MECHANISMS OF LIVER REGENERATION AFTER ACETAMINOPHEN-INDUCED HEPATOTOXICITY IN MICE

By

Bharat Bhushan,
M.S. (Pharm), National Institute of Pharmaceutical Education and Research, 2008

Submitted to the graduate degree program in
Toxicology and the Graduate Faculty of the University of Kansas
Medical Center in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

_____
Chairperson: Udayan Apte, PhD

_____
Hartmut Jaeschke, PhD

_____
Steven A. Weinman, MD, PhD

_____
Wen-Xing Ding, PhD

_____
Luciano DiTacchio, PhD

Date defended:

10th June 2016

The Dissertation Committee for Bharat Bhushan
certifies that this is the approved version of the following dissertation:

MECHANISMS OF LIVER REGENERATION AFTER ACETAMINOPHEN-INDUCED HEPATOTOXICITY IN MICE

Chairperson: Udayan Apte, PhD

Date Approved: 10th June 2016

ABSTRACT

Overdose of acetaminophen (APAP) is the major cause of acute liver failure (ALF) in the western world with very limited treatment options. Recent studies suggest that liver regeneration is a critical determinant of final recovery and overall survival following APAP overdose. Stimulating liver regeneration in patients of APAP-induced ALF holds a great therapeutic potential. However, development of novel regenerative therapies for ALF is hampered because the mechanisms of liver regeneration after APAP-induced ALF are not completely known. The focus of this research was to investigate the mechanisms of liver regeneration after APAP-induced hepatotoxicity in mice.

Firstly, we identified potential regulators of liver regeneration following APAP-induced hepatotoxicity using a novel incremental dose model. Liver injury and regeneration were studied in C57BL/6 mice treated with either 300 mg/kg (APAP300) or 600 mg/kg (APAP600) APAP. Mice treated with APAP300 developed extensive liver injury initially, followed by robust liver regeneration, resulting in resolution of the injury. In contrast, mice treated with APAP600 exhibited significant liver injury, but substantially delayed and attenuated liver regeneration resulting in sustained injury and decreased survival. The inhibition of liver regeneration in APAP600 group was associated with cell cycle arrest and decreased Cyclin D1 expression. Further analysis revealed that several known regenerative pathways including IL-6/STAT-3, growth factor signaling via EGFR/c-Met/MAPK pathways were activated even in APAP600 group where regeneration was inhibited. However, canonical Wnt/β-catenin and NF-κB pathways were activated only in APAP300 treated mice, where liver regeneration was stimulated.

Next, we investigated role of Wnt/β-catenin in further detail. ChIP analysis revealed decreased binding of β-catenin to Cyclin D1 promoter in APAP600 group correlating with decreased cyclin D1 induction and impaired liver regeneration. Overexpression of a stable form of β-catenin (S45D) in mice resulted in improved liver regeneration following APAP overdose. Inactivation of GSK3, an upstream negative regulator β-catenin, was found to be positively associated with β-catenin activation and liver regeneration. GSK3 inactivation was remarkably reduced in APAP600 group, where liver regeneration was attenuated and delayed. Treatment with a selective GSK3 inhibitor (L803-mts), as late as 4 hr after APAP600, resulted in early initiation of liver regeneration and improved survival in mice. Early hepatocyte proliferation after GSK3 inhibition was due to rapid induction of cyclin D1 secondary to increased activation of β-catenin signaling.

Finally, we investigated the role of EGFR signaling in liver regeneration, which was dose-dependently activated after APAP overdose. Surprisingly, early EGFR inhibition (1 hr post-APAP) by treatment with an irreversible EGFR inhibitor, canertinib, strikingly attenuated APAP hepatotoxicity suggesting role of EGFR in development of APAP hepatotoxicity. Delayed EGFR inhibition, 12 hr post-APAP, did not alter initial injury but caused cell cycle arrest, remarkable impairment of liver regeneration and decreased survival after APAP overdose (APAP300) in mice.

Overall, these studies comprehensively investigated the mechanisms of liver regeneration after APAP-induced ALF and will pave the road for future therapeutic and mechanistic research on liver regeneration after APAP-induced ALF.

ACKNOWLEDGEMENTS

This PhD would have not been possible without the support of so many people whom I met over these last 6 years, but also people from way before who have inspired me towards a scientific career. I feel generously blessed to have met all these people and be given this opportunity that has shaped me as a person.

Firstly, I would like to take this opportunity and thank Dr. Apte for his staunch belief in my abilities and also supporting me in every possible way, to pursue this dissertation work. Thank you for giving me the chance to grow both personally and professionally. I will forever remember what I have learnt from you and am grateful to you for imparting these life-long lessons.

Secondly, I want to thank all my committee members – Dr. Hartmut Jaeschke, Dr. Steven Weinman, Dr. Wen-Xing Ding, Dr. Luciano DiTacchio and Dr. Xiaochao Ma – for their time and valuable suggestions throughout the duration of this work and for always looking out the best for me. I sincerely appreciate for the time and efforts you have invested in me. I specifically wanted to thank Dr. Jaeschke for directing me to the Apte lab for pursuing my PhD, which truly has been a blessing in every way.

I want to thank the past and current members of Apte lab – Dr. Chad Walesky, Dr. Kevin Beggs, Prachi Borude, Ian Huck, Steven McGreal, Samikshya Paudel, Genea Edwards, Tara Gallagher, Michael Manley, Dr. Lina Sun– for their lovely company, friendship and support through scientific trials and tribulations. I also would like to thank Cody, Dorothy, Elizabeth and Stacy for providing administrative assistance over the past years.

I also want to thank Dr. Xiaobo Zhong and the Zhong lab members for their support in nourishing my scientific endeavors, while I was in Zhong lab. Further, I want to acknowledge Pharmacology & Toxicology Department for supporting my PhD journey throughout.

I want to thank my past mentor – Dr. Deepak K. Majumdar – for teaching me what scientific research means and inspiring me to pursue a scientific career. I want to thank all my friends at KUMC who have inevitably formed my family here, for their support throughout this dissertation work, by never making me feel that I am away from home. Last but not the least; I want to take this opportunity to thank them, without them I would not have made it so far, both as a person and as a PhD student. I want to thank my parents for their unconditional love and support; for sowing in me the values of sincerity, commitment and hard work. I want to thank my brother, sister and wife for being an endless source of encouragement and inspiration.

Bharat Bhushan

CONTENTS

CHAPTER I: INTRODUCTION

1.1 ACETAMINOPHEN (APAP) AND ITS HEPATOTOXICITY

Acetaminophen (APAP) is the most commonly used over-the-counter (OTC) analgesic and anti-pyretic drug across the world. APAP is present in numerous formulations either alone or in combination with other active ingredients. Although, APAP is considered very safe at therapeutic doses, overdose of APAP can cause severe liver damage and acute liver failure (ALF). In fact, APAP overdose is the foremost cause of ALF in the US accounting for approximately 50% of all the ALF cases (Lee 2012, Bernal, Lee et al. 2015). APAP overdose is associated with more than 78,000 emergency room visits, 33,000 hospitalizations and around 500 deaths every year in the US (Budnitz, Lovegrove et al. 2011, Manthripragada, Zhou et al. 2011). While majority of APAP overdose cases are intentional (around 70%), cases of therapeutic misadventures are also frequent (Budnitz, Lovegrove et al. 2011). Extensive research has been done to study the mechanism of APAP toxicity, but the current treatment options are very limited. N-acetylcysteine (NAC) is the current standard of care for APAP overdose patients, which is effective only at an early stage (Yang, Miki et al. 2009, Bernal, Lee et al. 2015). Ultimate option is liver transplantation, which is complicated by issues such as donor availability, graft rejections, long-term immunosuppression and exorbitant costs (Keeffe 2001).

At therapeutic doses, majority of the APAP (>85%) undergoes conjugation reaction with glucuronide or sulfate followed by excretion. Remaining APAP (5-15%) is metabolized to a reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) by CYP450 enzymes (mainly CYP2E1 and CYP1A2) (Nelson 1990, Zaher, Buters et al. 1998). This

metabolite is toxic, but gets rapidly conjugated to glutathione and converted into non-toxic metabolite. In case of APAP overdose, there is excess production of NAPQI, which depletes cellular glutathione and form adducts with cellular proteins by reacting with sulfhydryl groups of cysteine residues. Mitochondria are the critical target of APAP toxicity and APAP adduct formation with mitochondrial proteins is considered important for toxicity initiation (Meyers, Beierschmitt et al. 1988, Tirmenstein and Nelson 1989, Jaeschke, McGill et al. 2012). This leads to mitochondrial dysfunction and generation of reactive oxygen species (ROS) in mitochondria. Additionally, superoxide radicals react with nitric oxide to form the highly potent oxidant, peroxynitrite, in mitochondria. Initial mitochondrial stress is considered not sufficient to cause hepatocyte necrosis and is exacerbated by a plethora of intracellular signaling events, which are not completely understood. c-Jun N-terminal kinase (JNK) activation, its mitochondrial translocation and interaction with the mitochondrial outer membrane protein Sab (SH3BP5) is considered one of such important signaling events (Hanawa, Shinohara et al. 2008, Win, Than et al. 2011, Jaeschke, Xie et al. 2014). Mitochondrial dysfunction and oxidative damage eventually lead to opening of the mitochondrial permeability transition (MPT) pore. This results in release of endonucleases such as apoptosis-inducing factor (AIF) and endonuclease G from the mitochondrial intermembrane space into the cytosol that cause DNA damage. The ultimate consequence is hepatocellular necrosis specifically in the centrilobular region of the liver (Jaeschke, McGill et al. 2011, Jaeschke, McGill et al. 2012, Jaeschke, Xie et al. 2014).

1.2 LIVER REGENERATION

Liver has an extraordinary capacity to regenerate upon loss of liver tissue. Liver regeneration can occur as a compensatory response to toxin-induced liver injury, surgical resection, infection or trauma. Liver regeneration is seen in all vertebrates even after losing considerable amount of liver mass. Regenerative capacity of liver is long known and described even in ancient Greek mythology. Liver regeneration was first experimentally described by Higgins and Anderson in 1931, using rodent model of two-third partial hepatectomy (PH). PH is still the most widely studied model of liver regeneration in which two-third of the liver is surgically removed and liver re-grows precisely to its original size within few days (5-7 days in rats and mice). After PH, virtually all the remaining cells undergo 2-3 round of replication to restore original liver mass by compensatory hyperplasia (Michalopoulos 2007, Michalopoulos 2013, Michalopoulos 2014).

The mechanisms of liver regeneration after PH have been extensively studied for decades. The process of liver regeneration is highly orchestrated and involves complex network of, often redundant, signaling pathways involving cytokines (such as tumor necrosis factor-α: TNFα and interleukin 6: IL6) and growth factors (such as hepatocyte growth factor: HGF, epidermal growth factor: EGF and transforming growth factor-α: TGFα). In brief, there are two different schools of thoughts regarding the mechanism of liver regeneration after PH. According to paradigm put forth by Nelson Fausto, cytokines initiate the process by priming the hepatocytes so that they are readily responsive to proliferative stimuli. This is followed by growth factors mediated entry into cell cycle and

progression through cell cycle leading to proliferation (Fausto 2000, Fausto, Campbell et al. 2006). In comparison, according to the theory put forth by George Michalopoulos, there is no demarcation in the time frame of action of growth factors and cytokines. Rather, there are some primary mitogens (such as HGF and ligands of EGF receptor), which can directly stimulate hepatocytes proliferation even in serum free media stand-alone. Others are secondary mitogens such as IL6 and TNFα, which assist hepatocytes proliferation leading to orchestrated liver regeneration events. It is interesting to note that abolition of any of these signaling pathways alone does not inhibit liver regeneration permanently (except HGF/met) but only delays it, highlighting the redundancy of the pathways involved in liver regeneration (Michalopoulos and DeFrances 1997, Michalopoulos 2007). Apart, from classic regenerative response to loss of liver tissue, liver growth also occurs in adaptive physiological events such as during pregnancy. Further, many xenobiotics (such as phenobarbital and fibrate drugs) can stimulate hepatocyte proliferation and cause liver enlargement in absence of liver injury via activation of nuclear receptors (such as constitutive androstane receptor: CAR and peroxisome proliferator-activated receptor-α: PPAR-α). Despite decades of extensive research to understand the phenomenon of liver regeneration, the exact mechanism are still not completely understood; especially the events that terminates liver regeneration precisely after attaining original size of liver following PH (Michalopoulos 2007, Michalopoulos 2010).

1.3 LIVER REGENERATION: CRITICAL DETERMINANT OF FINAL OUTCOME AFTER CHEMICAL-INDUCED LIVER TOXICITY

Liver regeneration is also observed as a compensatory response to liver injury mediated by toxicants such as thioacetamide (Mangipudy, Chanda et al. 1995), carbon tertrachloride (Rao, Mangipudy et al. 1997), chloroform (Anand, Soni et al. 2003) and acetaminophen (Bajt, Knight et al. 2003, Apte, Singh et al. 2009). Data on hepatotoxicants, in general, suggest that liver regeneration follows the principles of dose-response (Mehendale 2005). Studies indicate that liver regeneration following toxicant-induced liver injury increases proportionately to the extent of liver injury, but only up-to a threshold dose. Doses higher than the threshold dose actually inhibit liver regeneration (Mangipudy, Chanda et al. 1995, Anand, Murthy et al. 2003, Mehendale 2005). In fact, liver regeneration plays a critical role in determination of final outcome of injury such that timely stimulation of regeneration leads to regression of injury but delayed or inhibited regeneration culminates in progression of injury and death (Mehendale 2005). Importance of liver regeneration in toxic injury is especially highlighted by experiments in which liver regeneration was manipulated. For example, inhibition of liver regeneration, with anti-mitotic agents such as colchicine, following liver injury induced by several toxicants resulted in exacerbation of injury. Similarly, stimulation of liver regeneration in these cases inhibited progression of injury resulting in better survival (Mehendale 2005).

Similar to other hepatotoxicants, APAP-induced liver injury is followed by compensatory liver regeneration, where in, the hepatocytes in closest proximity to the necrotic zones

divide and replace dead cells leading to recovery. Several lines of evidence indicate the important of role of liver regeneration in determining outcome after APAP-induced liver injury. For instance, treatment with IL6 (James, Lamps et al. 2003), stem cell factor (Hu and Colletti 2008), VEGF (Donahower, McCullough et al. 2010) and glutathione (Bajt, Knight et al. 2003) enhanced liver regeneration resulting in regression of injury or enhanced survival after APAP-induced toxicity in mice. Further, streptozotocin-induced diabetic mice were reported to be resistant to APAP-induced injury because of higher regeneration capacity and inhibiting regeneration by anti-mitotic agent (colchicine) in these mice resulted in increased mortality (Shankar, Vaidya et al. 2003). Furthermore, there are recent clinical evidences suggesting correlation of liver regeneration after APAP overdose with enhanced survival. Apte *et al.* demonstrated correlation of β-catenin activation with higher spontaneous liver regeneration, preventing liver transplantation in patients (Apte, Singh et al. 2009). Another report indicated association of α-feto protein (marker of liver regeneration) with better survival rate in patients with APAP-induced ALF (Schmidt and Dalhoff 2005). All these data indicate that stimulating liver regeneration in APAP-induced ALF patients is a plausible therapeutic option.

1.4 MECHANISMS OF LIVER REGENERATION AFTER APAP-INDUCED HEPATOTOXICITY

In order to develop regenerative therapy for APAP-induced ALF, we should first understand the mechanisms of liver regeneration after APAP-induced ALF. These mechanisms can be different from most widely studied PH model based on the fact that APAP-injury is mechanistically different from PH (Table 1). APAP injury involves

necrosis and widespread inflammation which is minimal in PH (Michalopoulos 2010, Jaeschke, Williams et al. 2012). Furthermore, liver regeneration is synchronous in case of PH and involves proliferation of virtually all the remaining hepatocytes. In contrast, in case of APAP injury mostly cells around necrotic zone proliferate (Bajt, Knight et al. 2003, Michalopoulos 2007). Although, APAP-induced liver injury is clinically significant model for ALF, mechanisms of liver regeneration after APAP-induced hepatotoxicity are highly underexplored compared to mostly commonly studied PH model of liver regeneration. Following part of this section reviews some of the studies, which investigated mechanisms of liver regeneration after APAP-induced liver injury.

Table 1. Differences between liver regeneration after partial hepatectomy and acute liver injury

Features	Partial Hepatectomy	Acute Liver Injury
Starting point	A known starting point (time of surgery)	Extended process with undefined starting point
Location	All hepatocytes in the remaining lobes	Mostly areas surrounding the necrotic zones
Cell cycle	Synchronous	Unsynchronized
Inflammation	Not significant	Extensive
Injury	Moderate	Extensive (dose-dependent)

Role of Cytokines: As mentioned previously, activation of cytokine pathways (IL-6/STAT-3 and TNF-α/NF-κB) is considered as important "priming event" that contribute

to initiation of early stages of liver regeneration after PH (Fausto 2000). There are some evidences indicating role of these cytokines pathways in liver regeneration after APAP-induced liver injury as well. TNF-α-mediated proliferative signaling in hepatocytes is majorly mediated by TNF receptor 1 (TNFR1). Two independent studies demonstrated that deletion of TNFR1 impaired liver regeneration after APAP overdose in mice (Chiu, Gardner et al. 2003, James, Kurten et al. 2005). TNFR1 KO mice were found to be more susceptible to APAP toxicity in one of these studies (Chiu, Gardner et al. 2003). Thus, inhibitory effects on liver regeneration by TNFR1 deletion could be mediated secondary to exaggerated injury. TNF-α binding to its receptor ultimately results in stabilization and nuclear translocation of transcription factor NF-κB. Previous reports showed increased hepatic TNF-α concentrations and NF-κB DNA binding after APAP treatment, which were correlated positively with increased cyclin D1 protein expression and liver regeneration in mice (Yang, Miki et al. 2009, Yang, Zhang et al. 2011, Yang, Zhang et al. 2012). In contrast, decreased serum TNF-α concentration, NF-κB DNA binding along decreased expression of cyclin D1 were reported following interventions that impaired liver regeneration after APAP-induced liver injury (Yang, Miki et al. 2009, Yang, Zou et al. 2012).

Similar evidences implicating role of IL6/STAT3 signaling in regeneration after APAP-induced liver injury have been reported. IL6 levels in liver and serum are reported to be increased after APAP overdose in mice (Bajt, Knight et al. 2003, James, McCullough et al. 2003, Masubuchi, Bourdi et al. 2003). Further, IL6-KO mice were reported to have impaired liver regeneration after APAP toxicity without any alteration of initial liver injury.

Impaired liver regeneration in IL6-KO was associated with prolonged elevation of AST levels (James, Lamps et al. 2003). Pretreatment with IL-6 in these KO mice resulted in restoration of liver regeneration parameters along with decrease in AST levels, indicating role of IL-6 in liver regeneration and recovery after APAP-induced hepatotoxicity (James, Lamps et al. 2003). IL-6 binding to its receptor at cellular membrane leads to phosphorylation and nuclear translocation of STAT-3, which ultimately results in transcription of its target genes. Impaired liver regeneration in TNFR1 KO mice was associated with delayed phosphorylation of STAT-3 suggesting cross-talk of TNF-α/NF-κB and IL-6/STAT-3 signaling in liver regeneration after APAP-induced liver injury, as known to exist in case of PH model (Chiu, Gardner et al. 2003).

Role of growth factors: Growth factors such as EGFR ligands and HGF are considered direct mitogens for hepatocytes and are critical for liver regeneration after PH. In fact, EGFR ligands (such as EGF and TGFα) and HGF are the only known direct mitogens for hepatocytes (Michalopoulos 2007). EGFR ligands and HGF act primarily through activation of EGF receptor (EGFR) and c-Met receptor, respectively. Genetic ablation or siRNA-mediated downregulation of cMet caused almost complete blockage of liver regeneration after PH highlighting importance of this pathway in liver regeneration (Michalopoulos 2007). Although, growth factors signaling plays central role in liver regeneration and hepatocyte proliferation after PH, their role in liver regeneration after APAP-induced liver injury is relatively unknown. In a study using sixty-two beagle dogs, administration of hepatic stimulatory substance alone or in combination with TGF-α, insulin-like growth factor II and insulin did not affect survival or liver regeneration after lethal dose (LD70) of APAP (Francavilla, Azzarone et al. 1993). In another study,

plasma HGF levels were found to be strikingly elevated in APAP-induced ALF patients. However, plasma HGF levels were significantly higher in non-survivors compared to survivors after APAP-induced ALF (Hughes, Zhang et al. 1994). These evidences indicate that stimulation with additional direct mitogens after APAP-induced ALF may not be beneficial. However, further mechanistic studies are required to delineate potential role of these direct mitogens in hepatocyte proliferation after APAP-induced ALF.

Vascular endothelial growth factor (VEGF) is a mitogen for endothelial cells and is known to play an important role in angiogenesis during liver regeneration after PH. Further, it can indirectly cause hepatocyte proliferation via production of HGF from endothelial cells (Michalopoulos 2007). Several reports have demonstrated important role of VEGF and its receptor VEGFR in liver regeneration after APAP-induced hepatotoxicity. Hepatic VEGF levels and expression of its receptors (VEGFR1, VEGFR 2, and VEGFR 3) increases after APAP overdose in mice and rats (Donahower, McCullough et al. 2006, Papastefanou, Bozas et al. 2007, Kato, Ito et al. 2011). Whereas, treatment with VEGFR inhibitor in mice impaired hepatocyte proliferation (Donahower, McCullough et al. 2006), administration of human recombinant VEGF increased hepatocyte regeneration after APAP overdose in mice, without altering initial hepatotoxicity (Donahower, McCullough et al. 2010). Further, VEGFR1 KO mice exhibited impaired restoration of microvasculature, diminished hepatocyte proliferation and expression of growth factors such as HGF and FGF, associated with decreased survival after APAP overdose (Kato, Ito et al. 2011).

Role of progenitor cells: Role of progenitor cells in liver regeneration has been a topic of intense debate. In most of the scenarios, proliferation of remnant hepatocytes to produce new hepatocytes is sufficient for liver regeneration. However, in case of severe hepatic injury where hepatocyte proliferation is inhibited, cells of biliary ductular origin (specifically terminal bile ductules called canals of Herring) can give rise to bipotent progenitor cells (known as the oval cells). These cells have ability to differentiate into hepatocytes restoring liver regeneration (Michalopoulos 2011). A dose and time-dependent bi-phasic oval cell reaction was also reported after administration of sub-lethal doses of APAP in mice, which was largely restricted to smallest portal tracts (Kofman, Morgan et al. 2005). Interestingly, the time-course of oval cell reaction in mice was closely correlated to previously documented temporal changes in serum stem cell factor (SCF) after APAP-induced liver injury, suggesting potential role of SCF in progenitor cell activation (Simpson, Hogaboam et al. 2003, Kofman, Morgan et al. 2005). Another study reported increased expression of SCF and its receptor c-kit (progenitor cell marker) in liver after APAP overdose in mice (Hu and Colletti 2008). While, mice treated with anti-SCF antibody and SCF-deficient mice exhibited increased mortality, treatment with exogenous SCF increased hepatocyte proliferation and improved survival after APAP-induced liver injury (Simpson, Hogaboam et al. 2003, Hu and Colletti 2008). Further, recent studies have suggested hepatic stellate cells as 'stem cell niche' for hepatic progenitor cells. Depletion of activated stellate cells using gliotoxin resulted in impaired liver regeneration after APAP overdose in mice, which was associated with decreased oval cell reaction (Shen, Chang et al. 2011). Similarly, progenitor cell response was also observed in APAP-induced ALF patients with severe

liver necrosis (Theise, Saxena et al. 1999). Furthermore, α-feto protein (marker of fetal phenotype and progenitor cells) positive cells were found in APAP-induced ALF patients (Theise, Saxena et al. 1999) and serum AFP levels were correlated with better prognosis and survival in APAP-induced ALF patients (Schmidt and Dalhoff 2005). All these studies suggest potential role of progenitor cells in liver regeneration after APAP-induced ALF, which should be explored in future for the standpoint of regenerative therapy and biomarker development.

1.5 WNT/ β -CATENIN SIGNALING AND ITS ROLE IN LIVER REGENERATION

Wnt/β-catenin signaling is known to play an important role in liver development and maturation (Thompson and Monga 2007). β-catenin is a transcription factor, which under un-stimulated state is bound to destruction complex consisting of Axin, Adenomatous polyposis coli (APC), Glycogen synthase kinase-3β (GSK3β) and Casein kinase 1 (CK1). CK1 first phosphorylates β-catenin at Ser45, which primes β-catenin. Primed β-catenin is recognized by GSK3β, which phosphorylates β-catenin at Thr41 residue and subsequently at Ser33 and Ser37 residues. Phosphorylated β-catenin is recognized by β-TrCP (an E3 ubiquitin ligase subunit), and subsequently ubiquitinated and targeted for proteosomal-mediated degradation (Fig. 1.5.1) (Clevers 2006, Kaidanovich-Beilin and Woodgett 2011). During canonical Wnt signaling, Wnts binds to Frizzled (Fzd) receptor which associates with LRP 5/6 and recruits Dishevelled (Dvl), which leads to phosphorylation of LRP 5/6. Phospho-LRP 5/6 binds to Axin leading to dissociation of β-catenin destruction complex and inactivation of GSK3β. This results in stabilization of β-catenin and its nuclear translocation. In nucleus, β-catenin binds with T

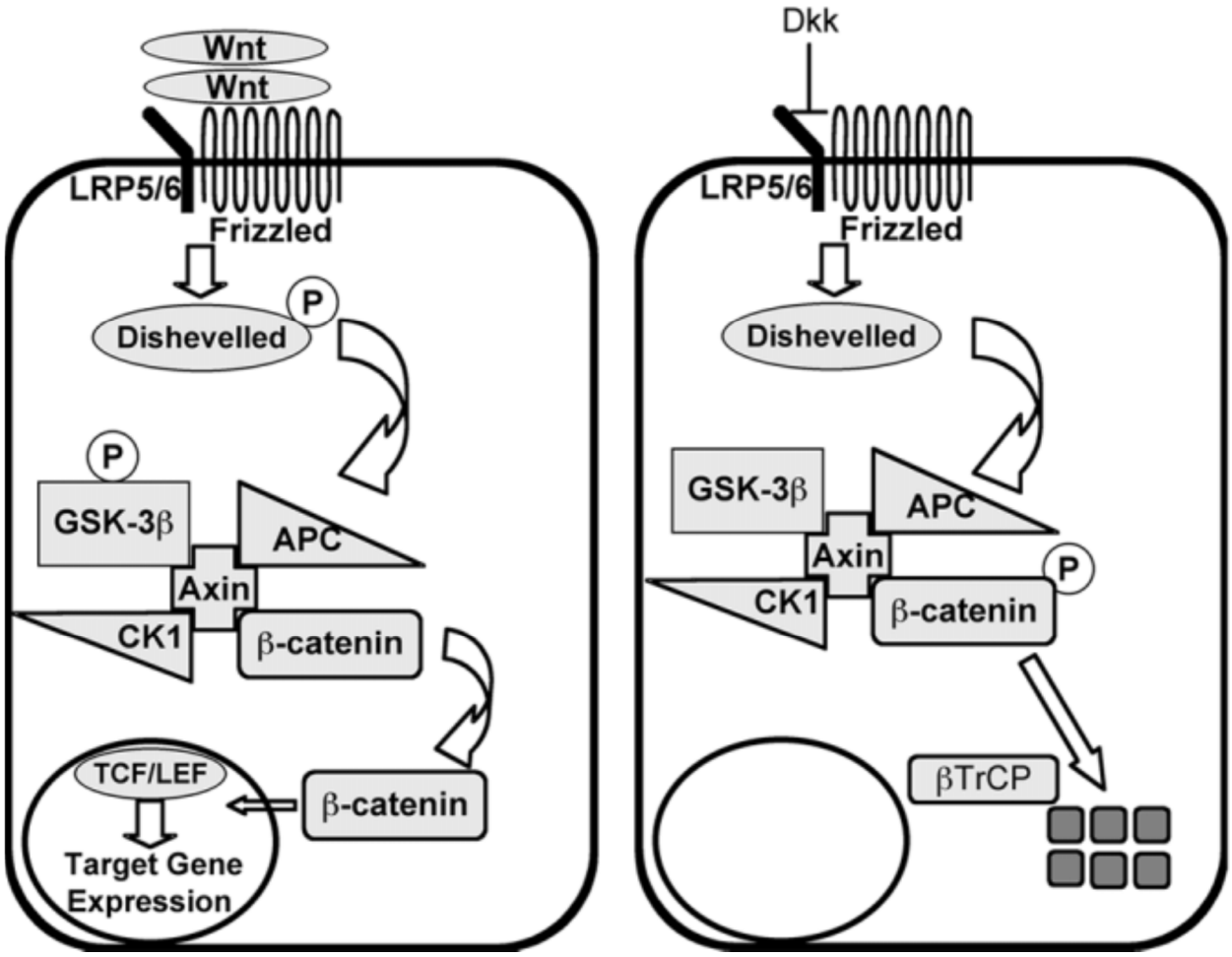

Figure 1.5.1. Schematic representation of canonical Wnt/β-catenin signaling pathway. Wnts binding to Frizzled receptor leads to inactivation of β-catenin destruction complex, hypo-phosphorylation and stabilization of β-catenin, which translocates to nucleus and cause transcriptional activation of its target genes (left panel). Under un-stimulated state, β-catenin is bound by its destruction complex, which phosphorylate β-catenin and target it for proteosomal degradation (Right panel). Adapted from (Thompson and Monga 2007)

cell factor/lymphoid-enhancing factor (TCF/LEF) family of DNA binding proteins and cause transcriptional activation of its targets such as axin2, glutamine synthetase, cyclin D1 and lect2 (Clevers 2006, Nejak-Bowen and Monga 2011) (Fig. 1.5.1). Independent of Wnt signaling, β-catenin also associates with cytoplasmic domain of E-cadherin as a component of cell-cell adherens junction. This bound pool of β-catenin is stable and not available for degradation by destruction complex (Clevers 2006, Thompson and Monga 2007).

Previous literature suggests important role of Wnt/β-catenin signaling in liver regeneration after PH (Nejak-Bowen and Monga 2011). β-catenin gets activated very early during liver regeneration after PH in rats (Monga, Pediaditakis et al. 2001). Knockdown of β-catenin in rats using antisense oligonucleotide resulted in decreased liver regeneration (Sodhi, Micsenyi et al. 2005). Further, in two independent studies, liver-specific deletion of β-catenin in mice caused delayed liver regeneration, which was correlated with decreased cyclin D1 induction (Tan, Behari et al. 2006, Sekine, Gutierrez et al. 2007). In contrast, mice over-expressing Ser45 mutated β-catenin displayed accelerated liver regeneration after PH, which was correlated with increased expression of cyclin D1. Furthermore, direct gene delivery of Wnt-1 in mice showed similar increase in hepatocyte proliferation (Nejak-Bowen, Thompson et al. 2010). β-catenin activation has also been observed during liver regeneration following treatment with sublethal dose of APAP in mice (Apte, Singh et al. 2009). Activation was specifically observed in the areas surrounding the centrilobular necrotic areas, which was correlated with increased cyclin D1 expression and liver regeneration. Liver specific deletion of β-catenin in mice compromised expression of enzymes (CYP2E1 and

CYP1A2) involved in metabolic activation of APAP resulting in attenuated APAP toxicity. In order to study direct role of β-catenin on liver regeneration after APAP-induced liver injury, CYPs were induced in mice and equitoxic doses were compared in β-catenin KO and control mice. In this equitoxic dose study, liver regeneration was found to be decreased in β-catenin KO mice suggesting role of β-catenin in liver regeneration after APAP-induced ALF (Apte, Singh et al. 2009). Moreover, analysis of liver samples from APAP-induced ALF patients showed correlation of β-catenin activation with higher spontaneous liver regeneration, avoiding need for liver transplantation (Apte, Singh et al. 2009). Further investigation in clean experimental models will help to confirm role of β-catenin in liver regeneration after APAP-induced liver injury.

1.6 GLYCOGEN SYNTHASE KINASE-3 (GSK3) SIGNALING AND ITS ROLE IN LIVER REGENERATION

Glycogen synthase kinase-3 (GSK3) is a ubiquitously expressed serine/threonine protein kinase, which is constitutively active in cells under resting state and primarily regulated by inactivation. In mammals, GSK-3 exists in two isoforms GSK-3α and GSK-3β which share 98% sequence homology in kinase domain leading to almost redundant functionality (Kaidanovich-Beilin and Woodgett 2011). GSK3 lie downstream of number of major signaling pathways including growth factors, insulin and Wnt signaling, which negatively regulate GSK3 activity. GSK3 activity is primarily regulated by phosphorylated-mediated inactivation, but other methods of inactivation such as sequestration and subcellular localization are also known (Jope and Johnson 2004). Initially discovered to regulate glycogen synthase, GSK3 is now known to regulate a

wide spectrum of proteins and affect a number of cellular functionalities including proliferation, differentiation and survival (Jope and Johnson 2004). GSK3 functions by phosphorylation-mediated inactivation/ degradation of its substrates. As described in previous section, GSK3 causes phosphorylation mediated targeting of β-catenin for proteosomal degradation, thus negatively regulating β-catenin signaling (Doble and Woodgett 2003). Apart from β-catenin, GSK3 can regulate many other cell proliferation mediators such as Cyclin D1, c-Myc, c-Jun, and eIF2B (Sutherland 2011). For instance, GSK3β can directly regulate Cyclin D1 protein by its phosphorylation, proteolysis and decreased nuclear localization (Diehl, Cheng et al. 1998).

Role of GSK3 in liver regeneration has not been as extensively studied as compared to β-catenin. GSK3 inhibitor had reported to induce in-vitro expansion of mice primary hepatocytes (Ito, Kamiya et al. 2012). Further, inhibition of GSK3 facilitated survival signaling in primary human hepatocytes via activation of β-catenin (Gotschel, Kern et al. 2008). Moreover, GSK3 negatively regulates β-catenin, which is known to play an important role in liver regeneration. Counterintuitively, several studies indicate that GSK3 may play a pro-regenerative role after PH (Chen, Yang et al. 2007, Jin, Wang et al. 2009, Sekiya and Suzuki 2011). Treatment with GSK3 inhibitor or siRNA mediated downregulation of GSK3 has reported to impair liver regeneration in mice and rats after PH (Chen, Yang et al. 2007, Jin, Wang et al. 2009, Sekiya and Suzuki 2011). However, role of GSK3 in compensatory liver regeneration after APAP-induced liver injury is not known and can be different from PH model based on mechanistic difference between these models. Nevertheless, increased inactivation of GSK3β (Ser9 phosphorylation) was observed during regenerative phase after APAP-induced liver injury in mice, which

was correlated with increased liver regeneration (Apte, Singh et al. 2009). Silencing of GSK3β resulted in inhibition of APAP-induced injury, but this was attributed to role of GSK3β in initiation of APAP toxicity. However, increased Ser9 phosphorylation of GSK3β (inactive form) was observed after APAP administration in this study as well (Shinohara, Ybanez et al. 2010). This inactivation of GSK3β may have role to play in liver regenerative, which was not investigated in this report. Thus, further investigations are required to establish role GSK3 in compensatory liver regeneration after APAP-induced liver injury.

1.7 EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) AND ITS ROLE IN LIVER REGENERATION

Epidermal growth factor receptor (EGFR) or ErbB-1 is a prototypical tyrosine kinase receptor of the ErbB family, which is highly expressed in liver and known to be involved in hepatocytes proliferation, liver regeneration and hepatocellular carcinogenesis (Michalopoulos and DeFrances 1997, Carver, Stevenson et al. 2002, Michalopoulos and Khan 2005). Other ErbB family members include ErbB-2, ErbB-3 and ErbB-4. ErbB-2 does not have any known ligand and its expression greatly decreases in adult liver; ErbB-3 is present in adult liver but lacks intrinsic kinase activity; ErbB-4 is not expressed in adult or embryonic liver (Carver, Stevenson et al. 2002, Michalopoulos and Khan 2005). EGFR is a cell surface receptor with an extracellular ligand-binding domain, a transmembrane domain and a cytoplasmic domain with tyrosine kinase activity. EGFR can be activated by variety of extracellular ligands, including EGF, transforming growth factor alpha (TGF-α), heparin-binding EGF (HB-EGF) and

amphiregulin. Ligand binding results in dimerization of EGFR and activation of its intrinsic tyrosine kinase domain. This subsequently results in auto-phosphorylation of tyrosine residues in the cytoplasmic domain. These resultant phosphorylation residues act as docking sites for multiple proteins leading to activation of wide spectrum of cell signaling pathways (Normanno, De Luca et al. 2006).

Apart from HGF, EGFR ligands are the only known direct mitogens which can cause hepatocyte proliferation in serum free medium, highlighting an important role of EGFR in hepatocyte proliferation (Michalopoulos 2007). *In vivo* administration of EGFR ligands such as EGF and TGF-α also lead to hepatocyte proliferation along with increased liver size (Bucher, Patel et al. 1977) . EGFR is activated very rapidly after 2/3rd liver resection i.e. partial hepatectomy (PH) and is known to play an important role in promoting timely liver regeneration (Michalopoulos and Khan 2005, Michalopoulos 2007, Fausto, Campbell et al. 2012). Genetic deletion of EGFR in mouse liver or shRNA mediated downregulation of EGFR in rats resulted in impaired and delayed liver regeneration after PH, but final recovery of liver mass was not affected due to compensatory mechanisms (Natarajan, Wagner et al. 2007, Paranjpe, Bowen et al. 2010). In another study, hepatocyte specific deletion of EGFR in mice produced weaker impairment on liver regeneration after PH, but no alteration was observed on liver regeneration after CCl_4-induced liver necrosis (Scheving, Zhang et al. 2015). Mice deficient in EGFR ligands - amphiregulin and HB-EGF – or sialadenectomized mice that have lower level of serum EGF displayed deficient liver regeneration. Further, overexpression or injection of HB-EGF in rodents increased liver regeneration

(Michalopoulos and Khan 2005, Michalopoulos 2007, Fausto, Campbell et al. 2012). Role of EGFR in liver regeneration after APAP-induced liver injury is entirely unknown.

1.8 STATEMENT OF PURPOSE

Current treatment options for APAP overdose patients are very limited and warrant development of novel therapeutic strategies. Recent studies suggest that liver regeneration is a critical determinant of final recovery and survival following APAP overdose (Hu and Colletti 2008, Apte, Singh et al. 2009, Donahower, McCullough et al. 2010). Thus, stimulating liver regeneration in patients of APAP-induced ALF holds a great therapeutic potential. Although mechanisms of liver injury after APAP overdose have been extensively studied, our knowledge regarding mechanisms of compensatory liver regeneration following APAP-induced injury is extremely limited, hindering development of novel regenerative therapies for ALF. Our long-term goal is to develop regenerative therapies based on stimulation of liver regeneration for APAP-induced ALF. The objective of this dissertation work was to investigate mechanisms of liver regeneration and identify targets to stimulate liver regeneration after APAP-induced liver injury. We used an incremental dose model to delineate the mechanisms of liver regeneration following APAP-induced ALF with hypothesis that liver regeneration should remain intact after lower dose, but impaired at higher dose. Thus, differential regulation of regenerative pathways after APAP-induced toxicity could be analyzed and potential targets to stimulate regeneration after APAP overdose could be identified. Further, based on previous literature, we hypothesized that β-catenin and GSK3 play important role in liver regeneration such that stimulation of β-catenin or inhibition of GSK3 after

APAP-induced ALF would result in improved liver regeneration. This was investigated by overexpression β-catenin and pharmacological inhibition of GSK3 in mice. Lastly, EGFR is known to play important role in liver regeneration after liver resection but its role liver regeneration after APAP-induced liver injury is completely unexplored. Thus, role of EGFR in liver regeneration after APAP-induced liver injury was investigated using pharmacological intervention to inhibit EGFR signaling in mice.

CHAPTER II: ANALYSIS OF PRO-REGENERATIVE SIGNALING AFTER ACETAMINOPHEN-INDUCED HEPATOTOXICITY IN MICE USING A NOVEL INCREMENTAL DOSE MODEL

This section is reproduced from:

"Pro-regenerative signaling after acetaminophen-induced acute liver injury in mice identified using a novel incremental dose model." Bhushan et al. 2014, American Journal of Pathololgy 184(11): 3013-3025, with permission from publisher.

2.1 ABSTRACT

Acetaminophen (APAP) overdose results in acute liver failure (ALF) and has very limited treatment options. Previous studies show that stimulating liver regeneration is critical for survival after APAP overdose but the mechanisms remain unclear. In this study, we identified major signaling pathways involved in liver regeneration following APAP-induced acute liver injury using a novel incremental dose model. Liver injury and regeneration were studied in C57BL/6 mice treated with either 300 mg/kg (APAP300) or 600 mg/kg (APAP600) APAP. Mice treated with APAP300 developed extensive liver injury and robust liver regeneration. In contrast, APAP600 treated mice exhibited significant liver injury but substantial inhibition of liver regeneration resulting in sustained injury and decreased survival. The inhibition of liver regeneration in APAP600 group was associated with cell cycle arrest, decreased cyclin D1 expression and sustained p21 expression. Several known regenerative pathways including IL-6/STAT-3, EGFR/c-Met/MAPK pathway were activated even at APAP600 where regeneration was inhibited. However, canonical Wnt/β-catenin and NF-κB pathways were activated only in APAP300 treated mice where liver regeneration was stimulated. Next, we investigated role of Wnt/β-catenin in further detail. ChIP analysis revealed increased binding of β-catenin to cyclin D1 promoter specifically at APAP300 correlating with higher cyclin D1 induction. Furthermore, overexpression of a stable form of β-catenin (S45D) in mice resulted in increased hepatocyte proliferation and improved liver regeneration following APAP overdose. Improved liver regeneration in S45D was associated with increased expression of Cyclin D1. Taken together, our incremental dose model has identified differential role of several signaling pathways in liver regeneration after APAP overdose

and highlighted β-catenin as a potential target for regenerative therapies for APAP-induced ALF.

2.2 INTRODUCTION

Acetaminophen (APAP) is one of the most widely used over-the-counter analgesic and anti-pyretic drug in the world. APAP is safe at therapeutic doses but overdose can cause acute liver failure (ALF). In fact, APAP overdose is associated with more than 78,000 emergency room visits and 33,000 hospitalizations every year in the US (Budnitz, Lovegrove et al. 2011, Manthripragada, Zhou et al. 2011). The only pharmacological intervention, at present, is N-acetylcysteine (precursor of glutathione), which is successful only if given within few hours after APAP overdose (Athuraliya and Jones 2009). Ultimate option is liver transplantation, which is complicated by issues such as donor availability, long term immunosuppression and exorbitant costs (Keeffe 2001).

Previous studies suggest that liver regeneration following APAP overdose plays a critical role in determination of outcome of injury (Schmidt and Dalhoff 2005, Hu and Colletti 2008, Apte, Singh et al. 2009, Donahower, McCullough et al. 2010). α-Feto protein, a marker of liver regeneration, was found to be associated with better survival rate in patients with APAP-induced ALF (Schmidt and Dalhoff 2005). Several other studies in animal models suggest that timely stimulation of liver regeneration, such as with stem cell factor (Hu and Colletti 2008) and VEGF (Donahower, McCullough et al. 2010), improves survival after APAP overdose in mice. These studies highlight stimulating liver regeneration in APAP-induced ALF patients as a plausible therapeutic option. However, the mechanisms of liver regeneration after APAP-induced ALF are not well known. Whereas liver regeneration has been extensively studied in past

(Michalopoulos 2007), most of the studies are based on partial hepatectomy (PH) model, a mechanistically different model from APAP-induced ALF.

Data on hepatotoxicants, in general, suggest that liver regeneration follows the principles of dose-response(Mehendale 2005). Studies indicate that liver regeneration following toxic injury to liver increases proportionately to injury but only up-to a threshold dose. Doses higher than the threshold dose actually inhibit liver regeneration resulting in progression of injury to acute liver failure and death (Mangipudy, Chanda et al. 1995, Anand, Murthy et al. 2003, Mehendale 2005). These studies have suggested that at higher doses regeneration is inhibited due to blockade in critical pro-regenerative signaling pathways (Mangipudy, Chanda et al. 1995, Apte, Limaye et al. 2003, Mehendale 2005). Based on this principle, we developed a novel incremental dose model to delineate the mechanisms of liver regeneration following APAP-induced acute liver injury (ALI). We used two doses of APAP, a lower dose (300 mg/kg) following which liver regeneration is intact and a higher dose (600 mg/kg) after which liver regeneration is inhibited. We performed a comprehensive analysis of several signaling pathways known to be involved in liver regeneration and identified pathways, which are potentially important for liver regeneration after APAP-induced ALI and can be targeted therapeutically.

2.3 MATERIALS AND METHODS

Animals, Treatment and Tissue Harvesting

Two-month-old male C57BL/6 mice purchased from Jackson Laboratories were used in these studies. Mice overexpressing stable form of β-catenin, where Serine 45 is mutated to Aspartic Acid (S45D) have been described before (Nejak-Bowen, Thompson et al. 2010). Details of generation and characterization of these mice have been published previously (Nejak-Bowen, Thompson et al. 2010). All animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care-accredited facilities at the University of Kansas Medical Center under a standard 12-hr light/dark cycle with access to chow and water *ad libitum*. The Institutional Animal Care and Use Committee at University of Kansas Medical Center approved all studies. Mice were fasted 12 hr before administration of APAP. APAP was dissolved in warm 0.9% saline and mice were treated with either 300 or 600 mg/kg APAP, intraperitoneally (i.p.). Food was returned to the mice after APAP treatment. Mice (n = 3 to 7) were sacrificed at 0, 0.5, 1, 3, 6, 12, 24, 48, 72 and 96 hr following APAP treatment by cervical dislocation under isofluorane anesthesia, and blood and livers were collected. The S45D and respective wild-type (WT) littermates were treated with either 300 or 600 mg/kg APAP and sacrificed at 6 and 24 hours (APAP300 study) or 24 and 48 hours (APAP600 study) after APAP administration, followed by blood and liver collection. Serum samples were obtained from the blood and used for analysis of alanine aminotransferase (ALT) activity using commercially available kits (ThermoFisher Scientific, Pittsburgh PA). Liver

sections, nuclear and cytoplasmic protein extracts were prepared as described previously (Wolfe, Thomas et al. 2011).

Antibodies

All primary antibodies obtained from Cell Signaling Technologies (Danvers, MA) for Western blot analysis. Active β-catenin antibody was obtained from EMD Millipore Corporation (Billerica, MA). All secondary antibodies used for Western blot analysis were obtained from Cell Signaling Technologies. Biotinylated secondary antibodies for immunohistochemistry were purchased from Jackson Immunoresearch (West Grove, PA).

Protein Isolation and Western Blotting

Protein estimation and western blot analysis was performed using pooled samples of protein extracts prepared from frozen liver tissues as previously published without any modifications (Wolfe, Thomas et al. 2011).

PCNA Immunohistochemistry

Paraffin-embedded liver sections (4 μm thick) were used for immunohistochemical detection of PCNA as described before (Wolfe, Thomas et al. 2011).

Real Time PCR

Total RNA was isolated from APAP300 and APAP600 livers using Trizol method according to the manufacturer's protocol (Sigma, St. Louis, MO) and converted to cDNA as previously described (Apte, Singh et al. 2009). mRNA levels of various genes were

determined using Real Time PCR analysis using commercially available TaqMan Gene Expression Assays (Life Technologies previously known as Applied Biosystems, Carlsbad, CA) on the Applied Biosystems Prism 7300 Real-time PCR Instrument according to manufacturer's protocol. Rplp0 gene expression in the same samples was used for data normalization and data was expressed as fold change compared to 0 hr.

Chromatin Immunoprecipitation (ChIP)

ChIP was done using whole liver tissue from APAP300 and APAP600 animals for 12 hr time point as described previously (Walesky, Gunewardena et al. 2013). Chromatin was isolated using approximately 200 mg of frozen liver tissue from three mice of each group. Isolated chromatin was then incubated with either no antibody, β-catenin (5 µg; BD transduction, Catalogue # 610154) or p65 (5 µg; Cell Signaling, Catalogue # 8242) antibodies for immunoprecipitation. Real-time PCR analysis was done using primers designed to cover known p65 and β-catenin binding sites on *Ccnd1* promoter. Data was normalized with input DNA and represented as fold enrichment with respect to no antibody control. Primers sequence: Forward – 5'-GCAGGACTTTGCAACTTCAAC-3'; Reverse – 5'-TTTCTCTGCCCGGCTTTG-3'

Statistical Analysis

Data presented in the form of bar/line graphs show mean ± SEM. Significant difference between groups was determined using Student's T-test. Difference between groups was considered statistically significant at $P<0.05$. Statistically significant difference was

represented as * in graphs when compared between the two doses and represented as # when compared with respect to basal levels (0 hr) wherever specified.

2.4 RESULTS

Sustained injury and inhibited recovery after higher dose of APAP

We compared liver injury at the two doses of APAP (APAP300 and APAP600) over the time-course of 0 to 96 hr by studying serum ALT and histopathological analysis of liver sections (scoring sections for percent necrosis) (Fig. 2.4.1A, B and C). Liver injury increased after APAP treatment and peaked at 12 hr after treatment in both the doses. Liver injury was characterized by necrosis in the centrilobular region, hallmark of APAP toxicity. Interestingly, there was no significant difference in injury up to 24 hr in both the groups except area of necrosis was significantly higher at 12 hr in APAP600 compared to APAP300 group (Fig. 2.4.1C). Overall trend showed moderately higher injury in APAP600 group up to 24 hr. However, there was marked difference in progression of injury at later time points. APAP300 showed slight regression of injury at 48 hr and complete recovery at 72 and 96 hr as evidenced by decreased serum ALT levels, complete recovery from necrosis and 100% survival. In contrast, approximately 25% animals died at various time points from 48 to 96 hr in APAP600 group (Fig. 2.4.1B). Remaining animals showed sustained injury and were not recovered up to 96 hr. Injury was strikingly higher at 72 and 96 hr in APAP600 group compared to APAP300 group.

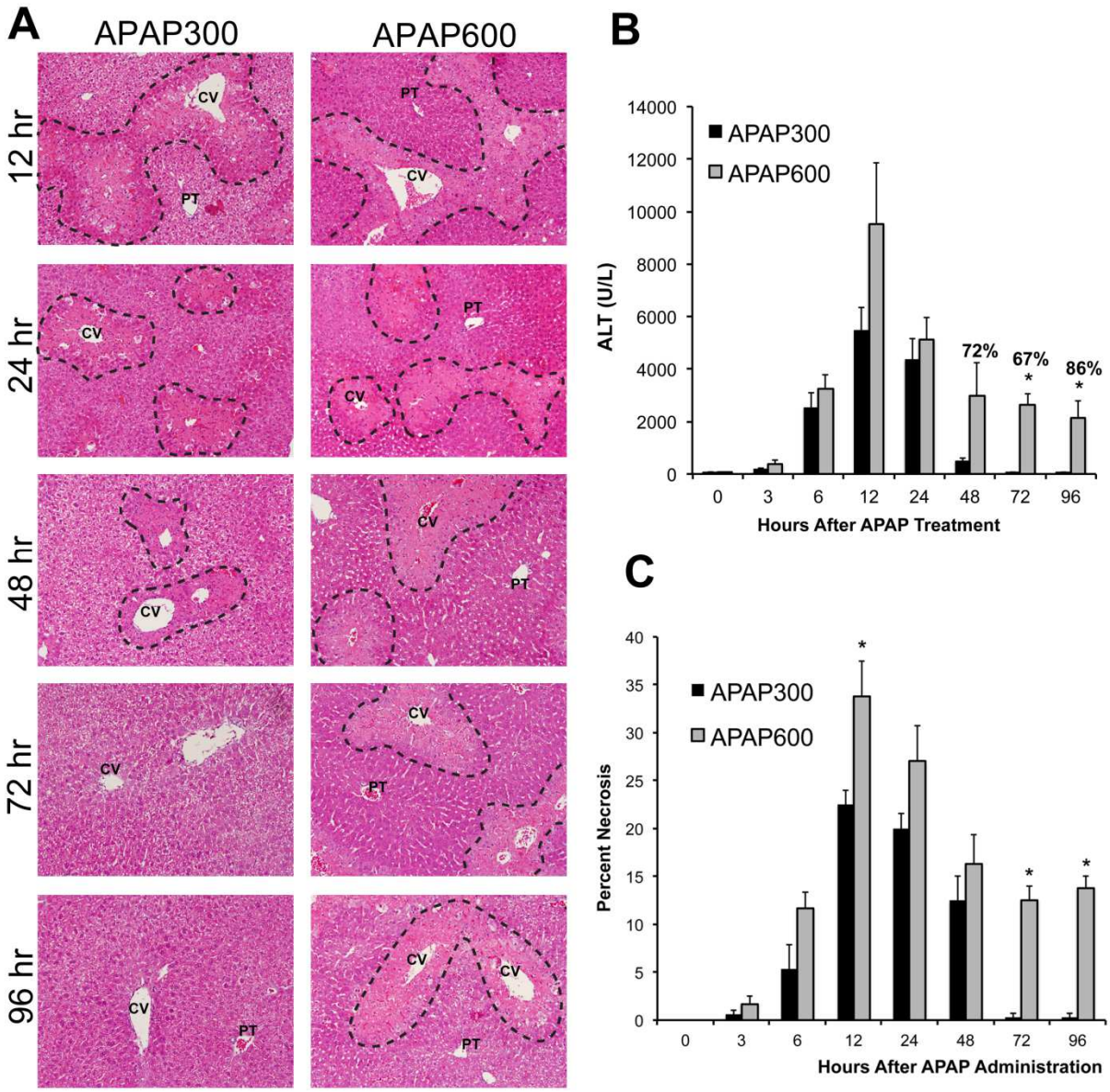

Figure 2.4.1. Sustained liver injury and inhibited recovery after higher dose of APAP. (A) Representative photomicrographs of H&E stained liver sections with necrotic area outlined (CV denotes central vein and PT denotes portal triad) (B) Bar graph showing serum ALT levels with percent survival specified over bars at time points where any mortality was observed. (C) Bar graph showing percent necrosis area based on H&E stained liver sections. All samples were collected from mice (n = 3-7) treated with either 300 or 600 mg/kg APAP at various time points up-to 96 hr after APAP treatment. * indicate significant difference between two doses at $p<0.05$

Inhibited liver regeneration and cell-cycle arrest correlated with sustained injury and inhibited recovery at higher dose

Next, we studied liver regeneration at the two doses of APAP using PCNA immunohistochemistry (Fig. 2.4.2A). Quantification of total PCNA positive cells (Fig. 2.4.2B) showed evidence of a few cells entering cell cycle as early as 3 hr in APAP300, but robust proliferation response with dramatic increase in PCNA positive cells was observed only from 12 hr after APAP treatment specifically around the necrotic areas. PCNA positive cells remains elevated up to 48 hr and then progressively decreased at 72 and 96 hr. In contrast, APAP600 showed almost no PCNA positive cells up to 24 hr after APAP administration. PCNA positive cells started appearing at much delayed time point of 48 hr onwards in APAP600 group and were significantly lower compared to APAP300 group. The PCNA data were corroborated by Ki-67 immunofluorescence staining (Fig. 2.4.3). Overall, there was significantly delayed and attenuated liver regeneration in APAP600 group which correlated with sustained injury, delayed recovery and decreased survival at the higher dose.

Interestingly, even at the time point of peak injury (12 hr after APAP treatment) at higher dose, more than 50% of hepatocytes were still intact as demonstrated by necrosis scoring. This suggests that marked decrease in regeneration in the APAP600 dose is not due to lack of viable hepatocyte in the higher dose. This was further confirmed by HNF4α staining as a marker of intact hepatocytes (Fig. 2.4.4). In fact, overall cellular death was not strikingly different at the two doses at early time points where regeneration was initiated at lower dose. Further, closer look at individual animal data revealed even though there was no difference in liver injury in some animals

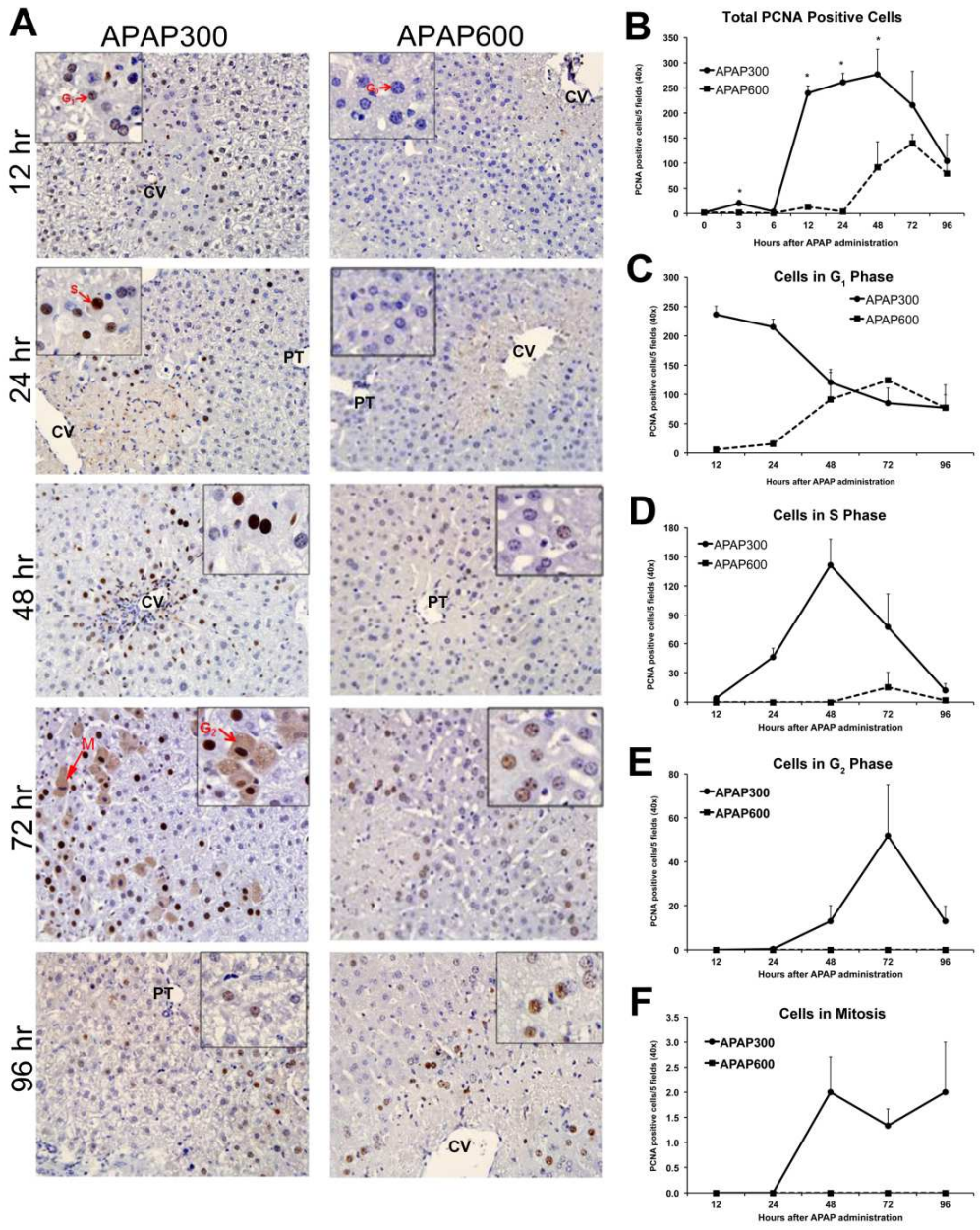

Figure 2.4.2. Inhibition of liver regeneration following higher dose of APAP. (A) Representative photomicrographs of PCNA stained liver sections. Insets show cells in specific phase of cell cycle as denoted by arrows, with arrows pointing out different phases of cell cycle. G0 cells: blue nuclear staining; G1 cells: light brown nuclear staining; S phase cells: deep brown nuclear staining; G2 cells: diffused brown cytoplasmic staining; M phase cells: deep-blue chromosomal staining. (B) Line graph showing total number of PCNA positive cells per five high power (40x) fields. Line graphs showing total number of cells in (C) G1, (D) S, (E) G2 and (F) M phases per five high power (40x) fields demonstrating cell cycle progression. All samples were collected from mice (n = 3-5) treated with either 300 or 600 mg/kg APAP over a time course of 0 to 96 hr after APAP treatment. CV denotes central vein and PT denotes portal triad. * indicate significant difference between two doses at $p<0.05$

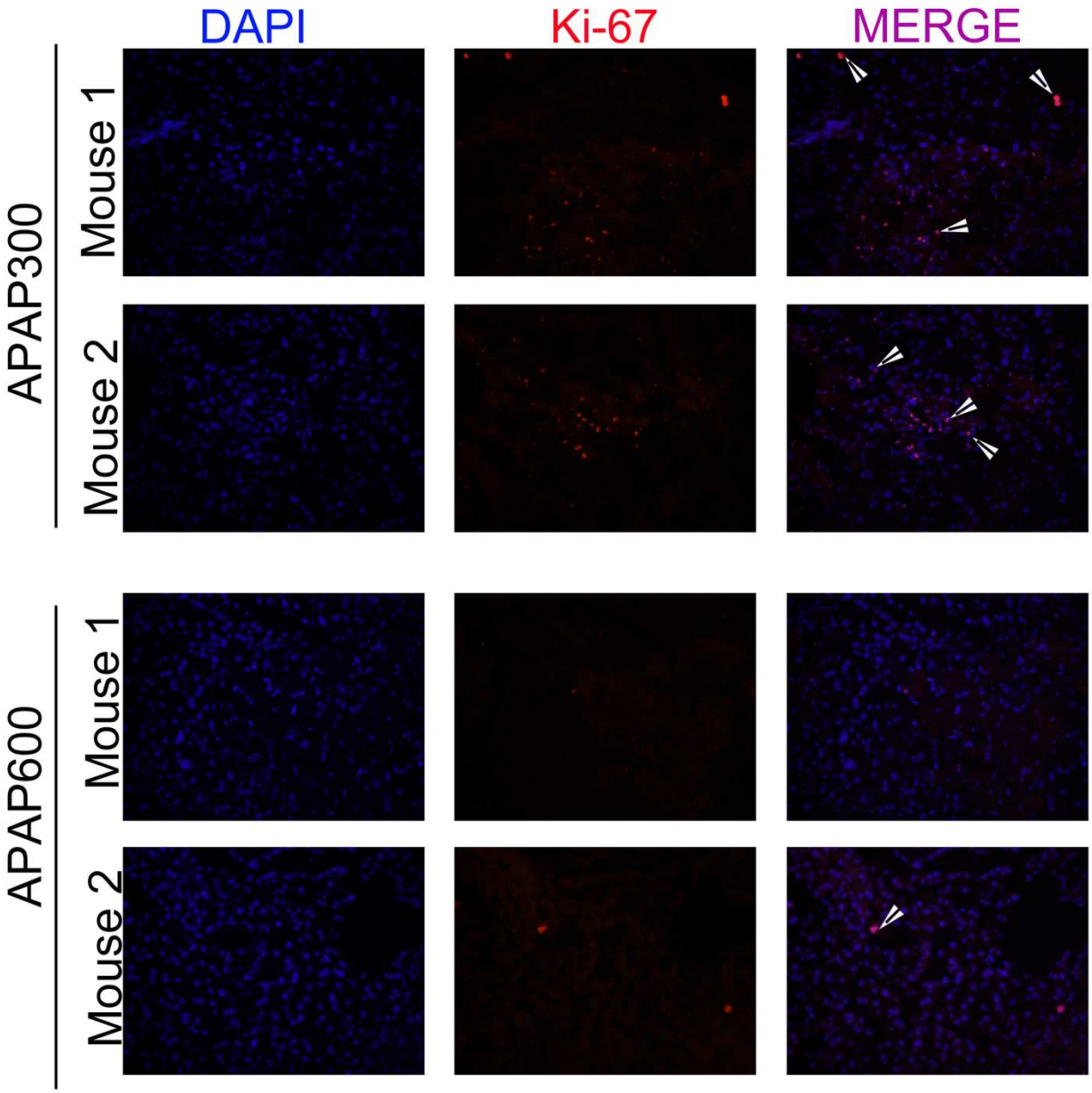

Figure 2.4.3. Inhibition of liver regeneration following higher dose of APAP. Representative photomicrographs showing Ki-67 immuno-fluorescence performed on frozen liver sections of mice treated with either 300 or 600 mg/kg APAP and scarified at 24 hr after APAP treatment. (Ki-67: Red; DAPI: Blue; Merge: Pink)

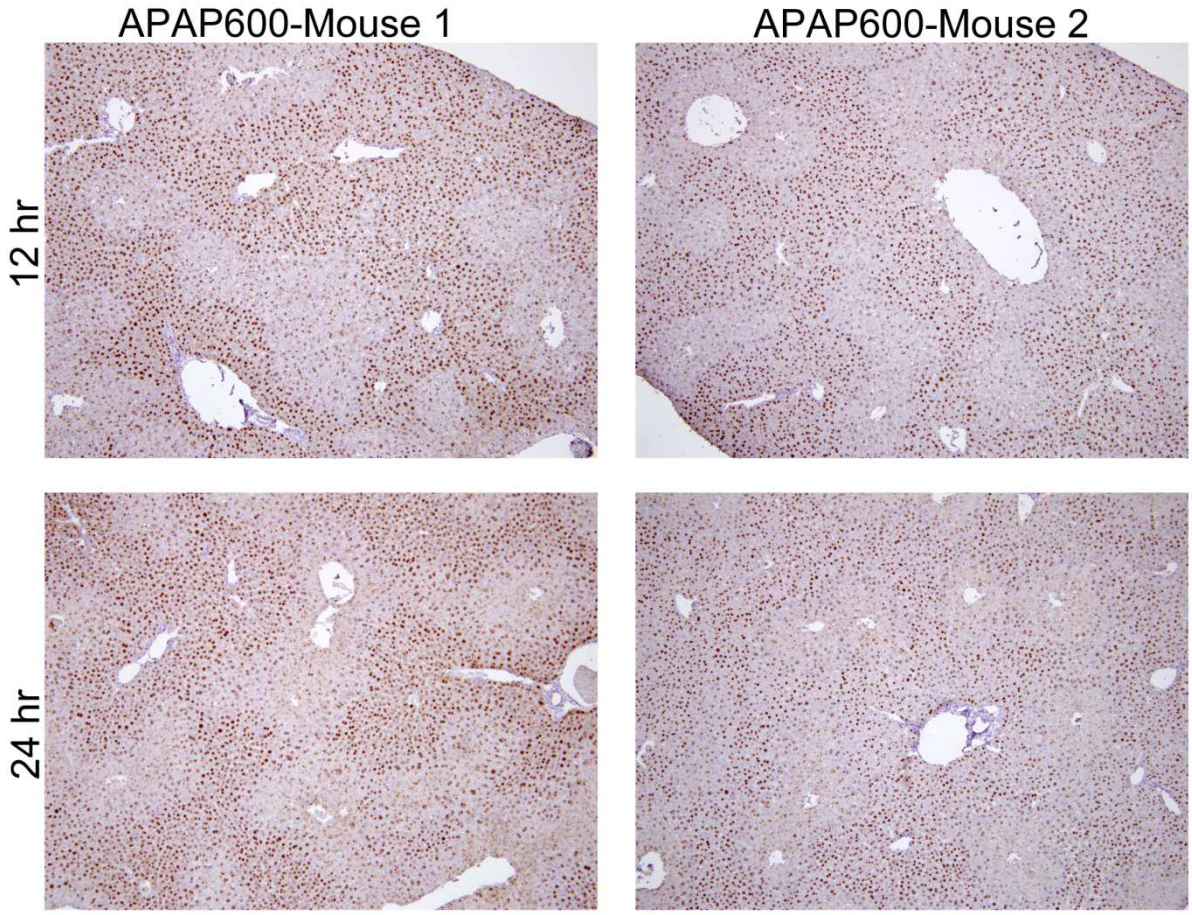

Figure 2.4.4. HNF4α staining showing intact hepatocytes following higher dose of APAP. HNF4α immunohistochemistry on paraffin-embedded liver sections from mice treated with 600 mg/kg APAP and scarified at 12 and 24 hr after APAP treatment.

between groups but there was striking difference in regenerative response (data not shown).

Next, we quantified cells in specific cell cycle phase by studying PCNA stained sections in detail to further look into progression of cell cycle (Fig 2.4.2C-F). In APAP300 group, cells started entering cell cycle at 12 hr with numerous cells entering into G_1 phase from G_0 phase. At 24 hr, most of the cells were still in G_1 phase with many cells progressed to S phase. Majority of the cells progressed to S phase at 48 hr and further to G_2 phase by 72 hr. Mitoses were evident between 48 to 96 hr. By 96 hr, majority of cells returned to quiescent state. In contrast, in APAP600 group, almost all the cells stayed in G_0 phase up to 24 hr. A few cells started entering G_1 phase only at much delayed time point of 48 hr and further these cells do not progress through cell cycle at later time points and remain arrested at G_1 phase. No mitotic activity was observed at any time points in APAP600 group. These results suggest that inhibition of entry into cell cycle and early cell cycle arrest in the remaining viable hepatocytes at higher dose is the reason for inhibited liver regeneration rather than mere higher cell death.

Cell cycle regulators correlated with arrested cell cycle and inhibited liver regeneration at higher dose

We further studied expression of various core cell cycle proteins, which regulate cell cycle entry. We first determined Cyclin D1 levels, induction of which is the critical step which is required by hepatocytes for progressing through G_1 phase and committing to DNA replication (Fausto 2000). There was an early 2-fold induction of Cyclin D1 mRNA at 3 hr followed by a sharp peak (6-fold induction) at 12 hr specifically in

APAP300 group correlating with time of onset of regenerative phase. Cyclin D1 mRNA level rapidly declined at 24 hr to around 2.5-fold and reached to basal level by 96 hr. In contrast, Cyclin D1 mRNA did not increase significantly in APAP600 group at any time point but showed slight induction in some animals at much delayed time points of 48 and 96 hr (Fig. 2.4.5A). Moreover, we observed significant decrease in Cyclin D1 mRNA levels at 6 hr compared to basal levels in APAP600 group suggesting active inhibition of cell cycle. Western blot analysis further corroborated mRNA data with early and overall much higher increase in Cyclin D1 protein only in APAP300 dose correlating with PCNA protein expression (Fig. 2.4.5B). Cyclin D1 binds to CDK4 and this complex in-turn causes phosphorylation and inactivation of Rb, which ultimately leads to transcription of many cell cycle genes (Fausto 2000). CDK4 protein and phosphorylation of Rb followed trend similar to Cyclin D1 expression (Fig. 2.4.5B).

We further analyzed dynamics of cyclin dependent kinase inhibitors, p21 and p27 to elucidate if active cell cycle inhibition is involved at higher dose (Fig. 2.4.5C and D). Interestingly, p21 mRNA was markedly induced at both the doses as early as 3 hr after APAP. However, induction was much higher and sustained up to 96 hr at higher dose but decreased remarkably after 24 hr at lower dose (Fig 2.4.5C). Protein expression of p21 also displayed similar trend. p27 protein also showed moderate induction at both doses showing pattern similar to p21 with notable higher expression at 6 hr after APAP treatment at higher dose, the time point preceding onset of normal liver regeneration response in regenerative dose (Fig 2.4.5D). These data suggest that active cell cycle inhibition is involved at higher dose of APAP where liver regeneration is inhibited.

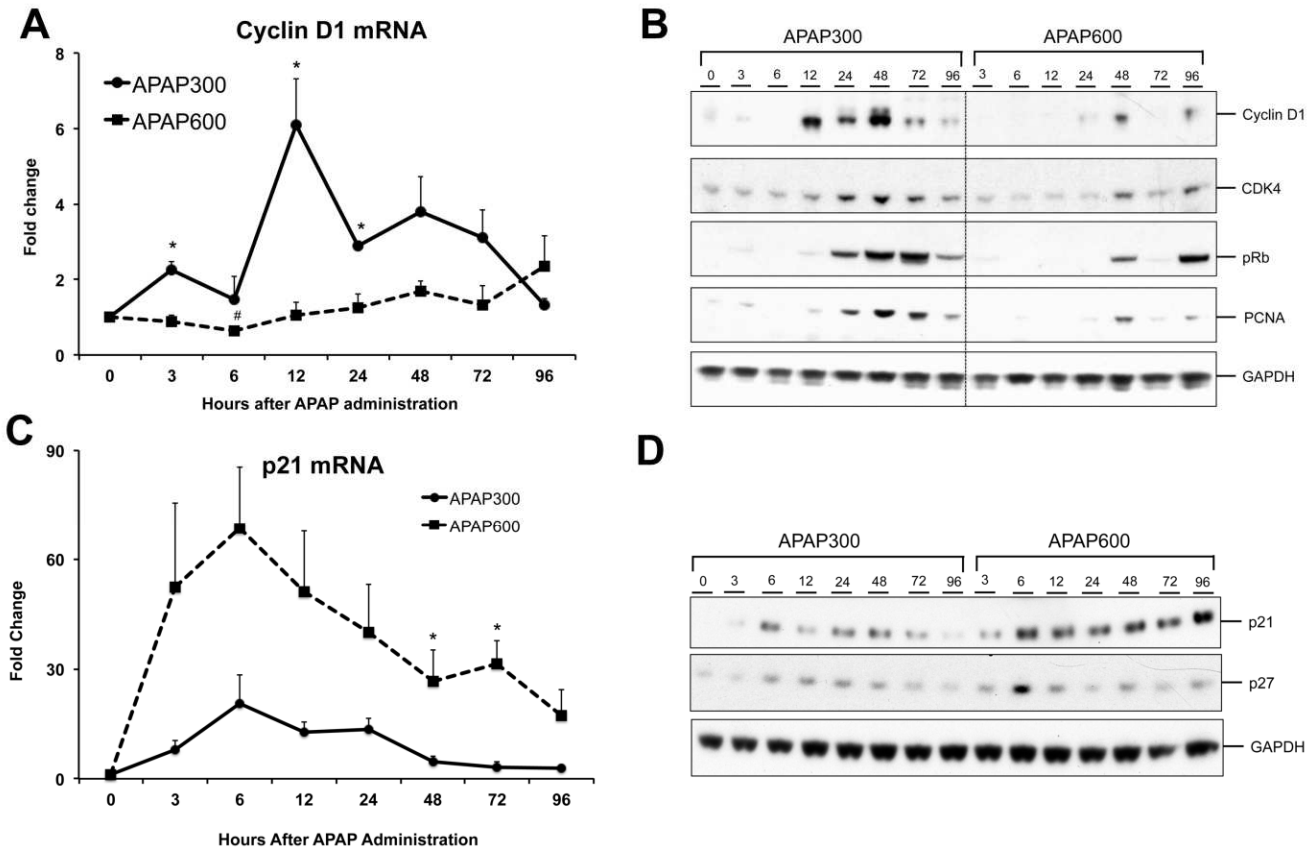

Figure 2.4.5. Cell cycle regulators correlated with inhibited liver regeneration at higher dose. (A) Cyclin D1 mRNA expression (B) Cyclin D1, CDK4, phospho-Rb, PCNA, (C) p21 mRNA and (D) p21 and p27 protein in liver of mice treated with either 300 or 600 mg/kg APAP. All samples were collected over a time course of 0 to 96 hr after APAP treatment (n = 3-5). * indicate significant difference between two doses at $p<0.05$

Overall, early and robust induction of cyclin D1 at lower dose resulted in timely liver regeneration and recovery. Inhibition of such robust and timely response and sustained activation of cell cycle inhibitors at higher dose correlated with cell cycle arrest, inhibited liver regeneration, progression of injury and decreased survival. Next, we studied major upstream pathways, which are known to induce cyclin D1 and stimulate liver regeneration. For these studies, based on the time course of regenerative response, we focused on time points up to 24 hr after APAP treatment considering importance of early regenerative response for successful recovery.

Growth factor signaling via EGFR and c-Met and MAPKs signaling remains highly activated following high dose of APAP

Epidermal growth factor (EGF) and hepatocyte growth factor (HGF) are considered the primary mitogens for hepatocytes and are critical for liver regeneration after PH (Michalopoulos 2007). EGF and HGF act primarily through activation of EGF receptor (EGFR) and c-Met receptor, respectively (Michalopoulos 2007). This leads to, among others, activation of MAPK signaling culminating in cell proliferation. However, importance of these growth factors in liver regeneration following APAP-induced ALI is not known. We found that the protein expression of EGFR and c-Met was increased at both the doses (Fig 2.4.6A). To determine activation status of these receptors, we investigated the expression of phosphorylated forms of EGFR and c-Met. There was remarkable activation of EGFR as early as 0.5 hr after APAP300 treatment attaining peak at 6 hr, the time point preceding onset of marked liver regeneration response and

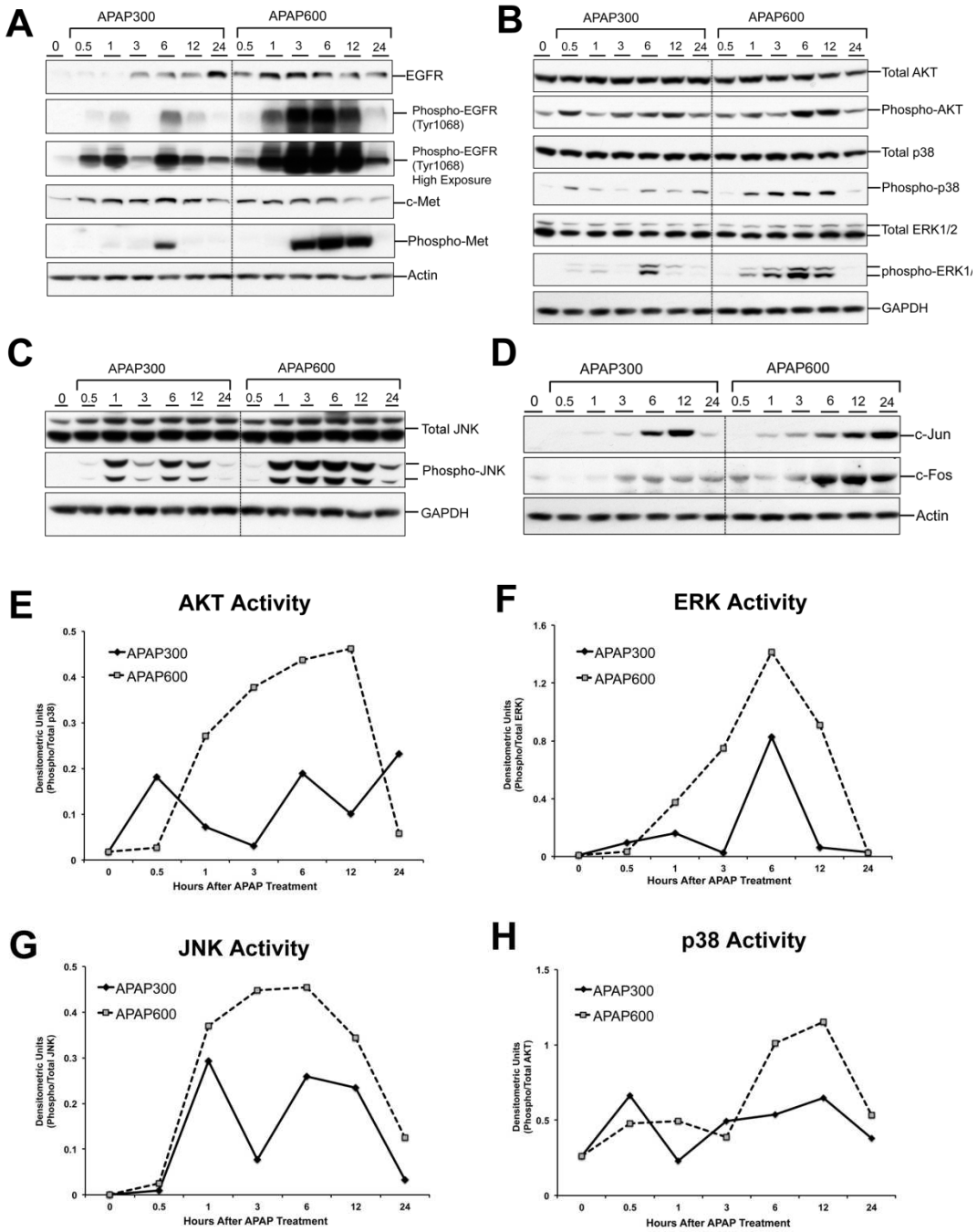

Figure 2.4.6. Growth factor signaling via EGFR/ c-Met and MAPKs signaling remains highly activated following high dose of APAP. Western blot analysis of (A) EGFR, phospho-EGFR (normal and high exposures), c-Met, phospho-Met, (B) total AKT, phospho-AKT, total p38, phospho-p38, total ERK1/2, phospho-ERK 1/2, (C) total JNK, phospho-JNK, (D) c-Jun and c-Fos using total liver extract of mice liver treated with either 300 or 600 mg/kg APAP. (E), (F), (G) and (H) Densitometric analysis showing p38, ERK 1/2, JNK and AKT activation respectively. All samples were collected over a time course of 0 to 24 hr after APAP treatment (n = 3-5).

then progressively decreased at later time points. Interestingly, similar trend was observed for APAP600 group with much higher and sustained response in dose-dependent manner (Fig 2.4.6A). Similar results were observed for c-Met activation for which we found marked phosphorylation only at 6 hr after APAP300 treatment but much higher and sustained phosphorylation after APAP600 treatment starting as early as 3 hr and maintained till 12 hr (Fig 2.4.6A). Further studies on downstream MAPK signaling revealed that AKT, p38, ERK1/2 and JNK total protein levels remained unchanged after both the doses. However, a dose dependent increase in phosphorylation (activation) of all these kinases including AKT, p38, ERK1/2 and JNK was observed after APAP treatment (Fig. 2.4.6 B, C, E-H). Activation of all these kinases was higher in APAP600 group as compared to APAP300 group. Further, similar trend was observed for expression of downstream transcription factors c-Jun and c-Fos (Fig. 2.4.6D). Overall, these results suggest that signaling through growth factor and downstream MAPKs remain activated even at the dose of APAP where liver regeneration is inhibited. Thus, lack of activation of these signaling may not be the reason for failed liver regeneration at higher dose.

It should be noted that apart from involvement in cell proliferation and liver regeneration pathways (Schwabe, Bradham et al. 2003), JNK activation is also considered as one of the important steps in mediating APAP overdose induced liver injury (Jaeschke, McGill et al. 2012). Phosphorylation of JNK and its translocation to mitochondria early in APAP toxicity leads to exacerbation of mitochondrial oxidant stress (Jaeschke, McGill et al. 2012). Despite previous attempts, the role of JNK

activation in liver regeneration after APAP overdose remains undefined and should be further evaluated (Bourdi, Korrapati et al. 2008).

Differential activation of cytokine pathways (IL-6/STAT-3 and TNF-α/NF-κB) during liver regeneration after APAP overdose

Activation of cytokine pathways (IL-6/STAT-3 and TNF-α/NF-κB) is considered as important "priming event" that contribute to initiation of early stages of liver regeneration after PH (Fausto 2000). However, their role in liver regeneration after APAP overdose is not known. IL-6 mRNA was induced at both the doses of APAP as early as 3 hr after APAP treatment and remains induced up to 24 hr showing peak at 12 hr after APAP treatment (Fig 2.4.7A). Interestingly, IL-6 induction was greater at higher dose of APAP where regeneration was inhibited. IL-6 binding to its receptor at cellular membrane leads to phosphorylation and nuclear translocation of STAT-3, which ultimately results in transcription of many target genes. Corroborating IL6 data, we observed significantly higher phosphorylation of STAT-3 in APAP600 mice, which showed less regeneration (Fig 2.4.7B). While, STAT-3 phosphorylation was transient (appeared at 6 and 12 hr) in APAP300 group, it started early (at 3 hr) and maintained till 24 hr in APAP600 group. Whereas total STAT-3 levels were induced after APAP treatment in both doses, no significant difference between the groups was observed. Overall these data suggest that, similar to growth factor signaling, IL-6 signaling remain intact even at non-regenerative dose of APAP thus may not explain failed liver regeneration at higher dose.

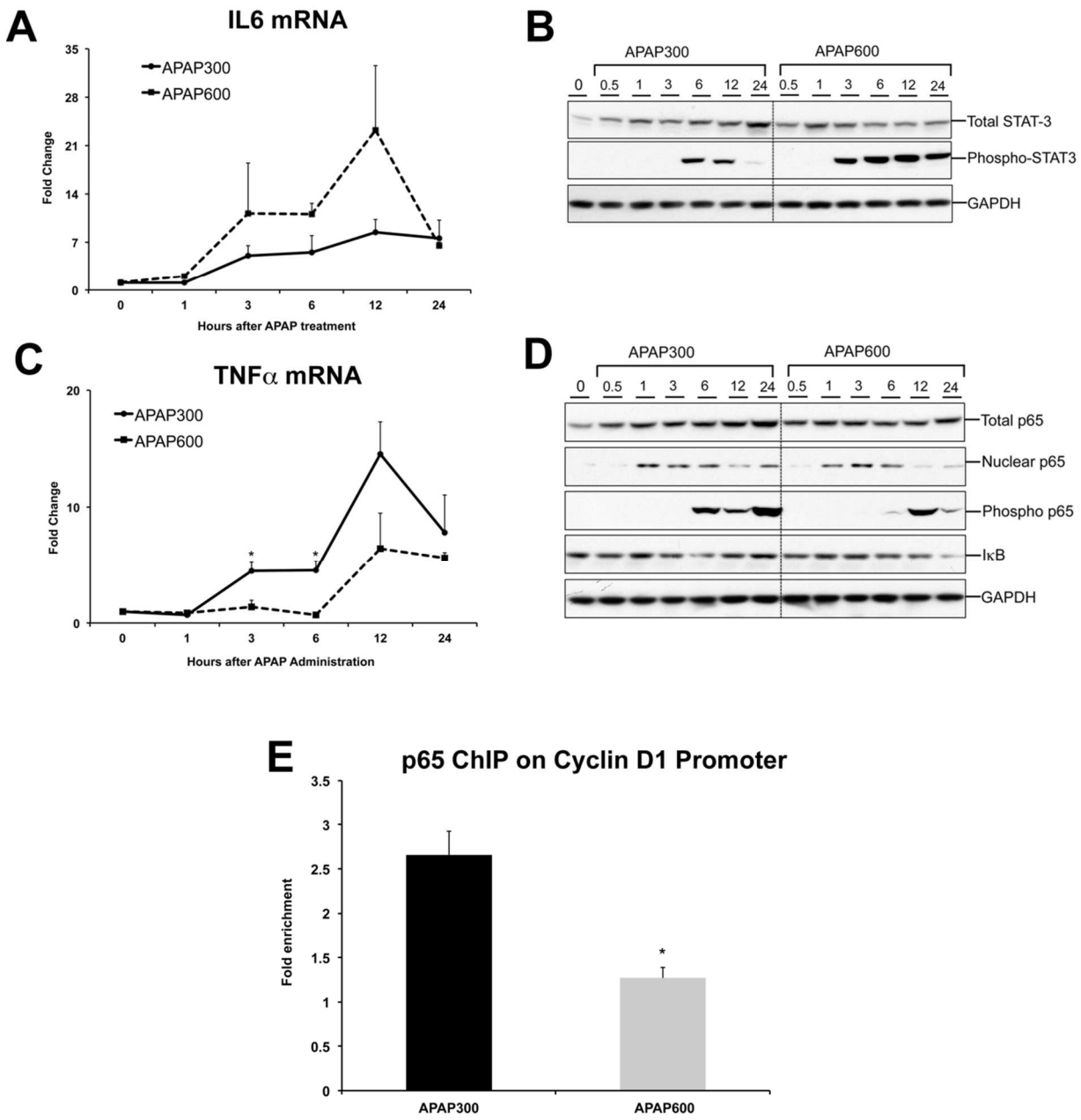

Figure 2.4.7. Differential activation of cytokine pathways during liver regeneration after APAP overdose. (A) IL-6 and (C) TNF-α mRNA expression in liver of mice treated with either 300 or 600 mg/kg APAP. Western blot analysis of (B) total STAT-3, phospho STAT-3, (D) total, nuclear, phosphorylated p65 and IκB using total liver extract (unless specified) of mice treated with either 300 or 600 mg/kg APAP. All samples were collected over a time course of 0 to 24 hr after APAP treatment (n = 3-5). (E) ChIP analysis showing p65 binding to Cyclin D1 promoter at 12 hr after either 300 or 600 mg/kg APAP treatment (n=3). * indicate significant difference between two doses at $p<0.05$

Next, we determined the status of NF-κB signaling during liver regeneration after APAP-induced ALI using our incremental dose model (Fig. 2.4.7C and D). TNF-α binding to its receptor ultimately results in stabilization and nuclear translocation of transcription factor NF-κB (consisting p65 as one of its subunits). *Cyclin D1* is a known target of NF-κB (Guttridge, Albanese et al. 1999). Interestingly, TNF-α was induced at early time points (3 and 6 hr), preceding regenerative phase, specifically in lower (regenerative) dose and its mRNA level peaked at 12 hr after APAP treatment. In contrast, TNF-α was not induced until 12 hr after APAP in higher dose and overall showed much lower induction compared to APAP300 dose (Fig. 2.4.7C). Further, there was marked increase in phosphorylation of p65 at Ser536, a site known to enhance transactivation potential of p65(Viatour, Merville et al. 2005), in APAP300 group preceding regenerative phase (6 hr after APAP) and sustained until 24 hr (Fig. 2.4.7D). Whereas, APAP600 group also showed increase in Ser536 phosphorylation, it was only at 12 hr post-APAP and was overall lower than in APAP300 group. While, marked nuclear translocation of p65 was observed at both the doses, the increase in nuclear p65 was sustained in APAP300 group till 24 hr after APAP but declined sharply at 12 hr and 24 hr after APAP in APAP600 group. Total p65 protein expression was increased in both the groups at all the time points compared to basal levels and IκB protein level did not appear to change at any dose (Fig. 2.4.7D).

Overall, activation of NF-κB signaling correlated with stimulation of liver regeneration at lower dose and we observed indication of inhibited activation of NF-κB signaling at higher dose where liver regeneration was inhibited. Considering *Cyclin D1*

is a known target of p-65, we wanted to directly compare p-65 binding to *Cyclin D1* promoter at the two doses using ChIP assay (Fig 2.4.7E). Interestingly, we observed around 2.5-fold higher binding of p-65 to *Cyclin D1* promoter in APAP300 compared to APAP600 group, 12 hr after APAP treatment, which is the time point of peak induction of Cyclin D1. All these data suggest that inhibited activation of NF-κB signaling may be one of reason for inhibited cyclin D1 induction and inhibited liver regeneration at higher dose.

Activation of Wnt/β-catenin signaling is inhibited following higher dose of APAP

Previous report have demonstrated involvement of β-catenin activation in liver regeneration after PH and APAP induced ALI (Nejak-Bowen and Monga 2011), (Apte, Singh et al. 2009). Here, we further analyzed the role of Wnt/ β-catenin signaling in detail using our incremental dose model. Whereas total β-catenin protein remained unchanged in both the groups at all time points, a substantial increase in nuclear β-catenin was observed at 12 hr and 24 hr after APAP overdose in APAP300 group (Fig. 2.4.8A). In contrast, APAP600 exhibited initial increase in nuclear β-catenin at 0.5 and 1 hr after APAP treatment, which decreased at later time points. The decline in nuclear β-catenin was consistent with substantial increase in two inactive forms of β-catenin (Ser45/Thr41-phosphorylated and Ser33/37/Thr41-phosphorylated) in APAP600 mice. To further study the upstream mechanism, we determined expression of total and phosphorylated (inactive) form of glycogen synthase kinase-3β (GSK3β), which regulates β-catenin degradation in canonical Wnt signaling (Fig. 2.4.8B). Data indicate significant inactivation of GSK3β as shown by marked increase in phospho-GSK3β

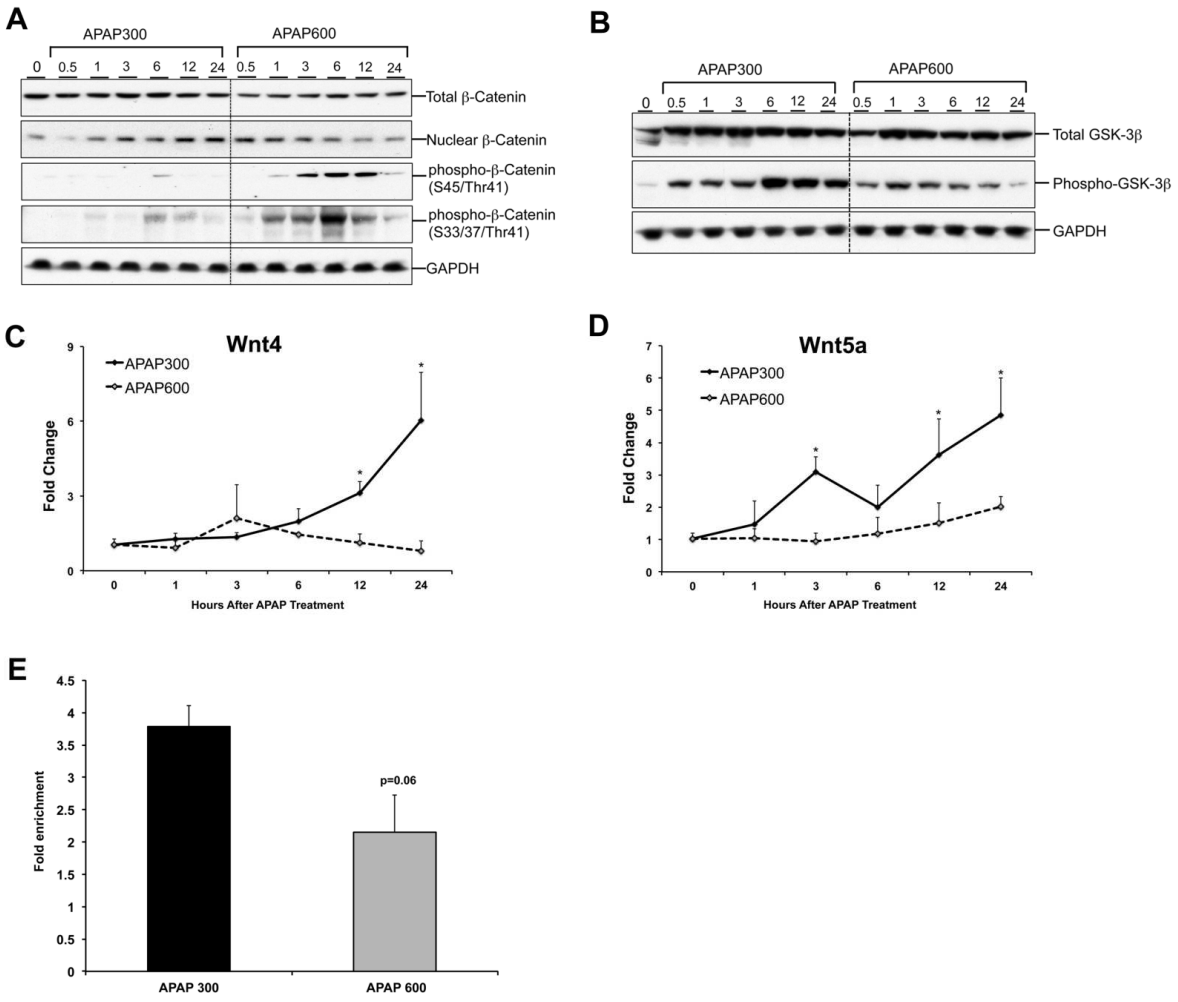

Figure 2.4.8. **Activation of Wnt/β-catenin signaling is inhibited following higher dose of APAP.** Western blot analysis of (A) total β-catenin, nuclear β-catenin, phospho-β-catenin (S45/Thr41), phospho-β-catenin (S33/37/Thr41), (B) total GSK-3β and phospho-GSK-3β. Total liver extract (unless specified) of mice treated with either 300 or 600 mg/kg APAP were used for Western blots. mRNA expression of (C) Wnt4 and (D) Wnt5a in liver of mice treated with either 300 or 600 mg/kg APAP. All samples were collected over a time course of 0 to 24 hr after APAP treatment (n = 3-5). (E) ChIP analysis showing β-catenin binding to *Cyclin D1* promoter at 12 hr after either 300 or 600 mg/kg APAP treatment (n = 3). * indicate significant difference between two doses at $p<0.05$

protein in APAP300 mice between 6 hr to 24 hr after APAP overdose, correlating with β-catenin activation. Interestingly, higher dose showed initial GSK3β inactivation to some extent (less compared to APAP 300), but this inactivation markedly decreased during later phase. The decreased inactivation (or increased activation) of GSK3β at 6 to 24 hr after APAP treatment in APAP600 group are consistent with increase in phosphorylated β-catenin species (inactive). Next we determined protein expression of Dvl, which acts upstream of GSK3β in canonical Wnt signaling and is activated by Wnt binding to Frizzled (Fzd) receptor. A significant increase in Dvl expression was observed in APAP300 group at 12 and 24 hr post-APAP. In contrast, Dvl expression increased early after APAP600 dose but declined during the regenerative phase (Fig. 2.4.9).

To determine if expression of any of the Wnts and Fzds is induced during APAP-induced liver regeneration, we quantified mRNA of all 11 Wnts and 10 Fzld genes known to be expressed in the liver (Zeng, Awan et al. 2007). The data indicate that Wnt4 and Wnt5a mRNA exhibited significant induction in APAP300 group during regenerative phase (12 and 24 hr) (Fig. 2.4.8C and D). However, none of the Wnt mRNAs were induced at any time point following APAP600 dose. Among the Fzd genes, Fzd7 was significantly induced only in APAP300 group between 3 to 12 hr, while Fzd8 showed opposite trend with slight but significant induction in APAP600 group at 12 and 24 hr (Fig. 2.4.9). All other Fzd genes remained unaffected in both groups.

Overall, we observed stimulation of Wnt/β-catenin signaling specifically in regenerating dose correlating with induction of Cyclin D1 which is a known target of β-catenin (Torre, Benhamouche et al. 2011). Further, activation of Wnt/β-catenin signaling

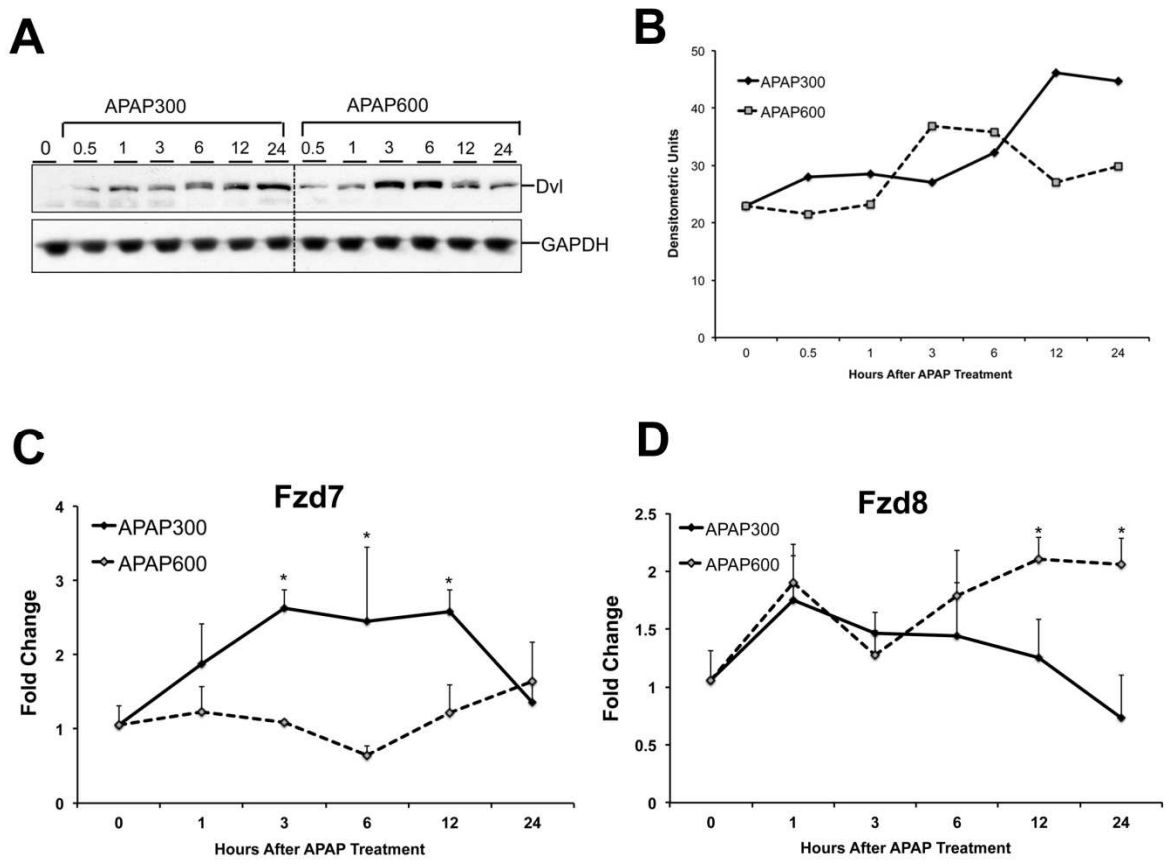

Figure 2.4.9. (A) Western blot and (B) densitometric analysis of Dvl using total liver extracts from mice treated with 300 mg/kg or 600 mg/kg APAP. Real time PCR analysis of (C) Fzd7 and (D) Fzd8 mRNA in livers of mice treated with 300 mg/kg or 600 mg/kg APAP (n = 3-5).

was inhibited at higher dose correlating with inhibited Cyclin D1 induction. Finally, we directly checked if there is difference in β-catenin binding to Cyclin D1 promoter at the two doses using ChIP assay. We observed around 2-fold higher enrichment of β-catenin binding to Cyclin D1 promoter in APAP300 compared to APAP600 group, 12 hr after APAP treatment, which is the time point of peak induction of Cyclin D1 (Fig. 2.4.8E). Taken together, these data suggest that inhibited activation of Wnt/β-catenin signaling may be one of reason for inhibited cyclin D1 induction and inhibited liver regeneration at higher dose.

Increased liver regeneration in mice over expressing stable form (S45D) of β-catenin after APAP overdose

Our studies using the incremental dose model identified Wnt/β-catenin signaling as potential targets for stimulating liver regeneration after APAP overdose. We further confirmed the role of β-catenin in stimulation of liver regeneration after APAP overdose using mice over expressing a stable (S45D) form of β-catenin. Preliminary analysis showed moderately higher total β-catenin in the S45D mice (Fig. 2.4.10A). Further analysis indicated no significant difference in hepatic CYP2E1, the main enzyme involved in APAP bioactivation, in S45D mice as compared to WT mice (Fig. 2.4.10A). Preliminary studies indicated that the WT controls mice for the S45D strain, which are the S45D negative littermates, are more susceptible to APAP than C57BL/6 mice and exhibited slightly different time course of liver injury development. Therefore, we initially used a dose of 300 mg/kg APAP in this study, which was sufficient to show attenuated liver regeneration response in WT mice. WT and S45D mice were treated with 300

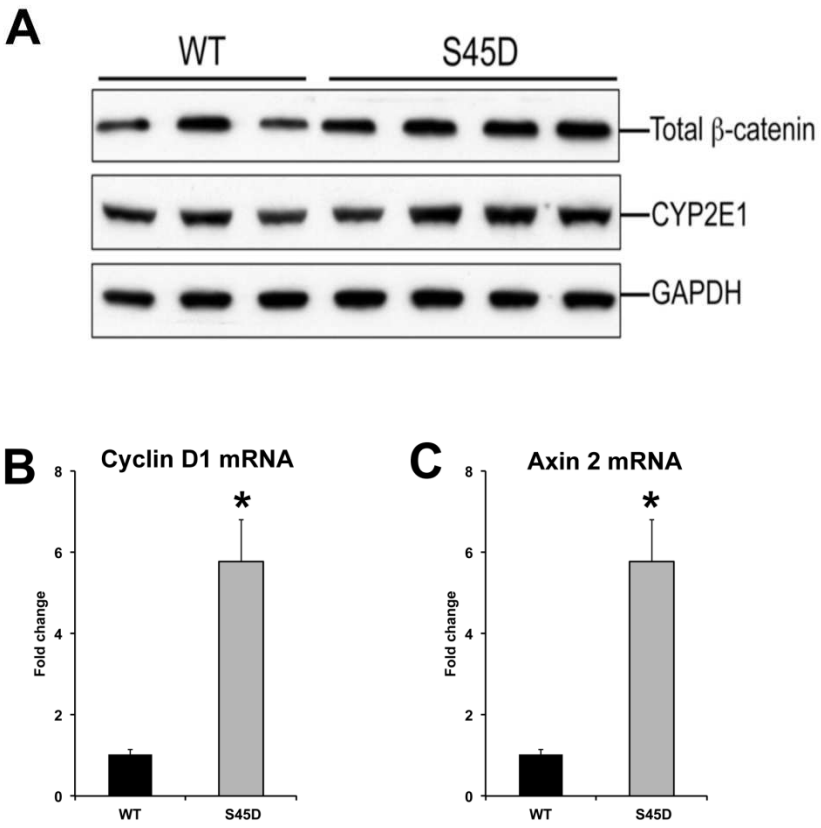

Figure 2.4.10. (A) Western blot analysis of total β-catenin and CYP2E1 in WT and S45D mice before APAP treatment (n=3-4). Changes in (B) Cyclin D1 and (C) Axin2 mRNA at 24 hr after treatment with 300 mg/kg APAP in WT and S45D mice (n=5).

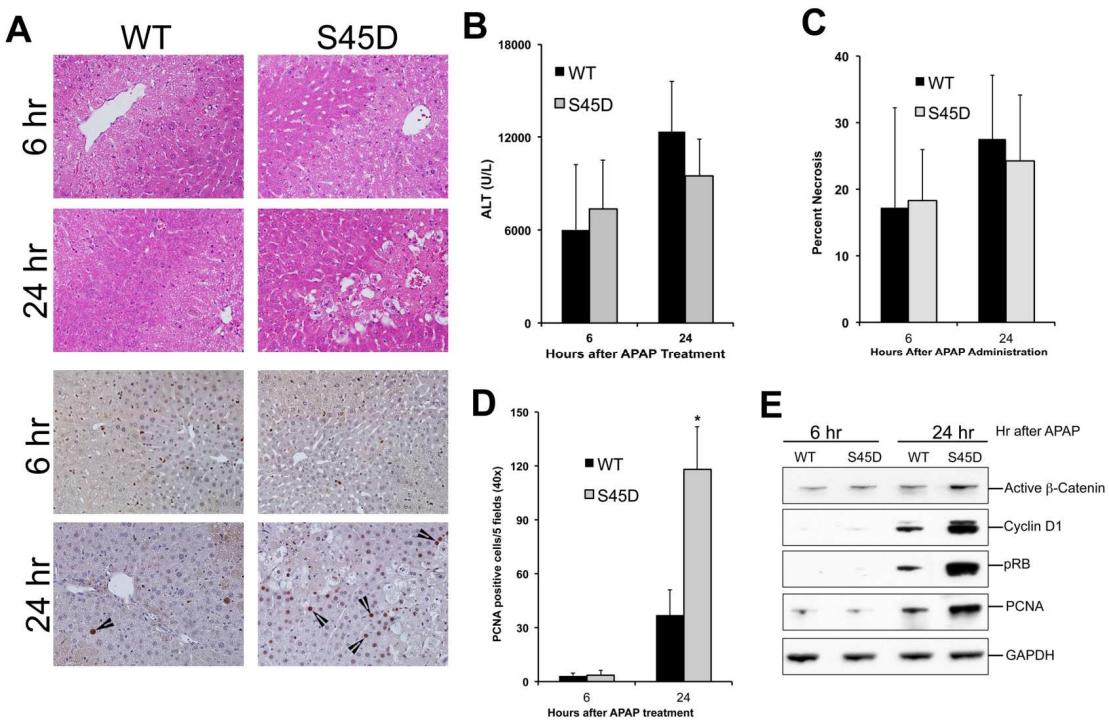

Figure 2.4.11. **Over expression of stable form of β-catenin improves liver regeneration after 300 mg/kg APAP.** (A) Representative photomicrographs of H&E stained (upper panel) and PCNA-stained (lower panel) liver sections from WT and S45D mice treated with 300 mg/kg APAP. Bar graphs showing (B) Serum ALT levels, (C) percent necrosis area and (D) PCNA counts in WT and S45D mice treated with 300 mg/kg APAP. (E) Western blot analysis of active β-catenin, cyclin D1, pRb and PCNA using total liver extracts in WT and S45D mice treated with 300 mg/kg APAP (n=5). * indicate significant difference between WT and S45D group at $p<0.05$. Arrow head represents PCNA positive cells.

mg/kg APAP and liver injury and regeneration were analyzed at 6 and 24 hr after APAP treatment. Serum ALT levels and necrosis scoring demonstrated similar liver injury in WT and S45D mice at both the time points after APAP (Fig. 2.4.11A-C) suggesting injury process is not altered in S45D mice. While, WT mice showed only a few PCNA positive hepatocytes at 24 hr after APAP treatment, liver regeneration was remarkably improved in S45D mice as indicated by higher PCNA positive hepatocytes in S45D mice at 24 hr after APAP treatment compared to WT mice (Fig. 2.4.11D). This was further substantiated by PCNA protein expression, which displayed similar pattern (Fig. 2.4.11E). Western bot analysis indicated significantly higher activated β-catenin in S45D mice at 24 hr after APAP treatment (Fig. 2.4.11E). Increase in activated β-catenin was accompanied by increased cyclin D1 and phosphorylation of Rb protein in S45D mice at 24 hr after APAP overdose (Fig. 2.4.11E). Real Time PCR analysis indicated increase gene expression of Cyclin D1 and Axin 2, the known targets of β-catenin, in S45D mice following APAP treatment as compared to WT mice at 24 hr after APAP overdose (Fig. 2.4.10B and C). Next, we studied liver regeneration in S45D mice after higher dose of APAP (600 mg/kg) (Fig. 2.4.12A, D and E). Both WT and S45D mice did not show any significant liver regeneration at 24 hr after APAP. However, S45D mice showed higher PCNA protein expression (with higher PCNA positive cells) compared to WT mice (Fig. 2.4.12A and D) at 48 hr after APAP treatment. This was further substantiated by higher cyclin D1 protein expression and downstream phosphorylation of Rb protein in S45D mice compared to WT mice at 48 hr after APAP treatment (Fig. 2.4.12E). Similar to APAP300 dose, both WT and S45D mice showed similar injury at 24 and 48 hr after APAP600 treatment (Fig. 2.4.12A- C). These data show that overexpression of β-

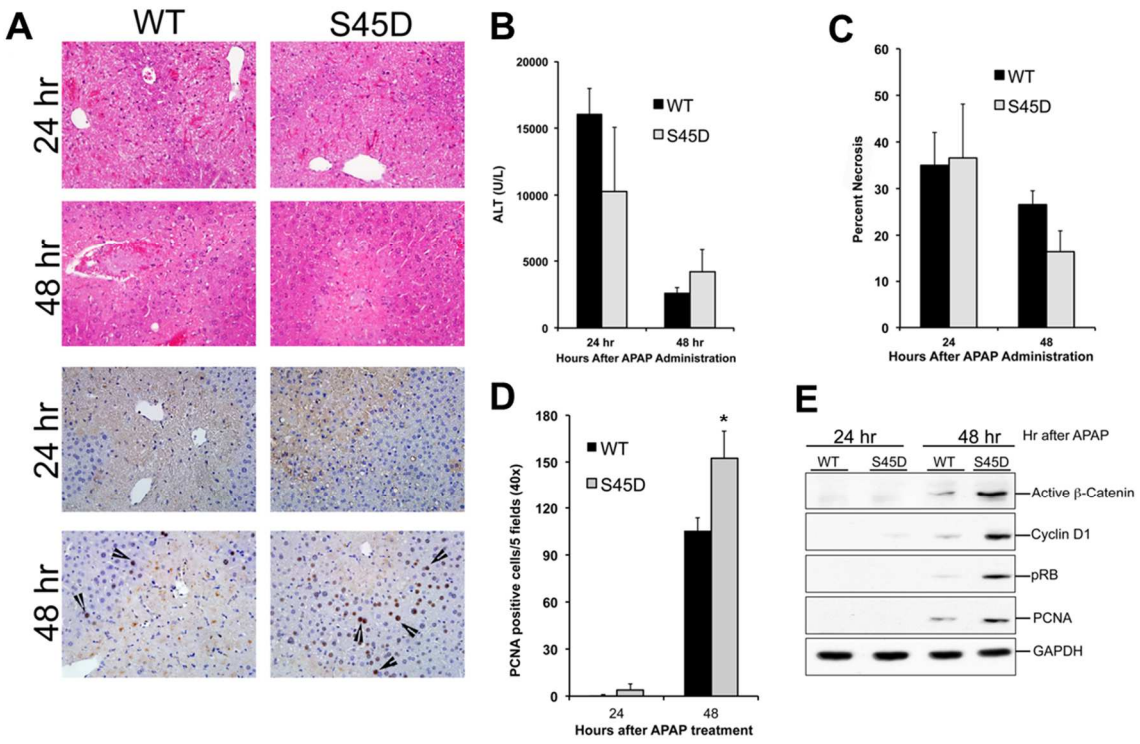

Figure 2.4.12. Over expression of stable form of β-catenin improves liver regeneration after 600 mg/kg APAP. (A) Representative photomicrographs of H&E stained (upper panel) and PCNA-stained (lower panel) liver sections from WT and S45D mice treated with 600 mg/kg APAP. Bar graphs showing (B) Serum ALT levels, (C) percent necrosis area and (D) PCNA counts in WT and S45D mice treated with 600 mg/kg APAP. (E) Western blot analysis of active β-catenin, cyclin D1, pRb and PCNA using total liver extracts in WT and S45D mice treated with 600 mg/kg APAP (n=5). * indicate significant difference between WT and S45D group at $p<0.05$. Arrow head represents PCNA positive cells.

catenin resulted in faster and higher liver regeneration after APAP overdose.

2.5 DISCUSSION

APAP overdose is the foremost cause of ALF in the US contributing to around 46% of all ALF cases (Lee, Squires et al. 2008). Treatment options after APAP-induced ALF are extremely limited. Extensive research on chemicals and drugs that induce liver injury has shown that compensatory liver regeneration plays a critical role in determination of final outcome of injury. It is known that timely stimulation of regeneration leads to regression of injury while failing to regenerate culminates in progression of injury and death (Mangipudy, Chanda et al. 1995, Anand, Soni et al. 2003, Mehendale 2005). Several recent clinical and animal studies showing similar findings after APAP overdose suggest that stimulating liver regeneration can be a potential treatment option for APAP-induced ALF (Hu and Colletti 2008, Apte, Singh et al. 2009, Donahower, McCullough et al. 2010). However, our knowledge regarding mechanisms of liver regeneration after APAP-induced injury is extremely limited and there is a critical need to study these mechanisms systematically.

Dynamics of liver regeneration after toxic insult has been extensively studied for many toxicants such as thioacetamide (Mangipudy, Chanda et al. 1995), carbon tertrachloride (Rao, Mangipudy et al. 1997), and chloroform (Anand, Soni et al. 2003). These studies demonstrated that liver regeneration follows classic rules of dose-response such that regeneration increases with dose of toxicant proportionate to injury. This occurs until a threshold dose beyond which the ability of liver to regenerate declines leading to progression of injury (Mehendale 2005). Based on this principle, we

developed an incremental dose model to study mechanisms of liver regeneration after APAP-induced ALI. In this study, we used two doses of APAP, a lower dose (300 mg/kg) that caused extensive liver injury but also significant compensatory regeneration leading to regression of injury and spontaneous recovery and a higher dose (600 mg/kg) that caused sustained injury, compromised recovery and decreased survival. We observed marked inhibition of liver regeneration at higher dose highlighting importance of liver regeneration in overall survival after APAP overdose supporting previous findings (Hu and Colletti 2008, Apte, Singh et al. 2009, Donahower, McCullough et al. 2010). It should be noted that the marked inhibition of regeneration was not due to lack of viable hepatocytes at the higher dose. Necrosis score analysis and immunohistochemical staining for HNF4α, which stains for viable hepatocytes, demonstrated that more than 50% hepatocytes were viable in APAP600 dose at peak injury. In fact, overall cellular death was not strikingly different at the two doses at early time points where regeneration was initiated at lower dose and inhibited at higher dose. These data and further analysis of cell cycle phases indicated that the inhibition of regeneration was not simply because of too much cell death but due to inhibited cell cycle entry and cell cycle arrest at very early stage in viable cells surrounding necrotic zone at higher dose. Higher cellular stress in viable hepatocytes may be a possible explanation for this inhibited liver regeneration at higher dose. This observation also distinguishes the APAP incremental dose model from 90% partial hepatectomy model, where the primary reason behind decreased liver regeneration is lack of critical mass required to initiate regeneration.

Our most remarkable finding was that Cyclin D1 mRNA and protein expression increased in the lower dose preceding the start of regenerative phase but this was completely inhibited in the higher dose. These data further support that initial entry into cell cycle is inhibited at higher dose underscoring the importance of induction of Cyclin D1 in regulation of liver regeneration following APAP-induced ALI. Further, we found higher and sustained induction of p21 protein at higher dose supporting possible contribution of active inhibition of cell cycle in cell cycle arrest at higher dose due to elevated cellular stress. Similar results are reported previously after ethanol treatment in PH model, where ethanol inhibited Cyclin D1, induced p21 and impaired liver regeneration (Koteish, Yang et al. 2002). Interestingly, there was moderate induction of p21 even at regenerative dose suggesting possible role of p21 in coordinating liver regeneration. Recent study demonstrating role reversal of p21 in liver regeneration depending upon degree of injury certainly supports this possibility (Buitrago-Molina, Marhenke et al. 2013). Further studies are required to determine the exact role of p21 in liver regeneration after APAP overdose.

Several regenerative pathways such as growth factors/MAPKs, TNFα/NF-κB, Wnt/β-catenin and IL6/STAT-3, are known to induce Cyclin D1 and hepatocyte proliferation (Michalopoulos 2007). We determined status of some these pathways in our model to look further into mechanism behind differential expression of CyclinD1 at both the doses. There was remarkable activation of growth factor receptors (c-Met and EGFR), downstream MAPKs (ERK1/2, p38 and JNK) and IL-6/STAT-3 signaling at regenerative dose but, interestingly, activation was much higher and sustained at non-regenerative dose. Previous studies suggest that some of these pathways may be

important for normal liver regeneration after APAP overdose where animals spontaneously regenerate. For instance, liver regeneration was found to be impaired in IL-6 knock out mice after moderate APAP overdose.(James, Lamps et al. 2003) Further confirmatory studies are required to demonstrate role of some of these pathways in liver regeneration after APAP overdose. However, our data clearly indicate, for the first time, that inability to activate these signaling pathways is not limiting regeneration at higher dose where animals are not able to recover spontaneously, as these pathways are already highly activated at this dose. Additional stimulation of these pathways may not be a good strategy from therapeutic standpoint. This also questions administration of growth factors as a therapeutic strategy after APAP-induced ALF. This observation is consistent with previous reports that administration of growth factors did not affect liver regeneration in dogs after APAP-induced ALF (Francavilla, Azzarone et al. 1993). Also patients who died after APAP-induced ALF were found to have higher amount of HGF circulating in plasma (Hughes, Zhang et al. 1994).

These data give rise to two possibilities. First, an active inhibitory signaling is blocking liver regeneration at higher dose counteracting these regenerative signaling. Activation of cell cycle inhibitors (e.g. p21) is one such signaling pathway that we observed in this study. Moreover, some of the known regenerative pathways, which are highly activated in APAP600, may be actively involved in inhibiting regeneration. For instance, over-activation of p38 has been associated with decreased regeneration in PH model (Koteish, Yang et al. 2002, Horimoto, Fulop et al. 2004). Also, p38 can directly inhibit Cyclin D1 expression (Lavoie, L'Allemain et al. 1996). Similarly impaired regeneration after PH in ob/ob mice with fatty liver or in mice with hyper-stimulated IL6

signaling was correlated with sustained and over-activation of STAT3 (Wustefeld, Rakemann et al. 2000, Torbenson, Yang et al. 2002). Further, sustained activation of these mediators was correlated with induction of p21 and inhibition of Cyclin D1, which is consistent with our data.

The second possibility is that specific critical pathways apart from the growth factor-mediated pro-regenerative signaling regulate liver regeneration after APAP overdose. Previous studies have shown that Wnt/β-catenin pathway plays a critical role in liver regeneration in general and after APAP over dose in particular (Apte, Singh et al. 2009, Nejak-Bowen and Monga 2011). Consistent with these data, we observed activation of canonical Wnt signaling at regenerative dose. More interestingly, β-catenin activation was inhibited at higher dose where liver regeneration was inhibited. We also observed TNF-α/NF-κB signaling activation at regenerative dose supporting previous studies showing role of TNF receptor in liver regeneration after APAP overdose(Chiu, Gardner et al. 2003, James, Kurten et al. 2005). Further, previous data showed increased NF-κB DNA binding after APAP treatment, which was correlated positively with regeneration in mice (Yang, Miki et al. 2009, Yang, Zhang et al. 2011). Interestingly, similar to β-catenin signaling, our results indicated that activation of TNF-α/NF-κB signaling is inhibited at higher dose where recovery is compromised. Both, β-catenin and NF-κB are known to transcriptionally regulate and induce Cyclin D1 directly (Guttridge, Albanese et al. 1999, Torre, Benhamouche et al. 2011). Our ChIP data support important role of such regulation in liver regeneration after APAP overdose. Our findings implicate that inhibited activation of Wnt/β-catenin and TNF-α/NF-κB signaling

may be contributing factor to inhibited liver regeneration after severe APAP overdose and thus could be potential target for stimulating liver regeneration therapeutically. Over activation of beta-catenin is known to accelerate liver regeneration after PH (Nejak-Bowen, Thompson et al. 2010, Nejak-Bowen and Monga 2011). Further, our previous study showed correlation of beta-catenin activation with higher spontaneous liver regeneration and survival in APAP-induced ALF patients (Apte, Singh et al. 2009). In the current study, we observed that mice overexpressing mutated form of β-catenin have better liver regeneration after low and high dose of APAP. Our data for the first time indicated that stimulating canonical Wnt signaling could be a viable approach for improving liver regeneration in APAP-induced ALF patients.

In summary, our findings using a novel comparative model have demonstrated that high doses of APAP will inhibit liver regeneration due to active inhibition of cell cycle progression and/or by lack of stimulation via critical pro-mitogenic pathways. Some interesting potential pathways that may be involved in active inhibition of liver regeneration include p38 MAPK and IL-6 signaling and require further detailed investigation. Further, this study has revealed canonical Wnt signaling and NF-κB signaling as potential therapeutic targets to stimulate liver regeneration after APAP overdose. Finally, our studies indicate that concomitant inhibition of several inhibitory pathways and activation of various stimulatory pathways may have promising future in developing regenerative therapies for APAP-induced ALF patients.

CHAPTER III: INHIBITION OF GSK3 ACCELERATED LIVER REGENERATION AFTER ACETAMINOPHEN-INDUCED HEPATOTOXICITY IN MICE

3.1 ABSTRACT

Overdose of acetaminophen (APAP) is the leading cause of acute liver failure (ALF) in the US with very limited treatment options. Timely initiation of compensatory liver regeneration after APAP-induced hepatotoxicity is critical for final recovery and survival. Stimulating liver regeneration in patients of APAP-induced ALF holds a great therapeutic potential, but development of novel regenerative therapies for ALF is hampered because the mechanisms of liver regeneration after APAP-induced ALF have not extensively explored yet. Here we investigated the role of glycogen synthase kinase-3 (GSK3) in liver regeneration after APAP-induce hepatotoxicity, which is currently unknown. GSK3 was rapidly inactivated after moderate APAP overdose (300 mg/kg) in mice, which corresponded to timely stimulation of compensatory liver regeneration. GSK3 inactivation was remarkably reduced after higher dose of APAP (600 mg/kg), where liver regeneration was attenuated and delayed. Treatment with selective GSK3 inhibitor (L803-mts), starting from 4 hr after 600 mg/kg dose of APAP, resulted in early initiation of liver regeneration without any alteration of APAP-induced hepatotoxicity in mice. Interestingly, early liver regeneration after GSK3 inhibition was associated with improved survival. Early cell cycle initiation in hepatocytes after GSK3 inhibition was due to rapid induction of cyclin D1 and phosphorylation of retinoblastoma protein. This was associated with increased activation of β-catenin signaling after GSK3 inhibition. Taken together, our study has revealed a novel role of GSK3 in liver regeneration after APAP overdose and identified GSK3 as a potential therapeutic target to improve liver regeneration after APAP-induced ALF.

3.2 INTRODUCTION

Liver injury caused by acetaminophen (APAP) overdose is the leading cause of acute liver failure (ALF) in the western world, accounting nearly half of all the ALF cases (Lee, Squires et al. 2008, Lee 2012). Despite decades of research, current treatment options after APAP-overdose are extremely limited. N-acetylcysteine (NAC) is the current standard of care for APAP overdose patients, which is effective only at an early stage (Athuraliya and Jones 2009, Bernal, Lee et al. 2015). Mechanisms of liver injury after APAP overdose have been extensively investigated in past in order to develop novel therapeutic strategies. However, many patients seek medical attention late after liver injury is already established and difficult to manipulate (Larson 2007). Liver injury after APAP-overdose is subsequently followed by compensatory liver regeneration, which is critical for recovery. Previous studies from our laboratory and others have demonstrated that liver regeneration is a critical determinant of survival after APAP overdose (Schmidt and Dalhoff 2005, Hu and Colletti 2008, Apte, Singh et al. 2009, Donahower, McCullough et al. 2010). Liver regeneration is a parameter that can be potentially modulated even at a late stage in pathogenesis of APAP-induced ALF and stimulating liver regeneration in patients of APAP-induced ALF holds a great therapeutic potential. However, development of novel regenerative therapies for ALF is hampered because the mechanisms of liver regeneration after APAP-induced ALF have not extensively explored yet. Mechanisms of liver regeneration are mostly studied in partial hepatectomy (PH) model (Michalopoulos 2007), which is considerably different from APAP-hepatotoxicity, the model of clinical ALF (Bhushan, Walesky et al. 2014).

Glycogen synthase kinase-3 (GSK3) is a ubiquitously expressed serine/threonine protein kinase, which is constitutively active in cells under resting state and primarily regulated by inactivation (Kaidanovich-Beilin and Woodgett 2011). GSK3 is downstream of a number of major signaling pathways including growth factors, insulin and canonical Wnt signaling, which negatively regulate GSK3 activity. GSK3 activity is primarily regulated by phosphorylation-mediated inactivation, but other methods of inactivation such as sequestration and subcellular localization are also known (Jope and Johnson 2004). Initially discovered to regulate glycogen synthase, GSK3 is now known to regulate a wide spectrum of proteins and affect a number of cellular functionalities including proliferation, differentiation and survival (Jope and Johnson 2004). GSK3 functions by phosphorylation-mediated inactivation/ degradation of its substrates. As a subunit of β-catenin destruction complex, GSK3 causes phosphorylation mediated targeting of β-catenin for proteosomal degradation, thus negatively regulating β-catenin signaling (Doble and Woodgett 2003). Apart from β-catenin, GSK3 can regulate many other cell proliferation mediators such as Cyclin D1, c-Myc, c-Jun, and eIF2B (Sutherland 2011).

Inhibitors of GSK3 have been reported to induce *in-vitro* expansion of mouse primary hepatocytes (Ito, Kamiya et al. 2012) and facilitate survival signaling via activation of β-catenin in them (Gotschel, Kern et al. 2008). Further, GSK3 negatively regulates β-catenin, which plays a critical role in liver regeneration (Tan, Behari et al. 2006, Sekine, Gutierrez et al. 2007, Nejak-Bowen, Thompson et al. 2010, Nejak-Bowen and Monga 2011). Interestingly, other studies indicate that GSK3 may play a pro-regenerative role after PH (Chen, Yang et al. 2007, Jin, Wang et al. 2009, Sekiya and Suzuki 2011).

Treatment with GSK3 inhibitor or siRNA mediated downregulation of GSK3 has reported to impair liver regeneration in mice and rats after PH (Chen, Yang et al. 2007, Jin, Wang et al. 2009, Sekiya and Suzuki 2011). Antisense oligonucleotides mediated silencing of GSK3β has reported to inhibit APAP-induced injury, attributed to role of GSK3β in initiation of APAP toxicity (Shinohara, Ybanez et al. 2010). However, role of GSK3 in compensatory liver regeneration after APAP-induced liver injury is not known. In previous chapter, we established an incremental dose model in mice to study mechanisms of liver regeneration after APAP-induced ALF, which has revealed a number of signaling pathways involved in liver regeneration after APAP overdose. These data also showed that inactivation of GSK3β is positively correlated with early initiation of liver regeneration after APAP overdose. Further, overexpression of β-catenin resulted in improved liver regeneration after APAP-induced liver injury. Here, we investigated direct role of GSK3 in compensatory liver regeneration after APAP overdose using pharmacological inhibitor of GSK3. Based on our previous findings, we hypothesized that inhibition of GSK3 after APAP treatment will result in enhanced liver regeneration. Here we report that treatment with L803-mts, a selective GSK3 inhibitor, accelerate liver regeneration after APAP overdose via early activation of β-catenin and induction of Cyclin D1.

3.3 MATERIALS AND METHODS

Animals, treatments and tissue harvesting

Eight weeks old, C57BL/6J mice were purchased from Jackson Laboratories and used in all the studies. All animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facilities at the University of Kansas Medical Center under a standard 12-hr light/dark cycle with access to chow and water *ad libitum*. All studies were approved by The Institutional Animal Care and Use Committee at the University of Kansas Medical Center. APAP (Sigma, St. Louis, MO) was dissolved in warm 0.9% saline and administered intraperitoneally (i.p.). Mice were fasted overnight before APAP administration. For study with incremental doses of APAP, mice (n = 3 to 7) were treated with either 300 or 600 mg/kg APAP, and sacrificed at 0, 3, 6, 12, 24, 48, 72 and 96 hr following APAP treatment. For GSK3 inhibition studies**,** a substrate competitive peptide inhibitor of GSK3, L803-mts: N-myristol-GKEAPPAPPQS(p)P (Genemed Synthesis, San Francisco, CA) was used. GSK3 inhibitor (GSK3i) was dissolved in warm phosphate-buffered saline (PBS) and administered 4 hr after APAP (600 mg/kg) treatment, followed by treatments every 24 hr until sample collection. For preliminary study, GSK3i was administered at doses of 400 and 800 nmol per mouse and animals (n = 3-4) were euthanized 24 hr after APAP treatment. In the following studies, 800 nmol/mouse dose of GSK3i was administered and mice (n=4-12) were sacrificed at 18, 24, 48 and 72 hr after treatment with APAP. Euthanasia was performed by cervical dislocation under isoflurane anesthesia, and blood and livers were collected. Serum samples were obtained from the blood and used

for further analysis. Liver sections were prepared for histological analysis as described previously (Wolfe, Thomas et al. 2011).

Histological analysis and serum ALT measurement

Paraffin-embedded liver sections (4 μm thick) were used for H&E staining and scored for percentage necrotic area. Serum alanine aminotransferase (ALT) was measured using the Infinity ALT kit (ThermoFisher Scientific, Pittsburgh, PA), according to the manufacturer's protocol.

Immunohistochemistry

Paraffin-embedded liver sections (4 μm thick) were used for immunohistochemical detection. PCNA staining was performed for cell proliferation analysis as described before (Wolfe, Thomas et al. 2011). Anti-PCNA antibody was purchased from Cell Signaling Technologies (Danvers, MA). Biotinylated secondary antibody was purchased from Jackson Immunoresearch (West Grove, PA).

Protein isolation and western blot analysis

Total cell lysate was prepared from frozen liver tissues as described previously (Bhushan, Borude et al. 2013). Protein estimation and western blot analysis was performed using individual or pooled samples of protein extracts as previously described in detail (Wolfe, Thomas et al. 2011). All primary and secondary antibodies were obtained from Cell Signaling Technologies (Danvers, MA), unless stated otherwise. Active β-catenin antibody was purchased from EMD Millipore (Billerica, MA). Image J software was used for densitometric analysis of western blot images.

Statistical Analysis

Data presented in the form of bar/line graphs show mean ± SEM. Significant difference between two groups was determined using Student's t-test and between three or more groups using one-way analysis of variance (ANOVA) with Tukey's post-hoc test. Difference between groups was considered statistically significant at $P<0.05$.

3.4 RESULTS

Rapid inactivation of GSK3β associated with early and robust liver regeneration at lower dose of APAP, which was attenuated at higher dose

Our work in previous chapter established an incremental dose model to study mechanisms of liver regeneration after APAP-induced ALF. Two doses of APAP (300 mg/kg and 600 mg/kg) were administered in separate groups of mice. Lower dose caused extensive liver injury, but injury was followed by robust compensatory liver regeneration leading to recovery. In contrast, at higher dose, liver regeneration was lower and delayed leading to decreased survival. Interestingly, inactivation of GSK3β was higher in regenerating dose treated mice and positively correlated with compensatory liver regeneration after APAP overdose. In the current study, inactivation of GSK3β was examined after incremental doses of APAP (300 mg/kg and 600 mg/kg) in mice over an extended time-course up to 96 hr (Fig. 3.4.1A and B). Interestingly, rapid increase in phosphorylation at Ser 9 residue of GSK3β (which is known to cause inactivation of GSK3β) was observed at the earliest investigated time point of 3 hr and peaked at around 6 to 12 hr after treatment with lower dose of APAP. Phosphorylation of GSK3β was gradually decreased at later time and reached around basal levels at 96 hr. Some phosphorylation of GSK3β was also observed at 3 to 12 after higher dose of APAP, but remarkably lesser compared to lower dose after 3 hr. In fact, phosphorylation of GSK3β rapidly decreased to basal levels at 24 hr and remained at similar low levels at later time points up to 96 hr after higher dose of APAP (Fig. 3.4.1A and B). As reported in previous chapter, increase in expression of proliferating cell nuclear antigen

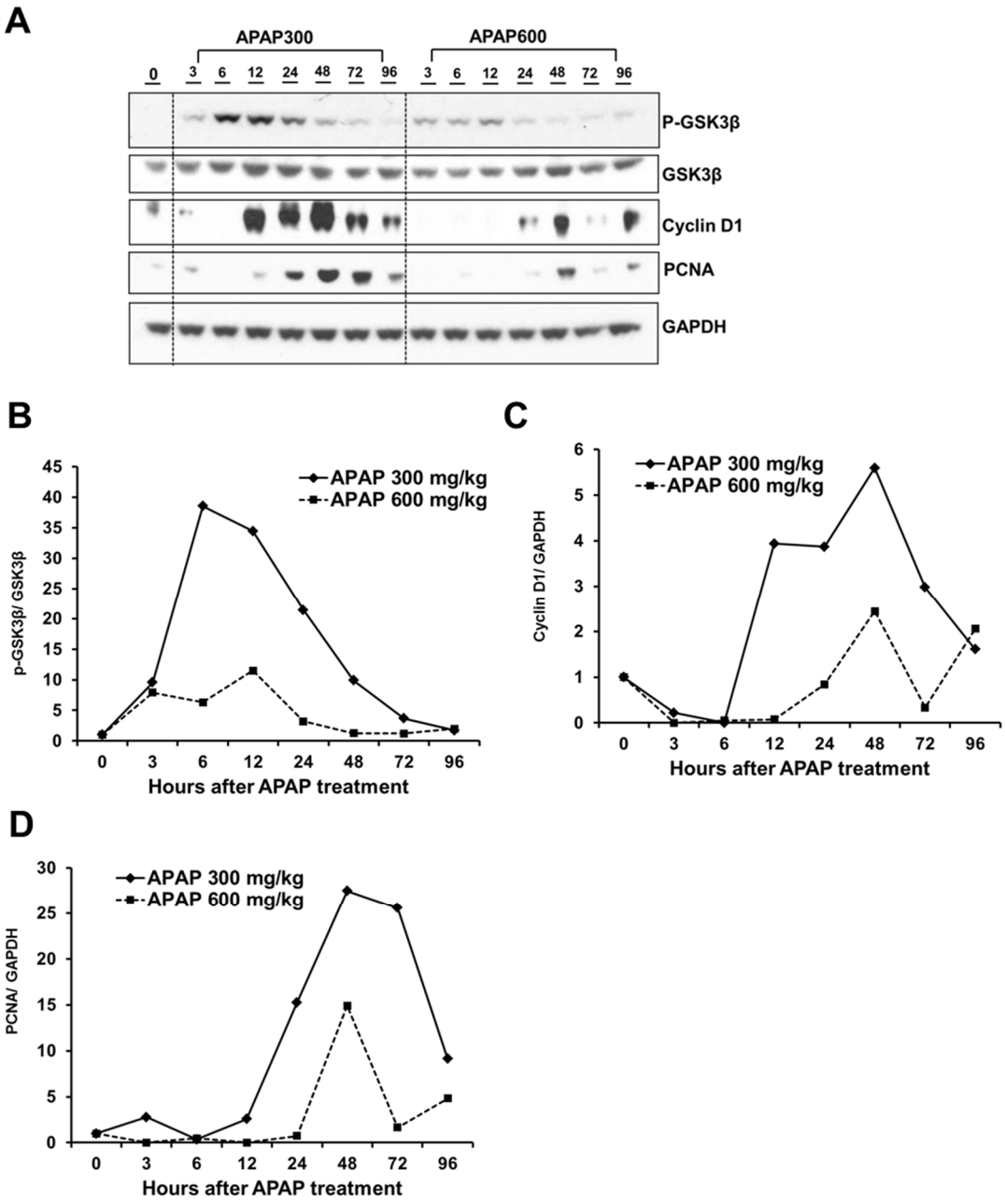

Figure 3.4.1. Rapid inactivation of GSK3β associated with early and robust liver regeneration at lower dose of APAP, which was attenuated at higher dose. (A) Western blot analysis of phospho-GSK3β (Ser9), GSK3β, Cyclin D1 and PCNA in total cell lysate of liver at various time points after administration of 300 mg/kg and 600 mg/kg APAP in mice (n=3-5). Densitometric analysis showing (B) GSK3β inactivation, (C) Cyclin D1 and (D) PCNA protein expression based on western blot images shown in (A).

(PCNA), which is a maker of cell proliferation, was remarkably lower and delayed at higher dose compared to lower dose (Fig. 3.4.1A and D). Similar pattern was observed for protein expression of core cell cycle protein, Cyclin D1, which governs entry into cell cycle (Fig. 3.4.1A and C). Thus, inactivation of GSK3β was found to be positively correlated with early initiation of liver regeneration after APAP overdose.

Pharmacological inhibition of GSK3 caused dose-dependent improvement in proliferation parameters without altering APAP hepatotoxicity

In the initial dose finding study, L803-mts, a highly selective GSK3 inhibitor (GSK3i), was administered 4 hr after treatment with high dose of APAP (600 mg/kg) in mice. L803-mts (N-myristol-GKEAPPAPPQS(p)P) is a substrate-competitive fatty acid-conjugated peptide inhibitor of GSK3. It contains a phosphorylated site that specifically mimics primed GSK3 substrate providing it selectivity. Selectivity is the major issue for protein kinase inhibitors which are mostly ATP-competitive (Plotkin, Kaidanovich et al. 2003). Further, fatty acid moiety of the inhibitor provides cell permeability and stability *in vivo* (Plotkin, Kaidanovich et al. 2003). All analysis was performed 24 hr after APAP treatment. Two doses of GSK3i (400 nmol and 800 nmol) were used based on previous *in vivo* studies in mice (Plotkin, Kaidanovich et al. 2003, Rao, Hao et al. 2007). APAP treatment caused significant liver necrosis in the centrilobular region and elevation of serum ALT levels (Fig 3.4.2A and B). APAP-mediated liver injury was not altered by treatment with any of the two doses of GSK3i as indicated by histopathological analysis of liver sections (Fig 3.4.2A) and serum ALT levels (Fig 3.4.2B). Next, Cyclin D1 and

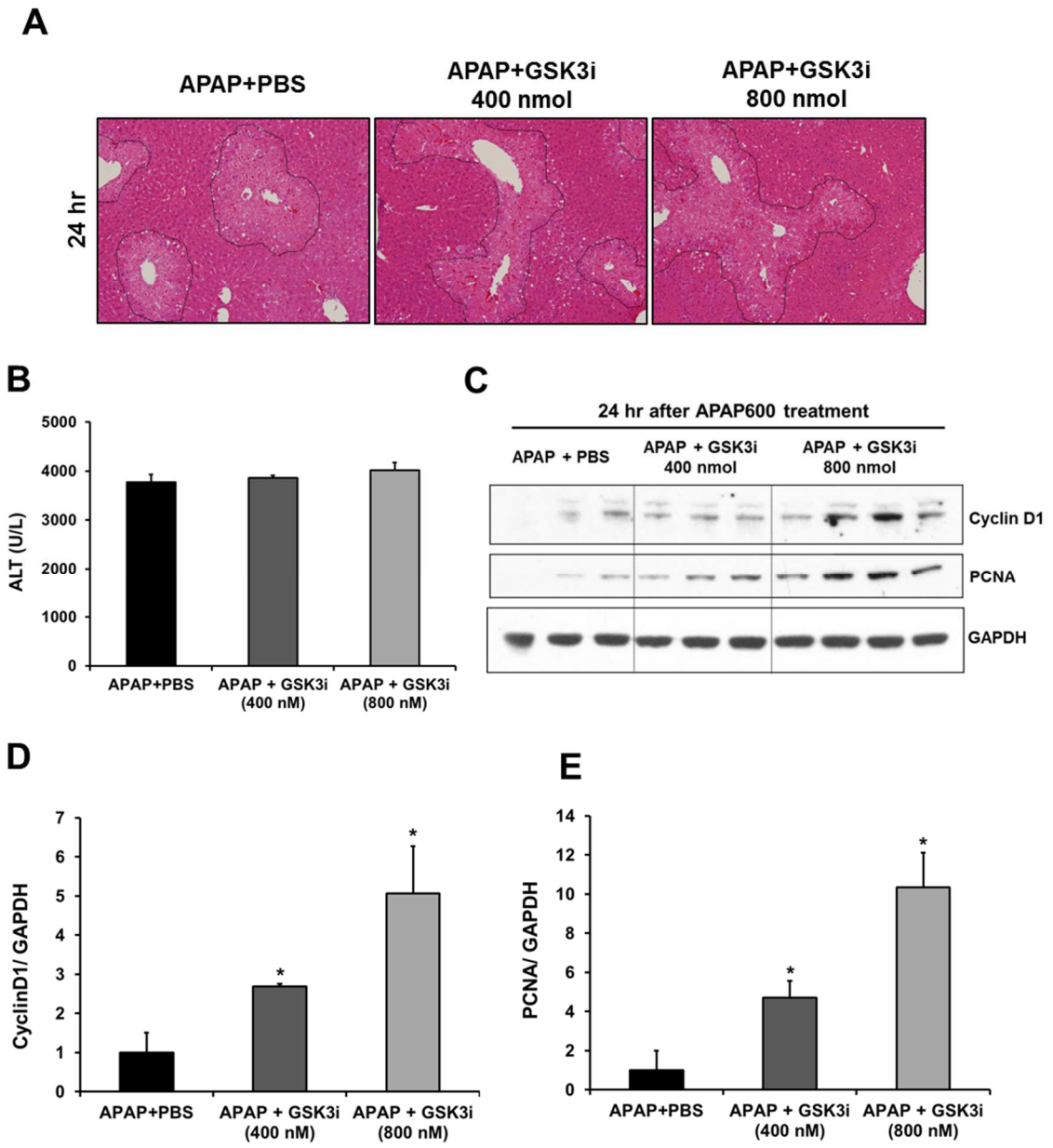

Figure 3.4.2. Pharmacological inhibition of GSK3 resulted in dose dependent improvement in liver regeneration parameters without alteration of APAP-induced hepatotoxicity. (A) Representative photomicrographs of H&E stained liver sections with necrotic area outlined; (B) Serum ALT levels; (C) Western blot analysis of Cyclin D1 and PCNA in liver lysate with their densitometric analysis shown in (D) and (E), respectively. All samples were collected at 24 hr after treatment with 600 mg/kg APAP. Vehicle (PBS) and L803 mts (400 and 800 nmol/mouse) were administered 4 hr after treatment with APAP (n=3-4). * indicate significant difference with respect to vehicle treated group at $p<0.05$.

PCNA protein expression levels were studied as a measure of liver regeneration using western blot analysis. Despite similar injury, GSK3i treatment caused significant increase in both CyclinD1 and PCNA protein expression at 24 hr after APAP treatment (Fig 3.4.2C-E). Further, increase in expression of these proteins was dose-dependently higher after treatment with 800 nmol compared to 400 nmol dose of GSK3i. 800 nmol dose of GSK3i was selected for following comprehensive time-course analysis of liver injury and regeneration.

Inhibition of GSK3 did not alter APAP-induced hepatotoxicity but improved survival

Next, 800 nmol of GSK3i was administered starting from 4 hr after treatment with 600 mg/kg APAP in mice. Liver injury was studied over a time-course of 18 to 72 hr to cover both peak toxicity and liver regeneration time-points. Liver injury was studied using histopathological analysis of H&E stained liver sections and serum ALT levels (Fig 3.4.3A-C). APAP caused extensive liver necrosis (around 50% percentage necrotic area) at 18 hr after APAP treatment (Fig 3.4.3A and B). Liver necrosis was sustained at all later time points up to 72 hr as reported in previous chapter for high dose of APAP treatment. Treatment with GSK3i did not significantly alter APAP-induced liver necrosis at any of the investigated time points (Fig 3.4.3A and B). Further, serum ALT analysis corroborated the histopathological results (Fig 3.4.3C). Some decrease in serum ALT levels with time was observed in both the groups despite sustained necrotic areas. This is expected due to limited half-life of ALT in serum and is consistent with our results in previous chapter. Very interestingly, despite no alteration in development of

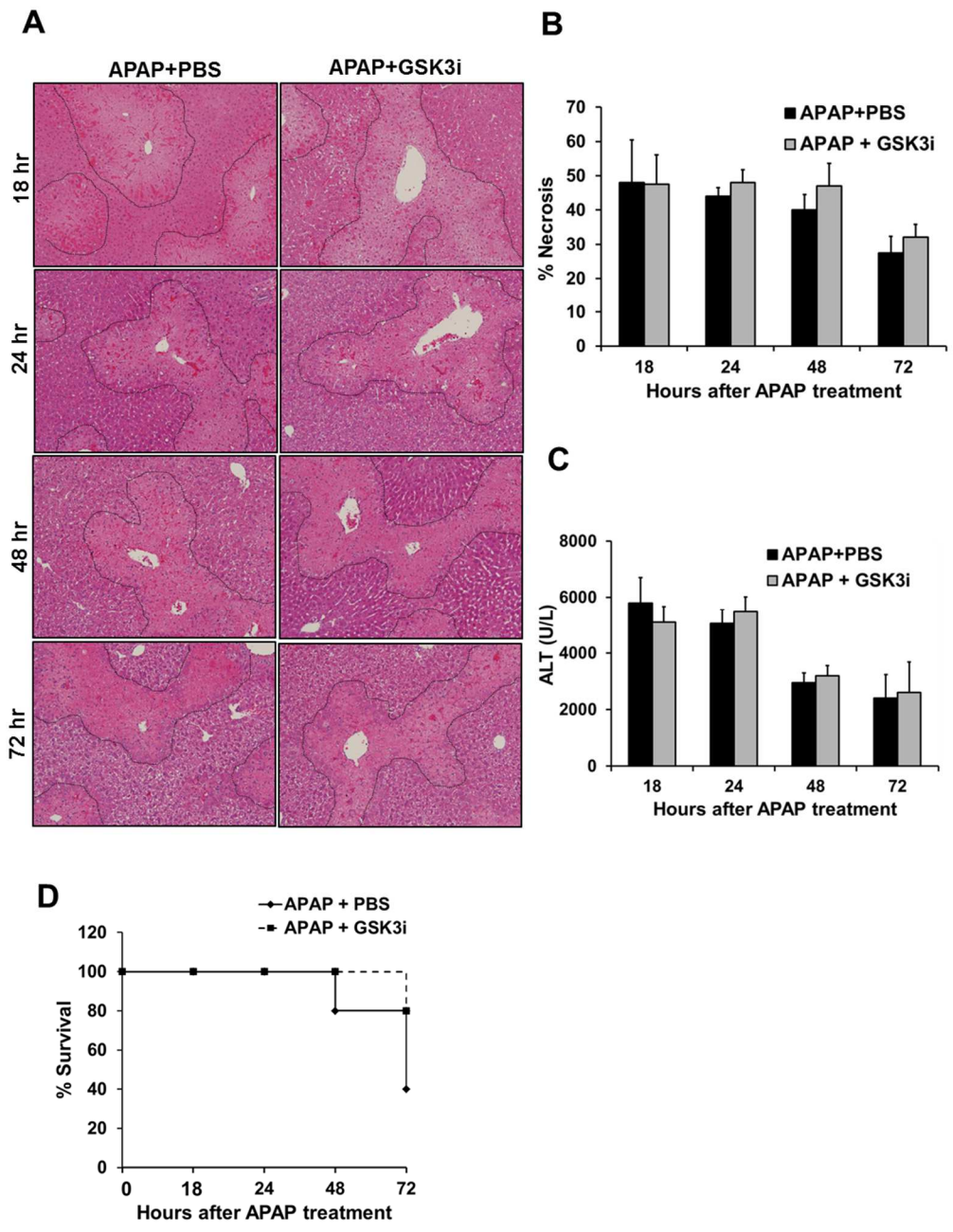

Figure 3.4.3. Inhibition of GSK3 did not alter APAP-induced hepatotoxicity but improved survival. (A) Representative photomicrographs of H&E stained liver sections with necrotic area outlined, (B) percentage necrosis area based on H&E stained liver sections, (C) serum ALT levels (n=4-5), and (D) survival analysis of mice treated with 600 mg/kg APAP followed by treatment with L803-mts (800 nmol/mouse) or PBS (n=4-12). L803-mts or PBS was administered 4 hr post-APAP followed by every 24 hr until sample collection. All samples were collected and survival was recorded at 18, 24, 48 and 72 hr after APAP treatment.

APAP-induced liver injury, GSK3i treatment improved survival (Fig. 3.4.3D). No mortality was observed in any of the two groups up to 24 hr after APAP treatment. 20% and 60% mortality was observed in APAP-alone treatment group at 48 and 72 hr, respectively. In contrast, no mortality was observed in GSK3i-treated group at 48 hr and only 20% mortality was observed at 72 hr (Fig. 3.4.3D).

Inhibition of GSK3 caused early hepatocyte proliferation and liver regeneration after APAP overdose

Next, we investigated effect of GSK3 inhibition on compensatory liver regeneration after APAP-induced liver injury. Liver regeneration was studied by immunohistochemical analysis of PCNA over the time-course of 18 to 72 hr after APAP administration with or without treatment of GSK3i (Fig. 3.4.4A and B). Very few PCNA-positive cells were observed at 18 and 24 hr after 600 mg/kg APAP treatment consistent with our results in previous chapter. Significant number of positive cells was observed only at 48 and 72 hr in APAP-alone treated group. Interestingly, GSK3i inhibition caused early entry into cell cycle with PCNA positive cells observed surrounding the necrotic zones even at 18 hr. Drastic increase in number of PCNA-positive cells was observed at 24 hr in GSK3i-treated group. However, number of PCNA-positive cells were comparable to APAP-alone treated group at 48 and 72 hr (Fig. 3.4.4A and B). These data were corroborated with protein expression of PCNA as analyzed using western blot (Fig. 3.4.5A and C; Fig. 3.4.6A-C). Protein expression of PCNA was increased starting from 24 hr in GSK3i-treated group, while PCNA expression was increased only after 48 hr in APAP-alone treated group. Significantly higher number of PCNA-positive cells and higher PCNA

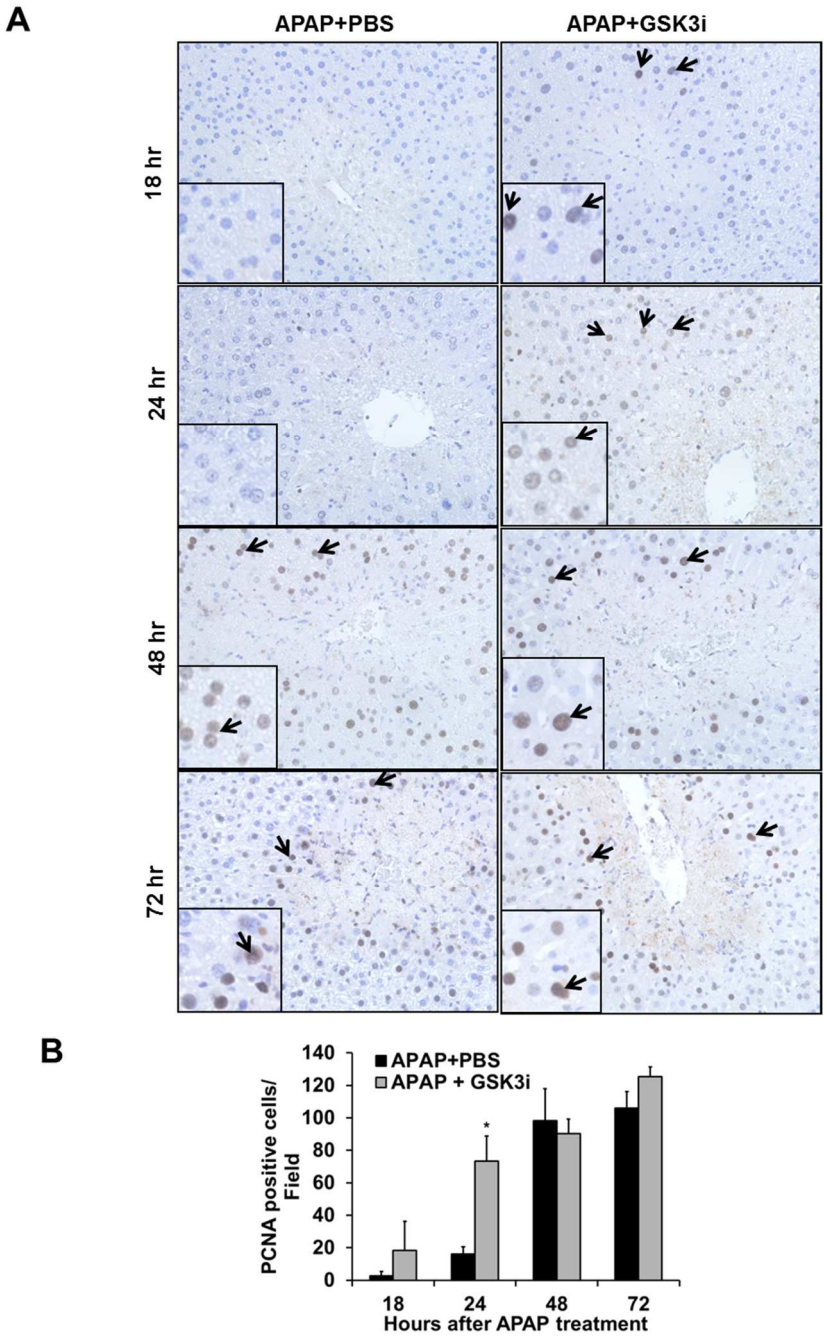

Figure 3.4.4. Inhibition of GSK3 caused early hepatocyte proliferation and liver regeneration after APAP overdose. (A) Representative photomicrographs of PCNA stained liver sections with arrows indicating cells with nuclear PCNA staining (brown). (B) Total number of PCNA-positive cell per high power field (x40). Liver section were obtained from mice (n=4-5) treated with 600 mg/kg APAP followed by treatment with L803-mts (800 nmol/mouse) or PBS. L803-mts or PBS was administered 4 hr post-APAP followed by every 24 hr until sample collection. All samples were collected at 18, 24, 48 and 72 hr after APAP treatment. * indicate significant difference between groups at $p<0.05$.

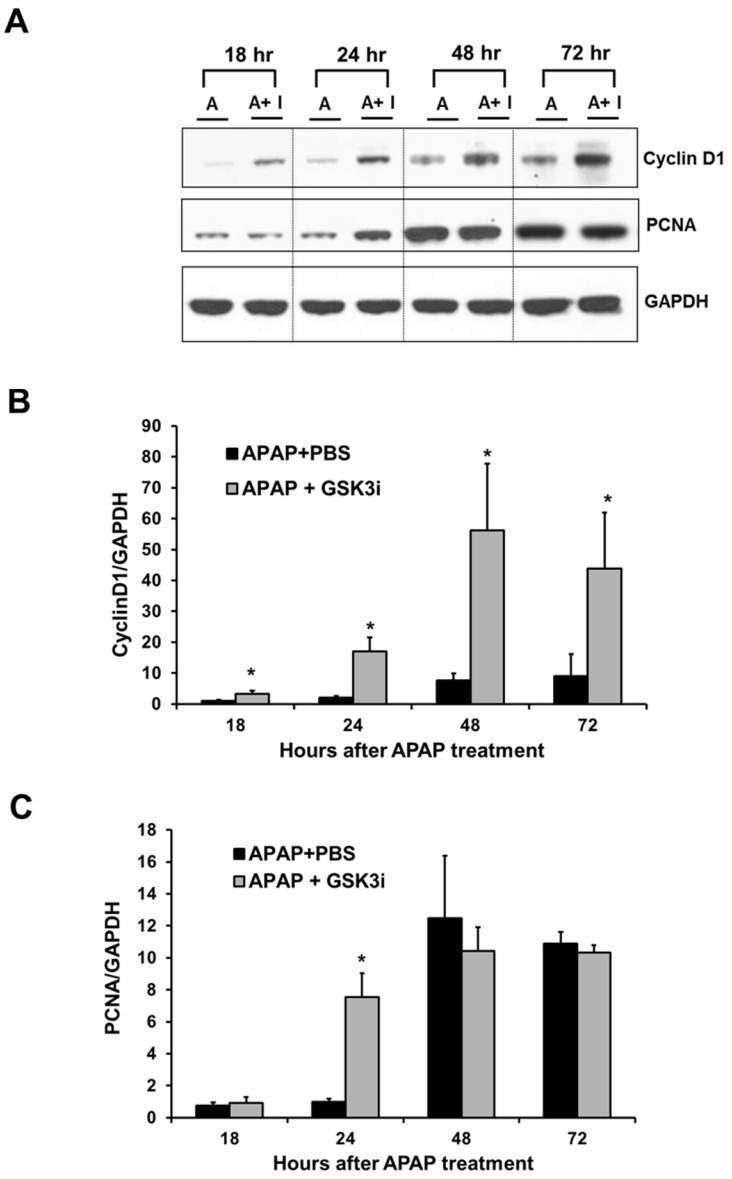

Figure 3.4.5. GSK3 inhibition caused early expression of CyclinD1 and PCNA after APAP overdose. (A) Western blot analysis of Cyclin D1 and PCNA in pooled total cell lysate with their densitometry based on analysis of individual samples shown in (B) and (C), respectively. All liver samples were obtained from mice (n=4-5) treated with 600 mg/kg APAP followed by treatment with L803-mts (800 nmol/mouse) or PBS. L803-mts or PBS was administered 4 hr post-APAP followed by every 24 hr until sample collection. All samples were collected at 18, 24, 48 and 72 hr after APAP treatment. * indicate significant difference between groups at $p<0.05$.

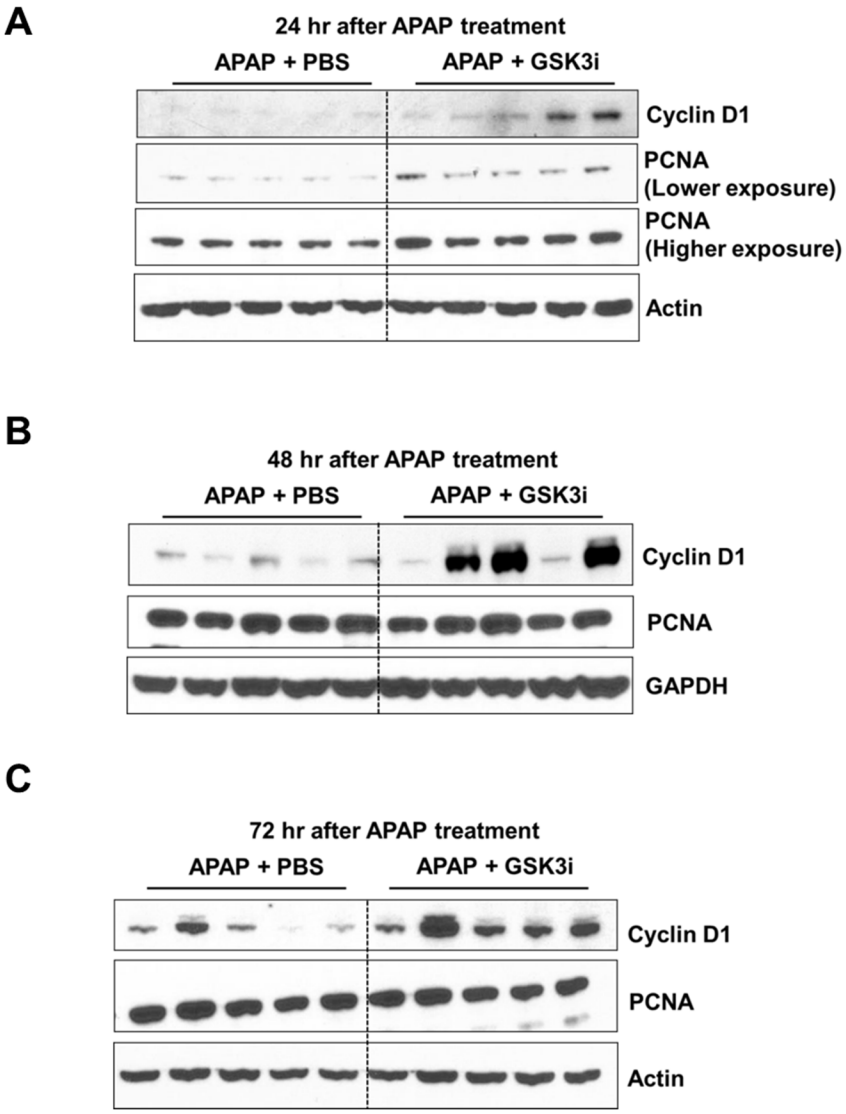

Figure 3.4.6. Western blot analysis of Cyclin D1 and PCNA in total cell lysate at (A) 24 hr, (B) 48 hr and (C) 72 hr after APAP treatment. All liver samples were obtained from mice (n=5) treated with 600 mg/kg APAP followed by treatment with L803-mts (800 nmol/mouse) or PBS. L803-mts or PBS was administered 4 hr post-APAP followed by every 24 hr until sample collection.

protein expression was observed at 24 hr after APAP administration in GSK3i-treated group. These data demonstrated that inhibition of GSK3i resulted in early liver regeneration after APAP-induced liver injury.

GSK3 inhibition caused early and sustained expression of CyclinD1 after APAP overdose

Next, we sought to investigate mechanism of early increase in liver regeneration after GSK3 inhibition. Induction of Cyclin D1 is a critical limiting factor that controls entry into cell cycle and commitment to cell cycle progression in hepatocytes (Fausto 2000). Cyclin D1 protein expression was 3-fold higher in GSK3i-treated group at 18 hr post-APAP and 8-fold higher at 24 hr compared to APAP-alone treated group (Fig 3.4.5A and B). This correlated with early hepatocytes proliferation after GSK3 inhibition. Further, Cyclin D1 expression increased with time in GSK3i-treated group. Increase in Cyclin D1 expression was also observed in APAP-alone treated group but at delayed time point of 48 and 72 hr. However, Cyclin D1 expression consistently remained remarkably higher in GSK3i-treated group at all the investigated time points compared to APAP-alone treated group (Fig 3.4.5A and B; Fig. 3.4.6A-C).

Inhibition of GSK3 resulted in increased activation of β-catenin after APAP overdose

Finally, we investigated the signaling mechanism(s) involved in early increase in cyclin D1 expression and increase in liver regeneration after GSK3 inhibition. GSK3 is known to negatively regulate β-catenin signaling (Kaidanovich-Beilin and Woodgett 2011) and

we reported in previous chapter that stimulation of β-catenin signaling can improve liver regeneration after APAP-induced liver injury. Therefore, we investigated effect on β-catenin signaling in our study. We analyzed the earliest time point of 18 hr that preceded robust increase in liver regeneration response in GSK3i-treated group. GSK3 causes phosphorylation of β-catenin at Thr41, Ser33 and Ser37 ultimately leading to proteosomal-mediated degradation of β-catenin (Kaidanovich-Beilin and Woodgett 2011). Phosphorylation of β-catenin was found to be significantly decreased in GSK3i-treated group, 18 hr after APAP administration, as studied using antibody that specifically recognizes Thr41, Ser33 or Ser37 phosphorylation of β-catenin (Fig. 3.4.7A and B). This was corroborated with 5-fold increase in active β-catenin levels (dephosphorylated form) in GSK3i-treated group (Fig. 3.4.7A and C). Total β-catenin levels were not altered by GSK3i treatment (Fig. 3.4.7A). Stable (unphosphorylated) β-catenin translocates to nucleus and induces its target genes, which includes cell proliferation genes such as Cyclin D1. Increase in β-catenin signaling was consistent with observed early increase in Cyclin D1 levels at 18 hr after APAP treatment in GSK3i group (Fig. 3.4.5A and B, 3.4.7A and D). Cyclin D1 binds with CDK4 and causes its activation, which then phosphorylates Rb protein, ultimately leading to induction of several key cell cycle genes (Fausto 2000). Further, we observed increased phosphorylation of Rb protein in GSK3i group at 18 hr after APAP treatment corresponding with increased Cyclin D1 expression (Fig. 3.4.7A and E). Thus our results demonstrated that early activation of β-catenin after GSK3i inhibition might be involved in early entry into cell cycle and accelerated liver regeneration after APAP-induced liver injury.

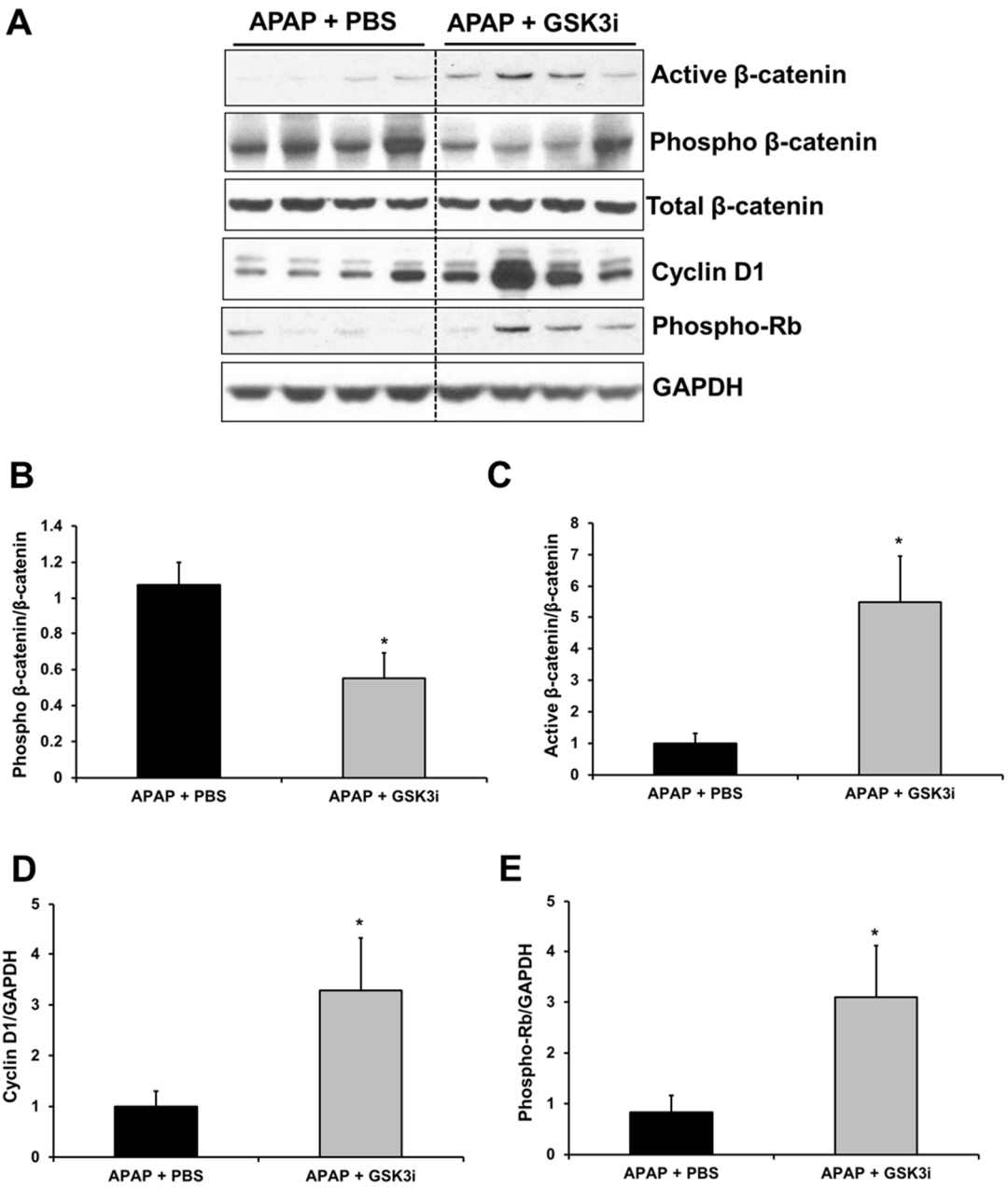

Figure 3.4.7. Inhibition of GSK3 resulted in increased activation of β-catenin after APAP overdose. (A) Western blot analysis of active β-catenin, phospho β-catenin (Thr41/Ser33/Ser37), total β-catenin, Cyclin D1 and phospho-Rb in total cell lysate with their densitometric analysis shown in (C), (B), (D) and (E), respectively. All liver samples were obtained from mice (n=4) treated with 600 mg/kg APAP followed by treatment with L803-mts (800 nmol/mouse) or PBS. L803-mts or PBS was administered 4 hr post-APAP and samples were collected at 18 hr after APAP treatment. * indicate significant difference between groups at $p<0.05$.

3.5 DISCUSSION

Although mechanisms of APAP hepatotoxicity have been extensively studied leading to considerable progress in our knowledge, mechanisms of compensatory liver regeneration that follows liver injury after APAP overdose have just begun to be explored, which have great potential to be utilized to find novel therapeutic targets. Here we investigated role of GSK3 in liver regeneration after APAP-induce hepatotoxicity, which is currently unknown.

GSK3β was rapidly inactivated preceding robust liver regeneration response after moderate APAP overdose. Inactivation of GSK3β was greatly diminished at higher dose of APAP, where liver regeneration was remarkably attenuated and delayed. This positive correlation of GSK3β inactivation with early liver regeneration suggested a possible role of GSK3 inactivation in liver regeneration after APAP-induced hepatotoxicity. Consistent with this hypothesis, pharmacological inhibition of GSK3 using selective inhibitor of GSK3 caused dose-dependent early hepatocyte proliferation and liver regeneration after APAP-induced hepatotoxicity. These data suggest that GSK3 may inhibit liver regeneration after APAP overdose and an inability to cause timely inactivation of GSK3 may be responsible for delayed liver regeneration at higher dose of APAP. The pro-proliferative role of GSK3 inhibition is consistent with previous report where inhibitor of GSK3 induced in-vitro expansion of hepatocytes (Ito, Kamiya et al. 2012). However, our finding is contradictory to few previous reports where inhibition or siRNA mediated downregulation of GSK3 resulted in impaired hepatocyte proliferation after PH (Chen, Yang et al. 2007, Jin, Wang et al. 2009, Sekiya and Suzuki

2011). These discrepancies in findings may be due to inherent differences in models used to study of liver regeneration. APAP overdose has significant mechanistic difference from PH including presence of significant cell death, liver injury, oxidative stress, and asynchronous nature of cell cycle. Our study highlights the notion that same signaling pathway may have different and sometimes opposite role in different models of regeneration. These studies also underscore the importance of investigating mechanisms of liver regeneration after APAP overdose separately from PH to determine the inherent model associated differences.

We further investigated the potential mechanisms underlying role of GSK3 inactivation in causing early initiation of liver regeneration after APAP-induced hepatotoxicity. Inhibition of GSK3 caused consistent increase in cyclin D1 expression. Thus, early initiation of liver regeneration after GSK inhibition was most possibly due to early induction of cyclin D1 (which governs entry into cell cycle) and subsequent phosphorylation of Rb protein. Further, our results demonstrate that early activation of β-catenin after GSK3 inhibition might be involved in early induction in cyclin D1 and accelerate liver regeneration after APAP-induced liver injury. This corroborated our findings in previous chapter that overexpression of stable form of β-catenin signaling in mice increased cyclin D1 expression and improved liver regeneration; and β-catenin KO mice showed decreased liver regeneration after APAP-induced liver injury (Apte, Singh et al. 2009). Similar to our findings in APAP model, overexpression of β-catenin in mice resulted in early initiation of liver regeneration in PH model with increased expression of cyclin D1 without altering peak liver regeneration (Nejak-Bowen, Thompson et al. 2010). Further, liver-specific β-catenin deletion in mice caused delayed liver regeneration after

PH, which was correlated with decreased cyclin D1 induction (Tan, Behari et al. 2006, Sekine, Gutierrez et al. 2007). Apart from negative regulation of β-catenin, GSK3 can directly regulate several other cell proliferation mediators including cyclin D1, c-Myc, c-Jun and eIF2B (Kaidanovich-Beilin and Woodgett 2011, Sutherland 2011). Thus, GSK3 could also potentially alter liver regeneration after APAP overdose in β-catenin-independent manner, which remained to be investigated.

Shinohara et al. recently reported an attenuation of APAP hepatotoxicity upon antisense oligonucleotides mediated silencing of GSK3β (Shinohara, Ybanez et al. 2010). This was attributed to role of GSK3β in liver injury initiation through JNK activation and Mcl-1 degradation. In our study, treatment with GSK3i did not alter APAP-induced hepatotoxicity at any of the investigated time points. Further, we did not observe any alteration of APAP-mediated JNK activation by GSKi treatment (data not shown). These discrepancies are due to differences in experimental design. In our study, GSK3i was administered 4 hr after APAP such that injury initiation events have already taken place, while pretreatment strategy was utilized in the previous study. In fact, we purposefully used post-treatment strategy such that APAP injury initiation was not interfered. This is because, any alteration in injury can have secondary effects of liver regeneration and the purpose of the study was to study direct role of GSK3 in liver regeneration. Thus our study indicated that effect of accelerated liver regeneration is directly mediated by GSK3 inhibition and is not secondary to altered injury.

Very interestingly, survival was improved after GSK3 inhibition without any alteration of injury. It should be noted that all the existing clinically used markers for prediction of

outcome in ALF patients are solely based on injury parameters and are far from reliable in predicting outcome (Lee 2012). Thus, it is not surprising that survival was not correlated with liver injury in our model. Recent studies have demonstrated critical role of timely liver regeneration in determining final outcome after APAP overdose (Hu and Colletti 2008, Apte, Singh et al. 2009, Donahower, McCullough et al. 2010). Further, markers of liver regeneration such as α-Feto protein were reported to correlate with final outcome in APAP-induced ALF patients (Schmidt and Dalhoff 2005). On similar line, in our study early liver regeneration response after GSK3 inhibition was associated with increased survival. Further, this is corroborated by our previous finding where β-catenin activation was correlated with higher spontaneous liver regeneration, avoiding need for liver transplantation in ALF patients (Apte, Singh et al. 2009). In spite of these correlations, further studies are required to establish causal role of early liver regeneration in improved survival observed in our study.

In the past, the primary focus of research in the area of APAP-inflicted liver pathophysiology has been mechanisms of initiation APAP hepatotoxicity and targeting liver injury for development of therapeutic strategies. However, many of the APAP overdose patients typically seek medical attention late after APAP overdose such that injury is already established (Larson 2007). Even after decades of research, NAC is still the only standard therapy for APAP overdose which is effective only at very early stage (Bernal, Lee et al. 2015). In mice, NAC does not provide protection when administered 4 hr post-APAP (James, McCullough et al. 2003). Liver regeneration is a parameter that can be potentially modulated even at the late stage in the APAP-induced ALF and thus is an attractive therapeutic target. In our study, initiating GSK3i treatment 4 hr after

APAP overdose in mice showed indication of improved liver regeneration parameters and survival. Time window of therapeutic effectiveness of GSK3i is expected to be much more in humans considering that the pathogenesis of APAP hepatotoxicity is generally delayed in humans compared to mice (Xie, McGill et al. 2014). Our study has highlighted the potential therapeutic value of improving liver regeneration even after a delayed intervention following APAP overdose. Thus, future investigations on similar lines are warranted to identify novel therapeutic targets to stimulate live regeneration after APAP-induced hepatotoxicity, which are currently underexplored.

In conclusion, our study revealed novel inhibitory role of GSK3 in timely liver regeneration response after APAP-induced hepatotoxicity. Inhibition of GSK3 accelerated liver regeneration after APAP overdose associated with early induction of Cyclin D1, increased β-catenin signaling and improved survival. Thus, our study suggested inhibition of GSK3 as a potential therapeutic strategy to improve liver regeneration after APAP-induced ALF.

CHAPTER IV: ROLE OF EPIDERMAL GROWTH FACTOR RECEPTOR IN LIVER INJURY AND REGENERATION AFTER ACETAMINOPHEN OVERDOSE IN MICE

4.1 ABSTRACT

Epidermal growth factor receptor (EGFR) plays a crucial role in hepatocyte proliferation. Its role in acetaminophen (APAP)-mediated hepatotoxicity and subsequent liver regeneration is completely unknown. We studied role of EGFR after APAP overdose using pharmacological inhibition strategy. Rapid, sustained and dose-dependent activation of EGFR was noted after APAP treatment in mice, which was triggered by glutathione depletion. EGFR activation was also observed in primary human hepatocytes after APAP treatment, preceding elevation of toxicity markers. Treatment of mice with an EGFR inhibitor (EGFRi), Canertinib, 1 hr post-APAP resulted in robust inhibition of EGFR activation and a striking reduction in APAP-induced liver injury. Metabolic activation of APAP, formation of APAP-protein adducts, APAP-mediated JNK-activation and its mitochondrial translocation were not altered by EGFRi. However, EGFRi treatment prevented APAP-mediated mitochondrial dysfunction. Finally, delayed treatment with EGFRi, 12 hr post-APAP, did not alter peak injury but caused remarkable impairment of liver regeneration resulting in sustained injury and decreased survival after APAP overdose in mice. Impairment of liver regeneration was due to complete inhibition of cyclinD1 induction, subsequent phosphorylation of retinoblastoma protein and cell cycle arrest. In conclusion, our study has revealed a novel role of EGFR both in initiation of APAP injury and in stimulation of subsequent compensatory liver regeneration after APAP overdose.

4.2 INTRODUCTION

Epidermal growth factor receptor (EGFR) is a prototypical tyrosine kinase receptor of the ErbB family, which is highly expressed in liver and known to be involved in hepatocytes proliferation, liver regeneration and hepatocellular carcinogenesis (Michalopoulos and DeFrances 1997, Carver, Stevenson et al. 2002, Michalopoulos and Khan 2005). EGFR can be activated by variety of extracellular ligands, including EGF, transforming growth factor alpha (TGF-α), heparin-binding EGF (HB-EGF) and amphiregulin (Normanno, De Luca et al. 2006). Ligand binding leads to dimerization of EGFR and auto-phosphorylation of tyrosine residues in its cytoplasmic domain, which then act as a hub for numerous cell signaling pathways. Apart from hepatocyte growth factor (HGF), EGFR ligands are the only known direct mitogens which can cause hepatocyte proliferation in serum free medium, emphasizing an important role of EGFR in hepatocyte proliferation (Michalopoulos 2007). *In vivo* administration of EGFR ligands such as EGF and TGF-α also lead to hepatocyte proliferation along with increased liver size (Bucher, Patel et al. 1977). EGFR is activated very rapidly after 2/3rd partial hepatectomy (PH), and is known to play important role in promoting timely liver regeneration (Michalopoulos and Khan 2005, Michalopoulos 2007, Fausto, Campbell et al. 2012). Genetic deletion of EGFR in mice liver or shRNA-mediated downregulation of EGFR in rats resulted in delayed liver regeneration (Natarajan, Wagner et al. 2007, Paranjpe, Bowen et al. 2010). Further, mice deficient in EGFR ligands, amphiregulin and HB-EGF, displayed deficient liver regeneration and overexpression of HB-EGF increased liver regeneration (Michalopoulos and Khan 2005, Michalopoulos 2007, Fausto, Campbell et al. 2012).

Apart from the well-known role of EGFR in cell proliferation, its role in cell death signaling has also been reported, but remained largely ignored (Arany 2008, Reinehr and Haussinger 2009). Other than ligand-dependent activation of EGFR, ligand-independent activation of EGFR by hydrophobic bile acids, CD95 ligand and hyperosmolarity has been reported in hepatocytes as well as hepatic stellate cells (HSCs), which was found to be involved in mediating cell death signaling (Reinehr, Graf et al. 2003, Reinehr, Schliess et al. 2003, Reinehr, Becker et al. 2004, Reinehr, Becker et al. 2005, Reinehr, Becker et al. 2005, Reinehr and Haussinger 2009, Sommerfeld, Reinehr et al. 2009, Reinehr and Haussinger 2012). An unexpected role of EGFR activation in cell death has also been reported in kidney (Arany 2008). Nephrotoxic insults such as cisplatin and cyclosporine A treatment were shown to activate EGFR in proximal tubular cells, which was involved in toxicity rather than survival (Arany, Megyesi et al. 2004, Sarro, Tornavaca et al. 2008).

Acetaminophen (APAP) is the most commonly used analgesic worldwide and its overdose is the major cause of acute liver failure (ALF) in the western world, accounting for almost 50% of all the ALF cases. Current treatment options after APAP overdose are extremely limited with liver transplantation as the final resort (Bernal, Lee et al. 2015). APAP toxicity is initiated by metabolism to a reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which then binds to cellular proteins (particularly mitochondrial proteins). This mitochondrial distress is exacerbated by a plethora of intracellular signaling events resulting in massive mitochondrial oxidative damage/dysfunction and liver cell necrosis (Jaeschke, McGill et al. 2012). Extensive research in the last few decades has revealed that APAP hepatotoxicity involves highly

sophisticated pathways, but the exact intracellular signaling events are still not completely known (Jaeschke, McGill et al. 2012). Liver toxicity after APAP overdose is also followed by compensatory liver regeneration, which is a critical determinant of final recovery (Bhushan, Walesky et al. 2014). Identifying targets to stimulate liver regeneration holds great potential to develop novel therapeutic strategies (Hu and Colletti 2008, Donahower, McCullough et al. 2010, Bhushan, Walesky et al. 2014). Unfortunately, mechanisms of liver regeneration are mostly studied in PH model, which is considerably different from the APAP hepatotoxicity model.

The role of EGFR in APAP-mediated acute liver injury and subsequent liver regeneration is completely unknown, which was investigated in the current study. Our work in chapter-II established an incremental dose model in mice to study mechanisms of liver regeneration after APAP-induced ALF. Paradoxically, dose-dependent activation of EGFR was observed after APAP overdose, such that activation of EGFR was remarkably greater at higher doses of APAP, where liver regeneration was inhibited. Here we report that early inhibition of EGFR activation by pharmacological intervention in mice remarkably attenuate APAP hepatotoxicity, delayed inhibition of EGFR activation lead to impaired compensatory liver regeneration, suggesting a dual role of EGFR in both injury initiation and subsequent liver regeneration after APAP overdose.

4.3 MATERIALS AND METHODS

Animals, treatments and tissue harvesting

Eight weeks old, C57BL/6J mice were purchased from Jackson Laboratories and used in all the studies. All animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facilities at the University of Kansas Medical Center under a standard 12-hr light/dark cycle with access to chow and water *ad libitum*. All studies were approved by The Institutional Animal Care and Use Committee at the University of Kansas Medical Center. For all APAP experiments, mice were fasted overnight and APAP (Sigma, St. Louis, MO) was dissolved in warm 0.9% saline and administered intraperitoneally (i.p.) next morning. For study with incremental doses of APAP, mice (n = 3 to 7) were treated with either 300 or 600 mg/kg APAP, and sacrificed at 0, 0.25, 1, 3, 6, 12, 24, 72 and 96 hr following APAP treatment. For studies with EGFR inhibitor (EGFRi), canertinib, dihydrochloride salt (80 mg/kg, i.p.) (LC Laboratories, Woburn, MA) was dissolved in warm phosphate-buffered saline (PBS). EGFRi or PBS (vehicle) was administered 1 or 12 hr after 300 mg/kg APAP and mice (n=3-12) were sacrificed at 3, 6, 12 and 24 hr or 24 and 48 hr after treatment with APAP, respectively (represented by scheme shown in Fig 4.4.2A and 4.4.8A). For mitochondrial respiration analysis, EGFRi or PBS (vehicle) was administered 2 hr before or 1 hr after 300 mg/kg APAP and mice (n=3) were sacrificed at 1.5 hr or 3 hr after treatment with APAP, respectively (represented by scheme shown in Fig 4.4.5C and F). For N-acetylcysteine (NAC) experiments, NAC (500 mg/kg, dissolved in PBS), EGFRi (80 mg/kg, i.p.) or PBS (vehicle) was administered 1.5 hr after APAP (300 mg/kg, i.p.) and mice (n=3) were sacrificed 6 hr after treatment with APAP. For

glutathione depletion study, phorone (200 mg/kg, i.p., dissolved in corn oil) was administrated in mice (n=5) without fasting and mice were sacrificed 2 hr later. Euthanasia was performed by cervical dislocation under isoflurane anesthesia, and blood and livers were collected. Serum samples were obtained from the blood and used for further analysis. Liver sections were prepared for histological analysis as described previously (Wolfe, Thomas et al. 2011).

Primary human hepatocytes (PHH) experiments

Primary human hepatocytes (PHH) were isolated as described previously (Xie, McGill et al. 2014), without any modification, after informed consent of patient and study approval by University of Kansas Medical Center Institutional Review Board. Experiments were repeated 4 times, independently, with hepatocytes obtained from 4 liver donors. Cells were washed with sterile PBS, approximately 3 hr after seeding on collagen-coated dishes and treated with 10 mM APAP dissolved in serum free Hepatocyte Maintenance Medium (Lonza, Walkersvill, MD), supplemented as described previously (Xie, McGill et al. 2014). Cells were harvested at 0, 3, 6, 15, 24, 36 and 48 hr after treatment with APAP and used for further downstream analysis as described previously (Xie, McGill et al. 2014).

Histological analysis and serum ALT measurement

Paraffin-embedded liver sections (4 μm thick) were used for H&E staining and scored for percentage necrotic area. Serum alanine aminotransferase (ALT) was measured using the Infinity ALT kit (ThermoFisher Scientific, Pittsburgh, PA), according to the manufacturer's protocol.

Immunohistochemistry

Paraffin-embedded liver sections (4 μm thick) were used for immunohistochemical detection. PCNA and nitrotyrosine staining were performed for cell proliferation analysis and for assessment of nitrotyrosine (NT) protein adducts, respectively, as described before (Wolfe, Thomas et al. 2011). Anti-PCNA antibody was purchased from Cell Signaling Technologies (Danvers, MA) and anti-nitrotyrosine antibody was purchased from Life Technologies (Grand Island, NY). Biotinylated secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA).

Isolation of subcellular fractions, protein isolation and western blotting

Total cell lysate was prepared from frozen liver tissues as described previously (Wolfe, Thomas et al. 2011). Mitochondria and cytosolic fractions were prepared from freshly isolated liver using differential centrifugation as described previously without any modification (Xie, Williams et al. 2013). Protein estimation and western blot analysis was performed using individual or pooled samples of protein extracts as previously described in detail (Wolfe, Thomas et al. 2011). All primary and secondary antibodies were obtained from Cell Signaling Technologies (Danvers, MA) unless stated otherwise. Anti-nitrotyrosine antibody was purchased from Upstate (Lake Placid, NY). Anti-APAP-protein adduct antibody was a kind gift from Dr. Lance Pohl, NHIBL, NIH. Image J software was used for densitometric analysis of western blot images.

Isolation of mitochondria from mouse liver and analysis of mitochondrial respiration

Mitochondria were isolated from freshly obtained mice livers by differential centrifugation method. All the steps of the preparation were performed on ice. Briefly, the liver was rinsed 2X with mitochondria isolation buffer (MSHE+BSA: 70 mM sucrose, 210 mM mannitol, 5 mM HEPES, 1 mM EGTA and 0.5% (w/v) fatty acid-free BSA; pH 7.2) and minced using dounce homogenizer in ~10 volumes of MSHE+BSA. Homogenate was centrifuged twice at 1000 g for 10 min at 4°C. The supernatant was passed through 2 layers of cheesecloth to remove fat/lipid layer. The supernatant was centrifuged at 8000 g for 10 min at 4°C. The pellet was washed once with MSHE+BSA and resuspended in a minimal volume of MSHE+BSA. Total protein (mg/ml) was determined using BCA protein assay.

All mitochondrial respiration assays were performed using an XF24–3 Extracellular Flux Analyzer by Seahorse Bioscience (North Billerica, MA). The XF24 sensor cartridge was hydrated with 1 ml calibration buffer per well overnight at 37°C. Isolated liver mitochondria were first diluted ten times in cold Mitochondrial assay solution (1X MAS: 70 mM sucrose, 220 mM mannitol, 10 mM KH2PO4, 5 mM MgCl2, 2 mM HEPES, 1 mM EGTA and 0.2% (w/v) fatty acid-free BSA, pH 7.2 at 37°C) with substrate (10 mM succinate + 2 μM rotenone), then subsequently diluted to 2 mg/ml and 50 μl of this mitochondrial suspension was plated to each well (except for background correction wells) while the plate was on ice. The plate was transferred to a centrifuge equipped with a swinging bucket and spun at 2000 g for 20 minutes at 4°C. After centrifugation, 450 μl of 1X MAS + substrate (at room temperature) was added to each well. The

mitochondria were viewed briefly under a microscope to ensure consistent adherence to the well, then placed at 37°C for 10 minutes to allow the plate to warm. The sensor cartridge was loaded with port A, 40 mM ADP (4 mM final); port B, 25 µg/ml oligomycin (2.5 µg/ml final); port C, 40 µM FCCP (4 µM final); and port D, 40 µM antimycin A (4 µM final) to measure the bioenergetic profile. The plate was then transferred to the XF24 instrument and the respiration was sequentially measured in a coupled state with substrate present (basal respiration), followed by State 3 (phosphorylating respiration, in the presence of ADP and substrate), State 4o (Leak state induced with the addition of oligomycin - inhibitor of ATP synthase), and then maximal uncoupler (FCCP)-stimulated respiration (State 3u). At the end, antimycin A (complex III inhibitor) is added to inhibit mitochondrial respiration completely.

Glutathione (GSH and GSSG) analysis

Total hepatic GSH and GSSG levels were measured in liver homogenates by kinetic assay with a modified method of Tietze and as described previously (Xie, Ramachandran et al. 2015).

Statistical Analysis

Data presented in the form of bar/line graphs show mean ± SEM. Significant difference between two groups was determined using Student's t-test and between three or more groups using one-way analysis of variance (ANOVA) with Tukey's post-hoc test. Difference between groups was considered statistically significant at $P<0.05$.

4.4 RESULTS

Rapid and sustained activation of EGFR in mice and primary human hepatocytes after APAP overdose

Our work in chapter-II established an incremental dose model to study mechanisms of liver regeneration after APAP-induced ALF. Two separate groups of mice were administered either a 'regenerating' (300 mg/kg) or a "non-regenerating' (600 mg/kg) dose of APAP, respectively. The lower dose caused extensive liver injury, followed by robust compensatory liver regeneration leading to recovery. In contrast, at higher dose, liver regeneration was inhibited leading to sustained injury and decreased survival. EGFR signaling is considered crucial for hepatocyte proliferation. Paradoxically, activation of EGFR was remarkably greater at the higher dose of APAP, where liver regeneration was inhibited. To further follow up, in the current study, activation of EGFR was examined after incremental doses of APAP (300 mg/kg and 600 mg/kg) in mice over an extended time-course of 15 min to 96 hr (Fig. 4.4.1A and B). A striking increase in phosphorylation of EGFR at the Tyr-1068 residue (i.e. activation) was observed within 15 minutes after treatment with 300 mg/kg APAP and was sustained up-to 96 hr, with peak activation at around 6 hr. Overall activation of EGFR was remarkably higher after treatment with 600 mg/kg APAP up to 24 hr, with peak activation at 3 hr, but both EGFR expression and phosphorylation were significantly downregulated from 24 to 96 hr at the higher dose. On the contrary, EGFR activation remained sustained at the lower dose and was greater compared to the higher dose after 24 hr (Fig. 4.4.1A and B). Remarkable liver injury, as indicated by elevation of serum ALT levels, was observed beyond 3 hr after treatment with both doses of APAP, suggesting activation of EGFR

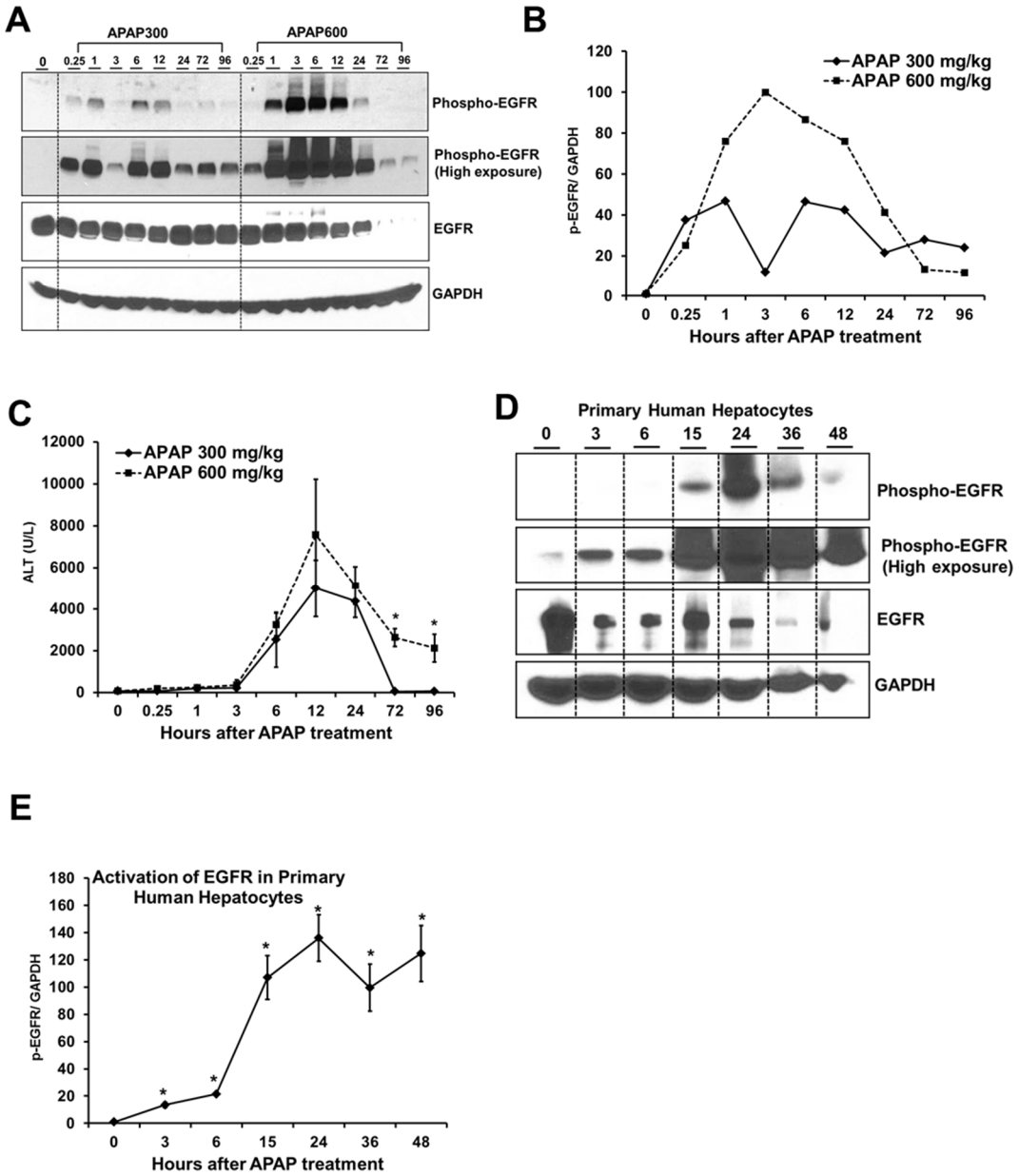

Figure 4.4.1. Rapid and sustained activation of EGFR in mice and primary human hepatocytes after APAP-treatment. (A) Western blot analysis of phospho-EGFR (Tyr1068) and EGFR in total cell lysate and (C) ALT levels in serum, at various time points after administration of 300 mg/kg and 600 mg/kg APAP in mice (n=3-7). (B) Densitometric analysis showing activation (phosphorylation) of EGFR based on western blot image shown in (A). (D) Western blot analysis of phospho-EGFR and EGFR in total cell lysate at various time points after treatment of primary human hepatocytes (PHH) with 10 mM APAP. (E) Densitometric analysis showing activation of EGFR in PHH with data representing mean ± SEM of independent western blot analysis from 4 liver donors. * indicate significant difference between groups at $p<0.05$.

preceded liver injury in mice (Fig. 4.4.1C). As reported in chapter-II, injury was sustained at 72 and 96 hr in the higher dose group, but mice treated with lower dose recovered at these time points (Fig. 4.4.1C).

We also examined EGFR activation in primary human hepatocytes (PHH) following APAP treatment. PHH were cultured in serum free hepatocyte maintenance medium without any addition of EGFR ligands or any other primary mitogens and were treated with 10mM APAP for various time points from 0 to 48 hr. Similar to the mouse data, significant EGFR activation was observed in PHH starting as early as 3 hr (about 20-fold activation at this time point) and activation remained sustained up to 48 hr, with peak activation observed at 24 hr (Fig. 4.4.1D and E). Previous studies have also shown that earliest signs of mitochondrial damage i.e. loss of mitochondrial membrane potential occurs between 6 and 15 after APAP treatment (Xie, McGill et al. 2014). These data indicate that EGFR activation occurs very rapidly after a toxic dose of APAP, both in mice and PHH, preceding development of injury.

Dramatic attenuation of APAP hepatotoxicity by pharmacological inhibition of EGFR (1 hr post-APAP)

Next, the role of EGFR activation in APAP-induced liver injury was investigated. Mice were treated with the highly potent and selective pan-EGFR inhibitor (canertinib, 80 mg/kg) (Slichenmyer, Elliott et al. 2001),1 hr after administration of APAP, and an extended time-course of 3 to 24 hr after APAP was examined (as represented by scheme shown in Fig 4.4.2A). APAP treatment caused significant liver necrosis in the centrilobular region and elevation of serum ALT levels, with injury progressing with time

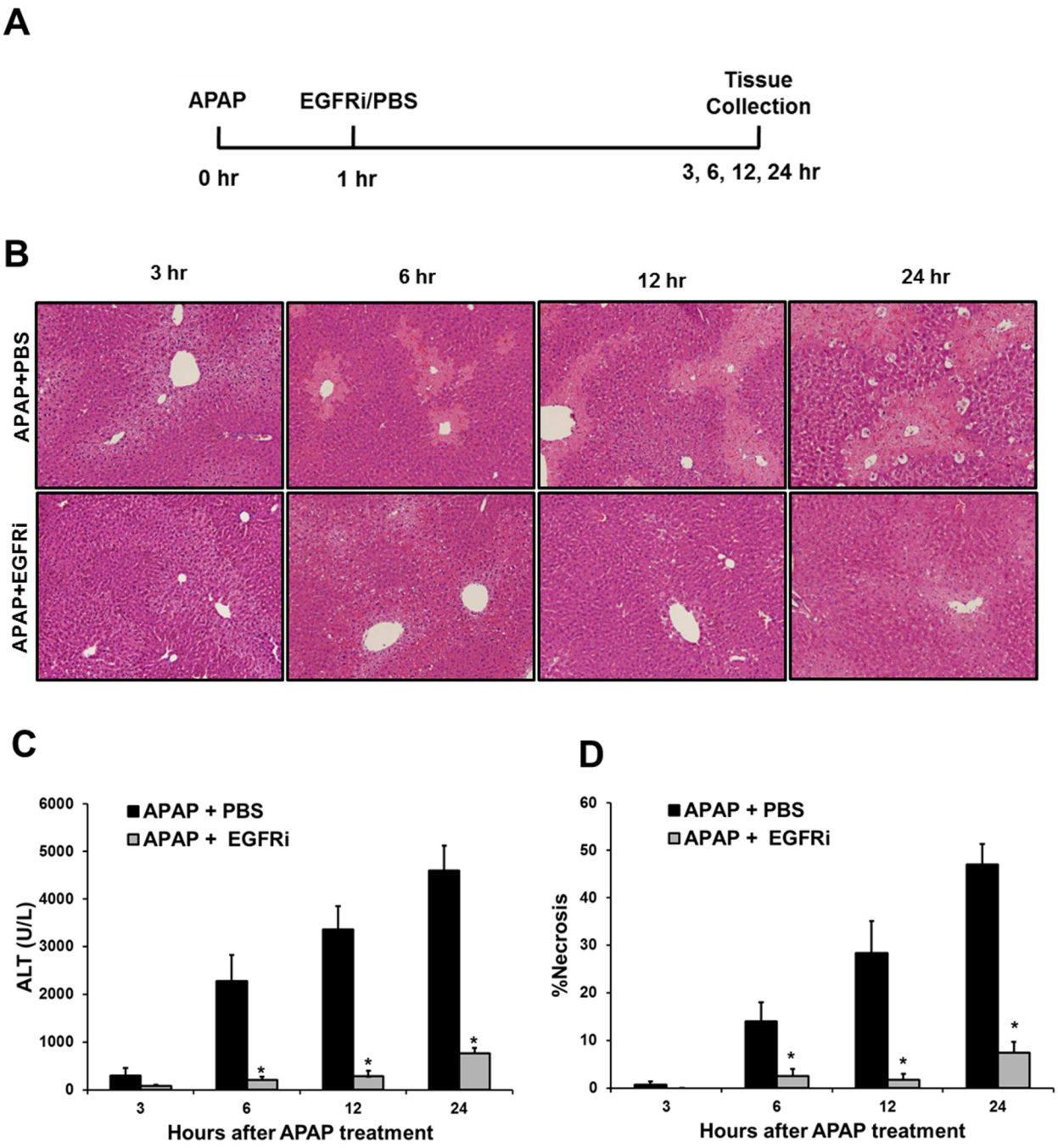

Figure 4.4.2. Treatment with EGFR inhibitor (1 hr post-APAP) remarkably attenuated APAP-induced hepatotoxicity. (A) Schematic showing experimental design. (B) Representative photomicrographs of H&E stained liver sections, (C) serum ALT levels and (D) percentage necrosis area based on H&E stained liver sections of mice (n=3-12) treated with 300 mg/kg APAP followed by canertinib (80 mg/kg) or PBS, 1 hr post-APAP. All samples were collected at various time points up-to 24 hr after APAP treatment. * indicate significant difference between groups at $p<0.05$.

from 3 to 24 hr (Fig. 4.4.2B-D). Treatment with EGFR inhibitor (EGFRi) caused a dramatic reduction of the area of necrosis, with almost no observable necrosis up to 12 hr and very small zones of necrosis at 24 hr (Fig. 4.4.2B and D). A similar trend was also observed for serum ALT levels at all the investigated time points (Fig. 4.4.2C). As expected, APAP treatment caused significant activation of EGFR and treatment with EGFRi decreased EGFR activation to basal levels (Fig. 4.4.3A). This dramatic effect with almost complete abolishment of liver injury after pharmacological inhibition of EGFR activation suggested crucial role of EGFR in APAP-induced hepatotoxicity.

EGFR inhibition does not alter APAP-mediated glutathione depletion and APAP-protein binding

Next, we investigated several major steps involved in the development of APAP toxicity to determine the mechanisms of EGFRi-mediated reduction in APAP toxicity. APAP metabolism in liver results in its bio-activation by formation of a reactive metabolite, NAPQI. NAPQI is conjugated with glutathione in the liver, followed by excretion of the conjugated product. This results in rapid depletion of glutathione and binding of excess NAPQI to cellular proteins (especially mitochondrial proteins) leading to formation of APAP-protein adducts (Jaeschke, McGill et al. 2012). APAP caused significant depletion of glutathione when measured at 3 hr after APAP administration, which was not altered by treatment with EGFRi (1 hr post-APAP) (Fig. 4.4.3B). Recovery of glutathione levels after initial depletion can regulate progression of injury by decreasing APAP-induced oxidative stress. Recovery of glutathione levels at later time points (6 to 24 hr) was not increased by EGFRi treatment (Fig. 4.4.3B). Further, APAP-protein

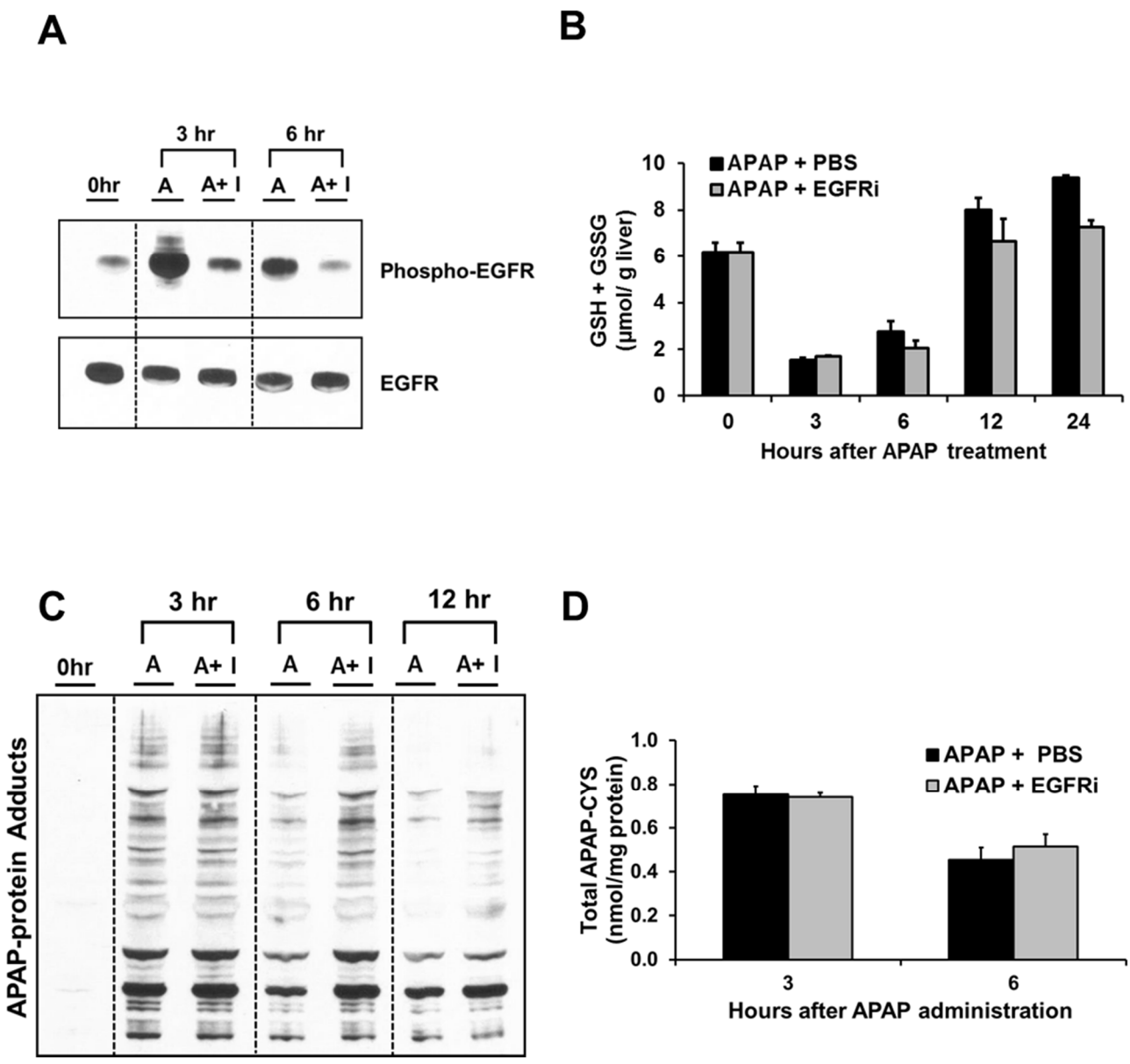

Figure 4.4.3. Robust inhibition of EGFR activation without any alteration of APAP metabolism and protein-adduct formation by EGFR inhibitor. (A) Western blot analysis of phospho-EGFR and EGFR, (B) total glutathione levels in liver extract; APAP-protein adducts levels as measured by (C) western blot analysis and (D) HPLC-MS/MS. Mice (n=3-5) were treated with 300 mg/kg APAP followed by canertinib (80 mg/kg) or PBS, 1 hr post-APAP. All samples were collected at various time points up-to 24 hr after APAP treatment.

adduct levels were measured by both western blot analysis (Fig. 4.4.3C) and HPLC based method (Fig. 4.4.3D). No significant difference was observed in APAP-protein adduct levels by treatment with EGFRi. These data indicate that EGFRi treatment does not alter early injury initiation events of APAP toxicity, namely, metabolic activation of APAP to reactive metabolite NAPQI, subsequent glutathione depletion and APAP-protein adducts formation.

JNK activation, its mitochondrial translocation and signaling through PKC not altered by EGFR inhibition

APAP-protein adducts formation is very crucial for initiation of APAP toxicity, but not sufficient to cause hepatocyte death and necrosis (Jaeschke, McGill et al. 2012). Initial mitochondrial distress is exacerbated by a plethora of intracellular signaling events, which are still under investigation. JNK is considered one of the important mediators of APAP toxicity and can be potentially activated by EGFR signaling. Phosphorylation mediated activation of JNK and its mitochondrial translocation is known to exacerbate mitochondrial oxidative stress during APAP toxicity (Jaeschke, McGill et al. 2012). As expected, APAP treatment caused remarkable activation (phosphorylation) of JNK at 3 and 6 hr followed by rapid decrease in activation at 12 hr, when examined using total cell lysate. Interestingly, EGFRi treatment did not alter APAP-mediated JNK activation (Fig. 4.4.4A). We further investigated mitochondrial levels of JNK and p-JNK. Mitochondrial translocation of JNK and increase in mitochondrial levels of activated JNK were observed at 3 and 6 hr after APAP treatment, which was not decreased by treatment with EGFRi. In fact, mitochondrial levels of p-JNK were significantly higher in

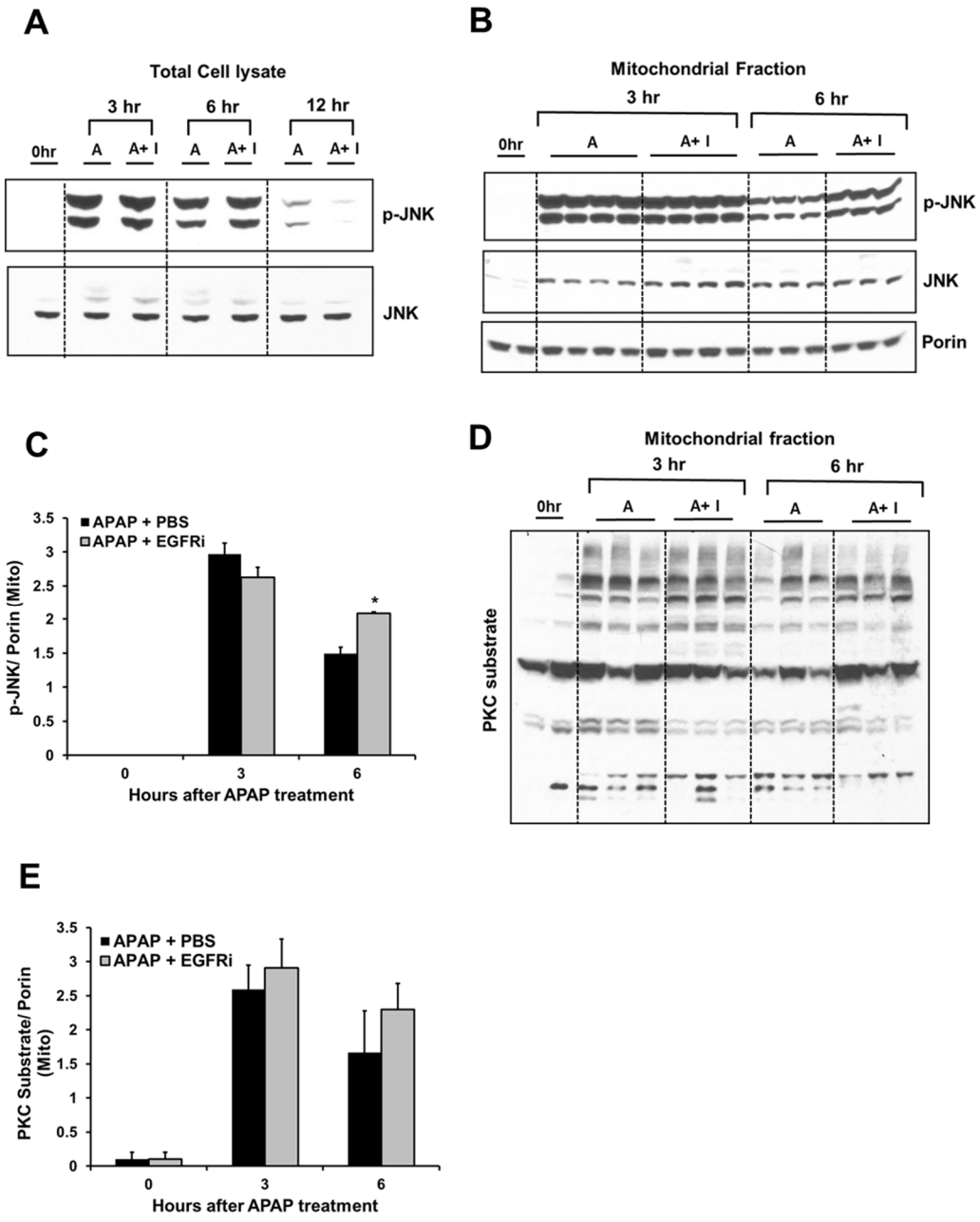

Figure 4.4.4. JNK activation, its mitochondrial translocation and signaling through PKC not altered by EGFR inhibition. Western blot analysis of p-JNK and JNK in (A) total cell lysate and (B) mitochondrial fraction with densitometric analysis of mitochondrial activated JNK shown in (C). (D) Western blot analysis showing PKC activation (studied using antibody against phosphorylated PKC substrates in mitochondria) with its densitometric analysis shown in (E). All analysis were done on liver samples collected at various time points after treatment with 300 mg/kg APAP + PBS or APAP + canertinib (80 mg/kg, 1 hr post-APAP) (n=3-4). * indicate significant difference between groups at $p<0.05$.

EGFRi treated group compared vehicle-treated control at 6 hr after APAP (Fig. 4.4.4B and C). Recently, protein kinase C (PKC) was reported to phosphorylate mitochondrial proteins and regulate APAP-induced liver injury in JNK-dependent and independent manner (Saberi, Ybanez et al. 2014). As PKC is one of the downstream effector of EGFR, we investigated if EGFR inhibition can alter APAP-mediated PKC activation. Consistent with the previous report, activation of PKC signaling (as studied by measuring phosphorylated PKC substrates in mitochondria) was observed at 3 and 6 hr after APAP administration and was not altered by EGFRi treatment (Fig 4.4.4D and E).

APAP caused mitochondrial translocation of EGFR and mitochondrial dysfunction, which was restored by EGFR inhibitor

EGFR has been reported previously to translocate to mitochondria, in endocytosis dependent manner. EGFR-mediated phosphorylation was reported to cause alteration of mitochondrial electron transport chain and subsequently mitochondrial function (Boerner, Demory et al. 2004, Demory, Boerner et al. 2009). Interestingly, in our study, EGFR levels increased remarkably in mitochondria at 1.5 hr after treatment with APAP in mice, suggesting APAP-mediated translocation of EGFR to mitochondria (Fig. 4.4.5A). Similar mitochondrial translocation was also observed for activated EGFR after APAP treatment. Further, mitochondrial translocation of both EGFR and activated EGFR was completely abolished by pretreatment with EGFRi (2 hr prior to APAP) (Fig. 4.4.5A). Our results were corroborated in PHH, where remarkable translocation of EGFR into mitochondria was observed at 6 and 15 hr after APAP treatment, along with a similar increase in mitochondrial levels of activated EGFR (which was more evident at

15 hr) (Fig. 4.4.5B). Our results coincide with previous observations, where mitochondrial dysfunction was found to be initiated between 6 and 15 hr after APAP treatment in PHH (Xie, McGill et al. 2014).

Next, we investigated the effect of EGFR inhibition on alteration of APAP-mediated mitochondrial dysfunction using Seahorse extracellular flux analyzer. Oxygen consumption parameters were analyzed in freshly isolated mitochondria 1.5 hr after treatment with APAP in mice with or without EGFRi (2 hr prior to APAP) (as represented by scheme shown in Fig 4.4.5C). Significant reduction in ADP-stimulated phosphorylating mitochondrial respiration and FCCP (uncoupler)-induced maximal mitochondrial respiration was observed within 1.5 hr after APAP administration. Interestingly, EGFRi treatment completely restored both these parameters (Fig. 4.4.5D and E). Similar restoration of mitochondrial functional parameters was observed at 3 hr after APAP treatment in mice when EGFR inhibitor was administered 1 hr post-APAP (Fig. 4.4.5F, G and H). These data indicate that mitochondrial translocation of EGFR and its activity in mitochondria may be involved in early mitochondrial dysfunction after APAP overdose preceding overt necrosis, which was prevented by treatment with the EGFR inhibitor.

Decreased oxidative stress, mitochondrial protein nitration and release of endonucleases from mitochondria by EGFR inhibitor

Initial mitochondrial dysfunction leads to a vicious circle of mitochondrial oxidative and nitrosative-stress and subsequent mitochondrial damage during APAP-induced liver

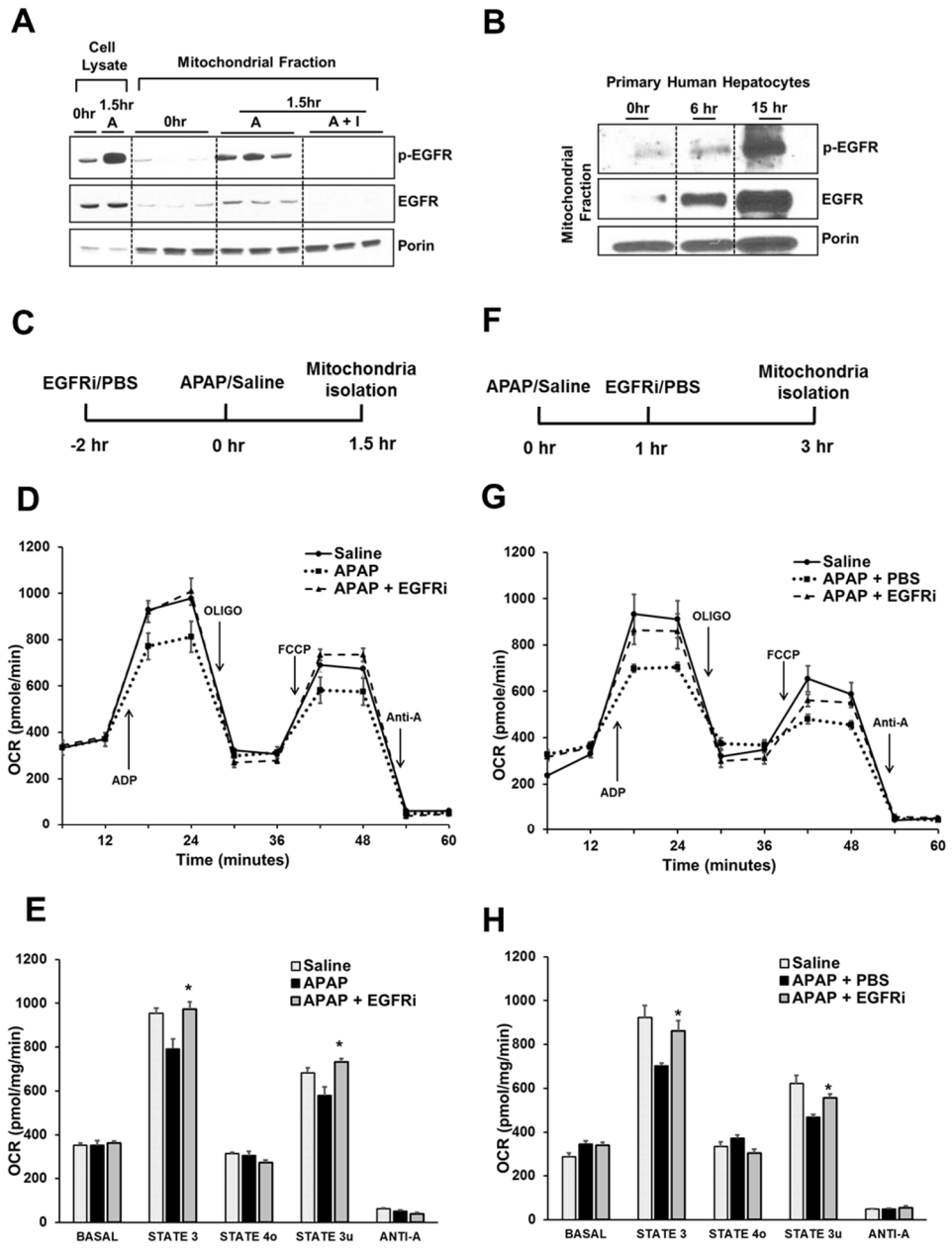

Figure 4.4.5. APAP caused mitochondrial translocation of EGFR and mitochondrial dysfunction, which was restored by EGFR inhibitor. Western blot analysis of phospho-EGFR and EGFR in mitochondrial fraction at various time points, (A) after administration of APAP (300 mg/kg) in mice pretreated (2hr prior to APAP) with canertinib (80 mg/kg); (B) after treatment of primary human hepatocytes (PHH) with 10 mM APAP. (C-H) Oxygen consumption rate analysis in freshly isolated mitochondria from mice (n=3) treated with APAP (300 mg/kg) + PBS, APAP (300 mg/kg) + canertinib (80 mg/kg) or without any treatment (control), using Seahorse extracellular flux analyzer. (D) and (E) Canertinib was administered 2hr before APAP and analysis was done 1.5 hr after APAP treatment, as represented by scheme in (C). (G) and (H) Canertinib was administered 1hr post-APAP and analysis was done 3 hr after APAP treatment, as represented by scheme in (F) . OXPHOS was manipulated with injection of ADP (4 mM), oligomycin (2.5 µg/ml), FCCP (4 µM), and antimycin A (4 µM). Respiration was sequentially measured in a coupled state with substrate present (basal respiration), followed by State 3 (phosphorylating respiration, in the presence of ADP and substrate), State 4o (induced with the addition of oligomycin), and then maximal uncoupler-stimulated respiration (State 3u). * indicate significant difference w.r.t. to APAP+PBS group at $p<0.05$.

necrosis (Jaeschke, McGill et al. 2012). Oxidized glutathione levels and its percentage with respect to total glutathione levels (marker of oxidative stress) were measured at various time points after treatment with APAP with and without EGFRi. A significant decrease in oxidized glutathione levels (Fig. 4.4.6A) and the percentage of oxidized glutathione (Fig. 4.4.6B) was observed in EGFRi-treated group compared to vehicle-treated group at 12 and 24 hr after APAP-treatment. Superoxide radicals react with nitric oxide to form the potent oxidant peroxynitrite, which is generated mainly in mitochondria during APAP hepatotoxicity (Jaeschke, McGill et al. 2012). Peroxynitrite then reacts with tyrosine residues of proteins to form nitrotyrosine protein adducts, which further results in mitochondrial damage. Staining liver sections using nitrotyrosine antibody revealed significant reduction in APAP-mediated formation of nitrotyrosine adducts at 6 hr after treatment with EGFRi (Fig. 4.4.6C). Further, western blot analysis of mitochondrial fraction revealed that APAP-mediated nitrotyrosine adducts formation was significantly reduced in mitochondria after treatment with EGFRi (Fig. 4.4.6D and E). Mitochondrial dysfunction and oxidative damage ultimately lead to opening of mitochondrial permeability transition (MPT) pore and release of endonucleases such as apoptosis-inducing factor (AIF) and endonuclease G from mitochondrial intermembrane into the cytosol. These endonucleases cause DNA damage that ultimately results in liver necrosis (Jaeschke, McGill et al. 2012). As expected, APAP treatment caused remarkable release of mitochondrial intermembrane endonucleases, AIF and endonuclease G, as well as SMAC into the cytosol (Fig. 4.4.6F-G). EGFR inhibition completely abolished release of AIF at 3 hr after APAP-treatment and cytosolic AIF remained very low even at 6 hr (Fig. 4.4.6F-G). Similarly, significant decrease in early

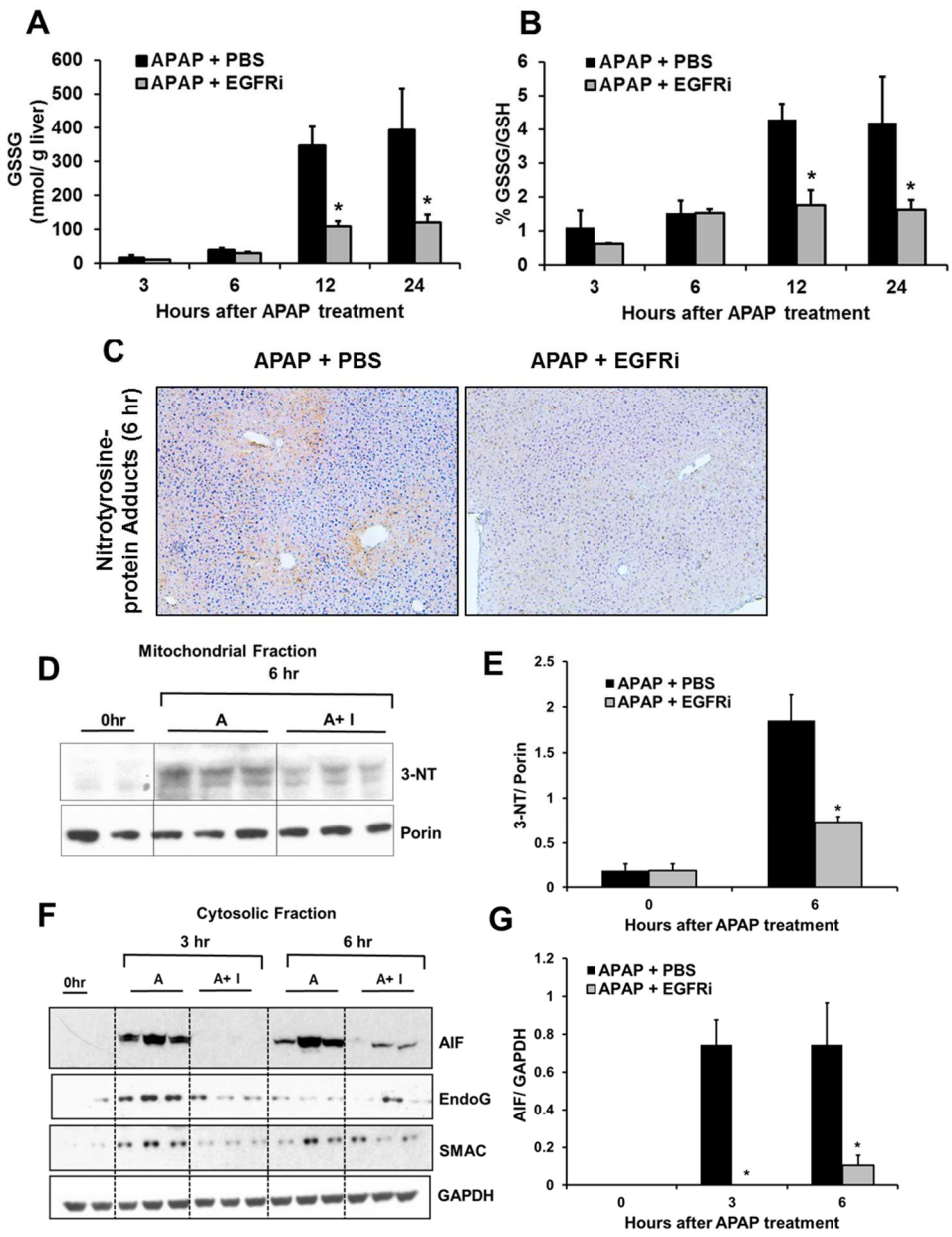

Figure 4.4.6. Decreased oxidative stress, mitochondrial protein nitration and release of endonucleases from mitochondria by EGFR inhibitor. (A) Oxidized glutathione levels and (B) percentage of oxidized glutathione with respect to total glutathione level in total liver extract. (C) Representative photomicrographs of liver sections stained for nitrotyrosine-protein adducts. (D) Western blot analysis of nitrotyrosine adducts in mitochondrial fraction with its densitometric analysis shown in (E). (F) Western blot analysis of AIF, endonuclease G and SMAC in cytosolic fraction with densitometric analysis of AIF as shown in (G). All analysis were done on liver samples collected at various time points after treatment with APAP (300 mg/kg) + PBS or APAP (300 mg/kg) + canertinib (80 mg/kg, 1 hr post-APAP) (n=3-5). * indicate significant difference between groups at $p<0.05$.

release of endonuclease G was observed after EGFRi treatment (Fig. 4.4.6F). These data suggest that EGFR plays an important role in development of mitochondrial oxidative stress and mitochondrial damage during pathogenesis of APAP hepatotoxicity.

Role of glutathione depletion in rapid activation of EGFR by toxic dose of APAP

Early activation of EGFR was observed after APAP treatment, even in primary human hepatocytes, when cultured in chemically defined serum free medium with no EGFR ligands or other primary mitogens. This suggested a possible role of ligand-independent intercellular events in triggering of EGFR activation, as reported previously in liver cells after other toxic insults (Reinehr and Haussinger 2009). Glutathione depletion is one of the earliest events after APAP overdose. In order to investigate if glutathione depletion, *per se,* can cause activation of EGFR, mice were treated with phorone (200 mg/kg, i.p.), a glutathione depleting agent. As expected, phorone treatment caused remarkable depletion of glutathione within 2 hr (Fig. 4.4.7A). Interestingly, glutathione depletion by treatment with phorone was accompanied by robust activation of EGFR and downregulation of EGFR (Fig. 4.4.7B and C). To further substantiate the role of glutathione depletion in activation of EGFR after APAP overdose, APAP-mediated depleted glutathione was replenished by treatment with NAC (N-acetylcysteine, 500 mg/kg, i.p.), 1.5 hr after APAP (Fig. 4.4.7D). Treatment with NAC caused remarkable decrease in APAP-mediated EGFR activation, as analyzed 6 hr after APAP treatment, correlating with recovery of glutathione levels (Fig. 4.4.7E and F). These data suggest that glutathione depletion after APAP overdose may be involved in early activation of EGFR.

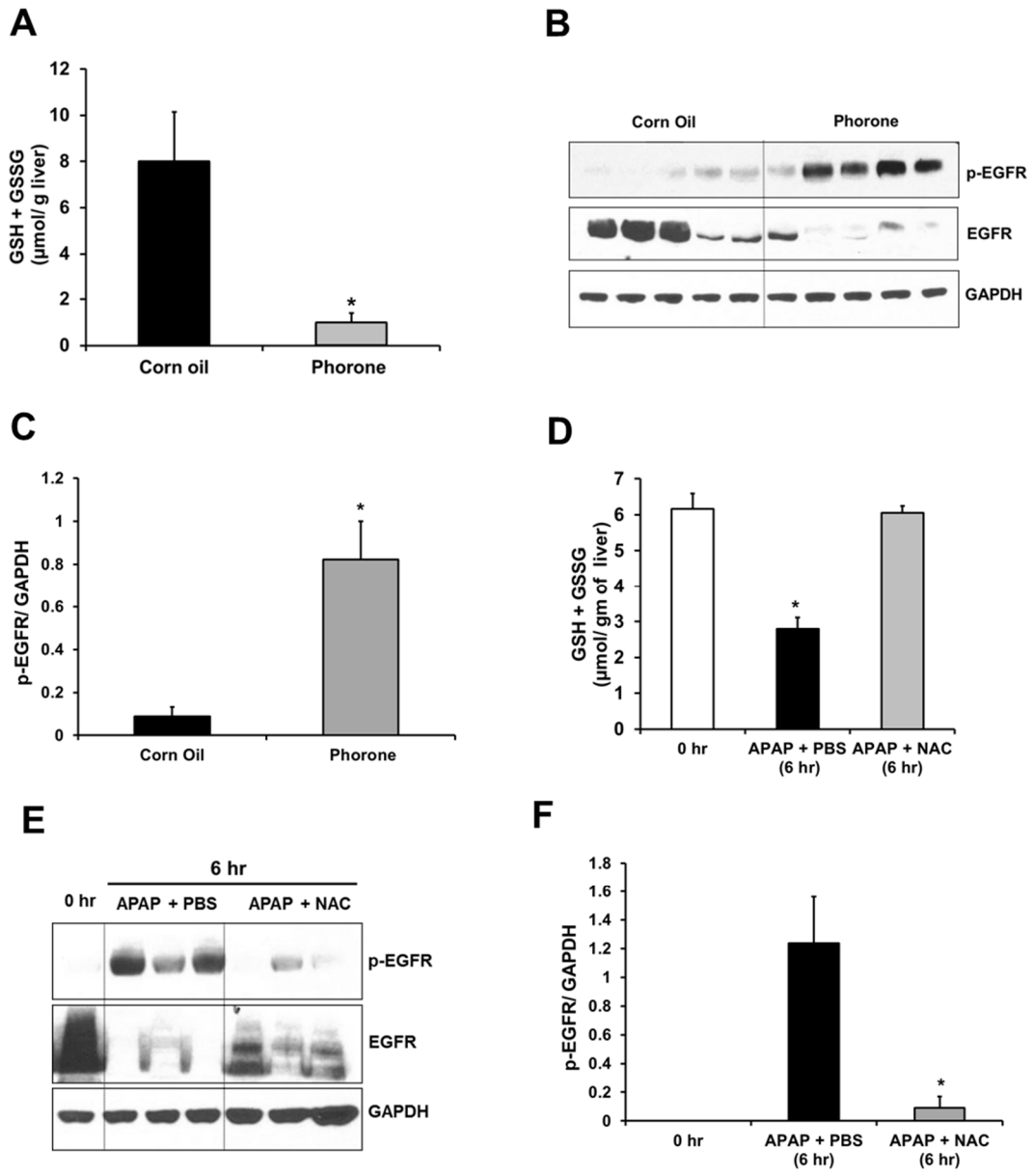

Figure 4.4.7. Role of glutathione depletion in rapid activation of EGFR by APAP. (A) Total glutathione levels in liver extract and (B) western blot analysis of phospho-EGFR and EGFR in total cell lysate from liver samples obtained 2 hr after treatment with Phorone (200 mg/kg in corn oil) or corn oil (control) in mice (n=5). (C) Densitomertic analysis showing EGFR activation based on western blot image shown in (B). (D) Total glutathione levels in liver extract and (E) western blot analysis of phospho-EGFR and EGFR in total cell lysate from liver samples obtained 6 hr after treatment with APAP (300 mg/kg) + PBS or APAP (300 mg/kg) + NAC (N-acetylcysteine: 500 mg/kg, 1.5 hr post-APAP) in mice (n=3). (F) Densitomertic analysis showing EGFR activation based on western blot image shown in (E). * indicate significant difference between groups at $p<0.05$.

Increased progression of injury, impaired recovery and decreased survival after delayed treatment with EGFR inhibitor (12 hr post-APAP)

Several studies using partial hepatectomy (PH) model have demonstrated a crucial role of EGFR signaling in liver regeneration after liver re-section (Michalopoulos 2007, Fausto, Campbell et al. 2012). Thus, the role of EGFR activation was investigated in compensatory liver regeneration after APAP-induced liver injury. Since liver regeneration is a compensatory response to liver injury after APAP toxicity, any alteration in liver injury can indirectly affect regeneration. To investigate a direct role of EGFR on liver regeneration, EGFRi was administered 12 hr post-APAP, with notion that injury is already established by this time and liver regeneration is just initiated based on previously characterized dynamics of liver injury and regeneration after APAP overdose (as described in chapter-II) . Parameters related to liver injury and regeneration were studied at 24 and 48 hr after treatment with APAP (as represented by the scheme shown in Fig 4.4.8A). As expected, liver injury at 24 hr after APAP treatment was not altered by EGFRi-treatment as indicated by percentage necrotic area (Fig. 4.4.8B and D) and serum ALT levels (Fig. 4.4.8C). Similar to our results in chapter-II, the injury was significantly resolved at 48 hr compared to 24 hr in APAP-treated group. However, liver injury did not improve at 48 hr compared to 24 hr in EGFRi-treated group (Fig. 4.4.8B-D) and resulted in 50% decrease in survival at 48 hr post-APAP (Fig. 4.4.8E).

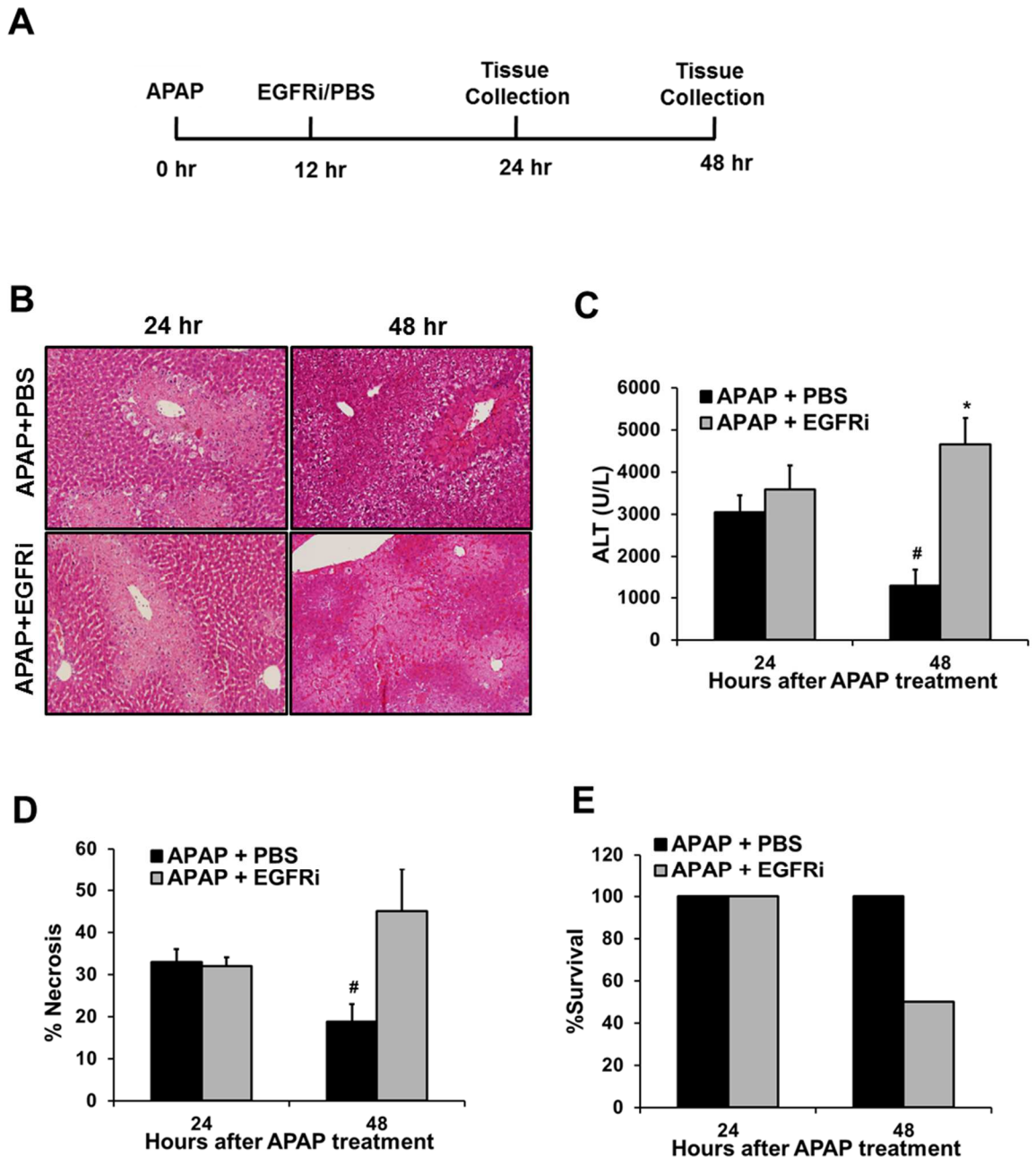

Figure 4.4.8. Increased progression of injury, impaired recovery and decreased survival after delayed (12 hr post-APAP) treatment with EGFR inhibitor. (A) Schematic showing experimental design. (B) Representative photomicrographs of H&E stained liver sections, (C) serum ALT levels, (D) percentage necrosis area based on H&E stained liver sections and (E) survival analysis of mice treated with 300 mg/kg APAP followed by treatment with canertinib (80 mg/kg) or PBS, 12 hr post-APAP. All samples were collected and survival was recorded at 24 and 48 hr after APAP treatment (n=5-6). * indicate significant difference between groups at $p<0.05$. # indicates significant difference w.r.t. 24 hr time point within same treatment group.

Impaired liver regeneration by delayed treatment with EGFR inhibitor (12 hr post-APAP)

Next, we investigated effect of delayed treatment with EGFRi on compensatory liver regeneration following APAP overdose. Liver regeneration was studied using PCNA staining (Fig. 4.4.9A), which is a marker of cell proliferation. Consistent with our results in chapter-II, numerous PCNA-positive cells were observed surrounding the zones of necrosis, at 24 and 48 hr after treatment with APAP, suggesting robust liver regeneration. Interestingly, minimal PCNA-positive cells were observed at both time points in the EGFRi group, indicating compensatory liver regeneration response was almost completely abolished after treatment with EGFRi (Fig. 4.4.9A and B). Quantification of PCNA-positive cells was further corroborated by analysis of PCNA protein expression using western blot (Fig. 4.4.10A and E).

Cell cycle regulators correlated with impaired liver regeneration by delayed treatment with EGFR inhibitor (12 hr post-APAP)

Induction of Cyclin D1 is a key initial step that regulates entry into the cell cycle. APAP-treatment caused a remarkable increase in the expression of Cyclin D1 at 24 and 48 hr. APAP-mediated increase in protein expression of CyclinD1 was dramatically decreased at both time points after delayed treatment with EGFRi (Fig. 4.4.10A and B). Cyclin D1 binds to CDK4 and causes its activation, which then phosphorylates Rb protein, ultimately leading to induction of several key cell cycle genes. CDK4 protein expression followed a pattern similar to Cyclin D1 in both groups (Fig. 4.4.10A and C). Further, remarkable phosphorylation of Rb protein was observed at 48 hr in APAP-treated

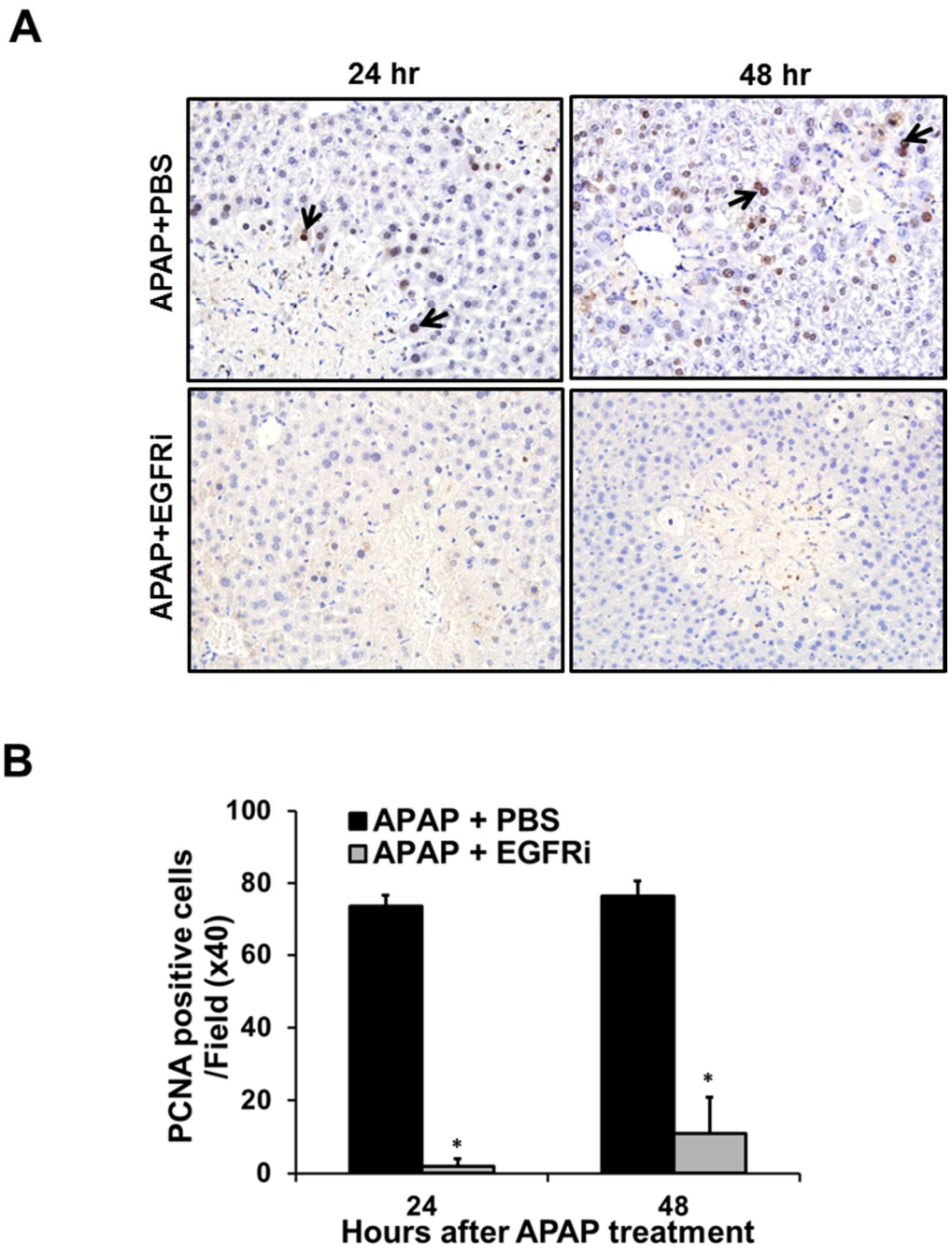

Figure 4.4.9. Impaired liver regeneration by delayed (12 hr post-APAP) treatment with EGFR inhibitor. (A) Representative photomicrographs of PCNA stained liver sections with arrows indicating cells in S-phase with nuclear PCNA staining (brown). (B) Total number of PCNA-positive cell per high power field (x40). Mice (n=3-5) were treated with 300 mg/kg APAP. Canertinib (80 mg/kg) or PBS was administered 12 hr post-APAP. All samples were collected at 24 and 48 hr after APAP treatment. * indicate significant difference between groups at $p<0.05$.

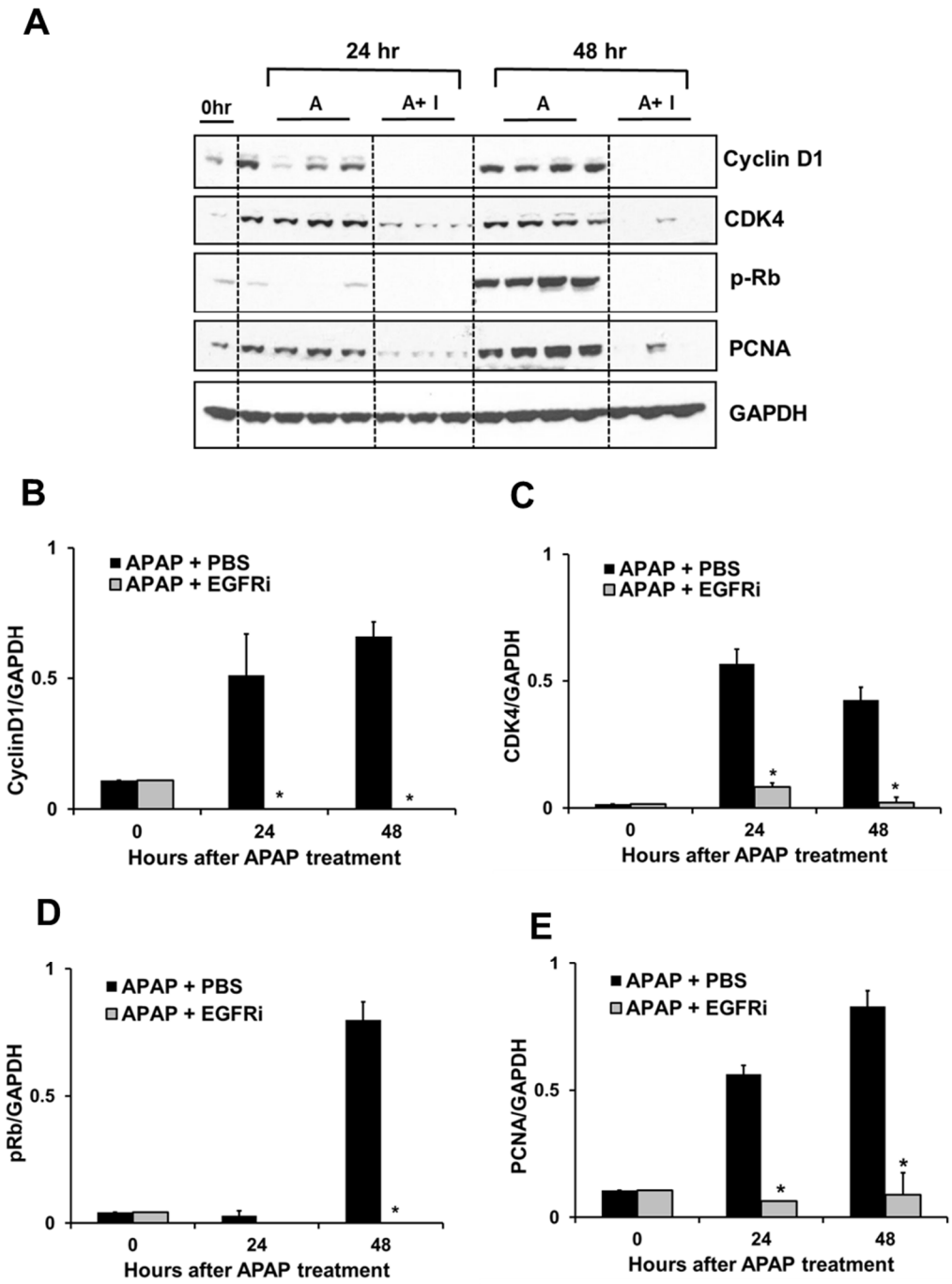

Figure 4.4.10. Cell cycle regulators correlated with impaired liver regeneration after delayed treatment with EGFR inhibitor (12 hr post-APAP). (A) Western blot analysis of cyclinD1, CDK4, phospho-Rb and PCNA using total liver extract with their densitometric analysis shown in (B), (C), (D) and (E), respectively. Mice (n=3-4) were treated with 300 mg/kg APAP. Canertinib (80 mg/kg) or PBS was administered 12 hr post-APAP. All samples were collected at 24 and 48 hr after APAP treatment. * indicate significant difference between groups at $p<0.05$.

group, which was completely abolished after treatment with EGFRi (Fig. 4.4.10 A and D). These data demonstrate that delayed treatment with EGFRi (12 hr post-APAP) prevented cell cycle progression and caused complete inhibition of liver regeneration leading to decreased recovery and reduced survival after APAP overdose.

4.5 DISCUSSION

Although mechanisms of APAP hepatotoxicity have been extensively studied leading to considerable progress in our knowledge, the exact intracellular signaling events still not completely known (Jaeschke, McGill et al. 2012). On the contrary, mechanisms of compensatory liver regeneration that follows liver injury after APAP overdose have just begun to be explored. EGFR signaling is well known to play critical role in hepatocyte proliferation and normal liver regeneration after PH (Michalopoulos 2007, Fausto, Campbell et al. 2012), but a few reports also suggest its role in cell death signaling in liver (Arany 2008, Reinehr and Haussinger 2009). The role of EGFR in acetaminophen (APAP)-mediated acute liver injury and subsequent liver regeneration is completely unknown, which was investigated in this study.

Similar to our work in chapter II, dose-dependent activation of EGFR was observed after toxic doses of APAP in mice such that activation was remarkably greater at a higher non-regenerating dose of APAP. Interestingly, EGFR activation starts very rapidly, within 15 min. after APAP treatment. Similar rapid and consistent activation of EGFR was also observed in primary human hepatocytes after APAP treatment. This dose-dependent early activation of EGFR, preceding injury, with paradoxical inverse correlation with liver regeneration, suggested a possible role of EGFR activation in the development of injury. Indeed, inhibition of EGFR both pre- and post-APAP treatment by using a highly specific pan-EGFR inhibitor (Slichenmyer, Elliott et al. 2001), canertinib, almost completely abolished APAP toxicity consistently and reproducibly, suggesting a crucial role of EGFR activation in APAP hepatotoxicity. This paradoxical

role of EGFR activation in cell death is not completely unprecedented. Hydrophobic bile acids and CD95 ligand have been previously reported to activate EGFR in both hepatocytes and HSCs (Reinehr, Graf et al. 2003, Reinehr, Schliess et al. 2003, Reinehr, Becker et al. 2004, Reinehr, Becker et al. 2005, Reinehr, Becker et al. 2005, Reinehr and Haussinger 2009, Sommerfeld, Reinehr et al. 2009, Reinehr and Haussinger 2012). EGFR activation in these studies was involved in apoptosis through tyrosine phosphorylation of death receptor (CD95) and stabilization of the death-inducing signaling complex and inhibition of EGFR activation resulted in decreased cell death (Reinehr and Haussinger 2012). Similarly, EGFR was activated after treatment with the nephrotoxic insults cisplatin and cyclosporine A in proximal renal tubular cell lines and inhibition of EGFR activation resulted in diminished cell death (Arany, Megyesi et al. 2004, Sarro, Tornavaca et al. 2008).

We further investigated the potential mechanisms underlying role of EGFR activation in mediating APAP hepatotoxicity. Metabolic activation of APAP, reactive metabolite-mediated glutathione depletion and subsequent covalent binding to cellular proteins were not altered by EGFRi treatment indicating that the early events involved in initiation of APAP hepatotoxicity were not changed by EGFRi treatment. Initial mitochondrial dysfunction and its feed-forward accentuation by oxidative stress play a central role in APAP hepatotoxicity with underlying signaling pathways not completely known (Jaeschke, McGill et al. 2012). Our study revealed, for the first time, that EGFR translocated to mitochondria and its activated form is increased in mitochondria very rapidly after APAP treatment. This was associated with very early decline in mitochondrial respiratory capacity, preceding overt necrosis after APAP overdose.

Mitochondrial levels of both activated EGFR and EGFR, were drastically decreased by EGFR inhibition, resulting in complete restoration of mitochondrial function. Subsequently, EGFR inhibition also prevented mitochondrial oxidative stress and release of mitochondrial intermembrane proteins, especially AIF, into the cytosol, which is important for nuclear DNA damage and necrosis. These data have revealed a highly novel role of EGFR in APAP toxicity via modulating mitochondrial dysfunction. The exact mechanisms how mitochondrial EGFR activity can be involved in very early mitochondrial dysfunction remain unknown. EGFR can be involved, directly or indirectly via one of its effectors, in phosphorylation or alteration of mitochondrial proteins involved in APAP-associated mitochondrial dysfunction such as Sab. A previous study reported that activated EGFR can be translocated to mitochondria and phosphorylate mitochondrial cytochrome c oxidase subunit II (CoxII) leading to decrease in its activity and ATP depletion (Demory, Boerner et al. 2009). Further detailed studies are necessary to determine the exact interacting proteins of EGFR in mitochondria during APAP hepatotoxicity.

The mechanism of rapid activation of EGFR is another interesting aspect which was investigated in this study. Early activation of EGFR in primary human hepatocytes in maintenance medium, where no EGFR ligands were present, suggested a possible role of ligand-independent intracellular mechanism in activation of EGFR. Such a mechanism has been demonstrated in liver cells after apoptotic-stimuli and was specifically involved in cell death (Reinehr and Haussinger 2009). These studies also demonstrated that oxidative stress was responsible for phosphorylation of EGFR via activation of Yes kinase (Reinehr and Haussinger 2009). In our study, investigations

with the glutathione depleting agent (phorone) and glutathione replenishing agent (NAC) indicated a potential role of glutathione depletion in EGFR activation. This is in concordance with the fact that glutathione depletion is one of the very early events that occur after APAP overdose similar to EGFR activation and glutathione is required physiologically to scavenge ROS and limit oxidative stress. The role of GSH depletion in EGFR activation is supported by rapid activation of EGFR in serum free conditions where no EGFR ligands are present. However, our studies do not rule out possibility of ligand-dependent activation of EGFR after APAP overdose, especially at later time points. TGF-α can be produced by hepatocytes and activate EGFR receptor on hepatocytes in an autocrine fashion (Michalopoulos and Khan 2005). Whether this autocrine loop by *de novo* TGF-α synthesis may be involved in activation of EGFR remains to be tested.

In order to investigate a direct role of EGFR activation on liver regeneration after APAP overdose, a delayed EGFRi treatment strategy (12 hr post-APAP) was utilized, such that APAP hepatotoxicity was already established. Delayed treatment with EGFRi almost completely abolished compensatory hepatocyte proliferation after APAP overdose. Failure of cell cycle progression was due to complete inhibition of cyclin D1 induction. This suggested that apart from the role in liver injury after APAP overdose, EGFR signaling plays a critical function in liver regeneration as well. Similarly, a dual role of EGFR was reported previously in HSCs, where activation of EGFR upon stimulation with bile acids caused cell proliferative signaling, which shifted to cell death signaling upon sustained JNK activation-mediated coupling of EGFR with the CD95 death receptor (Reinehr and Haussinger 2009, Sommerfeld, Reinehr et al. 2009). A

similar dual role of EGFR has been proposed in the case of nephrotoxic insults, where EGFR activation can lead to renal injury or recovery depending on degree, duration and type of stress (Arany 2008). A number of previous studies in the PH model of liver regeneration show that inhibition or elimination of a single pathway (including EGFR signaling) can only delay or diminish liver regeneration but cannot permanently abolish liver regeneration due to upregulation of compensatory pathways (Michalopoulos 2007). In contrast, in our study, delayed inhibition of EGFR after APAP overdose resulted in almost complete inhibition of liver regeneration, resulting in progression of injury and decreased survival. These data suggest that the dynamics of liver regeneration is very different in the APAP-induced ALF model (compared to PH), which advances very rapidly and involves progression of injury, such that a timely regenerative response is imminently critical for final recovery. Since most of these studies so far have been focused on studying mechanisms of liver regeneration after PH, there is a critical need to understand mechanisms of liver regeneration in acute liver injury models, which are more relevant for clinical drug-induced ALF.

Our data regarding deleterious effect of EGFR inhibition on liver regeneration suggest that it will be challenging to develop EGFR inhibition as a therapeutic strategy for APAP toxicity as depending upon the time of inhibition, outcome can be completely opposite. This is particularly important aspect to consider in case of APAP overdose, where the exact time of overdose is not usually known in patients. Further comprehensive studies in future, to investigate temporal dynamics of signaling pathways affected by EGFR activation during pathogenesis of APAP hepatotoxicity can assist in delineating toxicity

effectors from regenerative signaling for targeted development of anti-injury or pro-regenerative therapy.

To conclude, early inhibition of EGFR activation after APAP overdose in mice attenuated liver injury by preventing mitochondrial dysfunction and delayed inhibition of EGFR resulted in impairment of liver regeneration after APAP toxicity by arresting cell cycle activation. Thus our study indicated that EGFR activation can play a dual role in APAP overdose and is involved in both initiation of APAP-induced injury and in stimulating subsequent liver regeneration. Further, our study supports the emerging notion that signaling pathways that regulate liver injury and subsequent tissue repair are intricately linked and similar to death receptors, growth factor receptor such as EGFR can couple to both cell death and proliferation signaling in a context-dependent manner.

CHAPTER V: CONCLUSIONS AND FUTURE DIRECTIONS

Acetaminophen (APAP) overdose is the leading cause of acute liver failure (ALF) in the western world, accounting nearly 50% of all the ALF cases in the US and 60% in the UK (Bernal and Wendon 2013, Bernal, Lee et al. 2015). Despite decades of research, current treatment options after APAP-overdose are extremely limited. N-acetylcysteine (NAC) is the current standard of care for APAP overdose patients, which is effective only at an early stage (Athuraliya and Jones 2009, Bernal, Lee et al. 2015). Mechanisms of liver injury after APAP overdose have been extensively investigated in past in order to develop novel therapeutic strategies. However, most of the patients seek medical attention late, such that injury is already established and difficult to treat (Larson 2007). Liver injury after APAP-overdose is subsequently followed by compensatory liver regeneration, which promotes recovery. Previous studies have demonstrated that liver regeneration is a critical determinant of survival after APAP overdose (Schmidt and Dalhoff 2005, Hu and Colletti 2008, Apte, Singh et al. 2009, Donahower, McCullough et al. 2010). Liver regeneration can be potentially modulated even at a late stage in the pathogenesis of APAP-induced ALF and stimulating liver regeneration in patients of APAP-induced ALF holds a great therapeutic potential. However, development of novel regenerative therapies for ALF is hampered because the mechanisms of liver regeneration after APAP-induced ALF have not extensively explored yet. The mechanisms of liver regeneration are mostly studied in the partial hepatectomy (PH) model (Michalopoulos 2007), which is considerably different from APAP-hepatotoxicity, the model of clinical ALF. The focus of the studies outlined in this dissertation was to investigate the mechanisms of liver regeneration after APAP-induced liver injury. After initial studies to establish a model to identify potential

regulators of liver regeneration after APAP overdose in mice, our later studies were focused on investigating the role of β-catenin, GSK3 and EGFR in liver regeneration after APAP-induced liver injury.

5.1 INCREMENTAL DOSE MODEL TO STUDY LIVER REGENERATION AFTER APAP OVERDOSE IN MICE

Liver regeneration following toxic injury to liver is known to follow dose-response principles. Liver regeneration increases with dose of toxicant proportionate to injury until a threshold dose, beyond which the ability of liver to regenerate is impaired (Mehendale 2005). Based on this principle, we developed a novel incremental dose model to study mechanisms of liver regeneration after APAP-induced hepatotoxicity utilizing two doses of APAP in mice. A lower dose (300 mg/kg) that caused extensive liver injury but also significant compensatory regeneration leading to regression of injury and spontaneous recovery; and a higher dose (600 mg/kg) that inhibited liver regeneration resulting in sustained injury and decreased survival. We performed a comprehensive analysis of several signaling pathways known to be involved in liver regeneration and identified pathways which are potentially important for liver regeneration after APAP-induced hepatotoxicity. The approach of using two doses not only identified proliferative signaling pathways that correlated with robust liver regeneration after moderate APAP overdose, but more importantly provided us insight about potential mechanisms for impaired liver regeneration after the higher dose of APAP. Understanding the mechanism of impaired liver regeneration after high doses of APAP is more relevant

clinically, as high doses simulate pathophysiology of patients which cannot recover spontaneously after APAP overdose and require liver transplantation.

Very interestingly, growth factor signaling via EGFR and c-Met and downstream MAPKs (ERK, p38 and JNK), were dose-dependently activated and remain highly activated even after the higher dose of APAP, where regeneration was inhibited. Growth factors such as EGFR ligands and HGF (ligand of c-Met) are known to play a critical role in hepatocyte proliferation and liver regeneration after PH (Michalopoulos 2007). In fact, EGFR ligands and HGF are the only known direct mitogens, which can cause hepatocyte proliferation in serum free medium. Our results question administration of growth factors as a therapeutic strategy after APAP-induced ALF. This observation is consistent with previous reports that administration of growth factors did not affect liver regeneration in dogs after APAP-induced ALF (Francavilla, Azzarone et al. 1993). Also patients who died after APAP-induced ALF were found to have higher amount of HGF circulating in the plasma (Hughes, Zhang et al. 1994). Similarly, IL-6/STAT-3 signaling has been reported to be important for liver regeneration after APAP-induced hepatotoxicity using IL6 KO mice (James, Lamps et al. 2003). However, in our incremental dose model, IL6/STAT-3 signaling remained highly activated even after the higher dose of APAP, where regeneration was inhibited.

Our study revealed that marked inhibition of regeneration at the higher dose is not due to lack of viable hepatocytes as more than 50% hepatocytes were viable at this dose even at the peak injury. In fact, peak injury was not remarkably different at two doses, while liver regeneration was remarkably impaired only at the higher dose.

Impaired liver regeneration at the higher dose even in presence of pro-proliferative signaling indicated possibility that some anti-proliferative signaling is blocking liver regeneration at higher dose counteracting the pro-regenerative signaling. Activation of cell cycle inhibitors such as p21 and cell cycle arrest at the higher dose supported this possibility. Moreover, some of the known regenerative pathways, which are highly activated in APAP600, may be actively involved in inhibiting regeneration. For instance, over-activation of p38 (Koteish, Yang et al. 2002, Horimoto, Fulop et al. 2004) and IL6/STAT3 signaling (Wustefeld, Rakemann et al. 2000, Torbenson, Yang et al. 2002) has been associated with decreased regeneration in the PH model. Further, sustained activation of these mediators was correlated with induction of p21 and inhibition of Cyclin D1, which is consistent with our data. Comprehensive studies focused on investigating role of cell cycle inhibitors in impaired liver regeneration after severe APAP overdose will be conducted in future. These studies will elucidate mechanisms of cell cycle arrest and facilitate discovering strategies to overcome the barriers of liver regeneration.

Apart from activation of anti-proliferative signaling at the higher dose, some specific proliferative signaling pathways were inhibited at the higher dose. These pathways were specifically activated after the lower dose of APAP correlating with robust regenerative response. These included canonical Wnt/β-catenin and TNF-α/NF-κB signaling pathways. This was further supported by our ChIP analysis, which demonstrated that the binding of transcription factors β-catenin and NF-κB to Cyclin D1 was diminished at higher dose of APAP, correlating with decreased induction of Cyclin D1. Our study suggested that stimulating these pro-regenerative pathways may be a potential strategy

for development of novel regenerative therapies of APAP overdose, which warrant further investigation. Role of β-catenin signaling was comprehensively studied in later part of this dissertation work. Role of TNF receptor in liver regeneration after APAP overdose (Chiu, Gardner et al. 2003, James, Kurten et al. 2005) and positive correlation of NF-κB DNA binding with liver regeneration after APAP overdose has been reported previously (Yang, Miki et al. 2009, Yang, Zhang et al. 2011, Yang, Zhang et al. 2012). One the future direction of this work will be to investigate if stimulation of TNF-α/NF-κB pathway after the higher dose of APAP can improve liver regeneration, recovery and outcome.

Overall, our work established a novel model to study liver regeneration after APAP-induced toxicity in mice. This is first systematic study, to our knowledge, which comprehensively investigated the mechanisms of liver regeneration after APAP-induced hepatotoxicity. Further, our work with incremental doses of APAP revealed dose-dependent differential regulation of pro-regenerative pathways after APAP hepatotoxicity which greatly improved our understanding of the mechanism of liver regeneration compared to single dose models. Our worked identified several potential regulators of liver regeneration after APAP-overdose which will be investigated comprehensively in future for potential development of regenerative therapies. In future, more doses of APAP can be included in this model of regeneration (to make it more robust) in order to gain more insights on differential dose-dependent alteration of liver regeneration and underlying mechanisms. Finally, our studies also indicate that concomitant inhibition of anti-proliferative pathways and activation of pro-regenerative

pathways may have a promising future in developing regenerative therapies for APAP-induced ALF patients.

5.2 ROLE OF β-CATENIN SIGNALING IN LIVER REGENERATION AFTER APAP OVERDOSE

β-catenin has been emerged to be an important player in liver regeneration after PH (Nejak-Bowen and Monga 2011). Various studies in rodent model have demonstrated that liver specific deletion of β-catenin diminishes liver regeneration (Tan, Behari et al. 2006, Sekine, Gutierrez et al. 2007), while overexpression of β-catenin accelerates liver regeneration after PH (Nejak-Bowen, Thompson et al. 2010). Further, our previous work showed that liver regeneration after APAP overdose is impaired in hepatocyte specific β-catenin KO mice (Apte, Singh et al. 2009). Our incremental dose model of APAP demonstrated that β-catenin signaling is inhibited after severe APAP overdose in mice, where liver regeneration is delayed and inhibited. Therefore, we investigated whether stimulation of β-catenin signaling can improve liver regeneration parameters after severe APAP-induced hepatotoxicity. We utilized a transgenic mouse model that overexpresses a stable form of β-catenin in the liver. Our work demonstrated that overexpression of β-catenin can improve liver regeneration parameters even after severe APAP hepatotoxicity secondary to timely induction of Cyclin D1. Importantly, liver injury parameters were not altered by overexpression of β-catenin suggesting regeneration can be modulated independent of injury. Our data show that stimulating β-catenin could be a viable approach for improving liver regeneration in APAP-induced ALF patients. One of the future directions of this work is to screen repurposing libraries

to find novel stimulators of β-catenin signaling and to investigate their potential to improve liver regeneration after APAP hepatotoxicity for development of novel regenerative therapy.

5.3 ROLE OF GSK3 SIGNALING IN LIVER REGENERATION AFTER APAP OVERDOSE

Although our work using transgenic mice demonstrated a role of β-catenin signaling in liver regeneration after APAP-induced hepatotoxicity, we wanted to investigate if we can modulate liver regeneration after APAP overdose by pharmacological interventions. Such translational investigations would be more significant from clinical perspective. GSK3 inhibitors are known to activate β-catenin signaling and are currently under preclinical and clinical investigation for various other pathological conditions (Doble and Woodgett 2003, Kaidanovich-Beilin and Woodgett 2011). However, apart from β-catenin, GKS3 can regulate a myriad of other cell proliferation mediators in both positive and negative manner (Jope and Johnson 2004, Kaidanovich-Beilin and Woodgett 2011, Sutherland 2011). Previous studies using PH model have demonstrated deleterious effect of GSK3 inhibition/ downregulation on liver regeneration (Chen, Yang et al. 2007, Jin, Wang et al. 2009, Sekiya and Suzuki 2011). In contrast, GSK3 inhibition also reported to mediate *in vitro* expansion of hepatocytes (Ito, Kamiya et al. 2012). Our incremental dose model demonstrated that GSK3β is rapidly inactivated preceding robust liver regeneration response after moderate APAP overdose. Inactivation of GSK3β was greatly diminished at the higher dose of APAP, where liver regeneration was remarkably attenuated and delayed. Thus, GSK3β inactivation positively correlated

with β-catenin activation and robust liver regeneration. Therefore, we investigated if inactivation of GSK3β by pharmacological intervention would improve liver regeneration after severe APAP overdose. We demonstrated that treatment with L803-mts, a substrate-competitive GSK3 inhibitor, as late as 4 hr after APAP, can lead to early initiation of liver regeneration after severe APAP overdose. Early cell cycle initiation in hepatocytes after GSK3 inhibition was associated with increased activation of β-catenin signaling. Interestingly, early liver regeneration after GSK3 inhibition was also associated with improved survival. Time window of therapeutic effectiveness of GSK3 inhibitor is expected to be much more in human considering the pathogenesis of APAP hepatotoxicity is generally delayed in humans compared to mice (Xie, McGill et al. 2014). Thus, our study highlights inhibition of GSK3 as a potential therapeutic strategy to improve liver regeneration after APAP-induced ALF. In future, other clinically promising GSK3 inhibitors will be investigated for potential pro-regenerative effects. From mechanistic standpoint, GSK3 KO strategy will be utilized in mice to further substantiate role of GSK3 in liver regeneration after APAP overdose. Further, role of other cell proliferation proteins regulated by GSK3, apart from β-catenin, will be investigated in liver regeneration after APAP overdose.

5.4 ROLE OF EGFR SIGNALING IN LIVER REGENERATION AFTER APAP OVERDOSE

Our incremental dose model revealed that EGFR signaling was remarkably activated after moderate APAP overdose, where injury was followed by robust liver regeneration. However, EGFR signaling was activated even more after higher dose of APAP, where

regeneration was inhibited. Although, this suggested that activated EGFR signaling is not sufficient to promote liver regeneration after severe APAP overdose, activated EGFR signaling may still be playing important role in compensatory liver regeneration after moderate APAP overdose that result in spontaneous recovery. In order to elucidate that, we investigated effect of EGFR inhibition (using specific pan-EGFR inhibitor, Canertinib) on liver regeneration after APAP overdose. To our surprise, early EGFR inhibition (1 hr post-APAP) strikingly attenuated APAP hepatotoxicity suggesting role of EGFR in development of APAP hepatotoxicity. Since liver regeneration is a compensatory response to liver injury after APAP toxicity, any alteration in liver injury can indirectly affect regeneration. Thus, direct role of EGFR inhibition on liver regeneration could not be tested. In order to investigate a direct role of EGFR activation on liver regeneration after APAP overdose, a delayed EGFR inhibition strategy (12 hr post-APAP) was utilized, such that APAP hepatotoxicity was already established. We demonstrated that delayed inhibition of EGFR almost completely abolished compensatory hepatocyte proliferation and decreased survival after moderate APAP overdose, which normally results in robust liver regeneration and spontaneous recovery. Failure of cell cycle progression was due to complete inhibition of cyclin D1 induction. Thus, our study established a critical role of EGFR signaling in liver regeneration after moderate APAP overdose. A number of previous studies in the PH model of liver regeneration show that inhibition or elimination of a single pathways can only delay or diminish liver regeneration but cannot permanently abolish liver regeneration (Michalopoulos 2007). Along similar lines, genetic ablation of EGFR or siRNA-mediated downregulation resulted in delayed liver regeneration after PH, which was eventually

compensated by upregulation of other pathways (Natarajan, Wagner et al. 2007, Paranjpe, Bowen et al. 2010). In contrast, in our study, delayed inhibition of EGFR after APAP overdose resulted in almost complete inhibition of liver regeneration, resulting in decreased survival. Thus, our data further highlight that the dynamics and mechanisms of liver regeneration are very different in APAP-induced ALF model (compared to PH), which advances very rapidly such that a timely regenerative response is imminently critical for final recovery.

5.5 CONCLUDING REMARKS

Several recent studies have demonstrated a critical role of timely liver regeneration in determining the final outcome after APAP overdose (Hu and Colletti 2008, Apte, Singh et al. 2009, Donahower, McCullough et al. 2010). Thus, stimulating liver regeneration in patients of APAP-induced ALF holds a great therapeutic potential. However, development of novel regenerative therapies for ALF is hampered because the mechanisms of liver regeneration after APAP-induced ALF have not extensively explored yet. Based on the dose-response principles, our work established a novel incremental dose model to identify potential regulators of liver regeneration after APAP overdose in mice. Our incremental dose model of APAP overdose showed that robust liver regeneration after moderate APAP overdose was associated with complete spontaneous recovery, while inhibited and delayed liver regeneration after severe APAP overdose was associated with impaired recovery and decreased survival. Further, several pro-regenerative signaling pathways (including β-catenin signaling) were differentially activated after moderate APAP overdose correlating with spontaneous

recovery and inhibited after severe APAP overdose correlating with impaired liver regeneration. Stimulating these pathways could prove to be a viable approach for improving liver regeneration and recovery after severe APAP hepatotoxicity, where spontaneous recovery is impaired. Indeed, our work demonstrated that stimulation of β-catenin signaling in mice can improve liver regeneration after severe APAP overdose. Moreover, our work established that inhibition of GSK3 (which is a negative regulator of β-catenin signaling) can lead to early liver regeneration and improvement in survival after APAP overdose in mice.

Surprisingly, our incremental dose model revealed that several other known regenerative pathways (including growth factors signaling pathways such as EGFR signaling) were dose-dependently activated and remain highly activated even after severe APAP overdose, where liver regeneration is impaired. This suggests that activation of these pathways is not sufficient to promote liver regeneration after severe APAP overdose. These signaling pathways may still be playing important role in compensatory liver regeneration after moderate APAP overdose, which normally results in spontaneous recovery. Indeed, our work demonstrated that inhibition of EGFR after moderate APAP overdose can lead to impairment of liver regeneration and decreased survival.

Overall, our incremental dose model will be critical in improving mechanistic understanding of the differences between spontaneous transplant free survival vs transplant-assisted survival and ALF-related deaths observed in APAP overdose patients. Further, our work demonstrates that stimulating liver regeneration could be a

viable therapeutic strategy and justifies further investigation of novel therapeutic strategies to stimulate liver regeneration after APAP-induced hepatotoxicity, which are currently extremely underexplored. Regenerative therapies at least could be helpful in bridging the time-gap before liver is available for transplantation to ALF patients. To conclude, this is the first systematic study to our knowledge, which comprehensively investigated the mechanisms of liver regeneration after APAP-induced ALF and will pave the road for future therapeutic and mechanistic research on liver regeneration after APAP-induced ALF.

CHAPTER VI: REFERENCES

Anand, S. S., S. N. Murthy, V. S. Vaidya, M. M. Mumtaz and H. M. Mehendale (2003). "Tissue repair plays pivotal role in final outcome of liver injury following chloroform and allyl alcohol binary mixture." Food Chem Toxicol 41(8): 1123-1132.

Anand, S. S., M. G. Soni, V. S. Vaidya, S. N. Murthy, M. M. Mumtaz and H. M. Mehendale (2003). "Extent and timeliness of tissue repair determines the dose-related hepatotoxicity of chloroform." Int J Toxicol 22(1): 25-33.

Apte, U., S. Singh, G. Zeng, B. Cieply, M. A. Virji, T. Wu and S. P. Monga (2009). "Beta-catenin activation promotes liver regeneration after acetaminophen-induced injury." Am J Pathol 175(3): 1056-1065.

Apte, U. M., P. B. Limaye, D. Desaiah, T. J. Bucci, A. Warbritton and H. M. Mehendale (2003). "Mechanisms of increased liver tissue repair and survival in diet-restricted rats treated with equitoxic doses of thioacetamide." Toxicol Sci 72(2): 272-282.

Arany, I. (2008). "Dual role of the activated epidermal growth factor receptor in renal tubular cells during stress." Kidney Int 73(1): 5-7.

Arany, I., J. K. Megyesi, H. Kaneto, P. M. Price and R. L. Safirstein (2004). "Cisplatin-induced cell death is EGFR/src/ERK signaling dependent in mouse proximal tubule cells." Am J Physiol Renal Physiol 287(3): F543-549.

Athuraliya, T. N. and A. L. Jones (2009). "Prolonged N-acetylcysteine therapy in late acetaminophen poisoning associated with acute liver failure--a need to be more cautious?" Crit Care 13(3): 144.

Bajt, M. L., T. R. Knight, A. Farhood and H. Jaeschke (2003). "Scavenging peroxynitrite with glutathione promotes regeneration and enhances survival during acetaminophen-induced liver injury in mice." J Pharmacol Exp Ther 307(1): 67-73.

Bernal, W., W. M. Lee, J. Wendon, F. S. Larsen and R. Williams (2015). "Acute liver failure: A curable disease by 2024?" J Hepatol 62(1 Suppl): S112-120.

Bernal, W. and J. Wendon (2013). "Acute liver failure." N Engl J Med 369(26): 2525-2534.

Bhushan, B., P. Borude, G. Edwards, C. Walesky, J. Cleveland, F. Li, X. C. Ma and U. Apte (2013). "Role of Bile Acids in Liver Injury and Regeneration following Acetaminophen Overdose." American Journal of Pathology 183(5): 1518-1526.

Bhushan, B., C. Walesky, M. Manley, T. Gallagher, P. Borude, G. Edwards, S. P. Monga and U. Apte (2014). "Pro-regenerative signaling after acetaminophen-induced acute liver injury in mice identified using a novel incremental dose model." Am J Pathol 184(11): 3013-3025.

Boerner, J. L., M. L. Demory, C. Silva and S. J. Parsons (2004). "Phosphorylation of Y845 on the epidermal growth factor receptor mediates binding to the mitochondrial protein cytochrome c oxidase subunit II." Mol Cell Biol 24(16): 7059-7071.

Bourdi, M., M. C. Korrapati, M. Chakraborty, S. B. Yee and L. R. Pohl (2008). "Protective role of c-Jun N-terminal kinase 2 in acetaminophen-induced liver injury." Biochem Biophys Res Commun 374(1): 6-10.

Bucher, N. L., U. Patel and S. Cohen (1977). "Hormonal factors concerned with liver regeneration." Ciba Found Symp(55): 95-107.

Budnitz, D. S., M. C. Lovegrove and A. E. Crosby (2011). "Emergency department visits for overdoses of acetaminophen-containing products." Am J Prev Med 40(6): 585-592.

Buitrago-Molina, L. E., S. Marhenke, T. Longerich, A. D. Sharma, A. E. Boukouris, R. Geffers, B. Guigas, M. P. Manns and A. Vogel (2013). "The degree of liver injury determines the role of p21 in liver regeneration and hepatocarcinogenesis in mice." Hepatology 58(3): 1143-1152.

Carver, R. S., M. C. Stevenson, L. A. Scheving and W. E. Russell (2002). "Diverse expression of ErbB receptor proteins during rat liver development and regeneration." Gastroenterology 123(6): 2017-2027.

Chen, H., S. Yang, Z. Yang, L. Ma, D. Jiang, J. Mao, B. Jiao and Z. Cai (2007). "Inhibition of GSK-3beta decreases NF-kappaB-dependent gene expression and impairs the rat liver regeneration." J Cell Biochem 102(5): 1281-1289.

Chiu, H., C. R. Gardner, D. M. Dambach, S. K. Durham, J. A. Brittingham, J. D. Laskin and D. L. Laskin (2003). "Role of tumor necrosis factor receptor 1 (p55) in hepatocyte proliferation during acetaminophen-induced toxicity in mice." Toxicol Appl Pharmacol 193(2): 218-227.

Clevers, H. (2006). "Wnt/beta-catenin signaling in development and disease." Cell 127(3): 469-480.

Demory, M. L., J. L. Boerner, R. Davidson, W. Faust, T. Miyake, I. Lee, M. Huttemann, R. Douglas, G. Haddad and S. J. Parsons (2009). "Epidermal growth factor receptor translocation to the mitochondria: regulation and effect." J Biol Chem 284(52): 36592-36604.

Diehl, J. A., M. Cheng, M. F. Roussel and C. J. Sherr (1998). "Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization." Genes Dev 12(22): 3499-3511.

Doble, B. W. and J. R. Woodgett (2003). "GSK-3: tricks of the trade for a multi-tasking kinase." J Cell Sci 116(Pt 7): 1175-1186.

Donahower, B., S. S. McCullough, R. Kurten, L. W. Lamps, P. Simpson, J. A. Hinson and L. P. James (2006). "Vascular endothelial growth factor and hepatocyte regeneration in acetaminophen toxicity." Am J Physiol Gastrointest Liver Physiol 291(1): G102-109.

Donahower, B. C., S. S. McCullough, L. Hennings, P. M. Simpson, C. D. Stowe, A. G. Saad, R. C. Kurten, J. A. Hinson and L. P. James (2010). "Human recombinant vascular endothelial growth factor reduces necrosis and enhances hepatocyte regeneration in a mouse model of acetaminophen toxicity." J Pharmacol Exp Ther 334(1): 33-43.

Fausto, N. (2000). "Liver regeneration." J Hepatol 32(1 Suppl): 19-31.

Fausto, N., J. S. Campbell and K. J. Riehle (2006). "Liver regeneration." Hepatology 43(2 Suppl 1): S45-53.

Fausto, N., J. S. Campbell and K. J. Riehle (2012). "Liver regeneration." J Hepatol 57(3): 692-694.

Francavilla, A., A. Azzarone, G. Carrieri, U. Cillo, D. Van Thiel, V. Subbottin and T. E. Starzl (1993). "Administration of hepatic stimulatory substance alone or with other liver growth factors does not ameliorate acetaminophen-induced liver failure." Hepatology 17(3): 429-433.

Gotschel, F., C. Kern, S. Lang, T. Sparna, C. Markmann, J. Schwager, S. McNelly, F. von Weizsacker, S. Laufer, A. Hecht and I. Merfort (2008). "Inhibition of GSK3 differentially modulates NF-kappaB, CREB, AP-1 and beta-catenin signaling in hepatocytes, but fails to promote TNF-alpha-induced apoptosis." Exp Cell Res 314(6): 1351-1366.

Guttridge, D. C., C. Albanese, J. Y. Reuther, R. G. Pestell and A. S. Baldwin, Jr. (1999). "NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1." Mol Cell Biol 19(8): 5785-5799.

Hanawa, N., M. Shinohara, B. Saberi, W. A. Gaarde, D. Han and N. Kaplowitz (2008). "Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury." J Biol Chem 283(20): 13565-13577.

Horimoto, M., P. Fulop, Z. Derdak, J. R. Wands and G. Baffy (2004). "Uncoupling protein-2 deficiency promotes oxidant stress and delays liver regeneration in mice." Hepatology 39(2): 386-392.

Hu, B. and L. M. Colletti (2008). "Stem cell factor and c-kit are involved in hepatic recovery after acetaminophen-induced liver injury in mice." Am J Physiol Gastrointest Liver Physiol 295(1): G45-G53.

Hughes, R. D., L. Zhang, H. Tsubouchi, Y. Daikuhara and R. Williams (1994). "Plasma hepatocyte growth factor and biliprotein levels and outcome in fulminant hepatic failure." J Hepatol 20(1): 106-111.

Ito, H., A. Kamiya, K. Ito, A. Yanagida, K. Okada and H. Nakauchi (2012). "In vitro expansion and functional recovery of mature hepatocytes from mouse adult liver." Liver Int 32(4): 592-601.

Jaeschke, H., M. R. McGill and A. Ramachandran (2012). "Oxidant stress, mitochondria, and cell death mechanisms in drug-induced liver injury: lessons learned from acetaminophen hepatotoxicity." Drug Metab Rev 44(1): 88-106.

Jaeschke, H., M. R. McGill, C. D. Williams and A. Ramachandran (2011). "Current issues with acetaminophen hepatotoxicity--a clinically relevant model to test the efficacy of natural products." Life Sci 88(17-18): 737-745.

Jaeschke, H., C. D. Williams, A. Ramachandran and M. L. Bajt (2012). "Acetaminophen hepatotoxicity and repair: the role of sterile inflammation and innate immunity." Liver Int 32(1): 8-20.

Jaeschke, H., Y. Xie and M. R. McGill (2014). "Acetaminophen-induced Liver Injury: from Animal Models to Humans." J Clin Transl Hepatol 2(3): 153-161.

James, L. P., R. C. Kurten, L. W. Lamps, S. McCullough and J. A. Hinson (2005). "Tumour necrosis factor receptor 1 and hepatocyte regeneration in acetaminophen toxicity: a kinetic study of proliferating cell nuclear antigen and cytokine expression." Basic Clin Pharmacol Toxicol 97(1): 8-14.

James, L. P., L. W. Lamps, S. McCullough and J. A. Hinson (2003). "Interleukin 6 and hepatocyte regeneration in acetaminophen toxicity in the mouse." Biochem Biophys Res Commun 309(4): 857-863.

James, L. P., S. S. McCullough, L. W. Lamps and J. A. Hinson (2003). "Effect of N-acetylcysteine on acetaminophen toxicity in mice: relationship to reactive nitrogen and cytokine formation." Toxicol Sci 75(2): 458-467.

Jin, J., G. L. Wang, X. Shi, G. J. Darlington and N. A. Timchenko (2009). "The age-associated decline of glycogen synthase kinase 3beta plays a critical role in the inhibition of liver regeneration." Mol Cell Biol 29(14): 3867-3880.

Jope, R. S. and G. V. Johnson (2004). "The glamour and gloom of glycogen synthase kinase-3." Trends Biochem Sci 29(2): 95-102.

Kaidanovich-Beilin, O. and J. R. Woodgett (2011). "GSK-3: Functional Insights from Cell Biology and Animal Models." Front Mol Neurosci 4(40): 16.

Kato, T., Y. Ito, K. Hosono, T. Suzuki, H. Tamaki, T. Minamino, S. Kato, H. Sakagami, M. Shibuya and M. Majima (2011). "Vascular endothelial growth factor receptor-1 signaling promotes liver repair through restoration of liver microvasculature after acetaminophen hepatotoxicity." Toxicol Sci 120(1): 218-229.

Keeffe, E. B. (2001). "Liver transplantation: current status and novel approaches to liver replacement." Gastroenterology 120(3): 749-762.

Kofman, A. V., G. Morgan, A. Kirschenbaum, J. Osbeck, M. Hussain, S. Swenson and N. D. Theise (2005). "Dose- and time-dependent oval cell reaction in acetaminophen-induced murine liver injury." Hepatology 41(6): 1252-1261.

Koteish, A., S. Yang, H. Lin, J. Huang and A. M. Diehl (2002). "Ethanol induces redox-sensitive cell-cycle inhibitors and inhibits liver regeneration after partial hepatectomy." Alcohol Clin Exp Res 26(11): 1710-1718.

Larson, A. M. (2007). "Acetaminophen hepatotoxicity." Clin Liver Dis 11(3): 525-548, vi.

Lavoie, J. N., G. L'Allemain, A. Brunet, R. Muller and J. Pouyssegur (1996). "Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway." J Biol Chem 271(34): 20608-20616.

Lee, W. M. (2012). "Acute liver failure." Semin Respir Crit Care Med 33(1): 36-45.

Lee, W. M. (2012). "Liver: determining prognosis in acute liver failure." Nat Rev Gastroenterol Hepatol 9(4): 192-194.

Lee, W. M., R. H. Squires, Jr., S. L. Nyberg, E. Doo and J. H. Hoofnagle (2008). "Acute liver failure: Summary of a workshop." Hepatology 47(4): 1401-1415.

Mangipudy, R. S., S. Chanda and H. M. Mehendale (1995). "Tissue repair response as a function of dose in thioacetamide hepatotoxicity." Environ Health Perspect 103(3): 260-267.

Manthripragada, A. D., E. H. Zhou, D. S. Budnitz, M. C. Lovegrove and M. E. Willy (2011). "Characterization of acetaminophen overdose-related emergency department visits and hospitalizations in the United States." Pharmacoepidemiol Drug Saf 20(8): 819-826.

Masubuchi, Y., M. Bourdi, T. P. Reilly, M. L. Graf, J. W. George and L. R. Pohl (2003). "Role of interleukin-6 in hepatic heat shock protein expression and protection against acetaminophen-induced liver disease." Biochem Biophys Res Commun 304(1): 207-212.

Mehendale, H. M. (2005). "Tissue repair: an important determinant of final outcome of toxicant-induced injury." Toxicol Pathol 33(1): 41-51.

Meyers, L. L., W. P. Beierschmitt, E. A. Khairallah and S. D. Cohen (1988). "Acetaminophen-induced inhibition of hepatic mitochondrial respiration in mice." Toxicol Appl Pharmacol 93(3): 378-387.

Michalopoulos, G. K. (2007). "Liver regeneration." J Cell Physiol 213(2): 286-300.

Michalopoulos, G. K. (2010). "Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas." Am J Pathol 176(1): 2-13.

Michalopoulos, G. K. (2011). "Liver regeneration: alternative epithelial pathways." Int J Biochem Cell Biol 43(2): 173-179.

Michalopoulos, G. K. (2013). "Principles of liver regeneration and growth homeostasis." Compr Physiol 3(1): 485-513.

Michalopoulos, G. K. (2014). "Advances in liver regeneration." Expert Rev Gastroenterol Hepatol 8(8): 897-907.

Michalopoulos, G. K. and M. C. DeFrances (1997). "Liver regeneration." Science 276(5309): 60-66.

Michalopoulos, G. K. and Z. Khan (2005). "Liver regeneration, growth factors, and amphiregulin." Gastroenterology 128(2): 503-506.

Monga, S. P., P. Pediaditakis, K. Mule, D. B. Stolz and G. K. Michalopoulos (2001). "Changes in WNT/beta-catenin pathway during regulated growth in rat liver regeneration." Hepatology 33(5): 1098-1109.

Natarajan, A., B. Wagner and M. Sibilia (2007). "The EGF receptor is required for efficient liver regeneration." Proc Natl Acad Sci U S A 104(43): 17081-17086.

Nejak-Bowen, K. N. and S. P. Monga (2011). "Beta-catenin signaling, liver regeneration and hepatocellular cancer: sorting the good from the bad." Semin Cancer Biol 21(1): 44-58.

Nejak-Bowen, K. N., M. D. Thompson, S. Singh, W. C. Bowen, Jr., M. J. Dar, J. Khillan, C. Dai and S. P. Monga (2010). "Accelerated liver regeneration and hepatocarcinogenesis in mice overexpressing serine-45 mutant beta-catenin." Hepatology 51(5): 1603-1613.

Nelson, S. D. (1990). "Molecular mechanisms of the hepatotoxicity caused by acetaminophen." Semin Liver Dis 10(4): 267-278.

Normanno, N., A. De Luca, C. Bianco, L. Strizzi, M. Mancino, M. R. Maiello, A. Carotenuto, G. De Feo, F. Caponigro and D. S. Salomon (2006). "Epidermal growth factor receptor (EGFR) signaling in cancer." Gene 366(1): 2-16.

Papastefanou, V. P., E. Bozas, M. G. Mykoniatis, A. Grypioti, S. Garyfallidis, C. S. Bartsocas and P. Nicolopoulou-Stamati (2007). "VEGF isoforms and receptors expression throughout acute acetaminophen-induced liver injury and regeneration." Arch Toxicol 81(10): 729-741.

Paranjpe, S., W. C. Bowen, G. C. Tseng, J. H. Luo, A. Orr and G. K. Michalopoulos (2010). "RNA interference against hepatic epidermal growth factor receptor has suppressive effects on liver regeneration in rats." Am J Pathol 176(6): 2669-2681.

Plotkin, B., O. Kaidanovich, I. Talior and H. Eldar-Finkelman (2003). "Insulin mimetic action of synthetic phosphorylated peptide inhibitors of glycogen synthase kinase-3." J Pharmacol Exp Ther 305(3): 974-980.

Rao, P. S., R. S. Mangipudy and H. M. Mehendale (1997). "Tissue injury and repair as parallel and opposing responses to CCl4 hepatotoxicity: a novel dose-response." Toxicology 118(2-3): 181-193.

Rao, R., C. M. Hao, R. Redha, D. H. Wasserman, O. P. McGuinness and M. D. Breyer (2007). "Glycogen synthase kinase 3 inhibition improves insulin-stimulated glucose metabolism but not hypertension in high-fat-fed C57BL/6J mice." Diabetologia 50(2): 452-460.

Reinehr, R., S. Becker, A. Eberle, S. Grether-Beck and D. Haussinger (2005). "Involvement of NADPH oxidase isoforms and Src family kinases in CD95-dependent hepatocyte apoptosis." J Biol Chem 280(29): 27179-27194.

Reinehr, R., S. Becker, A. Hongen and D. Haussinger (2004). "The Src family kinase Yes triggers hyperosmotic activation of the epidermal growth factor receptor and CD95." J Biol Chem 279(23): 23977-23987.

Reinehr, R., S. Becker, V. Keitel, A. Eberle, S. Grether-Beck and D. Haussinger (2005). "Bile salt-induced apoptosis involves NADPH oxidase isoform activation." Gastroenterology 129(6): 2009-2031.

Reinehr, R., D. Graf and D. Haussinger (2003). "Bile salt-induced hepatocyte apoptosis involves epidermal growth factor receptor-dependent CD95 tyrosine phosphorylation." Gastroenterology 125(3): 839-853.

Reinehr, R. and D. Haussinger (2009). "Epidermal growth factor receptor signaling in liver cell proliferation and apoptosis." Biol Chem 390(10): 1033-1037.

Reinehr, R. and D. Haussinger (2012). "CD95 death receptor and epidermal growth factor receptor (EGFR) in liver cell apoptosis and regeneration." Arch Biochem Biophys 518(1): 2-7.

Reinehr, R., F. Schliess and D. Haussinger (2003). "Hyperosmolarity and CD95L trigger CD95/EGF receptor association and tyrosine phosphorylation of CD95 as prerequisites for CD95 membrane trafficking and DISC formation." FASEB J 17(6): 731-733.

Saberi, B., M. D. Ybanez, H. S. Johnson, W. A. Gaarde, D. Han and N. Kaplowitz (2014). "Protein kinase C (PKC) participates in acetaminophen hepatotoxicity through c-jun-N-terminal kinase (JNK)-dependent and -independent signaling pathways." Hepatology 59(4): 1543-1554.

Sarro, E., O. Tornavaca, M. Plana, A. Meseguer and E. Itarte (2008). "Phosphoinositide 3-kinase inhibitors protect mouse kidney cells from cyclosporine-induced cell death." Kidney Int 73(1): 77-85.

Scheving, L. A., X. Zhang, M. C. Stevenson, D. W. Threadgill and W. E. Russell (2015). "Loss of hepatocyte EGFR has no effect alone but exacerbates carbon tetrachloride-induced liver injury and impairs regeneration in hepatocyte Met-deficient mice." Am J Physiol Gastrointest Liver Physiol 308(5): G364-377.

Schmidt, L. E. and K. Dalhoff (2005). "Alpha-fetoprotein is a predictor of outcome in acetaminophen-induced liver injury." Hepatology 41(1): 26-31.

Schwabe, R. F., C. A. Bradham, T. Uehara, E. Hatano, B. L. Bennett, R. Schoonhoven and D. A. Brenner (2003). "c-Jun-N-terminal kinase drives cyclin D1 expression and proliferation during liver regeneration." Hepatology 37(4): 824-832.

Sekine, S., P. J. Gutierrez, B. Y. Lan, S. Feng and M. Hebrok (2007). "Liver-specific loss of beta-catenin results in delayed hepatocyte proliferation after partial hepatectomy." Hepatology 45(2): 361-368.

Sekiya, S. and A. Suzuki (2011). "Glycogen synthase kinase 3 beta-dependent Snail degradation directs hepatocyte proliferation in normal liver regeneration." Proc Natl Acad Sci U S A 108(27): 11175-11180.

Shankar, K., V. S. Vaidya, U. M. Apte, J. E. Manautou, M. J. Ronis, T. J. Bucci and H. M. Mehendale (2003). "Type 1 diabetic mice are protected from acetaminophen hepatotoxicity." Toxicol Sci 73(2): 220-234.

Shen, K., W. Chang, X. Gao, H. Wang, W. Niu, L. Song and X. Qin (2011). "Depletion of activated hepatic stellate cell correlates with severe liver damage and abnormal liver regeneration in acetaminophen-induced liver injury." Acta Biochim Biophys Sin (Shanghai) 43(4): 307-315.

Shinohara, M., M. D. Ybanez, S. Win, T. A. Than, S. Jain, W. A. Gaarde, D. Han and N. Kaplowitz (2010). "Silencing glycogen synthase kinase-3beta inhibits acetaminophen hepatotoxicity and attenuates JNK activation and loss of glutamate cysteine ligase and myeloid cell leukemia sequence 1." J Biol Chem 285(11): 8244-8255.

Simpson, K., C. M. Hogaboam, S. L. Kunkel, D. J. Harrison, C. Bone-Larson and N. W. Lukacs (2003). "Stem cell factor attenuates liver damage in a murine model of acetaminophen-induced hepatic injury." Lab Invest 83(2): 199-206.

Slichenmyer, W. J., W. L. Elliott and D. W. Fry (2001). "CI-1033, a pan-erbB tyrosine kinase inhibitor." Semin Oncol 28(5 Suppl 16): 80-85.

Sodhi, D., A. Micsenyi, W. C. Bowen, D. K. Monga, J. C. Talavera and S. P. Monga (2005). "Morpholino oligonucleotide-triggered beta-catenin knockdown compromises normal liver regeneration." J Hepatol 43(1): 132-141.

Sommerfeld, A., R. Reinehr and D. Haussinger (2009). "Bile acid-induced epidermal growth factor receptor activation in quiescent rat hepatic stellate cells can trigger both proliferation and apoptosis." J Biol Chem 284(33): 22173-22183.

Sutherland, C. (2011). "What Are the bona fide GSK3 Substrates?" Int J Alzheimers Dis 2011: 505607.

Tan, X., J. Behari, B. Cieply, G. K. Michalopoulos and S. P. Monga (2006). "Conditional deletion of beta-catenin reveals its role in liver growth and regeneration." Gastroenterology 131(5): 1561-1572.

Theise, N. D., R. Saxena, B. C. Portmann, S. N. Thung, H. Yee, L. Chiriboga, A. Kumar and J. M. Crawford (1999). "The canals of Hering and hepatic stem cells in humans." Hepatology 30(6): 1425-1433.

Thompson, M. D. and S. P. Monga (2007). "WNT/beta-catenin signaling in liver health and disease." Hepatology 45(5): 1298-1305.

Tirmenstein, M. A. and S. D. Nelson (1989). "Subcellular binding and effects on calcium homeostasis produced by acetaminophen and a nonhepatotoxic regioisomer, 3'-hydroxyacetanilide, in mouse liver." J Biol Chem 264(17): 9814-9819.

Torbenson, M., S. Q. Yang, H. Z. Liu, J. Huang, W. Gage and A. M. Diehl (2002). "STAT-3 overexpression and p21 up-regulation accompany impaired regeneration of fatty livers." Am J Pathol 161(1): 155-161.

Torre, C., S. Benhamouche, C. Mitchell, C. Godard, P. Veber, F. Letourneur, N. Cagnard, S. Jacques, L. Finzi, C. Perret and S. Colnot (2011). "The transforming growth factor-alpha and cyclin D1 genes are direct targets of beta-catenin signaling in hepatocyte proliferation." J Hepatol 55(1): 86-95.

Viatour, P., M. P. Merville, V. Bours and A. Chariot (2005). "Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation." Trends Biochem Sci 30(1): 43-52.

Walesky, C., S. Gunewardena, E. F. Terwilliger, G. Edwards, P. Borude and U. Apte (2013). "Hepatocyte-specific deletion of hepatocyte nuclear factor-4alpha in adult mice results in increased hepatocyte proliferation." Am J Physiol Gastrointest Liver Physiol 304(1): G26-37.

Win, S., T. A. Than, D. Han, L. M. Petrovic and N. Kaplowitz (2011). "c-Jun N-terminal kinase (JNK)-dependent acute liver injury from acetaminophen or tumor necrosis factor (TNF) requires mitochondrial Sab protein expression in mice." J Biol Chem 286(40): 35071-35078.

Wolfe, A., A. Thomas, G. Edwards, R. Jaseja, G. L. Guo and U. Apte (2011). "Increased activation of the Wnt/beta-catenin pathway in spontaneous hepatocellular carcinoma observed in farnesoid X receptor knockout mice." J Pharmacol Exp Ther 338(1): 12-21.

Wustefeld, T., T. Rakemann, S. Kubicka, M. P. Manns and C. Trautwein (2000). "Hyperstimulation with interleukin 6 inhibits cell cycle progression after hepatectomy in mice." Hepatology 32(3): 514-522.

Xie, Y., M. R. McGill, K. Dorko, S. C. Kumer, T. M. Schmitt, J. Forster and H. Jaeschke (2014). "Mechanisms of acetaminophen-induced cell death in primary human hepatocytes." Toxicol Appl Pharmacol 279(3): 266-274.

Xie, Y., A. Ramachandran, D. G. Breckenridge, J. T. Liles, M. Lebofsky, A. Farhood and H. Jaeschke (2015). "Inhibitor of apoptosis signal-regulating kinase 1 protects against acetaminophen-induced liver injury." Toxicol Appl Pharmacol 286(1): 1-9.

Xie, Y., C. D. Williams, M. R. McGill, M. Lebofsky, A. Ramachandran and H. Jaeschke (2013). "Purinergic receptor antagonist A438079 protects against acetaminophen-induced liver injury by inhibiting p450 isoenzymes, not by inflammasome activation." Toxicol Sci 131(1): 325-335.

Yang, R., K. Miki, X. He, M. E. Killeen and M. P. Fink (2009). "Prolonged treatment with N-acetylcystine delays liver recovery from acetaminophen hepatotoxicity." Crit Care 13(2): 9.

Yang, R., S. Zhang, A. Cotoia, N. Oksala, S. Zhu and J. Tenhunen (2012). "High mobility group B1 impairs hepatocyte regeneration in acetaminophen hepatotoxicity." BMC Gastroenterol 12: 45.

Yang, R., S. Zhang, H. Kajander, S. Zhu, M. L. Koskinen and J. Tenhunen (2011). "Ringer's lactate improves liver recovery in a murine model of acetaminophen toxicity." BMC Gastroenterol 11: 125.

Yang, R., X. Zou, M. L. Koskinen and J. Tenhunen (2012). "Ethyl pyruvate reduces liver injury at early phase but impairs regeneration at late phase in acetaminophen overdose." Crit Care 16(1): R9.

Zaher, H., J. T. Buters, J. M. Ward, M. K. Bruno, A. M. Lucas, S. T. Stern, S. D. Cohen and F. J. Gonzalez (1998). "Protection against acetaminophen toxicity in CYP1A2 and CYP2E1 double-null mice." Toxicol Appl Pharmacol 152(1): 193-199.

Zeng, G., F. Awan, W. Otruba, P. Muller, U. Apte, X. Tan, C. Gandhi, A. J. Demetris and S. P. Monga (2007). "Wnt'er in liver: expression of Wnt and frizzled genes in mouse." Hepatology 45(1): 195-204.