Osteopontin promotes rat hepatocyte proliferation both in vitro and in vivo

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\section*{ABSTRACT}
\textbf{Aim:} This study aimed to examine the effects of osteopontin (OPN) on hepatocyte growth and liver regeneration (LR).

\textbf{Methods:} A recombinant lentivirus expressing OPN and OPN-siRNAs were used to treat BRL-3A cells, while the adenovirus expressing OPN or OPN-targeted shRNA were applied for rat primary hepatocytes. Moreover, rrOPN and OPN-Ab were added to treat BRL-3A. Next, rrOPN was administrated into rat regenerating livers. Then \textit{in vitro} and \textit{in vivo} assays were performed to evaluate the biological function of OPN in hepatocyte growth and LR.

\textbf{Results:} OPN overexpression facilitated proliferation and viability of BRL-3A cells and primary hepatocytes, while OPN silencing reversed these effects. Similarly, rrOPN stimulated cell cycle progression and viability, but OPN-Ab led to cell cycle arrest and decreased viability. OPN overexpression induced the expression of p-STAT3, p-AKT and CCND1, and OPN siRNA led to reduction of p-AKT and CCND1. Furthermore, rrOPN promoted the expression of p-STAT3 and p-AKT, while OPN-Ab and PI3K/Akt inhibitor LY294002 both inhibited the expressions of p-AKT and Bcl2. Moreover, LR rate, serum IL-6 and TNF-\textalpha; Ki-67\textsuperscript{+} proportion and the phosphorylation of STAT3, AKT and p65 were augmented by rrOPN treatment.

\textbf{Conclusion:} OPN promotes hepatocyte proliferation both \textit{in vitro} and \textit{in vivo} through STAT3 and AKT signaling pathways.

\section*{Introduction}
Following massive damage induced by environmental toxins, infections, alcohol, etc., hepatocytes, the primary cell type of the liver, although present in the G0 phase of mitosis in normal conditions, they rapidly enter the G1 phase and undergo mitosis, and this is the process involved in liver regeneration (LR). Mature hepatocytes are always the first and the most important resource for liver repair and LR [1], and the proliferation capacity of endogenous or transplanted hepatocytes ultimately affects the pathological development of various liver diseases [2].

Osteopontin (OPN), which is also known as secreted phosphoprotein 1 (SPP1), exists in various hepatic cells such as biliary epithelial cells (BECs), Kupffer cells, activated hepatic stellate cells (HSCs), natural killer T (NKT) cells, injured hepatocytes and hepatocellular carcinoma (HCC) cells, and promotes inflammatory response, cell activation, cell proliferation or migration during the development of a variety of inflammatory liver diseases [3,4]. However, there is some controversy on whether OPN can directly affect hepatocyte proliferation. According to the study conducted by Wen et al., no proliferative advantage was observed in hepatocytes treated with recombinant mouse OPN (rmOPN) \textit{in vitro} [5], whereas Wang et al. reported biliary epithelial cell proliferation and HSC activation, but decreased hepatocyte proliferation without altering cell viability in mice treated with recombinant human OPN (rhOPN) [6]. Therefore, it is still necessary to confirm whether this discrepancy is due to different types of recombinant OPN (mouse and human) or not. Arai et al. first described the important role of OPN in LR due to induction and activation of hepatic stem cells [7,8]. Furthermore, Wen et al. demonstrated that OPN knockout (KO) mice exhibited delayed LR, which was attributed to attenuated activation of macrophages, decreased IL-6 levels, and then reduced activation of Stat3 in hepatocytes of OPN\textsuperscript{-/-} mice [5]. Our \textit{in vivo} study preliminarily showed that OPN silencing inhibited LR rate as well as the expressions of PCNA and CCL2, but augmented the expression of BAX by injecting OPN-shRNA expression plasmid through rat tail vein [9]. However, it still remains to be elusive whether OPN overexpression...
facilitates in vivo hepatocyte proliferation during liver repair and regeneration.

Our previous studies have revealed that OPN mRNA was up-regulated at multiple time points during 2–72 h in isolated hepatocytes, Kupffer cells, and dendritic cells from rat regenerated livers after partial hepatectomy (PH) [10], and most of the genes involved in OPN-mediated signaling pathways were significantly changed in rat regenerated livers, and were mainly associated with stress response, cell activation, proliferation, adhesion and migration [11]. In view of the important roles of OPN during LR and the results of our previous studies, we hypothesized that OPN can directly promote rat hepatocyte proliferation. On the other hand, it is generally acknowledged that OPN could perform its functions though PI3K/Akt, MAPK, NF-κB and JAK/STAT signaling pathways by binding to a number of integrin receptors or CD44 in various cancer cells [12–14]. Wen et al have confirmed the role of OPN-mediated IL-6/Stat3 signaling pathway during the early phase of mouse LR [5]. However, it should be further studied whether OPN could affect hepatocyte proliferation and LR though some other pathways.

Consequently, this study has chosen rat BRL-3A cells and primary hepatocytes to investigate the in vitro effects of OPN, and further applied recombinant rat OPN (rrOPN) for detecting the impact of OPN overexpression in vivo. This in turn helps to confirm whether OPN could promote hepatocyte proliferation both in vitro and in vivo, and lay a foundation for its potential use in promoting liver repair in various liver diseases.

Materials and methods

Primary cell isolation and cell culture

The hepatocytes were isolated from Sprague–Dawley (SD) rats by a two-step collagenase perfusion, filtration and centrifugation (50 × g for 5 min) as described by Shen et al. [15]. The freshly isolated hepatocytes were seeded in DMEM ( Dulbecco’s modification of Eagle’s medium, Hyclone, USA) containing 10% FBS (fetal bovine serum, Gibco, USA) and 5 ng/mL HGF (hepatocyte growth factor, Thermo, USA). The cells were then inoculated (5 × 10⁴ cells/well) in a 24-well plate for 4 h in a humidified atmosphere with 95% air and 5% CO₂ at 37 °C. The growth medium was replaced with fresh culture medium every 2 days. Rat liver cell line BRL-3A was obtained from the Cell Bank of Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences (GNR10) and cultured in low glucose DMEM containing 10% FBS and supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Hyclone, USA) in a humidified atmosphere in 5% CO₂ at 37 °C.

Plasmids construction and lentivirus and adenovirus package

Full-length cDNA encoding rat OPN was amplified from pEGFP-N1-OPN vector (previously constructed in our lab), and then was successfully inserted into pCDH-CMV-MCS-EF1-copGFP vector to obtain OPN expression plasmid (pCDH-OPN). Furthermore, pCDH-OPN or its control vector plasmid (pCDH-GFP) was co-transfected with two packaging plasmids PSPAX2 and PMD2G into HEK293T cells (purchased from the Cell Bank of Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences) to produce Lenti-OPN or Lenti-GFP lentivirus particles, respectively. Lentivirus supernatant was collected 60 h after transfection. Meanwhile, the recombinant OPN overexpression (Ad-OPN) and its control (Ad-RFP) adenovirus, and three Ad-shOPNs (shRNA1, GCUCUACGGACUGAGGAUA; shRNA2, GGATGAACACCGTGAAAC; shRNA3, GGATGA CTTTAAGCAAGAACTCTT) adenovirus and a control Ad-scramble adenovirus were prepared by Hanbio (Shanghai, China).

Cell transfection and treatment

For OPN overexpression in BRL-3A cells, 5 × 10⁴ cells/well were cultured in a 24-well plate, and infected with Lenti-OPN or control Lenti-GFP lentivirus supernatant at a multiplicity of infection of 100 with 10 mg/ml polybrene (Sigma). The transfection efficiency was measured by detecting GFP expression under fluorescence microscope (80i, Nikon, Japan). Subsequently, some GFP positive cells were selected according to the fluorescence intensity in cells, and then were expanded by limiting the dilution cloning to produce a monoclonal cell line for Lenti-OPN or control Lenti-GFP group. Moreover, BRL-3A cells were transfected with OPN-specific siRNAs (siRNA1, GCUUACGGACUGAGGUAU; siRNA2, GGAUGAACAAGCGGUGAAG) and a negative control (scramble RNA, UUCUCGAACGUGUCAGUTT) (RiboBio, Guangzhou, China) by using Lipofectamine 2000 (Invitrogen, CA) according to the procedures provided by RiboBio Corporation. On the other hand, rat primary hepatocytes were infected with Ad-OPN or its control Ad-RFP, and Ad-shOPN or its control Ad-scramble recombinant adenovirus (MOI = 100). After transfection for 6 h, the medium was replaced with fresh medium followed by cell culturing for an additional 24 h. Then the expression level of RFP in primary hepatocytes was detected to determine the transfection efficiency under fluorescence microscope. Recombinant rat OPN (rOPN, 6359-OP, diluted to 1 and 2 μg/mL in PBS) or mouse osteopontin neutralizing antibody (OPN-Ab, AF808, diluted to 5 μg/mL in PBS) (both are obtained from R&D Systems) were used to treat BRL-3A cells. Furthermore, to identify one possible signaling pathway mediated by OPN, BRL-3A cells were pre-treated with DMSO or 25 μM LY294002 (PI3K/Akt inhibitor LY294002, Beyotime, China) for 30 min, and subsequently treated with PBS or 5 μg/ml OPN-Ab for 48 h. The expressions of OPN and other related genes/proteins in target cells were detected by qRT-PCR and western blotting at indicated time points.

Partial hepatectomy (PH) rat model

Thirty healthy eight-week-old male SD rats (200 ± 20) g were selected and randomly divided into 6 groups (n = 5) in each group (3 control groups and 3 experimental groups). All groups were subjected to 2/3 PH as described by Higgins and
Anderson [16]. The rats in the experimental groups immediately received single injection of 4 μg rrOPN (2 μg/mL), (3 rrOPN groups) and the control groups were injected with 2 mL of PBS (3 PBS groups) via tail vein. After the rats in each group were sacrificed at 3, 24 and 72 h after PH, their body and liver tissues were accurately weighted, and then the liver tissues from the middle part of the right lobe were obtained and cut in half. One part was stored at −70°C in a deep freezer for qRT-PCR and western blot analysis, and the other part was flash frozen on a freezing microtome for further histopathological analysis and immunohistochemistry staining. Meanwhile, serum was collected for subsequent ELISA tests. LR rate is defined as (the liver weight per 100 g of the body weight at sacrifice/preoperative estimated liver weight per 100 g of the body weight) × 100. The preoperatively estimated total liver weight was calculated from the resected liver weight, and was estimated as (weight of 70% rec/70) ×100 after removing 70% liver tissue [17]. All operations and handling procedures were carried out in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, issued by Henan Normal University, and all animal studies were approved by the Ethics and Animal Welfare Committee at Henan Normal University.

**Cell viability**

The viability of BRL-3A liver cells and primary hepatocytes was measured by methyl thiazolyl tetrazolium (MTT) and cell counting kit-8 (CCK8) assays, respectively. Briefly, BRL-3A cells (3 × 10^4) were seeded into 96-well plates for MTT assay, and the Ad-OPN or Ad-RFP, and Ad-shOPN or Ad-scramble adenovirus-transfected hepatocytes were seeded into a 96-well plate with 3 × 10^3 cells/well in DMEM supplemented with 10% FBS and 5 mg/mL HGF for CCK8 assay. At 24, 48 and 72 h, 10 μL of MTT reagent (5 mg/mL, Geneview, USA) or 10 μL of CCK8 (Roche Diagnosis, Mannheim, Germany) was added to each well. Then the mixture was cultured for another 4 h or 3 h in the dark at 37°C. Subsequently, the medium was replaced with 100 μL of dimethylsulfoxide (DMSO) (Sigma, USA) for MTT assay. Finally, the absorbance was measured at 490 nm and 450 nm by Biotek reader (ELx800, USA) for MTT and CCK8 assays, respectively. Three independent experiments were performed in three duplicates.

**Cell proliferation assay**

After transfection with OPN-specific siRNAs and scramble RNA, the proliferation capacity of BRL-3A liver cells was detected by BrdU-incorporation assay. In brief, 5 × 10^4 BRL-3A cells/well were cultured on glass coverslips in a 24-well plate for 24 h, and then transfected with OPN-specific siRNAs and scramble RNA by using Lipofectamine 2000 according to the procedure provided by Ribobio corporation. After another 42 h, BrdU (Sigma, USA) was added into the medium at a final concentration of 10 μM at 37°C for 6 h. The cells were then dried and fixed, and the cellular DNA was denatured with FixDenat solution (Roche) for 30 min at room temperature. Next, the anti-BrdU antibody (Boster, China, 1:1000) was incubated for 40 min, followed by FITC-secondary antibody (Boster, China, 1:1000) incubation for 35 min at 37°C. Finally, cell nuclei were stained with propidium iodide (PI) (20 μg/ml, Sigma, USA). Thereafter, five random non-overlapping visual fields were selected, and the number of BrdU-positive cells and the total nuclei in the identical optical fields were counted using Image-Pro Plus 6.0 software (Media Cybernetics, USA).

### Immunofluorescence

The subcellular location of OPN protein was determined by immunofluorescence assay. Firstly, the BRL-3A cell line in blank control, and the selected GFP-positive BRL-3A cell line in Lenti-GFP and Lenti-OPN groups were seeded and cultured on glass coverslips in 24-well plates (5 × 10^4 per well) for 48 h. The cells were then fixed with 4% paraformaldehyde for 15 min to enhance membrane permeability with 0.3% Triton X-100. After blocking with bovine serum albumin (BSA) for 30 min, the cells were incubated with a mouse anti-OPN primary antibody (Santa Cruz, 1:100) for overnight at 4°C. After washing with PBS buffer, the cells were incubated with a Cy3-conjugated goat anti-mouse IgG secondary antibody (Santa Cruz, 1:100) for 1 h at room temperature. The cells were then stained with Hoescht 33258 to observe the nuclei. In addition, the proliferation of primary hepatocytes was detected by Ki-67 immunofluorescence assay. In brief, the rat primary hepatocytes were transfected with Ad-OPN or Ad-RFP, and Ad-shOPN or Ad-scramble for 48 h, and then were fixed with 10% paraformaldehyde for 15 min. Subsequently, the slices were incubated with rabbit anti-rat Ki-67 (Abcam, 1:100) for overnight at 4°C, followed by one-hour staining with goat anti-rabbit FITC (Boster, China, 1:1000) at 37°C. Thereafter, the cell nuclei were stained with PI to visualize the nuclei. Finally, the images were captured under a Nikon 80i fluorescence microscope.

### Flow cytometry (FCM) analysis

Cell cycle was evaluated by using flow cytometry (FCM) method. The selected GFP-positive BRL-3A cells in Lenti-GFP or Lenti-OPN group were cultured for 48 h, harvested, and then collected after treatment with OPN siRNA, rrOPN or OPN-Ab at indicated time points. In addition, rat primary hepatocytes were seeded in a 6-well plate with a density of 2 × 10^5 cells/well and treated with Ad-OPN or control Ad-RFP, and Ad-shOPN or control Ad-scramble adenovirus for 48 h. Then rat primary hepatocytes were harvested by centrifugation and washed with PBS. The cell samples were fixed with pre-cold 70% ethanol and then incubated in PBS with 0.2 mg/mL RNase A (Solarbio, China) at 37°C, followed by staining with PI in dark for 15 min for evaluating the cell cycle. Finally, the scattered signals of cells were detected by FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). All assays were independently repeated thrice.
**Biochemical analysis**

Fasting blood from the orbital venous plexus was collected and analyzed by biochemical analyzer ADVIA 2400 (SIEMENS, Germany), including alanine aminotransferase (ALT) and aspartate aminotransferase (AST). IL-6 and TNF-α levels were measured with specific enzyme-linked immunosorbent assay (ELISA) kit (NeoBioscience, China) according to the manufacturer’s instructions.

**Histopathological detection**

Small cuboids from the right lobe of the liver were flash frozen and sectioned on a freezing microtome. Some slices were stained with hematoxylin and eosin to assess the pathological changes induced by rOPN administration, while some other slices were stained with Sudan IV, which were used to stain lipid droplets in lesion areas [18]. As for Ki-67 immunohistochemistry staining, the slices were incubated with rabbit anti-rat Ki-67 (Abcam, 1:100) for overnight at 4 °C, followed by one-hour staining with goat anti-rabbit FITC (Boster, China, 1:1000) at 37 °C. Thereafter, the cell nuclei were stained with PI (Sigma, USA). Finally, histopathologic examinations of liver sections were conducted by a pathologist and peer-reviewed, and the number of Ki-67-positive cells and total nuclei in five random non-overlapping visual fields was counted using Image-Pro Plus 6.0 software.

**Quantitative RT-PCR**

Total RNA was extracted from frozen liver tissues by using Trizol reagent (Invitrogen, USA) according to the manufacturer’s instructions and treated by DNase I, and then 2 μg of total RNA was reverse-transcribed using random primers and Reverse Transcription Kit (Promega, USA). The obtained first-strand cDNA samples were then subjected to quantitative PCR amplification by using SYBR® Green I on the Rotor-Gene 3000 A (Corbett Robotics, Australia). All PCR cycling conditions were modified to 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. Relative changes in mRNA expression were calculated using the 2^(-ΔΔCT) method and normalized by β-actin. Detection for each group was independently repeated thrice. The primers were designed according to mRNA sequences and synthesized by Shanghai Generay Biotech Co., Ltd (Table 1).

**Western blotting (WB)**

Whole cell lysates from cells and liver tissues were prepared in lysis buffer (Solarbio, China) with protease/phosphatase inhibitor cocktail (100×, Cell Signaling Technology, USA), and then the extracted protein concentration was calculated by BCA protein assay kit (Solarbio, China). After the equal amounts of total proteins (20 μg) were separated by SDS-PAGE and then transferred onto a nitrocellulose membrane, the membrane was incubated with some primary antibodies against β-actin, CCND1, BCL2, BAX, p-STAT3 (Tyr705), p-AKT (Ser473) and p-NF-κB p65 (Ser536) (CST, USA) at a dilution of 1:2500, followed by horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies (Sigma, USA) at a dilution of 1:10000 and then developed using enhanced chemiluminescence substrate (Western Lightning® Plus-ECL), and the relative abundance of target proteins was measured by using ImageQuant TL software. β-actin or GAPDH (CST, 1:2000) served as an internal reference in this study [19].

**Statistical analysis**

Statistical significance between groups was assessed by unpaired two-tailed Student’s t test. Multiple comparisons between more than two groups were conducted by one-way ANOVA. Data is presented as means ± standard error of the mean (SEM). * indicates p < .05 and ** indicates p < .01. The level of statistical significance was set at p < .05.

**Results**

**The expression changes of endogenous OPN affected liver cell proliferation**

To study the effect of OPN on liver cell proliferation, rat BRL-3A cells were transfected with Lenti-OPN or Lenti-GFP, and the monoclonal BRL-3A cells were obtained by limiting dilution cloning in both Lenti-OPN and control Lenti-GFP groups. As shown in Figure 1D, the overexpression of endogenous OPN affected the cell proportions in monoclonal BRL-3A cell lines from different groups were seeded at 48 h, immunofluorescence assay demonstrated localization of OPN mainly in the cytoplasm of BRL-3A cells, and the expression of OPN (as indicated by red fluorescence intensity) was significantly higher in Lenti-OPN group than that in blank control and Lenti-GFP groups (Figure 1A). Also OPN was significantly overexpressed at both mRNA and protein levels in Lenti-OPN group compared with that in Lenti-GFP group (Figure 1B and C). These data indicated that OPN was successfully overexpressed in Lenti-OPN group. As shown in Figure 1D, the overexpression of OPN obviously promoted the cell viability of BRL-3A cells.

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**Table 1. Genes and their primer sequences used in qRT-PCR.**

| Genes   | Primer sequences | Products (bp) |
|---------|------------------|---------------|
| Opn     | FP: 5′-CATCGATGGTCTCTCATGAC-3′ | 206           |
| Ccn1    | FP: 5′-GATGTTGTCGCTGCTGAC-3′   | 199           |
| Bcl2    | FP: 5′-GAGACCTGTTGATCTTCTG-3′   | 136           |
| Bax     | FP: 5′-TTTCACTCGAGATGACTCAG-3′   | 263           |
| Il-6    | FP: 5′-AGTCATTATCTCTGTTGCCT-3′   | 277           |
| Tnf-α   | FP: 5′-TCTACTCCCAGCTCTCTGATTG-3′ | 101           |
| Actb    | FP: 5′-GTGCAGGGCAGGCGGCTATTCT-3′ | 109           |

FP: forward primer; RP: reverse primer.
at both 48 and 72 h ($p < .01$). Thus, 48 h was selected as the time-point for the following experiments. After that, the percentage of G0/G1 phase cells was significantly decreased, while the proportion of S and G2/M phase cells were increased in Lenti-OPN group at 48 h (Figure 1E and Supplemental Figure S2A and B).

Similarly, for detecting the effects of OPN overexpression in primary hepatocytes, the adenoviruses Ad-RFP and Ad-OPN were used to infect the rat primary hepatocytes, and the transfection efficiency was assessed by detecting the expression of RFP in hepatocytes under fluorescence microscope after 24 h of adenoviral transfection. As shown in Figure 1(F), both Ad-RFP and Ad-OPN groups showed strong fluorescence signals, and the proportion of RFP-positive cells in the two groups reached 90% according to visual inspection under fluorescent microscope, indicating efficient transfection of Ad-RFP and Ad-OPN in rat hepatocytes. Further WB detection showed that the OPN protein levels were obviously up-regulated at 48 h after transfection in Ad-OPN group of hepatocytes when compared to those in Ad-RFP group (Figure 1G). Next, we found that Ad-OPN led to marked increase of hepatocyte viability at 48 and 72 h after transfection by CCK8 assay (Figure 1H). At 48 h after transfection, Ki67 immunofluorescence staining showed that cell proliferation proportion was increased in Ad-OPN group (20.60 ± 0.73%) than that in Ad-RFP group (15.20 ± 0.25%, $p < .05$, Figure 1I), and FCM analysis demonstrated that cell population in G2/M phase showed significant increase in Ad-OPN group (6.40 ± 0.79%) when compared with Ad-RFP group (3.90 ± 0.01%, $p < .05$, Figure 1J and Supplemental Figure S2C, D). These results suggested that overexpression of OPN facilitated proliferation of primary hepatocytes. Therefore, OPN could promote the proliferation of both liver cell line and primary hepatocytes in vitro.

**OPN down-regulation inhibited cell proliferation**

To further confirm the effects of OPN on cell proliferation, the expression of OPN in BRL-3A cells was silenced. The results of qRT-PCR and agarose gel electrophoresis showed that siRNA1 and 2 knocked down the expression of OPN, and the suppression efficiency of siRNA2 was obviously higher than siRNA1 at 24 h after transfection (Figure 2A). Accordingly, BRL-3A cells were further transfected with siRNA2. As expected, the cell viability was significantly attenuated than that of the scramble group at 24 and 48 h (Figure 2B). After that, we chose transfection after 48 h as detection time point for further experiments. Accordingly, FCM detection results showed that the proportion of G0/G1 phase cells in OPN-siRNA group was significantly increased (Figure 2C, Supplemental Figure S2E, F), and BrdU incorporation assay...
further confirmed the suppression of OPN expression caused by OPN-siRNA, inhibiting the proliferation of BRL-3A cells (Figure 2D, Table 2).

Similarly, rat primary hepatocytes were infected with Ad-shOPNs or Ad-scramble adenovirus. qRT-PCR analysis and WB detection showed that Ad-shOPN3 obviously knocked down the OPN expression at 24 and 48 h after transfection, respectively (Figure 2E and F). Therefore, Ad-shOPN3 was selected for transfecting primary hepatocytes in the following experiments. As expected, the proportion of RFP-positive cells in the two groups reached more than 90% at 24 h after transfection, indicating that Ad-shOPN and Ad-scramble were both efficiently transfected in rat hepatocytes (Figure 2G). CCK8 assay showed that the viability of hepatocytes was not significantly attenuated until 72 h in Ad-shOPN group (Figure 2H), whereas Ki67 immunofluorescence staining showed that the ability of hepatocyte proliferation was decreased at 48 h in Ad-shOPN group (Figure 2I). Furthermore, FCM analysis demonstrated that cell population in G2/M phase were significantly increased at 48 h after transfection in Ad-shOPN group (1.45 ± 0.34%) when compared with Ad-scramble group (4.01 ± 0.66%, p < .01, Figure 2J and Supplemental Figure S2G, H). These results suggested that decreased OPN led to inhibition of proliferation of primary hepatocytes via arresting cell-cycle progression. Therefore, down-regulation of OPN inhibited the proliferation of both liver cell line and primary hepatocytes in vitro.

Table 2. The impact of OPN-siRNA treatment on proliferation of BRL-3A rat liver cells.

|                      | No. of total cells | No. of BrdU⁺ cells | Cell proliferation proportion (%) |
|----------------------|--------------------|--------------------|----------------------------------|
| scramble             | 180.7 ± 3.2        | 72.3 ± 8.0         | 40.00 ± 3.90                     |
| OPN-siRNA            | 187.3 ± 13.9       | 54.3 ± 7.2*        | 28.93 ± 2.08*                    |

*p < .05 vs scramble group.

Endogenous OPN stimulated the activation of its downstream signaling pathways

As shown in Figure 1D and E, OPN overexpression promoted the viability and cell cycle progression of BRL-3A cells at 48 h after seeding the positive monoclonal Lenti-OPN and
Lenti-GFP BRL-3A cell line. Subsequently, WB detection and corresponding densitometry analysis revealed that the expressions of the downstream p-STAT3, p-AKT and CCND1 in Lenti-OPN group were significantly induced when compared with those in Lenti-GFP group at 48 h (Figure 3A). However, surprisingly, OPN siRNA led to marked reduction of p-AKT and CCND1 but not p-STAT3 at 48 h after transfection (Figure 3B). Furthermore, we also detected the expression of apoptotic proteins BCL2 and BAX, but did not observe their significant expression changes (data not shown). Consistently, the mRNA levels of Ccnd1 were significantly increased in Lenti-OPN group and attenuated by OPN siRNA (Figure 3C), accounting for obvious changes in the protein levels. Therefore, we inferred that AKT and STAT3 signaling were involved in OPN-mediated cell proliferation promotion.

**rrOPN and OPN-Ab treatment markedly affected cell cycle progression**

OPN acts as a secreted protein, activates multiple signaling pathways upon binding to several receptors, and ultimately participates in regulating cell physiological functions. To further study the direct effects of exogenous OPN on cell growth, BRL-3A cells were treated with different concentrations of rrOPN (0, 1, and 2 μg/mL) according to some previous studies [6,20], and the MTT data showed that 1 μg/mL rrOPN had no obvious effect on cell viability. However, 2 μg/mL rrOPN showed no significant effect on cell viability at 48 h, but significantly promoted cell viability at 72 h when compared with control (*p* < .05, Figure 4A). Thus, 2 μg/mL was selected as the concentration of rrOPN and 72 h as the time-point for the following experiments. Thereafter, the cell population in G0/G1 phase was significantly down-regulated than that in control group (*p* < .05), while the cell population in S + G2/M phase was significantly up-regulated (*p* < .05), indicating that 2 μg/mL rrOPN could stimulate cell cycle progression at 72 h after treatment (Figure 4B). In contrast, after treatment with 5 μg/mL OPN-Ab for 48 h, the viability of BRL-3A cells was significantly decreased when compared with that treated with PBS (*p* < .01, Figure 4C). Flow cytometry analysis further showed that the cell proportion in G0/G1 phase was significantly increased (*p* < .05), while in S + G2/M phase was decreased at 48 h after treatment with OPN-Ab when compared to that in control group (*p* < .05, Figure 4D).

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**Figure 3.** Endogenous OPN affected the activation AKT and STAT3 signaling pathways. (A) Western blots (left panel) and corresponding densitometry (right panel) showed up-regulated expressions of p-STAT3, p-AKT, and CCND1 (left panel) in Lenti-OPN group at 48 h after positive monoclonal BRL-3A cell lines were seeded. *p* < .05, **p** < .01 vs Lenti-GFP group. (B) Western blots (left panel) and corresponding densitometry (right panel) analysis showed the expressions of p-STAT3, p-AKT, and CCND1 in BRL-3A cells at 48 h after transfection with OPN-siRNA. *p* < .05 vs scramble group. (C) qRT-PCR analyzed the expression of Ccnd1 gene in BRL-3A at 48 h after Lenti-OPN and Lenti-GFP were seeded or transfected with OPN-siRNA. ††*p* < .01 vs respective control group. The data are presented as mean ± SEM, and β-actin was used as a loading control.
Thus, OPN-Ab may lead to cell cycle arrest at G0/G1 phase, and 5 μg/mL OPN-Ab and 48 h were chosen for the following experiments.

**OPN stimulated the activation of its downstream signaling pathways**

The WB analysis of the BRL-3A cells after treatment with rOPN for 72 h demonstrated that the expressions of both p-STAT3 and p-AKT were significantly augmented, while the expression of p-AKT exhibited obvious down-regulation when BRL-3A cells were treated with OPN-Ab for 48 h (Figure 5A). Furthermore, the expression of BCL2 protein was induced by rOPN treatment, while obviously reduced by OPN-Ab (Figure 5A). Also, the expression ratio of apoptosis-related BCL2 to BAX at mRNA level was increased after rOPN treatment and decreased after OPN-Ab treatment (Figure 5B). Unexpectedly, the protein levels of CCND1 showed no marked changes (figure not shown). Subsequently, we further confirmed the relationship between OPN and p-AKT by pretreating the BRL-3A liver cells with PI3K/Akt inhibitor LY294002 for 30 min, and then with 5 μg/ml OPN-Ab for 48 h. The results showed that LY294002 treatment reduced AKT phosphorylation as OPN-Ab did, and the combination of LY29400 and OPN-Ab attenuated AKT phosphorylation. Concomitantly, the combination of LY294002 and OPN-Ab significantly inhibited the expression of BCL2 protein, while induced BAX proteins than that treated by LY294002 or OPN-Ab alone in rat BRL-3A liver cells (Figure 6). Collectively, the above results suggested that exogenous OPN could induce cell proliferation and survival through AKT signaling pathway activation, which might also affect STAT3 signaling pathway.

**rrOPN facilitated liver regeneration and hepatocyte proliferation**

To further confirm the direct effects of OPN overexpression on LR, rrOPN was immediately injected into the livers of rats after PH. Wen et al have supported the unique role of OPN during the priming phase of mouse LR by using OPN−/− mice [5], and one of our previous studies has preliminarily demonstrated the role of OPN during the termination stage of rat LR by applying OPN shRNA plasmids [9]. Hence, this study selected 3, 24 and 72 h after rat PH as time points, which not only included the priming and proliferation stages but also fitted with the cut-off points of termination stage for rat LR [21]. As a result, this study demonstrated that LR rate was significantly higher until 72 h in rrOPN group than that in control group (Figure 7A). Normally, hepatocytes are the most important kind of liver cells that are mobilized in LR, and to further detect whether rrOPN could impact hepatocyte proliferation, the expression of Ki67 in liver tissues was further measured by immunofluorescent assay. For PBS-administration group, the Ki67-positive cell proportion
reached its peak at 24 h after PH, and the Ki67-positive cell proportions showed no significant changes at 3 and 24 h after PH between rrOPN and PBS group (Figure 7B and Supplemental Figure S3). At 72 h, the Ki67-positive cell proportions showed significant increase in rrOPN group when compared with those in PBS group (Figure 7B,C), coinciding with significant increase in LR rate at this time point and indicating that administration of rrOPN facilitated hepatocyte proliferation after PH, and ultimately promoting LR.

Furthermore, to reveal the underlying molecular mechanism of how rrOPN affects LR, the contents of IL-6 and TNF-α in serum were measured by ELISA. The results showed that the levels of IL-6 at 3 h and the levels of TNF-α at 3 and 24 h in serum showed significant increase when compared with that in control group (Figure 7D). Correspondingly, the mRNA expression levels of IL-6 and TNF-α in liver tissues were stimulated by administration of rrOPN at 3 h after PH (Figure 7E). However, the expressions of IL-6 and TNF-α protein in liver tissues were gradually reinforced from 3 h to 72 h after PH in rrOPN group (Figure 7F). Moreover, the serum levels of ALT and AST showed slight elevation at 3 h and decrease at 24 h after administration of rrOPN when compared with that in control group (Figure 7G). However, no significant difference in serum ALT or AST levels was observed between the two groups. Thereafter, HE staining was performed to assess the livers histopathologically. The results showed that the damage of hepatic lobule was aggravated in both groups at 24 h after PH (Figure 7H, middle panel). We then further analyzed the damage of liver tissue by Sudan IV staining, and found that some lipid droplets were obviously stained in deep red in the hepatic cells around central vein and portal area, inferring to microvesicular steatosis in these areas (Supplemental Figure S4). The liver morphology at 72 h was almost restored with clear nuclei in the two groups, and the number of hepatic cells was increased, and the hepatic cells were arranged more orderly in rrOPN group (Figure 7H, right panel). Therefore, rrOPN did not induce liver tissue injury and necrosis, but led to the secretion of IL-6 and TNF-α.

Subsequently, to evaluate the signaling pathways activated by rrOPN, the changes in the expression of OPN and downstream proteins were detected in liver tissues at 3, 24 and 72 h after PH and PBS or rrOPN administration. As shown in Figure 8A, the contents of OPN in liver tissues were gradually increased from 3 to 72 h in both groups, and rrOPN administration led to significant up-regulation of OPN contents at all time points when compared with PBS group. In addition, the expression of p-AKT was reinforced at 3 and 24 h, while p-STAT3 and p-p65 (one of NF-κB subunits) were
at 3 h following PH in rrOPN group when compared with those in PBS group. Hence, AKT, STAT3 and NF-κB signaling pathways were activated by administration of rrOPN during the initiation of LR. Interestingly, the expression of p-p65 was down-regulated at 24 and 72 h. Our data also showed that the expression of target protein CCND1 was highly induced at 3 and 72 h, and the expression of BAX was obviously attenuated at 3 h without marked changes of BCL2 protein (Figure 8B). This might be due to the activation of several above-mentioned signaling pathways. To sum up, rrOPN injection elevated the contents of IL-6 and TNF-α in serum, regulated the expressions of CCND1 and BAX through STAT3, AKT and NF-κB signaling pathways, and ultimately promoted hepatocyte proliferation and LR rate during rat LR.

**Discussion**

The present study was designed to confirm the role of OPN in liver cell proliferation and LR, and the results finally demonstrated that OPN could affect hepatocyte proliferation in vitro, and promote hepatocyte proliferation during rat LR through both STAT3 and AKT signaling pathways. This in turn laid a foundation for its potential use in promoting LR during liver repair.

Several studies have focused on the stimulatory role of endogenous OPN in cancer cell proliferation and migration during carcinogenesis [22–24]. Similarly, regarding the role of OPN in the growth of normal liver cells, Xiao et al revealed that the change in the OPN expression level impacted not only the proliferation of human HSC cell LX-2 but also the TGF-β1-mediated HSC activation [25,26]. Also Wen et al pointed out that OPN depletion attenuated the activation of Kupffer cells during LR [5]. However, the effect of endogenous OPN on the growth of hepatocytes remains to be elucidated. This study used lentiviruses containing OPN to induce stable overexpression of OPN in rat BRL-3A cells, and found that they promoted both cell viability and cell cycle progression. More importantly, we further revealed that the proportion cell proliferation and cell populations in S and G2/M phases were both increased after transfection with Ad-OPN when using rat primary hepatocytes. Furthermore, the expressions of p-STAT3 and p-AKT were obviously enhanced in response to OPN endogenous overexpression. Previous studies have showed that activated STAT3 induces c-Myc and then transcriptionally regulates CCND1 [27], and Akt also stimulates cell replication by causing nuclear accumulation of cyclin D1 [28]. Accordingly, our study results showed that the expression of downstream CCND1 was significantly induced. In contrast, OPN-siRNA inhibited cell viability and proliferation. It has been previously shown that OPN siRNA...
suppressed the growth of gastric cancer cells through down-regulation of p-AKT [29], and the expressions of p-AKT and its downstream CCND1 in BRL-3A cells were significantly attenuated when treated with OPN-siRNA in this study, revealing the inhibitory mechanism of OPN siRNA on hepatocyte proliferation.

To validate the direct effect of OPN on the growth of hepatocytes, rrOPN was used to treat rat BRL-3A liver cells, and the results demonstrated direct stimulatory role of OPN on cell proliferation when treated with rrOPN and OPN-Ab. Interestingly, these results are distinct from those when mouse primary hepatocytes were treated with rmOPN [5] or rhOPN [6]. Given that the mouse primary hepatocytes are different from rat hepatocyte cell line, we hypothesized that this difference might be due to differences in the cell types used as materials for studying. Furthermore, the expression of p-STAT3 was significantly attenuated after rrOPN treatment, which is not contrary to the results of some previous studies, where OPN could activate STAT3 signaling pathway and drive a positive feedback with STAT3 signaling pathway in some cancer cells [14,30]. Of note, the expression of p-AKT was obviously changed in response to either rrOPN or OPN-Ab treatment, and WB analysis results after pretreatment with PI3K/Akt inhibitor LY294002 confirmed the involvement of AKT signaling in mediating the role of OPN. Li et al found that the proliferation of human endometrial carcinoma cells HEC-1A could be promoted by rhOPN through AKT signaling pathway [12], which partially supported our results. As a whole, the expression of p-Akt was changed more significantly than that of p-STAT3 in Figures 3 and 5, which may be due to the fact that OPN can affect Akt pathway either through integrin or CD44 cell surface receptor [31,32], but affect STAT3 pathway only through integrin receptor [30]. However, the downstream BCL2 and BAX proteins, but not CCND1, showed variations after treatment with rrOPN or OPN-Ab. In line with the study conducted by He et al., rhOPN could induce the expressions of p-AKT and Bcl-2 and significantly inhibited Bax [33], suggesting that rrOPN or OPN-Ab treatment also could regulate cell survival.

After determining the in vivo effects of rrOPN on hepatocyte proliferation though Ki67 immunofluorescent assay, the present study found that Ki67-positive cell proportion reached its peak at 24 h after PH in PBS group. This could be explained by the fact that the first peak of DNA synthesis occurred in the parenchymal cells at 24 h after resection in rat [34]. Furthermore, the Ki67-positive cell proportions and LR rates were all significantly elevated at 72 h after PH in rrOPN group when compared with those in PBS group, and this was in line with continuous upregulation of OPN from 3–72 h during rat LR [9] and fitted with the cut-off point of termination stage for rat LR [21]. On the other hand, the changes in liver histopathology and production and secretion of various proteins were mainly observed at 3 h, while most
of them showed no significant differences at 72 h after PH between rrOPN and PBS group, suggesting that the selection of time points for detection is relatively appropriate. However, our previous study results revealed that OPN shRNA delayed rat LR rate at 168 h [9], indicating that the exogenous OPN may impact LR more rapidly and directly than OPN shRNA plasmids. Interestingly, the liver/body ratio in OPN/−/− mice was suppressed at 48 h [5], i.e., when the first hepatocyte proliferation wave occurred in mice [35]. In view of different expression profiles of OPN between rat (two expression peaks at 12 and 72 h) [9] and mouse (one expression peak at 12 h) [5] regenerating livers, we inferred that the function of rat OPN was not reflected until 72 h or later than 72 h by LR rate or liver/body ratio, and this was different from the results of mouse LR [5].

After rrOPN was administrated into rat liver after PH, the IL-6 contents as detected by ELISA, qRT-PCR and WB showed significant changes, which was in agreement with the obvious changes observed in IL-6 mRNA levels and protein secretion between WT and OPN/−/− mice [11]. Of note, TNF-α mRNA levels were increased, while the contents of TNF-α protein in liver tissue was decreased when compared with control group, implying that majority of newly synthesized proteins were secreted into serum. This could be due to the enhanced TNF-α protein levels in serum as detected by ELISA. Though rrOPN did not induce obvious liver tissue injury and necrosis, this led to the secretion of IL-6 and TNF-α. These are always considered to be the most important initiators in LR, and further led to the initiation of hepatocyte proliferation during LR [36]. In line with the findings from Wen et al. [5], serum ALT and AST levels were greatly increased at 24 h but showed no significant differences between rrOPN and PBS groups, which might be due to the large individual variations among experimental rats. Though microvesicular steatosis in liver tissues was observed at 24 h, the liver cells presented no significant abnormality at 72 h after PH in both rrOPN and PBS groups. Thus, rrOPN administration did not result in apparent liver injury, and this was in contrast to the results of our preliminary in vivo study [9]. In view of the volume of 10 ml PBS containing OPN-expressing plasmids in the previous study, we suspected that the use of big volume of administered liquid might contribute to the infiltration of immune cells as only 2 ml PBS containing rrOPN was applied in the present study.

As mentioned above, rrOPN administration resulted in greater induction of IL-6, and further robust phosphorylation of STAT3 was observed at 24 h after PH in rrOPN group, which was in accordance with the results from the study by Wen et al that utilized OPN/−/− mouse [5]. Moreover, the expression of p-AKT was more rapid and robust at 3 and 24 h in rrOPN group, which was in contrast to the fact that AKT was activated by phosphorylation in the regenerating liver [37,38]. NF-κB transcriptionally activated CCND1 and then promoted the G1-S phase transition [39]. In view of the significant increase of p-p65 at 3 h, we inferred that it might contribute to the upregulation of CCND1 at 3 h after PH. However, a previous study revealed that OPN silencing blocked NF-κB activation, and increased Bax expression [40], and this study indicated that rrOPN significantly suppressed Bax expression at 3 h. This might be due to NF-κB activation (the expression of p-p65) induced by rrOPN at 3 h after PH. Moreover, Fan et al. have indicated that overexpression of intracellular OPN (iOPN) inhibited the activation of NF-κB, and played a critical role in anti-inflammatory response in DEN-induced hepatocarcinogenesis murine model [41]. Notably, as the content of iOPN indicated by WB was increasing, the expression of p-p65 was down-regulated at 24 and 72 h, suggesting that OPN-mediated NF-κB signaling pathway might be implicated in controlling the inflammatory response after priming LR, although NF-κB triggered innate immune response and promoted earlier cell proliferation during LR [42]. However, the role of OPN-mediated NF-κB signaling pathway during LR needs to be further studied.

Collectively, OPN could affect hepatocyte proliferation in vitro, and promote hepatocyte proliferation in vivo through both STAT3 and AKT signaling pathways. In fact, this in vitro study for the first time demonstrated the direct stimulatory effect of exogenous OPN on the proliferation of rat liver cells BRL-3A. But it would be more convincing if the primary hepatocytes were also utilized. In addition, some specific inhibitors for STAT3 and NF-κB signaling pathways should be added to further confirm the functional mechanism of OPN. It is certainly worth studying as to how OPN balances its roles between hepatocyte proliferation and inflammatory response during LR, and whether it could play a similar role in liver repair under other pathological conditions.

Ethics statement

All experiments complied with the guidelines for the care and use of experimental animals of the Ministry of Science of the People’s Republic of China.

Author contributions

Conceptualization, Gaiping Wang; Investigation, Peipei Chu; Methodology, Peipei Chu, Meng Chen and Shasha Chen; Resources, Xiaofang Li and Shasha Chen; Software, Liya Cheng and Ganggang Yang; Supervision, Cuifang Chang; Writing-original draft, Gaiping Wang; Writing-review & editing, Congcong Zhao.

Disclosure statement

No potential conflict of interest was reported by the authors.

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