WNT/BETA-CATENIN SIGNALING IN LIVER HOMEOSTASIS AND REGENERATION

by

Jing Yang

MD, Peking University, 2010

Submitted to the Graduate Faculty of

the School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2014

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

This dissertation was presented

by

Jing Yang

It was defended on

August 25, 2014

and approved by

Chairperson: Dr. Wendy M. Mars, PhD, Associate Professor, Department of Pathology

Dr. Youhua Liu, PhD, Professor, Department of Pathology

Dr. George Michalopoulos, MD, PhD, Professor and Chair, Department of Pathology

Dr. Donghun Shin, PhD, Assistant Professor, Department of Developmental Biology

Thesis Advisor: Dr. Satdarshan P.S. Monga, MD, Professor and Vice Chair, Division of Experimental Pathology, Department of Pathology

WNT/BETA-CATENIN SIGNALING IN LIVER HOMEOSTASIS AND REGENERATION

Jing Yang, MD

University of Pittsburgh, 2014

Liver-specific *β-catenin* knockout (β-catenin-LKO) mice have revealed an essential role of β-catenin in metabolic zonation where it regulates pericentral gene expression and in initiating liver regeneration (LR) after partial hepatectomy (PH), by regulating expression of Cyclin-D1. However what regulates β-catenin activity in these events remains an enigma. Here, we investigate to what extent β-catenin activation is Wnt-signaling dependent and the potential cell source of Wnt ligands. We studied liver-specific *Lrp5/6* KO (Lrp-LKO) mice where Wnt-signaling was abolished in hepatocytes while the *β-catenin* gene remained intact. Intriguingly, like β-catenin-LKO mice, Lrp-LKO exhibited a defect in metabolic zonation observed as lack of glutamine synthetase (GS), Cyp1a2 and Cyp2e1. Lrp-LKO also displayed a significant delay in initiation of LR due to absence of β-catenin-TCF4 association and lack of Cyclin-D1. Given the important role of Wnt signaling, we further aimed to address the cellular source of Wnt ligands in liver. Q-PCR analysis showed distinct Wnt expression pattern in individual hepatic cell types before and after PH. To elucidate the role of different liver cell populations in Wnt secretion, we investigated conditional *Wntless* (*Wls*) KO mice, which lacked ability to secrete Wnts from hepatic cell populations, including liver epithelial cells (*Wls*-LKO), hepatic stellate cells (*Wls*-SKO), macrophages including Kupffer cells (*Wls*-MKO), and endothelial cells (*Wls*-EKO). *Wls*-LKO, *Wls*-SKO and *Wls*-MKO did not show any defect in hepatic zonation. *Wls*-EKO had decreased mRNA expression of zonal genes, which suggests that endothelial cells derived Wnts

may be important for maintaining zonation. After PH, *Wls*-LKO and *Wls*-SKO showed normal initiation of LR; however, *Wls*-MKO and *Wls*-EKO showed a significant deficit in LR. At later time points after PH, *Wls*-LKO had temporarily enhanced LR. Our experiments show that hepatocytes are capable of secreting Wnt5a during late stage of LR, which has an antagonistic role on Wnt/β-catenin pathway, while the hepatocytes from *Wls*-LKO cannot. Conclusion: Wnt-signaling is the major upstream effector of β-catenin activity in pericentral hepatocytes and during LR. Hepatocytes, cholangiocytes, hepatic stellate cells or macrophages are not the source of Wnts in regulating hepatic zonation, while endothelial cells may be involved in this process. On the other hand, Kupffer cells and endothelial cells are major contributing sources of Wnt secretion necessary for β-catenin activation during LR. Hepatocytes might secrete inhibitory Wnts involved in the termination of LR.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

Acknowledgements

Four years of graduate study is coming to the end; it has been a long way but everything I have been through stays fresh in my mind as just happened yesterday. I was a medical student with very limited research experience and flew thousands miles to come here to pursue my PhD, with enthusiasm, courage and curiosity towards the unknown. The four-year graduate study is not only a training process where I learned how to be an independent researcher, but also an invaluable experience in my life where I learned how to be a strong and mature person.

First of all, I would like to express sincere gratitude to my mentor, Dr. Paul Monga. I feel really fortunate to have him as my advisor, since his knowledge, vision, kindness and support have always helped bring out the best in myself. He has always been encouraging and supportive throughout my graduate study. His professional guidance and support on my project enabled me to conquer difficulties and finish high quality research, which I truly and deeply appreciate. He taught me to explore the unknown with the greatest enthusiasm and patience, while enjoying research with an optimistic mind. He has built an encouraging and supportive environment in the lab where trainees really enjoy working. Thanks to him, the four-year graduate study has been a very enjoyable journey for me.

Secondly, I would like to thank the current and past members of the Monga lab. It is the teamwork but rather my individual work that makes this dissertation possible. In particular, I would like to thank Dr. Hirohisa Okabe for his valuable advice and support of my research. He has spent countless hours helping me with experiments and troubleshooting problems. His input on my project and the approaches and techniques he taught me truly benefited my research tremendously.

And last, on a more personal level, I would like to express my sincerest love and appreciation to my parents, for the unconditional love and sacrifice they've offered in my life. Despite the physical distance between us during the past four years, I have always felt their support, which has made it possible for me to be able to complete my PhD studies.

1.0 INTRODUCTION

β-catenin is a transcriptional co-activator that regulates genes critical for cell proliferation and differentiation. β-catenin signaling plays a critical role in liver development, liver metabolism & regeneration, and aberrant β-catenin signaling is also associated with liver cancer [1]. The major pathway that activates β-catenin is the canonical Wnt/β-catenin signaling pathway. Additional signaling pathways can also activate β-catenin in a Wnt-independent manner. Wnt ligands can also transduce signals through downstream effectors other than β-catenin. β-catenin has been demonstrated to be a crucial regulator for maintaining liver homeostasis and initiating liver regeneration after partial hepatectomy, while the upstream signals that activate β-catenin in these processes are not fully understood.

1.1 THE WNT/BETA-CATENIN PATHWAY

β-catenin is a transcriptional co-activator that regulates genes critical for cell proliferation and differentiation. Numerous pathways can activate β-catenin, the major one being the canonical Wnt/β-catenin signaling. Additional signaling, such as receptor tyrosine kinases (RTK) and protein kinase A (PKA), can also activate β-catenin. Other than β-catenin, Wnt ligands also activate other pathways (non-canonical Wnt signaling pathway).

1.1.1 The canonical Wnt/β-catenin signaling pathway

Wnt/β-catenin signaling is referred to as the "canonical pathway" which is characterized as the major pathway that activates β-catenin. It is a highly regulated pathway controlled by multiple factors in this cascade (Figure 1). Wnts are secreted cysteine rich glycoproteins from epithelial, mesenchymal, and endothelial cells. Upon binding of Wnts to their receptor, the signaling cascade is activated, resulting in the activation of β-catenin.

In most normal adult cells, the Wnt/β-catenin pathway is turned off. This homeostasis is ensured by the absence of Wnts and the presence of the destruction complex that degrades β-catenin. Extracellular Wnts can interact with antagonists like secreted frizzled-related protein (sFrp) and dickkopf (Dkk) family members, preventing the activation of the signaling cascade. Cytoplasmic β-catenin is bound in a degradation complex with Axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1) and glycogen synthase kinase-3β (GSK-3β). β-Catenin is phosphorylated by CK1 and GSK-3β at specific serine/threonine residues [2]. The phosphorylated β-catenin is targeted for ubiquitination.

Binding of Wnts to the Frizzled receptor (Fz) induces association with low density lipoprotein receptor-related protein 5 and 6 (Lrp5/6). The Fz-Lrp5/6 co-receptor complex triggers activation of the canonical Wnt/β-catenin pathway. Dishevelled (Dsh) is recruited to Fz, and the Fz/Dsh complex further recruits Axin to Lrp5/6 [3]. GSK-3β and CK1 then phosphorylates Lrp5/6 instead of β-catenin, which results in the inactivation of GSK-3β [4-6]. The dephosphorylated β-catenin is released from the degradation complex and accumulates in the cytoplasm, further β-catenin translocates into the nucleus where it binds to T-cell factor/lymphoid enhancing factor-4 (TCF/LEF4), and triggers the transcription of target genes [7]. Numerous factors playing crucial

roles in cell proliferation and survival have been shown to be the downstream targets of β-catenin, such as cyclin-D1, c-Myc and so on.

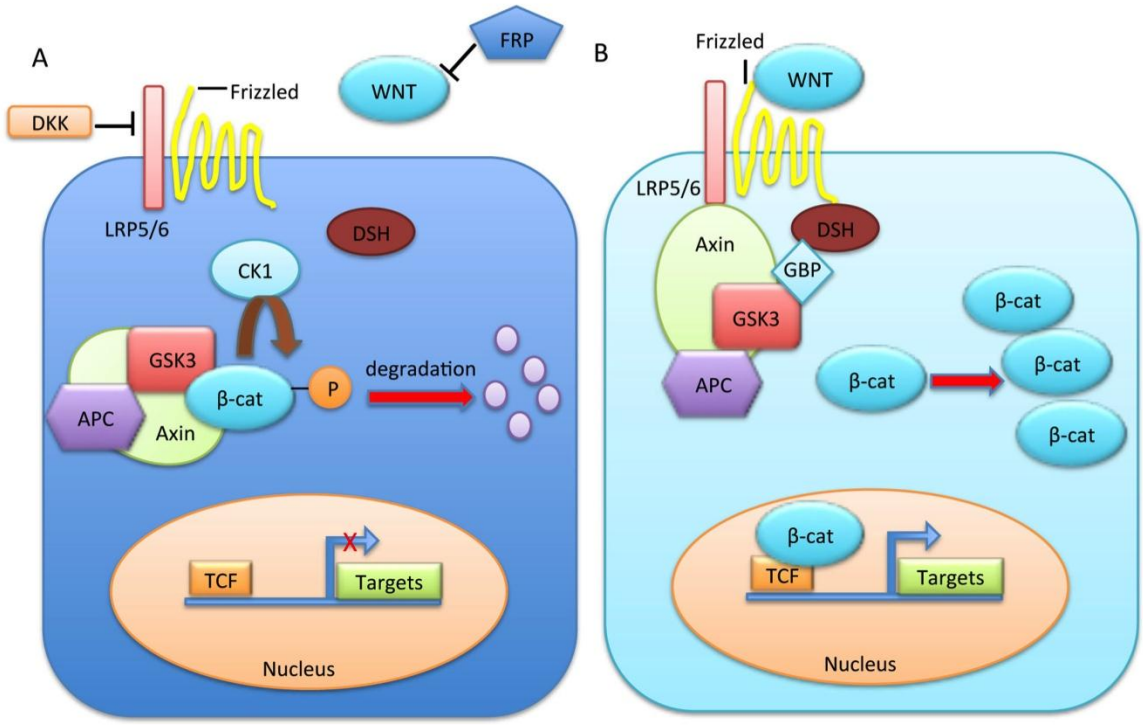

Figure 1. The canonical Wnt/β-catenin signaling pathway

(A) In homeostasis, the pathway is turned off. (B) Active signaling. APC, adenomatous polyposis coli; β-cat, β-catenin; CK, casein kinase; DKK, dickkopf; DSH, dishevelled; GBP, GSK3-binding protein; GSK, glycogen synthase kinase; LRP, LDL receptor-related protein; P, phosphorylation; sFRP, secreted Frizzled-related protein; TCF, T-cell factor.

1.1.2 Wnt-independent pathways that activate β-catenin

β-catenin can also interact with several receptor tyrosine kinases (RTKs) (Figure 2). Hepatocyte growth factor (HGF), a known mitogen for the liver [8], can activate β-catenin. This

effect is mediated through its receptor, MET, a RTK expressed in hepatocytes. Binding of HGF to its receptor activates the kinase activity of MET. Phosphorylated Met further phosphorylates β-catenin at tyrosine residues 654 and 670, which stabilizes β-catenin and results in its translocation to the nucleus and up-regulation of target genes [9, 10]. β-catenin also appears to interact with the epidermal growth factor receptor (EGFR), which is also a RTK. The ligand for EGFR, epidermal growth factor (EGF), is another known hepatocyte mitogen. Like MET, EGFR phosphorylates β-catenin at tyrosine residue 654 and stabilizes it [11]. Constitutive tyrosine phosphorylation of β-catenin by EGFR has been implicated in the metastasis of cancers [12, 13].

Cyclic AMP (cAMP)-dependent protein kinase (protein kinase A [PKA]) can also activate β-catenin by stabilizing it (Figure 2). Activation of PKA increased the cytoplasmic and nuclear β-catenin level, leading to TCF dependent transcription through β-catenin. Phosphorylation of β-catenin at Serine 675 and 552 by PKA inhibits ubiquitination of β-catenin, causing β-catenin to accumulate and target gene expression to be turned on [14, 15]. Mutation of Serine 675 of β-catenin attenuates the inhibition of ubiquitination of β-catenin by PKA, confirming that PKA stabilizes β-catenin by phosphorylating it [14, 15].

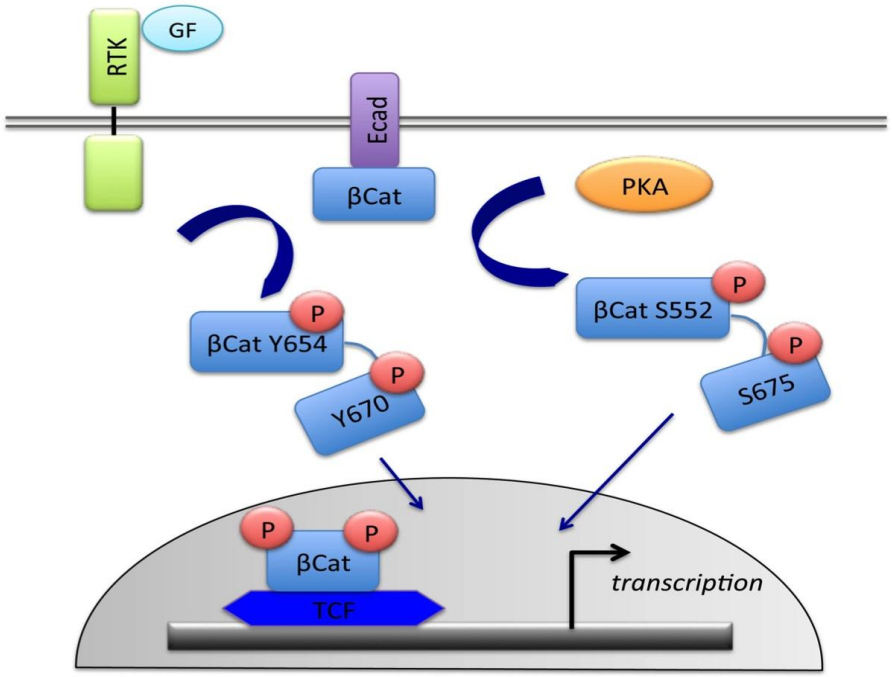

Figure 2. Wnt-independent pathways that activate β-catenin

Upon binding of growth factors (GFs) to receptor tyrosine kinase (RTK), RTK phosphorylates β-catenin at Tyrosine 654 and 670. Protein kinase A (PKA) can also phosphorylate β-catenin at Serine 552 and 675. The phosphorylation makes β-catenin stable and leads to its translocation into the nucleus.

1.1.3 The non-canonical Wnt pathway

Wnts can transduce signals through both canonical and non-canonical signaling pathways. The non-canonical Wnt signaling pathway includes those pathways that use downstream effectors other than β-catenin–TCF/LEF, often referred to as the β-catenin-independent pathway [16]. These non-canonical Wnt pathways play important roles in development, but their relationship to the canonical Wnt/β-catenin signaling remains elusive [17]. These pathways can be divided into two

distinct branches, the planar cell polarity pathway (PCP pathway) and the Wnt/Ca^{2+} pathway (Figure 3).

1.1.3.1 The PCP and Wnt/Ca^{2+} pathway

The best-characterized β-catenin independent pathway is the planar cell polarity (PCP) pathway. In PCP signaling, Wnt-frizzled complex activates small GTPases RAC1 and Ras homolog gene family, member A (RHOA), and c-Jun N-terminal kinase (JNK) is the downstream effector in this signaling cascade (Figure 3A). This pathway regulates cell polarity in morphogenetic processes in vertebrates, such as gastrulation and neural tube closure [18-20].

Another non-canonical Wnt pathway is the Wnt/Ca^{2+} pathway (Figure 3B). Upon binding of Wnts to frizzled receptor, heterotrimeric G protein is activated, which in turn activates phospholipase C (PLC). PLC further triggers Ca^{2+} release from intracellular store, which further activates cell division control protein 42 homolog (CDC42) and calcineurin [17, 21]. CDC42 mediates tissue separation and cell movements during gastrulation. Calcineurin activates transcription factor NF-AT to regulate ventral cell fate by suppressing canonical Wnt/β-catenin during axis formation [22].

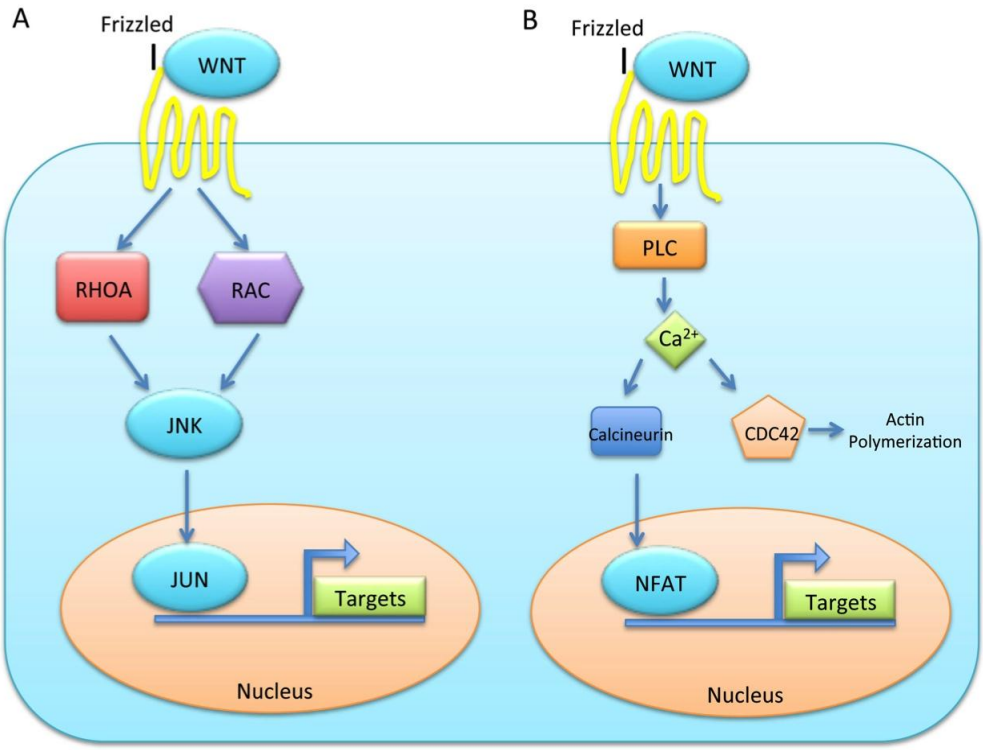

Figure 3. Non-canonical Wnt signaling

(A) Planar cell polarity (PCP) pathway, (B) Wnt-Ca^{2+} pathway.

CDC, cell division cycle related; JNK, c-Jun N-terminal kinase; JUN, jun proto-oncogene; NFAT, nuclear factor of activated T cells; PLC, phospholipase C; RAC, Ras related GTPase; RHOA, Ras homolog gene family, member A.

1.1.3.2 Wnt5a: a non-canonical Wnt ligand

Wnt5a is classified as a non-canonical Wnt family member [21, 23]. Wnt5a can activate the Wnt/Ca^{2+} pathway in the presence of Frizzled receptors [24-26]. It has been shown that Wnt5a stimulates the frequency of calcium fluxes in zebrafish embryos. The *Wnt5a* mutant exhibited reduced Ca^{2+} release, increased β-catenin accumulation and activation of downstream targets,

therefore suggesting the antagonistic role of Wnt/Ca^{2+} pathway against canonical Wnt/β-catenin activity [27]. Meanwhile, Wnt5a can activate the canonical Wnt/β-catenin pathway leading to the expression of downstream genes in the presence of Frizzled 4 receptor [28]. Other than these two signaling pathways, Wnt5a also transduces signals through receptor Ror2. Ror2 is a member of the Ror-family of receptor-tyrosine kinases. Ror2 mediates Wnt5a signaling by inhibiting the canonical Wnt/β-catenin pathway and activating Wnt/c-Jun N-terminal kinase (JNK) pathways [29]. This pathway is involved in the regulation of various cellular functions [30-32]; however, it remains elusive how this pathway regulates expression of the downstream targets.

The effect of Wnt5a signaling on canonical Wnt/β-catenin signaling is still controversial; however various studies have demonstrated an antagonistic role of Wnt5a on the β-catenin pathway. Wnt5a inhibits the activities of Wnt1 in Xenopus development [33]. *Wnt5a* knockout mice exhibit an enhanced β-catenin pathway [34, 35]. It has also been shown that Wnt5a acts as a tumor suppressor by inhibiting the Wnt/β-catenin signaling pathway. In thyroid cancer, Wnt5a suppresses tumors by inhibiting c-myc, a well-known oncogene and downstream target of the Wnt/β-catenin pathway [36]. In colon cancer, Wnt5a can inhibit constitutively active β-catenin in APC-mutated cells in an autocrine manner [37]. However, the function of Wnt5a in liver homeostasis and regeneration has not been addressed.

1.2 THE ROLE OF BETA-CATENIN IN LIVER HOMEOSTASIS

1.2.1 Hepatic zonation

The smallest functional unit in the liver is the hepatic lobule. The hepatic lobule appears to be histologically homogeneous; in fact, hepatocytes exhibit a striking functional heterogeneity. Metabolic processes are arranged in a gradient based on the functional heterogeneity of hepatocytes, which is known as hepatic zonation [38-40]. Blood enters the hepatic lobule through the hepatic artery and portal vein at the portal trial, and travel towards the central vein in the middle. Based on the location within the hepatic lobule, periportal, midzonal and pericentral hepatocytes are defined (Figure 4). Intriguingly, hepatocytes in different zones are functionally different due to differences in gene expression levels.

A hepatic lobule is divided into three zones: pericentral zone, which surrounds the central vein, periportal zone, which surrounds the portal triad (contains hepatic artery, portal vein and bile duct), and midzonal hepatocytes in between (Figure 4). Periportal and pericentral hepatocytes can be distinguished according to differences in their function. It is known that genes that encode for enzymes critical for ammonia and xenobiotic metabolism are predominantly expressed in pericentral hepatocytes. On the contrary, periportal hepatocytes express genes important for urea synthesis [41-44]. Consequently, the major metabolic functions of the liver exhibit differences in activity within the pericentral and periportal zones. It is generally accepted that the homeostatic function of the liver depends on the functional compensation of the two zones.

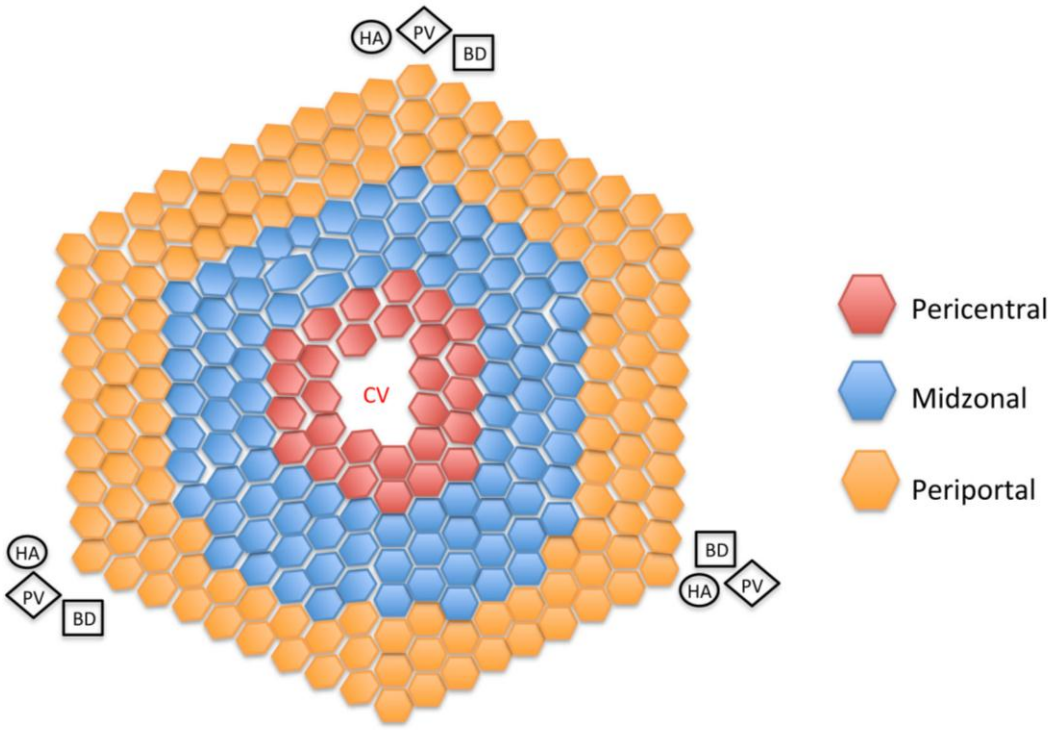

Figure 4. Schematic diagram of the hepatic lobule

The structure of hepatic lobule resembles a roughly hexagonal shape. Hepatocytes radiate out from the central vein. According to the location of hepatocytes within the lobule, pericentral, midzonal and periportal hepatocytes are defined. CV: Central vein, HA: hepatic artery, PV: portal vein, BD: bile duct.

1.2.2 The role of β-catenin in regulating hepatic zonation

Observations in liver tumors first indicated a role for β-catenin in regulating hepatic zonation. It has been found that liver tumors in mice harboring activating β-catenin mutations exhibit altered hepatic zonation, which is characterized by glutamine synthetase (GS) expression throughout the lesion [45]. Such findings in liver tumors suggest that β-catenin may be required for

mediating the expression of zone-specific genes.

Follow-up studies have unequivocally established the critical role of β-catenin signaling in hepatic zonation. It is believed that the differential zonal expression and activation of β-catenin and APC determines the pericentral versus periportal phenotype. In liver homeostasis, the activation of β-catenin signaling is confined to the pericentral zone. Pericentral hepatocytes express cytoplasmic and nuclear β-catenin in addition to membranous localization. It is known that genes that encode for enzymes critical in ammonia and xenobiotic metabolism are predominantly expressed in pericentral hepatocytes. These genes include GS, cytochrome P450 family members such as Cyp2e1 and Cyp1a2, which are downstream targets of β-catenin. On the contrary, periportal hepatocytes show higher expression of APC that can sequester β-catenin and as a consequence lower unbound, dephosphorylated β-catenin [46, 47]. These hepatocytes express genes encoding for urea synthesis such as arginase 1 and carbamoylphosphate synthase (CPS).

The key role of β-catenin signaling in dictating hepatic zonation was confirmed by a hepatocyte specific, β-catenin knockout mice model. Liver specific loss of β-catenin resulted in loss of GS, Cyp1a2 and Cyp2e1 expression from pericentral hepatocytes [48, 49]. Blocking β-catenin signaling repressed the pericentral genetic program, with the periportal genetic program expressed throughout the lobule [46, 48, 50]. On the other hand, using mice with liver-targeted inactivation of APC, it has been shown that the activation of β-catenin signaling induced the expression of a pericentral genetic program throughout the entire lobule, together with repression of the periportal genetic program [46] (Figure 5).

Although the interaction of β-catenin and APC is of essence in dictating hepatic zonation, it may not fully explain the striking demarcation between different zones. Immunohistochemistry showed a striking demarcation between the GS (a pericentral gene) and CPS (a periportal gene)

expressing zones [46, 49]. However, if a gradient of β-catenin/APC truly exists, a more gradual change in the expression pattern of zonation markers would be predicted. Therefore, additional signaling pathways or other components of the Wnt/β-catenin pathway may also be involved in the regulation of hepatic zonation.

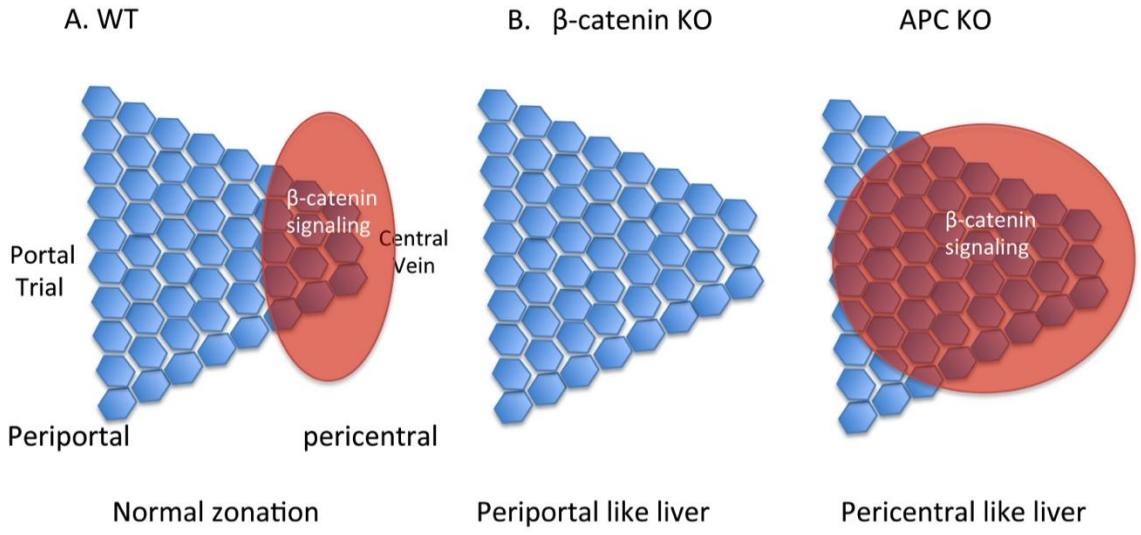

Figure 5. Opposing effects of the activation and inactivation of β-catenin signaling in mouse liver

(A) Wild-type liver, β-catenin signaling is active in pericentral hepatocytes. (B) β-catenin KO and APC-KO livers. β-catenin KO liver is periportal like, with periportal genetic program expressed throughout the lobule; APC-KO liver is pericentral like, with pericentral genetic program expressed throughout the lobule.

1.3 THE ROLE OF BETA-CATENIN IN LIVER REGENERATION

1.3.1 Introduction of liver regeneration

Liver is an intriguing organ with unique capacity to regenerate in response to the loss of liver mass or injuries. As a biochemical defense against toxic chemicals and re-processor of absorbed substrates, liver may be periodically exposed to harmful insults. The capability to regenerate has evolved to safeguard the wide range of functions performed by liver and serves as a mean to restore and repair liver function after injuries.

Liver regeneration (LR) is not dependent on a small group of progenitor cells, but instead carried out by proliferation of all the existing mature cell populations composing the entire organ, including hepatocytes, cholangiocytes, sinusoidal endothelial cells, Kupffer cells and stellate cells [51]. During liver regeneration, genes involved in protein synthesis, posttranslational processing and cytoskeletal assembly are upregulated early, followed by genes associated with metabolism [52, 53].

1.3.2 Clinical implication of studying liver regeneration

The only cure for end-stage liver disease is liver transplantation, which is a great technological achievement. However, donor organ availability cannot meet demand and many patients die on the waiting list. For patients who have no access to liver transplantation, successful regeneration leads to the restoration of liver function and survival, whereas insufficient regeneration results in liver failure and death. For this reason, it is necessary to understand the

mechanisms controlling LR, to identify factors and signaling pathways that could be therapeutically targeted and to develop methodologies to stimulate LR.

Enhancing LR pharmacologically by targeting some critical factors or signaling pathways would have far reaching implications and benefit a considerable amount of patients. Studies on the molecular basis of LR have revealed a series of transcription factors and signaling pathways associated with the initiation of LR. Pharmacologically inducing such pro-regenerative factors or activating pro-regenerative pathways could potentially enhance LR. Therefore, it is one of crucial aspects how understanding of the molecular mechanism controlling LR might be beneficial for clinical scenarios.

1.3.3 Liver regeneration induced by partial hepatectomy

LR is regulated by an intricate signaling network involving the interplay between various liver cell populations. *In vitro* models could be helpful for the study of isolated signal pathway, however, they always need to be validated using *in vivo* studies so as to simulate the interactions between the various cell populations in the liver. Currently, there is a broad range of experimental animals of different species and strategies for inducing LR. Since cell proliferation in the healthy liver is a rare event, the first step to initiate the regeneration response is to induce a liver injury in these animal models. Surgical resection has been widely used to experimentally induce LR.

1.3.3.1 Two thirds partial hepatectomy

Surgical resection is one of the most frequently and widely used techniques to initiate LR. The induction of LR by partial hepatectomy (PH) depends on the removal of functional liver mass.

The model of 2/3 PH in rodents was introduced by Higgins and Anderson [54]. The rodent liver has multiple lobes, and removing two thirds of the total liver mass can be easily accomplished by a surgical procedure, without damaging the remaining lobes. The residual lobes undergo proliferation and regain the original liver mass. This process is finished within one week in rats, about two weeks in mice [55].

PH in rodents involves ligation and resection of 2 from 4 lobes [56]. PH is well tolerated by rodents when appropriately performed. Induction of LR by PH bears some unique advantages. Since the rodents have uniform and consistent anatomy, the procedure has high accuracy and reproducibility. In addition, surgical resection can be performed in minutes, the induction of regeneration begins at one point in time, and therefore, the regenerative process can be precisely timed. The reproducibility and standardization of PH makes this model highly popular and intensively studied. Furthermore, the residual liver is not directly manipulated at the time of the surgery, so regeneration occurs without being exposed to the influence of necrosis or inflammation. However, along with the advantages, there are some limitations innate to this model. PH model may simplify the regeneration process too much to make an adequate accounting of the human disease condition. In human patients, LR is usually associated with inflammation, innate immunity, tissue healing through removal of necrotic tissue and so on [56, 57]. The PH model allows distinct understanding of the regenerative process purely as a response to the loss of liver mass, but for a better understanding of the regenerative process in the context of human liver diseases, some of the pathological factors that could have influences on the regeneration process need to be eventually integrated with the PH model.

1.3.3.2 Cellular basis of liver regeneration after partial hepatectomy

LR after PH features the proliferation of adult hepatic cell types. Hepatocytes are the first to proliferate. The kinetics of hepatocyte proliferation varies slightly in different species. The first peak of DNA synthesis in hepatocytes occurs around 24 hours after PH in rats and approximately 36 hours in mice [55]. Hepatocytes can secrete growth stimuli and induce proliferation of the other cell types in liver. Cholangiocytes and stellate cells begin to synthesize DNA about 12 hours after the hepatocytes, reaching a peak at about 48 hours in rat [58]. Three days after PH, as a consequence of cell proliferation, liver is characterized by cell clusters containing 10-14 hepatocytes. By day 4, stellate cells send processes between hepatocytes in the clusters and start producing several types of laminin. Following the penetration of stellate cells, sinusoidal endothelial cells also invade into the clusters, and in this manner the normal liver vasculature is restored [59].

1.3.3.3 Molecular basis of liver regeneration after partial hepatectomy

PH triggers a wide variety of gene expression and a series of cell signaling pathways that are under tight control. The expression of some early genes is induced during the first few hours after PH, which is termed the "priming phase". Many of the genes are involved in cytokine networks, such as TNF-α and IL-6. These cytokines can induce some critical transcription factors in the liver, including Stat3 and NF-κB, which in turn activate the genes that are responsible for promoting the quiescent hepatocytes to enter into the cell cycle [60, 61]. Cell cycle progression is then driven by growth factors, such as HGF and EGF [53, 62]. Some signaling pathways also become activated and play important roles in driving LR. Wnt/β-catenin signaling is one of the critical players activated following PH.

1.3.3.4 The role of Wnt/β-catenin signaling in triggering liver regeneration after partial hepatectomy

β-catenin signaling is a pro-proliferative and pro-survival signal that plays a vital role during LR after PH. β-catenin has been known to have a positive role in liver growth. Mice overexpressing β-catenin in the liver have increased hepatocyte proliferation and a 15% increase in liver size [63]. Transgenic mice expressing an oncogenic form of β-catenin developed hepatomegaly with increased hepatocyte proliferation without any compensatory apoptosis [64]. Given the growth advantage of β-catenin overexpressing liver, the question of whether β-catenin signaling also positively regulates LR after PH has been raised. Studies show that β-catenin signaling is rapidly activated following PH. In a rat model, there was a 2.5-fold increase in β-catenin protein a few minutes after PH [65]. The increase in β-catenin during early stages of LR contributes to the increased expression of cyclinD1 and c-myc, which are both downstream targets of β-catenin and play an important role in driving cell cycle progression and cell proliferation [66, 67] (Figure 6).

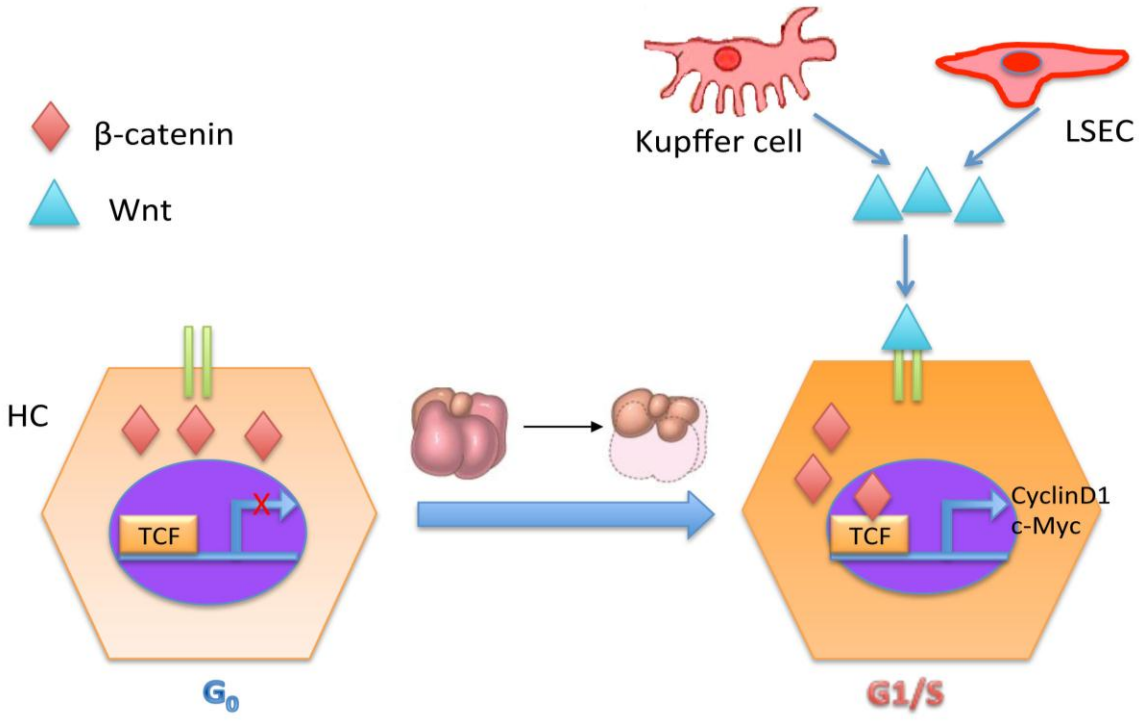

Figure 6. The role of β-catenin in liver regeneration after PH

Following PH, β-catenin signaling pathway becomes active. β-catenin translocates into the nucleus where it binds to TCF and turns on the expression of Cyclin-D1 and c-Myc, which is critical for the hepatocytes to enter into the cell cycle. Wnt ligands secreted from various nonparenchymal cell populations within the liver are important mitogenic signals that activate β-catenin. LSEC, liver sinusoidal endothelial cell.

The importance of β-catenin signaling in LR is further validated in knockdown studies. Ablation of β-catenin by antisense oligonucleotides compromised LR after PH [68]. Hepatocyte-specific disruption of β-catenin led to suboptimal liver regeneration after PH, with deficient expression of Cyclins-A, D, and E [49]. Hepatic DNA synthesis was also delayed in β-catenin knockout mice after PH [69]. While deletion of β-catenin reveals defects in LR, activation of this pathway has demonstrated the positive role of β-catenin signaling in LR. Zebrafish harboring

defects with APC, a negative regulator of β-catenin, display enhanced LR after PH, similar to mice with APC mutation [70]. Transgenic mice expressing constantly active forms of β-catenin also show a growth advantage during LR after PH [71].

LR after PH is an intricate process that is tightly regulated by a complex signaling network. Delays in LR can always be observed by blocking some pro-proliferative signals or pathways, such as β-catenin, HGF, EGF and so on; however, not a single kind of signaling alone can drive the regeneration process. There is enormous redundancy and overlap in the function of those proliferative signals, so that regeneration can still complete itself in case that some pathways are missing or blocked. Understanding individual signaling contributors during LR process is of essential importance, which could help optimize this process and ensure the maximal efficiency of LR after PH.

1.3.3.5 Termination of liver regeneration after partial hepatectomy

After PH, the LR process terminates when the normal liver weight to body weight ratio has been re-established. Since hepatocytes have seemingly unlimited proliferative capacity [72, 73], there must exist some regulatory mechanisms to stop the LR process. How does the liver sense that the hepatic mass has been restored, and what are the signals that terminate LR after PH? Unlike the initiation of LR which has been extensively studied, the mechanisms leading to proper termination of LR are not well understood. Some of the factors and signaling pathways have been shown to play a role in the termination of LR.

Transforming growth factor beta (TGFβ) has anti-proliferative effects and contributes to the termination of LR after PH. TGFβ can suppress hepatocyte proliferation in vitro [74]. Administration of TGF-β in hepatectomized rats inhibits the early proliferative response and leads to delay in hepatocyte DNA synthesis [75]. Disruption of TGF-β signaling combined inhibition of

activin A (a member of TGFβ family) in the liver enhances proliferation and leads to prolonged LR [76].

Extracellular matrix (ECM) signaling via integrins has an important role in proper termination of liver regeneration. ECM is a rich source of signaling to surrounding cells and such signaling is mediated through integrins [77]. Integrin-linked kinase (ILK) can interact with the cytoplasmic domains of β integrins and participates in the signaling transduction between ECM and cells [78]. Recent studies have revealed the role of ILK in regulating LR after PH. ILK deficient mice fail to terminate LR properly after PH, which renders larger livers reaching a size 59% greater than the original liver [79].

The ECM is a complex mixture of proteins and glycosaminoglycans. Glypican-3 (GPC3) is one of the components. GPC3 functions as a growth suppressor, and is involved in the termination of LR. Studies show that GPC3 level begins to increase 2 days after PH and peaks by day 5 [80]. Blocking GPC3 expression promotes hepatocyte growth in culture [80], on the contrary, overexpression of GPC3 in mice suppresses hepatocyte proliferation and LR after PH [81].

A series of pro-proliferative signaling turns on to initiate liver regeneration soon after PH, while turns off at later stages to prevent liver overgrowth and to terminate liver regeneration. Identifying the termination mechanisms of pro-proliferative signaling could be helpful to understand how LR stops. β-catenin is an essential pro-proliferative signaling that triggers LR after PH. In a rat model, β-catenin protein levels increase within several minutes after PH, concomitant with its translocation to the nucleus. However, β-catenin activation after PH was transient; as regeneration proceeds, this pathway is downregulated by activation of the destruction complex for β-catenin, so the protein level of β-catenin returns to normal by 48h, which is after the wave of hepatocyte proliferation [65]. This timely regulation allows β-catenin signaling to be activated to

trigger LR in early stages while limiting its sustained activation at later stages, thereby ensuring proper termination of LR processes. Thus, identification of the negative regulators of β-catenin signaling during late stages of LR may also be a component of the hepatostat. β-catenin is just one example among the numerous pro-proliferative signals whose activation and termination is under spatiotemporal constrains, and thus, exploring the termination mechanisms of these signaling pathways might provide useful insights into the understanding of eventual termination of LR.

1.4 WNT SECRETION IN LIVER

Wnt proteins are highly conserved palmitoylated glycoproteins secreted by epithelial, mural, or endothelial cells. The adult liver expresses numerous Wnts. All major cell types in the liver, hepatocytes, hepatic stellate cells (HSC), biliary epithelial cells (BECs), liver sinusoidal endothelial cells (LSECs) and Kupffer cells, express various kinds of Wnts [82]. However, the Wnt/β-catenin pathway is normally turned "off" in adult liver, thus this wide expression of Wnts might be in fact a balance between the "stimulatory" and "inhibitory" Wnts [83]. In homeostasis, only the β-catenin in the pericentral zone is active, but under stressed circumstances, such as PH, the Wnt/β-catenin pathway is rapidly activated to trigger liver regeneration (LR). Therefore, Wnt secretion must be tightly regulated to ensure the function of β-catenin in both liver homeostasis and during liver LR.

1.4.1 Wntless: a critical regulator of Wnt secretion

There are proteins involved in Wnt protein processing and secretion from producing cells. Wntless (Wls) is a multipass transmembrane protein required in the secretory pathway to promote the release of Wnts from producing cells [84] (Figure 7).

Wls localizes to the components of the secretory pathway, especially the Golgi [85-87] and the plasma membrane [88]. An interaction between Wls and Wnts has been predicted on the basis of colocalization and co-immunoprecipitation experiments [85]. The interaction between Wls and Wnts, and their localization to both the Golgi and the plasma membrane, suggest that they associate in the Golgi where they are incorporated into vesicles and transported to the plasma membrane. The current theory is that Wnts are palmitated by a membrane-bound acyltransferase, Porcupine, in the ER [89, 90]. Wls binds to the palmitate group of Wnts in the Golgi, and further associates with Wnts in the secretion vesicle. Wls acts as a cargo receptor, assisting Wnts to reach the plasma membrane, so that Wnts can be secreted to the extracellular matrix (Figure 7). The importance of Wls in Wnt secretion has been confirmed by depletion studies. Defect of *Wls* results in Wnt loss-of-function phenotypes [86, 87, 91, 92], suggesting that Wls is indispensable in Wnt secretion.

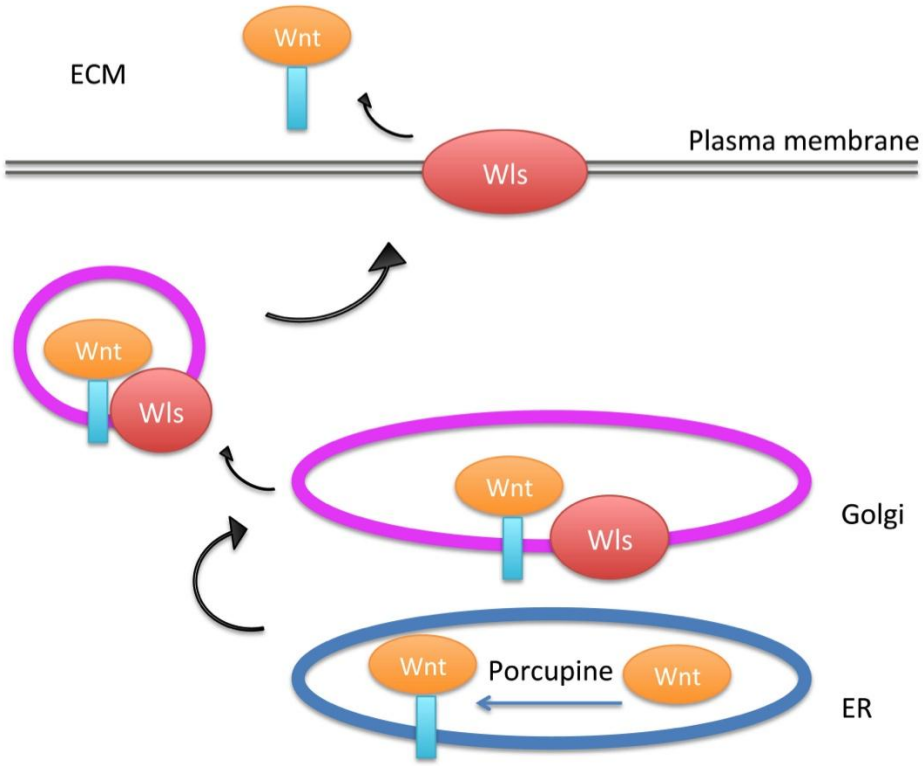

Figure 7. The role of Wls in Wnt signal secretion

Wnt is palmitoylated by Porcupine in the lumen of the endoplasmic reticulum (ER). Upon reaching the Golgi, Wntless (Wls) associates with Wnt in vesicles bound for secretion, leading to secretion of Wnt into the extracellular matrix (ECM).

The Wnt family consists of 19 secreted, cysteine-rich glycoproteins essential for embryogenesis and homeostasis. Wls appears to control the release of all Wnts, deletion of *Wls* leads to embryonic lethality in mice due to impairment of the developing body axis [93], therefore mice with a conditional null allele for *Wls* were generated. The utilization of *Wls* conditional mutants could be a powerful tool to study Wnt secretion under spatial and temporal control [94]. Cell-specific *Wls* deletion has divulged important roles of Wnt ligands in multiple organs [95-98].

The crucial role of Wnt ligands in bone development and homeostasis has been revealed by generating osteoblast-specific *Wls*-deficient mice. Bone-mass accrual was significantly inhibited in knockout mice, with a dramatic reduction of both trabecular and cortical bone mass. The knockout animals had spontaneous fractures and a high frequency of premature lethality [96]. Wnts also regulates retina angiogenesis. Deletion of the *Wls* in retinal myeloid cells results in increased angiogenesis in the deeper layers and defective remodeling [95]. A mouse model with a defect of *Wls* in epidermis revealed functional roles of Wnt ligands in fibroblast proliferation and in the epidermal hair follicle initiation program [97]. Wnt ligands are also required for maintaining stem cell activity, while deletion of *Wls* in nail stem cells results in failure to regenerate the nail or digit [98].

Taken together, the tissue specific genetic manipulation of *Wls* has been widely utilized to address the role of Wnt ligands in various organ systems under both physiological and pathological condition. However, the role of Wnt ligands secreted by different hepatic cell populations in liver homeostasis and regeneration remained unexplored; therefore, deletion of *Wls* in individual hepatic cell types might be of essence to elucidate this fundamental question.

1.4.2 The role of different hepatic cell populations in Wnt secretion

The liver sinusoid is the most basic functional unit of the liver (Figure 8). The liver sinusoid is a capillary lined by liver sinusoidal endothelial cells (LSECs). Hepatocytes, the parenchymal cells in the liver, are separated from the sinusoids by a small space called the space of Disse. Non-parenchymal cells are also located inside the sinusoids. Hepatic stellate cells (HSCs) can help to maintain the extracellular matrix, and Kupffer cells can take up and destroy foreign

materials. Bile canaliculi are small channels formed between adjacent hepatocytes to collect bile secreted by hepatocytes [99].

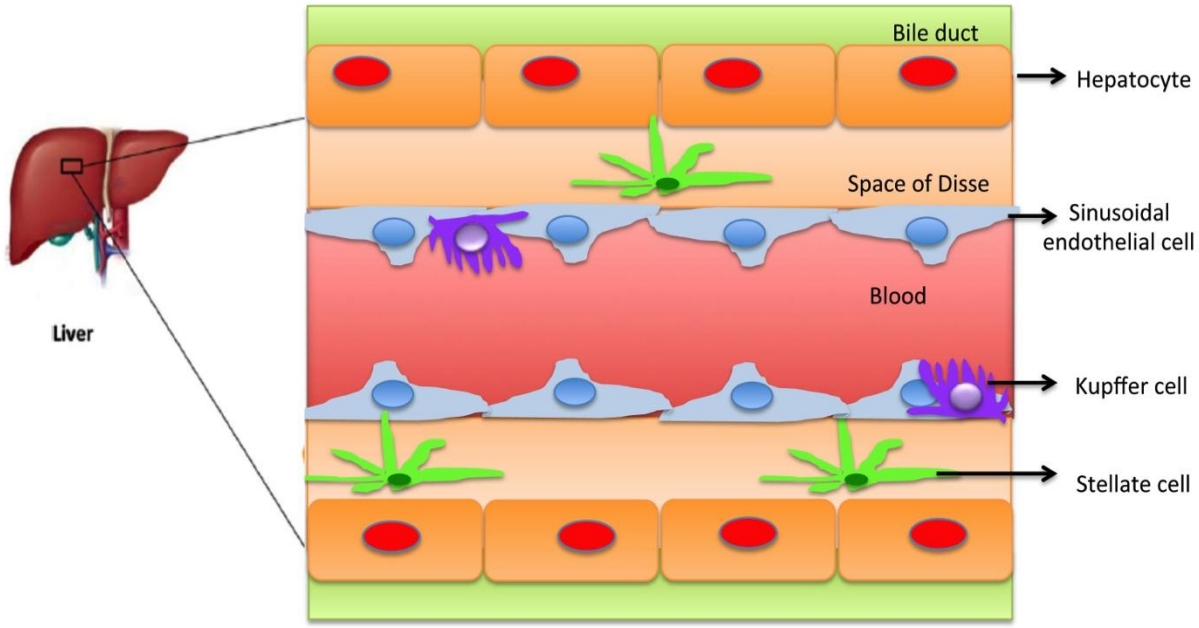

Figure 8. The liver sinusoid functional unit

All major cell types in the liver, including hepatocytes, HSCs, cholangiocytes, LSECs and Kupffer cells, express various kinds of Wnts [82]. It has been widely accepted that non-parenchymal cells (NPCs) in the liver play important roles in maintaining liver homeostasis and regulating liver growth [100, 101]. Recent studies further show that NPCs are a source of proliferative signals during LR, secreting various factors including Wnt ligands. It has been reported that LSECs secrete Wnt2 and HGF to stimulate LR. Diminished expression of LSEC derived Wnt2 and HGF leads to defect of LR, while restoring their expression rescues the phenotype [102]. Macrophages are also a source of Wnt proteins in regenerating adult liver.

Macrophages induce Wnt3a expression and β-catenin activation to promote LR during chronic liver injury [103]. HSCs also seem to play an important role in LR by producing HGF to trigger cell proliferation [104].

Although the role of β-catenin in liver homeostasis and LR has been intensively studied, the upstream signals that regulate β-catenin activity in these processes remains elusive. Wnt ligands are the most important upstream signals that activate β-catenin; however, the regulation of Wnt secretion and the cellular source of Wnt ligands that modulate β-catenin activity are not fully understood. Recent studies suggest that NPCs might be a critical source of several Wnt ligands during LR. However, there are still gaps in our knowledge that need to be filled in to fully understand the cellular source of Wnt proteins and their functions in the liver.

2.0 CENTRAL HYPOTHESIS AND RATIONALE

There is accumulating evidence showing that β-catenin plays a critical role in maintaining normal liver function and in the process of liver regeneration (LR). Canonical Wnt-signaling is the major pathway that induces β-catenin activation [3]. Binding of Wnt to its cell surface receptor Frizzled and co-receptor Lrp5/6 stabilizes β-catenin protein that in turn translocates to the nucleus to bind to the T-cell factor (TCF) family of transcription factors in order to activate gene expression of tissue- and context-specific targets. Other than Wnt-signaling, β-catenin can also be activated in a Wnt-independent manner. Some examples include β-catenin activation by hepatocyte growth factor (HGF) by phosphorylation at tyrosine-654 (Y654) [9, 10], epidermal growth factor (EGF) by phosphorylation at Y654 [11], Flt3 also by phosphorylation at Y654 [105], and protein kinase A (PKA) by phosphorylation at serine-552 (S552) and -675 (S675) [14].

The relative contribution of Wnt-dependent and Wnt-independent pathways to β-catenin activation in physiological context has not been addressed. Therefore, we aim to investigate **to what extent β-catenin signaling in liver is Wnt dependent in both normal liver homeostasis and during LR, and identify the source of Wnts that modulate β-catenin activation in these processes**.

Aim 1: To investigate whether the function of β-catenin in maintaining liver homeostasis and triggering LR is Wnt-signaling dependent or not.

β-Catenin is a transcriptional co-activator that plays a critical role in liver biology. In liver

homeostasis, hepatocytes exhibit molecular heterogeneity based on their location within the hepatic lobule, which is known as hepatic zonation. β-catenin is one of the key molecules regulating the zonation pattern. Pericentral hepatocytes express cytoplasmic and nuclear β-catenin in addition to membranous localization, and regulate the expression of genes such as GS, and certain cytochrome P450 enzymes, such as Cyp1a2 and Cyp2e1 [106]. In addition, β-catenin signaling is also essential for the initiation of LR. Liver has the unique capacity to regenerate following partial hepatectomy (PH). During LR, a series of cell signaling pathways and cascades are triggered that are tightly regulated. β-Catenin signaling is one such pathway that is activated very early after PH [65].

Indeed, we and others have previously shown that a *liver-specific β-catenin knockout (β-catenin-LKO)* lacking this protein in both hepatocytes and cholangiocytes, shows defective pericentral gene expression and a 24 hour delay in the entry of hepatocytes to S-phase after PH with a peak at 72 hours and not 40 hours [48, 49]. However what the upstream regulator of β-catenin is in these events remains unknown.

We have now generated *liver-specific Lrp5/6 double knockout mice (Lrp-LKO)* where upstream Wnt-signaling is abolished while β-catenin expression and downstream signaling is intact. Thus β-catenin in Lrp-LKOs can still be activated by signaling pathways other than Wnts (Figure 9). With this model, we can distinguish between Wnt-dependent and -independent β-catenin signaling. In this aim, we test whether Lrp-LKOs phenocopy β-catenin-LKOs in terms of defective hepatic zonation and delay in LR, and address to what extent β-catenin activation is Wnt signaling dependent in these processes.

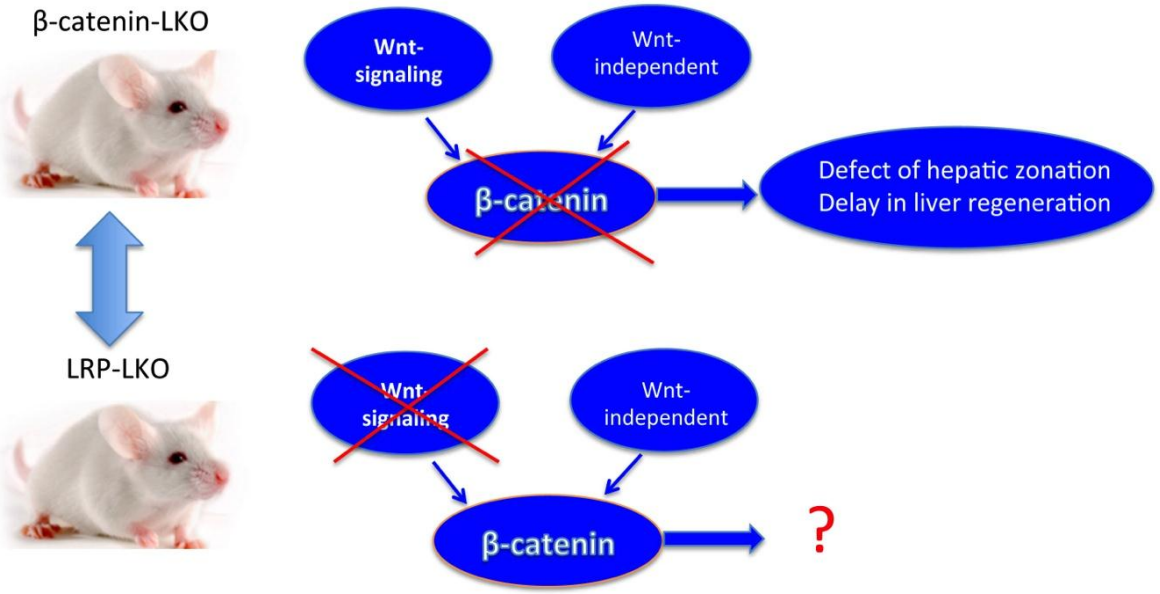

Figure 9. Comparison of liver specific β-catenin knockout and liver specific Lrp5/6 knockout mice

In liver specific β-catenin knockout (β-catenin-LKO), both Wnt-dependent and Wnt-independent β-catenin signaling are abolished, and it results in defect of hepatic zonation and delay in liver regeneration. In liver specific Lrp5/6 knockout (LRP-LKO), only Wnt-dependent signaling is abolish, while the Wnt-independent pathways remain intact and can activate β-catenin.

Aim 2: To identify the cellular source of Wnt ligands that modulate the activation of β-catenin in dictating hepatic zonation and triggering LR.

Various cell types in the liver express Wnts, including hepatocytes, hepatic stellate cells (HSCs), cholangiocytes, liver sinusoidal endothelial cells (LSECs) and Kupffer cells [107]. Studies have shown that non-parenchymal cells, such as HSC, LSEC and macrophages, secrete factors to stimulate LR [102-104]. However, what specific cell population secretes Wnts that modulate β-catenin activity in liver homeostasis and LR is unknown. *Wntless (Wls)* encoding a multipass

transmembrane protein has been identified as a regulator for proper sorting and secretion of Wnts [108]. Deficiency in *Wls* leads to Wnt secretion defects and failure to activate β-catenin [93]. In order to study Wnt secretion from different hepatic populations, we generated cell specific *Wls* knockout mice that lack the ability of a specific cell population to secrete Wnt ligands. *Wls* conditional KO for liver epithelial cells (Wls-LKO), endothelial cells (Wls-EKO), macrophages including Kupffer cells (Wls-MKO) and stellate cells (Wls-SKO) were generated. Hepatic zonation and LR kinetics were analyzed in all animal models to elucidate the cellular source of Wnt ligands playing critical roles in these processes (Figure 10).

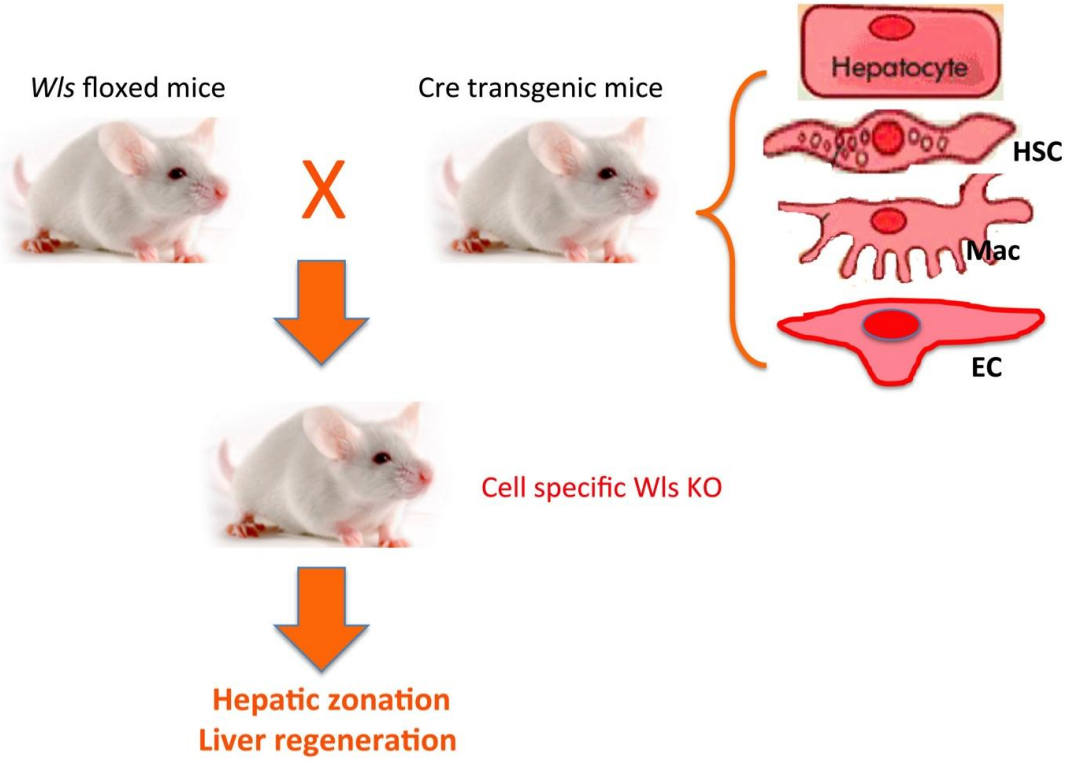

Figure 10. Generation of cell specific *Wls* knockout

Wls double floxed mice are bred with *Cre* transgenic mice driven by different cell specific promoters for hepatocytes, hepatic stellate cells (HSCs), macrophages (Mac) and endothelial cells (EC), to generate cell specific *Wls* knockouts.

Hepatic zonation and liver regeneration were analyzed in individual knockout models, to identify the cellular source of Wnt ligands playing important roles in these processes.

3.0 MATERIALS AND METHODS

3.1 MICE AND BREEDING

Homozygous *Lrp5/6* double-floxed mice were reported recently [109]. To conditionally delete *Lrp5* and *Lrp6* from hepatocytes and cholangiocytes, homozygous *Lrp5*/*6* double-floxed mice (*Lrp5*$^{flox/flox}$*Lrp6*$^{flox/flox}$) were bred with *Cre* transgenic mice driven by an *albumin* promoter (*Albumin-Cre*) (Jackson Laboratories, Bar Harbor, ME) [110]. The offspring carrying homozygous *Lrp5* floxed alleles, a floxed *Lrp6* allele and an *albumin-Cre* allele (*Lrp5*$^{flox/flox}$*Lrp6*$^{flox/wt}$*Alb-Cre*$^{+/-}$) were then bred to homozygous *Lrp5*/*6* double-floxed mice (*Lrp5*$^{flox/flox}$*Lrp6*$^{flox/flox}$). The mice with genotype *Lrp5*$^{flox/flox}$*Lrp6*$^{flox/flox}$*Alb-Cre*$^{+/-}$ represent liver-specific *Lrp5/6* KO or *Lrp*-LKO mice. Other genotypes *Lrp5*$^{flox/wt}$*; Lrp6*$^{flox/wt}$*; Alb-Cre*$^{-/-}$ *& Lrp5*$^{flox/flox}$*; Lrp6*$^{flox/lwt}$*; Alb-Cre*$^{-/-}$ *& Lrp5*$^{flox/wt}$*; Lrp6*$^{flox/flox}$*; Alb-Cre*$^{-/-}$ *& Lrp5*$^{flox/flox}$*; Lrp6*$^{flox/flox}$*; Alb-Cre*$^{-/-}$ were used as controls (*Con).* No phenotype was observed in *Con*. Genotyping was performed by polymerase chain reaction (PCR) analysis using genomic DNA isolated from a tail clipping.

In order to generate liver-specific *Wls* KO, homozygous *Wls* floxed mice (*Wls*$^{flox/flox}$) were bred with *Albumin-Cre*$^{+/-}$ mice (Jackson Laboratories, Bar Harbor, ME) [110]. The offspring carrying floxed *Wls* allele and *Albumin-Cre* (*Wls*$^{flox/wt}$*; Alb-Cre*$^{+/-}$) were bred to homozygous *Wls* floxed mice (*Wls*$^{flox/flox}$). Mice with genotype *Wls*$^{flox/flox}$*; Alb-Cre*$^{+/-}$ represent *Wls*-LKO mice. Mice with genotypes *Wls*$^{flox/flox}$*; Alb-Cre*$^{-/-}$ *and Wls*$^{flox/wt}$*; Alb-Cre*$^{-/-}$ were used as *Con*.

To generate liver specific *Wnt5a* KO mice, homozygous *Wnt5a* floxed mice (*Wnt5a*$^{flox/flox}$) were bred with *Albumin-Cre*$^{+/-}$ mice [110]. The offspring carrying floxed *Wnt5a* allele and *Albumin-Cre* (*Wnt5a*$^{flox/wt}$*; Alb-Cre*$^{+/-}$) were bred to homozygous *Wnt5a* floxed mice (*Wnt5a*$^{flox/flox}$). Mice with genotype *Wnt5a*$^{flox/flox}$*; Alb-Cre*$^{+/-}$ represent *Wnt5a*-LKO mice. Mice with genotypes *Wnt5a*$^{flox/flox}$*; Alb-Cre*$^{-/-}$ *and Wnt5a*$^{flox/wt}$*; Alb-Cre*$^{-/-}$ were used as *Con*.

To generate stellate cell-specific *Wls* KO mice, *Wls*$^{flox/flox}$ mice were bred with *GFAP-Cre*$^{+/-}$ mice (Jackson Laboratories, Bar Harbor, ME) [111] using similar strategy as described above. Mice with genotype *Wls*$^{flox/flox}$*; GFAP-Cre*$^{+/-}$ represent *Wls*-SKO. Mice with genotypes *Wls*$^{flox/flox}$*; GFAP-Cre*$^{-/-}$ *and Wls*$^{flox/wt}$*; GFAP-Cre*$^{-/-}$ were used as *Con.*

To generate macrophage-specific *Wls* KO mice, *Wls*$^{flox/flox}$ mice were bred with *Lyz2-Cre*$^{+/-}$ (also called LyzM-Cre) mice (Jackson Laboratories, Bar Harbor, ME) [112] using similar strategy as described above. Mice with phenotype *Wls*$^{flox/flox}$*; Lyz2-Cre*$^{+/-}$ represent *Wls*-MKO. Mice with genotypes *Wls*$^{flox/flox}$*; Lyz2-Cre*$^{-/-}$ *and Wls*$^{flox/wt}$*; Lyz2-Cre*$^{-/-}$ were used as *Con.*

To generate endothelial cell specific *Wls* knockout mice, *Wls*$^{flox/flox}$ mice were bred to *Tie2-Cre*$^{+/-}$ mice (Jackson Laboratories, Bar Harbor, ME) [113] in the same manner as above to obtain *Wls*$^{flox/flox}$*; Tie2-Cre*$^{+/-}$ mice. Since, no viable pups for *Wls*$^{flox/flox}$*; Tie2-Cre*$^{+/-}$ were obtained, mice with genotype *Wls*$^{flox/wt}$*; Tie2-Cre*$^{+/-}$ were used for experiments and mice with genotypes *Wls*$^{flox/flox}$*; Tie2-Cre*$^{-/-}$ *and Wls*$^{flox/wt}$*; Tie2-Cre*$^{-/-}$ were used as *Con*.

Further, to generate viable endothelial cell specific *Wls* knockout mice, *Wls*$^{flox/flox}$ mice were bred to *VE-cadherin-CreER*^{T2} mice [114] in the same manner as above. Mice with phenotype *Wls*$^{flox/flox}$*; VE-cadherin-CreER*$^{T2+/-}$ represent *Wls*-EKO. Mice with genotypes *Wls*$^{flox/flox}$*; VE-cadherin-CreER*$^{T2-/-}$ *and Wls*$^{flox/wt}$*; VE-cadherin-CreER*$^{T2-/-}$ were used as *Con*.

3.2 TAMOXIFEN INJECTION

Tamoxifen was injected to adult *Wls*-EKO and control mice in order to induce Cre recombinase activity. Tamoxifen (Sigma) was dissolved in corn oil (Sigma) to make the final concentration 50 mg/ml. Six- to eight-week old control and *Wls*-EKO mice were injected with 7.5 mg tamoxifen every 48 hours, 3 injections in total, and the livers were harvested at one week, two weeks or four weeks after the last injection.

3.3 MORPHOMETRIC ANALYSIS

All experiments on mice were performed under the strict guidelines of the National Institutes of Health and the Institutional Animal Use and Care Committee at the University of Pittsburgh. Age- and sex-matched conditional KO and *Con* littermates were weighed and killed (n=3–5). Livers were isolated and the wet weights were recorded to calculate the liver weight to body weight ratio (LW/BW X100). The means for the 2 groups were compared for statistically significant differences by Student t test.

3.4 PARTIAL HEPATECTOMY

Twelve-week-old male Con or KO were subjected to partial hepatectomy (PH) [115]. Equal numbers of KO and Con mice were killed by cervical dislocation after Isoflurane anesthesia at different time points: 4 hours (n=3), 24 hours (n=3), 40 hours (n≥3), 3 days (n≥3), 4 days (n=3)

and 5 days (n=3) after PH. The regenerating livers were harvested and used for protein extraction, and paraffin embedding as described elsewhere [65, 68].

3.5 ACETAMINOPHEN LIVER TOXICITY AND MORTALITY

Twelve-week-old *Lrp*-LKO mice (n=5) and Con mice (n=5) received an intraperitoneal injection of acetaminophen (600 mg/kg dissolved in 0.45% NaCl). Animals were sacrificed 24 hours after acetaminophen administration. Blood samples were collected from orbital sinus, and serum alanine aminotransferases (ALT) and aspartate aminotransferase (AST) levels determined by automated methods at the University of Pittsburgh Medical Center Clinical Chemistry laboratory.

3.6 PROTEIN EXTRACTION AND WESTERN BLOTS

Whole-cell lysate preparation was performed using RIPA buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150mmol/L sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate [pH adjusted to 7.4]) containing fresh protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO) as described previously [65]. 40-50 μg of protein was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis using the mini-PROTEIN 3 electrophoresis module assembly (Biorad, Hercules, CA) and transferred to immobilon-polyvinylidene difluoride membranes (Millipore, Billerica, MA). Proteins were detected by Super-Signal West Pico Chemiluminescent Substrate (Pierce, Rockford,

IL) and visualized by autoradiography.

Primary antibodies used in this study were against β-catenin (BD Transduction); TCF4 (Cell Signaling), Lrp5 (Cell Signaling), Lrp6 (Cell Signaling), Cyclin-D1 (Thermo Marker); γ-catenin (Cell Signaling), E-cadherin (Santa Cruz Biotechnology), Regucalcin (Santa Cruz Biotechnology), Glutamine Synthetase (Santa Cruz Biotechnology), Cyp1a2 (Santa Cruz Biotechnology), Cyp2e1 (Millipore), Wls (Millipore), Actin (Chemicon); and GAPDH (Santa Cruz Biotechnology) at 1:1000. Horseradish-peroxidase-conjugated secondary antibodies (Chemicon) were used at 1:20,000 to 1:50,000 dilutions.

3.7 HISTOLOGY AND IMMUNOHISTOHEMISTRY

Liver sections from the age- and sex-matched KO and Con mice were analyzed by immunohistochemistry (IHC) for β-catenin (Santa Cruz Biotechnology), Glutamine Synthetase (Santa Cruz Biotechnology), Cyp1a2 (Santa Cruz Biotechnology), Cyp2e1 (Millipore) and PCNA (Santa Cruz Biotechnology) to determine their expression and/or localization using the indirect immunoperoxidase technique as described previously [116]. Briefly, 4 μm paraffin sections were passed through xylene, graded ethanol, and rinsed in phosphate-buffered saline (PBS). 3% hydrogen peroxide (Sigma) was used to inactivated endogenous peroxide. Slides were microwaved in citrate buffer for 8 minutes and blocked in super block (ScyTek Laboratories) for 10 minutes followed by 1-hour incubation at room temperature in the primary antibody. After 3 washes with PBS, the sections were incubated in the secondary horseradish-peroxidase-conjugated antibody (Chemicon) for 30 min at room temperature and signal was detected using the ABC Elite kit (Vector Laboratories, Burlingame, CA) following the manufacturer's instructions. Sections were

counterstained with Shandon (Thermo Scientific) for 1 minute and passed through the dehydration process followed by cover slipping and mounting using DPX (Fluka Labs, St. Louis, MO). Slides were viewed under an Axioskop 40 (Zeiss) upright research microscope and digital images were obtained by Nikon Coolpix camera.

For proliferation assay, indirect IHC was performed for PCNA that recognizes cells in the cell cycle. The positive cells were counted in 3 low-power fields (200X) in 3 sections from 3 different knockout or control livers. The average numbers of positive cells were compared and statistical assessment was made by the Student t test. A p value of less than 0.05 was considered significant.

3.8 IMMUNOPRECIPITATION

Five hundred μg of lysate in a 1-ml volume (in the presence of protease and phosphatase inhibitors) was precleared using appropriate control IgG together with 12 μl of protein A/G agarose for 30 min at 4°C (Santa Cruz Biotechnology). The supernatant obtained after centrifugation (1000 × *g*) at 4°C was incubated with primary antibody overnight at 4°C using end-over-end rotation, followed by 20 μl of resuspended protein A/G agarose for 1 h. The pellets were collected by centrifugation (1000 × *g*) and washed four times for 5 min each with RIPA buffer at 4°C. The pellets were resuspended in an equal volume of standard electrophoresis loading buffer with SDS and fresh β-mercaptoethanol and boiled for 5 min. 15 μl of the samples were resolved on ready gels. Antibodies used for immunoprecipitation were mouse anti-β-catenin (BD Transduction) and rabbit anti-TCF4 (Cell Signaling).

3.9 SEPARATION OF HEPATOCYTES, ENDOTHELIAL CELLS AND MACROPHAGES

Mouse liver cells were isolated by 2-step collagenase perfusion [71]. Hepatocytes were isolated as a pellet after centrifugation (500rpm) for 5 minutes. The supernatant was centrifuged (1500 rpm) for 10 minutes, and the pellet containing mostly non-parenchymal cells (NPCs) was collected. Endothelial cell and macrophage positive selection from NPCs was performed using QuadroMACS column separation Kit (Miltenyi Biotech, Cambridge, MA). Anti-mouse CD31, anti-mouse F4/80 antibody (Biolegend, San Diego, CA) and specific microbeads were used according to the manufacture's instruction. After the column separation, RNA was extracted from the cell pellet, and cytospin was performed with the cell suspension. Cells were centrifuged at 500 rpm for 5 minutes on glass slides followed by fixation with 4% paraformaldehyde for 10 minutes when used for immunofluorescence staining.

3.10 SEPARATION OF HEPATIC STELLATE CELLS

HSCs were isolated from Con mice and *Wls*-SKO as described [117, 118]. Briefly, livers were digested with collagenase- and protease-containing HBSS. Cells were suspended and incubate with collagenase and DNase at 37^0C for 45 min. The suspension was filtered through nylon mesh. The filtrate was centrifuged (50g) 3-4 times for 1 min to remove hepatocytes and cell debris, and the supernatant was collected and centrifuged (1400g) for 7 min. The pellet containing NPCs was suspended in HBSS, and after purification via Nycodenz gradient centrifugation, HSCs were suspended (1×10^6 cells/ml) in DMEM containing 10% FBS, 10% horse serum. Cells were seeded

on gelatin (0.1% in PBS)-coated plates at a density of $0.5\times10^6/cm^2$. Twenty minutes later, loosely adherent HSCs were harvested and RNA was harvested.

3.11 IMMUNOFLUORESCENCE STAINING

The staining was done using the protocol described before [65]. Briefly, cells were blocked in 2% bovine serum albumin in PBS for 45 min at room temperature. Primary antibody, rat anti-mouse F4/80 antibody (AbD Serotec, Raleigh, NC) at 1:100 dilution, was added to sections for 1 h at room temperature. After being washed, the fluorescently tagged secondary antibodies were applied to the sections for 1 h at room temperature. After three washes with PBS, the nuclei were counterstained using 0.001% Hoechst dye (bis-benzimide). The slides were coverslipped using gelvatol.

3.12 RNA ISOLATION AND QUANTITATIVE POLYMERASE CHAIN REACTION

Cellular RNA was obtained by micro RNA isolation kit (Ambion). Total RNA was reverse-transcribed after DNase treatment using the TURBO DNAse Kit (Ambion) as per the manufacturer's instructions. The RNA was used for quantitative polymerase chain reaction (q-PCR) analysis as described elsewhere [119]. The comparative delta Ct (ΔCT) method was used for analysis of the data, and calculations were made with the StepOne software (Applied Biosystems, Grand Island, NY). The primers used in the study are listed (Table. 1).

Table 1. Primer sequences used for q-PCR

Gene	Forward primer	Reverse primer
Alb	tgacccagtgttgtgcagag	ttctccttcacaccatcaagc
CD68	gacacttcgggccatgtt	gaggaggaccaggccaat
Lyz	gaatggaatggctggctact	cgtgctgagctaaacacacc
Tie2	cataggaggaaacctgttcacc	gcccccacttctgagctt
Vegfr2	cagtggtactggcagctagaag	acaagcatacgggcttgttt
Gfap	tcgagatcgccacctacag	gtctgtacaggaatggtgatgc
Desmin	gcgtgacaacctgatagacg	tggatttcctcctgtagtttgg
Gapdh	acccagaagactgtggatgg	cacattgggggtaggaacac
Wls	cccagccatgagcaaagt	gcatgaggaacttgaacctga
GS	ctcgctctcctgacctgttc	ttcaagtgggaacttgctga
Cyp1a2	cctggactgactcccacaac	aacgccatctgtaccactga
Cyp2e1	ccaccagcacaactctgagata	cccaataaccctgtcaatttctt
Wnt1	atccatctctcccacctcctac	gaatctttctctcaccctctgg
Wnt2	tctgtctatcttgggcattctg	ttccttcgctatgtgatgtttc
Wnt2b	accttcctctaccctcaatcct	tcactcagcctcctaaatccat
Wnt3	gtctgctaatgctggcttgac	taggaagggatgggaggtgt
Wnt3a	tcggcgatggtggtagagaaac	tcgcagaagttgggtgagg
Wnt4	agaactggagaagtgtggctgt	aaaggactgtgagaaggctacg
Wnt5a	gtcctttgagatgggtggtatc	acctctgggttagggagtgtct
Wnt5b	tgtcagttgtatcaggagcaca	gtgaaggcagtctctcggcta
Wnt6	tttacaccagcccacgaaag	actcacccatccatcccagta
Wnt7a	ggatgctcacagggaaagaac	gcaggaaacccagaataccc
Wnt7b	gctccttcctactcgctctgt	ggtccctttgtggttcacttt
Wnt8a	acggtggaattgtcctgagcatg	gatggcagcagagcggatgg
Wnt8b	gtttgcttgggaccgttg	tccatttcgggagtcatca
Wnt9a	atggtgtgtctggctcctg	cagtggcttcattggtagtgct
Wnt9b	gggtgtgtgtggtgacaatct	ggtccttgcttcctctcttg
Wnt10a	tcctgttcttcctactgctgct	acgcacacacacctccatc
Wnt10b	ccactacagcccagaacctc	ggagagacccttttcaacaactg
Wnt11	ccctggaaacgaagtgtaaatg	aggtagcgggtcttgaggtc
Wnt16	gctgtaacctcctctgctgtg	gtggacatcggtcatactttca

3.13 ENZYME-LINKED IMMUNOSORBENT ASSAY

48 hours after PH, liver was perfused and hepatocytes were cultured for 24 hours. Conditioned medium was collected and concentrated by centrifugal filter devices (Millipore). Wnt5a level was measured by using the mouse Wnt5a enzyme-linked immunosorbent assay (ELISA) kit (CUSABIO). Experiments were performed according to the manufacturer's instructions. In brief, standard and samples were loaded onto 96-well microplates pre-coated with antibody specific for Wnt5a, and incubated for 2 hours at 37°C. Each well was aspirated and then the plate was incubated with biotin-conjugated antibody specific for Wnt5a for 1 hour at 37°C. After washing, avidin conjugated Horseradish Peroxidase (HRP) was added to the wells and incubated for 1 hour at 37°C. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells and incubated for 15-30 minutes at 37°C to induce a color reaction. The reaction was stop by adding Stop Solution to each well. Optical density of each well was determined by microplate reader at wavelength 450 nm.

4.0 RESULT

4.1 RESULT (AIM1): BETA-CATENIN ACTIVATION IN LIVER IS WNT-SIGNALING DEPENDENT

To address to what extent β-catenin activation is Wnt-signaling dependent in liver homeostasis and during liver regeneration (LR), we generated liver-specific *Lrp5/6* null mice (*Lrp-LKO*) where only Wnt-signaling is abolished, while Wnt-independent signaling remains intact, and compared them with liver-specific *β-catenin* null mice (*β-catenin*-LKO).

4.1.1 Generation and characterization of conditional *Lrp5/6* null mice

After strategic breeding, liver-specific *Lrp5/6* null mice (*Lrp-LKO*) were generated [120]. Genotyping was performed by PCR (Figure 11A), using primer P1 and P3 as described previously [109]. Livers from *Lrp*-LKO exhibited a significant decrease in Lrp5 and Lrp6 protein compared to control mice (*Con*) (Figure 11A). Eight-month-old *Lrp-LKO* and *Con* mice had comparable and normal levels of serum AST, ALT and albumin (data not shown). While total bilirubin was within normal range, average in controls was 0.26 mg/dl (n=5) as compared to 0.5 mg/dl (n=5) in *Lrp-LKO* (p=0.009). *Lrp*-LKO also displayed smaller livers and LW/BW ratio was significantly lower in *Lrp*-LKO compared to *Con* (Figure 11B). To address the discrepancy in size, we assessed

hepatocytes in S-phase by PCNA immunohistochemistry (IHC). *Lrp*-LKO livers had significantly less PCNA positive cells at baseline (Figure 11C, 11D). Concomitantly, lower Cyclin-D1 expression was also evident in KO by western blots (WB) (Figure 11E). Intriguingly, all the above observations were similar to *β-catenin*-LKO mice [49].

4.1.2 β-Catenin at adherens junctions in the *Lrp-LKO* livers at baseline

Next, we assessed β-catenin levels and levels of other key components at adherens junctions in the *Lrp*-LKO livers [120]. WB shows *Lrp*-LKO and *Con* to have comparable levels of β-catenin, γ-catenin and E-cadherin (Figure 11E).

4.1.3 Disruption of metabolic zonation in conditional *Lrp5/6* null mice

Next, we assessed β-catenin localization by IHC in Con and Lrp-LKO livers [120]. Con livers show predominantly membranous β-catenin except in pericentral hepatocytes where there was enhanced cytosolic labeling as well (Figure 11F). However in Lrp-LKO, cytosolic β-catenin localization in pericentral hepatocytes was notably lacking with occasional periportal hepatocyte showing some cytoplasmic staining (Figure 11F). Lrp-LKO livers also showed notably low protein expression of multiple downstream targets of β-catenin, such as Regucalcin, GS, Cyp1a2 and Cyp2e1 (Figure 11E). IHC also showed loss of pericentral expression of GS, Cyp2e1 and Cyp1a2 in Lrp-LKO livers (Figure 12A).

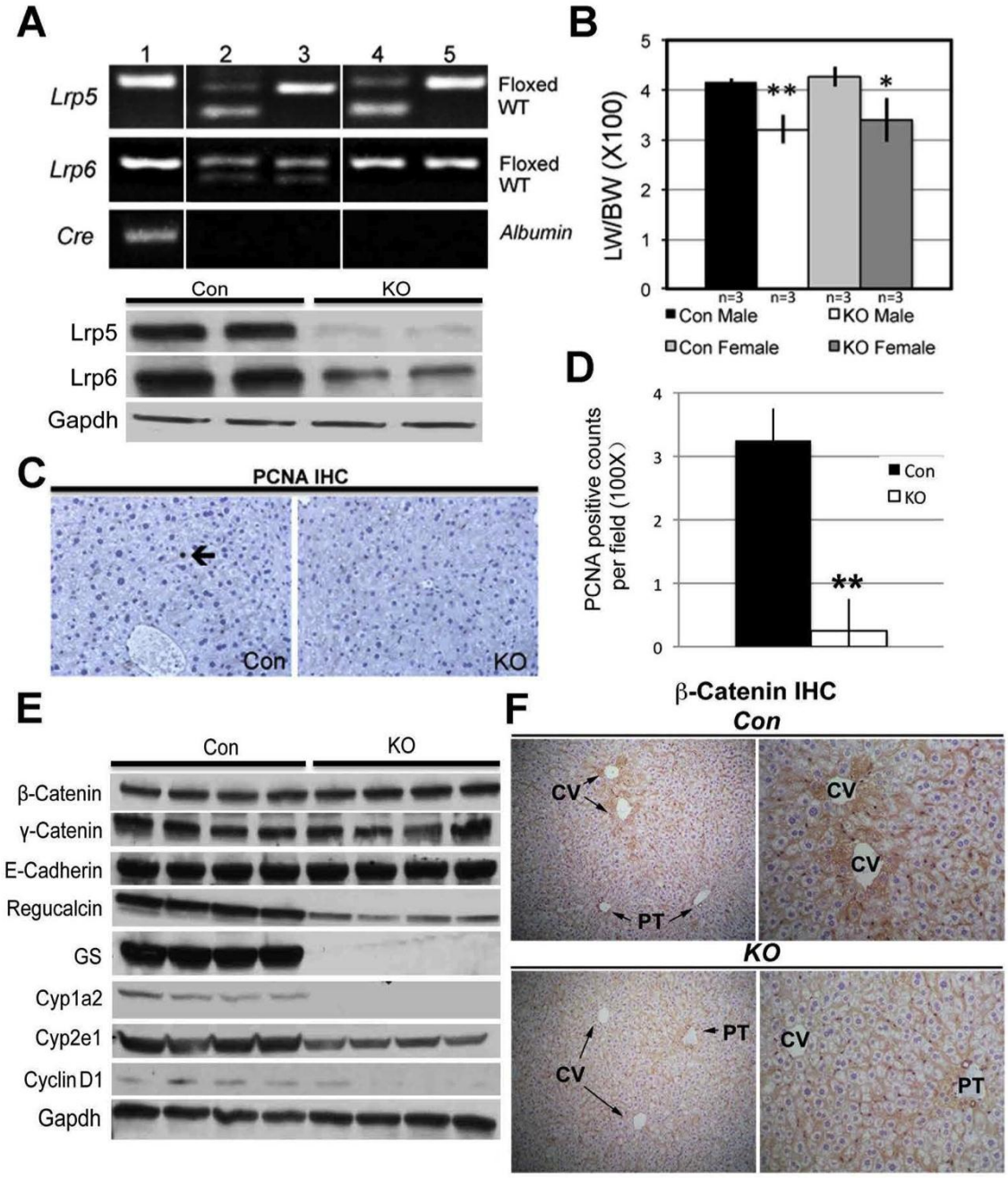

Figure 11. Successful deletion of Lrp5 and Lrp6 in liver leads to alterations in downstream β-catenin signaling (*p<0.05, **p<0.01)

A. Representative PCR (upper panel) shows genotype of mice for identifying Lrp-LKO with genotype $Lrp5^{flox/flox}$; $Lrp6^{flox/flox}$; $Cre^{+/-}$ (lane 1). Mice with other genotypes (lane 2-5) were used as Con. Representative WB (lower panel) shows a dramatic decrease in Lrp5 and Lrp6 in the tissue lysates from the Lrp-LKO livers.

B. Decreased LW/BW ratio in 8-month old Lrp-LKO. This difference was around 20% in both males and females and significant.

C. IHC for PCNA shows an occasional positive hepatocyte (arrow) in a representative section from Con liver at baseline, while none were detected in Lrp-LKO.

D. Quantification of PCNA staining shows significantly fewer positive cells in Lrp-LKO.

E. Representative WB shows no change in total levels of β-catenin, γ-catenin and E-cadherin in Lrp-LKO livers as compared to Con. However several downstream targets of β-catenin were downregulated in Lrp- LKO livers. Prolonged exposure of WB film enabled us to observe baseline Cyclin-D1 in Con, which was notably reduced in Lrp-LKO livers.

F. IHC for β-catenin shows predominantly membranous localization in both Lrp-LKO and Con livers. However, in Con, cytosolic localization of β-catenin was seen in hepatocytes around central vein (CV) and not portal triads (PT). Lrp-LKO show absence of cytosolic β-catenin around CV with a few hepatocytes around PT showing some cytoplasmic localization. (left panel Con and KO-100x; right panel Con and KO-200x).

The functional loss of Cyp1a2 and Cyp2e1 was further addressed by acetaminophen (APAP) toxicity study [120]. Cyp1a2 and Cyp2e1 metabolize APAP to a toxic metabolite, N-acetyl-p-benzo-quinone imine (NAPQI), which is highly reactive and causes liver cell necrosis through glutathione depletion [121, 122]. *β-Catenin*-LKO mice are protected from APAP overdose due to absent Cyp2e1 and Cyp1a2 [48, 49]. Likewise *Lrp*-LKO when administered intra-peritoneal injection of APAP (600mg/kg), showed significantly lower serum AST and ALT levels as compared to *Con* (Figure 12B). Therefore, despite normal β-catenin levels in *Lrp*-LKO, ablation of *Lrp5/6* to disrupt Wnt signaling impaired β-catenin activity and zonation.

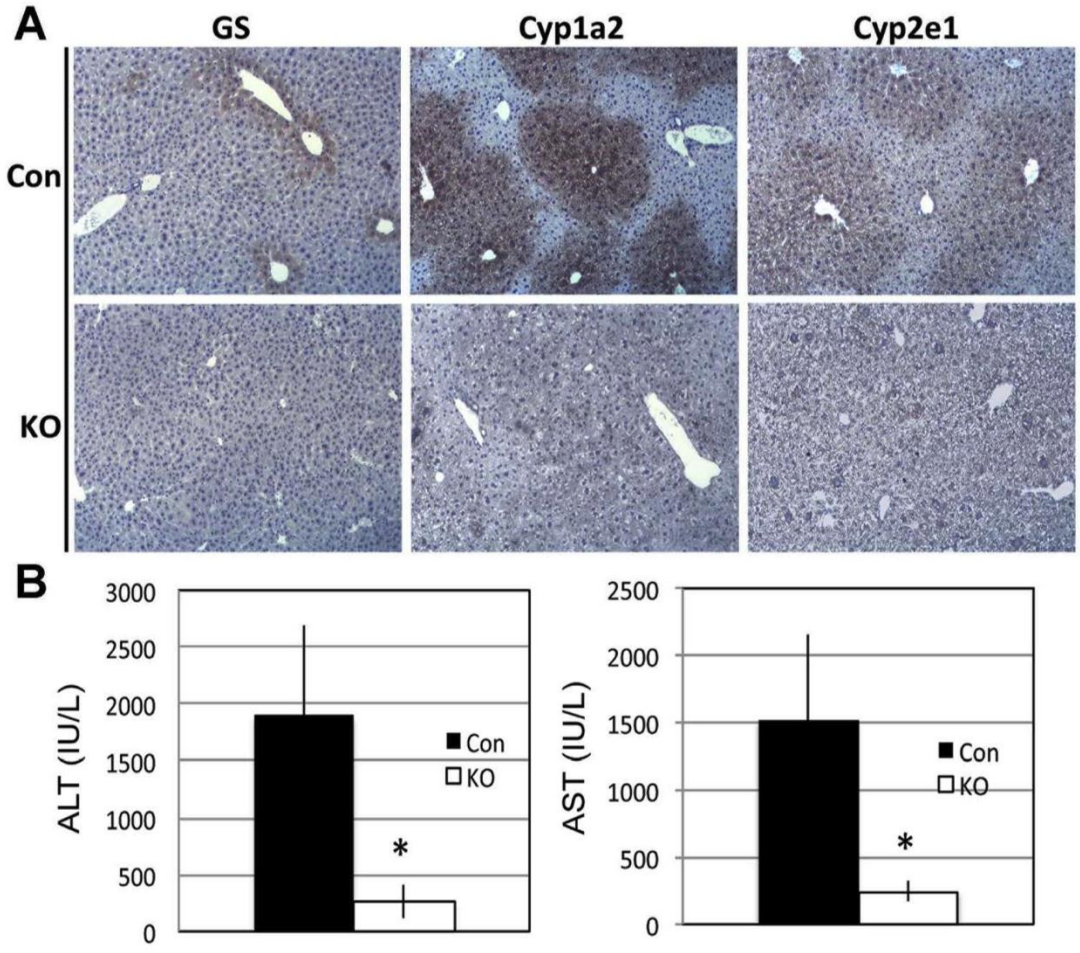

Figure 12. Loss of Lrp5/6 in liver compromises pericentral zonation

A. IHC shows normal pericentral expression of GS, Cyp1a2 and Cyp2e1 in Con livers, while absence of these proteins is observed in littermate Lrp-LKO livers.

B. When injected with lethal dose of APAP (600 mg/kg body weight), Lrp-LKO showed significantly lower ALT and AST levels than Con after 24 hours (*$p<0.05$).

4.1.4 Retarded liver regeneration in *Lrp-LKO* mice after partial hepatectomy

Next, *Lrp*-LKO and *Con* were subjected to PH. As expected, *Con* mice showed abundant hepatocytes in S-phase at 40 and 72 hours after PH with decline at later time-points (Figure 13A, 13B) [120]. Intriguingly, *Lrp*-LKO livers exhibited significantly fewer PCNA-positive hepatocytes at both 40 and 72 hours, although more PCNA-positive hepatocytes were observed at 72 than 40 hours (Figure 13A, 13B). In addition, numbers of mitotic figures as assessed by analysis of H&E images from Lrp-LKO and Con showed a notably lower mitosis in hepatocytes in the former at 72 hours (Figure 14A, 14B). β-Catenin target Cyclin-D1 that regulates G1-S phase transition during LR [123] was notably lower in *Lrp*-LKO at 40 hours with progressive increase at 72 hours although it was still lower than *Con* (Figure 13D).

Next, we wanted to address if β-catenin could be activated by alternate signals in *Lrp*-LKO than *Con* after PH. Intriguingly, no differences in Y654-, S552- or S675-β-catenin were evident between the *Lrp*-LKO and *Con* at 40 or 72 hours after PH (Figure 13C).

Since β-catenin binding to TCF4 precedes Cyclin-D1 expression after PH [49, 71], we assessed their association by immunoprecipitation at 4 hours after PH [120]. TCF4-β-catenin complex was observed in *Con* but absent in *Lrp*-LKO (Figure 13E).

Taken together, these data suggest that *Lrp*-LKO phenocopy *β-catenin*-LKO after PH, thereby indicating that only Wnt signaling is responsible for β-catenin activation during LR after PH.

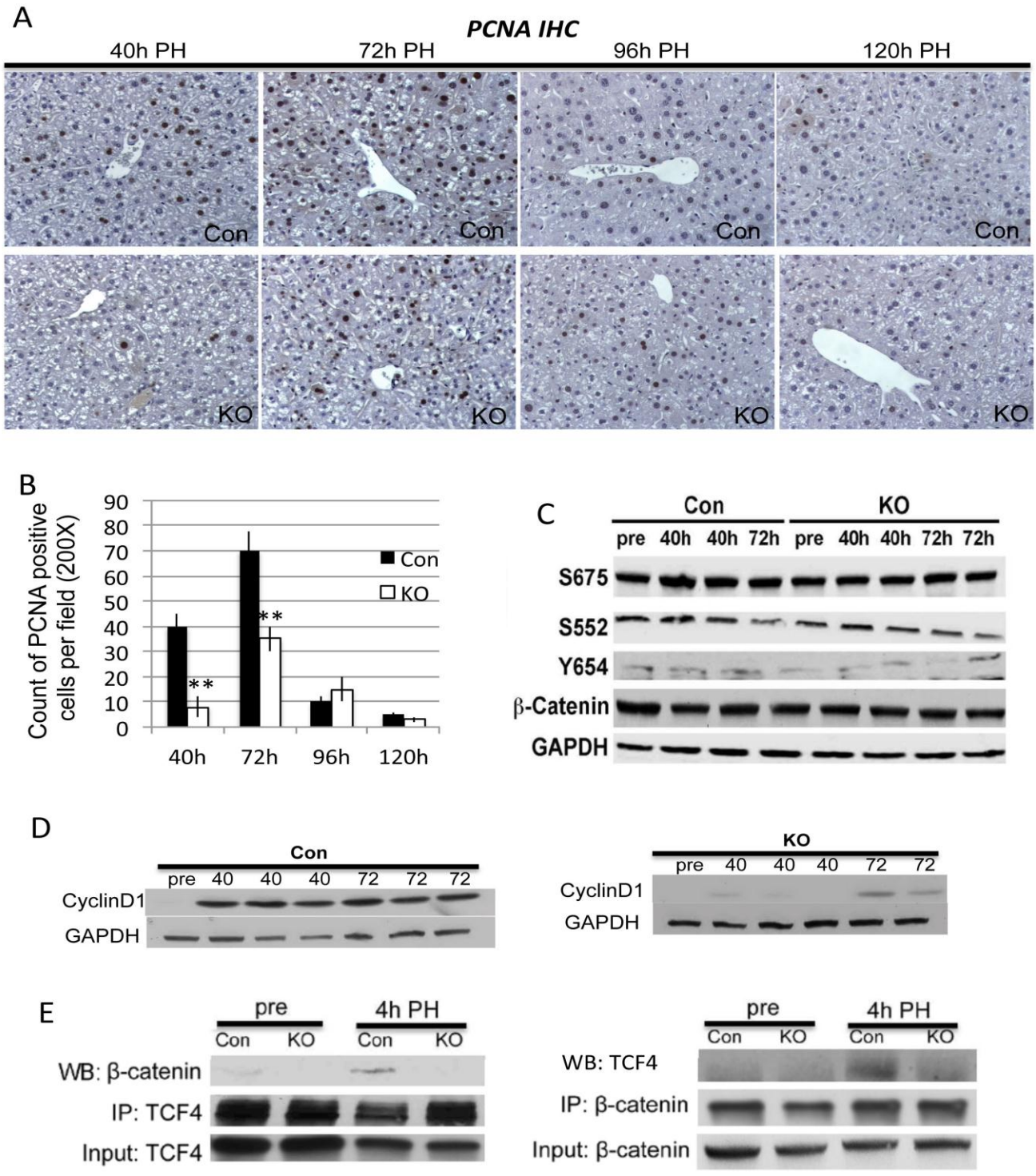

Figure 13. Abolishing Wnt/β-catenin signaling through Lrp5/6 ablation in liver impairs LR after PH

A. IHC for PCNA identifies several hepatocytes in S-phase at 40 and 72 hours after PH, while only a few trailing hepatocytes were PCNA-positive at 96 and 120 hours. In contrast, Lrp-LKO showed dramatically fewer PCNA-positive hepatocytes at 40 hours with an increase at 72 hours. Like Con, only a few PCNA positive hepatocytes were detected at later times in Lrp-LKO (200x).

B. PCNA quantification shows a gradual increase in positive hepatocytes in Lrp-LKO from 40 to 72 hours although these are lower than Con at both times (**$p<0.01$).

C. Representative WB shows comparable levels of S675-β-catenin, S552-β-catenin, Y654-β-catenin and total β-catenin in Con and Lrp-LKO livers after PH.

D. WB shows low protein expression of Cyclin-D1 after PH in Lrp-LKO compared to Con at both 40 and 72 hours although its levels increase in Lrp-LKO at 72 hours.

E. Representative WB shows β-catenin and TCF4 association at 4 hours after PH in Con and not Lrp-LKO livers by immunoprecipitation studies. Immunoprecipitation studies were performed by pull down of either β-catenin or TCF4. Respective input controls are included in analysis as well.

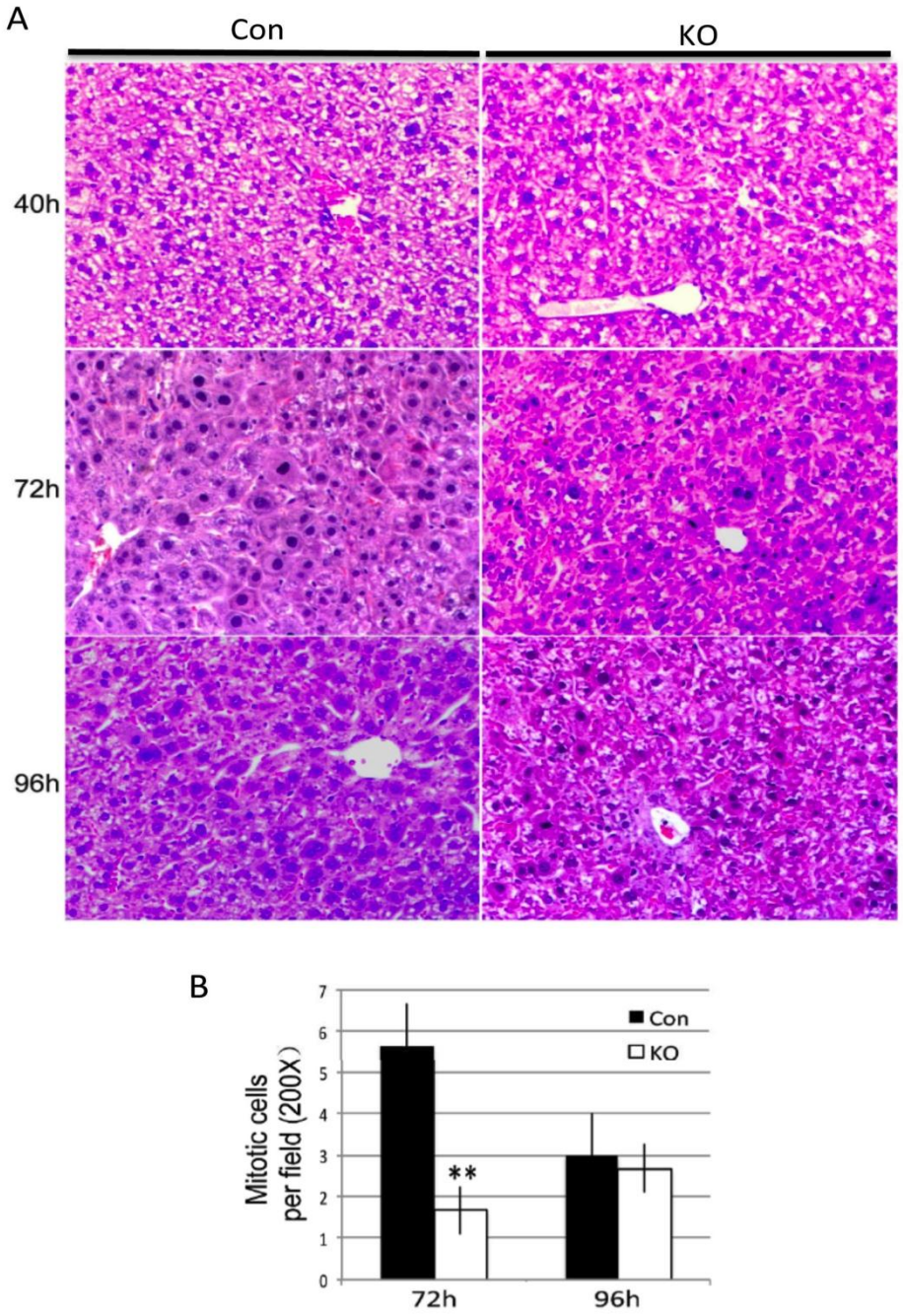

Figure 14. Mitotic figures show impaired liver regeneration in *Lrp*-LKO

A. Mitotic figures of Con and Lrp-LKO at 40, 72 and 96 hours after PH.

B. Quantification of mitotic figures shows a significantly lower number in Lrp-LKO as compared to Con at 72 hours after PH. (**p<0.01)

4.2 RESULT (AIM2): THE CELLULAR SOURCE OF WNT LIGANDS

Our work demonstrated that Wnt-signaling is the predominant pathway that regulates β-catenin effects in both liver homeostasis and during liver regeneration (LR) [120]. We hence next aimed to investigate the cellular source of Wnt ligands in the liver. By isolating major hepatic cell populations, we analyzed cell specific Wnt expression in liver homeostasis and during LR. By genetic manipulation of *Wntless (Wls*), which is specific and necessary for Wnt secretion [124], we generated multiple cell-specific *Wls* deletion mice, which lack the ability of a specific hepatic cell population to secrete Wnt ligands, and analyzed their hepatic zonation and LR kinetics, to elucidate the role of different liver cell types in Wnt secretion and β-catenin activation.

4.2.1 Wnt expression in individual hepatic cell populations in liver homeostasis and regeneration

Our lab has previously shown that major cell types in the liver express various Wnt ligands in normal liver [82]. In order to get deeper insight into the role of different cell types in Wnt secretion and address the cellular source of Wnt proteins in the liver, we isolated different liver cell types after collagenase perfusion, and analyzed Wnt expression at baseline and after partial hepatectomy (PH), to address the potential cellular source of Wnt ligands in liver homeostasis and during LR.

4.2.1.1 Wnt expression profile in individual liver cell types at baseline

Different liver cell populations, including hepatocytes, endothelial cells, macrophages and stellate cells, were isolated from two-months-old male mice as described in the methods. The high purity of isolated cells was confirmed by quantitative PCR (q-PCR) using primers for cell specific markers (Figure 15).

The Wnt expression profile in hepatocytes, endothelial cells and macrophages was determined by q-PCR (Figure 16). The expression of eight out of the nineteen Wnts was detectable in hepatocytes, and eleven out of nineteen Wnts were detectable in endothelial cells, and ten out of nineteen in macrophages. Among the Wnts that can be detected (Figure 16), the expression pattern appears to be distinct in individual cell types. Wnt2 and Wnt2b were highly expressed in endothelial cells and macrophages. Some Wnt ligands, such as Wnt4, Wnt9b and Wnt16 were mainly expressed in endothelial cells, while hepatocytes have more abundant Wnt5a and Wnt5b expression.

Previous studies have demonstrated various roles of Wnt ligands in different aspects of liver biology. Wnt2 has been shown to be a pro-proliferative signal that is induced after PH to trigger LR [102]; Wnt2b signaling positively regulates liver specification during liver development [125]; Wnt4 upregulation has been shown to activate stellate cells [126]. While Wnt5a activate noncanonical β-catenin-independent pathways [127], it is expressed in mature hepatocytes and cholangiocytes [82], but the physiological functions of Wnt5a in liver are not clear. The different expression pattern of Wnt ligands in individual hepatic cell populations might be crucial for maintaining liver homeostasis, or might be related to the functions of specific cell populations in response to PH or liver injuries.

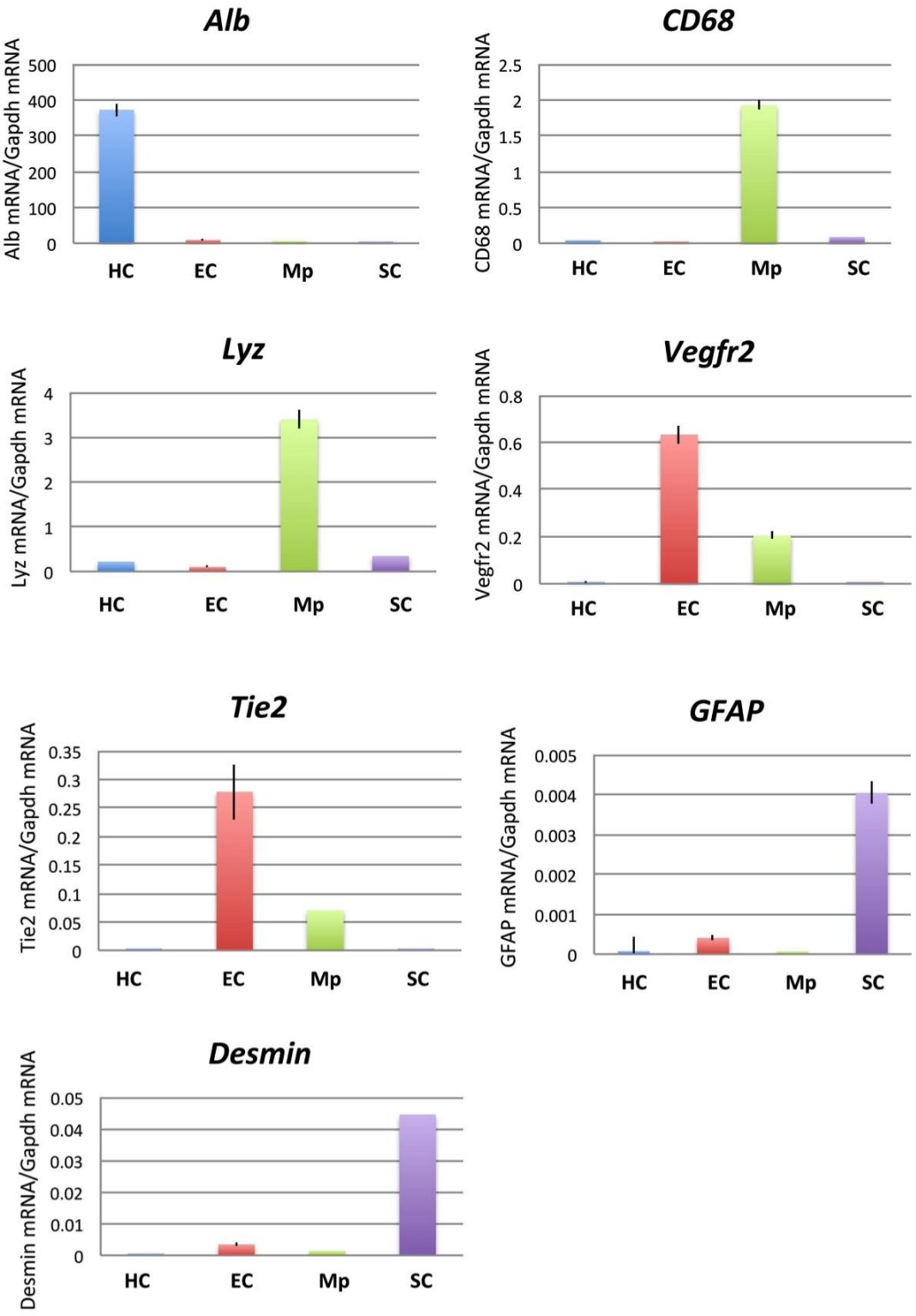

Figure 15. Q-PCR analysis of cell specific markers in individual hepatic cell populations

Four major hepatic cell populations were isolated with high purity. The cell specific marker expression was determined by q-PCR. Relative expression levels were normalized to *Gapdh*. HC, hepatocytes; EC, endothelial cells; Mp, macrophages; SC, stellate cells.

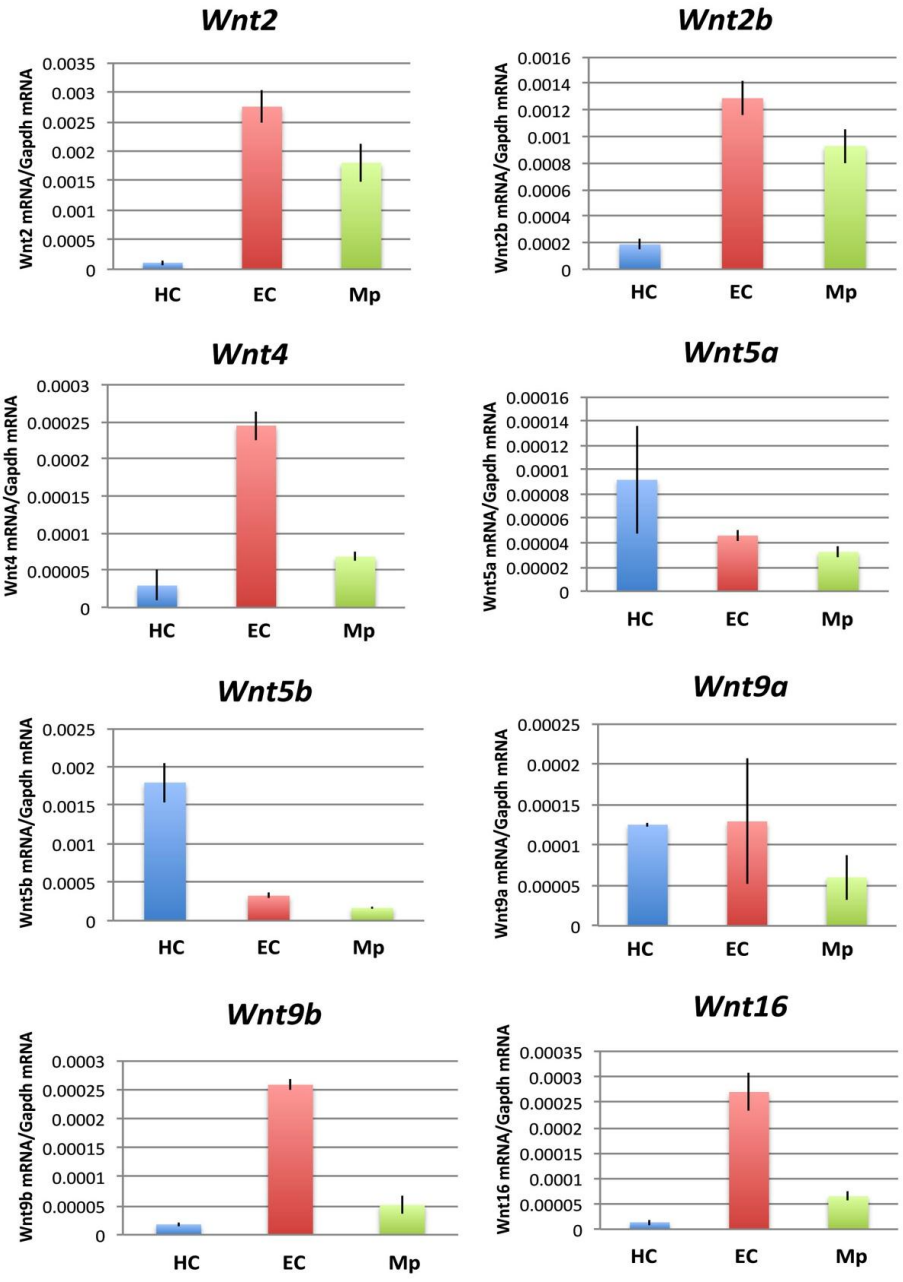

Figure 16. Wnt expression analysis in individual cell populations at baseline

Hepatocytes (HC), endothelial cells (EC) and macrophages (Mp) were isolated from normal mouse liver, and Wnt expression in individual cell populations was determined by q-PCR. The expression of Wnt was normalized to Gapdh.

4.2.1.2 Wnt expression profile in individual liver cell types during liver regeneration

Next, Wnt expression was examined in different cell populations after PH. Fold changes of Wnt expression 12 hours after PH versus before PH was analyzed in hepatocytes, endothelial cells and macrophages (Figure 17). The Wnt expression pattern differed in individual cell populations; notably, *Wnt2* and *Wnt9b* expression were dramatically induced in both endothelial cells and macrophages after PH, but the increase was moderate in hepatocytes (Figure 17). There was a 45-fold induction of *Wnt2* expression and a 16-fold increase of Wnt9b expression in endothelial cells. In macrophages, the induction of *Wnt2* and *Wnt9b* was also dramatic; while in hepatocytes, the increase was marginal (Table 2). *Wnt2* and *Wnt9b* expression in resting and regenerating livers (Figure 18) further confirmed this finding.

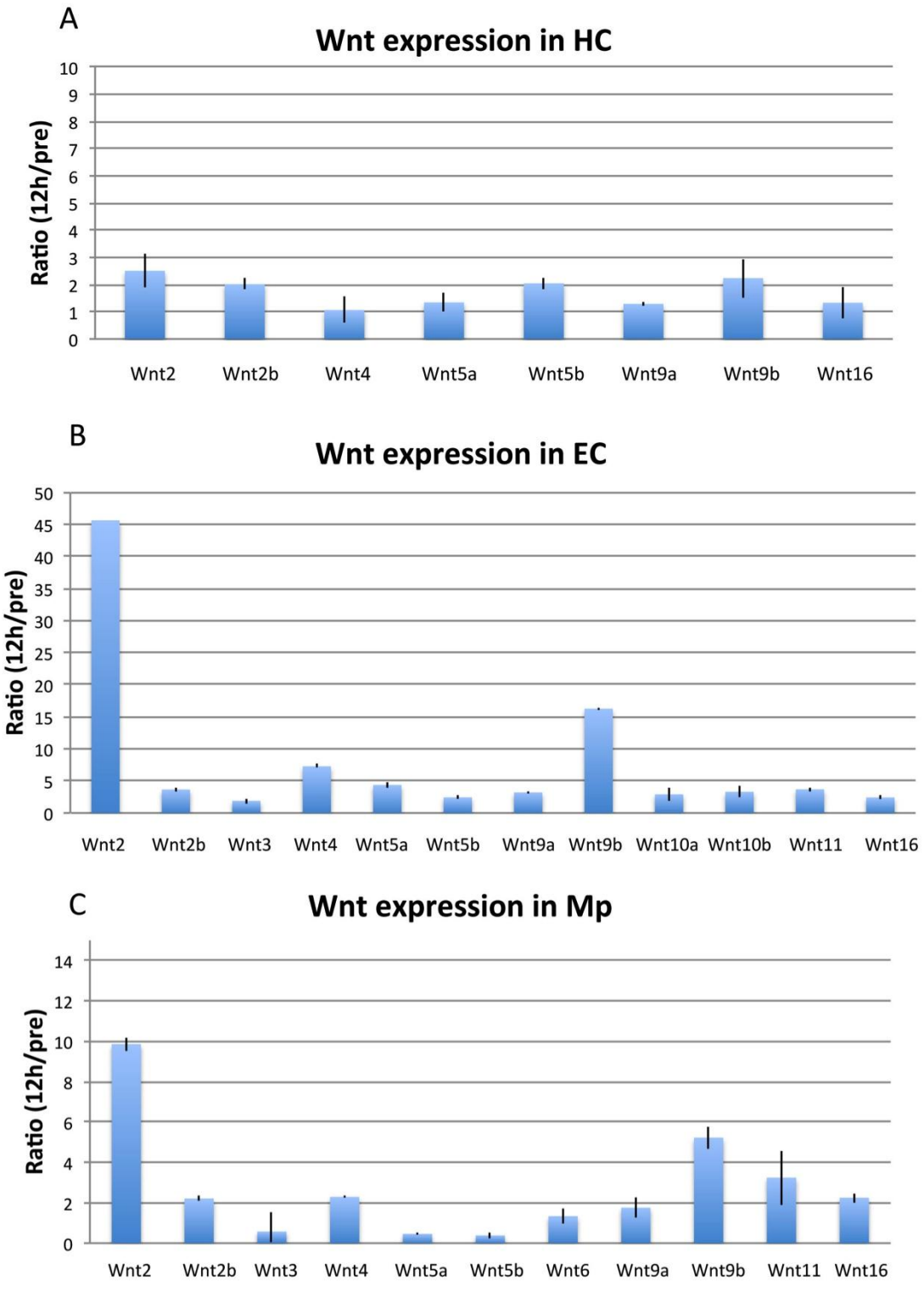

Figure 17. *Wnt* expression in individual cell populations after PH

Wnt expression was analyzed in hepatocytes (A), endothelial cells (B), and macrophages (C) 12 hours after PH. The expression levels at 12 hours after PH were normalized to the levels prior to PH (pre).

HC, Hepatocytes; EC, Endothelial cell; Mp, Macrophages.

Table 2. Ranking of fold changes of *Wnt* expression after PH in individual cell populations

Endothelial cell			**Macrophage**		
Ranking	**Gene**	**Fold change**	**Ranking**	**Gene**	**Fold change**
1	*Wnt2*	45	1	*Wnt2*	9.8
2	*Wnt9b*	16	2	*Wnt9b*	5.2
3	*Wnt4*	7.3	3	*Wnt11*	3.2
4	*Wnt5a*	4.4	4	*Wnt4*	2.3
5	*Wnt11*	3.7	5	*Wnt16*	2.2
6	*Wnt2b*	3.7	6	*Wnt2b*	2.2
7	*Wnt10b*	3.3	7	*Wnt9a*	1.75
8	*Wnt9a*	3.2	8	*Wnt6*	1.33
9	*Wnt10a*	2.9	9	*Wnt3*	0.57
10	*Wnt5b*	2.4	10	*Wnt5a*	0.46
11	*Wnt16*	2.4	11	*Wnt5b*	0.38
12	*Wnt3*	1.89			

Hepatocyte		
Ranking	**Gene**	**Fold change**
1	*Wnt2*	2.5
2	*Wnt9b*	2.3
3	*Wnt5b*	2
4	*Wnt2b*	2
5	*Wnt5a*	1.34
6	*Wnt16*	1.33
7	*Wnt9a*	1.3
8	*Wnt4*	1.1

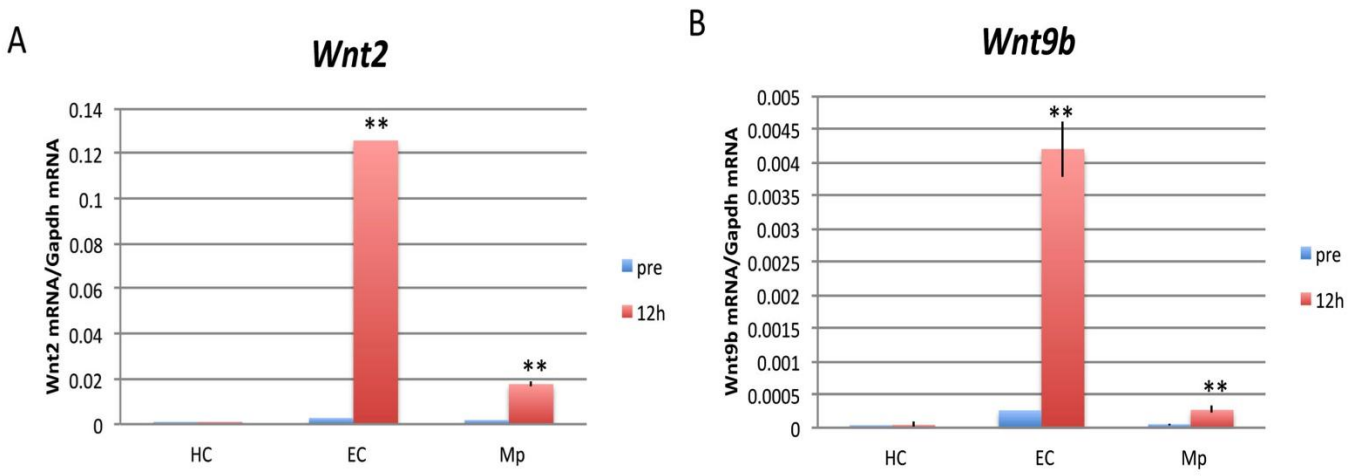

Figure 18. *Wnt2* and *Wnt9b* expression in individual cell populations before and after PH

Wnt2 (A) and *Wnt9b* (B) expression was analyzed in different cell types before and 12 hours after PH. HC, Hepatocytes; EC, Endothelial cell; Mp, Macrophages.

Wnt2 has been shown to be an autocrine growth factor in liver sinusoidal endothelial cells (LSECs). Wnt2 induces endothelial cell proliferation and differentiation by stimulating VEGF signaling [128]. After PH, Wnt2 is secreted by LSECs to trigger LR [102]. Our finding that Wnt2 expression is highly upregulated in endothelial cells after PH further confirms LSECs being a source of Wnt2 during LR. More importantly, our finding that *Wnt2* is significantly induced in macrophages after PH suggests a potential role of macrophages being a source of stimulatory Wnts during LR. We also found that the expression of Wnt9b was dramatically induced in endothelial cells and macrophages after PH; however, the function of Wnt9b in liver biology is not well understood.

Taken together, the Wnt expression profile after PH indicates that endothelial cells and macrophages could both be the source of Wnt ligands in response to PH, while hepatocytes might

not play an important role. However, the q-PCR analysis only indicates the *Wnt* expression but not protein secretion, and therefore, needs to be validated in the context of Wnt secretion.

4.2.2 The role of liver epithelial cells in Wnt secretion

Q-PCR analysis of *Wnt* expression suggests individual hepatic cell populations might have different roles in Wnt secretion. To further study Wnt protein secretion, we utilized *Wls*-floxed mice. *Wntless (Wls)* encodes a multipass transmembrane protein that is specific and necessary for Wnt transport from Golgi to the membrane for secretion [85]. In order to fully elucidate the cellular source of Wnt proteins directing β-catenin activity for zonation and LR, we generated cell type specific *Wls* knockout mice that lack the ability of a specific cell population to secrete Wnts. Hepatocyte and cholangiocyte specific (or liver-specific) *Wls* knockout mice (*Wls*-LKO) were first generated to address the role of liver epithelial cells in Wnt secretion.

4.2.2.1 Generation and baseline characterization of liver-specific *Wls* knockout mice

Hepatocyte and cholangiocyte specific (or liver-specific) *Wls* KO (*Wls*-LKO) were generated by interbreeding *Albumin*-Cre and *Wls*-floxed mice. PCR confirmed the concomitant presence of floxed allele and cre-recombinase in *Wls*-LKO (Figure 19A) using primers P2 and P4 as described previously [94]. WB from *Wls*-LKO liver lysates shows less Wls relative to Con (Figure 19B). However, appreciable Wls persisted in *Wls*-LKO suggesting that other non-parenchymal cells also express *Wls*, and which would not be affected by *Albumin*-Cre. Indeed, cell fractionation into parenchymal and non-parenchymal cells by percoll gradient after collagenase

perfusion showed notably higher *Wls* expression in the non-parenchymal cell compartment (data not shown).

Eight-month-old *Wls*-LKO mice had comparable and normal levels of serum AST, ALT, bilirubin, and albumin levels to Con (data not shown). *Wls*-LKO showed marginally bigger livers than Con (Figure 19C), although the difference was not statistically significant. Baseline proliferation examined by IHC for PCNA did not reveal any differences (Figure 19D, 19E).

4.2.2.2 Normal adherens junctions and hepatic zonation in *Wls*-LKO mice

Next, we investigated changes in β-catenin, its downstream signaling and junctional proteins in *Wls*-LKO mice. β-Catenin, γ-catenin and E-cadherin were unchanged in *Wls*-LKO (Figure 19F). The expression of the downstream targets of β-catenin signaling was comparable between *Wls*-LKO and Con (Figure 19F). IHC for pericentral expression of β-catenin targets showed no differences in staining for GS, Cyp1a2 and Cyp2e1 between *Wls*-LKO and Con (Figure 20A). Therefore, blocking Wnt secretion from hepatocytes or cholangiocytes does not affect regulation of zonation by β-catenin signaling in hepatocytes.

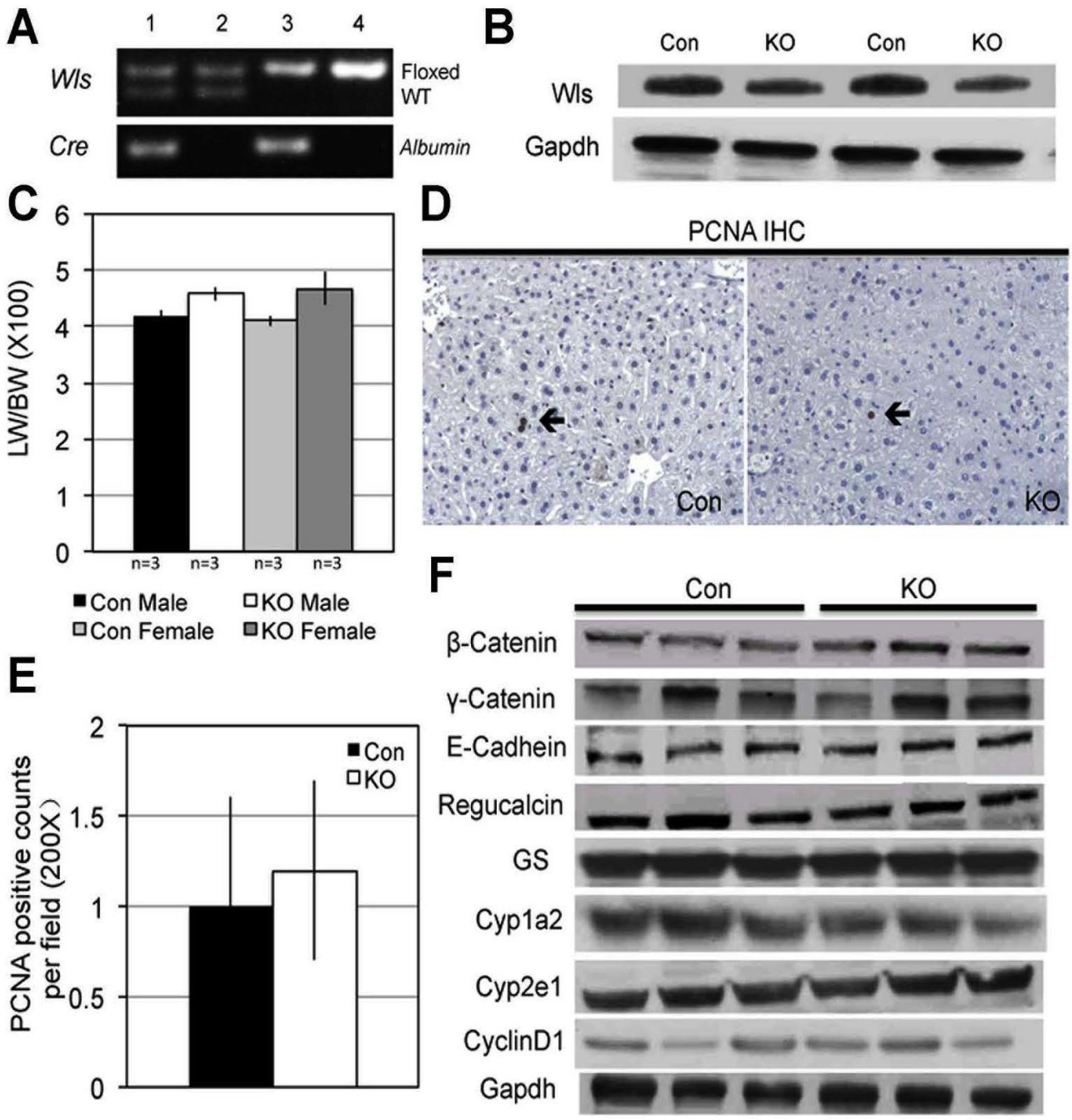

Figure 19. Successful deletion of *Wls* in *Wls*-LKO mice

A. Representative PCR shows genotype of mice for identifying the *Wls*$^{flox/flox}$; *Albumin*-Cre+/- (*Wls*-LKO) (lane 3). Mice with genotype *Wls*$^{flox/wt}$; *Albumin*-Cre-/- & *Wls*$^{flox/flox}$;*Albumin*-Cre-/- (lanes 2 & 4) were used as Con.

B. WB shows a modest decrease in Wls in liver of *Wls*-LKO compared to Con.

C. Higher LW/BW ratio in 8-month old *Wls*-LKO as compared to Con although difference was not significant.

D. IHC shows occasional PCNA-positive hepatocyte in Con and *Wls*-LKO liver (200X).

E. Quantification of PCNA staining (200X) shows comparable number of positive hepatocytes between Con and *Wls*-LKO.

F. Representative WB shows comparable expression of β-catenin, relevant junctional components and β-catenin downstream, targets in *Wls*-LKO and Con livers. Prolonged exposure of WB film enabled us to observe baseline Cyclin-D1 in Wls-LKO and Con, which was comparable in the two groups.

4.2.2.3 Normal initiation of liver regeneration in *Wls*-LKO mice

The role of hepatocyte and cholangiocyte-derived Wnt proteins in LR was examined next by subjecting the *Wls*-LKO and Con to PH. Comparably higher numbers of PCNA-positive hepatocytes were observed in *Wls*-LKO and Con mice at 40 hours post-PH (Figure 20B). Quantification of PCNA staining showed no significant differences (Figure 20C). Likewise, *Wls*-LKO had comparable Cyclin-D1 levels to Con at 40 hours (Figure 20D). Therefore, lack of any differences in regeneration kinetics at early time points after PH indicates that β-catenin activation during LR is not dependent on hepatocyte- or cholangiocyte-derived Wnts.

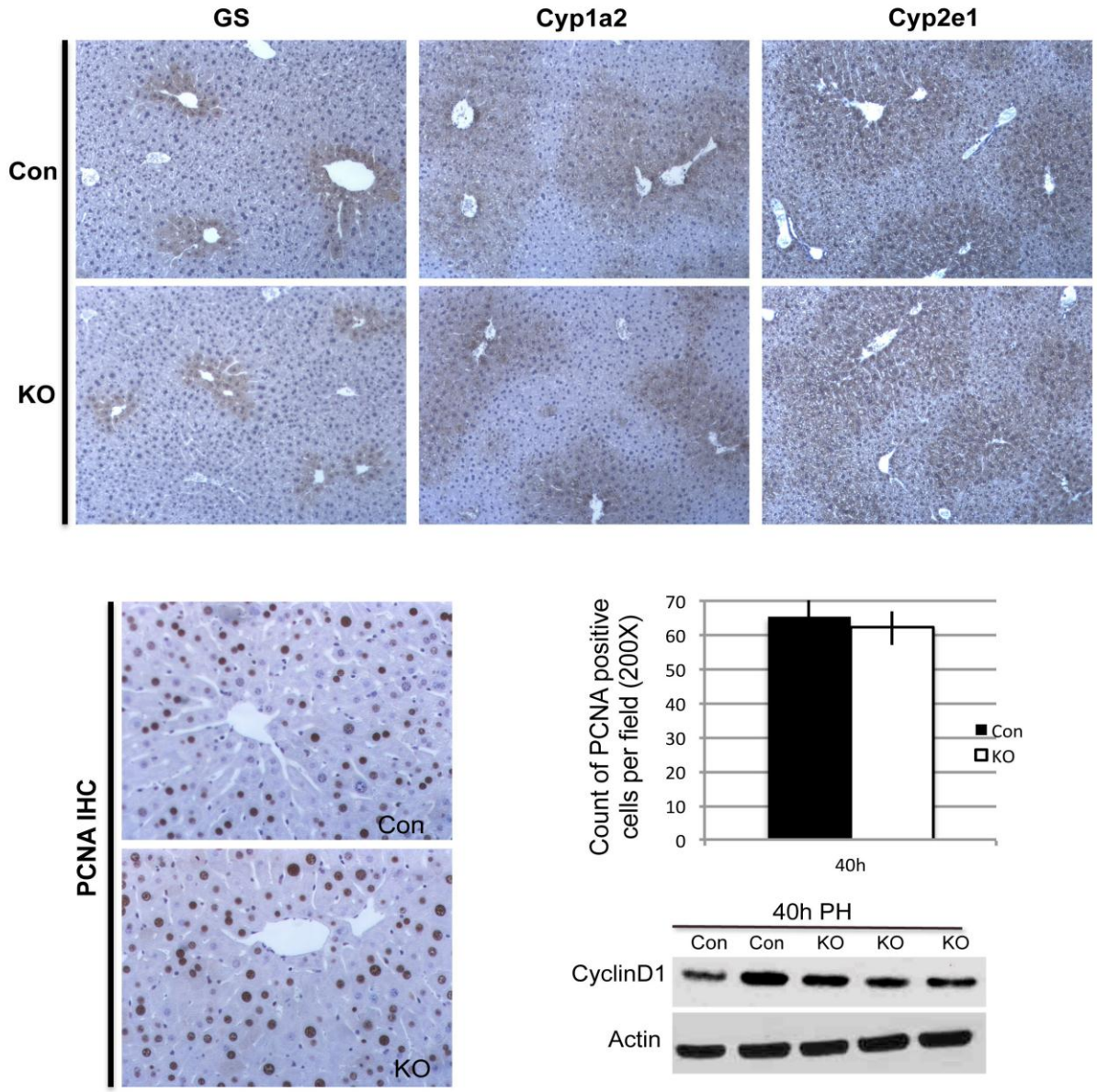

Figure 20. Ablation of *Wls* in liver epithelial cells has no effect on hepatic zonation or initiation of LR

A. IHC for GS, Cyp1a2 and Cyp2e1 in Con and *Wls*-LKO shows normal pericentral localization in both groups (100x).

B. PCNA IHC shows comparable numbers of PCNA-positive hepatocytes in Con and *Wls*-LKO livers at 40 hours after PH (200X).

C. Quantification of PCNA staining confirms similar numbers of PCNA-positive hepatocytes in Con and *Wls*-LKO at

40 hours after PH.

D. Representative WB shows comparable Cyclin-D1 expression at 40 hours after PH in both Con and *Wls*-LKO livers.

4.2.2.4 Continuous proliferation in *Wls*-LKO at late times after PH

Temporarily enhanced LR in Wls-LKO after PH

LR kinetics were further analyzed at later time points after PH, including 3 to 14 days after PH. PCNA staining showed comparably higher numbers of positive hepatocytes at 3 days after PH (Figure 21A). Intriguingly, 4 days after PH, when LR has already been attenuated in the Con livers as suggested by low numbers of PCNA positive cells, considerable amounts of proliferating cells were present in the *Wls*-LKO (Figure 21A). However, 5 days after PH, no differences in the number of positive cells were observed. At day 14, LR was completely terminated in both Con and *Wls*-LKO (Figure 21A). Quantification of PCNA staining showed a significantly higher number of proliferating cells in *Wls*-LKO 4 days after PH, with no differences after day 5 (Figure 21B). Consistently, WB showed higher levels of Cyclin-D1 in *Wls*-LKO at 4 days after PH, but the level went down at day 5 (Figure 21C). Immunoprecipitation showed enhanced β-catenin/TCF4 binding in the knockout at both 40 hours and 3 days after PH, suggesting that there was a delay in the termination of active β-catenin signaling in the *Wls*-LKO (Figure 21D).

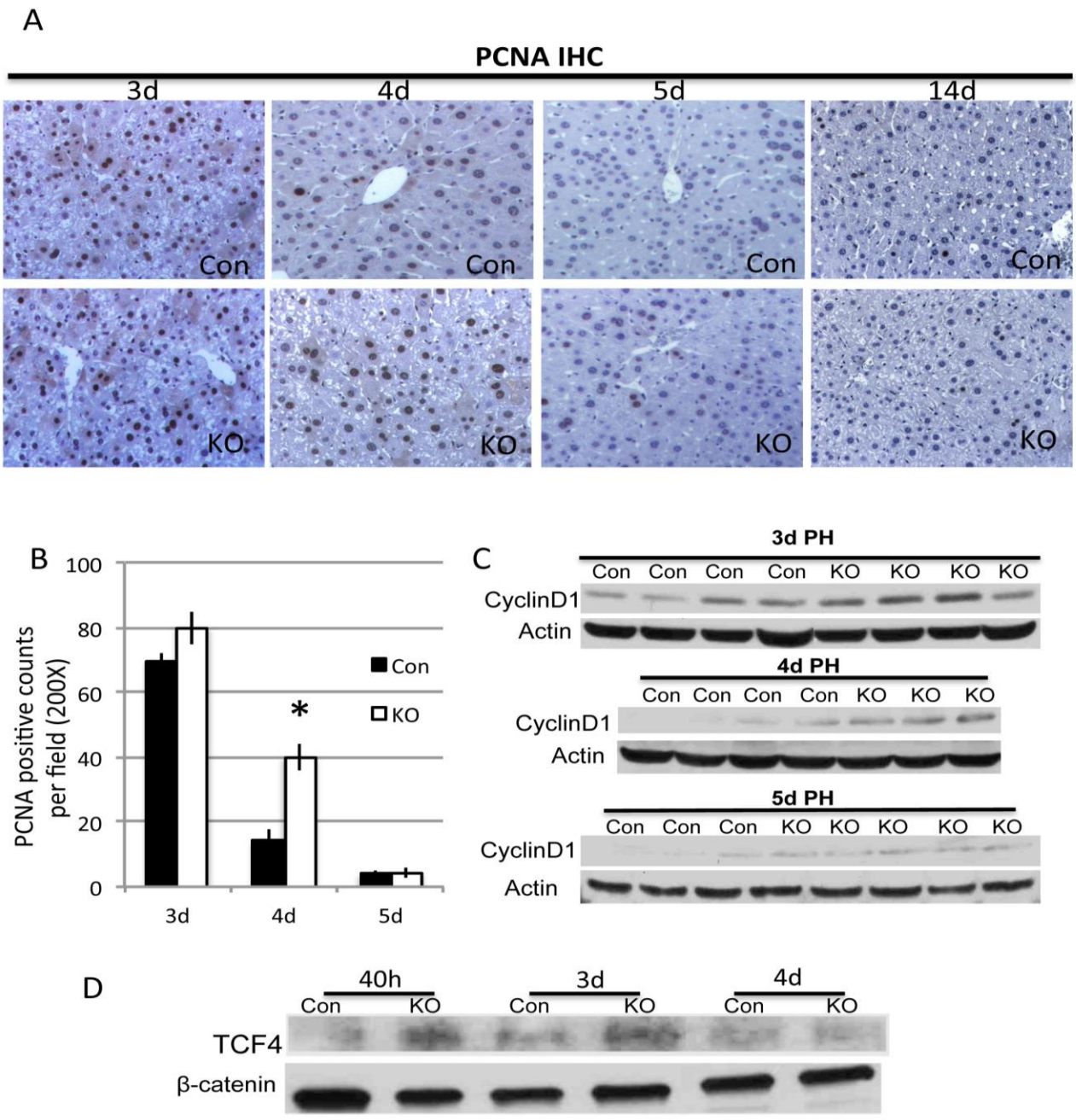

Figure 21. Temporarily enhanced LR in *Wls*-LKO at later times after PH

A. IHC for PCNA shows comparably high numbers of positive cells at 3 days after PH, a notably higher number of positive cells in *Wls*-LKO 4 days after PH, and comparably low numbers of positive cells at 5 days and 14 days after PH (200X).

B. Quantification of PCNA staining confirms significantly higher number of positive cells in *Wls*-LKO 4 days after PH (*p<0.05).

C. Representative WB shows higher Cyclin-D1 expression at 4 days after PH in *Wls*-LKO livers.

D. Immunoprecipitation shows enhanced β-catenin and TCF4 binding in *Wls*-LKO at 40 hours and 3 days after PH.

Induction of Wnt5a signaling pathway at later time points after PH

Given the fact that *Wls*-LKO had enhanced LR at late time points after PH and a delay in the termination of active β-catenin signaling, we hypothesized that hepatocytes might be able to secrete some inhibitory Wnt ligands involved in the termination of LR, while hepatocytes of *Wls*-LKO were not capable of Wnt secretion, leading to a delay in the termination of LR. Wnt5a is a classic non-canonical Wnt ligand that has been shown to have an antagonistic role on Wnt/β-catenin signaling [33-37]. Unpublished data from our lab also showed that Wnt5a can inhibit β-catenin signaling in both primary hepatocytes and hepatoma cell lines, therefore we further examined Wnt5a signaling using *Wls*-LKO (Figure 22). WB with total liver lysates showed an induction of Wnt5a expression at 40 hours after PH (Figure 22A). At day 3, there were much less Wnt5a proteins in the *Wls*-LKO compared with Con, so as receptor Ror2. However, no obvious difference was observed at day 4. In order to address the role of hepatocytes in Wnt5a secretion during LR, primary hepatocytes from hepatectomized Con and *Wls*-LKO were cultured and the conditioned medium were collected 24 hours later. ELISA was performed to measure Wnt5a concentration in the conditioned medium. As expected, Wnt5a concentration in the Con conditioned medium was 2.5 fold higher than in the *Wls*-LKO medium (Figure 22B). Therefore, our experiments suggest that there is an induction of Wnt5a signaling after PH, which might be involved in the termination of LR. Hepatocytes from Con livers can secrete Wnt5a after PH, while

the hepatocytes from *Wls*-LKO have a defect in Wnt5a secretion, which might be the cause of enhanced LR in *Wls*-LKO.

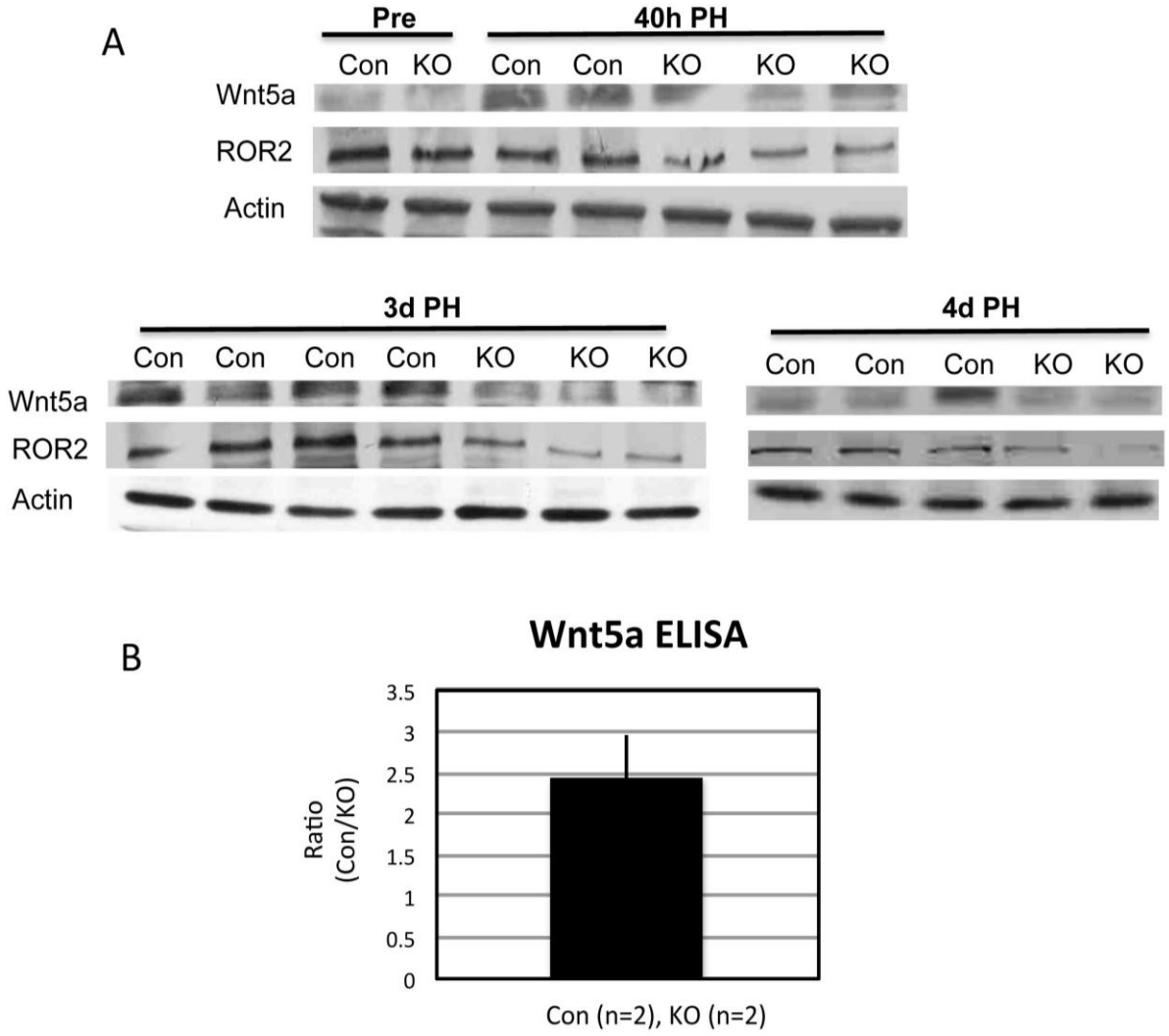

Figure 22. Defect of Wnt5a secretion from hepatocytes in the *Wls*-LKO

A. WB shows induction of Wnt5a expression in the Con livers 40 hours after PH. At 3 days after PH, there is much less Wnt5a proteins in *Wls*-LKO compared with Con, so as Ror2.

B. ELISA was performed to measure Wnt5a concentration in the conditioned medium. Wnt5a concentration in the Con medium is 2.5 fold higher than in the *Wls*-LKO medium.

Generation of liver specific Wnt5a knockout mice

Our lab has shown that Wnt5a inhibited primary hepatocyte and HepG2 cell proliferation by suppressing β-Catenin signaling (unpublished data). Next we generated hepatocyte and cholangiocyte specific (or liver specific) *Wnt5a* knockout mice (*Wnt5a*-LKO) to elucidate the function of Wnt5a *in vivo*. *Wnt5a*-LKO were generated by interbreeding *Albumin*-Cre and *Wnt5a*-floxed mice [129]. PCR confirmed the concomitant presence of floxed allele and cre-recombinase in *Wnt5a*-LKO (Figure 23A). WB from *Wnt5a*-LKO liver lysates shows less Wnt5a expression relative to Con (Figure 23B). Eight-month-old *Wnt5a*-LKO mice had comparable and normal levels of serum AST, ALT, bilirubin, and albumin levels to Con (data not shown). *Wnt5a*-LKO showed normal liver size similar to Con (Figure 23C). Baseline proliferation examined by IHC for PCNA did not reveal any differences (Figure 23D, 23E). β-Catenin and its downstream target Cyclin-D1 were unchanged in *Wnt5a*-LKO (Figure 23F). IHC for pericentral expression of β-catenin targets showed no differences in staining for GS, Cyp1a2 and Cyp2e1 between *Wnt5a*-LKO and Con (Figure 23G). Therefore, hepatocyte or cholangiocyte specific *Wnt5a* knockout mice display no alteration in β-Catenin signaling pathway at baseline.

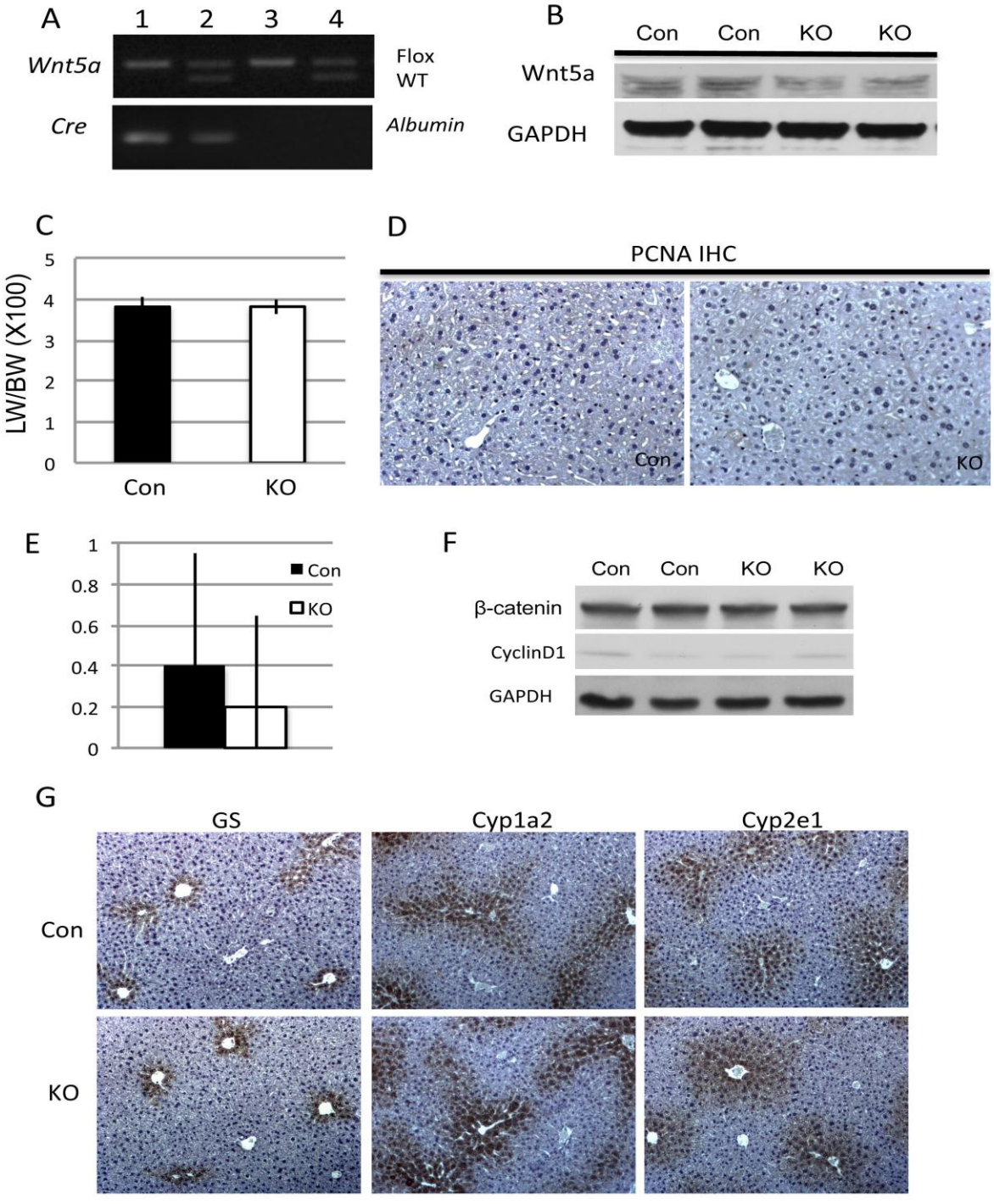

Figure 23. Baseline characterization of *Wnt5a*-LKO mice

A. Representative PCR shows genotype of mice for identifying the *Wnt5a*$^{flox/flox}$; *Albumin*-Cre+/- (*Wnt5a*-LKO) (lane 1). Mice with genotype *Wnt5a*$^{flox/flox}$; *Albumin*-Cre-/- &*Wnt5a*$^{flox/wt}$; *Albumin*-Cre-/- (lanes 3 & 4) were used as Con.

B. WB shows a modest decrease in Wnt5a expression in livers of *Wnt5a*-LKO compared to Con.

C. Normal LW/BW ratio in 8-month old *Wnt5a*-LKO as compared to Con.

D. IHC shows occasional PCNA-positive hepatocyte in Con and *Wnt5a*-LKO liver (200X).

E. Quantification of PCNA staining (200X) shows no difference in the number of positive hepatocytes between Con and *Wnt5a*-LKO.

F. Representative WB shows comparable expression of β-catenin and Cyclin-D1 in *Wnt5a*-LKO and Con livers.

G. IHC for GS, Cyp1a2 and Cyp2e1 shows normal zonation in *Wnt5a*-LKO livers.

Liver specific Wnt5a knockout mice have normal LR kinetics

Next, to address the role of *Wnt5a* in LR, *Wnt5a-LKO* and Con mice were subjected to PH. PCNA staining at different time points after PH did not reveal any differences in LR kinetics between Con and *Wnt5a-LKO* (Figure 24A, 24B). WB also showed comparable Cyclin-D1 levels at all the time points (Figure 24C). Therefore, deletion of *Wnt5a* in liver epithelial cells appears to have no notable effect on LR. To understand the reason for the lack of phenotype, we further investigated the Wnt5a expression pattern during LR in both Con and *Wnt5a-LKO*. Q-PCR analysis with total liver tissue showed that there were mild but significant differences in *Wnt5a* expression between Con and *Wnt5a-LKO* at baseline and early time points after PH, while no differences at the late time points (Figure 24D). Therefore, it is likely that liver epithelial cells are not the only source of Wnt5a. Other cell populations can also secrete Wnt5a during LR to compensate for the loss of epithelial cell derived Wnt5a. Other signaling pathways involved in the termination of LR, such as TGF-β [75, 76], ILK [79] and GPC3 [80, 81], might also be activated to ensure the proper termination of LR in the *Wnt5a-LKO*.

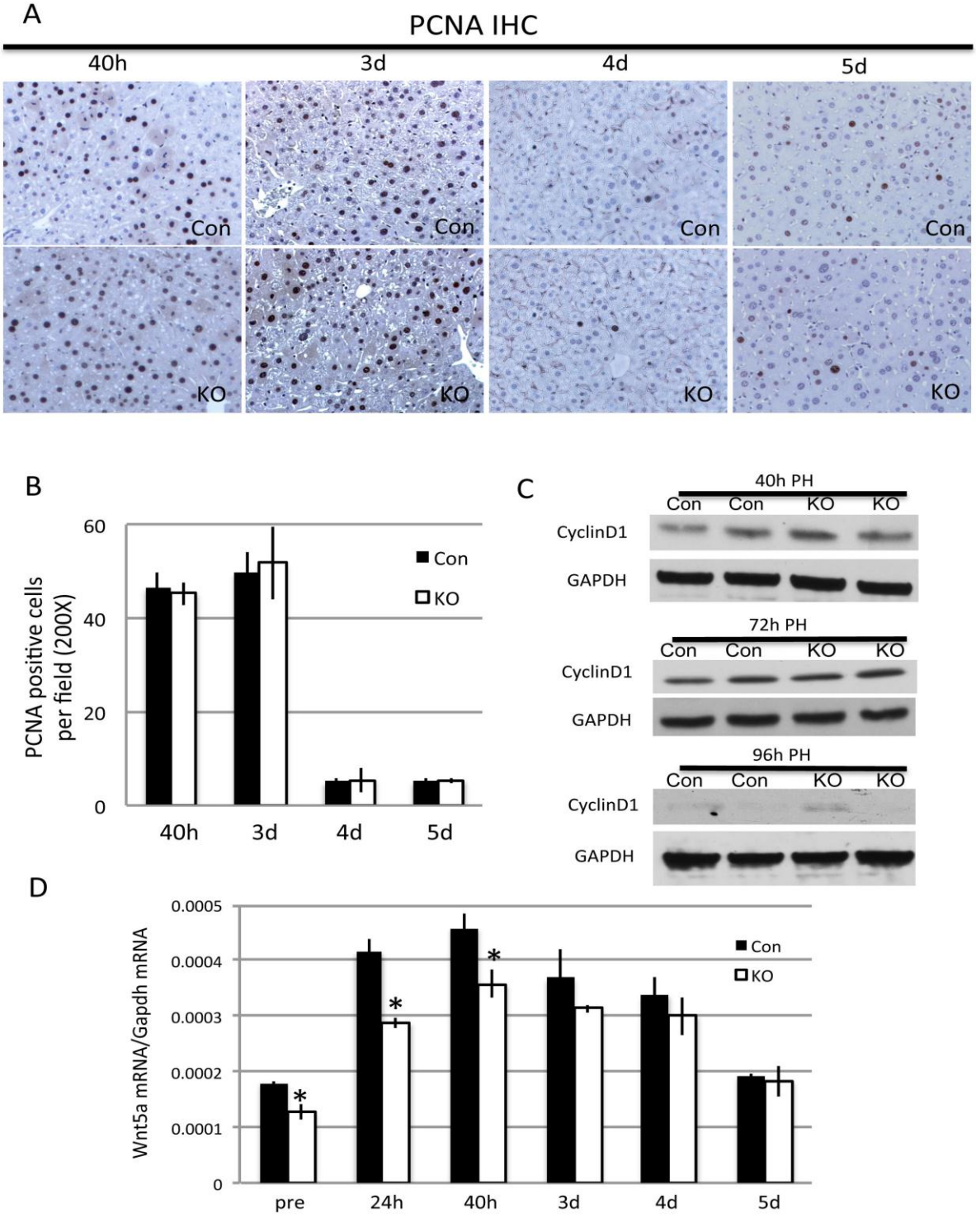

Figure 24. Ablation of *Wnt5a* in liver epithelial cells has no effect on liver regeneration

A. PCNA IHC shows comparable numbers of positively cells at all the time points after PH (200X).

B. Quantification of PCNA staining reveals no differences in the number of positive cells between Con and knockouts.

C. WB shows comparable levels of Cyclin-D1 at 40, 72 and 96 hours after PH.

D. Q-PCR analysis of *Wnt5a* expression in total liver tissue shows significant differences between Con and KO at baseline and 24 & 40 hours after PH, while no differences at later times (*$p<0.05$).

4.2.3 The role of hepatic stellate cells in Wnt secretion

After ruling out the role of hepatocytes and cholangiocytes as a source of Wnt proteins that regulate β-catenin activation in liver homeostasis and LR, we next tested the possibility of hepatic stellate cells (HSC) being a relevant source of Wnt ligands. Stellate cell specific *Wls* knockout mice (*Wls*-SKO) were generated to address the role of hepatic stellate cells in Wnt secretion.

4.2.3.1 Generation and baseline characterization of stellate cell-specific *Wls* knockout mice

The *Wls*-floxed mice were bred with mice that express *Cre* recombinase under control of the glial fibrillary acidic protein (GFAP) promoter [111], to generate stellate cell specific *Wls* knockout mice ($Wls^{flox/flox}$; *GFAP*-$Cre^{+/-}$), referred to as (*Wls*-SKO). PCR was used to identify the *Wls*-SKO and Con (Figure 25A). Decreased levels of Wls protein were evident by WB from Wls-SKO livers (Figure 25B). Although *GFAP*-Cre mice have been widely used to generate HSC specific null animal models [130-132], there is some controversy as to whether *GFAP*-Cre can effectively target HSCs [133], therefore, in order to validate this model, we isolated HSCs from Con and *Wls*-SKO livers (as described in the method section), with the high purity of HSCs confirmed by q-PCR (Figure 15). *Wls* expression in HSCs from Con and *Wls*-SKO was determined

by q-PCR. As expected, there was a dramatic reduction in *Wls* expression in HSCs from *Wls*-SKO (Figure 25C).

Eight-month-old *Wls*-SKO and Con mice had comparable and normal serum levels of AST, ALT, bilirubin and albumin (data not shown). *Wls*-SKO displayed smaller livers and LW/BW ratio was significantly lower in *Wls*-SKO compared to *Con* (Figure 25D). To address the discrepancy in liver size, we assessed hepatocytes in S-phase by PCNA IHC. Surprisingly, *Wls*-SKO livers had comparable PCNA positive cells with Con at baseline (Figure 25E, 25F). Cyclin-D1 expression was also comparable between *Wls*-SKO and Con by WB (Figure 25G). Since GFAP is also expressed in other cell populations, such as enteric glial cells of the small intestine [134] and astrocytes of the brain [111, 135], the reduction in liver size might be subsequent effect caused by the defect of *Wls* in other organ systems rather than in the liver.

4.2.3.2 Normal adherens junctions and hepatic zonation in *Wls*-SKO null mice

Next, we investigated changes in β-catenin, its downstream signaling and junctional proteins in *Wls*-SKO mice. β-Catenin, γ-catenin and E-cadherin were unchanged in *Wls*-SKO (Figure 25G). The expression of the downstream targets of β-catenin signaling was comparable between *Wls*-SKO and Con (Figure 25G). IHC for pericentral expression of β-catenin targets showed no differences in staining for GS, Cyp1a2 and Cyp2e1 between Wls-SKO and Con (Figure 26A). Therefore, blocking Wnt secretion from hepatic stellate cells does not affect regulation of zonation by β-catenin signaling.

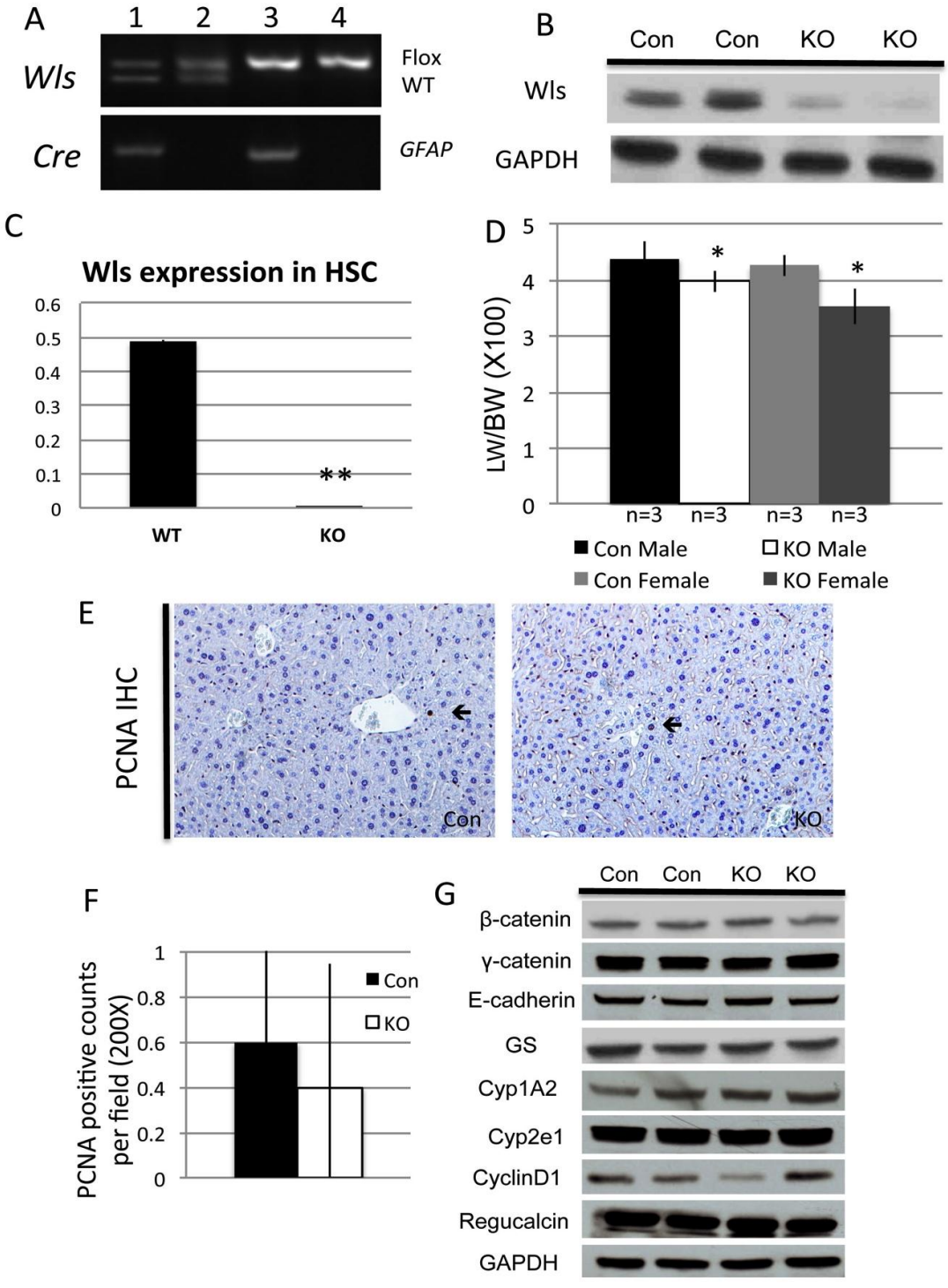

Figure 25. Successful deletion of *Wls* in *Wls*-SKO mice

A. Representative PCR shows genotype of mice for identifying the *Wls*$^{flox/flox}$; *GFAP*-Cre+/- (*Wls*-SKO) (lane 3). Mice with genotype *Wls*$^{flox/wt}$; *GFAP*-Cre-/- & *Wls*$^{flox/flox}$;*GFAP*-Cre-/- (lanes 2 & 4) were used as Con.

B. WB shows an evident decrease in Wls in liver of *Wls*-SKO compared to Con.

C. *Wls* expression in isolated hepatic stellate cells shows a significant reduction of *Wls* mRNA level in *Wls*-SKO compared to Con, **p<0.01.

D. Significantly lower LW/BW ratio in 8-month old *Wls*-SKO as compared to Con, *p<0.05.

E. IHC shows occasional PCNA-positive hepatocyte in Con and *Wls*-SKO liver (200X).

F. Quantification of PCNA staining (200X) shows comparable number of positive hepatocytes between Con and *Wls*-SKO.

G. Representative WB shows comparable expression of β-catenin, relevant junctional components and β-catenin downstream, targets in *Wls*-SKO and Con livers. Prolonged exposure of Cyclin-D1 WB film shows comparable Cyclin-D1 in Wls-SKO and Con at baseline.

4.2.3.3 Normal initiation of liver regeneration in *Wls*-SKO mice

The role of hepatic stellate cell-derived Wnt proteins in LR was examined next by subjecting the *Wls*-SKO and Con to PH. PCNA staining showed comparably high numbers of positive cells in *Wls*-SKO and Con mice at 40 hours post-PH (Figure 26B). Quantification of PCNA staining showed no significant differences (Figure 26C). Likewise, *Wls*-SKO had comparable Cyclin-D1 levels to Con at 40 hours (Figure 26D). Analysis at 96 hours after PH also revealed no difference in PCNA-positivity between the *Wls*-SKO and Con group (Figure 26B and 26C). Therefore, lack of any differences in LR kinetics indicates that β-catenin activation during LR is not dependent on stellate cell-derived Wnts.

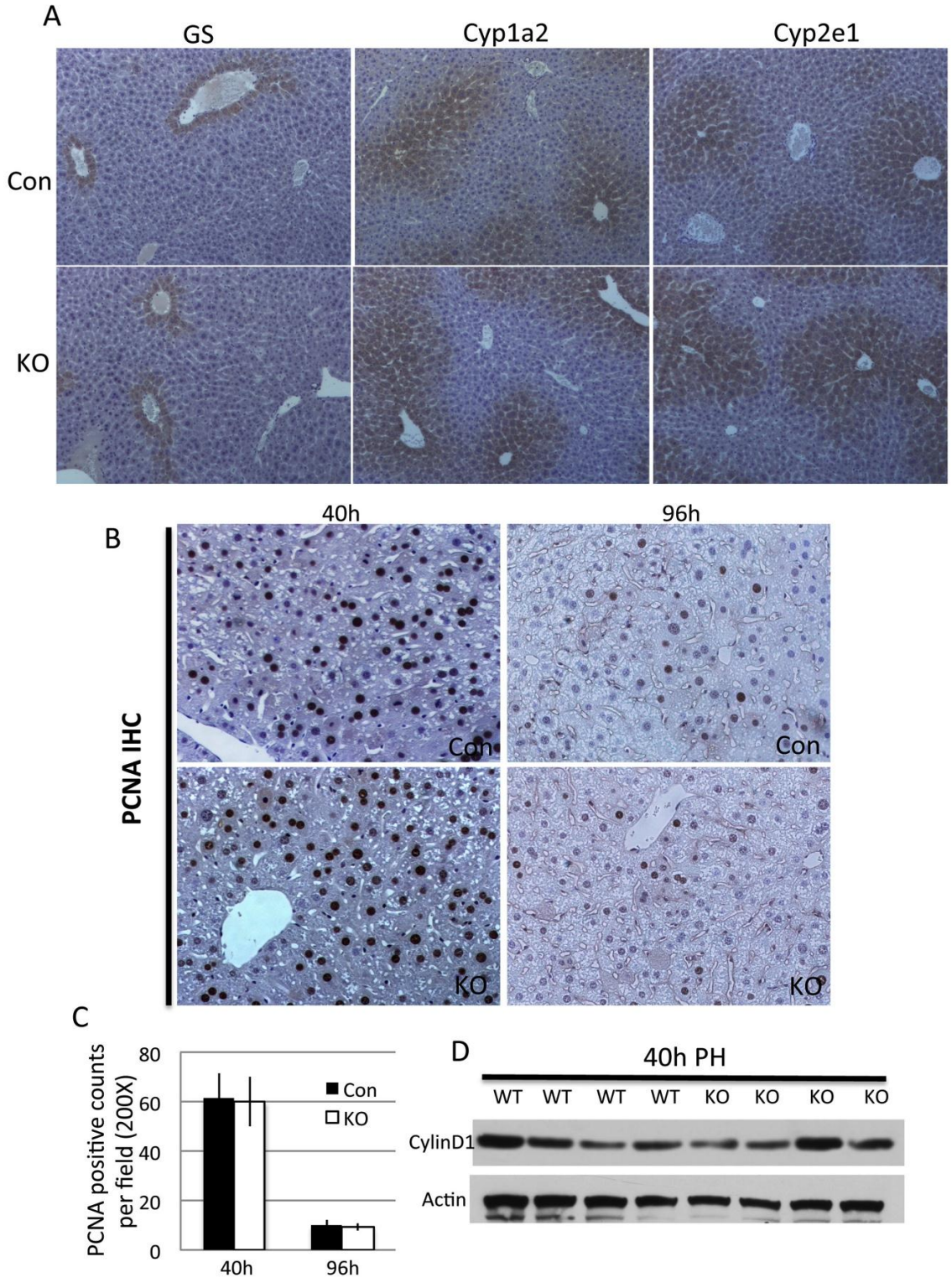

Figure 26. Ablation of *Wls* in liver stellate cells has no effect on hepatic zonation or initiation of LR

A. IHC for GS, Cyp1a2 and Cyp2e1 in Con and *Wls*-SKO shows normal pericentral localization in both groups (100x).

B. PCNA IHC shows comparable numbers of PCNA-positive hepatocytes in Con and *Wls*-SKO livers at 40 hours and 96 hour after PH (200X).

C. Quantification of PCNA staining confirms similar numbers of PCNA-positive hepatocytes in Con and *Wls*-SKO at 40 hours and 96 hours after PH.

D. Representative WB shows comparable Cyclin-D1 expression at 40 hours after PH in both Con and *Wls*-SKO livers.

4.2.4 The role of macrophages in Wnt secretion

After ruling out the role of hepatocytes, cholangiocytes and hepatic stellate cells as a source of Wnt proteins that regulate β-catenin activation in liver homeostasis and LR, we further we generated macrophage specific *Wls* KO to address the role of macrophages in Wnt secretion and β-catenin activation.

4.2.4.1 Generation and baseline characterization of macrophage-specific *Wls* knockout mice

To generate macrophage-specific *Wls* null mice (*Wls*-MKO), we bred the *Wls*-floxed mice to *Lyz2*-Cre mice [136]. PCR was used to identify the *Wls*-MKO and Con (Figure 27A). Decreased level of Wls protein from *Wls*-MKO livers was evident by WB (Figure 27A). To verify successful loss of *Wls*, Kupffer cells were isolated by QuadroMACS column separation as discussed in methods and stained for marker F4/80 to demonstrate a notable enrichment (Figure 27B). Q-PCR performed on enriched Kupffer cell-fractions showed a significant decrease in *Wls* expression in *Wls*-MKO as compared to Con (Figure 27B).

Eight-month-old *Wls*-MKO and Con mice had comparable and normal serum levels of AST, ALT, bilirubin and albumin (data not shown). *Wls*-MKO mice (n=5) had marginally smaller livers than Con (n=3); however the difference did not reach statistical significance (p=0.079) (Figure 27C). IHC for PCNA identified fewer positive cells in *Wls*-MKO, although differences were not statistically significant (Figure 27D, 27E). Intriguingly, a notable decrease in baseline Cyclin-D1 protein expression was evident in *Wls*-MKO (Figure 27F). Hence, we conclude that macrophages may secrete Wnts and contribute to baseline hepatocyte turnover to govern liver size.

4.2.4.2 Normal adherens junctions and hepatic zonation in *Wls*-MKO mice

Next, the effect of abolishing Wnt secretion from macrophages on β-catenin levels and other related junctional proteins was assessed. WB showed no notable changes in the levels of β-catenin, γ-catenin or E-cadherin in *Wls*-MKO when compared to Con livers (Figure 27F). WB also showed comparable levels of Regucalcin, GS, Cyp1a2 & Cyp2e1 in *Wls*-MKO and Con livers (Figure 27F). More importantly, comparable GS, Cyp1a2 and Cyp2e1 localization confined to the pericentral area was evident in *Wls*-MKO and Con livers (Figure 28). Thus, blocking Wnt secretion from macrophages does not have any effect on β-catenin at adherens junctions or in metabolic zonation.

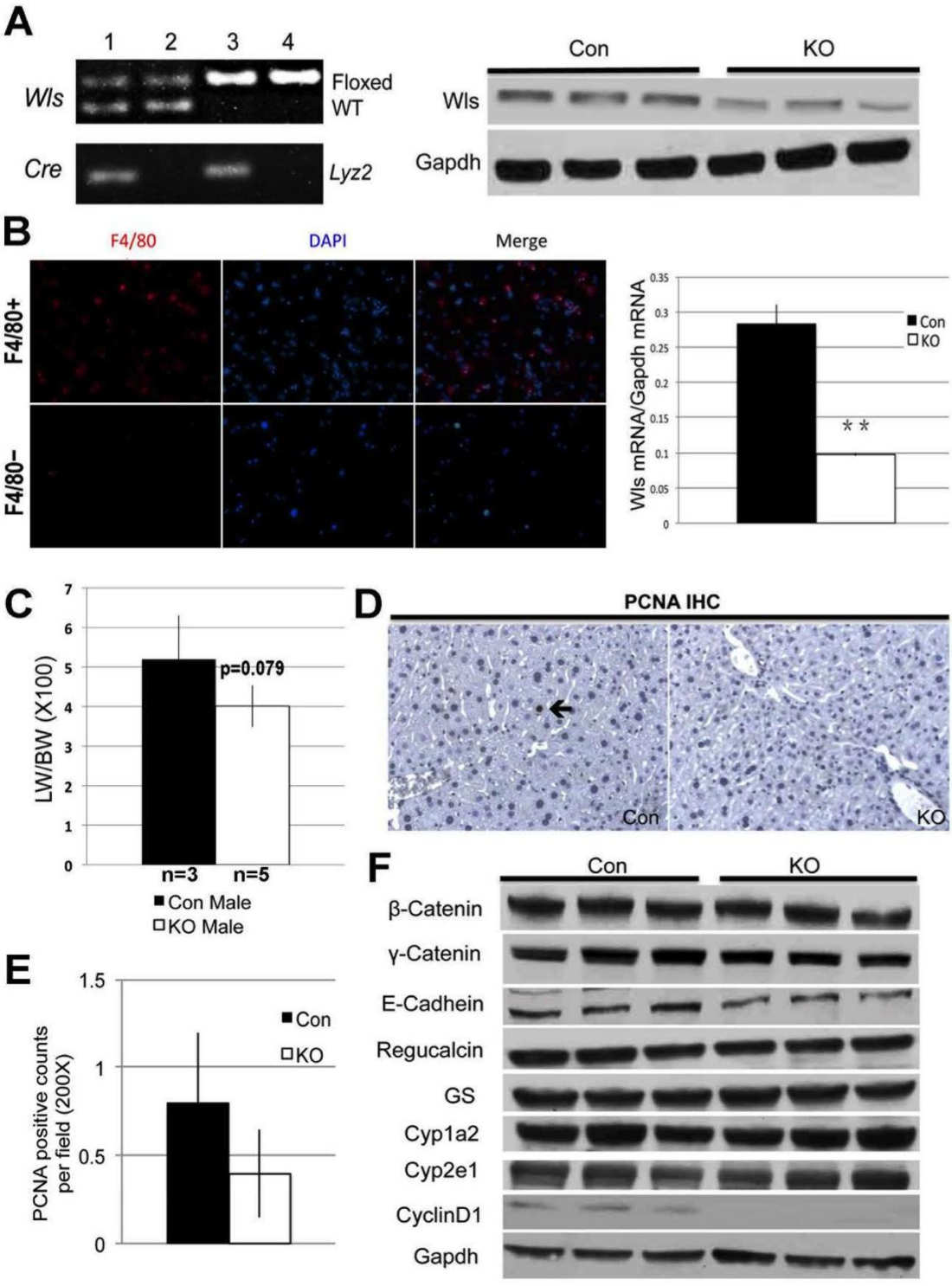

Figure 27. Impact of deletion of *Wls* in Kupffer cells on hepatic homeostasis

A. Representative PCR (left) showing genotype of *Wls*-MKO ($Wls^{flox/flox}$; *Lyz2*-Cre+/-) (lane 3) and Con with genotype $Wls^{flox/wt}$; *Lyz2*-Cre-/- & $Wls^{flox/flox}$;*Lyz2*-Cre-/- (lanes 2 & 4). Representative WB shows a notable decrease in Wls in *Wls*-MKO liver lysate.

B. Enrichment for Kupffer cells after collagenase perfusion and column separation of non-parenchymal cells is verified by immunocytochemistry on cytospin for F4/80 (red) showing positive staining in the F4/80-positive cell fraction and not in F4/80-negative fraction (left). *Wls* mRNA expression in F4/80+ cells isolated from Con and *Wls*-MKO, shows significantly lower expression in the latter (**$p<0.001$).

C. Bar graph depicts marginally lower LW/BW ratio in *Wls*-MKO, although difference was not statistically significant.

D. IHC (200X) shows an occasional PCNA-positive hepatocytes in Con at baseline (arrow), while no positive cell is observed in a representative view in *Wls*-MKO.

E. Quantification of PCNA staining showing less positive cells in *Wls*-MKO than Con at baseline, although differences were insignificant.

F. WB shows no alteration in proteins levels of β-catenin, junctional proteins and pericentral targets. However Cyclin-D1 was notably lower in *Wls*-MKO livers.

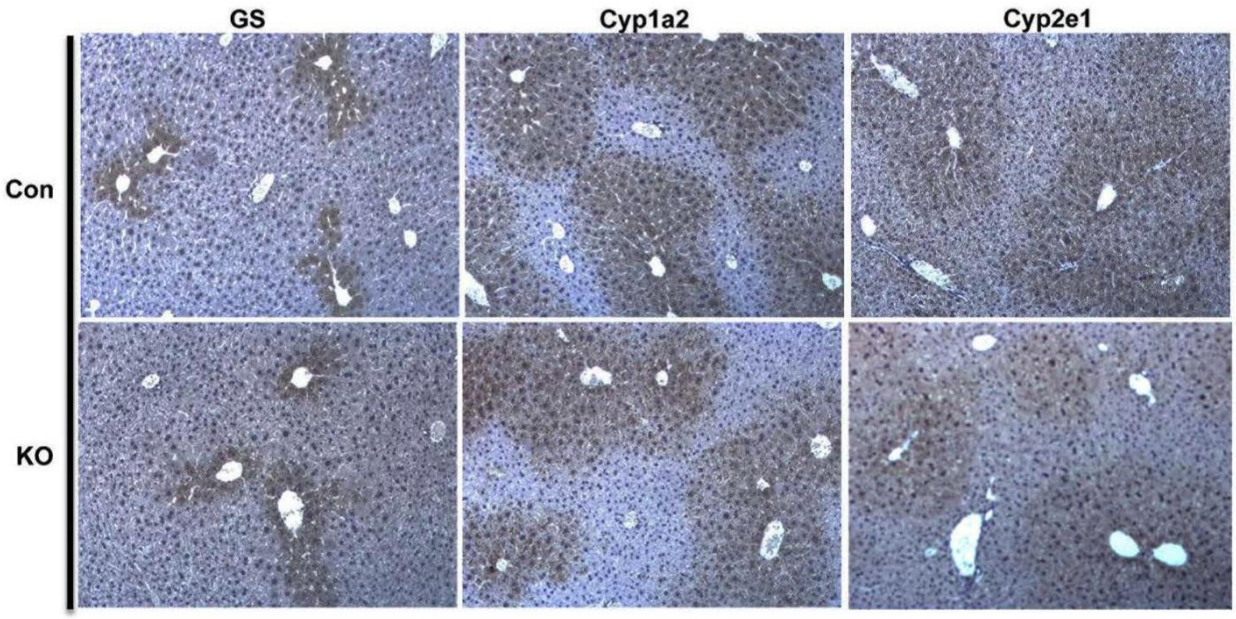

Figure 28. No defect in pericentral zonation in *Wls*-MKO

Immunohistochemistry shows normal pericentral localization of GS, Cyp1a2 and Cyp2e1 in Con livers. In *Wls*-MKO livers, comparable expression pattern was evident.

4.2.4.3 Suboptimal liver regeneration in *Wls*-MKO mice

To address the role of macrophage derived Wnts in LR, *Wls*-MKO and Con mice were subjected to PH. At 40 hours after PH, a notable decrease in PCNA-positive hepatocytes was observed in *Wls*-MKO, however at 72 hours no difference was seen (Figure 29A). Upon quantification, this deficit at 40 hours was found to be around 33% and significant (Figure 29C). In addition, numbers of mitotic figures as assessed by analysis of H&E images from *Wls*-MKO and Con showed significantly lower mitosis in hepatocytes in the former at 40 hours (Figure 29B, 29D).

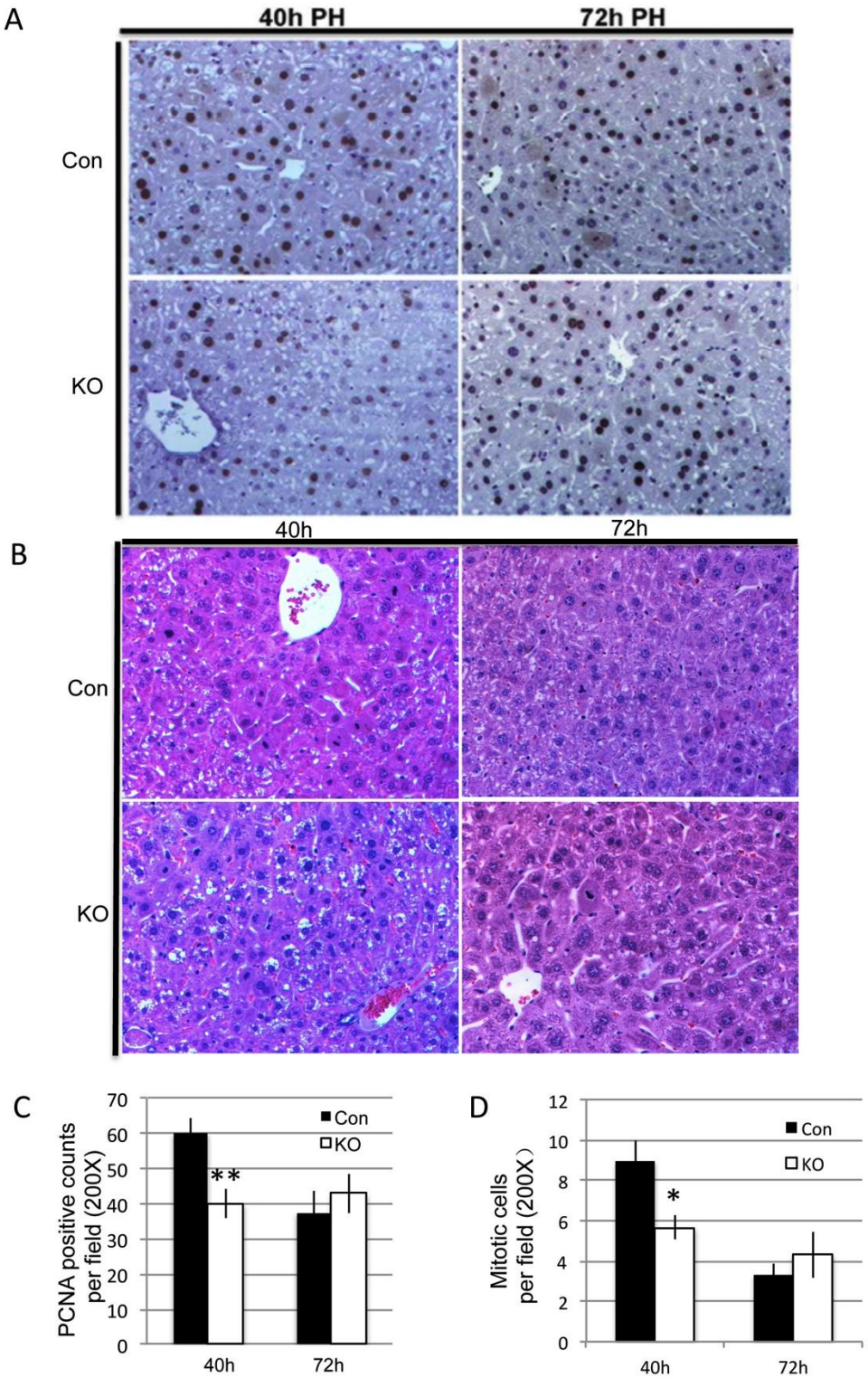

Figure 29. Dampened LR in *Wls*-MKO after PH

A. IHC (200X) showing many PCNA-positive hepatocytes in Con livers at 40 hours and 72 hours; however several PCNA-negative hepatocytes were observed in *Wls*-MKO at 40 hours.

B. Mitotic figures show less cells undergoing mitosis in *Wls*-MKO at 40 hours after PH.

C. Quantification of PCNA staining showing a 33% and significant decrease in the number of hepatocytes in S-phase in *Wls*-MKO at 40 hours (**p<0.01).

D. Quantification of mitotic figures shows a significantly lower numbers in *Wls*-MKO as compared at Con at 40 hours after PH. (*p<0.05).

WB showed a concomitant decrease in Cyclin-D1 at 40 hours in *Wls*-MKO (Figure 30A). Co-precipitation studies showed a notable decrease in β-catenin-TCF4 complex in *Wls*-MKO livers at 4 hours after PH (Figure 30B). This indicates that β-catenin activation in hepatocytes after PH is at least in part regulated by Wnts derived from Kupffer cells.

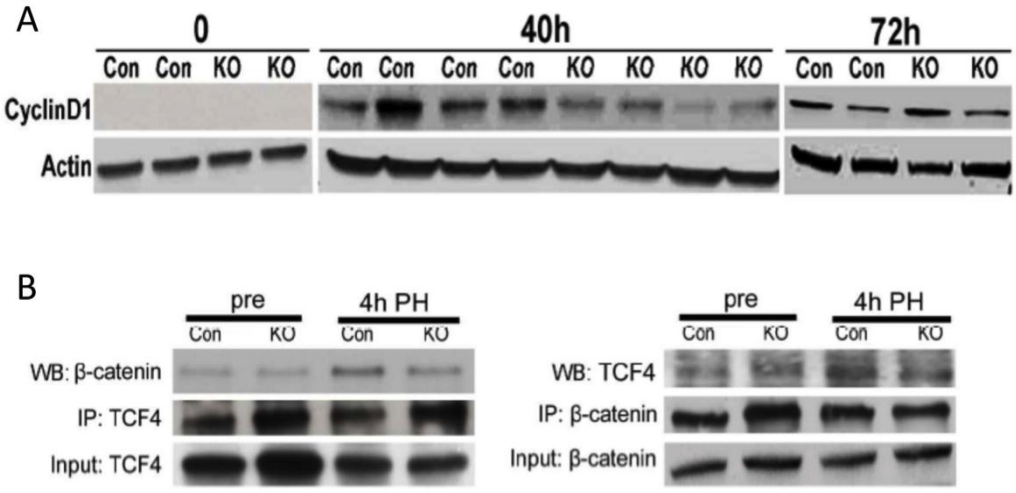

Figure 30. Impaired β-catenin activation in *Wls*-MKO after PH

A. Representative WB shows lower Cyclin-D1 levels in *Wls*-MKO at 40 hours after PH.

B. A representative immunoprecipitation study shows decreased TCF4-β-catenin complex formation at 4 hours after PH in *Wls*-MKO when compared to Con. Immunoprecipitation studies were performed by pull down of either β-

catenin or TCF4. Respective input controls are included in analysis as well.

4.2.5 The role of endothelial cells in Wnt secretion

Liver endothelial cells are known to have important roles in maintaining hepatic zonation and triggering LR [102, 137]. Our q-PCR data shows a dramatic induction of Wnt expression in liver endothelial cells after PH (Figure 17, Table 2), which suggests that endothelial cells might be playing a critical role in Wnt secretion. To test our hypothesis, we generated endothelial cell specific *Wls* null mice, and analyzed their zonation and LR kinetics.

4.2.5.1 Generation of endothelial cell-specific *Wls* null mice using *Tie2*-Cre

Deletion of *Wls* using *Tie2*-Cre [138] led to no viable pups suggesting embryonic lethality. Heterozygous mice that lacked an allele of *Wls* in endothelial cells showed no decrease in Wls protein and these livers did not show any decrease in pericentral targets (Figure 31). When subjected to PH, no defect in LR was observed in heterozygous knockout animals (Figure 31).

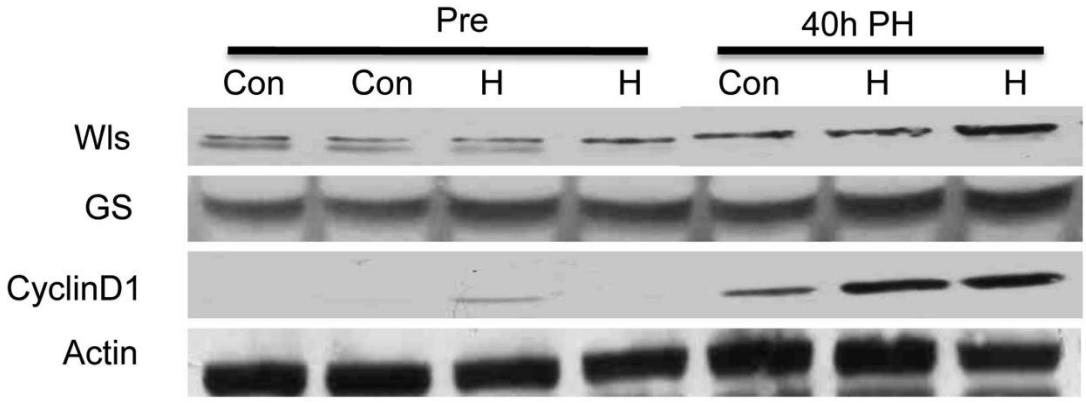

Figure 31. Heterozygous endothelial cell specific *Wls* knockouts have normal zonation and LR

No difference was observed in Wls and GS protein levels between Con and heterozygous knockouts (H). After PH, the heterozygous knockouts had sufficient Cyclin-D1 induction.

4.2.5.2 Generation of endothelial cell-specific *Wls* null mice using *VE-cadherin*-CreER^{T2}

To study Wnt secretion from endothelial cells in adult mice, we bred *Wls*$^{flox/flox}$ mice with *VE-cadherin*-CreER$^{T2+/-}$ to generate viable endothelial cell specific *Wls* null mice (*Wls*-EKO) [114]. *VE-cadherin*-CreER^{T2} mice have been widely used to generate inducible endothelial cell specific knockout models, and it has been shown that Cre-recombinase activity is present in the liver endothelial cells, including both the endothelial cells lining the central vein and the sinusoidal endothelial cells [114]. This therefore allowed us to elucidate the role of liver endothelial cells in Wnt secretion. PCR was performed to identify knockout and control animals (Figure 32). Mice with genotype *Wls*$^{flox/flox}$;*VE-cadherin*-CreER$^{T2+/-}$ are homozygous inducible *Wls* knockouts, referred to as *Wls*-iEKO, and mice with genotype *Wls*$^{flox/flox}$;*VE-cadherin*-CreER$^{T2-/-}$ and *Wls*$^{flox/wt}$;*VE-cadherin*-CreER$^{T2-/-}$ are used as control (Con). Six- to eight-week old *Wls*-iEKO and Con mice were treated with Tamoxifen (Tm) to induce Cre-recombinase activity, generating *Wls*-EKO mice. All the animals appear to be normal after Tm treatment.

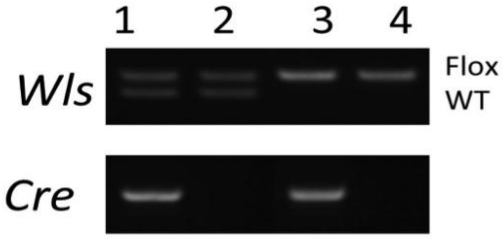

Figure 32. PCR shows genotype of mice for identifying *Wls*-iEKO

Representative PCR (upper panel) shows genotype of mice for identifying *Wls*-iEKO with genotype *Wls*$^{flox/flox}$;*VE-*

cadherin-CreER$^{T2+/-}$ (lane 3). Mice with genotypes *Wls*$^{flox/wt}$;*VE-cadherin*-CreER$^{T2-/-}$ (lane 2) and *Wls*$^{flox/flox}$;*VE-cadherin*-CreER$^{T2-/-}$ (lane 4) are used as control (Con).

4.2.5.3 Baseline characterization of *Wls*-EKO after Tamoxifen treatment

Livers were harvested one week, two weeks and four weeks following the last injection of Tm. A dramatic reduction in Wls protein level was observed in livers from *Wls*-EKO at all the time points after Tm treatment, which suggests that Tm treatment has successfully deleted *Wls* from endothelial cells. However, no obvious alterations were observed in the components of the β-catenin signaling pathway except for lower Cyp1a2 expression in the *Wls*-EKO animals (Figure 33). Immunohistochemistry (IHC) on liver tissues one week after Tm treatment showed normal GS and Cyp2e1 expression in the pericentral zone, with lower Cyp1a2 expression in *Wls*-EKO (Figure 34).

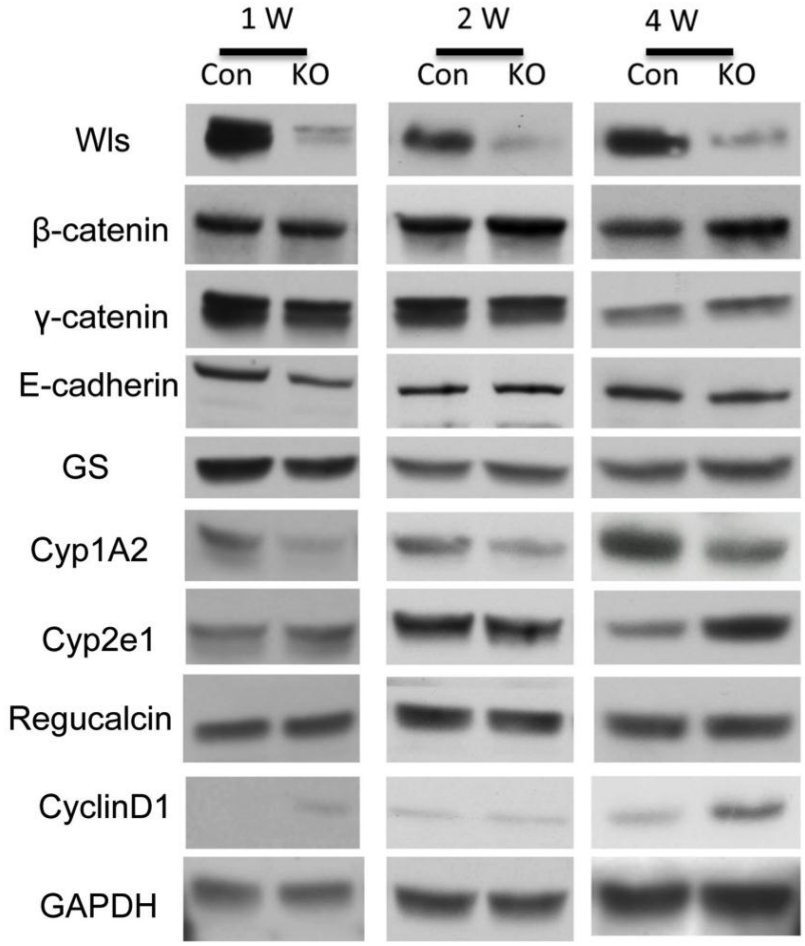

Figure 33. Baseline characterization of *Wls*-EKO after Tamoxifen treatment

Western blot shows a dramatic reduction of Wls protein expression in the *Wls*-EKO one week, two weeks and four weeks after Tm treatment, while no alteration in β-catenin signaling pathway was observed except for lower Cyp1a2 expression in the *Wls*-EKO.

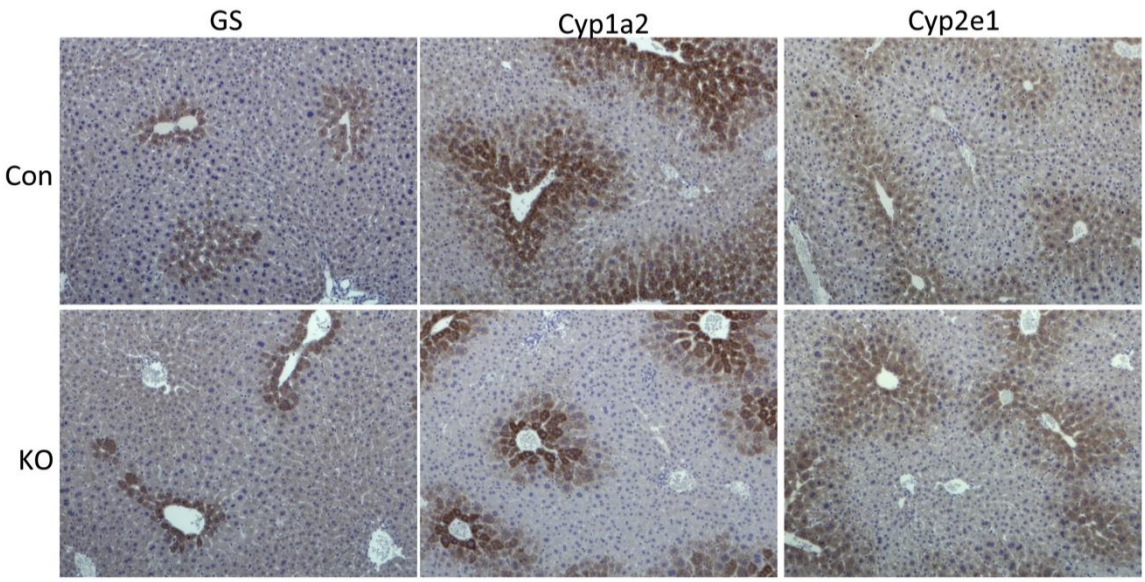

Figure 34. Zonation of Wls-EKO one week after Tamoxifen treatment

IHC on liver tissues of mice one week after Tm treatment shows comparable staining for GS and Cyp2e1 in Wls-EKO and Con, while Cyp1a2 expression was notably reduced in *Wls*-EKO compared to Con.

Next, q-PCR was performed to examine the pericentral gene expression at different time points after Tm treatment (Figure 35). An obvious reduction in the mRNA expression of GS, Cyp1a2 and Cyp2e1 was observed in *Wls*-EKO, one week and two weeks after Tm treatment. However, three and four weeks after treatment, there was an increase in the expression of zonation markers in the *Wls*-EKO (Figure 35).

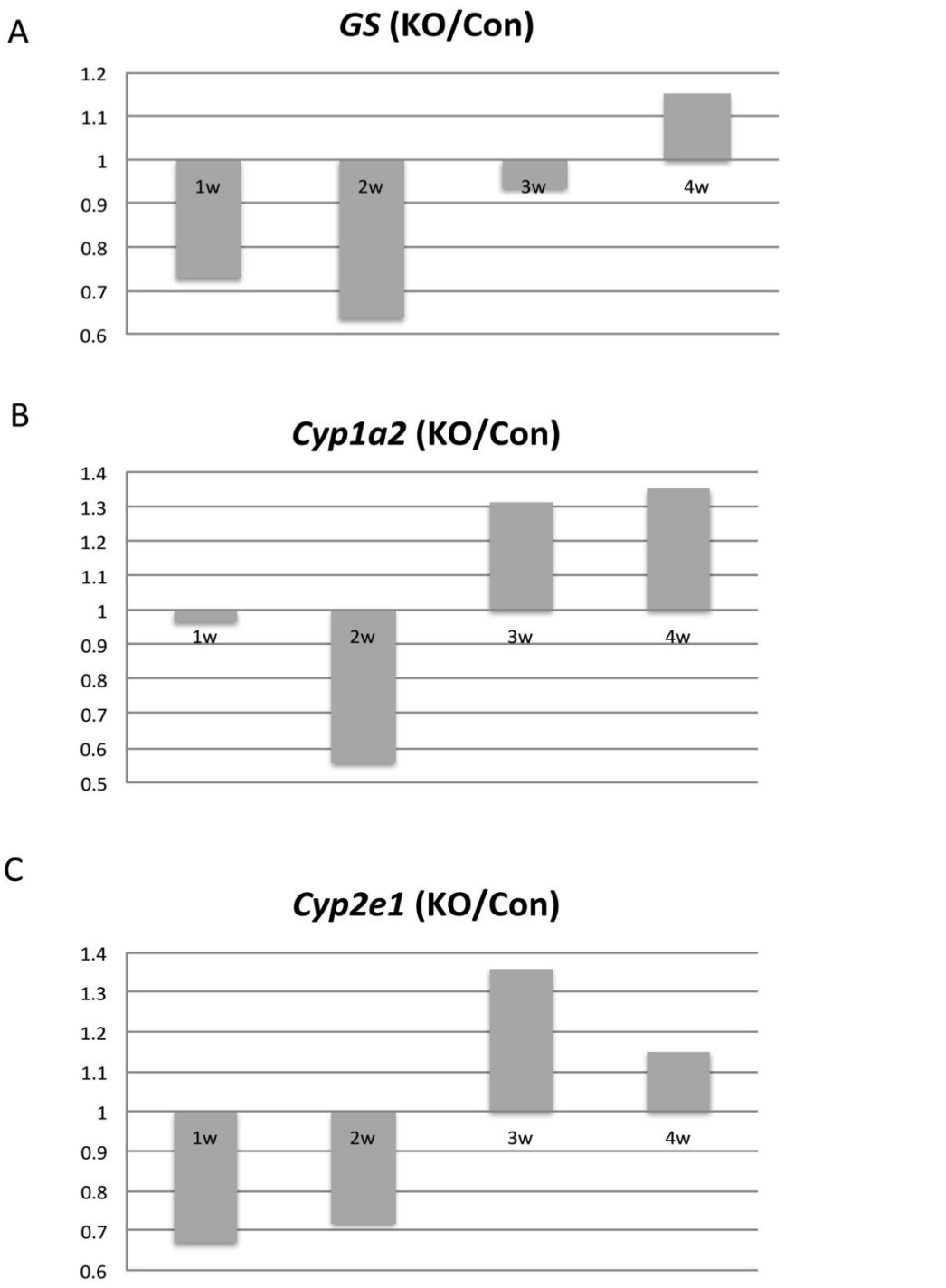

Figure 35. Changes of the expression of zonation genes in *Wls*-EKO after Tm treatment

(A) Ratio of *GS* expression in *Wls*-EKO over Con shows decreased expression of *GS* in *Wls*-EKO one and two weeks after Tm treatment, however, three weeks and four weeks after Tm treatment, *GS* expression begins to increase in the *Wls*-EKO. The expression of *Cyp1a2* (B) and *Cyp2e1* (C) shows the same trend.

One and two weeks after Tm treatment, the reduction in mRNA expression of the zonation genes suggests that endothelial cell derived Wnts may play an important role in maintaining hepatic zonation. However, we did not observe a decrease in GS and Cyp2e1 protein level. Since the half-life of these enzymes in mouse liver is unknown, it could be that the half-life is longer than we expected, therefore the residual proteins persist when we analyzed zonation one or two weeks after Tm injection. However, three to four weeks after Tm treatment, the increased expression of zonation genes in *Wls*-EKO might be due to repopulation of endothelial cells derived from progenitor cell populations or the compensation of Wnt secretion from other cell types in the liver.

4.2.5.4 Suboptimal liver regeneration in *Wls*-EKO one week after Tamoxifen treatment

To address the role of endothelial cell derived Wnts in LR, PH was performed on Con and *Wls*-EKO one week following the Tm treatment, and LR kinetics were analyzed by PCNA and Cyclin-D1 staining. At 40 hours after PH, a notable decrease in PCNA and Cyclin-D1 positive hepatocytes was observed in *Wls*-EKO (Figure 36A). Upon quantification, this deficit at 40 hours was found to be around 50% and significant (Figure 36B, 36C). This indicates that β-catenin activation in hepatocytes after PH is in part regulated by Wnts derived from endothelial cells.

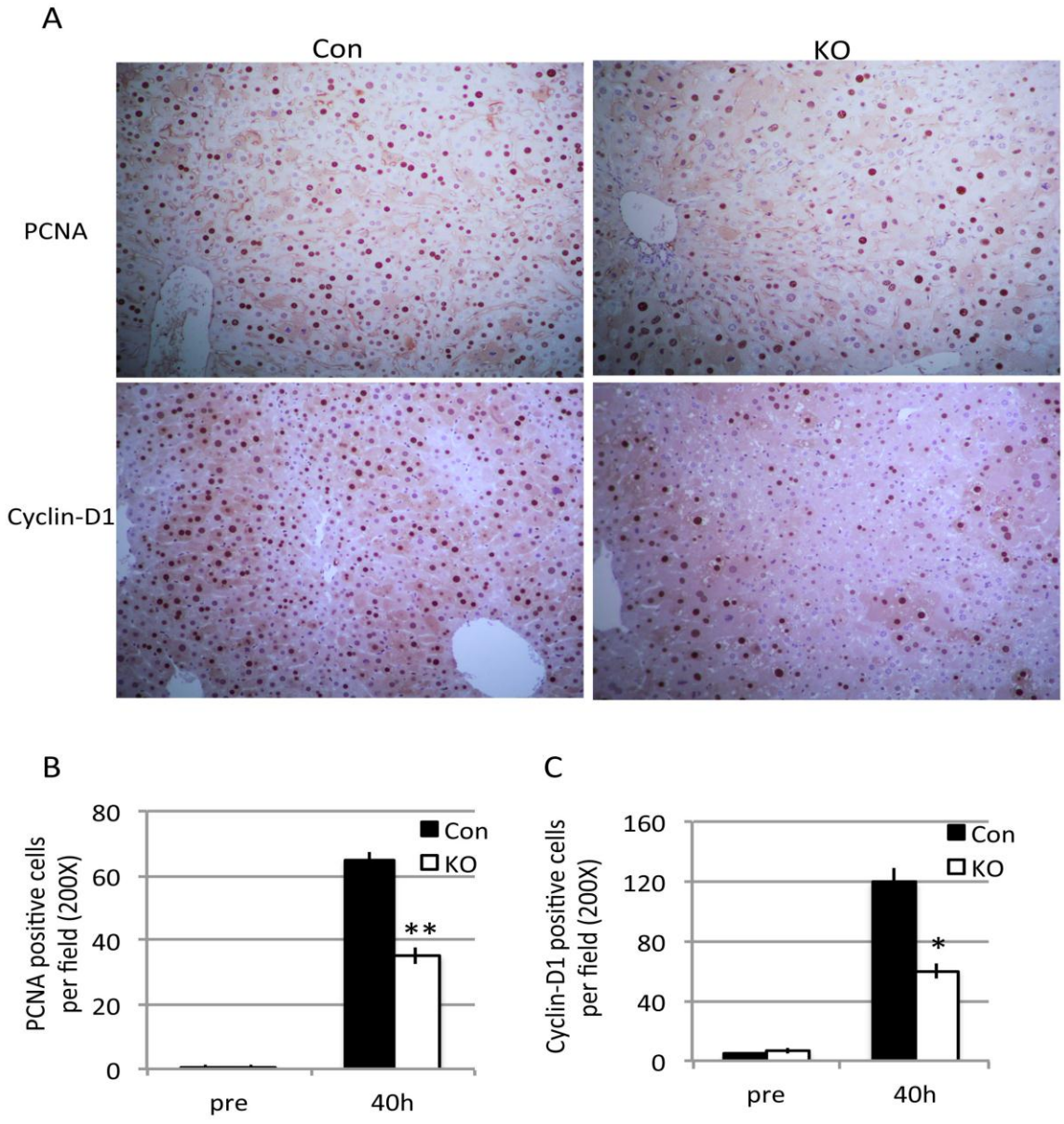

Figure 36. Suboptimal LR in *Wls*-EKO after PH

A. PCNA and CyclinD1 staining showing less positive cells in *Wls*-EKO 40 hours after PH compared to Con (100X).

B. Quantification of the PCNA staining (200X) shows a significant reduction in the number of PCNA positive cells 40 hours after PH in *Wls*-EKO (**$p<0.01$).

C. Quantification of the Cyclin-D1 staining (200X) shows a significant reduction in the number of Cyclin-D1 positive cells 40 hours after PH in *Wls*-EKO (*$p<0.05$).

5.0 DISCUSSION

5.1 THE ROLE OF WNT/BETA-CATENIN SIGNALING IN LIVER HOMEOSTASIS AND LIVER REGENERATION

β-Catenin, a critical downstream effector of Wnt signaling, acts as a coactivator for TCF family of transcription factors to regulate expression of several target genes in a tissue specific manner. Deletion of *β-catenin* in hepatocytes has yielded two major phenotypes in the liver [48, 49]. The first observation at baseline is a defect in metabolic zonation as *β-Catenin*-LKO mice lack several downstream targets in pericentral hepatocytes such as genes encoding for GS (ammonia metabolism), Cyp2e1, Cyp1a2 (xenobiotic metabolism) and others involved in glycolytic pathway and Tricarboxylic acid (TCA) cycle [139]. The second phenotype in *β-catenin*-LKO mice was a defect in LR after PH. The role of β-catenin signaling in LR is now well established in rodents, zebrafish and patients [70, 106]. Activation of β-catenin is relatively early with its nuclear translocation evident in minutes to hours after PH, where it complexes with TCF4 to regulate the expression of *Ccnd1*. Cyclin-D1 is critical for driving G1 to S phase cell cycle progression and marks the point of no return for cell proliferation [140]. In fact absence of β-catenin in hepatocytes in *β-Catenin*-LKO led to a significant lag in the initiation of LR with hepatocyte S-phase peaking at 72 hours instead of 40 hours due to decreased Cyclin-D1 [49, 69].

Endogenous or exogenous activation of β-catenin has been shown to improve LR in animal

models as well as in patients [71, 141, 142]. However, for translational exploitation of β-catenin signaling in regenerative medicine, it will be critical to understand the cellular and molecular circuitry upstream of β-catenin activation in hepatocytes at baseline and during LR. This is of special relevance since β-catenin activation has also been shown to occur downstream of non-Wnt mechanisms such as those mediated by HGF, EGF, Flt3 and PKA, which have been independently shown to be playing a significant role in LR after PH [143].

In the current study using genetic mouse models we address the key regulators of β-catenin in hepatic zonation and LR. We used genetic knockout of *Lrp5* and *Lrp6* to disrupt Wnt signaling in hepatocytes and cholangiocytes using *Albumin*-Cre, which was also used by us to delete *β-catenin* in the same two cell-types previously [49, 144]. Deleting both *Lrp5* and *Lrp6* has been shown to prevent any compensation and hence assures complete abrogation of Wnt signaling [109], while it has no effect on Wnt-independent pathways. β-Catenin protein levels and localization were not altered in *Lrp*-LKO, therefore allowing us to investigate the upstream signaling that regulates the activation of β-catenin in liver homeostasis and LR.

The breach in Wnt signaling in *Lrp*-LKO prevented pericentral β-catenin activation and led to defect of hepatic zonation. Expression of pericentral markers, GS, Cyp1a2 and Cyp2e1, were abolished in *Lrp*-LKO, which phenocopied *β-catenin*-LKO. This finding demonstrates that the pericentral zonation function of β-catenin is fully regulated by Wnt signaling (Figure 37).

A more surprising result came from the analysis of LR in this model. Despite β-catenin activation by many non-Wnt dependent mechanisms and despite the physiological activation of such pathways as HGF, EGF and PKA after PH, there was a considerable deficit in LR in the *Lrp*-LKO similar to *β-catenin*-LKO mice as seen by decreased PCNA at 40 and 72 hours and reduced mitosis at 72 hours [49, 63]. Intriguingly, while *β-catenin*-LKO showed comparable numbers of

hepatocytes in S-phase versus Con at 72 hours, the *Lrp*-LKO continued to show fewer hepatocytes in S-phase at this time as compared to their littermate controls. However the numbers of hepatocytes in S-phase were notably greater at 72 hours than the 40-hour time-point, suggesting redundant mechanisms that compensate, which as what was observed in *β-catenin*-LKO as well. An increase in Cyclin-D1 at 72 hours in regenerating *Lrp*-LKO livers over baseline and 40 hours suggests sufficient LR to restore the smaller baseline hepatic mass in *Lrp*-LKO. No alternate mechanism of β-catenin activation in regenerating *Lrp*-LKO livers was observed at any time. Although β-catenin remains intact, it fails to be activated after PH, as shown by lack of β-catenin and TCF4 complex in *Lrp*-LKO. Thus Wnt signaling strictly regulates β-catenin during the process of LR (Figure 37).

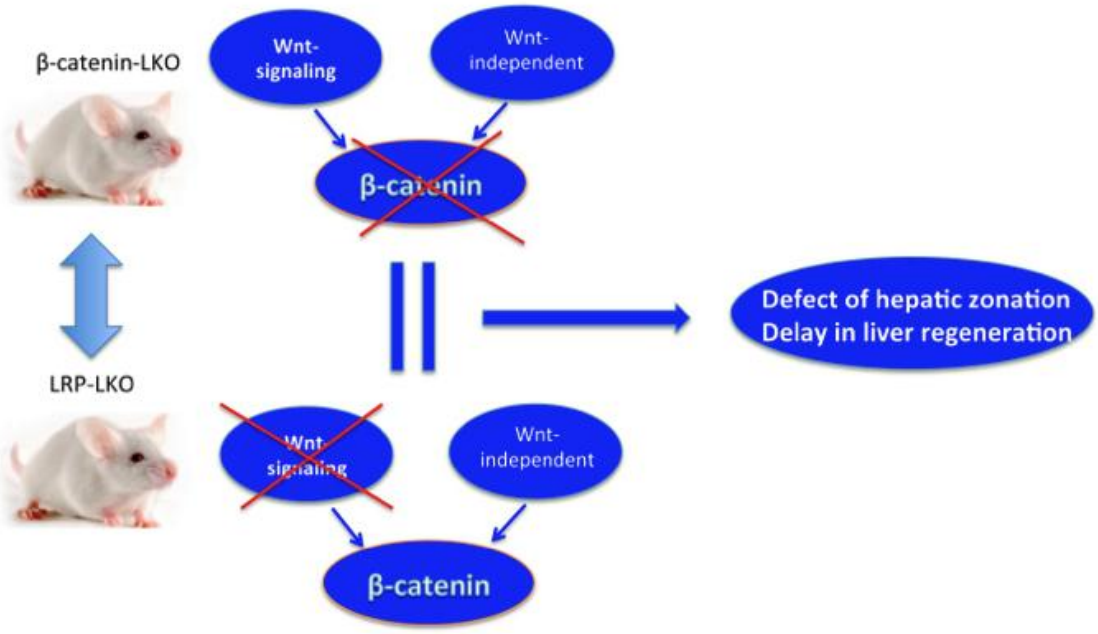

Figure 37. Wnt-signaling is the determinant pathway that regulates β-catenin activation

In *β-catenin*-LKO, β-catenin is deleted from hepatocytes and cholangiocytes, which abolishes both Wnt-dependent and Wnt-independent signaling; while in *LRP*-LKO, only Wnt-signaling is abrogated, β-catenin remains intact and can be

activated by Wnt-independent pathways. *Lrp*-LKO phenocopied *β-catenin*-LKO in defective liver zonation and delay in liver regeneration, suggesting that β-catenin activation in these processes is strictly regulated by Wnt-signaling.

5.2 THE ROLE OF INDIVIDUAL HEPATIC CELL POPULATIONS IN WNT SECRETION

To determine the cellular source of Wnt ligands at baseline and after PH is challenging as several hepatic cell types express multiple Wnts [82]. An exciting development to address the role of global Wnt signaling has been to interfere with either their biological activity or prevent their secretion from a cell. Deletion of the gene encoding for porcupine, which is responsible for glycosylation and acylation of Wnt proteins, has been used to disrupt Wnt function [145]. Another relevant protein, Wntless (Wls), has been shown to be indispensable for Wnt secretion [85]. In fact, conditional deletion of *Wls* has been used in various organ systems to address cell-specific Wnt secretion under spatial and temporal control [94]. Studies show that deletion of *Wls* using Wnt1-Cre phenocopies Wnt1-null abnormalities in brain [95, 146]. Conditional deletion of *Wls* has revealed the crucial role of Wnt ligands in bone development and homeostasis, retina angiogenesis, maintaining stem cell activity and other important biological events [95-98]. Therefore, depletion of *Wls* from individual hepatic cell populations, including hepatocyte, cholangiocytes, Kupffer cells, stellate cells and endothelial cells, would be a powerful tool to address the source of Wnt ligands in the liver.

5.2.1 The role of liver epithelial cells in Wnt secretion

Utilizing *Wls*-floxed mice, we blocked Wnt secretion from hepatocytes and cholangiocytes. Western blot shows only a modest reduction in Wls protein level in the *Wls*-LKO livers, which suggests that the other hepatic cell populations also express *Wls* and are capable of Wnt secretion. Indeed, cell fractionation into parenchymal and non-parenchymal cells showed notably higher *Wls* expression in the nonparenchymal cell compartment.

5.2.1.1 The role of liver epithelial cell derived Wnts in hepatic zonation and initiation of LR

Our results showed no alteration in either pericentral gene expression, or in the initiation of LR when Wnt-secretion was ablated from hepatocytes and cholangiocytes, therefore, rule out the possibility that hepatocytes and cholangiocytes as the source of Wnt ligands that regulate β-catenin activation in these processes. Our q-PCR analysis of *Wnt* expression in hepatic cell populations also shows very modest induction of stimulatory Wnt ligands (such as Wnt2) after PH in the hepatocytes, with robust induction in the endothelial cells and macrophages, further confirming that liver parenchymal cells might not be the source of activating Wnt ligands during LR.

Liver regeneration after PH features the proliferation of adult hepatic cell types. Hepatocytes have enormous proliferative potential, and they are the first to proliferate after PH. The first peak of DNA synthesis in hepatocytes occurs around 24 h after PH in rats and approximately 36 h in mice [55]. Cholangiocytes, stellate cells and endothelial cells all start to proliferate after hepatocytes [55, 58, 59]. β-Catenin plays an essential role in triggering hepatocyte proliferation after PH. β-catenin activation in hepatocytes can be observed very shortly after PH [65], while a defect of β-catenin leads to a delay in hepatocyte proliferation [49]. Our findings suggest that hepatocytes are not the source of Wnt ligands that activate β-catenin during LR.

However, hepatocytes can respond to the Wnt signals secreted by other cell populations and proliferate earlier than them. Therefore, there might be some regulatory mechanisms by which hepatocytes are the first to respond to the activating Wnt signals after PH. Gene array analysis in individual hepatic cell populations before and after PH might help to identify the genes playing important roles in this process.

5.2.1.2 The role of liver epithelial cell derived Wnts in the termination of LR

Our experiments showed a temporarily enhanced LR and prolonged activation of β-catenin signaling in *Wls*-LKO at later stages of LR. In Con, there was an induction of Wnt5a signaling, an inhibitor of the Wnt/β-catenin pathway, which was absent in *Wls*-LKO. Therefore, we hypothesized that hepatocytes might be able to secrete inhibitory Wnt ligands such as Wnt5a to terminate LR, while *Wls*-LKO has a defect in Wnt secretion from hepatocytes which leads to sustained LR. By ELISA, we did confirm that Con hepatocytes were capable of secreting Wnt5a after PH, while the hepatocytes from *Wls*-LKO failed to, which further supports our hypothesis.

Next, we aimed to investigate the role of Wnt5a in LR, and thus liver epithelial cell specific *Wnt5a* knockout mice were generated. *Wnt5a*-LKO livers had slightly less Wnt5a protein and mRNA levels at baseline, suggesting that other liver cell populations also express Wnt5a. Surprisingly, *Wnt5a*-LKO exhibited no alteration in LR kinetics after PH. Q-PCR analysis for *Wnt5a* expression in total liver tissue showed mild but significant differences between Con and *Wnt5a*-LKO 24 and 40 hours after PH, while no differences at later time points, which indicates that other liver cell populations can secrete Wnt5a to compensate for the loss of liver epithelial cell derived Wnt5a. Other signaling pathways involved in the termination of LR, such as TGFβ, ILK and GPC3, may also become active to ensure the proper termination of LR in *Wnt5a*-LKO.

However, there is some discrepancy in phenotypes between the two animal models: *Wls*-LKO exhibits sustained LR, while the *Wnt5a*-LKO has normal LR kinetics. Numerous reasons could cause the discrepancy. All Wnt secretion from liver epithelial cells is blocked in *Wls*-LKO, while only *Wnt5a* is deleted in the *Wnt5a*-LKO. Liver epithelial cells might be able to secrete other inhibitory Wnt ligands to terminate LR, and thus ablation of *Wnt5a* alone is not sufficient to cause any phenotype. Analyzing the expression of all the Wnt ligands at different time points after PH in Con and knockouts might help identifying candidates of inhibitory Wnts being upregulated during later stage of LR to inhibit β-catenin activation and terminate LR. Another possible reason is that ablation of liver epithelial cell derived Wnt5a might be compensated by the Wnt5a secreted by other liver cell populations, therefore *Wls*-LKO only show temporarily enhanced LR, while *Wnt5a*-LKO have normal LR kinetics. Examining *Wnt5a* expression in individual cell populations during late stages of LR might be helpful to address the source of Wnt5a in both Con and knockout animals. Finally, the *Wls*-LKO and *Wnt5a*-LKO were generated in different genetic background. The strain difference might also contribute to the observed discrepancy in phenotypes.

5.2.2 The role of hepatic stellate cells in Wnt secretion

To elucidate the role of hepatic stellate cells in Wnt secretion, we generated stellate cell specific *Wls* knockout (*Wls*-SKO) by breeding *Wls* floxed mice to *GFAP*-Cre mice [111]. GFAP is believed to be a marker for hepatic stellate cells [147-150], and *GFAP*-Cre mice have been widely used to generate HSC specific null animal models [130-132]. However, there is still controversy on whether *GFAP*-Cre can efficiently target HSCs [133]. Therefore, to verify successful loss of

Wls in HSCs, we compared *Wls* expression in HSCs isolated from Con and *Wls*-SKO livers. As expected, there was a dramatic reduction in *Wls* expression in HSCs from *Wls*-SKO, which confirmed that our animal model was valid.

We further characterized the *Wls*-SKO at baseline. *Wls*-SKO livers appear to be histologically normal and do not have any liver injuries (data not shown). No alteration was observed in *Wls*-SKO, except for slightly lower LW/BW ratio compared to Con. To address the mechanism for this phenotype, we examined the baseline proliferation by PCNA staining and Cylin-D1 expression. Surprisingly, no obvious alteration was found in *Wls*-SKO. However, GFAP is not only expressed in hepatic stellate cells, but also in several other cell populations, such as enteric glial cell of small intestine [134] and astrocytes in the brain [111, 135]. Therefore the smaller liver size could be consequent effect resulted from the defect of *Wls* in other organs rather than in the liver.

Next, we examined hepatic zonation and LR. Our results show no alteration in either pericentral gene expression, or in the initiation of LR when Wnt-secretion was ablated from hepatic stellate cells. Therefore, our findings rule out HSCs as the source of Wnt proteins that regulate β-catenin activation in liver homeostasis and LR.

HSCs are the main source of extracellular matrix (ECM), thus playing an essential role in liver fibrosis. In chronic liver diseases, injuries activate and transform quiescent HSCs which then become activated myofibroblasts, and it is the central pathogenic mechanism behind liver fibrosis [151, 152]. Meanwhile, Wnt/β-catenin signaling has been demonstrated to play important roles in HSC development and liver fibrosis [153, 154]. Activation of the Wnt/β-catenin pathway by a Wnt agonist activates HSCs and promotes liver fibrosis [155], while inhibition of Wnt/β-catenin signaling attenuates liver fibrosis [156]. Although our study suggests that Wnt ligands secreted by

HSCs are dispensable for the activation of β-catenin in liver homeostasis and during LR, a more important role of the HSC derived Wnt ligands might lie in liver fibrogenesis. Therefore, we further plan to address the role of HSC derived Wnt proteins in the context of liver fibrosis. Since inhibition of the activation and function of HSCs has become the most important treatment strategy for liver fibrosis [152], our new study will aim to elucidate the upstream signaling leading to HSC activation and how it might bear significant clinical relevance.

5.2.3 The role of Kupffer cells in Wnt secretion

To elucidate the role of Kupffer cells in Wnt secretion, we generated macrophage specific *Wls* knockout (*Wls*-MKO) by breeding *Wls* floxed mice to *Lyz2*-Cre mice [136]. *Lyz2*-Cre mice have been widely used to generate myeloid cell specific conditional knockouts [136]. To verify successful loss of *Wls*, Kupffer cells were isolated. Q-PCR performed on enriched Kupffer cell-fractions showed a significant decrease in *Wls* expression in *Wls*-MKO as compared to Con.

Based on our study, abrogating Wnt secretion from Kupffer cells results in no defects in metabolic zonation. GS, Cyp1a2 and Cyp2e1 expression were not altered in pericentral hepatocytes. However, when the animals were subjected to PH, a 33% decrease in S-phase hepatocytes and hepatocyte mitosis was evident in *Wls*-MKO at 40 hours after PH. This coincided with a notable decrease in β-catenin-TCF4 complex and Cyclin-D1 expression after PH. These observations are consistent with the dramatic induction of *Wnt2* expression in the Kupffer cells 12 hours after PH (Table. 2). Taken together, our findings suggest that Kupffer cells are an important source of Wnt proteins that activate hepatocyte β-catenin in a paracrine manner during LR.

Indeed, the important role of Kupffer cells in liver regeneration has been well established. Studies suggest that Kupffer cells are crucial for LR because of their ability to produce growth factors and cytokines contributing to LR [157]. Depletion of Kupffer cells in rodents results in delayed liver regeneration, caused by reduced expression of important growth factors or an imbalanced hepatic cytokine expression [158, 159]. Our study further confirms the importance of Kupffer cells in LR by elucidating their role in Wnt secretion and β-catenin activation during LR.

5.2.4 The role of endothelial cells in Wnt secretion

Endothelial cells are believed to have critical roles in maintaining liver homeostasis and triggering LR [102, 137]. In normal liver, β-catenin is activated in pericentral hepatocytes, which dictates pericentral zonation. Wnt signals emanating from the endothelial cells of the central vein may activate β-catenin in the pericentral hepatocytes [137]. During LR after PH, liver sinusoidal endothelial cells secrete pro-proliferative signals, such as HGF and Wnt2, to induce hepatocyte proliferation [102]. Our Wnt expression analysis in individual liver cell populations shows a dramatic induction of *Wnt2* and *Wnt9b* expression in the endothelial cells after PH, which again suggests an important role of the endothelial cells in Wnt secretion.

To address the role of endothelial cells in Wnt secretion, we first utilized *Tie2-Cre* to deplete *Wls* from endothelial cells. *Tie2-Cre* mediated *Wls* deletion led to embryonic lethality, which was not surprising because of the many important roles of Wnt/β-catenin signaling in vasculogenesis and angiogenesis [160, 161]. While embryonic lethality precluded us from addressing the role of hepatic sinusoidal endothelial cells and of endothelial cells lining the hepatic

vessel walls, viable heterozygous knockouts did not show any notable decrease in Wls. Simultaneously, no defects in hepatic zonation or LR were observed.

Next, we generated Tamoxifen (Tm) inducible-conditional *Wls*-iEKO to elucidate the endothelial-hepatocyte paracrine interactions in the liver. WB shows a dramatic reduction of Wls expression in livers from *Wls*-EKO after Tm treatment, which suggests that *Wls* is deleted in the endothelial cells. Zonation and LR were analyzed at different time points after Tm treatment. Our experiments show an obvious reduction in the mRNA expression of all zonation markers in *Wls*-EKO one and two weeks after Tm treatment. Therefore, endothelial cell secreted Wnts might play an important role in regulating zonal gene expression. However, three and four weeks after treatment, there is an increase in the mRNA expression of zonation genes in the knockouts. Numerous reasons can explain this phenotype and need to be validated in future experiments.

Compensation of Wnt secretion from other cell populations could be a possible cause for the increased expression of zonation markers at later time points after Tm. Endothelial cell derived Wnts might be important for maintaining zonation; however, other cell populations in the liver can also secrete Wnts and might be able to compensate for the lack of Wnts secreted by endothelial cells. If so, animal models with *Wls* deleted from two or more hepatic cell populations must be generated, to address two or more cell populations together being the source of Wnt ligands that dictate liver zonation. Another potential reason for this phenotype is the repopulation of endothelial cells from progenitor cells. Residing progenitor cells may give rise to endothelial cells, and *Wls* might be expressed in the newly repopulated endothelial cells three or four weeks after Tm treatment, as there is no effective Tm to activate Cre-recombinase activity. To test this possibility, endothelial cells can be isolated from *Wls*-EKO livers at earlier and later time points

after Tm injection. Analysis of *Wls* expression will answer the question whether there is repopulation of *Wls* expressing endothelial cells at later time points after Tm injection.

Given the fact that zonal gene expression increases in *Wls*-EKO three and four weeks after Tm treatment, one or two weeks after injection should be best time points to address the role of endothelial cell derived Wnts in regulating zonation. However, one and two weeks after Tm, we only observed a decrease in Cyp1a2 protein level, while GS and Cyp2e1 protein level did not change. Since the half-life of these enzymes in mouse liver is unknown, it could be that the half-life is longer than we expected. Therefore, the residual proteins might persist when we analyzed zonation one or two weeks after Tm injection, even though the mRNA level was already affected by the lack of endothelial cell derived Wnts. In order to fully address the effect of the lack of endothelial cell derived Wnts on zonation, we might have to deplete the original store of the enzymes at one or two weeks after Tm injection, and analyze zonation afterwards. We plan to inject the mice with a sub-lethal dose of acetaminophen to consume Cyp1a2 and Cyp2e1 [121, 122, 141], or feed the animals with a high protein diet to consume GS [162]. Con mice should be able to replenish the store of enzymes efficiently, whereas *Wls*-EKO should not, thereby leading to a more pronounced difference in zonation between Con and *Wls*-EKO.

The role of endothelial cells in LR after PH was more apparent. PCNA staining after PH showed a notable decrease in the number of positively stained hepatocytes in *Wls*-EKO 40 hours after PH. This indicates that β-catenin activation in hepatocytes after PH is at least in part regulated by Wnts derived from endothelial cells. Further, we plan to examine LR kinetics at different time points after PH and examine β-catenin activation in both Con and *Wls*-EKO, to fully address the role of endothelial cell derived Wnts in LR.

5.2.5 Summary: The role of hepatic cell populations in Wnt secretion

Our current study demonstrates the regulation of β-catenin in two fundamental processes inherent to the liver: hepatic zonation and liver regeneration (LR). In both cases, β-catenin is under the control of Wnt signaling only, as abolishing Wnt-dependent signaling compromises pericentral gene expression and results in delayed LR. To address the upstream signals that control β-catenin activation in these processes, conditional knockout animals that lack the ability of a specific cell population to secrete Wnts were generated. Our studies rule out the role of hepatocytes, cholangiocytes, stellate cells and Kupffer cells as the source of Wnt proteins that regulate zonation, and suggest a potential role of the endothelial cells derived Wnt ligands in this process (Figure 38). On the other hand, it became apparent that Wnt secretion from the non-parenchymal cell compartment of Kupffer cells and endothelial cells, was required for timely β-catenin activation in hepatocytes to initiate LR after PH, while there is some evidence to suggest that hepatocytes might secrete inhibitory Wnt ligands involved in the termination of LR (Figure 38).

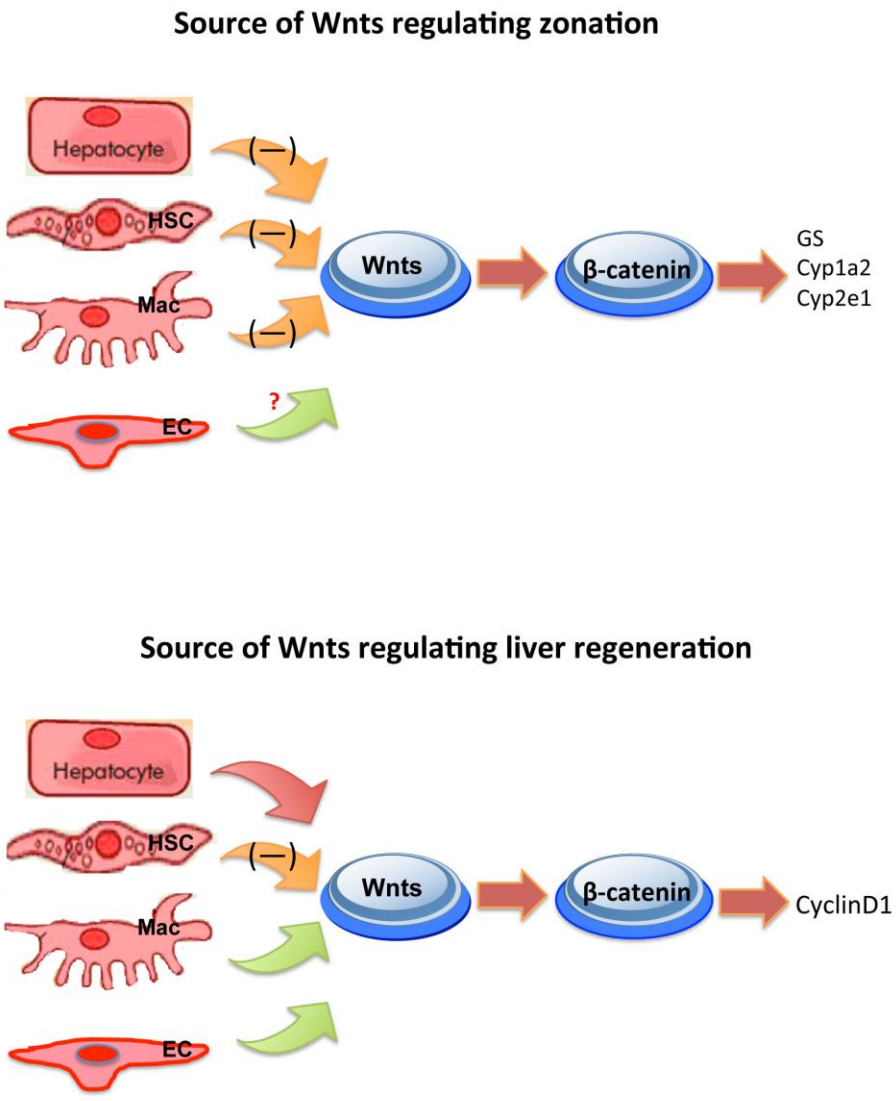

Figure 38. Source of Wnt ligands regulating hepatic zonation and liver regeneration

Diagrams showing the source of Wnts in regulating zonation and LR. For zonation, hepatocytes, HSCs and macrophages are not the source of Wnts. Endothelial cells might play an important role, but the exact role of endothelial cells needs to be further investigated. For LR, both macrophages and endothelial cells secrete activating Wnt ligands to trigger LR after PH, while hepatocytes might secrete inhibitory Wnts that suppress β-catenin activation during late stages of LR.

BIBLIOGRAPHY

1. Nault, J.C. and J. Zucman-Rossi, *Genetics of hepatobiliary carcinogenesis.* Seminars in liver disease, 2011. **31**(2): p. 173-87.
2. Clevers, H., *Wnt/beta-catenin signaling in development and disease.* Cell, 2006. **127**(3): p. 469-80.
3. Cliffe, A., F. Hamada, and M. Bienz, *A role of Dishevelled in relocating Axin to the plasma membrane during wingless signaling.* Current biology : CB, 2003. **13**(11): p. 960-6.
4. Zeng, X., et al., *Initiation of Wnt signaling: control of Wnt coreceptor Lrp6 phosphorylation/activation via frizzled, dishevelled and axin functions.* Development, 2008. **135**(2): p. 367-75.
5. Zeng, X., et al., *A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation.* Nature, 2005. **438**(7069): p. 873-7.
6. Cadigan, K.M. and Y.I. Liu, *Wnt signaling: complexity at the surface.* Journal of cell science, 2006. **119**(Pt 3): p. 395-402.
7. Logan, C.Y. and R. Nusse, *The Wnt signaling pathway in development and disease.* Annual review of cell and developmental biology, 2004. **20**: p. 781-810.
8. Zarnegar, R., *Regulation of HGF and HGFR gene expression.* EXS, 1995. **74**: p. 33-49.
9. Monga, S.P., et al., *Hepatocyte growth factor induces Wnt-independent nuclear translocation of beta-catenin after Met-beta-catenin dissociation in hepatocytes.* Cancer research, 2002. **62**(7): p. 2064-71.
10. Zeng, G., et al., *Tyrosine residues 654 and 670 in beta-catenin are crucial in regulation of Met-beta-catenin interactions.* Experimental cell research, 2006. **312**(18): p. 3620-30.
11. Shibata, T., et al., *Dominant negative inhibition of the association between beta-catenin and c-erbB-2 by N-terminally deleted beta-catenin suppresses the invasion and metastasis of cancer cells.* Oncogene, 1996. **13**(5): p. 883-9.
12. Bonvini, P., et al., *Geldanamycin abrogates ErbB2 association with proteasome-resistant beta-catenin in melanoma cells, increases beta-catenin-E-cadherin association, and decreases beta-catenin-sensitive transcription.* Cancer research, 2001. **61**(4): p. 1671-7.
13. Wang, K., et al., *Geldanamycin destabilizes HER2 tyrosine kinase and suppresses Wnt/beta-catenin signaling in HER2 overexpressing human breast cancer cells.* Oncology reports, 2007. **17**(1): p. 89-96.
14. Hino, S., et al., *Phosphorylation of beta-catenin by cyclic AMP-dependent protein kinase stabilizes beta-catenin through inhibition of its ubiquitination.* Molecular and cellular biology, 2005. **25**(20): p. 9063-72.
15. Taurin, S., et al., *Phosphorylation of beta-catenin by cyclic AMP-dependent protein kinase.* The Journal of biological chemistry, 2006. **281**(15): p. 9971-6.

16. Gomez-Orte, E., et al., *Multiple functions of the noncanonical Wnt pathway.* Trends in genetics : TIG, 2013. **29**(9): p. 545-53.
17. Niehrs, C., *The complex world of WNT receptor signalling.* Nature reviews. Molecular cell biology, 2012. **13**(12): p. 767-79.
18. Axelrod, J.D., *Progress and challenges in understanding planar cell polarity signaling.* Seminars in cell & developmental biology, 2009. **20**(8): p. 964-71.
19. Kikuchi, A., et al., *New insights into the mechanism of Wnt signaling pathway activation.* International review of cell and molecular biology, 2011. **291**: p. 21-71.
20. Simons, M. and M. Mlodzik, *Planar cell polarity signaling: from fly development to human disease.* Annual review of genetics, 2008. **42**: p. 517-40.
21. De, A., *Wnt/Ca2+ signaling pathway: a brief overview.* Acta biochimica et biophysica Sinica, 2011. **43**(10): p. 745-56.
22. Saneyoshi, T., et al., *The Wnt/calcium pathway activates NF-AT and promotes ventral cell fate in Xenopus embryos.* Nature, 2002. **417**(6886): p. 295-9.
23. Kuhl, M., et al., *The Wnt/Ca2+ pathway: a new vertebrate Wnt signaling pathway takes shape.* Trends in genetics : TIG, 2000. **16**(7): p. 279-83.
24. Kuhl, M., et al., *Antagonistic regulation of convergent extension movements in Xenopus by Wnt/beta-catenin and Wnt/Ca2+ signaling.* Mechanisms of development, 2001. **106**(1-2): p. 61-76.
25. Veeman, M.T., J.D. Axelrod, and R.T. Moon, *A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling.* Developmental cell, 2003. **5**(3): p. 367-77.
26. Kikuchi, A. and H. Yamamoto, *Tumor formation due to abnormalities in the beta-catenin-independent pathway of Wnt signaling.* Cancer science, 2008. **99**(2): p. 202-8.
27. Westfall, T.A., et al., *Wnt-5/pipetail functions in vertebrate axis formation as a negative regulator of Wnt/beta-catenin activity.* The Journal of cell biology, 2003. **162**(5): p. 889-98.
28. Mikels, A.J. and R. Nusse, *Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context.* PLoS biology, 2006. **4**(4): p. e115.
29. Oishi, I., et al., *The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway.* Genes to cells : devoted to molecular & cellular mechanisms, 2003. **8**(7): p. 645-54.
30. Nomachi, A., et al., *Receptor tyrosine kinase Ror2 mediates Wnt5a-induced polarized cell migration by activating c-Jun N-terminal kinase via actin-binding protein filamin A.* The Journal of biological chemistry, 2008. **283**(41): p. 27973-81.
31. Nishita, M., et al., *Filopodia formation mediated by receptor tyrosine kinase Ror2 is required for Wnt5a-induced cell migration.* The Journal of cell biology, 2006. **175**(4): p. 555-62.
32. Enomoto, M., et al., *Autonomous regulation of osteosarcoma cell invasiveness by Wnt5a/Ror2 signaling.* Oncogene, 2009. **28**(36): p. 3197-208.
33. Torres, M.A., et al., *Activities of the Wnt-1 class of secreted signaling factors are antagonized by the Wnt-5A class and by a dominant negative cadherin in early Xenopus development.* The Journal of cell biology, 1996. **133**(5): p. 1123-37.
34. Topol, L., et al., *Wnt-5a inhibits the canonical Wnt pathway by promoting GSK-3-independent beta-catenin degradation.* The Journal of cell biology, 2003. **162**(5): p. 899-908.
35. Sato, A., et al., *Wnt5a regulates distinct signalling pathways by binding to Frizzled2.* The EMBO journal, 2010. **29**(1): p. 41-54.

36. Kremenevskaja, N., et al., *Wnt-5a has tumor suppressor activity in thyroid carcinoma.* Oncogene, 2005. **24**(13): p. 2144-54.
37. MacLeod, R.J., M. Hayes, and I. Pacheco, *Wnt5a secretion stimulated by the extracellular calcium-sensing receptor inhibits defective Wnt signaling in colon cancer cells.* American journal of physiology. Gastrointestinal and liver physiology, 2007. **293**(1): p. G403-11.
38. Christoffels, V.M., et al., *A mechanistic model for the development and maintenance of portocentral gradients in gene expression in the liver.* Hepatology, 1999. **29**(4): p. 1180-92.
39. Gebhardt, R., *Metabolic zonation of the liver: regulation and implications for liver function.* Pharmacology & therapeutics, 1992. **53**(3): p. 275-354.
40. Jungermann, K. and N. Katz, *Functional specialization of different hepatocyte populations.* Physiological reviews, 1989. **69**(3): p. 708-64.
41. Gebhardt, R. and D. Mecke, *Heterogeneous Distribution of Glutamine-Synthetase among Rat-Liver Parenchymal-Cells Insitu and in Primary Culture.* Embo Journal, 1983. **2**(4): p. 567-570.
42. Gebhardt, R., et al., *Hepatocellular heterogeneity in ammonia metabolism: demonstration of limited colocalization of carbamoylphosphate synthetase and glutamine synthetase.* European journal of cell biology, 1991. **56**(2): p. 464-7.
43. Haussinger, D., *Hepatocyte Heterogeneity in Glutamine and Ammonia Metabolism and the Role of an Intercellular Glutamine Cycle during Ureogenesis in Perfused-Rat-Liver.* European Journal of Biochemistry, 1983. **133**(2): p. 269-275.
44. Haussinger, D., *Nitrogen-Metabolism in Liver - Structural and Functional-Organization and Physiological Relevance.* Biochemical Journal, 1990. **267**(2): p. 281-290.
45. Hailfinger, S., et al., *Zonal gene expression in murine liver: lessons from tumors.* Hepatology, 2006. **43**(3): p. 407-14.
46. Benhamouche, S., et al., *Apc tumor suppressor gene is the "zonation-keeper" of mouse liver.* Developmental cell, 2006. **10**(6): p. 759-70.
47. Benhamouche, S., et al., *[Wnt/beta-catenin pathway and liver metabolic zonation: a new player for an old concept]*. Medecine sciences : M/S, 2006. **22**(11): p. 904-6.
48. Sekine, S., et al., *Liver-specific loss of beta-catenin blocks glutamine synthesis pathway activity and cytochrome p450 expression in mice.* Hepatology, 2006. **43**(4): p. 817-25.
49. Tan, X., et al., *Conditional deletion of beta-catenin reveals its role in liver growth and regeneration.* Gastroenterology, 2006. **131**(5): p. 1561-72.
50. Cavard, C., et al., *Wnt/beta-catenin pathway in hepatocellular carcinoma pathogenesis and liver physiology.* Future oncology, 2008. **4**(5): p. 647-60.
51. Michalopoulos, G.K. and M.C. DeFrances, *Liver regeneration.* Science, 1997. **276**(5309): p. 60-6.
52. Arai, M., et al., *Gene expression profiling reveals the mechanism and pathophysiology of mouse liver regeneration.* The Journal of biological chemistry, 2003. **278**(32): p. 29813-8.
53. White, P., et al., *Identification of transcriptional networks during liver regeneration.* The Journal of biological chemistry, 2005. **280**(5): p. 3715-22.
54. Higgins, G.M. and R.M. Anderson, *Experimental pathology of the liver I Restoration of the liver of the white rat following partial surgical removal.* Archives of Pathology, 1931. **12**(2): p. 186-202.
55. Michalopoulos, G.K., *Liver regeneration.* Journal of cellular physiology, 2007. **213**(2): p. 286-300.

56. Palmes, D. and H.U. Spiegel, *Animal models of liver regeneration.* Biomaterials, 2004. **25**(9): p. 1601-11.
57. Michalopoulos, G.K., *Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas.* The American journal of pathology, 2010. **176**(1): p. 2-13.
58. Grisham, J.W., *A morphologic study of deoxyribonucleic acid synthesis and cell proliferation in regenerating rat liver; autoradiography with thymidine-H3.* Cancer research, 1962. **22**: p. 842-9.
59. Martinez-Hernandez, A. and P.S. Amenta, *The extracellular matrix in hepatic regeneration.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 1995. **9**(14): p. 1401-10.
60. Fausto, N., *Liver regeneration.* Journal of hepatology, 2000. **32**(1 Suppl): p. 19-31.
61. Taub, R., *Liver regeneration 4: transcriptional control of liver regeneration.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 1996. **10**(4): p. 413-27.
62. Su, A.I., et al., *Gene expression during the priming phase of liver regeneration after partial hepatectomy in mice.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(17): p. 11181-6.
63. Tan, X., et al., *Epidermal growth factor receptor: a novel target of the Wnt/beta-catenin pathway in liver.* Gastroenterology, 2005. **129**(1): p. 285-302.
64. Cadoret, A., et al., *Hepatomegaly in transgenic mice expressing an oncogenic form of beta-catenin.* Cancer research, 2001. **61**(8): p. 3245-9.
65. Monga, S.P., et al., *Changes in WNT/beta-catenin pathway during regulated growth in rat liver regeneration.* Hepatology, 2001. **33**(5): p. 1098-109.
66. Makino, R., K. Hayashi, and T. Sugimura, *C-myc transcript is induced in rat liver at a very early stage of regeneration or by cycloheximide treatment.* Nature, 1984. **310**(5979): p. 697-8.
67. Albrecht, J.H., M.Y. Hu, and F.B. Cerra, *Distinct patterns of cyclin D1 regulation in models of liver regeneration and human liver.* Biochemical and biophysical research communications, 1995. **209**(2): p. 648-55.
68. Sodhi, D., et al., *Morpholino oligonucleotide-triggered beta-catenin knockdown compromises normal liver regeneration.* Journal of hepatology, 2005. **43**(1): p. 132-41.
69. Sekine, S., et al., *Liver-specific loss of beta-catenin results in delayed hepatocyte proliferation after partial hepatectomy.* Hepatology, 2007. **45**(2): p. 361-8.
70. Goessling, W., et al., *APC mutant zebrafish uncover a changing temporal requirement for wnt signaling in liver development.* Developmental Biology, 2008. **320**(1): p. 161-74.
71. Nejak-Bowen, K.N., et al., *Accelerated liver regeneration and hepatocarcinogenesis in mice overexpressing serine-45 mutant beta-catenin.* Hepatology, 2010. **51**(5): p. 1603-13.
72. Overturf, K., et al., *Serial transplantation reveals the stem-cell-like regenerative potential of adult mouse hepatocytes.* The American journal of pathology, 1997. **151**(5): p. 1273-80.
73. Overturf, K., et al., *The repopulation potential of hepatocyte populations differing in size and prior mitotic expansion.* The American journal of pathology, 1999. **155**(6): p. 2135-43.
74. Houck, K.A. and G.K. Michalopoulos, *Altered responses of regenerating hepatocytes to norepinephrine and transforming growth factor type beta.* Journal of cellular physiology, 1989. **141**(3): p. 503-9.

75. Russell, W.E., et al., *Type beta transforming growth factor reversibly inhibits the early proliferative response to partial hepatectomy in the rat.* Proceedings of the National Academy of Sciences of the United States of America, 1988. **85**(14): p. 5126-30.
76. Oe, S., et al., *Intact signaling by transforming growth factor beta is not required for termination of liver regeneration in mice.* Hepatology, 2004. **40**(5): p. 1098-105.
77. Hynes, R.O., *Integrins: Bidirectional, allosteric signaling machines.* Cell, 2002. **110**(6): p. 673-687.
78. Wu, C.Y., *Ilk Interactions.* Journal of Cell Science, 2001. **114**(14): p. 2549-2550.
79. Apte, U., et al., *Enhanced Liver Regeneration Following Changes Induced by Hepatocyte-Specific Genetic Ablation of Integrin-Linked Kinase.* Hepatology, 2009. **50**(3): p. 844-851.
80. Liu, B.W., et al., *Investigation of the Role of Glypican 3 in Liver Regeneration and Hepatocyte Proliferation.* American Journal of Pathology, 2009. **175**(2): p. 717-724.
81. Liu, B., et al., *Suppression of Liver Regeneration and Hepatocyte Proliferation in Hepatocyte-Targeted Glypican 3 Transgenic Mice.* Hepatology, 2010. **52**(3): p. 1060-1067.
82. Zeng, G., et al., *Wnt'er in liver: expression of Wnt and frizzled genes in mouse.* Hepatology, 2007. **45**(1): p. 195-204.
83. Peifer, M. and P. Polakis, *Wnt signaling in oncogenesis and embryogenesis--a look outside the nucleus.* Science, 2000. **287**(5458): p. 1606-9.
84. Nusse, R., et al., *Wnt signaling and stem cell control.* Cold Spring Harbor symposia on quantitative biology, 2008. **73**: p. 59-66.
85. Banziger, C., et al., *Wntless, a conserved membrane protein dedicated to the secretion of Wnt proteins from signaling cells.* Cell, 2006. **125**(3): p. 509-22.
86. Belenkaya, T.Y., et al., *The retromer complex influences Wnt secretion by recycling wntless from endosomes to the trans-Golgi network.* Developmental cell, 2008. **14**(1): p. 120-31.
87. Franch-Marro, X., et al., *Wingless secretion requires endosome-to-Golgi retrieval of Wntless/Evi/Sprinter by the retromer complex.* Nature cell biology, 2008. **10**(2): p. 170-7.
88. Bartscherer, K., et al., *Secretion of Wnt ligands requires Evi, a conserved transmembrane protein.* Cell, 2006. **125**(3): p. 523-33.
89. Tanaka, K., Y. Kitagawa, and T. Kadowaki, *Drosophila segment polarity gene product porcupine stimulates the posttranslational N-glycosylation of wingless in the endoplasmic reticulum.* The Journal of biological chemistry, 2002. **277**(15): p. 12816-23.
90. Takada, R., et al., *Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion.* Developmental cell, 2006. **11**(6): p. 791-801.
91. Port, F., et al., *Wingless secretion promotes and requires retromer-dependent cycling of Wntless.* Nature cell biology, 2008. **10**(2): p. 178-85.
92. Yang, P.T., et al., *Wnt signaling requires retromer-dependent recycling of MIG-14/Wntless in Wnt-producing cells.* Developmental cell, 2008. **14**(1): p. 140-7.
93. Fu, J., et al., *Reciprocal regulation of Wnt and Gpr177/mouse Wntless is required for embryonic axis formation.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(44): p. 18598-603.
94. Carpenter, A.C., et al., *Generation of mice with a conditional null allele for Wntless.* Genesis, 2010. **48**(9): p. 554-8.
95. Stefater, J.A., 3rd, et al., *Regulation of angiogenesis by a non-canonical Wnt-Flt1 pathway in myeloid cells.* Nature, 2011. **474**(7352): p. 511-5.

96. Zhong, Z., et al., *Wntless functions in mature osteoblasts to regulate bone mass.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(33): p. E2197-204.
97. Chen, D., et al., *Dermal beta-catenin activity in response to epidermal Wnt ligands is required for fibroblast proliferation and hair follicle initiation.* Development, 2012. **139**(8): p. 1522-33.
98. Takeo, M., et al., *Wnt activation in nail epithelium couples nail growth to digit regeneration.* Nature, 2013. **499**(7457): p. 228-32.
99. Kang, Y.B., et al., *Layered long-term co-culture of hepatocytes and endothelial cells on a transwell membrane: toward engineering the liver sinusoid.* Biofabrication, 2013. **5**(4): p. 045008.
100. Bouwens, L., et al., *Liver cell heterogeneity: functions of non-parenchymal cells.* Enzyme, 1992. **46**(1-3): p. 155-68.
101. Malik, R., C. Selden, and H. Hodgson, *The role of non-parenchymal cells in liver growth.* Seminars in cell & developmental biology, 2002. **13**(6): p. 425-31.
102. Ding, B.S., et al., *Inductive angiocrine signals from sinusoidal endothelium are required for liver regeneration.* Nature, 2010. **468**(7321): p. 310-5.
103. Boulter, L., et al., *Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease.* Nature medicine, 2012. **18**(4): p. 572-9.
104. Chen, L., et al., *HSCs play a distinct role in different phases of oval cell-mediated liver regeneration.* Cell biochemistry and function, 2012.
105. Kajiguchi, T., et al., *Y654 of beta-catenin is essential for FLT3/ITD-related tyrosine phosphorylation and nuclear localization of beta-catenin.* European journal of haematology, 2012. **88**(4): p. 314-20.
106. Nejak-Bowen, K.N. and S.P. Monga, *Beta-catenin signaling, liver regeneration and hepatocellular cancer: sorting the good from the bad.* Seminars in cancer biology, 2011. **21**(1): p. 44-58.
107. Lade, A.G. and S.P. Monga, *Beta-catenin signaling in hepatic development and progenitors: which way does the WNT blow?* Developmental dynamics : an official publication of the American Association of Anatomists, 2011. **240**(3): p. 486-500.
108. Bartscherer, K. and M. Boutros, *Regulation of Wnt protein secretion and its role in gradient formation.* EMBO reports, 2008. **9**(10): p. 977-82.
109. Zhong, Z., et al., *Lrp5 and Lrp6 play compensatory roles in mouse intestinal development.* Journal of cellular biochemistry, 2012. **113**(1): p. 31-8.
110. Postic, C., et al., *Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase.* The Journal of biological chemistry, 1999. **274**(1): p. 305-15.
111. Zhuo, L., et al., *hGFAP-cre transgenic mice for manipulation of glial and neuronal function in vivo.* Genesis, 2001. **31**(2): p. 85-94.
112. Clausen, B.E., et al., *Conditional gene targeting in macrophages and granulocytes using LysMcre mice.* Transgenic Res, 1999. **8**(4): p. 265-77.
113. Kisanuki, Y.Y., et al., *Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo.* Dev Biol, 2001. **230**(2): p. 230-42.
114. Monvoisin, A., et al., *VE-cadherin-CreERT2 transgenic mouse: a model for inducible recombination in the endothelium.* Developmental dynamics : an official publication of the American Association of Anatomists, 2006. **235**(12): p. 3413-22.

115. Ye, H., et al., *Premature expression of the winged helix transcription factor HFH-11B in regenerating mouse liver accelerates hepatocyte entry into S phase.* Molecular and cellular biology, 1999. **19**(12): p. 8570-80.
116. Micsenyi, A., et al., *Beta-catenin is temporally regulated during normal liver development.* Gastroenterology, 2004. **126**(4): p. 1134-46.
117. Dangi, A., et al., *Selective expansion of allogeneic regulatory T cells by hepatic stellate cells: role of endotoxin and implications for allograft tolerance.* Journal of immunology, 2012. **188**(8): p. 3667-77.
118. Thirunavukkarasu, C., S.C. Watkins, and C.R. Gandhi, *Mechanisms of endotoxin-induced NO, IL-6, and TNF-alpha production in activated rat hepatic stellate cells: role of p38 MAPK.* Hepatology, 2006. **44**(2): p. 389-98.
119. Behari, J., *The Wnt/beta-catenin signaling pathway in liver biology and disease.* Expert review of gastroenterology & hepatology, 2010. **4**(6): p. 745-56.
120. Yang, J., et al., *Beta-catenin signaling in murine liver zonation and regeneration: A Wnt-Wnt situation!* Hepatology, 2014.
121. Raucy, J.L., et al., *Acetaminophen activation by human liver cytochromes P450IIE1 and P450IA2.* Archives of biochemistry and biophysics, 1989. **271**(2): p. 270-83.
122. Patten, C.J., et al., *Cytochrome P450 enzymes involved in acetaminophen activation by rat and human liver microsomes and their kinetics.* Chemical research in toxicology, 1993. **6**(4): p. 511-8.
123. Albrecht, J.H., et al., *Regulation of G(1) cyclin-dependent kinases in the liver: role of nuclear localization and p27 sequestration.* Am J Physiol, 1999. **277**(6 Pt 1): p. G1207-16.
124. Das, S., et al., *Wntless in Wnt secretion: molecular, cellular and genetic aspects.* Frontiers in biology, 2012. **7**(6): p. 587-593.
125. Ober, E.A., et al., *Mesodermal Wnt2b signalling positively regulates liver specification.* Nature, 2006. **442**(7103): p. 688-91.
126. Cheng, Q., et al., *Distinct mechanism of small-for-size fatty liver graft injury--Wnt4 signaling activates hepatic stellate cells.* American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons, 2010. **10**(5): p. 1178-88.
127. Kikuchi, A., et al., *Wnt5a: its signalling, functions and implication in diseases.* Acta physiologica, 2012. **204**(1): p. 17-33.
128. Klein, D., et al., *Wnt2 acts as a cell type-specific, autocrine growth factor in rat hepatic sinusoidal endothelial cells cross-stimulating the VEGF pathway.* Hepatology, 2008. **47**(3): p. 1018-31.
129. Yamaguchi, T.P., et al., *A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo.* Development, 1999. **126**(6): p. 1211-1223.
130. Hernandez-Gea, V., et al., *Autophagy releases lipid that promotes fibrogenesis by activated hepatic stellate cells in mice and in human tissues.* Gastroenterology, 2012. **142**(4): p. 938-46.
131. Mochizuki, A., et al., *Hepatic stellate cells orchestrate clearance of necrotic cells in a hypoxia-inducible factor-1alpha-dependent manner by modulating macrophage phenotype in mice.* Journal of immunology, 2014. **192**(8): p. 3847-57.
132. Scholten, D., et al., *Genetic labeling does not detect epithelial-to-mesenchymal transition of cholangiocytes in liver fibrosis in mice.* Gastroenterology, 2010. **139**(3): p. 987-98.

133. Mederacke, I., et al., *Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology.* Nature communications, 2013. **4**: p. 2823.
134. Stenkamp-Strahm, C., et al., *High-fat diet and age-dependent effects on enteric glial cell populations of mouse small intestine.* Autonomic neuroscience : basic & clinical, 2013. **177**(2): p. 199-210.
135. Moore, S.A., et al., *Deletion of brain dystroglycan recapitulates aspects of congenital muscular dystrophy.* Nature, 2002. **418**(6896): p. 422-5.
136. Clausen, B.E., et al., *Conditional gene targeting in macrophages and granulocytes using LysMcre mice.* Transgenic research, 1999. **8**(4): p. 265-77.
137. Burke, Z.D. and D. Tosh, *The Wnt/beta-catenin pathway: master regulator of liver zonation?* BioEssays : news and reviews in molecular, cellular and developmental biology, 2006. **28**(11): p. 1072-7.
138. Koni, P.A., et al., *Conditional vascular cell adhesion molecule 1 deletion in mice: impaired lymphocyte migration to bone marrow.* The Journal of experimental medicine, 2001. **193**(6): p. 741-54.
139. Torre, C., C. Perret, and S. Colnot, *Transcription dynamics in a physiological process: beta-catenin signaling directs liver metabolic zonation.* The international journal of biochemistry & cell biology, 2011. **43**(2): p. 271-8.
140. Albrecht, J.H. and L.K. Hansen, *Cyclin D1 promotes mitogen-independent cell cycle progression in hepatocytes.* Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research, 1999. **10**(6): p. 397-404.
141. Apte, U., et al., *Beta-catenin activation promotes liver regeneration after acetaminophen-induced injury.* The American journal of pathology, 2009. **175**(3): p. 1056-65.
142. Fanti, M., et al., *Tri-iodothyronine induces hepatocyte proliferation by protein kinase a-dependent beta-catenin activation in rodents.* Hepatology, 2013.
143. Michalopoulos, G.K., *Principles of liver regeneration and growth homeostasis.* Comprehensive Physiology, 2013. **3**(1): p. 485-513.
144. Apte, U., et al., *Wnt/beta-catenin signaling mediates oval cell response in rodents.* Hepatology, 2008. **47**(1): p. 288-95.
145. Barrott, J.J., et al., *Deletion of mouse Porcn blocks Wnt ligand secretion and reveals an ectodermal etiology of human focal dermal hypoplasia/Goltz syndrome.* Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(31): p. 12752-7.
146. Brault, V., et al., *Inactivation of the beta-catenin gene by Wnt1-Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development.* Development, 2001. **128**(8): p. 1253-64.
147. Maschmeyer, P., M. Flach, and F. Winau, *Seven steps to stellate cells.* Journal of visualized experiments : JoVE, 2011(51).
148. Tennakoon, A.H., et al., *Characterization of glial fibrillary acidic protein (GFAP)-expressing hepatic stellate cells and myofibroblasts in thioacetamide (TAA)-induced rat liver injury.* Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie, 2013. **65**(7-8): p. 1159-71.
149. Lim, M.C.C., G. Maubach, and L. Zhuo, *Glial fibrillary acidic protein splice variants in hepatic stellate cells - Expression and regulation.* Molecules and Cells, 2008. **25**(3): p. 376-384.

150. Carotti, S., et al., *Glial fibrillary acidic protein as an early marker of hepatic stellate cell activation in chronic and posttransplant recurrent hepatitis C.* Liver Transplantation, 2008. **14**(6): p. 806-814.
151. Ebrahimkhani, M.R., A.M. Elsharkawy, and D.A. Mann, *Wound healing and local neuroendocrine regulation in the injured liver.* Expert reviews in molecular medicine, 2008. **10**: p. e11.
152. Bona, S., et al., *Effect of antioxidant treatment on fibrogenesis in rats with carbon tetrachloride-induced cirrhosis.* ISRN gastroenterology, 2012. **2012**: p. 762920.
153. Nusse, R., *Wnt signaling.* Cold Spring Harbor perspectives in biology, 2012. **4**(5).
154. Cheng, J.H., et al., *Wnt antagonism inhibits hepatic stellate cell activation and liver fibrosis.* American journal of physiology. Gastrointestinal and liver physiology, 2008. **294**(1): p. G39-49.
155. Yin, X., et al., *R-Spondin2 Activates Hepatic Stellate Cells and Promotes Liver Fibrosis.* Digestive diseases and sciences, 2014.
156. Li, W., et al., *Pokeweed antiviral protein down-regulates Wnt/beta-catenin signalling to attenuate liver fibrogenesis in vitro and in vivo.* Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver, 2011. **43**(7): p. 559-66.
157. Amemiya, H., H. Kono, and H. Fujii, *Liver Regeneration is Impaired in Macrophage Colony Stimulating Factor Deficient Mice After Partial Hepatectomy: The Role of M-CSF-Induced Macrophages.* Journal of Surgical Research, 2011. **165**(1): p. 59-67.
158. Meijer, C., et al., *Kupffer cell depletion by CI2MDP-liposomes alters hepatic cytokine expression and delays liver regeneration after partial hepatectomy.* Liver, 2000. **20**(1): p. 66-77.
159. Takeishi, T., et al., *The role of Kupffer cells in liver regeneration.* Archives of histology and cytology, 1999. **62**(5): p. 413-22.
160. Cattelino, A., et al., *The conditional inactivation of the beta-catenin gene in endothelial cells causes a defective vascular pattern and increased vascular fragility.* The Journal of cell biology, 2003. **162**(6): p. 1111-22.
161. Dejana, E., *The role of wnt signaling in physiological and pathological angiogenesis.* Circulation research, 2010. **107**(8): p. 943-52.
162. Boon, L., et al., *High protein diet induces pericentral glutamate dehydrogenase and ornithine aminotransferase to provide sufficient glutamate for pericentral detoxification of ammonia in rat liver lobules.* Histochemistry and cell biology, 1999. **111**(6): p. 445-52.

APPENDIX A

CURRICULUM VITAE

Name: Jing Yang

Tel: 1-412-576-2643

Email: jiy36@pitt.edu
yangjing928@126.com

EDUCATION

◆2004. 9—2010. 8 MD, Peking University

◆2010. 8—Present PhD in progress, Cellular and Molecular Program,
Department of Pathology, University of Pittsburgh
Expected graduation: Sep 2014

ACADEMIC HONORS

◆Experimental Pathologist in Training Award, American Society of Investigative Pathology, 2014

◆Abraham D. Sobel Award, American Society of Investigative Pathology, 2013

◆Excellent Student Award, Peking University, 2008, 2009

◆Scholarship for outstanding medical students, Peking University, 2007, 2008

PEER REVIEWED PUBLICATION

1. **Yang J**, Mowry LE, Nejak-Bowen KN, Okabe H, et al. Beta-catenin signaling in murine liver zonation and regeneration: A Wnt-Wnt situation! *Hepatology*. 2014.
2. **Yang J**, Okabe H, Monga SP. Liver Development, Regeneration, and Stem Cells. Accepted for publication by *Pathobiology of Human Disease*. 2014.
3. Delgado ER, **Yang J**, et al. Identification and Characterization of a Novel Small-Molecule Inhibitor of beta-Catenin Signaling. *Am J Pathol*. 2014.
4. Okabe H, Delgado E, Lee JM, **Yang J**, et al. Role of leukocyte cell-derived chemotaxin 2 as a biomarker in hepatocellular carcinoma. *PLoS One.* 2014;9(6):e98817.
5. Lee JM, **Yang J,** et al. Beta-Catenin signaling in hepatocellular cancer: Implications in inflammation, fibrosis, and proliferation. *Cancer Lett*. 2014;343(1):90-7.
6. Delgado E, Bahal R, **Yang J**, et al. β-Catenin Knockdown in Liver Tumor Cells by a Cell Permeable Gamma Guanidine-based Peptide Nucleic Acid. *Curr Cancer Drug Targets.* Oct 13, 2013 (8):867-78.

PUBLISHED ABSTRACTS

1. **Yang J**, et al. Wnt/ β-Catenin signaling in liver regeneration. *FASEB J* April 2014 28:398.5
2. **Yang J**, et al. Wnt signaling in liver homeostasis and regeneration. *FASEB J* April 9, 2013 27:257.1
3. **Yang J**, et al. Beta-Catenin suppression induces CYP2b10 expression by activating CAR activity. *FASEB J* March 29, 2012 26:145.2

PROFESSIONAL EXPERIENCE

University of Pittsburgh Department of Pathology

Graduate Student July.2012-present

Supervisor: Dr. Paul Monga

β-catenin plays crucial role in liver homeostasis and regeneration. Wnt-signaling is the major pathway that activates β-catenin, while there are Wnt-independent pathways that can also activate β-catenin. The goal of the project is to address to what extent β-catenin activation is Wnt-dependent in both liver homeostasis and during liver regeneration, and elucidate where do the Wnts come from.

◆Generated LRP5/6 double KO where Wnt-signaling is abolished, while Wnt-independent signaling is intact and can activate β-catenin, and compare the phenotype with β-catenin KO where β-catenin signaling is totally abolished.

◆LRP5/6 KO phenocopied β-catenin KO in defective hepatic zonation and delay in liver regeneration (LR), therefore addressed that Wnt-signaling is the major pathway that activates β-catenin in both liver homeostasis and regeneration.

◆Investigated the source of Wnts in liver by generating different cell population specific Wls KO that lack the ability of a specific population to secrete Wnts.

◆Address a potential role of endothelial cells derived Wnt ligands in regulating basal pericentral β-catenin activation in liver, and rule out the role of hepatocytes, cholangiocytes, stellate cells and Kupffer cells as the source of Wnt proteins that regulate zonation.

◆Address Kupffer cells and endothelial cells are required for timely β-catenin activation in hepatocytes to initiate LR after PH, while hepatocytes and hepatic stellate cells derived Wnt ligands are not required in this process.

University of Pittsburgh Department of Pathology

Graduate Student July.2011-July.2012

Supervisor: Dr. Paul Monga

Work on the project of identification of biomarkers indicating β-catenin mutation in hepatocellular carcinomas. It has been found that in 30% of HCC patients, β-catenin is mutated. Biomarkers indicating β-catenin mutation have strong clinical implications for this subset of HCC patients.

◆Generated stable cell lines transfected with constitutively activated β-catenin mutation constructs.

◆Addressed that cell lines transfected with β-catenin mutants have distinct downstream targets.