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REVIEW

Shifting cell death modes in hepatic steatosis: from apoptosis to necroptosis

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Abstract. In the liver, hepatocyte death occurs during the regeneration process following injury. While hepatocyte death triggers regeneration through hepatocyte proliferation in the non-steatotic liver, it impairs this process in the steatotic liver. Both the number and mode of hepatocyte death during regeneration change in the steatotic liver, affecting regeneration and thereby contributing to the progression of acute liver injury and metabolic dysfunction-associated steatotic liver disease (MASLD). Apoptosis, a non-inflammatory mode of cell death, predominantly occurs during liver regeneration. As hepatic steatosis progresses, sporadic and scattered apoptotic cell death increases, leading to delayed regeneration. In severe steatotic livers undergoing regeneration, the mode of cell death shifts to pro-inflammatory necroptosis. This transition leads to inflammation around the dead hepatocytes, resulting in zonal hepatocyte death and further impairing regeneration, thus exacerbating acute liver injury and MASLD. The integrated stress response (ISR), mediated by phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α), plays a crucial role in regulating hepatocyte death during steatotic liver regeneration. The ISR-induced transcription factor C/EBP homologous protein (CHOP) promotes apoptosis, thereby delaying regeneration. When ISR is further enhanced, activating transcription factor 3 (ATF3) is upregulated, inducing the expression of receptor-interacting protein kinase 3 (RIPK3), which shifts cell death mode from apoptosis to necroptosis. While treatments for MASLD targeting apoptosis have shown limited success, future therapies targeting necroptosis and its regulatory molecules may provide novel therapeutic strategies.

Key words: MASLD/MASH, Apoptosis, Necroptosis, Integrated stress response, Regeneration

Introduction

The liver has a remarkable regenerative capacity. In rodents, liver mass recovers within just one week following a 70% partial hepatectomy [1, 2]. The 70% partial hepatectomy model in rodents allows analysis of liver regeneration without the confounding influence of injury etiology, such as hepatic toxins (*e.g.*, carbon tetrachloride) [2, 3]. Moreover, because partial hepatectomy can be performed within a few minutes, the time course of liver regeneration can be precisely monitored [4]. In the healthy non-steatotic liver, hepatocytes are typically in the G0 phase of the cell cycle and rarely undergo division [1]. However, hepatectomy induces hepatocyte proliferation following a priming phase that lasts approximately five hours [5, 6]. During this priming phase, levels of hepatocyte growth factor, tumor necrosis factor-α (TNFα), and interleukin-6 increase, promoting entry of most hepatocytes into the cell cycle [1, 6]. After hepatectomy, DNA synthesis begins at 12 hours, and hepatocyte proliferation peaks at 24 hours in rats and at 36–48 hours in mice, ultimately resulting in an average of 1.6 cycles of replication before liver mass is restored (Fig. 1) [1, 3, 5].

Hepatocyte death, alongside proliferation, also occurs during liver regeneration. In the non-steatotic liver, hepatocyte death appears in a scattered pattern in the remnant liver during regeneration, which peaks at 24 hours after partial hepatectomy in mice (Fig. 1) [7]. In the steatotic liver, hepatocyte death increases during liver regeneration. While hepatocyte death during regeneration promotes hepatocyte proliferation in non-steatotic livers [8], its increase in steatotic livers impairs regeneration, leading to delayed recovery and exacerbation of liver injury [9, 10]. Because impaired liver regeneration in hepatic steatosis contributes to the progression of drug-induced liver injury and metabolic dysfunction-associated steatotic liver disease (MASLD) [9, 10], a thorough understanding

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of hepatocyte death during the regenerative process is essential for elucidating their pathogenesis.

The shift in the role of hepatocyte death—from triggering regeneration to contributing to its failure—is closely linked to both the "number" and "mode" of cell death. Compared to non-steatotic livers, the number of hepatocyte death during regeneration increases significantly in steatotic livers. In a steatotic liver model induced by high-fat diet feeding, hepatocyte death during regeneration after 70% partial hepatectomy increases in accordance with the severity of hepatic steatosis [11]. Not only the "number" but also the "mode" of hepatocyte death changes in accordance with the severity of hepatic steatosis. In mice with moderate hepatic steatosis (30–60% of hepatocytes with lipid droplets), hepatocyte death after partial hepatectomy occurs *via* non-inflammatory apoptosis. However, in severe hepatic steatosis (where more than 60% of hepatocytes contain lipid droplets), pro-inflammatory necroptosis occurs, leading to extensive zonal cell death, which exacerbates liver injury (Fig. 2) [11, 12]. In regenerating severely steatotic livers, necroptosis likely triggers a vicious cycle of cell death and inflammation around dying hepatocytes, further worsening liver injury.

Apoptosis and necroptosis are known to coexist in the liver [13], and progress is being made to elucidate the mechanisms that regulate each mode of cell death and the transitions between them. In this article, we focus on how the number and mode of hepatocyte death are regulated during steatotic liver regeneration, with particular emphasis on the apoptosis-to-necroptosis transition as hepatic steatosis severity increases.

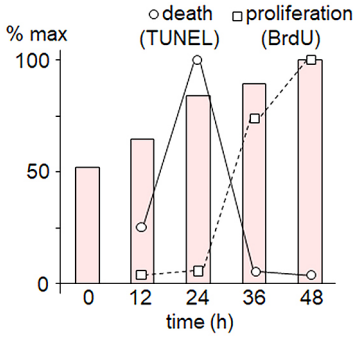


Fig. 1 Early hepatic response after partial hepatectomy

Changes in liver weight, hepatocyte death, and hepatocyte proliferation up to 48 hours after 70% partial hepatectomy. This figure was modified and reconstructed as a conceptual illustration based on the data presented in the publication of [7]. The bar graph represents the liver-to-body weight ratio (L/B ratio); open circles represent hepatocyte death (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells); and open squares represent hepatocyte proliferation (BrdU-positive cells), all shown as a percentage of the maximum value within 48 hours.

Non-Steatotic Liver Regeneration and Apoptosis

Apoptosis is a representative mode of cell death occurring during liver regeneration. It is a caspase-dependent, energy-requiring process characterized by cell shrinkage, plasma membrane blebbing, and chromatin condensation [14-16]. These morphological changes typically occur within 2–12 hours after apoptosis induction, during which apoptotic cells are phagocytosed by scavengers, such as Kupffer cells and stellate cells [17, 18]. Since plasma membrane integrity is preserved and apoptotic cells are rapidly cleared, only minimal amounts of pro-inflammatory intracellular molecules, known as damage-associated molecular patterns (DAMPs), are released, making apoptosis a non-inflammatory process [17, 19]. However, in cases of extensive apoptosis, if apoptotic cells are not efficiently phagocytosed and persist, secondary necrosis may occur—characterized by rupture of the plasma membrane, cell swelling, and the release of DAMPs [18, 20].

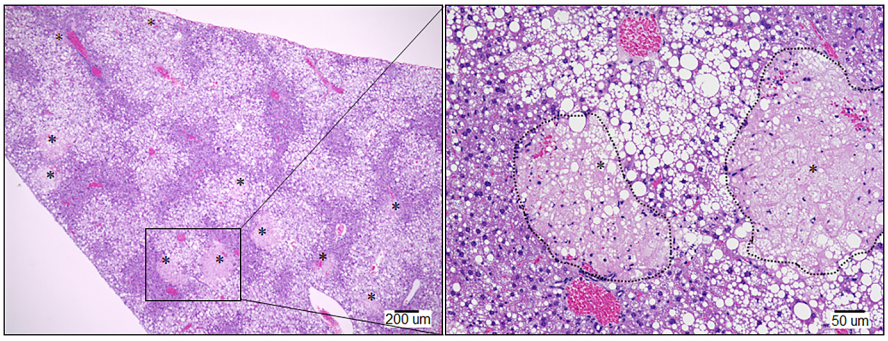


Fig. 2 Zonal hepatocyte death in severe steatosis after hepatectomy

Liver histology at 48 hours after partial hepatectomy in mice fed a high-fat diet for 16 weeks. Haematoxylin-eosin staining. Scale bars, 200 μm (left) and 50 μm (right). Asterisks indicate hepatocellular death foci.

Apoptosis is induced *via* two main mechanisms: the intrinsic (mitochondrial) pathway and the extrinsic (death receptor-mediated) pathway (Fig. 3) [19, 21]. In the intrinsic pathway, activation of pro-apoptotic B-cell lymphoma-2 (BCL-2) family proteins, such as BCL-2-associated X protein (BAX) and BCL-2 homologous antagonist/killer (BAK), induces mitochondrial outer membrane permeabilization, leading to the release of cytochrome c and other intermembrane space (IMS) proteins into the cytosol [17, 19, 21]. In the cytoplasm, cytochrome c activates caspase-9 by promoting the formation of apoptosome. Then, caspase-9 activates executioner caspases, such as caspase-3, ultimately inducing apoptosis [17, 19, 21]. In the extrinsic pathway, death ligands such as Fas ligand, TNFα, and TNF-related apoptosis-inducing ligand (TRAIL) bind to death receptors, activating caspase-8. Caspase-8 activates executioner caspases and induces mitochondrial dysfunction, both of which lead to apoptosis [17, 19, 21].

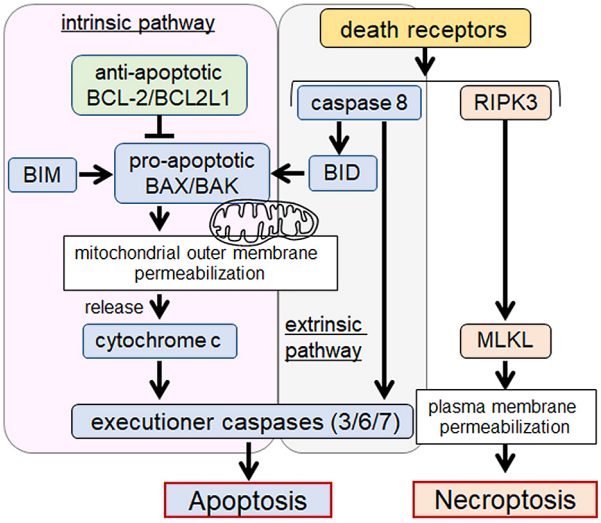


Fig. 3 Molecular mechanisms regulating apoptosis and necroptosis
The mechanisms that induce apoptosis include the intrinsic pathway and the extrinsic pathway. In the intrinsic pathway, activation of pro-apoptotic B-cell lymphoma-2 (BCL-2) family proteins, such as BCL-2-associated X protein (BAX) and BCL-2 homologous antagonist/killer (BAK), induces mitochondrial outer membrane permeabilization, leading to the release of cytochrome c into the cytosol. Cytochrome c activates executioner caspases, ultimately inducing apoptosis. In the extrinsic pathway, ligand binding to death receptors activates caspase-8, which both activates executioner caspases and induces mitochondrial dysfunction, leading to apoptosis. Necroptosis is a form of regulated cell death involving receptor-interacting protein kinase 3 (RIPK3) as a regulatory molecule and mixed lineage kinase domain-like (MLKL). Ligands binding to death receptors lead to the phosphorylation and activation of RIPK3. Activated RIPK3 phosphorylates MLKL, and the phosphorylated MLKL forms oligomers, which disrupt the cell membrane and induce cell death.

In the non-steatotic liver, apoptosis during regeneration plays a crucial role in inducing hepatocyte proliferation [8]. In non-steatotic mouse liver, apoptosis, detected by active caspase-3 staining, occurs from 12 to 48 hours after 70% partial hepatectomy, peaking around 24 hours (Fig. 1) [7, 11]. Following this apoptosis, hepatocyte proliferation, indicated by bromodeoxyuridine (BrdU) staining, begins approximately 36 hours after the resection (Fig. 1). Indeed, caspase-3/7 double knockout delays liver regeneration after partial hepatectomy, demonstrating the importance of apoptosis in this process [22]. Apoptotic cells promote hepatocyte proliferation by secreting interleukin-11 and prostaglandin E2 [3, 8]. In the partial hepatectomy model, the extrinsic pathway appears to mediate apoptosis, as TNFα gene expression transiently increases within 1–24 hours post-hepatectomy [7, 23]. Furthermore, neutralizing TNFα before hepatectomy suppresses hepatic regeneration [24].

Impaired Regeneration and Apoptosis in Steatotic Liver

In the steatotic liver, apoptosis increases during regeneration after partial hepatectomy in proportion to the severity of steatosis, thereby impairing liver regeneration, unlike in the non-steatotic liver, where it triggers regeneration [7, 11]. In leptin receptor-deficient db/db mice and metabolic dysfunction-associated steatotic liver (MASL) model mice fed a high-fat (HF) diet, active caspase-3 levels increase after partial hepatectomy, and liver regeneration is likewise impaired [11, 25]. In MASL mice, apoptosis occurs sporadically and is scattered throughout the hepatic lobules [11]. Apoptosis increases not only during acute liver regeneration but also in MASLD, where chronic liver regeneration persists [10]. Plasma cytokeratin-18 (CK-18) fragments, a biomarker of apoptosis, are reportedly elevated in patients with metabolic dysfunction-associated steatohepatitis (MASH) [26]. Liver biopsy samples from MASH patients show that the number of apoptotic cells positive for active caspase-3/7 correlates with hepatic fibrosis and inflammation [27].

Both the extrinsic and intrinsic pathways contribute to apoptosis induction in steatotic liver [13]. Gene expression of death receptors and their ligands, which mediate the extrinsic pathway, is upregulated in this condition. In MASH model mice fed a high-fat, high-fructose, high-cholesterol (FFC) diet, hepatic expression of genes encoding TRAIL receptor-2, Fas, and TNF receptor 1, along with their ligands, is increased [28]. Similarly, in steatohepatits model mice induced by a methionine- and choline-deficient diet (hereafter referred to as MCD mice),

both hepatic gene expression of TNFα [11] and protein expression of TRAIL receptor-2 are elevated [29]. However, hepatocyte-specific deletion of caspase-8, which is essential for the extrinsic pathway, did not affect the number of active caspase-3-positive cells in the livers of MCD mice [30]. This finding suggests that the intrinsic pathway, in addition to the extrinsic pathway, plays an important role in hepatocyte apoptosis in steatohepatitis. Indeed, oxidative stress and endoplasmic reticulum (ER) stress, both of which are increased in steatotic liver, influence the expression of pro- and anti-apoptotic BCL-2 family proteins involved in the intrinsic pathway [31]. In MCD mice, expression of the anti-apoptotic BCL-2 family protein BCL2L1 is reduced [29]. In contrast, in MASL mice fed a HF diet, *Bcl2l1* gene expression remains unchanged, whereas the pro-apoptotic *Bax* gene is upregulated [7, 32].

In MASLD, apoptosis-dependent delay of regeneration is widely thought to result in maladaptive regeneration, contributing to persistent inflammation and fibrosis [10]. Accordingly, caspases, which drive this process, have been considered therapeutic targets. The pan-caspase inhibitor emricasan was expected to be a promising treatment for MASH. In MASH mouse models fed a high-cholesterol, high-fat diet, emricasan reduced hepatocyte apoptosis, serum transaminase levels, and hepatic inflammation and fibrosis-related gene expression [33]. However, clinical trials in MASH patients revealed that emricasan did not improve hepatic inflammation or fibrosis and, in some cases, even exacerbated hepatocellular injury and fibrosis [34, 35]. These findings suggest that, in addition to apoptosis, other modes of cell death contribute to MASH progression and impaired regeneration in the steatotic liver.

Necroptosis during Steatotic Liver Regeneration

Necroptosis, a mode of cell death morphologically similar to necrosis, is characterized by cell membrane rupture and swelling of the cell and organelles [9, 36]. Due to cell membrane rupture, necroptotic cells release large amounts of DAMPs, which trigger a strong inflammatory response and may induce secondary cell death in the surrounding tissues [9, 36]. However, unlike necrosis, necroptosis is a regulated cell death, involving the receptor-interacting protein kinase 3 (RIPK3) as a regulatory molecule and mixed lineage kinase domain-like (MLKL), which forms pores in the membrane, as the effector molecule [9, 36, 37]. Necroptosis is triggered by ligand binding to death receptors, which initiates a signaling cascade involving RIPK3 phosphorylation and activation. Activated RIPK3 then phosphorylates MLKL, which then forms oligomers that disrupt the cell membrane and induce cell death (Fig. 3) [9, 37]. Whether a signal from death receptors results in apoptosis or necroptosis depends on the activation of caspase-8 and RIPK3. Caspase-8 negatively regulates necroptosis by cleaving and inactivating necroptosis-related molecules such as RIPK3. Conversely, when caspase-8 activity is inhibited, necroptosis is preferentially induced [9, 14, 38]. In addition to death receptors, innate immune receptors such as Toll-like receptor 3/4 are also known to induce necroptosis [9, 14, 38, 39]. These receptors trigger necroptosis through RIPK3 activation, but simultaneous activation of caspase-8 can suppress necroptosis. Thus, the balance between caspase-8 and RIPK3 activities determines whether a cell undergoes apoptosis or necroptosis under these signaling conditions [9, 14, 38].

In the liver, RIPK3 expression is generally low, and necroptosis occurs infrequently [40]. In fact, in both healthy non-steatotic and even advanced steatotic livers, RIPK3 expression is low, and the predominant form of cell death is apoptosis. In the MASL model induced by a HF diet, systemic *Ripk3* deficiency leads to a phenotype characterized by increased apoptosis in adipose tissues, where RIPK3 is expressed. This results in aggravated hepatic steatosis, driven by insulin resistance and glucose intolerance induced by adipose inflammation [41, 42]. Additionally, in moderate hepatic steatosis, RIPK3 expression does not increase during the regenerative process following partial hepatectomy. Instead, only an increase in apoptosis is observed, with no necroptosis occurring [11].

Oxidative and ER stress—such as that occurring during liver regeneration in severe steatosis—induces RIPK3 expression, enabling hepatocytes to undergo necroptosis. Indeed, in MCD mice, the number of RIPK3-positive hepatocytes correlates with the hepatic regenerative response, namely the number of proliferating hepatocytes [30]. In severe steatosis, RIPK3 expression is minimal before partial hepatectomy, but its expression significantly increases post-resection, leading to necroptosis [11]. During the regeneration process following resection in severe steatosis, both spotty and zonal cell death occur across hepatic lobules (Fig. 2), with over half of the spotty cell death identified as necroptosis based on negative staining for active caspase-3 [11]. Notably, *Ripk3* knockdown in hepatocytes abolishes zonal and spotty non-apoptotic cell death, leaving only apoptosis, as indicated by active caspase-3 staining [11]. Although RIPK3 expression is induced during regeneration in severe hepatic steatosis, activation of RIPK3 is required for the execution of necroptosis. Indeed, administration of a TNFα-neutralizing antibody at 12 hours after partial hepatectomy in severe steatosis suppresses RIPK3 phosphorylation and consequently abolishes zonal cell death

[11].

In MCD mice, liver-specific *Ripk3* knockdown or knockout reduces hepatocyte death and alleviates liver inflammation and fibrosis [11, 30]. Furthermore, systemic *Ripk3* knockout has also been reported to reduce liver injury induced by acetaminophen and alcohol [43, 44]. Unlike RIPK3, MLKL is expressed in the healthy non-steatotic liver and is critical for the occurrence of hepatic necroptosis. Systemic *Mlkl* knockout, which confers resistance to HF diet-induced weight gain and insulin resistance, alleviates steatosis and liver inflammation induced by an FFC diet [45-47]. These findings suggest that necroptosis in the liver plays a crucial role in the acute and chronic damage of steatotic liver, including in MASH.

Regulation of Hepatocyte Death by Integrated Stress Response

The shift from apoptosis to necroptosis during liver regeneration is regulated by phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α), a key component of the integrated stress response (ISR). The ISR is typically triggered by intracellular conditions such as oxidative and ER stress [48]. Phosphorylation of eIF2α suppresses general protein translation while selectively enhancing the translation of activating transcription factor 4 (ATF4), which regulates the transcription of stress-responsive genes. ATF4 induces stress-responsive transcription factors, including C/EBP homologous protein (CHOP) and ATF3, thereby initiating the cellular stress response (Fig. 4) [49]. Notably, CHOP functions as a pro-apoptotic transcription factor by upregulating genes such as TRAIL receptor-2 and the pro-apoptotic BCL-2 family member BCL-2-interacting mediator of cell death (*Bim*), while downregulating anti-apoptotic genes like *Bcl2* and *Bcl2l1* [50-52]. Indeed, CHOP-deficient cells exhibit resistance to ER stress-induced apoptosis [53].

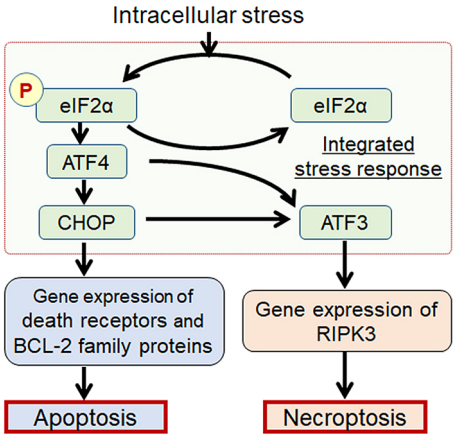


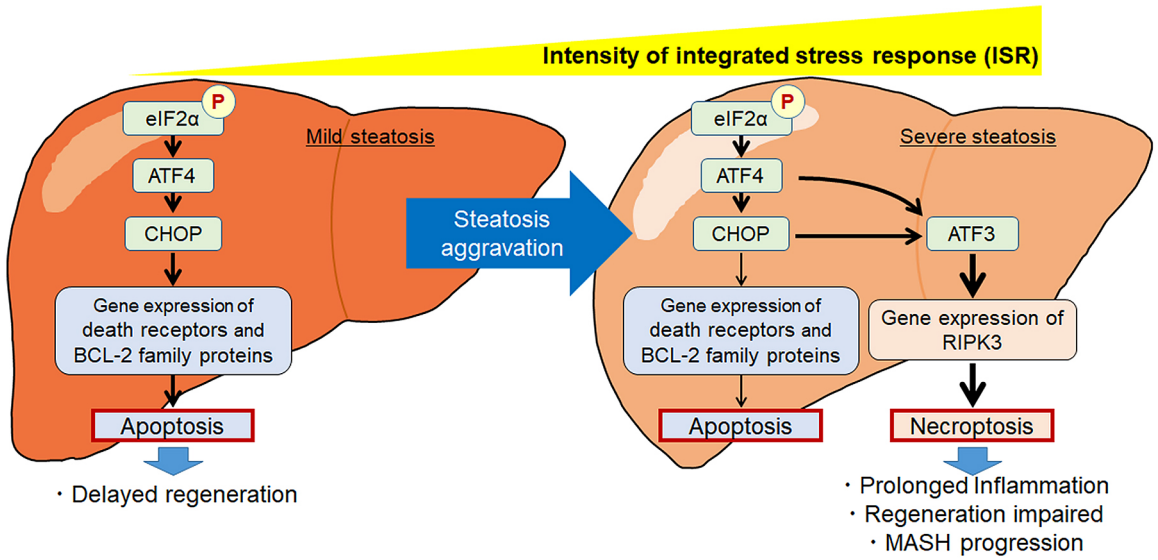
Fig. 4 The integrated stress response (ISR)

The ISR-induced C/EBP homologous protein (CHOP) promotes apoptosis. When ISR is enhanced, activating transcription factor 3 (ATF3) is upregulated, inducing the expression of receptor-interacting protein kinase 3 (RIPK3), which results in necroptosis.

In the steatotic liver, ISR is activated, with a marked increase observed during the regenerative process following injury [7, 11]. ER stress activates stress response pathways mediated by inositol-requiring enzyme 1α (IRE1α) and ATF6 [52]. Although these two pathways are also activated in a manner similar to ISR, they do not show further upregulation during the regenerative phase after injury [7]. The enhancement of ISR during liver regeneration plays a critical role in the induction of apoptosis. When ISR is suppressed by overexpressing growth arrest and DNA damage-inducible 34 (*Gadd34*)—a phosphatase that dephosphorylates eIF2α—in the moderately steatotic liver prior to partial hepatectomy, apoptosis is reduced, thereby alleviating regenerative impairment [7].

The intensity of ISR during liver regeneration is a key factor in determining the shift in cell death mode from apoptosis to necroptosis. In severe steatosis, zonal hepatocyte death occurs during liver regeneration after resection, accompanied by a significant increase in ISR [7, 11]. In moderate steatosis, apoptosis increases during regeneration; however, hepatocyte-specific knockdown of *Gadd34*, which enhances ISR, induces necroptosis [7]. Variations in ISR intensity influence hepatocyte death modes through the regulation of ATF3 expression. Specifically, excessive ISR activation in hepatocytes increases ATF3 expression, which directly binds to the *Ripk3* promoter and induces its transcription (Fig. 4) [11]. In *Atf3*-deficient mice, necroptosis does not occur during regeneration, even after resection of severely steatotic liver [11]. In cultured hepatocytes, TNFα stimulation typically induces apoptosis, characterized by cell shrinkage, whereas overexpression of ATF3 shifts TNFα-induced cell death to necroptosis, which is characterized by cell swelling [11]. Beyond partial hepatectomy, necroptosis also exacerbates liver injury induced by acetaminophen or alcohol [43, 44]. These liver injuries are associated with increased ATF3 expression [54-56], suggesting that ATF3-mediated RIPK3 induction may also contribute to these pathological processes.

ATF3-dependent RIPK3 induction in hepatocytes is closely associated with MASH pathogenesis. In the liver of MCD mice and human MASH patients, most ATF3-positive hepatocytes are also RIPK3-positive, and conversely, most RIPK3-positive hepatocytes are ATF3-positive [11]. Furthermore, in ATF3-deficient mice, RIPK3



Graphical Abstract

expression is reduced, and liver inflammation and fibrosis remain minimal despite MCD diet feeding [11].

Conclusion

Hepatocyte death during regeneration influences liver disease progression, regardless of the underlying cause of injury. It has been proposed that various forms of hepatocyte death coexist in the liver [13]. This review focused on apoptosis and necroptosis, two representative modes of cell death. In steatotic liver regeneration, non-inflammatory apoptosis and pro-inflammatory necroptosis coexist, yet their roles differ significantly. Therapies targeting apoptosis alone have shown limited efficacy in treating MASH. In steatotic liver, the mode of hepatocyte death shifts from apoptosis to necroptosis depending on the intensity of ISR (Graphical Abstract). Accordingly, necroptosis may represent a potential therapeutic target in MASH. Indeed, systemic *Ripk3* deficiency ameliorates steatohepatitis in MCD mice, where RIPK3 is strongly expressed in the liver [30]. In contrast, in models such as MASL, where hepatic RIPK3 expression is low, *Ripk3* deficiency exacerbates hepatic steatosis due to its effects on adipose tissue [41, 42]. These findings suggest that, when targeting RIPK3, it may be necessary to assess hepatic RIPK3 expression and implement organ-specific inhibition. Alternatively, the RIPK3-inducing transcription factor ATF3 or the downstream of RIPK3, the necroptosis effector MLKL, may serve as novel therapeutic targets.

In recent years, increasing attention has been directed toward other modes of hepatocyte death, such as pyroptosis and ferroptosis [36, 37], which may also play important roles in liver pathology. A deeper understanding of the regulatory mechanisms underlying diverse forms of regulated cell death may lead to the development of novel therapeutic strategies for hepatic steatosis-related diseases.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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