Original Article

GPRC5A regulates proliferation and oxidative stress by inhibiting the STAT3/Socs3/c-MYC pathway in hepatocellular carcinoma

Lixia Zhang,[1,2] Weibing Yang,[3] Jin Yang,[1,*] and Fu Sun[4]

[1]Department of Medical Oncology and [3]Department of Ultrasound, The First Affiliated Hospital of Xi'an Jiaotong University, No. 277, Yanta West Road, Xi'an, Shaanxi 710061, China
[2]Department of Oncology, Xi'an Daxing Hospital, Xi'an, Shaanxi 710016, China
[4]Department of Orthopedics, The First Affiliated Hospital of Xi'an Medical University, Xi'an, Shaanxi 710000, China

(Received 25 October, 2022; Accepted 10 December, 2022; Released online in J-STAGE 1 July, 2023)

The G protein-coupled receptor, class C, group 5, member A (GPRC5A) plays a key role in various diseases, but its effect on hepatocellular carcinoma (HCC) and the potential underlying mechanisms remains unclear. In the present study, we explored the effect of GPRC5A on the progression of HCC and further explored its mechanism of action. The results revealed that the expression of GPRC5A was lower in HCC tissues and cells. Overexpression of GPRC5A suppressed the proliferation and epithelial-mesenchymal transition (EMT) of HCC cells. In addition, overexpression of GPRC5A induced oxidative stress and apoptosis. Further study showed that overexpression of GPRC5A inhibited the expression of STAT3/Socs3/c-MYC related-protein and the NLRP3 inflammasome. Moreover, the STAT3/Socs3/c-MYC and NLRP3 inflammasome was involved in the effect of GPRC5A on HCC cells. These results suggest that GPRC5A suppresses proliferation and EMT, induces oxidative stress and leads to apoptosis of HCC cells, potentially by regulating STAT3/Socs3/c-MYC signalling and the NLRP3 inflammasome. These findings suggest that GPRC5A has an anti-tumor effect in the formation of HCC, and the molecular therapy of GPRC5A provides a theoretical basis for treating HCC.

Key Words: **GPRC5A, hepatocellular carcinoma, STAT3/Socs3/c-MYC, NLRP3**

Liver cancer is a kind of malignant tumour with a high mortality, second only to gastric and oesophageal cancer, and can be divided into both primary and secondary liver cancer.[1] Hepatocellular carcinoma (HCC) belongs to the group of primary liver cancer, accounting for about 90% of liver cancers. HCC can be induced by a variety of causes, with HBV infection being the main cause. HCC is asymptomatic in early stages and is characterised by pain, fever and fatigue in the liver region in late stages.[2] At present, treatment of HCC includes surgery, chemotherapy, radiotherapy, and biotherapy.[3]

The G protein-coupled receptor, class C, group 5, member A (GPRC5A) is a member of the 3-G protein coupled receptor family.[4] GPRC5A is mainly located in the plasma membrane, endoplasmic reticulum, Golgi body and extracellular vesicles.[5] Previous studies have found that GPRC5A is dysregulated in a variety of malignant tumours, suggesting that GPRC5A may be involved in tumour progression.[6,7] Results gained from GPRC5A gene knockout mice showed that GPRC5A had an anti-tumor effect on lung adenocarcinoma. Moreover, a high mutation rate of the GPRC5A gene was found in breast cancer patients.[8] At present, there are few reports about the effect of GPRC5A on HCC cells, so it is necessary to further explore its role and mechanism in these cells.

Signal transducer and activator of transcription 3 (STAT3), a member of the signal transducer and activator of transcription family, is an immunosuppressive carcinogen.[9] More and more studies show that STAT3 has activity in a variety of malignant tumours, including leukaemia, lung cancer, gastric cancer and colorectal cancer.[10–12] Studies have shown that knockout of STAT3 improves the efficacy of immunogenic chemotherapy by stimulating tumour cells to produce type 1 interferon.[13] The suppressor of cytokine signal transduction 3 (Socs3) protein is a kind of protein that can weaken cytokine signal transduction.[14] Socs3 dysfunction leads to a variety of diseases, including inflammation, allergies, autoimmune diseases and cancer.[15] The existing evidence shows that the JAK/STAT signalling pathway not only regulates the immune response in cells, but can also rapidly activate the expression and biological function of Socs3.[16] C-myc is one of the important members of the myc family.[17] It is widely involved in various biological cellular processes, such as promoting cell proliferation and migration, which are related to the occurrence and development of a variety of tumours.[18] In recent years, the role of the STAT3/c-myc signalling pathway in various cancers has been studied, including in colorectal cancer, gastric cancer and oesophageal cancer.[19–21]

In the present research, we found that GPRC5A inhibits proliferation and EMT, and induces oxidative stress and apoptosis of HCC cells, potentially by regulating STAT3/Socs3/c-MYC signalling and the NLRP3 inflammasome. Our results suggested that GPRC5A inhibits the progression of HCC. Therefore, GPRC5A may be a key target in the clinical treatment of HCC.

Materials and Methods

Tissue. From October 2017 to May 2019, 48 cases of hepatocellular carcinoma were treated, aged 32–70 years (average 56 years). Pathological examination confirmed the diagnosis in all cases. In addition, fresh tumour tissue samples were collected from all these patients and matched adjacent tissue samples were used as controls. The study was approved by the local Ethics Committee and written consent was obtained from all selected patients.

Cell. Human normal hepatocyte cell line IHHA-1 and hepatoma cells HuH-7, HCCLM3 and MHCC-97H, as well as human hepatoblastoma HepG2 were purchased from American Type Culture Collection (ATCC, Manassas, VA). IHHA-1 and

*To whom correspondence should be addressed.
E-mail: jiny6646@sina.com

doi: 10.3164/jcbn.22-125

HuH-7, HepG2, HCCLM3 and MHCC-97H were cultured in DMEM cell culture medium containing 10% foetal bovine serum and 1% penicillin in an incubator at 37°C and 5% CO_2.

Reagents. Antibodies, including anti-GPRC5A, anti-VEGF, anti-caspase-3, anti-caspase-9, anti-NLRP3 and anti-STAT3 anti-Socs3, anti-c-MYC, anti-COX-2, anti-E-cadherin, anti-vimentin, and anti-iNOS were purchased from Cell Signaling Technology (CST, Boston, MA). Anti-GAPDH was purchased from Abcam Inc. (Cambridge, UK).

Plasmids. The plasmids used for overexpression in HepG2 and HCCLM3 cells were pcDNA3.1-GPRC5A, pcDNA3.1-STAT3 and pcDNA3.1-NLRP3. GPRC5A, STAT3 and NLRP3 coding sequences were cloned into the plasmid pcDNA3.1 (Agilent, Santa Clara, CA) using polymerase chain reaction (PCR). pcDNA3.1 without the GPRC5A, STAT3 and NLRP3 genes was used as a negative control (1 μg/ml). pcDNA3.1-GPRC5A, pcDNA3.1-STAT3 and pcDNA3.1-NLRP3 were transfected into HepG2 and HCCLM3 cells (6-well plate, $1 \times 10^6/cm^2$).

Transfection. The HepG2 and HCCLM3 cells were inoculated with 1×10^5 cells/well on a 24 well plate. When the cell growth convergence rate reached about 60%, pcDNA3.1-GPRC5A was transfected into HepG2 or HCCLM3 cells according to the instructions of LipofectamineTM 3000 transfection reagent. After culturing for 48 h at 37°C and 5% CO_2 in an incubator, cells from each group were collected for follow-up experiments.

MTT assay. The cell proliferation of HepG2 and HCCLM3 cells was measured using an MTT assay kit (Abcam, Cambridge, UK). Cell proliferation was measured by spectrophotometry at specified time points using a microplate reader (Thermo Fisher Scientific, Waltham, MA) at 490 nm.

ELISA. The TNF-α (BioSite, Paris, France), NO (BioSite) and IL-6 (BioSite) concentrations in the supernatants were detected using corresponding ELISA kits according to the instructions. The absorbance was measured at 450 nm using a microplate reader. The above experiments were repeated three times and the average value was obtained.

Cell apoptosis analysis. HepG2 and HCCLM3 cells were incubated in 6-well plates for 24 h. Finally, the cells were collected and incubated with Annexin-V and propidium iodide (PI) for 20 min. The incidence of apoptosis was evaluated by the flow cytometer (FCM).

Measurement of NAD^+. Intracellular NAD^+ levels were measured using the EnzyChrom NAD^+/NADH assay kit (BioAssay Systems, Hayward, CA). Cells were washed with PBS, and then lysed with the supplied NAD extraction buffer. NAD^+ was extracted from the lysate according to the manufacturer's protocol. The measurement of NAD^+ is based on an alcohol dehydrogenase cycling reaction. The change in absorbance at 565 nm for 15 min at room temperature was measured.

Measurement of ATP. Cells were culture for 24 h, and then the culture medium was removed, stored, and replaced by HEPES buffer. After washing, the initially stored culture medium was added to cells for 1 h. The cells were lysed with 10 mM Tris-HCl (pH 7.8), and ATP content was determined using a quantitative bioluminescent assay (Sigma, St. Louis, MO) according to the instructions of the manufacturer and an iMark microplate absorbance reader (Bio-Rad, Hercules, CA).

Measurement of ROS. Briefly, HepG2 and HCCLM3 cells were incubated in 6-well plates overnight. The cells were collected and incubated with ROS indicator DCFH-DA (10 μM) in PBS for 30 min at 37°C. The fluorescence was analysed using an Accuri C6 plus flow cytometer (BD Biosciences, Flanklin Lakes, NJ).

RT-PCR. Total RNA samples from IHHA-1, HuH-7, HepG2, HCCLM3 and MHCC-97H cells were isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA). Using specific primers for the reverse transcription reaction (Invitrogen), the thermocycling conditions of RT-qPCR were as follows: 95°C for 1 min; 32 cycles of 95°C for 20 s, 55°C for 30 s, and 72°C for 20 s. Relative transcriptional levels were calculated using the $2^{-\Delta\Delta CT}$ method with GAPDH as a normalising gene.

Western blotting. HepG2 and HCCLM3 cells were collected and lysed with Ripa buffer (Sigma) for 15 min. The supernatant was collected by centrifugation (10,000 × *g*, 30 min, 4°C). A BCA protein concentration test kit (Solarbio, Beijing, China) was used to determine the total protein concentration. Then, polyacrylamide gel electrophoresis (SDS-PAGE, 10%, Bio-Rad) was used to separate the total protein which was transferred to a PVDF membrane (Millipore, Boston, MA). The membrane was placed in 5% zero fat milk powder and incubated with the corresponding primary antibody and anti-β-actin monoclonal antibody. After cleaning, the membrane was incubated with the HRP secondary antibody (Thermo Fisher Scientific). Finally, protein complexes were displayed by enhanced chemiluminescence (ECL) substrate and detected by a Carestream molecular imaging system (Bio-Rad). The primary antibodies used in this study were as follows: anti-GPRC5A, anti-VEGF, anti-caspase-3, anti-caspase-9, anti-NLRP3 and anti-STAT3 anti-Socs3, anti-c-MYC, anti-COX-2, anti-E-cadherin, anti-vimentin and anti-iNOS; all were purchased from CST.

Statistical analyses. Data are represented as means ± SD and each experiment was performed in triplicate. One-way ANOVAs and a paired *t* test were used to analyse statistical significance. Pearson correlation coefficient to analyze the correlation between STAT3 and GPRC5A. All statistical analyses were performed by SPSS 20.0 software (SPSS, Inc., Chicago, IL). *P* values <0.05 were considered to be significant.

Results

The expression of GPRC5A in HCC tissue and cells was significantly decreased. In order to explore whether there was a differential expression of GPRC5A between normal tissues and cells and hepatoma tissues and hepatoma cells, we analysed the expression of GPRC5A by qRT-PCR and Western blot. As shown in Fig. 1A, the mRNA expression of GPRC5A was significantly decreased in tumour tissues as compared to adjacent tissues (Fig. 1A). As expected, the expression of GPRC5A protein in HCC tissues was lower than that of adjacent tissues (Fig. 1B). In addition, the expression of GPRC5A mRNA and protein in HCC cells was significantly down-regulated compared with normal cells (Fig. 1C and D).

Overexpression of GPRC5A inhibited proliferation and epithelial-mesenchymal transition (EMT) in HCC cells. In order to explore the effect of GPRC5A on the proliferation and migration of hepatoma cells, both the number of cells and the expression of migration-related proteins were measured. HepG2 and HCCLM3 cells were transfected with the control pcDNA3.1 (pcDNA3.1 group) or pcDNA3.1-GPRC5A (p-GPRC5A group), respectively. The expression of GPRC5A protein was significantly increased in the pcDNA3.1-GPRC5A group (Fig. 2A). In addition, pcDNA3.1-GPRC5A significantly inhibited cell proliferation to a greater extent than the pcDNA3.1 group in HepG2 and HCCLM3 cells (Fig. 2B). Moreover, the expression of VEGF protein was significantly down-regulated in the p-GPRC5A group (Fig. 2C). Similarly, the epithelial-mesenchymal transition-related protein N-cadherin and Vimentin were drastically down-regulated when transfected with pcDNA3.1-GPRC5A, and E-cadherin were drastically up-regulated in p-GPRC5A group (Fig. 2D–G).

Overexpression of GPRC5A reduced the level of inflammatory factors in HCC cells. In order to further explore the function of GPRC5A, the effect of GPRC5A on inflammatory

doi: 10.3164/jcbn.22-125

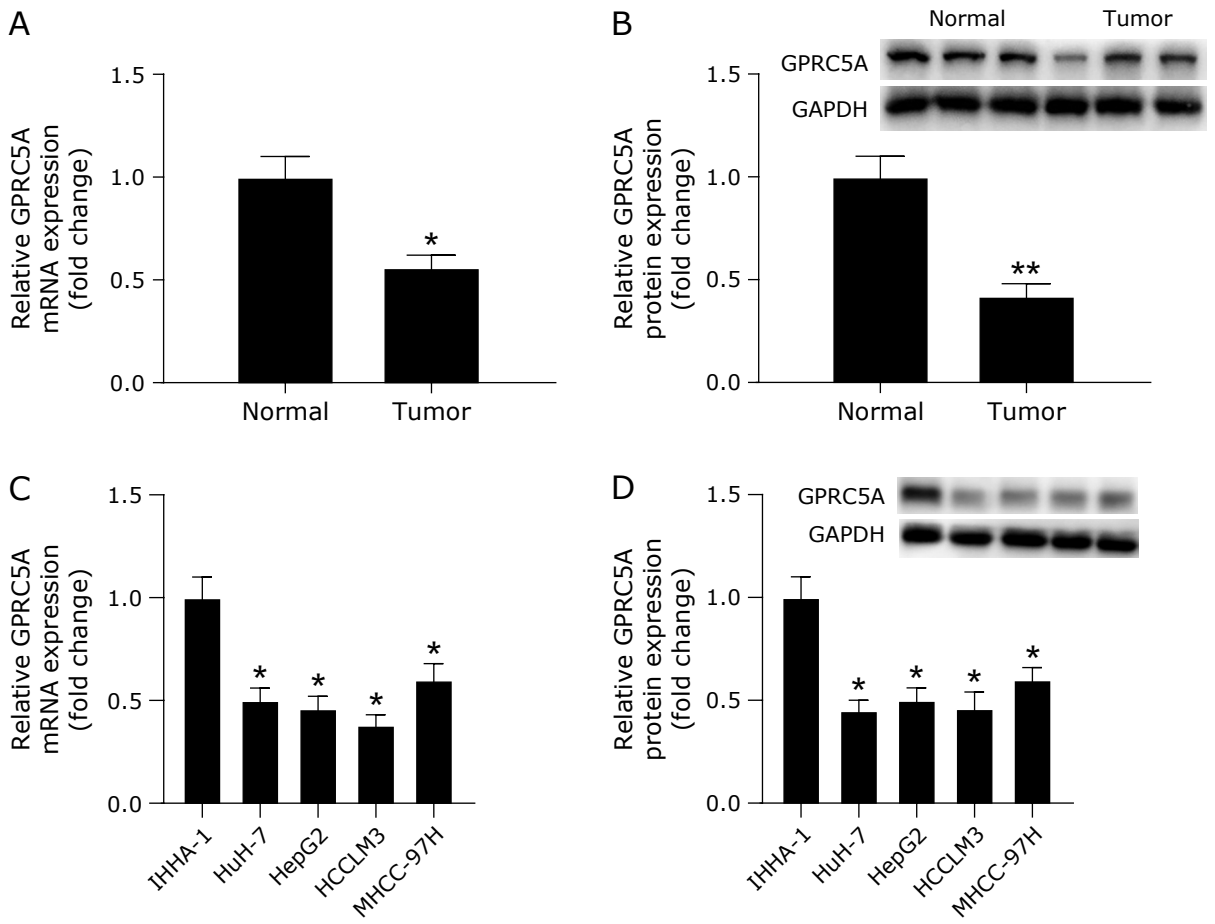

Fig. 1. The expression of GPRC5A in HCC tissue and cells is decreased significantly. (A) The mRNA expression of GPRC5A in adjacent and HCC tumour tissues. (B) The protein expression of GPRC5A in adjacent and HCC tumour tissues. (C) GPRC5A mRNA expression in normal hepatocytes IHHA-1 and HCC cells HuH-7, HepG2, HCCLM3 and MHCC-97H. (D) GPRC5A protein expression in normal hepatocytes IHHA-1 and HCC cells HuH-7, HepG2, HCCLM3 and MHCC-97H. * indicates $p<0.01$ compared with IHHA-1 group or Adjacent group.

Table 1. Relevance between clinicopathological characteristics of HCC patients and GPRC5A expression

Clinical and pathological characteristics	GPRC5A expression			
	Cases	High	Low	*p* value
Age				0.265
<50	12	2	10	
≥50	36	8	28	
Sex				0.595
Female	20	3	17	
Male	28	6	22	
TNM stage				0.175
I–II	14	5	9	
III–IV	34	4	30	
Tumor size				0.045*
<5	18	7	11	
≥5	30	9	21	
Metastasis				0.047*
Yes	32	6	26	
No	16	5	11	

factors was determined. Our results showed that the levels of IL-6, TNF-α and NO were significantly decreased when HCC cells were transfected with pcDNA3.1-GPRC5A (Fig. 3A–C). In addition, pcDNA3.1-GPRC5A decreased the expression of COX-2 and iNOS (Fig. 3D).

Overexpression of GPRC5A induced oxidative stress and apoptosis in HCC cells. We further explored the effect of GPRC5A on oxidative stress in hepatoma cells. As shown in Fig. 4A, the level of ROS was significantly up-regulated after treatment with pcDNA3.1-GPRC5 as compared to HCC cells transfected with pcDNA3.1 (Fig. 4A). However, pcDNA3.1-GPRC5A significantly decreased the levels of NAD^+/NADH and ATP in HepG2 and HCCLM3 cells (Fig. 4B and C). More importantly, pcDNA3.1-GPRC5A significantly increased apoptosis in HepG2 and HCCLM3 cells (Fig. 4D). Moreover, the expression of caspase-3 and caspase-9 was significantly up-regulated in the pcDNA3.1-GPRC5A group (Fig. 4E).

Overexpression of GPRC5A inhibited the activation of STAT3/Socs3/c-MYC and the NLRP3 inflammasome. We further explored the mechanisms involved in GPRC5A-regulated proliferation, EMT and oxidative stress. As shown in Fig. 5A, the expression of GPRC5A protein was significantly increased in the pcDNA3.1-GPRC5A group (Fig. 5A). We found that overexpression of GPRC5A significantly inhibited the expression of p-STAT3, Socs3 and c-MYC in HepG2 cells (Fig. 5B–D). Moreover, the expression of NLRP3 was significantly decreased when cells were transfected with pcDNA3.1-GPRC5A (Fig. 5E and F).

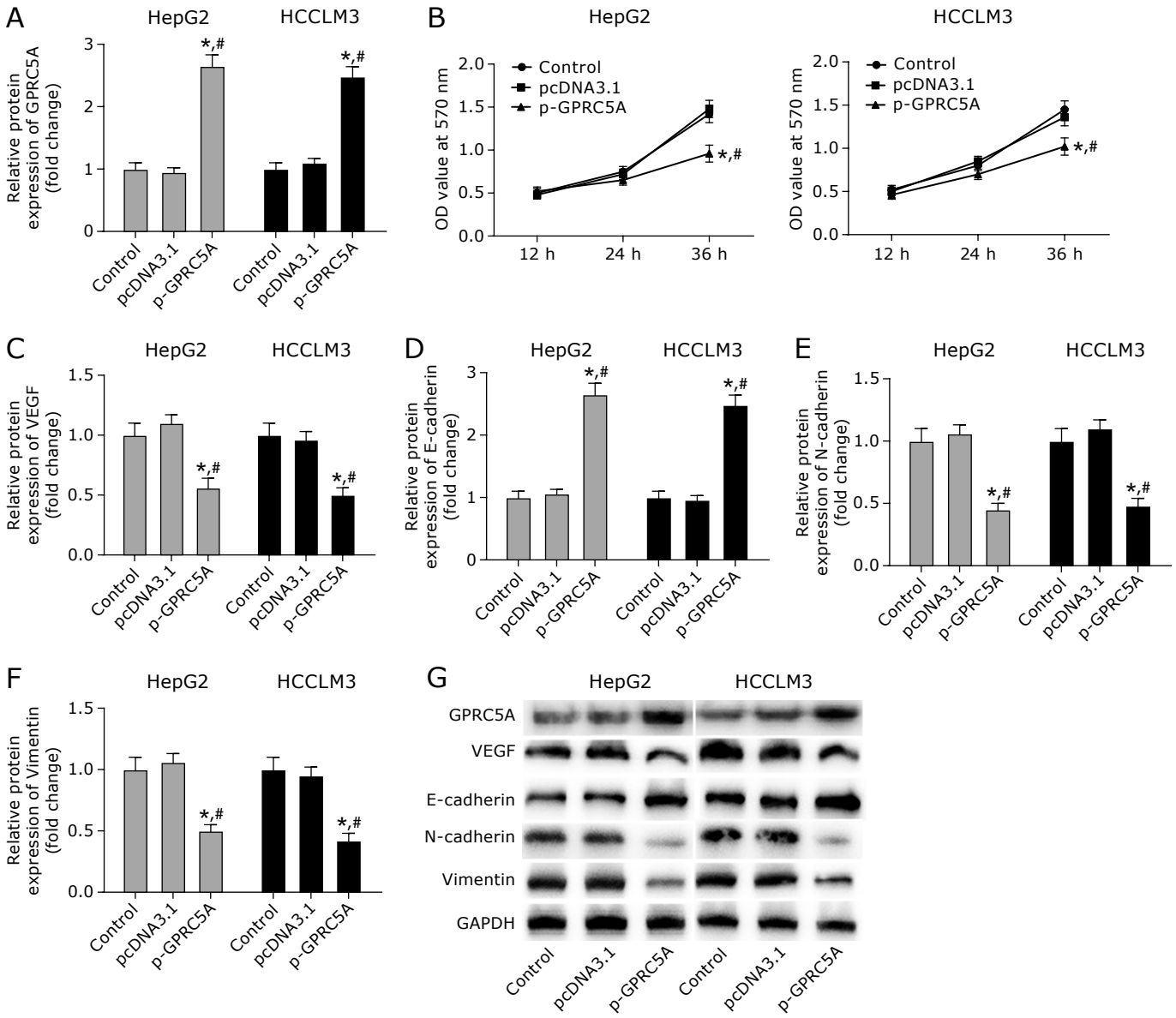

Fig. 2. Overexpression of GPRC5A inhibits proliferation and migration of HCC cells. HepG2 and HCCLM3 cells were transfected with control pcDNA3.1 (pcDNA3.1 group) or pcDNA3.1-GPRC5A (p-GPRC5A group), respectively. (A) GPRC5A protein expression in the control group, pcDNA3.1 group and p-GPRC5A group. (B) Cell proliferation of HepG2 and HCCLM3 cells in the control group, pcDNA3.1 group and p-GPRC5A group. (C) VEGF protein expression in control group, pcDNA3.1 group and p-GPRC5A group. (D) E-cadherin protein expression in control group, pcDNA3.1 group and p-GPRC5A group. (E) N-cadherin protein expression in control group, pcDNA3.1 group and p-GPRC5A group. (F) Vimentin protein expression in control group, pcDNA3.1 group and p-GPRC5A group. (G) Western blot was used to measure the protein expression. * indicates $p<0.05$ compared with control group, and # indicates $p<0.05$ compared with pcDNA3.1 group. GAPDH was used as an invariant internal control for calculating protein-fold changes.

The STAT3/Socs3/c-MYC and NLRP3 inflammasome were involved in the mechanism of GPRC5A on HCC cells. HepG2 cells were transfected with pcDNA3.1-GPRC5A, pcDNA3.1-STAT3, pcDNA3.1-NLRP3, pcDNA3.1-Socs3 or pcDNA3.1-c-MYC, respectively. We found that the expression of VEGF was significantly down-regulated after transfection with pcDNA3.1-GPRC5A. Meanwhile, STAT3, NLRP3, Socs3 or c-MYC significantly increased the expression of VEGF and reversed the inhibition of GPRC5A (Fig. 6A). As expected, overexpression of STAT3, NLRP3, Socs3 or c-MYC significantly down-regulated the apoptosis of HepG2 cells compared with the untreated group (Fig. 6B). In addition, the level of IL-6 was significantly increased when the cells were treated with pcDNA3.1-STAT3, pcDNA3.1-NLRP3, pcDNA3.1-Socs3 or pcDNA3.1-c-MYC as compared to the untreated group (Fig. 6C). More importantly, pcDNA3.1-STAT3, pcDNA3.1-NLRP3, pcDNA3.1-Socs3 or pcDNA3.1-c-MYC significantly increased the expression of E-cadherin (Fig. 6D). Moreover, the level of ROS was significantly decreased after treatment with pcDNA3.1-STAT3, pcDNA3.1-NLRP3, pcDNA3.1-Socs3 or pcDNA3.1-c-MYC, and reversed the role of GPRC5A (Fig. 6E).

Discussion

Abnormal expression of GPRC5A is closely related to the occurrence of malignant tumours.[22] It has been found that GPRC5A is preferentially expressed in lung tissue, and GPRC5A deficiency can promote the development of lung cancer.[23] In

doi: 10.3164/jcbn.22-125

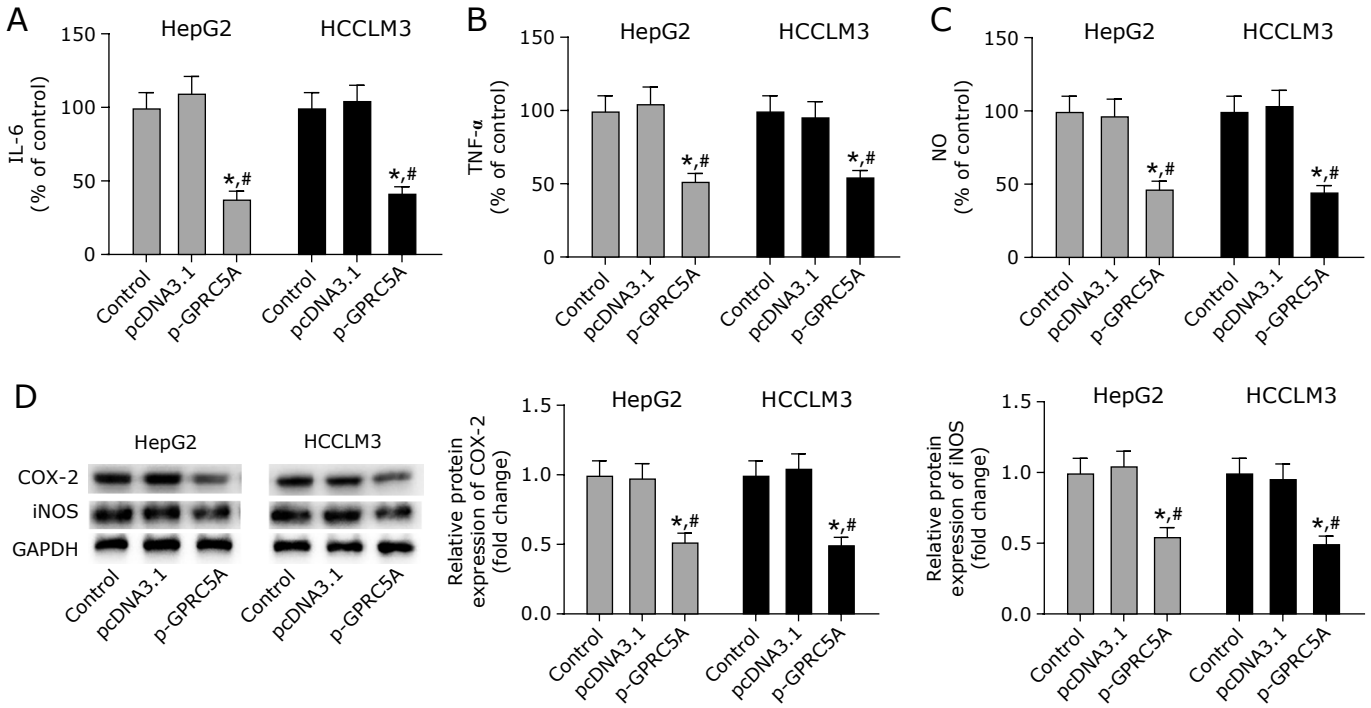

Fig. 3. Overexpression of GPRC5A reduces the level of inflammatory factors in HCC cells. HepG2 and HCCLM3 cells were transfected with control pcDNA3.1 (pcDNA3.1 group) or pcDNA3.1-GPRC5A (p-GPRC5A group), respectively. (A–C) An ELISA assay was used to measure the level of IL-6, TNF-α and NO in control group, pcDNA3.1 group and p-GPRC5A group. (D) Protein expression of COX-2 and iNOS in control group, pcDNA3.1 group and p-GPRC5A group. * indicates $p<0.05$ compared with control group, and # indicates $p<0.05$ compared with pcDNA3.1 group. GAPDH was used as an invariant internal control for calculating protein-fold changes.

addition, GPRC5A has been shown to be down-regulated in breast cancer cells, and plays an anti-cancer role by inhibiting EGFR and its downstream pathway.[24] However, some authors have come to the opposite conclusion about the role of GPRC5A in human cancer. Sawada *et al.*[4] found that GPRC5A promoted cell proliferation and bone metastasis in prostate cancer by regulating the cell cycle. Coincidentally, Zhang *et al.*[25] reported that the expression of GPRC5A increased in colorectal cancer tissues and promoted tumour progression, through VNN-1-induced oxidative stress. At present, there are no consistent conclusions about the role of GPRC5A in the occurrence and development of human cancer. Our study showed a low expression of GPRC5A in HCC tissues and cells. Furthermore, GPRC5A inhibited proliferation and EMT and induced oxidative stress and apoptosis in HCC cells. Once, we believe that GPRC5A may play an important role in the development of HCC, and provide theoretical basis for clinical diagnosis. Maybe GPRC5A can be used as a diagnostic biomarker. Moreover, we found that GPRC5A decreased ATP and the NAD^+/NADH ratio, indicating that perhaps GPRC5A influences energy homeostasis, which may be one of the ways in which GPRC5A plays a role. In addition, overexpression of GPRC5A can increase ROS production. Perhaps GPRC5A regulates ROS production by changing the energy balance of cells, and this mechanism is worthy of further study.

Recent studies have shown that GPRC5A dysfunction occurs in a variety of human cancer-related pathways, including NF-κB, FAK/SCR and STAT3 pathways.[23] STAT3 is considered an important target for cancer treatment, and it is easily activated in various malignant tumours.[26] STAT3 regulates the glycolysis process of HCC through the HK2 pathway, and provides multiple potential therapeutic targets for HCC treatment through glycolysis intervention.[27] STAT3 and Twist co-mediate EMT and induce invasion and metastasis of HCC, and activated STAT3, Twist and EMT markers can be used as potential molecular targets to prevent and treat invasion and metastasis of HCC.[28] In addition, Jiang *et al.*[29] found that STAT3, as a downstream target gene of miR-500a-3p, promotes the characteristics of tumour stem cells in HCC and further promotes the deterioration of HCC. In addition, GPRC5A overexpression suppressed the IL-6 induced activation of STAT3, inhibited cell growth and blocked the further deterioration of head and neck squamous cell carcinoma (HNSCC). Likewise, in the present study, we found that GPRC5A inhibited the expression of STAT3, and STAT3 promoted proliferation and EMT in HCC cells, which indicates that STAT3 plays an important role in promoting the development of HCC.

It has been reported that STAT3/Socs3, activated by IL-6, promotes the development of pancreatic intraepithelial neoplasia and pancreatic cancer.[30] Similarly, Geng *et al.*[31] found that STAT3 and Socs3 were increased in T-cell lymphoma tissues and cells, and inhibition of STAT3 and Socs3 expression inhibited the progress of T-cell lymphoma. In addition, the authors believe that there is a negative feedback loop between STAT3 and SOCS3, which may play a tumorigenic role in T-cell lymphoma by inhibiting the production of specific cytokines and apoptosis signals. Furthermore, STAT3 and c-MYC were up-regulated in human gastric cancer tissues and cells, and knockouts of c-MYC inhibited proliferation and glycolysis in gastric cancer cells, suggesting that STAT3/c-MYC may be a potential therapeutic target for gastric cancer.[19] Therefore, targeting the STAT3/Socs3/c-MYC pathway may be a potential therapy for human cancer.

Lu *et al.*[32] found that the NLRP3 inflammasome was activated by chenodeoxycholic acid (CDCA) in a dose-dependent manner, leading to activation of the NLRP3-mediated pathway and promoting the occurrence and development of hepatic fibrosis. It has been reported that the NLRP3 inhibitor, MCC950,

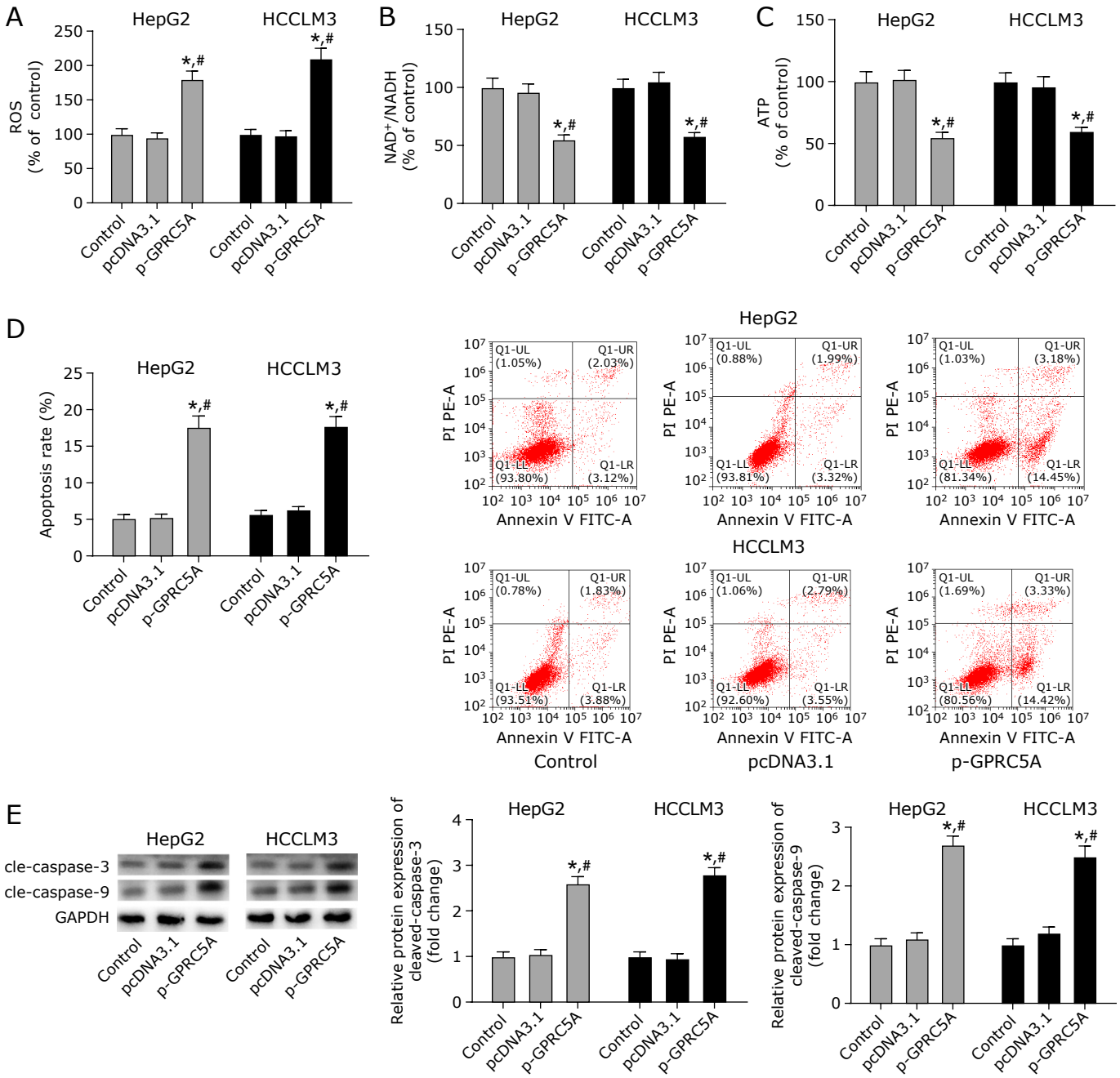

Fig. 4. Overexpression of GPRC5A induces oxidative stress and apoptosis in HCC cells. HepG2 and HCCLM3 cells were transfected with control pcDNA3.1 (pcDNA3.1 group) or pcDNA3.1-GPRC5A (p-GPRC5A group), respectively. (A) The ROS level in control group, pcDNA3.1 group and p-GPRC5A group. (B) The NAD^+/NADH level in control group, pcDNA3.1 group and p-GPRC5A group. (C) The ATP level in control group, pcDNA3.1 group and p-GPRC5A group. (D) The apoptosis rate (%) of HepG2 and HCCLM3 in control group, pcDNA3.1 group and p-GPRC5A group. (E) The protein expression of cleaved-caspase-3 and cleaved-caspase-9 in control group, pcDNA3.1 group and p-GPRC5A group. * indicates $p<0.05$ compared with control group, and # indicates $p<0.05$ compared with pcDNA3.1 group. GAPDH was used as an invariant internal control for calculating protein-fold changes.

improves the NAFLD pathology of obese diabetic mice and reduces liver damage. In addition, MCC950 improves liver fibrosis in mice. Targeting NLRP3 is a reasonable way to treat liver diseases.[33] In this study, we found that the expression of NLRP3 was significantly decreased when transfected with pcDNA3.1-GPRC5A. Indeed, there are literature reports "Deregulation of the NLRP3 inflammasome in hepatic parenchymal cells during liver cancer progression".[34] We believe that the occurrence and development of liver cancer is a complex process, involving a variety of regulatory factors and regulatory pathways. Therefore, due to different factors, the expression of a certain factor changes, which is also very common. Therefore, the study of liver cancer is still not deep enough and needs to be further explored.

In conclusion, our current study reveals that GPRC5A inhibits the occurrence and development of HCC by regulating the STAT3-mediated Socs3/c-MYC signalling pathway and NLRP3 inflammasome, which suggests that GPRC5A might be a new biomarker related to cell proliferation, EMT, oxidative stress and apoptosis in HCC. In the future, identifying a reasonable mecha-

doi: 10.3164/jcbn.22-125

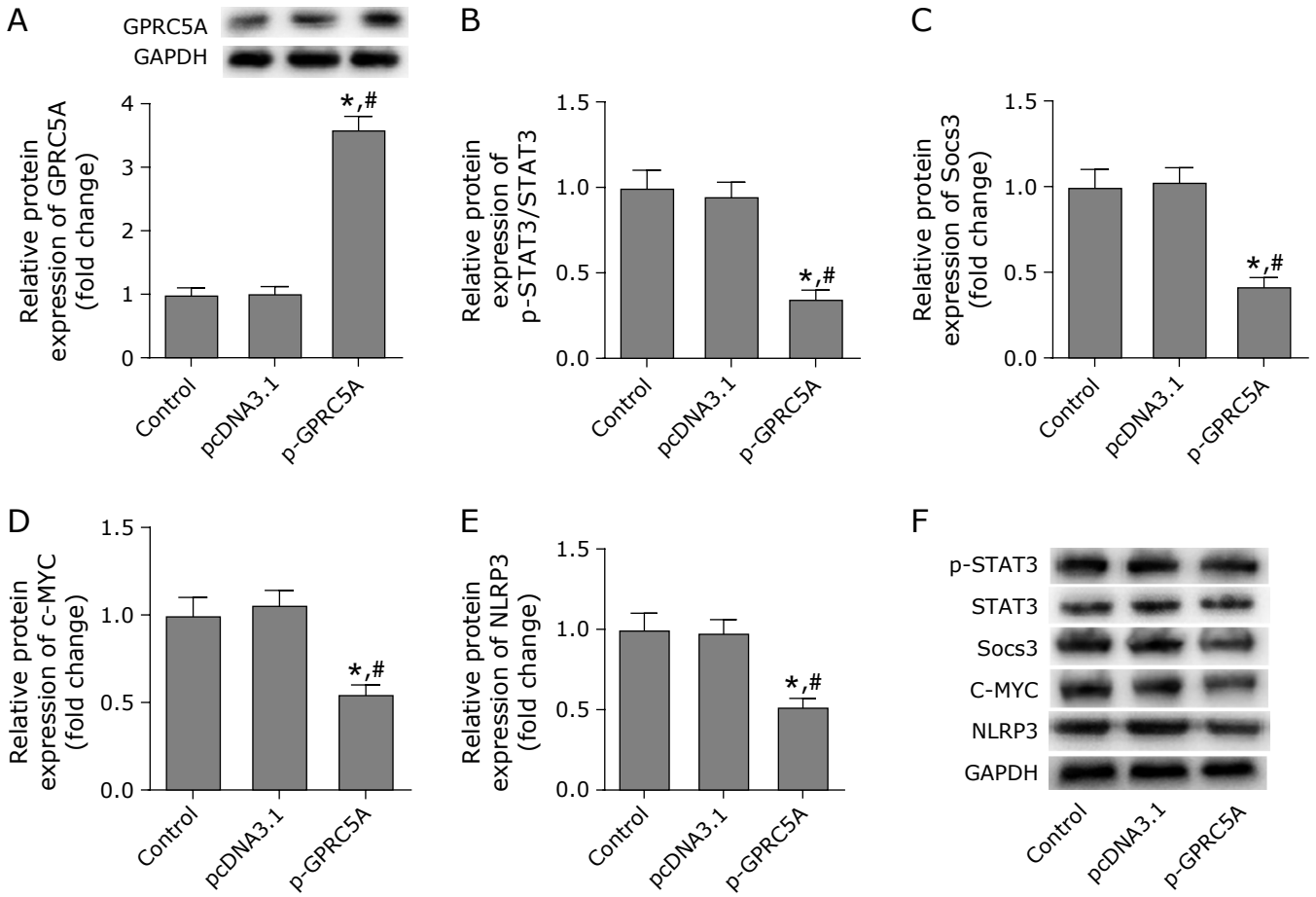

Fig. 5. Overexpression of GPRC5A inhibits the activation of STAT3/Socs3/c-MYC and NLRP3 inflammasome. The HepG2 cells were transfected with control pcDNA3.1 (pcDNA3.1 group) or pcDNA3.1-GPRC5A (p-GPRC5A group), respectively. (A) GPRC5A protein expression in the control group, pcDNA3.1 group and p-GPRC5A group. (B) The expression of p-STAT3 and STAT3 protein in control group, pcDNA3.1 group and p-GPRC5A group. (C) The expression of Socs3 protein in control group, pcDNA3.1 group and p-GPRC5A group. (D) The expression of c-MYC protein in control group, pcDNA3.1 group and p-GPRC5A group. (E) The expression of NLRP3 protein in control group, pcDNA3.1 group and p-GPRC5A group. (F) Western blot was used to measure the protein expression. * indicates $p<0.05$ compared with control group, and #indicates $p<0.05$ compared with pcDNA3.1 group. GAPDH was used as an invariant internal control for calculating protein-fold changes.

nism for the role of GPRC5A will help us to understand its function more comprehensively and, finally, find a new way to treat human malignant tumours. It is reported that GPRC5A has abnormal expression and mutation in various cancers.[8] Similarly, we found abnormal expression of GPRC5A in HCC. We will continue to explore whether GPRC5A mutation exists in HCC in the following experiments, so as to provide the basis for clinical diagnosis of HCC. This study is only a preliminary study on the role and mechanism of GPRC5A in HCC. As we all know, the occurrence and development of HCC is a complex process. Similarly, the role and molecular mechanism of GPRC5A are very complex. Therefore, it still needs to be further explored. In the following research, we will use recombinant GPRC5A to further explore, so as to provide theoretical basis for clinical diagnosis and treatment of HCC.

Author Contributions

JY conceived, designed the research, edited and revised the manuscript. LZ drafted and revised the manuscript; WY performed experiments; FS analyzed data and prepared figures. All authors approved the final version of the manuscript.

Acknowledgments

None.

Financial Disclosure

None.

Conflict of Interest

No potential conflicts of interest were disclosed.

Ethics Approval

All experiments on human subjects were conducted in accordance with the Declaration of Helsinki and that all procedures were carried out with the adequate understanding and written consent of the subjects, and this study was formally approved by the Human Subjects Review Board of The First Affiliated Hospital of Xi'an Jiaotong University.

Availability of Data and Materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

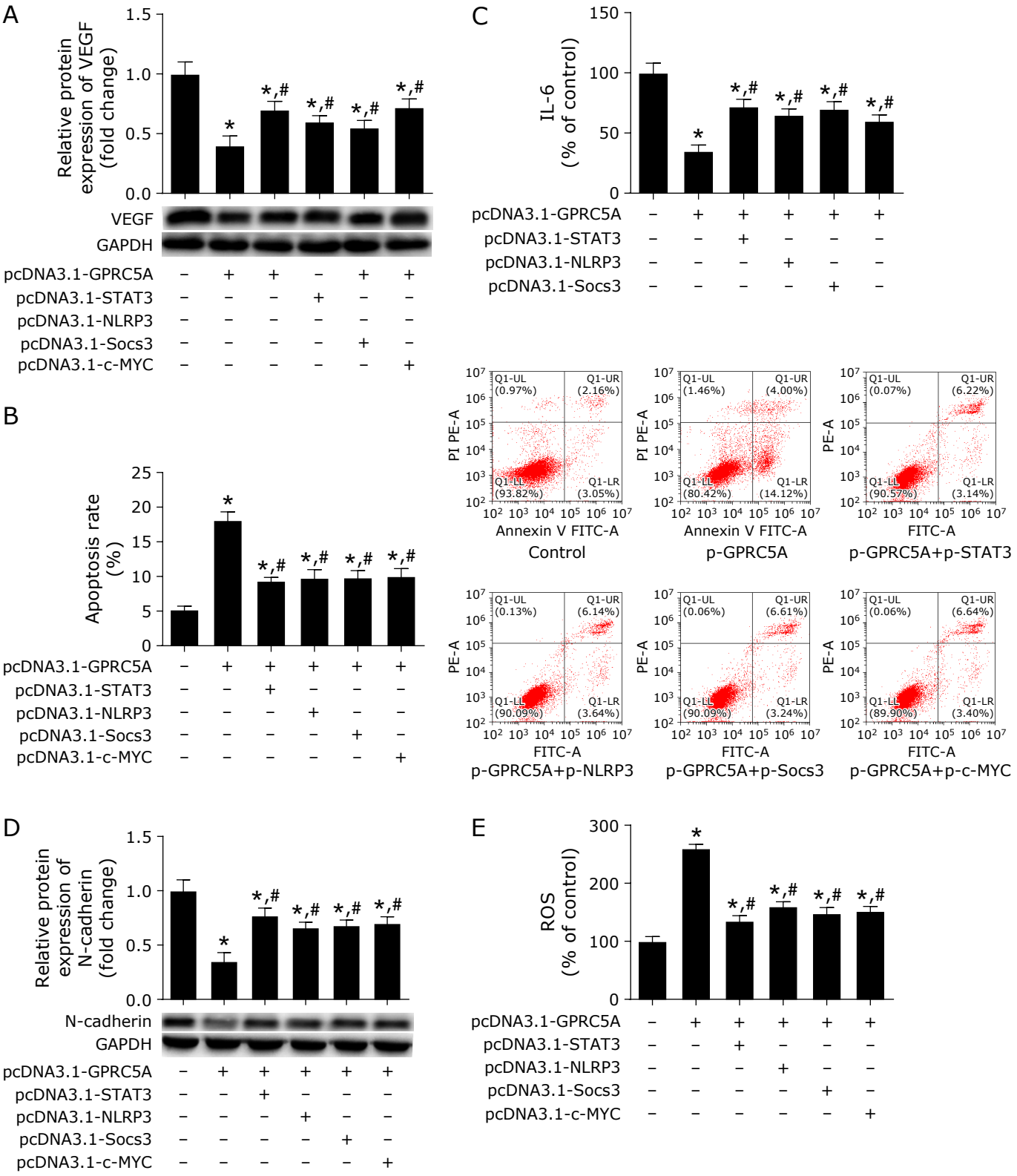

Fig. 6. The STAT3/Socs3/c-MYC and NLRP3 inflammasome are involved in the mechanism of GPRC5A on HCC cells. HepG2 cells were transfected with pcDNA3.1-GPRC5A, pcDNA3.1-STAT3 or pcDNA3.1-NLRP3, respectively. (A) The expression of VEGF protein in each group. (B) The apoptosis rate (%) of HepG2 in each group. (C) The level of IL-6 in each group. (D) The expression of N-cadherin protein in each group. (E) The ROS level in each group. * indicates $p<0.05$ compared with untreated group, and # indicates $p<0.055$ compared with pcDNA3.1-GPRC5A group. GAPDH was used as an invariant internal control for calculating protein-fold changes.

doi: 10.3164/jcbn.22-125

References

1 Anwanwan D, Singh SK, Singh S, Saikam V, Singh R. Challenges in liver cancer and possible treatment approaches. *Biochim Biophys Acta Rev Cancer* 2020; **1873**: 188314.
2 Villanueva A. Hepatocellular Carcinoma. *N Engl J Med* 2019; **380**: 1450–1462.
3 Reynaert H, Colle I. Treatment of advanced hepatocellular carcinoma with somatostatin analogues: a review of the literature. *Int J Mol Sci* 2019; **20**: 4811.
4 Sawada Y, Kikugawa T, Iio H, *et al*. GPRC5A facilitates cell proliferation through cell cycle regulation and correlates with bone metastasis in prostate cancer. *Int J Cancer* 2020; **146**: 1369–1382.
5 Zhou H, Rigoutsos I. MiR-103a-3p targets the 5' UTR of GPRC5A in pancreatic cells. *RNA* 2014; **20**: 1431–1439.
6 Liu H, Zhang Y, Hao X, *et al*. GPRC5A overexpression predicted advanced biological behaviors and poor prognosis in patients with gastric cancer. *Tumour Biol* 2016; **37**: 503–510.
7 Yang Z, Li C, Fan Z, *et al*. Single-cell sequencing reveals variants in ARID1A, GPRC5A and MLL2 driving self-renewal of human bladder cancer stem cells. *Eur Urol* 2017; **71**: 8–12.
8 Sokolenko AP, Bulanova DR, Iyevleva AG, *et al*. High prevalence of GPRC5A germline mutations in BRCA1-mutant breast cancer patients. *Int J Cancer* 2014; **134**: 2352–2358.
9 Pencik J. STAT3-regulated ARF suppresses prostate cancer metastasis. *Pathologe* 2016; **37 (Suppl 2)**: 173–174. (in German)
10 Jing N, Tweardy DJ. Targeting Stat3 in cancer therapy. *Anticancer Drugs* 2005; **16**: 601–607.
11 Liu RY, Zeng Y, Lei Z, *et al*. JAK/STAT3 signaling is required for TGF-β-induced epithelial-mesenchymal transition in lung cancer cells. *Int J Oncol* 2014; **44**: 1643–1651.
12 Sanchez-Lopez E, Flashner-Abramson E, Shalapour S, *et al*. Targeting colorectal cancer via its microenvironment by inhibiting IGF-1 receptor-insulin receptor substrate and STAT3 signaling. *Oncogene* 2016; **35**: 2634–2644.
13 Yang H, Yamazaki T, Pietrocola F, *et al*. STAT3 inhibition enhances the therapeutic efficacy of immunogenic chemotherapy by stimulating type 1 interferon production by cancer cells. *Cancer Res* 2015; **75**: 3812–3822.
14 Bonetto A, Camperi A, Aydogdu T, *et al*. SOCS3 is a novel bi-functional regulator of muscle growth and wasting. *Poster session presented at IUPUI Research Day 2015.*
15 Gordon P, Okai B, Hoare JI, Erwig LP, Wilson HM. SOCS3 is a modulator of human macrophage phagocytosis. *J Leukoc Biol* 2016; **100**: 771–780.
16 Yin Y, Liu W, Dai Y. SOCS3 and its role in associated diseases. *Hum Immunol* 2015; **76**: 775–780.
17 Stine ZE, Walton ZE, Altman BJ, Hsieh AL, Dang CV. MYC, metabolism, and cancer. *Cancer Discov* 2015; **5**: 1024–1039.
18 Nebbioso A, Carafa V, Conte M, *et al*. c-Myc modulation and acetylation is a key HDAC inhibitor target in cancer. *Clin Cancer Res* 2017; **23**: 2542–2555.
19 Gao S, Chen M, Wei W, *et al*. Crosstalk of mTOR/PKM2 and STAT3/c-Myc signaling pathways regulate the energy metabolism and acidic microenvironment of gastric cancer. *J Cell Biochem* 2019; **120**: 1193–1202.
20 Subramaniam KS, Omar IS, Kwong SC, *et al*. Cancer-associated fibroblasts promote endometrial cancer growth via activation of interleukin-6/STAT-3/c-Myc pathway. *Am J Cancer Res* 2016; **6**: 200–213.
21 Zhang S, Li J, Xie P, *et al*. *STAT3/c-Myc* axis-mediated metabolism alternations of inflammation-related glycolysis involve with colorectal carcinogenesis. *Rejuvenation Res* 2019; **22**: 138–145.
22 Zhou H, Telonis A, Jing Y, *et al*. Abstract A41: GPRC5A acts as a potent oncogene in pancreatic cancer. *Cancer Res* 2016; **76 (24-Supplement)**: A41.
23 Jiang X, Xu X, Wu M, *et al*. GPRC5A: an emerging biomarker in human cancer. *Biomed Res Int* 2018; **2018**: 1823726.
24 Yang L, Ma T, Zhang J. GPRC5A exerts its tumor-suppressive effects in breast cancer cells by inhibiting EGFR and its downstream pathway. *Oncol Rep* 2016; **36**: 2983–2990.
25 Zhang L, Li L, Gao G, *et al*. Elevation of GPRC5A expression in colorectal cancer promotes tumor progression through VNN-1 induced oxidative stress. *Int J Cancer* 2017; **140**: 2734–2747.
26 Xie L, Zeng Y, Dai Z, *et al*. Chemical and genetic inhibition of STAT3 sensitizes hepatocellular carcinoma cells to sorafenib induced cell death. *Int J Biol Sci* 2018; **14**: 577–585.
27 Li M, Jin R, Wang W, *et al*. STAT3 regulates glycolysis via targeting hexokinase 2 in hepatocellular carcinoma cells. *Oncotarget* 2017; **8**: 24777–24784.
28 Zhang C, Guo F, Xu G, Ma J, Shao F. STAT3 cooperates with Twist to mediate epithelial-mesenchymal transition in human hepatocellular carcinoma cells. *Oncol Rep* 2015; **33**: 1872–1882.
29 Jiang C, Long J, Liu B, *et al*. miR-500a-3p promotes cancer stem cells properties via STAT3 pathway in human hepatocellular carcinoma. *J Exp Clin Cancer Res* 2017; **36**: 99.
30 Lesina M, Kurkowski MU, Ludes K, *et al*. Stat3/Socs3 activation by IL-6 transsignaling promotes progression of pancreatic intraepithelial neoplasia and development of pancreatic cancer. *Cancer Cell* 2011; **19**: 456–469.
31 Geng L, Lu K, Li P, *et al*. GLI1 inhibitor GANT61 exhibits antitumor efficacy in T-cell lymphoma cells through down-regulation of p-STAT3 and SOCS3. *Oncotarget* 2017; **8**: 48701.
32 Gong Z, Zhou J, Zhao S, *et al*. Chenodeoxycholic acid activates NLRP3 inflammasome and contributes to cholestatic liver fibrosis. *Oncotarget* 2016; **7**: 83951–83963.
33 Mridha AR, Wree A, Robertson AAB, *et al*. NLRP3 inflammasome blockade reduces liver inflammation and fibrosis in experimental NASH in mice. *J Hepatol* 2017; **66**: 1037–1046.
34 Wei Q, Mu K, Li T, *et al*. Deregulation of the NLRP3 inflammasome in hepatic parenchymal cells during liver cancer progression. *Lab Invest* 2014; **94**: 52–62.