Original Paper

Enhanced Liver Regeneration After Partial Hepatectomy in Sterol Regulatory Element-Binding Protein (SREBP)-1c-Null Mice is Associated with Increased Hepatocellular Cholesterol Availability

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Key Words

Liver regeneration • Srebp-1c-/- mice • Cholesterol • Triglyceride

Abstract

Background/Aims: Transient lipid accumulation within hepatocytes preceding the peak proliferative process is a characteristic feature of liver regeneration. However, molecular mediators responsible for this lipid accumulation and their functions are not well defined. Sterol regulatory element-binding proteins-1c (SREBP-1c) are critical transcriptional factors that regulate lipid homeostasis in the liver. We hypothesized that SREBP-1c deficiency induced alterations of lipid metabolism may influence hepatocyte proliferation and liver regeneration.

Methods: 2/3 partial hepatectomy (PH) was performed in wild type C57BL/6J (WT) and *Srebp-1c*-/- mice. The lipid contents in serum and liver were measured by enzymatic colorimetric methods. Hepatic lipid droplets were detected by Oil Red O staining and immunohistological staining. Hepatic expression of genes involved in lipid metabolism and cellular proliferation was determined by real-time PCR and/or immunoblot. Hepatocyte proliferation and liver regeneration were assessed by BrdU staining and the weight of remanent liver lobes in *Srebp-1c*-/- mice, respectively. **Results:** *Srebp-1c*-/- mice displayed reduced triglyceride and fatty acids but increased cholesterol in the liver before PH. In response to PH, hepatocellular DNA synthesis was elevated and cell cycle progression was prolonged in *Srebp-1c*-/- mice, which was associated with enhanced liver regeneration. However, *Srebp-1c*-/- mice had comparable

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triglyceride and fatty acid contents and expressions of related genes compared with WT mice during the liver regeneration. In contrast, SREBP-1c-deficiency-induced alteration of cholesterol metabolism was retained during the liver regeneration after PH. *Srebp-1c*^{-/-} mice exhibited higher cholesterol contents and enhanced expression of SREBP-2 and 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR) in the liver than WT mice after PH. Moreover, downregulation of genes involved in cholesterol elimination was observed after PH in *Srebp-1c*^{-/-} mice. **Conclusion:** SREBP-1c deficiency in mice did not interfere with triglyceride and fatty acid metabolism but was associated with significant changes in cholesterol profiles during liver regeneration after PH. These results suggest that increased hepatocellular cholesterol storage and cholesterol availability with the enhanced liver regeneration are identified in *Srebp-1c*^{-/-} mice. This study also shows that providing requisite cholesterol levels to proliferating hepatocytes and keeping appropriate cholesterol metabolism are required for normal liver regeneration.

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Introduction

Mammalian liver regeneration is quickly activated upon loss of liver mass, even 2/3 partial hepatectomy (PH) occurs [1]. It has long been recognized that in early stages of liver regeneration hepatocytes transiently accumulate significant amounts of lipid droplets, which are mainly composed of triacylglycerol (TG), cholesteryl esters (CE) and fatty acids [2-6]. Furthermore, hepatic mRNAs during early PH-stimulated liver regeneration are highly related to transient steatosis [7, 8]. The proliferative response surges within the first 3 days, and the liver mass is restored within a week in rodents [9, 10]. Therefore, hepatic lipids are considered to be critical for the process as cholesterol acts as the source of the new cell membrane [11] while TGs and FAs are energy sources required for hepatocyte proliferation [7, 12]. However, specific molecular mechanisms that regulate transient hepatic steatosis are not well defined. How alteration of hepatic lipid contents, especially levels of hepatic TGs affecting proliferation are still controversial. Either insufficient or excessive hepatic TG contents may have negative effects on the normal process of liver regeneration (Saw review [13]). In addition, cholesterol as a structural lipid is essential for completion of mitosis which is critical for cellular proliferation [14]. Promoters of sterol-regulated genes include sterol regulatory element (SRE), which is bound by sterol regulatory element-binding protein (SREBP) transcriptional factors in response to lipid alteration. SREBPs regulate more than 30 genes encoding proteins that mediate the synthesis and uptake of cholesterol as well as unsaturated fatty acids, TG or phospholipids [15, 16].

Three isoforms of SREBPs are found in mammalian cells. SREBP-1a and SREBP-1c are translated from a single gene with alternate promoters in which transcripts having different first exons. SREBP-2 is encoded by a separate gene. It is well known that SREBP proteins are initially bound to the rough endoplasmic reticulum (ER) membrane (Pre-SREBP). On sterol deprivation, SREBP is cleaved to liberate the N-terminal portion (nSREBP) and enters the nucleus where it binds to its target genes and activates their expressions related to lipid biosynthesis and uptake. SREBP-1a is a potent activator of all SREBP-responsive genes including those that mediate the synthesis of cholesterol, fatty acids, and triglycerides. While roles of SREBP-1c and SREBP-2 are more restricted than that of SREBP-1a. SREBP-1c, the predominant SREBP-1 isoform in adult liver, preferentially enhances expressions of genes required for synthesis of fatty acids but not cholesterol. Whereas SREBP-2 preferentially activates genes required for cholesterol synthesis [17-19]. As the critical roles of lipid on liver regulation, the roles of SREBPs on hepatocyte proliferation have not been intensively explored. However, SREBP-1a and SREBP-2 are required for normal embryogenesis [20], while selective disruption of SREBP-1c does not cause embryonic lethality. In order to understand the uncertain relationship between lipid regulation and liver regeneration, the roles of SREBP-1c in the liver and hepatic lipids handling in regenerative livers, we studied the lipid dynamics and the regenerative response to 2/3 PH in SREBP-1c-null mice.

Materials and Methods

Experimental animals

C57BL/6J mice and SREBP-1c-null mice (*Srebp-1c*^{-/-}) were purchased from The Jackson Laboratory. These *Srebp-1c*^{-/-} mice own a total ablation of the SREBP-1c transcript and show only a slight reduction in the level of the SREBP-1a transcript. All experimental procedures were carried out under a protocol approved by the Animal Care and Use Review Committee of Shenzhen University Health Science Center. The study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

Animal surgery

All mice were male with 8-10 weeks of age, which were kept on 12 h dark-light cycle and maintained on standard chow and water before and after the surgery. According to the technique described by Mitchell C and Willenbring H [21], two-thirds of the liver (median and left lobes) was removed. Surgery was performed between 9:00 and 11:00 AM to minimize the effect of diurnal variations on regeneration. To identify the SREBPs alteration and the systemic metabolic response to PH, we first determined the chronology of biochemical and molecular parameters at various time points after PH in C57BL/6J mice. 96 healthy male mice were randomly divided into 12 groups: 6 PH groups and 6 sham-operation (Sham) groups. Each group consisted of eight mice. The sham groups were subjected to the same procedure as the PH groups without the liver removal. After the surgery, the mice were sacrificed at different time points: 0, 0.5, 1, 2, 3 and 7 day(s), respectively. Then we performed PH in *Srebp-1c*^{-/-} mice (KO, n=36) and its wild type (WT, n=40). The mortality rate is less than 5% for both the WT and *Srebp-1c*^{-/-} mice. After PH, the mice were killed at 0, 1, 2, 3 and 7 day(s) and the remnant liver tissues were harvested. BrdU (50 mg/kg body weight) was administered intraperitoneally 2 h prior to tissue harvest [22]. The regenerative bottom right lobe was snap-frozen into liquid nitrogen and stored in -80°C for further analysis. For histological analysis, the upper right lobe was fixed in 4% neutral paraformaldehyde (PFA) at 4°C and then cryoprotected overnight in 20% sucrose solution or embedded in Optimal Cutting Temperature medium for future processing. The percentage of liver regeneration was calculated following the formula: Percentage of liver regeneration = weight of non-removed lobes/total body weight of mice × 100% [23].

Serum chemistry assays

Serum was separated by centrifugation after the sacrifice of mice. Serum glucose levels were measured by glucose oxidase method. Serum triglycerides (TG), total serum cholesterol (TC), cholesteryl esters (CE) and free cholesterol (FC), serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were all determined by enzymatic colorimetric methods using commercial kits (BioSino, Inc., China). Serum insulin levels were measured by ELISA (American Laboratories Product Co., USA) via following manufacturer's instructions. Free fatty acids (FFAs) were measured by commercial kits (Wako Pure Chemical Industries, Ltd., Japan).

Hepatic lipid assays

For determination of liver lipids, 50 mg aliquot of the liver was homogenized and then lipids were extracted. Levels of FFA in liver tissues were determined using a serum FFA determination kit (Wako Pure Chemical Industries, Ltd., Japan). Liver contents of TG, TC, CE, and FC were assayed using a tissue triglyceride and cholesterol assay kit (Applygen Technologies Inc., China), respectively.

Liver histological analysis

For the visualization of hepatic lipid content, 10 µm frozen sections were rehydrated and lipid droplets deposition was detected by Oil Red O-staining (Sigma-Aldrich, St Louis, USA) with a standard procedure. For H&E and immunohistological staining, the PFA-fixed tissues were sectioned at a thickness of 3 µm. Then the sections were stained with the BrdU labeling for BrdU positive nuclei by detection kit (Invitrogen Corporation, CA, USA) according to manufacturer's instructions. Hepatocellular nuclear BrdU labeling and mitoses were quantified by examination of at least three random 200 × fields with at least 300 cells and nuclei in each tissue section via light microscopy (Nikon, ECLIPSE Ci, Japan). Data analysis was conducted by using the morphometry software ImageJ.

RNA isolation and quantitative real-time PCR

Total RNAs were extracted from frozen liver tissue using TRIzol reagent (Invitrogen, USA) and then purified. Purified total RNA concentrations were determined using a Nanodrop LITE spectrophotometer at 260/280 nm or 260/230 nm ratios (Thermo Scientific, USA). Total RNA integrity was checked by electrophoresis on agarose gel. cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). For quantitative real-time PCR, cDNA was amplified using qTOWER 2.2 System (Analytik Jena, German) with SYBR Green Master Mix (TransGen Biotech, China). mRNA levels were normalized to beta-2 microglobulin (B2m) which is commonly used as a reference gene in analyses of regulation of gene expressions during liver regeneration [24]. It exhibits expression pattern homologous to other genes used for this purpose (e.g. beta-actin [25] and glyceraldehyde-3-phosphate dehydrogenase [26]). The standardized data was further used to calculate fold-differences in gene expressions. Specific primers were designed from sequences available in GeneBank and synthesized by Invitrogen (Guangzhou, China). Some gene primers were: B2m: 5'-CTCGGTGACCCTGGTCTTTC-3' and 5' GGATTCAATGTGAGGCGGG-3', beta-actin: 5'-TGTTACCAACTGGGACGACA-3' and 5'- GGGGTGTTGAAGGTCTCAAA- 3', SREBP-1a: 5'- ACTTTTCCTTAACGTGGGCCT-3' and 5'- CATCTCGGCCAGTGTCTGTT- 3', SREBP-1c: 5'- CCTGACAGGTGAAATCGGCG- 3' and 5'- TGTCTCACCCCGAGCATAGG-3', SREBP-2: 5'-AGCTGGGCGATGGATGAGAG-3' and 5'- TCAGGGAACCTCCCCTTGA-3'. Other special primers were not shown here.

Western blotting

Total proteins were extracted from frozen liver using RIPA lysis buffer (Applygen Technologies Inc., China) supplemented with a protease and phosphatase inhibitor cocktail (Thermo Scientific, USA). Protein concentrations were quantitated by using BCA assay (Thermo Scientific, USA). After that, proteins were denatured and prepared for western blot. Equivalent protein amounts were resolved electrophoretically on a 10% sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk in Tris-buffered saline (0.1% Tween-20) for 1 h. Blots were incubated overnight at 4°C with primary antibodies, followed by incubation with a horseradish peroxidase-conjugated secondary antibodies (ZSGB-BIO, Inc., China). Signals were visualized using an enhanced chemiluminescence detection system (ImageQuant LAS 400 mini, GE, Japan). Protein band densities were analyzed by ImageJ software. The expression of beta-actin was used as internal standard. All primary antibodies were bought from commercial corporations (Cell Signaling Technology, Abcam and Santa Cruz Biotechnology, USA).

Statistical analysis

Statistical analysis was conducted using SPSS 16.0 software. All values were expressed as means $\pm$ S.E. Difference between means was analyzed by using Student's t-test. A difference was considered significant when $p < 0.05$.

Results

Cholesterol and triglyceride profiles during liver regeneration in C57BL/6J mice

Systemic metabolic responses to PH, total body mass, remnant liver mass, biochemical and molecular parameters were measured at serial time points after surgery in wild type mice. Our initial characterization of serum and hepatic biochemical profiles and gene expression patterns after PH in the wild type mice recapitulated previous studies (see review [13]) (Fig. 1-2, (for all online suppl. material, see www.karger.com/doi/10.1159/000490030) Fig. S1-S4). Briefly, the maximum loss of body weight occurred 12 h after surgery with subsequent recovery to baseline about 1 week (Fig. 2A). From 12 h to 1 day after surgery, accumulation of lipids including TG and CE was developed in the regenerating mouse liver which was then returned to baseline two days after PH (Fig. 2C and D). Coincidentally, the maximum loss of the mass of epididymal and subcutaneous adipose tissues occurred 1 day after PH (see online suppl. material, Fig. S1 B,C). However, the contents of hepatic free cholesterol were dramatically decreased to the minimum level one day after PH (Fig. 2 F). Interestingly, mice subjected PH had much lower levels of serum glucose and triglyceride through the regenerative

process, compared with the sham group. In the meantime, levels of total cholesterol in serum were rapidly reduced 12 h after liver resection which was recovered to the equal level of the sham group two days after surgery (Fig. 1B). It has a higher probability that lipid alteration provided energy and materials to cell proliferation which were necessary for liver regeneration. As rapid decrease of free fatty acids and recovery of free cholesterol in regenerative livers two days after PH (Fig. 2E and F), peak hepatocellular DNA replication and mitotic progression occurred concomitantly 2-3 days after liver resection (see online suppl. material, Fig. S2, Fig. 3A, Fig. 4C). In addition, the maximum recovery of liver function to baseline occurred 2-3 days after PH (Fig. 1E and F). Thus, early lipid accumulation after PH could be beneficial for liver regeneration.

Expressions of SREBP-1 and genes involved in triglyceride and fatty acid metabolism during liver regeneration

SREBP-1 play critical roles in lipid metabolism in the mammalian liver. We first examined expression patterns of two forms of SREBP proteins in the total liver homogenates from wild type mice subjected to PH. We found that the level of total SREBP-1 protein was largely decreased from 12 h to 1 day after operations in both shams operated and PH mice. SREBP-1 was recovered from 1-2 days after surgery in these two groups of mice. Furthermore, the level of SREBP-1 in 12 h -1 day after surgery was

Fig. 1. Serum biochemical parameters during PH-induced liver regeneration in wild type mice. (A) Serum TG, (B) Serum TC, (C) Serum FFA, (D) Serum Glucose, (E) Serum alanine aminotransferase activity and (F) Serum aspartate aminotransferase activity of wild type mice at indicated time points after sham-operation or PH (n = 6-8 at each time point). Results were shown as mean $\pm$ S.E. *P<0.05, **P<0.01 for sham-operated mice vs. PH mice at each time point.

Fig. 2. Hepatic lipid contents during PH-induced liver regeneration in wild type mice. (A) Liver to body weight ratio of wild type mice at indicated time points after sham-operation or PH (n = 6-8 at each time point). (B) Total cholesterol, (C) TG, (D) CE, (E) free fatty acids, (F) free cholesterol in livers of wild type mice at indicated time points after sham-operation or PH (n = 6-8 at each time point). Results were calculated as mean $\pm$ S.E. *P<0.05, **P<0.01 for sham-operated mice vs. PH mice at each time point.

almost undetectable in livers from mice with PH, compared with the same group. However, the level of SREBP-1 protein in the regenerative liver went up in 2 days after PH which was much higher than the sham group of mice (Fig. 3A). Moreover, mRNA expression patterns of SREBP-1c and SREBP-1a and their target genes involved in lipogenesis including fatty acid synthase (FAS), acetyl-Coenzyme A carboxylase (ACC)-1 and stearoyl-Coenzyme A desaturase (SCD)-1 correlated with SREBP-1 protein expression after the surgery (Fig. 3B; see online suppl. material, Fig. S3 A-C). Expressions of genes involved in lipolysis and beta-oxidation in the liver from mice subjected to PH were significantly higher than those of the sham operated mice through all the time (see online suppl. material, Fig. S3 D-G).

Expressions of SREBP-2 and genes involved in cholesterol metabolism during liver regeneration

Next, we investigated the expression of SREBP-2 and genes involved in cholesterol biosynthesis, transport, and catabolism. The protein level of SREBP-2 in the regenerative liver was markedly higher 1 day after PH than that of the sham mice (Fig. 3A). However, the mRNA level of SREBP-2 was different from the protein level at the corresponding time point (Fig. 3B). Moreover, the mRNA level of 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR), the key enzyme of cholesterol synthesis, was similar to SREBP-2 during the regeneration (see online suppl. material, Fig. S4 A). The expression of HMGCR was significantly increased 2 days after PH when maximum hepatocellular DNA replication was reached, as indicated by a DNA synthesis marker--proliferating cell nuclear antigen (PCNA) (Fig. 3A). In the meantime, real-time PCR showed that LXR-targeted genes, the ATP-binding cassette sub-family G (ABCG)-5/8, which play key roles in cholesterol secretion were dramatically down-regulated after PH (see online suppl. material, Fig. S4 E). Our results indicated that cholesterol biosynthesis was stimulated during hepatocellular DNA replication whereas cholesterol catabolism is repressed which was in agreement with previous studies [3]. Taken together, profiles of the systemic and hepatic lipids and expressions of lipid-related genes (see online suppl. material, Fig. S3 and S4) during normal regeneration revealed that alteration of lipid levels was enough to supply to DNA replication and mitosis of remnant hepatocytes. As regeneration proceeds, these perturbations in hepatic and systemic metabolism resolve.

Fig. 3. Expression levels of SREBPs during liver regeneration in wild type mice. (A) Western blotting of the precursors of SREBPs in total liver homogenates of bottom right liver lobes of wild type mice at indicated time points after sham-operation or PH (n = 4 at each time point). (B) Quantitative RT-PCR analysis of mRNA levels of three isoforms of SREBPs in wild-type mice at indicated time points after sham-operation or PH. (n = 6-8 at each time point). Results were shown as mean ± S.E. *P<0.05, **P<0.01 for sham-operated mice vs. PH mice at each time point.

Fig. 4. Hepatocyte proliferation and liver regeneration in response to PH were enhanced in *Srebp-1c*^{-/-} mice. (A) Liver to body weight ratio of wild type (WT) or *Srebp-1c*^{-/-} mice (KO) at indicated time points after PH (n = 5-8 at each time point). (B) Mean body weight of WT or *Srebp-1c*^{-/-} mice (KO) before and after PH. (C) Immunohistochemical analysis of BrdU incorporation at 1, 2, 3 and 7 day(s) post-PH in wild-type or *Srebp-1c*^{-/-} mice (KO). Three random magnification 200 × fields (n = 4) were obtained. (D) Western blotting of cell proliferation related factors in total liver homogenates of bottom right liver lobes from wild type (WT) or *Srebp-1c*^{-/-} mice (KO) at indicated time points after PH (n = 4-6 at each time point). (E) Percentage of BrdU positive cells in wild-type or *Srebp-1c*^{-/-} mice (KO) at indicated time points after PH. Results were shown as mean ± S.E. *P<0.05, **P<0.01 for WT mice vs. KO mice at each time point.

Srebp-1c deficiency enhanced hepatocyte proliferation and liver regeneration in response to PH

Considering the dynamical alteration of lipid profiles and SREBPs during liver regeneration, we decided to use *Srebp-1c*^{-/-} mice to dissect the role of SREBPs in hepatic lipid dynamics and cell proliferation during liver regeneration in response to PH. We performed 2/3 PH in wild type and *Srebp-1c*^{-/-} mice. Mice were sacrificed at 0, 1, 2, 3 and 7 day(s) after the surgical procedure. Wet liver remnant weight, together with the total body weight, were used to calculate the hepatic regenerative rate. In WT mice, the liver to body weight ratios augmented from 1.9% at day 1 to 2.5% at day 3 after PH, while the upward trend in hepatic regenerative rate was 1.8% at day 1 to 3.1% at day 3 after PH in *Srebp-1c*^{-/-} mice. SREBP-1c gene deficiency was associated with enhanced liver regeneration from day 2 to day 3, especially at day 3 after PH, compared to WT mice (Fig. 4A). To further investigate the association between deficiency in SREBP-1c and enhanced liver regeneration, we studied expression levels of the cell cycle and proliferative markers (Cyclin B1, Cyclin D1, Cyclin A, and PCNA) and analyzed liver sections by BrdU in WT and *Srebp-1c*^{-/-} mice. In WT mice, BrdU positive nuclei were detectable at day 1 (5.3% per total nuclei) and peaked at day 2 (29.4% per total nuclei). After that, the value reduced markedly at day 3 to day 7 (6.9%-3.6% per total nuclei) post-PH. Interestingly, BrdU incorporation was increased by 8.1% at day 1 and 40% at day 2 post-PH and then it was maintained at a significantly higher positive ratio (26.2% per total nuclei) at day 3 in *Srebp-1c*^{-/-} mice, compared with that in WT mice at the same time point (Fig. 4C,E). These results were suggestive of prolonged cell-cycle progression, increased DNA replication and enhanced hepatocellular proliferation in *Srebp-1c*^{-/-} mice subjected to PH. Correspondingly, the induction of Cyclin B1, key mediators of cell cycle progression at G2/M [27], were significantly increased in remnant livers of *Srebp-1c*^{-/-} mice after 2 or 3 days of PH compared with WT mice. Similarly, the expression of PCNA,

Fig. 5. Levels of triglyceride, fatty acids and associated gene alterations in *Srebp-1c*^{-/-} mice or WT mice during liver regeneration after PH. (A) Serum TG, (B) Serum FFA, (C) Serum glucose were quantified in wild type mice (WT) or *Srebp-1c*^{-/-} mice (KO) at indicated time points after PH (n = 5-8 at each time point). (D) Microscopic examination of oil-O-red stained liver sections from wild type mice (WT) or *Srebp-1c*^{-/-} mice (KO) at indicated time points after PH. Three random magnification 200 × fields (n = 4) were obtained. (E) Western blotting of FAS, pACC, ACC and ADRP in total liver homogenates of bottom right liver lobes in wild type (WT) or *Srebp-1c*^{-/-} mice (KO) at indicated time points after PH (n = 4 at each time point). (F) Hepatic contents of TG and (F) FFA in wild type mice (WT) or *Srebp-1c*^{-/-} mice (KO) at indicated time points after PH (n = 5-8 at each time point). Results were shown as mean ± S.E. *P<0.05, **P<0.01 for WT mice vs. KO mice at each time point.

the marker of proliferating hepatocytes, was also significantly higher in *Srebp-1c*^{-/-} mice compared with WT mice after 2 or 3 days of PH. Expressions of cyclins A and the relevant cyclin-dependent kinase 2 (CDK2), which mediates cell-cycle at G1 and G1/S, were higher at day 1 post PH in *Srebp-1c*^{-/-} mice than WT mice (Fig. 4D and see online suppl. material, Fig. S6). Cyclin D1, one of the mediator of the cell cycle at G1, also showed higher levels at day 3 after PH in *Srebp-1c*^{-/-} mice (Fig. 4D), although the expression of the matched CDK4 was no differences between these two groups (see online suppl. material, Fig. S6). Thus the current data indicated that *Srebp-1c*^{-/-} mice showed enhanced hepatocyte proliferation and liver regeneration in response to PH.

SREBP-1c deficiency did not impact the expressions of cyclin-dependent kinase inhibitors p21 and p27 during liver regeneration after PH

It has reported that SREBPs could impact cell growth and cell cycle through direct acting on the expression of the universal inhibitors of cyclin-dependent kinases p21 and p27 [28-30]. Then we examined whether expressions of cyclin-dependent kinase inhibitors in livers of *Srebp-1c*^{-/-} mice after PH were different from WT mice. As showed in Fig. S6, the protein levels of p21 and p27 were higher in the liver of *Srebp-1c*^{-/-} mice before PH, compared with WT mice. CDK2 and CDK4 were essentially increased through the whole regeneration process, while p21 and p27 were in reduction at day 1 post PH then went up 1-2 day(s) after PH in both kinds of mice. Although the expressions of p21 and p27 kept slightly higher at day 1 post PH in *Srebp-1c*^{-/-} mice, we did not find any significant alterations in the levels of p21 and p27 between *Srebp-1c*^{-/-} mice and WT mice during the liver regeneration 2 days after PH.

SREBP-1c deficiency did not interfere with triglyceride or fatty acid metabolism during liver regeneration after PH. SREBP-1c is a predominant subtype of SREBP-1 in the liver which mainly favors for fatty acid and triglyceride synthesis [31]. To confirm whether the absence of SREBP-1c affect triglyceride and cholesterol metabolism during liver regeneration after PH as well as mechanisms for enhanced liver regeneration in *Srebp-1c*^{-/-} mice, we examined lipid profiles of livers and mRNAs of rate-limiting and regulatory genes essential for lipid metabolism from WT mice and *Srebp-1c*^{-/-} mice at indicated time points after PH.

Before the liver resection, *Srebp-1c*^{-/-} mice had much lower levels of serum TG and FFA (Fig. 5A,B) while no differences in serum glucose level (Fig. 5C), compared with WT mice. Moreover, TG and FFA in the liver of *Srebp-1c*^{-/-} mice were significantly decreased (Fig. 5F,G). Correspondingly, livers of *Srebp-1c*^{-/-} mice manifested reductions in the expression of genes or proteins involved in fatty acid and triglyceride synthesis including FAS, SCD-1 (see online suppl. material, Fig. S7) and ACC (Fig. 5E). *Srebp-1c*^{-/-} mice also displayed a decrease in SREBP-1a mRNA level (see online suppl. material, Fig. S7 A). The precursor of SREBP-1 was detectable but significantly lower in *Srebp-1c*^{-/-} mice liver compared with WT mice (Fig. 6E). This suggested that SREBP-1a was normally expressed in *Srebp-1c*^{-/-} mice livers with a slight decrease of mRNA level (see online suppl. material, Fig. S7 A). However, *Srebp-1c*^{-/-} mice displayed comparable serum and hepatic TG and FFA compared with WT mice within 3 days after PH (Fig. 5A,B,F,G). Oil-red-O staining of liver sections also revealed similar contents of neutral fat in *Srebp-1c*^{-/-} mice and WT mice (Fig. 5D). Furthermore, mRNAs of SREBP-1a and *de novo* lipogenesis related genes were not different between *Srebp-1c*^{-/-} mice and WT mice (see online suppl. material, Fig. S7). We also defined the morphology of lipid droplets (LD) in remnant liver sections by H&E staining. Both *Srebp-1c*^{-/-} mice and WT mice appeared homogeneous microvesicular fat droplets 1 day after PH with reduced LD formation 2-3 days after PH (see online suppl. material, Fig. S8). However, slightly decreased levels of adipose differentiation related protein (ADRP) levels were found in *Srebp-1c*^{-/-} mice

Fig. 6. Cholesterol levels and expressions of SREBPs in *Srebp-1c*^{-/-} mice or WT mice during liver regeneration after PH. (A) Serum total cholesterol, (B) Hepatic contents of TC, (C) Hepatic contents of CE, (D) Hepatic contents of FC were quantified in wild type mice (WT) or *Srebp-1c*^{-/-} mice (KO) at indicated time points after PH (n = 5-8 at each time point). (E) Western blotting of precursors or nuclear form of SREBPs in hepatocellular homogenates of bottom right liver lobes from wild type (WT) or *Srebp-1c*^{-/-} mice (KO) at indicated time points after PH (n = 4-6 at each time point). Results were shown as mean ± S.E. *P<0.05, **P<0.01 for WT mice vs. KO mice at each time point.

at day 1 post-PH (Fig. 5E). We also found that *Srebp-1c*^{-/-} mice exhibited similar expression levels of genes involved in fatty acid beta-oxidation, and comparable TG secretion compared with WT mice within 3 days after hepatectomy (data were not shown). There was also no difference in the mass of adipose tissue alterations after PH, between WT mice and *Srebp-1c*^{-/-} mice (see online suppl. material, Fig. S9). The recuperative capacities in triglyceride and fatty acid synthesis in *Srebp-1c*^{-/-} mice during PH-induced liver regeneration may result from aggravated SREBP-1a synthesis, as the unaltered nuclear form of SREBP-1 in *Srebp-1c*^{-/-} mice livers (Fig. 6E). Given that changes in triglyceride of *Srebp-1c*^{-/-} mice after PH was not different from that in WT mice, we deduced that enhanced hepatocellular proliferation and liver regeneration in *Srebp-1c*^{-/-} mice is not related to the changes of triglyceride and fatty acid metabolism induced by the deficiency of SREBP-1c before liver resection.

SREBP-1c deficiency induce alteration of cholesterol profiles during liver regeneration after PH

Although *Srebp-1c*^{-/-} mice own a total ablation of the SREBP-1c transcript and only a slight reduction in levels of the alternate SREBP-1a transcript. There is a 50% increase in the level of SREBP-2 transcript and increases in transcripts of enzymes utilized in cholesterol biosynthesis [32]. Current data also showed there was a much higher expression of SREBP-2 and higher cholesterol contents in the liver of *Srebp-1c*^{-/-} mice (Fig. 6 and see online suppl. material, Fig. S10 A). Contrary to the triglyceride metabolism in *Srebp-1c*^{-/-} mice after PH, serum and hepatic cholesterol in un-hepatectomized *Srebp-1c*^{-/-} mice remained after liver resection. As shown in Fig. 6, total hepatic cholesterol (TC) and cholesterol ester (CE) were higher in *Srebp-1c*^{-/-} mice than WT mice 1 day after PH (Fig. 6B,C). The levels of cholesterol were gradually returned close to initial level in both groups 2 days after PH. The change of CE during liver regeneration in *Srebp-1c*^{-/-} mice was consistent with the mRNA levels of HMGCR (see online suppl. material, Fig. S10 C) and nuclear SREBP-2 (Fig. 6E). Free cholesterol (FC) in livers of *Srebp-1c*^{-/-} mice did not differ from that in WT mice 1, 2, or 3 day(s) after PH (Fig. 6D) which may be associated with altered expressions of ABCG5/8 and unstable de-esterification speed of hepatic CE in *Srebp-1c*^{-/-} mice compared with WT mice at different time points (see online suppl. material, Fig. S10).

We also measured cholesterol metabolism related-genes in *Srebp-1c*^{-/-} mice during liver regeneration. We noticed that mRNAs of Cyp7a1 and Cyp8b1, two key enzymes catalyzing bile acid synthesis from cholesterol, were significantly decreased in *Srebp-1c*^{-/-} mice before

Fig. 7. Protein levels of farnesoid X receptor (FXR) and small heterodimer partner (SHP) in *Srebp-1c*^{-/-} mice or WT mice during liver regeneration after PH. Western blotting of FXR and SHP in total liver homogenates of bottom right liver lobes from wild type (WT) or SREBP-1c^{-/-} mice (KO) at indicated time points after PH (n = 4-6 at each time point). Results were shown as mean ± S.E. *P<0.05, **P<0.01 for WT mice vs. KO mice at each time point.

and 1 day after liver resection (see online suppl. material, Fig. S10 H and I). Consistently, we also found that the direct target of farnesoid X receptor (FXR), small heterodimer partner (SHP) which is a critical negative regulatory factor of Cyp7a1 and Cyp8b1 [33], was largely increased in *Srebp-1c*^{-/-} mice during liver regeneration (Fig. 7). Therefore, we speculated that all of these results including increased nuclear SREBP-2 expression and changes of genes related to cholesterol synthesis, metabolism and secretion facilitate more appropriate liver cholesterol storage and improve cholesterol availability, which could provide requisite cholesterol to hepatocellular proliferation and mitosis after PH.

Discussion

In the present study, we repeated hepatectomy induced-transient steatosis in the liver of wild type mice. Remarkable triglyceride and cholesterol ester accumulation developed in regenerative livers from 12 h to 1 day after PH. Then lipids levels dramatically returned to baseline after 2 days when hepatocellular DNA replication reached the peak. Systematically, the *de novo* lipogenesis in the liver may provide the initial supply of fatty acids for TG synthesis at the early phase (0–6 h) of liver regeneration and subsequent uptake of systemically derived fatty acids from adipose tissues (see online suppl. material, Fig. S1 B,C) serve as a source of hepatic lipids in mice.

SREBP-1, especially the subtype of SREBP-1c, mainly targets expressions of *de novo* lipogenesis in mammalian livers [18, 34]. In wild type mice subjected to PH, coincidentally with expressions of FAS, ACC and SCD-1, mRNAs of the hepatic precursor of two SREBP-1 subtypes also reduced to a barely detectable level at 12 h to 1 day after hepatectomy and recovered after 2 days, which is the time point of peak hepatocellular DNA replication. We concluded that significantly increased FFA inflow in remnant livers should inhibit hepatic SREBP-1 expression after liver resection through a feedback regulation. Correspondingly, expressions genes of *de novo* lipogenesis were down-regulated till the consumption of FFA reached the peak 2 days post-PH (Fig. 2E). These results indicate that SREBP-1 mediated-lipid synthesis may provide sufficient amount of FFA and energy to support hepatocyte proliferation at day 2 after PH. However, the mechanisms and functional significance of the dynamics of SREBP-1 and its targeted gene expressions in the resected liver is not clear. It has previously reported that combined systemic elimination of receptor tyrosine kinases MET and epidermal growth factor receptor (EGFR) signaling (MET knockout +EGFR-inhibited mice) completely abolished liver regeneration, which may be partly explained by a dramatic decrease in expression of SREBP-1 and lipid synthesis in METKO-EGFRi mice [35]. On other hand, decreased expression of SREBP-1 in METKO-EGFRi mice indicated that MET and EGFR signals could regulate the expression of SREBP-1 gene. The initiation and termination of the signaling of MET and EGFR are accompanied by the progress of hepatocyte proliferation, which may be a possible reason for the dynamic alteration of SREBP-1 during liver regeneration. Above all, it's reasonable to presume that SREBP-1 may play important roles on the balance between the fatty acid synthesis and β -oxidation during the liver regeneration via cooperating with other metabolism-regulated factors.

However, we found that SREBP-1c null mice displayed enhanced liver regeneration after PH which agreed with the down-regulation of SREBP-1 in wild type mice after PH. *Srebp-1c*^{-/-} mice display decreased serum and hepatic TG and FFA levels as well as lower levels of *de novo* lipogenesis in livers. However, only SREBP-1c deficiency in mice did not interfere with triglyceride and fatty acid metabolism and lipid droplet formation during liver regeneration. Expression of genes involved in the hepatic *de novo* fatty acid synthesis and β -oxidation was also no difference between SREBP-1c null mice and wild type mice after PH (Fig. 5 and see online suppl. material, Fig. S7). Before the liver resection, total ablation of the SREBP-1c transcript in *Srebp-1c*^{-/-} mice only results in a slight reduction in levels of the SREBP-1a transcripts (see online suppl. material, Fig. S7) [32]. However, there were no significant distinctions in SREBP-1a and hepatic nuclear SREBP-1 expressions between

Srebp-1c^{-/-} mice and wild type mice from 2 to 3 days after PH. Hence, the recuperative profiles of triglyceride and fatty acid metabolism in *Srebp-1c*^{-/-} mice during PH-induced liver regeneration may be derived from aggravated SREBP-1a processing after PH. These data suggested that the prolonged hepatocellular DNA replication and enhanced regeneration may be not related to the alteration of triglyceride metabolism in *Srebp-1c*^{-/-} mice. One possibility is that SREBP-1c could directly regulate the factors involving in cell cycle over its lipid-regulated roles. However, Inoue et al [29], had reported that SREBP-1a and SREBP-2 but not SREBP-1c strongly activate the promoter activity of the major CDK inhibitor p21^{WAF1/CIP1} gene by a novel SREBP binding site in the promoter. In SREBPs transgenic mice, sustained SREBP-1a activation markedly increased hepatic p21 mRNA and protein levels in SREBP-1a-expressing livers. In contrast, constitutive activation of SREBP-2 only modestly elevated p21 expression in transgenic livers, whereas SREBP-1c was essentially inactive for p21 [29]. Another published paper has also reported that SREBP-1a induces various CDK inhibitors such as p27 and p16, in addition to p21, leading G1 cell-cycle arrest in cultured cell growth [30]. Consistently, overexpression of nuclear SREBP-1a in mouse liver also causes marked impairment in liver regeneration after PH [30]. In our study, the levels of nuclear SREBP-1a were similar during liver regeneration in *Srebp-1c*^{-/-} mice and wild type mice (Fig. 6E). And the ablation of SREBP-1c did not influence the protein contents of p21 and p27 in the regenerating liver (see online suppl. material, Fig. S6), which is agreed with the results about that SREBP-1c does not activate the promoter of p21 and p27 [29, 30]. Surprisingly, trends of increasing hepatic proteins of p21 and p27 in un-hepatectomized *Srebp-1c*^{-/-} mice were obvious (see online suppl. material, Fig. S6) and may be related to compensated activation of SREBP-2, as increased nuclear SREBP-2 in the liver (Fig. 6E). However, the increased trends of p21 and p27 were not stably remained after liver resection, though SREBP-2 was still higher in the regenerating liver in *Srebp-1c*^{-/-} mice. Therefore, the present data, at least, implied that SREBP-1c and SREBP-2 may not directly regulate cell cycle and cell growth *in vivo*, though we can't exclude the possibility that other cell-cycle related factors could be only regulated by SREBP-1c or SREBP-2.

However, another factor linked lipid metabolic regulation of SREBPs and liver regeneration is cholesterol. Cholesterol is the structural component of biological membranes that influences properties such as permeability and fluidity while also contributing to the modulation of the membrane proteins, protein trafficking, and transmembrane signalings. Importantly, cholesterol is also a precursor of steroids and bile acids which contribute to the solubilization of other lipids and functions as signal transducers [36]. It has been demonstrated that cholesterol starvation resulted in cell cycle arrest specifically at G2 phase, thus it can be inferred that cholesterol acts as a regulator of cell cycle progression [14, 37]. In the present study in wild type mice, PH caused a remarkable increase in hepatic CE from 12 h to 1 day after the surgery and recovered to the baseline level after 2 days subsequently when the liver function nearly recovered to the level of sham operated mice. In the meanwhile, the hepatic FC dramatically reduced and then recovered at corresponding time points. We concluded that massive FC was firstly esterified to CE as a non-toxic storage form preparing for new hepatocytes production. Furthermore, it is speculated that the increase in cholesterol concentration in the resected livers occurred mostly at the expense of cholesterol from the circulation [38] before the initiation of the hepatocyte mitosis, as the serum cholesterol was significantly decreased (Fig. 1B) while the expression of HMGCR was also down-regulated (see online suppl. material, Fig. S4 A) from 12 h to 1 day after PH. Despite expressions of genes related to cholesterol uptake including LDLR and SR-BI was not significantly altered before and after PH (see online suppl. material, Fig. S4), hepatocellular cholesterol may be also obtained from the sinusoidal domain via transcytosis [36]. And the present data cannot reflect post-transcriptional regulations of cholesterol receptors during liver regeneration. Notably, PH induced a significant decrease in LXRα-targeted genes - ABCG5/8 (see online suppl. material, Fig. S4 E), which encode for proteins involved in cholesterol efflux. This result is in agreement with the study that PH attenuates LXR-driven cholesterol efflux and catabolism pathways, which increases cholesterol availability and positively modulate

hepatocyte regenerative capacity [39]. Accordingly, after the first post-hepatectomy phase (0- 1 day) consisting of the initiation of DNA synthesis, regenerative livers undergo significant alterations in cholesterol homeostasis. Maintaining requisite cholesterol levels could be important for hepatocyte proliferation during liver regeneration [14].

Hydroxy-methylglutaryl CoA reductase (HMGCR), the rate-limiting enzyme of cholesterol synthesis, plays a vital role in the cholesterol homeostasis in livers. In our study, the expression of HMGCR was down-regulated prior to 2 days after PH while its mRNA level was significantly higher at 2 day than sham control (see online suppl. material, Fig. S4 A). In agreement with previous studies [39-41], which suggested that the pre-existing cholesterol storage or the delivered lipoprotein cholesterol may be insufficient to meet the cellular required demand soon after the start of cell division in the regenerating liver (2-3 days after PH) while higher expression of HMGCR and its activity and strong new cholesterol synthesis are required. Interestingly, in the present study, we found that precursors of SREBP-2 were increased after PH (Fig. 3) while its mRNA levels were similar with the HMGCR through the liver regeneration. Reasons for these differences between genes and protein levels of SREBP-2, especially during 12 h to 1 day after PH were not clear. Post-transcriptional and post-translational modifications of SREBP-2 may also lead to the difference. Based on this, SREBP-2 and associated cholesterol alterations may play important roles in the preparation for hepatocellular proliferation after PH. Coincidentally, in SREBP-1c null mice, there was a 50% increase in the level of SREBP-2 transcripts and increase in transcripts of enzymes utilized in cholesterol biosynthesis. Liver cholesterol content is increased while serum cholesterol levels are reduced [32]. Expressions of ABCG5/8 was slightly upregulated at day 2 and 3 in *Srebp-1c*^{-/-} mice (see online suppl. material, Fig. S10 F and G), indicating increased cholesterol efflux is in order to prevent high cholesterol-induced cytotoxicity. Cholesterol metabolic profiles in *Srebp-1c*^{-/-} mice were kept during the liver regeneration after PH. *Srebp-1c*^{-/-} mice displayed higher cholesterol storage in the liver compared with wild type mice 1 day after PH. And the transformation of CE to FC was more pronounced from day 2 to day 3 after PH in *Srebp-1c*^{-/-} mice compared with WT ones (Fig. 6), suggesting more FC for hepatocellular proliferation and mitosis in *Srebp-1c*^{-/-} mice.

We also found that the expression of HMGCR was higher before and after the mitosis in *Srebp-1c*^{-/-} mice which partly indicated that higher activity of HMGCR throughout the whole liver regeneration (see online suppl. material, Fig. S10 C). Interestingly, administration of pravastatin, a HMGCR inhibitor, followed by compensatory induction of HMGCR activity in rats led to greater liver DNA synthesis after PH [42]. As discussed above, increased HMGCR activity could provide sufficient cholesterol for cell division thus favoring hepatocellular proliferation. In contract, direct cholesterol-rich administration had no beneficial or harmful effect on the initiation of liver regeneration in rats after PH [42, 43], which may due to cholesterol overload that may induce cytotoxicity in regenerated liver. In addition, Cyp7a1 and Cyp8b1, the limiting-rate enzyme of bile acids (BAs) synthesis, were decreased after PH in *Srebp-1c*^{-/-} mice (see online suppl. material, Fig. S10 H,I). It's well known that bile acid/FXR signaling pathway is required for normal liver re-generation. When liver is injured, FXR activation results in the suppression of CYP7a1 by inducing its negative regulator SHP, which help rapidly decrease hepatic BA levels to protect the liver from apoptosis and necrosis induced by elevated BA concentrations [33, 44, 45]. Although we are not clear how deficiency of SREBP-1c caused induction of SHP (Fig. 7), the down-regulation of Cyp7a1 may root in high levels of SHP in livers of *Srebp-1c*^{-/-} mice and favor to the liver regeneration. Most importantly, decreased expression of Cyp7a1 and Cyp8b1 did suggest that decreased cholesterol catabolism into BAs in SREBP-1c^{-/-} mice after PH, which may contribute to appropriate cholesterol storage in the regenerated liver. Due to the potential roles of SHP [46, 47] and suppression of Cyp7a1 [48] on cell proliferation, the present data cannot exclude the direct contributions of increased SHP and decreased Cyp7a1 to liver regeneration in *Srebp-1c*^{-/-} mice. However, we speculate that all of these cholesterol metabolism-related alterations including increased nuclear SREBP-2 expression and changes of genes involving in cholesterol synthesis and catabolism caused more appropriate liver cholesterol storage and improved

cholesterol availability in *Srebp-1c*^{-/-} mice. This could be beneficial for hepatocellular proliferation and mitosis after PH and associated with enhanced liver regeneration of *Srebp-1c*^{-/-} mice. Additional experiments such as the effect of direct statins administration or additional cholesterol in diet during PH are needed to confirm our conclusion. These experiments would be helpful to figure out the reason of transient enhancement of liver regeneration in *Srebp-1c*^{-/-} mice is due to increased availability of cholesterol.

In conclusion, in our study, we showed that SREBP-1c gene deficiency in mice was not associated with quantitative or kinetic differences in the accumulation of hepatic triglyceride contents or in lipid droplet formation after PH. However, enhanced liver regeneration was observed in *Srebp-1c*^{-/-} mice after PH. This event could be explained by the more appropriate liver cholesterol storage and improved cholesterol availability during liver regeneration in *Srebp-1c*^{-/-} mice. This study also shows that providing requisite cholesterol levels to proliferating hepatocytes and keeping appropriate cholesterol metabolism are required for normal liver regeneration.

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Disclosure Statement

The authors declare no competing interests.

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