Research Paper

Autophagy regulates turnover of lipid droplets via ROS-dependent Rab25 activation in hepatic stellate cell

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ABSTRACT

Activation of hepatic stellate cells (HSCs) is a pivotal event in liver fibrosis, characterized by dramatic disappearance of lipid droplets (LDs). Although LD disappearance has long been considered one of the hallmarks of HSC activation, the underlying molecular mechanisms are largely unknown. In this study, we sought to investigate the role of autophagy in the process of LD disappearance, and to further examine the underlying mechanisms in this molecular context. We found that LD disappearance during HSC activation was associated with a coordinate increase in autophagy. Inhibition or depletion of autophagy by Atg5 siRNA impaired LD disappearance of quiescent HSCs, and also restored lipocyte phenotype of activated HSCs. In contrast, induction of autophagy by Atg5 plasmid accelerated LD loss of quiescent HSCs. Importantly, our study also identified a crucial role for reactive oxygen species (ROS) in the facilitation of autophagy activation. Antioxidants, such as glutathione and N-acetyl cysteine, significantly abrogated ROS production, and in turn, prevented autophagosome generation and autophagic flux during HSC activation. Besides, we found that HSC activation triggered Rab25 overexpression, and promoted the combination of Rab25 and PI3KCI, which direct autophagy to recognize, wrap and degrade LDs. Down-regulation of Rab25 activity, using Rab25 siRNA, blocked the target recognition of autophagy on LDs, and inhibited LD disappearance of quiescent HSCs. Moreover, the scavenging of excessive ROS could disrupt the interaction between autophagy and Rab25, and increase intracellular lipid content. Overall, these results provide novel implications to reveal the molecular mechanism of LD disappearance during HSC activation, and also identify ROS-Rab25-dependent autophagy as a potential target for the treatment of liver fibrosis.

1. Introduction

Liver fibrosis represents a naturally occurring wound healing reaction in a wide range of chronic liver injuries [1–3]. As the pathogenesis progresses without effective management, it will lead to formation of liver fiber nodules and disruption of normal liver structure and function, finally culminating in cirrhosis and hepatocellular carcinoma (HCC) [1–3]. Liver fibrosis and early cirrhosis are dynamic and reversible, thus efforts to modulate the fibrogenesis process are important for preventive treatment of cirrhosis and hepatic failure [4]. The transdifferentiation of hepatic stellate cells (HSCs) into contractile, matrix-producing myofibroblasts (MFs) is a central event in hepatic fibrogenesis [5–7]. In healthy liver, quiescent HSCs store 80% of total liver retinols and normally maintain lipocyte phenotype characterized by storage of lipid droplets (LDs) in the cytoplasm [8]. Quiescent HSCs release LDs depending on the extracellular retinol status [8]. However, as a consequence of liver injuries, activated HSCs lose their retinols and display a myofibroblastic phenotype accompanied by excessive production of extracellular matrix (ECM), subsequently leading to liver fibrosis [9,10]. We previously reported that recovering the accumulation of LDs could inhibit the activation of HSCs [11]. The current work was aimed to evaluate the underlying mechanisms of LD disappearance during HSC activation.

It is recognized that the classical triglyceride packaging or lipolysis pathway is involved in LD turnover [12–14]. Previous studies [11,15], including ours [11], have reported that Diacylglycerol acyltransferase 1
(DGAT1), Adipose triglyceride lipase (ATGL), or Hepatic lipase (LIPC) are all new target molecules, and attract extensive concern of numerous scholars. Moreover, transcriptional regulation is also required to maintain the adipogenic phenotype of HSCs [17–19]. The major transcription factors involved in cell adipocyte differentiation include PPARα [16], PPARγ [17], Nrf2 [18], LXR [19], and so on. However, these findings are not enough to fully elucidate the molecular mechanisms of LD disappearance. Studying on underlying mechanism mediated by others can provide new perspectives for the development of therapeutic targets and measures of liver fibrosis. Recently, Friedman SL et al. reported that autophagy released lipid that promoted fibrogenesis by activated hepatic stellate cells in mice and in human tissues [20]. While the canonical pathway for LD catabolism involves cellular lipases that dock to the LD surface, catalyzing the hydrolysis of triacylglycerol and downstream metabolites under normal conditions, autophagy refers to intracellular pathway for a degradative process in which the LDs are engulfed by an autophagosome that subsequently fuses with lysosomes to form autolysosomes [21]. Within the autolysosome, acid lipases break down triacylglycerol into its glycerol and fatty acid components [21]. Interestingly, studying the role of autophagy in pathological conditions will provide a brand new perspective to reveal the pathological mechanism and find the effective diagnostic signs and therapeutic targets in abnormal lipids metabolism.

Autophagy delivers LDs to lytic compartments for degradation, and is mediated through actions of autophagic proteins and lipid droplet-associated proteins [22–28]. Possible candidates to mediate LD targeting are the soluble NSF attachment protein receptors (SNAREs) [22]. Long implicated in LD fusion, SNAREs have recently been described to mediate autophagosome biogenesis [22]. Another possibility is LC3, which is a protein critical for autophagosome membrane formation [23]. The finding that LC3 associates with LDs in the apparent absence of an autophagosomal membrane, suggests that this protein may function in LD recognition [23]. Recently, some significant evidence suggested that lipid droplet-associated proteins Rab7, Perilipin A, TIP47 and ADRP may be responsible for lipid degradation in the course of autophagic internalization of membrane-bound organelles into the vacuole [24–26]. In addition, autophagic proteins Atg18, Atg21 and Atg40 were also reported to be involved in LD biosynthesis [27,28]. These findings provide supports for the role of autophagy in LD catabolism while providing an alternative mechanism by which cells mobilize fat in response to cellular needs and external stimuli.

In the present study, we elucidated the role of autophagy in the process of LD disappearance during HSC activation, and to further examine the underlying mechanisms in this molecular context. We found that autophagy regulated LD turnover via ROS-dependent Rab25 activation during HSC activation. Our study provides a rationale for the identification of autophagy as a new pharmacologic target for the treatment of liver fibrosis.

2. Materials and methods

2.1. Reagents and antibodies

Glutathione (GSH), N-acetyl cysteine (NAC), hydrogen peroxide (H2O2), sodium pyruvate (Na Py), catalase (Cat), superoxide dismutase (SOD), chloroquine (CQ), 3-methyl adenine (3-MA), Bafilomycin A1, dimethyl sulfoxide (DMSO), Mito Peroxy Yellow 1 (MitoPY1), anti-rabbit IgG, and anti-mouse IgG were purchased from Sigma-Aldrich (St Louis, MO, USA). Dulbecco’s modified essential medium (DMEM), Opti MEM medium, phosphate buffered saline (PBS), trypsin-EDTA, and fetal bovine serum (FBS) were bought from Gibco BRL (Grand Island, NY, USA). Oxidation sensitive 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR, USA). Primary antibodies against α-SMA, Desmin, GFAP, LC3-I/II, ULK1, p-ULK1, mTOR, p-mTOR, Atg5, Atg7, Atg9, Atg14, P62, P38 Kinase Class III (PI3KCIII), and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). Primary antibody against α1(I) procollagen was purchased from Epitomix (San Francisco, CA). Primary antibodies against Perilipin A, Rab25, and Fibronectin were purchased from Abcam Technology (Abcam, Cambridge, UK). Atg5 siRNA, Rab25 siRNA, negative control siRNA, Atg5 plasmid constructs and negative control vectors were purchased from Hanbio (Shanghai, China). MegaTran 1.0 transfection reagent was from OriGene (Rockville, MD, USA).

2.2. Animals and experimental design

Male ICR mice (ages 6–8 weeks) were purchased from Nanjing Medical University (Nanjing, China). All mice were bred and maintained in a specific pathogen-free condition. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine (Nanjing, China). A mixture of carbon tetrachloride (CCl4; 0.5 ml per 100 g bodyweight) and olive oil (1: 9 (v/v)) was used to induce liver fibrosis in mice by i.p. injection. Forty mice were randomly divided into four groups of ten animals each with comparable mean bodyweight. Mice of Group 1 were served as a vehicle control and intraperitoneally (i.p.) injected with olive oil. Mice of group 2 were i.p. injected with CCl4 and given administration of Ad.Fc (a control adenovirus encoding IgG2 α Fc fragment). Mice of group 3 were i.p. injected with CCl4 and given administration of Ad.shAtg5 (adenovirus encoding mouse Atg5 shRNA for inhibiting autophagy pathway). Mice of group 4 were i.p. injected with CCl4 and given administration of Ad.shRab25 (adenovirus encoding mouse Rab25 shRNA for inhibiting Rab25). Mice of groups 2–4 were i.p. injected with CCl4 every other day for 4 weeks. Adenoviruses (2.5×10⁷pfu/g, once per 2 weeks) were injected into mice by tail vein.

At the end of the experiment, mice were sacrificed after anesthetization with an injection of 50 mg/kg pentobarbital. A small portion of the liver was removed for histopathological and immunohistochemical studies by fixation with 10% formalin and subsequent embedding with paraffin. The remaining liver was cut in pieces and rapidly frozen with liquid nitrogen for extraction of total RNA and hepatic proteins.

2.3. Histological analysis

Harvested liver tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Liver slices of 5 µm thick were prepared and stained with hematoxylin and eosin and Masson’s trichrome stain by using standard methods. For Sirius red collagen staining, thin sections were deparaffinized and stained with picro-sirius red for 1 h at room temperature. After washes, sections on the slides were dehydrated in 100% ethanol and in xylene, and then they were mounted in Permount. Sirius Red and Masson-stained areas from 10 fields from 3 to 6 mice/group were quantified with ImageJ.

2.4. Cell isolation and cell culture conditions

Primary HSCs were isolated from male ICR mice (Nanjing Medical University, Nanjing, China) as described [29]. Isolated HSCs were cultured in DMEM with 10% fetal bovine serum, 1% antibiotics, and maintained at 37°C in a humidified incubator of 5% CO2 and 95% air. Cell morphology was assessed using an inverted micro-scope with a Leica Qwin System (Leica, Germany).

2.5. Plasmid transfection

Atg5 siRNA, Rab25 siRNA, negative control siRNA, Atg5 plasmid constructs, and negative control vectors were transfected into HSCs using MegaTran 1.0 transfection reagent according to manufacturer’s instructions [30]. Cells were treated with selenite or PBS as a solution control. The transfection efficiency was confirmed by western blot analysis.
2.6. RNA isolation and real-time PCR

RNA was isolated from HSCs using TRIzol (Life Technologies, Waltham, MA) and reverse transcribed into cDNA by Revert Aid reverse transcriptase (Thermo, Fisher Scientific, Waltham, MA). Real-time PCR was performed on a Bio-Rad CFX384™ real-time PCR detection system using iTaq™ Universal SYBR® Green Super mix (Bio-Rad). The following genes were probed with quantitative PCR using β-actin gene as loading control: Perilipin A, ADRP, LC3, Atg3, and Atg6/Beclin1. Primer sequences were as follows: β-actin, 5′-GGTACACCTGGGAGCA-CA′-3′ and 5′-GGGTTGTTGAGGTCTCAAA-3′; Perilipin A, 5′-AACCTGTCGTGGAGGAC-3′ and 5′-GACCTTGTCAAGGTTGCTTG-3′; ADRP, 5′-CTGGAAGGAAAGGACCAAGTCTG-3′ and 5′-ACCAGCTGTGAGGCTCTG-3′; Beclin1, 5′-TATCCAGCAGGTGAAGAT-3′ and 5′-CAAGCGACCCAGTCTGAAAT-3′; Atg3, 5′-GCAAACAAGAACCTATGAC CGT-3′ and CTTCATACATGTGCTCAACTG-3′.

2.7. Western blot analysis and immunoprecipitation

Cells were lysed using mammalian lysis buffer (Sigma St. Louis, MO, USA) and western blot was performed as per the manufacturer’s guidelines (Bio/Rad, Hercules, CA, USA) [30]. Densitometry analysis was performed using Image J software (NIH, Bethesda, MD, USA). For immunoprecipitation, cells were lysed with RIPA buffer for 30 min on ice. PI3KCIII or Rab25 was immunoprecipitated with specific antibodies. Immunoprecipitated complexes were then subjected to Western blot analysis. Specific proteins were immunoprecipitated or detected using antibodies against PI3KCIII or Rab25. β-actin was detected with anti-β-actin (Danvers, MA, USA) for use as loading controls.

2.8. Transmission electron microscopy

Cells were seeded onto 4-chambered coverglass (Lab-Tek Chambered Coverglass System) (Nalgene/Nunc, Rochester, NY, USA) at a density of 2×10^4 cells/ml (14,000 cells/well). Images were acquired using the Olympus EM208S transmission electron microscope.

2.9. Immunofluorescence analysis

Immunofluorescence staining with treated cells was performed as we previously reported [30]. 4′,6-Diamidino-2-phenylindole (DAPI) was used to stain the nucleus of HSCs in vitro. All the images were captured with the fluorescence microscope and representative images were shown. The software Image J was used to quantitate the fluorescent intensity on the micrographs.

2.10. Oil red O staining and Nile Red staining

HSCs were seeded in 6-well plates and cultured in DMEM with 10% FBS for 24 h, and then were treated with drugs for the indicated time periods. HSCs were stained with oil red O reagents (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), or Nile Red reagents (Bioengineering Institute, Nanjing, China), or Nile Red (9-(diethyl amino) benz(a)phenoxazin-5(5H)-one, Sigma-Aldrich) to visualize the lipids with a light microscope (×100 amplification). Images were taken in a blinded fashion at random fields. Results were from triplicate experiments.

2.11. Biochemical analysis

The levels of retinol, triglyceride, and total cholesterol in HSCs were determined by commercially available kits (Nanjing Jiancheng Bioengineering Institute) according to manufacturer’s instructions [11].

2.12. Measurement of intracellular ROS levels

Intracellular ROS level was measured using oxidation-sensitive fluorescent probe DCFH-DA as described previously [30]. To investigate the involvement of particular ROS in autophagy activation, different ROS scavengers such as GSH (10 mM), NAC (10 mM), Na Py (10 mM), Cat (5000 U/ml) and SOD (1000 U/ml) were used. All ROS scavengers were added to the well for 1 h.

2.13. Determination of GSH/GSSG ratio

Intracellular GSH and GSSG levels were determined according to the manufacture protocol [30]. Briefly, cells were lysed in 150 μl of 5% sulfosalicylic acid by freeze and thawing. After centrifugation (10000×g for 10 min), 25 μl of supernatant was mixed with 150 μl of potassium phosphate buffer, PH 7.0, containing 5 mM EDTA, 1.5 mg/ml DTNB and 6 U/ml glutathione reductase. Finally, 50 μl of (0.16 mg/ml) NADPH in potassium phosphate buffer was also added. The absorbance was then quantified at 412 nm using Perkin Elmer (Vicor X3) microplate reader. For the measurement of (GSSG), samples and GSSG standards were treated with 1 M 2-vinylpyridine (10 μl/100 μl sample) and triethanolamine (6 μl/100 μl sample) at room temperature for 1 h. Further reaction was carried out in a similar manner to the total GSH assay.

2.14. Detection of mitochondrial H_{2}O_{2}

MitoPY1 was used to evaluate the mitochondrial-derived H_{2}O_{2} as described previously [30]. Cells (0.5×10^6 cells/well) were seeded in 6-well dishes with complete medium and allowed to grow for 24 h. The cells were then exposed to MitoPY1 (10 mM) for 1 h. The MitoPY1-containing medium was then removed. After the treatment, cells were washed with PBS for three times and the change in the mitochondrial H_{2}O_{2}-derived fluorescence was examined under Olympus DP71 fluorescence microscope.

2.15. Calculations and statistics

Individual culture experiments and animal experiments were performed in duplicate or triplicate and repeated three times using matched controls, and the data were pooled. Results were expressed as either SD or mean ± standard error of the mean (SEM). The statistical significance of differences (*p < 0.05) was assessed by t-test.

3. Results

3.1. LDs disappear in cytoplasm during HSC activation in vitro

Previous studies [30,31], including ours [30], have confirmed that HSC activation in vivo, as a result of different liver injuries, could be mimicked in vitro by plating freshly isolated HSCs on plastic tissue culture dishes. In agreement with previous findings [30,31], after 7 days in culture, HSC activation markers like α-SMA, procollagen 1α1, Desmin, GFAP, and Fibronecin were significantly up-regulated showing that HSCs undergo an activation process in vitro as well (Fig. 1A) (Supplementary Fig. 1A). Next, we investigated the changes of LDs during HSC activation. Nile red staining showed that quiescent HSCs cultured for 3 days dramatically decreased LDs, and after 7 days in culture, LDs almost disappeared in cytoplasm (Fig. 1B). Moreover, Oil red O (ORO) staining and absorbance were also used to determine intracellular lipid content [32]. Freshly isolated HSCs cultured in vitro for 3 days could lose 50% of the intracellular lipid content, while quiescent HSCs decreased more than 80% for 7 days in culture (Fig. 1C). Importantly, it is well known that LDs of HSCs mainly
content retinol, triglycerides and cholesterol [5–10]. Therefore, we detected the level of retinol, triglycerides and cholesterol from quiescent HSCs to activated HSCs, respectively. As expected, retinol, triglycerides and cholesterol decreased 80%, 70%, and 75% in HSCs cultured for 7 days, respectively (Fig. 1D, E). Lastly, lipid droplet-associated proteins Perilipin A and ADRP, which appear to be intrinsic to the surfaces of intracellular lipid storage droplets [33], were detected by Real-time PCR. We found that the mRNA expression of Perilipin A and ADRP was decreased with the activation of HSCs in vitro (Supplementary Fig. 1 B). Overall, these results indicate that LD disappearance is considered one of the hallmarks of HSC activation.

3.2. The autophagosome generation and autophagic flux are increased during HSC activation in vitro

Numerous studies [34–36] in hepatocytes have shown a crucial role for autophagy in LD metabolism. In this study, we hypothesized that autophagy could play a pivotal role in LD disappearance during HSC activation. To test this hypothesis, we employed different biological methods to investigate the changes of autophagy in freshly isolated HSCs from day 0 till day 7 in culture. First of all, extensive cell-based studies of levels of LC3 conjugated to LC3-II (Fig. 2A) and other indicators of autophagy including p-ULK1, ULK1, p-mTOR, and mTOR confirmed that the formation of autophagosomes was increased and these responses were associated with an increase in p-ULK1 activity and a decrease in p-mTOR activity (Fig. 2B). In addition, seven important pro-autophagic proteins in formation of autophagosomes were detected by western blot and Real-time PCR analysis in both quiescent and activated HSCs, respectively 1 and 5 days in culture. The results revealed that many components of autophagosome generation including LC3-I/II, Atg3, Atg5-Atg12, Atg6/Beclin1, Atg7, Atg9 and Atg14 were all upregulated in 5-day cultured HSCs compared to 1-day cultured HSCs (Supplementary Fig. 2A and B). Moreover, immunofluorescent staining was performed on quiescent and activated HSCs, respectively 0, 1, 3, 5 and 7 days in culture, using an Atg5 specific antibody. The results showed that the expression of Atg5 was increased with HSC activation (Fig. 2C). Lastly, immunofluorescent staining of ectopic GFP-LC3 (data not shown) and endogenous LC3-II (Fig. 2D) confirmed that the autophagosome generation was increased during HSC activation.

Next, we employed four different methods to assess autophagic flux in freshly isolated HSCs cultured for 7 days, respectively. Firstly, we assessed the levels of p62 from quiescent HSCs to activated HSCs. p62 accumulates when autophagy is inhibited, and is decreased when there is autophagic flux [37]. Immunofluorescent staining of endogenous p62 showed significant decrease from day 0 till day 7 in culture, indicating increased autophagic induction (Fig. 2E). Secondly, the protein expression of p62 was also determined at different time points. Western
blot analysis showed that 5-day cultured HSC resulted in a significant inhibitory effect, by which referred to an increase of autophagic flux in activated HSCs (Fig. 2F). Thirdly, we observed an increase in LC3-II level in cells which were cultured for 5 days followed by chloroquine (CQ) treatment compared with cells which were cultured for 5 days alone, suggesting that autophagic flux is increased during HSC activation (Fig. 2G). Lastly, the transmission electron microscopy (TEM) was used to observe autophagy. As expected, we observed the presence of a high level of autophagosomes or lysosomes in 7-day cultured HSCs. In contrast, it was difficult to observe autophagosomes or lysosomes in control HSCs (Fig. 2H). Collectively, these results support that autophagic flux is increased during HSC activation.

3.3. The inhibition of autophagy blocks LD disappearance of quiescent HSCs and reverses lipocyte phenotype of activated HSCs in vitro

To determine whether autophagy activation is directly involved in LD disappearance of quiescent HSCs, we used Atg5 siRNA to block autophagosome formation. Western blot analysis confirmed that Atg5 siRNA not only reduced Atg5 levels but also significantly decreased the expression of LC3-II (Fig. 3A). Then, ORO colorimetric absorbance was preformed to measure its effects on LD disappearance in freshly isolated HSCs, respectively 1, 3, and 5 days in culture. As expected, Atg5 siRNA blocked LD disappearance and maintained intracellular
lipid content of quiescent HSCs (Fig. 3B). Furthermore, retinol and triglyceride content were also determined in quiescent HSCs cultured from 1 day to 5 days. The results showed Atg5 siRNA not only maintained lipocyte phenotype of quiescent HSCs, but also increased intracellular retinol and triglyceride content of 1-day cultured HSCs (Fig. 3C). In addition, Nile red staining was performed on quiescent and activated HSCs, respectively 1, 3 and 5 days in culture. Our data revealed that Atg5 siRNA prevented the loss of LDs in freshly isolated HSCs cultured for 5 days (Fig. 3D). Besides, lipid droplet-associated proteins Perilipin A and ADRP were also detected by Real-time PCR. We found that the treatment of Atg5 siRNA impaired the decrease of Perilipin A and ADRP expression in the same 5-day culture (Supplementary Fig. 3A and B).

Next, we investigated whether inhibition of autophagy can reverse the lipocyte phenotype of activated HSCs. Activated (day 5) HSCs were treated with selective inhibitors 3-MA, Baﬁlomycin A1 or Atg5 siRNA for 24 h. ORO colorimetric absorbance showed that intracellular lipid content was increased by above 3 folds in Atg5 siRNA or autophagy inhibitors treated HSCs compared to control HSCs (Fig. 3F). Furthermore, main components of LDs, retinol, triglyceride and cholesterol [5–10], were determined to reflect changes of LD content in activated HSCs. A nearly 1.5-fold increase in retinol, triglyceride and cholesterol content could be evidenced (Fig. 3G). The mRNA expression of lipid droplet-associated proteins Perilipin A and ADRP also showed consistent results in activated HSCs treated by autophagy inhibitors 3-MA, Baﬁlomycin A1 or Atg5 siRNA (Supplementary Fig. 3C and D). Lastly, TEM observation revealed that LDs in control-siRNA treated HSCs were almost wrapped by autophagic vesicle, which was an important process for the degradation of LDs (Fig. 3E). By contrast, LDs in Atg5-siRNA treated HSCs reappeared in the cytoplasm, and double membrane structures of autophagy almost disappeared, suggesting that activated HSCs restored the lipocyte phenotype (Fig. 3E). Taken together, these findings demonstrate that inhibition of autophagy blocks LD disappearance of quiescent HSCs and reverses lipocyte phenotype of activated HSCs.

3.4. The induction of autophagy accelerates LD disappearance in quiescent HSCs in vitro

To assess the impact of autophagy activation on intracellular lipid content, freshly isolated HSCs were treated with Atg5 plasmid (Plasmid construction encoding Atg5). As shown in Fig. 4A, Atg5 plasmid not only increased Atg5 levels but also significantly up-regulated the expression of LC3-II. Then, ORO colorimetric absorbance was preformed to measure its effects on LD disappearance in freshly isolated HSCs cultured for 5 days (Fig. 4D). Besides, lipid droplet-associated proteins Perilipin A and ADRP were also detected by Real-time PCR. We found that the treatment of Atg5 siRNA impaired the decrease of Perilipin A and ADRP expression in the early stage of HSC activation, which is a well-characterized indicator of fibrosis (Fig. 4C). Importantly, immunofluorescent

Fig. 3. The inhibition of autophagy blocks LD disappearance of quiescent HSCs and reverses lipocyte phenotype of activated HSCs. freshly isolated HSCs were treated with control-siRNA or Atg5-siRNA, and then were cultured for 5 days. (A) Western blot and densitometric analysis showing LC3-I/II levels. (B) ORO absorbance showing the changes of lipid droplets content. (C) Retinol and triglycerides measurement showing the changes of lipid droplets content. (D) Nile red staining showing the changes of cellular lipid content. Activated HSCs were treated with control-siRNA, control-vehicle, Atg5-siRNA, 3-MA or Baﬁlomycin A1. (E) TEM displaying the content of lipid droplets. (F, G) ORO absorbance, retinol, triglycerides and cholesterol measurement showing the changes of lipid droplets content. For the statistics of each panel in this figure, data are expressed as mean ± SD (n=3); *P < 0.05 versus control, **P < 0.01 versus control, ***P < 0.001 versus control.

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staining was performed on quiescent HSCs from day 0 till day 5 in culture, using lipid droplet-associated protein Perilipin A specific antibody. The expression of Perilipin A was observed in 1-day cultured HSCs with control-Vector treatment, while the treatment of Atg5 plasmid promoted their disappearance in the same 1-day cultured HSCs (Fig. 4C). Overall, these data suggest that the induction of autophagy accelerates LD disappearance in quiescent HSCs in vitro.

3.5. The overexpression of Rab25 is required for autophagy to degrade LDs during HSC activation in vitro

Accumulating evidence suggests that autophagy delivers LDs to lytic compartments for degradation, which is mediated through actions of autophagic proteins and lipid droplet-associated proteins [22–28]. It has been reported that the small GTPase Rab7 was highly activated under nutrient deprivation and was required to promote direct physical interactions between autophagy and LDs in live hepatocellular carcinoma cells [24]. Besides, Rab18 has also been reported to be relevant Rab-GTPase for stellate cells as it dynamically modulated both LD size and cellular activation [19]. Interference with Rab18 may therefore have significant therapeutic benefit in ameliorating liver fibrosis [19]. In the current study, we hypothesized that Rab25 could play a pivotal role in autophagy-dependent LD disappearance during HSC activation. To test this hypothesis, the protein expression of Rab25 was detected in quiescent HSCs from day 0 till day 7 in culture. As shown in Fig. 5A, we observed robust upregulation of Rab25 in 7-day culture period, which was consistent with the LD disappearance of freshly isolated HSCs. Then, Rab25 siRNA was used to down-regulate Rab25 expression for reverse verification. ORO colorimetric absorbance showed that the treatment of Rab25 siRNA blocked LD disappearance and maintained intracellular lipid content of quiescent HSCs (Fig. 5B). Furthermore, retinol and triglyceride content were also determined in quiescent HSCs cultured from 1 day to 5 days. Our data revealed that Rab25 siRNA prevented the loss of LDs in freshly isolated HSCs cultured for 5 days (Fig. 5C). In addition, Nile red staining was performed on quiescent and activated HSCs, respectively 1, 3 and 5 days in culture. The results showed that Rab25 siRNA maintained lipocyte phenotype of quiescent HSCs (Fig. 5D). Collectively, these results prompt that Rab25 serves as an important mediator of LDs loss and acquisition of fibroblastic phenotype.

Attractively, previous studies in other cells have shown that interaction between Rab-GTPase and PI3KCIII played an important role in the development of a variety of diseases [38,39]. Thus, we speculated that the binding of PI3KCIII to Rab25 direct autophagy to recognize, wrap and degrade LDs during HSC activation. Indeed, as shown in immunoprecipitation assay, the binding of PI3KCIII to Rab25 increased gradually with the activation of HSCs (Fig. 5E). Besides, the co-localization of PI3KCIII and Rab25 further showed that the interaction between Rab25 and PI3KCIII increased in quiescent HSCs cultured from 1 day to 5 days (Fig. 5F). Interestingly, in order to further verify the hypothesis that overexpression of Rab25 is required for autophagy to degrade LDs during HSC activation, both Rab25 siRNA and Atg5 plasmid were used to treat quiescent HSCs. As shown in Fig. 5G, the treatment of control-Vector did not result in LD
disappearance in Rab25-siRNA treated HSCs, following 5-day culture. Importantly, the treatment of Atg5-plasmid also did not lead to LD loss in 5-day cultured quiescent HSCs treated by Rab25 siRNA (Fig. 5G). Taken together, these data support that Rab25 mediates recognition of autophagy on LDs during HSC activation.

3.6. ROS generation triggers autophagy activation and Rab25 overexpression during HSC activation in vitro

Several reports have recently provided strong evidence for the involvement of ROS in the induction of autophagy in response to chemotherapy-induced stress [40,41]. We assumed that ROS generation triggered autophagy activation and Rab25 overexpression during HSC activation. To evaluate this assumption, we first examined the ROS content in quiescent HSCs from day0 till day7 in culture. As expected, cellular ROS gradually occurred to accumulation in the process of HSC activation (Fig. 6A). Next, freshly isolated HSCs were treated with thiold-based ROS scavengers such as GSH and NAC, following by 5-day culture. ROS content, autophagosome generation, autophagic flux, Rab25 expression, and intracellular lipid content were all determined. The results showed that treatment with GSH and NAC completely abrogated the ROS accumulation (Fig. 6B), and in turn, impaired LC3-II conversion (Fig. 6C), autophagosome generation (Fig. 6C), autophagic flux (Fig. 6D), the binding of P38KCI to Rab25(Fig. 6E), and LD disappearance (Fig. 6F). Overall, these results suggest that ROS generation plays an important role in the autophagy-dependent LD disappearance.

3.7. Mitochondrial H$_2$O$_2$ production and reduction in GSH/GSSG ratio trigger autophagy activation and LD disappearance during HSC activation in vitro

It is agreed that mitochondrial respiratory chain is a major source of ROS production in eukaryotic cells [42]. To directly assess whether HSC activation could induce the production of mitochondrial-derived H$_2$O$_2$, a mitochondrial specific fluorescent probe that selectively turn-on fluorescence enhancement in response to H$_2$O$_2$ was used [43]. As shown in Fig. 7A, an increase in the Mito-PY1-derived fluorescence was observed in activated HSCs (Day 5) or quiescent HSCs (Day 0) treated with H$_2$O$_2$ (used as positive control) compared to quiescent HSCs. To confirm the role of H$_2$O$_2$ production in autophagy activation and LD disappearance, Na Py (sodium pyruvate), a potent intracellular scavenger of H$_2$O$_2$ was used. The results showed that pretreatment with Na Py completely blocked the MitoPY1-derived fluorescence induced by HSC activation ( Fig. 7A). Furthermore, we found that Na Py prevented LC3-II conversion (Fig. 7B), autophagosome generation (Fig. 7B), autophagic flux (Fig. 7C), and LD loss (Supplementary Fig. 4A, B) in activated HSCs. To investigate whether O$_2$ - also contributes to autophagy activation and LD disappearance, Na Py (sodium pyruvate), a potent intracellular scavenger of H$_2$O$_2$ was used. The results showed that pretreatment with Na Py completely blocked the MitoPY1-derived fluorescence induced by HSC activation (Fig. 7A). Furthermore, we found that Na Py prevented LC3-II conversion (Fig. 7B), autophagosome generation (Fig. 7B), autophagic flux (Fig. 7C), and LD loss (Supplementary Fig. 4A, B) in activated HSCs. To investigate whether O$_2$ - also contributes to autophagy activation and LD disappearance, SOD (a specific scavenger of O$_2$ -) and Cat (a specific scavenger of H$_2$O$_2$) were used in the further study. Similarly, we found that pretreatment with Cat significantly blocked the LC3-II conversion (Fig. 7D) and LD loss (Supplementary Fig. 4C, D) in activated HSCs. However, pretreatment with SOD failed to induce the same effect (Fig. 7D) (Supplementary Fig. 4C, D). Altogether, these results clearly show that mitochondrial H$_2$O$_2$ production triggers autophagy activation and LD disappearance during HSC activation in vitro. Lastly, we assessed the changes of GSH/GSSG ratio during HSC activation. Interestingly, the results
revealed that quiescent HSCs decreased more than 50% in the cellular GSH/GSSG ratio for 7 days in culture (Fig. 7E). Next, we analyzed the role of H2O2 in the modulation of GSH/GSSG ratio using Na Py or Cat. As expected, pretreatment with Na Py or Cat effectively inhibited the reduction in GSH/GSSG ratio in activated HSCs (Fig. 7E). Collectively, these data demonstrate that the reduction in GSH/GSSG ratio can trigger autophagy activation and LD disappearance during HSC activation in vitro.

3.8. Rab25-dependent autophagy regulates LD turnover of HSCs in vivo

We further examined whether disruption of autophagy could affect LD turnover of activated HSCs in vivo. Forty mice were randomly divided into four groups of ten animals each with comparable mean bodyweight. Mice of four groups were administrated with Vehicle control, Ad.Fc, Ad.shAtg5 or Ad.shRab25, respectively, throughout the 4-week period of CCl4 treatment. First and foremost we evaluated the effect of interrupting autophagy on liver injury in vivo. Gross examination showed that morphological changes pathologically occurred in the mouse liver exposed to CCl4 compared with the normal liver, but Ad.shAtg5 or Ad.shRab25 treatment improved the pathological changes in livers (Fig. 8A). Liver fibrosis was also demonstrated by histological analyses. Hematoxylin and eosin (H & E), Masson and picro-Sirius red staining showed that Ad.shAtg5 or Ad.shRab25 treatment significantly ameliorated histopathological feature of liver fibrosis characterized by decreased collagen deposition, whereas livers derived from mice treated with Ad.Fc plus CCl4 exhibited more severe liver fibrosis (Fig. 8A). Next, primary HSCs were isolated for detection of intracellular lipid content. Western blot and immunofluorescence analysis showed that Ad.shRab25 down-regulated the expression of Rab25, while Ad.shAtg5 inhibited Atg5 expression (Fig. 8B), LC3-II conversion (Fig. 8B), and autophagic flux (Fig. 8C) in freshly isolated HSCs. Then, we evaluated the effect of interrupting autophagy on LD disappearance of HSCs in vivo. Retinol and triglyceride content were determined in primary HSCs isolated from CCl4-treated mouse liver. Our data revealed that the treatment of Ad.shAtg5 or Ad.shRab25 alleviated CCl4-induced LD loss of primary HSCs (Fig. 8D). Lastly, Nile red staining further showed that CCl4 treatment promoted HSCs to loss LDs, while the treatment of Ad.shAtg5 or Ad.shRab25 maintained lipocyte phenotype of quiescent HSCs (Fig. 8E). Collectively, these data reveal that Rab25-dependent autophagy regulates LD turnover of HSCs in vivo.

4. Discussion

HSCs were initially termed “fat-storing cells”, which derives from the large amount of neutral lipids that are stored in numerous LDs in the cytosol [5–8]. Generally, it is agreed that retinol and triglyceride are present at similar concentrations in LDs obtained from primary rat
HSCs isolated from well-nourished rats [5-8]. Besides, cholesterol, cholesteryl ester, phospholipids and free fatty acids are also reported to be present in rat HSC LDs [5-8]. However, following liver injury, whether due to chronic alcohol consumption, viral infection, xenobiotic exposure, iron overload or another less common insult, HSCs undergo a process known as activation, which is a transition of quiescent cells into proliferative, fibrogenic and contractile myofibroblasts [9,10]. This is accompanied by a series of changes in gene expression patterns that collectively give rise to responses that include cell proliferation and contractility, fibrogenesis, chemotaxis, new and altered ECM deposition, cytokine release and LD loss [9,10]. Free fatty acids released by LD degradation are thought to serve as energy sources for transdifferentiation of HSCs into myofibroblast-like cells to produce fiber including α-SMA and collagen, suggesting that LD degradation closely correlates to HSC activation [9,10]. Despite the fact that LD accumulation represents the most characteristic feature of quiescent HSCs and that their loss is a hallmark of HSC activation, their biological roles in HSC activation and the underlying signaling events remain to be elucidated. Recently, Friedman SL et al. reported that autophagy released lipid that promoted fibrogenesis by activated hepatic stellate...
cells in mice and in human tissues [20]. Eichmann TO et al. demonstrated that ATGL and CGI-58 were lipid droplet proteins of the hepatic stellate cell line HSC-T6 [44]. Rat homologue of ATGL not only hydrolyzed triglyceride, but also degraded retinol in the presence of rat CGI-58, and thus may participate in the breakdown of both neutral lipid esters in HSCs [44]. Furthermore, Yuen JJ et al. also reported that diacylglycerol O-acyltransferase 1 (DGAT1) catalyzed the final step of triglyceride synthesis, transferring an acyl group from acyl-CoA to diacylglycerol, and thus had a role in retinoid storage and metabolism in HSCs [45]. Interestingly, we previously investigated the role of the canonical Wnt/β-catenin pathway in regulation of HSC lipogenesis [11]. In the current study, we evaluated the role of autophagy in the process of LD disappearance, and further examined the underlying mechanisms in this molecular context.

Autophagy maintains cellular homeostasis by degrading proteins, lipids and organelles in lysosomes. Turnover of nonfunctional cellular components by autophagy ensures quality control and recycling of degraded products provides energy. In addition to the classic pathway of lipid metabolism by cytosolic lipases, LDs are sequestered in autophagosomes that fuse with lysosomes for the breakdown of lipid droplet components by lysosomal enzymes [21]. The ability of autophagy to respond to changes in nutrient supply allows the cell to alter LD metabolism to meet cellular energy demands. Pathophysiological changes in autophagic function can alter cellular lipid metabolism and promote disease states. Autophagy therefore represents a new cellular target for abnormalities in lipid metabolism and accumulation [21]. Recently, Hernandez-Gea V and Friedman SL demonstrated that inhibition of autophagy resulted in lipid accumulation in HSCs, indicating a quiescent phenotype, and attenuated liver fibrosis in vivo [46]. Besides, Miyamae Y et al. also found that tetrandrine-induced lipid accumulation through blockade of autophagy in a hepatic stellate cell line could alleviate liver injury [47]. In the current study, we showed that autophagosome generation and autophagic flux were both increased during HSC activation, and these responses were associated with an increase in p-ULK1 activity and a decrease in p-mTOR activity (Fig. 2). Inhibition or depletion of autophagy by Atg5 siRNA impaired LD disappearance of quiescent HSCs, and also restored lipocyte phenotype of activated HSCs (Fig. 3). In contrast, induction of autophagy by Atg5 plasmid accelerated the LD loss of quiescent HSCs (Fig. 4). Altogether, these results supported that targeting inhibition of autophagy in HSCs could reverse lipocyte phenotype of activated HSCs and then ameliorate liver injury and fibrosis.

Fig. 8. Rab25-dependent autophagy regulates LD turnover of HSCs in vivo. Forty mice were randomly divided into four groups of ten animals each with comparable mean bodyweight. Mice of four groups were administrated with Vehicle control, Ad.Fc, Ad.shAtg5 or Ad.shRab25, respectively, throughout the 4-week period of CCl4 treatment. (A) The pathological changes of the liver were observed by Gross examination, Scale bar, 1 cm. Liver sections were stained with hematoxylin and eosin, Masson reagents and Sirius red. Representative photographs are shown. (B) Primary HSCs were isolated for detection of Rab25, Atg5, and LC3-I/II expression. (C) P62 immunostaining was used to show autophagic flux in isolated HSCs. (D) Retinol and triglycerides measurement were used to show the changes of lipid droplets content in isolated HSCs. (E) Nile red staining was performed to show the changes of cellular lipid content. For the statistics of each panel in this figure, data are expressed as mean ± SD (n=6); **P < 0.01 versus CCl4 treatment, ***P < 0.001 versus CCl4 treatment; ##P < 0.01 versus vehicle control, ###P < 0.001 versus vehicle control.

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alcoholic fatty liver disease in mice [52]. Taken together, these data reveal that targeting inhibition of autophagy in hepatocytes could induce lipid accumulation, impair β-oxidation, and aggravate liver injury. Autophagy plays a different role in different pathogenesis and different liver diseases. The effect of targeting autophagy on the whole liver depends on the specific disease state.

Autophagy delivers LDs to lytic compartments for degradation, which is mediated through actions of autophagic proteins and lipid droplet-associated proteins [22–28]. Recently, accumulating evidence suggests that Rab GTPase family plays a key role in the recognition of LDs by autophagy [19,24]. Rab GTPases are members of the Ras GTPase superfamily that broadly control budding, motility and fusion of vesicles in most cell types [53]. Rab proteins interconvert between active, GTP-bound form and inactive, GDP-bound form. In their active conformation, they interact with various effector molecules to carry out diverse functions [53]. Rab GTPases are usually small containing only a C-terminal prenylation site for membrane anchoring [53]. Interestingly, Schroeder B et al. reported that Rab GT-Pase Rab7 was highly activated under nutrient deprivation and was required to promote direct physical interactions between autophagy and LDs in live hepatocellular carcinoma cells [24]. Moreover, Wang et al. showed that Rab32 is important for autophagy and lipid storage in drosophila [54]. Besides, Rab18 has also been reported to be relevant Rab-GT-Pase for stellate cells as it dynamically modulated both LD size and cellular activation [19]. In the current study, for the first time, we determined the importance of Rab25 in LD disappearance by autophagy. We found that HSC activation triggered Rab25 overexpression, and promoted the combination of Rab25 and PI3KCh, which direct autophagy to recognize, wrap and degrade LDs. Down-regulation of Rab25 activity, using Rab25 siRNA, blocked the target recognition of autophagy on LDs, and inhibited LD disappearance of quiescent HSCs (Fig. 5). To our knowledge, this is the first report that the over-expression of Rab25 is required for autophagy to degrade LDs during HSC activation. Attractively, since Rab25 shares 63% homology with the ubiquitous Rab11A, Rab25 function is thought to mirror that of Rab11A [55,56]. Casanova JE et al. reported that interaction between Rab25 and Rab11A was associated with the apical recycling system of polarized Madin-Darby canine kidney cells [57]. Besides, the competition assay by Lall P et al. revealed that Rab25 can out-compete Rab14 for binding to FIP2 in vitro [58]. Moreover, He H et al. reported that Rab25 expression was upregulated in a case series of 11 hepatocellular carcinomas and a cholangiohepatoma along with dysregulated expression of a number of other Rab proteins including Rab18, Rab4B, Rab10, Rab22A and Rab24 [59]. In view of the above-mentioned facts, we conservatively hypothesized that Rab25 may interact with other Rab GTPases to play a function in HSCs. In the further research, we will study the interaction between Rab25 and other Rab proteins in LD disappearance during HSC activation.

Importantly, we identified ROS as upstream inducers that trigger autophagy activation and Rab25 overexpression during HSC activation. Treatment with Antioxidants, such as glutathione and N-acetyl cysteine, significantly abrogated ROS production, and in turn, prevented autophagosome generation and autophagic flux during HSC activation (Fig. 6). Interestingly, the scavenging of excessive ROS could also disrupt the interaction between autophagy and Rab25, and increase intracellular lipid content (Fig. 6). Our results indicate a similar function of ROS in HSCs in consistent with previous reports [60,61]. Rodriguez-Vargas JM et al. reported that ROS-induced DNA damage and PARP-1 were required for optimal induction of starvation-induced autophagy [60]. Furthermore, Kongara S et al. showed that ROS accumulation upregulated autophagy levels, which likely contributed to their tumor-initiating capacity [61]. Although our data suggested direct connection between ROS and autophagy as a likely cause of LD disappearance, we could not eliminate other indirect effects that may mediate HSC activation. Interestingly, it is well established that endoplasmic reticulum (ER) stress can affect various stages of autophagy, including autophagy induction, vesicle nucleation, and elongation of the phagophore [62,63]. Gao W et al. reported that autophagy induction in response to fluctuation in the cytosolic Ca2+ was directly regulated by ER stress in different conditions [62]. Qin L et al. also showed that ER stress resulted in the inactivation of the Akt pathway, contributing to the decrease of mTOR activity and subsequent autophagy induction [63]. It is interesting to study the role of ER stress in autophagy activation and LD loss during HSC activation.

In summary, these results provide the first mechanistic evidence that HSC activation-induced LD disappearance in liver fibrosis is associated with ROS-Rab25-dependent activation of autophagy. These findings are especially noteworthy since it is possible that pharmacological or hormonal regulation of autophagy may be useful therapeutic strategies for liver fibrosis.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2016.12.021.

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