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RESEARCH PAPER

PAF enhances cancer stem cell properties via β-catenin signaling in hepatocellular carcinoma

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ABSTRACT

Increasing proofs have declared that liver cancer stem cells (CSCs) are the main contributors to tumor initiation, metastasis, therapy resistance, and recurrence of hepatocellular carcinoma (HCC). However, the molecular mechanisms underlying CSCs regulation remain largely unclear. Recently, PCNA-associated factor (PAF) was identified to play a key role in maintaining breast cancer cell stemness, but its role in liver cancer stem cells has not been declared yet. Herein, we found that both mRNA and protein expression levels of PAF were significantly higher in HCC tissues and cell lines than normal controls. CSC-enriched hepatoma spheres displayed an increase in PAF expression compared to monolayer-cultured cells. Both loss-of-function and gain-of-function experiments revealed that PAF enhanced sphere formation and the percentage of $CD133^+$ or $EpCAM^+$ cells in HCCLM3 and Huh7 cells. In the xenograft HCC tumor model, tumor initiation rates and tumor growth were suppressed by knockdown of PAF. Mechanistically, PAF can amplify the self-renewal of liver CSCs by activating β-catenin signaling. Taken together, our results demonstrate that PAF plays a crucial role in maintaining the hepatoma cell stemness by β-catenin signaling.

Abbreviations: CSCs: cancer stem cells; HCC: hepatocellular carcinoma; PAF: pCNA-associated factor.

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KEYWORDS
PAF; hepatocellular carcinoma cells; β-catenin signaling

1. Introduction

Hepatocellular carcinoma (HCC), comprising over than 70% of primary liver cancer cases, has a high fatality rate [1–3]. In spite of the rapid progresses that have been achieved in HCC detection and treatment, the overall survival rate is still poor due to frequent recurrence and therapy resistance [4].

A great number of studies have declared that a small cluster of cells with increasing capabilities of tumor-initiating and self-renewal, known as cancer stem cells (CSCs), located in various kinds of tumor including hematologic [5], brain [6], breast [7], prostate [8], liver [9], pancreas [10] and colon cancer [11]. These cells are considered to be the primary cause of metastasis, relapse, and therapy resistance of HCC [12,13]. Based on the differentiation stage, liver CSCs express specific markers, such as epithelial cell adhesion molecule (EpCAM), cluster of differentiation 133 (CD133), CD44, CD13 and CD24 [14–17]. Therefore, liver CSCs are becoming attractive targets for liver cancer therapy and prevention strategies. Kaori et al. recently reported that engineered biological nanoparticles containing small interfering RNA targeting β-catenin significantly reduced the tumor growth rate by suppressing the proliferation of hepatic cancer stem cells [18]. However, because the mechanisms underlying maintaining the self-renewal of liver CSCs are poorly understood, therapeutic strategies directly targeting stem cells in HCC are few until now.

PAF (also known as KIAA0101 and OEATC-1) is defined as a proliferating cell nuclear antigen (PCNA)–interacting protein [19]. PAF participates in an extensive range of biological activities like DNA replication and cell cycle progression by interacting with PCNA [20]. Overexpression of PAF was closely related with tumor development and weak prognosis in a broad spectrum of human cancers, which acted as an oncogene by enhancing cancer cell proliferation, migration and invasion

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[21–24]. A recent study has reported that PAF exerts functions in maintaining the stemness properties of breast cancer cell by positively modulating Wnt signaling [25]. Nevertheless, the exact role of PAF in liver CSCs remains largely unknown.

The present study aimed to determine whether PAF regulates the stemness of hepatocellular carcinoma cells. Our results demonstrated that PAF mRNA and protein expression were both up-regulated in HCC tumors and liver CSCs. To evaluate the effects of changes in PAF expression on the stemness of CSCs, we constructed lentiviral vectors able to inhibit or increase PAF gene expression in HCC cell lines. By knockdown or overexpression of PAF, our results revealed that PAF enhanced the self-renewal ability in liver CSCs via activating β-catenin signaling.

2. Materials and methods

2.1 HCC specimens

HCC and normal control liver tissues were acquired from 20 HCC patients who accepted surgery at Shaanxi Provincial Cancer Hospital, from 2010 to 2016. After histopathology confirmation, these tissues were stored at −80°C for further use. This study was authorized by the Ethics Committee of Shaanxi Provincial Cancer Hospital, and all patients or their guardians signed the informed consent form.

2.2 Cells

The human HCC cell lines: HCCLM3, PLC/PRF/5, Huh7, and Hep3B, and normal control cell line THLE-2 were purchased from Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai Institute of Cell Biology), and maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, USA) including 10% fetal bovine serum (AccuRef Scientific, Xi'an, China) at 37°C in an atmosphere containing 5% CO_2.

2.3 Plasmid construction, lentivirus production, and siRNA transfection

The CDS sequence of PAF was generated by PCR and inserted into pcDNA3.1 vector (Invitrogen) to construct PAF overexpression vector. HCCLM3 and Huh7 cells with 60% confluence were transfected with lentivirus-PAF-shRNA or lentivirus-PAF-shNC for 24 h and then selected for a week in a medium supplemented with puromycin (Sigma, USA) to obtain stable expression cells. Small interfering RNA (siRNA) for silencing β-catenin was purchased from GenePharma (Shanghai, China). The sequence was as follows: si-β-catenin sense: 5′-UGGUUGCCUUGCUCAACAA-3′; si-β-catenin anti-sense: 5′-ACCAA CGGAACGAGUUGUU-3′; si-NC sense: 5′-UUCUCCGAACGUGUC ACGUUU-3′; si-NC anti-sense, 5′-ACGUGA CACGUUCGGAGAAUU-3′.

2.4 RNA extraction and qRT-PCR

Total RNAs were separated from HCC cells or tissues using Trizol reagent (Invitrogen, USA) and reverse transcription reaction was conducted by using Super M-MLV reverse transcriptase (Tiangen, Beijing, China) and random primers. The Bestar™ Real-time PCR Master Mix was used for quantitative PCR analysis using the SYER Green Method. β-actin was used as the internal control. The primers were obtained from Sangon Biotech and listed as following: PAF, 5′-ATGGTGCGGACTAAAGCAGAC-3′ (forward) and 5′-CCTCGATGAAACTGATGTCGAAT-3′ (reverse); β-actin, 5′-GGGACCTGACT GACTACCTC-3′ (forward) and 5′-TCATACTCCTGCTTGCTGAT-3′ (reverse).

2.5 Western blot analysis

The levels of protein expression were analyzed by western-blot analysis as previous described [26]. Briefly, proteins were collected from cells or tissues using RIPA (AccuRef Scientific). After quantification by the BCA Protein Assay kit (EXPrecision, Xi'an, China), equal amounts of protein were isolated by 12% SDS-PAGE gels, transferred to the PVDF membrane. The PVDF membranes were cut into small pieces containing target protein bands as designated by a protein ladder (AccuRef Scientific). After blocking with 1% BSA (Sigma-Aldrich) for 1 h at room temperature, the membranes were incubated with anti-PAF (1:2000; ab226255; Abcam), anti-t-Akt (1:500; ab8805; Abcam), anti-p-Akt

(1:500; ab38449; Abcam), antip-GSK3β (1:500; ab68476; Abcam), GSK-3β (1:1,000; ab32391; Abcam), anti-β-catenin (1:5000; ab32572; Abcam), or anti-β-actin (1:5,000; ab179467; Abcam), respectively, overnight at 4°C. Following incubating with horseradish peroxidase-labeled secondary antibodies at room temperature for 1 h, the blots were visualized by chemiluminescent gel imaging system of ChemiDoc XR (Bio-RAD). β-actin was acted as an internal control.

2.6 Spheroid formation assay

HCC cell lines HCCLM3 and Huh7 at a density of $1x10^3$/ml were seeded in an ultra-low attachment 6-well plate (Corning Incorporated, USA) including 2% B27 (Invitrogen), 20 ng/ml basic fibroblast growth factor (bFGF, Sigma), and 20 ng/ml rhEGF (PeproTech, USA). After 7 days, the tumor spheres with a diameter larger than 50 μm were photographed and calculated.

2.7 Flow cytometry

HCCLM3 and Huh7 cells washing with PBS containing 2% fetal bovine serum were centrifuged at 1000 rpm for 5 min. Then, cell suspension with a concentration of 10^7 cells/mL was incubated with APC-conjugated-EpCAM antibody or PE-conjug-CD133 antibody (BD Biosciences, San Diego, CA, United States) for 30 min at 4°C. IgG antibody labeled with APC or PE was served as isotype control. Following that, the proportions of $CD133^+$ or $EpCAM^+$ HCC cells were analyzed on a Becton-Dickinson FACS Calibur flow cytometer.

2.8 Experiment animal models

To evaluate the tumor formation ability, cells were mixed with Matrigel (Becton Dickinson) at a volume ratio of 1:1 and injected subcutaneously into NOD/SCID mice (6-week-old, Shanghai, China) with indicated number. The mice were sacrificed 7-week post-inoculation, and the incidence of tumor was recorded.

To observe whether PAF promoted sphere cell growth *in vivo*, ten of mice were randomly divided into two groups with Huh7 sh-NC or Huh7 sh-PAF sphere cell injection subcutaneously. After that, the mice were measured every 3 days and tumor volumes were calculated by $V = (a^2 \times b)/2$. The mice were sacrificed on day 40 after first injection. The xenograft tissues were then removed and weighed. All experiments using animals were obeyed the guidelines of the animal care and use committees of Shaanxi Provincial Cancer Hospital.

2.9 Statistics analysis

All results were displayed as mean ± SEM and analyzed using Prism 6.0 software. Significant between two-group differences was evaluated by Student's t-test. When comparing the differences more than two groups, one-way ANOVA was performed. The significance was set as $p < 0.05$.

3. Results

3.1 PAF was up-regulated in HCC tissues and cells

The level of PAF in 20 HCC tumor specimens and adjacent control specimens was measured by qRT-PCR analysis. Our result demonstrates that the mRNA level of PAF is remarkably increased in HCC tissues compared to controls (Figure 1a). Western blot results also revealed that PAF protein level was significantly up-regulated in four cases of HCC tumor tissues compared with the relevant normal controls (Figure 1b). Moreover, the expression of PAF in normal control cell THLE-2 and HCC cell lines was evaluated by qRT-PCR and western blot analysis. A notable elevation of PAF level was observed in Huh7, HCCLM3, Hep3B and PLC/PRF/5 cells (Figures 1c and 1d) by comparison with THLE-2 cells,

3.2 PAF expression was elevated in liver CSCs

Next, we cultured spheres of Huh7 and HCCLM3, and detected the expression of stemness markers of hepatoma spheres. The representative photos of spheres from two HCC cell lines are shown in Figure 2a. CD133 and EpCAM), the two markers of stemness, were significantly higher in spheroids generated from HCCLM3 and HUH7 cells compared to adherent states (Figure 2b), suggesting that sphere culture could enrich liver CSCs. We

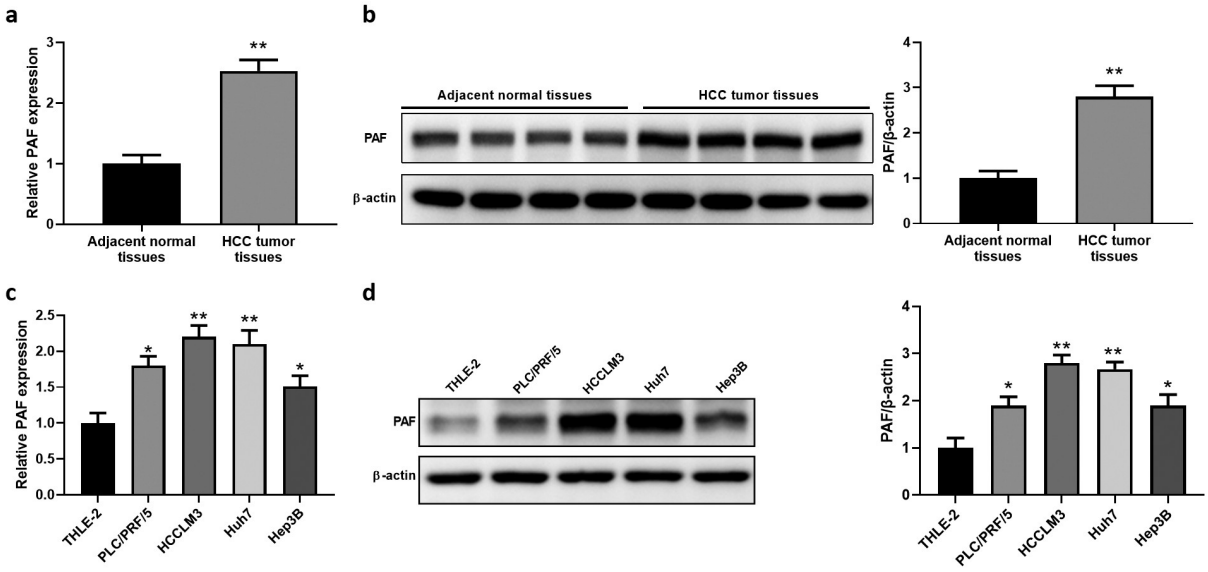

Figure 1. PAF expression is up-regulated in HCC samples and cell lines.

A. Analysis of the average PAF mRNA expression levels in HCC tumor tissues and the corresponding normal tissues by qRT-PCR.B. The protein levels of PAF in four cases HCC tumor tissues and the corresponding normal tissues were detected by western blot analysis. C Analysis of PAF mRNA expression levels in HCC cell lines (Huh7, HCCLM3, PLC/PRF/5, Hep3B) and the normal liver cell lines (THLE-2) by qRT-PCR.D. Western-blot analysis of PAF expression in HCC cell lines and normal liver cell lines.

further detected PAF expression in spheroids from HCC cells. We found that both mRNA and protein levels of PAF were markedly elevated in spheres in contrast to adherent cells (Figures 2c and 2d), indicating that PAF might play a role in liver CSCs.

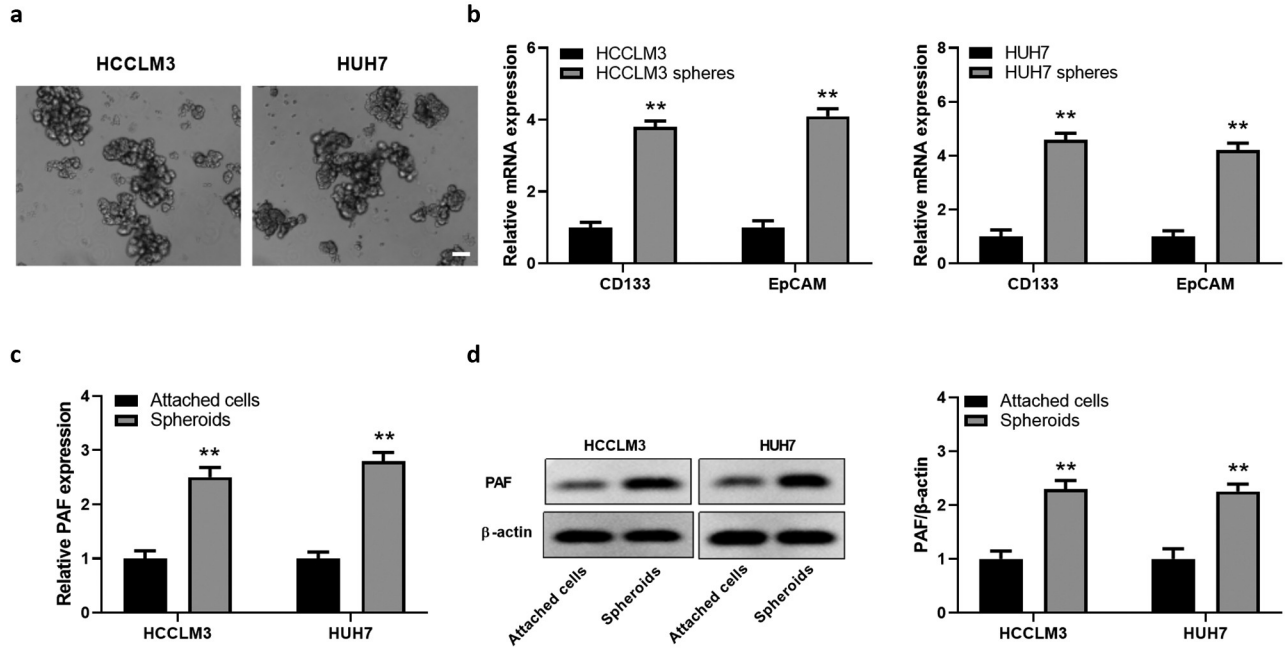

Figure 2. PAF expression levels are elevated in spheres derived from HCC cell lines.

A. The representative photos of spheroids formed from two HCC cell lines (Huh7 and HCCLM3). Scale bar = 100 μm.B. The mRNA levels of CD133 and EpCAM, two markers of stemness, were measured in spheroids and adherent cells by qRT-PCR analysis.C. PAF mRNA expression was measured in spheroids and adherent cells by qRT-PCR analysis.D. PAF protein expression was measured in spheroids and adherent cells by western-blot.

3.3 PAF enhanced the self-renewal ability of liver CSCs

To further determine the function of PAF in CSCs regulation, HCCLM3 and HUH7 cells were transfected with sh-PAF to knock down their expression. The sh-PAF stable transfectants were established, as verified by examining PAF mRNA and protein levels (Figures 3a and 3b). Compared with control, depletion of PAF resulted in an obvious decrease in the number of spheroids (Figure 3c). FCM results displayed a reduced percentage of $CD133^+$ or $EpCAM^+$ cells (which represented CSCs) in PAF-deleted HCCLM3 and Huh7 cells (Figure 3d). Next, we established HCC cell lines over-expressing PAF by transfecting PAF-expression vector into HCCLM3 and Huh7 cells, which is confirmed by measuring PAF expression with qRT-PCR and western-blot analysis (Figures 4a and 4b). HCC cells overexpressing PAF caused an obvious increase in the number of spheroids and percentage of liver CSCs by comparison with

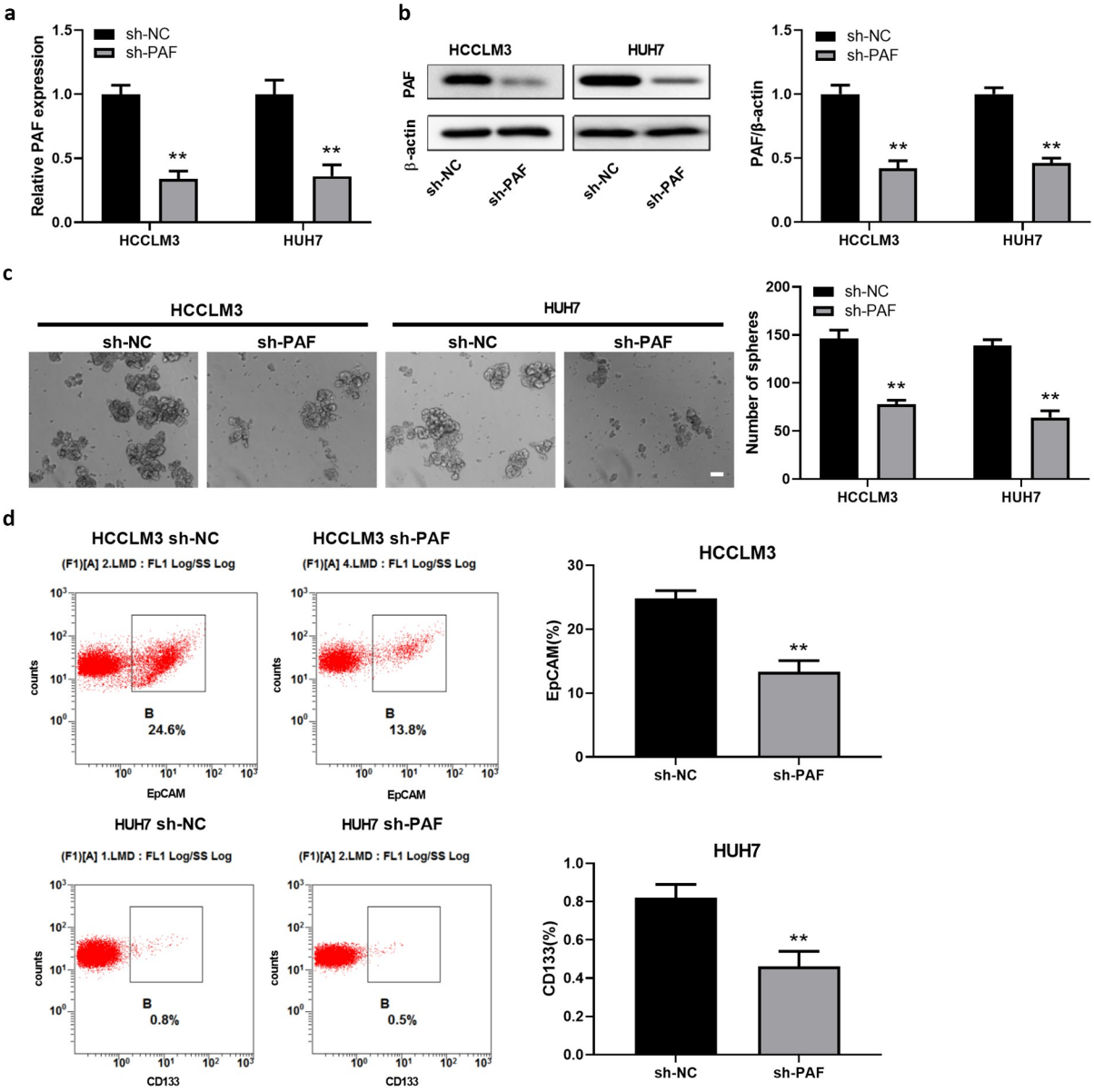

Figure 3. PAF knockdown significantly impairs self-renewal ability of liver CSCs.

A. qPCR analysis validated the effects of PAF knockdown in HCC cells.B. Western-blot analysis confirmed the effects of PAF knockdown in HCC cells.C. Representative images of spheres after PAF knockdown (sh-NC was scramble control). Scale bar = 100 μm.D. The proportion of CD133+ or EpCAM+ HCC cells in PAF knockdown transfectants was evaluated using flow-cytometric assays.

control cells (Figures 4c and 4d). These data revealed that PAF may be involved in HCC development via enhancing the self-renewal ability of CSCs.

3.4 PAF promoted tumor initiation and growth *in vivo*

One of the key features of CSCs is their tumor formation capability. To further test the effect of PAF on tumorigenicity, Huh7 cells expressing sh-PAF or sh-NC were injected into NOD-SCID mice subcutaneously followed by evaluating tumor occurrence after inoculation. We found that Huh7 cells expressing sh-PAF displayed lower tumor incidences than control cells, whether mice were inoculated with 1×10^3, 1×10^4, or 1×10^5 cells (Table 1). Next, the action of PAF on sphere cell growth *in vivo* was evaluated by implanting the Huh7 sphere cells expressing sh-PAF into NOD-SCID mice. The representative photos of xenografts are shown in Figure 5a.

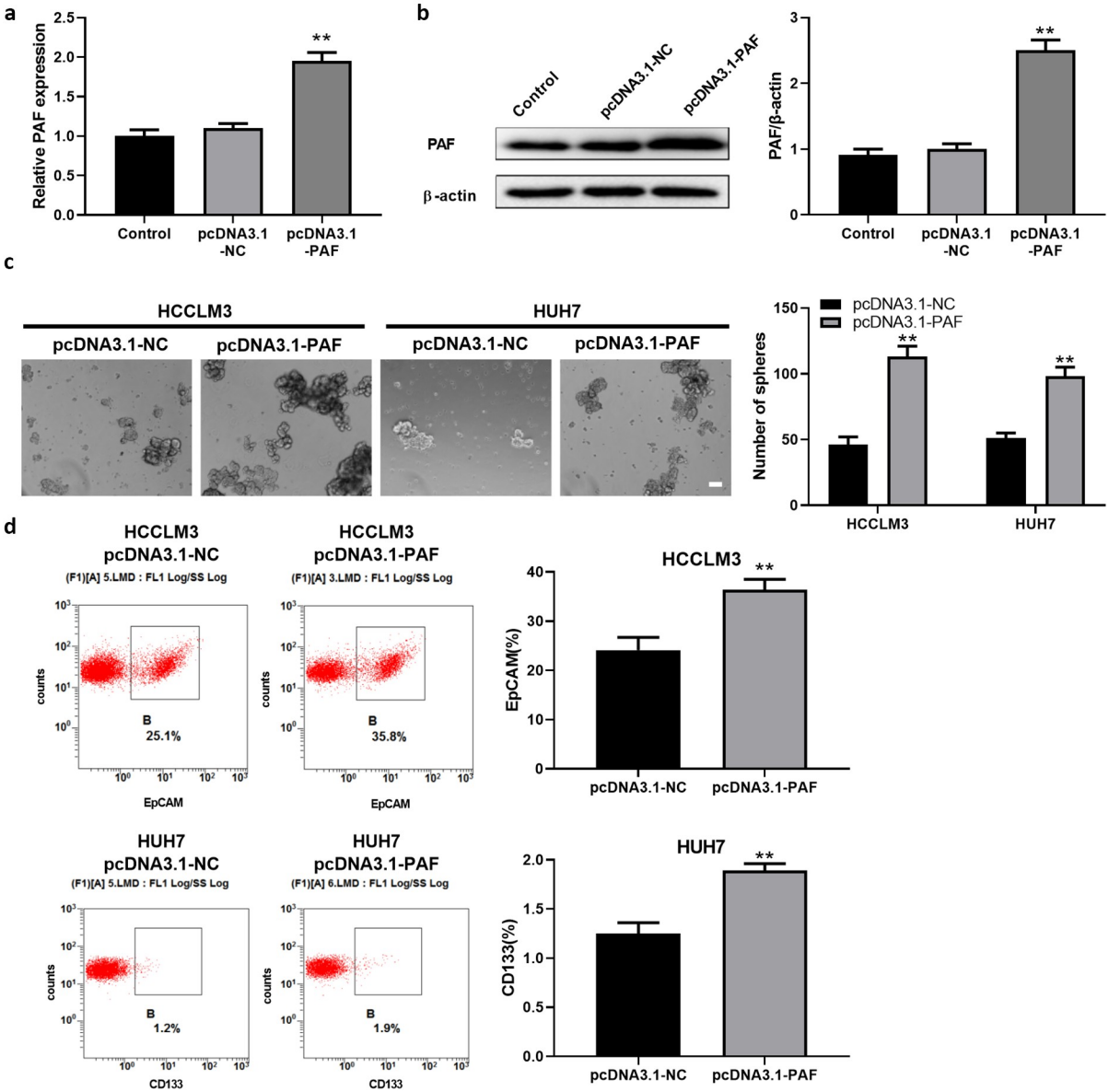

Figure 4. PAF over-expression promotes sphere formation of HCC cells.

A. qPCR analysis validated the effects of PAF over-expression in HCC cells.B. Western-blot analysis confirmed the effects of PAF over-expression in HCC cells.C. Representative images of spheres after PAF over-expression (pcDNA3.1 as a control). Scale bar = 100 μm.D. The proportion of $CD133^+$ or $EpCAM^+$ HCC cells in PAF over-expression transfectants was evaluated by flow-cytometric assay.

PAF deletion significantly reduced the average tumor volume and weight of xenografts (Figures 5b and 5c). QRT-PCR analysis revealed that PAF level in xenografts was much lower in sh-PAF group compared to control group (Figure 5d).

3.5 The enhancement of self-renewal ability in liver CSCs was β-catenin dependent

It has been reported that PAF activates Wnt/β-catenin signaling to maintain the stemness of breast cancer; thus, we detected β-catenin level in liver CSCs. We found that PAF ablation significantly reduced the expression of β-catenin in spheroids from HCCLM3 and Huh7 cells (Figure 6a). Moreover, depletion of β-catenin by specific siRNA abolished the difference in the number of spheroids between sh-PAF transfected HCCLM3 and Huh7 cells and their corresponding control cells (Figure 6b). In accordance with the above observation, we found that the discrepancy in the proportion of liver CSCs between sh-PAF transfected HCC cells and control cells was also eliminated (Figure 6c). Therefore, these findings suggested that PAF could regulate self-renewal of liver CSCs via a β-catenin dependent way.

Table 1. Tumor initiation of HUH7 cells in NOD-SCID mice.

Number of cells injected	HUH7 sh-NC	HUH7 sh-PAF
10^3	2/5	0/5
10^4	5/6	3/6
10^5	6/6	4/6

NOD-SCID mice: non-obese diabetic-severe combined immune deficiency mice
HUH7 cells: human hepatocellular carcinoma cell lines

3.6 PAF promotes the expression of β-catenin via activation of Akt/GSK3β signaling

Previous study had reported that Akt signaling was involved in the regulation of β-catenin [27]. To investigate the potential molecular mechanism by which PAF is able to modulate β-catenin signaling, we performed western blot assay to detect the levels of Akt and its downstream protein GSK3β. As shown in Figure 7, depletion of PAF significantly decreased the phosphorylated Akt and phosphorylated GSK3β, while total Akt and total GSK3β remained unchanged when compared with negative control. As expected, the downregulation of PAF also decreased the protein level of β-catenin (Figure

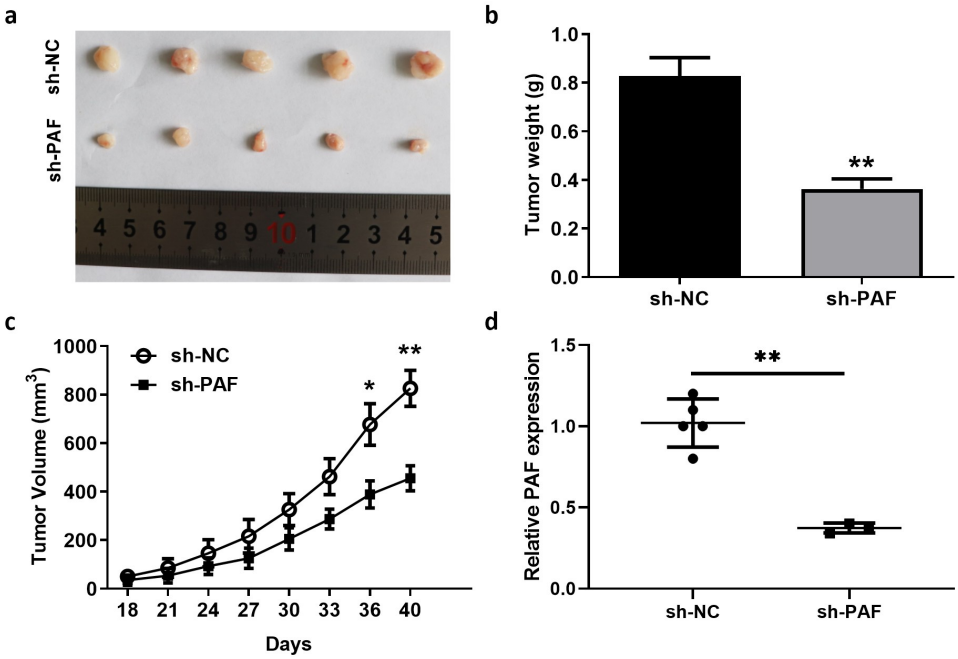

Figure 5. PAF knockdown significantly represses the tumor formation and growth of HCC *in vivo*. NOD-SCID mice were subcutaneously injected with Huh7 sh-PAF or control sphere cells (1×10^4).

A. On day 40 after initial injection, the xenograft tissues were isolated and shown.B and C. Tumor weight (B) and volumes (C) were measured.D. The PAF mRNA level in xenograft tissues was detected by qRT-PCR analysis.

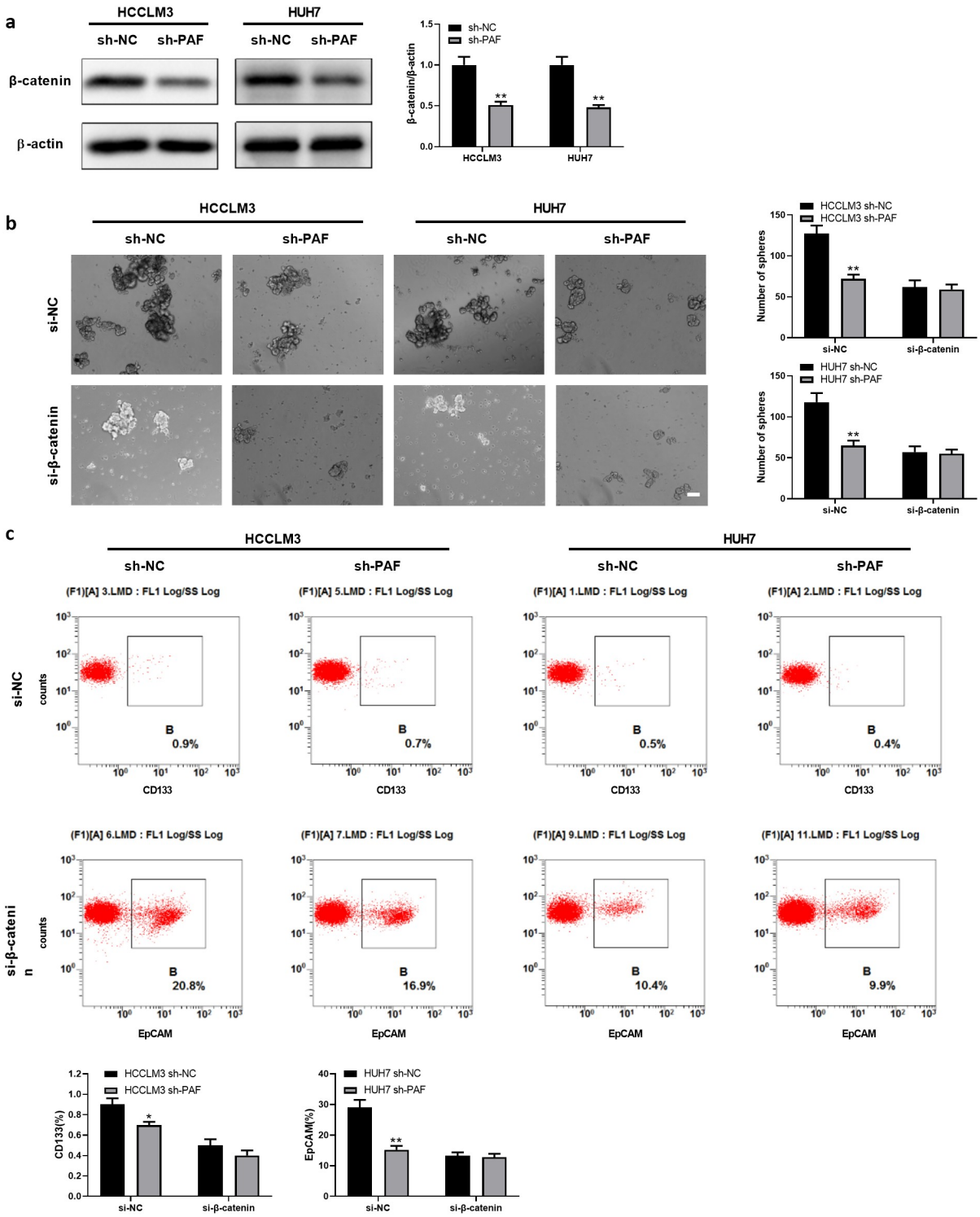

Figure 6. PAF facilitates self-renewal ability of liver CSCs via activating β-catenin.

A. The β-catenin expression in spheroids from Huh7 and HCCLM3 cells transfected with sh-PAF or sh-NC were examined by western blot assay.B. The spheroid formation assays were performed in Huh7 or HCCLM3 cell transfection with si-β-catenin or si-NC, and the number of spheroids was calculated. Scale bar = 100 μm.C. The proportion of CD133+ or EpCAM+ HCC cells in Huh7 or HCCLM3 cell transfection with si-β-catenin or si-NC were determined by flow-cytometric assay.

7). Taken together, these data suggested that PAF modulated the expression of β-catenin by regulating Akt/GSK3β signaling.

4. Discussion

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer. Of all cancers,

HCC ranks the second most lethal tumor worldwide. However, with the advances in methods of diagnosis and treatment, the survival rate of HCC patients is poor. Hepatocellular carcinoma with a high rate of recurrence and heterogeneity comprised heterogeneous cell populations [28,29], among which cancer stem cells (CSCs) are key drive force for tumorigenesis, chemo-resistance, and recurrence of HCC [30]. HCC patients with higher numbers of CSCs predicted poorer outcomes. Thus, the discovery of a novel molecular modulating stemness of CSCs could contribute to the advancement of HCC treatment. In the present study, we demonstrated that PAF exerted important functions in liver CSCs self-renewal and could be a novel target in HCC therapy.

PAF was initially identified as a PCNA-associated factor by using a yeast two-hybrid screen system [20], which acted as an oncogene in several types of tumors including hepatocellular carcinoma. In this study, PAF levels in HCC tissues and cells were measured. Both mRNA and protein expression of PAF were notably up-regulated, which was consistent with the previous studies. Due to the key role of CSCs in tumor development and recurrence, we explored the effect of PAF on liver CSCs. CD133 and EpCAM are two cell surface markers expressed in CSCs that modulate stem cell properties in liver CSCs. Using two HCC cell lines, HCCLM3 and Huh7, we set up suspension spheroid culture condition and detected the expression of CD133 and EpCAM. Our results showed that the level of CD133 and EpCAM were both higher in spheroids compared to adherent states, verifying that liver CSCs were enriched by spheroid culture of cancer cells. Moreover, we observed that PAF mRNA and protein expression were obviously higher in spheroids. These findings indicated that PAF might be involved in liver cancer stem cell regulation.

PAF has been reported to play a crucial role in maintaining the stemness features of breast cancer cells via regulating cell plasticity [25]. However, its function in liver CSCs is largely obscure. In the present study, through knockdown or overexpression of PAF, we demonstrated that PAF increased spheroid formation and the percentage of $CD133^+$ or $EpCAM^+$ hepatoma cells. Xenograft model further supported that PAF promoted sphere propagation in *vivo*. These data indicate that PAF is needed for liver CSC expansion by enhancing liver CSCs self-renewal and may provide a hopeful target for HCC therapy.

Wnt/β-catenin was identified as an important signaling pathway to regulate self-renewal of liver CSCs

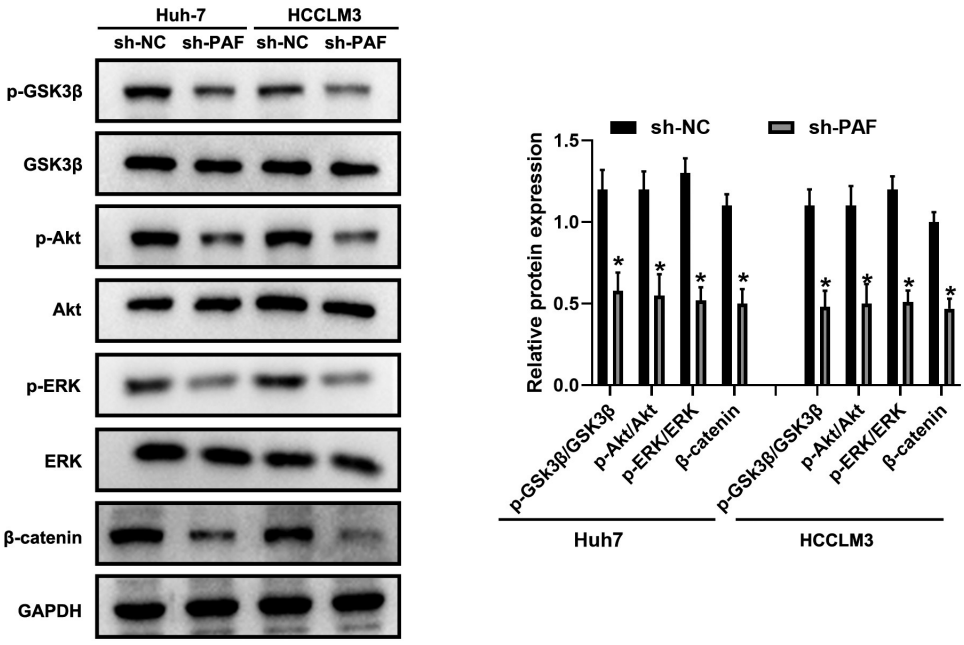

Figure 7. Downregulation of PAF deactivates Akt/GSK3β signaling.

HCC cells transfected with either sh-PAF or sh-NC vector were harvested and the total protein was extracted for western blot analysis of Akt, p-Akt, GSK3β, p-GSK3β, and β-catenin. β-actin was used as loading control. Gray-scale quantification data from three biological repeats are shown as mean ± SD in the right panel.

in human HCC. It has been reported that anti-miR -181 inhibitors could inhibit stemness-related gene level and tumorigenicity in EpCAM$^+$ HCC via blocking Wnt/β-catenin pathway [31]. To explore the downstream signaling pathway underlying the modulation of CSCs by PAF, we assessed the level of β-catenin in hepatoma spheroids. Consistent with previous studies, we found that PAF upregulated β-catenin expression, thus resulting in the enhanced self-renewal of liver CSCs. Numerous studies have reported that the activation of Akt signaling plays a critical role in the pathogenesis of hepatic cancer [32–34]. Activation of Akt suppresses GSK3β by phosphorylation at Ser9 [35], resulting the increment of β-catenin [36]. Using RNA interference, we found that downregulation of PAF significantly reduced phosphorylated Akt and phosphorylated GSK3β, by which the expression of β-catenin was inhibited (Figure 7), indicating Akt signaling is potentially involved in the PAF-regulated β-catenin expression. Consistently, Liu et al. found that Akt activation promotes the expression of β-catenin, resulting in the progression of hepatocellular carcinoma [37]. A recent study demonstrated that PAF was significantly increased in the high-grade serous ovarian cancer and silencing PAF could markedly decrease the phosphorylation of PI3K, AKT, and mTOR and promoting cisplatin sensitivity of ovarian cancer cells [38]. This evidence suggests that PAF might regulate the GSK3β/β-catenin pathway in HCC via Akt activation.

In conclusion, the findings of our study demonstrated that it is a novel regulator of PAF. PAF levels were obviously increased in HCC tumors and liver CSCs. In addition, PAF overexpression enhanced liver CSC properties via activating β-catenin signaling. Therefore, targeting PAF could be a hopeful strategy for CSC therapy in HCC.

Acknowledge

Not applicable

Disclosure statement

No potential conflict of interest was reported by the authors.

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No Funding was received.

Data availability

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

Authors' contributions

LM and LZB conceived and designed the experiments. MXD, SJR and ZPT performed the experiments. CY and LY analyzed and interpreted the data. MXD wrote the manuscript. LM and LZB revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and informed consent

Written informed consent was obtained from all patients and the study protocol was approved by the Ethics Committee of Shaanxi Provincial Cancer Hospital. Animal care and study were approved by the Institutional Animal Care and Use Committee of Shaanxi Provincial Cancer Hospital.

Patient consent for publication

Not applicable.

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