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The role of 11β-hydroxysteroid dehydrogenase type 1 in liver fibrosis and inflammation in non-alcoholic fatty liver disease

Xiantong Zou

Doctor of Philosophy
University of Edinburgh
2013


Declaration

I declare that this thesis and the work described within are solely my own work with the following exceptions:

Liver function tests were undertaken by Dr. Forbes Howie in the MRC Centre for Reproductive Health. Routine formalin fixed liver tissue processing and paraffin embedding were performed in the Queen’s Medical Research Institute Histology service.

This work has not been submitted previously at this or any other university for a higher degree.

Xiantong Zou
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Dedication

I would like to dedicate this work to my grandmother, Ms Huilan Liu, who has been patient, loving, and gracious towards me for the past 28 years.

谨以此文献给一直在关心我，爱护我，支持我的外婆，刘慧兰女士。
Abstract

Non-alcoholic fatty liver disease (NAFLD) is a worldwide health problem which includes steatosis (triglyceride accumulation alone), non-alcoholic steatohepatitis (NASH, with liver inflammation), fibrosis, cirrhosis and hepatocellular carcinoma. Liver fibrosis, which is a reversible response, is the final phase of most chronic liver disease and is characterized by accumulation of extracellular matrix (ECM) from activated hepatic stellate cells (HSCs). Glucocorticoids (GCs) regulate many aspects of metabolism involved in NAFLD. Also, GCs limit HSC activation in vitro. Tissue GC levels are regulated by 11β-hydroxysteroid dehydrogenase-1 (11β-HSD1) which converts inactive 11-dehydrocorticosterone (DHC) into active corticosterone. Previous studies demonstrate that 11β-HSD1 deficiency improves fatty liver in obesity models, but the role of 11β-HSD1 in mechanisms involved in the progression and/or resolution of hepatic injury is largely unknown. I hypothesized that 11β-HSD1 modulates fibrotic and inflammatory responses during hepatic injury and/or the resolution phase.

First I sought to address if the levels of 11β-HSD1 during different models of liver injury are dysregulated. In mice, 11β-HSD1 was down-regulated in choline deficient diet (CDD) induced steatosis, methionine and choline deficient diet (MCDD) induced NASH, carbon tetrachloride (CCL4) induced liver fibrosis and thioacetamide (TAA) induced liver fibrosis. In CCL4 injured livers, the down regulation of 11β-HSD1 was observed around the scar area. To test if 11β-HSD1 plays a key role in modulating liver inflammation and fibrosis responses in NAFLD and liver fibrosis I used initially11β-HSD1 knockout (KO) mice. 11β-HSD1 KO showed higher HSC activation only in the High fat feeding model but not in CDD and MCDD models. In the CCL4 injury model, despite reduced hepatocellular injury, 11β-HSD1 KO mice showed enhanced collagen deposition during peak injury and increased fibrotic gene expression during the early resolution phase although unaltered inflammatory markers during both peak injury and resolution. To further dissect cell-specificity on the effect of 11β-HSD1, I repeated the CCL4-injury model using the hepatocyte-specific 11β-HSD1 KO (Alb-HSD1). Alb-HSD1 mice did not show increased susceptibility to fibrosis compared to control littermates suggesting that the 11β-
HSD1 possibly modulates fibrotic response by affecting HSC function. To mechanistically address how GCs inhibit HSC activation in vitro I studied the effects of 11β-HSD1 on HSC in vitro. 11β-HSD1 expression was down-regulated during ‘spontaneous’ HSC activation, and 11β-HSD1 deficiency enhanced susceptibility to activation. The GC (11-DHC)’s inhibitory effect on HSC activation was reversed by 11β-HSD1 inhibition.

Finally, to address the clinical relevance of 11β-HSD1 in hepatic injury and/or resolution a selective 11β-HSD1 inhibitor, UE2316, was used. UE2316 induced a pro-fibrotic phenotype in ob/ob mice and CCL4-treated C57BL/6 mice, but had no effect when administered only during injury resolution.

In conclusion, 11β-HSD1 deficiency causes increased activation of HSCs following diet and chemical injury and promotes liver fibrosis. Effects of 11β-HSD1 inhibitors, which are a potential treatment for metabolic syndrome, are perhaps offset by adverse outcomes in liver.
MEETING ABSTRACTS


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<tr>
<td>11β-HSD</td>
<td>11β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>A</td>
<td>11-dehydrocorticosterone</td>
</tr>
<tr>
<td>Acta2 (αSMA)</td>
<td>actin alpha 2 gene encoding αSMA</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
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<td>ALT</td>
<td>alanine aminotransferase</td>
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<tr>
<td>cDNA</td>
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<td>COL-1 and -3</td>
<td>collagen type I and III</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Cp</td>
<td>crossing point</td>
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</table>
IL interleukin

IκBα nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

KO knockout

LPL lipoprotein lipase

LXα Liver X receptor-α

Ly6C lymphocyte antigen 6C

MCDD methionine and choline deficient diet

MCP-1 monocyte chemotactic protein-1

MMP matrix metalloproteinases

MR Mineralocorticoid receptor

mRNA messenger ribonucleic acid

NAD nicotinamide adenine dinucleotide

NADP(H) nicotinamide adenine dinucleotide phosphate (reduced)

NAFLD non-alcoholic fatty liver disease

NASH non-alcoholic steatohepatitis

NEFA non-esterified fatty acids

NFκB nuclear factor kappa-light-hain-enhancer of activated B cells

PAI-1 plasminogen activator inhibitor type 1

PCR polymerase chain reaction
Chapter 1. Introduction
This thesis concerns the effect of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) on inflammation and fibrogenesis in non-alcoholic fatty liver disease (NAFLD). This chapter will introduce some basic background of NAFLD especially fibrosis; pathogenesis of fibrosis; glucocorticoids background and effect; and the regulation and effect of 11β-HSD1 as well as the relationship between 11β-HSD1 and NAFLD.

1.1. Non-Alcoholic Fatty Liver Disease (NAFLD)

NAFLD is characterized by hepatic steatosis (simple fat accumulation), steatohepatitis (fat accumulation plus inflammation), fibrosis, cirrhosis and in some cases hepatocellular carcinoma in the absence of excessive alcohol consumption (typically a threshold of <20 g a day for women and <30 g a day for men) (Dancygier, 2010, Anstee et al., 2011). NAFLD is strongly associated with metabolic syndrome which is characterized by impaired insulin sensitivity and associated with central obesity, hypertension, impaired glucose tolerance, dyslipidaemia and microalbuminuria. Liver fibrosis is a final stage of most chronic liver disease (CLD). The main causes of liver fibrosis in industrialized countries include chronic Hepatitis C virus (HCV) infection, alcohol abuse, and non-alcoholic steatohepatitis (NASH) (Bataller and Brenner, 2005). Cirrhosis is defined as an advanced stage of fibrosis, characterised by nodules of liver parenchyma which are separated by fibrotic septa and is associated with major angio-architectural changes (Pinzani and Rombouts, 2004).

1.1.1. Epidemiology

The prevalence of NAFLD is increasing along with improved living standards. It is now the most widespread liver disease in the world. The prevalence rate of NAFLD varies from 20% to 30% in different areas (Table 1.1). Age, gender, race and ethnicity, metabolic conditions including type 2 diabetes, polycystic ovarian syndrome (PCOS) and obesity are independent risk factors for NAFLD (Table 1.2).
### Table 1.1 Incidence of NAFLD in different areas in the world

<table>
<thead>
<tr>
<th>Area</th>
<th>Incidence of NAFLD</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brazil</td>
<td>35.2% in Brazil middle-aged and older population</td>
<td>(Karnikowski et al., 2007)</td>
</tr>
<tr>
<td>China</td>
<td>17.29% in general adult Shanghai population</td>
<td>(Fan et al., 2005)</td>
</tr>
<tr>
<td>India</td>
<td>16.6% in general adult Indian population</td>
<td>(Amarapurkar et al., 2007)</td>
</tr>
<tr>
<td>Europe</td>
<td>20% in general Italian population</td>
<td>(Bedogni et al., 2005)</td>
</tr>
<tr>
<td>Japan</td>
<td>29% in apparently healthy middle-aged Japanese adults</td>
<td>(Jimba et al., 2005)</td>
</tr>
<tr>
<td>US</td>
<td>33% in general population</td>
<td>(Browning et al., 2004)</td>
</tr>
</tbody>
</table>
Table 1.2 Independent risk factors of NAFLD prevalence

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Influence on NAFLD distribution</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Increased NAFLD incidents with age</td>
<td>(Frith et al., 2009)</td>
</tr>
<tr>
<td>Gender</td>
<td>42% in white men and 24% in white women</td>
<td>(Browning et al., 2004)</td>
</tr>
<tr>
<td>Race and Ethnicity</td>
<td>NAFLD incident varied in different races: 45% in Hispanics; 33% in whites; 24% in black</td>
<td>(Browning et al., 2004)</td>
</tr>
<tr>
<td>Type 2 Diabetes</td>
<td>69% of diabetic patients showed NAFLD tested by ultra sound</td>
<td>(Leite et al., 2009)</td>
</tr>
<tr>
<td>Polycystic ovarian syndrome (PCOS)</td>
<td>55% patient with PCOS showed hepatic steatosis</td>
<td>(Gambarin-Gelwan et al., 2007)</td>
</tr>
<tr>
<td>Obesity</td>
<td>63% obese patients (mean BMI 42+/-6) were diagnosed with NAFLD</td>
<td>(Boza et al., 2005)</td>
</tr>
</tbody>
</table>

As an advanced stage of NAFLD, the incidence of NASH among the general population is about 2-3% (Falck-Ytter et al., 2001). Insulin resistance, systemic hypertension and increased serum aminotransferase level independently predict the progression from steatosis to NASH (Dixon et al., 2001). In addition, NASH is considered to be a major cause for cryptogenic cirrhosis (Powell et al., 1990, Caldwell et al., 1999). The progression rate from steatosis to NASH varies from 0 to 23% (Table 1.3). Studies indicate the percentage of NASH patients developing
complications of end stage liver disease during long term follow-up is 5.4% (Argo and Caldwell, 2009, Ekstedt et al., 2006). Serum markers including AST, ALT, GTT and ferritin were used to predict the progression of steatosis to NASH and cirrhosis (Fracanzani et al., 2008). However, recent studies found there is no optimal serum ALT level to predict NASH and advanced fibrosis (Verma et al., 2013, Khosravi et al., 2011).

Table 1.3 Natural progression from steatosis to NASH

<table>
<thead>
<tr>
<th>Study</th>
<th>Patient number</th>
<th>Follow up (years)</th>
<th>NASH</th>
<th>Fibrosis/cirrhosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Adams et al., 2005b)</td>
<td>420 steatosis patients</td>
<td>7.6</td>
<td>49 (11.7%)</td>
<td>21 (5%)</td>
</tr>
<tr>
<td>(Wong et al., 2010)</td>
<td>13 steatosis patients</td>
<td>3</td>
<td>3 (23%)</td>
<td>None</td>
</tr>
<tr>
<td>(Teli et al., 1995)</td>
<td>40 steatosis patients</td>
<td>7-16</td>
<td>None</td>
<td>1 (2.5%)</td>
</tr>
</tbody>
</table>

The mortality rate of NAFLD is higher than non-NAFLD people and even higher in advanced stages of NAFLD (Ong et al., 2008). Liver disease is the third leading cause of death among patients with NAFLD (Ong et al., 2008). NASH patients have more liver-related mortality compared to patients with only steatosis in studies with up to 18-year follow-up (Matteoni et al., 1999, Ekstedt et al., 2006). The mortality rate of cirrhosis increased in Britain during 1990s. Between the periods 1987–1991 and 1997–2001, cirrhosis mortality in men doubled (104% increase) in Scotland and rose by over two-thirds (69%) in England and Wales (Leon and McCambridge, 2006). Liver failure is the main cause of the mortality of NASH associated cirrhosis.
(9 of the 23 patients in 10-year follow-up) (Hui et al., 2003). Against this background, understanding the progression of NAFLD is critically important.

1.1.2. Histology

1.1.2.1. Steatosis

As an early form of NAFLD, steatosis is histologically manifest by triglyceride (TG) droplet deposition within the cytoplasm of hepatocytes (Puri et al., 2007). In adult patients, steatosis is most commonly observed in the perivenular, acinar zone 3 hepatocytes (Figure 1.1); in some adult and pediatric cases, steatosis may occupy the entire acinus (Tiniakos et al., 2010).

1.1.2.2. NASH

The histologic criteria for the diagnosis of adult NASH includes steatosis, hepatocellular injury (usually in the form of ballooning), and lobular inflammation (infiltration of inflammatory cells including neutrophils, lymphocytes and macrophages) predominantly in zone 3 (Figure 1.1). The diagnosis of steatohepatitis does not require fibrosis, but peri-sinusoidal fibrosis can be present. With progression, portal and peri-portal fibrosis may develop in conjunction with the zone 3 peri-sinusoidal fibrosis (Brunt, Janney et al. 1999).

1.1.2.3. Fibrosis

Liver fibrosis is characterized by accumulation of hepatic extracellular matrix (ECM) with predominant deposition of fibrillar collagens. Although liver fibrosis/cirrhosis is the final stage of most chronic liver disease, the pattern of fibrosis varies according to the underlying disease. Viral hepatitis causes scarring in peri-portal areas whilst NAFLD fibrosis is located in peri-central or peri-sinusoidal areas (Brunt, 2001). To score the severity of fibrosis, METAVIR and Knodell (Histological activity index, HAI) (Knodell et al., 1981) scores are commonly used for fibrosis that has evolved from viral hepatitis and portal obstruction, whereas NASH is generally scored by a system recommended by Brunt et al. (Brunt et al., 1999). For both of them, fibrosis can be classified into 4 histological stages: scar without bridging (Stage 1); scar with
little bridging (Stage 2); scar with intensive bridging (Stage 3); and cirrhosis which shows nodules of regenerative parenchyma surrounded by fibrotic septa (Stage 4) (Bedossa and Poynard, 1996, Brunt et al., 1999).

Figure 1.1 NAFLD histology

Representative trichrome stain pictures of human liver sections (originally ×200) in steatosis, NASH and fibrosis as well as a cartoon illustration for key histology features are shown. The hepatic acinus is divided into 3 zones according to their distance from the portal triads and central vein (CV). Perisinusoidal space (the space of Disse) is the space between sinus and hepatocytes. Steatosis is characterized by perivenous lipid deposition (histology picture from (Paradis and Bedossa, 2008)). NASH is characterized by steatosis plus inflammatory cell infiltration, with/without peri-venous fibrosis (histology picture from (Adams et al., 2005a)). Fibrosis developed from NASH has scars located in peri-central and perisinusoidal area with or without bridging (histology picture from (Hernandez-Gea and Friedman, 2011)). Arrows point to steatosis and fibrosis.
1.1.3. Pathophysiology

1.1.3.1. Steatosis

The accumulation of lipids in the liver results from an imbalance between the synthesis and disposal of triglycerides in the liver. The source of hepatic lipid droplets seen in NAFLD patients has been estimated to be 59.0% from serum non-esterified fatty acids (NEFA), 26.1% from de novo lipogenesis (DNL) and 14.9% from diet (Donnelly et al., 2005). Insulin resistance plays a significant role in the pathogenesis of NAFLD by affecting glucose production, glucose disposal, lipolysis and lipid oxidation (Angulo, 2002). NAFLD patients have significantly higher hyperglycaemia, hyperinsulinemia and hypertriglyceridemia compared to healthy controls (Marchesini et al., 1999, Donnelly et al., 2005).

1.1.3.2. Non-alcoholic steatohepatitis (NASH)

Day et al. suggested a two ‘hit’ theory that if lipid droplet deposition is the first ‘hit’, the progression from steatosis to steatohepatitis requires a second ‘hit’ (Day and James, 1998). This theory is now widely accepted and it helps to explain why only a certain percentage of steatosis patients develop into NASH and also why it is difficult to induce NASH and fibrosis in obese models. The second ‘hit’ can be persistent insulin resistance, enhanced oxidative stress from lipid peroxidation, or increased inflammatory mediators including adipokines, cytokines and inflammatory cells (Sanyal et al., 2001, Berson et al., 1998). Insulin resistance accelerates the development of NASH in a dietary rat model possibly through the TGFB related pathway (Ota et al., 2007). Lipotoxicity, a term used to describe the toxic effect of free fatty acid (FFAs) on the survival of non-adipose tissue, especially pancreas beta cells, can cause decreased cell viability and increased caspase activation and apoptosis in hepatocytes (Li et al., 2009). Adipokines, primarily pro-inflammatory cytokines such as tumour necrosis factor α (TNFα) and interleukin 6 (IL-6), are released by white adipose tissue and involved in promoting an inflammatory response in the liver, facilitating the development from steatosis to NASH (Marra and Bertolani, 2009). Lipid peroxidation markers are significantly increased in both rodent NASH models and human NASH patients (Leclercq et al., 2000, Chalasani et al., 2004). Blocking
lipid peroxidation significantly reduced NASH in rodent models (Leclercq et al., 2000) suggesting reactive oxygen species (ROS) provides a key stimulus of NASH progression. Endotoxin derived from enteric bacteria and absorbed through the gut also contributes to promoting the inflammation response in NAFLD (Bergheim et al., 2008). A schematic view of possible factors contribute to NASH progression is presented in Figure 1.2.
Figure 1.2 Mechanisms in progression of steatosis to NASH

Systemic, especially hepatic, insulin resistance contributes to the progression of steatosis. Adipose tissue also plays an important part in promoting hepatic inflammation and fibrosis in NASH. In obese patients, adipose tissue secretes adipokines including leptin, IL-6 and TNFα. Systemic high levels of these adipokines can stimulate the pro-inflammatory response in the liver. Increased lipolysis releases FFA causing hepatic injury. Peroxidation of FFA and lipids from DNL in the liver contributes to the production of ROS which is the key stimulus of hepatic fibrotic response. Endotoxin from the gut also contributes to the initiation of hepatic inflammation in NASH.
1.1.3.3. **Fibrosis**

1.1.3.3.1. **Fibrogenesis**

Liver fibrosis can be initiated by liver injury caused by alcoholic exposure, bile duct obstruction and viral hepatitis. In NAFLD, high levels of glucose, free fatty acid and adipokines can all be triggers to pro-fibrotic response in the liver (Basaranoglu et al., 2013). Hepatocytes undergo apoptosis and even necrosis if the stimuli persist. Hepatocyte necrosis triggers an inflammatory response by recruiting neutrophils (Jaeschke, 2006). Hepatocyte apoptosis is not only a reaction to injury but also releases fibrogenic mediators including reactive oxygen species (ROS), growth factors, cytokines and chemokines, triggers inflammation and initiates the activation of hepatic stellate cells (HSCs) (Jaeschke, 2002, Canbay et al., 2004). HSCs are the major myofibroblast precursors in the liver, which release extra cellular matrix (ECM) after activation. Normal ECM, which consists of type IV collagen, heparin sulphate proteoglycan and laminin is replaced by fibrillar collagens (predominantly collagen I and III) (Friedman, 1993), causing the formation of the scar. The ECM content is regulated by a balance between matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). During the fibrogenesis process, activated HSCs produce TIMPs which inhibit the MMP dependent degradation of ECM favouring scar formation. The hepatic inflammatory response contributes to promote scar formation by releasing pro-fibrotic mediators, enhancing injury and stimulating HSC activation (Figure 1.3).

1.1.3.3.2. **Fibrosis resolution**

The idea that liver fibrosis/cirrhosis is a reversible process has been widely accepted after 1979 (Perez-Tamayo, 1979). Spontaneous resolution, characterized by less ECM deposition than peak injury, is found in human fibrosis developed from bile duct obstruction, Hepatitis C and NASH (Dixon et al., 2004, Arthur, 2002, Hammel et al., 2001) as well as in animal fibrosis models including bile duct ligation and carbon tetrachloride (CCL₄) induced fibrosis (Iredale et al., 1998, Issa et al., 2001). When the source of liver injury source is removed, fibrosis starts to resolve. During resolution, hepatocytes undergo regeneration and this regeneration facilitates the
reversal of liver fibrosis (Ueki et al., 1999). HSCs undergo apoptosis (Iredale et al., 1998, Issa et al., 2001) and de-activation (Kisseleva et al., 2012). TIMP-1 level is reduced whereas overall MMP activity is increased during fibrosis resolution (Issa et al., 2004). The degradation of fibrillar type I collagen is critical to HSC apoptosis and hepatocyte regeneration during recovery (Issa et al., 2003). Inflammatory cells such as macrophages, especially anti-fibrotic macrophages (Section 1.1.3.3.6), facilitate this process (Figure 1.3).
Figure 1.3 Scheme of fibrogenesis and resolution

During the onset of liver fibrosis, hepatocytes are injured. After injury, ROS and cytokines secreted (majorly transforming growth factor β (TGFβ) and platelet-derived growth factor (PDGF)) in the liver activate the quiescent HSCs to myofibroblast-like cells (α smooth muscle actin positive (α-SMA +)). Activated HSCs are the major source of the ECM. The deposition and degradation of ECM depends on the balance of MMPs and TIMP. During injury, macrophages are recruited, classically activated and contribute to the activation of HSCs. During the resolution phase, hepatocytes regenerate. HSCs undergo apoptosis and de-activation. Resolving macrophages produce MMPs and clear the apoptotic cells in the liver but the mechanism of the phenotype switch is largely unknown.
**Hepatic stellate cells (HSCs)**

HSCs are the cells storing vitamin A in the form of retinoid in the Disse space (perisinusoidal area) in normal liver. They were called liver lipocytes in the past and the ratio of HSCs: hepatocytes is 3.6:100 in normal liver (Horn et al., 1986). HSCs express an array of cell markers among which desmin (Yokoi et al., 1984) and glial fibrillary acidic protein (GFAP) (Gard et al., 1985) are widely used as markers for both quiescent and activated HSCs. The expression of the cytoskeletal protein α smooth muscle actin (αSMA), which is not detected in quiescent HSCs, accompanies the activation of HSCs (Rockey et al., 1992).

After activation, HSCs differentiate into myofibroblast-like cells, which are proliferating, ECM producing, and contractile. ROS, transforming growth factor β (TGFβ), platelet-derived growth factor (PDGF) and connective tissue growth factor (CTGF) are all strong stimuli for HSC activation (Friedman, 2008a). During fibrogenesis, HSCs release fibrillar collagens, particularly collagen type I and III, which can in turn further activate HSCs (Friedman et al., 1989).

HSCs are the major but not the only myofibroblast precursors in the liver. Other cells such as bone marrow derived myofibroblasts (Forbes et al., 2004), bone marrow derived fibrocytes (Kisseleva et al., 2006) and portal fibroblasts (Wells et al., 2004) also contribute to the synthesis of ECM during fibrosis yet the proportion of them is much smaller compared to HSCs.

**Key pro-fibrotic mediators**

Pro-fibrotic mediators play an important part in fibrogenesis. ROS consists of superoxide and hydroxyl radicals, hydrogen peroxide and aldehydic end products which come from lipid peroxidation and can be produced not only by hepatocytes but also HSCs, macrophages and other inflammatory cells (Sanchez-Valle et al., 2012). The development of fibrosis in NASH is associated with increased ROS level (Kohli et al., 2010) whilst anti-oxidative treatment can reduce key pro-fibrotic and pro-inflammatory genes including pro-collagen type I, MCP-1, TIMP-1 possibly via suppression of activator protein 1 (AP-1) and nuclear factor kappa-light-chain-
enhancer of activated B cells (NF-kB) pathways (Leclercq et al., 2004, Vizzutti et al., 2010).

Growth factors favour the activation of HSCs. TGFβ is the major stimulus to HSCs during the onset of fibrogenesis. TGFβ is increased in fibrotic livers in both human (Castilla et al., 1991) and rodents (Czaja et al., 1989). The source of TGFβ are macrophages (Matsuoka and Tsukamoto, 1990) and activated HSCs during liver injury (Gong et al., 1998). Signalling of TGFβ is mediated by three receptor types: TGFβ receptor types I, II and III (TβRI, TβRII, TβRIII (Border and Noble, 1994)). TGFβ regulates pro-fibrotic effects predominantly through Smad related signalling pathway in HSCs (Dooley et al., 2000). The phosphorylation of Smad2/3 is induced by TGFβ during HSC activation (Liu et al., 2003a). Targeted disruption of Smad3 and over expression of Smad7, which antagonizes the Smad2/3 pathway, both alleviate liver fibrosis in vivo suggesting prominent but opposite roles of these Smads in liver fibrosis (Latella et al., 2009, Dooley et al., 2003). TGFβ stimulates collagen production (Armendariz-Borunda et al., 1992) and TIMP-1 expression (Knittel et al., 1999) in HSCs facilitating fibrosis response.

PDGF, which is the strongest mitogen for HSCs, stimulates the proliferation, migration rate and MMP expressions of HSCs in vitro (Seki et al., 2012, Yang et al., 2003). PDGF receptor β expression is either low or undetectable in quiescent HSCs but greatly enhanced during HSC activation (Shah et al., 2013, Wong et al., 1994). PDGFβ overexpression significantly stimulates liver fibrosis, pro-collagen type I, MMP-2, MMP-9 and TIMP-1 expression in vivo without affecting the TGFβ related pathway suggesting PDGFβ is an independent stimulus to liver fibrosis (Czochra et al., 2006).

Connective tissue growth factor (CTGF) is also a key pro-fibrotic stimulus released by hepatocytes and HSCs (Gressner et al., 2007). CTGF is significantly increased in fibrotic livers and activated HSCs in vitro (Williams et al., 2000a) and the silencing of CTGF leads to significantly reduced serum pro-collagen III, hepatic collagen deposition and liver fibrosis staging suggesting an important role of this cytokine (Li et al., 2006).
Metalloproteinases (MMPs) and their tissue inhibitors (TIMPs)

TIMP1 is the major component of tissue inhibitors of MMPs during liver fibrosis. Although TIMP1 is mainly secreted by HSCs, it has been reported to come from other sources including other myofibroblast precursors and macrophages (Iredale et al., 2012). The expression of TIMP-1 is a strong indicator of HSC activation during fibrogenesis and is possibly associated with pro-collagen I and collagenase expression (Iredale et al., 1996). TIMP-1 level is greatly increased during fibrogenesis and reduced during resolution (Hemmann et al., 2007). Importantly, serum TIMP-1 level has become a clinical indicator for cirrhosis (Rosenberg et al., 2004).

The MMPs are a family of zinc-dependent metallo-endoproteinases which can degrade different types of ECM in the liver (Hemmann et al., 2007). MMP expression is closely associated with HSC activation. MMP-13, which is a rodent equivalent enzyme to human MMP-1, can be secreted by HSCs (Knittel et al., 1999) as well as macrophages (Fallowfield et al., 2007). During the fibrogenesis process, MMP-13 can be a marker for early activation of HSCs, however, the expression of MMP-13 is attenuated when the HSCs are fully activated (Iredale et al., 2012). Gelatinase MMP-2, which can also degrade collagenase IV, is largely produced by HSCs (Milani et al., 1994). Similar to MMP-13, activated HSCs express MMP-9 in the early stage but fail to maintain this level of MMP-9 when the HSCs are fully activated (Han et al., 2004). MMP-2 also facilitates the further activation of HSCs by degrading normal sub-endothelial matrix to allow a disproportionate increase of fibrillar collagens (Olaso et al., 2001).

During fibrogenesis, the up-regulation of MMP activity is necessary to degrade normal ECM whereas during fibrosis resolution, MMP activity is also required to degrade fibrillar ECM. However, the expression pattern of each MMP might be different from each other. Table 1.4 summarizes the pattern of key MMP changes during fibrogenesis and fibrosis resolution in vivo.
<table>
<thead>
<tr>
<th>Function</th>
<th>Name</th>
<th>Source</th>
<th>Fibrogenesis</th>
<th>Fibrosis resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenase</td>
<td>MMP-1</td>
<td>Inflammatory cells (Benyon et al., 1996)</td>
<td>↑ in severe cirrhosis (Lichtinghagen et al., 2003)</td>
<td>↑ (Guido et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>MMP-13</td>
<td>HSC and macrophage (Knittel et al., 1999, Fallowfield et al., 2007)</td>
<td>↑ and then ↓ (Watanabe et al., 2000, Yan et al., 2005)</td>
<td>↑/unchanged (Watanabe et al., 2000)</td>
</tr>
<tr>
<td>Gelatinase</td>
<td>MMP-2</td>
<td>Predominantly HSC (Knittel et al., 1999)</td>
<td>↑ (Benyon et al., 1996)</td>
<td>↑ (Knittel et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>MMP-9</td>
<td>HSC and Macrophage (Han et al., 2007)</td>
<td>↑/unchanged (Zhou et al., 2004, Kossakowska et al., 1998)</td>
<td>↑ (Knittel et al., 2000)</td>
</tr>
<tr>
<td>Stromelysins</td>
<td>MMP-3</td>
<td>Prominent in HSC (Vyas et al., 1995)</td>
<td>↑ and then ↓ (Knittel et al., 1999)</td>
<td>?</td>
</tr>
<tr>
<td>Elastinase</td>
<td>MMP-12</td>
<td>Macrophage (Pellicoro et al., 2012)</td>
<td>↑ (Pellicoro et al., 2012)</td>
<td>?</td>
</tr>
</tbody>
</table>
1.1.3.3.6. **Fibrosis and inflammatory cells**

Neutrophils are recruited to fibrotic regions rapidly after liver injury (Saito et al., 2003). They produce MMP-8 which is another important collagenase for collagen I, but their role is not key to fibrosis (Siller-Lopez et al., 2004). It was demonstrated that liver fibrosis resulting from bile duct ligation (BDL) is not affected by neutrophil depletion (Saito et al., 2003). Also, a reduction in neutrophil numbers in chemokine receptor type 2 (CXCR-2, the receptor for chemokine ligand 2) deficient mice, which had 50% less neutrophil infiltration after α-naphthylisothiocyanate (ANIT) administration, has indistinguishable fibrotic response from their wild type littersmates (Xu et al., 2004).

Macrophages are key inflammatory cells involved in wound healing. The role of macrophages during fibrosis is complicated since functionally distinct subpopulations of macrophages play different roles during different stages of fibrosis. The depletion of macrophages during the process of fibrogenesis results in less HSC activation and ECM deposition whereas lack of macrophages during the fibrosis resolution phase leads to a failure of ECM degradation in toxin induced liver fibrosis (Imamura et al., 2005, Duffield et al., 2005). Macrophages can stimulate HSC activation by releasing stimuli such as TGFβ (Matsuoka and Tsukamoto, 1990) and also promote HSC death through TNF related apoptosis-inducing ligand (TRAIL) (Fischer et al., 2002). Macrophage mediated phagocytosis of apoptotic cells contributes to the resolution of fibrosis (Popov, 2010).

Different macrophage populations play different roles in liver fibrosis. Nowadays, identification of pro-fibrotic and anti-fibrotic macrophage populations has become a key goal in order to develop new therapies for liver fibrosis. Classically activated macrophages (M1) are pro-inflammatory, and activated by LPS and classical Th1 (T-helper type 1) cytokines such as IL-1 and TNFα. Alternatively activated macrophages (M2) are primarily anti-inflammatory macrophages, activated by Th2 (T-helper type 2) cytokines such as IL-4 and IL-12 (Goerdt and Orfanos, 1999, Gordon, 2003). In liver fibrosis, both M1 macrophages and M2 macrophages have potentially harmful and beneficial effects in fibrogenesis/resolution (Novak and Koh, 2013, Henderson and Iredale, 2007, Song et al., 2000) suggesting that isolating
additional macrophage subsets within each macrophage subtype will be necessary to identify pro-fibrotic and anti-fibrotic macrophages, if such exist.

Macrophages differentiated from Ly6C monocytes have a phenotype of classically activated macrophages and are pro-fibrotic. They are attracted to the liver by chemokine ligand 2 (CCL-2) and secrete cytokines including TGFβ to activate HSCs (Karlmark et al., 2009). More recently, a CD11b^{hi}F4/80^{int}Ly6C^{lo} subset of macrophages has been identified as the key subset in resolving liver fibrosis induced by CCL4 in mice (Ramachandran et al., 2012). The resolving macrophages are predominantly alternatively activated macrophages and produce MMP-9, MMP-12 (Ramachandran et al., 2012) and MMP-13 (Fallowfield et al., 2007), thus facilitating fibrosis resolution.

The adaptive immune response also plays an important part in liver fibrosis. Depletion of B cells attenuates CCL4 induced liver fibrosis, suggesting B cells can promote a fibrotic response (Novobrantseva et al., 2005). Inhibiting the effect of T_h2 cells with an IL-13 inhibitor also prevents liver fibrosis (Chiaramonte et al., 1999). However, inhibiting CD4^{+} or CD8^{+} T cells individually showes no effect on liver fibrosis (Novobrantseva et al., 2005).

1.1.3.3.7. **Association of liver fibrosis and tissue repair in other systems**

Liver fibrosis shares markedly similar processes with tissue repair that involves controlled inflammation and scar formation. The tissue repair process involves a series of complicated cascades including haemostasis, inflammation, cell proliferation, tissue remodelling and scar formation (Enoch and Leaper, 2008). Fibrosis can happen in many organs including lung, kidney and skin. Myofibroblasts, characterized by the expression of the highly contractile protein α-smooth muscle actin, are considered as the major source of ECM during tissue repair/fibrosis process. During normal tissue repair, including skin wound healing, activation of myofibroblasts contributes to restore the integrity of impaired tissue (Hinz et al., 2007). However, once the activation of myofibroblasts is un-restrained, a normal tissue repair process turns into harmful fibrogenesis.
As described in section 1.2.2.3, HSCs are the major myofibroblast precursor in the liver (Kisseleva et al., 2012). Local pericytes, bone marrow derived fibroblasts, and possibly resident epithelial cells are all considered as myofibroblast precursors in the kidney (Humphreys et al., 2010, Lin et al., 2008); Local fibroblasts as well as bone marrow derived fibroblasts can develop into myofibroblasts in the lung (Zhang et al., 1994, Phillips et al., 2004); keratinocytes can differentiate into fibroblasts and further develop into myofibroblasts in the cornea (Fini, 1999); scar fibroblasts, which are developed from normal dermal fibroblasts, differentiate to myofibroblasts causing further scar formation in skin (van der Smissen et al., 2013); and adventitial fibroblasts exhibit the characteristics of trans-differentiation into myofibroblasts in atherosclerotic lesions (Xu et al., 2013). Since all myofibroblasts share the same pathway of fibrogenesis, modifying myofibroblast activation is now a target for drug development for fibrosis in multiple tissues. Cytokines (such as IL-13 and IL-1), growth factors (such as PDGF and TGFβ), peroxisome proliferator activated receptors (PPARs) and caspases are identified as important regulators of fibrosis and are being investigated as potential targets of anti-fibrotic drugs across tissue and organs (Wynn and Ramalingam, 2012).
1.1.4. Models for NAFLD

1.1.4.1. Steatosis and NASH

Generally, rodent models for steatosis and NASH mimic some of the major pathophysiology of the human disease including insulin resistance and lipid deposition in the liver. Fat induced steatosis models such as high fat diet induced obesity (Table 1.5) mimic the natural disease process in human obesity and metabolic syndrome. However, they rarely induce inflammation and fibrosis (Buettner et al., 2006). Methionine and choline deficient diet (MCDD) can induce extensive inflammation in the liver but its underlying mechanism is not directly relevant to any human disease. Table 1.5 presents the advantages and disadvantages of the use of rodent models of steatosis and NASH.
<table>
<thead>
<tr>
<th>Description</th>
<th>Metabolic effects</th>
<th>Liver changes</th>
<th>Pros</th>
<th>Cons</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>High fat diet</td>
<td>high fat chow (58% calories from fat)</td>
<td>obesity, hyperinsulinemia, insulin resistance</td>
<td>steatosis, minimal inflammation and fibrosis</td>
<td>models human diet-induced NAFLD</td>
<td>minimal fibrosis and inflammation (Buettner et al., 2006)</td>
</tr>
<tr>
<td>CDD</td>
<td>fat accumulation in the liver without causing inflammation</td>
<td>unaltered or decreased blood insulin level; no systemic obesity</td>
<td>Steatosis</td>
<td>no obesity, not a good model for human NAFLD</td>
<td>(Velayudham et al., 2009, Koteish and Diehl, 2001)</td>
</tr>
<tr>
<td>MCDD</td>
<td>fat accumulation due to impaired VLDL transport</td>
<td>weight loss without metabolic syndrome steatohepatitis with pro-fibrotic response</td>
<td>hepatic inflammation</td>
<td>profound weight loss, no obesity</td>
<td>(Velayudham et al., 2009, Koteish and Diehl, 2001)</td>
</tr>
<tr>
<td>Diet/Condition</td>
<td>Description</td>
<td>Associated Features</td>
<td>Clinical Findings</td>
<td>References</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>-------------</td>
<td>---------------------</td>
<td>-------------------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>High fructose/sucrose with HFD</td>
<td>Diets high in saturated fat and fructose leading to hepatic oxidative stress</td>
<td>Obesity, hyperinsulinemia, insulin resistance, Steatosis and NASH, intensive inflammation and fibrosis, metabolic features</td>
<td>Only 50% of mice show fibrosis indicating the large variance of effect of this diet</td>
<td>Kohli et al., 2010</td>
<td></td>
</tr>
<tr>
<td>ob/ob</td>
<td>Leptin deficient mice</td>
<td>Obesity, hyperglycemia, hyperinsulinemia and hyperlipidemia, Steatosis (only develop NASH with LPS challenge), models metabolic syndrome and NAFLD</td>
<td>Leptin deficiency is rare in humans; leptin deficiency could reduce fibrotic susceptibility</td>
<td>Li et al., 2003, Yang et al., 1997</td>
<td></td>
</tr>
<tr>
<td>db/db</td>
<td>Leptin receptor mutation leading to genetic obesity</td>
<td>Obesity, hyperglycemia, hyperinsulinemia and hyperlipidemia, Steatosis</td>
<td>Models metabolic syndrome and NAFLD, no inflammation and fibrosis in the liver</td>
<td>Koteish and Diehl, 2001</td>
<td></td>
</tr>
<tr>
<td>db/db + MCDD</td>
<td>Genetic obesity plus hepatic oxidative stress</td>
<td>Obesity, hyperglycemia, hyperinsulinemia and hyperlipidemia, Steatohepatitis with pro-fibrotic response, NASH with less reduction in body weight than MCDD alone</td>
<td>Not natural human process</td>
<td>Sahai et al., 2004</td>
<td></td>
</tr>
</tbody>
</table>
1.1.4.2. Fibrosis

Carbon tetrachloride (CCL\textsubscript{4}) is a widely used model for liver fibrosis (Duplantier et al., 2004, Iredale et al., 1998). The CCL\textsubscript{4} causes liver injury by secreting free radicals including CCL\textsubscript{3}* and CCL\textsubscript{3}OO* metabolized by cytochrome P450 (CYP450) (Weber et al., 2003) (Figure 1.4). Acute CCL\textsubscript{4} administration (single dose) causes necrosis and inflammation in the liver whilst chronic CCL\textsubscript{4} administration (more than 4 weeks) induces liver fibrosis and even cirrhosis (Henderson et al., 2006). Administration of CCL\textsubscript{4} to rats for 4 weeks leads to reversible fibrosis after the withdrawal of CCL\textsubscript{4}, whilst administration for 8 weeks or 12 weeks leads to early cirrhosis or micro-nodular cirrhosis, respectively (Pellicoro et al., 2012). CCL\textsubscript{4} treatment for 16 weeks causes up to 40% of animals to die and the remaining animals shows obvious cirrhotic nodules (Gomez-Hurtado et al., 2011).
Figure 1.4 The mechanism of CCL₄ induced liver damage

CCL₄ is metabolized by CyP450 including CYP2E1, CYP2B1, CYP2B2 and possibly CYP3A to form the trichloromethyl radical, CCL₃*. CCL₃* damages nucleic acids and protein through halo-alkylation and impairs critical cellular processes. CCL₃* can also be metabolized to CCL₃OO*, a highly reactive species, to induce ROS mediated liver damage.
Thioacetamide (TAA) is also widely used in inducing liver fibrosis (Safadi et al., 2004). Since TAA is soluble in water, it can be administered through either injecting intra-peritoneally or dissolving in the drinking water. After administration, TAA is bioactivated into its sulfoxide and sulfone forms by CYPs, primarily CYP2E1, in the liver (Wang et al., 2000) (Figure 1.5). TAA metabolic products bind covalently to liver macromolecules and induce oxidative stress (Porter and Neal, 1978), inducing DNA and protein damage, necrosis and inflammatory infiltration (Diez-Fernandez et al., 1993, Ledda-Columbano et al., 1991).

**Figure 1.5 Mechanism of TAA induced liver fibrosis**

*TAA is bioactivated by CYP2E1 in the liver and its metabolic products TAA sulfoxide and TAA sulfone bind to liver macromolecules to produce reactive oxygen species (ROS), leading to oxidative stress in the liver. The oxidative stress causes cell necrosis/apoptosis and periporal inflammation and promotes liver fibrosis.*
Bile duct ligation (BDL) is also a widely used model for fibrosis. BDL stimulates the proliferation of biliary epithelial cells and hepatocyte progenitors, resulting in portal inflammation and fibrosis (Iredale et al., 1996). Liver pathology following BDL is also potentially reversible after the removal of bile duct obstruction (Eken et al., 2006).

1.1.4.3. Experimental model selection

The ‘3Rs’ concept (reduce, refine, replace) should be taken into consideration for protecting and avoiding unnecessary use of experimental animals according to the ethical concerns of animal welfare. When selecting experimental models, the smallest animal numbers should be used to achieve significant difference and in vitro techniques should be used to replace animal models if possible.

In this thesis, I have chosen to test my hypothesis using CDD, MCDD, high fat diet and CCL₄ to induce NAFLD with varying features of NASH and fibrosis in mice, and by studying NAFLD in ob/ob mice. Also, primary mouse HSCs in vitro were used to test HSC activation. The rationale for each of these models is provided in the relevant chapters describing these experiments.

1.2. Glucocorticoids

1.2.1. Key Features

Glucocorticoids (GC) are a set of steroid hormones that are synthesised in the adrenal gland cortex and regulate metabolic and immune pathways. GCs are important stress hormones that respond to stressors ranging from mild psychological dysfunction to intensive physical trauma (Axelrod and Reisine, 1984). GCs bind to glucocorticoid receptors (GR) which are present in almost all mammalian cells with also affinity to mineralocorticoid receptor (MR, as described in Section 1.2.4.4). The importance of GCs in clinical use was illustrated by Hench et al. who isolated cortisone (‘compound E’) and treated patients with rheumatoid arthritis (Hench et al., 1949). This work won the Nobel Prize for Medicine in 1950.
Dysregulation of GC levels can be hazardous. Cushing’s syndrome, which is caused by excessive activation of glucocorticoid receptors, is most commonly iatrogenic (chronic GC administration). Patients with Cushing’s syndrome may develop symptoms of abdominal obesity, weakness of proximal thigh muscles, and hypertension (Newell-Price et al., 2006). In contrast, in primary adrenal insufficiency, which is called Addison’s disease, patients present with chronic features of hyponatraemia, hypotension and occasionally hypoglycaemia (Napier and Pearce, 2012).

GCs are synthesised from the precursor cholesterol within the zona fasciculate, catalysed by a series of enzymes which are located in either the smooth endoplasmic reticulum or mitochondria. The basic structure of cholesterol, which contains 3 cyclohexane rings and 1 cyclopentane ring, is preserved all the way through cortisol biosynthesis (Figure 1.6). Due to the absence of 17α-hydroxylase in the adrenal, the primary active GC in rodents is corticosterone rather than cortisol which predominates in human (Chung et al., 1987, de Kloet, 2003).
Figure 1.6 The synthesis of glucocorticoids

Paths illustrating GC production in the adrenal gland. HSD= hydroxysteroid dehydrogenase. Adapted from Recent Advances in Endocrinology and Metabolism (by J.L.H. O'Riordan, 1982).
1.2.2. **Physiological and therapeutic effects of GCs**

1.2.2.1. **Metabolism**

Overall glucocorticoids (GCs) induce an anti-insulin effect during metabolism and increase plasma glucose and NEFAs (Andrews and Walker, 1999). GCs induce blood glucose level through many pathways. In the liver, GCs increase hepatic glucose output by transcriptionally up-regulating phosphoenol pyruvate carboxykinase (PEPCK) (Watts et al., 2005), glucose 6-phosphatase (G6Pase) (Van Schaftingen and Gerin, 2002) and tyrosine aminotransferase (TAT) (Schacke et al., 2002), which are the key enzymes regulating gluconeogenesis. They can induce both glycogenesis and glycogenolysis in the liver (Stalmans and Laloux, 1979) to ensure the body has effective release of fuel during times of stress or starvation (Macfarlane et al., 2008). In peripheral tissues such as adipose tissue and muscle, GCs inhibit glucose uptake and oxidation (Olefsky, 1975) resulting in peripheral insulin resistance. Also, GCs can increase blood glucose by increasing other hormones such as catecholamines and glucagon.

GCs also play key roles in lipid metabolism. GCs can either increase lipolysis in immature adipocytes or promote *de novo* lipogenesis (DNL) especially in the liver (Peckett et al., 2011). Free fatty acid level can be increased by GCs since they increases lipoprotein lipase (LPL) activity in adipose tissues (Appel and Fried, 1992). Hormone-sensitive lipase (HSL) (Slavin et al., 1994) and adipose triacylglyceride lipase (ATGL) (Serr et al., 2011), which are main enzymes involved in the mechanism of lipolysis (TGs to FFA), can be induced by GCs. GCs affect body fat distribution by ‘favouring’ deposition of fat in central or visceral adipose tissue, as seen in Cushing’s Syndrome, due to decreased lipolysis and increased LPL activity in these tissues (Rebuffe-Scrive et al., 1988).

Glucocorticoids can regulate protein metabolism by decreasing the rate of protein synthesis and increasing the rate of protein degradation in skeletal muscle (Tomas et al., 1979). Mechanisms of glucocorticoid induced proteolysis involve primarily the up-regulation of the ubiquitin-proteasome pathway and possibly calcium-dependent protein degradation (Auclair et al., 1997, Wang et al., 1998).
1.2.2.2. **Inflammation**

GCs suppress immune responses and that is why they are used for the treatment of many autoimmune and inflammatory conditions such as rheumatoid arthritis and asthma. They have complex roles in different immune cells. Serum lymphocyte, monocyte and eosinophil numbers are reduced whilst neutrophil counts are increased after GC administration (Jennings et al., 1990). Although GCs induce apoptosis of lymphocytes and eosinophils, they promote the survival of neutrophils (Nittoh et al., 1998). Similarly, GCs induce apoptosis of human monocytes and reduce their ability to release pro-inflammatory cytokines such as IL-1 (Schmidt et al., 1999), but this effect is monocyte subtype dependent (Ehrchen et al., 2007). In addition to this anti-inflammatory role, physiological GCs can promote the phagocytosis ability of macrophages favouring the resolution of inflammation (Gilmour et al., 2006).

Another anti-inflammatory role of GCs is the ability to suppress cytokines released from inflammatory cells. By inhibiting key trans-repression factors for nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and activator protein (AP-1), GCs inhibit expression of cytokines including interleukin-1 (IL-1), TNFα, granulocyte-macrophage colony-stimulating factor (GM-CSF), chemokines including interleukin-8 (IL-8) and macrophage inflammatory protein 1 α (MIP-1α) and adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1). Additionally, GCs up-regulate the expression of anti-inflammatory genes such as IκBα and IL-1 receptor antagonist to facilitate inflammatory resolution (McColl et al., 2007).

1.2.2.3. **Tissue repair and fibrosis**

GCs are used as treatments for human idiopathic pulmonary fibrosis (Mapel et al., 1996), autoimmune hepatitis induced fibrosis (Czaja, 2009) and skin keloid (Kauh et al., 1997). GCs’ anti-fibrotic effect is usually regarded as being closely associated with their anti-inflammatory effect. However, the anti-fibrotic effect is also associated with GCs’ intrinsic role in inhibiting the activation of myofibroblast
precursors. GCs inhibit the activation of HSCs (Bolkenius et al., 2004), human
dermis fibroblasts (Carroll et al., 2002, Terao et al., 2011) and lung fibroblasts
(Goulet et al., 2007).

Moreover, it is proven that glucocorticoids can decrease the TGFβ stimulated pro-
collagen 1 and pro-collagen 3 synthesis in fibroblasts (Meisler et al., 1995, Cutroneo
and Sterling, 2004). GCs have profound effects to suppress pro-fibrotic genes such as
TGFβ-1 and -2, MMP-1, -2, -9, and -10 and TIMP-2 in epidermal keratinocytes,
although they unexpectedly reduced the expression of apoptotic genes and prevented
UV induced keratinocytes apoptosis (Stojadinovic et al., 2007). Suppression of pro-
collagen gene expression by glucocorticoids is mediated by decreasing the binding of
the TGFβ activator protein complex to the TGFβ element in the distal promoter of
the pro-collagen 1 gene (Meisler et al., 1995). Table 1.6 summarizes *in vivo* and *in
vitro* studies of GCs’ effect in tissue repair and fibrosis.
Table 1.6 GC effect in tissue repair and fibrosis

<table>
<thead>
<tr>
<th>Organ</th>
<th>Myofibroblast precursors</th>
<th>GC effect <em>in vitro</em></th>
<th>GC effect <em>in vivo</em></th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>HSCs</td>
<td>Inhibit TGFβ induced profibrotic response in HSCs</td>
<td>Improve primary biliary cirrhosis and autoimmune hepatitis in human</td>
<td>(Bolkenius et al., 2004, Czaja, 2009, Leuschner et al., 1999)</td>
</tr>
<tr>
<td>Kidney</td>
<td>Kidney pericyte</td>
<td>?</td>
<td>Blunt aldosterone induced kidney fibrosis in mice</td>
<td>(Brem et al., 2010)</td>
</tr>
<tr>
<td>Skin</td>
<td>Dermal fibroblast</td>
<td>Inhibit TGF-β1 production by human dermal fibroblasts</td>
<td>Reduce pro-collagen expression in patients after keloid excision; suppress TGFβ transcripts in wounds</td>
<td>(Terao et al., 2011, Carroll et al., 2002, Kauh et al., 1997, Frank et al., 1996)</td>
</tr>
<tr>
<td>Cornea</td>
<td>Cornea Keratinocyte/Fibroblasts</td>
<td>Induce keratinocyte apoptosis</td>
<td>Reduce αSMA deposition in subablation stroma after photorefractive keratectomy in cat</td>
<td>(Buhren et al., 2009, Bourcier et al., 1999)</td>
</tr>
<tr>
<td>Lung</td>
<td>Pulmonary fibroblast</td>
<td>Inhibit αSMA expression in lung fibroblasts; reduce human lung fibroblast activation induced by TGFβ</td>
<td>Used as a treatment for pulmonary fibrosis</td>
<td>(Goulet et al., 2007, Sabatini et al., 2012, Mapel et al., 1996)</td>
</tr>
</tbody>
</table>
1.2.2.4. Effects on other organs

In the cardiovascular system, GCs can increase blood pressure by changing vascular sensitivity to pressor agents and increasing the renin-angiotensin-aldosterone system response (Saruta et al., 1986). GC excess induces osteopenia and osteoporosis attributable to GCs’ inhibition of osteoblast function (Canalis, 1996). Osteonecrosis is one of the most severe complications of GC treatment. Basal cortisol elevation causes hippocampal damage and impaired hippocampus-dependent learning and memory in humans (Lupien et al., 1998).

1.2.3. Central regulation of glucocorticoids

Circulating levels of GCs are under the control of the hypothalamus-pituitary-adrenal (HPA) axis (Figure 1.7). The paraventricular nucleus (PVN) and supraoptic nucleus (SON) in the hypothalamus release corticotropin-releasing hormone (CRH). CRH, along with arginine vasopressin (AVP), stimulates the anterior pituitary to release pro-opiomelanocortin (POMC), the precursor of adrenocorticotropic hormone (ACTH). ACTH targets the cortex of the adrenal gland to promote the synthesis and secretion of glucocorticoids, mineralcorticoids (primarily aldosterone) and adrenal androgens (dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulphate (DHEAS) and androstenedione). Increased serum GC levels have a negative effect on the release of both ACTH and CRH to restore GC levels (de Kloet, 2003). This negative feedback is GR dependent. Mutations in GR result in increased serum ACTH and cortisol levels (Sapolsky et al., 2000).

Serum ACTH levels fluctuate in a circadian, and also an ultradian pulsatile, pattern with high frequency and low amplitude oscillations (Horrocks et al., 1990). Serum GC levels followed the same pattern as ACTH which peaks in the morning and is lowest in the evening in animals active in daytime. Conversely, GC levels are lowest in the morning and highest in the evening in nocturnal animals such as rodents (Windle et al., 1998). Stressors including emotional stress, injury and inflammation activate the HPA axis (de Kloet, 2003). HPA axis responsiveness is also impaired in certain inflammatory disorders e.g. arthritis (Crofford, 2002), Crohn’s disease (Crofford, 2002) and allergic conditions (Buske-Kirschbaum, 2009).
Pharmacological GC administration suppresses the HPA axis with delay in restoring normal function even after months of withdrawal.

The bioavailability of GCs can also be regulated by the level of corticosteroid-binding globulin (CBG). Under basal conditions, only 6% of plasma cortisol is free, 14% is bound to albumin (Keenan et al., 2004) and 80-90% is bound to CBG (Siiteri et al., 1982). Besides binding to GCs, CBG may play a part in providing an adequate endocrine response to stress (Richard et al., 2010).
Figure 1.7 The HPA axis

CRH, which is regulated in a diurnal rhythm, is released by SON and PVN into the portal circulation and promotes the anterior pituitary to secrete ACTH. ACTH stimulates the adrenal cortex (zona fasciculata) to secrete GCs. GCs suppress the secretion of CRH and ACTH via a negative feedback loop.
1.2.4. Local regulation of glucocorticoids

1.2.4.1. \textit{11\textbeta-}Hydroxysteroid dehydrogenase type 1 (11\textbeta-HSD1)

1.2.4.1.1. Biochemistry of 11\textbeta-HSD1

The local effect of GCs is amplified by 11\textbeta-HSD1 which converts the inactive GC precursors (cortisone in human and 11-dehydrocorticosterone (11-DHC) in rodents) to active form of GCs (cortisol in human and corticosterone in rodents). 11\textbeta-HSD1 mainly acts as a reductase and this reductase activity is dependent on a high ratio of nicotinamide adenine dinucleotide phosphate in its reduced form (NADPH) relative to its oxidized form (NADP) \textit{in vivo} and in cultured cells (Dzyakanchuk et al., 2009). The high ratio of NADPH/NADP is maintained by hexose-6-phosphate dehydrogenase (H6PDH), which converts glucose 6-phosphate (G6P) to 6-phosphogluconolactone (6-PGL) in a reaction that regenerates NADPH from NADP (Atanasov et al., 2004) (Figure 1.8). 11\textbeta-HSD1 can also catalyse dehydrogenase activity in the presence of high concentration of NADP in homogenates and microsomes (Lakshmi and Monder, 1988) as well as in some transfected cells (Atanasov et al., 2004). A recent study showed both reductase and dehydrogenase activities of 11\textbeta-HSD1 were detected in muscle and splanchnic circulations, and the dehydrogenase activity was more readily detected than reductase activity in adipose tissue in human (Hughes et al., 2012).

11\textbeta-HSD1 is located in the endoplasmic reticulum (ER) with its N-terminus anchored to the ER membrane and the C-terminus projecting into the ER lumen (Mziaut et al., 1999). H6PDH is also located in the ER, where it has direct protein-protein interaction with the N-terminal 39 residues of 11\textbeta-HSD1 (Atanasov et al., 2008). Some patients with cortisone reductase deficiency (CRD), who fail to regenerate cortisol from cortisone via 11\textbeta-HSD1, carry inactivating mutations in the H6PDH gene (Lavery et al., 2008). H6PDH deficient mice lack the reductase activity of 11\textbeta-HSD1, which converts 11-DHC to corticosterone, but have an increased conversion of corticosterone to 11-DHC (Lavery et al., 2006).
Figure 1.8 The reaction catalysed by 11β-hydroxysteroid dehydrogenase type 1 and 2

11β-HSD1 activity is highly dependent upon the ratio of co-factor nicotinamide adenine dinucleotide phosphate/reduced nicotinamide adenine dinucleotide phosphate (NADP/NADPH), while 11β-HSD2 is an exclusive nicotinamide adenine dinucleotide (NAD+) dependent dehydrogenase. 11β-HSD1 acts as a reductase in the presence of a high ratio of NADPH/NADP and converts the inactive form of GC (cortisone in human) to active form of GC (cortisol in human). 11β-HSD1 can also exhibit dehydrogenase activity which catalyses the reverse reaction when the NADH predominates. The high ratio of NADPH/NADP in cells is maintained by the conversion of NADP to NADPH by H6PDH which catalyses conversion of glucose 6-phosphate (G-6-P) to 6-phosphogluconolactonase (6-PGL). 11β-HSD2 only inactivates cortisol with NAD as co-factor.
1.2.4.1.2. **11β-HSD1 distribution and regulation**

11β-HSD1 is widely distributed in many organs with abundant expression in liver, adipose tissue, lung and lower expression in brain, gonads and colon (Rajan et al., 1995). In whole tissue homogenates, liver has the highest 11β-HSD1 expression compared to the lung, brain and kidney (Brereton et al., 2001, Tannin et al., 1991). The distribution pattern of 11β-HSD1 in a given tissue varies between cell types. In the brain, 11β-HSD1 is most highly expressed in the cerebral cortex, hippocampus, hypothalamic medial preoptic area and arcuate nuclei and anterior pituitary (Moisan et al., 1990). In the lung, 11β-HSD1 is expressed at highest levels in the interstitial fibroblast and less in the type II pneumocytes (Brereton et al., 2001). In the liver, although 11β-HSD1 is highly expressed in hepatocytes (Jamieson et al., 1995), 11β-HSD1 distribution shows a zonal difference: higher around central vein and lower in portal area (Brereton et al., 2001). During development, 11β-HSD1 is not present until embryonic day 15 (E15) in mouse (Hundertmark et al., 1995, Speirs et al., 2004).

11β-HSD1 is expressed in many immune cells including lymphocytes (Zhang et al., 2005), mast cells (Coutinho et al., 2013) and cells from the myeloid lineage (Chapman et al., 2006). The level of 11β-HSD1 in monocytes is low but increases remarkably after differentiating to dendritic cells (Freeman et al., 2005) or macrophages (Thieringer et al., 2001). Notably, whether 11β-HSD1 level is higher in M1 or M2 macrophages is still disputed since different studies showed opposite results (Thieringer et al., 2001, Martinez et al., 2006).

11β-HSD1 was first cloned in 1989 (Agarwal et al., 1989) and its protein (288aa with a molecular weight of 34kDa) was isolated in rat liver (Lakshmi and Monder, 1988). The 11β-HSD1 gene was transcribed from 3 promoters: P1 (Bruley et al., 2006), P2 and P3 (Moisan et al., 1992). P1 is located 23 kb 5' to P2 and both promoters are detected in liver, lung, adipose tissue, and brain. However, P1 (encoding exon 1A) predominates in lung and P2 (encoding exon 1B) predominates in liver, adipose tissue, and brain (Bruley et al., 2006). P3 promoter, which locates within the intron between exon 2 and 3, encodes a truncated protein (26kD) which lacks both reductase and dehydrogenase activity (Mercer et al., 1993).
11B-HSD1 \textit{in vivo} is regulated during inflammatory status or metabolic disorders. In obesity, most studies show increased 11B-HSD1 levels in adipose tissue and decreased 11B-HSD1 in the liver in humans (Rask et al., 2001, Rask et al., 2002). Elevated 11B-HSD1 in adipose tissue and the liver reduction was confirmed in Zucker obese rats, a leptin-resistant rodent models of obesity (Livingstone et al., 2009). In diabetic patients, 11B-HSD1 activity is reduced in skeletal muscle (Jang et al., 2007), potentially conferring metabolic protection by reducing intracellular cortisol generation. 11B-HSD1 activity is significantly increased in inflammatory diseases including colitis (Bryndova et al., 2004) and rheumatoid arthritis (Hardy et al., 2008), suggesting enhanced GC activation for controlling inflammation.

At a cellular level, 11B-HSD1 can be stimulated by cytokines, hormones and metabolically active drugs. Pro-inflammatory cytokines IL-1 and TNF\(\alpha\) increased 11B-HSD1 expression in many cell types including synovial fibroblasts (Hardy et al., 2006) and human pre-adipocytes (Tomlinson et al., 2001), suggesting an underlying mechanism of 11B-HSD1 to restrain inflammation. During the differentiation from monocytes to macrophages, IL-4, which initiated the alternative activation, induced \(\approx 10\) fold increase in 11B-HSD1 expression whereas LPS, a classical activation stimulus, increased 11B-HSD1 expression only by \(\approx 4\) fold (Thieringer et al., 2001). GCs themselves induce 11B-HSD1 expression in cells. 11B-HSD1 level is up-regulated by GCs in human fibroblasts (Hammami and Siiteri, 1991), rat hepatocytes (Jamieson et al., 1995) and A549 cells (Sai et al., 2008). Insulin significantly induced, whereas growth hormone (GH) and insulin-like growth factor (IGF-1) suppressed, 11B-HSD1 activity in fully differentiated 3T3 adipocytes in a time dependent manner (Morita et al., 2009, Balachandran et al., 2008). Additionally, salicylate down-regulates 11B-HSD1 in fully differentiated SGBS adipocytes (Nixon et al., 2012).

Transcription factors such C/EBPs enhance 11B-HSD1 in hepatocytes and adipocytes (Esteves et al., 2012, Williams et al., 2000b). The activation of other transcription factors such as PPAR\(\alpha\) in the liver (Hermanowski-Vosatka et al., 2000), PPAR\(\gamma\) in adipocytes (Berger et al., 2001), HNF-1\(\alpha\) in the liver (Shih et al., 2001) and LXR\(\alpha\) in adipocytes (Stulnig et al., 2002) decreased 11B-HSD1 mRNA although the pathway is not clear yet.
1.2.4.1.3. **Effects of 11β-HSD1**

1.2.4.1.3.1. Transgenic models to study 11β-HSD1

The most widely used model of 11β-HSD1 manipulation is 11β-HSD1 knockout mice (Kotelevtsev et al., 1997). 11β-HSD1 knockout (KO) mice were produced by replacing genomic 11β-HSD1 with vectors in embryos, which were originally made on a mixed background then backcrossed more than 8 generations on a C57BL/6 background. 11β-HSD1 KO mice showed complete KO effect in the liver and brain although a 30% leak was found in the lung (Kotelevtsev et al., 1997, Morton et al., 2001). To isolate the effect of 11β-HSD1 on different target tissues, tissue specific 11β-HSD1 over expressing/KO mice are developed including liver over expressing 11β-HSD1 mice (Paterson et al., 2004), adipose tissue specific 11β-HSD1 overexpression mice (Masuzaki et al., 2001) and pancreatic islet β cell specific 11β-HSD1 overexpression mice (Turban et al., 2012) with a series of tissue specific KO mice in development.

1.2.4.1.3.2. Effects of 11β-HSD1 in metabolism and obesity

11β-HSD1 KO mice show a cardio-protective phenotype (Seckl, 2004). 11β-HSD1 KO mice have attenuated activation of key hepatic gluconeogenic enzymes and less hyperglycaemia provoked by obesity or stress (Kotelevtsev et al., 1997). 11β-HSD1 KO mice also had significantly lower plasma triglyceride level and improved glucose tolerance (Morton et al., 2001) suggesting a role of 11β-HSD1 KO in improving insulin sensitivity. However, 11β-HSD1 KO mice showed a subtle HPA axis activation (Harris et al., 2001, Kotelevtsev et al., 1997) although this activation seems to be strain dependent and is not present on a C57BL/6 genetic background (Carter et al., 2009).

The role of 11β-HSD1 in obesity and metabolic syndrome could be a result of effects in multiple organs. The improved lipid profile and glucose tolerance in 11β-HSD1 KO mice is attributed to the induction of fat metabolism in the liver and beneficial alterations on adipose tissue distribution and function (Morton et al., 2001, Morton et al., 2004). Hepatic 11β-HSD1 overexpression leads to increased lipid synthesis and
transport enzymes as well as insulin resistance although the mice are not obese or glucose intolerant (Paterson et al., 2004). Transgenic aP2/11β-HSD-1 Tg mice provide a model for fat specific 11β-HSD1 overexpression, and exhibited intra-abdominal obesity and increased fat cell size, insulin-resistant diabetes and dyslipidaemia with lower serum adiponectin levels, a key insulin-sensitising factor (Masuzaki et al., 2001). This indicates 11β-HSD1 in the liver and adipose tissue is key to insulin resistance.

1.2.4.1.3.3. Effect of 11β-HSD1 in inflammation

11β-HSD1 is important for ‘controlling’ inflammation as a local amplifier of GC action. 11β-HSD1 KO mice had more severe inflammation than their wild type controls in models of acute injury (Table 1.7). However, in atherosclerosis and obesity involved inflammation, 11β-HSD1 deficiency results in reduced inflammatory mediators (Wamil et al., 2011, Hermanowski-Vosatka et al., 2005), suggesting different mechanisms whereby 11β-HSD1 is involved in regulating inflammatory response.
Table 1.7 Effects of global 11β-HSD1 deficiency on inflammation in different *in vivo* models

<table>
<thead>
<tr>
<th>Model</th>
<th>Mediator</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute endotoxinemia</td>
<td>increased TNFα, IL-6 and IL-12 from macrophages and B cells after LPS challenge</td>
<td>(Zhang and Daynes, 2007)</td>
</tr>
<tr>
<td>Acute myocardial infarct healing</td>
<td>increased neutrophils and macrophages (especially alternatively activated) and tissue MCP-1 level</td>
<td>(McSweeney et al., 2010)</td>
</tr>
<tr>
<td>Acute sterile peritonitis</td>
<td>decreased macrophage phagocytosis of apoptotic neutrophils; increased inflammatory cell filtration</td>
<td>(Gilmour et al., 2006, Coutinho et al., 2012)</td>
</tr>
<tr>
<td>Chronic atherosclerosis in apoE-/- mice</td>
<td>reduced macrophage and T cell number in lesion site</td>
<td>(Garcia et al., 2013, Kipari et al., 2013)</td>
</tr>
<tr>
<td>Chronic arthritis</td>
<td>early onset and impaired resolution</td>
<td>(Coutinho, Gray et al. 2012)</td>
</tr>
<tr>
<td>Chronic adipose inflammation in obesity</td>
<td>reduced systemic MCP-1 level and T cell number</td>
<td>(Wamil et al., 2011)</td>
</tr>
</tbody>
</table>

11β-HSD1 has a significant role in regulating inflammatory cells *in vitro*. GCs’ effect in promoting macrophage phagocytosis of apoptotic neutrophils is 11β-HSD1 dependent since 11β-HSD1 KO macrophages fail to exhibit enhanced phagocytosis.
ability in the presence of 11-DHC (Gilmour et al., 2006). Also, by generating active GC, 11β-HSD1 tonically suppresses mast cell degranulation which is important to allergy responses (Coutinho et al., 2013).

1.2.4.1.3.4. Effects of 11β-HSD1 in tissue repair and fibrosis

Although GCs showed prominent effect in regulating tissue repair and fibrosis, few studies have investigated 11β-HSD1’s role specifically in tissue repair and fibrosis processes. 11β-HSD1 KO mice show increased collagen level in atherosclerotic lesions (Kipari et al., 2013) and aged induced skin defects (Tiganescu et al., 2011). Also, 11β-HSD1 inhibited mice showed increased collagen production in skin wound healing (Terao et al., 2011) suggesting 11β-HSD1 deficiency can stimulate collagen deposition.

11β-HSD1 can possibly modulate tissue repair and fibrosis by stimulating myofibroblast precursors. 11β-HSD1 is present in many fibroblasts, which have the potential ability to differentiate into myofibroblasts, and the expression is induced after differentiation (Hardy et al., 2006). Cytokines such as IL-1 and TNFα can promote 11β-HSD1 expression in mouse embryonic fibroblasts (Ahasan et al., 2012). 11β-HSD1 is expressed in epidermal keratinocytes (NHEKs) and normal human dermal fibroblasts (NHDF) and its level is increased after the differentiation of NHEKs. However, whether 11β-HSD1 contributes to fibrosis (of lung, liver or kidney) in vivo has not been investigated thoroughly.

1.2.4.1.3.5. Effect of 11β-HSD1 in other systems

11β-HSD1 also regulates angiogenesis and cognitive impairments. 11β-HSD1 is expressed in vessel walls and vascular smooth muscle cells (Walker et al., 1991, Cai et al., 2001). There is a strong positive correlation between vascular 11β-HSD1 gene expression and coronary artery disease in patients with metabolic syndrome (Atalar et al., 2012). 11β-HSD1 deficiency promotes angiogenesis in many models including acute myocardial infarct healing, implanted sponge in vivo and cutaneous surgical wounds (Small et al., 2005). Genetic analyses showed that specific haplotypes 11β-HSD1 gene is related to 6 fold of risk of Alzheimer’s disease compared to wild type
control (de Quervain et al., 2004). $11\beta$-HSD1 KO mice showed alleviated age-related learning impairments (Yau et al., 2001).

1.2.4.1.4. Pharmacological $11\beta$-HSD1 inhibition

$11\beta$-HSD1 inhibitors were developed as therapeutic medications as well as model tools. The non-selective $11\beta$-HSD1 inhibitor carbenoxolone (CBX), which was a licensed drug for oesophageal ulceration and inflammation in the UK, in human studies, improves the insulin sensitivity in male type 2 diabetic patients, altered the lipid turnover in obesity and improved the cognitive status in healthy elderly men and type 2 diabetics (Walker et al., 1995, Andrews et al., 2003, Sandeep et al., 2004, Sandeep et al., 2005). In rodents, CBX also shows reduced hepatic steatosis and improved lipid profile and insulin sensitivity (Nuotio-Antar et al., 2007). However, CBX showed side effects including raising blood pressure and lowering plasma potassium in humans due to an inhibitory effect on $11\beta$-HSD2 activity (Andrews et al., 2003).

$11\beta$-HSD1 specific inhibitors have been developed to achieve the same effect as CBX but with fewer side effects from inhibiting $11\beta$-HSD2. $11\beta$-HSD1 specific inhibitors are now under investigated in clinical trials. PF-00915275 has passed the phase 1 clinical trial for type 2 Diabetes and is proved to be safe and well tolerated in all doses tested (Courtney et al., 2008). INCB13739-202 improves the fasting glucose level, haemoglobin A1c (HbA1c) level and homeostasis model assessment (HOMA)-insulin resistance in type 2 diabetic patients and reduces plasma triglycerides in patients with hyperlipidaemia in a phase 2 clinical trial (Rosenstock et al., 2010). However, a limitation of this drug is a mild activation of the HPA axis and testosterone elevation in woman (Rosenstock et al., 2010). In rodents, $11\beta$-HSD1 specific inhibitors improve triglyceridemia, and reduce body weight, insulin and fasting glucose in diet-induced obesity (Berthiaume et al., 2007, Hermanowski-Vosatka et al., 2005).
11β-Hydroxysteroid dehydrogenase (11β-HSD2)

11β-HSD2 is an exclusive dehydrogenase which converts the active form of GCs to their 11-keto metabolites. The effect of 11β-HSD2 is nicotinamide adenine dinucleotide (NAD\(^+\))-dependent (Walker et al., 1992) (Figure 1.8). 11β-HSD2 is largely restricted to mineralocorticoid targeted tissues, including aorta, distal convoluted tubule of the nephron, colon and salivary glands, to prevent non-selective mineralocorticoid receptors (MRs) from binding to glucocorticoids which have a concentration 1000 times higher than aldosterone in serum (Ferrari, 2010). Additionally, in the placenta, which is not a classic mineralocorticoid target tissue, abundant 11β-HSD2 protects the foetus from exposing to high levels of maternal glucocorticoids (Edwards et al., 1993).

In the syndrome of apparent mineralocorticoid excess (SAME) in human, renal 11β-HSD2 deficiency causes severe hypertension, sodium retention and hypokalaemia by allowing GCs instead of mineralocorticoids to bind to MRs (Stewart et al., 1987). 11β-HSD2/-/- mice showed hypokalaemia, hypotonic polyuria and severe hypertension (Kotelevtsev et al., 1999). Inhibition of 11β-HSD2 reduces birth weight and programmes hypertension, increased HPA axis reactivity, and increased anxiety-related behaviour of the offspring in rats (Welberg et al., 2000, Lindsay et al., 1996).

5α-reductases and 5β-reductase

5α-Reductases can catalyse the reduction of Δ4,5 double bonds in steroids to 5α-dihydro metabolites. These metabolites can be further catalysed by 3α-hydroxysteroid dehydrogenases to 3α,5α-tetrahydrod steroids. 5α- reductase type 1 (5αR1) is mainly expressed in non-reproductive tissues including liver, brain and skin whilst 5α- reductase type 2 (5αR2) is predominantly expressed in male reproductive tissues including prostate and epididymis (Normington and Russell, 1992, Thigpen et al., 1993). 5α-Reductases metabolize steroids including aldosterone (Morris, 1993), progesterone (Rapkin et al., 1997) and glucocorticoids (McInnes et al., 2004), producing 5α-reduced metabolites which are still bioactive since they can bind to their parent receptors. 5α-Reduced glucocorticoid metabolites are now considered as a new therapeutic target since 3α,5α-tetrahydrocorticosterone showed
similar anti-inflammatory effect as corticosterone without metabolic side effects (Yang et al., 2011).

5β-reductase (5βR) catalyses reduction of Δ4,5 double bonds in steroids to 5β-dihydro metabolites, which can be further metabolized into 3α,5β-tetrahydrosteroids. 5βR is primarily expressed in the liver (Berseus et al., 1965) with minor expression detected in the kidney (Quinkler et al., 2003). The metabolites of 5βR are bio-inactive since they fail to activate their parent receptors (McInnes et al., 2004).

1.2.4.4. Glucocorticoid receptor (GR)

As lipophilic compounds, GCs are able to diffuse through membranes freely to bind to their receptors. They can either bind to glucocorticoid receptors (GR, low affinity type II) or mineralocorticoid receptors (MR, high affinity type I). GRs are the main receptors for GCs and are expressed in most cells whilst MRs are expressed only in a few tissues and usually co-localized with 11β-HSD2 (Ferrari, 2010). GR, a ligand-regulated transcription factor that belong to the superfamily of nuclear receptor, binds GCs and regulates target genes positively or negatively (Mangelsdorf et al., 1995). The structure of GR contains three major domains: a variable N-terminal region (containing a transactivation domain, activation function-1 (AF-1)), a short and well-conserved cysteine-rich central domain (also called central DNA-binding domain (DBD)), and a relatively well-conserved C-terminal (ligand-binding domain (LBD)) (Beato, 1989).

GRs are inactive and held by a family of heat-shock proteins (Hsp) in the cytoplasm before binding to GCs. Hsp70 and Hsp90 are the two main chaperone molecules involved in this system (Liberman et al., 2007). When GCs bind, GRs are released from the chaperones and trans-located to the nucleus, where the transcriptional regulation of target genes occurs.

GRs can regulate target genes either in a positive (transactivation) or negative (trans-repression) manner. The genes that are positively regulated by GCs have a consensus sequence on their DNA, glucocorticoid response elements (GREs), usually in the promoter area. The transactivation effect of GRs is involved in regulating metabolic gene products and anti-inflammatory proteins (Beck et al., 2009). GRs can trans-
activate target genes in many ways, of which the following three are most common. In a simple way, GRs can bind to GREs directly as a homodimer. In composite sites, GRE and other transcription factor (TF) binding sites bind to their ligands to function simultaneously. Also, GRs can activate tethering GRE sites without direct contact with the DNA but through a protein to protein interaction (Newton and Holden, 2007) (Figure 1.9).

The trans-repression effect of GR is very important in restraining inflammatory cytokines during inflammation (De Bosscher and Haegeman, 2009). Negative glucocorticoid response elements can bind to the monomeric GR directly through DNA-protein interaction to suppress target gene expression (Drouin et al., 1993). In a composite or tethering way, GRs and/or TFs bind to their binding cites to inhibit the transcription of target genes (De Bosscher et al., 2003) (Figure 1.9).

Also, non-genomic actions of GCs can also be mediated by GR. GC induced inhibition of T-cell-receptor (TCR) signalling in T cells by reducing the phosphorylation of lymphocyte-specific protein tyrosine kinases (Lck/Fyn) (Lowenberg et al., 2005). Targeted disruption of GR significantly blocked this inhibitory effect (Lowenberg et al., 2006).
GRs are inactive in quiescent state and bound to Hsps (Hsp70 and Hsp90). When GCs come through to the cytoplasm, they bind to GRs and enter the nucleus. In the nucleus, GRs regulate transcription positively (transactivation) or negatively (transrepression). (1) GRs can bind to glucocorticoid responsive elements (GRE) and activate target genes as dimers; (2) GRs along with other transcription factors bind to GRE in composite with another transcription factor binding site (TFBS) to promote target gene expression; (3) GRs can also bind to other TFs without contact with DNA in a tethering way. Similarly, the transrepression effect of GR can be achieved through composite (5) and tethering (6) mechanisms. However, to repress target gene expression, GR binds to negative GREs (nGREs) directly in a monomeric form (4).
1.3. Relationship between GCs and NAFLD

1.3.1. NAFLD’s influence on GC regulation

In association with NAFLD, the central regulation of GCs is altered. Circulating cortisol concentrations are markedly less suppressed by an overnight low-dose of dexamethasone administration in obese healthy individuals with NAFLD than those in individuals without NAFLD, suggesting a subtle activation on HPA axis in steatosis patients (Zoppini et al., 2004). In addition, higher serum cortisol levels significantly predicted the presence of NAFLD and the progression of liver fibrosis, independently of potential confounders analysed by a logistic regression (Targher et al., 2006). This indicates GCs and NAFLD are closely associated with each other in metabolic syndrome.

NAFLD changes not only central GC regulation but also local GC regulation. During steatosis, local enzyme activities are changed, potentially providing a compensatory mechanism of reducing hepatic GC output to protect the liver from GC induced hepatic fat accumulation. *In vivo* measurements regarding urinary steroid metabolites and cortisone to cortisol conversion suggest 11β-HSD1 activity is reduced (Rask et al., 2001, Stewart et al., 1999) and 5α- and 5β-reductase activities are up-regulated (Andrew et al., 1998) in obese patients. However, hepatic 11β-HSD1 activity and 5α-reductase were not associated, whilst an increase of 5β-reductase was significantly associated, with liver fat accumulation independently of obesity in a small group of lean males (Westerbacka et al., 2003). Notably, 5β-reductase catalyses the reaction that converts cholesterol into bile acids (Danielsson and Sjovall, 1975) of which the hydrophobic ones are toxic to hepatocytes. The increase of 5β-reductase may contribute to altered bile acid and cholesterol metabolism in NASH (Westerbacka et al., 2003). The expression pattern of GC regulating enzymes is less studied in NASH and liver fibrosis patients *in vivo* and the results are variable. One recent study showed 5α- and 5β-reductase activity was increased, whereas 11β-HSD1 activity was unchanged in NASH (Konopelska et al., 2009). Another study with 8 NASH and 8 steatosis patients suggested there was an increase 11β-HSD1 activity with unaltered 5α-reductase activity in NASH (Ahmed et al., 2012).
Due to the difficulty of sample collection of liver biopsies, hepatic enzyme mRNA levels were less investigated. Hepatic 11β-HSD1 mRNA level showed no difference in one study (Konopelska et al., 2009), but increased in another (Ahmed et al., 2012) in NASH patients compared to healthy controls.

A similar pattern is shown in rodent models of obesity. Both obese Zucker rats and high fat fed rats show reduced 11β-HSD1 activity and increased 5α-reductase 1 activity (Livingstone et al., 2000, Drake et al., 2005). Few studies have investigated the pattern of 11β-HSD1 and 5α- and 5β-reductases in NASH and liver fibrosis models although preliminary data in our laboratory showed decreased transcripts of 11β-HSD1 and 5α- reductases with similar 5β-reductase mRNA level in mice fed CDD or MCDD (Macfarlane et al. unpublished). In this thesis,

In this thesis, I will investigate the pattern of 11β-HSD1 in NASH and fibrosis models to fill this gap.

1.3.2. GCs’ influence on NAFLD

1.3.2.1. Effects of GCs in steatosis

Evidence that glucocorticoids increase the incidence of steatosis has been obtained from patients with Cushing’s syndrome. 20% of Cushing’s syndrome patients (Rockall et al., 2003), compared with 9.7% of unselected hospital patients (el-Hassan et al., 1992) are diagnosed with steatosis by computed tomography (CT), which is an insensitive diagnostic technique. In systemic lupus erythematosus (SLE) patients treated with GCs, high dose GC administration is a significant factor in the aetiology of severe steatosis (Matsumoto et al., 1992).

As detailed above, GCs’ effect on steatosis is plausibly associated with known effects of GCs on metabolism. Glucocorticoids induce steatosis by inducing insulin resistance and causing hepatic fat accumulation by disturbing the balance of lipid synthesis and export (Andrews and Walker, 1999, Angulo, 2002). Increased de novo lipogenesis (DNL) and impaired lipid β-oxidation induced by dexamethasone result in an increase in steatosis (Dolinsky et al., 2004, Letteron et al., 1997).
1.3.2.2. **Effect of GCs effect in NASH**

GCs’ impact on NASH is disputable due to limited study numbers. In a follow-up study with 48 SLE patients, 6% of patients receiving high dose GCs were diagnosed with NASH (Matsumoto et al., 2007). However, acute alcoholic steatohepatitis was significantly improved by treating with glucocorticoids (Forrest et al., 2007).

The mechanism of GCs’ role on the progression of steatosis to NASH and fibrosis is poorly addressed. A study showed high fat diet and long term GC administration caused significantly increased hepatic fat accumulation, fibrosis and plasma alanine aminotransferase levels compared with normal chow and placebo (D'Souza A et al., 2012). However, the pathway regulates the progression was largely unknown.

1.3.2.3. **Effect of GCs effect in liver fibrosis**

GCs can be used for patients with fibrosis in autoimmune hepatitis and primary biliary cirrhosis. Prednisone, alone or at a lower dose in combination with azathioprine, increases the 20-year life expectancy to 80% and prevents or reduces hepatic fibrosis in 79% of patients in autoimmune hepatitis (Czaja, 2009). Combination therapy of ursodeoxycholic acid (UDCA) and budesonide significantly improve liver histology score compared to UDCA alone and placebo in patients with primary biliary cirrhosis (Leuschner et al., 1999). However, GCs are not used in other chronic liver diseases including viral hepatitis because of their immune-suppressive effect.

Despite the clinical use of GCs in liver fibrosis, the mechanism of GCs’ effect in liver fibrosis is still poorly understood. GCs may regulate tissue remodelling with or without reduced liver fibrosis. Dexamethasone significantly decreased serum AST and ALT levels and the fibrosis histology score after bile duct ligation, a model for liver fibrosis (Eken et al., 2006). Dexamethasone significantly down-regulated Timp1 and Mmp8 mRNA level in a rat bile duct obstruction and recovery model although ECM deposition was not affected suggesting a possible role of glucocorticoid in tissue remodelling (Muratore et al., 2009).
Effect of GCs in key cells involved in liver fibrosis

The effect of GCs on hepatocyte regeneration is controversial. GCs stimulated the expression of plasminogen activator inhibitor type 1 (PAI-1) and CTGF in hepatocytes treated by TGFβ (Wickert et al., 2007), suggesting a pro-fibrotic role of GCs. Dexamethasone mildly inhibited DNA synthesis in cultured hepatocytes by inhibiting EGF pathways (Scheving et al., 2007). Conversely, dexamethasone enhanced hepatocyte DNA synthesis in the presence of EGF (McGowan, 1988). Dexamethasone also suppressed the apoptosis of hepatocytes induced by either serum deprivation (Evans-Storms and Cidlowski, 2000) or TGFβ (Wanke et al., 2004). Whether these observations represent physiological effect of GCs on hepatocyte regeneration and pro-fibrotic effect is unclear.

As in other myofibroblast precursors, GCs can inhibit the activation of HSC induced by TGFβ by suppressing Smad pathways in vitro (Bolkenius et al., 2004). GCs also stimulated TGFβ type III receptor expression in HSCs suggesting GCs are an important regulator of the TGFβ pathway (Wickert et al., 2004). In this case, GCs can be possibly anti-fibrotic by restraining myofibroblast activation.

Any selective effect of glucocorticoids on pro-fibrotic macrophages and anti-fibrotic macrophages (as described in section 1.1.3.3.6) is largely unknown. Selective delivery of dexamethasone to hepatic macrophages increased collagen deposition and TIMP-1 expression, although accompanied by a decrease in reactive oxygen species (ROS) producing cells (Melgert et al., 2001), suggesting a complex role of glucocorticoids in macrophages during liver fibrosis.

11β-HSD1 and NAFLD

11β-HSD1 gene polymorphisms and NAFLD in human

11β-HSD1 gene polymorphisms are associated with altered 11β-HSD1 activity (Malavasi et al., 2010, Draper et al., 2002). In this case, the studies on 11β-HSD1 gene polymorphisms provide evidence for potential relevance between 11β-HSD1 and diseases. There are no direct data suggesting any 11β-HSD1 gene mutation is related with incidence of NAFLD. However, some key features of NAFLD show
relevance with 11\(\beta\)-HSD1 gene polymorphisms. Elevated serum alanine aminotransferase (ALT), is associated with two HSD11B1 polymorphisms (at rs12086634 and rs1000283) in human (Moon et al., 2013). This indicates the possible association between 11\(\beta\)-HSD1 and hepatocellular injury. Also, a novel HSD11B1 variant (at rs3753519) is associated with obesity markers (BMI, waist circumference and serum cholesterol) and serum \(\gamma\)-glutamyl transpeptidase (GGT) suggesting a possible underlying correlation between 11\(\beta\)-HSD1 and NAFLD in children (Olza et al., 2012). A weak association was detected between two polymorphic (CA)\(_n\) repeats in HSD11B1 and waist: hip ratio, suggesting 11\(\beta\)-HSD1 could be possibly associated with central obesity (Draper et al., 2002). Association of HSD11B1 polymorphism and Type 2 diabetes is found in Pima Indians (Nair et al., 2004) although not in Koreans (Moon et al., 2011).

1.3.2.4.2. **Role of 11\(\beta\)-HSD1 in NAFLD**

GC administration leads to enhanced hepatic steatosis whilst blocking GC effect ameliorates steatosis along with metabolic syndrome. Exaggerating hepatic GC action leads to dyslipidaemia, steatosis and hepatic insulin resistance (Paterson et al., 2004). On the other hand, blocking the effect of GC leads to relieved hepatic fat accumulation. 11\(\beta\)-HSD1 inhibition leads to increased lipid \(\beta\)-oxidation and improved VLDL transport, resulting in reduced hepatic fat accumulation in rats (Berthiaume et al., 2007). Participants treated with selective 11\(\beta\)-HSD1 inhibitors have significantly reduced hepatic TG and increased transcripts of several genes coding for enzymes of mitochondrial and peroxisomal \(\beta\)-oxidation (Berthiaume et al., 2010).

There is no direct evidence suggesting 11\(\beta\)-HSD1’s role in NASH development. However, blocking GCs’ effect in this stage could still be beneficial because limiting fat accumulation and improving insulin resistance in the liver may restrain the development of NASH even in the face of exaggerated pro-inflammatory pathways. Insulin resistance is independently associated with NASH (Dixon et al., 2001). Hepatic lipid oxidation gene PPAR\(\alpha\) protects mice from diet induced NASH (Abdelmegeed et al., 2011). The leptin pathway stimulates the progression of NASH (Sahai et al., 2004). Conversely, it was suggested that inhibiting TG synthesis
relieves steatosis but could stimulate the progression of steatohepatitis in mouse dietary NASH models (Yamaguchi et al., 2007). 11β-HSD1 KO mice have significantly increased insulin sensitivity, hepatic PPARα which leads to stimulated lipid oxidation in the liver, and decreased serum leptin level (Morton et al., 2001). This suggests 11β-HSD1 KO can impede NASH development not only by improving insulin sensitivity but also by improving lipid profile. However, as an important immune-suppressing reagent, blocking GCs’ effect may lead to exaggerated inflammation in the liver which would promote NASH progression. In this study, I will further investigate the effect of 11β-HSD1 KO in a rodent NASH model.

11β-HSD1’s effect in liver fibrosis was largely unknown. 11β-HSD1 KO mice had significant reduced hepatic fibrinogen mRNA suggesting a possible role of 11β-HSD1 in regulating matrix degradation (Morton et al., 2001). However, whether 11β-HSD1 KO would stimulate collagen deposition in the liver, as it did in atherosclerosis and a skin wound healing model, or influence HSC activation is largely unknown. In this thesis, I will investigate the 11β-HSD1 KO’s effect on liver fibrosis and HSC activation.

1.4. Hypothesis

I hypothesize that 11β-HSD1 deficiency increases hepatic susceptibility to fibrosis and inflammation in NAFLD.

1.5. Aims

- To test if 11β-HSD1 levels are altered in the liver in different models of NAFLD and liver fibrosis (Chapter 3)
- To investigate the effect of 11β-HSD1 deficiency (transgenic models) on the progression and/or resolution of steatosis, NASH and fibrosis (Chapter 4 -5)
- To test the influence of pharmacological inhibition of 11β-HSD1 during liver injury and/or resolution (Chapter 6)
- To understand the mechanism of GC mediated inhibition of HSC activation (Chapter 7)
Chapter 2.  Materials and Methods
All chemicals were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated. Radioactive chemicals were purchased from Amsham (Bucks, UK). 11β-HSD1 inhibitor UE2316 was developed in house and used as previously described (Turban et al., 2012). Culture equipment was from Lonza (Slough, UK) unless specifically mentioned. Solvents were purchased from Fisher (Loughborough, UK) in HPLC grade. Solvents were stored at room temperature (18-22°C, RT), 4°C or -20°C. Detailed information of supplier information is listed in the Appendix.

### 2.1. Reagents

**Table 2.1 Cell culture reagents**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-Dehydrocorticosterone (A)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Collagenase IV</td>
<td>Roche</td>
</tr>
<tr>
<td>Corticosterone (B)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Dextran coated charcoal</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Dimethyl Sulphoxide (DMSO)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>DMEM/F12 Glutmax medium</td>
<td>GIBCO/Invitrogen</td>
</tr>
<tr>
<td>Dnase I</td>
<td>Roche</td>
</tr>
<tr>
<td>Fetal bovine serum (FBS)</td>
<td>Lonza</td>
</tr>
<tr>
<td>HBSS</td>
<td>Lonza</td>
</tr>
<tr>
<td>L-Glutamine (200mM)</td>
<td>Lonza</td>
</tr>
<tr>
<td>Mifepristone (RU486)</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>
NucleoCassette 

NucleoCounter 

Penicillin (10,000U/ml)/streptomycin (10,000 µg/ml) 

PRMI 1640 

TGFβ 

UE2316* 

*UE2316 ([4-(2-chlorophenyl-4-fluoro-1-piperidinyl][5-(1H-pyrazol-4-yl)-3-thienyl]-methanon) was kindly supplied by McBride et al. and used in cells as previously described (Turban et al., 2012).

<table>
<thead>
<tr>
<th>Species</th>
<th>IC50 (nM)</th>
</tr>
</thead>
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<tr>
<td>Human</td>
<td>42</td>
</tr>
<tr>
<td>Mouse</td>
<td>126</td>
</tr>
<tr>
<td>Rat</td>
<td>80</td>
</tr>
<tr>
<td>Monkey</td>
<td>72</td>
</tr>
</tbody>
</table>

IC50 was determined in assays of HEK293 cells stably transfected with the full length hsd11b1 genes for each species as previously described (Webster et al., 2007).
### Table 2.3 Immunostaining reagents

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Protein block</td>
<td>Dako</td>
</tr>
<tr>
<td>Antibody diluent D</td>
<td>Dako</td>
</tr>
<tr>
<td>Avidin/biotin blocking kit</td>
<td>Vector</td>
</tr>
<tr>
<td>Bio-Rad Protein Assay Kit</td>
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<tr>
<td>DL-Dithiothreitol (DTT)</td>
<td>Sigma-Aldrich</td>
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<td>Fast green</td>
<td>Sigma-Aldrich</td>
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<td>Formalin solution (4%)</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Harris' haematoxylin</td>
<td>Thermo</td>
</tr>
<tr>
<td>Hydrogen peroxide (30%)</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Liquid diaminobenzidine (DAB) + substrate chromogen system</td>
<td>Dako</td>
</tr>
<tr>
<td>Phosphate buffered saline (PBS) tablets</td>
<td>Oxoid</td>
</tr>
<tr>
<td>R.T.U. vectorstain kit (ABC reagent)</td>
<td>Vector</td>
</tr>
<tr>
<td>Molecular biology reagent</td>
<td>Manufacturer</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>[³⁵S]-dUTP</td>
<td>GE healthcare</td>
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<tr>
<td>BIOTAQ Red DNA Polymerase kit</td>
<td>Gentaur</td>
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<td>Diethyl phyocarbonate (DEPC)</td>
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<td>Low electroendosmosis (LE) agarose</td>
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<tr>
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<td>Roche</td>
</tr>
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<td>Nuclease-free water</td>
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<td>Invitrogen</td>
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<td>RNaseZAP</td>
<td>Ambion</td>
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<td>Promega</td>
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<td>SuperScript™ III Reverse Transcriptase</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>T7 RNA polymerase</td>
<td>Promega</td>
</tr>
<tr>
<td>QIAzol Reagent</td>
<td>Qiagen</td>
</tr>
</tbody>
</table>
2.1.1. **Buffers and solutions**

**Table 2.5 Buffer recipe**

<table>
<thead>
<tr>
<th>RNA Work</th>
<th>DEPC Water</th>
<th>1 drop DEPC (Sigma, Dorset, UK) was dissolved in 100ml of de-ionised water and stood overnight before autoclaving.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular biology</td>
<td>DNA lysis buffer</td>
<td>100nM ethylenediamine tetra-acetic acid (EDTA), 50nM Tris/HCl pH7.6 and 200mM NaCl was adjusted with dH20 and sterilized by filtering through 0.2µm filter.</td>
</tr>
<tr>
<td>Molecular biology</td>
<td>TE buffer</td>
<td>10mM Tris pH 8.0 and 1mM EDTA was dissolved in dH20. The buffer was autoclaved before use.</td>
</tr>
<tr>
<td>Molecular biology</td>
<td>TBE Buffer</td>
<td>Tris base (890mM), boric acid (890mM) and EDTA (0.5M) were dissolved in distilled water (800ml). The pH was adjusted to 8.0 through addition of NaOH (10M) and volume was adjusted to 1L with distilled water. The solution was autoclaved and stored at room temperature. The buffer was diluted 20 times before using.</td>
</tr>
<tr>
<td>Western blot</td>
<td>Protein Lysis Buffer</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 1M, pH 7.5), NaCl (137mM), NaF (10mM), EDTA (2mM) and glycerol (10% v/v) were dissolved in distilled water (500ml) and the pH was adjusted to 7.6. Solution was stored at 4°C. Final lysis buffer was made by adding proteinase inhibitors in following dilutions: benzamidine (1M) was diluted 1:100; orthovanadate (200mM, pH10) was diluted 1:100; leupeptin (1mg/ml) was diluted 1:500; aprotinin was added 1:500; PMSF (100mM) was diluted 1:100.</td>
</tr>
<tr>
<td>Western blot</td>
<td>Resolving (lower) Gel Buffer</td>
<td>300mM Tris and 2.8mM SDS was dissolved in dH₂O and the pH was adjusted to 8.8.</td>
</tr>
<tr>
<td>Western blot</td>
<td>Stacking (upper) Gel Buffer</td>
<td>50mM Tris and 1.4mM SDS was dissolved in dH₂O and the pH was adjusted to 6.8.</td>
</tr>
<tr>
<td>Western blot</td>
<td>Western Running</td>
<td>Tris base (250mM), sodium dodecyl sulphate (SDS, 350mM) and Glycine (2M) were dissolved in distilled water (1L) and stored at RT. A working solution was used by diluting this solution 1:10.</td>
</tr>
<tr>
<td>Buffer</td>
<td>20× transfer buffer was made by dissolving Tris base (250mM) and glycine (1.92M) in distilled water. 1× Transfer buffer was made by diluting the 20× solution 50ml with 850ml water and 100ml methanol.</td>
<td></td>
</tr>
<tr>
<td>--------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Western blot</td>
<td>TBS and TBST buffer</td>
<td></td>
</tr>
<tr>
<td>Western blot</td>
<td>10× solution: Tris base (250mM), KCl (27mM) and NaCl (1.5M) were dissolved in distilled water (800ml). The pH was adjusted to 7.4 with concentrated HCl (37%), before the volume was adjusted to 1L with distilled water and stored at RT. Solution was diluted 1:10 before using. TBST buffer was made by adding 0.05% v/v Tween-20 to 1×TBS.</td>
<td></td>
</tr>
<tr>
<td>Western blot</td>
<td>Blocking Milk</td>
<td></td>
</tr>
<tr>
<td>Western blot</td>
<td>Non-fat dry milk (Rio-Rad, Herts, UK, 5g) was dissolved in 1x TBST (100ml) and stored at 4°C.</td>
<td></td>
</tr>
<tr>
<td>Steroid assay</td>
<td>Homogenizing buffer for steroid extraction</td>
<td></td>
</tr>
<tr>
<td>Steroid assay</td>
<td>4.9mM Tris base, 1.27mM EDTA and 20% w/v glycerol was dissolved in dH₂O and pH was adjusted to 7.5. Solution was stored in 4°C. 0.05mM DTT was added to solution right before use.</td>
<td></td>
</tr>
<tr>
<td>Steroid assay</td>
<td>Buffer</td>
<td><strong>25mM sodium acetate, 1.27mM EDTA and 10% w/v glycerol was added to dH₂O and the pH was adjusted to 6.0. Solution was stored in 4°C before using.</strong></td>
</tr>
<tr>
<td>---------------</td>
<td>---------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Steroid assay</td>
<td>Modified C Buffer</td>
<td><strong>50mM Tris base, 300mM NaCl, 12.6% w/v glycerol and 1.27mM EDTA was dissolved in dH₂O and pH was adjusted to 7.7. Solution was stored at 4°C before using.</strong></td>
</tr>
<tr>
<td>In situ hybridization</td>
<td>Pre- Hybridization Buffer</td>
<td><strong>2× solution: 5.88ml DEPC H₂O, 2.4ml 5M NaCl, 200μl 1M Tris (pH 7.5), 400μl 50x Denhardt’s solution (Sigma, Dorset, UK), 80μl 250mM EDTA, 1ml 10mg/ml sonicated salmon sperm DNA (Sigma, Dorset, UK), and 40μl 50mg/ml yeast tRNA (Sigma, Dorset, UK),</strong></td>
</tr>
<tr>
<td>In situ hybridization</td>
<td>Hybridization Buffer</td>
<td><strong>6.68ml DEPC H₂O, 2.4ml 5M NaCl, 200μl 1M Tris (pH 7.5), 400μl 50x Denhardt’s solution, 80μl 250mM EDTA, 200μl 10mg/ml sonicated salmon sperm DNA (Sigma, Dorset, UK), 40μl 50mg/ml yeast tRNA (Sigma, Dorset, UK), and 2.0g dextran sulphate (Sigma, Dorset, UK).</strong></td>
</tr>
<tr>
<td>In situ hybridization</td>
<td>RNASE buffer</td>
<td><strong>1ml 5M NaCl, 100μl 1M Tris and 40μl 250mM EDTA, made up to 10ml with DEPC H₂O</strong></td>
</tr>
</tbody>
</table>
**In situ hybridization**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraformaldehyde</td>
<td>4% paraformaldehyde was dissolved in DEPC water and mixed at 80°C until for 1 hour. Sample was autoclaved before storing at 4°C. Solution is stable for up to 1 month.</td>
</tr>
<tr>
<td>Saline sodium citrate buffer (SSC)</td>
<td>3M NaCl and 0.3M trisodium citrate were mixed and dissolved in dH2O to make 20×SSC solution. The pH was adjusted to 7.0 and the buffer was autoclaved for further use. Working solutions were 2×SSC (1:10) and 0.1×SSC (1:200).</td>
</tr>
</tbody>
</table>

**Immunohistochemistry**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Citrate (0.01M)</td>
<td>1.92 g citric acid (anhydrous) was made up to 1L with dH2O and the pH was adjusted to 6.0.</td>
</tr>
<tr>
<td>H2O2</td>
<td>30% H2O2 (Sigma, Dorset, UK) was diluted 1:10 using 1×PBS in 1:10 dilution.</td>
</tr>
</tbody>
</table>

**Staining**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Picrosirius red solution</td>
<td>0.5g Sirius red F3B (C.I. 35782) (Direct red 80 from Sigma, Dorset, UK) was dissolved in 500ml saturated picric acid solution (Sigma, Dorset, UK) and stirred for 30 minutes. Additional sirius red F3B</td>
</tr>
</tbody>
</table>
was added to ensure the presence the precipitation when necessary.

<table>
<thead>
<tr>
<th>Staining</th>
<th>Oil Red O</th>
<th>Oil Red O working solution was made by diluting 30ml of Oil Red O solution (0.5% in 2-propanol, Sigma, Dorset, UK) in 20ml distilled water and allowed to stand for 10 minutes. Solution is filtered through filter paper into a Coplin jar with cover.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staining</td>
<td>Phosphomolybdic acid</td>
<td>0.2% w/v phosphomolybdic acid was dissolved in distilled water and stored at room temperature.</td>
</tr>
<tr>
<td>Cell culture</td>
<td>Heat inactivated FBS</td>
<td>De-frosted FBS was incubated at 55°C for 30 minutes and stored at -20 °C for use.</td>
</tr>
<tr>
<td>Cell culture</td>
<td>Charcoal stripped FBS</td>
<td>Dextran coated charcoal (Sigma, Dorset, UK) was put into 500ml heat inactivated FBS (5g/500ml) and mixed overnight at 4°C. Charcoal/serum mix was filtered through 5µm, 1.22µm, 0.45µm and 0.22 µm filters with the final filtration in sterile hood.</td>
</tr>
</tbody>
</table>
2.2. Animals

All experiments were carried out under a UK Home Office animal license (project licence: Jonathan Seckl: 60/3962; personal licence: 60/12620). Animals were housed in standard housing cages in BRR Unit in the Chancellor’s Building of the University of Edinburgh. The environment was controlled in a condition of 12 hours light and 12 hour dark at 18-22°C. All mice were fed on a standard chow (RM1 diet, 801002, Special Diet Services, Witham, UK) and drinking water after weaning. Experimental mice were male mice of C57BL/6 background aged 8-12 weeks old or otherwise stated. Mice were bred in house or purchased from Jackson Laboratories (Bar Harbor, ME, US) with a UK animal importing licence.

2.2.1. Transgenic models

2.2.1.1. Global knockout mice

Global 11β-HSD1 knockout (KO, 11β-HSD1/-) mice, which are homozygotes containing a targeted disruption of the hsd11b1 gene encoding 11β-HSD1 (Backcrossed more than 8 generations on a C57BL/6 background) were provided by our group as described before (Morton et al., 2001). Age matched C57BL/6 mice were used as control mice unless specified.

2.2.1.2. Ob/ob mice

Ob/ob mice were purchased from Jackson Laboratories (Bar Harbor, ME, US). Frozen liver samples from ob/ob mice were kindly provided by Andrew McBride as a gift.

2.2.1.3. Liver specific 11β-HSD1 KO mice

Hepatocyte specific 11β-HSD1 KO (Albumin Cre<sup>Tg<sub>+</sub></sup> 11β-HSD1<sup>Flox/Flox</sup>, Alb-HSD1 mice) were developed by Manwani et al. in house and kindly provided by Prof. K. Chapman as a gift (unpublished). Cre-LoxP technique was used to knockout hsd11b1
gene specifically in the liver (mechanism in Figure 2.1). Albumin CreTg/+ mice, which showed specificity and efficiency in knocking down target genes in the liver (Kenerson et al., 2013, Inoue et al., 2004), were purchased from Jackson Laboratories (Backcrossed 8 times to C57BL/6 mice according to supplier’s information, Bar Harbor, ME, US). 11β-HSD1Flox/Flox mice were developed in house by Prof. K. Chapman’s group. Albumin CreTg/+11β-HSD1Flox/+ offspring of Albumin CreTg/+ mice and 11β-HSD1Flox/Flox mice were further crossed with 11β-HSD1Flox/Flox mice. Albumin CreTg/+11β-HSD1Flox/Flox (Alb-HSD1) mice were selected as experimental mice and 11β-HSD1Flox/Flox mice were used a control.

![Figure 2.1 Cre-LoxP deletion of 11β-HSD1 in the liver](image)

**Figure 2.1 Cre-LoxP deletion of 11β-HSD1 in the liver**

Two LoxP sites were integrated to hsd11b1 gene to flank exon 3. Cre recombinase under an Albumin enhancer was produced specifically in hepatocytes. Cre recombinase recognises the LoxP sites and cut through both loxP sites leading to a deletion of the floxed segment.

### 2.2.2. Chemical induced liver injury

#### 2.2.2.1. Acute carbon tetrachloride (CCL4) induced liver injury

1.6µl/g 25% CCL4 dissolved in olive oil was administrated to the intra-peritoneal space (i.p.) of the mice which was fasted overnight as previously described
(Henderson et al., 2006). The mice were re-fed after injection and livers were harvested at 24 hours after last CCL₄ injection (peak injury).

### 2.2.2.2. Chronic CCL₄ induced liver fibrosis

A reversible model of CCL₄ induced chronic liver fibrosis was undertaken as previously described (Issa et al., 2004). 1.6µl/g 25% CCL₄ dissolved in olive oil was injected i.p. twice a week for 12 weeks to induce liver fibrosis. Mice killed 24 hours after last CCL₄ administration were considered as representing peak fibrosis; mice killed 72 hours and 8 days after last CCL₄ administration were defined as in the resolution phase according to previous data (Ramachandran et al., 2012).

### 2.2.3. Diet models

#### 2.2.3.1. High fat diet (HFD) induced obesity

Samples from genetically 11β-HSD1 deficient mice on HFD were kindly provided by Michailidou et al. and pharmacologically 11β-HSD1 inhibited mice on HFD were kindly provided by McBride et al. During diet induced obesity (DIO), mice aged 8-10 weeks received normal chow or HFD, 58% calories as fat (D12331) (Research Diets, New Brunswick, US), for 18 weeks.

#### 2.2.3.2. Choline deficient and methionine-choline deficient diet for NAFLD

To induced steatosis and NASH without obesity, mice were fed control (518574), choline deficient (CDD, 518753) or methionine-choline deficient diet (MCDD, 518810) from Dyets (Bethlehem, PA, US) for 2 weeks as previously described (Macfarlane et al., 2011). The diets were calorie matched and detailed information is listed in Table 2.6.
Table 2.6 Constitution of Control diet, CDD and MCDD

<table>
<thead>
<tr>
<th></th>
<th>Control diet (Control)</th>
<th>Choline deficient diet (CDD)</th>
<th>Methionine and choline-deficient diet (MCD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kcal/gram</td>
<td>4.3</td>
<td>4.3</td>
<td>4.3</td>
</tr>
<tr>
<td>Kcal%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (as L-amino acids)</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>55</td>
<td>55</td>
<td>55</td>
</tr>
<tr>
<td>Fat</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Choline Bitartate (g/kg)</td>
<td>14.48</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L-Methionine (g/kg)</td>
<td>1.7</td>
<td>1.7</td>
<td>0</td>
</tr>
</tbody>
</table>

2.2.3.3. *Thioacetamide (TAA) induced liver fibrosis*

600 mg/L TAA (Sigma, Dorset, UK) was dissolved in drinking and administered to C57BL/6 aged 12 weeks for 1 year. Water supply was switched to normal water after TAA termination. Frozen liver samples were kindly provided by Pellicoro et al as a gift.

2.2.3.4. *11β-HSD1 inhibitor diet*

UE2316 diet was made by mixing 0.15% w/w UE2316 into RM1 diet by DBM Food Hygiene Supplies Ltd. Mice were administered with this diet throughout the experiment to achieve 11β-HSD1 inhibition in the liver (Sooy et al. unpublished data). C57BL/6 mice receiving normal RM1 diet were used as control mice.
2.3. Cells

2.3.1. Primary culture of mouse HSCs

2.3.1.1. Cell preparation

Primary mouse hepatic stellate cells were collected from wild type C57BL/6 mice or 11β-HSD1 KO mice. Each prep was made from 2-4 mice aged 9-10 weeks culled by schedule one. Each liver was perfused with 5ml saline from inferior vena cava (until the livers were pale after the perfusion). Livers were harvested and dissected into 2×2 mm pieces. For each prep, the pieces were digested at 37°C for 20 minutes by 15ml 10mg/ml DNASE I (Roche, East Essex, UK), 5ml 10mg/ml Collagenase B (Roche, East Essex, UK) and 5ml 100mg/ml Pronse (Sigma, Dorset, UK). Enzyme solutions were freshly prepared in HBSS and filter sterilized through 0.2µm filters. After incubation, the digest mix was poured through 70µm strainers and further washed through by 5ml of HBSS with additional 5ml 10mg/ml DNASE I. Cells were spun at 572g for 7 minutes and the supernatant was discarded. Gradient Optiprep medium was prepared and added to 15ml falcon tube: bottom layer with 2.4ml neat Optiprep (Sigma, Dorset, UK) plus 1.6ml HBSS; middle layer with 2.9ml neat Optiprep and cell palettes suspended with 4.5ml HBSS; upper layer with 0.5ml HBSS. The tube was spun at 4°C at 1100g for 20 minutes with medium acceleration rate and minimum de-acceleration rate. The top layer was collected as HSCs followed by a further wash with 50ml HBSS and 1.5ml DNASE I. Cell number was counted by Nucleocounter (Allerød, Denmark) according to manufacturer’s protocol. 1×10^6 cells per well were seeded into 6 well plates and cultured in 2ml DMEM with 16% FBS 100 U/ml penicillin/ 100mg/ml streptomycin and 2mM L-glutamine. For 24 well plates, 1×10^5 cells were seeded into each well with 0.5ml culture medium. Cell medium was refreshed every two days after seeding.

2.3.1.2. Spontaneous primary HSC activation

Primary HSCs can be activated on non-sticky plastics with the presence of serum (Issa et al., 2004). Wild type control and/or KO cells from each prep were plated in triplicates and harvested in day 2, 5, 8, 11 and 13 after seeding. For pharmacological
studies, cells were treated with combination of vehicle, Corticosterone (B, 500nM), 11-dehydrocorticosterone (A, 500nM), UE2316 (10µM) and RU486 (10 µM) and harvested at day 8 after seeding.

2.3.2. LX-2 Cells

LX-2 cells are immortal human HSC cells which are a useful tool to study liver fibrosis response in vitro (Xu et al., 2005). LX-2 cells are highly viable in serum-free medium as primary HSCs and show strong similarity in gene expression with semi-activated primary HSCs (98.7%). LX-2 cells can be further activated by TGF-β or hypoxia (Shi et al., 2007). Cells were maintained in DMEM with 10% FBS, 100 U/ml penicillin/ 100mg/ml streptomycin and 2mM L-glutamine.

2.3.2.1. TGF-β activation of LX-2 cells

5×10^5 LX-2 cells were plated in each well of 6 well plates. After cells reached 70% confluence, medium was changed to serum free medium after wash the cells with PBS twice. Cells were incubated with 2ng/ml TGF-β or vehicle for 16 hours. Cells were harvested and stored at -80°C for further analysis.

2.3.3. Cell Lysate preparation

During harvest, 2ml of cell medium was transferred into eppendorfs and frozen on dry ice. Each well was washed with 1ml PBS. For protein analysis, 60µl protein lysis buffer (recipe in Table 2.5) was added to the cells and left for 5 minutes at room temperature. For RNA extraction, 350µl RLT buffer (Qiagen,Crawley,UK) were added to the cells and left for 5 minutes. Cell lysates were collected by scraping the cells off using cell scrapers (Corning, Amsterdam, the Netherlands). All cell lysates were transferred to eppendorfs and froze on dry ice.

2.3.4. Cell fixation

Cells seeded in 24 well plates were used for immunochemistry staining. Cells were washed with PBS after culture medium was removed. Cells were further incubated with 0.5ml cold methanol for 10 minutes at -20°C and stained directly after fixing.
2.3.5. Flow cytometry of non-parenchymal cells

2.3.5.1. Cell isolation

Livers were perfused with 5ml of saline through inferior vena cava during harvesting and 200-300mg of liver was stored in PRMI1640 medium (Lonza, Slough, UK). Liver pieces were mashed and digested in PRMI 1640 medium with Dnase I (final concentration 25 mg/ml, Roche, East Essex, UK) and Collagenase B (final concentration 15 mg/ml, Roche, East Essex, UK) on a rotator at 37°C for 45 minutes. Cells were spun at 50g for 5 minutes and the pellets, of which the majority were hepatocytes, were discarded. The supernatant was collected and spun at 350G for 15 mins twice. Pellets containing macrophages were collected and washed in PBS. Live and dead Marker (eBiomedicine, New castle, UK) were added to each sample and left for 25 minutes at 4°C to stain the dead cells. Cells were washed in PBS twice and blocked in 10% mouse serum (Sigma, Dorset, UK) for 30 minutes. Fluorescent antibodies were added to each sample (details of antibodies is presented in Table 2.7) and left for 30 minutes at 4°C. After washing in PBS, the cells were fixed in 10% formalin and stored at 4°C for further analysis. Randomly selected cells were not stained or stained with each single antibody for adjusting the fluorescence compensation of flow cytometry using BD LSR Fortessa (BD, Oxford, UK) as manufacturer instructed.
Table 2.7 Information for antibodies used in flow cytometry

<table>
<thead>
<tr>
<th>Name</th>
<th>Florecense</th>
<th>Company</th>
<th>Cat#</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ly6G</td>
<td>Pacific blue</td>
<td>Biolegend</td>
<td>127612</td>
<td>1:100</td>
</tr>
<tr>
<td>CD11b</td>
<td>AF488</td>
<td>eBioscience</td>
<td>53-0112-82</td>
<td>1:100</td>
</tr>
<tr>
<td>F4/80</td>
<td>APC</td>
<td>eBioscience</td>
<td>45-5932-82</td>
<td>1:50</td>
</tr>
<tr>
<td>CD45.2</td>
<td>AF700</td>
<td>Biolegend</td>
<td>109821</td>
<td>1:100</td>
</tr>
<tr>
<td>Ly6C</td>
<td>Perp cy 5.5</td>
<td>Biolegend</td>
<td>127607</td>
<td>1:100</td>
</tr>
<tr>
<td>CD11c</td>
<td>PE</td>
<td>eBioscience</td>
<td>12-0114-81</td>
<td>1:100</td>
</tr>
<tr>
<td>Live&amp;Dead</td>
<td>AF780</td>
<td>eBioscience</td>
<td>65-0865-18</td>
<td>1:100</td>
</tr>
</tbody>
</table>

2.3.5.2. Flow cytometry analysis

The flow cytometry was conducted on a BD LSR Fortessa (BD, Oxford, UK). Cells were analysed as previously described (Ramachandran et al., 2012). Compensation was calculated automatically according to the results of all single stained samples according to manufacturer’s protocol. Side scatter (SSC) and forward scatter (FSC) were used to identify singlets and cells. 10,000 events of cells were collected from each preparation. Cells with negative Dead stain (AF780⁻) were defined as live cells. Live CD45.2⁺ cells were defined as inflammatory cells. Among CD45.2⁺ cells, Ly6G⁺CD11b⁺ (Pacific blue positive and AF488 positive) cells were gated as neutrophils. Ly6G⁻CD11b⁻ cells were defined as non-neutrophils inflammatory cells. CD11bintF4/80hi cells were gated as resident macrophages and CD11bhiF4/80int cells were gated as macrophages derived from monocytes. CD11bint F4/80hi cells were used to further analyse the Ly6C subsets. Data was analysed by flow jo 6.0. Results were expressed as the ratio of target cell numbers over all cell numbers. Detailed gating information is illustrated in Figure 2.2.
**Figure 2.2 Flow cytometry gating strategy**

In this sampling gating singlets were selected with linear forward scatter height (FSC-H) against forward scatter area and FSC-A (FSC-A). Events fitted into a logarithm curve plotted in side scatter area (SSC-A) against forward scatter area (FSC-A) axis were then gated as cells. Cells negative of Live/Dead marker were selected for further analysis. CD45.2 positive cells were selected as bone marrow derived cells. Among these cells, Ly6G^+CD11b^+ cells were defined as neutrophils. Other bone marrow derived cells were plotted with CD11b and F4/80. CD11b^{int}F4/80^{hi} cells were resident macrophages (Mϕ). CD11bhiF4/80^{int} cells were monocyte derived macrophages (Mϕ). Ly6C^{hi} and Ly6C^{lo} subsets were gated according to the Ly6C expression of CD11b^{hi}F4/80^{int} cells.

**2.4. Histology**

**2.4.1. Preparations of liver sections**

**2.4.1.1. Formalin fixed sections**

Formalin fixed sections were used for immunohistochemistry and picrosirius red (PSR) staining. 200-300mg fresh liver portions were fixed in formalin for 16 hours at room temperature. The fixed tissue was dehydrated and embedded in paraffin in the histology department (the University of Edinburgh, the Queen’s Medical Research Institution). The blocks were cut into sections of 4 µm by microtome (Thermo, Runcorn, UK) in the histology department.

**2.4.1.2. Frozen sections**

Frozen sections were used for immunofluorescence, In situ hybridization and Oil red O staining. A piece of tissue covered in foil was snap-frozen in liquid nitrogen. Sections were cut by cryostat (Leica, Bucks, UK) at -20 °C into 10 µm sections and stored at -80 °C.
2.4.2. Immunohistochemistry

2.4.2.1. Immunohistochemistry on paraffin embedded tissue

Formalin fixed paraffin slides of liver samples were de-waxed in xylene for 10 minutes and rehydrated in 100% ethanol, 95% ethanol, 80% ethanol and 60% ethanol in turn for 1 minute each. Antigen retrieval was used to unmask the antigen covered by protein-cross links in formalin fixed tissue. Slides were incubated in pre-heated boiling sodium citrate buffer (recipe in Table 2.5) for 15 minute. After a briefly washing with water, slides were immersed in 3% hydrogen peroxide (H$_2$O$_2$ recipe in Table 2.5) with agitation for 10 minutes. The slices were then rinsed in PBS and mounted in Shandon Sequenza racks (Fisher, Loughborough, UK). After three washes of PBS, 3 drops of Adivin block (Vector Labs, Burlingame, CA, USA) were added to each slide and allowed to incubate for 15 minutes at room temperature. After another 2 washes of PBS, slides were incubated with 3 drops of Biotin block (Vector Labs, Burlingame, CA, USA) for 15 minutes at room temperature. Primary antibody was added at stated concentration (Table 2.8) directly after slides were incubated in 120µl diluted serum (1 part normal serum with 4 part PBS added by 0.5% Tween-20) (Vector Labs, Burlingame, CA, USA) or protein block (Vector Labs, Burlingame, CA, USA) for 30 minutes at room temperature. The second antibody diluted in serum or antibody diluent was added after 3 washes of PBS and allowed to incubate for 30 minutes at room temperature. After three further washes in PBS, slides were incubated with 3 drops of Vector Avidin and Biotin horseradish peroxidase macromolecular Complex (RTU ABC, Vector Labs, Burlingame, CA, USA) for 30mins at room temperature. The colour was developed by adding 3 drops of diaminobenzidine reagent (DAB, DAKO, Ely, UK) for 5-10 minutes at room temperature. The colour development was terminated by removing the slices from the Sequenza racks. Slices were left in Harris’s haematoxylin (Sigma, Dorset, UK) for 5 seconds and rinsed in tapping water. Colour for nuclei was further developed by incubating the slides in Scot’s tap water (Fisher, Loughborough, UK). After a brief wash in running water, slices were incubated in graded ethanol (65%, 75%, 100% and 100%) for 20 seconds each. Slides were mounted by Di-N-Butyle Phthalate in Xylene mountant (DPX, Sigma, Dorset, UK) after being incubated in xylene for 10
minutes. The nuclei were blue and the positive cells were brown under the microscope.
Table 2.8 Antibodies and dilutions for IHC

<table>
<thead>
<tr>
<th>Protein target</th>
<th>1stAb source</th>
<th>Dilution</th>
<th>2ndAb source</th>
<th>Block&amp;Ab dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen1 (COL-1)</td>
<td>Southernbiotech 1310-01 Goat anti type I collagen</td>
<td>1:300 overnight at 4ºC</td>
<td>Biotin linked rabbit anti- goat antibody (DAKO, Ely, UK) 1:300 at room temperature for 30 minutes</td>
<td>Protein block and antibody Diluent (DAKO, Ely, UK)</td>
</tr>
<tr>
<td>Collagen3 (COL3)</td>
<td>Southernbiotech 1330-01 Goat anti type III collagen</td>
<td>1:300 overnight at 4ºC</td>
<td>Biotin linked rabbit anti- goat antibody (DAKO, Ely, UK) 1:300 at room temperature for 30 minutes</td>
<td>Protein block and antibody Diluent (DAKO, Ely, UK)</td>
</tr>
<tr>
<td>αSMA</td>
<td>Abcam ab7817 Anti-alpha smooth muscle Actin antibody</td>
<td>1:4000 overnight at 4ºC</td>
<td>Biotin linked goat anti-mouse antibody (DAKO, Ely, UK) 1:300 at room temperature for 30 minutes</td>
<td>Goat serum (Vector Labs, Burlingame, CA, USA)</td>
</tr>
<tr>
<td>F4/80</td>
<td>Abcam ab6640 rat anti mouse F4/80 monoclonal antibody</td>
<td>1:300 2 hours at room temperature</td>
<td>Biotin linked rabbit anti-rat antibody (DAKO, Ely, UK) 1:300 at room temperature for 30 minutes</td>
<td>Rabbit serum (Vector Labs, Burlingame, CA, USA)</td>
</tr>
<tr>
<td>GR1 (Ly6G)</td>
<td>Cambridge Biosciences 108413 Leaf purified Rat anti-mouse GR1 antibody</td>
<td>1:200 at 4ºC overnight</td>
<td>Biotin linked rabbit anti-rat antibody (DAKO, Ely, UK) 1:300 at room temperature for 30 minutes</td>
<td>Rabbit serum (Vector Labs, Burlingame, CA, USA)</td>
</tr>
</tbody>
</table>

2.4.2.2. Immunohistochemistry on cells in culture

Cells fixed in methanol were washed in PBS for 3 times. No antigen retrieval was performed before the staining. Cells in each well were incubated in 0.5ml H₂O₂, Advin Block, Biotin block, Protein block, primary antibody, secondary antibody, ABC vector and DAB in turn as described in section 2.4.2.1. Cells were incubated in
haematoxylin for 30 second and washed by running water. Cells were incubated in PBS and stored at 4°C for up to a week.

2.4.3. Immunofluorescence

2.4.3.1. Dual staining immunofluorescence on frozen sections

Frozen sections of liver were fixed in cold acetone for 10 minutes then briefly washed by PBS and placed into a well humidified and dark chamber. Protein block (DAKO, Ely, UK) was added and allowed to incubate for 30 minutes. 11β-HSD1 antibody (developed in house, and a gift from Dr Scott Webster) was added as primary antibody to incubate the sections overnight at 4°C (details in Table 2.9). The next day, anti-sheep secondary antibody conjugated with fluorescent 488 was added to the sections and left for 1 hour at room temperature (details in Table 2.9). Sections were washed in PBS and re-blocked by protein block. αSMA antibody was added to the left in room temperature for 2 hours (details in Table 2.9). After 3 PBS washes, anti-mouse secondary antibody conjugated with fluorescent 555 was added to the sections and allowed to incubate for 1 hour. 4',6-Diamidino-2-Phenylindole, Dihydrochloride mounting medium (DAPI, Vector labs, Burlingame, CA, USA) was used to mount and counter stain the sections. Slides were kept at 4°C in the dark for further analysis.

Table 2.9 Antibody information for immunofluorescence

<table>
<thead>
<tr>
<th>Name</th>
<th>1st Ab</th>
<th>Condition</th>
<th>2nd Ab</th>
<th>Block &amp; Ab dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>11β-HSD1</td>
<td>Sheep anti 11β-HSD1 antibody (In house)</td>
<td>1:700 overnight at 4°C</td>
<td>Alexa Fluor® 488 Donkey Anti-Sheep IgG (Invitrogen, Paisley, UK)</td>
<td>Both using Protein block and Antibody Diluent (DAKO, Ely, UK)</td>
</tr>
<tr>
<td>αSMA</td>
<td>Abcam ab7817 Anti-alpha smooth muscle Actin antibody</td>
<td>1:2000 2 hours at room temperature</td>
<td>Alexa Fluor® 555 Goat Anti-Mouse IgG (H+L) Antibody (Invitrogen, Paisley, UK)</td>
<td>—</td>
</tr>
</tbody>
</table>
2.4.4. Hematoxylin and eosin stain (H&E stain)

Liver sections were dipped into Hematoxylin solution for 5 seconds and washed in running water for 1 minute. Sections were further developed in Scot’s tap water for 10 second to develop the blue colour of the nuclei. Sections were incubated in Eosin solution for 1 minute followed by 1 minute in running water. Cells were incubated in graded ethanol before mounted in DPX.

2.4.5. Picrosirius red stain (PSR)

Paraffin embedded sections were de-waxed in xylene for 10 minutes and rehydrated through graded alcohols (as described in Section 2.4.2) and then incubated in 0.2% v/v phosphomolybdc acid (Sigma, Dorset, UK). Slides were incubated in saturated picrosirius red solution (recipe in Table 2.5) for 1.5 hours and rinsed in 0.5% acetic acid two times. Slides were then incubated in 0.1% w/v fast green (Sigma-Aldrich) dissolved in 0.5% acetic acid for 1 minute. After rinsing in 0.5% acetic acid for 2 times, slides were incubated in 100% ethanol for 1 minute with agitation twice and then incubated in xylene for 1 minute before mounted in DPX. Images were taken under microscope (details in Section 2.4.7) and the background was green and collagens were red under a microscope.

2.4.6. Oil Red O stain

Frozen liver sections (prepared as Section 2.4.1.2) were air dried and then fixed by 4% paraformaldehyde (recipe in Table 2.5). Slides were briefly washed with running tap water and rinsed with 60% 2-propanol. Slides were stained with freshly made Oil Red O solution (recipe in Table 2.5) for 15 minutes at room temperature. After a brief rinse with 60% 2-propanol, slides were stained with Mayer’s haematoxylin for 1 minute at room temperature. After a brief rinse in distilled water, sections were mounted by humid mounting media aqueous mounting medium (Simga, Dorset, UK).

2.4.7. Image analysis for histology

Axiostar plus microscope (Carl Zeiss, Welwyn Garden City, UK) was used to take pictures of stained sections. For PSR, αSMA and collagen I, 30-40 pictures of
neighbouring fields (avoiding big vesse) in ×80 magnification were taken and analysed in Photoshop (Adobe photoshop CS3 version 10.0, Adobe systems Inc., San Jose, CA, USA). Pixels of brown or red area were selected by the software and the results were expressed as pixel numbers. 10 pictures from randomly selected samples were used to adjust the settings which allowed all positive areas to be selected in these pictures. The percentage of area positive for collagen fibres or αSMA staining among pixels of a whole picture was calculated as a quantitative value on a continuous scale. For αSMA staining in livers with fat deposition, data were corrected for nuclei numbers per picture.

2.5. Genotyping

2.5.1. DNA extraction from liver tissue

Liver tissue (<10mg) was digested with 400µl DNA lysis buffer (recipe in Table 2.5) supplemented with 1:20 20% SDS and 1:20 10mg/ml Proteinase K at 55°C overnight. 160µl 5M NaCl was added and mixed well. Supernatant was collected after spinning the mixture at 15,781g at room temperature for 10 minutes. 400µl 2-propanal was added to precipitate the DNA. Supernatant was discarded after spinning the mixture at 15,871g at room temperature for 10 minutes. DNA pellet was further washed with 70% ethanol and excess liquid was moved after spin the samples at 15,871g at room temperature for 10 minutes. Samples were left at 55°C over with lid open for 15 minutes to allow complete evaporating of excess ethanol. 50µl TE (recipe in Table 2.5 for recipe) buffer was added to solve the DNA. DNA samples were stored at 4°C for further use.

2.5.2. PCR for target genes

Detailed PCR reaction recipe and condition was listed in Table 2.10. Primers were pre-designed and ordered from Invitrogen (UK). Primers were dissolved with TE buffer and stored at stock concentration of 100pmol/µl. Before use, Primers were diluted with dH₂0 to 10pmol/µl. Taq enzyme, buffers and MgCl₂ (Biotaq amplification kit, Gentaur) were added according to the manufacturer’s protocol. Detailed PCR recipe and running conditions are listed in Table 2.10.
<table>
<thead>
<tr>
<th></th>
<th>11β-HSD1 Flox</th>
<th>Albumin Cre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>ctgcatatgtttggtgg</td>
<td>gacegtacacaaatgcttg</td>
</tr>
<tr>
<td>Reverse</td>
<td>cccacaatgcatgtttggaacatt</td>
<td>ttacgtatctctggcagegatc</td>
</tr>
<tr>
<td>Buffer (µl)</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>25mM MgCl2 (µl)</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Forward Primer (µl)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Reverse Primer (µl)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>dNTP (µl)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Taq (µl)</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>H₂O (µl)</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Total (µl)</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Pre-Denature</td>
<td>94°C 10 min</td>
<td>94°C 10 min</td>
</tr>
<tr>
<td>Repeat</td>
<td>32 cycles</td>
<td>32 cycles</td>
</tr>
<tr>
<td>Denaturing</td>
<td>94°C 1 min</td>
<td>94°C 1 min</td>
</tr>
<tr>
<td>Annealing</td>
<td>64°C 1 min</td>
<td>64°C 1 min</td>
</tr>
<tr>
<td>Reaction</td>
<td>Temperature</td>
<td>Time</td>
</tr>
<tr>
<td>----------------</td>
<td>-------------</td>
<td>-------</td>
</tr>
<tr>
<td>Elongating</td>
<td>72°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Elongating</td>
<td>72°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Final</td>
<td>72°C</td>
<td>10 min</td>
</tr>
<tr>
<td>Final</td>
<td>72°C</td>
<td>10 min</td>
</tr>
<tr>
<td>Product size</td>
<td>WT 255bp</td>
<td>Flox 385bp</td>
</tr>
<tr>
<td></td>
<td>465 bp</td>
<td></td>
</tr>
</tbody>
</table>

### 2.5.3. Agarose gel electrophoresis for target genes

10μl DNA product was loaded onto a 1.5% agarose gel (0.1% Gel red, Cambridge Biosciences, Cambridge, UK) and electrophoresed at 90 volts for 1 hour. BXT-20 UV visualize system (Uvitec, Cambridge, UK) was used to visualize the gel.

### 2.6. mRNA quantification

#### 2.6.1. RNA extraction

Before RNA extraction, working bench surface and pipettes were treated with RNaseZap to avoid Rnase contamination (Invitrogen, Paisley, UK). Liver weighing 10-20mg was homogenized in Qiazol reagent 600μl (Qiagen, Crawley, UK) by a homogenizer (Ika, Staufen, Germany). 200μl chloroform was added to the homogenized cell lysates and shaken vigorously for 15 seconds. The mixture was spun at 4°C for 15 minutes at 13,523g. RNA was extracted from the clear lysate using Qiagen RNeasy mini extraction kit (Qiagen, Crawley, UK) according to the instructions of the manufacturer. To check the RNA quality, an aliquot of RNA sample was loaded on a 1.5% agarose gel with 0.1% Gel Red. BXT-20 UV visualize system (Uvitec, Cambridge, UK) was used to visualize the 28S and 18S rRNA bands, which indicating the integrity of the RNA. RNA concentration was measured with a Nano-drop spectrophotometer (NanoDrop ND-100, Fisher, Loughborough, UK) and A260/A280 was used to monitor the RNA quality. RNA samples were stored at -80°C for further analysis.
Cell lysates (section 2.3.3) were homogenized by spinning through Qia shredder (Qiagen, Crawley, UK). RNA was extracted from homogenized cell lysates using Qiagen RNeasy Micro extraction kit (Qiagen, Crawley, UK) according to the manufacturer’s protocol. Nanodrop was used to control the RNA quality and measure RNA concentration. RNAs were stored at -80 for further analysis.

2.6.2. **Reverse transcription (RT)**

1µg RNA was reverse-transcribed into single-stranded cDNA using Superscript III reserve transcriptase kit (Invitrogen, Paisley, UK) with a volume of 20µl according to the manufacturer’s instruction. 1µg RNA (adjust by Rnase free water to 8µl), 1µl DNASE I (Invitrogen, Paisley, UK) and 1µl DNASE I Buffer were incubated at room temperature for 15 minutes. This reaction is terminated by adding 1µl EDTA and incubating the mix at 65°C for 10 minutes. 1µl Random primer (50ng/dl) and 1µl dNTP (10mM) were added and the mixed solution was denatured at 65°C for 5 minutes and put onto ice for at least 1 minute. 4µl First Strand buffer (5 times), 2µl DTT (0.1mM), 0.5µl RNasin plus Rnase inhibitor (40u/µl) as well as 0.5µl SuperScript III were added to reach 20µl final volume in each reaction tube. The RT PCR program used was 25°C for 5 min, 50°C for 60 min and 70°C for 15 min in PCR programmed machine (Rio-Rad, Herts, UK). In each RT PCR, negative controls were included: one without the RNA template and the other without the transcriptase enzyme.

2.6.3. **Realtime quantitative PCR**

The gene transcripts of interest were measured using the Roche UPL (Roche, East Essex, UK) system or ABI analysing system (Applied Biosystems, Warrington, UK). For the UPL analysis system, 0.1µl probe (Roche, East Essex, UK), 0.1µl each primer (Invitrogen, Paisley, UK), 5µl master mix (Roche, East Essex, UK), 2.7µl PCR graded water (Roche, East Essex, UK) and 2µl of sample (diluted 1:20) were added into each well of the real-time PCR plate. For the ABI assay, each reaction contains 0.5µl probe mix (Applied Biosystems, Warrington, UK), 5µl master mix (Roche, East Essex, UK), 2.5µl PCR graded water (Roche, East Essex, UK) and 2µl of sample (diluted 1:20). The real-time PCR was performed using the Lightcycler
480 machine (Roche, East Essex, UK) as follows: initially denatured at 95°C for 5 minutes, and then underwent 50 cycles of PCR amplification (60°C×15 second, 95°C×30 second and 60°C×15 second). All samples were analysed in triplicates and standards for each gene was generated by serially diluting cDNA pooled from all subjects (Neat, 1:2,1:4;1:8;1:16;1:32;1:64;1:128;1:256). cDNA sample from each subject was diluted 1:20 for analysis. The crossing point (Cp) value of each reaction was identified automatically by the Lightcycler 480. Standard curve (y axis Cp value, x axis log concentration) fitted with a straight line was also calculated automatically. Triplicates were regarded as acceptable if the standard deviation of the Cp value was less than 0.5 cycle. The standard curve was regarded as reliable if the reaction efficiency was between 1.8 and 2.2 and the error was less than 0.05. The reverse-transcription was regarded reliable if both the transcriptase free sample and RNA free sample were not detectable. The mean value of each target gene transcript was normalized to 18s if no group difference were detect between 18S level. Table 2.11 provides detailed information for primers and probes.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward primer</th>
<th>Backward primer</th>
<th>Probe #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rn18s(^1)</td>
<td>ctcaacacgggaacacctca</td>
<td>egctccaccaactaagaaeg</td>
<td>77</td>
</tr>
<tr>
<td>Col3a1</td>
<td>tccectggaatctgtgaatc</td>
<td>tgaagtgaattggggaagat</td>
<td>49</td>
</tr>
<tr>
<td>Col1a1</td>
<td>cagttcagcttggttgacct</td>
<td>gcagctgacttccaggatgt</td>
<td>15</td>
</tr>
<tr>
<td>Mmp2</td>
<td>taacctggtgacgctgctg</td>
<td>ttcaggaataaagccaccttgaa</td>
<td>77</td>
</tr>
<tr>
<td>Mmp9</td>
<td>aacgacataagccccatctca</td>
<td>gctgtggctagttggttcg</td>
<td>19</td>
</tr>
<tr>
<td>Mmp12</td>
<td>tggattgatatccacactgtgaggt</td>
<td>aatactgctggggttaagca</td>
<td>51</td>
</tr>
<tr>
<td>Mmp13</td>
<td>gcccagaaacttccacactac</td>
<td>tcagagccagaactttctcc</td>
<td>89</td>
</tr>
<tr>
<td>Timp1</td>
<td>tcagagccagactttctcc</td>
<td>aagctgagagaactttctcc</td>
<td>76</td>
</tr>
<tr>
<td>Mcp1</td>
<td>cctttgagttgtggacaaaa</td>
<td>ggctggagagctacaagagg</td>
<td>62</td>
</tr>
<tr>
<td>Tgfb1</td>
<td>ttggagcaacatgtggaactc</td>
<td>cagcagggctttactacag</td>
<td>72</td>
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<tr>
<td>Il1</td>
<td>tgaatgaaagacggcatcacc</td>
<td>ttcattttggtattgcttgg</td>
<td>78</td>
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<tr>
<td>Cxcl1</td>
<td>gactccagccacactcacc</td>
<td>tggaggtgctcctttcttc</td>
<td>83</td>
</tr>
<tr>
<td>Hsd11b1</td>
<td>tctacaatgaagagttcagaccag</td>
<td>gcccgtagtacaacttttt</td>
<td>1</td>
</tr>
<tr>
<td>GR</td>
<td>caaggtgcgctatctatgtgaa</td>
<td>ctttggtcttcaagctccttc</td>
<td>91</td>
</tr>
<tr>
<td>Gilz</td>
<td>cctggattggggataacagtgc</td>
<td>ggttcttcaaggtctgctctc</td>
<td>49</td>
</tr>
<tr>
<td>Acta2(^2)</td>
<td>acctttcctcaggcatctttca</td>
<td>ataggtgttctctggatge</td>
<td>58</td>
</tr>
<tr>
<td>Ym1</td>
<td>n/a ABI assay NM_009892.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1: Ribosomal RNA.
2: Acta2 encoding αSMA.


2.7. Protein analysis

2.7.1. Protein extraction

2.7.1.1. Protein extraction

50-100µg liver samples were homogenized in protein lysis buffer (1ml/100µg tissue, recipe in Table 2.5). Cell lysates (Section 2.3.3) and liver homogenates were rotated for 45 minutes and spun at 15,871g for 15 minutes at 4°C. The supernatant was collected and stored in -80 °C for further use.

2.7.1.2. Protein concentration measurement

Protein concentration was measured by the Lowry methods using Protein Assay Package (Rio-Rad, Herts, UK) according to the manufacturer’s instruction. The absorbance of Coomassie® Brilliant Blue G-250 dye binding to proteins at wavelength of 690 is proportional to the concentration of protein. Standard curve of sequential BSA concentrations (0, 0.2µg/ml, 0.4µg/ml, 0.6µg/ml, 0.8µg/ml, 1µg/ml, 1.2 µg/ml and 1.5 µg/ml) was prepared in protein lysis buffer (recipe in Table 2.5). 5µl samples (diluted 1:5 in cell protein samples; diluted 1:50 in liver protein samples) or standards were added in duplicates to a 96 well plates before adding 25µl reagent A’ (made by adding reagent S in reagent A in a 1:20 dilution). 200µl reagent B was added to each well and plates were read in 15-60 minute when the colour developed was stable. Plates were read under OPTI max microplate reader (Molecular Devices Corporation, CA, USA) and the standard curve (y axis Concentration and x axis absorbance) was fitted in line using Softmax Pro (Molecular Devices Corporation, CA, USA). The standard curve was regarded as reliable if the r >0.98 and the SD of each sample duplicates < 10% mean value. Sample value was calculated automatically by the software according to standard value.

2.7.1.3. Protein sample preparation

Proteins were diluted with protein lysis buffer to a final concentration of 10µg/ml if applicable. For cell samples with neat concentration around 1-2 µg/ml, sample concentration were diluted according to the least concentrated sample. NuPAGE LDS
sample loading dye (Invitrogen, Paisley, UK) was added in 1:4 dilution and NuPAGE sample reducing agent (Invitrogen, Paisley, UK) was added in 1:10 dilution. Protein samples were stored at -20 °C for further analysis.

2.7.2. Western blot

2.7.2.1. Polyacrylamide gel making

Table 2.12 provides the information for gel preparation. Gels were cast within pre-cleaned gel making cassettes (Rio-Rad, Herts, UK). Lower gels were first cast and allowed to coagulate for 30 minutes with dH₂O topped to stop evaporating. The top layer of dH₂O was removed before upper gels were casted. Upper gel was cast and inserted with a 10 well comb. The comb was removed 30 minutes after casting. Gels were applied to an electrophoresis device, Mini-Protean Tetra Cell (Rio-Rad, Herts, UK) before use.
Table 2.12 Polyacrylamide gel recipe

<table>
<thead>
<tr>
<th>30% Acrylamide</th>
<th>Buffer</th>
<th>water</th>
<th>10% APS</th>
<th>TEMED</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% lower gel</td>
<td>4ml</td>
<td>3ml</td>
<td>5ml</td>
<td>120µl</td>
</tr>
<tr>
<td>(Upper gel buffer)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper gel</td>
<td>1.2ml</td>
<td>2.5ml</td>
<td>6.3ml</td>
<td>120µl</td>
</tr>
<tr>
<td>(Lower gel buffer)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.7.2.2. *Gel electrophoresis*

Samples with loading dye and reducing buffer were defrosted and denatured at 95°C for 5 minutes. After a brief spin, 20µl protein samples (Section 2.7.1.3) loaded to each well and separated by electrophoresis on a 10% SDS-PAGE running at constant current 20mA/gel for 1.5 hours.

2.7.2.3. *Gel transfer*

Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Watford, UK), which were activated by incubating with methanol for 30 seconds. A gel sandwich was prepared within the cassette in a sequence of fibre pad, filter paper, gel, PVDF membrane, filter paper and fibre pad. The gel sandwich was made within a tank containing transfer buffer (recipe in Table 2.5). Air bubbles were carefully squeezed out when applying the layers. After setting up the sandwich (with the gel facing the negative electrode and the membrane facing the positive electrode), transfer was run at 280mV for 2 hours with an ice cube to cool down the system.

2.7.2.4. *Immunoblotting*

After transfer, 5% milk was used to block the PVDF membrane transferred with protein for non-specific binding at room temperature for 30 minutes. Blots were incubated with primary antibody at 4°C overnight. After 3 washes with TBST for 5
minutes each time, blots were incubated with secondary antibodies (either HRP linked antibody or fluorescent linked antibody) at room temperature for 1 hour and washed 3 times with TBST for 5 minutes each time. Blots were ready for development after washes. The detailed information for immunoblotting antibodies and conditions is presented in Table 2.13.

2.7.2.5. Blot development

Both chemical development and fluorescent detection were used to visualize the blot. For HRP linked secondary, ECL solution (GE, Bucks, UK) was added to all the blots and left on for 3 minutes. Chemiluminescence sensitive films were put against the membrane in the dark and exposed for 1 minute, 5 minutes and 10 minutes. Films were processed by the Konica SRX-101 X-Ray developer (Essex, UK). For fluorescence assays, fluorescence linked secondary antibodies were added to the membrane. Membranes were rinsed in TBST and visualized in the Odyssey Clx system (Li-cor, Cambridge, UK).
### Table 2.13 Antibodies for Western blotting

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Primary Ab</th>
<th>Secondary Ab</th>
<th>Block</th>
<th>MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>αSMA</td>
<td>Abcam ab7817 Anti-alpha smooth muscle Actin antibody; 1:5,000 at 4ºC overnight</td>
<td>HRP linked anti-mouse IgG (Abcam), 1:10,000 for 1 hour at room temperature</td>
<td>5% milk</td>
<td>46kDa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>For Fluorescent blotting: IRDye 800CW Goat anti-Mouse IgG (Li-Cor) 1:10,000 for 1 hour at room temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smad3p</td>
<td>Millipore 04-1042 rabbit anti phosphorylated Smad3 antibody; 1:2000 at 4ºC overnight</td>
<td>HRP linked anti-rabbit IgG (DAKO, Ely, UK), 1:2000 for 1 hour at room temperature</td>
<td>1% BSA</td>
<td>60kDa</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11β-HSD1</td>
<td>In house</td>
<td>Alexa Fluor® 680 donkey anti sheep antibody 1:10,000 for 1 hour at room temperature</td>
<td>5% milk</td>
<td>36kDa</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Millipore AB2302 anti GAPDH antibody; 1:100,000 for 1 hour at room temperature</td>
<td>HRP linked anti-mouse IgG (Abcam), 1:10,000 for 1 hour at room temperature</td>
<td>5% milk</td>
<td>38kDa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>For Fluorescent blotting: IRDye 800CW Goat anti-Mouse IgG (Li-Cor) 1:10,000 for 1 hour at room temperature</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.8. **In situ mRNA hybridization**

In situ hybridization was carried out following a standard protocol used in the lab as described previously (Seckl et al., 1990).

2.8.1. **Sections**

Rnase free slides were prepared by sub-bedding the slides with poly-L-lysine (Sigma, Dorset, UK). Cryostat machine (Leica, Bucks, UK) was set to -20ºC and pre-cleaned
with RNase Zap (Ambion, Warrington, UK). Frozen livers were fixed on to the cryostat tissue holder (Company) using OCT (Fisher, Loughborough, UK) at -20°C. Tissues were cut into 10 µm sections and thaw-mounted on to the slides. Sections were stored at -80°C for further use.

2.8.2. Probe preparation

11β-HSD1 plasmid was produced in vitro from a PVL105 plasmid encoding a 470bp 11β-HSD1 mouse cDNA (plasmid was kindly provided by Val Kelly et al. as a gift). The plasmid was digested with Bam H1 restriction enzyme (Promega, Southampton, UK) for sense probe and Hind III restriction enzyme (Promega, Southampton, UK) for antisense probe. The product was purified by elecrophoresis in a low melting point agarose gel and DNA fragment was collected under UV light. DNA product was extracted using QIAquick Gel Extraction Kit (Qiagen, Crawley, UK) according to the manufacturer’s protocol. Sense probe was made by transcribing the DNA strand with SP6 RNA polymerase, and antisense probe was transcribed with T7 RNA polymerase (Promega, Southampton, UK). Probes were made by adding 1µl 10mM dATP, 1µl 10mM dCTP, 1µl 10 mM dGTP and 4µl 1mM \[^{35}\text{S}\]-dUTP (Amersham, Bucks, UK), 0.5µl 200mM DTT, 0.4µl Rnas e Inhibitor (Invitrogen, Paisley, UK) with 1µl RNA polymerase. Probes were purified by Nick column (Amersham, Bucks, UK) according to manufacturer’s protocol. Probe activity was measured by counting a 1µl aliquot in 1ml scintillation fluid (Pico-fluor 40, Canberra Packard, Berkshire, UK) in a β-counter (Wallac 1450 Microbeta Plus liquid scintillation counter, Milton Keynes, UK). The probes were stored at -20°C and used within one month after made.

2.8.3. Pre-hybridization

Air dried sections were incubated in Rnase free paraformaldehyde (recipe in Table 2.5) for 10 minutes for fixation. After two washes in PBS, sections were incubated in freshly made 1% triethanolamine (Sigma, Dorset, UK) for 10 minutes and then dehydrated in graded ethanol. 20ml of hybridization box buffer (20% 20×SSC (recipe in Table 2.5), 30% DEPC water and 50% deionised formamide (Sigma, Dorset, UK)) was added into each box containing two layers of filter papers.
Sections were added with 200µl pre-hybridization mix (50% 2× pre-hybridization and 50% deionised formamide) and left in the hybridization box at 55°C for 2.5 hours.

2.8.4. Hybridization

Hybridization mix per slide was made by adding 50% v/v deionised formamide and 49% v/v radioactive probe at the concentration of 10MBq/ml (diluted by 2× hybridization buffer). The mix was denatured at 70°C for 10 minutes, cooled on ice for 1 minute and added with 1% (v/v) 1M DTT. Slides were drained and dried by lens paper without disturbing the sections. 200µl final hybridization mix was added to each slide to ensure all the sections were covered in liquid. All slides were put back to the hybridization box and incubated at 55°C overnight.

2.8.5. Removal of accessory mRNA

To avoid too much background, Rnase was added to degrade unspecific mRNAs. Sections were washed in 2×SSC buffer three times and RNAse buffer (recipe in Table 2.5) was added to Rnase box containing 1 filter paper. 200µl RNase (final concentration 30µg/ml) dissolved in Rnase buffer (recipe in Table 2.5) were added to each slides and left in the Rnase box for 1.5 hours at 37°C. After additional washes in 2×SSC and 0.1×SSC, the slides were dehydrated in gradient ethanol (60%, 75% and 90% in ammonium acetate) and then air dried.

2.8.6. Visualization and quantification of in situ hybridization

To quantify the signal of 11β-HSD1 mRNA probe binding, sections were put against MR films (Kodak, Herts, UK), which are radiation sensitive, in a light tight cassette for 2 days. Films were developed by X-ray film developer (GE Sensing, Billerica, US). Sections were then blotted with antisense probes and were mounted with photographic emulsion (NTB2, Kodak) for 4 weeks. The slides were developed in D19 developer for 3 minutes, rinsed in dH2O for 5 minutes, fixed in Amfix fixative for 3 minutes and washed in water for 5 minutes at 50°C. Sections were counter stained with H&E (Section 2.4.4) and mounted by DPX. Pictures (×400) were taken under the microscope in bright field. The intensity of radioactivity was analysed by densitometry measurement using KS300 (Carl Zeiss, Welwyn Garden City, UK).
Background was set by counting the silver grain number per µm² by selecting 1 circular area (radius 40 µm) in the unstained fields on each section (e.g. inside the vessel lumen). Average grains per area (µm²) was counted by randomly selecting 5 areas (radius 40 µm) per field in each ×400 magnification field. The final result was calculated by deducting the background value from the measurement value. 10 fields per section were measured.

2.9. **11β-HSD1 activity assays**

2.9.1. **Synthesis of tritiated -11-dehydrocorticosterone ([³H]-A)**

[³H]-A was made from [³H]-corticosterone ([³H]-B) as previously described (Low et al., 1994). Rat placenta which expresses 11β-HSD2 with very low 11β-HSD1 level was used for synthesis of [³H]-A. 150µl [³H]-B (12.8µM, Amersham, Bucks, UK) was air dried and incubated with 300µl placenta homogenates in the presence of 200µl 25mM NAD in 4.45ml C buffer (recipe in Table 2.5) in a 37°C incubator overnight. The next day, 6ml of ethyl acetate was added and the mix was vortexed well. After a spin at 700g for 15 minutes at 4°C, the upper layer was transferred into a glass tube and blown down under nitrogen then reconstituted by 150µl ethanol. [³H]-A activity was measured by counting a 1µl aliquot in 1ml scintillation fluid (Pico-fluor 40, Canberra Packard, Berkshire, UK) in a β-counter (Wallac 1450 Microbeta Plus liquid scintillation counter, Milton Keynes, UK). [³H]-A concentration was calculated by multiplying [³H]-A concentration by [³H]-A/[³H]-B radioactivity ratio. The purity of [³H]-A was checked by running [³H]-A and [³H]-B on HPLC (Section 2.9.5). [³H]-A was stored at -20°C for further experiments.

2.9.2. **11β-HSD1 reductase activity in HSCs**

980nM A and 5nM [³H]-A were added to each well of HSCs (2 days, 5 days and 8 days after plating) in 6-well plates for 24 hours in 37°C incubator. 24 hour incubation was selected after a series of conditions were tested to identify the optimal condition for 10%-40% [³H]-A conversion rate. Cell culture medium and blank controls were harvested into glass tubes. Cell numbers were counted after trypsinizing (1ml trypsin (Lonza, Slough, UK)) the cells at 37 °C for 5 minutes.
Steroids were extracted by Sep-pak columns (Waters, Milford, US) as the manufacturer instructed. Briefly, Sep-pak columns were activated by methanol and 1.5ml cell culture medium was added to the columns. After a wash with 5ml water, steroids were collected to glass tubes by running methanol through the columns and blown dried before being dissolved in 250µl water then extracted with 4ml ethyl acetate. Ethyl acetate was blown dry using nitrogen on a heat block at 60ºC. The samples were stored at -20ºC.

2.9.3. 11β-HSD1 dehydrogenase activity in liver tissue

11β-HSD1 in homogenized tissue has both dehydrogenase activity and reductase activity. Normally, the reaction product of dehydrogenase assay is purely [\(^{3}\)H]-B (from [\(^{3}\)H]-A), but the reaction product of reductase reaction consists of metabolites from not only 11β-HSD1 but also from 5α- and 5β-reductases. In this case, dehydrogenase assay was used to measure 11β-HSD1 activity in tissue. 50-100 mg liver tissue was homogenized by Ultra-Turrax T8 auto-homogenizer (Ika, Staufen, Germany) in homogenizing buffer (recipe in Table 2.5). Protein levels of liver lysates were tested as described in 2.7.1. Liver homogenate (2µg/ml protein), 10nM [\(^{3}\)H]-B and 400µM NADP were incubated in C buffer (recipe in Table 2.5) at 45ºC for 20 minutes. The 20 minute incubation was the optimal condition for a 10%-40% conversion rate of [\(^{3}\)H]-B, after testing a range of incubation time (20, 40 and 60 minutes). Steroids were extracted by ethyl acetate and stored in -20 ºC.

2.9.4. 11β-HSD1 reductase activity in liver tissue

For 11β-HSD1 inhibition experiment, reductase activity was measured. 50-100 mg liver tissue was homogenized by Ultra-Turrax T8 auto-homogenizer (Ika, Staufen, Germany) in homogenizing buffer (Table 2.5). Protein levels of liver lysates were tested as described in 2.7.1. Liver homogenate (2µg/ml protein), 10nM [\(^{3}\)H]-A and 400µM NADPH were incubated in C buffer (recipe in Table 2.5) at 45ºC for 20 minutes. The 20 minute incubation was the optimal condition for a 10%-40% conversion rate of [\(^{3}\)H]-A, after testing a range of incubation time (20, 40 and 60 minutes). Steroids were extracted by ethyl acetate and stored in -20 ºC.
2.9.5. **High-performance liquid chromatography (HPLC) for \[^3\text{H}\]-A and \[^3\text{H}\]-B**

Steroid samples stored at -20°C were re-suspended with 400μl mobile phase solution (60% water, 25% methanol and 15% acetonitrile). Samples were sealed in HPLC assay tubes and analysed by ASI 300 HPLC machine with dual λ absorbance detector (Waters 2487, Waters, Milford, USA) and a flow scintillation analyser (150TR, Packard Instruments, Downers Grove, USA) at the solvent flow speed of 1.5ml/minute at 45°C. Samples were run for 25 minutes (steroids extracted from cell medium for reductase assay and steroids extracted from livers for dehydrogenase assay) or 35 minutes (steroids extracted from tissues for reductase assay) to make sure both peaks of \[^3\text{H}\]-A and \[^3\text{H}\]-B and any other unspecific peaks was shown completely. Peaks of \[^3\text{H}\]-A and \[^3\text{H}\]-B were measured according to similar position to standard \[^3\text{H}\]-A and \[^3\text{H}\]-B. All other obvious peaks were also measured. The reductase activity of \(^{11}\beta\)-HSD1 in cells was expressed as the percentage of \[^3\text{H}\]-A/(\[^3\text{H}\]-A +\[^3\text{H}\]-B)/100,000 cells/hour; dehydrogenase activity of \(^{11}\beta\)-HSD1 in liver samples were expressed as the percentage of \[^3\text{H}\]-B/(\[^3\text{H}\]-A +\[^3\text{H}\]-B) and converted to mmol/mg protein/min; due to the presence of other peaks, reductase activity of \(^{11}\beta\)-HSD1 in liver samples were expressed as the percentage of \[^3\text{H}\]-B+ other peaks/(\[^3\text{H}\]-A +\[^3\text{H}\]-B+ other peaks) and converted to mmol/mg protein/min.

2.10. **Biochemistry assays**

2.10.1. **Liver triglycerides**

100mg liver was homogenised in 20 volumes of 2-propanol. The homogenate was shaken in an orbital shaker for 45 minutes at 500 rpm and then spun at 3000g for 10 minutes at 4°C and the supernatant assayed using a commercial triglyceride kit (Thermo, Runcorn, UK) according to the manufacturer’s protocol. Results were converted to μmol/g (liver). Briefly, triglycerides are enzymatically hydrolysed by lipase to free fatty acids and glycerol; the glycerol is phosphorylated by adenosine triphosphate (ATP) with glycerol kinase (GK) to produce glycerol-3-phosphate and adenosine diphosphate; glycerol-3-phosphate is oxidized by dihydroxyacetone
phosphate (DAP) by glycerolphosphate oxidase producing H₂O₂. In a trinder type colour reaction catalysed by peroxidase, the H₂O₂ reacts with 4-aminoantipyrine (4-AAP) and 3,5-dichloro-2-hydroxybenzene sulfonate (DHBS) to produce a red coloured dye. The absorbance of this dye is proportional to the concentration of triglycerides present in the sample.

A calibration standard was made by sequential diluting 2.5mmol/l triglyceride standarded solution provided by the kit (2.5mmol/l, 2.0mmol/l, 1.8 mmol/l, 1.5 mmol/l, 1 mmol/l, 0.5 mmol/l, 0.2 mmol/l and 0 mmol/l). 2µl standards and samples were pipetted into each well of a 96-well plate and 200µl assay reagent provided by the kit was added. The mixture was incubated at 37°C for 5 minutes and a plate reader was used to measure absorbance at 500nm. Plates were read under OPTI max microplate reader (Molecular Devices Corporation, CA, USA) and the standard curve (y axis Concentration and x axis absorbance) was fitted in line using Softmax Pro (Molecular Devices Corporation, CA, USA). The standard curve was regarded as reliable if the r²>0.98 and SD of sample duplicates <10% mean value. Sample value was calculated automatically by the software according to standard value.

### 2.10.2. Picrosirius red

A commercial Sircol Collagen assay kit was used to measure collagen level of frozen livers (Biocolor, Belfast, UK). Sirius Red is an anionic dye with sulphuric acid side chain groups. These groups react with the side chain groups of the basic amino acids present in collagen. The specific affinity of the dye for collagen, under the assay conditions is due to the elongated dye molecules becoming aligned parallel to the long, rigid structure of native collagens that have intact triple helix organisation. The absorbance of this dye is proportional to the concentration of collagens present in the sample at wavelength 540 nm.

100mg frozen liver was homogenised in 1ml 0.5M acetic acid. Samples were rotated on a rotator for 45 minutes at 4°C for complete extraction of the soluble collagens. Samples were spin at 15,871g for 15 minutes at 4 °C and the supernatant was collected. Standards were made by sequentially dilute the standard stock provided by the kit (0, .5, 1, 2.5, 5 µg/ml). 100µl of samples and standards was added with 1ml
Sircol Dye reagent provided by the kit and mixed in a mechanical shaker for 30 minutes at room temperature. The mix was then spun at 9,391g for 10 minutes at room temperature and then the supernatant was removed completely. To each tube was added 1ml Alkali reagent provided by the kit and vortexed well. Samples and standards were loaded on to a 96-well plate with duplicates and the absorbance at 540nm of the samples was measured using OPTI max microplate reader (Molecular Devices Corporation, CA, US). The standard curve was regarded as reliable if the $r^2>0.98$ and SD of each duplicate measurements $<10\%$ mean value. Sample value was calculated automatically by the software Softmax Pro (Molecular Devices Corporation, CA, US).

2.10.3. **Liver function tests (LFTs)**

Plasma samples were collected when the mice were decapitated and stored at -80 °C for further analysis. Liver functions tests were kindly undertaken by Dr. Forbes Howie in the MRC Centre for Reproductive Health.

2.10.3.1. **Alanine aminotransferase (ALT)**

ALT was measured using the method described by Bergmeyer et al. (Bergmeyer et al., 1978), utilising a commercial kit (Alpha Laboratories Ltd., Eastleigh, UK) according to manufacturer’s protocol. A Cobas Fara centrifugal analyser (Roche, East Sussex, UK) was used to run the samples.

2.10.3.2. **Aspartate aminotransferase (AST)**

AST was determined by a commercial kit (Randox Laboratories, UK) adapted for use on a Cobas Fara centrifugal analyser (Roche Diagnostics Ltd, Welwyn Garden City, UK) according to manufacturer’s protocol.

2.10.3.3. **Albumin**

Plasma albumin measurements were determined using a commercial serum albumin kit (Alpha Laboratories Ltd., Eastleigh, UK) adapted for use on a Cobas Fara
centrifugal analyser (Roche Diagnostics Ltd, Welwyn Garden City, UK) according to manufacturer’s protocol.

2.10.3.4. Alkaline phosphatase (ALP)

ALP was determined by a commercial kit (Randox Laboratories, UK) adapted for use on a Cobas Fara centrifugal analyser (Roche Diagnostics Ltd, Welwyn Garden City, UK) according to manufacturer’s protocol.

2.11. Statistical analysis

All data are expressed as Means ± SEM. Two-way ANOVA was used to compare the effect of genotype and treatment. Post-hoc LSD test was used to compare each group when the variance was equal otherwise data were logarithmically transformed to achieve equal variance. One-way ANOVA followed by post-hoc Tukey test were used to compare means between more than 2 groups. Student’s t tests were used to compare 2 groups. For 11β-HSD1 inhibitor diet treated mice with CCL₄ induced liver injury, one-way ANOVA followed by post-hoc Tukey test was used to test differences between time points within vehicle and drug treated groups; significant differences between treatment within a time point was tested by Student’s t test (2 groups) or one-way ANOVA (3 groups) followed by post-hot Tukey test if applicable. If variance was not equal, data were logarithmically transformed for equal variance. Graphs were drawn by Graphpad Prism 5. Data were analysed by Statistica 7, SPSS15.0 or Graphpad Prism 5.0.
Chapter 3. The Pattern of 11β-HSD1 Expression in NAFLD Models


3.1 Introduction

11β-HSD1 is expressed in the liver in both humans and rodents (Seckl and Walker, 2001). Human studies have shown that 11β-HSD1 was down regulated or unchanged in fatty liver (Rask et al., 2002, Stewart et al., 1999). Although many studies have investigated the pattern of 11β-HSD1 expression during, the pattern of 11β-HSD1 in the advanced stages of NAFLD including NASH and fibrosis is still poorly understood. Recently it was shown that 11β-HSD1 activity and expression were reduced in patients with steatosis yet increased or unaltered in patients with NASH (Ahmed et al., 2012, Konopelska et al., 2009). It was also suggested 11β-HSD1 mRNA level was increased in human chronic liver disease (mostly cirrhotic) from a complicated aetiologies (Ahmed et al., 2008).

In most studies of rodent models of obesity, 11β-HSD1 expression and activities are reduced in the liver and increased in adipose tissue (Rask et al., 2001, Livingstone et al., 2000). However, there is no specific study on how hepatic 11β-HSD1 is regulated in rodent models for steatosis (without complication of obesity), NASH and liver fibrosis.

There are several animal models of NAFLD and liver fibrosis available. Among them, choline deficient diet (CDD) and methionine and choline deficient diet (MCDD) are reliable models to induce rodent liver steatosis and NASH, respectively. CDD induces liver fat accumulation without affecting body weights or adipose deposition in mice. Moreover, it attenuates insulin resistance and glucose intolerance in mice fed a high-fat diet by increasing hepatic expression of free fatty acid esterification genes (Raubenheimer et al., 2006). MCDD is an established model of liver fat accumulation and inflammation. Animals on MCDD have dramatic weight loss leading to improved peripheral insulin resistance yet increased hepatic insulin resistance (Leclercq et al., 2007, Rinella and Green, 2004). Although CDD induces no inflammatory response in the short term in the liver, long-term CDD administration can cause severe fibrosis (32 week) and carcinoma (70 week) in rats (Nakae, 1999).
Liver fibrosis can be induced by intra-peritoneal injection of carbon tetrachloride (CCL\textsubscript{4}), which causes oxidative stress and chemical injury in the liver (Williams and Burk, 1990). A single dose of CCl\textsubscript{4} leads to peri-venous necrosis and steatosis whereas prolonged administration leads to profound peri-venous liver fibrosis which is revisable (Pierce et al., 1987, Iredale et al., 1998). Centri-lobular necrosis and peri-portal inflammatory cell infiltration in rodent livers can be induced by dissolving thioacetamide (TAA) into drinking water (Salguero Palacios et al., 2008) or by injecting TAA intra-peritoneally (Yogalakshmi et al., 2010).

From the above, I hypothesize that \( \beta \)-HSD1 is down regulated in mouse models of steatosis and up-regulated in mouse models of NASH and liver fibrosis.

3.2 Aims

- To test whether \( \beta \)-HSD1 level is altered in CDD, MCDD, CCl\textsubscript{4}, and TAA induced liver injury
- To test what cell type leads to the changes of \( \beta \)-HSD1 in these liver injury models

3.3 Methods

3.3.1 Animal models

3.3.1.1 Steatosis and NASH models

Since previous studies have investigated the hepatic expression of \( \beta \)-HSD1 in HFD fed mice, which develop steatosis recapitulating ‘natural’ human process, we chose to study \( \beta \)-HSD1 in CDD and MCDD, which have not been studied previously. Although CDD and MCDD are non-physiological models, they are capable of inducing hepatic steatosis and NASH despite systemic deregulation so they are also useful to reveal the relationship between NAFLD and \( \beta \)-HSD1 in the liver. 12 week-old C57BL/6 mice were fed either control diet (Control), choline deficient diet (CDD) or methionine-choline deficient diet (MCDD) (Dyets Inc.,
Bethlehem, PA) for 2 weeks (detailed diet information in Section 2.2.3.2). Mice were culled by decapitation and their livers were snap frozen and stored at -80°C for analysis.

### 3.3.1.2 Liver fibrosis models

CCL₄ administration recapitulate toxin induced liver fibrosis in human, which is very rare nowadays. However, the cascade of injured hepatocyte-activated HSCs, and stimulated inflammation is consistent with human liver fibrosis developed from hepatitis, NASH, and biliary obstruction. Vehicle or 25% CCL₄ (1.6µl/g body weight) was injected to the intra-peritoneal space of C57BL/6 mice 10-12 weeks of age twice per week for 12 weeks. Mice were culled 48 hours (peak injury), 1 week and 1 month (recovery phases) after the last CCL₄ injection (details see Section 2.2.2.2). Vehicle treated mice were harvested at the 1-month time point as control. For the TAA induced liver injury model, 600mg/l TAA or vehicle was added in the drinking water of C57BL/6 mice for a year and the mice were harvested immediately (peak fibrosis) or 1 week after TAA withdrawal (resolving phase) (details in Section 2.2.3.3). Vehicle treated mice were harvested at 1-week time point as control. Mice were culled by schedule 1 killing and livers were snap frozen and stored at -80°C for analysis. Liver samples from mice injured with CCL₄ and TAA were kindly provided by Dr. A. Pellicoro.

#### 3.1.1.1. Sample size

In order to detect significance between diet or chemical treatments, sample size was calculated with a statistical power of 0.8 and type I error α=0.05 in Power & Sample Size Calculator (Statistical Solutions). N=6 was selected according to previous data in CDD, MCDD and CCL₄ models and N=3 was selected in TAA model according to previous data in the lab (Macfarlane et al., 2011, Pellicoro et al., 2012).

### 3.3.2 Lab techniques

Methods for *in situ* 11β-HSD1 mRNA hybridization on livers from mice fed control diet, CDD and MCDD as well as mice treated with CCL₄ are presented in Section 2.8. mRNA extraction from whole liver homogenates and target gene analysis is
performed as described in Section 2.6. Western blot for protein analysis of liver homogenates of CCL₄ administered mice is presented in Section 2.7. 11β-HSD1 activity measurement (as dehydrogenase) in homogenized frozen liver tissues is measured as described in Section 2.9. H&E staining on CCL₄ injured liver sections and dual immunofluorescence on CCL₄ injured liver sections are presented in Section 2.4.4 and Section 2.4.3.1, respectively.

3.1.2. Statistics

To compare multiple groups (more than 2), one-way ANOVA followed by post-hoc Tukey test was used in Graphpad Prism 5.0.
3.4 Results

3.4.1 11β-HSD1 is decreased in mouse steatosis and NASH models

First, to test how 11β-HSD1 is regulated during liver injury, 11β-HSD1 expression was measured in a variety of mouse NAFLD and liver fibrosis models. CDD and MCDD were used to induce steatosis and NASH, respectively. In C57BL/6 mice 1) body weight was reduced; 2) liver weights were unchanged; 3) hepatic triglyceride content was markedly increased; 4) plasma AST and ALT were unchanged; 5) hepatic total collagen staining was unchanged compared with control diet feeding (Figure 3.1) during 2-week CDD feeding. During 2-week MCDD feeding, 1) body weights were dramatically reduced; 2) liver weights were unchanged; 3) hepatic triglyceride content was increased; 4) plasma AST and ALT were greatly enhanced; 5) hepatic total collagen staining was increased compared with control diet feeding (Figure 3.1).

To localize 11β-HSD1 in the liver I used in situ mRNA hybridization on sections of livers from mouse fed control diet, CDD or MCDD using an ‘antisense’ [35S]-cDNA probe complementary to mouse 11β-HSD1 mRNA. From the autoradiographs, there was no signal of control probe ‘sense’ but the signal of ‘antisense’ was strong after a 2-day exposure to the film (Figure 3.2 A) showing the specificity of the 11β-HSD1 probe. There was a significant decrease in 11β-HSD1 mRNA expression in the livers of CDD and MCDD compared with control diet (Figure 3.2 C).
Figure 3.1 CDD induces steatosis and MCDD induces NASH in C57BL/6 mice

A: The percentage of weight changes at termination of the diets normalized to starting weights in C57BL/6 mice fed with control diet (control), CDD and MCDD. B: The percentage of liver weights relative to body weights. C: Hepatic triglyceride (TG) content. D and E: Plasma AST (D) and ALT (E) level. F: Quantification graph of picrosirius red (PSR) staining showing the percentage of positive staining among all pixels per picture. Results were presented as Mean ± SEM, N=10/group. Significance was tested by one-way ANOVA followed by post-hoc Tukey test. *p<0.05, **p<0.01 and ***p<0.001 versus control diet; +p<0.05, ++p<0.01 versus CDD.
Figure 3.2 Decreased Hsd11b1 mRNA level in CDD and MCDD treated mice

A: Representative autoradiographs showing in situ mRNA hybridization of Hsd11b1 mRNA in uninjured C57BL/6 livers with arrows indicating representative cells hybridized with sense [35S]-labelled Hsd11b1 cRNA probe (negative control) and antisense [35S]-labelled Hsd11b1 cRNA probe. B: Representative images showing positive binding to antisense [35S]-labelled Hsd11b1 cRNA probe (×400 magnification). Small dots are silver grains hybridized to Hsd11b1 and arrows indicate representative cells with positive hybridization. C: Quantification graph showing average grain counts per area. Results were presented as Mean ± SEM, N=6/group. Significance was tested by one-way ANOVA followed by post-hoc Tukey test. *p<0.05, **p<0.01 and ***p<0.001 versus control diet.
3.4.2 11β-HSD1 is decreased in mouse liver fibrosis models

In addition, I investigated the 11β-HSD1 mRNA expression in liver fibrosis models. TAA and CCL4 administration induced profound fibrosis deposition in the liver in C57BL/6 mice (Pellicoro et al., 2012). Liver fibrosis recovered from 1 week post CCL4 termination whereas unaltered at 1 week after TAA withdrawal (Pellicoro, unpublished data). After TAA administration for 1 year, 11β-HSD1 mRNA was reduced markedly. Moreover, this down-regulation of 11β-HSD1 persisted after 1 week recovery (Figure 3.3 A). In the CCL4 induced liver fibrosis and resolution model, 11β-HSD1 mRNA levels were reduced at 48 hours after the last CCL4 injection (peak fibrosis) and recovered at 1 week and 1 month post CCL4 termination (resolution phases) (Figure 3.3 B). A similar pattern was found in the 11β-HSD1 protein levels (Figure 3.3 C and D). The down-regulation of 11β-HSD1 mRNA was further confirmed by in situ hybridization of 11β-HSD1 cDNA probes in non-necrotic cells i.e. those which have normal nuclei (Figure 3.4). Changes in 11β-HSD1 at the mRNA and protein levels were not reflected at the 11β-HSD1 activity level in whole liver homogenates between uninjured and CCL4 injured livers (Figure 3.5).
Figure 3.3 Decreased 11β-HSD1 expression in liver fibrosis models

A: Hsd11b1 mRNA level in mice treated with vehicle, TAA or 1 week recovered from TAA administration. Results are presented as Mean ± SEM, N=3/group. Significance was tested by one-way ANOVA followed by post-hoc Tukey test; * p<0.05 and ** p<0.01 versus untreated group. B: Hsd11b1 mRNA level in mice treated with vehicle or harvested at 48 hours (Peak injury), 1 week and 1 month (resolving phases) post CCL4 termination. C: Representative picture of blots and quantification graph of 11β-HSD1 protein level corrected for GAPDH. Results are presented as Mean ± SEM, N=6/group. Significance was tested by one-way ANOVA followed by post-hoc Tukey test; * p<0.05, ** p<0.01 and *** p<0.001 versus vehicle treated group (Veh); + p<0.05 and +++ p<0.001 versus 48 hour time point.
Figure 3.4 Hsd11b1 mRNA in CCL₄ induced liver fibrosis measured by in situ hybridization

A: Representative pictures in CCL₄ injured livers showing positive binding to antisense $[^{35}S]$-labelled Hsd11b1 cDNA probe (× 400 magnification). Small dots are silver grains hybridized to Hsd11b1 and arrows indicate representative cells with positive hybridization. B: Quantification graph of average grain counts per area. Results are presented as Mean ± SEM. N=6/group. Significance was tested by one-way ANOVA followed by post-hoc Tukey test; *** p<0.001 versus vehicle treated group (veh); +++ p<0.001 versus 48 hours group.
Figure 3.5 11β-HSD1 activity was unchanged in CCL₄ induced liver fibrosis

A: Representative HPLC curves showing [³H]-B (peaked at about 15 minutes) and [³H]-A (peaked at about 10 minutes) in whole liver homogenates of mice harvested at 48 hours (peak injury) and 1 week (recovery) post CCL₄ termination. B: Quantification graph showing the conversion rate (nmol/mg protein/min) of [³H]-B (peaked at 15 minutes) to [³H]-A (peaked at 11 minutes) in liver homogenate. Results are presented as mean ± SEM, N=6. Significance was tested by one-way ANOVA followed by post-hoc Tukey test.
3.4.3 11β-HSD1 distribution in hepatocytes, HSCs and macrophages in CCL4 induced liver fibrosis

Hepatocytes, HSCs and macrophages are key players during liver injury. In order to understand if a specific cell type contributes to the 11β-HSD1 changes in CCL4 induced liver injury, 11β-HSD1 expression in hepatocyte, HSC and macrophage was investigated. 11β-HSD1 was down regulated in ‘healthy’ hepatocytes according to in situ hybridization measurements (Figure 3.4). Hepatocyte death, identified by karyolysis and eosinophilic cytoplasm in H&E staining, was found around the scars in peak fibrosis (Figure 3.6). This was also confirmed by 11β-HSD1 immunofluorescence. 11β-HSD1 was reduced around the scars in peak fibrosis and recovered in the resolution phases (Figure 3.7).

Dual immunohistochemistry of 11β-HSD1 and αSMA showed that in peak fibrosis αSMA was up-regulated in the scar area where the loss of 11β-HSD1 was observed. 11β-HSD1 staining in αSMA+ was not as intense as in ‘healthy’ hepatocytes (Figure 3.7).

11β-HSD1 was present in macrophages since macrophages showed positive 11β-HSD1 counts during CCL4 injury by in situ hybridization (Figure 3.8 A). However, 11β-HSD1 staining in F4/80 positive cells was of variable intensity making it difficult to establish whether macrophage 11β-HSD1 was reduced with liver injury. Both 11β-HSD1-high and 11β-HSD1-low macrophages can be found in both uninjured and injured livers according to dual staining of F4/80 and 11β-HSD1 (Figure 3.8 B).
Figure 3.6 Hepatocyte death in mice injured with CCL₄

Representative pictures of H&E stained mouse liver sections (×80 magnification) in mouse liver harvested from vehicle-treated (uninjured) and 48 hours (peak injury) post CCL₄ termination are shown. Arrows indicate hepatocyte necrosis (karyolysis, eosinophilic cytoplasm and inflammatory infiltration) around scar areas.
Figure 3.7 Immunohistochemistry pattern of expression of 11β-HSD1 in HSCs after CCL4 injury

Representative pictures of 11β-HSD1 (green) and αSMA (red) dual staining in Vehicle treated (Veh) and CCL4 injured mice at 48 hour, 1 week and 1 month post last CCL4 administration (×80 magnification). Arrows are pointing to reduced 11β-HSD1 expression around scar area. Left panel: 11β-HSD1 staining; middle panel αSMA staining; right panel: merged pictures with DAPI showing the nuclei.
Figure 3.8 Expression of 11β-HSD1 in macrophages and co-staining of 11β-HSD1 and macrophage in CCL4 injured livers

A: Representative pictures showing in situ hybridizations with an antisense [\textsuperscript{35}S]-labelled 11β-HSD1 cDNA probe at 48 hours after the last CCL4 administration (peak fibrosis) and at 1 week of recovery. Macrophages are indicated with black arrows. B: Representative pictures of dual staining of 11β-HSD1 (Green) and F4/80 (Red) with DAPI showing the nuclei in livers either uninjured or during peak injury. Macrophages higher in 11β-HSD1 (orange) are indicated with yellow arrows and macrophages with low 11β-HSD1 (red) are indicated with red arrows.
3.5 Discussion

In this chapter, I have shown that 11β-HSD1 was down regulated not only in steatosis but also in steatohepatitis and fibrosis in a number of mouse models. In the liver fibrosis model, 11β-HSD1 down-regulation may be attributed to hepatocyte death.

With respect to the down-regulation of 11β-HSD1 observed in the mouse model of NAFLD, this is consistent with models of the metabolic syndrome. In metabolic syndrome, the reduction in hepatic 11β-HSD1 may be a compensatory mechanism to reduce the local intrahepatic glucocorticoid load (Seckl, 2004) since local glucocorticoid can cause more lipid deposition in the liver and it has been shown that a failure of down-regulate 11β-HSD1 activity facilitates the development of dyslipidaemia, insulin resistance and obesity (Valsamakis et al., 2004). The reduction in cortisol can be achieved either by reduced 11β-HSD1 activity or by increased activity of 5α/5β-reductases. In steatosis patients liver fat accumulation is associated with both increased 5α-reductase conversion and down regulation of hepatic 11β-HSD1 (Westerbacka et al., 2003).

In this chapter, CDD significantly induced hepatic triglyceride deposition without affecting inflammatory/fibrosis response whilst MCDD induced not only hepatic triglyceride content but also hepatic injury and fibrosis suggesting they are good models for steatosis and NASH. Here I have shown that the down-regulation of 11β-HSD1 in mice fed CDD followed the same pattern as steatosis models with metabolic syndrome (Livingstone et al., 2000). The mechanism of CDD induced steatosis is still unclear. It was proposed that CDD and MCDD models cause liver fat accumulation by impairing VLDL export (Lombardi et al., 1968). However, recent studies show the CDD inhibited neither synthesis of phosphatidylcholine, which is a major component of VLDL, nor apoB secretion (Kulinski et al., 2004). Studies in our group find that CDD mice had no significant changes in triglyceride secretion, fatty acid turnover or de novo lipogenesis in the liver (Macfarlane et al., 2011). Transcript levels of genes involved in FFA esterification such as 1-acyl-sn-glycerol-3-phosphate acyltransferase alpha (AGPAT-1) and diacylglycerol acyltransferase 2 (DGAT-2) are up-regulated in CDD fed mice suggesting increased FFA
esterification might be the mechanism of CDD induced steatosis (Raubenheimer et al., 2006). Glucocorticoids (GCs) promote fatty liver in experimental animals by increasing de novo triglyceride synthesis and reducing intracellular lipolysis (Dolinsky et al., 2004). 11β-HSD1 knockdown, by administrating an antisense oligonucleotide, significantly improved Western diet induced steatosis by reducing synthesis and secretion of triglycerides (Li et al., 2011). Therefore, down regulation of 11β-HSD1 in the CDD model could be a compensating mechanism to produce less active GCs, further reducing TG deposition in the hepatocytes.

Few studies have looked at the hepatic 11β-HSD1 expression in NASH although some reports indicate 11β-HSD1 level in NASH is either unchanged or increased (Ahmed et al., 2012, Konopelska et al., 2009). Our study is the first to show that 11β-HSD1 mRNA is significantly reduced in MCDD treated mice, a diet model for steatohepatitis. Unlike CDD, MCDD mice have impaired VLDL transport of fat in the liver with the presence of increased inflammatory cell infiltration and increased hepatocyte damage (Macfarlane et al., 2011). Moreover, the peripheral insulin sensitivity in MCDD mice is significantly increased due to the severe loss of peripheral fat (Rinella and Green, 2004). Nevertheless, hepatic insulin resistance is increased since mice fed MCDD showed impaired insulin signalling (Leclercq et al., 2007). MCDD leads to a reduction in insulin induced insulin receptor substrate 1 (IRS-1), insulin receptor substrate 2 (IRS-2), and Akt phosphorylation, which are key factors in the insulin signalling pathway, confirming decreased hepatic insulin sensitivity in this model (Schattenberg et al., 2005). In this case, the reduction of 11β-HSD1 in the liver of MCDD fed mice can contribute to reduce hepatic insulin resistance in this model.

In the CCL₄ induced liver injury, the transcripts of 11β-HSD1 tested by both real-time PCR and in situ hybridization as well as the 11β-HSD1 protein level were down-regulated at peak fibrosis. However, 11β-HSD1 activity in whole liver lysates remained unchanged. A possible post-transcriptional control of enzyme activity might exist. Similar discrepancy between mRNA level and 11β-HSD1 activity was found by Ricketts et al. (Ricketts et al., 1998). Unstable enzyme activity in the samples was considered as the reason for the discrepancy in that experiment.
In CCL₄ induced liver fibrosis, fibrosis resolution starts from 48 hours after the last CCL₄ administration. 11β-HSD1 followed the opposite pattern to the fibrosis deposition: reduced during peak fibrosis and recovered during resolution. Down regulation of 11β-HSD1 mRNA and protein levels were also observed in TAA induced fibrosis, further confirming the pattern of 11β-HSD1 in rodent liver fibrosis. However, 11β-HSD1 transcripts were not unregulated at 1 week after TAA withdrawal. This could be attributed to the TAA induced fibrosis not having resolved after 1 week recovery (Pellicore et al. unpublished data). Both liver fibrosis models confirm that 11β-HSD1 levels are negatively associated with liver fibrosis.

Several lines of evidence suggest that the loss of 11β-HSD1 could be attributed to hepatocyte death. Firstly, 11β-HSD1 is highly expressed in hepatocytes; secondly, hepatocyte necrosis occurs during peak fibrosis whilst regeneration occurs in the resolution phases, paralleling changes in 11β-HSD1; thirdly, other cell types such as HSCs or macrophages failed to show robust 11β-HSD1 expression in the liver compared with hepatocytes. However, other mechanisms may also contribute to the loss of 11β-HSD1 in these models. The in situ hybridization data showed that even in non-necrotic cells 11β-HSD1 transcript was reduced suggesting a functional switch in these ‘healthy’ hepatocytes in CCL₄ induced liver injury.

In this study, toxins TAA and CCL₄ reduced 11β-HSD1 level. Hepatic cytochrome P450 enzymes (CyP450s) are required for the bioactivation of both TAA and CCL₄. Hepatic CYP2E1, which is a key factor responsible for the bio-activation of numerous compounds, appears to be primarily involved in bio-activation of TAA (Wang et al., 2000, Ramaiah et al., 2001). The essential role of 11β-HSD1 in protecting organisms from the damage of toxic carbonyl compound accumulation is generally accepted (Maser and Oppermann, 1997). For examples, 11β-HSD1 acts as a microsomal quinone reductase to reduce the oxidative stress caused by the oxidation of quinone (Wermuth et al., 1986, Maser and Oppermann, 1997). The down regulation of 11β-HSD1 could facilitate the damage of these toxins. This could be further tested by inhibit/knockout 11β-HSD1 to investigate whether this damage was amplified ( Chapter 5 and Chapter 6).
Apart from hepatocytes, HSCs are also key cells involved in the pathogenesis of NAFLD. After CCL₄ injury, activated HSCs showed less 11β-HSD1 compared with hepatocytes nearby. Whether 11β-HSD1 is present in HSCs is unknown because immunofluorescence of αSMA and 11β-HSD1 can only identify activated HSCs. Also, there is a possibility that 11β-HSD1 is weakly expressed in activated cells but the staining was ‘masked’ by strong hepatocyte staining. Markers for both quiescent and activated HSCs such as desmin and GAFP can be used to further investigate the expression of 11β-HSD1 in both uninjured and fibrotic livers. Also, isolating HSCs by flow cytometry and then testing 11β-HSD1 expression can help to reduce the ‘masking’ effect of hepatocytes. To my knowledge there is neither direct evidence that 11β-HSD1 is present in HSCs nor information on how 11β-HSD1 is regulated in these cells prior to my work. However, in other myofibroblast precursors, 11β-HSD1 expression is detected (Torday et al., 1985, Brereton et al., 2001), suggesting a possibility that 11β-HSD1 is present in HSCs. This is addressed further later in the thesis.

My study showed 11β-HSD1 was present in macrophages, in accordance to previous findings that 11β-HSD1 is expressed in inflammatory cells including macrophages (Chapman et al., 2009). I also found that macrophages showed two populations: 11β-HSD1 high and 11β-HSD1 low expressing population in the liver. This can be explained by differing stages of differentiation of macrophages since fully differentiated macrophages express 10 times higher 11β-HSD1 than undifferentiated macrophages (Thieringer et al., 2001).

Data from rodent and human studies regarding the regulation of 11β-HSD1 are sometimes inconsistent. In some human studies 11β-HSD1 mRNA level is increased or unchanged in NASH and liver cirrhosis caused by mixed liver diseases (Ahmed et al., 2012, Konopelska et al., 2009). More specifically, here I have shown in CCL₄ induced liver injury that 11β-HSD1 immunostaining was reduced near the scar area whereas a study on human normal formalin fixed liver sections revealed the 11β-HSD1 staining was intensified in hepatocytes in peri-septal areas (Ahmed et al., 2012). It is very difficult to compare human versus mouse models of disease since available models could not faithfully represent human NAFLD. Additionally, human
livers in these studies came from mixed pathologies including cryptogenic cirrhosis, hepatitis C cirrhosis, hepatitis B cirrhosis, and primary biliary cirrhosis (Ahmed et al., 2008) whereas we specifically concentrated on toxin induced fibrosis. Although the injury type might be different, the potential mechanisms involving injured hepatocytes and activation of HSCs and recruited inflammatory cells are nevertheless common processes both in the rodent model and in human pathophysiology of NAFLD.

In summary, \(11\beta\text{-HSD1}\) is down regulated in NAFLD models suggesting a compensating mechanism, which involves increasing hepatic insulin sensitivity and decreasing local lipids, is required throughout the pathogenesis and development of NAFLD. However, in this chapter, I failed to clarify whether \(11\beta\text{-HSD1}\) is present in HSCs. Also, whether this down-regulation of \(11\beta\text{-HSD1}\) contributes to the progression of NAFLD is unknown. To further address these issues, \(11\beta\text{-HSD1}\) knockout mice were used in NAFLD models in Chapter 4 and Chapter 5 and the \(11\beta\text{-HSD1}\) expression pattern in HSCs will be further investigated in Chapter 7.
Chapter 4. Effect of 11β-HSD1 Deficiency on Steatosis, Inflammation and Fibrosis in Dietary Models of NAFLD
4.1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by hepatic fat accumulation with or without inflammation. Steatosis is the early stage of NAFLD characterized by fat deposition which can progress to steatohepatitis with fat deposition and additional inflammation after a second ‘hit’ (Day and James, 1998).

GCs regulate metabolic and inflammatory processes and are in turn controlled by central and local regulation. Central regulation of GCs primarily involves the hypothalamic–pituitary–adrenal (HPA) axis. Locally in tissues, GC levels are regulated by glucocorticoid receptor (GR), GC amplifying enzyme 11β-HSD1, GC inactivating enzymes such as 5α- and 5β-reductases. 11β-HSD1, which is expressed in many tissues in both human and rodents, generates active glucocorticoid within tissues. It is well established that global 11β-HSD1 deficiency has ‘cardio-protective’ effects and a healthier metabolic profile during DIO, with reduced fat accumulation, insulin resistance and adipose scarring (Kotelevtsev et al., 1997, Morton et al., 2001, Michailidou et al., 2012). On the other hand, tissue-specific hepatic 11β-HSD1 over expression leads to fatty liver and reduced hepatic lipid clearance without obesity (Paterson et al., 2004) and adipose-specific 11β-HSD1 over expression mice causes obesity and metabolic syndrome with hyperglycaemia (Masuzaki et al., 2001).

11β-HSD1 regulates inflammatory responses in a variety of acute and chronic inflammatory models (reviewed by (Chapman et al., 2009)) and has been shown to regulate fibrotic response in some tissues, for example the skin (Terao et al., 2011). Nevertheless it is still widely unknown how 11β-HSD1 deficiency affects hepatic inflammation and pro-fibrotic response in NAFLD. In Chapter 3 I showed that 11β-HSD1 levels were reduced in all NAFLD model tested suggesting that glucocorticoid deficiency is a feature of NAFLD, and consistent with the hypothesis that 11β-HSD1 deficiency plays a pro-fibrotic role in NAFLD.

To test this hypothetical role of 11β-HSD1 in the development and/or resolution of NAFLD, I used global 11β-HSD1 knockout (KO) mice (Morton et al., 2001) as characterised previously. In addition to previous models tested in chapter 3, I used high fat diet feeding to induce steatosis in association with obesity. High fat diet has
been used commonly to induce obesity with increased insulin resistance, hyperlipidaemia and steatosis in C57BL/6 mice (Buettner et al., 2006).

From the above, I hypothesize that 11β-HSD1 deficiency increases the susceptibility to hepatic inflammation and fibrosis in high fat diet and choline deficient diet induced steatosis and methionine-choline deficient diet induced steatohepatitis.

4.2. Aims

- To address whether global 11β-HSD1 deficiency changes hepatic inflammation and fibrosis in diet induced obesity (DIO) model.
- To address whether global 11β-HSD1 deficiency changes hepatic inflammation and fibrosis in CDD and MCDD models.

4.3. Methods

4.3.1. C57BL/6 (wild type) and 11β-HSD1 KO mice in NAFLD models

11β-HSD1 KO mice (Morton et al., 2001) were developed in the lab as previously described. 8 to 10-week-old 11β-HSD1 KO mice and age matched C57BL/6 mice were fed high fat diet (HFD, 58% calories as fat, D12331 from Research Diets, New Brunswick, US) for 18 weeks and compared with wild type mice fed normal chow. In addition, 18 to 20-week-old 11β-HSD1 KO and wild type C57BL/6 control mice were fed control diet, CDD and MCDD for 2 weeks. N=4 for each cohort on control diet, N=6 for each cohort on CDD and MCDD diet (detailed CDD and MCDD information is presented in Section 2.2.3.2). Animals were culled by decapitation and plasma samples were collected at the meantime. Livers were harvested and snap frozen in liquid nitrogen before moved to -80°C.

In order to detect significance between diets and genotype, sample size was calculated with a statistical power of 0.8 and type I error α=0.05 in Power & Sample Size Calculator (Statistical Solutions). N=6 was selected according to previous data.
in HFD, CDD and MCDD models according to previous data in the lab (Macfarlane et al., 2011, Morton et al., 2004). However, due to limited supply of knock out animals, N=4 was used in control diet groups.

4.3.2. Lab techniques

Methods for RNA extraction from whole liver homogenates, reverse transcription and real time PCR for target genes method are presented in Section 2.6. Picrosirius red staining, immunohistochemistry and Oil red O staining on formalin fixed liver tissues were conducted as described in Section 2.4.5, Section 2.4.2 and Section 2.4.6, respectively. The method for biochemical assay for triglycerides is described in Section 2.10.1. Flow cytometry and gating example are presented in section 2.3.5. Liver function tests were done by F. Howie in the MRC Centre for Reproductive Health as described in Section 2.10.3.

4.3.3. Statistics

To compare multiple groups (more than 2), one-way ANOVA followed by post-hoc Tukey test was used. Grouped data were tested by two-way ANOVA followed by post-hoc LSD test. Graphpad Prism 5.0 and Statistica 7.0 were used for statistical tests.
4.4. Results

4.4.1. 11β-HSD1 KO mice have higher HSC activation after HF feeding

HFD significantly induced weight gain compared to normal chow. The weight gain during 18-week high fat feeding was similar between genotypes (Michailidou et al., 2012). High fat diet for 18 weeks significantly increased hepatic lipid accumulation, however, there was no difference in liver triglyceride content between 11β-HSD1 KO mice and control mice (Figure 4.1). Total collagen deposition and αSMA deposition were similar between wild type on normal chow and HFD. However, 11β-HSD1 KO mice had significantly higher αSMA staining despite similar collagen deposition in the liver compared with wild type mice after HFD feeding (Figure 4.2). There were no significant changes in mRNA levels of pro-fibrotic genes (Col1a1, Col3a1, Timp1, Acta2 (αSMA) and Tgfb1) and inflammatory genes (Cxcl1 and Mcp1) between either diet treatments or genotypes (Figure 4.3).
Figure 4.1 Increased liver lipid content during high fat feeding is similar in wild type and 11β-HSD1 KO mice

A: Representative pictures of oil red O staining in livers of wild type mice (WT) fed control diet (CD) or WT and 11β-HSD1 KO mice fed high fat diet (HF). B: Quantification of liver triglycerides (TG) by biochemical assays. Results are presented as Mean ± SEM, N=4 in wild type untreated mice; N=6 in high fat diet treated groups. Significance was tested by one-way ANOVA followed by post-hoc Tukey tests. +++ p<0.001 versus WT mice fed control diet.
Figure 4.2 Increased HSC activation in 11β-HSD1 KO mice with HFD feeding

A: A panel of representative pictures of picrosirius red (PSR) staining (left panel, ×80 magnification) and αSMA immunohistochemistry staining (right panel, ×200 magnification) in the livers of wild type mice (WT) fed control diet and WT and 11β-HSD1 fed high fat diet. B: Quantification of the percentage of positive pixels of PSR in the livers. C: Quantification of the percentage of positive pixels of αSMA corrected with nucleus numbers per picture. Results are presented as Mean ± SEM. N=4 in wild type mice on control diet; N=6 in high fat diet treated groups. Significance was tested by one-way ANOVA followed by post-hoc Tukey test. * p<0.05 versus wild type fed HFD. + p<0.05 versus wild type mice fed control diet.
Figure 4.3 Unaltered pro-fibrotic and pro-inflammatory gene transcripts in 11β-HSD1 KO mice fed HFD

mRNA levels of pro-fibrotic (Collα1, Collα3α1, Actα2(αSMA), Tgfβ1, Timp1) and pro-inflammatory genes (Cxcl11 and Mcpl) normalized to 18S. Results are presented as Mean ± SEM. N=4 in wild type mice on control diet; N=6 in high fat diet treated groups. Significance was tested by one-way ANOVA followed by post-hoc Tukey test. No significant difference was detected.
4.4.2. **11β-HSD1 deficiency does not affect fibrosis and inflammatory responses in the CDD model**

CDD feeding for 14 weeks had significantly increased the bodyweights and liver weights in both C57BL/6 and 11β-HSD1 KO mice to a similar degree compared with control diet groups (Figure 4.4 A-C). CDD induced hepatic triglyceride content compared with control diet, but the levels were significantly higher in 11β-HSD1 KO mice (Figure 4.4 D).

Mice fed CDD had less total collagen and collagen I deposition, but similar αSMA staining compared with control diet fed mice irrespective of genotype (Figure 4.5). In contrast, CDD induced Acta2 (αSMA) transcripts, but had no effect on Tgfb1 mRNA levels regardless of genotype.

Mice fed CDD showed increased serum ALT levels but unaltered AST, ALP and albumin levels compared with control diet irrespective of genotype (Figure 4.7). Mice fed CDD had similar hepatic neutrophil number and increased hepatic macrophage number compared with mice fed control diet (Figure 4.8). After CDD feeding, 11β-HSD1 KO showed a trend of higher neutrophil numbers but no effect in macrophage numbers compared with control mice. Hepatic inflammatory genes Cxcl2, Mcp1, Il1 and Cxcl1 were all unaffected by CDD (Figure 4.9 A-D). However, CDD increased Ym1 transcript levels compared with control diet group (Figure 4.9 E). 11β-HSD1 deficiency had no effect in CDD fed mice but significantly reduced Il1 transcripts in control diet group (Figure 4.9). Macrophage subset analysis suggested both pro-fibrotic macrophages (Ly6C<hi>) and anti-fibrotic macrophages (Ly6C<lo>) were induced by CDD irrespective of genotype (Figure 4.10).
4.4.3. 11β-HSD1 deficiency decreases HSC activation and inflammatory response in MCDD model

Mice fed MCDD for 2 weeks had significant weight loss compared with those fed control diet and CDD regardless of genotype (Figure 4.4 A and B). Both liver weights corrected for body weight and liver triglyceride content were unaltered in MCDD compared with control diet but lower compared with CDD irrespective of genotype (Figure 4.4 C and D).

MCDD fed mice showed no increase in total collagens or collagen I deposition compared with control diet fed mice (Figure 4.5 B and C). However, MCDD significantly induced αSMA deposition compared to control diet (Figure 4.5D). 11β-HSD1 KO had no effect in total collagen/collagen I deposition but markedly reduced αSMA deposition in MCDD fed mice (Figure 4.5). MCDD also induced hepatic Acta2 (αSMA) and Tgfb1 mRNA levels compared with control diet (Figure 4.6). However, unlike an inhibition in αSMA deposition, 11β-HSD1 KO showed no effect on the mRNA levels of Acta2 (αSMA) and Tgfb1 despite the reduced hepatic αSMA deposition.

MCDD significantly induced serum ALT, AST and ALP levels with no effect on albumin irrespective of genotype (Figure 4.7). MCDD fed mice showed similar hepatic neutrophil numbers, but increased macrophage numbers compared with control diet and CDD irrespective of genotype (Figure 4.8). MCDD significantly induced hepatic inflammatory gene transcripts including Cxcl2, Mcp1, Il1 and Ym1 with no effect on Cxcl1 compared with both control diet and CDD (Figure 4.9). 11β-HSD1 KO markedly reduced mRNA levels of Il1 and Ym1 in MCDD fed groups. Macrophage subset analysis suggested both pro-fibrotic macrophages (Ly6C<sup>hi</sup>) and anti-fibrotic macrophages (Ly6C<sup>lo</sup>) were increased in MCDD compared with control diet and CDD (Figure 4.10). 11β-HSD1 deficiency showed no effect in regulating these subsets (Figure 4.10).
Figure 4.4 Body weights and hepatic lipids in wild type and 11β-HSD1 KO mice fed CDD and MCDD

A: Body weight changes throughout the 2 week duration on diets in 11β-HSD1 KO and wild type control mice (WT) fed control diet (N=4), CDD (N=6) and MCDD (N=6). B: Body weight changes at termination of experiment. C: Liver weights corrected for body weights. D: Hepatic triglyceride level (µmol/mg liver weight). Results are presented as Mean ± SEM. Significance was tested by two-way ANOVA followed by post-hoc LSD test; tables next to the graphs show the p values of source of variance from diet, genotype and interactions tested by two-way ANOVA; *** p<0.001: significant difference between genotypes (geno) within a diet group; although post-hoc significant differences between diets were detected, they are omitted for clarity.
Figure 4.5 Unaltered collagen levels but reduced αSMA deposition in livers of 11β-HSD1 KO mice fed MCDD but not CDD

A: A panel of representative pictures of picrosirius red (PSR) staining and collagen I and αSMA immunohistochemistry (×80 magnification). B-C: Graphs quantifying the percentage of positive stained pixels of PSR (B) and collagen I (C). D: Quantification of the percentage of positive pixels of αSMA corrected with nucleus numbers per picture in 11β-HSD1 KO and wild type mice (WT) fed control diet (N=4), CD (n=6) and MCDD (n=6). Results are presented as Mean ± SEM. Significance was tested by two-way ANOVA followed by post-hoc LSD test; tables next to the graphs show the p values of source of variance tested by two-way ANOVA; ** p<0.01: significant difference between genotypes within a diet group; although post-hoc significant differences between diets were detected, they are omitted for clarity.
mRNA levels of hepatic pro-fibrotic gene Acta2 (αSMA) (A) and Tgfb1 (B) normalized to 18S in 11β-HSD1 KO and wild type control mice (WT) fed control diet (N=4), CDD (N=6) and MCDD (N=6). Results are presented as Mean ± SEM. Significance was tested by two-way ANOVA followed by post-hoc LSD test; tables next to the graphs show the p values of source of variance tested by two-way ANOVA; no post-hoc significance was detected between genotypes; although post-hoc significant differences between diets were detected, they are omitted for clarity.
Figure 4.7 Unaltered liver function tests (LFTs) in 11β-HSD1 KO mice on CDD and MCDD

Graphs for plasma ALT (A), AST (B), ALP (C) and albumin (D) levels in 11β-HSD1 KO and wild type control mice fed control diet (N=4), CDD (N=6) and MCDD (N=6). Results are presented as Mean ± SEM. Significance was tested by two-way ANOVA followed by post-hoc LSD test; tables next to the graphs show the p values of source of variance tested by two-way ANOVA; no post-hoc significant difference was detected between genotypes within any diet group; although post-hoc significant differences between diets were detected, they are omitted for clarity.
Figure 4.8 Unaltered liver inflammatory cells in 11β-HSD1 KO mice treated with either CDD or MCDD

A: A panel of representative flow cytometry pictures selecting F4/80$^+$CD11b$^+$ cells (macrophages, Mac) and Ly6G$^+$Cd11b$^+$ cells (neutrophils, Neutro). B and C: Quantification graphs of inflammatory cells as the percentage of macrophages (B) and neutrophils (C) among all non-parenchymal cells in the liver in 11β-HSD1 KO and wild type control mice (WT) fed control diet (N=4), CDD (N=6) and MCDD (N=6). Results are presented as Mean ± SEM. Significance was tested by two-way ANOVA followed by post-hoc LSD test; tables next to the graphs show the p values of source of variance tested by two-way ANOVA; no difference between genotypes was detected; although post-hoc significant differences between diets were detected, they are omitted for clarity.
Figure 4.9 Decreased Il1 and Ym1 mRNA levels in livers of 11β-HSD1 KO mice fed MCDD

mRNA level of Cxcl2 (A), Mcp1 (B), Il1 (C), Cxcl1 (D) and Ym1 (note there was no Ym1 signal detected in control diet groups) (E) normalized to 18S in 11β-HSD1 KO and wild type control mice (WT) fed control diet (N=4), CDD (N=6) and MCDD (N=6). Results are presented as Mean ± SEM. Significance was tested by two-way ANOVA followed by post-hoc LSD test; tables next to the graphs show the p values of source of variance tested by two-way ANOVA; * p<0.05: significant difference between genotypes within a diet group; although post-hoc significant differences between diets were detected, they are omitted for clarity.
Figure 4.10 No alteration in liver macrophage subsets in 11β-HSD1 KO mice treated with CDD and MCDD

A: A panel of representative flow cytometry pictures showing Ly6C^{hi} macrophages and Ly6C^{lo} macrophages. B and C: Quantification graphs showing the percentage of Ly6C^{hi} macrophages (B) and Ly6C^{lo} macrophages (C) among all non-parenchymal cells in the liver in 11β-HSD1 KO and wild type control mice fed control diet (N=4), CDD (N=6) and MCDD (N=6). Results are presented as Mean ± SEM. Significance was tested by two-way ANOVA followed by post-hoc LSD test; tables next to the graphs show the p values of source of variance tested by two-way ANOVA; no post-hoc significant difference was detected between genotypes within a diet group; although post-hoc significant differences between diets were detected, they are omitted for clarity.
4.1 Discussion

In this chapter, I have demonstrated that 11β-HSD1 had an effect in HSC activation and inflammation in some rodent NAFLD models. 11β-HSD1 KO increased HSC activation in HFD fed mice, had no effect in CDD fed mice, and unexpectedly decreased HSC activation and inflammatory gene transcripts in MCDD fed mice. This study discovered a potential role of 11β-HSD1 in regulating HSCs and inflammatory responses in the liver.

In chapter 3, I have shown fully activated HSCs had less 11β-HSD1 expression than healthy hepatocytes by dual staining of 11β-HSD1 and αSMA. However, in this chapter, 11β-HSD1 manipulation significantly affected HSC activation in both HFD and MCDD fed mice, but in spite of the different pattern observed in these models. This suggests 11β-HSD1 can be potentially expressed in HSCs and also involved in cell activation regulation. However, further characterising of 11β-HSD1 in HSCs, which will be described in Chapter 7, is necessary.

Although no significant effect of 11β-HSD1 KO on the progression of liver fibrosis was observed, whether 11β-HSD1 affects liver fibrosis may not have been adequately assessed in these experiments. Activation of HSCs is a very important process initiating fibrosis cascade. 11β-HSD1 KO could potentially influence liver fibrosis by affecting HSC activation. However, across the NAFLD models I tested, no evidence of 11β-HSD1 KO’s role on liver fibrosis was found. This could be a limitation of the models. HFD and CDD are mild liver injury models which would make fibrosis quantification very difficult; although MCDD induced much higher inflammation in the liver, it failed to increase liver fibrosis compared with control diet in this study. The effect of 11β-HSD1 KO in liver fibrosis could be further investigated in a specific liver fibrosis model with profound scar deposition.

High fat diet feeding is a well-established technique to induce obesity, steatosis and metabolic syndrome (Morton et al., 2004, Buettner et al., 2006), but fibrosis in the liver is weak in this model. Mice fed high fat diet had increased inflammatory markers such as MCP-1 and IL-6 in plasma and adipose tissue suggesting an exaggerated systemic and adipose tissue inflammatory response in this model (Gu et
al., 2013, Meng et al., 2013). Whether this model elevates hepatic inflammation is detatable. Some studies suggest after 8-week high fat diet feeding, mice develop remarkably increased hepatic transcripts of TNF\(\alpha\), IL-6 and elevated hepatic inflammatory cell numbers (Meng et al., 2013, Wang et al., 2011). However, Kohli et al. failed to observe any increase in hepatic inflammation or liver fibrosis in 16-week-high-fat diet fed mice despite a rise in plasma AST and ALT level was detected (Kohli et al., 2010). This low-grade inflammation with/without fibrosis model makes it difficult to find out 11\(\beta\)-HSD1’s effect in fibrosis progression.

The HSC-activating effect of 11\(\beta\)-HSD1 deficiency in a DIO model raises an interesting question: whether 11\(\beta\)-HSD1 deficiency’s effect on HSCs is a consequence of its metabolic effect? From the data so far, there was no direct link between HSC activation and metabolic regulating effect of 11\(\beta\)-HSD1 deficiency. Since NAFLD and obesity are highly linked, adipose tissue is a very important regulator on NAFLD development. Adipokines, such as leptin and adiponectin are important regulators on liver fibrosis. Leptin is required for HSC activation and leptin deficiency worsens liver fibrosis induced by toxin (Ikejima et al., 2001); adiponectin has an adverse effect on liver fibrosis since adiponectin ameliorated liver fibrosis induced by toxin (Kamada et al., 2003). 11\(\beta\)-HSD1 KO significantly decreases adipose leptin and increases adiponectin (Morton et al., 2004). These observations suggest the metabolic effect of 11\(\beta\)-HSD1 may be anti-fibrotic rather than pro-fibrotic. In our study, the HSC-activating effect of 11\(\beta\)-HSD1 deficiency overrides the benefits of metabolic effects in DIO model, suggesting a direct new role of 11\(\beta\)-HSD1 deficiency in the liver.

Similar with HFD, CDD is a model for steatosis, in which the inflammation and fibrosis are too mild to study. Interestingly, 11\(\beta\)-HSD1 KO in CDD mice did not improve the steatosis but caused more TG deposition in the liver. This is surprising given previous studies that 11\(\beta\)-HSD1 KO increased the transcripts of hepatic lipid metabolic genes in obese mice that were insulin resistant (Morton et al., 2001). However, CDD is a steatosis model with increased hepatic insulin sensitivity. It causes lipid deposition by increasing hepatic lipid esterification genes such as acyl-CoA synthetase, long chain 1 (ASCL-1) and diacylglycerol acyltransferase-2
(DAGAT-2) and had no effect on hepatic lipid oxidative related genes such as PPARα and carnitine palmitoyl-transferase 1α (CPT-1α) (Raubenheimer et al., 2006). However, 11β-HSD1 deficiency alters hepatic lipid primarily by up-regulating sterol regulatory element-binding protein-1c (SREBP-1c) and lipid oxidative PPARα at the transcriptional level (Morton et al., 2001). In this case, 11β-HSD1 KO may have no effect or even adverse effect on lipid metabolism in CDD induced steatosis.

In my hands, MCDD worked as a robust model to induce higher inflammatory cell infiltration, hepatocellular injury, Tgfb1 transcript and HSC activation although not fibrosis in the liver compared with control diet and CDD. As described before, MCDD is a very robust model to induce NASH and significantly increase liver fibrosis compare to control diet (Leclercq et al., 2000). However, profound fibrosis is found in studies using this diet for 4 up to 12 weeks (Tomita et al., 2013, Leclercq et al., 2004). In my study, mice were only fed MCDD for 2 weeks because they needed to be terminated if body weights dropped by 20% under the terms of the Home Office project licence. In this case, MCDD in this study failed to cause severe enough liver fibrosis leaving the question ‘Whether 11β-HSD1 KO influences liver fibrosis?’ unsolved.

In my study, 11β-HSD1 KO had an opposite effect on HSC activation in MCDD compared with HFD fed mice, suggesting a model-dependent role of 11β-HSD1 KO on HSC activation. In HFD, the HSC-stimulating effect of 11β-HSD1 KO was independent of its role in inflammation because no inflammatory marker tested was up-regulated. Decreased HSC activation in 11β-HSD1 KO mice fed MCDD is consistent with our previous finding that treating MCDD mice with dexamethasone up-regulated pro-fibrotic markers in the liver (unpublished data). According to the pathways I investigated, decreased HSC activation was not subject to differences in hepatocyte injury because AST and ALT levels as well as liver TG content were not different with 11β-HSD1 deficiency. The inhibiting effect of 11β-HSD1 KO on HSC activation in MCDD fed mice was also not due to improved fibrosis-resolving factors. This is because 1) the resolving macrophage subset, Ly6Clo macrophages (Ramachandran et al., 2012) remained similar between genotypes and 2) YM-1, which is a marker for alternative activated macrophages, which possibly facilitates
the resolution of inflammation (Lopez-Navarrete et al., 2011), is even decreased in 11β-HSD1 KO. In this case, the reduced activation of HSCs could result from reduced pro-inflammation response in 11β-HSD1 KO since Il1 transcripts were significantly reduced although other pro-inflammatory markers as CXCL-1 and MCP-1 remained unchanged. To clarify the effect of 11β-HSD1 in HSCs, other liver injury models *in vivo* and cells *in vitro* should be investigated.

In my study, 11β-HSD1 deficiency had no effect on hepatic inflammatory response in DIO. In the CDD model, neutrophil numbers showed a trend to be higher in 11β-HSD1 KO. However, pro-inflammatory gene *Il1* and alternative macrophage activation marker *Yml* was reduced in 11β-HSD1 KO mice fed MCDD. Many pieces of evidence suggest that 11β-HSD1 KO mice have an exaggerated inflammatory response in many acute inflammatory models (Zhang and Daynes, 2007, Gilmour et al., 2006). However, opposite results are found in other models. 11β-HSD1 KO showed reduced circulating MCP-1 level in a model of atherosclerosis (Kipari et al., 2013). Also, 11β-HSD1 KO mice show suppressed inflammatory signalling and lower adipocyte MCP-1 secretion with strikingly reduced cytotoxic T-cell and macrophage infiltration, predominantly in visceral fat after high fat feeding (Wamil et al., 2011). Thus, the pro-inflammatory effect of 11β-HSD1 deficiency can be tissue and model dependent.

This study has a limitation of small group number of mice on control diet. This may have limited statistic power of the whole study. However, what I focused on in this study was the difference between wild type and knockout mice in different models of NAFLD. The small control group is only used for validating the primary effect of HFD, CDD and MCDD. HFD and CDD both induced significant triglyceride deposition in the liver suggesting they worked well as models for steatosis. However, MCDD failed to increase hepatic TG albeit it increased inflammatory markers. This could be due to 1) limited MCDD exposure time as discussed above and 2) the use of aged mice (20-week-old) may affect the efficiency of MCDD.

In conclusion, I have shown that 11β-HSD1 deficiency affects HSC activation in different models of NAFLD. So little attention has been paid to the fibrotic response in previous studies of 11β-HSD1 deficiency that our study leads the research of 11β-
HSD1 to a new angle. However, the effect of 11β-HSD1 deficiency on HSCs was not reproducible in all models suggesting a need to investigate other models. Also, 11β-HSD1’s effect on fibrosis progression is still unclear because the pro-fibrotic responses were too subtle in the models we have investigated. For this reason, I decided to investigate 11β-HSD1’s effect in a more severe fibrotic response in toxin induced fibrosis. These data are presented in the next chapter.
Chapter 5. Effect of 11β-HSD1 Deficiency on Liver Fibrosis Induced by CCL₄
5.1. Introduction

Liver fibrosis, characterized by the accumulation of extracellular matrix (ECM) in the liver, is the final stage of most chronic liver disease. During the pathogenesis of liver fibrosis, hepatocytes are injured and undergo apoptosis and hepatic stellate cells (HSCs) undergo activation and express myofibroblast markers such as α-smooth muscle actin (αSMA). Activated HSCs are the major source of ECM which is regulated by metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) (reviewed by (Brenner, 2009)). In the resolution phase of chemical induced liver fibrosis, hepatocytes regenerate and HSCs undergo apoptosis (Iredale et al., 1998) or de-activation (Troeger et al., 2012). Macrophages play key roles in the fibrosis response: they stimulate the fibrosis response at the start of the injury (Duffield et al., 2005) and also facilitate the spontaneous resolution of liver fibrosis (Fallowfield et al., 2007).

Glucocorticoids (GCs) are key players that regulate metabolism and inflammatory responses in many tissues. Unlike the GCs’ role in pulmonary fibrosis (Sabatini et al., 2012), there are limited data suggesting an anti-fibrotic role of GCs in the liver although they are used as complementary treatment in primary biliary cirrhosis (Raikhel'son et al., 2012). GCs could potentially inhibit liver fibrosis by: a) inhibiting hepatocyte injury, since pharmacological doses of GCs protected rodent primary cultured hepatocytes from death receptor-mediated apoptosis in vitro (Oh et al., 2006); b) inhibiting TGFβ stimulated HSC activation through the Smad3 pathway (Bolkenius et al., 2004); and c) inhibiting the pro-inflammatory response and promoting an anti-inflammatory response by stimulating macrophages to phagocytose (Liu et al., 1999).

In Chapter 4 I showed 11β-HSD1 deficiency increased HSC activation in DIO fed mice but decreased HSC activation in MCDD fed mice models. However, whether 11β-HSD1 KO affects liver fibrosis is still unclear by studying these NAFLD models because they failed to stimulate intensive liver fibrosis. CCL4 induced liver injury is a well-established model for liver fibrosis: acute CCL4 administration (single dose) causes necrosis and inflammation in the liver and chronic CCL4 administration (more than 4 weeks) induces liver fibrosis and even cirrhosis (Henderson et al., 2006).
Although CCL₄ induced liver injury/fibrosis does not represent the natural process of liver fibrosis developed from NAFLD, key factors including hepatocyte death, HSC activation and macrophage infiltration is consistent between this model and natural human disease. Besides, in Chapter 3 I found 11β-HSD1 level was reduced in peak fibrosis and recovered during resolution in CCL₄ induced liver fibrosis suggesting a possible association between 11β-HSD1 and CCL₄ induced liver injury. In this case, I used CCL₄ induced liver fibrosis to further investigate the effect of 11β-HSD1 KO on liver fibrosis.

**In this chapter I hypothesize that 11β-HSD1 deficiency worsens liver fibrosis in CCL₄ induced liver fibrosis.**

### 5.2. Aims

- Investigate the influence of 11β-HSD1 deficiency on the response to acute CCL₄ induced liver injury
- Investigate the influence of global 11β-HSD1 deficiency on the fibrotic and inflammatory response to chronic CCL₄ administration
- Investigate the influence of hepatocyte specific 11β-HSD1 deficiency on the fibrotic and inflammatory response to chronic CCL₄

### 5.3. Methods

#### 5.3.1. CCL₄ induced liver injury

##### 5.3.1.1. Single dose CCL₄ administration in wild type and 11β-HSD1 KO mice

11β-HSD1 global KO mice were developed in the lab as previously described (Morton et al., 2001). 11β-HSD1 KO mice and aged matched wild type (WT) C57BL/6 mice (N=6 in each group) were fasted overnight with free access to water. 0.4µl/g CCL₄ was administrated into the intra-peritoneal space. The mice were re-fed
after the CCL4 injection and harvested at 24 hours after the CCL4 injection (peak injury).

5.3.1.2. **Chronic administration of CCL4 in wild type and 11β-HSD1 KO mice**

12 to 14-week-old 11β-HSD1 KO mice and age matched C57BL/6 mice were injected with 0.4μl/g CCL4 intra-peritoneally twice a week for 12 weeks as previously described (Ramachandran et al., 2012). Mice in each group were harvested at 24 hours, 72 hours and 8 days after the last CCL4 injection to study peak fibrosis, early fibrosis resolution phase and late fibrosis resolution phase, respectively.

5.3.1.3. **Chronic CCL4 administration on hepatocyte specific 11β-HSD1 KO mice**

Hepatocyte specific 11β-HSD1 KO mice, Albumin Cre<sup>Cre</sup>/11β-HSD1<sup>Flox/Flox</sup> (Alb-HSD1) mice, were developed by Manwani et al. in the lab (unpublished data) (breeding information in Section 2.2.1.3). Alb-HSD1 mice showed 90% 11β-HSD1 knock down in the liver with no effect in other tissues compared with 11β-HSD1<sup>Flox/Flox</sup> mice (Manwani et al., unpublished). In this study, 8-10-week-old Alb-HSD1 mice (N=8) and aged matched Cre negative 11β-HSD1 homo floxed mice (11β-HSD1<sup>Flox/Flox</sup>) (control group, N=7) were injected with 0.4μl/g CCL4 intraperitoneally twice a week for 12 weeks. Mice were harvested at 24 hours after the last CCL4 administration (peak injury).

5.3.1.4. **Sample size calculation**

In order to detect significance between chemical treatment and genotype, sample size was calculated with a statistical power of 0.8 and type I error α=0.05 in Power & Sample Size Calculator (Statistical Solutions). N=6 was selected according to previous data in CCL4 treated mice (Morton et al., 2001, Pellicoro et al., 2012). However, due to limited supply of knockout mice, N=5 was used in each cohort of peak injury (24 hours post CCL4 termination in chronic CCL4 liver fibrosis model).
5.3.2. **Lab techniques**

Methods for RNA extraction from frozen livers, reverse transcription and real time PCR for target genes is presented in Section 2.6. Picrosirius red (PSR) staining, immunohistochemistry and H&E staining in formalin fixed liver sections are undertaken as described in Section 2.4.5, Section 2.4.2 and Section 2.4.4, respectively. Flow cytometry method and gating example are presented in section 2.3.5. Genotyping method is presented in Section 2.5. Liver function tests were undertaken by F. Howie in MRC Centre for Reproductive Health as described in Section 2.10.3.

5.3.3. **Statistics**

Student’s t test was used to compare differences between two groups. Grouped data were tested by two-way ANOVA followed by post-hoc LSD test. Graphpad Prism 5.0 and Statistica 7.0 were used for statistical tests.

5.4. **Results**

5.4.1. **11β-HSD1 KO mice show decreased liver injury but increased collagen deposition after acute CCL4 injury**

Single CCL4 administration led to increased total collagen deposition measured by PSR staining in 11β-HSD1 KO mice compared with control mice (Figure 5.1 A-B). Although H&E staining showed no differences in necrosis and inflammation in the liver of both genotypes, hepatocyte injury indicated by hepatocyte oedema was observed (Figure 5.1 C), 11β-HSD1 KO mice had decreased serum ALT, AST and ALP levels compared with wild type controls (Figure 5.1 D-G).
Figure 5.1 Increased total collagen deposition but reduced hepatocyte injury in 11β-HSD1 KO mice after single CCL$_4$ administration

A: Representative picture with arrows indicating positive staining of PSR showing total collagen (magnification ×80). B: Quantification graph for positive PSR staining in wild type (WT) C57BL/6 control mice and 11β-HSD1 KO mice at 24 hours post CCL$_4$ injection. C: Representative pictures of H&E staining (magnification ×80). Arrows indicate hepatocyte oedema. D-F: Plasma liver function test (LFT) levels including ALT (D), AST (E), ALP (F) and albumin (G). Results are presented as Mean ± SEM, N=6 in each group. Significance was tested by Student’s t tests after logarithmically transforming the data for equal variance in figure D, E and F. * p<0.05 and *** p<0.001
5.4.2. **Effect of 11β-HSD1 deficiency on response to chronic CCL4 injury**

5.1.1.1 *Increased liver fibrosis in 11β-HSD1 KO mice after chronic CCL4 administration*

In the 12-week CCL4-injury model, liver weights peaked at 72 hours and dropped at 8 days post CCL4 termination in wild type control mice. Liver weights were higher in 11β-HSD1 KO mice at 24 hours and 8 days after CCL4 termination (Figure 5.2).

Total collagen deposition as indicated by PSR staining peaked at 24 hours after the termination of CCL4 administration, and at 72 hours the resolution of injury was apparent although there was no further resolution at 8 days after the last CCL4 administration in wild type mice. Deposition of COL-1, COL-3 and αSMA all peaked at 24 hours post CCL4 termination and started to resolve at 72 hours with complete resolution at 8 days after the last CCL4 administration in wild type mice in accordance with previous studies (Ramachandran et al., 2012) (Figure 5.3). 11β-HSD1 deficiency caused a) a trend of increase in total collagen at 24 hours (Figure 5.3 B), b) a significant increase at 24 hours and a trend of increase at 8 days (p=0.076) in COL-1 deposition (Figure 5.3 C), c) unaffected COL-3 at all time points (Figure 5.3D) and d) a trend of decrease in αSMA at 24 hours and a trend of increase in αSMA 8 days post CCL4 termination (Figure 5.3 E).

Pro-fibrotic genes also showed time dependent resolution in wild type control mice. The higher fibrotic response in 11β-HSD1 KO mice during peak injury was also confirmed by significantly elevated mRNA levels of *Coll1a1* at 24 hours (Figure 5.4 A). Transcripts of *Acta2* (αSMA), *Timp1* and *Mmp9* at 72 hours after the last CCL4 injection (Figure 5.4 B-D) were significantly increased in 11β-HSD1 KO mice. αSMA protein level failed to resolve in wild type mice (Figure 5.4 E and F). The protein level of αSMA was significantly higher in 11β-HSD1 KO than wild type mice at 24 hours and 8 days after last CCL4 injection (Figure 5.4 E and F). *Tgfb1* mRNA level showed a time dependent resolution whereas Smad3 phosphorylated...
protein (Smad3\(^p\)) showed no differences between time points in wild type control mice. Neither \(Tgfb1\) mRNA nor Smad3\(^p\) protein level were elevated in 11\(\beta\)-HSD1 KO mice (Figure 5.5).
Figure 5.2 Increased liver weights in 11β-HSD1 KO mice after chronic CCL₄ administration

Liver weights (A) and liver weights corrected for body weights (B) at 24 hours (N=5), 72 hours (N=6) and 8 days (N=6) after the last CCL₄ injection. Results are presented as Mean ± SEM. Significance was tested by two-way ANOVA followed by post-hoc LSD test; tables next to the graphs show the p values of source of variance tested by two-way ANOVA; *p<0.05, ** p<0.01, *** p<0.001: significant difference between genotypes (geno) within a time point; although post-hoc significant differences between time points were detected, they are omitted for simplicity.
Figure 5.3 Increased peak fibrosis in 11β-HSD1 KO mice in chronic CCL4 induced liver injury

Representative pictures (A) and quantification graphs of PSR (B), COL-1 (C), COL-3 (D) and αSMA (E) staining showing the percentage of positive stained pixels among all pixels per picture at 24 hours (N=5), 72 hours (N=6) and 8 days (N=6) after the last CCL4 administration in wild type (WT) and 11β-HSD1 KO mice. Significance was tested by two-way ANOVA followed by post-hoc LSD test; tables next to the graphs show the p values of source of variance tested by two-way ANOVA; *p<0.05: significant difference between genotypes (geno) within a time point; although post-hoc significant differences between time points were detected, they are omitted for simplicity.
Figure 5.4 Increased pro-fibrotic gene mRNA and protein levels in 11β-HSD1 KO mice after chronic CCL₄ administration

A-D: Liver mRNA levels of pro-fibrotic genes including Acta2 (αSMA) (A), Colla1 (B), Timp1 (C), Mmp9 (D) normalized to 18S at 24 hours (N=5), 72 hours (N=6) and 8 days (N=6) after last CCL₄ administration in WT and 11β-HSD1 KO mice. E and F: Representative blots (E) and quantification graph (F) showing αSMA corrected for GAPDH. Significance was tested by two-way ANOVA followed by post-hoc LSD test; in B-D data were logarithmically transformed for equal variance; tables next to the graphs show the p values of source of variance tested by two-way ANOVA; *p<0.05: significant difference between genotypes (geno) within a time point; although post-hoc significant differences between time points were detected, they are omitted for clarity.
Figure 5.5 Lack of measurable changes in TGFβ pathway in 11β-HSD1 KO mice in CCL4 induced liver fibrosis

A: Tgfb1 mRNA level normalized to 18S at 24 hours (N=5), 72 hours (N=6) and 8 days (N=6) after the last CCL4 administration in WT and 11β-HSD1 KO mice. B and C: Representative immune-blots (B) and quantification graph showing Smad3p protein level corrected for GAPDH. Results are presented as Mean ± SEM, N=3 in each group. Significance was tested by two-way ANOVA followed by post-hoc LSD test; tables next to the graphs show the p values of source of variance tested by two-way ANOVA; no significant difference was detected between genotypes (geno) within a time point; although post-hoc significant difference between time points are detected, they were omitted for simplicity.
5.1.1.2 11β-HSD1 KO mice show similar inflammatory responses compared with control mice after chronic CCL4 administration

Liver function tests showed plasma aminotransferases decreased in a time dependent way (Figure 5.6 A and B). Alkaline phosphatase (ALP) and Albumin showed no difference between time points (Figure 5.6 C and D). Plasma AST an ALT levels were lower in 11β-HSD1 KO mice at 24 hours and similar levels at 72 hour and 8 days after the last CCL4 administration compared with control mice (Figure 5.6).

Macrophage and neutrophil numbers measured by flow cytometry showed a time-dependent decrease in wild type mice (Figure 5.7 B and C). 11β-HSD1 KO showed no effect in macrophage number across all time points although a trend of neutrophil number increase was found at 72 hours after the last CCL4 administration (Figure 5.7 B and C). Immunohistochemistry staining showed no altered macrophage number (F4/80) in wild type control mice across all time points, but neutrophil numbers measured by GR-1 staining decreased in a time-dependent manner (Figure 5.7 E and F). 11β-HSD1 KO had decreased F4/80+ macrophage staining at 24 hours after the last CCL4 administration but no effect in neutrophil staining (Figure 5.7 E and F). Hepatic transcripts of inflammatory gene Mcp1 and II1 dropped in a time dependent manner regardless of genotype (Figure 5.8).

Hepatic Ly6C^{hi} macrophage (pro-fibrotic) numbers peaked at 24 hours and dropped from 72 hours after CCL4 termination and Ly6C^{lo} macrophage (anti-fibrotic) numbers were not decreased until 8 days after CCL4 termination in wild type mice (Figure 5.9 B and C) in accordance with previous data (Ramachandran et al., 2012). Ly6C^{lo}/Ly6C^{hi} ratio was highest at 72 hours but decreased again at 8 days after CCL4 termination in wild type mice (Figure 5.9 D). Both Ly6C^{hi} and Ly6C^{lo} macrophage numbers were similar between wild type and KO mice (Figure 5.9).
Figure 5.6 Decreased plasma aminotransferase levels in 11β-HSD1 KO mice in CCL₄ induced liver fibrosis

Plasma AST (A), ALT (B), ALP (C) and Albumin (D) levels at 24 hours (N=5), 72 hours (N=6) and 8 days (N=6) after the last CCL₄ administration in wild type control (WT) and 11β-HSD1 KO mice. Results are presented as Mean ± SEM. Significance was tested by two-way ANOVA followed by post-hoc LSD test; tables next to the graphs show the p values of source of variance tested by two-way ANOVA; * p<0.05 and *** p<0.001: significant difference between genotypes (geno) within a time point; although post-hoc significant differences between time points were detected, they are omitted for clarity.
Figure 5.7 Inflammatory cell numbers in 11β-HD1 KO mice and wild type control mice in CCL4 induced liver fibrosis

A-C: A panel of representative flow cytometry pictures of F4/80+CD11b+ cells (macrophages) and Ly6G+CD11b+ cells (neutrophils) (A) and quantification graphs showing the percentage of macrophages (Mϕ) (B) and neutrophils (C) among all hepatic non-parenchymal cells in wild type (WT) and 11β-HSD1 KO mice at 24 hours (N=5), 72 hours (N=6) and 8 days (N=6) after the last CCL4 administration. D-F: Representative picture of Gr1 (Neutro, neutrophils) and F4/80 (macrophages) (D, magnification ×200) and quantification graphs showing numbers of macrophage (Mac, E) and neutrophil (Neutro, F) per field. Results are presented as Mean ± SEM. Significance was tested by two-way ANOVA followed by post-hoc LSD test; tables next to the graphs show the p values of source of variance tested by two-way ANOVA; *** p<0.001: significant difference between genotypes (geno) within a time point; although post-hoc significant differences between time points were detected, they are omitted for simplicity.
Figure 5.8 Unaltered inflammatory gene mRNA levels in 11β-HSD1 KO in CCL4 induced liver fibrosis

Liver mRNA levels of pro-inflammatory gene Mcp1 (A) and Il1 (B) normalized to 18S in wild type (WT) and 11β-HSD1 KO mice at 24 hours (N=5), 72 hours (N=6) and 8 days (N=6) after the last CCL4 administration. Results are presented as Mean ± SEM. Significance was tested by two-way ANOVA followed by post-hoc LSD test; tables next to the graphs show the p values of source of variance tested by two-way ANOVA; no significant difference was detected with between genotypes (geno) within a time point; although post-hoc significant differences between time points were detected, they are omitted for simplicity.
Figure 5.9 Unaltered macrophage subsets in 11β-HSD1 KO mice in CCL4 induced liver fibrosis

CD11b\textsuperscript{hi}F4/80\textsuperscript{int} macrophages were divided into two subsets according to Ly6C level. A: Representative pictures showing Ly6C\textsuperscript{hi} (pro-fibrotic macrophages) and Ly6C\textsuperscript{lo} (anti-fibrotic) cells. B and C: Quantification graphs showing the percentage of Ly6C\textsuperscript{hi} (B) and Ly6C\textsuperscript{lo} (C) among all hepatic non-parenchymal cells at 24 hours (N=5), 72 hours (N=6) and 8 days (N=6) after the last CCL\textsubscript{4} administration. D: Ratio of Ly6C\textsuperscript{lo} macrophages over Ly6C\textsuperscript{hi} macrophages. Results are presented as Mean ± SEM. Significance was tested by two-way ANOVA followed by post-hoc LSD test; tables next to the graphs show the p values of source of variance tested by two-way ANOVA; no significant difference was detected between genotypes (geno) within a time point; although post-hoc significant differences between time points were detected, they are omitted for simplicity.
5.4.3. Hepatocyte specific 11β-HSD1 KO does not recapitulate the pro-fibrotic phenotype of global KO

To address whether the pro-fibrotic effect of 11β-HSD1 deficiency is attributable to the loss of this enzyme within hepatocytes, hepatocyte specific 11β-HSD1 KO mice (Alb-HSD1) and 11β-HSD1<sup>FloX/FloX</sup> (control) mice were investigated in CCL<sub>4</sub> induced liver fibrosis. Alb-HSD1 mice were Cre positive whereas control mice were Cre negative measured by genotyping (Figure 5.10 A). 11β-HSD1 transcripts were reduced by 93 % in the liver (p=0.0002) in Alb-HSD1 mice compared with control mice (Figure 5.10 B). 11β-HSD1 protein level was undetectable in whole liver homogenates (Figure 5.10 C).

Alb-HSD1 mice did not recapitulate the pro-fibrotic phenotype of global 11β-HSD1 KO mice. Unlike global 11β-HSD1 KO mice, Alb-HSD1 mice showed neither increased collagen deposition nor decreased serum AST or ALT level compared with control mice (Figure 5.11 A-F). The inflammatory cell numbers were similar between Alb-HSD1 mice and control mice (Figure 5.11 G).
Figure 5.10 Genotyping and 11β-HSD1 expression in Alb-HSD1 and control mice

A: Genotyping for Albumin Cre of DNA extracted from the livers of Alb-HSD1 and control mice (11β-HSD1 homo floxed mice). B: mRNA expression of 11β-HSD1 normalized to 18S in homogenized whole liver samples in Alb-HSD1 (N=8) and control (N=7) mice. Results are presented as Mean ± SEM, *** p<0.001 tested by Student’s t test. C: Immuno-blot showing 11β-HSD1 protein levels in homogenized whole liver samples (N=3).
Figure 5.11 Alb-HSD1 mice show similar fibrotic response at peak injury to control littermates

A-D: Representative pictures (A, magnification×80) and quantification graphs of PSR (B), COL-1 (C) and αSMA (D) staining showing the percentage of positive stained pixels among all pixels per picture from liver sections of Alb-HSD1 (N=8) and control mice (N=7). E-F: Plasma ALT (E) and AST (F) level. G: Representative picture (G) of Gr-1 (neutrophils, Neutro) and F4/80 (macrophages, Mϕ) staining with arrows indicating positive stained cells. H and I: quantification graphs showing neutrophil (H) and macrophage (I) numbers per area. Results are presented as Mean ± SEM. Significance was tested by Student’s t test. No differences were detected between genotypes.
5.5. Discussion

These data show that genetic 11β-HSD1 deficiency promoted fibrogenesis in the liver. This novel finding is not driven by measurable changes in hepatocyte injury or liver inflammation but appeared more closely related to HSC activation.

In this study, acute CCL4 injection failed to induce a histologically quantifiable necrosis in the liver. This is different from a previous study showing that single dosing of CCL4 leads to hepatic necrosis and inflammation (usually marked by hepatocyte death and inflammatory cell infiltration) at 24 hours to 48 hours post administration (Erickson et al., 2006). This could be due to a mild dose (0.4µl/mg) rather than a moderate dose (1µl/mg) (Henderson et al., 2006) was used to match chronic dosing. However, this model still caused hepatocyte damage since hepatocyte oedema was observed and serum AST and ALT levels were greatly induced (abnormal relative to normal level in uninjured mice, <50 U/L (Palipoch and Punsawad, 2013)). In this case, we can conclude that hepatocyte injury is reduced during 11β-HSD1 deficiency.

In chronic CCL4 induced liver fibrosis, total collagen deposition failed to show the same time dependent resolution pattern as collagen I and collagen III suggesting a possibility that some other collagens (e.g. collagen IV) may not contribute to fibrosis resolution in CCL4 induced fibrosis. Nevertheless, this should not interfere with 11β-HSD1 KO’s effect in liver fibrosis. Although 11β-HSD1 KO stimulated collagen I deposition, collagen III was unaffected. Total collagen showed a strong trend but not a significant increase in 11β-HSD1 KO mice suggesting 11β-HSD1’s effect on total collagens is weakened by un-altered collagen III. It is suggested systemic GCs decreased the synthesis of both collagen I and collagen III in skin and tendon (Autio et al., 1994, Wei et al., 2006). Our study showed the provoking effect of 11β-HSD1 KO was only on collagen I but not collagen III suggesting 11β-HSD1 is not associated with collagen III metabolism during liver fibrosis.

Here I showed that 11β-HSD1 deletion leads to augmented scar formation and the accumulation of collagens was not due to increased hepatocyte injury since a) both acute CCL4 injection and chronic CCL4 administration leads to increased collagen
deposition and less serum transaminase levels; b) Alb-HSD1 mice showed no
difference in fibrosis with wild type control mice although they induced profound
11β-HSD1 deficiency in the liver. Also, the pro-fibrotic effect of 11β-HSD1 KO was
less likely to be associated with enhanced inflammatory stimulus since the key
inflammatory player, macrophages, showed no increase in either total number or pro-
inflammatory subset (Ly6C^hi). This suggests that 11β-HSD1 KO’s effect on liver
fibrosis could be a direct result of 11β-HSD1 KO’s effect on HSC activation. To test
this, pericyte specific 11β-HSD1 KO mice (PDGFβrCreTg/+ 11β-HSD1^{Flox/Flox},
PDGFr-HSD1) have been developed by crossing PDGFβrCreTg/+ and 11β-
HSD1^{Flox/Flox}. HSCs are the liver specific pericytes which express PDGFR\(\beta\) (Pinzani
et al., 1992). In the time available, I have not completed testing the response to
chronic CCL\(_4\) administration in these mice but this will be an important experiment
to complete in future.

Augmented collagen deposition during 11β-HSD1 deficiency is observed in other
models. Scar thickness in the lesion of post-myocardial infarction is increased in
11β-HSD1 KO mice (McSweeney et al., 2010). Also, total collagen level in coronary
artery plaques is increased in 11β-HSD1 deficient ApE^-/- mice (Kipari et al., 2013,
Iqbal et al., 2012). My data contribute to enlarge the frame of 11β-HSD1’s effect in
tissue repair and fibrosis.

In this study, collagen deposition only showed a trend of increase in 11β-HSD1 KO
mice at 8 days post CCL\(_4\) termination. This suggests 11β-HSD1 KO had a higher
resolving speed compared with wild type controls. This was not due to apoptosis/de-
activation of HSCs because \(\alpha\)SMA level remained high in late recovery phase. This
was also not due to enhanced anti-fibrotic macrophage behaviour because anti-
fibrotic macrophage (Ly6C^lo)/pro-fibrotic macrophage (Ly6C^hi) ratio showed no
difference between genotypes. Whether this was due to increased hepatocyte
regeneration is beyond the realm of this thesis but worth investigating.

The majority of the pro-fibrotic genes I tested showed significant increase in 11β-
HSD1 KO mice at early resolution phase in CCL\(_4\) induced fibrosis. Among these
pro-fibrotic genes, only Cola1 showed up-regulation by 11β-HSD1 at 24 hours post
CCL\(_4\) termination in accordance of histological finding that COL-1 deposition is
increased. The up-regulation of *Acta2* (αSMA), *Timp1* and *Mmp2*, which were upregulated by 11β-HSD1 KO at 72 hours post CCL₄ termination, strongly indicates HSC activation. αSMA is a well-established marker for stellate cell activation; TIMP-1 as well as MMP-2 levels show positive correlation with HSC activation (Iredale, 1997, Friedman, 2008b). Although a variety of pro-fibrotic genes were increased, a primary fibrogenesis gene *Tgfb1* and Smad 3p signalling were not upregulated by 11β-HSD1 KO. It is possible that TGFβ is not regulated by 11β-HSD1 since we find 11β-HSD1 KO fail to alter TGFβ in adipose tissue fibrosis (Michailidou et al., 2012).

In Chapter 4, I showed HSC activation was increased in HFD fed mice but decreased in MCDD fed mice. In this chapter, I demonstrated 11β-HSD1 KO affected HSC activation in a more advanced liver fibrosis model. A discrepancy was found in αSMA deposition, mRNA level and protein level (Table 5.1). Overall, the αSMA expression is increased except at 24 hours post the last CCL₄ administration. Usually, αSMA levels measured by immunohistochemistry, real-time PCR and western blot match each other (Henderson et al., 2006). In this study, a discrepancy subject to measurements indicates the effect of 11β-HSD1 in HSC activation may be mild. With evidence of pro-fibrotic genes that are strongly associated with HSC activation, 11β-HSD1 KO increases HSC activation in the resolving phases. Why αSMA deposition showed a trend of decrease at the peak injury in chronic CCL₄ induced liver fibrosis is unknown. A possible explanation could be 11β-HSD1 KO mice had a different pattern of HSC activation: that the HSC level was higher in an earlier stage, started to resolve before peak injury, and failed to completely resolve at late resolving phase. However, whether that is true should be further tested by using an earlier time point.
Different from its fibrogenic role in the liver, 11β-HSD1 deficiency played an anti-fibrotic role in adipose tissue. This anti-fibrotic role resulted from the enhanced angiogenesis in the adipose tissue of 11β-HSD1 KO mice (Michailidou et al., 2012). The angiogenesis role of 11β-HSD1 deficiency was demonstrated both in vivo (McSweeney et al., 2010) and in vitro (Small et al., 2005). However, in liver fibrosis, angiogenesis correlates with the severity of fibrosis and usually becomes apparent when the liver develops severe cirrhosis (Taura et al., 2008, Corpechot et al., 2002). In our study, 12-week CCL4 administration has not caused end stage cirrhosis, so the effect of 11β-HSD1 deficiency on fibrogenesis overrides 11β-HSD1’s effect on angiogenesis.

Inflammation plays an important role in liver fibrogenesis (Bataller and Brenner, 2005). Usually liver injury follows the same pattern as liver fibrosis (Ji et al., 2011a). However, in our model, there is a significant separation between liver injury (decreased serum aminotransferase levels) and fibrosis severity indicating 11β-HSD1’s role in fibrosis is less associated with 11β-HSD1’s role in inflammation.

In our model, only neutrophil numbers showed a trend of increase in 11HSD1 KO mice at 72 hours after last CCL4 injection. Increased neutrophil number in lesion sites is observed with 11β-HSD1 deficiency during the resolution phase of a myocardial infarction model (McSweeney et al., 2010). Also, the neutrophil number in 11β-HSD1 KO mice increased in an early resolving time point (72 hours after last

### Table 5.1 αSMA expression pattern in CCL4 induced liver fibrosis

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<th>24h</th>
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CCL₄ injection) is consistent with the fact that the clearance of neutrophils is impaired in 11β-HSD1 KO mice (Gilmour et al., 2006). Although GC administration inhibits the apoptosis of neutrophils (Meagher et al., 1996), GCs can limit neutrophil infiltration by suppressing pro-inflammatory cytokines that are regulated by NF-κB and AP-1 (Barnes, 1998). Nevertheless, this should not have much impact on ECM deposition in liver fibrosis since neutrophil numbers are not key players in fibrogenesis. Neither anti-neutrophil serum treatment nor genetic neutrophil impaired mice showed any alterations in liver fibrosis models (Saito et al., 2003, Xu et al., 2004).

Macrophages are key players in liver fibrosis which can either promote or improve liver fibrosis (Duffield et al., 2005, Fallowfield et al., 2007). In my study, total macrophage number tested by immunohistochemistry was decreased in peak injury in 11β-HSD1 KO mice. Unlike previous data (Ramachandran et al., 2012), the flow cytometry of macrophages only showed monocyte derived macrophages (CD11b⁻F4/80⁻) but failed to show the numbers of resident macrophage (CD11b⁺F4/80⁺) possibly due to batch difference of the F4/80 antibody. However, this weakness of the experiment is overcome by the immunohistochemistry of F4/80, which primarily measures resident macrophage (CD11b⁻F4/80⁻) since these cells would show strong F4/80 staining. Reduction of macrophages in 11β-HSD1 KO mice is intriguing but not surprising. 11β-HSD1 showed decreased MCP-1 expression and macrophage numbers in adipose tissue after high fat feeding (Wamil et al., 2011). Similarly, the pro-inflammatory markers of macrophages was down regulated by 11β-HSD1 inhibition in J774.1 cells (Ishii et al., 2007). There can be a tissue-dependent role of macrophage number inhibiting of 11β-HSD1 deficiency.

This thesis has not investigated the role of 11β-HSD1 KO on adaptive immune response, which is also important in liver fibrosis. CD8+ T-cell, CD4+ T-cells and B cells are all associated with liver fibrosis development (Safadi et al., 2004, Chiaramonte et al., 1999, Novobrantseva et al., 2005). Glucocorticoids regulate adaptive immune response as well. Endogenous GCs down-regulate immune response by affecting lymphocyte development (Ashwell et al., 2000). Chronic GC deficiency by Adrenalectomy (ADX) significantly results in increased thymus size.
and cellularity (Jaffe, 1924, Purton et al., 2000). Exposure to GCs induces both T cell and pre-B cell apoptosis (Igarashi et al., 2005). 11β-HSD1 is expressed in CD4+ T cells, CD8+ T cells and B cells (Zhang et al., 2005) and 11β-HSD1 inhibition leads to impaired releasing of type 1 cytokines by T-cells residing within certain lymphoid organs (Hennebold et al., 1996). I cannot exclude the possibility that 11β-HSD1 deficiency could stimulate inflammatory response in liver fibrosis by promoting T cell and B cell maturation.

How 11β-HSD1 deficiency protected hepatocyte from CCL4 induced injury is a very interesting issue to address. Surprisingly, this protection effect was not due to 11β-HSD1 deficiency in hepatocytes since Alb-HSD1 mice showed no effect on serum AST and ALT levels. Whether this effect was due to reduced hepatic inflammation in 11β-HSD1 KO was unknown. Although I had shown there was reduced resident macrophage by immunohistochemistry at peak injury in 11β-HSD1 KO mice, other inflammatory markers showed either no difference or increase. Also, the systemic inflammatory profile is unaddressed. There is a possibility that 11β-HSD1 KO mice had a decreased systemic inflammatory profile in CCL4 induced liver fibrosis leading to a reduced inflammatory damage on hepatocytes. To test that possibility, serum cytokine levels including IL-6 and TNF-α could be measured.

11β-HSD1 deficiency’s hepatocyte protective effect contradicts with 11β-HSD1’s protective role in hepatic detoxifying (Maser and Oppermann, 1997). However, it is compatible with the fact that Cushing’s syndrome leads to elevated serum transaminase levels in human (Guven et al., 2007) and corticosterone treatment increases serum transaminase levels in rodents (D’Souza A et al., 2012) possibly due to enhanced steatosis. Hepatocytes, which are very important metabolic cells, highly express 11β-HSD1 (Seckl, 2004) and respond to GCs in many ways. The protective effect of 11β-HSD1 KO can be subject to GC’s effect in inhibiting hepatocyte proliferation (Kimura et al., 2011, Scheving et al., 2007). However this is debatable since Yamamoto et al. found dexamethasone inhibit TGFβ induced hepatocyte apoptosis by increasing Bcl-xL, which is an anti-apoptotic molecule, in two identical hepatocyte cell lines (Yamamoto et al., 1998).
In conclusion, loss of GCs regenerated within the liver by 11β-HSD1 may contribute to unrestrained activation of HSCs following chemical injury and promote liver fibrosis. This contrasts with anti-fibrotic effects of 11β-HSD1 deficiency in adipose. The pro-fibrotic phenotype of global 11β-HSD1 KO was not due to the effect of 11β-HSD1 deficiency in hepatocytes, but possibly due to the 11β-HSD1 KO’s effect in HSC activation. However, more time points of CCL₄ induced liver fibrosis should be tested to answer why HSC activation showed a trend of decrease at peak fibrosis but increased in the resolving phases. Also, since 11β-HSD1 inhibitors are now developed as a possible treatment to metabolic syndrome, it is very important to find out whether pharmacological 11β-HSD1 deficiency also has pro-fibrotic effect in the liver. In addition, this experiment failed to show whether the un-resolved pro-fibrotic gene was due to 11β-HSD1 KO’s effect during the onset or due to the 11β-HSD1 KO’s effect during the resolving alone. To further investigate the role of pharmacological 11β-HSD1 inhibition and to dissect the timing of the effect of 11β-HSD1 on fibrogenesis and fibrosis resolution, an 11β-HSD1 inhibition model will be used in Chapter 6.
Chapter 6. Effects of Pharmacological 11β-HSD1 Inhibition in NAFLD Models
6.1. Introduction

11β-HSD1 inhibitors were originally developed for metabolic syndrome since genetically 11β-HSD1 deficient mice are protected from metabolic syndrome. The non-selective 11β-HSD1 inhibitor carbenoxolone (CBX) enhanced insulin sensitivity in humans (Walker et al., 1995) and attenuates liver triglyceride and free cholesterol in rodents (Nuotio-Antar et al., 2007). However, CBX shows side effects including raising blood pressure and lowering plasma potassium in human due to inhibitory effect on 11β-HSD2 activity (Andrews et al., 2003).

Selective 11β-HSD1 inhibitors were developed to avoid side effects and achieve maximum inhibition. Compounds such as triazoles, sulphonamides, adamantyl carboxamides, thiazolones and antisense oligonucleotides have been developed for human and rodent use as selective 11β-HSD1 inhibitors (Webster et al., 2010). Selective 11β-HSD1 inhibitors improve obesity, and lipid metabolic profile in a DIO model (Wang et al., 2006, Wang et al., 2012, Hermanowski-Vosatka et al., 2005) and ameliorate glucose tolerance in ob/ob mice (Park et al., 2011). Some compounds have progressed to phase II clinical trial for metabolic syndrome and cognitive disorders (Anagnostis et al., 2013, Webster et al., 2007). Selective 11β-HSD1 inhibitors are generally well tolerated and show controlled blood glucose level and modest improvements in haemoglobin A(1c) in type 2 diabetic patients (Rosenstock et al., 2010, Feig et al., 2011).

In the previous chapters, I showed that global 11β-HSD1 deficiency stimulated HSC activation in a DIO model and persistant HSC activation in CCL4 induced liver injury. Also, 11β-HSD1 KO enhanced pro-fibrotic response at peak injury but only showed a trend of enhanced fibrosis during resolution, suggesting 11β-HSD1 KO mice had a quicker resolving speed compared with control mice. It would be useful to investigate whether 11β-HSD1 inhibitors recapitulate the same phenotype as global KO. Also, as a useful tool of 11β-HSD1 inhibition, 11β-HSD1 inhibitors can be used as an acute 11β-HSD1 inhibition technique to dissect the role of 11β-HSD1 deficiency in both fibrogenesis and fibrosis resolution.
In this chapter, I hypothesize that pharmacological $11\beta$-HSD1 deficiency can recapitulate the effects of global $11\beta$-HSD1 KO in mouse NAFLD and fibrosis models. Also, $11\beta$-HSD1 inhibition affects liver fibrosis at both onset and resolution.

6.2. Aims

- To verify whether pharmacological $11\beta$-HSD1 inhibition causes augmented fibrosis response in mouse NAFLD and fibrosis models.
- To test whether the fibrogenic effect of $11\beta$-HSD1 deficiency in CCL$_4$ induced liver injury is due to the effect of $11\beta$-HSD1 deficiency in fibrogenic stage or resolution phases.

6.3. Method

6.3.1. $11\beta$-HSD1 inhibitor

The compound UE2316 ([4-(2-chlorophenyl-4-fluoro-1-piperidinyl][5-(1H-pyrazol-4-yl)-3-thienyl]-methanon) which was synthesized in house according to the published synthetic method (Sooy et al., 2010), was kindly provided by Dr. S. Webster as a gift. In vitro screening of UE2316 potency for the median inhibitory concentration (IC50) was determined in HEK293 cells as previously described (Webster et al., 2007) (details in Table 2.2).

6.3.2. Pharmacological $11\beta$-HSD1 inhibition in HFD fed mice and ob/ob mice

Frozen liver samples of pharmacological $11\beta$-HSD1 inhibition in HFD fed mice and ob/ob mice were kindly provided by Andrew McBride as a gift. C57BL/6 mice aged 10-12 weeks were put on to high fat diet (Section 2.2.3.1) for 18 weeks. In the last 4 weeks of HFD treatment, 10 mg/kg/day UE2316 or vehicle was administered to the mice using subcutaneous osmotic mini-pumps (Model 2004, Alzet, CA, USA) until the harvest of the mice. Leptin deficient ob/ob mice were purchased from Jackson Laboratories (Bar Harbor, ME, US) under a UK Home Office importing license.
ob/ob mice aged 12 weeks were treated with 10 mg/kg/day UE23126 or vehicle using subcutaneous osmotic mini-pumps (Model 2004, Alzet, CA, USA) for 4 weeks until harvest. Mini-pump administration of UE2316 induced hepatic 11β-HSD1 inhibition to 6.5 ± 5.6 % of controls (McBride et al., Unpublished data).

6.3.3. Pharmacological 11β-HSD1 inhibition in CCL4 induced fibrosis

C57BL/6 mice aged 10-12 weeks were treated with 0.4µl/g CCL4 intra-peritoneally twice a week for 12 weeks. 0.15% UE2316 was supplemented to mice diet to make UE2316 diet. From the start of CCL4 administration, mice in Veh group and mice in UE group received control diet or UE2316 diet, respectively. Mice in UE2316 recovery (UE-R) group received control diet from the beginning of the injection until 48 hour after the last injection and switched to UE2316 diet until the culls to provide inhibition of 11β-HSD1 only during the resolution phases. Mice in each diet group were harvested at 6 hours (pre-peak injury, to capture any early changes of HSC activation), 24 hours (peak injury), 72 hours (early resolution phase) and 8 days (late resolution phase) after the last CCL4 injection if applicable. Food intake of UE2316 achieved fast 11β-HSD1 inhibition in the liver 2-4 hours after administration (Soo et al., Unpublished data).
Figure 6.1 Diagram of 11β-HSD1 inhibition in CCL4 induced liver fibrosis

Livers were harvested at 6 hours, 24 hours, 72 hours and 8 days after the last injection. Veh group were fed normal chow; UE group were fed normal chow supplemented with 0.15% w/w UE2316 from the beginning of CCL4 injury; UE2316 recovery (UE-R) group were fed normal chow during CCL4 and then switched to UE2316 diet at 48 hours after the last CCL4 injection for 11β-HSD1 inhibition during fibrosis resolving phase since normally the resolution phase started before 48 hours after last CCL4 injection.

6.3.3.1. Sample size calculation

In order to detect significance between chemical treatments, sample size was calculated with a statistical power of 0.8 and type I error $\alpha=0.05$ in Power & Sample Size Calculator (Statistical Solutions). N=6 was selected in CCL4 treated mice according to previous data (Pellicoro et al., 2012) and N=8 was selected in HFD treated and ob/ob mice (Livingstone et al., 2009, Sooy et al., 2010, Morton et al., 2001).

6.3.4. Lab techniques

RNA extraction from frozen liver samples, reverse transcription and real time PCR were undertaken as described in Section 2.6. Methods for Picrosirius red staining and immunohistochemistry on formalin fixed liver sections are described in Section 2.4.5 and Section 2.4.2, respectively. Flow cytometry method, example for gating and information for antibodies are presented in 2.3.5. 11β-HSD1 reductase activity was measured as described in Section 2.9.4. Liver function tests were undertaken by F. Howie in the MRC Centre for Reproductive Health as described in Section 2.10.3.
6.3.5. Statistics

For 11β-HSD1 inhibition in HFD fed and ob/ob mice, Student’s t test was used to test the significant differences between vehicle treated and UE2316 treated groups. If variance was different between two groups, data were logarithmically transformed for equal variance. For 11β-HSD1 inhibition in CCL₄ induced liver injury, one-way ANOVA followed by post-hoc Tukey test was used to detect the differences between time points in vehicle treated groups. Differences between vehicle treated (Veh), UE2316 treated (UE) and UE2316 in recovery (UE-R) groups were analysed by Student’s t test (two group time points) and one-way ANOVA (three group time points) followed by post-hoc Tukey test if applicable.
6.4. Results

6.4.1. Pharmacological 11β-HSD1 inhibition results in unaltered pro-fibrotic response in C57BL/6 mice fed high fat diet

Unlike previous reports that 11β-HSD1 inhibition improves lipid profile in a DIO model (Hermanowski-Vosatka et al., 2005), there was no significant difference in body weights and liver weights in UE2316 treatment compared with vehicle in mice fed HFD (McBride et al. unpublished). The mRNA levels of hepatic Acta2 (αSMA), Col1a1, Tgfb1 and Mcp1 were similar between vehicle and UE2316 treated mice in DIO model (Figure 6.2).

![Figure 6.2 Unaltered pro-fibrotic gene transcripts in UE2316 treated mice fed HFD](image)

*A panel of mRNA levels of Acta2 (αSMA), Col1a1, Tgfb1 and Mcp1 relative to 18S in livers from vehicle and UE2316 treated mice on HFD. Results are presented as Mean ± SEM, N=8 in each group. Significance was tested by Student’s t test.*
6.4.2. Pharmacological 11β-HSD1 inhibition promotes pro-fibrotic gene transcripts in ob/ob mice

ob/ob mice treated with UE2316 showed a significant decrease in body weights (p<0.01) although no effect in liver weights compared with ob/ob mice treated with vehicle (McBride et al., unpublished). Also, in ob/ob mice, 11β-HSD1 inhibition failed to reduce liver triglyceride content (Figure 6.3 A). Total collagen content quantified by biochemical assay in ob/ob mice showed no difference between vehicle and UE2316 treatment (Figure 6.3 B). However, pro-fibrotic gene Colla1 mRNA level in whole liver lysates showed a significant increase in the UE2316 treated group. Similarly, Tgfb1 mRNA level showed a strong trend towards an increase after UE2316 treatment (p=0.053). Other transcripts of pro-fibrotic genes and inflammatory genes including Acta2 (αSMA), Mmp13, Mmp9, Timp1, Il1, Cxcl2 and Mcp1 showed the same pattern of increase after UE2316 treatment although they were not statistically significant (Figure 6.4).
Figure 6.3 Unchanged hepatic triglycerides and collagen level in UE2316 treated ob/ob mice

Quantification of hepatic triglycerides (TGs) (A) and total collagens (B) in liver homogenates in ob/ob mice treated with vehicle or UE2316 by biochemical analysis. Significance was tested by Student’s t test. No significant difference was detected between vehicle and UE2316 treatment.
Figure 6.4 Increased Col1a1 mRNA levels but unaltered other fibrotic genes in UE2316 treated ob/ob mice

A panel of graphs showing pro-fibrotic gene mRNA levels normalized to 18S in Vehicle and UE2316 treated ob/ob mice. Results are presented as Mean ± SEM, N=8 in each group. Significance was tested by Student’s t test after logarithmically transforming the data to achieve equal variance. * p<0.05
Pharmacological $11\beta$-HSD1 inhibition increases hepatic fibrogenesis and slows the resolution of fibrosis after CCL$_4$ induced liver injury

In C57BL/6 mice treated with UE2316, the drug intake fluctuated in the first week but remained constant in the following weeks at around 25 mg/kg/day. During the 12-week CCL$_4$ injury, UE2316 diet treatment showed significant decrease in body weights compared with vehicle diet treatment (Figure 6.5 A). Body weights at termination showed no difference between time points in Veh group (Figure 6.5B). Liver weights were highest at 72 hours after the last CCL$_4$ (Figure 6.5 C) in Veh group although this difference was undetectable when the liver weights were normalized by body weights (Figure 6.5 D). UE and UE-R group showed no difference in either body weights or liver weights compared to Veh group among all time points (Figure 6.5 B-D). $11\beta$-HSD1 reductase activity showed no difference between 24 hour and 8 day time point. There was no difference among all diet treatment in $11\beta$-HSD1 reductase activity in the measured time points (Figure 6.5 E).

In vehicle treated groups, total collagen deposition was similar among all time points (Figure 6.6 A and B). However, COL-1 deposition reduced in a time dependent manner at 24 hours, 72 hours and 8 days post CCL$_4$ termination (Figure 6.6 C and D). $\alpha$SMA peaked at 24 hours and reduced at 72 hours and 8 days post CCL$_4$ termination (Figure 6.6 E and F).

Higher collagen deposition was detected in UE2316 treated mice (UE group) compared with vehicle treated mice (Veh group) after CCL$_4$ administration. Specifically, total collagen deposition was significantly increased at 24 hours after CCL$_4$ termination and remained high at 8-day time point ($p=0.057$) in UE2316 group (Figure 6.6 A and B). Collagen 1 deposition showed a trend of increase at 24 hours after last injection ($p=0.076$), and was significantly higher at 8 days after CCL$_4$ termination (Figure 6.6 C and D). UE2316 induced an increase in $\alpha$SMA staining level at as early as 6 hours after last CCL$_4$ injection, but showed unaltered $\alpha$SMA deposition at other time points (Figure 6.5 E and F).
UE2316 administration during the resolution phase (UE-R group), did not recapitulate the exaggerated fibrosis in UE group (Figure 6.6 A-D). Moreover, UE-R showed significantly less αSMA deposition at 8 days after CCL4 termination (Figure 6.6 E and F).
Figure 6.5 UE2316 reduces body weights but does not affect liver weights in CCL₄ induced liver fibrosis

A: body weights change among 12-week experimental time. B: body weights at termination. C: liver weights at termination. D: liver weights at termination corrected for body weights. Please note data for 6 hour and 24 hour time points are not applicable for the UE-R group. E: Quantification graph showing the conversion rate (nmol/mg protein/min) of [³H]-A to [³H]-B in liver homogenate at 24 hours and 8 days after CCL₄ termination. Results are presented as Mean ± SEM, N=6 in each group. Significance of Veh groups between time points was tested by one-way ANOVA followed by post-hoc Tukey test; significance of each diet treatment within a time point was tested by Student’s t test or one-way ANOVA followed by post-hoc Tukey test if applicable. No significant difference between diet treatments within a time point was detected; although post-hoc significant differences between time points were detected, they are omitted for clarity.
Figure 6.6 Pharmacological 11β-HSD1 inhibition shows worse hepatic fibrosis at peak phase of injury and slower resolution in CCL₄ induced liver fibrosis

Representative pictures (magnification ×80) and quantification graphs of picrosirius red (PSR) (A and B), collagen I (COL-1) (C and D) and αSMA (E and F) in the liver at 6 hours (6h), 24 hours (24h), 72 hours (72h) and 8 days (8d) after CCL₄ termination. Percentage of positive staining among all pixels per picture was quantified. Please note data for 6 hour and 24 hour time points are not available for the UE-R group. Results are presented as Mean ± SEM, N=6 in each group. Significance of Veh groups between time points was tested by one-way ANOVA followed by post-hoc Tukey test; significance of each diet treatment within a time point was tested by Student’s t test or one-way ANOVA followed by post-hoc Tukey test if applicable. * p<0.05, *** p<0.001 showing differences between diet treatments within a time point; although post-hoc significant differences between time points were detected, they are omitted for simplicity.
6.4.4. **Pharmacological 11β-HSD1 inhibition reduces inflammatory response in CCL4 induced liver injury**

Plasma liver function tests (LFTs) suggested the liver injury in Veh groups peaked at 24 hours and reduced during the resolution time points (Figure 6.7). Similarly to global 11β-HSD1 KO mouse, plasma AST and ALT were reduced in UE group at 24 hours after CCL4 termination (Figure 6.7). No difference between Veh, UE and UE-R group was found in the resolution phases (Figure 6.7).

In Veh group, neutrophil number decreased during resolution phase confirming the previous pattern in wild type mice (Figure 6.8 B). UE group had decreased hepatic neutrophil number across all time points compared with Veh group. UE-R group had reduced hepatic neutrophil number at 72 hour time point but failed to maintain the reduction at 8 day time point compared with Veh group (Figure 6.8 B).

In vehicle treated mice, monocyte derived macrophage (CD11b^hi\ F4/80^int) numbers showed a trend of increase at 72 hours and dropped at 8 days after the last CCL4 administration compared with 6 hour and 24 hour time points. Resident macrophage (CD11b^int\ F4/80^hi) numbers showed a time dependent increase and reached the highest level at 8 days post CCL4 termination. Total macrophage numbers showed a trend of increase at 72 hours after the last CCL4 injection (Figure 6.9 B-D).

UE group had a) significantly decreased monocyte derived macrophages only at 72 hours after CCL4 termination (Figure 6.9 B), b) similar resident macrophages at all time points (Figure 6.9 C) and c) significantly reduced total macrophages at only 72-hour time point (Figure 6.9 D) compared with Veh group. UE-R group only showed a) modest reduction in monocyte derived macrophages at 72 hours and 8 days compared with Veh group (Figure 6.9 B), b) similar resident macrophages across all time points compared with Veh and UE groups (Figure 6.9 C), and c) modest reduction in total macrophages at 72 hours and 8 days after CCL4 termination compared with Veh group (Figure 6.9 D).
Figure 6.7 Prior 11β-HSD1 inhibition decreases plasma aminotransferase levels at 24 hours post CCL₄ termination

Plasma aspartate aminotransferase (AST, A) and alanine aminotransferase (ALT, B) levels at 6 hours (6h), 24 hours (24h), 72 hours (72h) and 8 days (8d) after CCL₄ termination. Please note data for 6 hour and 24 hour time points are not applicable for the UE-R group. Results are presented as Mean ± SEM, N=6 in each group. Significance of Veh groups between time points was tested by one-way ANOVA followed by post-hoc Tukey test; significance of each diet treatment within a time point was tested by Student’s t test or one-way ANOVA followed by post-hoc Tukey test if applicable. *** p<0.001 showing differences between diet treatments within a time point; although post-hoc significant differences between time points were detected, they were omitted for simplicity.
Within the gate: neutrophils

A) Veh | UE

| 6h  | 19.0% | 10.6% |
| 24h | 18.7% | 8.53% |
| Ly6G| 5.07% | 2.73% |
| 72h | 3.37% | 3.93% |
| 8d  | 11.2% | 3.27% |

CD11b

B) Neutrophils (%)

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1-way ANOVA of Veh group p=0.0106
Figure 6.8 UE2316 treatment decreases hepatic neutrophil numbers in CCL$_4$ induced liver fibrosis

A: A panel of representative flow cytometry pictures shows neutrophils (Ly6G$^+$CD11b$^+$) at 6 hours (6h), 24 hours (24h), 72 hours (72h) and 8 days (8d) post CCL$_4$ termination. B: A quantification graph showing the percentage of neutrophils among all hepatic non-parenchymal cells. Please note data for 6 hour and 24 hour time points are not applicable for the UE-R group. Results are presented as Mean ± SEM, N=6. Significance between time points in Veh groups was tested by one-way ANOVA followed by post-hoc Tukey test; significance of each diet treatment within a time point was tested by Student’s t test or one-way ANOVA followed by post-hoc Tukey test if applicable. * p<0.05 showing the significant differences between diet treatments within a time point; although post-hoc significant differences between time points were detected, they are omitted for simplicity.
A) Monocyte derived Macrophages

#### Veh

- 6h: 13.8% (7.02%)
- 24h: 45.1% (6.09%)
- 72h: 18.6% (22.2%)
- 8d: 17.4% (40.3%)

#### UE

- 6h: 17.2% (7.10%)
- 24h: 24.3% (7.03%)
- 72h: 7.78% (7.40%)
- 8d: 5.67% (24.0%)

#### UE-R

- 6h: 7.10%
- 24h: 7.03%
- 72h: 7.40%
- 8d: 28.7%

CD11b

F4/80
Figure 6.9 UE2316 treatment shows decreased monocyte-derived macrophages and total macrophages during early resolution in CCL4 induced liver fibrosis

A: A panel of representative flow cytometry pictures selecting CD11b$^{hi}$F4/80$^{int}$ cells as monocyte derived macrophages (Mϕ) and CD11b$^{int}$F4/80$^{hi}$ cells as resident macrophages at 6 hours (6h), 24 hours (24h), 72 hours (72h) and 8 days (8d) after CCL4 termination. B-D: Quantification graphs showing the percentage of monocyte derived macrophages (B), resident macrophages (C) and total macrophages (D) among all hepatic non-parenchymal cells. Please note data for 6-hour and 24-hour time points are not applicable for the UE-R group. Results are presented as Mean ± SEM, N=6. Significance between time points in Veh groups was tested by one-way ANOVA followed by post-hoc Tukey test; significance of each diet treatment within a time point was tested by Student’s t test or one-way ANOVA followed by post-hoc Tukey test if applicable. * $p<0.05$ showing the significant differences between genotypes within a time point; although post-hoc significant differences between time points were detected, they are omitted for simplicity.
6.4.5. **Pharmacological 11β-HSD1 inhibition reduces the number of resolving macrophages (Ly6C\textsuperscript{lo} subset) in CCL\textsubscript{4} induced liver injury**

In Veh group, Ly6C\textsuperscript{hi} macrophage numbers peaked at 24 hours after CCL\textsubscript{4} termination and Ly6C\textsuperscript{lo} macrophage numbers showed a trend of peaking at 72 hours after last CCL\textsubscript{4} injury as previously described (Figure 6.10 B and C) (Ramachandran et al., 2012). UE group showed a) reduced Ly6C\textsuperscript{lo} macrophages at 72 hours after CCL\textsubscript{4} termination (Figure 6.10 B), b) equivalent Ly6C\textsuperscript{hi} macrophages across all time points (Figure 6.10 C), and c) decreased Ly6C\textsuperscript{lo}/Ly6C\textsuperscript{hi} ratio at 72 hour time compared with Veh group (Figure 6.10 D). UE-R group showed a) modest reduction in Ly6C\textsuperscript{lo} macrophages at 72 hours after CCL\textsubscript{4} termination (Figure 6.10 B), b) equivalent Ly6C\textsuperscript{hi} macrophages across all time points (Figure 6.10 C) and c) modest reduction in Ly6C\textsuperscript{lo}/Ly6C\textsuperscript{hi} ratio at 72 hour time point compared with Veh group (Figure 6.10 D).
Figure 6.10 UE2316 reduces hepatic Ly6C<sup>lo</sup> macrophages at 72 hours after last CCL<sub>4</sub> injection without affecting Ly6C<sup>hi</sup> macrophages

A: Representative pictures showing liver Ly6C<sup>hi</sup> and Ly6C<sup>lo</sup> macrophages at 6 hours (6h), 24 hours (24h), 72 hours (72h) and 8 days (8d) after CCL<sub>4</sub> termination. B: Quantification graphs showing percentage of Ly6C<sup>hi</sup> macrophages (B) and Ly6C<sup>lo</sup> macrophages (C) among all hepatic non-parenchymal cells. D: Ratio of Ly6C<sup>lo</sup> over Ly6C<sup>hi</sup> macrophages. Please note data for 6 hour and 24 hour time points are not applicable for the UE-R group. Results are presented as Mean ± SEM, N=6. Significance between time points in Veh groups was tested by one-way ANOVA followed by post-hoc Tukey test; significance of each diet treatment within a time point was tested by Student’s t test or one-way ANOVA followed by post-hoc Tukey test if applicable. * p<0.05; ** p<0.01 showing the significant differences between diet treatments within a time point; although post-hoc significant difference between time points were detected, they are omitted for simplicity.
6.5. Discussion

In this chapter, I have investigated the effect of pharmacological $1\beta$-HSD1 inhibition in steatosis and liver fibrosis models. Pharmacological $1\beta$-HSD1 inhibition recapitulated the phenotype of global $1\beta$-HSD1 deficiency in both ob/ob mice and CCL$_4$ induced liver fibrosis model, and indicated the pro-fibrotic effect of $1\beta$-HSD1 deficiency occurs primarily at the onset of fibrosis rather than the resolution phase of fibrosis.

Treatment for obesity is a possible application of $1\beta$-HSD1 inhibitors. Since pro-fibrotic effect of $1\beta$-HSD1 deficiency in CCL$_4$ induced liver injury was found in Chapter 5, it was important to test whether this effect can cause any liver risks of $1\beta$-HSD1 inhibitors. In HFD induced steatosis model, UE2316 administration failed to improve lipid metabolic profile (Andrew McBride, unpublished). Also, no profibrotic gene was up-regulated by UE2316 administration. This is consistent with $1\beta$-HSD1 KO mice on HFD (Chapter 4). However, to further investigate whether pharmacological $1\beta$-HSD1 inhibition promoted HSC activation in HFD fed mice, $\alpha$SMA IHC staining could be analysed.

In our study, 4-week $1\beta$-HSD1 inhibition caused a profound increase in a series of hepatic pro-fibrotic and inflammatory genes in ob/ob mice. Although hepatic $1\beta$-HSD1 is down-regulated in ob/ob mice (Liu et al., 2003b), inhibiting $1\beta$-HSD1 still provokes fibrogenesis in the liver. This suggests that down regulation of $1\beta$-HSD1 is required to reduce hepatic lipid load in NAFLD whilst $1\beta$-HSD1 even at a low level limits the fibrogenesis process in the liver.

ob/ob mice are widely used for studies in obesity and metabolic syndrome since they develop obesity because of leptin deficiency (Perfield et al., 2013). Liver-specific interaction of leptin with $1\beta$-HSD1 is involved in the development of obesity and insulin resistance in ob/ob mice (Liu et al., 2003b). UE2316 administrated by minipump for 4 weeks decreased body weights in ob/ob mice (McBride et al., unpublished), and another $1\beta$-HSD1 inhibitor is reported to improve glucose tolerance and serum lipid profile in ob/ob mice (Park et al., 2011). However, the up-
regulation of pro-fibrotic genes by UE2316 administration may limit the use of 11β-HSD1 inhibitors in obesity.

Pharmacological 11β-HSD1 deficiency throughout the whole CCL4 injury (UE group) recapitulated the phenotype of 11β-HSD1 KO (Chapter 5) with reduced liver injury during peak injury, increased collagen deposition (total collagen) during peak injury and unresolved fibrosis at late recovery time point. This confirms that the use of 11β-HSD1 inhibitors should be closely monitored during studies with liver injury. Mice treated with UE2316 only during fibrotic resolving phase (UE-R) failed to increase scar deposition as UE group suggesting the pro-fibrotic effect of 11β-HSD1 deficiency relies on the onset of pro-fibrotic response. When pro-fibrotic response escalates, inhibiting 11β-HSD1 would have no effect in fibrosis.

In this chapter, to further address the HSC activation pattern during CCL4 induced liver injury, a new time point 6 hours after last CCL4 termination was investigated. During CCL4 induced liver injury in 11β-HSD1 KO mouse, HSC activation showed a trend of decrease although collagen deposition was increased during peak injury (Chapter 5). This was due to early stimulation in HSCs since the highest αSMA level was at 6 hours after the last CCL4 administration. This suggests HSCs during 11β-HSD1 deficiency/inhibition have an earlier activating pattern compared with control mice (Figure 6.11). In this case, augmented activation of HSCs is prior to increased scar deposition during peak fibrosis. αSMA level during resolution is more complicated. UE group had similar, whereas UE-R group had less, αSMA deposition compared with control group. A double role of 11β-HSD1 in HSC activation can be possible: stimulating HSC activation during onset but also stimulating HSC apoptosis/de-activation accurately during resolving. However, this should be further investigated in HSCs in vitro and HSC specific 11β-HSD1 KO mice should be investigated. Moreover, the HSC activation pattern was different from ECM deposition pattern during 11β-HSD1 inhibition, and similar to global 11β-HSD1 KO mice. This is consistent with previous studies that show ECM deposition and HSC activation proceed over different time scales (Iredale et al., 1998, Henderson et al., 2006).
Figure 6.11 Earlier activation and delayed recovery pattern of HSC activation in 11β-HSD1 deficient/inhibited mice during CCL4 induced liver fibrosis

11β-HSD1 deficient mice showed early and higher activation and delayed resolution pattern compared with control mice.

UE2316 administration leads to reduced neutrophils at all time points. A trend of this inhibition effect can be observed even if only inhibiting 11β-HSD1 during the resolving phase suggesting an acute anti-neutrophil effect of 11β-HSD1 inhibition. However, this was not observed in 11β-HSD1 KO mice (Chapter 5). A difference between 11β-HSD1 inhibition and 11β-HSD1 KO suggests a possible role of 11β-HSD1 in immune development since 11β-HSD1 inhibited mice have normal immune system development compared with 11β-HSD1 KO mice. Evidence of inhibited neutrophil infiltration can be found in other models. The nonspecific 11β-HSD inhibitor Carbenoxolone (CBX) limited lung neutrophil numbers as well as MCP-1 and TNFalpha transcripts in bronchial alveolar lavage fluid during endotoxin instillation induced inflammation (Suzuki et al., 2004). It is plausible this effect came from 11β-HSD1 inhibition since 11β-HSD1 is highly expressed in lung whilst 11β-
HSD2 is less expressed in adult lung (Seckl et al., 2004). Also, systemic GC administration inhibits neutrophil apoptosis in human (Meagher et al., 1996). In this case, abolishing GCs’ effect by inhibiting 11β-HSD1 could possibly lead to more neutrophil apoptosis in liver. However, neutrophils are not key players of liver fibrosis (Saito et al., 2003), impaired neutrophil numbers should not contribute to augmented scar deposition.

In UE2316 treated mice, both monocyte derived macrophage numbers and total macrophage numbers are decreased. This is different from 11β-HSD1 KO mice which showed no difference in liver monocyte derived macrophages. Again, differences in immune system development between 11β-HSD1 inhibition and 11β-HSD1 deficiency can possibly be the reason. Similar to our study, 11β-HSD1 inhibition leads to a modest reduction in macrophage infiltration in atherosclerotic lesions (Kipari et al., 2013) indicating a potential anti-inflammatory role of 11β-HSD1 inhibitors in certain models.

In UE2316 treated mice, the reduction of hepatic macrophage results from the decreased Ly6C\textsuperscript{lo} macrophage subset rather than changes of other macrophage subsets. The impaired Ly6C\textsuperscript{lo} macrophage subset recruitment could be a reason that the UE group had unresolved scars at 8 days after CCL\textsubscript{4} termination. Ly6C\textsuperscript{hi} macrophages and/or monocytes are proven to promote fibrotic response in both lung fibrosis and liver fibrosis (Gibbons et al., 2010, Ramachandran et al., 2012). 11β-HSD1 inhibition showed no effect in promoting Ly6C\textsuperscript{hi} numbers suggesting the elevation of fibrosis during peak injury was not due to increased pro-fibrotic macrophages. Ly6C\textsuperscript{lo} macrophages were believed to be resolving macrophages in CCL\textsubscript{4} induced liver injury as well as in other inflammatory models (Nahrendorf et al., 2007, Ramachandran et al., 2012). Reduced anti-fibrotic macrophages led to a robust phenotype of unresolved fibrosis at 8-day time point in 11β-HSD1 inhibited mice. UE-R group did not show unresolved fibrosis although UE-R group showed a mild reduction in anti-fibrotic macrophages. This is because a) UE-R had less fibrosis than UE at peak injury and b) a mild Ly6C\textsuperscript{lo} macrophage reduction does not influence resolution of fibrosis. Interestingly, 11β-HSD1 KO mice only showed a weak phenotype of un-resolved fibrosis possibly due to unaltered hepatic Ly6C\textsuperscript{lo}
macrophage number. This can be possible due to the difference of immune system development as discussed above. Also, Ly6C<sup>lo</sup> macrophages are derived from Ly6C<sup>hi</sup> macrophages (Ramachandran et al., 2012, Arnold et al., 2007). The reduction in Ly6C<sup>lo</sup>/Ly6C<sup>hi</sup> macrophage ratio indicated 11β-HSD1 might be involved in the phenotype switch (Ly6C<sup>hi</sup> to Ly6C<sup>lo</sup>) providing a new mechanism of macrophage phenotype switch for further studies.

The fact that 11β-HSD1 inhibition leads to reduced anti-fibrotic macrophage subsets (Ly6C<sup>lo</sup>) in the liver is consistent with other findings that GCs are involved in promoting anti-inflammatory macrophages. In a murine peritonitis model, dexamethasone administration significantly promotes the CD11b<sup>lo</sup> macrophages, which are characterised as resolving macrophages in that model (Schif-Zuck et al., 2011). In this case, GC’s anti-inflammation/anti-fibrosis role can be 11β-HSD1 dependent.

The fact that impairment anti-fibrotic response leads to unresolved fibrosis is in accordance with GC physiology. It is well established that GCs promote the phagocytosis behaviour of macrophages facilitating the resolution of inflammation (Giles et al., 2001, Liu et al., 1999). 11β-HSD1 played an important in this mechanism since 11β-HSD1 KO mice showed impaired clearance of apoptotic neutrophils (Gilmour et al., 2006). The removal of apoptotic bodies by macrophages contributed to the resolution of fibrosis (Popov, 2010). This indicates the lack of 11β-HSD1 might be associated with impaired resolution because of impaired apoptotic body clearance.

Pharmacological 11β-HSD1 inhibition and 11β-HSD1 KO were all good models to induce systemic 11β-HSD1 deficiency (Kotelevtsev et al., 1997, Sooy et al., 2010). Although 11β-HSD1 inhibitors partly inhibit 11β-HSD1, the inhibition rate was low in organs that have high clearance rate of this compound. Food intake of UE2316 showed no difference in hepatic reductase activity. This may relate to a difficulty of collecting samples immediately after food intake since this compound was cleared in the liver in up to 4 hours after food administration. However, the inhibition of 11β-HSD1 is adequate since the pharmacological inhibition model recapitulated the profibrotic phenotype of 11β-HSD1 KO mice. To further characterize 11β-HSD1
inhibition of UE2316 diet, systemic 11β-HSD1 activity can be measured by serum 11-DHC and corticosterone ratio.

Human and animal studies show 11β-HSD1 inhibitors are potential treatments for metabolic syndrome and cognitive impairment. In some companies, 11β-HSD1 inhibitors are being developed for NAFLD related diseases such as steatosis, obesity and metabolic syndrome. My studies indicate the potential fibrogenic effect of 11β-HSD1 inhibition requires careful monitoring during those trials especially in those with co-existing liver disease. Also, my study revealed the pro-fibrotic effect of 11β-HSD1 inhibition occurs at the onset of injury rather than the recovery of injury suggesting a new mechanism of 11β-HSD1 and fibrosis. 11β-HSD1 KO/inhibition had a model and time point dependent effect on HSC activation. From these in vivo studies, 11β-HSD1 manipulation had variable results which may or may not be mediated by effects on HSCs. For that reason, validating 11β-HSD1’s role in HSCs is necessary. In addition to the in vivo model we have used in Chapter 4, 5 and 6, I will further investigate 11β-HSD1’s role in HSCs in vitro. This work will be presented in next chapter.
Chapter 7. Role of 11β-HSD1 in HSC Activation
7.1. Introduction

Hepatic stellate cells (HSCs) are the major source of extracellular matrix (ECM) accumulation during liver fibrosis. In uninjured liver, HSCs locate in the Disse space and store vitamin A. During liver injury, they are activated and differentiate from a vitamin A-rich cell into a proliferating, fibrogenic, and contractile cell which is a myofibroblast-like cell type ($\alpha$-SMA$^+$) (Friedman, 2008a). Besides producing ECM, activated HSCs express MMP-2 (Arthur et al., 1992), MMP-9 (Han et al., 2007) and MMP-13 (Schaefer et al., 2003) as well as TIMP-1 (Iredale, 1997) which are key factors regulating ECM deposition.

TGF$\beta$ is regarded as the key mediator of liver fibrosis in humans (Gressner et al., 2002). The secretion of TGF$\beta$ is increased in activated HSCs (Bissell et al., 1995). TGF$\beta$ treated HSCs show not only stimulated ECM synthesis but also increased TIMP-1 expression (Shek and Benyon, 2004). TGF-$\beta$ in the serum was also considered as the stimulus of the ‘spontaneous activation’ of HSCs on uncoated plastics. HSCs can also be activated by other stimuli such as hypoxia (Copple et al., 2011), reactive oxidative species (Svegliati Baroni et al., 1998), and connective tissue growth factor (Gao and Brigstock, 2004).

HSCs, as well as other myofibroblast precursors, can be inhibited by GCs. GCs inhibit rodent HSC activation by reducing TGF$\beta$-Smads pathway (Bolkenius et al., 2004). GCs’ effect on HSCs is regarded to be glucocorticoid receptor (GR) dependent since a GR blocker mifepristone, blocks the inhibitory effect of dexamethasone on TGF-$\beta$ secretion (Bolkenius et al., 2004). Whether local GCs’ inhibitory effect of HSC activation can be modulated by the GC amplifying enzyme 11$\beta$-HSD1 can be tested by genetic deletion or pharmacological inhibition. There are no published data on whether 11$\beta$-HSD1 regulates GC signalling in these cells.

Previously I showed a) 11$\beta$-HSD1 expression in activated HSCs was lower compared with hepatocytes in vivo, b) HSCs were more activated during 11$\beta$-HSD1 deficiency in a range of NAFLD models including ob/ob mice, HFD fed mice and CCL$_4$ injured mice (only at certain time points); c) HSCs were less activated in MCDD fed 11$\beta$-HSD1 KO mice; d) administration of 11$\beta$-HSD1 inhibitors to mice.
caused earlier HSC activation compared with control mice. In order to investigate whether the pro-fibrotic phenotype we saw in 11β-HSD1 deficient mice is due to the effect of 11β-HSD1 in HSCs, I further investigated the effect of GCs in HSCs in vitro.

In this chapter, I hypothesize that GCs inhibit HSC activation and this effect is amplified by 11β-HSD1 in vitro.

7.2. **Aims**

- To identify 11β-HSD1 expression pattern in HSCs
- To confirm GCs’ effect on HSCs
- To test whether GCs’ effect of HSCs are amplified by 11β-HSD1 and are GR dependent

7.3. **Methods**

7.3.1. **Cell culture**

7.3.1.1. **Primary culture of HSCs**

Mouse primary hepatic stellate cells were isolated from fresh livers from male C57BL/6 or 11β-HSD1 KO mice mice aged 8-10 weeks as previously described (Iredale et al., 1998). Livers were digested with Dnase I, pronase and collagenase IV then density separated. Mouse cells were cultured in DMEM (Invitrogen, Paisley, UK) with 16% FBC, 100 U/ml penicillin/ 100mg/ml streptomycin and 2mM L-Glutamine unless specified. Cells were plated on to uncoated 6-well plates in a density of 1×10^6/ well (details in Section 2.3.1).

LX-2 cells were kindly provided by Rebecca Aucott as a gift and maintained in DMEM with 10% FBS, 100 U/ml penicillin/ 100mg/ml streptomycin and 2mM L-glutamine. LX-2 is an immortal line of cells that recapitulate most characters of human semi-activated HSCs: expressing pro-fibrotic genes, viable in serum free medium and sensitive to TGF-β activation (Xu et al., 2005).
7.3.1.2. **Activation and pharmacological treatment of HSCs**

7.3.1.2.1. **Spontaneous activation of primary mouse HSCs**

Primary rodent HSCs can be self-activated by culturing in medium supplemented with serum (Schnabl et al., 2001). In my study, primary mouse HSCs were harvested from wild type C57BL/6 mice or 11β-HSD1 KO mice and seeded in $1 \times 10^6$/well onto uncoated 6 well plates directly to allow spontaneous cell activation. Culture medium was refreshed every 2 days. Cells were harvested for RNA and protein analysis at day 2, 5, 8, 11 and 13 post seeding.

For pharmacological treatment of spontaneously activated wild type mouse primary HSCs, cells were cultured in medium supplemented with charcoal-stripped serum (recipe in Table 2.5), treated with combination of vehicle, corticosterone (B, 500nM), 11-dehydrocorticosterone (A, 500nM), UE2316 (10µM) and RU486 (10 µM), and harvested at day 8 after seeding.

7.3.1.2.2. **TGF-β activation of LX-2 cells**

LX-2 cells were used in passage 20-24. Cells were seeded in $5 \times 10^5$/well onto 6 well uncoated plates. Cells were incubated with GCs (500nM cortisol, F) overnight before the medium was changed to serum free medium. Vehicle or TGF-β (2ng/ml) with or without GCs were added to cells and left for 20 hours. Protein and mRNA were harvested for analysis.

7.3.2. **Protein and mRNA measurements**

Cell lysate preparation is described in Section 2.3.3. RNA extraction, reverse transcription and real time PCR method are illustrated in Section 2.6. Western blot for protein quantification method is presented in Section 2.7. Immunohistochemistry in HSCs was undertaken as presented in Section 2.4.2. 11β-HSD1 activity measurement in cultured cells was done as described in Section 2.9.2.
7.3.3. Statistics

Student’s t test was used to compare two groups. One-way ANOVA followed by post-hoc Tukey test was used to compare differences between multiple groups. Grouped data were analysed by two-way ANOVA followed by post-hoc LSD tests. If variance was not equal, data were logarithmically transformed for equal variance.
7.4. Results

7.4.1. 11β-HSD1 levels are reduced during HSC activation

Primary mouse HSCs cultured in serum can ‘spontaneously’ activate on uncoated plastics with the presence of serum as previously described (Issa et al., 2004). During ‘spontaneous’ mouse HSC activation, αSMA protein level increased dramatically increased after 8 days in culture (Figure 7.1 A). 11β-HSD1 mRNA, protein and activity were measurable in HSCs at all time points and changed in a time dependent manner. 11β-HSD1 mRNA and protein level were reduced and plateaued after 5 days in culture (Figure 7.1 B and C). 11β-HSD1 activity was also decreased after activating spontaneously in culture (Figure 7.1 E-F). 11β-HSD1 mRNA was also present, and decreased after TGF-β activation, in LX-2 cells (Figure 7.2). Corticosterone induced 11β-HSD1 mRNA by 2.3 fold in primary mouse HSCs (Figure 7.3 A) and cortisol induced 11β-HSD1 mRNA level by 7.6 fold in LX-2 cells (Figure 7.3 B).
A) αSMA protein (A.U.)

B) 11β-HSD1 protein (A.U.)

C) Hsc70 mRNA (A.U.)

E) Conversion %/cell/hour
Figure 7.1 11β-HSD1 expression is decreased during mouse primary HSC activation in vitro

A: Representative immune-blots and quantifying graph showing αSMA level corrected for GAPDH in HSCs during 2-13 days in culture (N=3). B: Representative immune-blots and quantifying graph showing 11β-HSD1 protein level corrected for GAPDH in HSCs during 2-13 days in culture. N=3 from an individual prep. C: mRNA level of Hsd11b1 in primary C57BL/6 HSCs during 2-13 days culture. Results are presented as Mean ± SEM. Experiment was performed in triplicates and repeated 3 times (N=9). D: Representative picture of peaks for tritiated-11-dehydrocorticosterone ([3H]-A, peak at 12 minutes) and tritiated-corticosterone ([3H]-B, peak at 17 minutes) at day 2, 5 and 8 in HSCs in culture. E: Quantification graph of the percentage of [3H]-A to [3H]-B conversion per 100,000 HSCs in 24 hours. Results are presented as Mean ± SEM; N=3 from an individual prep. Significance was tested by one-way ANOVA followed by post-hoc Tukey test.* p<0.05, ** p<0.01 and *** p<0.001 versus 2-day time point; ++ p<0.01 and +++ p<0.001 versus 5 day time point.
Figure 7.2 Reduced \textit{Hsd11b1} during LX-2 cell activation

\textit{Hsd11b1} mRNA levels in LX-2 cells treated with vehicle or 2ng/ml TGF-\(\beta\) corrected for 18S. Results are presented as Mean \(\pm\) SEM; \(N=3\). Significance was tested by student’s \(t\) test after log transforming the data for equal variance. \(*\) \(p<0.05\)

Figure 7.3 GCs induce \textit{Hsd11b1} transcripts in HSCs

\textit{Hsd11b1} mRNA levels corrected for 18S in primary mouse HSCs treated with 500nM Corticosterone (figure A) and LX-2 cells treated with 500nM cortisol (figure B). Results are presented as Mean \(\pm\) SEM; experiment was repeated 3 times in triplicates (\(N=9\)). Significance was tested by Student’s \(t\) test. \(*\) \(p<0.05\); \(***\) \(p<0.001\)
7.4.2. Sensitivity to spontaneous activation in 11β-HSD1 KO HSCs

During the ‘spontaneous’ activation, transcripts of pro-fibrotic genes Acta2 (aSMA), Col1a1 and Timp1 were increased in a time dependent manner until day 11 in culture in wild type mouse primary HSCs (Figure 7.4). Mmp9 transcripts were highest at 5 days and reduced after 8 days in culture in wild type primary HSCs. Transcripts of Acta2 (aSMA), Mmp9 and Timp1 tended to be higher in 11β-HSD1 KO HSCs at day 8 in culture and Col1a1 mRNA level showed a trend of increase at 11 days in culture (Figure 7.4 A-D). Gross examination under the microscope showed a time dependent increase of aSMA staining in wild type HSCs. aSMA staining was more intense at day 8 in 11β-HSD1 KO compared to wild type HSCs confirming the mRNA pattern of Acta2 (aSMA) (Figure 7.5).
Figure 7.4 pro-fibrotic gene transcripts in 11β-HSD1 KO HSCs

mRNA levels of Acta2 (αSMA) (A), Col1a1 (B), Mmp9 (C) and Timp1 (D) normalized to 18S in wild type (WT) and 11β-HSD1 KO primary mouse HSCs cultured in medium supplemented with FBS from day 2 to day 13 post harvest. HSC cultured for 2 days were regarded as the baseline (before activation) and cells began to activate afterwards. Results are represented as Mean ± SEM. Experiment was repeated in 3 individual preps in triplicates (N=3). Significance was tested by two-way ANOVA followed by post-hoc LSD tests; tables next to the graphs showing the result of two-way ANOVA; no significant difference between genotypes within a time point was detected; significant differences between time points were detected but omitted for simplicity.
Figure 7.5 αSMA staining in wild type and 11β-HSD1 KO HSCs during spontaneous activation

Images (×200 magnification) of primary mouse wild type (WT) and 11β-HSD1 KO HSCs in culture stained with αSMA at 2 days, 5 days, 8 days, 11 days and 13 days post harvest. Arrows point to brown stained cells indicating more αSMA staining at 8 days in culture.
7.4.3. 11β-HSD1 amplifies GC effects on HSC activation

The previous experiments indicated that HSCs were activated at day 8 in culture, but this activation had not reached a plateau, so this time point was selected for pharmacological treatment of HSCs. After 8 days in culture, Acta2 (αSMA), Colla1, Mmp9 and Mmp13 mRNAs showed a trend of reduction by 11-dehydrocorticosterone (A) and corticosterone (B). In the absence of steroid, 11β-HSD1 inhibitor UE2316 showed a trend of decreasing Acta2 (αSMA) mRNA level without affecting other gene transcript levels. UE2316 prevented the down-regulating effect of A on Acta2 (αSMA), Colla1, Mmp9 and Mmp13 mRNAs to the level of vehicle. GR blocker RU486 also showed a trend of reducing Acta2 (αSMA) and Mmp9 transcripts in the absence of added steroid without affecting Colla1 and Timp1. RU486 did not block the effect of B for all the genes tested (Figure 7.6). αSMA protein level showed no effect of A or B (Figure 7.6 E).

Although RU486 failed to block corticosterone’s effect on HSCs, the GR downstream reporting gene Gilz can be induced by corticosterone and this induction was blocked by RU486 (Figure 7.8 A). GR mRNA levels were reduced by corticosterone and this reduction was also blocked by RU486 (Figure 7.7 B). Mr (MR) mRNA level was undetectable in these cells (not shown).
Figure 7.6 11-DHC’s effect on HSCs is 11β-HSD1 dependent

Figure A-D: mRNA level of Acta2 (αSMA) (A), Coll1a1 (B), MMP9 (C) and TIMP1 (D) normalized to 18S in primary wild type HSCs cultured for 8 days. Cells were treated with combination of vehicle (Veh), 11-DHC (A, 500nM), Corticosterone (B, 500nM), UE2316 (U, 10µM) and RU486 (R, 10µM) as stated. Results are presented as Mean ± SEM. Experiment was repeated 3 times in triplicates (N=3). Figure E: Representative immune-blots and quantification graph showing αSMA protein level corrected for GAPDH. Results were represented as Mean ± SEM. N=3 from an individual group.
Figure 7.7 GR expression in primary mouse HSCs

mRNA level of Gilz (A) and GR (B) corrected for 18S in primary mouse HSCs. Cells were treated with vehicle (Veh) or corticosterone (500nM) with or without RU486 (10µM) for 8 days. Results are represented as Mean ± SEM; experiment was repeated twice in triplicates. Significance was tested by one-way ANOVA followed by post-hoc Tukey test; * p<0.05 and *** p<0.001 versus Veh group; +++ p<0.001 versus corticosterone treated group.
7.5. Discussion

In this chapter I showed that GCs’ inhibition effect on HSCs is amplified by 11β-HSD1 since 11-dehydrocorticosterone is converted to corticosterone in these cells, and UE2316 blocked 11-dehydrocorticosterone’s effect on HSCs. This appears physiologically important since 11β-HSD1 KO HSCs tended to activate more in vitro. This is the first study to identify a role for 11β-HSD1 in HSCs.

In this chapter, 11β-HSD1 expression was detected in HSCs. Although there is no previous evidence of 11β-HSD1 expression in HSCs, 11β-HSD1 expression has been tested in other myofibroblast precursors. 11β-HSD1 is expressed in dermal mesenchymal cells (fibroblasts) although there is no evidence how 11β-HSD1 respond to TGF-β mediated cell differentiation (Tiganescu et al., 2011). Lung fibroblasts activated by TGF-β had significantly lower 11β-HSD1 level (Yang et al, data unpublished).

The more HSCs were activated, the less 11β-HSD1 level was. This is consistent with my previous data that activated HSCs showed weak 11β-HSD1 staining in vivo (Chapter 3). However, this is different from many cell types including stromal cells and inflammatory cells. 11β-HSD1 level is greatly increased when these cells are activated by cytokines (Ahasan et al., 2012). Similarly, in macrophage/monocyte cell line THP-1 and J774.1, LPS greatly induces the cell activation and 11β-HSD1 level (Ishii et al., 2007). The activation of 11β-HSD1 in these cells could be a compensating mechanism restraining further inflammation. Contrary to this mechanism, the loss of 11β-HSD1 in activated HSCs could possibly contribute to HSC self-activating.

In this study, HSC activation down regulated 11β-HSD1 level while ligands such as GCs enhanced 11β-HSD1 level and restrained HSC activation. This fits the hypothesis that HSC activation is characterised by the loss of adipogenic transcriptional regulation, which is required for the maintaining of HSC quiescence (Cheng et al., 2008). 11β-HSD1 plays an important part in regulating fat metabolism and can be regulated by metabolic transcriptional factors such as PPARs and Liver X receptor-α (LXRα) (Nakano et al., 2007, Stulnig et al., 2002). It is suggested that
activation of HSCs significantly decreases lipid-metabolic related genes including PPARs, C/EBPs, LXRα and SREBP-1c whilst treating HSCs with adipocyte differentiation mixture (isobutylmethylxanthine, dexamethasone, and insulin) or increasing ectopic PPAR and SREBP-1c expression restores the quiescent phenotype of activated HSCs (She et al., 2005, Hazra et al., 2004). The regulation of 11β-HSD1 in HSCs adds to this hypothesis and suggests a new target to inhibit HSC activation and restore HSC quiescence.

In this study I found that 11β-HSD1 can be induced by GCs in both primary mouse HSCs and LX-2 cells. This can be observed in many cell types including adipose stromal cells (Bujalska et al., 1999), osteoblasts (Cooper et al., 2002), adipocytes (Engeli et al., 2004) and human chorionic trophoblast (Sun et al., 2002). Similar results can be found in other myofibroblast precursors as well. In cultured human amnion fibroblasts, dexamethasone treatment induced up-regulation of 11β-HSD1 (Sun and Myatt, 2003). In human dermal fibroblasts, GCs induced 11β-HSD1 expression in a dose dependent manner (Tiganescu et al., 2011).

In this study, GCs inhibited spontaneous activation of HSCs since pro-fibrotic gene transcripts were down regulated. This is consistent with previous findings that GCs inhibit TGF-β induced activation HSCs (Bolkenius et al., 2004). GCs are proven to be a strong regulator to inhibit myofibroblast activation. GCs significantly inhibited the differentiation from fibroblasts to myofibroblasts in the lung (Sabatini et al., 2012). However, GCs could only inhibit ECM deposition in lung fibroblasts activated by TGF-β in combination with long-acting beta (2)-agonists but failed to work on their own (Goulet et al., 2007).

11β-HSD1 KO HSCs tend to be more prone to ‘spontaneous’ activation. Although ‘spontaneous’ activation is not a perfect model to mimic in vivo pathogenesis, TGFβ related pathway, which is very common in fibrogenesis in vivo, is involved in this in vitro model. Normal serum contains TGFβ and also the substrate of 11β-HSD1 (11-DHC) although both in a very low amount. In this case, a trend of augmented HSC activation in 11β-HSD1 KO HSCs is consistent with our previous in vivo data that HSC activation was found in 11β-HSD1 KO/inhibited mice in many NAFLD models (Chapter 4 and 6). The fact that Acta2 (αSMA) was modestly up-regulated at day 8 in
culture and Col1a1 was up-regulated modestly at day 10 in culture also confirms that 11β-HSD1 KO mice had an early HSC activation compared with wild type mice and enhanced αSMA deposition and COL-1 deposition did not occur at the same time point in 11β-HSD1 inhibited/deficient mice (Chapter 5 and 6). However, this experiment has a limitation of small N numbers which can possibly result in non-significant results. More N numbers are necessary in further studies.

It was not new that the activation of HSCs can be inhibited by GCs (Bolkenius et al., 2004). However, it is the first time that it has been shown that this effect is amplified by 11β-HSD1. UE2316 blocked the effect of 11-DHC. This is consistent with a study on normal human epidermal keratinocytes (NHEKs) (Terao et al., 2011). Although RU486 did not completely block the effect of corticosterone it partially impaired the effect of corticosterone suggesting the GCs’ effect is at least in part GR dependent.

GR was detected in HSCs in a previous study (Bolkenius et al., 2004, Raddatz et al., 1996). Dexamethasone treatment induces nuclei/cytoplasm GR fluorescence ratio indicating HSCs respond to glucocorticoid through GR (Raddatz et al., 1996). Also, GR not only exits in HSC but also functions as it does in other cells: binding Hsp90 and NF-kB to regulate gene expression (Myung et al., 2009). However, the possibility that GCs affect HSCs through MR could not be excluded. Although we did not test any signal of MR mRNA expression, MR was shown present in HSCs since the pro-fibrotic effect of aldosterone can be blocked by spironolactone (Ji et al., 2011b). This could be further investigated by dosing manipulation of RU486 and the use of spironolactone.

11β-HSD1 inhibitor UE2316 (10µM) and RU486 (10µM) induced decrease of αSMA in the absence of added steroids. 11β-HSD1 KO HSCs were cultured in medium with normal serum, providing a source of substrate for the enzyme to amplify GC action, whereas for the drug treatment experiment, UE2316 and RU486 were added to a medium with stripped serum which lacks GCs, so their effects are independent of steroid. This could be due to high dosing of inhibitors with direct effects on cell proliferation as observed in other studies (Terao et al., 2011). Also, this off-target effect can be mediated by other substrates of 11β-HSD1. 11β-HSD1 can also metabolize 7β-hydroxy- and 7-keto-cholesterol in vitro (Mitic et al., 2013).
Inhibition of 11βHSD1 leads to the dysregulation of these sterols can possibly contribute to the attenuated activation of HSCs.

Pro-fibrotic genes regulate HSC activation and function. *Acta2* (αSMA) and *Col1a1* are very important genes that were up-regulated during HSC activation (Iredale et al., 2012). In mouse HSCs, we found they were increased by 11β-HSD1 KO and inhibited by 11-DHC which can be blocked by an 11β-HSD1 inhibition dependent way. MMP-2, MMP-9 and MMP-13 are important MMPs up-regulated after HSC activation although MMP-13 only increase during the onset of activation (Milani et al., 1994, Han et al., 2007); TIMP-1 is significantly increased during HSC activation, and the presence of TIMP1 inhibits the apoptosis of HSCs (Iredale et al., 1996, Murphy et al., 2002). *Mmp9* transcripts were affected by both 11β-HSD1 KO and 11β-HSD1 inhibition, whereas *Timp1* mRNA level only tended to elevating at 8 days in culture during 11β-HSD1 deficiency but did not show any changes during 11β-HSD1 inhibition (data unshown). It is plausible that MMP-9 is a GC responding gene since the expression of MMP-9 can be trans-regulated by NF-KB and AP-1 in HSCs indicating a possible transcriptional regulation by GC through GR dependent pathway on MMP-9 (Takahra et al., 2004). A similar pattern of MMPs and Timp-1 was found in TGF-β activated lung-fibroblasts: budesonide significantly affected MMP-9, but had no effect on TIMP-1 and MMP-2 (Todorova et al., 2009).

Although GCs inhibited αSMA at transcriptional level, αSMA protein was unaffected by GCs. I have just investigated the 8 day time point of ‘spontaneous’ activation which may not be the time point for robust protein level changes. To find out GCs’ effect on the protein expression pattern of HSCs, a time course should be analysed. However, 11β-HSD1 inhibition showed a trend of enhanced αSMA expression suggesting the lack of 11β-HSD1 stimulates HSC activation.

In conclusion, 11β-HSD1 expression is reduced when HSCs are activated. Lack of 11β-HSD1 tend to increase ‘spontaneous’ HSC activation, potentially explaining the profibrotic phenotype of 11β-HSD1 KO mice.
Chapter 8. General Discussion and Future Directions
8.1. Effect of 11β-HSD1 on fibrotic and inflammatory response in the liver

In this thesis, I investigated a series of NAFLD models to study the effect of 11β-HSD1 in liver fibrosis and inflammation. Table 8.1 summarizes the major effects that 11β-HSD1 deficiency/inhibition had on NAFLD models.

Table 8.1 Effects of 11β-HSD1 deficiency/inhibition in NAFLD models

<table>
<thead>
<tr>
<th>Model</th>
<th>collagen deposition</th>
<th>HSC activation</th>
<th>Pro-fibrotic inflammatory response</th>
<th>Anti-fibrotic inflammatory response</th>
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<td>Ob/ob</td>
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<tr>
<td>MCDD</td>
<td>↔</td>
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<td>↓</td>
<td>↔</td>
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<tr>
<td>CCL4 induced peak injury</td>
<td>↑</td>
<td>↑/↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>Resolution of CCL4 induced fibrosis</td>
<td>↑/↔</td>
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<td>↓/↑</td>
<td>↓/↔</td>
</tr>
</tbody>
</table>

The role of 11β-HSD1 deficiency/inhibition in NAFLD is model dependent. 11β-HSD1 did not show measurable changes of fibrosis/collagen deposition in steatosis and NASH models which only had limited hepatic fibrosis. The pro-fibrotic effect of 11β-HSD1 deficiency was only confirmed in CCL4 induced injury which had a pronounced fibrosis in the liver. This corroborates 11β-HSD1 deficiency’s effect on
stimulating collagen deposition during tissue repair and fibrosis in other organs including skin wound healing, aortic atherosclerosis and myocardial infarction (Kipari et al., 2013, McSweeney et al., 2010, Terao et al., 2011). Collagen synthesis is required during the start of tissue repair but deregulation of collagen synthesis promotes fibrosis (Klingberg et al., 2013). It is important to distinguish between the beneficial effect of 11β-HSD1 deficiency during the onset of wound-healing and its harmful effect on un-controlled ECM deposition in future studies.

Overall, 11β-HSD1 deficiency promoted HSC activation. 11β-HSD1 deficiency/inhibition promoted HSC activation in most of NAFLD models tested. Intriguingly, a reduction of HSC activation was observed in the MCDD model in 11β-HSD1 KO mice suggesting 11β-HSD1’s effect is model dependent. The in vitro studies also confirmed 11β-HSD1 amplified the inhibitory effect of GCs. GCs’ inhibitory effect is also found in many myofibroblast precursors including fibroblasts in the lung, skin and cornea (Carroll et al., 2002, Bourcier et al., 1999, Goulet et al., 2007). This work highlights a potential generalised role of 11β-HSD1 in regulating myofibroblast behaviour.

Hepatic pro-inflammatory mediators showed discrepancies between NAFLD models suggesting a model dependent impact of 11β-HSD1 deficiency. This is not surprising because 11β-HSD1 have different roles in regulating neutrophil numbers, total macrophages and pro-inflammatory mediators in different systems (Gilmour et al., 2006, Coutinho et al., 2012, Wamil et al., 2011).

Investigating anti-fibrotic macrophage subsets helped to dissect the role of 11β-HSD1 during fibrogenesis and fibrosis resolution. A reduction in anti-fibrotic macrophage subsets led to unresolved fibrosis during 11β-HSD1 inhibition and CCL4 induced liver fibrosis recovery. These data are in accordance with previous studies that 11β-HSD1 is required for the macrophage’s phagocytosis of apoptotic cells (Gilmour et al., 2006), a process that is necessary for the resolution of liver fibrosis (Popov, 2010). This effect provided a new angle to study 11β-HSD1’s role in fibrosis/inflammation resolution.
8.2. 11β-HSD1 deficiency: ‘hepatic protective’ or ‘hepatic adverse’?

As a very important complication of metabolic syndrome, the prevalence of NAFLD parallels the occurrence of type 2 diabetes and cardiovascular disease (Mavrogiannaki and Migdalis, 2013). NAFLD increases the incidence of cardiovascular disease possibly through enhanced inflammatory, increased fibrinogen and plasminogen activator inhibitor-1 (PAI-1), increased serum triglycerides and impaired HDL (Targher et al., 2010). 11β-HSD1 KO mice have reduced serum triglycerides, diminished VLDL, raised ‘beneficial’ HDL and improved hepatic insulin sensitivity all compatible with a ‘cardio protective’ phenotype (Morton et al., 2001). Also, the changing of insulin sensitivity in the liver protects the liver from fat accumulation suggesting 11β-HSD1 deficiency may be ‘hepatic protective’ in steatosis.

11β-HSD1 deficiency can potentially be ‘hepatic protective’ during fibrosis development due to the metabolic profiles it improves. Many metabolic factors are involved in the development of liver fibrosis. Liver fibrosis is alleviated by PPARα ligands and adiponectin and enhanced by leptin and pro-inflammatory cytokines (Manautou et al., 1998, Ikejima et al., 2001, Saxena et al., 2002, Kamada et al., 2003, Pradere et al., 2013, Polyzos et al., 2010). 11β-HSD1 deficiency/inhibition results in enhanced hepatic PPARα and adipose tissue adiponectin transcripts as well as reduced serum leptin and pro-inflammatory cytokines in obesity models (Morton et al., 2001, Wamil et al., 2011). If obesity and fibrogenesis occur at the same time, 11β-HSD1 deficiency can be ‘hepatic protective’ in limiting fibrogenesis (Figure 8.1).

Studies in this thesis suggest, however, that 11β-HSD1 deficiency is ‘hepatic adverse’ because 11β-HSD1 KO/inhibition stimulated HSCs activation and ECM deposition in liver fibrosis (Figure 8.1). This HSC stimulating effect is not limited to chemical induced liver fibrosis, but also happens during steatosis modelled by high fat diet fed mice and ob/ob mice. This indicates the direct HSC activating effect overrides the beneficial effect of improved metabolic profile during 11β-HSD1 deficiency in
NAFLD. However, 11β-HSD1 KO showed reduced HSC activation in NASH (at least in the MCDD model). This could be partly due to the NASH model we used did not involve peripheral insulin resistance and obesity, which are main characters of human metabolic syndrome. Also, it is plausible that 11β-HSD1 influence steatosis-NASH-fibrosis development differently according to the stages of the disease.

In my study, in spite of the increased fibrosis during chemical liver injury, 11β-HSD1 deficiency protected hepatocytes from chronic toxin induced hepatocyte injury, as evidenced by lower serum transaminases. Also, pharmacological 11β-HSD1 deficiency reduced pro-inflammatory cells (neutrophils and macrophages). In this case, 11β-HSD1 deficiency is ‘hepatic-protective’. Nevertheless, the inhibitory effect on an anti-fibrotic macrophage subset reduced the protective effect of 11β-HSD1.
Figure 8.1 11β-HSD1 KO’s effect on NAFLD

Adipose tissue secretes adipokines including leptin, adiponectin and inflammatory cytokines. Leptin and adiponectin inhibit hepatic fat accumulation. Leptin is essential for HSC activation whilst adiponectin prevents HSC activation. Pro-inflammatory cytokines including IL-6, IL-1, MCP-1, TNFα and TGFβ stimulate liver inflammatory response. 11β-HSD1 deficiency can reduce the release of leptin and pro-inflammatory cytokines and increase the transcripts of adiponectin, possibly ameliorating NAFLD. Also, 11β-HSD1 KO reduces hepatic fat accumulation. In our study, 11β-HSD1 KO directly activates hepatic HSC activation leading to enhanced ECM deposition in the liver. 11β-HSD1 showed a possible inhibitory effect on hepatic inflammation. Green arrows showing promoting effect; red arrows with a stop line showing inhibiting effect; blue arrow showing promoting effect discovered by this thesis; blue arrow with red stop line showing inhibiting effect discovered by this thesis.
8.3. Clinical aspects

Since our study showed a pro-fibrotic role of 11β-HSD1 deficiency, glucocorticoids can possibly be a treatment for liver fibrosis. Glucocorticoid treatment alone or in combination with immune suppressor azathioprine prevents or reduces hepatic fibrosis in a large portion of autoimmune hepatitis patients (Czaja, 2009). This primarily involves the immune-suppressive effect of GCs. The usage of GCs in NAFLD is limited due to their immune-suppressive effect and metabolic effect. However, new types of GCs which have significant anti-inflammatory effect and reduced metabolic effect are in development. 5α-reduced corticosterone exhibits dissociated anti-inflammatory and metabolic effect showing similar anti-inflammatory effect without weight loss, hypertension or hyperinsulinemia compared with corticosterone (Yang et al., 2011). Modified GCs has been put in to clinical trials and may possibly be used in more liver fibrosis diseases.

The development of 11β-HSD1 inhibitors shows great potential in regulating metabolic syndrome, atherosclerosis and age-related cognitive impairment. This thesis demonstrated that despite reduced hepatocyte injury and inflammatory cells, 11β-HSD1 inhibitor worsened fibrosis in chemical injured mouse models. Although in mild liver injury steatosis induced by HFD and ob/ob mice, 11β-HSD1 inhibition only stimulated an increase of HSCs without promoting collagen deposition, the response could escalate when liver injury accumulates. Nowadays, all 11β-HSD1 inhibitors are developed for early stages of NAFLD (steatosis) but this thesis highlights a potentially important side effect in the unpredictable group in whom NAFLD may progress. It is also less feasible to monitor transcription level of pro-fibrotic genes in these studies. However, once 11β-HSD1 inhibitors have been used widely in clinical treatments, usage of these drugs on patients with severe liver disease such as viral hepatitis and alcoholic liver disease should be closely monitored or avoided.

8.4. Future Work

This work opened the field of the role of 11β-HSD1 in hepatic fibrogenesis. Following the study, there are some interesting areas to be investigated.
8.4.1. Role of 11β-HSD1 in myofibroblasts

Although our research strongly suggested that 11β-HSD1 deficiency in HSCs leads to the pro-fibrotic phenotype in global 11β-HSD1 KO mice, there is no direct evidence supporting that this in vivo effect is driven by 11β-HSD1 deficiency in HSCs. An HSC specific 11β-HSD1 deficient model should be investigated. At the time of submission of this thesis, a pericyte specific 11β-HSD1 knockout mouse is in development using CRE-Lox techniques (PDGFr-HSD1). Preliminary data on PDGFr-HSD1 mice suggested these mice have reduced 11β-HSD1 level in HSCs. However, due to the time limit, phenotype analysis of this model has not been completed. Also, since 11β-HSD1 may have the same effect in many myofibroblast precursors, we can use PDGFr-HSD1 mice to investigate how 11β-HSD1 influences kidney pericytes and lung fibroblasts in kidney fibrosis models and pulmonary fibrosis models. By this means, the ultimate role of 11β-HSD1 in tissue repair and fibrosis can be discovered.

8.4.2. Other GC regulators that might modulate myofibroblast functions

Although we have investigated 11β-HSD1’s effect in HSCs and liver fibrosis, other GC regulating molecules such as 5α- and 5β- reductases could be further investigated. Also, the role of MR and 11β-HSD2 in HSCs can also be further investigated. Although our study did not detect any signal of MR transcripts and there was a report suggesting that MR is expressed in these cells (Ji et al., 2011b) and is possibly involved in aldosterone induce HSC activation (Caligiuri et al., 2003). It would be worth investigating the role of MR and possibly 11β-HSD2 in HSCs to understand the mechanism of the inhibitory role of GCs and pro-fibrotic role of aldosterone.

8.4.3. Role of 11β-HSD1 and GCs in hepatocyte injury

The role of GCs in metabolic responses in hepatocytes has been extensively addressed previously. Here I showed data supportive of a protective role of 11β-HSD1 deficiency on hepatocyte injury. However, hepatocyte specific 11β-HSD1 deficiency failed to recapitulate this phenotype. Additionally, the same protective
The effect of global 11β-HSD1 deficiency was not observed in a NASH model MCDD indicating this protective effect depends on the type of injury. The effect of GCs on hepatocyte apoptosis is complex and depends on the doses of GCs and the nature of the injury (Kimura et al., 2011, Oh et al., 2006). It will be interesting to understand how 11β-HSD1 deficiency affects hepatocyte apoptosis, necrosis or proliferation.

8.4.4. 11β-HSD1 expression pattern in NAFLD and liver fibrosis in human

In this thesis I showed decreased 11β-HSD1 expression in NAFLD models and liver fibrosis models in mice. However, human studies provided inconsistent results with some showing increased (Ahmed et al., 2012) and others showing unaltered (Konopelska et al., 2009) 11β-HSD1 level during NASH and fibrosis. For 11β-HSD1 activity, blood and urine samples can be used to analyse the global 11β-HSD1 activity which mainly comes from hepatic 11β-HSD1 activity. However, analysing 11β-HSD1 protein and transcripts are necessary to further test the 11β-HSD1 level in the liver. In this case, new techniques including small molecule visualising system and stable isotope tracers with arteriovenous sampling might be used for these studies. Well designed and statistically efficient studies are needed to measure 11β-HSD1 activity in NASH and fibrosis.

8.5. Conclusions

In this study, I demonstrated that genetic deficiency and pharmacological inhibition of 11β-HSD1 increased liver susceptibility to chemical (CCL4) induced liver fibrosis in mice in both peak fibrosis time point and resolving phases. This effect was not due to increased hepatocyte injury and exaggerated hepatic inflammation but possibly due to a direct activation of HSCs. Hepatocyte specific 11β-HSD1 deficiency failed to recapitulate the global KO phenotype indicating it is the HSCs that are affected by 11β-HSD1 deficiency. This was further confirmed by the fact that the inhibitory effect of GCs is amplified by 11β-HSD1 in vitro. This thesis advances the field of a novel role of 11β-HSD1 in pro-fibrogenic process and suggests that the use of 11β-HSD1 inhibitor should be carefully considered in patients with fibrotic liver disease.
## Appendix: Supplier addresses

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<td>Bio-Rad House, Maylands Avenue, Hemel Hempstead, Herts., HP2 7TD, U.K.</td>
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<tr>
<td>Chemometec</td>
<td>Gydevang 43, DK-3450 Allerod, Denmark</td>
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<td>Dyets, Inc.</td>
<td>2508 Easton Avenue, P. O. Box 3485, Bethlehem, PA 18017, US</td>
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<td>Oxoid Ltd.</td>
<td>Wade Road, Basingstoke, Hampshire, RG 24 8PW, UK</td>
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<td>Promega Ltd.</td>
<td>Delta house, Chilworth Research Centre, Southampton, SO1 7NS, U.K.</td>
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<td>Skelton House Lloyd Street, North Manchester M15 6SH, U.K.</td>
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<td>Roche Diagnostics Ltd.</td>
<td>Bell Lane, Lewes, East Sussex, BN7 1LG, U.K.</td>
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<td>Fancy Road, Poole, Dorset, BH12 4OH, U.K.</td>
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<td>Special Diet Services Limited</td>
<td>Unit 1 Stepfield, Industrial Estate East, Witham Essex CM8 3TH, U.K.</td>
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<td>Thermo</td>
<td>93-96 Chadwick Road, Astmoor, Runcorn, Cheshire, WA7 1PR, UK</td>
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<td>Vector Laboratories Ltd.</td>
<td>3 Accent Park, Bakewell Road, Orton Southgate, Peterborough, PE2 6XS, UK</td>
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<td>Carl Zeiss Ltd.</td>
<td>511 Coldhams Ln, Cambridge, Cambridgeshire CB1 3JS</td>
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