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Hepassocin Regulates Cell Proliferation of the Human Hepatic Cells L02 and Hepatocarcinoma Cells Through Different Mechanisms

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ABSTRACT

Hepassocin (HPS) is a specific mitogenic active factor for hepatocytes, and inhibits growth by overexpression in hepatocellular carcinoma (HCC) cells. However, the mechanism of HPS regulation on growth of liver-derived cells still remains largely unknown. In this study, we found that HPS was expressed and secreted into the extracellular medium in cultured L02 human hepatic cells; conditional medium of L02 cells promoted proliferation of L02 cells and this activity could be blocked by anti-HPS antibody. Moreover, we identified the presence of receptor for HPS on L02 cells and HepG2 human hepatoma cells. Overproduction of truncated HPS, which signal peptide was deleted, significantly inhibited the proliferation of HCC cells and induced cell cycle arrest. These findings suggest that HPS promotes hepatic cell line L02 cells proliferation via an autocrine mechanism and inhibits HCC cells proliferation by an intracrine pathway. J. Cell. Biochem. 112: 2882–2890, 2011.

KEY WORDS: HEPASSOCIN; AUTOCRINE; HCC; INTRACRINE

Various previous studies have revealed that growth of hepatocytes is regulated by locally produced growth factors that function through autocrine or paracrine mechanisms. For example, TGF-α is produced by hepatocytes and provides a mitogenic stimulus through an autocrine mechanism [Mead and Faust, 1989]. EGF provides a mitogenic stimulus on hepatocytes through the endocrine system [Skov Oslen et al., 1988]. FGF-1 and VEGF are also produced by regenerating hepatocytes and affect growth and differentiation of endothelial cells through paracrine mechanisms [Kan et al., 1989; Mochida et al., 1996]. HGF, as the most effective mitogen, stimulates hepatocytes proliferation and protects hepatocytes from apoptosis by paracrine mechanisms [Kakazu et al., 2004]. Moreover, the existence of liver-specific growth factors has been extensively reported in liver regeneration.

Hepassocin (HPS), also name hepatocyte-derived fibrinogen-related protein (HFREP-1) and fibrinogen-like 1(FGL-1), is a liver-specific expression gene and has mitogenic activity on isolated hepatocytes [Hara et al., 2000]. Human HPS has specific mitogenic activity on primary hepatocytes and human hepatic cell line, but have no stimulating activity on non-liver derived cells; silencing of HPS by RNA interference induces growth inhibition of hepatocytes, which suggested that the human HPS may be an endogenous physiological regulator of hepatocyte proliferation. Additionally, we demonstrated that HPS induced phosphorylation of ERK1/2 in a

Abbreviations used: TGF-α, transforming growth factor-α; EGF, epidermal growth factor; FGF-1, fibroblast growth factor-1; VEGF, vascular endothelial growth factor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; CM, conditional medium.

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time-dependent manner and inhibition of ERK1/2 pathway blocked HPS-induced hepatocyte proliferation, which suggested that ERK1/2 pathway was involved in HPS-induced proliferation effect. Recently, we demonstrated that HPS stimulated the hepatocyte proliferation in vivo and prevented the rat liver injury induced by D-galactosamine and CCl_4. During liver regeneration, the expression of HPS mRNA was strongly up-regulated. After 70% hepatectomy of mouse liver, HPS was induced at 2 h and the second peak arrived at 24 h. The expression of HPS maintained high until 72 h and declined to the basal level thereafter. These results indicated that HPS might function as a regulator of hepatocyte growth and be involved in liver regeneration [Li et al., 2010]. On the other hand, studies also have revealed that HPS was associated with progression of liver tumors. Expression of the HPS/LFIRE-1 was frequently down-regulated or lost in hepatocellular carcinoma (HCC) tissues. Exogenous HPS expression in human HCC cells inhibited their anchorage-dependent or -independent growth in vitro, and down-regulation of HPS by antisense approach enhanced cancer cells proliferation and colony formation in soft agar [Yan et al., 2004]. Taken together, these results suggested that HPS played an important role in the development and physiological function of liver, and associated with progression of liver tumors. However, the molecular mechanisms of regulation hepatocyte derived cells growth by HPS still remain largely unknown.

In the present study, we confirmed the existence of HPS receptor on human hepatic cell line L02, and found that HPS promoted L02 cell proliferation via an autocrine mechanism. However, in a HCC cell line HepG2, HPS inhibited cells growth by an intracrine pathway.

MATERIALS AND METHODS

CELLS CULTURES AND REAGENTS

Human hepatoma cell line HepG2, human hepatic cell line L02, human embryonic kidney cell line HEK293, and human chronic myeloid leukemia cell line K562 were obtained from Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) or RPMI Medium 1640 (Gibco, Carlsbad, CA) supplemented with penicillin (100 U/ml), streptomycin (10 μg/ml), and 10% fetal bovine serum (MD, St. Louis, MO) at 37°C in a humidified incubator with 5% CO_2 in air and split 1:2 at confluence. Recombinant human HPS (rhHPS) was produced as described previously [Li et al., 2010]. G418 was purchased from Calbiochem (San Diego, CA). Normal mouse IgG was purchased from Santa Cruz Biotech. (Santa Cruz, CA).

PLASMID CONSTRUCTIONS

The human full-length HPS was amplified by polymerase chain reaction (PCR) from the human liver cDNA, and cloned into the pcDNA3.1/Myc-HisB vector (Invitrogen, Carlsbad, CA) and pEGFP-N1 vector (Clontech, Palo Alto, CA), respectively. The HPS mutant lacking N-terminal 22 amino acids of HPS was inserted into pcDNA3.1/Myc-HisB vector or pEGFP-N1 vector to generate pcDNA-ΔN22 and ΔN22-GFP, respectively. For antibody production, N-terminal 22 amino acids deleted human HPS cDNA was cloned into pET 28b (Merck Biosciences, Bad Soden, Germany). The DNA sequences of all constructs were confirmed by sequence analysis.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

This assay was performed as described [Thomas et al., 1986]. Briefly, this assay used polyclonal antibody against rhHPS for capture and monoclonal mouse antibody against rhHPS for detection. A reporter system used anti-mouse IgG-HRP (Santa Cruz Biotech.) as the second antibody and o-PD (Merck Biosciences) as the substrate, and the absorbance was measured in a microplate spectrophotometer at 490 nm (Model 680XR; Bio-Rad, Hercules, CA).

WESTERN BLOTTING

Cells were collected and lysed with RIPA buffer containing protease inhibitor for 30 min on ice and debris were removed by centrifugation at 12,000 rpm for 25 min, and the lysate was quantitated with Bradford kit. The lysates were resolved by SDS–PAGE and transferred polyvinyldifluoride (PVDF) membrane and probed antibodies at following concentrations: β-actin, 1:1,000; ERK, 1:1,000; p-ERK, 1:1,000, p53, 1:1,000; p27, 1:500; cyclin D1, 1:500 (Santa Cruz Biotech.), and HPS, 1:500 (R&D systems, Minneapolis, MN) and chemiluminescent detection was conducted using the supersignal substrate (Pierce, Rockford, IL) according to the manufacturer's specifications.

MTS ASSAY

2×10^3 cells were seeded into each well of 96-well plates. After 24 h, the medium was changed to fresh serum-free medium containing different reagent and incubated for 48 h. Absorbance were determined by using CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI) according to the manufacturer's protocol at indicated time points. All experiments were conducted at least three times.

PREPARATION OF CELL CONDITIONAL MEDIUM (CM)

Cells (5×10^6) were seeded in a 75 cm^2 cell culture flask (Corning, NY) and cultured for 24 h. After washed with PBS at three times, 10 ml fresh serum-free medium was added and cultured for 48 h. The medium was collected and concentrated using ultrafiltration (Millipore, Bedford, MA) with a 10,000 MW cut-off to 0.5 ml and stored at −70°C.

FITC-LABELED HPS-BINDING ASSAY

FITC-labeling of HPS was performed as described [Ghiran et al., 2000]. Cells in 100 μl of PBS were incubated with increasing concentration of FITC-labeled HPS with or without a 50-fold excess of unlabeled HPS at 37°C for 1 h. Cells were washed three times with PBS, and suspended with 100 μl of PBS. Then cells were added into flat bottom 96-well plate and fluorescence intensity at 520 nm was measured separately for the excitation wavelength of 490 nm using a Multi-Mode Microplate reader (SPECTRAmax M5; Molecular Devices. Inc, Silicon valley, CA). The standard curve was obtained between different fluorescence intensity (Y) and corresponding concentration of FITC-labeled HPS (X) with the same method.

CELL CYCLE ANALYSIS

Cells were collected by trypsinazation, pelleted, and resuspended in 1% polyformaldehyde for 15 min. After fixation, cells were washed twice with PBS, then fixed in 70% ethanol overnight at −20°C. Subsequently, cells were precipitated, washed with PBS and

incubated in 100 μl 1 mg/ml RNase A for 30 min at 37°C. Propidium iodide was added for 10 min and DNA content was analyzed by flow cytometry (BD Biosciences, San Jose, CA). For each analysis 20,000 gated events were collected to permit cell cycle analysis of both green fluorescence protein (GFP)-positive and GFP-negative cell subpopulations, and the population of nuclei in each phase of the cell cycle was determined using established CellQuest analysis software (BD Biosciences).

COLONY FORMATION ASSAY

2×10^5 cells were seeded in six-well plates and allowed to adhesion overnight. Cells were transfected with 5 μg of wild-type HPS, HPS mutant expression plasmid or pcDNA3.1 vector, by transfection reagent (Vigorous Biotech Co., Beijing, China), according to the manufacturer's instruction. After G418 (1,400 μg/ml) selection for 3 weeks, positive colonies were fixed with methanol and stained with crystal violet for enumeration. Each transfection experiment was performed in triplicate.

STATISTICAL ANALYSIS

Statistical significance was calculated using the unpaired *t*-test (two-tailed) using Graphpad Prism (GraphPad software, Inc., San Diego, CA) unless otherwise noted. A *P*-value of <0.05 was considered to indicate statistical significance.

RESULTS

HUMAN HPS IS SECRETED TO EXTRACELLULAR CULTURE DEPENDENT ON N-TERMINAL 22 AMINO ACIDS

Previous studies have reported that mouse HPS was secreted to extracellular with a signal peptide mechanism and the N-terminal 22 amino acids of human HPS was predicted as the signal peptide sequence [Hara et al., 2000; Hara and Yoshimura, 2001]. To confirm this prediction, HEK293 cells were transfected with the pcDNA-HPS vector which contained full-length human HPS cDNA, pcDNA-ΔN22 vector which contained mutant HPS lacking 1–22 amino acids, or pcDNA3.1 control vector. After 48 h of transfection, cells lysates and CM were subjected to Western blotting with HPS antibody. As shown in Figure 1A, HPS proteins were detected in the cell lysates from the cells transfected with pcDNA-HPS and pcDNA-ΔN22 vectors. However, HPS was detected only in the CM from cells transfected with full-length HPS expression vector, indicating that N-terminal 1–22 amino acids sequence was required for the secretion of HPS to extracellular culture. Then, we assayed the effect of CM containing secreted HPS on the proliferation of L02 cells. As shown in Figure 1B, CM from cells transfected with full-length HPS expression vector significantly induced cell proliferation in a dose-dependent manner. However, CM from cells transfected with HPS mutant lacking N-terminal 1–22 amino acids expression vector did not affect L02 cell proliferation. Our previous study reported that prokaryotic expressing rhHPS-induced cell proliferation was depended on ERK1/2 pathway. Here, we confirmed this conclusion using HPS secreted from HEK293 cells on hepatic cells with an inhibitor experiment. As shown in Figure 1C, L02 cells proliferation induced by CM from HEK293 cells transfected with full-length HPS was inhibited by U0126 (ERK1/2 inhibitor) but not LY294002 (PI3K inhibitor). Accordingly, Western blotting showed that HPS-induced ERK activity was inhibited by U0126 (5 nM) but not LY294002 (10 nM) when AKT was inhibited by LY294002 (Fig. 1D). In addition, AKT pathway was not activated by HPS while ERK pathway was activated.

PREPARATION OF NEUTRALIZING ANTIBODY AGAINST HPS

To develop antibodies against HPS, HPS protein was expressed fused with His-tag and purified from *E. coli*. Four colonies of HPS monoclonal antibody (named A–D, respectively) were obtained from experimental mice by immunization of the HPS fusion protein. After purification of antibodies with chromatograph of protein G, Western blotting was performed and both prokaryotic and eukaryotic expressing rhHPS proteins confirmed that the prepared antibodies were able to be recognized by all the four colonies antibody (data not shown). To confirm the ability of antibodies to neutralize HPS, we investigated whether they blocked the induced proliferation of L02 cells by exogenous HPS. Specifically, CM from cells transfected with HPS expression vector was preincubated with different HPS antibodies at 4°C for 3 h and then was added to cultured L02 cells, and cell proliferation was monitored by MTS assay. As a control, CM was preincubated with mouse IgG. As shown in Figure 1E, the antibody-C completely blocked the mitogenic effect of rhHPS, suggesting that antibody-C could be used as a neutralizing antibody. Furthermore, antibody-C also blocked HPS-induced phosphorylation of ERK1/2 in L02 cells (Fig. 1F).

IDENTIFICATION OF HPS RECEPTOR ON LIVER CELLS

To identify receptor of HPS, FITC-labeled HPS-binding assay was performed. Increasing concentrations of FITC-labeled HPS co-incubated with L02 cells in the presence or absence of excess amounts of unlabeled HPS at 37°C for 1 h, and fluorescence intensity was analyzed with fluorescence microplate reader. Figure 2A,B showed a typical saturation curve of HPS–FITC binding to cultured L02 cells. Further, L02 cells were incubated with HPS–FITC and varied concentration of unlabeled HPS for 1 h, FACS analysis showed about 60% inhibition in binding was observed with a 50-fold excess of unlabeled HPS (Fig. 2C). These suggested that presence of a receptor of HPS. Moreover, by fluorescence-binding assay, we also found that HepG2 hepatoma cells contained HPS receptor (data not shown). But for the non-hepatocytes derived from other tissue such as K562 cells no HPS receptor was detected (data not shown).

To determine whether the ERK1/2 was activated by HPS through its specific receptor, L02, HepG2, and K562 cells were starved in free serum for 24 h and then treated with 100 ng/ml rhHPS for the indicated time. Phosphorylation of ERK1/2 was analyzed by Western blotting. As shown in Figure 3A,B, exogenous HPS induced phosphorylation of ERK1/2 at 5 min in both L02 and HepG2 cells. However, phosphorylation of ERK1/2 was not induced by HPS in K562 cells (Fig. 3C), suggesting that HPS affected the ERK1/2 activation in liver cells through its specific receptor.

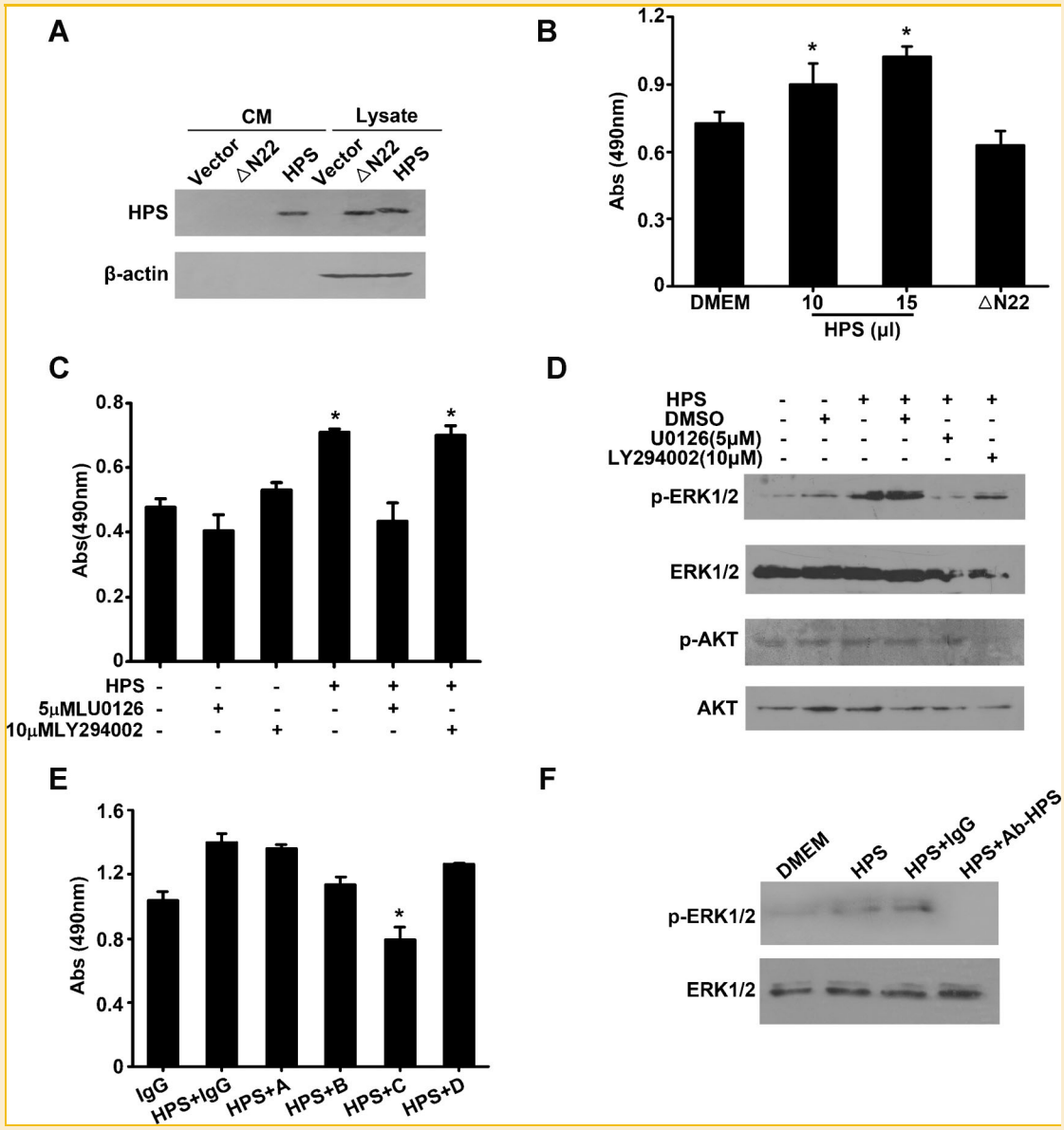

Fig. 1. HPS is secreted dependent on N-terminal 22 amino acids. A: HEK293 cells were transfected with pcDNA3.1, pcDNA-HPS, pcDNA-ΔN22, respectively. Forty-eight hours after transfection, cells were lysed and analyzed by Western blotting. B: 2×10^3 L02 cells were cultured in serum-free medium for 24 h, then cells were treated with CM from HEK293 cells transfected with pcDNA3.1, pcDNA-HPS, pcDNA-ΔN22, respectively. Cells were cultured for 2 days and the proliferation was analyzed with MTS. C: L02 cells were pretreated with U0126 (ERK1/2 inhibitor) or LY294002 (PI3K inhibitor) at indicated concentrations for 1 h, followed by addition of 15 μl CM from HEK293 cells transfected with pcDNA-HPS for 24 h in serum-free medium. The cell proliferation was measured by MTS. D: L02 cells were pretreated with U0126 (5 nM) or LY294002 (10 nM) for 1 h followed by addition of CM from HEK293 cells transfected with pcDNA3.1 or pcDNA-HPS for 24 h in serum-free medium. Ten minutes later, total cell lysates were prepared and phosphorylated ERK (p-ERK) and phosphorylated AKT (p-AKT) were analyzed by Western blotting. E: 2×10^3 L02 cells were cultured in serum-free medium for 24 h, then cells were treated with 15 μl CM from HEK293 cells transfected with pcDNA-HPS, which was preincubated with 300 μg/ml mouse IgG or 300 μg/ml HPS antibodies at 4°Cfor 3 h. After 2 days, the cell proliferation was analyzed with MTS. Error bars indicate SD for triplicate samples of experiments. The statistical difference between the samples was demonstrated as $^{*}P \leq 0.05$ or $^{**}P \leq 0.01$. F: L02 cells were starved in serum-free for 24 h, then cells were treated with 15 μl CM from HEK293 cells transfected with pcDNA-HPS, which was preincubated with 300 μg/ml mouse IgG or anti-HPS antibody-C at 4°C for 3 h, and was harvested at 10 min. p-ERK was analyzed by Western blotting.

CM FROM HEPATIC CELL LINE L02 PROMOTES L02 CELLS PROLIFERATION

To determine whether HPS is expressed in liver-derived cells and secreted into the CM, total cell lysates and the serum-free CM from different cell lines were subjected to Western blotting analysis. As shown in Figure 4A, HPS production was detected in total cell lysates and CM of both L02 and HepG2 cells. To quantitate HPS level secreted from cells, we developed a sandwich ELISA method using HPS antibody-C. Standard curve of sandwich ELISA generated by rhHPS yielded a consistent $r = 0.999$, which detectable protein

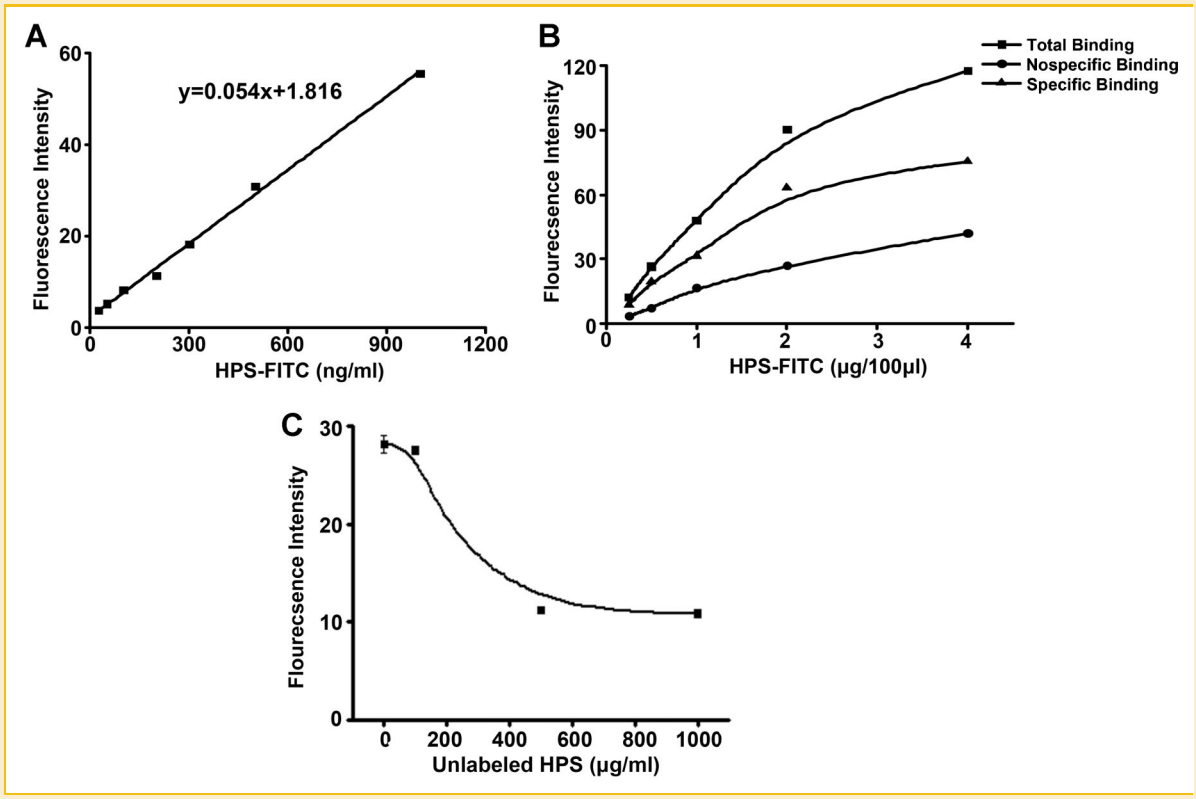

Fig. 2. Identification of HPS receptor in L02 cells. A: FITC-labeled HPS was diluted with PBS at the indicated concentrations, and 100 μl was added into 96-well flat measurement plate, and fluorescence intensity was calibrated using a fluorescence microplate reader. B: 3×10^5 L02 cells were incubated with increasing concentrations of HPS–FITC in the presence or absence of a 50-fold excess of unlabeled HPS at 37°C for 1 h. Specific binding was calculated by subtraction of the values for binding in the presence of excess unlabeled HPS from the values for total binding. C: 5×10^5 L02 cells were incubated with 20 μg/ml HPS–FITC with varied concentration of unlabeled HPS (0–1,000 μg/ml) at 37°C for 1 h. After cells were washed with and resuspended with PBS, fluorescence intensity was measured with flow cytometry.

amount ranged from 1 to 25 ng (Fig. 4B). As shown in Figure 4C, the concentrations of HPS protein in L02 and HepG2 CMs ranged from 5 to 12 ng/ml and the level of HPS in L02 CM was higher than that in HepG2 cells. These results suggested that HPS were expressed and released into extracellular medium in L02 and HepG2 cells. To rule out the possibility of its leakage from cells, cellular damage was assessed by trypan blue staining, which indicated that more than 99% of the cells were viable (data not shown).

We further determine whether the CM from L02 cells can induce the proliferation of L02 cells itself. As Figure 5A showed the CM of

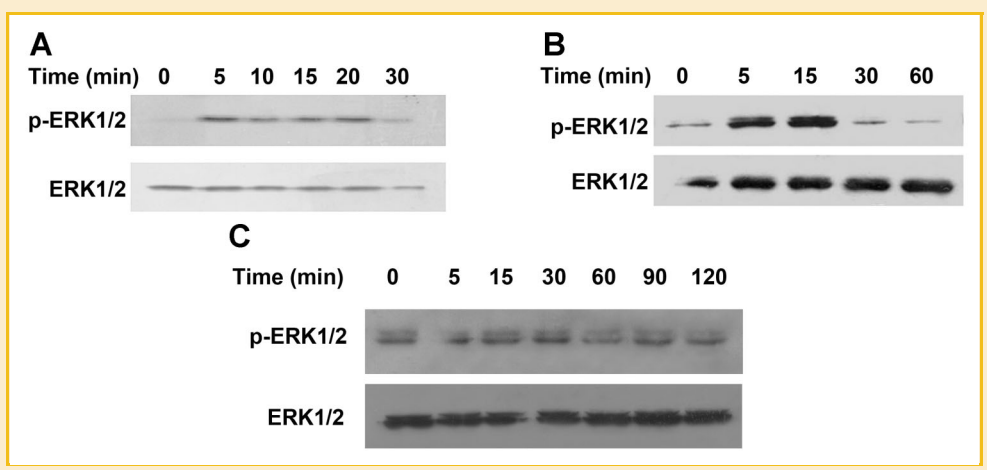

Fig. 3. Activity of ERK1/2 induced by HPS in different cells. Cells were cultured in serum-free medium for 24 h, then the cells were treated with 100 ng/ml rhHPS in serum-free medium, and harvested at the indicated times. p-ERK was analyzed by Western blotting (A: L02 cells; B: HepG2 cells; C: K562 cells).

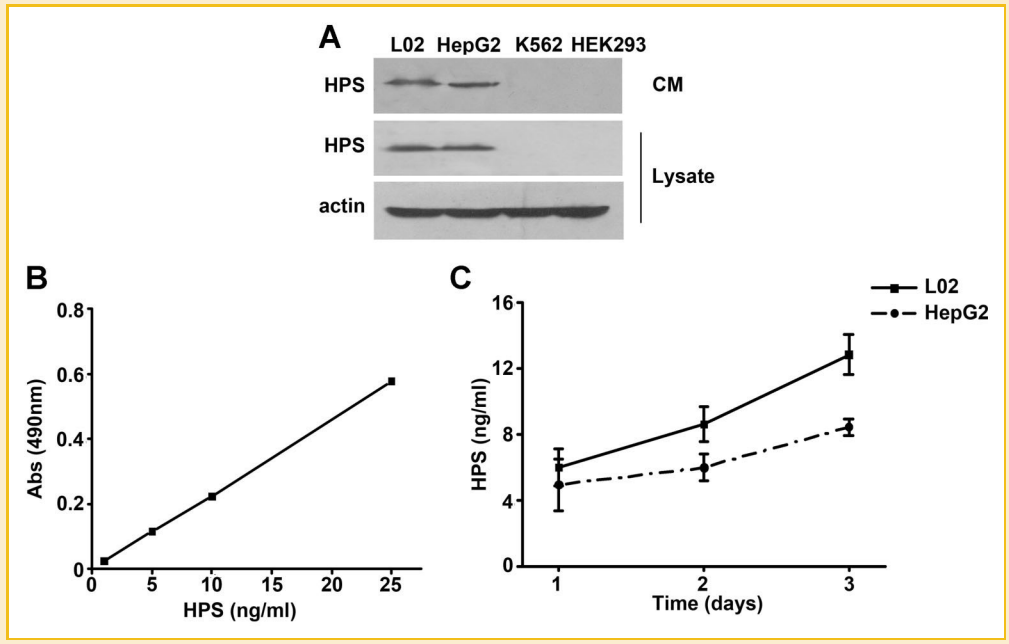

Fig. 4. HPS can be secreted by hepatic cell lines. A: L02, HepG2, K562, and HEK293 cells were cultured in free-serum medium for 48 h. Twenty-fold concentrated medium and lysates were analyzed by Western blotting. B: Standard curve of ELISA was generated with different concentrations of rhHPS. C: 2×10^6 L02 or HepG2 cells were cultured in free-serum medium for 24, 48, and 72 h, after concentrated to 20-fold, the medium were analyzed with sandwich ELISA.

L02 cells significantly stimulated proliferation of L02 cells, while the CM from HEK293 cells had no effect on L02 cells proliferation. To figure out the possible role of HPS in the stimulatory effect of L02 cells CM, CM was preincubated with anti-HPS antibody or mouse IgG at 4°C for 3 h and added to cultured L02 cells, then the cell proliferation was monitored. As shown in Figure 5B, the induced proliferation of L02 cells by CM was significantly attenuated when preincubated with neutralizing antibody against HPS ($P < 0.01$), whereas mouse IgG had no effect on the induction. This finding indicated that HPS played an important role in stimulatory effect of L02 CM.

INHIBITION OF L02 CELLS PROLIFERATION BY NEUTRALIZING ANTIBODIES AGAINST HUMAN HPS

To determine whether endogenous HPS protein regulated the cell proliferation, L02 cells were maintained in serum-free medium for 24 h in the presence or absence of anti-HPS antibody, and cell proliferation was measured at 4th day. As Figure 5C showed autonomous proliferation of L02 cells in serum-free medium was significantly inhibited by presence of neutralizing antibody against human HPS ($P < 0.01$). In addition, treatment of HPS antibody inhibited ERK1/2 phosphorylation of cultured L02 cells (Fig. 5D). These results indicated that endogenous secreted HPS promoted L02 cells proliferation.

HPS INHIBITS PROLIFERATION OF HCC CELLS THROUGH AN INTRACRINE PATHWAY

To determine whether HPS affected proliferation of hepatoma cells, four hepatoma cell lines were treated with rhHPS, and the growth of these cells were not affected (data not shown), which agreed with the previous studies [Hara and Yoshimura, 2001; Li et al., 2010], suggesting a different mechanism of HPS in the regulation HCC cell growth. To confirm this hypothesis, we transfected HPS expression vector (pcDNA-HPS) or HPS mutant (pcDNA-ΔN22) into HepG2 cells, and the growth of G418-resistant clones were measured. As shown in Figure 6A,B, full-length HPS expression resulted in a 31.5% reduction of colony formation, and HPS mutant ΔN22 reduced 70.0% colony formation, compared to transfection of control vector. To evaluate the effects of HPS expression on cell cycle progression, we next generated two green fluorescent protein-tagged HPSs (full-length HPS-GFP and 1–22 amino acids deleted HPS-GFP(ΔN22-GFP)). In both constructs, HPS was placed at the N-terminus of GFP and expression of HPS was confirmed by Western blotting (data not shown). The vectors HPS-GFP, ΔN22-GFP, or GFP control were transiently transfected into HepG2 cells, and after 48 h, about 40% of cells manifested GFPs (data not shown). Then, living cells were subjected to DNA staining with propidium iodide followed by FACS analysis. The cell cycle of GFP-positive as well as GFP-negative cells from the same plate by gating with the use of the medium intensity green fluorescence signal was analyzed. As shown in Figure 6C,D, expression of full-length HPS-GFP in HepG2 cells resulted in an increased proportion of cells in G0/G1 phase, whereas this effect was more intensive when ΔN22-GFP was expressed in HepG2 cells. These results suggested that intracellular HPS involved in the inhibition of HCC proliferation, which was different from extracellular HPS with an alternative mechanism.

Further, overexpression of wild-type HPS in HepG2 cells increased phosphorylated form of ERK, but p-ERK level in cells transfected with 1–22 amino acids deleted HPS construct was comparable to control cells transfected with pcDNA3.1 (Fig. 6E),

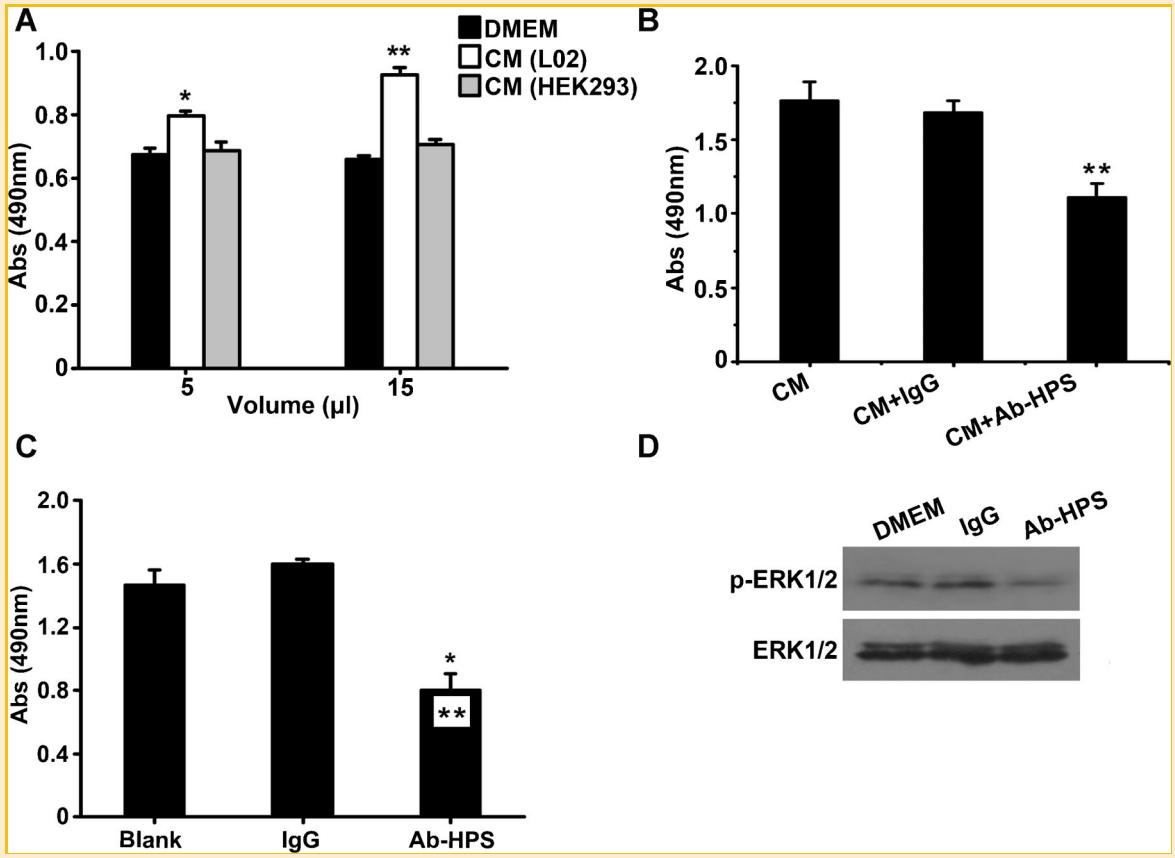

Fig. 5. Inhibition of L02 cells by neutralizing antibody against human HPS. A: 2×10^3 L02 cells were cultured in serum-free medium for 24 h, then the cells were treated with DMEM or CM from L02 or HEK293 cells as indicated volume for 2 days. The proliferation of cells were analyzed with MTS. B: 2×10^3 L02 cells were cultured in serum-free medium for 24 h, then the cells were treated with 15 μl CM from L02 cells, which was preincubated with 300 μg/ml mouse IgG or anti-HPS antibody-C at 4°C for 3 h. After 4 days, the proliferation of cells were analyzed with MTS. C: 2×10^3 L02 cells were cultured in serum-free medium for 24 h, then changed into serum-free medium with 300 μg/ml mouse IgG or anti-HPS antibody-C in 100 μl medium for 4 days. The cell proliferation was measured by MTS. Error bars indicate SD for triplicate samples of experiments. The statistical difference between the samples was demonstrated as $^{*}P \leq 0.05$ or $^{**}P \leq 0.01$. D: L02 cells were starved in serum-free for 24 h, then cells were treated with 300 μg/ml mouse IgG or 300 μg/ml anti-HPS antibody-C for 24 h. p-ERK level were analyzed by Western blotting.

which indicated that ERK1/2 phosphorylation was activated by extracellular HPS that secreted from the cells, not intracellular HPS, and HPS-induced inhibition proliferation was not associated with ERK pathway. To further elucidate the mechanism by which HPS blocked cell cycle progression, we examined the expression of cell cycle regulation protein when different dose of full-length HPS were transfected into HepG2 cells, and p53, p27 and cyclin D1 were analyzed. As shown in Figure 6F,G, p53 and p27 were increased and cyclin D1 was decreased in a dose-dependent manner. Furthermore, the change of these proteins was enhanced in cells transfected with full-length HPS and much stronger in cells transfected with truncated HPS. These results suggested that HPS could regulate p53, p27, and cyclin D1 leading to cell cycle arrest in G0/G1 phase.

DISCUSSION

In this study, we have presented evidence for the presence of an autocrine HPS loop in L02 human hepatic line on the basis of three criteria: (1) HPS was expressed and secreted into the extracellular medium in cultured L02 cells; (2) L02 cells expressed HPS-specific receptor; and (3) L02 CM induced proliferation of L02 cells and this function was inhibited by anti-HPS antibody. The autonomous proliferation of L02 cells in serum-free medium was significantly inhibited by presence of neutralizing antibody against HPS, suggesting that HPS secreted by L02 cells was probably attributable to its proliferative ability. Importantly, we have demonstrated that silencing of HPS by RNA interference result in L02 liver cells growth inhibition and ERK inhibitor (U0126) block hepatic cells proliferation induced by HPS [Li et al., 2010]. These results showed that HPS might play an important role in hepatocytes proliferation through an autocrine mechanism.

In this study, we reported that presence of receptor for HPS on L02 cells and human hepatoma cells. This is the first report about the existence of a cellular receptor for HPS. We also found that the distribution of HPS receptor was certainly specific. Non-hepatocytes derived from other tissue had no HPS receptor (data not shown). These suggested that HPS stimulated hepatocyte proliferation by binding to specific receptor on the cell surface. Although both of human hepatocyte cells L02 and human hepatoma cells HepG2 have

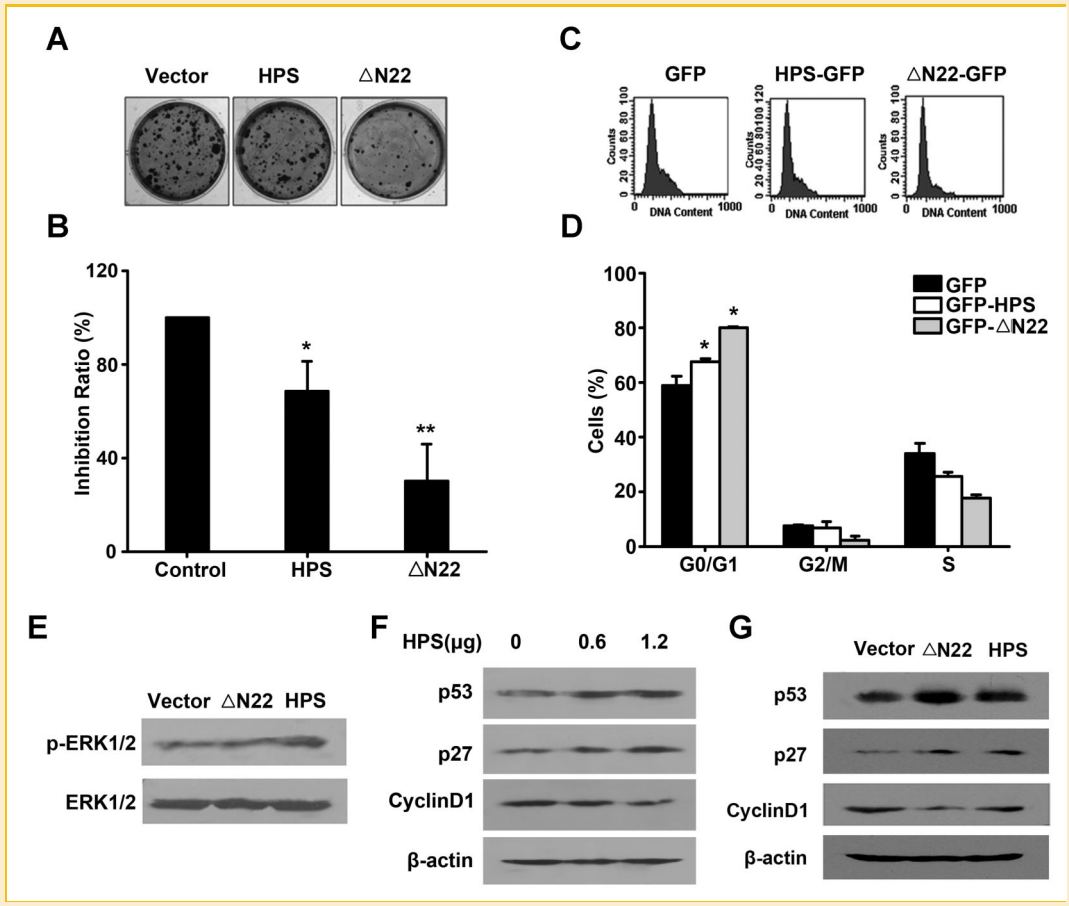

Fig. 6. HPS inhibits growth of HCC through an intracrine pathway. A: 2×10^5 HepG2 cells were transfected with 5.0 μg pcDNA-HPS, pcDNA-ΔN22 or control pcDNA3.1 vectors, respectively. G418-resistant colonies were stained 20 days later. B: Histograms of colony counts from colony formation assay. Error bars indicate SD for triplicate samples. C: HepG2 cells were transfected with GFP, HPS-GFP, ΔN22-GFP, respectively. At 48 h after transfected, cell cycles were analyzed by flow cytometry. D: Summary of cell cycle analysis. Error bars indicate SD for triplicate samples. The statistical difference between the samples was demonstrated as $^{*}P \leq 0.05$ or $^{**}P \leq 0.01$. E: HepG2 cells were transfected with pcDNA3.1, pcDNA-HPS, pcDNA-ΔN22, respectively. The p-ERK levels of cells were analyzed by Western blotting at 48 h after transfection. F: HepG2 cells were transfected with pcDNA3.1, pcDNA-HPS as indicated, respectively. The expression of p53, p27, and cyclin D1 were analyzed by Western blotting at 48 h after transfection. G: HepG2 cells were transfected with pcDNA3.1, pcDNA-HPS, and pcDNA-ΔN22, respectively. The expression of p53, p27, and cyclin D1 were analyzed by Western blot at 48 h after transfection.

the receptor for HPS, and treatment of exogenous HPS induced ERK1/2 phosphorylation, it is different in proliferation response to HPS, this may be due to different of number and affinity of the receptor for HPS and signaling pathways downstream of receptor in the human HCC cell line. Moreover, autocrine growth factors appear to be important in transformation and malignancy [Di Marco et al., 1989; Westermark and Heldin, 1991; Jeffers et al., 1996; Mange et al., 1999]. Our results also suggested that HPS autocrine loop may be not play a key role in regulation of HCC cells proliferation. However, it remained to be further investigated whether that transfection of a full-length HPS cDNA into hepatocytes can induce transformation. Although exogenous HPS has no effect for growth in HepG2 cells, HPS could active ERK pathway in HepG2 cells, and it hinted HPS might play other role in HepG2 cells.

Although our and other's results [Hara and Yoshimura, 2001] have showed that exogenous HPS had no effect on growth of HCC cell line cells, previous studies have reported that overexpression of human HPS in HCCs inhibits their growth in vitro and tumorigenicity in vivo, knockdown of endogenous HPS by antisense approach results in increase HCC cells proliferation, these results suggested that HPS might serve as a candidate tumor suppressor and regulate HCC growth in intracrine mechanism. Indeed, the secretion of HPS after transfection of a full-length HPS cDNA into cells was demonstrated, but 1–22 amino acids deleted HPS was almost exclusively located within cells, and no secretion of HPS was detected in CM. This result suggested that secretion of HPS is a signal peptide dependent. Furthermore, we found that the overproduction of truncated HPS, by means of the transient or stable transfection of 1–22 amino acids deleted HPS expression construct into HepG2 cells significantly inhibited the proliferation and induced cell cycle arrest, compared to transfection of full-length HPS cDNA vector. Taken together, our observation raised the possibility that HPS inhibited HCC cells growth in an intracrine mechanism. Intracrine activities of cytokines have been reported in several physiological and pathological situations, including erythropoietin (EPO) in erythroid progenitors [Pech et al., 1993], PDGF in v-sis transformed

cells [Keating and Willianms, 1988] and also IL-6 in myeloma cells and renal cell carcinoma cells [Levy et al., 1991; Alberti et al., 2004]. Intracrines tend to be synthesized in multiple isoforms, some destined for secretion, some destined to function in the cells that synthesized them. These isoforms are generated through the use of alternate transcription start sites, alternative message splicing, alternative translation start sites, and even alternative ribosomal entry sites [Re and Cook, 2006]. HPS expression is mainly regulated at the transcriptional level [Yu et al., 2009], whether present different promoters, alternative, splicing, and multiple polyadenylation signals generate different HPS transcripts in HCC still need further study.

It has been reported that cell cycle arrest causes cell growth inhibition [Braun-Dullaeus et al., 1998] and a possible mechanism by which HPS overexpression inhibited proliferation in HepG2 cells was by modulation of some key regulators of the cell cycle. The tumor suppressor protein p53 mainly activated CDKs inhibitor p21 transcriptionally [Xiong et al., 1993], p27 is another CDKs inhibitor which is a member of the Kip/Cip family [Denicourt and Dowdy, 2004]. p21 and p27 regulate the cell cycle progression by binding with active CDK4–cyclin D1 complex and thereby inhibiting their kinase activities resulting in cell cycle arrest in G0/G1 phase [Toyoshima and Hunter, 1994; Cayrol et al., 1998]. Our study showed that intracellular HPS increased p53 and p27, and decreased cyclin D1, which suggested that HPS played an important role in hepatoma as a regulator of cell proliferation through the control of key cell cycle modulators and arrest in G1/S phase transition. Cell immortalization and uncontrolled division lead to malignant tumor, and cell cycle control play an important role in tumor growth and development. HPS as a specific expression protein and growth inhibitor, might be a potential target for hepatoma.

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