Cell Death in Biology and Diseases

Wen-Xing Ding
Xiao-Ming Yin *Editors*

# Molecules, Systems and Signaling in Liver Injury

Springer

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*Series Editors*

Xiao-Ming Yin
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# Molecules, Systems and Signaling in Liver Injury

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Cell Death in Biology and Diseases
ISBN 978-3-319-58105-7 ISBN 978-3-319-58106-4 (eBook)
DOI 10.1007/978-3-319-58106-4

Library of Congress Control Number: 2017945288



Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

# Series Preface

Cell death, or conversely cell survival, is a major biological phenomenon. Just as with cell proliferation and cell differentiation, cell death is a choice that a cell has to make, sometimes voluntarily, other times accidentally. As such, cell death serves a purpose in the biology of a multicellular organism. The machinery of cell death and that of cell protection are evolutionarily conserved, and their elements can even be found in single-celled organism. The disruption of cell death mechanisms can often cause developmental abnormalities. Factors that can trigger cell death are diverse, and the cell death process is intricately connected with other biological processes. Cell death directly contributes to the pathogenesis of many diseases, including cancer, neurodegenerative diseases, and tissue injury in organ failure.

The study of cell death and cell survival has become a multidisciplinary subject, which requires expertise from all fields of the modern biology. Exploring the role of cell death in disease development and the modulation of cell death for the prevention and treatment of devastating disease demands constant updating of our knowledge through the broadest interactions among all investigators, basic and clinical. The rapid expansion of our knowledge in this field has gone beyond what could be summarized in a single book. Thus, this timely series Cell Death in Biology and Diseases summarizes new developments in different areas of cell death research in an elaborate and systemic way. Each volume of this series addresses a particular topic of cell death that either has a broad impact on the field or has an in-depth development in a unique direction. As a whole, this series provides a current and encyclopedic view of cell death.

We would like to sincerely thank the editors of each volume in the series and the authors of each chapter in these volumes for their strong commitment and great effort towards making this mission possible. We are also grateful to our team of professional Springer editors. They have worked with us diligently and creatively from the initiation and are continuing this on the development and production of each volume of the series. Finally, we hope the readers will enjoy the reading, find the content helpful to their work, and consider this series an invaluable resource.

Indianapolis, IN, USA — Xiao-Ming Yin
Augusta, GA, USA — Zheng Dong

# Preface

Cell death is a fundamental biological phenomenon. It is evolutionarily conserved but can assume different forms under different conditions. While apoptosis, necrosis, and necroptosis are perhaps best studied, less known forms of cell death such as pyroptosis, ferroptosis, parthanatos, and entosis are increasingly found in various conditions or organisms. In multicellular organisms, cell death is important for development, shaping organ size, reforming tissue architecture, promoting functional differentiation, and determining mitochondrial inheritance. In the post--development stage, cell death determines the severity in tissue injury and the degree of subsequent response in inflammation, fibrosis, repair, regeneration, and tumorigenesis. The pathological changes in a complex organ, such as the liver, can be greatly affected by the death program in its cellular components. As the major organ for metabolism and detoxification, the liver is constantly under challenges from both internal and external sources. Viral infection and xenobiotics are the two major environmental stimuli that can cause significant hepatocyte death. Metabolic disturbance (such as in autophagy function) and special food components (such as lipids, ethanol, cholesterol, sugars) are the major internal stress that can also take a significant token on the hepatocytes that leads to cell death. Furthermore, the liver may experience traumatic injury as in cholestasis or ischemia, which often leads to tissue injury to various degrees. The liver is composed of hepatocytes (the majority), cholangiocytes, stellate cells, fibroblasts, macrophages/Kupffer cells, sinusoidal cells, and many more. They respond to these insults with different sensitivities and contribute to the overall liver pathology in various ways. Thus different signaling pathways may be involved in a cell type-specific and/or in a stimulus-specific way, which triggers subcellular organelle (mitochondria and endoplasmic reticulum as well as lysosomes) stress to induce cell death. Significant progresses have been made in the past decades to characterize these cell death pathways and the cells that are involved in liver injury. We are thus preparing two books devoted to the mechanisms of cell death in the liver and liver diseases. While the first volume mainly discusses liver injury and cell death caused by the various external and internal stimuli, the second volume discusses in great detail the type of cells and intracellular organelles involved in cell death as well as several major signaling pathways

involved. We have paid particular attention to the interaction of different cells and aimed to provide a global view of the liver pathology that connects injury/cell death to other pathological changes, such as inflammation, fibrosis, and tumor development. Notably we will also discuss the interaction between the liver and the intestine where microbiota can both influence and be influenced by the liver pathology. Authors who are invited to contribute to the two volumes are respected experts in the subject, which should greatly enhance the authority of the chapter and the book. The volume editors are thus indebted to the authors for their outstanding contributions that make these two books so informative and thought-provoking.

Kansas City, KS, USA — Wen-Xing Ding
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# Chapter 1
# Lipotoxicity in Non-parenchymal Liver Cells

**Edward N. Harris and Justin L. Mott**

## Abbreviations

| | |
|---|---|
| α-SMA | Alpha-smooth muscle actin |
| DR5 | TRAIL death receptor 5 |
| eNOS | Endothelial nitric oxide synthase |
| FasL | Fas ligand |
| GGT | Gamma-glutamyl transpeptidase |
| Hh | Hedgehog |
| HSCs | Hepatic stellate cells |
| KCs | Kupffer cells |
| LSECs | Liver sinusoidal endothelial cells |
| LXRs | Liver X receptors |
| NAFLD | Nonalcoholic fatty liver disease |
| NASH | Nonalcoholic steatohepatitis |
| PD-1 | Programmed death 1 receptor |
| PD-L1/2 | Programmed death 1 receptor ligands |
| PNPLA3 | Patatin-like phosholipase domain-containing 3 |

**Financial Support** This chapter was supported in part by the National Institutes of Health and National Institute of General Medical Science grant P20GM104320 (to JLM) and the Fred & Pamela Buffett Cancer Center. The contents of this manuscript are solely the responsibility of the authors.

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W.-X. Ding, X.-M. Yin (eds.), *Molecules, Systems and Signaling in Liver Injury*,
Cell Death in Biology and Diseases, DOI 10.1007/978-3-319-58106-4_1

X.-M. Yin and Z. Dong (Series eds.), *Cell Death in Biology and Diseases*

| | |
|---|---|
| TGF-β | Transforming growth factor-β |
| TNFR | Tumor necrosis factor receptors |
| VEGF | Vascular endothelial growth factor |

## 1.1 Introduction

Liver injury in the presence of metabolic alterations is becoming a major cause of morbidity and mortality. Nonalcoholic fatty liver disease (NAFLD) includes simple steatosis and the more severe NASH (Goh and McCullough 2016; Demir et al. 2015). NASH-associated cirrhosis and end-stage liver disease are currently the second leading indication for liver transplantation and are the fastest-growing cause for transplantation in the USA (Wong et al. 2015; Charlton et al. 2011). Altered metabolic states associated with NAFLD and NASH are obesity, insulin resistance, metabolic syndrome, and type 2 diabetes. Obviously, there is considerable overlap among these conditions. Development of metabolic syndrome is commonly associated with obesity, and increasing rates of obesity have paralleled increased NASH incidence. It is estimated that 34–40% of the adult population in the USA is obese (body mass index over 30 kg/m$^2$). Over 9 million adults in the USA have NASH, and over 100 million have NAFLD (Angulo 2002). The burden of this disease is hard to overstate.

Histologic findings of NASH include lipid droplet accumulation in hepatocytes (steatosis), hepatocellular injury and ballooning, and lobular inflammation. While not required for the diagnosis of NASH, fibrosis is the best predictor of disease outcomes (Angulo et al. 2015; Ekstedt et al. 2015). In stage 2 and higher fibrosis, there is portal fibrosis in addition to perisinusoidal lobular fibrosis. Another unscored histologic finding is ductular reaction with proliferation of hepatic progenitor cells in the lobule expressing markers of biliary epithelial cells and ductular reaction with a related expansion of biliary epithelial cells in the portal tract (Richardson et al. 2007). The degree of ductular reaction correlated with fibrosis stage in NASH with the greatest area of ductular reaction in stage 4 fibrotic NASH livers (Richardson et al. 2007; Gadd et al. 2014). Portal inflammation with CD68-positive macrophages and CD3- or CD8-positive lymphocytes also correlated with stage of fibrosis (Gadd et al. 2014). Ultrastructurally, liver sinusoidal endothelial cells (LSECs) lose their fenestrae and become capillarized.

Metabolic changes associated with NASH suggest a role for lipid signaling in disease. Indeed, genome-wide association studies have demonstrated that the lipase patatin-like phosholipase domain-containing 3 (PNPLA3) is associated with NAFLD (Romeo et al. 2008). Circulating free fatty acids are increased in NASH patients, and patients with stage 3 or 4 fibrosis had significantly higher free fatty acid levels than patients with stage 0–2 fibrosis. Interestingly, serum free fatty acids did not correlate with the degree of steatosis (Nehra et al. 2001). The predominant circulating fatty acid is palmitic acid, a 16-carbon saturated fatty acid. Saturated free fatty acids such as palmitic and stearic acid are cytotoxic to several cell types, termed lipoapoptosis. Monounsaturated free fatty acids such as oleate or palmitoleate are generally not toxic and even offer protection from lipoapoptosis (Welters

et al. 2004; Akazawa et al. 2010; Mei et al. 2011; Ahn et al. 2013). Monounsaturated free fatty acids still promote lipid droplet accumulation, consistent with the concept that steatosis per se is not cytotoxic.

Here, we will discuss pathologic changes in NASH associated with lipid mediators. For some liver cell types, this includes lipoapoptosis; for others the lipotoxicity is manifested by altered function. The various cell types in the liver each contribute to NASH, and these roles will be discussed.

## 1.2 Hepatocytes

Apoptosis of hepatocytes in NASH is covered in depth elsewhere in this volume and will not be covered here at length. We note that hepatocyte apoptosis is a hallmark of NASH (Feldstein et al. 2003; Wieckowska et al. 2006) and free fatty acids can induce lipoapoptosis in cultured hepatocyte-derived cells (Ji et al. 2005; Malhi et al. 2006a; Cazanave et al. 2014). The term lipoapoptosis is used here to indicate apoptotic cell death caused by a lipid mediator and is not necessarily dependent on cellular accumulation of lipid droplets, a condition called steatosis. Steatosis is histologically striking in NAFLD and NASH, though may be absent in later stages of NASH, resulting in the historical term "cryptogenic cirrhosis" (Brunt 2005). On the other hand, apoptotic cell death is not readily apparent on general stains (such as H&E staining). Apoptotic hepatocytes can be identified on occasion in histologic sections, and their prevalence is better estimated using special staining such as TUNEL to detect cleaved DNA ends or cleaved caspase-3 to identify cells with activated caspases. In NASH, hepatocyte apoptosis is generally in the range of 2–5%, representing about a fivefold increase over normal liver (Feldstein et al. 2003).

## 1.3 Cholangiocytes

Evidence for biliary involvement in NASH is currently circumstantial. Pathological assessment of NASH is scored by steatosis, lobular inflammation, and hepatocyte ballooning, as well as unscored histologic findings such as fibrosis (Brunt et al. 2011). Biliary changes are not part of the histologic criteria for NAFLD or NASH. Bile duct epithelial cells do not accumulate histologically significant amounts of triglycerides in the form of lipid droplets. Because cholangiocytes do not become steatotic, it is tempting to overlook their potential role in nonalcoholic fatty liver disease. However, storing neutral lipids in the form of triglycerides and cholesterol esters in lipid droplets is not required to sense and respond to circulating lipids like free fatty acids.

Though not part of the scoring criteria, there are biliary histologic changes in NASH. For example, a common finding in NASH is the occurrence of the ductular reaction, a marked expansion of biliary epithelial cells (Roskams et al. 2003; Richardson et al. 2007; Gadd et al. 2014). Ductular reaction in NASH was most commonly associated with worse fibrosis (Richardson et al. 2007; Gadd et al. 2014).

Increasing fibrosis stages correlated with increasing area of ductular reaction (Richardson et al. 2007). Interpreting the significance of the ductular reaction is complicated by the observation that ductular reaction may be present from different mechanisms. For instance, ductular reaction may indicate hepatocyte injury in combination with impaired hepatocyte proliferation (Roskams et al. 2003; Falkowski et al. 2003; Clouston et al. 2005). Alternatively, ductular reaction may indicate a proliferative response to direct biliary damage (Xu et al. 2004; Munshi et al. 2011). Some contribution from each is also feasible.

Is there evidence of direct biliary damage? The biliary marker gamma-glutamyl transpeptidase (GGT) is released into the serum in biliary injury. Elevated serum GGT is correlated with metabolic syndrome (Rantala et al. 2000), risk of developing type 2 diabetes (Perry et al. 1998), histologic fibrosis (Tahan et al. 2008), and likelihood of NASH (Neuschwander-Tetri et al. 2010). However, GGT is not specific for injury to bile duct epithelial cells and can be found at canaliculi of hepatocytes as well (Hanigan and Frierson 1996; Irie et al. 2007). Patients with a cholestatic phenotype of NAFLD (elevated GGT, elevated alkaline phosphatase) were matched to patients with non-cholestatic NAFLD for body mass index and markers of hepatocyte injury (alanine and aspartate aminotransferases) and were assessed for microscopic evidence of biliary injury. Histologic assessment demonstrated bile duct swelling, duct loss, ductular proliferation (ductular reaction), and portal fibrosis. Notably, those patients with increased markers of biliary injury were more likely to have worse fibrosis or even cirrhosis than the matched cohort (Sorrentino et al. 2005). To date, these markers of injury may still reflect either primary biliary injury or secondary biliary damage due to signaling in other cells in the liver. Notably, primary biliary cholangitis was worsened by concomitant overweight, with more steatosis, higher inflammatory grade, and worse bile duct damage found in overweight patients suggesting that lipotoxicity may contribute in the biliary tree, although other metabolic traits were not correlated with severity (Hindi et al. 2013). Additionally, the same single nucleotide polymorphism in PNPLA3 (I148M) that increases NAFLD risk was found to be associated with worse transplant-free survival in patients with primary sclerosing cholangitis. This effect was found in men with severe fibrotic disease manifested by a dominant stenosis (Friedrich et al. 2013). The molecular mechanism behind worsening of disease is unknown. The communication between cells that is necessary for normal liver function means that perturbation in one cell type can, and often does, alter another cell type.

The location of fibrosis in early NASH is generally pericentral, suggesting that cells in the portal tract may contribute less to disease (Brunt et al. 2011). Certainly, hepatocyte injury, altered endothelial function, and activation of Kupffer and hepatic stellate cells result in fibrosis. Still, in pediatric NASH, fibrosis is commonly found predominantly in the portal tract, sometimes referred to as type 2 NASH (Schwimmer et al. 2005; Takahashi et al. 2011). Sequential liver biopsies have demonstrated that patients with periportal fibrosis (stage 2 or 3) can transition to a pattern of pericentral fibrosis (Adams et al. 2005), demonstrating that the region of fibrosis may not be constant or may be different depending on sampling of the liver by biopsy. Whether bile duct epithelial injury promotes portal fibrosis in NASH remains an

open question, but biliary fibrosis in other conditions implies that the cholangiocyte could contribute.

Developmentally, hepatocytes and cholangiocytes arise from a bipotential common precursor cell. The cells generally are few in number in the healthy adult liver but likely contribute to the ductular reaction under conditions of injury. Because of this shared developmental origin, it is likely hepatocytes and cholangiocytes retain common signaling pathways. Also because of this shared development, and the fact that the albumin promoter becomes active in precursor cells, it is important to carefully interpret studies in which the albumin promoter has been used to drive liver-specific genetic changes. For example, it is common to employ a cell-type-specific promoter to express Cre recombinase allowing for genetic rearrangement at loxP sites in a subset of somatic cells, but not in all cells. These conditional genetic systems can be used to selectively delete genes that have floxed exons only in cells expressing Cre. Alternatively, the Cre system can be used to selectively induce expression of a constitutively silent transgene that contains a lox-stop-lox cassette upstream of the transcriptional start site. Recombination in Cre-positive cells then removes the stop cassette and allows expression in a cell-type-specific manner. In the liver, either the albumin promoter (Alb-Cre) or the albumin promoter and alpha-fetoprotein enhancers (Alfp-Cre) have been used. These systems are liver specific (Postic et al. 1999; Kellendonk et al. 2000) but should not be considered hepatocyte specific. Both Alb-Cre and Alfp-Cre result in recombination in cholangiocytes as well as the expected hepatocytes (Tchorz et al. 2009; Kellendonk et al. 2000), i.e., in both epithelial cell types in the liver.

Given these caveats, deletion of the apoptosis-related TRAIL death receptor (DR5) in liver epithelial cells reduced NASH injury in a mouse model (Idrissova et al. 2015). The TRIAL death receptor is upstream of the initiator caspase-8. Cre-mediated deletion of caspase-8 in liver epithelial cells also decreased injury and fibrosis in methionine−/choline-deficient mice (Hatting et al. 2013). These findings implicate death receptor signaling in lipoapoptosis and severity of liver injury. In hepatocyte-derived cell lines, interfering with mRNA for DR5, caspase-8, or FADD reduced lipoapoptosis induced by treating hepatocytes with saturated free fatty acids (Cazanave et al. 2011). The same study also found that human cholangiocyte-derived cell lines underwent apoptosis upon exposure to saturated free fatty acids and that inhibition of DR5 or caspase-8 prevented lipoapoptosis (Cazanave et al. 2011).

Based on this study, and the shared developmental origin of cholangiocytes and hepatocytes, we have begun to study cholangiocyte lipoapoptosis. Cultured cholangiocyte-derived cell lines undergo caspase-dependent lipoapoptosis upon treatment with stearate or palmitate. Apoptosis increased with increasing concentration of these fatty acids and was partially dependent upon activation of the transcription factor FoxO3 (Natarajan et al. 2014). In contrast to hepatocyte lipoapoptosis (Malhi et al. 2006a), cholangiocytes did not require JNK activation for cell death. This cell culture model employed cultured cells and individual fatty acids at relatively high concentrations. The serum free fatty acid content is increased in obesity and metabolic syndrome as well as in NASH patients, so the free fatty acid concentrations were chosen to mimic pathophysiologic, not physiologic, levels. These limitations

should be recognized in considering the potential role of cholangiocyte lipoapoptosis in NASH. We hypothesize that cholangiocyte lipoapoptosis, in combination with activation of the surviving cholangiocytes, can promote or worsen fibrogenesis, inflammation, and NASH progression.

## 1.4 Kupffer Cells

Kupffer cell apoptosis does not appear to cause worsening of NASH. However, it is apparent Kupffer cells are an active cell type determining the severity of inflammation and injury. Specifically, in a series of needle biopsy samples, Kupffer cells were found to be aggregated in NASH but not in healthy liver samples or livers with steatosis (Lefkowitch et al. 2002). There was even sporadic evidence of intracellular lipids in Kupffer cells, which may have represented Kupffer cell lipid droplets or phagocytosed lipid-laden hepatocytes (Lefkowitch et al. 2002).

Experimental depletion of Kupffer cells, preferentially large periportal Kupffer cells, can be accomplished by treating animals with gadolinium chloride which forms aggregates above pH 6 and is taken up by KCs causing their death (Hardonk et al. 1992). Alternatively, the hydrophilic bisphosphonate clodronate can be packaged into liposomes facilitating uptake into macrophages by phagocytosis where it induced apoptosis (Van Rooijen and Sanders 1994). Using these tools revealed that Kupffer cells are necessary for short-term steatosis and hepatic insulin resistance. Depletion of Kupffer cells for 1 week prevented hepatic steatosis due to high-fat feeding (Stienstra et al. 2010). An acute, 3-day high-fat diet induced hepatic insulin resistance that was prevented by Kupffer cell depletion (Lanthier et al. 2010). Gadolinium chloride-mediated depletion of Kupffer cells prevented steatosis induced by 2 weeks of either high-fat or high-sucrose feeding (Huang et al. 2010). Additionally, Kupffer cell depletion prevented hepatic insulin resistance tested by insulin clamp. During this short dietary intervention, there was no change in fasting insulin or fasting glucose levels (Huang et al. 2010). Weekly Kupffer cell depletion with liposomal clodronate prevented glucose intolerance during a 4-week high-fat diet (Lanthier et al. 2011). Similarly, twice-weekly gadolinium improved systemic insulin resistance and hepatic steatosis in mice fed with a high-fat diet for 3 weeks (Neyrinck et al. 2009). Weekly clodronate-mediated depletion in mice fed with a methionine−/choline-deficient diet for 3 weeks that causes NASH-like liver changes without insulin resistance or obesity reduced steatosis and markers of fibrosis and inflammation (Rivera et al. 2007). However, a 16-week dietary challenge with high fat did not benefit from Kupffer cell depletion during the last 10 days (Lanthier et al. 2011). This suggests that once metabolic changes are present, Kupffer cells are not required to maintain these alterations.

In summary, lipid-induced apoptosis of liver macrophages is unlikely to contribute to NASH, while prevention of Kupffer cell activation (by killing off these cells) has a beneficial, preventative effect. Therapeutic benefit from Kupffer cell depletion after metabolic changes lacks experimental support.

## 1.5 Liver Sinusoidal Endothelial Cells

Liver sinusoidal endothelial cells (LSECs) are unique endothelial-type cells lining the capillary bed of the liver and serve as the blood barrier between general circulation and the space of Disse which allows access to stellate cells and hepatocytes. The three defining characteristics that make LSECs unique from other endothelia are (1) the lack of an underlying basal lamina (Wisse et al. 1985), (2) numerous cytoplasmic holes or fenestrae organized as sieve plates, and (3) a very high endocytic (fluid phase) capacity. Each fenestra is 100–175 nm in diameter and allows plasma components including small chylomicron and chylomicron remnants to pass through this single-cell layer and come in direct contact with hepatocytes (Braet and Wisse 2002; Wisse 1970). Formation of fenestrae occurs by an actin-dependent fusion between opposite sheets of the plasma membrane when there is a lack of intramembrane particles (Taira 1994). In addition, formation and overall numbers of fenestrae are maintained by adequate levels of vascular endothelial growth factor (VEGF), and loss of VEGF reduces fenestrae (Xie et al. 2012). Due to the dynamic nature of fenestrae, various external stimuli cause the number of fenestra to increase, including hypoxia (Frenzel et al. 1976) and pressure (Nopanitaya et al. 1976; Fraser et al. 1980), or decrease including ethanol (Mak et al. 1984; Jacobs et al. 2009; Sarphie et al. 1997) and nicotine (Fraser et al. 1988). It has been observed that normal aging decreases the size and number of fenestrae, termed pseudo-capillarization, and affects lipid metabolism due to reduced chylomicron contact with the hepatocytes (Le Couteur et al. 2001; McLean et al. 2003; Warren et al. 2005). LSECs are very efficient in the internalization of extracellular matrix and waste materials from blood. Rapid ligand internalization and receptor recycling are owed in part to a net-like distribution of clathrin heavy chains associated with clathrin-coated vesicles in the endocytic processes of internalization, vesicle maturation, and ligand trafficking (Falkowska-Hansen et al. 2007). It is believed that few cells of the mammalian body can match or exceed the endocytic capacity of LSECs.

Tumor necrosis factor receptors (TNFRs) are very low to absent in the normal liver, but in lipotoxic conditions, TNFR expression is increased in hepatocytes, LSECs, cholangiocytes, and KCs (Volpes et al. 1992). Fas receptor, of the TNFR family, is a 45-kDa receptor on the plasma membrane belonging to the tumor necrosis factor family. Fas ligand (FasL) is expressed on activated immune cells (T cells, monocytes, and neutrophils) inducing apoptosis in Fas-expressing cells (Depraetere and Golstein 1997). Normally, the expression of Fas on LSECs is quite low, but that increases under conditions of NASH or lipotoxicity (Malhi et al. 2006b; Muschen et al. 1998). Despite the increase in Fas expression, LSECs are more resistant to apoptosis in the presence of FasL than hepatocytes and stellate cells, though higher physiological doses of FasL did, eventually, induce apoptosis in LSECs (Fig. 1.1). Low doses of FasL or anti-Fas antibodies during in vitro studies have demonstrated an increased expression of ICAM-1 and VCAM-1 instead of apoptosis suggesting that alternative pathways stimulated by Fas are operative in LSECs (Cardier et al. 1999). It is speculated that an increase in adhesion molecules may be a signal for

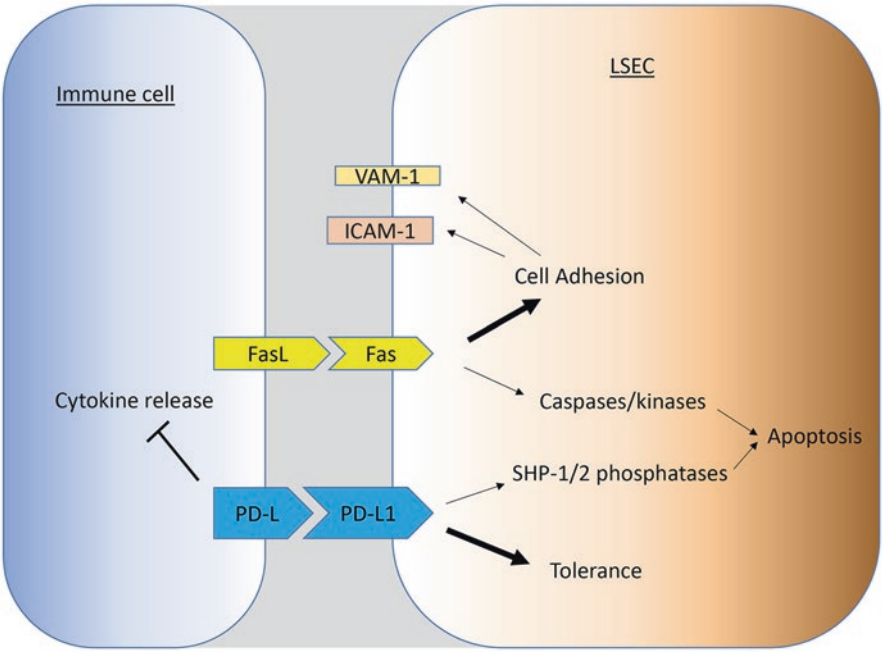


**Fig. 1.1** Interaction of activated immune cells and LSECs. LSECs interact with both quiescent and activated immune cells. LSECs express Fas and PD-L1 receptors which induce parallel pathways that induce apoptosis. However, the dominant outcomes for each pathway are illustrated by the thicker arrow, and apoptosis is only induced after an ill-defined threshold of stimulation has been met. Overall, LSECs tend to dampen the inflammatory response in many environments including NAFLD/NASH

recruitment for liver-specific Kupffer cells (KCs). Clearance of cell debris and other particles is primarily performed by KCs as these cells are professional phagocytes, whereas LSECs internalize material through rapid pinocytosis. It is estimated that macromolecules suitable for LSEC internalization are less than 0.23 μm, which shifts the burden of phagocytosis to KCs (Shiratori et al. 1993; Park et al. 2008). KCs and LSECs coordinate controlled and tempered responses in chronic inflammatory responses that prove to be hepatoprotective in which both cell types are required to keep tissue damage in check. For instance, depletion of KCs prior to acetaminophen-induced liver injury results in exacerbated liver injury, particularly to the hepatic endothelium (Holt et al. 2010). Similarly in a sepsis model, LSEC apoptosis and Fas expression were increased after a depletion of KCs with the use of clodronate liposomes (Hutchins et al. 2013a). Both of these experimental examples show that KCs are necessary for the viability and well-being of LSECs in a background of escalating inflammation.

LSECs are unique antigen-presenting cells and interact with immune cells including the resident KCs and transient immune cells via the programmed death 1 receptor (PD-1) and its ligands (PD-L1/2; Fig. 1.1) (Keir et al. 2008). LSECs express

PD-L1 which is the ligand for PD-1 expressed by cells within the sinusoids, KCs and CD8+ T cells. This interaction promotes immune tolerance and dampens liver inflammation which is important as the liver sinusoids are directly downstream from gut circulation, containing bacterial and food antigens, via the portal vein (Schurich et al. 2010). Furthermore, PD-1/PD-L1 interactions between LSECs and $T_H1$ and $T_H17$ suppress cytokine release from these activated T cells when comparing these activated T cells with dendritic cells, strengthening the hypothesis that LSECs are critical for immune tolerance within the liver (Carambia et al. 2013). PD-1/PD-L1 increases in expression to keep inflammation in check under a variety of simulants including viral infection (Xie et al. 2009; Larrubia et al. 2009), hepatitis, and hepatocellular carcinoma (Wang et al. 2011). Immune tolerance does have its limits in that an undefined threshold may be breached. In mice with sepsis-induced injury (punctured cecum), LSECs and KCs had increased PD-L1 and PD-1 expression, respectively. This resulted in LSEC cell death, with lower cell numbers overall when isolated from the liver, and the few harvested LSECs had lower VEGFR2 expression (a marker for angiogenesis and maintenance of healthy tissue) (Hutchins et al. 2013b). These animals succumbed to septic shock along the same pathways as those exhibiting acute liver failure. The characteristics of PD-1−/PD-L1-induced injury and cell death are very similar to Fas−/FasL-mediated injury model mentioned previously. These pathways are not connected but run in parallel and may synergize each other under the right conditions. The differences between the Fas/FasL and PD-1/PD-L1 are that the former utilizes death receptor-associated kinases and caspases (Wajant 2002) in contrast to the latter which is CD28 dependent and utilizes SHP-1 and SHP-2 phosphatases (Sheppard et al. 2004; Okazaki et al. 2001).

LSECs contain several scavenger receptors that "sense" the extrahepatic environment and respond accordingly (Weigel et al. 2012). These receptors include Stabilin-1, Stabilin-2, mannose receptor, and FC-gamma/CD32b receptors (Sorensen et al. 2015). One of the ligands for Stabilin-1 is SPARC or secreted protein acidic and rich in cysteine which may also serve as a ligand for VCAM-1 (Kzhyshkowska et al. 2006; Kelly et al. 2007). SPARC (also known as osteonectin and BM-40) is a secreted extracellular matrix-associated protein with a major role in the wound healing response, tissue remodeling (Bradshaw et al. 2001), and liver fibrosis (Atorrasagasti et al. 2013). SPARC expression is increased in acute-on-chronic alcohol-induced hepatitis. Using a SPARC knockout mouse, the investigators demonstrated decreased CD4+ T-cell infiltration in the liver and decreased apoptosis of the hepatic endothelium induced by TNF-alpha and IL-6 (Peixoto et al. 2015). This suggests that increased signaling through the Stabilin-1 receptor and, possibly VCAM-1, via SPARC activates LSECs, promotes leukocyte infiltration, and further stimulates death receptors such as Fas/TNFR.

Accumulation of free fatty acids and neutral fats arising from metabolic disorders promotes cellular damage and cell death in many tissues including the liver (Malhi and Gores 2008). It is well known that fat-storing hepatocytes suffer from lipotoxic conditions leading to ballooning and cell death; but how do these metabolic conditions affect LSECs? In most cell-based studies, free fatty acids activate several pathways that lead to cell death including BAX-dependent apoptosis and

lysosomal permeabilization, over-generation of reactive oxygen species, activation of p53, and hyperstimulation of the JNK pathway (Malhi and Gores 2008; Brenner et al. 2013). In line with this, rodents fed with a high-fat diet had LSECs with decreased numbers and porosity of fenestrae, also known as capillarization, compared with rodents fed with a balanced chow control diet (Cogger et al. 2016). In contrast, rats that were fasted for 48 h had an increase in LSEC fenestration by 10% (O'Reilly et al. 2010), and rats that were calorically restricted had an increase of 60% of LSEC fenestration and porosity (Jamieson et al. 2007) suggesting that deprivation of calories increases LSEC porosity for nutrient absorption by the hepatocytes, whereas overnutrition decreases LSEC porosity. However, LSECs do not store fatty acids in NAFLD and NASH conditions (author's unpublished observations), and high levels of certain free fatty acids may be neutral or beneficial for LSEC survival that is contrary for hepatocytes and cholangiocytes (Hang et al. 2012). Recent evidence supports this idea in that in vitro cultures of LSECs supplemented with oleic acid promote maintenance of fenestrae and modulate the Akt/PKB signaling followed by MAPK/ERK signaling to promote survival (Hang et al. 2012). Notably, oleic acid is largely nontoxic to hepatocytes and cholangiocytes except on very high concentrations. Overall, these results show that fenestrae of LSECs are a good marker for cell health and survival, and the amount and type of free fatty acids in vivo or in vitro will affect survival and health of LSECs.

Loss of fenestrae or capillarization is not the only marker for LSECs under stressful conditions. In both fatty liver rodent models and human patients, the size of the liver increases by up to ~20%. The increase in size is primarily attributed to the fat stored in the hepatocytes. Excessive fat storage compresses the sinusoidal space within the capillary bed of the liver and increases portal blood pressure (DeLeve et al. 2008b). Increased portal pressure and other associated factors of lipotoxicity will involve KCs and HSCs to recruit inflammatory cells into the liver and foster conditions for inflammation and deposition of extracellular matrix within the space of Disse (fibrosis) (Farrell et al. 2008; McCuskey et al. 2004). Physical parameters within the sinusoids are just as important in cellular health and maintenance as the biochemical signaling molecules. More research needs to be performed to determine the influence of geometric/physical conditions on LSEC autocrine/paracrine signaling.

Capillarization of LSECs is a hallmark of LSEC injury and a precursor for LSEC death. The mechanism for capillarization is beginning to be understood in that hedgehog (Hh) signaling is a primary driver of this phenomenon. Generally, Hh signaling regulates vascular development and increases during all types of liver injury. In addition to hepatocytes, LSECs express Hh receptor, ligands, and the ligand antagonist, Hhip. Using primary cells in vitro, capillarization was stimulated with increased Hh receptor signaling and inhibited with Hhip. Capillarization was compared in parallel with Hh target genes, Gli1/2 and Ptch1, LSEC migration, and tube formation (Xie et al. 2013). Since capillarization is the start of LSEC injury, does it occur before or after stellate cell activation which promotes inflammation and the pre-fibrotic phenotype in NASH? The current paradigm suggested that capillarization of LSECs precedes HSC activation, and decapillarization (healthy

fenestrae phenotype) promotes quiescence of HSCs (Deleve et al. 2008a). During liver injury, Hh signaling is increased as a normal repair response for organ regeneration. Injury associated with lipotoxicity (or chronic injury) induces aberrant Hh signaling which induces capillarization.

Liver X receptors (LXRs), oxysterol-activated nuclear receptors for lipogenesis, are found in the liver and other tissues with high lipid metabolism and may play a role in LSEC physiology. There is recent evidence indicating that LXR and Hh pathways cross talk in the development of LSEC capillarization. In LXR-deficient mice, Hh signaling was increased (measured by monitoring expression of Gli2 and CD31) in LSECs in parallel with LSEC capillarization in a carbon tetrachloride injury mouse model (Xing et al. 2016). The conclusions of all these experimental data are that LSEC fenestration is promoted by LXR under normal conditions and shifts to capillarization upon induction of the Hh signaling pathways during severe or chronic injury.

What are the outcomes of LSEC cell death and injury? In chronic injury situations, LSECs become capillarized reducing the oxygen content in the liver. Hypoxia stimulates VEGF production in hepatocytes, HSCs, and LSECs in both autocrine and paracrine manners. Overstimulation by VEGF increases the number of sinusoidal vessels, but these vessels have varying diameter, shape, and flow pattern for blood. While increased blood vessels may serve to decrease portal pressure by increasing avenues of blood flow, the full outcome of angiogenesis in the liver is correlated to portal hypertension and promotion of fibrogenesis (Thabut and Shah 2010). Activated HSCs are in intimate contact with capillarized LSECs which increase the number of HSCs around the blood vessel and also restricts the expansion of the blood vessel when expansion is needed to relieve fluid pressure. As HSCs tighten around the LSECs, portal pressure is increased which exacerbates chronic inflammation (Lee et al. 2007). All of these conditions are forward feeding in that a tenuous chronic inflammation may become more acute with the onset of fibrosis and cirrhosis from either physical or molecular injuries (Gentile and Pagliassotti 2008). Rats fed with a cafeteria diet high in saturated fat and sucrose developed hypertension, obesity, insulin resistance, and elevated triglycerides (i.e., features of metabolic syndrome). Plasma free fatty acids increased from 1600 to 3200 μM. Portal blood flow was decreased and hepatic vascular resistance more than doubled. The dietary intervention was continued for 30 days and resulted in steatosis, but fibrosis did not develop in this short time. In this early stage of disease, LSEC function was impaired with reduced vasodilation response and decreased endothelial nitric oxide synthase (eNOS) phosphorylation and enzymatic function (Pasarin et al. 2012).

In conclusion, LSECs are dynamic gatekeepers of the liver allowing select solutes of the blood to come in contact with the hepatocytes. As a regulatory function, they continually filter out macromolecules from the plasma. LSECs, in coordination with other non-parenchymal cells, keep a check on the immune system to prevent runaway inflammation and destruction of the tissue and promote immune tolerance. The loss of these cells is a precursor for liver failure.

## 1.6 Hepatic Stellate Cells

Hepatic stellate cells (HSCs) are the major site of storage for fat-soluble retinol esters and retinol in the body (Hendriks et al. 1985). HSCs reside in the space of Disse between LSECs and hepatocytes. HSCs have a transcription factor profile that is similar to adipocytes, consistent with their fat-storing function (She et al. 2005). Their name, stellate, comes from their shape with a triangular cell body containing the nucleus and long, delicate cytoplasmic processes extending into the space of Disse. Transdifferentiation of HSCs into a myofibroblastic phenotype results in loss of lipid droplets, expression of alpha-smooth muscle actin (α-SMA), development of contractile properties, and production of type I and III collagen. This process is referred to as activation of HSCs and is an integral part of hepatic fibrogenesis.

Activation of HSCs is due to cell-cell communication and changes in the extracellular matrix in NASH. For example, a major fibrogenic stimulus that activates HSCs is transforming growth factor-β (TGF-β) produced by HSCs and Kupffer cells (De Bleser et al. 1997; Friedman 2000; Bataller and Brenner 2005). Additionally, HSCs can be activated by conditioned medium from steatotic cultured hepatoma cells. A sub-toxic concentration of palmitic acid (200 μM) was added to primary human hepatocytes or immortalized hepatoma cell lines for 24 h and the conditioned culture medium then transferred to primary HSCs or to the HSC cell line hTERT-HSC, respectively. Conditioned medium increased HSC production of profibrogenic TGF-β, remodeling proteins matrix metalloproteinase-2, tissue inhibitor of metalloproteinase-1 and metalloproteinase-2 and type I collagen, and the contractile protein α-SMA. Importantly, conditioned medium also promoted resistance of HSCs to apoptosis, suggesting that steatotic hepatocytes may promote survival of fibrogenic HSCs in the liver. Lack of toxicity in the palmitic acid-treated hepatocytes was confirmed by the absence of nuclear changes (Hoechst) and lack of release of lactate dehydrogenase or aminotransferases (Wobser et al. 2009). This study demonstrated that hepatocytes release an activating factor upon steatogenesis. The authors used size-exclusion chromatography with a 5-kD cutoff spin column and observed that the removal of proteins over 5 kDa abolished the activating effect. While free fatty acids are smaller than this cutoff, they are also generally bound to proteins in solution, so it is possible that some residual free fatty acids were in the conditioned medium.

Hepatocyte apoptosis is a hallmark of NASH and may provoke HSC activation. The HSC cell line LX-1 was found to have phagocytic capacity to take up apoptotic bodies from dead hepatocytes. This uptake was microtubule dependent and was specific to HSCs as hepatocytes themselves did not engulf apoptotic bodies. Uptake then resulted in HSC activation, measured by α-SMA and type I collagen expression as well as production of TGF-β (Canbay et al. 2003). In this study, apoptosis was induced in hepatoma cells (HepG2 cells) by exposure to UV light, eliminating the possible carry-over of the toxic stimulus in the crude preparation of apoptotic bodies. Apoptotic bodies were prepared by low-speed centrifugation of the collected medium (2000 *g*), so this preparation is not expected to contain soluble cell-free signaling mediators and probably represents a distinct profibrogenic signal.

Activation of HSCs by conditioned medium from steatotic hepatocytes and by hepatocyte apoptotic bodies is relevant in the liver where these two cell types are in close proximity. The predictable result is increased production of extracellular matrix proteins, especially types I and III collagen, resulting in fibrosis and liver dysfunction. Biopsy samples of liver samples from patients with NASH support a role for activated HSCs in fibrosis, with 97% of NASH patients in one study showing an increase in activated HSCs (Washington et al. 2000). Activated HSCs in that study were correlated with the severity of fibrosis but not the degree of steatosis or iron deposition. Resolution of fibrosis requires either deactivation of HSCs or apoptosis of activated HSCs. Evidence supports apoptosis of these cells during fibrosis resolution. Increased HSC apoptosis was observed, for example, in the first 2 days after surgical reversal of bile duct obstruction in the initial stages of fibrosis resolution (Issa et al. 2001). Similarly, non-parenchymal apoptosis was increased during days 3–7 after the last carbon tetrachloride injection in a chemical model of fibrosis and resolution (Iredale et al. 1998). Thus, induction of HSC apoptosis is expected to have a beneficial effect on fibrosis. Indeed, inducing HSC apoptosis in bile duct-ligated mice reduced markers of activated HSCs (Anan et al. 2006a) and reduced the severity of injury (Anan et al. 2006b).

Toxic lipid mediators in NASH appear to indirectly activate HSCs and indirectly promote the survival and fibrogenic potential of these cells. HSC apoptosis would presumably have a beneficial effect on NASH fibrosis, but regrettably lipid mediators seem to act indirectly on HSCs to promote their activation. Apoptosis in this cell compartment may be a therapeutic goal.

## 1.7 Conclusions

Lipotoxicity in the liver involves induction of cell death (lipoapoptosis) in hepatocytes and potentially cholangiocytes in NASH (Fig. 1.2). Cell death in these epithelial cells may lead to slight elevations of serum markers of hepatobiliary injury but more importantly promote activation of fibrogenic HSCs and inflammatory Kupffer cells. Additionally, lipotoxicity to LSECs leads to capillarization, impaired LSEC function, increased vascular resistance, and ultimately cell death. Increased LSEC and epithelial cell death plus activation and increased survival of Kupffer and stellate cells mean that global attempts to prevent cytotoxicity in the liver may not yield therapeutic benefits. Instead, increasing death of Kupffer cells and HSCs while protecting hepatocytes and cholangiocytes may be a better, if more difficult, goal.

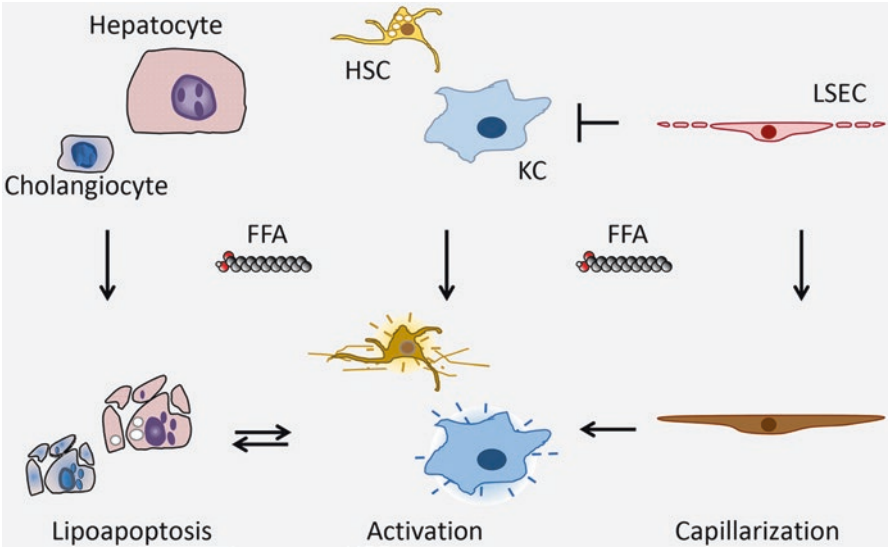


**Fig. 1.2** Lipotoxicity in the liver. Healthy cells, hepatocyte, cholangiocyte, hepatic stellate cell (*HSC*), Kupffer cell (*KC*), and liver sinusoidal endothelial cell (*LSEC*) are shown at the top of the figure. Upon pathologic signaling, including by free fatty acids (*FFA*), the normal cell functions and interactions are disrupted. LSEC capillarization and injury as well as lipoapoptosis of hepatocytes and possibly cholangiocytes promote activation of KCs and HSCs. The combined effects include injury, inflammation, and fibrogenesis representing key features of nonalcoholic steatohepatitis

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# Chapter 2
# The Role of Cholangiocyte Cell Death in the Development of Biliary Diseases

**April O'Brien, Chad Hall, Laurent Ehrlich, Tianhao Zhou, Fanyin Meng, Gianfranco Alpini, and Shannon S. Glaser**

## Abbreviations

| | |
|---|---|
| ADPKD | Autosomal dominant polycystic kidney disease |
| AEA | Anandamide |
| AKT | Protein kinase B |
| AMA | Antimitochondrial antibodies |
| BA | Biliary atresia |
| BDL | Bile duct ligation |
| CBDL | Common bile duct ligation |
| CCA | Cholangiocarcinoma |
| CD | Cluster of differentiation |
| DEN | Diethylnitrosamine |
| FADD | Fas-associated death domain |
| FFA | Free fatty acid |
| HSC | Hepatic stellate cells |
| IFN-γ | Interferon- γ |
| IL | Interleukin |
| JNK | c-Jun N-terminal kinase |
| LMBDL | Left median bile duct ligation |

This material is the result of work supported by resources at the Central Texas Veterans Health Care System. The content is the responsibility of the author(s) alone and does not necessarily reflect the views or policies of the Department of Veterans Affairs or the United States Government.

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W.-X. Ding, X.-M. Yin (eds.), *Molecules, Systems and Signaling in Liver Injury*, Cell Death in Biology and Diseases, DOI 10.1007/978-3-319-58106-4_2

X.-M. Yin and Z. Dong (Series eds.), *Cell Death in Biology and Diseases*

| | |
|---|---|
| NAFLD | Nonalcoholic fatty liver disease |
| NF-kB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NK | Natural killer |
| NOD | Nucleotide-binding oligomerization domain |
| PAMPs | Pathogen-associated molecular patterns |
| pANCA | Peri-neutrophil cytoplasmic antibody |
| PBC | Primary biliary cholangitis |
| PKD | Polycystic kidney disease |
| PNPLA3 | Patatin-like phospholipase domain-containing protein 3 |
| PRR | Pattern-recognition receptors |
| PSC | Primary sclerosing cholangitis |
| PUMA | p53 upregulated modulator of apoptosis |
| STAT3 | Signal transducer and activator of transcription 3 |
| TLRs | Toll-like receptors |
| TNF | Tumor necrosis factor |
| TRAIL | Tumor necrosis factor-related apoptosis-inducing ligand |
| UDCA | Ursodeoxycholic acid |

## 2.1 Introduction

The liver is comprised primarily of two types of epithelia: hepatocytes and cholangiocytes. Hepatocytes make up approximately 70% of the endogenous population, whereas cholangiocytes constitute only 3–5% (Glaser et al. 2009). Cholangiocytes are heterogeneous, varying in size, shape, and function, and are generally categorized as either small or large based upon both their morphology and functionality. While both kinds of cholangiocytes line the biliary tree, large cholangiocytes are more columnar in shape and line larger bile ducts, whereas small cholangiocytes tend to be cuboidal and are found in smaller bile ducts (Maroni et al. 2015). In addition, small cholangiocytes are less specialized with a higher nucleus/cytoplasm ratio. Large cholangiocytes have a smaller nucleus/cytoplasm ratio and are ciliated, allowing them to act as both chemo- and mechanosensors (Maroni et al. 2015). Together, their primary role is to secrete, modify, and transport the bile into the duodenum. Cholangiocytes also facilitate repair following hepatic injury, sense, and respond to inflammatory signals and are the target of cholangiopathies. Effective targeted therapies for this subset of biliary diseases are lacking; therefore, it is critical to establish a thorough understanding of the biological pathways and mechanisms of cholangiocyte death that may play a role in the pathogenesis of cholestatic liver diseases.

## 2.2 Cholangiopathies

Cholangiopathies are diseases of the biliary epithelium and commonly include primary sclerosing cholangitis (PSC), primary biliary cholangitis (PBC), biliary atresia, polycystic liver disease, and cholangiocarcinoma (Lazaridis and LaRusso 2015).

Cholangiopathies can be categorized, but are not limited to the following groups: autoimmune, drug- or toxin-induced, ischemic, idiopathic, infectious, and genetic (O'Hara et al. 2013). The five major cholangiopathies – PSC, PBC, autosomal dominant polycystic kidney disease (ADPKD), biliary atresia, and cholangiocarcinoma – affect both genders and all ages worldwide.

## 2.3 Primary Sclerosing Cholangitis

PSC is a chronic, progressive disease that results in periductular inflammation and fibrosis that progresses to end-stage liver diseases (Primary Biliary Cirrhosis 2016). Unlike other cholestatic liver diseases, PSC affects both the small and large bile ducts with 20% of small-duct PSC presenting patients progressing to large-duct PSC (Primary Biliary Cirrhosis 2016). Additionally, the majority of PSC patients will also have inflammatory bowel disease (Trivedi and Chapman 2012). PSC predominantly strikes males (66%) more than females and ranges from adolescence to advanced ages with the average age of a PSC patient being 41 years at diagnosis (Primary Biliary Cirrhosis 2016). Currently there are no effective treatments that halt disease progression, and liver transplantation is often necessary (Hirschfield et al. 2013; Karlsen et al. 2010). Complicating the need for liver transplantation is the reoccurrence of the disease, the predominance toward hepatobiliary cancers and secondary autoimmune disorders.

### *2.3.1 Primary Biliary Cholangitis*

PBC is a chronic inflammatory cholestatic disease of autoimmune origins that affects the small interlobular and septal bile ducts. Without treatment, PBC will progress to cirrhosis and end-stage liver failure (Corrigan and Hirschfield; Czul and Levy 2016). It presents more frequently in women than men, and individuals with a diagnosis before 50 years of age have a 50% chance that treatment options will fail (Corrigan and Hirschfield). Approximately 30% of PBC patients will also be diagnosed with additional autoimmune disorders, the most common being Sjögren's syndrome (17.4%) and Raynaud's syndrome (12.5%) (Primary Biliary Cirrhosis 2016). Currently the treatment of choice is ursodeoxycholic acid (UDCA) which aids in slowing the disease progression and delays transplantation necessity or death (Qian and Dinghang 2014). Due to the pruritus that accompanies PBC, antihistamines will often be prescribed to improve the patient's quality of life. In addition, anti-inflammatories may help reduce the ductal inflammation allowing for better bile flow (Primary Biliary Cirrhosis 2016).

### 2.3.2 Autosomal Dominant Polycystic Kidney Disease

ADPKD is an inherited renal disorder characterized by the development and growth of cysts in the kidneys, liver, pancreas, and spleen (Ali et al. 2015; Srivastava and Patel 2014; Tong et al. 2015). ADPKD is the most common form of genetic renal failure in adults. ADPKD can result from two types of genetic mutations: ADPKD-1 sufferers carry a genetic mutation on the gene-encoding protein polycystin-1 found on chromosome 16, while ADPKD-2 is caused by a gene mutation on chromosome 4 that encodes the protein polycystin-2 (Erickson et al. 2008; Tong et al. 2015). ADPKD-1 mutations tend to be more severe with patients entering end-stage renal disease at a younger median age. It also accounts for 85% of clinical cases overall (Ali et al. 2015; Srivastava and Patel 2014). Both mutations result in the development of cysts, resembling grape clusters and primarily filled with serous fluid, blood, and urine. These cysts eventually compromise normal renal function through crowding and compression of the renal tubes (Srivastava and Patel 2014). Within the liver, cysts arise from cholangiocytes and ultimately replace healthy liver tissue to a point where they may compress adjacent organs, leading to discomfort and anorexia (Gordon 2015). Consequently many patients remain asymptomatic until the sheer size and number of cysts necessitate medical intervention, generally not until adulthood. Treatment options include cyst fenestration, partial hepatectomy, or liver transplantation (Drenth 2010).

### 2.3.3 Biliary Atresia

Biliary atresia is a congenital disorder that affects more females than males and predominantly children of Asian or African American descent. There are two types of biliary atresia: fetal and perinatal. Of the two, fetal biliary atresia poses a greater threat to the infant as it is often accompanied by defects of the heart, spleen, or intestines. (Hartley et al. 2009). Models of biliary atresia and livers from affected children express increased levels of proapoptosis molecules such as caspase 1 and 4 and TNFα (Petersen and Davenport 2013). While there are no known hereditary components, several theories have been proposed to explain this phenomenon, including viral or bacterial infections, autoimmunity, or dysregulated prenatal liver and bile duct development. A Kasai procedure is often required to restore enteric drainage of bile; however, many of these patients will go on to require hepatic transplantation or die from complications of the disease (Hartley et al. 2009).

### *2.3.4 Cholangiocarcinoma*

Cholangiocarcinoma is a rare malignancy of the biliary epithelium that commonly presents late in the disease progression. The malignancy is difficult to treat due to the advanced stage at presentation and is often fatal with an overall 5-year survival rate of less than 15% (Ciombor and Goff 2013). Cholangiocarcinomas are categorized by the tumor's origins, either arising from the bile ducts lying within the liver (intrahepatic CCA) or outside the liver (extrahepatic CCA) (Keller and Schub 2016). Difficulties in the treatment of cholangiocarcinoma stem from the fact that it is most often diagnosed at an advanced stage limiting available treatment options. To date, the most palliative option is liver resection, which carries additional risks of bile leakage, abdominal abscesses, and liver failure (Ciombor and Goff 2013).

## 2.4 Animal Models

Currently there are many commercially available live animal models of cholestatic liver disease that include mechanical, chemical, genetically modified, and parasitic etiologies (Primary Biliary Cirrhosis 2016). Bile duct ligation (BDL) (mechanical), Mdr2$^{-/-}$ mice (genetic modification), IL-12p35$^{-/-}$ (genetic modification), and dnTGFβRII (genetic modification) will be discussed in further detail. BDL is a well-studied mechanical representation of liver fibrosis used to model cholestatic liver disease. This is achieved through surgical ligation of the common bile duct, which impedes the flow of bile, inducing a strong fibrotic response after 21–28 days (Tag et al. 2015). Recent years have seen the technical advancements of partial BDL or complete BDL with surgical reanastomosis. Bile is a necessary component of the digestive system to emulsify fats and digest lipids while excreting bilirubin and additional wastes. Blockage of the common bile duct causes toxic buildup of waste products resulting in inflammatory cascades and upregulation of fibrotic gene expression. This model has the added benefit of being highly reproducible.

Another reliable animal model of fibrosis is the generation of Mdr2 (Abcb4)$^{-/-}$ mice, which, as a result of a canalicular phospholipid flippase deficiency, spontaneously develop liver injury and present with features of PSC. The resultant cascade of events includes bile leakage into the portal tract due to a disruption of both the tight junctions and basement membranes of the biliary epithelium (Popov et al. 2005). It is also thought that the presence of these phospholipids emulsifies certain molecules such as bile acids, reducing their overall toxicity.

PBC, like biliary atresia, is notoriously difficult to mimic in its pathogenesis. Previous models demonstrate the development of the hallmark antimitochondrial antibodies (AMA) yet lack in their replication of the trademark fibrosis or cirrhosis,

the underlying cause for liver transplantation in PBC patients (Popov 2013). Recently, a double-mutant mouse generated by crossing two PBC mouse models, dnTGFβRII and IL-12p35$^{-/-}$, to generate an IL-12p35$^{-/-}$; dnTGFβRII strain, features both circulating AMA and periportal fibrosis (Popov 2013). This advancement represents an outstanding opportunity for researchers to test novel therapies and targets in order to gain insight into this devastating disease.

Two rat models with ADPKD are utilized: one where the animals exhibit spontaneous manifestations of polycystic kidney disease (PKD) through heritable traits and the other where genetic modifications mimic the mutation of human orthologous genes (Erickson et al. 2008). In the spontaneous hereditary model Han:SPRD-Cy, rats form renal cysts as a result of a missense mutation. This mutation shares the human PKD phenotype through non-orthologous genes. Homozygous rats will have initial cysts discoverable in the neonate stage, whereas the heterozygotes develop the disease at a much slower rate. PKD rats carry a mutation orthologous to the ADPKD gene in humans, and these animals express this gene mutation in the kidney, liver, and pancreas (Erickson et al. 2008). Both the PKD and Cy/+ strains have an approximate life span of 1.5 years.

Historically, several animal models have been developed to mimic biliary atresia, from lampreys and zebra fish to rhesus monkeys and mice. Lampreys have been found to naturally develop degeneration of the biliary system. Although it has proven to be useful for the study of bile duct transport, it lacks the disease pathology of human biliary atresia. Zebra fish are advantageous for studying biliary atresia because of their rapidly developing gastrointestinal system and clear elucidation of biliary development. Rhesus monkeys provided the first evidence that viral infections play a role in the etiology of biliary atresia when infant monkeys with biliary pathology tested positive for reovirus type 3. Hepatic viral-induced mice have provided further details into the pathogenic pathways, but to date no individual animal model has been discovered that truly mimics the disease pathology of biliary atresia in human infants (Petersen 2012).

Cholangiocarcinomas arise from chronic cholestasis, which ultimately contributes to the overall hepatocellular injury. One way to effectively model this pathogenesis is through a combination of left median bile duct ligation (LMBDL) and diethylnitrosamine (DEN). Due to the catastrophic insult suffered by the liver after common bile duct ligation (CBDL), many animals display high mortality with severe liver damage or greater survival rates associated with a significant decrease in liver damage (Yang et al. 2011). Intraperitoneally injected diethylnitrosamine, a known carcinogen, initiates liver cancer formation, and when coupled with the LMBDL procedure, a model of cholestasis results in a working model of human cholangiocarcinoma which effectively mimics the disease progression and chronic cholestasis and fibrosis (Yang et al. 2011).

## 2.5 Cell Death

### *2.5.1 Innate Immunity in Cholangiocytes*

The liver's unique histological structure provides the basis for its role as the first line of defense against xenobiotics and infection (Ishibashi et al. 2009). The functional unit of the liver is called the hepatic lobule consisting of a hexagonal-like sheet of cells surrounding a central vein with portal triads at the periphery. Blood rich in metabolites, toxins, hormones, and immune cells returning from splanchnic and mesenteric circulation enters the liver from the portal vein and flows into the sinusoids lined by fenestrated endothelial cells. Here hepatocytes, along with Kupffer cells, hepatic stellate cells (HSCs), endothelial cells, and various lymphocytes, coordinate immune surveillance and detoxify as the blood flows to the central vein. Additionally, hepatocytes and cholangiocytes secrete and modify the bile into bile canaliculi, which flows the opposite direction from the central vein to the portal triad and is drained by the biliary tree into the gallbladder and duodenum (Gao et al. 2008; Turcotte and Jarnagin 2015).

Cholangiocytes play a major role in hepatobiliary innate immunity. As opposed to the fenestrated endothelial cells lining the sinusoid, cholangiocytes maintain an epithelial barrier through regulation of tight junction proteins. Cholangiocytes secrete antimicrobial peptides such as defensins and secretory IgA as well as mucosal layer that protects the epithelial surface (Chen et al. 2008). Cholangiocytes also express primary cilia that extend into the biliary lumen whose function is both mechanical and sensory. Defects in primary cilia are associated with ADPKD and BA (Chu et al. 2012). Furthermore, cholangiocytes express pattern-recognition receptors (PRR) that recognize pathogen-associated molecular patterns (PAMPs) to initiate a localized, nonspecific inflammatory response (Fig. 2.1). Examples of PRR include surface Toll-like receptors (TLRs) and cytoplasmic NOD and RIG receptors (Gao et al. 2008; Turcotte and Jarnagin 2015).

PAMP recognition by these receptors can lead to programmed gene expression changes and coordinated defense against pathogens through secretion of inflammatory cytokines (TNF-α, IFN-γ, IL-1, IL-6, and TGF-β) and recruitment of immune cells (Kupffer cells, neutrophils, and various lymphocytes) (Gao et al. 2008; Turcotte and Jarnagin 2015). Cholangiocytes also respond in various ways to cytokines secreted by other cells including, but not limited to, changes in proliferation, apoptosis, cytotoxicity, and expression of surface adhesion ligands (Chen et al. 2008). It is widely believed that adhesion ligands can facilitate direct cell-to-cell cytotoxicity between cholangiocytes and immune cells.

Natural killer (NK) cells are a major player in innate immunity and are implicated in cholangiopathies (Gao et al. 2008). Recent evidence revealed that cholangiocytes express surface CD1d ligands that recruit NKT cells (a subtype of NK cells) and initiate cell death (Schrumpf et al. 2015). NK cells are crucial in fighting viruses, intracellular pathogens, and tumors because a) they do not express antigen receptors like T and B cells and b) they target cells with decreased expression of

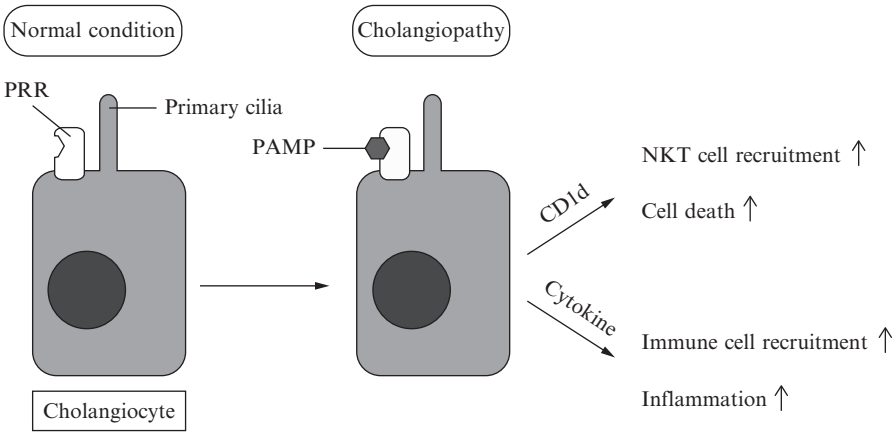


**Fig. 2.1** Cholangiocyte innate immunity in cholangiopathies. Cholangiocytes express primary cilia and pattern-recognition receptors (*PRR*) as part of the innate immune system. Defects in primary cilia or uncontrolled activation of PRR can lead to cholangiopathies

MHC class I molecules (presumably due to evasive actions of the disease state) (Vivier et al. 2008). NK cells can also have a protective effect through clearing of profibrotic, proinflammatory cells. NK cells target activated hepatic stellate cells (HSCs) for cell death, but not quiescent HSCs (Gao et al. 2008), and are responsible for clearing self-recognizing T cells and dendritic cells (Tian et al. 2013).

Cholangiocytes also play a role in maintaining homeostasis between innate and adaptive immunity. Both cytokine secretion and cognate interaction via MHC class II molecules can drive activation and differentiation of various T and B lymphocytes. Dysregulation of this balance can lead to cholangiopathies. PBC is characterized by the presence of antimitochondrial autoantibodies (AMA) that drive sustained TLR signaling, increased proinflammatory Th1 and Th17 and decreased Treg phenotypes, and increased cholangiocyte cytotoxicity and HSC activation (Syal et al. 2012; Wang et al. 2016). PSC has a similar pathophysiology however with a few key differences. For instance, p-ANCA autoantibodies predominate instead of AMA, and cholangiocyte senescence is more prolonged (Nakanuma et al. 2015).

### *2.5.2 Apoptosis in Cholangiocytes*

Cholangiopathies are rare disorders with no common genetic defects, yet chronic inflammation seems to be a universal contributing factor. Apoptosis is an important regulatory mechanism to keep cellular transformation in check through the removal of damaged cells and reduction in malignant progression (Humphreys et al. 2010). Apoptosis also serves to maintain homeostasis and promote liver tissue regeneration

as apoptotic bodies release growth signals that in turn stimulate the progenitor cells to initiate proliferation (Guicciardi et al. 2013). Apoptosis is the process by which a cell exposes a phosphatidylserine on the outer leaflet while condensing its chromatin and fragmenting its DNA. As a result, the cell shrinks and blebs and is reduced to smaller membrane-enclosed organelles called apoptotic bodies which are then marked for phagocytosis (Guicciardi et al. 2013). This fragmentation and signaling cascade, through a family of caspases, act upon various molecules to regulate normal cellular turnover and maintenance during times of stress.

Cholangiocytes undergo apoptosis through a variety of molecular pathways. Common pathways include Fas (CD95), TRAIL-R1/2, TNFR1/2, and CD40 (Fig. 2.2). In addition to the aforementioned receptors, there is evidence that autophagy and lipoapoptosis are also key players in the apoptotic regulatory system. Fas (CD95) receptors, a member of the TNF receptor superfamily, are widely expressed in the liver at low levels, which dramatically increase during inflammation, via its ligand FasL (CD178). In PBC, there is cross-linking of the ligand, resulting a progressive loss of the bile ducts (Guicciardi et al. 2013). Furthermore, there is evidence that both hepatocytes and cholangiocytes form regenerative clusters that overexpress death receptor Fas and mito-inhibitory protein glypican 3 (Hattoum et al. 2013). Additionally, previous research has shown that anandamide (AEA), an endocannabinoid, is both antiproliferative and proapoptotic. Cannabinoid receptors, Cb1 and Cb2, are found in cells of the central nervous system, various peripheral tissues, the gastrointestinal tract, and the immune system. However, blocking these G-protein-coupled receptors does not alter the effects of AEA on cholangiocarcinomas. This is due, in part, to the reaction of AEA with other receptor-independent microdomains that recruits lipid rafts, along with Fas and FasL, in the outer leaflet of the plasma membrane. Disruption of these rafts with methyl-β-cyclodextrin mitigated the AEA suppression of cellular proliferation (DeMorrow et al. 2007).

Another indispensable member of the TNF receptor superfamily is the TNF-related apoptosis-inducing ligand, or TRAIL, additionally noted as death receptor 4/5. In healthy cells, proapoptotic pathways are blocked, and TRAIL activates NF-kB and JNK to promote cellular differentiation and proliferation (Ishimura et al. 2006). However, in cholestatic liver disease, such as human PSC and PBC, TRAIL expression and apoptosis are significantly increased causing cholangiocyte cell death and cholangitis (Takeda et al. 2008). In this pathway, the ligand binds to the death receptors TRAIL-R1/2 and forms a trimeric structure. It is this structure that recruits the Fas-associated death domain (FADD) and procaspases 8 and 10. Through self-activation apoptosis is initiated (Ishimura et al. 2006).

Tumor necrosis factor alpha (TNF-α) is a necessary immunological cytokine that plays a critical role in the inflammatory response of the innate immune system. It is produced in response to viruses, parasites, bacterial endotoxins, and various cytokines. Its course of action is determined by its cell surface receptors, TNF-α receptor 1 (TNF-αR1) and TNF-α receptor 2 (TNF-αR2). TNF-αR1 is widely distributed

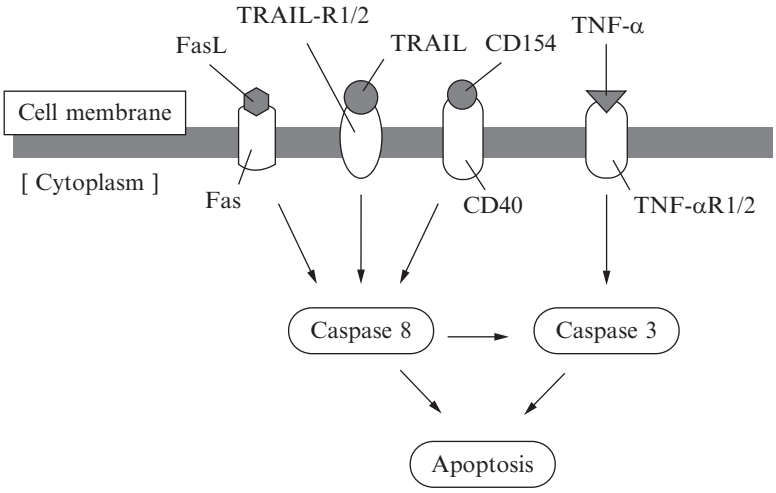


**Fig. 2.2** Extrinsic apoptosis signaling in cholangiocytes. Various external signaling pathways that lead to caspase cascade and apoptosis

and responsible for the majority of the TNF-α cellular responses, including apoptosis (Idriss et al. 2014). Previous research has found that this pathway operates through caspase 3, a common end point caspase in the apoptosis cascade. Generally, cholangiocytes are markedly resistant to TNF-α and proliferate in response to ductal secretions of secretin in an autocrine and paracrine manner following injury. However, cholestatic injury, a primary feature of cholangiopathies, sensitizes cholangiocytes to TNF-α signaling in a way that alters ductular secretions and increases apoptosis (Alpini et al. 2003).

CD40 is a major player in cholangiocyte apoptosis through a variety of signaling pathways such as Fas-mediated signaling, STAT3 upregulation, and CD154-bearing Kupffer cells. Fas, or CD95, is a typical death receptor of the TNF family that, in response to cross-linking with its ligand FasL, results in cholangiocyte apoptosis and subsequent bile duct loss. However, research has demonstrated that Fas-FasL cross-linking on its own is not enough to induce apoptosis in either normal or malignant cells. Additionally, Fas has been shown to be downregulated as the tumor moves toward genetic instability. Thus, it appears that CD40, a cell surface glycoprotein, is the main route for apoptosis signaling. In addition to autocrine and paracrine death induction, cholangiocytes are also susceptible to immune-related death through the CD154-expressing Kupffer cells. In chronic liver diseases, circulating macrophages may be activated by bacterial endotoxins or proinflammatory cytokines. These activated macrophages then overexpress CD154 directly inducing apoptosis through binding its cognate receptor, CD40 (Alabraba et al. 2008). Other key players also regulate CD40 signaling in apoptotic

cell death. For example, inhibition of STAT3 yields a 50% reduction in overall cholangiocyte cell death (Ahmed-Choudhury et al. 2006). While this reduction is significant and offers a promising therapeutic target, it also indicates there are other, equally important, players to be explored along the CD40 axis.

In addition to cholangiocyte innate death receptors, nonconventional pharmaceutical treatments have also been found to increase apoptosis in cholangiocarcinoma cell lines. For example, Simvastatin, a cholesterol-lowering drug readily used in the prevention and treatment of atherosclerosis, has been shown to increase apoptosis in cholangiocarcinoma cell lines while simply inhibiting proliferation in normal cholangiocytes with no effect on apoptosis (Miller et al. 2011). Several cholesterol pathway intermediates facilitate proper membrane localization and activity of the Rho family of GTPases, including Rac1. Simvastatin interferes with cholangiocyte's ability to localize Rac1 to cholesterol rafts, presumably through inhibition of these crucial intermediates, subsequently decreasing Rac1 activity and increasing apoptosis. Specifically, simvastatin was shown to increase caspase 3 and caspase 7 activities. These results were completely reversible when the cells were pretreated with cholesterol (Miller et al. 2011). As cholangiocytes are typically quiescent unless under duress, the treatment poses no threat to the healthy epithelial cells (Maroni et al. 2015).

### *2.5.3 Senescence and Autophagy*

Recent studies in biliary physiology have shown that cholangiocytes use senescence and autophagy in response to biliary injury, including fibrosing cholangiopathies, such as PSC and PBC (Nakanuma et al. 2015; Wang 2015). Cellular senescence is a cellular process by which proliferation halts and cells become arrested in the G1 phase of the cell cycle. Senescent cells remain metabolically active; however, they do not respond to external stimuli (Kuilman et al. 2010). Cellular senescence can occur following numerous insults, such as oxidative stress and oncogene activation, and is thought to be a protective mechanism against additional insults (Nakanuma et al. 2015). Senescent cells have also been shown to take on a secretory phenotype, which persistently secrete cytokines, chemokines, growth factors, and profibrotic factors (Kuilman et al. 2010; Lawless et al. 2010).

Autophagy is a regulated program of self-degradation that allows cells to remove long-lived or damaged proteins and/or organelles. When autophagy is activated during cellular stress, organelles and proteins become sequestered in autophagosomes and digested through fusion with lysosomes (Mizushima 2007). These mechanisms can act as a protective response to cellular stress and enable cells to maintain cellular viability and homeostasis (Kroemer et al. 2010). These cellular mechanisms, along with apoptosis, play a critical role in cholangiocyte response to injury. Regulation of these cellular processes occurs through a balance between the tumor suppressor p53 and expression of anti-apoptotic signaling, specifically the AKT/mTOR pathway (Wang 2015). The cross talk between p53 and AKT/mTOR determines whether or not cells survive injury (Nakanuma et al. 2015).

Previous studies have shown that these mechanisms are implicated in PBC and may be associated with the progressive bile duct loss that is characteristic of the disease. For example, cholangiocytes from PBC patients exhibit shortened telomeres and increased expression of SA-β-Gal, $p16^{INK4a}$, and $p21^{WAF1/Cip}$, which are characteristics of senescent cells (Sasaki et al. 2005, 2008b). It is hypothesized that the senescent cholangiocytes in PBC maintain a secretory phenotype, which promotes the release of cytokines and chemokines into the periductular environment, creating an inflammatory reaction resulting in biliary injury (Sasaki et al. 2008a). Additionally, studies have shown that the autophagy markers are upregulated in bile ducts from PBC patients (Sasaki et al. 2012). This includes microtubule-associated protein light chain 3β (LC3) and p62, a protein involved in the ubiquitin-bound cargo to autophagosomes (Wang 2015). Accumulation of p62 is suggestive of deregulated autophagy and insufficient processing of damaged proteins, which could be a contributing factor in the pathogenesis of PBC (Nakanuma et al. 2015).

Additionally, studies have shown that senescence and autophagy may also contribute to the pathogenesis of PSC. Cultured cholangiocytes isolated from PSC patients demonstrate a senescent phenotype, such as overexpression of $p16^{INK4a}$ and SA-β-Gal. These cholangiocytes also express a secretory phenotype, such as in PBC, which may contribute to the recruitment of inflammatory cells and modulation of the periductal environment (Tabibian et al. 2014a, b). Less is known about the role of autophagy in PSC; however, it has been suggested that the deregulated autophagy in respect to processing microbial pathogens from the intestinal flora may contribute to the development of peri-neutrophil cytoplasmic antibody (pANCA), an autoantibody commonly found in PSC patients (Nakanuma et al. 2015; Schwarze et al. 2003). These findings suggest that senescence and autophagy may play a role in the progression of PBC and PSCs, but more research is needed to elucidate the differences between the two etiologies of biliary injury.

### *2.5.4 Lipoapoptosis*

Nonalcoholic fatty liver disease (NAFLD) is not recognized in the cholangiopathy family, but it is the most common chronic liver disease in Western countries (Gautheron et al. 2014). The involvement of cholangiocyte damage in the pathogenesis of NAFLD has not been thoroughly investigated. A recent study has demonstrated that the PNPLA3 rs738409 variant (I148M) that is associated with an increased risk for the development of NAFLD and alcoholic liver disease due to hepatocyte lipotoxicity is also a risk factor for reduced survival in male PSC patients with dominant bile duct stenosis presumably due to lipotoxicity at the cholangiocyte level (Friedrich et al. 2013; Romeo et al. 2008; Tian et al. 2010). A recent in vitro study has demonstrated that saturated FFAs palmitate and stearate induced cholangiocyte lipoapoptosis via caspase activation, nuclear translocation of FoxO3, and increased proapoptotic PUMA expression (Natarajan et al. 2014). These findings may help explain why a subset of NAFLD patients with symptoms of

cholestasis have biliary injury not consistent with what is normally observed in the majority of patients with NAFLD. Further in vivo model studies are necessary to clearly elucidate the role of cholangiocyte lipoapoptosis in the pathogenesis of NAFLD.

## 2.6 Conclusions and Future Directions

Cholangiocytes play an integral role in the overall function of the liver and its ability to clear toxins, aid in the digestion and absorption of fats, and expel waste through the bile. Cholangiopathies continue to carry a large disease burden due to the lack of effective treatment strategies despite extensive research over the past two decades. The use of transgenic mice to mimic these diseases will hopefully provide more insight into the mechanisms of liver injury. Studies suggest that cholangiocytes use numerous mechanisms to respond to hepatic injury including apoptosis, autophagy, and senescence. A better understanding of the biological functions and cell death pathways of cholangiocytes in both normal and disease states will aid in the development of novel targeted therapies for cholangiopathies.

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# Chapter 3
# Cell Death and Autophagy in Hepatic Stellate Cell Activation and Function

**Fatemeh P. Parvin-Nejad and Scott L. Friedman**

## Abbreviations

| | |
|---|---|
| aHSC | Activated hepatic stellate cell |
| AMPK | Adenosine monophosphate-activated protein kinase |
| ASK1 | Apoptosis signal-regulating kinase 1 |
| ATF6 | Activating transcription factor 6 |
| BiP | Binding immunoglobulin protein |
| ECM | Extracellular matrix |
| ER | Endoplasmic reticulum |
| ERK | Extracellular signal-regulated kinase |
| FasL | Fas ligand |
| HIF-1α | Hypoxia-inducible factor 1 alpha |
| HSC | Hepatic stellate cell |
| IFNγ- | Interferon gamma |
| IP3 | Inositol 1,4,5-trisphosphate |
| IRE1 | Inositol-requiring kinase 1 |
| JNK | c-Jun-N-terminal kinase |
| MAPK | Mitogen-activated protein kinase |

**Grant support** Fatemeh Parvin-Nejad is supported by a Howard Hughes Medical Institute Medical Research Fellows grant. Scott L. Friedman is supported by grant NIDDK RO156621.

**Author contributions** FPN– wrote the initial draft and designed figures. SLF –reviewed and finalized text, modified figures.

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W.-X. Ding, X.-M. Yin (eds.), *Molecules, Systems and Signaling in Liver Injury*, Cell Death in Biology and Diseases, DOI 10.1007/978-3-319-58106-4_3

X.-M. Yin and Z. Dong (Series eds.), *Cell Death in Biology and Diseases*

| | |
|---|---|
| MMP | Matrix metalloproteinase |
| mTOR | Mammalian target of rapamycin |
| NF-κB | Nuclear factor-kappa B |
| NGF | Nerve growth factor |
| NK cell | Natural killer cell |
| Nrf2 | NF-E2-related factor 2 |
| PDGF | Platelet-derived growth factor |
| PERK | Protein kinase-like endoplasmic reticulum kinase |
| PI3K | Phosphatidylinositol 3-kinase |
| PKCθ | Protein kinase C theta |
| PUMA | p53-upregulated modulator of apoptosis |
| qHSC | Quiescent hepatic stellate cell |
| TGF-β | Transforming growth factor beta |
| TIMP | Tissue inhibitor of metalloproteinases |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| TRAIL | TNF-related apoptosis-inducing ligand |
| UPR | Unfolded protein response |
| VEGF | Vascular endothelial growth factor |
| XBP1 | X-box binding protein 1 |

## 3.1 Introduction

Hepatic fibrosis contributes to a significant burden of end-stage liver disease worldwide. Tremendous advances in uncovering mechanisms of fibrosis have begun to yield significant progress in defining therapeutic targets and developing new treatments. Some of this success is based on the observations that hepatic fibrosis is often reversible (Iredale et al. 1998), but the underlying mechanisms have not yet been fully elucidated. Understanding these mechanisms may unearth additional therapeutic targets to prevent or even reverse fibrosis in patients with hepatic disease, since despite the remarkable progress, there are still no approved antifibrotic drugs.

Central to the pathogenesis of hepatic fibrosis is the hepatic stellate cell. This resident perisinusoidal mesenchymal cell is the primary storage site for vitamin A in normal liver, contained within cytoplasmic droplets as retinyl esters. In liver injury of any cause, stellate cells undergo a characteristic “activation” in which they transdifferentiate from a resting nonproliferative cell to a highly fibrogenic, proliferative, and contractile cell type that orchestrates hepatic inflammation and fibrogenesis (Lee et al. 2015). This article summarizes emerging knowledge about the contributions of cell death and autophagy to the pathways driving conversion of quiescent (qHSC) to activated (aHSC) hepatic stellate cells. This summary is especially timely, since clarification of cell death

and autophagy in other tissues and biological contexts has begun to illuminate our understanding of stellate cell biology.

## 3.2 Apoptosis and Autophagy in aHSCs

Two mechanisms of cellular recycling and energy homeostasis in aHSCs—apoptosis and autophagy—have been implicated in the regulation of aHSC function and survival and may prove to be central in the balance of fibrogenesis and fibrosis resolution. Apoptosis, or programmed cell death, has a clear role in the resolution of fibrosis; in fact, the fraction of apoptotic cells undergoing apoptosis increases in resolving hepatic injury (Iredale et al. 1998). Importantly, aHSCs are at increased susceptibility to apoptosis mediated by the soluble Fas ligand, possibly related to decreased expression of anti-apoptotic proteins Bcl-2 and Bcl-xl (Gong et al. 1998). On the other hand, autophagy, which displays increased rates of flux during liver injury (Hernández-Gea et al. 2012), drives activation of HSCs (Thoen et al. 2011), ultimately contributing to increased fibrogenesis.

Autophagy is a mechanism of intracellular energy homeostasis, degrading intracellular organelles and proteins to provide energy substrates for cellular processes (Czaja 2011). In activated stellate cells, autophagy degrades lipid stored within cytoplasmic droplets to provide energy supporting their activation (Hernández-Gea et al. 2012). Here we examine the signaling pathways driving cell death and autophagy that contribute to stellate cell activation and fibrogenesis.

## 3.3 HSC Activation and Apoptosis

Apoptosis of aHSCs contributes to the spontaneous resolution of hepatic fibrosis, as best characterized in carbon tetrachloride-induced liver injury in rodents (discussed in detail below) (Iredale et al. 1998). Apoptosis is induced in HSCs through several mechanisms, setting in motion a negative feedback loop that can attenuate fibrogenesis. Moreover, an apoptotic fate in activated HSCs is controlled by the balance between a complex array of pro-apoptotic and anti-apoptotic factors that include:

### *3.3.1 Natural Killer Cell-Mediated Apoptosis*

One mechanism by which aHSCs undergo apoptosis is through natural killer (NK) cell-mediated killing. NK cells, a component of the innate immune system, can induce apoptosis of target cells through a variety of mechanisms, including the transfer of cytoplasmic granules from NK cells to their cellular targets as well as the

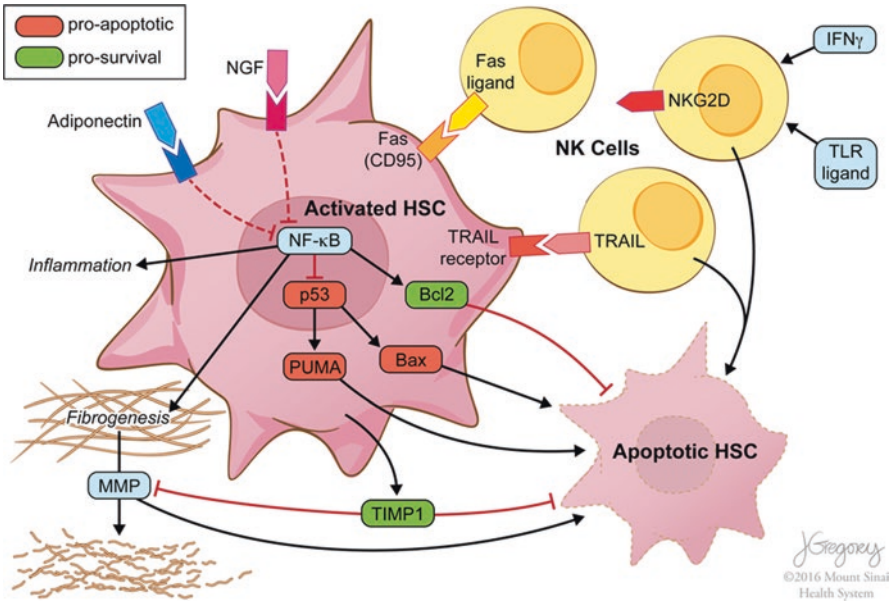


**Fig. 3.1** Apoptotic pathways in activated HSCs. Activated HSCs (aHSCs) negatively regulate their own fibrogenic function through several pathways of apoptosis (factors colored green are pro-survival; those colored red are pro-apoptotic). Natural killer (NK) cells, activated by interferon gamma and TLR ligands, recognize aHSCs through NKG2D, Fas ligand, and TRAIL, acting either through the release of cytoplasmic granules or the caspase 8/caspase 3 pathway to provoke aHSC apoptosis. MMPs, which can drive fibrinolysis, also lead to apoptosis, whereas their inhibitors, TIMPs, are anti-apoptotic, contributing to persistent fibrogenesis from surviving aHSCs. NF-κB, which is inhibited by downstream effectors of adiponectin and NGF, modulates transcription in the nucleus, upregulating the anti-apoptotic Bcl2 while downregulating pro-apoptotic proteins Bax and PUMA by reducing expression of p53. NF-κB also contributes to inflammation and fibrogenesis (Printed with permission from ©Mount Sinai Health System)

engagement of NK cell-expressed Fas ligand (FasL) or tumor necrosis factor (TNF) with their respective effectors on target cells, Fas (also known as CD95) and TNF receptor (TNFR) (Warren and Smyth 1999). In the liver, NK cells can target and kill aHSCs, thus reducing hepatic fibrosis (Radaeva et al. 2006). Activated by interferon gamma (IFNγ) and the Toll-like receptor (TLR) ligand polyinosinic:polycytidylic acid, NK cells induce apoptosis of aHSCs through cell receptors NKG2D, TNF-related apoptosis-inducing ligand (TRAIL) (Radaeva et al. 2006), and FasL (Fig. 3.1) (Saile et al. 1997). Fas and TRAIL receptor can also induce apoptosis mediated by the caspase-8/caspase-3 pathway (Iredale 2001). Interestingly, while Fas is expressed ubiquitously on both hepatocytes and HSCs, TRAIL receptor is upregulated only on aHSCs, thereby presenting a more specific target for future therapeutics that could potentially attenuate fibrosis without damaging the hepatic parenchyma (Taimr et al. 2003; Fasbender et al. 2016; Oh et al. 2016). NK cells can also mediate apoptosis of senescent HSCs, leading to reduced fibrogenesis (Krizhanovsky et al. 2008).

### 3.3.2 Matrix Metalloproteinases and Their Inhibitors: Linking Fibrinolysis and Apoptosis

Besides NK cell-mediated killing, HSC apoptosis is also regulated by the pathways that control fibrosis resolution, or fibrinolysis, which in turn depends on the balance between matrix metalloproteinases (MMPs), which cleave collagen and ECM materials, and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs). TIMP-1, which is produced by aHSCs (Ramachandran et al. 2015), has direct anti-apoptotic activity in addition to its inhibitory function on MMPs, some of which are in fact pro-apoptotic (Fig. 3.1) (Iredale 2001). However, TIMP-1 and TIMP-2 levels decrease in association with the resolution of liver injury (Iredale et al. 1998). This reduction in TIMP-1 levels relieves the inhibition on both MMP function and aHSC apoptosis, not only increasing the degradation of extracellular matrix (ECM) already deposited but also decreasing further fibrogenesis by reducing the number of aHSCs.

### 3.3.3 Nuclear Factor-Kappa B: An Anti-Apoptotic Factor

Nuclear factor-kappa B (NF-κB) regulates aHSC survival by inhibiting apoptotic factors in the nucleus. Levels of transcriptionally active NF-κB rise sharply during HSC activation, leading to the inhibition of apoptosis as well as the expression of several pro-inflammatory genes leading to increased fibrogenesis. NF-κB's anti-apoptotic function is achieved through the continued expression of the anti-apoptotic protein Bcl-2 and the inhibition of pro-apoptotic factors Bax and PUMA via the suppression of cell cycle regulator p53 (Fig. 3.1). NF-κB is inhibited by nerve growth factor (NGF), a neurotrophin, as well as by adiponectin through downstream AMPK pathway activation (Hernandez-Gea and Friedman 2011).

## 3.4 Autophagy and HSC Activation

Autophagy plays a key role in the activation of HSCs. Autophagic flux increases during liver injury and HSC activation, whereas inhibiting autophagy partly inhibits HSC activation (Thoen et al. 2011). Increased autophagic activity is thought to liberate energy from lipid droplets contained within HSCs, thereby providing the required energy for HSC activation; in fact, in HSCs from an autophagy-knockout mouse strain, activation and resulting fibrogenesis can be restored by adding oleic acid as an energy substrate (Hernández-Gea et al. 2012). The engagement of autophagy to provide fuel for extracellular matrix (ECM) production occurs in fibrogenic cells from the kidney and lung as well, suggesting that autophagy is part of a core pathway of fibrogenesis across several tissue types (Hilscher et al. 2013). Several pathways and processes mediate the

activation of HSCs via autophagy, including an mTOR-dependent pathway and the hypoxia-inducible factor 1 alpha (HIF-1α) pathway following hypoxia, among other pathways.

### 3.4.1 mTOR-Dependent Autophagy

The mTOR pathway induces autophagy leading to HSC activation in periods of metabolic (Hilscher et al. 2013) as well as hypoxic stress (Jin et al. 2016). Under nutrient-depleted conditions, the inhibitory function of mTOR complexes (mTORC) 1 and 2 on their usual target, the ULKs-Atg13-FIP200 complex (a mammalian autophagy complex), is relieved, leading to activation of the target complex and induction of autophagy (Fig. 3.2) (Hosokawa et al. 2009). mTORC1 is regulated by several factors, including the phosphatidylinositol 3-kinase (PI3K) complex, components of which have opposing effects: class I PI3K inhibits autophagy through Akt signaling, while class III PI3K (Vps34) stimulates autophagy by increasing concentrations of phosphatidylinositol 3-phosphate. Other kinases influencing the effect of mTOR on autophagy include adenosine monophosphate-activated protein kinase (AMPK), IκB kinase, and mitogen-activated protein kinase (MAPK) pathway components, including extracellular signal-regulated kinase (ERK), p38, and c-Jun-N-terminal kinase (JNK) (Hilscher et al. 2013); the functions of p38 and JNK with respect to endoplasmic reticulum (ER) stress are discussed below. Hypoxic stress also provokes autophagy and HSC activation in rats through the calcium-dependent AMPK-mTOR and protein kinase C-theta pathways (Fig. 3.2) (Jin et al. 2016). Interestingly, calcium release mediated by a different factor, inositol 1,4,5-trisphosphate (IP3), leads to the *inhibition* of autophagy through a $Ca^{2+}$-calpain-Gsα pathway (Hilscher et al. 2013).

### 3.4.2 HIF-1α: The Role of Hypoxia in Autophagy and HSC Activation

HIF-1α, a protective factor in periods of hypoxic stress, regulates autophagy-mediated activation of HSCs. HIF-1α can be induced by several pathways, including hypoxia, growth factors (such as platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), and TNF-α), oncogenic signaling pathways (p53 and Ras), and vascular endothelial growth factor (VEGF) (through a positive feedback loop involving PI3K and MAPK), as well as epigenetic mechanisms (Fig. 3.2) (Zhan et al. 2015). In conditions of hypoxic stress, HIF-1α stimulates the activation of HSCs (Wang et al. 2013), which is attributable to HIF-1α-induced autophagic activity (Deng et al. 2014). HIF-1α is also implicated in the self-perpetuating cycle of HSC activation and fibrogenesis. Hypoxia, oncogenic

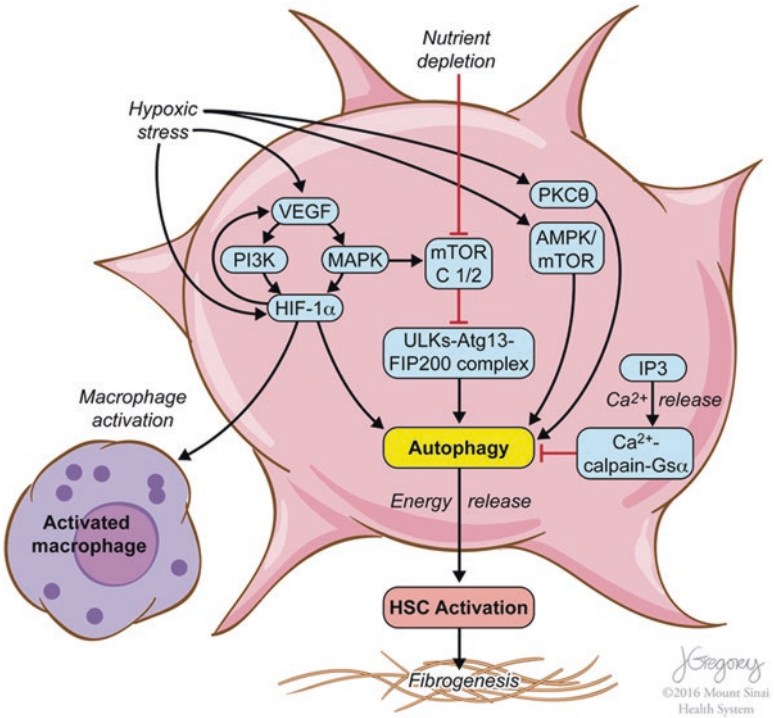


**Fig. 3.2** Autophagy-inducing pathways leading to HSC activation. In HSCs, autophagy generates energy through cleavage of retinyl esters to yield fatty acids, which support HSC activation and fibrogenesis. Under conditions of nutrient depletion, mTOR complex activity is inhibited, relieving its inhibition of the ULKs-Atg13-FIP200 complex, which leads to autophagy. Conditions of hypoxic stress lead to activation of several other pathways triggering autophagy, including PKCθ, the calcium-dependent AMPK/mTOR pathway, and HIF-1α. Conversely, calcium release also allows IP3 to activate the *anti-autophagic* $Ca^{2+}$-calpain-Gsα complex. HIF-1α is regulated by a positive feedback loop: hypoxia stimulates VEGF, which upregulates HIF-1α through the actions of PI3K and MAPK. The upregulated HIF-1α in turn further induces VEGF. MAPK also activates the anti-autophagic mTORC, providing an internal balance mechanism. HIF-1α also has an anti-fibrogenic function, activating macrophages which clear TGF-β-secreting necrotic hepatocytes during liver injury, thus removing a stimulus for fibrogenesis (Printed with permission from ©Mount Sinai Health System)

stimuli, and growth factors can lead to increased VEGF expression, which triggers PI3K- and MAPK-dependent stimulation of HIF-1α, which in addition to provoking autophagic-mediated HSC activation feeds back to further stimulate VEGF production and continue the cycle (Zhan et al. 2015). Furthermore, the VEGF feedback loop may comprise part of a common pathway between HIF-1α and mTOR (which is also regulated by PI3K and MAPK as described above) in the induction of autophagy. Notably, HIF-1α may have an anti-fibrogenic role as well (Fig. 3.2). In knockout mice lacking HIF-1α only in HSCs, loss of HIF-1α increases collagen I production and reduces conversion of macrophages to a more active phenotype

(which clear necrotic hepatocytes) following carbon tetrachloride-induced hepatic injury. These findings suggest that increased fibrogenesis following liver injury could be mediated by exposure of HSCs to necrotic products from inadequately cleared, dying hepatocytes, stimulating TGF-β-dependent collagen production (Mochizuki et al. 2014).

## 3.5 Interplay Between Autophagy and Apoptosis

Of particular interest are signals that regulate *both* apoptosis and autophagy in their roles in HSC activation and fibrogenesis, including the ER stress response, TGF-β signaling, Hedgehog signaling, and the oncogenic regulator p62. These signals integrate HSC activation, fibrogenesis, and liver remodeling and may prove to be key in identifying factors that can distinguish between these different fates, thus providing therapeutic targets to promote hepatic regeneration in the absence of fibrogenesis.

### *3.5.1 ER Stress: Linking Autophagy and Apoptosis*

One well-described pathway that moderates both apoptosis and autophagy is the unfolded protein response (UPR) following ER stress. Oxidative stress has already been shown to activate HSCs (Svegliati-Baroni et al. 2001). Conditions of oxidative stress and increased protein synthesis may overwhelm ER folding capacity, leading to activation of the UPR, a compensatory mechanism which functions to restore ER homeostasis (Hernández-Gea et al. 2013). In this mechanism, unfolded proteins sequester a regulatory factor known as binding immunoglobulin protein (BiP), releasing and thus activating its three targets, all of which are ER transmembrane-signaling proteins. These proteins—activating transcription factor 6 (ATF6), inositol-requiring kinase 1 (IRE1), and protein kinase-like endoplasmic reticulum kinase (PERK)—provide signals to upregulate genes encoding proteins which contribute to protein folding and otherwise help reduce the burden on the overloaded ER (Fig. 3.3) (Schröder and Kaufman 2005). Of these, ATF6 and IRE1α can upregulate autophagy and resulting collagen 1 expression (Hernández-Gea et al. 2013; de Galarreta et al. 2016), whereas the PERK pathway has been implicated in HSC activation and fibrogenesis via overexpression of SMAD2 (an effector of the TGF-β pathway) (Koo et al. 2016). Conversely, IRE1 mediates ER stress-induced aHSC apoptosis after treatment with cannabidiol (Lim et al. 2011) as well as after exposure to the senescence-inducing factor CCN1 (Borkham-Kamphorst et al. 2016).

Given the multitude of downstream effects of IRE1α signaling, its role must be further clarified. IRE1α acts through apoptosis signal-regulating kinase 1 (ASK1) to activate downstream stress kinases JNK and p38 MAPK (Borkham-Kamphorst et al. 2016).

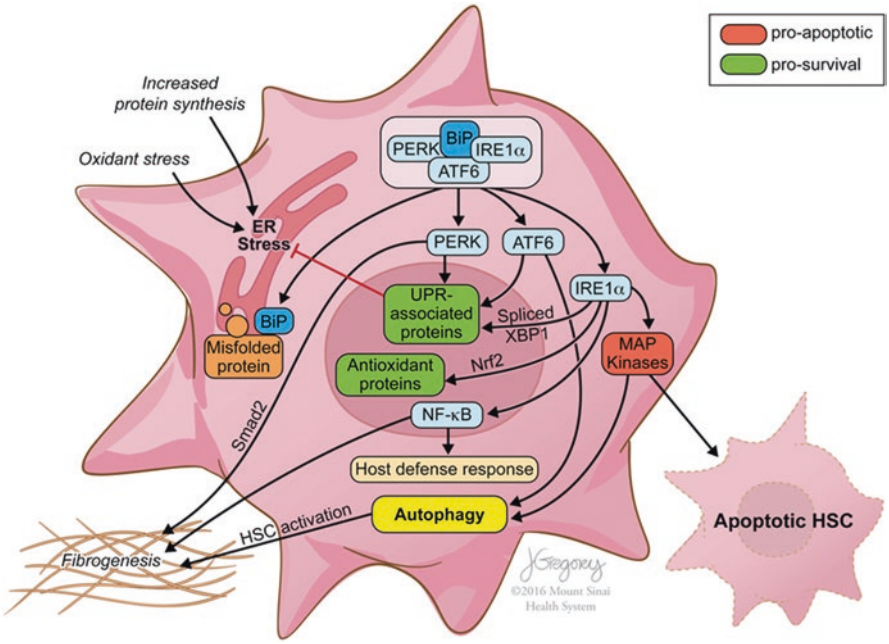


**Fig. 3.3** ER stress and the unfolded protein response in HSCs. Oxidants and conditions requiring increased protein synthesis increase stress on the ER, leading to the misfolding of proteins (factors colored green are pro-survival; those colored red are pro-apoptotic). Misfolded proteins trigger the compensatory unfolded protein response (UPR) by sequestering the inhibitory BiP away from the three UPR effectors, PERK, ATF6, and IRE1α. These three arms of the UPR increase the transcription and expression of UPR-associated proteins such as molecular chaperones, which improve protein folding and reduce protein expression, reducing the burden on the ER in an effort to adapt to changing demands on the cell. The effectors also modulate autophagy, apoptosis, and fibrosis. PERK induces the overexpression of Smad2 by destabilizing downregulatory transcription factors, leading to Smad2-mediated fibrogenesis. ATF6 has also been implicated in stimulating autophagy. IRE1α is the most complex effector arm, leading to *adaptive* responses such as increasing expression of UPR-associated proteins (by splicing XBP1) and antioxidant proteins (by upregulating Nrf2), *alarm* responses such as activating NF-κB (which initiates a host defense response as well as contributing to fibrogenesis), and, when these strategies prove unsuccessful in maintaining cellular viability, the *apoptotic* response through various MAP kinases (some of which also contribute to autophagy) (Printed with permission from ©Mount Sinai Health System)

Yet the functions of these effectors remain to be fully distinguished. While the autophagic and fibrogenic properties of IRE1α in activated HSCs are dependent on p38 MAPK (Hernández-Gea et al. 2013), some studies have found increased levels of activated JNK as well (though no causality in the autophagy pathway) (de Galarreta et al. 2016), and JNK has been independently implicated in autophagy induction in other cell types. In starvation models using HeLa and breast cancer cell lines, JNK1 phosphorylates Bcl-2, releasing Beclin 1 to stimulate autophagy (Wei et al. 2008). Both factors appear to have a role in HSC apoptosis as well. The overexpression of CCN1 (which induces senescence in cutaneous myofibroblasts during

wound healing) in HSC and myofibroblast cell lines leads to HSC apoptosis associated with an increase in JNK and reduced collagen 1 expression, but no change in p38 MAPK levels (Borkham-Kamphorst et al. 2016). Yet thapsigargin-induced ER stress acts through JNK *and* p38 MAPK to induce apoptosis (Huang et al. 2014). Parallel to its autophagic and apoptotic functions, IRE1α also triggers the transcription of antioxidant proteins through the action of Nrf2, which is also associated with fibrogenesis (Hernández-Gea et al. 2013); hydrogen peroxide-detoxifying enzymes produced in this response play a protective role against apoptosis (Dunning et al. 2013). Additionally, IRE1α activates cell survival factor NF-κB, which initiates the production of host defense mechanisms (Borkham-Kamphorst et al. 2016).

These many functions can be conceptualized according to the adaptation-alarm-apoptosis ER stress model for understanding the complicated cellular response via IRE1α and the involvement of both pro-survival and pro-apoptotic pathways. The initial response to ER stress involves an attempt to adapt to increased demands through the UPR effectors IRE1α, PERK, and ATF6, which improve ER folding capacity and reduce the quantity of proteins to be folded (Borkham-Kamphorst et al. 2016). At this point, autophagy may provide additional energy for activation of the cell while stimulating fibrogenesis as well. When this attempt at adaptation fails, the cell next signals alarm through NF-κB, representing one last checkpoint to maintain cell viability through host defenses before the balance ultimately shifts to apoptosis, which reduces the population of fibrogenic cells and contributes to the resolution of fibrosis (Borkham-Kamphorst et al. 2016). This adaptation-alarm-apoptosis model may provide a framework for designing future studies, which must better delineate a temporal sequence of function for the various components of the ER stress response in order to reconcile the conflicting conclusions of the current literature.

### *3.5.2 TGF-β: A Fibrotic Stimulus Regulating Autophagy and Apoptosis*

Another possible common pathway is that mediated by TGF-β, which reduces apoptosis and increases autophagy, leading to HSC activation and increased fibrogenesis. TGF-β is well-described as an important fibrotic stimulus, triggering the production of ECM in HSCs (Friedman 2000). In the liver, TGF-β1 is primarily produced by macrophages and can activate HSCs (Karlmark et al. 2009), causing enhanced expression of TIMPs (Wynn and Barron 2010) and thereby contributing to the persistence of ECM. TGF-β has both pro-apoptotic and anti-apoptotic functions, mediated by Smad family proteins as well as other mediators, as seen in a number of cell types and developmental contexts (Heldin et al. 2009). One study reports the downregulation of apoptosis in HSCs treated with TGF-β, which was

reversed upon the application of bafilomycin, an autophagy blocker, or anti-LC3 siRNA, an inhibitor of the autophagy pathway (Fu et al. 2014). However, further studies are needed to confirm this effect.

### 3.5.3 *miR-148a and Hedgehog Signaling: Incorporating Autophagy and Apoptosis into Liver Regeneration*

The Hedgehog signaling pathway is yet another node linked to both autophagy and apoptosis in HSC regulation. Hedgehog signaling has a central role in embryonic development, but is also significantly activated during chronic liver injury to mediate hepatic repair (Machado and Diehl 2015). Hedgehog ligands contribute to the activation of HSCs, which also produce these ligands once activated (Machado and Diehl 2015). The Hedgehog pathway also inhibits autophagy, a function which is relieved by miR-148a, a micro-RNA which itself inhibits Hedgehog signaling. Treatment of HSC cell lines with miR-148a also increases apoptosis, suggesting another common pathway of regulation (Liu et al. 2015). Interestingly, Hedgehog also activates HIF-1α, which stimulates the epithelial-to-mesenchymal transition in HSCs, facilitating their function in remodeling the injured liver (Chen et al. 2012). A better understanding of the contributions of this signaling pathway may provide insight into the molecular switches regulating regeneration versus fibrosis in the liver.

### 3.5.4 *p62: A Linchpin of Autophagic and Apoptotic Balance*

p62 has recently emerged as a key factor integrating autophagy, apoptosis, and HSC activation and function. Through its scaffolded protein structure, it activates NF-κB by interacting with TRAF6, Nrf2 by sequestering the inhibitory Keap1, and mTORC1 by interacting with mTORC1 regulators at its PB1 domain (Moscat et al. 2016). Through these multiple interactions, p62 is connected to the inflammatory, fibrogenic, and anti-apoptotic roles of NF-κB, the antioxidant activity of Nrf2, and the anti-autophagic function of mTORC1. Whereas the global overexpression of p62 leads to the development of hepatocellular carcinoma (Umemura et al. 2016), the function of p62 specifically in HSCs, acting through the vitamin D receptor, corresponds to the inhibition of HSC activation and produces anti-fibrogenic and anti-tumorigenic effects (Duran et al. 2016). Thus, p62, along with the ER stress response, TGF-β, and Hedgehog signaling, may belong to a panel of multi-regulatory factors preserving the delicate balance of autophagy and apoptosis in HSCs and contributing to liver injury and fibrosis when dysregulated.

## 3.6 Conclusion

The activation of HSCs and their resulting fibrogenesis are regulated by a number of pathways involving autophagy and apoptosis. Here we have examined the contributions of NK cells, MMPs, and NF-κB on apoptosis and of mTOR signaling and HIF-1α on autophagy, as well as the integrated actions of the ER stress response, TGF-β, Hedgehog signaling, and p62. Much remains to be learned about the function of HSCs in fibrosis and healing of the liver, and carefully detailing the influences of these pathways may be key to our understanding of liver function and the future development of therapeutic agents aimed at healing liver injury without causing fibrosis.

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# Chapter 4
# The Switch: Mechanisms Governing Macrophage Phenotypic Variability in Liver Disease

**John Marentette and Cynthia Ju**

## Abbreviations

| | |
|---|---|
| AILI | Acetaminophen-induced liver injury |
| ALA | Amebic liver abscess |
| ALD | Alcoholic liver disease |
| AMI | Acute myocardial infarction |
| APAP | Acetaminophen |
| Arg1 | Arginase 1 |
| CCL2 | Chemokine (C-C motif) ligand 2 |
| CCR2 | C-C chemokine receptor type 2 |
| CGD | Chronic granulomatous disease |
| COX-2 | Cyclooxygenase-2 |
| CSF | Colony-stimulating factor |
| CTGF | Connective tissue growth factor |
| $CX_3CR1$ | CX3C chemokine receptor 1 |
| DHA | Docosahexaenoic acid |
| EGFR | Epidermal growth factor receptor |
| EPA | Eicosapentaenoic acid |
| GFP | Green fluorescent protein |
| GM-CSF | Granulocyte/macrophage colony-stimulating factor |
| HBV | Hepatitis B virus |
| HCC | Hepatocellular carcinoma |

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W.-X. Ding, X.-M. Yin (eds.), *Molecules, Systems and Signaling in Liver Injury*, Cell Death in Biology and Diseases, DOI 10.1007/978-3-319-58106-4_4

X.-M. Yin and Z. Dong (Series eds.), *Cell Death in Biology and Diseases*

| | |
|---|---|
| HCV | Hepatitis C virus |
| HIF | Hypoxia-inducible factor |
| IFNγ | Interferon γ |
| IGF | Insulin-like growth factor |
| IL | Interleukin |
| iNOS | Inducible nitric oxide synthase |
| $LTB_4$ | Leukotriene $B_4$ |
| LPS | Lipopolysaccharide |
| Ly6C | Lymphocyte antigen 6 complex |
| SIRS | Systemic inflammatory response syndrome |
| SREBP-1c | Sterol regulatory element-binding protein 1c |
| TLR | Toll-like receptor |
| TNFα | Tumor necrosis factor α |
| NALD | Non-alcoholic liver disease |
| NGF | Nerve growth factor |
| NO | Nitric oxide |
| Nr4a1 | Nuclear receptor subfamily 4 group A member 1 |
| PDGF | Platelet-derived growth factor |
| $PGE_2$ | Prostaglandin $E_2$ |
| PHD | Prolyl hydroxylase |
| PPARα | Peroxisome proliferator-activated receptor α |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| SPM | Specialized pro-resolving mediators |
| TGFβ | Transforming growth factor β |
| Treg | Regulatory T cell |
| VEGF | Vascular endothelial growth factor |
| VHL | Von Hippel-Lindau |

## 4.1 Introduction

Macrophages are key effectors of innate immune response to pathogens and the initiation of inflammation, and they contribute to maintaining tissue homeostasis, tissue repair, and development (Wynn et al. 2013b). Macrophages also help shape the adaptive immune response through the presentation of foreign antigens and production of chemokines which facilitate the trafficking of adaptive immune cells to sights of inflammation and tissue damage (Newson et al. 2014). Macrophages are classified as either tissue resident (i.e., liver Kupffer cells, brain microglia, and alveolar macrophages in the lung) or recruited (monocyte−/bone marrow-derived macrophages). In mice, monocytes are subdivided based on their expression of Ly6C and CCR2. $Ly6C^{hi}$ $CCR2^{+}$ monocytes rapidly infiltrate tissue upon injury (Karlmark et al. 2009). During acute or chronic liver injury, $Ly6C^{hi}$ monocyte-derived macrophages are the predominate macrophage subtype (Tacke and Randolph 2006;

Karlmark et al. 2009). Ly6C$^{low}$CCR2$^{-}$ monocytes have been shown to exhibit a "patrolling" behavior and have been found crawling along the hepatic endothelium (Carlin et al. 2013). While monocyte-derived Ly6C$^{hi}$ initially exert tissue-destructive properties, they can differentiate into Ly6C$^{low}$ monocyte-derived macrophages which promote tissue repair and resolution of inflammation (Dal-Secco et al. 2015). The human monocyte counterparts are classified as classical (CD14$^{hi}$CD16$^{-}$), intermediate (CD14$^{+}$CD16$^{+}$), and nonclassical (CD14$^{dim}$CD16$^{+}$) (Ingersoll et al. 2010). Mouse and human monocytes share much similarity but differ in several aspects that must be taken into consideration when translating mouse and human studies. For instance, based on receptor expression CD16$^{-}$ and Ly6C$^{hi}$, monocytes highly express CCR2, whereas CD16$^{hi}$ and Ly6C$^{lo}$ monocytes express high levels of $CX_3CR1$ (Geissmann et al. 2003; Tacke et al. 2007). However, in regard to FcγR1 expression, mRNA and protein expression is conserved between CD16$^{-}$ and Ly6C$^{hi}$ monocytes, whereas human CD16$^{+}$ monocytes lose FcγR1 surface protein expression which is retained on Ly6C$^{low}$ monocytes (Ingersoll et al. 2010). Furthermore, Ly6C$^{low}$ monocytes are more instrumental in phagocytosis, whereas in humans, CD16$^{-}$ monocytes are more phagocytic (Wildgruber et al. 2009; Tacke et al. 2006). Macrophages display a remarkable ability to alter their phenotype based on tissue microenvironmental cues such as hypoxia, cytokines, lipids, and exposure to dead cells. In acute and chronic liver diseases, various subsets of hepatic macrophages have been found to be involved in disease pathogenesis. In this review, we will summarize the development, heterogeneity, and plasticity of macrophages in response to microenvironmental stimuli in physiological and pathophysiological conditions of the liver.

## 4.2 Types and Origins of Hepatic Macrophages

In normal healthy liver, tissue-resident Kupffer cells comprise 90% of the macrophage population (Ju and Tacke 2016). The remaining population of liver macrophages under homeostatic conditions are comprised of monocyte-derived, Ly6C$^{low}$, blood vessel patrolling macrophages (Fig. 4.1). The origin of Kupffer cells has been a long-standing debate, and recent fate mapping studies have provided some insights. The developmental fate of macrophages has been deduced using time course analysis of *Csf1r*-, *Flt3*-, and *Tie2*-expressing cells. It was shown that adult tissue-resident macrophages in the brain, liver, lung, skin, and spleen arise almost exclusively from a *Tie2* progenitor pathway (Gomez Perdiguero et al. 2014). Furthermore, the data indicate that liver-resident Kupffer cells develop from fetal yolk-sac progenitor cells during embryonic day 6.5 (E6.5)–E8.0 and are only marginally replaced by hematopoietic stem cells during development (Gomez Perdiguero et al. 2014). Additionally, analysis of mice utilizing green fluorescent protein (GFP) reporter or Cre recombinase in their $CX_3CR1$ gene (Yona et al. 2013) and parabiosis experiments (Hashimoto et al. 2013) have shown that during homeostatic scenarios, monocytes do not significantly contribute to most tissue-resident

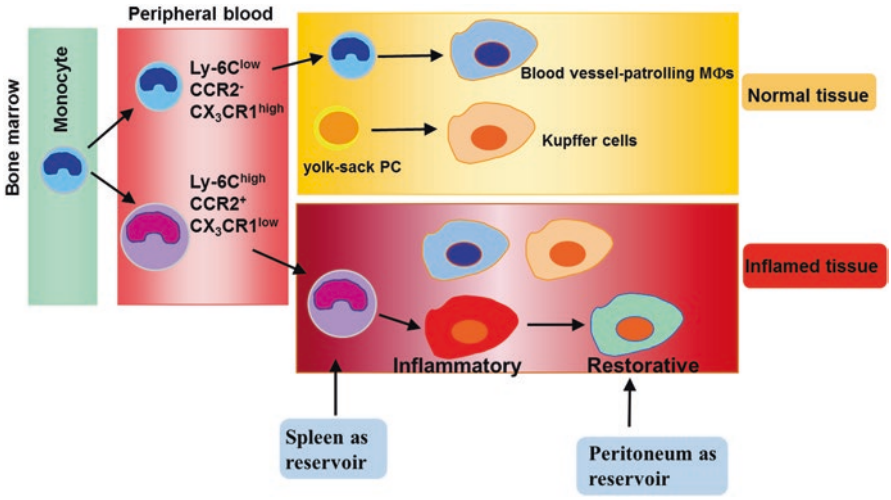


**Fig. 4.1** Macrophages in normal and inflamed tissues. Monocyte development in the bone marrow and reside in the blood as Ly6C$^{high}$ monocyte and Ly6C$^{low}$ monocytes. In normal liver tissue, the two predominant types of macrophages are tissue yolk-sac progenitor-derived tissue-resident Kupffer cells and blood vessel patrolling Ly6C$^{low}$ monocyte-derived macrophages. Following injury and subsequent inflammation, the liver is rapidly infiltrated by Ly6C$^{hi}$ monocytes that differentiate into pro-inflammatory Ly6C$^{hi}$ macrophages. Additional Ly6C$^{hi}$ monocytes reside in the spleen and can be mobilized following injury. As inflammation progresses, the Ly6C$^{hi}$ pro-inflammatory macrophages convert to the tissue-restorative Ly6C$^{low}$ macrophage phenotype to promote resolution of the inflammatory response. Additionally, a population of Ly6C$^{low}$ tissue-restorative macrophages in the peritoneum can be rapidly recruited into the liver during injury

macrophage populations. Several studies have indicated that tissue-resident macrophages develop from different lineage depending on the cells tissue fate. For instance, microglial cells arise predominately from yolk-sac-derived macrophages (Ginhoux et al. 2010), while Langerhans cells mostly derive from fetal liver progenitors but retain yolk-sac-derived components (Hoeffel et al. 2012). Additionally, it has been proposed that macrophages of the lung, heart, kidney, and liver derive from fetal liver-derived progenitors (Guilliams et al. 2013; Epelman et al. 2014; Ginhoux and Jung 2014). Finally it has been shown that tissue macrophages can replenish themselves (Hashimoto et al. 2013).

Kupffer cells are principally involved in maintaining tissue homeostasis by serving as an immune sentinel. They sense antigens, pathogens, or damaged cells through interaction with a number of cell surface receptors (such as complement receptor, toll-like receptors (TLR), scavenger receptors, and Fcγ receptor) to initiate and potentiate the inflammatory response (Sica et al. 2015). The roles of macrophages are elicited through the production of trophic factors [i.e., macrophage colony-stimulating factor (CSF), granulocyte/macrophage CSF (GM-CSF), insulin-like growth factor (IGF), nerve growth factor (NGF)], immune mediators [i.e., inter-

feron γ (IFNγ), tumor necrosis factor α (TNFα), chemokine (C-C motif) ligand 2 (CCL2), interleukin-4 (IL-4)], and effector signaling molecules [inducible nitric oxide synthase (iNOS)] (Gautier et al. 2012; Ju and Tacke 2016). In addition, Kupffer cells are involved in the development of immune tolerance, acting as an antigen-presenting cell. Studies have shown that Kupffer cells are involved in liver-mediated tolerance to allografts (Kamei et al. 1990; Sato et al. 1996), to induce tolerance to soluble antigens (Ju et al. 2003) and suppress dendritic cell-mediated T cell proliferation (You et al. 2008).

During liver injury, resident Kupffer cells mount initial responses by rapid production of inflammatory cytokines, such as IL-1β and TNFα, and chemokines (CCL2) which facilitate the recruitment of additional inflammatory cells, such as monocytes. Ly6C$^{hi}$ monocytes are recruited to inflamed tissue and differentiate into Ly6C$^{hi}$ macrophages which promote inflammatory progression (Serbina et al. 2012) (Fig. 4.1). The trafficking of monocytes to inflamed tissue is facilitated through the interaction of monocyte CCR2 with CCL2. Several studies using either infection or sterile tissue damage have demonstrated the influx of Ly6C$^{hi}$ monocytes (Lin et al. 2009; Morias et al. 2015; Helk et al. 2013; Karlmark et al. 2009; You et al. 2013; Serbina et al. 2012). The Ly6C$^{+}$ cells switch to a tissue-restorative Ly6C$^{low}$ macrophage as injury resolves (Wang et al. 2014). The Ly6C$^{low}$ macrophages are instrumental in the resolution of the inflammatory and wound healing response. Ample evidence supports the concept that Ly6C$^{low}$ macrophages are derived from Ly6C$^{hi}$ macrophages in injured tissues rather than recruited from circulation (Nahrendorf et al. 2007; Ramachandran et al. 2012; Ludovic et al. 2007; Lin et al. 2009; Hilgendorf et al. 2014; Varga et al. 2013). In an animal model of kidney injury, it was shown that the generation of Ly6C$^{low}$ monocytes during disease progression is from differentiating Ly6C$^{high}$ monocytes and that this phenotypic conversion counteracts inflammation and facilitates tissue repair (Lin et al. 2009). This concept has been validated in studies utilizing Nr4a1$^{-/-}$ mice, in which the absence of Nr4a1 inhibits Ly6C$^{low}$ monocyte production in the bone marrow (Hanna et al. 2011). In these studies focused on myocardial infarction and muscle injury, it was observed that wild-type and Nr4a1$^{-/-}$ mice display similar accumulation of Ly6C$^{hi}$ and Ly6C$^{low}$ macrophages (Hilgendorf et al. 2014; Varga et al. 2013). These observations suggest that Ly6C$^{hi}$ macrophages give rise to Ly6C$^{low}$ macrophages.

Aside from the bone marrow, the spleen serves as an immediate reservoir for the mobilization of large quantities of Ly6C$^{hi}$ monocytes following injury (Swirski et al. 2009). It was shown that following myocardial ischemia, the spleen harbors fewer monocytes in comparison with spleens in non-ischemic animals, and the number of monocytes in the blood significantly increased indicating a flux of splenic monocytes into circulation. Furthermore, GATA6$^{+}$ peritoneal macrophages, which are Ly6C$^{low}$ and are important in facilitating tissue repair, have recently been shown to infiltrate directly into the liver within 1 h of liver injury in response to signals from necrotic cells and that depletion of peritoneal macrophages increases mortality in models of acute liver injury (Wang and Kubes 2016) (Fig. 4.1).

## 4.3 Tissue Microenvironmental Cues Governing Macrophage Phenotypes and Functions

Besides the heterogeneity, the phenotype and functions of macrophages change based on the local tissue environment. The plasticity of macrophages is coordinated through microenvironmental cues and the subsequent response of macrophages. Several inciting factors (hypoxia, cytokines, lipids, and dead cells) have been elucidated that stimulate the diverse array of macrophage functions.

### *4.3.1 Impact of Tissue Hypoxia on Macrophages*

Inflammatory signaling typically results in a severely oxygen-deprived tissue environment due to increased oxygen demand, as well as increased consumption by infiltrated inflammatory cells. For example, neutrophils consume copious amounts of oxygen for respiratory burst (Eltzschig and Carmeliet 2011). Macrophages often accumulate in hypoxic areas and macrophage functions are strongly influenced by hypoxia (Murdoc et al. 2005). The expression of hypoxia-inducible factor (HIF) allows for organismal adaptation to hypoxia by increasing oxygen delivery or allowing for the adaptation of the organism to decreased oxygen availability (Wenge 2002). HIFs consist of a constitutively expressed β-subunit and an oxygen-sensitive α-subunit, whose function is controlled via prolyl hydroxylase (PHD) and von Hippel-Lindau (VHL)-mediated ubiquitination and degradation (Ohh et al. 2000; Maxwell et al. 1999). The two predominant α-subunit isoforms of HIF are HIF1α and HIF2α. Increased HIF1α and HIF2α protein expressions have been reported in several liver diseases including non-alcoholic liver disease (NALD), alcoholic liver disease (ALD), and hepatocellular carcinoma (HCC) (Bangoura et al. 2004; Corpechot et al. 2002; Novo et al. 2012). Additionally, HIFs may contribute to fibrosis development as hypoxic areas co-localize with those of fibrosis, and HIF stabilization has been reported in liver fibrosis models (Corpechot et al. 2002; Moon et al. 2009). Critical cellular processes such as glycolysis, oxygen homeostasis, fat metabolism, tissue remodeling, angiogenesis, and cell proliferation are influenced by HIF1α signaling (Semenza 2001; Hirota 2002). Several pathological processes in which macrophage are involved (i.e., inflammation, wound healing, tumor development) are characterized by hypoxia (Semenza 2014). Macrophages rely on HIFs for energy production and activity (Burke et al. 2002). HIF1α and HIF1β have been shown to be increased during the differentiation of monocytes to macrophages in vitro and from human peripheral blood monocytes (Oda et al. 2006). Both HIF1α and HIF2α accumulate in primary human macrophages and mouse bone marrow-derived macrophages under hypoxic conditions in vitro (Griffiths et al. 2000; Talks et al. 2000). HIF1α protein has been shown to be stabilized in pro-inflammatory macrophages and is linked to nitric oxide (NO) production (Takeda et al. 2010). HIF2α has been shown to be stabilized in tissue-restorative

macrophages and is linked to arginase 1 (Arg1) expression (Takeda et al. 2010). These studies highlight the importance of HIF expression in regulating macrophage phenotype and function.

### 4.3.2 Macrophage Responses to Cytokines

Cytokine-induced macrophage activation and cytokines produced by activated macrophages have been used to group macrophages into two distinct groups, the classically activated and alternatively activated macrophages (Murray et al. 2014). LPS either alone or in combination with cytokines (i.e., IFNγ, TNFα, and GM-CSF) can induced classically activated macrophages, which produce immune-stimulatory cytokines (TNFα, IL-1β, IL-6, IL-12, and IL-23) as well as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Steevels and Meyaard 2011). IL-4, IL-10, IL-13, and transforming growth factor β (TGFβ) have been shown to promote alternatively activated macrophages. In a model of muscle injury and repair, ablation of regulatory T cells (Treg) produced a more severe injury due the inability of the infiltrated, pro-inflammatory $Ly6C^{hi}$ macrophages to switch to the tissue-restorative $Ly6C^{low}$ macrophages. Among transcripts shown to be downregulated due to the ablation of Tregs was IL-10, a known polarizer of the tissue-restorative $Ly6C^{low}$ macrophages (Burzyn et al. 2013). Similarly, in a mouse model of acute myocardial infarction (AMI) depletion of Tregs exacerbated the infarct area and delayed resolution (Weirather et al. 2014). This observation was attributed to the Treg-deficient mice displaying mainly pro-inflammatory macrophages compared to control-treated AMI mice, indicative of a delayed transition to the tissue-restorative macrophage phenotype (Weirather et al. 2014). These observations are further supported in a mouse model of angiotensin II-induced kidney injury, in which Treg depletion resulted in more pro-inflammatory macrophage polarization and increased injury. As in the studies focused on muscle injury and repair, the injurious phenotype was attributed to reduced anti-inflammatory cytokine expression, such as IL-10 (Mian et al. 2016). While cytokine stimulation generally activates macrophages to one phenotype or another, certain pathogens exploit cytokine stimulation as a means of dampening immune responses. Infection with *Salmonella typhimurium* (*S. typhimurium*) resulted in a delayed immune response and increased bacterial burden due to macrophage necroptosis. This observed macrophage necroptosis was mediated through the induction of IFNγ (Robinson et al. 2012).

### 4.3.3 Effects of Lipid Mediators on Macrophages

Lipid mediators such as resolvins, protectins, and maresins embody a class of molecules termed specialized pro-resolving mediators (SPM) which facilitate the resolution of inflammatory responses (Dalli and Serhan 2016). In the process of

resolution, pro-inflammatory macrophages undergo phenotypic changes to that of the tissue-restorative macrophages. SPMs have been shown to influence the ability of conversion to the tissue-restorative macrophages, thereby promoting inflammation resolution (Serhan and Savill 2005; Dalli and Serhan 2016). During the initiation of inflammation, arachidonic acid-derived lipid mediators actively facilitate the egress of leukocytes to a site of inflammation. Prostaglandin $E_2$ ($PGE_2$) and leukotrienes $B_4$ ($LTB_4$) which are generated via metabolism of arachidonic acid play vital roles in endothelial changes and diapedesis of neutrophils to inflammatory sites (Levy et al. 2001; Serhan and Savill 2005). During inflammatory response progression, lipid-derived mediators switch from the inflammation-inducing leukotrienes to the inflammation-prohibitive lipoxins $A_4$ and $B_4$ (Serhan and Savill 2005). Lipoxin $A_4$ increases TGF-β1 expression during resolution thereby mediating the resolution response (Bannenberg et al. 2005). Serhan et al. have shown that resolution of inflammation is a biochemically active process involving the biosynthesis of a set of SPMs including lipoxins, E-series resolvins derived from eicosapentaenoic acid (EPA), D-series resolvins derived from docosahexaenoic acid (DHA), protectins, and maresins (Bannenberg et al. 2005; Serhan and Savill 2005). Although their actions are specific, each class of SPMs participates in limiting neutrophil recruitment, limiting microbial invasion and enhancing the uptake of cellular debris and apoptotic neutrophils in resolution (Bannenberg et al. 2005).

### *4.3.4 Phagocytosis of Dead Cells by Macrophages*

Cell death helps shape the inflammatory response and the programming of macrophages. The recognition of apoptotic cells by macrophages has a profound impact on altering the macrophages response and promoting tissue restoration (Gordy et al. 2011; Goren et al. 2009). During inflammation resolution, uptake of apoptotic neutrophils by macrophages is an essential step in the reduction of inflammation (Savill 1997). Uptake of apoptotic cells may stimulate macrophages to release anti-inflammatory mediators, such as TGF-β1, which suppress inflammation (Huynh et al. 2002; Lucas et al. 2003). Efferocytosis is the process by which macrophages ingest and remove apoptotic and necrotic cells. Efferocytic receptors on macrophages sense surface changes on apoptotic cells, such as the flipping of phosphatidylserine from the inner to the outer leaflet of the cell membrane, to distinguish apoptotic cells from healthy ones (Fadok et al. 1992). Effective removal of dead cells is paramount for the resolution of the inflammatory response by preventing the release of intracellular contents (such as proteases and inflammatory mediators from neutrophils) of dying cells into the tissue environment perpetuating inflammation (Vandivier et al. 2006). Efferocytosis of dead hepatocytes has been shown to promote the transition from the $Ly6C^{hi}$ macrophages to the tissue-restorative $Ly6C^{low}$ (Wang et al. 2014). Recognition and removal of neutrophils following the initial response to an injury help shape the inflammatory response toward a restorative process. Exposure of macrophages to apoptosing neutrophils

suppresses pro-inflammatory cytokine and chemokine production through the production of TGFβ and IL-10 (Bratton and Henson 2011; Korns et al. 2011). Studies using a mouse model of chronic granulomatous disease (CGD), in which autoimmunity and excessive inflammation are known to occur, have shown that macrophage efferocytosis of neutrophils is impaired resulting in hyperinflammation (Fernandez-Boyanapalli et al. 2009). Macrophages from CGD mice displayed characteristics of pro-inflammatory macrophages with impaired efferocytosis compared to macrophages from wild-type (WT) mice which display a tissue-reparative phenotype. In addition, WT macrophages expressed more IL-4 compared to CGD macrophages, and the impaired efferocytosis of CGD macrophages was attenuated by pretreatment with IL-4 (Fernandez-Boyanapalli et al. 2009). Recent studies have demonstrated the impact of Kupffer cell death on mediating infiltrating macrophage phenotype. In this study, infection with *Listeria monocytogenes* resulted in rapid Kupffer cell necroptosis within the first hours of infection. Kupffer cell death triggered the release of IL-33, which triggers production of IL-4 (Pecaric-Petkovic et al. 2009), resulting in recruited monocyte-derived macrophages to proliferate and switch from a pro-inflammatory to an anti-inflammatory phenotype (Blériot et al. 2015).

## 4.4 Hepatic Macrophages in Acute and Chronic Liver Injury

The heterogeneous phenotype of liver macrophages has been exemplified in numerous studies of both acute and chronic liver injury. As described below, macrophages exert opposing roles in a variety of liver injury models. They can facilitate the perpetuation of the inflammatory response or act to dampen inflammation and promote tissue restoration. However, the functions of both pro-inflammatory and tissue-restorative macrophages must remain in check as excessive responses from either macrophage population can contribute to disease progression in both acute and chronic liver diseases.

### *4.4.1 Acetaminophen-Induced Liver Injury (AILI)*

Acetaminophen (APAP) overdose causes severe damage to the liver which can progress to liver failure. Following hepatocyte damage from APAP, resident Kupffer cells are activated and release a number of pro-inflammatory mediators, TNFα and IL-1β, and chemoattractants, CCL2, which promote inflammation and a massive influx of circulating monocytes (Zigmond et al. 2014; Holt et al. 2008). In addition to activation of Kupffer cells, CCR2-depenent hepatic accumulation of circulating monocytes has been observed (Holt et al. 2008). Recent studies have also shown that both $Ly6C^{hi}$ and $Ly6C^{low}$ monocyte-derived macrophages are present in the liver during AILI (Zigmond et al. 2014). In the early resolution phase, the infiltrating

Ly6C$^{hi}$ monocytes differentiate into Ly6C$^{lo}$ macrophages and become the predominant liver macrophage phenotype (Zigmond et al. 2014). Mice lacking CCR2 or those treated with anti-CCR2 antibodies exhibit delayed recovery following APAP administration suggesting a prominent role of monocyte-derived macrophages in tissue repair following acute injury (Zigmond et al. 2014; Holt et al. 2008). Interestingly, it was shown that the number of resident Kupffer cells significantly reduces after APAP administration but recovers during the resolution phase by self-renewal (Zigmond et al. 2014). When both hepatic macrophage populations, resident and infiltrating, are depleted, the recovery from APAP-induced liver injury is even more delayed further highlighting the influence of hepatic macrophages in recovery after acute liver damage (You et al. 2013). Moreover, it has been shown that enhancing both infiltrating and resident macrophage populations via administration of CSF1 improved AILI (Stutchfield et al. 2015). However, debate still continues as to whether yolk-sac-derived tissue-resident Kupffer cells repopulate the liver or if the reestablishment of macrophage populations is from circulating monocytes.

### *4.4.2 Parasite-Induced Liver Inflammation*

Liver macrophages contribute to the severity and protection against parasite-induced liver inflammation by producing tissue-destructive TNFα as well as being the major source of anti-inflammatory cytokine, IL-10. In a mouse model of *Trypanosoma congolense* infection, three distinct myeloid cell subsets were observed including Ly6C$^{hi}$ "inflammatory" monocytes that produce TNFα and contribute to the development of systemic inflammatory response syndrome (SIRS); Ly6C$^{-}$ "patrolling" monocytes, which produce IL-10 limiting TNFα-mediated tissue damage; and IL-10-producing macrophages (Morias et al. 2015). In these studies, it was shown that 7 days postinfection, C57Bl/6 mice displayed high accumulation of Ly6C$^{hi}$ monocytes which corresponded to a peak in pro-inflammatory immune response. After 21 days postinfection, the number of Ly6C$^{hi}$ monocytes reduced, and the predominant myeloid cell type was that of Ly6C$^{low}$ monocytes and macrophages. By the use of CCR2$^{-/-}$ mice, the investigators determined that following *T. congolense* infection, CCR2$^{-/-}$ mice display less Ly6C$^{hi}$ monocytes, Ly6C$^{low}$ monocytes, and macrophages compared to their wild-type counterparts (Morias et al. 2015). This data shows during parasite infection that the majority of Ly6C$^{low}$ monocytes and macrophages arise from infiltrating Ly6C$^{hi}$ monocytes and that the Ly6C$^{low}$ cells promote this phenotypic switch. In addition to infiltrating monocytes/macrophages, Kupffer cells have been shown to influence the severity of parasite-induced liver inflammation. In a mouse model of *Entamoeba histolytica* infection, amebic liver abscess (ALA) formation was almost completely abolished by depletion of Kupffer cells by gadolinium chloride or clodronate liposomes (Helk et al. 2013). Kupffer cells were shown to substantially contribute to liver damage during ALA formation. In addition to Kupffer cells, Ly6C$^{hi}$ monocytes were shown to be instrumental in

abscess formation (Helk et al. 2013). In CCR2$^{-/-}$ mice, it was observed that abscess formation was almost abolished following infection and was accompanied by a significant reduction in Ly6C$^{hi}$ monocytes. Abscess formation was restored following adoptive transfer of CD115$^{+}$ monocytes into CCR2$^{-/-}$ mice (Helk et al. 2013). These studies further show the dual role of tissue destruction and restoration in monocyte/macrophage populations.

## 4.5 Chronic Liver Disease

Multiple etiological contributors have been shown to result in the development of chronic liver disease such as viral infection, alcohol abuse, metabolic disorders, and autoimmune disease. With the exception of viral hepatitis, chronic liver injury stems from the accumulation of fat in the liver (steatosis) and the development of liver inflammation (hepatitis) which over time progresses to liver fibrosis with continued fibrosis resulting in cirrhosis and finally progressing to hepatocellular carcinoma. Macrophages play major roles in each stage in the progression of chronic liver injury.

### *4.5.1 Non-alcoholic and Alcoholic Liver Disease (NALD and ALD)*

Non-alcoholic and alcoholic liver disease are leading causes of liver-related morbidity and mortality in western countries (Gao and Bataller 2011; Sakaguchi et al. 2011). The pathogenesis is similar between both diseases starting with fatty liver and developing into hepatitis. Kupffer cells have been shown to play a major role in hepatocytes metabolic adaptation by regulating the oxidation of fatty acids and promoting lipid storage in hepatocytes (Olefsky and Glass 2010). Kupffer cells also become activated in response to overload of lipids and cholesterol in the liver (Dixon et al. 2013) and produce high levels of TNFα and IFNγ (Bergheim et al. 2008). The earliest response of the liver to alcohol abuse is the development of steatosis in hepatocytes. Early studies show that alcohol intake disrupts mitochondrial β-oxidation of fatty acids resulting to steatosis development (Baraona and Lieber 1979). Additionally, ethanol increases fatty acid synthesis in hepatocytes by upregulating sterol regulatory element-binding protein 1c (SREBP-1c) (You et al. 2002) and inhibits peroxisome proliferator-activated receptor (PPAR)-α reducing fatty acid oxidation (Yu et al. 2003). Ablation of Kupffer cells has been shown to inhibit the development of fatty liver after alcohol exposure (Adachi et al. 1994) and in response to a high-fat diet (Leroux et al. 2012).

A large number of people develop fatty liver without any other complications; however, others progress from steatosis to hepatitis. Persistent inflammation and

injury can lead to scaring in the liver which can progress to cirrhosis (Marra et al. 2008). Accumulation of fat in liver cells has been shown to induce hepatocyte apoptosis through mitochondrial impairment which is associated with oxidative stress and the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Rubio et al. 2007). The cytotoxic effects of oxidative stress on hepatocytes lead to cell death via apoptosis and necrosis further promoting inflammation and fibrosis (Feldstein et al. 2003; Wieckowska et al. 2006). The major source of inflammatory mediators, TNFα, IL-1β, and ROS, during non-alcoholic liver disease pathogenesis is from Kupffer cells and bone marrow-derived macrophages (Ramadori and Armbrust 2001; Rivera et al. 2007). In a mouse model of NALD, using the methionine-choline-deficient (MCD) diet, it was observed that conventional Kupffer cells were lost early in disease development and were replaced by monocyte-derived macrophages. Characterization of the infiltrating macrophages revealed high CCR2 expression, low Ly6C expression, and reduced expression of genes associated with anti-inflammatory macrophages. This is conflicting to the typical view of macrophages with high CCR2 expression also displaying high Ly6C expression. This data further exemplifies the plasticity of macrophage phenotype in a given microenvironment.

In patients with alcoholic hepatitis and fibrosis, clinical observations support the notion that macrophages promote disease progression (Tapia-Abellán et al. 2012). Macrophage inflammatory genes are upregulated in cirrhosis patients (Tapia-Abellán et al. 2012), and several indicators of macrophage activation are elevated in ALD patients. Circulating monocytes from ALD patients are more susceptible to lipopolysaccharide (LPS) stimulation (Gobejishvili et al. 2006; Zhang et al. 2001). Activation of hepatic macrophages via ethanol administration has been shown to enhance the production of TNFα, IL-6, CCL2, and ROS in animal model of ALD (Enomoto et al. 2000; Petrasek et al. 2012). Depletion of hepatic macrophages reduces alcohol-induced inflammation (Koop et al. 1997). Pro-inflammatory Kupffer cells have been shown to perpetuate the disease progression in ALD, and reducing pro-inflammatory Kupffer cells is protective against ALD (Wan et al. 2014). Furthermore, it was demonstrated that tissue-restorative Kupffer cells promote apoptosis of pro-inflammatory Kupffer cells thereby limiting ALD progression (Wan et al. 2014). Both resident Kupffer cells and infiltrating macrophages are involved in ALD pathogenesis in animal studies. The infiltrating macrophages consist of the $Ly6C^{low}$ and $Ly6C^{+}$ subsets. Upon phagocytosis, the pro-inflammatory $Ly6C^{hi}$ macrophages appear to switch phenotype to the tissue-restorative $Ly6C^{low}$ phenotype (Wang et al. 2014). In the context of ALD, macrophages actively participate in tissue inflammation and damage as well as the resolution of fibrosis rendering therapeutic targeting of macrophage populations difficult. However, further understanding of the microenvironmental cues which govern the switch between tissue-destructive and tissue-restorative macrophages may elucidate additional therapeutic avenues.

### 4.5.2 Liver Fibrosis

Chronic liver inflammation ultimately leads to liver fibrosis, and macrophages have been shown to exert dual and opposing roles in fibrosis signaling. During the early phase of fibrosis initiation, Ly6C$^{hi}$ monocytes differentiate into pro-inflammatory Ly6C$^{hi}$ macrophages that interact with HSCs to promote fibrosis. Pro-fibrotic macrophages secrete various pro-fibrotic mediators, such as IL-1β, platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), and TGFβ (Kolb et al. 2001; Seki et al. 2007; Kinnman et al. 2000; Paradis et al. 2002). By the use of CCR2$^{-/-}$ mice and in mice-administered pharmacological inhibitors of CCL2, it was shown that blockade of the CCL2/CCR2 pathway inhibits Ly6C$^{hi}$ monocyte recruitment and attenuates liver fibrosis (Karlmark et al. 2009; Ehling et al. 2014). In addition to promoting fibrosis progression, macrophages have been shown to be instrumental in fibrosis resolution through the production of IL-10, Arg1, and matrix metalloproteinases (MMP) (Pesce et al. 2009; Wilson et al. 2007; Ramachandran et al. 2012; Wynn et al. 2013a). Ramachandran et al. characterized the macrophage subset that facilitates fibrosis resolution as the Ly6C$^{low}$ macrophage. Furthermore, they demonstrated the role of the tissue-restorative Ly6C$^{low}$ macrophages in fibrosis resolution through the use of transgenic CD11B-DTR mice in which administration of diphtheria toxin selectively ablates Ly6C$^{low}$ macrophages. Depletion of the Ly6C$^{low}$ macrophages in a $CCl_4$-induced hepatic fibrosis model resulted in impaired fibrosis resolution (Ramachandran et al. 2012). Therefore, further understanding the mechanisms involved in the conversion of pro-fibrogenic macrophages to tissue-restorative macrophages could provide therapeutic insight into managing liver fibrosis through promoting macrophage-elicited fibrosis resolution.

### 4.5.3 Viral Hepatitis

Infection with either hepatitis B virus (HBV) or hepatitis C virus (HCV) can result in chronic liver disease and the progression to HCC (Ganem and Prince 2004; Lauer and Walker 2001). Macrophages have been shown to play an important role in viral hepatitis-induced liver damage. While only limited studies have been conducted showing the impact of HBV infection of Kupffer cells, HCV and its proteins have been shown to activate Kupffer cells. Following infection with HCV, increases in the number of Kupffer cells as well as the expression level of Kupffer cell activation markers such as CD163 and CD33 have been observed (Burgio et al. 1998; Khakoo et al. 1997; McGuinness et al. 2000). In addition, Kupffer cells release pro-inflammatory cytokines IL-1β, IL-6, IL-18, and TNFα (Tu et al. 2010; Hosomura et al. 2011; Shrivastava et al. 2013). The cytokines released by Kupffer cells exhibit opposing effects on modulating antiviral activities with TNFα promoting cellular entry of HCV (Boltjes et al. 2014) and IL-6, IL-18, and IFNγ inhibiting viral

replication (Broering et al. 2008; Zhu and Liu 2003; Zhu et al. 2004). In addition, Kupffer cells have been shown to produce pro-fibrogenic factors which promote the progression of liver injury toward fibrosis/cirrhosis (Wallace et al. 2008). Due to the difficulty in distinguishing Kupffer cells from infiltrating macrophages, the available information in regard to the role of infiltrating macrophages in viral hepatitis liver injury is limited. Therefore, to date, the response observed in viral-induced liver injury has been attributed to that of the Kupffer cells. However, future research to elucidate the contributions of the different macrophage population in viral hepatitis will help understand which immune cells actively participate in disease progression and therefore provide potential therapeutic targets.

### *4.5.4 Hepatocellular Carcinoma (HCC)*

Persistent inflammation and fibrosis in the liver ultimately ends with the development of HCC which is the most common type of primary liver cancer and the third leading cause of cancer-related death worldwide (Capece et al. 2013). During primary tumor development in HCC, Kupffer cells are the predominant macrophages cell type, but once the primary tumor is established, infiltrating macrophages are more instrumental in disease progression (Wu et al. 2012; Ju and Tacke 2016). To facilitate the growth and progression of HCC, macrophages produce a number of key signaling molecules associated with angiogenesis, cell growth, and metastasis. These signaling molecules include growth factors (vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR) ligands, PDGFβ, and TGFβ), cytokines (IL-6, TNFα, and IL-10), factors involved in matrix remodeling, and metastasis (MMPs and cyclooxygenase-2 (COX-2)) (Capece et al. 2013). Effectively targeting macrophages could prove beneficial in HCC management.

## 4.6 Conclusion

Macrophages represent a diverse and heterogeneous population of innate immune cells which depending on the microenvironment actively facilitate tissue damage as well as tissue restoration. An imbalance in the conversion of pro-inflammatory macrophages to tissue-restorative macrophages promotes a chronic inflammatory phenotype which can develop into fibrosis and cirrhosis and ultimately may progress to liver cancer. Hepatocyte damage caused by various insults lead to tissue inflammation, in which pro-inflammatory macrophages play an important role. In the normal process of wound healing, the microenvironment changes and promotes the phenotypic conversion to the tissue-restorative, inflammation-resolving macrophage subset, which plays a critical role in tissue repair. However, during chronic liver injury and inflammation, an imbalance in the conversion of pro-inflammatory macrophages

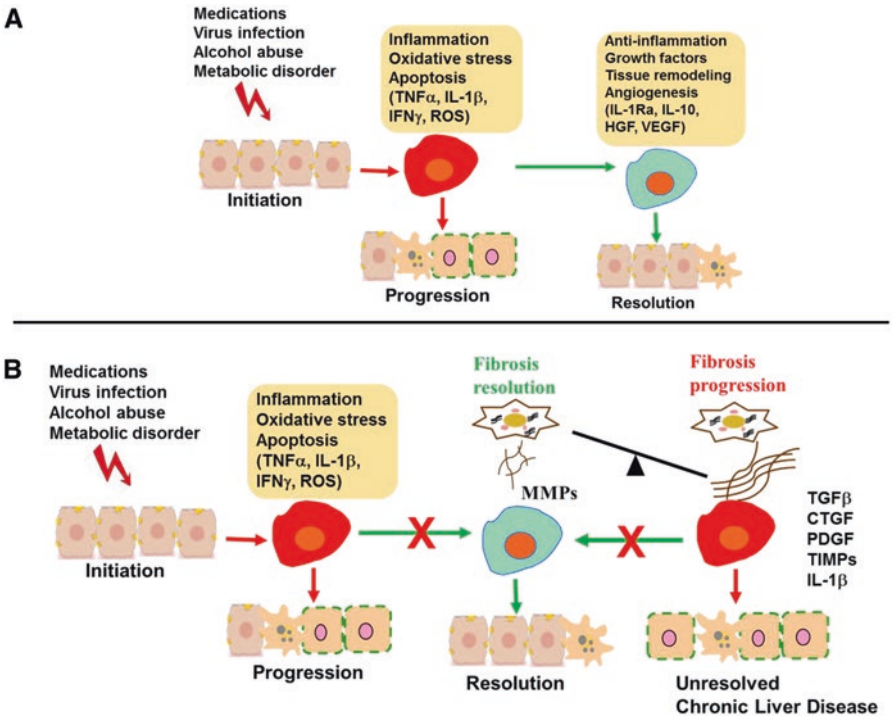


**Fig. 4.2** Role of macrophages in inflammation and fibrosis. (**a**) Following insult to hepatocytes, pro-inflammatory macrophages release inflammatory cytokine which facilitates the removal of damage cells and cellular debris. In the normal process of inflammation resolution, pro-inflammatory macrophages undergo a phenotypic conversion to the tissue-restorative, inflammation-resolving macrophage subset. (**b**) When inflammation resolution becomes unbalanced, chronic inflammation can ensue and result in fibrosis development

to tissue-restorative macrophages promotes a chronic inflammatory phenotype which can develop into fibrosis and cirrhosis and ultimately may progress to liver cancer (Fig. 4.2). Potential therapeutic targets to managing excess inflammation in the liver and therefore potentially limiting the development of liver fibrosis consist of blocking recruitment of Ly6C$^{hi}$ monocytes from the vasculature via blockade of the CCL2/CCR2 pathway. Additionally, stimulating the influx of Ly6C$^{low}$ macrophages from the peritoneum could provide another means of modulating the macrophage populations to promote a tissue-restorative environment. Further studies elucidating the molecular pathways governing the conversion of the Ly6C$^{hi}$ pro-inflammatory macrophages to that of the tissue-restorative Ly6C$^{low}$ macrophages will be important in identifying potential therapeutic target to treat liver diseases with exuberant inflammation.

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# Chapter 5
# Regulation of Apoptosis by Bcl-2 Family Proteins in Liver Injury

**Hayato Hikita and Tetsuo Takehara**

## Abbreviations

| | |
|---|---|
| ALT | Alanine aminotransferase |
| BH | BCL-2 homology |
| ER | Endoplasmic reticulum |
| HCC | Hepatocellular carcinoma |
| LPS | Lipopolysaccharide |
| MOMP | Mitochondrial outer membrane permeability |
| NASH | Non-alcoholic steatohepatitis |
| TNF | Tumour necrosis factor |

## 5.1 Introduction

Cell death is traditionally divided into three types according to morphology: type 1, type 2 and type 3 (Green and Llambi 2015). Apoptosis, classified as type 1 cell death, is caspase activity-dependent and requires ATP. Apoptotic cells shrink, and their nucleus becomes fragmented. Type 2 and type 3 cell death are known as autophagic cell death and necrotic cell death, respectively (Green and Llambi 2015). Apoptosis is a mechanism of programmed cell death in which cells actively progress to death. In 1985, Bcl-2 was reported as an oncogene (Tsujimoto et al. 1985)

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W.-X. Ding, X.-M. Yin (eds.), *Molecules, Systems and Signaling in Liver Injury*, Cell Death in Biology and Diseases, DOI 10.1007/978-3-319-58106-4_5

X.-M. Yin and Z. Dong (Series eds.), *Cell Death in Biology and Diseases*

and revealed to be an anti-apoptotic protein (Vaux et al. 1988). After Bcl-2 was reported, similar proteins with the same BCL-2 homology (BH) 1–4 domains were successively found and called Bcl-2 family proteins (Aouacheria et al. 2015). These proteins also participate in apoptosis regulation (Tsujimoto 2003). In this section, we describe the regulation of apoptosis by Bcl-2 family proteins in the liver.

## 5.2 Mechanisms by Which Bcl-2 Family Proteins Control Apoptosis

Apoptosis is regulated by Bcl-2 family proteins (Tsujimoto 2003), which consist of pro-apoptotic and anti-apoptotic proteins. Currently, five anti-apoptotic Bcl-2 family proteins have been reported, including Bcl-2, Bcl-xL, Mcl-1, Bcl-w and Bfl-1. All anti-apoptotic Bcl-2 family proteins have BH1, BH2, BH3 and BH4 domains. In contrast, pro-apoptotic proteins have been further subdivided into multidomain proteins and BH3-only proteins (Aouacheria et al. 2015; Tsujimoto 2003). Among pro-apoptotic proteins, Bak and Bax have BH1, BH2 and BH3 domains and are considered multidomain proteins. BH3-only proteins have only the BH3 domain. The canonical BH3-only proteins, which are confirmed to have pro-apoptotic effects, consist of eight proteins: Bid, Bim, PUMA, Noxa, Bmf, Bad, Bik and Bad (Aouacheria et al. 2015). Various apoptotic stimuli activate Bak/Bax via the activation of BH3-only proteins. Two pathways can activate BH3-only proteins. One is the extrinsic pathway, which is initiated by the activation of death receptors including the tumour necrosis factor (TNF)-α, Fas and TRAIL receptors. The stimulation of cell death receptors activates caspase-8. While activated caspase-8 directly activates caspase-3 in type 1 cells, such as lymphocytes, in type 2 cells, including hepatocytes, activated caspase-8 cleaves Bid to form truncated Bid, an active form of Bid, leading to Bak/Bax activation (Guicciardi et al. 2013; Scaffidi et al. 1998). The other pathway is the intrinsic pathway, which is initiated by various types of intracellular stress. For example, an increase in endoplasmic reticulum (ER) stress activates Bim and PUMA (Cazanave et al. 2010, 2011), which activate Bak/Bax. Genotoxic stress activates PUMA and Noxa via p53, leading to Bak/Bax activation (Green and Llambi 2015; Khoo et al. 2014). Thus, various apoptotic stimuli result in Bak/Bax activation though the action of BH3-only proteins. Inactive Bax exists not only on the outer membrane of mitochondria but also in the cytosolic fraction, and activated Bax moves to the outer membrane of mitochondria. However, both inactive Bak and activated Bak exist on the outer membrane of mitochondria. Anti-apoptotic Bcl-2 family proteins inhibit Bak/Bax activation.

The identity of the mechanism by which BH3-only proteins activate Bak/Bax is an ongoing controversy. The “direct activation model” states that BH3-only proteins directly activate Bak/Bax (Zheng et al. 2016). In this model, the BH3-only proteins Bid, Bim and PUMA are traditionally thought to be capable of Bak/Bax activation (Edwards et al. 2013; Kuwana et al. 2002, 2005), and the other BH3-only proteins act as sensitizers by neutralizing anti-apoptotic Bcl-2 family proteins. Recently, all BH3-only proteins have been shown to be capable of Bak/Bax activation (Du et al. 2011; Vela et al. 2013). The “indirect activation model” states that Bak/Bax can be

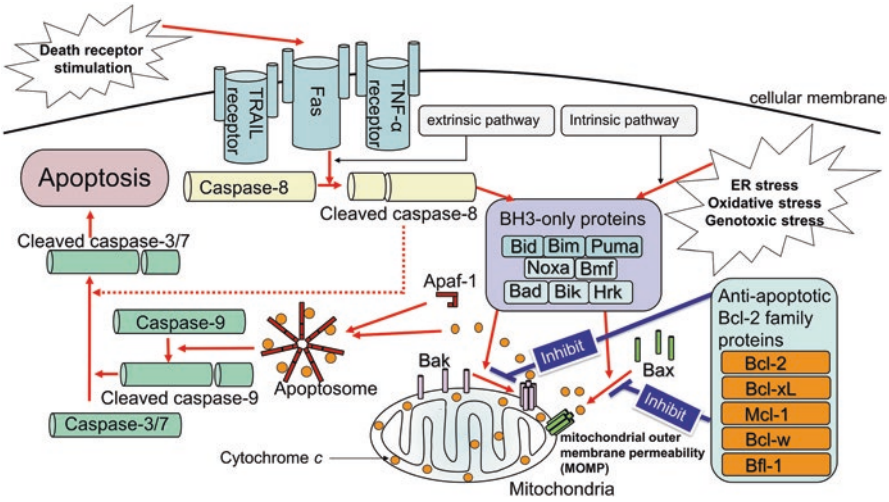


**Fig. 5.1** Apoptosis signalling pathway

activated without any stimulation and that their activation is inhibited by anti-apoptotic Bcl-2 family proteins. Activated BH3-only proteins inhibit anti-apoptotic Bcl-2 family proteins, leading to Bak/Bax activation (Willis et al. 2007). Both models are based on scientific evidence, and other developing or combined models have also been proposed (García Sáez and Villunger 2016; Llambi et al. 2011; Lovell et al. 2008). Thus, the precise mechanisms of Bak/Bax activation remain unclear and are the subject of ongoing study (Doerflinger et al. 2015).

When Bak/Bax are activated, they oligomerize to form pores on the outer mitochondrial membrane, resulting in mitochondrial outer membrane permeability (MOMP) (Luna-Vargas and Chipuk 2016). After MOMP occurs, cytochrome c, which is located between the outer and inner mitochondrial membrane, is released into the cytoplasm through the pores. Released cytochrome c forms a complex with Apaf-1, called the apoptosome, in the presence of ATP. The apoptosome activates pro-caspase-9 by cleaving it into caspase-9, which in turn activates caspase-3/7 and CAD to initiate apoptosis (Fig. 5.1). Protein synthesis is not required in the apoptotic signalling cascade that occurs as a result of the sequential activation of Bak/Bax and caspase-3/7. Therefore, apoptosis rapidly occurs after Bak/Bax activation, and it is important to directly or indirectly inhibit anti-apoptotic Bcl-2 family proteins to prevent Bak/Bax activation and subsequent apoptosis.

### *5.2.1 Bcl-xL and mcl-1 in Hepatocytes*

Five Bcl-2 family proteins have been reported to be anti-apoptotic, including Bxl-2, Bcl-xL, Mcl-1, Bcl-w and Bfl-1. In mice, deficiencies in Bcl-2 (Veis et al. 1993), Bcl-w (Print et al. 1998; Ross et al. 1998) or Bfl-1 (Hamasaki et al. 1998) do not produce any phenotype in the liver. In contrast, hepatocyte-specific Bcl-xL

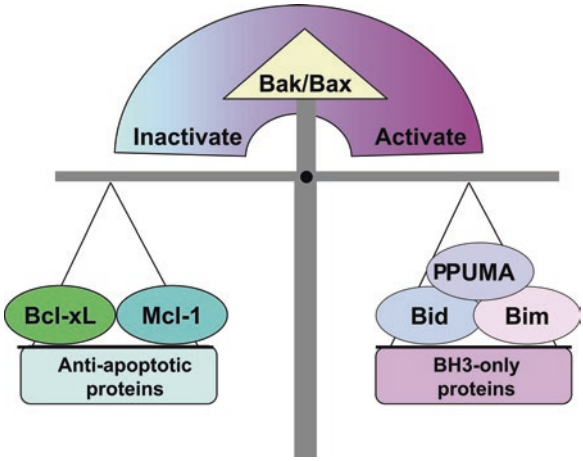


**Fig. 5.2** Schematic of hepatocyte apoptosis regulated by Bcl-2 family proteins

knockout mice undergo continuous hepatocyte apoptosis, as evidenced by haematoxylin and eosin (HE) staining, terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining and immunohistochemistry of cleaved caspase-3 performed in liver sections (Takehara et al. 2004). Similarly, hepatocyte-specific Mcl-1 knockout mice undergo continuous hepatocyte apoptosis (Hikita et al. 2009b; Vick et al. 2009). Interestingly, serum alanine aminotransferase (ALT) levels in both types of knockout mice increase to approximately 200–800 IU/L, suggesting that not only hepatocyte necrosis but also hepatocyte apoptosis can increase ALT levels. Continuous hepatocyte apoptosis, as well as the increased serum ALT levels, in hepatocyte-specific Bcl-xL knockout mice or hepatocyte-specific Mcl-1 knockout mice was completely inhibited by further knockout of Bak and Bax (Hikita et al. 2009a, b). These results also support the idea that the loss of Bcl-xL or Mcl-1 activates Bak/Bax, leading to hepatocyte apoptosis (Fig. 5.2). Furthermore, hepatocyte-specific Bcl-xL and Mcl-1 double-knockout mice show a loss of hepatocytes in the foetal liver, where most cells are haematopoietic. Mice were born but died within 1 day due to liver failure (Hikita et al. 2009b). While a hetero-deficiency of Bcl-xL or Mcl-1 did not produce any phenotype in the liver, double hetero-deficiency of Bcl-xL and Mcl-1 caused continuous hepatocyte apoptosis, similar to that in Bcl-xL or Mcl-1-deficient mice (Hikita et al. 2009b). Thus, Bcl-xL and Mcl-1 cooperate to protect hepatocytes from apoptosis (Fig. 5.2). In hepatocytes, among the five anti-apoptotic Bcl-2 family proteins, Bcl-xL and Mcl-1 are the key molecules that inhibit apoptosis by producing pro-apoptotic signals under physiological conditions.

### *5.2.2 BH3-Only Proteins in Hepatocytes*

Among the BH3-only proteins, eight have been confirmed to exhibit a pro-apoptotic effect: Bid, Bim, PUMA, Noxa, Bmf, Bad, Bik and Hrk. These proteins act as sensors for various apoptotic stimuli. Bid is activated by caspase-8, which is activated

by death receptor signalling mediated by Fas ligand, TRAIL and TNF-α stimulation. Bim is activated by ER stress. However, it is unclear whether sensor proteins are active or inactive under physiological conditions. In mouse experiments, hepatocyte apoptosis observed in Bcl-xL or Mcl-1 knockout mice is suppressed by the further ablation of Bid or Bim (Hikita et al. 2009a, b; Kodama et al. 2013) and is completely suppressed by the ablation of both Bid and Bim (Kodama et al. 2013). These results suggest that Bid and Bim are activated even under physiological conditions and participate in the regulation of apoptosis by the Bcl-2 family proteins. Recently, we found that the ablation of PUMA suppresses hepatocyte apoptosis in Bcl-xL knockout mice (unpublished data), suggesting that PUMA also participates in the regulation of apoptosis under physiological conditions. The liver is located downstream of gut blood flow, which contains lipopolysaccharide (LPS), a component of Gram-negative anaerobic bacteria. The liver contains many resident immune T or NK cells, which stimulate Fas or TRAIL receptors. Therefore, it is logical that Bid is activated under physiological conditions. Hepatocytes synthesize many proteins and suffer from ER stress. Therefore, it is reasonable to conclude that Bim or PUMA are also activated under physiological conditions (Fig. 5.2).

### *5.2.3 Bcl-2 Family Proteins in Hepatitis*

Hepatocyte apoptosis is frequently observed in pathological conditions, and many Bcl-2 family proteins regulate this process. Hepatocyte apoptosis is frequently observed in the livers of patients with chronic hepatitis C or B (Guicciardi and Gores 2005; Hayashi and Mita 1997) and is also detected in the livers of non-alcoholic steatohepatitis (NASH) patients (Feldstein et al. 2003). Caspase inhibitors efficiently decrease serum ALT levels in patients with chronic hepatitis C, chronic hepatitis B and NASH (Anstee et al. 2010; Pockros et al. 2007; Ratziu et al. 2012; Shiffman et al. 2010), suggesting that the increased ALT level in chronic hepatitis is dependent on hepatocyte apoptosis. Many hepatocytes undergoing apoptosis are detected in the livers of patients with fulminant hepatitis (Leifeld et al. 2006). Collectively, hepatocyte apoptosis is one of the characteristic features of hepatitis. Many Bcl-2 family proteins are involved in the mechanisms by which hepatocyte apoptosis occurs.

In viral and autoimmune hepatitis, lymphoid cells in the liver induce hepatocyte apoptosis. T cells or NK cells activate Fas or TRAIL receptors, and monocytes or macrophages activate TNF-α receptors on hepatocytes (Malhi et al. 2010). These death receptor stimuli activate caspase-8, followed by Bak/Bax activation in hepatocytes (Guicciardi et al. 2013). Bak/Bax subsequently activate downstream caspase-3/7, resulting in apoptosis (Fig. 5.1). Indeed, high levels of Fas ligand are detected in the serum of patients with fulminant hepatitis, and Fas is highly expressed in their hepatocytes. Fas agonistic antibodies or Fas ligands consistently induce fulminant hepatitis in mice (Nagata 1996; Ogasawara et al. 1993). Fas activates caspase-8, which in turn activates Bid, leading to apoptosis through Bak/Bax activa-

tion (Guicciardi et al. 2013). A Bid deficiency inhibits Fas-induced fulminant hepatitis in mice (Yin et al. 1999); this finding clearly indicates that hepatocytes are type 2 cells. A double deficiency in Bak and Bax also inhibits Fas-induced fulminant hepatitis in mice, suggesting that Fas-mediated apoptosis in hepatocytes is dependent on mitochondria. However, more precise observations have revealed that Fas slowly induces hepatocyte apoptosis in Bak/Bax double-knockout mice (Hikita et al. 2011). Death receptor-mediated apoptosis, which is observed under normal conditions, is thought to be mediated by Bid-Bak/Bax activation in hepatocytes (Scaffidi et al. 1999). However, this result suggests that caspase-8 can directly activate not only Bid but also caspase-3, even in type 2 cells such as hepatocytes. This direct activation of caspase-3 serves as a backup system for the Bak/Bax-mediated apoptosis pathway in the liver (Fig. 5.1).

Hepatocyte apoptosis is induced by several mechanisms in patients with steatohepatitis (Hirsova et al. 2016). One such mechanism is the increase in LPS from the gut, which stimulates toll-like receptors on hepatocytes and macrophages in the liver (Roh and Seki 2013). This mechanism increases the levels of inflammatory cytokines and chemokines and induces the accumulation of monocytes and lymphocytes in the liver, leading to the stimulation of the death receptors on hepatocytes. TNF-α, an inflammatory cytokine, also stimulates TNF-α receptors on hepatocytes. In a mouse model, a TNF-α deficiency (Mukai et al. 2016) or antibiotics (Hu et al. 2015) can suppress steatohepatitis. Lymphocyte activation by inflammation activates Fas or TRAIL receptors on hepatocytes. Free fatty acids increase hepatocyte apoptosis in the presence of insulin, although this increase is blocked by an FAS deficiency (Sommerfeld et al. 2015). In mice, deletion of the TRAIL receptor suppresses steatohepatitis (Werneburg et al. 2007, 2012). Fatty acids can also directly activate TRAIL receptors on hepatocytes (Akazawa et al. 2013). Death receptor activation via various mechanisms followed by Bid activation leads to Bak/Bax activation. Moreover, TRAIL stimulation also activates JNK-dependent Bim, leading to Bak/Bax activation (Cazanave et al. 2010, 2011). Thus, the death receptor-mediated (extrinsic) pathway is one mechanism by which hepatocyte apoptosis is induced in steatohepatitis. However, palmitate and unsaturated free fatty acids inhibit autophagy, leading to increased ER stress in hepatocytes (Tanaka et al. 2016). Increased ER stress activates Bim and PUMA via the activation of CHOP or JNK (Cazanave et al. 2010, 2011). Palmitate also increases the level of reactive oxygen species (ROS) in hepatocytes (Cazanave et al. 2009). This increase in ROS activates JNK (Luedde et al. 2014), leading to Bim or PUMA activation. Thus, Bim or PUMA activation via the intrinsic pathway leads to Bak/Bax activation. PUMA knockout mice are resistant to steatohepatitis (Cazanave et al. 2009). Taken together, the aforementioned findings suggest that the BH3-only proteins Bid, Bim and PUMA are activated by extrinsic and intrinsic pathways and are involved in apoptosis observed in patients with steatohepatitis (Akazawa et al. 2010).

### 5.2.4 *Bcl-2 Family Proteins in Hepatocellular Carcinoma (HCC)*

HCC tumours suffer from various stresses, including genotoxic stress, ER stress or death receptor signalling from host immune cells and are required to overexpress anti-apoptotic proteins to evade apoptosis (Hanahan and Weinberg 2011). Indeed, one-third of HCC tumours exhibit Bcl-xL overexpression (Takehara et al. 2001), and Mcl-1 is overexpressed in half of HCCs (Sieghart et al. 2006). The suppression of Bcl-xL deamidation and an increase in let-7 microRNAs are involved in the mechanisms by which functional Bcl-xL is overexpressed in HCC tumours (Shimizu et al. 2010; Takehara and Takahashi 2003). The overexpression of anti-apoptotic proteins in HCC directly increases the growth speed of tumours (Hikita et al. 2010). Interestingly, hepatocyte-specific Bcl-xL or Mcl-1 knockout mice accumulate oxidative stress in the liver and develop HCC (Hikita et al. 2012, 2015; Weber et al. 2010). These HCC tumours in Bcl-xL or Mcl-1 knockout mice maintain the deficiency of Bcl-xL or Mcl-1. However, HCC tumours in Bcl-xL knockout mice overexpress Mcl-1, and HCC tumours in Mcl-1 knockout mice overexpress Bcl-xL (Hikita et al. 2012). These data also support the idea that upregulation of anti-apoptotic proteins is essential for HCC development. Similar to hepatocytes under physiological conditions, HCC tumours are also dependent on the existence of Bcl-xL and Mcl-1. The suppression of Bcl-xL and Mcl-1 is a promising potential HCC treatment. However, Bcl-xL and Mcl-1 knockdown in hepatocytes also results in apoptosis, leading to massive hepatitis, resulting in death in mice (Hikita et al. 2010). An HCC-specific knockdown of Bcl-xL and Mlc-1 is required. Sorafenib, a multi-kinase inhibitor, suppresses Mcl-1, and this phenomenon is HCC specific (Hikita et al. 2010). Combination therapy with sorafenib and an anti-Bcl-xL drug, ABT-737 or ABT-263, is a candidate treatment for HCC. Currently, many small molecules that target anti-apoptotic proteins are under investigation (Delbridge et al. 2016; Luna-Vargas and Chipuk 2016). In addition, death receptor-targeted drugs or these drugs in combination with small molecules that target anti-apoptotic proteins are other potential treatments for HCC. TRAIL activation therapy has recently been reported (Wahl et al. 2013). Thus, targeted therapy of Bcl-2 family proteins is a potential candidate for HCC treatment.

## 5.3 Conclusion

Hepatocytes suffer from various stresses, including death receptor stimuli and ER stress. These stresses activate corresponding BH3-only proteins, including Bid, Bim and PUMA, which activate Bak/Bax. Bcl-xL and Mcl-1 cooperate to protect hepatocytes from apoptosis. Many Bcl-2 family proteins participate in the regulation of hepatocyte apoptosis not only under pathological liver but also physiological conditions. The balance between anti-apoptotic Bcl-2 family proteins and pro-apoptotic

Bcl-2 family proteins controls Bak/Bax activation to determine the fate of hepatocytes (Fig. 5.2). The modulation of Bcl-2 family proteins has potential for hepatitis treatment, preventative therapy for avoiding liver carcinogenesis and HCC treatment.

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# Chapter 6
# Beta-Catenin and the Survival of Hepatocytes

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## Abbreviations

| | |
|---|---|
| ActD | Actinomycin D |
| APC | Adenomatous polyposis coli |
| CBP | CREB-binding protein |
| CK1 | Casein kinase 1 |
| DC | Dendritic cell |
| DISC | Death-inducing signaling complex |
| Dkk-1 | Dickkopf-related protein 1 |
| Dvl | Dishevelled |
| FADD | Fas-associated death domain |
| FGF | Fibroblast growth factor |
| FRP | Frizzled-related protein |
| Fz | Frizzled |
| GalN | D-Galactosamine |
| GPCR | G-protein-coupled receptor |
| GS | Glutamine synthetase |
| GSK3β | Glycogen synthase kinase 3β |
| HCC | Hepatocellular carcinoma |
| HGF | Hepatocyte growth factor |
| HIF-1 | Hypoxia-inducible factor-1 |

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W.-X. Ding, X.-M. Yin (eds.), *Molecules, Systems and Signaling in Liver Injury*, Cell Death in Biology and Diseases, DOI 10.1007/978-3-319-58106-4_6

X.-M. Yin and Z. Dong (Series eds.), *Cell Death in Biology and Diseases*

| | |
|---|---|
| HSC | Hepatic stellate cell |
| I/R | Ischemia/reperfusion |
| IAP | X-linked inhibitors of apoptosis |
| iNOS | Inducible nitric oxide synthase |
| JNK | c-Jun $NH_2$-terminal kinase |
| KO | Knockout |
| LECT2 | Leukocyte cell-derived chemotaxin 2 |
| LEF | Lymphoid enhancer-binding factor |
| LNA | Locked nucleic antisense |
| LNP | Lipid nanoparticle |
| LPS | Lipopolysaccharide |
| LRP | Low-density lipoprotein receptor-related protein |
| LSEC | Liver sinusoidal endothelial cell |
| NFAT | Nuclear factor associated with T cells |
| NF-κB | Nuclear factor kappa B |
| PKA | Protein kinase A |
| PTEN | Phosphatase and tensin homolog deleted on chromosome 10 |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| RTK | Receptor tyrosine kinase |
| Ser | Serine |
| SMP30 | Senescence marker protein-30 |
| T3 | Triiodothyronine |
| TCF | T cell-specific transcription factor |
| TGF-β | Transforming growth factor-β |
| Thr | Threonine |
| TNF | Tumor necrosis factor |
| TRADD | TNFR-associated death domain |
| TUNEL | Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling |
| WIF | Wnt inhibitory factor |
| Wls | Wntless |

## 6.1 The Wnt/β-Catenin Signaling Pathway

The Wnt/β-catenin signaling pathway is a highly evolutionarily conserved regulatory system that is essential in controlling developmental decisions as well as growth and maintenance of adult tissues. The past three decades' worth of extensive research has increased our understanding of this pathway enormously, demonstrating its indispensable importance in processes such as cell fate, stem cell control, differentiation, growth, survival, proliferation, regeneration, and self-renewal (Cadigan and Nusse 1997; Wodarz and Nusse 1998; Peifer and Polakis 2000; Willert et al. 2003; Logan and Nusse 2004; Nusse 2005; Niehrs 2012). Given its diverse

roles, then, it is not surprising that mutations in this signaling pathway have also been associated with various diseases. In particular, dysregulation of Wnt/β-catenin signaling has been observed in the initiation and progression of several different types of cancers, including those in the colon and liver (Peifer and Polakis 2000; Polakis 2000; Paul and Dey 2008).

Although aberrant β-catenin activation was noted in a subset of human hepatocellular carcinomas (HCCs) as early as the late 1990s (de La Coste et al. 1998; Miyoshi et al. 1998; Satoh et al. 2000), in the last decade or so this pathway has become the focus of intense interest and research in the field of hepatic pathophysiology. These studies have contributed much to our understanding of the essential role of Wnt/β-catenin signaling in both liver health and disease (Apte et al. 2008; Monga 2015). This pathway has been shown to be critical in the highly dynamic environment of the developing liver, where it regulates the processes of patterning and specification, as well as hepatoblast proliferation and maturation (Nejak-Bowen and Monga 2008; Monga 2014). Wnt/β-catenin signaling is also active during the postnatal hepatic growth spurt and can be reactivated in an adult liver under conditions of experimentally induced controlled growth, such as in liver regeneration after partial hepatectomy (Nejak-Bowen and Monga 2011). Pathologically, this pathway is also involved in hepatic disorders such as acute liver injury, diet-induced steatohepatitis, fibrosis, cholestasis, and progenitor cell activation (Myung et al. 2007; Apte et al. 2008, 2009; Cheng et al. 2008; Behari et al. 2010; Thompson et al. 2010; Yeh et al. 2010; Nejak-Bowen et al. 2013; Ge et al. 2014). The present chapter will be an in-depth discussion on the recently identified role of Wnt/β-catenin signaling in cell survival and apoptosis during liver injury.

### *6.1.1 The Canonical Wnt Pathway*

Wnt proteins are secreted glycoproteins approximately 40 kDa in size (Tanaka et al. 2002). Importantly, certain posttranslational modifications are required in order for Wnts to be secreted and signal efficiently (Bartscherer and Boutros 2008). For example, Porcupine, located in the endoplasmic reticulum, is essential for palmitoylation and glycosylation of Wnt proteins (Kadowaki et al. 1996; Zhai et al. 2004; Barrott et al. 2011), while Wntless (Wls), a multipass transmembrane protein, conveys the posttranslationally modified Wnts from the Golgi to the membrane for secretion (Banziger et al. 2006; Bartscherer et al. 2006). Once secreted, Wnts can act in either a paracrine or an autocrine fashion, but predominantly Wnts are short-distance signals, meaning that the signal occurs adjacent to the secreting cell, rather than over a long range (Clevers and Nusse 2012).

In the absence of Wnt proteins, Wnt signaling is inactive. This state is characterized by degradation of β-catenin, the central effector in the canonical pathway. A destruction complex, which consists of Axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3β (GSK3β), binds free β-catenin in the cytoplasm. When Wnt signaling is absent, β-catenin

is phosphorylated by CK1 and GSK3β at the N-terminal region of the protein, specifically at serine 45 (Ser45), threonine 41 (Thr41), Ser37, and Ser37 (Behrens et al. 1998; Amit et al. 2002; Liu et al. 2002; Yanagawa et al. 2002; Clevers 2006). This sequential phosphorylation targets β-catenin for ubiquitination by β-TrC, which ultimately leads to degradation by the proteasome (Aberle et al. 1997; MacDonald et al. 2009). These events maintain the pathway in "OFF" mode by preventing β-catenin nuclear translocation and keeping β-catenin protein levels in the cell low. Inactivation of canonical Wnt signaling can also occur if Wnts are sequestered or prevented from binding to their receptors. One such modulator is Frizzled-related proteins (FRPs), which are smaller proteins (30kD) with Fz-like cysteine-rich domain that bind and sequester Wnts (Rattner et al. 1997). Similarly, Wnt inhibitory factors (WIFs) bind Wnts to inactivate the pathway (Hsieh et al. 1999).

Binding of Wnt to the seven-pass transmembrane Frizzled (Fz) receptor and co-receptor low-density lipoprotein receptor-related protein (LRP) 5/LRP6 on the surface of cells triggers activation of the canonical Wnt pathway (Bhanot et al. 1996; Pinson et al. 2000; Tamai et al. 2000; Wehrli et al. 2000) (Fig. 6.1). Dishevelled (Dvl) interacts with the Frizzled receptor (Wong et al. 2003; Cadigan and Liu 2006), and the Fz/Dvl complex in turn relocates Axin to LRP5/LRP6 (Cliffe et al. 2003). This removes GSK3β and CK1 from the cytoplasmic complex, resulting in hypophosphorylation of β-catenin; these proteins then also phosphorylate LRP5/LRP6 to activate canonical β-catenin signaling (Zeng et al. 2005, 2008). The release of β-catenin from the destruction complex results in the accumulation of cytoplasmic and active β-catenin. β-Catenin then translocates to the nucleus via an as-yet unknown mechanism that may involve Rac1 activation (Esufali and Bapat 2004; Wu et al. 2008; Schlessinger et al. 2009) or direct interaction with components of the nuclear pore complex (Yokoya et al. 1999; Henderson and Fagotto 2002). In the nucleus, β-catenin displaces the transcriptional inhibitor Groucho and binds to lymphoid enhancer-binding factor/T cell-specific transcription factor (LEF/TCF), allowing for initiation of target gene transcription (Molenaar et al. 1996; Cavallo et al. 1998; Logan and Nusse 2004). CREB-binding protein (CBP), which is a known coactivator for several transcription factors, as well as another related acetyltransferase, p300, acts as transcriptional coactivators in the β-catenin-TCF transcription machinery (Hecht et al. 2000; Takemaru and Moon 2000). More recently, P300 and CBP have shown to have differential and sometimes opposite effects on target gene promoters (Ma et al. 2005).

One interesting aspect of β-catenin-TCF target gene expression is that it seems to be tissue specific as well as temporally regulated, which is reflected in the number and diversity of the genes regulated. A partial listing of some notable Wnt/β-catenin-regulated genes is shown in Table 6.1. Importantly, Wnt/β-catenin signaling regulates the expression of many liver-specific genes, which are involved in processes as diverse as metabolism, drug detoxification, zonation, differentiation, and proliferation (Monga 2015).

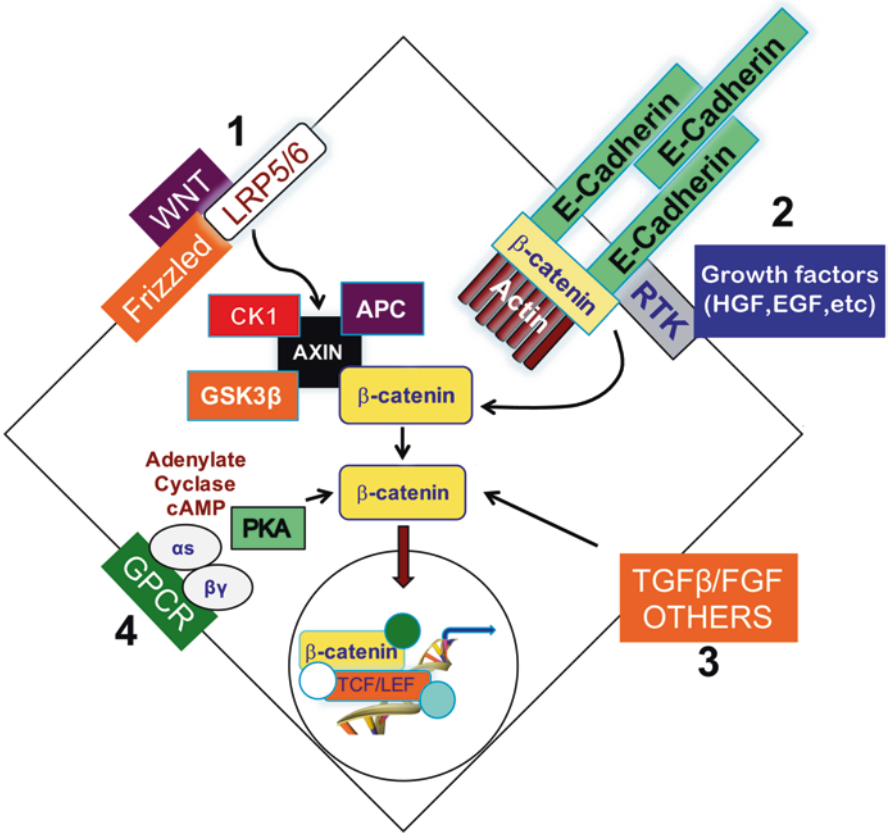


**Fig. 6.1** β-catenin signaling in the hepatocyte. (*1*) Canonical Wnt/β-catenin signaling. Upon binding of Wnt to Frizzled receptor and co-receptor LRP5 or LRP6, the signal is transduced to the β-catenin degradation complex comprised of GSK3β, CK1, Axin, and APC to inactivate it. β-Catenin dissociates from the multimeric complex to eventually accumulate in the cytoplasm and eventually translocate to the nucleus where it acts as a cofactor for the TCF/LEF family of transcription factors. (*2*) β-Catenin at the cell surface. β-Catenin mediates cell-cell adhesion by forming a bridge between the actin cytoskeleton and E-cadherin present at the cell surface. β-Catenin also associates with receptor tyrosine kinases (*RTKs*) at the cell surface. In the presence of growth factors, β-catenin is phosphorylated and translocates to the nucleus to activate genes important in proliferation and morphogenesis. (*3*) TGF-β and fibroblast growth factors (*FGFs*) can also activate β-catenin-mediated transcription through multiple mechanisms that are not yet fully elucidated. (*4*) Signaling through G-protein-coupled receptors (*GPCRs*) can stimulate β-catenin activation through production of cyclic AMP (*cAMP*) and PKA

### *6.1.2 Noncanonical Wnt signaling*

Wnt proteins can also activate several pathways that are independent of β-catenin. These noncanonical Wnt signaling pathways utilize other methods of signaling downstream of Wnt/Fz, which may intersect or overlap one another (Behari et al. 2010;

**Table 6.1** A partial list of Wnt/β-catenin target genes

| General Wnt target genes | Effect of Wnt signaling | Reference |
|---|---|---|
| Gastrin | Increase | (Koh et al. 2000) |
| Survivin | Increase | (Zhang et al. 2001) |
| MMP7 | Increase | (Brabletz et al. 1999) |
| Epidermal growth factor receptor | Increase | (Tan et al. 2005) |
| Met | Increase | (Boon et al. 2002) |
| Jagged | Increase | (Rodilla et al. 2009) |
| Fibroblast growth factors (FGF) | Increase | (Shimokawa et al. 2003; Chamorro et al. 2005; Hendrix et al. 2006) |
| Vascular endothelial growth factor (VEGF) | Increase | (Zhang et al. 2001) |
| Claudin-1 | Increase | (Miwa et al. 2001) |
| Sox9 | Increase/ decrease | (Blache et al. 2004; Yano et al. 2005) |
| Nanog | Decrease | (Pereira et al. 2006) |
| E-cadherin | Decrease | (Jamora et al. 2003) |
| *Wnt/β-catenin pathway repressors* | | |
| Axin2 | Increase | (Leung et al. 2002) |
| βTrCP | Increase | (Spiegelman et al. 2000) |
| Dkk1 | Increase | (Chamorro et al. 2005) |
| Frp2 | Increase | (Lescher et al. 1998) |
| Naked | Increase | (Wharton et al. 2001) |
| *Wnt/β-catenin positive regulators* | | |
| Frizzled | Increase/ decrease | (Cadigan et al. 1998; Willert et al. 2002) |
| LRP6 | Decrease | (Khan et al. 2007) |
| TCF | Increase | (Roose et al. 1999) |
| LEF1 | Increase | (Hovanes et al. 2001) |
| Rspo2 | Increase | (Kazanskaya et al. 2004) |
| *Cell-cycle regulators/oncogenes* | | |
| Cyclin D1 | Increase | (He et al. 1998; Shtutman et al. 1999; Tetsu and McCormick 1999) |
| c-myc | Increase | (He et al. 1998) |
| *Liver-related genes* | | |
| Glutamine synthetase | Increase | (Cadoret et al. 2002) |
| Glutamate transporter-1 | Increase | (Cadoret et al. 2002) |
| Glutathione S-transferase | Increase | (Giera et al. 2010) |
| Ornithine aminotransferase | Increase | (Cadoret et al. 2002) |
| Cytochrome P450 enzymes | Increase | (Loeppen et al. 2005; Gougelet et al. 2014) |
| Regucalcin | Increase | (Nejak-Bowen et al. 2009) |
| Constitutive androstane receptor | Increase | (Gougelet et al. 2014) |
| Glucose-6-phosphatase | Increase | (Liu et al. 2011) |
| Phosphoenolpyruvate carboxykinase (PEPCK) | Increase | (Liu et al. 2011) |
| Lect2 | Increase | (Ovejero et al. 2004) |
| Nitric oxide synthase 2 | Increase | (Du et al. 2006) |

Niehrs 2012). Among these alternate signaling mechanisms is the planar cell polarity pathway, which regulates changes in cell polarity and motility through activation of JNK (Boutros et al. 1998; Bartscherer and Boutros 2008; Tada and Kai 2009), and the Wnt/$Ca^{2+}$ pathway, which is mediated by the activation of heterotrimeric G proteins and nuclear factor associated with T cells (NFAT) (Slusarski et al. 1997; De 2011). Additional pathways include the Wnt5-ROR pathway, the Wnt-PKA pathway, the Wnt-mTOR pathway, and others (Semenov et al. 2007; Sugimura and Li 2010; Ho et al. 2012). All of the noncanonical Wnt pathways are discussed further in several recent reviews (Seifert and Mlodzik 2007; De 2011); the remainder of this chapter, however, will focus primarily on the canonical Wnt signaling pathway.

### *6.1.3 Interaction of β-Catenin with Other Pathways*

Apart from its role as a transcriptional cofactor in the canonical Wnt signaling pathway, β-catenin also plays an important role in the maintenance of epithelial cell adherens junctions. β-Catenin forms a bridge between the actin cytoskeleton and E-cadherin present at the cell surface (Aberle et al. 1994; Orsulic et al. 1999; Lilien and Balsamo 2005). The β-catenin/E-cadherin interaction mediates cell-cell adhesion and is regulated by the phosphorylation of β-catenin at tyrosine residue 654 (Roura et al. 1999; Piedra et al. 2001) (Fig. 6.2). Following tyrosine phosphorylation of β-catenin, the β-catenin/E-cadherin complex dissociates, increasing the cytosolic pool of β-catenin, which eventually results in increased transcriptional activity (Piedra et al. 2001; Kam and Quaranta 2009). Specifically, in the liver this dissociation also causes subsequent degradation of E-cadherin, resulting in a loss of adherens junctions and impaired apical trafficking in hepatocytes (Huber and Weis 2001; Theard et al. 2007). Loss of adhesion may also contribute to motility, which is an important component of the cellular response in processes such as development, regeneration, and cancer growth. Recently, however, the role of β-catenin at adherens junctions was reported to be redundant, as liver-specific β-catenin conditional knockout (KO) mice lacking β-catenin in hepatocytes continued to show maintenance of adherens junctions due to an upregulation of γ-catenin or plakoglobin in the KO mice (Wickline et al. 2011, 2013).

β-Catenin also interacts with several receptor tyrosine kinases in the liver. Met is the receptor for hepatocyte growth factor (HGF), a known mitogen, motogen, and morphogen for the liver (Zarnegar 1995). Our laboratory has previously shown that Met and β-catenin associate at the surface of hepatocytes (Monga et al. 2006) (Fig. 6.2). Binding of HGF to Met induces its phosphorylation, which then in turn phosphorylates β-catenin at tyrosine residues 654 and 670, resulting in translocation of β-catenin to the nucleus (Monga et al. 2002; Zeng et al. 2006) (Fig. 6.1). Injecting the human HGF gene into mice also causes dissociation of the Met/β-catenin complex and induction of the β-catenin pathway, resulting in hepatomegaly (Apte et al. 2006). Other reports have demonstrated that HGF positively regulates β-catenin-TCF transactivation, albeit via different mechanisms (Papkoff and Aikawa 1998; Hiscox and

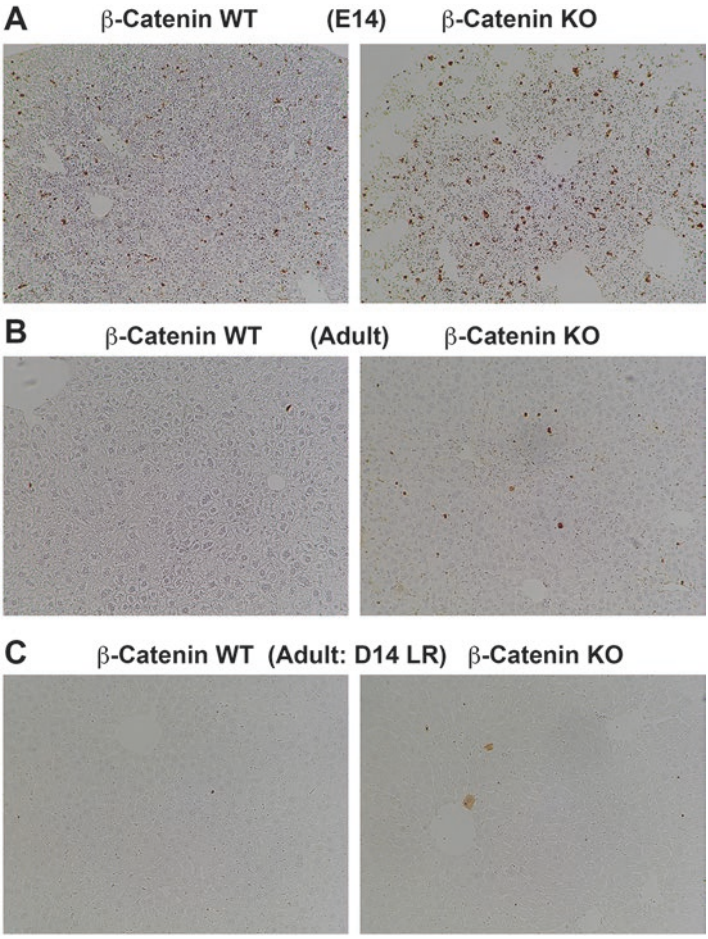


**Fig. 6.2** Increased apoptosis in the absence of hepatocyte β-catenin. Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) immunohistochemistry staining shows increased apoptosis in the livers of mice lacking β-catenin in hepatocytes at E14 (**a**), 4 months after birth at homeostasis (**b**), and 14 days after partial hepatectomy (**c**), compared to wild-type controls

Jiang 1999; Danilkovitch-Miagkova et al. 2001). These findings have important implications for liver cancer. Simultaneous activation of Met and a mutated active form of β-catenin have been found in a subset of human HCC, which has recently been replicated in a genetic model (Tward et al. 2007; Tao et al. 2016a). Hepatoblastomas, which frequently have deletions or missense mutations in exon 3 of the β-catenin gene, also express high levels of Y654 β-catenin, which is induced by HGF/Met signaling (Purcell et al. 2011). Similarly, a direct interaction between β-catenin and the epidermal growth factor pathway has been reported as well (Hoschuetzky et al. 1994; Kanai et al. 1995; Takahashi et al. 1997). ErbB2, a member of this family, can phosphorylate β-catenin at the same tyrosine residue (Y654) as Met, which may increase cell motility (Shibata et al. 1996; Bonvini et al. 2001) (Fig. 6.2).

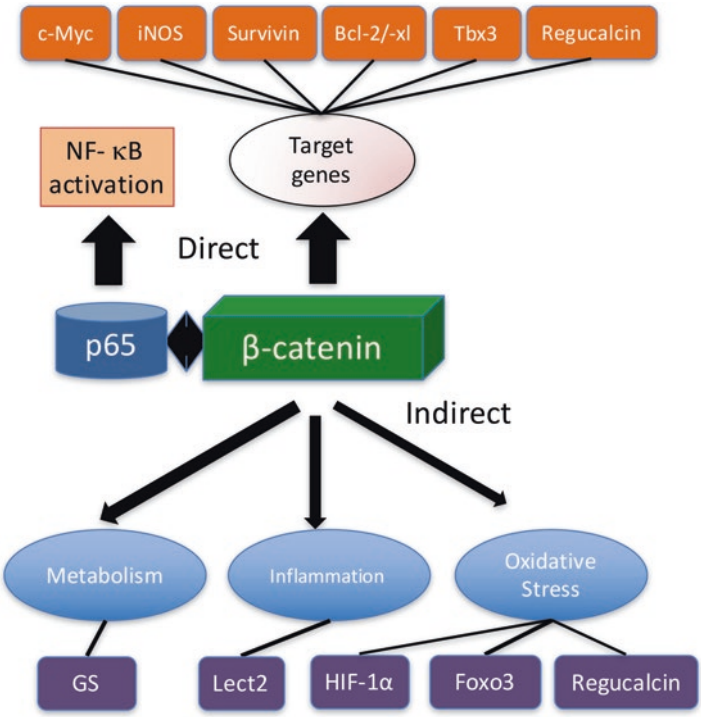


**Fig. 6.3** Role of β-catenin in the survival of hepatocytes. β-Catenin can regulate the survival of hepatocytes through multiple mechanisms. It directly regulates the expression of several genes which encode factors with proven roles in regulating cell death. Physical interaction of β-catenin with p65 subunit also directly plays a role in directly regulating NF-κB activity and hence in cell survival. In addition, to β-catenin's role as a cofactor for the TCF family of transcription factors, it can also act as a cofactor for FoxO3 and HIF-1α to regulate the expression of distinct target genes. Through such interactions and regulation of target genes, β-catenin can modulate processes like cell metabolism, inflammation, and oxidative stress and hence can indirectly have an impact on cell survival

Transforming growth factor-β (TGF-β), a known regulator of self-renewal that has also been implicated in the modulation of HCC (Tang et al. 2008), can impact β-catenin signaling as well (Fig. 6.3). TGF-β-mediated loss of E-cadherin results in the release of β-catenin from cell-cell contacts and its subsequent translocation to the cytoplasm. Active β-catenin thus leads to the increased cell motility and invasive phenotype seen in gastrointestinal and liver cancers (Peinado et al. 2003; Katuri et al. 2006). Additionally, the β-catenin-TCF complex physically interacts with Smads 2, 3, and 4, mediators of TGFβ signaling; such interactions prevent β-catenin degradation, facilitate nuclear translocation, or synergistically enhance transcription of a subset of Wnt target genes (Labbe et al. 2000; Lei et al. 2004; Warner et al. 2005; Hirota et al. 2008; Romero et al. 2008; Zhang et al. 2010a).

Lastly, protein kinase A (PKA) has been shown to activate β-catenin downstream of G-protein-coupled receptor (GPCR)/cAMP signaling (Fig. 6.4). PKA has been

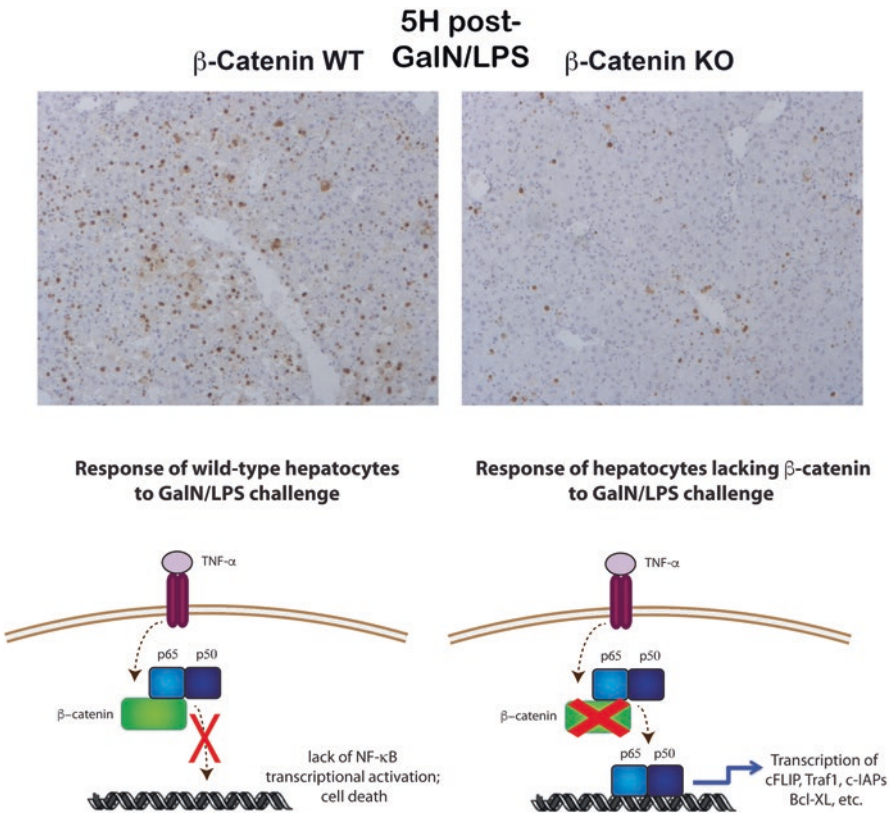


**Fig. 6.4** Role of β-catenin in regulating NF-κB/p65 activation in liver injury. *Top*: apoptosis is decreased in livers lacking β-catenin compared to wild-type livers after GalN/LPS injury. *Bottom*: in acute liver injury, administration of LPS results in production of TNF-α; however, the presence of β-catenin prevents p65 from translocating to the nucleus and activating target gene expression. However, in the absence of β-catenin, there is early, robust, and protracted activation of NF-κB when the TNF-α pathway is stimulated, which leads to activation of pro-survival genes and protection from apoptosis

shown to phosphorylate β-catenin at specific residues in the C-terminus (Ser552 and Ser675) to lead to β-catenin nuclear translocation and activation (Taurin et al. 2006).

## 6.2 Mechanisms of Apoptosis and Cell Survival

Apoptosis, also known as programmed cell death, is a critical component in liver injury and can occur via two major pathways: the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway (Ding and Yin 2004). The death receptor pathway is activated by binding of death receptor ligands to receptors on the cell

surface, while the mitochondrial pathway is initiated by intracellular stresses such as DNA damage or changes of intracellular $Ca^{2+}$. Of these, the death receptor pathway has been extensively studied in hepatocytes, since hepatocytes express an abundance of death receptors (Yoon and Gores 2002). This is presumably due to evolutionary pressure which favored enhanced death receptor expression in the liver to help eliminate pathogens and cells damaged by toxins (Akazawa and Gores 2007). Thus, death receptor-mediated apoptosis in the liver is a critical component of homeostasis, as it targets damaged or abnormal cells for elimination without disrupting liver physiology (Nagata 1997; Akazawa and Gores 2007). Of the six identified death receptors, Fas and TNF-α are considered to have pathologic significance in the liver (Yin and Ding 2003). Importantly, much of our current knowledge on the subject of hepatocyte apoptosis has been derived from experimental induction of liver failure (Galanos et al. 1988).

### *6.2.1 Fas Pathway*

Fas (also known as CD95) is a member of the TNF receptor family which is primarily activated by Fas ligand (FasL), a membrane-spanning protein typically expressed on the surface of activated T cells, although soluble forms of FasL are also present in serum (Berke 1995; Yoon and Gores 2002). Experimentally, the Fas pathway can be induced in mice via intravenous injection of Jo-2 antibody, a Fas agonist which causes death of the animal through massive hepatocyte apoptosis (Ogasawara et al. 1993). Activation of the receptor by FasL or Jo-2 causes trimerization of the receptor, which leads to formation of the death-inducing signaling complex (DISC) (Chinnaiyan et al. 1995; Leist et al. 1996; Ashkenazi and Dixit 1998; Peter and Krammer 2003). This complex consists of the Fas-associated death domain (FADD), which contains a death effector domain (Boldin et al. 1995) that is required for caspase 8 recruitment. Caspase 8, also called the initiator caspase for its ability to activate executioner caspases such as caspases 3, 6, and 7, is activated at the DISC (Thornberry and Lazebnik 1998; Thorburn 2004). In type II cells such as hepatocytes, additional activation of the mitochondrial pathway by caspase 8 is required for apoptosis (Scaffidi et al. 1998). Caspase 8 cleavage of Bid, a proapoptotic protein that translocates to the mitochondria, triggers the release of cytochrome c, which activates caspase 9 and amplifies the apoptotic signal (Luo et al. 1998; Yin et al. 1999).

Hepatocytes can be protected from the effects of Fas by the antiapoptotic Bcl-2 family of proteins, including Bcl-2, myeloid cell leukemia factor-1, and Bcl-$x_L$, which inhibit the mitochondrial pathway (Lacronique et al. 1996; Rodriguez et al. 1996; de la Coste et al. 1999; Scaffidi et al. 1999). Alternatively, apoptosis can be inhibited at the level of caspase 8 by c-FLIP, a structural mimetic of caspase 8 that lacks proteolytic activity and binds directly to either caspase 8 or FADD (Irmler et al. 1997; Tschopp et al. 1998; Scaffidi et al. 1999). Other inhibitors of Fas-mediated apoptosis include X-linked inhibitors of apoptosis (IAPs) and serine protease inhibitors such as antitrypsin (Deveraux et al. 1997; Daemen et al. 2000).

Recently, it has been shown that Met sequestration of the Fas receptor can also prevent Fas-mediated apoptosis in hepatocytes (Zou et al. 2007). Mice lacking Met were hypersensitive to Fas-induced apoptosis (Huh et al. 2004); similarly, high doses of HGF sensitize apoptosis-resistant bid-deficient hepatocytes to Fas-induced cell death through increased dissociation of Fas from Met (Zhao et al. 2007). Further, YLGA, a peptide derived from the Met α-chain, inhibits Fas-mediated apoptosis in fatty liver disease (Zou et al. 2007). The Met/Fas interaction has been demonstrated to regulate apoptosis in endothelial cells as well (Smyth and Brady 2005).

Upregulation of Fas-mediated apoptosis has been implicated in the progression of many human liver diseases. For example, apoptosis is increased in patients with alcoholic hepatitis, and the number of apoptotic cells correlates with disease severity (Natori et al. 2001). Both the Fas receptor and FasL are strongly expressed in patients with this alcoholic hepatitis, which suggests that FasL-positive hepatocytes can induce apoptosis in adjacent Fas-expressing hepatocytes (Natori et al. 2001; Zhou et al. 2001; Yoon and Gores 2002). Another disease in which apoptosis occurs primarily through Fas upregulation is nonalcoholic steatohepatitis (Feldstein et al. 2003). Toxic bile salt accumulation in cholestatic liver disease results in induction of apoptosis in a Fas-dependent manner independent of FasL (Faubion et al. 1999). Fas signaling is also an essential component in hepatocyte injury and apoptosis in Wilson's disease (Strand et al. 1998). In viral hepatitis, apoptosis, which is regulated either by the pathogen itself or the host cytotoxic T cell-mediated immune response, occurs primarily through the Fas pathway (Kountouras et al. 2003). Fas receptor expression has been correlated with areas of inflammation and necrosis in hepatitis B (Mochizuki et al. 1996) and is also present in livers of patients with hepatitis C (Hiramatsu et al. 1994).

### *6.2.2 TNF-α Pathway*

Tumor necrosis factor (TNF)-α is a pleiotropic cytokine that is critical to many diverse cellular processes in the liver, including proliferation, inflammation, and cell death (Schwabe and Brenner 2006). Execution of these functions occurs when TNF-α binds to its major receptor TNF-R1, causing a conformational change in the receptor that initiates DISC formation similar to that formed during Fas-mediated apoptosis. In the case of TNF-α-mediated signaling, this complex is composed of TNF/TNF-R1 trimers and the TNFR-associated death domain (TRADD) (Hsu et al. 1995, 1996; Chen et al. 2002). TRADD then recruits FADD, TNF-associated factor 2 (Traf-2), and receptor-interacting protein-1 (RIP-1) (Micheau and Tschopp 2003). If antiapoptotic signals are blocked, FADD activates caspase 8, which cleaves other downstream effector caspases like caspase 3 as well as activates proapoptotic members of the Bcl-2 family such as Bax and Bak (Wei et al. 2001). Thus, TNF-α-mediated apoptosis utilizes the same downstream apoptotic machinery as the Fas pathway, albeit independently of Bid (Stennicke et al. 1998; Harper et al. 2003; Chen et al. 2007).

A common means of inducing TNF-α signaling other than administration of TNF-α itself is through the use of lipopolysaccharide or LPS, an endotoxin derived from gram-negative bacteria (Lehmann et al. 1987; Shiratori et al. 1990; Yin and Ding 2003). The interaction of LPS with macrophages stimulates the release of TNF-α, which induces downstream signaling as described before. Unlike Fas-mediated apoptosis, induction of apoptosis via the TNF-α pathway requires hepatocyte sensitization, which is usually accomplished by pretreatment with either D-galactosamine (GalN) or actinomycin D (ActD) before administration of either TNF-α ligand or LPS. GalN is a hepatotoxic agent that depletes UTP, thus inhibiting de novo RNA synthesis and sensitizing hepatocytes to the effects of endotoxin (Lehmann et al. 1987). ActD is a different class of transcriptional inhibitor that binds DNA duplexes and prevents synthesis of protective proteins (Leist et al. 1994). In addition, administration of recombinant TNF-α directly after sensitization causes hepatotoxicity identical to that seen in LPS-treated mice, which also suggests that TNF-α is the major mediator of endotoxin lethality (Lehmann et al. 1987). Cell death caused by activation of the TNF-α pathway is believed to occur primarily by apoptosis, followed by necrosis.

In addition to initiating the cell death machinery, binding of TNF-α to its receptor stimulates formation of signaling complexes that prevent cell death, culminating in the activation of nuclear factor kappa B (NF-κB) (Guicciardi and Gores 2009). NF-κB is a group of dimeric transcription factors that initiate a wide variety of cellular programs in response to inflammation and injury. Inactive NF-κB is sequestered in the cytoplasm by members of the IκB family, which become phosphorylated and degraded by the IKK kinase complex in response to TNF-α signaling (Wajant et al. 2003; Israel 2010). Loss of the IκB complex liberates NF-κB, which then translocates to the nucleus, where it becomes phosphorylated and fully activated (Chen and Greene 2004). NF-κB has also been recognized as a key apoptosis inhibitor through its activation of genes including IAPs, c-FLIP, TRAFs, and Bcl family members, among others (Wang et al. 1998; Karin and Lin 2002). Expression and activation of these proteins suppress caspase activation and thus allow the cell to avoid undergoing apoptosis. Activation of NF-κB by LPS or TNF-α can directly antagonize the proapoptotic effects of the latter (Karin and Lin 2002), as demonstrated in experimental models where p65 is required for protection from TNF-α-mediated apoptosis (Beg and Baltimore 1996; Van Antwerp et al. 1996; Wallach et al. 1999). However, treatment with LPS or TNF-α alone does not cause any hepatocyte injury and thus requires agents like GalN, whose pretreatment prevents NF-κB-mediated pro-survival target gene expression for up to 3–4 hours. In summary, signaling through the TNF-α pathway can result in either life or death for the cell depending on physiological context and pathway cross talk, which favors activation of one pathway over another (Kyriakis 2001).

Like Fas, the TNF-α pathway is also dysregulated in a wide range of liver diseases. In addition to Fas, TNF-R1 is upregulated in the livers of patients with alcoholic liver disease (Ramalho et al. 2006; Akazawa and Gores 2007). Serum levels of TNF-α are also increased in patients with alcoholic steatohepatitis and

correlate with disease progression and mortality, suggesting that this pathway plays a role in the pathogenesis of this disease (Bird et al. 1990). Oxidative stress caused by glutathione depletion can exacerbate the severity of alcoholic steatohepatitis by sensitizing hepatocytes to TNF-α (Colell et al. 1998). TNF-R1 and TNF-α are upregulated in both hepatitis B and hepatitis C, probably as a result of inflammation due to viral infection, and are associated with disease progression and severity (Marinos et al. 1995; Fang et al. 1996; Kallinowski et al. 1998; Tokushige et al. 2000). TNF-α-mediated apoptosis also plays a role in ischemia/reperfusion (I/R) injury and fulminant hepatic failure, likely through overaction of the immune system and generation of reactive oxygen intermediaries (Malhi et al. 2010).

### *6.2.3 Other Cell Signaling Pathways Involved in Cell Survival and Apoptosis*

It is also worth mentioning that dysregulation of cell growth and cell cycle checkpoint pathways also contribute to hepatocyte survival and/or apoptosis, especially in HCC. The proto-oncogene c-Myc is a transcription factor that has an important role in regulating apoptotic signaling at the mitochondria, as well as through the death receptor pathways mentioned above (Hoffman and Liebermann 2008). c-Myc also regulates p53, a transcription factor that represses antiapoptotic proteins such as survivin and Bcl-2, resulting in growth arrest (Wu et al. 2001; Hoffman et al. 2002). This pathway can be suppressed by growth factors and is also commonly mutated, deleted, or otherwise activated in HCC, resulting in resistance to apoptosis (Muller et al. 1997; Hainaut and Hollstein 2000; Henriksson et al. 2001; Kodama et al. 2011). Prolonged activation of c-Jun $NH_2$-terminal kinase (JNK) can also contribute to hepatocyte cell death (Chen et al. 1996; Chang et al. 2006; Das et al. 2009), although its target, the transcription factor c-Jun, protects hepatocytes from apoptosis (Eferl et al. 2003; Fuest et al. 2012). Activation of the TGF-β pathway is generally proapoptotic, although TGF-β can also activate survival signals such as Akt and EGFR ligands (Ohno et al. 1995; Sanchez et al. 1996; Samson et al. 2002; Valdes et al. 2004; Yang et al. 2006; Caja et al. 2007). Finally, dysregulation of growth factor receptor pathways can result in constitutive activation of the signaling cascade, conferring resistance to apoptosis. For example, hepatitis viruses protect hepatocytes from apoptotic cell death by promoting the activation of Ras/PI3K/Akt/mTOR survival pathway (Steelman et al. 2011), and overexpression of components of these growth factor pathways can promote cell survival (Huynh et al. 2003; Liu et al. 2006).

## 6.3 β-Catenin in Cell Survival and Apoptosis

### *6.3.1 Cell Death in the Absence of β-Catenin*

β-Catenin signaling appears to play a role in modulating cell survival especially in the liver. Deletion of β-catenin or inhibition of Wnt signaling has been shown to induce apoptosis in several in vivo and in vitro models. We will specifically discuss examples during prenatal and postnatal hepatic development.

β-Catenin gene and protein expression peaks at embryonic days E10–E14 in mouse embryonic liver, and during this time β-catenin is localized to the nucleus, cytoplasm, and membrane in different epithelial cells and coincided with ongoing cell proliferation (Micsenyi et al. 2004). Mouse embryonic livers from E9.5–E10 stages cultured in the presence of a β-catenin antisense oligonucleotides showed decreased proliferation and a simultaneous increase in apoptosis, two processes vital to hepatic morphogenesis that follows hepatic specification and induction (Monga et al. 2003). These findings were corroborated by a subsequent study that demonstrated overexpression of β-catenin in developing chicken livers leads to a threefold increase in liver size, which is due at least in part to an expanded hepatoblast population (Suksaweang et al. 2004). In the same study, blocking β-catenin expression through overexpression of pathway inhibitors resulted in decreased liver size and altered liver shape. Characterization of embryos where β-catenin was deleted from hepatoblasts using β-catenin-floxed mice and Foxa3-cre verified the above findings as β-catenin-deficient livers showed a notable decrease in liver size and associated mortality at around E16–E18 (Tan et al. 2008). Among other defects, a dramatic increase in death of resident hepatoblasts was apparent in knockouts (KOs) as shown by immunohistochemistry for TUNEL (Fig. 6.2a). Interestingly, an earlier study employing GSK3β gene knockouts also demonstrated a phenotype of increased liver cell death and liver degeneration that resulted in embryonic lethality (Hoeflich et al. 2000). This seems paradoxical since GSK3β loss would lead to β-catenin activation, which appears to be mitogenic and pro-survival. However, the liver degeneration evident in this model was consistent with excessive TNF-α toxicity and was rescued by neutralizing TNF-α. Thus, GSK3β loss prevented NF-κB activation and led to enhanced cell death in this model and was not due to β-catenin activation. Of further relevance here is that untimely and excessive β-catenin stabilization during hepatic development also has deleterious consequences. APC deletion during liver development also led to a dramatic increase in cell death and a counterintuitive decrease in cell proliferation; however, this was associated with untimely differentiation of hepatoblasts into biliary cells (Decaens et al. 2008). Although not directly tested, this could be a consequence of enhanced β-catenin levels which could modulate cell death through NF-κB modulation as discussed in the forthcoming section.

Our laboratory has also previously shown an increase in hepatocyte apoptosis in adult livers following hepatocyte-specific deletion of β-catenin (Tan et al. 2006). We observed a basal increase in hepatocyte survival as detected by TUNEL staining

(Fig. 6.2b). The exact basis of this observation still remains unclear. Similarly, we have reported an increase in hepatocyte cell death during liver regeneration at both early and late times (Fig. 6.2c) (Tan et al. 2006). Therefore, overall β-catenin appears to contribute to the anti-cell death phenotype in basal hepatocytes.

### *6.3.2 Mechanisms by Which Wnt/β-Catenin Signaling Regulates Hepatocyte Survival*

The regulation of cell survival by Wnt/β-catenin signaling can occur at multiple levels, as described in the next several sections. β-Catenin can exert an inhibitory effect on the transcriptional activity of the NF-κB subunit p65 through physical association, which has implications in both acute liver injury and cancer. Several genes involved in cell survival, such as survivin and regucalcin, are direct transcriptional targets of Wnt/β-catenin signaling. Finally, β-catenin can also play an indirect role in cell survival through regulation of cell metabolism, inflammation, and oxidative stress (Fig. 6.3).

#### 6.3.2.1 Direct Mechanisms

Physical Association

Many studies have addressed the complex cross regulation of the NF-κB and Wnt/β-catenin pathways in other cell and tissue types. For example, loss of E-cadherin in melanoma cells leads to an increase in the amount of cytoplasmic β-catenin, which in turn induces activation of NF-κB through a p38-mediated mechanism (Kuphal et al. 2004). Another study showed that β-catenin exists in a complex with IKKα, a key regulator of NF-κB activity, and that this kinase can phosphorylate β-catenin (Lamberti et al. 2001). Furthermore, inhibition or downregulation of IKKα inhibits growth of prostate cancer and multiple myeloma cell lines through decreased expression and activity of β-catenin (Albanese et al. 2003; Hideshima et al. 2009). Activation of β-catenin is downstream of the IKKα/β/NF-κB pathway in proximal colonic crypts as well (Umar et al. 2009). Other studies have identified the ubiquitin ligase receptor β-TrCP as a convergence point between these two pathways, as it regulates turnover of both β-catenin and NF-κB. Induction of β-TrCP by β-catenin, for example, leads to increased NF-κB transactivation; conversely, inhibition of β-TrCP impairs NF-κB activity while simultaneously causing nuclear accumulation of β-catenin (Spiegelman et al. 2000; Nakayama et al. 2003). From these studies, then, it can be concluded that in hepatocytes, β-catenin may interact with the NF-κB pathway at multiple points in the signaling cascade as well.

The physical association between the β-catenin and the NF-κB survival pathways was first described in colon and breast cancer. In that study, direct binding of

β-catenin to NF-κB prevented its translocation and target gene activation in a colon cancer cell line (Deng et al. 2002). Furthermore, a correlation was found between activated β-catenin and inhibition of NF-κB target gene expression in colon and breast cancer tissues, highlighting the clinical relevance of this cross regulation between the β-catenin and NF-κB pathways. Mechanistically, another group has demonstrated that E-cadherin mediates the association of p65 with β-catenin, and thus NF-κB transcriptional activity is inhibited not by the nuclear pool of β-catenin but by the β-catenin associated with the adherens junction complex (Solanas et al. 2008). A direct interaction between the NF-κB p50 subunit and β-catenin was also described in bacterial-colonized HCT116 intestinal epithelial cells. Interestingly, the expression of mutated, constitutively active β-catenin also prevents IκBα degradation, thus inhibiting NF-κB activity by both direct and indirect mechanisms (Sun et al. 2005). Finally, co-immunoprecipitation in colon cancer cells showed a direct interaction between β-catenin and both p50 and p65 proteins. Further, as in the other tissue and cell types mentioned above, manipulation of β-catenin levels inversely influences NF-κB-mediated target gene expression (Du et al. 2009). Collectively, these findings support previous studies in which overexpression of β-catenin is pro-apoptotic and that this property is independent of TCF4 and the transcriptional function of β-catenin (Kim et al. 2000; Deng et al. 2004).

Conversely, p65 can also downregulate the β-catenin pathway by suppressing β-catenin nuclear translocation or activation (Masui et al. 2002; Saegusa et al. 2007). In colon cancer cells, treatment with a nonsteroidal anti-inflammatory drug inhibits Wnt/β-catenin signaling via NF-κB activation and its subsequent physical association with β-catenin (Cho et al. 2005). p65 was also shown to repress β-catenin-dependent cyclin D1 transcription, possibly through a protein-protein interaction (Hwang et al. 2010). Indeed, another group has validated this hypothesis by showing that activation of NF-κB in colon cancer cells blocks β-catenin binding to the cyclin D1 promoter through increased association between β-catenin and p65 (Abe et al. 2014). Thus, p65 inhibits β-catenin activity in much the same way as β-catenin inhibits p65 activity.

In the liver, the importance of this complex was first demonstrated in human HCC tissues. β-Catenin expression inversely correlated with the expression of either Fas and inducible nitric oxide synthase (iNOS), which are both targets of the NF-κB signaling pathway, in liver tumors (Du et al. 2009). Our laboratory has recently demonstrated that liver-specific β-catenin KO mice unexpectedly exhibit prolonged survival and reduced injury to TNF-α induced apoptosis. We elucidated this to be due to the existence of an inhibitory p65/β-catenin complex, which resides in the cytoplasm of hepatocytes. During the process of liver injury, this complex undergoes dynamic regulation, and its absence in the β-catenin KO mice led to earlier and robust activation of NF-κB target gene expression (Nejak-Bowen et al. 2013) (Fig. 6.4). In recent work from our laboratory, we also found the converse – that inhibition of p65 in culture can activate β-catenin – although the implications for cell survival after liver injury are unknown.

Adding to the complexity of these pathway interactions, another group found that GSK3β and APC, which are major upstream regulators of β-catenin, can regulate

NF-κB in colon and breast cancer cells and that this effect was mediated by β-catenin (Deng et al. 2004). In hepatocytes, however, inhibition of GSK3β, which would normally suppress NF-κB activity and promote cell death, instead fails to induce apoptosis after TNF-α challenge. This protection from apoptosis is due to induction of Wnt/β-catenin signaling and production of antiapoptotic factors, which ultimately lead to cell survival (Gotschel et al. 2008). Importantly, the authors found that contrary to previous publications, β-catenin does not directly mediate suppression of NF-κB activity. The differences between this finding and the studies mentioned in the previous paragraphs may be the level at which β-catenin and/or NF-κB is activated or suppressed, as well as the selectivity of the inhibitors or activators for their target protein(s).

The recent investigations into the function of the β-catenin/NF-κB complex and its role in cell survival have implications beyond LPS/TNF-α-induced apoptosis, as the role and regulation of this complex in other acute liver injuries such as acetaminophen toxicity and alcoholic liver injury remain to be explored. Additionally, it will be important to elucidate the role of this complex in cell survival and apoptosis during physiological process such as liver development, growth, and regeneration.

## Transcriptional Targets of β-Catenin

Apoptosis can also be suppressed through transcriptional activation of the Wnt/β-catenin pathway. Chen et al. first demonstrated that Wnt/β-catenin signaling inhibits apoptosis in fibroblast cells, but experiments in hepatocytes and HCC cell lines soon followed (Chen et al. 2001). For example, the expression of hepatitis B x antigen in hepatoma cells was found to correlate with upregulation of β-catenin and enhanced cell survival (Lian et al. 2006). Stabilization of β-catenin in AML12 cells also resulted in protection from TNF-α-induced apoptosis (Shang et al. 2004). On the other hand, degradation of β-catenin was observed in hepatoma cells treated with chemotherapeutic agents designed to induce apoptosis (Emanuele et al. 2004). Transfection of hepatoma cells with a tumor-suppressing gene also resulted in β-catenin degradation, concomitant with apoptosis (Yoshibayashi et al. 2007). Additionally, overexpression of miR-122 promoted apoptosis in hepatoma cells, likely through downregulation of Wnt1 and β-catenin (Xu et al. 2012). Although none of these studies identified the downstream targets responsible for β-catenin-mediated cell survival, an oligonucleotide array analysis of β-catenin siRNA-treated HeLa cells identified a differential regulation of genes implicated in cell survival pathways such as PI3K/AKT, NF-κB, Fas, and p53 (Huang et al. 2006). Specific to the liver, other investigators have identified a reduction in the expression of angiogenesis genes, matrix metalloproteinase genes, and anti-apoptosis genes when β-catenin is silenced in HCC cells (Wang et al. 2010). Thus, the diversity of survival pathways affected by Wnt/β-catenin activation highlights its global role in cell signaling and provides strong support for its pro-survival function, independent of its role in regulating NF-κB activity.

One of the prototypical β-catenin target genes is c-Myc, which regulates cell proliferation and differentiation, and is also frequently activated in cancer (Dang 1999, 2012, Klaus and Birchmeier 2008). Interestingly, c-Myc can also induce or sensitize cells to apoptosis, which is a fail-safe program to prevent inappropriate cell proliferation (Hoffman and Liebermann 1998, 2008). In hepatocyte cell lines, c-Myc-induced apoptosis was a result of oxidative stress (Xu et al. 1997). c-Myc is also partly responsible for IFNγ-induced apoptosis in cultured hepatocytes (McCullough et al. 2007). Additionally, overexpression of c-Myc in hepatocytes led to an increase in apoptosis (Nevzorova et al. 2013).

On the other hand, deletion of c-Myc perinatally in vivo also induced apoptosis and oxidative stress in hepatocytes (Baena et al. 2005). Inhibition of c-Myc expression in rat hepatocyte cell lines also sensitizes hepatocytes to TNF-α cytotoxicity (Liu et al. 2000). Interestingly NF-κB transcriptional activity was also decreased in the absence of c-Myc, although these two effects were independent. Another study showed that NF-κB cooperates with c-Myc in promoting hepatocyte survival in a mouse hepatocyte cell line (Bellas and Sonenshein 1999). Thus, c-Myc is an important component in altering the balance between apoptosis and survival, although the molecular regulators influencing this cell fate decision are unclear. Additionally, although not all of these effects may be attributable to β-catenin-regulated transcriptional control of c-Myc, there is evidence that Wnt signaling can directly inhibit c-Myc-induced apoptosis in fibroblasts (You et al. 2002).

Inducible nitric oxide synthase (iNOS, also known as NOS2) is induced by proinflammatory cytokines in the setting of oxidative stress. In the liver, the antioxidant properties of iNOS are hepatoprotective and contribute to cell survival (Kuo et al. 1997). Du et al. were the first to identify that the iNOS gene was directly regulated by Wnt/β-catenin signaling in liver cells (Du et al. 2006). Intriguingly, in a follow-up paper, the same group demonstrated that β-catenin can also regulate iNOS expression indirectly through a physical association with NF-κB. Overexpression or mutation of β-catenin in colon and liver cancer cell lines led to a decrease in cytokine-mediated NF-κB transcriptional activity and a subsequent decrease in iNOS promoter activity; conversely, inhibition or lack of β-catenin induced NF-κB activity and iNOS expression (Du et al. 2009). These findings indicate that β-catenin can regulate the oxidative stress response through an inverse relationship with NF-κB. On the other hand, another group found that activation of β-catenin led to increased transcription of β-TrCP, which in turn degrades IκB. This leads to NF-κβ activation, which results in transcription of iNOS (Bandino et al. 2008). This direct relationship between β-catenin activation and NF-κB transcription of iNOS would appear to be in contrast to Du et al.'s findings; however, in the former, activation of β-catenin was the primary event and occurred due to disruption of adherens junctions and loss of association with E-cadherin, whereas in the latter, cytokine production and activation of NF-κB was the initiating stimulus. Thus, whether iNOS is activated or suppressed by β-catenin may be context-dependent.

Survivin, an antiapoptotic protein that inhibits caspase activation, is a β-catenin target gene (Zhang et al. 2001). Deletion of survivin in hepatocytes during postnatal

liver development results in mitotic arrest and cell death (Li et al. 2013). Additionally, survivin is frequently overexpressed in HCC and may play an important role in survival and progression of this disease (Ito et al. 2000; Liu et al. 2008; Peroukides et al. 2010; Yang et al. 2011). Treatment of HCC cell lines with the small-molecule β-catenin inhibitor FH535 reduces survivin expression as well as cell proliferation, suggesting that this therapy may be effective at several levels (Gedaly et al. 2014). Small-molecule antagonists of the TCF4/β-catenin complex also resulted in decreased c-Myc and survivin expression, concomitant with decreased cell survival and increased apoptosis in vitro as well as in HCC xenografts (Wei et al. 2010). Additionally, the proteasomal inhibitor MG132 induced apoptosis in HCC cell lines secondary to increased β-catenin degradation and decreased survivin expression (Cervello et al. 2004). Mutated or constitutively active β-catenin also results in upregulation of the antiapoptotic proteins Bcl-2 and Bcl-$x_L$, albeit through indirect mechanisms (Oltersdorf et al. 2005; Xie et al. 2005; Li et al. 2007a, b; Mott and Gores 2007). Thus, Wnt/β-catenin signaling can positively impact cell survival through direct upregulation of antiapoptotic genes, although due to the diverse biological effects of this signaling pathway, other mechanisms are likely to contribute as well.

Tbx3 is a member of the T-box gene family that plays a role in patterning events during liver development through transcriptional repression (Davenport et al. 2003). Recently, Renard et al. showed that mutated β-catenin can induce the expression of Tbx3 in human and mouse HCC models as well as HCC cell lines (Renard et al. 2007). After confirming that Tbx3 transcription is directly regulated by β-catenin, they investigated the role of Tbx3 in cellular function. They found that inhibiting Tbx3 blocks β-catenin-mediated cell survival and renders cells sensitive to apoptosis, initiating a sequence of events ultimately leading to downregulation of p53 (Renard et al. 2007).

Regucalcin, also known as senescence marker protein-30 (SMP30) (Fujita 1999), is a $Ca^{2+}$-binding protein that has been implicated in maintaining cell homeostasis and function (Yamaguchi 2000; Yamaguchi 2005). In the liver, the overexpression of regucalcin in hepatoma cells suppresses cell proliferation and the expression of several known oncogenes, such as c-Myc (Tsurusaki and Yamaguchi 2003). Regucalcin overexpression also has a suppressive effect on apoptosis induced by TNF-α or thapsigargin (Izumi and Yamaguchi 2004). An important role of regucalcin has also been reported in vitamin C biosynthesis (Kondo et al. 2006). Through chromatin immunoprecipitation and in vitro promoter deletion mutants, we recently identified regucalcin to be a novel target of β-catenin signaling in the liver (Nejak-Bowen et al. 2009). Indeed β-catenin KO mice show significantly lower ascorbate levels secondary to decreased expression of both regucalcin and L-gulonolactone oxidase, both critical in vitamin C synthesis in murine hepatocytes (Linster and Van Schaftingen 2007). Furthermore, hepatocytes from β-catenin KO mice, which normally undergo massive apoptosis within 48 hours of culture, can be rescued by vitamin C supplementation. These findings are in agreement with previous studies where regucalcin overexpression has been shown to suppress cell death (Izumi and Yamaguchi 2004). Our results are also concordant with previous studies where mice containing a germline null mutation of SMP30 have been shown to be highly susceptible to both TNF-α and Fas-mediated apoptosis (Ishigami et al. 2002; Matsuyama et al. 2004).

Interestingly, we have also shown that loss of regucalcin promotes apoptosis in HepG2 cells, although the increase in apoptosis was not nearly as pronounced as in the murine hepatocytes (Nejak-Bowen et al. 2009). Moreover, the apoptosis in regucalcin siRNA-transfected hepatoma cells was rescued by supplementation with either ascorbic acid or N-acetylcysteine, a potent antioxidant. This is relevant because human cells lack L-gulonolactone oxidase and are unable to synthesize vitamin C; thus, the increased cell death seen in human hepatoma cells with decreased regucalcin expression cannot be due to restoration of vitamin C biosynthesis. These observations suggest that β-catenin might be important in human hepatocyte survival through regucalcin-dependent regulation of the redox state of the cell, independent of regucalcin's vitamin C biosynthetic function. In fact, previous publications from our lab have documented increased oxidative stress and apoptosis in prenatal β-catenin KO livers (Tan et al. 2008), MCD-induced liver injury (Behari et al. 2010), and adult livers from β-catenin conditional KOs (Tan et al. 2006; Zhang et al. 2010b). Thus, Wnt/β-catenin signaling plays a direct role in protection from apoptosis through regulation of the regucalcin gene, which contributes to cell survival by several mechanisms.

#### 6.3.2.2 Indirect Mechanisms

Oxidative Stress

Reactive oxygen species (ROS), reactive nitrogen species (RNS), and free radicals are critical for physiology. They are a normal by-product of aerobic metabolism and also serve important functions as messenger molecules both inside and outside the cell. Reactive oxygen species generated by growth factors and cytokines are implicated in mediating cellular responses through signal transduction (Lander 1997). However, when the balance between ROS generation and antioxidant protection is disrupted, cells undergo oxidative stress (Sies 1997). Oxidative stress can cause damage through inflammation, ischemia, apoptosis, and necrosis and is a common feature of many liver injuries noted in the previous sections (Diesen and Kuo 2010).

The Wnt/β-catenin signaling pathway can regulate cell survival indirectly through regulation of oxidative stress-induced apoptosis. An interaction between β-catenin and hypoxia-inducible factor-1 (HIF-1) has been described in colorectal cancer which promotes adaptation to hypoxia or insufficient oxygen and nutrients (Kaidi et al. 2007). In the liver, loss of β-catenin in hepatocytes renders mice more susceptible to I/R injury, resulting in increased apoptosis; conversely, overexpression of Wnt-1 confers resistance to hypoxia and I/R injury (Lehwald et al. 2011). In both studies, under conditions of oxygen deprivation, HIF-1α competes with TCF4 for direct binding with β-catenin to induce the expression of genes that regulate adaptation and survival under hypoxic conditions. Therefore, interactions between β-catenin and HIF-1α depend on the redox state of a cell and in turn influence adaptation and survival.

Another mechanism of Wnt/β-catenin-regulated protection against oxidative stress involves the FoxO transcription factors, which control processes such as metabolism, proliferation, apoptosis, and survival. β-Catenin binds directly to FoxO in response to oxidative stress and enhances its transcriptional activity in *C. elegans* and in colon carcinoma cells (Essers et al. 2005; Hoogeboom et al. 2008). However, in the liver, Wnt/β-catenin signaling is important for reduction of oxidative stress-induced apoptosis through suppression, rather than enhancement, of FoxO3. During oxidative liver injury, β-catenin in hepatocytes inhibits FoxO-mediated apoptosis through transcription of serum/glucocorticoid-regulated kinase 1, which inactivates FoxO3, thus preventing transcription of such proapoptotic targets as p27 and Bim (Tao et al. 2013). Thus, regulation of oxidative stress by Wnt/β-catenin signaling appears to be context and tissue specific.

Oxidative stress also contributes to the development of HCC. The absence of β-catenin in hepatocytes promotes enhanced hepatocarcinogenesis in a mouse tumor model; this finding was secondary to sustained oxidative stress, which led to inflammation and apoptosis (Zhang et al. 2010b). Treatment of these β-catenin knockout mice with an antioxidant protects these mice from developing tumors, further supporting a role for β-catenin in regulating oxidative injury during tumorigenesis. Furthermore, identification of the association of CTNNB1 mutations with mutations in the gene encoding NRF2, a transcription factor that is critical in cellular redox homeostasis, using high-resolution copy number analysis of tumors not only suggests cooperation between these two pathways in HCC but also a role for Wnt/β-catenin signaling in the management of oxidative stress (Guichard et al. 2012). Thus, these studies identify a role for β-catenin as a potential tumor suppressor through its antioxidant properties.

## Inflammation

Like NF-κB activation, inflammation can be a double-edged sword for the liver. Acute inflammation in response to insult or injury is an important part of immunity; however, chronic inflammation, such as that found in hepatitis and cirrhosis, is a risk factor for the development of HCC (Hagemann et al. 2007). Leukocyte cell-derived chemotaxin 2, or LECT2, was recently identified as a direct target of β-catenin and is mainly synthesized in the liver (Yamagoe et al. 1998; Ovejero et al. 2004). LECT2 was first characterized as a chemotactic factor for neutrophils (Yamagoe et al. 1996). However, it appears that in the liver, LECT2 regulates the homeostasis of natural killer T cells, as mice lacking LECT2 have increased hepatic natural killer T cells. Interestingly, these mice display more severe hepatic injury than controls in response to concanavalin A, characterized by increased apoptotic hepatocytes and elevation in secreted cytokine levels (Saito et al. 2004). These findings implicate LECT2, and thus β-catenin, in the pathogenesis of hepatitis and other inflammatory liver diseases. Another study found that oncogenic β-catenin induces a pro-inflammatory program that is permissive to tumorigenesis. In this model, LECT2 was identified as a key effector involved in this process, as deletion of this gene resulted in a dramatic

aggravation of the inflammatory response (Anson et al. 2012). Interestingly, these authors also found that tumors with constitutively active β-catenin also had activated NF-κB as well, although whether that was a result of increased cytokine production or through direct regulation of the pathway by β-catenin is unknown. Accordingly, LECT2 has been utilized as a biomarker for β-catenin-activated hepatocytes in mouse models of HCC and in human HCCs irrespective of β-catenin mutational status (Colnot et al. 2004; Okabe et al. 2014). Serum LECT2 levels have also been used as prognostic indicators in recovery from acute liver failure and in liver regeneration after living donor liver transplantation (Sato et al. 2004a, b). These findings once again emphasize the essential role of β-catenin in regulating hepatocyte survival, even through secondary processes such as inflammation.

Cell Metabolism

As shown in Table 6.1, β-catenin is involved in glutamine metabolism through regulation of several genes including the glutamate transporter GLT-1 as well as glutamine synthetase (GS) (Cadoret et al. 2002). GS catalyzes conversion of glutamate to glutamine, which is required for protein and nucleotide synthesis. Glutamine can also be an alternative energy source for proliferating cells, particularly in tumor cells where the concentrations of reactive oxygen species are high and the citric acid cycle may be impaired (Cederbaum and Rubin 1976; Fukuda et al. 2002; Wan et al. 2015). In fact, a subset of tumors becomes addicted to glutamine as a substrate for maintenance of cell viability (Wise et al. 2008). Because of its dependence on β-catenin signaling, GS has been shown to be a surrogate target for β-catenin-activating mutations in HCC (Cadoret et al. 2002; Zucman-Rossi et al. 2007; Cieply et al. 2009; Lee et al. 2014). To assess if this had any physiological function, Tardito et al. treated various HCC cell lines with GS inhibitors. They found that only the β-catenin-mutated HCC cells were sensitive to glutamine depletion and the resulting inhibition of GS resulted in increased autophagy and apoptosis (Tardito et al. 2011). A follow-up study by this same group showed that glutamine depletion also hinders the growth of HCC xenografts (Chiu et al. 2014). Thus, β-catenin-dependent dysregulation can cause alterations in cell metabolism that have indirect consequences for cell survival.

### *6.3.3 Wnt/β-Catenin Signaling and Cell Survival in Other Liver Cell Types*

In addition to its well-established contribution to hepatocyte cell survival, the Wnt/β-catenin signaling pathway also plays a role in preventing apoptosis in other liver cell types. Although the role and regulation of Wnt/β-catenin signaling are just beginning to be understood in these populations, hepatic stellate cells, endothelial cells, and inflammatory cells have all been shown to utilize this pathway to promote

cell survival during liver injury. Together, these findings illustrate the cross talk and interdependency between cell types during liver injury, as well as the redundancy of critical pathways such as Wnt/β-catenin in regulating cell survival regardless of type.

#### 6.3.3.1 Hepatic Stellate Cells

Hepatic stellate cells (HSCs) are fat-storing mesenchymal cells of the liver and are responsible for diverse functions including vitamin A storage, maintenance of sinusoidal vascular tone, and wound healing. During liver injury, HSCs are activated, resulting in deposition of extracellular matrix; in chronic injury, this process ultimately culminates in hepatic fibrosis. Thus, as the major liver cell type that responds to hepatocellular damage, HSCs are a vital component of the liver's response to injury and repair (Friedman 2008). Survival of quiescent, nonactivated HSC is dependent on Wnt signaling (Subramaniam et al. 2012). Wnt signaling is also involved in HSC activation and subsequent fibrosis, as Wnt antagonism restores quiescence in HSC (Cheng et al. 2008; Zhu et al. 2010). Interestingly, high doses of Dickkopf-related protein 1 (Dkk-1), an inhibitor of the β-catenin pathway, also induce apoptosis in cultured activated HSC. Studies by other groups have shown that treatment of hepatic stellate cells with Wnt3a suppresses TRAIL-induced apoptosis and contributes to cell survival (Myung et al. 2007) and that Wnt signaling is downregulated during HSC apoptosis (Shin et al. 2009), again suggesting a positive role for Wnt/β-catenin signaling in HSC survival and activation. On the other hand, several groups have suggested a role for noncanonical Wnt signaling in HSC activation (Jiang et al. 2006; Corbett et al. 2015). Furthermore, treatment with noncanonical Wnt5a can also protect activated HSC from apoptosis, as well as stimulate the expression of profibrogenic mediators from neighboring Kupffer cells. Thus, noncanonical Wnt signaling is implicated in the survival and profibrogenic phenotype of activated HSC through two mechanisms (Corbett et al. 2015). The complex regulation of HSC by canonical or noncanonical Wnt signaling thus needs to be further elucidated, as do the target genes responsible for HSC activation and liver fibrosis.

Liver Sinusoidal Endothelial Cells

Liver sinusoidal endothelial cells (LSECs), in addition to forming a continuous lining of the liver sinusoids, also contribute to repair after liver injury (DeLeve 2013). Although few direct studies exist on the role of Wnt/β-catenin signaling in hepatic endothelial cells, this pathway has been shown to induce proliferation in other endothelial cell types (Wright et al. 1999; Masckauchan et al. 2005). Further studies have demonstrated that the expression of mutated active β-catenin can promote cell survival after growth factor deprivation; activation of the Wnt/β-catenin pathway also promotes angiogenesis by induction of IL-8 (Masckauchan et al. 2005). Noncanonical Wnt signaling can also promote survival of endothelial cells in the

absence of growth factors, albeit through a distinct mechanism (Masckauchan et al. 2006). Finally, Gedaly et al. showed that the β-catenin inhibitor FH535 reduced survivin levels in LSEC as well as in HCC cell lines (Gedaly et al. 2014). As with other cell types in which the activation of β-catenin contributes to survival, it will be important to determine the downstream targets and pathways responsible for this protection.

#### 6.3.3.2 Inflammatory and Immune Cells

I/R injury triggers a complex cascade of inflammatory mediators that encompasses Kupffer cell activation, cytokine release, increased expression of cellular adhesion molecules and chemokines, and neutrophil transmigration, culminating in hepatocellular injury and death (Lentsch et al. 2000; Tsung et al. 2005). Knockdown of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which regulates the innate immune response, results in activation of β-catenin through Akt, which led to resistance against I/R injury through enhancement of antiapoptotic pathways (Kamo et al. 2013). The regulation of these immune networks by β-catenin may occur primarily in macrophage cells, as these cells secrete many cytokines including TNF-α that contribute to injury and inflammation (Colletti et al. 1994; Meng and Lowell 1997; Bruck et al. 2003; Giakoustidis et al. 2003; Wang et al. 2013). Furthermore, activation of β-catenin after PTEN knockdown also inhibited TLF4/NF-κB signaling, and the subsequent decrease in pro-inflammatory response may play a critical role in promoting survival as well (Kamo et al. 2013). Another mechanism by which Wnt/β-catenin signaling may regulate inflammation is found in a follow-up paper, which describes a role for β-catenin in reprogramming dendritic cells (DCs) during I/R injury. In this case, activated β-catenin in DC inhibits PTEN and promotes PI3K/Akt signaling, which in turn downregulates DC maturation and function through inhibition of TLR4. These findings are linked to a decrease in hepatocellular damage, inflammation, and apoptosis after I/R injury (Ke et al. 2013). Thus, enhancement of Wnt/β-catenin signaling in immune cells may ameliorate liver damage through suppression of inflammation.

## 6.4 Conclusions

Because apoptosis is a common feature in the pathology of many different liver diseases, therapeutic inhibition of this process has the potential to reduce injury and improve patient survival (Yoon and Gores 2002). Several therapies aimed at reducing apoptosis are already being utilized in preclinical and clinical trials in the treatment of liver disease. Infliximab, a monoclonal antibody raised against TNF-α, has been shown to attenuate hepatic injury in a pilot study (Spahr et al. 2002). Administration of caspase inhibitors lowered aminotransferase activity and decreased liver damage in NASH and hepatitis C patients and also protects against

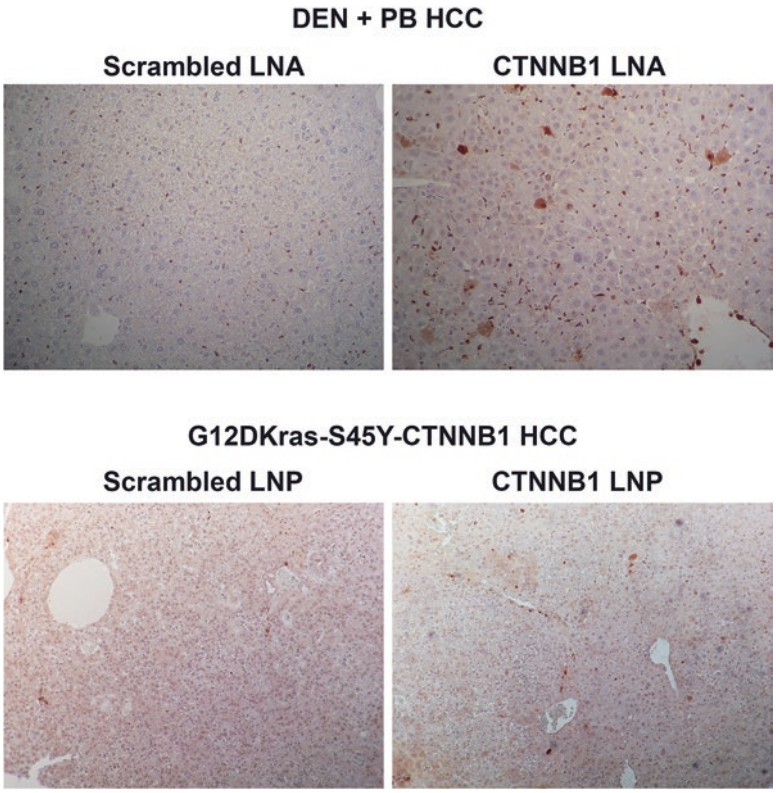


**Fig. 6.5** Increased apoptosis in HCC after treatment with β-catenin inhibitors. (**a**) TUNEL staining shows induction of apoptosis in diethylnitrosamine/phenobarbital-induced HCC after treatment with β-catenin-locked nucleic acid antisense (*LNA*). (**b**) Increased apoptosis in Kras/β-catenin-mutated HCC treated with a lipid nanoparticle (*LNP*) loaded with a Dicer substrate siRNA targeting β-catenin

apoptotic injury during liver transplantation (Valentino et al. 2003; Baskin-Bey et al. 2007; Pockros et al. 2007; Masuoka et al. 2009; Ratziu et al. 2012). Chronic cholestatic liver disease is commonly treated with ursodeoxycholic acid, which is believed to attenuate both oxidative stress and apoptosis (Kumar and Tandon 2001; Rodrigues and Steer 2001). Exogenous antioxidant therapy has also been utilized to treat liver injury and apoptosis related to ROS; however, the results of these studies have been mixed (Diesen and Kuo 2010).

Other novel antiapoptotic therapies may include activators of pro-proliferative pathways, which would shift the balance toward cell survival and away from death. The Wnt/β-catenin pathway is one such example of a pro-survival pathway that may be amenable to manipulation. Our group and others have recently shown that triiodothyronine (T3) can activate β-catenin in hepatocytes and induce proliferation; however, further studies will be needed to elucidate the role of T3 in protection from liver injury (Fanti et al. 2014; Alvarado et al. 2016).

An overall useful exploitation of the observation that β-catenin is pro-survival in hepatocytes is to be able to suppress β-catenin signaling in liver tumors to induce tumor cell death. Indeed, β-catenin suppression using modalities like locked nucleic antisense (LNA), or siRNA packaged in liver tumor cell-specific EnCore lipid nanoparticles (LNPs), has shown a dramatic decrease in β-catenin activity and, as a consequence, a profound decrease in tumor burden in various models. For example, using LNA against β-catenin gene (CTNNB1) led to a complete response in a chemical carcinogenesis model where CTNNB1 mutations are drivers of the disease (Delgado et al. 2015). This was in part due to enhanced numbers of apoptotic nuclei as shown by staining for TUNEL (Fig. 6.5a). In another model of HCC, where sleeping beauty transposon and transposase were used to stably express mutant Kras and mutant β-catenin in a subset of hepatocytes, the use of CTNNB1 lipid nanoparticles (LNPs) led to a notable decrease in HCC burden as compared to the controls that received Scrambled LNP (Tao et al. 2016b). Again, the liver sections from the CTNNB1-LNP-treated Kras-β-catenin mice showed enhanced cell death as also detected by increased TUNEL staining (Fig. 6.5b). Thus, anti-β-catenin therapies may be of relevance in the treatment of a subset of HCC cases where unequivocal β-catenin activation is evident due to mutations in CTNNB1, in β-catenin degradation complex components such as AXIN1/AXIN2, or in other mechanisms (Fig. 6.6). The impact of β-catenin suppression on HCC burden is at least in large part predicted to be through the decrease in its direct transcriptional

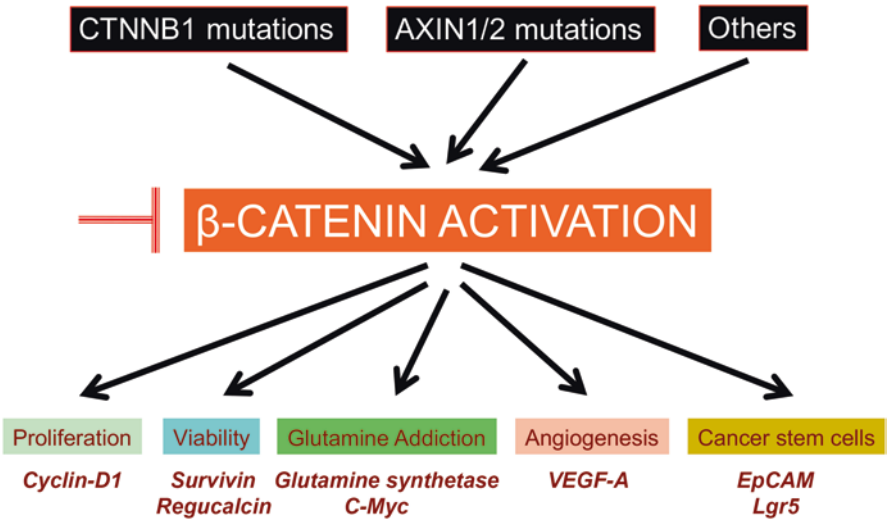


**Fig. 6.6** Exploiting β-catenin's pro-survival role in hepatic tumors for therapeutics. β-Catenin activation in tumors like hepatocellular cancers can occur due to mutations in CTNNB1, AXIN1/AXIN2, or other causes. Once activated, β-catenin activates several target genes through its cofactor function and hence can regulate several processes critical in tumor growth and development. Suppression of β-catenin in tumors could lead to tumor cell death due to effects on multiple mechanisms like cell viability, tumor metabolism, and angiogenesis

targets, which have been discussed and hence will impact multiple and critical aspects of tumor biology (Fig. 6.6). In fact, disruption of even one of the various biological functions due to β-catenin downregulation followed by a decrease in its target genes affects another biological function, and hence the effects are multiplied. For example, the effect on tumor metabolism affects tumor cell survival and proliferation, as well as angiogenesis.

Other novel therapies for treatment of acute liver injury may include activators of pro-proliferative pathways such as NF-κB. Paradoxically, in these situations, inhibition of β-catenin, rather than activation, may be necessary. As discussed above, the inhibitory β-catenin/p65 complex prevents NF-κB activation and results in cell death and morbidity after TNF-α treatment, while loss of β-catenin from hepatocytes promotes survival. In recent years, several small-molecule inhibitors of the Wnt/β-catenin pathway have been identified. These antagonists have been touted as the next generation of cancer therapeutics because of their antiproliferative and pro-apoptotic effects in HCC with aberrant Wnt pathway activation (Emami et al. 2004; Lepourcelet et al. 2004; Behari et al. 2007; Handeli and Simon 2008; Delgado et al. 2015; Tao et al. 2016b ).

Repurposing these inhibitors of β-catenin to prevent apoptosis in certain cases of liver injury or acute liver failure may thus be a relevant therapeutic strategy.

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# Chapter 7
# Nuclear DAMPs in Hepatic Injury and Inflammation

**Rui Kang and Daolin Tang**

## 7.1 Introduction

The liver is composed of hepatocytes (parenchymal cells) and non-parenchymal cells (e.g., Kupffer cells, sinusoidal endothelial cells, and stellate cells). As the largest gland and solid organ in the body, the liver plays a critical role not only in the metabolism of carbohydrates, proteins, and fat but also in the clearance of drugs and other harmful substances from the blood. Abnormal cell death, including regulated and nonregulated forms, is implicated in the pathologic presentation of almost all types of liver disease (Luedde et al. 2014). Nonregulated cell death is uncontrolled, whereas regulated cell death (RCD) is controlled by intracellular signals or proteins (Ashkenazi and Salvesen 2014). According to the current classification by the Nomenclature Committee on Cell Death, there are 11 forms of RCD (viz., anoikis, autophagic cell death, apoptosis, cornification, entosis, ferroptosis, mitotic catastrophe, necroptosis, NETosis, parthanatos, and pyroptosis) with different morphological, biochemical, and genetic features (Galluzzi et al. 2015).

In addition to the direct loss of normal cell numbers, abnormal RCD can drive inflammatory injury or immune suppression by releasing damage-associated molecular pattern molecules (DAMPs) (Green et al. 2009). Different from pathogen-associated molecular pattern molecules (PAMPs) generated from foreign pathogens, DAMPs are endogenous molecules released from dead or dying cells (Bianchi 2007; Tang et al. 2012b). The list of DAMPs is rapidly increasing and can be divided into nuclear (e.g., high-mobility group box 1 [HMGB1], histone, and nuclear DNA), mitochondrial (e.g., mitochondrial transcription factor A and mito-

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W.-X. Ding, X.-M. Yin (eds.), *Molecules, Systems and Signaling in Liver Injury*,
Cell Death in Biology and Diseases, DOI 10.1007/978-3-319-58106-4_7

X.-M. Yin and Z. Dong (Series eds.), *Cell Death in Biology and Diseases*

chondrial DNA), and cytosolic DAMPs (e.g., heat shock protein and S100) and others. PAMPs trigger infection, whereas DAMPs induce sterile inflammation. Both PAMPs and DAMPs can act as danger signals to regulate the inflammatory response by binding to pattern recognition receptors (PRRs), mainly in the immune system (Takeuchi and Akira 2010). These PRRs include toll-like receptors (TLRs), NOD-like receptors, RIG-I-like receptors, and the receptor for advanced glycation end products (RAGE). Meanwhile, the interplay between PAMPs and DAMPs may accelerate and amply the inflammatory response. Thus, danger signals and their receptors are emerging targets in inflammation-associated human diseases, including liver disease (Chen et al. 2013). In this chapter, we will focus on nuclear DAMPs (HMGB1 and histone) in RCD and their roles in liver injury and inflammation.

## 7.2 Regulated Cell Death in Liver Injury and Inflammation

### *7.2.1 Apoptosis*

#### 7.2.1.1 The Process of Apoptosis

The term "apoptosis" was first used by three pathologists, Kerr, Wyllie, and Currie, in 1972 to describe morphologically distinct ultrastructural changes of RCD (Kerr et al. 1972). As a naturally occurring type of physiological cell death, apoptosis is required for the elimination of unnecessary and unwanted cells to maintain tissue homeostasis. In contrast, abnormal or excessive apoptosis contributes to tissue injury under various pathological conditions. Two apoptotic processes, including the extrinsic and intrinsic pathways, are well documented at molecular levels (Elmore 2007). The extrinsic pathway is mostly mediated by death receptors (DRs), which belong to the tumor necrosis factor (TNF) receptor gene superfamily. After binding to ligands, DRs including the Fas receptor (FasR/CD95), TNF receptor 1, lymphotoxin receptor, DR3, and DR4/DR5, can activate caspase-8 and subsequently caspase-3 to induce apoptosis. Unlike the DR-mediated extrinsic apoptosis pathway, the intrinsic pathway is associated with dynamic mitochondrial changes, including mitochondrial membrane potential loss and subsequent mitochondrial apoptosis-inducing protein release (Wang and Youle 2009). In particular, release of cytochrome c (Liu et al. 1996), second mitochondria-derived activator of caspases (Du et al. 2000), and HtrA serine peptidase 2 (van Loo et al. 2002) from the mitochondria to the cytoplasm can activate caspase-9 and subsequently caspase-3 to induce apoptosis. Thus, caspases (a family of protease enzymes) play central roles in triggering apoptosis, although a caspase-independent apoptotic pathway exists (McIlwain et al. 2015). In addition to caspase, the pro- (e.g., Bax, Bak, Bid, and PUMA) and anti-(e.g., Bcl-2, Bcl-xL, and Mcl-1) apoptotic Bcl-2 family members control mitochondrial membrane potential in apoptosis (Shamas-Din et al. 2013). Notably, caspase 8-mediated cleavage of Bid promotes intrinsic

mitochondrial pathway activation, suggesting a direct molecular mechanism linking the extrinsic and intrinsic pathways in the induction of apoptosis (Gross et al. 1999; Li et al. 1998).

#### 7.2.1.2 Apoptosis in Hepatic Pathology

Apoptosis is observed in almost all types of liver injury caused by extrinsic (e.g., viruses, alcohol, and drugs) and intrinsic (e.g., toxic bile acids, free fatty acids, and ischemia/reperfusion [I/R]) factors (Malhi and Gores 2008; Wang 2014). Inflammation mediators from activation of lymphocytes, macrophages, and neutrophils in liver injury also trigger apoptosis in hepatocytes (Lawson et al. 1998). Natural killer cells, a type of cytotoxic lymphocyte, can release perforin/granzyme to induce apoptosis in hepatocytes and hepatic stellate cells (Radaeva et al. 2006; Takeda et al. 2001). Excessive apoptotic death of hepatocytes and non-parenchymal cells contributes to liver injury (Guicciardi and Gores 2005). Apoptotic cell death is generally recognized as a noninflammatory process due to an intact apoptotic body plasma membrane and the rapid clearance of apoptotic cells by phagocytes (e.g., macrophages and dendritic cells). However, engulfment of apoptotic bodies by Kupffer cells (the macrophages in the liver) and hepatic stellate cells has been demonstrated to enhance death ligand production and stimulate the fibrogenic activity (Canbay et al. 2003; Oberhammer et al. 1992; Takehara et al. 2004). These changes exacerbate hepatic fibrosis. In addition, nuclear DAMPs such as histone, nuclear DNA, and HMGB1 can be released by apoptotic cells, which mediates inflammation, the immune response, and cell proliferation (Bell et al. 2006; Chen et al. 2014).

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver and usually develops from hepatitis, hepatic fibrosis, and cirrhosis. The regulation of apoptosis plays crucial roles not only in the chemotherapeutic treatment of HCC, but also in tumorigenesis of HCC. One hallmark of cancers, including HCC, is intrinsic or acquired resistance to apoptosis (Fabregat 2009). Pharmacologic induction of apoptosis inhibits HCC tumor growth and advancement. Surprisingly, recent studies of $CD95^{-/-}$ and $PUMA^{-/-}$ mice indicate that apoptosis may promote tumor formation at the early stage by promoting stem cell proliferation or activation of the JNK pathway in the tumor microenvironment (Chen et al. 2010; Labi et al. 2010; Michalak et al. 2010; Ranger et al. 2003). Similarly, hepatocyte-specific BID-deficient mice are resistant to diethylnitrosamine (DEN)-induced hepatocarcinogenesis by suppressing inflammation-related compensatory proliferation (Wree et al. 2015). Hepatocyte-specific Bcl-xL or Mcl-1 knockout mice display increased hepatocyte apoptosis and spontaneous HCC formation (Hikita et al. 2012; Weber et al. 2010). One possible underlying mechanism of apoptosis-mediated tumorigenesis is that oxidative injury from apoptotic hepatocytes may promote gene mutation in liver tumorigenesis (Hikita et al. 2015). Collectively, dysregulated apoptosis plays a dual role in HCC tumorigenesis, depending on the tumor stage (Tang et al. 2011c).

## *7.2.2 Necroptosis*

### 7.2.2.1 The Process of Necroptosis

Necrosis, also termed "oncosis," has been long considered a passive and unregulated form of cell death characterized by nuclear/plasma membrane leakage and adenosine triphosphate (ATP) depletion (Zong and Thompson 2006). Recent studies have highlighted that some cases of necrosis are also regulated and programmed (Galluzzi et al. 2012; Silke et al. 2015; Vanden et al. 2014). This form of necrosis is termed necroptosis and can be traced back to the finding in 1965 that TNF (also called cytotoxin) can induce cell death (Kolb and Granger 1968). We now know that TNF and other DR ligands (e.g., TNF-related apoptosis-inducing ligand) trigger necroptosis when the expression or activity of caspase-8 is inhibited by genetic (e.g., knockout of caspase-8 or Fas-associated protein with death domain) and pharmacological (e.g., ZVAD-FMK) methods (Kaiser et al. 2011; Welz et al. 2011). Additionally, interferons (IFNs), viral nucleic acid, and murine cytomegalovirus can induce necroptosis by IFN-receptor, TLR3, and Z-DNA-binding protein 1, respectively (Pasparakis and Vandenabeele 2015). These stimuli can finally activate receptor-interacting serine-threonine kinase (RIPK)-3 and subsequently target mixed lineage kinase domain-like (MLKL) by protein phosphorylation in the necrosome (He et al. 2009; Murphy et al. 2013; Newton et al. 2014; Sun et al. 2012). Finally, MLKL translocates to the plasma and cytoplasmic membranes, where it triggers necroptosis by modulating ion channel activities and $Ca^{2+}$ influx (Cai et al. 2014). Of note, TNF-mediated RIPK1 phosphorylation is required for RIPK3 activation in necroptosis. In contrast, TLR adaptor molecule 1 (but not RIPK1) is required for viral nucleic acid-mediated RIPK3 activation in necroptosis. The underlying mechanism of IFN-mediated RIPK3 activation remains elusive.

### 7.2.2.2 Necroptosis in Hepatic Pathology

A number of DAMPs, such as HMGB1, S100A9, IL-33, and mitochondrial DNA, are released under necroptotic conditions and contribute to the subsequent inflammatory response in vitro and in vivo (Duprez et al. 2011; Kovalenko et al. 2009; Lau et al. 2013). Genetic and pharmacologic inhibition of necroptosis by using knockout mice (e.g., RIPK1−/−, RIPK3−/−, and MLKL−/−), necrostatin-1 (a RIPK1 inhibitor), GSK843/872 (a RIPK3 inhibitor), and necrosulfonamide (a MLKL inhibitor) have been demonstrated to provide protection against tissue injury in multiple inflammatory-associated diseases (Linkermann and Green 2014; Zhou and Yuan 2014). Of note, ethanol and acetaminophen increase RIPK3 expression in mouse and human livers. RIPK3−/− and RIPK1−/− mice are more resistant to ethanol-, acetaminophen-, high-fat choline-, or methionine and choline-deficient diet-induced liver injury with decreased proinflammatory cytokine expression (Afonso et al. 2015; Deutsch et al. 2015; Ramachandran et al. 2013; Roychowdhury et al. 2013). Necrostatin-1 not only reduces ethanol- and acetaminophen-induced

liver injury but also limits concanavalin A-induced hepatitis in mouse models. However, RIPK1- and RIPK3-deficient mice display a different phenotype in concanavalin A-induced experimental hepatitis. One study showed that loss of RIPK3 limits, whereas loss of RIPK1 exacerbates concanavalin A-induced hepatitis and animal death (Deutsch et al. 2015). Other studies have shown that RIPK3−/− mice are not protected against concanavalin A-induced experimental hepatitis (Weinlich et al. 2013). The reason for these inconsistencies is still unresolved. Liver Kupffer cells can release DAMPs and cytokines in necroptosis to induce type 1 microbicidal inflammation and type-2-mediated liver repair upon infection (Bleriot et al. 2015). Thus, necroptosis pathway impairment contributes to alcohol-related liver disease, drug-induced liver injury, and hepatitis (Bakhautdin et al. 2014; Csak et al. 2011; Deutsch et al. 2015; Li et al. 2014a; Liedtke et al. 2011; Roychowdhury et al. 2013; Wang et al. 2016). Induction of necroptosis can improve anticancer therapies in apoptosis-resistant cancer cells (Chen et al. 2016). A recent study indicated that necroptosis-mediated CXCL1 release promotes pancreatic cancer tumorigenesis through inhibition of antitumor immunity (Seifert et al. 2016). However, the role of necroptosis in HCC tumorigenesis remains unclear.

### *7.2.3 Autophagic Cell Death*

#### 7.2.3.1 Autophagy and Autophagic Cell Death

Autophagy is a highly-conserved and lysosome-dependent degradation pathway in various species (Mizushima and Levine 2010). Currently, it includes three major forms, namely, macroautophagy, microautophagy, and chaperone-mediated autophagy. Among them, macroautophagy (hereafter referred to as autophagy) is well studied and divided into selective (e.g., mitophagy and xenophagy) and nonselective autophagy. Transmission electron microscopy assay has revealed three membrane-associated structures (phagophore, autophagosome, and autolysosome) in the dynamic process of autophagy (Klionsky and Emr 2000). The phagophore is the chief organelle that is vital for initiation of the autophagic cascade. After engulfing the cytosolic materials, the phagophore grows into a sealed autophagosome with the expression of autophagy-related marker microtubule-associated protein light chain 3-II (Mizushima et al. 2010). Finally, autolysosomes are generated after autophagosomes fuse into lysosomes, which facilitates the degradation of the engulfed material. At molecular levels, autophagy-related (ATG) family members can form different protein complexes involved in the regulation of formation and maturation of membrane-associated autophagic structures (Xie et al. 2015). These complexes are also important for the interplay between autophagy and other forms of RCD (Kang et al. 2011; Marino et al. 2014; Wong et al. 2013). In many cases, induction of autophagy promotes cell survival in response to harmful environments (Kroemer et al. 2010). In contrast, excessive autophagy may lead to cell death and tissue injury in some cases (Liu and Levine 2015). In particular, autosis has been

recently identified as a specific form of autophagic cell death triggered by activation of $Na^+/K^+$-ATPase (Liu et al. 2013). The molecular switches between autophagy-mediated survival and death remain largely unidentified (Das et al. 2012).

#### 7.2.3.2 Autophagic Cell Death in Hepatic Pathology

Autophagic dysfunction has been demonstrated to be involved in a number of liver pathologies. Induction of autophagy can maintain the energetic balance of the liver, alleviate endoplasmic reticulum stress, and remove unused or damaged hepatic proteins and organelles (Czaja et al. 2013; Madrigal-Matute and Cuervo 2016; Martinez-Lopez and Singh 2015; Schneider and Cuervo 2014; Yin et al. 2008). Thus, genetic inhibition of autophagy by using liver conditional knockout mice (e.g., ATG5−/− and ATG7−/−) has demonstrated that autophagy deficiency accelerates tissue injury and the inflammatory response in ethanol and acetaminophen-induced liver injury and high-fat diet-induced lipid accumulation, as well as I/R injury (Ding et al. 2010; Ezaki et al. 2011; Hernandez-Gea et al. 2012; Kim et al. 2013; Liu et al. 2015; Ni et al. 2012, 2016, 2014; Seok et al. 2014; Taguchi et al. 2012; Toshima et al. 2014; Xiao et al. 2016; Yang et al. 2010b). Pharmacological induction of autophagy by rapamycin significantly attenuates acetaminophen- and alcohol-induced liver injury (Lin et al. 2013). In contrast to rapamycin, autophagy inhibitors such as chloroquine can cause liver injury (Lin et al. 2013). Of note, the activity of chloroquine may work in an autophagy-independent manner (Maes et al. 2014). Autophagy plays a dual role in cancer biology, depending on tumor stage and type (White 2015). Autophagy inhibits tumorigenesis in HCC, a finding based on studies of autophagy-deficient mice. For example, mice with heterozygous disruption of ATG5 and ATG7 are shown to develop spontaneous liver tumor partly by activation of the nuclear factor (erythroid-derived 2)-like 2 (NRF2) pathway (Ichimura et al. 2013; Inami et al. 2011; Komatsu et al. 2010; Ni et al. 2014; Riley et al. 2010; Taguchi et al. 2012; Takamura et al. 2011). In contrast, autophagy may exert a pro-carcinogenic effect in HCC by promoting cell death or by inducing expression of factors that stimulate cell invasion and metastasis (Di Fazio et al. 2016; Li et al. 2013). Although autophagy has been well studied as a cell survival mechanism providing protection from liver injury, the function of autophagic cell death in liver disease still needs to be better understood.

### *7.2.4 Pyroptosis*

#### 7.2.4.1 The Process of Pyroptosis

The term “pyroptosis” was originally coined to indicate proinflammatory (“pyro”) programmed cell death (“ptosis”) during bacterial infection in macrophages (Brennan and Cookson 2000). It now refers to a type of RCD in immune cells mediated by the activation of inflammasome. The canonical inflammasomes include NLR (e.g., NLRP1, NLRP3, and NLRC4) and non-NLR (e.g., AIM2) types, which

are mediated by activation of caspase-1 in response to PAMPs (e.g., lipopolysaccharide [LPS] and bacterial flagellin) and DAMPs (e.g., ATP, uric acid, DNA, and RNA) (Bergsbaken et al. 2009; Martinon et al. 2009; Rathinam and Fitzgerald 2016; Schroder and Tschopp 2010). In contrast, noncanonical inflammasome is triggered following the binding of intracellular LPS to caspase-11 (in mice) or caspase-4 or caspase-5 (in humans) (Hagar et al. 2013; Kayagaki et al. 2013; Man and Kanneganti 2016; Shi et al. 2014). Gram-negative bacteria secretion of outer membrane vesicles is responsible for the delivery of LPS to the host cell cytosol to bind caspase-11, which will cause cleavage of gasdermin-D (Vanaja et al. 2016). The N-terminal of gasdermin-D can trigger subsequent cell death as well as canonical NLRP3-inflammasome activation (He et al. 2015; Kayagaki et al. 2015; Shi et al. 2015). Thus, cleavage of gasdermin-D links the interplay between noncanonical and canonical inflammasomes (Wallach et al. 2016). Inflammasome activation is usually associated with secretion of proinflammatory cytokines (e.g., IL-1β, IL-1α, IL-18, IL-33, and HMGB1) and thereby triggers injury.

#### 7.2.4.2 Pyroptosis in Hepatic Pathology

Increased caspase-1-dependent pyroptosis has been observed in both Kupffer cells and hepatocytes during the development of nonalcoholic steatohepatitis, alcoholic steatohepatitis, and I/R (Kamo et al. 2013; Petrasek et al. 2012). IL-1β release from inflammasome activation not only directly induces hepatic steatosis and injury but also enhances LPS-mediated TNF-α production, which further exacerbates liver injury (Qiao et al. 2015). Global knockout caspase-1 ameliorates, whereas global knockin NLRP3 increases inflammasome-dependent liver injury in mice (Wree et al. 2014). In contrast, myeloid cell-derived NLRP3 activation resulted in a less severe liver phenotype in the absence of detectable pyroptotic liver cell death (Wree et al. 2014). Thus, NLRP3 inflammasome plays an important role in triggering inflammatory injury and fibrosis in both hepatocytes and non-parenchymal cells (Ganz et al. 2011; Imamura et al. 2009; Zhu et al. 2011). Moreover, heat stroke-induced liver injury requires HMGB1-mediated NLRP3 inflammasome activation (Geng et al. 2015). Caspase-11 also contributes to hepatic inflammation by activation of Kupffer cells (Hendrikx et al. 2013). Autophagy deficiency triggers inflammasome activation in macrophages, which promotes liver injury (Ilyas et al. 2016). Thus, inhibition of pyroptosis with drugs is an alternative approach to the treatment of inflammation in liver disease (Heymann and Tacke 2016; Hoque et al. 2014; Szabo and Petrasek 2015; Yang et al. 2016).

### *7.2.5 NETosis*

#### 7.2.5.1 The Process of NETosis

NETosis is a unique form of RCD that is characterized by the release of decondensed chromatin (e.g., DNA, HMGB1, and histone) and granular contents to the extracellular space (Steinberg and Grinstein 2007; Yipp et al. 2012). This process

also termed neutrophil extracellular trapping, which was first observed in neutrophil activation in infection (Brinkmann et al. 2004). In addition to neutrophils, NETosis can be induced in endothelial and cancer cells under pathologic conditions such as hypoxia (Demers and Wagner 2014; Remijsen et al. 2011). NADPH oxidase-mediated reactive oxygen species (ROS) production is an early event in the induction of NETosis. Peptidylarginine deiminase 4 (PAD4) is an enzyme responsible for protein citrullination. PAD4-mediated histone citrullination is a late event in chromatin decondensation and release in NETosis (Branzk and Papayannopoulos 2013). Thus, genetic or pharmacologic inhibition of PAD4 could inhibit NETosis (Branzk and Papayannopoulos 2013; Hahn et al. 2013). However, PAD4-independent NETosis may exist and needs further identification (Metzler et al. 2014).

#### 7.2.5.2 NETosis in Hepatic Pathology

NETosis-associated nuclear DAMP release and tissue damage is implicated in the pathologies of inflammatory and autoimmune disorders such as systemic lupus erythematosus, rheumatoid arthritis, asthma, vessel vasculitis, and psoriasis (Branzk and Papayannopoulos 2013). More recently, formation of NETosis has been reported in liver I/R injury (Huang et al. 2015). Increased NETosis in hepatocytes induces Kupffer cell activation and subsequent proinflammatory cytokine release, which contributes to sterile inflammation. In contrast, blocking NETosis by neutrophil depletion or using PAD4 inhibitor reduces liver I/R injury in a DAMP-dependent manner (Huang et al. 2015). In addition to I/R injury, NETosis-mediated DAMP release also contributes to adhesion, proliferation, migration, and invasion of HCC cells by activation of TLR9 (Tohme et al. 2016). Further studies are needed to elucidate the potential role of NETosis in other types of liver injury such as viral infection.

### *7.2.6 Ferroptosis*

#### 7.2.6.1 Process of Ferroptosis

Ferroptosis is a new form of RCD and was first reported in cancer cells (Dixon et al. 2012). Morphologic, biochemical, and genetic studies highlight the unique aspects of ferroptosis compared to other forms of RCD (e.g., apoptosis, necroptosis, and autophagic cell death). The classical inducer of ferroptosis is erastin, which displayed anticancer activity in an oncogenic Ras-dependent manner in an original study (Dolma et al. 2003). In contrast, other recent studies indicate that erastin triggers ferroptosis in both a RAS-dependent and RAS-independent manner (Nils Eling et al. 2015; Schott et al. 2015; Yagoda et al. 2007; Yu et al. 2015b). In addition to erastin, several clinical drugs (e.g., sulfasalazine, sorafenib, and artesunate) have been demonstrated to trigger ferroptosis not only in cancer cells but also in certain

normal cells (e.g., kidney cells, neurons, fibroblasts, and T cells) (Xie et al. 2016; Yang and Stockwell 2015). Oxidative injury from iron accumulation and lipid peroxidation is responsible for ferroptosis induction. Several proteins are involved in the regulation of ferroptosis by targeting two iron metabolism and oxidative stressors, although the molecular mechanism and death-effector remain largely unknown. For example, glutathione peroxidase 4 (Yang et al. 2014), NRF2 (Sun et al. 2016b), metallothionein-1G (MT-1G) (Sun et al. 2016a), and heat shock protein beta-1 (Sun et al. 2015) function as negative regulators of ferroptosis by limiting lipid ROS production or reducing cellular iron uptake. By contrast, NADPH oxidase and p53 (Jiang et al. 2015) act as positive regulators of ferroptosis by promoting lipid ROS production and inhibiting expression of SLC7A11 (a specific light chain subunit of the cystine/glutamate antiporter), respectively. In addition, activation of MAPK (Yagoda et al. 2007) and PKCα (Do Van et al. 2016) also contributes to ferroptosis in different cells. Together, these findings reveal a complex molecular and signaling mechanism in ferroptosis.

#### 7.2.6.2 Ferroptosis in Hepatic Pathology

Ferroptosis has emerged as a new regulatory mechanism for liver injury. For example, acetaminophen has been demonstrated to induce ferroptosis in primary liver cells, and ferroptosis inhibitors such as ferrostain-1 can inhibit acetaminophen-induced liver injury (Lorincz et al. 2015). Ferroptosis has been shown to promote ischemic liver damage, and ferroptosis inhibitor liproxstatin-1 can protect against liver I/R in mice (Friedmann Angeli et al. 2014). Sorafenib, a multiple kinase inhibitor, is now the only approved systemic therapy for advanced HCC. Recent studies indicate that ferroptosis mediates the anticancer activity of sorafenib in HCC cells. Sorafenib-mediated cell death in human (HepG2, Hep3B, and SNU-182) and mouse (Hepa1–6) HCC lines is blocked by ferrostatin-1 (a ferroptosis inhibitor), but not ZVAD-FMK (an apoptosis inhibitor) and necrosulfonamide (a necroptosis inhibitor) (Sun et al. 2016b). Activation of the NRF2 pathway protects against ferroptosis in human and mouse HCC cells (Sun et al. 2016b). Upon exposure to ferroptosis-inducing compounds (e.g., erastin, sorafenib, and buthionine sulfoximine), p62/SQSTM1 (an adaptor protein) expression prevents NRF2 degradation and enhances subsequent NRF2 nuclear accumulation through inactivation of Kelch-like ECH-associated protein 1 (Keap1) (Sun et al. 2016b). Genetic or pharmacologic inhibition of NRF2 expression/activity in HCC cells increases the anticancer activity of erastin and sorafenib in HCC cells in vitro and in tumor xenograft models (Sun et al. 2016b). Moreover, Sorafenib specifically induces MT-1G expression by activation of NRF2 in human HCC cells (Sun et al. 2016a). Suppression of MT-1G expression enhances sorafenib sensitivity by induction of ferroptosis (Sun et al. 2016a). These findings not only identify a novel mechanism of sorafenib resistance but also suggest a new link between the NRF2-MT-1G pathway and ferroptosis in human HCC cells.

## 7.3 Nuclear DAMPs in Hepatic Injury and Inflammation

### *7.3.1 HMGB1*

#### 7.3.1.1 HMGB1 in Cell Death

HMGB1 is the most abundant nonhistone nuclear protein and belongs to the HMG family. In the nucleus, HMGB1 can bind and bend DNA to regulate multiple chromatin-associated events such as gene transcription and DNA repair. As a classical DAMP with cytokine and chemokine functions, HMGB1 is actively secreted by immune cells (e.g., macrophages and neutrophils) or passively released in various types of cell death (e.g., necrosis, apoptosis, necroptosis, autophagic cell death, pyroptosis, NETosis, and ferroptosis) (Kang et al. 2014a). Oxidative stress plays a central role in the regulation of HMGB1 secretion and release (Yu et al. 2015a). Once released, the activity of HMGB1 is regulated at multiple levels such as receptor, partner, and redox status. In general, RAGE (Kokkola et al. 2005), TLR2 (Park et al. 2004), TLR4 (Park et al. 2004), and TLR9 (Tian et al. 2007) are receptors required for the HMGB1-mediated immune response. In contrast, CD24 (Chen et al. 2009) and TIM-3 (Chiba et al. 2012) are negative binding receptors that limit HMGB1 activity in the immune response. Extracellular HMGB1 is a sticky protein and can bind DAMPs (e.g., histone, DNA, and IL-1) and PAMPs (e.g., LPS) to amply the inflammatory response. The oxidative state of released HMGB1 can fine-tune HMGB1 activity (Tang et al. 2012a, b; Venereau et al. 2012; Yang et al. 2010a). In general, deduced all-thiol-HMGB1 displays chemokine activity, whereas disulfide-HMGB1 exhibits cytokine activity. In contrast, oxidized HMGB1 loss displays both chemokine and cytokine activity (Venereau et al. 2012).

Intracellular HMGB1 is generally an antiapoptotic protein in response to ultraviolet radiation, TNF, and TRAIL in vitro (Brezniceanu et al. 2003). Intracellular HMGB1 inhibits apoptosis in both transcriptional-dependent (e.g., regulation of Bcl-2 family protein expression) and transcriptional-independent (e.g., regulation of autophagy and p53 location) manners. In vivo, loss of HMGB1 in the pancreas increases L-arginine-induced apoptosis and necrosis due to oxidative injury (Kang et al. 2014b). Compared with its antiapoptotic role, HMGB1 is a pro-autophagy protein in many cells. HMGB1 promotes autophagy also in both transcriptional-dependent and transcriptional-independent manners. For example, HMGB1 can transcriptionally regulate heat shock protein β-1 (HSPB1) expression, which affects autophagosome and autolysosome formation in response to mitochondrial injury (Tang et al. 2011a). Cytosolic HMGB1 regulates autophagosome formation by direct binding to Beclin-1, a key regulator of autophagy (Tang et al. 2010b). The binding of HMGB1 with Beclin-1 is positively regulated by unc-51-like kinase 1 and nucleus accumbens-1 (Zhang et al. 2012), whereas it is negatively regulated by p53 (Livesey et al. 2012), SNCA/α-synuclein (Song et al. 2014), and lysosomal thiol reductase (Chiang and Maric 2011). Moreover, inhibition of HMGB1 expression by miR34A (Liu et al. 2014a) or miR22 (Li et al. 2014b) limits autophagy in

chemotherapy. In contrast, increased HMGB1 translocation from the nucleus to the cytosol by activation of poly [ADP-ribose] polymerase 1 (PARP1) promotes TRAIL resistance by induction of autophagy (Yang et al. 2015). Compared with intracellular HMGB1, extracellular HMGB1-mediated autophagy requires RAGE-mediated PI3KC3 activation (Tang et al. 2010a). In addition to HMGB1-dependent autophagy regulating cell death and inflammation in vitro and in vivo, HMGB1-independent autophagy may exist in the liver and heart (Huebener et al. 2014; Sun and Tang 2014). Induction of proptosis by PAMPs increases HMGB1 release in macrophages (Lu et al. 2012). Released HMGB1 in turn accelerates inflammasome activation and the release of IL-1β and IL-18, again implicating the involvement of DAMPs and PAMPs in systematic inflammation.

#### 7.3.1.2 Role of HMGB1 in Hepatic Pathology

Extracellular HMGB1 plays a significant pathogenic role in liver injury, and serum HMGB1 is a useful biomarker of liver disease. The involvement of extracellular HMGB1 in liver injury was first described in a liver warm I/R mouse model (Tsung et al. 2005; Watanabe et al. 2005). Activation of ROS and the calcium/calmodulin-dependent protein kinase signaling pathway is required for HMGB1 release by hepatocytes in I/R. TLR4 contributes to HMGB1-mediated proinflammatory cytokine generation and organ damage in warm hepatic I/R injury (Tsung et al. 2005). Blocking HMGB1 release and activity protects against I/R injury. In addition to TLR4, TLR9 (Bamboat et al. 2010) and RAGE (Zeng et al. 2009) also mediate liver I/R injury, suggesting that HMGB1 may interact with multiple receptors to mediate warm liver I/R injury. HMGB1 is also released by hepatocytes in hepatitis C virus or hepatitis B virus (HBV) infection, which mediates the antiviral response in a TLR4-dependent pathway (Jung et al. 2011; Zhou et al. 2011). In addition to viral hepatitis, HMGB1 release has been significantly observed in nonalcoholic fatty liver disease, hepatic fibrosis, and alcohol- and drug-induced liver injury. Once released, HMGB1 promotes the inflammation and proliferation response via TLR4-MyD88 or the MAPK-NF-κB or inflammasome pathways (Geng et al. 2015). Similarity, hypoxia-induced HMGB1 release in HCC cell lines can activate TLR4- and RAGE-signaling pathways to induce inflammasome-dependent secretion of IL-1β and IL-18. These changes in the tumor microenvironment promote HCC invasion and metastasis (Yan et al. 2012). Serum HMGB1 levels were significantly higher in HCC and HBV patients and are associated with poor outcomes in HCC patients (Cheng et al. 2008; Jiang et al. 2012).

The role of intracellular HMGB1 in the liver was recently investigated using hepatocyte-specific HMGB1 conditional knockout mice (Sun and Tang 2015). HMGB1 depletion does not affect liver development and function under physiological conditions. However, different groups observed a dual role of HMGB1 in the liver under pathological conditions. Conditional knockout of HMGB1 in the liver increases nuclear and mitochondrial injury and nuclear DAMP release in response to I/R injury (Huang et al. 2014). In contrast, mice with hepatocyte-specific HMGB1

depletion were more resistant to acetaminophen- and alcohol-induced liver injury by inhibition of inflammation, fatty acid synthesis, and fatty acid β-oxidation(Ge et al. 2014; Huebener et al. 2015). Understanding the tissue- and cell-specific roles of HMGB1 in stress is important for HMGB1-based target therapy (Chen et al. 2013; Tang et al. 2014).

## *7.3.2 Histone*

### 7.3.2.1 Role of Histone in Cell Death

Histones are highly alkaline proteins found in eukaryotic cells (Bhasin et al. 2006; Luger et al. 1997). Histones are the fundamental unit of chromatin, namely, the nucleosome. In eukaryotes, the nucleosome is composed of 147 bp DNA wrapped around eight core histone proteins, including two each of H2A, H2B, H3, and H4. The linker histones, H1 and its variant forms, have been implicated in the formation of higher orders of chromatin structure. Posttranslational modifications (PTMs) of histones at the amino-terminal tails, including acetylation, phosphorylation, methylation, and ubiquitination, are changed in response to various stimuli. These changes led to the generation of the histone code hypothesis, which plays a critical role in epigenetic regulation of gene expression in several cellular processes, including various types of cell death (Strahl and Allis 2000). Histone phosphorylation and acetylation have been suggested to affect chromatin function and structure during apoptosis (Hajji and Joseph 2010). For example, histone H2B phosphorylation at Ser14 has been proposed as an epigenetic marker of apoptotic cells associated with DNA degradation (Ajiro et al. 2010). Moreover, phosphorylation of the histone H2AX at Ser139 (termed γ-H2AX) is a hallmark of the DNA-damage response at the early stage of apoptosis (Sharma et al. 2012). Compared with these apoptotic histone codes (Huang et al. 2013; Sanders et al. 2013), recent studies have also described histone codes in autophagy (e.g., H3K56ac, H4K16ac, H3K4me3, H4K20me3, and H3K9me2) (Fullgrabe et al. 2014). The anticancer activity of the histone deacetylase (HDAC) inhibitor depends on induction of forms of RCD such as apoptosis, necroptosis, and autophagic cell death (Xu et al. 2007). HDAC-mediated epigenetic reprogramming also regulates pyroptosis in macrophages (Ha et al. 2014). How these histone PTMs contribute to the interplay between different kinds of RCD remains to be elucidated in detail.

Histones, a new type of nuclear DAMP, can be released by dead and dying cells in apoptosis, necrosis, and NETosis (Chen et al. 2014). In apoptosis, core histones (H2A, H2B, H3, and H4) and link histone (H1) separate from genomic DNA, translocate to the cytosol, and finally release into the extracellular space (Gabler et al. 2004; Gilthorpe et al. 2013; Wu et al. 2002). Both histone release and DNA fragmentation formation are important nuclear events in apoptosis. This process is primarily mediated by CAD (caspase-activated DNase)/DFF-40(DNA fragmentation factor) (Wu et al. 2002). Compared with histone release in apoptotic cells, histone

secretion by activated immune cells (e.g., basophils, mast cells, and neutrophils) is mediated by NETosis in response to pathogen infection (Brinkmann et al. 2004). In addition to histones, other nuclear DAMPs, including HMGB1 and DNA, are important components released in NETosis (Brinkmann et al. 2004). These nuclear DAMPs can capture and degrade invading pathogens and play an important role in antibacterial responses. In contrast, extracellular histones have been implicated as inflammatory mediators in lethal infections (e.g., sepsis (Xu et al. 2009)) and sterile inflammation (e.g., I/R injury (Allam et al. 2012; Bosmann et al. 2013; De Meyer et al. 2012; Huang et al. 2011; Wen et al. 2013; Xu et al. 2011) and pancreatitis (Kang et al. 2013)). Extracellular histones also induce disseminated intravascular coagulation by promoting coagulation and thrombosis (Semeraro et al. 2011). TLRs and RAGE are major receptors required for histone activity in tissue injury and inflammation (Kang et al. 2016; Xu et al. 2011). The nuclear DAMP complex can trigger apoptosis, autophagy, and pyroptosis in immune and nonimmune cells, which in turn regulates proinflammatory cytokine release in cell death (Liu et al. 2014b).

#### 7.3.2.2 Role of Histone in Hepatic Pathology

Several studies have shown that extracellular histone plays a significantly pathologic role in liver injury, including concanavalin A-induced hepatitis, acetaminophen-induced hepatotoxicity, liver I/R, and acute liver failure. Elevated serum histones have been observed in animal models with I/R and chemical-induced liver injury (Allam et al. 2012; Huang et al. 2011; Wen et al. 2013; Xu et al. 2011). Exogenous histones can induce proinflammatory cytokine (TNF-α and IL-6) release by binding to TLR2, TLR4, and TLR9 to activate NF-κB and the mitogen-activated protein kinase [MAPK]) pathway in the liver. Blocking histone-TLR signaling by using histone-neutralizing antibody or knockout of TLR2, TLR4, and TLR9 in mice limits liver injury. In addition to directly activating TLR9, extracellular histones also enhance nucleic acid–mediated inflammation through TLR9 (Xu et al. 2011). Downstream of TLRs, myeloid differentiation factor 88 (MyD88) also contributes to histone-mediated liver I/R injury and sterile inflammation (Huang et al. 2011). The extracellular histone-TLR9 pathway promotes ROS production, which is required for NLRP3 inflammasome activation in Kupffer cells during sterile inflammatory liver injury. In contrast, RAGE, but not TLRs, is required for histone/DNA-mediated AIM2 inflammasome activation in macrophages during sterile inflammatory pancreas injury (Liu et al. 2014b). These findings provide different receptor-dependent mechanisms of inflammasome activation by histones in the sterile inflammatory response.

In patients with multiple tumors, especially in advanced cancer, levels of serum nucleosome, including histone and DNA, are significantly higher compared with healthy populations (Holdenrieder et al. 2001; Trejo-Becerril et al. 2003). Recent evidence has shown that release of nuclear DAMPs, including HMGB1, DNA, and histone, promotes tumor metastasis to the liver after surgical stress in NETosis

(Tohme et al. 2016). In contrast, genetic or pharmacologic inhibition of PAD4 or use of DNAse limits nuclear DAMP-mediated tumor metastasis (Tohme et al. 2016). Thus, nuclear DAMPs from abnormal cell death are important contributors to tumor metastasis.

## 7.4 Concluding Remarks

The liver performs many essential functions related to digestion, metabolism, and immunity. Liver injuries not only cause various types of cell death but also stimulate sterile inflammation. DAMPs such as HMGB1 and histone are critical molecular mediators linking cell death and the sterile inflammatory response in liver injury. Blocking the interaction between DAMPs and their receptors (e.g., TLRs and RAGE) can protect against liver injury from drugs, alcohol, viruses, trauma, and cancer in animal models. Anti-DAMP-based therapeutic strategies may be useful in patients with liver disease. However, many challenges still remain with respect to understanding the structure, modification, and function of DAMPs in liver injury and inflammation. Emerging data also points to the importance of interplay among different types of RCD in the regulation of DAMP release and the inflammatory response. Future studies are required to fully clarify and distinguish between these mechanisms.

**Acknowledgments** We apologize to the researchers who were not referenced due to space limitations. We thank Christine Heiner (Department of Surgery, University of Pittsburgh) for her critical reading of the manuscript. This work was supported by the National Institutes of Health of the USA (R01GM115366 and R01CA160417 to D.T.), the National Natural Science Foundation of Guangdong (2016A030308 D.T.), and a Research Scholar Grant from the American Cancer Society (RSG-16-014-01-CDD to D.T.).

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# Chapter 8
# The Critical Role of Mitochondria in Drug-Induced Liver Injury

**Carl W. Decker, Joseph G. Casian, Kim Tho Nguyen, Luke A. Horton, Madhuri P. Rao, Kai H. Silkwood, and Derick Han**

## Abbreviations

| | |
|---|---|
| ALT | Alanine aminotransferase |
| AMPK | AMP-activated protein kinase |
| APAP | Acetaminophen |
| ARE | Antioxidant response element |
| ASK1 | Apoptosis signal-regulating kinase 1 |
| Bcl-2 | B-cell lymphoma-2 |
| Cu3 | Cullin-dependent E3 ubiquitin ligase complex |
| CYP450 | Cytochrome P450 |
| DILI | Drug-induced liver injury |
| Drp1 | Dynamin-related protein 1 |
| GCL | Glutamate cysteine ligase |
| GSH | Glutathione |
| GSK-3β | Glycogen synthase kinase-3β |
| GWAS | Genome-wide association studies |

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W.-X. Ding, X.-M. Yin (eds.), *Molecules, Systems and Signaling in Liver Injury*, Cell Death in Biology and Diseases, DOI 10.1007/978-3-319-58106-4_8

X.-M. Yin and Z. Dong (Series eds.), *Cell Death in Biology and Diseases*

| | |
|---|---|
| HLA | Human leukocyte antigen |
| JNK | c-Jun *N*-terminal kinase |
| Keap1 | Kelch-like ECH-associated protein 1 |
| LPS | Lipopolysaccharide |
| Mcl-1 | Induced myeloid leukemia cell differentiation protein |
| Mfn | Mitofusin |
| MLK3 | Mixed-lineage kinase-3 |
| MOMP | Mitochondrial outer membrane permeabilization |
| MPT | Mitochondrial permeability transition |
| NAPQI | *N*-Acetyl-*p*-benzo-quinoneimine |
| Nrf-2 | Nuclear factor (erythroid-derived 2)-like-2 |
| Opa1 | Optic atrophy 1 |
| PGC-1α | Proliferator-activated receptor gamma coactivator-1α |
| PKC | Protein kinase C |
| RIP1 | Receptor-interacting serine/threonine-protein kinase 1 |
| ROS | Reactive oxygen species |
| Sab | SH3 homology associated BTK-binding protein |
| SOD | Superoxide dismutase |
| TNF | Tumor necrosis factor-α |

## 8.1 Introduction

The liver is the major organ responsible for drug detoxification and consequently the major site of drug injury (Kaplowitz 2005; Han et al. 2006a). Drug-induced liver injury (DILI) is the leading cause of black box warnings, drug withdrawals, and clinical trial failures (Kaplowitz 2005; Thames 2004; Kaplowitz 2002). DILI describes a condition where the medical intake of a drug(s) causes abnormal liver tests (i.e., increased serum ALT levels), which may lead to catastrophic consequences, including acute liver failure (Ulrich 2007). While drugs that cause liver injury are not structurally or chemically related, most hepatotoxic drugs have been shown to "stress" mitochondria (Boelsterli and Lim 2007; Han et al. 2010, 2013). In most cases, mitochondrial stress caused by hepatotoxic drugs is not sufficient to cause liver injury. However, mitochondrial stress may alter signaling pathways including adaptation/survival pathways that sensitize hepatocytes to extrinsic factors, such as inflammation and the adaptive immune system, to trigger liver injury (Han et al. 2013). This review will examine the critical role mitochondria play in mediating DILI.

## 8.2 Overview of DILI

DILI is categorized into two broad categories: predictable and idiosyncratic. Acetaminophen (APAP) is one of the few marketed drugs that cause a predictable, dose-dependent hepatotoxicity in animal models and in patients. APAP, due to

unintentional or deliberate overdose, is the most common cause of drug-induced liver failure, accounting for ~46% of acute liver failure cases in the United States (Ostapowicz et al. 2002). Idiosyncratic DILI is the rare form of liver injury (1 in 1000–20,000) that occurs in patients taking therapeutic doses of drugs (Kaplowitz 2005; Holt and Ju 2010). Idiosyncratic DILI is not strictly dose dependent, but a dose threshold generally exists, as a minimum dosage of drug (50–100 mg daily) is required for liver injury to occur. Idiosyncratic DILI is generally not observed in animal models, as most drugs pass animal testing before being administered to patients. Idiosyncratic DILI is also generally not observed during clinical trials due to insufficient population sizes (~low thousands) (Han et al. 2010; Holt and Ju 2010). Consequently, only when a drug becomes consumed by a large number of patients does idiosyncratic DILI become unequivocally recognized. Idiosyncratic DILI accounts for ~13% of acute liver failure cases in the United States.

The underlying mechanisms of why a small subpopulation of patients develops liver injury with therapeutic drug doses, while the majority of patients do not, are complex. Idiosyncratic DILI results from the convergence of multiple risk factors, such as genetics, age, gender, diet, and infections (Ulrich 2007; Lee 2003). Recent genome-wide association studies (GWAS) have shown that patients with certain human leukocyte antigen (HLA; the human equivalent of MHC) haplotypes are susceptible to idiosyncratic DILI caused by a number of drugs, including lumiracoxib, lapatinib, ticlopidine, amoxicillin-clavulanate, flucloxacillin, and ximelagatran (Hautekeete et al. 1999; O'Donohue et al. 2000; Hirata et al. 2008; Daly et al. 2009; Singer et al. 2010; Lucena et al. 2011; Alfirevic et al. 2012). For example, the HLA-DRB1*1501 haplotype increases a patient's risk for amoxicillin-clavulanate-induced DILI, while HLA-DRB1*5701 increases the risk for flucloxacillin-induced DILI. The strong relationship between hepatotoxic drugs and HLA polymorphisms suggests that the adaptive immune system plays an important role in a large number of idiosyncratic DILI cases (Han et al. 2013). A recent study has shown that abacavir, which causes hypersensitive reactions, binds within the F pocket of the peptide-binding groove of the HLA receptor (HLA-B*57:01 haplotype) (Illing et al. 2012). Thus, patients with the HLA-B*57:01 haplotype have a greater potential for autoimmune reactions because the drug is presented on the HLA receptor to T-cells (Adam et al. 2014). Although patients with certain HLA haplotypes have increased risk for DILI, overall rates remain low for most drugs (Watkins 2009). Other extrinsic factors (infections, inflammation, diet) also play important roles in the development of idiosyncratic DILI (Ulrich 2007; Han et al. 2010).

## 8.3 Drugs that Cause DILI Stress Mitochondria

Mitochondria – as major generators of ATP, key regulators of apoptosis, and major sources of reactive oxygen species (ROS) – play a central role in hepatocyte survival (Han et al. 2013; Cadenas and Davies 2000). Stress or injury to mitochondria has profound effects on ATP and ROS levels and can trigger the release of pro-death

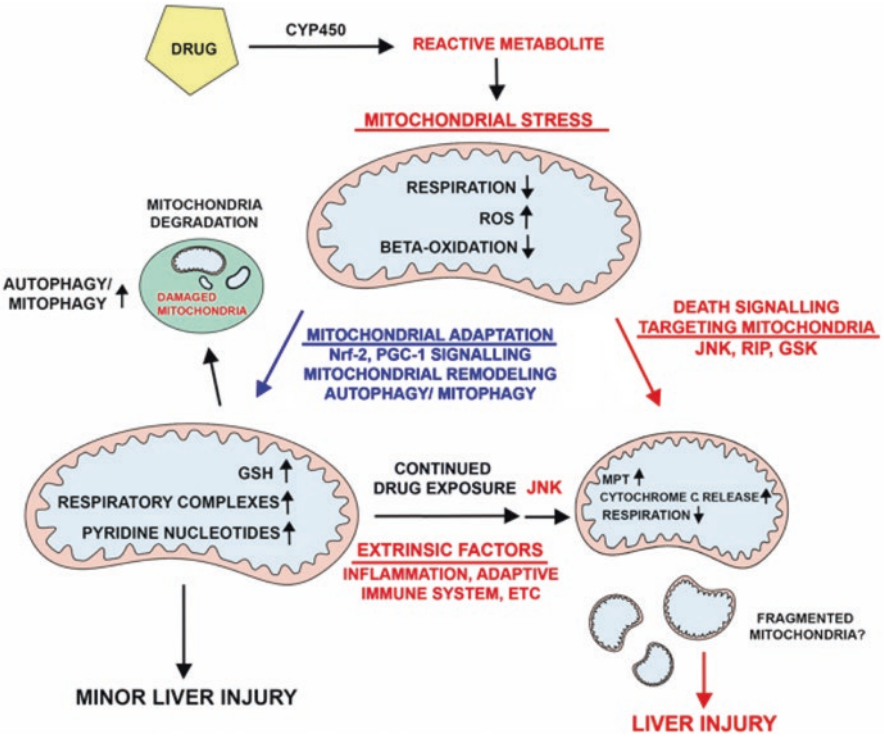


**Fig. 8.1** *Critical role of mitochondria in DILI.* Hepatotoxic drugs or drug metabolites formed from metabolism by cytochrome P450 (*CYP450*) have been shown to inhibit mitochondrial respiration, disrupt beta-oxidation, increase mitochondrial ROS generation, and cause other types of mitochondrial disruptions that stress or injure mitochondria. In most cases, mitochondrial stress induced by a drug is not sufficient to cause hepatotoxicity, because adaptation pathways including autophagy/mitophagy, mitochondrial remodeling, and mitochondrial fusion-fission become activated. Mitochondrial fusion-fission could also potentially play a deleterious role by initiating mitochondrial fragmentation that promotes hepatocyte death. A small number of patients may progress to severe liver injury due to failure to adapt resulting from inflammation and other extrinsic factors. When mitochondrial and hepatocellular injury reaches a critical threshold, death signaling pathways involving JNK that target mitochondria become activated. Activated JNK binds to Sab on the outer mitochondrial membrane to trigger the mitochondrial permeability transition (*MPT*), which causes mitochondrial swelling, and release of cytochrome *c* that results in hepatocyte death and liver injury

proteins such as cytochrome *c* (Kaplowitz 2002). Mitochondria play a critical role in DILI during all stages of the pathology (Kaplowitz 2005): (1) inhibition of mitochondrial function by drugs or reactive metabolites that can initiate DILI (Han et al. 2006a), (2) adaptation pathways involving mitochondria that help most patients tolerate drugs (Thames 2004), and (3) activation of death signaling pathways involving c-Jun *N*-terminal kinase (JNK) that targets mitochondria to cause hepatocyte death and liver injury (Fig. 8.1). Most drugs that cause DILI, predictable or idiosyncratic, have been shown to stress or injure mitochondria by disrupting mitochondrial function (Boelsterli and Lim 2007; Han et al. 2013; Jones et al. 2010). Hepatotoxic

drugs, or drug metabolites formed from metabolism by cytochrome P450 (CYP450), have been shown to inhibit mitochondrial respiration, disrupt beta-oxidation, deplete mitochondrial glutathione (GSH), increase mitochondrial ROS generation, and cause other mitochondrial disruptions that stress or injure mitochondria (Han et al. 2013). However, in most cases, mitochondrial stress induced by a drug is not sufficient to cause hepatotoxicity, and the majority of patients taking drugs do not develop liver injury. Most patients taking idiosyncratic hepatotoxic drugs may be "adaptors" that avoid liver injury by upregulating adaptation signaling pathways, many involving mitochondria. Mitochondrial adaptation pathways include autophagy/mitophagy, mitochondrial remodeling, and mitochondrial fusion-fission (Fig. 8.1). A small fraction of patients ("susceptibles") may progress to severe injury due to a failure of the liver to adapt because of inflammation and other extrinsic factors. When mitochondrial and hepatocellular injury reaches a critical threshold, death signaling pathways involving JNK become activated and target mitochondria (Han et al. 2013; Hanawa et al. 2008). The binding of activated JNK (phosphorylated JNK) to mitochondria triggers the mitochondrial permeability transition (MPT), which causes mitochondrial swelling and release of cytochrome *c* that results in hepatocyte death and liver injury (Han et al. 2013).

DILI has been difficult to study due to the rarity of the disease in patients and lack of animal models (Kaplowitz 2005; Boelsterli 2003). Because APAP is one of the few marketed drugs that cause a predictable, dose-dependent hepatotoxicity, most of our mechanistic knowledge on the role of mitochondria in DILI has come from work with APAP in cultured hepatocytes and in animals (Kaplowitz 2005). While APAP treatment causes very predictable liver injury, insights regarding idiosyncratic DILI have been obtained using APAP in animal models, since its hepatotoxicity is similarly affected by age, sex, diet, inflammation caused by bacteria and viruses, and genetic factors (Han et al. 2010; Maddox et al. 2010). Because the APAP animal model has revealed a great deal of information regarding the role of mitochondria in drug-induced hepatotoxicity, we will begin with an overview of the central role mitochondria play in APAP-induced liver injury.

## 8.4 Central Role of Mitochondria in APAP-Induced Liver Injury

Mitochondrial injury plays an essential role in APAP-induced liver injury. It has been demonstrated that serum levels of mitochondrial biomarkers (mtDNA, glutamate dehydrogenase) are predictive of patients surviving APAP-induced acute liver failure (McGill et al. 2014). Patients with greater mitochondrial injury that causes the release of mitochondrial constituents into the plasma are much more likely to suffer acute liver failure. Consequently, there has been increased research exploring mitochondria-targeted therapies, such as mitochondria-targeted antioxidants (Mito-TEMPO), and electron transport mediators, such as methylene blue, to protect against APAP-induced liver injury in animal models (Lee et al. 2015; Du et al. 2017).

### 8.4.1 Inhibition of Mitochondrial Bioenergetics by APAP

APAP hepatotoxicity can be directly attributed to the formation of *N*-acetyl-*p*-benzo-quinoneimine (NAPQI), a reactive metabolite generated during the metabolism of APAP by CYP450, primarily by the CYP2EI isoform (Dahlin et al. 1984; Chen et al. 1998). NAPQI is a highly reactive metabolite that forms covalent bonds with protein thiols and nonprotein thiols including glutathione (GSH), the major antioxidant in cells. At hepatotoxic doses of APAP, NAPQI causes severe depletion of GSH in the cytoplasm and mitochondria, which contain separate GSH pools (Han et al. 2006a; Kaplowitz et al. 1985). APAP treatment has also been shown to decrease levels of glutamate cysteine ligase (GCL), the rate-limiting enzyme in GSH synthesis, further depleting GSH in the liver (Shinohara et al. 2010). The depletion of mitochondrial GSH is considered the critical event in APAP hepatotoxicity, as it results in the partial inhibition of mitochondrial respiration and enhanced mitochondrial ROS generation – important in activating JNK death signaling. NAPQI may directly bind thiols in the respiratory complexes to inhibit mitochondrial respiration (Burcham and Harman 1991), with complex II being particularly sensitive to NAPQI inhibition (Lee et al. 2015). APAP treatment enhances mitochondrial ROS generation by (Kaplowitz 2005) reducing the efficacy of GSH peroxidase, which utilizes GSH to detoxify $H_2O_2$, and (Han et al. 2006a) by inhibiting mitochondrial respiratory complexes, which generally enhance superoxide generation (Hanawa et al. 2008; Han et al. 2003a, b). There is a critical threshold for mitochondrial GSH depletion that determines whether liver injury occurs. If the threshold is reached (>90%), extensive NAPQI inhibition of mitochondrial proteins and/or enhanced mitochondrial $H_2O_2$production will activate the JNK death signaling cascade that causes severe liver injury (Han et al. 2013). When the GSH threshold is not reached, adaptation pathways involving nuclear factor (erythroid-derived 2)-like-2 (Nrf-2) help mitochondrial and liver GSH levels recover, resulting in little liver injury (Copple et al. 2008). Low non-hepatotoxic doses of APAP have been shown to cause reversible mitochondrial depolarization in the liver, without hepatocyte necrosis or ALT release (Hu et al. 2016).

### 8.4.2 Adaptation of Mitochondria to Promote Survival in Response to APAP

Adaptive mechanisms in the liver, including mitochondrial adaptation, may be crucial in patients consuming even therapeutic doses of APAP (4 grams daily). One study reported that the majority of patients (up to 81%) receiving therapeutic doses of APAP initially develop elevated plasma ALT levels (Watkins et al. 2006). However, adaptation mechanisms appear to protect the majority of patients from APAP, and in most cases, continuous APAP intake does not result in liver injury. Mitochondrial adaptation mechanisms including enhancement of mitochondrial

GSH levels, autophagy/mitophagy, mitochondria remodeling, and alteration of mitochondrial fusion-fission have been characterized in various liver pathologies. We will next examine mitochondrial adaptation mechanisms that may be important in protecting against APAP hepatotoxicity.

#### 8.4.2.1 Enhancement of Mitochondrial GSH Levels

Mitochondria lack enzymes necessary for GSH synthesis, and consequently GSH must be transported into the mitochondrial matrix (Kaplowitz et al. 1985). Interestingly, once GSH is oxidized to GSSG, it appears to become trapped inside the mitochondrial matrix: GSSG transporters have not been observed in mitochondria (Olafsdottir and Reed 1988; Garcia et al. 2010). We have demonstrated that feeding respiratory substrates (i.e., glutamate/malate, succinate, etc.) into the electron transport chain generates NADPH, which is essential in maintaining GSH and protein thiols in their reduced forms, as well as reversing posttranslational modifications such as S-glutathionylation and S-nitrosylation (Garcia et al. 2010; Chang et al. 2014). During times of oxidative stress, respiratory substrates are critical in keeping the mitochondrial redox environment reduced and fully functional (Garcia et al. 2010).

The transcription factor Nrf-2 plays a central role in adaptation to APAP in the liver by replenishing cytoplasmic and mitochondrial GSH levels (Copple et al. 2010). Nrf-2 is usually bound in the cytoplasm to Kelch-like ECH-associated protein 1 (Keap1), a redox-sensitive anchoring protein. Also attached to Keap1 is a cullin-dependent E3 ubiquitin ligase complex (Cu3) that facilitates the ubiquitination of Nrf-2. Keap1 binds newly synthesized Nrf-2, which results in Cu3-dependent ubiquitination and proteasomal degradation (Copple et al. 2010; Tong et al. 2007). Consequently, Nrf-2 has an extremely short half-life (10–30 min). The binding of critical thiols in Keap1 (~25 cysteine residues) by NAPQI and other electrophiles causes a conformational change that prevents Nrf-2 ubiquitination and degradation (Han et al. 2013). Therefore, the NAPQI-induced conformational change of Nrf-2 keeps Keap1 occupied without being degraded, allowing newly synthesized Nrf-2 to translocate to the nucleus and bind to the antioxidant response element (ARE), a DNA sequence in the promoter of antioxidant enzymes such as GCL (Copple et al. 2008; Tong et al. 2007). Nrf-2 upregulation of GCL is essential in replenishing mitochondrial and cytoplasmic GSH following even therapeutic doses of APAP. Even low, nontoxic doses of APAP have been shown to activate Nrf-2 and increase GSH synthesis in the liver of mice (Goldring et al. 2004).

#### 8.4.2.2 Autophagy/Mitophagy

Autophagy is a lysosome-mediated process where intracellular proteins and organelles, including mitochondria, are degraded and recycled (Yin et al. 2008). Mitophagy is a selective form of autophagy, where damaged mitochondria are

removed and degraded by lysosomes (Ding and Yin 2012). Both autophagy and mitophagy are enhanced by various stresses, including starvation. Since APAP treatment is known to cause mitochondrial dysfunction, it is not surprising that both autophagy and mitophagy occur following hepatotoxic doses of APAP. Activation of autophagy and mitophagy is believed to be protective, as damaged mitochondria are removed, especially those with enhanced ROS generation (Ni et al. 2012). Pharmacological inhibition of autophagy aggravates APAP-induced liver injury (Baulies et al. 2015), while enhancement of autophagy using rapamycin protects against APAP-induced liver injury in mice. AMP-activated protein kinase (AMPK), which is regulated by protein kinase C (PKC) (Saberi et al. 2008), is believed to promote autophagy in the liver and help protect against APAP (Saberi et al. 2014). Mitophagy has not been extensively characterized during APAP hepatotoxicity but is believed to be mediated by Parkin in the liver (Ding et al. 2012a).

#### 8.4.2.3 Mitochondria Remodeling

The mitochondrial respiratory chain can undergo remodeling and biogenesis to increase respiratory capacity in response to injury (Han et al. 2013; Than et al. 2011). We observed that chronic alcohol feeding in mice and rats caused various types of mitochondrial remodeling, including alterations in respiratory complex proteins, increased mitochondrial NADH-NADPH levels, and increased mitochondrial respiration as adaptations to alcohol (Han et al. 2012, 2016). Mitochondrial remodeling is likely mediated by proliferator-activated receptor gamma coactivator-1α (PGC-1α), a coactivator and master regulator of mitochondria (Than et al. 2011; Han et al. 2012). Mitochondrial injury in the liver caused by the hepatotoxin $CCl_4$, which induces extensive lipid peroxidation, has similarly been shown to increase mitochondrial respiration and levels of respiratory complexes in the liver (Shiryaeva et al. 2008; Morimoto et al. 1988). Thus, APAP treatment, which injures mitochondria and enhances mitochondrial degradation by autophagy and mitophagy, is likely to activate PGC-1α and other mitochondrial biogenesis pathways to promote mitochondrial remodeling and biogenesis. However, to our knowledge a detailed study examining mitochondrial remodeling and biogenesis following APAP treatment has not been performed in the liver, and further studies are needed.

#### 8.4.2.4 Alteration of Mitochondrial Fusion-Fission

Mitochondria constantly undergo fusion-fission in order to exchange mitochondrial constituents including mtDNA, respiratory complexes, mitochondria proteins, and GSH (Otera and Mihara 2011; Reddy et al. 2011). Mitochondrial fusion and fission are primarily controlled by four highly conserved GTPases: mitofusins (Mfn1, Mfn2) in mitochondrial outer membranes promote fusion; optic atrophy (Opa1) in the inner membranes promotes fusion; and dynamin-related protein 1 (Drp1) from the cytoplasm translocates to mitochondria to initiate fission (Otera and Mihara

2011; Chan 2006). Stress can alter mitochondrial fusion-fission rates and mitochondrial morphology, which can promote cell death or cell survival depending on the circumstances. Mitochondrial fission mediated by Drp1 can promote mitochondrial fragmentation and cell death (Otera and Mihara 2011; Westermann 2010). Conversely, alterations in mitochondrial fusion-fission can promote cell survival, in cases such as starvation, where mitochondrial fission decreases to produce elongated mitochondria with greater cristae surface areas that increase respiration and ATP production (Gomes et al. 2011). APAP hepatotoxicity is associated with dramatic morphological alterations in mitochondria, including both fragmentation and elongation (Ruepp et al. 2002), as well as formation of spheroid-shaped mitochondria (Ding et al. 2012b; Ni et al. 2013). The implications of these mitochondrial morphological changes caused by hepatotoxic doses of APAP remain uncertain. It has been suggested that spheroid-shaped mitochondria formation mediated by Mfn1 or Mfn2 may be a protective mechanism of inactivating damaged mitochondria (Ding et al. 2012a), but further research is needed. ROS appear to be important in triggering mitochondrial morphological changes and spheroid-shaped mitochondria formation in hepatocytes (Ding et al. 2012b). Drp1 has been observed to translocate to mitochondria very early during APAP hepatotoxicity (Dara et al. 2015), which may help explain some observations of fragmented mitochondria during APAP hepatotoxicity. However, the importance of this Drp1 translocation to mitochondria during APAP hepatotoxicity requires further examination. Overall, mitochondrial morphological changes are very prominent during APAP-induced liver injury, but whether these changes play an adaptive, detrimental, or inconsequential role in hepatotoxicity needs further exploration.

## *8.4.3 Death Signaling Pathways Targeting Mitochondria*

### 8.4.3.1 JNK Activation and Translocation to Mitochondria

Despite adaptive mechanisms, if mitochondrial GSH depletion reaches a critical threshold, the death signaling pathway involving JNK becomes activated (phosphorylation). Activated JNK targets mitochondria and plays a critical role in promoting mitochondrial dysfunction and liver injury during APAP hepatotoxicity. JNK inhibition or silencing protects against APAP-induced liver injury, despite excessive mitochondrial GSH depletion and covalent binding in the liver (Hanawa et al. 2008). JNK plays an important role in stress response; however, when JNK activation is sustained, it promotes mitochondrial dysfunction and cell death (Han et al. 2010; Seki et al. 2012). Increased mitochondrial ROS generation mediated by NAPQI is believed to activate JNK during APAP-induced liver injury through a complex feed-forward mechanism. The maximal activation of JNK appears to involve a number of upstream kinases, including PKC-α, receptor-interacting serine/threonine-protein kinase 1 (RIP1), glycogen synthase kinase-3β (GSK-3β), mixed-lineage kinase-3 (MLK3), and apoptosis signal-regulating kinase 1 (ASK1)

(Shinohara et al. 2010; Saberi et al. 2014; Dara et al. 2015; Nakagawa et al. 2008; Sharma et al. 2012). Silencing any one of these kinases in mice inhibits JNK activation and translocation to mitochondria to protect against APAP hepatotoxicity. Activated JNK translocates to mitochondria and binds SH3 homology associated BTK-binding protein (Sab or SH3BP5), a scaffold protein on the outer membrane of mitochondria that contains a kinase interaction motif (Wiltshire et al. 2004; Win et al. 2014). The binding of JNK to Sab further impairs mitochondrial respiration and enhances mitochondrial ROS generation by activating a signaling cascade involving mitochondrial protein tyrosine phosphatase, nonreceptor type 6 (SHP1), and mitochondrial Src (Win et al. 2016). Once critical levels of JNK translocate to mitochondria, the mitochondrial permeability transition (MPT) is triggered, causing mitochondrial swelling and release of cytochrome *c* that results in hepatocyte death and liver injury. The exact mechanism of how JNK triggers MPT remains to be fully characterized.

APAP hepatotoxicity is an active process, since JNK signaling pathways must be activated for liver injury to occur. This is somewhat surprising, as APAP-induced liver injury occurs primarily through necrosis rather than apoptosis (Hanawa et al. 2008; Gunawan et al. 2006). Clinical studies have shown that circulating levels of full length K18 (Fl-K18), a marker of necrosis, were much higher (85%) than the cleaved K18 fragment (cK18), a marker of apoptosis (15%), in patients suffering from APAP hepatotoxicity (Antoine et al. 2012). These findings correspond with animal studies that have demonstrated that APAP hepatotoxicity occurs primarily through hepatocyte necrosis. The fact that JNK actively mediates hepatocyte death challenges the traditional view that APAP hepatotoxicity is a passive process due to overwhelming hepatocellular injury caused by NAPQI (Han et al. 2010; Han et al. 2013). APAP hepatotoxicity can therefore be viewed as a type of "programmed necrosis" (Han et al. 2013; Jones et al. 2010).

#### 8.4.3.2 Mitochondrial Targeting by Other Kinases

JNK is not the only kinase that has been shown to translocate to mitochondria during APAP-induced liver injury. PKC-α, GSK-3β, and RIP1 have all been shown to translocate to liver mitochondria following hepatotoxic doses of APAP. However, it has been difficult to decipher whether the translocation of these kinases contributes to mitochondrial dysfunction during APAP hepatotoxicity, since silencing these kinases inhibits JNK activation and translocation to mitochondria. For example, PKC-α translocates to mitochondria and phosphorylates mitochondrial proteins. However, silencing PKC-α inhibits JNK activation and translocation during APAP hepatotoxicity, suggesting that PKC-α is important in activating JNK. In other injury models, PKC-ε translocation to mitochondria appears to play a protective role in cardiac ischemic injury by phosphorylating mitochondrial proteins such as pyruvate dehydrogenase, while PKC-δ, which inhibits pyruvate dehydrogenase, appears to promote cardiac ischemic injury (Churchill and Mochly-Rosen 2007; Kheifets and Mochly-Rosen 2007; Budas et al. 2010; Gong et al. 2012). Similarly,

GSK-3β translocation to mitochondria is believed to be important in mediating ischemia-reperfusion injury in the heart by promoting MPT (Das et al. 2008; Maurer et al. 2006; Juhaszova et al. 2004). Therefore, the translocation of other kinases – such as PKC-α, GSK-3β, and RIP1 – to mitochondria following APAP treatment may play a role in APAP hepatotoxicity, but further studies are needed to delineate their effects from JNK (Shinohara et al. 2010).

#### 8.4.3.3 Mitochondrial Regulation by Bcl-2 Family Members

Mitochondria are the organelles where members of the B-cell lymphoma-2 (Bcl-2) family exert their apoptotic or anti-apoptotic effects. Bax, a proapoptotic member of the Bcl-2 family, has been shown to translocate to liver mitochondria during APAP hepatotoxicity, possibly through a JNK-dependent pathway (Hanawa et al. 2008). However, Bax knockout mice exhibit the same level of liver injury as control mice following APAP treatment, suggesting that Bax is not essential for mitochondrial dysfunction and hepatocyte death (Bajt et al. 2008). JNK translocation to mitochondria has been suggested to phosphorylate and inactivate Bcl-2 and Bcl-xl, two anti-apoptotic proteins on the outer membrane of mitochondria (Hanawa et al. 2008; Latchoumycandane et al. 2007). Induced myeloid leukemia cell differentiation protein (Mcl-1), an anti-apoptotic Bcl-2 family member, is also degraded in the liver during APAP hepatotoxicity (Shinohara et al. 2010). Silencing Mcl-1 in the liver causes spontaneous apoptosis in hepatocytes (Vick et al. 2009), suggesting that the loss or inactivation of anti-apoptotic Bcl-2 family members from mitochondria may contribute to hepatocyte death following APAP treatment. Taken together, many proteins (kinases, Bcl-2 family members) translocate to mitochondria, while anti-apoptotic Bcl-2 members become inactivated in mitochondria. All of these events may be contributing to mitochondrial dysfunction during APAP hepatotoxicity, but currently only JNK translocation to mitochondria has been shown to be critical in causing mitochondrial dysfunction essential for liver injury.

## 8.5 Role of Mitochondria in Idiosyncratic DILI

Idiosyncratic DILI likely involves mitochondrial stress, mitochondrial adaptations, and death signaling pathways targeting mitochondria as observed with APAP (Fig. 8.1). While APAP is unique in that its toxicity is dependent on severe GSH depletion, many other features of APAP hepatotoxicity are likely to occur during liver injury caused by other hepatotoxic drugs (Han et al. 2013). Thus, hepatotoxic drugs may have many general shared characteristics, as well as some unique drug-specific features (i.e., metabolism, reactive metabolites, etc.). Since idiosyncratic DILI is rare and lacks good animal models, our overall knowledge is limited and more open to speculation.

### 8.5.1 Inhibition of Mitochondrial Homeostasis by Idiosyncratic Hepatotoxins

Most drugs that cause idiosyncratic DILI have been shown to stress or injure mitochondria. Reactive metabolites formed during drug metabolism, rather than the drug itself, may often be disruptive to mitochondria in hepatocytes (Kaplowitz 2005; Holt and Ju 2010). Many reactive metabolites can also directly cause oxidative stress. For example, troglitazone forms a reactive metabolite that forms adducts with molecules like GSH (Alvarez-Sanchez et al. 2006). Table 8.1 shows idiosyncratic hepatotoxic drugs that disrupt mitochondrial function in hepatocytes or isolated mitochondria. A wide range of mitochondrial functions including mitochondrial electron transport, beta-oxidation, and protein synthesis can be inhibited by hepatotoxic drugs. The majority of drugs listed in Table 8.1 do not cause liver injury (i.e., increased ALT, histology) when given to healthy animals, though mitochondrial stress can sometimes be observed in the liver. For example, chronic treatment of nimesulide to mice increased cytochrome *c* release from mitochondria in the liver, but ALT levels and histology remained similar to control (Ong et al. 2006). In most cases, mitochondrial inhibition by idiosyncratic hepatotoxic drugs listed in Table 8.1 is only observed in cultured hepatocytes or with isolated liver mitochondria. Antimicrobials cause ~46% of idiosyncratic DILI cases, possibly because mitochondria are of bacterial ancestry and still share many genetic and structural similarities with bacteria. A limited amount of human data suggests that mitochondrial abnormalities occur in the liver during idiosyncratic DILI. Tolcapone-induced DILI has been observed to be associated with mitochondrial swelling in the livers of patients (Spahr et al. 2000). Electron microscopy studies have similarly demonstrated that aspirin-induced Reye's syndrome is linked with abnormal mitochondria in the liver (Iancu et al. 1977; Osterloh et al. 1989).

Large screening studies have also confirmed that inhibition of mitochondrial homeostasis occurs for the majority of idiosyncratic hepatotoxic drugs. Screening for drugs (300 tested) that cause oxidative stress and disrupt mitochondrial homeostasis (ROS, mitochondrial membrane potential, etc.) in human hepatocytes revealed that 50–60% of idiosyncratic hepatotoxic drugs caused mitochondrial perturbations, whereas non-hepatotoxic drugs were shown not to affect mitochondria (0–5%) (Xu et al. 2008). Similarly, screenings utilizing isolated mouse liver mitochondria showed that most drugs that cause idiosyncratic DILI disrupt mitochondrial function (i.e., membrane potential, respiration, cytochrome *c* release, etc.; 89% positive predictive value) (Porceddu et al. 2012). Thus, these types of mitochondrial screening studies, in combination with "omics" technologies (genomics, metabolomics, etc.) may better identify idiosyncratic hepatotoxins during drug development (Winnike et al. 2010; Daly 2012).

If a majority of hepatotoxic drugs disrupt mitochondrial homeostasis, the question remains why liver injury only occurs in a small number of patients, while the majority remains unaffected. It is clear that mitochondrial stress alone is not enough to cause liver injury in most cases, and other factors contribute to DILI. The mechanism of idiosyncratic DILI is multifactorial (genetics, age, gender, diet, infections),

**Table 8.1** Drugs associated with mitochondrial dysfunction

| Drugs | Model system | Effect on mitochondria |
|---|---|---|
| Acetaminophen | In vivo – mice liver, cultured hepatocytes | Depletion of mitochondrial GSH, inhibition of respiration, increased ROS generation |
| Amineptine | In vivo – mice liver | Inhibition of beta-oxidation |
| Amiodarone, dronedarone | Isolated liver mitochondria, cultured hepatocytes | Increased ROS generation, inhibition of respiration, inhibition of beta-oxidation |
| Aspirin | In vivo – mice liver, isolated liver mitochondria, cultured hepatocytes | MPT, mitochondrial uncoupling, inhibition of beta-oxidation |
| Benzarone, Benzbromarone | Cultured hepatocytes, isolated liver mitochondria | Inhibition of respiration, mitochondrial uncoupling |
| Diclofenac | Cultured hepatocytes, isolated mitochondria | Mitochondrial uncoupling, inhibition of ANT and ATPase, MPT, Bax-induced MOMP |
| Diflunisal | Isolated liver mitochondria | Mitochondrial uncoupling, inhibition of medium-chain acyl-CoA synthetase |
| Fialuridine | In vivo – woodchuck and rat liver mitochondria, HepG2 cells | Enlarged mitochondria, decreased mtDNA, reduced cristae |
| Isoniazid | HepG2 cells, cultured hepatocytes, isolated liver mitochondria | Inhibition of complex I, decreased membrane potential, increased ROS generation |
| Mefenamic acid | Isolated kidney or liver mitochondria | Mitochondrial uncoupling, inhibition of various acyl-CoA synthetase |
| Nefazodone | Human hepatocytes, isolated liver mitochondria, HepG2 cells | Inhibition of mitochondrial respiration (complex I and IV) |
| Nimesulide | Isolated liver mitochondria, rat hepatocytes | Mitochondrial uncoupling, increased ROS generation, MPT |
| Perhexiline | Cultured hepatocytes, isolated liver mitochondria | Mitochondrial uncoupling, inhibition of mitochondrial respiration (complex I and II), inhibition of beta-oxidation |
| Stavudine | Liver of patients, HepG2 cells, cultured hepatocytes | Inhibition of beta-oxidation, increased ROS generation, reduced and abnormal cristae |
| Tamoxifen | Isolated liver mitochondria | Inhibition of mitochondrial respiration (complex I), mitochondrial uncoupling, increased ROS generation |
| Tianeptine | In vivo – mice liver | Inhibition of beta-oxidation (short- and medium-chain fatty acid) |
| Tolcapone | Isolated liver mitochondria, in vivo – rat liver | Mitochondrial uncoupling, mitochondrial swelling |
| Trazodone | Human hepatocytes | Loss of mitochondrial membrane potential |
| Troglitazone | HepG2 cells, isolated liver mitochondria | Promotes PGC-1 degradation to decrease mitochondrial mass, decreased mitochondrial respiration, MPT |
| Valproate | In vivo – mice liver, cultured hepatocytes, submitochondrial particles | Inhibition of beta-oxidation, inhibition of mitochondrial pyruvate uptake, inhibition of mitochondrial respiration, depletion of mitochondrial GSH |

and it is possible that the same drug could cause DILI through different mechanisms in different patients. Genetic alterations that predispose patients to oxidative stress appear to cause liver injury in a number of idiosyncratic hepatotoxins. Mice that are heterozygous for Mn-superoxide dismutase (SOD $2^{+/-}$mice) represent an animal model with greater mitochondrial oxidative stress, due to reduced levels of mitochondrial SOD responsible for clearing superoxide in the matrix. Two idiosyncratic hepatotoxic drugs (troglitazone, flutamide) were shown to cause liver injury in SOD $2^{+/-}$mice but not in wild-type mice (Ong et al. 2007; Kashimshetty et al. 2009). It must be noted that troglitazone-induced liver injury is somewhat controversial in this model (Fujimoto et al. 2009). Patients with mutations in Mn-SOD or mutations in GSH peroxidase (GPX1), responsible for detoxification of $H_2O_2$, are at greater risk for developing cholestatic DILI (Lucena et al. 2010). Similarly, patients, predominantly women, with mutations in glutathione S-transferase (GST) have an increased risk for developing idiosyncratic DILI regardless of the type of drug involved (Lucena et al. 2008). Genetic alterations represent only one contributing factor to idiosyncratic DILI, and the failure of adaptation pathways due to extrinsic factors, such as infections, may also be important.

### *8.5.2 Mitochondrial Adaptation During Idiosyncratic DILI*

Clinical studies suggest that the liver readily adapts to chronic drug intake. Idiosyncratic hepatotoxic drugs often cause higher rates of mild, asymptomatic, and transient elevated serum ALT levels that disappear with continuous drug treatment (Watkins 2009; Watkins 2005). Drug screening studies in primary hepatocytes have shown that idiosyncratic toxicants (troglitazone, trovafloxacin) alter gene expression patterns much more than their non-hepatotoxic counterparts, possibly due to the mitochondrial stress induced by hepatotoxic drugs (Kier et al. 2004; Liguori et al. 2005). Many of these signaling changes in hepatocytes likely involve similar adaptation mechanisms previously characterized for APAP. Unfortunately, very little information regarding mitochondrial adaptation is available for most drugs that cause DILI due to the lack of reliable animal models. There is some evidence that many hepatotoxic drugs cause changes in mitochondrial morphology. In primary hepatocytes, chloramphenicol treatment induces the formation of large mitochondria (mega-mitochondria) associated with lower respiration (Karbowski et al. 1999). Mega-mitochondria with enhanced $H_2O_2$generation were observed with troglitazone treatment, but not with its non-hepatotoxic counterparts (pioglitazone, ciglitazone) (Shishido et al. 2003). Finally, trovafloxacin treatment has been shown to downregulate many mitochondrial genes, including Mfn1, suggesting that mitochondrial morphological changes occur in hepatocytes (Liguori et al. 2005). Given that many drugs that cause DILI inhibit mitochondrial respiration and/or induce oxidative stress – both of which are known to trigger mitochondrial morphological changes – these observations of mitochondrial morphological alterations are not surprising. Whether these mitochondrial morphological changes are protective, detrimental, or insignificant remains to be explored.

#### 8.5.2.1 Failure of Adaptation Signaling Pathways Due to Drug Intake

Inflammation caused by bacteria or viruses is a clinically important risk factor for the development of idiosyncratic DILI (Ulrich 2007; Roth and Ganey 2010). Some idiosyncratic hepatotoxic drugs may promote liver injury by inhibiting signaling pathways involved in adaptation to inflammation, particularly cytokines such as tumor necrosis factor-α (TNF) released during inflammation (Han et al. 2009). TNF binding to TNF receptors in hepatocytes simultaneously activates a cell death signaling pathway and a survival/adaptation pathway. The death signaling pathway involves JNK activation and translocation to mitochondria that perpetuate a self-amplifying loop, similar to that observed during APAP hepatotoxicity (Han et al. 2009). TNF is not normally toxic to hepatocytes because TNF also activates survival/adaptation pathways involving NF-κB, which transcribes genes (GAPDD45b, XIAP, A20) and antioxidant proteins (Mn-SOD, ferritin) responsible for turning off JNK and promoting cell survival (Han et al. 2009; Wong et al. 1989; Wullaert et al. 2006). Consequently, NF-κB activation allows hepatocytes to adapt to TNF released during inflammation, and JNK activation is transient. However, oxidative stress and/or inhibition of mitochondrial respiration associated with many idiosyncratic hepatotoxins has been shown to inhibit NF-κB activation and sensitize hepatocytes to TNF-induced apoptosis (Nagai et al. 2002; Matsumaru et al. 2003; Han et al. 2006b; Lou and Kaplowitz 2007). Thus, it is not surprising that APAP and the idiosyncratic toxicant chlorpromazine have been shown to sensitize cultured hepatocytes to TNF-induced apoptosis (Gandhi et al. 2010).

The failure of hepatocytes to adapt to TNF has also been observed in vivo. Idiosyncratic hepatotoxic drugs (chlorpromazine, trovafloxacin, ranitidine) that do not normally cause liver injury in rats will induce liver injury when co-treated with a sublethal dose of lipopolysaccharide (LPS), which activates the innate immune system and promotes TNF secretion (Shaw et al. 2007; Tukov et al. 2007; Luyendyk et al. 2003). The sensitizing effects of LPS to idiosyncratic hepatotoxic drugs were shown to be mediated by TNF released by the innate immune system (Liguori et al. 2010). Therefore, drugs that induce mitochondrial stress and/or enhanced ROS generation are likely blocking adaptation signaling pathways (NF-κB) and/or helping to sustain JNK activation to sensitize hepatocytes to TNF-induced apoptosis in vivo (Han et al. 2013). With atorvastatin, a statin associated with idiosyncratic liver injury, the opposite phenomenon is observed: inflammation inhibits drug-induced adaptation pathways (Wu et al. 2016). In ApoE-/- mice, atorvastatin treatment was shown to induce Nrf-2 translocation to the nucleus and upregulate antioxidant enzymes (catalase, SOD). Inflammation induced by casein injection was observed to inhibit Nrf-2 activation induced by atorvastatin, leading to liver injury including hepatic steatosis and fibrosis. Given that both hepatotoxic drugs and inflammation are associated with oxidative stress that can inhibit adaptation signaling pathways (Nrf-2, NF-κB) and sustain JNK activation, it is easy to see how these two factors may work synergistically to promote liver injury. Surprisingly, the therapeutic potential of antioxidants to protect against idiosyncratic DILI has not been extensively explored, especially since vitamin E has been shown to protect against non-alcoholic steatosis (NASH) in patients (Sanyal et al. 2010).

The increased risk of DILI that occurs with certain HLA haplotypes suggests that the adaptive immune system is important in mediating DILI caused by some drugs. Certain haplotypes of HLA, which bind drugs or reactive metabolites, may promote the adaptive immune system to attack the liver (Holt and Ju 2010). These drug-induced autoimmune reactions may increase during inflammation, when the immune system is in a heightened state. Drug-induced mitochondrial dysfunction may also be sensitizing hepatocytes to components of the adaptive immune system, such as cytotoxic T-cells (Han et al. 2013). It is also possible that idiosyncratic hepatotoxic drugs may inhibit adaptation to TNF and other cytotoxic agents (i.e., FasL, perforin, granulysin, granzyme) released by the adaptive immune system. Clearly the role the immune system plays in idiosyncratic DILI merits further investigation.

### *8.5.3 Mitochondria Targeting by Death Signaling Pathways During Idiosyncratic DILI*

Idiosyncratic DILI caused by drug intake and extrinsic factor(s) is likely to involve death signaling pathways, such as JNK, once substantial mitochondrial and hepatocyte injury occurs. JNK is a strong candidate to mediate DILI caused by a wide range of drugs since it plays a central role in many liver pathologies. JNK plays a critical role in hepatocellular injury caused by bile acids, fatty acids, ER stress, TRAIL-Fas, ischemia/reperfusion, and TNF-induced liver injury in vivo (Con A plus D-galactosamine, LPS plus D-galactosamine, and TNF plus D-galactosamine) (Seki et al. 2012; Schwabe 2006; Ibrahim et al. 2011; Win et al. 2011, 2014, 2015 ). At sufficient levels, JNK translocation to mitochondria will induce either MPT or mitochondrial outer membrane permeabilization (MOMP) to trigger hepatocyte death. Along with JNK, Bcl-2 family members including Bax and Bcl-xl are likely to determine if MPT or MOMP occurs. While we hypothesize that JNK plays a central role in mediating idiosyncratic DILI caused by many drugs, further studies are needed.

## 8.6 Concluding Remarks

DILI appears to involve mitochondria during all stages of the disease (Xu et al. 2008; Porceddu et al. 2012). Although idiosyncratic hepatotoxic drugs are not structurally or chemically related, most drugs that cause DILI “stress” mitochondria. In most cases, hepatocytes adapt to the drug-induced mitochondrial stress by activating adaptive signaling pathways, including mitochondrial adaptation responses such as autophagy/mitophagy, mitochondrial remodeling, and alterations in mitochondrial fusion-fission. While mitochondrial stress, such as increased mitochondrial ROS generation, may not be sufficient to trigger hepatocyte death, it may

inhibit adaptation/survival signaling pathways (NF-κB, Nrf-2) needed to survive extrinsic factors such as inflammation. After a critical threshold of mitochondrial and hepatocellular injury occurs, JNK is activated and targets mitochondria to induce MPT or MOMP, leading to cell death. Thus, mitochondria are the central points where oxidative injury, pro-death proteins, and adaptation pathways converge to determine the fate of hepatocytes during DILI.

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# Chapter 9
# Mitochondrial Damage and Mitophagy in Ischemia/Reperfusion-Induced Liver Injury

**Kristina L. Go, Sooyeon Lee, Kevin E. Behrns, and Jae-Sung Kim**

## Abbreviations

| | |
|---|---|
| ALCAT1 | Lysocardiolipin acyltransferase 1 |
| ALLM | Acetyl-Leu-Leu-methioninal |
| AMPK | Adenosine monophosphate-activated protein kinase |
| ANT | Adenine nucleotide translocase |
| ATG | Autophagy-related proteins |
| ATP | Adenosine triphosphate |
| BECN1 | Beclin-1 |
| CsA | Cyclosporine A |
| CVP | Central venous pressure |
| ER | Endoplasmic reticulum |
| FUNDC1 | FUN14 domain-containing protein-1 |
| HCC | Hepatocellular carcinoma |
| IPC | Ischemic preconditioning |
| I/R | Ischemia/reperfusion |
| JNK1 | c-Jun N-terminal protein kinase 1 |
| KAT | Lysine acetyltransferase |
| KDAC | Lysine deacetylase |
| LC3 | Microtubule-associated protein 1 light chain 3 |
| MFN | Mitofusin |
| MPT | Mitochondrial permeability transition |
| mtDNA | Mitochondrial DNA |

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W.-X. Ding, X.-M. Yin (eds.), *Molecules, Systems and Signaling in Liver Injury*,
Cell Death in Biology and Diseases, DOI 10.1007/978-3-319-58106-4_9

X.-M. Yin and Z. Dong (Series eds.), *Cell Death in Biology and Diseases*

| | |
|---|---|
| mTOR | Mammalian target of rapamycin |
| $NAD^+$ | Oxidized nicotinamide adenine dinucleotide |
| NAFLD | Nonalcoholic fatty liver disease |
| NASH | Nonalcoholic steatohepatitis |
| PI | Propidium iodide |
| PI3KIII | Class III phosphatidylinositol 3-kinase |
| PINK1 | PTEN-induced putative kinase protein 1 |
| RIPC | Remote ischemic preconditioning |
| ROS | Reactive oxygen species |
| SIRT1 | Sirtuin 1 |
| TMRM | Tetramethylrhodamine methyl ester |
| ULK1 | UNC-51-like kinase 1 |
| VDAC | Voltage-dependent adenine nucleotide |

## 9.1 Introduction

Chronic liver disease is a consequence of many etiologies including viral infection, congenital disorders, repeated ethanol exposure, pharmacological toxicities, and metabolic disorders. The spectrum of chronic liver disease ranges from simple steatosis to steatohepatitis which can progress to fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). Chronic liver disease is currently the fifth leading cause of death worldwide. Furthermore, in comparison to other types of cancer, the incidence and rates of cancer-related deaths from HCC continue to rise in both genders (Ryerson et al. 2016). Although conservative strategies are an important component of therapy for chronic liver disease, the only strategies that can provide long-term cure for most patients are surgical resection and liver transplantation. As ischemia/reperfusion (I/R) injury inevitably occurs during these surgical treatments, patients with chronic liver disease, who have compromised liver function, are at higher risk of developing hepatic failure after surgery.

In this chapter, we review and update current information on liver I/R injury, including the unique anatomy of the liver associated with its vulnerability to I/R injury, types of I/R injury, and the central role of mitochondrial dysfunction and defective autophagy in hepatocellular death secondary to I/R. Moreover, we discuss how underlying patient comorbidities such as aging and steatosis further impact I/R injury and describe proposed methods to mitigate hepatic I/R injury.

## 9.2 Liver Anatomy and I/R Injury

The evolution of hepatic surgery in the last century can be attributed in part to increased understanding of internal biliary and vascular anatomy of the liver. Where hepatic trauma and liver resection were once associated with significant morbidity

and mortality, techniques of vascular clamping first described by J. H. Pringle in the 1900s (Pringle 1908) and later refined in Jean-Louis Lortat-Jacob's description of a right hepatectomy in the 1950s (Lortat-Jacob et al. 1952) have shifted the landscape of liver surgery from a rare operation to a regular but complex procedure. While hepatic resection has become commonplace, operative blood loss remains an independent predictor of perioperative morbidity and mortality (Jarnagin et al. 2002); therefore, an understanding of hepatic anatomy is cardinal in improving surgical outcomes. Likewise, evaluation of gross and cellular anatomy is a vital component in the study of hepatic I/R injury.

### *9.2.1 Vascular Anatomy*

Blood flow through the liver ranges from 1,500 to 2,000 ml/min and accounts for approximately 25% of the cardiac output. The substantial blood volume that enters the liver can be inferred by examining the organ's dual vascular inflow: the portal venous supply provides 75% of hepatic inflow, which is greater than 50% of the liver's oxygen requirements (Vollmar and Menger 2009). The hepatic artery provides 25% of hepatic inflow to meet the remaining metabolic and functional needs of the liver. Blood enters the liver through terminal portal venules and hepatic arterioles, flows through the sinusoids, and drains through a central vein to the hepatic vein and, ultimately, to the suprahepatic inferior vena cava.

Vascular anatomy of the liver is highly variable, and care must be taken to examine the vasculature preoperatively and intraoperatively to ensure the safest approach during resection. The portal vein arises behind the neck of the pancreas and is formed by the confluence of the superior mesenteric and splenic veins. From the neck of the pancreas, it ascends posteriorly to the common bile duct and hepatic artery into the hilum of the liver. Here, the portal vein bifurcates into a shorter, more vertical, right portal branch, dividing further into the right anterior and posterior branches, and a longer, more horizontal left portal branch. The left portal branch enters the umbilical fissure to supply the left liver. In a common anatomical variation, the right anterior and posterior branches may come directly from the portal vein, and the right portal branch may be absent.

Variation also exists in the hepatic arterial system. In approximately 50% of patients, the hepatic artery arises as a branch of the celiac artery, becomes the proper hepatic artery after giving off gastroduodenal and right gastric arteries, courses through the hepatoduodenal ligament, and branches into the right and left hepatic arteries. The most common variations seen include a replaced right hepatic artery, which originates from the superior mesenteric artery, and a replaced left hepatic artery, which arises from the left gastric artery.

### 9.2.2 Functional Surgical Anatomy

Historically, the liver was divided into the right and left lobes by the falciform ligament; however, division of the liver into sections based on either arteriobiliary segmentation (Healey and Schroy 1953) or Couinaud segmentation is now widely adopted (Couinaud 1999). Following Couinaud's nomenclature, the liver is divided into four sections based on the three main branches of the hepatic veins. Each segment drains bile via a bile duct and receives blood flow from a branch of the hepatic artery and portal vein resulting in eight segments. Controversy in nomenclature and segmentation, however, exists as to whether the caudate lobe should be divided into two segments. While Couinaud's segmentation is often used, several attempts have been made to standardize terminology used during hepatic resection including the development of Brisbane terminology (Celinski and Gamblin 2010).

### 9.2.3 Microcirculatory Anatomy of the Liver

Specific aspects of the liver's histologic architecture contribute to its unique functions and the pathogenesis of an array of liver diseases. The smallest functional unit of the liver is the acinus, which is derived from the liver's blood supply. The dimension of the unit is based on blood flow that originates from terminal arterioles and portal venules, passes through sinusoids, and drains from a central vein to the hepatic vein. Thus, the acinus is routinely divided into zones with relation to the portal vein (periportal zone) and the central vein (perivenous, pericentral, centrilobular) and is often subdivided based on differential expression of key enzymes.

Unidirectional blood flow through the acinus creates unique gradients of metabolites and oxygen that can impact the functions of hepatocytes as well as nonparenchymal cells. A partial pressure of oxygen in periportal blood (60–65 mmHg) is substantially greater than that in perivenous blood (30–35 mmHg) (Jungermann and Kietzmann 1996). The zonal difference in oxygen concentration plays an important role in both physiology and pathology of the liver. For example, hepatocytes near the periportal area have greater capacity for oxidative metabolism, gluconeogenesis, ureagenesis, beta-oxidation, and cholesterol synthesis, as compared to pericentral counterparts. In contrast, pericentral hepatocytes demonstrate higher rates of lipogenesis, glycolysis, and drug detoxification than periportal cells. Besides this zonal dependency in basal functions, the oxygen gradient markedly influences the onset of hepatocellular injury that is often confined to a particular zone of the liver lobule. Patients with steatosis and hemochromatosis exhibit prominent lipid peroxidation and hemosiderin accumulation in the pericentral area (MacDonald et al. 2001; Niemelä 2001). In addition, hepatocytes close to the pericentral region are the first to incur hypoxic injury due to the intralobular oxygen gradient (Jungermann and Kietzmann 2000). In contrast, after partial hepatectomy or drug-induced hepatocyte damage, regeneration of liver begins in the periportal area and extends to the pericentral zone afterward (Fabrikant 1968; Lee et al. 1998).

## 9.3 Hepatic I/R Injury: Clinical Context and Cellular Mechanisms

### *9.3.1 Types of I/R Injury*

I/R injury can occur in a myriad of clinical states either secondary to low-flow states such as hypovolemia, sepsis, or cardiogenic shock, sinusoidal obstruction syndrome, Budd-Chiari syndrome, or other veno-occlusive dysfunction or as a consequence of surgical resection. Despite advances in understanding hepatic anatomy, improved surgical and anesthetic techniques, and more prudent patient selection, operative blood loss remains a major concern during hepatectomy. For patients undergoing liver resection, high operative blood loss is an independent risk factor of morbidity and mortality (Shimada et al. 1994). Intraoperative blood loss more than 1000 mL has been shown to be an independent predictor of hyperbilirubinemia (>5 mg/dL) after resection (Fukumori et al. 2011). Among patients with HCC, increased blood loss heightens morbidity and mortality (Shimada et al. 1998), and the extent of blood loss independently predicts risk recurrence and survival (Katz et al. 2009).

Several techniques exist in the current perioperative armamentarium to limit blood loss during hepatectomy. Due to the absence of valves in the hepatic veins, venous bleeding in the liver directly correlates to central venous pressure (CVP). Higher CVP results in distended veins and higher blood loss. By limiting intraoperative fluids during resection, a target CVP of less than 5 mmHg minimizes backbleeding during parenchymal transection (Celinski and Gamblin 2010). Once resection and hemostasis are achieved, the patient may be resuscitated to euvolemia. Low CVP anesthetic technique is well tolerated by patients and minimizes intraoperative blood loss and transfusion rates (Melendez et al. 1998).

In addition to low CVP anesthesia, various vascular occlusion techniques have been developed to limit bleeding and may be further categorized as strategies to occlude hepatic inflow or maneuvers that occlude both vascular inflow and outflow. During hepatic pedicle clamping, also known as Pringle's maneuver, a vascular clamp or loop encircles the hepatoduodenal ligament containing the portal vein and hepatic artery. Disappearance of the distal pulse of the hepatic artery indicates successful occlusion. Pringle's maneuver may be done continuously or intermittently throughout the procedure. In addition to I/R, risks of clamping include splanchnic congestion and air embolus (Zhou et al. 2008), which becomes higher when Pringle's maneuver is used in conjunction with a low CVP. Selective inflow occlusion has also been described wherein inflow occlusion is applied to the branches of the hepatic artery and portal vein that feed the hemi-liver that is to be resected. Although this technique minimizes I/R injury to remnant liver and reduces splanchnic congestion, significant hilar dissection is required, and risk of blood loss to the non-clamped hemi-liver still exists (Abdalla et al. 2004).

Hepatic ischemia can be categorized into warm and cold ischemia. Whereas warm I/R is observed as a consequence of vascular occlusion and low-flow states,

cold I/R is unique to liver transplant where the explanted graft is subjected to a hypothermic, ischemic, preservation phase prior to warm reperfusion after vascular reanastomosis (Papadopoulos et al. 2013). While cellular death is the common outcome from either type of I/R injury, the mechanisms of injury are distinct. Cold I/R injures sinusoidal endothelium and non-parenchymal cells (Klune and Tsung 2010), whereas hepatocytes are a major cell type experiencing warm I/R injury (Cursio et al. 2015).

#### 9.3.1.1 Warm I/R Injury

The mechanisms underlying warm I/R injury are multifactorial and occur in a progressive manner. Soon after reperfusion, calcium overloading and reactive oxygen species (ROS) accumulation in mitochondria result in onset of the mitochondrial permeability transition (MPT), mitochondrial dysfunction, and necrotic death of hepatocytes. Continued reperfusion activates non-parenchymal cells like Kupffer cells and propagates further ROS production, leading to stimulation of chemokines and cytokines that actively recruit neutrophils (Jaeschke 1998; Klune and Tsung 2010). During the late stage of reperfusion, uncontrolled neutrophil infiltration causes irreversible inflammation (Zhai et al. 2011). Although inflammation plays an important role in late reperfusion injury, hepatocyte damage at the early stage of reperfusion is the prominent pathological event that instigates permanent hepatic injury later.

Three major biochemical changes occur during warm ischemia; (1) anoxia, (2) nutrient depletion, and (3) cytosolic acidosis. Interruption of blood flow limits oxygen delivery to mitochondria and depletes adenosine triphosphate (ATP) synthesis from the mitochondrial electron transport chain, leading to disruption of energy-dependent metabolic processes (Cursio et al. 2015). ATP-dependent channels and exchangers are likewise compromised, and dysregulation of sodium, chloride, and calcium homeostasis results (Cursio et al. 2015). Halted supply of nutrient and energy substrates resulting from impeded blood flow also potentiates ATP loss. Moreover, anoxia lowers cytosol pH due to the accumulation of lactate and hydrogen ion, limiting the efficacy of enzymes that optimally operate at a neutral pH. While prolonged tissue acidosis will eventually cause liver cell death, acidosis during acute ischemia confers cytoprotection due to blockade of MPT onset and inactivation of injurious catabolic enzymes (Kim et al. 2003b). However, damage initiated during ischemia is aggravated upon restoration of blood flow and the return of physiologic pH, a phenomenon called pH paradox (Kim et al. 2003b).

#### 9.3.1.2 Cold I/R Injury

In contrast to warm I/R injury, endothelial and non-parenchymal cells are major contributors to reperfusion injury after cold ischemia (Klune and Tsung 2010). As such, pathological manifestations observed in cold I/R reflect aberrant functions of

non-parenchymal cells, including decreased graft microcirculation, increased platelet activation, vasoconstriction, and upregulation of adhesion molecules. Of importance, these alterations further activate Kupffer cells, recruit neutrophils, and enhance production of ROS, ultimately potentiating hepatocyte death (Peralta et al. 2013). Because cold I/R is unique to orthotopic liver transplantation, while warm I/R is observed in a multitude of clinical settings, we will focus on the mechanisms, clinical implications, and therapies involved in warm I/R injury.

## 9.4 Mitochondrial Dysfunction After I/R

The liver plays a central role in the clearance of toxins, synthesis of hormones and proteins, vitamin storage, blood sugar regulation, bile synthesis, ketone body synthesis, and lipid homeostasis. Besides these multiple functions, the liver's regenerative capacity further demonstrates the need for hepatocytes to generate high amounts of ATP to meet energy demands for hepatic regrowth. Mitochondria, a cell's power plant, generate more than 90% of cellular ATP and are composed of two membranes with differential permeability, an outer membrane with more permissive permeability and an inner membrane, which is nearly impermeable to all solutes except those with specific carriers or exchangers. Structural integrity of mitochondrial membranes is critical for mitochondrial functions and cell survival. Hence, it is not surprising to observe that alterations in structural barrier in mitochondria often precede hepatocellular injury and death. In particular, loss of mitochondrial inner membrane permeabilization and subsequent onset of mitochondrial dysfunction are a key causal mechanism of hepatocyte death after I/R. In this section, we will describe the role of mitochondrial injury after I/R and discuss how underlying mitochondrial dysfunction is involved in aging and fatty liver disease, all of which predispose hepatocytes to further I/R injury.

### *9.4.1 Mitochondrial Permeability Transition (MPT)*

Mitochondrial membrane integrity is prerequisite for the maintenance of proton motive force across mitochondria and can be challenged by prolonged opening of MPT pores. Though further studies are warranted to elucidate its precise molecular composition, the general consensus is that MPT pores have three major components: an adenine nucleotide translocator (ANT) on the inner mitochondrial membrane, voltage-dependent anion channels (VDAC) on the outer mitochondrial membrane, and cyclophilin D located in the mitochondrial matrix (Kim et al. 2003a). Cyclosporine A (CsA) blocks cyclophilin D, a peptidyl-prolyl *cis-trans* isomerase that catalyzes conformational changes in ANT leading to pore opening. Bongkrekic acid, a toxin generated from fermented coconut products, inhibits ANT (Halestrap et al. 2002). In addition, $Mg^{2+}$, acidic pH, and trifluoroperazine are

known to inhibit MPT opening (Lemasters et al. 1998). Under basal conditions, transient opening and closing of these high-conductance permeability transition pores stochastically take place to temporarily create a burst of superoxide production, matrix alkalinization, and oscillation of the mitochondrial membrane potential, suggesting that mitochondrial flashes may be intimately integrated with the regulation of intramitochondrial $Ca^{2+}$ and ROS (Wang et al. 2008; Hou et al. 2013, 2016). However, under pathological conditions like I/R, permanent opening of MPT pores ensues, resulting in either necrotic or apoptotic cell death. During ischemia, there is a profound drop in ATP with a concomitant increase in inorganic phosphate that stimulates glycolysis and causes a buildup of lactate. Although this intracellular acidosis prevents opening of high-conductance permeability transition pores of the mitochondrial inner membrane, it loads the cell with $Na^+$ through the $Na^+/H^+$ antiporter. As low ATP levels during ischemia also reduce $Na^+/K^+$ ATPase activity, $Na^+$ overloading becomes persistent and eventually reverses $Na^+/Ca^{2+}$ antiporter which culminates in excess $Ca^{2+}$ loading. Upon reperfusion, recovery of oxygen and energy substrates together with a compromised electron transport chain outbursts ROS and incites MPT onset in mitochondria. Thus, the combination of oxidative stress, ATP depletion, elevated inorganic phosphate, and increased matrix calcium after reperfusion permanently opens pores to initiate the onset of MPT (Qian et al. 1997; Halestrap and Richardson 2015). The instigation of MPT collapses the mitochondrial inner membrane permeability barrier, allowing solutes up to 1,500 Da to diffuse freely across the mitochondrial inner membrane (Kim et al. 2003a, 2006a, 2008a, 2012). Free diffusion into the mitochondrial matrix induces swelling of mitochondria and uncoupling of oxidative phosphorylation and depolarization of mitochondria (Go et al. 2015). In the end, the loss of mitochondrial membrane potential and collapse of pH gradient nullify mitochondrial proton motive force, causing depletion of ATP and hepatocellular death.

Near-complete depletion of ATP during prolonged ischemia makes the occurrence of apoptosis less likely than necrosis. Using confocal microscopy, the MPT, mitochondrial depolarization, and necrotic cell death after reperfusion can be visualized in live hepatocytes. Calcein, a green-fluorescing fluorophore, is a polyanionic solute of 623 Da that is impermeable to the mitochondrial inner membrane (Fig. 9.1). As a result, confocal images of calcein-loaded hepatocytes have a honeycomb appearance with numerous dark round or oval voids corresponding to individual respiring mitochondria. Co-loading with tetramethylrhodamine methyl ester (TMRM), a red-fluorescing cationic fluorophore that accumulates electrophoretically in mitochondria, confirms that virtually every dark void is a polarized mitochondrion. TMRM does not quench calcein fluorescence to cause these voids. After 4 h of simulated ischemia when isolated rodent hepatocytes are subjected to anoxic, nutrient-depleted, and acidotic conditions, mitochondria depolarize and release TMRM due to lack of oxygen, but do not become permeable to calcein, as shown by the persistence of dark voids in calcein images, indicative of lack of MPT onset during ischemia. After reperfusion (reoxygenation at pH 7.4), mitochondria begin to repolarize rapidly. The cell, however, depolarizes after 10 min (loss of TMRM). Concomitant with depolarization, calcein redistribution into some of the

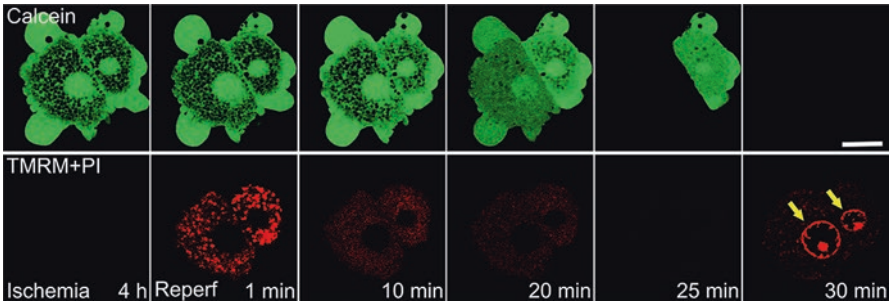


**Fig. 9.1** The onset of MPT and hepatocyte death after reperfusion. Confocal images of calcein (*green*), TMRM (*red*), and propidium iodide (PI, *red*) after 4 h of simulated ischemia of rodent hepatocytes. During ischemia, calcein was excluded by mitochondria, indicative of permeability pore closure. After 1 min of reperfusion, mitochondria repolarized without calcein redistribution. After 10 min, calcein redistributed into some mitochondria due to MPT onset, and mitochondria began to depolarize, as evidenced by loss of TMRM fluorescence. Within 20 min, widespread onset of MPT occurred in nearly all mitochondria. Later, calcein fluoresce disappeared because of the loss of plasma membrane integrity, concomitant with nuclear labeling of PI (*arrows*), denoting cell death. Scale bar, 10 μm

mitochondrial matrices is evident, leading to the disappearance of the dark voids of the mitochondria (onset of the MPT). One noticeable event at this time point is that redistribution of calcein is confined to a discrete population of mitochondria, suggesting that some mitochondria are more susceptible to MPT induction. A few minutes later, all mitochondria become permeable to calcein, and cells completely lose the mitochondrial membrane potential. After 30 min, cell viability is lost, as shown by total loss of calcein and nuclear staining with propidium iodide (PI). When cells are reperfused in conditions that block the MPT, including CsA at pH 7.4 or acidic pH, mitochondria continue to repolarize after reperfusion, mitochondrial calcein voids remain intact, and cell viability is sustained (Qian et al. 1997). Hence, these observations indicate that reperfusion causes CsA-sensitive mitochondrial inner membrane permeabilization and depolarization, leading to cell death. Thus, onset of the MPT is a key mechanism underlying pH-dependent necrosis after I/R in hepatocytes. While necrosis is the dominant fate of cell death during I/R, apoptosis can occur in the liver after I/R. Studies with isolated rodent hepatocytes have demonstrated that the onset of MPT induces apoptotic cell death in the presence of glycolytic energy supply (Qian et al. 1999; Kim et al. 2003a, b, c). After MPT onset, mitochondrial swelling ruptures the mitochondrial outer membranes and releases cytochrome *c*. Normally sequestered in the mitochondrial intermembrane space, this 12 kDa protein is released into the cytosol, binds to apoptosis-inducing factor-1, and pro-caspases to form the apoptosome. As activation of caspases is an energy-dependent process, the presence of ATP following mitochondrial membrane permeabilization triggers apoptotic signaling (Lemasters 1999; Lemasters et al. 2002; Kim et al. 2003a). In sharp contrast, ATP-deficient hepatocytes undergo necrotic cell death in spite of the presence of upstream proapoptotic signals. Thus, the onset of MPT and glycolytic ATP availability govern the fate of hepatocyte death after reperfusion as to whether cells after I/R undergo necrosis or apoptosis (Kim et al. 2003b).

### *9.4.2 Mitophagy: Mitochondrial Quality Control Machinery*

#### 9.4.2.1 Autophagy

Autophagy is an evolutionarily conserved pathway that removes protein aggregates, surplus cytoplasmic contents, or dysfunctional organelles through lysosomal digestion (Baehrecke 2005). First described by Christian de Duve as a cellular "self-eating" process (Yang and Klionsky 2010), three types of autophagy exist: chaperone-mediated autophagy, microautophagy, and macroautophagy (Mizushima et al. 2008). Chaperone-mediated autophagy specifically targets soluble proteins with the KFERQ pentapeptide motif. Proteins with this motif are recognized by 70 kDa molecular chaperone heat shock protein. After interacting with lysosome-associated membrane protein 2A, cargo is delivered to the lysosome for degradation (Massey et al. 2006). Microautophagy delivers cytosolic substrates directly to the endosomal or lysosomal membrane where cellular cargos are invaginated into the lumen for degradation (Santambrogio and Cuervo 2011). Mechanistic details of microautophagy are largely unknown. Macroautophagy, herein referred to as "autophagy," is the most common form of autophagy. Two types of macroautophagy have been described: canonical, where multiple autophagy-related proteins (ATG) interact to form a cup-shaped double membrane complex called the phagophore, and the less understood noncanonical autophagy that can occur in the absence of some key ATG (Codogno et al. 2012). To date, over 30 ATG have been identified, and 18 ATG are essential to formation of the autophagosome (Vlada et al. 2015).

Five key steps occur during autophagy: initiation, nucleation, elongation, fusion, and degradation (Kim and Lee 2014). During basal conditions where autophagy occurs infrequently, mammalian target of rapamycin complex 1 (mTORC1) phosphorylates UNC-51-like kinase 1 (ULK1), the mammalian ortholog of yeast ATG1, at the Ser757 residue. When phosphorylated by mTORC1, ULK1 is dissociated from adenosine monophosphate-activated protein kinase (AMPK), which prevents autophagy initiation but promotes cellular proliferation and growth. Reduction in energy content, such as starvation or hypoxia, drives AMPK-mediated phosphorylation of Ser 317, 555, and 777 residues of ULK1 (Jung et al. 2010; Kuma and Mizushima 2010; Rabinowitz and White 2010; Kim et al. 2011; Settembre et al. 2011; Sanchez et al. 2012; Rubinsztein et al. 2012). By binding RAPTOR, phosphorylated ULK1 suppresses the kinase activity of mTORC1 (Jung et al. 2011). Additionally, activated ULK1 phosphorylates other targets including ATG13 and focal adhesion kinase family interacting protein of 200 kDa (FIP200), forming the ULK1-ATG13-FIP200 complex on the surface of the double-membrane phagophore. It has been reported that membranes of the phagophore are originated from endoplasmic reticulum (ER), Golgi, or mitochondria (Mijaljica et al. 2006; Hara et al. 2008; Hosokawa et al. 2009; Kuma and Mizushima 2010). In parallel with initiation events, further phosphorylation of Beclin-1 (BECN1) by stress-responsive c-Jun N-terminal protein kinase 1 (JNK1) liberates BECN1 from its B-cell lymphoma 2 (Bcl-2)-associated complex (Wei et al. 2008; Dai et al. 2011). Free BECN1

enables the recruitment of ATG14, vacuolar protein sorting 34 (VPS 34), and VPS15 to generate the BECN1-ATG15-VPS34-VPS15 complex (Russell et al. 2013). Other proteins such as VPS34, a class III lipid kinase, are likely involved in the modulation of autophagosomal membranes and vesicle trafficking (Backer 2008; Obara and Ohsumi 2011). The phagophore is further shaped by several other factors including UV radiation resistance-associated gene (UVRAG) (Liang et al. 2006), endophilin B-1 (BIF-1) (Takahashi et al. 2007), ATG14L (Itakura et al. 2008), and RUN domain Beclin-1-interacting and cysteine-rich domain-containing protein (RUBICON) (Matsunaga et al. 2009), corroborating the complexity of autophagy initiation.

Elongation of the autophagosome is the coordinated orchestration between ATG12 and microtubule-associated protein 1 light chain 3 (LC3). ATG12, covalently linked to ATG5 through the E2-like ubiquitin carrier protein ATG10, becomes activated by ATG7, an E1-ubiquitin-like enzyme. The ATG12-ATG5 complex ultimately interacts with ATG16L1 (Mizushima et al. 2011). Parallel to formation of the ATG12-ATG5-ATG16LI complex, ATG7 and ATG3 conjugate phosphatidylethanolamine to LC3-I to generate LC3-II, a biomarker of autophagy. While unconjugated LC3-I predominantly resides in the cytosol, LC3-II localizes to autophagosomal membranes (Ravikumar et al. 2010; Mizushima et al. 2010). ATG4B, a cysteine protease, cleaves the C-terminus of LC3 for conversion of LC3-I into LC3-II, but also possesses the ability to release the phosphatidylethanolamine group from LC3-II (Levine and Klionsky 2004). Removal of this phospholipid group and release of LC3 from the autophagosome induce fusion of the autophagosome with endosomes or lysosomes. Thus, ATG4B is essential both in recycling LC3-II and in the fusion and degradation of autophagosomal contents.

Fusion between the mature autophagosome and a lysosome generates the autolysosome (Ao et al. 2014). Components that mediate fusion include Ras-like GTPase RAB7, which tethers the autophagosome to the lysosome (Chua et al. 2011), and the UVRAG-BECN1-class III phosphatidylinositol 3-kinase (PI3KIII) and class C vacuolar protein-sorting complexes, whose interaction likely facilitates Rab7-mediated fusion (Moreau et al. 2013). Soluble N-ethylmaleimide-sensitive fusion factors also affect autolysosome formation. During degradation, catabolic enzymes residing in autolysosomal lumen including acidic proteases, lipases, nucleases, and glycosidases degrade cellular cargos into amino acids, which reenter the cell for other metabolic purposes (Weber et al. 1998; Rabinowitz and White 2010; Moreau et al. 2013).

Growing evidence indicates that autophagy may operate either selectively or nonselectively. The degradation of specific cellular constituents and organelles occurs during selective autophagy and includes pexophagy (peroxisomes) (Dunn et al. 2005), mitophagy (mitochondria) (Lemasters 2014), ribophagy (ribosomes) (Kraft et al. 2008), reticulophagy (endoplasmic reticulum) (Hamasaki et al. 2005), lipophagy (lipid droplets) (Singh et al. 2009), and ferrintinophagy (iron) (Mancias et al. 2014).

#### 9.4.2.2 Types of Mitophagy

Mitochondria are the major source of ROS generation, and mitochondrial DNA (mtDNA) is distinctly prone to ROS-mediated injury (Shadel and Clayton 1997). Aberrant alterations in mtDNA are implicated in a broad range of human diseases due to its high susceptibility to oxidative damage and its limited DNA repair capability, as compared to nuclear DNA (Penta et al. 2001). Therefore, accumulation of abnormal mitochondria resulting from defective mitochondrial clearance causes uncontrolled ROS formation, mtDNA mutation, energetic failure, and ultimately cell death. As MPT onset and mitochondrial dysfunction are causative events attributing to hepatocellular death after I/R, timely clearance of abnormal or dysfunctional mitochondria is pivotal to hepatocellular survival after reperfusion. Accordingly, factors that impair the removal of damaged mitochondria promote I/R injury, and factors that improve clearance may prevent I/R injury. Mitophagy is a selective autophagy that sequesters and degrades damaged or abnormal mitochondria in a timely manner (Fig. 9.2). Mitophagy is also involved in normal mitochondrial turnover. Lemasters has recently proposed three distinct types of mitophagy (Lemasters 2014). Type I mitophagy follows the mechanisms similar to the canonical autophagic pathway described previously and is phosphatidylinositol-3-kinase (PI3K) dependent. In this type of mitophagy, the autolysosome initially sequesters polarized mitochondria that later become depolarized as a consequence of MPT onset or the acidic microenvironment in the autophagic vesicles (Czaja et al. 2013). Type I mitophagy is evident in response to nutrient starvation or acute ischemic events (Fig. 9.2).

In contrast, dissipation of mitochondrial membrane potential by mitochondrial uncouplers such as carbonyl cyanide *m*-chlorophenyl hydrazine or carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone initiates the PI3K-independent and PTEN-induced putative kinase protein 1 (PINK1)-dependent type II mitophagy. Accumulating evidence indicates that differential localization to the inner or outer mitochondrial membranes regulates PINK1 stability and function. In healthy mitochondria, PINK1 remains in a cleaved state due to robust serine protease activity of presenilin-associated rhomboid-like protein (PARL) in the mitochondrial

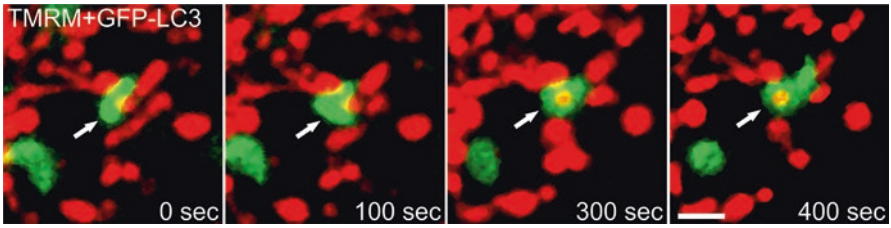


**Fig. 9.2** Visualization of mitophagy. Time-lapse images of confocal microscopy with GFP-LC3 and TMRM in isolated mouse hepatocytes. Under autophagy-stimulating conditions such as nutrient depletion and starvation, hepatocytes develop mitophagy wherein green-fluorescing autophagosome completely sequesters a mitochondrion within 5 min after the phagophore formation (*arrow*). Note that the mitochondrion in the lumen of autophagosome remains polarized at 400 s. Scale bar, 2 μm

intermembrane space (Jin et al. 2010; Deas et al. 2011). As PARL activation is electrogenic, mitochondrial depolarization abrogates PARL activity, resulting in inhibition of the constitutive degradation of PINK1 and accumulation of the 63-kDa full-length form on the outer mitochondrial membrane, where it binds to BECN1 and recruits PARKIN to depolarized mitochondria. Subsequently, the E3 ubiquitin ligase activity of PARKIN ubiquitinates substrates including p62 and VDAC (Michiorri et al. 2010; Geisler et al. 2010; Okatsu et al. 2012). Transcription factor p62 has been shown to link autophagic contents to phagophores or autophagosomes (Kirkin et al. 2009). Among the factors that influence type II mitophagy, changes in membrane potential rather than pH or ATP levels appear to prompt PARKIN accumulation and the onset of type II mitophagy (Narendra et al. 2009). Additionally, mitochondrial receptor Bcl2/adenovirus EB 19 kDa interacting protein 3 (BNIP3) has been implicated in mitophagy (Feng et al. 2013). BNIP3 expression changes in response to levels of hypoxia-inducible factor 1α (HIF-1α), and increased translocation of BNIP3 to the mitochondrial outer membrane is observed in the onset of type II mitophagy (Chen et al. 1997; Bruick 2000; Bellot et al. 2009). Of importance, execution of type II mitophagy appears to follow a canonical autophagy pathway (Ray et al. 2000; Zhu et al. 2013). Other factors are likely involved in type II mitophagy. Recently, it has been demonstrated that mitochondrial depolarization activates FUN14 domain-containing protein-1 (FUNDC1), a mitochondrial outer membrane protein that accumulates at the ER-mitochondria contact sites (Liu et al. 2012; Wu et al. 2014). FUNDC1-mediated mitophagy occurs through the coordinated dephosphorylation of Ser13 by mitochondrial phosphatase PGAM family member 5 (Chen et al. 2014) and phosphorylation of Ser17 by ULK1 (Wu et al. 2014). It has been proposed that WXXL motif of BNIP3 and/or FUNDC1 binds to LC3 prior to mitophagy induction (Chen et al. 2008; Sandoval et al. 2008; Feng et al. 2013).

Type III mitophagy is another form of mitophagy where damaged and oxidized components of the mitochondria bud off as vesicles and transit into multivesicular bodies (Soubannier et al. 2012). Generation of mitochondria-derived vesicles increases as an early response to oxidative stress. Type III mitophagy has been proposed to complement mitophagy as delivery to the lysosomes through this pathway is independent of mitochondrial membrane potential, ATG5 and LC3. In addition, silencing of dynamin-1-like protein (DRP1), a mitochondrial fission protein enhancing mitophagy (Twig et al. 2008), does not affect vesicle formation and transport. Hence, type III mitophagy may be distinct from DRP1-dependent mitophagy. It is, however, unknown whether type III mitophagy relies on PI3K activation or not.

#### 9.4.2.3 Role of Mitophagy in Hepatic I/R Injury

MPT onset and mitochondrial failure after reperfusion amass toxic mitochondrial by-products and precipitate I/R-induced hepatocyte death, insinuating that reperfused hepatocytes might have defective or insufficient mitophagy. Biochemical, genetic, and imaging approaches have indeed revealed that reperfusion of ischemic

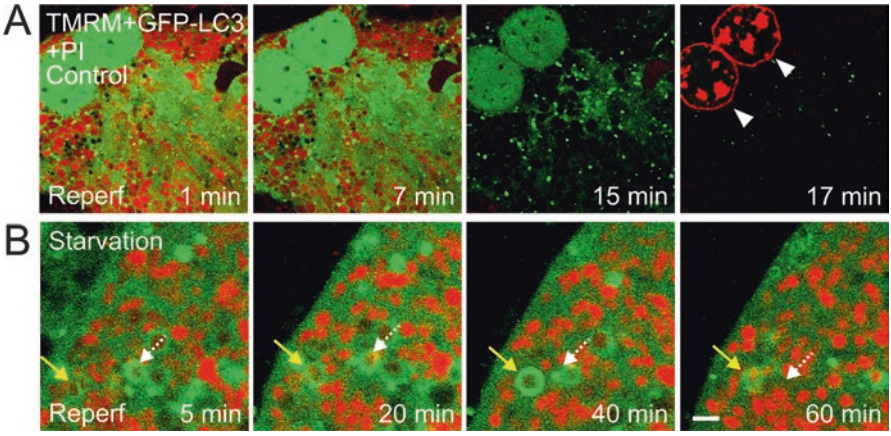


**Fig. 9.3** Impaired autophagy after I/R. Visualization of autophagy and mitophagy after reperfusion. Hepatocytes labeled with GFP-LC3 and TMRM were subjected to 4 h of simulated ischemia. Within 7 min of reperfusion, mitochondria repolarized. Although some green punctate autophagosomes were visible, they were unable to enwrap abnormal mitochondria (*top panels*). The cell lost mitochondrial membrane potential and cell viability after 17 min, as indicated by PI labeling in the nuclei (*arrow heads*). In stark contrast, when hepatocytes were exposed to starvation prior to ischemia, cells executed mitophagy, as shown by green-fluorescing ring structure surrounding a mitochondrion (*dotted white arrow*). More importantly, the cell sustained mitochondrial membrane potential and viability after reperfusion (*bottom panels*). Solid yellow arrow displays an initiation of new mitophagy. Scale bar, 2 μm

hepatocytes impairs autophagy and mitophagy (Fig. 9.3). Autophagic impairment after I/R stems from two factors: energy insufficiency and loss of key autophagy proteins. Autophagy is a highly energy-dependent process. While nutrient depletion and starvation are powerful stimuli for autophagy induction in normal livers, the formation of autophagic vesicles during ischemia is nonetheless halted due to ATP depletion. Lack of functional autophagy can be assessed biochemically by monitoring the changes in LC3-II, a biomarker of autophagy, before and after administration of lysosomal inhibitors like bafilomycin or chloroquine. Since blockade of autolysosomal degradation of autophagic cargos by these agents accumulates autophagosomes, the increase in LC3-II after lysosomal inhibition reflects the extent of autophagic flux. Similarly, fluorescence imaging of GFP-tagged LC3 can visualize autophagosomes that uniquely display punctate pattern of green fluorescence. Hepatocytes after 4 h of ischemia do not exhibit chloroquine-mediated increase in LC3-II, indicative of lack of autophagic flux. However, cell death under this condition is negligible since naturally occurring tissue acidosis prevents the onset of MPT and mitochondrial dysfunction. During the early stage of reperfusion, mitochondria transiently repolarize and exclude calcein, denoting that MPT pores remain closed (Fig. 9.1). At this time, as hepatocytes temporarily recover ATP synthesis, cells are able to execute autophagy and sustain viability. Nevertheless, the energy needed to eliminate swollen and damaged mitochondria during ischemia and early reperfusion begins to outweigh the capacity to generate adequate levels of

ATP. During the late stage of reperfusion, such imbalance becomes exacerbated, autophagy eventually ceases, and hepatocytes increasingly accumulate abnormal or dysfunctional mitochondria that release greater levels of $Ca^{2+}$ and ROS to neighboring mitochondria. Widespread onset of MPT occurs, and necrotic cell death ensues thereafter (Kim et al. 2008b, 2013; Wang et al. 2011b). Consistent with this observation, it has been demonstrated that MPT onset initially affects a subpopulation of mitochondria prior to extensive MPT, supporting the presence of nonuniform pool of mitochondria (Pacher and Hajnoczky 2001). The toxic metabolites and byproducts from a discrete subpopulation of mitochondria disperse damage to healthy adjacent mitochondria before systemic mitochondrial dysfunction develops. Thus, functional mitophagy in order to sequester and clear a mitochondrial subpopulation that is vulnerable to I/R injury may be pivotal to sustain both mitochondrial bioenergetics and cell survival.

Apart from energy insufficiency after reperfusion, depletion of key autophagy proteins is another factor contributing to autophagy failure after I/R. During ischemia, cytosolic $Ca^{2+}$ increases progressively without affecting mitochondrial $Ca^{2+}$ due to ATP depletion and collapse of $Na^+$ and $K^+$ gradients that inhibit ATP-driven $Ca^{2+}$ pumps and secondary ion exchangers (Kim et al. 2012). Moreover, mitochondrial depolarization during anoxia blocks mitochondrial $Ca^{2+}$ exchanger that is modulated by mitochondrial membrane potential (Gunter and Pfeiffer 1990). Cytosolic increase in $Ca^{2+}$ stimulates calpains that are known to maintain substantial activity even under acidic pH (Maddock et al. 2005), which in turn degrades ATG7 and BECN1, two key autophagy proteins involved in autophagosome formation (Wang et al. 2011b; Kim et al. 2008b, 2013). After 4 h of ischemia in isolated hepatocytes, ATG7 and BECN1 decrease to 8.5% and 31.9% of normal values, respectively (Kim et al. 2008a). During reperfusion, mitochondrial $Ca^{2+}$ increases substantially (Kim et al. 2012), calpains become further activated, and levels of both ATG7 and BECN1 decline by 90%. The importance of calcium and calpains in autophagy failure in reperfused hepatocytes is also substantiated by findings that pharmacological inhibitors like acetyl-Leu-Leu-methioninal (ALLM) or carbamazepine considerably suppress the loss of both ATG7 and BECN1 and promote autophagy and cell survival after reperfusion (Kim et al. 2008a, 2013). Of importance, overexpression of either ATG7 or BECN1 or calpain inhibitors all prevent the onset of MPT and cell death, indicating that calpain-mediated defective mitophagy precedes the onset of MPT and cell death. Taken together, with the combination of ATP depletion and ATG loss, the formation of autophagic vesicles is impeded, and autophagy becomes dysfunctional after I/R.

#### 9.4.2.4 Role of Sirtuin 1 (SIRT1) in Autophagy After I/R

Posttranslational modification of proteins by the covalent addition of an acetyl group to ε-lysine residues is referred to as acetylation. This covalent modification neutralizes the positive charge of lysine, affecting the total charge balance of target proteins and altering the steric environment of interaction sites. Acetylation/

deacetylation is involved in a variety of cellular processes such as DNA binding, enzyme activity, and the stability and localization of target proteins. Lysine acetyltransferases (KATs) and deacetylases (KDACs) are two major enzymes to support the balance between acetylation and deacetylation. Four subtypes of KDACs exist including class III KDACs, also known as sirtuins. Among seven mammalian sirtuin isoforms, SIRT1 requires oxidized nicotinamide adenine dinucleotide ($NAD^+$) as a cofactor (Finnin et al. 2001; Min et al. 2001). SIRT1 resides in both the cytosol and nucleus (Tanno et al. 2007) and regulates a myriad of cellular events such as energy metabolism (Rodgers et al. 2005; Gerhart-Hines et al. 2007; Purushotham et al. 2009), mitochondrial biogenesis (Lagouge et al. 2006; Aquilano et al. 2010), and autophagy (Lee et al. 2008; Hariharan et al. 2010; Jang et al. 2012; Fang et al. 2014). As implied by its nuclear location, SIRT1-mediated deacetylation is a central chemical modification in regulation of various gene transcriptions. Some examples encompass peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) (Rodgers et al. 2005), CREB-regulated transcription coactivator 2 (Liu et al. 2008), forkhead transcription factors (FOXO) (Park et al. 2010), fibroblast growth factor (FGF21) (Li et al. 2014), and signal transducer and activator of transcription (STAT3) (Nie et al. 2009). There exists growing evidence that acetylation/deacetylation impacts autophagy at the transcriptional, translational, and posttranslational level. Inhibition of ATG7 acetylation with spermidine increases autophagy (Kuma and Mizushima 2010; Sanchez et al. 2012). Conversely, overexpression of acetyltransferase p300 inhibits autophagy by increasing acetylation of ATG5, ATG7, ATG8, and ATG12 and its interaction with ATG7 (Lee and Finkel 2009). It has also been suggested that SIRT1 can interact directly with ATG5, ATG7, and LC3 during nutrient insufficiency (Lee et al. 2008). The importance of acetylation/deacetylation in autophagy is further supported by recent studies demonstrating that acetylated BECN1 suppresses autophagosome maturation and endocytic trafficking (Sun et al. 2015) and that the acetylation status of LC3 governs its distribution between the nucleus and cytoplasm (Huang et al. 2015). While deacetylation of ATG, in general, promotes autophagy, acetylation can also augment autophagy. Using cell lines, Lin et al. have demonstrated that acetylated ULK1 drives autophagy initiation under the condition of serum deprivation (Lin et al. 2012). Interestingly, when cells are exposed to glucose insufficiency, a condition stimulating autophagy, ULK1 becomes phosphorylated instead, implicating the presence of an intimate cross talk between acetylation/deacetylation and phosphorylation in autophagy regulation.

SIRT1 is critical in hepatocyte survival after I/R. We have recently reported that I/R depletes the liver of SIRT1, which in turn contributes to mechanistic chains of defective autophagy, mitochondrial dysfunction, and hepatocyte injury (Biel et al. 2016). In this study, human liver biopsies collected after 15 min of inflow occlusion during hepatectomy decreases SIRT1 expression by 30%. Similar to patient findings, both murine liver homogenates obtained from an in vivo model of I/R and isolated hepatocytes subjected to a simulated in vitro I/R show a significant reduction in SIRT1, corroborating the sensitivity of SIRT1 to I/R. SIRT1 depletion is likely, at least in part, mediated by calpain activation secondary to $Ca^{2+}$ overloading

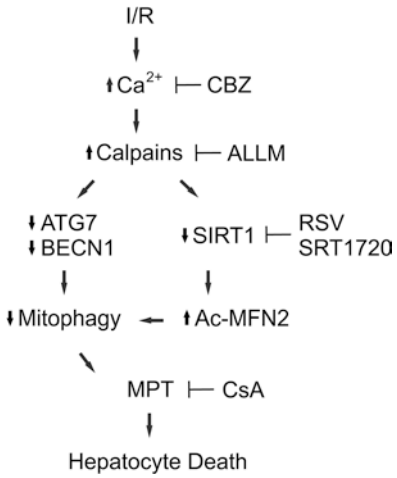


**Fig. 9.4** Scheme of I/R-induced autophagy impairment and cell death. After reperfusion, hepatocytes are overloaded with calcium, which in turn stimulates calpains. These enzymes subsequently hydrolyze ATG7 and BECN1, causing defective autophagy. Since impaired autophagy fails to clear dysfunctional or abnormal mitochondria, hepatocytes accumulate ROS and calcium, toxic precursors inducing the MPT, and ultimately lose viability. At the same time, activated calpains degrade SIRT1, leading to accumulation of acetylated MFN2 (Ac-MFN2) and suppression of mitophagy. Suppression of calcium elevation, inhibition of calpains, activation of SIRT1, or blockade of the MPT all attenuate MPT-dependent hepatocyte death after reperfusion. (Abbreviations: *ALLM* acetyl-Leu-Leu-methioninal, *CBZ* carbamazepine, *CsA* cyclosporine A, *MPT* mitochondrial permeability transition, *RSV* resveratrol)

during I/R since pharmacological inhibition of calpains considerably suppresses the loss of SIRT1 as well as autophagy defects and necrotic cell death. Genetic overexpression of SIRT1 or pharmacological activation with resveratrol or SRT1720 also maintains mitochondrial structural integrity, enhances mitophagy, and prevents MPT onset. Conversely, hepatocytes from SIRT1 conditional knockout mice are vulnerable to I/R injury, as evidenced by impaired autophagic flux, rapid MPT onset, and increased cell death (Fig. 9.4). Collectively, SIRT1 depletion is an important causal mechanism to heighten reperfusion injury.

About 35% of all mitochondrial proteins are acetylated (Anderson and Hirschey 2012), and 24% of acetylated mitochondrial proteins are involved in energy homeostasis during fasting/starvation (Kim et al. 2006b). This suggests that the acetylation status in mitochondria may be a core element to command mitochondrial function and bioenergetics. In fact, hyperacetylation of mitochondrial proteins has been reported in obesity (Hirschey et al. 2011) and chronic alcohol consumption (Fritz et al. 2012), both of which are closely associated with mitochondrial malfunction. We have recently proposed a mechanism as to how cytosolic SIRT1 modulates mitochondrial function (Biel et al. 2016). Biochemical and genetic approaches reveal that cytosolic SIRT1 interacts with and deacetylates a mitochondrial outer membrane protein, mitofusin (MFN). Because either knockdown or deletion

mutants of MFN2 abolish SIRT1-mediated autophagy and cytoprotection against I/R injury, MFN2 is necessary for SIRT1-dependent autophagy. Furthermore, as no changes are observed in the acetylation status of MFN1 after SIRT1 overexpression, MFN2, but not MFN1, is a substrate for SIRT1. Therefore, the acetylation status of this mitochondrial outer membrane protein may be an important molecular switch to facilitate the cross talk between the cytoplasm and mitochondria in the liver.

## 9.5 Mitochondrial Injury in Other Liver Diseases

The prevalence of chronic liver disease continues to rise. Although viral hepatitis and alcohol-related liver disease are among the most common causes of chronic liver disease, the growing obesity epidemic, a rising population of the elderly, and the impact of subacute or chronic use of medications all contribute to liver injury. It is also intuitively expected that the response of patients having liver diseases to I/R will differ greatly from healthy individuals.

### *9.5.1 Mitochondrial Dysfunction in Aging*

In the United States, the population of individuals older than 65 is anticipated to rise to 89 million people by 2050 (Cellier et al. 2016). The growing geriatric population affects multiple aspects of liver disease. Aged individuals are more vulnerable to acute liver injury and are at increased risk to develop hepatic fibrosis and cirrhosis that can proceed to HCC; moreover, older age is a poor prognostic factor in chronic liver disease of many etiologies. Despite these findings, hepatic resection for the elderly with HCC has become increasingly accepted as a main stay of treatment (Kim et al. 2015a) and may provide a more curative outcome when compared to their young counterparts (Mizuguchi et al. 2015). Concurrently, older donor livers are more susceptible to ischemic injury during and following transplantation. Thus, understanding how aging impacts hepatocellular function under basal conditions and after I/R remains paramount.

Ultrasound studies suggest that liver volume decreases by 20–40% with age and that compared to patients younger than 40, patients older than 65 have a 35% decrease in blood volume through the liver. Moreover, functional studies likewise allude to a decrease in the mass of functional liver cells with age. In rodent models, older rats had less regenerative capacity after hepatectomy than their younger counterparts (Tsukamoto et al. 1993).

From a cellular standpoint, both hepatocytes and non-parenchymal cells are equally affected by aging. Sinusoidal epithelial cells become thicker and lose their fenestrations to acquire morphology akin to small capillaries (Huang et al. 2009; Kim et al. 2015b). In combination with an accumulation of oxidative stress and decreased oxygen flow, pseudo-capillarization in aged sinusoids diminishes the efficiency of

filtration and clearance of multiple types of drugs (Le Couteur et al. 2008; Kim et al. 2015a). Evaluation of the hepatic immune cells also suggests that inflammatory responses and antitumor responses are altered with age (Kawabata et al. 2008). Observations of patient biopsies and samples from rodent models have indicated that with advancing age, hepatocytes undergo multiple intracellular alterations. Lipofuscin, an autofluorescent pigment from oxidized proteins and lipids that accumulate in lysosomes, is associated with enhanced free radical formation and a failure to clear waste products (Le Couteur et al. 1999, 2008). Lipid droplets and vesicles are abundant in aged hepatocytes, and a marked decline is evident in smooth ER surface area in aged livers which may mirror reduced hepatic microsomal protein concentrations and enzymatic activity (Zhang and Cuervo 2008; Hoare et al. 2010). However, overall function of aged livers remains intact with advancing age.

From a structural perspective, the mitochondria from aged murine livers appear more swollen and have fewer cristae, shorter cristae, and reduced density (Zhang and Cuervo 2008). Functionally, the mitochondria from senescence-accelerated murine models demonstrate more uncoupling of oxidative phosphorylation and less efficient energy production, suggesting that aged mitochondria may not be as functionally efficient as young mitochondria (Zhang and Cuervo 2008; Khraiwesh et al. 2014). These observations are well aligned with the mitochondrial free radical hypothesis of aging. First described by Harman in the 1950s, it posits that mtDNA is more susceptible to damage due to an absence of histones and their location in the free radical-enriched inner mitochondrial membrane. DNA damage in mitochondria further creates a vicious cycle of free radical propagation that ultimately harms cellular macromolecules and other organelles (Nakahara et al. 1998; Kolosova et al. 2001; Okatani et al. 2002). However, it is worth noting the controversy of this hypothesis. In particular, studies in *C. elegans* refute free radical hypothesis due to unexpected observations that life span is not shortened in *C. elegans* mutants that lack superoxide dismutase (Harman 1956, 1972, 1981) and that mitochondrial ROS can paradoxically increase longevity in *C. elegans* (Van Raamsdonk and Hekimi 2012). In spite of the controversy, solid lines of evidence, however, exist that mitochondrial dysfunction plays a central role in aging. The mitochondrial genome contains multiple copies within each mitochondrion. It encodes 13 subunits of the electron transport chain, two rRNAs, and 22 tRNAs and also possesses a noncoding regulatory region (Yang and Hekimi 2010; Yee et al. 2014). Given that specific mutations occur with aging especially in the sixth segment of a noncoding region of mtDNA (Fraga et al. 1990; Hamilton et al. 2001; Cavallini et al. 2007) and large deletion mutations are frequently found in aged mtDNA (Michikawa et al. 1999), erroneous replication or ineffective repair of mtDNA may also contribute to aging (Trifunovic et al. 2004). Accumulation of damaged protein and decreased membrane fluidity are likewise contributing factors to age-dependent decline in cellular dysfunction. As young cells efficiently eliminate surplus or unnecessary cellular constituents with autophagy, age-mediated increase in abnormal proteins and lipids may manifest decreased capacity of cellular clearance with aging. Zhang and Cuervo have shown that autophagic capacity in the liver subsides in old rats (Zhang and Cuervo 2008). Other work also supports the importance of autophagy in protein

quality control with age. In human kidney cell lines and fibroblasts, aged cells favor autophagy over proteasomal degradation to turnover polyubiquitinated proteins (Gamerdinger et al. 2009). Of interest, although basal levels of autophagy are relatively unchanged in aged cells, they are unable to display a robust autophagic response to additional stresses, inferring that autophagic adaptation declines with advancing age (Donati et al. 2001, 2008). Consistent with these studies, decreased autophagic capacity has been shown to be attributable to age-mediated sensitivity to I/R injury (Wang et al. 2011). In 3-month- and 26-month-old mouse livers, the number of polarized mitochondria and their mitochondrial membrane potential are indistinguishable between two age groups. Moreover, basal levels of autophagic flux are comparable between young and old livers, and hepatocytes from both young and aged mice have a robust autophagic flux under mildly stressed conditions such as normoxia and starvation, supporting that alterations in hepatic parenchymal structure and basal function are minimal with age. However, when aged livers are subjected to additional stress such as short I/R, a condition that young livers tolerate well, they rapidly develop autophagy failure, MPT onset, and cell death. Immunoblotting, genetic, and imaging approaches in both isolated hepatocytes and in vivo livers show that calpain activation after short I/R degrades ATG4B, a key autophagy protein that activates and recycles LC3, in aged livers. As a corollary, aged livers lose autophagic responsiveness to reperfusion and increasingly accumulate dysfunctional mitochondria that provokes widespread onset of MPT and ultimately cell death. Overexpression of ATG4B mitigates this age-dependent vulnerability to reperfusion injury. Hence, despite seemingly minimal impact of aging per se on hepatocellular basal autophagy, aged livers are unable to elicit a proper autophagic response to I/R.

### *9.5.2 Mitochondrial Dysfunction in the Fatty Liver*

The rising obesity epidemic is quickly making nonalcoholic fatty liver disease (NAFLD) the most common cause of chronic liver disease. NAFLD's clinical spectrum ranges from simple hepatic steatosis, where lipids account for greater than 5% of the wet weight of the liver, to hepatic inflammation seen in nonalcoholic steatohepatitis (NASH), to cirrhosis, and to HCC (Starley et al. 2010). About 29% of patients with severe NASH will develop cirrhosis and HCC within 10 years of diagnosis (Argo and Caldwell 2009); thus, understanding the underlying mechanisms of NAFLD development and implications of I/R is a growing priority.

The progression from simple steatosis to NASH has been described through a "two-hit" hypothesis by Day and colleagues (Day and James 1998). Excess hepatic triglycerides and cholesterol sensitize the liver to a second insult, hallmarked by oxidative stress, inflammation, and the induction of insulin resistance (Matsuzawa-Nagata et al. 2008). A meticulous review of the mechanisms underlying the evolution from steatosis to NASH is beyond the scope of this chapter; however, the

interplay between mitochondrial dysfunction, autophagy, and I/R injury both at the bench and in the clinical setting warrants further examination.

Fat over-intake can alter mitochondrial structure and function. In comparison to rats fed with a high-fish oil diet, those fed with a high-lard diet clearly demonstrate increased mitochondrial fission, as evidenced by decreased MFN2 (Wang et al. 2015) and increased DRP1 and mitochondrial fission 1 protein (FIS1) expression (Lionetti et al. 2014). Composition of mitochondrial membranes is likewise changed with surplus fat consumption, in part by increased cardiolipin content (Aoun et al. 2012a, b). Cardiolipin, a unique phospholipid localized in the mitochondrial inner membrane, plays a pivotal role in the maintenance of membrane fluidity (Unsay et al. 2013), ion trafficking (Subramani et al. 2016), protein translocation (Gebert et al. 2009), mitochondrial bioenergetics (Beyer and Nuscher 1996), and mitochondrial-dependent apoptosis (Lutter et al. 2001). Recently, relocation of cardiolipin from the inner to the outer membrane has been proposed to mediate fusion between mitochondria and lysosomes, an integral part of mitophagy (Chu et al. 2013). In parallel with altered mitochondrial structure, excess lipid intake can adversely affect mitochondrial function, including fatty acid oxidation, expression of uncoupling protein 2 (UCP-2) (Jiang et al. 2008; Hodson et al. 2010), and proton leak across the inner mitochondrial membrane (Rolfe et al. 1994). Liver mitochondria isolated from high-fat-fed rats exhibit reduced mitochondrial respiration and increased ROS production (Kathirvel et al. 2010; Vial et al. 2011). mtDNA mutation rates also increase in high-fat diet-fed mice, compared to their normal chow counterparts (Yang et al. 2010)

Multiple murine models have been developed to demonstrate that autophagy is negatively impacted with steatosis (Yang et al. 2010). Steatotic livers manifest a profound ER stress and decreased levels of BECN1, ATG5, and ATG7 (Yang et al. 2010). Excessive lipids can be, however, removed by lipophagy (Singh et al. 2009). Onset of lipophagy requires the interaction between ancient ubiquitous protein 1 (AUP1) and E2 ubiquitin conjugase G2 (UBE2G2) at the lipid droplet surface (Spandl et al. 2011), which induces clustering of lipid droplets (Lohmann et al. 2013). Conversion of LC3-I to LC3-II in a RAB7-dependent manner occurs on the surfaces of lipid droplet aggregates or small, individual lipid droplets prior to autophagosome engulfment (Singh et al. 2009; Schroeder et al. 2015). RAB7, a small GTPase, appears to be a key mediator in lipophagy because its depletion voids lipophagic efficiency and modifies the morphology of multivesicular bodies, lysosomes, and autophagosomes (Schroeder et al. 2015). Autophagy inhibition via 3-methyladenine, knockdown of ATG5, and liver-specific ATG7 knockout all cause the aberrant accumulation of lipids, further emphasizing an integral role of lipophagy in hepatic lipid metabolism (Koga et al. 2010).

Dysfunctional mitophagy is also associated with the pathogenesis of NAFLD. The hepatic expression of lysocardiolipin acyltransferase 1 (ALCAT1), an acyl CoA-dependent enzyme catalyzing the remodeling of cardiolipin in obesity, diabetes, and cardiovascular disease, is significantly upregulated by high-fat diet and leptin deficiency. Wang et al. have shown that elevated levels of ALCAT1 observed in animal models of NAFLD are mechanistically linked to autophagic arrest,

oxidative stress, mitochondrial dysfunction, and insulin resistance in hepatocytes, which can be reversed by specific knockout of ALCAT1 (Wang et al. 2015). ALCAT1-null mice improve mitochondrial respiration and autophagy in the presence of high-fat diet.

Mitochondrial dysfunction secondary to excess lipid exacerbates hepatic I/R injury (Selzner et al. 2000). ATP deficiency resulting from increased expression of UCP-2 in fatty livers makes mitochondria more vulnerable to I/R injury (Evans et al. 2008). Decreased glutathione and thioredoxin levels are common in steatotic hepatocytes, reflecting reduced antioxidant capacity (Zhang et al. 2014). From a surgical perspective, the presence of steatosis has been associated with increased morbidity and mortality during hepatectomy, including longer operative times, increased intraoperative blood loss, and increased blood transfusions (Behrns et al. 1998; Veteläinen et al. 2007; de Meijer et al. 2010). In addition, much literature has discussed the impact of steatotic donor livers in the context of transplantation. Macrosteatosis has been known to reduce graft survival 1 year after transplant (Spitzer et al. 2010) and increase the likelihood of primary non-function and initial poor function after transplant (Ploeg et al. 1993).

## 9.6 Strategies to Reduce I/R Injury

Although preclinical animal studies have offered numerous therapeutic strategies to mitigate hepatic I/R injury, promising outcomes have not been achieved in the clinical setting. This could be largely due to incomplete understanding of multifactorial nature of I/R injury. In this section, we discuss cellular and surgical approaches that may attenuate hepatocellular injury.

### *9.6.1 Pharmacological Approaches*

MPT onset is a principal step toward hepatocyte necrosis after warm I/R, and strategies to prevent the MPT have been intensively evaluated (Fig. 9.4). CsA is the most well-studied inhibitor of the MPT and has been shown to suppress hepatic I/R injury. However, this cyclophilin D blocker has a narrow therapeutic window at nanomolar concentration ranges. In addition to its nephrotoxic effects, this drug inhibits calcineurin, a $Ca^{2+}$- and calmodulin-dependent phosphatase, resulting in immunosuppression. NIM811, a non-immunosuppressive CsA derivative, mitigates hepatic I/R injury and improves regeneration after hepatectomy in rodent models (Waldmeier et al. 2002; Zhong et al. 2007, 2008; Rehman et al. 2011), but has yet to be tested clinically. TRO40303, which binds to the cholesterol site of the mitochondrial benzodiazepine receptor to block the MPT, did not show noticeable benefit after cardiac ischemia in clinical trials (Atar et al. 2015), and studies in liver have not been pursued. Importantly, more fundamental issues on CsA-based therapy

are that despite its capacity to prevent mitochondrial pore opening, this agent does little to mitigate upstream events of the MPT such as ROS and $Ca^{2+}$ accumulation. Therefore, cells could continuously amass ROS and $Ca^{2+}$ in the presence of MPT blockers. Additionally, hepatocytes can develop CsA-insensitive MPT when mitochondria encounter high dose of MPT inducers (He and Lemasters 2002).

After reperfusion, accumulation of calcium and ROS stimulates calpains, which ultimately impair the autophagic machinery and causes mitochondrial inner membrane permeabilization. Use of steroids, $Ca^{2+}$ chelators, and protease inhibitors has been proposed to ease reperfusion injury (Uchida et al. 1994; Yamashita et al. 2001; Gurusamy et al. 2010; Pan et al. 2012; Akhtar et al. 2013). Other agents include hemin and calcium chelators (Yun et al. 2014; Go et al. 2015), glutathione (Suyavaran et al. 2015), and *N*-acetylcysteine (Fukuzawa et al. 1995; Nakano et al. 1995). Attempts to enhance autophagy either through calpain inhibition or sustaining autophagic machinery have also been evaluated in the preclinical setting. Carbamazepine, an FDA-approved anticonvulsant drug, confers cytoprotection after I/R in mouse livers by preventing calpain activation and depletion of ATG7 and BECN1 (Kim et al. 2013). ALLM, a membrane permeable peptide that blocks calpains and cathepsins, promotes autophagy after I/R by preventing cytosolic SIRT1 degradation (Biel et al. 2016). Other SIRT1 activators such as SRT1720 and resveratrol could impart a protective effect after I/R through autophagy enhancement (Biel et al. 2016). In addition to SIRT1, autophagy inducers such as cisplatin or rapamycin have been demonstrated to attenuate hepatocellular necrosis after I/R (Cardinal et al. 2009a, b; Evankovich et al. 2012; Zhu et al. 2015).

### *9.6.2 Surgical Approaches*

Ischemic preconditioning (IPC) is a procedure whereby repeated short episodes of occlusion and release of the portal vein and hepatic artery mitigate the risk of I/R injury (Murry et al. 1990). Mechanisms underlying IPC cytoprotection are multifactorial and affect both hepatocytes and non-parenchymal liver cells. Mitochondrial integrity is better maintained, and cell death is reduced by IPC (Yadav et al. 1999; Clavien et al. 2003; Selzner et al. 2003b; Lee and Lee 2006; Ko et al. 2013). Autophagy may also be enhanced by IPC in a heme oxygenase 1-dependent manner (Liu et al. 2014). However, clinical benefits of IPC remain controversial, and there exists a disparity between IPC protocols and the heterogeneity of patients evaluated within and among studies (Clavien et al. 2000; Ye et al. 2014).

Similar to IPC, intermittent clamping utilizes shorter periods of occlusion and reperfusion (Selzner et al. 2003a). A prospective randomized study comparing prolonged Pringle to intermittent clamping demonstrates that intermittent clamping reduces peak transaminase levels and is associated with lower incidence of postoperative liver failure (Belghiti et al. 1999). Petrowsky et al. have reported that either IPC or intermittent clamping confers a comparable protection as measured by peak transaminase levels, duration of stay in the intensive care unit, total hospital length

of stay, and complication rate, although IPC presents somewhat lower intraoperative blood loss and shorter transection times (Petrowsky et al. 2006). Murine models further interrogating IPC and intermittent clamping suggest that beyond 75 min of continuous occlusion, intermittent clamping confers better cytoprotection than IPC (Rüdiger et al. 2002). Other studies with animal models have indicated that IPC and intermittent clamping provide equivalent protection in steatotic (Saidi et al. 2007) and cirrhotic livers (Jang et al. 2008). In contrast, intermittent clamping has been proposed as a better strategy to restore bile flow and reduce hepatocellular injury in aged livers (Schiesser et al. 2009). It is, however, unknown how intermittent clamping confers such benefits.

Whereas IPC and intermittent clamping primes the liver by direct occlusion and release of blood flow into the liver, remote ischemic preconditioning (RIPC) inflicts brief episodes of I/R to distant tissues or organs prior to liver resection (Gho et al. 1996). Beneficial outcomes of RIPC through occlusion of limb or intestine have been reported in the liver (Abu-Amara et al. 2011; Kageyama et al. 2015). The mechanisms by which RIPC provides protection against hepatic I/R injury remain to be elucidated, but heme oxygenase 1 may be an important player (Lai et al. 2006, 2007; Jia et al. 2015; Kageyama et al. 2015). Future studies are warranted to evaluate whether these promising preclinical results can be implemented in the clinical setting.

## 9.7 Concluding Remarks and Future Perspectives

As aging, obesity, diabetes, cancer, and metabolic syndrome prevail, a number of patients with acute and chronic liver disease continue to rise. It is anticipated that hepatic diseases will be a major socioeconomic burden in the future. Although liver resection and transplantation are still the most effective lifesaving interventions for patients with advanced liver disease, I/R injury is inevitable during these surgical treatments. To date, strategies to improve liver function after I/R have not been successful mostly due to an incomplete understanding of the pathogenesis of I/R injury. Dysfunctional autophagy and subsequent onset of mitochondrial malfunction are critical events contributing to reperfusion injury in the liver. Mitophagy selectively targets and removes damaged mitochondria laden with ROS, $Ca^{2+}$, and oxidative stress. Despite its therapeutic potentials for ameliorating hepatic I/R injury, our understanding of mitophagy remains limited. In addition, visible gaps are apparent between animal models and clinical settings. Thus, it is imperative to develop improved animal models that accurately reflect the patient population such as obese or aged patients. Future studies on mechanistic insights into mitophagy and its translational applications could lead to development of viable treatments for patients.

**Acknowledgments** This work was supported in part by US National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases grant DK079879 and DK090115 (J-S Kim) and National Institute on Aging AG028740 (J-S Kim).

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# Chapter 10
# Role of Endoplasmic Reticulum Stress in Hepatic Injury

**Cheng Ji, Neil Kaplowitz, and Hui Han**

## Abbreviations

| | |
|---|---|
| AA | Amino acid |
| ABCA1 | ATP-binding cassette transporter member 1, also known as the cholesterol efflux regulatory protein (CERP) |
| ACC | Acetyl-CoA carboxylase |
| ACL | ATP citrate lyase |
| ADH | Alcohol dehydrogenase |
| AERR | Alcohol-induced ER stress response |
| AKT | Also known as protein kinase B (PKB), is a serine−/threonine-specific protein kinase |
| ALD | Alcohol-induced liver disease |
| APAP | Acetaminophen |
| Apo-B/E | Apolipoprotein B/E |
| ARα | Androgen receptor-α |
| ASK1 | Apoptosis signal-regulating kinase 1 |
| ASMase | Acidic sphingomyelinase |
| ATF | Activating transcription factor |
| BAX | bcl-2-like protein 4 |
| BHMT | Betaine-homocysteine methyltransferase |
| BiP | Also known as *GRP78*, glucose-regulated protein 78 |
| C/EBP | CCAAT-enhancer-binding protein |
| CaMKII | Calmodulin-dependent protein kinase II |

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W.-X. Ding, X.-M. Yin (eds.), *Molecules, Systems and Signaling in Liver Injury*,
Cell Death in Biology and Diseases, DOI 10.1007/978-3-319-58106-4_10

X.-M. Yin and Z. Dong (Series eds.), *Cell Death in Biology and Diseases*

| | |
|---|---|
| CBS | Cystathionine β-synthase |
| CDIPT | CDP-diacylglycerol-inositol 3-phosphatidyltransferase |
| CHOP | C/EBP homologous protein |
| COX | Cyclooxygenases |
| CREB | Cyclic AMP response element-binding protein |
| Creld2 | Cysteine rich with EGF-like domains 2 |
| CYP2E1 | Cytochrome P4502E1 |
| DGAT2 | Diacylglycerol acyltransferase-2 |
| DR5 | Death receptor 5 |
| EDEM | ER degradation-enhancing mannosidase-like protein |
| eIF2α | Eukaryotic initiation factor 2α |
| ER | Endoplasmic reticulum |
| ERAD | ER-associated degradation |
| ERK | Extracellular signal-regulated kinases |
| ERO1 | ER oxidase 1 |
| ERα | Estrogen receptor-α |
| FAS | Fatty acid synthase |
| GADD34 | Growth arrest and DNA damage-inducible protein 34 |
| GGH | Ground glass hepatocyte |
| GSH | Glutathione |
| GSK3β | Glycogen synthase kinase-3β |
| HBV | Hepatitis B |
| HCA | Hepatocellular adenomas |
| HCC | Hepatocellular carcinoma |
| HCV | Hepatitis C |
| HDL | High-density lipoprotein |
| HepG2 | A perpetual cell line derived from human liver carcinoma cells |
| HERP | Homocysteine-induced ER protein |
| HFD | High-fat diet |
| HHcy | Hyperhomocysteinemia |
| HIV PIs | Anti-HIV protease inhibitors |
| HNF1 | Hepatocyte nuclear factor 1 α |
| Huh7 | A cell line derived from hepatic cellular carcinoma cells |
| IP10 | Interferon gamma-inducible protein 10 |
| IRE1 | Inositol-requiring enzyme 1 |
| JNK | c-Jun N-terminal kinases |
| LDLT | Living donor liver transplantation |
| LPS | Lipopolysaccharide |
| MCD | Methionine-choline deficient |
| MDDC | Monocyte-derived dendritic cells |
| MEOS | Microsomal ethanol oxidizing system |
| NADH | Nicotinamide adenine dinucleotide |
| NAFLD | Nonalcoholic fatty liver disease |
| NASH | Nonalcoholic steatohepatitis |
| NF-κB | Nuclear factor κB |
| NNRTI | Non-nucleoside reverse transcriptase inhibitors |

| | |
|---|---|
| NRF2 | NF-E2-related factor 2 |
| NRTI | Nucleoside/nucleotide reverse transcriptase inhibitors |
| NSAIDs | Nonsteroidal anti-inflammatory drugs |
| PERK | PKR-like ER-localized eIF2α kinase |
| PGC1 | PPAR gamma co-activator 1 |
| PP2Ac | Protein phosphatase 2Ac |
| PPAR | Peroxisome proliferator-activated receptor |
| PTEN | Phosphatase and tensin homolog |
| RIDD | Regulated IRE1-dependent mRNA decay |
| ROS | Reactive oxygen species |
| S1P | Site 1 protease |
| S2P | Site 2 protease |
| SAH | S-adenosylhomocysteine |
| SAM | S-Adenosylmethionine |
| SCD | Stearoyl-CoA desaturase |
| SERCA | Sarco/ER calcium ATPase |
| SHP | Small heterodimer partner |
| SREBP | Sterol regulatory element-binding protein |
| STAT3 | Signal transducer and activator of transcription 3 |
| sXbp1 | Unconventionally spliced Xbp1 |
| TRAF2 | TNF receptor-associated factor 2 |
| TRAIL | TNF-related apoptosis-inducing ligand |
| TRB3 | Tribbles 3 |
| TUDCA | Tauroursodeoxycholic acid |
| UDCA | Ursodeoxycholic acid |
| UPR | The unfolded protein response |
| VEGF | Vascular endothelial growth factor |
| VLDL | Very low-density lipoprotein |
| XBP1 | X-box binding protein 1 |

## 10.1 Introduction

The endoplasmic reticulum (ER) is a type of organelle in the cells of eukaryotes. The ER was first discovered in 1943 (Claude 1943), and its fundamental structure and function in protein synthesis were identified 2 years later (Porter et al. 1945). There are two types of ER, rough ER and smooth ER. The rough ER is an extensive membranous network of cisterns, branched tubules, and flattened sacs with the cytosolic face studded with ribosomes that are the sites of protein synthesis. The smooth ER lacks ribosomes and functions in synthesis of lipid and steroid hormones and drug detoxification. The rough ER consists of oxidizing conditions and a high calcium concentration, which are necessary for the formation of disulfide bonds and proper protein folding (Gaut and Hendershot 1993; Bulleid 2012). During protein synthesis, nascent proteins are translated into the ER lumen where they are

co-translationally and/or posttranslationally modified with oligosaccharyl residues for recognition by ER chaperones and subsequent extracellular secretion or intracellular distribution to other organelles (Ogata et al. 2006). Since its discovery, the ER has been shown to be essential in maintaining cellular protein homeostasis through integrating various intracellular and extracellular signals including growth, differentiation, inflammatory, and metabolic signals. Accumulation of misfolded/unfolded proteins will stress the ER and trigger the unfolded protein response (UPR), a unique set of molecular responses that are conserved among various eukaryotic organisms (Walter and Ron 2011; Mori 2015). The ER stress response contributes to development and progression of many human disorders, including but not limited to neurodegeneration, atherosclerosis, diabetes, infectious and inflammatory disease, and cancer (Ozcan and Tabas 2012; Wang and Kaufman 2012). The liver is particularly vulnerable to the ER stress as it consists of cells with abundant ER of microsomal structures, which assume synthesis of a large amount of secretory and membrane proteins (Palade 1995; Palade and Siekevitz 1956). The ER stress contributes to a spectrum of liver injuries, which are reviewed in this chapter.

## 10.2 Molecular Mechanisms of ER Stress Response

The ER stress is a state in which protein folding slows and unfolded or malfolded proteins accumulate in the ER (Mori 2000; Patil and Walter 2001; Schröder and Kaufman 2005; Ji and Kaplowitz 2006; Ron and Walter 2007; Ron and Hubbard 2013), which results from perturbations of ER homeostasis such as inhibition of glycosylation, alterations of ER redox environment, calcium depletion, lipid overloading, or viral infection. The ER stress activates the UPR, which constitutes a series of ER-to-nucleus signaling pathways that are mediated by three ER-resident transmembrane sensor proteins: inositol-requiring protein 1 (IRE1), dsRNA-activated protein kinase R PKR-like ER-localized eIF2α kinase (PERK), and activating transcription factor 6 (ATF6) (Fig. 10.1). The three sensors are activated upon dissociation from their inhibitory binding with the chaperone GRP78/BiP. IRE1, which has kinase and endoribonuclease activities, is activated by trans-autophosphorylation. The RNAse activity of IRE1 under low-activation conditions participates a process known as regulated IRE1-dependent mRNA decay (RIDD), which degrades RNA and reduces protein synthesis and loading in the ER (Maurel et al. 2014). The activated IRE1 processes mRNA of the transcription factor X-box binding protein 1 (XBP1) via unconventional splicing to form transcriptionally active spliced XBP1 (sXBP1). sXBP1, often known as a UPR marker, activates a number of UPR target genes, including chaperone genes that are responsible for restoring ER folding capacity and genes of the ER-associated degradation (ERAD) pathway that are important for cell survival (Hampton 2002). The second sensor PERK phosphorylates the eukaryotic initiation factor 2α (eIF2α) subunit, leading to an inhibition of the initiation of translation and a global attenuation in protein translation. Phosphorylation of eIF2α selectively activates activating transcription factor 4 (ATF4), which regulates genes for ER

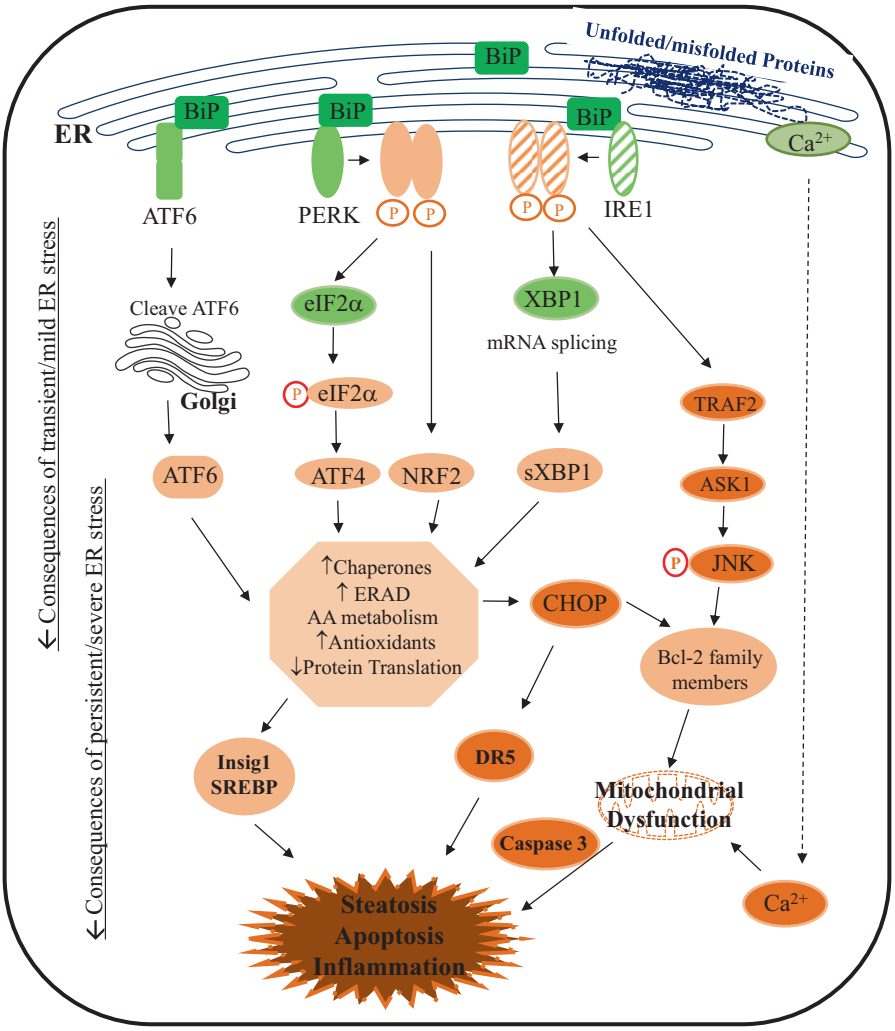


**Fig. 10.1** Canonical unfolded protein response (UPR) and consequences. Accumulation of unfolded/misfolded proteins activates the three ER sensors, ATF6, PERK, and IRE1. ATF6 is translocated from the ER to the Golgi apparatus where it is activated by proteolysis. Activated PERK phosphorylates either eIF2, which enables translation of ATF4 or NRF2. Active IRE1 is a kinase as well as an endonuclease that regulates the unconventional splicing of the transcription factor XBP1 into sXBP1. Cleaved ATF6, ATF4, NRF2, and sXBP1 are all nuclear transcription factors that upregulate the expression of UPR target genes/proteins which relates to ER chaperones, translational attenuation, the ERAD pathway, antioxidant responses, and amino acid metabolism resulting in the restoration of the homeostasis. However, prolonged and severe ER stress modulates Insig1/SREBP, CHOP/DR5, JNK/Bcl-2 family proteins, cytosolic $Ca^{2+}$ concentration, and mitochondrial function, leading to steatosis, apoptosis, and inflammation

chaperones, ERAD, and amino acid metabolism, and the transcription factor (C/EBP homologous protein (CHOP) (Oyadomari and Mori 2004). PERK also activates NRF2 that regulates cellular antioxidant responses. The third sensor ATF6 is cleaved in the Golgi to form a transcriptionally active fragment that traffics to the nucleus to activate UPR target genes. Therefore, the UPR in general results in reduced synthesis of proteins, increased capacity of folding, and increased turnover of unfolded proteins, which lead to restoration of ER homeostasis.

However, under prolonged or severe ER stress, the UPR provokes a complex network of interacting and parallel responses contributing to pathological consequences such as apoptosis, inflammation, and fat accumulation (Fig. 10.1) (Hotamisligil 2010; Ozcan and Tabas 2012; Wang and Kaufman 2012). The ER stress-induced apoptosis is mediated by a few factors. CHOP regulates GADD34 (growth arrest and DNA damage-inducible protein). GADD34 binds protein phosphatase-1 and enhances eIF2α dephosphorylation, leading to premature restoration of protein translation that further stresses the ER. CHOP also regulates expression of the TRAIL receptor DR5 and pro- and antiapoptotic Bcl-2 family proteins Bim, Bax, and Bcl-2 that modulate cell death (Tabas and Ron 2011). Sustained activation of IRE1 enables recruitment of the adaptor protein TRAF2 and activates ASK1, JNK, and NF-κB, which mediate apoptosis (Kitamura 2011). In addition, disturbance of ER calcium homeostasis, upregulation of ER oxidase 1 (ERO1) by CHOP, activation of caspase-12 (in animals) or caspase-4 (in humans), phosphorylation of AKT, and activation of GSK3β by tribbles 3 (TRB3) are other mechanisms underlying ER stress-induced inflammation and apoptosis (Ron and Hubbard 2013; Kim et al. 2015). Excessive lipid accumulation is also a main pathological feature of prolonged ER stress, and each of the three ER stress sensors has direct molecular effects on lipogenesis. The IRE1α-XBP1 branch regulates C/EBPα and C/EBPβ that directly control the expression of genes involved in de novo fatty acid biosynthesis (Rutkowski et al. 2008). The ATF6 branch is involved in phospholipid biosynthesis, fatty acid oxidation, and lipoprotein secretion (Zeng et al. 2004; Oyadomari et al. 2008). Severe or prolonged ER stress increases expression of eIF2α-specific phosphatase GADD34, which compromises the PERK-eIF2α signaling and reduced expression of C/EBPα/β and downstream transcription factor PPARγ resulting in interference with hepatic lipogenesis (Bobrovnikova-Marjon et al. 2008). The PERK-eIF2α signaling also controls activation of SREBP that regulates de novo lipid synthesis (Ji et al. 2006).

## 10.3 ER Stress in Alcohol-Induced Liver Disease

Alcohol is mainly metabolized in the liver. Partial role of ER in alcohol metabolism was initially realized decades ago when NADH from the hepatic oxidation of ethanol to acetaldehyde by alcohol dehydrogenase (ADH) was found to support also microsomal ethanol oxidations (Cinti et al. 1973; Lieber 1987). The inducible microsomal ethanol oxidizing system (MEOS) is associated with proliferation of

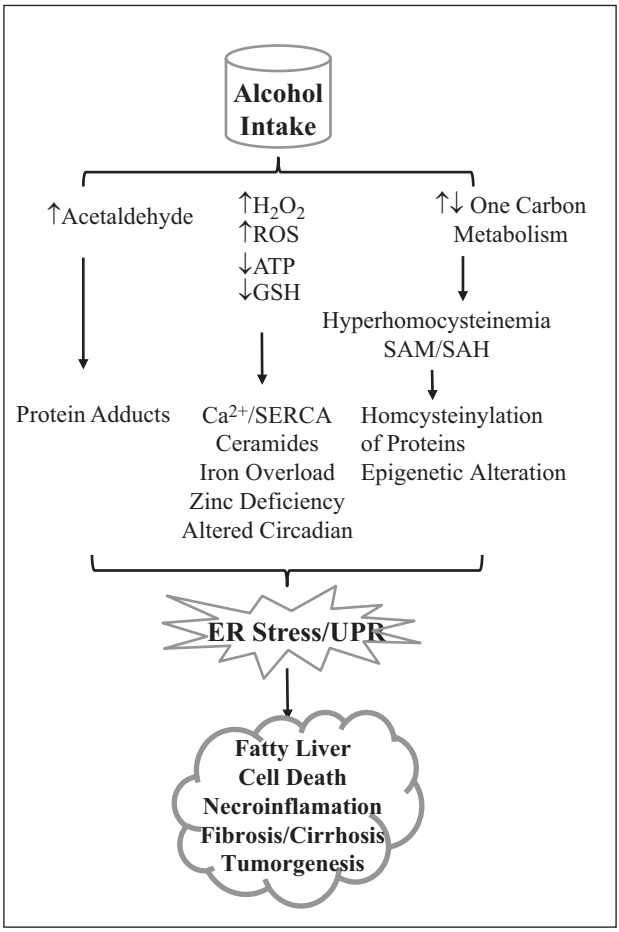


**Fig. 10.2** Multiple factors involved in alcohol-induced ER stress and liver injury. Alcohol, alcohol metabolites, and alterations of cellular redox and one carbon metabolism modify proteins that disturb ER protein homeostasis and cause ER stress response resulting in liver injury

the ER and a concomitant induction of cytochrome P4502E1 (CYP2E1) in rats and humans. Free radical release as a consequence of CYP2E1 function in the ER and subsequent oxidative stress and lipid peroxidation generally contribute to ALD (Cederbaum et al. 2009). However, alcohol-induced ER stress response (AERR) was not recognized until recently (Fig. 10.2). Molecular evidence for an impaired UPR was first found in the intragastric alcohol-fed mice using microarray gene expression profiling (Ji and Kaplowitz 2003). The alterations of selected ER stress markers were associated with severe steatosis, scattered apoptosis, and necroinflammatory foci. Moderate upregulation of expression of SREBP-1c and SREBP-2 and their responsive genes was detected by immunoblotting (Ji and Kaplowitz 2003; Ji et al. 2006). SREBP-1c knockout mice were protected against triglyceride

accumulation (Ji et al. 2006). Knocking out CHOP resulted in minimal alcohol-induced apoptosis in mouse liver (Ji et al. 2005). In a mouse model with moderate obesity and alcohol infusion, there are synergistic effects of accentuated ER and mitochondrial stress and nitrosative stress on hepatic necroinflammation and steatohepatitis (Xu et al. 2011). In micropigs fed alcohol, liver steatosis and apoptosis were shown to be accompanied by increased CYP2E1, GRP78, SREBP-1c, CYP2E1, and caspase-12 (Esfandiari et al. 2005), which were correlated with elevated transcripts of lipogenic genes such as fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and stearoyl-CoA desaturase (SCD). Further, alcohol consumption increases gut-derived lipopolysaccharide (LPS), which is linked to impair UPR and advanced hepatic injury. Full UPR as indicated by activation of IRE1α, ATF-6, and eIF2α was detected in cirrhotic livers of rats challenged with LPS, whereas only eIF2α was activated in the basal state in the cirrhotic rats without the LPS challenge (Tazi et al. 2007).

Besides rodents and micropigs, other species including humans have ER stress response to alcohol consumption. Zebrafish larvae metabolize alcohol that is simply added to the water (Passeri et al. 2009). Upon alcohol challenge, zebrafish larvae developed signs of acute ALD, including hepatomegaly and steatosis accompanied by changes in the expression of genes for SREBP-mediated hepatic lipogenesis. Further, the ER stress response appeared much robust in zebrafish deficient in CDP-diacylglycerol-inositol 3-phosphatidyltransferase (CDIPT) (Thakur et al. 2011). In the species, *Caenorhabditis elegans* without a liver for alcohol digestion, little AERR has been detected (Ient et al. 2012). In baboon fed alcohol orally, upregulation of calpain 2 and calpain p94 and downregulation of eIF2α were detected (Seth and Leo 2003). The most clinically relevant studies regarding AERR are from human cells and patients. AERR is reported in human monocyte-derived dendritic cells (MDDC) (Boukli et al. 2010), HepG2 cells expressing human CYP2E1 (Magne et al. 2011), and primary human hepatocytes (Kao et al. 2012). Striking upregulation of multiple ER stress signaling molecules was detected in human patients with ALD (Longato et al. 2012; Ramirez et al. 2013).

A major cause for AERR is alcohol-induced hyperhomocysteinemia that is often seen in rodents and humans (Barak et al. 1987; Hultberg et al. 1993; Blasco et al. 2005; Lutz 2008). A few lines of molecular evidence support the homocysteine mechanism. First, betaine is a methyl donor for re-methylation of homocysteine to methionine by betaine-homocysteine methyltransferase (BHMT). Simultaneous betaine feeding or transgenic expression of BHMT in alcohol-fed mice decreased elevated homocysteine and abrogated AERR (Ji and Kaplowitz 2003; Ji et al. 2004, 2008). Second, an intragastric infusion with both high fat and alcohol induced moderate obesity and much severe ALD (Xu et al. 2011), which resulted from synergistic effects of an accentuated ER stress by elevated homocysteine in combination with mitochondrial stress and adiponectin resistance. Third, rats with alcohol infusion expressed BHMT normally and did not develop a significant hyperhomocysteinemia (Shinohara et al. 2010). Consequently, the alcohol-infused rats had a minimal ER stress response and were more resistant to ALD. Fourth, in a study with 14 inbred mouse strains with alcohol infusion, profound differences in ALD were

observed among the strains in spite of consistently high levels of urine alcohol (Tsuchiya et al. 2012). ER stress-related genes were induced only in strains with the most liver injury, which were closely associated with expression patterns of methionine metabolism-related genes and plasma homocysteine levels. Thus, abnormal protein modifications by excessive homocysteine are likely responsible for the alcoholic ER stress and UPR in alcohol-infused mice that lack a sufficient upregulation of BHMT.

Multiple factors can cause AERR during ALD. Factors other than homocysteine include direct alcohol metabolites, epigenetic changes, genetic predisposition, iron overload, zinc deficiency, circadian clock, ceramide, and disrupted $Ca^{2+}$ homeostasis (Fig. 10.2). First, alcohol metabolites, e.g., acetaldehyde, or oxidants were found contributing to AERR and development of fatty liver in a study examining effects of a selective inhibition of CYP2E1 on the development of ALD in alcohol-infused rats (Ronis et al. 2010). Second, epigenetic and genetic alterations are related to AERR. Severe steatohepatitis and upregulations of hepatic ER stress signals were associated with decreased SAM/SAH ratio in alcohol-infused mice deficient in heterozygous cystathionine β-synthase (CBS) (Esfandiari et al. 2010). The low SAM/SAH ratio was correlated with decrease levels of suppressor chromatin marker trimethylated histone H3 lysine-9 (3meH3K9) in the promoter regions of ER stress markers. Such epigenetic mechanism for AERR also occurs in human alcoholics. Epigenetic DNA hypermethylation of the promoter of the ER stress-responsive HERP gene has been reported to downregulate its mRNA expression in patients with alcohol dependence (Bleich et al. 2006; Han et al. 2013). With respect to effects of genetic defect on AERR, a robust ER stress response was observed in the mice with a liver-specific deletion of BiP and fed moderate doses of alcohol (Ji et al. 2011), which was accompanied by much aggravated hepatic steatosis and fibrosis. This suggests that the liver BiP deletion represents a genetic predisposition that unmasks a distinct mechanism by which alcohol induces ER stress, one that normally is largely obscured by compensatory changes in normal animals or presumably in the majority of human population who have low-to-moderate drinking. Third, evidence for a causal effect of iron overload on AERR was reported in mice deficient in the hemochromatosis gene ($Hfe^{-/-}$). Co-feeding ad libitum with alcohol and a high-fat diet (HFD) was found to lead to increased XBP1 splicing, activation of IRE1α and PERK, and CHOP protein expression, which were associated with profound steatohepatitis and fibrosis (Tan et al. 2013). In contrast to iron overload, alcohol induces zinc deficiency in alcoholics, which links to ER stress and cell death damages. In Wistar rats fed chronic alcohol, it was found recently that increased expression of hepatic p-eIF2α, ATF4, and CHOP and activation of caspase-3 were associated with increased cell death and reduced hepatocyte ER zinc levels. The alcohol-induced ER stress and cell death could be inhibited by zinc supplement but not by antioxidant treatment (Sun et al. 2015). Fourth, circadian clock has recently been found to be associated with AERR and fatty liver injury (Hiroyuki et al. 2014). Alcohol disturbs the nuclear receptor small heterodimer partner (SHP)-mediated circadian clock and thus impairs oscillations of ER stress response and lipid accumulation. Fifth, alterations of lipid composition of the cellular membranes by alco-

hol can be a direct cause for AERR. One of the common phospholipids of membrane bilayers is sphingomyelin. Acid sphingomyelinase (ASMase) hydrolyzes sphingomyelin into ceramides, which, in addition to their critical structural function in membrane bilayers, regulate apoptosis, cellular stress response, inflammation, and metabolism (Marí et al. 2014). Alcohol feeding increases ASMase expression and activity in both experimental models and patients with acute alcoholic hepatitis (Fernandez et al. 2013). Particularly, incubation of HepG2 cells with exogenous ASMase disrupts ER $Ca^{2+}$ homeostasis. Since SERCA contributes to $Ca^{2+}$ homeostasis in the ER (Pinton et al. 2001), it is likely that ASMase activation and subsequent ceramide production disrupt physical properties of the ER membrane, which modulates SERCA activities. Thus, SERCA activities might be a key element in AERR and liver injury. Results from other earlier studies also support the role of SERCA in AERR. In mice, alcohol exposure aggravates the inhibitory effects on SERCA and $Ca^{2+}$ homeostasis by some anti-HIV drugs (Kao et al. 2012). In a model for dendritic regression of Purkinje neurons from the cerebellar cortex of ethanol-fed rats, dilation of the extensive smooth ER and altered SERCA activities were shown to precede activation of ER-resident caspase-12 (Dlugos 2014).

In brief, alcohol-induced hepatic ER stress occurs in the livers of several species including mouse, rat, minipigs, zebrafish, and humans, which can be caused by multiple factors and contributes significantly to ALD.

## 10.4 ER Stress in NAFLD/NASH

Lipid accumulation in the absence of alcohol consumption characterizes nonalcoholic fatty liver disease (NAFLD). When members of the lipid family such as free fatty acids and free cholesterol become excessive, they can cause apoptosis of hepatocytes, which advances NAFLD to severe nonalcoholic steatohepatitis (NASH). NASH is a risk factor for downstream hepatic inflammation and various degrees of fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). NAFLD and NASH become increasingly common worldwide. It is a recent research focus to understand the molecular mechanisms responsible for the development of NAFLD and progression of NAFLD to NASH. The ER stress is likely an important initial factor contributing to the hepatic lipid accumulation, and lipotoxicity is central to the subsequent development of steatohepatitis. The role of non-proteasomal ER pathways in lipid metabolism was initially noticed when the ER stress-inducing agent, tunicamycin, inhibited N-linked glycosylation and interfered with the production of apolipoprotein B-100 (apoB-100) in HepG2 cells (Liao and Chan 2001) and another ER stress-inducing agent, thapsigargin (TG), activated the ER-resident caspase-12 and impaired calcium homeostasis in Huh7 cells, which was inhibited by tauroursodeoxycholic acid (TUDCA) (Xie et al. 2002). The role of ER stress in lipid accumulation was further established when alcohol-induced fatty liver disease was reported to be associated with ER stress and UPR in vivo animal models (Ji and Kaplowitz 2003) and the ER was reported to be the site of cholesterol-induced cytotoxicity

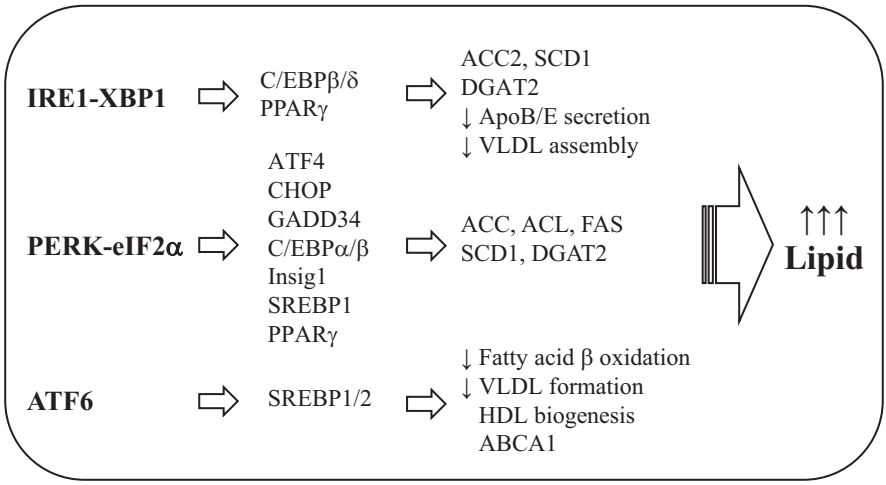


**Fig. 10.3** The UPR and hepatosteatosis. Each of the three UPR branches is directly involved in regulating transcription factors and/or enzymes for lipogenesis resulting in fat accumulation in the liver under ER stress conditions

(Feng et al. 2003). Following these earlier reports, the induction of ER stress was first described in the livers of genetic and diet-induced models of NAFLD/NASH (Ozcan et al. 2004). Moreover, chemical chaperones inhibited ER stress as well as hepatic lipid accumulation in a murine model of hepatic steatosis (ob/ob mice) (Ozcan et al. 2006). These findings have since been confirmed in several other animal models with obesity or without obesity (e.g., a methionine-choline-deficient (MCD) diet-induced hepatic steatosis) (Wang et al. 2006a, b; Rahman et al. 2007; Yang et al. 2007; Sreejayan et al. 2008). Of clinical relevance, alterations of several UPR components including increased sXBP-1, increased phosphorylation of eIF2α and JNK, and activation of ATF4 and CHOP were further found in the livers of patients with NAFLD/NASH (Puri et al. 2008). Taken together, these reports highlight the importance of ER stress in the development of NAFLD/NASH.

How ER stress regulates the lipid metabolism in the liver is under intense investigations. Several lines of molecular evidence indicate that each of the three ER stress-sensing pathways (IRE1α/XBP1, PERK/eIF2α, and ATF6α) plays a direct role in regulating the development of microvesicular steatosis in the liver (Fig. 10.3). For the role of the IRE1α-XBP1 pathway in steatosis, a hepatocyte-specific deletion of IRE1α in mice has been demonstrated to repress expression of key metabolic transcriptional regulators, including C/EBPβ, C/EBPδ, and PPARγ and enzymes involved in triglyceride biosynthesis resulting in severe hepatic steatosis under ER stress (Zhang et al. 2011). IRE1α was required for efficient secretion of apolipoproteins upon disruption of ER homeostasis in the liver of IRE1α-deficient mice. XBP1 of this pathway regulates hepatic lipogenesis through directly binding to the promoters of lipogenic genes including SCD1, diacylglycerol acyltransferase-2 (DGAT2), and ACC2, which activates expression of these genes in hepatocytes (Lee et al. 2008). De novo lipid biosynthesis was reduced in the livers of mice with an

XBP1 deletion. A recent study demonstrated that the IRE1α-XBP1 pathway plays a critical role in the assembly and secretion of very low-density lipoprotein (VLDL) in the liver. Mice with the genetic ablation of IRE1α in hepatocytes demonstrated much severe hepatosteatosis upon metabolic challenges such as fasting and a high fructose diet (Wang et al. 2012), suggesting that the IRE1α-XBP1 pathway appears to be required for the maintenance of hepatic lipid homeostasis under ER stress conditions. For the role of the PERK-eIF2α-ATF4 pathway in steatosis, a deletion of PERK has been shown to inhibit the sustained expression of the lipogenic enzymes FAS, ATP citrate lyase, and SCD1 (Bobrovnikova-Marjon et al. 2008). ATF4 has been shown to activate C/EBPα and C/EBPβ regulating hepatic lipogenesis (Rahman et al. 2007; Millward et al. 2007). A heterozygous ATF4 deletion not only resulted in significantly decreased liver and white adipose tissue expression of lipogenic genes, such as PPARγ, SREBP-1c, ACC, and FAS, but also protected mice from diet-induced hepatic steatosis (Seo et al. 2009; Wang et al. 2010; Xiao et al. 2013). ATF4 also modulates CHOP functions regulating key genes involved in lipid metabolism (Chikka et al. 2013). Further, overexpression of GADD34 downstream of this pathway selectively dephosphorylated eIF2α, which compromised eIF2α-mediated signaling under ER stress conditions and resulted in diminished hepatosteatosis in animals fed a high-fat diet (Oyadomari et al. 2008). In addition, protein kinase-mediated p-eIF2α increases ATF4 translation. Thus, the PERK-eIF2α-ATF4 pathway plays an important role in promoting lipogenesis. For the role of the ATF6 pathway in steatosis, activated nuclear ATF6α has also been shown to interact with the nuclear form of SREBP2 thus inhibiting its transcriptional activity and reducing hepatic lipid accumulation (Zeng et al. 2004). In response to pharmacological ER stress, ATF6α-ablated mice developed hepatic steatosis as a result of reduced fatty acid β-oxidation and attenuated VLDL formation (Rutkowski et al. 2008; Yamamoto et al. 2010)). Moreover, in response to feeding of a high-fat diet, the ATF6α-deficient mice developed greater degrees of hepatic steatosis and glucose intolerance in association with increased expression of SREBP1c (Usui et al. 2012). Therefore, the ATF6 pathway also plays a direct role in the ER stress-induced lipid accumulation.

Under conditions of chronic ER stress, inflammatory response such as activation of NF-κB and JNK can be initiated generally by unfolded protein that caused ROS and oxidative stress and by PERK, ATF6, or the IRE1α-TRAF2 complex (Urano et al. 2000). More significantly, the inflammation can be contributed by lipotoxic members of the lipid family during NAFLD. Several lines of evidence indicate that excessive hepatic lipid accumulation interferes with ER stress response and promotes cell death and progression of steatosis to steatohepatitis. ER stress is reported to impair hepatic ABCA1 function, HDL biogenesis, and cholesterol efflux and synthesis in human hepatic cells (Röhrl et al. 2014). Excess cellular cholesterol loading increases cholesterol trafficking to ER membranes, depletes ER calcium stores, and activates the UPR and CHOP leading to apoptosis (Feng et al. 2003; Lluis et al. 2003). Studies have also shown that palmitate and other saturated fatty acids activate the UPR and apoptosis via the PERK/ATF4/CHOP pathways in human liver cells (Wei et al. 2006; Cao et al. 2012). Deletion of CHOP protects

hepatocytes from palmitate-induced apoptosis in vitro via inhibition of JNK activation (Pfaffenbach et al. 2010; Zhang et al. 2015). Further, unsaturated fatty acids rescue palmitate-induced apoptosis by channeling palmitate into triglyceride pools and away from pathways leading to apoptosis (Wei et al. 2006). Particularly, palmitoleate can block ER stress-associated increases of the BH3-only proteins Bim and PUMA and inhibits lipoapoptosis in human and mouse primary hepatocytes and the Huh7 and Hep 3B cell lines (Cazanave et al. 2010), which is probably through protecting the hepatocytes against downstream death mediator Bax. Furthermore, saturated fatty acids and cholesterol have been shown to work synergistically to promote ER stress and cell death (Pineau et al. 2009), which could be attenuated by short-term exposure to unsaturated fatty acids that enables more efficient triglyceride synthesis and VLDL secretion (Listenberger et al. 2003; Fu et al. 2012). However, the lipotoxic effect of fatty acids and cholesterol on ER stress and cell death could not be rescued under chronic conditions as chronic exposure to oleic acid, a more physiologically relevant model of metabolic disease, induces ER stress and suppresses VLDL secretion (Ota et al. 2008). Under obese conditions, there are disruptions in ER calcium retention and shift in lipid composition (Fu et al. 2011), which could promote ER stress and result in an irreversible progression of NAFLD to NASH. Most interestingly, these lipotoxic effects resulting from chronic ER stress apparently occur in human patients with NASH (Lake et al. 2014).

In summary, the UPR signaling directly regulates lipid metabolism which contributes to lipid accumulation in the liver. Excessive lipids resulted from chronic/severe ER stress upregulate genes involved in apoptosis, which may further contribute to the liver transformation from simple steatosis to NASH.

## 10.5 ER Stress in Drug-Induced Hepatotoxicity

Many drugs target the liver, cellular enzymes of which such as cytochromes P450 can metabolize drugs generating numerous reactive electrophilic metabolites and free radicals that cause damages to the liver. Such drug-induced liver injury is a major concern for the well-being of both patients and pharmaceutical companies because it is one of the leading causes for drug failure, interruption of clinical trials, or withdrawal from the market after approval (Lee 2003; Kaplowitz 2005; Labbe et al. 2008; Senior 2014). To prevent or manage the drug-induced injury, it is fundamental to understand the molecular mechanism underlying the drug-induced hepatotoxicity. Multiple factors including binding of toxic drug metabolites to proteins/lipids/DNA, glutathione depletion, mitochondrial dysfunction, oxidative stress, lipid peroxidation, cell death, and impaired immune response are known to be involved in the liver toxicity (Gunawan and Kaplowitz 2007). In the recent years, ER stress has begun to be recognized playing an important role in the drug-induced liver toxicity, which has helped identify additional risk factors.

A number of pharmacological drugs induce ER response in the liver. First, nonsteroidal anti-inflammatory drugs (NSAIDs) such as cyclooxygenase (COX)

inhibitors, diclofenac and indomethacin, are used in the treatment of acute and chronic inflammation. Apoptosis is associated with side effects of NSAIDs. Impaired UPR is found to be responsible for the NSAID-induced cell death in Huh7 cell lines (Franceschelli et al. 2011). Either diclofenac or indomethacin is able to activate only the PERK pathway that regulates the expression of the proapoptotic CHOP protein. The indomethacin-induced apoptosis could be suppressed in CHOP-deficient mice and in cultured guinea pig gastric mucosal cells by expression of the dominant-negative form of CHOP. These results indicate that ER stress-related CHOP may be involved in NSAID-induced apoptosis (Tsutsumi et al. 2004).

Second, thiazolidinediones (TZDs) such as ciglitazone and troglitazone are synthetic PPAR ligands that promote increased insulin sensitivity in type II diabetic patients. However, ciglitazone and troglitazone were found to induce CaMKII phosphorylation in liver epithelial cells, which correlated with induction of ER stress as indicated by eIF2α or PERK activation. A selective CaMKII inhibitor attenuated MKK3/6 and p38 as well as PERK and eIF2α phosphorylation (Gardner et al. 2005). Macelignan, a natural compound isolated from *Myristica fragrans*, reduced ER stress and JNK activation in the liver and adipose tissue of db/db mice and subsequently increased insulin signaling (Han et al. 2008). These data provide evidence supporting that TZDs likely deplete ER calcium, activate CaMKII, and phosphorylate PERK or eIF2α leading to ER stress response, which exerts antiproliferative effects and subsequent hepatic cell death.

Third, antalgic and antipyretic acetaminophen (APAP) is a popular drug for the management of pain and hyperthermia. However, APAP often induces hepatic injury, which is mediated by several mechanisms including mitochondrial alterations, ATP depletion, JNK activation, oxidative stress, and increased cytosolic calcium (Burcham and Harman 1988; Jaeschke 1990; Aubert et al. 2012; Hur et al. 2012). Recent in vivo and in vitro evidence indicates that APAP is also able to induce ER stress response that could contribute to hepatic cell death (Lorz et al. 2004; Nagy et al. 2007; Uzi et al. 2013; Kalinec et al. 2014). Mice with CHOP deficiency are resistant to a lethal dose of APAP (1 g/kg). However, at a lower dose (500 mg/kg), the APAP-induced ER stress was a relatively late event (Hur et al. 2012; Uzi et al. 2013), suggesting that the ER stress-mediated hepatotoxicity by APAP could be either significant at extremely high doses or secondary to mitochondrial dysfunctions at medium to high doses (Macanas-Pirard et al. 2005).

Fourth, drug resistance is common in HCC, which can be a major problem in HCC chemotherapy. One of the underlying mechanisms that mediate the drug resistance involves drug-induced ER stress and surviving effects of the UPR. For instance, anticancer cisplatin treatment triggers the UPR in HCC cells, which subsequently increases Hsp27-mediated autophagy activation and inhibits cisplatin-induced apoptosis (Chen et al. 2011). The cisplatin-induced ER stress is reported to mediate chemoresistance after living donor liver transplantation (LDLT) for patients, in which HCC tumor recurrence is an obstacle and anticancer treatment is often needed after the liver surgery. The cisplatin treatment has been shown to activate ATF6/GRP78 signaling and cause overexpression of interferon gamma-inducible

protein 10 (IP10) that promoted HCC cell proliferation and tumor growth (Geng et al. 2015). Similarly, certain antioxidants are also shown to be inducers of hepatic ER stress that compromises the anti-HCC treatment (Schonthal 2012). Baicalein is a flavonoid that activates eIF2α and IRE1α and autophagy and downregulates the pro-survival Bcl-2 family, increases intracellular calcium, and activates JNK and CHOP. These mixed pro-survival and pro-death effects may limit baicalein's anti-HCC activity (Wang et al. 2014). Thus, combination of the anticancer drugs with other inhibitors against survival effects of UPR and autophagy may result in better therapeutic effects against HCC.

Fifth, antiretroviral drugs such as HIV protease inhibitors (HIV PIs) and non-nucleoside reverse transcriptase inhibitor (NNRTI) are used in the highly effective treatment for HIV-infected patients. However, antivirals singly or in combination increase the risk for HIV-associated liver disease (Núñez et al. 2001; Jones and Núñez 2012). There are reports indicating that some PIs such as atazanavir, lopinavir, and ritonavir and NNRTI such as efavirenz induce ER stress response, which is associated with altered lipid metabolism, inflammation, and apoptosis in hepatocytes and mouse liver (Cao et al. 2010; Apostolova et al. 2013; Taura et al. 2013). Moreover, the HIV PI-induced ER stress indicated by major ER stress markers can be worsened synergistically by alcohol exposure in mice and primary mouse and human hepatocytes (Kao et al. 2012). These reports support that ER stress is a pathogenic mechanism for the liver toxicities of some antiretroviral drugs. However, the precise mechanisms by which the PIs induce ER stress are still under investigations. One possible mechanism could be nonspecific inhibition of proteasome activity by the peptide mimetic PIs. Proteasome inhibition has been shown for atazanavir, lopinavir, and ritonavir (Schmidtke et al. 1999; Pajonk et al. 2002; Piccinini et al. 2002; Bono et al. 2012). Another possibility is that PI-induced oxidative stress could trigger the ER stress response (Touzet and Philips 2010; Taura et al. 2013). In addition, the PIs could interact directly with ER membrane proteins and the UPR components. For instance, inhibition of the ER-resident SERCA that regulates calcium homeostasis by lopinavir and ritonavir has been reported (Kao et al. 2012; Hu et al. 2015; Han et al. 2017).

## 10.6 ER Stress in Hepatitis Virus Infections

In hepatitis virus infections, viruses subvert the cellular translation machinery to produce large amounts of viral proteins, which leads to an overwhelming load of unfolded or misfolded proteins, causes ER stress, and triggers the UPR in the host hepatocytes. The UPR in the host interacts with the viruses, and the outcome can either be host cell protective, i.e., restricting viral protein production through translation attenuation, or host cell apoptosis and proviral, i.e., enabling viral protein production through chaperone production. The diverse role of ER stress and UPR in hepatitis virus infections and pathogenesis has often been demonstrated. In cirrhotic and carcinoma liver biopsies from HBV-infected patients, HBV infection was found

to alter subcellular structures and induce a marked hypertrophy of the ER within hepatocytes (Hadziyannis et al. 1973). These hepatocytes termed "ground glass hepatocyte" (GGH) were found to have unusual expression and intracellular accumulation of HBV surface proteins (Hadziyannis et al. 1973; Popper 1975). Further, the accumulation of the viral proteins and the development of the GGH morphology were linked to mutations in the entire pre-S region that are secretion incompetent (Xu and Yen 1996; Tai et al. 2002). The accumulation of HBV surface proteins in host hepatocytes specifically activates ER stress signaling pathways as indicated by the upregulation of GRP78, GRP94, and ER-resident kinases (Wang et al. 2006a, b). These studies demonstrated that the ER stress induced by the pre-S mutants plays a key role in progression of HBV pathogenesis and the virus may have evolved through mutation to manipulate host UPR signaling pathways to promote its translation and persistence in infected cells.

Interactions of specific UPR components with unfolded viral proteins can trigger the UPR response during viral infection. It is reported that the chaperone GRP78 was associated transiently and non-covalently with the unfolded or immature glycoproteins during HCV infection, which were not transported to the plasma membrane but persist as complexes in the ER for a long period of time before degradation (Ng et al. 1989; Choukhi et al. 1998). On the other hand, in virus-infected cells, core and NS3 proteins of HCV are inserted into the ER and transported to cell surface and activate the expression of GRP78 (Liberman et al. 1999). HCV replicons expressing only nonstructural proteins are also capable of stimulating GRP78 expression (Tardif et al. 2002). These studies suggest that either the process of viral replication or the production of a specific viral protein in the ER is capable of inducing UPR response.

HCV can also promote its replication through suppressing the pro-survival UPR pathways and impairing antiviral pathways. It is reported that HCV replicon induces sXBP-1 but inhibits ERAD in Huh7 (Tardif et al. 2004; Zheng et al. 2005). HCV gene expression correlates the translocation of ATF6 cytoplasmic domain to the nuclei of cells that are expressing HCV subgenomic replicons. ATF6 activates the IRE1-XBP1 pathway by upregulating the transcription of XBP1. Both XBP1-spliced mRNA and sXBP1 protein were elevated in the HCV replicon-expressing cells (Tardif et al. 2004; Zheng et al. 2005), indicating that IRE1-catalyzed splicing is enhanced in the cells that host HCV gene expression. However, the downstream activity of the XBP1 is somehow repressed in the HCV-infected cells which prevents transcriptional induction of the ER degradation-enhancing mannosidase-like protein (EDEM) and inhibits ERAD (Tardif et al. 2004). Similarly, in Hep3B cells infected with HBV, expression of the multifunctional regulatory protein of HBV (HBx protein) alone is sufficient to activate both the ATF6 and IRE1-XBP1 pathways, and silencing HBx blocks their activation induced by the constitutive replication of HBV (Li et al. 2007). Thus, the protective UPR signaling in virus-infected cells is somehow interrupted and fails to further remove the excessive viral proteins. In addition, HCV has been reported to stimulate fatty acid synthesis for virus replication and maturation (Ke and Chen 2012; Targett-Adams et al. 2010). This has been speculated to be an adaptive mechanism employed by the viruses to use the

UPR signaling pathways for their own benefit given that activation of the IRE1/XBP1 pathway could increase the surface area of the ER to support the buildup of glycoproteins and enable virion budding or expand ER to reduce ER stress resulted from virus maturation. Thus, regulation of the host UPR by HCV and HBV may be an important pathophysiologic process utilized by the viruses.

The hepatitis virus can evade the host UPR resulting in pathological consequences. For instance, expression of HCV replicon has been reported to decrease glycosylation and prevented assembly of MHC-1, which allow continued virus production in host cells under severe ER stress and avoidance of immune clearance resulting in apoptosis and liver injury (Fournillier et al. 2001; Tardif and Siddiqui 2003; Fang et al. 2006; Lin et al. 2014). HCV or HBV protein expression in liver cells induces ER stress response resulting in $Ca^{2+}$ release from the ER, which has been demonstrated to activate cyclic AMP response element-binding protein (CREB) (Benali-Furet et al. 2005, Christen et al. 2007). The activated CREB upregulates protein phosphatase 2Ac (PP2Ac), which impairs cell cycle regulation leading to apoptosis and hepatocarcinogenesis. In addition, expression of HCV core in Huh7 or HepG2 triggers hyper-expression of GRP78, GRP94, and calreticulin, CHOP induction, BAX translocation to mitochondria, cytochrome c release, and cleavages of caspase-3 and PARP (Benali-Furet et al. 2005). In the liver of transgenic mice, conditional expression of HCV structural proteins increased CHOP and cleaved caspase-3 and hepatic apoptosis (Sreejayan et al. 2008). These reports support that there is a direct link between hepatitis virus infection, ER stress, and liver injury.

## 10.7 ER Stress in Liver Cancer

During initiation, transformation, and establishment of liver cancer, the UPR can be activated by increased protein folding and assembly in the ER of liver cancer cells that rapidly grow and proliferate, by accumulations of mutant proteins that cannot be correctly folded, and by poor vascularization that creates hypoxia, glucose deprivation, and oxidative stress. The pro-survival capabilities of the UPR may provide chronic support for continuous proliferation and survival of liver cancer cells even under adverse conditions. Recent evidence indicates that nearly all the branches of the UPR signaling pathways are associated with liver cancer. In human HCC, elevated GRP78, ATF6, and IRE1 activations were observed (Shuda et al. 2003; Hetz et al. 2006; Al-Rawashdeh et al. 2010). Elevated Grp78 level has been reported to correlate well with higher pathologic grade, recurrence rate, and poor survival in patients with liver cancer (Luo and Lee 2013). Similarly, animal studies demonstrated that XBP1 is required for tumor growth in vivo. Knocking out Xbp1 blocked tumor formation in mice even though their growth rate and secretion of vascular endothelial growth factor (VEGF) were not decreased (Romero-Ramirez et al. 2004). Overexpression of XBP1s is associated with HCC in patients (Carrasco et al. 2007). Moreover, the contributory role of each of the UPR components to liver

tumor initiation and development can be dynamic. For example, in a mouse model of HCC, increased expression of ER-resident chaperones was observed during tumor initiation and tumor progression (Vandewynckel et al. 2014). The IRE1 signaling pathway was activated only during tumor initiation, whereas the ATF6 pathway was activated only after tumor initiation. Interestingly, the PERK pathway was activated during tumor progression and continued to rise in the tumors, which make the PERK pathway the most promising target for HCC therapy (Vandewynckel 2014; Bu and Diehl 2016). In addition, ER stress can induce other antiapoptotic responses during tumorigenesis. Activated GSK3β by ER stress has been reported to phosphorylate p53, increase its degradation, and therefore protect cancer cells from p53-dependent apoptosis (Goldstein and Li 2009). NF-κB is activated during ER stress to regulate antiapoptotic responses (Kitamura 2011). Major oncogenes including Bcl-2, Bcl-XL, Mcl-1, Akt, and Ras and tumor suppressors such as p53, PTEN, and Beclin 1 directly regulate SERCA (Bittremieux et al. 2016), which alters $Ca^{2+}$ signaling, promotes a high tolerance toward cell stress and damage, and favors malignancy.

Despite the above firm association of ER stress with liver cancer, the complete role of the UPR in liver carcinogenesis, especially how it mediates two conflicting outcomes, has yet to be defined. The dual role of GRP78/BiP in tumorigenesis is a typical example (Ji et al. 2011; Lau et al. 2013; Zhu and Lee 2015). BiP controls early tumor development through suppressive mechanisms such as the induction of cell cycle arrest, reprograming of lipid metabolism, or tumor dormancy via activating PERK (Weston and Puthalakath 2010). On the other hand, at more advanced stages of tumor progression, during which cells are exposed to more severe stressors, BiP suppresses caspase-7 activation and interacts with ER stress-induced chaperone proteins such as clusterin to promote cell survival and tumor development (Wang et al. 2013a, b). In addition, spontaneous hepatocellular adenomas (HCA) in aged female are found in mice with a liver-specific BiP deletion and under constitutive ER stress (Han et al. 2013; Lau et al. 2013). Active ATF6, CHOP, GSK3β, and Creld2 (cysteine rich with EGF-like domains 2) are increased in the knockout, indicative of continuous ER stress response. None of p53, HNF1α, or GP130 is significantly changed compared between wild type and knockout. Interestingly, cyclin D is reduced in the tumor portion of the knockout mice, which is specifically associated with overexpression of a variant of estrogen receptor-α (ERα). In contrast, there were no significant changes in the expression of ERβ, androgen receptor-α (ARα), cyclin E, or cyclin G. These findings indicate that inhibition of cyclin D and overexpression of an estrogen receptor variant promote the liver tumor development in the female BiP knockouts (Lau et al. 2013). Moreover, pathways of ERK1/2, Stat3, and p38 indicative of high malignancy are activated in these knockout mice treated with additional stresses such as high-fat diet or alcohol exposure (Han et al. 2013; Lau et al. 2013; Ji 2015). Therefore, the role of ER stress in liver cancer is more complex than initially anticipated, which may be determined by multiple endogenous and exogenous factors that change the delicate balance between the UPR-influenced oncogenes and tumor suppressors.

## 10.8 ER Stress in Inter-organelle Cross Talk

The ER, Golgi apparatus, and lysosomes are major components of the endomembrane system of hepatocytes, which play critical roles in cell homeostasis. Although each of the membranous organelles forms a single functional unit and their membranes cannot fuse with each other directly, they communicate through vesicle transport or inter-organellar connections (Daniele and Schiaffino 2014; Senft and Ronai 2015). The inter-organellar connections also exist between membranes of the ER, the nuclei, and the mitochondria. These connections enable inter-organellar information exchange, pathway coordination, or cross talk during stress responses, which leads to either recovery of cellular homeostasis or facilitation of disease development. There are several potential pathways for the inter-organelle cross talk. First, the activation of ATF6 requires both the ER and Golgi apparatus. Upon ER stress, phosphorylated ATF6 is transported to the Golgi in a COP-II vesicle and sequentially cleaved by S1P and S2P proteases, resulting in release of the cytoplasmic domain of ATF6 (Ye et al. 2000). The activated ATF6 mediates the UPR survival response involving an arrest of general protein synthesis and selected synthesis of chaperone proteins needed for ER homeostasis (Walter and Ron 2011; Wang and Kaufman 2012). Second, CHOP was initially found to mediate ER stress-caused cell death (Ji et al. 2005; Ron and Walter 2007; Hu et al. 2015). However, CHOP is also increased upon accumulation of unfolded proteins in the mitochondrial matrix, which mediates the mitochondrial UPR ($UPR^{mt}$) (Aldridge et al. 2007; Horibe and Hoogenraad 2007). Third, the expression of the ER-resident HSP47 can be induced by the Golgi dysfunction, which protects cells from Golgi stress-induced apoptosis (Miyata et al. 2013). Fourth, it is known that acute or chronic alcohol inhibits the ER-resident SERCA in human and mouse hepatocytes (Kao et al. 2012) and in neurons of mouse spinal cord and rat Purkinje (Dlugos 2014). Since there is a SERCA-mediated intricate relationship between the ER and mitochondria in intracellular calcium homeostasis, the alcoholic inhibition of SERCA could accommodate a massive transfer of calcium from the ER to mitochondria exacerbating oxidative stress. Similarly, palmitate treatment of primary hepatocytes resulted in calcium efflux from the ER, leading to mitochondrial dysfunction and oxidative stress (Egnatchik et al. 2014). In NASH, palmitate-induced ER stress also inhibits SERCA activity and causes ER calcium release into cytosol. High cytosolic levels of calcium in the hepatocytes inhibit autophagosome-lysosome fusion and protective autophagy (Czaja 2015; Dubois et al. 2016). Fifth, decreased mitochondrial $O_2$ consumption due to an altered metabolism can lead to an increase in reactive oxygen species (ROS) and reduction in ATP generation, both of which could worsen ER stress-caused cell death (Mohammad et al. 2012; Softic et al. 2016). In addition, the enzyme CYP2E1 is known to hydrolyze various small molecules such as fatty acid and alcohol into by-products, which is implicated in cellular stress-induced hepatocyte injury and progression to NASH by promoting oxidative stress, inflammation, and protein modification (Abdelmegeed et al. 2012). Recent evidence indicates that hepatic CYP2E1 undergoes transport from ER to mitochondria where it has

distinctly different enzyme activities, which complicate pathological outcomes (Aubert et al. 2011; Hartman et al. 2015). Therefore, these inter-organellar interactions lead to the pleiotropic effects of ER stress that are often observed and potentially impact the interpretation and prediction of UPR signaling outputs.

## 10.9 Conclusions

The UPR in the liver is essentially a defense mechanism to attenuate ER stress and maintain the protein homeostasis. Under pathophysiological conditions, the molecular components of the UPR signaling have diverse biological functions in the hepatocytes and interact with other cellular signaling pathways, including oxidative stress, inflammation, autophagy, and mitochondrial function. Persistent ER stress overwhelms the distinct defensive capacity of the UPR and generally leads to hepatic cell death, which plays contributory roles in nearly all forms of liver disease. In alcohol-induced hepatic ER stress and injury, persistence of ER stress can be caused by alcohol metabolites/derivatives, elevated homocysteine, ceramides, increased SAH, and disruptions of calcium, iron, or zinc homeostasis. In NAFLD, lipid accumulation may initially trigger the ER stress response, which further regulates lipid metabolism and accumulation. Excessive lipids upregulate genes involved in hepatic cell death, which help transform the liver from simple steatosis to NASH. In drug-induced hepatotoxicity, ER stress inducers can be drugs and their derivatives that idiosyncratically interact with the ER components or the UPR pathways. In hepatitis virus infections, ER stress and the UPR in the host hepatocytes are most likely attributed by the persistent production of large amounts of viral proteins. In liver carcinogenesis, the UPR pro-survival signals may be preferentially activated in ER-stressed hepatocytes, which interact with multiple endogenous and exogenous factors that change the delicate balance between oncogenes and tumor suppressors. Therefore, the pathophysiological role of ER stress and the UPR demonstrated for the development of various forms of liver disease will provide us unique therapeutic opportunities. Drugs targeting the UPR signaling will be developed, and more clinical trials in humans toward treating the ER stress-induced liver diseases are anticipated.

**Acknowledgments** This work is supported by the US National Institutes of Health (NIH) grants R01AA018612 (NCE), R21AA023952, and R01DA042632 (to C. Ji). H. Han is a PhD student in Ji's lab. Dr. Ji is grateful to the graduate students and postdoctoral fellows who contributed to the studies in the lab. The USC Research Center for Liver Diseases (P30DK48522) and the Southern California Research Center for Alcoholic Liver and Pancreatic Diseases and Cirrhosis (P50AA011999-20) provided technical assistance for the studies.

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# Chapter 11
# The Impact of Gut Microbiota on Liver Injury

**Wenke Feng and Craig McClain**

## Abbreviations

| | |
|---|---|
| 4-HNE | 4-Hydroxynonenal |
| AC | Alcoholic cirrhosis |
| ACLF | Acute-on-chronic liver failure |
| ALD | Alcoholic liver disease |
| ALT | Alanine transaminase |
| AST | Aspartate transaminase |
| AXOS | Arabinoxylanoligosaccharide |
| BCAA | Branched chain amino acid |
| BCFA | Branched chain fatty acid |
| CDR | Cirrhosis dysbiosis ratio |
| CRAMP | Cathelicidin-related antimicrobial peptide |
| DCA | Deoxycholic acid |
| DEN | Diethylnitrosamine |
| FMT | Fecal microbiota transplantation |
| FOS | Fructooligosaccharide |
| FXR | Farnesoid X receptor |
| GLP-1 | Glucagon-like peptide-1 |
| GOS | Galactooligosaccharide |
| HCC | Hepatocellular carcinoma |
| HE | Hepatic encephalopathy |
| HFD | High-fat diet |
| HOMA | Homeostasis model assessment |

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W.-X. Ding, X.-M. Yin (eds.), *Molecules, Systems and Signaling in Liver Injury*,
Cell Death in Biology and Diseases, DOI 10.1007/978-3-319-58106-4_11

X.-M. Yin and Z. Dong (Series eds.), *Cell Death in Biology and Diseases*

| | |
|---|---|
| IFALD | Failure-associated liver disease |
| LAB | Lactic acid bacteria |
| LGG | *Lactobacillus rhamnosus* GG |
| LPS | Lipopolysaccharide |
| MCD | Methionine-choline deficiency |
| MDA | Malondialdehyde |
| MELD | Model for end-stage liver disease |
| MHE | Minimal HE |
| NAFLD | Nonalcoholic fatty liver disease |
| NASH | Nonalcoholic steatohepatitis |
| NF-kB | Nuclear factor kB |
| NLRP | Nod-like receptor protein |
| NOD2 | Nucleotide-binding oligomerization domain 2 |
| OFC | Oligofructose |
| PAMP | Pathogen-associated molecular pattern |
| PPARγ | Peroxisome proliferator-activated receptor-γ |
| sAH | Severe alcoholic hepatitis |
| SBP | Spontaneous bacterial peritonitis |
| SCFA | Short-chain fatty acid |
| SIBO | Small intestinal bacterial overgrowth |
| TJ | Tight junction |
| TLR | Toll-like receptor |
| γGT | Gamma glutamyl transferase |

## 11.1 Dysbiotic Factors Contributing to Liver Disease, in General

The human intestinal tract harbors over 100 trillion bacteria in a diverse and complex community, which equates to tenfold more bacteria than human body cells. Studies of gut flora initially relied on culture-dependent techniques, but over 80% of the gut microbes are not culturable by standard methods. Because of recent advancements in high-throughput next-generation sequencing technology, we are beginning to gain more details about the gut microbiota. We now know that the collective microbiota genome (microbiota) has 100-fold more genes than the human genome, as determined by sequencing. Gut bacteria coevolve with human beings (symbiosis), and the changes of the bacterial population and structure can have important consequences, both beneficial and harmful, to the host. Bacterial metabolism and microbiota-host interactions influence the pathophysiology of the host. The gut microbiota composition is tightly regulated. Genetic factors, the mucosal immune system, dietary factors, and environmental factors are essential regulators of gut microbiota homeostasis.

**Genetic factors** It is clear that host genetics and gut microbiota both have an impact on metabolic phenotypes. Several twin studies revealed that genetic heritage has an influence on the human gut bacterial composition (Dicksved et al. 2008; Lee

et al. 2011; Turnbaugh et al. 2009). A study including 10 monozygotic twin pairs with Crohn's disease and 8 healthy twin pairs demonstrates that fecal microbial communities are similar, and this similarity is disrupted when the twins are discordant for Crohn's disease. A larger twin study including 154 individuals revealed that the composition of the human gut microbiota is shared among family members. Testing core measurable microbiota in mice identified 18 host quantitative trait loci which are linked to bacterial abundance, suggesting that host genetics shape individual microbiota diversity (Turnbaugh et al. 2009). It has been found that an increased abundance of *Enterobacteriaceae* is strongly associated with the nucleotide-binding oligomerization domain 2 (NOD2) risk allele for inflammatory bowel disease (Knights et al. 2014). Intestinal inflammation causes intestinal barrier dysfunction and increases permeability leading to a translocation of bacteria and their products to the liver, and this is an important contributor to liver disease. In the liver, NOD2 variants are genetic risk factors for death and spontaneous bacterial peritonitis (SBP) in cirrhosis (Appenrodt et al. 2010; Bruns et al. 2012), and genetic polymorphisms of NOD2 are associated with increased mortality in liver transplant patients with nonalcoholic liver diseases (Saner et al. 2014). An alteration of gut microbiota noted in NOD-like receptor protein 6 (NLRP6) and NLRP3 inflammasome-deficient mice is associated with exacerbated hepatic steatosis and inflammation through regulation of TLR4 and TLR9 that drives NASH (nonalcoholic steatohepatitis) progression (Henao-Mejia et al. 2012). In a more specific aspect, a recent study showed that the *Christensenellaceae* family is a heritable taxon which forms a co-occurrence network with other heritable taxa (Goodrich et al. 2014). These bacteria families are enriched in individuals with low body mass index (Goodrich et al. 2014). Despite recent studies investigating genetic contributions to the gut microbiota changes, future carefully designed studies to determine whether known genetic risk alleles for liver disease have an impact on gut microbiota and how environmental factors interact with genetic factors are needed.

**Environmental factor** Environmental factors are important contributors shaping gut bacterial composition. People with similar cultural factors share similarities in microbiota. Diet, hygiene habits, antibiotic usage, and environmental exposure are among the most important contributors. In particular, emerging evidence suggests that environmental pollutants could interact with gut microbiota (Liu et al. 2014; Choi et al. 2013; Ribiere et al. 2016). Gut bacteria can metabolize environmental chemicals from various categories, either increasing or decreasing their toxicity to the host. Conversely, these chemicals may also influence the gut flora homeostasis and function. However, the relevance of the microbiota-xenobiotics/toxicants interactions in both experimental or human liver disease needs more detailed studies.

**Dietary factors** Energy from the diet is extracted by gut bacteria, and the nutrients are provided to both bacteria and the host. In newborns, their feeding pattern has a strong impact on gastrointestinal bacterial communities. *Bifidobacteria* dominate the microbiota in babies that are breast-fed until weaning, while babies that are formula-fed have a more diverse microbiota (Magne et al. 2006). Solid food

induces changes in the microbiota, and a relatively stable, healthy-like microbiome assembles by the age of three (Scholtens et al. 2006). In adults, major macronutrients, including carbohydrate, protein, and fat, can have a significant impact on the microbial community composition.

There are three main classes of carbohydrate: resistant starches (RS), non-starch polysaccharides (NSP) and oligosaccharides, including di- and monosaccharides. Intake of high amounts of carbohydrate, especially fructose, has been considered as a contributing factor promoting nonalcoholic fatty liver disease (NAFLD) (Jin and Vos 2015). Changes in the amount and type of carbohydrate profoundly influence microbiota composition and the microbial metabolites in adult human volunteers. Recent studies revealed that high fructose consumption changed intestinal microbiota in humans and in experimental animals (Ferrere et al. 2016). Commonly used prebiotics including fructooligosaccharides (FOS), galactooligosaccharides (GOS), and lactulose are classified as carbohydrates. Many studies have shown that following dietary supplementation with GOS and FOS, there was an increase in *Bifidobacteria*, which is positively correlated with healthy status. In a humanized rat (inoculated with a human microbiota) model, arabinoxylanoligosaccharide (AXOS) supplementation increased numbers of *Roseburia/E. rectale* group, which have been recognized as butyrate-producing bacteria (Van den Abbeele et al. 2011). Butyrate is one of the major short-chain fatty acids (SCFAs), and it has beneficial effects in NAFLD and alcoholic liver disease (ALD) through multiple mechanisms. In an arsenic−/high-fat diet-induced liver injury mouse model, supplementation with oligofructose (OFC) showed a protective effect, partially through microbiota alteration (Massey et al. 2015). OFC feeding increased the relative abundance of *Bacteroidetes* with a concomitant decrease in *Firmicutes*. In addition, OFC restored the growth of *Tenericutes* which was lost in animals that received high-fat diet (Massey et al. 2015).

Protein malnutrition is strongly associated with more advanced liver disease. Protein turnover in the colon provides nitrogen and amino acids for certain bacterial growth, which in turn affects protein fermentation. High-protein feeding increased cecal branched chain amino acids (BCAAs) in conventional rats compared to germ-free rats (Lhoste et al. 1996). Most studies so far focus on the effects of altered fermentation products on microbiota composition, rather than dietary protein itself. Gut flora humanized rats fed meat diets had lower levels of SCFAs and higher levels of branched chain fatty acids (BCFAs), greater ammonia, and higher pH in cecal contents compared to pea protein-fed animals (Lhoste et al. 1998; Scott et al. 2013).

It is well-established that high-fat (HF) consumption is a major contributing factor to NAFLD and ALD. HF diet feeding significantly reduced *Bifidobacteria* counts and fecal SCFAs, including butyrate, concentrations compared to low-fat (LF) feeding in human subjects (Brinkworth et al. 2009). HF diet-induced microbiota alterations seem to be reversible. In mice, a 12-week HF diet induced obesity and changed microbiota composition, while the mouse phenotype became indistinguishable from control diet-fed animals after switching the HF diet to a normal diet for additional 10 weeks (Zhang et al. 2012a). This reversibility of microbiota in

obesity provides a rationale for developing approaches to influence microbiota in order to treat metabolic diseases, including NASH and obesity, if in fact it plays an etiologic role in these diseases (Zhang et al. 2012a).

## 11.2 Microbiota and Alcoholic Liver Disease

ALD encompasses hepatic steatosis, steatohepatitis, and more serious forms including fibrosis, cirrhosis, and liver cancer (Gao and Bataller 2011). Consumption of alcohol may induce small and large intestinal bacterial overgrowth, particularly Gram-negative bacteria and alterations in bacterial composition. Alcohol metabolism in the intestine also causes intestinal epithelial barrier dysfunction resulting in the increased translocation of bacterial products, including endotoxin, bacterial DNA, and pathogen-associated molecular patterns (PAMPs), from the gut lumen to the liver. Those toxic microbial-related products trigger the inflammatory cascade and the induction of reactive oxygen species and other pro-inflammatory/toxic factors in Kupffer cells, hepatocytes, and other liver cells. Clinical studies show that human subjects with alcoholic cirrhosis have higher levels of serum bacterial products than healthy controls (Parlesak et al. 2000; Bajaj et al. 2014b).

Intestinal dysbiosis and bacterial overgrowth have long been observed in human alcoholic subjects. Alcohol abuse-induced intestinal bacterial overgrowth in humans was documented over three decades ago (Bode et al. 1984). Anaerobic and aerobic cultures of samples from jejunal juice displayed significantly higher bacterial counts in alcoholics compared to healthy controls, indicating small intestinal bacterial overgrowth might contribute to functional abnormalities of the small intestine in patients with chronic alcohol abuse. This same group of the investigators further showed a higher prevalence of small intestine bacterial overgrowth in chronic alcoholics compared to controls using a breath test (Bode et al. 1993). The observation was later confirmed by other groups in alcoholic cirrhosis patients (Morencos et al. 1995; Kirpich et al. 2008; Bhonchal et al. 2007). Recently, Bajaj et al. studied intestinal bacterial composition in 244 alcoholic cirrhotic patients using a novel index, namely, cirrhosis dysbiosis ratio (CDR, a low number indicating dysbiosis). The authors found that intestinal dysbiosis was more severe in decompensated cirrhotics compared to compensated cirrhotics (Bajaj et al. 2013). Gut microbiota are not only affected by excessive alcohol consumption; moderate drinking has also been shown to be a strong risk factor for small intestine bacterial overgrowth (Gabbard et al. 2014). Mutlu and colleagues investigated the mucosa-associated colon microbiota in alcoholics with and without cirrhosis and in controls (Mutlu et al. 2012). Pyrosequencing analysis of colon biopsy samples revealed that mucosa-associated bacteria were persistently altered in a subset of alcoholics, and this was correlated with endotoxemia (Mutlu et al. 2012). Although studies on bacterial overgrowth and dysbiosis are increasing, as yet there is no specific intestinal bacterial pattern identified to have a proven etiologic role in the development of ALD. However, the fact that alcohol consumption

causes bacterial overgrowth and dysbiosis provides an opportunity for the treatment and/or prevention of ALD by targeting intestinal microbiota.

Another human study showed that alcohol-dependent subjects developed gut leakiness after 3 weeks of alcohol abstinence together with fecal dysbiosis and changes in colonic microbiota (Leclercq et al. 2014). The functional changes in microbiota have also been studied. Alcohol-dependent subjects with high intestinal permeability showed higher phenol and lower 4-methyl-phenol concentration in feces than the subjects with low intestinal permeability, while the indole and 3-methyl-indole concentrations were oppositely changed. These metabolite alterations could be associated with liver injury from alcohol abuse.

Compared with clinical observations, studies in experimental animal models of ALD provide more detailed information. In an intragastric alcohol-feeding mouse model, Yan et al. (2011) showed that 3 weeks of alcohol ingestion led to bacterial overgrowth in the proximal small intestine and to dysbiosis, which was associated with the suppression of antimicrobial peptides, Reg3b and Reg3g. Alcohol-fed mice displayed an increase in *Bacteroidetes* and *Verrucomicrobia* abundance and a decrease in *Firmicutes* level compared to pair-fed animals. Interestingly, an overgrowth of *Akkermansia muciniphila* was observed in alcohol-fed mice, and this is believed to be responsible for mucin degradation. Moreover, the population of *Lactobacilli* was depleted in alcohol-fed mice, and *Lactobacilli* are generally considered to be a beneficial bacterial group.

In a study using the Lieber-DeCarli alcohol-feeding mouse model (Bull-Otterson et al. 2013), metagenomics analysis demonstrated a decline in the abundance of both *Bacteroidetes* and *Firmicutes* phyla, with a proportional increase in the Gram-negative *Proteobacteria* and Gram-positive *Actinobacteria* phyla. Genera analysis showed the greatest expansion in Gram-negative alkaline-tolerant *Alcaligenes* and Gram-positive *Corynebacterium*. These alterations were accompanied by the changes in colonic pH and liver steatosis (Bull-Otterson et al. 2013).

Alcohol exposure-associated gut bacterial alterations were also studied in mice with various genetic backgrounds. Wild-type mice have higher alcohol-induced hepatic steatosis and injury than mucin knockout mice ($Muc2^{-/-}$) (Hartmann et al. 2013), and mucin plays an important role in intestinal mucus homeostasis. $Muc2^{-/-}$ mice are protected from intestinal bacterial overgrowth after alcohol feeding and have a higher expression of antimicrobial peptides, Reg3b and Reg3g. Interestingly, overexpression of Reg3g in the intestinal epithelial cells restricts bacterial colonization of the mucosal surface, reduces bacterial translocation, and protects mice from alcohol-induced steatohepatitis (Wang et al. 2016b). Although wild-type and $Reg3b^{-/-}$ mice had a similar increase in gut microbiota after alcohol feeding, $Reg3b^{-/-}$ mice showed significantly higher numbers of mucosa-associated bacteria in the mucus and epithelial layer of the small intestine than wild-type mice. Alcohol-induced bacterial colonization in the intestine was also demonstrated by another group who observed significant changes in the mucosa-associated microbiota in the colon in mice after a 10-week alcohol-feeding regimen (Mutlu et al. 2012). The authors concluded that alcohol appears to impair control of the mucosa-associated microbiota, and the subsequent breach of the mucosal barrier facilitates progression of alcoholic liver disease.

Germ-free mice were used to study the impact of alcohol on gut microbiota homeostasis with somewhat conflicting results. Surprisingly, the absence of the microbiota (germ-free) in mice did not protect but, in fact, exacerbated ALD (Chen et al. 2015a), suggesting that microbiota are required for hepatic homeostasis including ethanol metabolism, oxidative stress, and bile acid metabolism. Germ-free mice exhibited dysregulation of ethanol-metabolizing enzymes, as a small dose of alcohol could induce liver damage (Chen et al. 2015a). The requirement of microbiota for liver homeostasis has also been demonstrated in other types of liver disease (Tabibian et al. 2016; Mazagova et al. 2015). A study of ALD using germ-free mice showed that 7-day alcohol feeding in the drinking water caused less inflammation in the liver and did not cause liver injury in germ-free mice compared with alcohol-fed conventional mice. Interestingly, fecal transplantation from conventional mice fed alcohol to germ-free mice induced injury and inflammation in both liver and intestine, suggesting that alcohol consumption caused dysbiosis. A more recent work extended the study using germ-free mice. Humanization of mice using human intestinal microbiota transplants from alcoholic patients showed significant differences between germ-free and conventional mice (Llopis et al. 2016). Two patient donors had distinct microbiota compositions, with a large amount of *Bifidobacteria* and *Streptococci* in the patient with severe alcoholic hepatitis (sAH) compared to the patient without severe alcoholic hepatitis (noAH). The germ-free mice receiving the intestinal microbiota from a patient with sAH developed more severe liver inflammation, higher liver necrosis, greater intestinal permeability, and higher translocation of bacteria than mice harboring the intestinal microbiota from an alcoholic patient with noAH. Interestingly, in conventional mice humanized with the intestinal microbiota from an sAH patient, a second subsequent transfer of intestinal microbiota from the noAH patient improved alcohol-induced liver lesions.

Investigators at two nearby institutions in France incurred a reproducibility problem in creating experimentally-induced ALD (Ferrere et al. 2017). The mice were from the same vendor, and the investigators used the same Lieber-DeCarli liquid alcohol diet at both facilities. The group of alcohol-sensitive animals developed classic ALD and disruption of gut barrier function, while the alcohol-resistant mice from the other institution did not develop liver disease. The investigators evaluated why they could not generate the same liver injury at both institutions, and their final conclusion was gut microbiota. They ultimately performed fecal transplantation of fresh feces from alcohol-resistant donor mice to alcohol-sensitive mice three times a week. Another group of mice received dietary pectin during the entire alcohol-feeding period. Both of these interventions prevented liver steatosis/inflammation and improved gut homeostasis in the sensitive mice. Analysis of the intestinal microbiota showed that the proportion of *Bacteroides* was significantly lower in the alcohol-sensitive mice. Principal coordinate analysis showed major differences between the intestinal microbiota of sensitive and resistant mice. These data demonstrate the importance of gut flora and the gut-liver axis in the development and reproducibility of experimental ALD. If these results can be extrapolated to humans, it may also help explain why some people who drink heavily develop ALD.

The effect of the types of dietary fat on gut microbiota homeostasis has been evaluated in mice with ALD. Saturated fat (medium chain triglycerides enriched) feeding reduced *Proteobacteria* and *Actinobacteria* and increased *Bacteroidetes* in feces of mice exposed to 8 weeks of alcohol feeding (Kirpich et al. 2016). A reduction in endotoxemia and liver steatosis and injury was seen in animals fed the saturated fat diet. In addition, supplementation of saturated long-chain fatty acids maintained intestinal eubiosis and reduced alcohol-induced liver injury (Chen et al. 2015b).

## 11.3 Microbiota and Nonalcoholic Liver Disease

NAFLD is pathologically similar to alcohol-induced fatty liver disease in subjects without an alcohol drinking history. It is regarded as the most common liver disease worldwide. The prevalence of NAFLD is greatly increased in patients with the metabolic syndrome-obesity, type 2 diabetes, and hyperlipidemia. In NAFLD patients, approximately 25% may progress to NASH (nonalcoholic steatohepatitis) which may result in liver fibrosis and cirrhosis. Patients with liver cirrhosis are at increased risk for hepatocellular carcinoma.

The importance of microbiota in hepatic steatosis was elucidated clinically as early as three decades ago when Drenick et al. (1982) studied patients undergoing gastric bypass surgery. The authors found that hepatic steatosis coincided with bacterial overgrowth, and treatment with antibiotics in these patients reduced hepatic steatosis. Later on, it was demonstrated that small intestine bacterial overgrowth is more prevalent in patients with NASH than in healthy controls (Wigg et al. 2001). Since then, increasing evidence in animals and humans has improved our understanding of the role of microbiota homeostasis in the development of NAFLD (Abu-Shanab and Quigley 2010).

Several clinical studies have shown that alterations of the gut microbial community are closely associated with the prevalence and development of NAFLD. Significant shifts of the intestinal microbiota community, such as overrepresentation of selected members of *Firmicutes* and *Lactobacillus* species, and decreased bacteria of phylum *Firmicutes* were found in NAFLD patients (Raman et al. 2013). Microbiota samples from patients with NAFLD or NASH exhibited a lower abundance of Ruminococcaceae family members. Obese children with NASH have a significantly disproportionate *Escherichia* in their intestinal microbiota. Adult NAFLD patients had a significant lower percentage of *Clostridium coccoides* than NASH patients in their gut, indicating a possible role for *Clostridium coccoides* in the development of NAFLD to NASH.

In a 6-month longitudinal study involving 16 NASH patients and 22 healthy controls (Wong et al. 2013a), fecal samples displayed a lower abundance of *Faecalibacterium* and *Anaerosporobacter* but higher abundance of *Parabacteroides* and *Allisonella* in the patients. In a cross-sectional study involving 11 biopsy-proven NALFD, 22 NASH, and 17 living liver donors as controls (Mouzaki et al. 2013), PCR measurement showed a lower abundance of *Bacteroides* and *Prevotella* and

higher fecal *Clostridium coccoides* in NASH patients compared with patients with simple steatosis. It is concluded that there is an inverse and diet−/BMI-independent association between the *Bacteroidetes*/*Prevotella* bacteria and the presence of NASH. Similarly, a recent study of 57 patients with biopsy-proven NAFLD revealed that NAFLD patients with fibrosis had a significant increase in *Bacteroides* and *Ruminococcus* and a decrease in *Prevotella* (Boursier et al. 2016). It appears that *Bacteroides* are independently associated with NASH, and *Ruminococcus* are independently associated with significant fibrosis.

NAFLD often occurs in obese patients, but it is not rare in non-obese adults, especially in Asian populations. A study (Wang et al. 2016a) aimed at understanding whether abnormal intestinal microbiota are associated with NAFLD in non-obese patients found that the NAFLD patients had lower diversity and changes in phylum levels in fecal microbiota. Specifically, 20% more of phylum *Bacteroidetes* and 24% less of *Firmicutes* were found in fecal samples of non-obese NAFLD patients compared to non-obese healthy controls. In addition, four families and their eight genera within *Firmicutes*, which were SCFA-producing and 7α-dehydroxylating bacteria, were significantly decreased. Furthermore, dysbiosis-associated metabolic markers were found to be correlated with non-obese NAFLD.

In a recent pediatric NAFLD study, 61 children diagnosed with NAFLD, NASH, or obesity and 54 healthy children were recruited (Del Chierico et al. 2017). A multivariate analysis of the stool microbiota and volatile organic compounds found that a combination of *Oscillospira*, *Rikenellaceae*, *Parabacteroides*, *Bacteroides fragilis*, *Sutterella*, *Lachnospiraceae*, 4-methyl-2-pentanone, 1-butanol, and 2-butanone could serve as a marker to classify NAFLD patients and healthy subjects.

However, studies comparing intestinal microbiota in NAFLD and NASH patients have produced inconsistent and even contradictory results. To better determine whether patients with NAFLD and NASH have distinct microbiota profiles, studies with larger and better characterized patient cohorts are needed. Identification of specific microbial alterations of NAFLD and NASH patients will promote our understanding of the role of gut contents in liver pathophysiology and could lead to biomarker discovery and strategies for the treatment of NAFLD and NASH.

The role of gut microbiota in high-fat diet (HFD)-induced NAFLD has been widely studied in animal models. HFD feeding increases obesity, but not all obese subjects develop NAFLD. A 10-week HFD feeding increased body weight, hepatic fat, inflammatory cell infiltration, and inducible nitric oxide synthase concentration in mice along with an increase in fecal *Lactobacillus* species (Zeng et al. 2013). The increased lactobacillus may have an effect on lipid metabolism via bile acid metabolism, which contributes to the fatty liver formation. In a mouse model (Le Roy et al. 2013), germ-free mice were colonized with intestinal microbiota from two distinct mice fed HFD. One of the donor mice developed hyperglycemia with higher concentrations of pro-inflammatory cytokines (responder), while another mouse was normoglycemic and had less systemic inflammation (non-responder). The germ-free mice that received microbiota from the responder developed hyperglycemia and hepatic steatosis, while the germ-free mice that received microbiota from the non-responder remained normoglycemic and did not develop NAFLD. The

microbial composition showed distinct differences at the phylum, genus, and species levels between the recipients from the responder and the recipients from the non-responder. This study demonstrates that NAFLD is transmissible to germ-free mice, and the gut microbiota contribute to the development of NAFLD independent of obesity.

In children with NAFLD or NASH, fecal microbiota were compared to healthy controls (Zhu et al. 2013). Blood alcohol concentrations were similar between healthy subjects and NAFLD children, but higher in NASH patients with increased abundance of alcohol-producing bacteria. The authors postulate that distinct composition of gut microbiota in NASH, obese and healthy subjects could offer targets for intervention in the disease. In a mouse study (Cope et al. 2000), the authors showed that endogenous alcohol concentration was increased in obese mice without exogenous alcohol exposure. Neomycin treatment reduced blood alcohol concentrations together with inducing an improvement of NALFD, suggesting a role for the microbiota in the production of endogenous alcohol and the development of NAFLD.

Inflammasome-mediated gut microbiota homeostasis in NAFLD and obesity progression has been studied in a mouse model (Henao-Mejia et al. 2012). The NLRP6 and NLRP3 inflammasome-deficient mouse model revealed that changes in the composition of the gut microbiota are associated with exacerbated hepatic steatosis and inflammation. Gut microbiota-associated influx of bacterial products into portal circulation act as TLR4 and TLR9 agonists leading to enhanced hepatic TNF-α expression that drives NASH progression. Co-housing of inflammasome-deficient mice with wild-type mice, or transfer of the dominant microbiota from the deficient mice to wild-type mice, resulted in exacerbation of hepatic steatosis and the progression of NAFLD. This study highlights the central role of the microbiota in the pathogenesis of unrelated systemic auto-inflammatory and metabolic disorders.

Results from human studies suggest that in addition to high-fat diets, the intake of sugar, particularly fructose, may be a risk factor for the development of NAFLD (Jin and Vos 2015). Mice were either fed with 30% fructose in drinking water/tap water with or without antibiotics for 8 weeks (Wagnerberger et al. 2012). Hepatic steatosis found in the livers of fructose-fed mice was associated with a significant induction of TLR1-4 and TLR6-8. The deleterious effects of fructose were attenuated in antibiotic-treated mice, indicating that microbiota homeostasis and increased intestinal translocation may be involved in the onset of fructose-induced NAFLD. Another mouse study showed that fructose feeding caused steatosis and dyslipidemia, with significantly decreased *Bifidobacterium* and *Lactobacillus* and increased endotoxemia (Jegatheesan et al. 2016).

## 11.4 Microbiota and Liver Cancer

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and one of leading causes of cancer mortality. Approximately 80% of the liver cancer patients also have liver cirrhosis. Among the various etiologies, alcohol abuse and hepatitis C/B virus are the most common and most studied risk factors. Patients with liver cirrhosis and HCC exhibited a significant increase in serum endotoxin levels (Zhang et al. 2012b), indicating a correlation of gut homeostasis with cirrhosis and HCC. The association of gut microbiota and HCC has been described in recent animal studies (Li et al. 2016b; Xie et al. 2016a, b). These studies have implications for human liver cancer risk assessment and prevention.

In a rat model (Zhang et al. 2012b), chronic diethylnitrosamine (DEN) administration was associated with an imbalance of subpopulations of gut microbiota. A significant suppression of *Lactobacillus* species, *Bifidobacterium* species, and *Enterococcus* species was found in feces of the rats with DEN-induced HCC. The changes in gut microbiota were associated with intestinal inflammation. Induction of enteric dysbiosis or intestinal inflammation by penicillin or dextran sulfate sodium, respectively, also significantly promoted tumor formation.

Obesity is increasingly recognized as a major risk factor for several common types of cancer, including HCC. In a murine model, Yoshimoto and colleagues (2013) showed that dietary or genetic obesity induced gut dysbiosis which led to the increase of deoxycholic acid (DCA) levels, a gut bacterial metabolite known to cause DNA damage. DCA in the circulation provokes the senescence-associated secretory phenotype in hepatic stellate cells, which, in turn, produce inflammatory factors in the liver, thus facilitating HCC development in mice after exposure to the chemical carcinogen. Blocking DCA production or reducing gut bacteria prevents HCC development in obese mice. Gut microbiota alterations and dysregulated bile acids were also described in another murine model of NASH-HCC (Xie et al. 2016a), in which dysbiosis was closely correlated with altered bile acid concentrations and the progression of liver disease from NASH to HCC (Xie et al. 2016b).

The role of specific intestinal microbiota in HCC development has been studied (Fox et al. 2010). Helicobacter-free C3H/HeN mice were inoculated with the food-borne contaminant, aflatoxin B1 (AFB), and/or *Helicobacter* hepaticus. Intestinal colonization by *H. hepaticus* was sufficient to promote aflatoxin- and HCV transgene-induced HCC. Neither bacterial translocation to the liver nor induction of hepatitis was necessary. In summary, enteric microbiota directly relates to HCC risk in mice exposed to carcinogenic chemicals or hepatitis virus transgenes.

In another murine study, Dapito et al. demonstrated that intestinal microbiota exerted a profound influence on HCC promotion through the LPS (lipopolysaccharide)-TLR4 pathway (Dapito et al. 2012). HCC development in chronically injured livers depended on the intestinal microbiota and TLR4 activation in non-bone-marrow-derived resident liver cells, which is distinct from previous studies in which bone-marrow-derived cells such as macrophages were believed

to be responsible for inflammatory cytokine production and genotoxic HCC. This study suggests that the intestinal microbiota and TLR4 represent potential therapeutic targets for HCC prevention in advanced liver disease.

Grat et al. (2016) determined the gut microbiota profile in 15 patients with HCC and 15 non-HCC patients matched according to etiology of cirrhosis and for model for end-stage liver disease (MELD) scores, all of whom underwent liver transplantations. Gut microbiota analysis showed that the presence of HCC was associated with significantly increased fecal counts of *Escherichia coli* (*E. coli*). Intestinal overgrowth of *E. coli* may contribute to hepatocarcinogenesis.

## 11.5 Microbiota and Other Liver Diseases

Gut flora and bacterial translocation play an important role in the pathogenesis of the cirrhosis and its complications, including hepatic encephalopathy (HE) and spontaneous bacterial peritonitis (SBP, the most common type of infection). Bacterial infections promote dysregulated intestinal immunity and bacterial overgrowth/dysbiosis, contributing to acute-on-chronic liver failure (ACLF) and death in cirrhosis.

Bacterial infection is a severe complication in the course of cirrhosis. Increased endotoxemia has been shown in patients with advanced stage cirrhosis, and this is associated with the activation of the cytokine cascade in SBP. To study the alterations in gut microbiota in cirrhosis and their longitudinal changes with decompensation, stool samples from 244 age-matched compensated/decompensated cirrhotic patients and controls were analyzed (Bajaj et al. 2014b). The CDR (low score indicates more severe disease) was highest in controls followed by compensated and decompensated patients. These worsening dysbiosis was correlated with the increasing endotoxin levels.

Hepatic encephalopathy (HE) is a common neurocognitive impairment in cirrhosis (Bajaj et al. 2009) that is mechanistically associated with gut bacteria and colonic mucosal microbiota composition and inflammation in the setting of intestinal barrier dysfunction (Bajaj et al. 2012b). Dysbiosis has been seen in cirrhotic patients with HE with a higher abundance of potentially pathogenic bacteria and a reduction of commensal bacteria (Bajaj et al. 2012c). A study involving 69 cirrhotic patients with minimal HE (MHE) showed substantial derangements in the gut microecology (Liu et al. 2004), with significant fecal overgrowth of potentially pathogenic *E. coli* and Staphylococcal species compared to cirrhotic patients without HE. Synbiotic treatment (combination of pre- and probiotics) modified the gut flora and was associated with a significant reduction in blood ammonia levels and endotoxemia. Another MHE study (Zhang et al. 2013) showed an overrepresentation of two bacterial families, *Streptococcaceae* and *Veillonellaceae*, in cirrhotic patients with and without MHE, compared with normal individuals. These studies suggest that targeting the gut microbiota with novel agents may be an alternative form of management of minimal HE in patients with cirrhosis.

The salivary microbiota have also been studied in cirrhotic patients with HE (Bajaj et al. 2015). Cirrhotic patients with HE-associated gut dysbiosis have a reduction in autochthonous bacteria and changes in bacterial function, which are

also present in saliva. Dysbiosis in saliva is associated with a higher risk of further hospitalization owing to liver-related complications.

## 11.6 Mechanisms Linking Gut Microbiota and Liver Disease

**Intestinal integrity** It is becoming increasingly clear that intestinal barrier dysfunction contributes to most of the liver diseases studied thus far. The integrated intestinal barrier has a vital role in preventing translocation of harmful bacteria and their products into the portal circulation. The epithelial cells form a lining with the paracellular space sealed by tight junctions (TJ) and adherens junctions (Rao 2009), and this is covered by a protective mucus layer that physically blocks most particles from direct contact with the epithelial cells (Turner 2009). Intestinal IgA, a major class of antibody secreted by the gut mucosa, is an important contributor to gut barrier function (Conley and Delacroix 1987). Dietary factors in ALD and NAFLD-caused dysbiosis directly affect the gut intestinal barrier at multiple levels including tight junctions, production and stabilization of mucin, recruitment and activation of inflammatory cells, and reduction of IgA secretion leading to the increased intestinal permeability and the exposure of mucosal and liver cells to potentially harmful bacteria and products (Hooper and Macpherson 2010; Wei et al. 2011). However, a recent study demonstrates that absence of IgA does not affect the development of ALD, likely due to a compensated increase in IgM, which limits bacterial translocation in ALD (Inamine et al. 2016).

Increased intestinal oxidative stress in ALD and NAFLD subjects could damage intestinal barrier function. Although the majority of ingested alcohol is metabolized in the liver, a significant amount of alcohol is also metabolized in the intestine, leading to increased ROS mediated by Cyp2E1 (Forsyth et al. 2014). In addition, gut bacteria-produced endogenous alcohol in NAFLD subjects contributes to ROS production causing barrier dysfunction.

Intestinal mucosa experiences profound fluctuations in blood flow and metabolism (Glover et al. 2016). Intestinal tissue hypoxia induces protective mechanisms including increased hypoxia-inducible factor (HIF) expression, and HIF is a master transcription factor regulating many genes in intestinal homeostasis (Saeedi et al. 2015). Studies have shown that the expression of several HIF-targeting genes, including intestinal trefoil factor, p-glycoprotein, antimicrobial peptides [Reg3 and cathelicidin-related antimicrobial peptide (CRAMP)], and tight junction claudin-1, is reduced in the intestines in ALD (Wang et al. 2011, 2012) and NAFLD subjects. Deleting HIF in the intestine causes dysbiosis and exacerbates liver injury (Shao et al. 2016), suggesting that HIF signaling is important for gut microbiota homeostasis and intestinal barrier function.

The tight junction protein, occludin, was decreased by both alcohol and high-fructose diet due to dysbiosis. It was shown that intestinal inflammation-induced upregulation of TNF-α increased microRNA 122a (miR122a), which regulates occludin expression (Ye et al. 2011). Overexpression of miR122a decreased occludin protein levels and increased permeability in epithelial Caco-2 cells (Zhao et al. 2015).

Clinical and animal studies have shown that liver steatosis and injury induced by alcohol and high-fat diet are associated with dysbiosis, increased permeability, and circulating endotoxins. The effect of LPS is not limited to the liver but also affects other tissues/organs, such as adipose and pancreas, that contribute to hepatic fat accumulation. Several mechanisms have been proposed to be responsible for the translocated bacteria and products contributing to the pathogenesis of fatty liver disease. The TLR pathway is a well-known target for the effects of increased LPS, which activates TLR4 in hepatic Kupffer cells and stellate cells to stimulate pro-inflammatory and profibrotic pathways (Seki and Brenner 2008). Mucosal TLR activation might also contribute to hepatic steatosis via intestinal epithelial MYD88 and inflammasome activation (Everard et al. 2014).

**Short-chain fatty acids** In the intestine, SCFAs are produced in the distal small intestine and colon where nondigestible carbohydrates such as resistant starch, dietary fiber, and other low-digestible polysaccharides are fermented by saccharolytic bacteria including the phyla *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. Acetate and propionate are the main products of the *Bacteroidetes* phylum, and butyrate is mainly produced by the *Firmicutes* phylum. As an energy precursor, SCFAs are implicated in the pathogenesis of NAFLD because of their possible contribution to obesity. The first evidence regarding SCFAs was from a study by Turnbaugh et al. (2006) showing that the cecum in ob/ob mice has an increased concentration of SCFAs and that transplantation of germ-free mice with the gut microbiota from ob/ob mice caused greater fat gain than from transplants from lean animals. In humans, increased production of SCFAs by the gut microbiota was also observed in overweight and obese people, compared to lean subjects (Schwiertz et al. 2010). Using metagenomics analysis, the majority of studies showed that ob/ob mice (Ley et al. 2005) and obese patients (Ley et al. 2006) exhibit reduced abundance of *Bacteroidetes* and proportionally increased abundance of *Firmicutes*. However, how these ratio changes affect energy imbalance leading to obesity and its complications including NAFLD needs further study. In fact, generally, SCFAs have more beneficial effects than obesity-causing effects (den Besten et al. 2013). Beneficial effects of SCFAs are through several pathways, including immunoregulation, enhanced intestinal barrier function, acting as a histone deacetylase 1 (HDAC) inhibitor, decreased expression of lipogenic genes, increased carnitine palmitoyltransferase 1A expression (den Besten et al. 2013), and a peroxisome proliferator-activated receptor-γ (PPARγ)-dependent mechanism, thereby shifting metabolism in adipose and liver tissue from lipogenesis to fatty acid oxidation (den Besten et al. 2015).

**Bile acids** Gut microbiota produce numerous metabolites to regulate host metabolism through binding to their cognate receptors. Bile acids are produced in the liver and metabolized in the intestine by gut bacteria. The role of bile acids in ALD and NAFLD is complex (Rinella and Sanyal 2015). Farnesoid X receptor (FXR) and G protein-coupled receptor 5 (Zhu et al. 2016; Yuan and Bambha 2015; Yamada et al. 2016; McMahan et al. 2011, 2013) are considered as the major regulators of bile acid metabolism, as they are involved in numerous metabolic pathways in the host.

Bile acids can regulate gut microbiota through activation of innate immune genes in the intestine (Wahlstrom et al. 2016). Hepatic FXR activation by bile acids suppresses NF-κB signaling and decreases hepatic inflammation in ALD (Manley and Ding 2015), NAFLD (Carr and Reid 2015), and fibrosis (Verbeke et al. 2016). Accumulating data demonstrate that both alcohol and high-fat diet consumption inhibit FXR activation. Intestinal FXR activation by bile acids inhibits bacterial overgrowth and mucosal damage (Inagaki et al. 2006). In contrast, deletion of FXR promotes bacterial overgrowth (Swennes et al. 2014) and intestinal epithelial barrier dysfunction (Inagaki et al. 2006). A recent clinical trial using an FXR ligand, obeticholic acid, in patients with non-cirrhotic NASH (FLINT trial) showed that activation of FXR by obeticholic acid improved histological NASH (Neuschwander-Tetri et al. 2015). However, another experimental NASH study showed that FXR antagonism might also be beneficial for NASH (Jiang et al. 2015). Manipulation of the gut microbiota resulted in an alteration of intestinal bile acid composition leading to intestinal FXR inactivation. This FXR antagonism reduced ceramide synthesis and de novo lipogenesis in the liver. Therefore, FXR regulation is complex with a broad range of effects, and gut microbiota could regulate FXR activity through bile acid metabolism. It should be noted that human and animal bile acid metabolism are different. Caution should be taken when attempting to explain experimental results that are in conflict.

**Endogenous ethanol** ALD and NAFLD share a high similarity in histological features and might have some similar pathways in their pathogenesis. Although dietary alcohol is the primary cause of liver injury in ALD, the bacterial-produced ethanol cannot be overlooked, due to its substantial production. Several studies suggest that endogenous alcohol might be involved in the development of NAFLD (Cope et al. 2000; Zhu et al. 2013). *Proteobacteria* (especially *E. coli* and other *Enterobacteriaceae*) is the major phyla of gut bacteria producing alcohol (Zhu et al. 2013). For example, 1 g of *E. coli* produces 0.8 g of ethanol per hour under anaerobic conditions (Dawes and Foster 1956). In NASH patients, those bacteria have been found to be increased in the gut, as shown by an increase in breath ethanol concentration (Nair et al. 2001) and an upregulation of alcohol metabolizing capacity (Baker et al. 2010). Gut bacteria-produced ethanol might contribute to increased intestinal permeability and endotoxemia, leading to TLR and inflammasome activation. Absorbed endogenous ethanol also may have a direct toxic effect on the liver.

## 11.7 Treatment Targeting Microbiota in Liver Disease

Manipulating gut microbiota to counteract the deleterious effects of pathogenic bacteria has been widely used in the management of liver disease. Probiotics, prebiotics, and synbiotics have been shown to be beneficial to the health of intestinal microbiota.

**Probiotics** Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host, according to the FAO/WHO definition (Fooladi et al. 2013). The beneficial effects of probiotics have been widely investigated in multiple animal models and clinical studies of a variety of disease conditions in the gastrointestinal system such as inflammatory bowel disease, NASH, cirrhosis, and ALD (O'Mahony et al. 2005; Kirpich and McClain 2012). Ideal probiotic strains for this kind of application should be resistant to bile, hydrochloric acid, and pancreatic juice; be able to tolerate stomach and duodenum conditions and gastric transport; and have the ability to stimulate the immune system, thereby improving intestinal function via adhering to and colonizing the intestinal epithelium. In addition, probiotic strains must be able to survive during manufacture and storage in order to exert considerable healthful outcomes (Lin et al. 2006). Currently, the most often used probiotics are *Bifidobacteria*, lactic acid bacteria (LAB), *Propionibacteria*, yeasts (*Saccharomyces boulardii*), and the Gram-negative *Escherichia coli* strain (Nissle 1917). *Lactobacilli*, major contributors to the LAB group, also are frequently used probiotics. Various species and strains of *Lactobacilli* have been used in animals and humans, including *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, and *Lactobacillus helveticus*. Most of these species belong to phylum *Firmicutes*. *Bifidobacterium*, which produces lactic acid, is another commonly used probiotic genus and belongs to the *Actinobacteria* phylum. To date, a large number of probiotics have been reported to be suitable for the treatment of a variety of diseases, and this number is still growing.

**Prebiotics** Unlike probiotics, prebiotics, which have also been frequently used for disease treatment, are not live bacteria but rather nondigestible carbohydrates. Prebiotics serve as an energy source for"good" bacteria and stimulate the growth and activities of specific bacteria in the gut (Yan et al. 2011). SCFAs, in particular, acetate (Perry et al. 2016) and butyrate (Berni Canani et al. 2012), have been recognized as beneficial metabolites associated with many biological functions in the gut. One of the important functions of butyrate is its ability to regulate gene expression through epigenetic mechanisms (Berni Canani et al. 2012). Butyrate enhances cell proliferation and inhibits cell apoptosis in normal cells but not in the transformed cells (Hass et al. 1997).

**Synbiotics** Using prebiotics and probiotics in combination is usually described as synbiotics. The net health effect of synbiotics is usually synergistic (Pineiro et al. 2008). Synbiotics practically have been used in a large body of clinical and experimental studies on liver disease.

**Probiotic treatment/prevention of ALD** Probiotics have been used in experimental animals and, to some extent, in humans, to modulate gut microbial homeostasis and to manage ALD. Probiotics have been used in various experimental ALD animal models, including chronic alcohol exposure, single-dose acute alcohol exposure, multiple-dose alcohol exposure, and alcohol exposure plus LPS challenge. A variety of probiotic strains have been used, such as *Lactobacillus rhamnosus* GG,

*Lactobacillus acidophilus*, *Lactobacillus helveticus*, *Bifidobacterium*, VSL#3, heat-killed *Lactobacillus brevis* SBC8803, and *Lactobacillus rhamnosus* GG supernatant.

Among those, *Lactobacillus rhamnosus* GG (LGG) is the most frequently used strain. LGG is a Gram-positive bacterial strain of the *Lactobacillus rhamnosus* species that was isolated in 1983 by Barry R. Goldin and Sherwood L. Gorbach. In several models of ALD in rats and mice, LGG administration showed significant protective effects. LGG reduced plasma endotoxin level, improved liver enzymes alanine transaminase (ALT) and aspartate transaminase (AST), and reduced hepatic steatosis and injury.

Nanji and coworkers were one of the earliest groups demonstrating the effectiveness of LGG in experimental ALD (Nanji et al. 1994). LGG was administrated to Wistar rats at $10^{10}$ CFU (colony forming units), and this probiotic reduced alcohol-induced endotoxemia and liver injury. In another study, a combination treatment using *Lactobacillus acidophilus*, *Lactobacillus helveticus*, and *Bifidobacterium* in rats with alcohol pancreatitis-related liver damage effectively protected against endotoxin/bacterial translocation, as well as liver damage in the course of acute pancreatitis and concomitant heavy alcohol consumption (Marotta et al. 2005). Additional studies using LGG in rats demonstrated reduced alcohol-induced gut leakiness, oxidative stress, and inflammation in both intestine and liver (Forsyth et al. 2009) kĸand improved intestinal dysbiosis (Mutlu et al. 2009). Another frequently used probiotic mixture, VSL#3, was shown to be effective in modulating gut microbiota and protecting against alcohol-induced intestinal barrier dysfunction in rats (Chang et al. 2013).

Our group fed mice with the Lieber-DeCarli liquid diet containing 5% alcohol for 8 weeks to produce hepatic fatty liver and injury. These mice were treated with LGG culture broth at $10^9$ CFU for the final 2 weeks along with continued chronic alcohol administration. LGG supplementation reversed established alcoholic hepatic steatosis and injury (Wang et al. 2011). This beneficial effect was associated with a reduction in circulating LPS and improved intestinal barrier function mediated, at least in part, by intestinal HIF-modulated mucus layer regulation.

While many reports have studied the effects of probiotics in experimental ALD, clinical trials are limited. Stadlbauer and coworkers evaluated the effectiveness of the probiotic *Lactobacillus casei* Shirota on alcoholic cirrhosis (AC) patients ($n$ = 12) and healthy controls ($n$ = 13) in a small open-labeled study (Stadlbauer et al. 2008). Compared to the baseline before treatment, cirrhotic patients who received the probiotics for 4 weeks had a significantly lower TLR4 expression and IL-10, sTNFR1 (soluble TNF receptor), and sTNFR2 levels, along with a restored neutrophil phagocytic activity, suggesting that the probiotic is safe and may be effective in the treatment of patients with defective immunity.

In a brief report, Loguercio et al. (2002) showed that treatment of 10 alcoholic cirrhosis patients (who were all persistent alcohol users with a median daily intake of pure ethanol of 150 g) with a synbiotic mixture of different bacteria strains and a prebiotic significantly improved liver damage and function compared to basal values. Patients were treated with the synbiotics for 2 months, followed by 1 month of

a washout period. The ALT and γGT (gamma glutamyl transferase) levels were slightly, but not significantly, increased after the washout period. These results indicate that the effects of synbiotic treatment partially persisted beyond the end of treatment. The same group (Loguercio et al. 2005) also reported that a commonly used probiotics mixture, VSL#3, was beneficial in liver disease. This open-labeled study involved 22 NAFLD and 20 alcoholic cirrhosis (AC) patients and 36 hepatitis C virus (HCV)-positive patients with and without liver cirrhosis for comparison. VSL#3 treatment significantly improved plasma levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in NAFLD and AC patients, but cytokines (TNF-α, IL-6, and IL-10) improved only in AC patients.

More recently, Dhiman et al. (2014) reported that probiotic VSL#3 treatment reduced liver disease severity and hospitalization in a double-blind trial in patients with cirrhosis including AC ($n = 89$, 46 probiotics, 43 placebo; patients who had an alcohol use history in the previous 6 weeks were excluded). Lata and colleagues (2007) in a double-blind, randomized study in 34 cirrhosis patients (19 on probiotics; 15 on placebo), who had an alcoholic etiology of their cirrhosis, showed that treatment with the probiotic *Escherichia coli* Nissle for 42 days improved colonic colonization and liver function with probiotic treatment. In an open-labeled, randomized study which involved 66 patients who were diagnosed with alcoholic psychosis and liver disease as well as 24 matched healthy controls, Kirpich et al. (2008) demonstrated that after 5 days of treatment with *Bifidobacterium bifidum* and *Lactobacillus plantarum* 8PA3, mild alcoholic hepatitis patients had a significant end-of-treatment reduction of ALT and AST, lactate dehydrogenase, and total bilirubin. Compared to standard therapy, probiotic treatment significantly reduced serum ALT. This liver function improvement was associated with changes in the fecal commensal bacteria *Bifidobacteria* and *Lactobacilli*.

Taken together, clinical studies suggest that targeting the gut-liver axis through the use of probiotics may have a therapeutic role in the treatment of patients ranging from those with mild alcoholic hepatitis to those with severe alcoholic cirrhosis. As noted, further rigorous studies with larger sample sizes for testing the effects of probiotics on ALD are needed. Developing novel probiotic strains and related products (including isolating new probiotic bacteria with improved potency for inhibiting pathogenic bacterial growth, strengthening intestinal barrier function, and improving immunoregulation and engineered probiotic bacteria producing specific metabolites) will provide more selectivity for treating ALD patients at different disease stages (Li et al. 2016a).

Accumulating evidence demonstrates the protective effect of probiotics on multiple pathological disorders. However, these treatments are not always effective because, in many cases, live bacteria must colonize the gut to confer their beneficial effects. The spectrum of pathogenic bacteria varies from patient to patient. Drugs, in particular antibiotics, used by patients may be harmful to live probiotics leading to an unstable and variable effect of the live probiotics. Moreover, the clinically recommended dose of probiotics usually consists of billions of live bacteria. Generally, probiotics are considered safe, but several reports have raised safety concerns about ingesting such large amounts of bacteria, especially when the intestinal

function and/or the patient's immune response are compromised (Pereg et al. 2011; Tandon et al. 2009; Wiest et al. 2003; Bauer et al. 2002). In an attempt to address these potential concerns, soluble factors secreted from live probiotics and dead probiotics have been used in the treatment of several diseases/conditions, such as inflammatory bowel disease, colitis, and arthritis (Nowak et al. 2012; Zakostelska et al. 2011; Yan and Polk 2012). Yan et al. demonstrated that soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth (Yan et al. 2013). Interestingly, the beneficial effects of probiotics on ALD do not appear to be restricted to viable probiotic bacteria. Segawa and colleagues demonstrated that oral administration of heat-killed *Lactobacillus brevis* SBC8803 induced the expression of cytoprotective heat shock proteins and improvement of intestinal barrier function leading to amelioration of experimental ALD (Segawa et al. 2008). Recently, we evaluated the effectiveness of LGG culture supernatant in the prevention of acute and chronic alcohol-induced hepatic steatosis and liver injury (Zhang et al. 2015; Zhao et al. 2015; Wang et al. 2012). Pretreatment with LGG supernatant (LGG-s) reduced hepatic fat accumulation in mice subsequently exposed to acute-binge alcohol (Wang et al. 2012). Furthermore, co-administration of LGG supernatant with alcohol in the Lieber-DeCarli liquid diet for 4 weeks significantly prevented alcohol-induced intestinal barrier dysfunction, endotoxemia, fatty liver, and inflammation in mice (Zhang et al. 2015; Zhao et al. 2015). The use of probiotic culture supernatant opens a new avenue for the probiotic use. Further characterization of the LGG-s active components will enhance our understanding of the protective effect of probiotics in ALD and advance the development of new therapeutic strategies for ALD.

**Probiotic treatment/prevention of NAFDL/NASH** Evidence of the beneficial effects of probiotics and symbiotics on NAFLD is extensively derived from rodent models. In a pioneering study of an animal model of NAFLD (Li et al. 2003), VSL#3 probiotic mixture (a multi-strain preparation composed of *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium infants*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. bulgaricus, and *Streptococcus thermophilus*) was used to treat high-fat diet-induced NAFLD. Treatment with VSL#3 improved liver histology, reduced hepatic total fatty acid content, and decreased serum ALT levels. Mechanistic analysis showed that VSL#3 treatment reduced the activity of Jun N-terminal kinase (JNK), a TNF-regulated kinase that promotes insulin resistance, and decreased the DNA binding activity of NF-κB, the target of IKKβ, another TNF-regulated enzyme that causes insulin resistance. Consistent with treatment-related improvements in hepatic insulin resistance, fatty acid β-oxidation and uncoupling protein (UCP)-2 expression decreased after treatment with VSL#3. The beneficial effects of VSL#3 treatment were comparable to anti-TNF antibody treatment. In a possibly more relevant mouse model (Ma et al. 2008) to diet-induced obesity in humans, the beneficial effects of VSL#3 supplementation on the prevention of the development of hepatic steatosis was also attributed to the regulation of hepatic natural killer T cells (NKT cells) and suppression of the TNF-α/IKK-β signaling pathway. Moreover, VSL#3

exhibited anti-inflammatory activity through a reduction of nuclear factor κB (NF-κB) activation, decreasing hepatic TNF-α production, and cyclooxygenase and inducible nitric oxide synthase expression (Ma et al. 2008). In a rat model of NASH, 4 weeks of VSL#3 treatment attenuated high-fat diet-induced oxidative and inflammatory liver damage (Esposito et al. 2009). In a methionine-choline deficiency (MCD) diet-induced steatohepatitis model, VSL#3 attenuated fibrosis, but did not reduce fatty liver and steatohepatitis (Velayudham et al. 2009).

Several strains of *Lactobacillus* and *Bifidobacterium* have shown protective effects on NAFLD. Administration of *Lactobacillus rhamnosus* PL60, a probiotic bacterium of human origin which produces conjugated linoleic acid, showed anti-obesity effects and improved liver steatosis in a mouse model of diet-induced obesity (Lee et al. 2006). *Lactobacillus acidophilus* and *Lactobacillus casei* administration for 8 weeks demonstrated antioxidant effects in the liver and pancreatic tissues in high-fructose diet-fed mice (Yadav et al 2007). Recent studies also showed that *Lactobacillus* probiotic treatments prevented fructose excess-induced (Wagnerberger et al. 2013; Yadav et al. 2007; Ritze et al. 2014) or choline deficiency-induced hepatic steatosis in animals (Okubo et al. 2013). The beneficial effects of probiotics in NAFLD have been linked to restoration of gut microbiota, gut barrier integrity, and attenuation of inflammatory cytokine expression in the liver (Ritze et al. 2014; Xu et al. 2012). *Lactobacillus* probiotics have also been used to prevent hepatic cholesterol and triglyceride accumulation in rats fed high-cholesterol diet (Wang et al. 2009).

*Lactobacillus paracasei* F19 significantly attenuated liver injury induced by ischemia-reperfusion and a methionine-restricted/choline-deficient diet in rats by restoring gut microbiota and reducing inflammation and steatosis (Nardone et al. 2010). Of note, administration of different strains of *Lactobacillus* and *Bifidobacterium* in an acute liver injury rat model has shown different effects on bacterial translocation and hepatocellular damage (Adawi et al. 2001). In addition, in a study comparing *Bifidobacterium longum* and *Lactobacillus acidophilus*, Xu et al. (2012) showed that *Bifidobacterium longum* was more effective in preventing NAFLD development, further supporting the importance of careful evaluation of probiotic strains in therapeutic use.

Prebiotic preparations have also been used to treat NAFLD in animals. ob/ob mice treated with prebiotic with non-prebiotic carbohydrate as control showed that the beneficial effect of the prebiotic was mediated by attenuation of liver inflammation in obese mice through a glucagon-like peptide-2 (GLP2)-dependent effect on the gut barrier (Cani et al. 2009). In another mouse model, prebiotic FOS administration reduced liver fat accumulation associated with cecal bacterial alterations (Pachikian et al. 2013).

Despite a large body of preclinical studies on probiotics and NAFLD, high-quality clinical studies are still limited. A double-blind clinical trial (Vajro et al. 2011) using *Lactobacillus* GG in obese children with NAFLD showed significant decreases in serum ALT values and in anti-peptidoglycan-polysaccharide antibodies, which are used as an indirect indicator of intestinal bacterial overgrowth and translocation. Loguercio et al. (2005) demonstrated that VSL#3 supplementation in

patients affected by several types of chronic liver diseases, including NAFLD, may reduce liver damage and improve serum levels of various biomarkers. A NAFLD-related study endpoint was a reduction of ALT and AST accompanied by a reduction of pro-inflammatory cytokines.

A small, randomized, placebo-controlled study of NAFLD in children (Alisi et al. 2014) showed that supplementation with probiotics significantly improved their liver disease. They showed that 4-month supplementation of VSL#3 improved both fatty liver and BMI. A previous study (Yadav et al. 2013) showed that the same probiotic preparation suppressed body weight gain and insulin resistance in mice. Interestingly, VSL#3 supplementation in NAFLD children showed a clear trend toward increasing concentrations of total and activated glucagon-like peptide-1 (GLP-1), suggesting that the VSL#3-dependent GLP-1 increase could be responsible, at least in part, for these beneficial effects. A recent randomized triple-blind trial study (Famouri et al. 2017) including 64 obese children with NAFLD showed that 12-week supplementation with a probioitic mixture (*Lactobacillus acidophilus* ATCC B3208; *Bifidobacterium lactis* DSMZ 32269; *B. bifidum* ATCC SD6576; *L. rhamnosus* DSMZ 21690) significantly reduced serum levels of ALT, AST, cholesterol, LDL-C, and triglycerides, further suggesting that this probiotic mixture can be effective in improving pediatric NAFLD.

A randomized study on the use of a synbiotic that contains five probiotics (*L. plantarum*, *L. delbrueckii* spp. *bulgaricus*, *L. acidophilus*, *L. rhamnosus*, *B. bifidum*) and a prebiotic (inulin) over 6 months in adults with NASH produced a significant decrease in hepatic steatosis (Wong et al. 2013b). Another clinical study showed that synbiotic treatment (*L. casei*, *L. rhamnosus*, *S. thermophilus*, *B. breve*, *L. acidophilus*, *B. longum*, *L. bulgaricus*, and FOS) of NAFLD patients over 28 weeks inhibited NF-κB-induced TNF-α production (Eslamparast et al. 2014).

Several meta-analyses on the effectiveness of probiotics in NAFLD have been published. A meta-analysis (Ma et al. 2013) of four randomized controlled trials (Wong et al. 2013b; Malaguarnera et al. 2012; Vajro et al. 2011; Aller et al. 2011), selected from 475 items published from 1996 to 2013, on probiotics in patients with NAFLD/NASH concluded that probiotic therapies can reduce liver aminotransferase levels, serum cholesterol, and TNF-α and improve insulin resistance in patients with NAFLD. This suggests that modulation of the gut microbiota using probiotics may represent a unique method of treating or preventing NAFLD. Although this meta-analysis introduces difficulty in obtaining unequivocal correlations between biochemical changes and pharmacological treatment or lifestyle modifications, such as the inclusion of probiotics in diet, it is informative since it demonstrates the complexity of probiotic treatment of NAFLD.

A recent meta-analysis including nine international randomized controlled trials of probioitcs in childhood and adult NAFLD showed that probiotics provided improvements in the outcomes of homeostasis model assessment (HOMA), total cholesterol, high-density lipoprotein, and TNF-α in any NAFLD patient and in triglycerides in Italian and Spanish patients, but there were no improvements in the outcomes of BMI, glucose, or insulin in adult NAFLD patients (Gao et al. 2016).

A more comprehensive meta-analysis summarized the effects of probiotics and synbiotics on obesity, insulin resistance syndrome, type 2 diabetes, and NAFLD (Saez-Lara et al. 2016). Current scientific evidence shows that effects of probiotics in patients with NAFLD are primarily an improvement in liver tests and metabolic parameters. Some clinical results reported beneficial effects using both probiotic and synbiotic supplementation. However, there are studies that did not show any significant effect of probiotic administration on these chronic diseases. These contradictory effects might be related to inappropriate design such as diversity, the use of several strains, and the small number of individuals receiving some interventions. The authors suggest that further studies to evaluate the best dose-response effect of probiotics and synbiotics are needed.

**Treatment of cirrhosis** The first-line treatment of the cirrhotic with PSE is lactulose. However, treatment with lactulose showed only minimal impact on microbiota composition (Bajaj et al. 2012a). The microbial functionality may be more important than composition in HE. A recent study using LGG in patients with cirrhosis showed promising results (Bajaj et al. 2014a). In this randomized placebo-controlled double-blind Phase I trial involving 30 patients with MHE, LGG treatment reduced gut dysbiosis and endotoxemia with improved gut microbiota-metabolome linkage. In an animal study, VSL#3 has been shown to decrease bacterial translocation and improve intestinal permeability in rats with cirrhosis (Adawi et al. 2001).

It is obvious that antibiotics decrease gut bacterial growth and changes the composition. The effects on antibiotic alterations on gut microbiota appear to be more sustained than previously recognized (Cox and Blaser 2015). In animal studies, antibiotics corrected liver function induced by multiple pathogenic conditions. In clinical studies, however, antibiotics have been primarily employed in the treatment and prevention of several complications of liver disease (Rimola et al. 1985). Prophylactic antibiotics have been used mainly on bacterial infections which target the most common pathogenic microorganisms in cirrhosis (Wong et al. 2005). There is no research thus far to support the use of antibiotics as the primary therapy of ALD and NAFLD or related liver diseases such as intestinal failure-associated liver disease (IFALD) where small intestinal bacterial overgrowth (SIBO) is an important pathogenic factor (Barclay et al. 2011).

**Fecal microbiota transplantation** Fecal microbiota transplantation (FMT) is a technique in which intestinal microbiota are transferred from a healthy donor to the patient to introduce or restore healthy microbiota in the gut. Numerous studies have demonstrated that FMT is an effective therapeutic strategy for *Clostridium difficile* infection. There also is strong evidence that FMT can be used to treat NAFLD and ALD in animal models. In a NAFLD murine model study (Le Roy et al. 2013), the microbiota from mice that developed NAFLD from HFD feeding were transferred to germ-free mice, which subsequently developed steatosis and harbored a large number of *Barnesiella* and *Roseburia*. However, the mice which received the microbiota from the HFD mice that did not develop NAFLD did not develop steatosis. This indicates that FMT might be useful in NAFLD treatment. Similar results have also been found in ALD mice (Ferrere et al. 2017). Although there are promising

data from rodents, a clinical study of FMT for liver disease is lacking. We need a better understanding of the host-intestine interactions to determine the optimal intestinal preparation and environment for FMT. Appropriate standardization and characterization of the FMT preparation and a simplified regulatory mechanism for utilizing FMT need to be established for it to become an alternative therapy for liver diseases associated with gut microbiota.

## 11.8 Conclusions

There is increasing evidence supporting the concept that gut microbiota play a central role in liver diseases such as NAFLD, ALD, cirrhosis, and liver cancer. Microbiota can impact the onset and progression of these diseases. Many of these studies were conducted in preclinical animal models in which the disease phenotypes could be altered by gut microbiota manipulation and antibiotics could reverse the disease. Further human study is urgently needed. A major difficulty of studying the human microbiota is the variation in the microbiota composition and functionality from individual to individual. In the past, multiple procedures have been used to assess the microbiota homeostasis, and they might produce different results. There is need to develop standardized and more sophisticated techniques to analyze microbiota and their functional activity.

It is clear that many factors affect gut microbiota and liver. In particular, dietary factors such as lipid type, fructose, and alcohol, as well as lifestyle factors such as exercise and sleep, have been studied. Manipulation of these factors may have preventive/therapeutic potential for liver disease. Probiotics, prebiotics, synbiotics, and FMT can have specific gut effects on microbiota and liver pathophysiology that impact on development of liver disease and offer therapeutic opportunities.

Many elegant studies have identified a number of regulatory mechanisms, such as bile acid metabolism, SCFA production, endogenous alcohol generation, increased intestinal barrier dysfunction, and dysregulated immune function in the gut and liver. These factors might be simultaneously involved as "multiple hits," but the individuals and disease types may determine the relative importance of those mechanisms. Furthermore, it is important to emphasize that the metabolic effects of dysbiosis play a central role. A combination of microbiota metagenomics and metabolomics, and possibly proteomics, genomics, lipidomics, and other high-throughput omics techniques, will provide insights into mechanisms and novel therapeutic strategies. Such a comprehensive study may enable a precision medicine approach based on patient's prognostic biomarkers, clinical observations, and pathogenic pathways.

Further studies should also focus on the identification of specific pathogenic microbiota species, complete description of the microbiota, microbiota functionality, and the interaction with diets, environmental factors, and lifestyles via longitudinal studies. Such an approach could reveal how microbiota influence disease phenotype and enable us to design specific therapeutic approaches targeting individual microbiota for liver diseases.

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W.-X. Ding, X.-M. Yin (eds.), *Molecules, Systems and Signaling in Liver Injury*,
Cell Death in Biology and Diseases, DOI 10.1007/978-3-319-58106-4

X.-M. Yin and Z. Dong (Series eds.), *Cell Death in Biology and Diseases*