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*Original Article*

# Pigment epithelium-derived factor (PEDF) peptide promotes the expansion of hepatic stem/progenitor cells via ERK and STAT3-dependent signaling

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**Abstract:** Hepatic stem/progenitor cells (HPC) have been considered as a potential cell source of an alternative to liver transplantation. Production of large numbers of autologous HPC from small pieces of live tissue is crucial for the application of HPC-based liver therapy. In this study, we demonstrated that a synthetic 44-amino acid peptide (44-mer) derived from pigment epithelium-derived factor (PEDF) can facilitate the production of a large number of actively dividing HPC from normal adult rat livers in a 35-day culture period. The phenotypic properties of HPC were characterized by morphological observation, colony formation and high expression of classical HPC markers including epithelial cell adhesion molecule (EpCAM), leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) and tumor-associated calcium signal transducer (TROP2). The 44-mer stimulated HPC proliferation in vitro and in mouse livers injured by a single intraperitoneal injection of carbon tetrachloride. In addition, the 44-mer induced the phosphorylation of ERK1/2 and STAT3 in HPC. Blocking the activity of ERK or STAT3 with pharmacological inhibitors attenuated the effects of the 44-mer on the induction of HPC proliferation. The long-term expanded HPC still possessed a bipotent ability to differentiate towards bile duct cells and mature hepatocytes. These results imply that the PEDF peptide may be a simple and effective agent to improve HPC-based liver therapy.

**Keywords:** PEDF, peptide, growth factor, liver, stem cell

## Introduction

The liver performs many vital functions, including homeostasis, synthesis and storage of glucose and proteins, detoxification and immune defense [1]. At the end stage of liver diseases such as fibrosis and cirrhosis, when the liver loses its naturally regenerative capacity; liver transplantation is a feasible and effective therapeutic strategy for treatment [1]. However, the option of liver transplantation is restricted by the lack of suitable donors. Cell-based therapies have attracted a lot of attention as alternatives to liver transplantation [2]. Mature primary hepatocytes cannot be used as a supportive cell source for liver transplantation because they cannot be expanded effectively in vitro [2]. For example, rat hepatocytes gradually died in the first two weeks after isolation [3, 4]. Hepatic stem/progenitor cells (HPC) persist in the adult liver and have the bipotent ability to differentiate into hepatocytes and cholangiocytes [5, 6]. HPC have been proposed recently as one of the major candidate cell sources for cell therapy in liver failure [7, 8]. HPC infusion in a rodent model has been shown to efficiently repopulate a damaged liver and to improve liver function [9]. A recent study, in which over 98% of hepatocytes were deleted in mice, demonstrated that the remaining HPC can make a massive contribution to restoration of the liver parenchyma, suggesting HPC as a potential remedy for liver failure [6]. Although hepatocytes can also be generated from embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, and other adult stem cells, HPC remain a potential source of hepatocytes because of their orthotopic nature [8]. However, the practical application of

HPC faces technical problems. Previous reports indicate that rat HPC isolated from normal adult livers have a limited proliferative capacity and expansion in culture is restricted to a period of 21~35 days after isolation [4, 10]. This has been partially overcome by the development of defined medium containing growth factors for the expansion and differentiation of HPC [11, 12].

Pigment epithelium-derived factor (PEDF) is a 50 kDa glycoprotein secreted by the liver. Previously, we reported that PEDF is an intrinsic anti-fibrotic factor [13]. Hepatic PEDF synthesis decreased dramatically in the liver following $CCl_4$ administration but over-expression of PEDF via a viral vector halted the progression of liver fibrosis in an experimental animal [13]. PEDF has multiple biological functions and these functions are executed by its various functional domains [14]. A human PEDF 44-mer (amino acid positions Val78-Thr121) has neurotrophic and neuroprotective activities [14, 15]. Our previous studies showed that the 44-mer stimulates the proliferation of limbal stem cells and muscle satellite cells in vitro or in damaged tissues [16, 17]. Recently, we performed direct intraperitoneal injection of the 44-mer peptide to efficiently ameliorate $CCl_4$-induced acute liver injury in mice [18]. Moreover, the 44-mer protects primary rat hepatocytes against apoptosis induced by serum deprivation and TGF-β1, supporting its hepatocellular protective function [18]. However, whether the 44-mer modulates the self-renewal of HPC isolated from adult rat liver remains unclear. In this study, we investigated the effect of the synthetic 44-mer on HPC proliferation in vitro and in acute liver injury. We provide evidence that the 44-mer peptide are effective mitogens for HPC.

## Materials and methods

### *Materials*

The PEDF peptides 44-mer and 34-mer (Asp44-Asn77) were synthesized, modified by acetylation at the $NH_2$ termini and amidation at the COOH termini for stability, and characterized by mass spectrometry (>90% purity) at GenScript (Piscataway, NJ). Peptide was preserved in solvent composed of 10 mM citrate buffer (pH 6.0) and 10 mM NaCl. MCDB 201 medium, Nicotinamide and SITE liquid medium were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-pan Cytokeratin antibody (panCK; ab215838), Anti-EpCAM antibody (ab71916), and anti-desmin (ab32362) were purchased from abcam (Cambridge, UK). Anti-BrdU antibody (GTX42641), anti-HNF-3β (GTX100309) and anti-albumin (GTX102419) were from GeneTex (San Antonio, Tex, USA). Phospho-Stat3 (Tyr-705), STAT3, phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) and ERK antibody purchased from Cell Signaling Technology (Danvers, MA). SB203580, PD98059, LY294002, SN-50 and STAT3 inhibitor (No. 573096) were purchased from Calbiochem (La Jolla, CA, USA).

### *Animal study and acute liver injury induced by $CCl_4$*

Experimental procedures were approved by the Mackay Memorial Hospital Review Board and conducted according to national animal welfare regulations. 6-wk-old female C57BL/6 mice (six mice per experimental condition) were injected intraperitoneally with $CCl_4$ solution at a dose of 5 ml/kg body weight (1:4 mixture with olive oil). Immediately, the 44-mer peptide and 34-mer control peptide at 10 mg/kg were administered by intraperitoneal injection, twice a day, with an interval of 8 hours. After $CCl_4$ administration for 48 h, mice were anesthetized by an intraperitoneal injection of a mixture of zoletil (6 mg/kg) and xylazine (3 mg/kg), the abdominal cavity was opened, and blood was removed by cardiac puncture from each mouse. Liver specimens were collected for histological analysis.

### *HPC isolation and culture conditions*

Liver cells were harvested from Sprague Dawley rats using a 2-step in situ collagenase perfusion technique as described previously [4]. The cells were then washed in phosphate buffered saline and centrifuged (50 *g* for 5 minutes) twice at room temperature. Cell viability was determined by trypan blue exclusion and was typically 85-90%. Isolated hepatocytes were resuspended in William's E medium (WEM; Life Technologies, Inc., Grand Island, NY) supplemented with 10% FBS, 0.01 U/ml insulin, 5.5 mM glucose, 50 μg/ml gentamicin, and plated at a concentration of $5 \times 10^4$ cells/$cm^2$ in collagen coated plates (Corning Costar Corporation, Cambridge, Mass.). Cell incubations were performed in a humidified incubator at 5% $CO_2$ and 37°C temperature. After plating for 24 h, non-adherent cells and debris were removed by washing with 10% FBS-WEM.

**Table 1.** Primers used in the quantitative real-time RT-PCR

| Primers | Accession number | Sequences (5'-3') |
|---|---|---|
| Lgr5 | NM_001106784.1 | F: CGTAGGCAACCCTTCTCTTATC |
| | | R: GTAATTTGCGAGGCACCATTC |
| EpCAM | NM_138541.1 | F: GTGGTGGTGTTAGCAGTCATT |
| | | R: GGCATTGAGCTCTCTGTGTATC |
| TROP2 | NM_001009540.2 | F: GAGAAGCGAACCTAGCTTGTAG |
| | | R: GTTTGTGGAGAGAGAAGGAAGAG |
| GAPDH | NM_017008.4 | F: AGACAGCCGCATCTTCTTGT |
| | | R: CTTGCCGTGGGTAGAGTCAT |

**Table 2.** Primers used in the RT-PCR

| Primers | Accession number | Sequences (5'-3') |
|---|---|---|
| EpCAM | NM_138541.1 | F: cgcagctcagaaagactgtg |
| | | R: tcgtcacactcgggatcata |
| Afp | NM_012493.2 | F: acctgacagggaagatggtg |
| | | R: gcagtggttgataccggagt |
| Albumin | BC085359 | F: cttcaaagcctgggcagtag |
| | | R: gcactggcttatcacagcaa |
| Cyp11a1 | NM_017286.3 | F: gctggaaggtgtagctcagg |
| | | R: cactggtgtggaacatctgg |
| HNF1b | NM_001308148.1 | F: gacactcctcccatcctcaa |
| | | R: acatcaaccacctccctctg |
| CK19 | NM_199498.2 | F: agtaacgtgcgtgctgacac |
| | | R: agtcgcactggtagcaaggt |
| GAPDH | NM_017008.4 | F: agacagccgcatcttcttgt |
| | | R: cttgccgtgggtagagtcat |

To expand HPC, freshly isolated hepatocytes were incubated with a basal medium (75% Dulbecco's modified Eagle's medium (DMEM) and 25% MCDB 201 medium supplemented with 1% FBS, 100 μM of β-mercaptoethanol, 25 mM HEPES, 5 mM of Nicotinamide, 1% SITE liquid medium, and 100 μg/ml penicillin/streptomycin) with or without PEDF-derived peptides (10 μM), changing the medium every 3 days.

*Quantitative real-time RT-PCR*

The total RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase I (Qiagen, Santa Clarita, CA) to remove genomic DNA and then purified with an RNA purification kit (Dynabeads; Invitrogen). Synthesis of cDNA was performed by Superscript III (Invitrogen). Quantitative real-time PCR was performed in a GeneAmp 7700 sequence detection system (Applied Biosystems, Foster City, CA). Amplification was carried out in a total volume of 40 ml containing 3 pmol of primers, serially diluted RT product and SYBR Green PCR core reagents (Applied Biosystems). Primers used in the experiment were listed in **Table 1**. The step-cycle program was set for denaturing at 95°C for 15 s, and annealing and extension at 62°C for 1 min, for a total of 40 cycles. All determinations were measured in triplicate. The cycle threshold (*Ct*) values corresponded to the PCR cycle number at which fluorescence emission in real time reaches a threshold above the base-line emission were analyzed using GeneAmp 7700 SDS software. The *Ct* value of the PCR product of interest and a control mRNA (GAPDH) were then used to calculate relative quantities of mRNA between samples.

*Semi-quantitative reverse transcriptase (RT)-PCR*

The total RNA was extracted from cells using the TRIzol (Invitrogen). Synthesis of cDNA was performed with 1 μg of total RNA at 50°C for 50 min using oligo (dT) primers and reverse transcriptase (Superscript III; Invitrogen). The amplification mixture contained 1 × Taq polymerase buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 μM primer pair, and 0.5 U of Taq DNA polymerase. Primer sequences are shown in **Table 2**. cDNA was synthesized in an 18-22 cycle amplification reaction (denaturation, 20 s, 94°C; annealing, 30 s, 57°C; and polymerization, 40 s, 72°C). The number of cycles for the primer set was chosen to be in the linear range of amplification. The PCR products were electrophoresed in a 2% agarose gel containing ethidium bromide and visualized by UV illumination.

*Immunohistological staining*

Formalin-fixed, paraffin-embedded liver specimens were deparaffinized in xylene and rehydrated in a graded series of ethanol concentrations. Slides were blocked with 10% goat serum for 60 min and then incubated with primary antibody against panCK and EpCAM (1:100 dilution) at room temperature (RT) for 2 h. The slides were subsequently incubated with the FITC-donkey anti-rabbit IgG and Alexa

Fluor® 647 Goat anti-mouse IgG (1:500 dilution; BioLegend, San Diego, CA) for 20 min and then counterstaining with Hoechst 33258 for 6 min, and viewed with Zeiss epifluorescence microscope.

*Immunocytochemistry*

Cells were fixed with 4% paraformaldehyde and then treated at 4°C with methanol for 2 min, and blocked with 1% goat serum and 5% BSA for 1 h. Cells were stained with antibodies to EpCAM, desmin, HNF-3β or albumin (1:100 dilution) at RT for 2 h. The slides were subsequently incubated with the FITC-donkey anti-rabbit IgG (1:500 dilution) for 20 min and then counterstaining with Hoechst 33258 for 6 min. To quench autofluorescence in FITC channel (visualizing at excitation of 473 nm), slides were incubated with the Sudan Black B (0.1% in 70% ethanol; Sigma-Aldrich) for 30 min [19]. Slides were rinsed with PBS for 1 minute. Finally, the sections were mounted with FluorSave™ reagent (Calbiochem), coverslipped and viewed with a Zeiss epifluorescence microscope.

*BrdU labeling*

HPC were incubated in culture medium containing 10 μM PEDF peptide and BrdU (final, 10 μM) for one day. After fixing with 4% paraformaldehyde, cells were exposed to cold methanol for 2 min, and then treated with 1 N HCl at RT for 1 h, Cells were blocked with 10% goat serum for 15 min and then incubated with anti-BrdU monoclonal antibody (1:100 dilution; GeneTex) for 2 h at RT. Subsequently, cells are incubated with the dunkey anti-mouse FITC immunoglobulin (1:500 dilution) for 20 min and counterstaining with Sudan Black B for 30 min.

*Immunoblot analysis*

Cell lysis, SDS-PAGE, and antibodies use for immunoblotting were as described in our previous study [20]. The band intensity in immunoblots was evaluated with a Model GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, CA) and analyzed using Labworks 4.0 software.

*Hepatocyte and cholangiocyte differentiation from HPC*

HPC were harvested from the cultures at day 35 by tapping the dishes gently to avoid contaminating the samples with adherent cells. Cells were plated on collagen-coated tissue culture plates at a density of $1 \times 10^5$ cells/cm$^2$ in expansion medium supplemented with 10% FBS, 20 ng/mL of hepatocyte growth factor (HGF; R&D Systems, Minneapolis, MN), and 100 nM of dexamethasone), as described previously [4]. The medium was changed every 3~5 days. Cultures were analyzed between days 30 and 40. To induce cholangiocyte differentiation, HPC were grown in Matrigel™ (In Vitro Technologies, Cat. 354234) for 12 days in WEM with supplements including 2 mM Glutamine, 5% fetal bovine serum, 20 ng/ml epidermal growth factor (In Vitro Technologies), 30 ng/ml insulin-like growth factor-II (PeproTech Inc, Rocky Hill, NJ, USA) as previously described [21].

*Albumin and urea assay*

Albumin and urea levels in the cell culture supernatant were determined by ALB ELISA kit and Urea Assay Kit (Abcam) according to the manufacturer's protocol. No urea or ammonia was detected in culture medium alone. Due to the nature of the culture conditions, data for HPC reflect the levels in 2 weeks old media, whereas data for primary hepatocytes were obtained from 24 hours old cultures. The ALB and urea levels of HPC, HPC-derived hepatocytes and rat primary hepatocytes were normalized to cell numbers.

*Statistical analysis*

Data are expressed as mean ± standard deviation (SD). The Mann-Whitney U test was used to determine statistically significant differences. $P$ values <0.05 were considered significant.

## Results

*The 44-mer enhances the expansion of rat HPC in culture*

To verify the function of PEDF-derived short peptides (34-mer and 44-mer), primary rat liver cells were exposed to basal medium supplemented with the 34-mer, 44-mer or peptide vehicle (solvent). When primary rat liver cells were plated, they quickly attached to the dishes and formed monolayer cultures within 2 days. When cultured for 11 days, approximately 90%

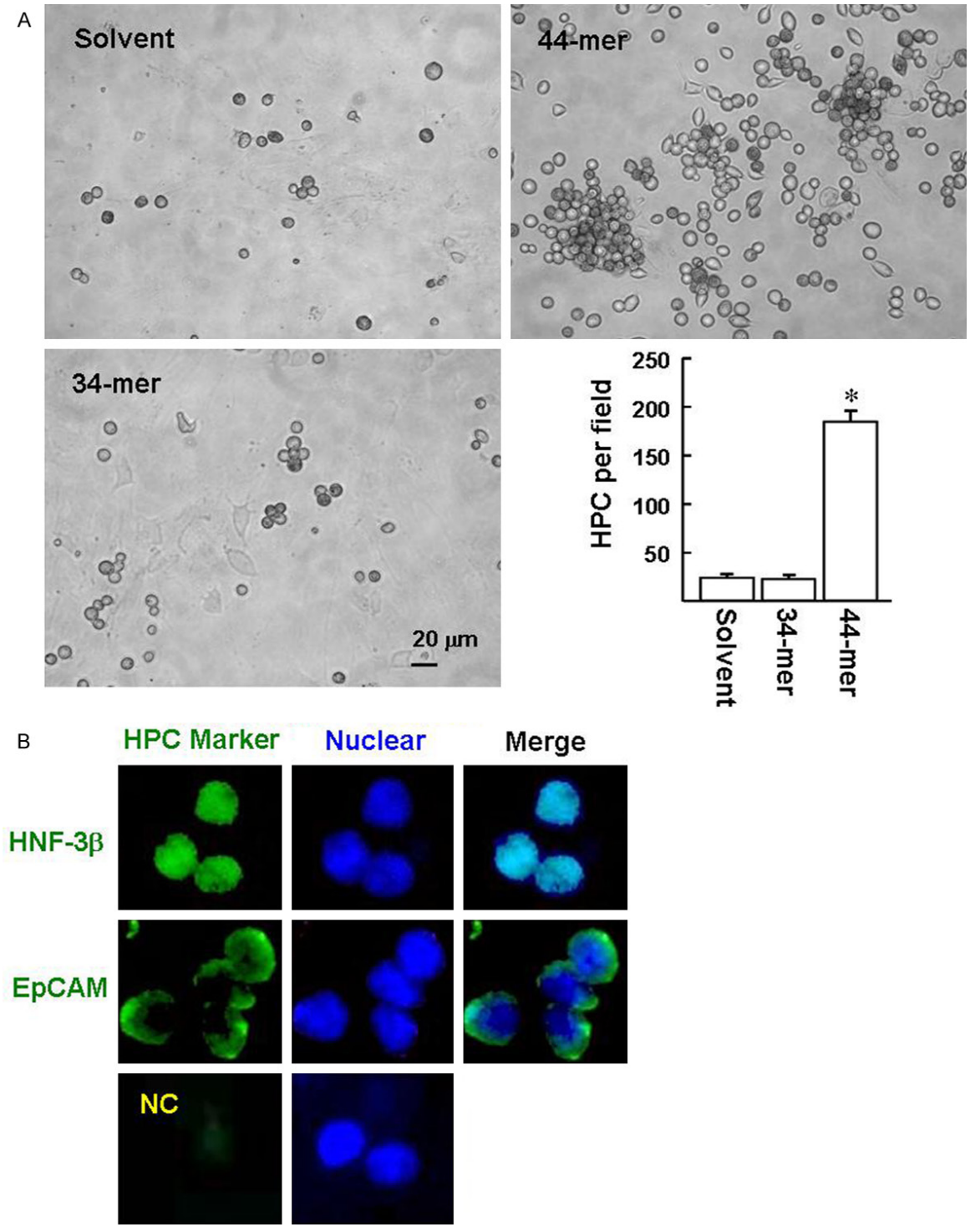


**Figure 1.** Proliferative capacities of HPC under PEDF-derived short peptides. Liver cells were seeded (about 1 × $10^5$ per well in 6-well plates) and cultivated in basal medium or basal medium supplemented with either 10 μM 34-mer or 10 μM 44-mer or peptide solvent for 35 days. Cells were fed with fresh medium every 3 days. A. The clonogenic capacity of the HPC was monitored by a Zeiss phase-contrast microscopy using 400 × high power field. Data represent three independent experiments. *$P$<0.0001 *versus* 34-mer-treated cells. B. At day 35, the expanded HPC was determined by immunocytochemistry with antibody against HNF-3β or EpCAM and then counterstaining with Sudan black B. Nuclei were located by counterstaining with Hoechst 33258. Immunostaining with FITC-conjugated secondary antibody alone as negative control (NC). Sections were observed under a Zeiss epifluorescence microscope with a CCD camera (× 400, 10 fields/sample).

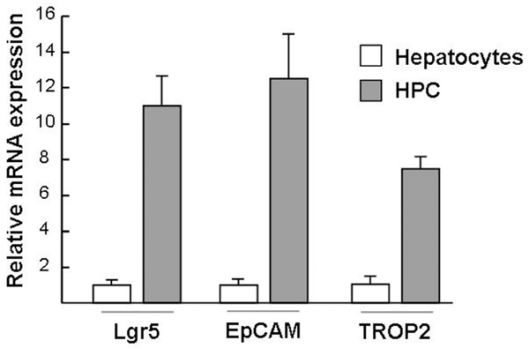


**Figure 2.** Quantitative real-time PCR analysis of *Lgr5*, *EpCAM* and *TROP2* expression. The relative expression was calculated using the ΔΔCt method and normalized to *gapdh* expression levels. Results are expressed as mean ± SE of 3 independent experiments.

of the primary large hepatic epithelial cells died gradually, while HPC with round to oval morphology emerged markedly among the liver cells as previously described [4]. However, the expansion of HPC had ceased almost completely at day 21, when the HPC were cultured in basal medium [4]. On the other hand, the 44-mer stimulated HPC expansion beyond 21 days. At day 35, liver cells stimulated with the 44-mer formed colonies of HPC (consisting of about 15-50 cells; **Figure 1A**). Quantitatively, 44-mer-treated liver cells had higher numbers of HPC (or oval-shaped cells) compared to the liver cells continuously cultivated in basal medium supplemented with peptide solvent and the 34-mer (HPC per microscope field: 183.8 ± 11.8 versus 23.3 ± 4.0 and 21.8 ± 4.2).

Also, as depicted in the **Figure 1B**, we used immunocytochemistry to characterize the phenotype of oval-shaped cells. They were positive for the epithelial cell adhesion molecule (EpCAM, a HPC marker) [22, 23] and hepatocyte nuclear factor-3 beta (HNF-3β; a key transcription factor to enhance the expression of EpCAM gene) [24]. In addition, they are negative for desmin (a stellate cell marker), and hepatocyte marker albumin expression (data not shown).

Next, we used real-time PCR analyses to compare the expression of HPC marker in freshly purified hepatocytes and expanded HPC after the 44-mer treatment for 35 days (**Figure 2**). The mRNA levels of the HPC marker Lgr5 [25], EpCAM and TROP2 [26] were enriched in the HPC by 11, 13 and 8-fold, compared to the expression in primary rat hepatocytes. The results suggest that the 44-mer not only promotes HPC proliferation but the cells also retain the features of stem cells in culture.

*The 44-mer induces HPC proliferation in livers injured by $CCl_4$*

It has been demonstrated that mouse HPC shows almost no expansion in response to acute toxic liver injury induced by administration of a single dose of $CCl_4$ [27]. To seek evidence of the effect of the 44-mer on HPC expansion in vivo, we monitored HPC expansion following the $CCl_4$-induced liver injury by double immunostaining for EpCAM and pancytokeratin (panCK) as previously reported [28, 29]. As shown in **Figure 3A**, numerous EpCAM/panCK-double positive HPC, concentrated around portal areas, were identified in the $CCl_4$/44-mer-treated mice, compared to the $CCl_4$ and $CCl_4$/34-mer treated mice (HPC per microscope field: 21 ± 3.3 versus 3 ± 0.9 and 3 ± 1.0; **Figure 3B**). The 44-mer has been demonstrated to alleviate $CCl_4$-induced acute liver injury by reducing hepatocyte death and oxidative stress in mouse liver [18]. This work further suggests that the 44-mer may stimulate HPC proliferation to contribute to liver mass recovery after an acute liver injury induced by $CCl_4$.

*The mitogenic effect of the 44-mer on HPC is modulated by activation of ERK and STAT3*

To determine the influence of the 44-mer on the proliferative capacity of HPC, primary rat liver cells were cultured in basal medium for 11 days to expand HPC and then exposed to the 44-mer for 24 h. Cell purity was verified to be approximately 98% by HNF-3β staining (**Figure 4**). HPC proliferation was detected by BrdU pulse-labeling (24 hours) and analyzed by immunostaining by anti-BrdU antibody (*green* color) and counterstained with Sudan Black B to mask HPC *green* autofluorescence. The results revealed that the 44-mer treatment (5, 10, and 20 μM) increased BrdU-positive HPC levels by ~1.9, 3.0, and 3.0-fold in a dose-dependent fashion, compared with solvent-treated cells.

Next, we used pharmacological inhibitors to explore the molecular basis of HPC proliferation induced by the 44-mer. BrdU pulse-label-

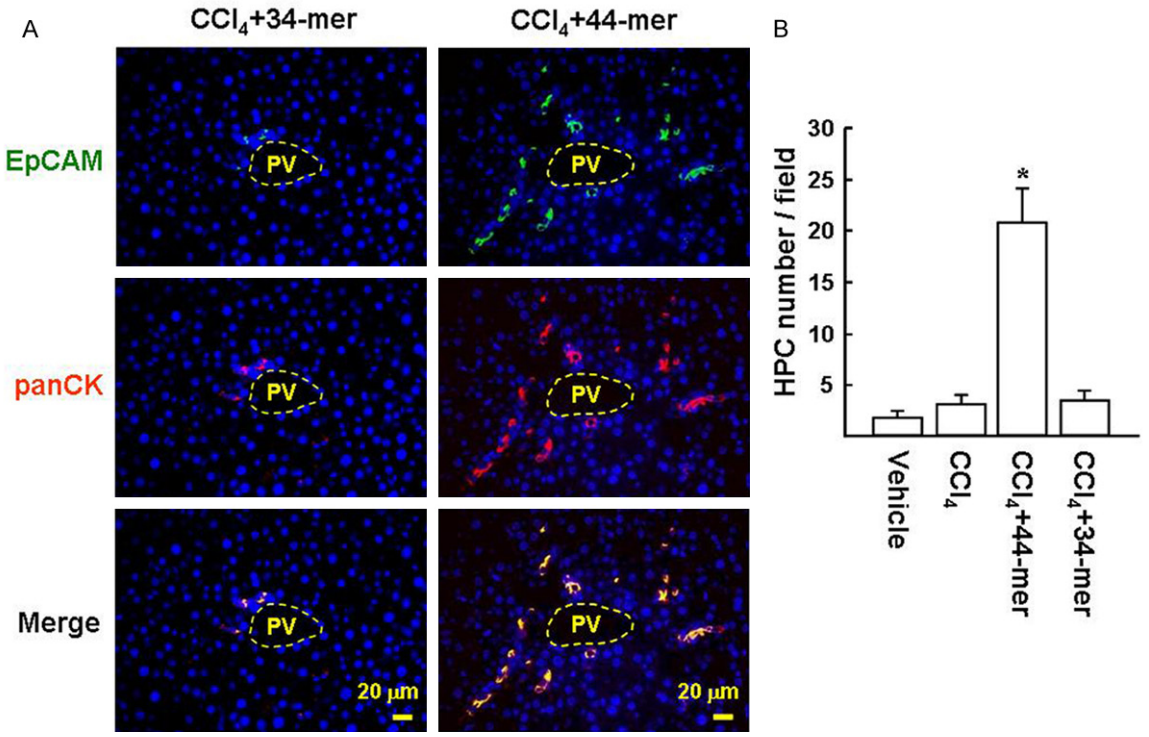


**Figure 3.** The 44-mer induces HPC expansion in liver of $CCl_4$-treated mice. A. Liver tissues were quantified 48 h after administration of a single dose of $CCl_4$. Representative images of EpCAM (*green*) and panCK (*red*)-stained liver tissues from 3 independent experiments are shown. The fluorescent dye Hoechst 33258 was used to mark nuclei (*blue*). PV: portal vein. B. The numbers of HPC were quantified by EpCAM and panCK-double staining and digital image analysis was performed blinded on an average of 12 randomly selected 400 × magnification fields from each section using a Zeiss epifluorescence microscope and Zeiss software. *P<0.02 versus $CCl_4$+34-mer treated group.

ing assays revealed that pretreatment with ERK or STAT3 inhibitor suppressed the 44-mer-induced cell proliferation from 31.4 ± 1.9% to 9.2 ± 1.7% and 8.4 ± 1.3%, respectively (**Figure 5A**). Inhibition of p38 MAPK, PI3K/Akt and NF-κB had no such effect. Cyclin D1 is a key regulator of cell proliferation [30]. HPC was treated with the 44-mer for 24 h, and then harvested for western blotting using the anti-cyclin D1 antibody. The results revealed that the 44-mer induced a 2.7-fold of the cyclin D1 protein, compared to solvent-treated cells (**Figure 5B**). Meanwhile, the 44-mer-mediated cyclin D1 induction was significantly blocked by pretreatment with ERK or STAT3 inhibitor. These data suggest that simultaneous activation of ERK and STAT3 is required for the 44-mer to stimulate HPC proliferation. We also examined whether the 44-mer can induce phosphorylation of ERK and STAT3 in HPC. HPC was expanded by basal medium for 11 days and then treated with 44-mer for intervals ranging from 5 to 30 min. The immunoblots revealed that the 44-mer caused a rapid and transient increase in phospho-ERK1/2 (p-ERK1/2) levels at 5~10 min and p-STAT3 levels at 5~30 min. The peak phosphorylation of STAT3 occurred 15 min after the 44-mer stimulation (**Figure 5C** and **5D**). However, the 34-mer did not lead to increase in p-ERK1/2 and p-STAT3. Collectively, the findings imply that the 44-mer induces HPC expansion in vitro via activation of ERK and STAT3 signaling pathways.

*After expansion by the 44-mer, HPC are able to differentiate into hepatocytes and ductal cells*

We examined whether HPC, after continuous treatment with the 44-mer for 35 days, retained their bipotent developmental potential, giving rise to mature hepatocytes and biliary epithelial cells. At day 35, very few hepatocytes remain viable on culture plate but HPC form clones by the 44-mer stimulation as shown in **Figure 1A**. These HPC clones were harvested gently by a dropper and re-plated onto a Type I collagen plate in medium supplemented with 10% FBS and HGF for further 30 days. Most of the dif-

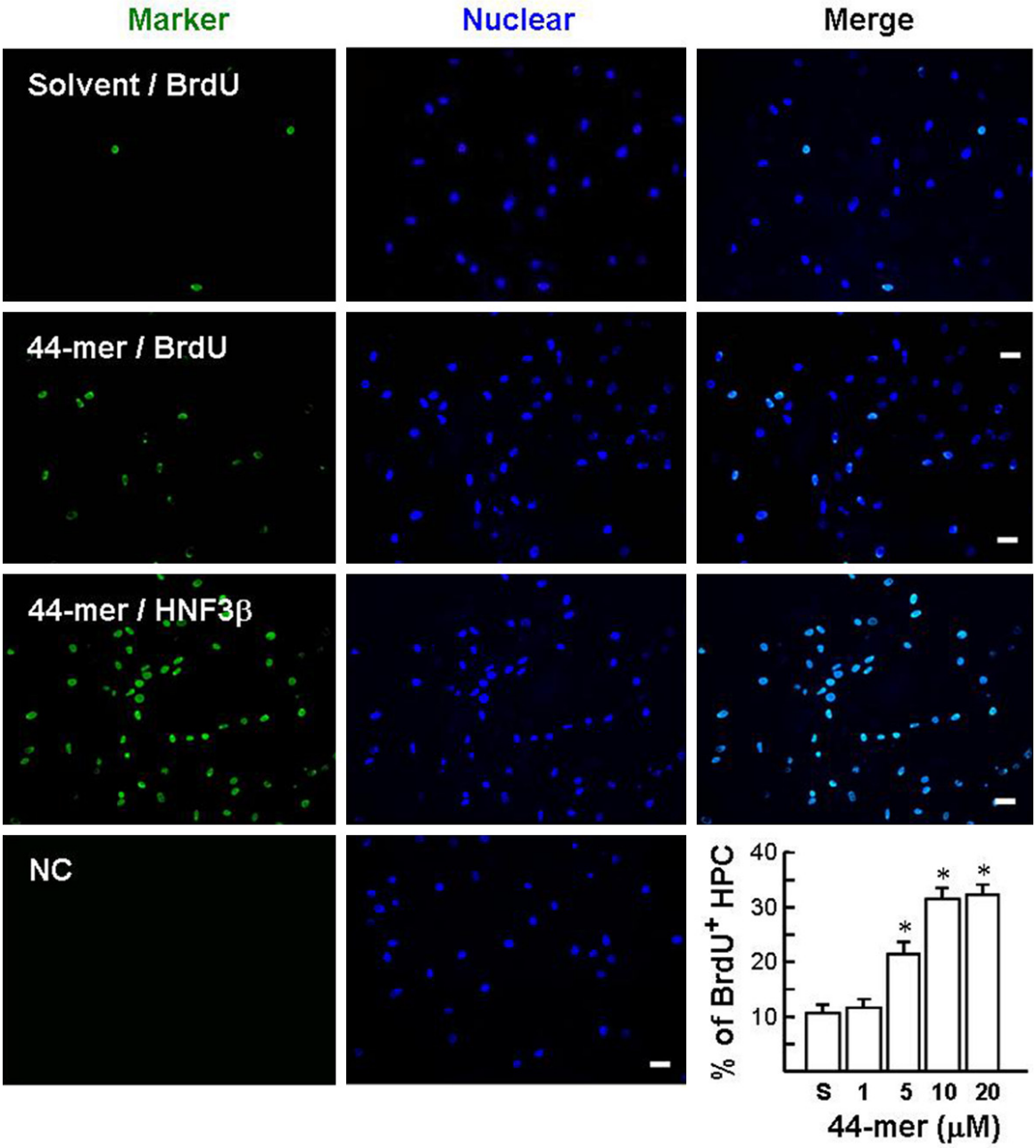


**Figure 4.** Dosage effect of the 44-mer on HPC proliferation. Primary rat liver cells were cultured in basal medium for 11 days to expand HPC and then incubated with medium supplemented with 44-mer combined with 10 μM BrdU for a further 24 h. Representative images of three independent experiments show immunofluorescence staining of cell replication by anti-BrdU antibody (*green* labeling). HPC was verified by HNF-3β staining. NC: immunofluorescence staining with FITC-conjugated secondary antibody alone. Ten randomly selected fields were photographed for each treatment and the percentage of BrdU-positive HPC per total HPC was calculated as proliferation index (%). Results are expressed as mean ± SE of 3 independent experiments. *P<0.05 versus solvent (S)-treated cells. Scale bar = 20 μm.

ferentiated cells were stained positive for hepatocyte marker albumin (**Figure 6A**). RT-PCR analysis revealed the expression levels of the hepatocyte marker genes, *albumin* and *Cyp-3a11* (cytochrome P450 3a11), were expressed at lower levels in the HPC cultured in growth medium as compared to HPC cultured in hepatogenic differentiation medium for 30 days. It

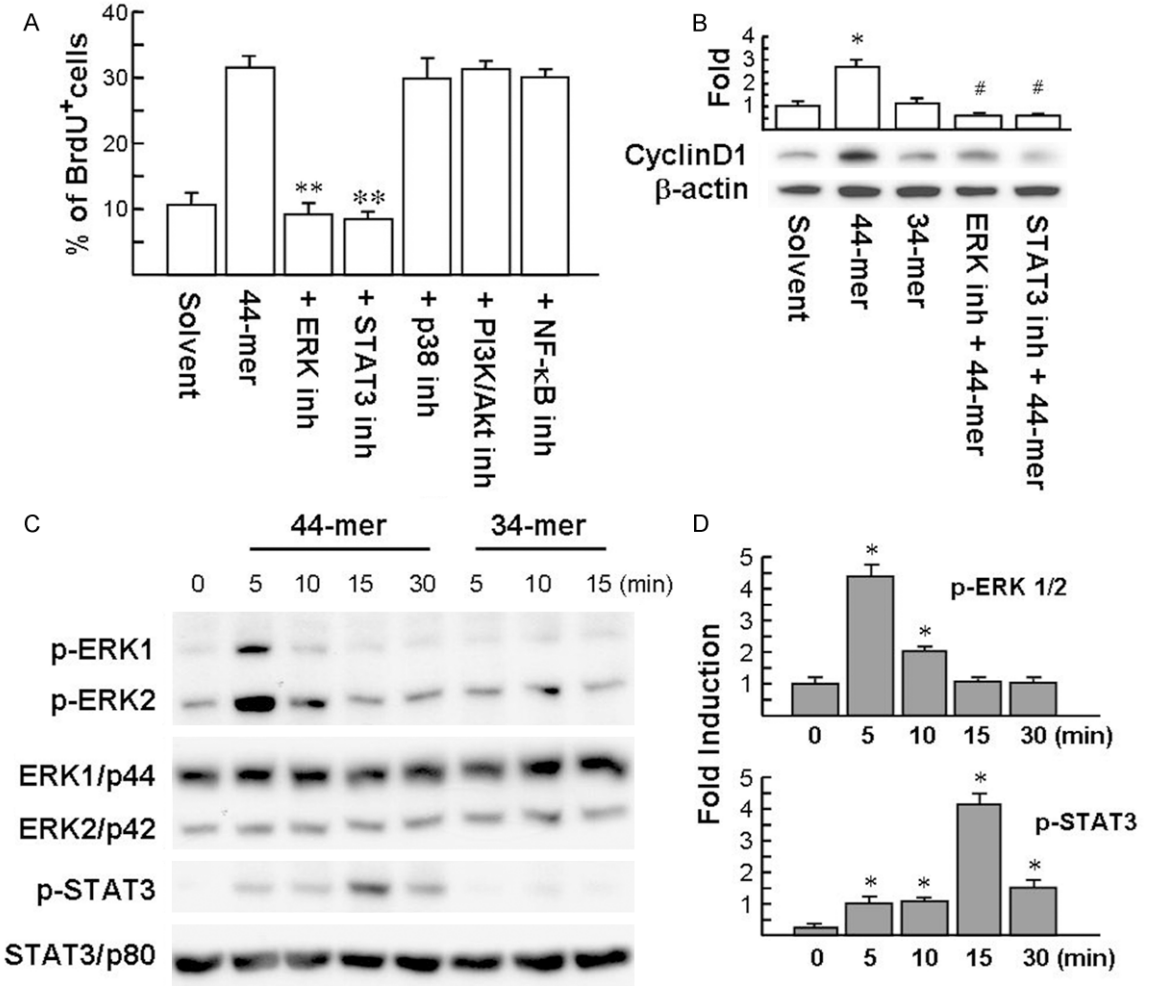


**Figure 5.** The 44-mer induces HPC proliferation through the ERK and STAT3 signaling. HPC was confirmed by HNF-3β staining as described in legend of **Figure 4**. A: Cells were pretreated with PD98059 (10 μM; ERK inhibitor), 50 μM STAT3 inhibitor, SB203580 (10 μM; p38 MAPK inhibitor), LY294002 (10 μM; PI3K/Akt inhibitor) or SN50 (10 μM; NF-κB inhibitor) for 2 h before treatment with 10 μM 44-mer and 10 μM BrdU for another 24 h. Results are expressed as mean ± SE of 3 independent experiments. **P<0.001 versus 44-mer-treated cells. B: Western blot analysis of the expression of cyclin D1 in HPC. Cell treatment was performed as above described. Representative blots and densitometric analysis with the mean ± SE from three independent experiments are shown. *$P$<0.05 versus solvent treated cells. C and D: The 44-mer induce phosphorylation of ERK1/2 and STAT3 in expanded HPC in a time-dependent fashion. Immunoblotting was performed to detect the active phosphorylated forms (upper panels) and the unphosphorylated forms (lower panels) by re-incubation with the antibodies indicated. Representative blots and densitometric analysis are shown from three independent experiments. *P<0.05 versus untreated cells (time 0).

also led to a commensurate decrease in abundance of *Afp* (alpha fetoprotein) and *EpCAM* transcript (**Figure 6B**). Albumin synthesis and urea production are classical assays for the study of liver-specific function [31]. These hepatocytes secreted high amounts of albumin and urea into the culture medium (**Figure 6C**), although HPC themselves did not produce urea or albumin in vitro. These results indicate that HPC expanded by the 44-mer can express hepatocyte-specific genes and biosynthesize urea and albumin.

To evaluate the potential for differentiation into cholangiocytes, the expanded HPC were grown in Matrigel. After 11 days of culture, we observed cells forming tubules by phase microscopy, which had a branched morphology and lumen as previously reported (**Figure 6D**) [21, 32]. In addition, RT-PCR revealed that the expression of cholangiocytic marker gene, *Hnf1b* and *CK19* (cytokeratin-19), were upregulated after HPC exposed to differentiation medium (**Figure 6E**), conforming the differentiation of cholangiocytes from expanded HPC.

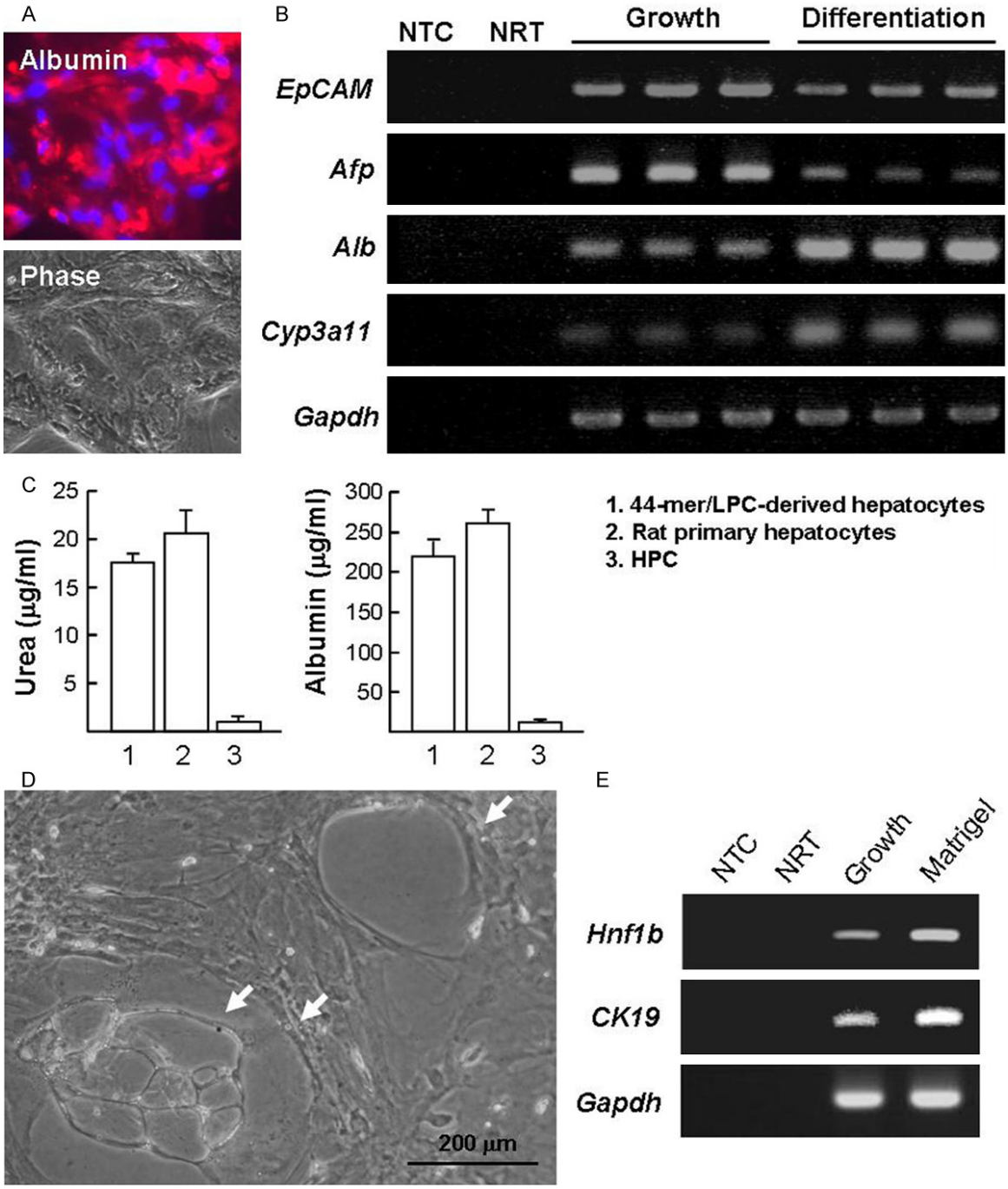


**Figure 6.** Characterization of HPC after culture in hepatogenic differentiation medium. HPC were treated continuously with the growth medium supplemented with 44-mer for 35 days. The expanded HPC were then seeded in hepatogenic differentiation medium for 30 days. A. Immunofluorescence analysis shows the expression of albumin (*red*) and phase contrast image in hepatocytes. Nuclei were visualized by Hoechst 33258 staining *(blue*). Data represent three independent experiments. B. RT-PCR analysis of the HPC markers *EpCAM, Afp* and the hepatocyte markers *Alb, Cyp3a11. Gapdh* was used as a loading control. Non-template (NTC) and no reverse transcriptase enzyme (NRT) were included as controls. C. Secretion of urea and albumin. Primary culture of rat hepatocytes was used for assay within 24 hours of isolation. The results are shown as the mean ± SE of three independent experiments. D. The expanded HPC were seeded in Matrigel for 11 days to induce differentiation along the cholangiocyte lineage. Phase contrast image shows the tubules and branching morphology as indicated by arrows. E. RT-PCR analysis of the expression of biliary markers *hnf1b* and *CK19* after cholangiocytic differentiation for 11 days. *gapdh* was used as a loading control.

Collectively, these findings imply that HPC expanded by the 44-mer can differentiate into mature hepatocytes and cholangiocytes.

## Discussion

The production of adequate numbers of HPC from small pieces of live tissue is critical for development of HPC-based therapies for liver cell transplantation [7, 8]. The rat has been used widely for the study of liver regeneration [9]. However, rat HPC isolated from adult live tissue rapidly lose their capacity for self-renewal in culture [4, 10]. Therefore, an effective rat HPC culture system would be useful for study of rat HPC in regenerative medicine. Here, we demonstrated that a PEDF-derived short peptide, 44-mer, can facilitate the self-renewal of rat HPC extensively in culture, shown by morphological observation of an increase of round or oval-shaped cells, colony formation and expression of multiple biomarkers, including autofluorescence and *Lgr5*, *EpCAM*, *HNF3β,* and *AFP* gene expression, as reported previously [4, 9, 22-25], while the expanded HPCs still retain the capacity to differentiate towards mature hepatocytes. In addition, the 44-mer peptide can be injected directly into the peritoneal cavity to efficiently stimulate HPC expansion in mouse livers injured by $CCl_4$. Our findings imply that the 44-mer peptide may be a valuable agent to improve HPC-based liver therapy.

Several signaling pathways have been implicated in the proliferative responses of HPC to various growth factors. Epidermal growth factor (EGF) has been shown to induce Sca-1-positive HPC proliferation by stimulating phosphorylation of ERK1/2 [11]. Interleukin (IL)-22 activates STAT3 to promote EpCAM-positive HPC proliferation in vitro [12]. The present study revealed that the mitogenic activity of the 44-mer on HPC involved activation of the ERK and STAT3 pathways. Our recent studies also support the hypothesis that ERK and STAT3 signaling is involved in the PEDF peptide-mediated mitogenic effect on HPC. We found that activation of the ERK and STAT3 pathways, essential for the proliferation of limbal stem cells and muscle satellite cells, is stimulated by PEDF and the 44-mer [16, 17]. Moreover, cyclin D1, a key regulator of cell cycle progression, was identified as a molecular target of PEDF/ERK/STAT3 signaling in muscle satellite cells [17]. Our recent studies also found that PEDF and the 44-mer induced Akt phosphorylation in C2C12 myoblasts [17]. However, HPC pretreated with PI3K/Akt inhibitor had no inhibitory effect on the mitogenic activity of the 44-mer. A plausible explanation of this phenomenon is that PEDF/44-mer fails to affect Akt phosphorylayion in HPC. At present, we do not understand how 44-mer induces phosphorylation of Akt, primarily owing to the fact that the linkage between PEDF receptor and Akt signaling pathway remains elusive. In addition, the Wnt/β-catenin pathway is important for HPC stem cell potential and necessary for *Lgr5* gene expression [25]. We found high levels of *Lgr5* gene expression in HPC after long-term 44-mer stimulation. This suggests that the Wnt/β-catenin pathway maintains a permissive level in long-term 44-mer stimulation. PEDF and the 44-mer can induce STAT3 activation in different types of stem cells including limbal stem cells and muscle satellite cells [16, 17]. It is noticed that STAT3 activation by IL-6 can significantly increase the levels of β-catenin in pancreatic cancer cells [34]. These suggest that the 44-mer regulates Wnt/β-catenin signaling depending on STAT3 activation.

The liver is a highly regenerative organ, mainly dependent on the duplication of hepatocytes and/or cholangiocytes, the main parenchymal cells of the liver, constituting ~80% of the mass [35]. HPC are defined as bipotent hepatic progenitor cells and constitute a small population (~1-2%) in the liver [36]. The role of HPC in liver homeostasis and repair remains largely unknown. However, a recent study indicated that massive hepatocyte death in mice could be restored to near normal function by expansion of resident HPC in the damaged liver [6]. In addition, in advanced liver damage, hepatic regeneration can occur through proliferation of the HPC population, such as defined by immunostaining of pancytokeratin (panCK)-positive HPC [27, 28]. Another key finding of this study is that the 44-mer can induce panCK-positive HPC proliferation in injured liver. Our recent study also indicates that the 44-mer ameliorates chronic liver injury induced by repeated $CCl_4$ injection in mice [18]. However, the importance of HPC expansion induced by the 44-mer on improvement of the structure of chronically damaged liver awaits further examination.

Our study implies that PEDF may facilitate production of adequate amounts of autologous HPC from small pieces of liver tissues and the possibility of their application in clinical practice. Our previous study has demonstrated that the 44-mer can induce proliferation of limbal stem cells derived from human, rabbit and mouse. This implies that the 44-mer effect on improving stem cell self-renewal in experimental animals can be extended to human. Our current studies try to evaluate if the 44-mer modulates the proliferation of rodent HPC. The results may have preclinical impact to further study human HPC, which is at present the main aim of our laboratory.

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## Disclosure of conflict of interest

None.

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