INVESTIGATING THE ROLE OF BETA1 INTEGRIN IN
HEPATIC STELLATE CELL ACTIVATION AND
LIVER FIBROSIS

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

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LIST OF ABBREVIATIONS

ADAM  A disintegrin and metalloproteinase domain
A HSC  Activated hepatic stellate cells
ANP   Atrial naturetic peptide
APS   Ammoniumpersulphate
Arp2/3 Actin-related 2/3 complex
αSMA  Alpha smooth muscle actin
Bcl2  B cell lymphoma 2
BDL   Bile duct ligation
Brdu  Bromodeoxyuridine
Bp    Base pairs
BSA   Bovine serum albumin
cAMP  Cyclic adenosine monophosphate
CCl4  Carbon tetrachloride
ChIP  Chromatin immunoprecipitation
ColI  Type I collagen
CTGF  Connective tissue growth factor
CXCL9 Chemokine (C-X-C motif) ligand 9
DAPI  4', 6-diamidino-2- phenylindole
DDR   Discoidin domain receptor
DMEM  Dulbecco’s Modified Eagle Medium
DMSO  Dimethyl sulfoxide
dNTPs Deoxynucleotide triphosphates
ECM   Extracellular matrix
EDTA  Ethylenediaminetetraacetic acid
EGF   Epidermal growth factor
ELF   Enhanced liver fibrosis
EMT   Epithelial-mesenchymal transition
ET-1  Endothelin-1
FAK   Focal adhesion kinase
FBS   Foetal bovine serum
FERM  Band four-point-one/ezrin/radixin/moesin
FGF  Fibroblast growth factor
FITC  Fluorescein isothiocyanate
FXR  Farnesoid X receptor
GFAP  Glial fibrillary acidic protein
GusB  Glucuronidase, beta
HBSS-  Hank’s balanced salt solution without calcium or magnesium
HBSS+  Hank’s balanced salt solution with calcium and magnesium
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSC  Hepatic stellate cell
Hsp 1a/b  Heat shock proteins 1a/b
IgG  Immunoglobulin G
IKBα  Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha
IL-1  Interleukin 1
IL-10  Interleukin 10
ILK  Integrin linked kinase
iNOS  Inducible nitric oxide synthetase
IP  Immunoprecipitation
Itga  Integrin alpha
Itgb  Integrin beta
LFTs  Liver function tests
LOXL2  Lysyl oxidase-like 2
LPS  Lipopolysaccharide
LRAT  Lecithin-retinol acyltransferase
MEF  Mouse embryonic fibroblast
mHSCs  Mouse hepatic stellate cells
MLC  Myosin light chain
MLCK  Myosin light chain kinase
MKL1  Megakaryoblastic leukaemia factor-1
MMP  Matrix metalloproteinase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide</td>
</tr>
<tr>
<td>MYL9</td>
<td>Myosin light chain 9, regulatory</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NFKB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NS</td>
<td>Non-significant</td>
</tr>
<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>Platelet derived growth factor receptor β</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PIC</td>
<td>Phosphatase inhibitor cocktail</td>
</tr>
<tr>
<td>PLA</td>
<td>Proximity ligation assay</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator activated receptor</td>
</tr>
<tr>
<td>PSI</td>
<td>Plexin-semaphorin-integrin</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>Q HSCs</td>
<td>Quiescent hepatic stellate cells</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>rHSCs</td>
<td>Rat hepatic stellate cells</td>
</tr>
<tr>
<td>ROCK</td>
<td>RhoA associated kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SHARPIN</td>
<td>SHANK-associated RH domain interactor</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>Smad</td>
<td>Mothers against decapentaplegic homologs</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SOX9</td>
<td>Sex-determining region Y box 9</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted protein, acidic and rich in cysteine</td>
</tr>
<tr>
<td>SRF</td>
<td>Serum response factor</td>
</tr>
<tr>
<td>Sry</td>
<td>Sex determining region Y</td>
</tr>
<tr>
<td>TAZ</td>
<td>Transcriptional co-activator with PDZ-binding motif</td>
</tr>
<tr>
<td>TEAD</td>
<td>SV40 transcriptional enhancer factor</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>Transforming growth factor beta1</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitors of metalloproteinases</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumour necrosis factor-related apoptosis inducing ligand</td>
</tr>
<tr>
<td>TWEAK</td>
<td>Tissue necrosis factor-like weak inducer of apoptosis</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>YAP</td>
<td>Yes-associated protein</td>
</tr>
</tbody>
</table>
**ABSTRACT**

The University of Manchester

Katherine Martin

Degree: Doctor of Philosophy (PhD)

Thesis Title: Investigating the Role of Beta1 Integrin in Hepatic Stellate Cell Activation and Liver Fibrosis

Date: 20\(^{th}\) March 2015

Liver fibrosis is characterised by progressive deposition of type 1 collagen-rich extracellular matrix in response to iterative liver injury. The progressive accumulation of scar tissue within the liver predisposes to the development of hepatocellular carcinoma, and ultimately leads to organ failure.

Liver fibrosis is an increasing cause of morbidity and mortality. However, current treatment options are limited, and the only curative option for end stage liver disease is transplantation. Anti-fibrotic agents are urgently needed to halt, or reverse, the fibrotic process; however, to date, they have remained frustratingly elusive.

The primary cell type responsible for laying down the pathological fibrotic matrix is the hepatic stellate cell (HSCs). In the healthy liver, HSCs are quiescent vitamin A storing cells, however, in response to liver injury they are activated into proliferative, migratory and contractile myofibroblasts. A number of cytokines and transcription factors are implicated in this activation process; in addition, biomechanical forces have emerged as an important regulator. The integrins are a family of cell surface receptors, which are predominately involved in mediating cellular interactions with the microenvironment.

The work presented in this thesis demonstrates that beta1 integrin (Itgb1) plays an important role in HSC activation through regulation of the actin myosin cytoskeleton, and thereby, the cell’s ability to sense and respond to changes in the biomechanical microenvironment.

By identifying and investigating downstream effectors of Itgb1 in HSC activation, the group I p21-activated kinases (Paks) were discovered as potential therapeutic targets in liver fibrosis. In addition, alpha11 integrin was identified as a fibroblast-specific partner of Itgb1 in HSCs, which crucially, may allow HSC-specific targeting of Itgb1.

Taken together, the data presented in this thesis suggest that disrupting Itgb1 signalling in HSC activation may be a novel therapeutic avenue for liver fibrosis. In particular, pharmacological inhibition of the downstream effectors, the group I Paks, has shown promise as an anti-fibrotic both *in vitro* and *in vivo*. 
DECLARATION AND COPYRIGHT STATEMENT

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1 INTRODUCTION

1.1 Liver Anatomy

The liver is the largest visceral organ, weighing around 1.2 – 1.5 kg and comprising 2% of the total body weight in the adult (Lefkowitch, 2011). It is situated in the right upper quadrant of the abdomen, with its superior surface in direct contact with the diaphragm. The liver is divided into two main lobes, right and left, by the falciform ligament, with two additional smaller lobes (caudate and quadrate) located on the posterior and inferior surfaces of the right main lobe. However, it is more clinically useful to divide the liver into functional segments defined by their blood supply and biliary drainage (Figure 1.1) (Lefkowitch, 2011). This is particularly relevant when considering surgical resection of the liver.

The liver has a dual blood supply: it receives venous blood from the intestines via the portal vein, and arterial blood via the hepatic artery. Both these vessels enter the liver through the porta hepatis, on the inferior surface of the right lobe, where they divide into branches supplying the right and left lobes. After passing through the hepatic microcirculation, blood is collected into the right and left hepatic veins, which emerge from the liver and drain directly into the inferior vena cava. Bile leaves the liver via the right and left bile ducts, which merge to form the common hepatic duct, at the porta hepatis.
The liver can be divided into functional segments based on blood supply and biliary drainage. The hepatic veins divide the liver into four sectors: the middle hepatic vein runs in Cantlie’s line (a plane between the middle of the gallbladder fossa anteriorly and the inferior vena cava posteriorly) and divides the liver into functional right and left hemi-livers; the right hepatic vein divides the right hemi-liver into anterior and posterior sectors; and the left hepatic vein divides the left hemi-liver into medial and lateral sectors. The Couinaud classification describes eight functional liver segments, with segments I – IV making up the left hemi-liver, and segments V – VIII making up the right hemi-liver. Segment I corresponds to the caudate lobe, located on the posterior aspect of the liver. (Farid et al., 2013)

1.2 Liver Microanatomy

Classically, the liver microarchitecture is described as hexagonal lobules, each with a central vein and portal tracts at the peripheries (Lefkowitch, 2011). Portal tracts consist of a portal vein, a hepatic arteriole and a bile duct, enveloped in connective tissue. Blood empties from the afferent portal vein into the sinusoids, flows down a pressure gradient, and drains into the efferent central vein. The terms ‘periportal’ and ‘pericentral’ are often used when referring to regions of the parenchyma.

A more useful concept is the functional ‘liver acinus’, which was introduced by Rappaport in 1954 (Rappaport et al., 1954). The acinus is triangular, with a terminal tributary of the hepatic vein at its apex, and a portal venule at its base (Figure 1.2).
Blood perfuses from the portal venule towards the terminal hepatic vein. The parenchyma is divided into zones dependent on the direction of the blood flow. Thus, zone 1 is proximal to the portal venule; zone 2 is in the middle; and zone 3, equivalent to the pericentral region, is adjacent to the terminal hepatic vein.

**Figure 1.2 Diagrammatic representation of the liver acinus**

Blood flows from the afferent vessels (branches of the portal vein, with a lesser contribution from arterioles arising from the hepatic artery) towards the efferent central vein. Hepatocytes in acinar zone 1 receive first pass blood, whilst those in the pericentral zone 3 receive less oxygenated blood. Hepatocyte function, ECM composition and density varies depending on location within the acinus.

As blood flows through the sinusoids it is modified by the actions of hepatocytes, through the uptake and secretion of macromolecules. The specific function of hepatocytes varies depending on their zonal location. Hepatocytes in zone 1 are predominately responsible for gluconeogenesis and bile-salt dependent bile formation, whereas hepatocytes in the relatively oxygen-poor zone 3 have the highest concentrations of the drug metabolising P450 enzymes, and are particularly vulnerable to hepatotoxic reactions (Lefkowitch, 2011).

Hepatocytes are separated from the sinusoidal endothelial cells by the space of Disse. There is no basement membrane; however, there is a sparse extracellular matrix (ECM), which does not impede diffusion between the plasma and the hepatocytes. The amount and composition of the ECM varies depending on the distance from the portal tracts. In the periportal regions, the matrix is predominately composed of type IV collagen, laminin and heparin sulphate proteoglycan (Reid *et al.*, 1992). Towards the pericentral region, the matrix volume decreases with replacement of the basal lamina components by fibrillar collagens, fibronectin, chondroitin sulphate proteoglycan and dermatan sulphate proteoglycan (Reid *et al.*, 1992).
1.3 Liver Cell Types

1.3.1 Hepatocytes

Hepatocytes, the liver parenchymal cells, are the body’s chemical powerhouses. They account for around 70% of the total liver cell population and perform many varied functions (Si-Tayeb et al., 2010). In addition to the production and excretion of bile, they produce nearly all plasma proteins; are involved in lipid metabolism and cholesterol synthesis, as well as glycogen storage and glucoenogenesis; and are responsible for detoxification. Their specific function depends on their location within the acinus.

They develop from the liver diverticulum, which arises from the foregut endoderm during the third week of embryogenesis. The liver diverticulum is divided into two parts, hepatic and biliary. Cells within the hepatic part develop into the liver and intrahepatic biliary ducts, whilst those within the biliary part form the extrahepatic ducts and the gallbladder (Lemaigre, 2009). By week seven, the bipotential hepatoblasts within the hepatic part invade into the septum transversum mesenchyme, and begin to differentiate into hepatocytes and cholangiocytes. Oncostatin M signalling is thought to drive hepatocyte differentiation and maturation, whilst Notch and transforming growth factor beta (TGF-β) stimulate cholangiocyte differentiation (Lemaigre, 2009).

The hepatocytes are arranged into plates, of double cell thickness in the neonate, decreasing to single cell thickness by adulthood (Lefkowitch, 2011). Their basal membrane surface area is increased by numerous microvilli, which extend into the sinusoidal space. The apical plasma membranes of adjacent hepatocytes form a narrow lumen, or bile canaliculus. Bile is secreted by hepatocytes into the canalicula, which empty into interlobular bile ducts within the portal tracts. Hepatocytes are often polyploidal, and binuclear; this is thought to signify terminal differentiation (Guidotti et al., 2003).

The relationship of hepatocytes to the sinusoidal space and other resident liver cells is depicted in Figure 1.3.
1.3.2 Cholangiocytes

In addition to hepatocytes, bipotential hepatoblasts also give rise to cholangiocytes. Cholangiocytes are the biliary epithelial cells, which line the intrahepatic and extrahepatic bile ducts. They are responsible for the modification of bile produced by the hepatocytes (Lefkowitch, 2011). However, they have different functions depending on their location within the biliary tract, similar to the metabolic zonation of hepatocytes.

1.3.3 Hepatic Progenitor Cells

Hepatocytes proliferate to repopulate the liver following injury. However, prolonged or severe liver injury can overwhelm the proliferative capacity of hepatocytes, and it is in this setting, that hepatic progenitor cells are thought to be activated (Duncan et al., 2009). Hepatic progenitor cells are bipotential precursors, with the ability to differentiate into both hepatocytes and cholangiocytes, and are thought to reside within the biliary tree (Sackett et al., 2009). Severe liver injury is associated with the development of ductular reactions, which contain the hepatic progenitor cells. Moreover, the number of ductular reactions correlates with the severity of liver disease (Lowes et al., 1999).
The nature of the liver injury and the microenvironment are thought to influence the fate of hepatic progenitors, and direct their differentiation into hepatocytes or cholangiocytes (Español-Suñer et al., 2012). Hepatic progenitor cell-derived mature hepatocytes have been demonstrated in models of liver injury by lineage tracing studies, however, whether they are limited to the periportal areas or are found throughout the parenchyma is controversial (Español-Suñer et al., 2012; Furuyama et al., 2011). The hepatic progenitor cell response is stimulated by macrophages and tissue necrosis factor-like weak inducer of apoptosis (TWEAK) signalling, linking the inflammatory response to regeneration (Bird et al., 2013; Tirnitz-Parker et al., 2010).

Ductular reactions show similarities to the embryonic ductular plate, this describes the ring-like arrangement of cholangiocytes which arises during liver development, and is then remodelled to form the bile ducts (Lemaigre, 2009). Moreover, lineage tracing experiments have suggested that this is the developmental origin of hepatic progenitor cells (Carpentier et al., 2011).

### 1.3.4 Sinusoidal Endothelial Cells

Sinusoidal endothelial cells are derived from existing blood vessels within the septum transversum mesenchyme during liver development (Si-Tayeb et al., 2010). They differ from capillary endothelial cells due to the lack of a basement membrane, and the presence of fenestrations (Si-Tayeb et al., 2010). The diameter and frequency of the fenestrations is dependent on the distance from the portal tracts, and is regulated by the ECM (McGuire et al., 1992). Sinusoidal cells also have a huge capacity for endocytosis (Lefkowitch, 2011).

### 1.3.5 Kupffer Cells

Kupffer cells are liver specific macrophages; they reside within the sinusoid and can exhibit both classically activated (M1) and alternatively activated (M2) phenotypes (Murray and Wynn, 2011).

M1 macrophages are pro-inflammatory; they are important for defence against pathogens, but are also implicated in a number of inflammatory and autoimmune diseases (Murray and Wynn, 2011). M2 macrophages are anti-inflammatory and promote wound healing. They secrete pro-fibrotic cytokines including TGF-β1 and
platelet derived growth factor (PDGF) and may initiate myofibroblast activation. They can also independently remodel the ECM through secretion of matrix degrading enzymes (Murray and Wynn, 2011) and stimulate apoptosis of M1 macrophages through secretion of the cytokine interleukin 10 (IL10) (Wan et al., 2014). On the other hand, there is evidence that M2 macrophages ameliorate fibrosis (Wynn and Ramalingam, 2012).

Interestingly, it has been suggested that the ratio of M2:M1 Kupffer cell subtypes influences the progression of liver disease, with a preponderance of M2 macrophages favouring more limited disease (in alcoholic liver disease and non-alcoholic fatty liver disease) (Wan et al., 2014). Moreover, a high Kupffer cell M2:M1 ratio is protective against alcohol induced liver injury in mice (Wan et al., 2014).

However, more recently, it has been argued that the M1-M2 classification of macrophage subtypes is overly simplified, and that a ‘spectrum’ of macrophage phenotypes more accurately describes the characteristics and functions of macrophage populations seen in vivo (Mosser and Edwards, 2008). Mosser and Edwards have suggested a classification based around three functions: host defence, wound healing and immune regulation, with some macrophages sharing more than one of these characteristics (Mosser and Edwards, 2008).

In line with this, Ramachandran et al, have identified that restorative Ly6C<sup>lo</sup> macrophages, which are critical for liver fibrosis resolution, have a pattern of gene expression which does not fit into either the M1 or M2 category, but instead shows features of both (Ramachandran et al., 2012). Restorative Ly6C<sup>lo</sup> macrophages are derived from recruited Ly6C<sup>hi</sup> monocytes which undergo a phenotypic switch within the liver (Ramachandran et al., 2012). Whereas recruited Ly6C<sup>lo</sup> monocytes contribute to the repopulation of the resident macrophages (Kupffer cells) during late fibrosis resolution (Ramachandran et al., 2012).

**1.3.6 Hepatic Stellate Cells**

The hepatic stellate cell was first identified by Kupffer in 1876, who described them as ‘sternzellen’ (Friedman, 2008a). Since then, they have been variably known as lipocytes, Ito cells, perisinusoidal cells and fat storing cells. However, hepatic stellate cell (HSC) is now the accepted nomenclature. In the healthy liver, HSCs account for
around 10% of the total cell population (Kisseleva et al., 2012). They reside in the subendothelial space of Disse, in close association with the sinusoidal endothelial cells. They have numerous dendritic cytoplasmic processes which wrap around the sinusoids, leading to their comparison with pericytes in other organs (Lee et al., 2007).

The developmental origin of HSCs has been obscure, however, there is now convincing evidence that they are derived from the septum transversum mesenchyme (Asahina et al., 2011). Previously, a neural crest origin was proposed, based on the expression of neural cell markers glial fibrillary acidic protein (GFAP), nestin and p75 neurotrophin receptor (Niki et al., 1996; Niki et al., 1999; Trim et al., 2000). However, lineage tracing experiments did not support this, and it therefore seems unlikely (Cassiman et al., 2006).

In the healthy liver, HSCs are a retinoid store, with characteristic vitamin A-rich lipid droplets within the cytoplasm (Friedman, 2008a). There is also evidence that they contribute to the hepatic immune response (Wilson et al., 2014). However, most interest has focussed on the activation of HSCs in response to liver injury. Activated HSCs lose their retinoid droplets and develop into contractile, alpha smooth muscle actin (\(\alpha\)Sma) expressing myofibroblasts, which secrete the damaging, type I collagen-rich matrix that characterises liver fibrosis.

1.3.7 Portal Fibroblasts

Portal fibroblasts are resident fibroblasts within the portal tract; relatively little is known about their functions in both the healthy and injured liver. It seems likely that they develop into myofibroblasts during biliary fibrosis; however, their relative contribution to matrix deposition is controversial (Beaussier et al., 2007; Mederacke et al., 2013). Other proposed roles for portal fibroblasts include regulation of cholangiocyte proliferation and providing structural support to the biliary ducts and portal vessels (Wells, 2014).

1.4 Liver Fibrosis: an Overview

Fibrosis is an important part of the normal wound healing response, and maintains tissue integrity after injury. However, an excessive or dysregulated fibrotic response, accompanied by failure of resolution, can be pathological. Fibrotic disease can affect
Liver fibrosis is a chronic wound healing response, which results in the accumulation of ECM following liver injury. In the early stages this is largely asymptomatic, however, over time this can progress to cirrhosis, which is associated with significant morbidity and mortality. Cirrhosis is a pathological description, and is defined by the appearance of thick fibrotic septae and nodules of regenerative parenchyma. This leads to distortion of the hepatic architecture, and is associated with vascular changes, which can result in portal hypertension. Increases in the portal pressure underlie the development of many of the complications of cirrhotic liver disease, including variceal bleeding, ascites formation and hepatorenal syndrome. In addition, the stiffened, fibrotic microenvironment is conducive to the development of hepatocellular carcinoma (Hernandez-Gea et al., 2013).

Current management of liver fibrosis is limited to treatment of the underlying cause and of any complications. Liver transplantation is reserved for those with end stage disease, but this is limited by the availability of donor organs; and is associated with operative and peri-operative risks, as well as the complications of immunosuppression. Ideally, anti-fibrotic drugs are needed to treat patients at earlier stages, before cirrhosis develops. However, despite intensive research efforts these have remained frustratingly elusive.

Broadly speaking, there are two main reasons for this. Firstly, there are a number of challenges to the clinical translation of promising targets identified through in vitro studies. It is impossible to recapitulate the complex hepatic microenvironment in vitro, and animal models developed to address this do not faithfully reproduce the disease seen in humans. For example insulin resistance is strongly associated with non-alcoholic fatty liver disease in humans; however, mice fed on a methionine -choline deficient diet (a model of fatty liver disease) do not develop insulin resistance (Rinella and Green, 2004). In addition, fibrosis in humans takes years to decades to develop, and therefore clinical trials require lengthy follow up. They are further hampered by the lack of robust, sensitive, non-invasive methods to determine fibrosis stage (Schuppan and Pinzani, 2012).
Secondly, many aspects of the underlying molecular mechanisms remain poorly understood, and further research is required to gain better insight into the pathological processes, and reveal new therapeutic targets.

1.5 Liver Injury

Liver fibrosis results from chronic iterative liver injury, of which there are many causes. The commonest are alcohol, viral hepatitis and non-alcoholic fatty liver disease. Others include autoimmune disease, iron overload disorders such as hereditary haemochromatosis, Wilson’s disease (an autosomal recessive condition leading to accumulation of copper within the liver and brain), α1-antitrypsin deficiency (a serpinopathy resulting in retention of polymerised mis-folded α1-antitrypsin within hepatocytes) and drug reactions.

Regardless of the aetiology, liver injury results in hepatocyte necrosis and apoptosis, leading to loss of functioning parenchymal tissue, and triggering an inflammatory cascade. In addition, some agents cause deleterious effects on other cell types, for example, acetaldehyde, a product of alcohol metabolism has been shown to directly activate HSCs (Svegliati-Baroni et al., 2001).

1.5.1 Immune Response

Hepatocellular damage leads to the release of cell contents and reactive oxygen species, which directly activate HSCs and attract tissue macrophages. Activated HSCs and macrophages secrete pro-inflammatory mediators, which recruit T cells and neutrophils to the site of injury, and drive further activation of HSCs.

1.5.1.1 Adaptive Immunity

Classically, fibrosis was thought to be regulated by the balance of T helper 1 (T\textsubscript{H1}) to T helper 2 (T\textsubscript{H2}) cells, with a more prominent T\textsubscript{H2} response favouring fibrosis (Pellicoro et al., 2014). However, additional T\textsubscript{H} cell subsets have now been discovered which complicate the picture. For example, T\textsubscript{H17} cells secrete IL-17, which directly stimulates type I collagen production in HSCs; and regulatory T cells (T\textsubscript{Reg}) have been shown to have both anti-fibrotic and pro-fibrotic effects in different models of liver disease (Pellicoro et al., 2014).
In addition, Novobrantseva et al demonstrated that B-cell deficient mice have reduced hepatic collagen deposition compared with wild type controls after repetitive liver injury (Novobrantseva et al., 2005). Interestingly, B-cell deficiency was associated with reduced hepatic macrophage infiltration and HSC activation after acute liver injury, although this difference was no longer apparent with repetitive injury (Novobrantseva et al., 2005).

1.5.1.2 Toll-Like Receptor Signalling

Toll-like receptors (TLRs) are a family of cell surface receptors involved in activating the immune response to invading pathogens. TLR4 recognises lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, and is expressed by Kupffer cells and HSCs (Seki et al., 2007).

Liver injury is associated with increased intestinal permeability, resulting in bacterial translocation and increased exposure of the liver to LPS (Seki and Schnabl, 2012). Seki et al propose that TLR4 signalling provides a ‘molecular link’ between inflammatory and fibrogenic signalling in liver disease (Seki et al., 2007). They demonstrated that TLR4 enhances hepatic inflammation and fibrogenesis (Seki et al., 2007). Moreover, they showed that the fibrogenic effects of TLR4 are primarily mediated by HSCs, through a mechanism involving enhanced TGFβ1-mediated activation and collagen production (Seki et al., 2007).

In keeping with this, several single nucleotide polymorphisms (SNPs) within the TLR4 gene have been shown to be associated with risk of fibrosis progression in patients with chronic hepatitis C infection (Li et al., 2009).

1.6 Molecular Mechanisms of Fibrosis

The concept of ‘core’ and ‘regulatory’ pathways in fibrosis has been proposed (Mehal et al., 2011). Core pathways describe the molecular mechanisms that are necessary to initiate fibrosis in response to a trigger, and are likely to be conserved across organ systems. As such, targeting core pathways may be more effective in limiting fibrosis, but may be associated with off-target effects (Mehal et al., 2011). Therefore, one approach may be to inhibit core pathways, but target therapy to a specific cell or tissue type.

Wound healing and fibrosis are orchestrated by contractile myofibroblasts, which
develop from precursor cells within the tissue, in response to injury. Following activation, the myofibroblasts lay down the fibrillar collagen matrix, which characterises fibrosis, and contraction of the fibrotic scar results in further distortion of the tissue architecture. In many different organ systems, myofibroblast precursors have been identified as mesenchyme-derived perivascular cells; in the liver, these cells are the HSCs (Duffield, 2012).

1.6.1 HSC Activation

Activation of HSCs can be conceived as two phases along a continuum: initiation followed by perpetuation (Friedman, 2008a). Initiation describes the initial changes in gene expression that prime the HSC to respond to pro-fibrotic stimuli. These early changes are mediated by paracrine signaling from damaged hepatocytes and non-parenchymal cells including Kupffer and endothelial cells, in addition to changes within the ECM (Friedman, 2008a). Once primed, HSCs maintain their activation through paracrine and autocrine signaling, and develop the phenotypic characteristics of myofibroblasts. This is known as perpetuation, and is associated with at least six changes in HSC function including: retinoid loss, proliferation, fibrogenesis, contractility, chemotaxis and matrix degradation (Figure 1.4) (Friedman, 2008b).
Liver injury causes hepatocyte damage and Kupffer cell activation, which leads to the release of reactive oxygen species (ROS), TGF-β1 and other inflammatory and pro-fibrotic cytokines. This results in the activation of HSCs, which transform from quiescent vitamin A storing cells to proliferative, contractile and fibrogenic myofibroblasts. Adapted from (Friedman, 2008a).

1.6.1.1 Retinoid Loss

HSC activation is associated with loss of their characteristic cytoplasmic retinoid and lipid droplets. The reasons for this are unclear. Hernández-Gea et al have shown that release of HSC lipid droplets occurs by autophagy following liver injury, and provides energy for activation. Furthermore, blocking autophagy in HSCs resulted in retention of lipid droplets, and reduced liver fibrosis in vivo (Hernández-Gea et al., 2012). Conversely, Kluwe et al did not see a protective advantage from liver fibrosis in lecithin-retinol acyltransferase (LRAT)-null mice, whose HSCs lack lipid droplets owing to a defect in retinol metabolism (Kluwe et al., 2011).

1.6.1.2 Proliferation

The most potent HSC mitogen is platelet derived growth factor (PDGF); it signals via its tyrosine kinase receptors to activate the mitogen activated protein kinase (MAPK) and phosphoinositide-3-kinase Akt (PI3K-Akt) pathways (Friedman, 2008b). The PDGF receptor β (PDGFRβ) is upregulated following HSC activation,
however, there is now convincing evidence that quiescent HSCs also express PDGFRβ, suggesting that they are capable of responding to this mitogen even in their inactive state (Henderson et al., 2013).

1.6.1.3 Fibrogenesis

Activated HSCs are believed to be the major source of myofibroblasts during liver fibrosis; this has been demonstrated in both hepatotoxic and biliary models of fibrosis (Kisseleva et al., 2012; Mederacke et al., 2013). They are responsible for the excessive production and deposition of the fibrillar ECM that characterises fibrosis.

TGF-β1 is the most important pro-fibrotic cytokine; once activated it binds to its transmembrane receptor complex of TGF-β receptor types I and II. TGF-β1 binds to the type II receptor, which activates phosphorylation of the receptor-activated mothers against decapentaplegic homologs (R-Smads) 2 and 3 by the type I receptor (Wrana et al., 1992). Phosphorylated R-Smads 2 and 3 then form a complex with the common mediator Smad 4 (Co-Smad 4) and translocate to the nucleus, where they activate transcription of pro-fibrotic genes (Leask and Abraham, 2004). The inhibitory Smads 6 and 7 can prevent R-Smad phosphorylation, and nuclear translocation of the R-Smad Co-Smad complex.

The fibrotic ECM is predominantly composed of fibrillar forming type I collagen, along with type III collagen and fibronectin (Hernandez-Gea and Friedman, 2011). Type I collagen molecules are formed from two collagen alpha-1(I) chains (COL1A1) and a collagen alpha-2(I) chain (COL1A2). Following translation the pro-alpha chains are modified within the endoplasmic reticulum, before forming a triple helical structure (Kadler et al., 1996). Procollagen is converted into collagen by cleavage of the C- and N-terminal propeptides; this can occur within the cell in plasma membrane protrusions known as fibropositors (Kadler et al., 2007). Collagen then self-assembles into fibrils and is stabilised in the extracellular space by lysyl oxidase mediated cross-linking.

In fibroblasts, overexpression of Smads 3 and 4 activate the COL1A2 promoter, whilst overexpression of Smad 7 blocks TGF-β1 stimulated COL1A2 promoter activity (Chen et al., 1999). Interestingly, TGF-β1 upregulates expression of the inhibitory Smad 7 in fibroblasts, and therefore, may negatively regulate its own
actions (Chen et al., 1999).

1.6.1.4 Contractility

Myofibroblasts express αSma and are highly contractile. This is important for wound closure during normal wound healing. However, in fibrosis, contractility of activated HSCs is associated with pathological consequences.

1.6.1.4.1 Contractile Machinery

The cellular contractile machinery consists of actin and myosin proteins. Soluble, globular actin monomers (G-actin) are polymerised to form actin filaments (F-actin). F-actin is polarised, and polymerisation takes place at the free barbed end. This process is limited by capping proteins, which bind to the barbed end and inhibit further exchange of G-actin subunits.

New actin filaments are initiated by an actin nucleation core; examples include the actin-related 2/3 complex (Arp2/3) which nucleates branched actin filaments; and the formins, which initiate spontaneous actin polymerisation (Pollard, 2007). In addition, there are a number of other regulatory actin-binding proteins, including profilin-1, which can both inhibit and promote actin polymerisation depending on its relative concentrations to G-actin and free barbed ends (Ding et al., 2012); and the actin-severing protein cofilin (Bravo-Cordero et al., 2013).

The myosins are a family of motor proteins. The conventional myosin, myosin II, is involved in smooth muscle contraction. It is formed from two heavy chains, and four light chains, which are subtyped into regulatory and essential. Contraction occurs through a cycle of actin-myosin cross-bridging and adenosine triphosphate (ATP) hydrolysis. In smooth muscle, this can be initiated by phosphorylation of the regulatory myosin light chain (MLC) (Reynaert et al., 2002).

1.6.1.4.2 Signalling Pathways Mediating Contraction

Cellular contraction is regulated either by changes in intracellular calcium (Ca\(^{2+}\)) or by RhoA associated kinase (ROCK) signalling (Reynaert et al., 2002). In smooth muscle cells, contraction is initiated by an increase in intracellular Ca\(^{2+}\), resulting from influx through voltage gated channels, or release from intracellular stores in response to hydrolysis of inositol lipids (Reynaert et al., 2002). This leads to Ca\(^{2+}/\)calmodulin dependent phosphorylation of MLC kinase (MLCK), which in turn
phosphorylates the regulatory MLC (Follonier Castella et al., 2010). Alternatively, signalling by the small GTPase RhoA leads to activation of ROCK. ROCK both inhibits MLC phosphatase (MLCP), and directly phosphorylates MLCK (Follonier Castella et al., 2010). There is evidence that both these signalling pathways play a role in HSC contraction, however, it has been suggested that Ca\(^{2+}\) signalling becomes more important as HSCs are activated (Reynaert et al., 2002).

1.6.1.4.3 Regulation of Sinusoidal Resistance

As liver specific pericytes, HSC contraction may result in increased sinusoidal resistance, contributing to the development of portal hypertension. This is supported by the findings of Thimgan et al, who demonstrated that HSC contraction in response to the vasoconstrictor endothelin-1 (ET-1) was sufficient to increase sinusoidal resistance *in vitro* (Thimgan and Yee, 1999); and Zhang et al, who demonstrated that ET-1 stimulated sinusoidal contraction at sites co-localising with HSCs in the intact liver (Zhang et al., 1994). Moreover, Bhathal et al demonstrated that in the cirrhotic rat liver around 20 to 30% of the increased intrahepatic resistance is reversible by vasodilators, with a pattern of response consistent with the effects of vasodilators on myofibroblasts *in vitro* (Bhathal and Grossman, 1985).

The potent vasoconstrictor ET-1 is secreted by HSCs following liver injury, and acts through its G-protein coupled receptors, type A (ET\(_A\)) and type B (ET\(_B\)) (Rockey et al., 1998). The ET\(_A\) receptor predominately mediates vasoconstriction, whilst the ET\(_B\) receptor is associated with a number of biological effects (Reynaert et al., 2002). Both receptor types are expressed on HSCs; however, receptor ET\(_A\) is predominant during early HSC activation (Pinzani et al., 1996). Therefore, ET-1 may act in an autocrine manner to stimulate HSC contraction following liver injury.

The vasoconstrictive effects of ET-1 are antagonised by nitric oxide (NO), a messenger molecule and potent vasodilator. It is synthesised from L-arginine by three isoforms of nitric oxide synthetase (NOS): inducible NOS (iNOS) is expressed by various cell types, including HSCs and hepatocytes; whilst endothelial and neuronal cells express the constitutive isoforms, eNOS and nNOS, respectively (Reynaert et al., 2002).

In cirrhotic liver disease there is a pathological imbalance in NO bioavailability: excessive NO in the systemic and splanchnic circulation promotes the development
of a hyperdynamic circulation; whilst NO deficiency in the liver, contributes to increased intrahepatic resistance (Reynaert et al., 2002).

A number of other vasoactive mediators acting upon HSCs have been identified; these are listed in Table 1.1.

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<td>ANP</td>
<td>Relaxation</td>
</tr>
<tr>
<td>Adrenomedullin</td>
<td>Relaxation</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>Relaxation</td>
</tr>
<tr>
<td>PGI2/PGE2</td>
<td>Relaxation</td>
</tr>
<tr>
<td>Y-27632 (rho kinase inhibitor)</td>
<td>Relaxation</td>
</tr>
</tbody>
</table>

NO, nitric oxide; ANP, atrial natriuretic peptide; PG, prostaglandin

Table 1.1 Mediators of HSC contractility
Adapted from (Reynaert et al., 2002)

1.6.1.4.4 Matrix Interaction
Contraction is also important in allowing HSCs to sense and respond to changes within the ECM. During differentiation into myofibroblasts, precursor cells develop actin stress fibres and supermature focal adhesions in response to mechanical stress (Gabbiani, 2003). This is dependent on an intact actin-myosin cytoskeleton, as inhibiting myosin with blebbistatin is associated with a reduction in stress fibres and
focal adhesions in HSCs (Liu et al., 2010b). In addition, increased matrix stiffness and the ability to generate tension are prerequisites for HSC activation (Olsen et al., 2011).

Furthermore, myofibroblast contraction is an important mechanism by which latent TGF-β1, sequestered in the matrix, is activated (this is discussed further in section 1.11.9.1); this leads to perpetuation of the myofibroblast phenotype, and a positive feedback loop driving fibrotic progression.

Finally, HSC mediated contraction of the fibrotic scar results in further distortion of the liver architecture, which in turn impairs organ function.

1.6.1.4.5 Chemotaxis
HSCs are capable of migrating towards a number of cytokines, for example, PDGF has been shown to stimulate HSC chemotaxis, facilitating migration of HSCs to the site of injury (Melton and Yee, 2007).

1.6.1.4.6 Matrix Degradation
HSCs are capable of secreting matrix metalloproteinases, a family of proteins involved in matrix degradation. During early fibrogenesis, the normal basement membrane is degraded, and is subsequently replaced by the type I collagen-rich matrix characteristic of fibrosis. Matrix metalloproteinases and matrix degradation and production in fibrosis are discussed further in section 1.8.1.

1.6.2 Transcriptional Regulation
A wide variety of transcriptional regulators are implicated in the activation of HSCs, including both those that maintain HSC quiescence, and must therefore be downregulated; and those that directly activate transcription of pro-fibrotic genes.

The nuclear hormone receptors are a superfamily of ligand-activated transcription factors, and include the peroxisome proliferator-activated receptors (PPARs). Several PPAR isoforms have been identified (α, β, γ and δ); they are ubiquitously expressed and are important in the regulation of lipid and glucose metabolism. PPARγ is associated with adipocyte differentiation, and is highly expressed by quiescent HSCs (She et al., 2005). Its expression must be downregulated to allow HSC activation, and conversely, HSC activation can be partially reversed by forced
expression of PPARγ (Galli et al., 2000; She et al., 2005). Furthermore, the synthetic PPARγ ligand, pioglitazone, prevents HSC activation and is anti-fibrotic in vivo (Kon et al., 2002).

PPARγ expression is upregulated by another member of the nuclear hormone receptor superfamily, the bile acid activated farnesoid X receptor (FXR) (Fiorucci et al., 2005). Data on the expression of FXR in HSCs are conflicting, however, there is agreement that FXR agonists are anti-fibrotic in vivo (Fickert et al., 2009; Fiorucci et al., 2004; Mudaliar et al., 2013; Verbeke et al., 2014). In addition, the FXR agonist obeticholic acid reduces intraheaptic resistance and portal pressures in two rat models of liver fibrosis; an effect thought to be mediated by increased eNOS (Verbeke et al., 2014).

The LIM homeobox gene Lhx2 is also required to maintain a quiescent HSC phenotype. Lhx2 knockout in mice results in accumulation of activated HSCs and progressive liver fibrosis, which is lethal in utero (Wandzioch et al., 2004). Moreover, expression of Lhx2 in the human HSC line LX-2 results in downregulation of the pro-fibrotic genes αSma and collagen I α(I) (Wandzioch et al., 2004).

On the other hand, a number of transcription factors positively regulate HSC activation. For example, the Smad family of transcription factors mediate TGF-β signaling, one of the most potent pro-fibrotic cytokines (this is discussed further in section 1.6.1.3). MyoD, a class B basic helix-loop-helix protein and myogenic transcription factor, regulates skeletal muscle cell differentiation and is also expressed by activated HSCs, where it is thought to regulate contractility (Mann and Mann, 2009). The transcription factor sex-determining region Y box 9 (Sox9) regulates ECM deposition during development (Pritchett et al., 2010), and is ectopically expressed by activated HSCs, where it regulates type I collagen transcription (Piper Hanley et al., 2007). Nuclear factor kappa B (NF-κB) is a family of transcription factors which are important in the regulation of immune and inflammatory responses. NF-κB includes five subunits, which can homo- or heterodimerise, and is inhibited by the IkαB protein (Mann and Mann, 2009). During HSC activation, there is an increase in NF-κB signaling; this is important for two reasons: the transcription of pro-inflammatory and pro-fibrotic genes, and resistance
to apoptosis (Mann and Mann, 2009).

1.6.3 Epigenetic Regulation

Epigenetics describes heritable modifications to the chromosome leading to altered gene expression, which are not due to changes within the DNA sequence, and includes: DNA methylation, histone modification and regulation of gene expression by non-coding RNAs (Mann, 2014).

In a fascinating study, Zeybel et al demonstrated that an ancestral history of liver fibrosis protects future generations from fibrogenesis, in response to chronic hepatotoxic injury. The protective benefit was associated with a reduction in activated HSCs, and epigenetic changes including hypomethylation of the quiescence-associated gene peroxisome proliferator activated receptor γ (PPAR γ), and hypermethylation of TGF-β (Zeybel et al., 2012).

Interestingly, this intergenerational protective benefit is at odds with the findings of Kisseleva et al, who demonstrated that a prior episode of liver fibrosis predisposes individuals to a more aggressive fibrotic response to subsequent episodes of liver injury (Kisseleva et al., 2012).

1.6.4 Alternative Sources of Myofibroblasts

There is now convincing evidence that activated HSCs are the major source of myofibroblasts in liver fibrosis (Kisseleva et al., 2012; Mederacke et al., 2013). However, there are other potential sources of hepatic myofibroblasts, and these include portal fibroblasts, bone marrow derived fibrocytes and mesenchymal stem cells (Iwaisako et al., 2012).

1.6.4.1 Portal Fibroblasts

Portal fibroblasts are thought to differentiate into myofibroblasts in response to biliary injury, but their contribution to liver fibrosis, and their role in liver fibrosis from other aetiologies, is controversial (Dranoff and Wells, 2010; Mederacke et al., 2013).
1.6.4.2 Fibrocytes

Fibrocytes are a population of circulating bone marrow derived, CD45+, collagen producing cells, which migrate to the site of tissue injury and contribute to the fibrotic response. They are recruited to the liver following both biliary and hepatotoxic injury, however, their contribution to the hepatic myofibroblast population is minor (approximately 3 to 5% of hepatic myofibroblasts are thought to be derived from fibrocytes) (Kisseleva et al., 2006; Scholten et al., 2011). This is in contrast to other organ fibrosis, where they play a more major role: for example around 25% to 34% of lung myofibroblasts are thought to originate from bone marrow cells (Kisseleva and Brenner, 2008).

1.6.4.3 Bone Marrow Derived Mesenchymal Stem Cells

Bone marrow derived mesenchymal stem cells can also differentiate into myofibroblasts within the injured liver and contribute to the fibrotic response (Russo et al., 2006). However, more recently, Higashiyama et al demonstrated only a negligible contribution to type I collagen production by bone marrow derived cells in two different mouse models of liver fibrosis (Higashiyama et al., 2009).

On the other hand, allogenic bone marrow transplantation in mice subjected to carbon tetrachloride (CCl₄) is associated with a reduction in liver fibrosis (Sakaida et al., 2004); and clinical trials of stem cell therapy in chronic liver disease are ongoing (Forbes and Newsome, 2012).

1.6.4.4 Epithelial to Mesenchymal Transition

Epithelial to mesenchymal transition (EMT) describes the process by which epithelial cells lose their intracellular adhesions and polarity; detach from the basement membrane; migrate into the ECM; and lose their epithelial markers, whilst acquiring those of mesenchymal cells. It has been proposed that hepatocytes and cholangiocytes undergo EMT in liver fibrosis, and contribute to the myofibroblast population (Brenner et al., 2012). In support of this, Zeisberg et al have shown evidence of hepatocyte EMT in CCl₄-induced liver fibrosis (Zeisberg et al., 2007); whilst Omenetti et al have shown gene expression data supporting EMT of cholangiocytes in cholestatic liver disease (Omenetti et al., 2008). However, this is disputed, and more recent lineage tracing studies from the Wells and Brenner groups
suggest that neither hepatocytes nor cholangiocytes undergo EMT in liver fibrosis (Chu et al., 2011; Scholten et al., 2010; Taura et al., 2010).

1.6.5 HSCs in Fibrosis Resolution

The association between HSC apoptosis and fibrosis resolution was first described many years ago (Iredale et al., 1998). Activated HSCs secrete type I collagen and tissue inhibitors of metalloproteinases (TIMP) -1 and -2; therefore, eliminating activated HSCs should tip the balance of ECM turnover away from production and towards degradation (discussed further in section 1.8.1). This is supported by the finding that expression of pro-collagen 1, TIMP-1 and TIMP-2 decline during spontaneous recovery from fibrosis, whilst collagenase activity increases (Iredale et al., 1998). Furthermore, inducing apoptosis in HSCs using gliotoxin, attenuated fibrosis in rats treated with CCl4 (Wright et al., 2001).

Depleting macrophages during recovery from liver fibrosis is associated with a failure to remodel the fibrotic scar (Duffield et al., 2005). Therefore, macrophages are critical mediators of fibrosis resolution; one potential mechanism by which they may promote fibrosis resolution, is the induction of HSC apoptosis through secretion of pro-apoptotic tumour necrosis factor-related apoptosis inducing ligand (TRAIL) and matrix metalloproteinase-9 (MMP-9) (Ramachandran and Iredale, 2012).

More recently, Kisseleva et al have proposed the intriguing concept that following cessation of liver injury, a proportion of activated HSCs revert to an ‘inactive’ phenotype, and are primed to respond to future insults more rapidly (Kisseleva et al., 2012). These inactivated HSCs adopted a quiescent-like phenotype with downregulation of pro-fibrotic genes. However, they failed to upregulate the quiescence-associated genes adipose differentiation relate protein (Adfp), Adipor1 and GFAP; and when cultured in vitro, they exhibited a more robust response to TGF-β1 stimulation, than quiescent HSCs. This suggested that inactivated HSCs are primed to respond to future insults more vigorously, and is supported by the finding that mice treated with a second course of CCl4 following a recovery period, developed more severe fibrosis than mice treated with a single course. Finally, they suggest that inactivated HSCs are resistant to apoptosis due to upregulation of anti-apoptotic heat shock proteins 1a/b (Hspa1a/b) (Kisseleva et al., 2012). These findings are supported by Troeger et al, who have also demonstrated that HSCs are
inactivated, and primed to respond to future fibrogenic stimuli, following fibrosis resolution (Troeger et al., 2012).

1.6.6 Targeting HSCs for Therapy

The ability to specifically target HSCs for the treatment of liver fibrosis would be advantageous, as this would enable inhibition of core fibrotic pathways whilst avoiding potentially deleterious effects in other organs. As such, engineering compounds to facilitate their specific uptake by HSCs is an active area of research, and several strategies have been suggested.

Beljaars et al have pioneered one of the most promising approaches, using mannose-6-phosphate modified albumin as a drug carrier (Beljaars et al., 1999). Mannose-6-phosphate modified albumins are specifically taken up by activated HSCs via the mannose-6-phosphate/insulin-like growth factor receptor. Using this approach, the angiotensin type I receptor blocker, losartan; a Rho kinase inhibitor; and a TGF-β receptor type I (activin-like kinase 5; ALK5) inhibitor, have been targeted to activated HSCs in rodent models of liver fibrosis, with an associated reduction in collagen deposition (Moreno et al., 2010; van Beuge et al., 2011; van Beuge et al., 2013).

In addition, peptides with affinity for the PDGFRβ, which is highly expressed by activated HSCs, have been used to modify an albumin carrier and liposome to target interferon γ to activated HSCs (Bansal et al., 2011; Li et al., 2012).

An alternative mechanism for targeting HSCs was developed by Douglass et al. They synthesised a single chain antibody (C1-3) against the membrane protein synatophysin, which is expressed on activated HSCs in the liver, and confirmed that this specifically targets myofibroblasts in liver fibrosis. In addition, they demonstrated attenuation of CCl4-induced liver fibrosis in mice treated with C1-3-conjugated gliotoxin compared with free gliotoxin (Douglass et al., 2008).

Finally, Sato et al used vitamin-A coupled liposomes to deliver siRNA targeted against a collagen-specific chaperone molecule to HSCs, and demonstrated a significant reduction in fibrosis in three different rat models of liver fibrosis (Sato et al., 2008).
1.7 The Extracellular Matrix in Fibrosis

The extracellular matrix provides structural integrity, and maintains the differentiation and functional specificity of the cells it supports, through both its biochemical and biomechanical properties. In the normal liver, ECM synthesis and degradation are maintained in equilibrium, however, in liver fibrosis, there is an imbalance favouring ECM production. Both the quantity and quality of the ECM are altered, with replacement of the basal membrane-like components by dense fibrillar collagens (Hernandez-Gea and Friedman, 2011). Progressive fibrosis is associated with collagen cross-linking and elastin deposition, which confer greater resistance to degradation (Issa et al., 2004).

1.7.1 Capillarisation

Obliteration of the sinuosity space by fibrotic matrix results in a physical barrier to diffusion, and impairs exchange between the hepatocytes and plasma. Moreover, the sinusoidal endothelial cells lose their fenestrations, and hepatocytes their microvilli, in a process termed ‘capillarisation’ (Hernandez-Gea and Friedman, 2011). Hepatocyte function is further impaired by a reduction in metabolic capacity; this can be observed in vitro by a reduction in albumin secretion and cytochrome P450 enzymes in hepatocytes cultured on type I collagen, compared with a basement membrane-like matrix (Bissell et al., 1987). Together, these changes result in impaired organ function and increased portal pressure.

1.7.2 Growth Factors and Matricellular Proteins

The ECM also acts as a reservoir for growth factors and matricellular proteins, which can be tapped during fibrosis.

For example, the most potent regulator of fibrosis, TGF-β1, is sequestered within the matrix as a latent complex, and can be activated by contractile myofibroblasts (Worthington et al., 2011). This mechanism can generate a positive feed forward loop, leading to amplification and progression of the fibrotic process. Other examples of matrix-bound, pro-fibrogenic growth factors include the fibroblast growth factors 1 and 2 (Yu et al., 2003).

The term ‘matricellular’ describes non-structural proteins present within the ECM, which modulate cell-matrix interactions and cell function. Relevant examples include
connective tissue growth factor (CTGF, CCN2), osteopontin and secreted protein, acidic and rich in cysteine (SPARC); these have been shown to play a central role in fibrosis in a number of organ systems (Bornstein and Sage, 2002). Their effects are mediated through several mechanisms, for instance CTGF has been shown to activate myofibroblasts and induce TGF-β1 (Lipson et al., 2012); osteopontin enhances proliferation and migration of myofibroblasts (Kohan et al., 2009; Xiao et al., 2012); and SPARC is important for collagen fibril assembly, and also modulates TGF-β1 activity (Atorrasagasti et al., 2013; Rentz et al., 2007). Levels of these proteins are increased in liver disease, and their abrogation protects against fibrosis (Atorrasagasti et al., 2013; Coombes et al., 2014; Xiao et al., 2012).

1.7.3 Mechanical Stiffness

In addition to its biochemical properties, biomechanical forces within the ECM are important in the progression of liver fibrosis and its clinical consequences. Increased portal pressure underlies many of the clinical complications of liver disease and is related to changes in intrahepatic resistance to blood flow, according to the analogy of Ohm’s law \( P = Q \times R \), where \( P \) is the change in pressure, \( Q \) is the flow and \( R \) is the resistance to flow (Hernandez-Gea and Friedman, 2011). Resistance to blood flow through the sinusoids is increased by deposition of fibrotic ECM within the sinusoidal space, along with ‘capillarisation’. Thus, fibrosis results in mechanical changes within the liver, which have important clinical consequences.

The mechanical stiffness of the matrix is determined by its components (collagens, proteoglycans and other matrix proteins), in addition to their post-translational modifications, organisation and cross-linking (Wells, 2008). Stiffness of a material can be calculated using Young’s modulus (also known as the tensile or elastic modulus): this is a ratio of stress to strain, and materials with a high Young’s modulus are rigid. Normal liver tissue has a Young’s modulus of 0.4 – 0.6 kPa, whilst cirrhotic liver measures from 3 kPa upwards of 12 kPa (Olsen et al., 2011). As a comparator, the Young’s modulus of plastic is \( \sim 10^6 \) kPa (Janmey and Miller, 2011). In the clinic, liver stiffness can be measured using transient elastography, and has been shown to correlate with fibrosis stage; and in some studies, portal hypertension and the development of oesophageal varices (Foucher et al., 2006; Rockey, 2008).
On a cellular level matrix stiffness is an important regulator of cell behaviour and phenotype. For example, cells migrate along a stiffness gradient, towards areas of greater rigidity; a phenomenon termed ‘durotaxis’ (Lo et al., 2000); and of relevance to liver fibrosis, matrix stiffness has been shown to regulate the activation of HSCs (Olsen et al., 2011). This was described by Olsen et al, who cultured HSCs on a range of increasingly stiff gel supports, and demonstrated that HSC activation is positively correlated with matrix stiffness, independent of the matrix composition and the pro-fibrotic cytokine, TGF-β1 (Olsen et al., 2011). In addition to a stiff matrix, activation of HSCs also depends on their ability to generate tension (Olsen et al., 2011). Tension is generated through myosin II activity when a cell’s actin cytoskeleton is anchored to a fixed support within the ECM, via a complex known as a focal adhesion (Gardel et al., 2010). Increased traction forces result in maturation of focal adhesions, and direct cell migration towards a stiffer ECM (Gardel et al., 2010).

Importantly, matrix stiffness has been shown to increase early following liver injury and before the development of fibrosis, possibly as a result of lysyl oxidase mediated matrix cross-linking (Georges et al., 2007). Furthermore, inhibition of lysyl oxidase-like 2 attenuates liver fibrosis (Barry-Hamilton et al., 2010).

Taken together, these findings are consistent with the concept of matrix stiffness as an early regulator of HSC activation.

1.8 ECM Turnover

1.8.1 Matrix Metalloproteinases

The matrix metalloproteinases (MMPs) are a family of 23 (24 in mouse) zinc-dependent proteolytic enzymes, which are capable of degrading the ECM (Klein and Bischoff, 2011). They can be sub-divided into soluble and membrane anchored types, and classified by their substrate specificity.

Accordingly, the soluble MMPs are divided into four groups: the collagenases (MMP-1, -8 and -13); the gelatinases (MMP-2 and -9); the stromelysins (MMP-3, -10 and -11); and a heterogenous group consisting of matrilysin (MMP-7), metalloelastase (MMP-12), enamelysin (MMP-20), endometase (MMP-26) and epilysin.
MMPs are potentially destructive, and so it is important that their function is regulated at several levels. Transcription of MMP mRNA is controlled by a number of cytokines and growth factors, and there is also evidence for epigenetic regulation (Klein and Bischoff, 2011). In addition, most MMPs are secreted as zymogens and require activation by cleavage of the pro-peptide (Klein and Bischoff, 2011). Lastly, their enzymatic activity can be inhibited by binding to their soluble inhibitors, known as tissue inhibitors of metalloproteinases (TIMPs).

TIMPs act to both stabilize the pro-enzyme and block the catalytic activity of the active enzyme (Iredale, 1997). Although non-convalent, binding between TIMPs and MMPs is essentially irreversible under physiological conditions (Iredale, 1997). However, if separation does occur, TIMP inhibitory activity is retained (Iredale, 1997). To date, four TIMPs have been identified (TIMP-1, -2, -3 and -4); although there are some differences in inhibitory activity, they bind to most MMPs (Brew et al., 2000).

Within the liver, inflammatory cells, HSCs and hepatocytes secrete MMPs and TIMPs. The relative ratio of MMPs to TIMPs alters during the progression and resolution of liver fibrosis, tipping the balance to favour ECM production or degradation. During early fibrosis, activated HSCs secrete MMPs 2 and 9, which act against type IV collagen and degrade the normal basement membrane-like matrix (Arthur et al., 1992; Han et al., 2007; Iredale, 2007). As fibrosis progresses, HSC expression of TIMP-1 and -2 increases, inhibiting MMPs and favouring ECM deposition, and the accumulation of fibrotic scar (Iredale, 2007). Roderfeld et al demonstrated a crucial role for TIMP-1 in liver fibrosis: they showed that using a mutant MMP-9 to scavenge TIMP-1, protects against liver fibrosis in a mouse model (Roderfeld et al., 2006). In human disease, TIMP-1 levels correlate with the severity of fibrosis (Yata et al., 1999), and are a component of the Enhanced Liver Fibrosis (ELF) score, which is validated to estimate fibrosis severity (Parkes et al., 2011; Rosenberg et al., 2004), and predict clinical outcomes (Parkes et al., 2010). During fibrosis resolution, removal of the injurious agent is associated with downregulation
of TIMP-1 and -2, and the MMP:TIMP balance tips to favour ECM degradation (Figure 1.5) (Iredale, 2007).

![Diagram of ECM degradation and production during fibrosis]

**Figure 1.5 The balance of ECM degradation and production during fibrosis**

During fibrosis progression, HSC expression of TIMP-1, TIMP-2 and type I collagen is increased, favouring ECM production. However, during fibrosis resolution, TIMP-1, TIMP-2 and type I collagen are downregulated, tipping the balance in favour of ECM degradation.

### 1.8.2 A Disintegrin and Metalloproteinase Domain Proteins

A disintegrin and metalloproteinase domain (ADAM) proteins are a zinc dependent, largely membrane bound family of proteases (Nyren-Erickson *et al.*, 2013). To date, 40 different ADAM proteins have been described, of which 21 are thought to be functional in humans (Duffy *et al.*, 2009). All ADAMs contain a disintegrin domain through which they are capable of binding to integrins; and can modulate cell adhesion and migration, in an ADAM- and integrin-specific manner (Duffy *et al.*, 2009).

### 1.9 Fibrosis Resolution

Historically, clinical dogma was that cirrhosis was irreversible. However, evidence suggests that this is not the case, and it is now known that cirrhosis is a dynamic disease with the potential for regression (Bonis *et al.*, 2001). Clinically, regression of fibrosis has been observed following treatment in patients with liver disease from a variety of aetiologies. For example, in hepatitis B and C infection following antiviral therapy (Arthur, 2002; Malekzadeh *et al.*, 2004); alcoholic liver disease following abstinence from alcohol (Pares *et al.*, 1986); autoimmune hepatitis following immunosuppressive treatment (Dufour *et al.*, 1997); primary biliary cirrhosis following methotrexate (Kaplan *et al.*, 1997); secondary biliary cirrhosis following biliary decompression (Hammel *et al.*, 2001); and, in non-alcoholic fatty liver...
disease (NAFLD) following weight loss (Dixon et al., 2004). Similar findings have been confirmed in animal models, with remodeling of the ECM to almost normal architecture following cessation of CCl₄ treatment (Iredale et al., 1998) or bilio-jejunal anastomosis following bile duct ligation (Issa et al., 2001). However, whether advanced cirrhosis can be reversed is controversial, and it seems likely that there is a point of no return, at which stage architectural changes are so extensive and the collagen scar densely cross linked, that remodeling is not possible (Iredale, 2007).

1.10 Vascular Changes

Structural and functional changes in the vasculature are important in the pathogenesis of cirrhosis. Intrahepatic shunts can develop between the afferent and efferent vessels, allowing blood to bypass the liver parenchyma and enter the systemic circulation without being processed by hepatocytes (Desmet and Roskams, 2004). In addition, tissue hypoxia stimulates angiogenesis within the liver parenchyma, and this has been linked to the progression of fibrosis.

Vascular endothelial growth factor (VEGF) is one of the most potent stimulators of angiogenesis. Its expression is induced in response to hypoxia by the oxygen sensitive transcription factor Hypoxia-Inducible Factor 1alpha (HIF1α), and is increased in liver fibrosis (Friedman, 2008b; Yoshiji et al., 2003). In vitro, VEGF stimulates type I collagen production and proliferation in activated HSCs; whilst, in in vivo models of liver fibrosis, inhibition of VEGF signalling by blockade of its receptors VEGFR-1 and VEGFR2, is associated with a significant decrease in fibrosis (Yoshiji et al., 2003).

However, whilst VEGF promotes fibrogenesis, there is also evidence that it is required for fibrosis resolution. Yang et al demonstrated that blockade of VEGF with a neutralising antibody impaired ECM remodelling in two mouse models of fibrosis resolution (Yang et al., 2014). They suggest that during fibrosis resolution, VEGF promotes monocyte recruitment to the liver by increasing vascular permeability and stimulating monocyte migration. The infiltrating monocytes differentiate into macrophages within the liver and enhance fibrosis resolution through a chemokine (C-X-C motif) ligand 9 (CXCL9) – MMP13 axis (Yang et al., 2014). This is in keeping with the findings of Duffield et al, who demonstrated that macrophages are critical mediators of fibrosis resolution (Duffield et al., 2005).
1.11 Cell Matrix Interaction

1.11.1 Integrins

Interactions between cells and their microenvironment are primarily mediated by a family of cell surface receptors, the integrins. Integrins are heterodimers formed from an alpha and a beta subunit. They primarily bind ECM proteins; however, some bind soluble ligands such as fibronectin, or membrane proteins on adjacent cells (Hynes, 2002). In addition, they form a mechanical link between the ECM and the intracellular actin cytoskeleton.

To date 18 alpha and 8 beta subunits have been identified, forming 24 distinct integrin heterodimers. These can be classified according to their ligand specificity: collagens (α1β1, α2β1, α10β1 and α11β1); laminin (α3β1, α6β1, α7β1 and α6β4), RGD (all αV integrins, α5β1, α8β1 and αIIbβ3) and LVD (α4β1, α4β7, α9β1, αEβ7 and all β2 integrins) (Campbell and Humphries, 2011). However, there is redundancy within this system, as individual integrins are capable of binding several ligands (Hynes, 2002).

Alternatively, they can be grouped by subunit; this is particularly relevant when considering genetic knockout models. By this classification, the beta1 (Itgb1) subfamily is the largest, with 12 identified alpha subunit partners. Of particular relevance to fibrosis, all four collagen receptors are Itgb1 heterodimers, and αVβ1 is thought to play an important role in activation of TGF-β1 (discussed further in section 1.11.9.1).
Table 1.2 Integrins classified by beta subunit
There are 8 beta subunits and 18 alpha subunits, which associate to form 24 different integrin heterodimers. Itgb1 associated with 12 different alpha subunits, forming the largest subfamily. Adapted from (Hynes, 2002).

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<th>β1</th>
<th>β2</th>
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<th>β4</th>
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<td>αlbb</td>
<td>α6</td>
<td>αV</td>
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Table 1.3 Collagen integrin receptors
Adapted from (Eckes et al., 2010).

Collagen Integrin Receptors

Collagen I  α1β1, α2β1, α11β1
Collagen II  α10β1
Collagen III α1β1, α2β1, α11β1
Collagen V  α1β1, α2β1
Collagen I fibrils α2β1, α11β1
Figure 1.6 Integrin classification
Diagram demonstrating integrin alpha-beta heterodimers and their ligand specificities. Adapted from (Hynes, 2002).
Integrins are generally expressed on the cell surface in an inactive state, with low affinity for ligand binding. They are activated in response to cell-specific cytoplasmic signalling, which results in a conformational change within the extracellular domain and exposure of the ligand binding site. The ability of a cell to quickly switch integrins ‘on and off’ is required for cell migration, and is also necessary to avoid potential catastrophe arising from excessive adhesion (Cox et al., 2010). The importance of this ‘on – off’ mechanism is illustrated by the platelet integrin αIIbβ3. This is expressed on the platelet membrane in a constitutionally inactive state, and requires platelet activation following injury to ‘switch on’ (Hynes, 2002). Following activation, αIIbβ3 binds to its ligands fibrinogen, von Willenbrand factor and fibronectin, resulting in platelet aggregation and haemostasis (Hynes, 2002). However, without this regulation, uncontrolled platelet adhesion would result in excessive thrombosis, which is potentially life threatening.

1.11.2 Discoidin Domain Receptors

The discoidin domain receptors, DDR1 and DDR2, are a second family of cell surface receptors responsible for mediating cell-matrix interactions (Leitinger, 2014). They are activated by collagens, which leads to receptor autophosphorylation and initiation of intracellular signalling pathways, regulating many aspects of cell behaviour. They have also been shown to modulate integrin signalling (Leitinger, 2014).

DDR2 expression is increased in rodent models of chronic liver injury; however, deleting DDR2 exacerbates liver fibrosis in the CCl₄ model of chronic liver injury, in part due to increased TGF-β expression in HSCs and modulation of HSC-macrophage interactions (Olaso et al., 2011; Zhang et al., 2010).

1.11.3 Structure of Integrins

Integrin subunits are formed from a large extracellular domain, a single-pass transmembrane region and a short, unstructured cytoplasmic tail (Campbell and Humphries, 2011). The integrin heterodimer can be visualised as a ligand binding ‘head’ on top of two ‘legs’, and this anatomical analogy is used when describing the extracellular structures of the subunits.
The alpha subunit consists of a β-propeller domain supported by a ‘leg’ including a thigh and two calves, with a hinge point (‘knee’ or genu) located between the thigh and first calf (Campbell and Humphries, 2011). The beta subunit contains a β-I domain, with a more flexible ‘leg’ formed from a hybrid region, a plexin-semaphorin-integrin (PSI) domain, four cysteine-rich epidermal growth factor (EGF) repeats and a β-tail (Campbell and Humphries, 2011). A flexible bend is located in a similar position to the alpha subunit ‘knee’, such that the integrin can bend at this point.

Nine of the alpha subunits contain an α-I domain within the β-propeller, and this is the site of ligand binding in these integrins (Hynes, 2002). For those integrins without an α-I domain, the ligand binding site is formed by an association of the β-propeller region of the alpha subunit with the β-I domain of the beta subunit (Hynes, 2002).

1.11.4 Integrin Activation: Inside-Out Signalling

Integrins can switch between a ‘v-shape’ to an upright position, by flexing or extending at the knee. This conformational change gives rise to the ‘switchblade’ model of integrin activation (Beglova et al., 2002). In this model, the bent form is inactive, with the ligand binding site in close proximity to the cell membrane and inaccessible. Integrin activation occurs when intracellular signalling leads to a conformational change in the extracellular domain, with the integrin extending into an upright position, revealing the ligand binding site (Beglova et al., 2002).
Integrins can flex at the genu located between the thigh and calf 1 domains of the alpha subunit, corresponding with a flexible bend within the beta subunit. In the ‘switchblade’ model of integrin activation, integrins in the upright position are active, whereas those in the bent conformation are inactive. EGF, epidermal growth factor; PSI, plexin-semaphorin-integrin. Adapted from (Campbell and Humphries, 2011)

However, it has been argued that integrin activation can occur in the bent conformation, and that straightening of the integrin occurs after ligand binding (Xiong et al., 2003; Ye et al., 2008). This led to the proposal of an alternative model of integrin activation: the ‘deadbolt’ model (Xiong et al., 2003).

The deadbolt model suggests that in the resting state, the β-tail interacts with the β-I domain, preventing ligand binding (Xiong et al., 2003). Integrin activation occurs when intracellular signalling leads to a conformational change within the transmembrane regions, resulting in release of the β-I domain, and increased affinity for ligand binding. In this model, activation does not depend on an increase in height of the integrin; this is thought to happen after ligand binding (Xiong et al., 2003).

Following activation, integrins are more laterally mobile within the cell membrane and can group into clusters through interactions between the transmembrane domains of adjacent integrin subunits (Qin et al., 2004). Clustering increases avidity for ligand binding and can also activate downstream intracellular signalling cascades (Qin et al., 2004; Ye et al., 2014).
This ability of integrins to regulate their affinity for ligand binding is termed ‘inside-out signalling’, and is associated with separation of the cytoplasmic tails in response to intracellular signalling (Qin et al., 2004). Although it is generally accepted that this results from disruption of a salt bridge between the alpha and beta subunits, this may not be true for all integrins, as Czuchra et al found no change in activation of Itgb1 following genetic disruption of the alpha-beta salt bridge (Czuchra et al., 2006; Moser et al., 2009).

1.11.5 Talin

The cytoplasmic protein talin plays an important role in integrin activation. It is expressed in two isoforms talin 1 and talin 2, and consists of an N-terminal head domain with a flexible C-terminal rod (Moser et al., 2009). The head contains a band-four-point-one/ezrin/radixin/moesin (FERM) domain, which binds to the membrane proximal NPxY motif within the beta subunit cytoplasmic tail, resulting in separation of the alpha and beta tails (Calderwood et al., 2002; Moser et al., 2009; Qin et al., 2004). In the resting state, talin is autoinhibited, with masking of the head by the flexible rod (Goksoy et al., 2008). Before binding to the beta subunit can occur, talin must be activated to expose the FERM domain. Yan et al demonstrated that calpain cleaves talin into its head and rod, and that this is associated with increased binding to the beta subunit (Yan et al., 2001). In addition, binding to the lipid second messenger, phosphatidylinositol-4,5-bisphosphate, can induce a conformational change in talin, which reveals the integrin binding site (Goksoy et al., 2008). Furthermore, binding of talin to the beta subunit and the phospholipid membrane results in an increase in the tilt angle of the beta subunit transmembrane domain; which in turn, leads to destabilisation of the interaction between the alpha and beta transmembrane domains, favouring integrin activation (Kim et al., 2012; Ye et al., 2014).

Talin binding is inhibited by tyrosine phosphorylation of the integrin cytoplasmic tail (Anthis et al., 2009), and is negatively regulated by the scaffolding proteins filamin and docking protein 1 (Dok1), which compete with talin for binding to the beta subunit (Kiema et al., 2006; Wegener et al., 2007). In addition, the cytosolic protein SHANK-associated RH domain interactor (SHARPIN) inhibits recruitment of talin,
and the kindlins (discussed in the following section), to Itgb1 by binding to the membrane proximal region of alpha subunit cytoplasmic tails (Rantala et al., 2011).

Talin is also able to bind to the cytoskeleton through actin and vinculin binding sites within the rod domain (Moser et al., 2009). It has been suggested that in the activation of Itgb1 heterodimers, binding of talin to the cytoskeleton is more important than disruption of the alpha-beta interaction (Margadant et al., 2011).

1.11.6 Kindlins

The kindlins are a family of three FERM-domain containing proteins (kindlin-1, -2 and -3). Kindlin-2 is the most widely expressed and is found in most tissues, whilst kindlin-1 is primarily expressed in epithelial cells, and kindlin-3 is restricted to haemopoietic cells (Moser et al., 2009). Kindlins bind to the distal NxxY motif within beta subunit tails, and act synergistically with talin to activate integrins (Moser et al., 2009). However, their mechanism of action is not completely understood, and it is thought that their effects may be integrin and cell type specific (Ye et al., 2014).

1.11.7 Outside in Signalling

Activated integrins aggregate into clusters and their cytoplasmic tails associate with a variety of cytoskeletal proteins, forming an intracellular complex or focal adhesion (Clark and Brugge, 1995). Focal adhesions couple integrins to the actin cytoskeleton, and facilitate the transmission of signals from the ECM to the cell interior. This is known as ‘outside-in signalling’ (Qin et al., 2004).

Despite regulating many cellular functions, integrins themselves have no intrinsic enzymatic activity, and rely on catalytic proteins within the focal adhesion to initiate intracellular signalling cascades. For example, focal adhesion kinase (FAK) associates with the cytoplasmic tails of beta subunits (Clark and Brugge, 1995), and the adaptor proteins paxillin and talin (Mitra and Schlaepfer, 2006). Binding of integrins to their ECM ligands results in autophosphorylation of FAK within the focal adhesion (Clark and Brugge, 1995). Next, the Src family of kinases are able to form a complex with FAK, possibly by binding to the FAK autophosphorylation site through their SH2 domains. Signalling downstream of the FAK-Src complex promotes cell migration, proliferation and survival (Mitra and Schlaepfer, 2006).
Likewise, integrin-linked kinase (ILK), a serine/threonine kinase, localises to focal adhesions and transduces signals from activated integrins (Cabodi et al., 2010).

Together, FAK-Src and ILK complexes initiate many intracellular signalling cascades, resulting in effects on cell survival, proliferation, migration and invasion, gene transcription and cytoskeletal organisation (Cabodi et al., 2010). Integrin-stimulated signalling pathways converge with those triggered by growth factor receptors, and synergy between ECM and growth factor activated signalling may explain the attachment-dependent growth of normal cells (Hynes, 1992).

Finally, FAK and ILK mediated signalling pathways are central to fibrosis in several organs, including the lung, liver and kidney, suggesting their involvement in a core fibrotic pathway (Lagares et al., 2012; Li et al., 2003; Shafiei and Rockey, 2011; Zhang et al., 2006). Of specific relevance to liver fibrosis, FAK is critical for proliferation and migration of HSCs (Reif et al., 2003), whilst ILK regulates αSma and type I collagen expression (Zhang et al., 2006).

1.11.8 Integrin Trafficking and Recycling

Integrins are internalised by both clathrin-dependent and clathrin-independent mechanisms; once internalised they are either redistributed to the plasma membrane, or degraded through the lysosomal pathway (Margadant et al., 2011).

1.11.9 Integrins and Mechanotransduction

Integrins provide a physical link between the ECM and the actin cytoskeleton, and as such, can relay information about changes in the mechanical stiffness of the ECM to the cell interior. This important mechanism, by which cells can sense and respond to mechanical changes within their environment, is known as mechanotransduction or mechanosensitive signalling.

1.11.9.1 Mechanotransduction and Activation of TGF-β1

One of the most widely reported mechanisms by which integrin-mediated signalling potentiates fibrosis, is by the activation of TGF-β1 (Henderson and Sheppard, 2013). This can be termed ‘extrinsic mechanotransduction’, as it happens outside the cell (Huang et al., 2012).
Extracellular TGF-β1 is non-covalently associated with latency-associated peptide (LAP), forming the inactive small latent complex (Worthington et al., 2011). Frequently, this small latent complex is covalently bound to latent TGFβ-binding protein to form the large latent complex (LLP), which is incorporated within the ECM (Worthington et al., 2011).

Integrins capable of binding to the RGD motif within the latency-associated peptide can activate TGF-β1 by two mechanisms. Firstly, proteolytic activation of TGF-β1 is mediated by integrin αVβ8, which binds to the latent complex and localises it with the membrane bound matrix metalloproteinase (MMP), MT-1 MMP. This facilitates the proteolytic cleavage of the LAP, and release of active TGF-β1 (Wipff and Hinz, 2008). A similar mechanism is proposed for αVβ3 with MMP-2 and MMP-9 (Wipff et al., 2007).

Secondly, αVβ3, αVβ5, αVβ6 and an unidentified Itgb1 heterodimer can activate TGF-β1 independently of protease activity, by inducing a conformational change within the LLP, resulting in release of active TGF-β1 (Munger et al., 1999; Wipff and Hinz, 2008; Wipff et al., 2007). This depends on an intact cytoskeleton, cell contraction and an inert, non-compliant ECM (Wipff and Hinz, 2008) (Figure 1.7).
Figure 1.8 Mechanical activation of TGF-β1 by integrins

Latent TGF-β1 is sequestered within the ECM as a large latent complex (LLC), comprising TGF-β1, latency associated peptide (LAP) and latent TGFβ binding protein (LTBP-1). By contracting against a non-compliant ECM, myofibroblasts expressing integrins capable of binding to the RGD motif within the LAP, can induce a conformational change within the LLC resulting in release of active TGF-β1. Adapted from (Wipff and Hinz, 2008; Wipff et al., 2007; Worthington et al., 2011)

Recently, Henderson et al have established the importance of integrin-mediated TGF-β1 activation in driving fibrosis (Henderson et al., 2013). Using a genetic knockout model, they demonstrated that myofibroblast-specific deletion of αV integrin protects against fibrosis in the lung, liver and kidney. In vitro, they confirmed reduced expression of pro-fibrotic genes in αV-null activated HSCs, as well as in control activated HSCs treated with an αV blocking antibody. Both control and αV-null HSCs expressed similar levels of TGF-β1, however, αV-null HSCs were significantly less able to activate TGF-β1. In keeping with this, reduced levels of phosphorylated Smad 3 were seen in αV-knockout livers compared with controls.

Next, they investigated the effects of deleting four of the five beta subunits (beta3, beta5, beta6 and beta8) known to heterodimerise with αV, and surprisingly, found no change in liver fibrosis. The role of Itgb1 could not be studied due to the lack of a suitable HSC-specific Cre recombinase driver. Therefore, the authors conclude that the fibrogenic effects of αV must rely on the contribution of multiple αV integrins, or be due to αVβ1 alone.
This is supported by their finding that the small molecule alphaV antagonist CWHM12 attenuates fibrosis in the lung and liver (Henderson et al., 2013); whilst in a previous study, the small molecule αVβ3 and αVβ5 antagonist cilengitide, was associated with exacerbation of liver fibrosis (Patsenker et al., 2009).

More recently, Reed et al developed a specific small molecule inhibitor of αVβ1, and demonstrated that αVβ1 inhibition resulted in a significant reduction in both lung and liver fibrosis, similar to that observed with pan-alphaV inhibition (Reed et al., 2015). This suggests that αVβ1 may be the major alphaV heterodimer driving HSC activation and fibrosis (Reed et al., 2015).

1.11.9.2 **Intrinsic Mechanotransduction**

‘Intrinsic mechanotransduction’ has been described as actin cytoskeleton mediated intracellular signalling in response to increased matrix stiffness, leading to the activation of mechanosensitive transcription factors (Huang et al., 2012). As integrins form the physical link between the ECM and the actin cytoskeleton, it seems very likely that they play a central role in regulating mechanotransduction.

Two mechanosensitive transcriptional programs have been identified: the co-activator megakaryoblastic leukaemia factor-1 (MKL1), which mediates the transcriptional regulator serum response factor (SRF); and the transcriptional regulators yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) (Janmey et al., 2013). The MKL1/SRF pathway is regulated by changes in actin polymerisation in response to mechanical tension, which in turn is regulated by the integrin effectors, RhoA and ROCK (Huang et al., 2012).

MKL1 is bound to G-actin in the cytoplasm, and as G-actin is polymerised into F-actin, MKL1 is released. Free MKL1 then translocates to the nucleus, where it associates with SRF to activate a program of fibrotic gene transcription (Huang et al., 2012). The MKL1/SRF pathway has been shown to directly regulate lung myofibroblast activation relevant to pulmonary fibrosis (Huang et al., 2012; Zhou et al., 2013).

YAP/TAZ appear to be directly regulated by cytoskeletal tension, and are not dependent on G-actin levels (Dupont et al., 2011). Following activation, YAP and TAZ are dephosphorylated and localise to the nucleus, where they act with the TEA
domain (TEAD) family of transcription factors to regulate gene expression; a well characterised target genes is CTGF (Shimomura et al., 2014).

The mechanism by which cytoskeletal tension regulates YAP and TAZ is unknown. However, it has been suggested that a YAP/TAZ inhibitor may be sequestered within the F-actin cytoskeleton and released during cytoskeletal remodelling; or alternatively, that a YAP/TAZ activator may be revealed following a conformational change associated with actin contractility (Halder et al., 2012).

Increased matrix stiffness and the ability to generate mechanical tension are prerequisites of HSC activation, independent of TGF-β1 (Olsen et al., 2011). Therefore, intrinsic mechanotransduction and mechanosensitive transcriptional regulation may be an important mediator of HSC activation.
The co-activator megakaryoblastic leukaemia factor-1 (MKL1) is sequestered within the cytoplasm when bound to G actin. Actin polymerisation results in release of MKL1, which translocates to the nucleus to activate transcription of fibrosis-associated genes. The transcriptional regulator yes-associated protein (YAP) is activated by cytoskeletal tension. This leads to YAP dephosphorylation and translocation to the nucleus, where it acts with the TEAD family of transcription factors to regulate transcription of fibrosis-associated genes.


1.11.9.3 **Itgb1 in Fibrosis**

Itgb1 is the largest integrin subfamily, comprising twelve different integrin heterodimers, which are widely expressed (Hynes, 2002). Therefore, it is not surprising, that global Itgb1 knockout is lethal in utero, during the peri-implantation stage of embryogenesis (Fässler and Meyer, 1995). Consequently, investigating the role of Itgb1 in fibrosis relies upon a targeted approach to gene deletion, for example, by using specific drivers of Cre recombinase to delete Itgb1 in mice with the Itgb1 gene flanked by loxP sites.

Although PDGFRβ is specific to HSCs in the liver, using the PDGFRβ-Cre to delete Itgb1 in vivo results in aneurysmal vascular malformation, and is fatal during the early post-natal period (Abraham et al., 2008; Henderson et al., 2013). However, Itgb1 has been successfully deleted from skin fibroblasts in vivo, using the tamoxifen-inducible Cre recombinase, under the control of the proc2(I) collagen gene. Deleting Itgb1 in skin fibroblasts prevented their activation into myofibroblasts, and protected mice from developing bleomycin-induced skin fibrosis (Liu et al., 2009). In conflict with these data, Gerber et al demonstrated that an Itgb1 activating antibody suppressed expression of collagens type I and III in systemic sclerosis skin fibroblasts in vitro, and prevented skin fibrosis in the stiff skin syndrome mouse model (Gerber et al., 2013). It is difficult to reconcile these two studies.

In the kidney, Yeh et al demonstrated that Itgb1 blocking antibodies attenuated fibrosis in the unilateral ureteric obstruction mouse model (Yeh et al., 2010).

Therefore, there is some evidence to support of role of Itgb1 in fibrosis; however, this needs further confirmation, and the role of Itgb1 in liver fibrosis is unknown.

1.11.10 **Integrins as Therapeutic Targets**

Integrins are implicated in many pathological processes including fibrosis, inflammation and cancer, and this coupled with their accessibility on the cell surface, makes them attractive therapeutic targets in a wide range of diseases (Goodman and Picard, 2012).

However, due to the involvement of integrins in many developmental and physiological processes, unwanted off-target effects are a real concern. On the other
hand, redundancy in ligand binding may limit the effectiveness of integrin antagonists. In addition, the development of neutralising antibodies against antibody-based integrin antagonists may result in reduced efficacy, whilst small molecule inhibitors may have agonist-like effects at low doses, for example at the plasma concentration nadir, and this has implications for dosing (Cox et al., 2010).

Despite these caveats, integrin antagonists have been developed and are used in clinical practice. The best established is abciximab, which blocks αIIbβ3 and inhibits platelet aggregation. It is routinely used in the management of high risk acute coronary syndrome, and has been shown to reduce mortality following percutaneous coronary intervention (De Luca et al., 2005).

The alpha4 inhibitor, natalizumab, has been used in the treatment of the inflammatory conditions Crohn’s disease and multiple sclerosis (Hoeper et al., 2014). However, due to its immunosuppressive effects in the central nervous system it has been linked to the development of progressive multifocal leukoencephalopathy (PML), and because of this it was withdrawn from the treatment of Crohn’s disease (Cox et al., 2010).

More recently, the selective α4β7 antagonist, vedolizumab, has been developed for the treatment of inflammatory bowel disease, and so far, this has not resulted in the significant off-target effects associated with pan-alpha4 inhibition (Gilroy and Allen, 2014).

In the cancer field, the α5β1 inhibitor volociximab is undergoing clinical trials in a number of malignancies including non-small cell lung and ovarian cancers (Bell-McGuinn et al., 2011; Besse et al., 2013); and the αVβ3 and αVβ5 antagonist cilengitide has been investigated in the treatment of glioblastoma. Although it initially showed promise, a recent phase III trial (CENTRIC) failed to show benefit in overall survival or progression free survival for combination therapy with cilengitide, compared to standard treatment (Soffietti et al., 2014).

Cilengitide has also been investigated in liver fibrosis; however, opposing results were seen in vitro and in vivo. In vitro, cilengitide inhibited pro-fibrotic gene expression and migration in HSCs, suggesting its potential as an anti-fibrotic agent (Patsenker et al., 2007). However, in vivo, cilengitide exacerbated liver fibrosis; the
study authors suggest that this may be a consequence of its anti-angiogenic effects and tissue hypoxia (Patsenker et al., 2009).

Therefore, integrin antagonists are used in the clinical management of inflammatory and thrombotic disorders, and show promise in the treatment of malignancies; however, until recently, their therapeutic potential in fibrosis has remained untapped. Within the last year, Henderson et al have published exciting data, which demonstrate that the small molecule αV inhibitor, CWHM 12, significantly attenuates fibrosis in the lung and liver (Henderson et al., 2013). This suggests that integrin antagonism is a viable therapeutic strategy in fibrotic disease.
1.12 This Thesis

Understanding the role of Itgb1 in HSC activation may lead to the identification of novel therapeutic targets in liver fibrosis. This project aims to investigate the role of Itgb1 during HSC activation; and to identify downstream effectors, which may be targetable for the treatment of liver fibrosis. A short summary of each chapter is detailed below.

1.12.1 Itgb1 Regulates HSC Activation via the Actomyosin Cytoskeleton and Mechanotransduction

This chapter explores the role of Itgb1 in HSC activation and describes a pro-fibrotic circuit involving Itgb1, the actin myosin cytoskeleton and the mechanosensitive transcriptional regulator Yap.

1.12.2 Investigating the Role of Group I Paks in HSC Activation and Liver Fibrosis

In this chapter, a family of protein kinases, the group I p21-activated kinases (Paks), are identified as novel downstream effectors of Itgb1 in HSC activation. Inhibition of group I Paks attenuates both HSC activation \textit{in vitro}, and liver fibrosis \textit{in vivo}.

1.12.3 Itga11 Shows Promise as a Fibroblast-Specific Partner of Itgb1 Regulating HSC Activation

Itga11 is dramatically upregulated in activated HSCs. Immunoprecipitation and immunofluorescence are used to demonstrate co-localisation of Itga11 with Itgb1 in activated HSCs. Depletion of Itga11 in activated HSCs is associated with a reduction in type I collagen.
2 METHODS

2.1 Extraction of the Rat Liver

Adult male Sprague-Dawley rats, weighing less than 500g, were euthanized by Home Office Schedule 1 methods. Immediately following sacrifice, the abdomen was opened by midline incision and the liver exposed. The portal vein was cannulated with a 22-gauge needle and 1000 units of heparin sulphate (LEO Pharma, Denmark) in 2 mls of Hanks Balanced Salt Solution without calcium or magnesium (HBSS-; Invitrogen, Life Technologies) were injected. The cannula was secured in situ with two sutures and attached to a perfusion pump primed with HBSS- at 37°C; HBSS- was infused at a starting rate of 10ml/minute. After approximately 5 seconds the inferior vena cava was incised, and digital pressure applied intermittently above the incision to facilitate perfusion and drainage of the liver. After perfusion with 100ml, HBSS- was exchanged for HBSS with calcium and magnesium (HBSS+; Invitrogen, Life Technologies) and 120mg Pronase (Roche, UK) in 10ml HBSS+ was added. When around 150ml of perfusate remained, 20mg Collagenase (Roche, UK) in 10ml HBSS+ was added. Once the liver had been perfused with all the HBSS+, the cannula was removed and the liver was dissected out and placed into 35mg Pronase in 10ml HBSS+.

2.2 Isolation of Rat HSCs

In a primary cell culture hood, the rat liver was homogenised by passage through a sterile 125 µm Nyboldt mesh (John Staniar, UK) into a 500ml beaker; HBSS+ and DNase I (10mg DNase (Roche, UK) in 10mls HBSS+) were added to facilitate this, and to make up the final filtrate volume to 200ml. The filtrate was divided into four 50ml tubes and centrifuged at 1800 rpm for 7 minutes. The supernatant was discarded; the pellet was re-suspended in 2 mls DNase I and the volume made up to 50 mls with HBSS+. The samples were then centrifuged for a second time at 1800 rpm for 7 minutes. The supernatant was discarded and the pellets were combined and re-suspended in 8ml of DNase I. The final volume was made up to 44ml with HBSS+; this was gently mixed with an Optiprep gradient (17ml Optiprep (Axis-Shield Diagnostics Ltd) and 13ml HBSS+) and divided between two 50ml tubes. Using a Pasteur pipette, 5ml of HBSS+ was carefully added to the top of each
gradient mix; they were the centrifuged at 2115 rpm (1500g) for 23 minutes at 4°C without brake.

Due to their high lipid content, HSCs formed a cloudy suspension, which was transferred using a Pasteur pipette to a 50ml tube. 2ml of DNase I were added, and the volume was made up to 50ml with HBSS+. The sample was centrifuged at 2000 rpm for 7 minutes; the supernatant discarded and the pellet re-suspended in 10ml of HSC culture medium. HSC culture medium consisted of Dulbecco’s modified eagle’s medium with high glucose (4.5g/l), glutamine and sodium pyruvate (DMEM; Sigma, UK) with 16% heat inactivated foetal bovine serum (FBS; Invitrogen, Life Technologies), 2mM L-glutamine (Invitrogen, Life Technologies) and 1% penicillin/streptomycin (100units/ml penicillin and 100µg/ml streptomycin; Invitrogen, Life Technologies). Cells were counted using a haemocytometer with trypan blue (Invitrogen, Life Technologies) exclusion of non-viable cells. A proportion of the cell suspension (equating to around 3 million cells) was taken for RNA and protein extraction, and the remaining cells were plated out at 5 million cells per T75 flask (Corning) in 15ml of culture medium. The culture medium was fully changed on the following day.

2.3 Isolation of Mouse HSCs

Adult Itgb1/CreER+/+ or wildtype mice were euthanized by Home Office Schedule 1 methods. Immediately following sacrifice, the abdomen was opened by midline incision and the liver dissected out and placed in HBSS+. Due to the smaller size of livers of between two to five mice were combined per HSC preparation.

In a primary cell culture hood, the livers and HBSS+ were transferred to a 10cm dish (Corning) with the addition of Pronase and Collagenase. Livers were cut up into small pieces using iris scissors and then incubated for 30 minutes at 37°C. The macerated livers were then homogenised by passage through a 125 µm Nyboldt mesh into a 500ml beaker; HBSS+ and DNase I were added to facilitate this and make up the final volume to 200ml. The procedure then followed that described for the isolation of rat HSCs.
2.4 **Primary HSC Culture**

Rat and mouse HSCs were cultured over 7 to 14 days, during which they were activated into myofibroblasts. Cells were maintained in an incubator at 37°C with 5% CO₂, and were observed microscopically to assess for the development of a myofibroblast-like morphology and to evaluate their confluency. Once the cells reached around 80 to 90% confluency they were passaged.

2.5 **LX-2 Cells**

LX-2 cells are an immortalised human HSC cell line, and were kindly provided by Professor SL Friedman. LX-2 cells were generated by spontaneous immortalisation in low serum conditions (Xu *et al.*, 2005). They have the retinoid phenotype of HSCs, and express αSMA, vimentin and GFAP (Xu *et al.*, 2005). Microarray analysis showed high correlation of gene expression between primary human HSCs and LX-2 cells (Xu *et al.*, 2005). In addition, LX-2 cells are highly transfectable (Xu *et al.*, 2005).

LX-2 cells were maintained in culture medium at 37°C with 5% CO₂. LX-2 culture medium consisted of DMEM supplemented with 1% FBS, 2mM L-glutamine and 1% penicillin/streptomycin (100units/ml penicillin and 100μg/ml streptomycin). LX-2 cells were activated by culturing for 24-48 hours in medium supplement with 10% FBS.

2.6 **Cell Passage**

To passage cells, the culture medium was removed and the cells were incubated in 10ml of TrypLE Express (Gibco, Life Technologies) for 5 minutes, or until cells were no longer adherent to the flask surface. 10ml of culture medium was then added and the cell suspension transferred to 50ml tube and centrifuged at 2000 rpm (7 minutes) or 1500 rpm (5 minutes), for primary HSCs and LX-2 cells respectively. The supernatant was removed and the cells re-suspended in 10ml culture medium and divided between two T75 flasks with an additional 10ml of culture medium per flask. The medium was half changed on the following day, and twice weekly for all cultured cells.
2.7 Freezing and Thawing Cells

Cells were frozen and stored in liquid nitrogen at -196°C. Cells were detached and pelleted as described in section 2.6; then re-suspended in 1ml freezing medium and transferred to a cryotube. Freezing medium consisted of DMEM containing 50% FBS and 10% dimethylsulphoxide (DMSO, Sigma, UK). Cryotubes were placed in a cryogenic freezing vessel containing isopropanol at -80°C overnight, and then transferred into liquid nitrogen tanks for long-term storage.

For use, cells were removed from liquid nitrogen and thawed at 37°C. The cell suspension was diluted with 10ml culture medium and centrifuged at 2000 rpm (7 minutes) or 1500 rpm (5 minutes) for HSCs and LX-2 cells respectively. The supernatant was removed and the cell pellet was re-suspended in culture medium and transferred to a T75 flask.

2.8 Cell Treatments

2.8.1 Tamoxifen

Tamoxifen (4-hydroxytamoxifen; Sigma, UK) was dissolved in ethanol (Fisher Scientific, UK) to a concentration of 100µM. This was added to the culture medium in a 1:1000 dilution to give a final concentration of 100nM.

HSCs isolated from Itgb1
tg/fl CreER+-/- mice were treated with either 100nM tamoxifen or the equivalent volume of ethanol vehicle control on the day of plating on to plastic (day 0). The following day (day 1) the medium was changed with the addition of 100nM tamoxifen or the equivalent volume of ethanol vehicle. Four days later (day 5) the medium was fully changed, without addition of tamoxifen or ethanol.

2.8.2 Verteporfin

Verteporfin (Sigma, UK) was dissolved in DMSO (Sigma, UK) to a concentration of 10mM. This was added to the culture medium in a 1:1000 dilution to give a final concentration of 10µM. Quiescent HSCs were isolated from wildtype mice and culture activated with addition of 10µM verteporfin or equivalent volume DMSO vehicle control. The medium was changed on day 1 and day 5, with the addition of
10μM verteporfin or DMSO. HSCs were cultured for 8 days and then harvested for RNA extraction.

2.8.3 IPA3

IPA3 (Tocris Bioscience, UK) was dissolved in DMSO to a concentration of 30mM. This was added to the culture medium in a 1:4000, 1:2000 or 1:1000 dilution to give a final concentration of 7.5, 15 or 30μM.

IPA3 or equivalent volume of DMSO vehicle control was added to the culture medium of activated HSCs for 24 hours. HSCs were then harvested for protein extraction.

2.9 Cell Transfections

The process of introducing nucleic acids into eukaryotic cells is termed ‘transfection’. Small interfering RNA (siRNA) was transfected into primary HSCs and LX-2 cells by two methods.

In primary HSCs, electroporation was used. This technique uses a brief electrical field pulse to facilitate RNA entry by two mechanisms: firstly, generating temporary aqueous pores within the lipid bilayer; and secondly, increasing the transmembrane potential by 0.5-1.0 mV, to drive charged particles through the pores (Weaver, 1995).

In LX-2 cells, liposome transfection was used. In this technique, cationic lipids form a vesicle with the negatively charged RNA, fuse with the cell membrane and facilitate delivery of genetic material to the cell interior.

Transfection of siRNA was used to study the effects of post-transcriptional silencing of specific genes. siRNA are 20-25 base pair sequences of double stranded RNA which are incorporated into the RNA-induced silencing complex to inhibit translation, and induce degradation of complimentary mRNA. siRNA transfection can result in non-specific effects by inducing an innate immune response; or off-target effects by the inadvertent downregulation of genes with partial complementarity. To control for these effects, a non-specific siRNA with no known homology to any mammalian gene (‘scrambled’ siRNA) was used as a negative control (AllStars Negative Control, Qiagen). siRNA sequences (Qiagen) used in this study are listed in table 2.1.
<table>
<thead>
<tr>
<th>siRNA target gene</th>
<th>Cat. No.</th>
<th>Species</th>
<th>Target sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Itga11</td>
<td>SI00244566</td>
<td>mouse</td>
<td>CACGCCCTATCTGGACCTATA</td>
</tr>
<tr>
<td>Itga11</td>
<td>SI02699046</td>
<td>mouse</td>
<td>TACGACCTTTACTGTCAGAAAA</td>
</tr>
<tr>
<td>Pak1</td>
<td>SI01368598</td>
<td>mouse</td>
<td>CTGGGCATTATGGCAATTGAA</td>
</tr>
<tr>
<td>Pak3</td>
<td>SI01368682</td>
<td>mouse</td>
<td>CCCACTGAGGATGAAACAGTAA</td>
</tr>
<tr>
<td>Pak3</td>
<td>SI04418967</td>
<td>mouse</td>
<td>TAGCAGCACATCAGTGAATAC</td>
</tr>
<tr>
<td>Pak3</td>
<td>SI04418974</td>
<td>mouse</td>
<td>CCGAGACTCTTCAGCACTCAA</td>
</tr>
</tbody>
</table>

Table 2.1 siRNA sequences

2.9.1 Transfection of Primary HSCs

Activated primary HSCs were released from the culture flask and pelleted as described in section 2.6. The cell pellet was re-suspended in 5ml culture medium and the cells counted with a haemocytometer. Cell suspension containing 5x10^5 cells was aliquoted into 15ml tubes and centrifuged at 2000 rpm for 7 minutes. The supernatant was removed and the cells re-suspended in 100µl Nucleofector Solution (Lonza, UK) with addition of target siRNA (Qiagen) or scrambled siRNA (All Stars Negative Control, Qiagen) to a final concentration of 10nM. The cell-siRNA solution was transferred into a cuvette (Amaxa® Basic Nucleofector Kit T, Lonza) and placed into the Lonza Nucleofector Device. Electroporation was achieved using programme U-25. Approximately 500µl warmed culture medium was added to the cuvette, and the cell suspension was transferred to a 6-well plate (Corning) using a supplied pipette (Amaxa® Basic Nucleofector Kit T, Lonza). The final volume was made up to 2ml with culture medium. The following day the medium was fully changed, and cells were harvested after 48 hours for protein or RNA extraction.

2.9.2 Transfection of LX-2 Cells

LX-2 cells were plated at a density of 150x10^3 per well in 6-well plates. The following day, a transfection solution was made consisting of:

- 100µl serum free DMEM
- 0.6µl siRNA (20µ M stock) / 0.3µl each of two siRNAs
- 12µl HiPerFect (Qiagen, UK)
The solution was mixed and incubated at room temperature for 5-10 minutes. A negative control with scrambled siRNA (using 0.6µl of 20µM stock) was also prepared.

The culture medium was removed and replaced with 2.3ml of warmed culture medium per well. The transfection solution was then added to each well using a filter-tip pipette. The following day, the siRNA transfection was repeated, and 24 hours after this, the cells were harvested for protein or RNA extraction.

### 2.10 Genotyping

Itgb1\(^{fl/fl}\) CreER\(^{+/-}\) were ear punched for the dual purposes of identification and providing a tissue sample for genotyping. Genomic DNA was extracted from tissue or HSCs and genotyping was performed by non-quantitative PCR reaction. The result were visualised by agarose gel electrophoresis (see section 2.10.3).

#### 2.10.1 DNA Extraction and PCR from Tissue

DNA extraction and PCR from tissue samples (ear clippings) were carried out using the REDExtract-N-Amp™ Tissue PCR Kit (Sigma, UK). To extract DNA, 100µl extraction solution and 25µl tissue preparation solution were added to each sample, mixed by vortex mixer, and incubated for 10 minutes at room temperature. The mixture was then heated to 95°C for 5 minutes, followed by the addition of 100µl neutralisation B solution. The extract was then ready for use in PCR, as follows:

- DNase-free H\(_2\)O 2µl
- REDExtract-N-Amp PCR Reaction Mix 10µl
- 5µM Forward Primer 2µl
- 5µM Reverse Primer 2µl
- DNA sample 4µl

REDExtract-N-Amp PCR Reaction Mix is a 2x solution specifically optimised for use with the kit extraction solutions. It comprises of a buffer, salts, dNTPs, Taq polymerase and RED Taq® dye, to allow direct loading of the PCR product on to an agarose gel.
For genotyping of Itgb1^{fl/fl} CreER+/- mice the primers used are listed in table 2.2 and the reaction conditions detailed below.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Itgb1 Forward</td>
<td>TTC TGC AAG TGT GGT G</td>
</tr>
<tr>
<td>Itgb1 Reverse</td>
<td>TGC CAC TCC AAA CAT AGA GC</td>
</tr>
<tr>
<td>β-Actin CreER Forward</td>
<td>AAC CTG GAT AGT GAA ACA GGG GC</td>
</tr>
<tr>
<td>β-Actin CreER Reverse</td>
<td>GGA ACC GAC TTG ACG TAG CCA GC</td>
</tr>
</tbody>
</table>

**Table 2.2 PCR primers used for genotyping**

PCR conditions for the Itgb1 primers were as follows:

94°C for 5 minutes

35 cycles of

94°C for 1 minute

60°C for 1 minute

72°C for 90 seconds

Then hold at 72°C for 7 minutes

PCR conditions for the β-Actin CreER primers were as follows:

94°C for 5 minutes

30 cycles of

94°C for 30 seconds

60°C for 30 seconds

72°C for 1 minute

Then hold at 72°C for 7 minutes
Expected PCR product sizes were ~ 900 base pairs for the $Igββ$ allele and ~ 600 base pairs for the $β$-Actin CreER transgene.

### 2.10.2 DNA Extraction and PCR from Cells

DNA extraction from HSCs was performed using a DNeasy Blood and Tissue Kit (Qiagen). Cells were pelleted as described in section 2.6, and then re-suspended in 200μl phosphate buffered saline (PBS) supplemented with 20μl proteinase K. The sample was then mixed with 200μl Buffer AL by vortex mixer, and incubated at 56°C for 10 minutes. 200μl ethanol (96-100%) were then added to the sample, and mixed by vortex mixer. The solution was applied to a DNeasy Mini Spin column placed in a 2ml collection tube, and centrifuged at 8000 rpm for 1 minute. Subsequently, 500μl Buffer AW1 and 500μl Buffer AW2 were each applied to the spin column, each followed by centrifugation. Finally, the membrane was dried by centrifuging at 13000 rpm for 3 minutes. DNA was eluted by transferring the spin column to a 1.5ml tube, applying 200μl Buffer AE to the membrane, allowing to stand for 1 minute at room temperature and then centrifuging at 13000 rpm for 1 minute.

The DNA sample was then used in PCR as follows:

- DNase-free $H_2O$ 8.9μl
- 5x Green GoTaq® Reaction Buffer (Promega) 4μl
- dNTPs 2μl
- GoTaq® Polymerase (Promega) 0.3μl
- 5μM Forward Primer 0.4μl
- 5μM Reverse Primer 0.4μl
- MgCl$_2$ 2μl
- DNA sample 2μl
The 5x Green GoTaq® Reaction Buffer (Promega) contains a blue and yellow dye which separate during electrophoresis, allowing progress to be monitored. The primers and reaction conditions were the same as those detailed in section 2.10.1.

2.10.3 Agarose Gel Electrophoresis

1-2% agarose gels were created to allow separation of DNA fragments by electrophoresis. Agarose (Melford labs, UK) was dissolved in TBE buffer (80mM Tris, 80mM Boric acid, 2mM Na2EDTA) by boiling. The gel solution was then cooled, mixed with SafeView (New England Biolabs) at a 1:10 dilution, and poured into a gel-setting tray. A comb was inserted to create wells and gels were allowed to set at room temperature. Subsequently, the comb was removed and the gel was submerged in TBE buffer in a gel-running tank. DNA samples were loaded into the wells, alongside DNA markers (Hyperladder I and IV; Bioline). If necessary, DNA samples were mixed with 5x loading buffer (Bioline) prior to loading. DNA was electrophoresed at 100V for 60-80 minutes. The gel was then removed and DNA visualised by UV light using a Molecular Imager Gel Doc XR+ System with Image Lab Software (Biorad).

2.11 Complimentary DNA Synthesis from RNA

2.11.1 Extraction of Cellular RNA

An RNeasy Mini Kit (Qiagen) was used for RNA extraction, following the manufacturer’s instructions. Cells were washed twice with ice-cold phosphate buffered saline (PBS), lysed in 350µl of RLT buffer with 1:100 dilution β-mercaptoethanol (Sigma, UK) and collected with a cell scraper. The lysate was mixed with an equal volume of 70% ethanol, applied to a RNeasy Mini Spin Column and centrifuged at 10,000 rpm for 15 seconds. RNA bound to the silicon gel spin column membrane was then washed with buffers RW1 (700 µl) and RPE (500 µl x 2). The membrane was dried by centrifuging at full speed for 1 minute. Finally, RNA was eluted with 30µl of RNase-free water.

Any contaminating DNA was removed using a DNase Kit (Sigma, UK), following the manufacturer’s instructions. 1.75µl DNase1 and 3.5µl of 10x buffer were added to 30µl of RNA solution, and the mixture was incubated for 15 minutes at room
temperature. Finally, 15µl of Stop solution were added and the mixture was incubated for 10 minutes in a water bath at 70°C.

2.11.2 RNA Quantification

The RNA concentration and purity were assessed using a Nanodrop 3300 (Thermo Scientific) spectrophotometer. 1 µl of sample was applied, and the concentration was determined using absorbance at 260nm. The ratio of absorbance at 260 and 280nm was used to determine purity.

2.11.3 Complimentary DNA Preparation

Complimentary DNA (cDNA) was synthesised using a reverse transcriptase (RT) High Capacity RNA-to-cDNA Kit (Applied Biosystems). For each RNA sample a positive RT reaction and negative RT control (without RT enzyme) was generated. The total volume per reaction was 20µl with 10µl of 2x buffer mix (MgCl2, dNTPs, RNase inhibitors, Oligo(dT) primer and stabilizers) and 1µl 20x reverse transcriptase enzyme with a total of 1µg of RNA (up to a total volume of 9µl). A Biorad Tetrad thermal cycler was used to heat the reaction mixture to 37°C for 60 minutes and then to 95°C for 5 minutes to terminate the reaction.

2.11.4 Quantitative PCR

2.11.4.1 Primer Design

Primers used for quantitative PCR (qPCR) were identified from publications or were designed using NCBI Primer BLAST software. The gene sequence was uploaded and primers generated using the following criteria: product size of 70-120 base pairs, primer size between 18-23 base pairs and an intron-spanning sequence (where possible). All primers were ordered from Eurofins MWG Operon (Ebersberg, Germany). Primers used in this study are listed in Appendix 8.3.

2.11.4.2 Quantitative Real Time PCR

Real time PCR was performed in 96-well Fast Optical Reaction Plates (Applied Biosystems, UK) using SYBR Green Mastermix (Primer Design, Southampton) and a StepOne Plus Real Time PCR System thermocycler (Applied Biosystems).
SYBR Green Mastermix comprised a thermo-stable TAQ Polymerase, buffer, dNTPs, MgCl₂ at concentrations optimised for the enzyme and the SYBR Green dye (no further information provided by manufacturer).

Each reaction consisted of:

SYBR Green Mastermix 10μl
10μM Forward Primer 2μl
10μM Reverse Primer 2μl
cDNA 1μl
DNase-free H₂O to 20μl

Experiments were performed in triplicate with a negative control (minus-RT reaction) and two reference genes per sample (glucuronidase-β (GUSB) and β-Actin). The plate was sealed and inserted into the thermocycler; reaction conditions were as follows:

50°C for 2 minutes hold
95°C for 2 minutes hold
40 cycles of:
95°C for 15 seconds
58°C for 30 seconds
72°C for 45 seconds

A melt-curve step was included to confirm amplification of a single product.

2.11.4.3 qPCR Data Analysis

SYBR green fluoresces when bound to double stranded DNA; this can be quantified to give an estimation of the amount of PCR product. Data analysis was performed using StepOne Software v2.1. The cycle threshold (Ct) value was calculated at the cycle number at which the fluorescent signal became exponential. Relative levels of
gene expression were calculated as a fold change from control using the ΔΔCt method, as summarised below:

Fold change = $2^{\Delta \Delta Ct}$

Where, $\Delta \Delta Ct = \Delta Ct_{\text{experimental}} - \Delta Ct_{\text{control}}$

$\Delta Ct_{\text{experimental}}$ and $\Delta Ct_{\text{control}}$ were normalised to the reference genes ($GUSB$ and $\beta$-Actin)

### 2.12 3' RNA Microarray

HSCs were extracted from Itgb1fl/fl CreER+ mice and divided into three: RNA was isolated from quiescent HSCs on the day of extraction; Itgb1-null and control HSCs were cultured for eight days with the addition of tamoxifen or vehicle control for the first 48 hours (see section 2.8.1). Cells were pelleted and RNA was extracted as described in section 2.11.1. 0.5μg of RNA was converted to cDNA, to allow verification of Itgb1 deletion, by qPCR (see sections 2.11.2 - 4). The remaining RNA was stored at -80°C and transferred to the Microarray Facility at the University of Manchester. RNA quality was assessed Agilent Bioanalyser prior to performing the microarray analysis. An Affymetrix Mouse Genome 430 2.0 Array platform was used. The results were analysed, and principal component assay and clustering analysis were performed by Leo Zeef in the Bioinformatics Department at University of Manchester.

### 2.13 Immunoblotting

#### 2.13.1 Preparation of Cell Lysate for Protein Analysis

Cell culture plates were placed onto ice, medium was removed and the cells were washed three times with ice cold PBS. Cells were then lysed in RIPA buffer (Sigma, UK) with 1 x EDTA free Protease Inhibitors (Roche), 1 x Inhibitor Cocktails 1 and 2 (Sigma) and distilled H₂O to volume) using a cell scraper. The volume of RIPA buffer used was dependent on the dimensions of the culture plate; for 6 well plates, 100μl of RIPA was used per well. The cell lysate was collected into 1.5ml Eppendorf
tubes and centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatant volume was measured and assayed for protein content.

2.13.2 Protein Quantification by Bradford Assay

Whole cell lysate was subjected to Bradford assay to determine the protein concentration. 5μl of cell lysate and 200μl of diluted Bradford reagent (Bradford protein assay, Biorad; diluted 1 in 5 parts with deionised H2O) was added to a well of clear, flat-bottomed 96-well plate. Each sample was assayed in triplicate alongside a standard dilution series of bovine serum albumin (BSA; Sigma) from 0-1 mg/ml. The absorbance measured at 595nm with an automated plate reader (EL800; BioTek, Potton, UK). The data was exported to an Excel file, and a standard curve was generated from the BSA dilution series; samples were compared with this to quantify protein concentration. Protein concentrations were equilibrated between paired samples by dilution with RIPA buffer where necessary.

2.13.3 SDS-PAGE Gel

Protein samples were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using Bio-rad minigel electrophoresis equipment (Bio-Rad; Hemel Hempstead, UK). Gels comprised a lower resolving gel (1M Tris (Fisher Scientific, UK) pH 8.8 2.5 ml, 20% SDS (Fisher Scientific, UK) 50μl, 40% acrylamide (38.67% acrylamide and 1.33% bis-acrylamide; Amresco) 2-3ml (for 8-12.5% gel), 2.89-3.89ml H2O (for 8-12.5% gel), 10% ammoniumpersulphate (APS; Sigma) 300μl, tetramethylethylenediamine (TEMED; Sigma) 12.5μl) and an upper stacking gel ((1M Tris pH 6.8 625μl, 20% SDS 25μl, 40% acrylamide 525μl, 10% APS 150μl, TEMED 12.5μl, 3.66ml H2O). Sample loading lanes were created by a plastic comb, which was placed into the stacking gel until the gel had set.

2.13.4 Protein Electrophoresis

Whole cell lysates were mixed with 5x protein loading buffer (320mM Tris-HCl pH6. 5% SDS, 0.4% bromo-phenol blue (Sigma), 25% glycerol (Fisher Scientific, UK) 0.5% β-mercaptoethanol (Sigma), H2O to volume), boiled for 5 minutes and electrophoresed in 8-12% SDS-polyacrylamide gels to resolve proteins by molecular mass. A prestained protein marker was electrophoresed alongside samples (7 kDa – 175 kDa; Colourplus Prestained Protein Marker, Broad Range; New England
Biolabs, UK). Gels were immersed in running buffer (250mM Tris, 2M Glycine, 35mM SDS, to volume with dH$_2$O) and proteins resolved by 200V for one hour.

### 2.13.5 Transfer to Nitrocellulose Membrane

Gels were placed onto a nitrocellulose membrane (G.E. Healthcare), and sandwiched between filter paper and sponges within a cassette. This was carried out whilst submerged in deionised H$_2$O. The gel and membrane containing cassette was then immersed in transfer buffer (25mM Tris, 200mM Glycine, 10 % Methanol (Fisher Scientific, UK)), and proteins transferred at 100V for 90 minutes. Transfer of proteins to the nitrocellulose membrane was confirmed by staining with ponceau red (0.2g ponceau red (Sigma, UK), 10ml acetic acid (Fisher Scientific, UK) and 100ml dH$_2$O).

### 2.13.6 Protein Detection

The nitrocellulose membrane was blocked with 5% non-fat milk (Sigma) in a solution of 0.1% Tween (Sigma) in phosphate buffered saline (PBS; Sigma) for two hours, and then incubated with the primary antibody diluted in 5% milk-0.1% Tween-PBS at 4°C overnight.

The following morning, the membrane was washed three times for five minutes in 0.1% Tween-PBS, and then incubated with the appropriate HRP-conjugated secondary antibody, diluted in 5% milk-0.1% Tween-PBS at room temperature for one hour.

The membrane was then washed three times for five minutes in 0.1% Tween-PBS and then incubated with a chemiluminescent solution for five minutes in the dark. For β-actin ECL western blotting substrate (Pierce) was used; and for all other antibodies, Amersham ECL western blotting reagent (GE Healthcare, UK).

The signal was then detected using an automated detection system (ChemiDoc; Biorad), and densitometric analysis was carried out using QuantityOne software.

### 2.14 Co-Immunoprecipitation

Co-immunoprecipitation was used to identify proteins forming a complex with Itgb1 in activated HSCs.
2.14.1 Preparation of Cell Lysate

Activated rHSCs were cultured to approximately 90% confluence in 10 cm culture dishes (Corning). The culture dish was then placed on ice, the culture medium was removed and cells were washed three times with ice cold PBS. Cells were then scraped in 250 µl NETN buffer (100 mM NaCl, 20 mM Tris-Cl, 0.5 mM EDTA, 0.5% (v/v) Nonidet P-40 (Calibochem), 1 x EDTA free Protease Inhibitors, 1 x Inhibitor Cocktails 1 and 2), transferred to a 1.5 ml tube and rotated at 4°C for twenty minutes. The cell lysate was then centrifuged at 3500 rpm for five minutes at 4°C, and the supernatant transferred to a new 1.5 ml tube. A proportion (40 µl) of the total cell lysate was removed, mixed with 5x protein loading buffer (320mM Tris-HCl pH6. 5% SDS, 0.4% bromo-phenol blue, 25% glycerol, 0.5% β-mercaptoethanol, H2O to volume) and boiled for five minutes.

2.14.2 Sample Pre-Clearance

The cell lysate sample was pre-cleared with magnetic protein G coated beads (New England Biolabs) and rat IgG (Purified rat IgG, R&D Systems) to avoid non-specific protein binding during the immunoprecipitation.

The supernatant from 50µl of resuspended beads was removed using a magnetic separation rack. The beads were resuspended in the cell lysate with 1µl of non-specific IgG, and incubated for one hour at 4°C. The supernatant was then removed using a magnetic separation rack, and split equally between two 1.5ml tubes. The beads were discarded.

2.14.3 Antibody Binding to Magnetic Beads

The supernatant was removed from two 25µl aliquots of resuspended beads using a magnetic separation rack. The beads were then resuspended in 100µl of 0.1% BSA in PBS with addition of either 1µl of rat IgG (negative control) or 1µl of Itgb1 antibody (Millipore), and incubated for ten minutes with rotation at room temperature. The beads were then washed by gentle pipetting with 200µl of 0.1% BSA in PBS; the wash buffer was removed by magnetic separation rack, and discarded.
2.14.4 Immunoprecipitation

The antibody (IgG or anti-Itgb1) coated beads were then mixed with equal volumes of the cell lysate, and incubated for one hour with rotation at 4°C. The supernatant was removed by magnetic separation rack, transferred to a 1.5 ml tube and mixed with 5x protein loading buffer.

The beads with bound antibody-antigen complex were then washed three times by gentle pipetting with 200μl 0.1% BSA in PBS. Between washes, the wash buffer was removed by magnetic rack separation. The first wash sample was transferred to a 1.5ml tube and mixed with 5 x protein loading buffer.

Protein was eluted from the beads by mixing by vortex with 10μl 5x loading buffer and 10μl 1% SDS and boiled for five minutes. The supernatant was then retrieved by magnetic separation rack.

The total lysate, supernatant, first wash and immunoprecipitation samples for both the Itgb1 immunoprecipitation and the IgG negative control were then subjected to SDS-PAGE, as described earlier. The nitrocellulose membranes were probed with antibodies against Itgb1 to confirm immunoprecipitation of Itgb1 protein, and Itga11 to determine if this protein forms a complex with Itgb1 in activated rHSCs.

![Diagram](image)

**Figure 2.1 Diagrammatic representation of immunoprecipitation for Itgb1 and associated proteins**
2.15 Immunofluorescence

Activated HSCs were plated onto chamber slides (Fisher Scientific, UK) at a density of 5000 cells per well and incubated overnight at 37°C. Then, the medium was removed; cells were washed twice with ice-cold PBS (Sigma, UK) and fixed in 4% paraformaldehyde (PFA; Sigma, UK) for 10 minutes. PFA was removed and the cells washed with PBS.

The chamber was removed and antigen retrieval was achieved by boiling the slides in 10mM sodium citrate buffer pH 6 (Sigma, UK) for 10 minutes. Slides were allowed to cool for 20 minutes in the sodium citrate, and then washed three times for 5 minutes in PBS. A wax border was drawn round each section (ImmEdge Hydrophobic Barrier Pen, Vector Laboratories, UK). Primary antibodies were diluted in PBS with 0.1% Triton-X 100 (Sigma, UK) with 3% serum (from the species in which the secondary antibody was raised; Vector Laboratories). A table of antibodies and their dilutions is included in Appendix I.

Cells were incubated with primary antibody in a humidified container at 4°C overnight. The following day slides were washed three times for 5 minutes in PBS. Cells were then incubated with a secondary antibody diluted in PBS with 0.1% Triton-X 100 for 2 hours in a humidified container. Where available, the secondary antibody was directly conjugated to a fluorochrome (Alexa Fluor® antibodies, Invitrogen). However, a biotinylated secondary antibody was used when detecting anti-Itgb1 raised in rat, and a FITC-conjugated secondary antibody was used when detecting anti-Itga11 raised in sheep, as anti-rat and anti-sheep Alexa Fluor® antibodies were not available in our laboratory. A table of secondary antibodies and their dilutions is included in Appendix I.

Slides were then washed three times for 5 minutes in PBS, and if a biotinylated secondary antibody was used then cells were incubated with texas red streptavidin (Vector Laboratories) diluted 1:200 in PBS with 0.1% Triton-X 100 for one hour at 4°C in a humidified container. Slides were then washed three times for 5 minutes in PBS and the process was repeated with a second primary antibody and appropriate secondary antibody.
Finally, slides were washed three times for 5 minutes in PBS and rinsed in dH₂O. Coverslips were mounted with VectorShield mounting medium containing 4’, 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories), and the edges sealed with nail varnish.

Images were obtained using Axiovert (Zeiss) imaging system and Axiovision 4.7 software (Zeiss), and collated with Photoshop (Adobe Systems, Uxbridge, UK).

2.16 F- and G-Actin Staining

Itgb1-null and control HSCs were cultured on chamber slides (Fisher Scientific, UK) and treated with 0.1% TritonX-100 for 5 minutes. The slides were then washed twice with PBS and then incubated with 1µl deoxyribonuclease I, Alexa Fluor® 594 Conjugate (Invitrogen) and 13.9µl phalloidin Alexa Fluor® 488 linked (Invitrogen) were diluted in 540µl PBS with 1% bovine serum albumin (Sigma) for 20 minutes. The slides were then washed with dH₂O, and coverslips were mounted with VectorShield mounting medium containing DAPI (Vector Laboratories), and the edges sealed with nail varnish.

2.17 Functional Assays

2.17.1 Cell Migration

HSCs were plated into 24-well plates (Corning) at a density of 5000 cells per well, and incubated overnight at 37°C. The following day the 24-well plate was transferred to the bioimaging facility. Images were acquired at 10 minute intervals over a 24 hour period with 10x magnification on an AS MDW live cell imaging system (Leica) using a 63x/1.30 Plan Apo glycerine objective, the BGR filter set (Chroma [61002]) and a (red (DS Red)) Precise LED fluorescent light source. Imaging software Image Pro 6.3 by Media Cybernetics LtD. Point visiting was used to allow multiple positions to be imaged within the same timecourse and cells were maintained at 37°C with 5% CO₂. The images were collected using a Coolsnap HQ (Photometrics) camera with a Z optical spacing of 0.2µm and only the maximum intensity projections of these images are shown in the results.

Image sequences were analysed using ImageJ software. The MTrackJ plug-in was used to track cell movement over the 24 hour period. To determine relative cell
migration, the total length of the cell track was generated for multiple cells, averaged and normalised to control. For individual cells, the co-ordinates at each 10 minute interval were made relative to a starting point of (0,0) and plotted to give examples of cell migration tracks. Measurements in ImageJ were calculated in inches and were converted to μm as follows:

Images were taken at a 0.86 μm per pixel. In ImageJ 1 inch was equivalent to 300 pixels. Therefore, in ImageJ 1 inch was equivalent to 258 μm (300 x 0.86 = 258).

2.17.2 Cell Proliferation

Bromodeoxyuridine (BrdU) is a synthetic analog of thymidine, and is incorporated into newly synthesised DNA during cell replication, in place of thymidine. Subsequently, it can be detected by immunocytochemistry to determine cell proliferation.

HSCs were plated onto chamber slides (Fisher Scientific, UK) at a density of 5000 cells per well and incubated overnight at 37°C. The following day the culture medium was changed with the addition of 30μM BrdU (Sigma, UK). The cells were incubated for 4 hours at 37°C. Then, the medium was removed; cells were washed twice with ice-cold PBS (Sigma, UK) and fixed in 4% paraformaldehyde (PFA; Sigma, UK) for 10 minutes. PFA was removed and the cells washed with PBS.

Subsequently, cells were treated with 2M hydrochloric acid (HCl; Sigma, UK) for 45 minutes and then washed three times with 0.1M boric acid (pH8.5; Fisher Scientific Chemicals, UK) for 10 minutes. Finally, cells were washed with PBS for 15 minutes.

Cells were incubated overnight with sheep anti-BrdU antibody (ab1893; Abcam, UK) diluted 1:1000 in 0.1% Titron® X-100 (Sigma, UK) in PBS supplemented with 3% donkey serum (Sigma, UK), at 4°C in a humidified container. The following day, the cells were washed three times in PBS for 5 minutes, and then incubated with donkey anti-sheep fluorescein isothiocyanate (FITC)-conjugated antibody (Abcam, UK) diluted 1:500 in 0.1% Triton® X-100 in PBS, for 2 hours at 4°C in a light-protected, humidified container. Finally, cells were washed three times in PBS, rinsed in de-ionised H2O, and coverslips (Appleton Woods) were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, UK). The
edge of the coverslip was sealed with nail varnish, and allowed to dry at room temperature, in the dark.

Images were captured from two areas of each chamber well at 10x magnification using an Axio Al Imager and Axiovision software. BrdU and DAPI positive nuclei were counted using Adobe Photoshop CS4 software. The percentage of proliferating cells was calculated by dividing the number of BrdU positive nuclei by the total number of DAPI positive nuclei.

2.17.3 Collagen Gel Contraction

HSC contraction was assessed by type I collagen gel contraction assay. In brief, HSCs suspended in culture medium were mixed with type I collagen, and aliquoted into a 12-well culture plate. Once polymerised, gels were detached from the sides of the well, and allowed to freely float in culture medium for 24 hours. Contraction was assessed by measuring the change in gel surface area.

2.17.3.1 Titration of NaOH

Rat tail type I collagen is supplied in a 0.2M acetic acid solution, and therefore, the required volume of NaOH to neutralise pH and allow collagen polymerisation was determined prior to starting the collagen gel contraction assay.

Eight clear 1.5ml tubes were sequentially labelled. 720μl of culture medium (with phenol red) and 280μl 3.56mg/ml type I collagen solution (BD Bioscience) were added to each tube. Immediately following, 1 to 8 μl 1M NaOH (Sigma, UK) solution was added and the sample mixed by vortex mixer. Colour change and gel polymerisation were observed, and the required volume of NaOH was determined by normalisation of culture medium colour and adequate gel polymerisation.

2.17.3.2 Collagen Gel Synthesis

Itgb1fl/fl CreER+ HSCs were cultured in the presence of 100nM tamoxifen or ethanol vehicle control. Cells were pelleted as described in section 2.6, and then resuspended in 1ml culture medium. Cells were counted using a haemocytometer and cell suspensions were diluted with culture medium, where necessary, to a concentration of 1 million cells/ml. In a 15 ml tube, 1674μl type I collagen solution were mixed with 3750μl culture medium with the addition of 18μl 1M NaOH. This
solution was divided into two 15 ml tubes, and 75μl cell suspension (tamoxifen or ethanol treated Itgb1fl/fl CreER+ HSCs) and 225μl culture medium were added to each. 775μl of this final mixture were aliquoted per well in a 12-well plate (3 wells per cell sample). The plates were incubated at 37°C for 1 hour to allow gels to polymerise. Gels were then detached from the well using a pipette tip and carefully transferred to a 6 well plate, with 3 ml warmed culture medium per well.

2.17.3.3 Contraction Analysis
The free floating collagen gels were imaged at time 0, using a Biorad Chemidoc with white light filter. The gels were then incubated at 37°C overnight, and re-imaged at 24 hours. The gel surface area was measured using ImageJ, and the percentage contraction of the gel calculated by 100 – ((Gel surface area at time 24 hours/Gel surface area at time 0)*100).

2.17.4 Cell Viability
The MTT assay relies on the activity of mitochondrial enzymes in viable cells to convert MTT to water insoluble purple formazan. The purple formazan was then dissolved in 0.1M HCl in isopropanol and absorbance read at 560 nm using a spectrophotometric plate reader.

2.18 In Vivo Models of Liver Fibrosis
Two commonly used in vivo models of liver fibrosis were used: repeated exposure to the hepatotoxin carbon tetrachloride (CCl₄), and bile duct ligation (BDL). CCl₄ results in hepatocyte damage, necrosis and apoptosis, with an associated inflammatory reaction. Advanced fibrosis develops over 8 weeks with twice-weekly intraperitoneal injection of CCl₄ (Iredale, 2007). BDL results in cholestatic liver injury, with proliferation of bile ductules, portal inflammation and fibrosis within two weeks (Iredale, 2007). For both models, methods were adapted from those used by Professor Mann and Drs Mann and Oakley at Newcastle University.

2.18.1 CCl₄ Administration
Adult male C57BL/6J mice were injected intraperitoneally with 2μl/g body weight of a 1:3 dilution of CCl₄ (Sigma, UK) in olive oil (Sigma, UK) or olive oil alone, twice-weekly for 8 weeks. Treatment with IPA3 (Tocris Bioscience, UK) or DMSO
(Sigma, UK) was added for the final 4 weeks. IPA3 was dissolved in DMSO to a concentration of 4µg/µl; 1µl/g body weight was injected intraperitoneally three times per week (on alternate days to CCl4 or olive oil). Control mice were injected with DMSO. Injections were performed in a fume hood using dedicated 100µl glass syringes (Hamilton, Switzerland) and disposable 26-gauge hypodermic needles (Becton Dickinson, Ireland).

In summary, there were four treatment groups: olive oil with DMSO; olive oil with IPA3; CCl4 with DMSO; and CCl4 with IPA3. There were 5 animals in each of the olive oil groups, and 10 in each of the CCl4 groups.

2.18.2 Bile Duct Ligation

Adult male C57BL/6J mice were anaesthetised by isoflurane (Baxter, UK) inhalation, injected subcutaneously with 150µl buprenorphine solution (6µg/ml in buprenorphine in 0.9% saline) and shaved in the abdominal region. The anaesthetised animal was then transferred to the operating table, and anaesthesia maintained by continuous isoflurane inhalation. The abdomen was sterilised with iodine solution (Videne) and then opened by a limited mid-line incision. The bile duct was exposed, with assistance of retainers. Ligatures (Ethicon) were tied around the bile duct in two regions. The abdominal incision was then closed in two steps: with discontinuous sutures (Ethicon) for the muscle layer; and discontinuous buried sutures for the cutaneous layer. Sham-operated animals underwent the same procedure but without ligation of the bile duct.

Following closure of the abdomen, isoflurane was switched off, and the animal allowed to recover from the anaesthetic whilst receiving supplemental oxygen. Once awake, the animal was placed on to tissue paper in a cage within a warming cabinet.

The following day BDL and sham-operated mice were given a further subcutaneous injection of 100µl buprenorphine. In the second post-operative week, mice were given alternate day subcutaneous injections of 100µl 0.9% saline (Baxter). Throughout the post-operative period, mice were housed within a warming cabinet with a soft mash diet.
In the second post-operative week, experimental mice were given three intraperitoneal injections with 1µl/g body weight of 4µg/µl IPA3 in DMSO, or DMSO alone.

In summary, there were four treatment groups: Sham with DMSO; Sham with IPA3; BDL with DMSO; and BDL with IPA3.

2.18.3 Collection of Liver Tissue and Serum

After 8 weeks treatment with CCl₄ or 2 weeks post-BDL, mice were euthanized by Home Office Schedule 1 methods. Immediately following sacrifice, the mice were weighed and the thoraco-abdominal cavity was opened by midline incision. The heart and inferior vena cava were incised and blood was allowed to collect in the inferior aspect of the thoracic cavity. A 1000µl pipette was used to transfer the blood into a 1.5ml tube. The liver was then dissected out in its entirety, weighed, and sections were cut from each lobe using a scalpel blade. The initial capsular section was discarded. Subsequent sections were placed into 4% paraformaldehyde (PFA) for histology; smaller sections were placed into RLT buffer (Qiagen) or snap frozen in liquid nitrogen, for RNA and protein extraction, respectively. A sample of the spleen was taken and placed into 4% PFA.

2.18.4 Preparation of Serum and Liver Function Test Analysis

Clotted blood samples were centrifuged at 8000 rpm for 10 minutes at 4°C. The supernatant was transferred to a 1.5ml tube; the volume was measured and 0.9% saline (Baxter) added to a make up the volume to 200µl if necessary. If dilution with 0.9% saline was required, the dilution factor was recorded. Serum samples were stored at -80°C until required for analysis of liver function tests (LFTs). Analysis of LFTs was undertaken by Alison Gaskill in Clinical Biochemistry at Manchester Royal Infirmary, and a final adjustment was made for the dilution factor.

2.18.5 Tissue Embedding and Sectioning

Tissue samples were fixed in 4% PFA with gentle rocking at room temperature for 4 hours. The PFA was then exchanged for 70% ethanol, and samples were left at room temperature overnight, with gentle rocking. The following day tissue samples were
dehydrated in a series of increasingly concentrated ethanol solutions (80%, 90% and 100%; 1-2 hours in each concentration, at room temperature with gentle rocking). Finally, tissue samples were cleared in chloroform (Fisher Scientific, UK) overnight at room temperature, with gentle rocking.

The next day, the chloroform was removed and the tissue samples were infiltrated with liquefied paraffin wax. The paraffin wax was changed twice, each with a 1-2 hour incubation at 72°C in a vacuum chamber. During the final incubation, vacuum pressure of 100mBar was applied for 2 hours.

The tissue samples were then embedded in paraffin wax, and once hardened, 5μm sections were cut using a steel blade mounted in a microtome (Leica Biosystems, UK). Sections were mounted on to glass microscope slides (X-tra® Adhesive Slides; Leica Biosystems, UK) and dried overnight at 37°C in a heated cabinet.

2.18.6 Picro-Sirius Red Staining

Sections were de-waxed by two sequential immersions in zylene (Fisher Scientific, UK) for three minutes each, and then rehydrated by immersion in 100% and 90% ethanol for two minutes each, followed by a rinse in dH2O. Sections were then immersed in picro-sirius red (Sirius red 0.5 g in 500ml of saturated aqueous solution of picric acid (Sigma)) for 1 hour and 15 minutes. Subsequently, sections were briefly rinsed in 0.5% acetic acid (Fisher Scientific) in dH2O, dehydrated in 100% ethanol and cleared in zylene. Coverslips were mounted with Entellan (Merck).

2.18.6.1 Quantification of Picro-Sirius Red

Images were acquired using a [20x/0.80 Plan Apo] objective using the 3D Histech Pannoramic 250 Flash II slide scanner. Ten images, from all three liver lobes, at 10x magnification, were selected from each slide at random, and analysed using Adobe Photoshop. The Colour Range tool was used to select red pixels; the number of selected pixels was recorded and expressed as a fraction of the total number of pixels, and this was averaged for the 10 images per slide to give a quantification of sirius red staining.
2.18.7 Hydroxyproline Assay

2.18.7.1 Hydroxyproline Assay

Hydroxyproline assay was carried out using the QuickZyme Biosciences Total Collagen Assay and Total Protein Assay kits (QuickZyme Biosciences, The Netherlands), and following the manufacturer’s instructions. The QuickZyme Total Collagen Assay is based on the detection of hydroxyproline, an amino acid predominantly incorporated into the triple helix of collagen. They have demonstrated sensitivity of this method in determining collagen per protein content in 5 to 10 10μm sections of formalin-fixed, paraffin-embedded tissue, without the need for paraffin removal.

Ten 10μm tissue sections were transferred to a 1.5ml screw-capped tube with addition of 150μl 6M HCl and incubated at 95°C in a calibrated oven for 20 hours to hydrolyse collagen. The collagen standard was prepared at the same time by mixing 125μl of the supplied 1200μg/ml in 0.02M acetic acid with 12M HCl in a screw-capped tube and incubating at 95°C for 20 hours.

Following incubation, the samples were cooled to room temperature and then centrifuged at 13000g for 10 minutes. The supernatant (below the liquid paraffin layer) was transferred to a 1.5ml tube and diluted with 4M HCl for further analysis. The collagen standard was diluted with 4M HCl to generate an 8 sample standard line with concentrations ranging from 300μg/ml to 0μg/ml. A pilot study analysis was then performed with dilution of experimental samples ranging from 1:2, to 1:8 to determine the appropriate dilution factor.

To perform the assay, 35μl of known standard or diluted experimental sample were added to appropriate wells of a 96 well clear, flat bottomed plate. 75μl assay buffer was added to each well and plate was sealed with an adhesive plate seal, and incubated for 20 minutes at room temperature with shaking. During this time, a suitable volume of detection reagent was prepared by mixing detection reagents A and B in a 2:3 ratio. 75μl of detection reagent was added to each well, the plate was sealed with an adhesive plate seal, mixed by shaking and incubated at 60°C for 1 hour in an oven. Subsequently, the plate was cooled on ice to room temperature, and the absorbance measured at 570nm with an automated plate reader (EL800; BioTek,
Potton, UK). The data was exported to an Excel file, and a standard curve was generated from the collagen dilution series; samples were compared with this to quantify collagen concentration.

A 1:2 sample dilution was determined as appropriate, and the assay was repeated using a 1:2 dilution with 4M HCl for all experimental samples. The assay was performed in duplicate for each sample.

2.18.7.2 Protein Assay

In order to express liver collagen (or hydroxyproline) content per total amount of protein, a total protein assay was also performed on the hydrolysed samples using the QuickZyme Total Protein Assay kit.

The protein standard was prepared by reconstituting the supplied dry protein reagent with 400μl 6M HCl resulting in a 3mg/ml protein stock solution. This was then diluted with 6M HCl to generate an 8 sample standard curve ranging from 300μg/ml to 0μg/ml.

The hydrolysed samples were diluted with 6M HCl and a pilot study was performed, with dilution factors ranging from 1:2 to 1:16.

To perform the assay 15μl of each standard protein sample, and 15μl of each experimental sample were added to the appropriate wells of a 96-well clear, flat bottomed plate. 120μl assay buffer were added to each well, and the contents mixed by shaking. The colour reagent working solution was prepared by mixing the colour reagent stock solution with reagent buffer in a 1:9 ratio and mixed well by vortex. 15μl of colour reagent working solution were added to each well, the plate was sealed with an adhesive plate seal and mixed by plate shaker. The plate was then incubated at 95°C for 45 minutes in an oven. Subsequently, the plate was cooled to room temperature, and the absorbance measured at 570nm with an automated plate reader (EL800; BioTek, Potton, UK). The data was exported to an Excel file, and a standard curve was generated from the protein dilution series; samples were compared with this to quantify protein concentration.
A 1:16 sample dilution was determined as appropriate, and the assay was repeated using a 1:16 dilution with 6M HCl for all experimental samples. The assay was performed in duplicate for each sample.

Finally, the hydroxproline content was expressed as a fraction of the total protein content for each sample.

### 2.19 DNA Cloning

#### 2.19.1 Generation of Vectors

##### 2.19.1.1 DNA Cloning

#### 2.19.1.1.1 Generation of Insert DNA

The TEAD binding site of the *MYL9* gene was cloned into the pGL3-promotor plasmid vector (Promega, UK), and the *YAP* coding sequence was cloned into the expression vector pcDNA3.1 for luciferase assay. DNA for ligation was generated by PCR of genomic DNA for the *MYL9* gene, and the pcDNA Flag YAPI vector (plasmid 18881, Addgene, Cambridge USA). Primers were designed around the region of interest and to contain recognition sequences for restriction enzymes, KpnI and XhoI for the *MYL9* gene, and KpnI and XbaI for the *YAP* gene. Restriction enzymes were chosen after confirming the absence of binding sites within the insert sequence using NEBcutter v2 (New England Biolabs). Following amplification, DNA products were separated by agarose gel electrophoresis (see section 2.10.3). The electrophoresed DNA was visualised using a UV transilluminator (Uvitec). Bands of the appropriate size were excised using a razor blade and transferred to a pre-weighed 15ml centrifuge tube (Falcon). The tube and gel were then weighed together, and the original tube weight subtracted to calculate the gel weight.

DNA was extracted from the gel using a Gel Extraction kit (Qiagen), following the manufacturers instructions. The gel was dissolved in 3 volumes of QG buffer for 10 minutes at 50°C with mixing by vortex. When fully dissolved, the solution was mixed with 1 volume of isopropanol and added to a QIAquick spin column. The spin column was centrifuged at 13,000rpm for 1 minute; then washed with 750μl buffer PE and centrifuged at 13,000rpm for further 1 minute. The flow-through was discarded and the spin column was centrifuged at 13,000rpm for a further 1 minute to
remove all liquid. Finally, the spin column was placed into a clean 1.5ml microcentrifuge tube, and DNA was eluted in 30μl of RNase and DNase free H$_2$O (Sigma).

DNA was quantified using a spectrophotometer, and then digested with the appropriate restriction enzymes at 37°C for 1 hour. Digested DNA was separated by agarose gel electrophoresis and then gel extracted.

2.19.1.1.2 Preparation of Vector DNA

pGL3-Promoter and pcDNA3.1 vectors were digested with the appropriate restriction enzymes at 37°C for 1 hour, and then dephosphorylated by incubation with calf intestinal phosphatase (New England Biolabs) at 37°C for 30 minutes. The vector DNA was quantified using a spectrophotometer.

2.19.1.1.3 Ligation of Insert and Vector

The digested insert and vector DNAs, 1μl of T4 DNA ligase (Roche), and 2μl DNA ligase buffer (Roche) were made up to a 20μl volume with DNase and RNAase free H$_2$O. Ratios of insert:vector DNA were calculated for efficient ligation. DNA and H$_2$O were first incubated at 65°C for 10 minutes to linearise the insert and vector DNA. The ligase and buffer were then added, and the mixture incubated at 16°C for 16 hours, followed by 65°C for 10 minutes to inactivate the ligase.

2.19.1.1.4 Mutation of MYL9 Insert DNA

As a negative control, a mutated version of the MYL9 insert DNA, missing 6 base pairs of the binding site, was created using the cloned vector. Primers were generated missing these base pairs and a series of two PCRs were carried out using the known pGL3-promoter primers (RVprimer3 and GLprimer2) and the mutated MYL9 primers missing 6 base pairs. This resulted in two template strands with an overlap of 15 base pairs (Figure 2.2).
A mutated MYL9 insert was generated as a negative control. In round 1 PCR, two reactions were set up with pGL3 promoter vector with MYL9 insert DNA digested with KpnI and XhoI. One with primers RVprimer3 and reverse mutant MYL9 missing 6 base pairs (bp) (shown in red); and one with primers forward mutant MYL9 missing 6 bp and GLprimer2 (shown in green). Products from these two reactions were used together in the round 2 PCR with the MYL9 cloning primers (shown in blue). This generated a single product, similar to the original MYL9 insert but missing the 6 bp required for YAP binding.
Reaction conditions were as follows:

94°C 3 minutes

30 cycles of:

94°C 45 seconds

55°C 45 seconds

72°C 1 minute

Then hold at 72°C for 8 minutes

Each reaction mixture was then incubated for 15 minutes at 37°C with 5 units of Klenow (New England Biolabs). DNA products were then separated by agarose gel electrophoresis and gel extracted. The extracted DNA was then subjected to a second PCR, using the alternating ratios of the two products and the MYL9 primers used to create the original insert.

The reaction conditions were as follows:

94°C for 3 minutes

72°C for 15 minutes

30 cycles of:

94°C 45 seconds

55°C 45 seconds

72°C for 1 minute

Then hold at 72°C for 8 minutes

The DNA product was digested with restriction enzymes KpnI and XhoI, separated by agarose gel electrophoresis and gel extracted. The mutated insert was ligated into the pGL3-promoter vector as previously described in section 2.19.1.1.3.
2.19.1.2 Plasmid Preparation

2.19.1.2.1 Bacterial Transformation

1-5μl DNA were added to 20μl chemically competent bacteria (DH5α; Invitrogen), and incubated on ice for 30 minutes. The mixture was then heatshocked at 42°C for 20 seconds and then cooled for 2 minutes on ice. Following which, 950μl pre-warmed LB buffer were added (Tryptone 1%, Yeast extract 0.5%, Agar 1.5%, NaCl 8.6mM, Glucose 20mM), and the mixture was incubated with shaking at 200rpm for 1 hour at 37°C.

Subsequently, the turbid mixture was centrifuged at 5000rpm for 3 minutes, and all but approximately 75μl of the supernatant discarded. The cell pellet was then resuspended in the remaining supernatant and plated out onto LB-Agar with ampicillin (100µg/ml; Sigma). Inoculated plates were inverted and incubated overnight at 37°C.

The following morning, colonies were selected using a sterile pipette tip and inoculated into 5ml LB with ampicillin. Cultures were incubated at 37°C with shaking at 200rpm overnight.

The following morning, the cells were pelleted by centrifugation at 8000rpm for 3 minutes, and plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen) to allow confirmation of the bacterial transformation prior to preparing a larger quantity of plasmid DNA.

Bacterial cells were resuspended in 250μl buffer P1 and transferred to a microcentrifuge tube. 250μl buffer P2 were then added and the solutions mixed by tube inversion. Subsequently, 350μl buffer N3 were added, the solutions mixed by tube inversion, followed by centrifugation at 13,000rpm for 10 minutes. The supernatant was then added to a QIAprep spin column and centrifuged for 1 minute at 13,000rpm. The spin column was then washed with 750μl buffer PE, and centrifuged at 13,000rpm for 1 minute. Any residual wash buffer was removed by centrifugation at 13,000rpm for an additional 1 minute. The spin column was then transferred to a clean 1.5ml microcentrifuge tube and DNA eluted with 30μl DNase and RNase free H₂O.
The plasmid DNA was digested with the appropriate restriction enzymes, and then separated by agarose gel electrophoresis and visualised by UV light using a Molecular Imager Gel Doc XR+ System with Image Lab Software (Biorad) to confirm the correct insert and vector size.

2.19.1.2.2 Preparation of Plasmid DNA

To prepare a larger quantity of plasmid DNA, appropriate colonies were selected with a sterile pipette tip and inoculated into 5ml LB with ampicillin and incubated at 37°C for 8 hours with shaking at 200rpm. Subsequently, the starter cultures were added to 250ml sterile LB with ampicillin and incubated overnight at 37°C with shaking at 200rpm.

The following morning, bacterial cells were pelleted by centrifuging at 2400rpm for 15 minutes, and resuspended in 10mls buffer P1. 10 mls buffer P2 were added to the cell suspension, mixed by inversion, and incubated for 5 minutes at room temperature. Then 10ml pre-chilled buffer P3 were added and mixed by inversion. The mixture was transferred to a QIAfilter Cartridge and incubated for 10 minutes. Subsequently, the cell lysate was filtered into an equilibrated HiSpeed Tip. The HiSpeed Tip was then washed with 60ml buffer QC. The DNA was eluted with 15ml buffer QF and precipitated with 10.5ml isopropanol using a QIAprecipitator, and finally, was washed with 70% ethanol and eluted with 1ml buffer TE.

The plasmid DNA was quantified using a spectrophotometer, and sequenced at the University of Manchester DNA Sequencing core facility. For the pGL3-promoter vector the RVprimer3 primer (Promega: 4952-4971 on pGL3-promoter vector sequence) was used for the sense sequence and GLprimer2 primer (Promega; 281-303 on pGL3-promoter vector sequence) for the anti-sense sequence. For the pcDNA3 vector the T7 and Sp6 primers were used.

2.20 Luciferase Assay

To assay activation of the MYL9 gene enhancer region by the transcriptional regulator YAP, a firefly luciferase reporter assay was used. Plasmids containing the cloned or mutated TEAD binding site within the MYL9 gene were transfected into LX2 cells along with vector containing the YAP gene or an empty vector.
Transfection efficiency was controlled for by simultaneous transfection with a plasmid containing Renilla reniformis.

### 2.20.1 Transfection

Transfections were performed in 12 well plates with LX2 cells at approximately 70% confluency. A transfection mix was made up consisting of 1μg DNA, 3μl Transfast (Promega) and 0.5ml serum-free culture medium, mixed by vortex and incubated for 15 minutes at room temperature. Culture medium was then removed from each well, and replaced by the transfection mix. The cells were incubated for 1 hour at 37°C, and then an additional 2 mls of culture medium was added to each well.

In addition, LX2 cells were transfected with the wild type MYL9 TEAD-binding region with YAP expression vector or empty vector control in the presence of 10μM verteporfin or DMSO vehicle control.

### 2.20.2 Luciferase Assay

The luciferase assay was performed 24 hours after transfection. The culture medium was removed and cells were washed with PBS. Cells were then incubated for 15 minutes at room temperature in 1x Passive Lysis Buffer (PLB; Promega; 250μl per well) with gentle rocking. Subsequently, 20μl of the supernatant from each well were added to a 96-well opaque luminometer plate (Greiner) and light production was measured with an Orion L Luminometer (Berthold Technologies). The luminometer was programmed to inject 100μl of luciferase assay reagent II (Promega) per well and to measure the light produced over 10 seconds. Subsequently, renilla was assayed by addition of 100μl Stop and Glo reagent (Promega) per well and measurement of light produced over 1 second. Luciferase relative light units were normalised to renilla relative light units to control for transfection efficiency, and made relative to empty vector controls.

### 2.21 ChIP

#### 2.21.1 Cell Culture, Fixation and Chromatin Isolation

Activated mHSCs were cultured in 10cm dishes (Corning) until approximately 80% confluent. The culture medium was removed and cells were washed with ice cold
PBS (Sigma) and fixed in 1% formaldehyde in DMEM for 10 minutes at room temperature with gentle rocking. Cells were then washed with PBS and incubated in STOP fix (0.125M glycine in PBS) for 5 minutes at room temperature. Cells were then washed with PBS, scraped in 2ml cell scrape solution (1.25mM phenylmethylsulfonyl fluoride (PMSF) in PBS) and transferred to a 15ml tube. The cell solution was then centrifuged for 10 minutes at 720g at 4°C. The supernatant was discarded, and the cell pellet was resuspended in 2ml ice-cold cell lysis buffer (10mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH6.5, 0.5mM EGTA (ethylene glycol tetraacetic acid), 10mM EDTA (Ethylenediaminetetraacetic acid), 0.25% Triton X 100 in H2O) with 5µl phosphatase inhibitor cocktail (Sigma). The cell lysate was incubated on ice for 5 minutes and then centrifuged for 10 minutes at 720g at 4°C. The supernatant was discarded and the cell pellet was resuspended in 2ml ice-cold nuclei was buffer (10mM HEPES pH6.5, 0.5mM EGTA, 1mM EDTA. 200mM NaCl in H2O) with 10µl phosphatase inhibitor cocktail. The solution was centrifuged for 10 minutes at 720 rcf at 4°C, the supernatant discarded, and the pellet resuspended in 500µl ice-cold nuclei lysis buffer (50mM Tris-HCl pH8.1, 10mM EDTA, 1% SDS in H2O) with 2µl phosphatase inhibitor cocktail and transferred to a 1.5ml tube.

2.21.2 Chromatin Shearing

The chromatin was sheared by sonication using a Bioruptor® Standard (Diagenode) with level high, alternating sonication on/off in 30 second cycles at 8°C for 10 minutes.

2.21.3 DNA Extraction

DNA was extracted with phenol/chloroform. An equal volume of phenol:chloroform:isoamly alcohol (Sigma) was added to the sample, mixed by vortex mixer for 10 seconds, and centrifuged at 13,000 rpm for 1 minute. The upper aqueous layer was carefully transferred to a clean microcentrifuge tube. An equal volume of chloroform (Fisher Scientific) was added to the sample, mixed by vortex mixer for 10 seconds, and centrifuged at 13,000 rpm for 30 seconds. The upper aqueous phase was carefully transferred to a clean microcentrifuge tube. 3M sodium acetate pH 5.2 was added to the sample, at a 1:10 dilution, and mixed. Subsequently, 20µg glycogen (Sigma) were added to the sample with mixing, followed by 2.5
volumes of ice cold 100% ethanol with mixing. The sample was then incubated for 1 hour at -80°C and then centrifuged at 13,000 rpm for 20 minutes at 4°C to pellet the DNA. The ethanol was carefully removed and replaced with an equal volume of ice cold 70% ethanol. The sample was centrifuged at 13,000 rpm for 5 minutes at 4°C. Finally, the ethanol was carefully removed and the pellet allowed to air dry for 10 – 15 minutes at room temperature, before resuspension in H₂O.

2.21.4 Immunoprecipitation

The sheared chromatin was pre-cleared with 50µl magnetic, ChIP-grade, protein G-coated beads (Cell Signalling) for 1 hour at 4°C with rotation. Subsequently, for each immunoprecipitation, 50µl sheared chromatin were added to 450µl IP dilution buffer (16.7mM Tris-HCl pH8.1, 1.2mM EDTA, 167mM NaCl, 0.01% SDS, 1.1% Triton X 100 in H₂O) and incubated with 50µl protein G beads, 3µg antibody or IgG and 1µl phosphatase inhibitor cocktail at 4°C overnight with rotation. 20µl sheared chromatin was taken as the input.

A magnetic separation rack was used to facilitate washing of the antibody-bead complexes three times with 1ml wash buffer 1 (20mM Tris-HCl pH8.1, 2mM EDTA, 50mM NaCl, 0.1% SDS, 1% Triton X 100 in H₂O), three times with 1ml wash buffer 2 (10mM Tris-HCl pH8.1, 1mM EDTA, 250mM LiCl, 1% NP40, 1% deoxycholate in H₂O) and three times with 1ml TE buffer (10mM Tris-HCl pH8.1, 1mM EDTA in H₂O).

Protein-DNA complexes were eluted by shaking for 45 minutes at room temperature in 300µl elution buffer (1% SDS, 100mM NaHCO₃ in H₂O). A magnetic separation rack was used and the supernatant transferred to a 1.5ml tube; the beads were discarded. Protein-DNA crosslinks were reversed by incubation with 12µl 5M NaCl at 65°C for 2.5 hours. Subsequently, protein contamination was cleared by incubation with 2µl proteinase K (10mg/ml proteinase K (Sigma), 50mM Tris HCl pH8.1, 10mM CaCl₂ in H₂O) for 1 hour at 37°C. Finally, 6µl protease inhibitor PMSF were added to each sample.
3 RESULTS: ITGB1 REGULATES HSC ACTIVATION VIA THE ACTOMYOSIN CYTOSKELETON AND MECHANOTRANSDUCTION

3.1 Introduction
Liver fibrosis results from an aberrant wound healing response to iterative liver injury, and is characterised by excessive deposition of type I collagen-rich ECM. This results in distortion of the liver architecture leading to impaired organ function, and predisposing to liver failure and hepatocellular carcinoma. One of the major cell types implicated in liver fibrogenesis is the HSC. In the healthy liver, quiescent HSCs reside in the subendothelial space of Disse, where they act as a vitamin A store. However, following liver injury, they become activated and develop the characteristics of contractile myofibroblasts: they proliferate, migrate into the liver parenchyma and secrete the damaging, fibrotic ECM.

A number of cytokines, matricellular proteins and transcription factors are implicated in this activation process (discussed in sections 1.6.2-3 and 1.7.2-3). However, it is becoming increasingly apparent that the mechanical microenvironment plays an important role in influencing cell fate and phenotype. In the context of liver fibrosis, increased matrix stiffness is a prerequisite for HSC activation (Olsen et al., 2011). Activated HSCs deposit type I collagen-rich ECM and contract the fibrotic scar, leading to further matrix stiffening; and therefore, a potential feed forward loop is established, amplifying HSC activation and fibrogenesis (Janmey et al., 2013).

One of the main routes by which cells sense changes within their mechanical microenvironment, is via a family of heterodimeric cell surface receptors, the integrins. Integrins are formed from an alpha and a beta subunit. There are eighteen alpha and eight beta subunits (Hynes, 2002). Itgb1 is known to associate with twelve different alpha subunits, forming the largest integrin subfamily (Hynes, 2002).

3.1.1 Itgb1 Maturation
Itgb1 is initially synthesised as an 86 kDa peptide core, which is then partially glycosylated within the endoplasmic reticulum, forming a 115 kDa biosynthetic precursor (Bellis et al., 1999). Precursor Itgb1 is retained within the endoplasmic
reticulum, forming a pool from where it either partners with an alpha subunit and undergoes maturation, or is degraded (Heino et al., 1989; Lenter and Vestweber, 1994). When partnered with an alpha subunit, precursor Itgb1 is transported to the Golgi where it is further glycosylated to form the 130 kDa mature Itgb1, and is subsequently expressed on the cell surface as an alpha-beta heterodimer (Heino et al., 1989).

The rate of Itgb1 maturation is determined by the availability of alpha subunits, and is enhanced by TGF-β1 stimulation. In unstimulated fibroblasts, the rate of Itgb1 maturation is slow (t_{1/2} ~10 hours), whereas stimulation with TGF-β1 increases both the rate of maturation (t_{1/2} ~3 hours) and the expression of Itgb1 mRNA (Heino et al., 1989). Variation in Itgb1 glycosylation has been proposed as a regulatory mechanism of integrin function (Bellis, 2004). For example, during terminal differentiation of keratinocytes, inhibition of Golgi-mediated glycosylation, together with a reduction in integrin trafficking, results in decreased cell surface expression of Itgb1-containing heterodimers (Hotchin and Watt, 1992). Conversely, inhibition of the alkaline ceramidase 2-sphingosine pathway, enhances maturation and cell surface expression of Itgb1, and promotes cell adhesion to both fibronectin and collagen coated surfaces (Sun et al., 2009).

3.1.2 Itgb1 Ligands

Itgb1 heterodimers bind a wide variety of ECM proteins: along with fibronectin (αVβ1, α4β1, α5β1, α8β1) and collagen (α1β1, α2β1, α10β1, α11β1), they also bind to laminin (α1β1, α2β1, α3β1, α6β1, α7β1, α10β1), tenascin (α8β1, α9β1), vitronectin (α8β1) and osteopontin (αVβ1, α4β1, α5β1, α8β1, α9β1) (Humphries et al., 2006). In addition, the cell adhesion molecules vascular cell adhesion molecule 1 (VCAM-1) and mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) are recognised by selected Itgb1 heterodimers (α4β1 and α9β1; and α4β1 alone, respectively) (Humphries et al., 2006).

3.1.3 Itgb1 in Development

Itgb1 heterodimers are widely expressed, and are essential for normal development. Global deletion of Itgb1 is embryonic lethal (Fässler and Meyer, 1995); more specific, targeted gene deletion reveals diverse roles for Itgb1, which depend on the
cell and tissue type. For example, conditional deletion of Itgb1 in skin epithelial cells is associated with severe blistering secondary to defective basement membrane assembly and hemidesmosome instability, along with reduced epidermal proliferation (Raghavan et al., 2000). Likewise, in mammary epithelia, loss of Itgb1 is associated with reduced epithelial cell proliferation and increased apoptosis (Faraldo et al., 2001). Whereas, in the intestinal epithelium, ablation of Itgb1 is associated with increased epithelial cell proliferation, defective enterocyte differentiation and postnatal lethality due to severe malnutrition; but not increased epithelial cell apoptosis or changes in basement membrane structure (Jones et al., 2006). In endothelial cells, Itgb1 is required for survival, adhesion and migration during angiogenesis, with loss of Itgb1 resulting in abnormal vascular development and death during embryogenesis (Carlson et al., 2008). In the central nervous system, deletion of Itgb1 from neurons and glia cells results in severe defects in brain development, and defective assembly and remodelling of the meningeal basement membrane (Graus-Porta et al., 2001).

Importantly, Itgb1 regulates ECM organisation during chondrogenesis, with loss of Itgb1 in chondrocytes resulting in abnormal collagen fibril assembly, and a reduction in the fibrillar density of cartilage (Aszodi et al., 2003). In addition, Itgb1 is required for normal wound healing in the skin, shown by reduced ECM production in mice with a fibroblast-specific deletion of Itgb1 (Liu et al., 2010a). Taken together, these data suggest that Itgb1 plays an important role in physiological ECM organisation and wound healing, and provide clues that Itgb1 signalling may be relevant to fibrosis.

Accordingly, deletion of Itgb1 from skin fibroblasts in mice protects against bleomycin-induced skin fibrosis (Liu et al., 2009), and kidney fibrosis is prevented by Itgb1-blocking antibodies in the unilateral ureteric obstruction model (Yeh et al., 2010). These studies suggest that Itgb1 may be an important regulator of organ fibrosis; however, its role in liver fibrosis is undetermined.

3.1.4 Mechanotransduction

The concept that mechanical forces can influence tissue remodelling and development was first proposed during the late 19th century (Eyckmans et al., 2011). However, mechanobiology did not receive much attention until the late 20th century,
when important discoveries were made demonstrating the role of physical forces in regulating bone development and vascular function (Eyckmans et al., 2011). There is now increasing interest in the role of biomechanical forces in influencing cell behaviour and fate, particularly with relevance to disease processes such as cancer and fibrosis.

Integrins form a physical link between the extracellular environment and the cell interior, by connecting to the cellular actomyosin cytoskeleton via focal adhesions (Hynes, 2002). Focal adhesions act as both a ‘physical anchor’ and a ‘signalling hub’, allowing the conversion of mechanical stimuli into biochemical signalling cascades (Janoštiak et al., 2014). This ability of cells, to sense and respond to changes within their physical microenvironment, has been termed mechnosensitive signalling, or mechanotransduction (Huang et al., 2012). Recently, evidence for Itgb1 as a mechanosensor in vivo was presented by Kanasaki et al, who demonstrated that deletion of Itgb1 from the bladder urothelium results in abnormal signalling responses to bladder wall stretch (Kanasaki et al., 2013).

Cells respond to changes in their mechanical microenvironment by adapting their function, behaviour and phenotype; these changes are effected by alterations in intracellular signalling pathways, and also through changes in gene transcription. Two distinct mechnosensitive pathways regulating gene transcription have been described, MKL1/SRF and YAP/TAZ, and these are discussed further in the following section (Janmey et al., 2013). In addition, NF-κB is activated in response to biomechanical forces, and has been implicated in HSC survival and liver fibrosis (Nam et al., 2009; Oakley et al., 2005; Young et al., 2010). Finally, exposure to physiological mechanical stresses is characteristic of several tissue types, for example, compressive forces in bone and cartilage, tensional forces in the skin, and shear forces in the vascular endothelium; and a number of different transcriptional regulators have been identified as mechnosensitive in these tissues (Janmey et al., 2013).

3.1.4.1 MKL1/SRF
MKL1 is a member of the myocardin and related transcription factors family, which co-activate SRF to promote transcription of pro-fibrotic genes, including αSma (Janmey et al., 2013). Myocardin is constitutively nuclear, whereas in fibroblasts, the
myocardin-related transcription factors shuttle between the cytoplasm and the
nucleus in response to changes within the actin cytoskeleton (Pipes et al., 2006).
MKL1 is sequestered in the cytoplasm when bound to monomeric (G-) actin, however, as actin is polymerised (F-actin), MKL1 is released and translocates to the
nucleus, where it interacts with SRF to regulate gene transcription (Janmey et al.,
2013).

The ratio of G-actin to F-actin is influenced by matrix stiffness, with increased
matrix stiffness favouring actin polymerisation. In lung fibroblasts, culture on a stiff
matrix substrate is associated with increased actin polymerisation, nuclear
translocation of MKL1 and αSma transcription (Huang et al., 2012). Moreover, in
vivo, ablation of MKL1 inhibits lung fibroblast to myofibroblast transition, and
protects against bleomycin-induced lung fibrosis (Zhou et al., 2013). Likewise, in
wildtype mice, inhibiting Rho kinase leads to actin depolymerisation and
deactivation of MKL1 nuclear signalling, with a reduction in bleomycin-induced
lung fibrosis (Zhou et al., 2013). In contrast, isoxazole, a small molecule activator of
MKL1 signalling, promotes myofibroblast differentiation in dermal fibroblasts, and
enhances cutaneous wound healing (Velasquez et al., 2013). MKL1 is also
implicated in the development of ocular (Yu-Wai-Man et al., 2014), intestinal
(Johnson et al., 2013), renal (Elberg et al., 2008) and cardiac fibrosis (Small et al.,
2010); however its role in liver fibrosis is unknown.

3.1.4.2 YAP/TAZ

YAP and TAZ are transcriptional co-activators; they do not bind DNA directly, but
associate with other transcription factors to regulate gene expression. In mammals,
YAP and TAZ are reported to partner with a number of different transcription
factors, but their effects are primarily mediated by members of the TEAD/TEF
family (Pan, 2010). Classically, YAP is regulated by the Hippo pathway, a serine
kinase cascade that ultimately leads to phosphorylation and retention of YAP within
the cytoplasm (Gumbiner and Kim, 2014). Inhibition of YAP via the Hippo pathway
is an important negative regulator of tissue growth and organ size; the corollary that
YAP is oncogenic, is supported by reports of YAP overexpression in several
different cancer types (Pan, 2010).
More recently, a role for YAP in mechanotransduction has been elucidated. Dupont \textit{et al} demonstrated that YAP activity is regulated by matrix stiffness, via a mechanism involving stress fibres and cytoskeletal tension, independent of Hippo signalling (Dupont \textit{et al}., 2011). Unlike MKL1/SRF signalling, YAP activity was not influenced by alterations in the ratio of monomeric to filamentous actin (Dupont \textit{et al}., 2011).

After establishing that YAP activity is sensitive to changes in matrix stiffness, the authors next demonstrated that YAP mediates cellular responses to the mechanical microenvironment (Dupont \textit{et al}., 2011). Mesenchymal stem cells differentiate into osteoblasts when cultured on a stiff substrate, and other cell types including adipocytes, when cultured on a soft matrix. However, depletion of YAP allowed adipogenic differentiation of mesenchymal stem cells cultured on a stiff substrate, demonstrating that YAP is required to effect cellular responses to a stiff matrix environment (Dupont \textit{et al}., 2011). In cancer associated fibroblasts (CAFs) YAP, but not TAZ, is required for contractility and fibrotic matrix production; YAP-depleted CAFs are less able to remodel matrices to enhance cancer cell invasion (Calvo \textit{et al}., 2013). Taken together, these studies suggest that YAP is both activated by, and promotes formation of, stiff fibrotic matrices. Therefore, it is plausible that YAP plays a role in fibrotic disease progression, although this has not yet been investigated.

\subsection{3.1.4.3 Activation of TGF-\(\beta\)1}

It is important to note that there is an established link between integrins, mechanotransduction and fibrosis, involving the mechanical activation of TGF-\(\beta\)1.

TGF-\(\beta\)1 is the most potent pro-fibrotic cytokine. It is sequestered in the ECM within a large latent complex, and can be mechanically activated by specific integrin heterodimers (discussed in section 1.11.9.1). To date, the \(\alpha V\) integrin heterodimers \(\beta 3, \beta 5, \beta 6\) and \(\beta 8\) have been reported to activate TGF-\(\beta\)1 by binding to the RGD sequence within the latency-associated peptide (Wipff and Hinz, 2008). However, \textit{Itgb}1 is also implicated, as inactivation of \textit{Itgb}1 in fibroblasts by either genetic ablation or neutralising antibodies, is associated with a reduction in TGF-\(\beta\)1 activation (Liu \textit{et al}., 2010a; Wipff \textit{et al}., 2007).
3.1.5 Summary

There is now a body of evidence that suggests that the mechanical environment is an important determinant of fibrosis progression. Integrins form a physical link between the extracellular environment and the cell interior, and are therefore well placed to transduce mechanical signals. Itgb1 is the largest integrin subfamily, and is implicated in fibrotic disease in the skin and kidney. There is evidence to support a role for Itgb1 in the activation of the pro-fibrotic cytokine TGF-β1. However, we hypothesise that Itgb1 signalling may also lead to the activation of mechanosensitive transcription factors to promote fibrosis.
3.2 Aims

In this chapter, we investigated the hypothesis that Itgb1 is important in HSC activation. The specific aims were:

1. To characterise expression of Itgb1 during HSC activation

Expression of Itgb1 mRNA and protein was analysed by qPCR and immunoblotting, respectively, in culture-activated HSCs.

2. To investigate the consequences of Itgb1-deletion in HSCs

The CreER-LoxP system for conditional gene deletion was used to investigate the consequences of Itgb1 ablation during HSC activation in vitro.

3. To investigate the role of Itgb1 in mechanosenstive signalling in activated HSCs

Localisation of mechanosensitive transcriptional regulators Mkl1 and Yap was examined by immunocytochemistry in control and Itgb1-null HSCs. The role of Yap in regulating the contractile protein Myl9 in activated HSCs was investigated by chromatin immunoprecipitation and luciferase assay.
3.3 Results

3.3.1 Itgb1 is Upregulated in Activated HSCs

Itgb1 expression was investigated in quiescent and activated HSCs (Figure 3.1). Itgb1 protein was detected as both the 115 kDa precursor and 130 kDa mature forms. Throughout this study, quantification is of total Itgb1 protein (precursor and mature). A timecourse activation demonstrated that in quiescent rat HSCs Itgb1 protein was undetectable, whereas by day 7 of activation there was an increase in precursor Itgb1, which was then glycosylated to mature Itgb1 (Figure 3.1A and B). By day 10 of activation there was a significant increase in total Itgb1 protein, alongside the profibrotic proteins αSma and type I collagen (Col1). Itgb1 mRNA was upregulated 3.4 fold in activated rat HSCs (Figure 3.1C). Itgb1 protein expression in LX-2 cells (a human HSC line) was investigated as a second model. Total Itgb1 protein was increased by 4.4 fold in LX-2 cells cultured in high serum (activated) versus low serum (quiescent), but this did not reach statistical significance (Figure 3.1D).

Co-localisation of Itgb1 with αSma and the pro-fibrotic transcription factor Sox9 was demonstrated by immunocytochemistry in activated mouse HSCs (Figures 3.1 E and F). αSma was incorporated into stress fibres, whilst Itgb1 was expressed throughout the cell and was present at the protruding edge of stress fibres, consistent with its inclusion in focal adhesions.

Itgb1 was also expressed in activated mouse HSCs with nuclear Sox9, a transcription factor that has been shown to regulate HSC activation (Piper Hanley et al., 2007; Pritchett et al., 2011).
Figure 3.1 Itgb1 is upregulated in activated HSCs.

**A, B.** Quantification and representative immunoblots of a timecourse activation of rat HSCs (rHSCs), showing increase in protein levels for Itgb1, αSma and Col1. Protein levels were normalised to Day 0. **C.** Quantitative PCR of Itgb1 in quiescent (Q) and activated (A) rHSCs. Quantification was normalised to β-actin and GusB. **D.** Quantification and representative immunoblot of Itgb1 protein in LX-2 cells (human HSC line) cultured in low and high serum medium. **E, F.** Immunofluorescence in A mouse HSCs (mHSCs) showing expression of Itgb1 with αSma (E) and Sox9 (F). Scale bars represent 50 μm. All immunoblotting quantification was standardised to β-actin; n ≥ 3. Error bars are SEM. Significance was determined using a two-tailed Student T test; *p<0.05, **p<0.01.
3.3.2 Genetic Inactivation of *Itgb1* in HSCs

Deletion of *Itgb1* from skin fibroblasts is associated with a reduction in αSma and Col1 expression, along with a reduced ability to contract collagen gels and migrate on ECM (Liu *et al.*, 2010a). Therefore, we hypothesised that *Itgb1* inactivation in HSCs might be associated with maintenance of a quiescent-like phenotype.

To investigate this, we used HSCs extracted from *Itgb1*^{fl/fl} βactinCreER+ mice to allow genetic inactivation of *Itgb1 in vitro*. In *Itgb1*^{fl/fl} βactinCreER+ mice the first coding exon (exon 2) of the *Itgb1* gene is flanked by loxP sites. Excision is mediated by the bacteriophage P1 Cre recombinase, which recombines between the two loxP sites. Cre is fused to the mutated ligand binding domain of the oestrogen receptor (ER) to form CreER, allowing temporal control of Cre activity. The mutated ligand binding domain of CreER recognises the synthetic ligand 4-hydroxytamoxifen (tamoxifen) but not endogenous oestrogens (Feil *et al.*, 1996). In the absence of tamoxifen, CreER is sequestered within the cytoplasm bound to heat shock protein-90 (Hayashi and McMahon, 2002). Ligation by tamoxifen results in activation of CreER, which then translocates to the nucleus and recombines between loxP sites. In *Itgb1*^{fl/fl} βactinCreER+ mice, CreER is globally expressed under control of the βactin promoter. Breeding was arranged to maintain homozygosity of the *Itgb1*^{fl/fl} allele and heterozygosity for the CreER transgene.

![Diagrammatic representation of Cre-LoxP mediated gene excision](image)

**Figure 3.2 Diagrammatic representation of Cre-LoxP mediated gene excision**

Ligation of CreER by tamoxifen (1) leads to its activation and translocation to the nucleus (2), where Cre recombines between loxP sites resulting in excision of the target gene (3).
The Itgb1^{fl/fl} βactinCreER+ mice were developed and characterised by the Streuli group (Jeanes et al., 2012). They demonstrated that addition of 100 nM tamoxifen to the cell culture medium resulted in complete loss of Itgb1 protein at 48 hours (Jeanes et al., 2012). Similarly, we have shown in HSCs extracted from Sox9^{fl/fl} ROSA26CreER+ mice that addition of tamoxifen to the culture medium for 48 hours results in efficient deletion of Sox9 protein by 72 hours (unpublished data).

Likewise, we were able to show that addition of 100 nM tamoxifen to the cell culture medium for the first 48 hours following isolation of Itgb1^{fl/fl} βactinCreER+ HSCs, resulted in efficient recombination of the Itgb1 gene (Figure 3.2A). PCR primers were designed, such that PCR of the intact gene generated a 900 base pair product, whereas PCR of the recombined gene generated a 200 base pair product (Figure 3.2B). Thus, there is evidence of tamoxifen-independent recombination, as PCR with primers 1 and 2 generated a 200 base pair product in both the tamoxifen-treated and control Itgb1^{fl/fl} βactinCreER+ HSCs (Figure 3.2A). However, despite this, tamoxifen treatment resulted in a significant reduction in both Itgb1 mRNA and protein (Figures 3.2C and D).

In a second model, Itgb1 was inactivated in passaged activated Itgb1^{fl/fl} βactinCreER+ HSCs. Tamoxifen-induced loss of Itgb1 protein from passaged activated Itgb1^{fl/fl} βactinCreER+ HSCs was confirmed by immunocytochemistry (Figure 3.2E) and immunoblotting (Figure 3.5).
Itgb1^fl/fl CreER+ HSCs isolated from Itgb1^fl/fl CreER+ mice were culture activated with addition of 100 nM tamoxifen to the culture medium for 48 hours from the day of isolation to delete Itgb1 from quiescent (Q) HSCs, or following passage of HSCs to delete Itgb1 from A HSCs. A. PCR using primers 1 and 2 with DNA extracted from tamoxifen-treated Itgb1^fl/fl CreER+ HSCs or controls. Tamoxifen induced recombination and deletion of exon 2 of the Itgb1 gene, with loss of the 900 bp product. There was a post-recombination 200 bp product in both tamoxifen-treated and control HSCs, suggesting that there was some Cre activity in the absence of tamoxifen. B. Diagrammatic representation of primers 1 and 2 in relation to the loxP sites. C. and D. Tamoxifen-treatment of Q Itgb1^fl/fl CreER+ HSCs resulted in significant reduction in Itgb1 mRNA and protein. E. Immunofluorescence showing loss of Itgb1 protein (green) in tamoxifen-treated Itgb1^fl/fl CreER+ A HSCs. DAPI (blue) nuclear stain shown in all images. Scale bar represents 50 μm. PCR quantification normalised to β-actin and GusB. Immunoblotting quantification was standardised to β-actin; n=3. Error bars are SEM. Significance was determined by two tailed Student T test, ***p<0.001.
3.3.3 *Itgb1* Deletion in HSCs is Associated with a Reduction in Pro-Fibrotic Proteins

Following liver injury, HSCs are activated into pro-fibrotic myofibroblasts, characterised by *de novo* expression of αSma, and production of Col1. The transcription factor Sox9 is ectopically expressed by activated HSCs, and regulates transcription of Col1 (Piper Hanley *et al.*, 2007). The ability to inhibit HSC activation would be advantageous, as theoretically, this would result in an attenuated fibrotic response to chronic liver injury. This is supported by the findings of Puche *et al.*, who demonstrated that HSC depletion is associated with a reduction in liver injury and collagen deposition in experimental models of liver fibrosis (Puche *et al.*, 2013).

HSC activation can be modelled *in vitro*, by culturing HSCs on plastic. We detected a significant reduction in the pro-fibrotic proteins αSma, Col1 and Sox9 in culture activated Itgb1-null HSCs (Figure 3.3A and B). Importantly, Col1 the predominant protein in the fibrotic ECM, was reduced by 70% in Itgb1-null HSCs. In control HSCs, αSma, the classical marker of myofibroblasts, was incorporated into stress fibres and localised with Itgb1, whereas in Itgb1-null HSCs, there was a deficiency of both Itgb1 and αSma, as detected by immunocytochemistry (Figure 3.3C). In the absence of CreER, tamoxifen treatment had no effect on the expression of Itgb1, αSma, Col1 or Sox9 (Figure 3.3D). Taken together, these results suggest that Itgb1 deficiency is associated with an impaired ability of HSCs to activate into myofibroblasts.
**Figure 3.4** *ltgb1* deletion is associated with a reduction in pro-fibrotic proteins

**A, B.** Quantification and representative immunoblots of control and *ltgb1*-null AHSCs showing decrease in protein levels for *ltgb1*, αSma, Col1 and Sox9. Immunoblotting quantification was standardised to β-actin; n=3. Error bars are SEM. Significance was determined by two tailed Student T test, *p<0.05; ***p<0.001.**

**C.** Immunofluorescence in control and *ltgb1*-null AHSCs showing localisation of *ltgb1* (red; top) with αSma (green; top) in control cells; and loss of *ltgb1* (red; bottom) and αSma (green; bottom) in null cells. DAPI (blue) nuclear stain shown in merged image. Scale bars 50 μm.

**D.** Immunoblot showing that in the absence of CreER, tamoxifen had no effect on *ltgb1*, αSma, Col1 or Sox9 expression.
3.3.4 *Itgb1* Deletion in Activated HSCs is Associated with a Reduction in Pro-Fibrotic Proteins

We next investigated the effect of deleting *Itgb1* in activated HSCs. We anticipated a less robust response, as any reduction in pro-fibrotic protein expression would be associated with a reversal of cell phenotype, as opposed to the prevention of HSC activation and *de novo* expression. We were able to show that deleting *Itgb1* in activated HSCs was associated with a significant reduction in Col1 and Sox9, and a non-significant reduction in αSma expression (Figure 3.5). As we expected, this response was less striking than the effects of *Itgb1* deletion prior to HSC activation (e.g. 39% versus 70% reduction in Col1).

![Figure 3.5](image)

**Figure 3.5 Itgb1 deletion from activated HSCs results in a reduction of fibrotic markers**

Passaged *Itgb1*fl/fl*CreER*+ A HSCs were treated with 100nM tamoxifen for 48 hours to induce deletion of *Itgb1*. Quantification and representative immunoblots following *Itgb1* knockout in A HSCs, showing a decrease in protein levels for *Itgb1*, αSma, Col1 and Sox9. Immunoblotting quantification was standardised to β-actin; n ≥ 3. Error bars are SEM. Significance was determined by two tailed Student T test, *p<0.05; ***p<0.001.
3.3.5 Itgb1-null HSCs are Less Migratory

Quiescent HSCs reside within the subendothelial space of Disse; however, following liver injury, activated HSCs migrate into the liver parenchyma and secrete type I collagen-rich ECM, to produce the fibrotic scar. Cell migration relies on the dynamic turnover of integrin-mediated adhesions to facilitate the protrusion and adhesion of the cell’s leading edge, and the detachment and retraction of the cell’s rear (Huttenlocher and Horwitz, 2011). Therefore, we hypothesised that the absence of Itgb1 would be associated with a reduced ability of activated HSCs to migrate. Using live cell imaging to track cells over 24 hours, we detected a significant reduction in the distance covered by activated Itgb1-null HSCs (Figure 3.5). This was true, when Itgb1 was deleted both prior to, and following, HSC activation (Figure 3.5A, B, C and D).
Figure 3.6 *Itgb1*-null HSCs are less migratory

Cell migration quantification and example distribution of the trajectories travelled by cells (track length, µm) taken over 24 hours using single cell tracking measurements in control and *Itgb1*-null HSCs. Relative track length was significantly reduced in *Itgb1*-null HSCs with *Itgb1* knockout both before (A, B) and after (C, D) HSC activation. Track lengths were averaged and normalised to control samples. Data was obtained from three biological repeats of the experiment, and in each case 30-77 cells were manually tracked. Significance determined by two-tailed Student T test, * p<0.05; ** p<0.01.
3.3.6 Itgb1-null HSCs are Less Proliferative and Less Contractile

Contractility is an important attribute of activated HSCs, contributing to the sinusoidal resistance and increased matrix stiffness which characterise liver fibrosis (Bhathal and Grossman, 1985; Thimgan and Yee, 1999). Others have shown that Itgb1-null fibroblasts are less able to contract a collagen gel (Liu et al., 2010a). Therefore, we hypothesised that depleting Itgb1 in activated HSCs would result in a less contractile phenotype. We observed that Itgb1-null HSCs were less proliferative, and confirmed this by bromodeoxyuridine (BrdU) incorporation assay in HSCs with Itgb1 deletion following activation. We detected a 2.9 fold reduction in nuclear BrdU in Itgb1-null HSCs compared with control HSCs over a four hour exposure (Figure 3.6A and B).

In order to perform a type I collagen gel contraction assay, large cell numbers were required and therefore the assay was performed in HSCs with deletion of Itgb1 following activation. We detected a significant reduction in the ability of activated Itgb1-null HSCs to generate contractile forces in free floating type I collagen gel matrices over 24 hours (Figure 3.6C and D). The addition of mitomycin C, to inhibit cell proliferation and eliminate this as a confounding factor, attenuated the reduction in contraction slightly, but this remained significant (Figure 3.6C and D).
Figure 3.7 Itgb1-null HSCs are less proliferative and contractile

Cell proliferation in control and Itgb1-null AHSCs following BrdU incorporation. A Quantification of BrdU (green) positive cells shown as a percent of total cells (blue DAPI staining). B Representative immunofluorescent image. C and D. HSCs were embedded within a free floating type I collagen gel for 24 hours. Contraction was quantified as a percentage change in gel surface area relative to control (C). Control HSCs were able to contract a collagen gel relative to Itgb1-null HSCs. Addition of mitomycin C to eliminate cell proliferation as a confounding factor attenuated the reduction in contraction slightly. n=3 without mitomycin C; n=2 with mitomycin C. Representative images (D). Significance was determined by two-tailed Student T test; * p<0.05; ** p<0.01.
3.3.7 Loss of Itgb1 is Not Associated with Decreased Cell Viability or Increased Apoptosis

During fibrogenesis, activated HSCs are resistant to apoptosis, and conversely, fibrosis resolution is associated with HSC apoptosis (Iredale et al., 1998; Mann and Mann, 2009; Wright et al., 2001). Loss of Itgb1 has been linked to increased apoptosis in some cell types, but not in others (Faraldo et al., 2001; Jones et al., 2006; Wu et al., 2009). Therefore, we next investigated the viability of Itgb1-null HSCs.

To determine cell viability, Itgb1-null and control HSCs were grown to confluence and then incubated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for four hours. The MTT assay relies on the activity of mitochondrial enzymes in viable cells to convert MTT to water insoluble purple formazan. The purple formazan was then dissolved in 0.1M HCl in isopropanol and absorbance read at 560 nm using a spectrophotometric plate reader. No significant difference was detected between control and Itgb1-null HSCs, suggesting that loss of Itgb1 does not affect cell viability (Figure 3.8A).

To determine if Itgb1 deficiency was associated with an increase in HSC apoptosis, we carried out immunoblotting for cleaved caspase 3. Caspase 3 is an effector caspase, and is activated by cleavage via both the intrinsic and extrinsic apoptosis pathways. We did not detect the 17 kDa cleaved caspase 3 in control or Itgb1-null HSCs, suggesting that loss of Itgb1 is not associated with apoptosis in HSCs (Figure 3.8B).
Figure 3.8 Loss of Itgb1 is not associated with decreased cell viability or increased apoptosis in HSCs

A. MTT assay for cell viability in control and Itgb1-null AHSCs. Absorbance was detected at 560 nm by spectrophotometric plate reader. n = 3. Non-significance (NS) was determined by two-tailed Student T test.

B. Immunoblotting for full length (35 kDa) and cleaved (17 kDa) caspase 3. Apoptosis is unaltered between control (-Tamoxifen) and Itgb1-null (+ Tamoxifen) AHSCs (Itgb1fl/fl;CreER+). Positive control lysate is UV treated rHSCs.
3.3.8 Itgb1 is Important for HSC Activation and Maintenance of a Myofibroblastic Phenotype

Taken together, these results suggest that Itgb1 is important for HSC activation, and the maintenance of a myofibroblastic phenotype. Importantly, loss of Itgb1 in activated HSCs was associated with a reduced ability to produce type I collagen and contract collagen gel matrices, both determinants of matrix stiffness. Increased matrix stiffness and the ability to generate tension are prerequisites for HSC activation (Olsen et al., 2011). Moreover, others have shown that Itgb1 acts as a mechanosensor *in vivo* (Kanasaki et al., 2013). Therefore, we postulated that loss of Itgb1 may impair the ability of HSCs to sense changes in their mechanical microenvironment, resulting in the disruption of mechanosensitive signalling pathways. Two mechanosensitive transcriptional regulatory pathways have been described, MKL1 and YAP. We investigated if MKL1 and YAP were active in HSCs, and if they were disrupted by loss of Itgb1.

3.3.9 Loss of Itgb1 is Associated with Reduced Stress Fibres and Reduced Nuclear Mk11 Staining

The transcription factor MKL1 co-activates SRF to regulate fibrotic gene expression (Janmey et al., 2013). In activated HSCs, SRF is upregulated, and RNA interference-mediated knockdown is associated with a reduction in αSma expression (Herrmann et al., 2007). MKL1 belongs to the family of myocardin and related transcription factors. Myocardin has been implicated in the activation of HSCs (Shimada et al., 2010), but to our knowledge, the role of MKL1 in HSC activation is unknown.

We detected active nuclear Mk11 expression in some, but not all, activated control HSCs, whereas in Itgb1-null HSCs we did not detect nuclear Mk11 (Figure 3.9A). Mk11 activation is regulated by changes in the ratio of monomeric (G-actin) to polymerised (F-actin). Mk11 is sequestered within the cytoplasm when bound to monomeric G-actin, but is released as actin is polymerised, and translocates to the nucleus (Janmey et al., 2013).

In control HSCs, we detected robust F-actin stress fibre formation with evidence of G-actin in both the cytoplasm and nucleus (Figure 3.9B). In contrast, Itgb1 depletion was associated with a marked reduction in F-actin stress fibres, and intense nuclear
staining for G-actin (Figure 3.9B). This is consistent with the findings of Pendleton et al., who demonstrated that in mast cells, disruption of F-actin by latrunculin B was associated with nuclear translocation of actin (Pendleton et al., 2003). The authors speculate that nuclear accumulation of actin might be involved in a cellular stress response (Pendleton et al., 2003). Whilst it is established that nuclear actin plays a role in the regulation of gene transcription, a detailed mechanistic understanding is lacking (Grosse and Vartiainen, 2013).

MKL1 requires monomeric actin for its export from the nucleus (Grosse and Vartiainen, 2013), and therefore, increased nuclear monomeric actin would be consistent with reduced nuclear Mkl1 in Itgb1-null HSCs. However, given that we did not detect robust nuclear Mkl1 staining in control activated HSCs, we next investigated the transcription factor, YAP.
Figure 3.9 Loss of Itgb1 is associated with reduced F-actin stress fibre formation and reduced nuclear Mkl1 staining

Immunofluorescence in control and Itgb1-null AHSCs. A. Top, nuclear localisation of Mkl1 (red) indicated by white arrows; nuclei identified by DAPI (blue). Bottom, reduced expression and cytoplasmic distribution of Mkl1 in Itgb1-null AHSCs. Scale bar 50 μm. B. Detection of F- (green) and G- (red) actin by fluorescent labelling of phalloidin and DNase I, respectively, in control and Itgb1-null HSCs. Bottom, reduced F-actin stress fibre formation and intense nuclear staining for G-actin in Itgb1-null HSCs. Scale bar 50μm.
3.3.10 The Mechanosensitive Transcriptional Regulator
YAP is Reduced in Itgb1-null HSCs

YAP is a mechanosensitive transcriptional regulator which acts with the TEAD/TEF family of transcription factors to mediate cell responses to increased matrix stiffness (Dupont et al., 2011). It is activated via a mechanism involving stress fibre formation and cytoskeletal tension, but unlike MKL1, YAP activation is not dependent on alterations in the ratio of monomeric to filamentous actin (Dupont et al., 2011). In cancer associated fibroblasts, YAP mediates contractility and fibrotic matrix production; however, its role in the regulation of myofibroblasts in fibrotic disease is unknown.

We detected a seven-fold increase in total Yap protein in activated HSCs (Figure 3.10A). In contrast, in Itgb1-null activated HSCs total Yap protein was barely detectable (Figure 3.10B). The subcellular localisation of YAP is thought to be regulated by phosphorylation. Increased phosphorylation correlates with reduced nuclear YAP (Wada et al., 2011); whilst phosphorylation at serine 127 (S127) enhances YAP binding to the scaffolding protein 14-3-3, and promotes its retention within the cytoplasm (Basu et al., 2003).

In Itgb1-null activated HSCs, we detected a 3.8 fold increase in the ratio of S127 phosphorylated Yap to total Yap (Figure 3.10C). In line with this, Yap was sequestered within the cytoplasm in Itgb1-null activated HSCs, whereas it localised to the nucleus in control activated HSCs (Figure 3.10D).

These results suggest that the transcriptional co-activator YAP is a potential regulator of HSC activation, and might be activated by Itgb1 signalling.
Figure 3.10 The mechanosensitive transcriptional regulator Yap is reduced in Itgb1-null HSCs

A, B and C. Quantification and representative immunoblots. (A) Yap protein levels increased in ArHSCs compared to QrHSCs and (B) decreased in Itgb1-null AHSCs. (C) Increase in inactive phosphorylated Yap (PhosphoYap) in Itgb1-null AHSCs. D. Immunofluorescence in control and Itgb1-null AHSCs. Top, nuclear localization of Yap (green, left) indicated by DAPI nuclear stain (blue, middle) and composite image (right). Bottom, reduced expression and cytoplasmic distribution of YAP in Itgb1-null AHSCs. Scale bar 50 µm. All immunoblotting quantification was standardised to β-actin. n ≥ 3. Significance determined by two-tailed Student T test. Error bars are SEM.
3.3.11 Yap Regulates Myl9 in Activated HSCs

During liver fibrogenesis the ECM is remodelled by activated HSCs, with degradation of the basement membrane-like matrix and deposition of a type I collagen-rich fibrotic scar. This, together with HSC-mediated contraction of the scar, results in increased matrix stiffness, which potentiates further HSC activation.

Similarly, in cancer, activated fibroblasts are capable of remodelling the matrix, resulting in increased stromal stiffness and promoting cancer cell invasion and metastasis. The matrix remodelling ability of cancer-associated fibroblasts is dependent on YAP-mediated regulation of the contractile protein, myosin light polypeptide 9 regulatory (MYL9). Therefore, we postulated that the same might be true in activated HSCs.

Accordingly, we were able to show that expression of Myl9 protein is increased 9.3 fold in activated HSCs (Figure 3.11A); whereas loss of Itgb1 was associated with a 72% reduction in Myl9 (Figure 3.11B). By dual immunocytochemistry, we detected Myl9 protein aligned along stress fibres with nuclear expression of Yap in control activated HSCs. In contrast, in Itgb1-null activated HSCs, both Myl9 and Yap were reduced (Figure 3.11 C).

Myl9 is a myosin regulatory light chain (MRLC), and is highly expressed in smooth muscle (Park et al., 2011). Two MRLCs associate with two myosin heavy chains and two essential light chains to form the contractile protein complex, myosin II. In non-muscle cells, the activity of myosin II is regulated by MRLCs. In fibroblasts, knockdown of all three murine MRLCs is associated with disruption of actin fibres and reduced contractility (Park et al., 2011). Therefore, loss of Myl9 may, at least in part, account for the reduced actin stress fibre formation and contractility of Itgb1-null activated HSCs (Figures 3.9B and 3.7C).
Figure 3.11 The contractile protein Myl9 is increased in activated HSCs

A and B. Quantification and representative immunoblots. Myl9 protein levels increased in A rHSCs relative to Q rHSCs (A) and decreased in response to Itgb1 loss in A HSCs compared to control (B). All immunoblotting quantification is standardised to β-actin. n = 3. Significance determined by two-tailed Student T test.

C. Immunofluorescence in control and Itgb1-null A HSCs. Top, Myl9 (green) is organised along stress fibres and co-localises with nuclear Yap (red) in control HSCs. Bottom, loss of both Myl9 and Yap in Itgb1-null A HSCs. Scale bar represents 50μm.
3.3.12 YAP Regulates Expression of MYL9 in Activated HSCs

We next investigated if YAP directly regulates MYL9 in activated HSCs. YAP is a transcriptional co-activator, and does not bind DNA directly but interacts with the TEAD/TEF family of transcription factors. Using in silico analysis software (dcode.org.), we identified a conserved TEAD/TEF binding motif within the 3′ untranslated region (UTR) of the MYL9 gene (Figure 3.12A). By chromatin immunoprecipitation (ChIP) using a Yap antibody, we were able to demonstrate enrichment for this region by PCR in activated HSCs (Figure 3.12B). This suggests that the YAP/TEAD complex binds to the 3′ UTR of the MYL9 gene.

Transcriptional activity was determined by luciferase assay in LX-2 cells following the synthesis of a pGL3-promotor vector containing the TEAD binding site of MYL9 in the presence or absence of YAP (Figure 3.12C). We detected a 1.9 fold increase in activity of the pGL3-promotor vector containing the TEAD binding site with the addition of a YAP expression vector. In contrast, we detected no change in activity of a pGL3-promotor vector containing the mutated TEAD binding site in the presence or absence of YAP (Figure 3.12D). In addition, we repeated the luciferase assay with the pGL3-promotor vector containing the TEAD binding site of MYL9 in the presence or absence of YAP, with the addition of 10 μM verteporfin or DMSO vehicle control. Verteporfin has been shown to inhibit YAP activity, by binding to and changing the configuration of YAP, and thereby disrupting its interaction with TEAD (Liu-Chittenden et al., 2012), and therefore, we hypothesised that verteporfin would inhibit transcription of MYL9. We were able to show a trend towards reduced luciferase activity in the presence of verteporfin, but this did not reach statistical significance (Figure 3.12E).

Finally, we culture activated mouse HSCs in the presence of 10 μM verteporfin, or vehicle control, to inhibit Yap activity; and demonstrated that verteporfin treatment was associated with a significant reduction in Itgb1, Col1, and Myl9 mRNA and a non-significant reduction in the well-characterised Yap target, Ctgf (Figure 3.13).

Taken together, these results suggest that the YAP/TEAD complex is able to bind to and regulate transcription of the MYL9 gene in activated HSCs. Moreover, Yap inhibition is associated with a significant reduction in Itgb1 and Col1, suggesting that
there may be a positive feed forward loop involving Itgb1, activation of Yap and expression of Col1.
Figure 3.12 YAP regulates transcription of MYL9 in HSCs

A. Alignment of Myl9 3' UTR showing conserved TEAD-binding motif indicated by asterisk (*). B. ChIP assay for TEAD-binding element in conserved region. Myl9 gene enriched using the core TEAD co-factor YAP in ArHSCs. Negative control is immunoglobulin (IgG) and positive control is input (diluted 10-fold). C and D. Luciferase activity in LX2 cells transfected with enhancer constructs containing the wild type (C) or mutated (mut) (D) Myl9 TEAD-binding region. Luciferase activity presented (in relative light units; RLU) following co-transfection with a YAP expression vector or an empty vector control. Results were normalised to renilla luciferase control plasmid and are relative to control, set at 1. E. Luciferase activity in LX2 cells transfected with enhancer constructs containing wild type Myl9 TEAD-binding region. Luciferase activity presented (in relative light units; RLU) following co-transfection with a YAP expression vector or an empty vector control in response to DMSO or Verteporfin (VP) treatment. Results were normalised to renilla luciferase control plasmid and are relative to control, set at 1.
Figure 3.13 Yap regulates fibrotic gene expression in A HSCs
Quantitative PCR of transcript abundance following inhibition of the Yap-Tead interaction using verteporfin in A mHSCs. Quantification was normalised to β-actin and GusB. n = 3. Error bars are SEM. Significance was determined by two-tailed Student T test * p < 0.05; ** p <0.01; *** p < 0.001.
3.4 Discussion

Itgb1-containing heterodimers form the largest integrin subfamily, and play an important role in mediating cell-matrix interactions (Hynes, 2002). As a mechanosensor, Itgb1 is capable of transmitting mechanical signals from the extracellular microenvironment to the cell interior (Kanasaki et al., 2013). Fibrosis is associated with significant changes in the biophysical characteristics of the ECM, with deposition of type I collagen and contraction of the fibrotic scar resulting in stiffening of the matrix. Increased matrix stiffness potentiates further activation of pro-fibrotic myofibroblasts, establishing a feed forward loop and amplification of fibrogenesis (Olsen et al., 2011). Taking this into account, it is not surprising that Itgb1 has been implicated in fibrotic disease in both the skin and kidney (Liu et al., 2009; Yeh et al., 2010). However, the role of Itgb1 in liver fibrosis was undetermined.

In this study, we present evidence that Itgb1 is important in the activation of HSCs, a pivotal step in the pathogenesis of liver fibrosis. Moreover, we place Itgb1 within a pro-fibrotic circuit involving the contractile cytoskeleton and activation of the mechanosensitive transcriptional regulator YAP.

3.4.1 Itgb1 is Upregulated in Activated HSCs

HSC activation can be modelled in vitro by culturing on plastic: over 7 to 14 days HSCs acquire the characteristics of myofibroblasts, develop a stellate-like morphology and express αSma and Col1 (Rockey et al., 1992). In this study, Itgb1 was upregulated in culture activated HSCs, paralleling expression of αSma and Col1, and localised with nuclear Sox9. This is consistent with our group’s previous finding that the transcription factor SOX9 is ectopically expressed in activated HSCs and regulates COL1 (Piper Hanley et al., 2007; Pritchett et al., 2011). Others have also shown that Itgb1 is upregulated in activated HSCs (Carloni et al., 1996; Levine et al., 2000). However, Dodig et al reported no difference in Itgb1 protein levels between quiescent and activated HSCs (Dodig et al., 2007).
3.4.2 Loss of Itgb1 is Associated with Impaired HSC Activation

In skin fibroblasts, loss of Itgb1 results in impaired myofibroblast differentiation, with reduced expression of αSma and Col1 (Liu et al., 2009; Liu et al., 2010a). In this study, we used HSCs extracted from Itgb1<sup>fl/fl</sup> CreER<sup>+</sup> mice to investigate the effects of Itgb1 deletion in HSCs in vitro. We observed similar effects to those seen by Liu et al in skin fibroblasts. Loss of Itgb1 was associated with reduced expression of the pro-fibrotic proteins αSma, Col1 and Sox9. This was apparent when Itgb1 was deleted both prior to, and following, HSC activation; although, the effect was greatest when Itgb1 was deleted from quiescent HSCs.

In addition, we observed that Itgb1 deficiency altered the myofibroblastic characteristics of activated HSCs. Itgb1-null HSCs were less proliferative, migratory and contractile. These results were consistent with those shown by other groups, who have demonstrated reduced migration and contraction in Itgb1 deficient fibroblasts (Liu et al., 2010a; Schiller et al., 2013).

Collectively, these results suggest that Itgb1 is required for HSC activation, and that loss of Itgb1 is associated with a less fibrotic phenotype.

3.4.3 Loss of Itgb1 is Associated with Disruption of the Contractile Cytoskeleton

HSC activation is associated with cytoskeletal re-organisation and the development of extended stress fibres (Kato et al., 1999). In Itgb1-null HSCs, we observed impaired formation of actin stress fibres, comparable with the effects of Itgb1 depletion in skin fibroblasts and chondrocytes (Liu et al., 2010a; Raducanu et al., 2009). In addition, we detected reduced levels of the contractile protein Myl9. Perturbation of the contractile cytoskeleton in Itgb1-null HSCs suggests that their ability to generate tension is impaired. This prompted us to investigate whether loss of Itgb1 resulted in disrupted mechanosensitive signalling in HSCs.
3.4.4 Loss of Itgb1 is Associated with Reduced Activation of the Mechanosensitive Transcriptional Regulator Yap

Two mechanosensitive transcriptional regulatory pathways have been described MKL1/SRF and YAP/TAZ (Janmey et al., 2013). MKL1 co-activates SRF resulting in the transcription of fibrosis-associated genes including αSma. In addition, MKL1 has been shown to interact with other transcription factors relevant to fibrosis, including NF-kB (Fang et al., 2011), Smad3 (Morita et al., 2007) and Specificity Protein 1 (Sp1) (Luchsinger et al., 2011). MKL1 is implicated in fibrogenesis in a number of different organs (Elberg et al., 2008; Johnson et al., 2013; Small et al., 2010; Yu-Wai-Man et al., 2014). However, its role in liver fibrosis is unknown.

We detected nuclear localisation of MKL1 in some, but not all, activated HSCs. Therefore, we did not investigate MKL1 further, but focussed on the alternative mechanosensitive transcriptional regulator YAP. However, following completion of this study, Tian et al have published data suggesting that MKL1 (alternatively known as MRTF-A) plays an important role in HSC activation and liver fibrosis (Tian et al., 2014). They demonstrated that MRTF-A/MKL1 was upregulated during HSC activation and localised to the nucleus; whereas deletion of MRTF-A/MKL1 was associated with downregulation of fibrotic markers in vitro, and attenuated liver fibrosis in vivo (Tian et al., 2014).

Classically, YAP is negatively regulated by the Hippo pathway, to control tissue growth and organ size (Pan, 2010). However, recently an alternative mechanism of YAP activation involving stress fibre formation and cytoskeletal tension, has been described (Dupont et al., 2011). Moreover, YAP has been shown to regulate fibrotic matrix production and contractility in cancer-associated fibroblasts (Calvo et al., 2013).

In line with this, we demonstrated that, in addition to impaired stress fibre formation and contractility, Itgb1 depletion in HSCs is associated with reduced nuclear localisation of Yap. Moreover, we were able to show that YAP regulates transcription of MYL9 in HSCs, suggesting that there is a pro-fibrotic circuit involving Itgb1, the contractile cytoskeleton and activation of YAP. Similarly, Calvo et al demonstrated regulation of MYL9 by YAP in cancer-associated fibroblasts, but not at the level of transcription (Calvo et al., 2013). They also proposed a positive
feed forward loop involving YAP and the contractile cytoskeleton, leading to perpetuation of the cancer-associated fibroblast phenotype (Calvo et al., 2013).

They and others have demonstrated, by inhibition of stress fibres with latrunculin A and inhibition of myosin with blebbistatin, that YAP activation is dependent on the cell’s ability to generate cytoskeletal tension (Calvo et al., 2013; Dupont et al., 2011). Therefore, it is tempting to speculate that inhibition of YAP in Itgb1-null HSCs is a consequence of the defective contractile cytoskeleton. However, further experiments are required to conclusively prove this; including assessment of YAP activation following disruption of the actomyosin cytoskeleton in activated HSCs.

A further mechanism of YAP activation involving the mevalonate pathway has recently been uncovered (Sorrentino et al., 2014). Sorrentino et al demonstrated that inhibition of the mevalonate pathway with statins resulted in inactivation of YAP. This is interesting, because statins have previously been shown to inhibit HSC activation and liver fibrosis (Klein et al., 2012; Trebicka et al., 2010; Wang et al., 2013).

Finally, the compound verteporfin has also been shown to inhibit YAP activity, by disrupting the interaction of YAP with TEAD transcription factors (Liu-Chittenden et al., 2012). In this study, we demonstrated that verteporfin reduced expression of Itgb1, Col1, Myl9, and the established YAP target, Ctgf during HSC activation. This reinforces our proposal that Itgb1, the contractile cytoskeleton and YAP are involved in a pro-fibrotic circuit in activated HSCs.

3.4.5 Summary

Liver fibrosis is a major health and economic burden. In the UK, liver disease is the fifth leading cause of mortality, with an average age of death of just 59 years (Moore et al., 2009). Worryingly, there is an upward trend, with both hospital admissions and deaths due to liver disease increasing by 8 to 10% each year (Moore et al., 2009). The increasing prevalence of liver fibrosis underscores the urgent need for anti-fibrotic therapies. Collectively, our data suggest that targeting the Itgb1 - contractile cytoskeleton - YAP pro-fibrotic circuit in HSCs may be a means of dampening HSC activation and fibrogenesis (Figure 3.12).
Itgb1 is widely expressed and is required for the development and homeostasis of many tissue types. Therefore, global inhibition of Itgb1 is likely to be associated with significant off-target effects, and would not be a viable therapeutic strategy. More precise targeting could be achieved by identifying an HSC-specific Itgb1-containing heterodimer, or downstream mediator of Itgb1 signalling. Alternatively, a compound such as verteporfin could be used to directly inhibit YAP.

Figure 3.14 Diagrammatic representation of the proposed pro-fibrotic circuit, involving ITGB1, the contractile cytoskeleton and YAP in HSC activation

Model outlining the role of ITGB1 in mechanotransduction and HSC activation. ITGB1 is required for development of the contractile cytoskeleton and activation of YAP. YAP regulates expression of COL1, ITGB1 and MYL9 to promote matrix stiffening, and thereby a positive feed-forward loop is established. Potential strategies for therapeutic targeting of this pro-fibrotic circuit are highlighted in red.
4 RESULTS: INVESTIGATING THE ROLE OF GROUP I PAKS IN HSC ACTIVATION AND LIVER FIBROSIS

4.1 Introduction

Activation of HSCs into contractile myofibroblasts is a pivotal step within the pathogenesis of fibrosis, and is driven by a variety of factors (Friedman, 2008a). In addition to well-established soluble mediators, such as TGF-β1, these include the biophysical characteristics of the cellular microenvironment (Olsen et al., 2011). Integrins are cell surface receptors, which form a physical link between the extracellular environment and the cell interior; and consequently, can relay mechanical signals between the two.

In the previous chapter, we outlined a role for Itgb1 as a mechanosensor signalling via the contractile cytoskeleton to activate the transcriptional regulator YAP during HSC activation. We hypothesised that disrupting this pro-fibrotic circuit might be a novel therapeutic strategy for liver fibrosis. However, global inhibition of Itgb1 is likely to be associated with significant off-target effects, and therefore, identifying a downstream mediator of Itgb1 in activated HSCs may allow more precise targeting.

In this chapter, we conducted a microarray analysis to compare gene expression between Itgb1-null and control activated HSCs. We interrogated the dataset to identify downstream effectors of Itgb1 known to regulate the contractile cytoskeleton, and uncovered a potential role for p21-activated kinases (PAKs) in HSC activation.

4.1.1 PAK Structure and Activation

PAKs are a family of serine/threonine kinases, which are involved in a multitude of cell processes and behaviours. There are six mammalian isoforms, and these are categorised into two groups based on their structure and regulation. Group I comprises PAK1-3, and group II includes PAK4-6 (Bokoch, 2003).

4.1.1.1 Group I PAKs

The Group I PAKs were originally discovered during a screen for Rho GTPase binding proteins in rat brain cytosol (Manser et al., 1994). They are closely related in
structure, each having an N-terminal protein binding domain, which overlaps with an auto-inhibitory domain, and a C-terminal kinase domain (Dammann et al., 2014; Lei et al., 2000). The kinase domain is highly consistent between PAK1-3, with over 90% sequence homology (Van Eyk et al., 1998).

In the resting state, group I PAKs form auto-inhibitory homodimers, with the auto-inhibitory domain of one molecule masking the kinase domain of the other, and vice versa (Parrini et al., 2002). Binding of GTP-bound, but not GDP-bound, Cdc42 or Rac1 to the protein binding domain, disrupts the dimerization, and allows the auto-phosphorylation and activation of the kinase (Manser et al., 1994; Parrini et al., 2002). PAK activation is associated with phosphorylation at multiple residues (Gatti et al., 1999; Manser et al., 1997; Zhan et al., 2003). A notable example is phosphorylation at threonine 423 in PAK1, which is required for full catalytic activity, and also maintains the activation state by preventing auto-inhibition (Zenke et al., 1999).

In addition, PAK1 is activated by sphingolipids, independently of Cdc42 or Rac1 binding (Bokoch et al., 1998); and PAK2 is activated by caspase-mediated proteolytic cleavage of the N-terminal regulatory domain (Rudel and Bokoch, 1997).

4.1.1.2 Group II PAKs

The group II PAKs were more recently discovered (Jaffer and Chernoff, 2002). They differ from the group I PAKs in both structure and regulation. For example, only PAK5 has an identifiable auto-inhibitory domain (Ching et al., 2003); and the kinase domain of PAK4 shares only 53% sequence homology with PAK1-3 (Abo et al., 1998). In addition, the role of Cdc42 in the activation of PAK4-6 is controversial (Wells and Jones, 2010). Abo et al demonstrated that Cdc42 was not required for activation of PAK4, but instead regulated its subcellular localisation (Abo et al., 1998); whereas, Ching et al showed that Cdc42 was able to activate auto-phosphorylation of PAK5 (Ching et al., 2003).
4.1.2 PAK Function

4.1.2.1 Genetic Knockout Models

Genetic knockout models have been developed, allowing greater insight into the role of PAKs in vivo.

4.1.2.1.1 PAK1

PAK1 has a wide tissue distribution, but is predominantly expressed in the brain, muscle and haemopoetic cells (Allen et al., 2009; Arias-Romero and Chernoff, 2008). PAK1 knockout mice are viable, fertile and have a normal lifespan (Allen et al., 2009; Asrar et al., 2009). However, they exhibit defects in the immune system, glucose homeostasis and neuronal function.

In the immune system, PAK1 knockout is associated with a reduced cutaneous allergic response, due to defective mast cell degranulation (Allen et al., 2009). In vitro studies in bone marrow derived-mast cells demonstrated that this results from a failure to disassemble cortical F-actin in response to allergen stimulation, and in turn, this prevents mast cell granules from accessing the plasma membrane (Allen et al., 2009). In macrophages, loss of PAK1 is associated with formation of numerous, unstable lamellipodia, although this has no effect on migration or chemotaxis in vitro (Smith et al., 2008).

In addition, PAK1 knockout is associated with glucose intolerance, resulting from impaired insulin secretion coupled with peripheral insulin resistance (Wang et al., 2011). The peripheral insulin resistance is linked to a reduction in insulin-stimulated Glucose Transporter Type 4 (GLUT4) translocation in skeletal muscle, which is reminiscent of the failure to traffic granules to the cell surface in allergen-stimulated mast cells (Allen et al., 2009; Wang et al., 2011).

In the central nervous system, loss of PAK1 does not result in any gross anatomical or locomotor abnormalities (Asrar et al., 2009). However, there are defects in the synaptic plasticity of hippocampal neurons, suggesting that learning and memory may be impaired (Asrar et al., 2009).
4.1.2.1.2 PAK2

PAK2 is widely expressed, and global knockout is lethal in early embryogenesis due to multiple developmental abnormalities (Arias-Romero and Chernoff, 2008).

Conditional PAK2 knockout mice have been developed, and so far, have revealed roles for PAK2 in the immune system. In mast cells, PAK2 opposes PAK1, by inhibiting mast cell degranulation (Kosoff et al., 2013); and in T lymphocytes, PAK2 is required for normal development and maturation (Phee et al., 2014).

4.1.2.1.3 PAK3

PAK3 is predominately expressed in the brain, but is also present in cartilage, liver, spleen and testis (Arias-Romero and Chernoff, 2008).

PAK3 knockout mice are viable and fertile, with no detectable abnormalities in behaviour or central nervous system structure (Meng et al., 2005). However, they exhibit defects in hippocampal synaptic plasticity, which translate to impairments in learning and memory (Meng et al., 2005).

In humans, loss of function mutations in PAK3 are associated with X-linked intellectual disability (Allen et al., 1998; Peippo et al., 2007).

4.1.2.1.4 PAK4

PAK4 is widely expressed, and its knockout results in early embryonic lethality, associated with defects in the heart, central nervous system and vasculature (Qu et al., 2003; Tian et al., 2009).

4.1.2.1.5 PAK5 and PAK6

PAK5 and PAK6 are predominately expressed in the brain, and knockout of either one does not appear to result in a phenotype (Nekrasova et al., 2008). However, combined knockout is associated with impaired locomotion and learning, suggesting that there is redundancy between PAK5 and PAK6 (Nekrasova et al., 2008).

4.1.2.2 Cellular Function

On a cellular level, PAKs are involved in a number of functions, including regulation of actin cytoskeletal dynamics, migration and contraction (Bokoch, 2003). However, the effects appear to be cell type specific.
4.1.2.2.1 Actin Cytoskeletal Dynamics
Regulation of actin cytoskeletal dynamics by PAK1 is well-described (Bokoch, 2003). Mechanistically, PAK1 is capable of phosphorylating and activating the Lin-11 Ist-1 and Mec3 kinases (LIMK), which in turn phosphorylate and inactivate the actin depolymerisation factor coflin (Edwards et al., 1999). Accordingly, PAK1 knockout in neurons is associated with reduced F-actin and impaired coflin phosphorylation following NMDA-stimulation (Asrar et al., 2009). However, in mast cells, loss of PAK1 results in persistence of the cortical F-actin ring following antigen stimulation, suggesting the involvement of alternative signalling pathways (Allen et al., 2009).

4.1.2.2.2 Migration
In fibroblasts, PAK1 is required for directional motility (Sells et al., 1999). However, in endothelial cells, expression of both constitutively active and dominant negative PAK1 result in decreased migration, suggesting that cell migration depends on controlled regulation of PAK1 activity, in a cell-type specific manner (Kiosses et al., 1999).

4.1.2.2.3 Contraction
Regulation of contraction by PAK is complex, and appears to be dependent on cell type and context (Bokoch, 2003). A number of investigators have demonstrated that PAK1-3 are capable of phosphorylating MRLC in vitro, resulting in increased contractility (Kiosses et al., 1999; Sells et al., 1999; Van Eyk et al., 1998; Zeng et al., 2000). On the contrary, others have argued that PAK1 inhibits contraction by phosphorylating and inactivating MLCK, resulting in an overall decrease in MRLC phosphorylation (Sanders et al., 1999). However, more importantly, are in vivo and ex vivo studies, which demonstrate that PAK1 is required for airways smooth muscle contraction and reactivity (Hoover et al., 2012); and ML2/MYL9 phosphorylation and cardiac contractility during ischaemia-reperfusion injury (Monasky et al., 2012).

4.1.3 PAKs and Fibrosis
The role of PAKs in regulating the contractile cytoskeleton suggests their potential as mediators of mechanosensitive signalling. Indeed, Zhang et al demonstrated that in Caenorhabditis elegans, PAK1 is part of a mechanosensory-signalling pathway and is activated by mechanical tension arising from muscle contraction (Zhang et al.,
2011). Similarly, in kidney tubular cells, PAK1 has been shown to drive nuclear localisation of the mechanosensitive transcriptional regulator MRTF, and activate transcription of pro-fibrotic αSma (Sebe et al., 2008). These findings, coupled with the role of mechanosensitive signalling in myofibroblast activation (discussed in section 1.7.3), suggest that PAKs may be implicated in the pathogenesis of fibrotic disease.

Accordingly, Chen et al demonstrated that in kidney fibrosis, PAK1 is activated in response to increased intraglomerular pressure, and regulates mechanical stretch-induced pro-fibrotic cytokine production in mesangial cells (Chen et al., 2013). In addition, Wilkes et al demonstrated that fibroblasts require PAK2 for TGF-β stimulated proliferation and myofibroblast-like morphological change (Wilkes et al., 2003). However, in contrast, Liu et al showed that mice with a cardiomyocyte specific PAK1 knockout are more susceptible to cardiac hypertrophy and fibrosis following mechanical stress or neuroendocrine agonist stimulation (Liu et al., 2011).

Despite these findings, there are surprisingly few publications on the role of PAKs in fibrosis; and to our knowledge, none on the role of PAKs in liver fibrosis.

4.1.4 PAK Inhibitors

In contrast to the sparsity of publications on PAKs and fibrosis, there are many on the role of PAKs in cancer (Radu et al., 2014). PAKs are implicated in the pathogenesis of cancer through a number of mechanisms including promoting cell proliferation, survival, invasion and metastasis (Radu et al., 2014). The involvement of PAKs within several different oncogenic signalling pathways, suggests their potential as therapeutic targets in cancer (Radu et al., 2014). Consequently, interest in the discovery of PAK inhibitors has increased, and several small molecule antagonists have been identified. For example, ATP-competitive PAK inhibitors have been developed including the pan-PAK inhibitor, PF-3758309, and the group I specific inhibitor, FRAX-597 (Chow et al., 2012). They both show effectiveness against skin cancer in vivo (Chow et al., 2012); however, PF-3758309 is limited by its pharmacological properties, and both inhibit off-target kinases (Radu et al., 2014).

Deacon et al discovered the allosteric PAK inhibitor IPA3 (2,29- dihydroxy-1,19-dinaphthyldisulfide), which targets the PAK auto-inhibitory domain (Deacon et al., 2008). IPA3 selectively inhibits group I PAKs, with greatest efficacy against PAK1;
but is not able to inhibit pre-activated PAKs (Deacon et al., 2008). A disadvantage of IPA3 is the continuous reduction of its disulphide bond, which may lead to inactivation in the intracellular environment, and limits its use clinically (Deacon et al., 2008; Radu et al., 2014).

In addition to the newly discovered PAK inhibitors, drugs in current clinical use may exhibit PAK inhibitory activity, and therefore, might be suitable for drug repositioning. For example, the anti-inflammatory 5-amino salicylic acid, mesalazine, used in the treatment of ulcerative colitis, has been shown to inhibit PAK1 in colonic epithelial cells resulting in increased cell adhesion (Khare et al., 2013). Oakley et al have previously investigated the effects of the mesalazine precursor, sulfasalazine, in HSC apoptosis and liver fibrosis (Oakley et al., 2005). Although they detected increased HSC apoptosis and improved liver fibrosis resolution in sulfasalazine treated mice, they did not detect any effect of mesalazine on HSC apoptosis (Oakley et al., 2005). However, PAK-specific inhibition, and effects on other fibrogenic properties of activated HSCs, have yet to be investigated.
4.2 **Aims**

In this chapter our specific aims were:

1. **Identify downstream mediators of Itgb1 signalling in HSC activation**

To do this we carried out a microarray analysis comparing the gene expression of Itgb1-null and control activated HSCs, and interrogated the dataset by cluster analysis using online bioinformatics tools.

2. **Analyse expression of group I Paks in activated HSCs**

3. **Determine the effects of group I Pak inhibition on HSC activation *in vitro*, and liver fibrosis *in vivo***

To do this, we used siRNA-mediated gene silencing and pharmacological inhibition with IPA3.
4.3 Results

4.3.1 Gene Expression Microarray Comparing Itgb1-null and Control HSCs

To identify downstream mediators of Itgb1 signalling in HSC activation, we carried out a microarray analysis comparing the gene expression profiles of Itgb1-null with control HSCs.

HSCs were extracted from Itgb1<sup>fl/fl</sup> CreER<sup>+</sup> mice and divided into three: RNA was isolated from quiescent HSCs on the day of extraction; Itgb1-null and control HSCs were cultured for eight days with the addition of tamoxifen or vehicle control for the first 48 hours (Figure 4.1A). The whole experiment was repeated to generate a biological replicate. Efficiency of Itgb1 mRNA depletion in tamoxifen-treated HSCs was assessed by qPCR. We detected a reduction in Itgb1 mRNA by 72% and 83% in the tamoxifen-treated HSCs (Figure 4.1B). RNA quality was assessed by Agilent Bioanalyser prior to performing the microarray analysis.

Principal component analysis demonstrated that the greatest differences in gene expression in Itgb1-null and control HSCs were between HSC groups, rather than between biological replicates (Figure 4.1C).
Figure 4.1 Preparation of samples for gene expression microarray

A. HSCs were extracted from Itgb1$^{fl/fl}$ CreER+ mice and divided into three: RNA was isolated from Q HSCs on the day of extraction; Itgb1-null and control HSCs were cultured for eight days with the addition of tamoxifen or vehicle control for the first 48 hours.

B. Efficiency of Itgb1 mRNA depletion in tamoxifen-treated HSCs was assessed by quantitative PCR. We detected a reduction in Itgb1 mRNA by 72% and 83% in the tamoxifen-treated HSCs. PCR quantification was normalised to β-actin and GusB.

C. Principal component analysis demonstrated that the greatest differences in gene expression in Itgb1-null and control HSCs were between HSC groups, rather than between biological replicates.
Changes in gene expression were compared between activated control and quiescent HSCs; and Itgb1-null and activated control HSCs. The top ten up- and downregulated genes are listed in tables 4.1 – 4.4. Reassuringly, Itgb1 was one of the top ten downregulated genes in Itgb1-null HSCs (Table 4.4).

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Fold Change A v Q</th>
<th>Regulation A v Q</th>
<th>Gene Description</th>
</tr>
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<tbody>
<tr>
<td>Col11a1</td>
<td>86.8</td>
<td>Up</td>
<td>collagen, type XI, alpha 1</td>
</tr>
<tr>
<td>Lox</td>
<td>73.8</td>
<td>Up</td>
<td>lysyl oxidase</td>
</tr>
<tr>
<td>Caica</td>
<td>68.9</td>
<td>Up</td>
<td>calcitonin/calcitonin-related polypeptide, alpha</td>
</tr>
<tr>
<td>Timp1</td>
<td>67.6</td>
<td>Up</td>
<td>tissue inhibitor of metalloproteinase 1</td>
</tr>
<tr>
<td>Hs5st2</td>
<td>62.3</td>
<td>Up</td>
<td>heparan sulfate 6-O-sulfotransferase 2</td>
</tr>
<tr>
<td>Cdh2</td>
<td>61.9</td>
<td>Up</td>
<td>cadherin 2</td>
</tr>
<tr>
<td>Col12a1</td>
<td>58.4</td>
<td>Up</td>
<td>collagen, type XII, alpha 1</td>
</tr>
<tr>
<td>Col11a1</td>
<td>54.8</td>
<td>Up</td>
<td>collagen, type XI, alpha 1</td>
</tr>
<tr>
<td>Fam110c</td>
<td>54.0</td>
<td>Up</td>
<td>family with sequence similarity 110, member C</td>
</tr>
<tr>
<td>Plod2</td>
<td>51.2</td>
<td>Up</td>
<td>procollagen lysine, 2-oxoglutarate 5-dioxygenase 2</td>
</tr>
</tbody>
</table>

Table 4.1 Top 10 upregulated genes in activated (A) versus quiescent (Q) HSCs

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Fold Change A v Q</th>
<th>Regulation A v Q</th>
<th>Gene Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amy2a1/2/3/4/5</td>
<td>2069.0</td>
<td>Down</td>
<td>amylase 2a1/2/3/4/5/</td>
</tr>
<tr>
<td>Mup1</td>
<td>1907.2</td>
<td>Down</td>
<td>major urinary protein</td>
</tr>
<tr>
<td>Cela3b</td>
<td>1492.2</td>
<td>Down</td>
<td>chymotrypsin-like elastase family, member 3B</td>
</tr>
<tr>
<td>Cips</td>
<td>1083.3</td>
<td>Down</td>
<td>colipase, pancreatic</td>
</tr>
<tr>
<td>Try4 / 5</td>
<td>880.3</td>
<td>Down</td>
<td>trypsin 4 / 5</td>
</tr>
<tr>
<td>Igj</td>
<td>692.7</td>
<td>Down</td>
<td>immunoglobulin joining chain</td>
</tr>
<tr>
<td>Serpina1a</td>
<td>676.9</td>
<td>Down</td>
<td>serine (or cysteine) peptidase inhibitor, clade A, member 1A</td>
</tr>
<tr>
<td>Fabp1</td>
<td>645.1</td>
<td>Down</td>
<td>fatty acid binding protein 1, liver</td>
</tr>
<tr>
<td>Cpb1</td>
<td>640.5</td>
<td>Down</td>
<td>carboxypeptidase B1 (tissue)</td>
</tr>
<tr>
<td>Cela2a</td>
<td>636.6</td>
<td>Down</td>
<td>chymotrypsin-like elastase family, member 2A</td>
</tr>
</tbody>
</table>

Table 4.2 Top 10 downregulated genes in activated (A) versus quiescent (Q) HSCs
### Table 4.3 Top 10 upregulated genes in Itgb1-null versus control activated HSCs

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Fold Change</th>
<th>Regulation</th>
<th>Gene Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cxcl5</td>
<td>5.3</td>
<td>Up</td>
<td>chemokine (\text{C-C superfamily}) ligand 5</td>
</tr>
<tr>
<td>Scn3a</td>
<td>5.2</td>
<td>Up</td>
<td>sodium channel, voltage-gated, type III, alpha</td>
</tr>
<tr>
<td>Serpina3g</td>
<td>4.3</td>
<td>Up</td>
<td>serine (or cysteine) peptidase inhibitor, clade A, member 3G</td>
</tr>
<tr>
<td>Car8</td>
<td>4.1</td>
<td>Up</td>
<td>carbonic anhydrase 8</td>
</tr>
<tr>
<td>Cc11</td>
<td>3.7</td>
<td>Up</td>
<td>chemokine (\text{C-C superfamily}) ligand 11</td>
</tr>
<tr>
<td>Aldh1a3</td>
<td>3.3</td>
<td>Up</td>
<td>aldehyde dehydrogenase family 1, subfamily A</td>
</tr>
<tr>
<td>Angpt2</td>
<td>3.1</td>
<td>Up</td>
<td>angiopoietin 2</td>
</tr>
<tr>
<td>Cdh1</td>
<td>3.0</td>
<td>Up</td>
<td>cell adhesion molecule with homology to L1CAM</td>
</tr>
<tr>
<td>Emscn</td>
<td>3.0</td>
<td>Up</td>
<td>endomucin</td>
</tr>
<tr>
<td>Khl4</td>
<td>3.0</td>
<td>Up</td>
<td>kelch-like 4 (Drosophila)</td>
</tr>
</tbody>
</table>

### Table 4.4 Top 10 downregulated genes in Itgb1-null versus control activated HSCs

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Fold Change</th>
<th>Regulation</th>
<th>Gene Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stmn2</td>
<td>4.0</td>
<td>Down</td>
<td>stathmin-like 2</td>
</tr>
<tr>
<td>Gabra4</td>
<td>3.3</td>
<td>Down</td>
<td>gamma-aminobutyric acid (GABA) A receptor, subunit alpha 4</td>
</tr>
<tr>
<td>Col11a1</td>
<td>3.2</td>
<td>Down</td>
<td>collagen, type XI, alpha 1</td>
</tr>
<tr>
<td>Itgb1</td>
<td>3.0</td>
<td>Down</td>
<td>integrin beta 1 (fibronectin receptor beta)</td>
</tr>
<tr>
<td>Fam19a2</td>
<td>2.9</td>
<td>Down</td>
<td>family with sequence similarity 19, member A2</td>
</tr>
<tr>
<td>Bicd1</td>
<td>2.8</td>
<td>Down</td>
<td>bicaudal D homolog 1 (Drosophila)</td>
</tr>
<tr>
<td>Emx2</td>
<td>2.8</td>
<td>Down</td>
<td>empty spiracles homolog 2 (Drosophila)</td>
</tr>
<tr>
<td>Plac1</td>
<td>2.8</td>
<td>Down</td>
<td>placental specific protein 1</td>
</tr>
<tr>
<td>Zbtb8b</td>
<td>2.7</td>
<td>Down</td>
<td>zinc finger and BTB domain containing 8b</td>
</tr>
<tr>
<td>Dox4</td>
<td>2.7</td>
<td>Down</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 4</td>
</tr>
</tbody>
</table>
4.3.2 Functional Clustering Analysis of Gene Expression Microarray

The data set was filtered using parameters of p value < 0.05 and fold change > 1.5 in the Itgb1-null versus control activated HSC group. This generated a list of differentially expressed genes (915 probesets), which were then organised into seven clusters based on similarity of expression profile across the dataset (quiescent, activated control and Itgb1-null HSCs) using a $\kappa$-means clustering algorithm (Figure 4.2A).

In the previous chapter, we concluded that Itgb1-null HSCs have a less fibrotic, and thus, more quiescent-like, phenotype. Therefore, we were particularly interested in genes which were upregulated between activated control and quiescent HSCs, but downregulated between Itgb1-null and activated control HSCs, and vice versa. Accordingly, clusters 1 and 4 were rejected as the direction of change in gene expression was discordant between Itgb1-null and quiescent HSCs (Figure 4.2A). The remaining five clusters were analysed using the online bioinformatics resource Database for Annotation, Visualisation and Integrated Discovery (DAVID) version 6.7. Functional annotation clustering was carried out with medium stringency; functional clusters with an enrichment score > 2 were selected, and are proportionately represented by enrichment score (Figure 4.2B). Analysis of clusters 5 and 6 did not reveal any functional annotation clusters with an enrichment score > 2.
Figure 4.2 Functional clustering analysis of gene expression microarray  

A. Heat map showing significant gene expression changes (p <0.05; fold change >1.5) in Itgb1-null versus control activated HSCs. Quiescent HSCs indicated for comparison and similarity with Itgb1-null HSCs. Seven gene clusters were identified by similarity of expression change. Colour indicates genes upregulated (red), downregulated (blue) and intermediate regulation (yellow). Clusters 1 and 4 were rejected as the direction of change in gene expression was discordant between Itgb1-null and Q HSCs.  

B. Gene Ontology analysis of clusters 1, 3, 5, 6 and 7, with enrichment score > 2; there was no over-representation in clusters 5 and 6. Charts show proportional representation of cellular functions by enrichment score.
Functional annotation clustering and Gene Ontology analysis revealed that cluster 2 was over-represented with genes involved in proliferation, wound healing and the cell membrane. In addition, cluster 3 was over-represented by genes involved in contraction, the cytoskeleton and ECM. Finally, cluster 7 was over-represented by genes involved in adhesion, proliferation, migration, cell surface, plasma membrane, leucocyte activation and proliferation, blood vessel development, response to wounding, immune response, response to virus and protein dimerization. (Figure 4.2B)

Thus, our unbiased analysis of statistically significant array targets identified a cluster of genes involved in the contractile cytoskeleton and ECM. This was consistent with our earlier findings, that Itgb1-null HSCs have a defective actomyosin cytoskeleton and are less contractile (Figures 3.7C, 3.9B, 3.11B and C).

Genes identified in cluster 3 and associated with contraction, the cytoskeleton and ECM are listed in tables 4.5 - 4.7.

<table>
<thead>
<tr>
<th>Contraction Associated Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAP, adenylate cyclase-associated protein 2 (yeast)</td>
</tr>
<tr>
<td>FERM, RhoGEF and pleckstrin domain protein 2</td>
</tr>
<tr>
<td>FH2 domain containing 1</td>
</tr>
<tr>
<td>LIM domain binding 3</td>
</tr>
<tr>
<td>PDZ and LIM domain 3</td>
</tr>
<tr>
<td>PDZ and LIM domain 5</td>
</tr>
<tr>
<td>actin, alpha 2, smooth muscle, aorta</td>
</tr>
<tr>
<td>actin, gamma 2, smooth muscle, enteric</td>
</tr>
<tr>
<td>actin-binding Rho activating protein</td>
</tr>
<tr>
<td>anillin, actin binding protein</td>
</tr>
<tr>
<td>calponin 1</td>
</tr>
<tr>
<td>desmin</td>
</tr>
<tr>
<td>junctophilin 1</td>
</tr>
<tr>
<td>myosin, heavy polypeptide 2, skeletal muscle, adult; myosin, heavy polypeptide 1, skeletal muscle, adult</td>
</tr>
<tr>
<td>myosin, light polypeptide 9, regulatory</td>
</tr>
<tr>
<td>syncoilin</td>
</tr>
<tr>
<td>transgelin</td>
</tr>
<tr>
<td>tropomyosin 1, alpha</td>
</tr>
<tr>
<td>tropomyosin 2, beta</td>
</tr>
</tbody>
</table>

Table 4.5 Contraction associated genes identified from gene expression cluster 3
<table>
<thead>
<tr>
<th>Cytoskeleton Associated Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD-2 associated protein</td>
</tr>
<tr>
<td>ESF1, nucleolar pre-rRNA processing protein, homolog (S. cerevisiae)</td>
</tr>
<tr>
<td>FERM, RhoGEF and pleckstrin domain protein 2</td>
</tr>
<tr>
<td>LIM domain binding 3</td>
</tr>
<tr>
<td>PDZ and LIM domain 3</td>
</tr>
<tr>
<td>actin, alpha 2, smooth muscle, aorta</td>
</tr>
<tr>
<td>actin, gamma 2, smooth muscle, enteric</td>
</tr>
<tr>
<td>actin-binding Rho activating protein</td>
</tr>
<tr>
<td>alpha thalassemia/mental retardation syndrome X-linked homolog (human)</td>
</tr>
<tr>
<td>anillin, actin binding protein</td>
</tr>
<tr>
<td>centrosomal protein 55</td>
</tr>
<tr>
<td>cyclin-dependent kinase inhibitor 2A</td>
</tr>
<tr>
<td>desmin</td>
</tr>
<tr>
<td>family with sequence similarity 110, member C</td>
</tr>
<tr>
<td>heat shock protein family, member 7 (cardiovascular)</td>
</tr>
<tr>
<td>kinesin family member 23</td>
</tr>
<tr>
<td>microtubule associated monooxygenase, calponin and LIM domain containing 2</td>
</tr>
<tr>
<td>myosin, heavy polypeptide 2, skeletal muscle, adult; myosin, heavy polypeptide 1, skeletal muscle, adult</td>
</tr>
<tr>
<td>myosin, light polypeptide 9, regulatory</td>
</tr>
<tr>
<td>ninin-1-like</td>
</tr>
<tr>
<td>non-protein coding RNA 153</td>
</tr>
<tr>
<td>nuclear fragile X mental retardation protein interacting protein 2</td>
</tr>
<tr>
<td>phosphodiesterase 4D interacting protein (myomegalin)</td>
</tr>
<tr>
<td>syncofilin</td>
</tr>
<tr>
<td>transgelin</td>
</tr>
<tr>
<td>tropomyosin 1, alpha</td>
</tr>
<tr>
<td>tropomyosin 2, beta</td>
</tr>
</tbody>
</table>

Table 4.6 Cytoskeleton associated genes identified from gene expression cluster 3
<table>
<thead>
<tr>
<th>ECM Associated Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 12</td>
</tr>
<tr>
<td>arylsulfatase J</td>
</tr>
<tr>
<td>collagen triple helix repeat containing 1</td>
</tr>
<tr>
<td>collagen, type I, alpha 1</td>
</tr>
<tr>
<td>collagen, type IV, alpha 6</td>
</tr>
<tr>
<td>collagen, type V, alpha 2</td>
</tr>
<tr>
<td>collagen, type XI, alpha 1</td>
</tr>
<tr>
<td>coxsackie virus and adenovirus receptor</td>
</tr>
<tr>
<td>cysteine rich protein 61</td>
</tr>
<tr>
<td>fibronectin 1</td>
</tr>
<tr>
<td>growth arrest specific 1</td>
</tr>
<tr>
<td>insulin-like growth factor binding protein 3</td>
</tr>
<tr>
<td>integrin, alpha 10</td>
</tr>
<tr>
<td>matrilin 2</td>
</tr>
<tr>
<td>met proto-oncogene</td>
</tr>
<tr>
<td>myosin, light polypeptide 9, regulatory</td>
</tr>
<tr>
<td>nephronectin</td>
</tr>
<tr>
<td>osteoglycin</td>
</tr>
<tr>
<td>p21 protein (Cdc42/Rac)-activated kinase 3</td>
</tr>
<tr>
<td>placental specific protein 1</td>
</tr>
<tr>
<td>protease, serine, 23</td>
</tr>
<tr>
<td>sema domain, immunoglobulin domain (ig), short basic domain, secreted, (semaphorin) 3A</td>
</tr>
<tr>
<td>similar to fibrillin 2; fibrillin 2</td>
</tr>
<tr>
<td>sine oculis-related homeobox 4 homolog (Drosophila)</td>
</tr>
<tr>
<td>tissue inhibitor of metalloproteinase 3</td>
</tr>
<tr>
<td>twisted gastrulation homolog 1 (Drosophila)</td>
</tr>
<tr>
<td>versican</td>
</tr>
</tbody>
</table>

Table 4.7 ECM associated genes identified from gene expression cluster 3
4.3.3 Ingenuity Pathway Analysis of cluster 3 genes

In the previous chapter, we postulated that disruption of the contractile cytoskeleton in HSCs might be an effective therapeutic strategy in liver fibrosis. Therefore, as downstream targets of Itgb1 and potential regulators of the contractile cytoskeleton, genes identified in cluster 3 were taken forward for Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com).

Reassuringly, Ingenuity Pathway Analysis revealed that ‘Hepatic fibrosis - HSC activation’ was the top canonical pathway (Table 4.8). However, having already identified that Itgb1-null HSCs are less migratory (Figure 3.6), we narrowed our focus to the pathway ‘Regulation of actin based motility by Rho’ (Figure 4.3).

This highlighted that actin and MLC/Myl9 were downregulated in Itgb1-null HSCs, consistent with our previous findings; and revealed a potential role for group I PAKs upstream of actomyosin assembly and contraction (Figure 4.3 and Tables 4.5 – 4.7).

Group I PAKs are well described regulators of the actin cytoskeleton (Bokoch, 2003), and have been shown to positively regulate contraction by phosphorylation of MRLC, such as MYL9 (Kiosses et al., 1999; Sells et al., 1999; Van Eyk et al., 1998; Zeng et al., 2000). Moreover, they have been implicated in mechanosensitive signalling in the pathogenesis of kidney fibrosis (Chen et al., 2013). However, it is unclear whether PAKs play a role in liver fibrosis. Therefore, we decided to investigate the group I PAKs in HSC activation and liver fibrosis.
Table 4.8 Top 20 canonical pathways represented by genes in Cluster 3

Ingenuity Pathway Analysis was used to determine the top canonical pathways represented by genes listed in cluster 3. Pathways were ranked by the negative log of the p-value calculated by Fisher’s exact test to determine enrichment.
Ingenuity Pathway Analysis revealed that genes listed in cluster 3 were over represented in the canonical signalling pathway ‘Regulation of actin based motility by Rho’. Genes are outlined in a colour corresponding to their regulation in Itgb1-null compared with control A HSCs; and filled in with a colour corresponding to their regulation in A compared with Q HSCs, downregulated (green); upregulated (red). Genes outlined in black were not identified in cluster 3. Blue arrows highlight a potential signalling pathway Itgb1 and the contractile cytoskeleton. The signalling pathway was generated through the use of QIAGEN’s Ingenuity Pathway Analysis (IPA®, QIAGEN, Redwood City, www.qiagen.com/ingenuity).
4.3.4 Loss of Itgb1 is Associated with Reduced Levels of Pak1 and Pak3 Proteins

We determined the protein levels of Paks 1–3 in quiescent, activated and Itgb1-null HSCs (Figure 4.4). Pak1 was highly upregulated in activated HSCs, with levels increasing by 14 fold above those in quiescent HSCs. Loss of Itgb1 was associated with a 74% reduction in Pak1 protein. Levels of Pak2 protein did not appear to alter between quiescent and activated HSCs, and therefore, we did not investigate Pak2 further. Pak3 was upregulated in activated HSCs, but to a lesser extent than Pak1; loss of Itgb1 resulted in a 46% reduction in Pak3 protein.

![Graph showing Pak1-3 protein levels in activated and Itgb1-null HSCs](image)

Figure 4.4 Pak1-3 protein levels in activated and Itgb1-null HSCs

Quantification and representative immunoblots showing increased protein levels of Pak1 and 3 in A rHSCs relative to Q rHSCs (A), and a reduction in both proteins following loss of Itgb1 (B). Immunoblotting quantification was standardised to β-actin; n=3. Error bars are SEM. Significance was determined by two tailed Student T test, *p<0.05; **p<0.01
4.3.5 Pak1 and Pak3 Deficiency is Associated with Reduced Expression of Fibrotic Markers in Activated HSCs

Next, we used siRNA to investigate the effects of Pak1 and Pak3 depletion in activated HSCs. Using siRNA targeted against Pak1 mRNA we achieved a robust reduction in Pak1 protein in activated HSCs. We detected that a 74% reduction in Pak1 protein resulted in a 63% decrease in Col1 and a 38% decrease in Sox9 (Figure 4.5A). Although we did not detect a decrease in αSma, we found a significant reduction in phosphorylated Myl9 (pMyl9) (Figure 4.5A). Myl9 is a MRLC, and phosphorylation results in its activation and generation of contractile forces. We did not detect a change in Itgb1. These findings suggest that in activated HSCs, Pak1 is important in the regulation of contraction and Col1 production.

Pak3 siRNA mediated knockdown was also associated with a significant, but less dramatic, reduction in Col1. A 63% decrease in Pak3 protein resulted in a 51% reduction in Col1 (Figure 4.5B).

These results suggest that Pak1 and, to a lesser extent, Pak3 may play a role in regulating the fibrotic response of activated HSCs. Our observation that pMyl9 is reduced in Pak1 depleted HSCs suggest that this may include regulation of HSC contractility.
Figure 4.5 siRNA-mediated Pak1 and Pak3 knockdown in activated HSCs is associated with a reduction in fibrotic markers

Quantification and representative immunoblots showing reduced relative protein levels of fibrotic markers following abrogation of Pak1 (A) and Pak3 (B) using siRNA in AmHSCs relative to control scrambled siRNA. A single siRNA was used against Pak1, and multiple siRNA against Pak3. All immunoblotting was normalised to β-actin. Significance was determined by two-tailed Student T test, * p<0.05; ** p<0.01; *** p<0.001. n ≥ 3. Error bars are SEM.
4.3.6 The Group I PAK Inhibitor IPA3 Decreases Expression of Fibrotic Proteins in Activated HSCs

Next, we used the group I PAK antagonist IPA3, to determine if pharmacological inhibition of Pak1 and Pak3 in activated HSCs results in a reduction in pro-fibrotic markers, similar to that observed in Pak1 and Pak3-depleted HSCs.

IPA3 causes a conformational change in pre-activated PAK, which renders it catalytically inactive and resistant to phosphorylation (Deacon et al., 2008). It is specific for group I PAKs, but is primarily effective against PAK1 (Deacon et al., 2008).

Based on the knowledge that 30μM IPA3 inhibits Pak kinase activity in mouse embryonic fibroblasts (Deacon et al., 2008), we initially tested a concentration gradient ranging from 7.5μM to 30μM in mouse activated HSCs, and analysed Col1 protein levels as a read-out.

We detected a robust, concentration-dependent decrease in Col1 levels in IPA3 treated activated mouse HSCs (Figure 4.6A). Based on the robust reduction in Col1 (81%), and less potential for toxicity than with higher concentrations, we selected the 15μM concentration to test in a second model (activated rat HSCs). We observed a similar reduction in Col1 in IPA3 treated activated rat HSCs, alongside a significant but less striking, reduction in Sox9 (Figure 4.6B).
Figure 4.6 The group I PAK inhibitor IPA3 attenuates expression of fibrotic markers in activated HSCs

**A.** Quantification and representative immunoblots of Col1 protein levels in A mHSCs treated with 7.5, 15 or 30μM IPA3 relative to DMSO vehicle control. n = 2.

**B.** Quantification and representative immunoblots for fibrotic markers in A rHSCs treated with 15μM IPA3 relative to DMSO vehicle control. n = 3. Immunoblotting quantification was standardised to β-actin. Error bars are SEM. Significance was determined by two tailed Student T test, * p<0.05.
4.3.7 Investigating IPA3 in *In Vivo* Models of Liver Fibrosis

Based on our finding that treatment with IPA3 resulted in a significant reduction in Col1 in activated HSCs *in vitro*, we hypothesised that IPA3 would attenuate liver fibrosis *in vivo*.

The clinical utility of IPA3 is thought to be limited by its disulphide bond, which is presumably reduced under physiological conditions (Yi *et al.*, 2010). However, IPA3 has been investigated, and shown to be effective, in *in vivo* animal models.

Wong *et al* treated hepatocellular carcinoma zenografted nude mice with 2mg/kg or 4mg/kg IPA3, or DMSO vehicle control, administered three-times weekly by intraperitoneal injection over four weeks (Wong *et al.*, 2013). IPA3 treatment was well tolerated with no significant weight loss. However, there was a dramatic reduction in tumor size in the 4mg/kg treatment group.

Likewise, Hoover *et al* showed a significant response to IPA3 *in vivo*. They treated mice with nebulized 5mM IPA3 or DMSO vehicle control prior to bronchial challenge with inhaled acetylcholine, and demonstrated a significant reduction in airways reactivity in IPA3 treated mice (Hoover *et al.*, 2012).

Therefore, we decided to investigate the effect of IPA3 in liver fibrosis *in vivo*. We used two models to induce liver fibrosis by different mechanisms. Firstly, repetitive administration of CCl₄, which causes iterative hepatocellular damage, and results in an inflammatory response and fibrosis. Secondly, bile duct ligation (BDL), which causes cholestatic injury, and results in bile duct proliferation, biliary infarcts, portal inflammation, and fibrosis (Kountouras *et al.*, 1984).

Based on the work of Wong *et al*, we used a dose of 4mg/kg IPA3, administered three-times weekly by intraperitoneal injection (Wong *et al.*, 2013). To determine whether IPA3 was an effective therapeutic agent in established liver fibrosis, we initiated IPA3 treatment four weeks after commencing CCl₄, or one week after BDL (Figure 4.7).

IPA3 treatment was well tolerated and was not associated with any weight loss (Figure 4.8B and C).
Figure 4.7 Timecourse of IPA3 injections in models of liver fibrosis
A CCl₄ (or olive oil vehicle control) was administered twice weekly by intraperitoneal injection for 8 weeks. In the final four weeks, 4mg/kg IPA3 (or DMSO vehicle control) was administered three times per week by intraperitoneal injection. B Cholestatic liver fibrosis was induced by BDL (or sham control) for two weeks. In the final week, 4mg/kg IPA3 (or DMSO vehicle control) was administered three times by intraperitoneal injection.
Figure 4.8 Characterisation of animal weight following IPA3 treatment in models of liver fibrosis

Final mouse weights are expressed as a percentage of their initial weights. IPA3 treatment was not associated with weight loss compared to DMSO control in CCl₄ or BDL models of liver fibrosis.
4.3.7.1 CCl₄ Induced Liver Fibrosis

4.3.7.1.1 Serum Liver Enzymes

CCl₄ is a hepatotoxin, and repeated administration results in iterative hepatocellular damage and necrosis, leading to an inflammatory response and fibrosis. Serum alanine aminotransferase (ALT) is commonly used as a biomarker for hepatocyte damage and necrosis. ALT is a cytosolic enzyme which catalyses the transfer of alanine to the keto group of α-ketoglutaric acid to form pyruvic acid. This reaction is important in gluconeogenesis. ALT is predominantly expressed in the liver; however, it is also present in the heart, kidney and skeletal muscles. Serum ALT levels are quantified by measuring the catalytic activity, and are expressed as units/litre (U/l).

In humans, serum ALT normally ranges between 5 – 40 U/l. Elevated serum ALT may indicate hepatocellular damage, however, there are alternative causes such as myopathy. Serum ALT > 1000 U/l generally indicates acute hepatocellular damage resulting from toxins, such paracetamol, acute viral hepatitis or ischaemia.

In this study, we did not find any difference in ALT levels between groups (Figure 4.9). This was surprising because CCl₄ causes hepatocyte injury, and therefore, one would predict that ALT levels would be elevated in mice receiving CCl₄. Indeed, others have shown that serum ALT is elevated by 10³ orders of magnitude in mice treated with CCl₄ (Henderson et al., 2013; Saiman et al., 2014). However, this was apparent when measurements were taken 24 to 36 hours following CCl₄ injection. Therefore, it is likely that the elevated ALT reflects the acute CCl₄-induced hepatocellular injury, and not necessarily any ongoing hepatocyte damage arising from the fibrotic process itself. In line with this, Saiman et al demonstrated that 72 hours post-CCl₄ injection, serum ALT levels have almost normalised (Saiman et al., 2014).

In this study, serum ALT was measured 96 hours following the final CCl₄ injection, and therefore, we missed the window of opportunity to detect an elevation in ALT. Primarily, this would be important to determine whether IPA3 causes hepatocellular injury itself, or modulates the effect of CCl₄-induced hepatocyte injury.

Alkaline phosphatase (ALP) is also routinely measured as part of the biochemical liver profile. The majority of serum ALP is derived from the liver or bone, however,
it is also present in the placenta, ileal mucosa and kidney. In the liver, ALP is expressed in the cytosol of cells associated with the sinusoidal and canalicular membranes. It is predominantly elevated in cholestasis due to increased synthesis; however, more minor elevations are sometimes seen in association with hepatocellular injury. In this study, we did not detect any difference in ALP levels between CCl$_4$ treated and control mice (Figure 4.9).

Figure 4.9 Characterisation of serum liver enzymes in CCl$_4$-induced liver fibrosis
Serum alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were measured 96 hours after the final CCl$_4$ injection. There was no difference in ALT and ALP levels between treatment groups.
4.3.7.1.2 Collagen Quantification

We utilised digital image analysis to quantify collagen staining in liver sections from control and CCl$_4$-treated mice. Liver sections were stained with picro-sirius red, which colours collagen red and the cytoplasm yellow. The area of red was calculated as a percentage of the total area, to give a measurement of collagen content. As expected, by histological quantification, we found increased collagen in livers from CCl$_4$-treated mice compared with controls (Figure 4.10A). Qualitatively, we detected expansion of the portal areas with portal to portal, and portal to central bridging, consistent with Ishak grade 4 fibrosis (Figure 4.10B).

We hypothesised that IPA3 would attenuate liver fibrosis, and by histological quantification, we were able to show a significant reduction in collagen content in IPA3 treated mice with CCl$_4$-induced liver fibrosis (Figure 4.10A).

As an additional method of collagen quantification, we measured the hydroxyproline content of CCl$_4$-induced fibrotic livers (Figure 4.10C). There was a trend towards a reduction in the hydroxyproline content of IPA3 treated livers compared with controls; however, this did not reach statistical significance.
Figure 4.10 IPA3 attenuates CCl₄-induced liver fibrosis in mice

Fibrosis induced for 4 weeks with twice weekly injections of olive oil or CCl₄, followed by a further 4 weeks of oil or CCl₄ injections plus control DMSO or IPA3 treatment. A and B. Sirius red (SR) staining (collagen deposition; red) and quantification in liver tissue. A. Morphometric quantification of SR staining in IPA3 treated CCl₄ animals versus controls. B. Representative images of SR staining of liver tissue; olive oil control (top) or CCl₄ induced fibrosis (bottom) following treatment with DMSO control (left) or IPA3 (right). Magnification x5. C. Hydroxyproline quantification in CCl₄-induced fibrotic liver tissue in DMSO control versus IPA3 treatment. A non-significant reduction in hydroxyproline shown following IPA3 treatment. (n = 5 in olive oil + control; n = 3 in olive oil + IPA3; n = 5 in CCl₄ + control; n = 4 in CCl₄ + control group). Significance determined by two tailed Student T test. ** p<0.01.
4.3.7.2 BDL Induced Liver Fibrosis

We next investigated whether IPA3 would have a similar effect in a model of biliary fibrosis, BDL. BDL results in cholestatic injury, and therefore, the aetiology of liver fibrosis is different to that induced by CCl₄.

4.3.7.2.1 Liver to Body Weight Ratio

We detected a significant increase in the liver to body weight ratio in BDL mice compared with controls (Figure 4.11A). In comparison, we did not detect any difference in liver to body weight in CCl₄-induced liver fibrosis (Figure 4.11B).

An increase in liver to body weight ratio following BDL is widely reported, and is presumably due to the biliary duct proliferation, which is prominent following BDL (Bridle et al., 2009; Gäbele et al., 2009; Moal et al., 2006; Song et al., 2011). Others have reported attenuation in the increase of liver to body weight ratio following BDL with experimental treatments for liver fibrosis (Bridle et al., 2009). However, we did not detect any difference in the liver to body weight ratio between IPA3-treated and control BDL mice (Figure 4.11A).
Figure 4.11 Characterisation of liver weight following IPA3 treatment in models of liver fibrosis

Liver and final body weights were measured at the time of sacrifice. A and B. BDL was associated with a significant increase in liver to body weight ratio. CCl₄-induced liver fibrosis was not associated with any change in liver to body weight ratio. IPA3 treatment was not associated with a reduction in liver to body weight ratio in either BDL or CCl₄-induced liver fibrosis. (n = 5 in each of the sham groups; n = 7 in BDL + control; n = 8 in BDL + IPA3; n = 5 in olive oil + control; n = 3 in olive oil + IPA3; n = 5 in CCl₄ + control; n = 4 in CCl₄ + control group). Significance was determined by two-tailed Student T test; *** p < 0.001.
4.3.7.2.2 Serum Bilirubin and Liver Enzymes

Bilirubin is a product of haem catabolism; unconjugated, it is water insoluble and is transported in the plasma bound to albumin. It is then taken up by hepatocytes, which glucuronidate it, thereby making it water soluble, and excrete it into bile. Serum bilirubin is elevated in cholestatic disease, and also with severe hepatocellular damage.

As expected, we detected a marked increase in serum bilirubin in BDL mice compared with controls (Figure 4.12). We also detected an increase in ALP in the BDL mice (Figure 4.12). However, there was no difference in bilirubin or liver enzymes between IPA3-treated BDL mice and controls (Figure 4.12).
Figure 4.12 Characterisation of serum bilirubin and liver enzymes in BDL-induced liver fibrosis

Mice were sacrificed two weeks after BDL or sham operation, and serum bilirubin, ALT and ALP were analysed at the Manchester Royal Infirmary. Bilirubin was below the lower limit of detection in sham mice. BDL was associated with increased bilirubin and ALP. IPA3 treatment was not associated with any difference in bilirubin or liver enzymes compared with DMSO controls. (n = 5 in each of the sham groups; n = 7 in BDL + control; n = 8 in BDL + IPA3)
4.3.7.2.3 Collagen Quantification

We next quantified collagen in the BDL and control liver tissues as a measure of fibrosis. We used both digital image analysis to quantify picro-sirius red staining of liver sections, and hydroxyproline content. In contrast to CCl₄-induced fibrosis, we were unable to demonstrate a significant reduction in BDL-induced liver fibrosis between IPA3-treated and control mice (Figure 4.13).
Figure 4.13 IPA3 treatment does not attenuate BDL-induced liver fibrosis

Animals were maintained for 2 weeks following BDL (or sham operative control) to induce liver fibrosis. In the second week animals were treated with IPA3 or DMSO vehicle control A and B. Sirius red (SR) staining (collagen deposition; red) and quantification in liver tissue. A. Morphometric quantification of SR staining in IPA3 treated BDL animals versus controls. B. Representative images of SR staining in of liver tissue; operative control (sham; top) and BDL (bottom) following treatment with DMSO control (left) or IPA3 (right). Magnification x5. C. Hydroxyproline quantification in BDL-induced fibrotic liver tissue in DMSO control versus IPA3 treatment. No significant differences in collagen deposition or hydroxyproline content were detected between IPA3 treated and DMSO control BDL mice. (n = 5 in each of the sham groups; n = 7 in BDL + control; n = 8 in BDL + IPA3)
4.4 Discussion

The activation of HSCs is considered to be the pivotal step in liver fibrogenesis, and as such, has been the focus of efforts in the search for anti-fibrotic drug targets. In the previous chapter, we identified that Itgb1 is important in the activation of HSCs, and is required for a functioning contractile cytoskeleton and nuclear localisation of the transcriptional regulator, YAP. Moreover, we hypothesised that disrupting the pro-fibrotic Itgb1 - contractile cytoskeleton - YAP circuit in activated HSCs may represent a novel therapeutic strategy for liver fibrosis. However, global inhibition of Itgb1 is likely to be associated with significant off-target effects, and therefore, we hypothesised that identifying downstream mediators of Itgb1 signalling in activated HSCs might allow more precise targeting.

In this chapter, we identified a role for the group I PAKs downstream of Itgb1 in HSC activation, and demonstrated their potential as novel targets for the treatment of liver fibrosis.

4.4.1 Gene Expression Microarray Analysis of Itgb1-null and Control Activated HSCs

To identify downstream mediators of Itgb1 signalling in activated HSCs, we carried out a gene expression microarray analysis comparing Itgb1-null with control activated HSCs. Significant targets were grouped into clusters based on similarity of gene expression. Clusters were then analysed for enrichment of Gene Ontology terms using an online bioinformatics tool, DAVID. Using this unbiased approach, we identified a cluster of genes involved in contraction, the cytoskeleton and ECM. Reassuringly, this was consistent with our findings in the previous chapter, where we demonstrated that Itgb1-null HSCs have a defective contractile cytoskeleton and are less able to generate contraction forces in collagen gel matrices.

Moreover, we hypothesised that destabilising the contractile cytoskeleton in activated HSCs would disrupt the pro-fibrotic Itgb1 - contractile cytoskeleton - YAP circuit, and diminish their fibrotic response. Consequently, we selected this cluster of genes for further analysis using Ingenuity Pathway Analysis. Encouragingly, this revealed ‘Hepatic fibrosis – HSC activation’ as the top canonical pathway. However,
based on our earlier discovery that Itgb1-null HSCs are less migratory, we narrowed our focus to the pathway: ‘Regulation of actin based motility by Rho’.

This uncovered a potential role for the group I PAKs as effectors of Itgb1 signalling in activated HSCs. The group I PAKs are involved in cytoskeletal re-organisation and regulation of contraction, both pertinent to HSC activation (Bokoch, 2003). Furthermore, group I PAKs are implicated in the pathogenesis of cancer, and drug discovery programmes have led to the development of small molecule inhibitors (Dammann et al., 2014; Yi et al., 2010). However, relatively little is known about the role of PAKs in fibrosis. Therefore, we decided to investigate the role of group I PAKs in liver fibrosis.

4.4.2 Pak1 and Pak3 are Upregulated in Activated HSCs

The group I Paks comprise of Pak1, Pak2 and Pak3. We assayed the expression of Pak1-3 in activated HSCs, and discovered that both Pak1 and Pak3 were significantly increased in activated HSCs, whilst levels of Pak2 did not change. Moreover, we detected that both Pak1 and Pak3 were significantly reduced in Itgb1-null HSCs. This verified the role of Pak1 and Pak3 as potential downstream effectors of Itgb1 signalling in activated HSCs.

Expression of Pak1 and Pak2 has previously been described in HSCs (Bandapalli et al., 2012; Rovida et al., 2008; Zhou et al., 2009); however, to our knowledge, this is the first report of Pak3 expression in activated HSCs.

4.4.3 Inhibition of Group I Paks is Associated with Reduced Expression of Fibrotic Proteins in Activated HSCs

To determine if group I Paks are involved in HSC-mediated fibrogenesis, we investigated the effects on fibrotic protein expression of group I Pak inhibition by two methods, siRNA-mediated gene silencing and small molecule blockade. We demonstrated that depletion of Pak1 was associated with a marked reduction in Col1, the predominant constituent of the fibrotic ECM, and a significant, but less profound, reduction in the fibrosis-associated transcription factor Sox9. In addition, we were able to show a significant reduction in phosphorylated Myl9. Phosphorylation of Myl9 results in its activation, and generation of contractile forces. Therefore, it is tempting to suggest that Pak1 deficiency impairs cellular contraction, resulting in
disrupted mechanosensitive signalling and, as a consequence, reduced Col1 expression. However, this would require further investigation.

Depletion of Pak3 was associated with a significant, but less dramatic, reduction in Col1. Whilst treatment with the small molecule inhibitor IPA3, resulted in a striking reduction in Col1, greater than that observed with Pak1-depletion alone. IPA3 antagonises all three group I PAKs, and therefore, this suggests that there might be some redundancy between Pak1-3 in the regulation of Col1.

Interestingly, the group I PAKs can be linked to activation of YAP, by the tumour suppressor, Merlin. Merlin is encoded by the Neurofibromatosis 2 (Nf2) gene, and its loss of function is associated with development of nervous system tumours, predominantly schwannomas and meningiomas. Merlin-deficiency is associated with PAK activation, and treatment of schwannoma cells with IPA3 has been shown to reduce cell spreading, adhesion and membrane ruffling (Flaiz et al., 2009). In addition, inhibition of group I PAKs in Merlin-deficient fibroblasts reduces proliferation, invasiveness and fusiform morphology (Chow et al., 2010). In brain development, Merlin-deficiency is associated with increased Yap activity, resulting from both increased Yap levels and enhanced nuclear localisation (Lavado et al., 2013). Whilst in vitro, Merlin-deficiency was shown to promote Yap stability (Lavado et al., 2013). Interestingly, these effects were thought to be independent of the Hippo pathway, as no change in Yap phosphorylation was detected (Lavado et al., 2013). Instead, the study authors proposed that Merlin might regulate Yap nuclear localisation via the actin cytoskeleton (Lavado et al., 2013). Collectively, these studies support a role for group I PAKs in the activation of YAP via the actin cytoskeleton.

In addition, PAK1 has been linked to activation of the mechanosensitive transcriptional regulator, MRTF. In kidney tubular cells, PAK1 is required for nuclear translocation of MRTF and activation of the αSma promoter, in response to disruption of cellular contacts (Sebe et al., 2008). Taking into account the findings of Tian et al., who demonstrated that MRTF is required for HSC activation in vitro and liver fibrosis in vivo, it is plausible that PAK1 acts via MRTF to regulate HSC activation (Tian et al., 2014). However, if this were the case, one would expect to see
a significant reduction in \(\alpha\)Sma, a well-described target gene of MRTF, with PAK1 inhibition, which we did not detect.

More broadly, it is likely that disruption of HSC contraction would result in reduced mechanical activation of TGF-\(\beta\)1, so-called ‘extrinsic mechanotransduction’ (Henderson et al., 2013; Huang et al., 2012).

Alternatively, the reduction in Col1 in Pak1-deficient HSCs might be mediated by decreased activity of Sox9, which has been shown to regulate *Col1* transcription in activated HSCs (Piper Hanley et al., 2007).

### 4.4.4 IPA3 Attenuates CCl\(_4\)-Induced Liver Fibrosis

We next investigated if treatment with IPA3 could attenuate liver fibrosis *in vivo*. Although the clinical utility of IPA3 is thought to be limited by its disulphide bond, it has been efficacious in *in vivo* models of asthma and hepatocellular carcinoma (Hoover et al., 2012; Wong et al., 2013). Clinically, patients often present with established liver fibrosis, and therefore, we elected to study IPA3 in a therapeutic model, in which liver fibrosis was induced prior to initiating treatment with IPA3.

We detected a significant reduction in CCl\(_4\)-induced liver fibrosis in IPA3 treated mice, as determined by histological quantification of collagen staining. We used hydroxyproline content as a second method of collagen quantification in CCl\(_4\)-induced liver fibrosis, and detected a trend towards reduction in IPA3 treated mice, although this did not reach statistical significance.

In contrast, we did not detect a difference in BDL-induced liver fibrosis between IPA3-treated and control mice. There are several potential explanations for this discrepancy. Firstly, the dose and interval of IPA3 treatment may not have been sufficient to allow detection of a significant reduction in fibrosis in the BDL model. Secondly, if IPA3 is excreted into bile, then interruption of the enterohepatic circulation by BDL may result in altered pharmacokinetics. This could lead to a reduction in plasma levels: for example, plasma levels of propranolol and exaproprolol were reduced in BDL rats compared with controls (Motheová et al., 1986). Thirdly, efficacy of IPA3 in liver fibrosis may be disease-specific. Although activated HSCs are considered to be the predominant fibrogenic cell type in liver fibrosis of all aetiologies (Mederacke et al., 2013), there is still debate over the role of portal...
fibroblasts in biliary fibrosis (Wells, 2014). It has been proposed that portal fibroblasts and myofibroblasts may play an important role early after biliary injury (Wells, 2014). Portal fibroblasts are a distinct liver cell population, and express different markers to those of HSCs (Wells, 2014). In keeping with this, Bandapalli et al have suggested that PAK2 is expressed in HSCs but not portal fibroblasts (Bandapalli et al., 2012). In sum, this provides a possible explanation for the differential effects of IPA3 in hepatotoxic and biliary-induced liver fibrosis.

It is important to note here, that we have not yet demonstrated that IPA3-mediated attenuation of CCl4-induced liver fibrosis is not a consequence of reduced hepatocellular injury. Therefore, it is possible that IPA3 is protective against hepatocellular, but not biliary, injury. Thus, explaining the differential effects observed with IPA3 treatment in CCl4- and BDL-induced liver fibrosis. To determine this, further experiments investigating the effect of IPA3-treatment on CCl4-induced hepatocellular injury are required. For example, measurement of serum ALT 24 hours after administration of CCl4 in IPA3-treated or control mice.

4.4.5 Summary

Advanced fibrosis and cirrhosis are associated with grave clinical complications, and yet, our treatment options are limited. This, coupled with the increasing prevalence of liver disease, underpins the urgent need for anti-fibrotic agents. Activated HSCs are the predominant fibrogenic cell type within the liver, and as such, are the ideal cellular target for anti-fibrotic therapies (Mederacke et al., 2013). Since their discovery over 100 years ago, many of the cytokine and growth factor stimulated signalling pathways involved in HSC activation have been elucidated (Friedman, 2008a). However, more recently, signalling arising from the mechanical microenvironment has emerged as an important regulator of HSC activation (Olsen et al., 2011).

In the previous chapter, we outlined a role for Itgb1 as a mechanosensor signalling via the contractile cytoskeleton to activate the transcriptional regulator YAP during HSC activation. We hypothesised that identifying a downstream mediator of Itgb1 signalling in HSC activation might allow more precise targeting of this pathway, and avoid the potential off-target effects likely to be associated with global inhibition of Itgb1.
In this chapter, we identified the group I PAKs as potential downstream effectors of Itgb1 in activated HSCs. Moreover, we demonstrated that group I Pak inhibition attenuates Col1 expression in activated HSCs in vitro, and hepatotoxin-induced liver fibrosis in vivo. As a promising therapeutic strategy for liver fibrosis, group I PAK inhibition warrants further investigation.
5 RESULTS: ITGA11 SHOWS PROMISE AS A FIBROBLAST-SPECIFIC PARTNER OF ITGB1 REGULATING HSC ACTIVATION

5.1 Introduction

Activated HSCs are the predominant fibrogenic cell type within the liver, and lay down the fibrillar collagen matrix that characterises fibrosis (Kisseleva et al., 2012; Mederacke et al., 2013). In the healthy liver, HSCs are quiescent vitamin A storing cells, located in the space of Disse and in close proximity to the sinusoidal blood vessels. However, following liver injury, they are activated and develop into profibrotic myofibroblasts. This response is not unique to the liver; similar mesenchyme-derived perivascular cells, which transform into myofibroblasts following tissue injury, have been identified in the kidney, lung, central nervous system, skin and muscle (Duffield, 2012; Greenhalgh et al., 2013). These observations led to the proposal of an overarching theory of fibrosis, in which perivascular cells are culpable, and therefore, may be the ideal cellular target for anti-fibrotic therapies (Duffield, 2012).

In this study, we have demonstrated that Itgb1 signalling is important in HSC activation, in keeping with the findings of Liu et al and Yeh et al, who have shown that Itgb1 is required for myofibroblast activation in skin and kidney fibrosis (Liu et al., 2009; Yeh et al., 2010).

Core fibrotic pathways are those essential to the development of fibrosis, and are shared between different organ types (Mehal et al., 2011). Thus, Itgb1 signalling in myofibroblasts and myofibroblast-precursors might represent a core fibrotic pathway. Targeting core pathways is likely to have a significant impact on fibrosis, but at the risk of potential adverse off-target effects (Mehal et al., 2011).

In this regard, global inhibition of Itgb1 would not be a viable therapeutic strategy, due to unacceptable effects in other cell types and organs. For example, Itgb1 depletion in hepatocytes is associated with impaired liver regeneration (Speicher et al., 2014); and loss of Itgb1 in intestinal epithelial cells results in severe malnutrition.
(Jones et al., 2006). Therefore, the ability to specifically target Itgb1 in myofibroblasts and myofibroblast-precursors would be advantageous.

5.1.1 Identifying a Specific Alpha Subunit Partner for Itgb1 in HSC Activation

Integrin inhibitors are used clinically in the treatment of thrombotic and inflammatory disorders, and antagonists targeting specific alpha-beta heterodimers have been developed (Cox et al., 2010). For example, pan-alpha4 inhibition in the treatment of inflammatory bowel disease is linked to the development of progressive multifocal leucoencephalopathy (PML), due to immunosuppressive effects in the central nervous system (Cox et al., 2010). Therefore, an α4β7 specific antibody has been developed, which does not cross-react with either monomer (Feagan et al., 2005). This appears to selectively target the intestinal immune system, and is efficacious in the treatment of ulcerative colitis, without adverse off-target effects (Bickston et al., 2014; Feagan et al., 2005). In addition, selective antagonists have been discovered for αIIbβ3 and αVβ3, despite these heterodimers sharing a subunit (Itgb3) and recognising the same ligand (RGD) (Cox et al., 2010).

To identify alpha subunit partners of Itgb1 that might regulate HSC activation, we assayed the expression of the twelve alpha subunits known to heterodimerise with Itgb1, in activated HSCs. This revealed dramatic upregulation of Itga11 in activated HSCs.

5.1.2 Itga11

5.1.2.1 Itga11 and Fibrosis

Itga11 is the most recently discovered alpha integrin subunit, and forms one of the four Itgb1 heterodimers (α1β1, α2β1, α10β1, α11β1) known to bind collagens. α11β1 preferentially binds to fibrillar type I collagen, suggesting that it may be important in cell-matrix interactions in fibrosis. In support of this, Itga11 has been shown to mediate cell adhesion to, and contraction of, type I collagen matrices in vitro (Barczyk et al., 2009; Popova et al., 2007a; Tiger et al., 2001). Furthermore, Carracedo et al demonstrated that, Itga11 is regulated by mechanical tension in fibroblasts, and in turn, regulates their differentiation into αSma-positive
myofibroblasts in vitro (Carracedo et al., 2010). Whilst, in vivo, Talior-Volodarsky et al demonstrated significant upregulation of Itga11 alongside αSma in cardiac fibroblasts isolated from rats with cardiac fibrosis, compared with controls (Talior-Volodarsky et al., 2012).

5.1.2.2 Itga11 Expression

During development, Itga11 is highly expressed in areas of organised interstitial collagens, and regions adjacent to cartilage formation (Tiger et al., 2001). This led to the suggestion that Itga11 might be regulated by the chondrogenesis-associated transcription factor, scleraxis (Tiger et al., 2001). However, subsequently, scleraxis knockout mice were shown to have normal expression of Itga11 (Popova et al., 2007b). Nevertheless, the expression of Itga11 in cells around the ribs, vertebra and intervertebral discs, suggests that it may be regulated by cartilage-derived signalling (Popova et al., 2007b).

In line with this, SOX9, a transcription factor involved in the regulation of ECM during development, is expressed in the intervertebral discs in a similar distribution to Itga11 in the human embryo (Pritchett et al., 2010; Tiger et al., 2001). Reactivation of developmental pathways is linked to disease pathogenesis, and accordingly, ectopic expression of SOX9 is implicated in fibrosis of the skin, kidney and vasculature (Pritchett et al., 2010). In addition, we have shown that SOX9 is ectopically expressed in activated HSCs, and regulates transcription of COL1 (Piper Hanley et al., 2007). Interestingly, in a microarray analysis of gene expression in Sox9-depleted HSCs, we detected a significant reduction in expression of Itga11 mRNA (unpublished data).

Collectively, these findings suggest that Itga11 may be involved in fibrogenic signalling, and might act downstream of the fibrosis-associated transcription factor, SOX9.

Finally, Lu et al demonstrated that Itga11 expression is restricted to fibroblasts (Lu et al., 2010); therefore, it is tempting to speculate that inhibition of Itga11 in fibrotic disease would be associated with minimal off-target effects.
5.1.2.3 Itga11 Deletion

The potential effects of Itga11 inhibition in vivo, can be inferred from the study of Itga11 knockout mice. Itga11-null mice develop proportional dwarfism in the first four weeks of life, along with reduced bone mineral density and bone strength (Blumbach et al., 2012). These abnormalities were identified as secondary to impairment in the growth hormone - insulin-like growth factor 1 axis (Blumbach et al., 2012). In addition, Itga11 deficiency was associated with impaired tooth eruption, secondary to defective incisor periodontal ligaments (Popova et al., 2007a). No abnormalities were detected in the cornea, intervertebral discs, intestine or skin (Popova et al., 2007a). Therefore, Itga11 inhibition may be associated with potential adverse effects, including growth hormone deficiency, osteoporosis and periodontal disease. Although, whether such defects would be apparent in mice with Itga11 knockout post-development, or indeed in humans, requires further investigation.

5.1.3 Summary

In summary, there is a body of evidence supporting a role for Itga11 in the regulation of myofibroblast differentiation and collagen organisation, suggesting that α11β1-mediated signalling may be important in fibrogenesis. However, despite this, the role of Itga11 in fibrotic disease is undetermined.
5.2 Aims

Our overall aim in this chapter was to identify a specific alpha subunit partner for Itgb1, involved in the regulation of HSC activation. To achieve this, our specific aims were:

1. To assay expression of the twelve alpha subunit partners of Itgb1 in quiescent and activated HSCs, by qPCR

   This revealed dramatic upregulation of Itga11 in activated HSCs, which stimulated our subsequent aims.

2. To determine if α11β1 is expressed in activated HSCs

3. To determine if Itga11 depletion in activated HSCs is associated with downregulation of fibrotic markers
5.3 Results

5.3.1 Identifying an Alpha Subunit Partner for Itgb1 in HSC Activation

To identify a specific alpha subunit partner for Itgb1 in HSC activation, we assayed the expression of the twelve alpha subunits known to heterodimerise with Itgb1, in quiescent and activated rat HSCs, by qPCR (Itga1-11), and immunoblotting (Itga11 and ItgaV). We detected a dramatic upregulation (~2800 fold) in expression of Itga11 mRNA in activated HSCs (Figure 5.1A). We next investigated expression of Itga11 and ItgaV proteins in quiescent and activated rat HSCs, and detected a ~40 fold increase in Itga11 protein in activated HSCs (Figure 5.1B).

A review of the literature uncovered evidence supporting a role for Itga11 in myofibroblast activation and fibrogenesis (discussed in section 5.1.2.1), and therefore, we pursued the role of Itga11 in HSC activation further.
Figure 5.1 Expression of alpha subunit partners of Itgb1 in activated HSCs
A. Quantification of mRNA expression of eleven alpha subunit partners of Itgb1. Assayed by qPCR in Q and A rHSCs. mRNA expression in A rHSCs is expressed relative to Q rHSCs, normalised to β-actin and GusB. n ≥ 2. Error bars are SEM. B. Quantification and example immunoblot of Itga11 and ItgaV proteins in Q and A rHSCs. Immunoblotting quantification was standardised to β-actin; n ≥ 3. Error bars are SEM. Significance was determined using a two-tailed Student T test; *p<0.05.
We confirmed association of Itga11 with Itgb1 in activated HSCs by co-immunoprecipitation, using an Itgb1 antibody to pull-down, and immunoblotting the isolated protein complexes for Itga11. We detected enrichment for Itga11 following immunoprecipitation with an Itgb1 antibody compared with IgG negative control, suggesting that Itga11 complexes with Itgb1 in activated HSCs (Figure 5.2A). In addition, we demonstrated co-localisation of Itga11 with Itgb1 in activated HSCs by dual immunofluorescence (Figure 5.2B). Taken together, these studies suggest that α1β1 is expressed by activated HSCs.

### Figure 5.2 Expression of α1β1 in activated HSCs

**A.** Co-immunoprecipitation of Itgb1 and Itga11 in activated rat HSCs, using an Itgb1 antibody (or IgG negative control) to pull-down and immunoblotting the isolated protein complexes for Itgb1 and Itga11. Itgb1 and Itga11 were enriched following pull-down with Itgb1 antibody compared with IgG negative control. A representative immunoblot of two independent experiments is shown. SN = Supernatant. IP = Immunoprecipitation. **B.** Dual immunofluorescence in A rHSCs demonstrated co-localisation of Itga11 (green) with Itgb1 (red). Nuceli are shown by DAPI staining (blue). Scale bar represents 50 μm.
5.3.2 Itga11 Depletion is Associated with Reduced Expression of Col1

Carracedo et al demonstrated that Itga11 was required for differentiation of fibroblasts into αSma-positive myofibroblasts in response to mechanical tension (Carracedo et al., 2010). Therefore, we next investigated whether depletion of Itga11 in activated HSCs was associated with a less fibrotic phenotype. We used siRNA to deplete *ITGA11* in LX-2 cells. We detected that a ~65% reduction in *ITGA11* mRNA was associated with a ~30% reduction in *COL1A1* and a ~50% reduction in *SOX9*.

We had difficulty in finding an antibody to reliably detect ITGA11 in human cells, and therefore, to investigate the effects of Itga11 depletion on levels of fibrotic proteins we used mouse activated HSCs. Using Itga11 targeted siRNA, we achieved a ~47% reduction in Itga11 protein, which was associated with a significant reduction in Col1 by ~35%, and non-significant reductions in Sox9 and Myl9, by ~30% and ~50% respectively.
Figure 5.3 Itga11 depletion is associated with reduced Col1

A and B. Abrogation of ITGA11 by siRNA in LX-2 cells compared scrambled siRNA negative control. A. mRNA quantification by qPCR for fibrotic markers showing significant decreases in COL1A and SOX9. qPCR was normalised to β-actin and GusB. n = 3. B. Protein quantification following ITGA11 knockdown and example immunoblot for fibrotic markers are shown. All immunoblotting was normalised to β-actin. n ≥ 3. Error bars are SEM. Significance was determined by two-tailed Student T test, * p<0.05; ** p<0.01; *** p<0.001.
5.4 Discussion

We have previously outlined a role for the mechanosensor Itgb1 in the regulation of HSC activation; and have demonstrated that Itgb1 deficiency in activated HSCs is associated with a less fibrotic phenotype. Activated HSCs are thought to be the prime cellular targets for anti-fibrotic therapies in liver fibrosis (Mederacke et al., 2013). The ability to inhibit HSC activation, or downgrade their fibrotic response would, in theory, result in attenuation of liver fibrosis.

However, global inhibition of Itgb1 is likely to be associated with significant off-target effects, precluding it as a viable therapeutic strategy. For example, Itgb1 depletion in hepatocytes is associated with impaired hepatocellular regeneration (Speicher et al., 2014); and loss of Itgb1 in intestinal epithelial cells results in severe malnutrition (Jones et al., 2006). Therefore, the ability to specifically target Itgb1 in HSCs would be advantageous, and may allow therapeutic targeting of pro-fibrotic Itgb1 signalling whilst avoiding deleterious effects in other cell types and organs.

More broadly, Itgb1 is implicated in the pathogenesis of skin and kidney fibrosis (Liu et al., 2009; Yeh et al., 2010), and therefore, the ability to target Itgb1 in myofibroblasts and myofibroblast precursors may be of advantage in the treatment of fibrosis in a number of organs.

Itgb1 is known to heterodimerise with twelve different alpha subunits, and therefore, identifying an HSC-specific Itgb1-containing heterodimer would allow more precise targeting. Encouragingly, there is precedent in targeting specific alpha-beta heterodimers in the clinical management of disease. For example, the selective $\alpha_4\beta_7$ antagonist vedolizumab has been used in the treatment of inflammatory bowel disease, and has so far not been associated with the harmful off-target effects linked to pan-alpha4 inhibition (Gilroy and Allen, 2014).

In this chapter, we assayed mRNA expression of the twelve alpha subunits known to heterodimerise with Itgb1, in quiescent and activated HSCs, and identified that Itga11 was dramatically upregulated during HSC activation. A review of the literature uncovered evidence supporting a role for Itga11 in mechanosensing, contraction and myofibroblast activation (Barczyk et al., 2009; Carracedo et al.,
2010; Popova et al., 2007a; Tiger et al., 2001); therefore, we investigated the role of Itga11 in HSC activation further.

5.4.1 Expression of α11β1 in Activated HSCs

We demonstrated that Itga11 was also increased at the protein level in activated HSCs; and by co-immunoprecipitation that Itga11 associated with Itgb1 in activated HSCs. To corroborate this, we used dual immunofluorescence to demonstrate co-localisation of Itga11 and Itgb1 in activated HSCs. However, to better resolve the association to within the same focal adhesion, confocal microscopy could be used. In addition, proximity ligation assay (PLA) would allow visualisation of α11β1 complexes, and their distribution on the cell membrane. In this technique, target proteins are labelled with primary antibodies raised in different species, which are then bound by species-specific secondary antibodies tagged with short DNA strands, (termed PLA probes). When in close proximity, the DNA strands interact, are connected by hybridising DNA oligonucleotides, and subsequently amplified by a polymerase. The replicated DNA circles are then labelled with fluorescent-tagged complementary oligonucleotides, and are visualised as a bright spot using a fluorescence microscope.

5.4.2 α11β1: the Specific Itgb1-Containing Heterodimer Regulating HSC Activation?

Itga11 is only known to heterodimerise with Itgb1 (Hynes, 2002); therefore, if α11β1 is the specific Itgb1-containing heterodimer involved in the regulation of HSC activation, one would expect Itga11 depletion to phenocopy loss of Itgb1 in activated HSCs.

However, we detected a significant reduction in Col1 only, in association with Itga11-depletion; whereas with Itgb1 deletion in activated HSCs, we demonstrated significant reductions in both Col1 and Sox9, and a non-significant reduction in αSma. Nevertheless, this does not preclude Itga11 as the specific partner to Itgb1 regulating HSC activation, as different techniques, with different efficiencies, were used to deplete the integrin subunits. We used the CreER-loxP system to delete the Itgb1, and achieved a ~80% reduction in Itgb1 protein; whereas we used siRNA to deplete Itga11, and only achieved a ~47% reduction in Itga11 protein.
An Itga11-null mouse strain has been developed by the Gullberg group, but unfortunately, we have not had access to this genetic knockout model (Popova et al., 2007a). However, the MRC Mouse Network and the International Mouse Phenotyping Consortium are developing a conditional Itga11 knockout mouse model, which we hope to use to determine the effects of Itga11 deletion in HSCs in vitro and liver fibrosis in vivo. These will be the critical experiments to determine whether α11β1 is the specific Itgb1-containing heterodimer regulating HSC activation, and if it is a potential therapeutic target in liver fibrosis.

It is important to note that following completion of this work, Reed et al have published compelling data suggesting that αVβ1 may be a major Itgb1-containing heterodimer regulating fibrosis, at least in the lung and liver (Reed et al., 2015). Despite this, it remains plausible that α11β1 may play an important role in fibrosis, either by acting synergistically with αVβ1, or perhaps at a different time-point within the fibrotic process. As we gain a better understanding of fibrosis and see the translation of anti-fibrotics to clinical practice, it is likely that different anti-fibrotic strategies will be required for different stages and aetiologies of fibrosis.

5.4.3 Summary

The ability to specifically target an Itgb1-containing heterodimer in activated HSCs is attractive, as this would allow disruption of pro-fibrotic Itgb1 signalling whilst avoiding the potential deleterious effects associated with global Itgb1 inhibition. In this study, we identified Itga11 as a partner for Itgb1 in activated HSCs. Itga11 is only known to heterodimerise with Itgb1, and its expression is restricted to fibroblasts (Hynes, 2002; Lu et al., 2010). Moreover, Itga11 has been shown to act as a mechanosensor and regulate myofibroblast activation (Carracedo et al., 2010). Collectively, these findings suggest that α11β1 is a promising candidate as the Itgb1-containing heterodimer regulating HSC activation. In line with this, we have presented some preliminary data demonstrating that Itga11 depletion is associated with a reduction in Col1. However, the definitive experiments utilising an Itga11 genetic knockout model are yet to be performed.
6 GENERAL DISCUSSION

6.1 Overview and Importance

Liver disease is a leading cause of morbidity and mortality. In the UK, deaths from liver disease are increasing, whilst all other major causes of mortality in the under 65s are in decline (Moore et al., 2009). Increasingly, liver disease is affecting young patients of working age. In the last 10 years there has been a five-fold increase in the development of cirrhosis in 35 - 55 year olds (Moore et al., 2009).

Liver fibrosis results from an aberrant wound healing response to chronic liver injury, due to a variety of insults. In the early stages, liver fibrosis is reversible, with cessation of the insult and injury. However, too often, liver fibrosis is not identified until it has progressed to advanced stages, by which point, reversal is often difficult, although not impossible (Iredale, 2007; Malekzadeh et al., 2004).

Currently, treatment options for liver fibrosis are limited, and there are no clinically approved anti-fibrotic agents. The only treatment for end-stage liver disease is liver transplantation; but this is limited by supply of donor organs, and is complicated by operative risks and long-term immunosuppression.

Clearly, the gold standard is prevention of liver injury. This requires public education on safe limits of alcohol consumption, dietary and exercise advice to prevent obesity and vaccination against hepatitis B. In addition, early detection of liver disease would allow treatment of the underlying cause, where possible, before advanced fibrosis develops. This would require better public awareness, and the availability of reliable, non-invasive biomarkers.

Once fibrosis has developed, the ability to halt or ameliorate the fibrotic process would be of great advantage in the clinical management of patients with liver disease. Reducing the burden of liver fibrosis would benefit individual patients, by lowering their risk of complications, such as variceal bleeding and hepatocellular carcinoma; and reduce hospital admissions and the socioeconomic costs associated with end-stage liver disease.
6.2 HSC Activation and Mechanotransduction

Activated HSCs are the predominant fibrogenic cell within the liver, and as such, they are the ideal cellular target for anti-fibrotic therapy (Mederacke et al., 2013). In the healthy liver, HSCs are quiescent vitamin A storing cells which reside within the space of Disse. However, following liver injury, they become activated and develop the characteristics of fibrogenic myofibroblasts. They proliferate, migrate into the liver parenchyma, and lay down the fibrillar ECM which characterises fibrosis (Friedman, 2008a). They are highly contractile, and contribute to the development of portal hypertension, an important clinical sequela of advanced liver fibrosis, by modulating sinusoidal resistance (Bhathal and Grossman, 1985; Thimgan and Yee, 1999; Zhang et al., 1994). In addition, contraction of the fibrotic scar by activated HSCs results in further distortion of the liver architecture.

Many of the humoral factors involved in the activation of HSCs have now been identified (Friedman, 2008a). However, more recently, the biophysical characteristics of the cellular microenvironment have emerged as an important regulator of HSC activity (Olsen et al., 2011). The translation of physical stimuli into biochemical signalling cascades has been termed ‘mechanotransduction’, and this can be considered as ‘extrinsic’ or ‘intrinsic’ to the cell (Huang et al., 2012). Extrinsic mechanotransduction refers to the mechanical activation of TGF-β1, which occurs when myofibroblasts bind to latent TGF-β1 sequestered within the ECM, and generate contractile forces in response to increased matrix stiffness (Huang et al., 2012; Worthington et al., 2011). This is mediated by the alphaV integrins, which recognise the RGD sequence within the latent TGF-β1 complex, and transmit the mechanical forces generated by the contractile cytoskeleton (Wipff and Hinz, 2008; Wipff et al., 2007). The critical importance of this pathway in fibrogenesis has recently been demonstrated by Henderson et al. They have shown that deletion of alphaV integrins in myofibroblasts protects against liver, kidney and lung fibrosis, by a mechanism involving reduced activation of TGF-β1 (Henderson et al., 2013). Moreover, they have demonstrated that targeting this pathway with a small molecule alphaV integrin inhibitor is effective in the treatment of liver and lung fibrosis (Henderson et al., 2013).
In this study, we have outlined an alternative intrinsic mechanotransduction pathway in HSC activation. We propose that matrix stiffening is sensed by Itgb1 and is translated, by downstream effectors, into development of the contractile cytoskeleton, and activation of the transcriptional regulator Yap. Moreover, we have shown that Yap regulates expression of Myl9, Col1 and Itgb1, suggesting that this pathway forms a positive feed forward loop leading to amplification of fibrogenesis. We concluded that targeting this pathway may represent a novel therapeutic avenue for liver fibrosis.

6.3 Targeting Itgb1 in Activated HSCs

Itgb1 is widely expressed and its global inhibition is likely to be associated with unacceptable off-target effects. Therefore, we utilised two strategies to discover more specific targets for anti-fibrotic therapy.

Firstly, we carried out a microarray analysis to identify downstream effectors of Itgb1 signalling in activated HSCs. This uncovered a role for the group I PAKs in HSC activation. We demonstrated that the group I PAKs regulate Col1 expression in activated HSCs in vitro; and, that the group I PAK inhibitor IPA3 attenuates hepatotoxin-induced liver fibrosis in vivo.

To our knowledge, this is the first report of IPA3 treatment in an in vivo model of fibrosis. However, others have shown that IPA3 inhibits growth of hepatocellular carcinoma cells in vivo (Wong et al., 2013). Hepatocellular carcinoma is a well-described complication of chronic liver disease, as the fibrotic microenvironment is conducive to hepatic carcinogenesis (Wallace and Friedman, 2014). Therefore, the ability to attenuate both fibrosis and hepatocellular carcinoma growth, would be particularly advantageous in the management of patients with advanced liver fibrosis and cirrhosis.

Secondly, we assayed the expression of the alpha subunit partners of Itgb1 in activated HSCs, and discovered that Itga11 was dramatically upregulated. Itga11 is currently thought to heterodimerise exclusively with Itgb1, and its expression is restricted to fibroblasts (Hynes, 2002; Lu et al., 2010). Therefore, targeting Itga11 would allow more selective inhibition of Itgb1 in fibrosis. In line with this, we have demonstrated that α11β1 is expressed by activated HSCs, and that Itga11 depletion is
associated with reduced Col1 expression in activated HSCs. The data presented here suggest that Itga11 is a promising therapeutic target in liver fibrosis. However, further studies are required, utilising an Itga11 knockout mouse model, to understand if Itga11 inhibition is protective against liver fibrosis in vivo.

6.4 Future Work

The data presented in this thesis have generated many further questions to be addressed in future work.

6.4.1 The Role of YAP in HSC Activation

Firstly, the importance of YAP as a mechanosensitive regulator of HSC activation requires confirmation by additional experiments. In this study, we used verteporfin to inhibit activation of Yap in HSCs, and demonstrated reduced expression of Itgb1, Col1 and Myl9. These findings require corroboration by an additional method of Yap inhibition, for example by siRNA-mediated gene silencing. In addition, we have so far only examined the effect of Yap inhibition on mRNA expression of fibrotic targets, and this will also need to be assessed at the protein level.

Sorrentino et al have demonstrated YAP inhibition by statins (Sorrentino et al., 2014); therefore, it would be interesting to determine the effect of statins on Yap activity in activated HSCs in vitro. This is particularly relevant as statins are already approved for clinical use.

Finally, the importance of Yap in liver fibrosis in vivo will be determined by verteporfin (or possibly statin) therapy in mouse models of liver fibrosis.

6.4.2 Mechanism of Action of Group I PAKs in HSC Activation

Secondly, the mechanism of action of group I PAKs in HSC activation requires elucidation. Our finding that phosphorylated Myl9 is reduced in Pak1 depleted HSCs, suggests that the group I PAKs play a role in HSC contraction. However, functional characterisation of Pak1-deficient HSCs is required, including an assessment of their ability to contract collagen gel matrices. A Pak1 knockout mouse model is available, and this would allow more robust assessment of Pak1-depletion in HSCs, compared with siRNA mediated gene silencing or small molecule inhibition.
In contrast, a Pak1 activating peptide has been developed (Wang *et al.*, 2014), and it would be interesting to determine if this enhances HSC activation, and if it is able to rescue the phenotype of Itgb1-null HSCs.

The results of IPA3 treatment in liver fibrosis presented in this study will be corroborated by investigating liver fibrosis in global Pak1 knockout mice, and HSC-specific Pak1 knockout mice. HSC-specific Pak1 knockout mice will be developed by crossing an available conditional Pak1 knockout mice with PDGFRβCre expressing mice.

Finally, through our collaboration with GSK, we have access to selective group I PAK inhibitors, which may be more potent than IPA3. These will be investigated in HSC activation *in vitro*, and potentially, in liver fibrosis *in vivo*.

6.4.3 The Role of Itga11 in HSC Activation and Liver Fibrosis

Thirdly, a conditional Itga11 knockout mouse is being developed, which will allow us to build on the results presented here, and more robustly examine the role of Itga11 in HSC activation *in vitro*. In addition, developing an HSC-specific Itga11 knockout mouse model will allow us to determine if Itga11 antagonism is a viable therapeutic strategy in liver fibrosis *in vivo*. Having determined this, we would seek to identify small molecule Itga11 inhibitors through our collaboration with GSK.

6.4.4 Summary

In this study we have outlined the role of Itgb1 in regulating HSC activation. We suggest that Itgb1 acts as a mechnosensor, leading to the activation of Yap via the contractile cytoskeleton. We have discussed potential therapeutic targets within this pathway, and have demonstrated that inhibition of the group I Paks, downstream of Itgb1, is a promising therapeutic strategy in liver fibrosis.
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# Appendix

## 8.1 Table of Primary Antibodies

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## 8.2 Table of Secondary Antibodies

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### 8.3 Table of Primers

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