



Cathepsin L-deficiency enhances liver regeneration after partial hepatectomy

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ABSTRACT

Aim: Cathepsin L (Ctsl) plays a pivotal role in lysosomal and autophagic proteolysis. Previous investigations revealed that partial hepatectomy (PH) decreases biosynthesis of cathepsins in liver, followed by suppression of lysosomal and autophagic proteolysis during liver regeneration. Conversely, it was reported that autophagy-deficiency suppressed liver regeneration. Thus, the purpose of this study is to determine if Ctsl deficiency affects liver regeneration after PH.

Methods: 70% of PH was performed in male Ctsl-deficient mice (Ctsl^{-/-}) and wild-type littermates (Ctsl^{+/+}) after PH. Mice were sacrificed and wet weight of the whole remaining liver was measured. Bromodeoxyuridine (BrdU)-immunostaining of liver sections was performed. Expression of cyclin D1, p62, LC-3, Nrf2, cleaved-Notch1, Hes1 was evaluated by western blot analysis. NQO1 mRNA expression was measured by realtime-PCR.

Results: After a 70% of PH, the liver mass was significantly restored within 5 days in Ctsl^{-/-} mice compared to wild-type. Ctsl-deficiency enhanced the increases in both the rate of BrdU-positive cells and cyclin D1 expression after PH more than wild-type mice. On the other hand, Ctsl-deficiency upregulated p62, cleaved-Notch1 and Hes1 expression after PH. Moreover, the protein level of Nrf2 in the nucleus and mRNA expression of NQO1 in the liver after PH was also up-regulated in Ctsl^{-/-} mice.

Conclusions: These findings suggest that accumulation of p62 due to loss of Ctsl plays an important role in liver regeneration through activation of Nrf2-Notch1 signaling. Taken together, Ctsl might be a new therapeutic target on disorder of liver regeneration.

1. Introduction

Liver regeneration proceeds through a series of complex signaling pathways [1]. Although hepatocytes rarely divide under normal circumstances, the liver has a remarkable ability to regenerate after surgical removal or after liver injury. Understanding the hepatic regenerative process has clinical interest, since the effectiveness of many treatments for chronic liver diseases, such as resection of tumors and donor liver transplantation, is dependent upon efficient liver regeneration. Post-hepatectomy liver insufficiency is one of the main problems associated with major hepatic resection in cirrhotic livers. Thus, liver resection in cirrhotic patients is a risky therapeutic decision. Accordingly, it is necessary to identify new therapeutic targets to stimulate liver regeneration.

Lysosomes play an essential role in protein turnover by degrading exogenous and endogenous proteins and supplying cells with amino acids necessary for protein synthesis or as an energy source [2]. Lysosomal proteolysis is caused mainly by cysteine proteinases such as cathepsin B, H, and L [2]. Cathepsin L (Ctsl) expressed in almost all types of eukaryotic cells, is primarily involved in turnover and degradation of intracellular proteins [2]. Hepatocytes also synthesize and secrete large amounts of Ctsl. It is well known that lysosomal proteolytic activities are inversely correlated with cell growth [3]. Lysosomal proteolysis is lower in transformed cells than in parent cells. Further insight into the relationship between lysosomal proteolysis and cell growth has been obtained from the studies using regenerating liver [4]. In regenerating liver, decreased protein degradation rather than increased protein synthesis, has been shown to be the main cause of the rapid increase in

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cell mass after hepatectomy. Lysosomal and autophagic proteolysis in regenerating liver is suppressed principally through a suppression of cathepsins expression [5]. As previous investigations have shown, the down-regulation of Ctsl activity by pharmacological inhibitors suppressed autophagic membrane turnover and the resulting Ctsl deficiency enhanced accumulation of the selective autophagic substrate p62 [6,7].

Autophagy is a catabolic process which degrades long-lived proteins and cellular organelles such as mitochondria and endoplasmic reticulum (ER) for an alternative energy source during nutrient deprivation [8]. Autophagosomes engulf organelles and then fuse with lysosomes to form mature autolysosomes, in which the sequestered proteins are digested into amino acids by lysosomal enzymes. Selective recruitment of p62 into the autophagosome is then degraded [9]. Thus, impaired autophagy caused accumulation of p62. Recent evidence demonstrated that the accumulation of p62 due to autophagy-deficiency plays a pivotal role in hepatoma development via persistent activation of nuclear factor erythroid 2-related factor 2 (Nrf2) [10]. The Nrf2-Keap1 system is one of the major cellular defense mechanisms against oxidative stress. It was reported that the accumulation of p62 promotes the stabilization of Nrf2 followed by transcriptional activation of Nrf2 target genes encoding antioxidant proteins. Previous reports demonstrated that liver regeneration after partial hepatectomy was down-regulated in Nrf2 null mice [11]. It was suggested that activation of Nrf2 plays a pivotal role in the regenerative response after partial hepatectomy. Therefore, in this study, we hypothesize that Ctsl-deficiency enhances liver regeneration after PH with an up-regulation of p62-associated Nrf2 signaling.

2. Materials and methods

2.1. Animals and experimental design in an in vivo model

Male *Cathepsin L*^{-/-} (Ctsl^{-/-}) [12] and wild-type littermates mice 8 weeks after birth were housed under specific pathogen-free conditions. All animals received humane care in compliance with the experimental protocol approved by the Committee of Laboratory Animals according to institutional guidelines (permit no. 290176). A 70% partial hepatectomy (PH) or sham procedure was performed on male Ctsl^{-/-} and wild-type littermates. Mice were sacrificed 1, 6, 12, 24, 48, 120 h after PH and wet weight of the remaining liver was measured after 2 and 5 days. The mortality rate after 70% partial hepatectomy was lower than 1%. Restored liver mass was expressed as the percentage of remaining liver weight compared to predicted-whole liver weight calculated as following, $R = 100 \times 0.7 \times (W2/W1)$, where W1 is weight the liver resected by the operation, and W2 is the weight of regenerating liver.

2.2. Immunohistochemistry for BrdU staining

To examine BrdU incorporation in hepatocyte nuclei, formalin-fixed and paraffin-embedded tissue sections were deparaffinized and incubated with 3% H₂O₂ for 10 min. Next, tissue sections were incubated with 2 N hydrochloric acid for 30 min. After blocking with normal horse serum for 60 min, tissue sections were incubated with a mouse monoclonal anti-BrdU antibody (Dako Cytomation Norden A/S, Glostrup, Denmark). After rinsing the primary antibody, the sections were incubated with secondary biotinylated anti-mouse immunoglobulin G antibody, and the specific binding was visualized with the avidin-biotin complex solution followed by incubation with a 3, 3'-diaminobenzidine tetrahydrochloride solution using Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). BrdU-positive hepatocytes were counted in five 100× fields on each slide to determine the average number BrdU-labeling index (BrdU-positive hepatocytes/ total hepatocytes).

2.3. Electron microscopic analysis

Livers were fixed with 3% glutaraldehyde (TAAB Laboratory Equipment Ltd., UK) in 0.05 M sodium cacodylate buffer at pH 7.2, post-fixed with 2% osmic acid, epon-embedded, sectioned at a thickness of 1 μm, and stained with toluidine blue. Ultrathin sections were observed using a Hitachi H7100 electron microscope. For morphometric analysis, a minimum of five random fields per hepatocytes were taken and examined in a blinded manner at ×3000 and ×7000 magnification. The term “autophagic vesicle” refers to an autophagosome or autolysosome.

2.4. Immunoblot analysis

Whole liver protein extracts were obtained by homogenizing liver samples in a buffer containing 66 mM Tris (pH 8.0), 5 mM ethylenediamine tetraacetic acid (EDTA), 1% Triton X-100 and protease/phosphatase inhibitors. Proteins (10 μg per lane) was separated on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were soaked with 5% nonfat dry milk in Tris-buffer saline containing 0.05% TritonX-100 (TBS-T), and then incubated with the following primary polyclonal antibodies: p62/SQSTM1 (p62) and microtubule-associated protein IA/IB light chain 3 (LC3) (Sigma-Aldrich), GAPDH, phosphorylated-p70S6K (p-p70S6K), phosphorylated-S6 ribosomal protein (p-S6), the nuclear factor-like 2 (Nrf2), cyclin D1, STAT3, phospho-STAT3, cleaved-Notch1 and Hes1 (Cell Signaling Technology USA). After washing with TBS-T, blots were developed using horseradish peroxidase-conjugated secondary antibodies and the ECL detection kit (Amersham Pharmacia Biotech Inc. UK).

2.5. Quantitative real-time polymerase chain reaction

Total RNA was isolated from liver samples using the RNA isolation Kit (GE Healthcare Japan Corporation, Japan) according to the manufacturer's protocol. The RNA concentration and purity were assessed using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Waltham, MA, USA). In total, 2 μg of RNA was reverse transcribed using a high-capacity cDNA Reverse Transcriptase (RT) kit (Thermo Fisher Scientific Waltham, MA, USA) according to the manufacturer's protocol. Real-time PCR was performed to amplify *Tnfa*, *Il-6*, *Nqo1* and *Gapdh* using a Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green dye and the following specific primers:

Mouse *tnf-α* (forward) 5'-AAGCCTGTAGCCCACGTCGTA-3' and (reverse) 5'-GGCACCAGTAGTTGGTTGCTTTG-3',

Mouse *IL-6* (forward) 5'-CCACTTCACAAGTCGGAGGCTTA-3' and (reverse) 5'-GCAAGTGCATCATCGTTGTTTCATAC-3',

Mouse *Nqo 1* (forward) 5'-CAGCCAATCAGCGTTCCGGTA-3' and (reverse) 5'-CTTCATGGCGTAGTTGAATGATGC-3',

Mouse *Gapdh* (forward) 5'-TGTGTCCGTCGTGGATCTGA-3' and (reverse) 5'-TTGCTGTTGAAGTCGCAGGAG-3'.

2.6. Adenosine-5'-triphosphate assay of liver tissue

Liver samples were collected and stored at -80 °C. The levels of adenosine-5'-triphosphate (ATP) in liver samples were measured using a firefly bioluminescence assay kit (AMERIC-ATP Kit; Wako Pure Chemical Industries) according to the manufacturer's instructions. The luciferase activity was measured using a plate reader (ARVO X5; PerkinElmer Inc.).

2.7. Statistical analysis

All data are expressed as mean ± standard error. Comparison

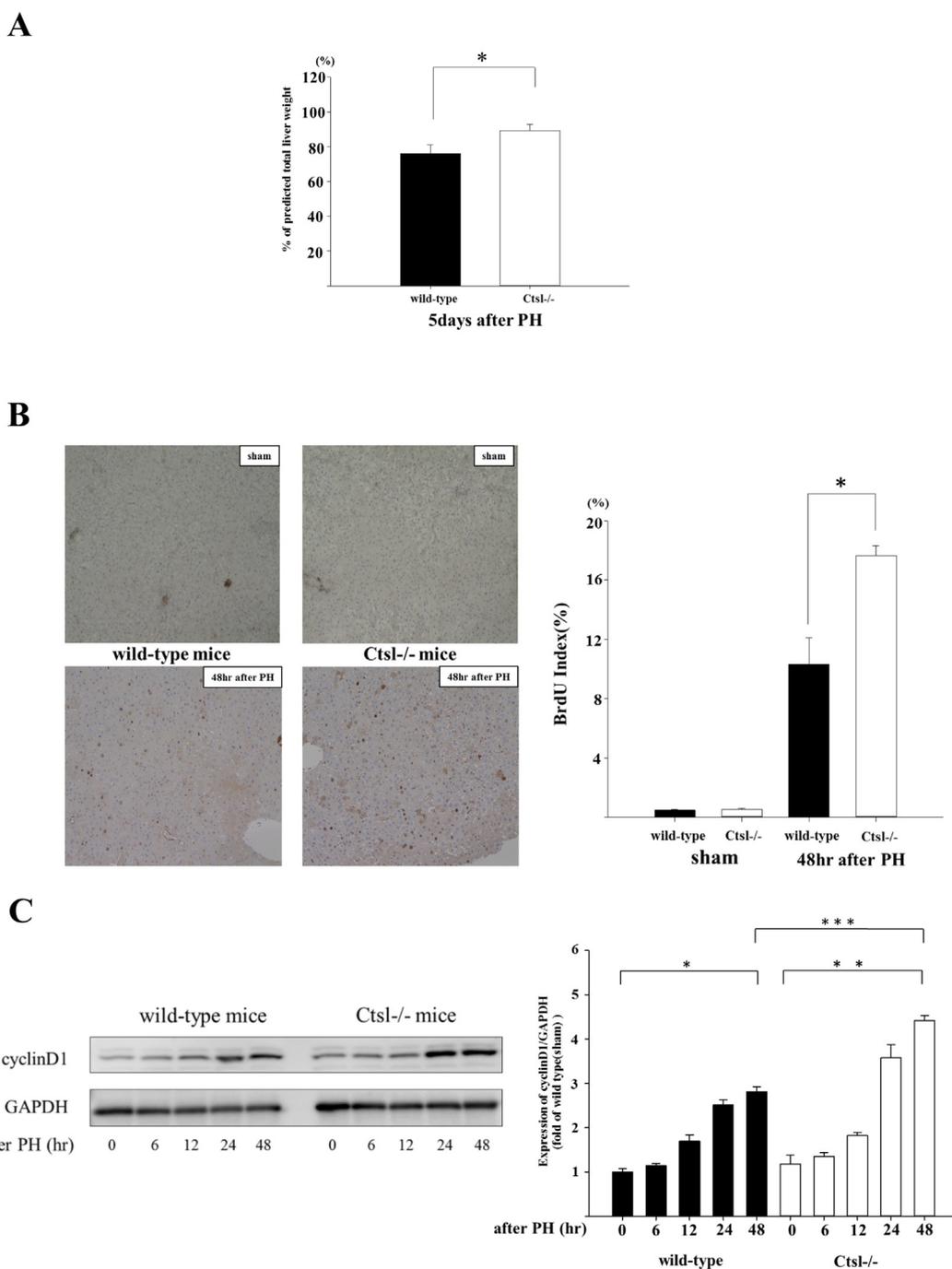


Fig. 1. Effect of cathepsin L-deficiency on regeneration of liver after 70% partial hepatectomy (A) Restored liver mass was expressed as the percentage of remaining liver weight compared to predicted-whole liver weight calculated by resected- liver weight. ($n = 6$; $*P < 0.05$ versus Wild-type mice 5 days after PH, by ANOVA on ranks). (B) Representative photomicrographs of BrdU staining 48 h after PH from wild-type and Ctsl^{-/-} mice are shown (original magnification 100 ×). Mean BrdU indices are plotted ($n = 5$; mean ± SEM, $*P < 0.05$, by ANOVA on ranks). (C) Hepatic cyclin D1 expression after PH was detected by western blotting. Representative photographs of specific bands from 5 individual experiments are shown. The results by densitometry for cyclin D1 (expressed as fold over sham wild-type mice) are plotted (mean ± SEM, $*P < 0.05$ versus sham wild-type, $***P < 0.05$ versus sham Ctsl^{-/-}, $***P < 0.05$ versus wild-type 48 h after PH, by ANOVA on ranks).

between groups was performed with one-way ANOVA followed by Turkey's post hoc test using Sigma plot software. P -values < 0.05 were considered significant.

3. Results

3.1. Liver regeneration after partial hepatectomy is up-regulated in Ctsl^{-/-} mice

In this study, we first investigated the differences in liver regeneration after 70% PH between wild-type mice and Cts^{-/-} mice. All mice survived after PH. Regenerated liver mass at 5 days after PH in

wild-type mice was 74% of total liver weight predicted by resected-liver weight; however, liver mass was increased to 89% of predicted total liver in *Ctsl*^{-/-} mice. Restoration of liver weight 5 days after PH in *Ctsl*^{-/-} mice was significantly higher than wild-type mice ($p = 0.008$) (Fig. 1A). To evaluate DNA synthesis during liver regeneration, we examined BrdU uptake in hepatocyte nuclei by immunohistochemistry (Fig. 1B). BrdU incorporation was observed in $10.5 \pm 1.79\%$ of hepatocytes 48 h after PH in wild-type mice; while BrdU incorporation rate reached to $17.5 \pm 0.49\%$ in *Ctsl*^{-/-} mice. To further confirm if up-regulated liver regeneration in *Ctsl*^{-/-} mice, hepatic expression of cyclin D1 was detected by western blot analysis (Fig. 1C). In wild-type mice, cyclin D1 expression 48 h after PH was increased almost 3-fold over basal levels; however, *Ctsl* deficiency more enhanced increase in cyclin D1 after PH to about 1.7-fold of wild-type. Taken together, these findings clearly indicate that *Ctsl* deficiency accelerates the regenerative response in the liver after PH.

3.2. Production of cytokines after partial hepatectomy is not changed in *Ctsl*^{-/-} mice

TNF α can promote liver regeneration shortly after PH; therefore, hepatic TNF α mRNA was measured by Real-time PCR (Fig. 2A). TNF α mRNA levels in the liver before PH were low, and no significant difference was observed between wild-type and *Ctsl*^{-/-} mice. After PH, hepatic TNF α mRNA was increased within 1 h to roughly the same level in wild-type mice and *Ctsl*^{-/-} mice. Since IL-6 and the Janus kinase (JAK)-STAT pathway play a pivotal role in liver regeneration, hepatic IL-6 mRNA after PH was also evaluated (Fig. 2A). In wild-type and *Ctsl*^{-/-} mice, IL-6 mRNA levels were elevated at 1 h after PH with values reaching about 7-fold of basal value. The activation of the downstream JAK-STAT pathway was evaluated by detecting phosphorylation of STAT3 (Tyr705) in the liver, using western blot analysis (Fig. 2B). In wild-type and *Ctsl*^{-/-} mice, the levels of STAT3 phosphorylation in the liver were increased 6 h after PH, followed by a gradual decrease.

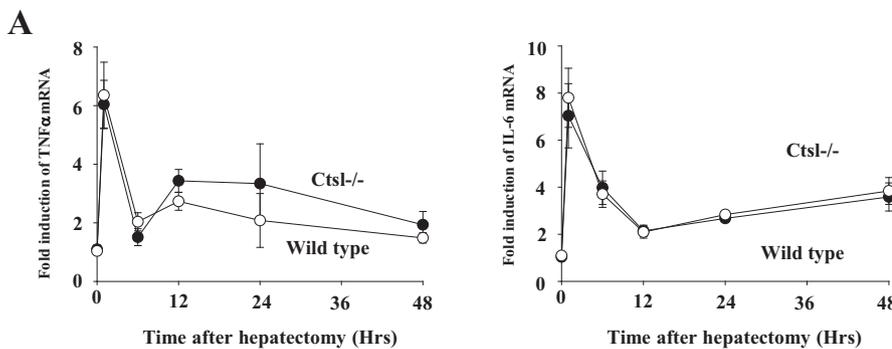
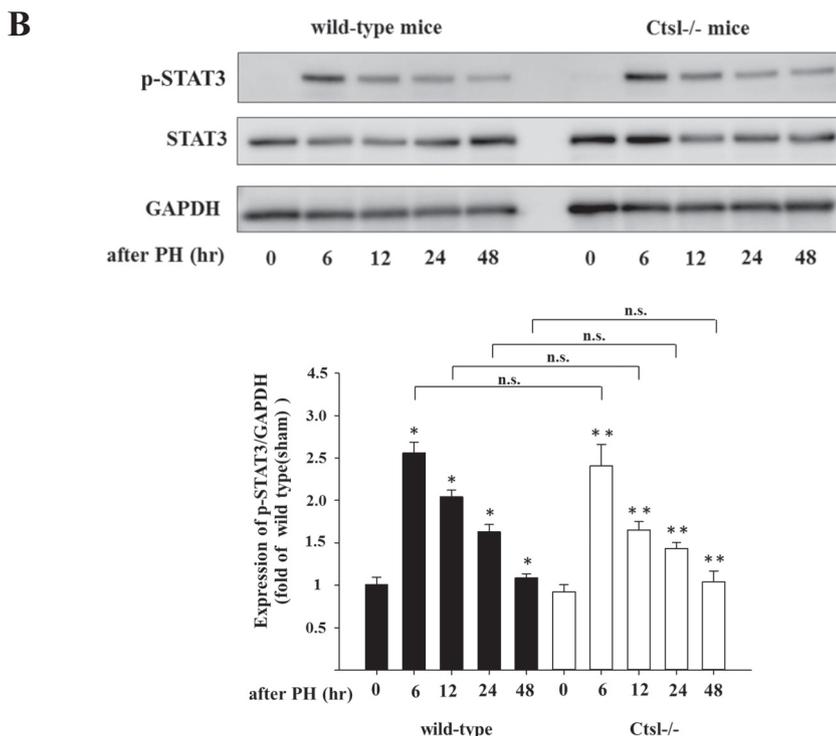
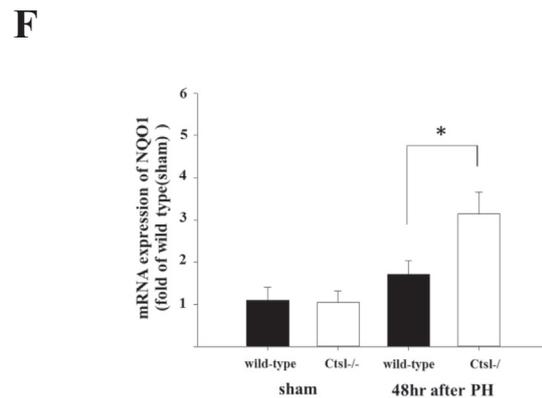
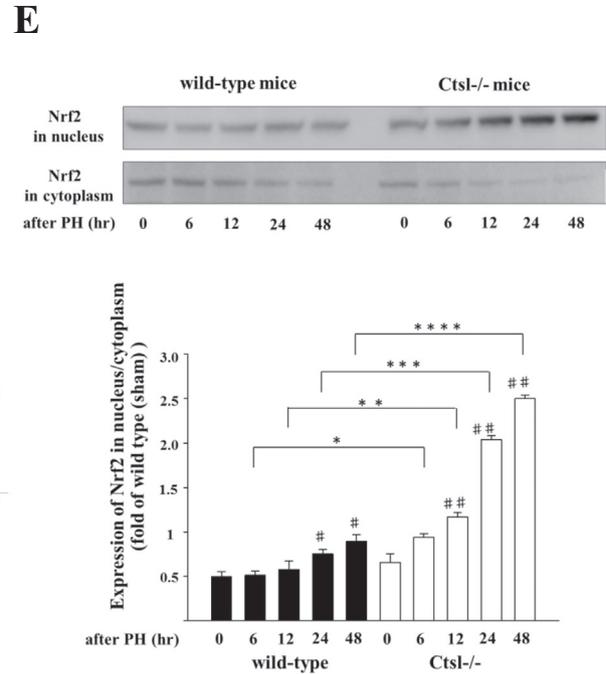
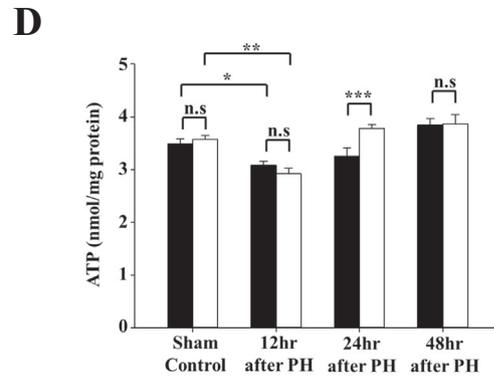
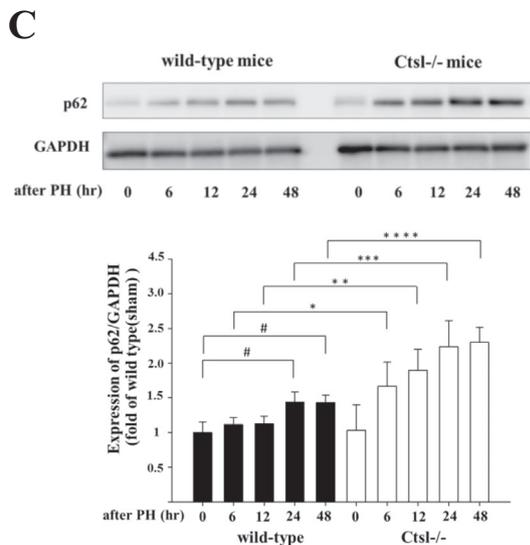
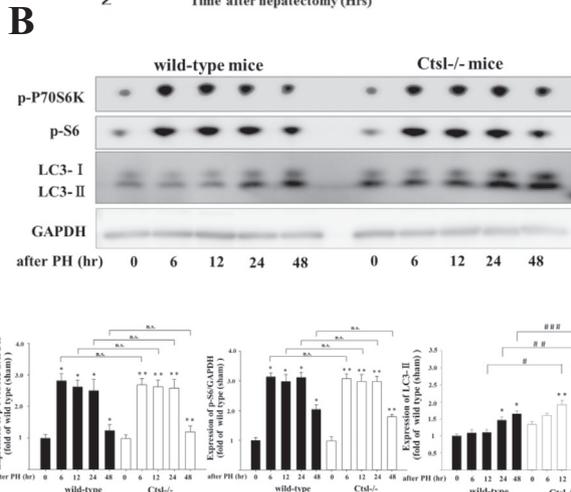
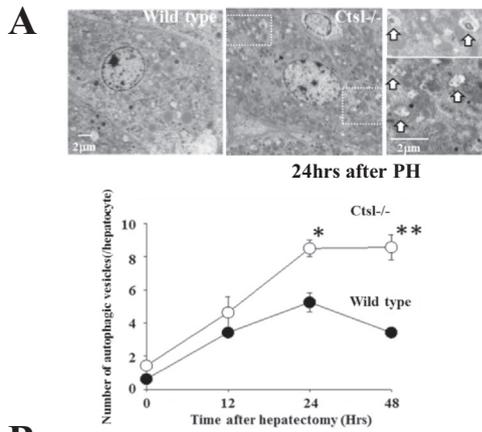


Fig. 2. Effects of cathepsin L-deficiency on hepatic TNF α and IL-6 mRNA and phosphorylation of STAT3 after partial hepatectomy.

(A) The levels of TNF α mRNA and IL-6 mRNA in the liver were determined by real-time RT-PCR ($n = 4$; mean \pm SEM). (B) Protein level of phosphorylated-STAT3 and total STAT3 in the liver were detected by western blotting. Representative photographs of specific bands for phosphorylated (p)-STAT3, and total STAT3 are shown. The results by densitometry for p-STAT3/GAPDH (expressed as fold over sham wild-type mice) are plotted ($n = 5$; mean \pm SEM, * $P < 0.05$ versus sham wild-type, ** $P < 0.05$ versus sham *Ctsl*^{-/-}, by ANOVA on ranks).





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3.3. Accumulation of autophagic vesicles after partial hepatectomy in Ctsl-/- mice

Following PH, an increase in the number of autophagosomes in wild-type mice peaked at 24 h, followed by a gradual decrease (Fig. 3A). In Ctsl-/- mice, the number of autophagosomes after PH increased more than wild-type mice, increasing autophagic vesicles was

maintained to 48 h after PH. Similarly, expression of autophagic membrane protein LC3-II, the lipidation form of LC3 proteins, was enhanced in Ctsl-/- mice after PH (Fig. 3B). PH activated mTOR substrate p70S6K and S6 ribosomal protein in liver from both wild type and Ctsl-/- mice after 6, 12 and 24 h. Activation of both p70S6K and S6 ribosomal protein due to PH in Ctsl-/- mice PH was almost equal to wild type mice. These findings imply that Ctsl deficiency did not

Fig. 3. Effects of cathepsin L-deficiency on autophagy in the regenerating liver.

(A) Autophagosomes in the regenerating liver. Arrows mark the autophagic vesicles. The number of autophagic vesicles in a hepatocyte at the indicated times after hepatectomy are shown (Scale bar, 2 μ m, n = 4; mean \pm SEM, **P* < 0.05 versus wild-type 24 h after PH, ***P* < 0.05 versus wild-type 48 h after PH, by ANOVA on ranks). (B) Expression of phosphorylated (p)-p70S6K, phosphorylated (p)-S6, the autophagy-related protein LC3 in wild-type and *CtSL*^{−/−} mice at 0–48 h after 70% PH (representative western blottings are shown). The results by densitometry for p-p70S6K/GAPDH, p-S6/GAPDH, LC3-II/LC3-I (expressed as fold over sham wild-type mice) are plotted (n = 5; mean \pm SEM, **P* < 0.05 versus sham wild-type, ***P* < 0.05 versus sham *CtSL*^{−/−}, #*P* < 0.05 versus wild-type 12 h after PH, ##*P* < 0.05 versus wild-type 24 h after PH, ###*P* < 0.05 versus wild-type 48 h after PH, by ANOVA on ranks). (C) Expression of the autophagy-related protein p62 in wild-type and *CtSL*^{−/−} mice at 0, 6, 12, 24, 48 h after 70% PH (representative western blot analysis are shown). The results for p62 by densitometry (expressed as fold over sham wild-type mice) are plotted (n = 5; mean \pm SEM, #*P* < 0.05 versus sham wild-type, **P* < 0.05 versus wild-type 6 h after PH, ***P* < 0.05 versus wild-type 12 h after PH, ****P* < 0.05 versus Wild-type 24 h after PH, *****P* < 0.05 versus Wild-type 48 h after PH, by ANOVA on ranks). (D) The levels of ATP in the liver was measured using a firefly bioluminescence assay (n = 4; mean \pm SEM, **P* < 0.05 versus sham wild-type, ***P* < 0.05 versus sham *CtSL*^{−/−}, ****P* < 0.05 versus wild type 24 h after PH by ANOVA on ranks). (E) Expression of Nrf2 of nucleus and cytoplasm in wild-type and *CtSL*^{−/−} mice at 0–48 h after 70% PH (representative western blottings are shown). The results for Nrf2 by densitometry (expressed as fold over sham wild-type mice) are plotted (n = 5; mean \pm SEM, **P* < 0.05 versus wild-type 6 h after PH, ***P* < 0.05 versus wild-type 12 h after PH, ****P* < 0.05 versus Wild-type 24 h after PH, *****P* < 0.05 versus Wild-type 48 h after PH, #*P* < 0.05 versus sham Wild-type, ##*P* < 0.05 versus sham *CtSL*^{−/−}, by ANOVA on ranks). (F) The levels of NQO1 mRNA in the liver was determined by real-time RT-PCR (n = 4; mean \pm SEM, **P* < 0.05 versus wild-type 48 h after PH, by ANOVA on ranks).

affect mTOR signaling. We next investigated the autophagic function after PH in *CtSL*^{−/−} mice. Expression of p62 in the liver from sham operated wild-type mice was nearly identical to sham *CtSL*^{−/−} mice. Expression of p62 was significantly increased in wild-type mice at 24 and 48 h after PH (Fig. 3C). On the other hand, *CtSL* deficiency enhanced the increase in p62 expression due to PH to a greater extent than wild-type mice. These results indicate that *CtSL* deficiency enhances both the accumulation of autophagic vesicles and p62 expression after PH.

It was known that PH was followed by considerable damage to mitochondria, reduced intrahepatic ATP generation. Thus, autophagy during the early phase of liver regeneration is critical for maintaining healthy mitochondria capable of producing ATP and prevents hepatocytes from becoming senescent. In our experiment, hepatic ATP value in *CtSL*^{−/−} was same as wild type mice without PH. As expected, PH decreased hepatic ATP levels in wild type mice after 12 h (Fig. 3D). Similarly, hepatic ATP was equally suppressed in *CtSL*^{−/−} mice 12 h after PH. Hepatic ATP value in *CtSL*^{−/−} mice was recovered to basal value 24 h after PH earlier than wild type mice.

3.4. Activation of Nrf2 signaling after partial hepatectomy in *CtSL*^{−/−} mice

Previous reports revealed that accumulation of p62 interacts with the Nrf2-binding site on Keap1, resulting in stabilization of Nrf2 followed by transcriptional activation of Nrf2 target genes. Thus, nuclear translocation of Nrf2 was evaluated by western blot analysis (Fig. 3E). The expression of Nrf2 in the nuclear fraction from wild-type mice liver was elevated 24 h after PH, whereas, expression of Nrf2 in nuclear protein was strongly up-regulated in *CtSL*^{−/−} mice 6 h after PH. Next, we evaluated mRNA expression of NQO1, which is one of the Nrf2 target genes. NQO1 mRNA levels were elevated after PH in wild-type mice by about 1.5-fold as compared to sham wild-type mice; however, *CtSL* deficiency enhanced the increase in NQO1 mRNA expression by 2-fold as compared to wild-type mice after PH (Fig. 3F). These data indicate that *CtSL* deficiency up-regulates transcriptional activation of Nrf2 after PH parallel to increasing p62 expression.

3.5. Activation of Notch signaling after PH in *CtSL*^{−/−} mice

Because Nrf2-Notch crosstalk plays an important role in liver regeneration, we evaluated the activity of Notch1 after PH (Fig. 4A). Cleaved Notch1 expression in liver from sham wild-type mice was the same as in sham *CtSL*^{−/−} mice; however expression was increased in wild-type mice 48 h after PH to about 2 fold of basal value. On the other hand, *CtSL* deficiency enhanced expression of cleaved-Notch1 to 1.5-fold of wild-type mice 48 h after PH. Expression of Hes1 regulated by Notch was analyzed to evaluate Notch activation after PH. Hes1 expression in the liver from *CtSL*^{−/−} mice after PH was increased to 1.5-fold of wild-

type mice (Fig. 4B). These findings suggested that *CtSL* deficiency enhances activation of Notch signaling after PH.

4. Discussion

The liver is one of the most complex organs, playing an important role in digestion, detoxification, blood sugar regulation, and fat metabolism. Liver regeneration induced by surgical injury is an orchestrated response. Here, we found that *CtSL* deficiency accelerates liver regeneration after 70% PH (Fig. 1A). After PH, *CtSL* deficiency accelerated cellular proliferation resulting in tissue remodeling and restoration to normal hepatic mass during shorter period than wild type mice (Fig. 1B). Hepatocyte proliferation in response to cytokines and growth factors plays a central role in liver regeneration. Increasing TNF α and IL-6 mRNA levels in the liver from *CtSL*-deficient mice after PH were same value as in wild type mice (Fig. 2A). Furthermore, in both wild-type and *CtSL*^{−/−} mice, the levels of STAT3 phosphorylation in the liver were increased 6 h after PH, followed by a gradual decrease (Fig. 2B). These findings suggest that *CtSL* deficiency did not affect cytokine production and signaling after PH.

CtSL deficiency enhances the accumulation of autophagic vesicles and p62 expression after PH (Fig. 3A, B, C). It has been reported that *CtSL* deficiency leads to decreased lysosomal proteolytic activity, impaired lysosomal fusion and/or autophagosomal content degradation, as well as autophagosome accumulation [13–15]. Self-degradation of autolysosomes and turnover of autophagic membranes are disturbed by *CtSL* deficiency, thereby autophagic vesicles were accumulated after PH in *CtSL*^{−/−} mice. Toshima et al. reported that autophagy deficiency impaired liver regeneration after PH [16]. Autophagy during the early phase of liver regeneration is critical for maintaining healthy mitochondria capable of producing ATP and prevents hepatocytes from becoming senescent [16]. A marked decrease in hepatic ATP levels and mitochondrial dysfunction by autophagy deficiency was associated with impairment of liver regeneration. In this autophagy-deficient model, as expected, hepatic ATP levels were significantly lower in autophagy-deficient mice than in control mice. Thus, all autophagy-deficient mice died 24 h after 90% PH and all wild-type mice survived [16]. In contrast, our findings showed that decreasing ATP values in *CtSL*^{−/−} mice during the early phase of liver regeneration were nearly same as wild type mice (Fig. 3D). Autophagy-deficiency causes hepatomegaly and swelling of hepatocytes because of deposition of aggregated-proteins; however, *CtSL* deficiency did not cause hepatomegaly and swelling of hepatocytes [9]. Furthermore, in *CtSL*^{−/−} mice, accumulation of deformed mitochondria was not observed in hepatocytes after PH, unlike in autophagy-deficient mice model (Fig. 3A). These findings indicate that *CtSL* deficiency did not cause the accumulation of damaged-mitochondria and a minimum level of autophagic function remains to maintain cellular homeostasis in *CtSL*^{−/−} mice; therefore, *CtSL* deficiency did not suppress liver regeneration after PH.

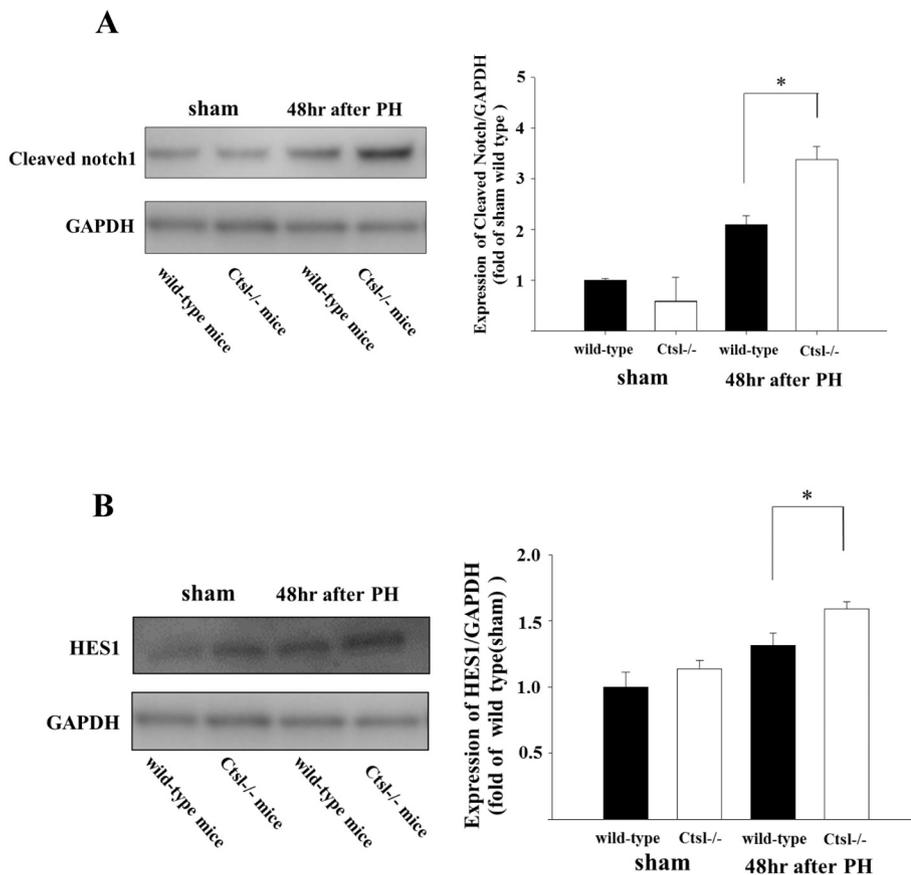


Fig. 4. Effect of cathepsin L deficiency on activation of Notch1 signaling after partial hepatectomy (A) Expression of cleaved-Notch1 in the liver from wild-type and Ctsl^{-/-} mice after 70% PH (representative western blottings are shown). The results for cleaved-Notch1 by densitometry (expressed as fold over sham wild-type mice) are shown (n = 5; mean ± SEM, *P < 0.05 versus Wild-type 48 h after PH, by ANOVA on ranks). (B) Expression of Hes-1 in the liver from wild-type and Ctsl^{-/-} mice after 70% PH (representative western blottings are shown). The results for Hes-1 by densitometry (expressed as fold over sham wild-type mice) are plotted (n = 5; mean ± SEM, *P < 0.05 versus wild-type 48 h after PH, by ANOVA on ranks).

During autophagy, autophagosomal membrane protein LC3-II which engulfs cytoplasmic components, also undergo degradation upon fusion of autophagosomes with lysosomes. In our study, Ctsl deficiency promoted increases in LC3-II expression and autophagic vesicles due to PH; however, activation of mTOR signaling after PH was not changed by Ctsl deficiency (Fig. 3A, B). Therefore, it was suggested that Ctsl deficiency does not affect induction of autophagy. It was reported that specific Ctsl-inhibitor effectively inhibited degradation of LC3-II [7]. Ctsl does not play a general role in the degradation of proteins in the lumen of autophagosomes, but rather is involved specifically in the degradation of autophagosomal membrane proteins. It was well-known that p62 is a LC3-binding protein. Thus, a direct interaction between p62 and accumulating LC3 may disturb the degradation of p62. Additionally, it was suggested that Ctsl deficiency delays the autophagic degradation process. Consequently, delayed-autophagic process might enhance p62 expression after PH in Ctsl^{-/-} mice.

Our results showed that Ctsl deficiency promoted both nuclear translocation of Nrf2 and transcription of the Nrf2-target gene NQO1 after PH (Fig. 3D, E). As reported by Komatsu et al., the autophagy system is utilized as an additional pathway to provide Nrf2 stabilization by controlling the turnover of Keap1 in a p62 dependent manner [9]. The accumulation of p62 by autophagy dysfunction interacts with the Nrf2-binding site on Keap1, and causes competitive inhibition of the Nrf2-Keap1 interaction, resulting in stabilization of Nrf2 followed by transcriptional activation of Nrf2 target genes. In Ctsl^{-/-} mice, nuclear translocation of Nrf2 is caused by PH accompanied by increases in p62 expression. Thus, it was suggested that Ctsl deficiency enhances p62 and accelerates Nrf2 signaling.

Nrf2 is highly activated in various tumor cells and promotes cellular proliferation through poorly understood mechanisms. It has been demonstrated that Nrf2 increases K-Ras-induced proliferation and Nrf2 regulates metabolic reprogramming by redirecting glucose and glutamine into anabolic pathways in cancer cells to support cancer cell

proliferation [17]. Our current study shows that Nrf2 is a novel regulator of hepatocyte mitosis during liver regeneration. As reported in previous publications, genetic deletion of Nrf2 dysregulates the components of the Wee1/Cdc2/cyclin B1 pathway which is essential for M phase entry [18,19]. Elevated oxidative stress and mitochondrial oxidative damage, observed in the liver after PH, is a fundamental component of liver regeneration. Nrf2 translocates into nucleus and regulates the transcription of genes involved in antioxidant genes [20], therefore, nuclear translocation of Nrf2 accelerated by Ctsl deficiency may suppress accumulation of excess oxidative stress and mitochondrial damage to facilitate liver regeneration. Interestingly, Ctsl deficiency promoted recovery from decrease in hepatic ATP value during liver regeneration after PH more than wild type mice (Fig. 3D).

Notch has been identified as Nrf2 direct target genes in regenerating livers [21]. Nrf2 and Notch signaling pathways are evolutionarily conserved in animal species, from *Drosophila* to *Homo sapiens*. The Notch signaling pathway plays a pivotal role on cell differentiation, survival/apoptosis, and cell cycle [22,23]. The cell cycle promoter *CyclinD1*, the proliferation-related gene *c-Myc*, the anti-apoptotic gene *Bcl2* and *Hes1* have been identified as Notch target genes [24]. In previous work, it has been shown that Notch signaling can be regulated by Nrf2, which in turn effects the rate of liver regeneration following partial hepatectomy [25]. Silencing Notch expression by injection of a Notch-siRNA expression vector suppresses the regenerative response after PH [26]. In contrast, liver specific Notch intracellular domain expression ameliorates liver regeneration in Nrf2-deficient mice [27]. Thus, Nrf2-to-Notch crosstalk is functional in the early phase of liver regeneration. In our experiment, Ctsl deficiency enhanced nuclear translocation of Nrf2 following activation of Notch (Fig. 3E, 4A). Additionally, expression of Hes1, which can directly modulate cellular proliferation, was also up-regulated by Ctsl-deficiency (Fig. 4B). These findings indicate that Nrf2 transcriptional activity is augmented by Ctsl deficiency and accelerated Notch signaling. Interaction of Nrf2-Notch

signaling plays an important role in up-regulation of liver regeneration under conditions of Ctsl deficiency. Interestingly, treatment with specific Ctsl inhibitor also enhanced liver regeneration after PH accompanied by increasing LC3-II and p62 (Supplementary Fig. 1). These findings supported the hypothesis that p62-mediated Nrf2-Notch signaling is essential for up-regulation of liver regeneration in Ctsl $-/-$ mice.

5. Conclusion

In summary, the potentiation of Nrf2 signaling promotes liver regeneration after PH through an accumulation of p62 in Ctsl deficiency. Suppression of cathepsin expression after PH is fundamental to liver regeneration. It is indicated that Ctsl is involved specifically in the degradation of autophagosomal membrane but not essential for autophagic function during liver regeneration after PH. Thus, Ctsl regulation might be a new therapeutic target for disorders of liver regeneration.

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