaluronic acid; AKT, protein kinase B; ERK, extracellular signal-regulated kinase.

the growth of HCC cells (27). A long non-coding RNA (lncRNA) called nuclear enriched abundant transcript 1 (NEAT1) is also required in CD44 expression for liver cancer (28). However, only limited research has been conducted on the role of CD44 in HCC migration and the relationship between CD44 and CXCR4. Previous studies have shown that the high expression of CD44 is correlated with a poor prognosis in bladder cancer patients (29,30). Similarly, previous study has shown that the downregulation of CD44 expression inhibited liver metastases in breast

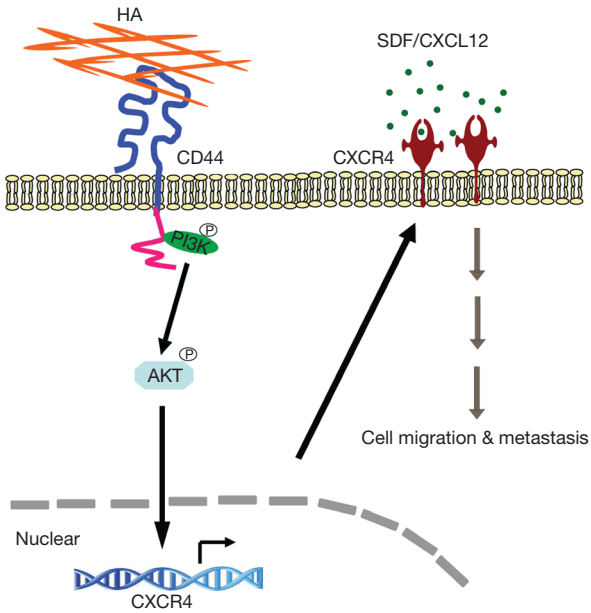

Figure 6 Graphical summary of the molecular mechanisms. CD44 fostered HCC cell migration and invasion by activating the AKT/ERK signaling pathway to upregulate CXCR4 expression. AKT, protein kinase B; ERK, extracellular signal-regulated kinase.

cancer patients (31). In the present study, HCC patients with high expressions of CD44 were prone to suffer from early recurrence and poor OS.

Our findings extend understandings of CD44-promoted invasion in HCC. The downregulation of CD44 resulted in the apparent decrease of CXCR4 among CXCR1-7 molecules. Given that SDF-1α, the ligand of CXCR4, is highly expressed in preferentially targeted organs (the lungs and bones) of HCC patients, we detected the expression of CXCR4 in CD44 high and low expression HCC cells, and found that HA/CD44 upregulated the expression of CXCR4, while the level of CXCR4 had little effect on the expression of CD44. Additionally, the CXCR4 inhibitor AMD3100 attenuated the HA-induced migration of CD44 high-expression ß HCC-LM3 cells. Further, the migration of HCC-LM3 increased significantly when HCC-LM3 was treated with SDF-1α/CXCL12, which was inhibited in HCC-LM3-shCD44. Notably, the migration of HCC-LM3-shCD44 was recovered when shCD44 insensitive CD44-cDNA was reintroduced. *In-vitro* results have shown that SDF-1α significantly stimulates the migration of CXCR4 high-expression HCC cells (32) and gastric carcinoma cells (33). To further investigate the role of CD44 in HCC progression, an *in-vivo* orthotopic mice model was adopted, and the results showed that compared to the HCC-LM3-shCTRL group, downregulating CD44 decreased HCC growth and prolonged survival significantly, while restoring the expression of CD44 led to higher tumor weights and shorter survival. Collectively, the results of the *in-vitro* and *in-vivo* experiments suggest that CD44 is crucial in HCC migration and metastasis, and the upregulation of CXCR4 might be a key step in maintaining its pro-metastasis role.

In addition to its adhesion function, CD44 also plays a key role in converting mechanical forces to biochemical signals. Sporadic studies have indicated that HA-CD44 mediates the migration of cancer cells, which may be related to the activation of AKT, ERK, and janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) (34-36). We found that the HA-CD44 axis was able to activate the AKT and ERK pathway and stimulate HCC-LM3 cell migration, and similar results have been found in relation to breast cancer (36). Notably, the inhibition of AKT and ERK by AKT and ERK inhibitors, respectively, significantly blocked the chemotactic responses of the HCC cells triggered by HA or SDF-1α. Thus, the CD44-promoted increase in CXCR4 may be AKT/ERK-activity dependent, and play an essential role in assisting in the migration and invasion of HCC.

In summary, we identified the importance of CD44 in underpinning the migration and invasion of HCC and showed the predictive role of CD44 in OS and recurrence after hepatectomy. Our results indicate that CD44 promotes HCC cell migration and invasion by activating the AKT/ERK signaling pathway to upregulate CXCR4 expression (see *Figure 6*). The capacity of CD44 to induce the expression of CXCR4 and the increased propensity of HCC to invade and metastasize to specific distant organs is consistent with the aggressive clinical characteristics of HCC. Our findings provide new insight into the roles of CD44 in CXCR4-mediated HCC progression, and CD44 may be a potential target for HCC therapy.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE and MDAR reporting checklists. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-2482/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-2482/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-2482/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Research Ethics Committee of the Zhongshan Hospital, Fudan University (No. 2006-653). Informed consent was taken from all the patients. Animal experiments were performed under a project license (No. 2020632) granted by the Animal Ethics Committee of Zhongshan Hospital, Fudan University, in compliance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" for the care and use of animals.

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