Curtin Medical School

The role of Wnt/β-catenin signalling in the regulation of liver progenitor cell responses in non-alcoholic fatty liver disease

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DECLARATION

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgement has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Human Ethics The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) – updated March 2014. The proposed research study received human research ethics approval from the Fremantle Hospital Human Research Ethics Committee (96/37).

Animal Ethics The research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes 8th edition (2013). The proposed research study received animal ethics approval from the Curtin University Animal Ethics Committee, Approval Number ARE2019-20.

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is one of the major contributors in an increasing global burden of chronic liver disease. Hepatic fat accumulation, also referred to as steatosis during the initial stage of NAFLD is benign. However, NAFLD represents a spectrum that progresses when steatosis is accompanied with hepatocellular injury and inflammation. This more severe stage is known as non-alcoholic steatohepatitis (NASH). In some cases, NASH is accompanied by fibrosis and may progress to cirrhosis and end-stage liver disease and cancer. Obesity, insulin resistance and other components of the metabolic syndrome are known risk factors for NAFLD. However, the exact underlying mechanisms that drive NAFLD spectrum progression are unknown. In addition, no pharmacotherapy is currently available to inhibit or reverse steatosis and NASH progression to further stages.

In case of acute liver injury, remaining healthy hepatocytes are able to proliferate and replace lost liver tissue. However, during most chronic liver diseases, stem-like liver progenitor cells (LPCs) emerge near the finest branches of the biliary tree in periportal areas. They migrate into the liver parenchyma to sites of injury in close association with fibrosis-driving hepatic stellate cells as well as inflammatory cells such as macrophages to establish an injury-regeneration niche, potentially orchestrated through cellular signalling crosstalk.

Numerous molecular signalling pathways govern the molecular and cellular behaviour of LPCs. A key candidate is the Wnt/β-catenin signalling pathway, which has demonstrated roles in liver development, adult liver homeostasis, and liver regeneration as well as in liver disease. However, the expression of the Wnt/β-catenin signalling pathway is highly context-specific, making it important to study its regulation in LPCs in an appropriate disease context, such as NAFLD.

The first aim of the studies presented in this thesis was to investigate the presence of LPCs and the expression of β-catenin in clinical specimens of NAFLD. Number of pan-cytokeratin-positive LPCs become more noticeable

with progression in the NAFLD stage. Additionally, the presence of cytoplasmic/nuclear β-catenin in subpopulations of LPCs indicated active Wnt/β-catenin signalling in these cells. Further, the expression of the Wnt/β-catenin signalling pathway was studied in the murine choline-deficient, ethionine-supplemented (CDE) chronic liver injury model. Hallmarks of NAFLD such as steatosis, inflammation, and fibrosis are observed with CDE administration. In addition, activation of LPC compartment can be reliably achieved with a CDE dietary regime. An upregulation in the upstream and downstream components of the Wnt/β-catenin signalling pathway in CDE-treated livers suggested overall activation of the pathway. Moreover, co-localisation of active β-catenin and the LPC markers A6, pan-cytokeratin and cytokeratin-19 confirmed that LPCs are Wnt-responsive and signal through the Wnt/β-catenin pathway. To investigate the expression of the Wnt/β-catenin pathway in LPCs which are exposed to a lipid-rich microenvironment, a novel LPC-focussed steatotic cell culture model was established using the well-characterised LPC line BMOL-TAT as well as palmitate and oleate to mimic NAFLD-like conditions. A significant upregulation in the β-catenin and other upstream components of the Wnt/β-catenin pathway such as Wnts, frizzled receptors at the transcript level was observed upon fat-loading. Moreover, nuclear/cytoplasmic localisation of active β-catenin was evident upon fluorescent staining. Overall, results indicated an active Wnt/β-catenin signalling pathway in fat-loaded LPCs. Finally, the effect of the Wnt/β-catenin pathway antagonist, PRI-724 on LPCs in NAFLD-like environments was studied *in vitro* and *in vivo*. PRI-724 treatment significantly inhibited the proliferation of BMOL-TAT cells in the lipid-loaded culture system. Additionally, a significant reduction in $A6^+$ LPCs was observed with PRI-724 administration for 23 days in chronically injured mice fed a CDE diet.

This thesis provides important new insights into the expression and regulation of Wnt/β-catenin signalling in (a) LPCs in human NAFLD patients, (b) fat-loaded LPCs *in vitro* and (c) LPCs proliferating in murine CDE-induced chronic liver injury. These novel data suggest the potential for future therapeutic targeting of LPCs in fatty liver disease conditions through targeting of the Wnt/β-catenin pathway.

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ABSTRACTS

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ABBREVIATIONS

Ac-Me	acetone-Methanol
ALT	alanine transaminase
APC	adenomatous polyposis coli
α-SMA	alpha-smooth muscle actin
BCA	bicinchoninic assay
BMOL	bipotential murine oval liver
BSA	bovine serum albumin
CBP	CREB-binding protein
CCR5	CC chemokine receptor 5
CDE	choline-deficient, ethionine-supplemented
C/EBPβ	CAAT enhancer binding protein β
CK	cytokeratin
CK1	casein kinase 1

CLD	chronic liver disease
CREB	cAMP response element binding protein
ddH_2O	double-deionised water
DDC	3,5-diethoxycarbonyl-1,4-dihydrocollidine
DKK	Dickkopf
DMAB	4-dimethylaminobenzaldehyde
DMSO	dimethyl sulfoxide
DRs	ductular reactions
Dvl	dishevelled
E-cadherin	epithelial-cadherin
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor

ERK	extracellular signal-regulated kinases
FBS	fetal bovine serum
FGF	fibroblast growth factor
Fn14	fibroblast growth factor-inducible 14
FFAs	free fatty acids
FFPE	formalin-fixed, paraffin-embedded
FOXO	forkhead box protein O
Fz	frizzled
GSK3β	glycogen synthase kinase 3β
H & E staining	haematoxylin and eosin staining
HCC	hepatocellular carcinoma
HGF	hepatocyte growth factor
HRP	horseradish peroxidase
HSCs	hepatic stellate cells

Hu	human
IF	immunofluorescence
IGF	insulin-like growth factor
IHC	immunohistochemistry
IL	interleukin
IR	insulin receptor
IRS	insulin receptor substrate
JNK	Jun N-terminal kinase
LEF	lymphoid enhancer factor
LGR5	leucine-rich repeat-containing G-protein coupled receptor 5
LPCs	liver progenitor cells
LRP	low-density lipoprotein receptor-related protein
LSECs	liver sinusoidal endothelial cells

MAFLD	metabolic dysfunction-associated fatty liver disease
MAPK	Ras-mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
MIP	macrophage inflammatory protein
mTOR	Akt-mammalian target of rapamycin
MTT	3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide
Mu	Murine
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor κ B
OCT	optimal cutting temperature
OV-6	oval cell marker

panCK	pan-cytokeratin
PBS	phosphate-buffered saline
PCP	planar cell polarity
PDGFB	platelet-derived growth factor subunit B
PEG	polyethylene glycol
PFA	paraformaldehyde
PHx	partial hepatectomy
PI3K	phosphatidylinositol 3-kinase
PNPLA3	patatin-like phospholipase domain-containing 3 protein
PPAR	peroxisome proliferator-activated receptor
PTEN	phosphatase and tensin homologue deleted on chromosome 10
RANTES	regulated upon activation T cells expressed and secreted
RIPA	radio-Immunoprecipitation Assay

ROR	receptor tyrosine kinase-like orphan receptor
RT-PCR	reverse transcription-polymerase chain reaction
SEM	standard error of the mean
sFRP	secreted frizzled-related proteins
sox9	sex determining region Y (SRY)-related high mobility group (HMG) box 9
STAT3	signal transducer and activator of transcription 3
TAT	tyrosine aminotransferase
TBS	tris buffered saline
Tbx3	t-box transcription factor 3
TCF	T cell factor
TGF	transforming growth factor
TNF	tumour necrosis factor
TWEAK	tumour necrosis factor-like weak inducer of apoptosis

WB	Western blotting
WIF1	Wnt inhibitory factor 1
Wnt/Ca^{2+}	Wnt/Calcium

Chapter 1

General Introduction

Parts of the general introduction have already been published and the article is included in the appendix (Shirolkar et al., 2018)

1.1 The liver

The liver is the largest internal organ in the body, situated in the right upper quadrant of the abdomen (Campbell, 2006; Ross & Pawlina, 1979). Anatomically, the liver can be divided into two large (the right and left) and two smaller lobes (the quadrate and caudate) (Ross & Pawlina, 1979; Rutkauskas, Gedrimas, Pundzius, Barauskas, & Basevicius, 2006). For clinical purposes, the liver structure is divided into eight segments (I to VIII), based on vascular and ductal patterns (Malarkey, Johnson, Ryan, Boorman, & Maronpot, 2005). Segments I-IV comprise left lobe whilst segments V-VIII make up the right lobe.

Microscopically, the liver parenchyma appears to be made up of repetitive structural and functional units termed lobules (Hailfinger, Jaworski, Braeuning, Buchmann, & Schwarz, 2006). There are a number of ways to characterise the structure of the liver lobule. The classic hexagonal lobule concept was first described by Kiernan in 1833 (Kiernan & Green, 1833; Malarkey et al., 2005). According to this concept, a hepatic lobule is a hexagonal mass of tissue with a central vein at the centre and the portal areas situated at the angles of a hexagon (Ross & Pawlina, 1979). The portal lobule concept was put forward by Mall in 1906, where the focus was placed on the portal triad (Malarkey et al., 2005). In 1954, Rappaport suggested the liver acinus theory (Malarkey et al., 2005). As per this concept, hepatocytes are divided into three zones in each liver acinus: zone 1, zone 2 and zone 3. Zone 1 is furthest from the central vein, whereas zone 3 is the closest and zone 2 is situated between zone 1 and 3 (Ross & Pawlina, 1979).

The liver has a unique dual blood supply system. Approximately, 75% of its blood supply comes from the hepatic portal vein, which carries oxygen-depleted blood from the digestive tract and abdominal organs to the liver to carry out metabolism-related processes. The remaining 25% of the blood supply comes from the hepatic artery, which is a branch of the coeliac trunk, carrying oxygenated blood (Mitra & Metcalf, 2012; Ross & Pawlina, 1979). In the liver lobule, blood coming from both the hepatic portal vein and the hepatic artery mix and pass through the sinusoids and travel towards the central vein (Ross & Pawlina, 1979; Vekemans & Braet, 2005). In contrast, bile flows from

the central region towards the bile duct situated in the portal triad (Karpen & Karpen, 2017).

The liver plays a crucial role in diverse physiological processes. Numerous plasma proteins such as albumins, lipoproteins, glycoproteins are produced and secreted by the liver. It is involved in carbohydrate, lipid and protein metabolism and controls uptake, storage and distribution of nutrients and vitamins. Various toxic substances and drugs are metabolised in the liver. In addition, the liver produces bile and also performs endocrine like functions by modifying the action of hormones, such as insulin, glucagon and growth hormone (Ross & Pawlina, 1979; Trefts, Gannon, & Wasserman, 2017).

1.1.1 Liver cell types

The liver is composed of a number of different, specialised parenchymal and non-parenchymal cell types, which together regulate all hepatic functions.

1.1.1.1 Hepatocytes

Hepatocytes are large (20 to 30 µm in dimension), polygonal cells, occupying almost 80% of the liver volume and they have exceptional capacity for regeneration. Hepatocytes contain numerous mitochondria, Golgi complexes, peroxisomes and extensive smooth endoplasmic reticula (Ross & Pawlina, 1979). Their intralobular location, either pericentral or periportal, dictates their functions, which are different but complimentary to each other (Hailfinger et al., 2006). During studies on carbohydrate metabolism, it was observed that periportal hepatocytes carry out gluconeogenesis, while glycolysis is executed by pericentral hepatocytes, simultaneously. The importance of this functional heterogeneity was explored by Jungermann, Katz and Sasse, and led to the conceptualisation of the ‘metabolic zonation’ theory. Later, markedly zonated expression of the glutamine synthetase enzyme and ammonia metabolism further strengthened the zonation theory (Gebhardt, 1992; Jungermann & Keitzmann, 1996). Gluconeogenesis, glycogen synthesis and β-oxidation processes mainly take place in periportal hepatocytes; while triglyceride

synthesis, lipogenesis or ketogenesis, and glycolysis are carried out by pericentral hepatocytes (Trefts et al., 2017).

1.1.1.2 Cholangiocytes

Cholangiocytes are the second most common hepatic epithelial cell population after hepatocytes. They form the lining of the intra- and extra hepatic ducts of the biliary system. Cholangiocytes contain organelle-scant cytoplasm and complete basal lamina. Tight junctions can be seen between adjacent cells. Each cholangiocyte possesses a primary cilium, which facilitates sensing of changes in the luminal flow (Ross & Pawlina, 1979; Trefts et al., 2017). A significant relationship is seen between the size of cholangiocytes and the area of the bile ducts. The larger bile ducts are lined by larger cholangiocytes, mainly columnar in shape, whereas smaller bile ducts are lined by smaller cholangiocytes, which are cuboidal in shape. The nucleus-to-cytoplasm ratio is higher in smaller cholangiocytes compared to their larger counterparts. This might be the reason for considering small cholangiocytes as undifferentiated, primitive cells and large cholangiocytes as differentiated cells (Marzioni et al., 2002). The major function of cholangiocytes is to modify and transport the hepatocyte-secreted bile (Tabibian, Masyuk, Masyuk, O'Hara, & LaRusso, 2013).

1.1.1.3 Kupffer cells

In 1876, von Kupffer identified liver-specific macrophages, which account for 15% of all liver cells, 35% of the non-parenchymal cells and more than 50% of resident macrophages in the body (Naito, Hasegawa, Ebe, & Yamamoto, 2004). Kupffer cells reside in the sinusoidal lumen anchored to the endothelial cells. The density of Kupffer cells is highest around the periportal area of the lobule (43%) and it decreases in the middle (28%) and central lobular areas (29%). Conventionally, they were viewed as stationary tissue macrophages but evidence indicates that they can migrate along the sinusoidal walls (Kolios, Valatas, & Kouroumalis, 2006). Kupffer cells are irregular in shape and contain

a number of lysosomes, phagosomes, well-developed endoplasmic reticulum and Golgi complexes. Kupffer cells are not very proliferative in normal conditions; however, their cellular turnover is greatly increased in the liver injury state. Apart from endocytosis, Kupffer cells perform a large variety of functions such as antigen processing, secretion of bioactive factors and cytotoxicity (Bouwens, De Bleser, Vanderkerken, Geerts, & Wisse, 1992).

1.1.1.4 Liver sinusoidal endothelial cells (LSECs)

LSECs are stationary cells, which form the lining of the vascular bed in the liver. They can be distinguished by the presence of fenestrations that are 150-175 nm in diameter (Bouwens et al., 1992). The fenestrations give them the ability to filter and control the transport and exchange of materials between the blood and the liver parenchyma. Endocytosis is another important function performed by the endothelial cells. LSECs, along with Kupffer cells, are the key cell types to first face the blood flow coming from the hepatic artery and the portal vein. They have the capacity to sense the difference between harmless and harmful antigens to avoid unnecessary immune responses. Therefore, the crosstalk between these cells and other parenchymal cells can play a crucial role in the development of chronic liver inflammation (Shetty, Lalor, & Adams, 2018; Wisse et al., 1996).

1.1.1.5 Hepatic stellate cells (HSCs)

HSCs were initially described as "*Sternzellen"* (star cells) by Kupffer and fat-storing cells by Ito (Wake, 1999). In 1995, it was recommended that these cells should be referred to as hepatic stellate cells by the international community of researchers ("Hepatic stellate cell nomenclature," 1996). HSCs are thought to be of mesenchymal origin and they reside in the space between sinusoidal endothelial cells and parenchymal cells. They account for 5-8% of the total liver cell population in the healthy liver (Geerts, 2001; Senoo, 2004). In physiological conditions, HSCs rarely proliferate and their main function is to store retinoids. They can store up to 80% of the total retinoids in the form of

retinyl palmitate in lipid droplets in the cytoplasm. However, in pathological conditions, they lose their retinoid stores and undergo transdifferentiation to become myofibroblast-like cells. These are also known as activated HSCs. The latter phenotype is associated with increased proliferation, motility, contractility and production of extracellular matrix components (Geerts, 2001; Hautekeete & Geerts, 1997; Pinzani, Marra, & Carloni, 1998). Numerous studies have confirmed the key role of activated HSCs in remodelling of extracellular matrix and its effect in the progression of chronic liver disease (CLD) (Sato, Suzuki, & Senoo, 2003).

1.1.2 Normal hepatic tissue turnover

In the absence of liver injury, a healthy liver has a very slow cell turnover rate and, only 0.2% to 0.5% of hepatic cells divide at any one time. The average life span of hepatocytes is between 200 to 300 days (Cordero-Espinoza & Huch, 2018; Tirnitz-Parker, Yeoh, & Olynyk, 2012). Various theories have been put forward to explain hepatocyte replication in healthy liver. In the earliest model of “the streaming liver”, the replication pattern of hepatocytes was thought to be similar to intestinal cell regeneration. It was based on the metabolic zonation theory. According to this model, the periportal hepatocytes start to proliferate and migrate towards the central area. These young hepatocytes, attain maturation during migration and upon reaching the central area, they start to express zone-specific genes (Duncan, Dorrell, & Grompe, 2009; Tirnitz-Parker, Yeoh, & Olynyk, 2012; Zajicek, Oren, & Weinreb JR., 1985). However, a lot of contradictory observations were made using different study designs, which negate the streaming liver theory. Using techniques that employ microlight guides and miniature oxygen electrodes, Thurman and Kauffman studied hepatic lobular compartmentation and its association with metabolic processes. They confirmed that metabolic zonation is indeed present, but this is not due to hepatocyte lineage migration but rather due to variation in the metabolite gradient across the liver lobule (Thurman & Kauffman, 1985). Further evidence against the streaming liver model came from studies performed by Bralet *et al*. where the authors introduced a β-

galactosidase-labelled marker gene in hepatocytes using retroviral-mediated gene transfer. The number of β-galactosidase-positive cells increased over time but the distribution pattern of positive cells in the liver lobule was not modified, even after one year. This confirmed that hepatocytes do not migrate from portal to central areas (Bralet, Branchereau, Brechot, & Ferry, 1994). Such findings by various research groups led to the conclusion that normal liver tissue turnover is mediated by randomly distributed hepatocytes and not by a specialised proliferative compartment (Tirnitz-Parker, Yeoh, & Olynyk, 2012). Interestingly, recent lineage-tracing experiments carried out by Wang and colleagues identified a proliferating and self-renewing hepatocyte population in zone 3 that expresses early liver progenitor markers such as t-box transcription factor 3 (Tbx3), Axin 2 and proliferates faster than other hepatocytes (Wang, Zhao, Fish, Logan, & Nusse, 2015).

As a result of such conflicting findings, the exact mechanism behind normal liver tissue regeneration remains debatable (Gilgenkrantz & Collin de l'Hortet, 2018).

1.2 Liver injury

In clinical practice, liver injury can be divided into acute or chronic, depending on the duration and extent of the liver injury.

Acute liver injury is promptly resolved after withdrawal of the injury insult and the liver restores its normal architecture and function (Malhi & Gores, 2008). Drug hepatotoxicity, viral hepatitis, ischemia-reperfusion and surgical resection are the commonest causes of acute liver injury (Nowatari, Murata, Fukunaga, & Ohkohchi, 2014). In Europe and the United States, the majority of acute liver injury cases can be attributed to drug-induced hepatotoxicity. On the other hand, in South Asia, viral hepatitis is responsible for most of the acute liver injury cases (Bernal, Lee, Wendon, Larsen, & Williams, 2015; McDowell Torres, Stevens, & Gurakar, 2010).

Chronic liver injury can be the result of extended acute liver injury. Several factors such as viral infection, metabolic-associated fatty liver disease, alcohol abuse, autoimmune disorders, drug use, cholestasis and less common metabolic diseases can lead to CLD (Nowatari et al., 2014). Consistent production and deposition of extracellular matrix components result in hepatic fibrosis, a hallmark of CLD. Significant hepatic fibrosis can lead to the development of cirrhosis or advanced CLD, which in turn may progress to hepatocellular carcinoma (HCC) (Fernandez-Iglesias & Gracia-Sancho, 2017; Schuppan & Afdhal, 2008).

Liver disease is responsible for approximately two million deaths per year, which constitutes 3.5% of all deaths globally. Of these, the highest number of deaths (1.16 million) are attributed to liver cirrhosis, making it the 11th most common cause of death worldwide (Asrani, Devarbhavi, Eaton, & Kamath, 2019).

Liver transplantation is the only available curative option for end-stage liver disease. However, it is associated with issues such as donor availability, surgery complications due to underlying conditions, organ rejection, high cost and only a minority of patients are eligible for this procedure (Nowatari et al., 2014).

1.3 Liver regeneration in acute versus chronic liver injury

The liver has an exceptional capacity to regenerate and recover the lost liver mass and function following acute liver injury. After surgical resection of up to 70% of the liver tissue, the remaining liver mass regrows within a week in rodents. The most studied model of liver regeneration, partial hepatectomy (PHx), was introduced by Higgins and Anderson (Manco, Leclercq, & Clerbaux, 2018; Nevzorova, Tolba, Trautwein, & Liedtke, 2015). It is important to note that the liver never restores the original structure during the regeneration process but undergoes compensatory hypertrophy or hyperplasia. In the case of 70% PHx, the initial regeneration surge includes hypertrophy of the hepatocytes, followed by hyperplasia of the parenchymal and non-parenchymal cells of the liver. In contrast, in 30% PHx, the liver regeneration is entirely dependent on the hypertrophy of hepatocytes (Kholodenko & Yarygin, 2017; Miyaoka et al., 2012; Miyaoka & Miyajima, 2013).

The liver regeneration via compensatory hyperplasia is a tightly regulated process that involves a number of growth factors, cytokines, transcription factors and cross-talk between hepatocytes and other hepatic cell types. The process of regeneration can be divided into three phases. The first phase (priming) prepares the remaining healthy hepatocytes for reception of growth factors; growth factor receptor activation takes place in the second phase, and the third phase corresponds to the termination of proliferation signals (Gilgenkrantz & Collin de l'Hortet, 2018).

The priming phase lasts for approximately 4 to 6 hours after PHx, during which notable gene expression changes take place in hepatocytes and non-parenchymal cells. Hepatocyte growth factor (HGF) is the first mitogen to stimulate hepatocytes and high levels of HGF are maintained throughout the regeneration by HSCs and LSECs. Apart from HGF, other growth factors such as epidermal growth factor (EGF), transforming growth factor (TGF) α, fibroblast growth factor (FGF), along with the inflammatory cytokines tumour necrosis factor (TNF) and interleukin (IL) 6 from Kupffer cells, play an important role in regeneration. Transcription factors such as nuclear factor κ B (NFκB),

signal transducer and activator of transcription 3 (STAT3), activating protein-1, CAAT enhancer binding protein β (C/EBPβ) are also activated during the priming phase. Transcription factor activation leads to cell cycle-related gene expression (Bilzer, Roggel, & Gerbes, 2006; Fausto, 2001; Manco et al., 2018; Mao, Glorioso, & Nyberg, 2014; Taub, 1996). The epidermal growth factor receptor (EGFR) and c-met are the crucial growth factor receptors required in the regeneration process (Gilgenkrantz & Collin de l'Hortet, 2018; Michalopoulos & DeFrances, 1997).

Once the correct liver-to-body mass ratio is achieved, termination of the proliferative phase takes place. Hippo, TGF-β and integrin signalling pathways inhibit hepatocyte proliferation (Manco et al., 2018; Michalopoulos, 2007).

The exact mechanism behind liver hypertrophy is not known. However, the Akt-mammalian target of rapamycin (mTOR) signalling axis may be involved in regulating the cell size (Miyaoka & Miyajima, 2013).

As explained earlier, chronic liver injury develops gradually due to repetitive and/or continuous insults to the liver. The liver attempts to cope up with this injury by continuous parenchymal and non-parenchymal cell proliferation, which leads to HSC activation and extracellular matrix protein production, mainly collagen. Persistent collagen deposition disrupts the liver architecture, where hepatocytes can no longer proliferate and they enter into replicative arrest. In such instances, the liver depends on stem cell-like liver progenitor cells (LPCs) to regulate the regeneration process (Manco et al., 2018).

1.4 Liver progenitor cells

In 1937, Kinosita reported the presence of oval-like cells in azo dye-treated rat livers (Kinosita, 1937). Later, Farber referred these small ovoid cells as “oval cells” that have a high nuclear-to-cytoplasm ratio (Farber, 1956a, 1956b). Wilson and Leduc were first to note the presence of bipotential liver progenitor or stem cells, as they documented the ability of ductular cells to give rise to hepatocytes and interlobular bile ducts in mice (Wilson & Leduc, 1958). These cells have been given different terminologies such as hepatic stem-like or progenitor cells, oval cells, transit-amplifying ductular cells and were identified as a part of ductular reactions (DRs) (Kohn-Gaone, Gogoi-Tiwari, Ramm, Olynyk, & Tirnitz-Parker, 2016b). The term LPCs will be used to address these cells throughout this thesis. The precise origin of LPCs is not known. Some researchers believe that they reside in the Canals of Hering and are a remnant part of the fetal hepatoblast pool. This may be due to their shared morphological characteristics and marker expression profiles with hepatoblasts. In contrast, some investigators consider LPCs to have originated from an exogenous source such as bone marrow cells (Petersen et al., 1999).

The origin of LPCs is still a controversial topic. In the field, LPCs are considered as bipotential stem-like cells that are not detected in a healthy liver and only appear in chronic liver injury, variably expressing biliary, hepatocytic and haematopoietic markers (Fausto & Campbell, 2003; Kohn-Gaone, Gogoi-Tiwari, Ramm, Olynyk, & Tirnitz-Parker, 2016a). Generally, a combination of different antibodies are used to identify and distinguish LPCs from hepatocytes or cholangiocytes. Commonly used markers include A6, sex determining region Y (SRY)-related high mobility group (HMG) box 9 (Sox9) ^{weak} and pan-cytokeratin (panCK) (Kohn-Gaone, Gogoi-Tiwari, et al., 2016a).

1.4.1 LPC-mediated regeneration

Although hepatocytes and LPCs require similar priming phases and they respond to the same growth factors and cytokine networks, LPC proliferation is more commonly observed in chronic liver injury. This may be attributable to the already suppressed hepatocyte response due to their replicative senescence (Fausto & Campbell, 2003).

Similar to hepatocyte-driven regeneration, TNF and IL-6 are key inflammatory cytokines involved in LPC growth. Knight and colleagues documented that TNF upregulation upon carcinogenic diet administration in mice is due to LPCs and inflammatory cells. They also noted a significantly impaired LPC response in a TNF receptor type 1 knockout animal model. In the same study, a small reduction in LPC numbers was noted in IL-6 knockout mice (Knight et al., 2000). Taken together, these findings suggest that TNF and IL-6 are important players in an optimal LPC response; however, they do not exclusively control it. Another TNF family member, tumour necrosis factor-like weak inducer of apoptosis (TWEAK) was identified as a mitogen for LPCs, which mediates its actions by binding to the fibroblast growth factor-inducible 14 (Fn14) receptor. The mitogenic potential of TWEAK was confirmed in various cell line and animal studies (Jakubowski et al., 2005; Tirnitz-Parker et al., 2010). Several other factors, such as interferon-γ and lymphotoxin β have been found to be upregulated in progenitor cell-mediated regeneration (Dwyer, Olynyk, Ramm, & Tirnitz-Parker, 2014; Fausto, 2005; Santoni-Rugiu, Jelnes, Thorgeirsson, & Bisgaard, 2005).

Upon activation, LPCs migrate into the liver parenchyma and form a niche in close association with activated HSCs and macrophages. Different cell types in a niche can influence each other's behaviour by establishing intra- and intercellular molecular signalling networks (Fausto & Campbell, 2003). Therefore, the concept of a DR was put forward to consider LPC compartment activation as part of the liver tissue remodelling happening during liver injury. DR can be defined as a phenomenon describing LPC proliferation, along with extracellular matrix, inflammatory and vascular changes induced in chronically injured livers (Gouw, Clouston, & Theise, 2011).

Using liver-specific gene knockout mouse models, signalling mechanisms regulating LPC activation, migration and differentiation have been identified. Experiments on liver specific c-met receptor knockout mice have confirmed the essential function of the HGF/c-met signalling pathway in LPC-mediated liver regeneration (Ishikawa et al., 2012; Suarez-Causado et al., 2015). The role of the Wnt/β-catenin and Notch pathways in regulating LPC differentiation towards hepatocytes or cholangiocytes is well established. In the case of biliary injury, HSCs secrete Notch ligand Jagged 1 that interacts with Notch receptors on LPCs and activates the downstream Notch signalling cascade, inducing differentiation of LPCs to cholangiocytes. On the other hand, in hepatocytic injury, activated macrophages produce Wnt ligands, which interact with corresponding Wnt receptors on LPCs to activate Wnt/β-catenin signalling. Numb, a downstream target of Wnt/β-catenin pathway acts as an inhibitor of Notch signalling and prompts the differentiation of LPCs into hepatocytes (Boulter et al., 2012; Itoh & Miyajima, 2014).

As LPCs proliferate in CLD conditions, they are continuously exposed to a dynamic pool of inflammatory cytokines, growth factors and reactive oxygen species, which make them easy targets for transformation. A subpopulation of transformed LPCs are thought to take part in the development of cancerous lesions. This hypothesis was supported when a subset of investigated HCC cells was identified to express LPC markers such as oval cell marker (OV-6) and alpha-fetoprotein (Libbrecht & Roskams, 2002). Further evidence of LPC involvement in HCC development came from a study, where short-term treatment with imatinib mesylate targeted to c-kit$^+$ LPCs led to a reduction in fibrosis, and long-term treatment resulted in impaired HCC formation in the choline-deficient, ethionine-supplemented (CDE) injury model (Knight, Tirnitz–Parker, & Olynyk, 2008). Moreover, 28-50% of human HCCs expressing LPC markers such as cytokeratin (CK) 7 and 19 are associated with poor patient outcomes (Mishra et al., 2009).

1.4.2 CDE murine model

Various mouse models have been developed to study LPC induction during CLD. However, every model exhibits distinct pathological patterns. Thus, depending on the research question, the appropriate model and time point must be carefully selected (Carlessi, Köhn-Gaone, Olynyk, & Tirnitz-Parker, 2019). The work undertaken in this PhD study utilised the CDE model, which is described below in detail.

Choline is an essential nutrient in mammals and its deficiency can give rise to liver and renal complications (Zeisel et al., 1991). Choline-deficient diet administration in rats was determined to affect hepatic triglyceride release, leading to fatty liver development (Lombardi, Pani, & Schlunk, 1968). Later it was found that the addition of DL-ethionine, a hepatocarcinogen, to the choline-deficient diet can lead to massive LPC compartment expansion (Shinozuka, Lombardi, Sell, & Iammarino, 1978; Steinberg, Hacker, Dienes, Oesch, & Bannasch, 1991). DL-ethionine is an antagonist of methionine and inhibits choline biosynthesis (Shinozuka et al., 1978). Long-term feeding (22-30 weeks) of CDE diet in rats was shown to give rise to HCC (Hacker, Steinberg, Toshkov, Oesch, & Bannasch, 1992; Shinozuka, Lombardi, Sell, & Iammarino, 1978). However, this conventional CDE diet can cause morbidity and mortality in mice. Therefore, modifications in the protocol were made to make it applicable to mice (Akhurst et al., 2001). Subsequently, many research groups carried out detailed mouse studies at various time points using modified CDE protocols to understand the liver toxicity generated by this model (Passman et al., 2015; Van Hul, Abarca-Quinones, Sempoux, Horsmans, & Leclercq, 2009). It is now clear that the CDE diet reliably induces steatosis, inflammation, LPC activation, fibrosis and HCC formation, making it an attractive model to study different pathological liver conditions (Kohn-Gaone, Dwyer, et al., 2016b).

1.4.3 Progenitor cells in human CLDs

Similar to LPCs in rodents, human LPCs variably express biliary (such as biliary-type CKs, OV6), fetal hepatocytic (such as α-fetoprotein, hepatocyte paraffin 1) and haematopoietic (such as c-kit, CD34) markers and can differentiate into biliary cells or hepatocytes (Roskams, Libbrecht, & Desmet, 2003). LPCs differentiate to biliary cells via formation of atypical reactive ductules, whereas intermediate hepatocyte-like cells appear during differentiation towards hepatocytic lineage (Libbrecht & Roskams, 2002).

In humans, LPC compartment activation has been observed in CLDs such as chronic viral hepatitis, alcoholic and non-alcoholic fatty liver disease, inherited metabolic diseases and HCC (Roskams et al., 2003). An increase in LPC numbers is associated with the progression in disease severity, irrespective of the type of injury (Lowes, Brennan, Yeoh, & Olynyk, 1999). A close correlation between the extent of injury, LPCs expansion and degree of fibrosis has also been revealed in many studies (Clouston et al., 2005; Prakoso et al., 2014; Roskams, 2008).

Expression of progenitor cell markers (such as α-fetoprotein, CK-7, 19, 14) has been found in more than half of HCCs and in more than 50% of HCC precursor lesions (Roskams et al., 2003). This may be an evidence for the potential role of LPCs in the development of HCC. Importantly, it has been reported that HCCs expressing progenitor cell markers are linked with poor outcomes (Libbrecht, 2006). Attempts were also made to use DR patterns as a marker to distinguish non-invasive versus invasive HCC (Park et al., 2007). Therefore, it is of clinical significance to further elucidate the relationship between LPCs and HCC.

1.5 Non-alcoholic fatty liver disease (NAFLD)

NAFLD is the most common CLD and comprises a spectrum of conditions. It begins with simple steatosis represented by fat accumulation in the liver parenchyma without inflammation. NAFLD may progress to non-alcoholic steatohepatitis (NASH), where fat accumulation is accompanied by inflammation and hepatocyte ballooning, which may induce fibrosis, eventually cirrhosis and other end-stage liver complications (Than & Newsome, 2015). It is estimated that 20 to 25% of the simple steatosis cases can progress to NASH, and 20% of NASH cases may further progress to fibrosis and cirrhosis (Than & Newsome, 2015). An epidemiological study carried out on United States veterans found that almost 13% of individuals with HCC did not have cirrhosis but they presented symptoms of metabolic syndrome, fatty liver or an unknown condition (Mittal et al., 2016). Therefore, it is important to note that simple steatosis has the potential to directly induce development of HCC without progressing to steatohepatitis, fibrosis and cirrhosis.

NAFLD is diagnosed when hepatic fat accumulation accounts for more than 5-10% of the total liver weight or when more than 5% of hepatocytes display steatosis in the absence of excessive consumption of alcohol (Weiss, Rau, & Geier, 2014). Acceptable alcohol intake for the diagnosis of NAFLD has been set to two standard drinks a day for men and one standard drink a day for women by the National Institutes of Health clinical research network on NAFLD/NASH (Farrell & Larter, 2006).

Various risk factors such as viral, toxic, genetic and metabolic syndrome can produce hepatic steatosis; and metabolic syndrome plays a critical role in the development and progression of NAFLD (Farrell, Teoh, & McCuskey, 2008). It shares a close and bidirectional association with metabolic syndrome, therefore NAFLD is also referred to as the hepatic manifestation of the metabolic syndrome (Pais et al., 2016). Among all metabolic syndromes, obesity and type 2 diabetes are the most common underlying conditions in NAFLD. Moreover, a sharp increase in NAFLD prevalence in recent times runs in parallel with obesity and type 2 diabetes epidemic (Vernon, Baranova, & Younossi, 2011). Recently, metabolic dysfunction-associated fatty liver

disease (MAFLD) has been recommended as a more relevant term to describe CLD with known underlying metabolic disorder compared to NAFLD (Eslam, Newsome, et al., 2020; Eslam, Sanyal, et al., 2020). According to this new proposed term, the diagnosis for MAFLD will include the confirmation of steatosis based on biopsy, imaging or blood biomarkers, in addition to the presence of one of the metabolic conditions: overweight/obesity, type 2 diabetes mellitus or indication of metabolic dysregulation (Eslam, Newsome, et al., 2020). The prevalence of NAFLD in the general population without underlying metabolic syndrome is approximately 16%, increasing to 43-60% in diabetic patients and 91% in obese individuals (Than & Newsome, 2015; Vernon et al., 2011). A Western Australian cohort study found that NAFLD prevalence in adolescents is 12.8% (Ayonrinde et al., 2011). Depending on the diagnostic method employed, prevalence of NAFLD can vary in different regions. Apart from metabolic conditions, old age, male gender and mutations in patatin-like phospholipase domain-containing 3 protein (PNPLA3) gene, apolipoprotein 3 gene have been identified as risk factors of NAFLD (Chitturi, Wong, & Farrell, 2011; Neuschwander-Tetri, 2017; Than & Newsome, 2015). The annual economic burden of this disease is estimated to be $103 billion in the United States and almost €35 billion in Europe-4 countries (Germany, France, Italy and United Kingdom) (Younossi et al., 2016).

Investigation for NAFLD is not straight-forward as most of the patients are either asymptomatic at the steatosis stage or complain of some common symptoms, which can easily be misdiagnosed as other diseases. The first step in the diagnosis of NAFLD is to eliminate the possibility of other liver diseases, particularly viral hepatitis and alcoholic liver disease. Secondly, hepatic ultrasound imaging can be conducted, which is the most convenient and non-invasive diagnostic method to document hepatic fat deposition. Increased liver echogenicity, vascular blurring and deep attenuation of ultrasound signal are ultrasound signs of fatty liver. Whilst liver biopsy remains the gold standard method to analyse the disease severity, its use is carefully considered given its invasive nature (Chitturi et al., 2011). Lifestyle modifications including healthy eating, weight reduction and regular exercise are still the only pragmatic treatment options for NAFLD management (Neuschwander-Tetri,

2017). The need for liver transplantation in end-stage NASH-driven cirrhosis has risen since 2001; and NAFLD recurrence has been observed in approximately 33% of the cases after transplantation (Canbay, Sowa, Syn, & Treckmann, 2016). Moreover, donor availability, surgery-related complications and economic burden make liver transplantation a less attractive option. There is no approved pharmacotherapy for NAFLD treatment. However, some candidates are currently in phase II and III trials such as obeticholic acid, elafibranor and selonsertib (Mahady & Adams, 2018).

1.5.1 Pathogenesis of NAFLD

Mechanisms behind development of steatosis and progression to further stages of the NAFLD spectrum are not clearly understood. A two-hit hypothesis model was proposed to describe the NAFLD pathogenesis. Increased visceral adiposity, insulin resistance and elevated levels of free fatty acids (FFAs) in the circulation disrupt the normal hepatic metabolism process, leading to triglyceride and FFA accumulation in hepatocytes. This development of fatty liver is recognised as the first hit. Lipid peroxidation, mitochondrial dysfunction and inflammation, initiated as a result of the first hit act as second hit and worsen hepatocyte damage, which in turn promotes disease progression (Malhi & Gores, 2008; Than & Newsome, 2015).

Some of the factors that contribute to progression of fatty liver disease are described below.

1.5.1.1 FFAs and NAFLD

Overfeeding and adipose tissue insulin resistance induce adipose tissue lipolysis and lipoprotein spill-over, resulting in elevation of circulating FFA levels. Plasma FFAs are reabsorbed in the liver and stored in the hepatocytes as triglycerides. Once the triglyceride storage threshold is reached, remaining excess FFAs generate toxic lipid metabolites such as ceramides, diacylglycerols and oxidised cholesterol metabolites, which lead to hepatocyte injury and production of reactive oxygen species (Magee, Zou, & Zhang, 2016; Petta et al., 2016).

1.5.1.2 Insulin signalling and NAFLD

The liver is a metabolically active organ and responds to the portal insulin influx to regulate the hepatic carbohydrate and lipid metabolism (Michael et al., 2000). In a healthy liver, insulin binds to the insulin receptor (IR), leading to the tyrosine phosphorylation of insulin receptor substrate (IRS) proteins (Leclercq, Da Silva Morais, Schroyen, Van Hul, & Geerts, 2007). Different isoforms of IRS proteins have been identified but IRS1 and IRS2 play the major roles in hepatic insulin signalling (Thirone, Huang, & Klip, 2006). Tyrosine phosphorylation of IRS proteins is a critical step, as it is associated with the activation of two downstream pathways: 1) Phosphatidylinositol 3-kinase (PI3K)-AKT pathway, and 2) Ras-mitogen-activated protein kinase (MAPK) pathway. The PI3K-AKT pathway is involved in achieving the metabolic actions of insulin, whereas the MAPK pathway is mainly responsible for cell growth and differentiation (Taniguchi, Emanuelli, & Kahn, 2006). An experiment on rats with short-term high-fat diet feeding reveals that hepatic fat accumulation leads to the blunting of IRS1 and IRS2, which can disrupt the insulin signalling cascade (Samuel et al., 2004), resulting in insulin resistance. Hepatic insulin resistance disturbs both gluconeogenesis and *de novo* lipogenesis in the liver, which leads to the imbalance between storage and export of lipids. Excess lipid storage can promote irreparable cell damage, pro-inflammatory pathway activation and increased oxidative stress (Petta et al., 2016).

1.5.1.3 Inflammation and NAFLD

Overnutrition results in adipose tissue expansion and hypertrophy of adipocytes. These hypertrophic adipocytes start secretion of pro-inflammatory cytokines, such as TNF, IL-6 and IL-1β. Continuous circulation of these cytokines create a chronic, low-grade inflammation state in the body. Such chronic inflammation has been shown to downregulate hepatic insulin sensitivity by initiating pro-inflammatory signalling pathways and directly affecting hepatic insulin signalling (Kitade, Chen, Ni, & Ota, 2017). TNF can phosphorylate IRS proteins at serine residues, leaving them unavailable for tyrosine phosphorylation (Hotamisligil, 2006). The presence of inflammatory cytokines, growth factors such as insulin, immune receptors and stress responses can activate NFκB, a nuclear transcription factor, associated with inflammation. Activation of NFκB initiates transcription of TNF, IL-6, IL-8 and IL-1 genes, which further exacerbate liver inflammation (Zeng, Tang, Yin, & Zhou, 2014).

The expression of cytokines is also important to consider because they are involved in the recruitment of Kupffer cells (Tiniakos, Vos, & Brunt, 2010). Kupffer cells can sense danger signals and undergo activation of M1 (classical/pro-inflammatory; expressing F4/80, CD11b, CD11c markers) or M2 phenotypes (alternative/anti-inflammatory; expressing F4/80, CD11b, $CD11c^{negative}$). The balance between expression of M1 and M2 forms play key roles in fatty liver disease progression or regression (Sica, Invernizzi, & Mantovani, 2014). M1 Kupffer cells are involved in the production of inflammatory cytokines, including chemokines such as monocyte chemoattractant protein (MCP)-1, which increases hepatic lipid accumulation (Kitade et al., 2017). In contrast, induction of M2 Kupffer cells, activated by IL-4 and IL-13, leads to the involvement of peroxisome proliferator-activated receptor (PPAR) that facilitates fatty acid-dependent aerobic metabolism, resulting in steatosis resolution (Olefsky & Glass, 2010; Sica et al., 2014). Moreover, M2 Kupffer cells can promote apoptosis of M1 cells, thus protecting the liver from further harm (Wan et al., 2014).

1.5.1.4 Oxidative stress and NAFLD

The imbalance between pro- and anti-oxidant levels generate oxidative stress. Increased oxidative stress hampers the regenerative capacity of hepatocytes, which in turn leads to the activation of LPC (Carpino, Renzi, Onori, & Gaudio, 2013). Nobili and colleagues made some interesting observations while working with paediatric NAFLD patient biopsies and found that oxidative stress generated in fatty liver disease impairs the ability of mature hepatocytes to replicate, thus activating the LPC compartment. This LPC pool expansion correlates with the degree of hepatocyte injury and fibrosis. Also, LPCs express certain adipokines, such as adiponectin, resistin and glucagon-like peptide-1; out of which, adiponectin expression is inversely proportional to the degree of steatosis, whereas the expression of the other two is enhanced with the progression in NAFLD (Nobili et al., 2012).

1.6 Wnt signalling

The Wnt pathway is evolutionarily well-conserved and has been demonstrated to play many roles in liver biology, ranging from fetal liver development, liver metabolism, LPC activation, and even in liver tumour formation (Nejak-Bowen & Monga, 2011). The extracellular Wnt proteins (1-19) can stimulate intracellular signalling cascades, either β-catenin-dependent or β-catenin-independent, upon binding with the frizzled (Fz) receptors. Expression of both of these pathways is strictly tissue-specific, and either complimentary or independent. The β-catenin-dependent pathway, also known as the Wnt/β-catenin pathway or canonical Wnt signalling pathway, has been extensively studied in liver biology (Komiya & Habas, 2008).

1.6.1 Wnt/β-catenin pathway (Canonical Wnt signalling)

In an 'OFF' state, when Wnt ligands are absent, non-phosphorylated β-catenin (active β-catenin) is prevented from accumulating in the cytoplasm by a protein degradation/destruction complex, comprising of scaffolding protein axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3β), which phosphorylates β-catenin at the serine and threonine residues. The phosphorylated β-catenin accumulated in the cytoplasm is then removed by proteasomal degradation (Behari, 2010).

In an 'ON' state, Wnt ligands bind to the transmembrane Fz receptors 1-10 and a co-receptor, low-density lipoprotein receptor-related protein (LRP) 5/6. This binding of Wnt-Fz-LRP5/6 leads to the enrolment of the dishevelled (Dvl) protein, phosphorylation of the LRP5/6, and recruitment of axin to the plasma membrane. Axin dislocation to the membrane disrupts the degradation complex, and in the absence of the functioning degradation complex, non-phosphorylated β-catenin accumulates in the cytoplasm. Accumulated active β-catenin eventually enters the nucleus, where it forms a complex with other coactivators and transcription factors such as T cell factor (TCF)/lymphoid enhancer factor (LEF) to activate target gene expression (Behari, 2010).

A number of antagonists and agonists have been identified that control the fine balance of ‘ON’ versus ‘OFF’ state and the expression of β-catenin-dependent and -independent pathways (Cruciat & Niehrs, 2013). Dickkopf (DKK) proteins 1-4, secreted frizzled-related proteins (sFRP) 1-5 and Wnt inhibitory factor 1 (WIF1) are examples of secreted antagonists and Norrin, R-spondins represent Wnt pathway agonists besides Wnt ligands (Cruciat & Niehrs, 2013).

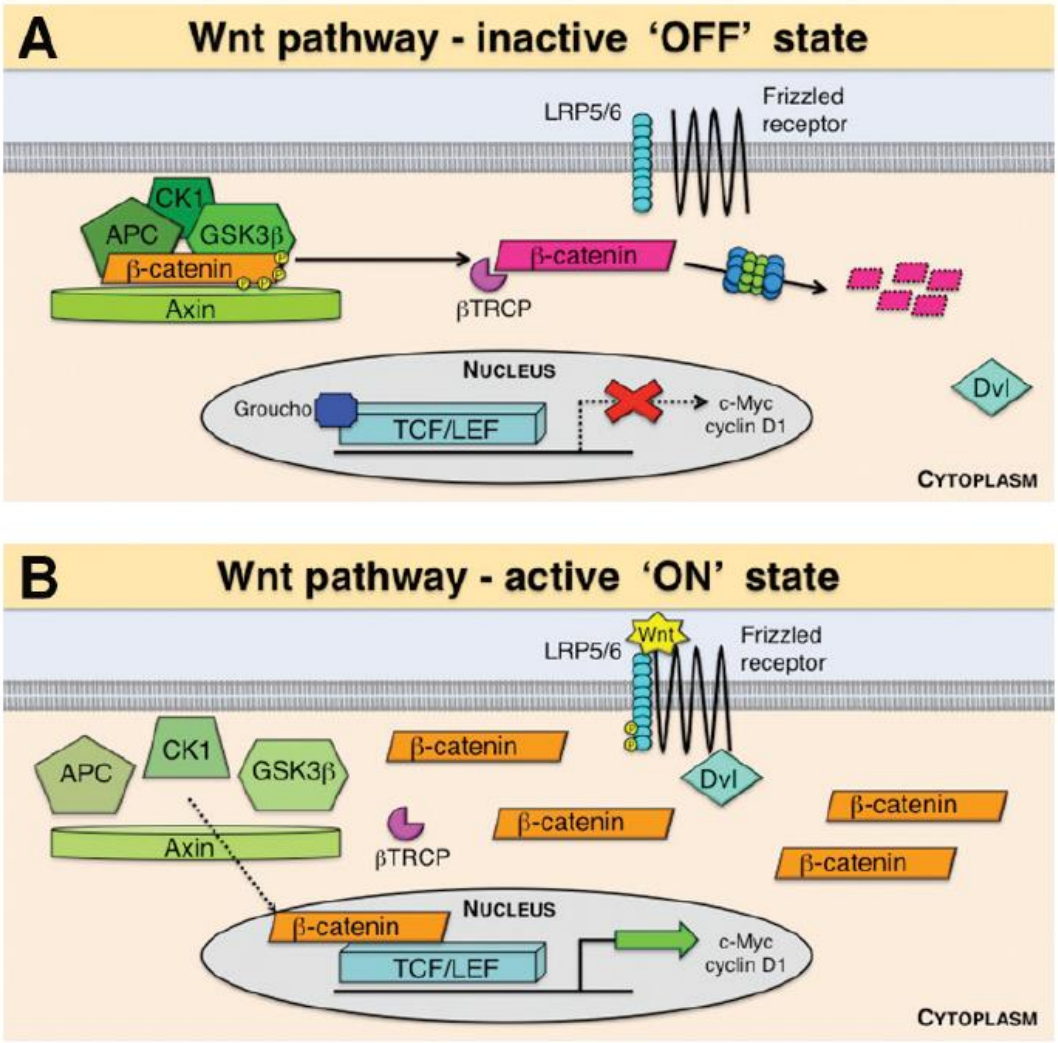


Figure 1.1: The canonical Wnt/β-catenin pathway.

In an 'OFF' state (A), the destruction complex, consisting of APC, GSK3β, CK1 and axin, binds and phosphorylates β-catenin, marking it for degradation through the proteasome. In this case, the repressor Groucho remains bound to TCF/LEF transcription factors, inhibiting the transcription of target genes such as c-Myc and cyclin D1. In an 'ON' state (B), a Wnt protein binds a Fz and LRP5/6 receptor, the Dvl protein activates a cascade, which eventually disrupts the destruction complex, leading to stabilisation, cytoplasmic accumulation and nuclear translocation of β-catenin and ultimately the transcription of target genes.

Image taken from (Shirolkar et al., 2018).

1.6.2 Non-canonical Wnt signaling

Non-canonical Wnt signalling exerts its downstream effects without the involvement of β-catenin. The non-canonical Wnt pathway can be further divided into the planar cell polarity (PCP) and the Wnt/Calcium (Wnt/Ca^{2+}) pathway (Russell & Monga, 2018).

The PCP pathway is involved in regulating cellular polarisation and directional cell movement. It comprises of non-canonical Wnt ligands, transmembrane receptor complex made up of Fz and the alternate receptors, receptor tyrosine kinase-like orphan receptor (ROR) 1/2, the intracellular small G-proteins Rac and Rho, and transcription factors. The PCP pathway is activated when non-canonical Wnt ligands such as Wnt5a bind with the Fz or alternate receptors and this receptor complex activates small G-proteins, which in turn activate Rho-associated kinase and Jun N-terminal kinase (JNK)-dependent transcription (Castañeda-Patlán, Fuentes-García, & Robles-Flores, 2018).

The Wnt/Ca^{2+} pathway utilises a similar extracellular Wnt ligand and receptor complex machinery as detailed for the PCP pathway. However, the intracellular downstream cascade involves activation of phospholipase C, which promotes production of diacylglycerol and inositol 1,4,5-triphosphate. Together they increase intracellular calcium levels, leading to activation of nuclear factor of activated T cells (NFAT). NFAT is a transcription factor that controls cell migration and proliferation (Castañeda-Patlán et al., 2018; Russell & Monga, 2018).

1.6.3 Role of Wnt signalling in the adult liver

The role of Wnt/β-catenin pathway in the regulation of liver zonation came to light when researchers studied expression patterns of the tumour suppressor gene APC and β-catenin within the liver lobule. In the periportal region, the expression of APC is high, whereas in perivenous hepatocytes, the expression of β-catenin is elevated (Benhamouche et al., 2006). The activity of β-catenin in pericentral hepatocytes is maintained by Wnt2 and Wnt9b, secreted by LSECs lining the central vein (Preziosi, Okabe, Poddar, Singh, & Monga, 2018;

Wang et al., 2015). This also explains why active Wnt/β-catenin pathway targets such as glutamine synthetase and the cytochrome P450 enzymes CYP2E1 and CYP1A2 are highly expressed in pericentral hepatocytes (Sekine, Lan, Bedolli, Feng, & Hebrok, 2006).

Beta-catenin regulates hepatic gluconeogenesis via interaction with forkhead box protein O (FOXO) 1 in insulin-resistant conditions. FOXO factors are sensitive to elevated insulin levels and the β-catenin-FOXO interaction can be detected in oxidative stress conditions (Liu et al., 2011; S. Monga, 2015; Rui, 2011).

Furthermore, β-catenin may be a key player in hepatic bile acid and cholesterol metabolism. Hepatic cholesterol accumulation and cholestasis was reported in mice with hepatocyte-specific β-catenin deletion and fed with a methionine- and choline-deficient diet (Behari et al., 2010).

1.6.4 Wnt signalling in liver regeneration

The role of Wnt/β-catenin signalling in liver regeneration has been comprehensively studied in the PHx model. An initial 2.5-fold increase in β-catenin protein and its nuclear translocation in hepatocytes was observed within five minutes of PHx, followed by a substantial decrease after five minutes. This early elevation in β-catenin protein may be responsible for the initiation of cell proliferation through activation of its target gene cyclin D1 (Monga, Pediaditakis, Mule, Stolz, & Michalopoulos, 2001). Later, confirmation of the role of β-catenin in governing the initial proliferative response in hepatic regeneration came from experiments performed in mice with hepatocyte-specific β-catenin deletion, which were subjected to PHx (Tan, Behari, Cieply, Michalopoulos, & Monga, 2006). In addition, it was noted that non-parenchymal cells such as Kupffer cells and LSECs are responsible for Wnt secretion due to a prompt increase of hepatocytic β-catenin after PHx (Yang et al., 2014). Termination of a regenerative response is a critical event. Interestingly, Wnt5a has been found to be a negative regulator of β-catenin-induced hepatocyte proliferation in *in vitro* and *in vivo* studies (Yang et al., 2015). Increased levels of Wnt5a, a classic non-canonical Wnt ligand and Fz

receptor 2 were observed at 24 hours post-PHx. This Wnt5a-Fz receptor 2 axis active in hepatocytes may be responsible for the termination of pro-proliferative signals activated by β-catenin during regeneration (Yang et al., 2015). Thus, Wnt pathway plays a critical role in activation as well as termination of the liver regeneration process.

Most of the postnatal hepatocyte-specific β-catenin knockout studies have followed up periods of less than 12 months. This follow up period is not sufficient to study the role of β-catenin in the regeneration of mature hepatocytes because they have a lifespan of approximately 300 days. Wang *et al*. studied the hepatocyte-specific β-catenin knockout model for a period of 18 to 20 months. They found that β-catenin-negative hepatocytes were functional throughout the study period; however, their proliferation capacity was compromised. From nine months, β-catenin-positive hepatocytes start appearing, which investigators thought may have originated from LPCs (Wang et al., 2011). Furthermore, Wnt signalling has been widely studied in the field of stem cell biology, as it is involved in the self-renewal, pluripotency and differentiation aspects of stem cells (Gougelet & Colnot, 2012). Therefore, Wnt signalling may be important in LPC activation, migration and differentiation during chronic liver injury.

The role of the Wnt/β-catenin pathway in the regulation of LPC responses was first investigated in rats treated with 2-acetylaminofluorine combined with PHx. The treatment led to an overall increase in total and active β-catenin protein in the cytoplasm and nuclei of LPCs. Moreover, the pathway expression was found to be under paracrine control, where Wnt1 was secreted by hepatocytes surrounding the LPCs and Fz receptor 2 expression was upregulated in LPCs (Apte et al., 2008). Additionally, to confirm the role of β-catenin in LPC activation, researchers used hepatocyte and bile duct specific β-catenin knockout mice fed with hepatotoxic 3,5-diethoxycarbonyl-1,4-dihydrocollidine to induce LPC responses. It was observed that β-catenin deletion significantly reduced $A6^+$ LPCs compared to control mice at early stages of injury (Apte et al., 2008). Other research group also documented similar findings (Hu et al., 2007). Additional evidence of active canonical signalling in LPCs came from Huch and colleagues who documented that expression of the Wnt target gene

leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) was not detected in control livers but upon a chronic liver injury stimulus induced by carbon tetrachloride injection, $LGR5^+$ cells appear near portal triads, where LPCs reside. Later, these $LGR5^+$ cells were found to be bipotential progenitor cells that can give rise to hepatocytes or biliary cells (Huch et al., 2013). Participation of the canonical pathway in LPC differentiation has been discussed previously in section 1.4.1. Upregulation of the Wnt/β-catenin pathway has also been reported in tumorigenic LPCs or liver cancer stem cells (Wang et al., 2016; W. Yang et al., 2008b).

1.6.5 Wnt signalling in liver disease

As explained earlier, canonical Wnt signalling plays a prominent role in hepatic zonation and metabolism. Dysregulation of Wnt signalling is implicated in metabolic diseases such as obesity, insulin resistance and NAFLD (Clevers & Nusse, 2012; Schinner, 2009). However, it is not clear whether upregulation or downregulation of Wnt signalling is responsible for disease progression in the NAFLD spectrum. One reason for this lack of clarity could be the complex interrelationship of canonical and non-canonical Wnt signalling and potential cross-talk of extra- and intracellular components of this pathway with other signalling pathways. Another reason may be the type of animal model incorporated into a study since pathway expression is context-specific (Russell & Monga, 2018).

An interesting study undertaken by Behari and colleagues, using liver-specific β-catenin knockout mice and liver-specific β-catenin transgenic mice, elucidated the role of β-catenin in diet-induced obesity. High-fat diet-fed transgenic mice developed obesity, hepatic insulin resistance and marked perivenous steatosis. In contrast, knockout mice fed a high-fat diet did not develop diet-induced obesity but displayed mild periportal steatosis, higher lobular inflammation and cellular toxicity (Behari et al., 2014). It is important to note that β-catenin did not directly participate in all the metabolic events; its role as one of the regulators of metabolic zonation cannot be neglected. Contribution of impaired Wnt/β-catenin signalling due to mutations in Wnt co-

receptor, LRP6 in dyslipidaemia and fatty liver development is well established (Singh et al., 2013). Interestingly, treatment with canonical Wnt3a in LRP6 mutant mice resulted in improvement in dyslipidaemia (Go et al., 2014). A study on the Wnt antagonist DKK3 concluded that high-fat diet-induced obesity, insulin resistance, and hepatic steatosis can be reduced by overexpression of DKK3 via Wnt/β-catenin-independent mechanisms (Xie et al., 2016).

Generally, canonical Wnts exert anti-inflammatory actions, whereas non-canonical Wnts are pro-inflammatory (Ackers & Malgor, 2018). Elevated levels of circulating non-canonical Wnt5a were reported in obese individuals (Catalan et al., 2014). Wnt5a-mediated JNK signalling activation promotes pro-inflammatory cytokine expression (Fuster et al., 2015), and upregulation of JNK signalling is associated with insulin resistance - a major contributor in fatty liver development (Samuel et al., 2004). Adipokine expression profiles of lean mice were compared to obese mice; and sFRP5 (inhibitor of Wnt proteins) concentration was decreased in the latter. When sFRP5-deficient mice were fed a high-fat diet, they exhibited a higher degree of steatosis compared to wildtype mice fed the same diet. Further analysis revealed that reduced sFRP5 level had no effect on the canonical Wnt signalling activity; however, non-canonical Wnt signalling was upregulated (Ouchi et al., 2010). Recently, it was shown that the effects of non-canonical Wnts in NAFLD development can be reversed via canonical Wnt administration (Wang et al., 2015). While working on the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) null mice, Debebe and colleagues proposed the contribution of steatosis in inducing a macrophage response, which secrete Wnts to drive the growth of tumorigenic LPCs and ultimately increases the tumour risk (Debebe et al., 2017).

The role of Wnt/β-catenin signalling in liver fibrosis is not very clear. Most of the studies support a role of canonical Wnt signalling in HSC activation (Cheng et al., 2008; Ge et al., 2014). However, a study from Kordes and colleagues reported active Wnt/β-catenin signalling in quiescent HSCs (Kordes, Sawitza, & Häussinger, 2008). Wnt5a expression has also been demonstrated in activated HSCs, suggestive of active non-canonical Wnt signalling (Corbett,

Mann, & Mann, 2015; Jiang, Parsons, & Stefanovic, 2006; Rashid et al., 2012). Interestingly, when HSC-specific Wntless knockout mice were subjected to fibrosis protocols, no effect on the development or resolution of fibrosis was observed (Zhang et al., 2019).

Activating mutations in the *CTNNB1* gene encoding β-catenin or inactivating mutations in negative regulators of the Wnt/β-catenin pathway such as *AXIN1, AXIN2* and *APC* lead to accumulation of β-catenin in the nucleus. Nuclear β-catenin accumulation is observed in approximately 40-70% of HCCs (Wang, Smits, Hao, & He, 2019) and a strong correlation exists between mutated β-catenin, size of the tumour and its invasive potential (Vilchez, Turcios, Marti, & Gedaly, 2016). Moreover, ≥20% of HCCs have upregulated *Wnt3/4/5A* and *Fz3/6/7* expression, which is associated with liver disease severity and tumour stage (Bengochea et al., 2008). Interestingly, strong cytoplasmic (non-nuclear) β-catenin overexpression has also been reported in poorly differentiated tumours (Khalaf et al., 2018; Wong, Fan, & Ng, 2001). Considering the notable histological representation of β-catenin in tumours, Lachenmayer *et al*. proposed molecular classification of HCC into a CTNNB1 class and Wnt-TGF-β class (Lachenmayer et al., 2012). The CTNNB1 class is identified by nuclear β-catenin staining, *CTNNB1* mutations and elevated liver-specific Wnt-target genes expression, such as glutamine synthetase and LGR5. On the other hand, the Wnt-TGF-β class is not associated with β-catenin mutations but exhibits increased levels of cyclin D1 and c-myc, which are common targets of the Wnt pathway (Lachenmayer et al., 2012). Interestingly, in mice, β-catenin overexpression in hepatocytes alone, does not lead to tumour formation; however, β-catenin stabilisation in fetal progenitors is capable of tumour development (Mokkapati et al., 2014). Numerous studies have confirmed the significant relationship between accumulated β-catenin in tumour cells and their proliferation status (Joo, Lee, & Kang, 2003; Nhieu et al., 1999; Schmitt-Graeff et al., 2005; Wang et al., 2012). A recent study demonstrated that in high-glucose conditions, Wnt inhibitor DKK4 downregulation leads to activation of Wnt/β-catenin signalling and proliferation of HCC cells (Chouhan et al., 2016). Different microenvironments can lead to activation of the Wnt/β-catenin pathway in different ways. Therefore, it is important to correctly identify the

factors and molecular interactions which lead to canonical Wnt pathway activation at a particular stage of CLD.

1.6.6 Cross-talk of Wnt/β-catenin signalling with other pathways

1.6.6.1 Cadherin-catenin

Beta-catenin is a crucial component of cadherin-based adherens junctions. Adherens junctions are responsible for maintaining cell-cell adhesion, which is necessary for tissue integrity. Cadherins are single-pass transmembrane glycoproteins, and a class of cadherins expressed in epithelial tissues is termed epithelial-cadherin (E-cadherin). The intracellular domain of E-cadherin binds to β-catenin, which links it to the actin cytoskeleton through α-catenin; thus, providing stability to the junction. Interestingly, cadherin-catenin binding protects β-catenin from degradation by the destruction complex (Campbell, Maiers, & DeMali, 2017; Russell & Monga, 2018; Valenta, Hausmann, & Basler, 2012).

1.6.6.2 HGF-Met pathway

Besides the cadherin-catenin interaction, a novel association between β-catenin and the HGF receptor c-Met was found in normal rat hepatocyte cultures. Approximately 30-40% of β-catenin is bound to c-Met at the inner side of the hepatocyte membrane and, upon stimulation by HGF this complex dissociates as c-Met and β-catenin undergo tyrosine phosphorylation. Moreover, growth factors such as HGF and EGF reduce GSK3β activity. Dissociation from the complex with c-Met and reduced GSK3β activity stabilise the β-catenin protein in the cytoplasm and encourage its nuclear translocation (Monga et al., 2002; Zeng, Apte, Micsenyi, Bell, & Monga, 2006).

1.6.6.3 PI3K/AKT-mTOR-MAPK network

The mTOR pathway governs life processes, which involve the use or generation of large amounts of energy and nutrients by both intracellular and extracellular signalling cascades. It plays an important role in cell growth, metabolism, proliferation and survival (Laplante & Sabatini, 2012). The mTOR protein belongs to the PI3K-related family of kinases. AKT, also known as protein kinase B is an important downstream target of PI3K signalling, which when activated, translocates to the nucleus and activates the mTOR complex (Matsuda, Kobayashi, & Kitagishi, 2013). The mTOR protein can further signal through two multi-protein complexes, mTOR complex (mTORC) 1 and 2 (Mathieu Laplante & Sabatini, 2009). The complex mTORC1 participates in protein and lipid synthesis and in autophagy, whereas mTORC2 is responsive to growth factors. Due to the close association of the PI3K/AKT/mTOR pathways and different metabolic processes, its dysregulation is often seen in diseases such as obesity and type 2 diabetes (Laplante & Sabatini, 2012). PTEN acts as an inhibitor of the PI3K/AKT/mTOR pathway. More evidence for the involvement of the PI3K/AKT/mTOR pathway in metabolic disorders came from the use of PTEN-deficient mice, which exhibit increased synthesis and storage of triglycerides in hepatocytes as a result of the upregulated PI3K pathway (Matsuda et al., 2013; Watanabe, Horie, & Suzuki, 2005). Activation of AKT phosphorylates GSK3β at Ser21/9 rendering it inactive. It can therefore no longer phosphorylate β-catenin, resulting in activation of canonical Wnt signalling (Mulholland, Dedhar, Wu, & Nelson, 2006). However, recent evidence suggests two separate reservoirs of GSK3α/β are present in the cytosol and the GSK3β pool, associated with the destruction complex in the Wnt/β-catenin pathway, and is not affected by PI3K/AKT signalling cascade (Wu & Pan, 2010). Alternatively, activation of the Wnt/β-catenin pathway upon mitogenic stimuli may be due to phosphorylation of LRP6 by MAPKs such as p38, extracellular signal-regulated kinases (ERK) 1/2, JNK1 (Červenka et al., 2011; Krejci et al., 2012). Furthermore, phosphorylation of β-catenin at Ser552 by activated AKT has been reported, which leads to stabilisation of cytoplasmic β-catenin reservoir (Fang et al., 2007).

1.7 Project aims

Chronic liver injury leads to an activation and expansion of LPCs. LPC-mediated liver regeneration is a tightly controlled process and is coordinated by a complex cross-talk between various liver cell types. NAFLD is the most common CLD and can progress to end-stage liver disease. Therapeutic options to halt the progression of this disease are not yet available. Wnt/β-catenin signalling pathway is well-studied in liver regeneration, different metabolic conditions and HCC. However, it remains unclear as to whether Wnt/β-catenin signalling regulates LPCs in the background of NAFLD.

Therefore, it has been hypothesised that the Wnt/β-catenin signalling regulates the liver progenitor cell responses in non-alcoholic fatty liver disease.

The aims of the studies included in this thesis were to investigate the role of the Wnt/β-catenin signalling pathway in a) NAFLD patient liver biopsy samples, b) the murine CDE chronic liver injury model and c) an *in vitro* LPC-focussed model of steatosis. The final aim was d) to study the effects of manipulation of the Wnt/β-catenin signalling pathway on LPCs in the same *in vitro* and *in vivo* model of NAFLD.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 General laboratory chemicals

All general chemicals were of analytical grade or equivalent, obtained from commercial suppliers. Supplier information has been provided for specialty reagents wherever necessary.

2.1.2 Water

Double-deionised water (ddH_2O) obtained from the Aquatec purification system (Aquatec, VIC, Australia) was used to prepare general solutions and buffers. UltraPure DNase/RNase-free water (Life Technologies, VIC, Australia) was used for molecular techniques involving RNA and microarray analyses.

2.1.3 Solutions and buffers

2.1.3.1 Phosphate-buffered saline (PBS)

PBS was prepared using 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.47 mM KH_2PO4, adjusted to pH 7.4 and stored at room temperature. For cell culture, commercial grade sterile PBS (Fisher Biotech, WA, Australia) was used.

2.1.3.2 Sodium citrate buffer (antigen retrieval)

A solution of 10 mM sodium citrate with 0.05% Tween 20 was prepared, pH adjusted to 6 and stored at room temperature for up to three months.

2.1.3.3 Acetone-methanol (ac-me) solution for fixation

A solution of acetone and methanol (ratio 1:1, v/v) was prepared and stored at -20^{0}C.

2.1.3.4 4% paraformaldehyde (PFA) solution for fixation

Ten g PFA (Sigma-Aldrich, NSW, Australia) was dissolved in 250 ml PBS by heating at 60^{0}C, aliquoted and stored at -20^{0}C.

2.1.3.5 Triton X-100 solution for permeabilisation

A 0.1% Triton X-100 solution was prepared in PBS from Triton X-100 (Sigma-Aldrich, NSW, Australia) and stored at 4^{0}C.

2.1.3.6 Oil Red O solution (0.5%) for triglyceride and lipid staining of tissue sections

A 0.5% Oil Red O solution was prepared by addition of a small volume of propylene glycol to 0.5 g of Oil Red O powder (Sigma-Aldrich, NSW, Australia). The volume was then gradually adjusted to 100 ml with propylene glycol while stirring continuously and heating the solution to 95^{0}C. Whatman coarse filter paper (GE Healthcare Life Sciences, NSW, Australia) was used to filter the warm solution. For further use, the solution was stored at room temperature.

2.1.3.7 Alanine transaminase (ALT) substrate solution for ALT assay

The ALT substrate solution was prepared by dissolving 0.2 M DL-alanine and 1.8 mM α-ketoglutarate in PBS, pH adjusted to 7.5 and stored at 4^{0}C.

2.1.3.8 ALT colour reagent for ALT assay

One mM 2, 4-dinitrophenylhydrazine in 1 M HCl was prepared by dissolving 20 mg of 2, 4-dinitrophenylhydrazine in 20 ml of 5 M HCl and the final volume was adjusted to 100 ml with ddH_2O.

2.1.3.9 Acetate citrate buffer for hydroxyproline assay

For collagen assessment through hydroxyproline assay, a solution of 0.88 M sodium acetate tri-hydrate, 0.24 M citric acid, 0.2 M acetic acid and 0.85 M NaOH was prepared. The pH was adjusted to 6.5 and stored at room temperature.

2.1.3.10 Chloramine-T solution for hydroxyproline assay

To make an oxidising buffer, 127 mg chloramine-T was added to 2 ml of 50% n-propanol and the final volume was adjusted to 10 ml using acetate citrate buffer (2.1.3.9).

2.1.3.11 Ehrlich's reagent for hydroxyproline assay

The Ehrlich's indicator reagent was prepared by dissolving 1.5 g 4-dimethylaminobenzaldehyde (DMAB) in 10 ml of n-propanol/perchloric acid mix (2:1 v/v).

2.1.3.12 PRI-724 solution for *in vivo* administration

A 5 mg/ml solution of PRI-724 was prepared by dissolving 5 mg PRI-724 (Selleckchem, VIC, Australia) in 2% dimethyl sulfoxide (DMSO; Sigma-Aldrich, NSW, Australia), 30% polyethylene glycol (PEG 300; OzVials Medical & Scientific, VIC, Australia), 5% (v/v) Tween 80 (Sigma-Aldrich, NSW, Australia) and the final volume was adjusted to 1 ml using ddH_2O. A solution of 2% DMSO, 30% PEG 300, 5% (v/v) Tween 80 in 1 ml ddH_2O was used as vehicle.

2.1.3.13 Williams' E maintenance medium for BMOL-TAT cell culture

Williams' E medium (Life Technologies, VIC, Australia) supplemented with 2.5 µg/ml fungizone (Life technologies, VIC, Australia), 48.4 ng/ml penicillin (Life technologies, VIC, Australia), 675 ng/ml streptomycin (Life Technologies, VIC, Australia), 292.3 ng/ml glutamine (Sigma-Aldrich, NSW, Australia), 15 ng/ml insulin-like growth factor (IGF)-II (GroPep, SA, Australia), 10 ng/ml EGF (BD Biosciences, CA, USA), 5 µg/ml insulin (Humulin; Eli Lilly, IN, USA) and 2% fetal bovine serum (FBS) (Serana, Pessin, Germany) was used as maintenance medium for BMOL-TAT cells. The FBS concentration was increased to 5% for only one passage after thawing.

2.1.3.14 Trypsin-ethylenediaminetetraacetic acid (EDTA) solution for cell culture

A 10X Trypsin-EDTA solution (Sigma-Aldrich, NSW, Australia) was diluted to 0.05% using sterile PBS, aliquoted and stored at -20^{0}C. Once thawed, aliquots were stored at 4^{0}C and used within a month.

2.1.3.15 Palmitate solution for the treatment of BMOL-TAT cells

A 1 mM palmitate solution for cell culture was prepared according to the instructions in the Seahorse Bioscience protocol, using bovine serum albumin (BSA) as a carrier and stabilising agent. Sodium palmitate (Sigma-Aldrich, NSW, Australia) and fatty acid-free BSA (Sigma-Aldrich, NSW, Australia) suitable for cell culture were used in the solution preparation. Afterwards, this solution was diluted using Williams' E maintenance medium (2.1.3.13), according to the experimental requirement.

2.1.3.16 Oleic acid solution for the treatment of BMOL-TAT cells

A 3 mM oleic acid-albumin-conjugated solution, suitable for cell culture, was purchased from Sigma-Aldrich, NSW, Australia. This solution was diluted

further using Williams' E maintenance medium (2.1.3.13), according to the experimental requirement.

2.1.3.17 PRI-724 solution for *in vitro* administration

A 10 mM PRI-724 solution was prepared by dissolving 5 mg PRI-724 (Selleckchem, VIC, Australia) in 0.9114 ml sterile DMSO. Afterwards, this solution was diluted using Williams' E maintenance medium (2.1.3.13), according to the experimental requirement.

2.1.3.18 MTT solution for MTT assay

A solution was prepared by dissolving 3-[4, 5-dimethylthiazolyl-2]-2, 5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, USA) in PBS at a concentration of 5 mg/ml.

2.1.3.19 Coverslip etching solution

To etch glass coverslips, a solution using ddH_2O (11.3 ml), ethanol (17.1 ml) and 3 g NaOH was prepared.

2.1.3.20 Oil Red O solution (0.5%) for triglyceride and lipid staining of cells

A 0.5% Oil Red O stock solution was prepared by dissolving 0.5 g of Oil Red O powder (Sigma-Aldrich, NSW, Australia) in 100 ml of isopropanol while stirring continuously and gently heating the solution in a water bath. For further use, the solution was stored at room temperature. For experiments, the working solution was prepared fresh by diluting 30 ml of stock solution with 20 ml of ddH_2O and filtered through Whatman coarse filter paper (GE Healthcare Life Sciences, NSW, Australia).

2.1.4 Antibodies

Primary and secondary antibodies used in experiments along with their experimental conditions are listed in Table 2.1 and Table 2.2, respectively. All antibodies were diluted in antibody diluent (Dako, NSW, Australia) before use.

Epitope	Host species	Reactivity	Clone	Dilution	Fixation	Antigen retrieval	Supplier details
A6	rat	mu		1:200 IF	Ac-Me		Gift from Dr Valentina Factor, National Cancer Institute, Bethesda, MD,USA
Active β-catenin	rabbit	mu	D13A1	1:200 IHC, 1:200 IF 1:2000 WB	Ac-Me	Sodium citrate	Cell Signaling Technology, MA, USA
β-catenin	rabbit	mu, hu	E247	1:200 IF 1:2000 WB		Sodium citrate	Abcam, MA, USA
β-tubulin	rabbit	mu	D2N5G	1:2000 WB			Cell Signaling Technology, MA, USA
CD11b	rat	mu	M1/70	1:200 IF	Ac-Me		eBioscience, CA,USA
CD45	rat	mu	30-F11	1:200 IF	Ac-Me		BD, NSW, Australia
CK19	rat	mu		1:200 IF	Ac-Me		TROMA-III, Developmental Studies Hybridoma Bank, IA, USA
Cyclin D1	rabbit	mu	92G2	1:1000 WB			Cell Signaling Technology, MA, USA
CYP2E1	rabbit	mu	HPA009128	1:100 IHC		Sodium citrate	Sigma-Aldrich NSW, Australia
Dvl2	rabbit	mu	30D2	1:1000 WB			Cell Signaling Technology, MA, USA
F4/80	rat	mu	BM8	1:200 IF	Ac-Me		eBioscience, CA, USA
Ki-67	rabbit	mu	D3B5	1:100 IF	Ac-Me 4% PFA		Cell Signaling Technology, MA, USA

LRP6	rabbit	mu	C47E12	1:1000 WB			Cell Signalling Technology, MA, USA
LGR5	rabbit	mu		1:100 IHC		Sodium citrate	Abcam, MA, USA
panCK	mu	hu	AE1/AE3	1:100 IF		Sodium citrate	Dako, NSW, Australia
panCK	rabbit	mu		1:200 IHC 1:200 IF	Ac-Me	Proteinase K	Dako, NSW, Australia
α-SMA	mu	mu	1A4	1:2000 IF	Ac-Me		Sigma-Aldrich NSW, Australia
GSK3β	rabbit	mu	27C10	1:2000 WB			Cell Signaling Technology, MA, USA

Table 2.1: List of primary antibodies. IHC, immunohistochemistry; IF, immunofluorescence; WB, Western blotting; mu, murine; hu, human.

Epitope	Host species	Reactivity	Dilution	Conjugate	Supplier details
IgG	goat	rabbit	1:500	Alexa Fluor 488	Life Technologies, VIC, Australia
IgG	goat	rabbit	1:500	Alexa Fluor 594	Life Technologies, VIC, Australia
IgG	goat	rat	1:500	Alexa Fluor 594	Life Technologies, VIC, Australia
IgG	goat	rat	1:500	Alexa Fluor 488	Life Technologies, VIC, Australia
IgG	goat	mu	1:500	Alexa Fluor 594	Life Technologies, VIC, Australia
IgG	goat	rabbit	1:2000	HRP-linked	Cell Signaling Technology, MA, USA

Table 2.2: List of secondary antibodies. HRP, horseradish peroxidase.

2.2 General methods

2.2.1 Animals

2.2.1.1 Animal handling

All animal experiments were performed according to the guidelines of the Australian code for the care and use of animals for scientific purposes at Curtin University, Perth, Australia with approvals from the local Animal Ethics Committee.

Animals were maintained under pathogen-free conditions in temperature- and 12-hour day/night cycle-controlled rooms. A maximum of six mice were placed together in a ventilated cage, provided with wheaten chaff bedding and *ad libitum* access to food and water.

2.2.1.2 Mouse strain

C57BL/6J wildtype mice were purchased from the Animal Resources Centre, Murdoch, Western Australia.

2.2.1.3 Experimental diet

Mice had *ad libitum* access to choline-deficient chow (MP Biomedicals, NSW, Australia) and drinking water containing 0.15% DL-ethionine (Sigma-Aldrich, NSW, Australia), termed the CDE diet. Control animals were given normal chow and drinking water.

2.2.1.4 PRI-724 administration

Animals received intraperitoneal injection of PRI-724 (2.1.3.12) (at a dose of 10 mg/kg body weight) or vehicle twice a week using a 0.3 ml Ultra-Fine II Short Needle insulin syringe (Becton Dickinson, WA, Australia). The amount of PRI-724 or vehicle was calculated according to the body weight of the individual animal. Animals were monitored daily to ensure their well-being.

2.2.1.5 Anaesthesia

A combination of ketamine (Provet, WA, Australia) at a dose of 100 mg/kg body weight and xylazine (Provet, WA, Australia) at a dose of 10 mg/kg body weight in PBS was administered intraperitoneally to anaesthetise the mice. The amount of anaesthetics was calculated according to the body weight of each animal. The depth of anaesthesia was confirmed using the leg withdrawal effect before proceeding further.

2.2.1.6 Serum extraction

After complete anaesthesia was attained, blood was extracted by cardiac puncture using a 27½ G needle (Terumo, NSW, Australia) connected to a 1 ml syringe (Terumo, NSW, Australia). Blood was allowed to clot at room temperature and then spun at 16,100 x g for 10 minutes at 4^0C. The supernatant containing serum was transferred to a new microcentrifuge tube and stored at -80^0C for further analysis.

2.2.1.7 Liver perfusion

Using a 27½ G needle (Terumo, NSW, Australia) attached to a 10 ml syringe (Terumo, NSW, Australia) the hepatic portal vein was cannulated to flush the liver with PBS. After visible blanching of the liver, the heart was cut and the liver was flushed with 5 ml of PBS using a steady flow rate.

2.2.1.8 Liver isolation, preservation and processing

After liver perfusion, the gall bladder was removed carefully and the liver was resected.

The liver tissue pieces were snap-frozen in liquid nitrogen and stored at -80^{0}C for subsequent molecular and biochemical analyses.

Two liver lobes were embedded in optimal cutting temperature (OCT) (Sakura Finetek, South Holland, Netherlands) medium, snap-frozen in liquid nitrogen and stored at -80^{0}C. Further, using a cryostat microtome (Leica CM1520, Leica Biosystems, VIC, Australia), liver sections of 7 µm thickness were cut, mounted onto SuperfrostPlus slides (Thermo Scientific, VIC, Australia) and stored at -80^{0}C for immunofluorescent and lipid staining experiments.

One liver lobe was kept in 10% formalin (Amber Scientific, WA, Australia) for 24 hours at room temperature and then transferred into 70% ethanol. Fixed tissues were processed in a tissue processor (Leica TP1020, Leica Biosystems, VIC, Australia), followed by embedding in paraffin using a tissue embedder (Leica EG1150C, Leica Biosystems, VIC, Australia). Using a microtome (Leica RM2235, Leica Biosystems, VIC, Australia), paraffin blocks were cut into 4 µm thick sections and mounted onto SuperfrostPlus slides (Thermo Scientific, VIC, Australia). Sections were kept in a hot room at 37^{0}C overnight and stored at room temperature.

2.2.1.9 Serum alanine transaminase assay (ALT assay)

Alanine transaminase is an enzyme that converts L-alanine and α-ketoglutarate to pyruvate and L-glutamate. The generated pyruvate reacts with 2,4-dinitrophenylhydrazine (colour reagent) to form hydrazone, which can be detected colourimetrically at 490 nm.

For this assay, 25 µl of serum was added to 100 µl of ALT substrate solution (2.1.3.7). The solutions were mixed gently and incubated for 1 hour at 37^0C. Then, 100 µl of ALT colour reagent (2.1.3.8) was added and solutions were incubated for 20 minutes at room temperature. The colour reaction was terminated by addition of 1 ml 0.4 M NaOH. Solutions were mixed by inversion and incubated at room temperature for 5 minutes. Finally, 200 µl of the assay solution was transferred into a clear 96-well plate (Thermo Fisher Scientific, VIC, Australia) to measure the absorbance at 490 nm using a plate reader (EnSight Multimode Plate Reader, PerkinElmer, VIC, Australia).

To generate the standard curve, a dilution series of 1.5 mM sodium pyruvate in PBS, diluted in ALT substrate solution, was used.

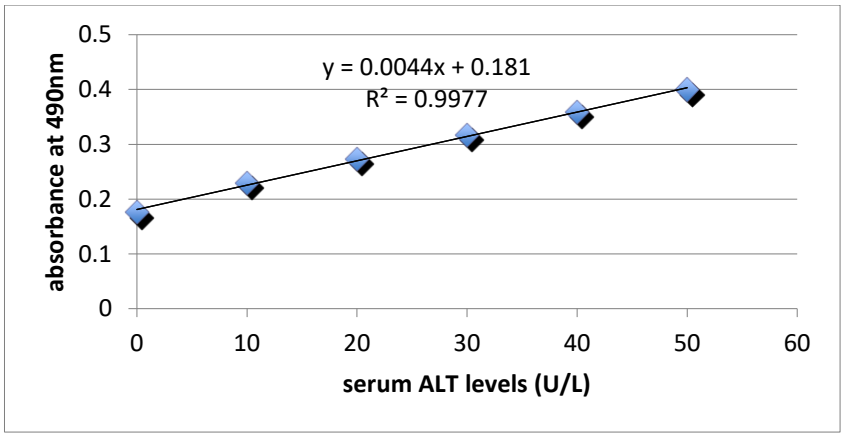


Figure 2.1: Typical standard curve for the ALT assay.

2.2.1.10 Hydroxyproline assay

A Hydroxyproline assay was performed to measure tissue collagen deposition. Approximately 125 mg of snap-frozen liver tissue was homogenised in 1 ml 6 M HCl at 95^{0}C for 20 hours, followed by centrifugation at 13,000 x g for 10 minutes. Then, 40 µl of supernatant was collected, to which 10 µl 10 M NaOH and 450 µl chloramine-T solution (2.1.3.10) were added. The mixture was incubated for 25 minutes at room temperature, before 500 µl DMAB (Ehrlich's) solution (2.1.3.11) was added and samples were incubated at 65^{0}C for 20 minutes. Finally, absorbance was measured at 560 nm using a plate reader (EnSight Multimode Plate Reader, PerkinElmer, VIC, Australia). To generate the standard curve, a dilution series of 0.5 mg/ml trans-4-hydroxy-L-proline (in H_2O), diluted in 6 M HCl, was used.

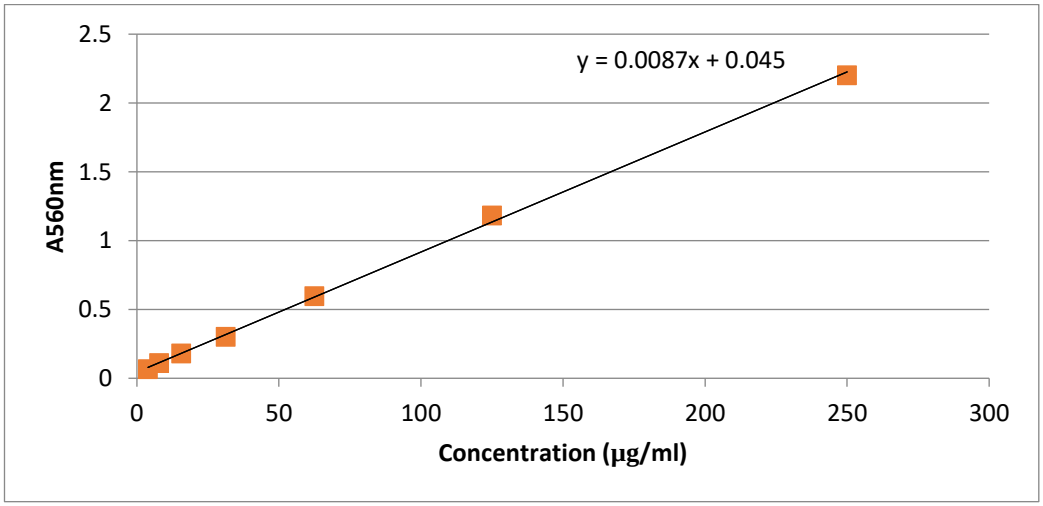


Figure 2.2: Typical standard curve for the hydroxyproline assay.

2.2.2 Histology techniques

2.2.2.1 Dewaxing and rehydration of formalin-fixed, paraffin-embedded sections (FFPE)

FFPE liver tissue sections were incubated three times in xylene, two times in 100% ethanol and once in 70% ethanol. Each incubation was performed for two minutes with gentle agitation and finally slides were placed in deionised water for 10 minutes before proceeding further.

2.2.2.2 Antigen retrieval

FFPE sections were exposed to heat-mediated antigen retrieval using sodium citrate buffer (2.1.3.2) or to proteolytic digestion using proteinase K (Dako, NSW, Australia) to unmask the epitopes. The type of antigen retrieval method used for each antibody is elucidated in Table 2.1.

2.2.2.3 Detection of antigens using immunohistochemistry

Once the dewaxing, rehydration and antigen retrieval steps were completed, paraffin sections were incubated in peroxidase blocking reagent (Dako, NSW, Australia) for 5 minutes to block the tissue-endogenous peroxidases and then washed in PBS for 5 minutes. Further, a biotin blocking system (Dako, NSW, Australia) was used to inactivate the tissue-endogenous biotin. Biotin blocking was performed by incubating the tissue sections in avidin solution for 10 minutes, followed by a PBS wash, and then in biotin solution for 10 minutes, followed by another PBS wash. Additionally, to prevent unspecific antibody binding, the tissue sections were incubated in serum-free protein blocking solution (Dako, NSW, Australia) for 30 minutes in a humidified chamber. Primary antibodies were diluted in antibody diluent (Dako, NSW, Australia) as per the concentrations listed in Table 2.1. Tissue sections were incubated with the primary antibody overnight at 4^0C in a humidified chamber. The next day,

tissue sections were washed in PBS prior to secondary antibody incubation. For primary antibodies raised in rabbit and mouse, the Dako LSAB+ System-HRP kit (Dako, NSW, Australia) was used for detection. Then, tissue sections were washed in PBS, before colour detection was performed using the liquid DAB+ Substrate Chromogen System (Dako, NSW, Australia). Haematoxylin solution (Dako, NSW, Australia) was used for nuclear counterstaining. Subsequently, sections were rinsed in running tap water, dehydrated and mounted (2.2.2.4). Tissue images were obtained using the Olympus BX51 microscope and the Olympus camera DP70 (Olympus, VIC, Australia). Positive cell counts were performed manually on blinded slides. A minimum of four and maximum of eight non-overlapping fields of view at x200 total magnification were quantitated using Image J.

2.2.2.4 Dehydration of tissue sections

For dehydration, tissue sections were incubated once in 70% ethanol, twice in 100% ethanol and three times in xylene for 30 seconds per incubation. Once the dehydration series was complete, tissue sections were mounted with Entellan New mounting medium (ProSciTech, QLD, Australia) and covered with a coverslip (Trajan Scientific and Medical, VIC, Australia).

2.2.2.5 Haematoxylin and eosin staining (H & E staining)

Once the dewaxing and rehydration of FFPE liver sections was complete, the sections were incubated in Gill's haematoxylin (Amber Scientific, WA, Australia) for 15 seconds, followed by rinsing in running tap water. Afterwards, dehydration was performed using one change of 95% ethanol for 1 minute. Then, sections were counterstained in 1% alcoholic eosin (Hurst Scientific, WA, Australia) for 30 seconds, followed by rinsing in one change of 90% ethanol and two changes of 100% ethanol for 1 minute each. Finally, the sections were incubated in three changes of xylene for 1 minute each, before mounting with mounting medium and coverslips.

2.2.2.6 Sirius Red staining

Picrosirius Red Stain Kit (Polyscience Inc., PA, USA) was used to perform Sirius Red staining for collagen assessment. Following dewaxing and dehydration, the sections were incubated in phosphomolybdic acid (Solution A) for 2 minutes, followed by rinsing in ddH_2O. Then, sections were incubated in picrosirius red solution (Solution B) for 1 hour, in 0.01 N hydrochloric acid (Solution C) for 2 minutes and finally immersed in 70% ethanol for 45 seconds. Subsequently, the sections were dehydrated, mounted and coverslipped. Sirius Red quantification was performed on a minimum of five and a maximum of eight non-overlapping x200 fields using Image J.

2.2.2.7 Fixation of frozen tissue sections

2.2.2.7.1 *Acetone-Methanol fixation*

Frozen tissue sections were placed in ice-cold ac-me solution (2.1.3.3) for 2 minutes for fixation, followed by air-drying for 1 hour. Afterwards, the sections were placed in PBS for at least 10 minutes prior to further experimentation.

2.2.2.7.2 *4% PFA fixation*

Frozen tissue sections were air-dried for 1 hour. Then, sections were incubated in 4% PFA solution (2.1.3.4) for 10 minutes, followed by washing in PBS for 5 minutes. Further, the sections were incubated in 0.1% Triton X-100 solution (2.1.3.5) for 10 minutes for permeabilisation and again washed in PBS for 5 minutes before proceeding further.

2.2.2.8 Immunofluorescent detection of antigens

For immunofluorescent detection of antigens, fixed frozen sections or antigen-retrieved paraffin sections were used. The tissue sections were incubated in serum-free protein blocking solution (Dako, NSW, Australia) for 30 minutes to avoid unspecific antibody binding. They were then incubated with primary antibodies diluted in antibody diluent (Dako, NSW, Australia) at 4^0C overnight or at room temperature for 1 hour. Slides were washed in PBS for 5 minutes before proceeding with secondary antibody incubation. The tissue sections were incubated with secondary antibodies, diluted in antibody diluent (Dako, NSW, Australia) at room temperature for 1 hour in the dark. The concentrations of secondary antibodies are listed in Table 2.2. Finally, the slides were washed in PBS for 5 minutes, dried and coverslipped using ProLong Gold antifade reagent with DAPI (Life technologies, VIC, Australia) to stain the nuclei. The fluorescent staining images were obtained using the microscope (Olympus BX51) and the Olympus camera DP70 (Olympus, VIC, Australia). Using Image J, positive cell counts were performed manually on blinded slides using a minimum of four and a maximum of eight non-overlapping fields of view at x200 total magnification.

2.2.2.9 Oil Red O staining

Frozen sections were air-dried for 30 minutes before fixing in ice-cold 10% formalin (Amber Scientific, WA, Australia) for 5 minutes. Then, the sections were rinsed with three changes of ddH_2O and air-dried for 10 minutes. Afterwards, the sections were placed in 1, 2-propanediol for 5 minutes, stained in pre-warmed Oil Red O solution (2.1.3.6) for 10 minutes and differentiated in 85% propylene glycol for 3 minutes. Then, sections were washed in two changes of ddH_2O and incubated in Gill's haematoxylin (Amber Scientific, WA, Australia) for 10 seconds to stain the nuclei. Subsequently, the sections were washed in running tap water and mounted using Dako Glycergel Mounting Medium (Dako, NSW, Australia). The images were taken using a microscope (Olympus BX51) and the Olympus camera DP70 (Olympus, VIC, Australia).

2.2.3 Cell culture

2.2.3.1 Thawing BMOL-TAT cells

Vials containing cell suspension were thawed in a glass beaker filled with warm (37^0C) water. A few drops of the cell maintenance medium (2.1.3.13) were added to the vial when the cell suspension was thawed to adapt cells to the medium change. Finally, the cell suspension was transferred to a T25 flask (Thermo Scientific, VIC, Australia) containing 4 ml of maintenance medium. Cell cultures were maintained at 37^0C, 5% CO_2 and 90% humidity in an IR Direct Heat CO_2 incubator (In Vitro Technologies, VIC, Australia).

2.2.3.2 Maintenance and subculturing of BMOL-TAT cells

For a T25 flask, 5 ml of cell maintenance medium (2.1.3.13) was used and medium was changed every second day. Cells were subcultured when they reached 80% confluence to maintain them in their growth phase. Trypsin-EDTA 0.05% solution (2.1.3.14) was used as a dissociation reagent.

2.2.3.3 Etching of coverslips

Coverslips were immersed in the chemical mixture (2.1.3.19) for 30 minutes, followed by thorough washing in ddH_2O. Coverslips were air-dried and UV-sterilised before proceeding with an immunofluorescent staining.

2.2.3.4 Immunofluorescent detection of antigens

Cells were grown on etched coverslips (2.2.3.3) and fixed using ice-cold acetone solution (2.1.3.3) for 4 minutes. Further, the cells were washed with PBS and blocked with serum-free protein blocking solution (Dako, NSW, Australia) for 20 minutes at room temperature. They were then incubated with primary antibodies (Table 2.1) diluted in antibody diluent (Dako, NSW, Australia) at 4^0C

overnight or at room temperature for 1 hour. The cells were washed three times with PBS before proceeding further with secondary antibody incubation in the dark for 1 hour. Secondary antibodies were diluted in antibody diluent (Dako, NSW, Australia), as listed in Table 2.2. Finally, the cells were washed in PBS three times and mounted with ProLong Gold antifade reagent with DAPI (Life technologies, VIC, Australia). The fluorescent staining images were taken using the Olympus BX51 microscope and the Olympus camera DP70 (Olympus, VIC, Australia).

2.2.3.5 Oil Red O staining

Cells were grown in clear 6-well plates (Thermo Scientific, VIC, Australia), washed with PBS and incubated in 10% formalin (Amber Scientific, WA, Australia) for 15 minutes. Afterwards, the cells were washed with ddH_2O and incubated with 60% isopropanol for 3 minutes, before incubation in Oil Red O solution (2.1.3.20) for 15 minutes and washing in three changes of ddH_2O. Finally, the cells were stained with Gill's haematoxylin (Amber Scientific, WA, Australia) for 15 seconds and rinsed in running tap water. Images were captured using the inverted Olympus IX51 microscope and the Olympus camera DP71 (Olympus, VIC, Australia).

2.2.3.6 MTT assay

This colorimetric assay is based on the ability of cellular oxidoreductase enzymes to convert the tetrazolium dye MTT (Sigma-Aldrich, USA) to insoluble, purple formazan. At the end of the treatment time point, cells were incubated with 10 µl of MTT solution (2.1.3.18) for 4 hours at 37^0C. To dissolve the crystals, the cells were treated with 100 µl of DMSO for 30 minutes, at room temperature, in the dark. Finally, the solution was mixed and the absorbance was measured at 590 nm using a plate reader (EnSight Multimode Plate Reader, PerkinElmer, VIC, Australia).

2.2.4 Molecular biology techniques

2.2.4.1 RNA extraction

Total RNA was extracted from approximately 30 mg of snap-frozen liver tissue or from 5×10^6 cultured BMOL-TAT cells using Isolate II RNA mini kit (Bioline, NSW, Australia), according to the manufacturer's instructions. Eluted RNA was dissolved in UltraPure DNase/RNase-free water (Life Technologies, VIC, Australia) and stored at -80^0C for future use. The RNA concentration was measured using the NanoDrop 1000 spectrophotometer (Thermo Scientific, VIC, Australia). RNA purity was assessed through $A_{260/230}$ and $A_{260/280}$ ratios. RNA samples with values greater than 1.7 for $A_{260/230}$ and greater than 1.8 for $A_{260/280}$ were used for further analysis.

2.2.4.2 Microarray analysis

cDNA synthesis and genomic DNA elimination from RNA samples for reverse transcription-polymerase chain reaction (RT-PCR) microarray analysis was performed using the RT2 First Strand Kit (Qiagen, VIC, Australia) for 0.5 µg of eluted RNA, according to the manufacturer's instructions.

The RT^2 Profiler PCR array kit (Qiagen, VIC, Australia) was used to perform mouse Wnt pathway-focussed (PAMM-043ZD) gene expression profiling. According to the manufacturer's recommendations, the RT^2 Profiler PCR Array Format D was selected, which was suitable for the in-house real-time cycler (Bio-rad CFX Connect Real-Time System, NSW, Australia). Each 96-well array plate contained primer assays for 84 pathway-associated genes and five housekeeping genes. Additionally, one genomic DNA control, three reverse-transcription controls and three positive PCR controls were also present on the array plate. C_T values generated at the end of the PCR run were exported to an Excel file and fold change/regulation for each gene on the array plate was calculated using a spreadsheet-based analysis tool, available on the Qiagen website (http://www.qiagen.com/geneglobe).

2.2.4.3 Protein extraction

Proteins were extracted from adherent BMOL-TAT cells and liver tissue pieces. Cells or tissues were washed with ice-cold PBS before incubation in ice-cold Radio-Immunoprecipitation Assay (RIPA) lysis buffer (Sigma-Aldrich, NSW, Australia), mixed with protease and phosphatase inhibitor cocktail (Cell Signaling Technology, MA, USA). Further, cells were scraped using a cell scraper and tissues were manually homogenised using sterile tissue homogenisers before subjecting them to homogenisation with a sonicator (Bioruptor Plus, diagenode, VIC, Australia). Finally, homogenised cells or tissues were centrifuged at 16,000 x g for 15 minutes. Supernatant, containing proteins, was collected, transferred to a fresh tube and stored at -80^{0}C.

2.2.4.4 Protein assay

The protein concentration of extracted samples was determined using the Pierce Bicinchoninic (BCA) protein assay kit (Thermo Scientific, VIC, Australia). For this colorimetric assay, diluted albumin standards and working reagent were prepared according to the manufacturer's instructions and the absorbance was measured at 562 nm, using a plate reader (EnSight Multimode Plate Reader, PerkinElmer, VIC, Australia).

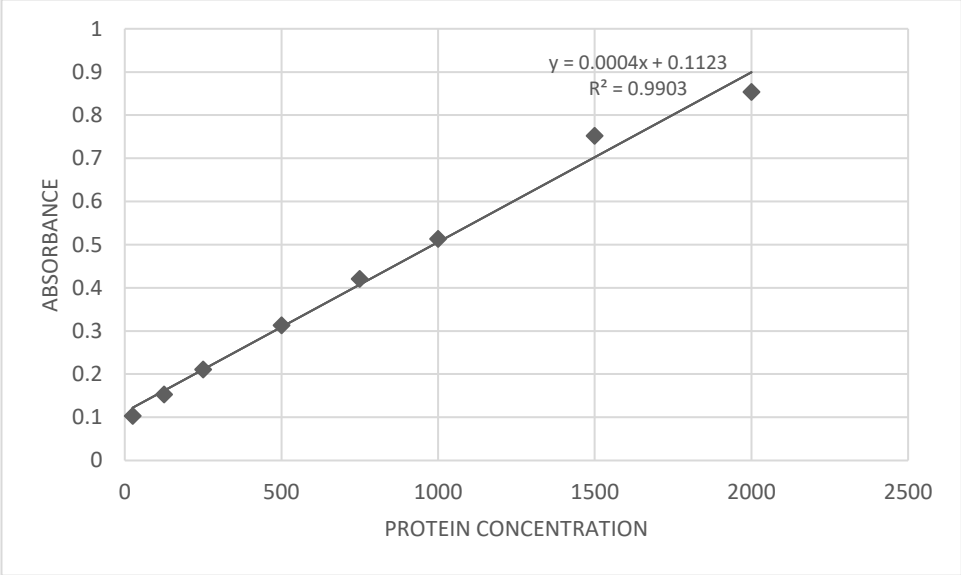


Figure 2.3: BCA protein assay standard curve.

2.2.4.5 Immunoblotting

The protein quantity was normalised in each sample and 25 µg of protein from each sample was used for immunoblotting. Samples were resolved using Bolt 4-12% Bis-Tris Plus precast gels (Invitrogen, VIC, Australia) and Bolt MES SDS Running Buffer (20x) (Life Technologies, CA, USA). Precision Plus Protein Kaleidoscope Standards (Bio-Rad, NSW, Australia) were used as molecular-weight size markers. Gels were then transferred onto a 0.2 µm nitrocellulose membrane (Bio-Rad, NSW, Australia) using Tris/Glycin buffer (Bio-Rad, NSW, Australia). Protein transfer was checked using REVERT Total Protein Stain (LI-COR, NE, USA). REVERT Total Protein Stain gel image can also be used as a loading control, according to manufacturer's recommendation. Afterwards, blots were washed with Tris-buffered saline (TBS)-Tween buffer (Bio-Rad, NSW, Australia), blocked with 5% instant skim milk powder in TBS-Tween buffer and incubated overnight with primary antibodies at 4^0C. The next day, blots were incubated with appropriate HRP-labelled secondary antibodies and the signal was detected using the enhanced chemiluminescence (ECL) kit (Bio-Rad, NSW, Australia) and ChemiDoc MP imaging system (Bio-Rad, NSW, Australia).

2.2.5 Statistical analysis

GraphPad Prism (version 8) software was used for graphs and statistical analyses. The data are represented as mean ± standard error of the mean (SEM) and *n* refers to biological replicates. Shapiro-Wilk test was performed to confirm the normality. Statistical significance was assessed using an unpaired Student's t-test (normal distribution of data), one-way ANOVA or Mann-Whitney U test (no normal distribution of data). Significance levels are denoted as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Chapter 3

The LPC-specific expression of β-catenin in NAFLD patient liver biopsies

3.1 Introduction

The presence of steatosis in non-alcoholic individuals with confirmed exclusion of other liver diseases leads to the diagnosis of NAFLD (Hashimoto, Taniai, & Tokushige, 2013). Non-invasive detection methods such as an ultrasound can determine the presence or absence of steatosis. However, to confirm the grade of NAFLD and in particular the presence of NASH or presence of fibrosis or cirrhosis, liver biopsy remains the preferred method (Tiniakos, 2010). Histopathological features of NAFLD include macrovesicular, microvesicular or a combination of macro- and microvesicular steatosis in more than 5% of hepatocytes. NASH is defined as the more progressed form of NAFLD, which is accompanied with hepatocellular ballooning and lobular inflammation (Kleiner & Makhlouf, 2016; Takahashi & Fukusato, 2014; Tiniakos, 2010). Apart from these common histologic features, some additional characteristics such as chronic portal inflammation, the presence of acidophil bodies, deposition of iron and the appearance of progenitor cells are also observed in NAFLD/NASH (Brunt, 2012).

CK7-positive cells, representing key components of DRs, have been observed in fatty livers without steatohepatitis and become more pronounced with the progression to NASH. The extent of DRs is proportional to the grade of NASH, grade of portal inflammation and the degree of hepatocyte replicative arrest (Gouw et al., 2011; Prakoso et al., 2014; Richardson et al., 2007). A study on 48 liver biopsy samples from NAFLD patients reported an increase in CK19-positive bile ductules which correlated with the severity of portal inflammation and fibrosis (Chiba et al., 2011). Inflammatory cells represent a critical part of the DRs. Macrophage-derived factors such as TWEAK and Wnt proteins may be responsible for DR heterogeneity, along with other candidates (Bird et al., 2013; Boulter et al., 2012). In addition, Gadd and colleagues reported that the extent of DRs in the NAFLD spectrum is only associated with the degree of portal, but not lobular inflammation (Gadd et al., 2014).

The prevalence of NAFLD-related HCC is rapidly increasing worldwide. Most of the non-cirrhotic HCC cases have been associated with an underlying metabolic syndrome and NAFLD (Desai, Sandhu, Lai, & Sandhu, 2019). At

present, the mechanisms behind the progression of steatosis and NASH to HCC are not very clear. To understand the mediators in the development of HCC from NAFLD, transcriptomic and metabolomic datasets from both NAFLD and HCC patients were compared by Clarke and colleagues. They hypothesised that Wnt signalling activation may be one of the critical events in the progression of NASH to HCC (Clarke et al., 2014). Moreover, Wnt/β-catenin signalling may be dysregulated in NAFLD-associated HCC due to epigenetic changes induced via metabolic pathways (Tian, Mok, Yang, & Cheng, 2016). Earlier, many studies have reported deregulated expression of β-catenin in HCC (Huang et al., 1999; Nhieu et al., 1999; Suzuki, Yano, Nakashima, Nakashima, & Kojiro, 2002); however, the expression of the β-catenin during the progressive stages of the NAFLD spectrum remains to be elucidated.

3.2 Study aims

The studies in this chapter were performed as proof-of-principle experiments to investigate the presentation of LPCs and the critical regulator of the Wnt/β-catenin signalling pathway, β-catenin, in different stages of human NAFLD. The first aim was to confirm the presence of LPCs in liver biopsies obtained from well-characterised subjects with NAFLD. This was followed by examining the overall expression and localisation of β-catenin in NAFLD. Lastly, using the same NAFLD samples, the potential expression of β-catenin in LPCs was studied.

3.3 Methodology

Human liver biopsy samples were retrieved from the Histopathology department of Fremantle Hospital (Fremantle, Australia). The cohort was comprised of 16 liver biopsies collected from subjects with different stages of the NAFLD spectrum. Eight subjects had steatosis-only, two had NASH, five had NASH and fibrosis whilst only one had cirrhosis. All other potential confounding causes of liver disease were excluded during clinical workup with routine standard-of-care biochemical and serological tests. LPC responses in all samples were assessed using immunofluorescence stainings of FFPE sections for the biliary and LPC marker panCK. To determine the presence and localisation of β-catenin, immunofluorescent labelling for β-catenin was performed on all FFPE sections. Finally, to determine whether LPCs express β-catenin, FFPE liver sections were immunofluorescently co-labelled for panCK and β-catenin.

3.4 Results

3.4.1 The detection of LPCs and characterisation of β-catenin expression in livers and in LPCs of NAFLD subjects

The positive immunofluorescence staining for the biliary cell and LPC marker panCK confirmed that LPCs were present in all biopsy samples of NAFLD patients, ranging from steatosis with no signs of fibrosis to cirrhosis. Representative images are shown for three individual patients who are at different stages of the NAFLD spectrum (Figure 3.1). The panCK$^+$ LPCs were primarily observed in periportal areas with occasional penetration into the parenchyma. They appeared as biliary structures, strings of bile duct epithelium or as small groups of cells. At the later stages of the NAFLD spectrum, panCK$^+$ LPCs became more evident.

Immunofluorescent labelling with β-catenin antibody detected the presence of this key regulator of the Wnt/β-catenin pathway in all biopsy samples. The presentation of β-catenin$^+$ cells was similar to panCK$^+$ LPCs. Positive β-catenin staining was mainly observed near the periportal areas, as identified by proximity to bile ducts in the portal triads. Localisation of β-catenin was either membranous or membranous and cytoplasmic/nuclear (Figure 3.2).

Finally, the co-localisation of β-catenin and panCK confirmed the expression of β-catenin in LPCs, as demonstrated in Figure 3.3. Although LPCs with positive β-catenin staining were identified in all the specimens, the localisation of the protein varied. In eight samples only membranous β-catenin staining was observed in LPCs whereas, in remaining eight specimens strong cytoplasmic/nuclear β-catenin was present in subset of LPCs, signifying active Wnt/β-catenin signalling in these subpopulations of LPCs. Interestingly, no correlation was found between the cytoplasmic/nuclear localisation of β-catenin in LPCs and the stage of NAFLD.

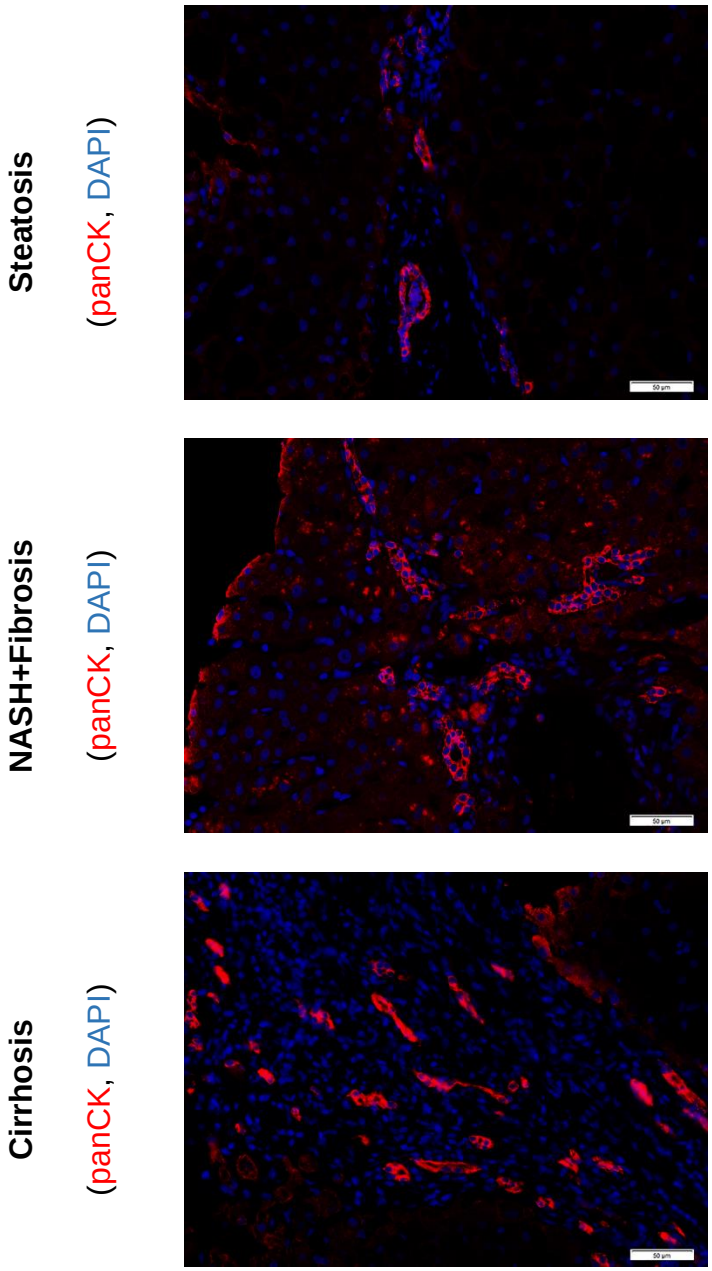


Figure 3.1: LPC proliferation in human NAFLD patients. FFPE liver sections from NAFLD patients were labelled for panCK using immunofluorescence to evaluate the presence of LPCs. panCK^{+} LPCs were detected at all stages of the NAFLD spectrum and they became more noticeable with progression in the disease stage. The scale bar depicts 50 µm.

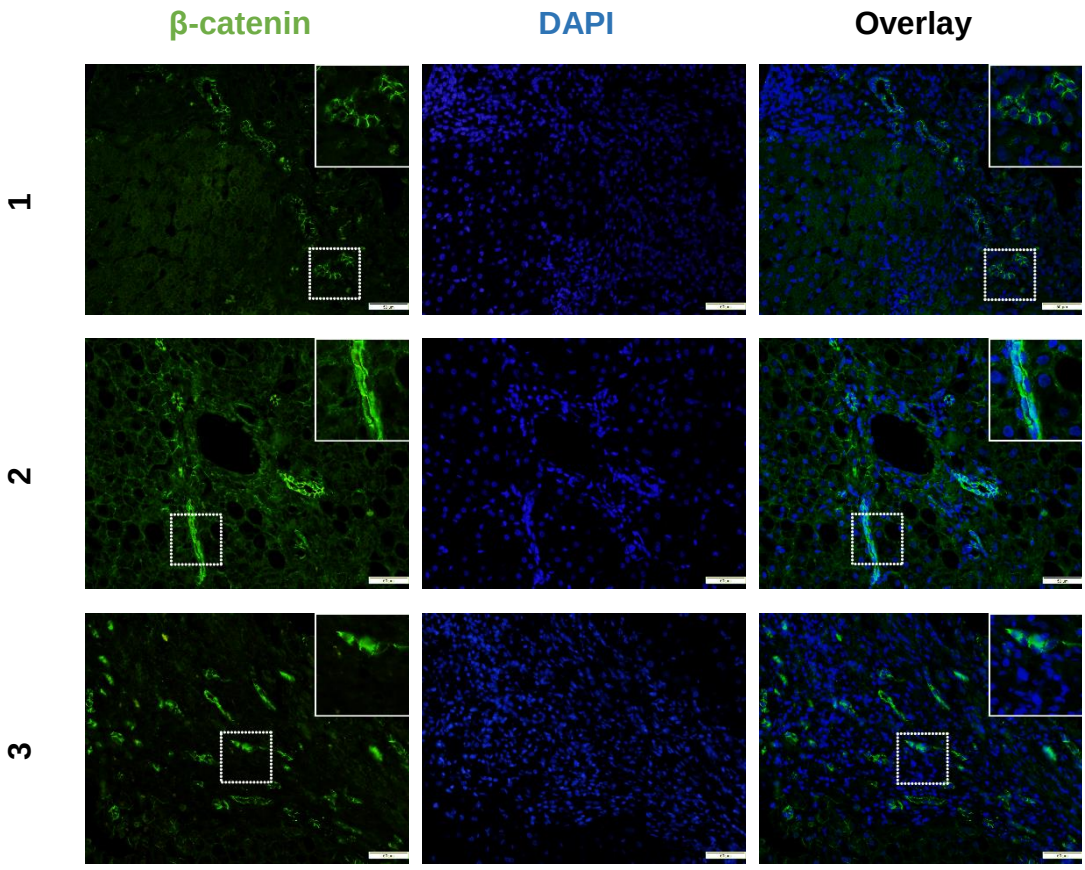


Figure 3.2: Membranous and cytoplasmic/nuclear staining of β-catenin. FFPE liver sections from NAFLD patients were labelled for β-catenin using immunofluorescence to assess the presence and localisation of β-catenin. Image 1 shows membrane-bound β-catenin and images 2 and 3 display membrane-bound as well as cytoplasmic/nuclear β-catenin in subset of cells. The scale bar depicts 50 µm.

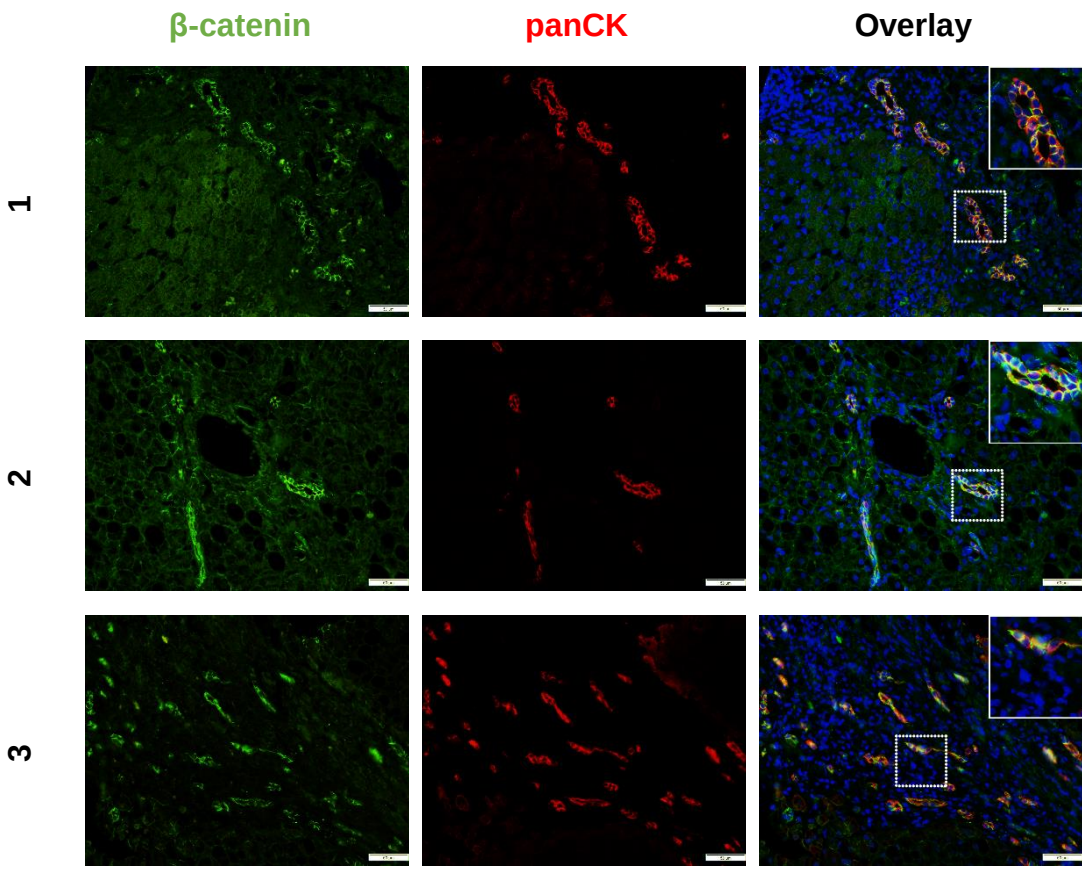


Figure 3.3: Liver progenitor cells express β-catenin. FFPE liver sections from NAFLD patients were co-labelled for β-catenin and panCK using immunofluorescence to confirm the presence of β-catenin$^+$ LPCs and ducts. panCK$^+$ LPCs expressed β-catenin; however, the localisation of β-catenin was variable. Image 1 displays LPCs with membrane-bound β-catenin and images 2 and 3 show subpopulation of LPCs expressing cytoplasmic/nuclear β-catenin in addition to membranous. The scale bar depicts 50 µm.

Patient number	Steatosis	NASH	Fibrosis	Cirrhosis	LPC numbers	Localisation of β-catenin in LPCS
S1	+				+	Membranous
S2	+				+	Mostly membranous and cytoplasmic/nuclear in subset of LPCs
S3	+				+	Mostly membranous and cytoplasmic/nuclear in subset of LPCs
S4	+				+	Membranous
S5	+				+	Mostly membranous and cytoplasmic/nuclear in subset of LPCs
S6	+				+	Membranous
S7	+				+	Membranous
S8	+				+	Mostly membranous and cytoplasmic/nuclear in subset of LPCs
S9		+	MILD		++	Membranous
S10	+	+	MODERATE		++	Mostly membranous and cytoplasmic/nuclear in subset of LPCs
S11		+	MILD		++	Membranous
S12	+	+	MILD		++	Membranous
S13		+			+	Mostly membranous and cytoplasmic/nuclear in subset of LPCs
S14				+	+++	Mostly membranous and cytoplasmic/nuclear in subset of LPCs
S15		+			+	Membranous
S16	+	+	MODERATE		++	Mostly membranous and cytoplasmic/nuclear in subset of LPCs

Table 3.1: Patient data and summary of β-catenin localisation in LPCs. This cohort of patients comprised of 16 subjects out of which eight had steatosis, two had NASH, five had NASH and fibrosis and one patient was reported with cirrhosis. LPCs become more evident as NAFLD stage progressed. No correlation was found between cytoplasmic/nuclear presentation of β-catenin and stage of the NAFLD spectrum.

3.5 Discussion

NAFLD is known as the hepatic manifestation of the metabolic syndrome and it shares a close relationship with obesity and insulin resistance (Benedict & Zhang, 2017). In the past decade, links between Wnt signalling and metabolic dysregulation have emerged (Schinner, 2009). For instance, failure in the recruitment of new adipocytes to manage metabolic influx can lead to obesity (Christodoulides, Lagathu, Sethi, & Vidal-Puig, 2009) and Wnt signalling was demonstrated to inhibit adipogenesis (Ross et al., 2000). In contrast, inhibition of Wnt signalling by sFRP4 upregulates adipogenic differentiation (Visweswaran et al., 2015). Recently, Chen *et al*. reported that rare gain-of-function mutations in *CTNNB1* can increase the obesity risk in humans (Chen et al., 2020). Mutations in *TCF7L2*, a Wnt signalling transcription factor, are linked to increased risk of type 2 diabetes (Grant et al., 2006). Geng and colleagues described the interaction between IRS1/2 and Dvl to promote canonical Wnt signalling (Geng et al., 2014). Furthermore, activation of hepatic Wnt/β-catenin signalling by insulin has been reported (Cabrae et al., 2020). The role of Wnt/β-catenin signalling in liver biology with respect to embryogenesis, maturation, zonation and hepatitis virus-induced hepatocarcinogenesis is well established (Perugorria et al., 2019). However, the specific hepatic cell types that mediate Wnt signalling effects in NAFLD-like conditions remain understudied.

This chapter investigated the presence of LPCs and β-catenin in archival liver biopsy samples of NAFLD subjects using histological methods. Most specimens had been collected at the early-to-mid stages of the NAFLD spectrum, primarily characterised as steatosis, NASH and fibrosis. This was particularly interesting as very few studies have examined β-catenin expression in the initial stages of NAFLD. More detailed experiments using molecular techniques such as western blotting or qPCR on upstream and downstream components of the canonical Wnt pathway were not possible at this stage, as the available patient samples were in the form of FFPE liver sections. These studies were undertaken using time course liver tissue generated in murine chronic liver disease (described in Chapter 4).

Initially, liver tissue was evaluated to document the expansion of the LPC compartment using the well-characterised marker panCK. A previous study on hepatitis C virus-infected patients before and after liver transplantation demonstrated that panCK reliably detects LPCs and DRs in human liver samples, with very similar specificity to the more commonly used biliary markers CK7 and CK19 (Prakoso et al., 2014). Our data confirmed the presence of LPCs in different stages of the NAFLD spectrum such as steatosis, NASH, fibrosis and cirrhosis, consistent with previous studies (Overi, Carpino, Franchitto, Onori, & Gaudio, 2020; Richardson et al., 2007).

Two reservoirs of β-catenin protein are present in the cell. The pool of β-catenin present at the cell membrane connected with E-cadherin does not take part in active Wnt signalling. The other non-phosphorylated pool of β-catenin, which is accumulated in the cytoplasm and nucleus, is the transcriptionally active form and the main regulator of canonical Wnt signalling (Clevers & Nusse, 2012). Therefore, positive cytoplasmic or nuclear staining of β-catenin is a reliable read-out of pathway activation. Importantly, very low levels of nuclear β-catenin are required to activate downstream targets, and they may lie below the detection limit of immunohistochemical or immunofluorescent techniques (Kalasekar et al., 2019). Hence, a strong cytoplasmic staining is also indicative of an activated pathway. Following initial characterisation of the presence of LPCs, detection of β-catenin was performed using immunofluorescent assessment of the liver samples. All patient samples displayed cluster of cells with positive β-catenin staining, which was either detected as membranous or membranous combined with strong cytoplasmic/nuclear. The presence of β-catenin as membrane-bound or cytoplasmic/nuclear was not found to be correlated with the stage of the disease. In all the samples, β-catenin positive cells were primarily localised to the periportal area which on occasion extended into the liver parenchyma.

The intralobular location of β-catenin$^+$ cells was similar to the usual distribution of panCK$^+$ cells. Thus, co-localisation studies were undertaken to determine whether LPCs are participants in the Wnt/β-catenin pathway during NAFLD-driven CLD. Immunofluorescent β-catenin and panCK double stainings confirmed that the respective signals co-localised in the same cells and

validated LPCs as being actively involved in β-catenin signalling in human NAFLD/NASH.

Taken together, the findings in this chapter support the hypothesis that in addition to hepatocytes, LPCs are amongst the drivers of β-catenin-mediated downstream effects during CLD. To study β-catenin expression within different grades of steatosis, NASH and fibrosis and understand if an increase in protein expression and the number of β-catenin^{+} LPCs positively correlates with disease severity, it will be necessary to evaluate a larger cohort of patients. The experiments in Chapter 3 provided some general knowledge as a basis for further, more detailed experiments that were undertaken using a mouse model of NAFLD (Chapters 4 and 6) and a novel cell culture system to study Wnt/β-catenin signalling in fat-loaded LPCs *in vitro* (Chapter 5).

Chapter 4

Wnt/β-catenin pathway expression in the murine CDE model of chronic liver injury

4.1 Introduction

NAFLD is a progressive heterogeneous disease, with greatly varying degrees of histopathological changes, depending on the disease severity and underlying contributing factors. Various animal models have been developed to reflect the heterogeneity of human NAFLD. The ideal animal model should exhibit hepatic steatosis, intralobular inflammation, hepatocellular ballooning, perisinusoidal fibrosis as well as susceptibility to cirrhosis and HCC, as seen in human NAFLD/NASH (Takahashi, Soejima, & Fukusato, 2012). Moreover, it is desirable to experimentally induce metabolic disruptions, which are commonly associated with NAFLD, such as obesity, insulin resistance, hyperlipidaemia etc. (Takahashi et al., 2012). However, an animal model that can demonstrate all characteristics of human NAFLD has not been reported to date. Generally, NAFLD models can be classified into diet-based models, genetic models or a combination of both (Fan & Qiao, 2009; Ninomiya, Kondo, & Shimosegawa, 2012). Dietary models can be developed by providing a high calorie diet, a nutrient-deficient diet and/or by administering hepatotoxins. Genetic animal models are generated by manipulating the appetite or metabolic regulation (Haczeyni et al., 2018). All currently used regimens come with certain advantages and limitations and the selection of an appropriate animal model largely depend on the aims and research focus areas of the study.

Choline is an important nutrient, which takes part in the maintenance of cell membrane integrity, transmembrane signalling, phosphatidylcholine synthesis, neurotransmission and methyl metabolism (Anstee & Goldin, 2006; Zeisel et al., 1991). Choline deficiency can hamper triglycerides assembly and export, leading to hepatic fat accumulation (Lombardi et al., 1968). Thus, a choline-deficient diet has been used in rodents to generate steatosis (Oliveira et al., 2003; Raubenheimer, Nyirenda, & Walker, 2006; Veteläinen, van Vliet, & van Gulik, 2007). Additionally, altered mitochondrial function and increased oxidative stress was found to be associated with choline deficiency-induced steatosis (Oliveira et al., 2003; Teodoro, Rolo, Duarte, Simões, & Palmeira, 2008). Together, these effects lead to repetitive cycles of liver cell injury, death and regeneration, finally resulting in the development of HCC in animals fed

with a choline-deficient diet long-term (Chandar & Lombardi, 1988; Lombardi, Chandar, & Locker, 1991). Ethionine is a hepatotoxin, which induces fatty liver upon short-term administration and hepatic carcinoma with long-term exposure (Epstein, Ito, Merkow, & Farber, 1967; Olivercrona, 1962; Sharma, Kuchino, & Borek, 1978). Importantly, co-administration of ethionine with a choline-deficient diet resulted in extensive proliferation of LPCs in rat livers (Shinozuka et al., 1978).

Later, modifications were made to the rat CDE protocol in order to make it suitable for feeding to mice (see Chapter 1, 1.4.2). Short-term CDE feeding induces steatosis, hepatocellular damage, inflammation, DRs and fibrosis in mice (Clerbaux L. et al., 2017; Van Hul et al., 2009), which makes it a relevant model to study LPC expansion associated with chronic fatty liver disease. The effect of CDE diet feeding on markers of liver injury, inflammation, DRs and fibrosis has been studied in great detail using a comprehensive time course. Briefly, injury parameters peak in the first week of CDE exposure and slowly decrease over time until they almost reach control levels after six weeks on the diet (Kohn-Gaone, Dwyer, et al., 2016a). Archival material from this previous time course were available for further studies, such as the experiments described below.

Even though the role of Wnt/β-catenin signalling in liver regeneration is well-studied (Russell & Monga, 2018), it is important to note that Wnt signalling activation is highly context- and tissue-specific. Therefore, it was necessary to investigate Wnt signalling expression in CDE-treated versus normal liver tissue at different time points of injury to establish a basis for later Wnt pathway manipulation experiments (see Chapter 6).

4.2 Study aims

The first aim of this chapter was to validate the CDE diet as a suitable model of murine NAFLD by investigating histopathological parameters, including steatosis, inflammation, fibrosis and LPC activation. The second aim was to characterise the expression of Wnt/β-catenin signalling in control versus CDE-treated livers using an archival six-week time course.

4.3 Methodology

Liver tissues used for experiments described in this chapter were obtained as part of a previous study. Six-week old male C57BL/6J mice were randomly grouped and subjected to control and CDE diet, with *ad libitum* access to food and water. Liver tissue was harvested on days 3, 7, 14, 21 and 42 and either cryo-preserved or FFPE.

Overall liver damage was assessed by H & E staining. The NAFLD spectrum hallmarks steatosis, inflammation and fibrosis were evaluated using Oil red O staining, fluorescent-labelling of $CD45^+$ inflammatory cells and more specifically $F4/80^+$ macrophages, and Sirius Red collagen staining, respectively. The CDE diet-induced LPC compartment activation was assessed using the biliary and LPC markers panCK and A6. Triple fluorescent staining for panCK, CD45 and the activated HSC marker, α-SMA evaluated the spatial association of these three key cell types in the injury and regeneration niche.

Distribution and localisation of non-phosphorylated (active) β-catenin in the liver at different time points was studied by immunohistochemistry and immunofluorescence techniques, while the expression of active β-catenin at the protein level was assessed by Western blotting.

Additionally, co-staining of active β-catenin with LPC markers (panCK, A6, CK19), F4/80 and α-SMA was performed to confirm the expression of active β-catenin in these cell types. Expression of different Wnt/β-catenin pathway components at the transcription level was assessed using a Qiagen Wnt RT-PCR array (PAMM-043ZD) for RNA isolated from one representative liver tissue sample of a 21-day control and a 21-day CDE-treated animal. Finally, the expression of the Wnt/β-catenin signalling pathway targets LGR5 and CYP2E1 was studied using immunohistochemistry.

4.4 Results

4.4.1 Histological analysis of control and CDE-treated livers

H & E staining of control and CDE-treated livers at different time points revealed the effects of a CDE diet on the liver architecture. At every time point, control animals presented a clearly defined parenchyma with evenly arranged cords of hepatocytes. In contrast, in CDE-treated animals, signs of liver damage were evident at all time points. The normal orderly arrangement of the liver parenchyma was disrupted with steatosis and basophilic cell infiltrates (Figure 4.1).

Steatosis is an important feature of NAFLD. Therefore, the livers were stained with Oil Red O, which binds to neutral lipids and fatty acids, to evaluate fat accumulation induced by the CDE treatment. There were no hepatic fatty changes in control diet-fed animals at any of the time points investigated. All CDE-treated livers displayed substantial macro-and microvesicular hepatic steatosis compared to control tissue. While macrovesicular steatosis dominated at earlier time points (day 3 and 7), later time points (day 21 and 42) mainly displayed microvesicular steatosis (Figure 4.2).

Inflammation is another hallmark in NAFLD spectrum progression. The inflammatory response to the CDE diet was characterised using immunofluorescent staining for the pan-inflammatory marker CD45 and the pan-macrophage marker F4/80. Compared to control diet fed mice, which had low and steady numbers of liver-resident inflammatory cells, CDE treatment led to an increase in numbers of $CD45^+$ and $F4/80^+$ inflammatory cells (Figure 4.3A and 4.3B).

Advanced stages of NAFLD are associated with fibrosis as a general regenerative response to chronic liver injury. Hence, it was of particular interest to study collagen deposition and the fibrosis pattern in the CDE model, which was undertaken through Sirius Red staining. While no signs of fibrosis were evident in control diet-fed animals, CDE administration resulted in mild portal fibrosis that was occasionally ‘chicken-wire’ in appearance. At times, when portal and central fields were in close proximity, the portal-to-central bridging fibrosis was also evident at later time points (Figure 4.4).

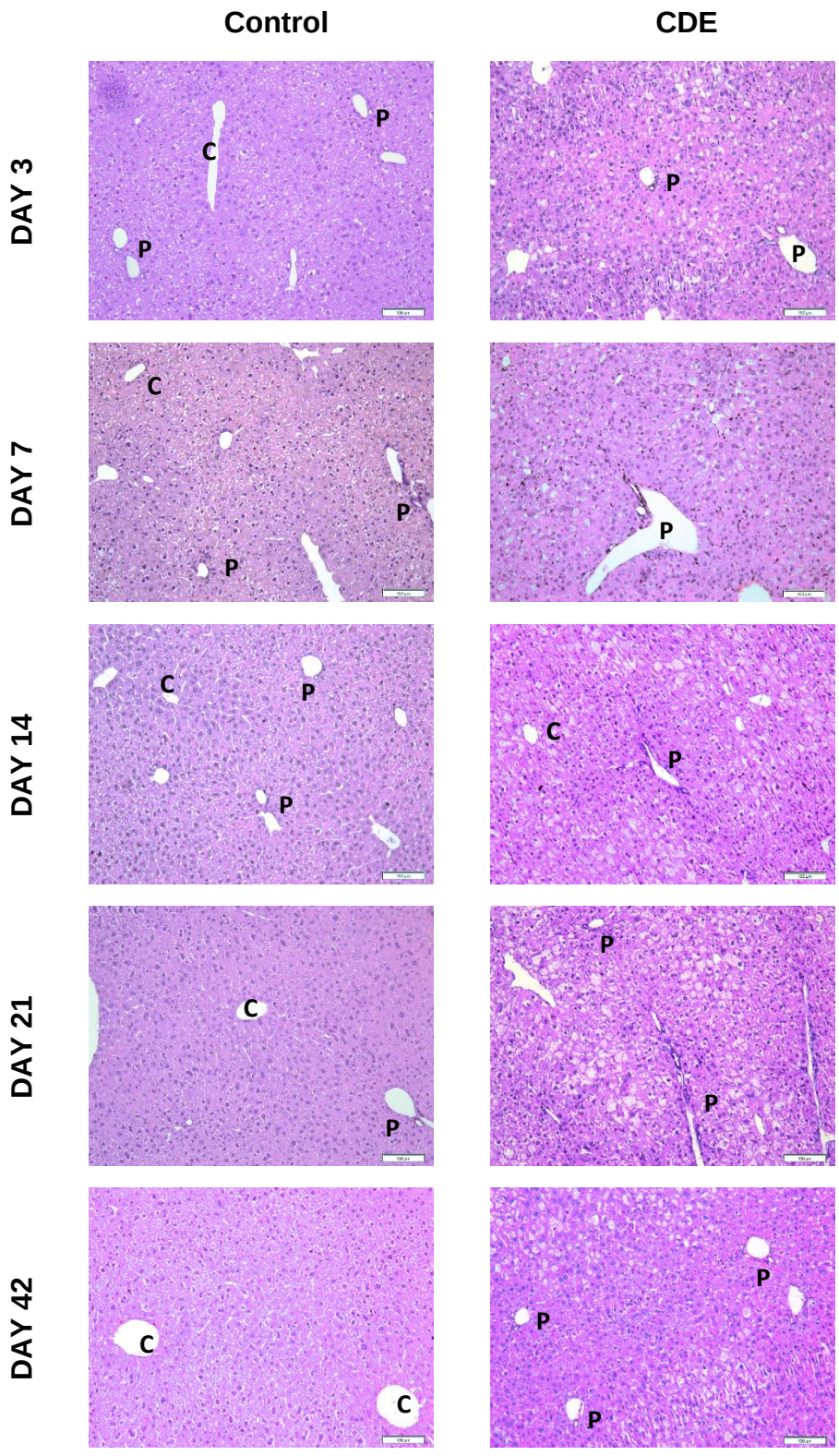


Figure 4.1: H & E staining of liver sections from control and CDE-treated mice. Mice on a control diet displayed normal liver architecture, while CDE-fed mice presented steatosis and increased numbers of basophilic periportal cells. The scale bar depicts 100 µm. C: central area, P: portal area.

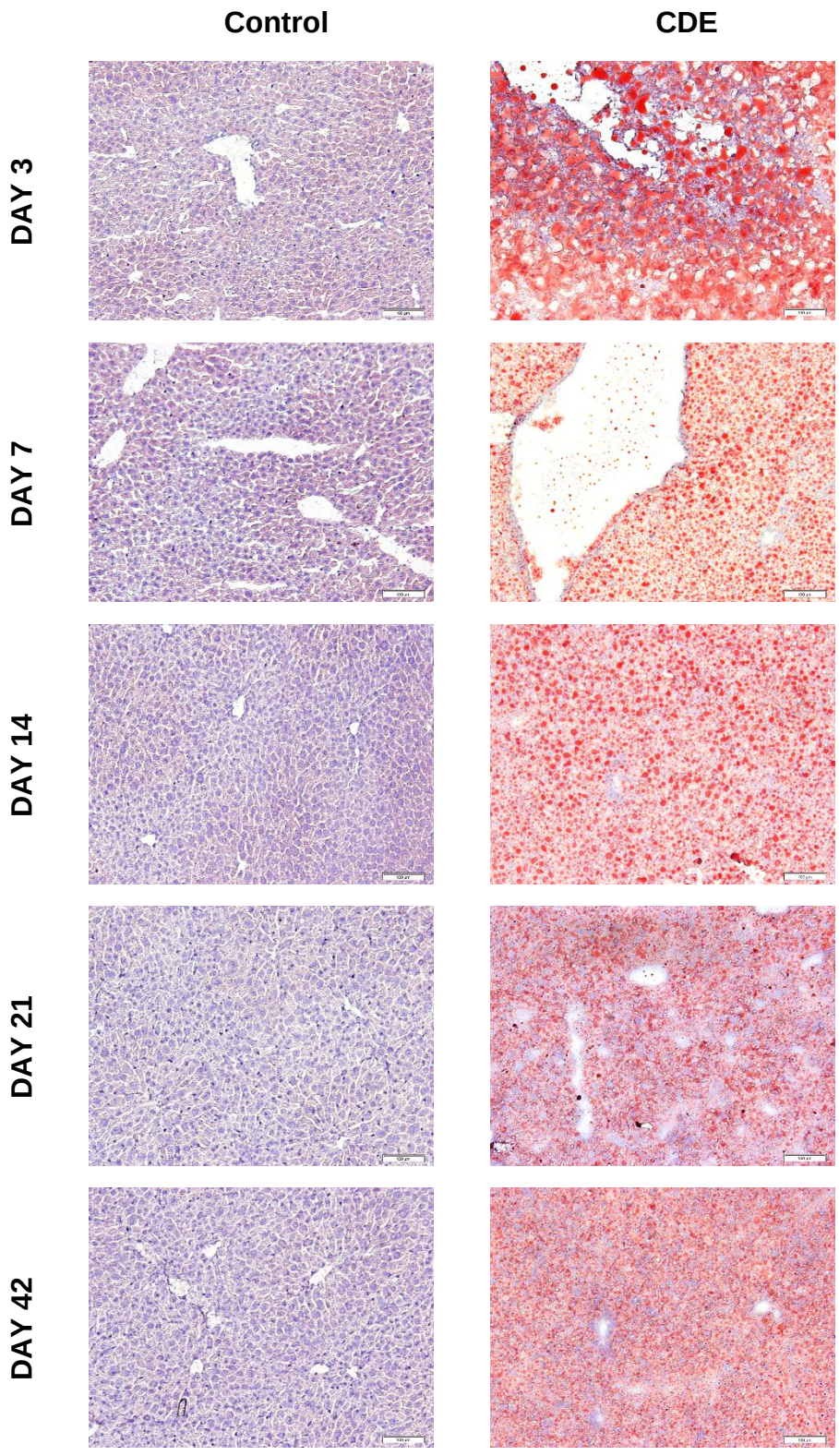


Figure 4.2: Oil red O staining of liver sections from control and CDE-treated mice. Mice on a control diet displayed limited fat deposition, whereas, mice on the CDE diet presented massive macro- and microvesicular fat deposition. The scale bar depicts 100 µm.

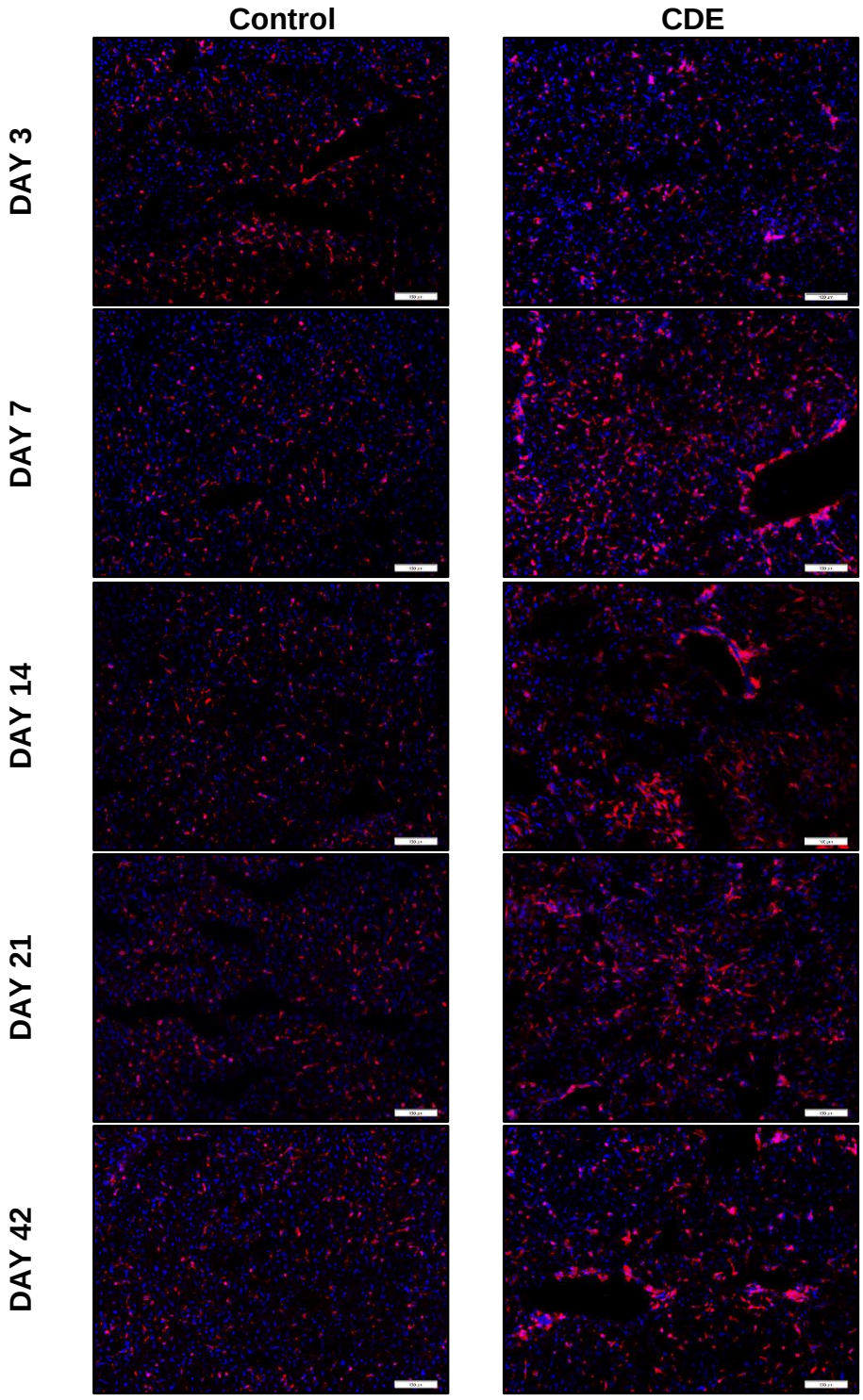


Figure 4.3A: Expression of CD45$^+$ cells in liver sections from control and CDE-treated mice. Immunofluorescent stainings demonstrated increased numbers of CD45$^+$ inflammatory cells in periportal areas in chronically injured compared to healthy livers. The scale bar depicts 100 µm.

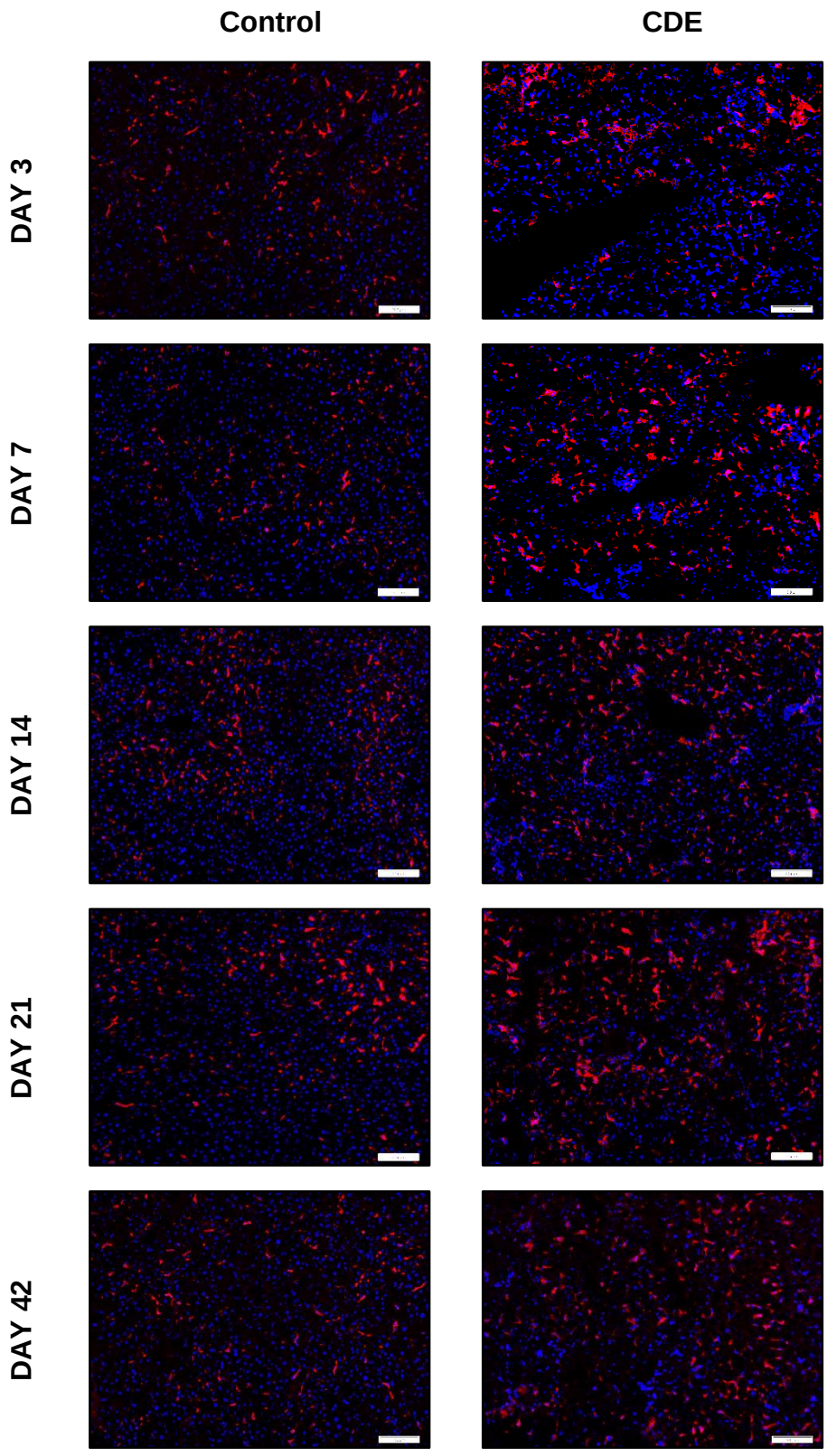


Figure 4.3B: Expression of F4/80^{+} cells in liver sections from control and CDE treatment mice. Immunofluorescent stainings demonstrate increased numbers of F4/80^{+} macrophages in chronically injured compared to healthy livers. The scale bar depicts 100 µm.

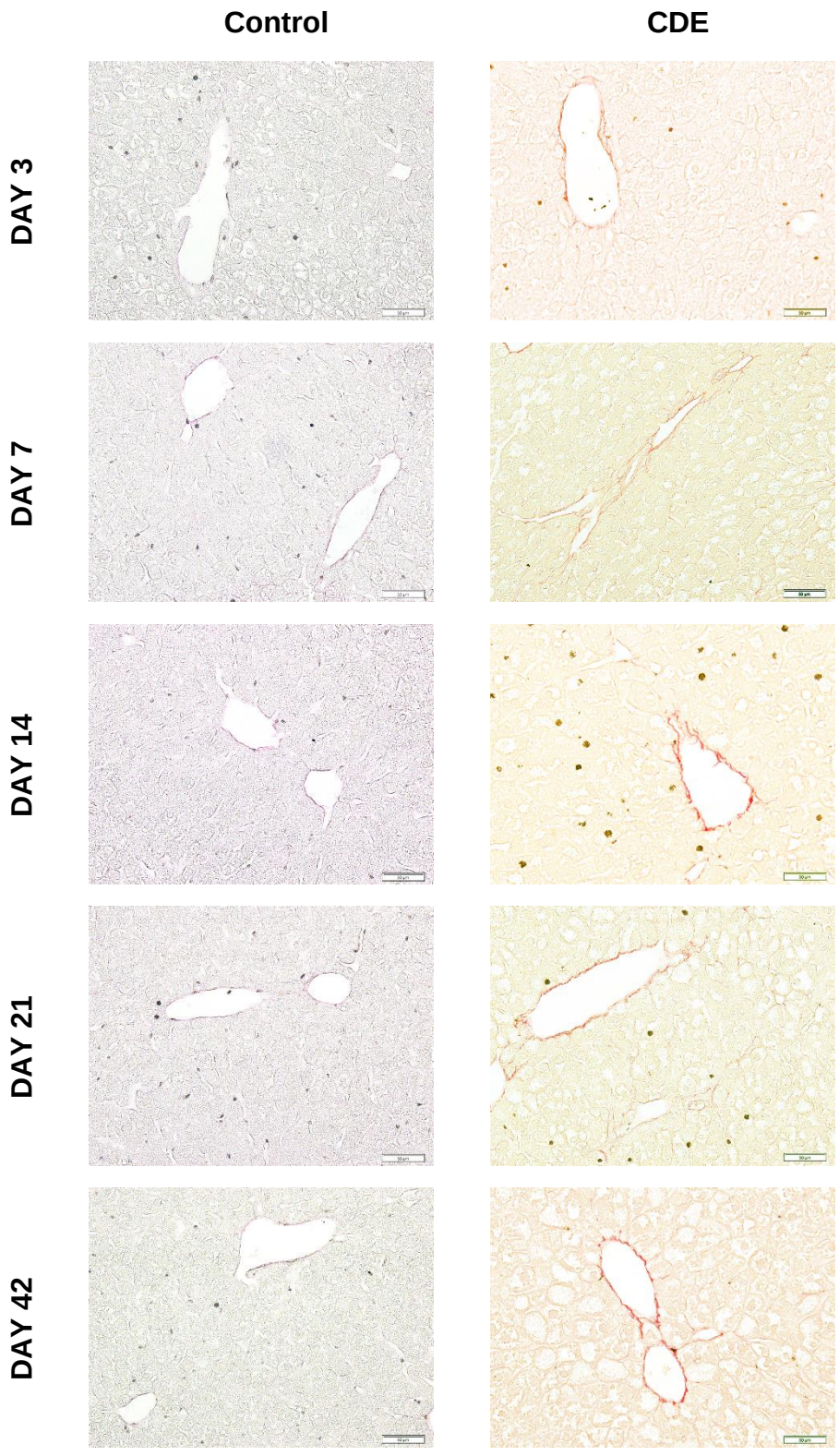


Figure 4.4: Sirius Red staining of liver sections from control and CDE-treated mice. CDE treatment induces collagen deposition around vessels with occasional mild “chicken-wire” fibrosis extending into the parenchyma. The scale bar depicts 50 µm.

4.4.2 Liver progenitor cell activation in CDE-treated animals

Chronic liver injury typically induces many cellular changes that accompany inflammatory and fibrotic changes, including the proliferation of LPCs as part of DRs.

Two different LPC markers were used to evaluate the activation of LPCs. In healthy control mice, panCK and A6 staining was restricted to the bile ducts. In contrast, in CDE-treated mice, the panCK$^+$ and A6$^+$ cell compartment expanded from the portal region and penetrated into the liver parenchyma at later time points. These cells were arranged in clusters of three to four cells, duct-like structures or as isolated single cells (Figures 4.5A and 4.5B). Quantitation of the panCK$^+$ cell population in all experimental groups revealed that numbers were significantly higher in CDE-treated animals compared to the healthy controls at all time points except for day 3, with a peak in cell numbers at day 21 (Figure 4.6). Immunofluorescent triple staining for panCK, CD45 and α-SMA demonstrates that LPCs closely associate with inflammatory cells and HSCs once injury is established, forming an injury and regeneration niche, as shown for day 21 of CDE feeding (Figure 4.7).

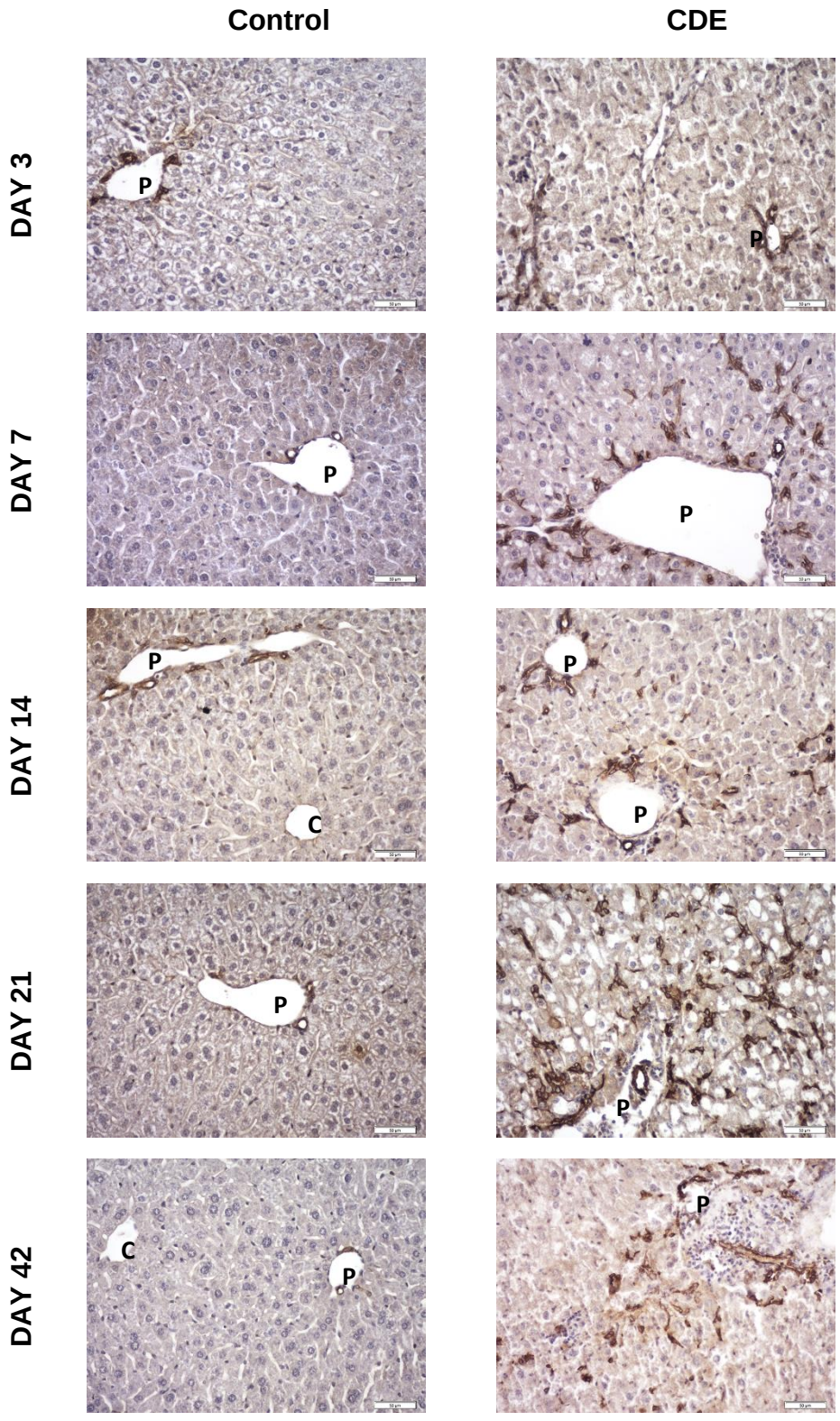


Figure 4.5A: LPC assessment in control versus CDE FFPE liver tissues. Expression of panCK is restricted to the bile ducts in control animals, while CDE-treated animals display panCK^{+} cells in ducts and migrating into the liver parenchyma. The scale bar depicts 50 µm. C: central area, P: portal area.

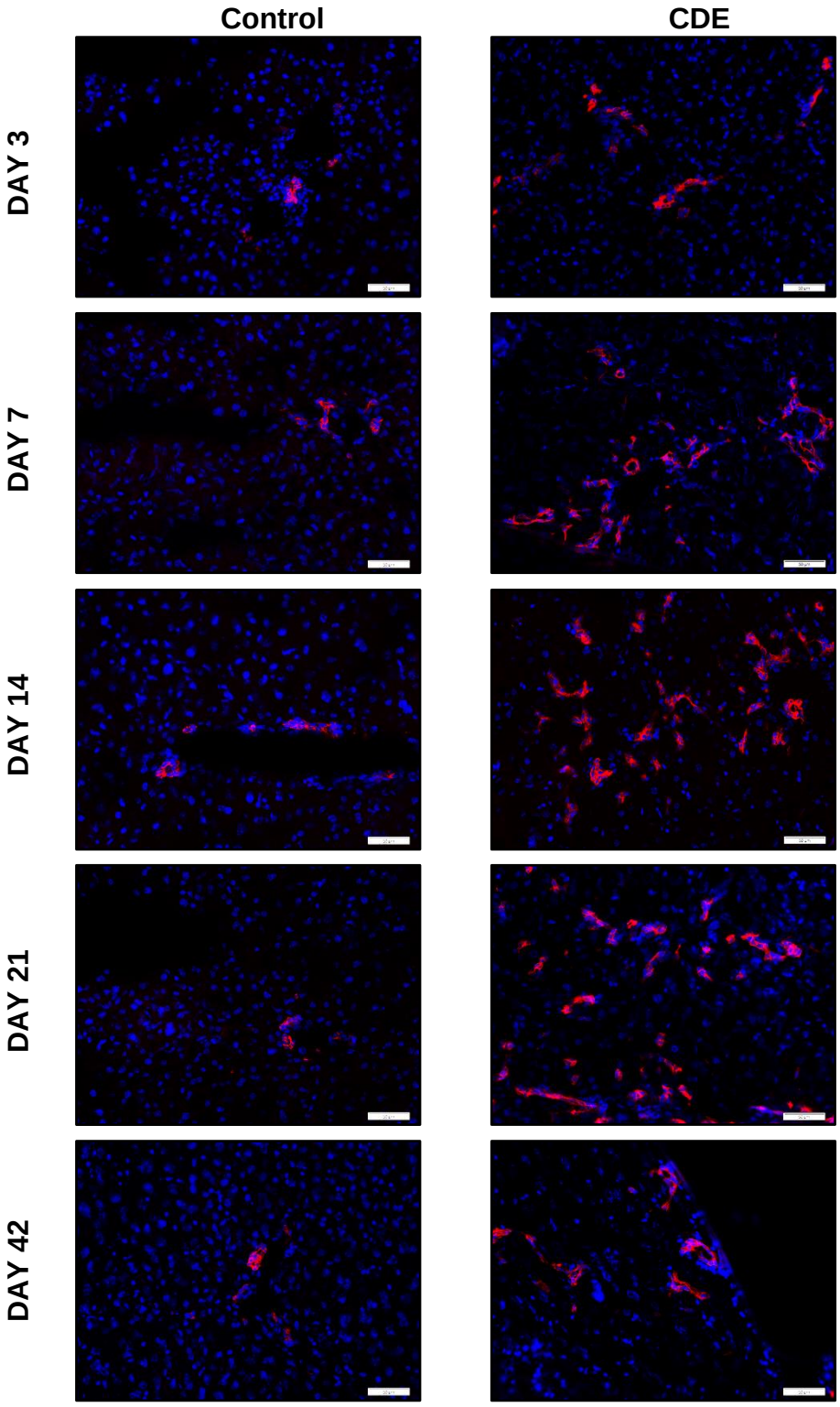


Figure 4.5B: LPC assessment in frozen control versus CDE liver tissues. Expression of A6 in frozen liver sections from control animals is restricted to the bile ducts. In contrast, CDE-treated animals display $A6^{+}$ cells in ducts as well as strings of cells or single cells in the liver parenchyma. The scale bar depicts 50 µm.

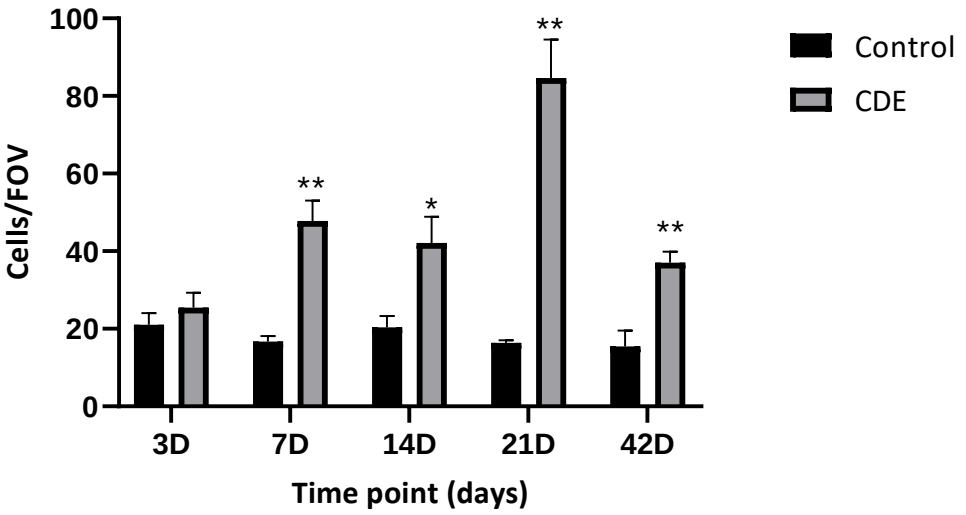


Figure 4.6: Quantitation of panCK⁺ cells in liver sections of mice on a control or CDE diet over a 42-day time course. Except for day 3, numbers of panCK⁺ cells in CDE-treated mice were significantly higher compared to healthy control mice throughout the time course. Data represent the mean ± SEM. *n*= 3-4 mice per group. **P* < 0.05, ***P* < 0.01 compared to control mice at the same time point. FOV: Fields of view.

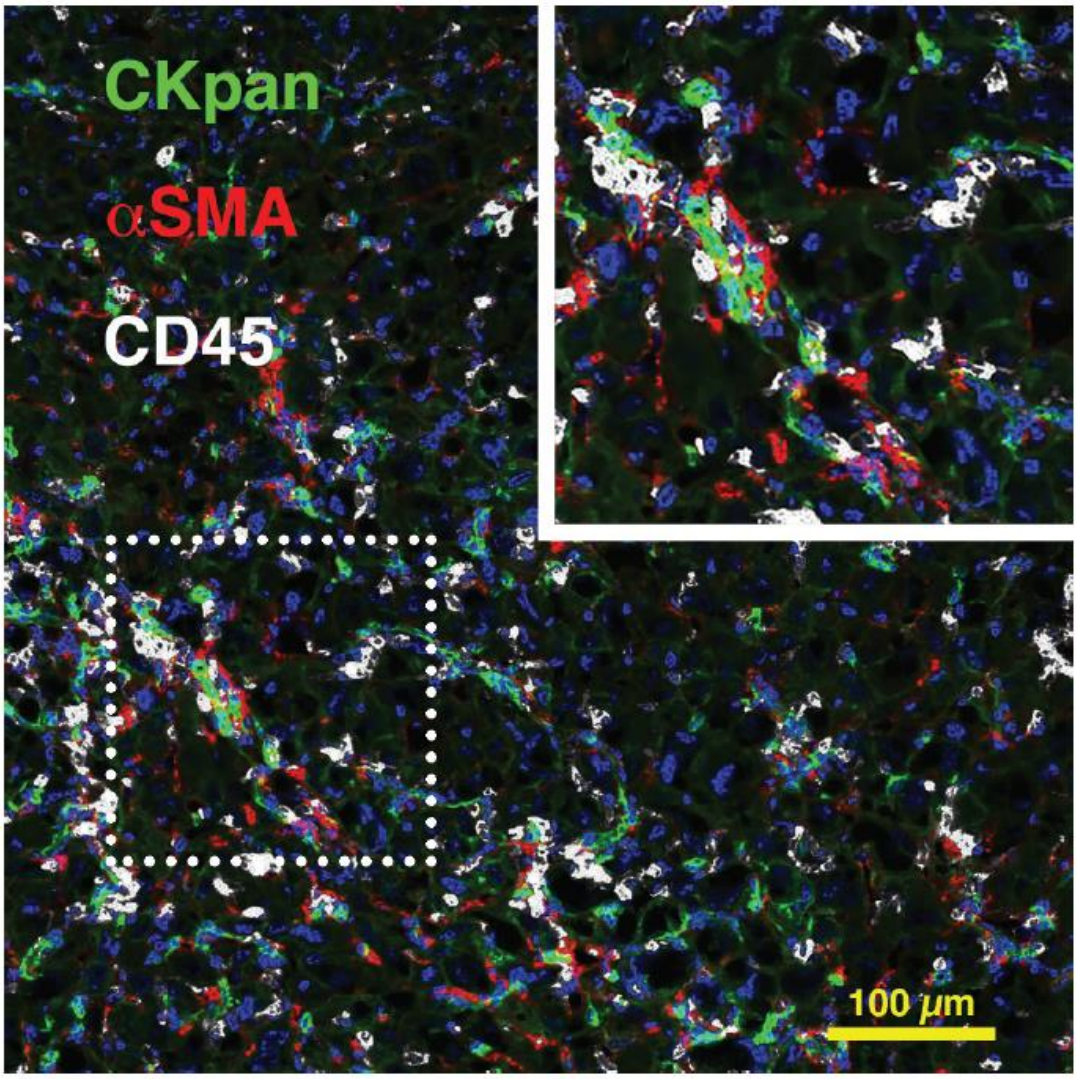


Figure 4.7: The injury and regeneration niche established by the CDE diet. panCK^{+} ductular cells and LPCs, α-SMA^{+} HSCs and CD45^{+} inflammatory cells are present in the injury and regeneration niche in close spatial organisation (Shirolkar et al., 2018). The scale bar depicts 100 μm.

4.4.3 Characterising Wnt/β-catenin pathway expression in CDE-treated animals

Immunohistochemical staining for the non-phosphorylated active form of β-catenin in control and CDE-treated animals at different time points revealed a distribution of positively stained cells that was similar to the staining pattern of panCK$^+$ LPCs (Figure 4.8). In control animals, strong membranous/cytopasmic staining was restricted to the bile ducts. However, in CDE-treated mice, strings of active β-catenin$^+$ cells can be seen around portal areas and penetrating into the parenchyma. The increase in active β-catenin$^+$ cell numbers in the CDE-treated animals reached significance for days 3, 14 and 21 (Figure 4.9). Moreover, similar to panCK$^+$ cells, the number of active β-catenin$^+$ cells peaked at day 21 and was reduced again at day 42 of CDE treatment. The increase in active β-catenin was also evident at the protein level in a 21-day CDE treated-liver compared to the respective control (Figure 4.10). Further, frozen liver sections from CDE-treated animals were stained with active β-catenin to assess the intracellular localisation of the protein. At all time points, hepatocytes displayed membranous active β-catenin staining, whereas a subset of bile duct cells and strings of cells around portal area demonstrated strong cytoplasmic/nuclear staining in addition to membranous staining (Figure 4.11). Additionally, double immunofluorescence staining was carried out to characterise the type of liver cells that express active β-catenin. Frozen CDE-treated liver sections were stained with various LPC markers such as A6, CK19 and panCK in combination with active β-catenin. The results showed that all three LPC subpopulations actively signal through the Wnt/β-catenin pathway (Figure 4.12). Double immunofluorescence staining of F4/80 and α-SMA with active β-catenin revealed that F4/80$^+$ macrophages and α-SMA$^+$ HSCs only express the membrane-bound form of active β-catenin in CDE-treated livers (Figure 4.13 and Figure 4.14).

It is essential to check the expression of other components of Wnt/β-catenin pathway to confirm that the pathway is active in CDE-treated animals. RT-PCR Wnt array analysis of 84 Wnt pathway-related genes revealed that β-catenin, cyclin D1 and cyclin D2 expression, along with the expression of a few other genes listed in Table 4.1, was upregulated in day 21 CDE-treated compared to day 21 healthy control liver, confirming Wnt/β-catenin signalling activity

(Figure 4.15). Co-localisation of the cell proliferation marker Ki-67 and the ductal/LPC marker A6 in a day 21 CDE-treated liver section confirms the presence of proliferating LPCs (Figure 4.16). Finally, increased expression of the Wnt/β-catenin pathway target genes CYP2E1 and LGR5 was evident in immunohistochemical stainings of day 21 CDE-treated compared to control livers (Figure 4.17).

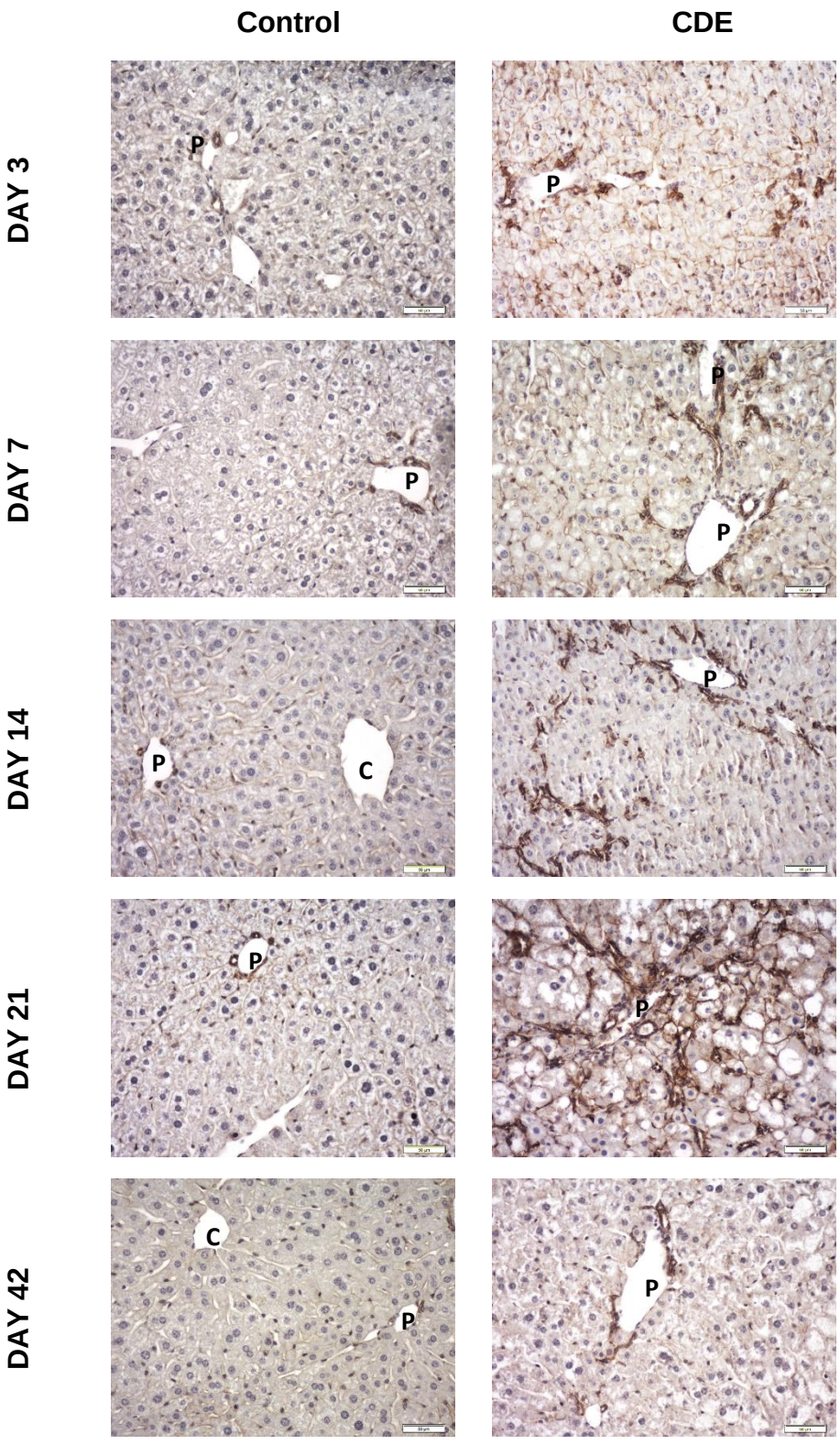


Figure 4.8: Expression of active β-catenin in FFPE control versus CDE liver tissues. Strings of active β-catenin⁺ cells can be seen penetrating the liver parenchyma in CDE-treated mice. In control mice, the staining is restricted to the bile ducts. The scale bar depicts 50 µm. C: central area, P: portal area.

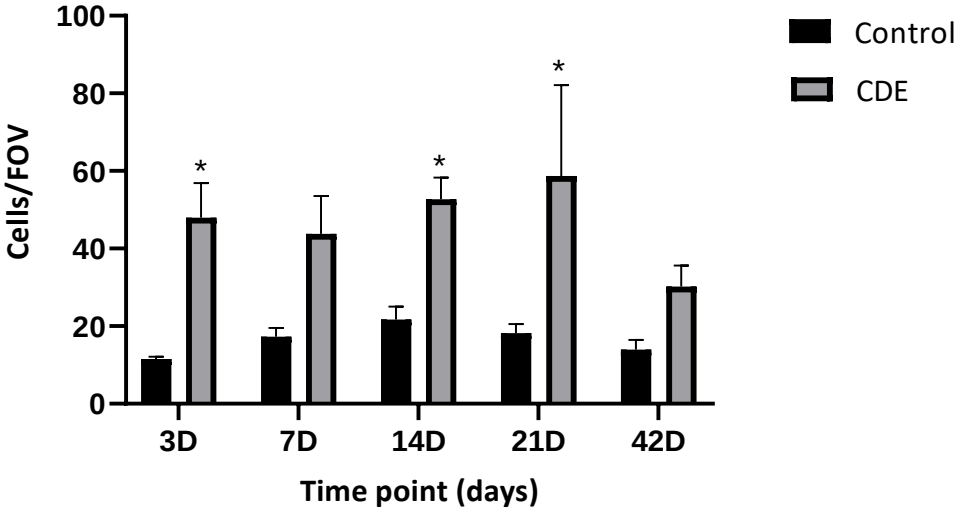


Figure 4.9: Quantitation of active β-catenin-positive cells in liver sections of mice on a control or CDE diet over a 42-day time course. Active β-catenin^{+} cells were significantly higher at days 3, 14 and 21 in CDE-treated livers compared to control livers. Data represent the mean ± SEM. *n*= 3-4 mice per group. * $P < 0.05$ compared to control mice at the same time point. FOV: Fields of view.

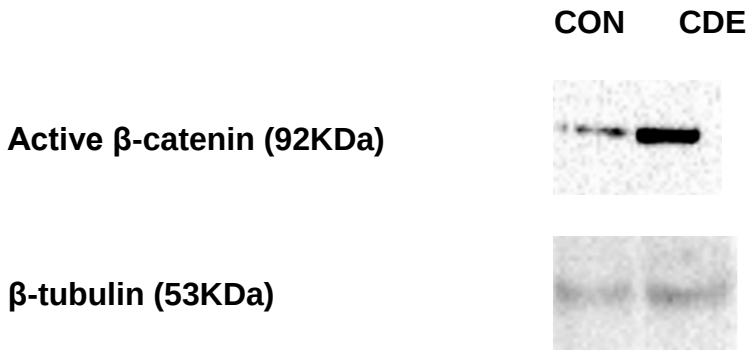


Figure 4.10: Presence of active β-catenin protein in control and CDE liver tissues at day 21. An increase in active β-catenin protein was evident in CDE diet-injured liver compared to control (CON) liver at day 21. *n*=1.

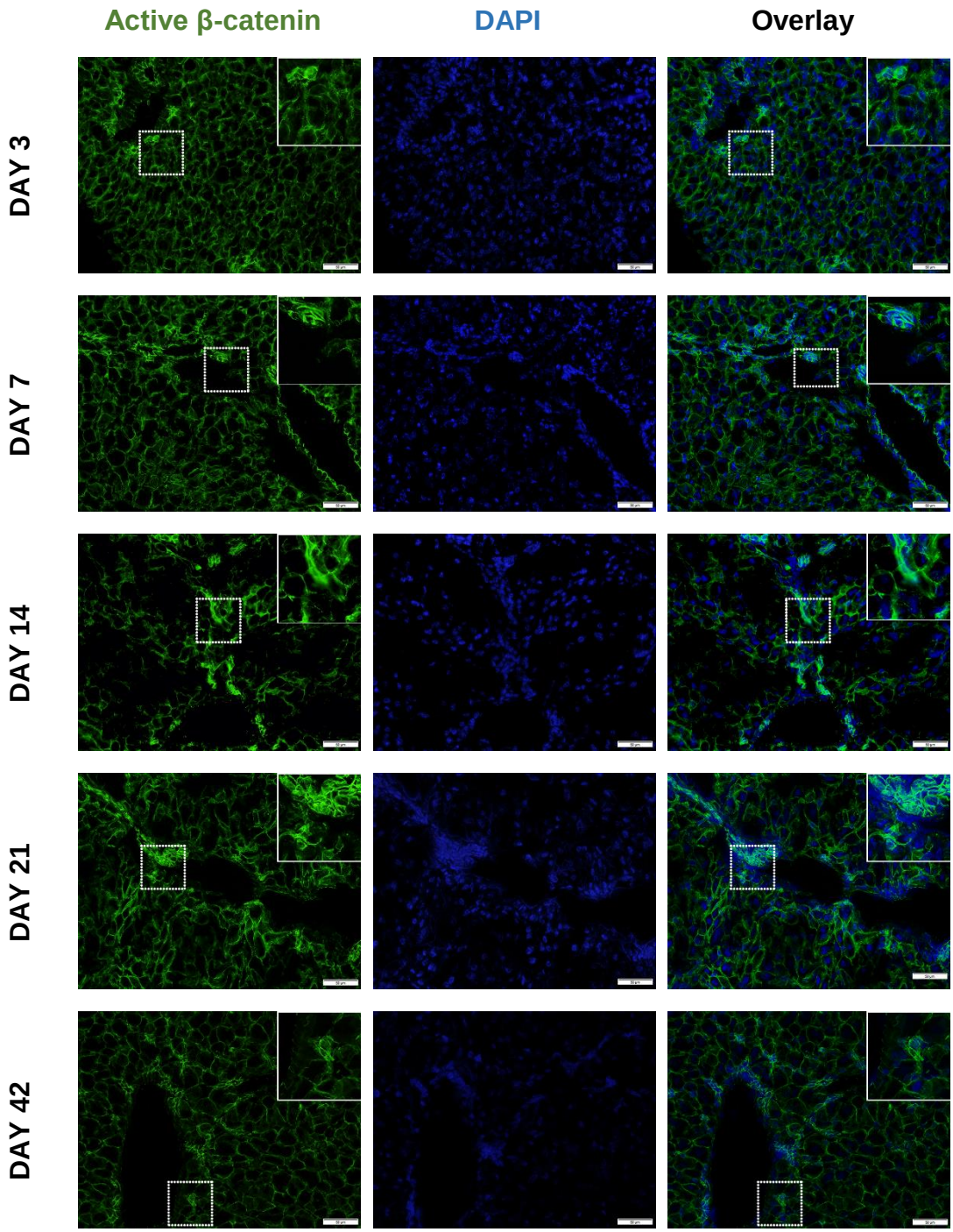


Figure 4.11: Intracellular localisation of active β-catenin in CDE-treated livers. Frozen liver sections from CDE-treated mice were labelled for active β-catenin using immunofluorescence to investigate the localisation of active β-catenin. Hepatocytes showed membranous, whereas, duct-like cells displayed strong cytoplasmic/nuclear active β-catenin^{+} staining. The scale bar depicts 50 µm.

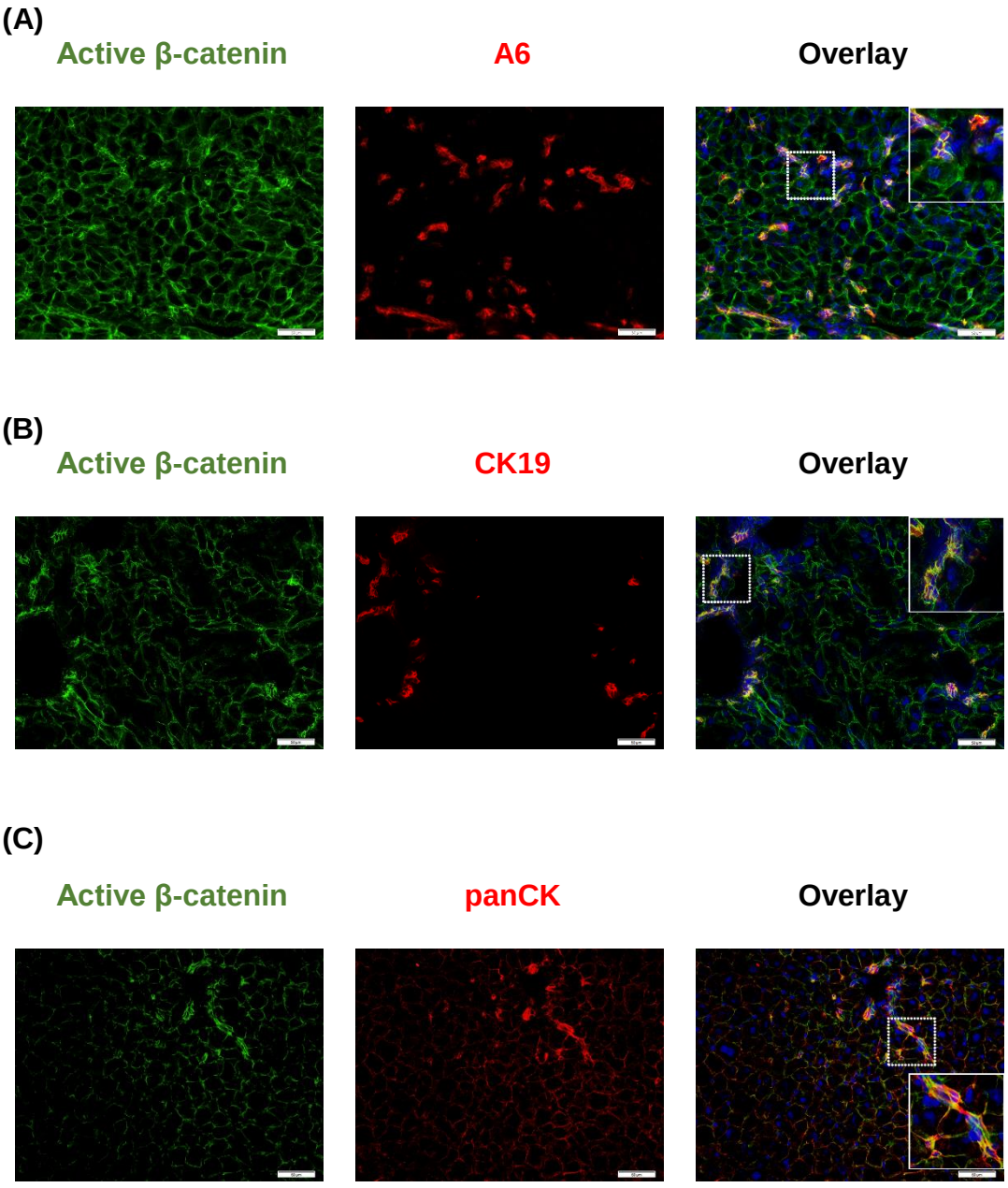


Figure 4.12: Expression of active β-catenin in LPCs. Frozen liver sections from day 21 CDE-treated mice were labelled for active β-catenin and the LPC markers A6 (A), CK19 (B) and panCK (C) to confirm the presence of active β-catenin in LPCs. Co-staining revealed the membranous localisation of active β-catenin in all LPCs and cytoplasmic/nuclear expression of active β-catenin in subset of LPCs. The scale bar depicts 50 μm.

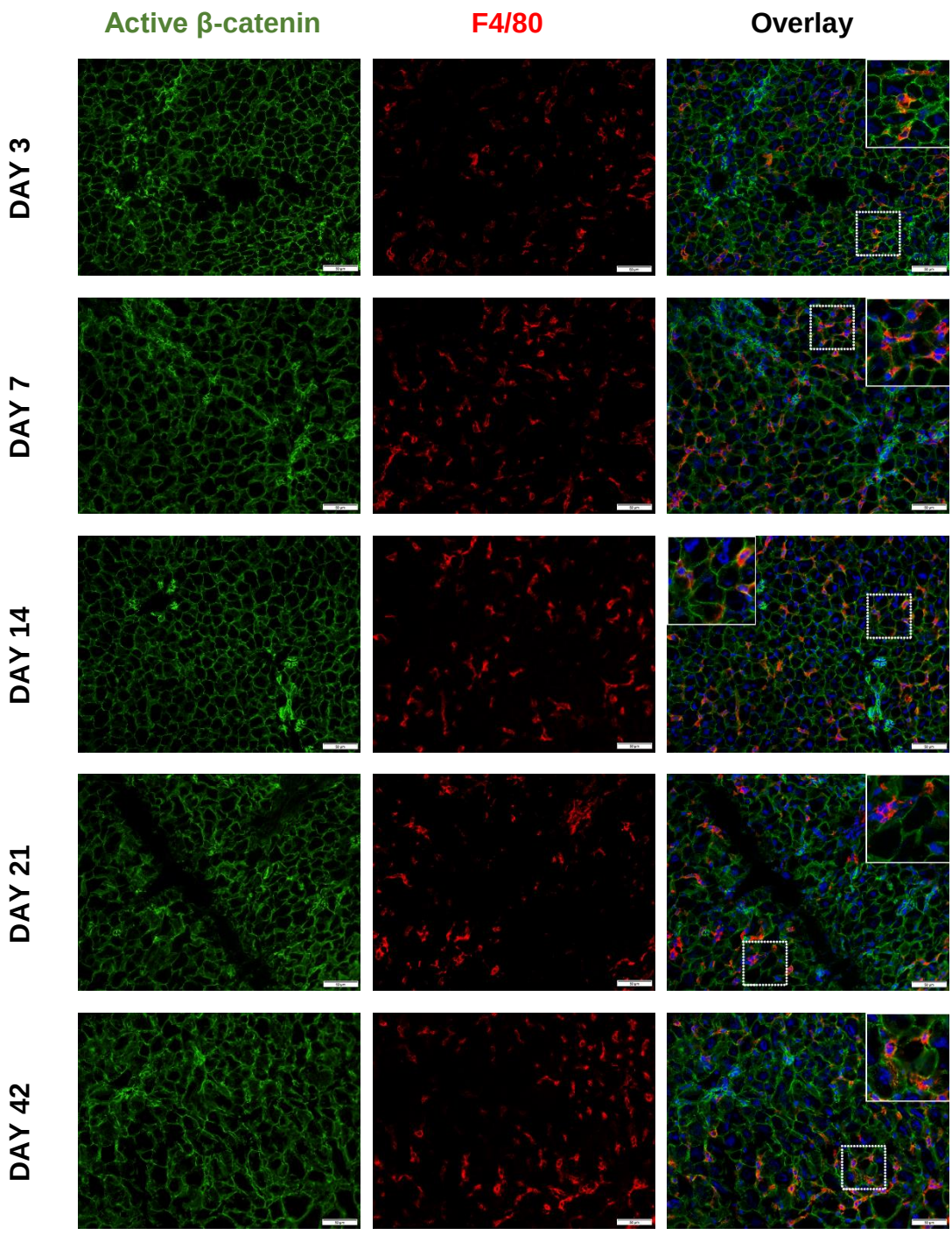


Figure 4.13: Active β-catenin expression in F4/80^{+} cells. Immunofluorescent double staining of frozen liver sections from CDE-treated mice for active β-catenin and F4/80 demonstrated membranous active β-catenin expression in subset of hepatic macrophages. The scale bar depicts 50 µm.

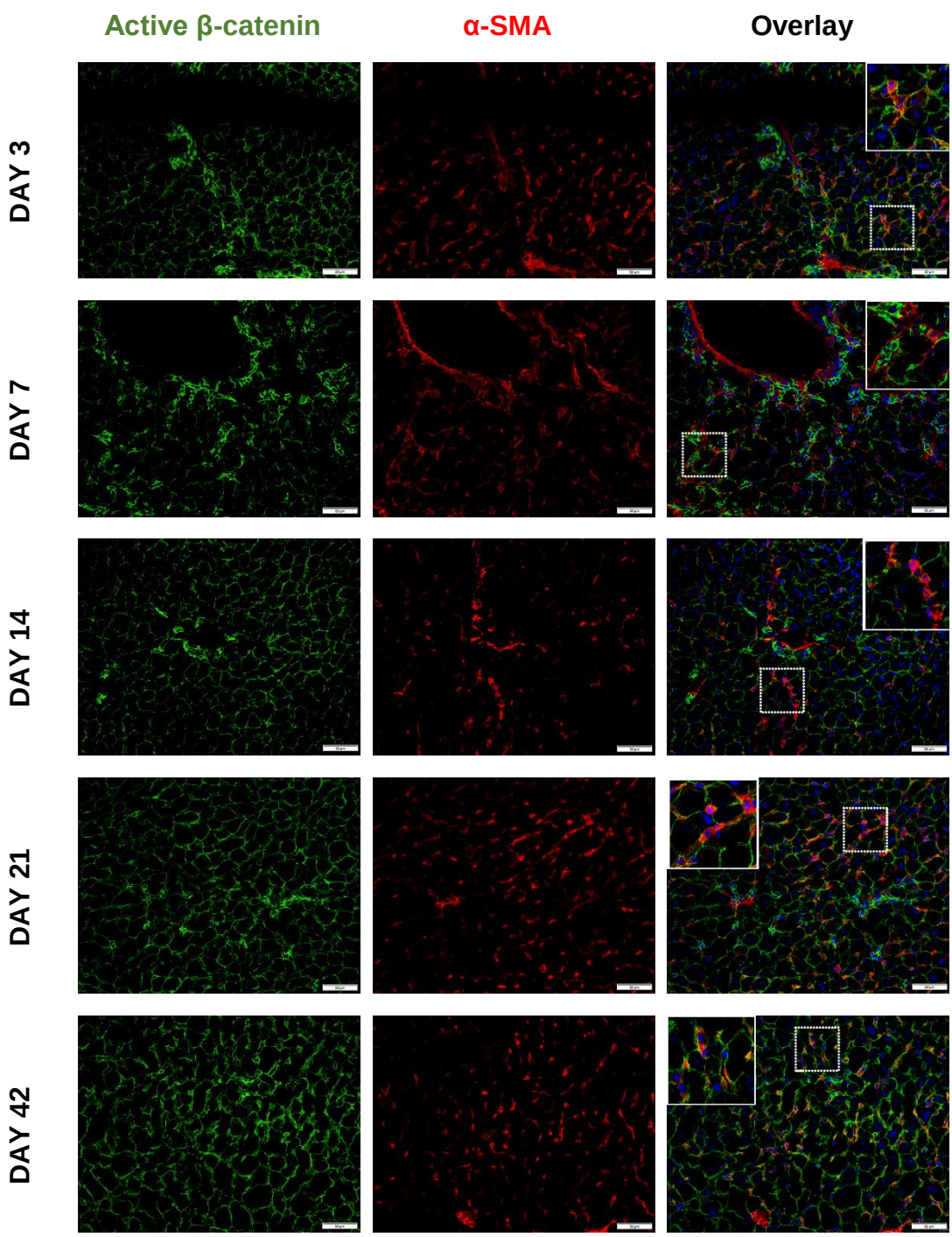


Figure 4.14: Active β-catenin expression in α-SMA$^+$ cells. Immunofluorescent double staining of frozen liver sections from CDE-treated mice for active β-catenin and α-SMA displayed membranous active β-catenin expression in subset of HSCs. The scale bar depicts 50 µm.

Scatter Plot

Test Group	Control Group	Fold Regulation Threshold
Group 1	Control Group	2

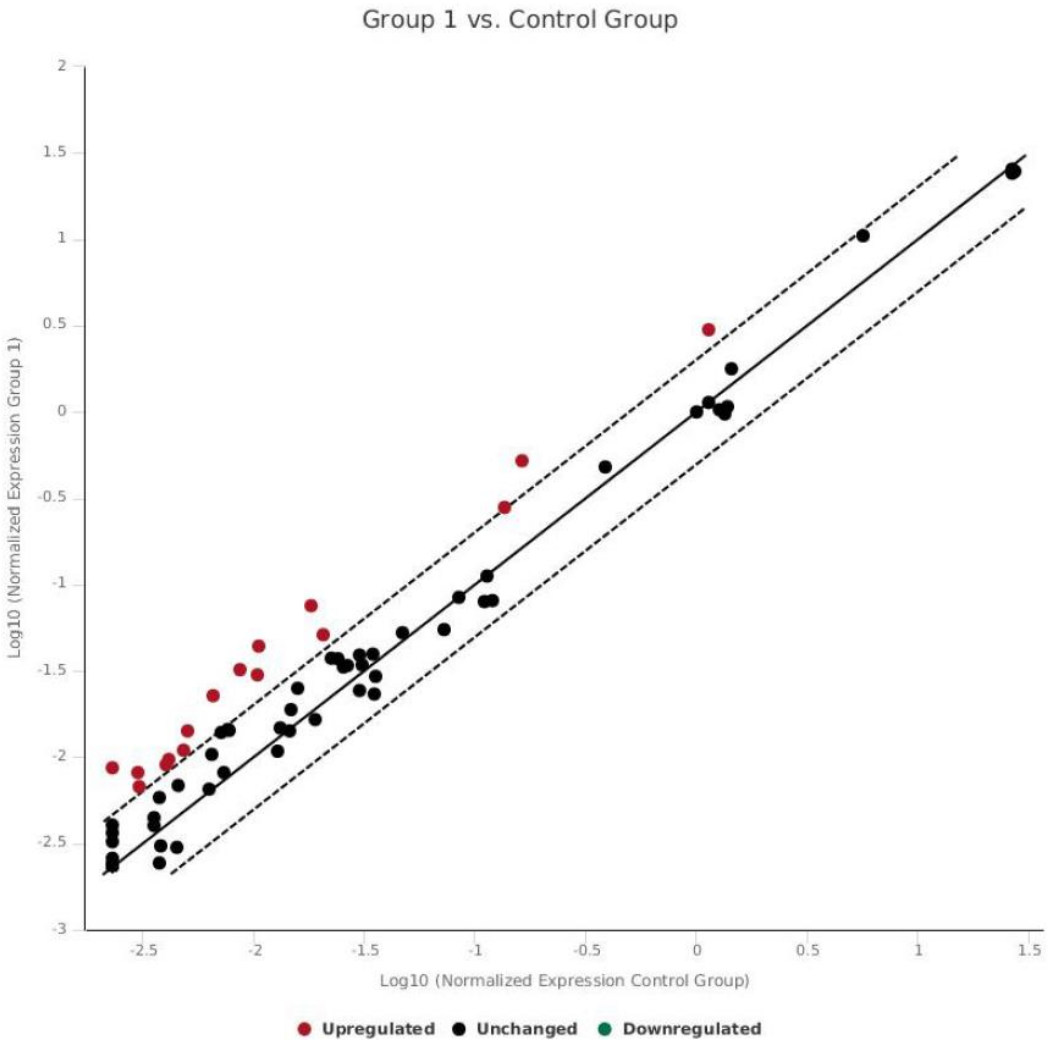


Figure 4.15: Wnt pathway expression at the transcription level in day 21 control versus day 21 CDE-injured liver tissue. RT-PCR Wnt array analysis revealed upregulation in 15 Wnt pathway-related genes in CDE-treated livers. Please refer to Table 4.1 on the next page for the list of upregulated genes. (Group 1: 21 day CDE diet-injured liver tissue, control group: 21 day control liver tissue).

Position	Gene Symbol	Fold Regulation
A06	Btrc	3.76
A07	Ccnd1	3.18
A08	Ccnd2	3.43
A10	Csnk2a1	4.14
A12	Ctnnb1	2.06
B01	Ctnnbip1	2.83
C04	Frzb	2.71
C05	Fzd1	2.25
C08	Fzd4	4.14
D01	Fzd9	2.27
D04	Kremen1	2.48
D11	Nfatc1	2.87
E03	Porcn	2.33
E08	Rhou	3.68
G06	Wnt5b	2.20
H03	Gapdh	2.62

Table 4.1: List of Wnt pathway upregulated genes in day 21 CDE-treated compared to day 21 control liver. (Csnk2a1: casein kinase 2, alpha 1 polypeptide, Fzd4: frizzled homolog 4, Btrc: beta-transducin repeat containing protein, Rhou: ras homolog gene family, member U, Ccnd2: cyclin D2, Ccnd1: cyclin D1, Nfatc1: nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1, Ctnnbip1: catenin beta interacting protein 1, Frzb: frizzled-related protein, Gapdh: glyceraldehyde-3-phosphate dehydrogenase, Kremen1: kringle containing transmembrane protein 1, Porcn: porcupine homolog, Fzd9: frizzled homolog 9, Fzd1: frizzled homolog 1, Wnt5b: Wingless-related MMTV integration site 5B, Ctnnb1: catenin (cadherin- associated protein), beta 1).

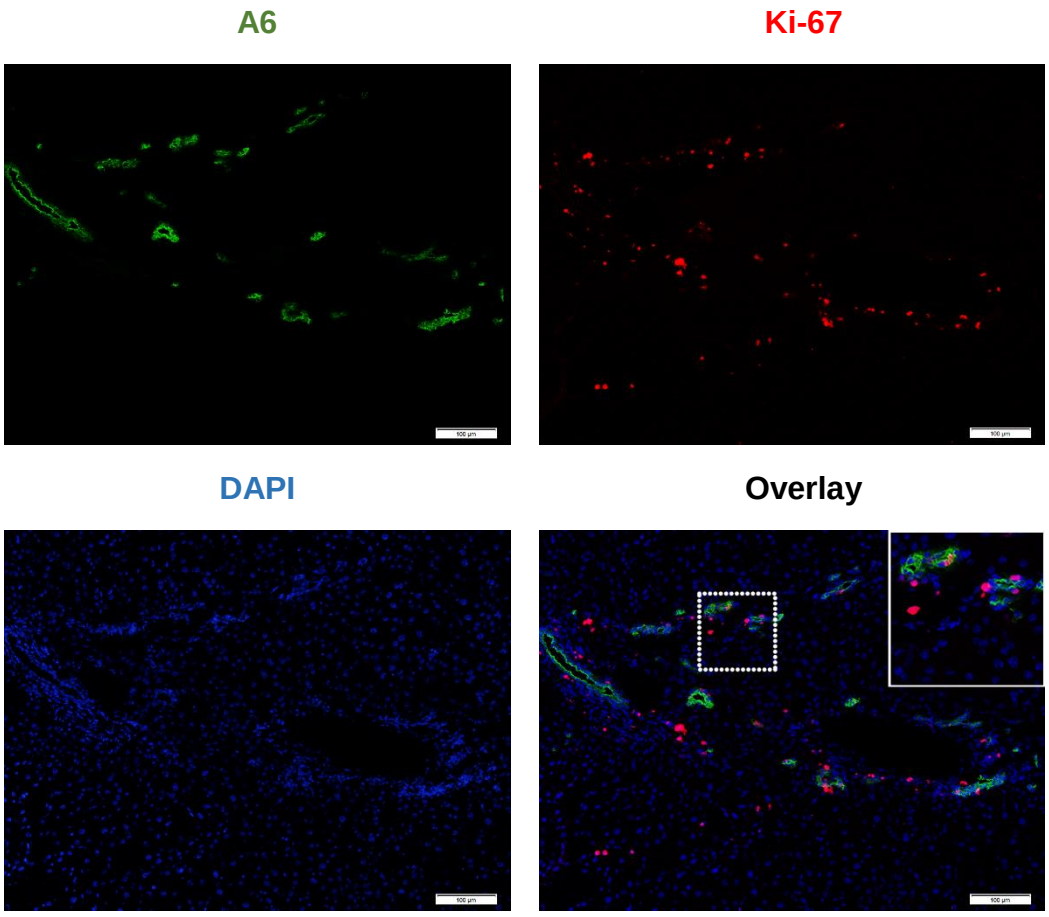


Figure 4.16: Double immunofluorescence staining of liver sections from day 21 CDE-treated mice for Ki-67 and A6. Proliferating LPCs confirmed by co-localisation of Ki-67 and A6. The scale bar depicts 100 µm.

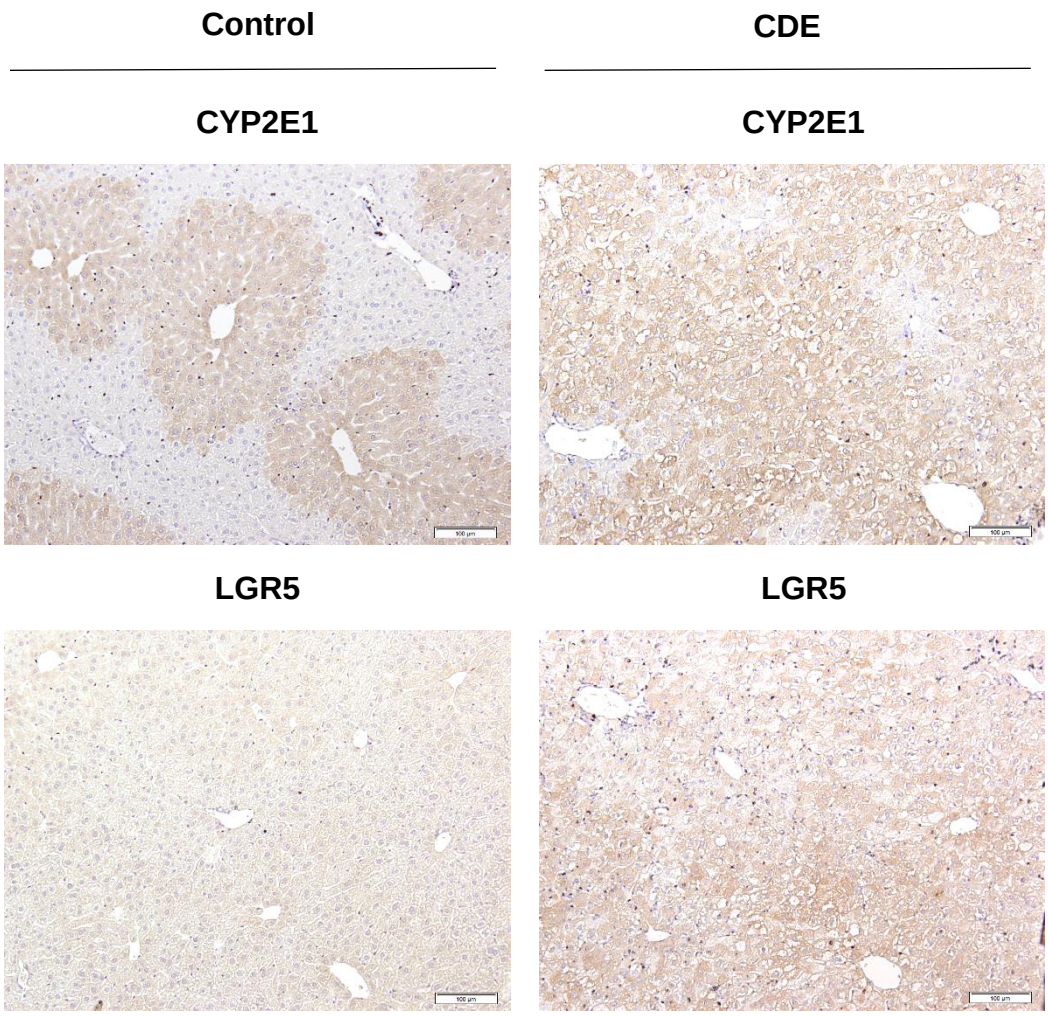


Figure 4.17: Expression of the Wnt/β-catenin pathway target genes CYP2E1 and LGR5 in control versus CDE liver tissues at day 21. Increased expression of the Wnt/β-catenin pathway target genes CYP2E1 and LGR5 in FFPE liver sections from 21-day CDE diet-fed animals was detected using immunohistochemistry. The scale bar depicts 100 µm.

4.5 Discussion

Animal models that can mimic human disease are an integral part of translational science. They provide valuable insights into understanding of the disease pathophysiology and facilitate discovery of novel therapeutic targets. The work presented in this chapter investigated the use of the CDE model to study NAFLD, LPC induction and the activation of the Wnt/β-catenin pathway in this murine model of CLD.

Upon CDE treatment, animals developed hepatic steatosis from as early as day 3, which changed from strong macro- to milder microvesicular steatosis after an initial adaptation period but remained detectable by Oil Red O staining. The number of $CD45^+$ and $F4/80^+$ inflammatory cells was increased at all time points in CDE-treated compared to healthy animals on a control diet. In addition, Sirius Red staining confirmed collagen deposition and fibrosis development in the CDE treatment group, which was particularly evident at day 21. Taken together, these results and a detailed study on CDE diet compared to thioacetamide administration carried out in our laboratory (Kohn-Gaone, Dwyer, et al., 2016a) suggest that the CDE diet reliably induces steatosis, inflammation and fibrogenic responses and is therefore considered a suitable model to study NAFLD-associated molecular and cellular processes. Another common feature of most human CLDs is the proliferation of LPCs as part of DRs. Importantly, their numbers directly correlate with the underlying disease severity (Lowes et al., 1999) making them a key cellular component of chronic injury progression. The CDE model is known to reliably activate the LPC compartment in portal areas, leading to the cells' proliferaration and migration into the liver parenchyma (Gogoi-Tiwari et al., 2017; Kohn-Gaone, Dwyer, et al., 2016a). Staining with the biliary cell/LPC markers panCK and A6 confirmed that LPCs were reproducibly activated in CDE-treated animals as early as day 3, with a peak in proliferation of $panCK^+$ cells detectable on day 21. The close temporal and spatial association of LPCs, HSCs and inflammatory cells in an injury and regeneration niche facilitates the exchange of various secreted cytokines and chemokines, functioning as mitogens, motogens or morphogens that direct LPC proliferation, migration and differentiation (Tirnitz-Parker et al., 2010; Van Hul et al., 2009; Williams, Clouston, & Forbes, 2014).

Wnt/β-catenin signalling plays an important role in liver development, homeostasis and repair. In contrast, abnormal activation of the pathway has been shown to play a role in liver pathogenesis (Perugorria et al., 2019). Wnt/β-catenin signalling is very complex, involving a plethora of ligands, receptors and co-factors. It is therefore important to understand the highly regulated activation status of the pathway in a disease setting before it can be utilised for potential therapeutic targeting. Thus, one of the aims of this chapter was to characterise the expression of the Wnt/β-catenin pathway in the context of the CDE model of chronic liver injury.

Canonical Wnt signalling activity is mostly evaluated through the expression and downstream effects of β-catenin, a central regulator of the pathway (Michalopoulos, 2013). In the absence of extracellular Wnts, cytoplasmic β-catenin is sequentially phosphorylated by CK1 on serine residue 45 and then by GSK3β on threonine residue 41 and serine residues 37 and 33, to prepare it for degradation (L. Wang, Liu, Gusev, Wang, & Fagotto, 2014). Hence, the form of β-catenin that is unphosphorylated at residues 33, 37 and 41 is considered an active form, which can trigger a downstream signalling cascade (Daugherty & Gottardi, 2007; Liu et al., 2002). In this study, immunohistochemical staining of active β-catenin in control and CDE-treated animals showed different expression patterns at all time points. Interestingly, it was observed that the distribution pattern of active β-catenin was similar to panCK expression. In addition, the number of active β-catenin^{+} cells peaked at day 21 as seen with panCK^{+} cells. Strong cytoplasmic/nuclear localisation of active β-catenin in a subset of cells was evident in all CDE-treated livers. By day 21, liver injury was well-established with substantial LPC proliferation present in the liver parenchyma. This time point was therefore selected for microarray studies focused on further, detailed Wnt/β-catenin pathway investigations. CDE-induced chronic liver injury led to significant upregulation of Wnt5b, Fz 1,4,9, β-catenin and cyclin D1,2 at the transcript level. Wnt5b upregulation has also been reported in hepatocytes harvested from methionine- and choline-deficient diet-treated mouse livers (Ju et al., 2020) and rats administered 2-acetylaminofluorene/carbon tetrachloride for 12 weeks (Chen et al., 2015). Although Wnt5b is traditionally considered a non-canonical Wnt (Zeng et al., 2007), the demonstrated upregulation of β-catenin

at the mRNA and protein level, cytoplasmic/nuclear localisation of active β-catenin as well as the transcriptional upregulation of the canonical Wnt pathway target cyclin D1 (Shtutman et al., 1999) confirm that the canonical Wnt/β-catenin signalling pathway is activated in CDE-treated livers. Earlier, Asgharpour et al. have reported an upregulation of a cell proliferation network involving β-catenin in a high-fructose/sucrose, high-fat Western diet model of NAFLD (Asgharpour et al., 2016). Moreover, increased expression of the Wnt target genes CYP2E1 and LGR5 (Huch et al., 2013; Tan, Behari, Cieply, Michalopoulos, & Monga, 2006) confirm the upregulation of the Wnt/β-catenin pathway with CDE treatment.

Previously, Boulter and colleagues have demonstrated that macrophage-induced Wnt/β-catenin signalling activation in LPCs direct their differentiation towards the hepatocytic lineage (Boulter et al., 2012). Moreover, in the macrophage-specific Wntless knockout mouse model, administration of hepatotoxic 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet promoted hepatobilliary injury through increased influx of inflammatory cells in conjunction with compromised hepatocyte proliferation (Jiang et al., 2019). Interestingly, a mouse model with deletion of Wntless in HSCs did not show any change in myofibroblast activation and the extent of fibrosis when subjected to carbon tetrachloride treatment (Zhang et al., 2019). In our CDE model, localisation of cytoplasmic/nuclear active β-catenin in a subset of cells, which express LPC markers confirms that LPCs signal through this pathway. $F4/80^+$ inflammatory cells and $\alpha\text{-}SMA^+$ HSCs were shown to express the membrane-bound active β-catenin, suggesting that inflammatory cells and HSCs are not regulated by canonical Wnt signalling at this point of chronic liver injury. This observation is particularly important as the antagonists used to manipulate the Wnt/β-catenin pathway activity, as described in Chapter 6, selectively affect LPCs in the CDE model.

Overall, the experiments presented in this chapter validated that (a) the CDE model of chronic liver injury is associated with upregulated Wnt/β-catenin signalling and (b) proliferating LPCs actively signal through this pathway.

Chapter 5

The analysis of Wnt/β-catenin signalling in a novel *in vitro* model of fat-loaded LPCs

5.1 Introduction

The previous chapter described the expression of LPCs and the Wnt/β-catenin pathway in the CDE model of chronic liver injury. The study of signalling pathways and interpretation of downstream effects in particular cell types can be difficult and complex in an *in vivo* situation. While mouse models provide insights into complex interplays of cells and allow us to mimic complex and dynamic microenvironments of the injured liver, it is often not possible to discern direct from indirect effects. Additionally, the presence of other non-parenchymal cells such as inflammatory cells and HSCs in close proximity to LPCs can affect pathway expression and modulation in LPCs. LPCs have recently been demonstrated to represent regulatory cells that can influence the fibrogenic status of neighbouring HSCs, suggesting their potential for therapeutic targeting (Gratte et al., 2021). However, for this potential to be realised, it is crucial to understand the key pathways that govern LPC behaviour in different injury microenvironments. While experiments described in Chapter 4 revealed that LPCs display the active form of β-catenin in their cytoplasm and nucleus when proliferating on the CDE diet, Chapter 5 will delineate the effects of lipids on Wnt/β-catenin signalling in LPCs in a NAFLD environment in isolation. Since there is currently no LPC-focussed *in vitro* model of steatosis available, it was necessary to establish and characterise a novel cell culture model for the *in vitro* studies of Wnt/β-catenin signalling effects in LPCs.

For this purpose, the well-characterised LPC line bipotential murine oval liver (BMOL)-tyrosine aminotransferase (TAT) was used. This clonal, spontaneously immortalised cell line was established from a long-term culture of CDE-induced LPCs, derived from transgenic mice carrying a TAT-glucocorticoid response element, allowing tracing of hepatocytic cell differentiation through β-galactosidase staining of TAT-expressing cells (Tirnitz-Parker, Tonkin, Knight, Olynyk, & Yeoh, 2007). BMOL-TAT cells express biliary and hepatocytic lineage markers, are non-transformed and display a typical epithelial-like phenotype (Tirnitz-Parker et al., 2007).

The role of FFAs in the pathogenesis of NAFLD is widely accepted. Dietary fatty acids, lipolysis of peripheral fat and *de novo* lipogenesis are the main

contributors of FFAs in fatty liver conditions (Chavez-Tapia, Rosso, & Tiribelli, 2011). The simplest approach to mimic the steatosis-like condition *in vitro* is treatment of cells with FFA. Various studies have reported increased levels of oleic, palmitic and palmitoleic acids in NAFLD patients (Feng et al., 2017; Gambino et al., 2016; Puri et al., 2009). Thus, palmitic and oleic acids are the most commonly used FFA to induce steatosis in cell culture systems. Palmitate is a saturated FFA, whereas oleate is a monounsaturated FFA (Mota, Banini, Cazanave, & Sanyal, 2016). It is well-documented in hepatocytic cell culture systems that oleate induces greater levels of steatosis but less apoptosis compared to palmitate (Gómez-Lechón et al., 2007; Malhi, Bronk, Werneburg, & Gores, 2006; Moravcová et al., 2015; Ricchi et al., 2009). Furthermore, a study by Joshi-Barve *et al*. reported that exposure of hepatic cells to palmitic acid resulted in increased expression of the pro-inflammatory cytokine IL-8 (Joshi-Barve et al., 2007). Additionally, when HSCs were exposed to the conditioned medium from steatotic hepatocytes, a significant upregulation in transcript levels of collagen type I was observed (Wobser et al., 2009). These data highlight the utility of *in vitro* models of lipid loading to understand the responses of particular cell types in steatotic conditions. To date, LPCs have not been studied in this context.

Wnt/β-catenin signalling is a key pathway for LPC-mediated liver regeneration (Lade & Monga, 2011; Yang et al., 2008a). Apart from regeneration, it has been well studied in metabolic disorders. Wnt signalling has been shown to regulate the process of adipogenesis (Chen & Wang, 2018; Visweswaran et al., 2015) and various studies have reported elevated levels of Wnt5a in obese and NAFLD patients (Catalan et al., 2014; Liang et al., 2017). Recently, a study carried out by Cabrae and colleagues revealed that the Wnt/β-catenin pathway in hepatocytes can be controlled by insulin via increase in stearoyl-CoA-desaturase 1 which leads to an activation of porcupine, a protein involved in activation and secretion of Wnts (Cabrae et al., 2020). These observations highlight the relevance of studies on Wnt/β-catenin signalling in *in vitro* models of steatosis.

5.2 Study aims

The first aim of this chapter was to establish an *in vitro* model of steatotic LPCs using BMOL-TAT cells treated with the two most common FFAs: palmitate and oleate. The second aim was to study the expression of Wnt/β-catenin pathway components in the palmitate- and oleate-treated BMOL-TAT cells.

5.3 Methodology

The LPC line BMOL-TAT was maintained in the maintenance medium (2.1.3.13). BMOL-TAT cells were cultured in the maintenance medium for at least three passages prior to further experiments. After the third passage, BMOL-TAT cells were treated with trypsin/EDTA (2.1.3.14) at 80% confluency and seeded at a density of 1,500 cells/100 µl per well in a 96-well plate. After 24 hours, the cells were subjected to different concentrations of palmitate (2.1.3.15) and oleate (2.1.3.16) for 72 hours with a medium change on every alternate day. BMOL-TAT cells cultured in maintenance medium were used as the control. Three technical replicates were included for each treatment concentration. MTT assays (2.2.3.6) were performed at the end of 72 hours to study the effect of various concentrations of palmitate and oleate on cell viability. MTT assays were repeated three times as per the above procedure. Results from the MTT assay determined the optimal working concentration of palmitate and oleate to be used in further experiments. The induction of steatosis in BMOL-TAT cells for the selected concentration of palmitate and oleate was confirmed using Oil Red O staining.

RNA extraction from three consecutive control, palmitate- and oleate-treated BMOL-TAT passages was performed for RT-PCR microarray analysis to evaluate the expression of Wnt/β-catenin signalling pathway components at the transcript level. Protein extraction from three consecutive control, palmitate-and oleate-treated BMOL-TAT cells was carried out to study the changes in the expression of Wnt/β-catenin pathway components at the protein level using western blot analysis. Finally, control, palmitate- and oleate-treated BMOL-TAT cells were cultured on etched coverslips and subjected to immunofluorescent staining to analyse the intracellular location of the active β-catenin protein.

5.4 Results

5.4.1 Induction of steatosis in BMOL-TAT cells

The aim of these experiments was to optimise the working concentrations of palmitate and oleate to achieve intracellular fat accumulation with minimal cytotoxicity. The viability of BMOL-TAT cells at different concentrations of palmitate and oleate was investigated by MTT assay after 72 hours of the respective FFA treatment. The results show that the higher concentrations of palmitate (200 µM, 100 µM and 50 µM) induced cytotoxic effects (Figure 5.1). More than 50% of BMOL-TAT cells were able to tolerate 25 µM and 12.5 µM of palmitate treatment for 72 hours. However, the viability was still significantly reduced compared to the control. The cell viability between the control and the palmitate concentration at 6.25 µM was not significantly different. Therefore, 6.25 µM of palmitate was selected for further experiments. On the other hand, with oleate treatment, no significant difference in cell viability was observed between control and any concentration of oleate (Figure 5.2). BMOL-TAT cells were able to tolerate all oleate doses for 72 hours. Hence, 100 µM was established as the final oleate working concentration for future experiments.

Oil Red O staining was performed on BMOL-TAT cells exposed to control medium, 6.25 µM palmitate and 100 µM oleate for 72 hours to confirm lipid accumulation with the selected palmitate and oleate concentrations (Figure 5.3).

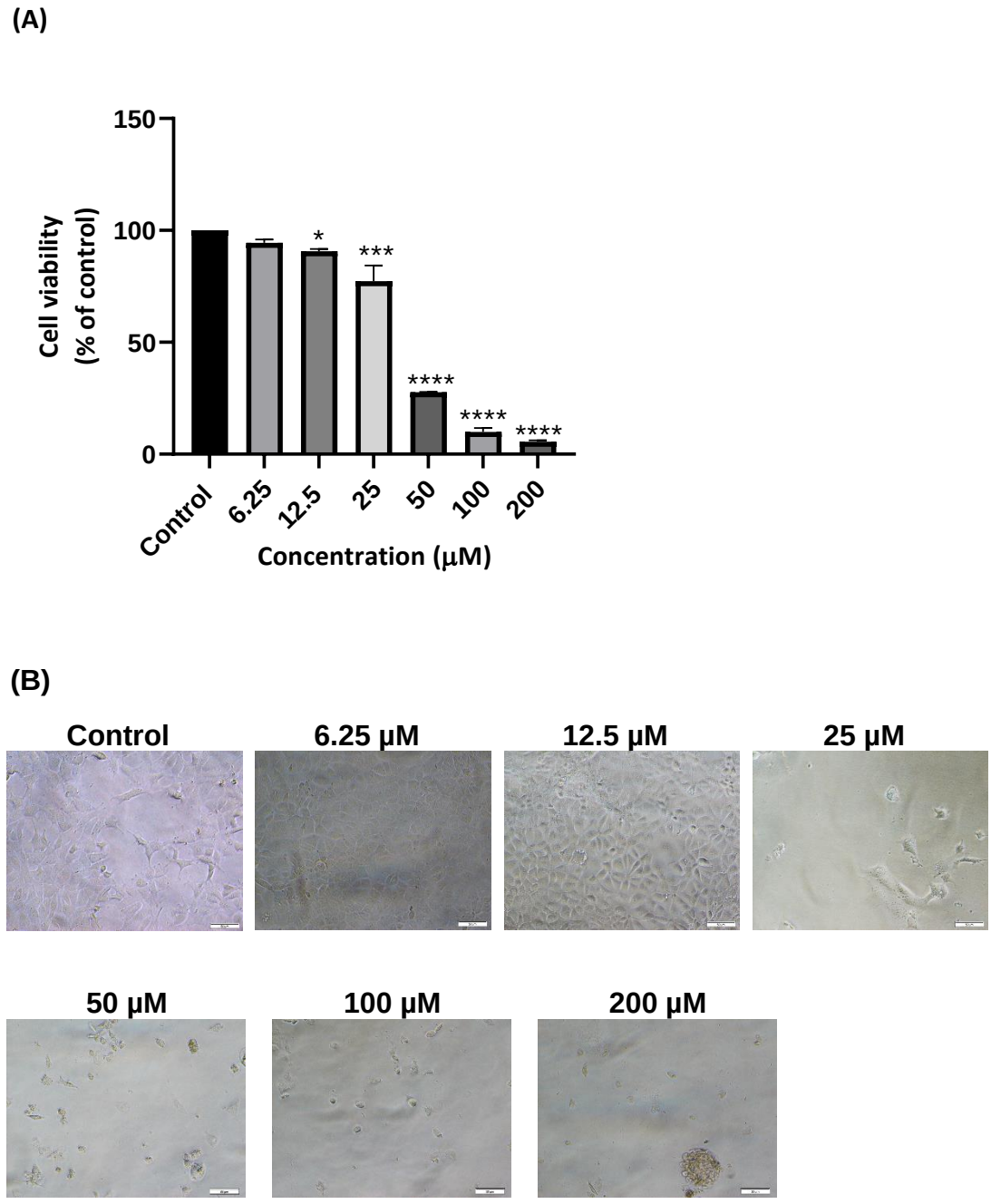
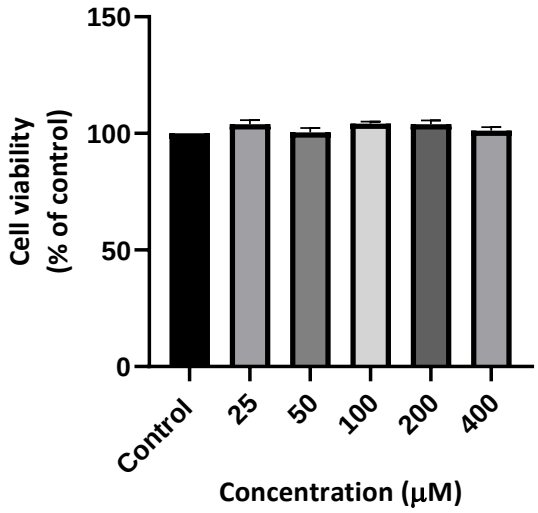


Figure 5.1: Effect of increasing palmitate concentrations on BMOL-TAT viability. BMOL-TAT cells were incubated with increasing concentrations of palmitate for 72 hours. BMOL-TAT cells incubated in normal maintenance medium for 72 hours served as control. (A) MTT assay revealed a significant dose-dependent decrease in cell viability with palmitate treatment. (B) Photomicrographs of control BMOL-TAT culture and BMOL-TAT cultures treated with different concentrations of palmitate. The scale bar depicts 50 µm. Data represent the mean ± SEM. n= 3. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 compared with controls.

(A)



(B)

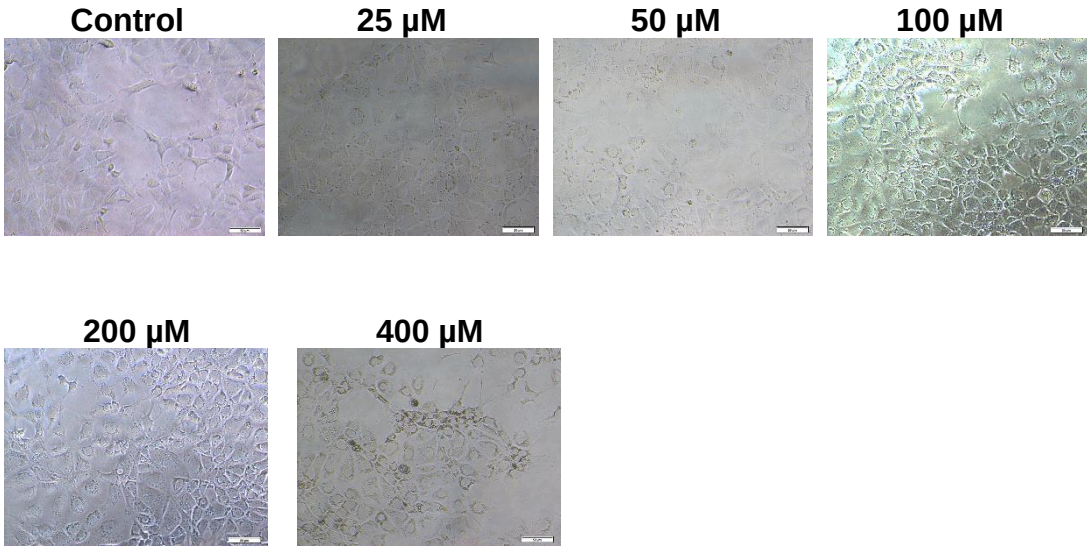


Figure 5.2: Effect of increasing oleate concentrations on BMOL-TAT viability. BMOL-TAT cells were incubated with increasing concentrations of oleate for 72 hours. BMOL-TAT cells incubated in normal maintenance medium for 72 hours served as control. (A) No significant effect on cell viability was observed with oleate treatment. (B) Photomicrographs of control BMOL-TAT culture and BMOL-TAT cultures treated with different concentrations of oleate. The scale bar depicts 50 µm. Data represent the mean ± SEM. *n*= 2.

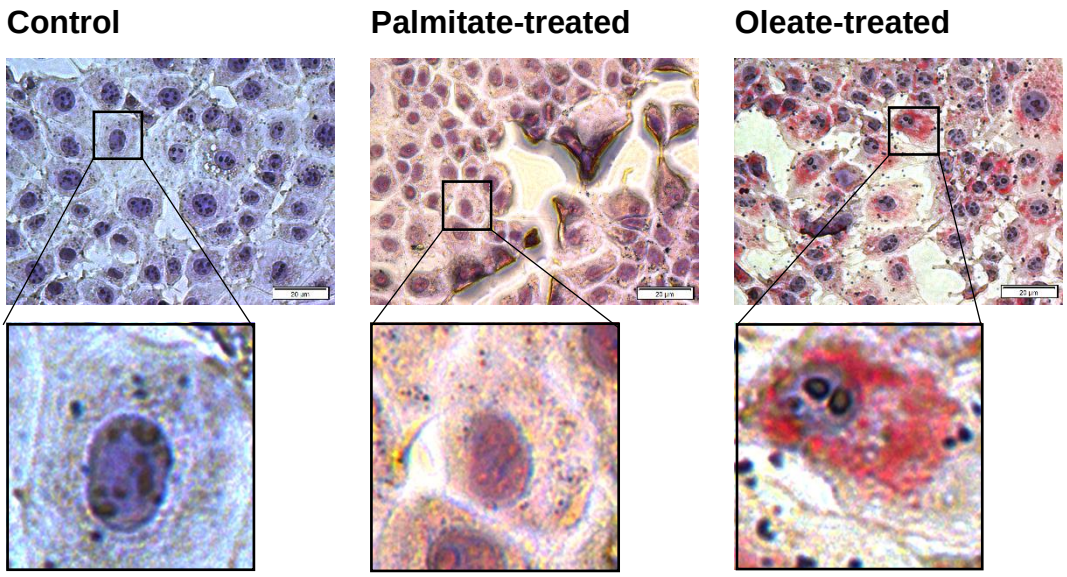


Figure 5.3: Oil Red O staining. Oil Red O staining performed on control, palmitate-treated (6.25 µM) and oleate-treated (100 µM) BMOL-TAT cells confirmed the lipid deposition in FFA-treated cells. The scale bar depicts 20 µm.

5.4.2 Expression of Wnt/β-catenin pathway components in palmitate- and oleate-treated BMOL-TAT cells

RT-PCR microarray analysis carried out on untreated and FFA-treated BMOL-TAT cells revealed that the palmitate and oleate treatments altered the expression of Wnt/β-catenin pathway components. Palmitate treatment led to a significant upregulation of Wnt1, Fz receptor 7 and axin1, whereas, Fz receptors 5, 6 and co-receptor LRP5 were significantly downregulated (Figure 5.4-5.7). On the other hand, Wnt7a and Wnt9a, Fz receptors 1 and 7, LRP5, Dvl1, axin1 and β-catenin were significantly upregulated at the mRNA level with oleate treatment, while Wnt5a and Wnt10a, Fz receptors 5 and 6, DKK1 and DKK3, WIF 1, sFRP 1, 3 and 4 were significantly downregulated in oleate-treated BMOL-TAT cells (Figure 5.8-5.11).

Next, the expression of some Wnt/β-catenin pathway proteins was detected by Western blot analysis. Palmitate treatment slightly increased total GSK3β and cyclin D1 protein expression; however, no effect on the expression of Dvl2, LRP6, total β-catenin and active β-catenin proteins was observed (Figure 5.12). In contrast, oleate treatment led to a slight increase in active β-catenin, Dvl2 and LRP6 proteins, while GSK3β, total β-catenin and cyclin D1 expressions remained unchanged (Figure 5.13).

Active β-catenin localisation was mostly membranous in untreated BMOL-TAT cells, whereas, exposure to palmitate and oleate treatment induced strong cytoplasmic/nuclear localisation of active β-catenin in a subpopulation of BMOL-TAT cells (Figure 5.14 and Figure 5.15).

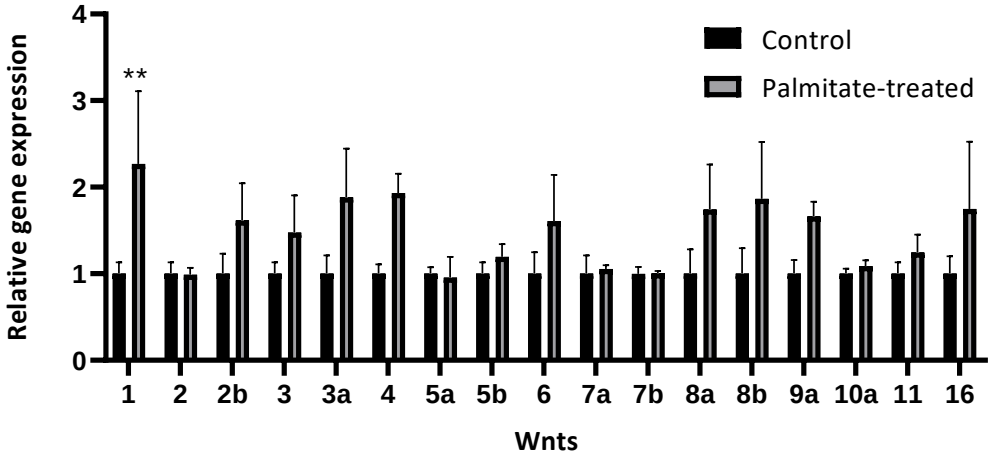


Figure 5.4: Effect of palmitate treatment on the expression of Wnts. Palmitate treatment led to significant upregulation in Wnt1. The data are represented as relative fold change in gene expression in treatment samples compared to controls. Data represent the mean ± SEM. *n*= 3. * $P < 0.05$, ** $P < 0.01$ compared with controls.

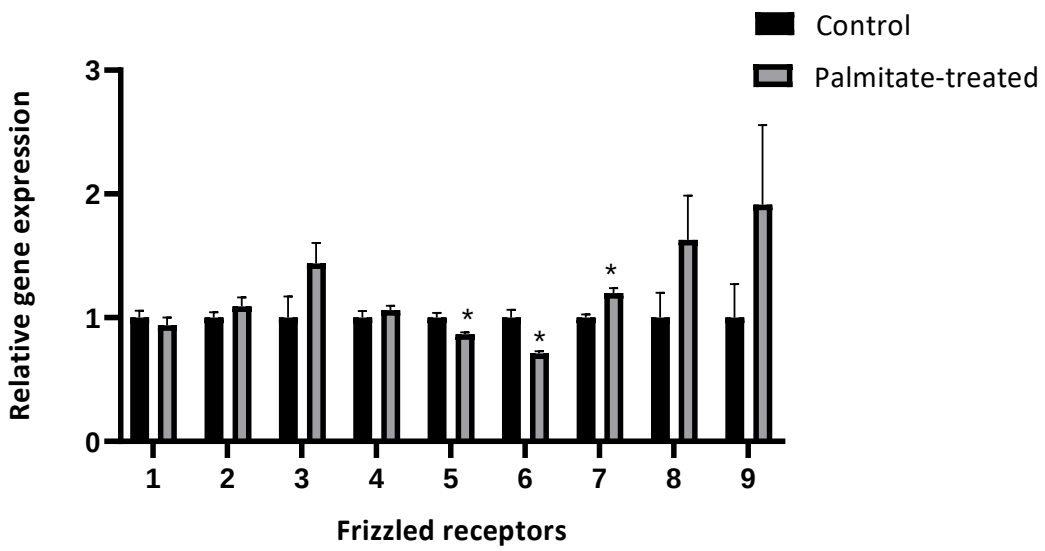


Figure 5.5: Effect of palmitate treatment on the expression of Fz receptors. Palmitate treatment led to significant downregulation in Fz receptors 5 and 6 and significant upregulation in Fz receptor 7. The data are represented as relative fold change in gene expression in treatment samples compared to control. Data represent the mean ± SEM. *n*= 3. * *P* < 0.05 compared with controls.

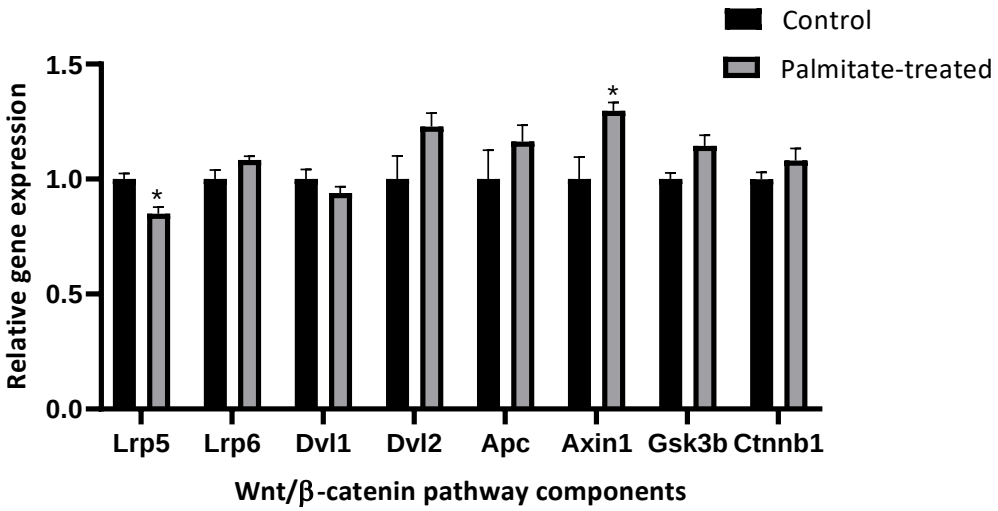


Figure 5.6: Effect of palmitate treatment on the expression of Wnt/β-catenin pathway components. Significant downregulation in LRP5 and significant upregulation in axin1 was observed with palmitate treatment. The data are represented as relative fold change in gene expression in treatment samples compared to control. Data represent the mean ± SEM. *n*= 3.
* *P* < 0.05 compared with controls.

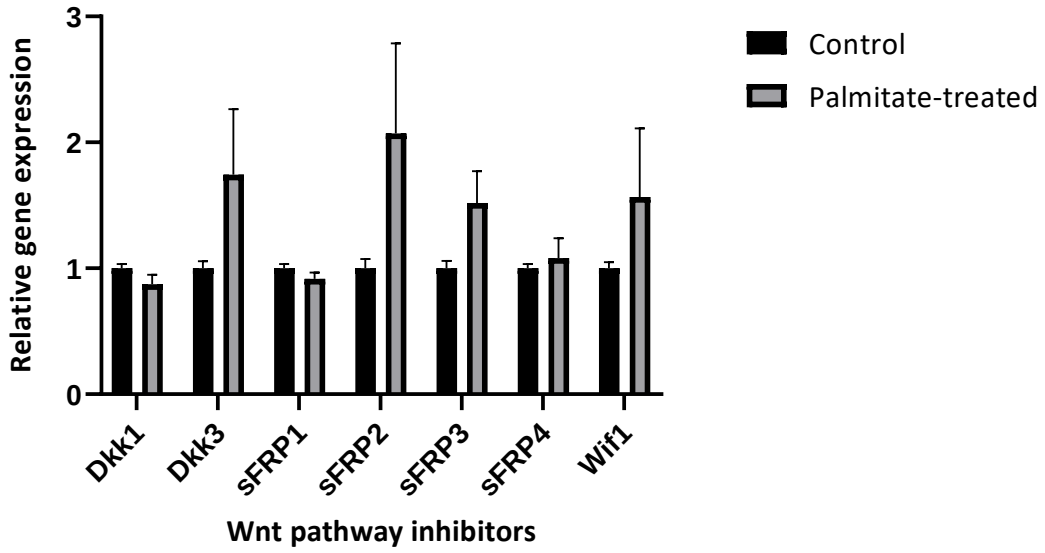


Figure 5.7: Effect of palmitate treatment on the expression of Wnt signalling pathway inhibitors. No significant effect on the expression of pathway inhibitors was observed with palmitate treatment. The data are represented as relative fold change in gene expression in treatment samples compared to control. Data represent the mean ± SEM. *n*= 3.

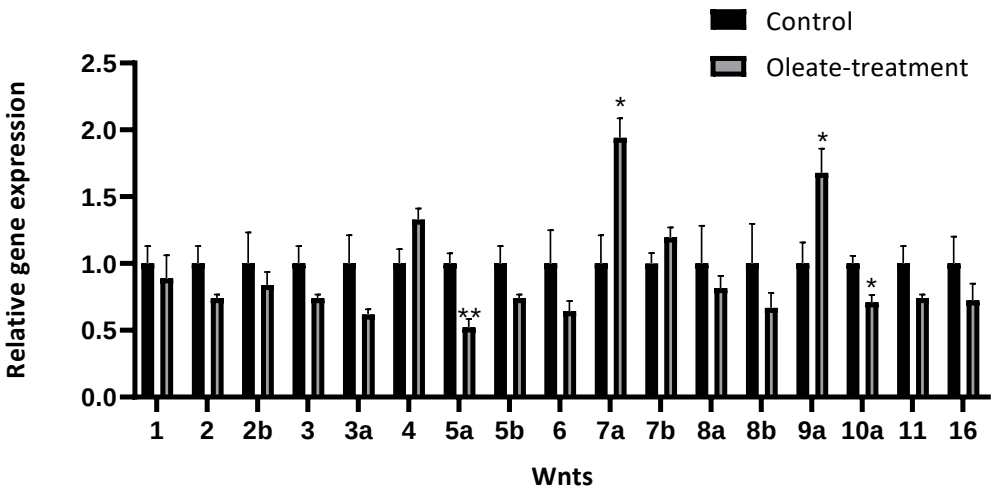


Figure 5.8: Effect of oleate treatment on the expression of Wnts. Oleate treatment led to significant downregulation in Wnt5a, Wnt10a and significant upregulation in Wnt7a and Wnt9a. The data are represented as relative fold change in gene expression in treatment samples compared to controls. Data represent the mean ± SEM. *n*= 3. * *P* < 0.05, ** *P* < 0.01 compared with controls.

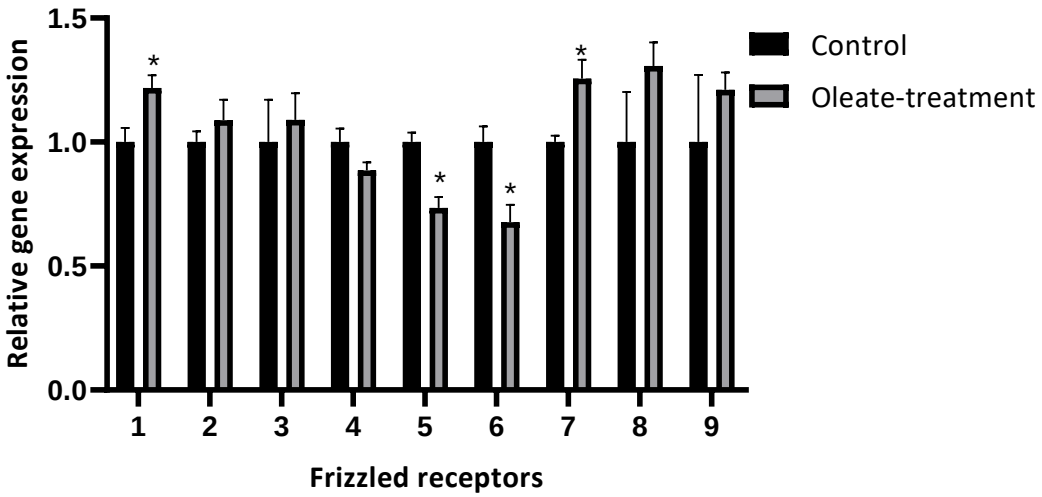


Figure 5.9: Effect of oleate treatment on the expression of Fz receptors. Oleate treatment led to significant upregulation in Fz receptors 1 and 7 and significant downregulation in Fz receptors 5 and 6. The data are represented as relative fold change in gene expression in treatment samples compared to control. Data represent the mean ± SEM. *n*= 3. * *P* < 0.05 compared with controls.

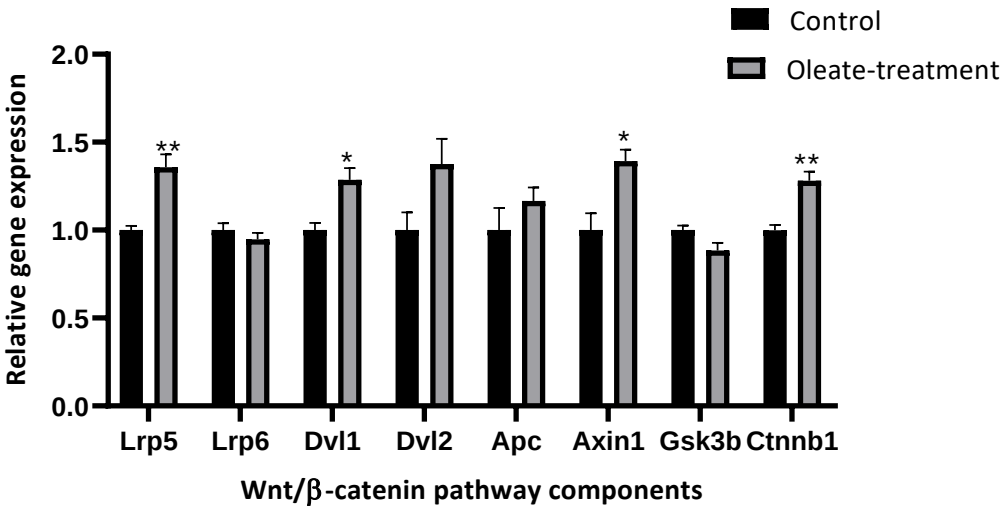


Figure 5.10: Effect of oleate treatment on the expression of Wnt/β-catenin pathway components. Significant upregulation in LRP5, Dvl1, Axin1 and β-catenin (Ctnnb1) was observed with oleate treatment. The data are represented as relative fold change in gene expression in treatment samples compared to control. Data represent the mean ± SEM. *n*= 3. * $P < 0.05$, ** $P < 0.01$ compared with controls.

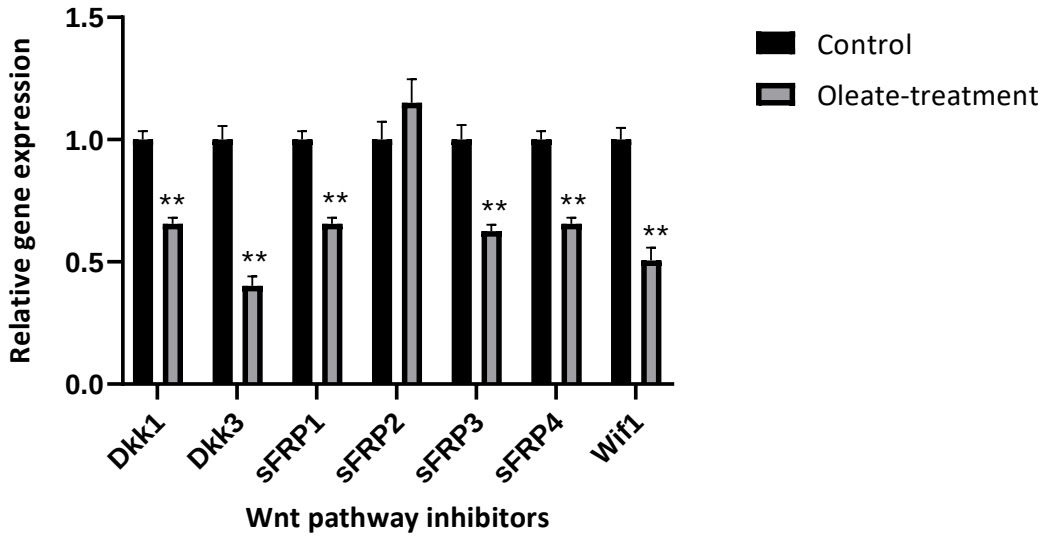


Figure 5.11: Effect of oleate treatment on the expression of Wnt signalling pathway inhibitors. Oleate treatment led to significant downregulation in the expression of DKK1, DKK3, sFRP1, sFRP3, sFRP4 and WIF1. The data are represented as relative fold change in gene expression in treatment samples compared to control. Data represent the mean ± SEM. *n*= 3. * $P < 0.05$, ** $P < 0.01$ compared with controls.

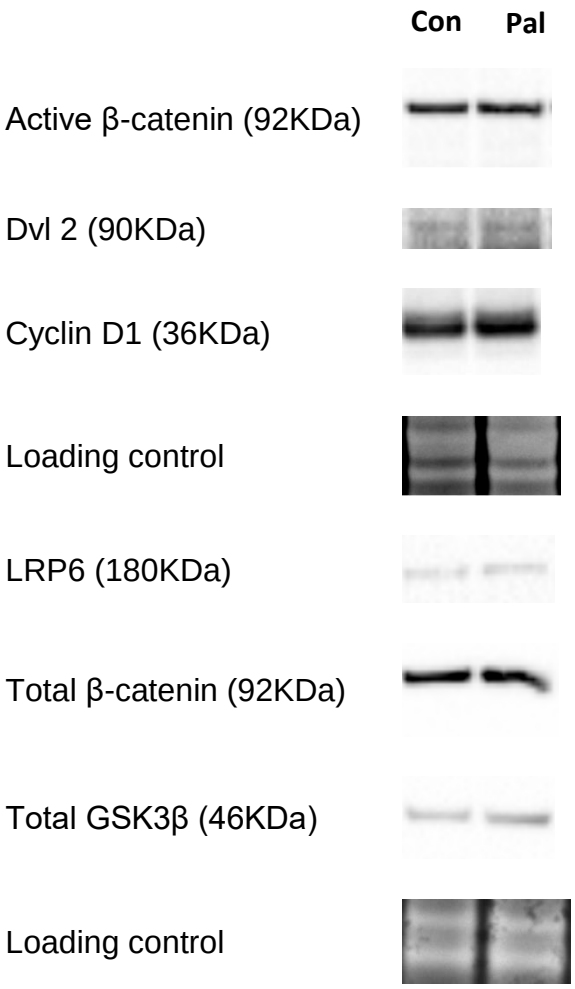


Figure 5.12: Expression of Wnt/β-catenin pathway components in palmitate-treated BMOL-TAT cells at the protein level. Shown here is representative western blot image for components of the Wnt/β-catenin pathway in control and treatment groups. A slight increase in total GSK3β and cyclin D1 proteins was observed with palmitate treatment. *n*=3. Con: control, Pal: palmitate treatment. Revert total protein stain was used as loading control.

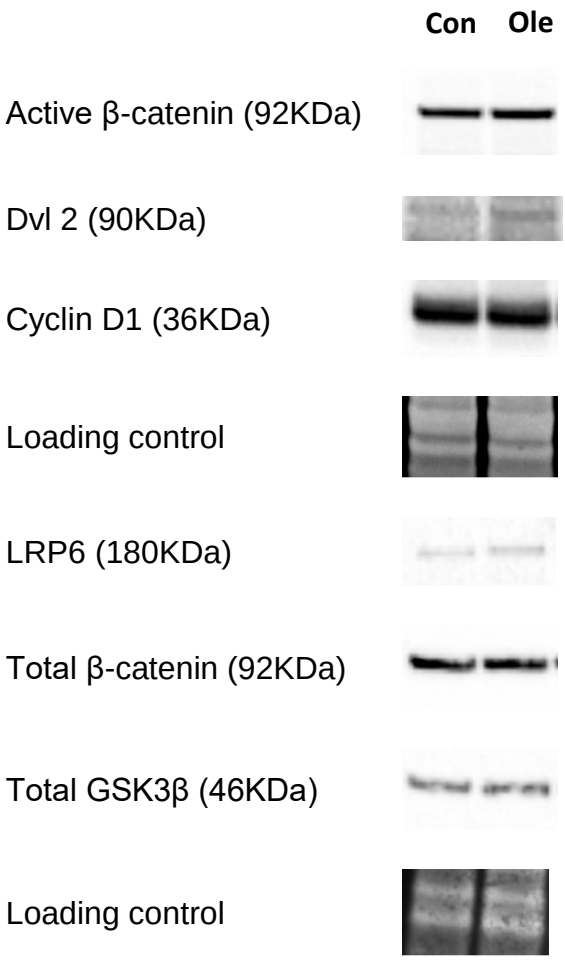


Figure 5.13: Expression of Wnt/β-catenin pathway components in oleate-treated BMOL-TAT cells at the protein level. Shown here are representative western blot images for components of the Wnt/β-catenin pathway in control and treatment groups. A slight increase in active β-catenin, Dvl2 and LRP6 proteins was noticed with oleate treatment. *n*=3. Con: control, Ole: oleate treatment. Revert total protein stain was used as loading control.

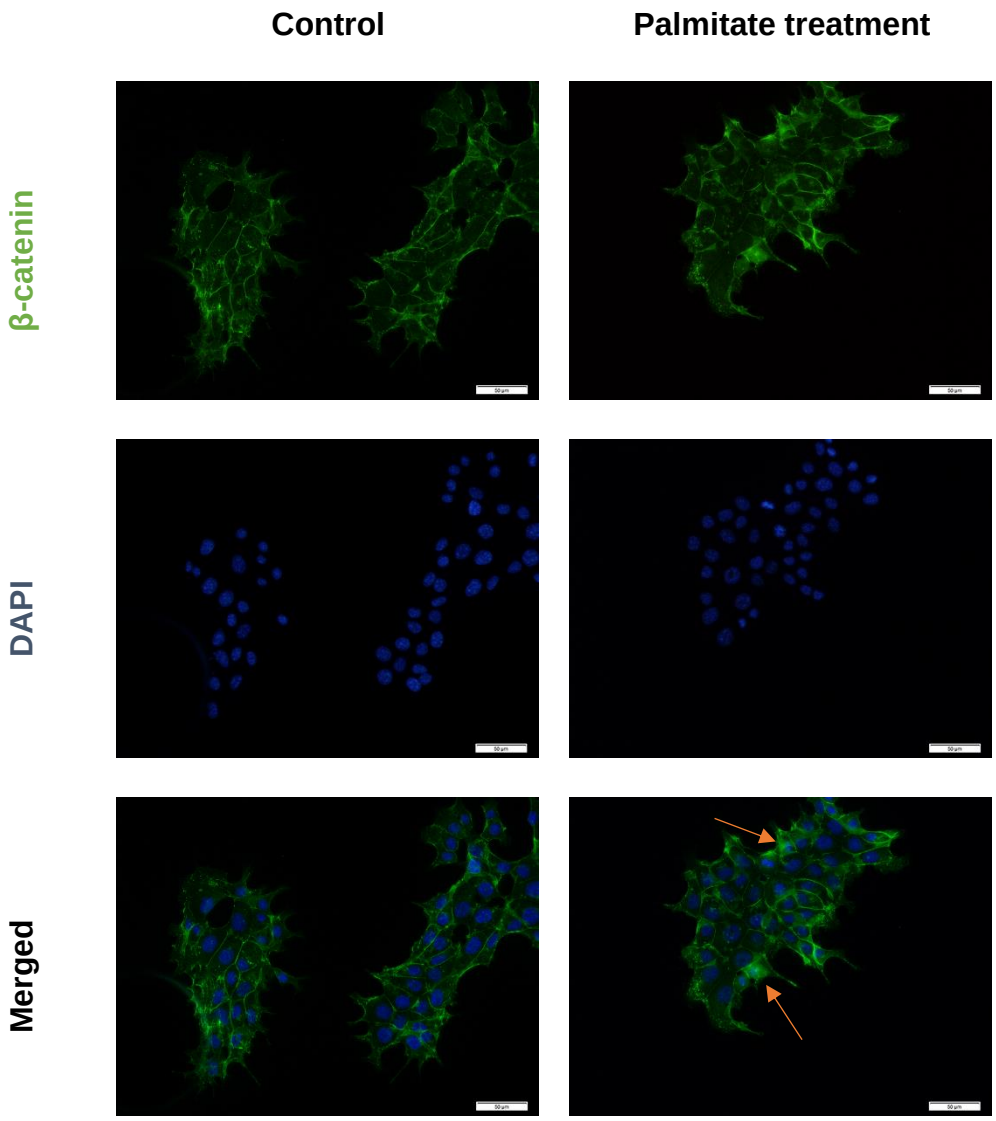


Figure 5.14: Expression of active β-catenin in palmitate-treated BMOL-TAT cells. The localisation of active β-catenin is mostly membranous in untreated control cells, whereas strong cytoplasmic and nuclear active β-catenin staining is evident in a subset of palmitate-treated cells, as indicated by arrows. The scale bar depicts 50 µm.

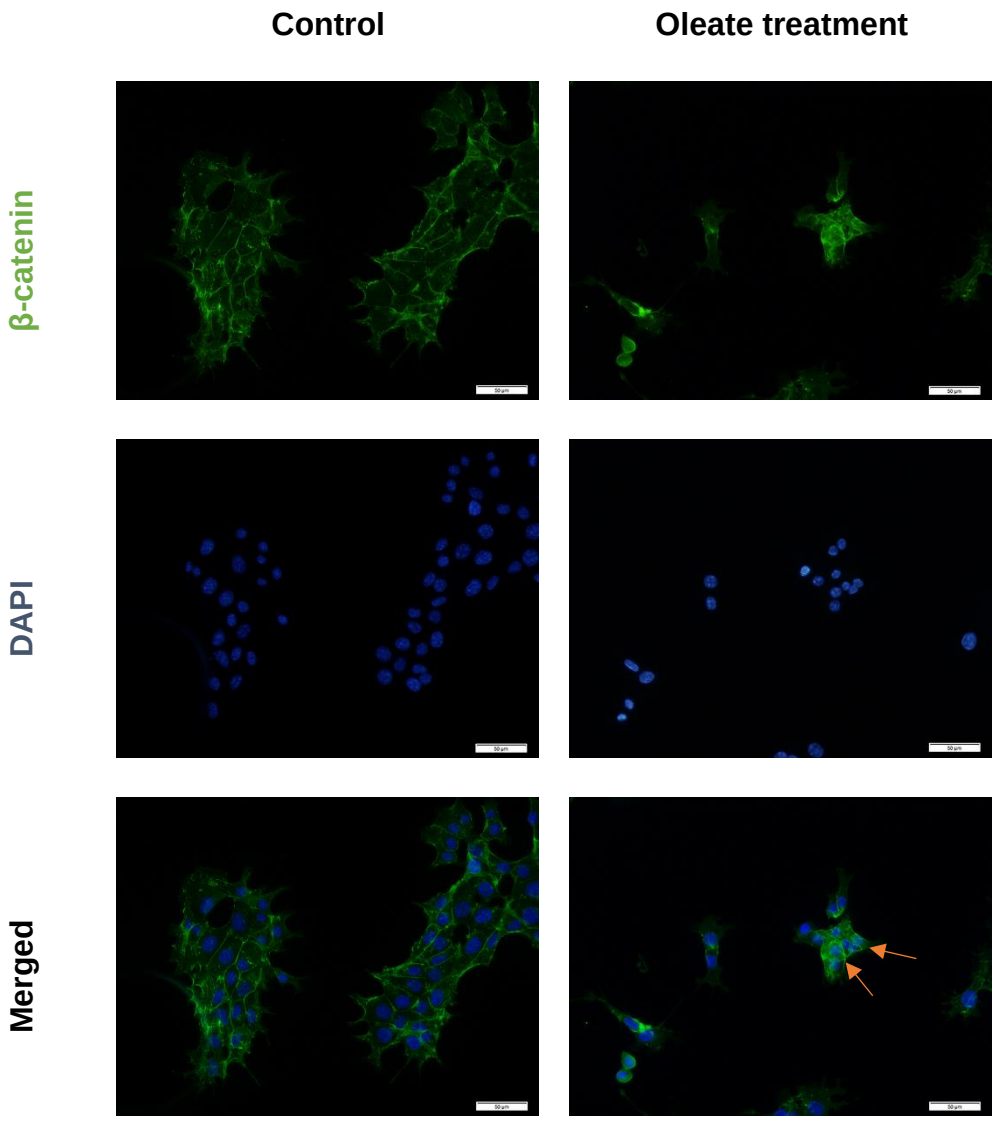


Figure 5.15: Expression of active β-catenin in oleate-treated BMOL-TAT cells. Active β-catenin is mostly membranous in untreated control cells, whereas strong cytoplasmic and nuclear active β-catenin staining is noticed in a subset of oleate-treated BMOL-TAT cells, as indicated by arrows. The scale bar depicts 50 µm.

5.5 Discussion

Ceramides, FFAs, free cholesterol, bile acids and lysophosphatidyl choline are examples of lipotoxic lipids described in the literature (Mota et al., 2016). Uptake of abundant circulating FFAs by hepatocytes plays an important role in the establishment and progression of NAFLD (Mota et al., 2016). In the majority of *in vitro* models of steatosis, cells are incubated with exogenous fatty acids using varying concentrations and time intervals (Green, Pramfalk, Morten, & Hodson, 2015). It is important to note that the fatty acid treatment can show variable cytotoxicity, depending on the type, concentration and exposure time of the respective fatty acid and type of cell (Alsabeeh, Chausse, Kakimoto, Kowaltowski, & Shirihai, 2018).

Treatment with saturated FFAs, such as palmitic acid is toxic and induce less steatosis in cells because it is poorly converted into triglyceride-enriched lipid droplets, which provide protection against lipocytotoxicity (Akazawa & Nakao, 2018). Moreover, palmitate treatment induces reactive oxygen species formation and apoptosis (Egnatchik et al., 2014). In contrast, oleic acid, a common unsaturated FFA, is less toxic and generates higher steatosis due to its ability for incorporation into triglycerides, which confer stability to the lipid droplets (Akazawa & Nakao, 2018). This effect was observed when BMOL-TAT cells were incubated with increasing concentrations of palmitate and oleate. Higher concentrations of palmitate were cytotoxic, whereas all tested oleate concentrations were well-tolerated by the BMOL-TAT cultures. Additionally, BMOL-TAT cells treated with oleate had more steatosis as shown by Oil Red O staining.

Primary hepatocytes harvested from human or rodent steatotic livers and liver cell lines such as HepG2, Huh-7, HepaRG are some of the most studied cell culture systems in NAFLD research (Green et al., 2015; Müller & Sturla, 2019). However, to date, no model has been described using LPC cultures as a research focus, despite the cells' well known role during CLDs including NAFLD. The experiments described in this chapter clearly demonstrate that steatosis can be induced in the LPC line BMOL-TAT using exogenous fatty acids, such as palmitate and oleate at doses that are not toxic for the cells. The effect of fat loading on the secretion of pro-inflammatory cytokines and

pro-fibrogenic gene activation in this *in vitro* model of steatosis has not yet been studied and will be the focus of future studies.

Earlier reports have demonstrated a role for active Wnt/β-catenin signalling in LPC-mediated regeneration as well as metabolism-related functions of the liver (Gougelet & Colnot, 2012). However, the specific contribution of LPCs in the activation or inhibition of the Wnt/β-catenin signalling in the underlying steatotic microenvironment is not very clear. Hence, the expression of different components of the Wnt/β-catenin pathway was examined in our lipid-loaded BMOL-TAT culture system.

Previously, it has been proposed that the expression of nuclear β-catenin and its transcriptional coactivator TCF4 were downregulated in an *in vitro* model of hepatic steatosis, established using palmitate-treated HepG2 cells (Seo et al., 2016). In our experiments, a strong cytoplasmic/nuclear staining was observed in a subset of palmitate-treated BMOL-TAT cells. However, palmitate-loading did not alter β-catenin expression at the transcript and protein level in spite of significant upregulation of Wnt1. This might be because the activation of the pathway in a subset of cells might not be sufficient to change the overall expression of the culture system.

Earlier, Wnt5a has been found to negatively regulate the Wnt/β-catenin signalling in *in vitro* and *in vivo* models of liver injury (Yang et al., 2015; Yuzugullu et al., 2009). Interestingly, in oleate-loaded BMOL-TAT cells Wnt5a expression was significantly downregulated. The stabilisation of β-catenin and activation of the downstream effectors of the pathway upon oleate-treatment through Wnt signalling-independent mechanisms was previously reported in clear cell renal cell carcinoma culture system (Kim et al., 2015). In oleate-treated BMOL-TAT cells, significant upregulation of β-catenin mRNA and a strong presence of active β-catenin in the cytoplasm/nucleus was observed along with Wnt7a and Wnt9a upregulation. Additionally, oleate treatment of BMOL-TAT cells led to a significant downregulation in the expression of Wnt pathway inhibitors such as sFRP1, sFRP3, sFRP4, DKK1, DKK3 and WIF1. From these observations, it can be concluded that BMOL-TAT cells are capable of producing Wnts and the active β-catenin accumulation in the cytoplasm/nucleus of subset of BMOL-TAT cells upon oleate treatment is via active Wnt/β-catenin pathway.

However, in our culture system, the expression of cyclin D1, a downstream target of the Wnt/β-catenin signalling pathway, was unchanged in oleate-loaded cells compared to controls. Cell viability assessment by MTT assay and manual cell counting also confirmed that oleate treatment had no significant effect on viability and cell proliferation of BMOL-TAT cells compared to untreated cells at the tested concentrations and time points. Of note, the main cell population for FFA uptake and metabolism are hepatocytes (Smith & Adams, 2011). Therefore, LPCs are not the obvious FFA target population. However, the proof-of-principle experiments described in this chapter demonstrate that LPCs are capable of oleate-induced Wnt/β-catenin signalling modulation, which may have other roles along with cell proliferation in this cell population.

Future experiments could focus on the use of TCF/LEF reporter assays to monitor the activity of the Wnt/β-catenin pathway in oleate-loaded BMOL-TAT cells. Moreover, performing additional cell culture assays assessing LPC differentiation, migration and signalling cross-talk to other hepatic cell populations upon exposure to a NAFLD-mimicking culture environment is warranted.

Chapter 6

In vitro and *in vivo* manipulation of Wnt/β-catenin signalling using the Wnt pathway inhibitor PRI-724

6.1 Introduction

The Wnt/β-catenin signalling pathway has been studied extensively in liver biology. In Chapter 1, components and functions of the Wnt/β-catenin pathway (1.6.1), its role in adult liver homeostasis (1.6.3), liver regeneration (1.6.4) and liver disease (1.6.5) were described in detail. As Wnt signalling plays a significant role in liver homeostasis, selecting the most appropriate agonists or antagonists for pathway manipulation is critical to achieve beneficial effects without disrupting normal liver physiology.

Antagonists targeting the Wnt/β-catenin signalling pathway can be broadly divided into five categories, depending on the site of action. They may inhibit 1) upstream regulators, 2) protein-protein interactions, 3) epigenetic regulators, 4) mediator complexes or 5) transcriptional outputs (Katoh, 2018). Many of these antagonists are associated with the risk of concurrently targeting β-catenin independent Wnt pathway activity (Kahn, 2014; Katoh, 2018). Therefore, in the context of liver biology, it is particularly important to find the appropriate Wnt pathway antagonist that can specifically target the deleterious effects of the pathway without affecting liver homeostasis.

Inside the nucleus, β-catenin recruits transcriptional co-activators such as p300 or cAMP response element binding protein (CREB)-binding protein (CBP) to generate a transcriptionally active complex, which then regulates the expression of target genes controlling proliferation, such as cyclin D1 (Tai et al., 2015). ICG-001, a first generation CBP/β-catenin interaction inhibitor, was identified while working on colon carcinoma cells using the TopFlash reporter gene system (McMillan & Kahn, 2005). PRI-724 is a second generation, small-molecule Wnt/β-catenin pathway antagonist, which exerts similar effects as ICG-001 by targeting the interaction between β-catenin and its transcriptional coactivator CBP (Lenz & Kahn, 2014). The toxicity profile of PRI-724 was first studied in patients with advanced solid tumours and trial results suggested that PRI-724 is associated with an acceptable toxicity profile (El-Khoueiry et al., 2013). Currently, four clinical trials at various phases using PRI-724 are either underway or completed (ClinicalTrials.gov identifiers: NCT01606579,

NCT01302405, NCT02195440, NCT01764477) (Lu, Green, Farr, Lopes, & Van Raay, 2016).

Some studies have been carried out to assess the effects of PRI-724 in animal models of CLDs. Osawa and colleagues reported that inhibition of the CBP/β-catenin interaction by PRI-724 led to liver fibrosis suppression in the carbon tetrachloride- and partial bile duct ligation-induced liver injury models. The authors suggested that this observed liver fibrosis reduction is due to inhibition of HSC activation, cell death of activated HSCs and the increased production of matrix metalloproteinases by macrophages (Osawa et al., 2015). Similar anti-fibrotic effects of PRI-724 were noted by Tokunaga and colleagues while working on a hepatitis C virus transgenic mouse model (Tokunaga et al., 2017). Further, the first clinical study involving hepatitis C virus-associated liver cirrhosis patients, reported encouraging anti-fibrotic activities of PRI-724 in these patients (Kimura et al., 2017). Moreover, ICG-001 combined with sorafenib showed improved anti-tumour effects of sorafenib in HCC cell lines and Huh7 xenografts (Lin et al., 2016). ICG-001 was also effective in reducing the tumour size and tumour number in a transgenic mouse model and a chemically-induced cholangiocarcinoma model in rats (Boulter et al., 2015).

Overall, the effect of PRI-724 and ICG-001 administration in different mouse models of liver fibrosis and HCC has been associated with reduced HSC activation and increased production of matrix metalloproteinase-8 by macrophages and neutrophils (Akcora, Storm, & Bansal, 2018; Osawa et al., 2015; Tokunaga et al., 2017). However, the effect of these inhibitors on LPCs, which are an important component of the injury and regeneration niche, has not yet been clarified.

In previous chapters, activation of the Wnt/β-catenin signalling pathway in LPCs in the CDE mouse model of chronic liver injury and in an LPC-focused steatosis cell culture model was described. Next, the effects of pathway manipulation through PRI-724 were studied *in vitro* and *in vivo*, applying knowledge obtained in previous experiments.

6.2 Study aim

The aim of this study was to evaluate the effects of the Wnt/β-catenin pathway manipulation using PRI-724 in an *in vitro* LPC-focused model of fat loading and in the CDE model of chronic liver injury.

6.3 Methodology

The LPC line BMOL-TAT was used for *in vitro* experiments described in this chapter. The MTT assay (2.2.3.6) was performed to confirm the effect of different concentrations of PRI-724 on the viability of palmitate-loaded (6.25 µM) BMOL-TAT cells. Cells were seeded at a density of 1,500 cells/100 µl per well and treated with palmitate (6.25 µM) and increasing concentrations of PRI-724 (6.25-100 µM) for 72 hours with medium change on every alternate day. PRI-724 was dissolved in DMSO (2.1.3.17). Therefore, BMOL-TAT cells maintained in a palmitate-loaded maintenance medium containing DMSO were used as treatment control. The viability percentage was calculated based on the absorbance values of the palmitate-loaded BMOL-TAT cells, without the addition of PRI-724 or DMSO. Similarly, MTT assays were performed to confirm the effect of different concentrations of PRI-724 on oleate-loaded (100 µM) BMOL-TAT cells. BMOL-TAT cells maintained in an oleate-loaded maintenance medium containing DMSO were used as treatment control. The viability percentage was calculated based on the absorbance values of the oleate-loaded BMOL-TAT cells, without the addition of PRI-724 or DMSO. Three technical replicates were included for each treatment concentration and the assay was repeated three times to confirm the results. The selected concentrations of palmitate and oleate were based on results described in Chapter 5 (see 5.4.1). The results from the MTT assay were used to determine the working concentrations of PRI-724 in palmitate- and oleate-loaded BMOL-TAT cells to be used for further experiments.

Protein extractions were carried out from BMOL-TAT cells that were in maintenance medium (control), palmitate- or oleate-loaded, palmitate+DMSO, oleate+DMSO, palmitate+PRI-724 and oleate+PRI-724 to evaluate the changes in the expression of active β-catenin and cyclin D1 proteins.

The experimental set up for *in vivo* experiments is shown in Figure 6.1. Six-week old male C57BL/6J mice were subjected to experimental conditions of the CDE diet (2.2.1.3) for 23 days. From day 10, mice were randomly grouped into vehicle or PRI-724 treatment groups. Final number of animals at each time point have been listed in Table 6.2. Both groups received either vehicle or PRI-

724 (2.1.3.12) at a dose of 10 mg/kg intraperitoneally on days 10, 13, 16, 19 and 22. The dose of PRI-724 was decided based on previous studies, compiled in Table 6.1. Since the CDE diet has liver-toxic effects, higher doses used in other disease models were not trialed to avoid cumulative toxicity. Liver tissues and sera from animals in both treatment groups were harvested on days 11, 17 and 23 for further investigations.

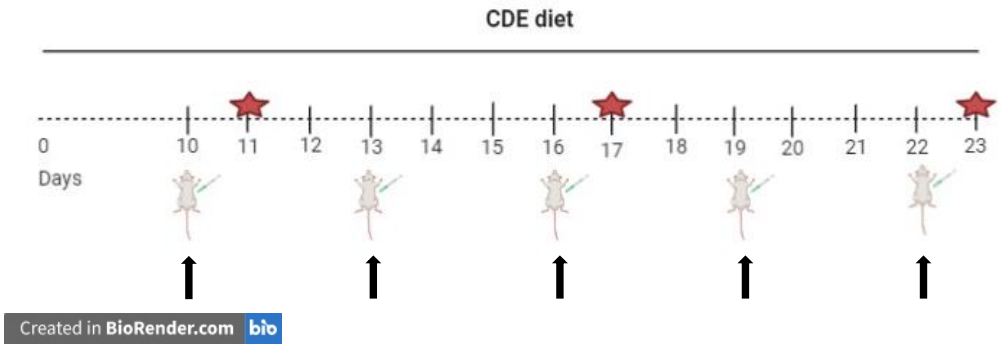


Figure 6.1: Schematic representation of vehicle or PRI-724 treatment of CDE-fed mice. The arrows highlight vehicle or PRI-724 injection days, while the red star depicts tissue and serum harvest time points.

Drug name	Mouse model	Dose	Adverse events	Reference
PRI-724	1) Transgenic hepatitis C virus mouse	1) 5 mg/kg/day 2) 5 mg/kg/day 3) 20 mg/kg/day 4) 15 mg/kg/ twice per week	No toxicity observed	(Tokunaga et al., 2017)
PRI-724	1) Carbon tetrachloride 2) Partial bile duct ligation	0.4 mg/mouse, 4 times a week	No significant toxicity	(Osawa et al., 2015)
ICG-001	1) Huh7 xenograft mouse model	20 or 50 mg/kg/3 times a week	No toxicity reported, even when combined with sorafenib	(Lin et al., 2016)
ICG-001	1) Thioacetamide supplementation 2) Intrahepatic cholangiocarcinoma xenograft model	5 mg/kg/3 times a week	No toxicity reported	(Boulter et al., 2015)
ICG-001	1) Carbon tetrachloride	5mg/kg/day	No toxicity reported	(Akcora et al., 2018)
ICG-001	1) *Pten*-null mice	0.8mg/day	No toxicity reported	(Debebe et al., 2017)

Table 6.1: Previous use of the Wnt/β-catenin inhibitors PRI-724 and ICG-001 in *in vivo* liver injury models.

Time point	Animal numbers	
	Vehicle treatment	PRI-724 treatment
Day 11	6	6
Day 17	6	6
Day 23	6	6

Table 6.2: Final animal numbers for each treatment group at the point of termination.

Overall liver damage was determined by body weight measurements, biochemical assessment of serum ALT levels and evaluation of general liver histology by H & E staining. Changes in LPC numbers were assessed by immunofluorescent staining and quantitation of the LPC markers panCK and A6. Expression of active β-catenin was studied in FFPE liver sections by immunohistochemical staining for active (non-phosphorylated) β-catenin. To examine the potential anti-proliferative effect of PRI-724, immunofluorescent Ki-67 staining was performed. Further, the inflammatory response was evaluated using fluorescent-labelling and quantitation of CD45-, F4/80- and CD11b-positive cells. Finally, Sirius Red staining and biochemical analysis of hydroxyproline levels were performed to analyse the potential anti-fibrogenic effect of PRI-724 treatment.

6.4 Results

6.4.1 Effect of PRI-724 on the viability of fat-loaded BMOL-TAT cells

The aim of these experiments was to confirm the responsiveness and effects of BMOL-TAT cells as a cell culture model of LPCs to increasing concentrations of PRI-724 prior to *in vivo* testing. As described above, PRI-724 specifically inhibits the Wnt/β-catenin target gene cyclin D1, which is involved in the regulation of cell proliferation.

The viability of palmitate-loaded BMOL-TAT cells was significantly reduced in a dose-dependent manner (6.25-100 µM PRI-724); however, no significant effect was observed at a PRI-724 concentration of 6.25 µM (Figure 6.2). Oleate-treated cells equally responded to PRI-724 treatment with significant dose-dependent reduction in viability at all tested concentrations (Figure 6.3). For further experiments, 12.5 µM and 6.25 µM of PRI-724 concentrations were selected for palmitate-loaded and oleate-loaded BMOL-TAT cells, respectively since these concentrations resulted in a viability reduction of approximately 50%.

Cyclin D1 and active β-catenin protein expression levels were slightly reduced with PRI-724 treatment in palmitate-loaded BMOL-TAT compared to control cells (Figure 6.4). In contrast, a clear reduction in cyclin D1 and active β-catenin protein levels were observed in oleate-loaded and PRI-724-treated BMOL-TAT cells compared to control and oleate-loaded BMOL-TAT cells (Figure 6.5).

These results suggest that oleate-loaded BMOL-TAT cells were more susceptible to PRI-724 treatment compared to the palmitate-loaded experimental group.

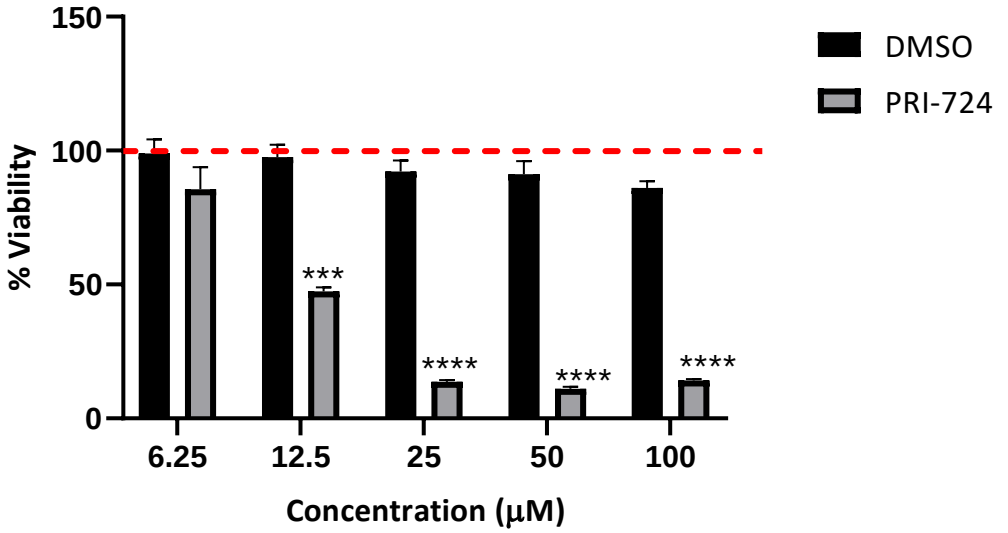


Figure 6.2: Effect of increasing concentrations of PRI-724 on the viability of palmitate-loaded BMOL-TAT cells. Dose-dependent reduction in viable palmitate-loaded BMOL-TAT cell numbers was evident with PRI-724 treatment. Data represent the mean ± SEM. *n*= 3. *** $P < 0.001$, **** $P < 0.0001$ compared with controls.

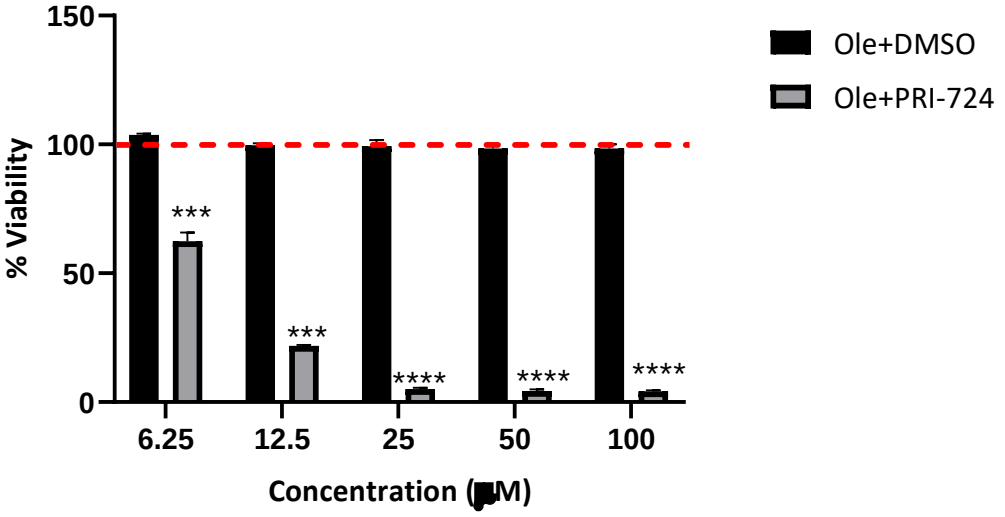


Figure 6.3: Effect of increasing concentrations of PRI-724 on the viability of oleate-loaded BMOL-TAT cells. PRI-724 treatment led to dose-dependent reduction in viable oleate-loaded BMOL-TAT cell numbers. Data represent the mean ± SEM. *n*= 3. *** $P < 0.001$, **** $P < 0.0001$ compared with controls.

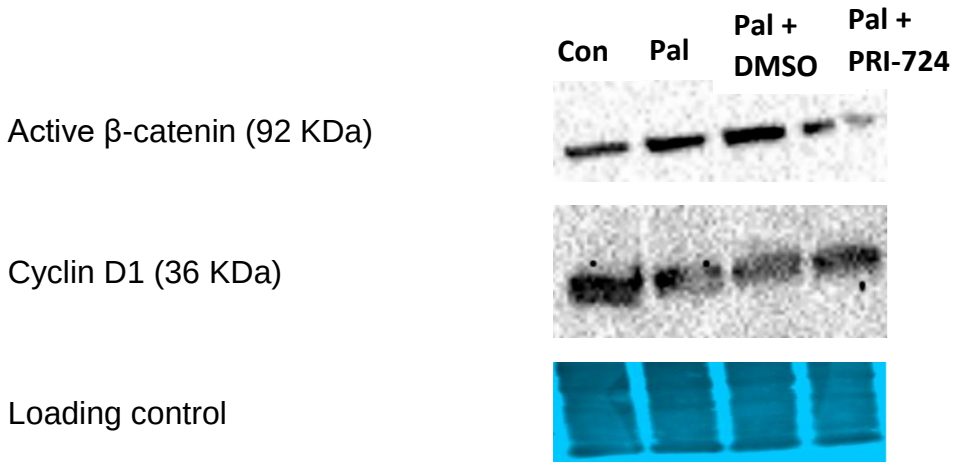


Figure 6.4: Expression of active β-catenin and cyclin D1 in palmitate-loaded+PRI-724-treated BMOL-TAT cells. Shown here are representative Western Blot images for active β-catenin and cyclin D1 in control and treatment groups. Cyclin D1 and active β-catenin protein expression levels were slightly reduced in palmitate-loaded+PRI-724-treated BMOL-TAT cells compared to control. *n*=1. Con: control, Pal: palmitate-loaded, Pal+DMSO: palmitate+DMSO-treated, Pal+PRI-724: palmitate+PRI-724-treated. Revert total protein stain was used as a loading control.

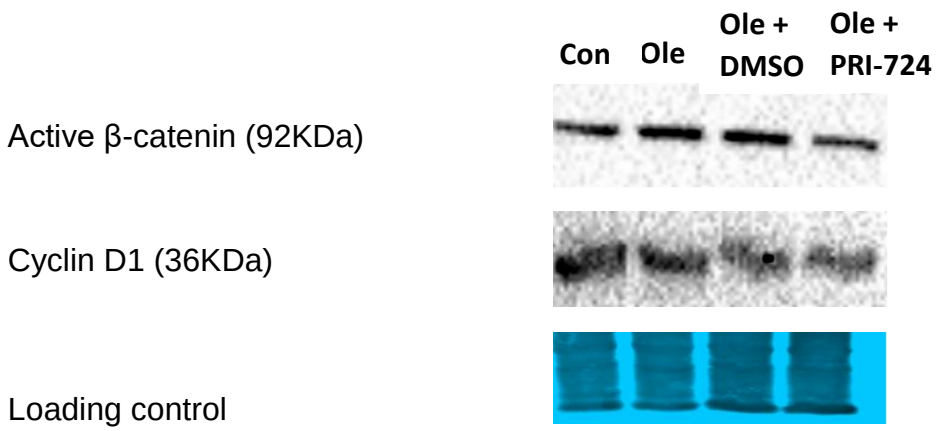


Figure 6.5: Expression of active β-catenin and cyclin D1 in oleate-loaded and PRI-724-treated BMOL-TAT cells. Shown here are representative Western Blot images for active β-catenin and cyclin D1 in control and treatment groups. A clear reduction in cyclin D1 and active β-catenin is observed in oleate-loaded and PRI-724-treated BMOL-TAT cells compared to the control and oleate-loaded BMOL-TAT cells. *n*=1. Con: control, Ole: oleate-treatment, Ole+DMSO: oleate+DMSO-treated, Ole+PRI-724: oleate+PRI-724-treated. Revert total protein stain was used as a loading control.

6.4.2 Assessment of liver tissue damage in vehicle- and PRI-724-treated livers

Disease parameters such as body weight, serum ALT levels and alterations to the liver architecture were assessed to compare the extent of liver tissue damage induced by vehicle and PRI-724 treatment in animals on the CDE diet.

The body weight is an important parameter of the CDE diet as mice weighing above 25-30 grams may be resistant to the diet and mice below 15 grams may not be able to tolerate the diet-related toxicity (Clerbaux L. et al., 2017). At the beginning of experimentation, animals in vehicle and PRI-724 treatment groups had similar starting body weights with an average weight of approximately 17 grams. During the time course, body weights of PRI-724-treated animals remained slightly lower than body weights of vehicle-treated mice, especially at the later time points of 19-23 days; however, this did not reach statistical significance (Figure 6.6). Other signs of physical discomfort, such as reduced activity levels, changes in the body posture and reduced hydration were not observed in any of the groups. Therefore, it can be concluded that PRI-724 was well-tolerated by all animals.

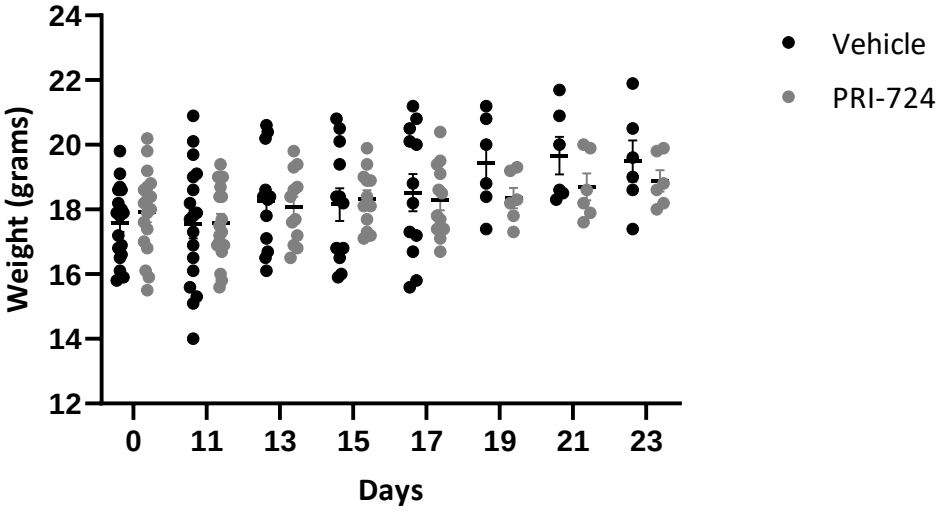


Figure 6.6: Body weight levels of vehicle- and PRI-724-treated mice on the CDE diet. Body weights of PRI-724-treated mice were slightly lower compared to the vehicle-treated controls throughout the duration of the experiment. Data represent the mean ± SEM.

Serum ALT levels indicate the extent of liver damage and hepatocyte injury. Mice in the PRI-724 treatment group displayed significantly increased ALT levels compared to the vehicle controls only at day 11, right after treatment induction. At the later time points of days 17 and 23, there was no significant difference in the two experimental groups (Figure 6.7).

Higher ALT levels at day 11 in the PRI-724 treatment group may be due to mild drug-related toxicity at first exposure. Nevertheless, equivalent ALT levels in PRI-724 and vehicle treatment groups at days 17 and 23 suggest that mice had adapted physiologically to the drug treatment.

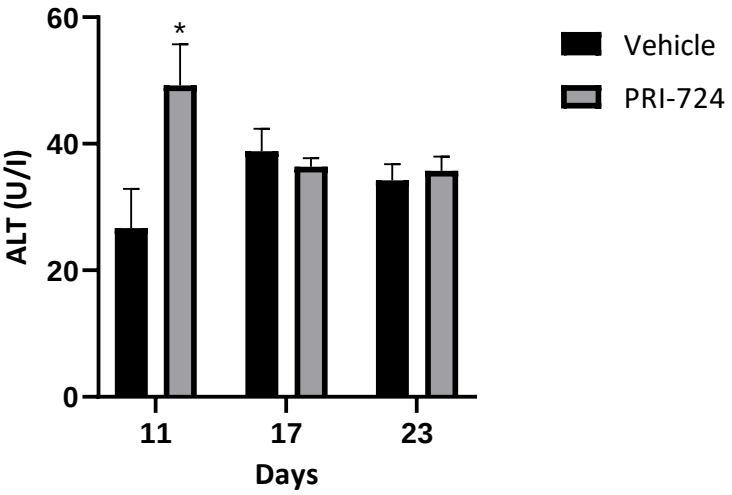


Figure 6.7: Serum ALT levels of vehicle and PRI-724-treated mice on the CDE diet. Twice weekly injections of PRI-724 resulted in significantly higher serum ALT levels than in vehicle-treated mice at day 11 but not days 17 or 23 of CDE feeding. Data represent the mean ± SEM. *n*= 6. * *P* < 0.05, compared with vehicle controls.

PRI-724 and vehicle treatment groups were both subjected to the CDE diet and, consequently both groups displayed detectable signs of steatosis, basophilic cell infiltration, hepatocyte injury in the form of swelling, rounding, ballooning, as expected. Interestingly, while livers harvested at earlier time points showed comparable liver histology in terms of liver architecture and cellular appearance, PRI-724-treated livers had reduced numbers of basophilic cells in periportal lobular areas on day 23 compared to vehicle-treated counterparts (Figure 6.8).

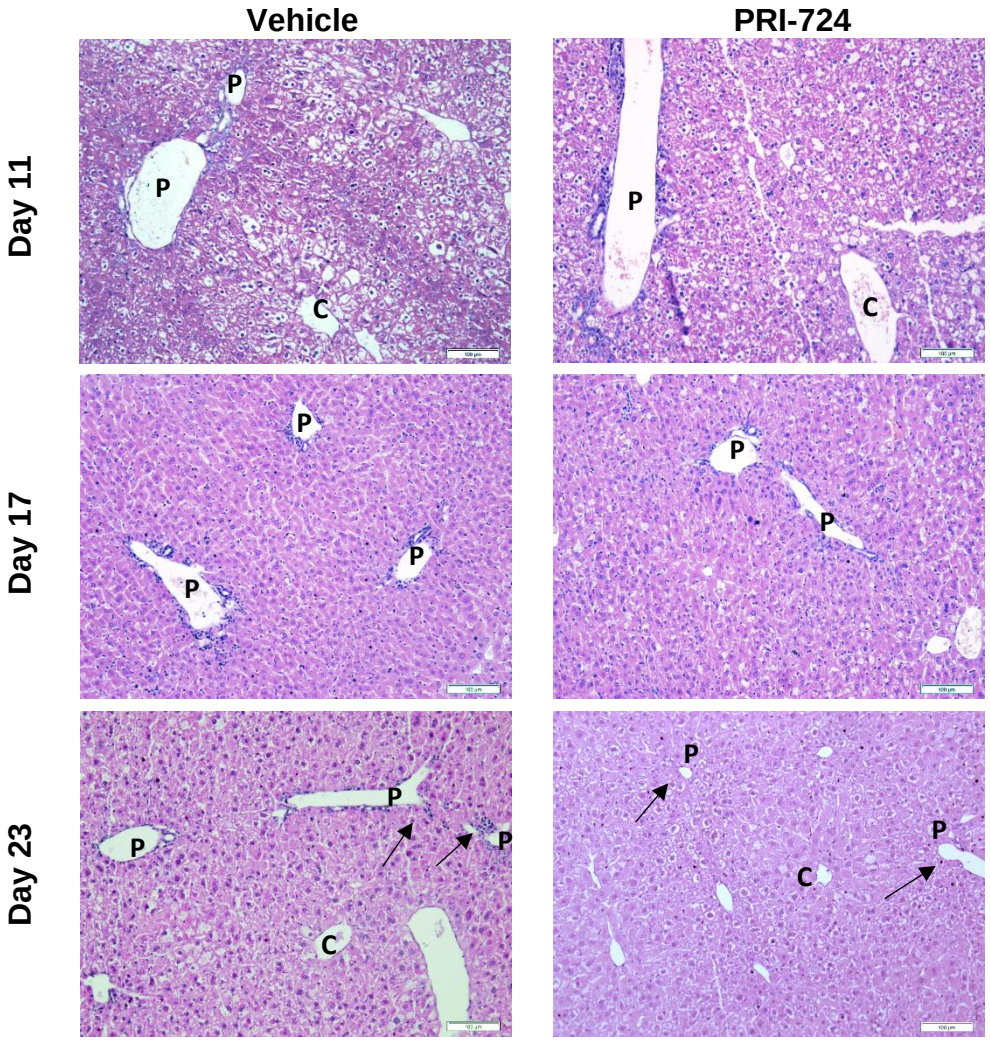


Figure 6.8: H & E staining of liver sections from vehicle- and PRI-724-treated chronically injured mice. Both treatment groups presented steatosis and increased basophilic periportal cells at days 11 and 17. However, at day 23, PRI-724-treated animals displayed less cell infiltrates near portal areas (see arrows), compared to vehicle-treated mice. The scale bar depicts 100 µm. P: portal area, C: central area.

6.4.3 Effect of PRI-724 and vehicle treatments on the proliferation of LPCs

To assess the proliferation of LPCs in PRI-724- and vehicle-treated mice, FFPE liver sections were immunohistochemically stained with the ductular and LPC marker panCK. In both PRI-724 and vehicle treatment groups, panCK$^+$ LPCs were present near periportal areas as single cells or strings of cells (Figure 6.9A). PRI-724 treatment led to a trend of a higher number of panCK$^+$ LPCs at day 11 compared to vehicle treatment. However, at the two later time points, PRI-724-treated livers displayed slightly lower numbers of panCK$^+$ LPCs compared to the vehicle controls. However, the differences using panCK as a general LPC marker did not reach statistical significance (Figure 6.9B). To corroborate these data, the last time point, day 23, was further evaluated by using A6 as a second marker, which is able to detect the biliary subpopulation of the LPC compartment in frozen tissue (Tirnitz-Parker et al., 2007). PRI-724-treated mice showed a significant reduction in A6$^+$ LPC numbers in periportal liver areas (Figure 6.10). Further, staining with the proliferative cell marker Ki-67 revealed that the reduction in A6$^+$ LPC numbers in PRI-724- compared to vehicle-treated livers was associated with a significant reduction in periportal cell proliferation at day 23 of CDE treatment (Figure 6.11).

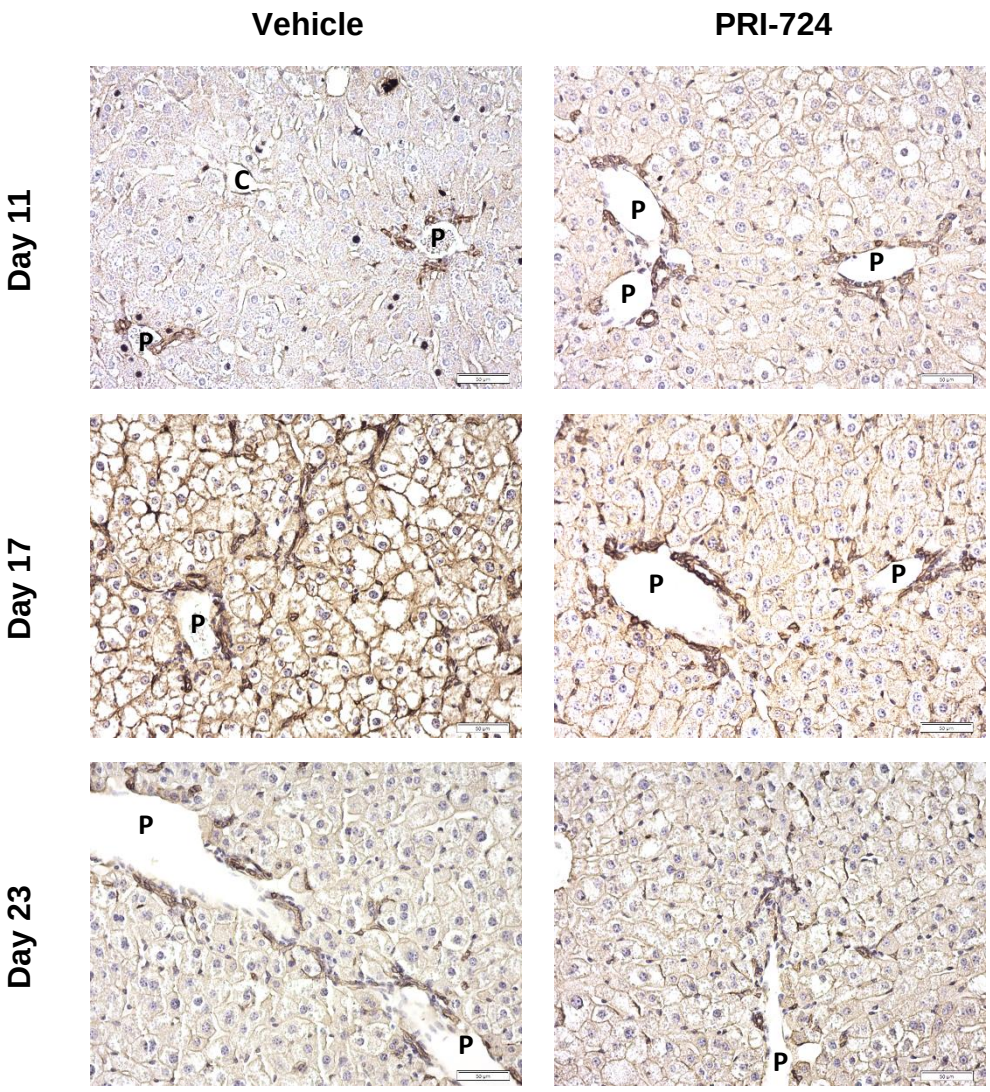


Figure 6.9 (A): Expression of panCK in vehicle- versus PRI-724-treated chronically injured liver tissues. In both treatment groups, panCK$^+$ cells can be seen as single cells or strings of cells around portal areas and at times penetrating the liver parenchyma. The scale bar depicts 50 µm. P: portal area, C: central area.

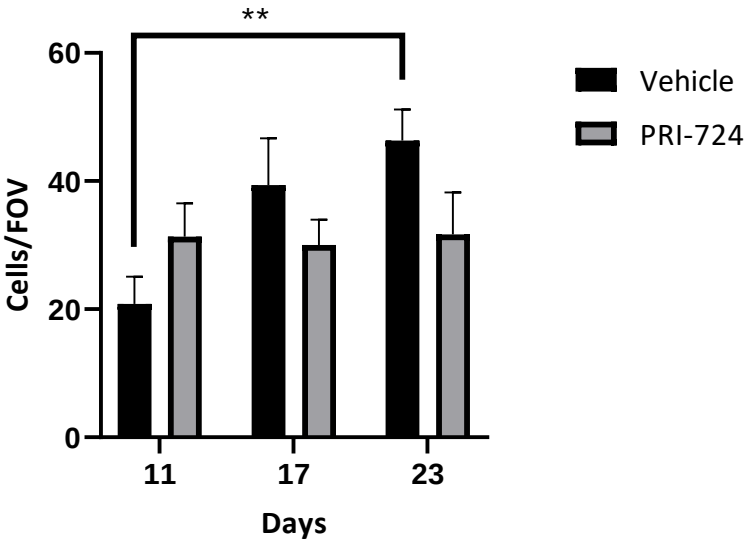
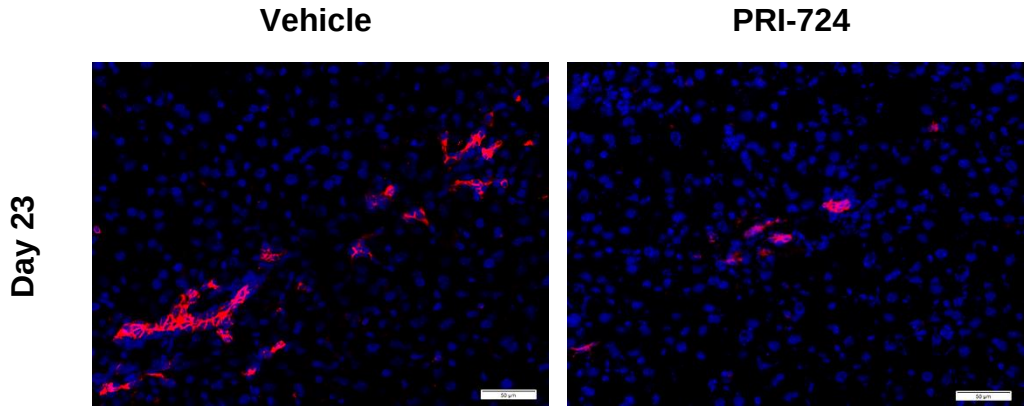


Figure 6.9 (B): Quantitation of panCK$^+$ cells in the liver sections of vehicle- and PRI-724-treated chronically injured mice. While numbers of panCK$^+$ cells increased over time in vehicle-treated mice and were statistically different at day 23 compared to day 11, they remained constant in the PRI-724 treatment group. Data represent the mean ± SEM. *n*= 5-6 mice per group. ***P* < 0.01 compared with controls. FOV: field of view.

(A)



(B)

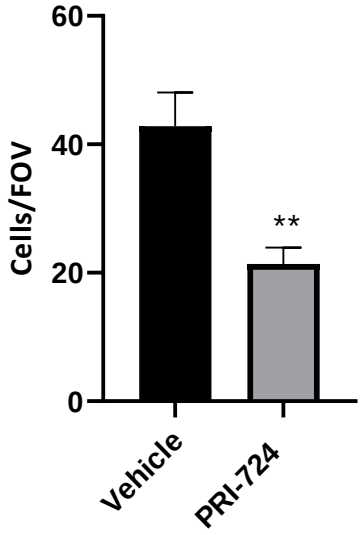


Figure 6.10: (A) Expression and (B) quantitation of A6$^+$ cells in vehicle- versus PRI-724-treated chronically injured liver tissues at day 23. Numbers of A6$^+$ cells were significantly lower in PRI-724- treated compared to vehicle-treated livers. The scale bar depicts 50 µm. Data represent the mean ± SEM. *n*=5-6 mice per group. ***P* < 0.01 compared to same time point controls. FOV: field of view.

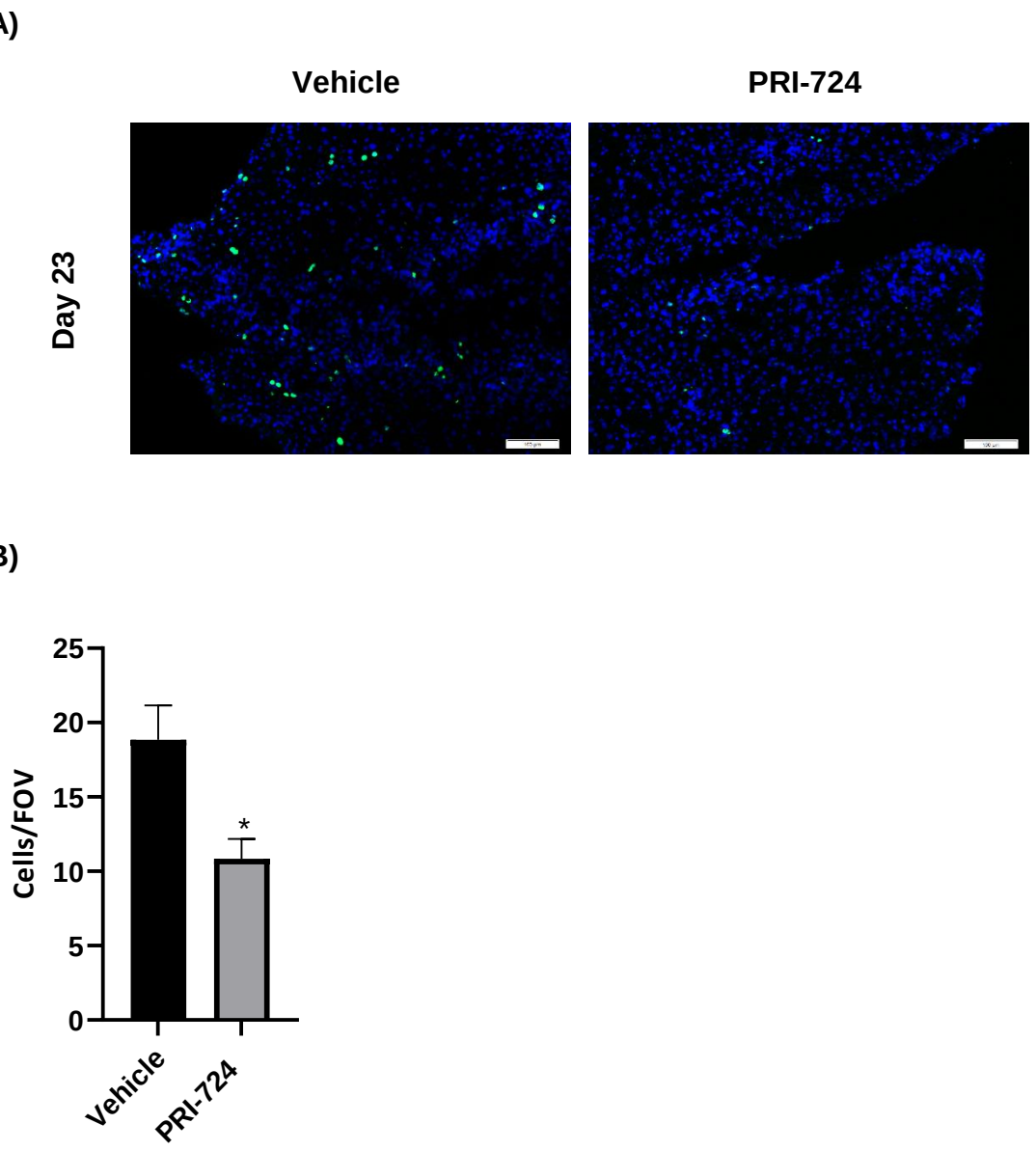
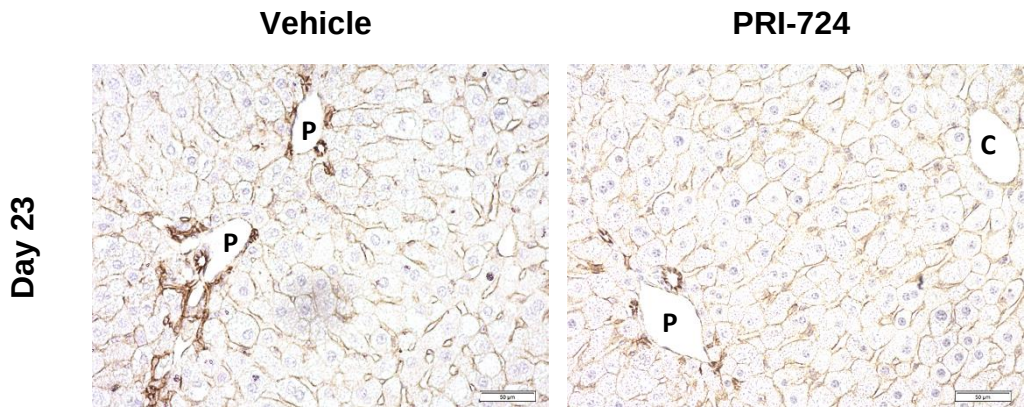


Figure 6.11: (A) Expression and (B) quantitation of Ki-67$^+$ cells in vehicle- versus PRI-724-treated chronically injured liver tissues at day 23. Numbers of Ki-67$^+$ proliferating cells were significantly reduced in PRI-724-treated compared to vehicle-treated livers. The scale bar depicts 100 µm. Data represent the mean ± SEM. *n*=6 mice per group. *P < 0.05 compared to same time point controls. FOV: field of view.

6.4.4 Effect of PRI-724 and vehicle treatments on the expression of active β-catenin

Immunohistochemical staining for active (non-phosphorylated) β-catenin revealed a similar staining pattern in both PRI-724- and vehicle-treated livers at day 23, with active β-catenin^{+} cells mainly present near the portal areas. Quantitation of positively stained cell numbers revealed no significant difference between both experimental groups.

(A)



(B)

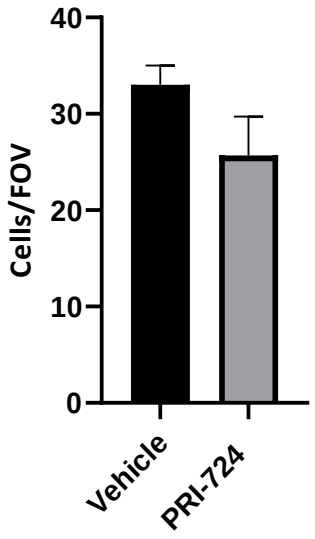
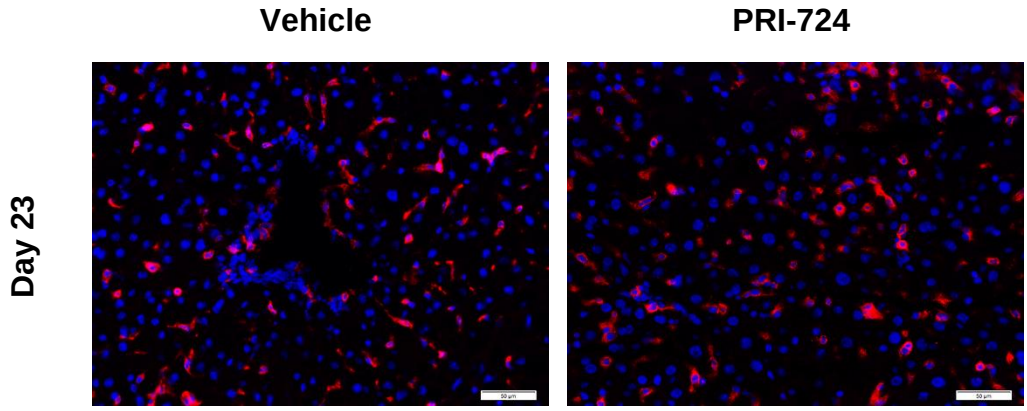


Figure 6.12: (A) Expression and (B) quantitation of active β-catenin$^+$ cells in vehicle- versus PRI-724-treated chronically injured liver tissues at day 23. Numbers of active β-catenin$^+$ cells present near portal areas were not statistically different in PRI-724-treated mice compared to the vehicle controls. The scale bar depicts 50 µm. P: portal area, C: central area. Data represent the mean ± SEM. *n*=6 mice per group. FOV: field of view.

6.4.5 Inflammatory signature of the PRI-724- and vehicle-treated mice

The inflammatory response at day 23 was assessed in both experimental groups by quantitation of $CD45^+$ inflammatory cells, $F4/80^+$ macrophages and $CD11b^+$ monocyte-derived macrophages in frozen liver sections. While cell numbers of $CD45^+$ and $F4/80^+$ cells were not statistically different in PRI-724-treated versus vehicle-treated mice (Figure 13 and Figure 14), numbers of infiltrating, monocyte-derived macrophages expressing CD11b antigen were significantly reduced in the PRI-724-treated animals compared to the vehicle controls (Figure 15).

(A)



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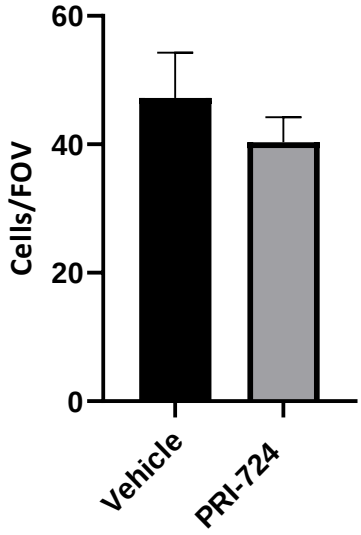
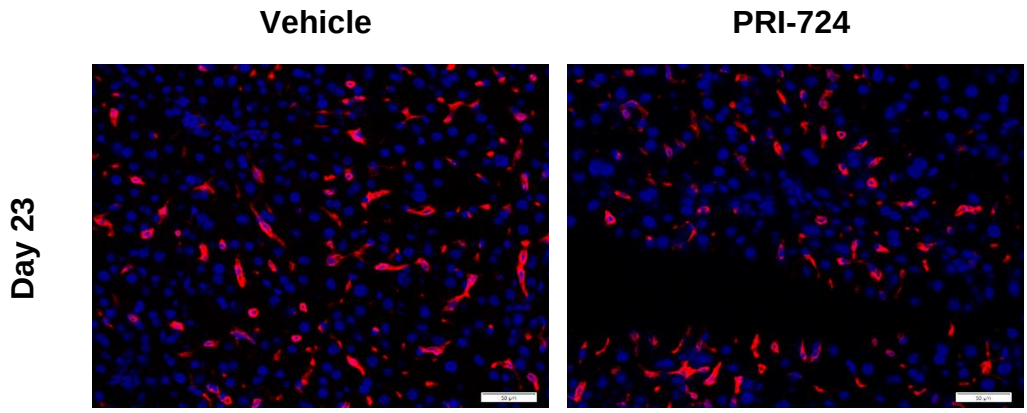


Figure 6.13: (A) Expression and (B) quantitation of CD45$^+$ cells in vehicle- versus PRI-724-treated chronically injured liver tissues at day 23. Numbers of CD45$^+$ inflammatory cells were not statistically different in PRI-724-treated compared to vehicle-treated animals. The scale bar depicts 50 µm. Data represent the mean ± SEM. *n*=5-6 mice per group. FOV: field of view.

(A)



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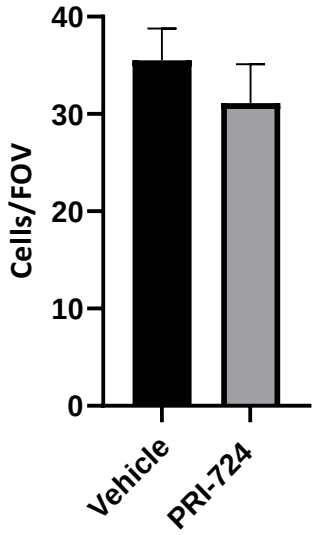


Figure 6.14: Expression and quantitation of F4/80$^+$ cells in vehicle- versus PRI-724- treated chronically injured liver tissues at day 23.

Numbers of F4/80$^+$ inflammatory cells were not statistically different in PRI-724-treated compared to vehicle-treated animals. The scale bar depicts 50 µm. Data represent the mean ± SEM. *n*=5 mice per group. FOV: field of view.

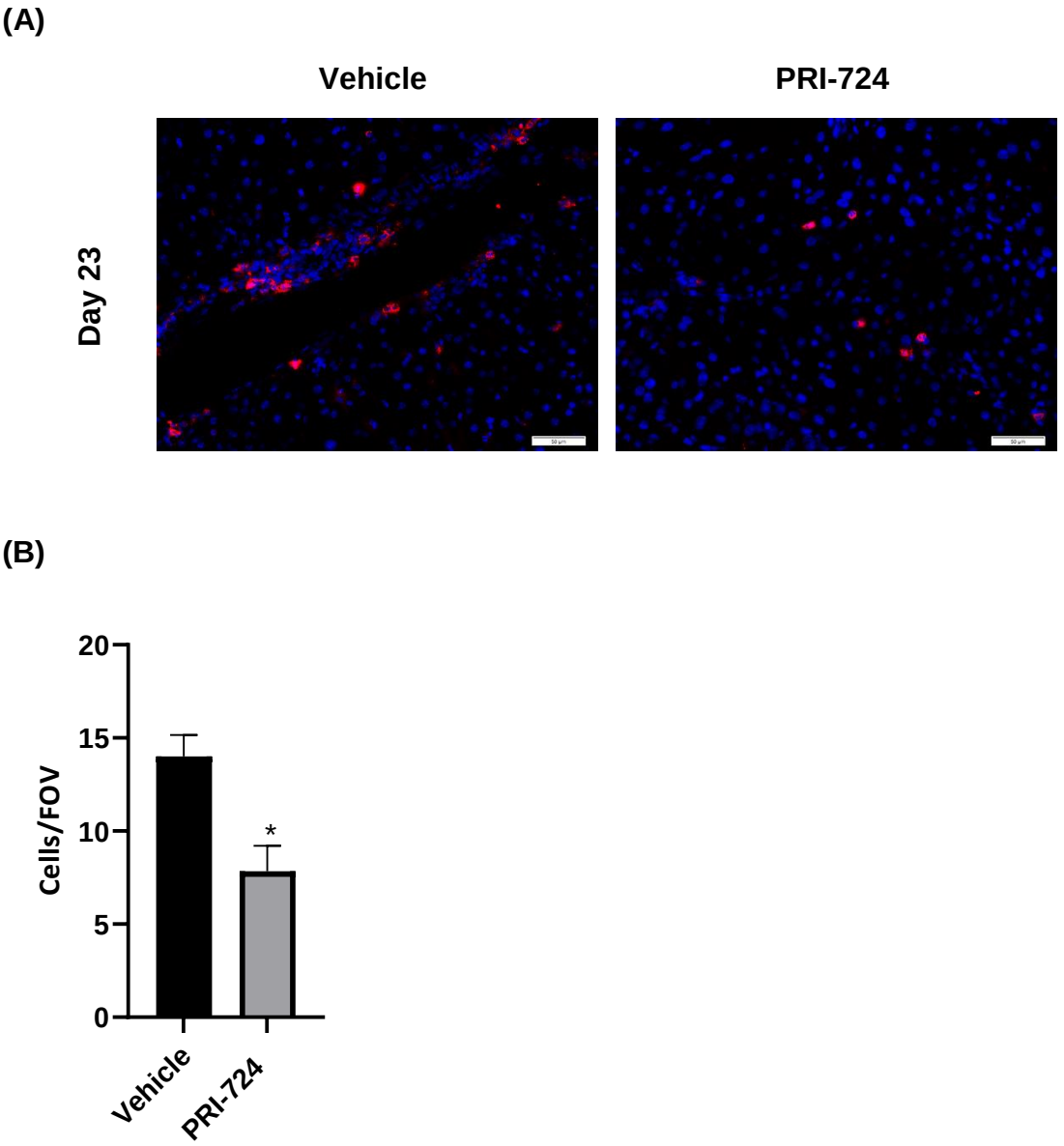


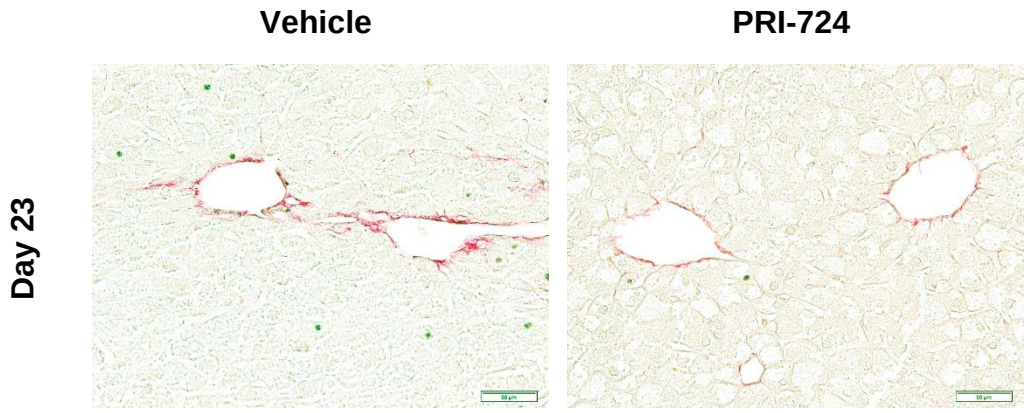
Figure 6.15: Detection and quantitation of CD11b$^+$ cells in vehicle- versus PRI-724-treated chronically injured liver tissues at day 23. A significant reduction in CD11b$^+$ cells was found in PRI-724-treated compared to vehicle-treated livers. The scale bar depicts 50 µm. Data represent the mean ± SEM. *n*=4-6 mice per group. **P* < 0.05 compared to same time point controls. FOV: field of view.

6.4.6 Effect of PRI-724 and vehicle treatment on fibrosis

Chronic liver injury induced by the CDE diet is associated with extracellular matrix remodelling and deposition of collagen, leading to hepatic fibrosis. Moreover, PRI-724 has been shown to have an effect on fibrosis resolution in some liver injury models, as described previously (see 6.1). Therefore, it was of interest to investigate the potential anti-fibrotic effect of PRI-724 treatment in the CDE model of chronic liver injury.

The extent of fibrosis in both treatment groups at day 23 was evaluated immunohistochemically by Sirius Red staining for the visual assessment of collagen deposition and fibrosis patterns and biochemically by hydroxyproline assay for the quantitation of a major collagen component. Vehicle-treated mice displayed higher levels of portal fibrosis compared to PRI-724-treated mice, as judged by positivity for Sirius Red. Quantitation of Sirius Red^{+} liver areas revealed a slight but not statistically significant reduction in collagen deposition in drug-treated animals compared to vehicle controls (Figure 6.16). These observations were consistent with data obtained through biochemical assessment of hydroxyproline, which showed a similar trend without statistical significance due to a relatively high degree of animal-to-animal variation (Figure 6.17).

(A)



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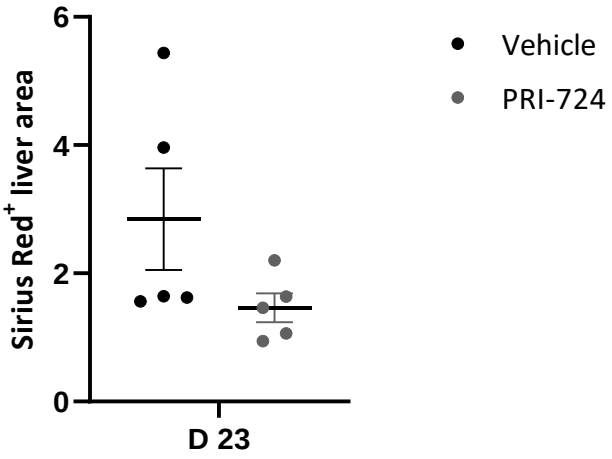

Figure 6.16: Sirius Red staining of liver sections from vehicle- and PRI-724-treated chronically injured mice. PRI-724 treatment led to a reduction in collagen deposition compared to vehicle treatment at day 23. The scale bar depicts 50 µm. Data represent the mean ± SEM. *n*=5 mice per group.

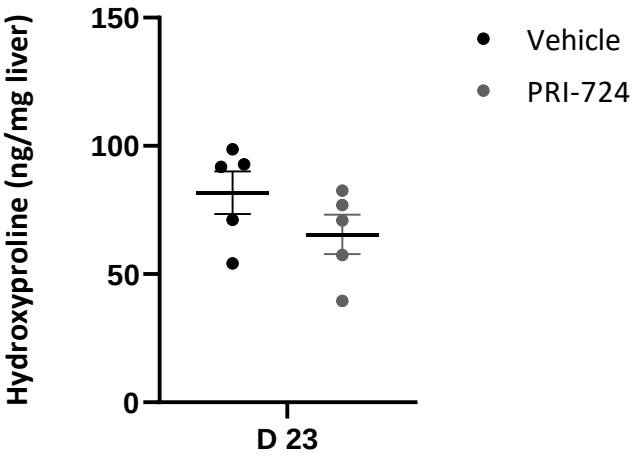

Figure 6.17: Hydroxyproline levels in vehicle- and PRI-724-treated chronically injured livers. Hydroxyproline levels, as a measure of collagen deposition, were slightly reduced in PRI-724-treated mice compared to the vehicle-treated controls at day 23. Data represent the mean ± SEM. *n*=5 mice per group.

6.5 Discussion

The Wnt signalling pathway plays a critical role in regulating fibrotic responses either through direct effects on the fibrogenic cells or indirectly through cross-talk with other signalling pathways such as the TGF-β pathway (Jarman & Boulter, 2020). The TGF-β pathway has a well-established role in driving fibrosis and, interestingly, it is known to promote activation of β-catenin by inhibiting GSK3β (Amini Nik, Ebrahim, Van Dam, Cassiman, & Tejpar, 2007). Since HSC activation is a crucial step in fibrosis initiation, the role of Wnt signalling in HSCs has been widely investigated. Various studies have reported the ability of Wnt signals to drive activation of HSCs as well as an upregulation in Wnt signalling by activated HSCs (Chen et al., 2020; Cheng et al., 2008; Jia et al., 2021; Jiang et al., 2006; Myung et al., 2007).

The activation status of HSCs during chronic liver injury is influenced by surrounding cell types, such as LPCs and inflammatory cells. Pozniak et al. used LPC and HSC cell lines to demonstrate that taurocholate-exposed LPCs secrete chemokines, such as macrophage inflammatory protein (MIP) 1α, MCP-1, regulated upon activation T cells expressed and secreted (RANTES) and platelet-derived growth factor subunit B (PDGFB), with MIP1α found to attract and recruit HSCs via CC chemokine receptor 5 (CCR5) (Pozniak et al., 2017). Another study demonstrated a receptor-ligand interaction between lymphotoxin β receptor^{+} HSCs and lymphotoxin β^{+} LPCs, which facilitated cellular cross-talk and induced HSCs to secret chemokines which further recruited LPCs, HSCs and leukocytes (Ruddell et al., 2009). Recently, *in vivo* studies using the LPC lines BMOL and BMOL-TAT (Tirnitz-Parker et al., 2007), in co-culture with primary activated HSCs or the HSC line LX-2, have revealed a regulatory role of LPCs by demonstrating modulation of fibrogenic processes in HSCs, most likely mediated by cellular cross-talk (Gratte et al., 2021). These data together suggest LPCs as key orchestrators of liver disease progression, and consequently as a potential therapeutic target, which was further investigated through Wnt/β-catenin inhibition studies *in vitro* and *in vivo*.

Testing of increasing concentrations of PRI-724 in cultures of palmitate- and oleate-loaded BMOL-TAT cells, as an *in vitro* model of LPCs, confirmed a dose-dependent reduction in cell viability. These treatments also served as proof-of-principle experiments, demonstrating that LPCs are a potential target population of PRI-724 treatment prior to further experiments that aimed to inhibit the Wnt/β-catenin pathway *in vivo*. Currently, no other studies reporting the use of PRI-724 in fat-loaded LPC culture systems are available. However, Gabata and colleagues recently demonstrated an anti-proliferative effect of C-82 (active form of PRI-724) in HCC cell lines, which have constitutively activated β-catenin (Gabata et al., 2020), supporting data obtained with palmitate- or oleate-loaded PRI-724-treated BMOL-TAT cells.

Next, the effects of PRI-724 treatment were studied in mice that had been subjected to the CDE diet to induce CLD. To increase the clinical relevance and significance of this *in vivo* pathway manipulation study, the administration of PRI-724 was commenced when chronic hepatic injury was already well established with detectable signs of steatosis, inflammation, fibrogenesis and LPC compartment activation. As previously reported in Chapter 4 of this thesis, steatosis, inflammation and fibrogenesis were observed at all time points on the CDE diet. Moreover, CDE feeding led to proliferation of LPCs as early as day 3 and peaked at day 21. Since the first week of CDE feeding is associated with the highest risk for adverse effects in the mice, PRI-724 treatment was started at day 10 of CDE feeding to avoid excessive toxicity and reduce the risks to the animals. As a consequence, no adverse events were recorded for this time course study of pharmacological Wnt/β-catenin pathway modulation of chronically injured mice.

To evaluate the cellular effects of PRI-724 treatment in mice subjected to the CDE diet, immunohistochemical and immunofluorescent stainings for LPC and inflammatory cell markers as well as histological and biochemical assesment of collagen as a fibrosis read out were undertaken. The data showed that inhibition of Wnt/β-catenin signalling by PRI-724 treatment resulted in a reduction of $A6^+$ LPCs and $CD11b^+$ infiltrating monocyte-derived macrophages, which most likely represented the $Ki67^+$ cells observed in

periportal lobular areas. These results are consistent with data reported by Köhn-Gaone and colleagues, who performed a detailed analysis of cellular dynamics in CDE-injured liver tissue and identified the portal liver areas as the lobular site that forms an injury and regeneration niche in response to CDE toxicity (Kohn-Gaone, Dwyer, et al., 2016a). PRI-724 treatment did not worsen the tissue damage induced by CDE diet but instead reduced LPC compartment activation and infiltration of bone marrow-derived macrophages.

A complete inhibition of LPCs through PRI-724 treatment was not anticipated, since only a subset of LPCs express strong cytoplasmic/nuclear β-catenin (see Chapter 4), and consequently only a subpopulation of LPCs is responsive to Wnt/β-catenin pathway inhibition by PRI-724. Moreover, Tan et al. demonstrated that conditional deletion of liver β-catenin leads to delayed regeneration but not complete suppression (Tan et al., 2006). Thus, β-catenin is an important but not critical regulator of liver regeneration. It is currently unknown which specific LPC population is responsive to PRI-724 and will require further study. The LPC pool is considered to represent a very heterogenous cell population that may contain (a) regenerative cells, which are thought to replace lost tissue by differentiation into either hepatocytes or cholangiocytes or (b) pathological cells that are associated with disease progression and may represent tumour-initiating cells or disease progressive cells through cellular cross-talk (Kohn-Gaone, Gogoi-Tiwari, et al., 2016b).

A previous study proposed that monocytes may promote LPC proliferation (Elsegood et al., 2015). The effect of PRI-724 administration on inflammatory cells was studied using three common inflammatory cell markers. The number of CD45- and F4/80-positive cells was slightly reduced with PRI-724 treatment; however, this was not statistically significant. Further experiments with an increased animal number may clarify this issue. Surprisingly, a significant reduction in $CD11b^+$ cells was observed with PRI-724 treatment, which was in contrast with an earlier study that reported an increase in $CD11b^+$ cells in hepatitis C virus transgenic mice (Tokunaga et al., 2017), highlighting the need for injury context-specific interpretation of results. As previous studies have reported PRI-724 as an anti-fibrotic agent, the effect of PRI-724 on fibrosis was

evaluated in our study using Sirius Red stainings and hydroxyproline assays. A slight reduction in collagen deposition was observed with PRI-724 treatment; however, this was not statistically significant due to relatively high animal-to-animal variability. Again, future experiments with increased animal numbers may be able to strengthen these data. In addition, additional experiments staining for markers of activated HSCs, including α-SMA, desmin and vimentin as well as additional Masson's trichrome stainings will be performed to gain more information on the potential anti-fibrotic effects of PRI-724 in CDE-injured mouse liver. Due to COVID-related laboratory shutdown periods, molecular examination of vehicle- versus PRI-724-treated livers could not be performed. Future experiments will investigate the molecular expression of pro-fibrotic mediatiors such as α-SMA, TGF-β, collagen type I, metalloproteinases and tisue inhibitor metalloproteinases as well as inflammatory cytokine and chemokine networks that may indirectly influence fibrogenesis. Lastly, long-term experiments with PRI-724 treatment using the CDE diet will inform about the potential inhibition or delay of tumorigenesis, which develops on a background of sustained inflammation and progressive fibrosis in this model.

Chapter 7
General Discussion

The global burden of CLD is substantial, affecting approximately 1.5 billion people (Moon, Singal, & Tapper, 2020). CLD-related mortality has increased by 46% between 1980 to 2010 worldwide (Younossi, 2019) and the factors responsible for this increase vary globally. The most common contributors to CLD are chronic hepatitis B virus, hepatitis C virus, NAFLD and alcoholic liver disease (Paik, Golabi, Younossi, Mishra, & Younossi, 2020). After the introduction of effective hepatitis B virus vaccination and potent antiviral treatments for hepatitis C virus infection, in the form of direct-acting antiviral agents, the global burden of chronic viral hepatitis is slowly declining. In contrast, the prevalence of NAFLD is increasing (Younossi, 2019). In the United States, the rise in CLD-associated mortality rates from 2007 to 2016 can be attributed mainly to NAFLD and alcoholic fatty liver disease (Kim et al., 2018). Similarly, in Australia, NAFLD-associated primary liver cancer cases have been on the rise and a substantial increase in NAFLD-related liver disease burden is predicted in the coming decade (Adams et al., 2020). Currently, NAFLD is the most common CLD in Western industrialised countries, with a community prevalence of 6-35% (Bellentani, 2017). Components of the very common metabolic syndrome, such as central obesity, type 2 diabetes mellitus, dyslipidaemia and insulin resistance, are closely associated with NAFLD (Bellentani, 2017).

NAFLD is a complex and heterogeneous disease, which progresses through various histopathological and pathophysiological stages (Lau, Zhang, & Yu, 2017). In 2020, an umbrella term MAFLD was introduced to more accurately capture the heterogeneity of the disease (Eslam, Sanyal, et al., 2020).

NAFLD is diagnosed when more than 5% of hepatocytes exhibit fat accumulation in the absence of significant alcohol consumption. It begins with simple steatosis and in some cases can progress to NASH, fibrosis, cirrhosis and finally HCC (Benedict & Zhang, 2017). Presently, the exact molecular mechanisms that drive the progression of this disease are not known. Therefore, the treatment options are limited to lifestyle modifications, such as dietary changes and exercise. These changes are not sufficient for a subset of patients, especially those with advanced and progressive NAFLD (Maurice

& Manousou, 2018). The only alternative for NASH-related end-stage liver disease is liver transplantation. However, it is not suitable for patients with severe underlying metabolic disorders due to a high risk of post-transplant complications (Leoni et al., 2018). Thus, there is an urgent need for therapeutic alternatives.

An appearance of DRs is a common feature observed in most CLDs (Sato et al., 2019). LPCs within DRs proliferate from the Canals of Hering and most likely also the smallest branches of the biliary tree, as part of a regenerative response in chronic liver injury (Dwyer et al., 2014). Traditionally, LPCs have been defined as immature stem-like cells, which emerge upon a chronic liver injury insult and have the potential to differentiate into biliary cells or hepatocytes, depending on the underlying injury stimulus (Kohn-Gaone, Gogoi-Tiwari, et al., 2016b). LPC numbers are proportional to the disease severity (Lowes et al., 1999) and, uncontrolled proliferation of LPCs may lead to HCC development (Tirnitz-Parker, Yeoh, & Olynyk., 2012). Knight and colleagues have shown a significant reduction in tumour formation by attenuating c-kit expressing LPCs using imatinib mesylate in the murine CDE model (Knight, Tirnitz-Parker, & Olynyk, 2008). Apart from their role in regeneration and carcinogenesis, LPCs can also act as regulatory cells (Gratte et al., 2021). LPC expansion occurs simultaneously with fibrogenesis and LPCs infiltrate the liver parenchyma in close temporal and spatial association with HSCs and inflammatory cells. Together these key cell types form a regenerative niche and cross-talk to each other to mediate disease-associated processes. Currently, our knowledge of regulatory molecular networks that orchestrate the chronic injury response is still limited.

A plethora of cytokines, growth factors and signalling mechanisms is known to control activation, proliferation and differentiation of LPCs during chronic liver injury. Depending on the injury stimulus and respective animal models utilised in the study, the expression of signalling pathways can vary (Sato et al., 2019). For the purpose of this project, it was therefore necessary to first investigate the Wnt/β-catenin candidate pathway in LPCs in isolation prior to more detailed

studies of potentially therapeutic pathway inhibition *in vivo*, where LPCs are embedded in a complex niche of molecular and cellular networks.

Wnt/β-catenin signalling represents one of the critical pathways that govern cell proliferation, cell polarity and cell fate determination during embryonic development and adult tissue maintenance (Logan & Nusse, 2004; MacDonald, Tamai, & He, 2009). Due to its role in controlling key biological processes, the Wnt signalling pathway has been associated with various diseases, such as cancer and degenerative genetic diseases (Nusse & Clevers, 2017). Among various intracellular branches of Wnt signalling, the Wnt/β-catenin signalling pathway, also known as the canonical Wnt signalling pathway, is the most studied (Kestler & Kühl, 2008). In the liver, Wnt/β-catenin signalling plays a role in development, homeostasis, regeneration and carcinogenesis (Shirolkar et al., 2018). Additionally, Wnt signalling is also associated with metabolic diseases, including obesity, diabetes, NAFLD and atherosclerosis (Ackers & Malgor, 2018).

However, the exact role of Wnt/β-catenin signalling in NASH and fibrosis progression is not clear, as contradictory findings have been observed previously (Monga, 2015). Myung *et al.* demonstrated that canonical Wnt signalling participates in HSCs activation and survival, while working with the immortalised human HSC line LX-2, treated with Wnt3a and sFRP1 (Myung et al., 2007). Further, a study by Yin and colleagues suggested that expression of the Wnt agonist R-spondin2 is upregulated in human fibrotic liver tissues and exogenous stimulation of mice HSCs with R-Spondin2 led to a dose-dependent increase in HSC proliferation and expression of α-SMA and collagen I proteins (Yin et al., 2014). In contrast, Corbett *et al*. reported minimal activation of canonical Wnt signalling in *in vitro* activated HSC systems, such as LX-2 cells and primary HSCs isolated from rat livers (Corbett et al., 2015). Another study demonstrated a similar finding, where Wnt5a promoted proliferation and cytokine production in LX-2 cells without canonical Wnt signalling participation (Dong et al., 2015). As the expression of Wnt/β-catenin signalling can vary under different conditions, more studies are required to characterise context-specific pathway expression.

Currently, the research for the development of anti-NASH agents revolves around the pathways that drive metabolic alterations in NAFLD or pathways that govern cell stress, apoptosis, inflammation and fibrogenesis (Banini & Sanyal, 2017). As LPCs and the Wnt/β-catenin signalling pathway are both closely associated with CLD-associated processes, the research described in this thesis sought to investigate the expression of the Wnt/β-catenin pathway in LPCs in NAFLD, using archival liver biopsy samples of NAFLD patients, an *in vivo* model of murine chronic liver injury and an *in vitro* LPC culture system.

The presentation of β-catenin^{+} hepatic cells in clinical specimens of NAFLD was studied using immunofluorescent techniques. The results described in Chapter 3 suggested an increase in LPC numbers with progression of the NAFLD spectrum in human liver biopsy samples. In addition, subsets of LPCs were found to express cytoplasmic/nuclear β-catenin, a central regulator of the Wnt/β-catenin pathway, indicating active signalling and suggesting LPC subpopulations as potential target cells of pharmacological Wnt/β-catenin signalling inhibition.

Chapter 4 of this thesis then characterised the CDE diet as a potential experimental model of NAFLD and NASH, and investigated the Wnt/β-catenin pathway at different stages of CDE-mediated liver injury.

An ideal animal model should reflect all stages of the human NAFLD spectrum and also capture the underlying metabolic dysfunction as observed in NAFLD patients. Moreover, it should be stable, reliable and reproducible with low mortality rates (Zhong, Zhou, Xu, & Gao, 2020). C57BL/6 is the preferred mouse strain in NAFLD studies due to its inherent predisposition to develop obesity, diabetes mellitus and NAFLD (Van Herck, Vonghia, & Francque, 2017). Administration of a high fat diet in different mouse strains has been demonstrated to lead to the development of hepatic steatosis. However, it does not produce liver injury or inflammation as observed in NASH. In contrast, feeding mice with a toxic and nutrient-deficient diet, such as the choline-deficient regimen, can generate a more severe, NASH-like pathology (Febbraio et al., 2019). Supplementing a choline-deficient diet with ethionine further accelerates the development of steatohepatitis (Tsurusaki et al., 2019).

Importantly, the choline-deficient model is clinically relevant as it has been reported that low choline intake in humans can lead to the development of hepatic steatosis (Corbin & Zeisel, 2012). However, a major drawback of choline-deficient diet administration is that it does not mirror metabolic alterations such as obesity and insulin resistance, which are commonly associated with human NAFLD (Hansen et al., 2017; Jahn, Kircher, Hermanns, & Geier, 2019).

The findings presented in Chapter 4, along with the data of Köhn-Gaone, Dwyer *et al.* indicate that the CDE diet is an appropriate mouse model to study hallmarks of NAFLD such as steatosis, inflammation and fibrosis (Kohn-Gaone, Dwyer, et al., 2016a). Although the CDE model captures the histopathological changes as observed in human NAFLD, the site of injury is different. In CDE, the injury is detected in periportal lobular regions, whereas, in adult human NAFLD, it is perivenular (Brunt & Tiniakos, 2010). However, this disparity may not be a major disadvantage for this project, as the focus of the research is on LPCs in NAFLD. LPC compartment activation starts in portal areas and the CDE diet reliably initiates LPC activation. Thus, it was an attractive model to study Wnt/β-catenin signalling in LPCs in NAFLD. The results presented in this chapter suggest an overall activation of the Wnt/β-catenin pathway in CDE-treated mice compared to controls. Additionally, among all the non-parenchymal cell types in the injury-regeneration niche, LPCs express active β-catenin and signal through this pathway. RT-PCR microarray data of the day 21 time point suggests upregulation in Wnt5b and Fz receptors 1,4 and 9 in CDE-treated compared to healthy livers. However, the cellular localisation of these Wnt ligands and Fz receptors was not studied due to the unavailability of sensitive antibodies. Powerful RNAscope technology has recently become available in our laboratory for future spatial investigations on the cell-specific expression of Wnt ligand and receptor transcripts.

Since it is difficult to determine the direct effects of lipid loading on Wnt/β-catenin signalling in LPCs in complex *in vivo* microenvironments, a novel LPC-focussed culture model of steatosis was established. Chapter 5 describes the

establishment of a steatotic LPC culture model using the BMOL-TAT cell line treated with palmitate and oleate, and the effect of lipid loading on the expression of the Wnt/β-catenin pathway.

The experimental conditions of lipid exposure without excessive cytotoxicity were defined by studying cell viability and fat accumulation. BMOL-TAT cells were able to induce steatosis at 6.25 µM of palmitate and 100 µM of oleate treatment in the absence of significant toxicity. Additionally, Wnt/β-catenin pathway activity in oleate-loaded cells was demonstrated by (a) a strong cytoplasmic/nuclear localisation of active β-catenin in a subset of BMOL-TAT cells, (b) the significant upregulation in the expression of pathway components such as Wnts, Fz receptors, LRP5, Dvl, β-catenin and (c) the significant downregulation in the expression of pathway inhibitors. In contrast, palmitate loading did not significantly upregulate the expression of β-catenin at the transcript and protein level, even though cytoplasmic/nuclear active β-catenin was present in a subset of cells. Oleate is a monounsaturated FFA, whereas palmitate is saturated FFA. This difference in the molecular structure and, thus, type of FFA may be responsible for the variation in the activation status of the pathway, in response to palmitate or oleate. Earlier studies have suggested the effects of nutrient availability on Wnt signalling activation. Anagnostou and Shepherd demonstrated that glucose induced autocrine activation of Wnt/β-catenin signalling in the macrophage cell lines J774.2 and RAW264.7 (Anagnostou & Shepherd, 2008). Palmitoylation of Wnt ligands is necessary for proper binding of the ligands with the receptors and their subsequent internalisation (Willert et al., 2003). In addition, availability of the lipids can influence the palmitoylation process and eventually have an effect on Wnt signalling activation (Sethi & Vidal-Puig, 2010). Although oleate loading modulated Wnt/β-catenin signalling expression in BMOL-TAT cells, it did not change the expression of the common Wnt target gene cyclin D1 significantly. This observation suggests that in oleate-loaded BMOL-TAT cells, Wnt/β-catenin signalling may be regulating other cellular processes and not just proliferation. In the future, it will be interesting to develop β-catenin-deleted BMOL-TAT cells to study β-catenin-independent effects of cellular lipid loading. Moreover, this steatotic cell culture model will also be used to perform

conditioned medium experiments with HSCs and inflammatory cells to understand the specific effects these cell types exert upon each other in NAFLD-like microenvironments.

Earlier stages of the NAFLD spectrum often remain undiagnosed until disease progression has reached a fibrotic stage. In addition, patients with advanced fibrosis have a higher risk to progress to end-stage liver disease compared to those with simple steatosis (Angulo et al., 2007). An earlier study reported that long-term suppression of hepatitis B virus replication with tenofovir disoproxil fumarate led to regression of fibrosis (Marcellin et al., 2013). However, such elimination of the liver injury insult is difficult in NASH-related fibrosis due to the complex aetiology of the disease. As a consequence, development of an anti-fibrotic therapy that can regress established fibrosis or at least halt fibrosis progression in NAFLD is an important research goal. The Wnt/β-catenin pathway antagonist PRI-724, which has previously been reported as an anti-fibrotic agent (Okazaki et al., 2019; Tokunaga et al., 2017; Whitlock et al., 2014) was tested in the novel lipid-loaded LPC culture model and in experimental CLD induced by CDE feeding.

The dose-dependent reduction in cell viability in oleate-loaded BMOL-TAT cells was achieved with PRI-724. Moreover, PRI-724 administration for 23 days to CDE-treated mice led to a significant reduction in Ki-67$^+$ proliferating cells and A6$^+$ ductal cells and LPCs as well as CD11b$^+$ monocyte-derived macrophages. These data confirm the responsiveness of LPCs to Wnt/β-catenin pathway modulation and demonstrate anti-proliferative effects of PRI-724 in LPCs and a subpopulation of inflammatory cells. Whether the effects on CD11b$^+$ cells are of a direct or indirect nature will have to be investigated in future studies.

Further, at day 23, a significant decrease in collagen deposition was not observed as judged by Sirius Red staining and hydroxyproline assay. However, a strong trend suggests that further investigation with a greater number of animals is required to establish whether PRI-724 exerts significant anti-fibrotic effects in the CDE model of chronic liver injury. In addition, future experiments will study the effect of PRI-724 on defined subsets of LPCs using

flow cytometry analysis to further define the specific PRI-724-responsive LPC populations.

The increasing global prevalence of NAFLD is worrisome, as it adds clinical and economic burdens on the healthcare systems. A lack of therapeutic options for the treatment of NAFLD warrants more studies to understand the underlying complex molecular signalling networks. LPCs represent a key regulatory cell type that gets activated in most chronic liver injury settings. They play important roles in cellular cross-talk with neighbouring cells, including fibrosis-driving HSCs and disease pathology-modifying inflammatory cells, and therefore signify attractive therapeutic target cells. Wnt/β-catenin signalling is one of the prominent pathways controlling cellular proliferation as well as metabolism in the liver and was demonstrated to play key roles in LPCs proliferating in the setting of NAFLD and NASH.

In conclusion, the studies presented in this thesis systematically show the activation of the Wnt/β-catenin pathway in a subset of LPCs (a) in NAFLD patients, (b) in the CDE mouse model of chronic liver injury and (c) in a fat-loaded LPC culture system. In addition, pharmacological pathway inhibition studies suggest a potential for therapeutic targeting of LPCs using the Wnt/β-catenin pathway inhibitor PRI-724.

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APPENDIX

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REVIEW ARTICLE

Wnt/β-Catenin Signalling during Liver Metabolism, Chronic Liver Disease and Hepatocarcinogenesis

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Abstract

Chronic liver diseases (CLDs) are increasing in prevalence and their end-stage complications, namely, cirrhosis, liver failure and hepatocellular carcinoma represent major global challenges. The most common initiators of progressive CLD are viral hepatitis and long-term alcohol abuse as well as steatosis and steatohepatitis. Irrespective of the underlying aetiology, a common feature of CLD is the formation of hepatic ductular reactions, involving the proliferation of liver progenitor cells (LPCs) and their signalling to fibrosis-driving hepatic stellate cells. The Wnt/β-catenin pathway has been found to regulate development, stemness and differentiation, and alterations in its activity have been associated with tumour development. Recent data highlight the role of Wnt/β-catenin signalling in hepatic metabolism, steatosis and cancer, and suggest targeting of this pathway as a promising molecular strategy to potentially inhibit CLD progression and hepatocarcinogenesis.

Keywords: chronic liver disease; hepatocellular carcinoma; liver progenitor cells; metabolic syndrome; Wnt/β-catenin signalling

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Introduction

Chronic liver disease (CLD) has become one of the most common causes of death globally with an estimated 1.03 million deaths per year, as reported in 2017. Excessive alcohol consumption, viral hepatitis and hepatic steatosis are the most prevalent risk factors for the initiation and progression of CLD (1). A UK report stated that standardised CLD mortality rates have increased by 400% since 1970, reflecting its growing burden and major challenge for global health (2). End-stage complications of CLD include cirrhosis, liver failure and malignancies, with hepatocellular carcinoma constituting 85–90% of all liver cancers (3). Current therapy options for hepatocellular carcinoma include surgical resection, radiofrequency ablation, transarterial chemoembolisation

and orthotopic liver transplantation. The multikinase inhibitors sorafenib and regorafenib are the only systemic treatments with proven survival benefits and they prolong the life expectancy of patients by 2 to 3 months (4). Immune-based approaches, including targeting of the immune checkpoint inhibitors programmed cell death (PD-1), programmed cell death ligand 1 (PD-L1) or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), represent novel, promising therapeutic strategies to prevent or treat hepatocellular carcinoma (5).

Chronic Liver Disease and the Ductular Reaction

CLD induces molecular and cellular processes, which are initially reparative but become detrimental in the prolonged setting. Damaged liver epithelial cells release pro-inflammatory signalling molecules, which recruit immune cells to the site of injury, induce collagen deposition or fibrosis and activate liver progenitor cells (LPCs) as part of the so-called 'ductular reactions' to restore lost liver tissue. The term ductular reaction describes the diverse histological phenomena occurring in response to chronic hepatic injury and encompasses the epithelial component as well as inflammatory and fibrogenic changes (6). Ductular reactions are observed in all forms of CLD with hepatocyte injury and replicative arrest. However, depending on the underlying aetiology, they display diverse morphologies, ranging from well-formed ductules to irregular strings of cells without obvious lumina (7). Irrespective of the injury stimulus, ductular reactions, activated biliary epithelial cells and LPCs are generally closely associated with inflammatory cell populations and fibrosis-driving, activated hepatic stellate cells, forming a very dynamic injury and regeneration niche (Figure 1). Although significant differences in injury and repair dynamics can be observed in different forms of CLD (8), epithelial, inflammatory and fibrogenic cells principally orchestrate liver regeneration versus disease progression through chemokine and cytokine crosstalk in all clinical settings (6, 9–13).

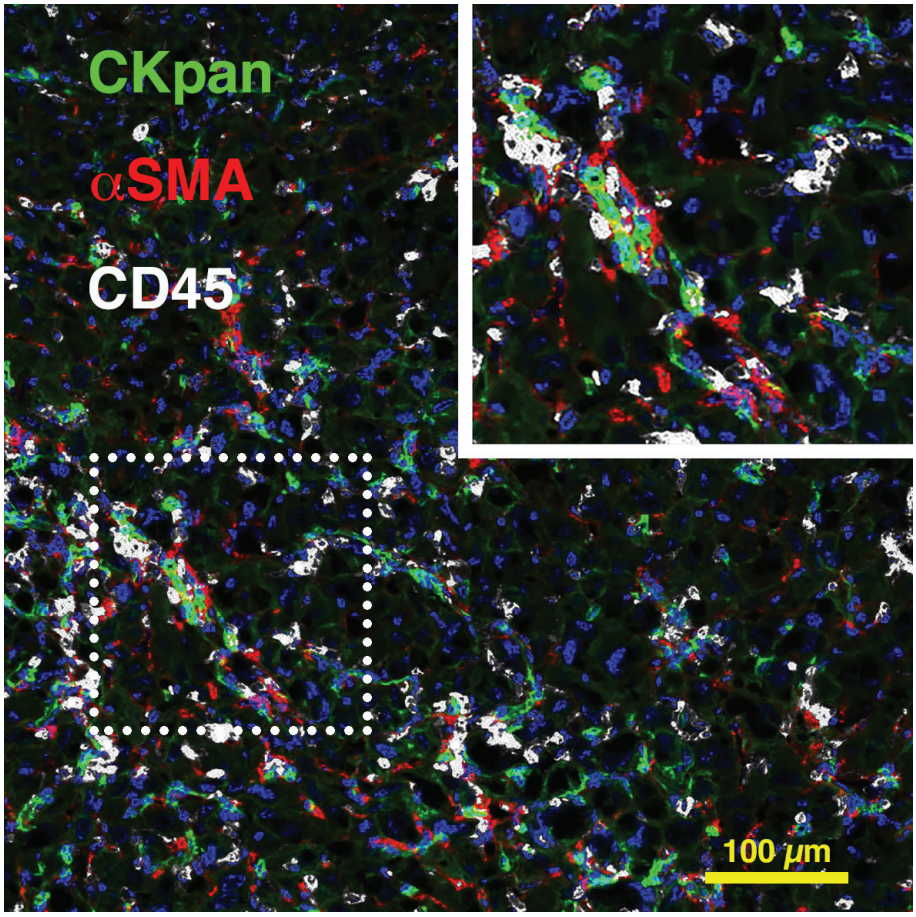

Figure 1. The injury and regeneration niche during chronic liver injury. Murine chronic liver injury induced by feeding a choline-deficient, ethionine-supplemented diet [15] leads to formation of an injury and regeneration niche, involving CKpan$^+$ ductular cells and LPCs (green), αSMA$^+$ hepatic stellate cells (red) and CD45$^+$ inflammatory cells (white). DAPI was used for nuclear localisation.

Liver Progenitor Cells and Cancer Stem Cells

LPCs are defined as a heterogeneous pool of immature, bipotential hepatic cells with diverse marker expression profiles and the ability to differentiate into either hepatocytes or biliary epithelial cells, depending on the underlying injury stimulus and thus tissue requirements. They are undetectable in healthy liver but upon injury emerge in portal areas near the Canals of Hering. Their origin and liver repopulation capacity have been controversially discussed (14). Studies using the choline-deficient, ethionine-supplemented model of chronic liver injury (15) reported that LPCs expressing osteopontin (16) or Foxl1 (17) contributed to hepatocellular regeneration. In addition, transplantation of clonogenic LPCs into hepatocyte-senescent murine livers, induced through deletion of the E3 ubiquitin ligase Mdm2, resulted in restoration of the hepatic parenchyma through generation of hepatocytic or biliary epithelia (18). The exact underlying mechanisms of LPC-mediated liver regeneration are not always clear; however, hepatocyte senescence seems to be a definite histological requirement (19).

The degree of LPC proliferation directly correlates with the severity of hepatocyte replicative arrest and the inflammatory and fibrogenic responses to CLD (20). Targeting of c-kit$^+$ LPCs through the multikinase inhibitor imatinib mesylate during experimental chronic liver injury resulted in reduced fibrogenesis and carcinogenesis (21). Moreover, the presence of hepatobiliary LPCs, marked by epithelial cell adhesion molecule (EpCAM) and cytokeratin 7 and 19, predicted an increased risk of tumour formation in cirrhotic, hepatitis C virus–infected patients (22). This suggests that some LPCs either indirectly influence tumour development by regulating the fibrogenic potential and chemotaxis of neighbouring hepatic stellate cells (9, 12, 13, 23) or directly as tumour-initiating or cancer stem cells (CSCs).

In general, CSCs are defined as undifferentiated cells that are capable to self-renew, initiate and maintain tumour growth and may be responsible for tumour recurrence after resection. Haraguchi and colleagues first postulated the existence of liver CSCs, based on the finding that the hepatocellular carcinoma cell lines HuH7 and Hep3B contained 0.9–1.8% of side population cells with the ability to efflux the fluorescent nucleic acid-binding dye Hoechst 33342 through high activity of adenosine triphosphate-binding cassette transporters (24). Similar side population cells successfully induced xenograft tumours upon transplantation into immunodeficient NOD/SCID mice, while no tumour formation was observed when non-side population cells were transplanted (25). Subsequently, numerous studies have focussed on the identification of reliable marker expression profiles for liver CSCs. The CD133$^+$ subpopulation of various hepatocellular carcinoma cell lines displayed a more immature, proliferative phenotype with greater colony formation capacity *in vitro* and upon xenotransplantation a higher tumorigenic potential compared to the CD133$^-$ cellular counterpart (26–28). Within the CD133$^+$ population, cells with the expression profile CD133$^+$CD44$^+$ have been described as more tumorigenic and metastatic than CD133$^+$CD44$^-$ cells (29, 30). Other studies have suggested the mucin-like cell surface glycoprotein CD24 (31) and the glycosylphosphatidylinositol-anchored glycoprotein CD90 or Thy-1 (32) as liver CSC markers. The transmembrane glycoprotein EpCAM is expressed by normal LPCs and CSCs and regulates cell–cell adhesion, proliferation, migration, differentiation and invasion (33). EpCAM is transcriptionally activated by the Wnt/β-catenin pathway, while inhibition of Wnt/β-catenin signalling was shown to suppress its expression (34). Interestingly, both CD44 and CD24 are direct Wnt target genes, marking this signalling pathway a key player in CSC biology and therefore a potential therapeutic target to prevent or treat hepatocellular carcinoma.

There is strong experimental evidence for Wnt signalling directly regulating the biology of LPCs and CSCs. Using the 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) model of chronic liver injury, Hu *et al*. demonstrated Wnt/β-catenin signalling activity in proliferating A6$^+$ LPCs and ductular reactions. Primary LPCs showed active, nuclear β-catenin and entered the cell cycle upon Wnt3a stimulation *in vitro* (35). A constitutively active β-catenin mutant was shown to promote LPC expansion in rodents subjected to the 2-acetylaminofluorene/partial hepatectomy model. In addition, the less differentiated, LPC-like, OV6$^+$ subpopulation of hepatocellular carcinoma cells displayed endogenously active Wnt/β-catenin signalling, coupled with a more aggressive phenotype, as judged by greater tumorigenicity and chemoresistance (36). Boulter and colleagues reported Wnt3a-induced expression of the ubiquitin ligase Numb, which is required to leave the biliary differentiation path, and hepatocyte nuclear factor 4α in the LPC line BMOL (37), together inducing its differentiation towards the hepatocyte lineage (38).

The Wnt/β-Catenin Signalling Pathway

The Wnt signalling pathway is highly conserved and has been associated with embryogenesis, proliferation, differentiation as well as carcinogenesis (39–41). It consists of 19 Wnt ligands, 10 Wnt receptors, referred to as frizzleds (FZD), a family of co-receptors, including low-density lipoprotein receptor-related proteins 5 and 6 (LRP5 and 6), and two branches of the pathway exist (42, 43). The non-canonical pathway comprises the planar cell polarity pathway (PCP) and the Ca^{2+} pathway. These are β-catenin-independent pathways that play roles in the regulation of the actin cytoskeleton and cytoskeletal rearrangement and will not be discussed further. In contrast, the canonical pathway is β-catenin-dependent and of particular interest therapeutically, as aberrant activation of this pathway has been postulated as a key driver in many malignancies such as prostate, colorectal, ovarian and liver cancer (41).

During the inactive 'off' state, there is no Wnt ligand bound to the FZD receptor, which results in the multi-protein destruction complex, consisting of glycogen synthase kinase 3β (GSK3β), casein kinase 1 (CK1), adenomatous polyposis coli (APC) and Axin, to bind β-catenin. The destruction complex then phosphorylates β-catenin in a sequential pattern on residues serine 33 (S33), serine 37 (S37) and threonine (T41). Beta-catenin is then ubiquitinated by the E3-ligase beta-transducin repeat containing protein (βTRCP) and marked for proteasomal degradation, preventing it from translocating to the nucleus. The transcription repressor Groucho remains bound to T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors, inhibiting transcription of target genes such as c-Myc and cyclin D1 (Figure 2) (40). Conversely, in the active 'on' state, a Wnt ligand binds to a FZD receptor, activating the protein dishevelled (Dvl), a cytoplasmic phosphoprotein crucial for Wnt signal transduction. Axin is recruited to the plasma membrane, binding to the co-receptor LRP5/6 and inhibiting GSK3β and the destruction complex. This allows unphosphorylated β-catenin to accumulate in the cytoplasm and translocate into the nucleus, where Groucho is displaced and unbound from TCF/LEF transcription factors. In this case, β-catenin is able to bind and activate downstream signalling (Figure 2) (40). It has been estimated that Wnt/β-catenin signalling regulates the expression of more than 80 target genes involved in cell fate determination, development, regeneration, zonation, metabolism, fibrosis and carcinogenesis of the liver (44, 45).

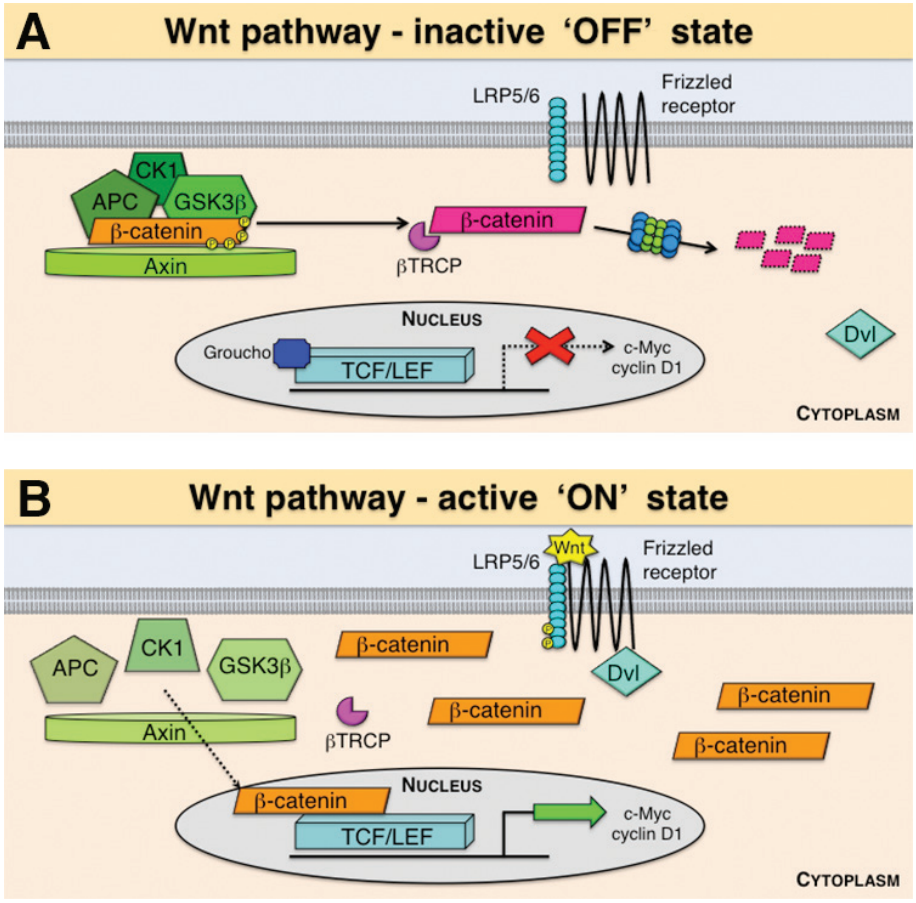

Figure 2. The canonical Wnt/β-catenin pathway. In the absence of a Wnt signal ('OFF' state), the destruction complex, consisting of adenomatosis polyposis coli (APC), glycogen synthase kinase 3-β (GSK3β), casein kinase 1 (CK1) and Axin, binds and phosphorylates β-catenin, marking it for ubiquitination by the E3 ubiquitin ligase subunit beta-transducin repeat containing protein (βTRCP) and degradation through the proteasome. In this case, the repressor Groucho remains bound to T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors, inhibiting transcription of target genes such as c-Myc and cyclin D1 (A). When a Wnt protein binds a Frizzled receptor and the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) in the 'ON' state, the protein dishevelled (Dvl) activates a cascade, which eventually disrupts the destruction complex, leading to stabilisation, cytoplasmic accumulation and nuclear translocation of β-catenin and ultimately the transcription of target genes (B).

Wnt/β-Catenin Signalling in Liver Metabolism

The liver regulates metabolic homeostasis by controlling glycogen storage, gluconeogenesis, plasma protein synthesis, lipoprotein synthesis and detoxification. To manage fluctuating metabolic demands, hepatic cells constantly alter the expression of respective regulatory pathways. Accordingly, hepatic Wnt signalling activity is modified under different physiological and pathophysiological conditions (45). In adult healthy hepatocytes, β-catenin is ubiquitously expressed, but it is more active in pericentral compared to periportal hepatocytes (44). Expression of β-catenin in periportal regions is inhibited by hepatocyte nuclear factor 4α (46). In contrast, pericentral hepatocytes display basal activation of β-catenin signalling, controlling expression levels of glutamine synthetase, ornithine aminotransferase and the glutamate transporter GLT-1, which together regulate glutamine metabolism (47). This heterogeneous distribution of metabolic function across the lobule reflects hepatic zonation and is necessary to achieve optimal metabolic regulation. Benhamouche and colleagues established that the Wnt/β-catenin pathway is a major control switch pathway for metabolic zonation by demonstrating that blocking of β-catenin in hepatocytes by infection with an adenovirus encoding the Wnt signalling antagonist Dickkopf-1 (Dkk-1) resulted in expansion of the periportal transcriptome and downregulation of perivenous genes. Conversely, constitutive activation of β-catenin through liver-induced disruption of the negative regulator APC reversed this gene expression profile and induced the perivenous gene expression programme (48).

The localisation and signalling activity of β-catenin becomes modified upon liver injury (Figure 3) (44, 49). Debebe and colleagues demonstrated recently that hepatic steatosis experimentally induced by feeding of a high fat diet, deletion of phosphatase and tensin homologue deleted on chromosome 10 (*Pten*) or transgenic expression of HCV core/NS5A protein, all resulted in macrophage-secreted Wnt activating $CD133^{+}CD49f^{+}$ tumour-initiating cells. These data strongly suggested a Wnt/β-catenin-mediated link between obesity and cancer (50). In addition, β-catenin was shown to regulate hepatic gluconeogenesis during starvation and insulin-resistant conditions via interaction with the transcription factor forkhead box protein O 1 (FoxO1). This interaction leads to a change in expression of genes encoding the enzymes glucose-6-phosphatase and phosphoenolpyruvate carboxykinase, which then determine the rate of hepatic gluconeogenesis (44, 51). During oxidative stress conditions, β-catenin interacts with FOXO and enhances FOXO transcriptional activity to induce expression of targets for detoxification of reactive oxygen species (52). FOXO factors are sensitive to increased insulin levels, hence the interaction of β-catenin and FOXO is particularly important in diseases associated with insulin resistance, such as non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH) and the metabolic syndrome in general (44). The metabolic syndrome, previously known as the insulin-resistance syndrome, has been defined as a clustering of the risk factors, namely, central obesity, hypertension, hypertriglyceridaemia, hyperglycaemia and low levels of high-density lipoprotein (53). Metabolic syndrome as well as NAFLD are associated with reduced insulin sensitivity and decreased insulin effects on glucose and lipid metabolism (54).

Numerous studies have established a role of the Wnt/β-catenin pathway in the metabolic syndrome since it was demonstrated that Wnt signalling represents a molecular switch to control adipogenesis. Activation of the canonical Wnt/β-catenin pathway through Wnt10b, inhibition of GSK3β or expression of dominant stable β-catenin prevented differentiation of preadipocytes and myoblasts through inhibition of the adipogenic transcription factors CCAAT/enhancer-binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) (55, 56). Kennell and MacDougald investigated *Xenopus* Wnt8 and FZD1 or FZD2 chimeras and established a role for both β-catenin-dependent and β-catenin-independent mechanisms in mesenchymal cell fate and adipogenesis (57). Conversely, in a recent report, inhibition of Wnt signalling through the Wnt-inhibitory molecule sclerostin led to spontaneous adipogenesis of pre-adipocytes and mesenchymal precursors (58), supporting the concept of Wnt signalling controlling the adipogenic switch.

Wnt/β-Catenin Signalling and Cancer

In hepatocellular carcinoma cells, the most frequent mutations occur in *TP53*, coding for the tumour suppressor p53, and *CTNNB1*, the β-catenin gene (59). Coste and colleagues suggested that 26% of human and 50% of mouse hepatocellular carcinomas carry activating mutations in *CTNNB1* (60). Subsequent studies supported these findings and reported up to 44% of hepatocellular carcinomas with *CTNNB1* mutations (61–64). Activation of β-catenin is hypothesised to be due to mutations in exon-3 at the serine and/or threonine sites near the NH_2 terminus in *CTNNB1*, which inhibit phosphorylation-dependent degradation of the β-catenin protein, leading to an aberrant activation of the canonical Wnt/β-catenin pathway (44). Simultaneous mutation of the β-catenin gene and the tumour suppressor H-ras or p21 by an adenovirus-mediated liver-specific Cre-expression system resulted in 100% tumour incidence with short latency of only several weeks (65). Interestingly, mutations in the β-catenin gene in hepatocellular carcinomas induce overexpression of β-catenin targets such as the glutamine synthetase gene *GLUL* (66, 67), whereas hepatocytes from glutamine synthetase-negative tumours are often *H-ras* or *BRAF* mutated and express E-cadherin, reflecting perivenous and periportal profiles, respectively (68). Loss-of-function in *APC* and *Axin* is mutually exclusive to *CTNNB1* mutations and has been detected in 1–3% and 8–15% of hepatocellular carcinoma cases (for review, see (69)).

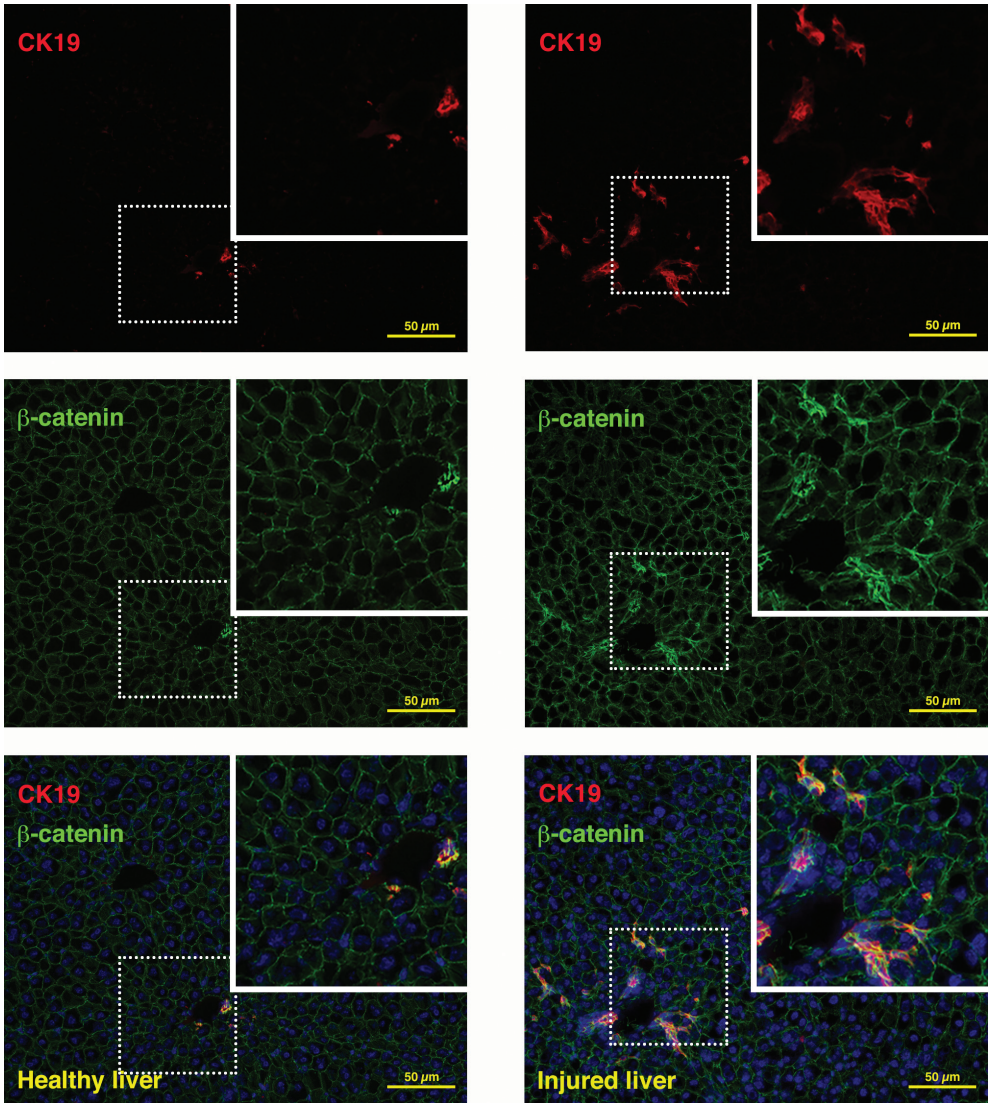

Figure 3. Beta-catenin and CK19 expression in healthy and injured liver. In healthy mouse liver, only ducts stain with an antibody targeting CK19 and show cytoplasmic and nuclear β-catenin expression, while β-catenin is exclusively membrane-bound in periportal hepatocytes (healthy liver, left panel). In injured liver (2-week treatment with a choline-deficient, ethionine-supplemented diet[15]), the CK19$^+$ compartment expands and demonstrates strong cytoplasmic and nuclear β-catenin, signifying active signalling (injured liver, right panel).

In a 2009 study, the Wnt ligands Wnt3, Wnt9a and Wnt10b were shown to be highly expressed in most hepatocellular carcinoma cell lines, irrespective of their differentiation status. Clear profiles were, however, observed with Wnt2b, Wnt4, Wnt5a, Wnt5b and Wnt7b, which were overexpressed in poorly differentiated cell lines, while Wnt8b and Wnt9b were only expressed in well-differentiated cell lines. These data suggested canonical Wnt signalling activity in well-differentiated cells, contributing to tumour initiation and its repression in poorly differentiated cell lines, which the authors hypothesised to regulate tumour progression (70). Other Wnt pathway components associated with hepatocellular carcinoma development include Wnt signalling antagonists such as secreted frizzled-related proteins (SFRPs), Wnt-inhibitory factor (WIF)-1 and Dickkopf (Dkk) proteins. SFRP1 has been suggested as a tumour suppressor gene, since its expression was downregulated due to promoter hypermethylation in 76.1% of hepatocellular carcinoma specimens at the RNA level and in 30% at the protein level (71). In hepatocellular carcinoma cell lines and clinical specimens, WIF-1 expression was equally found to be repressed by promoter hypermethylation, suggesting epigenetic inactivation as the primary cause

for WIF-1 loss during hepatocarcinogenesis (72). Inactivity of the negative Wnt regulators Dkk2 and Dkk3 has been reported in human gastrointestinal tumours (73). Fatima and colleagues observed significantly reduced mRNA expression of Dkk4 in almost half of all investigated hepatocellular carcinoma cases. Immunohistochemical data linked decreased Dkk4 expression to accumulation of β-catenin in hepatocellular carcinoma tissue. In addition, the authors showed that Dkk4 overexpression in hepatocellular carcinoma cell lines resulted in reduced cell proliferation, colony formation and cell migration, suggesting a tumour-suppressive role for Dkk4 (74). A recent study demonstrated that hepatocellular carcinoma cells proliferate upon stimulation in high glucose conditions as a result of Dkk4 downregulation, allowing Wnt3a-mediated β-catenin signalling and c-Myc upregulation (75), suggesting the Wnt pathway may be a therapeutic target in insulin-resistant conditions, leading to hepatocellular carcinoma.

Conclusion

Many diverse signalling pathways regulate liver development, homeostasis, regeneration and carcinogenesis. Given the strong evidence for an association of (i) progressive liver disease and LPCs, (ii) CSC-like LPCs and liver tumour formation and (iii) obesity, insulin resistance, hepatic steatosis and hepatocarcinogenesis, and the fact that the Wnt/β-catenin signalling seems to be playing major roles in all these processes, this pathway represents a particularly promising therapeutic target to prevent or treat hepatocellular carcinoma.

Conflict of interest statement

The authors report no conflict of interest with respect to research, authorship and/or publication of this article.

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