Research Square

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PCGF2 and PCGF4 Oppositely Drive Stem-like Properties in Hepatocellular Carcinoma

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Abstract

Background: PCGF4 as a cancer stem cells (CSC) marker displays stem cell-like properties, and drug resistance in hepatocellular carcinoma (HCC). PCGF2, a homologue of PCGF4, the effect of PCGF2 on liver CSCs (LCSCs) and drug resistance and the molecular mechanism of this effect have not been documented.

Methods: To measure the cell viability, CSCs properties of the cells, the Cell Counting Kit-8, spheroid assay, and flow cytometry assays were applied in the HCC cell lines, respectively. The self-renewal was determined by limiting dilution assay. Also, IHC and western blotting were used to detect protein expression of PCGF2 and PCGF4 in human HCC tissues, cell lines and the effects of PCGF2 overexpression on the p38 MAPK genes expression. Kaplan-Meier curves were generated for overall survival (OS) and disease-free survival (DFS). We performed KEGG analysis on target genes through the R language cluster profiler package.

Results: IHC showed that expression of PCGF4 and PCGF2 correlate inversely in HCC cell lines and HCC tumors. Overexpression of PCGF2 inhibited the stemness of HCC cells refected by decreasing sphere-forming and self-renewal capacities as well as the expression of CSCs markers. Interestingly, down-regulating PCGF4 led to similar results as up-regulating PCGF2. We also found that PCGF2 and PCGF4 oppositely regulated the stem-like properties driven by the p38 MAPK signalling pathway.

Conclusion: Our results suggest that PCGF2 inhibits the stem cell population, reduces the sphere formation ability in HCC cell lines, and increases sensitivity to sorafenib by targeting p38 MAPK signalling.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors, with high morbidity and mortality [1]. According to statistics, the recurrence rate of HCC is as high as 60–70% [2, 3]. Radical resection is the first choice of treatment for HCC, but the 5-year survival rate of patients postoperatively is only 30–50% [4]. Moreover, chemotherapy resistance reduces the efficacy of chemotherapeutic drugs, such as sorafenib, to a great extent. Malignant behaviours such as easy recurrence, poor prognosis and chemotherapy resistance of HCC can be attributed to the existence of liver cancer stem cells (LCSCs) [5] and exploring relevant diagnosis and treatment schemes for LCSCs is of great significance for treating HCC.

B lymphoma Mo-MLV insertion region 1 homologue (BMI1), also known as PCGF4, is a member of the polycomb repressive complex1 (PRC1) family of proteins [6]. Published articles have shown that CSCs are the main factor influencing tumor recurrence after treatment and that activation of some proto-oncogenes in CSCs, such as PCGF4, cause tumor recurrence [7, 8]. Moreover, PCGF4 has a key role in EMT (the epithelial-mesenchymal transition), cancer stem cells, and chemotherapy resistance [9–18]. In head and neck squamous cell carcinoma, PCGF4^{+} CSCs show cisplatin resistance, with possible relapse

after chemotherapy [9]. Atlasy found that PCGF4 was highly expressed in CD133 and EpCAM positive colorectal cancer CSCs [19]. Recent studies have demonstrated that carcinogenesis driven by PCGF4 is related to inhibition of the TGFβ-2/SMAD signal transduction axis [20] and targeting PCGF4 might inhibit the proliferation and increase the radio-sensitivity of HCC cells [21]. Down-regulation of PCGF4 inhibits the stemness of $CD133^+$ LCSCs by blocking the NF-κB signalling pathway [11]. Other work has indicated that HCC resistance can be overcome by co-delivering PCGF4 siRNA and cisplatin in cationic nanocapsules [22]. PCGF4 likely promotes invasion and migration in $CD133^+$ HepG2 cells by inducing EMT [23]. The polycomb gene PCGF4 contributes to the maintenance of tumor-initiating side population cells in hepatocellular carcinoma [24].

Another PRC1 member, melanoma nuclear protein 18 (Mel18), also known as PCGF2, is a homologous isomer of PCGF4 and plays different roles in various cancers. In some cancers, PCGF4 and PCGF2 synergistically promote cancer development [25–29]. It was found that expression of PCGF4 and PCGF2 in cancer tissues is abnormally up-regulated, inhibiting that of the REG3B protein, thereby affecting the STAT3 tumor-suppressor signalling pathway, promoting cell proliferation and reducing apoptosis in colorectal cancer [30]. In gastric cancer, low expression of PCGF2 is related to poor prognosis but correlates negatively with the gastric cancer stem cell markers Oct4, Sox2 and Gli1 [31]. Tao's study found that expression of PCGF2 in colorectal cancer is significantly lower than that in non-cancerous mucosa and that patients with high PCGF2 expression have longer disease-free survival times than others [32]. Furthermore, Won found that PCGF2 is a negative regulator of breast cancer CSCs that inhibits the stem cell population and self-renewal in vitro and in vivo by inactivating wnt-mediated notch signalling [33]. PCGF2 controls enrichment of tumor-initiating cells in the SP (side population) fraction in mouse breast cancer [34] and negatively regulates stem cell-like properties through down-regulation of miR-21 in gastric cancer cells [31]. Despite a large number of reports that PCGF4 is highly expressed in liver cancer and promotes the formation of liver cancer stem cells, the function of PCGF2 in liver cancer is unknown. In this study, we found that PCGF2 is down-regulated in HCC and highly expressed in adjacent tissues. We also observed that PCGF4 is highly expressed in cancer tissues and cancer cells and that PCGF2 has an effect opposite to that of PCGF4. Finally, preliminary exploration showed that PCGF2 and PCGF4 exhibit opposing stem-like properties driven by the p38 MAPK pathway in hepatocellular carcinoma.

2. Material And Methods

2.1 Tissue microarrays-Patients and tissues

Purchased tissue microarrays (Outdo Biotech Co., Ltd, Shanghai, China, LivH180Su06) containing recurrence time, survival time, biochemical indicators, and other clinical data to obtain tumor samples: including tissues and adjacent tissues from 90 patients who underwent surgical resection from February 2006 to May 2007 and were confirmed to be HCC by post operative pathology. All patients were followed up for 5–6 years. The study was approved by the Shanghai Outdo Biotech Company Ethics Committee

and informed consent was obtained from each patient under Institutional Reviewer Board protocols. The project number approved by the ethics committee is HLivH180Su06.

2.2 Immunohistochemistry-IHC

The same set of tissue microarray, the HCC tissue microarray from the same patient was separately stained with anti-PCGF2 (Santa Cruz biotechnology, H115) antibody and anti-PCGF4 antibody (Cell Signaling Technology, Beverly, 6964, USA) according to the previous description. Taking into account the intensity and percentage of staining, a semi-quantitative evaluation of the protein expression levels of PCGF2 and PCGF4 was performed by three experienced pathologists and for data analysis was referenced this work [35].

2.3 Cell culture and transfect

Human HCC cell lines Huh7 and HCCLM3 cells were purchased from Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM (Gibco, USA) containing 10% fetal bovine serum (Hyclone, Logan, Utah, USA), 1% penicillin and streptomycin in a 37°C incubator with 5% CO_2. PCGF2 over expression, PCGF4 knockdown and the corresponding negative control virus were produced from Genechem (Shanghai, China) and transfected according to the manufacturer's instructions, the stably transfected cells were cultured for subsequent experiments.

2.4 Cell proliferation assay

Cell proliferation was detected using cell Counting Kit-8 (CCK-8, Dojindo, Japan). In short, the above stably transfected cells were trypsinized (0.25% Trypsin EDTA, BI) and seeded at 2,000 cells / well on a 96-well plate (Corning, USA). Each group was set up with 6 replicates. After the cells were completely adhered, replace the complete medium with 100 μL of serum-free medium containing 10% (v/v) CCK8. After 2hr, the OD at 450 nm was measured and recorded as 0 day. Thereafter, the process was repeated every 24 hr until the 96 hr.

2.5 Protein extraction and western blot analysis

Cells treated as above and harvested at 48 h were washed three times in phosphate-buffered saline (PBS: pH 7.4; 0.15 mol/L). Total protein (~ 60μg), extracted in RIPA buffer from each subset, was exposed to SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% skim milk in Tris Buffered Saline and 1% Tween 20 (TBST) and incubated (overnight, 4°C) with primary antibody (1:1000) in TBS-T containing 1% skim milk. After three washings in TBS-T, membranes were incubated (room temp, 1 h) with secondary antibody (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and exposed to enhanced chemiluminescence reagent (Chemicon International, Temecula, CA, USA). To confirm equal protein loading, anti-β-actin antibody (1:2000; Sigma) was used to re-probe. All experiments were done in triplicate. Quantitative analysis of protein abundance using Image J software.

2.6 RT-PCR

RNA, primary cells was prepared by Trizol and reverse transcribed using TAKARA cDNA synthesis kit. The primers were designed and synthesized by Sangon Biotech (Shanghai, China), and the sequence is as follows: PCGF4, forward 5'-CCAGGGCTTTTCAAAAATGA-3', reverse 5'- GCATCACAGTCATTGCTGCT-3', PCGF2, forward 5'-GCATCTTGCCAAGTTTCTCC-3', reverse 5'-TCTGCAGGCAGTTCAAGCTA-3', respectively. GAPDH were used as internal controls. All experiments were done in triplicate.

2.7 Flow cytometry

Liver cancer stem cell markers such as anti-CD133 antibody, anti-CD13 antibody and anti-EpCAM antibody were screened by flow cytometry. In order to analyze the characteristics of samples, fluorescence activated cell sorting (FACS) analysis was carried out. For each well, 100 μL 1×10^6 cells were incubated with 2 μL anti-CD133-PE (cat. no.130-110-962), EpCAM-APC (cat. no. -111-000) and CD13-PE-Vio770 (cat. no. 130-120-727), purchased from Miltenyi Biotec (Bergisch Gladbac,Germany) in the dark at 4°C for 10 minutes. The cells were then washed twice with PBS and suspended in 500 μL PBS for analysis by flow cytometry.

2.8 Tumor sphere formation and in vitro limiting dilution assay

Huh7 and HCCLM3 cells at 10^3 cells/well were placed to the 6 well ultra low attachment plates (Corning Inc.) adding sphere medium for 5 day incubation. Sphere cells with diameter > 80 μm were counted. Sphere medium: DMEM-F12 medium supplemented with B27 (Invitrogen), 20ng/mL basic fibroblast growth factor, 20ng/mL epidermal growth factor) respectively.

For in vitro limiting dilution experiments, different HCCs were implanted into 96-well low-adhesion U-shaped plates with gradients of 12.5, 25, 50, and 100 cells per well, and each gradient was repeated for 24 wells. After 9 days of culture, determine the number of tumor spheres in each well, and use limiting dilution analysis (http://bioinf.wehi.edu.au/software/elda) to calculate the sphere formation efficiency.

2.9 Statistics

The data are expressed as average ± standard error. The data were analyzed using the Social Science Statistics package (SPSS, Chicago, Illinois, USA) version 17.0. Pearson and Spearman correlation coefficients were used to evaluate the correlation between gene expression and clinical and histopathological parameters. One-way ANOVA and t-test were used to compare between groups. The survival curve was drawn by Kaplan-Meier method and compared with logarithmic rank test. In all statistical analysis, $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Expression of PCGF4 and PCGF2 correlates inversely in HCC cell lines and HCC tumors

To determine PCGF2 and PCGF4 expression in HCC, we used IHC with a tissue microarray. The proportion of patients whose expression of PCGF2 in adjacent tissues was higher than that in cancer tissues was 66.26%. In contrast, the expression level of PCGF4 in cancer tissues was higher than that in adjacent tissues (51.76%) (Fig. 1A-1B). HCC tumor tissues expressed higher PCGF4 and lower PCGF2 (Fig. 1B). As expected, PCGF4 was up-regulated and PCGF2 down-regulated in HCC tissues. High expression of PCGF4 in many cancers predicts poor prognosis, and we assessed whether PCGF4 or PCGF2 affects the prognosis of HCC patients. A total of 76 patients with HCC (details in the material) were examined by tissue microarray, and expression levels of PCGF4 and PCGF2 were detected by IHC. Analysis of the relationship between PCGF4 expression and clinicopathological characteristics showed that up-regulation of PCGF4 is related to TNM staging (Table 1). Furthermore, Kaplan-Meier survival curves demonstrated that PCGF4 was an independent prognostic indicator of overall survival but not cumulative recurrence (Fig. 1C). Univariate analyses revealed that high expression of PCGF4 was significantly associated with decreased OS but not a high risk of postoperative recurrence in HCC patients (Table 2). Nevertheless, PCGF2 was not an independent prognostic indicator of overall survival (data not shown). In summary, rather than PCGF2, PCGF4 may be an independent factor in the prognosis of HCC. The same results were also found in cell lines. We used western blotting to detect expression of PCGF4 or PCGF2 in the normal liver cell line L02 and HCC cell lines Hep3B, Huh7, and HCCLM3. The results further revealed significantly higher expression of PCGF4 than L02, with the opposite results for PCGF2 (Fig. 1D). To further explore the role of PCGF2 in HCCs, we constructed different cell lines to study their functions.

Table 1
Relationship between tumor PCGF4 expression and clinicopathologic features in HCC patients

Characteristics		**PCGF4**		
		Negative	Positive	P*
Gender	Female	10	3	0.054
	Male	30	33	
Age	< 51	19	17	0.981
	> 51	21	19	
HBsAg	Negative	4	36	0.243
	Positive	7	29	
HCVAb	Negative	39	35	0.480
	Positive	0	1	
Cirrhosis	No	5	5	0.858
	Yes	35	31	
AFP (ng/ml)	< 20	14	9	0.343
	> 20	26	27	
ALT (U/L)	≤ 75	19	21	0.315
	> 75	13	23	
Tumor size (cm)	≤ 5	23	17	0.168
	> 5	15	21	
Tumor number	Single	34	6	0.274
	Multiple	9	27	
Vascular invasion	No	17	23	0.512
	Yes	18	18	
TNM	I	30	10	0.044*
	II-III	20	16	

Abbreviations: HCC, hepatocellular carinoma; HBsAg, hepatitis B surface antigen; HCVAb, hepatitis C virus antibody; AFP, α-fetoprotein; ALT, alanine transaminase; TNM, tumor-nodes-metastases; * P value < 0.05 was considered statistically significant. The Pearson Chi-square test was used.

Table 2
Univariate analyses of factors associated with overall survival of HCC patients

Variables	HR	95% CI	P*
PCGF4(High vs. low)	1.930	(1.094–3.404)	0.023*
Gender(male vs. female)	1.662	(0.746–3.702)	0.214
Age,years (> 51vs.≤51)	1.247	(0.706–2.202)	0.447
Tumor differentiation (poor vs. well)	0.868	(0.477–1.578)	0.642
Tumor size (cm) (> 5 vs.≤5)	1.692	(0.959–2.975)	0.048
Tumor number (multiple vs. single)	1.313	(0.670–2.573)	0.428
Vascular invasion (yes vs. no)	0.910	(1.052–3.289)	0.743
TNM(I vs. II-III)	1.860	(0.519–1.597)	0.033*
AFP ng/ml(≤ 20 vs.>20)	1.122	(0.603–2.085)	0.717
ALT(U/L)(≤ 75 vs.>75)	2.090	(1.155–3.781)	0.015*
Cirrhosis(Yes vs.No)	0.738	(0.330–1.648)	0.458
HCVAb(Negative vs. Positive)	1.780	(0.243–13.036)	0.570
HBsAg(Negative vs. Positive)	0.676	(0.316–1.445)	0.312

3.2 PCGF4 KD reduces tumor sphere formation and cell proliferation, and tumor sphere formation capacity is reduced after PCGF2 over-expression in HCC cells

To evaluate the effect of PCGF2 or PCGF4 on proliferation, PCGF4 expression was knocked down (PCGF4 KD) using siRNA in HCCLM3 and Huh7 cells, a confirmed by western blotting (Fig. 2A). As PCGF2 is expressed at low levels in HCC lines, we constructed PCGF2 over-expression (PCGF2 OE) cell lines, also verified by western blotting used FLAG antibody (Fig. 2A-2B). CCK-8 assays were used to analyze cell proliferation, and it was found that the cell proliferation rate decreased after PCGF4 KD (Fig. 2C, 2D). However, proliferation ability was not significantly suppressed by PCGF2 OE. Next, we explored the effect of PCGF4 or PCGF2 on the tumor sphere formation and found that PCGF4 KD decreased the size of tumor spheres and the number of spheres in HCCLM3 or Huh7 cells (Fig. 2E, F) and that sphere formation was decreased by PCGF2 OE in HCCLM3 and Huh7 cells (Fig. 2E, F).

To examine the effects of PCGF4 or PCGF2 on the frequency of sphere-forming cells, a limiting dilution assay was performed in which a greater number of cells were required to generate tumor spheres in the PCGF4 KD or PCGF2 OE group (Fig. 2G). These data indicate that PCGF4 blockade-mediated weakens tumor sphere formation in vitro, as does over-expression of PCGF2. These results indicate that sphere formation, as a characteristic of cells with a CSC phenotype, is reduced by PCGF4 KD or PCGF2 OE.

3.3 PCGF2 and PCGF4 opposite drives CD13 and EpCAM expression in HCC cells

To examine the role of PCGF2/PCGF4 in the regulation of human LCSCs, the specific gate strategy of FACS is illustrated in Fig. S1. For the HCCLM3 cell line, the number of $CD13^+$/$EpCAM^+$/$CD133^+$ cells among PCGF4 KD cells was significantly down-regulated ($P < 0.01$, $P < 0.01$, $P < 0.001$) (Fig. 3A). When PCGF4 expression was down-regulated in Huh7 cells, the number of $CD13^+$/$EpCAM^+$/$CD133^+$ cells was also reduced (Fig. 3B). In addition, flow cytometric analysis of $CD13^+$ and $EpCAM^+$ cell populations showed that PCGF2 OE had the same effect as PCGF4 knockdown. However, the number of $CD133^+$ cells was not significantly changed (Fig. 3A). Therefore, changes in PCGF2 were not obviously regulated by CD133 in HCCLM3 or Huh7 cell lines (Fig. 3A, 3B). Finally, we also found that the subpopulation of $CD13^+$ and $EpCAM^+$ cells increased after PCGF2 knockdown (data not shown). In summary, these results indicate that PCGF4 KD or PCGF2 OE significantly reduces expression of LCSC surface markers.

3.4 PCGF4 KD or PCGF2 OE enhances the sensitivity of cells to sorafenib

CSCs are a major cause of clinical drug resistance to chemical agents. Because it has been reported that LCSCs are resistant to sorafenib [36, 37], we compared the effect of sorafenib on the viability of PCGF4 KD or PCGF2 OE cells. According to the results presented in Fig. 2A, the proliferation rate of PCGF4 KD or PCGF2 OE cells within 24 hours was the same as that of NC cells. Therefore, sorafenib was immediately added after the cells adhered, and cell viability was detected within 24 hours. As expected, PCGF4 KD or PCGF2 OE cells were more sensitive to sorafenib at a concentration of 10 μM in HCCLM3 ($P < 0.001$) and Huh7 ($P < 0.001$) cells (Fig. 4A, 4B). In summary, these results reveal that PCGF2 or PCGF4 reversely regulates the stemness of LCSCs.

3.5 PCGF2/PCGF4 affects stem-like properties in HCC cells via p38 MAPK signalling

PCGF2 and PCGF4 are polycomb protein family PRC1 components. PRC1, considered a polycomb inhibitory complex, is a type of transcriptional repressor that regulates expression of target genes through chromatin modification. To clarify the downstream transcriptional regulatory network of PCGF2 or PCGF4, a comprehensive database of human transcription factor target genes hTF target and GTRD was applied. The number of overlapping PCGF4 genes (4496, the number of regulated target genes, Fig. 5A) was much greater than that of PCGF2 (939 genes, Fig. 5A), suggesting that PCGF4 shares a stronger role than PCGF2 in modulating biological events. PCGF4 and PCGF2 mainly showed MAPK signalling pathway enrichment (Fig. 5A). The MAPK pathway has three main branch routes: ERK, JNK and p38 MAPK. Among them, JNK and p38 have similar functions and are related to inflammation, apoptosis, and growth, p38 activity was reduced in PCGF4 KD or PCGF2 OE cells (Fig. 5B). Although the level of p-

ERK1/2 was down-regulated in HCCLM3 cells, there was no change in Huh7 cells. This may be caused by the heterogeneity of tumor cells.

4 Discussion

PCGF4 is an important regulator of cancer cell proliferation and CSC self-renewal, and it is considered an important therapeutic target [38]. Previous studies have found that PCGF4 is regulated by another PcG protein, PCGF2 [39]. Our study suggested that the two homologous proteins PCGF2 and PCGF4 reversely regulate the stem cell population through p38 pathway (Fig. 5C). Expression of PCGF2 and PCGF4 show an opposite trend in gastric cancer and breast cancer [39, 40]. However, regulation of the activity of LCSCs by PCGF2 or PCGF4 has not been verified. In this study, we observed that PCGF2 and PCGF4 negatively regulate the activity of LCSCs. It was also found that PCGF4 protein expression can be used as an independent prognostic indicator of overall survival but that PCGF2 expression cannot. However, inconsistent with other reports, we did not find that low expression of PCGF2 is associated with poor prognosis. This discrepancy may be due to the limited number of samples in our study or different types of cancer studied.

In addition, our data show that PCGF2 or PCGF4 not only play a role in the prognosis of HCC but are also related to the stemness and drug resistance of LCSCs. This reliable result indicates that low expression of PCGF4 or high expression of PCGF2 can reduce the population of LCSCs and sphere formation. Similar to HCC cells, LCSCs have heterogeneity, we used different LCSCs markers to compare effects on expression of PCGF2 and PCGF4 based on changes in LCSCs populations. Remarkably, low expression of PCGF4 reduced the abundance of $CD13^+$, $EpCAM^+$, and $CD133^+$ cells, but high expression of PCGF2 only affected that of $CD13^+$ and $EpCAM^+$ cells. In line with our findings, currently reported markers for LCSCs are not specific to LCSCs. To facilitate separation of LCSCs, various types of markers should be examined [41]. Our results indicate that PCGF2 or PCGF4 can alter the population and self-renewal ability of LCSCs. To determine whether low expression of PCGF2 leads to poor prognosis, we conducted IHC, and the results showed that PCGF2 is expressed at lower levels in HCC tissues than in paracancerous tissues but that PCGF2 cannot be used as an independent prognostic marker for patients with HCC.PCGF2, which is a component of the polycomb protein complex PRC1, regulates cell proliferation and senescence through transcriptional inhibition of PCGF4 and c-Myc oncoproteins [42]. However, no significant difference in the half-life of PCGF4 in control and PCGF2-over-expressing cells was observed, further indicating that PCGF4 is not regulated at the protein level by PCGF2 [42]. In our tumor samples, we did not detect a tendency for PCGF4 to be over-expressed in the same patient, but PCGF2 to be lower-expressed. It should be noted that we assessed the protein level and not the transcription level.

Regarding the HCC cell line, PCGF4 affects the proliferation of HCC cells, which is the same as the results of Wu et al [9, 10, 16, 22, 43, 44]. Over-expression of PCGF2 had no significant effect on the proliferation of HCC cells but enhanced sensitivity to sorafenib. Similarly, PCGF2 has been proposed as a tumor suppressor in breast cancer [45]. PCGF2 over-expression restored ER-α expression in triple-negative breast cancer (TNBC). Furthermore, mice bearing PCGF2-overexpressing MDA MB-468 TNBC cell tumors

acquired sensitivity to tamoxifen treatment, although no difference in tumor growth was observed between the control and PCGF2-overexpressing cell xenografts [46].

We also found that PCGF4 and PCGF2 negatively regulate tumor sphere formation. It is suggested that PCGF2, as a tumor-suppressor gene, also affects the maintenance of LCSCs stemness.

We demonstrated that PCGF4 KD or PCGF2 OE regulate the stem phenotype of LCSCs through the p38 pathway. Some study explored the role of p38 MAPK in maintenance of CSC phenotype, therapy resistance and DNA damage repair and response in HNSCC [47]. The role of p38 in cancer has been extensively studied. Some reports indicate that p38 acts as an anti-tumor factor, especially in promoting cell cycle arrest and differentiation, as a tumor suppressor. However, some articles discuss that p38 promotes cancer by enhancing tumor cell survival, migration, or resistance to stress and chemotherapy drugs [48]. There is a dual function of p38 MAPKs in colon cancer: inhibiting initiation of colitis-related tumors but promoting cancer cell survival [49]. Some studies demonstrate that p16Ink4a inhibits lung adenocarcinoma through PCGF4 when p38 is activated [50].

Our data indicated that the PCGF4 KD or PCGF2 OE could inhibit stem-like properties in vitro via downregulating the expression of p38 pathway. More importantly, PCGF4 KD or PCGF2 OEcould increase the sensitivity of HCC cells to sorafini. Therefore, further studies are required to develop a combination therapy as a potential therapeutic strategy.

Declarations

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Competing Interests

All the authors of this manuscript declare that they have no conflict of interest.

Author Contributions

JH, YZ, BC, XL: The overall design of the experiment, the completion of the experimental data. JH, YZ: Data analysis. JH, YZ, HF, YW: Draft manuscript. YX, KQ: Flow cytometry analysis. YC: IHC, immunohistochemical analysis. WM, BC: academic contributions and manuscript revision. XM, FL, JY: Technical guidance, experimental suggestions. XL and BC: Fund support.

Ethics approval

The study was approved by the Shanghai Outdo Biotech Company Ethics Committee.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent to publish

The authors affirm that human research participants provided informed consent for publication of the images in Figure(s) 1A, 1B and 1C."

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Figures

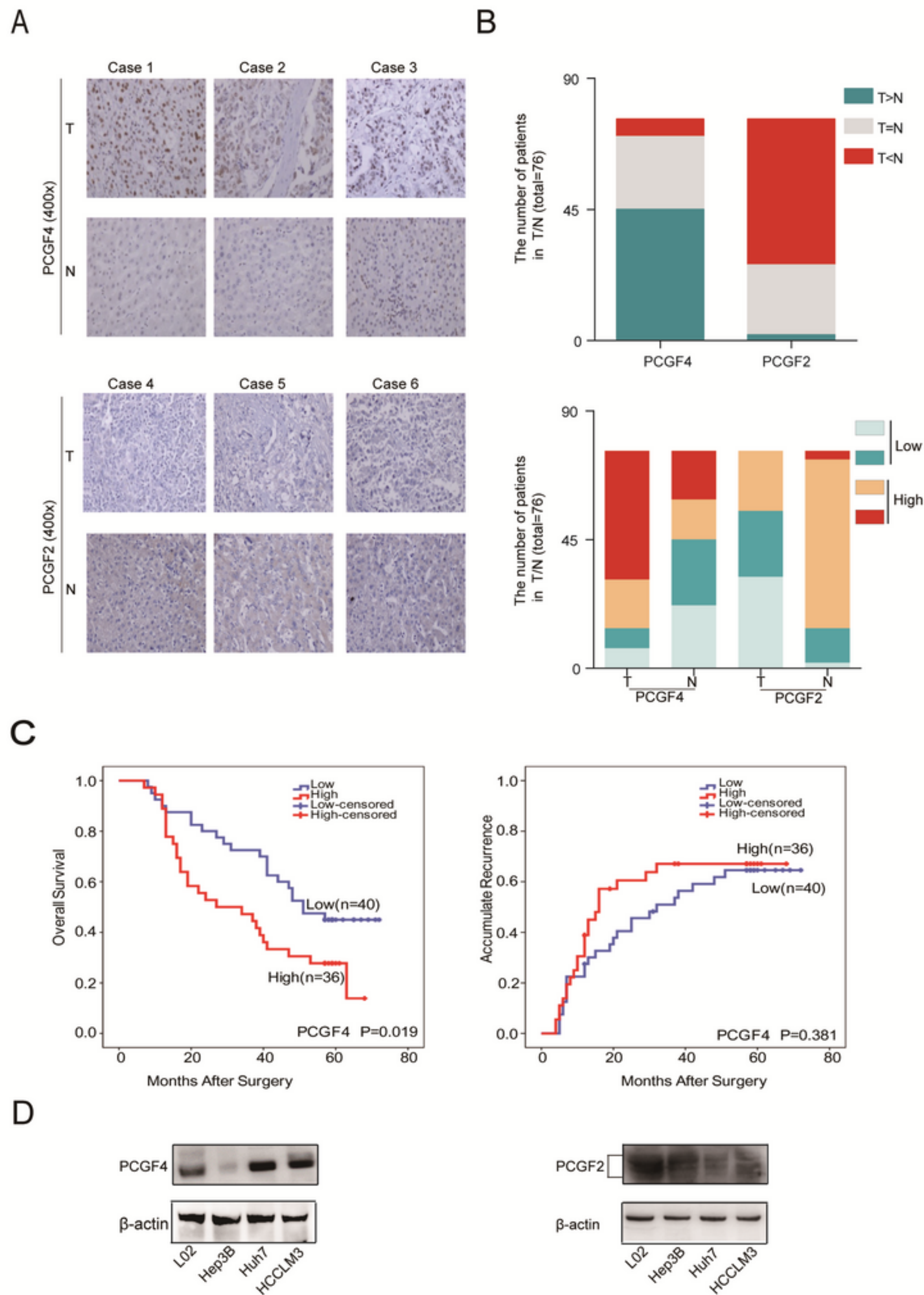
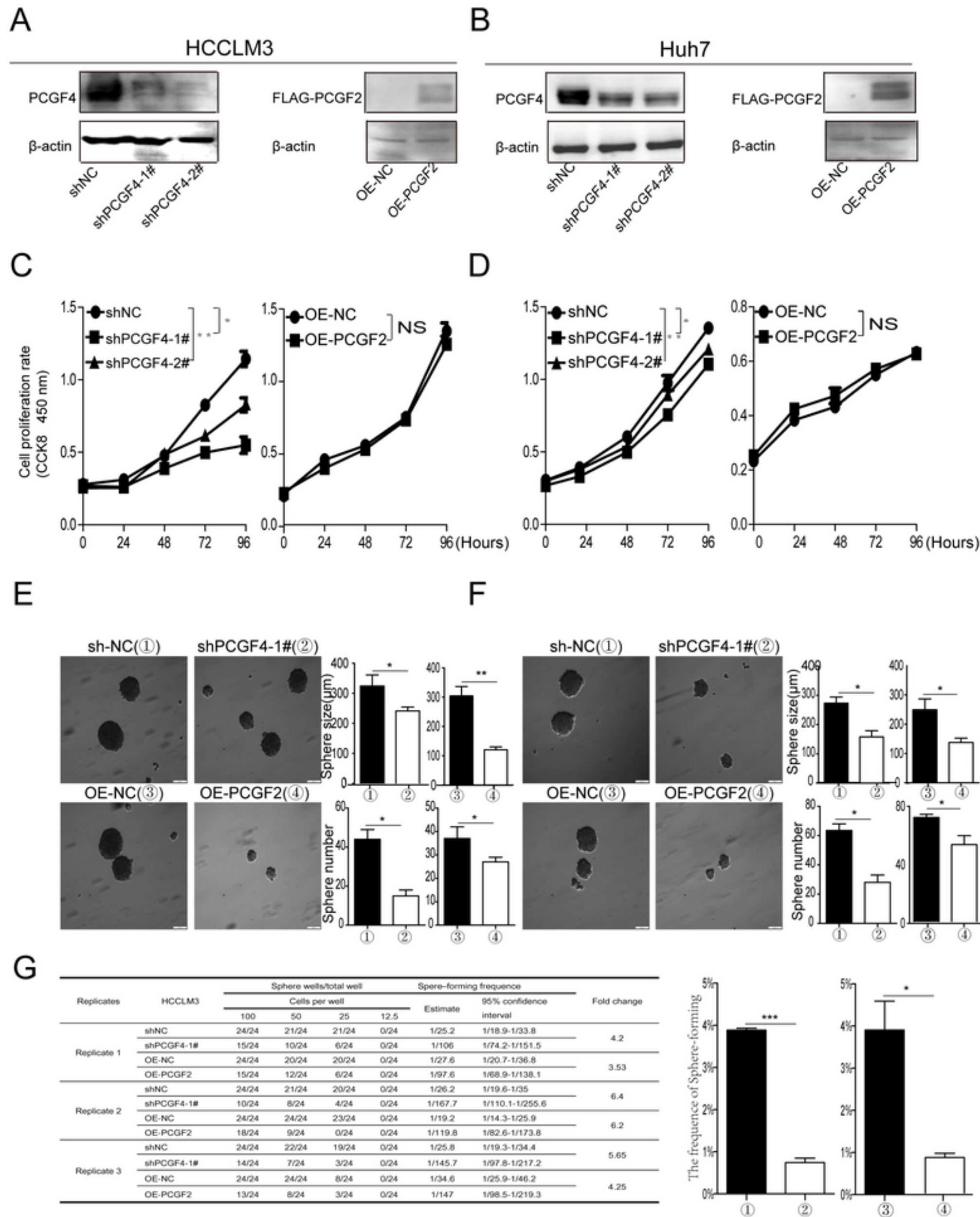


Figure 1

Expression of PCGF4 and PCGF2 correlated negatively in HCC patients and HCC cell lines. (A) Expression of PCGF2 and PCGF4 by IHC in 76 matched HCC and adjacent non-tumour tissues. (B) Bar graph showing statistics for staining intensity in training cohorts. (C) Kaplan-Meier curves for overall survival (P=0.019, Kaplan-Meier method (log-rank test)) and accumulated survival according to PCGF4 expression. (P =0.381, Kaplan-Meier method (log-rank test)). (D) Western blot analysis showed that

PCGF2 is expressed at low levels in cancer cells but that PCGF4 is highly expressed compared to L02 cells. Higher PCGF4 results in poor prognosis in HCC.

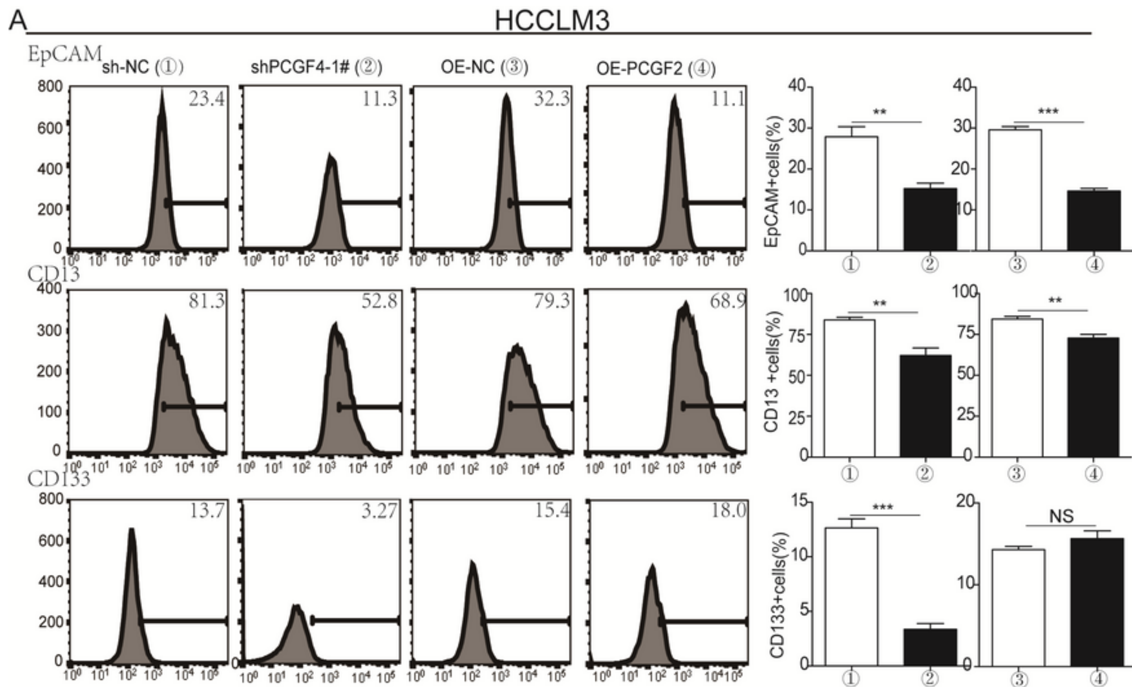


Replicates	HCCLM3	Sphere wells/total well				Spere-forming frequence		Fold change
		Cells per well				Estimate	95% confidence interval	
		100	50	25	12.5			
Replicate 1	shNC	24/24	21/24	21/24	0/24	1/25.2	1/18.9-1/33.8	4.2
	shPCGF4-1#	15/24	10/24	6/24	0/24	1/106	1/74.2-1/151.5	
	OE-NC	24/24	20/24	20/24	0/24	1/27.6	1/20.7-1/36.8	3.53
	OE-PCGF2	15/24	12/24	6/24	0/24	1/97.6	1/68.9-1/138.1	
Replicate 2	shNC	24/24	21/24	20/24	0/24	1/26.2	1/19.6-1/35	6.4
	shPCGF4-1#	10/24	8/24	4/24	0/24	1/167.7	1/110.1-1/255.6	
	OE-NC	24/24	24/24	23/24	0/24	1/19.2	1/14.3-1/25.9	6.2
	OE-PCGF2	18/24	9/24	0/24	0/24	1/119.8	1/82.6-1/173.8	
Replicate 3	shNC	24/24	22/24	19/24	0/24	1/25.8	1/19.3-1/34.4	5.65
	shPCGF4-1#	14/24	7/24	3/24	0/24	1/145.7	1/97.8-1/217.2	
	OE-NC	24/24	24/24	8/24	0/24	1/34.6	1/25.9-1/46.2	4.25
	OE-PCGF2	13/24	8/24	3/24	0/24	1/147	1/98.5-1/219.3	

Figure 2

Loss of PCGF4 inhibits cell proliferation and tumour sphere formation, and up-regulation of PCGF2 suppresses cell stemness in HCC. (A-B) Western blot analysis confirmed protein expression of PCGF2 or

PCGF4 in parental cells and stable cell lines. (C-D) CCK-8 assay detected the impact of PCGF2 up-regulation or PCGF4 down-regulation on the proliferation rate of Huh7 and HCCLM3 cell lines. (E-F) Cells were plated in low-adhesion plates, and the number of sphere cells with a diameter greater than 80 μm was counted. (G) In vitro limiting dilution assay of HCCLM3s in shNC, shPCGF4-1#, OE-NC, OE-PCGF2 groups. All experiments were performed in triplicate. Results are shown as mean ± SE. Scale bars =100 μm.



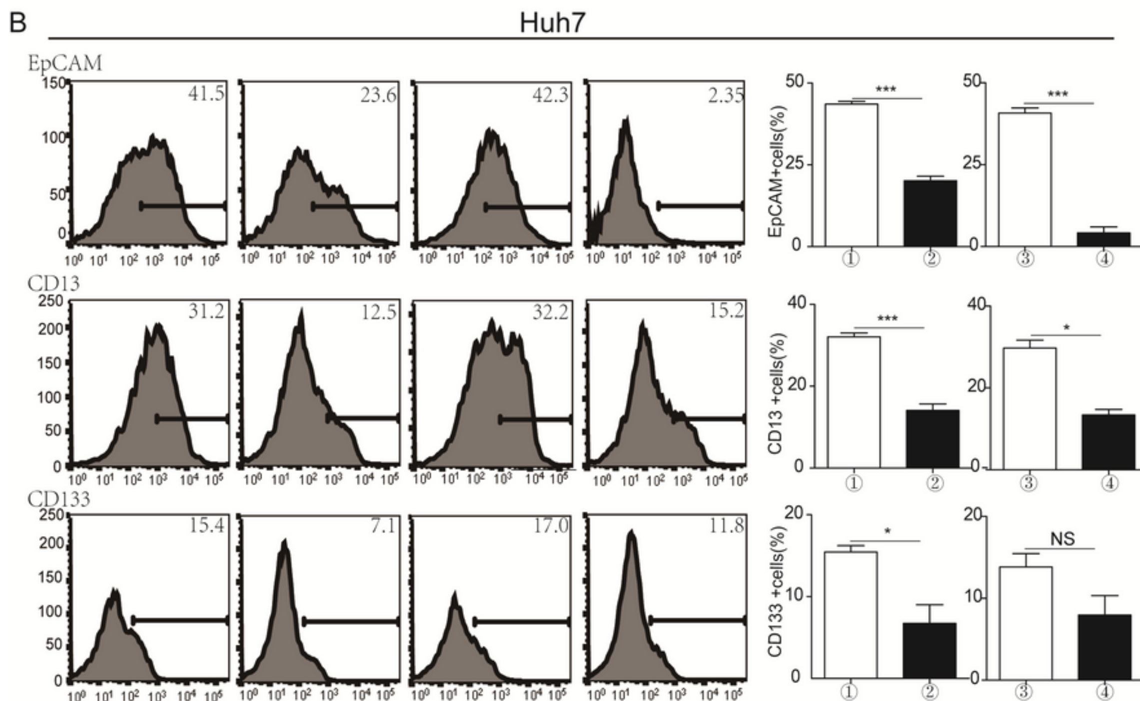


Figure 3

Decrease in CD13$^+$ and EpCAM$^+$ expression via down-regulated PCGF4 or up-regulated PCGF2. (A-B) HCCLM3 and Huh7 cells were transfected with NC or shPCGF4, and subpopulations with the CD13$^+$/CD133$^+$/EpCAM$^+$ phenotype in these cells were determined by FACS analysis. These cell populations in HCCLM3 or Huh7 cells over-expressing PCGF2 were also measured. Each plot is representative of 3 independent experiments. Results are shown as means± SD)

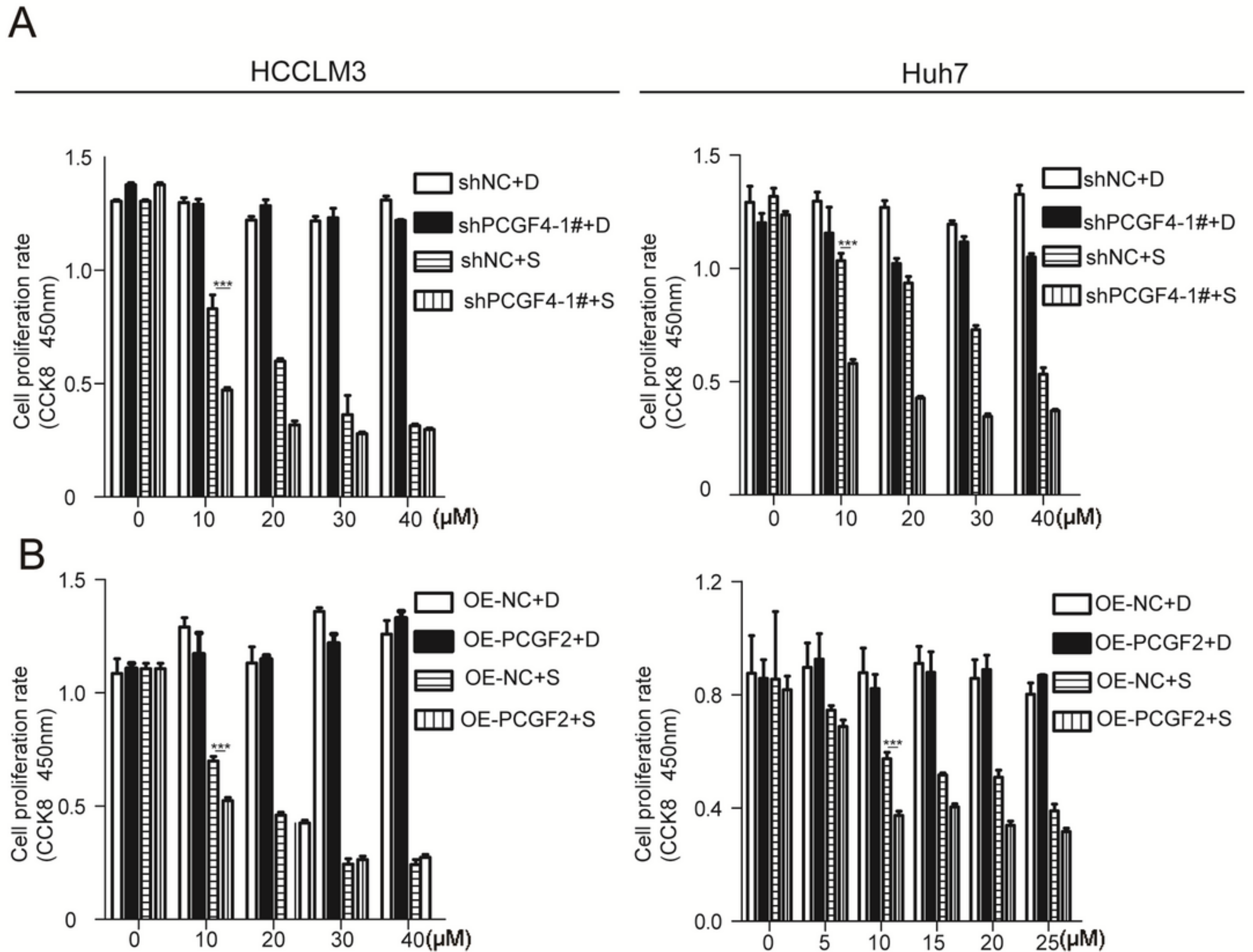


Figure 4

Cells with low PCGF4 expression or high PCGF2 expression are more sensitive to sorafenib. (A-B) The viability of Huh7 and HCCLM3 cells treated with 0-40 μM sorafenib for 24 hours was evaluated by CCK-8 assays. D: DMSO and S: sorafenib. Results are shown as the mean ± SD.

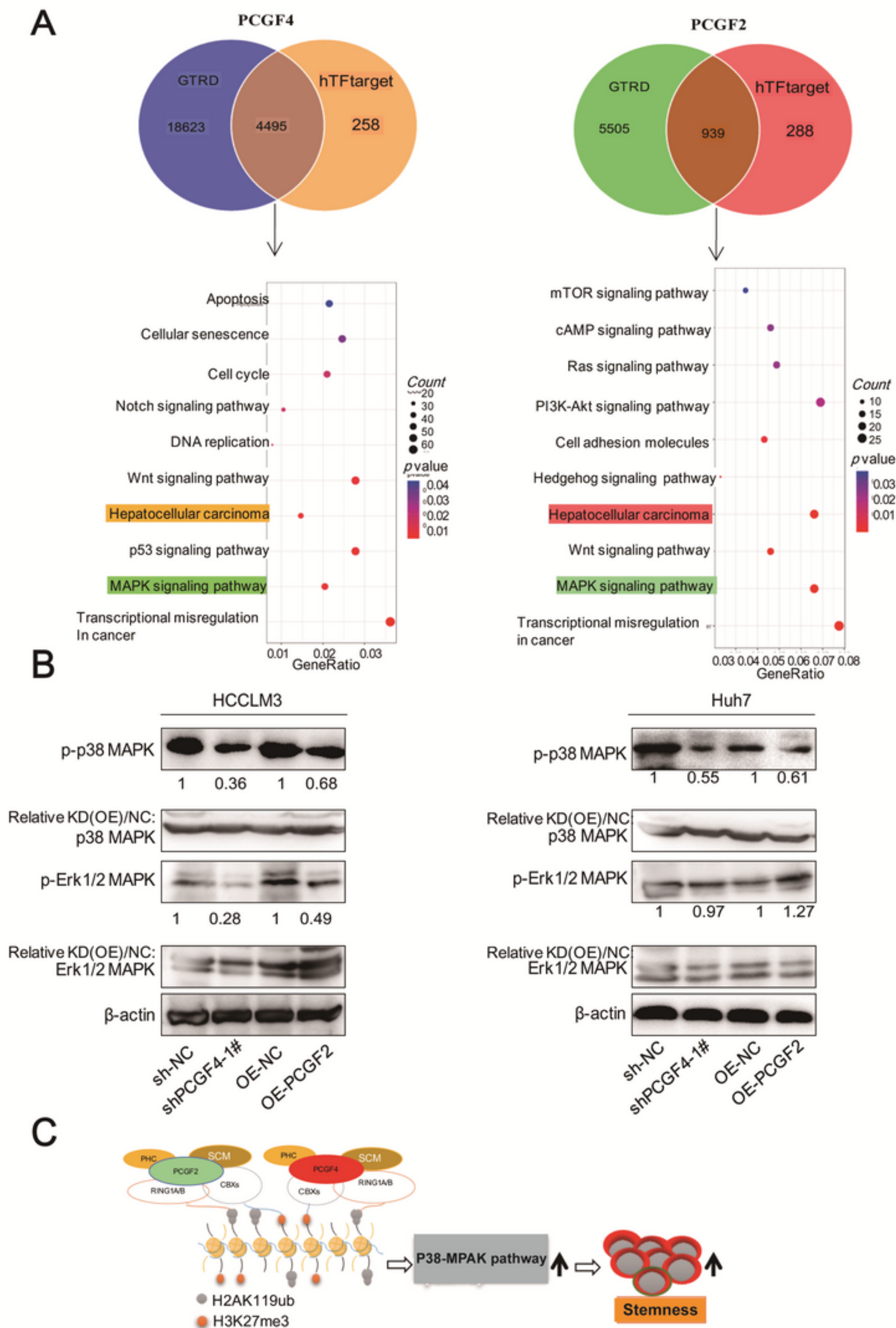


Figure 5

Functional enrichment analyse. (A) hTF target (http://bioinfo.life.hust.edu.cn/hTFtarget) is an online platform for exploring human transcription factor target genes. GTRD: a database of gene transcription regulation. We predicted target genes through the hTF target and GTRD databases, obtaining 939 and 4496 target genes for PCGF2 and PCGF4, respectively, and performed KEGG analysis on these target genes using the cluster Profiler package of the R language. (B) p38 activity was reduced in PCGF4

knockdown or PCGF2 over-expressing cells. Quantitative analysis of protein abundance was performed using Image J. The numbers under each western blot were calculated as follows: p38/total 38 density of each cell was measured, and the phospho-38/total p38 density ratio of NC cells was defined as 1.0; the shPCGF4/OE-PCGF2 cell density value divided by the phospho-p38/total p38 density ratio of NC cells. Erk1+2 as described in p38. (C) Proposed model for the regulatory mechanism of LCSCs by PCGF2 and PCGF4.

Supplementary Files

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- FigS1.tif