

# Reduced triglyceride accumulation due to overactivation of farnesoid X receptor signaling contributes to impaired liver regeneration following 50% hepatectomy in extra-cholestatic liver tissue

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**Abstract.** The aim of the present study was to investigate the role of triglyceride metabolism in the effect of obstructive cholestasis on liver regeneration following 50% partial hepatectomy (PH). Obstructive cholestatic rat models were achieved via ligation of the common bile duct (BDL). Following comparisons between hepatic pathological alterations with patients with perihilar cholangiocarcinoma, rats in the 7 day post-BDL group were selected as the BDL model for subsequent experiments. Liver weight restoration, proliferating cell nuclear antigen labeling index, cytokine and growth factor expression levels, and hepatic triglyceride content were evaluated to analyze liver regeneration post-PH within BDL and control group rats. The results of the present study revealed that obstructive cholestasis impaired liver mass restoration, which occurred via inhibition of early stage hepatocyte proliferation. In addition, reduced triglyceride content and inhibited expression of fatty acid  $\beta$ -oxidation-associated genes, peroxisome proliferator activated receptor  $\alpha$  and carnitine palmitoyltransferase, were associated with an insufficient energy supply within the BDL group post-PH. Notably, the expression levels of fatty acid synthesis-associated genes, including sterol-regulatory element-binding protein-1c, acetyl-coA carboxylase 1 and fatty acid synthase were also

reduced within the BDL group, which accounted for the reduced triglyceride content and fatty acid utilization. Further investigation revealed that overactivated farnesoid X receptor (FXR) signaling may inhibit fatty acid synthesis within BDL group rats. Collectively, the role of triglycerides in liver regeneration following PH in extra-cholestatic livers was identified in the present study. Additionally, the results indicated that overactivated FXR signaling-induced triglyceride reduction is associated with insufficient energy supply and therefore contributes to the extent of impairment of liver regeneration following PH within extra-cholestatic livers.

## Introduction

Obstructive cholestasis often arises as an incidental surgical symptom, and is caused by malignant biliary obstruction due to gallbladder carcinoma, intrahepatic cholangiocarcinoma and particularly perihilar cholangiocarcinoma (PHCC) (1,2). Extra-cholestasis status has been associated with the enrichment of hepatocellular hydrophobic bile acid and severe liver injury (3,4). Most patients with PHCC require ~50% hepatectomy to achieve radical resection (1); therefore, investigations into the effect of obstructive jaundice on liver mass and functional restoration following partial hepatectomy (PH) is of surgical importance (5,6). Previous studies have reported that high total bilirubin (TB) serum levels ( $>170 \mu\text{mol/l}$ ) cause post-hepatectomy liver failure and mortality (7,8). Therefore, biliary drainage is performed to reduce obstructive cholestasis-induced liver injury, which can also increase in-hospital morbidity and tract seeding (9,10). However, a meta-analysis did not report any benefits associated with preoperative biliary drainage (11). Research based on obstruction of various durations, the extent of liver sectioning and the option of biliary drainage has not provided any significant innovations into perioperative management of patients with PHCC, without surgical treatment. However, contradicting conclusions regarding impaired liver regeneration due to obstructive cholestasis post-PH have been reported (12-15). The effects of obstructive cholestasis on liver regeneration require further investigation and clinical confirmation.

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Hepatocellular triglyceride (TG) accumulation has been observed in the early phase of normal liver regeneration (16) and serves a crucial role in the maintenance of increasing energy demands via  $\beta$ -oxidation (17); metabolic factors involved in liver regeneration within extra-cholestatic liver tissue require further investigation. A previous study indicated that cholestasis decreased hepatic energy charge and increased hepatic lipoperoxide levels (18); alterations in TG metabolism may also mediate cholestatic liver inflammation and fibrotic processes in turn (19). Therefore, whether alterations in TG metabolism within extra-cholestatic conditions involve impairment of liver regeneration following PH remains to be determined.

In the present study, alterations in TG metabolism during liver regeneration within extra-cholestatic liver tissue were investigated. The results suggested that overactivation of farnesoid X receptor (FXR) signaling-induced reduction in TG may contribute to the impairment of liver regeneration in post-PH extra-cholestatic livers.

## Materials and methods

**Patients.** A total of 10 paraffin-embedded specimens of liver tissues from patients with PHCC were obtained from the Pathology Department of Nanjing Drum Tower Hospital (Nanjing, China) between April 2001 and May 2006. The diagnosis of PHCC was confirmed by histology or cytology. Patients did not have any history of biliary drainage. Preoperative serum TB levels within these patients were  $>170 \mu\text{mol/l}$  (mean  $\pm$  standard deviation,  $216.5 \pm 45.08 \mu\text{mol/l}$ ; range,  $171.4$ – $304.5 \mu\text{mol/l}$ ).

### Animals and surgeries

**Animals.** A total of 140 male Sprague-Dawley rats (230–250 g, 8 weeks-old) were purchased from the Laboratory Animal Centre of the Affiliated Drum Tower Hospital of Nanjing University Medical School (Nanjing, China). Rats were housed in a specific pathogen-free environment under a 12 h light/dark cycle, maintained in a temperature ( $22^\circ\text{C}$ ), air pressure- and humidity (60%)-controlled environment and fed *ad libitum*. All animal procedures were carried out in accordance with the Animal Care and Use Committee at the Model Animal Research Center of Nanjing University, (Nanjing, China). The present study was approved by the Ethics Committee of Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School.

**Ligation of the common bile duct (BDL) and sham operations.** Rats were injected intraperitoneally with sodium pentobarbital ( $70 \text{ mg/kg}$  body weight) for anesthetization prior to operations. Within the BDL group, two ligations of the distal common bile duct (CBD) were performed using 5-0 chinlon, and the CBD was cut between the ligations. Within the control (CTL) group, the same abdominal incision was made; however, the CBD and adjacent tissue were only touched with swabs; 3-0 chinlon was used to suture the abdomen.

**PH and internal biliary drainage.** PH was performed according to Nagai *et al* (20). Epidural catheters with 0.7 mm outer diameter were used to carry out internal biliary drainage.

Following 50% PH, the two ends of the epidural catheter were inserted into the duodenum and the dilated CBD; purse-string sutures were carried out for reinforcement.

Rats were randomly and equally separated into the CTL ( $n=5$ ), 7 day post-BDL (BDL-7 days,  $n=5$ ) and 14 day post-BDL (BDL-14 days,  $n=5$ ) groups; sham and BDL operations were performed as aforementioned. Rats were sacrificed on day 7 or 14 post-BDL and liver samples were obtained. Following comparisons of alterations within liver tissues from patients with PHCC, rats in the BDL-7 days group were selected for subsequent analyses. PH with internal biliary drainage was conducted 7 days following BDL operations in the BDL group and PH was performed following the sham operation within the CTL group. Rats were sacrificed on day 7 post-BDL and serum and liver samples were obtained (0 day).

For overall survival analysis, survival curves were calculated based on survival within 7 days post-PH in the CTL and BDL-7 days group using a novel group of rats ( $n=16$ ).

For studying the liver regeneration, a separate group of rats were sacrificed at day 1 ( $n=6$ ), day 3 ( $n=8$ ) and day 7 ( $n=8$ ) post-PH in the CTL and BDL-7 days group to obtain liver and plasma samples to determine liver regeneration status.

**Serum data analysis.** Blood samples were collected from the orbital venous plexus of rats and were subsequently centrifuged at  $3,000 \times g$  for 15 min (room temperature). Serum was obtained and analyzed in the Clinical Laboratory of the Affiliated Drum Tower Hospital of Nanjing University Medical School.

**Morphological analysis.** Liver tissue was fixed in 4% paraformaldehyde or 10% buffered formalin at  $4^\circ\text{C}$  for 24 h, followed by paraffin embedding and sectioned to  $5 \mu\text{m}$ . Subsequently, hematoxylin and eosin (H&E) staining was performed at room temperature (3 min for hematoxylin staining and 5 sec for eosin staining), and Sirius red staining was used to measure the hepatic collagen content in liver fibrosis. In brief, deparaffinised liver sections were incubated with Sirius red for 2 h at room temperature. Areas stained with Sirius Red were analyzed with ImageJ software (version 1.50; National Institutes of Health, Bethesda, MD, USA).

**Immunohistochemistry.** In brief, deparaffinised liver sections were blocked with 1% bovine serum albumin (Applygen Technologies, Inc., Beijing, China) for 1 h at room temperature and then incubated with a primary antibody against proliferating cell nuclear antigen (PCNA; 1:200) at  $4^\circ\text{C}$  overnight. Subsequently, they were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:100) for 3 h at room temperature. The localization and expression of PCNA within rat liver tissues were detected with DAB (Applygen Technologies, Inc.) as previously described (21). Localization and expression of PCNA were assessed and images were captured under an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan). The positive stained cells were assessed by Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). Primary antibodies against PCNA (cat. no. sc-7907) and horseradish peroxidase-tagged secondary antibody (cat. no. sc-2004) were purchased from Cruz Biotechnology, Inc. (Dallas, TX, USA).

**Terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL).** *In situ* DNA fragmentation within rat liver was assessed using the DeadEnd Colorimetric TUNEL system (Promega Corporation, Madison, WI, USA). In brief, deparaffinised and rehydrated liver sections were incubated with 1% proteinase K (Promega Corporation), then the *in situ* DNA fragmentation was assessed according to the manufacturer's protocol. At least 5 fields of view/slices were assessed and images were captured under an Olympus BX51 microscope (Olympus Corporation). The positive staining hepatocytes were assessed by Image-Pro Plus 6.0 (Media Cybernetics, Inc.).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to extract rat liver RNA. RT was performed using PrimeScript™ RT reagent (Takara Bio Inc., Otsu, Japan) according to the manufacturer's protocol. Subsequent RT-qPCR was carried out on an ABI-7300 (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR®-Green PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) as previously described (22). The thermocycling conditions were: 95°C for 1 min; 40 cycles of 95°C for 15 sec, 58°C for 15 sec and 72°C for 30 sec; with final heating at 72°C for 10 min. All quantification was performed in triplicate and normalized to  $\beta$ -actin. The relative expression level was calculated using the  $2^{-\Delta\Delta C_q}$  method (23). The primer sequences were: Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) forward, 5'-CATCCG TTCTCTACCCAGCC-3' and reverse, 5'-AATTCTGAGCCC GGAGTTGG-3'; interleukin (IL)-6 forward, 5'-CACTTCACA AGTCGGAGGCT-3' and reverse, 5'-TCTGACAGTGCATCA TCGCT-3'; hepatocyte growth factor (HGF) forward, 5'-CCT TCGAGCTATCGCGGTAAA-3' and reverse, 5'-GAATTT GTGCCGGTGTGGTG-3'; transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) forward, 5'-AGGGCTACCATGCCAACTTC-3' and reverse, 5'-CCACGTAGTAGACGATGGGC-3'; sterol-regulatory element-binding protein-1c (SREBP-1c) forward, 5'-CCC GGTTCCTCCAGGA ACTTT-3' and reverse, 5'-CTGTCT CACCCCCAGCATAG-3'; peroxisome proliferator activated receptor  $\alpha$  (PPAR $\alpha$ ) forward, 5'-TTCGTGGAGTCCTGGAAC TGA-3' and reverse, 5'-CCACAGAGCACCAATCTGTGA-3'; acetyl-coA carboxylase 1 (ACCI) forward, 5'-CTTGGGGTG ATGCTCCCATT-3' and reverse, 5'-GCTGGGCTTAAACCC CTCAT-3'; fatty acid synthase (FASN) forward, 5'-GCATTT CCACAACCCCAACC-3' and reverse, 5'-AACGAGTTGATG CCCACGAT-3'; carnitine palmitoyltransferase 1A (CPT1A) forward, 5'-CCTACCACGGCTGGATGTTT-3' and reverse, 5'-TACAACATGGGCTTCCGACC-3' and  $\beta$ -actin forward, 5'-GCAGGAGTACGATGAGTCCG-3' and reverse, 5'-ACG CAGCTCAGTAACAGTCC-3'.

**Tissue TG content.** Tissue TG reagents (Applygen Technologies, Inc., Beijing, China) were used to examine rat liver tissue TG content, according to the manufacturer's protocol.

**Western blot analysis.** Hepatocytes were isolated as described previously by Chen *et al* (24). Proteins were isolated from rat hepatocytes using radioimmunoprecipitation assay buffer containing protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) and quantified with bicinchoninic

acid assay kits (Applygen Technologies, Inc.); proteins were then boiled in loading buffer. Proteins were separated by 12% SDS-PAGE with 50  $\mu$ g total protein per lane and were then transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA), then the membranes were blocked with 5% skim milk for 1 h at room temperature. Membranes were incubated with primary antibodies for at least 8 h at 4°C. Protein levels were detected by incubation with HRP-conjugated secondary antibody for 1 h at room temperature and the signal was developed with ECL (EMD Millipore, Billerica, MA, USA) and visualized using a Tanon 5200 imaging system (Tanon Science and Technology Co., Ltd., Shanghai, China). Densitometric analysis was performed with ImageJ software (version 1.50; National Institutes of Health, Bethesda, MD, USA). Primary antibodies against small heterodimer partner (SHP; 1:100; cat. no. sc-30169), FXR (1:200; cat. no. sc-13063),  $\beta$ -actin (1:1,000, cat. no. sc-376421), goat anti-rabbit immunoglobulin (Ig)G-HRP and goat anti-mouse IgG-HRP (1:10,000; cat. nos. sc-2004 and sc-2005) were purchased from Santa Cruz Biotechnology, Inc.

**Statistical analysis.** Experiments were repeated  $\geq 3$  times; data are presented as the mean  $\pm$  standard deviation. Statistical calculations were performed with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) or SPSS statistical software 19 (IBM Corp., Armonk, NY, USA). Statistical significance was assessed by an unpaired two-tailed Student's t-test or one-way analysis of variance and Tukey's Highest significant difference test using. Differences in overall survival were evaluated by log-rank test.  $P < 0.05$  was considered to indicate a statistically significant difference and  $P < 0.01$  was considered to indicate a highly statistically significant difference.

## Results

**Hepatic morphological alterations within patients with PHCC are similar to those in BDL-7 days group rats.** To determine the appropriate duration for obstructive cholestatic development, morphological alterations within hepatic PHCC samples were analyzed with H&E and Sirius Red staining (Fig. 1). Moderate biliary hyperplasia and inflammatory cell infiltration within the portal area were observed within the BDL-7 days group and were more severe within the BDL-14 days group, compared with the sham group. Morphological alterations exhibited by the BDL-7 days group were similar to those within patients with PHCC (Fig. 1A). The aforementioned observations were confirmed via Sirius Red staining. Collagen deposition within the BDL-7 days group was similar to that of patients with PHCC (Fig. 1B). A  $\sim 2.2$ -fold increase in collagen deposition was observed in the BDL-14 days group compared with in the BDL-7 days group. Rats in the BDL-7 days group were selected for subsequent analysis of clinical obstructive cholestasis via PH with internal biliary drainage; PH alone was conducted within rats of the CTL group (Fig. 1D).

**Obstructive jaundice leads to hepatocyte proliferation and increased liver weight (LW).** Analysis of LW and body weight (BW) prior to PH revealed that obstructive cholestasis was associated with reduced body weight (BW) and malnutrition (12). Therefore, the LW/BW ratio was inapplicable to the

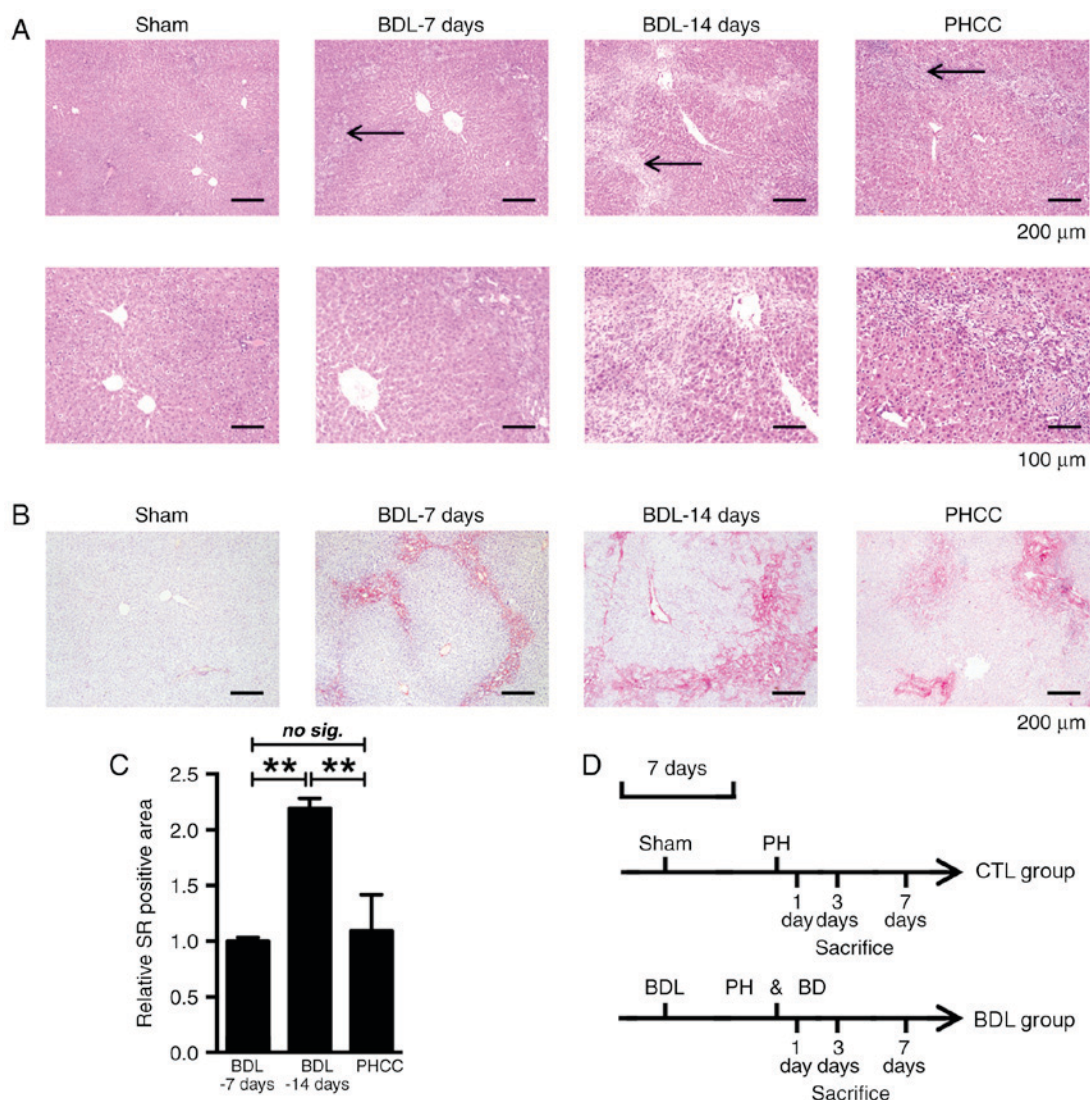


Figure 1. Hepatic morphological alterations in the BDL-7 days and BDL-14 days groups, and in patients with PHCC. (A) Biliary hyperplasia and inflammatory infiltration are presented by arrows and demonstrated by hematoxylin and eosin staining. These findings indicated that the severity of liver injury was greater within the BDL-14 days group compared with in the BDL-7 days group and in patients with PHCC. (B and C) Collagen deposition was analyzed via SR staining and quantified via ImageJ software. \*\* $P < 0.01$ . Data are expressed as the mean  $\pm$  standard deviation,  $n = 5$ . (D) Experimental schedule of the present study. BDL-7 days/14 days, 7/14 days post-ligation of the common bile duct; PHCC, perihilar cholangiocarcinoma; SR, Sirius Red.

evaluation of liver regeneration following PH as body weight was influenced by obstructive cholestasis status. Conversely, LW was increased, as was the LW/BW ratio (LW/BW; Fig. 2A); In addition to collagen deposition, biliary hyperplasia and inflammatory infiltration, hepatocellular behavior, including proliferation and apoptosis may also contribute to alterations in LW. Hepatocyte proliferation was determined by analyzing the number of PCNA-positive hepatocytes; 21% hepatocytes replicated pre-PH within the BDL-7 days group (Fig. 2B). Apoptotic rate was investigated using a TUNEL assay. As presented in Fig. 2C, apoptosis was increased within the BDL group compared with in the CTL group (2.1 vs. 8.2%). The findings of the present study indicated that obstructive jaundice was associated with hepatocyte proliferation, biliary hyperplasia, collagen deposition and inflammatory infiltration; therefore, LW and LW/BW increased.

*Obstructive cholestasis causes severe liver injury pre- and post-PH.* Serum data was collected to examine liver injury.

Increased alanine transferase (ALT), aspartate transaminase (AST), TB and total bile acids (TBA) levels were detected within the BDL group prior to PH (Fig. 3A-D), which was associated with severe liver injury. Following PH with internal biliary drainage, serum ALT levels were significantly decreased within the BDL group compared with in the CTL group; however, significant alterations in serum AST levels were not observed (Fig. 3A and B). Elevated TB and TBA serum levels rapidly decreased post-PH within the BDL group (Fig. 3C and D), which indicated that biliary drainage was successful. The findings of the present study suggested that obstructive cholestasis led to liver injury prior to and post-PH.

*Obstructive cholestasis inhibits liver mass restoration via hepatocyte proliferation suppression post-PH.* A decrease in overall survival was reported post-PH, which was associated with extra-cholestasis; ~56% BDL rats survived post-PH, whereas the CTL group exhibited 100% overall survival

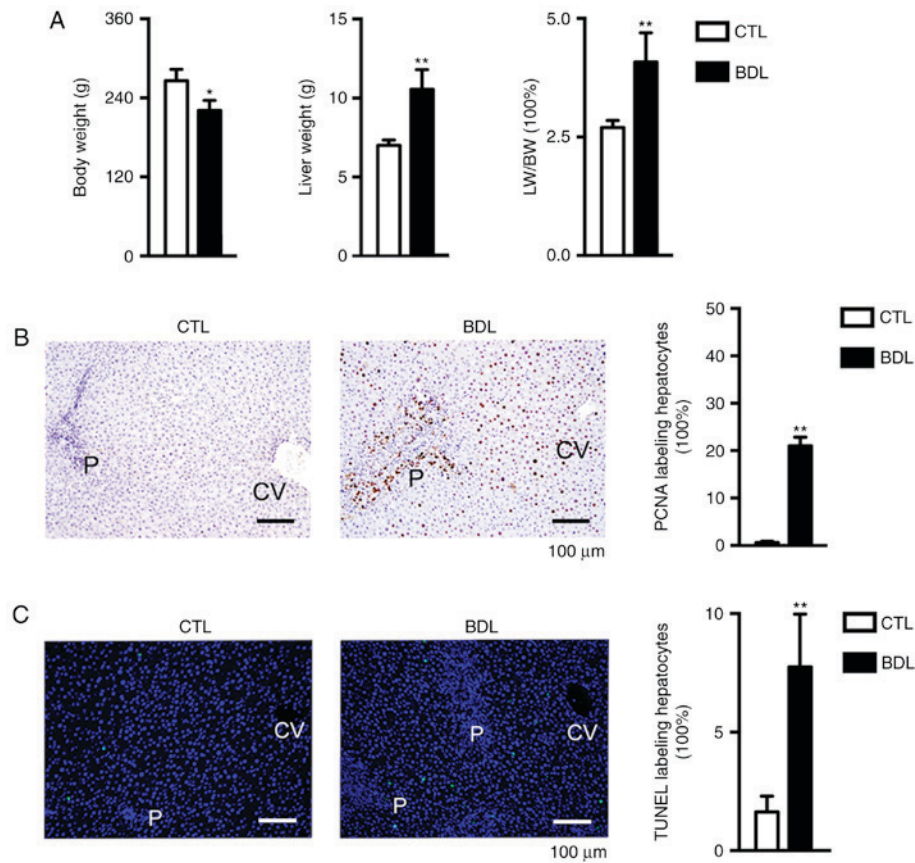


Figure 2. LW, BW and hepatocyte behavior in CTL and BDL groups pre-PH. (A) LW was significantly increased and BW was markedly decreased within the BDL group compared with in the CTL group; the LW/BW ratio pre-PH was significantly increased within the BDL group. \* $P < 0.05$ ; \*\* $P < 0.01$ . (B) Hepatocyte proliferation was detected, as determined by immunohistochemistry. The percentage of PCNA-positive hepatocytes was significantly increased within the BDL group compared with in the CTL group. \*\* $P < 0.01$ . (C) Hepatocyte apoptosis was analyzed via TUNEL assay; apoptosis was significantly increased within the BDL group compared with in the CTL group. \*\* $P < 0.01$ . Data are presented as the mean  $\pm$  standard deviation. BDL, ligation of the common bile duct; BW, body weight; CTL, control; CV, central vein; LW, liver weight P, portal area; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling.

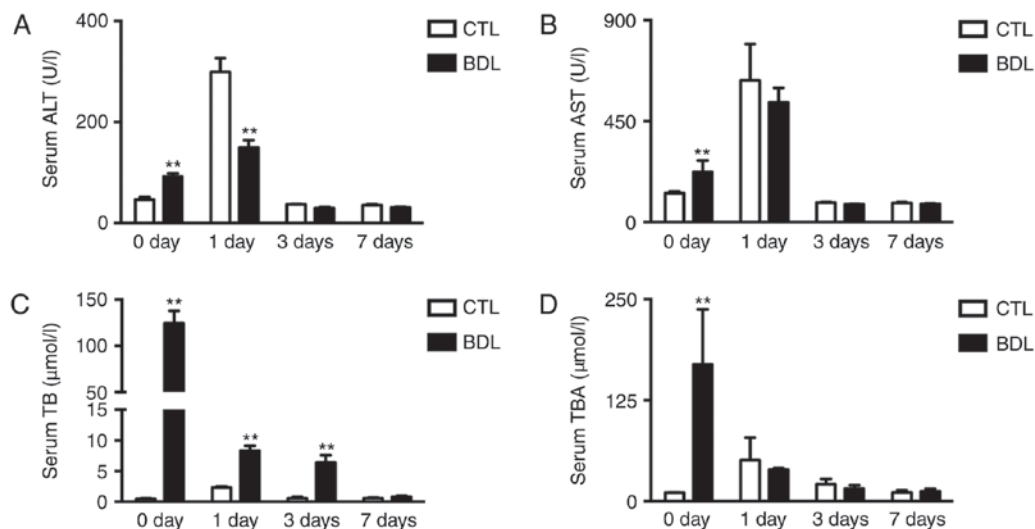


Figure 3. Obstructive cholestasis causes liver injury pre- and post-PH. Serum (A) ALT, (B) AST, (C) TB and (D) TBA levels within BDL and CTL groups pre- and post-PH. Data are expressed as the mean  $\pm$  standard deviation,  $n \geq 6$ ; \*\* $P < 0.01$ . ALT, alanine transaminase; AST, aspartate aminotransferase; BDL, ligation of the common bile duct; CTL, control; PH, partial hepatectomy; TB, total bilirubin; TBA, total bile acids.

( $P < 0.01$ ; Fig. 4A). Surgical-associated factors, including hemorrhage, re-obstruction, bile leakage and organ injury, were excluded, thus indicating that extra-cholestasis independently

decreased overall survival rate post-PH. In the present study, rat mortality was reported 2 days post-PH within the BDL group; acute liver failure following PH may have accounted

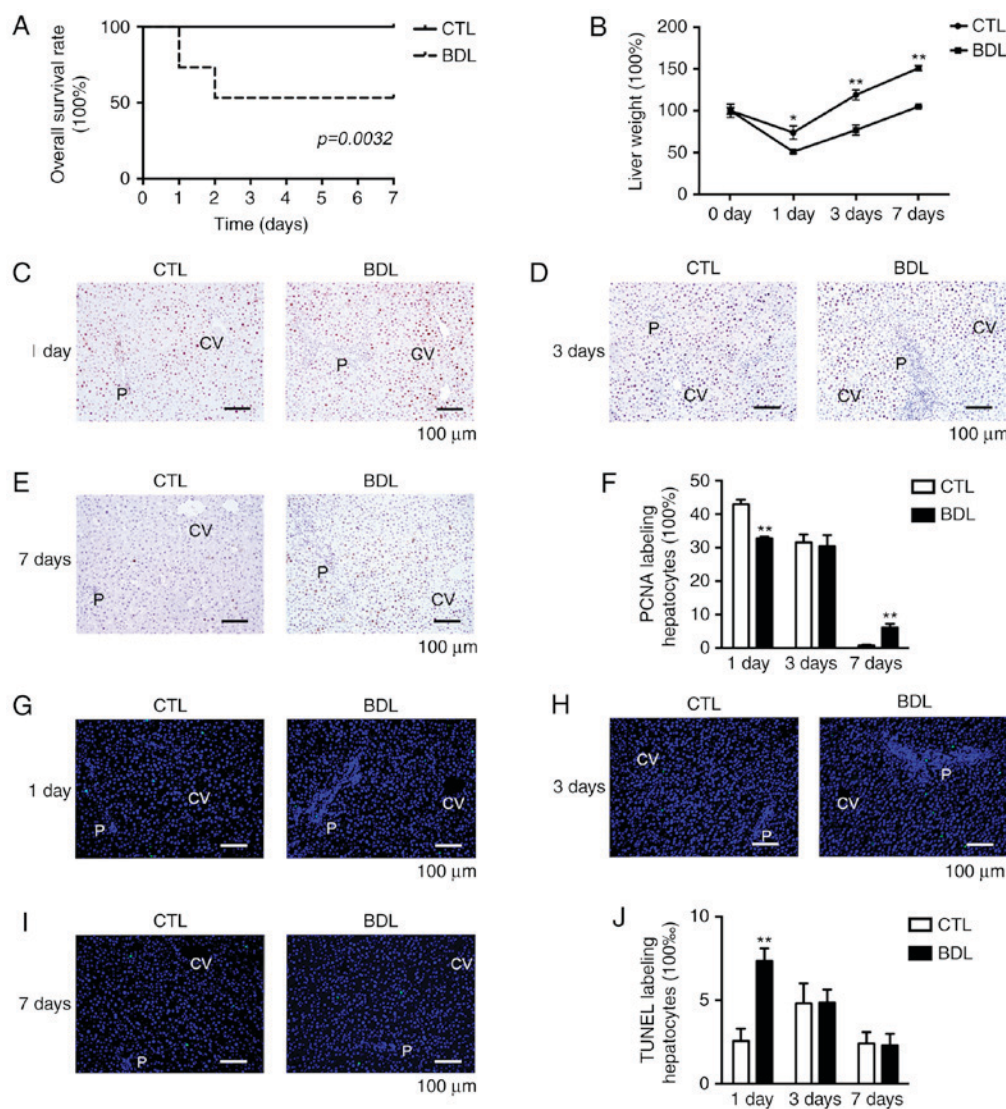


Figure 4. Obstructive cholestasis leads to impaired liver regeneration and mortality. (A) Decreased overall survival was observed in BDL group (56 vs. 100% within the CTL group). (B) Restoration of liver weight within BDL and CTL groups following PH. Hepatocyte proliferation (C) 1 day, (D) 3 days and (E) 7 days post-PH was detected by immunohistochemistry. (F) Percentage of PCNA-positive hepatocytes. Apoptosis of hepatocytes was measured (G) 1 day, (H) 3 days and (I) 7 days post-PH by TUNEL assay. (J) Percentage of TUNEL-positive hepatocytes. Data are expressed as the mean  $\pm$  standard deviation,  $n=16$ . \* $P<0.01$  and \*\* $P<0.05$ . BDL, ligation of common bile duct; CTL, control; CV, central vein; P, portal area; PCNA, proliferating cell nuclear antigen; PH, partial hepatectomy; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling.

for observed mortalities. The capacity of liver mass restoration was significantly impaired post-PH (Fig. 4B); therefore, post-PH liver failure may result from an impaired capacity for liver regeneration. LW of each time point post-PH was normalized to the pre-PH value.

Suppression of liver mass restoration may be associated with abnormal hepatocellular proliferative and apoptotic activities following PH. Compared with in the CTL group, the PCNA labeling index was markedly decreased at 1 day within the BDL group (43.2 vs. 33.7%; Fig. 4C-F). Analysis of hepatocyte apoptotic ability demonstrated a significant increase within the BDL group on day 1 compared with in the CTL group (2.5 vs. 7.2%; Fig. 4G-J). However, as apoptotic ability was weak between the groups, other factors may contribute to the inhibition of liver mass regeneration following PH. Therefore, extra-cholestasis may result in a reduction in overall survival and impaired liver mass restoration in response to early stage hepatocyte proliferation inhibition.

*Reduced accumulation of TG and altered cytokine and growth factor expression inhibits early stage post-PH-hepatocyte proliferation.* The process of liver regeneration is tightly regulated by cytokines, growth factors and metabolic factors (25). The expression levels of TNF- $\alpha$ , IL-6, HGF and TGF- $\beta$ 1 were analyzed by RT-qPCR. The results of the present study revealed a decrease in TNF- $\alpha$ , IL-6 and HGF expression levels on day 1 within the BDL group (Fig. 5A-C). Significant decreases in TGF- $\beta$ 1 expression levels were observed on days 3 and 7 within the BDL group (Fig. 5D), which may be associated with increased hepatocyte proliferative ability at day 7 in the BDL group (Fig. 4E and F). Inflammation infiltration was decreased on days 1, 3 and 7 in the BDL group. In addition, compared with the CTL group, TG accumulation was inhibited on days 1 and 3 within the BDL group (Fig. 5E-G). The findings of the present study were confirmed by hepatic TG analysis (Fig. 5H), which indicated that energy supplies were insufficient during the

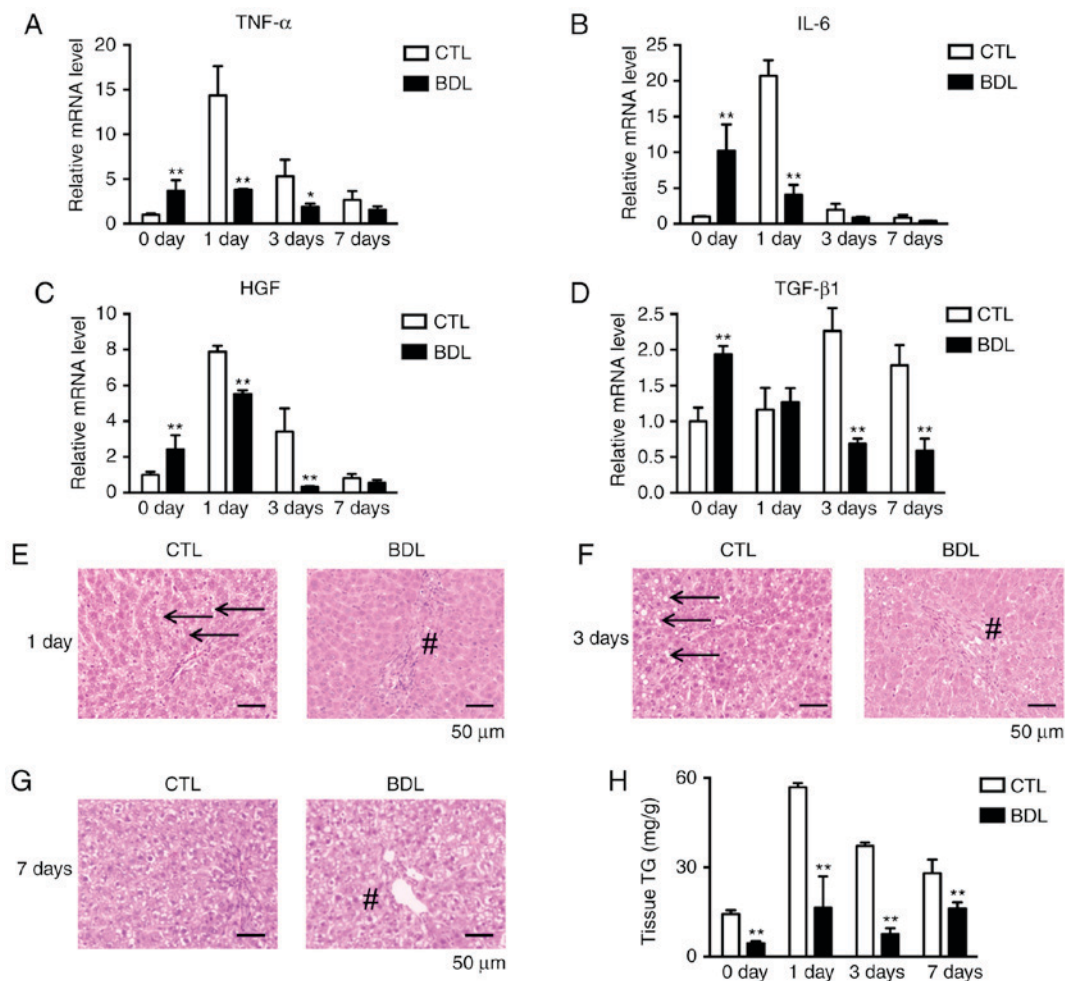


Figure 5. Cytokine, growth factor and metabolic factor expression levels associated with hepatocyte proliferation were evaluated pre- and post-PH. Quantification of hepatic (A) TNF- $\alpha$ , (B) IL-6, (C) HGF and (D) TGF- $\beta$  expression levels at the indicated time points pre- and post-PH. Expression was normalized to  $\beta$ -actin expression and calculated as the fold change compared with in the CTL group pre-PH. \* $P < 0.05$  and \*\* $P < 0.01$ . Morphological analysis of the CTL and BDL groups at (E) 1 day, (F) 3 days and (G) 7 days. Normal TG deposition was decreased post-PH (arrows) within the BDL group; biliary hyperplasia and inflammatory infiltration (#) in the portal area were decreased post-PH. (H) TG quantification. \*\* $P < 0.01$ . Data are expressed as the mean  $\pm$  standard deviation. BDL, ligation of common bile duct; CTL, control; IL-6, interleukin-6; HGF, hepatocyte growth factor; PH, partial hepatectomy; TG, triglycerides; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; TNF $\alpha$ , tumor necrosis factor- $\alpha$ .

liver regeneration process. Therefore, an insufficient energy supply and reduced TNF- $\alpha$ , IL-6 and HGF expression may be associated with impaired capacity of early stage liver regeneration.

*Overactivation of FXR signaling is associated with reduced TG accumulation post-PH within the BDL group.* TG metabolism is mediated by fatty acid metabolism, which mainly constitutes fatty acid synthesis and  $\beta$ -oxidation. The present study aimed to investigate the effect of TG metabolism in extra-cholestasis on impaired liver regeneration. Fatty acid  $\beta$ -oxidation is regulated by PPAR $\alpha$  and its target gene, CPT1A. RT-qPCR analysis revealed a significant decrease in PPAR $\alpha$  and CPT1A at 3 and 1 day post-PH, respectively (Fig. 6A and B), which was associated with reduced fatty acid utilization in early stage liver regeneration. In addition, the expression levels of fatty acid-synthesis regulators, including SREBP-1c, FASN and ACC1 were analyzed. SREBP-1c expression levels were inhibited at 0 and 1 day post-PH (Fig. 6C), which was associated with impaired *de novo* lipogenesis within the BDL group. Expression

levels of FASN and ACC1 were reduced during early stage regeneration following PH (Fig. 6D and E). Decreased SREBP-1c activity was associated with reduced tissue TG content and therefore reduced fatty acid utilization within the BDL group.

FXR signaling is required to mediate bile acid cytotoxicity induced by BDL (26,27) and serves as a critical regulator of TG metabolism by regulating SREBP-1c activity. In the present study, FXR signaling was analyzed via western blot analysis (Fig. 6F). Expression levels of SHP and FXR (Fig. 6G and H) were significantly increased 0 and 1 day post-PH; therefore, FXR signaling overactivation post-PH within the BDL group may account for impaired SREBP-1c activity. The findings of the present study indicated that obstructive cholestasis may impair liver regeneration due to altered cytokine and growth factor expression, and an insufficient energy supply. In addition, inhibition of the FXR signaling pathway may be associated with reduced TG accumulation during liver regeneration and impaired hepatocyte proliferation within obstructive cholestatic liver tissue.

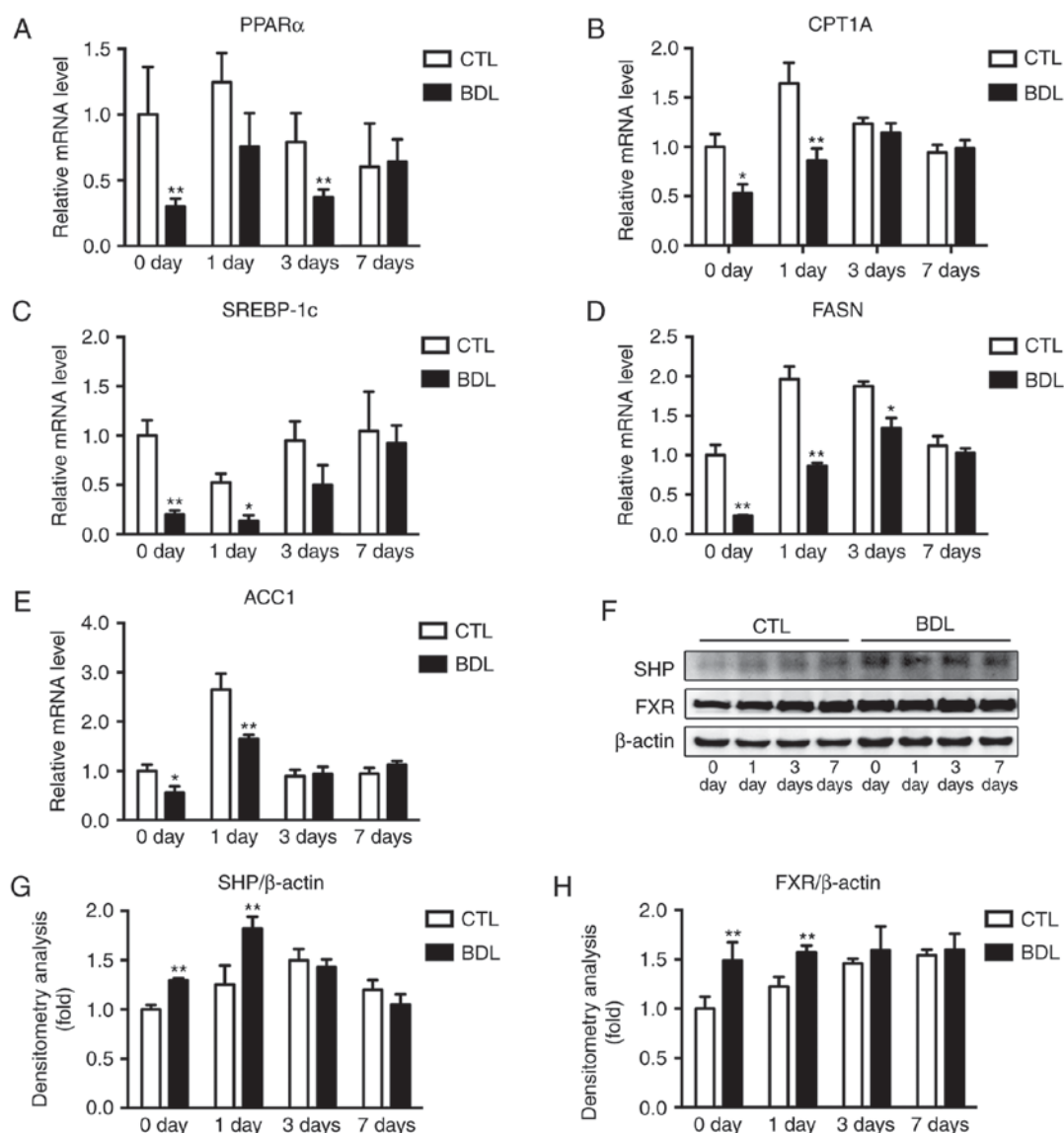


Figure 6. Fatty acid synthesis and  $\beta$ -oxidation, and FXR signaling were detected pre- and post-PH. Quantification of hepatic (A) PPAR $\alpha$ , (B) CPT1A, (C) SREBP-1c, (D) FASN and (E) ACC1 expression levels pre- and post-PH within the BDL and CTL groups. Expression levels were normalized to  $\beta$ -actin and calculated as the fold change compared with in the CTL group pre-PH. \* $P < 0.05$  and \*\* $P < 0.01$ . (F) Western blotting and (G and H) densitometry analyses of SHP and FXR were performed using the indicated antibodies pre- and post-PH. \* $P < 0.05$  and \*\* $P < 0.01$ .  $\beta$ -actin was used as the internal control. Expression levels were normalized to  $\beta$ -actin expression and calculated as the fold change compared with in the CTL group pre-PH. Data are expressed as the mean  $\pm$  standard deviation and calculated as the fold change. ACC1, acetyl-CoA carboxylase 1; BDL, ligation of common bile duct; CTL, control; FASN, fatty acid synthase; FXR, farnesoid X receptor; PPAR $\alpha$ , peroxisome proliferator activated receptor  $\alpha$ ; PH, partial hepatectomy; SHP, small heterodimer partner; SREBP-1c, sterol-regulatory element-binding protein-1c.

## Discussion

Liver failure is a common cause of postoperative mortality following major hepatectomy. Such surgical treatments are performed in cases of PHCC, which is characterized by malignant obstructive cholestasis and often requires major hepatectomy to cure (1,28). Extra-cholestasis has been reported to be a poor prognostic risk factor in postoperative analysis; however, the resection rate in PHCC is too low to investigate whether extra-cholestasis or cholangiocarcinoma affects overall survival (9). In addition, preoperative biliary drainage as a benefit for patients with PHCC remains controversial (29). Further investigation into whether cholestasis independently affects prognosis is required. In the present study, a decrease in overall survival rate was observed within the BDL group

following PH; therefore, severe cholestasis status may have independently affected overall survival. In order to reduce risks associated with cholestasis, appropriate measures, such as preoperative internal biliary drainage may be conducted (1). Preliminary studies demonstrated that biliary drainage itself did not have an effect on 7 day post-operation survival (data not shown).

Liver failure following major hepatectomy is mainly caused by impaired liver regeneration. The effect of obstructive cholestasis on liver regeneration post-PH was analyzed in the present study based on the BDL rat model; however, clinical significance based on various obstructive durations, liver sectioning extents and biliary drainage has not been reported in previous studies (12-15,30). The present study compared morphological alterations within patients with PHCC and BDL

rat models. PH and internal biliary drainage were performed to attain the clinical condition within rat models. Severe obstructive cholestasis was confirmed to impair liver regeneration. A previous study revealed that alterations in cytokine and growth factor expression levels affected liver regeneration within extra-cholestatic liver tissue (15). However, the role of metabolic factors in this process remains to be investigated, as previous studies have reported that cholestasis may influence systematic and hepatic TG metabolism, and restoration of hepatic energy stores (31,32). In the present study, the capacity of liver mass regeneration was inhibited within the BDL group. In addition to energy supplies, alterations in the expression levels of cytokines and growth factors, including TNF $\alpha$ , IL-6 and TGF- $\beta$ 1, suppressed hepatocyte proliferation. To the best of the authors' knowledge, the present study is the first to demonstrate a potential mechanism underlying cholestasis-induced impairments in liver regeneration, besides the altered expression of cytokines and growth factors (6). A previous study indicated that the application of  $\omega$ -3 fatty acids and the content of enteral nutritional suspensions, such as total protein-medium chain triglycerides, promoted liver regeneration within normal rats (33). In the clinical setting, whether enteral nutritional suspensions improve severe cholestasis-associated impairments post-PH remains to be investigated. A previous study indicated that deletion of the FXR gene within mice may reverse obstructive cholestasis-induced liver injury (26). In the present study, overactivated FXR signaling was also observed pre-PH and at 1 day post-PH. Therefore, FXR antagonists may serve to improve obstructive cholestasis-induced liver injury and promote liver regeneration following PH within extra-cholestatic liver tissue.

In conclusion, the results of the present study established the role of TG metabolism in impaired hepatocyte proliferation within cholestatic livers; impaired liver regeneration was induced by obstructive cholestasis due to altered cytokine and growth factor expression levels, and reduced energy supplies. Furthermore, a decrease in TG accumulation due to FXR-signaling overactivation may contribute to impairments in liver regeneration post-PH within extra-cholestatic liver tissue.

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