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Negative regulation of the hepatic fibrogenic response by Suppressor of Cytokine Signaling 1 (SOCS1)

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RÉSUMÉ

Régulation négative de la réponse fibrogénique hépatique par le suppresseur de la signalisation de cytokine 1 (SOCS1)

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Le suppresseur de la signalisation des cytokines 1 (SOCS1) est un régulateur indispensable de la signalisation de l'IFN-γ et a été aussi impliqué dans la régulation de la fibrose hépatique. Cependant, on ne sait pas si les fonctions anti-fibrotiques sont médiées directement dans le foie par SOCS1 ou par la modulation de l'IFN-γ, qui est connu pour son effet atténuateur de la fibrose hépatique. En outre, il est possible que SOCS1 contrôle la fibrose hépatique par la régulation des cellules stellaires hépatiques (CSH), un acteur clé dans la réponse fibrogénique. Alors que les voies d'activation des CSH ont été bien caractérisées, les mécanismes de régulation ne sont pas encore clairs. Les buts de cette étude étaient de dissocier la régulation de la réponse fibrogénique hépatique médiée par SOCS1 et celle dépendante de IFN-γ et d'élucider les fonctions régulatrices de SOCS1 dans l'activation des CSH. La fibrose hépatique a été induite chez des souris $Socs1^{-/-}Ifng^{-/-}$ par la diméthylnitrosamine ou le tétrachlorure de carbone.Les souris $Ifng^{-/-}$ et C57BL6 ont servi comme contrôles. Après les traitements fibrogéniques, les souris $Socs1^{-/-}Ifng^{-/-}$ ont montré des niveaux sériques élevés d' alanine aminotransférase (ALT) ainsi que l'augmentation de la fibrose du foie par rapport à des souris $Ifng^{-/-}$. Le dernier groupe a montré des niveaux plus élevés d'ALT et de fibrose par rapport aux souris C57BL6 contrôles. Les foies des souris déficientes en $Socs1$ ont montré une fibrose septale, qui a été associée à une augmentation de l'accumulation des myofibroblastes et à un dépôt abondant du collagène. Les foies déficients en SOCS1 ont montré une expression accrue de gènes codant pour l'actine musculaire lisse, le collagène et les enzymes impliquées dans le remodelage de la matrice extracellulaire, à savoir les métalloprotéinases de la matrice et l'inhibiteur tissulaire des métalloprotéinases. Les CSH primaires de souris déficientes en $Socs1$ ont montré une prolifération accrue en réponse à des facteurs de croissance tels que le HGF, EGF et le PDGF. Aussi, les foies fibrotiques de souris déficientes en $Socs1$ ont montré une expression élevée du gène PDGFB. Pris ensemble, ces données indiquent que SOCS1 contrôle la fibrose hépatique indépendamment de l'IFN-γ et qu'une partie de cette régulation peut se produire en régulant la prolifération des HSC et en limitant la disponibilité des facteurs de croissance.

Mots clés : SOCS1, Diméthylnitrosamine, Tétrachlorure de carbone, Cellules stellaires hépatiques, PDGF.

SUMMARY

Negative regulation of the hepatic fibrogenic response by suppressor of cytokine signaling

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Suppressor of cytokine signaling 1 (SOCS1) is an indispensable regulator of IFN-γ signaling and has been implicated in the regulation of liver fibrosis. However, it is not known whether SOCS1 mediates its anti-fibrotic functions in the liver directly, or via modulating IFN-γ, which has been implicated in attenuating hepatic fibrosis. Additionally, it is possible that SOCS1 controls liver fibrosis by regulating hepatic stellate cells (HSC), a key player in fibrogenic response. While the activation pathways of HSCs have been well characterized, the regulatory mechanisms are not yet clear. The goals of this study were to dissociate IFN-γ-dependent and SOCS1-mediated regulation of hepatic fibrogenic response, and to elucidate the regulatory functions of SOCS1 in HSC activation. Liver fibrosis was induced in $Socs1^{-/-}Ifng^{-/-}$ mice with dimethylnitrosamine or carbon tetrachloride. $Ifng^{-/-}$ and C57BL/6 mice served as controls. Following fibrogenic treatments, $Socs1^{-/-}Ifng^{-/-}$ mice showed elevated serum ALT levels and increased liver fibrosis compared to mice $Ifng^{-/-}$. The latter group showed higher alanine aminotransferase (ALT) levels and fibrosis than C57BL/6 controls. The livers of $Socs1$-deficient mice showed bridging fibrosis, which was associated with increased accumulation of myofibroblasts and abundant collagen deposition. $Socs1$-deficient livers showed increased expression of genes coding for smooth muscle actin, collagen, and enzymes involved in remodeling the extracellular matrix, namely matrix metalloproteinases and tissue inhibitor of metalloproteinases. Primary HSCs from $Socs1$-deficient mice showed increased proliferation in response to growth factors such as HGF, EGF and PDGF, and the fibrotic livers of $Socs1$-deficient mice showed increased expression of the $Pdgfb$ gene. Taken together, these data indicate that SOCS1 controls liver fibrosis independently of IFN-γ and that part of this regulation may occur via regulating HSC proliferation and limiting growth factor availability.

Keywords : SOCS1, Dimethylnitrosamine, Carbon tetrachloride, Hepatic stellate cells PDGF.

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LIST OF ABREVIATIONS

ALD	Alcoholic Liver Disease
ALT	Alanine AminoTransferase
APC	Antigen-Presenting Cells
CO	Carbon monoxide
CCl_4	Carbon tetrachloride
CLD	Chronic Liver Disease
CML	Chronic Myeloid Leukemia
CTGF	Connective Tissue Growth Factor
cAMP	Cyclic adenosine monophosphate
CDKN1A	Cyclin-Dependent Kinase Inhibitor 1A
CISH	Cytokine-inducible SH2-containing protein
DEN	Diethylnitrosamine
DC	Dendritic Cells
DMN	Dimethylnitrosamine
ECM	Extracellular Matrix
ER	Endoplasmic Reticulum
EGF	Epidermal Growth Factor
EMT	Epithelial Mesenchymal Transition
EPO	Erythropoietin
ET-1	Endothelin-1
FN	Fibronectin
GFAP	Glial Fibrillary Acidic Protein
GAG	Glycosaminoglycans
G-CSF	Granulocyte Colony- Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony-Simulating Factor
GH	Growth Hormone
HCC	Hepatocellular Carcinoma

HSC	Hepatic stellate cells
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HCV	Hepatitis C Virus
IFNα	Interferon Alpha
IFN-γ	Interferon Gamma
IRS1	Insulin Receptor Substrate
ILK	Integrin-Linked Kinase
IRF1	Interferon Response Factor 1
IL2	Interleukin 2
JAK	Janus Kinases
KIR	Kinase- Inhibitory Region
KCs	Kupffer cells
LF	Liver fibrosis
LSEC	Liver sinusoidal endothelial cells
LRAT	Lecithin-Retinol Acyltransferase
LOX	Lysyl oxidase
MMP	Matrix Metalloproteases
miRNA	MicroRibonucleicacid
MCP-1	Monocyte Chemotactic Protein
NOX	NADPH oxidase
NK	Natural Killer
NKT	Natural Killer T cells
NO	Nitric Oxide
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-alcoholic Steatohepatitis
NF-kβ	Nuclear factor kappa beta
RAR	Retinoic Acid Receptor
PDGF	Platelet-Derived Growth Factor
PRL	prolactin
PGs	Proteoglycans

RAG2	Recombinant Activating Gene2
ROS	Reactive Oxygen Species
RTKs	Receptor Tyrosine Kinases
RA	Retinoic Acid
RXR	Retinoid X Receptor
RBP	Retinol-Binding Protein
SNPs	Single Nucleotide Polymorphisms
STAT	Signal Transducer and Activator of Transcription
SOCS	Suppressor of Cytokine Signaling
TPO	Thrombopoietin
TIMP-1	Tissue Inhibitor of Matrix Metalloprotease-1
TLR-4	Toll-Like Receptor 4
TGF- β1	Transforming Growth Factor beta-1
TRAIL	Tumor Necrosis factor-related Apoptosis-inducing Ligand
WBC	White Blood Cell

1. INTRODUCTION

1. 1 The Liver

The liver is one of the major organs in our body. It is involved in diverse functions such as metabolism, synthesis of plasma proteins and storage of vitamins, carbohydrates and fats. The Liver as the main detoxifying organ, liver neutralizes toxic metabolites and removes toxins that enter portal vein circulation (Taub 2004; Fausto, Campbell, and Riehle 2006). The liver has a remarkable capacity to regenerate following physical, biological and toxic injuries (Miyajima, Tanaka, and Itoh 2014).

1.1.1 The Liver cells and functions

The liver is composed of parenchymal cells and nonparenchymal cells. The parenchymal cells are hepatocytes and cholangiocytes. Hepatocytes constitute about 94% of the total liver mass and carry out most functions in the liver (Papoulas and Theocharis 2009; Blomhoff R 1990). Cholangiocytes are epithelial cells that are located in intrahepatic and extra-hepatic biliary ducts. These are responsible for modification of the bile produced by hepatocytes (O'Hara et al. 2013; Alpini, McGill, and Larusso 2002).

Hepatic non-parenchymal cells include the sinusoidal endothelial cells, Kupffer cells (KCs), lymphocytes, hepatic stellate cells (HSCs) and biliary epithelial cells (Cholangiocytes)(Lemoinne et al. 2013). Kupffer cells are the liver resident macrophages that reside in sinusoids and play important roles in hepatic homeostasis, phagocytosis and host defense (M Naito, Hasegawa, and Takahashi 1997; Makoto Naito et al. 2004). These cells are derived from circulating monocytes and comprise approximately 20% of hepatic non-parenchymal cells (Ruck and Xiao 2002). Hepatic stellate cells (HSC) store retinoids and serve as precursors of liver myofibroblasts (Friedman 2008). Myofibroblasts are the main fibrogenic effector cells and are absent in the healthy liver. However, following the liver damage, HSCs differentiate and start producing extracellular matrix and collagen (Lemoinne et al. 2013). The liver sinusoidal endothelial cells (LSEC) line the capillaries and sinusoids and differ from the endothelium of big vessels in lacking a basement membrane and containing the fenestrae structures. (Bouwens et al. 1992; DeLeve 2013).

The liver contains large number of lymphocytes which include natural killer (NK), natural killer T cells (NKT), B cells, CD4 and CD8 T cells present around the portal tracts and throughout the hepatic parenchyma. NK and NKT cells provide first line immune defense against invading pathogens and also involved in recruitment of circulating lymphocytes (Racanelli and Rehermann 2006).

1.1.2 Liver lobule structure

The liver parenchyma is structurally divided into multiple functional units called lobules, which are hexagonal in shape and composed of a labyrinth of interconnected hepatocyte plates separated by sinusoidal blood vessels. Each lobule comprises of a central vein from where hepatocyte plates radiate out towards the perimeter of the lobule. Each lobule is surrounded and separated by connective tissue. **(Fig. 1a and b)** (Wallace, Burt, and Wright 2008).

The liver receives both oxygenated and non-oxygenated blood supply. The portal vein brings nutrient rich blood from the gut, which is partially oxygenated. About 30% of the hepatic blood comes via the hepatic artery. The two types of blood mix up at the edge of the portal tracts and circulate towards central vein through sinusoids. The central veins join together to form the hepatic vein, which drain into the inferior vena cava. The portal vein, hepatic artery and bile duct together are found at the angle of each lobule forming the portal triad (Wallace, Burt, and Wright 2008; Miyajima, Tanaka, and Itoh 2014).

B

Portal Triad
- Bile Duct
- Hepatic Artery
- Portal Vein

Bile canaliculus

Space of Disse

Central vein

Sinusoids

Blood flow
Bile flow

- Hepatocytes
- Canal of Hering
- Cholangiocyte
- Hepatic sinusoidal endothelial cell (HSEC)
- Kupffer cell
- Hepatic stellate cell (HSC)
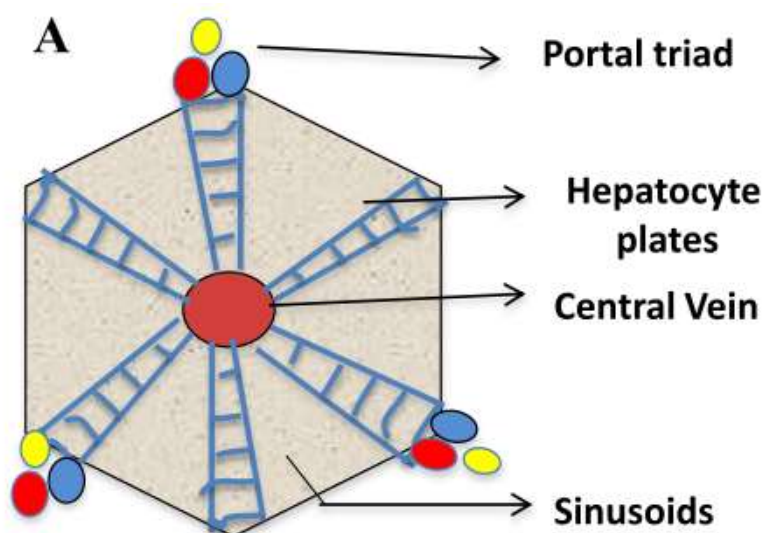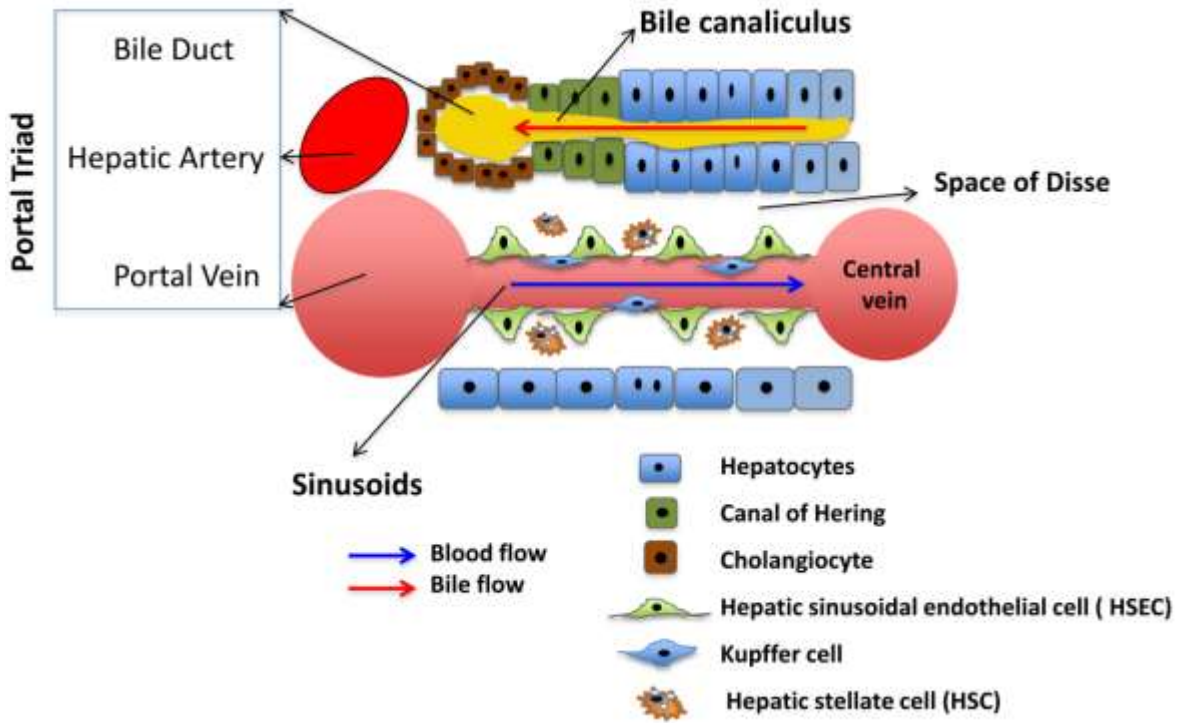
1.2 Chronic liver diseases (CLD) and Liver fibrosis

1.2.1 Epidemiology

The most common chronic liver diseases (CLD) that are accountable for the morbidity and mortality in humans are viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD), cirrhosis and hepatocellular carcinoma. These conditions are responsible for more than 95% of all deaths due to liver disease. Globally, more than 400 million individuals are chronically infected with hepatitis B virus (HBV), and above 170 millions of people are chronically infected with hepatitis C virus (HCV). About 9 to 36.9% of the population is affected with NAFLD, which is more prevalent in individuals with obesity and diabetes mellitus. Alcoholic liver disease (ALD) is prevalent in people who consume alcohol regularly (K. Zhou and Lu 2009). Other causes of CLD include overload of iron or copper absorption and autoimmune destruction of hepatocytes or bile duct epithelium (Guyot et al. 2006; Y. Liu et al. 2013).

According to Canadian Liver Foundation reports, the incidence of CLD in Canada is increasing alarmingly. It is estimated that one in ten Canadians, or approximately more than three million people, have some form of liver disease. Around half a million Canadians are chronically infected with Hepatitis B or C virus. NAFLD is also a common liver disease that afflicts as much as 25% of the Canadian population. Liver cirrhosis is recorded in about 5,000 deaths per year. Over 2,000 Canadians are expected to develop primary liver cancer and about 1,000 will die of this disease. It is noted that advanced forms of fibrosis and cirrhosis develop in about 20–40% of patients affected with CLD (Canadian Liver Foundation 2013). In general, progression of CLD takes several years or even decades to cause overt illness. For instance, in the case of hepatitis C infection, it takes up to 10 -15 years from the time of infection to develop fibrosis and cirrhosis (Massimo Pinzani and Macias-barragan 2016).

1.2.2 Liver fibrosis (LF)

Most chronic liver diseases are associated with liver fibrosis independent of its etiology (Massimo Pinzani and Macias-barragan 2016). Fibrosis is a wound healing response to any kind of injury to the liver and is characterized by the accumulation of interstitial or ''scar''

extracellular matrix (ECM) (Guo and Friedman 2007).

Liver fibrosis is a reversible process. In normal conditions, there is a balance between liver repair and scar formation. During acute injury, changes that occur in the liver are transient and reversible. On the other hand, chronic liver injury is associated with recurring replacement of hepatic parenchyma with fibrous tissue, which leads to cirrhosis. Cirrhosis is the end stage of liver fibrosis characterized by the loss of the lobular architecture and the appearance of regenerative nodules of liver parenchyma surrounded by fibrotic septa (U. E. Lee, Mstp, and Friedman 2011). The resulting loss of hepatic function is the cause of significant morbidity and mortality associated with cirrhosis (Iwaisako, Taura, and Koyama 2014). In cirrhosis, loss of functional hepatocytes and increased intrahepatic resistance to blood flow leads to hepatic insufficiency and portal hypertension, respectively (Tsochatzis, Bosch, and Burroughs 2014; D'Amico, Garcia-Tsao, and Pagliaro 2006). To compensate for the metabolic needs required by the liver, hepatocytes continuously undergo recurrent proliferation in an inflammatory milieu that could lead to the development of dysplastic nodules and eventually to hepatocellular carcinoma (HCC) (Kensler et al. 2003; Farazi and DePinho 2006). HCC is among the fifth most common cancer in the world, and the third leading cause of cancer death (M Pinzani and Rombouts 2004). It has been demonstrated that liver fibrosis is strongly associated with HCC, with ninety percent of HCC cases occurring in cirrhotic livers (D. Y. Zhang and Friedman 2014).

The ultrastructural pattern of fibrosis varies with the underlying etiology. In HBV or HCV infections, liver fibrosis is characterized by the formation of *portal–central* septa. In alcoholic and non-alcoholic steatohepatitis, deposition of the fibrillar matrix is concentrated around the sinusoids and around groups of hepatocytes like a chicken-wire pattern (Massimo Pinzani and Macias-barragan 2016). In chronic bile obstruction, *porto-portal septa* is observed due to the activation of portal fibroblasts (Guyot et al. 2006).

The development of fibrosis, and particularly cirrhosis, is associated with significant morbidity and mortality. Since it is the final common pathological pathway of chronic liver disease, regardless of etiology, developing antifibrotic strategies that target this pathway

would be an attractive approach to curtail the end stage of developing hepatocellular carcinoma. However, because fibrotic liver disease may not present clinically until an advanced or cirrhotic stage, development of methods for early detection of fibrosis and its reversal are essential issue for successful intervention (Bonis, Friedman, and Kaplan 2001).

1.3 Extra cellular matrix (ECM) in normal liver

Increased deposition of ECM is the main characteristic of liver fibrosis (Friedman 2010a). The main function of ECM is not only to provide structural support to cells, but also to regulate many cellular processes such as growth, migration, differentiation, survival, homeostasis and morphogenesis (Theocharis et al. 2015). ECM also serves as a reservoir for growth factors that are essential for liver regeneration such as the hepatocyte growth factor and epidermal growth factor. (Rozario and DeSimone 2010). The compositions of ECM vary from tissue to tissue. However, the major constituents of ECMs are fibrous-forming proteins, such as collagens, elastin, fibronectin (FN), and non–fiber-forming (interfibrillar) proteins such as glycoproteins, and proteoglycans (PGs) (Frantz, Stewart, and Weaver 2010).

Collagen is the most abundant fibrous protein which not only provides tensile strength but also promotes cell adhesion, support, chemotaxis, migration and tissue repair (Rozario and DeSimone 2010). To date, 28 types of collagen have been identified in vertebrates. Based on their structure and supramolecular organization, they are divided into fibril-forming collagens (Type I, II, III, V, XI), fibril-associated collagens (FACIT) (Type IX, XII, XIV, XVI, XIX), network-forming collagens (types X and VIII), anchoring fibrils type (VII), transmembrane collagens (type XIII, XVII), basement membrane collagens (Type IV) and others with unique functions (Gelse 2003). The structural hallmark of all collagens is their triple helix. For example, collagen 1 contains three α chains (2 α1, 1 α2) and series of Gly-X-Y repeats (where X and Y represent any amino acids but are frequently proline and hydroxyproline, which facilitate fibril formation (Mouw, Ou, and Weaver 2014).

Structurally, collagens are present in the homopolymers or heteropolymers. Type I collagen is the major component of ECM and is the product of two genes, *COL1A1*, and *COL1A2*. Synthesis of collagen type 1 involves a number of post-translational modifications. Hydroxylation of proline and lysine residues and glycosylation of lysine occur in the endoplasmic reticulum (ER) to form pro-collagen (Gordon and Hahn 2010). Pro-collagens are then secreted through the Golgi apparatus into the extracellular space where the N-terminal and C-terminal pro-peptides are cleaved by metalloproteinase enzymes to form collagens (Mouw, Ou, and Weaver 2014). Lysyl oxidase (LOX) is an enzyme involved in cross-linking collagens and elastins (Rodríguez, Rodríguez-Sinovas, and Martínez-González 2008).

Elastin fibers are formed by cross-linking of tropo-elastin molecules and provide recoil to tissues that undergo repeated stretch (Wise and Weiss 2009). Fibronectin is one type of fibrillar collagen, which regulates cell migration and adhesion activities through direct interactions with cell-surface integrin receptors. (Mao and Schwarzbauer 2005; Theocharis et al. 2015). Proteoglycans (PG) are major components of ECM that help in buffering, hydration and binding and provide swelling pressure to the tissue to withstand compressional forces (Yanagishita 1993; Järveläinen et al. 2009). PGs contain a central core protein which binds covalently to several glycosaminoglycan (GAG) chains such as hyaluronan, through which they interact with numerous growth factors, cytokines, chemokines and cell surface receptors (Kresse and Schönherr 2001).

Laminins are the family of glycoproteins present predominantly in basement membranes. They form interactions with cell surface receptors and are involved in various functions such as cell adhesion, migration, and differentiation and in wound repair (Malinda and Kleinman 1996; Durbeej 2010).

1.4 Cellular Sources of ECM in Liver Fibrosis

The ECM deposition in fibrosis is caused by the activation of myofibroblasts.

Myofibroblasts are usually not present in normal liver, but they are produced by transdifferentiation of liver resident cells, such as hepatic stellate cells (HSC) and portal fibroblasts (Lotersztajn et al. 2005). Many studies suggested that HSC are the major source of myofibroblasts in LF (Iwaisako, Brenner, and Kisseleva 2012). Portal fibroblasts are the major source of myofibroblasts in portal fibrosis or in cholestatic liver injury (Guyot et al. 2006). These hepatic myofibroblasts may also originate from bone marrow-derived mesenchymal cells and fibrocytes, which are recruited to the liver and differentiate into myofibroblasts. However, these cells make only a small contribution to the myofibroblast population in experimental liver fibrosis (Kisseleva et al. 2006; Bellini and Mattoli 2007; J. Xu et al. 2015).

It has been proposed that hepatic progenitor cells like hepatocytes, cholangiocytes, and hepatic sinusoidal endothelial cells differentiate into myofibroblasts through epithelial or endothelial-mesenchymal transition (EMT) and contribute to the progression of LF (Zeisberg et al. 2007; Robertson et al. 2007). But, two lineage tracing analysis studies showed that myofibroblasts originated from hepatocytes and cholangiocytes via EMT do not express any of the mesenchymal markers including α-SMA, FSP-1, Desmin and Vimentin in different LF-induced models (Taura et al. 2010; Chu et al. 2011). One recent study used *Lrat* (lecithin-retinol acyltransferase) Cre-transgenic mouse, which marks 99% of hepatic stellate cells (HSCs) in the liver, reported that stellate cells give rise to 82–96% of myofibroblasts in models of toxic, cholestatic and fatty liver disease (Mederacke et al. 2013). However, the relative contribution of other cellular sources may depend on the underlying etiology and region(s) of injury within the liver. (Friedman 2010b; M Pinzani and Rombouts 2004; Iwaisako, Taura, and Koyama 2014; Friedman 2010a).

Although hepatic myofibroblasts are heterogeneous in their origin, they show some similar cellular characteristics like the expression of α-SMA, collagen-α1 (J. Xu et al. 2015). Specific markers for cellular sources of myofibroblasts in the fibrotic liver are showed in **Table 1**.

Table 1 Sources of liver myofibroblasts and their markers

Quiescent HSC or Activated HSC	Portal fibroblasts	Fibrocytes	Hepatocytes	Cholangiocytes
Desmin+ CD146+ CD105+ GFAP+ LRAT+ *P75+* IL17RA+ Synemin+ Synaptophysin+ CD45- CD34- *Thy1.1-* Elastin	*Thy1.1+* Elastin+ Synemin+ CD105+ CD45- CD34- CD146- Desmin- GFAP**-**	CD45+ CD34+ CD11b+ Gr1+ or Ly6G+ MHCII+ ICAM1+ CCR2+ CCR7+ CXCR4+ CD80+ CD86+	CD45- Alb+ FSP1+	CD45- CK19+ FSP1+

1.5 Hepatic Stellate Cells

Hepatic stellate cells were first described by von Kupffer in 1876 as liver sternzellen (star-shaped cells) (Hellerbrand 2013). Ito and Nemoto described them as fat-storing cells, Suzuki as "interstitial cells, Bronfenmajer, Schaffner, and Popper as "lipocytes and Wake as peri-sinusoidal cells". To avoid the confusion with these synonyms, a standardized name is given to these cells as "Hepatic Stellate Cells (HSC)", which refers to the resting (quiescent) form of this cell type found in normal liver (Senoo et al. 2010).

HSC constitute about 1% of the total liver mass and 7% of the total number of liver cells. They reside in the space of Disse (sub endothelial space) which is the gap between hepatocytes and sinusoidal endothelial cells (Blomhoff R 1990). In the normal liver, stellate cells possess spindle-shaped cell bodies with long cytoplasmic processes. These processes wrap sinusoids and regulate circulation through the sinusoidal capillaries (Reynaert 2002). They also provide contacts with hepatocytes and other stellate cells, which help in the intercellular transport of soluble mediators and cytokines. The thorny projections on the

processes are involved in "sensing" chemotactic signals (Friedman 2008).

1.5.1 Retinoid Storage

In normal livers, HSC store vitamin A (retinoid) in their cytoplasm, which is a prominent characteristic feature of stellate cells in a quiescent state. About 80% of the total retinoids present in the entire body is stored in HSC of the liver in the form of retinyl esters in their lipid droplets (Senoo 2004; Weiskirchen and Tacke 2014). The lipid droplets of the stellate cells show intense but rapidly fading blue-green vitamin A autofluorescence when excited with the wavelength light of ~328 nm (Hellerbrand 2013). The number of droplets varies with the species and the abundance of vitamin A stores of the organism (Friedman 2008). Dietary retinoid intake but not triglyceride intake affects the number and size of HSC lipid droplets (Blaner et al. 2009). Dietary retinoids converts to retinol before absorbption in the small intestine (enterocytes), where they convert into retinyl esters and packaged into chylomicrons for transportation to the lymphatic circulation. Then, they are taken up by hepatocytes, where retinoids are hydrolyzed to retinol and bind with retinol-binding protein (RBP) to transfer to the HSCs for storage (Iwaisako, Taura, and Koyama 2014; Senoo et al. 2010). HSCs possess high levels of LRAT (lecithin: retinol acyltransferase) activity that is responsible for the esterification of retinol to retinyl ester and lipid droplet formation. It has been shown that $Lrat^{-/-}$ mice lack lipid droplets and retinyl esters in these cells but did not develop fibrosis suggesting a physiological role of LRAT in HSC (Fomby and Cherlin 2011; O'Byrne et al. 2005). In response to liver injury, stellate cells become activated which is characterized by the loss of their lipid droplets (Friedman 2008).

Vitamin A-storing cells are also present in other tissues such as pancreas, lungs, kidney, spleen, adrenal glands, testis, uterus, lymph nodes, thymus, bone and intestines, where they store lipid droplets and synthesize and secrete ECM components in these tissues. These cells are considered as extrahepatic stellate cells (Geerts 2001; Senoo et al. 2010). Among them, pancreatic stellate cells are similar to HSC in structure, storage of vitamin-A and expression of cytoskeletal markers and signaling pathways (Apte et al. 1998; Kordes, Sawitza, and Häussinger 2009).

1.5.2 Origin and Markers for identification of HSC

Embryonic origin of HSC is not yet clearly known since they express marker genes of all three germ layers. These cells are thought to derive from endoderm due to the expression of CD34 and cytokeratin-7/8 in fetal liver. The expression of mesodermal (Wilm's tumor suppressor gene (*Wt1*) and mesoderm posterior 1 gene (*Mesp1*)) suggests they have a mesodermal origin. Hepatic epithelial cells are thought to transdifferentiate into hepatic stellate cells in the injured liver through epithelial-mesenchymal transition (EMT) but, the contribution of EMT to the hepatic stellate cell lineage is highly controversial (Yin et al. 2013; Kordes, Sawitza, and Häussinger 2009). Some studies have suggested that HSC could also be derived from neural crest due to expression of neural cell type markers such as glial fibrillary acidic protein (GFAP), nestin, expression of neuronal cell adhesion molecule (N-CAM), synaptophysin, neurotrophins and neurotrophin receptor (Senoo et al. 2010). One study using double transgenic mouse line constitutively expressing yellow fluorescent protein (YFP) in the neural crest and their derivatives, showed that HSC in adult livers of these mice do not express YFP suggesting HSCs do not originate from the neural crest (Cassiman et al. 2006).

Markers to identify HSC are shown in Table1. Desmin has been widely used as a "gold standard" for the identification of the stellate cells in rodent liver, but its expression is not reliable in humans (Friedman 2008). α-SMA is one of the six actin isoforms which is expressed only when HSC are activated (D C Rockey, Boyles, et al. 1992). Other methods employed to determine HSC staining of lipid droplets using oil red O, Sudan red, toluidine blue or basic fuchsine, but these are not specific to HSC as hepatocytes and Kupffer cells also contain fat droplets (Geerts 2001).

1.6 Activation of HSC in liver fibrosis

Activation of HSC is a pivotal event in liver fibrosis where quiescent HSC transform into proliferative, fibrogenic, and contractile ''myofibroblasts'' (Friedman 2010a). Also, when primary HSC are seeded onto plastic plates in culture, they undergo an activation process that mimics the fibrogenic response in the liver. Activation of HSC is comprised of two phases, the initiation phase and the perpetuation phase (Figure 2).
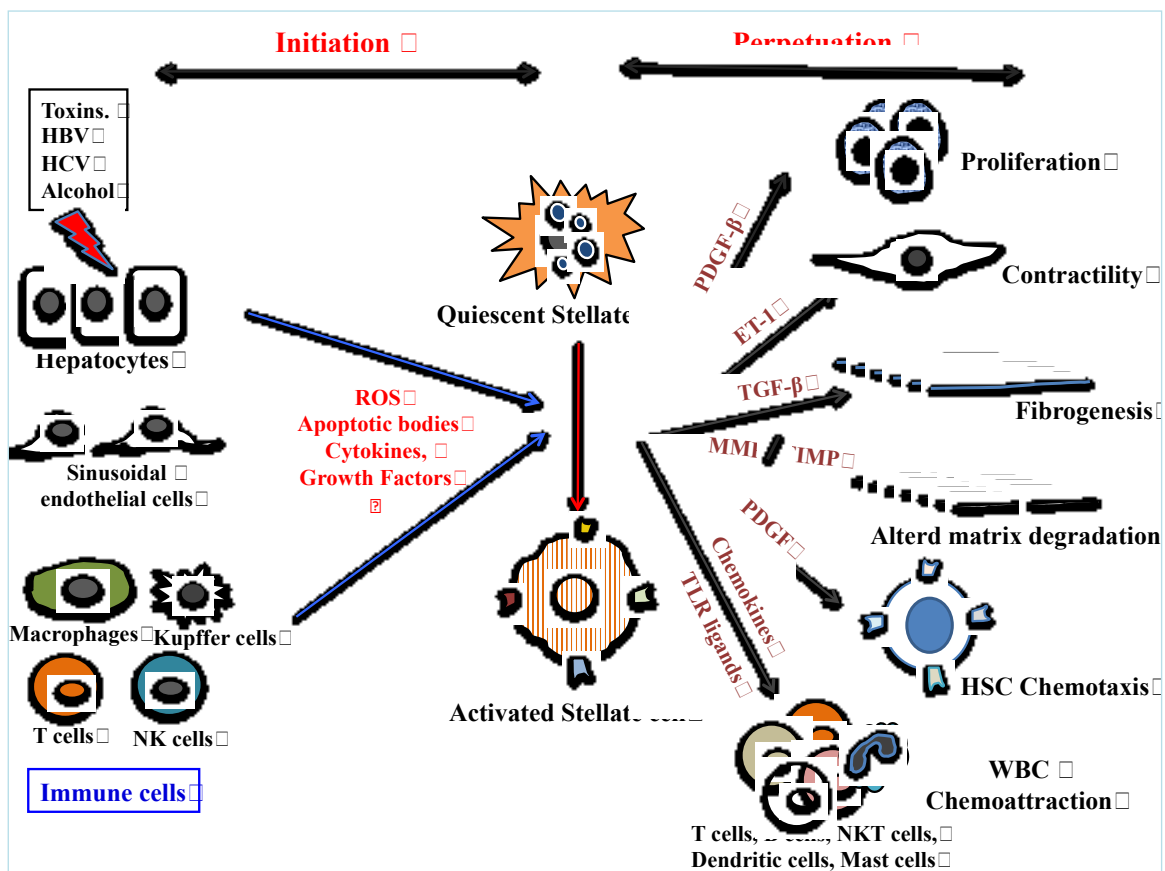
Figure 1 The activation of hepatic stellate cells: The pathways of HSC activation consist two phases; one is initiation phase (also referred to as pre-inflammatory) and perpetuation (continuous activation). Initiation is provoked by soluble stimuli such as oxidative stress (reactive oxygen intermediates), apoptotic bodies, LPS and paracrine stimuli from neighboring cell types including hepatocytes, Kupffer cells, sinusoidal endothelium and from infiltrating immune cells. In the perpetuation phase, the activated cells start expressing their own cytokines and growth factors that can act in autocrine and paracrine manner. The perpetuation phase is characterized by a number of specific phenotypic changes including proliferation, contractility, fibrogenesis, altered matrix degradation, chemotaxis and inflammatory signaling.

1.6.1 Initiating Pathways

The initiation phase consists a sequence of events, which make stellate cell respond to a wide range of cytokines and growth factors (Friedman 2010a). Initiating stimuli include paracrine signals such as reactive oxygen species (ROS), apoptotic bodies, cytokines released from injured liver resident cells and infiltrating inflammatory cells and changes in ECM composition (Brenner et al. 2000; Guo and Friedman 2007).

1.6.1.1 Oxidative stress

Most of the CLD like HCV infection, ALD and hemochromatosis are associated with oxidative stress, which enhances apoptosis or necrosis of hepatocytes, thereby amplifying the inflammatory response and fibrosis (Poli 2000). ROS include superoxide, hydrogen peroxide, hydroxyl radicals, reactive nitrogen species (RNS) and lipid peroxides derived from the damaged hepatocytes as well as partly from activated Kupffer cells and neutrophils that contain damaged mitochondria (Parola and Robino 2001; Friedman 2008). The activation of cytochrome P450 2E1 in hepatocytes leads to the generation of ROS that activates HSC through redox-sensitive intracellular signaling pathways, leading to increased collagen production (Kisseleva and Brenner 2007; Friedman 2010a). ROS also stimulate the production of profibrogenic mediators from Kupffer cells and other resident and circulating inflammatory cells (Sánchez-Valle et al. 2012).

1.6.1.2 Apoptosis of hepatocytes

Apoptosis is a programmed cell death process usually not associated with inflammation. In the case of CLD, continuous damage to hepatocytes leads to the deregulation of the apoptotic process, which results in the generation of apoptotic bodies (Canbay, Friedman, and Gores 2004). These are cleared by Kupffer cells and HSC by phagocytosis or engulfment (Guo and Friedman 2007). Phagocytosis by Kupffer cells leads to expression of a death ligand (Fas ligand) and the proinflammatory cytokine TNF-α, which accelerates hepatocyte apoptosis and inflammation (Canbay et al. 2003). The engulfment of apoptotic bodies by HSC also leads to the release of free radicals, up-regulation of TGF-β1 and collagen I expression. Continuous activation of these cells results in enhanced hepatocyte apoptosis, inflammation and sustained activation of HSC, ultimately leading to LF (S.-S.

Zhan et al. 2006; Guicciardi and Gores 2005).

1.6.1.3 Changes in ECM composition or quality and quantity

Changes in physical properties and chemical composition of ECM influence HSC activation (Friedman 2008). Increased stiffness caused by edema and inflammation also plays an important role in initiating fibrosis (Georges et al. 2007; Friedman 2010b).

Matrix stiffness affects the structure, proliferation, differentiation, motility and survival of cells (Wells 2008). Cells that grow on the soft matrices (Matrigel) show less proliferation, and are non-invasive and non-fibrogenic, whereas cells growing on stiff matrices (like fibrillar collagen) show opposite effects. However, these properties may vary with cell type (Wells 2005; Wells 2008). Type I collagen and stimulation of cells with growth factors such as PDGF, TGF-β and EGF enhance HSC migration and increased MMP-2 activity whereas the basement membrane ECM is inhibitory. These activities are mediated through α1β1 and α2β1 integrins, which are heterodimeric cell surface receptors that readily sense changes in the ECM composition (C.-Q. Yang et al. 2008). Integrin-linked kinase (ILK) is an important multi-domain focal adhesion protein involved in transducing signals from the ECM to HSC cytoskeleton. It has been shown that ILK plays an important role in HSC activation and LF (Y. Zhang et al. 2006; Shafiei and Rockey 2006). ILK mediates its functions through Rho and Gα12/13 signaling (Shafiei, Rockey, and Diseases 2014). Changes in ECM composition also lead to the release of growth factors, from reservoirs into the extracellular space, that promote cell proliferation and fibrogenesis (D. Schuppan et al. 2001; Friedman 2010b).

1.6.2 Perpetuating Pathways

The second stage of the HSC activation process is the perpetuation phase. Once these cells are activated by initiating stimuli, they respond to different cytokines and growth factors released from neighboring cells (by paracrine action) and also perpetuate their own activation by secreting cytokines, other mediators in an autocrine manner and by

upregulating their respective receptors (Moreira 2007).

The perpetuation phase is characterized by loss of the retinoid stores, enhanced proliferation, contractility, fibrogenesis, matrix degradation, chemotaxis and leukocyte chemoattraction (proinflammatory signaling), which collectively contribute to scar formation (Li and Friedman 1999; Friedman 2008).

1.6.2.1 Retinoid loss

Activated HSC are marked by loss of the Vitamin-A and lipid droplets in their cytoplasm. It has been shown that in vitro HSC cultures treated with retinoic acid (RA) stop proliferating and secrete TGF-β (Davis, Kramer, and Davidson 1990). HSC expresses nuclear retinoic acid (RAR) and retinoid X receptors (RXR) by which RA modulates proliferation and interstitial collagen expression (Hellemans et al. 2004). However, it is not yet clearly known whether the retinoid loss is a required for stellate cell activation.

1.6.2.2 Proliferation

One important feature of activated HSCs is their proliferative phenotype. The most potent mitogen of HSC is platelet-derived growth factor (PDGF). Other HSC mitogens include VEGF, thrombin, epidermal growth factor (EGF), TGF-α, keratinocyte growth factor and basic fibroblast growth factor (Don C Rockey 2006). Main sources of PDGF-β are endothelial cells and activated HSC. Quiescent HSC express the PDGF-α receptor subunit, but do not express the β-subunit until activation (Alcolado, Arthur, and Iredale 1997). The expression of PDGF-β and its receptor expression is enhanced during HSC activation (M Pinzani et al. 1994; Wong et al. 1994). PDGF-β over-expression enhances HSC proliferation and liver fibrosis without influencing TGF-β expression, indicating a TGF- β - independent mechanism (Czochra et al. 2006). Phosphatidyl inositol 3 kinase (PI3K), ERK and AKT downstream signaling pathways play an important role in mediating HSC proliferation and collagen deposition in response to PDGF-β (Bonner 2004). PDGF-β also activates NADPH oxidase (NOX) in HSC to produce ROS which further enhances HSC proliferation through the phosphorylation of p38 MAPK (Adachi et al., 1997). Besides,

PDGF-β mediates HSC proliferation by stimulating Na+/H+ exchanger (Di Sario et al. 1999). In cholestatic liver injury, PDGF induces a proliferative response in HSC, which precedes HSC phenotypic conversion into myofibroblasts (Kinnman et al. 2001).

1.6.2.3 Chemotaxis

Injury-associated stimuli induce migration of stellate cell towards damaged areas of the liver, where they undergo proliferation, synthesize extracellular matrix, and participate in tissue repair. These cells undergo apoptosis once injury resolves, but if injury persists; stellate cells drive the development of fibrosis and subsequent cirrhosis (Melton and Yee 2007). This migration of HSC depends on the extracellular matrix integrity, composition and cell-matrix interactions. It has been shown that stimulation of HSC with TGF-β1, PDGF-β, and collagen 1 results in an increase in their migratory capacity and it is MMP-2 and integrin dependent (C. Yang et al. 2003). Endothelin-1 (ET-1) and monocyte chemotactic protein (MCP-1) also induce chemotaxis of HSC (Ikeda et al. 1999; Marra et al. 1999; Ramón Bataller and Brenner 2001). Focal adhesion disassembly is necessary for stellate cell chemotaxis in response to PDGF (Melton et al. 2007). Adenosine and DNA from damaged hepatocytes that bind to A2 and TLR9 receptors respectively on HSC, inhibit PDGF-induced chemotaxis by blocking Ca^{+2} influx and up-regulating TGF-β and collagen I mRNA expression (Hashmi et al. 2007; Watanabe et al. 2007). Collagen type 4 has also been shown to inhibit HSC migration (C.Q. Yang et al., 2008).

1.6.2.4 Contractility of stellate cells:

HSC show contractility similar to myofibroblasts, mediated by α-smooth muscle actin (α–SMA) and myosin. The main factor involved in HSC contraction is endothelin-1, which regulates sinusoidal blood flow via ET-1 receptors expressed on HSC in the liver (Housset, Rockey, and Bissell 1993). Other molecules that induce contractility of HSCs include prostanoids, substance P, angiotensin II and arginine vasopressin (Don C Rockey 2006). Besides promoting contractility, ET- 1 and Angiotensin II also promote proliferation of HSC (Cho et al. 2000; Manuscript and Target 2009; R Bataller et al. 2000). Contractility of HSC is inhibited by vasodilator compounds such as nitric oxide (NO), carbon monoxide

(CO), PGE2, lipo-PGE1 and adrenomedullin (Don C. Rockey 2003). ET-1 exerts contractility through a Ca2+ dependent protein kinase (that is, PKC) and Rho pathway whereas NO exerts relaxation via the cyclic adenosine monophosphate (cAMP) pathway (Reynaert et al. 2002). In the normal liver, ET-1 is produced primarily by sinusoidal endothelial cells but, after injury, ET-1 is largely derived from HSC, which also up-regulate their receptors (Housset, Rockey, and Bissell 1993; Don C. Rockey 2003). Increased contractility by HSC contributes to increased intrahepatic resistance and portal pressure in cirrhotic livers (D C Rockey and Weisiger 1996).

1.6.2.5 Fibrogenesis

Transforming growth factor-β (TGF-β) is the major fibrogenic cytokine in many fibrotic diseases, including LF (A. M. Gressner 1996). Fibrotic diseases are associated with increased levels of TGF-β, which inhibit ECM degradation by down regulating the expression of MMP and upregulating the expression of tissue inhibitor of matrix metalloprotease-1 (TIMP-1) (K. Jeong 2008; Dooley and Dijke 2012). TGF-β is derived from paracrine sources such as sinusoidal endothelial cells and Kupffer cells as well as synthesized in an autocrine manner by HSC (Inagaki and Okazaki 2007).

TGF-β plays important roles in embryonic development, tissue remodeling, inflammation, angiogenesis, atherosclerosis, fibrosis and carcinogenesis (Mauviel 2005; Inagaki and Okazaki 2007). TGF-β is a potent growth inhibitor of hepatocytes and apoptosis of epithelial cells. Conversely, it stimulates fibroblasts to proliferate, produce ECM and induce a fibrotic response in various tissues *in vivo* (Leask and Abraham, 2004). In the liver, TGF-β enhances the transdifferentiation of HSC into myofibroblasts (A. M. Gressner 1996). The blockade of TGF-β signaling prevents progression of LF in experimental animals (Yata et al. 2002), whereas overexpression of TGF-β in transgenic models inhibited HSC apoptosis and induced HSCs to synthesize excessive amounts of matrix proteins, such as collagen types I, III, IV and fibronectin (Kanzler et al. 1999). Studies with a tetracycline-regulated gene expression system, in which plasma levels of TGF-β can be chemically regulated, showed that TGF-β inhibits MMP and up-regulates TIMP-1

expression and that progression of LF could be stopped by inhibiting TGF-β production (Ueberham et al. 2003).

1.6.2.5.1 TGF-β activation

TGF-β is a member of a large family of pleiotropic cytokines that includes bone morphogenetic proteins (BMPs), activins and other related factors. The TGF-β family contains three closely related isoforms (i.e. TGF-β1, TGF-β2, TGF-β3). They are secreted as latent precursor molecules (LTGF-β), which contain an N-terminal secretory signal sequence, a long precursor segment, *i.e.* the latency-associated peptide (LAP), and a C-terminal part corresponding to the mature TGF-β. The LTGF-β forms a complex with latent TGF-β-binding proteins (LTBP), activated by proteolytic cleavage by various proteases such as plasmin and thrombin. The biologically active form is a dimer of 25KDa in which the two subunits are linked by a disulfide-bridge (A. M. Gressner and Weiskirchen 2006; Y. Shi and Massague 2003) (Figure 3).

1.6.2.5.2 TGF- β receptors

TGF-β isoforms exert their biological effects through a distinct network of TGF-β type I (TβRI), type II (TβRII), and type III (TβRIII) cell-surface receptors. TβRI and TβRII are serine/threonine kinases that contain a cysteine-rich extracellular domain, a short hydrophobic transmembrane region, and a cytoplasmic region harboring the kinase motif. Ligand binding induces the assembly of type I and type II receptors into complexes, within which, TβRII phosphorylates and activates TβRI. This phosphorylation event is associated with activation of TβRI kinase and subsequent downstream signaling (A. M. Gressner and Weiskirchen 2006; Y. Shi and Massague 2003).

1.6.2.5.3 TGF-β signaling by Smad proteins

Signal transduction from activated TβRI to the nucleus is mediated predominantly via phosphorylation of cytoplasmic protein mediators Smads. The receptor-associated Smads (R-Smads; Smad1, 2, 3, 5 and 8) are recruited to activate TβRI by auxiliary proteins such as

Smad Anchor for Receptor Activation (SARA). Upon phosphorylation by activated TβRI on two serine residues, activated R-Smads form heteromeric complexes with a co-Smad, Smad4, and translocate into the nucleus where they induce the transcription of genes directly or indirectly involved in fibrogenesis namely, several fibrillar ECM proteins (collagen, fibronectin), matrix-degrading enzymes (MMPs) and some protease inhibitors (plasminogen activator inhibitor type 1, TIMP-1). They also induce genes regulating epithelial– mesenchymal cell transition, proliferation (cyclin-dependent kinase inhibitor p21) and apoptosis (caspases). The inhibitory Smads, Smad6 and Smad7 antagonize signaling by competing with R-Smads for binding to activated TβRI and thus inhibit the phosphorylation of R-Smads or recruiting E3 ubiquitin ligases to the activated TβRI (Verrecchia and Mauviel 2007; Verrecchia et al. 2010).
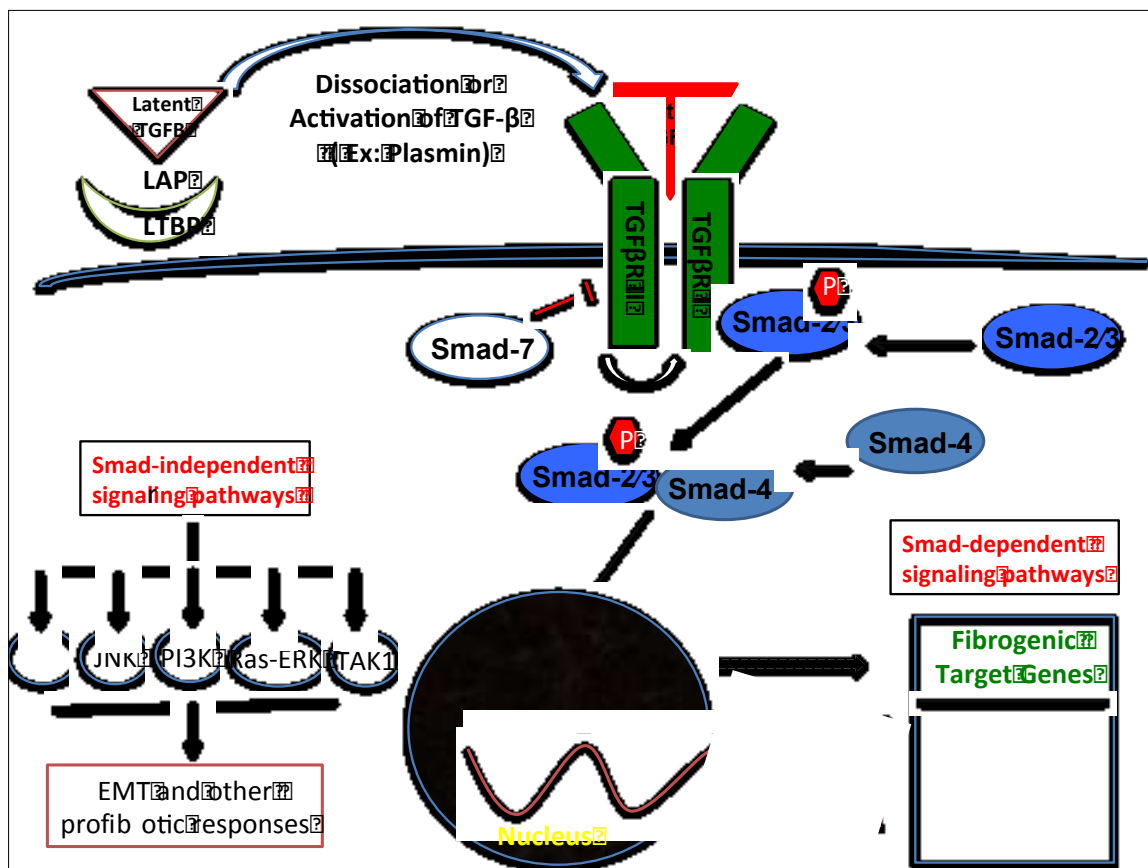
Figure 2: **The TGF-β signaling Pathway**: TGF-β is generally secreted as a large latent precursor molecule that is complexed with latency-associated peptide (LAP) and bound to latent-TGF-β-binding protein (LTBP). Latent TGF-β is activated when it dissociates from LAP and LTBP. The active TGF-β binds its receptor, initiates Smad-dependent and independent signaling. The Smad-dependent signaling pathway regulates fibrogenic target genes such as α-smooth muscle actin (SMA), collagen, connective tissue growth factor (CTGF), tissue inhibitor of metalloprotease (TIMP-1). TGF-β can also induce a number of Smad-independent pathways mediated by Ras, JNK, TGF-β-activated kinase (TAK), and phosphatidylinositol-3-kinase (PI3K) that promote EMT, apoptosis and fibrotic responses.

1.6.2.5.4 Differential Roles of Smads in liver fibrosis

Even though the structure of Smad proteins is similar, they show functional diversity (F. Xu et al. 2016). Smad2 and Smad3 are strongly activated in liver fibrosis, but smad3 is essential for the pathogenic process. TGF-β mediates activation of Smad2 primarily in in quiescent and intermediate cells, whereas Smad3 activation occurs primarily in transdifferentiated cells. (C. Liu et al. 2003). In activated HSC, TGF-β also regulates cytoskeletal organization via phosphorylation of Smad3 (Uemura et al. 2005), suggesting that the two Smads have distinct roles in the process of HSC activation. Consistently with this, $Smad3^{-/-}$ mice are much less susceptible to liver fibrosis than wild type mice indicating Smad3 is a main "fibrogenic mediator" in HSC (Latella et al. 2009; Cong et al. 2012b). Smad3 is also involved in the EMT process and generation of myofibroblasts (Masszi and Kapus 2011). Transient overexpression of Smad7 inhibits HSC transdifferentiation and experimental fibrosis. However, blockade of TGF-β signaling by Smad7 does not decrease α-SMA expression in cultured HSC (Dooley et al. 2003). TGF-β also induces the expression of connective tissue growth factor (CTGF), which stimulates ECM production by fibroblasts, via a functional Smad3 binding site in the CTGF promoter (Branton and Kopp 1999; Verrecchia et al. 2010; U. E. Lee, Mstp, and Friedman 2011).

TGF-β also mediates signaling by non-canonical, "non-Smad" signaling pathways via activation of mitogen-activated protein kinase (MAPK), Rho-like GTPase, p38 and c-Jun N-terminal kinases (JNKs) and phosphatidylinositol-3-kinase/AKT pathways. These pathways regulate EMT, apoptosis and other fibrogenic processes (Cong et al. 2012a; Y. E. Zhang 2009; Moustakas and Heldin 2005).

IFN-γ is a well-known anti-fibrotic cytokine that inhibits collagen synthesis (Granstein, Flotte, and Amento 1990). Smad7 induction by IFN-γ, blocks TGF-β/Smad signaling pathway through Jak1 and Stat1, and prevents the interaction of Smad3 with the TGF-β receptor (Ulloa, Doody, and Massagué 1999). IFN-γ promotes direct interaction of Y-box-binding protein (YB-1) with Smad3 and thus inhibits TGF-β–induced COL1A2 transcription (Higashi et al. 2003).

1.6.2.6 Inflammatory signaling

Diverse types of immune cells are implicated in the pathogenesis of LF as well as its resolution (Friedman 2010a).

(1) Infiltrating neutrophils do not have or have a minimal role in LF (J. M. Saito et al. 2003). Similarly, mast cells also have no role in the development of LF (Sugihara et al. 1999).

(2) HSC, like kupffer cells, express Toll-like receptors (TLR) on their surface. Activation by LPS upregulates IL-8 and MCP-1 gene expression and cell surface expression of ICAM-1 and VCAM-1 via a NF-kβ-dependent pathway (Paik et al. 2003). Exposure to bacterial products enhances a strong inflammatory response in HSC, characterized by IL-6 and MCP-1 secretion, which results in the recruitment of Kupffer cells (Brun et al. 2005). The TGF-β pseudo-receptor BMP and the activin membrane-bound inhibitor (Bambi) is a transmembrane glycoprotein induced by BMP (Bone morphogenetic protein) signaling and functions as a negative regulator of TGF-β signaling. TLR4 signaling in HSCs downregulates Bambi on quiescent HSC to enhance TGF-β signaling and LF (Seki et al. 2007; Guo et al. 2009).

(3) HSC also act as antigen-presenting cells (APC) as they express molecules for antigen presentation (MHC-I and MHC-II) and lipid-presenting molecules CD1b and CD1c. They internalize macromolecules and express molecules that modulate T-lymphocyte proliferation (CD40 and CD80) (Viñas et al, 2003). HSC process and present antigen to CD8$^+$ and CD4$^+$ T cells and CD1-restricted natural killer T (NKT) cells (Winau et al, 2007).

(4) Kupffer cells (KC) are liver resident macrophages derived from circulating monocytes (R. Xu, Zhang, & Wang, 2012). KCs are involved in LF by promoting HSC activation by secreting TGF-β and TNF-α. In mice, depletion of macrophages or inhibition of their recruitment to the liver by ablation of *Ccr2* inhibited LF, suggesting that macrophage

recruitment to the liver via CCR2 and activation of HSC are crucial for inducing LF (Duffield et al. 2005; Imamura et al. 2005; Seki et al. 2009; Miura et al. 2012). KCs elicit divergent effects on LF by promoting HSC activation during progression (mediated by TGF-β secretion) and by inducing HSC apoptosis via TRAIL during regression of LF (Friedman 2005).

 (5) NK cells inhibit LF by killing activated HSC in NKG2D-dependent, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)-dependent mechanisms and by producing IFN-γ (Gao, Radaeva, and Park 2009; Radaeva et al. 2006; Notas, Kisseleva, and Brenner 2009; Guo and Friedman 2007). HSCs express the CXCR3 chemokine receptor, whose ligand CXCL10 acts as pro-fibrotic factor by preventing NK cell activity (Hintermann et al. 2010). NKT cells display complex roles in liver injury, inflammation and fibrosis (Notas, Kisseleva, and Brenner 2009). Hepatic iNKT cells show inhibitory activity during early stage of LF but not at the later stages (Park et al. 2009; Gao, Radaeva, and Park 2009).

(6) CD8^{+} T lymphocytes promote fibrogenic activity of HSCs, whereas CD4^{+} T cells exert antifibrotic activity by stimulating NK cells via IL-2 mediated up-regulation of NKG2D (Safadi et al. 2004; Glässner et al. 2013). Regulatory T cells (Tregs) attenuate the anti-fibrotic activity of NK cells against HSC via both direct cell-contact-dependent inhibition and by down-regulating the NK cell activating ligands on HSCs (Langhans et al. 2015). B lymphocytes influence the development of LF in an antibody and T-cell-independent manner (Novobrantseva et al. 2005).

1.6.2.7 Altered matrix degradation or (MMPs and TIMPs imbalance)
Matrix metalloproteinases (MMPs) comprise a family of 25 zinc-dependent endopeptidases capable of degrading components of the ECM. MMPs also act on non-ECM substrates such as cytokines, chemokines and play regulatory functions in inflammation and immunity (Giannandrea and Parks 2014). MMPs are generally secreted into the extracellular

environment or tethered to the cell membranes (Duarte et al. 2015). MMPs are categorized into different groups according to their ECM substrate specificity: collagenases, gelatinases, membrane-type, stromelysins and matrilysins and others (Hemmann, Roderfeld, and Roeb 2007). MMPs are again subdivided into groups on the basis of differences in domain composition (Table2).

Table 2: MMPs nomenclature, their specific substrates

Enzyme nomenclature	MMP Nomenclature	Specific substrates
Collagenases	MMP-1 collagenase-1 (Interstitial collagenase) MMP-8 (Neutrophil collagenase) MMP-13 (Collagenase-3)	Fibrillar collagens: I, II, III, V, and XI,
Gelatinases	MMP-2 (Gelatinase-A) MMP-9 (Gelatinase-B)	Type IV, V, VII, X, XI, and XIV Collagens, Gelatin, Elastin, proteoglycans core proteins
Stromelysins	MMP-3 (Stromelysin 1) MMP-10 (Stromelysin 2)	Proteoglycans, Fibronectin, Laminin, Gelatins
Matrilysins	MMP-7 (Matrilysin 1) MMP-26 (Matrilysin 2)	Fibronectin, Laminin, Nidogen, Type IV collagen, Proteoglycans, β4-integrin
Membrane type MMPs	MT1-MMP or MMP-14, MT2-MMP or MMP-15, MT3-MMP or MMP-16, MT4- MMP or MMP-17, MT5-MMP or MMP-24, and MT6- MMP or MMP-25	Gelatin, Fibronectin, Laminin-1, Vitronectin, Cartilage proteoglycans, and Fibrillin-1
Secreted	MMP-11 MMP-28	Collagen IV

MMPs are synthesized by a wide range of cell types such as hepatocytes, HSC, Kupffer cells, neutrophils and recruited hepatic macrophages, and are secreted into the extracellular space in an inactive form called zymogen or pro-MMP (Page-mccaw, Ewald, and Werb 2007). MMP synthesis is induced by numerous mediators, which modulate their gene expression. The two key regulators of MMP production are IL-1 and TGF-β (Bruschi and Pinto 2013). The proteolytic activation of the pro-enzyme is controlled by other MMPs and serine proteinases such as plasmin (Hersznyi et al. 2012).

1.6.2.7.1 Tissue inhibitors of metalloproteinases (TIMPs)

The enzymatic activities of MMPs are controlled by endogenous tissue specific inhibitors called tissue inhibitors of metalloproteinases (TIMPs), which are secreted proteins. Four types of TIMPs (TIMP-1, 2, 3 and 4) inhibit MMP activity by binding reversibly to the catalytic site of MMPs in a 1:1 stoichiometric ratio (Manicone and McGuire 2008). Even though, TIMPs are capable of inhibiting all known MMPs, they differ in their affinity for specific MMPs (Arpino, Brock, and Gill 2015). Alterations in the MMP–TIMP balance lead to different pathologies such as delayed wound healing, tissue fibrosis, angiogenesis and tumor invasion (Duarte et al. 2015).

In the human liver, MMP-1 is the major interstitial collagenase. Until 2001, a homolog of MMP-1 had not been identified in rodents, MMP-13 was considered as the major interstitial collagenase in rodents, as it is capable of degrading type 1 collagen (Henriet, Rousseau, and Eeckhout 1992; Krane et al. 1996). Mmp1a, the true homolog of human MMP-1, discovered in 2001, demonstrates collagenase activity (Balbín et al. 2001). Several studies have shown that its expression is relatively low in healthy tissues but it is upregulated during pathological conditions (Foley and Kuliopulos 2014). Mmp1a is implicated in tumorigenesis and metastasis as shown by Mmp1a knockout mice and cancer cell lines (Foley et al. 2012). The activation of pro MMP-1 to active MMP-1 is a two-step reaction, which requires initial cleavage by plasmin and second cleavage by stromelysin. The profibrogenic cytokine TGF-β inhibits this cleavage process thereby inhibits ECM degradation, leading to its accumulation (Nagase et al. 1991).

In the early primary culture (days 0–3), HSCs express MMP-3 (stromelysin), MMP-1, MMP-13 (rat) and but not TIMP-1 and TIMP-2. The expression of MMPs during the early phase is affected by the method of HSC isolation, serum components and the culture substratum (Benyon and Arthur 2001). The pattern of expression of MMPs and TIMPs changes with the duration of culture: stromelysin and MMP-1/MMP-13 expression are downregulated with a marked increase in expression of both TIMP-1 and TIMP-2 (J. Iredale et al. 1996).

MMP-13 is expressed by macrophages and HSC; its expression in macrophages is relatively constant regardless of the stage or stimulus for activation in models of liver injury (John P. Iredale, Thompson, and Henderson 2013). MT1-MMP is involved in the conversion of pro MMP-2 to active MMP-2, and its activity is inhibited by excess TIMP-2 (Kinoshita et al. 1998). Fibrotic livers and cultured HSC show increased expression of MMP-2. Collagen-I enhances MMP-2 activation but not collagen-VI (Takahara et al. 1995; Théret et al. 1999). Collagen-I is the ligand for descoidin domain receptor 2 (DDR2) expressed on activated HSC, and this interaction upregulates MMP-2 expression. In fibrosis, a positive feedback mechanism between MMP-2 and collagen -1 enhances the pathogenic process (Olaso et al. 2001). TGF-β1 differentially affect MMP expression by downregulating interstitial collagenase expression and upregulating expression of gelatinase A, TIMP-1, and collagen-I (J P Iredale 1997).

1.7 Reversal of fibrosis

It has been shown in human and animal models that LF can be reversible. The mechanisms of regression of LF have been extensively studied in animal models and potential targets for antifibrotic therapy are being defined (Ramón Bataller and Brenner, 2001)**.** There are different pathways, which explain the molecular and cellular mechanisms of LF regression such as:

Pathway 1: Cessation of underlying liver injury: In patients, reversal of liver fibrosis is

usually achieved by removing underlying etiology factors such as viral agents (hepatitis C and B), alcohol, toxins and medication, or by treating the underlying disease. Treatment of the HBV or HCV infected patients with interferon-α or ribavirin and lamuvidines significantly reduces LF (Poynard et al. 2002; Alaluf and Shlomai 2016).

Pathway 2: Transition of the intrahepatic balance from inflammation to restoration Recovering hepatocytes and their neighboring non-parenchymal cells alter the microenvironment from a pro-inflammatory milieu to resolution, so that restorative and anti-inflammatory mediators become dominant. During resolution, macrophages show a restorative phenotypic switch characterized by low expression of Ly6C in mice and high expression of Mmp-9, Mmp-12, growth factors (favoring hepatocyte recovery) and phagocytosis-related receptors (Ramachandran et al. 2012; Hammerich et al. 2014). NK cells induce apoptosis of activated and senescent myofibroblasts via NKG2D and TRAIL (Langhans et al. 2015; Notas, Kisseleva, and Brenner 2009). During chronic injury, γδ T cells recruited to the liver by CCR6 activation, prevent excessive inflammation and fibrosis by inhibiting HSCs (Hammerich et al. 2014).

Pathway 3: Elimination of myofibroblasts or enhancing HSC apoptosis: Since HSC are the main source of ECM synthesis and the matrix degrading enzymes and their inhibitors in the liver, deactivation of myofibroblasts is key to fibrosis regression, which is achieved by senescence, apoptosis, and inactivation (Guo and Friedman 2007).

Senescent, activated HSC show activation of endogenous p53, reduced secretion of ECM components, enhanced secretion of degrading enzymes and increased susceptibility to NK cell-mediated apoptosis (Krizhanovsky et al. 2008). Activated myofibroblasts are able to return to a quiescent phenotype (Kisseleva et al. 2012), but these cells can be re-activated to myofibroblasts in response to fibrogenic stimuli and contribute to LF albeit less efficiently (Troeger et al. 2012; Mallat and Lotersztajn 2013).

Integrins are important for HSC survival. Inhibiting their adhesions with either a specific antibody or integrin antagonizing peptide results in HSC apoptosis associated with a reduction in the Bcl-2/Bax ratio as well as an increase in caspase 3 activity (X. Zhou et al. 2004; Iwamoto et al. 1999). TIMP-1 protects HSCs from apoptosis and inhibits MMP activity thereby favoring progression of LF (Hitoshi Yoshiji et al. 2002). Studies have shown that downregulation of TIMP production and administration of anti-TIMP-1 antibody increases ECM degradation and apoptosis of HSC (Murphy et al. 2002b; Parsons et al. 2004). TNF-α also exerts an anti-proliferative effect on HSC and induces HSC apoptosis in the presence of cycloheximide, an inhibitor of protein synthesis (Kisseleva and Brenner 2006). Activated HSC are more susceptible to TRAIL, since these cells express activation-dependent TRAIL-R2/DR5 receptors (Taimr et al. 2003). INFγ also induces HSC apoptosis (Saile et al. 2004). NF-kβ enhances survival of HSC, inhibition of NF-kβ or inhibition of the inhibitor of κβ kinase (Iκβ) with sulfasalazine or the fungal toxin gliotoxin promotes apoptosis of HSC (Oakley et al. 2005; Wright et al. 2001).

Pathway 4: Enhancing extracellular matrix degradation. Another approach for achieving reversibility of fibrosis is promoting the degradation of excess ECM. MMP-9 enhances apoptosis of HSC and inhibition of this protease resulted in increased HSC survival (X. Zhou et al. 2004). Collagen-I also provides a survival signal for activated HSCs, whereas enhanced activity of collagenase and degradation of collagen-I promotes the recovery from LF (Issa et al. 2003; J P Iredale et al. 1998; Tacke and Trautwein 2015).

1.8 Diagnosis of fibrosis

Accurate assessment of LF progression is essential for treatment of the disease. The different diagnostic approaches currently available to assess fibrosis progression are discussed below.

1.8.1 The Liver biopsy

The liver biopsy remains the "gold standard" method because it provides useful information on the current clinical status of the liver injury, allows to take a decision on type of therapy, and it reveals the stage of fibrosis that necessitates surveillance for hepatocellular carcinoma (HCC) and/or screening for viruses.

This biopsy is assessed for stage and grade of the liver injury. The staging refers to the extent of fibrosis and the presence of cirrhosis, whereas grading defines the extent of necroinflammatory activity (Goodman 2007). The main determinants of inflammatory activity are lymphocytic piecemeal necrosis, lobular necroinflammation and portal inflammation, which are graded from 0 to 4 in most classification systems. The main determinants of fibrosis are the length in the expansion of fibrotic areas between portal tracts and these changes are staged as 0 to 4 in the classification systems (Calculators 2014; Standish et al. 2006). Even though several scoring systems are available, the most commonly used are French METAVIR, the Batts-Ludwig, the International Association for the Study of the Liver (IASL) and the Ishak Scoring systems (Afdhal and Nunes 2004). However, in large clinical trials the METAVIR score is more commonly used.

Liver biopsy is direct, quick and has well-established staging systems (Ferrell 2013; Goodman 2007). It has some disadvantages such as sampling error and inter-observer variation among pathologists, with an average 20% error rate in assessment of fibrosis stage. Moreover, these diagnostic methods require frequent samplings, are expensive and are more fatal when compared to other noninvasive diagnostic methods (Regev et al. 2002).

1.8.2 Noninvasive methods

Over the past years, various non-invasive methods have become available to assess the severity of liver fibrosis. These are routine clinical parameters, such as physical examination findings, laboratory tests, radiographic tests, combinations of laboratory tests and specific serum markers. These methods can be divided into direct markers, indirect markers and imaging techniques.

1.8.2.1 Direct markers

The protein products of extracellular matrix synthesis or degradation are used as direct markers, which reflects the activity of the fibrotic process.

Markers of matrix deposition include procollagen I C-terminal fragment, procollagen III N-terminal fragment, tenascin, tissue inhibitor of metalloproteinase TIMP and TGF-β. On the other hand, markers of matrix removal include procollagen IV C-peptide**,** Procollagen IV N-peptide (7-S collagen), collagen IV, undulin**,** metalloproteinase MMP**,** urinary desmosine and hydroxylysylpyridinoline. Other non-specific, direct markers are hyaluronan**,** laminin, YKL-40 (O. A. Gressner, Weiskirchen, and Gressner 2007; Afdhal and Nunes 2004).

The FIBROSpect II is a commercially available test that combines hyaluronic acid, tissue inhibitor of a metalloproteinase-1 (TIMP-1), and alpha-2-macroglobulin in a predictive algorithm to determine the presence or absence of significant fibrosis. (Strauss 2010).

1.8.2.2 Indirect markers

Indirect markers are usually represented by algorithms, based on biochemical tests for markers that are commonly altered in CLD (Don C Rockey 2008). These include (1) Aspartate Aminotransferase-to-Platelet ratio index (APRI), **(**2) Forns index (combination of three biochemical markers AST, platelet count, and gamma globulin), (3) FT/FS (include age, gamma-glutamyltransferase (GGT), cholesterol, and platelet count) and (4) FibroTest (include patient age, gender and group of six biochemical markers associated with liver fibrosis: alpha-2-macroglobulin, haptoglobulin, GGT, apolipoprotein A1, total bilirubin, and ALT) (Denzer and Lüth 2009; Guidelines 2015; Calculators 2014).

These indirect markers are safe, convenient, inexpensive, non-invasive, reflect the status of the entire liver and can assess severity of fibrosis. These markers are also being used in combination with radiological tests to increase accuracy. However, these indirect tests are unable to discriminate between intermediate stages of fibrosis, staging scales vary between studies and cost varies with combinations (Winau et al. 2007; Das and Vasudevan 2008).

1.8.2.3 Imaging techniques

Imaging techniques are used to detect advanced CLD by recognizing surrogate markers of portal hypertension and structural changes in the liver. These are ultrasound, computed tomography, magnetic resonance imaging and transient elastography. They have high degree of sensitivity and specificity but are unable to diagnose and differentiate early stages of fibrosis.

Transient elastography (Fibroscan) measures the elasticity or stiffness of the liver by using ultrasound and low frequency elastic waves and can define the extent of fibrosis without the need for liver biopsy. It allows diagnosis of liver cirrhosis and significant fibrosis with accuracy (Press 2011; Castera 2011). This cannot be used in presence of ascites, obesity and are less accurate in low degree of fibrosis (Denzer and Lüth 2009).

Hepatic venous pressure gradient assessment (HVPG) measures presence and severity of portal hypertension. It is invasive and accurate in determining which patients with cirrhosis are at risk for decompensation, the stage at which the liver is extensively scarred and unable to function properly. People with decompensated cirrhosis develops symptoms like ascites, jaundice and complications that can be life threatening. It can be used only in patients with relatively advanced disease who are at risk of, or already have, portal hypertension (Friedman 2010b).

1.9 Current therapeutic approaches to liver Fibrosis

The various studies in understanding the cellular and molecular mechanisms involved in progression of LF has led to the development of new targets to treat the disease. This knowledge is also applicable to fibrosis of other organs such as the lung and kidneys. Similarly, advances in understanding the fibrogenic process in other organs are being evaluated in liver fibrosis. Some of the anti-fibrotic therapies being tested in experimental animals and in clinical trials to evaluate their safety and efficacy are discussed below.

Targeting myofibroblasts and the TGF-β pathway: Pirfenidone, (Esbriet and Pirespa) inhibits fibroblast proliferation and it is being used as a broad antifibrotic agent for the treatment of LF and other fibrotic diseases (King et al. 2014). Anti-TGF-β antibodies are being used to treat diseases of the newborn lung (Nakanishi et al. 2007). A Human Anti-Transforming Growth Factor-Beta (TGF-β) monoclonal antibody (fresolimumab) is also being explored by Genzyme (owned by Sanofi-Aventis) as a treatment for patients with interstitial pulmonary fibrosis (IPF) and myelofibrosis (Wynn and Ramalingam 2012). A humanized monoclonal antibody targeting Lysyl oxidase–like-2, an enzyme that catalyzes the cross-linking of collagen, is being explored by Gilead Sciences as a treatment for cardiac fibrosis, IPF and liver fibrosis. Bortezomib, a proteasomal inhibitor approved by the US Food and Drug Administration, induces apoptosis of hepatic stellate cells (Barry-Hamilton et al. 2010).

Targeting pro-inflammatory pathways is a valuable approach in situations where sustained inflammation is the major driving force in the development of fibrosis. IFN-γ is a well-known antifibrotic cytokine. Experimental studies and clinical studies suggested that IFN-γ1b is effective in patients with chronic HBV or HCV infection (H.-L. Weng et al. 2005) but a large clinical study showed little efficacy or no effect of IFN-γ in advanced LF (Pockros et al. 2007).

Tyrosine kinase inhibitor imatinib mesylate (Gleevec), which inhibits PDGF receptor signalling, did not affect survival or lung function in patients with IPF (Daniels et al. 2010), while another clinical study with the tyrosine kinase inhibitor, BIBF 1120, a triple angiokinase inhibitor (VEGFR, PRGFR, FGFR), showed improvement in lung function in patients with IPF (Richeldi et al. 2011). Ruxolitinib (NCB018424), a selective Janus kinase 1 (JAK1) and JAK2 inhibitor, is being used to treat myelofibrosis (Verstovsek et al. 2010).

Monoclonal antibodies against TNF-α (infliximab and etanarcept), CCL2, IL-1 and various

anti-inflammatory mediators, such as IL-10, Arg1, programmed death ligand-2 and Relm-α, or corticosteroids, to modulate the immune system, are being tested in clinical trials (Detlef Schuppan and Kim 2013; Wynn and Ramalingam 2012). One of the plant alkaloids used more commonly as a hepatoprotective agent is Silymarin, a flavonoid antioxidant extracted from *Silybum marianum*. It inhibits collagen deposition in animal models (Boigk et al. 1997) and some clinical tests reported its positive effect in alcoholic patients with early stage cirrhosis (Parés et al. 1998). Anti IL-17A monoclonal antibodies that disrupt IL-17 signaling have been shown to be beneficial for the treatment of pulmonary fibrosis (Mi et al. 2011). Chronic fibrotic diseases are also characterized by the excess production of IL-13 and increased expression of IL-13–inducible genes (Hershey 2003). Lebrikizumab, a humanized monoclonal antibody against IL-13 was shown to be effective in a subset of adults with poorly controlled asthma (Scheerens et al. 2014).

2.0. Suppressor of cytokine signaling proteins (SOCS)

Binding of most cytokines and growth factors to their respective receptors leads to activation of several intracellular signaling cascades, which mediate different cellular responses, such as proliferation, differentiation, survival and functional activation. Their excessive signaling can lead to loss of regulation and can promote a variety of diseases. Such unwanted excessive signalling by cytokines is controlled by many cell intrinsic mechanisms. One such mechanism involves a group of proteins known as Suppressor Of Cytokine Signaling (SOCS), which act in a negative feedback manner (Sullivan et al. 2007). The expression of SOCS family members induced by cytokines displays a tissue-specific pattern (Starr, Willson, and Viney 1997; Larsen 2002) and varies with respect to the cell line and tissue studied (Walter and Hospital 2001).

2.1 Structure of the SOCS family

SOCS proteins are relatively small proteins of 20 to 50 kDa (Trengove and Ward 2013). There are eight mammalian SOCS proteins: SOCS1 to 7 and Cytokine-inducible SH2-containing protein (CISH). Within each sub-family, pairs of SOCS proteins have similar structure and function: CISH/SOCS2, SOCS1/SOCS3, SOCS4/SOCS5 and SOCS6/SOCS7

(Trengove and Ward 2013). Even though SOCS proteins are able to regulate downstream signaling of different receptors, studies indicate that CISH and SOCS1-3 mostly regulate cytokine receptor signaling (Sullivan et al. 2007) while SOCS4-7 predominantly regulate growth factor receptor signaling (Segatto, Anastasi, and Alemà 2011).

SOCS family proteins possess three domains (Figure 4): a central Src-homology 2 (SH2) domain, a C- terminal domain known as the SOCS box that is highly conserved among SOCS proteins (Impson et al. 1999) and an N-terminal domain that is variable in length and sequence among SOCS family members and whose function remains largely unknown. SOCS proteins can be subdivided on the basis of a short (50-75 residues in CISH, SOCS1, 2 and 3) or long N-terminal region (270-385 residues in SOCS4, 5, 6, 7) (Cooney 2002; Linossi et al. 2013). SOCS1 and SOCS3 also contain a conserved 12-residue sequence called kinase- inhibitory region (KIR) within their N-terminal region, which is responsible for inhibition of cytokine receptor-associated Janus kinases (JAKs) (Tamiya et al. 2016; Walter and Hospital 2001). SOCS4 and SOCS5 possess a highly conserved region called N-terminal conserved region (NTCR) within their N-terminal domain but, the role of this sequence is not yet known. The N-terminal domains of SOCS6 and SOCS7 are required for their respective nuclear translocation (Cheng, Huang, Ma, Xu, Wang, and Zhang 2016; Walter and Hospital 2001)
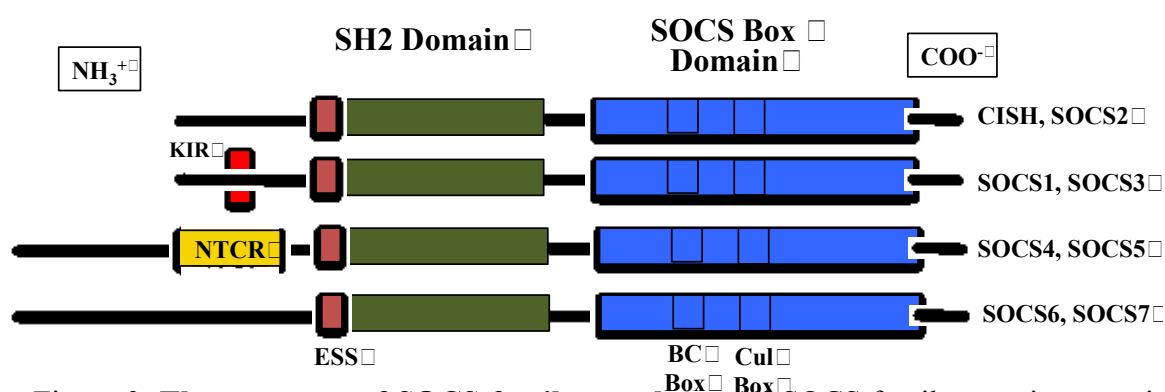
Figure 3. **The structure of SOCS family members.** All SOCS family proteins contain a central SH2 domain and a conserved C-terminal SOCS box domain. The SH2 domain is lengthened by the addition of an extended SH2 sequence (ESS). The SOCS box is composed of the BC box and the Cul box sub-domains. The N-terminal domain is highly variable among SOCS proteins. SOCS1 and SOCS3 contain a unique kinase inhibitory region (KIR) immediately upstream of the central SH2 domain. SOCS4 and SOCS5 harbor an N-terminal conserved region (NTCR).

2.2 Functional domains of SOCS proteins

The two main functional domains of SOCS proteins are the SH2 and the SOCS box domains. The central SH2 domain determines the target of each SOCS proteins. SH2 domain contains ~100 amino acids with two α-helixes and seven β-strands arranged in a βαββββαβ order. The SH2 domain can recognize phospo-tyrosine residues via a p-tyrosine binding pocket and a specificity determining region, which binds on the C-terminal side of the p-tyrosine (Machida and Mayer 2005).

One 12-amino-acid stretch N-terminal to the SH2 domain, called the Extended SH2 Subdomain (ESS), stabilizes the association of SOCS1 with the kinase activation loop of JAK2. An additional 12-amino-acid N-terminal segment, termed the Kinase Inhibitory Region (KIR), is required to inhibit JAK2 kinase activity (Yasukawa et al. 1999). The diversity of SOCS family functions also depends on the specificity of the SH2 domain. Even though, SOCS1 and SOCS-3 have a similar domain composition, SOCS1 SH2 binds to JAKs, while the SOCS-3 SH2 binds to receptors (Machida and Mayer 2005).

The SOCS box is a 40-amino acid motif with two core interaction sites: the BC-box and the Cul-box. Elongin B and Elongin C bind to BC- box (Linossi and Nicholson 2012). The SOCS box serves to assemble the ubiquitin ligase complex through interaction with Elongins B and C, Cullin-5 or Cullin-2, and Rbx-1 (Kamura et al. 2004; Cheng, Huang, Ma, Xu, Wang, and Zhang 2016; Walter and Hospital 2001). This culling Ring Ligase called CRL^{SOCS1} promotes ubiquitination of substrate proteins bound to SOCS via the SH2 domain or N-terminal sequences, and thereby promotes their degaradation by proteasomes **(Figure 5).** Notable SOCS1 ubiquitination substrates include the JAK kinases, cytokine receptor chains, p65 subunit of NF-kB, the MAL signalling adaptor of the LPS signalling complex and receptor tyrosine kinases such as MET (Ilangumaran, Ramanathan, and

Rottapel 2004; Yirui Gui et al. 2014).
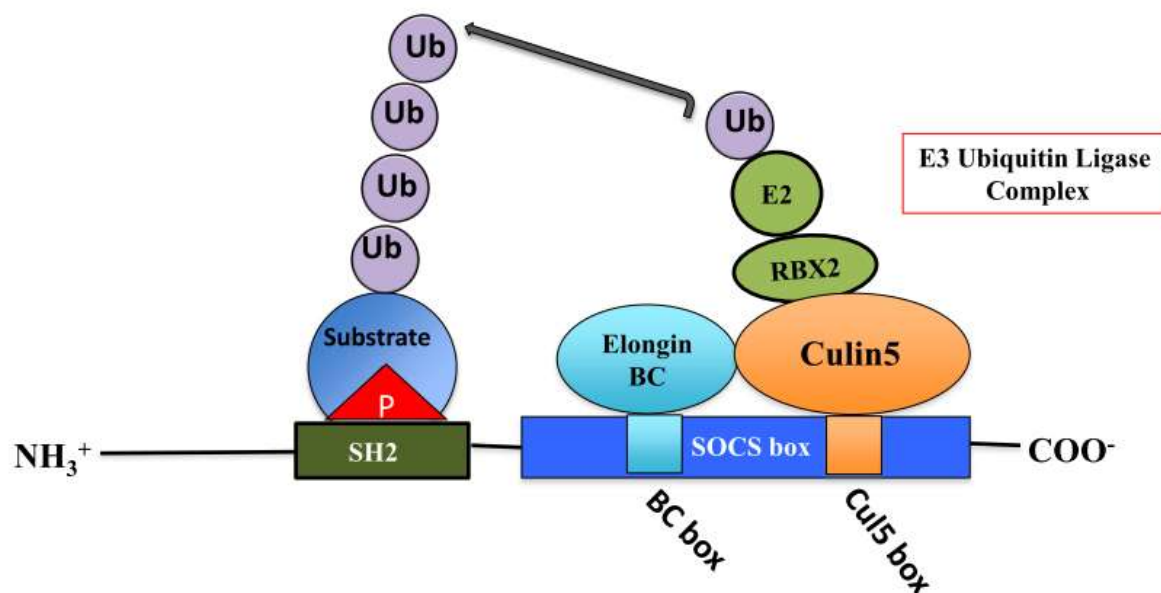
Figure 4. **Schematic illustration of the SOCS box domain and its function**. The SH2 domain of the SOCS protein recognizes the target protein (substrate) via its phospho-tyrosine (pY) residue. The SOCS protein recruits Elongin C/B (BCbox) and Cullin5 (Culin box) to its C-terminal SOCS-box domain and the whole complex functions as an E3 ubiquitin ligase called CRL^{SOCS}. This complex binds to RBX2 (RING-finger-domain-only protein-2) leading in turn to recruitment of the remaining components of an E3 ubiquitin ligase complex to mediate ubiquitination of target proteins and their subsequent proteasomal degradation.

2.3 Mechanisms of Action of SOCS proteins

The members of the Janus Kinase (JAK) family are JAK1, 2, 3 and TYK2. JAKs contain a FERM domain that mediates receptor association and regulates catalytic activity (Cheng, Huang, Ma, Xu, Wang, Zhang, et al. 2016). Binding of cytokines and growth factors to their receptors leads to the activation of receptor-associated Janus kinases (JAKs), which undergo transphosphorylation. The activated JAKs phosphorylate tyrosine residues on the cytoplasmic side of receptors that serve as docking sites to the SH2 domain of Signal Transducer and Activator of Transcription (STAT) proteins (Rawlings, Kristin, and

Harrison 2004).

There are 7 members in the STAT family (Levy and Darnell 2002). STATs are proteins containing amino-terminal, coiled-coil, SH2, linker, DNA binding, and transcriptional activation domains. The SH2 domain is required for STAT activation to dock the protein to tyrosine-phosphorylated receptor subunits to facilitate phosphorylation by JAKs. STATs possess conserved tyrosine residues near their C-terminus, which are phosphorylated by JAKs. Once STATs are phosphorylated, they undergo dimerization and translocate into the nucleus, where they induce the transcription of their target genes including SOCS proteins
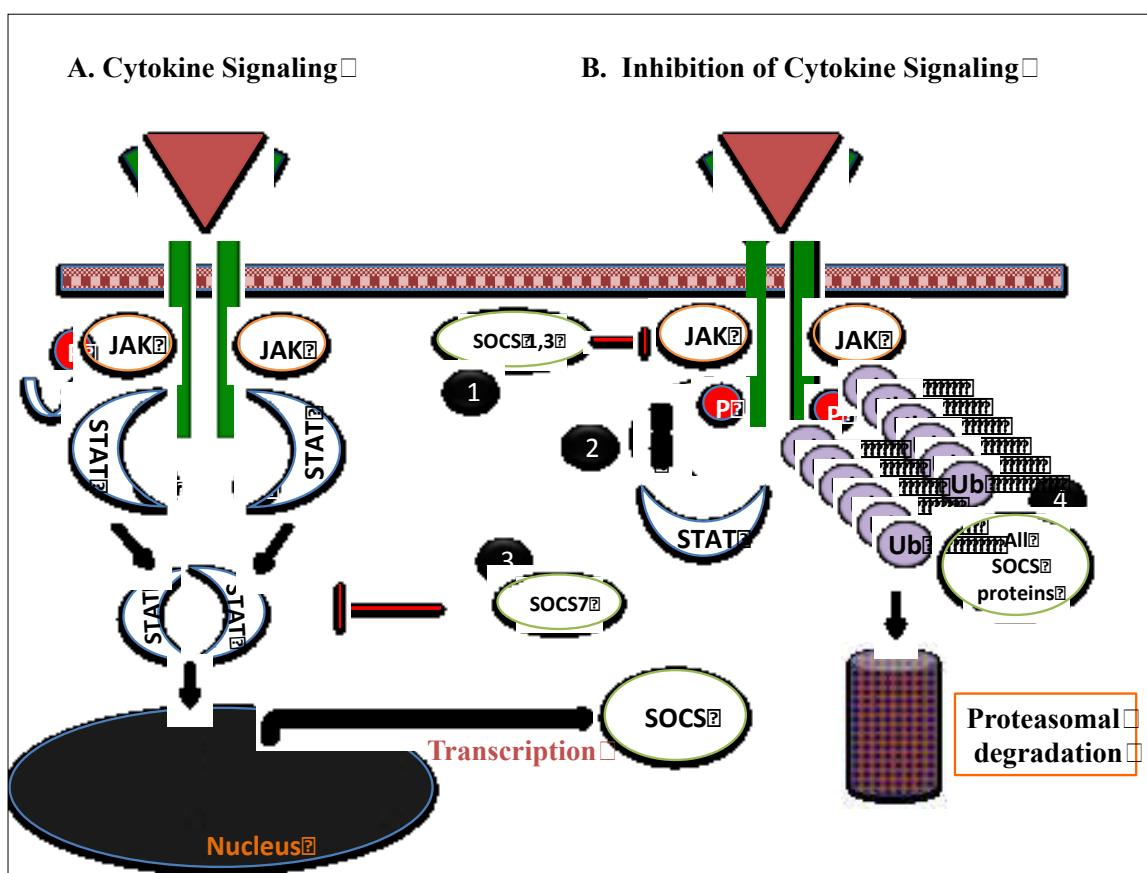
(Harrison 2012).

Figure 5 **JAK-STAT signaling pathway and its negative regulation by SOCS proteins:** (A). The binding of cytokine to its receptor leads to activation of the receptor. The members of JAK family undergo transphosphorylation and phosphorylate the tyrosine residues (pY) on the cytoplasmic segment of the receptor that acts as the docking sites for STAT proteins. Phosphorylated STAT proteins dimerize and translocate into the nucleus to promote transcription of target genes, including members of SOCS family. (B) SOCS proteins inhibit signaling by (1) inhibiting JAK activity (2) binding to pY residues via their SH2 domain and competitively block the JAK- STAT signalling axis (3) Preventing nuclear entry of STAT molecules. (4) By conjugating ubiquitin (Ub) monomers to the target receptor and/or JAKs and facilitate their subsequent proteasomal degradation.

The SOCS proteins can inhibit cytokine signaling using 3 different mechanisms, which vary among the SOCS family members (Nicola and Greenhalgh 2000), **(Figure 3).** First, by binding phosphotyrosines on the receptors SOCS physically block the recruitment of signal transducers, such as STATs, to the activated receptor. CISH, SOCS2, and SOCS3 also inhibit signaling via their ability to bind to phosphotyrosine residues typically on receptors, thereby blocking access of other SH2-containing signaling molecules (Trengove and Ward 2013; Starr, Willson, and Viney 1997). Second, SOCS proteins can bind directly to JAKs to specifically inhibit JAK kinase activity. SOCS1 and SOCS3 are able to directly inhibit JAK kinases, binding via their KIR domain to the JAK activation loop to inhibit kinase activity (Atsuo Sasaki et al. 1999). Third, SOCS interact with the elongin BC complex and promote ubiquitination of the SOCS-interacting signalling proteins including JAKs and receptor chains.

2.4. Functions of SOCS1 protein

2.4.1 SOCS1

Mouse and human SOCS1 proteins share 95–99% amino-acid identity. The mouse SOCS1 gene encodes a protein of 212 amino acids (Starr, Willson, and Viney 1997), while human SOCS1 is 211 amino acids long. The *SOCS1* gene lies within a large CpG island spanning 2.5 kb (Yoshikawa et al. 2001). SOCS1 was also known as SSI-1 and JAB, when it was discovered in 1997 by three groups using different experimental approaches: (i) as an IL-6-induced macrophage differentiation factor in murine monocytic leukemic M1 cells (Starr, Willson, and Viney 1997), (ii) as a protein named STAT-induced STAT-inhibitor (SSI), recognized by a monoclonal antibody directed against the STAT3 SH2 domain (Naka, Narazaki, and Hirata 1997) and (iii) as a protein that bound to the catalytic domain of JAK2 in a yeast two-hybrid screen that was named JAK-binding protein (JAB) (Endo and Masuhara 1997).

SOCS1 negatively regulates downstream signaling pathways of IFNα, IFN-γ, EPO, PRL, GH, LIF, TNF-α, IL-2, IL-3, IL-4, IL-6, IL-7, IL-12, IL-15, EPO, TPO, TSLP, Oncostatin

M (OSM) and Leptin as well as the receptors for insulin and IGF-1, and the TLRs (Walter & Hospital,2001). As discussed earlier, SOCS1 inhibits signaling by binding to JAK1, JAK2 and TYK2 and acts as a pseudosubstrate (Naka, Narazaki, and Hirata 1997; Endo and Masuhara 1997). Another mechanism is by proteasomal degradation of many signaling molecules including JAK1, JAK2, TEL-JAK2 and other substrates like the guanine nucleotide exchange factor VAV, insulin receptor substrate (IRS1 or IRS2) and focal adhesion kinase (FAK) (Piessevaux et al. 2008).

2.4.1.2 Role of SOCS1 in immunity

SOCS1 plays an important role in immunity. SOCS1-deficient mice die within 3 weeks of age before weaning due to fatty degeneration of the liver and infiltration of mononuclear cells in several organs. These mice exhibit decrease in the size of their thymus and loss of B lymphocytes in the bone marrow (Sciences 1998). The livers of SOCS1-deficient mice also show enhanced STAT1 activity and IFN-γ-inducible genes like iNOS and IRF-1 (Alexander et al. 1999). Abnormal T cell development and elevated IFN-γ levels are observed in SOCS1 deficient mice, suggesting that activated T cells are the source for IFN-γ. Hyper-responsiveness to IFN-γ signaling is the main cause of perinatal lethality in these mice and indicates that SOCS1 is a potent *in vivo* regulator of IFN-γ and T cell differentiation. These phenotypes were significantly reduced in *IFN-γ/SOCS1* double knockout mice and in SOCS1 knockout mice treated with anti-IFN-γ antibodies or by crossing *Socs1*-deficient mice with RAG2-deficient animals (RAG2, recombinant activating gene, is a key gene in lymphoid cell maturation (Marine et al. 1999; Nicola and Greenhalgh 2000; Cooney 2002; Alexander et al. 1999)). T cell conditional SOCS1 null mice show increased CD8+ differentiation as well as increased FOXP3$^+$ CD4$^+$ regulatory T cells in the thymus (Y. Zhan et al. 2009).

SOCS1 and SOCS3 play important regulatory roles in macrophages and dendritic cells (DCs) by modulating TLR signalling (Hanada et al. 2003; Nakagawa et al. 2002; Kinjyo et al. 2002). SOCS1 negatively regulates not only the JAK/STAT pathway, but also the TLR-

NFkB pathway (Ryo et al 2003 Molecular Cell; Yoshimura et al. 2004). SOCS1 KO mice also show enhanced insulin signaling (Jamieson et al. 2005). SOCS1 deficiency is associated with inflammatory pathologies such as arthritis, where exacerbated extent of joint destruction and synovial inflammation are observed due to deregulated pro-inflammatory cytokine signaling (Egan et al. 2003). Idiopathic pulmonary fibrosis is associated with enhanced inflammation and fibrosis, which is decreased by adenoviral delivery of SOCS1 (Nakashima et al., 2008). However, SOCS1 does not always confer protection against inflammatory or immune diseases. It has been shown that SOCS1 transgenic mice spontaneously develop colitis, with severe intestinal inflammation due to impaired expression of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), which is a negative regulator of T cell activation (A Sasaki et al. 2006).

2.4.1.2 SOCS1 as a tumor suppressor

SOCS1 is known as a tumor suppressor protein and is silenced by promoter hypermethylation and micro RNAs in various solid and non-solid cancers such as hepatocellular carcinoma (HCC), acute myeloid leukemia, glioblastoma multiforme, chronic myeloid leukemia (CML), breast cancer, ovarian cancer, cervical carcinogenesis and esophageal squamous cell carcinoma (Trengove and Ward 2013).

Aberrant methylation of CpG islands, which silences transcription of the genes, has been shown to be associated with cancer. The incidence of aberrant methylation of SOCS1 was found in up to 65% of human primary HCC tumor samples suggesting a tumor suppressor role of SOCS1 (Yoshikawa et al. 2001; B. Yang et al. 2003; Nagai et al. 2002). Heterozygous deletion of the *Socs1* gene in mice has shown enhanced carcinogenesis in a chemical (DEN) induced liver cancer model (T. Yoshida 2004) and confirms an antitumor role of SOCS1. This was subsequently confirmed in hepatocyte-specific *Socs1*-deficient mice (Yeganeh et al. 2016). Hepatitis C virus (HCV) is one of the causes of liver cancer. HCV core protein suppresses the expression of SOCS1 gene in human HCV-positive liver tissues. It is also reported that livers of HCV core protein gene transgenic mice and HepG2 cells expressing this protein down regulate the expression of SOCS1 (Miyoshi et al. 2005).

In addition to CpG methylation, micro RNAs have been implicated in downmodulating SOCS1 expression in tumors by directly binding to 3'-UTR of SOCS1. In breast cancer, miR-155 exerts its oncogenic role by negatively regulating *SOCS1* (S. Jiang et al. 2010). Similarly, miR-30d is implicated in downregulating SOCS1 expression in prostate cancer (Kobayashi et al. 2012).

The molecular mechanisms of tumor suppression by SOCS1 are far from clear. While attenuation of JAK-STAT signalling would be the key mechanism in controlling hematologic malignancies, other mechanisms may also operate. For instance, SOCS1 overexpression studies have reported SOCS1-mediated activation of the p53 tumor suppressor gene and inhibition of the paradoxical oncogenic functions of the cyclin-dependent kinase inhibitor 1A (CDKN1A, also known as p21) or by regulating Met receptor tyrosine kinase signaling as a tumor suppressor in cancer cells (Mallette et al. 2009; Warfel and El-deiry 2013; Y Gui et al. 2015; Yeganeh et al. 2016).

2.4.1.3 SOCS1 and its role in liver fibrosis (LF)

Cirrhotic nodules have long been considered to be premalignant lesions receding the pathogenesis of HCC. It has been suggested that genetic alterations accumulated during continuous regeneration of hepatocytes in an inflammatory milieu might lead to the development of HCC.

SOCS1 gene methylation was observed more frequently in HCC derived from cirrhotic livers than in those HCC that were not associated with cirrhosis (Okochi et al. 2003; Ogata et al. 2006). Yoshida and his group studied SOCS1 gene methylation in patients with chronic liver diseases to determine if SOCS1 genetic alteration is involved in LF progression as well as in HCC (Yoshida et al., 2004). This study reported a strong correlation between SOCS1 gene methylation and severity of LF. They also reported that Socs1$^{+/-}$ mice developed more severe LF than control mice following administration of the fibrogenic agent dimethylnitrosamine (DMN) suggesting a role for SOCS1 in controlling LF. However, it is not known how SOCS1 regulates the development of LF.

THESIS PREMISES

Liver fibrosis results from chronic damage to the liver characterized by the accumulation of ECM proteins, which is a typical feature of most types of chronic liver diseases. Continuous damage to the liver leads to the development of cirrhosis, which is associated with a significant mortality and morbidity. More than 90% of HCC is observed in cirrhotic livers (D. Y. Zhang and Friedman 2014). HCC is the 5th most common and 3rd most lethal cancer globally. Currently, there are a very few therapeutic options available to treat cancer. LF is reversible and is the final common pathological pathway of CLD regardless of its etiology. Therefore, understanding the cellular and molecular mechanisms involved in the progression of LF and development of rational antifibrotic strategies are considered the most promising approach in the fight against HCC.

Chronic damage to the liver results in the release of various cytokines and growth factors from liver resident cells as well as from recruited inflammatory cells, which are subsequently involved in the fibrogenic process. Cytokine and growth factor signaling is negatively regulated by cell intrinsic endogenous molecules such as the suppressor of cytokine signaling (SOCS) family proteins. It has been shown that the *SOCS1* gene is frequently suppressed in human HCC samples due to CpG methylation of its promoter region, suggesting an antitumor role of SOCS1 (Yoshikawa et al. 2001; B. Yang et al. 2003; Nagai et al. 2002). Yoshida *et al* observed methylation of SOCS1 gene in patients with CLD and found a strong correlation between SOCS1 gene methylation and the severity of LF (Takafumi Yoshida et al. 2004). Moreover, they also reported that SOCS1 haploinsufficient mice developed more severe LF than control mice following administration of the fibrogenic agent dimethylnitrosamine (DMN), suggesting a role of SOCS1 in the regulation of LF. Increased LF in these haploinsufficient mice was attributed to elevated STAT1 phosphorylation and interferon response factor 1 (IRF1) expression resulting from the increased IFN-γ signaling in the absence of SOCS1 (T. Yoshida 2004).

SOCS1 is an important regulator of IFN-γ signaling. Several experimental and clinical studies have shown that IFN-γ acts as an antifibrotic cytokine, (Czaja, Weiner, and Zern

1989; H. L. Weng, Cai, and Liu 2001; Z. Shi, Wakil, and Rockey 1997; Hong et al. 2002; W. Il Jeong et al. 2006; W.-I. Jeong, Park, and Gao 2008). However, another study reported that IFN-γ induces fibrosis (Knight et al. 2007). Thus, to clearly understand the role of SOCS1 and IFN-γ in LF, and whether SOCS1-regulated fibrogenic response is dependent on IFN-γ or not, we used $Socs1^{-/-}Ifng^{-/-}$ mice in models of chemical induction of LF.

Activated hepatic stellate cells (HSC) are the prime source for ECM production in the liver and play an important role in fibrogenesis (Friedman 2008). Though, activation pathways of HSCs have been well characterized, the regulatory mechanisms are not yet known. Thus, it is possible that SOCS1 might control LF by regulating HSC activation. In our study, we also examined the role of SOCS1 in HSC activation by isolating the primary HSC from $Socs1^{-/-}Ifng^{-/-}$, $Ifng^{-/-}$ and C57BL/6 mice.

HYPOTHESIS

SOCS1 is a critical regulator of hepatic fibrogenic response and part of this regulation may occur in HSC.

Objective of the study:

The specific aims of my research project are

1) To evaluate liver fibrosis in a *Socs1*-deficient mouse model.

I.   Induction of liver fibrosis with Dimethylnitrosamine (*DMN*) and Carbon tetrachloride (CCL$_4$)

II. Liver function test (measure ALT enzymes levels in the serum)

III. Gross anatomy and histopathological evaluation of the damaged liver (collagen deposition and accumulation of ECM)

IV. Quantitative Measurement of Collagen in the Liver by Hydroxyproline Content

V.   Evaluation of inflammatory cytokines, chemokines, and fibrogenic genes expression

VI. Immunohistochemical localization of α-SMA (specific marker of HSC)

VII. Western blot evaluation of α-SMA (HSC marker), p-SMADs (TGF-β signaling), IL-6 and IFN-γ downstream signaling molecules.

2. To determine the role of SOCS1 in HSC activation *in vitro*.

I.   Isolation of primary HSC from *Socs1*-deficient and control mice

II. Proliferation of HSC cells from *Socs1*-deficient and control mice by ^{3}H-(thymidine) incorporation assays.

Negative regulation of the Hepatic fibrogenic response by Suppressor of Cytokine Signaling 1

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Contribution:

Rajani Kandhi (myself) contributed to the 90% of the work presented. Subburaj Ilangumaran and Alfredo Menendez helped me in designing the experiments, analyzing the data and during the redaction of the manuscript. I carried out most of the experiments. Diwakar Bobbala, Mehdi Yeganeh and Marian Mayue provided technical assistance for the experiments.

Résumé :

Le suppresseur de la signalisation des cytokines 1 (SOCS1) est un régulateur indispensable de la signalisation de l'IFN-γ et a été aussi impliqué dans la régulation de la fibrose hépatique. Cependant, on ne sait pas si les fonctions anti-fibrotiques sont médiées directement dans le foie par SOCS1 ou par la modulation de l'IFN-γ, qui est connu pour son effet atténuateur de la fibrose hépatique. En outre, il est possible que SOCS1 contrôle la fibrose hépatique par la régulation des cellules stellaires hépatiques (CSH), un acteur clé dans la réponse fibrogénique. Alors que les voies d'activation des CSH ont été bien caractérisées, les mécanismes de régulation ne sont pas encore clairs. Les buts de cette étude étaient de dissocier la régulation de la réponse fibrogénique hépatique médiée par SOCS1 et celle dépendante de IFN-γ et d'élucider les fonctions régulatrices de SOCS1 dans l'activation des CSH. La fibrose hépatique a été induite chez des souris *Socs1*$^{-/-}$*Ifng*$^{-/-}$ par la diméthylnitrosamine ou le tétrachlorure de carbone. Les souris *Ifng*$^{-/-}$ et C57BL6 ont servi comme contrôles. Après les traitements fibrogéniques, les souris *Socs1*$^{-/-}$*Ifng*$^{-/-}$ ont montré des niveaux sériques élevés d'ALT ainsi que l'augmentation de la fibrose du foie par rapport à des souris *Ifng*$^{-/-}$. Le dernier groupe a montré des niveaux plus élevés d'ALT et de fibrose par rapport aux souris C57BL/6 contrôles. Les foies des souris déficientes en SOCS1 ont montré une fibrose septale, qui a été associée à une augmentation de l'accumulation des myofibroblastes et à un dépôt abondant du collagène. Les foies déficients en SOCS1 ont montré une expression accrue de gènes codant pour l'actine musculaire lisse, le collagène et les enzymes impliquées dans le remodelage de la matrice extracellulaire, à savoir les métalloprotéinases de la matrice et l'inhibiteur tissulaire des métalloprotéinases. Les CSH primaires de souris déficientes en SOCS1 ont montré une prolifération accrue en réponse à des facteurs de croissance tels que le HGF, EGF et le PDGF. Aussi, les foies fibrotiques de souris déficientes en SOCS1 ont montré une expression élevée du gène *Pdgfb*. Pris ensemble, ces données indiquent que SOCS1 contrôle la fibrose hépatique indépendamment de l'IFN γ et qu'une partie de cette régulation peut se produire en régulant la prolifération des HSC et en limitant la disponibilité des facteurs de croissance.

Mots clés : SOCS1, Diméthylnitrosamine, Tétrachlorure de carbone, Cellules stellaires hépatiques, PDGF.

Abstract

Suppressor of cytokine signaling 1 (SOCS1) is an indispensable regulator of IFNγ signaling and has been implicated in the regulation of liver fibrosis. However, it is not known whether SOCS1 mediates its anti-fibrotic functions in the liver directly, or via IFN□, which has been implicated in attenuating hepatic fibrosis. Additionally, it is possible that SOCS1 controls liver fibrosis by regulating hepatic stellate cells (HSC), a key player in fibrogenic response. While the activation pathways of HSCs have been well characterized, the regulatory mechanisms are not yet clear. The goals of this study were to dissociate IFN□-dependent and SOCS1-mediated regulation of hepatic fibrogenic response, and to elucidate the regulatory functions of SOCS1 in HSC activation. Liver fibrosis was induced in *Socs1$^{-/-}$Ifng$^{-/-}$* mice with dimethylnitrosamine or carbon tetrachloride. *Ifng$^{-/-}$* and C57BL/6 mice served as controls. Following fibrogenic treatments, *Socs1$^{-/-}$Ifng$^{-/-}$* mice showed elevated serum ALT levels and increased liver fibrosis compared to *Ifng$^{-/-}$* mice. The latter group showed higher ALT levels and fibrosis than C57BL/6 controls. The livers of SOCS1-deficient mice showed bridging fibrosis, which was associated with increased accumulation of myofibroblasts and abundant collagen deposition. SOCS1-deficient livers showed increased expression of genes coding for smooth muscle actin, collagen, and enzymes involved in remodeling the extracellular matrix, namely matrix metalloproteinases and tissue inhibitor of metalloproteinases. Primary HSCs from SOCS1-deficient mice showed increased proliferation in response to growth factors such as HGF, EGF and PDGF, and the fibrotic livers of SOCS1-deficient mice showed increased expression of the *Pdgfb* gene. Taken together, these data indicate that SOCS1 controls liver fibrosis independently of IFN□ □and that part of this regulation may occur via regulating HSC proliferation and limiting growth factor availability.

Keywords: liver fibrosis; SOCS1; dimethylnitrosamine; carbon tetrachloride; hepatic stellate cells; PDGF.

Highlights

- SOCS1 regulates hepatic fibrogenic response independently of IFN γ.

- SOCS1 modulates the expression of matrix metalloproteinases and their inhibitors in the liver.

- SOCS1 regulates hepatic stellate cell proliferation in response to growth factors.

1. Introduction

Liver fibrosis (LF)[1], which results from a deregulated tissue repair process, is a common feature of hepatitis virus infections and alcoholic liver disease [1-3]. LF also occurs in obesity, an important risk factor for hepatocellular carcinoma (HCC) [4]. These chronic inflammatory stimuli induce abnormal deposition of extra-cellular matrix (ECM), progressively replacing the liver parenchyma with fibrous scar tissue, leading to the end-stage disease called cirrhosis [1, 5]. Cirrhotic livers typically harbor regenerative nodules wherein hepatocytes undergo compensatory proliferation to restore the declining metabolic functions of the liver. The increased rate of hepatocyte proliferation in an inflammatory milieu leads to accumulation of genetic aberrations that give rise to hyperplastic and dysplastic nodules, and eventually to HCC [6]. Indeed, more than 90% of HCC occurs in cirrhotic livers [7].

Cirrhosis is a major healthcare burden and an important cause of global mortality, while HCC ranks as the 5th most common and 3rd most lethal cancer worldwide [8-11]. The pathogenesis of HCC spans over decades and the clinical onset is so insidious that very few therapeutic options are available to treat HCC [12]. On the other hand, the fibrogenic process that precedes cirrhosis is amenable to treatment, although the current therapeutic methods have not yet achieved the desired objective [7, 13, 14]. A greater understanding of the cellular and molecular underpinnings of liver fibrosis and HCC, and the normal regulatory controls of these pathogenic processes will accelerate the efforts to develop effective and targeted therapies for LF, cirrhosis and HCC [1, 7, 12, 15-17].

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[1] **Abbreviations:** ALT, alanine transferase; CCl4, carbon tetrachloride; DMN, dimethylnitrosamine; ECM, extra-cellular matrix; GBSS, Gey's Balanced Salt Solution; HCC, hepatocellular carcinoma; HSC, hepatic stellate cells; LF, Liver fibrosis; MMP, matrix metalloproteinase; NDP, nanazoomer Digital Pathology; RTK, receptor tryrosine kinase; SOCS, suppressor of cytokine signaling; SMA, alpha smooth muscle actin; TIMP, tissue inhibitors of MMP.

Cytokines and growth factors play crucial roles in hepatic fibrogenesis and compensatory proliferation of hepatocytes following liver damage [18-20]. An important regulatory mechanism of cytokine and growth factor signaling involves feedback inhibition by the suppressor of cytokine signaling (SOCS) family proteins [21-24]. The *SOCS1* gene is frequently repressed by epigenetic CpG methylation in up to 65% of HCC [25, 26], suggesting a tumor suppressor role of SOCS1 in the liver. This notion is supported by increased susceptibility of *Socs1*$^{+/-}$ mice to experimental HCC [27] and suppression of the *SOCS1* gene by hepatitis C virus [28].

SOCS1 is also implicated in controlling LF. Indeed, methylation of the *SOCS1* gene correlates with fibrosis and cirrhosis in human HCC [27, 29, 30]. Yoshida *et al*., have reported that *Socs1*$^{+/-}$ mice developed more severe LF than control mice following administration of the fibrogenic agent dimethylnitrosamine (DMN) [27]. This study did not use *Socs1*$^{-/-}$ mice as they die within two weeks after birth due to uncontrolled IFN□ signaling in the liver, necrosis of hepatocytes and fatty degeneration of the liver, all of which could be prevented by administration of neutralizing anti-IFN□ antibody or by deleting the *Ifng* gene [31, 32]. Yoshida *et al*., correlated the increased liver fibrosis in *Socs1*$^{+/-}$ mice to elevated STAT1 phosphorylation and interferon response factor 1 (IRF1) expression [27], presumably resulting from increased IFN□ signaling in the absence of SOCS1. However, IFN□ has also been shown to protect against liver fibrosis and ablation of the *Ifng* gene exacerbates liver fibrosis induced by DMN or carbon tetrachloride (CCl4) [33-37]. In agreement, mice lacking STAT1 developed accelerated CCl4-induced liver fibrosis, and the protective function of STAT1 was attributed to inhibition of HSC proliferation and stimulation NK-cell-mediated killing of HSCs [38]. In contrast to these reports, IFN□ has been shown to exacerbate liver fibrosis in mice fed with choline-deficient, ethionine-supplemented diet via enhancing hepatic progenitor cell response [39].

In the light of the above reports, increased hepatic fibrosis observed in *Socs1*$^{+/-}$ mice with intact *Ifng* gene is untenable with the protective functions of IFN□, which ought to be more efficient in these mice. Given that SOCS1 is an indispensable regulator of IFN□ signaling [31, 32], it is also possible that SOCS1 haplo-insufficiency might have exacerbated the pro-

fibrogenic role of IFN□ [39] in the absence of one *Socs1* allele. Alternatively, the protective functions of IFN□ may be mediated by SOCS1, requiring both alleles for efficient control of the fibrogenic response. Lastly, SOCS1 may be induced to exert its anti-fibrogenic functions independently of IFN□, probably by regulating other pro-fibrogenic pathways. To test these possibilities, we studied chemical-induced liver fibrosis in the absence of both alleles of SOCS1 without the influence IFN□□ by using *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice in two different liver fibrosis models. As controls, we used *Ifng*$^{-/-}$ and C57BL/6 mice. Our findings show that SOCS1 is a critical regulator of hepatic fibrogenic response and that part of this regulation may occur in HSCs.

2. Material and Methods

2.1. Animals

Socs1$^{-/-}$*Ifng*$^{-/-}$ and *Ifng*$^{-/-}$ mice in C57BL/6 background have been previously described [40]. Only male mice were used to induce HCC due to the protective effects of female sex hormones [41]. All experimental protocols on animals were carried out with the approval of the Université de Sherbrooke Animal Ethics Committee.

2.2. Induction of liver fibrosis

Liver fibrosis was induced in 8-10 week-old mice by intra-peritoneal injection of DMN or CCl4 following published methods [27, 42] (Fig. 1A). Briefly, DMN (Supelco, Bellefonte, PA; Cat #48552) was administered via intra-peritoneal (i.p) route (10□g/g body weight) every 2-3 days for three weeks. CCl4 (Sigma-Aldrich, Oakville, ON; 0.5□l/g body weight) was diluted in corn oil (1:2) and injected i.p. twice a week for five weeks. Treated mice were sacrificed three or four days after the last treatment. Serum samples and liver tissues were collected. Parts of the livers were fixed in buffered formalin or snap frozen and stored at -80ºC until use.

2.3. Gene expression analysis

RNA was extracted using RiboZol™ (AMRESCO, Solon, OH) and reverse transcribed using QuantiTect Kit (Qiagen). RNA purity was evaluated by UV absorption and RNA quality was tested by denaturing formaldehyde-agarose gel electrophoresis. The first complementary strand was made from 1µg total RNA using QuantiTect® reverse transcription kit (Qiagen). The primers for gene expression analysis (Supplementary Table 1) showed 90-100% efficiency and a single melting curve in the MyQi5® cycler (Bio-Rad, Mississauga, ON). Expression levels of the housekeeping gene *Gapdh* was used to calculate fold induction of the specific genes modulated by the presence or absence of SOCS1.

2.4. Histology and immunohistochemistry

Liver tissues were fixed in 10% buffered formalin and embedded in paraffin. 5µM thick liver sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E), Sirius red or Mason's trichrome stains following standard histochemical protocols. For immunohistochemistry of alpha smooth muscle actin (SMA), rehydrated liver sections were immersed in citrate buffer (pH 6.0) and microwaved intermittently for 10 min to retrieve antigenic epitopes. Slides were incubated in 3% hydrogen peroxide for 10min to eliminate endogenous peroxidase activity. After blocking with 5% BSA in Tris-buffered saline (TBS) containing 20% Tween-20 (TBS-T), the slides were incubated with mouse anti-SMA Ab (Abcam) at 4˚C overnight before applying horseradish peroxidase (HRP)-conjugated secondary Ab for 1h and developed with 3, 3'-diaminobenzidine (Sigma-Aldrich). The sections were counterstained with hematoxylin and mounted with a coverslip. Digital images of the stained sections were acquired using a Nanozoomer Slide Scanner and analyzed by the Nanozoomer Digital Pathology software (Hamamatsu Photonics, Japan). The NIH ImageJ software was used to quantify Sirius red staining and the SMA-positive areas.

2.5. Hydroxyproline assay

Hydroxyproline content was measured following published methods with slight modifications [43, 44]. Approximately 10 mg of liver tissue was homogenized in 1ml of 6N HCl using the bead mill MM 400 (Retsch, Hann, Germany), transferred to screw-capped glass tubes and topped up with 2ml of 6N HCl. The tubes were kept on a heat block at 110°C for 16 hours in a fume hood to hydrolyze proteins into amino acids. The hydrolysate was filtered through Whatman #1 filter paper, aliquots were evaporated on the heat block and the pellet was dissolved in 50% 2-propanol. Samples and hydroxyproline standards were distributed in a 96-well microtiter plate, and oxidized by adding chloramine T reagent (Sigma-Aldrich; dissolved in 50% isopropanol and adjusted to pH 6.5 with acetate/citrate buffer) and incubating for 25min at room temperature. Following the addition of Ehrlich reagent (*p*-dimethylaminobenzaldehyde dissolved in n-propanol/perchloric acid (2:1)), the chromophore was developed by incubating the samples at 50°C for 10min. Absorbance was read at 550nm using a spectrophotometer and the results were expressed as micrograms of hydroxyproline per gram of liver tissue.

2.6. Serum ALT level and ELISA

Serum alanine transferase (ALT) levels were measured using a kinetic assay (Pointe Scientific Inc, Brussels, Belgium). Serum IL-6 and TNF-α levels were measured by sandwich ELISA, using capture and detection Ab from BD Pharmingen Biosciences (San Diego, CA).

2.7. Western blot

Liver tissues were homogenized in a pH8.0 buffer containing 150mM NaCl, 50mM Tris-HCl, 1mM EDTA and protease and phosphatase inhibitor coctails (Roche, Indianapolis, IN) for 2min using the bead mill MM 400 (Retsch, Hann, Germany). Equal volume of the same buffer containing the detergents 0.2% SDS, 1% Triton X- 100 and 1% sodium deoxycholate was added and the lysates were kept on a shaker at 4°C for 30min. Following centrifugation at 15000g for 20min, the supernatant was collected and protein concentration determined using the RC-DC Protein Assay Kit (Bio-Rad, Mississauga, ON). Aliquots of

30-50µg proteins were analyzed by western blot using Ab listed in Supplementary Table 2. Secondary antibodies and enhanced chemiluminescence reagents were from GE Healthcare Life Sciences (Pittsburg, PA). Western blot images were captured by the VersaDOC 5000 imaging system (Bio-Rad).

2.8. Hepatic stellate cell isolation and proliferation assay

HSCs were isolated following published methods [38, 45]. Briefly, 12-16 week-old mice were anesthetized with an i.p. injection of a mixture of Ketaset® (100mg/kg, Wyeth) and Xylazine (10mg/kg, Rompun®, Bayer). The liver was first perfused with calcium-free Gey's Balanced Salt Solution (GBSS) containing 0.5mM EGTA at the rate of 7ml/min through the inferior vena cava. The liver was then digested by perfusion with collagenase type IV (100U/ml, Worthington, Lakewood, NJ) in GBSS supplemented with $CaCl_2$ (1.8mM). The liver was resected into a culture dish, the capsule was removed and minced to release the cells into medium. The cell suspension was filtered through 70□m nylon mesh and centrifuged at 500rpm (60*g*) for 5min three times to remove hepatocytes. The supernatant was centrifuged at 1600rpm (600*g*) for 10min at 4°C. After one washing step, the pellet was resuspended in 4 ml of 20% of Optiprep, 5ml of 11.5% Optiprep was gently overlaid and topped with 2ml of GBSS. The density gradient was centrifuged at 3000rpm for 17min without brake and stellate cells that collect at the interface of 11.5% optiprep and GBSS were centrifuged at 1600rpm for 10min at 4°C. The cells were washed in DMEM containing 20% fetal bovine serum (FBS) and cell number and viability were assessed by Tryptan blue exclusion. The cells were plated at a density of $1x10^5$ cells/well in uncoated 6-well tissue culture dishes and examined by phase contrast microscopy for the presence of lipid droplets and by fluorescence microscopy for auto-fluorescence from their vitamin A content.

To assess cell proliferation, HSCs were resuspended in serum-free DMEM and plated in 96-well flat bottom microtiter plates in the presence of the indicated growth factors (PDGF, EGF, TGF-β, HGF - 10ng/ml) and cytokines (IL-6, TNF-α - 10ng/ml) purchased from R&D systems (Minneapolis, MN) or Peprotech (Ricky Hill, NJ). One □Ci of methyl-[^{3}H]-

thymidine (NEN Life Sciences, Boston, MA) was added during the last 18h of the 72h culture period and the incorporated radioactivity measured as described elsewhere [40].

2.9. Statistical Analysis

Data were analyzed using the GraphPad Prism (San Diego, CA). Statistical significance was calculated by ANOVA or Mann-Whitney test, and p values <0.05 were considered significant.

3. Results

3.1. Fibrogenic treatment induces intrahepatic mononuclear cell infiltration in SOCS1-deficient mice

To evaluate the effect of SOCS1 deficiency on hepatic fibrogenic response, we first used the DMN-induced liver fibrosis model. Unlike $Socs1^{+/-}$ mice, which showed more than 50% mortality following DMN injection [27], three weeks of DMN treatment did not cause mortality in $Socs1^{-/-}Ifng^{-/-}$ mice. These mice did not show any overt illness, and their serum ALT levels were not significantly elevated compared to $Ifng^{-/-}$ and C57BL/6 control mice at the end of the treatment regimen (Fig. 1B). However, all groups of mice showed increased ALT levels after one week of DMN treatment that subsided after three weeks of exposure. Examination of the liver sections at the end of the treatment period by H&E staining did not reveal noticeable damage to the micro-architecture in the livers of $Socs1^{-/-}Ifng^{-/-}$ mice compared to the control mice (Fig. 1C). However, the SOCS1-deficient livers showed marked mononuclear cell infiltration around the portal triads and the central vein, which was not observed in the livers of control mice following DMN treatment.

We also examined liver fibrosis induced by CCl4, which has certain advantages over the DMN-induced fibrosis model [17]. DMN, which induces hemorrhagic centrilobular fibrosis, also displays mutagenic properties that could confound the fibrogenic response. This issue is not a concern with CCl4, which induces reversible centrilobular fibrosis. Besides, CCl4-induced fibrosis is more reproducible in C57BL/6 mice, which are in

general less susceptible to liver fibrosis [34]. In contrast to DMN treatment, serum ALT levels at the end of the CCl4 treatment regimen was significantly elevated in all three genotypes of the mice examined, and there was no significant difference between SOCS1-deficient and control mice (Fig. 1B). However, similarly to DMN, CCl4 induced marked mononuclear cell infiltration in SOCS1-deficient livers compared to controls, albeit to a lesser extent than DMN (Fig. 1C). Collectively, these results indicated that SOCS1 deficiency exacerbated the inflammatory response in the liver cause by fibrogenic stimuli.

3.2. SOCS1 deficiency increases the expression of inflammatory cytokines and chemokines in the liver and the circulating levels of IL-6 and TNF-α

SOCS1 is an important regulator of macrophage activation by TLR ligands and cytokine-dependent control of T lymphocyte homeostasis [21, 23]. Therefore, we examined whether the mononuclear cell infiltration in the livers of DMN- or CCL4- treated mice is associated with an increased inflammatory response. As shown in Fig. 2A, the transcript levels of inflammatory cytokines IL-6 and TNF-α were significantly elevated in SOCS1-deficient livers following DMN or CCl4 treatment. Accordingly, SOCS1 deficient mice showed increased levels of IL-6 and TNF-α in the peripheral circulation (Fig. 2B). Even though the induction of *Il6* and *Tnfa* genes in the livers of DMN- or CCl4- treated mice was comparable, the circulating levels of IL-6 and TNF-α were markedly elevated in CCl4-treated mice compared to DMN-treated mice.

CCL-2 (macrophage chemotactic protein 1), a chemokine secreted by HSCs and hepatocytes in fibrotic livers, is the principal mediator of monocyte influx into the inflamed liver [46]. The *Ccl2* gene was strongly induced by both DMN and CCl4, and SOCS1-deficient livers showed significantly high *Ccl2* gene expression compared to control mice (Fig. 2A). In contrast to serum ALT levels, which were increased to a comparable extent in *Socs1$^{-/-}$Ifng$^{-/-}$* and *Ifng$^{-/-}$* mice following CCl4 treatment, hepatic *Il6, Tnfa* and *Ccl2* gene expression and circulating IL-6 and TNF□ levels were significantly higher in *Socs1$^{-/-}$Ifng$^{-/-}$* mice than in *Ifng$^{-/-}$* mice. These results indicate that the absence of SOCS1 in the liver induces a strong inflammatory response that can occur independently of IFN-α.

3.3. SOCS1 deficient mice show increased hepatic fibrosis following chemical injury

Next, we examined liver sections by Sirius red staining to assess the extent and pattern of collagen deposition in the fibrotic livers. As shown in Fig. 3A, livers of $Socs1^{-/-}Ifng^{-/-}$ mice showed markedly strong fibrosis compared to $Ifng^{-/-}$ and C57BL/6 mice. $Ifng^{-/-}$ mice showed discernibly more fibrosis than C57BL/6 controls. While $Socs1^{-/-}Ifng^{-/-}$ mice showed extensive portal fibrosis with the formation of septa around many hepatic lobules, resembling the F2 stage fibrosis [47, 48], septal involvement was considerably less in $Ifng^{-/-}$ mice. Similar results were obtained following fibrosis induction with CCl4, which induced more pronounced fibrosis and produced more consistent results than DMN (Fig. 3A, bottom row). Moreover, CCl4 induced extensive septa formation in $Socs1^{-/-}Ifng^{-/-}$ mice, resembling the F3 stage fibrosis. Quantification of the Sirius red staining area showed significantly more fibrosis in $Socs1^{-/-}Ifng^{-/-}$ mice than in $Ifng^{-/-}$ mice (Fig. 3B). Mason's trichrome staining recapitulated the results of Sirius red staining with similarly high level of collagen deposition in $Socs1^{-/-}Ifng^{-/-}$ mice compared to control mice (Fig. 3C).

We quantified total collagen deposition in the liver parenchyma by measuring the hydroxyproline content of the fibrotic liver tissues (Fig. 4). Consistent with the histochemical staining data, the livers of $Ifng^{-/-}$ mice contained significantly more collagen than C57BL/6 mice. These results are in agreement with the anti-fibrotic role of IFN□ in DMN- or CCl4- induced hepatic fibrosis models [33-37]. However, $Socs1^{-/-}Ifng^{-/-}$ mice contained significantly more collagen than the livers of $Ifng^{-/-}$ mice (Fig. 3B). Collectively, the above results show that SOCS1 exerts a potent anti-fibrotic role in the liver, independently of IFN□.

3.4. SOCS1 deficient livers show increased accumulation of myofibroblasts and fibrogenic gene expression

During liver fibrosis, HSCs and other liver-resident and recruited mesenchymal cells become activated and differentiate into myofibroblasts that produce ECM [49]. To study the collagen-producing myofibroblats, we examined the liver sections of DMN- or CCl4-

treated mice by immunohistochemistry for smooth muscle actin (SMA), a marker of activated HSCs and myofibroblats. Consistent with the histochemical staining of collagen fibers, the livers of $Socs1^{-/-}Ifng^{-/-}$ mice showed more abundant SMA positive cells than the livers of $Ifng^{-/-}$ and C57BL/6 mice (Fig. 5A, 5B), indicating an important role for SOCS1 in controlling the differentiation and activation of collagen-producing myofibroblats.

To further characterize the SOCS1-dependent control the hepatic fibrogenic response, we studied the expression of *Acta2, Col1a1, Col3a1* genes coding for SMA and the fibrillar collagens I and III, respectively [50]. As shown in Fig. 6A, the expression of *Acta2*, *Col1a1* and *Col3a1* was significantly elevated in the livers of $Socs1^{-/-}Ifng^{-/-}$ mice, showing 2-3-fold higher induction than control mice. Induction of these genes was 3-6 fold more pronounced in CCl4-induced fibrosis than in DMN-induced fibrosis, in agreement with more severe fibrosis in the former model (Fig. 2). The key pro-fibrogenic cytokine gene *Tgfb* was also more strongly induced in the livers of $Socs1^{-/-}Ifng^{-/-}$ mice than in $Ifng^{-/-}$ mice in both DMN and CCl4 models. Strikingly, the induction of *Acta2, Col1a1, Col3a1 and Tgfb* genes was only marginally increased in $Ifng^{-/-}$ mice compared to wildtype mice.

3.5. Expression of genes coding for matrix remodeling are differentially regulated by SOCS1

In parallel to assessing the collagen genes, we quantified the expression of genes coding for enzymes involved in remodeling the extracellular matrix (ECM), namely, matrix metalloproteinases (*Mmp1, Mmp2, Mmp3, Mmp9*) and tissue inhibitors of MMPs (*Timp1* and *Timp2*). While TIMP-1 and TIMP-2 play a pro-fibrogenic role, the MMPs may exert pro- or anti-fibrotic roles at different stages of fibrosis [51, 52]. The expression of *Timp1* and *Timp2* genes was significantly higher in the livers of $Socs1^{-/-}Ifng^{-/-}$ mice than in $Ifng^{-/-}$ mice in both DMN and CCl4 models (Fig. 6B). $Ifng^{-/-}$ mice showed only marginal or negligible increase in expression of these genes compared to C57BL/6 controls. The expression of MMP genes showed profound variability. *Mmp1* was expressed at a significantly lower level in $Socs1^{-/-}Ifng^{-/-}$ mice than in $Ifng^{-/-}$ mice in both DMN and CCl4 models. *Mmp2* was marginally increased in SOCS1-deficient livers, and this increase was significant only in the DMN-induced fibrosis model. Strikingly, the expression of *Mmp3*

and *Mmp9* in SOCS1-deficient livers was differentially modulated in the two models (Fig. 6B), with a significant decrease in the DMN-induced model, but an increase in the CCl4 model. Similar to the collagen genes, IFN☐ deficiency alone caused only moderate, non-significant changes in the expression of the ECM remodeling genes. These results indicated that SOCS1 exerts a more critical control over the induction of fibrogenic genes than IFN☐.

3.6. *Fibrotic livers of SOCS1 deficient mice show increased phosphorylation of STAT3 and Smad3*

Increased DMN-induced hepatic fibrosis observed in *Socs1* heterozygous mice has been correlated to elevated STAT1 phosphorylation, increased IRF-1 expression and decreased STAT3 activation [27]. Previously, IFN☐ was shown to be the key cytokine in inducing STAT1 activation in the ConA-induced model of hepatitis [36]. Therefore, we examined phosphorylation of STAT1 and STAT3 in *Socs1*$^{-/-}$*Ifng*$^{-/-}$, *Ifng*$^{-/-}$ and control mice following the DMN and CCl4 regimens. The DMN-treated *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice did not show elevated STAT1 activation in the liver, but showed increased STAT3 phosphorylation compared to both *Ifng*$^{-/-}$ and C57BL/6 controls mice (Fig. 7A). *Ifng*$^{-/-}$ mice showed the same level of STAT3 phosphorylation in the liver as C57BL/6 control mice, but showed appreciable reduction in STAT1 phosphorylation, in agreement with the absence of IFN☐. Phosphorylation of AKT, which occurs downstream of cytokines and growth factors in hepatocytes, was not significantly different among the three groups of DMN-treated mice. In agreement with increase fibrosis and SMA staining, the livers of SOCS1 deficient mice showed increased levels of SMA and showed elevated Smad3 phosphorylation, which is the key signaling protein downstream of the fibrogenic cytokine TGF-β. These results indicated that unlike in other models, fibrosis induction in SOCS1-deificient is associated with elevated STAT3 activation.

3.7. *SOCS1 controls hepatic stellate cell proliferation*

Elevated levels of *Tgfb* transcripts (Fig. 6A), increased Smad3 phosphorylation (Fig. 7A), and increased proportion of SMA-staining cells (Fig. 5) in SOCS1-deficient livers in the

two fibrosis models suggested that quantitative increase in HSC numbers and their increased responsiveness to fibrogenic cytokines may underlie the severe hepatic fibrosis observed in these mice. Pathogenesis of liver fibrosis is associated with HSC proliferation, which can occur in response to several growth factors such as PDGF, FGF, EGF, and TGF-β that signal via receptor tryrosine kinases (RTK) [53, 54]. Cytokines such as IL-6 and IL-4 may also induce HSC proliferation directly or indirectly. In recent years, several reports have shown that several SOCS proteins including SOCS1 regulate not only JAK-STAT signaling, but also control signaling via RTKs [24]. We have shown that SOCS1 regulates HGF signaling via its receptor c-MET [40, 55]. Therefore, we evaluated proliferation of primary HSCs isolated from SOCS1-deficient and control mice in response to growth factor and cytokine stimulation. HSCs from both *Socs1*$^{-/-}$*Ifng*$^{-/-}$ and *Ifng*$^{-/-}$ mice showed significantly higher proliferation to PDGF than control cells. SOCS1 deficient HSCs also showed increased proliferation to EGF, TGF-β and HGF as well as IL-6.

Next, we examined the induction of PDGF gene expression in the livers following the DMN or CCl4 treatment. As shown in Fig. 8B, the livers of *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice showed significantly higher expression of the *Pdgfb* gene than *Ifng*$^{-/-}$ and C57Bl/6 mice. Together, these results suggest that SOCS1 may regulate hepatic fibrogenic response, at least partly, by controlling the proliferation of HSCs and indirectly by limiting the availability of growth factors.

Discussion

Pathogenesis of liver fibrosis involves chronic injury to hepatocytes, release of inflammatory mediators and activation of HSCs, Kupffer cells and lymphocytes. Many cytokines and growth factors induced during this inflammatory response impact the pathogenesis of liver fibrosis in a very complex, inter-connected manner. Specific gene knockout mice have shed light on the complex signaling pathways and control mechanisms in liver fibrosis. SOCS1 and SOCS3, which are important feedback negative regulators of many cytokines and growth factors, are implicated in hepatic fibrogenic response. However, the molecular mechanisms by which SOCS proteins control liver fibrosis remain unclear. Using two chemically-induced liver fibrosis models, here we show that (i) SOCS1

limits liver fibrosis, (ii) SOCS1 deficiency differentially affects the expression of ECM remodeling enzymes and (iii) the anti-fibrotic functions of SOCS1 occurs, at least partly, in HSCs.

Our results clearly show that SOCS1 deficiency exacerbates the hepatic fibrogenic response in an IFN□-independent manner. Yoshida et al., correlated the increased susceptibility of *Socs1*^{+/-} to DMN-induced liver fibrosis to STAT1 activation and IRF1 expression, and reduced STAT3 activation [27]. This idea was supported by an earlier study that showed a pathogenic role for STAT1 and a protective role for STAT3 in the ConA–induced hepatitis model [36]. This study showed that IFN-γ, produced by CD4^{+} T cells and NKT cells, activated STAT1, induced IRF1 and Bax, and promoted apoptosis of hepatocytes, while STAT3 activation downstream of IL-6 induced Bcl-xL and reduced hepatocyte injury caused by IFN□ [36]. Contrary to these reports, increased liver injury in *Socs1*^{-/-}*Ifng*^{-/-} mice occurred in the absence of elevated STAT1 activation but in the presence of increased *Il6* gene expression, elevated serum IL-6 levels and increased STAT3 phosphorylation in the liver following DMN or CCl4 treatment.

The elevated STAT1 activation reported in the livers of *Socs1*^{+/-} mice following DMN treatment most likely results from deregulated IFN□ signaling due to the limited availability of SOCS1. Even though DMN or CCl4-induced liver damage does not usually result in extensive T cell and NKT cell activation and IFN□ production as in the case of ConA-induced hepatitis [56], the lack of SOCS1 may exacerbate the basal IFN□ signaling that may be induced in these mice with intact *Ifng* alleles. Indeed several studies have shown a pro-fibrotic effect of IFN□ and STAT1 in DMN or CCl4 –induced fibrosis [33-38]. Our finding that *Socs1*^{-/-}*Ifng*^{-/-} mice develop severe liver fibrosis despite the absence of IFN□ and an appreciable increase in STAT1 activation highlight the critical requirement of SOCS1 to control pro-fibrogenic pathways that can occur independently of IFN□ or STAT1 activation. Besides, our findings indicate that SOCS3, which is also implicated in attenuating liver fibrosis [30], does not compensate for the loss of SOCS1, and that these two SOCS proteins may target distinct fibrogenic mechanisms.

The roles of STAT1 and STAT3, their main activators IFN-γ and IL-6, and their major regulators SOCS1 and SOCS3, respectively, have been extensively studied in the context of hepatitis, fibrosis and HCC [reviewed in [56]]. In this work, Gao and colleagues highlighted the dichotomous roles of STAT3 activation and IL-6 in liver injury, hepatic inflammation and fibrosis, and modulation of their functions in model-, cell-, and context-dependent manner. While STAT3 in hepatocytes confers protection against liver injury and fibrosis, it may play a pro-fibrogenic role in HSCs by promoting their survival and production of TIMP-1, and in Kupffer cells by increasing TGF-β production [57-59]. Similarly, while IL-6 protects hepatocytes from injury that may account for its anti-fibrotic role reported in some studies, several lines of evidence support a pro-fibrotic role of IL-6 by promoting HSC survival and proliferation [56]. Given that the livers of *Socs1*^{-/-}*Ifng*^{-/-} mice show increased expression of *Il6, Tgfb* and *Timp1* genes following DMN or CCl4 treatment, it is likely that the extensive fibrosis seen in these livers may result from increased IL-6-induced STAT3 activity. In agreement with this possibility, increased fibrosis in DMN-treated, hepatocyte-specific SOCS3-null mice has been attributed to increased STAT3 activation [30].

The increased expression of *Timp1* and *Timp2* in the livers of *Socs1*^{-/-}*Ifng*^{-/-} mice may also contribute to the exacerbated fibrogenic response in these mice. Both Timp-1 and Timp-2 promote fibrosis by inhibiting degradation and clearance of the fibrotic matrix [reviewed in [60]]. Besides, they may directly contribute to fibrosis by promoting survival of HSCs. As in the case of IL-6 and Stat3, Timp-1 seems to exert a dichotomous role in liver fibrosis. While an early study using transgenic model showed a pro-fibrogenic role for Timp-1, a recent study using Timp1-null mice showed an anti-fibrotic function [61, 62]. The latter study proposed that Timp-1 might promote liver fibrosis via stimulating HSC survival, while its anti-fibrotic function may occur via attenuating hepatocyte injury. Given the increased SMA positive staining (Fig. 5) and the quantity of SMA (Fig. 7) in the livers of DMN- or CCl4- treated SOCS1-null livers, it is likely that Timp-1 might have contributed to the survival of HSCs and their activation to myofibroblats in these mice, in addition to blocking ECM degradation.

A collective analysis of the roles of MMPs in liver, lung and kidney fibrosis models have clearly shown that MMPs exert both inhibitory and stimulatory activities in tissue fibrosis [52]. The physiological roles of MMPs include not only ECM remodeling, but also diverse other functions such as generation of bioactive ligands that act in autocrine and paracrine manner to module cell survival, proliferation, differentiation and migration [63]. In the liver, HSCs are the major source of MMPs [64]. Even though MMPs can cleave diverse collagens and other ECM substrates in vitro, whether all such activities occur *in vivo* is not yet clear. The *in vivo* biological activities of MMPs are influenced by their differential expression at different stages of fibrosis progression and resolution, and their ability to degrade the normal ECM versus the interstitial, fibrillar collagens that accumulate during fibrosis [50, 52, 64, 65]. While MMP-1 and MMP-2 are implicated in anti-fibrotic roles, MMP-3 is and MMP-9 (which is related to MMP-2) are implicated in promoting fibrosis [52]. While MMP-1 promotes degradation of collagen matrix, MMP-2 is implicated suppressing collagen-I expression, both contributing to the resolution of fibrosis [52, 60]. Consistent with these functions, *Mmp1* expression is drastically diminished in the fibrotic livers of SOCS1 deficient mice, while *Mmp2* was not significantly altered compared to control livers (Fig. 6B). Few studies have addressed the role of MMP-3 and MMP-9 in liver fibrosis. MMP-9 is an important mediator of leukocyte recruitment in acute liver injury [60]. A recent study showed that induction of miR155, which represses SOCS1, increased the expression of MMP-9 in HCC cells lines and increased their invasive potential [66]. Intriguingly, MMP-9 is differentially regulated in DMN- and CCl4-induced fibrosis in the livers of SOCS1-null mice (Fig. 6B). The significance of this differential expression in the two models, both of which initiate the fibrogenic response by inducing centrilobular necrosis, remains to be studied. Similarly, understanding the potential contribution of SOCS1-dependent modulation of cytokine and growth factor signaling to the induction of MMPs in liver fibrosis requires further investigation.

SOCS1 may exert direct control over the survival, proliferation and activation of hepatic stellate cells induced by growth factors that signal via RTKs [53, 54]. Even though SOCS molecules were originally discovered as feedback regulators of JAK-STAT signaling, accumulating evidence has shown their importance in regulating RTKs [24]. In

keeping with that, we have shown that SOCS1 attenuates HGF signaling in hepatocytes [40, 55]. Increased proliferation of SOCS1 deficient HSCs in response to many growth factors that signal via RTKs (PDGF, TGF-β, EGF and HGF), and increased expression of *Pdgfb* gene in the fibrotic livers of SOCS1-deficient mice suggest control of HSC proliferation and limiting the availability of growth factors are important mechanisms by which SOCS1 exerts its anti-fibrotic function.

Recent studies have shown that Sorafenib, which inhibits the RAF kinase downstream of many RTKs as well as their own enzymatic activity, displays a marked anti-fibrotic effect in vivo and this effect has been correlated to decreased HSC proliferation and collagen production [67, 68]. Repression of the *SOCS1* gene by promoter methylation frequently occurs in human liver fibrosis, and the frequency of methylation correlates positively with the severity of disease [27]. Even though various approaches such as DNA demethylases, gene therapy, peptide mimics, protein transfer, microRNA are being tested in various experimental disease settings, their optimization and use in human trials are still far from realization. Given that Sorafenib is an FDA approved drug, and that SOCS deficiency promotes HSC proliferation and fibrogenic gene expression in the mouse models, we propose that liver fibrosis patients with SOCS1 promoter methylation may benefit from Sorafenib therapy.

Authorship contributions

RK, AM and SI designed the experiments, analyzed data and wrote the manuscript. RK carried out most of the experiments. DB, MY and MM provided assistance to experiments.

Disclosure of Conflicts of Interest: The authors declare no potential conflicts of interest.

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Figure legends

Figure 1. *SOCS1 deficiency does not worsen liver damage caused by chemical injury, but promotes mononuclear cell infiltration.* (A) Induction of liver fibrosis using dimethylnitrosamine (DMN) or carbon tetrachloride (CCl4). (B) Serum ALT levels in SOCS1-deficient and control mice following DMN or CCl4 treatment. Data shown are mean ± standard error of mean (s.e.m). Mann-Whitney test: * $p < 0.05$. ** $p < 0.01$. (C) Representative hematoxylin and eosin-stained sections of the livers collected at the end of the DMN or CCl4 treatment regimens. Magnified images depict mononuclear cell infiltration in SOCS1-deficient livers. Bars are 100□M.

Figure 2. *SOCS1 deficiency increases the expression of inflammatory cytokine and chemokine genes in the liver and the circulating levels of IL-6 and TNF-α* (A) Real-time PCR quantification of *Il6*, *Tnfa* and *Ccl2* transcripts in the livers of SOCS1-deficient and control mice following DMN or CCL4 treatment. Fold induction was calculated by comparing the level of expression in treated mice versus untreated control mice within the same group. Mean ± s.e.m values from 6-8 mice per group are shown. (B) Levels of IL-6 and TNF-α in the peripheral blood of DMN- or CCL4- treated mice at the time of sacrifice. Numbers of mice in each group are indicated within parentheses. Data shown are mean ± s.e.m. Mann-Whitney test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Figure 3. *SOCS1 deficient mice show increased hepatic fibrosis following chemical injury.* (A) Representative images of Sirius red-stained sections of the livers from SOCS1-deficient and control mice at the end of the DMN or CCl4 treatment regimen. (B) Quantification of the Sirius red-stained areas of collagen deposition. Nanozoomer Digital Pathology software was used to select 20 random fields from 4 mice in each group, and the Sirius red staining area was quantified using the NIH Image J software. Data shown are mean ± s.e.m. Statistical significant between the groups are shown over the bar graphs. Statistical significance between treated and control groups are shown in the right panel. Mann-Whitney test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. (C) Representative images of

the liver sections from the above groups of mice following Masson's trichrome staining of collagen fibers.

Figure 4. *Increased collagen deposition in SOCS1-deficient mice following the induction of fibrogenesis.* Liver tissues from control and SOCS1-deficient collected at the end of the DMN or CCl4 treatment regimens were hydrolysed in acid and the hydroxyproline content was assessed by spectrophotometry. Data shown are mean ± s.e.m values from 5-6 mice per group. Mann-Whitney test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Figure 5. *SOCS1-deficient livers show increased accumulation of myofibroblasts and fibrogenic gene expression.* (A) Immunohistochemical staining of a-smooth muscle actin (SMA)-positive myofibroblasts in liver sections from SOCS1-deficient and control mice following DMN or CCl4 treatment. Representative images from four mice per group are shown. (B) Quantification of the SMA-staining. For each group, 20 digital images from 4 mice were analyzed as detailed in Fig. 3B. Data shown are mean ± s.e.m. Statistical significant between the groups are shown over the bar graphs. Statistical significance between treated and control groups are shown in the right panel. Mann-Whitney test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, *** $p < 0.0001$.

Figure 6. *SOCS1 deficiency profoundly modulates the expression of genes involved in hepatic fibrogenesis.* Induction of genes involved (A) in the fibrogenic response and (B) in the modulation of the extracellular matrix, in the livers of SOCS1-deficient and control mice following DMN or CCL4 treatment. Fold induction was calculated by comparing the level of expression in treated mice versus untreated control mice within the same group. Mean ± s.e.m values from 6-10 mice per group are shown. Mann-Whitney test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, n.s. not significant.

Figure 7. *Fibrotic livers of SOCS1 deficient mice show increased phosphorylation of STAT3 and Smad3.* Total lysate of SOCS1-deficient and control livers following the induction of hepatic fibrosis were evaluated for the phosphorylation and expression of the

indicated proteins. Results from one untreated control (C) and three treated mice (+) for each group are shown.

Figure 8. *Hepatic stellate cells from SOCS1 deficient mice show increase proliferation in response to growth factor stimulation.* (A) Primary hepatic stellate cells isolated from SOCS1-deficient and control mice were cultured in the presence of the indicated cytokines or growth factors and cell proliferation was evaluated by tritium-labeled thymidine incorporation assay. Cumulative data (mean + s.e.m.) from two different experiments carried out in triplicates are shown. (B) Expression of the *Pdgfb* gene was evaluated by qRT-PCR in the DMN- or CCl4- treated livers of SOCS1-deficient and control mice. Cumulative data from 6-8 mice per group are shown. Mann-Whitney test: * $p < 0.05$, ** $p < 0.01$, n.s. not significant.

Kandhi et al., Fig.1

A

DMN (10μg/g BW, i.p.)   Serum, Liver

CCl4 (0.5μl/g BW, 1:2 in corn oil, i.p.)   Serum, Liver

B

C
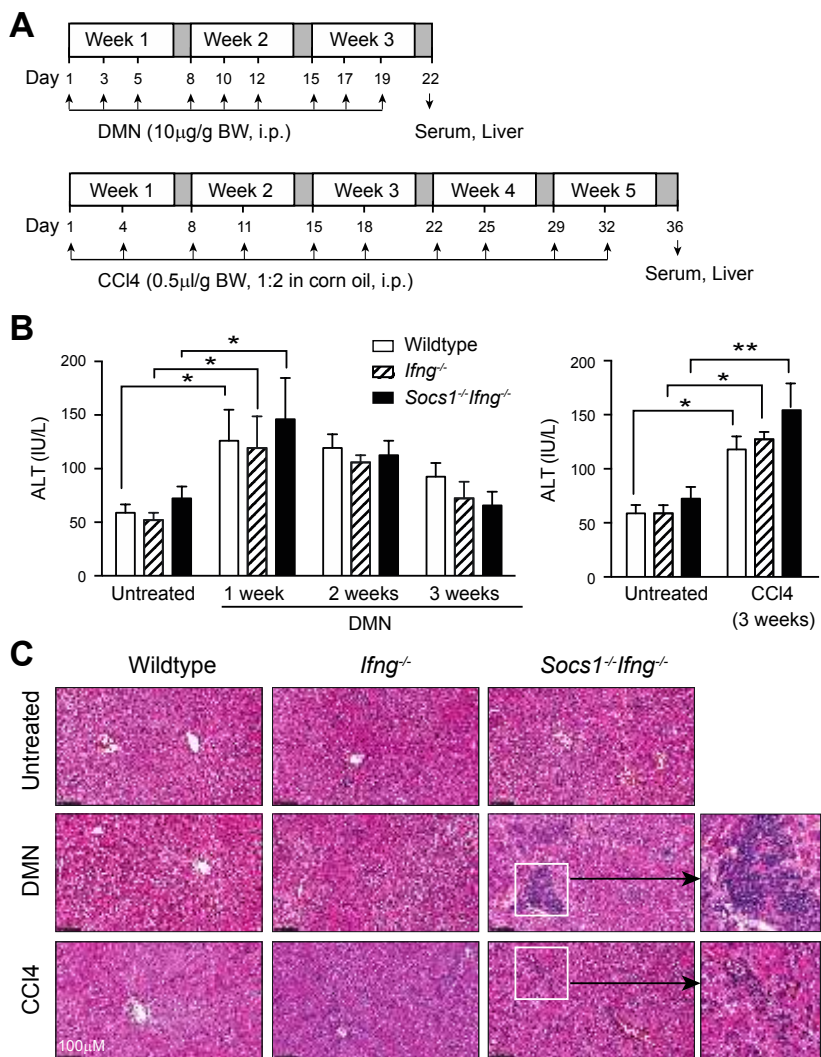
Kandhi et al., Fig.2

Kandhi et al., Fig.3

A

	Wildtype	*Ifng*−/−	*Socs1*−/−*Ifng*−/−
Control			
DMN			
CCl4			

500μM

Sirius Red

B

% Sirius red staining area

	Control vs	
	DMN	CCl4
Wildtype	**	**
Ifng−/−	***	***
Socs1−/−*Ifng*−/−	***	****

C

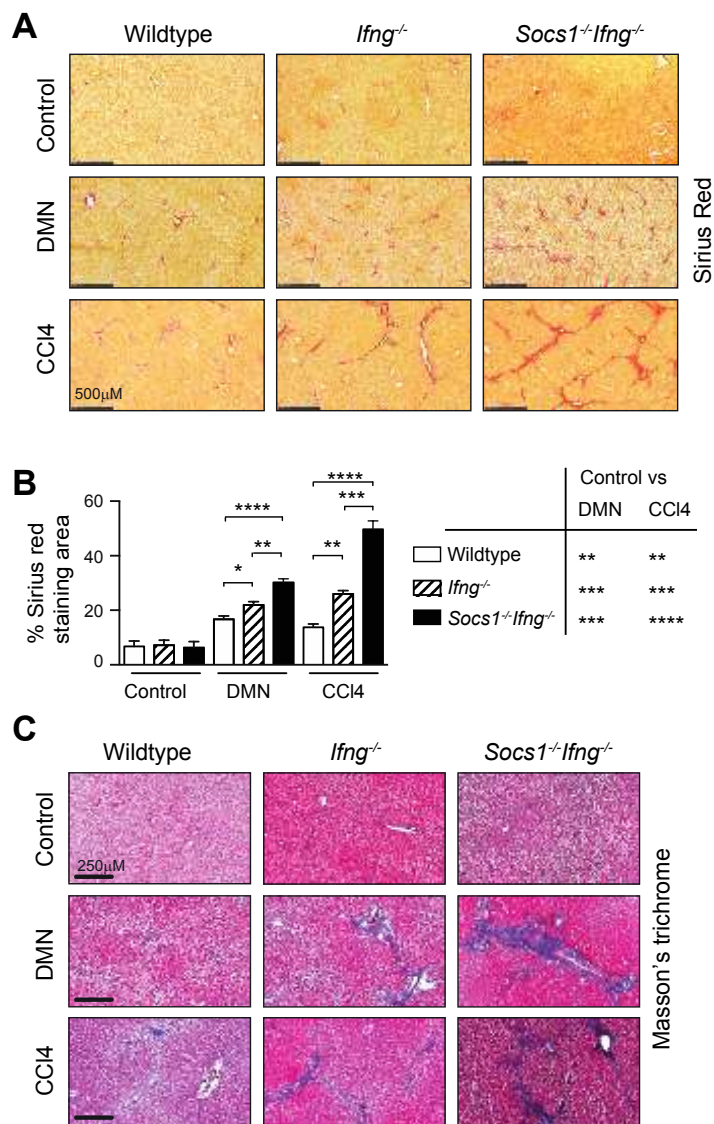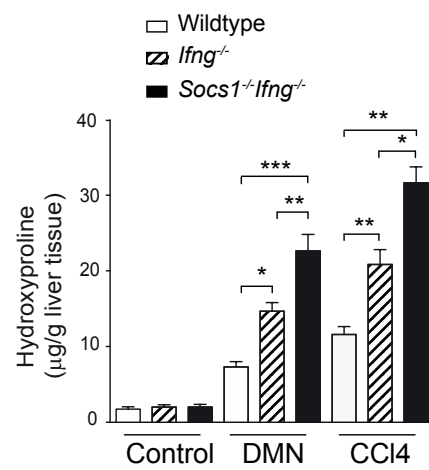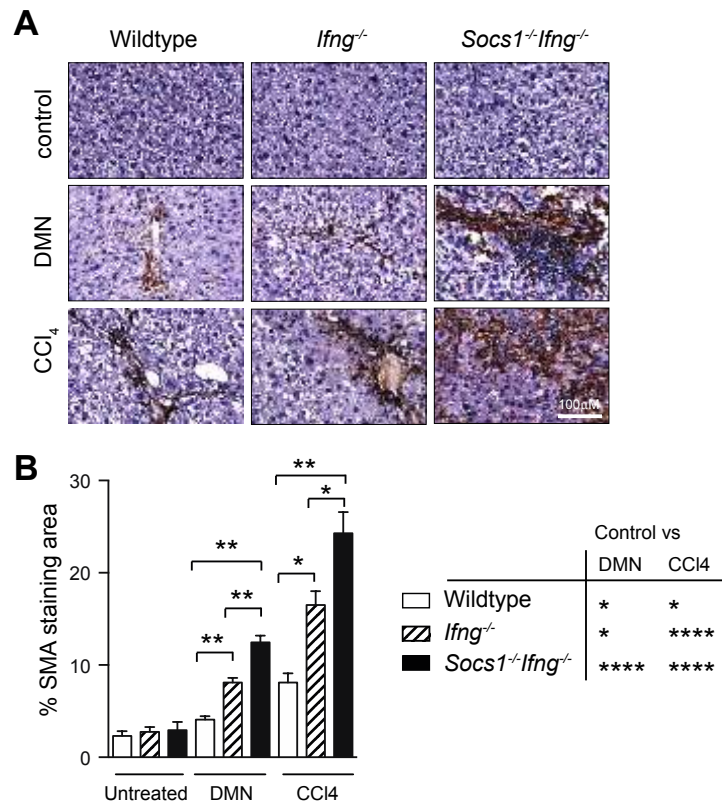|	Wildtype	*Ifng*−/−	*Socs1*−/−*Ifng*−/−
Control			
DMN			
CCl4			

250μM

Masson's trichrome
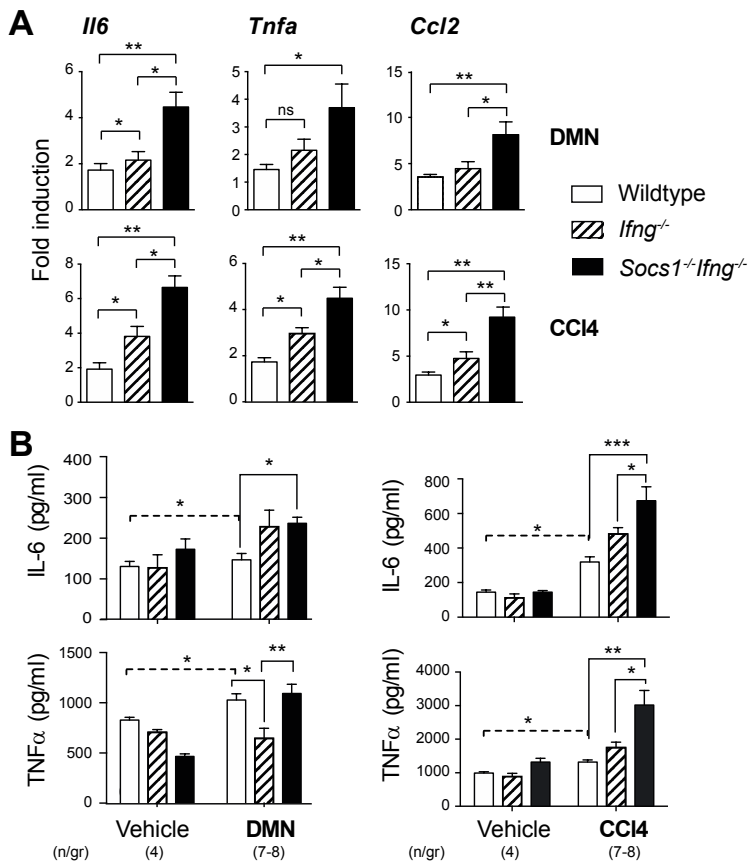
Kandhi et al., Fig. 4
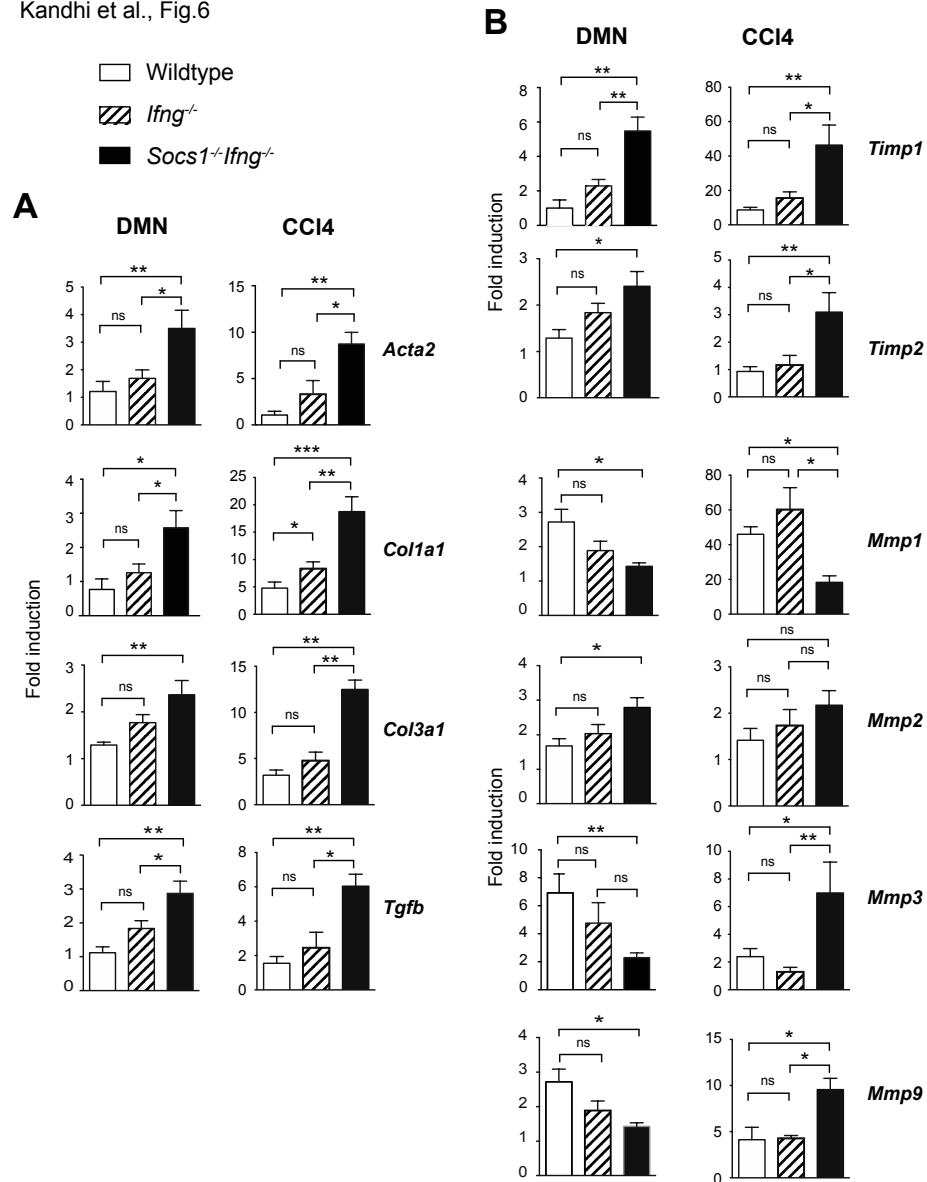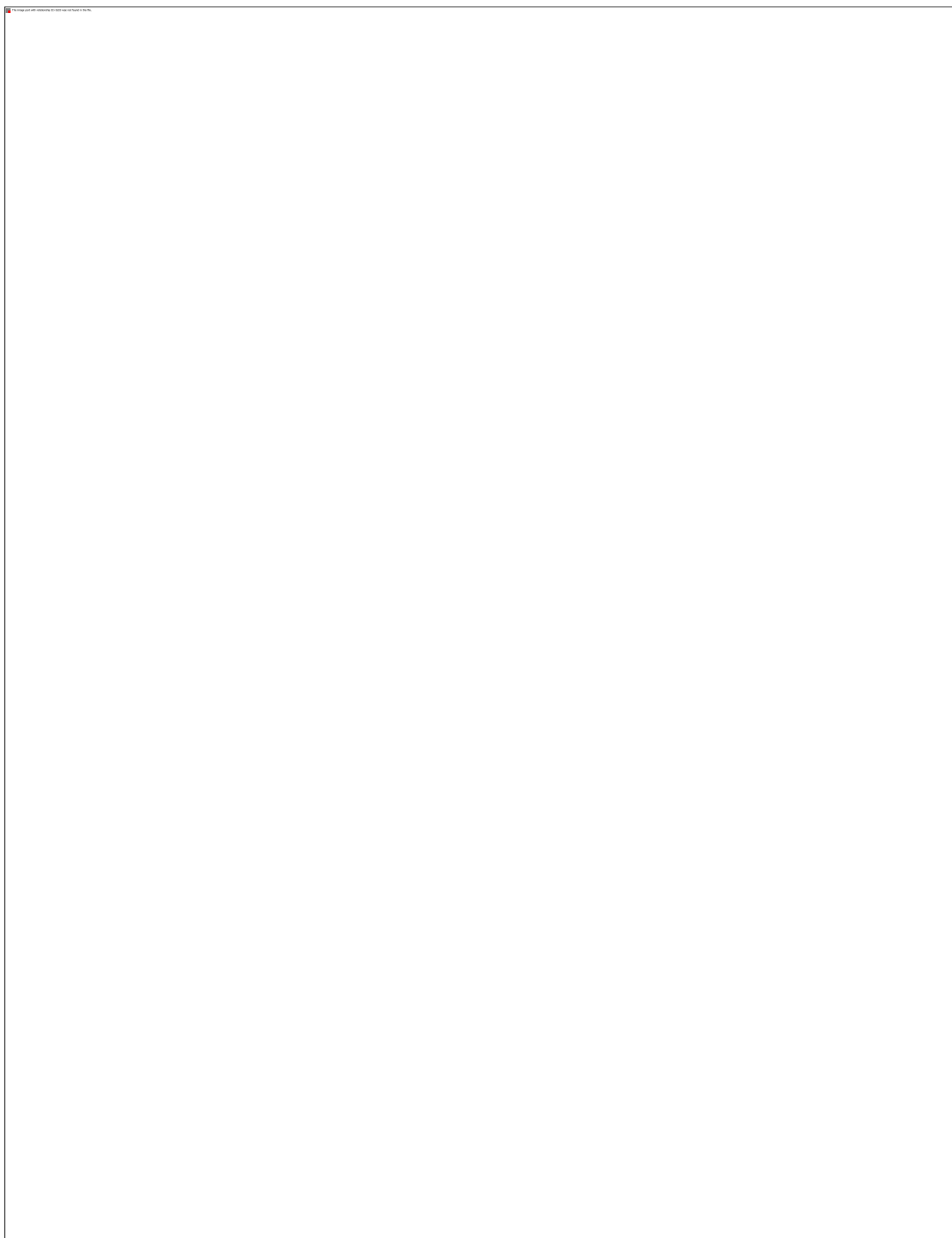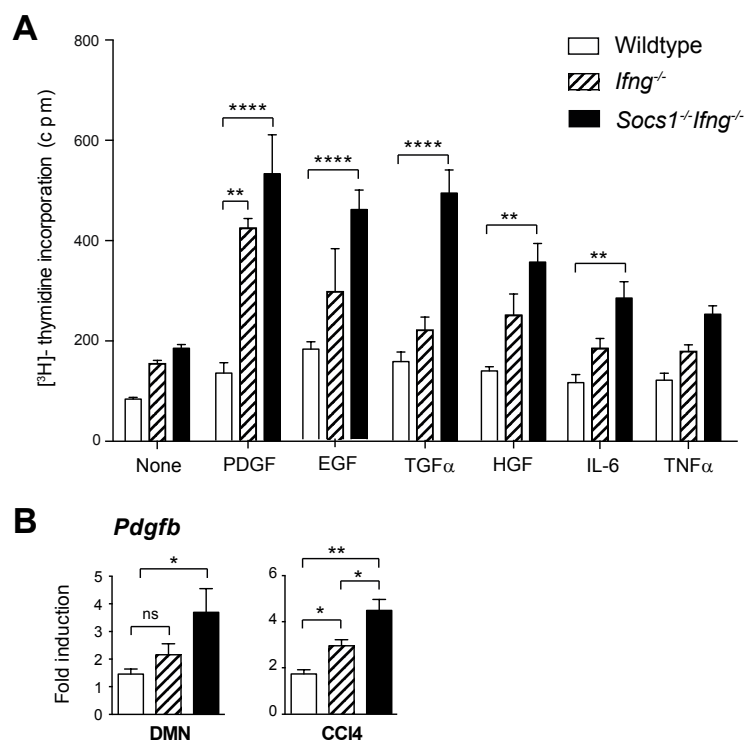
Kandhi et al., Fig. 5

Kandhi et al., Fig.6

Kandhi et al., Fig.8

A

B *Pdgfb*

Tables

Supplementary Table 1: List of qRT-PCR primers used in this study.

Gene name	Gene ID	Sense primer	Anti-sense primer	Amplicon Size
Acta2	NM_007392.3	AGTAATGGTTGGAATGG	GTGTCGGATGCTCTTCAGG	166
Col1a1	NM_007742.4	CTCCCAGAACATCACCTATCAC	ACTGTCTTGCCCCAAGTTCCG	171
Col3a1	NM_009930.2	AAGTCAAGGAGAAAGTGGTCG	CAGTCTCCCCATTCTTTCCAG	158
Il6	NM_031168.2	AGTCCGGAGAGGAGACTTCA	TTGCCATTGCACAACTCTTT	112
Ccl2	NM_011333.3	CAGGTCCCTGTCATGCTTCT	GTGGGGCGTTAACTGCAT	73
Mmp1a	NM_032006.3	TGTTGCTTCTCTGGGCTG	TCATCTCCTTGCCATTCACG	132
Mmp2	NM_008610.2	CAAGTTCCCCGGCGATGTC	TTCTGGTCAAGGTCACCTGTC	152
Mmp3	NM_010809	GATGAACGATGGACAGAGGATG	AAACGGGACAAGTCTGTGG	130
Mmp9	NM_013599.3	GATCCCCAGAGCGTCATTC	CCACCTTGTTCACCTCATTTTG	129
Pdgfb	NM_011057.3	CCTGCAAGTGTGAGACAGTAG	CTTTCGGTGCTTGCCTTTG	127
Tgfb1	NM_011577.1	ATACGCCTGAGTGGCTGTCT	CTGATCCCGTTGATTTCCA	129
Timp1	NM_011593.2	TTGCATCTCTGGCATCTGG	TGGTCTCGTTGATTTCTGGG	136
Timp2	NM_011594.3	CAGGAAAGGCAGAAGGAGATG	GATCATGGGACAGCGAGTG	130
Timp4	NM_080639.3	TTAAAGGGTTCGAGAAGGCC	TTCCATCACTGAGAATCTGGC	132
Tnfa	NM_013693.3	CGTCGTAGCAAACCACCAAG	GAGATAGCAAATCGGCTGACG	190
Gapdh	NM_001289726.1	ATGACATCAAGAAGGTGGTGAA	GTCTTACTCCTTGGAGGCCATGT	246

Gene names

Acta2, Actin, alpha 2
Col1a1, Collagen, type 1, alpha 1
Col3a1, Collagen, type 3, alpha 1
Il6, Interleukin-6
Ccl2, Chemokine (C-C motif) Ligand 2
Mmp1a, Matrix metalloproteinase 1a
Mmp2, Matrix metalloproteinase 2
Mmp3, Matrix metalloproteinase 3
Mmp9, Matrix metalloproteinase 9
Pdgfb, Platelet-derived growth factor, beta polypeptide
Tgfb1, Transforming growth factor, beta 1
Timp1, Tissue inhibitor of matrix metalloproteinase 1
Timp2, Tissue inhibitor of matrix metalloproteinase 2
Tnfa, Tumor necrosis factor alpha

Gapdh, Glycerolaldehyde-3-phosphate dehydrogenase

Supplementary Table 2: List of antibodies used in this study.

Molecule	Company	Cat. #
ERK1	Santa Cruz Biotechnology	sc-93
ERK2	Santa Cruz Biotechnology	sc-153
p-ERK	Cell Signaling Technology	#4695
α-SMA	Abcam	#7817
Smad-3	Cell Signaling Technology	#9513
p-Smad-3	Cell Signaling Technology	#9520
Stat1	Santa Cruz Biotechnology	sc-592
p-Stat1 (Y701)	Cell Signaling Technology	#9171
Stat3	Santa Cruz Biotechnology	sc-483
p-Stat3 (Y705)	Cell Signaling Technology	#9131

4. DISCUSSION

Cirrhosis is a pathological condition that results from chronic damage or scarring of the liver and is the end stage of liver fibrosis. It leads to cancer and is associated with significant morbidity and mortality. Cirrhosis is irreversible whereas fibrosis can be reversible, and thus is an important focus of HCC research. Fibrosis is the result of chronic inflammation characterized by excess deposition of extracellular matrix rich in collagen, produced by activated hepatic stellate cells. Chronic damage to the liver results in hepatocyte apoptosis and necrosis, recruitment of inflammatory cells, and release of cytokines and growth factors by liver resident and infiltrated cells resulting in activation of hepatic stellate cells into fibrogenic myofibroblasts.

Cell intrinsic regulatory molecules such as SOCS1 control cytokine and growth factor signaling. It has been extensively documented that the *SOCS1* gene is frequently repressed or silenced by methylation of the CpG islands on its promoter region in HCC and other cancers, suggesting an antitumor role of SOCS1. Besides, in patients with CLD, the *SOCS1* gene is silenced during fibrotic stages and there is a strong correlation between SOCS1 gene methylation and advancement of liver fibrosis (Takafumi Yoshida et al. 2004). Yoshida et al, showed for the first time, the antifibrotic role of SOCS1 by using SOCS1 heterozygous mice. They suggested that increased fibrosis observed in these mice was due to increased IFN-γ signaling and activation of the downstream signaling molecules STAT1 and IRF1 (T. Yoshida 2004). However, numerous experimental and clinical studies have reported that IFN-γ exerts antifibrotic functions (H. Weng et al. 2007; W. Il Jeong et al. 2006; Bansal et al. 2014; Ancini et al. 1996). Hence, we carried out this study in *Socs1*-deficient mice also lacking IFN-γ to clearly understand the mechanism by which SOCS1 regulates liver fibrosis.

Using two chemically induced liver fibrosis models we showed that 1) SOCS1 is a critical regulator of hepatic fibrogenic response that does not necessarily require IFN-γ to exhibit its antifibrotic functions. 2) A part of this SOCS1 regulation may be mediated by

controlling cytokines and growth factor responses of HSC and 3) SOCS1 differentially regulates the expression of ECM remodeling enzymes.

4.1 SOCS1 deficiency does not facilitate hepatocytes damage, but enhances hepatic inflammation

Alanine transaminase (ALT) is an enzyme present in hepatocytes. Upon damage, it is released into the blood, which can be used as an indicator to assess the extent of liver damage. Following treatment with DMN and CCl_4, we observed increased ALT levels in the serum of mice with all three genotypes when compared to that of vehicle-treated mice. However, we did not notice any statistical difference between *Socs1*-deficient mice and control groups at the end of the treatment indicating that the absence of SOCS1 does not worsen DMN- or CCl_4-induced hepatocyte damage.

In contrast, Yoshida *et al*., have shown that mortality rate and serum ALT levels were higher in DMN treated *Socs1*$^{+/-}$ mice and suggested that increased liver damage is observed in these mice due to elevated expression of STAT1 and pro-apoptotic factor IRF-1. Other studies also demonstrated the same by using hepatocyte-specific *Socs1* conditional knockout mice (Torisu et al. 2008; T. Yoshida 2004). But, this is not the case in our study, suggesting that at least part of the liver damage caused by DMN is mediated by increased sensitivity of SOCS1 deficient hepatocytes to IFN-γ, which was absent in our model of *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice.

Histological analysis of H&E-stained liver sections showed that the overall morphology of the liver was not affected, but infliltration of inflammatory cells was seen in the livers of *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice following treatment with DMN and CCl_4 (Fig.1C). In addition to this, *Socs1*-deficient mice also showed increased mRNA and protein levels of inflammatory cytokines like IL-6 and TNF-α, suggesting that SOCS1 deficiency enhances hepatic inflammation (Fig 2A, 2B). IL-6 and TNF-α are proinflammatory cytokines that come from different cellular sources including macrophages, Kupffer cells, T-cells, endothelial cells, fibroblasts, and hepatocytes. These cells could all be the cause of increased inflammatory

cytokines levels noticed in *Socs1*-deficient mice livers. However, the type of cells infiltrating into the livers of *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice following liver damage needs to be further characterized by using flow cytometry or by immunohistochemical staining (IHC) for Ly6G (neutrophils), CD3 (T cells), CD68 (macrophages) and B220 (B cells).

CCL2 (MCP-1) is a chemokine secreted by hepatocytes, HSC and biliary epithelial cells, which has been shown to recruit monocytes, macrophages, neutrophils and T-cells to the site of injury (Wasmuth, Tacke, and Trautwein 2010). It has been demonstrated that the inhibition of MCP-1 results in reduced infiltration of hepatic macrophages and decreased intrahepatic levels of pro-inflammatory cytokines (TNF-α, IFN-γ, and IL-6) in the injured liver (Baeck et al. 2012). Moreover, mice with a targeted deletion of its receptor CCR2, which is expressed on monocytes/macrophages, HSC and Kupffer cells, displayed reduced liver fibrosis in CCl$_4$ and bile duct ligation (BDL) models of liver fibrosis (Miura et al. 2012; Baeck et al. 2012; Seki et al. 2009). Accordingly, the livers of *Socs1*-deficient mice showed increased *Ccl2 (Mcp-1*) gene expression compared to those of control mice in both models (Fig.2A). This may be the reason for enhanced mononuclear cells infiltration and increase in IL-6 and TNF-α levels in the livers of *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice. From these observations, we suggest that the absence of SOCS1 induces a strong inflammatory response in the liver.

4.2 SOCS1 regulatory function occurs independently of IFN-γ

Cytokine signaling is stringently regulated by the SOCS family of proteins. Among them, SOCS1 is relatively skewed towards controlling STAT1, whereas SOCS3 towards regulating STAT3 (Kinjyo et al. 2002; Croker et al. 2002; Yasukawa et al. 2003). Yoshida *et al*., have reported that increased liver fibrosis observed in *Socs1*$^{+/-}$ mice is due to elevated STAT1 and pro-apoptotic factor IRF-1 expression as well as reduced STAT3 activation (T. Yoshida 2004). The increased STAT1 activation reported in the livers of *Socs1*$^{+/-}$ mice following treatment with DMN most likely results from deregulated IFN-γ signaling due to the limited availability of SOCS1. An earlier study using ConA-induced hepatitis model

also demonstrated that IFN-γ produced by CD4^{+} T-cells and NKT cells increased STAT1 activation, IRF1, and Bax, which induced apoptosis of hepatocytes, whereas STAT3 downstream of IL-6, protected hepatocytes from injury by inhibiting IFN-γ signaling and by induction of anti-apoptotic protein Bcl-xl (Hong et al. 2002).

In contrast to these reports, several studies have demonstrated that IFN-γ and its downstream signaling molecule STAT1 are one of the most important negative regulators of liver fibrosis. It has been shown in rodents that IFN-γ inhibits liver fibrosis induced by carbon tetrachloride (CCl4) or dimethylnitrosamine by inducing apoptosis of HSC (D C Rockey, Maher, et al. 1992; Ancini et al. 1996). IFN-γ also stimulates the cytotoxicity of NK cells against activated HSC by increasing the number of NK cells and upregulating tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) on NK cells (Radaeva et al. 2006; W. Il Jeong et al. 2006). Further, clinical studies have shown that IFN-γ treatment attenuates liver fibrosis in hepatitis B (HBV) and HCV patients (Muir, Sylvestre, and Rockey 2006; H.-L. Weng et al. 2005; H. L. Weng, Cai, and Liu 2001). IFN-γ did not suppress HSC activation in Stat1-deficient cells and failed to inhibit liver fibrosis in CCl4-treated $Stat1^{-/-}$ mice, suggesting that the antifibrogenic effects of IFN-γ are mediated by STAT1 (W. Il Jeong et al. 2006). Besides, IFN-γ and STAT1 are reported to inhibit HSC activation by inhibiting TGF-β/Smad signaling via increasing expression of Smad7 (Ulloa, Doody, and Massagué 1999). Taken together, these studies suggest that IFN-γ exerts its antifibrotic functions via STAT1.

In our study, we observed increased liver damage and fibrosis in $Socs1^{-/-}$ $Ifng^{-/-}$ mice without a significant change of STAT1 activation or IFN-γ signaling suggesting that SOCS1-mediated hepatoprotection or antifibrogenic response does not require IFN-γ or STAT1 activation. Besides, SOCS3, which is also implicated in inhibiting liver fibrosis (Ogata et al. 2006) did not compensate for the loss of SOCS1 suggesting that mechanisms by which SOCS1 and SOCS3 mediate their hepatoprotective activities are likely different. In fact, we observed increased $Il6$ gene expression, elevated serum IL-6 levels and increased STAT3 phosphorylation in $Socs1$-deficinet mice following DMN or CCl4

treatment. While STAT3 in hepatocytes confers protection against liver injury and fibrosis, it may play a pro-fibrogenic role in HSCs by promoting their survival and production of TIMP-1, and in Kupffer cells by increasing TGF-β production (Cao 2003; J P Iredale et al. 2010; J. X. Jiang et al. 2009). Therefore, our results clearly indicate that SOCS1 deficiency exacerbates the hepatic fibrogenic response in an IFN-γ-independent manner and that it may involve other mediators such as IL-6 (discussed in detail below).

4.3 SOCS1 deficiency is associated with enhanced fibrosis

Infiltrated immune cells and ongoing inflammation sustain the development of fibrosis. Since fibrosis is characterized by increased deposition of collagen, we have performed Sirius red and Masson's trichrome staining for liver tissue sections and quantified deposited collagen. We showed increased collagen deposition in the form of portal fibrosis with few septa (referred to as F2 stage) in DMN-treated *Socs1*-deficient livers, while CCl4 treated *Socs1*-deficient livers exhibited more severe fibrosis characterized by portal bridging fibrosis (F3 stage) compared with that of the control mice (Fig 3,4). Further, *Socs1*-deficient mouse livers also showed increased expression of collagen genes (*Col1a1* and *Col3a1)* (Fig 6A) and hydroxyproline levels (Fig 4) compared to *Ifn-γ*$^{-/-}$ and control mice. Our results are consistent with a previous study showing that SOCS1 insufficiency is associated with fibrotic changes in the liver (Takafumi Yoshida et al. 2004).

Socs1-deficient mouse livers also showed increased activation of myofibroblasts, which are the main source of collagen (Fig 5A, 5B). It has been demonstrated that fibroblasts from idiopathic pulmonary fibrosis (IPF) patients produce an excess of collagen due to the diminished expression of SOCS1, which has also been shown by using *Socs1*-deficient mouse fibroblasts. They also suggested that IFN-γ mediated inhibition of IL-4-induced collagen synthesis is mediated partly via SOCS1 (Shoda et al. 2007). The same group suggested that SOCS1 inhibits pulmonary inflammation and fibrosis using SOCS1 heterozygous mice (Nakashima et al. 2008). Similar to their reports, in liver fibrosis, cytokine-induced modulation of SOCS1 might regulate the activation of hepatic stellate cells or myofibroblasts and their collagen synthesis.

4.3.1 IL-6 / STAT3 and fibrosis

Several cytokines and growth factors are known to activate myofibroblasts, and deregulate collagen gene expression in liver fibrosis. In particular, IL-6 and TGF-β are known to act as profibrotic cytokines. However, the role of IL-6 and its downstream signalling mediator STAT3 in liver fibrosis is controversial. The function of STAT3 in liver inflammation and fibrosis is cell type-specific and model-dependent. STAT3 shows protective and proliferative functions in hepatocytes and a protective role in liver fibrosis (Taub 2003; H. Wang, Lafdil, Kong, et al. 2011; Deng et al. 2013). In contrast to these reports, other studies have demonstrated the opposite (pro-fibrotic) role of STAT3 in HSC. It has been shown that inhibition of the STAT3 pathway enhances apoptosis of HSC (J. X. Jiang et al. 2009) and HSC–specific STAT3 null mice mice are less susceptible to fibrosis (Meng et al. 2012). Accordingly, leptin and IL-6, the major activators of STAT3 in HSC, increase collagen mRNA expression during liver fibrosis (Saxena et al.,2002). IL-6 has also been shown to increase the expression of a-SMA and collagen in pancreatic stellate cells (Mews et al. 2002).

A recent study has found a correlation between IL-6 levels, p-STAT3 and liver fibrosis in HBV patients and demonstrated that Sorafenib shows antifibrotic effect through STAT3 inhibition in HSC (Su et al. 2015). Consistent with these findings, we also observed increased expression of IL-6 mRNA levels and protein levels and p-STAT3 in SOCS1 deficient livers in both DMN and CCl4 models. We speculate that IL-6 secreted from inflammatory cells or HSC themselves leads to hyperactivation of STAT3 in the absence of SOCS1 and promotes liver fibrosis by activating HSCs via paracrine and autocrine mechanisms. In addition, *Socs1*-deficient HSC showed increased proliferation in response to IL-6 (Fig 8A). In agreement with this possibility, increased fibrosis in DMN-treated hepatocyte-specific *Socs3*-null mice has been attributed to increased STAT3 activation (Ogata et al. 2006). The absence of SOCS1 in all cells (resident and recruited) of the liver, in our model, might lead to deregulated production of IL-6 from diverse cellular sources and activation of IL-6 downstream STAT3 signaling pathway. Although STAT3 signaling

can have a cell-dependent positive or negative effect on the development of fibrosis, the absence of SOCS1 in all cells might have tilted the net balance towards fibrosis. Further studies are required to understand the role of SOCS1 and STAT3 in hepatic stellate cells, Kupffer cells, sinusoidal cells, and inflammatory cells in the development of liver fibrosis. This can be addressed by using cell-specific $Socs1^{-/-}$ or $Stat3^{-/-}$ mice. Since SOCS1 is a negative regulator of IL-6 signaling, the absence of SOCS1 may enhance activation of stellate cells and collagen expression through STAT3 pathway. This possibility could be addressed by using $Socs1$-deficient HSC or by inhibiting or silencing STAT3 in HSC.

4.3.2 TGF-β/Smad3 and fibrosis

Numerous studies have demonstrated the pathogenic role of TGF-β in fibrotic diseases. Fibrotic diseases are often associated with increased levels of TGF-β, which inhibits ECM degradation by downregulating the expression of MMP and upregulating the expression of TIMP-1 (K. Jeong 2008; Dooley and Dijke 2012) TGF-β is known to be involved in proliferation and transdifferentiation of HSC into myofibroblasts in various tissues *in vivo* (Leask and Abraham, n.d.; A. M. Gressner 1996). It has been demonstrated in experimental animals that progression of LF can be prevented by the blockade of TGF-β signaling (Yata et al. 2002) whereas, the overexpression of TGF-β1 in a transgenic model inhibited HSC apoptosis and induced synthesis of excessive amounts of matrix proteins (Kanzler et al. 1999).

TGF-β mainly mediates its signaling through Smad-dependent and independent pathways. It has been shown that $Smad3^{-/-}$ mice are less susceptible to LF than wild type mice suggesting that Smad3 is a main "fibrogenic mediator" (Latella et al. 2009; Cong et al. 2012b). Consistent with these reports, in our study, we have demonstrated that $Socs1$-deficient mice showed increased expression of TGF-β, and its downstream signaling molecules p-Smad-3, collagen genes, TIMP-1 and fibrosis, compared to control mice. Based on these findings, we postulate that SOCS1 may play an essential role in regulating TGF-β production by diverse cells of the liver and/or its signalling in HSC. Further studies using cell-spcific $Socs1$ KO mice will elucidate how SOCS1 deficiency contributes to the

enhancement of the TGF-β pathway in hepatic fibrosis.

A crosstalk between IL-6 and TGF-β has been demonstrated in various models of fibrosis. Importantly, Ogata first revealed that TGF-β is a target gene of STAT3 and STAT3 enhances hepatic fibrosis through upregulation of TGF-β in hepatocytes and in liver fibrosis model in mice (Ogata et al. 2006). It has been reported using primary HSC and diethylnitrosamine (DEN)-induced rat fibrosis model that STAT3 enhances liver fibrosis through up-regulation of TGF-β and fibrotic product expression, this has also been reported in chronic hepatitis B (CHB) patients, (M.-Y. Xu et al. 2014).

Il6⁻/⁻ mice have decreased levels of TGF-β and reduced fibrosis in models of lung fibrosis (F. Saito et al. 2008). IL-6 trans signaling drives a STAT3-dependent pathway that leads to hyperactive TGF-β signaling (O'Reilly et al. 2014). However, IL-6 can also mediate pulmonary fibrosis independently of Smad3-dependent pathways through hyperactivation of STAT3 (O'Donoghue et al. 2012). In keloid scar tissue, and in cultured keloid fibroblasts the blockade of JAK2 inhibited phosphorylation of STAT3 and reduced collagen levels, cell proliferation and migration suggesting that JAK–STATs play a critical role in tissue fibrosis (Lim et al. 2006). It has also been reported that in activated pancreatic stellate cells, an autocrine loop exists between IL-6 and TGF-β through ERK and Smad2/3-dependent pathways (Aoki et al. 2006).

In the light of the above reports, we suggest that TGF-β could be a target gene of STAT3 and that the absence of SOCS1 may enhance JAK-STAT signaling and fibrosis. The crosstalk between IL-6/STAT3 and TGF-β/SMAD3 in HSC during liver fibrogenesis remains to be determined.

4.4 SOCS1 deficiency enhances the activity of tissue inhibitors of metalloproteinases (TIMPs)

An imbalance between matrix metalloproteinases (MMPs) and tissue inhibitors of

metalloproteinases (TIMPs) affects the synthesis and degradation of ECM, which plays an important role in the pathogenesis of liver fibrosis. TIMP-1, TIMP-2 participates in tissue remodeling by inhibiting various types of MMPs.

Similar to IL-6 and STAT3, TIMP-1 seems to exert a dichotomous role in liver fibrosis. The major sources for TIMP-1 are activated HSC, macrophages and hepatocytes. Previous studies using TIMP-1-deficient mice have shown that TIMP-1 displays a protective role in liver fibrosis (H. Wang, Lafdil, Wang, et al. 2011). However, several other studies demonstrated *in vitro* and *in vivo* that TIMP-1 exerts profibrotic activity by preventing the collagen degradation through inhibition of MMPS and also by inhibiting apoptosis of activated HSC (Murphy et al. 2002a; Benyon and Arthur 2001). Similar functions are further confirmed in *Timp-1* transgenic mice (H Yoshiji 2002). TIMP-1 and procollagen mRNA expression is increased during progression of liver disease, while interstitial collagenase (MMP-1) mRNA expression remained relatively unchanged (J. Iredale et al. 1996). Silencing TIMP-2 with siRNA in mice followed by CCl_4 injury showed reduced HSC activation and collagen deposition, suggesting its pro-fibrotic function of TIMP-2 (X. Liu et al. 2006).

We have observed increased mRNA expression of *Timp1, Timp2, and Col1a1, Col3a1* in the livers of $Socs1^{-/-}Ifng^{-/-}$ mice, indicating that these TIMPs might contribute to the increased deposition of collagen in *Socs1*-deficient mice. Similarly, an increase SMA positive staining (Fig. 5), the quantity of SMA (Fig. 7) and *Acta2* expression in the livers of DMN or CCl_4 treated *Socs1*-null mice, in addition to inhibiting MMP activity, suggests that increased Timp-1 activity might have contributed to the survival of HSC and their activation to myofibroblasts in the absence of SOCS1. Further studies are needed to understand how SOCS1 defciency leads to increased TIMP expression.

4.5 SOCS1 differentially regulates MMPs expression in Liver Fibrosis.

The biological functions of MMPs are complex because the same MMP can have opposing

effects based upon the cell type or tissue in which it is being expressed. Some MMPs are anti-fibrotic, whereas others show profibrotic functions. The choice of which MMPs to target for therapeutic purposes is still uncertain (Giannandrea and Parks 2014; Duarte et al. 2015).

We have observed reduced expression of *Mmp1* in the fibrotic livers of SOCS1 deficient mice in both DMN and CCl4-induced liver fibrosis models. The expression of *Mmp2* increased in DMN model whereas it was not significantly altered in the CCl4 model (Fig. 6B). MMP-1, also known as collagenase, degrades collagens type 1 and 3. Overexpression of human *MMP1* gene by adenovirus gene delivery inhibits liver fibrosis in a rat model while promoting hepatocyte proliferation (Iimuro et al. 2003). Similar findings have been reported in myocardial fibrosis upon over expression of *MMP1* (Foronjy et al. 2008). An antifibrotic role has been reported for MMP-2 (Gelatinase A or Type 4 collagenase) in both chemical and cholestasis induced fibrosis models using MMP-2 deficient mice and *in vitro* silencing of MMP-2 in stellate cells (Onozuka et al. 2011; Radbill et al. 2011).

MMP-3 and MMP-9 can activate latent TGF-β suggesting their profibrotic function (Giannandrea and Parks 2014). The role of MMP-9 in the liver is not clearly known. Some studies suggest a profibrotic function as it can activate TGF-β whereas some other studies attribute it an antifibrotic function (Cabrera et al. 2007; C. G. Lee et al. 2001). It has been suggested that MMP-3 promotes pulmonary fibrosis by inducing epithelial cells to undergo an epithelial-mesenchymal transition (EMT) to generate cells that are myofibroblast-like in function (Yamashita et al. 2011). Interestingly, MMP-3 and 9 expression is increased in CCl4-induced fibrosis and decreased in DMN- model in the livers of *Socs1* deficient mice (Fig. 6B). The significance of this differential expression in the two models, both of which initiate the fibrogenic response by inducing centrilobular necrosis, remains to be studied. Similarly, understanding the potential contribution of SOCS1-dependent modulation of cytokine and growth factor signaling to the induction of MMPs in liver fibrosis requires further investigation.

4.6 SOCS1 regulates HSC activation or proliferation in response to different cytokines
    and growth factors.

Liver fibrosis is characterized by increased proliferation of hepatic stellate cells. Several lines of evidence suggest that PDGF is the most potent mitogen for HSC. Pinzani *et al* in 1989 first reported the effect of growth factors on HSC proliferation. They found that PDGF, EGF, TGF-α, and βFGF induced a dose-dependent increase in DNA synthesis with a peak effect at 24 h. Among them, PDGF showed the maximum, 18-fold increase in DNA synthesis, followed by EGF, TGF-α, and βFGF, over control. PDGFβ and PDGFR mRNA expression is increased in the rat liver tissue following treatment with CCl_4 and in cirrhotic human livers (M Pinzani et al. 1996; M Pinzani et al. 1994). In a condition of chronic inflammation of the liver, the sustained release of PDGF by inflammatory cells and increased expression of PDGF receptors in activated FSC (earlier synonym for HSC) greatly contribute to the expansion of FSC population, and to the progression of fibrogenesis. TGF-β did not affect DNA synthesis of fat storing cells (FSC) or HSC but TGF-β enhanced the stimulatory effects of both EGF and PDGF (M Pinzani et al. 1989).

SOCS1 not only regulates JAK–STAT signaling but also RTKs signalling (Kazi et al. 2014). Previous study from our laboratory has shown that SOCS1 regulates HGF (c-Met) signaling in hepatocytes (Yirui Gui et al. 2011). To date, there is little evidence linking SOCS proteins in HSC with liver fibrosis and no previous report showing the role of SOCS1 in HSC. We have shown, for the first time, an increased expression of the *Pdgfβ* gene in fibrotic livers of *Socs1*-deficient mice and increased SOCS1-deficient HSC proliferation in response to PDGFβ and/or to other growth factors (TGF-α, EGF and HGF) (Fig. 8A). Our findings suggest that the antifibrotic function of SOCS1 is also mediated by regulating the availability of growth factors that promote HSC proliferation and/or by regulating growth factor signaling in HSC. Further studies are required to elucidate the molecular mechanisms by which SOCS1 regulates HSC proliferation.

The antifibrotic effect of IFN-γ is known to be achieved by directly inhibiting proliferation and activation of hepatic stellate cells (Ancini et al. 1996). It has been shown that IFN-γ

inhibits HSC proliferation via its downstream signaling molecule STAT1. STAT1-deficient HSC showed much greater cell proliferation in the presence of PDGFβ and PDGFβ mRNA expression than wild-type HSC, suggesting that STAT1 is a negative regulator for the proliferative effect of PDGF on HSC (W. Il Jeong et al. 2006). Given that, in this study, we showed that IFN-γ deficient HSCs showed enhanced proliferation in the presence of PDGFβ compared to wild type cells suggesting that IFN-γ inhibits PDGFβ induced proliferation. It is possible that the simultaneous absence of IFN-γ in SOCS1 deficient mice used in our study might relieve the inhibitory effects of IFN-γ –induced STAT1 activation. However, PDGFβ has also been shown to activate STAT1, STAT3, and ERK pathways (Massimo Pinzani 2002; M Pinzani et al. 1989), and STAT3 has been shown to be implicated in cell proliferation in different cell types (hirano, ishihara, & hibi, 2000). The increased proliferation of *Ifn-γ⁻/⁻/Socs1⁻/⁻* HSC and increased STAT3 activation in SOCS1 deficient livers indicate that PDGF likely mediates HSC proliferation via STAT3 pathway rather than diminished STAT1 activation in the absence of IFN-γ. Further studies using HSC-specific *Socs1*-null cells and mice are required to clarify the role of IFN-γ-induced STAT1 activation versus PDGF-induced STAT3 activation in HSC proliferation and liver fibrosis.

Sorafenib is a RAF (Rapidly Accelerated Fibrosarcoma) kinase inhibitor, downstream of many RTKs and is the only Food and Drug Administration-approved drug for the treatment of HCC. Recent studies reported the antifibrotic function of sorafenib, which reduces HSC proliferation and ECM synthesis by enhancing HSC apoptosis (Hong et al. 2013; Y. Wang et al. 2010). A recent study on the underlying molecular mechanisms of Sorafenib and its derivatives showed that their anti-fibrogenic effects are mediated via inhibition of STAT3 activation, down-regulation of TGF-β/Smad2/Smad3 signaling and downstream signalling pathways of PDGFR-β (Su et al. 2015). They also reported that correlation exists between p-STAT3 overexpression in HSC of chronic hepatitis B patients with advanced liver fibrosis score and high plasma IL-6 levels. In human liver fibrosis, SOCS1 gene is frequently suppressed by promoter methylation that correlates with disease severity (T. Yoshida 2004). Given that Sorafenib shows the antifibrogenic effect by inhibiting STAT3 activation and TGF-β and PDGF signalling pathways, and that SOCS1 deficiency increases

IL-6, pSTAT3, TGF-β, p-Smad-3 and PDGF expression in mouse models of fibrosis and enhances HSC proliferation, we propose that Sorafenib can be used as a therapeutic agent for the treatment of liver fibrosis in patients with *SOCS1* gene methylation.

5. CONCLUSIONS

In conclusion, our results suggest that absence of SOCS1 leads to acceleration of hepatic inflammation and fibrosis. SOCS1 regulates different cytokines and growth factors and thus inhibits fibrosis by attenuating HSC activation and proliferation. We also conclude that SOCS1 regulated fibrogenic response occurs in an IFN-γ independent manner. Further *in vitro* and *in vivo* studies on molecular mechanisms of SOCS1 in liver fibrosis will provide new insight in understanding its function and in predicting the disease progression and help in the development of targeted therapeutic strategies in preventing the progression of liver fibrosis.
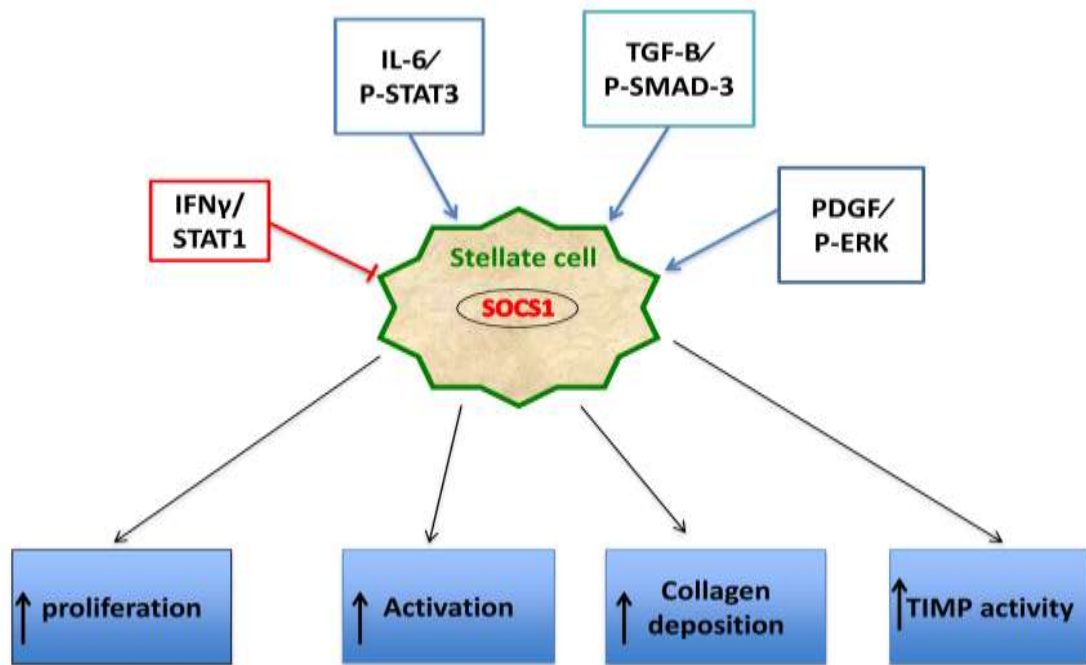
Figure 6 **Schematic diagram of SOCS1 role in liver fibrosis.** Hypothetical model illustrating the different cytokines and growth factors pathways in hepatic stellate cell (HSC) activation and fibrogenic response such as proliferation, activation, collagen deposition and final event fibrogenic response. Induction of SOCS1 in HSC may regulate these cytokines and growth factors signaling and thereby inhibit HSC activation and fibrogenic response.

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