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Pericrine regulation of physiological and pathological liver functions via hepatic stellate cell-derived RSPO3

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Table of contents

Acknowledgments.....	i
Table of contents	iii
List of Figures	vi
List of Tables	vii
Zusammenfassung	1
Summary	3
Contributions	5
1 Introduction	6
1.1 Anatomy and functions of the liver	6
1.2 Different cell types of the liver	7
1.2.1 Hepatocytes.....	8
1.2.2 Liver sinusoidal endothelial cells	9
1.2.3 Kupffer cells	10
1.2.4 Cholangiocytes	11
1.2.5 Hepatic stellate cells.....	12
1.2.6 Other cell types.....	14
1.3 Zonation of the liver	16
1.3.1 Hepatocyte zonation.....	16
1.3.2 LSEC zonation	17
1.3.3 KC zonation	17
1.3.4 HSC zonation	18
1.4 Liver regeneration and partial hepatectomy.....	18
1.5 Liver diseases	19
1.5.1 Viral hepatitis	19
1.5.2 Alcohol-associated liver disease (ALD)	21
1.5.3 Non-alcoholic fatty liver disease (NAFLD)	21
1.5.4 Liver fibrosis	22
1.5.5 Hepatocellular carcinoma (HCC)	22
1.5.6 Other liver diseases	23
1.6 Liver damage models.....	23
1.6.1 Chemically-induced models	24
1.6.2 Dietary models	26

Table of contents

1.6.3	Bile duct ligation	28
1.6.4	Immuno-induced models.....	28
1.6.5	Transgenic models.....	29
1.7	Wnt signaling and R-spondin 3 (Rspo3)	30
1.7.1	Wnt signaling.....	31
1.7.2	Rspo3	34
2	Aim of the study.....	35
3	Results.....	36
3.1	Characterization of Rspo3 expression in the liver	36
3.1.1	Enriching major hepatic cell populations from the mouse liver	36
3.1.2	HSC are major sources of Rspo3 in the liver	38
3.2	HSC-expressed Rspo3 exerts distinct functions in shaping liver homeostasis	38
3.2.1	Generation and characterization of conditional HSC-specific Rspo3 deficient mice.....	38
3.2.2	LSEC- and HSC-expressed Rspo3 do not compensate each other upon deletion and have no impact on the expression of LSEC-specific Wnt ligands in the liver	40
3.2.3	HSC-specific Rspo3 knockout mice show normal liver histology.....	42
3.2.4	HSC-specific Rspo3 knockout mice shows different liver zonation pattern compared to LSEC-specific Rspo3 knockout mice	42
3.2.5	HSC-specific Rspo3 deletion inhibits liver regeneration after partial hepatectomy.....	45
3.3	HSC-expressed Rspo3 is a key regulator in the pathogenesis of liver diseases	46
3.3.1	HSC-specific Rspo3 deletion protects the liver from CCl ₄ -induced liver fibrosis	46
3.3.2	Ablation of HSC-derived Rspo3 prevents liver cirrhosis and neoplastic transformation	50
3.3.3	HSC-expressed Rspo3 is dispensable in cholestasis-induced liver damage	51
3.3.4	Loss of HSC-specific Rspo3 alleviates APAP-induced acute liver injury	53
3.3.5	Deletion of Rspo3 in HSC primes aHSC to a pro-apoptotic/anti-proliferative status	56
3.3.6	HSC-derived Rspo3 exerts a pro-fibrotic phenotype independent of HSC Wnt secretion.....	56
3.3.7	HSC-specific Rspo3 deletion inhibits apoptotic signaling in hepatocytes	58
4	Discussion	59
4.1	Rspo3 from two cell types exerts different functions: angiocrine vs. pericrine	59
4.2	Rspo3 expressed HSC are key players in the pathogenesis of liver disease	61
4.3	Physiological functions of HSC-derived Rspo3: Is Rspo3 from HSC good or bad?	61
4.4	HSC-specific Rspo3 signaling cascade: Wnt-dependent or independent?	63

4.5	Closing remarks	64
5	Materials and Methods	65
5.1	Materials	65
5.1.1	Chemicals.....	65
5.1.2	Enzymes, growth factors, and proteins	65
5.1.3	Cells	65
5.1.4	Reagents and tools	66
5.1.5	Kits.....	67
5.1.6	Primers.....	68
5.1.7	TaqMan™ assays.....	68
5.1.8	Antibodies.....	68
5.1.9	Consumables	69
5.1.10	Devices and equipment	70
5.1.11	Software	71
5.1.12	Solutions and buffers	71
5.2	Methods	73
5.2.1	Animal studies	73
5.2.2	<i>In vivo</i> treatments	76
5.2.3	Sample processing	76
5.2.4	Flow cytometry and cell sorting	78
5.2.5	Staining and analysis.....	78
5.2.6	Cell culture	80
5.2.7	Molecular biology.....	82
5.2.8	Protein biochemistry	83
5.2.9	Bulk RNA-sequencing and data processing	85
5.2.10	Statistical analysis	85
6	Abbreviations	86
7	Publications.....	90
8	References.....	91

List of Figures

Figure 1. Anatomy of the liver lobule.....	7
Figure 2. Different cell types of the liver.	7
Figure 3. Wnt Signaling pathway.....	32
Figure 4. Scheme of isolating different liver cell types.	36
Figure 5. Purity of freshly isolated primary mouse HSC.	37
Figure 6. <i>Rspo3</i> expression in different liver cell types.	38
Figure 7. Generation of HSC-specific <i>Rspo3</i> inducible knockout mice.	39
Figure 8. Deletion of HSC-specific <i>Rspo3</i> does not affect <i>Rspo3</i> -dependent angiocrine regulation and vice versa.....	41
Figure 9. Characterization of <i>Rspo3</i> ^{ind-HSC} mouse livers.	43
Figure 10. Characterization of zone 2 and zone 3 liver zonation of <i>Rspo3</i> ^{ind-HSC} mice.	44
Figure 11. Characterization of zone 1 liver zonation of <i>Rspo3</i> ^{ind-HSC} mice.	45
Figure 12. HSC-specific <i>Rspo3</i> deletion inhibits hepatocyte proliferation and liver regeneration after PHx.....	46
Figure 13. HSC-specific <i>Rspo3</i> deletion protects the liver from CCl ₄ -induced acute liver injury.	48
Figure 14. HSC-specific <i>Rspo3</i> deletion protects the liver from CCl ₄ -induced chronic injury	49
Figure 15. Recovery of the liver from long-term CCl ₄ administration.	50
Figure 16. HSC-expressed <i>Rspo3</i> regulates neoplastic transformation in the liver.	51
Figure 17. Evaluation of C57BL/6N mice subjected to bile duct ligation.	52
Figure 18. HSC-derived <i>Rspo3</i> is dispensable in cholestasis-induced liver damage.....	53
Figure 19. Deletion of <i>Rspo3</i> from HSC alleviates APAP-induced liver injury.	54
Figure 20. RNA-seq analysis identified DEGs in HSC and mechanisms of the protective phenotype by HSC-specific <i>Rspo3</i> deletion.	54
Figure 21. Deletion of HSC-derived <i>Evi/Wls</i> does not protect the liver from CCl ₄ -induced liver injury.....	57
Figure 22. LSEC-specific <i>Rspo3</i> deletion does not protect the liver from CCl ₄ -induced liver injury.....	57
Figure 23. RNA-seq analysis identified DEGs in hepatocytes and mechanisms of the protective phenotype by HSC-specific <i>Rspo3</i> deletion.	58
Figure 24. Model of pericrine and angiocrine <i>Rspo3</i> regulation of liver zonation.....	60

List of Tables

Table 1: Chemical suppliers	65
Table 2: Enzymes, growth factors, and proteins.....	65
Table 3: Murine cell lines	65
Table 4: Cell culture reagents.....	66
Table 5: PCR reagents (RT, PCR, qPCR)	66
Table 6: Staining reagents.....	66
Table 7: Western blot reagents.....	67
Table 8: Reagents and tools for animal experimentation.....	67
Table 9: Kits	67
Table 10: Primers.....	68
Table 11: Murine TaqMan probes.....	68
Table 12: Primary antibodies	68
Table 13: Secondary antibodies.....	69
Table 14: Consumables	69
Table 15: Devices and equipment.....	70
Table 16: Software.....	71
Table 17: Solutions and buffers	71
Table 18: VE-cad Cre-ERT2 genotyping PCR.....	73
Table 19: iPDGFRb-P2A-CreERT2 genotyping PCR	74
Table 20: Rspo3 floxed genotyping PCR.....	74
Table 21: Gpr177 (Evi/Wls) floxed genotyping PCR.....	75
Table 22: PCR for mycoplasma detection.....	81
Table 23: qPCR reaction setup	83

Zusammenfassung

Die Leber übt zahlreiche wichtige Funktionen in unserem Körper aus, wie Verdauung, Entgiftung, Biosynthese und Speicherung verschiedener Nährstoffe. Diese Funktionen werden über ein komplexes Zusammenspiel verschiedener Zelltypen innerhalb der Leber ausgeführt, darunter Hepatozyten, die parenchymatösen Zellen der Leber, lebersinusoidale Endothelzellen (LSEC), Kupffer-Zellen (KC) und hepatische Sternzellen (HSC). Angesichts der vielfältigen Aufgaben der Leber sind die Leberfunktionen räumlich entlang des Leberläppchens verteilt, um die Stoffwechselprozesse effizient zu koordinieren und durchzuführen. Diese räumliche Aufteilung der Mikroumgebung in der Leber wird als „Leberzonierung“ bezeichnet. Die Zonierung der Leber wird durch verschiedene Faktoren der Mikroumgebung aufrechterhalten. Eine gestörte Zonierung der Leber führt zu pathologischen Veränderungen und verursacht Leberkrankheiten. Frühere Studien haben LSEC-abhängige Wnt-Signale und Rspo3 (ein Verstärker der Wnt-Signale) als wichtigste Faktoren für die Aufrechterhaltung der metabolischen Leberzonierung identifiziert. Der Verlust von Wnt-Liganden aus LSEC oder Rspo3 stört die Zonierung der Glutaminsynthetase (GS), eines wichtigen Enzyms, das den Leberstoffwechsel stabil hält. Obwohl LSEC die Hauptquelle für Wnt-Liganden in der Leber sind, produzieren auch andere nicht-parenchymale Zellen der Leber Wnt-Liganden, die die metabolische Architektur und Funktion der Leber regulieren können. In einer kürzlich durchgeführten Studie wurde eine Zonen-spezifische Expression von Rspo3 in HSC nachgewiesen. Dies deutet darauf hin, dass die aus den HSC stammende Rspo3/Wnt-Signalgebung eine spezifische Rolle bei der zonenspezifischen Regulierung der Leberfunktionen spielen könnte. Trotz dieser korrelativen Befunde wurden die Funktionen der perikrinen Wnt-Signalgebung in der Leber noch nicht systematisch untersucht.

Das Ziel dieser Arbeit war es, die Rolle der HSC-induzierten Wnt-Signalgebung bei der Regulierung der Leberfunktionen zu untersuchen. Zu diesem Zweck habe ich HSC-spezifische Rspo3- und Wnt-Transporter Evi/Wls (Wnt-Ligand-Sekretionsfaktor) Knockout-Mäuse erzeugt. Das Fehlen von HSC-produziertem Rspo3 führte nicht zu einer veränderten GS-Zonierung, wie dies bei LSEC-spezifischen Rspo3-Knockout-Mäusen gezeigt wurde. Stattdessen wurde eine veränderte Zonierung von Cytochrom P450, Enzymen, die am Stoffwechsel toxischer Produkte beteiligt sind, festgestellt. Darüber hinaus führte der Verlust von Rspo3 in den HSC zu einer beeinträchtigten Leberregeneration in einem 2/3-Teilhepatektomie-Modell. Pathologisch gesehen schützte die HSC-spezifische Deletion von Rspo3 die Leber vor CCl₄- und APAP-induzierten, aber nicht vor cholestatischen Leberschäden, die durch die Ligatur der Gallengänge verursacht wurden. Im Gegensatz dazu wurde dieser schützende Phänotyp bei LSEC-spezifischen Rspo3- oder HSC-spezifischen Evi/Wls-Knockout-Mäusen nicht beobachtet, was auf eine HSC- und Rspo3-Spezifität bei dieser Regulierung hinweist. Um die

zugrundeliegenden Mechanismen des schützenden Phänotyps bei der HSC-spezifischen Deletion von Rspo3 weiter aufzuklären, habe ich RNA-Sequenzierungen an HSC und Hepatozyten durchgeführt, die aus CCl₄-behandelten Mäusen isoliert wurden. Genexpressionsanalysen ergaben, dass die HSC-spezifische Deletion von Rspo3 aktivierte HSC auf eine pro-apoptotische/antiproliferative Signatur vorbereitete und apoptotische Signalwege in Hepatozyten hemmte.

Insgesamt bietet diese Studie eine umfassende Analyse von Rspo3, welches aus HSC stammt, bei der Regulierung sowohl der physiologischen als auch der pathologischen Leberfunktionen. Darüber hinaus zeigen die Daten, dass die Quelle und der Ort der Wnt-Signalagonisten die Leberfunktionen deutlich beeinflussen können. Außerdem gehen diese Ergebnisse über die pro-fibrotische Funktion der HSC hinaus und decken ihre Rolle bei der Aufrechterhaltung der homöostatischen Lebergesundheit auf.

Summary

The liver exerts multiple essential functions of our body, such as digestion, detoxification, biosynthesis, and storage of various nutrients. These functions are executed via a complex crosstalk among different cell types within the liver, including hepatocytes, the parenchymal cells of the liver, liver sinusoidal endothelial cells (LSEC), Kupffer cells (KC), and hepatic stellate cells (HSC). Given the diverse tasks performed by the liver, liver functions are spatially distributed along the lobule for efficiently coordinating and performing metabolic processes. This spatial division of labor in the liver microenvironment is termed “liver zonation”. Liver zonation is maintained via a complex regulation of various microenvironmental factors. Perturbed liver zonation drives pathological changes and causes liver diseases. Previous studies have identified LSEC-derived Wnt signaling and Rspo3 (a Wnt signaling enhancer) as primary environmental factors for maintaining liver metabolic zonation. Indeed, loss of LSEC-derived Wnt ligands or Rspo3 perturbs zonation of glutamine synthetase (GS), a critical enzyme that maintains liver metabolic stability. Although LSEC are the major source of Wnt ligands in the liver, other non-parenchymal cells of the liver also produce Wnt ligands that may, in turn, regulate metabolic architecture and function of the liver. Notably, a recent study revealed the zone-specific expression of Rspo3 in HSC. This suggests that HSC-derived Rspo3/Wnt signaling may play specific roles in regulating liver functions in a zone-specific manner. Despite these correlative findings, functions of HSC-derived Wnt signaling in the liver have not been systematically addressed.

This thesis aimed to study the role of HSC-derived Wnt signaling in regulating liver functions. To this end, I generated HSC-specific Rspo3 and Wnt transporter Evi/Wls (Wnt ligand secretion factor) knockout mice. Lack of Rspo3 from HSC did not lead to changes in GS zonation, as shown in LSEC-specific Rspo3 knockout mice. Instead, an altered zonation of cytochrome P450, enzymes that are involved in the metabolism of toxic products, was revealed. Furthermore, loss of Rspo3 in HSC led to impaired liver regeneration in a 2/3 partial hepatectomy model. Pathologically, HSC-specific Rspo3 deletion protected the liver from CCl₄- and APAP-induced, but not from cholestatic liver damage induced by bile duct ligation. In contrast, this protective phenotype was not seen in LSEC-specific Rspo3 or HSC-specific Evi/Wls knockout mice, indicating HSC and Rspo3 specificity in this regulation. To further elucidate underlying mechanisms of the protective phenotype upon HSC-specific Rspo3 deletion, I performed RNA-sequencing on HSC and hepatocytes isolated from CCl₄-treated mice. Gene expression analyses suggested that HSC-specific Rspo3 deletion primed activated HSC to a pro-apoptotic/anti-proliferative signature, and inhibited apoptotic pathways in hepatocytes.

Collectively, this study provides a comprehensive analysis of HSC-derived Rspo3 in regulating both physiological and pathological liver functions. Further, the data illustrate that the source and location of Wnt signaling agonists can distinctly affect liver functions. Moreover, these findings move beyond the pro-fibrotic function of HSC and uncover their role in sustaining homeostatic liver health.

Contributions

The overall study was conceptualized and designed under the guidance of my PhD supervisor Prof. Dr. Hellmut G. Augustin. All experiments outlined in this study were designed, performed, and analyzed by me. Moreover, all figures presented in this dissertation were generated by me.

I received technical support from Dr. Ki Hong Lee and Dr. Moritz Victor Jakab in cell sorting for RNA-sequencing experiments. I received assistance in sample preparation and data generation from the genomics and proteomics core facility at the DKFZ. The histological evaluation of fibrotic livers was performed by Prof. Dr. Carolin Mogler from TUM. For the analysis of sequencing data, I received technical assistance from Genevia Technologies (Tampere, Finland).

1 Introduction

1.1 Anatomy and functions of the liver

The liver is a unique metabolic organ of vertebrates. It is the largest internal organ that exerts various essential functions such as digestion, detoxification, biosynthesis of secreted proteins, regulation of blood coagulation, as well as the storage of nutrients. It is the only organ in humans with the capacity to fully regenerate to its original mass after resection. This capacity is based on compensatory growth, where the proliferation of all mature cell compartments is involved, rather than solely depending on progenitor/stem cells. As a result, the liver can regenerate to full mass, with the original shape getting lost during this process¹.

There are four different lobes in mice and humans, including the left lateral lobe, the right lateral lobe, the left medial lobe, and the right medial lobe². However, lobar architecture varies between the two species. The rodent liver is lobated, while humans have a non-lobated liver. Therefore, liver lobes can be ligated and removed easily in mice compared to humans. This characteristic makes partial hepatectomy in mice a powerful model for studying liver regeneration.

The basic functional unit of the liver is termed “lobule”. It is a hexagonal structure consisting of central veins located in the middle, with portal triads (portal vein, hepatic artery, and bile duct) sitting at each corner (**Figure 1**). These vessels together form a distinct vascular network with two inlets and one outlet, to maintain a special blood circulation³. The two inlet networks are the nutrition-rich portal vein (zone 1), which delivers around 75% of blood supply draining from the gastrointestinal tract, and the oxygen-rich hepatic artery, which delivers the remaining 25% of arterial blood⁴. Blood merges into the sinusoids, which distributes uniformly throughout the liver, and exits at inferior vena cava via the central vein (zone 3). Such gradient and segregation of liver functions along the lobule-radial axis is another distinct characteristic of the liver, termed “liver zonation”. As a third part of portal triads, bile ducts play crucial roles in the biliary tract. It is the path from the liver via which bile is secreted to the duodenum. These ducts receive blood from four arteries, including the cystic artery, the right hepatic artery, the pancreaticoduodenal artery, and the gastroduodenal artery, flowing from the proximal to the distal side⁵.

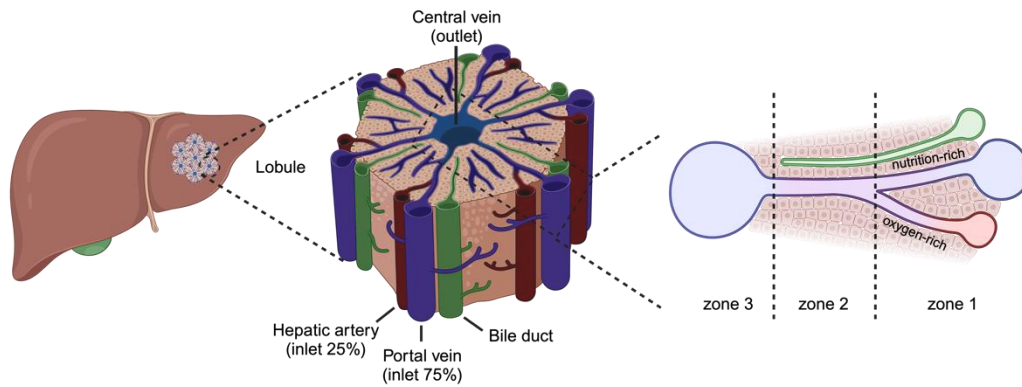


Figure 1. Anatomy of the liver lobule.

Liver lobule is the basic functional unit of the liver. It is a hexagonal column with central veins in the middle and portal triads on the side. Portal triads further consist of portal vein (blue), hepatic artery (red), and bile duct (green). Blood is delivered from portal side with 75% from portal vein and 25% from hepatic artery, merged at the sinusoids and exited via central veins.

1.2 Different cell types of the liver

The parenchymal cells of the liver are the hepatocytes. They are involved in most of the major functions of the liver, including metabolism, detoxification, and synthesis of various proteins. Non-parenchymal cells (NPCs) comprise liver sinusoidal endothelial cells (LSEC), hepatic stellate cells (HSC), Kupffer cells (KC), cholangiocytes, and other mesenchymal or liver-resident immune cells. The complex functions of the liver and its homeostasis are maintained by the coordination between all these parenchymal and NPCs via cell-cell interactions (**Figure 2**).

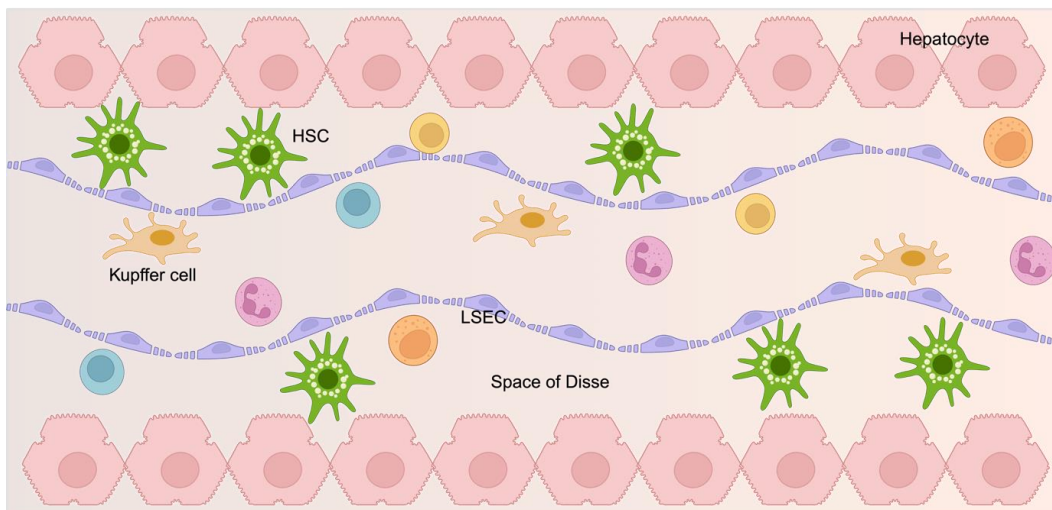


Figure 2. Different cell types of the liver.

The liver is composed of numerous types of cells, including hepatocytes as parenchymal and several different kinds of non-parenchymal cells (NPCs). NPCs mainly consist of LSEC, Kupffer cell, and HSC. LSEC are fenestrated endothelial cells in the liver that form blood vessels. Kupffer cells are tissue-resident macrophages in the liver, which are found in the lumen close to LSEC. HSC are pericyte-like cells in the liver and localized in Space of Disse. Furthermore, there are also diverse types of liver-resident immune cells, such as T-cells (yellow), B-cells (blue), NK cells (orange), neutrophils (purple), etc.

1.2.1 Hepatocytes

Hepatocytes are the parenchymal cells of the liver, which represent around 80% of total liver mass and 60% of total cell number⁶. They often have polyhedral shapes with a diameter of 20-30 micrometers. Different from most other cells, a typical and distinct feature of mammalian hepatocytes is polyploidy. However, the degree of polyploidization varies between species, with 70 to 80% in mice⁷ and only 30 to 40% in humans⁸. As dominant cell type of the liver, most functions of the liver are performed by hepatocytes. They are the manufacturing factory in the liver, which secrete proteins such as albumin⁹, fibrinogen¹⁰, and transferrin¹¹, as well as different complement factors¹².

Another major function of hepatocytes is metabolism. As the metabolic core, the liver is mainly responsible for lipid metabolism and detoxification of exogenous toxins. For this purpose, hepatocytes secrete cholesterol¹³, triglycerides¹⁴, and bile¹⁵. Upon carbohydrate intake, acetyl-CoA is converted to fatty acids via the glycolytic pathway in the cytoplasm. Next, glycerol, another product from this pathway, reacts with three fatty acids in the endoplasmic reticulum membrane and eventually converts to triglyceride, the final product of lipogenesis¹⁴. On the other hand, cholesterol is synthesized from acetate and is further oxidized by cytochrome P450 to be converted to primary bile acids via a multi-step process. Primary bile acids are then conjugated with glycine or taurine to form bile salts. Combined with water, bilirubin, fats, and inorganic salts, they become bile and support lipid digestion¹⁵.

Hepatocytes also play an essential role in the activation of innate immunity. Upon stimulation, hepatocytes secrete proteins that can either directly eliminate pathogens or act as antigen-presenting-like cells and trigger the immune system for further clearance. These proteins are called acute-phase proteins (APPs), whose expression changes strongly during infection by cytokines such as interleukins, interferon- γ , and tumor necrosis factor- α ¹⁶. The best characterized APPs are complements, C-reactive protein (CRP), serum amyloid A proteins (SAA), and serum amyloid P proteins (SAP)¹⁷. Another important defense mechanism is the secretion of pattern-recognition receptors (PRRs), including collectins, pentraxins, lipid transferases, and peptidoglycan-recognition proteins. They recognize pathogen-associated molecular patterns (PAMPs) that are expressed by pathogens and function via activation of complements or opsonization of phagocytosis¹⁸.

Given the remarkable capacity of hepatocytes for receptor-mediated endocytosis, they also serve as biological filter for portal blood¹⁹, the regulator of blood glucose and insulin²⁰, and gatekeeper of iron homeostasis of the body²¹. Taken together, hepatocytes exert several vital roles of the liver²², thereby serving as key regulators in maintaining liver homeostasis.

1.2.2 Liver sinusoidal endothelial cells

Liver sinusoidal endothelial cells (LSEC) are a specialized non-continuous endothelial cell (EC) population in the liver, acts as the barrier between the blood and hepatocytes. They represent around 15-20% of liver cells and approximately 3% of total liver mass²³. LSEC were first identified by transmission electron microscopy (TEM) in 1959²⁴ and were further described in detail in 1970²⁵. They do not have a basement membrane or diaphragm but form a thin layer throughout the smallest vessel in the liver, namely hepatic sinusoids. Most prominently, LSEC are characterized by pores, so-called “fenestrae”, making them one of the most distinct EC compared to EC in other organs. Working as sieves, LSEC facilitate free exchange of materials between blood and parenchyma. These materials, including proteins and plasma components, are absorbed by hepatocytes in the space between EC and hepatocytes called the “space of Disse”. Different from other vascular endothelial cells in the liver, cell junctions of LSEC are less organized. Notably, LSEC express lymphatic vessel endothelial hyaluronic acid receptor (Lyve1) and prospero homeobox protein 1 (Prox1), making them phenotypically and functionally similar to lymphatic endothelial cells²⁶.

LSEC serve as one of the most important scavengers in the body and play a key role in blood-borne waste clearance²⁷, due to their high endocytic capacity and large surface. This capacity is achieved by different scavenger and mannose receptors that are expressed by LSEC. These receptors are proteins that have broad binding capacity to ligands of various exogenous substances, including viruses and bacteria. They also modify endogenous sources such as oxidized lipoproteins or advanced glycation end-products. Based on their function and structure, scavenger receptors have been categorized into 11 classes by the National Institutes of Health in 2017²⁸. Among these receptors, LSEC are known to express SR-A, SR-B, SR-E, SR-H, FcγRIIb, and liver sinusoidal endothelial cell lectin (LSECtin)²⁹. On the other hand, the extraordinary scavenging capacity of LSEC is not always beneficial: With the diameter of the LSEC fenestrae going up to 200 nm, LSEC clear therapeutic drugs by clathrin-mediated endocytosis, making it challenging to develop pharmaceutical nanoparticle drugs²⁷.

LSEC also play an important role in regulating adaptive immune responses, beyond their regulatory function in innate immunity. However, rather than functioning as activators, LSEC prime an immunological tolerance environment of the liver. Due to constant exposure to low-dose inflammation from the gastrointestinal tract, immune tolerance is indispensable for maintaining a homeostatic liver microenvironment²⁹. The distinct immune tolerance of the liver is primarily achieved by the expression of mannose receptors on LSEC. CD44, a surface molecule expressed by activated T-cells, is known to be a LSEC-expressed ligand of LSECtin. Binding of LSECtin to CD44 inhibits T-cell proliferation and activation³⁰. Similarly, upregulation of LSEC-expressed programmed cell death 1 ligand 1 (PDL1) can

activate programmed cell death protein 1 (PD1) on naïve CD8⁺ T-cells and induce their tolerogenic response³¹. Such regulation highlights the antigen-presenting function of LSEC by cross-presenting soluble antigens on major histocompatibility complex (MHC) I molecule to T-cells. However, with high antigen concentrations during virus infections, LSEC directly contribute to pathogen immunity by inducing the activation of naïve T-cells to effector T-cells³⁰. The antigen presenting function of LSEC is also exerted by their low expression of MHC II, which promotes the differentiation of T-cells with regulatory potential, rather than into CD4⁺ helper T-cells^{32,33}. However, it has been shown that LSEC have limited effect on naïve T-cell proliferation and activation without exogenous co-stimulation³². Further investigations seem necessary for a better understanding of LSEC-regulated adaptive immunity.

LSEC also play a critical role during disease progression in the liver. A typical dysregulation of LSEC is capillarization, in which LSEC lose their fenestrations and start forming basement membrane. This often happens during liver fibrosis, followed by an upregulation of CD31 and VCAM1³⁴. During fibrosis, LSEC undergo a transcriptomic reprogramming process termed endothelial-to-mesenchymal transition (EndMT). During EndMT, LSEC lose their endothelial signature and gain mesenchymal morphology and functions. However, functional effect of EndMT on the development of liver fibrosis remains elusive. LSEC are also involved in viral infection³⁵, acute liver injury^{36,37}, as well as chronic liver diseases (CLD) such as nonalcoholic steatohepatitis (NASH)³⁸, liver cancer³⁹, and liver metastasis⁴⁰. Aging also impacts LSEC (change in cell thickness, loss of cell porosity, change in markers expression, etc.)^{41,42}, and may thereby lead to pathological changes.

1.2.3 Kupffer cells

Kupffer cells (KC) are liver-resident macrophages and represent around 30% of NPCs and 80-90% of tissue macrophages in the body. They have an amoeboid shape and stay in close proximity to LSEC⁴³. KC were discovered by electron microscopy from rat livers and were named after Karl Wilhelm von Kupffer. They were incorrectly defined as the integral parts of the endothelium⁴⁴. This was corrected 22 years later and re-identified as macrophages^{45,46}.

As the macrophage population of the liver, the primary function of KC is host defense. Similar to LSEC, Lectin or complements expressed by KC interacts with carbohydrate residues from the surface of bacteria. Thereafter, neutrophils adhere to KC and initiate the elimination of pathogens⁴³. This process is achieved by either phagocytosis (ingestion of solid particles) or pinocytosis (ingestion of liquid droplets), depending on the size of the particle. KC, LSEC, and dendritic cells consist of the reticuloendothelial system to clear PAMPs, as well as toxic products in the liver. Moreover, KC inhibit the activation of T-cells via prostaglandin E, 15-deoxy- Δ 12, and 14-PGJ2⁴⁷. KC also secrete IL-10 and transforming growth factor beta (TGF- β), to suppress T-cells^{48,49}.

With their enormous clearance capacity, KC are also responsible for oxidative degradation of heme released from senescent erythrocytes. Another critical role of KC is the regulation of hepatocyte metabolism. KC express peroxisome proliferator activated receptor (PPAR) δ and γ , which regulate the activation of M2-like KC. Loss of PPAR- δ leads to altered expression of oxidative phosphorylation (OXPHOS) and β -oxidation in the liver. This further influences the metabolism of hepatocytes and reduces the rate of fatty acid oxidation, leading to increased liver triglycerides and hepatic steatosis⁵⁰.

Apart from maintaining liver homeostasis, KC also play a critical role during pathological conditions. Activation of KC is the key determinant of peroxynitrite formation. This further leads to acetaminophen (APAP) toxicity⁵¹ and excessive formation of nitric oxide (NO). On the other hand, KC-derived NO can reduce ischemia-reperfusion injury as an endogenous defense mechanism due to its vasodilatory effect⁵². Furthermore, in alcoholic liver disease, KC are activated and secrete inflammatory chemokines and cytokines, including tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), etc⁴³. In non-alcoholic fatty liver disease (NAFLD), KC act as the key mediator of the initiation of different metabolic syndromes and insulin resistance (IR), which are hallmark characteristics of NAFLD transition⁴³. Little is known about the interaction between KC and hepatitis B virus (HBV)/hepatitis C virus (HCV), as they only infect hepatocytes⁵³. Nevertheless, it is suggested that KC induce apoptosis of hepatocytes in a fas- or CD95-dependent manner⁵⁴. Activated KC secrete cytokines that promote nuclear factor kappa B (NF- κ B) to inhibit HBV replication in hepatocytes and IL-6 that inhibits the replication of HCV⁵³.

KC also play critical roles during hepatocellular carcinoma (HCC) and liver metastasis. They have the ability to phagocytose circulating tumor cells in the liver^{53,54}. Activated KC also secrete NO, TNF- α , and prostaglandin E2 to defend tumor cells⁵⁴. Another indirect defense mechanism against tumors is the induction of natural killer cell (NK-cell) cytotoxicity by KC-secreted IL-12⁵⁴. Due to their anatomic location, KC are the first cell type to contact disseminated tumor cells. Depletion of KC prior to tumor challenge shows a protective feature of KC against hepatic metastasis⁵⁵. On the other hand, KC-derived hepatocyte growth factor (HGF) promotes the proliferation of tumor cells⁵⁶. Therefore, KC exert adverse functions against tumor in a context-dependent manner, where they inhibit tumor growth at an early stage and exhibit pro-tumor characteristics during the late stage⁵⁷.

1.2.4 Cholangiocytes

Cholangiocytes are epithelial cells that line up intra- and extrahepatic bile ducts. They represent around 3 to 5% of total liver mass⁵⁸. Their most important functions are formation and transportation of bile from the liver to small intestine. It is believed that cholangiocytes and hepatocytes share a common precursor, hepatoblast, which expresses both hepatocyte (HNF4 α) and cholangiocyte (CK19) markers⁵⁹. In case of liver damage, cholangiocytes can transdifferentiate into hepatocytes and vice

versa through an intermediate progenitor stage, enabling the regeneration of damaged area. There are two types of cholangiocytes along the biliary tree: small cholangiocytes and large cholangiocytes. The former are cuboidal, have proliferation potential, and show functional plasticity during disease, while the latter regulate bile secretion⁵⁸ and have columnar shape⁶⁰. Another difference between small and large cholangiocytes is that the latter have more organelles and a smaller nucleus-to-cytoplasm ratio⁶¹, indicating a higher differentiated status of large cholangiocytes compared to small cholangiocytes. Moreover, large cholangiocytes possess cilia that function as mechano- and chemo-sensor of bile duct lumen⁶².

Cholangiocytes contribute to around 40% of bile output⁵⁸. It is a complicated process regulated by gastrointestinal hormones that involves secretion and re-absorption of water, ions, and various solutes. The secretin-dependent cascade is one of the most studied pathways that regulates bile secretion. After binding of secretin to secretin receptors (SR) on the basolateral membrane, cyclic adenosine 3', 5'- monophosphate (cAMP) and the phosphorylation of cystic fibrosis transmembrane conductance regulator (CFTR) is induced. As a result, a chloride gradient is formed across the plasma membrane. This further activates apical Cl/HCO₃ anion exchanger 2 (AE2), initiates the secretion of bicarbonate into bile⁶³, and contributes to its alkalinity. At normal conditions, SR is exclusively expressed by larger cholangiocytes. In small cholangiocytes, Ca²⁺-dependent Cl⁻ secretion is more predominant and provides an alternative way for ductal fluid secretion via purinergic receptor activation⁶⁴. When large cholangiocytes are damaged, small cholangiocytes can also express SR *de novo* to regain large cholangiocytes characteristics⁶⁵.

Due to the anatomic location of cholangiocytes, they are constantly exposed to toxic agents and microbes. Therefore, another pivotal role of cholangiocytes is the detoxification of xenobiotics. Cholangiocytes may act as antigen-presenting cell (APC) and communicate with T-cells and lymphocytes. Recognition of PAMPs results in secretion of various cytokines and antimicrobial peptides, thereby involving cholangiocytes in both innate and adaptive immune responses⁶⁶.

1.2.5 Hepatic stellate cells

Hepatic stellate cells (HSC) are pericyte-like cell population in the liver. The percentage of quiescent HSC in total liver cell number is elusive and varies from 5- 15%⁶⁷⁻⁶⁹. They have a spindle-like shape and possess very long cell processes. HSC are the largest vitamin A reservoirs in the body⁷⁰. The history of HSC draws back to 1876, when Carl von Kupffer, the discoverer of KC, first described them to his colleague⁴⁴. Twenty-two years after his discovery, von Kupffer considered HSC as phagocytes and special EC of sinusoids. Thereafter, for almost 100 years, the real identity of HSC remained elusive. In 1971, Kenjiro Wake applied both gold chloride staining and silver impregnation and revealed that

“Sternzellen” described by von Kupffer and “lipid-laden fat-storing cells” are identical. They are different from Kupffer cells and sinusoidal endothelial cells⁷¹. HSC had more than 20 names during this long and tortuous history. In 1995, HSC were finally termed “hepatic stellate cells” by the international community⁷².

In contrast to their long history, the functions of HSC are unambiguous. As reservoir of vitamin A, HSC contain around 80% of total vitamin A of the body at quiescent stage⁷⁰. Upon stimulation, quiescent HSC (qHSC) are activated. They lose lipid droplets in the cytoplasm, transdifferentiate to myofibroblasts, and secrete extracellular matrix (ECM). These make them the key driver of liver fibrosis. As pericyte-like population of the liver, HSC exert mural cell features and have close contact with neighboring cells via their long cell processes. A single stellate cell may bridge 2 to 3 EC and 20 to 40 hepatocytes. Such cell group is called the “stellon” and represents the typical functional unit of HSC⁷³. By direct cell-cell contacts or secretion of soluble factors, HSC facilitate their intercellular transport and paracrine regulation, maintaining a homeostatic environment in the perivascular niche. Besides, qHSC also work as antigen-presenting cells in the liver and regulate the proliferation of NK cells⁷⁴.

HSC also play pivotal roles during liver regeneration. Liver regeneration is a complex multi-step process, which requires the intricate regulation of the initiation and the termination of re-growth. HSC constitutively secrete the multifunctional cytokine HGF, which is stored in an inactive form within the ECM. It enhances protein synthesis and promotes hepatic regeneration. Once needed, it is released in the active form and binds to cMet receptors on hepatocytes, EC, and biliary cells⁷⁵. On the other hand, HSC are also major source of TGF- β , which inhibits cell proliferation. Moreover, HSC may give rise to hepatocytes via mesenchymal to epithelial transition⁷⁶. Besides, HSC also secrete various cytokines, including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and angiopoietins. These factors enhance angiogenesis and promote neovascularization after damage⁷⁷. Therefore, HSC are one of the most important regulators during liver regeneration.

Besides their critical role in maintaining liver homeostasis and facilitating regeneration, HSC are also crucial mediators of cancer development, as around 80% of HCC develop on a fibrotic background⁷⁸. Moreover, activated hepatic stellate cells (aHSC) secrete soluble factors, including TGF- β , HGF, and VEGF, which directly promote tumor growth. On the other hand, HCC produces inflammatory chemokines and cytokines that in turn maintain HSC in an active stage⁷⁹. Such HSC-HCC interaction contributes to a pro-active tumor microenvironment, which promotes the survival and progression of HCC. Furthermore, HSC have immune-suppressive potential, where they induce apoptosis of active T-cells and expansion of regulatory T-cells, thereby resulting in a disturbed T-cell population⁸⁰. Therefore,

HSC have supportive functions in HCC development and progression, making them potential therapeutic targets against HCC.

1.2.6 Other cell types

Beyond above-mentioned cell types, there are also other types of cells in the liver, which only represent small subpopulations or only transiently exist during some specific stages.

1.2.6.1 Hepatic oval cells

Hepatic oval cells (HOC), the progenitor/stem cells within the liver, represent 1 to 3% of the total liver population⁸¹. They are approximately 10 μ m in size and have ovoid nuclei with a high nucleus-to-cytoplasmic ratio⁸². HOC were first discovered by in 1937 when a rat liver was stained by azo dye⁸³. They express both the hepatocyte marker albumin and the cholangiocyte marker Cytokeratin 19 (CK19). Apart from these two markers, OV-1 and OV-6 in rats and A6 in mice can be used as the golden standard for the detection of HOC. So far, none of existing markers represents all HOC, besides alpha-fetoprotein (AFP), a marker for immature hepatocytes or tumors⁸⁴. However, AFP is not expressed in mice⁸⁵. Upon liver injury, especially when the proliferation of hepatocytes is inhibited, HOC start to proliferate in peri-portal regions of the liver. HOC are located in the Canal of Hering, where hepatocytes connect to interlobular bile duct. However, the precise origin of them remains elusive⁸⁶.

1.2.6.2 Liver-associated fibroblasts

Fibroblasts have been first characterized as spindle-shaped cells that secrete collagen⁸⁷. They maintain tissue structure by secreting ECM and function differently depending on their origin and anatomic location⁸⁸. Among different types of fibroblasts, myofibroblasts are the best-studied and important cell type. Myofibroblasts are absent in normal adult liver. Upon injury, myofibroblasts emerge and are transiently activated. They form scars for tissue repair and are inactivated or cleared by apoptosis⁸⁹. Following continuous liver injury, aHSC transdifferentiate into myofibroblasts, secreting ECM, leading to liver fibrosis. However, aHSC are not the only origin of myofibroblasts in the liver. In liver fibrosis of different etiology, portal fibroblasts (PF) may also be a source of myofibroblasts.

PF are a quiescent fibroblast population located at the portal tract, underneath cholangiocytes⁹⁰. They were first described in 1961 when bile duct ligation (BDL) was performed in rats⁹¹. Due to a lack of specific markers, it remains challenging to distinguish PF from other mesenchymal cells in the liver. Thymocyte differentiation antigen 1 (Thy-1), an adhesion molecule, is absent in HSC and is expressed by PF⁹². However, its expression is not restricted to PF but also found in hematopoietic cells, neurons, EC, thymocytes, as well as many other cell types⁹³. Even in the liver, the expression of Thy-1 is not specific for PF, as HOC may also express Thy-1⁹⁴. Both HSC and PF give rise to ECM-secreting

myofibroblasts during liver fibrosis. However, PF only contributes in the early stage of cholestatic liver injury⁹⁵.

1.2.6.3 Liver resident and circulating immune cells

Due to its anatomic location, the liver constantly exposed to various stimulations, including toxic products and microorganisms. Liver resident immune cells are therefore pivotal to maintain a homeostatic hepatic environment and efficient immunological functions. Liver resident immune cells majorly consist of three types: lymphocytes, phagocytes, and granulocytes.

Lymphocytes in the liver include pit cells (liver-specific natural killer cells), natural killer T-cells, gamma delta T-cells ($\gamma\delta$ T cells), mucosal-associated invariant T-cells (MAIT cells), and B-cells⁹⁶. Pit cells were first discovered in 1976⁹⁷. They were named after their cytoplasmic granules. The functions of pit cells remained elusive until being identified as hepatic NK cells in 1983⁹⁸. They are found in close proximity to KC and LSEC⁹⁹. The cytotoxic activity of pit cells is highly dependent on KC and proceeds by the conjugation of pit cells to target cells, followed by the penetration of target cell cytoplasm and degradation regulated by integrins¹⁰⁰. Natural killer T-cells have a similar morphology as pit cells, with smaller and less abundant granules and lack of rod-cored vesicles¹⁰¹. They express higher levels of IL-12 receptors and are known to initiate and support the function of pit cells via interferon-gamma (INF- γ)¹⁰². Apart from natural killer T-cells, two further types of unconventional T-cells exist in the liver, namely $\gamma\delta$ T cells and MAIT cells. The liver is the richest source of $\gamma\delta$ T cells in the body¹⁰³. They are located in portal infiltrates, as well as spots where bile duct proliferation occurs⁹⁶. They produce TNF- α and INF- γ , and prime the formation of a long-lived memory population¹⁰⁴. MAIT cells were found only around 30 years ago¹⁰⁵ and were named in 2003¹⁰⁶. They are defined by their semi-invariant $\alpha\beta$ T-cell receptor (TCR) and act as the first firewall of the liver against bacterium¹⁰⁷. Different from $\gamma\delta$ T cells, they are highly enriched and widespread around liver lobules¹⁰⁸. MAIT cells are involved not only in maintaining homeostasis and regeneration processes of the liver^{109,110}, but in the pathogenesis of CLDs as well¹¹¹. Beyond T-cells, B-cells also represent an important part of hepatic lymphocyte population. They only represent up to 5% of intrahepatic lymphocytes in healthy livers⁹⁶. However, they are activated during infection and function via a germinal center-like structure surrounded by follicular dendritic cell network¹¹².

Phagocytes in the liver include macrophages, monocytes, and dendritic cells (DC). The liver-specific macrophages, namely KC, have been introduced above. Apart from KC, circulating monocytes are recruited to the liver and give rise to monocyte-derived macrophages (MoM ϕ) upon liver injury. MoM ϕ form a ring structure at the wound or accumulate in peri-portal regions and facilitate the wound healing process^{113,114}. In certain situations, such as viral infection and APAP-induced liver injury,

or when KC are depleted, MoM ϕ acquire KC characteristics, which are regulated by HSC and LSEC¹¹⁵⁻¹¹⁷. However, functionally, MoM ϕ and KC are still two distinct types of phagocytes. On the other hand, hepatic DC consist of myeloid DC and plasmacytoid DC, representing immature cells in healthy livers. They were first discovered and named in 1973¹¹⁸, based on their large nucleus, abundant cytoplasm, and dendrites. Myeloid DC act as APC that drive T-cell immune response, while plasmacytoid DC, on the other hand, produce interferons and regulate inflammatory responses¹¹⁹.

Granulocytes consist of neutrophils and eosinophils in the liver. Neutrophils are absent in the healthy liver. Upon infections and inflammations, inflammatory chemokines drive the migration of neutrophils from the blood to inflammation sites and start clearing via phagocytosis or secretion of proteolytic enzymes¹²⁰. Although the primary function of neutrophils is to produce reactive oxygen species (ROS) that cause hepatocyte damage and may exacerbate inflammation, an indispensable role in tissue revascularization and regeneration is also reported¹²¹. Eosinophils also originate from bone marrow and migrate through blood to damaged site, regulated by the crosstalk between other eosinophils and macrophages¹²². However, compared to neutrophils, function as well as the location of eosinophils is less established.

1.3 Zonation of the liver

The liver is a versatile organ consisting of different types of cells. These cells are not uniformly aligned next to each other, but are distributed following a gradient of nutrition, oxygen, and many other soluble factors, in line with blood flow along lobule radial axis, termed “liver zonation”. Liver zonation is important for an unperturbed liver function. Recent advances in single-cell transcriptomics have enabled the comprehensive study of cell heterogeneity at unprecedented high resolution. Hepatic zonation is by now well-established, showing all major cell types of the liver are spatially and/or functionally zoned.

1.3.1 Hepatocyte zonation

Metabolic zonation of hepatocytes has been characterized into three arbitrary regions in the early last century¹²³. Zone 1 represents hepatocytes around peri-portal regions, with a nutrition- and oxygen-rich microenvironment. They regulate gluconeogenesis, urea synthesis, secretory protein synthesis, biosynthesis of cholesterol, β -oxidation, and amino acid breakdown. In contrast, zone 3 represents hepatocytes around the central vein, with a nutrient-depleted and hypoxic environment. These hepatocytes are responsible for glutamine synthesis, glycolysis, and detoxification. Hepatocytes between zone 1 and zone 3 are zone 2 hepatocytes showing intermediate features. Besides metabolic heterogeneity, hepatocytes from the three zones also show distinct transcriptional signatures. By combining single-cell RNA sequencing (scRNA-seq) and single-molecule fluorescence in situ

hybridization (smFISH), half of hepatocyte-expressed genes have been shown either spatially or functionally zoned¹²⁴. Zoned metabolic functions and transcriptomic profile of hepatocytes are established shortly after birth and maintained throughout adulthood. One of the key determinants of the metabolic zonation of hepatocytes is the canonical Wntless-related integration site (Wnt) signaling from LSEC, namely “angiocrine Wnt signaling”¹²⁵. Ablation of angiocrine Wnt signaling leads to a defective hepatocyte zonation, while zonation is intact after ablation of Wnt signaling from epithelial cells or KC¹²⁶. Loss of angiocrine Wnt also leads to changes in cytochrome-related genes, liver growth, and disrupted lipid metabolism¹²⁷, suggesting a pivotal role of hepatocyte zonation for an undisturbed liver function.

1.3.2 LSEC zonation

Following the identification of hallmark genes for hepatocyte zonation, a distinct approach by performing scRNA-seq on pairs of hepatocyte and LSEC, termed “paired-cell sequencing”¹²⁸, was established. Localization of LSEC is identified and reconstructed using spatial information of attached hepatocyte. In this way, it is shown that around 35% of LSEC-expressed genes were similarly zoned as hepatocytes. The mechanisms of LSEC zonation are still not fully understood. Several molecules that are important for priming LSEC fate, such as GATA binding protein 4 (Gata4), transcription factor c-Maf (c-Maf), and Notch signaling, have been studied in terms of shaping LSEC zonation. Altered expression of such genes leads to loss of the original LSEC zonal signature¹²⁹⁻¹³¹. Interestingly, not only transcriptionally, a distinct proteomic and phosphoproteomic map of LSEC zonation¹³² was established. Furthermore, Fms-related tyrosine kinase 4 (Flt4), a flow-dependent regulator for vascular remodeling, was identified as one of the most significantly zoned proteins by phosphorylation.

1.3.3 KC zonation

KC heterogeneity has been reported decades ago¹³³. Unlike hepatocyte zonation, which is initiated in the first several weeks after birth, KC zonation is only established at the time of weaning. Zonation of KC is a dynamic process related to gut microbiota, mediated by LSEC in a chemokine (C-X-C motif) ligand 9 (CXCL9)-dependent manner. It plays a pivotal role in host defense process against bacterial dissemination and sepsis. The polarized distribution of KC along the liver lobule is called “immune zonation”. Anatomically, portal KC are larger than central and mid-zonal KC. Functionally, portal KC have higher lysosomal enzyme activity and phagocytic capacity than other KC. Portal KC produce more TNF- α , IL-1, and prostaglandinE2 (PGE2), while central KC secrete higher levels of NO⁴³. More KC are located on portal side than on central side¹³⁴. In a recent study¹³⁵, NK T-cells were revealed to exhibit a similar zonal enrichment pattern as KC.

1.3.4 HSC zonation

The anatomic heterogeneity of HSC was established in the late 20th century¹³⁶. During that time, the morphological zonation of HSC was heavily studied from numerous aspects, including desmin expression pattern, vitamin A storage, and cytoplasmic processes. Portal HSC show stronger desmin immunoreactivity than central HSC in a porcine model. The same pattern was also found for vitamin A storage. Interestingly, arctic animals show a different pattern of the vitamin A storage compared to pigs. These animals have the highest vitamin A storage in mid-zonal areas and the storage capacity gradually decreased toward portal and central regions¹³⁷. In rat HSC, cell processes in mid-zonal areas are longer than that in the central and portal regions.

The functional heterogeneity and transcriptomic zonation of HSC were only discovered recently. Intriguingly, the first single-cell atlas of HSC was generated in both healthy and fibrotic livers¹³⁸. qHSC show low heterogeneity and form a homogenous cell population^{139,140}. However, a computational method that involves independent component analysis identified and validated the existence of spatially variable genes along the liver lobule¹³⁸. Furthermore, several main trajectories of qHSC to aHSC representing different processes during fibrogenesis¹⁴¹ were demonstrated. This suggests a highly heterogeneous status of HSC in fibrotic livers. Similar to LSEC zonation, mechanisms of HSC zonation remains elusive.

1.4 Liver regeneration and partial hepatectomy

The liver possesses a remarkable capacity to regenerate. This capability is indispensable as the liver is constantly exposed to various toxins and pathogens. Liver regeneration is a multi-step process that involves all cell types within the liver. A strong coordination among hepatocytes and other cell types is required to determine the initiation and the termination phases of liver regeneration. Post-acute injury liver regeneration is a beneficial process that recovers the liver. However, in chronic inflammatory settings, loss of healthy hepatocytes leads to compensatory proliferation that is detrimental and may cause fibrosis or cancer.

Partial hepatectomy (PHx) is a technique, where parts of the liver are surgically removed. It was originally used to remove tumors from the liver of cancer-bearing patients. The first successful PHx was performed on a 44-year-old woman in 1911 to resect tumor from the right lobe¹⁴². Today, development of clinical technology have significantly improved post-operation survival rates. PHx and liver transplantation have become effective therapies to save the life of patients suffering from malignant liver diseases. Due to its high reproducibility, PHx has been widely used as a powerful tool to comprehensively study the mechanism of liver regeneration.

PHx on rodents for research purposes was proposed in 1931¹⁴³. The advantage of PHx is that this method does not introduce tissue injury or inflammation, thereby providing a physiological condition for liver regeneration. It is a highly reproducible and well-tolerated technique, with the initiation of regeneration process being well-controlled and defined. Normally, 2/3 of the liver is removed, and regeneration starts immediately after removal. The whole process lasts around a week, with total mass of the liver restored, while the removed lobe does not grow back.

There are two phases of liver regeneration after 2/3 PHx: The inductive phase and the angiogenic phase¹⁴⁴. During the inductive phase, hepatocyte proliferation is promoted by reduced TGF- β secreted by NPCs. Hepatocyte proliferation peaks at around 48 hours post-PHx and then declines to normal levels. On the other hand, proliferation of LSEC reaches the highest levels at approximately four days after PHx during the angiogenic phase¹. At the angiogenic phase, EC-derived Angiopoietin-2 functions as a spatiotemporal rheostat and regulates the switch between the inductive and the angiogenic phases¹⁴⁴. As a result, TGF- β expression is altered, which further determines the initiation and the termination of hepatocyte proliferation. Liver regeneration is a complex process that involves cell types beyond hepatocytes and EC. Hence, how the proliferation of other NPCs is regulated, remains to be characterized.

1.5 Liver diseases

A healthy liver with unperturbed functions requires a highly coordinated crosstalk among the different cell types within the organ. A perturbed microenvironment or miscommunication among different hepatic cells leads to malfunction of the liver and may cause liver disease. Liver disease causes 2 million deaths every year worldwide¹⁴⁵. Common symptoms of hepatopathy include fatigue, anorexia, jaundice, and ascites. However, these symptoms often appear at later-stages of diseases. Most of early-stage hepatopathies are asymptomatic. Therefore, liver diseases are frequently detected and diagnosed at late stage when effective interventions are limited. Hence, there is a high demand for establishing early detection techniques and novel therapies that are applicable to late-stage hepatopathy.

1.5.1 Viral hepatitis

Viral hepatitis is caused by virus infection. There are in total five different types of viral hepatitis: Hepatitis A, B, C, D, and E. Most of these hepatitis have no obvious symptoms, and are caused by different approaches.

Hepatitis A is caused by the hepatitis A virus (HAV). It is transmitted usually via food and drinks that had been contaminated by feces of patients or by direct contact with infectious persons. Due to its

way of transmission, HAV is more common in under-developed countries, where hygiene levels and penetration rates of vaccines are low. It has an incubation period of two to six weeks¹⁴⁶. The symptoms of Hepatitis A include jaundice, nausea, fever, and diarrhea. These symptoms often occur two months after infection. However, HAV is self-limiting and does not develop into a chronic phase. Children infected by HAV may not show symptoms and people recovered from HAV develop lifelong immunity. Therefore, it is less threatening than other types of viral hepatitis.

Hepatitis B is caused by the hepatitis B virus (HBV). It spreads via body fluids such as blood, saliva, semen, as well as horizontal transmission from mother to child. The symptoms of HBV are similar to that of HAV. Yet, unlike HAV, HBV has the potential to develop into chronic stages. This happens more frequently in young people than old ones. Once it becomes chronic, the continuously inflamed environment of the liver that HBV produces leads to further progression of the disease to cirrhosis and HCC. Although an effective vaccine has been available since 1982¹⁴⁷, hepatitis B is still a life-threatening disease that causes almost 1 million deaths annually worldwide.

Similar to HBV, hepatitis C caused by the hepatic C virus (HCV) can also become chronic and may lead to liver cirrhosis. In fact, unlike HBV, around half of people who are infected by HCV develop chronic infection. This accounts for 27% of cirrhosis and 25% of HCC¹⁴⁸. Unfortunately, there is still no effective vaccine for HCV so far. Therefore, the best way to prevent hepatitis C is to avoid its transmission. HCV transmits via blood and at low frequency from mother to kid. However, powerful treatments have been established: The World Health Organization (WHO) recommends direct-acting antivirals (DAA), such as sofosbuvir, as one of the most effective treatments for HCV¹⁴⁹.

Hepatitis D, also called “delta hepatitis”, is caused by the hepatic D virus (HDV). It is distinct among all five types of hepatitis, as its “symbiosis” with HBV and only occurs in HBV infected patients. Due to this special feature, HDV infections are split into two types: Coinfection, when a person is infected with both HBV and HDV at the same time; and superinfection, when HDV is infected after HBV infection. The reason for the satellite feature is that HBV is a prerequisite for the propagation of HDV¹⁵⁰. Infection of HDV after HBV is extremely dangerous, as acute infection may lead to liver failure. Furthermore, chronic HDV infection promotes the progression of cirrhosis and liver cancer¹⁵¹. Effective vaccines against HDV are still missing. However, HBV vaccine can prevent the infection of HDV due to their symbiosis phenotype.

Hepatitis E is caused by the hepatic E virus (HEV). Similar to HAV, HEV is found mostly in the feces of infected persons and transferred via fecal-oral transmission route. It is also self-limiting. So far, no effective treatment and vaccine has been established due to a lack of profitability in developed countries. However, scientists from China developed HEV 239 as a vaccine against HEV in 2012¹⁵²,

which is not yet recommended by WHO. Different from other four types of hepatitis viruses, HEV has four genotypes, of which type 1 and type 2 are restricted to humans, whereas type 3 and type 4 are also found in other species.

Besides the above-mentioned five types of hepatitis viruses, GB virus C (GBV-C), or hepatitis G virus (HGV), was identified in 1995¹⁵³. It does not cause human diseases. Torque teno virus (TTV), which was identified in 1997 in Japan, and has a high correlation with post-transfusion hepatitis. However, these two types of hepatitides are less characterized than the five major types of hepatitis viruses. Interestingly, liver injury after virus infection are not caused by the cytopathic effect of viruses, but by hepatitis B surface antigen (HBsAg) in the endoplasmic reticulum (ER) of hepatocytes. Therefore, it is sensitive to INF- γ -mediated destruction¹⁵⁴.

1.5.2 Alcohol-associated liver disease (ALD)

Alcohol-associated liver disease is liver damage caused by excessive alcohol consumption. There are three major phases: Alcoholic fatty liver disease, alcohol-associated hepatitis, and alcohol-associated cirrhosis. Fatty liver disease is also called steatosis. It is the first step of ALD and can be caused even by short-term alcohol abuse. Once excessive alcohol intake lasts over a long period, steatosis will progress to the next step: alcohol-associated hepatitis. At this stage, the liver is inflamed and liver cells are damaged. Such damage is reversible once alcohol intake is stopped, as damaged cells can regenerate. This wound-healing process may form scar tissues if it lasts for several years. Otherwise, scarring further leads to liver fibrosis and causes alcohol-associated cirrhosis. Cirrhosis is the last stage of ALD and is generally irreversible. However, stopping drinking can still prevent the liver from further damage. Cirrhosis may lead to liver failure and cause death. So far, no effective treatment for liver fibrosis and cirrhosis has been approved.

1.5.3 Non-alcoholic fatty liver disease (NAFLD)

Non-alcoholic fatty liver disease (NAFLD) is an umbrella term that describes different kinds of diseased conditions of the liver. In NAFLD, fat, instead of alcohol, plays a key role during disease development. As its name implies, there is excessive fat storage within the liver. Therefore, NAFLD is more common among overweight and obese people. Similar to ALD, NAFLD also develops following several steps, including fatty liver, non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis. Although NAFLD affects around a quarter of global population¹⁵⁵, the reason why fat accumulates in the liver and why it leads to liver damage is not fully understood^{156,157}. It is suspected that metabolic problems, lifestyle, and genetics could be factors causing NAFLD. Recently, the NAFLD nomenclature consensus group suggested that the term NAFLD is inappropriate because of its dependency on exclusionary confounders and has the potential to be stigmatizing¹⁵⁸. The new nomenclature, metabolic

dysfunction-associated steatotic liver disease, was widely supported because of its non-stigmatization, improved awareness, and better patient identification.

1.5.4 Liver fibrosis

Liver fibrosis is very common in CLD. It is a wound-healing response upon consistent liver damage. HSC are major sources of ECM in injured liver, thereby acting as key mediators of fibrogenesis. As liver fibrosis is a reversible process, early detection is essential for involving clinical intervention and to prevent the liver from further damage. Continuous challenge leads to further progression of the disease to cirrhosis and, for the final stage, cancer. The golden standard for liver fibrosis diagnosis is liver biopsy¹⁵⁹. However, it is an invasive method. So far, non-invasive assessment for liver fibrosis remains missing. After sampling, scoring systems with information on inflammatory activity and degree of fibrosis are established to evaluate the severity of diseases. Commonly used systems include Knodell score/Histological Activity Index (HAI), METAVIR score, and Ishak score. HAI is a histology activity index. It includes factors such as periportal & bridging necrosis, intralobular degeneration and focal necrosis, portal inflammation, and four degrees of fibrosis¹⁶⁰. Ishak score is a modified Knodell score, which has six stages of fibrosis. It mainly focuses on the expansion of fibrotic areas and bridging¹⁶¹. The METAVIR score, on the other hand, has five stages. It focuses on the existence of portal fibrosis and the number of septa¹⁶². Despite numerous studies showing effective therapies against fibrosis^{163,164}, no standard treatment has been formally approved. Low translation rates of anti-fibrosis therapies from fundamental research to bedside is due to a shortage of non-invasive markers to monitor and track the efficacy of novel drugs. The sensitivity of humans to anti-fibrosis therapies is also lower than in rodents¹⁶⁵. Thus far, liver transplantation remains as the only treatment to save the life of cirrhosis patients. Therefore, liver fibrosis and its final stage, liver cirrhosis, are still key challenges of global health that urgently need to be solved.

1.5.5 Hepatocellular carcinoma (HCC)

Hepatocellular carcinoma (HCC) is the most common primary cancer in the liver. It is at the third position of leading causes for cancer death worldwide¹⁶⁶. Cirrhosis, the common end stage of CLDs, is one of the major risk factors for HCC development. Hence, HCC mostly occurs after the symptoms of CLDs appeared. HCC can be diagnosed by biopsy test and magnetic resonance imaging (MRI). Several biomarkers have been identified for detection, diagnosis, and prognosis of HCC. Alpha-fetoprotein (AFP) is one of the best serum biomarkers for HCC. It is a glycoprotein expressed in the fetal liver, whose expression drops shortly after birth¹⁶⁷. AFP is not only a predictive factor, but also a prognostic factor. Elevated AFP levels indicate poor prognosis¹⁶⁸, as well as a high risk of recurrence¹⁶⁹. However, it is not perfect since the false-positive rate is high, especially in HCV patients¹⁷⁰.

HCC can develop from various kinds of CLDs, and has numerous types in terms of proliferation, pathology, differentiation, genetics, prognosis, and vascular invasion. Based on its proliferative phenotype, HCC has been classified into two major types: proliferative and non-proliferative type. The proliferative type is more aggressive and less differentiated. It normally has a high frequency of vascular invasion and high AFP levels and mainly develops in HBV-infected livers. In contrast, HCV and NAFLD-derived HCC are non-proliferative, with a differentiated (hepatocyte-like) cell status. It is less aggressive, and has lower levels of AFP¹⁷¹. HCC can be further classified into more subclasses based on mutational signatures, activated pathways, and immune phenotypes. Besides classification by molecular and immune features, another criterion, the Barcelona Clinic Liver Cancer (BCLC) classification, which evaluates cancer symptoms and the number of nodules, is also widely used¹⁷². BCLC divides HCC into five stages from: 0 (very early stage: single nodule, less than two centimeters), A (early stage: one to three nodules, all less than three centimeters), to D (terminal stage: severe liver damage). Based on this classification, treatments also vary from surgical resection, radiofrequency ablation, chemoembolization, targeted or immunotherapy drugs, to transplantation. Recently, a new drug combination of a previously accepted kinase inhibitor, sorafenib, and an angiogenesis inhibitor, bevacizumab, has entered phase three clinical trial. This combination therapy showed better outcomes than sorafenib alone¹⁷³.

1.5.6 Other liver diseases

Except for above-mentioned diseases, several other types of liver diseases can also lead to liver damage. Autoimmune hepatitis leads to an inflamed liver due to immune system disorders. Hemochromatosis causes iron overload that damages multiple organs. Primary biliary cholangitis damages bile ducts. Consequently, bile flows back and damages the liver, causing biliary cirrhosis. Similarly, primary sclerosing cholangitis narrows down and blocks bile ducts, to induce liver damage. Wilson's disease is a genetic disorder, which builds up copper in many organs, including the liver. Regardless of various etiologies of liver damage, all these diseases prime an inflamed hepatic microenvironment and lead to cirrhosis and HCC.

1.6 Liver damage models

To investigate mechanisms and to establish novel therapies for liver diseases of different etiologies, a suitable experimental model that best mimics corresponding damage is indispensable. As fibrosis is the leading risk factor for HCC, five types of *in vivo* experimental models for fibrosis research, including chemical, dietary, surgical, immune, and transgenic, are introduced.

1.6.1 Chemically-induced models

1.6.1.1 Carbon tetrachloride (CCl₄)

CCl₄ is one of the most commonly used hepatotoxins that induces liver fibrosis with high efficacy and reproducibility. Three or four phases of CCl₄ toxicity were discovered in Wistar rats in 1975¹⁷⁴. The first phase represents two to three weeks after CCl₄ administration with a necrotic phenotype accompanied by decreased pseudocholinesterase. The second phase starts in the following two to three weeks. A further necrotic liver and massive triglycerides accumulation, elevated aspartate transaminase (AST) levels, bromsulphalein (BSP) retention, and decreased pseudocholinesterase are detected in this phase. The third phase shows more increasing AST, BSP retention, triglycerides, as well as hydroxyproline, decreased pseudocholinesterase, and liver weight. These indicate the reduced ability of biosynthesis and atrophy of the liver. CCl₄-induced liver damage starts from the metabolism of CCl₄ by cytochrome P450 (CYP), a superfamily of monooxygenases. Metabolites from this process, trichloromethyl radical (CCl₃*), can react with nucleic acids, proteins, and lipids. This leads to disturbed lipid metabolism, oxidative stress, and lowers the permeability of organelle membranes¹⁷⁵. Administration methods of CCl₄ include intraperitoneal (i.p.), inhalation, and oral gavage. The majority of research groups prefer IP for its ease of handling, high reproducibility, and excellent survival rate. In contrast, inhalation is more difficult, as a specific hood and technique are required for this procedure. Recently, administration via gavage has also been performed, due to its lower intergroup variation¹⁷⁵. Short-term CCl₄ treatment starting from a single dose leads to reversible peri-central necrosis. Continuous insults result in development of peri-central fibrosis. In general, CCl₄ administration has advantages of being simple, low cost, short overall induction period, and highly reproducible. It can generate significant changes in liver pathology and is also capable of studying the regression of liver fibrosis. However, as treated animals do not become obese, this method is not suitable for research on NAFLD. The toxic and volatile features of CCl₄ also challenge researchers from safety aspects.

1.6.1.2 Acetaminophen (APAP)

APAP, more commonly known as Paracetamol, is widely used as an analgesic or antipyretic drug worldwide. Although safe at therapeutic doses, APAP overdose is dangerous and may cause acute liver failure¹⁷⁶. Similar to CCl₄, APAP administration induces peri-central liver damage. There are three different ways for ingested APAP to be metabolized in the body: 1) Up to 90% of APAP is transferred to innocuous glucuronidated and sulfated metabolites. They excrete from urine and are catalyzed by UDP-glucuronosyl transferases (UGT) and sulfotransferase (SULT)¹⁷⁷. 2) A small amount of (up to 2%) APAP funnels into the urine without being metabolized. 3) The rest of ingested APAP is metabolized by CYP, mainly CYP2E1 and to a lesser extent CYP3A4 and CYP1A2¹⁷⁸. These ingested APAP are oxidized

to either non-toxic 3-hydroxy-APAP (3-OH-APAP), or toxic N-acetyl-p-benzoquinone imine (NAPQI)¹⁷⁹. Further, NAPQI oxidizes the thiol groups of protein to make ROS¹⁸⁰. Glutathione (GSH) plays a pivotal role in detoxifying NAPQI by conjugation to form thiolate and cysteine compounds. They are further eliminated in the kidney¹⁸¹. During APAP overdoses, excessive NAPQI and ROS change the morphology and function of mitochondria, thereby inducing liver damage¹⁸². Unlike CCl₄, a second dose of APAP will initiate an adaptive process, which can alleviate injury phenotypes¹⁸³. This phenomenon is termed “liver memory” and requires further investigation for a better mechanistic understanding.

1.6.1.3 Alcohol

Experimental models for ALD using alcohol has been established since 1949¹⁸⁴. The feeding model is called *ad libitum*, where alcohol is administered via the drinking water. It is a very simple method and mimics drinking habits: intermittent and voluntary, with a normal diet. However, even though *ad libitum* drives liver damage and steatosis, it is still challenging to induce fibrosis due to natural aversion of mice against alcohol. To overcome such disadvantages, a diet (Lieber-DeCarli) that consists of high fat, alcohol, and nutrition components was designed in 1963 to achieve higher blood ethanol levels¹⁸⁵. Nevertheless, this model is still insufficient to induce fibrosis in the absence of a second insult¹⁸⁶. Hence, a chronic-binge model that combines chronic Lieber-DeCarli diet and one or multiple binges via gavage was introduced to achieve a more severe phenotype¹⁸⁷. This modified model is called Gao-Binge or National Institute on Alcohol Abuse and Alcoholism (NIAAA) model¹⁸⁸. Another model, Tsukamoto–French Model (TF Model), was developed in 1984¹⁸⁹ when fibrosis and cirrhosis were achieved for the first time. TF model is a hybrid model that starts with a Western diet followed by intragastric ethanol infusion and a high-fat liquid diet, together with binges¹⁹⁰. However, this model requires high technical support and high costs, making it challenging to implement in the laboratory.

1.6.1.4 Thioacetamide (TAA)

TAA is another widely used model for liver fibrosis. It is administered via IP or oral gavage. Unlike CCl₄ and APAP (induce central damage), TAA damages hepatocytes in both central and portal regions. Notably, it introduces more pronounced portal damage than other toxins¹⁹¹. TAA alone does not have hepatotoxicity. Its toxicity is initiated when metabolized by CYP and converted to TAA S-oxide. TAA S-oxide acts as ROS and binds to lipids and proteins, thereby causing cellular damage and necrosis of hepatocytes. The damage may last a few weeks, even after cessation of the administration. Correspondingly, it takes longer to induce significant fibrosis than other toxins¹⁹². As TAA is a carcinogen and mutagen¹⁹³, its long-lasting and slow reacting features make it challenging as a fibrosis model before the development of cholangiocarcinoma or HCC.

1.6.1.5 Diethylnitrosamine (DEN)

DEN is a highly toxic carcinogen that induces DNA damage/mutation and oxidative stress¹⁹⁴. It is introduced by IP injection or through the drinking water. Similar to TAA, damage occurs at both central and portal regions. The damage may further form fibrotic septa¹⁹¹. DEN has a long half-life period. Fibrosis retains and keeps developing even several months after the removal of DEN¹⁹⁵. As DEN-induced HCC develops at a low-dose with long-term exposure, DEN is a good model to investigate the progression of fibrosis to HCC. However, DEN-induced HCC shows big variabilities when it is performed between different species, genders, and ages. Therefore, careful adjustment of dosage and time course should be considered.

1.6.1.6 Other chemically-induced models

Besides toxins listed above, other hepatotoxins also exist and cause different kinds of liver damage. Arsenic is a hepatic carcinogen, which causes DNA damage and generates free radicals¹⁹⁶. These radicals further induce lipid peroxidation and damage the proper functions of cells. Fibrosis develops due to activation of HSC, repeated damage and wound healing of the liver. D-galactosamine (D-GaIN) is another robust toxin to the liver. Acute co-injection of lipopolysaccharide (LPS) and D-GaIN is a widely used model that induces acute liver injury. Liver fibrosis develops if co-injection is prolonged in a low-dose and long-term manner¹⁹⁷.

1.6.2 Dietary models

Compared to chemically-induced fibrosis, dietary models induce NAFLD or NASH. These diseases are normally the outcomes of perturbed metabolism caused by over nutrition from different kinds of diets. Dietary models mimic our daily food intake, hence providing a more natural and *ad libitum* way that is closer to real life than chemicals. In contrast, oral gavage is an intragastric technique. However, it introduces stress to animals and requires technical skills of handlers. Additionally, it should be considered that solvent used to dissolve chemicals may introduce extra lipids or water intake. In general, dietary models provide solid tools for investigating CLDs, especially for NASH or NAFLD, and simulate the pathology of human liver diseases.

1.6.2.1 High fat diet (HFD)

HFD is a widely used model for hepatic steatosis. It induces obesity, high insulin levels, and insulin resistance (IR). IR further promotes *de novo* lipogenesis, dysfunction of adipose tissues, and disorder of fatty acid output, thereby causing lipid accumulation in the liver¹⁹⁸. HFD accelerates the progression of NAFLD via downregulation of geranylgeranyl diphosphate synthase (GGPPS), which shifts fuel preference from fatty acid to glucose¹⁹³. Furthermore, liver-specific GGPPS knockdown leads to disturbed mitochondrial function and drives Warburg effect. It is an anaerobic glycolysis procedure

commonly seen in cancer cells, which creates a pro-fibrosis and inflammatory environment¹⁹⁹. However, several studies showed that HFD for less than four months failed to induce significant changes in fibrogenesis-related proteins^{200,201}. Although two typical risk factors (IR and obesity) were induced by HFD, gene expression profiling still only represents steatosis signature rather than NASH^{202,203}. Therefore, whether HFD is a good model for fibrosis remains elusive.

1.6.2.2 Western diet (WD)

WD is a diet model rich in fructose, cholesterol, and saturated fat. However, composition of WD may vary substantially. Further, the so-called WD model for rodents does not fully recapitulate “human Western diet” food pattern in real life. National Health and Nutrition Examination Survey showed that a typical American diet contains energy from around 49% carbohydrates, 35% fat, and 16% protein²⁰⁴. Based on this, a total Western diet that represents typical American nutrition intake is formulated²⁰⁵. However, its effect was mainly evaluated for colon cancer. The effectiveness of a total Western diet remains elusive. On the other hand, a diet that is closer to human diet may not lead to NAFLD or costs unreasonable time or money²⁰⁶. Due to these reasons, a well-defined composition of WD is still missing. Researchers often modify the recipe of “WD” depending on their specific research topics and purposes. Regardless of various kinds of WD used in the field, they require a shorter time than HFD to achieve steatosis²⁰⁷. Nevertheless, long-term WD treatment only causes mild damage to the liver²⁰⁸. Hence, the combination of WD with adjustments of the composition formula, such as sugar water or nutrient deficiency were applied, to get a better outcome for liver damage. WD alone was revealed to achieve only mild fibrosis²⁰⁹.

1.6.2.3 Other dietary models

Besides above-mentioned obesogenic models, nutrient-deficient models are also effective for inducing liver damage. Methionine- and choline-deficient diet (MCD) is a diet that contains high sucrose (40%) and fat (10%), with methionine and choline absent. These two components are essential for proper β -oxidation of mitochondria and biosynthesis of low-density lipoprotein²¹⁰. MCD induces a higher extent of inflammation, necrosis, and mitochondrial damage, but with milder levels of steatosis²¹¹. Mice fed with MCD may even lose up to 40% of their body weight²¹². Furthermore, serum insulin, leptin, and glucose levels are concurrently reduced¹⁹¹. Another diet is Choline-Deficient L-amino-defined (CDAA) diet. The composition formula of CDAA diet is adjusted from choline-deficient diet with proteins replaced by an equivalent amount of L-amino acids²¹³. CDAA diet takes longer to develop similar levels of histological changes as MCD. Since CDAA diet does not lead to IR or change in weight and displays an opposite metabolic profile to human disease²¹⁴, it is not a perfect model for metabolic studies. An atherogenic diet is a type of diet that is rich in cholesterol and cholate. It induces inflammation and

causes steatosis. However, it caused different metabolic change from NASH in humans. Other types of diets such as fructose diets and fast food diets are also frequently used to induce NASH. As a single type of diet alone is difficult to perfectly mimic all phenotypes and expression profiles of human NASH, combination of different types of diets normally leads to better outcomes. Nevertheless, a standardized dietary model that fully resembles every aspect of human NASH remains absent.

1.6.3 Bile duct ligation

Bile duct ligation (BDL) is a well-established and highly reproducible surgical model to induce liver damage. It introduces an obstructive cholestasis that leads to accumulation of bile acid within the liver through manipulation of the bile acid circulation. Bile duct obstruction was performed on rabbits and cats since 1872²¹⁵. The first study with rodents was performed forty years later²¹⁶. BDL is normally referred to as common bile duct ligation, where mid-ventral laparotomy is performed, followed by isolation of the common bile duct on top of the duodenum. Two ligations are introduced to the bile duct with a dissection between the two nodes²¹⁷. This triggers proliferation of biliary epithelial cells and ECM deposition²¹⁸. BDL mainly induces portal and sinusoidal fibrosis and causes severe inflammation and oxidative stress. Additionally, different from other models, mice with BDL show jaundice shortly after surgery. Recently, it was shown that PF, rather than HSC, is the major source of collagen at the early stages after BDL⁹⁵. Hence, BDL is a distinct and suitable model to investigate the roles of different mural cells during fibrogenesis. However, disadvantage of this model remains since the mortality rate of BDL is higher due to bile leakage or gall bladder rupture during the experiment. The pain led by the surgery also causes extra stress to the animals.

1.6.4 Immuno-induced models

Immuno-induced models were established based on autoimmune hepatitis (AIH), where immune cells induce self-attack to hepatocytes. AIH causes inflammatory necrosis and liver fibrosis. The cause of AIH remains elusive. There are two different types of AIHs: Type 1 is the most common form that can occur throughout lifespan. Such patients normally suffer also from other autoimmune disorders. Type 2 develops more frequently in young people and children. It is less frequent than type 1 AIH and is normally more severe. Type 1 and type 2 AIH involve different antibodies. Type 1 AIH involves antibody to smooth muscle cells, whereas type 2 involves antibodies to liver-kidney microsome or liver cytosol that target CYP2D6. So far, no cure is established for AIH. Medications mitigate the symptoms, but have high risk for relapse. Liver disease models that trigger immune reactions and cause liver damage include schistosomiasis, viruses, and several other stimuli.

1.6.4.1 Schistosoma

Schistosomiasis is primarily caused by three different types of *Schistosoma*, including *Schistosoma japonicum*, *Schistosoma mansoni*, and *Schistosoma haematobium*²¹⁹. Among them, *S. japonicum* is the most commonly used model for studying mechanisms or drug development for liver fibrosis, as fibrosis is one of the major pathological manifestations after infection²²⁰. *Schistosoma*-induced liver fibrosis is a complex process that involves immune inflammation, granuloma formation, and subsequential liver damage. Granuloma formation is the most critical step during this long process. It is initiated by egg-laying. Once the granuloma matures, it recruits ECM-secreting fibroblast to its outer zone. This further activates qHSC and leads to liver fibrosis.

1.6.4.2 Viruses

Viral hepatitis is one of the most important subtypes of liver disease. Therefore, developing effective models for studying viral hepatitis is an important topic for a better understanding of such life-threatening diseases. An adeno-associated virus serotype 8 (AAV8) - mediated model induces persistent HBV infection in mice²²¹. Another model based on recombinant covalently closed circular DNA (rcccDNA) from HBV that binds a specific DNA in the liver was developed. It also induces liver fibrosis after adenovirus delivery²²².

1.6.4.3 Other immune-induced models

Apart from *Schistosoma* and viruses, other stimuli may also induce immune responses and liver damage. Pig serum (PS) is a heterologous antigen that causes liver fibrosis. It initiates immune responses and stimulates the secretion of inflammatory cytokines to activate HSC²²³. PS injected rats phenocopied fibrosis in humans²²⁴. However, the timeframe is long, and animals may die due to allergic reactions. A knife bean phytoagglutinin, Concanavalin A, can also induce immune-mediated liver damage. It stimulates T-cell mitosis and secretion of several cytokines such as TGF- β and TNF- α after intravenous (i.v.) injection. The fibrosis it caused mimics autoimmune hepatitis, viral hepatitis, or other drug-induced immune-mediated liver fibrosis.

1.6.5 Transgenic models

Numerous transgenic models have been developed to induce liver damage. So far, most of established models show lipid accumulation in the liver, whilst inflammation and fibrosis are absent²¹². One of the most frequently used mouse lines is *ob/ob* mice. Due to a spontaneous point mutation in the leptin gene, *ob/ob* mice do not have functional leptin, which is secreted primarily by white adipose tissue (WAT) and acts as a negative food intake regulator. Therefore, *ob/ob* mice are often inactive and hyperphagic. They develop severe obesity, hyperglycemia, hyperlipidemia, hyperinsulinemia, and insulin resistance²²⁵. Interestingly, even with a restricted diet that maintains normal weight in lean

mice, *ob/ob* mice still gain excess body weight. Fat is redistributed from WAT to the liver and further induces hepatic lipotoxicity and lipoapoptosis (the apoptosis induced by excessive exposure to fatty acids)²¹². Nevertheless, *ob/ob* mice rarely develop spontaneous steatosis or severe liver damage. Another model, *db/db* mice, which are carriers for a spontaneous diabetic mutation in leptin receptor, show a similar albeit milder phenotype than *ob/ob* mice²²⁶. Both mouse lines require a secondary stimulus to develop NASH. However, compared to *ob/ob* mice, *db/db* mice develop significant fibrosis when fed with MCD diet²²⁷. *foz/foz* mice is a mouse model designed for NASH²²⁸. These mice have 11-base pair truncated in Alstrom syndrome 1 (*Alsm1*) gene. *foz/foz* mice develop spontaneous fatty liver and steatohepatitis, as well as pericellular fibrosis, when fed on a high-fat atherogenic diet²²⁹. Other mouse lines, including *KK-Ay/a* mice²³⁰, Phosphatase and Tensin Homolog (PTEN) mice²³¹, and PPAR α mice²³² have also been widely used to study liver diseases, especially NASH. Since most transgenic models alone do not progress to NASH, a combination of genetic modification and special diets is more frequently used.

HBV transgenic mice are another widely used model. Initially, mice expressing different viral proteins were used to evaluate the potential from virological and oncogenic aspects^{233,234}. To test the efficacy of antiviral agents, mice that express a complete HBV replicon were constructed²³⁵. However, these models were generated using immune-tolerant animals that do not develop liver damage such as fibrosis. Neither do they have the potential to clear viruses. Therefore, they are unsuitable for studying the mechanisms of infection or covalently closed circular DNA (cccDNA) synthesis²³⁶. Additionally, the lack of HBV receptors in murine hepatocytes makes it impossible for intrahepatic infection and further development of the disease.

1.7 Wnt signaling and R-spondin 3 (*Rspo3*)

Wnt signaling, especially the canonical β -catenin signaling cascade, plays indispensable roles in regulating liver functions. During development, lack of β -catenin leads to embryonic lethality²³⁷. At the late stage of liver development, β -catenin controls the fate of hepatoblasts, the common origin of hepatocytes and cholangiocytes. Hepatoblast-specific deletion of adenomatous polyposis coli (APC), an antagonist of Wnt signaling, leads to defects in hepatocyte differentiation²³⁸. Moreover, β -catenin regulates hepatocyte proliferation and maturation²³⁹. Mice lacking hepatocytic expression of β -catenin, LGR4/5, or LRP5/6 lead to decreased liver weight^{126,240,241}. In the homeostatic liver, Wnt/ β -catenin functions as key regulator establishing liver zonation. Loss of β -catenin in hepatocytes leads to an overall periportal phenotype throughout the liver²⁴². In turn, hepatocyte-specific β -catenin overexpression primes the liver to a pericentral identity²⁴³. During liver regeneration, β -catenin expression is transiently induced to promote hepatocyte proliferation. Besides, the Wnt/ β -catenin

pathway also regulates detoxification²⁴⁴, biosynthesis²⁴⁵, and even the development of HCC^{246,247}. Notably, experimental evidence indicates a role of β -catenin in liver pathobiology of various etiologies. However, the detailed mechanisms for each condition as well as the role of non-canonical Wnt signaling in liver physio-pathobiology, remains to be clarified. Therefore, this chapter summarized key findings of both canonical and non-canonical Wnt signaling, briefly reviewing the functions of Rspo3, one of the key regulators of Wnt signaling in the liver.

1.7.1 Wnt signaling

Wnt is named after Wingless (Wg) and integration (Int). It stands for “Wingless-related integration site”. Wg, a segment polarity gene, is indispensable for pattern generation during the development of adult drosophila wings²⁴⁸. In turn, int-1, the ortholog of Wg in humans and mice, was the first Wnt molecule identified as a proto-oncogene in a study about mouse mammary tumor virus (MMTV)-induced tumors in 1982²⁴⁹. As further findings of more int-1-related genes different from int-1 were discovered, the Wg/Int family was renamed as the Wnt family²⁴⁹. The Wnt signaling pathway is an evolutionarily highly conserved pathway across a myriad of species. Besides pivotal roles in carcinogenesis and development, Wnt signaling pathways regulate cell fate, cell proliferation, cell polarity, cell differentiation, and cell migration²⁵⁰. The Wnt family consists of 19 Wnt ligands²⁵¹ in vertebrates. 15 receptors, including frizzled (Fz), Low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) coreceptor, receptor tyrosine kinases Ryk and Ror families are also involved^{252,253}. Taken together, Wnt signaling can be divided into three different cascades: Canonical Wnt/ β -catenin pathway, Non-canonical planar cell polarity (PCP) pathway, and Non-canonical Wnt/calcium pathway (Figure 3).

1.7.1.1 Canonical Wnt/ β -catenin signaling

The hallmark of canonical Wnt signaling is the stabilization of the adherens junction associated-protein, β -catenin. In the absence of Wnt ligands, the levels of β -catenin remain low due to degradation by the destruction complex. The destruction complex consists of Axin, casein kinase 1 α (CK1 α), glycogen synthase kinase 3 β (GSK3 β), and adenomatous polyposis coli (APC). Among them, axin functions as scaffold protein and brings other components together to form the destruction complex. The destruction complex further mediates the phosphorylation of β -catenin at serine 45 by CK1 α and serines 33, 37, and threonine 41 by GSK3 β ²⁵⁴. Phosphorylated β -catenin can be detected by one of the components of E3 ubiquitin ligase complex, β -transducin repeat-containing protein (β TRCP). This leads to its ubiquitination and degradation²⁵⁵ to maintain the quiescence of the canonical Wnt pathway.

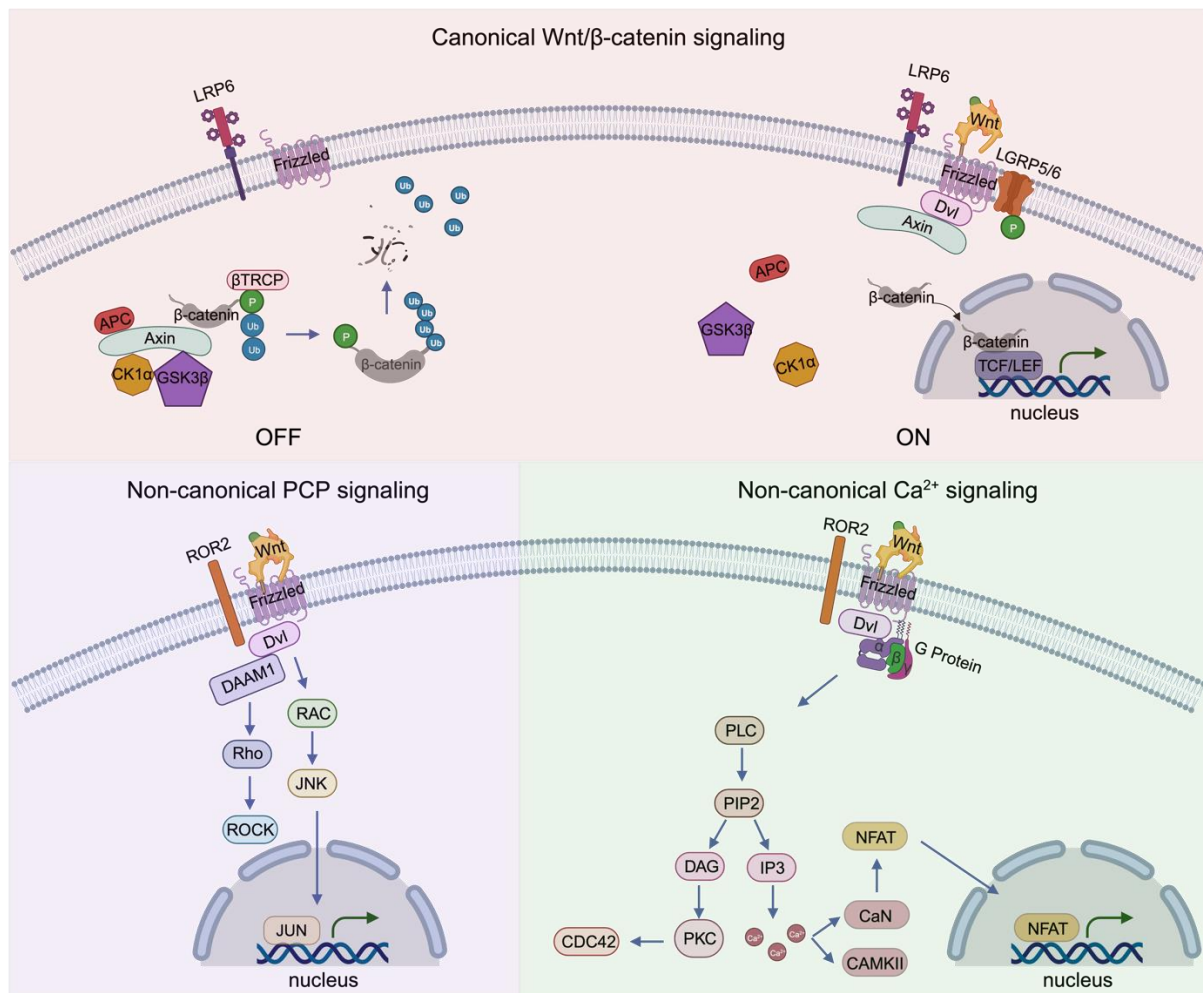


Figure 3. Wnt Signaling pathway.

Three major Wnt signaling pathways have been well characterized. The canonical Wnt pathway involves stabilization of β -catenin for further transcription. The destruction complex induces proteasome-based degradation of β -catenin by phosphorylation and prevents it from entering the nucleus. When Wnt ligand binds to Frizzled receptor, Dvl recruits Axin and disturbs the function of destruction complex. As a result, β -catenin translocates to nucleus and bind to TCF/LEF for the transcription of downstream signaling. Non-canonical Wnt signaling pathways do not involve β -catenin for regulation. The Wnt-PCP signaling requires Ror2. It recruits Dvl and its co-receptor DAAM1. Next, ROCK and Rac are activated to initiate signaling cascade. This signaling cascade regulates cell polarity, migration, and proliferation. Wnt-calcium signaling requires formation of Dvl, Frizzled, and trimeric G-Protein for the activation of PLC. PLC cleaves PIP2 to DAG and IP3. They induce the release of Ca^{2+} . As a result, activation of CDC42 and NFAT-related transcription are initiated.

Activation of β -catenin requires glycosylation and palmitoylation of Wnt ligands by porcupine in the ER²⁵⁶. During this process, a cargo receptor Wntless (Evi/Wls) mediates protein trafficking of Wnt from the Golgi apparatus to the cell membrane^{257,258}. Secreted Wnt subsequently binds to Fz receptors and LRP5/6 coreceptors, which further recruits the scaffolding protein Dishevelled (Dvl) and phosphorylates LRP5/6. Consequently, axin is recruited to the plasma membrane and disrupts the destruction complex to stabilize β -catenin. Non-phosphorylated/stabilized β -catenin then translocates to the nucleus and forms a complex with T-cell factor/lymphoid enhancer factor (TCF/LEF) to regulate

the expression of target genes as transcription factor²⁵⁹. R-spondin family-mediated enhancement of Wnt signaling transduction will be introduced in detail in 1.7.2.

1.7.1.2 Non-canonical Wnt signaling

The β -catenin-independent Wnt signals are referred to as non-canonical Wnt signaling. So far, two types of non-canonical Wnt signaling pathways: Wnt/PCP pathway, and Wnt/calcium pathway, have been characterized.

In the Wnt/PCP pathway, Wnt signaling is mediated by Fz receptors independent of LRP5/6. Instead, Ror2, Ryk, PTK7, and NRH1 serve as major co-receptors²⁶⁰⁻²⁶³, whose engagement leads to recruitment of Dvl. The PDZ and DIX domains of Dvl, together with co-receptor, form a complex with Dishevelled-associated activator of morphogenesis 1 (DAAM1). This further activates Rho GTPase through guanine exchange factor and its downstream Rho-associated kinase (ROCK). Concurrently, Dvl alone can activate Rac GTPase independent of DAAM1 and stimulate JNK activity. Additionally, DAAM1 also regulates actin polymerization via actin-binding protein, Profilin²⁶⁴. Taken together, these signaling cascades lead to modifications of the actin cytoskeleton, as well as gene expression that controls cell polarity, migration, and proliferation²⁶⁵. Notably, Rho and Rac have opposing functions. However, how their activities are coordinated remains largely unknown.

The Wnt/calcium pathway regulates the release of calcium from ER to control intracellular calcium levels. Wnt ligands bind and activate Fz receptor, and activate the PDZ and DEP domains of Dvl²⁶⁴. Consequently, a complex of Fz, Dvl, and a trimeric G-protein is formed. This leads to further activation of phospholipase C (PLC). Once PLC is activated, plasma membrane component phosphatidylinositol 4,5 biphosphate (PIP2) will be cleaved into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). DAG induces protein kinase C (PKC) activation, while IP3 directly binds to its receptor on ER to release calcium. As a result, increased calcium concentration and DAG further activate cell division control protein 42 homolog (CDC42) via PKC, a key regulator of ventral patterning, tissue separation, and cell movement during development²⁶⁴. Concurrently, increased intracellular calcium concentrations further activate calcium/calmodulin-dependent kinase II (CaMKII) and calcineurin (CaN). CaN stimulates the transcription factor nuclear factor of activated T-cells (NFAT), which regulates cell adhesion, cell migration, and tissue separation²⁶⁴. On the other hand, CaMKII triggers transforming growth factor β -activated kinase 1 (TAK1) and Serine/threonine protein kinase NLK signaling cascade that inhibits canonical Wnt signaling²⁶⁶. In turn, Dvl may also activate phosphodiesterase PDE, which inhibits protein kinase G (PKG), to inhibit calcium from releasing.

Recently, two novel non-canonical Wnt signaling pathways, Wnt/STOP²⁶⁷ and Wnt/TOR²⁶⁸, have been reported. The key mediator of Wnt/STOP pathway is Glycogen synthase kinase-3 beta (GSK3 β). It can phosphorylate proteins for proteasomal degradation²⁶⁹. Accumulation of GSK3 β target proteins is achieved by the sequestration of GSK3 β in multivesicular bodies after Wnt binds to its co-receptor. Wnt/STOP signaling pathway regulates cytoskeleton, cell division, and DNA remodeling²⁷⁰. Wnt/TOR pathway also involves GSK3 β to phosphorylate Tuberous Sclerosis Complex 2 (TSC2). Activation of TSC2 inhibits mammalian target of rapamycin complex 1 (mTORC1) function. Wnt ligands prevent phosphorylation of TSC2, hence activating mTORC1 signaling.

1.7.2 Rspo3

Rspo3 belongs to the R-spondin family, which consists of four secreted glycoproteins RSPO1-4. It was first identified in a human fetal brain cDNA library in 2002²⁷¹. The best-known role of Rspo3 is the potentiation of the Wnt/ β -catenin pathway. Rspo3 interferes with E3 ubiquitin ligase zinc and ring finger 3 (ZNRF3) and ring finger protein 43 (RNF43)-mediated negative feedback loop. This further leads to a failure in the degradation of LRP5/6 and Fz receptors, which enhances Wnt signaling²⁷². Additionally, Rspo3 is involved in Wnt/PCP pathway, where it binds to heparan sulfate proteoglycan (HSPG) syndecan-4 and LGR4/5 and regulates head cartilage morphogenesis and gastrulation²⁷³.

In the liver, Rspo3 expression is previously understood to be restricted to the pericentral endothelial compartment²⁷⁴. Furthermore, LSEC-derived Rspo3 serves as a key regulator of liver metabolic zonation. Loss of Rspo3 from endothelial cells leads to perturbed expression of the pericentral marker Glutamine synthetase (GS), phenocopying Evi knockout (KO) mouse liver^{126,274}. Pathologically, targeting canonical Wnt signaling via RSPO3 blockade provides promising therapeutic effect against liver fibrosis²⁷⁵. This highlighted a pivotal role of Rspo3 and Wnt signaling in the process of liver fibrogenesis. However, a recent study showed that Rspo3 is specifically produced by endothelial cells. It further showed that beyond endothelial cells, HSC also express Rspo3 in a pericentral zoned manner²⁷⁶. Given that HSC act as the key player in liver fibrosis and the importance of Wnt signaling in controlling liver function, it is critical to unveil whether HSC-expressed Rspo3 plays a role in these processes. Further, despite roles of LSEC-expressed Rspo3 in the liver being well-characterized, the majority of these discoveries are based on the prevailing notion that Rspo3 functions as a Wnt potentiator. Wnt-independent roles of Rspo3 are largely unknown. Indeed, HSC-secreted Wnt ligands are dispensable for shaping liver zonation and the development of liver fibrosis²⁷⁷. Moreover, as a short-range signal, mechanisms of how Wnt signaling is involved in distant regulation remains elusive. Undoubtedly, further investigation on unknown roles of Rspo3, especially HSC-derived Rspo3 in the liver, is required.

2 Aim of the study

The pathological roles of hepatic stellate cells (HSC), especially during the development of liver fibrosis, have been well characterized. However, functions of HSC under physiological conditions, especially for maintaining liver homeostasis, remain elusive. A recent study applied a HSC-depletion model and showed that loss of HSC resulted in the downregulation of Wnt-related pathways in the liver. Yet, the functions of HSC-derived Wnt signaling have not been fully addressed. Given that HSC-secreted Wnt ligands have no effect on regulating liver functions, it is plausible that other Wnt-related molecules are responsible in this process. Herein, I hypothesized that HSC-expressed Rspo3 is involved in the regulation of physiological and pathological liver functions.

The aims of this thesis consequently were I) to identify functions of HSC-derived Rspo3 in the liver, II) to compare different effects between LSEC- and HSC-expressed Rspo3 in regulating liver functions, and III) to re-evaluate the physiological/fibrosis-independent roles of HSC in maintaining liver homeostasis.

3 Results

3.1 Characterization of Rspo3 expression in the liver

3.1.1 Enriching major hepatic cell populations from the mouse liver

While functions of LSEC-derived Rspo3 are well established, our knowledge about HSC-derived Rspo3 remains elusive. Single-cell transcriptomics of the liver mesenchyme during fibrosis highlighted the peri-central expression of Rspo3 in HSC²⁷⁶. To gain further insight into hepatic Rspo3 expression, the expression patterns of all major cell types of the mouse liver were investigated. (Figure 4a).

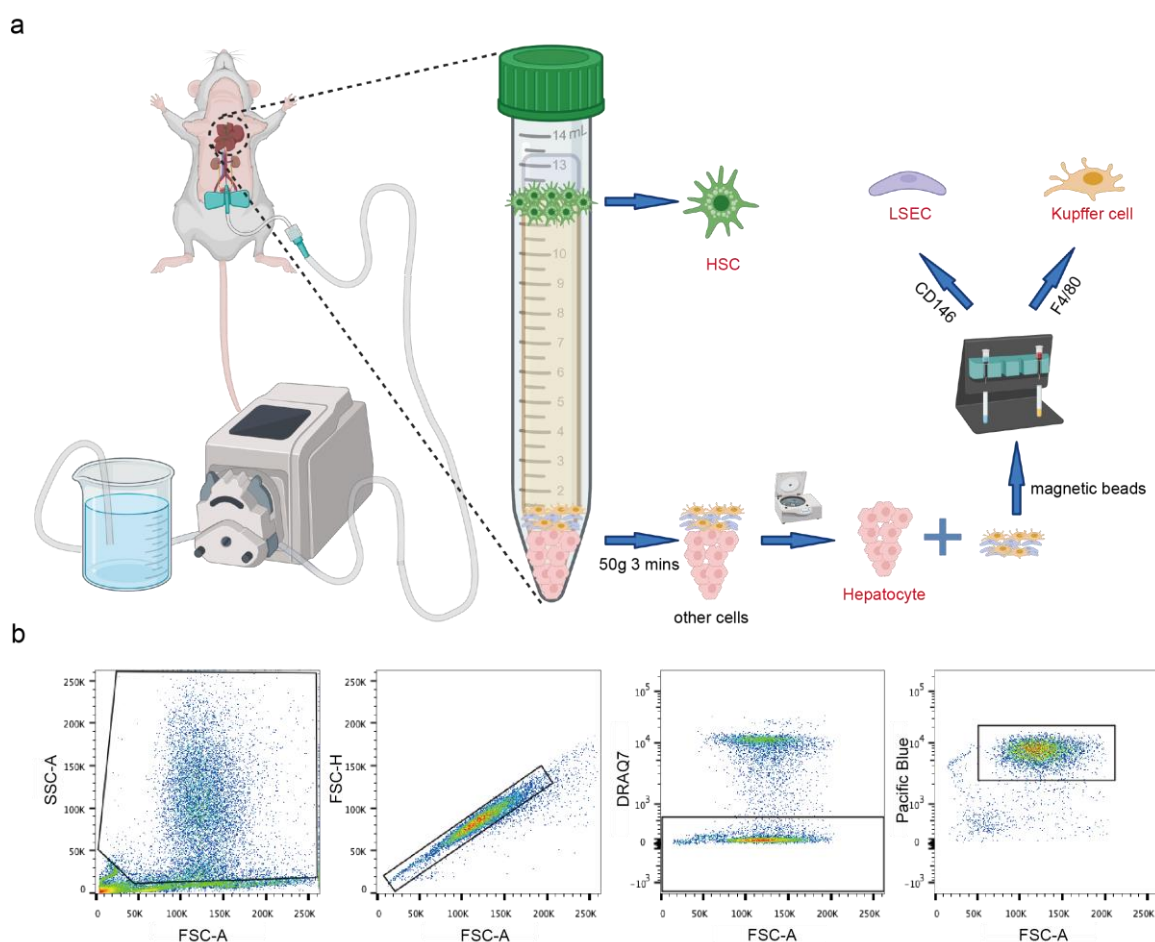


Figure 4. Scheme of isolating different liver cell types.

a) Mouse liver was perfused and digested using a peristaltic pump. HSC were first isolated by gradient centrifugation. Remaining cells were collected and hepatocytes were separated via low-speed centrifugation. Next, LSEC and KC were enriched by antibody-based magnetic beads. b) Gating strategy for HSC isolation. Enriched HSC were further purified by FACS sort via removal of debris, doublets, dead cells, and selected by their autofluorescence from vitamin A storage.

First, HSC were enriched via gradient centrifugation and Fluorescence-Activated Cell Sorting (FACS) (**Figure 4b**), taking advantage of vitamin A stored within their cytoplasm, based on a previously established protocol²⁷⁸. Thereafter, all remaining cells were collected, and hepatocytes were separated from other cells by low-speed centrifugation, benefiting from their large and heavy characteristics. Lastly, NPCs were isolated by ten minutes centrifugation followed by ACK incubation to lyse blood cells. LSEC and KC were positively selected from NPCs by antibody-coupled magnetic beads.

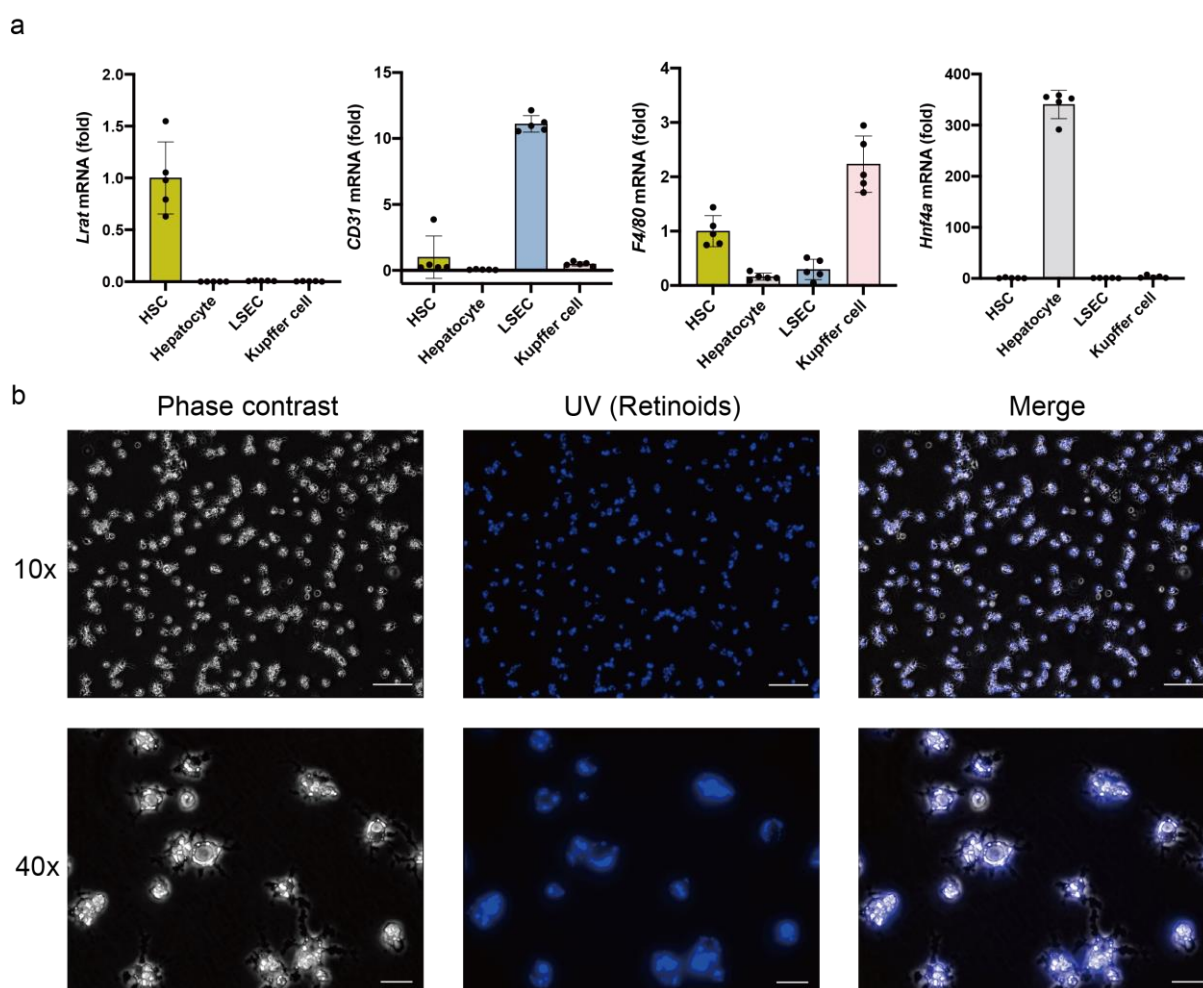


Figure 5. Purity of freshly isolated primary mouse HSC.

a) qPCR for landmark genes of different liver cell types *Lrat* (HSC), *CD31* (LSEC), *F4/80* (KC), and *Hnf4a* (Hepatocyte), normalized to *Actb*. b) Representative images of isolated HSC one day after seeding, visualized using phase contrast microscopy (left panel), retinoid fluorescence (middle panel), and merge (right panel). Scale bars, 10x: 100 μ m, 40x: 20 μ m.

To check the purity of isolated cells, expression of cell-specific markers was assessed by quantitative polymerase chain reaction (qPCR). Exclusive expression of landmark genes for each cell type (**Figure 5a**), indicating high purity of enriched cells. Additionally, autofluorescence of retinoids was detected in the cytoplasm of cells (**Figure 5b**). This signal rapidly bleached upon ultraviolet (UV) exposure, further confirming the ultra-purification of isolated HSC.

3.1.2 HSC are major sources of *Rspo3* in the liver

Next, expression of *Rspo3* in all four cell types was examined by qPCR. As expected, *Rspo3* was only expressed in LSEC and HSC, but not in hepatocytes and KC (**Figure 6a**). Notably, HSC expressed significantly higher levels of *Rspo3* than LSEC at the mRNA level. To further investigate the expression of *Rspo3* in LSEC and HSC at the protein level, Western blotting (WB) was performed with protein extracts from freshly isolated LSEC and HSC. Quantified bands showed higher albeit non-significantly different levels of *Rspo3* in HSC compared to LSEC (**Figure 6b-c**), suggesting that higher transcription did convert to higher protein amounts in HSC compared to LSEC. Nevertheless, these data showed that HSC represent unneglectable components of the *Rspo3* secretion in the liver. The high expression of *Rspo3* in HSC indicated that it may play critical roles in regulating liver functions.

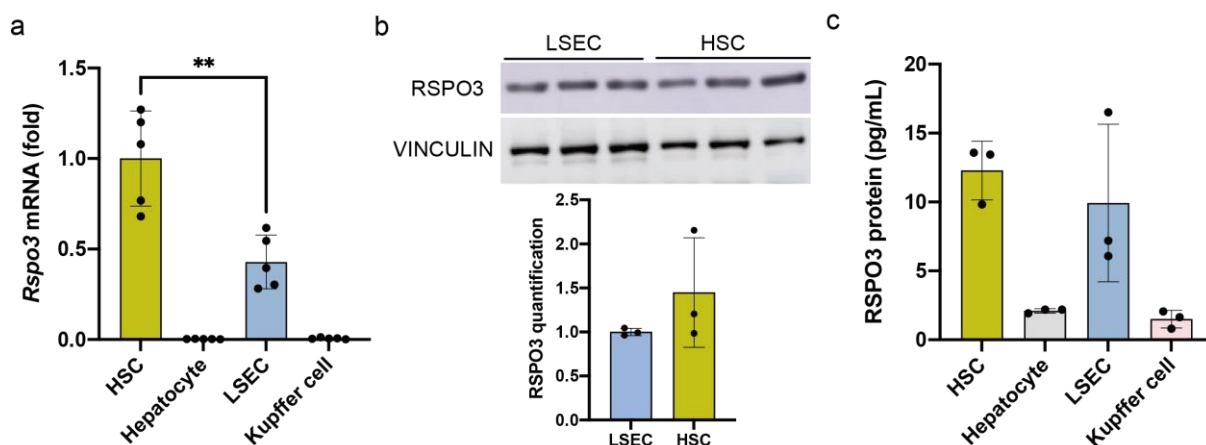


Figure 6. *Rspo3* expression in different liver cell types.

a) qPCR for *Rspo3* normalized to *Actb*. b) Western blot analysis of RSPO3 normalized to VINCULIN and quantification. c) RSPO3 ELISA in supernatants from primary mouse hepatic cells.

3.2 HSC-expressed *Rspo3* exerts distinct functions in shaping liver homeostasis

3.2.1 Generation and characterization of conditional HSC-specific *Rspo3* deficient mice

To unveil the functional consequences of HSC-expressed *Rspo3*, I generated HSC-specific *Rspo3* deficient mouse model. Despite that *Lrat-Cre* (constitutive) being widely used for HSC-specific loss of function studies, I crossed *Rspo3* floxed mice with *Pdgfrb-P2A-CreERT2* mice (*Rspo3*^{ind-HSC}) (**Figure 7a**).

As a postnatal deletion model is necessary to avoid compensatory effect caused by constitutive Cre during development. This also enables the deletion of *Rspo3* at a specific time of interest. To validate recombinant efficacy, mice were treated with tamoxifen (TAM) for five consecutive days by oral gavage (**Figure 7b**). qPCR analysis of *Rspo3* from isolated HSC suggested that *Rspo3* was successfully deleted from HSC in both male and female mice (**Figure 7c**).

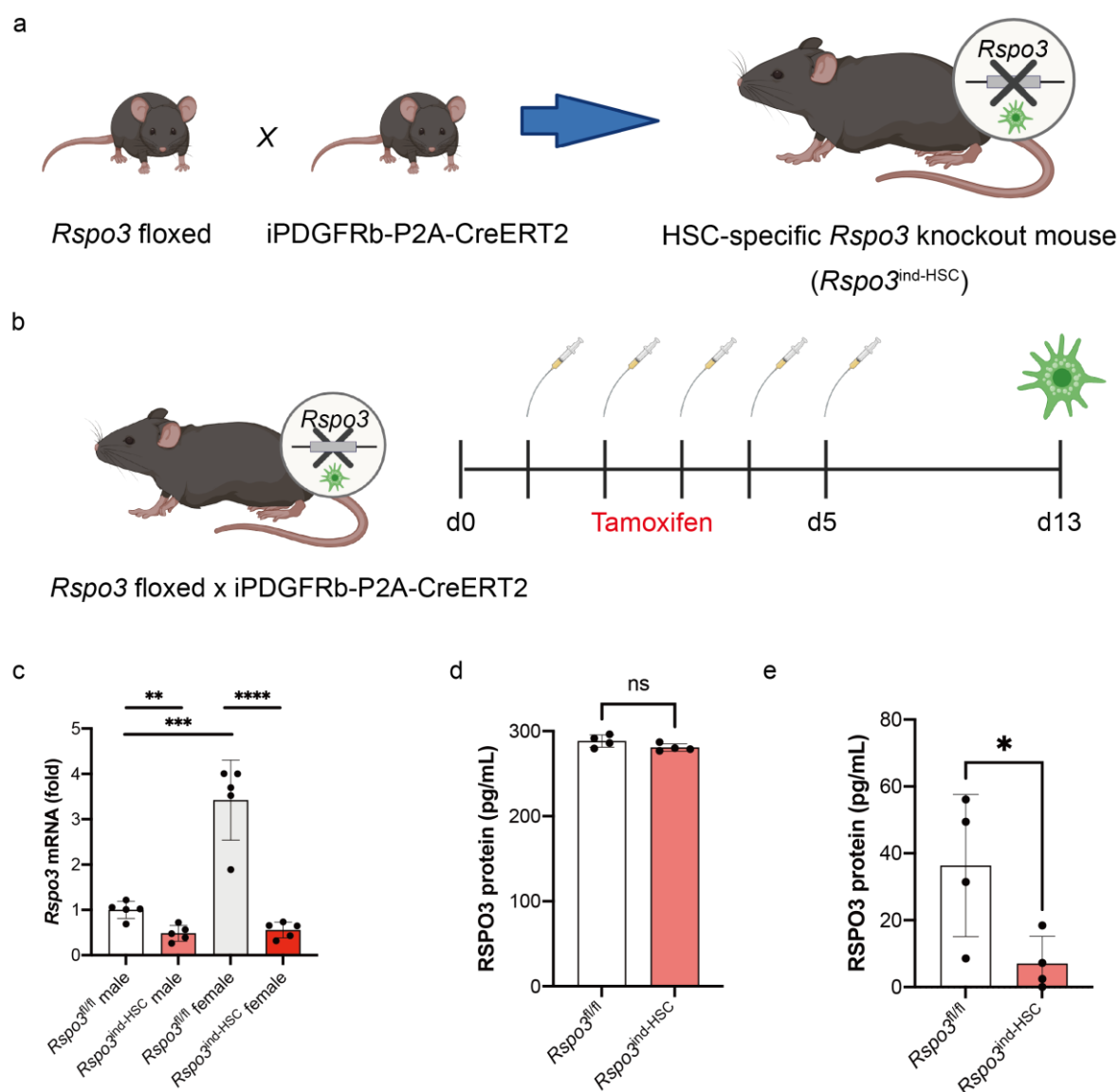


Figure 7. Generation of HSC-specific *Rspo3* inducible knockout mice.

a) Crossing scheme for generating *Rspo3*^{ind-HSC} mice. b) Schematic of tamoxifen-induced *Rspo3* deletion in HSC. c) qPCR for *Rspo3* normalized to *Actb* in male and female *Rspo3*^{ind-HSC} mice. d) ELISA for RSPO3 with protein from liver lysate in *Rspo3*^{ind-HSC} mice. e) ELISA for RSPO3 with supernatant of freshly isolated HSC cultured for 24 hours in *Rspo3*^{ind-HSC} mice.

Notably, although both genders showed comparable levels of *Rspo3* in *Rspo3*^{ind-HSC} animals, littermate control (*Rspo3*^{fl/fl}) female mice expressed higher *Rspo3* compared to *Rspo3*^{fl/fl} male mice. To further understand the contribution of HSC to total RSPO3 level, enzyme-linked immunosorbent assay (ELISA) of RSPO3 was performed with both proteins isolated from the liver lysate of snap-frozen lobe and supernatant of freshly isolated HSC that were cultured for 24 hours. Although RSPO3 protein levels were similar in liver lysates from *Rspo3*^{fl/fl} and *Rspo3*^{ind-HSC} mice (Figure 7d), supernatant from HSC of *Rspo3*^{ind-HSC} mice contained a significantly lower amount of RSPO3 than *Rspo3*^{fl/fl} mice (Figure 7e), suggesting a significant contribution of HSC-derived *Rspo3* to total RSPO3 protein levels.

3.2.2 LSEC- and HSC-expressed *Rspo3* do not compensate each other upon deletion and have no impact on the expression of LSEC-specific Wnt ligands in the liver

As both, LSEC and HSC express *Rspo3* in the liver, I further investigated whether *Rspo3* secretion is compensated upon its deletion in one cell type by the other. To this end, *Rspo3* expression was evaluated in HSC and LSEC after inducing HSC-specific *Rspo3* deletion. qPCR analysis of *Rspo3* in freshly isolated HSC showed significantly reduced *Rspo3* expression in HSC (Figure 8a). However, expression of *Rspo3* in LSEC did not change (Figure 8b).

Furthermore, LSEC-specific Wnt ligands, Wnt2 and Wnt9b, also showed comparable expression in LSEC from *Rspo3*^{ind-HSC} mice compared to *Rspo3*^{fl/fl} mice (Figure 8c). Next, HSC and LSEC were isolated from LSEC-specific *Rspo3* knockout mice (*Rspo3*^{iECKO}). Similarly, *Rspo3* expression was significantly reduced in LSEC (Figure 8e), whereas expression of Wnt2 and Wnt9b in LSEC (Figure 8f), as well as *Rspo3* in HSC, remained the same in *Rspo3*^{iECKO} compared to *Rspo3*^{fl/fl} mice (Figure 8d).

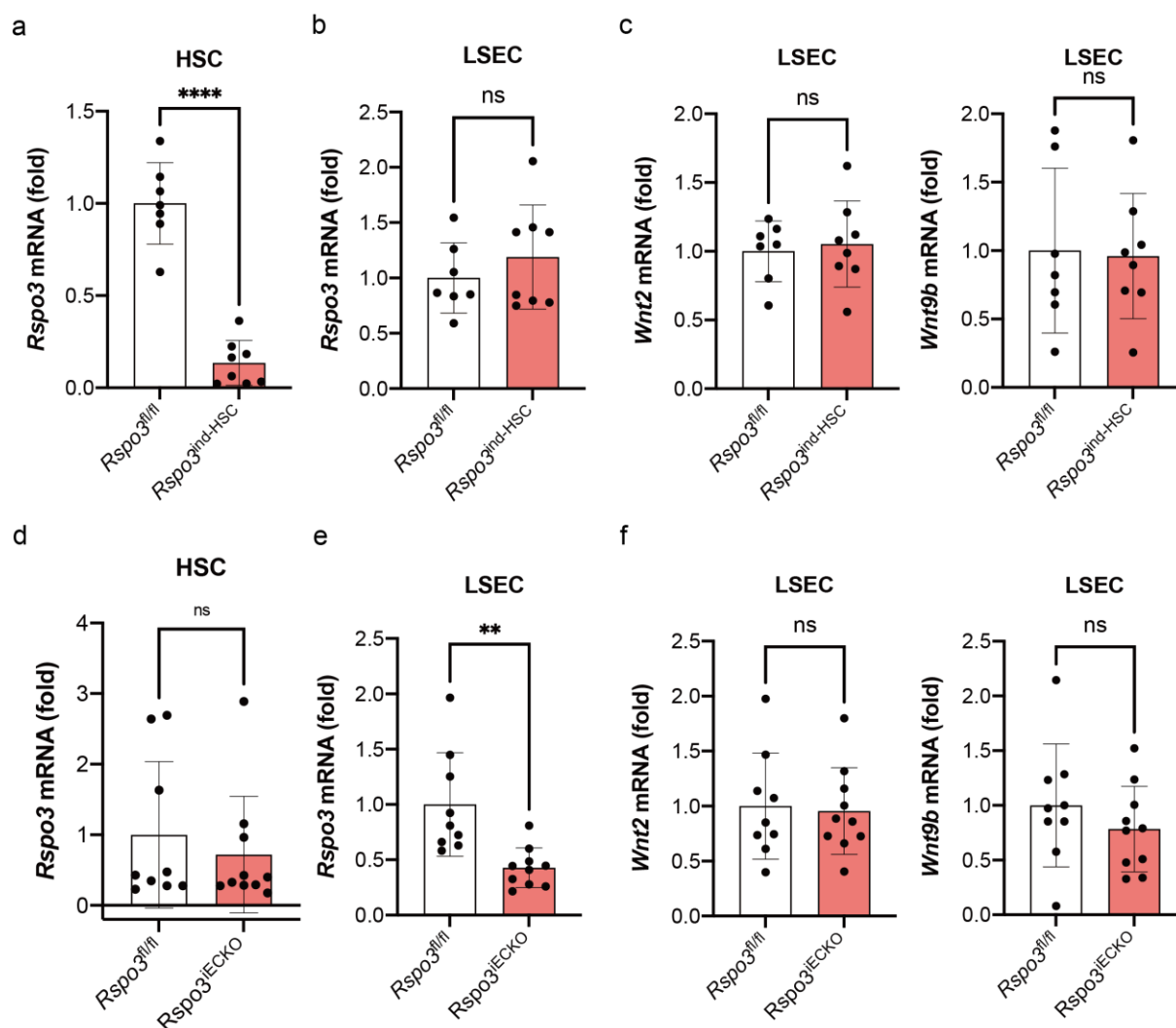


Figure 8. Deletion of HSC-specific *Rspo3* does not affect *Rspo3*-dependent angiocrine regulation and vice versa.

a) qPCR of *Rspo3* normalized to *Actb* in freshly isolated HSC from HSC knockout mice. b-c) qPCR of *Rspo3*, *Wnt2*, and *Wnt9b* normalized to *Actb* in freshly isolated LSEC from HSC knockout mice. d) qPCR of *Rspo3* normalized to *Actb* in freshly isolated HSC from EC knockout mice. e-f) qPCR of *Rspo3*, *Wnt2*, and *Wnt9b* normalized to *Actb* in freshly isolated LSEC from EC knockout mice.

3.2.3 HSC-specific *Rspo3* knockout mice show normal liver histology

First, I evaluated the role of HSC-derived *Rspo3* deletion in liver homeostasis. Plasma biochemistry analyses of alanine aminotransferase (ALT) and aspartate transaminase (AST) showed similar levels of both factors between two groups, indicating comparable liver function between *Rspo3^{fl/fl}* and *Rspo3^{ind-HSC}* animals (**Figure 9a**). Hematoxylin-Eosin (H&E) and Sirius Red (SR) staining on liver sections also showed normal histology with no fibrosis or inflammation (**Figure 9b**). Furthermore, to gain a better insight into the consequences of HSC-specific *Rspo3* deletion, number of HSC, status of HSC activation, and hepatocyte proliferation were further investigated by Desmin, alpha-smooth muscle actin (α SMA), and proliferating cell nuclear antigen (PCNA) staining. A comparable number and identical activation status of HSC was observed upon HSC-specific *Rspo3* deletion, indicated by immunohistochemistry (IHC) of Desmin and α -SMA (**Figure 9c**). Comparable numbers of proliferating hepatocytes at normal condition were identified by IHC staining of PCNA (**Figure 9d**). However, the liver-to-body weight ratio analysis showed a significantly reduced liver size in *Rspo3^{ind-HSC}* compared to *Rspo3^{fl/fl}* controls (**Figure 9e**). Taken together, deletion of *Rspo3* from HSC did not lead to an overt liver phenotype in histology, albeit resulting in decreased liver size in *Rspo3^{ind-HSC}* mouse.

3.2.4 HSC-specific *Rspo3* knockout mice shows different liver zonation pattern compared to LSEC-specific *Rspo3* knockout mice

Endothelial *Rspo3* is a key determinant of hepatic zonation²⁷⁴. Therefore, I sought to determine whether HSC-derived *Rspo3* has a similar function as LSEC-derived *Rspo3* in regulating liver zonation. As landmark genes for hepatocyte and LSEC zonation, GS and thrombomodulin (Thbd) were chosen, respectively, to examine potential effect of HSC-specific *Rspo3* deletion on liver zonation. Unlike LSEC-expressed *Rspo3*, HSC-specific deletion of *Rspo3* did not affect hepatocyte zonation as previously described, suggested by intact peri-central zonation of GS (**Figure 10a**). Concurrently, immunostaining of Thbd also showed a comparable pattern between *Rspo3^{fl/fl}* and *Rspo3^{ind-HSC}* mice (**Figure 10a**). Nevertheless, as a recent publication reported a potential role of HSC in regulating zone 2 hepatocytes²⁷⁹, I further examined two cytochrome P450 oxidases (*Cyp1a2* and *Cyp2e1*) that are enriched in zone 2 and zone 3. Interestingly, ablating *Rspo3* from HSC led to a significant change in the two genes (**Figure 10b**). Notably, LSEC-specific *Rspo3* deletion did not lead to similar changes in these two genes (**Figure 10c**). Next, since ectopic regulation of Wnt signaling showed indirect effect on zone 1 metabolism^{280,281}, I examined whether HSC-specific *Rspo3* deletion impacts also zone 1 metabolic zonation. Contrary to the downregulation of metabolic genes in zone 3, zone 1 genes, including *Cyp2f2* and *Hal*, were found significantly enhanced upon HSC-specific *Rspo3* deletion (**Figure 11a**), but remained the same after LSEC-specific *Rspo3* deletion (**Figure 11b**). Collectively, these results suggested that HSC-derived *Rspo3* regulates liver zonation differently from LSEC-expressed *Rspo3*.

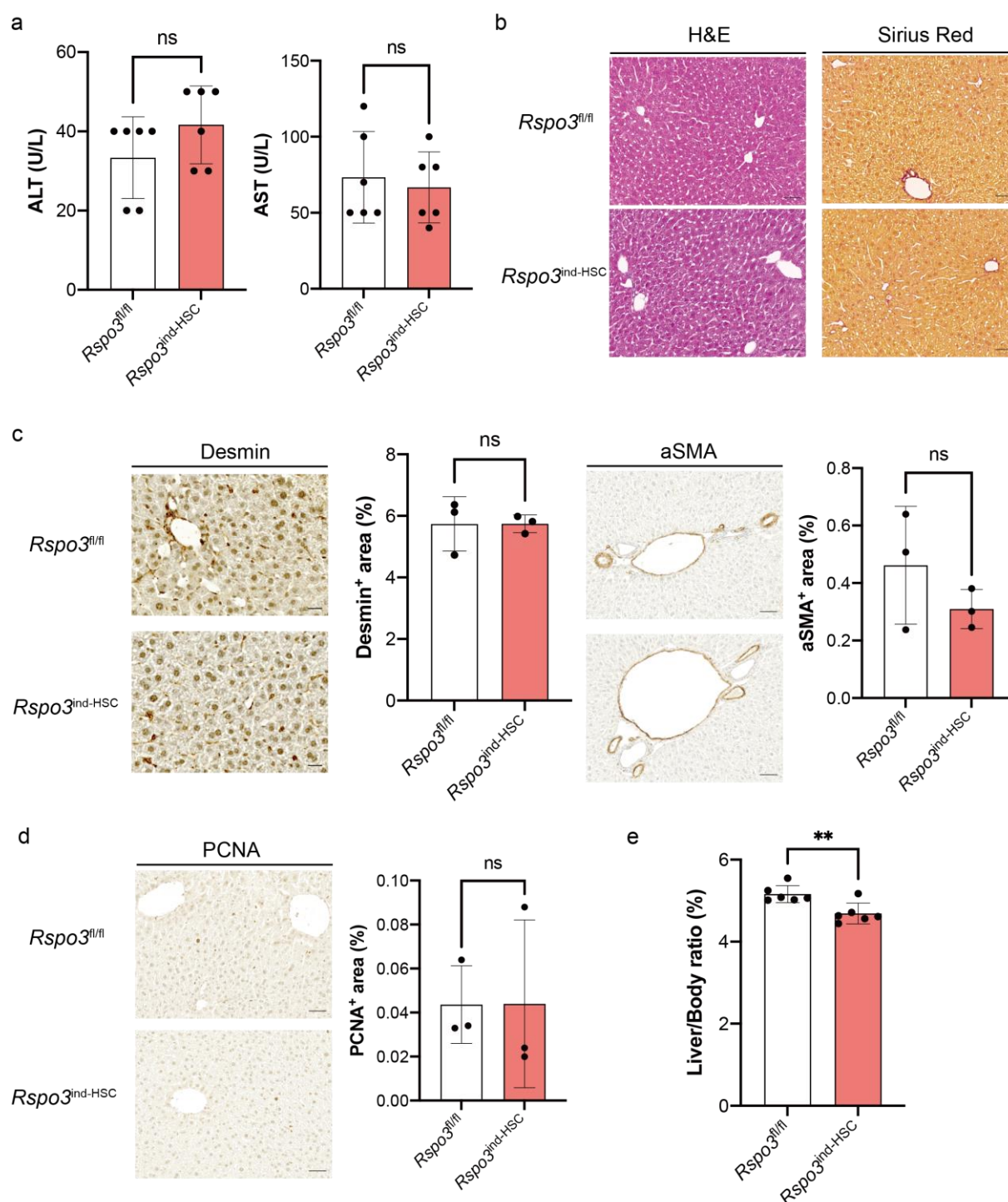


Figure 9. Characterization of *Rspo3*^{ind-HSC} mouse livers.

a) Plasma biochemistry of ALT and AST levels of *Rspo3*^{ind-HSC} compared to *Rspo3*^{fl/fl} mice. b) H&E and Sirius Red staining of the liver. c) IHC staining of desmin and α -SMA with quantification. d) IHC staining of PCNA and quantification. e) Liver-to-body weight ratio of *Rspo3*^{ind-HSC} mice and their *Rspo3*^{fl/fl} littermates. Scale bars, 100 μ m.

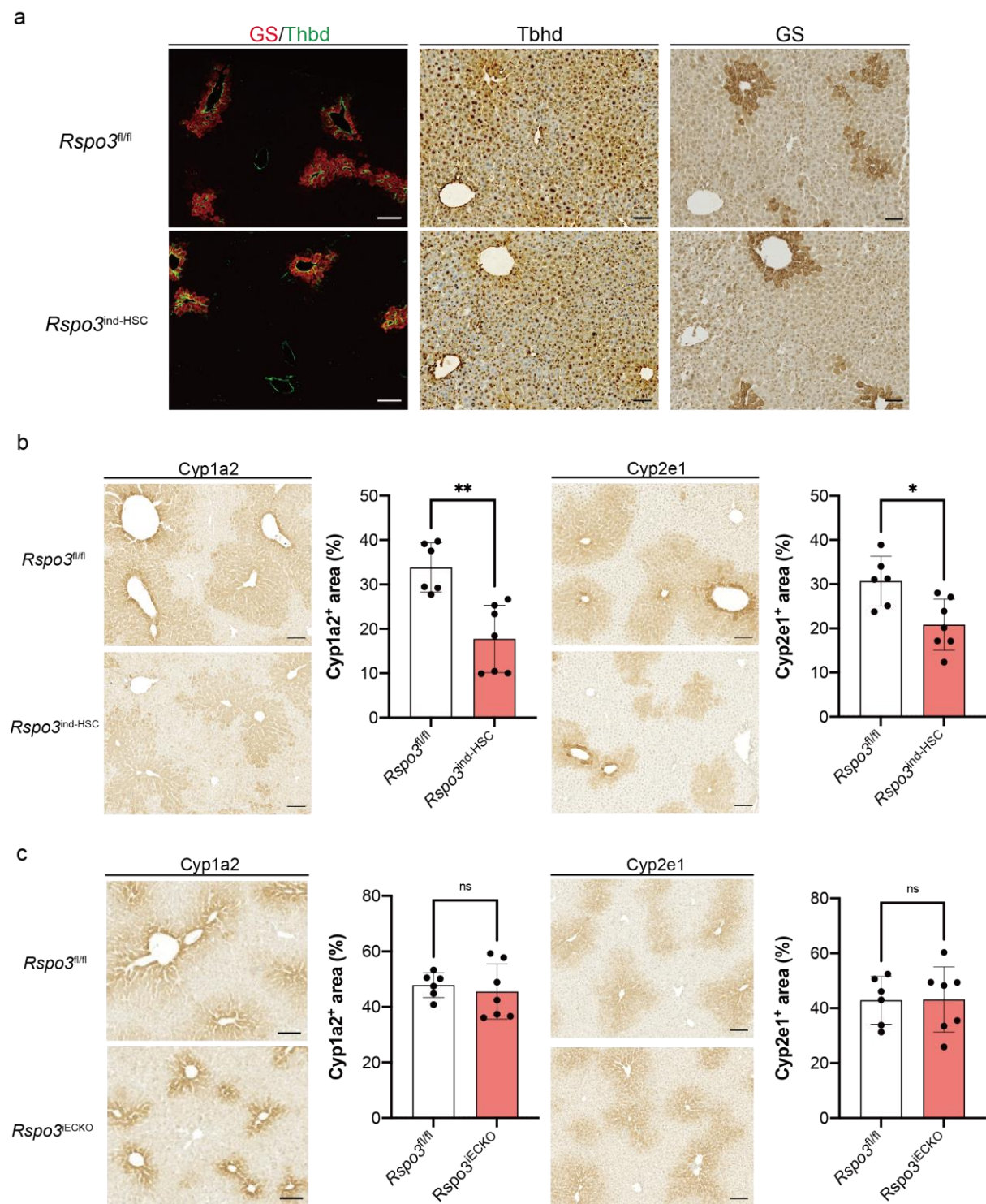


Figure 10. Characterization of zone 2 and zone 3 liver zonation of *Rspo3*^{ind-HSC} mice.

a) Immunostaining of GS and Tbh in *Rspo3*^{ind-HSC} mice. b) IHC of Cyp1a2 and Cyp2e1 in *Rspo3*^{ind-HSC}. c) IHC of Cyp1a2 and Cyp2e1 in *Rspo3*^{iECKO} mice. Scale bars, 100 μ m.

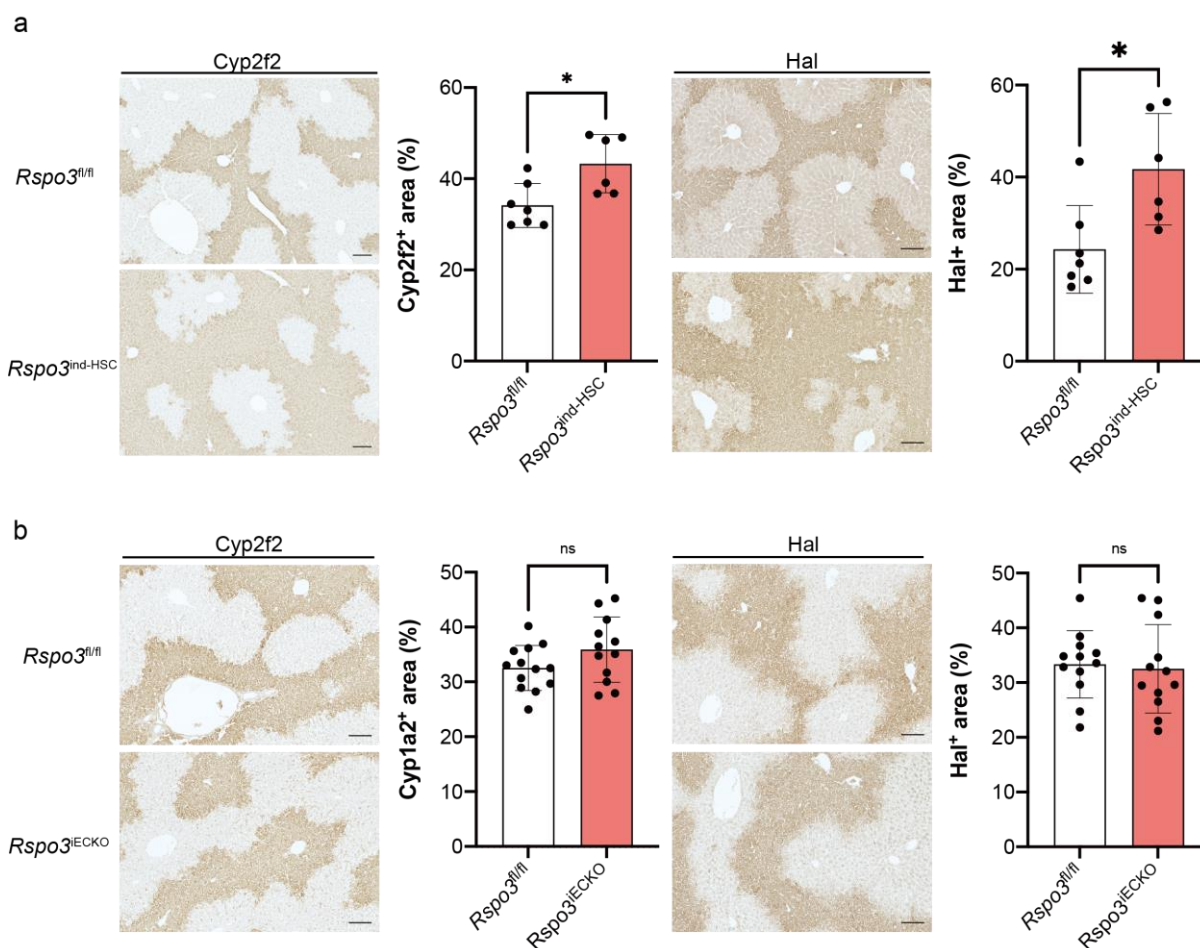


Figure 11. Characterization of zone 1 liver zonation of *Rspo3^{ind-HSC}* mice.

a) IHC of Cyp2f2 and Hal in *Rspo3^{ind-HSC}* mice. b) IHC of Cyp2f2 and Hal in *Rspo3^{iECKO}* mice. Scale bars, 100 μ m.

3.2.5 HSC-specific *Rspo3* deletion inhibits liver regeneration after partial hepatectomy

Multiple studies have suggested potential roles of different R-spondins in regulating cell proliferation and liver regeneration²⁸²⁻²⁸⁴. Moreover, HSC are also known to play critical roles during liver regeneration^{77,285,286}. Hence, it is conceivable to hypothesize that HSC-derived *Rspo3* may act as one of the key players in regulating liver regeneration. To validate this hypothesis, 2/3 PHx was performed on *Rspo3^{ind-HSC}* mice and their *Rspo3^{fl/fl}* littermates. As the liver reaches the proliferation peak within 48 hours, livers from operated animals were examined on the second day after PHx. A reduction of the percentage of Ki67⁺ hepatocytes was observed (**Figure 12a**). As a result, the liver-to-body weight ratio was significantly reduced in *Rspo3^{ind-HSC}* mice two days after PHx (**Figure 12b**). These data suggested an inhibited liver regeneration and less proliferating hepatocytes in *Rspo3^{ind-HSC}* mice compared to *Rspo3^{fl/fl}* control mice in the PHx hepatocyte regeneration model.

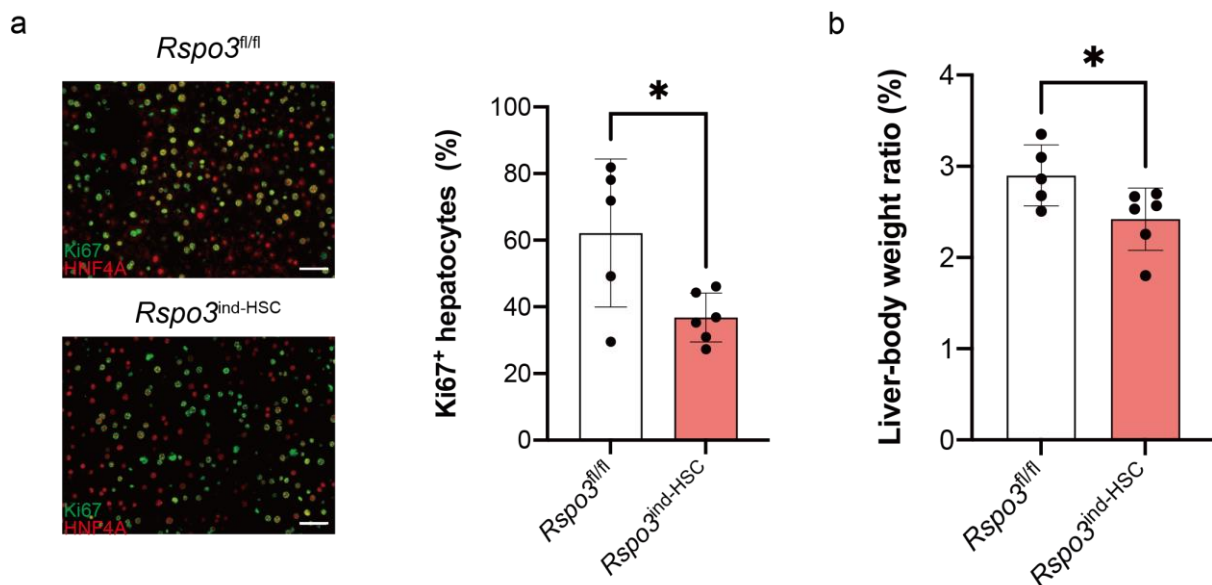


Figure 12. HSC-specific *Rspo3* deletion inhibits hepatocyte proliferation and liver regeneration after PHx.

a) Immunofluorescence staining of Ki67 and HNF4A of the liver at d2 after PHx. Scale bars, 100 μ m. b) Liver-to-body weight ratio at d2 after 2/3 PHx.

3.3 HSC-expressed *Rspo3* is a key regulator in the pathogenesis of liver diseases

HSC-related functions of *Rspo3* in the liver have so far been linked to two distinct processes, i) the activation and proliferation of HSC *in vitro*²⁸⁷, and ii) pro-fibrotic roles in CCl₄-induced liver fibrosis *in vivo*²⁷⁵. However, none of these published data further clarify the source of *Rspo3* with corresponding functions in the liver. Moreover, established functions of LSEC-expressed *Rspo3* are not consistent with these two findings^{127,273,274,288}. To this end, I hypothesized that HSC-derived *Rspo3* might be a key regulator of above-reported phenotypes. To validate this hypothesis *in vivo*, I subjected HSC-specific *Rspo3* knockout mice to different experimental challenges that activate HSC or induce liver fibrosis.

3.3.1 HSC-specific *Rspo3* deletion protects the liver from CCl₄-induced liver fibrosis

Among the experiment models in mice that induce HSC activation, CCl₄-induced fibrosis represents the most reproducible and most efficient experimental model to induce liver fibrosis^{175,289}. Hence, a single dose of CCl₄ was administered to mice by oral gavage after TAM-induced deletion of *Rspo3* from HSC to investigate the roles of HSC-derived *Rspo3* upon acute liver damage (**Figure 13a**). Two days after CCl₄ treatment, liver and plasma samples were collected for further analysis. Plasma biochemistry analyses of ALT and AST showed a significant increase of both markers after CCl₄ treatment, indicating successful induction of liver damage (**Figure 13b**). Remarkably, both ALT and AST levels were significantly reduced in *Rspo3^{ind-HSC}* mice, suggesting a less damaged liver in *Rspo3^{ind-HSC}* animals compared to their littermate controls (**Figure 13b**). Notably, although H&E and SR staining of

liver samples showed comparable histology and initiation of peri-central fibrosis (**Figure 13c**), reduced α -SMA⁺ areas were observed in *Rspo3*^{ind-HSC} compared to *Rspo3*^{fl/fl} mice, pointing towards a reduced activation of HSC after acute liver injury (**Figure 13d**). On the other hand, IHC for PCNA exhibited reduced PCNA⁺ cells in *Rspo3*^{ind-HSC} compared to *Rspo3*^{fl/fl} livers (**Figure 13e**). As PCNA levels may increase as a result of growth factor or DNA damage²⁹⁰, another cell proliferation marker, Ki67, was examined. Surprisingly, immunostaining of Ki67 indicated the absence of proliferating hepatocytes in both groups (**Figure 13f**). Taken together, these data indicated a protective phenotype of HSC-specific *Rspo3* deletion upon acute CCl₄ challenge.

To evaluate these findings in a chronic setting, CCl₄ was administered twice per week for four weeks, and hepatic parameters were assessed (**Figure 14a**). Consistent with the one-dose administration approach, liver function in *Rspo3*^{ind-HSC} was improved compared to *Rspo3*^{fl/fl} mice, which was evidenced by reduced plasma levels of ALT and AST two days after the last treatment (**Figure 14b**) and corroborated by histological analyses. H&E and SR staining showed abundant scar tissue, specifically close to peri-central regions of *Rspo3*^{fl/fl} livers, which are barely observed or completely absent in the livers of *Rspo3*^{ind-HSC} mice (**Figure 14c**). To evaluate whether fibrosis developed in one-month treated livers, Masson Trichrome (MT) staining was performed, highlighting a markedly reduced fibrotic area in the livers of *Rspo3*^{ind-HSC} mice compared to *Rspo3*^{fl/fl} mice (**Figure 14d**). Notably, several dark orange regions were marked near the fibrotic areas in SR staining of *Rspo3*^{fl/fl} livers. For a better identification of these regions, I performed *in situ* detection of apoptosis, Apop Tag, to validate the possibility that darker orange regions may be regions undergoing apoptosis. As expected, Apop Tag⁺ regions were identified in both, *Rspo3*^{fl/fl} and *Rspo3*^{ind-HSC} livers, with reduced apoptotic areas in *Rspo3*^{ind-HSC} group (**Figure 14e**). Interestingly, beyond hepatocytes, more apoptotic HSC were found in *Rspo3*^{ind-HSC} mice, which were less frequently observed in the livers of *Rspo3*^{fl/fl} mice (**Figure 14f**). As more profound fibrosis was identified in *Rspo3*^{fl/fl} mice, I quantified the collagen deposition in these mice. Hydroxyproline assays of plasma samples indicated a more than three-fold increase of collagen in *Rspo3*^{fl/fl} compare to *Rspo3*^{ind-HSC} mice (**Figure 14g**). Moreover, Differential Interference Contrast (DIC) microscopy technique revealed the existence of both, type I and type III collagen, indicated by red-yellow and green fibers, respectively (**Figure 14h**).

Results

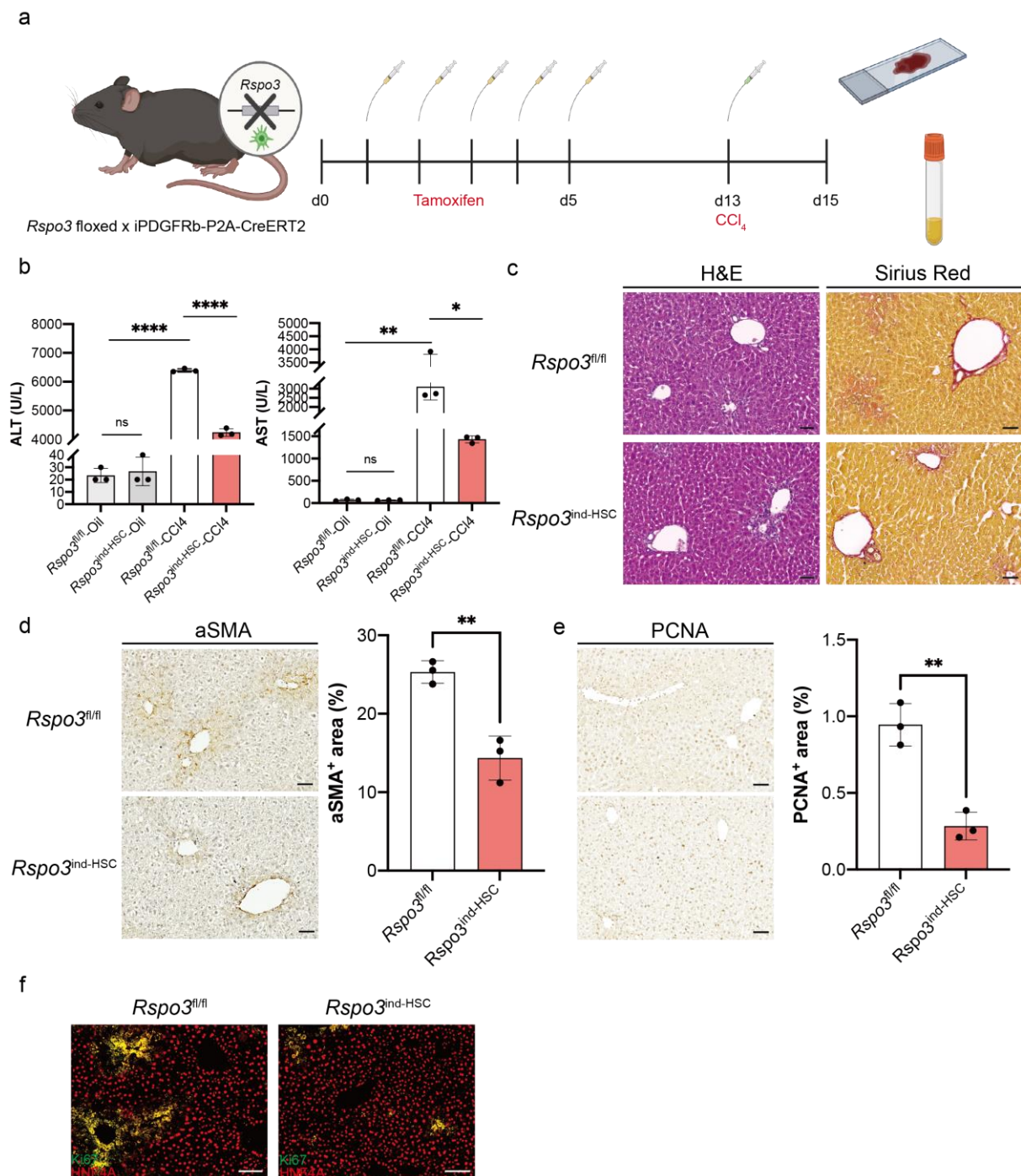


Figure 13. HSC-specific *Rspo3* deletion protects the liver from CCl₄-induced acute liver injury.

a) Experimental scheme of single dose CCl₄ administration. b) Plasma biochemistry of ALT and AST levels of *Rspo3*^{ind-HSC} compared to *Rspo3*^{fl/fl} mice. c) H&E and Sirius Red staining of the liver. d) IHC staining of α-SMA with quantification. e) IHC staining of PCNA with quantification. f) Immunofluorescence staining of Ki67 and HNF4A of liver at d2 after single dose CCl₄ administration. Scale bars, 100 μm.

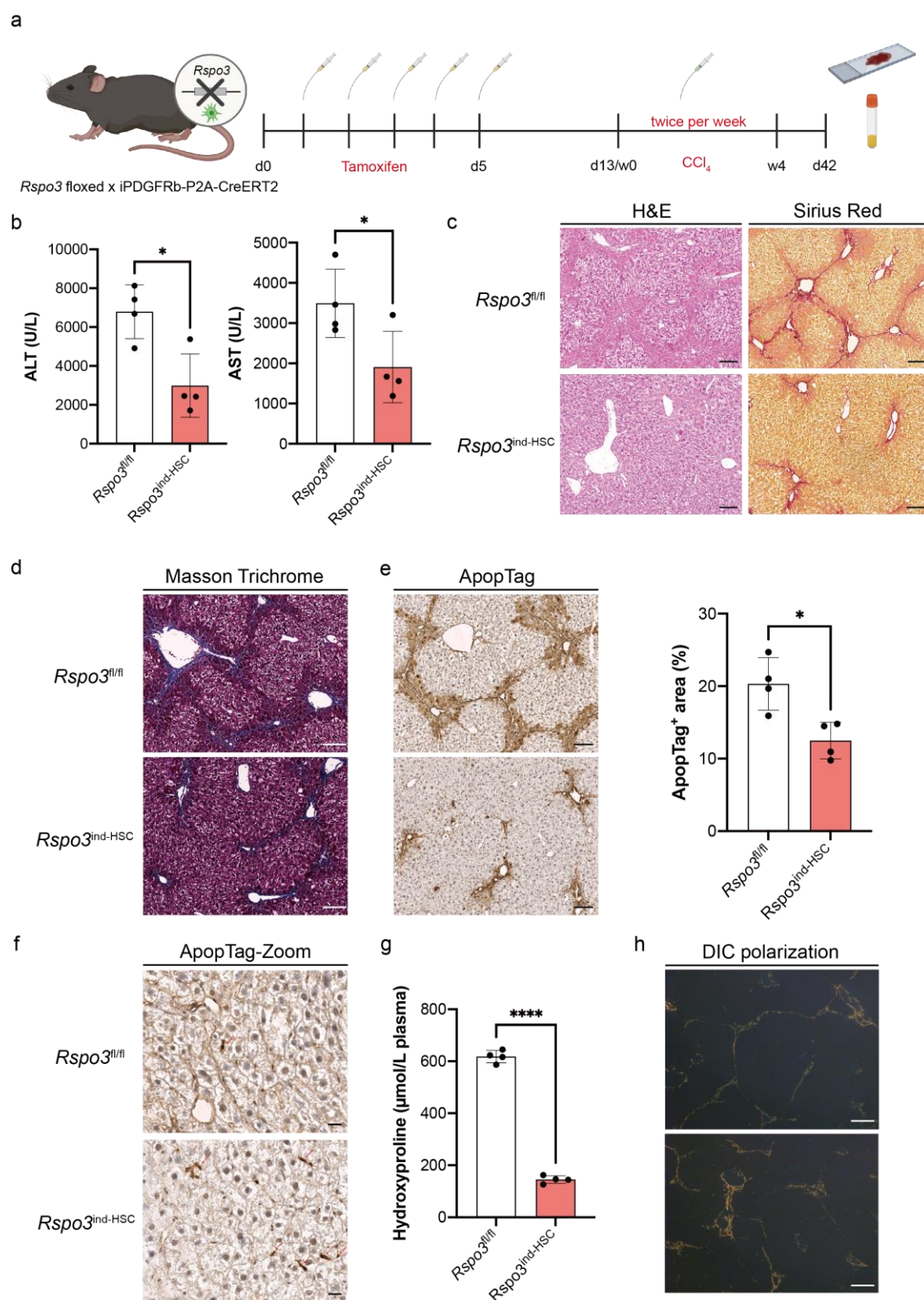


Figure 14. HSC-specific *Rspo3* deletion protects the liver from CCl_4 -induced chronic injury

a) Experimental scheme of multiple doses of CCl_4 administration. b) Plasma biochemistry of ALT and AST levels of *Rspo3*^{Ind-HSC} compared to *Rspo3*^{fl/fl} mice. c) H&E and Sirius Red staining of the liver. d) Masson Trichrome staining of the liver. e) ApopTag Peroxidase *in situ* apoptosis detection with quantification. f) Zoomed image of ApopTag for apoptotic HSC detection (red arrows). g) Hydroxyproline assay of plasmas. g) DIC polarization photos of Sirius Red-stained liver. Scale bars, 100 μm ; Zoom, 20 μm .

Considering the regeneration capacity of the liver, I also collected plasma and liver samples seven days after the last CCl₄ administration to evaluate whether the protective phenotype of HSC-specific *Rspo3* deletion was long-lasting or only a transient effect (**Figure 15a**). Plasma biochemistry of ALT and AST levels returned to normal in both groups (**Figure 15b**), indicating that liver function had recovered to an almost normal stage. Nevertheless, SR staining of the liver still suggested the existence of fibrosis, to a lesser extent in *Rspo3*^{ind-HSC} mice (**Figure 15c**).

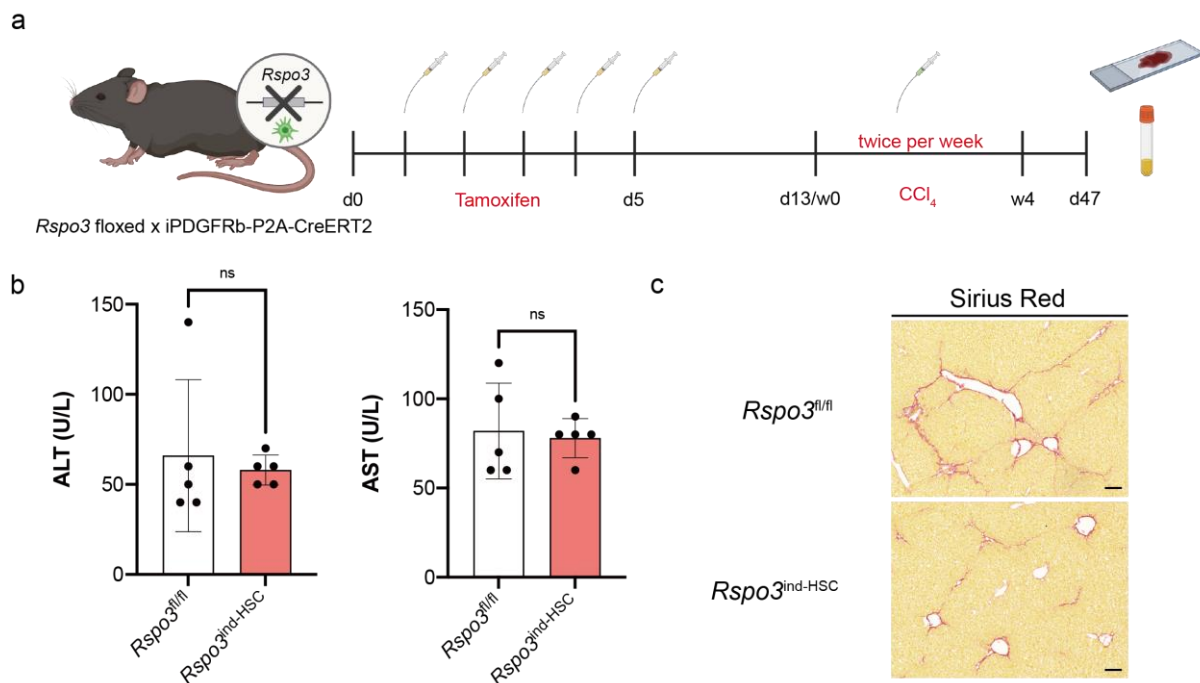


Figure 15. Recovery of the liver from long-term CCl₄ administration.

a) Experimental scheme of liver recovery after multiple CCl₄ administration. b) Plasma biochemistry of ALT and AST levels of *Rspo3*^{ind-HSC} compared to *Rspo3*^{fl/fl} mice. c) Sirius Red staining of the liver. Scale bars, 100 μ m.

3.3.2 Ablation of HSC-derived *Rspo3* prevents liver cirrhosis and neoplastic transformation

Over time, chronic liver damage such as liver fibrosis may further develop and exacerbate to liver cirrhosis and liver cancer. Hence, I investigated whether blockade of HSC-derived *Rspo3* has similarly a protective effect in the development of cirrhosis and, especially, liver cancer. To this end, I further extended the duration of CCl₄ administration to twenty weeks, when the liver typically displays tumorous foci. Two days after the last CCl₄ treatment, mice were sacrificed and their livers were examined (**Figure 16a**). As expected, an evaluation of the liver anatomy suggested higher inflammation and more developed fibrosis in *Rspo3*^{fl/fl} compared to *Rspo3*^{ind-HSC} liver (**Figure 16b**). Additionally, a significantly higher amount of collagen was deposited in the livers of *Rspo3*^{fl/fl} compared to *Rspo3*^{ind-HSC} mice, as evidenced by SR staining (**Figure 16c**). Most importantly, *Rspo3*^{ind-HSC}

mice displayed a marked reduction in the number of foci compared to the livers from *Rspo3^{fl/fl}* control mice (**Figure 16d-e**). These data support the hypothesis that blockade of HSC-derived *Rspo3* signaling may prevent the development of liver fibrosis and neoplastic transformation during CLD.

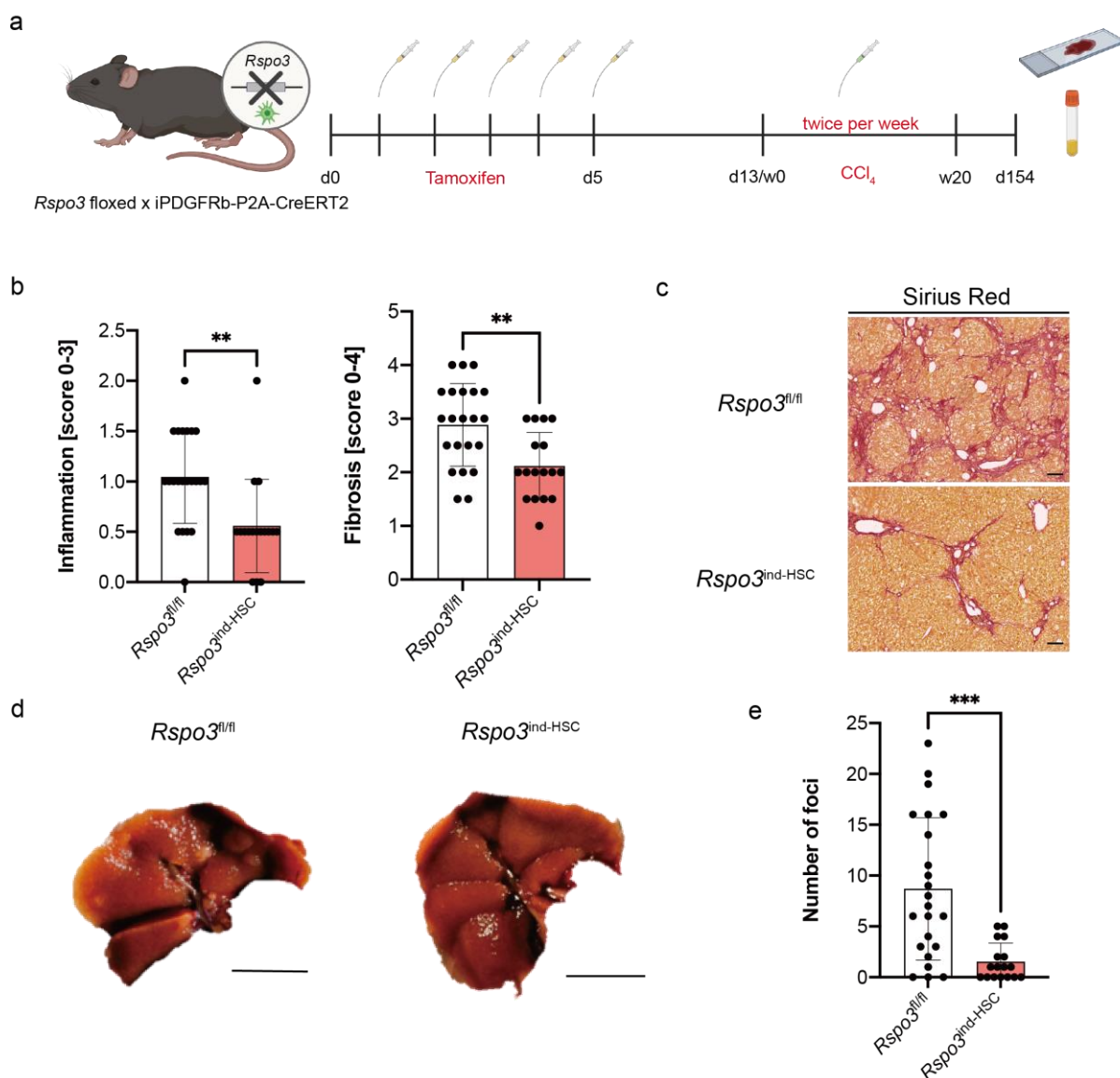


Figure 16. HSC-expressed *Rspo3* regulates neoplastic transformation in the liver.

a) Experimental scheme of CCl₄-induced liver cancer. b) Evaluation of inflammation and fibrosis of the liver. c) Sirius Red staining of the liver. d) Representative images of foci in the liver. Scale bars, 100 μ m; Photos, 1 cm. e) Number of foci in the liver.

3.3.3 HSC-expressed *Rspo3* is dispensable in cholestasis-induced liver damage

Besides hepatotoxins like CCl₄, surplus deposition of bile can also cause cholestatic liver damage. Bile duct ligation (BDL) is a widely used experimental model to study cholestasis-induced liver damage. To unveil potential roles of HSC-derived *Rspo3* in cholestatic liver injury, I performed BDL with C57BL/N wild-type mice to gain an overview of transcriptional dynamics of HSC-expressed *Rspo3* at different

time points after ligation (**Figure 17a**). Already one day after BDL, a drastic downregulation of *Rspo3* levels in HSC was observed that was accompanied by jaundice and an enlargement of gall bladder (**Figure 17b**). *Rspo3* expression levels remained low until they increased again when animals reached the humane endpoint (day 17 in this experiment) (**Figure 17c**). Liver damage caused by excessive accumulation of bile was reflected by elevated plasma ALT and AST levels, which at day 17 post-surgery started to revert again (**Figure 17d**). However, these changes did not lead to recovery or alleviation of sickness, as evidenced by more severe jaundice and further accumulation of bile inside swelling gall bladder.

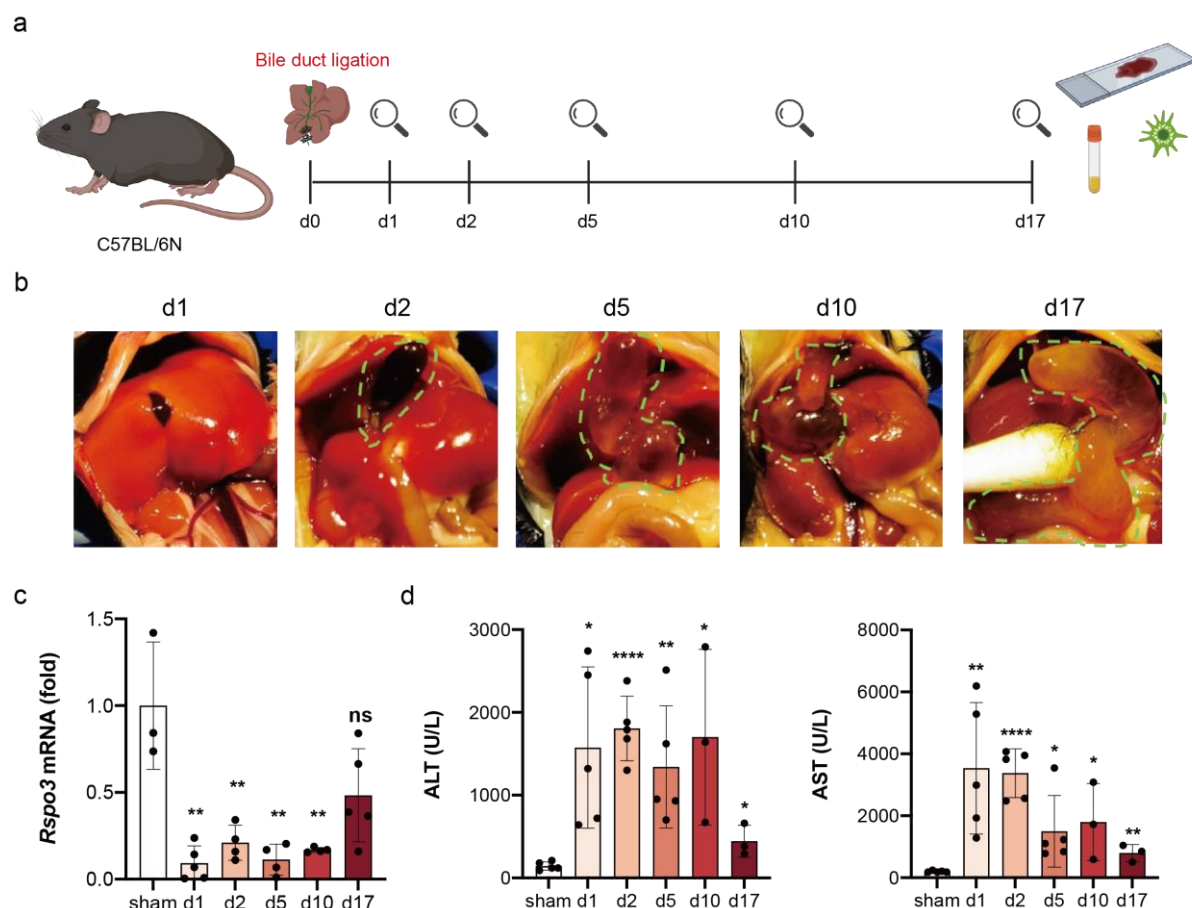


Figure 17. Evaluation of C57BL/6N mice subjected to bile duct ligation.

a) Experimental scheme of experimental approach for bile duct ligation. b) Representative photos of bile duct ligated liver at each time point with expanded gallbladder (green dashes) c) qPCR for *Rspo3* normalized to *Actb*. d) Plasma biochemistry of ALT and AST levels of mice subjected to bile duct ligation.

Next, I subjected HSC-specific *Rspo3* KO mice to BDL and euthanized them at two different time points: d5 when HSC are not actively involved, and d21 (humane end point is reached 4 days later than the previous experiment), based on *Rspo3* expression dynamics in C57BL/6N mice and previous experience⁹⁵. Plasmas and livers were collected for further analysis (**Figure 18a**). An increased, albeit comparable level of plasma ALT and AST were detected in *Rspo3*^{fl/fl} and *Rspo3*^{ind-HSC} mice at day 5 and

21 (**Figure 18b**). SR staining of the livers showed accumulation of bile into the liver at day 5, and an active proliferation of bile duct at day 21 (**Figure 18c,d**). However, no differences were observed between the two groups for either analysis (**Figure 18b-d**), indicating that HSC-expressed *Rspo3* did not play a role in regulating cholestatic liver damage in the presented model.

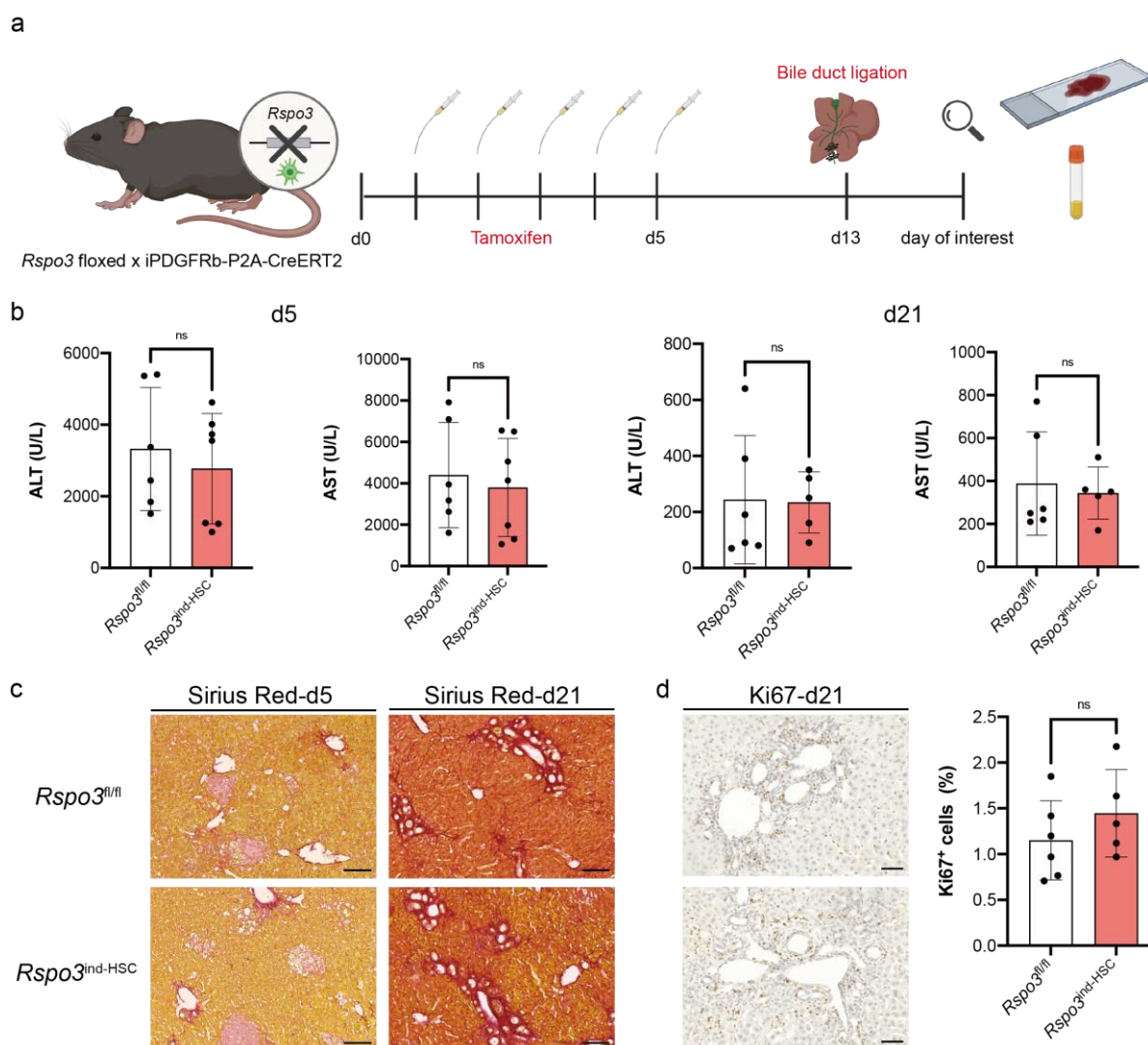


Figure 18. HSC-derived *Rspo3* is dispensable in cholestasis-induced liver damage.

a) Experimental scheme of experimental approach for bile duct ligation. b) Plasma biochemistry of ALT and AST levels of mice subjected to bile duct ligation at d5 and d21. c) Sirius Red staining of the liver. d) IHC staining of Ki67 of the liver at d21 after bile duct ligation with quantification. Scale bars, 100 μ m.

3.3.4 Loss of HSC-specific *Rspo3* alleviates APAP-induced acute liver injury

As deletion of *Rspo3* from HSC showed alleviated damage in CCl_4 model (peri-central liver injury model) but no effect in BDL model (peri-portal liver injury model), I hypothesize that HSC-derived *Rspo3* may only play critical functions during peri-central liver damage. Hence, to further support my previous findings, I treated mice with APAP, which induces oxidative stress and causes necrosis in peri-central regions of the liver. For this purpose, mice were fasted overnight and treated with APAP via oral

gavage at a semi-lethal dosage (300mg/kg) (**Figure 19a**). One day after the treatment, plasmas and livers were collected for further investigation. In line with CCl₄ treatments, plasma ALT and AST levels were significantly reduced in *Rspo3*^{ind-HSC} compared to *Rspo3*^{fl/fl} mice (**Figure 19b**). Consistently, H&E staining of the liver showed clear necrosis around peri-central regions in control animals, albeit almost absent in their KO littermates (**Figure 19c**). Collectively, these data demonstrate that diminished *Rspo3* secreted from HSC protects the liver from APAP-induced acute liver injury and support my hypothesis that HSC-derived *Rspo3* plays critical roles during peri-central liver damages, while has limited functions in peri-portal liver injuries.

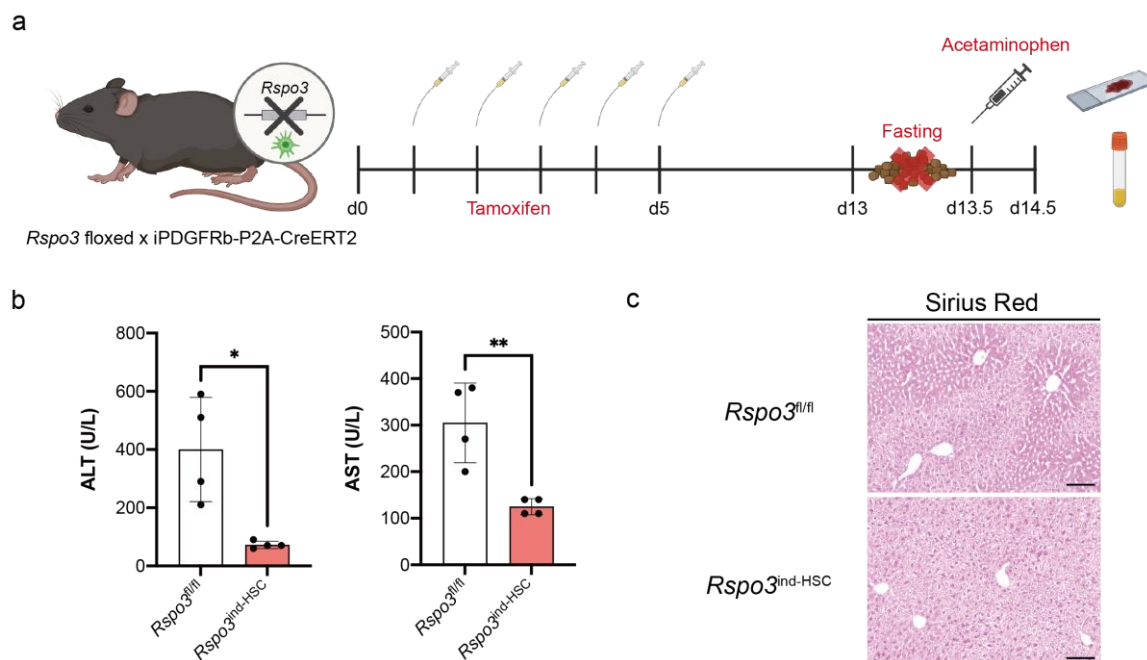


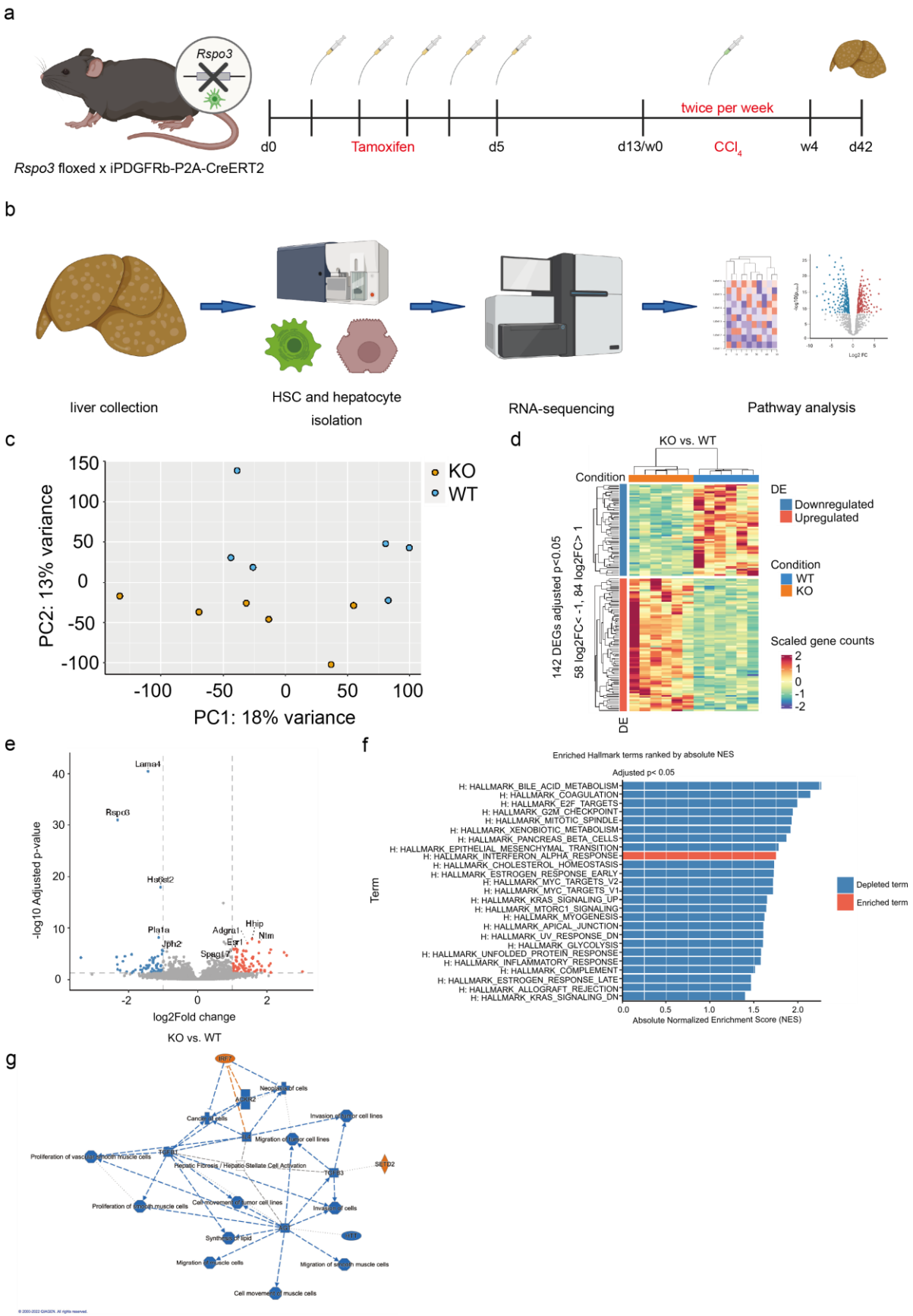
Figure 19. Deletion of *Rspo3* from HSC alleviates APAP-induced liver injury.

a) Experimental scheme of experimental approach for APAP administration. b) Plasma biochemistry of ALT and AST levels of mice subjected to APAP. c) H&E staining of the liver. Scale bars, 100 μ m.

Figure 20. RNA-seq analysis identified DEGs in HSC and mechanisms of the protective phenotype by HSC-specific *Rspo3* deletion.

a) Timeline of one-month CCl₄-treated mice for RNA-seq. b) Experimental scheme of RNA-seq analysis for HSC and hepatocytes. c) PCA of gene counts for WT and KO mice. d) Heatmap of DEGs comparing WT and KO mice treated with CCl₄ for one month. e) Volcano plot of DEGs, labeled genes are top five up- or down-regulated genes. f) GSEA analysis for depleted and enriched terms. g) IPA analysis for activated and inhibited pathways.

Figure 20. RNA-seq analysis identified DEGs in HSC and mechanisms of the protective phenotype by HSC-specific *Rspo3* deletion.



3.3.5 Deletion of *Rspo3* in HSC primes aHSC to a pro-apoptotic/anti-proliferative status

To decipher the underlying mechanisms of the liver protective phenotype against peri-central liver damage provided by HSC-specific *Rspo3* ablation, I subjected HSC and hepatocytes isolated from one-month CCl_4 -treated HSC-specific *Rspo3* KO mice and their WT littermates to RNA-sequencing (**Figure 20a-b**). A principle component analysis (PCA) revealed separation of KO and WT mice (**Figure 20c**). Differential gene expression analysis between knockout and control groups identified 142 differentially expressed genes (DEGs) in HSC (**Figure 20d-e**). Among these 142 DEGs, 84 genes were up-regulated and 58 were down-regulated in KO mice. The corresponding gene set enrichment analysis (GSEA) of these DEGs identified gene sets related to cell proliferation, such as mitotic spindle and G2M checkpoint, as well as myogenesis to be inhibited in *Rspo3*^{ind-HSC} mice (**Figure 20f**). Moreover, ingenuity pathway analysis predicted an inhibition of proliferation and migration of different mural cells (**Figure 20g**). These data indicate an intrinsic anti-proliferative and pro-apoptotic effect of HSC-specific *Rspo3* deletion to HSC in an autocrine manner.

3.3.6 HSC-derived *Rspo3* exerts a pro-fibrotic phenotype independent of HSC Wnt secretion

Given that *Rspo3* functions as a well-known Wnt-signaling potentiator, I examined the expression change of Wnt-related genes in HSC-specific *Rspo3* knockout mice. HSC-specific Wnt ligands, *Wnt4* and *Wnt5a*, were significantly reduced in RNA-seq analyses. To further investigate whether secretion of Wnt ligands is involved in the pro-fibrotic phenotype, HSC-specific Wnt transporter *Evi/Wls* knockout mice were subjected to four-month CCl_4 administration (**Figure 21a**). Plasma and liver samples were collected two days after the last treatment. Plasma biochemistry of ALT and AST showed a severe and comparable extent of liver damage in both WT and KO mice (**Figure 21b**). Similarly, H&E and SR staining of liver samples indicated same level of fibrotic scarring between two groups (**Figure 21c**).

As both, LSEC and HSC express *Rspo3* in the liver, I further confirmed whether diminished *Rspo3* from LSEC leads to a similar effect as HSC-specific *Rspo3* deletion. To achieve this, VE-Cadherin CreERT2 was used to delete *Rspo3* specifically in LSEC (iECKO). *Rspo3*-iECKO and control mice were treated with CCl_4 for four months (**Figure 22a**). Intriguingly, LSEC-specific *Rspo3* deletion did not provide any protective effect on the liver, as indicated by comparable plasma ALT and AST levels (**Figure 22b**) and similar degrees of histological liver damage (**Figure 22c**).

Collectively, these data showed that *Rspo3* deletion protects the liver from CCl_4 -induced liver damage in an HSC-specific manner, independent of Wnt ligand secretion from HSC.

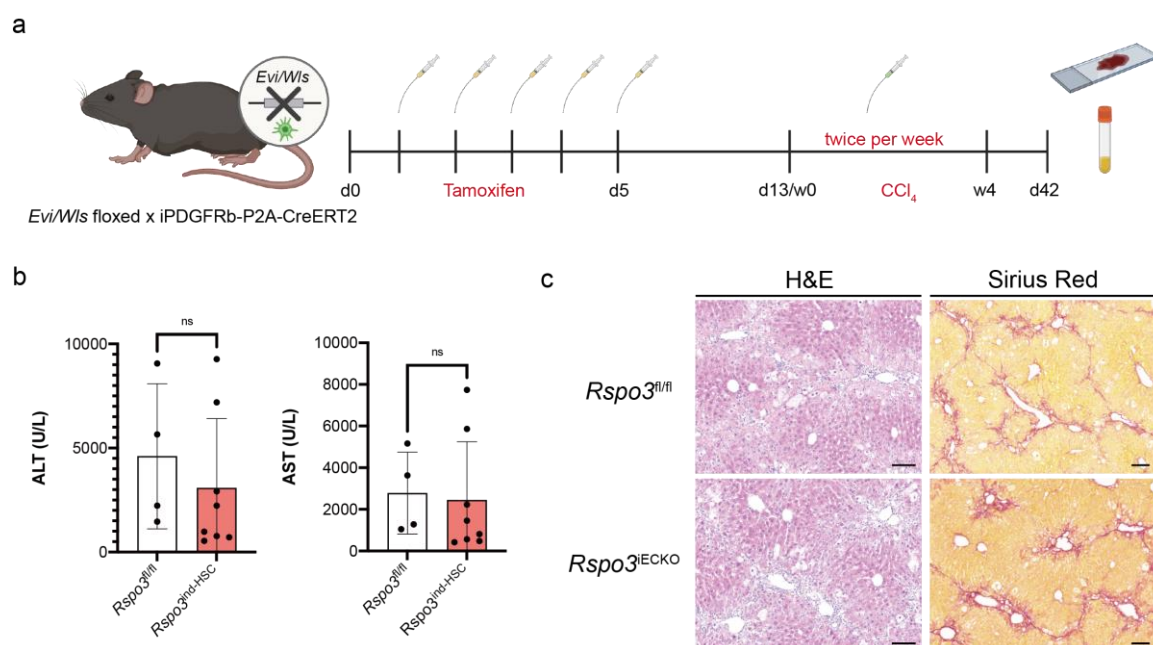


Figure 21. Deletion of HSC-derived *Evi/Wls* does not protect the liver from CCl₄-induced liver injury.

a) Experimental scheme of one-month CCl₄-treatment. b) Plasma biochemistry of ALT and AST levels of *Evi/Wls* KO mice. c) H&E and Sirius Red staining of the liver. Scale bars, 100 μ m.

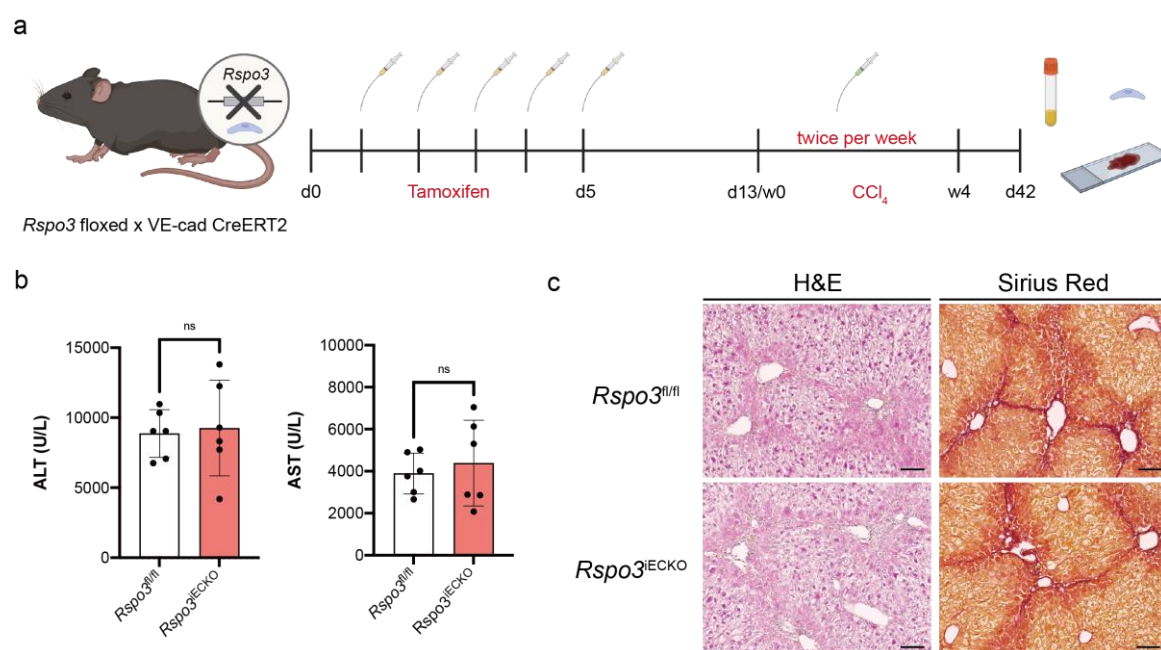


Figure 22. LSEC-specific *Rspo3* deletion does not protect the liver from CCl₄-induced liver injury.

a) Experimental scheme of one month CCl₄-treatment. b) Plasma biochemistry of ALT and AST levels of *Rspo3^{fl/fl}* mice. c) H&E and Sirius Red staining of the liver. Scale bars, 100 μ m.

4 Discussion

4.1 Rspo3 from two cell types exerts different functions: angiocrine vs. pericrine

Taking advantage of single-cell RNA-sequencing technique, the spatial heterogeneity of different cell types in the liver has been well characterized. Recent work has identified that all major cell types in the liver, including hepatocytes, LSEC, KC, and HSC are spatially and/or functionally zoned^{124,128,139,276,291}. Notably, Rspo3, a Wnt signaling enhancer, was listed as one of the top zoned molecules at the central vein regions in both LSEC and HSC. Functional studies on LSEC-derived Rspo3 had established the current angiocrine (EC-dependent regulation) Rspo3/Wnt-centered paradigm of liver zonation²⁷⁴. Furthermore, previous findings from our lab have shown that LSEC-expressed Rspo3 regulates vascular stability and pruning²⁸⁸. Additionally, Rspo3 was inhibited in tumor EC in both, mice and humans, and acted as a gatekeeper against tumor initiation in a murine HCC model. These data showed that Rspo3 from LSEC plays critical roles in regulating physiological and pathological liver functions. However, up until now, the functions of HSC-driven Rspo3, another Rspo3 producing cell population in the liver, remains poorly studied.

In current study, I established isolation protocols and enriched four major liver cell populations, including LSEC and HSC from the same liver. The work for the first time clearly show that HSC and LSEC, but not other cell types, are the main source of Rspo3 in the liver. Moreover, HSC expressed slightly higher levels of Rspo3 than LSEC. This indicated that HSC-expressed Rspo3 may affect liver functions to a broader extent. Indeed, while LSEC-derived Rspo3 only regulated the zonation of strictly central venous genes such as GS, HSC-derived Rspo3 was essential for the regulation of liver zonation throughout the liver. HSC-specific Rspo3 knockout led to an upregulation of zone 1 (Cyp2f2 and Hal) and downregulation of zone 2/3 (Cyp1a2 and Cyp2e1) genes. Furthermore, HSC-derived Rspo3 is indispensable for hepatocytes (primarily zone 2)²⁹² proliferation and liver regeneration after PHx. These data phenocopied the observation of the just eGFP death-inducing (Jedi) T-cell HSC depletion model, in which HSC were almost completely depleted from the whole mouse liver²⁷⁹. Additionally, reduced liver mass shown in the Jedi model was also observed in HSC-specific Rspo3 knockout mice. These data indicate that Rspo3-expressing HSC may be a key player for the phenotypes observed in the Jedi model.

Based on these findings, I hypothesized that Rspo3 may exert different functions in the liver, depending on its origin. The discrepancy of Rspo3 functions between HSC and LSEC may be potentially caused by its different zonation pattern in these cells. In LSEC, Rspo3 is expressed exclusively in the

central vein¹³⁴, whereas in HSC, although similarly zonated at the central vein area, the distribution of Rspo3 shows a gradient pattern, gradually decreasing from central vein to portal triads²⁹³. The complete absence of LSEC-derived Rspo3 in zone 1, but not Rspo3 from HSC, may partially explain why distinct phenotypes were observed upon LSEC- and HSC-specific Rspo3 deletion. At the central vein, angiocrine Rspo3 may be dominant over pericrine (HSC-dependent regulation) Rspo3 acting as a Wnt potentiator. Therefore, GS zonation was solely disturbed in LSEC-specific, but not HSC-specific Rspo3 knockout livers. While in mid-zones, HSC are the only source of Rspo3, thereby causing a reduced CYP metabolism and liver damage after CCl₄ or APAP treatment. At portal triads, reduced hepatic Wnt signaling led to increased zone 1 metabolism in HSC-specific Rspo3 knockout mice. Yet, minor changes after HSC-specific Rspo3 deletion compared to control (almost completely absent Rspo3 at portal regions from both LSEC and HSC) are still insufficient to drive protective phenotype during BDL-induced liver damage (**Figure 24**).

In summary, the functions of Rspo3 in the liver could differ, not only based on its origin, but more possibly depending on its location. Although both LSEC- and HSC-specific Rspo3 deletion lead to altered metabolism in the liver, changes in different genes will cause different outcomes. However, further investigation is needed to validate this hypothesis and to elucidate underlying mechanisms.

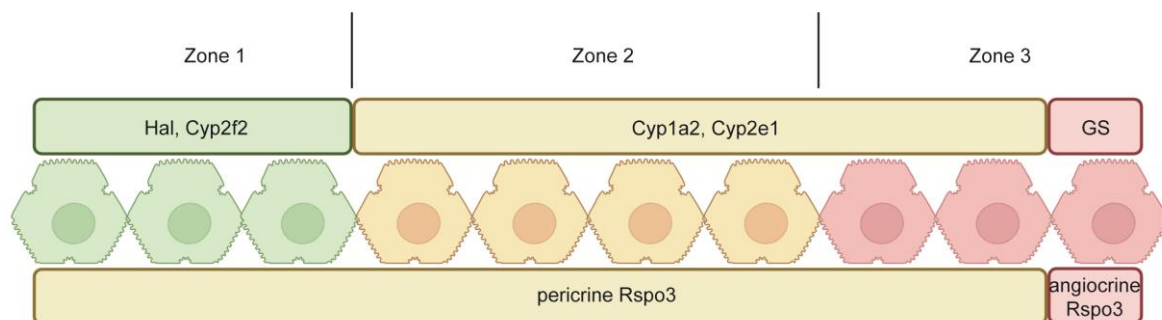


Figure 24. Model of pericrine and angiocrine Rspo3 regulation of liver zonation.

At zone 3, both LSEC and HSC express Rspo3. LSEC-derived Rspo3 is dominant over HSC-expressed Rspo3 and regulates the zonation of genes (such as GS) that are strictly expressed at central vein. At zone 2, only HSC express Rspo3 and regulate the zonation of zone 2 genes (Cyp1a2, Cyp2e1). At zone 1, both LSEC- and HSC-derived Rspo3 are almost absent, leading to an increase of genes (Hal, Cyp2f2) that are negatively regulated by Wnt signaling.

4.2 Rspo3 expressed HSC are key players in the pathogenesis of liver disease

During liver fibrosis, quiescent HSC are activated and transdifferentiate into collagen-producing cells, putting HSC at the center of fibrogenesis²⁹⁴. Despite increasing evidence unveiling mechanisms of liver fibrosis, only limited numbers of discoveries can be translated into clinical outcome. This unfortunate fact suggests, on the one hand, the existence of a gap between clinical and pre-clinical research, and on the other hand, indicates the possibility that a hitherto unknown function of HSC or HSC-derived molecules (such as HSC-expressed Rspo3) remains to be established.

Among the candidates that have been characterized as playing critical roles during fibrosis, Rspo3 is a promising target for anti-fibrotic therapy^{275,287}. However, such function is not executed by LSEC-expressed Rspo3. Therefore, I hypothesized that HSC-derived Rspo3, instead of Rspo3 from LSEC, may be responsible for this function. In this dissertation, a protective effect was identified in HSC-specific Rspo3 knockout liver upon acute and chronic CCl₄-induced liver damage. In a long-term setting, HSC-specific Rspo3 deletion further inhibited the development of neoplastic transformation in the liver. However, lack of Rspo3 from HSC did not protect the liver from BDL-induced liver damage. Considering the restricted expression of Rspo3 at zone 3, HSC-derived Rspo3 may function only in a short-range or direct cell-cell contact manner.

Additionally, RNA-sequencing of HSC from this study identified numerous DEGs between KO and WT animals. Among these genes, *Mest*, *Angptl6*, *Colec10*, *Vipr1*, *Colec11*, *Tmem56*, and *Ifitm1* represent a novel aHSC subtype identified in a previous study²⁹⁵. This HSC subpopulation is responsible for tissue repair, vasoactive, inflammation, and angiogenesis regulation, especially at late-stage liver injury. Therefore, HSC-specific Rspo3 deletion may primarily change the signature of this HSC subtype. Furthermore, *Hhip*, *Ntm*, *Colec10*, *Vipr1*, *Ptprt*, *Egfem1*, *Tmem100*, *Wfdc1*, *Gsg1l*, and *Rgs6* from my RNA-seq data were also down-regulated in the Jedi HSC depletion model²⁷⁹. This suggests that Rspo3-expressing HSC may be key players for phenotypes that were identified in the Jedi model. Further analyses such as zonation profiling and functional validation of these DEGs may provide further insight into the mechanisms of HSC-expressed Rspo3-dependent regulation after liver damage.

4.3 Physiological functions of HSC-derived Rspo3: Is Rspo3 from HSC good or bad?

In this study, the physiological and pathological roles of HSC-derived Rspo3 were evaluated using HSC-specific Rspo3 inducible knockout mice. The deletion of Rspo3 from HSC phenocopied HSC depletion using the Jedi²⁷⁹ model and displayed reduced liver-to-body weight ratio. Furthermore, although zonation of GS and Thbd was not changed, the expression of several other genes were significantly altered, as observed after HSC-specific Rspo3 deletion. Reduced Cyp1a2 and Cyp2e1 (zone 2 and 3),

the key cytochrome P450 responsible for the metabolism of toxic products to the liver, led to less generation of toxic metabolites from CCl₄ and APAP, contributing to better liver function in these liver injury models. In contrast, enhanced Cyp2f2 and Hal (zone 1, negatively regulated by Wnt) at portal regions indicated inhibited hepatic Wnt signaling in portal hepatocytes. These data unveiled hitherto unestablished functions of HSC-derived *Rspo3* in regulating physiological and pathological liver functions.

The current understanding of HSC functions is largely limited to their fibrogenic features in pathological settings. Beyond serving as a major vitamin A reservoir in the body, regulatory functions of HSC in a healthy liver, especially the communication with LSEC and hepatocytes, remain elusive. The data presented in this thesis took HSC-expressed *Rspo3* as an example, highlighted the importance of HSC in both pathological and physiological conditions. Yet, these new findings only represent a specific aspect of HSC-dependent regulation of liver function. For example, HSC-specific deletion of *Rspo3* led to less liver injury in CCl₄- and APAP-treated mice. However, these data do not suggest that HSC-derived *Rspo3* is detrimental to the liver. Instead, the less injured livers under pathological challenges faithfully reflect the changes of cytochrome P450 under physiological condition. Notably, female mice are more resistant to CCl₄-induced toxicity in the liver than male mice²⁹⁶. It should be taken into consideration that only male mice were used for CCl₄-related experiments in this study. Intriguingly, female mice express higher levels of *Rspo3* in HSC than male mice. Hence, less susceptibility to CCl₄ and higher expression of *Rspo3* in female mice compared to male mice reflect a protective nature of HSC-derived *Rspo3* in the liver. In humans, men are also more predisposed to liver disease compared to women. Previous study showed that female mice are protected from liver damage via estrogens^{297,298}. Therefore, it is thought-provoking whether there is a correlation between the protective function of HSC (or HSC-derived *Rspo3*) and gender differences. Furthermore, given that LSEC also express *Rspo3*, whether LSEC-derived *Rspo3* also fulfil a protective function to the liver remains to be investigated.

Another interesting finding of this study was that the expression of *Rspo3* in HSC was down-regulated *in vivo* in all liver injury models. However, the cause and mechanism of the changes in *Rspo3* expression remain elusive. Given that HSC-derived *Rspo3* led to a protective phenotype in mid-zonal liver damage models, the expression of *Rspo3* in HSC may be downregulated via certain feedback loops or adaptive responses. The downregulation of *Rspo3* in all liver injury models shown in this study, implicates a natural protective mechanism of the liver upon exogenous insults or damages. It is necessary to evaluate the expression dynamic of *Rspo3* and its correlation to liver disease progression.

Notably, the data shown in this study are solely based on Rspo3 loss-of-function experiments. Rspo3 gain-of-function experiments, which mimics the natural expression dynamics during disease progression, may be more beneficial to evaluate its clinical relevance. Furthermore, it is possible that other HSC-derived molecules, such as HGF, may also act as a mediator for regulating hepatocyte health and function²⁹⁹. Hence, regulation of liver homeostasis by HSC is a collaborative effect that involves multiple regulators, including Rspo3, HGF, etc. Identifying additional HSC-expressed factors could help to improve the understanding of HSC-dependent regulation of liver function under physiological conditions. Moreover, it should be considered that the RNA-seq analyses performed in this study were only based on the experiment model in a damaged setting (4 months of CCl₄ treatment). The question remains why HSC-specific Rspo3 knockout mice have smaller livers, regardless of similar cell proliferation in WT and KO livers. It is known that four members of Roof plate-specific spondins (RSPO) family, RSPO1-4, exert different functions. Notably, RSPO1 regulates cell proliferation and liver growth²⁸². However, HSC only express low levels of Rspo1. Sequencing of hepatocytes and HSC of HSC-specific Rspo3 knockout mice at normal conditions or investigating other members of the RSPO family may provide further insight to address such questions.

4.4 HSC-specific Rspo3 signaling cascade: Wnt-dependent or independent?

The Wnt signaling pathway plays important roles in numerous aspects of liver biology, including development, homeostasis, and pathogenesis. During development, β -catenin promotes hepatocyte proliferation and liver maturation²³⁹. Moreover, Wnt signaling is also a key regulator of liver zonation and liver regeneration^{242,300}. Pathologically, perturbed Wnt signaling leads to development of liver diseases and tumors^{301,302}. Nevertheless, little is known about the relationship between the Wnt signaling pathways and HSC in the liver³⁰³. Major findings about Wnt signaling in the liver are angiocrine related. Only few reports have shown that HSC-derived Wnt signaling is dispensable for liver zonation and fibrosis²⁷⁷. In this dissertation, the expression and function of HSC-derived Rspo3, another key regulator of Wnt signaling pathway, were evaluated. Employing different models, functions of the HSC-derived Rspo3 were identified under both pathological and physiological conditions. Notably, contrary to current paradigm that liver zonation is solely regulated in an angiocrine manner, the presented data showed that pericrine signaling is also a key regulator in this aspect. Furthermore, pathway analysis suggested that HSC-expressed Rspo3 may exert hitherto unknown functions during peri-central liver injury, independent of Wnt secretion from HSC. However, RNA-seq and pathway analyses performed in this study were based on data generated from HSC and hepatocytes, but not LSEC. It is therefore difficult to exclude the possibility that hepatocytes receive Wnt from LSEC when HSC-derived Wnt is absent. Interestingly, HSC-specific Rspo3 deletion led to

zonation changes of Wnt target genes, CYP and Hal, suggesting a potential involvement of Wnt signaling in this process. It is therefore surprising that HSC-specific Evi/Wls deletion did not phenocopy changes shown in HSC-specific Rspo3 knockout mice, whereas similar results were obtained in LSEC-specific Rspo3 and Evi/Wls knockout mice. On the other hand, the discrepancy between functions of HSC-expressed Rspo3 during CCl₄- or APAP-induced liver injury identified in this study and differential expression of HSC-derived Rspo3 between different genders underpins a counterintuitive mechanism of Rspo3 aside from the Wnt signaling pathway. Thus, whether HSC-expressed Rspo3 regulation of liver function is Wnt-dependent or independent remains elusive. Nevertheless, the data shown in this thesis pioneered a paradigm shift on HSC research from solely pathological-related direction to a wider aspect, and emphasized an imperative role of HSC-expressed Rspo3 in regulating liver functions.

4.5 Closing remarks

Taken together, the present study identified for the first time functions of HSC-derived Rspo3 under physiological and pathological conditions. In contrast to LSEC-derived Rspo3, HSC-expressed Rspo3 has limited effect on GS zonation, but regulates expression of cytochrome P450 genes specifically enriched in mid-zonal and portal hepatocytes. Altered Cyp1a2 and Cyp2e1 metabolism caused by HSC-derived Rspo3 deletion potentially leads to different tolerance of the liver against hepatotoxins. This further provides a protective benefit to the liver subjected to liver injuries. The comparison of cell type-specific Rspo3 knockout using same experimental models highlighted HSC-specificity of these phenotypes. The reason why the same molecule exerts different functions in the same organ and how cells distinguish the source of Rspo3 remains elusive and requires further investigation. Considering different zonation patterns of Rspo3 in LSEC and HSC, as well as zonal-specific phenotypes shows in this study, it is likely that anatomic positioning of Rspo3 plays a critical role in regulating Rspo3 functions in the liver.

Lastly, it needs to be considered that in this study, RNA-seq was performed and analyzed solely with CCl₄-treated mice. Considering changes of CYP at normal conditions after HSC-specific Rspo3 deletion, the function of HSC-derived Rspo3 under physiological condition requires further investigation. The necessity of HSC-derived Rspo3 in regulating hepatocyte metabolic zonation and its “harmful” phenotype under pathological challenges presented in this thesis underpin a double-edged sword-like feature of HSC-expressed Rspo3. This has to be considered carefully if Rspo3 is used as a therapeutic target for treating chronic liver diseases.

5 Materials and Methods

5.1 Materials

5.1.1 Chemicals

Chemicals that were used in this study were purchased from the following suppliers:

Table 1: Chemical suppliers

Company	Access
Abcam	www.abcam.com
AppliChem	www.applichem.com
BioLegend	www.biolegend.com
BIOZOL	www.biozol.de
Carl Roth	www.carlroth.com
Clear H ₂ O®	www.clearh2o.com
Gerbu	www.gerbu.de
Merck	www.merck.de
Qiagen	www.qiagen.com
R&D Systems	www.rndsystems.com
Roche	www.roche.com
Serva	www.serva.de
Sigma-Aldrich	www.sigmaaldrich.com
Thermo Fisher Scientific	www.thermofisher.com

5.1.2 Enzymes, growth factors, and proteins

Table 2: Enzymes, growth factors, and proteins

Enzyme	Company
DNase I	Sigma-Aldrich
Liberase	Roche
Pronase	Merck
Proteinase K	Qiagen

5.1.3 Cells

Table 3: Murine cell lines

Cell	Description	Source	Medium
Primary HSC	Freshly isolated HSC from the mouse liver	Perfusion-based isolation from C57BL/6 mouse	DMEM High Glucose + 10% FCS + 1% Pen/Strep + 50 µg/mL Gentamicin

5.1.4 Reagents and tools

Table 4: Cell culture reagents

Reagent	Company
Dimethylsulfoxide (DMSO)	AppliChem
Dulbecco's phosphate buffered saline (PBS)	Sigma Aldrich
Dulbecco's Modified Eagle medium - Glutamax (DMEM)	Thermo Fisher Scientific
Fetal Calf Serum (FCS, heat-inactivated)	Gibco
Gentamicin	Gibco
Penicillin/streptomycin (100x 104U/10mg/mL)	Sigma Aldrich
Trypan blue	Thermo Fisher Scientific
Trypsin-EDTA (10x)	Sigma Aldrich

Table 5: PCR reagents (RT, PCR, qPCR)

Reagent	Company
Direct PCR Lysis Reagent	PeqLab
DNase/RNase free H ₂ O	Gibco
QuantiTect Reverse Transcription Kit for cDNA Synthesis	Qiagen
RedTaq® ReadyMix™ PCR Reaction Mix	Sigma Aldrich
TaqMan® Fast Advanced PCR Master Mix Applied	Biosystems

Table 6: Staining reagents

Reagent	Company
Acetic acid glacial	Thermo Fisher Scientific
Biotin Blocking System	DAKO
Bovine Serum Albumin	PAA
CD31 MicroBeads, mouse	Miltenyi Biotech
DAB	Thermo Fisher Scientific
Direct Red 80	Sigma Aldrich
Donkey serum	Thermo Fisher Scientific
DRAQ7™	BioLegend
Elite® ABCkit, Peroxidase (standard)	BIOZOL
Eosin Y solution	Dako
F4/80 MicroBeads, UltraPure, mouse	Miltenyi Biotech
Fetal Calf Serum (FCS, heat-inactivated)	PAA
Fluorescent mounting medium	Dako
FxCycle Violet	Thermo Fisher Scientific
Histomount	Thermo Fisher Scientific
Hoechst Dye 33258	Merck
Hydrogen Peroxide, 3%	Thermo Fisher Scientific
M.O.M Immunodetection kit, Basic	BIOZOL
Mayers' Hematoxylin solution	Sigma Aldrich
Normal goat serum ready-to-use	Zymed
Roti-Histofix 4% (pH=7)	Carl Roth
Sucrose	Sigma Aldrich
Target Retrieval Solution 10x	DAKO
Trichrome Stain (Masson)	Sigma Aldrich

Table 7: Western blot reagents

Reagent	Company
Bovine Serum Albumin (BSA)	PAA
Nitrocellulose membrane	GE Healthcare
Orthovanadate	Merck
PageRuler Prestained Protein Ladder	Thermo Fisher Scientific
Pierce ECL Western Blotting Substrate	Thermo Fisher Scientific
Rotiphorese Gel 30	Carl Roth

Table 8: Reagents and tools for animal experimentation

Reagent/Tool	Company
Alcoholic iodine solution	WDT
Bepanthen eye cream	Roche
Corn oil	Sigma Aldrich
DietGel® Recovery	Clear H2O®
Ethicon suture silk 4-0	Johnson & Johnson MEDICAL
Ethicon suture silk 5-0	Johnson & Johnson MEDICAL
Gavage needle	Fine Science Tools
GBSS/B	Sigma Aldrich
Ketamin	Pfizer
Liver Digestion Medium	Metamizole
Metamizole/Metapyrin	Serumwerk
NaCl solution 0.9%	Braun
Na-heparin	Hirschmann
Rompun	Bayer
Surgical tools and equipment	Fine Science Tools
Tamoxifen	Sigma Aldrich
Winged Infusion Set	Terumo

5.1.5 Kits

Table 9: Kits

Kit	Company
ApopTag® Peroxidase in Situ Apoptosis Detection Kit	Millipore
Arcturus PicoPure RNA Isolation Kit GenElute	Thermo Fisher Scientific
DuoSet® Ancillary Reagent Kit 2	R&D Systems
DuoSet® Mouse R-Spondin 3	R&D Systems
GenElute Mammalian Total RNA Purification Kit	Sigma
Hydroxyproline Assay Kit	Cell Biolabs
Mouse RSPO3 ELISA Kit (Sandwich ELISA)	BIOZOL
Pierce BCA Assay Kit	Thermo Fisher Scientific
QuantiTect® Reverse Transcription Kit for cDNA Synthesis	Qiagen
Qubit RNA HS (High Sensitivity) Assay-Kit	Thermo Fisher Scientific
RNase-Free DNase	Qiagen
TriZol	Thermo Fisher Scientific
ViewRNA™ ISH Tissue Kit	Thermo Fisher Scientific

5.1.6 Primers

Table 10: Primers

Genotype	Primer name	Sequence (5'-3')
<i>Rspo3</i> floxed	Rspo3 OK 189	ATGCTTTGAGGCTTGTGACC
	Rspo3 OK 191	TACATTCTGGTTTCTCATCTGG
Evi/Wls floxed	Evifl/fl fwd #107	TGCTCCATTGAAGGCAAAACCTC
	Evifl/fl rev #109	AACTTGAATCACTGGTCTAAAATATGCC
YFP KI	mROSA-YFP_1 HL15	AAGACCGCGAAGAGTTTGTCC
	mROSA-YFP_2 HL54	TAAGCCTGCCCAGAAGACTCC
	mROSA-YFP_3 HL152	AAGGGAGCTGCAGTGGAGTA
iPDGFRb-P2A-CreERT2	iPDGFRbP2ACreERT2_Mut_R	ACATGTCCATCAGGTTCTTGC
	iPDGFRbP2ACreERT2_F	CCACCTTGAATGAAGTCAACAC
	iPDGFRbP2ACreERT2_Wt_R	AGCTTGTGGCAGTGTAGCTG
VE-cadCreERT2	VE-CAD-CreERT2 fwd	GCCTGCATTACCGGTCGATGCAACGA
	VE-CAD-CreERT2 rev	GTGGCAGATGGCGCGGCAACACCATT
	MB182F (Actin)	CAATGGTAGGCTCACTCTGGGAGATGATA
	MB182R (Actin)	AACACACACTGGCAGGACTGGCTAGG
Mycoplasma	MGSO	TGCCACCATCTGTCACTCTGTTAACCTC
	GPO	GGGAGCAAACAGGATTAGATACCTT

5.1.7 TaqMan™ assays

Table 11: Murine TaqMan probes

Gene	Assay ID
<i>Actb</i> (FAM)	Mm00607939_s1
<i>Rspo3</i>	Mm01188251_m1
<i>Evi/Wls</i>	Mm00509695_m1
<i>Hnf4α</i>	Mm01247712_m1
<i>Pecam1</i>	Mm01242584_m1
<i>Lrat</i>	Mm00469972_m1
<i>Emr1</i>	Mm00802529_m1

5.1.8 Antibodies

Table 12: Primary antibodies

Antigen	Species	Dilution	Company	Cat. No.	Application
CYP1A2	Mouse	1:100	Santa Cruz	sc-53241	IHC
CYP2E1	Rabbit	1:200	Abcam	ab28146	IHC
CYP2F2	Mouse	1:200	Santa Cruz	sc-374540	IHC
DESMIN	Rabbit	1:200	Abcam	ab15200-1	IHC
HAL	Rabbit	1:200	Sigma-Aldrich	HPA-038547	IHC
Glutamine Synthetase	Rabbit	1:5000	Abcam	ab49873	IHC
HNF4A	Rabbit	1:1000	Abcam	ab181604	IF

KI67	Rat	1:500	Invitrogen	14-5698-82	IF, IHC
PCNA	Mouse	1:100	Thermo Fisher	13-3900	IHC
THBD	Rat	1:50	Thermo Fisher	14-1411-82	IHC
α -SMA	Rabbit	1:200	Abcam	ab5694	IHC

Table 13: Secondary antibodies

Antigen	Species	Conjugate	Dilution	Company	Cat. No.
Rabbit	Goat	Alexa Fluor® 546	1:500	Invitrogen	A11071
Rat	goat	Alexa Fluor® 488	1:500	Invitrogen	A11006
Biotinylated antibody to rabbit	goat	Biotin	Ready to use	Zymed	A21450
Biotinylated antibody to mouse	goat	Biotin	Ready to use	Invitrogen	703-165-155
Biotinylated antibody to rat	goat	Biotin	1:500	VectorLabs	BA-9401

5.1.9 Consumables

Table 14: Consumables

Consumable	Company
384 well plates	4titude
96 well plates	Steinbrenner Laborsysteme
Biopsy punches	Stiefel
Blood glass capillary	VWR international
Cannula (18G, 19G, 27G)	BD
Cell culture dishes (6, 12, 24, 48 well plates, 10cm)	Sarstedt
Cell scraper	Corning
Cell strainer (100 μ m)	BD Falcon
Cotton swab	Edeka elcos face
Countess™ cell counting chamber slides	Invitrogen
Cryotubes	Carl Roth
FACS tubes	BD Biosciences
FACS tubes with strainer cap	Corning
Filter containing pipette tips	Sarstedt
Foam biopsy pads	Leica
Hamilton syringe (27G)	Hamilton
MACS MS columns	Miltenyi Biotec
Micronfine (29G, 30G)	BD
Microscope cover glasses	VWR international
Microscope glass slides	Menzel-Gläser
Microtome blade (N35)	PFM medical
Microtube 2ml, PP	Sarstedt

Pipette tips	Nerbe
Pipette tips - filter	Sarstedt
Pipette tips - multichannel	Rainin
Reaction tubes (0.2ml, 0.5ml, 1.5ml, 2ml)	Eppendorf
Reaction tubes (1.5ml) - DNA LoBind	Eppendorf
Reaction tubes (15ml, 50 ml)	Greiner
Sealing foil	Applied Biosystems
Sterile pipettes	Corning
Syringe - SoftJect 10ml	Thermo Fisher Scientific
Syringe - SoftJect 1ml	Thermo Fisher Scientific
Syringes	Dispomed
TB tube 2.5ml	Sarstedt
Tissue embedding cassettes	Kartell Spa

5.1.10 Devices and equipment

Table 15: Devices and equipment

Name	Company
Aria cell sorting platform	BD Biosciences
Axio ScanZ7.1 slide scanner	Zeiss
BioRad gel casting system	BioRad
BioRad gel running system	BioRad
BioRad Western Blotting equipment	BioRad
Cell culture hood	Thermo Fisher Scientific
Cell culture incubator	Thermo Fisher Scientific
Centrifuge	Eppendorf
Countess automated cell counter	Thermo Fisher Scientific
Freezing box	Thermo Fisher Scientific
Heating block	Eppendorf
HM355S microtome	Thermo Fisher Scientific
iMark™ Microplate Reader	BioRad
Light cycler 480	Roche
MasterCycler 384-Well	Eppendorf
Microm EC 350-2 (tissue embedding)	Thermo Fisher Scientific
Mixer mill MM200	Retsch
Mr. Frosty Freezing Container	Thermo Fisher Scientific
Multichannel pipette	Rainin
Multistep pipette	Eppendorf
Nanophotometer N60	Intas
Olympus IX 71	Olympus
Peristaltic pump	Ismatec
Pipetes	Eppendorf
Power supply	BioRad
QIAxcel Advanced System	Qiagen
QuadroMACS multistand	Miltenyi Biotech
Scale	Ohaus
Shaver	Moser
Special accuracy weighing device	Metler Toledo

Spin tissue processor STP120	Thermo Fisher Scientific
StepOnePlus Real-Time PCR System	Thermo Fisher Scientific
Table centrifuge (5417R)	Eppendorf
Thermocycler	Thermo Fisher Scientific
Vortex mixer	Neolab
Warm pad	ThermoLux
Water bath	Julabo

5.1.11 Software

Table 16: Software

Software	Company
Adobe illustrator	Adobe
FACSDiva™	BD
Fiji	ImageJ
FlowJo	Miltenyi Biotec
Graph Pad Prism (v8.0)	Graph Pad
Light Cycler 480 software	Roche
StepOne™ Software v2.1	Applied Biosystems
ZEN black	Zeiss
ZEN blue	Zeiss

5.1.12 Solutions and buffers

Table 17: Solutions and buffers

Solution/Buffer	Composition	
10x EGTA	8000 mg 400 mg 88.17 mg 151.02 mg 2380 mg 350 mg 190 mg 990 mg 1 L	NaCl KCl NaH ₂ PO ₄ ·H ₂ O Na ₂ HPO ₄ ·2H ₂ O HEPES NaHCO ₃ EGTA Glucose. H ₂ O H ₂ O
4% Paraformaldehyde (PFA)	4 % (m/v) ~1/400 1/10	Paraformaldehyde in VE water NaOH (until dissolved) adjust pH 7.2-7.4 10x PBS adjust pH 7.4
Ammonium chloride potassium buffer (ACK buffer)	150mM 10 M 100 mM	NH ₄ Cl KHCO ₃ Na ₂ EDTA Adjust pH 7.2-7.4
Blocking buffer (tissue staining)	10% 3%	Fetal calf serum Bovine serum albumin in PBS

Enzyme Buffer Solution	8000 mg 400 mg 88.17 mg 151.02 mg 2380 mg 350 mg 280 mg 5 L	NaCl KCl NaH ₂ PO ₄ ·H ₂ O Na ₂ HPO ₄ ·2H ₂ O HEPES NaHCO ₃ CaCl ₂ ·2H ₂ O H ₂ O
FACS buffer	5% (v/v)	Fetal calf serum in PBS
GBSS/A	370 mg 210 mg 70 mg 74.73 mg 30 mg 1090 mg 227 mg 225 mg 5 L	KCl MgCl ₂ ·6H ₂ O MgSO ₄ ·7H ₂ O Na ₂ HPO ₄ ·2H ₂ O KH ₂ PO ₄ Glucose. H ₂ O NaHCO ₃ CaCl ₂ ·2H ₂ O H ₂ O
Phosphate buffered saline (PBS)	1.34 M 27 mM 200 mM 4.7 mM	NaCl KCl Na ₂ HPO ₄ KH ₂ HPO ₄ Adjust pH 7.4
RIPA lysis buffer (modified)	50 mM 150 mM 1 mM 1 % 0.25 % 2 mM 1 x	50 mM 150 mM 1 mM 1 % 0.25 % 2 mM 1 x
Running buffer (1x)	192 mM 25 mM 0.1 %	Glycine Trizma Base SDS
Separating gel (12%) (1 Gel)	3.3 ml 2.5 ml 100 µl 4 ml 100 µl 4 µl	H ₂ O 1.5M Tris-HCl pH 8.8 10% SDS 30% Acrylamide APS TEMED
Stacking gel (5%) (1 Gel)	2.7 ml 0.5 ml 40 µl 0.67 ml 40 µl 4 µl	H ₂ O 1M Tris-HCl (pH 6.8) 10%SDS 30% Acrylamide APS TEMED
Tris-Buffered Saline Tween-20 (TBS-T)	10 mM 100 mM 0.1 %	Tris/HCl, pH 7.5 NaCl Tween-20

5.2 Methods

5.2.1 Animal studies

5.2.1.1 Animal husbandry

C57BL/6N mice purchased from Janvier Labs were used as wild-type animals in this study. Transgenic mouse lines, B6 *Rspo3* floxed x iPDGFRb-P2A-CreERT2, and B6 *Gpr177(Evi/Wls)* floxed x iPDGFRb-P2A-CreERT2, were bred in-house in the animal facility of the German Cancer Research Center. Transgenic mouse line B6 *Rspo3* floxed x VE-cad CreERT2 were provided from previous studies^{288,304}. All transgenic mouse lines are homozygous for the floxed allele. Cre-positive mice were heterozygous for Cre, and Cre-negative littermates were used as controls. Mice were kept in specific pathogen-free animal facilities and fed with autoclaved water and food *ad libitum* in sterile cages, with twelve hours of light and dark cycle, in a temperature-controlled room. Health of the animals was thoroughly checked daily for signs of potential diseases. Each animal was marked via ear punching and the biopsy was used for genotyping. *In vivo* experiments were performed with 8 to 12-week old mice, following German national guidelines on animal welfare and regulated by the regional council Karlsruhe, with approved permits G-251/20, DKFZ305, and DKFZ370.

5.2.1.2 Genotyping

Biopsy of ear punches or tail tips for the case of re-genotyping were lysed in 100µl Direct PCR Lysis Reagent containing 10µg Proteinase K overnight at 55 °C. Next, temperature was raised to 95 °C and incubated for 20 minutes to stop the reaction. Debris was removed via centrifugation, and supernatant was used for genotyping PCR. Remaining lysates were stored at 4 °C until checking the PCR results. Reaction mix was prepared with a Taq polymerase kit from Qiagen following tables below (Table 21-2x) depending on the genes and amplified on an Applied Biosystems Thermocycler. Results were analyzed with the QIAxcel Advanced System following the manufacturer's instructions.

Table 18: VE-cad Cre-ERT2 genotyping PCR

PCR Reaction mix (Qiagen)		PCR amplification program (Thermocycler)		
Reagent	Volume	Step	Temperature	Time
ddH ₂ O	7 µl	1	95 °C	2 min
REDTaq ReadyMix	10 µl	2	95 °C	30 sec
VE-CAD-CreERT2 fwd	1 µl	3	58 °C	45 sec
VE-CAD-CreERT2 rev	1 µl	4	72 °C	2 min
MB182F (Actin)	1 µl	Back to step 2: x35		

Materials and Methods

MB182R (Actin)	1 µl	5	72 °C	2 min
DNA	1 µl	6	4 °C	∞
Transgene: 400 bp, internal control: 300 bp				

Table 19: iPDGFRb-P2A-CreERT2 genotyping PCR

PCR Reaction mix (Qiagen)		PCR amplification program (Thermocycler)		
Reagent	Volume	Step	Temperature	Time
ddH ₂ O	15.3 µl	1	94 °C	5 min
Buffer 10x	2 µl	2	94 °C	15 sec
MgCl ₂	1 µl	3	60 °C	30 sec
dNTP	0.4 µl	4	72 °C	30 sec
iPDGFRbP2ACreERT2_Mut_R	0.4 µl	Back to step 2: x34		
iPDGFRbP2ACreERT2_F	0.4 µl	5	72 °C	5 min
iPDGFRbP2ACreERT2_Wt_R	0.4 µl	6	10 °C	∞
Taq-Polymerase	0.1 µl			
DNA	1 µl			
Transgene: 320 bp, internal control: 272 bp				

Table 20: Rspo3 floxed genotyping PCR

PCR Reaction mix (Qiagen)		PCR amplification program (Thermocycler)		
Reagent	Volume	Step	Temperature	Time
ddH ₂ O	9 µl	1	95 °C	2 min
REDTaq ReadyMix	10 µl	2	95 °C	15 sec
Rspo3 OK 189	1 µl	3	55 °C	30 sec
Rspo3 OK 191	1 µl	4	72 °C	30 sec
DNA	1 µl	Back to step 2: x34		
		5	72 °C	10 min
		6	4 °C	∞
Floxed: 410 bp, wildtype: 340 bp				

Table 21: Gpr177 (Evi/Wls) floxed genotyping PCR

PCR Reaction mix (Qiagen)		PCR amplification program (Thermocycler)		
Reagent	Volume	Step	Temperature	Time
ddH ₂ O	9 µl	1	95 °C	5 min
REDTaq ReadyMix	10 µl	2	95 °C	30 sec
Evifl/fl fwd #107	1 µl	3	50 °C	30 sec
Evifl/fl rev #109	1 µl	4	72 °C	1 min
DNA	1 µl	Back to step 2: x34		
		5	72 °C	10 min
		6	4 °C	∞
Floxed: 210 bp, wildtype: 340 bp				

5.2.1.3 Partial hepatectomy

Two-third partial hepatectomy (PHx) was performed based on the method described by Mitchell and Willenbring in Nature Protocols in 2008³⁰⁵. Briefly, mice were anesthetized i.p. with a mixture of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight). Anesthetized mouse was fixed on a temperature-controlled heating pad to keep intraoperative body temperature, with their eyes covered by eye cream (Bayer). After incising skin and peritoneum to expose the liver, left lateral lobe and median lobe were carefully ligated with 4-0 silk sutures (Ethicon) and resected. As post-surgical analgesia, metamizole (Serumwerk) was administrated via subcutaneous injection (200 mg/kg body weight) and drinking water *ad libitum* in the first 48 hours post-surgery. At the indicated time points, mice were euthanized to investigate liver regeneration.

5.2.1.4 Bile duct ligation

Common bile duct ligation was performed according to the method described by Tag et al. in Journal of Visualized Experiments in 2015³⁰⁶. In brief, mice were injected with Metamizole subcutaneously (200 mg/kg body weight) as pre-surgical analgesia. After anesthesia and being fixed on the heating pad with eyes covered by eye cream, skin and peritoneum were incised, and ventral side of the liver was lifted to expose hepatic hilus. Next, bile duct was carefully separated from the flanking portal vein and hepatic artery with forceps. Two surgical knots were introduced around bile duct with 5-0 silk sutures. As post-surgical analgesia therapy, metamizole was given *ad libitum* for an additional three days via drinking water. Mice were euthanized at the indicated time points by cervical dislocation.

5.2.2 *In vivo* treatments

5.2.2.1 Tamoxifen

Genetic deletion of the gene of interest was induced in eight to twelve-week-old mice by oral gavage of tamoxifen (Sigma Aldrich) dissolved in corn oil (2 mg in 100 μ l) for five consecutive days. A one-week washout period is followed before experimental use. For long-term experiments, mice were fed with irradiated tamoxifen food (Genobios, 400 mg/kg) until end of the experiment.

5.2.2.2 Carbon tetrachloride

To induce liver fibrosis, CCl₄ was dissolved in corn oil (400 μ g/ μ l) and delivered via oral gavage with a concentration of 1.6 g/kg body weight. Mice were treated once, or twice per week for four or twenty weeks. HSC were isolated two days after the last CCl₄ administration, and hepatocytes were isolated seven days after the last CCl₄ treatment.

5.2.2.3 Acetaminophen

To induce peri-central liver damage, APAP was dissolved in saline (15 μ g/ μ l) and delivered via i.p injection at the dose of 300 mg/kg body weight following twelve hours of fasting with drinking water *ad libitum*. After the treatment, food was given back, and mice were sacrificed 24 hours after APAP administration.

5.2.3 Sample processing

5.2.3.1 Blood withdrawal and plasma biochemistry analysis

For serological analysis, blood samples were taken at the beginning and the end of an experiment via cheek bleed with a lancet. For long-term experiments, blood was taken additionally every three weeks as an indicator of the extent of liver damage. Samples were collected with heparin-coated capillary, followed by a 13,000 x g centrifugation for 10 minutes at 4 °C to get supernatant (plasma). Plasma samples were stored at -20 °C until further analyses.

Plasma biochemistry analysis of ALT and AST was performed at the analysis center at the University Clinic of Heidelberg. Plasma samples were diluted 10 times with PBS with a final volume of 300 μ l and submitted for analysis.

5.2.3.2 HSC isolation

HSC were isolated from the mouse liver following the protocol optimized from the publication of Mederacke et al. in 2015 in Nature protocols²⁷⁸. In brief, mice were sacrificed by cervical dislocation and opened to expose the liver. A 27G winged infusion set (B. Braun) connected to a peristaltic pump (Ismatec; set at the speed of 5ml/min) was inserted into inferior vena cava, and portal vein was cut to

achieve *in situ* perfusion and enzymatic digestion of the liver. The liver was perfused with ethylene glycol tetraacetic acid solution (EGTA) at 37 °C for one minute. Next, the liver was digested with Pronase E (Merck Millipore, 0.4 mg/mL) at 37 °C for 5 minutes, followed by Liberase TM (Roche, 50 µg/mL) at 37 °C for 5 minutes. Digested liver was explanted and placed into a petri dish with 40 ml pre-warmed EBS solution for single-cell suspension. Single-cell solution was passed through a 100 µm cell strainer. After centrifuged at 580 x g for 10 minutes at 4 °C, cells were washed in Gey's balanced salt solution buffer (GBSS/B), and pellets were re-suspended in 32 ml GBSS/B and 16 ml Histodenz (Sigma Aldrich, 308.75 mg/mL in GBSS/A). After splitting into four 15 ml falcon tubes, 1.5 ml GBSS/B was added on top carefully. Last, suspension was centrifuged at 1,380 x g for 17 minutes at 4 °C without brake. HSC were enriched in the intermediate layer between GBSS/B and Histodenz. For better purity, enriched HSC were further sorted by FACS, based on autofluorescence of its vitamin storage in the cytoplasm.

5.2.3.3 Hepatocyte, LSEC, Kupffer cell, and HSC isolation from the same mouse liver

HSC were isolated as described above (1.1.2.2). All pellets from four 15 ml falcon tubes were collected and pooled into a 50 ml falcon tube. Hepatocytes were pelleted by centrifuging at 50 x g for 3 minutes at 4 °C. To enrich other NPCs from supernatant, hepatocyte residues were removed by centrifuging at same condition. Next, NPCs were collected as pellets after centrifugation at 300 x g for 10 minutes at 4 °C. To remove erythrocytes, NPCs were incubated in ACK buffer for 5 minutes and washed with FACS buffer by centrifuging at 1,500 revolutions per minute (rpm) for 5 minutes at 4 °C.

To enrich LSEC, NPCs were re-suspended in 200 µl FACS buffer and incubated with 20 µl mouse CD146 MicroBeads (Miltenyi Biotec) for 20 minutes in the dark on ice. After washing with 1 ml FACS buffer at 300 x g for 5 minutes at 4 °C, cells were re-suspended in 1 ml FACS buffer, filtered, loaded to a pre-equilibrated LS column (Miltenyi Biotec), and further processed according to the manufacturer's instruction. Enriched cells were stored at -80 °C until further investigation.

Flow through from previous step was collected and pelleted at 300 x g for 5 minutes at 4 °C. Next, cells were re-suspended in 200 µl FACS buffer and incubated with 20 µl mouse F4/80 MicroBeads (Miltenyi Biotec) for 20 minutes in the dark on ice. After incubation, cells were proceeded as described above to enrich Kupffer cells. Purified Kupffer cells were preserved at -80 °C until further investigation.

5.2.3.4 Hepatocyte isolation for RNA-sequencing

As the isolation procedure of HSC described above involved Pronase E-based digestion, which may introduce undesired hepatocyte death. Hence, for RNA sequencing of hepatocytes, another protocol was used for the isolation procedure. In brief, the liver was perfused with EGTA, as described above.

Next, the liver was digested with liver digestion medium (Gibco) supplemented with Liberase TM (Roche, 50µg/mL) at 37 °C for 5 minutes. Perfused liver was explanted and dissociated by gentle shaking in 40 ml RPMI medium (Gibco). After passing through a 100 µm cell strainer, hepatocytes were collected by centrifuging at 50 x g for 3 minutes at 4 °C. After FACS sorting, enriched cells were stored at -80 °C until submitted for sequencing.

5.2.4 Flow cytometry and cell sorting

For cell sorting, Aria I or Aria II sorter and BD FACS Diva Software (BD Bioscience) were used. Both HSC and hepatocytes were sorted with a 100 µm nozzle. Cell debris was gated out based on forward scatter area (FSC-A) and side scatter area (SSC-A). Forward scatter width (FSC-W) and forward scatter height (FSC-H) were used to exclude cell doublets. To remove dead cells, FxCycle™ violet staining (Invitrogen, 1:1,000) and DRAQ7 (BioLegend, 1:1,000) were added to cells, respectively. For HSC enrichment, an extra gating at Pacific blue channel based on the autofluorescence of vitamin A was performed. After sorting, enriched cells were preserved at -80 °C until further investigation.

5.2.5 Staining and analysis

5.2.5.1 Sample preparation

Freshly isolated left lateral lobe was used for tissue staining. Liver samples were fixed in 4% paraformaldehyde (PFA) at 4 °C overnight with slight agitation. Samples were thoroughly washed with PBS twice and transferred 70% ethanol until further processed in spin tissue processor STP120, being incubated in different reagents including ethanol gradient (70% - 85% - 96%), isopropanol, xylene, and paraffin. Next, samples were embedded into paraffin blocks before cutting.

Paraffin blocks with liver samples were cut into 7µm thickness with rotary microtome HM355S. Sessions were attached to glass slides and dried overnight in incubator at 37 °C. Dried slides were kept at room temperature (RT) before further staining.

5.2.5.2 Hematoxylin and eosin staining

To gain histological insights, liver sections were stained by hematoxylin and eosin. After deparaffinized and re-hydrated by incubating in Xylene, 99% - 85% - 70% ethanol gradient, and deionized water (VE water), slides were incubated in freshly filtered Hemalaun solution for 4 minutes and washed by running tap water for 10 minutes. Last, samples were stained in 1% ethanoic eosin solution for 2 minutes and washed three times in VE water, and dehydrated through decreasing ethanol gradient (70% - 85% - 99%), isopropanol, and Xylene. After mounting with histomount and drying for Xylene, stained slides were scanned in Zeiss AxioScan slide scanner and saved for further analysis.

5.2.5.3 Sirius Red staining

Sirius Red staining was performed to visualize and enhance intrinsic birefringence of collagen fibers in the damaged liver. Liver sections were deparaffinized, re-hydrated, and stained for nuclei with Hemalaun as described above (5.2.5.2). Next, sections were stained by picosirius red for one hour at room temperature. Subsequently, slides were washed twice with 0.5% acetic acid and dehydrated as described above. Last, stained slides were mounted with histomount, dried, and scanned by Zeiss Axioscan slide scanner or DIC microscopy.

5.2.5.4 Masson trichrome staining

Masson trichrome staining was used to stain collagen stored within the damaged liver. Sections were deparaffinized, re-hydrated, and incubated in Bouin's solution at 56 °C for 15 minutes. After cooling in tap water, Bouin's solution was washed away by washing in running tap water. Next, slides were stained in Weigert's Iron Hematoxylin solution for 5 minutes and washed again in running tap water for 5 minutes. Subsequently, slides were stained in Biebrich Scarlet-Acid Fuchsin for 5 minutes after rinsed in VE water and rinsed again after incubation. Last, slides were then incubated in Phosphomolybdic Acid solution and Aniline Blue solution for 5 minutes each and washed with 1% Acetic Acid for 2 minutes. Stained sections were rinsed and dehydrated through alcohol series and mounted before being scanned by Zeiss Axioscan slide scanner.

5.2.5.5 Immunohistochemistry staining

Liver sections were deparaffinized and re-hydrated. For antigen retrieval, slides were boiled in Citrate Buffer (Dako, pH=6) at 99 °C for 20 minutes in water bath and cooled down on ice for 30 minutes. After washing with TBS-T three times for 5 minutes, sections were blocked for peroxidase in 3% H₂O₂ for 10 minutes at RT. Next, slides were blocked in a blocking buffer containing 3% BSA in PBS and 1% Triton-X for 1 hour at RT. Last, sections were blocked in Avidin and Biotin for 5 minutes each, with three washing in between, and incubated with primary antibody at 4 °C overnight.

The next day, slides were washed for three times and incubated with biotinylated secondary antibody for 1 hour at RT. After the incubation and three times washing, sections were incubated in ABC solution (Vector Laboratories) for 30 minutes at RT, followed by three times washing. Subsequently, DAB was developed on the section until signals were clear. Reaction was stopped by VE water, followed by counterstain in Hematoxylin for 4 minutes, and washed with running tap water for 10 minutes. Last, sections were dehydrated and mounted in histomount.

For Cyp1a2 and Cyp2f2 staining, M.O.M.[®] immunodetection kit (Vector Laboratories) was used following the manufacturer's instructions.

5.2.5.6 Immunofluorescence staining

Tissue sections were first deparaffinized and re-hydrated as described. Antigen retrieval was achieved by incubating with Citrate Buffer (Dako, pH=9) at 99 °C for 20 minutes and then cooled down. After washed in PBS for 5 minutes, sections were blocked in the respective blocking reagent as indicated in Table 25 for 1 hour at RT. Subsequently, slides were incubated with primary antibody diluted with 10% goat serum in PBS (Zymed) overnight at 4 °C.

The next day, slides were washed three times with TBS-T with slight agitation for 5 minutes and stained by different secondary antibodies depending on the species of primary antibody, together with Hoechst 33342 (Sigma Aldrich, 1:2,000), for 1 hour at RT. Slides were mounted in DAKO mounting medium (Agilent) after three washes with TBS-T. Stained slides were preserved at 4°C in the dark before being scanned with a Zeiss AxioScan slide scanner.

5.2.5.7 Histological evaluation by histologist and hydroxyproline assay for fibrosis evaluation

To evaluate the extent of fibrosis, H&E and SR staining of fibrotic liver tissues were evaluated and scored by an experienced histologist at The Technical University of Munich, including semiquantative amount of fibrosis and their distribution in the liver (portal, with thin or large septae connecting between large veins, with architectural disturbance, or full cirrhosis). The extent of inflammation and fibrosis were scored from 0 to 3 and 0 to 4, respectively.

Hydroxyproline assay (Cell Biolabs) was performed using plasmas diluted with 12N HCl. After incubating at 95 °C for 24 hours, samples were cooled and mixed with activated charcoal and centrifuged at 10,000 x g for 5 minutes. Supernatant was transferred to a new tube and stored at 4 °C. Before measuring, samples were heated to 60 to 80 °C for 30 to 45 minutes until completely dried and incubated with Chloramine T Mixture for 30 minutes at RT. Next, Ehrlich's Reagent was added to samples and incubated at 60 °C for 45 minutes. After cooling for 5 minutes at 4 °C, samples were centrifuged at 6,000 x g for 15 minutes at RT. Supernatant was used for quantification following the manufacturer's protocol.

5.2.6 Cell culture

5.2.6.1 Cell maintenance and subculture

Human immortalized HSC cell line LX-2 was originally generated in Friedman's lab. When culturing, LX-2 was kept in Dulbecco's Modified Eagle Medium (DMEM) with high glucose (4.5g/L) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin in the incubator with 5% CO₂ in high humidity at 37 °C. Cells were passaged at 80 to 90% confluency. When splitting, cells were washed with PBS and detached with Trypsin-EDTA (Sigma Aldrich) at 37 °C for 3 minutes. Once cells were detached from the

plate, culturing medium was added to neutralize Tripsin and split at the ratio of 1:3 to 1:5, depending on the purpose.

Primary mouse HSC (mHSC) was freshly isolated from the mouse liver as previously described (5.2.3.2). After being enriched from the intermediate layer between GBSS/B and Histodenz, cells were counted manually with a counting chamber Neubauer. After counting, mHSC were seeded to culture dish with Dulbecco's Modified Eagle Medium (DMEM) with high glucose (4.5g/L) supplemented with 10% FCS, 1% penicillin/streptomycin, and 50 µg/mL Gentamycin at 37 °C for further investigation. mHSC was only used directly for experiment, without further subculture.

5.2.6.2 Mycoplasma detection

Cultured cells' identity was confirmed based on their morphology, and mycoplasma contamination was examined after being cultured for the first time by PCR. To do so, 1 ml culture medium from cells was collected and centrifuged to remove debris. Subsequently, 2 µl supernatant was used for Taq Polymerase kit (Qiagen) with the mixture and program shown in Table 26. Results were analyzed with QIAxcel Advanced System following the manufacturer's instructions.

Table 22: PCR for mycoplasma detection

PCR Reaction mix (Qiagen)		PCR amplification program (Thermocycler)		
Reagent	Volume	Step	Temperature	Time
ddH ₂ O	6.8 µl	1	95 °C	5 sec
Q-Solution	2.6 µl	2	95 °C	30 sec
Buffer 10x	1.25 µl	3	58 °C	30 sec
MgCl ₂	0.5 µl	4	72 °C	1 min
dNTPs (2.5 mM)	0.25 µl	Back to step 2: x40		
MGSO (10 µM)	0.25 µl	5	72 °C	10 min
GPO (10 µM)	0.25 µl	6	4 °C	∞
Taq (5U/µl)	0.1 µl			
Supernatant	2 µl			
Mycoplasma positive: 270 bp				

5.2.6.3 Cryopreservation

For long-term storage, cells were cryopreserved in a liquid nitrogen tank. Cultured cells were washed and detached and centrifuged at 200 x g for 5 minutes. Pellet was resuspended in cryopreserve medium that contains 50% culture medium, 40% FCS, and 10% DMSO, and aliquoted 1 ml each in cryotubes and gradually frozen in an isopropanol-containing box (Mr. Frosty freezing container, Thermo Fisher Scientific) at -80 °C overnight. Tubes were transferred to a liquid nitrogen tank for long-term preservation.

When recovering cells, cryotubes were warmed in a 37 °C water bath until completely thawed. 1 ml cell suspension was diluted in 6 ml cell culture medium and centrifuged at 200 x g for 5 minutes to remove DMSO. Next, cell pellets were resuspended in 10 ml cell culture medium and seeded in a 10 cm dish.

5.2.6.4 Cell counting

Cell number was counted before seeding to plates for experiments. Cells were washed and detached from culturing dish and transferred to a falcon tube. After mixing to a homogenous solution, 10 µl cell suspension was taken and mixed with 10 µl trypan blue. 10 µl mixture was applied to a counting chamber and counted by an automated cell counting machine (Countess, Thermo Fisher Scientific) following the manufacturer's instruction. Alternatively, primary isolated HSC were counted manually with a counting chamber Neubauer (Sigma Aldrich) following the manufacturer's manual.

5.2.7 Molecular biology

5.2.7.1 RNA isolation

RNA extraction from cultured cells was performed using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma Aldrich) following the manufacturer's instruction. RNA from sorted cells was isolated with an Arcturus™ Picopure™ RNA isolation kit (Thermo Fisher Scientific) based on the manufacturer's protocol. For snap-frozen tissues, small pieces of the organ were homogenized by TissueLyzer (Retsch) together with TriZol solution and purified using chloroform (Carl Roth). After centrifuged at 12,000 x g for 15 minutes at 4 °C, the upper aqueous phase was mixed with the same amount of 70% ethanol and applied to GenElute™ Mammalian Total RNA Miniprep Kit (Sigma Aldrich) according to the manufacturer's instruction. RNA was eluted in specific volumes of RNase-free water, depending on the expected RNA amount. Concentration of purified RNA was measured by NanoPhotometer N60 and immediately processed, leftover RNA was preserved at -80 °C for future use.

5.2.7.2 Reverse transcription of cDNA

After being quantified by NanoPhotometer N60, RNA (10 pg to 1 µg) was reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen) based on the manufacturer's instruction. In case of insufficient RNA amount, all samples were normalized to the sample with the lowest concentration. After reverse transcription, synthesized cDNA samples were diluted to 2.5 ng/µl with nuclease-free water and stored at -20 °C until use.

5.2.7.3 Quantitative PCR

Gene expression analysis was performed by quantitative PCR with TaqMan master mix and TamMan probes (Thermo Fisher Scientific) on either StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) for 96-well plates or Lightcycler 480 (Roche) for 384-well plates. Samples were pipetted to plates as indicated in Table 27. Samples were analyzed in either technical duplicated (96-well) or triplicated (384-well). Expression levels of genes were calculated using $2^{-\Delta\Delta Ct}$ algorithm, normalized to *Actb*.

Table 23: qPCR reaction setup

qPCR Reaction mix		Amplification program		
Reagent	Volume	Step	Temperature	Time
cDNA	4 µl	1. Pre-denaturation	95 °C	30 sec
TaqMan Fast Advanced Master Mix	4 µl	2. Denaturation	95 °C	2 sec
TaqMan probe	0.5 µl	3. Amplification	60 °C	20 sec
Nuclease-free water	0.5 µl	Go to step 2: x40		

5.2.8 Protein biochemistry

5.2.8.1 Protein extraction

Protein from cultured cells was extracted by lysed in modified RIPA buffer on ice for 15 minutes, with vortex every 5 minutes. Lysate was then centrifuged at 14,000 rpm at 4 °C for 15 minutes. Supernatant was transferred to new tubes, and protein concentration was measured by Pierce™ BCA assay kit following the manufacturer's instruction. For long-term storage, quantified protein was stored at -80 °C.

5.2.8.2 Western blotting

To make samples for Western blot, 20 to 25 μ g total protein was boiled in 5x laemmli buffer at 99 °C for 2 minutes and centrifuged at 11,000 x g for 1 minute at 4 °C. For long-term storage, samples were stored at -20 °C.

Samples were run on 12% SDS-PAGE gel using BioRad gel casting and running system in 1x running buffer. Protein was identified by its size with Page Ruler Prestained Protein ladder, which was applied to the gel together with samples. Samples were run at 100 voltages and 20-30 milliampere for around 90 minutes until expected bands reached the middle of the gel and laemmli buffer reached the bottom. Next, proteins were transferred to a nitrocellulose membrane at 50 voltages for 2 hours in 1x blotting buffer. After transferring, membrane was blocked in 5% skim milk dissolved in TBS-T for 1 hour at RT. Subsequently, membrane was cut at 70 kDa and incubated with primary antibodies (RSPO3: Invitrogen, PA5-117129; VINCULIN: Abcam, ab129002) at 4 °C overnight with slight agitation.

Following overnight primary antibody incubation, membrane was thoroughly washed in TBS-T three times for 5 minutes and incubated with horseradish peroxidase-conjugated secondary antibody (HRP goat anti-rabbit, DAKO, 703-095-155) at RT for 1 hour and washed again in TBS-T for three times. Proteins were visualized using Enhanced Chemiluminescence Western Blotting Substrate (ECL, Pierce) and scanned by Amersham imager 600 (GE). Quantification of bands was performed using densitometric analysis with Fiji software.

5.2.8.3 Enzyme-linked immunosorbent assay

RSPO3 protein concentrations were quantified using a DuoSet ELISA kit (R&D Systems) or sandwich ELISA kit (LSBio) following the manufacturer's protocols. Circulating RSPO3 levels and liver-specific RSPO3 concentration were measured with R&D kit, and RSPO3 contents in supernatants from cultured cells were determined with LSBio kit (with a higher detection range). Plasma samples were diluted 1:500 with 1x Reagent Diluent provided in the DuoSet ELISA kit. Protein lysate from snap-frozen liver sample was collected from the supernatant of homogenized tissue followed by 1,000 x g for 20 minutes and adjusted to 100 μ g/mL. Supernatant samples were collected 24 hours after plated from 0.1 million cells seeded in a 12-well plate and centrifuged at the same speed above. All samples were stored at -80 °C.

5.2.9 Bulk RNA-sequencing and data processing

HSC and hepatocytes were freshly isolated and sorted from 4 months CCl₄-treated HSC-specific Rspo3 KO mice and their littermates control as described above (5.2.3.2 and 5.2.3.4). Total RNA was extracted using an Arcturus Picopure RNA isolation kit (Thermo Fisher Scientific). Library for sequencing was generated from 10 ng total RNA with SMARTer Ultra Low RNA v4 kit (Clontech) and sequenced over four lanes on HiSeq 4000 single-read (HSC) and two lanes on NovaSeq 6000 (hepatocyte) paired-end platform, respectively.

Raw FASTQ files were processed via quality control and trimmed with default criteria (Phred>Q15, minimum read length 15 bp). Trimmed reads were aligned to mouse reference genome mm10 by STAR v2.7.10a³⁰⁷. Duplicate-marked and sorted alignment files were subjected to gene counting, and gene-level read counts were obtained with htseq-count v2.0.1³⁰⁸ with transcriptome annotation GTF file version GRCm38.96. Principle Component Analysis was computed with the top 1,000 most variable genes. Differential expression analysis was performed using DESeq2 with filtered genes with a minimum of total five raw counts across samples. Significant DEGs were defined using post-hoc analysis with BH-adjusted p-value < 0.05 and absolute log₂FC > 1. Heatmap was generated by clustering samples as columns and DEGs as rows with Euclidean distance with the “Ward.D” method using heatmap. The volcano plot was generated to highlight DEGs in blue (downregulated) and red (upregulated). Last, GSEA was performed with clusterProfiler package from R³⁰⁹, with top enriched (NES > 0 and p-adj < 0.05) and depleted (NES < 0 and p-adj < 0.05) terms visualized.

5.2.10 Statistical analysis

Statistical analysis was performed with GraphPad Prism (v9). Data were presented as mean ± standard deviation. Unpaired two-tailed Student's t-test was performed, and p-values less than 0.05 were considered significant (* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

6 Abbreviations

Abbreviation	Full name
3-OH-APAP	3-hydroxy-APAP
AAV8	Adeno-associated virus serotype 8
AE2	Anion exchanger 2
AFP	Alpha-fetoprotein
aHSC	Activated hepatic stellate cell
AIH	Autoimmune hepatitis
ALD	Alcohol-associated liver disease
ALT	Alanine aminotransferase
APAP	Acetaminophen
APC	Antigen-presenting cell
APC	Adenomatous polyposis coli
APPs	Acute-phase proteins
AST	Aspartate transaminase
AST	Aspartate transaminase
BDL	Bile duct ligation
bp	base pair
CaMKII	Calcium/calmodulin-dependent kinase II
cAMP	cyclic adenosine 3', 5' - monophosphate
CaN	Calcineurin
cccDNA	covalently closed circular DNA
CCl ₃ *	Trichloromethyl radical
CCl ₄	Carbon tetrachloride
CDAAs	Choline-Deficient L-amino-defined
CDC42	Cell division control protein 42 homolog
CFTR	Cystic fibrosis transmembrane conductance regulator
CK19	Cytokeratin 19
CK1 α	Casein kinase 1 α
CLD	Chronic liver disease
c-Maf	Transcription factor c-Maf
CRP	C-reactive protein
CXCL9	Chemokine (C-X-C motif) ligand 9
CYP	Cytochrome P450
DAAM1	Dishevelled-associated activator of morphogenesis 1
DAG	Diacylglycerol
DEGs	Differentially expressed genes
DEN	Diethylnitrosamine
D-GalN	D-galactosamine
DIC	Differential Interference Contrast
Dvl	Dishevelled
EC	Endothelial cells
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EndMT	Endothelial-to-mesenchyme transition
ER	Endoplasmic reticulum
Evi/Wls	Wntless

FACS	Fluorescence-activated Cell Sorting
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
Flt4	Fms-related tyrosine kinase 4
FSC-A	forward scatter area
FSC-H	Forward scatter height
FSC-W	forward scatter width
fwd	forward
Gata4	GATA binding protein 4
GBV-C	GB virus C
GGPPS	Geranylgeranyl diphosphate synthase
GS	Glutamine synthetase
GSH	Glutathione
GSK3 β	Glycogen synthase kinase 3 β
H&E	Hematoxylin-Eosin
HAI	Histological Activity Index
HAV	Hepatitis A virus
HBsAg	hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HEV	Hepatitis E virus
HFD	High fat diet
HGF	Hepatocyte growth factor
HGV	Hepatitis G virus
HOC	Hepatic oval cell
HSC	Hepatic stellate cell
i.p.	Intraperitoneal
iECKO	LSEC-specific knockout mice
IF	Immunofluorescence
IHC	Immunohistochemistry
IL	Interleukin
INF- γ	Interferon-gamma
Int	Integration
IP3	Inositol 1,4,5-triphosphate
IR	Insulin resistance
Jedi	Just eGFP death-inducing
KC	Kupffer cell
KO	Knockout
LEC	Liver endothelial cell
LPS	Lipopolysaccharide
LRP5/6	Low-density lipoprotein receptor-related proteins 5 and 6
LSEC	Liver sinusoidal endothelial cell
LSECtin	Liver sinusoidal endothelial cell lectin
Lyve1	Lymphatic vessel endothelial hyaluronic acid receptor
MAIT cells	Mucosal-associated invariant T-cells
MCD	Methionine- and choline-deficient diet
MHC	Major histocompatibility complex

Abbreviations

mHSC	primary mouse HSC (mHSC)
min	minute
MMTV	Mouse mammary tumor virus
MoMφ	Monocyte-derived macrophage
MRI	Magnetic resonance imaging
MT	Masson Trichrome
mTORC1	mammalian target of rapamycin complex 1
NAFLD	Non-alcoholic fatty liver disease
NAFLD	Non-alcoholic fatty liver disease
NAPQI	N-acetyl-p-benzoquinone imine
NASH	Nonalcoholic steatohepatitis
NASH	Non-alcoholic steatohepatitis
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa B
NIAAA	National Institute on Alcohol Abuse and Alcoholism
NK-cell	Natural killer cell
NO	Nitric oxide
NPCs	Non-parenchymal cells
PAMPs	Pathogen-associated molecular patterns
PBS	Dulbecco's phosphate buffered saline
PCA	Principle component analysis
PCNA	Proliferating cell nuclear antigen
PCP	Planar cell polarity
PD1	Programmed cell death protein 1
PDGF	Platelet-derived growth factor
PDL1	Programmed cell death 1 ligand 1
PF	Portal fibroblasts
PGE2	Prostaglandin E2
PHx	Partial hepatectomy
PIP2	Phosphatidylinositol 4,5 biphosphate
PKC	Protein kinase C
PKG	Protein kinase G
PLC	Phospholipase C
PPAR	Peroxisome proliferator activated receptor
Prox1	Prospero homeobox protein 1
PS	Pig serum
PTEN	Phosphatase and Tensin Homolog
qHSC	Quiescent hepatic stellate cell
qPCR	quantitative polymerase chain reaction
rcccDNA	recombinant covalently closed circular DNA
rev	reverse
RNF43	Ring finger protein 43
ROCK	Rho-associated kinase
ROS	Reactive oxygen species
rpm	revolutions per minute
RSPO	Roof plate-specific spondins
Rspo3	R-spondin 3
<i>Rspo3^{fl/fl}</i>	<i>Rspo3^{ind-HSC}</i> littermate contral
<i>Rspo3^{ind-HSC}</i>	inducible HSC-specific <i>Rspo3</i> deficient mice

RT	Room temperature
SAA	Serum amyloid a proteins
SAP	Serum amyloid p proteins
scRNA-seq	single-cell RNA sequencing
sec	second
siRNA	small interfering RNA
smFISH	single-molecule fluorescence in situ hybridization
SR	Secretin receptors
SR	Sirius Red
SSC-A	side scatter area
SULT	Sulfotransferase
TAA	Thioacetamide
TAM	Tamoxifen
TCF/LEF	T-cell factor/lymphoid enhancer factor
TCR	T-cell receptor
TEM	Transmission electron microscopy
TF Model	Tsukamoto–French Model
TGF- β	Transforming growth factor beta
Thy-1	Thymocyte differentiation antigen 1
TNF- α	Tumor necrosis factor- α
TSC2	Tuberous Sclerosis Complex 2
TTV	Torque teno virus
UGT	UDP-glucuronosyl transferases
UV	Ultraviolet
VE water	deionized water
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue
WB	Western blotting
Wg	Wingless
WHO	World Health Organization
Wnt	Wingless-related integration site
WT	Wild-type
ZNRF3	E3 ubiquitin ligase zinc and ring finger 3
α -SMA	alpha-smooth muscle actin
β TRCP	β -transducin repeat-containing protein
$\gamma\delta$ T cells	Gamma delta T-cells

7 Publications

Inverso D, Shi J, Lee KH, Jakab M, Ben-Moshe S, Kulkarni SR, Schneider M, **Wang G**, Komeili M, Vélez PA, Riedel M, Spegg C, Ruppert T, Schaeffer-Reiss C, Helm C, Singh I, Boutros M, Chintharlapalli S, Heikenwalder M, Itzkovitz S, Augustin HG. A spatial vascular transcriptomic, proteomic, and phosphoproteomic atlas unveils an angiocrine Tie-Wnt signaling axis in the liver. Dev Cell. 2021; 56(11):1677-1693

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