MECHANISM OF SOX9 ACTION AS A ROUTE TO DIAGNOSTIC STRATEGIES IN 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

2013

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LIST OF ABBREVIATIONS

AIH – Autoimmune hepatitis
ALD - Alcoholic Liver Disease
AMH - Anti-Mullerian Hormone
APRI - Aspartate Aminotransferase-To-Platelet Ratio Index
arHSCs - Activated rHSCs
α-SMA - α Smooth Muscle Actin
AUC – Area under curve
AUROC – Area under the receiver operator curve
Bcl2 - B-Cell Leukaemia/Lymphoma 2
BDL - Bile Duct Ligation
BMP – Bone Morphogenetic Protein
Brdu - 5-Bromo-2'-Deoxyuridine
bp – Base Pair
cAMP - Cyclic Adenosine Monophosphate
Ccl - Chemokine (C-C Motif) Ligand
Ccr - Chemokine (C-C Motif) Receptor
CCl4 - Carbon Tetrachloride
CD44 – Cluster of differentiation 44
HBV – Hepatitis B virus
HCV – Hepatitis C virus
CHC – Chronic hepatitis C
ChIP - Chromatin Immunoprecipitation
CNS - Central Nervous System
COL - Collagen
COMP - Cartilage Oligomeric Matrix Protein
Cxcr - Chemokine (C-X-C motif) Receptor
DAB - 3,3' -Diaminobenzidine
Dhh - Desert Hedgehog
DMEM - Dulbecco's Modified Eagle Medium
DMSO - Dimethylsulphoxide
dpc - Days Post Conception
E-cadherin – Epithelial Cadherin
ECM - Extracellular Matrix
EDGF - Endothelial Derived Growth Factor
ELF – European liver fibrosis panel
EMT - Epithelial Mesenchymal Transition
Eno1 – Enolase 1
EPIM - Epimorphin
ERK - Extracellular Regulated Kinase
FAK - Focal Adhesion Kinase
FBS - Foetal Bovine Serum
FGF - Fibroblast Growth Factor
FN1 - Fibronectin
Foxa2 - Forkhead Box A2
FSGS - Focal Segmental Glomerulosclerosis
GAPDH - Glyceraldehyde-3-Phosphate Dehydrogenase
GFP - Green Fluorescent Protein
Gli – Glioblastoma
GPNMB – Glycoprotein neuromedin B / osteoactivin
GusB - β-Glucuronidase
HA - Hyaluronan
HBSS- - Hanks’ Balanced Salt Solution minus Calcium and Magnesium
HBSS+ - Hanks’ Balanced Salt Solution plus Calcium and Magnesium
HCC - Hepatocellular Carcinoma
HH - Hedgehog
hHSCs - Human Hepatic Stellate Cells
HMG – High Mobility Group
HNF6 - Hepatocyte Nuclear Factor 6
HSCs - Hepatic Stellate Cells
IGF-II - Insulin-Like Growth Factor II
IgG - Immunoglobulin G
Ihh - Indian Hedgehog
IKBα - NF-κB Inhibitor Alpha
IL-1β - Interleukin 1β
iNOS - Inducible Nitric Oxide Synthase
Itga - Integrin α
LOXL2 - Lysyl Oxidase-Like 2
LPS - Lipopolysaccharide
Mb - Megabases
MBP-1 – cMyc binding protein 1
MCD - Methionine and Choline Deficient Diet
Shh - Sonic Hedgehog
shRNA - Short Hairpin RNA
siRNA - Small Interfering RNA
Smad - Smaller Mothers Against Decapentaplegia
Smo - Smoothened
SNARE - Soluble NSF Attachment Protein Receptor
SOX9 - SRY box-9
Sparc - Secreted Protein, Acidic, Cysteine-Rich
SP1 - Specificity Protein 1
SRY- Sex-Determining Region on the Y Chromosome
Stat - Signal Transducer and Activator of Transcription
TIMPs - Tissue Inhibitors of Metalloproteinases
TGF-α - Transforming Growth Factor alpha
TGF-β - Transforming Growth Factor beta
TM - Trans membrane
TNFα - Tumour Necrosis Factor α
uPA - Urokinase Plasminogen Activator
VEGF - Vascular Endothelial Growth Factor
VIM - Vimentin
wpc - Weeks Post Conception
ABSTRACT

The work presented in this thesis aims to understand the role of the Sry-box transcription factor (SOX9) in liver fibrosis as a route to novel diagnostic strategies for the disease. Fibrosis of the liver is a major cause of morbidity and mortality in the UK characterised by progressive accumulation of extracellular matrix (ECM) proteins. End-stage disease is treated by transplantation, but this is limited by donor numbers. Although potentially reversible if diagnosed early, current methods of diagnosis are invasive and prone to sampling error. There is a critical need to improve current methods or develop novel strategies to determine fibrotic activity and disease progression. To address this, better mechanistic understanding of liver fibrosis is urgently required. My supervisor (Dr Piper Hanley) previously discovered ectopic expression of SOX9 as a novel mechanism to underlie aspects of liver fibrosis. This discovery allowed me to investigate the molecular genetic network in which SOX9 operates. Given SOX9’s seemingly central role, I identified novel target genes of SOX9 action, some of which have already been highlighted as candidate biomarkers, for new diagnostic strategies in liver fibrosis. Moreover, investigating the expression of SOX9 directly during liver fibrosis in human biopsy samples highlighted the factor as a prognostic marker of the disease.

This thesis focused on four key research areas: Identification of the inflammatory glycoprotein Osteopontin (OPN) as a direct target of SOX9. In addition to OPN, identification of four other downstream SOX9 targets as potential biomarkers of liver fibrosis severity. SOX9 expression in liver biopsies is described and quantified, highlighting its role as a new method to assess liver fibrosis progression. This work is being patented as a novel prognostic test in liver fibrosis. Finally, the role of SOX9 mediating ECM through inhibition of collagenases (e.g. MMP13) is described.

Taken together, these data place SOX9 as a key mediator of liver fibrosis. However, translation of novel SOX9 targets as serum biomarkers of fibrosis and SOX9 as a marker of disease progression have ramifications on clinical practice, in particular assessing liver fibrosis progression.
DECLARATION AND COPYRIGHT STATEMENT

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ACKNOWLEDGMENTS

First and foremost, I would like to acknowledge my supervisors, Dr Karen Piper Hanley and Professor Neil Hanley, for their support, guidance and the opportunity to undertake this project. Supervision and guidance from Dr Neil Guha and Professor Will Irving at the Queens Medical Centre, University of Nottingham has been instrumental to this project, and their insightful wisdom and good company have made collaboration a genuine pleasure.

I could not have hoped to work with a better team than the groups of Karen and Neil. This work is a testament to their help and I look forward to lasting friendships. Specifically, I would like to thank James Pritchett and Emma Harvey for their infinite patience in teaching a clinician the techniques required for this project. I would also like to thank Grace Dolman who has provided invaluable help and Andy Berry for putting up with me sitting next to him and asking 101 ‘daft medic’ questions.

I would like to thank Manchester Biomedical Research Centre for initially funding my fellowship and the Medical Research Council for the Clinical Research Training Fellowship that has facilitated this work.

My friends and Tuesday pub quiz – you have been the sanctuary of normality.

Thank you Michelle, your unwavering support, encouragement and love have provided the foundation to build this project.

This is dedicated to BG and mum, who believe in learning.
1 INTRODUCTION

Fibrosis is present in all organs and is responsible for the majority of non-traumatic mortality. Liver fibrosis and cirrhosis are an eventual consequence of almost all chronic liver diseases (Friedman 2003). The incidence of mortality from liver disease is rising in the Western world and in the UK liver disease is now the fifth most common cause of mortality (Leon and McCambridge 2006). Causative agents include hepatotoxic viruses and alcohol consumption, both of which are an increasing challenge in the Western world (Sheron et al. 2011), and the growing epidemic of obesity, contributing to a significant growth in the prevalence of non-alcoholic steatohepatitis (Wallace et al. 2008). Transplant remains the only medical intervention for end-stage disease and donor organs are still supply limited. Strategies aimed at the fibrosis machinery remain lacking and are a vital challenge for medical research. To this end, understanding the processes of fibrosis is critical to both understand disease and identify potential anti-fibrotic therapies.

1.1 Structure of the liver

1.1.1 Anatomy of the liver

The liver is the largest visceral organ and second only to the skin, for size in the body. A normal, healthy liver weighs approximately 1.5kg (Skandalakis et al. 2004) and is traditionally divided into four lobes: right, left, caudate and quadrate. These can be divided into 8 functional segments, originally described by Couinaud (Couinaud 1954), according to vascular supply and biliary drainage. Unlike most other organs, two afferent vessels supply hepatic blood: the hepatic artery (25% of blood supply) and the portal vein (75%). Normal portal venous pressure ranges from 5-10mmHg, whilst mean arterial pressure in the hepatic artery is normally about 90mmHg. However, due to arteriole vasoconstriction, blood enters the hepatic sinusoidal capillaries at about 10mmHg (Abdel-Misih and Bloomston 2010). Blood draining into the liver from the portal vein and hepatic artery moves towards the central venuole, undergoing ultrafiltration and reabsorption. The central veins collect into
large vessels, ultimately draining into the three large hepatic veins (right, middle and left hepatic veins) and then into the inferior vena cava.

Bile salts are excreted by the liver, via the biliary canaliculi, and move into the bile ducts in the opposite direction to sinusoidal blood flow. The bile ducts coalesce into the common hepatic duct and bile is stored in the gallbladder until secreted, via the common bile duct, into the duodenum. The primary components of bile are bile salts (conjugated bilirubin, cholic acid, chenodeoxycholic acid, glutathione and other organic anions) and bicarbonate. Biliary excretion is an important function of the liver as it facilitates digestion of fats and lipids.

1.2 Function of the liver

The liver has a vast array of functions. More than half of a person’s reticulo-endothelial system is within the liver and the liver is responsible for bacterial destruction and removal of cellular debris entering the liver from the gut. Detoxification of the endogenous and exogenous substances is a vital role for the liver. This is done by hepatocytes, often by conjugation of the substance, and involves a complex system of enzymes, collectively known as cytochrome P450. The liver also performs a variety of metabolic functions, including carbohydrate storage as glycogen, manufacture of glucose (gluconeogenesis), fat metabolism and cholesterol synthesis, storage of fat-soluble vitamins (D, A and K), and protein synthesis (especially albumin and α-globulin). See Table 1-1.

1.3 The hepatic lobules and sinusoid

The liver parenchyma is organised into lobules and can be thought of as hexagonal structures around a central vein. The corners of the hexagon represent portal tracts, consisting of a bile duct, portal venuole and hepatic arteriole. Blood moves from the portal tract towards the centre, passing through the hepatic sinusoid. The hepatic sinusoid is a complex vascular channel with blood moving through at low pressure (Schaffner and Poper 1963). Rows of hepatocytes line the sinusoid and are separated from the blood by fenestrated endothelial cells and the sub-endothelial space, known
as the space of Dissé (See Figure 1-1). The various cells within the sinusoid perform distinct roles.

**Figure 1-1: The hepatic sinusoid.**
Diagrammatic representation of the liver sinusoid. Single-cell columns of hepatocytes are separated from blood flow by sinusoidal endothelial cells and the sub-endothelial space (space of Dissé). Kupffer cells and hepatic stellate cells reside within the space of Dissé. Bile flow is in the opposite direction to blood flow along bile canaliculi towards biliary epithelial cells. Adapted from Baxter et al.

1.4 **Cells within the liver**

The cell types within the hepatic sinusoid have different roles to allow the functioning of the liver.

1.4.1 **Hepatocytes**
Hepatocytes are the parenchymal cell of the liver and constitute approximately 80% of the mass of the liver and 60% of cells within the liver. The cells are often binucleate, complex chemical factories, abundant in rough and smooth endoplasmic reticulum, mitochondria, Golgi, lysosomes and peroxisomes. Within the adult
sinusoid the hepatocytes are lined up in vascular channels one cell thick. In the
developing foetus and at birth, hepatocytes are organised in two cell thick rows.
Hepatocytes have no basement membrane and are separated from endothelial cells
by the space of Dissé. Hepatocytes are involved in many metabolic processes and
these are outlined in Table 1-1. There is much heterogeneity between hepatocytes
and expression of CYPs depending in particular on the location in the hepatic lobule.

<table>
<thead>
<tr>
<th>Functions of Hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein Synthesis</strong></td>
</tr>
<tr>
<td>Albumin</td>
</tr>
<tr>
<td>Fibrinogen</td>
</tr>
<tr>
<td>Pro-thrombin</td>
</tr>
<tr>
<td>Complement</td>
</tr>
<tr>
<td>Many other examples</td>
</tr>
<tr>
<td><strong>Carbohydrate Metabolism</strong></td>
</tr>
<tr>
<td>Fatty acids from carbohydrates</td>
</tr>
<tr>
<td>Triglyceride synthesis from fatty acids and glycerol</td>
</tr>
<tr>
<td>Lipoprotein synthesis (VLDL, HDL)</td>
</tr>
<tr>
<td><strong>Gluconeogenesis</strong></td>
</tr>
<tr>
<td>Carbohydrate synthesis from lipids and amino acids</td>
</tr>
<tr>
<td><strong>Lipid Metabolism</strong></td>
</tr>
<tr>
<td>Cholesterol synthesis</td>
</tr>
<tr>
<td>Bile salt manufacture and secretion</td>
</tr>
<tr>
<td><strong>Detoxification</strong></td>
</tr>
<tr>
<td>Ammonia deaminated to produce urea</td>
</tr>
<tr>
<td>Drug (exogenous compounds) metabolism and conjugation</td>
</tr>
<tr>
<td>Steroid inactivation</td>
</tr>
</tbody>
</table>

Table 1-1: Functions of hepatocytes with specific examples.

1.4.2 Macrophages/Kupffer cells
Kupffer cells are non-parenchymal, monocyte, phagocytic cells of the liver. They are
resident macrophages found in the hepatic sinusoid between the endothelial cells and
within fenestrations. Kupffer cells are strategically positioned in liver sinusoids for
efficient phagocytosis of pathogens from portal and arterial blood, preventing
infection of various organs. Phagocytosis occurs by antibody-mediated clearance,
requiring fragment crystallisable (Fc) receptors or by complement activation,
specifically complement component 3 (C3) (Helmy et al. 2006). Activation of Kupffer cells can occur with a variety of agents, including ethanol, lipopolysaccharides (LPS), and dietary modifications (Smedsrod et al. 2009). Activation occurs by toll-like receptor 4 (TLR-4) and results in the release of reactive oxygen species (ROS) and multiple cytokines into the liver (Smedsrod et al. 2009).

1.4.3 Sinusoidal endothelial cells
Liver sinusoidal endothelial cells (SEC) separate the hepatocytes from blood. Crucially these cells contain fenestrations (approximately 100nm diameter), which allow bi-directional transfer of substances (Smedsrod et al. 2009). The porosity and diameter of the fenestrations is predominantly controlled by vascular endothelial growth factor (VEGF) released by hepatocytes, acting via SECs’ VEGF-receptor (Carpenter et al. 2005). SECs perform a vital scavenger role and have greatest endocytosis capacity of any cell in the body and clear an array of macromolecules by cell surface receptors facilitating endocytosis and then phagocytosis (Smedsrod et al. 1990).

1.4.4 Hepatic stellate cells
The hepatic stellate cell (HSC) is a mesenchymal cell that is found in the sub-endothelial space of Dissé. Originally described by Kupffer as sternzellen (star-shaped) cells in the peri-sinusoidal area after staining with gold-chloride to highlight vitamin A (Wake 1971). In normal, healthy liver HSCs have a spindle-shaped appearance and the projections of the cell wrap around SECs and hepatocytes (Friedman 2008). The storage of vitamin A is a hallmark of HSCs, a phenomenon that can be observed by auto-fluorescence of the droplets by light at approximately 320nm. HSCs have been described in all vertebrates from fish to humans (Kordes et al. 2009). Rodent HSCs have been shown to exhibit desmin and glial fibrillar acidic protein (GFAP), whereas human quiescent HSCs are devoid of desmin and not all express GFAP (Yokoi et al. 1984; Schmitt-Graff et al. 1991). However, even within a species there is much heterogeneity amongst HSCs. Rodent HSCs around the central vein (peri-central) can be negative for desmin whilst those around the portal tract (peri-portal) can be positive (Ballardini et al. 1994). They can also store variable amounts of vitamin A (Ballardini et al. 1994).
The embryonic origin of HSCs remains obscure and there is evidence to support their beginnings in both endoderm and septum transversum (Geerts 2004; Friedman 2008). Expression data in humans and rodents suggests that HSCs originate from mesenchymal cells of the septum transversum in humans and rodents (Enzan et al. 1997). Recent lineage tracing studies confirm these findings and provide further support of a septum transversum-derived mesothelial origin (Asahina et al. 2011).

An alternative, neural crest origin of HSCs was speculated, given their expression of neuronal markers, including GFAP, N-cadherin, brain-derived neurotropic factor (BDNF) and N-CAM, but this now appears far less likely (Cassiman et al. 2006). Interestingly, HSCs appear to be very similar to pancreatic stellate cells and transcriptome analysis of isolated cells has suggested that there is relatively little difference between gene expressions of these cells groups (Buchholz et al. 2005). This suggests a similar embryonic origin.

HSCs can be found within the progenitor cell niche around the Canals of Hering and, in culture models, HSCs promote the maturation of progenitors by direct cell-to-cell contact (Friedman 2008). HSCs also express the stem cell marker CD133, and this introduces the idea that HSC may have a pluripotent potential (Kordes et al. 2007). Similar to stem and progenitor cells, canonical Wnt signalling has been shown to maintain the quiescent phenotype of HSCs in-vitro (Kordes et al. 2008). In fact, other signalling pathways, such as Notch, Jak-Stat, Bmp and hedgehog, associated with undifferentiated cell types (i.e. regarded as stem or progenitor cells) have also been demonstrated in HSCs (Kordes et al. 2009). These data is in conflict with recent studies suggesting that HSCs are pericytes, mesenchyme derived cells common to fibrotic processes (Dulauroy et al. 2012). Pericytes are found in proximity to capillaries, which would be consistent with HSC’s distribution in the space of Dissé, as the hepatic sinusoid is in effect a hepatic capillary. Pericytes are considered myofibroblast progenitors (Duffield 2012), a feature that provides further proof of a shared lineage. Pericytes and their role in fibrosis are further discussed in Section 1.5.1.

The process of HSC activation can be thought of as a continuum and not simply as two diametric states. Upon stimulation, HSCs become activated towards a
myofibroblast-like phenotype and lose their vitamin A composition. Activation can be considered the process of change resulting in a proliferative, fibrogenic and contractile cell.

Recent work has cast doubt over the previously held paradigm that in the resolution of fibrosis, all activated HSCs undergo apoptosis (Iredale et al. 1998). Using different models of fibrosis and resolution coupled with HSC lineage tracing, it has been proposed that after removal of a fibrogenic insult, a proportion of activated HSCs inactivate into a quiescent like phenotype. In contrast to ‘normal’ HSCs, the inactivated HSCs respond rapidly to a new fibrogenic insult, resultant in an increase in fibrosis (Kisseleva et al. 2012; Troeger et al. 2012).

1.5 Liver fibrosis

1.5.1 Pathway of liver fibrosis
Fibrosis is the underlying cause in a substantial proportion of natural deaths and can be regarded as the process of aging in all organs (Wynn 2007). Despite recent advances this process is still not fully understood and, as yet, no approved anti-fibrotic drugs are in clinical use. Recent work has started to elucidate the biological processes involved in non-organ specific fibrosis and have highlighted the role of mesenchymal cells that are located around capillaries (Duffield 2012). Several names have been attributed to these cells, including peri-vascular cells, mural cells and pericyte-like cell. However, pericyte is now the preferred nomenclature (Duffield 2012). Recent studies have shown that pericytes are skin and muscle myofibroblast progenitors and in response to injury, adopt a migratory phenotype, become activated and deposit pathological matrix (Dulauroy et al. 2012). Visceral fibrosis follows a similar pathway and HSCs can be considered a pericyte, surrounding the hepatic sinusoid, the liver’s capillary. In this regard, understanding the process of fibrosis in the liver could provide a model to broader understanding of global fibrosis.

Liver fibrosis can be considered a wound-healing response. If the driving aetiology is removed, then the process can be entirely reversed (Ramachandran and Iredale 2012). However, perpetuation and chronic injury can result in persistent accumulation of
pathological extracellular matrix (ECM) and ultimately result in cirrhosis, associated with multiple complications and high mortality. A brief schematic outlining the process of fibrosis from insult to clinical outcomes is shown below in Figure 1-2.

![Figure 1-2: Pathway of liver fibrosis.](image)

Hepatotoxic factors initiate liver cellular injury through multiple mechanisms and toxic mediators/metabolites. Cellular injury cascades to activation of inflammatory processes and release of pro-inflammatory cytokines, resultant in the activation of HSCs. Activated HSCs have a large variety of pathological functions, including the deposition of ECM, causing cumulative fibrosis. Progressive fibrosis leads to cirrhosis and the associated complications.

### 1.5.2 Initial insults

Any external factor causing liver cellular death can be regarded as an insult; broadly divided into acute and chronic, depending on the length and persistence of the injury. Once over the initial phase of an acute insult, the liver architecture returns to normal and there is usually little evidence of the preceding injury. Chronic insult leads to chronic injury and this, in turn, provides the stimulus for the biological changes that ultimately result in fibrosis. The key feature of all insults is apoptosis and necrosis of liver cells. As hepatocytes are the most populace cell in the liver, they are the
predominant culprit, however, SEC apoptosis is also key in certain types of injury (e.g. ischaemia-reperfusion injury) (Malhi and Gores 2008). A list of causes of liver injury and the mediators that result in inflammation are shown in Table 1-2, with a brief description about the origin of the mediator.

<table>
<thead>
<tr>
<th>Causes of Liver Injury</th>
<th>Mediators</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol</td>
<td>Acetaldehyde</td>
<td>Toxic metabolites</td>
</tr>
<tr>
<td></td>
<td>Lipopolysaccharide (LPS)</td>
<td>Increased gut permeability</td>
</tr>
<tr>
<td>Hepatitis C virus (HCV)</td>
<td>HCV non-structural proteins</td>
<td>Protein cleavage</td>
</tr>
<tr>
<td>Cholestatic liver disease (e.g. PBC)</td>
<td>Bile acids</td>
<td>Decreased bile excretion</td>
</tr>
<tr>
<td>Non-alcoholic fatty liver disease (NAFLD)</td>
<td>Hyperglycaemia and ROS</td>
<td>Impaired glucose metabolism</td>
</tr>
<tr>
<td></td>
<td>Adipocytokines</td>
<td>Fat</td>
</tr>
<tr>
<td></td>
<td>Free fatty acids</td>
<td>Impaired lipid metabolism</td>
</tr>
</tbody>
</table>

Table 1-2: A list of hepatotoxic agents and the causative mediators of inflammation.

1.5.3 Inflammation

Chronic inflammation and fibrogenesis are inexorably linked in the pathogenesis of liver fibrosis. Broadly speaking, specific factors and pathogens (see Table 1-2) evade the liver’s physical barriers resulting in hepatic cellular apoptosis and necrosis. Chemical factors released by the injured cells drive the initial inflammatory response. A cellular response is instigated by resident leucocytes, including Kupffer cells, NK cells, polymorphonuclear cells and dendritic cells in response to the localised release of inflammatory factors (Szabo et al. 2007). Concurrent with this process, complement cascades are activated resulting in further release of inflammatory mediators, cellular recruitment and opsinisation of cells. This initial inflammatory response promotes migration and activation of non-resident neutrophils, macrophages and NK cells into the liver. These cells orchestrate phagocytosis of apoptotic and necrotic cells and also activate the adaptive immune response, bringing T-cells into the damaged liver. Alongside T-cells, there is a release of multiple cytokines, in response to the on-going injury, causing perpetuation of inflammation.
and activation of HSCs. A diagrammatic representation of this can be seen in Figure 1-3, with information of the key cytokines involved.

**Figure 1-3**: Initiation of inflammatory immune response and activation of HSCs.

Diagrammatic representation of inflammatory processes leading to activation of HSCs. Hepatotoxic injury to parenchymal and non-parenchymal liver cells initially provokes humeral response, followed by activation of Kupffer cells and recruitment of adaptive immune cells (T cells, NK cells and NKT cells). All of these processes produce cytokines capable of activating HSCs.

### 1.5.3.1 Inflammatory Mediators

TGF-β is widely recognised as a vital mediator of hepatic fibrosis (Gressner and Weiskirchen 2006). The three isoforms of TGF-β (TGF-β1, TGF-β2 and TGF-β3) have been investigated in liver fibrosis. TGF-β is produced in a latent form, with its cleaved pro-domain non-covalently bound to the active site. This regulates TGF-β’s bioavailability (Shi and Massague 2003). TGF-β can be held in complex with latent TGF-β binding proteins (LTBP) within the ECM and requires binding of the RGD-motif by integrin αv to facilitate activation. Activation requires more than integrin binding and cytokines, mechanical stress and ROS can be required to enable
activation (Shi et al. 2011). Macrophages primarily produce TGF-β and are instrumental in activation. In liver injury, TGF-β is produced by hepatocytes, Kupffer cells (resident macrophages), HSCs and SECs. The role of TGF-β has been extensively investigated, demonstrating its importance in activation of HSCs and prevention of ECM degradation (Armendariz-Borunda et al. 1992; Dooley and ten Dijke 2012). Hepatocytes secrete TGF-β in its latent form and non-parenchymal cells in both latent and active forms, to varying degrees. Once activated, TGF-β acts in both an autocrine and paracrine fashion, producing a positive feedback loop in HSCs (Bissell et al. 1995).

As a key mediator of fibrosis, the clinical utility of TGF-β measurement has been explored. In a clinical study of 38 patients using liver biopsy as gold standard, TGF-β has been reported to show correlation with increasing fibrosis in HCV (Kanzler et al. 2001). In particular, its ability to predict progressive disease was highlighted. However, clinical utility is limited due to TGF-β release from platelets in response to phlebotomy (Assoian et al. 1983).

The cytokine, platelet-derived growth factor (PDGF), is a potent proliferator of HSCs, inducing myofibroblastic change (Pinzani et al. 1989). PDGF binds to HSCs by the PDGF-receptor (PDGF-R) and causes functional change via phosphoinositol 3-kinase (PI3K) pathways. These pathways are also used by several other cytokines and integrins, including vascular endothelial growth factor (VEGF) (Hernandez-Gea and Friedman 2011). PDGF is found to be increased by immunohistochemistry stains in liver specimens from known cirrhotic livers, compared to controls (Ikura et al. 1997), but due to low specificity it is a poor biomarker of hepatic fibrosis.

1.5.3.2 Innate Immune Response
Necrotic cell death and the subsequent release of cell contents activate the innate immune system. The products released can be collective regarded as damage related molecular patterns (DAMPs) and include RNA, DNA, heat shock proteins and other factors (Figure 1-3) (Jaeschke 2011). As previously mentioned, the activation of macrophages and Kupffer cells is critical to the perpetuation of inflammation. The mode of activation has varying outcomes and classical activation occurs through toll-like receptor 4 (TLR-4). Kupffer cells strongly express TLR-4, the innate
immune receptor, and is activated by its main ligand, LPS and other factors. These include heat-shock proteins, IFN-α and other DAMPs (Jaeschke 2011). Downstream effectors include a host of cytokines and pro-inflammatory factors. TLR-9 activation of macrophages occurs with DNA fragments released from necrotic cells acting as the receptor ligand (Schwabe et al. 2006).

Alternative pathway activated (M2) macrophages are either recruited from the circulation (Holt et al. 2008) or activated in-situ as a result of a phenotype switch from M1 macrophages (Wynn and Barron 2010). IL-4 and IL-13 as opposed to IFN-γ and LPS, lead to alternative activation. M2 macrophages are considered to be predominantly anti-inflammatory and secrete regenerative factors (Wynn and Ramalingam 2012).

Kupffer cells release a multitude of signals, which can propagate fibrosis by HSC activation and differentiation into myofibroblasts, as well as aiding fibrosis resolution through inducing activated HSC apoptosis (Liu et al. 2010). The predominant macrophage effect is regulated by the stage of fibrogenesis and the interplay of cytokines (Xu et al. 2012).

In the majority of liver insults, the sterile inflammatory response is self-aggravating. Removal or inhibition of either the offending insult, down-regulation of the DAMPs released or inactivation of innate inflammatory cells (in particular macrophages) can attenuate the liver injury and oxidant stress.

LPS can be present at the site of injury from bacterial translocation and from pathogen associated release, in certain mechanisms of injury. LPS binds to TLRs in both Kupffer cells and HSCs. This results in activation of HSCs by two methods. Firstly, activating Kupffer cells causes a cascade of pro-inflammatory cytokines, including increased TGF-β, a key mediator of HSC activation. Secondly, LPS binding of TLR-4 on HSCs causes down regulation of the TGF-β pseudoreceptor BAMBI, effectively sensitising HSCs to the action of TGF-β (Seki et al. 2007).

Natural killer cells (NK cells) are a distinct group of innate immune cells and are rich in a variety of TLR receptors. The liver’s physiological anti-microbial role means
that it is abundant in these cells. In response to injury, NK cells induce apoptosis of activated HSCs and prevent their expansion, in effect limiting fibrosis (Jeong et al. 2006). Alcohol consumption inhibits NK cells, promoting fibrosis in any chronic, fibrotic liver disease (Gao et al. 2011).

1.5.3.3 Adaptive Immune Response

Endothelial adhesions molecules, chemokines released by parenchymal injury and the innate immune response recruit lymphocytes into the liver. The hepatic recruitment of effector cells and chemokines drives the latter phase of tissue damage. T cells enter the liver predominantly through the hepatic sinusoid even though they are capable of crossing at different regions of the microvasculature (Adams et al. 2010). Different subsets of T cells have varying functions in liver fibrosis. For example, T helper cells have both pro-fibrotic and anti-fibrotic roles. Th1 cells produce anti-fibrotic IL-2, whilst Th2 cells produce pro-fibrotic IL-4 and IL-6 (Xu et al. 2012).

Natural killer T (NKT) cells are a subset of T cells that exhibit some properties similar to NK cells. In contrast to NK cells in liver fibrosis, NKT cells’ function is less well understood and is thought to be generally pro-fibrotic. This is demonstrated in a study by Notas et al, whereby NKT depleted rodent livers demonstrated decreased fibrosis in response to BDL and CCl₄ injection (Notas et al. 2009).

B cell lymphocytes until recently were thought to have a very limited role in hepatic inflammation and fibrogenesis. However, in a study by Novobrantseva (Novobrantseva et al. 2005), they demonstrated attenuated fibrosis in B cell depleted murine models and suggested B cell IL-6 secretion promoted myofibroblast transdifferentiation and hence fibrosis.

Dendritic cells in the liver are believed to act as regulator of immunological tolerance and represent approximately 25% of the leucocyte population in fibrotic livers (Xu et al. 2012). In liver fibrosis, dendritic cell population is shown to increase five-fold and gain the ability to stimulate NK cells, HSCs and T cells (Connolly et al. 2009). Of further interest, this study demonstrated that dendritic cell depletion
resulted in complete abrogation of elevated levels of many inflammatory mediators (Connolly et al. 2009).

### 1.5.4 Activation of HSCs

The activation of stellate cells by inflammatory cytokines in response to an insult governs the change in matrix composition. This paracrine effect is particularly important in the initiation of HSC activation. Many of the cytokines and cells implicated in this stage have been outlined in section 1.5.3. After initiation, self-perpetuation and autocrine signalling become increasingly important drivers (See Figure 1-4).

**Figure 1-4: HSC activation and perpetuation.**

Pathway of HSC activation including mechanisms that provoke initiation and contribute to perpetuation. Neighbouring cells (Kupffer cells, SEC and hepatocytes) stimulate activation of HSCs by soluble factors. The perpetuating mechanisms include proliferation, contractility matrix degradation and chemotaxis. Fibrosis resolution occurs after removal of causative agents and activated HSCs undergo apoptosis, senescence and possibly revert back to a quiescent phenotype. From Friedman 2010.
As previously mentioned, HSCs undergo a continuum of phenotype change, initially becoming activated and, as the process proceeds, adopting the characteristics of a myofibroblast. In activation, HSCs become proliferative, fibrogenic, become chemotactic, contractile, loose their characteristic retinoids and attract other cells, in particular white blood cells.

1.5.4.1 Proliferation
PDGF and TGF-β are the most important factors in HSC activation and all forms of PDGF have been shown to be potent proliferative signals. Acting via tyrosine kinase receptors, PDGF-α and PI 3-K, they also facilitate the action of other tyrosine kinases, such as vascular endothelial growth factor (VEGF), adipokines and integrins (Friedman 2008).

1.5.4.2 Fibrogenesis
Fibrosis results in an increase in both in quantity of activated HSCs and an increase in matrix production, per cell (Iredale 2001). This is achieved by significant changes at the transcriptional level and post-transcriptionally (discussed in section 1.5.4.7). The dramatic alterations in the ECM that are the hallmark of fibrosis are discussed in section 1.5.5. The upstream signalling that perpetuates these changes, come from neighbouring cells and HSCs themselves. The TGF-β family, signalling through their receptors, regulates pro-collagen I and pro-collagen III production, via the intracellular SMA mothers against decapentaplegia (SMAD) proteins. Other fibrogenic stimuli are connective tissue growth factor (CTGF), VEGF, and lipid peroxides (ROS). These act in both TGF-β dependent and independent mechanisms (Friedman 2008).

1.5.4.3 Contractility
Contractility of HSCs has a variety of effects, including increased portal blood flow pressure; increasing mechanical stress and stiffness, perpetuating activation; and distortion of the local liver architecture. Recent studies have suggested that TGF-β, wingless integration 1-β-catenin (Wnt-β-catenin) and hyaluronan mediate this process in organ fibrosis by inducing EMT (Chen et al. 2011; Heise et al. 2011). As HSCs can be considered pericytes, it is understandable that they have additional roles in vascular permeability, stability and flow (Duffield 2012). With the
pathological dysregulation of fibrosis, angiogenic changes are witnessed. Regulation of this process is by nitrous oxide and the renin-angiotensin-aldosterone (RAA) system, specifically angiotensin II (Bataller et al. 2003).

1.5.4.4 Chemotaxis
Activated HSCs migrate into the parenchyma and act as chemo-attractants for other cells, especially leucocytes. This ensures that aHSCs are both at the site of inflammation and on-going fibrogenesis and attract other inflammatory cells to this region. A vast array of chemokines have been implicated in this process (Hernandez-Gea and Friedman 2011) and chief amongst them are PDGF and TGF-β.

1.5.4.5 Non-HSC myofibroblasts
Activated HSCs are not the only source of myofibroblasts within the liver. A variety of sources have been identified (shown in Figure 1-5). Portal fibroblasts have recently been highlighted as an important source of myofibroblasts, especially in the setting of biliary liver injury (Parola et al. 2008). The bone marrow has been postulated as a key source of MFs in liver cellular injury (Forbes et al. 2004; Russo et al. 2006). Additionally, GFP-labelled bone marrow cells have been found in the non-parenchymal liver cell population and found to be increased with CCl₄ liver fibrosis, in rodents (Baba et al. 2004). However, this has been recently questioned as bone marrow derived MFs contributed little Collagen I (Col1) to fibrosis in murine models (Higashiyama et al. 2009).
Figure 1-5: Sources of myofibroblasts in liver fibrosis.

Multiple sources of myofibroblasts have been suggested in liver fibrosis. The majority of MFs in liver fibrosis derive from activated HSCs, with portal fibroblasts and fibrocytes known to contribute. Influence of bone marrow derived myofibroblasts and EMT of hepatocytes remain controversial. From Hernandez-Gea and Friedman 2011.

1.5.4.6 Epithelial to Mesenchyme Transition

The process of epithelial to mesenchyme transition (EMT) in liver fibrosis, whereby epithelial cells detach from their basement membrane, become migratory and provide a mesenchymal MF population, is hotly debated. Proponents of EMT argue that in fibrosis, populations of cells with both mesenchyme (fibroblast-specific protein 1 (FSP-1 or S100A4) and $\alpha$-SMA) and epithelial (cytokeratin (CK) and E-cadherin) markers, suggesting an intermediate or transitional cell (Choi and Diehl 2009). Furthermore, known factors for EMT are also present in liver fibrosis, namely, growth factors PDGF and TGF-β, MMPs and hedgehog signalling (Zavadil and Bottinger 2005; Syn et al. 2009). Models of fibrosis have also demonstrated mesenchymal changes to mature hepatocytes, both in-vitro and in-vivo (Zeisberg et al. 2007). However, recent studies using lineage tracing of alpha foetal protein (AFP) positive liver cells have refuted fibrosis associated with EMT, finding no association of cells from an AFP positive lineage with mesenchyme markers, after inducing
fibrosis \textit{in-vivo} with BDL and CCl$_4$ (Taura et al. 2010; Wells 2010; Espanol-Suner et al. 2012).

1.5.4.7 Transcriptional Regulation

Mechanisms that facilitate a change in gene transcription are likely to alter the phenotypic profile of HSCs. Gene transcription is controlled by factors interacting with specific DNA binding sequences located within the regulatory areas of genes, such as the promoter or enhancer/repressor region. Transcriptional regulation of HSC activation is an area of considerable interest, with key transcription factors shown to either promote activation of HSCs or regulate collagen. A multitude of transcription factors have been identified and are involved in different mechanisms of HSC related fibrosis. These are shown in Table 1-3. As yet no single master controller of HSC activation / fibrosis perpetuation has been identified.

As shown in Figure 1-4, initiation and propagation of HSCs are regulated by different mechanisms. Broadly, transcriptional regulation can be divided into these two sub-groups. Initiation of HSC activation has been demonstrated by Kruppel-like factor 6 (KLF-6) and foxhead factors, Foxf1 and FoxO1 (Kalinichenko et al. 2003). This process is negatively regulated by several nuclear receptors, vitally peroxisome proliferator activated-\(\gamma\) (PPAR-\(\gamma\)) and pregnane X receptor (PXR) (Mann and Mann 2009). PXR induction maintains quiescence in human HSCs, but PXR is not expressed in rodent HSCs, suggesting that PXR independent pathways of activation exist (Haughton et al. 2006). Other nuclear receptors (PXR, PPAR-\(\gamma\), FXR, and RAR) have also been shown to promote quiescence and loss of these receptors is noted in up-regulation of collagen and ECM deposition (Mann and Mann 2009).

The PPAR family (\(\alpha\), \(\beta\), \(\gamma\) and \(\delta\)) are found in adipose cells and, in HSCs, PPAR-\(\gamma\) is thought to maintain adipogenic function and phenotype. Pioglitazone, a PPAR-\(\gamma\) agonist, has been shown to prevent HSC activation \textit{in-vitro} and ameliorate fibrosis \textit{in-vivo} (Bruck et al. 2009). Down-regulation of PPAR-\(\gamma\) expression perpetuates fibrosis by increased ECM production and fibrogenesis (Hazra et al. 2004).
The LIM homeobox transcription factor, Lhx-2, also appears to be essential for quiescence preservation of HSCs. Lhx-2 knockout is embryologically lethal due to neonatal liver fibrosis (Wandzioch et al. 2004)

The transcriptional profile of activated HSCs varies greatly from their quiescent state. Different transcription factors have been identified to induce different pathways of fibrosis perpetuation. SMAD 2, 3 and 4 have regulatory roles in different pathways of fibrosis, including ECM deposition, collagen production, and HSC proliferation (Dooley et al. 2001). SMAD-7, an inhibitor of TGF-β signalling, prevents HSC activation and liver fibrosis (Dooley et al. 2003). The Kruppel like factors, KLF-6 and Sp1, co-ordinate fibrosis perpetuation by driving collagen transcription, TGF-β signalling, and preventing ECM degradation by TIMP-1 expression (Table 1-3) (Mann and Mann 2009). KLF-6’s role in fibrosis perpetuation is less clear and recent studies examining the splice variants of KLF-6 have noted an increase in fibrosis in KLF-6 heterozygous mice (Ghiassi-Nejad et al. 2013). These data suggests that KLF-6 may be important to HSC activation but not in the regulation of fibrogenesis.
<table>
<thead>
<tr>
<th>Group</th>
<th>Transcription factor</th>
<th>Functional effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foxhead factors</td>
<td>Foxf1, FoxO1</td>
<td>HSC activation</td>
</tr>
<tr>
<td>Hedgehog</td>
<td>Gli-2</td>
<td>Osteopontin gene regulation</td>
</tr>
<tr>
<td>Kruppel-like factors</td>
<td>KLF-6, Sp1, BTEB</td>
<td>HSC activation, collagen gene regulation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collagen and TIMP-1 gene regulation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collagen gene regulation</td>
</tr>
<tr>
<td>Nuclear Receptors / Adipogenic signalling</td>
<td>PPAR-γ, Vitamin D Receptor, LXR, PXR, FXR</td>
<td>Quiescence, down-regulation of Collagen &amp; TGF-β synthesis</td>
</tr>
<tr>
<td>TGF-β signalling</td>
<td>SMAD (2, 3, 4), SMAD-3, SMAD-7</td>
<td>Collagen gene regulation</td>
</tr>
<tr>
<td>LIM Homeobox</td>
<td>Lhx-2</td>
<td>Quiescence</td>
</tr>
<tr>
<td>NF-κB signalling</td>
<td>NF-κB</td>
<td>Inflammation and apoptosis survival</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>AP-1, AP-2, Ets-1, SREB, C/EBP, Sox9, E Box binding proteins</td>
<td>TGF-β1, TIMP-1 gene regulation, Collagen synthesis, HSC activation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quiescence</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-apoptosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collagen synthesis</td>
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<tr>
<td></td>
<td></td>
<td>HSC activation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quiescence</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collagen synthesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collagen I, HSC activation</td>
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<tr>
<td></td>
<td></td>
<td>TGF-β gene regulation</td>
</tr>
</tbody>
</table>

**Table 1-3: List of transcription factors described in HSC activation.**

Transcription factors known to have roles in HSC activation. List produced from Mann and Smart 2002; Friedman 2006; Mann and Mann 2009.

The NF-κB transcription factor family is amongst the best studied. It is a complex family of 5 sub-units (p50, p52, p65, RelB, cRel), which form hetero or homodimers to create functional transcription factors. In an non-stimulated state, the classical dimer (p65:p50) is held within the cytoplasm by the inhibitory protein IκB-α. Stimulation can occur via TNF-α and LPS in the canonical pathway, with IκK
resulting in phosphorylation of IκB-α and nuclear translocation of the heterodimer, where it exerts its transcriptional effect. Non-canonical stimulation occurs by adipogenic signalling and removal of inhibition by PPAR-α. NF-κB is a key regulator of inflammation and has been described in hepatic fibrosis (Elsharkawy and Mann 2007). It has been shown to be induced in HSC activation and functions to promote the persistence of activated HSCs as a chronic wound healing response (Mann and Smart 2002). In this scenario, the normal negative feedback inhibition associated with increased NF-κB by its inhibitor IκB-β is diminished. The persistence of NF-κB signalling appears to be important in maintaining the activated HSC state (Lang et al. 2000). More recently, NF-κB has been shown to have a role in aHSCs survival by supressing JNK activation of p53 and preventing apoptosis (Watson et al. 2008). These effects will be further explored in section 1.6.

As shown in Table 1-3, many transcription factors have been described in diverse pathways of liver fibrosis. However, there remains a need to identify core factors regulating the process of fibrosis. Core pathways are likely to extend beyond an organ and be common to all fibrosis and be conserved across species (Mehal et al. 2011). SOX9 (sex-determining region Y-box 9) is a highly conserved transcription factor implicated in the development of many organs and ECM deposition during fibrosis (further information in Section 1.9) (Goldring et al. 2006). There are potent clues that SOX9 may be a core regulator of ECM with evidence of fibrosis in a variety of organs (Pritchett et al. 2011). For example, in liver fibrosis, attenuation of the Sox9 gene with small interfering RNA (siRNA) demonstrates reduction in both collagen I and α-SMA (Hanley et al. 2008). These data supports SOX9 as a potentially important transcription factor in the deposition of ECM in hepatic fibrogenesis. SOX9 and its role in fibrosis are further explored in Section 1.9.

1.5.4.8 Epigenetic Regulation

Gene expression can also be modulated without direct alteration to the transcriptome. This can occur in three ways: histone modification leading to alteration in chromatin structure and access to the genetic code, DNA methylation broadly silencing regions of genome and thirdly, silencing by non-coding microRNAs (Mann and Mann 2008). Histone modification and DNA methylation can confer cross-generational changes as they can be transferred in dividing cells. This has been demonstrated by decreased
susceptibility of offspring to fibrosis after CCl₄ was used to induce fibrosis in parents and grandparents (Zeybel et al. 2012). This study demonstrated that epigenetic factors ‘programme’ the genome to be more resistant to future insults and that these alterations are transferable between generations.

Small, non-coding RNAs, also known as microRNA or miRNA, bind messenger RNA causing alteration in their translation. Specifically, miR-27a and miR-27b have been shown to reverse activated HSCs into a quiescent phenotype (Ji et al. 2009) and is another complex layer of regulation beyond the genome.

1.5.5 Extracellular matrix

The normal composition of the liver basement membrane consists predominantly of laminin, heparansulfate proteoglycans, and the non-fibrillar collagen IV (Friedman 2008). Fibrillar collagen deposition is an integral component of fibrosis, with collagens I and III increasing with fibrosis. Initial synthesis is via a procollagen precursor. Enzymatic cleavage at both the carboxyl- and amino-terminal ends of the procollagen molecule by two distinct enzymes, procollagen C-proteinase and procollagen N-proteinase, results in the formation of collagen molecules (Laurent et al. 2007). Certain collagens, particularly collagens I and III in liver fibrosis, form insoluble fibrils. As fibrosis progresses the fibrils thicken into bands of scar, visible by light microscopy (Henderson and Iredale 2007).

1.5.5.1 Metalloproteinases

An important regulatory mechanism of ECM deposition and turnover involves matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitor of metalloproteinases (TIMPs). MMPs are a family of 25 ECM degrading enzymes. They are categorised by their substrate specificity, as outlined in Table 1-4. Some MMPs are membrane bound and referred to as metalloelastases or MT-MMPs (MMP-12, 14 and 15).

MMPs have a variety of functions and act on different ECM proteins, including collagens (fibrillar, gelatins, elastins), laminin, and fibronectin (Iredale et al. 2012). From gene transcription to active MMPs within the ECM, there are several different levels of regulation. There is initial, complex transcriptional control. For example,
MMP-9 is positively and negatively regulated by several micro-RNAs (Rutnam et al. 2012). Post-translation, most MMPs are secreted into the ECM as zymogens, where they are cleaved from a pro-form into an active protein. MMPs can also be functionally inactivated by TIMPs non-covalently binding to their active site. This bond is reversible under certain physiological conditions and both MMP and TIMP retain activity after separation (Iredale 1997). The N-terminal of TIMPs allows inactivation of active MMPs whilst the C-terminal has activity against pro-forms, preventing activation of the MMP zymogen (Iredale et al. 2012). Various cell types secrete different types and quantities of MMPs and TIMPs and subtle alterations in their relative ratios can alter quantity and quality of the surrounding ECM. Cellular sources of MMPs and TIMPs in the liver are shown in Table 1-4.

<table>
<thead>
<tr>
<th>Group</th>
<th>Name</th>
<th>Family</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenases</td>
<td>Collagenase-1</td>
<td>MMP-1</td>
<td>HSC</td>
</tr>
<tr>
<td></td>
<td>Collagenase-3</td>
<td>MMP13</td>
<td>HSC, Kupffer cells, SECs</td>
</tr>
<tr>
<td></td>
<td>Neutrophil Collagenase</td>
<td>MMP-8</td>
<td>Kupffer cells, neutrophils</td>
</tr>
<tr>
<td>Stromelysins</td>
<td>Stromelysin-1</td>
<td>MMP-3</td>
<td>HSC</td>
</tr>
<tr>
<td></td>
<td>Stromelysin-2</td>
<td>MMP-10</td>
<td>HSC</td>
</tr>
<tr>
<td></td>
<td>Stromelysin-3</td>
<td>MMP-11</td>
<td>Hepatocytes</td>
</tr>
<tr>
<td>Gelatinases</td>
<td>Gelatinase A</td>
<td>MMP-2</td>
<td>HSC, MF</td>
</tr>
<tr>
<td></td>
<td>Gelatinase A</td>
<td>MMP-9</td>
<td>HSC, Kupffer cells, Hepatocytes</td>
</tr>
<tr>
<td>Matrilysin</td>
<td>MT-MMP</td>
<td>MMP-7</td>
<td>HSC</td>
</tr>
<tr>
<td>Metalloelastases</td>
<td>MT-MMP-1</td>
<td>MMP-12</td>
<td>Macrophages, Kupffer cells</td>
</tr>
<tr>
<td></td>
<td>MT-MMP-2</td>
<td>MMP-14</td>
<td>HSC, Kupffer cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MMP-15</td>
<td>Hepatocytes, BEC</td>
</tr>
<tr>
<td>TIMPs</td>
<td>TIMP-1</td>
<td></td>
<td>HSC, MF, Kupffer cells, Hepatocytes</td>
</tr>
<tr>
<td></td>
<td>TIMP-2</td>
<td></td>
<td>HSC, MF, Kupffer cells</td>
</tr>
</tbody>
</table>

Table 1-4: MMPs and TIMPs in liver fibrosis.
A list of the main metalloproteinases involved in liver fibrosis and the source of their production. Adapted from Guo and Friedman 2007.

MMPs with interstitial collagenase are necessary for breakdown of the fibrillar collagens. MMP-1 (MMP13 in rodents), 2, 8 and 14 are implicated in the
degradation of collagen I and III, hence promoting resolution (Iredale 2004). These MMPs cleave collagen molecules making the ECM more susceptible to other MMPs for further degradation. MMP13 is expressed early in fibrosis by Kupffer cells, HSCs and SECs, stimulating a pro-fibrotic response by degrading native ECM surrounding cells. In rodent models MMP13 levels reduce as scar progresses; however, it has been shown to increase in resolution of fibrosis in both HSCs and Kupffer cells (Hemmann et al. 2007).

Collagenalytic activity decreases with progression of fibrosis. This is due to an increase in expression of TIMP-1 and 2 (Figure 1-6), and also as a consequence of ECM cross-linking making degradation of scar more difficult for proteinases. However, considering MMPs to be anti-fibrotic and TIMPs to be pro-fibrotic is too simplistic. For example, MMP-9 is present in the early stages of fibrosis and has been shown to activate latent TGF-β, promoting fibrosis and HSC activation and MMP-8 knockout mice have reduced fibrosis and TIMP-1 expression (Iredale 2004; Han 2006).

1.5.5.2 A Disintegrin and Metalloproteinases
A Disintegrin and Metalloproteinase (ADAM) are a family of membrane anchored metalloproteases capable of regulating ECM collagen deposition by cleaving MMPs, TIMPs, cytokines and collagens (Edwards et al. 2008). ADAM-8, 9, 12 and 28 are up regulated in activated HSCs and collagen producing myofibroblasts (Schwettmann et al. 2008). The expression of all these ADAMs was also noted to increase in histological liver samples of fibrosis from a variety of aetiologies. In particular, ADAM-12 positive pericytes have been shown to be positive for mesenchymal myofibroblast markers and producers of ECM, including Collagen I. Furthermore, knockout of ADAM-12 in muscle fibrosis models, led to ablation of fibrotic cells and limited collagen deposition (Dulauroy et al. 2012).
1.5.5.3 Therapeutic use of metalloproteinases

Due to the global function of MMPs in regulating ECM, non-targeted delivery is not a viable therapeutic option. However, adenovirus delivery of human MMP-1 (also known as collagenase-1) into rat livers resulted in reversal of fibrosis and degradation of type 1 collagen (Iimuro et al. 2003). These data is especially relevant as MMP-1 is not expressed in rodents but was still activated by cleavage from pro-form to active MMP-1. The transported MMP-1 was enzymatically active and delivery did not cause hepatic injury.

Using a similar adenovirus method of delivery, MMP-8 has also been shown to reverse cirrhosis in rodent models (Siller-Lopez et al. 2004). In this study, bile duct ligation and CCl₄ were used to induce cirrhosis prior to gene delivery into the liver. Extensive fibrosis reversal was observed coupled with improved liver function tests and proliferation of liver cells. Alteration in the balance of TIMPs and MMPs can
clearly have wide-ranging effects on fibrosis and ECM environment and provide an exciting opportunity in the development of anti-fibrotic therapy (Iredale 2004).

1.6 Fibrosis resolution

1.6.1 Mechanisms of fibrosis resolution

The paradigm that fibrosis or even cirrhosis was a non-reversible event has been widely refuted. In animal models of fibrosis, dramatic recovery has been noted, even after severe injury (Iredale 2007). This has been verified in liver biopsies from patients with fibrosis from a variety of aetiologies, showing improvement after removal of the causative agent (Ellis and Mann 2012). Cirrhosis is not a static endpoint and should be considered a dynamic and bi-directional process. Resolution of cirrhosis remains hotly debated. The reason for this is most likely the quantity of the fibrotic area and its insoluble quality, with complicated cross-linked regions proving inaccessible to degradation (Friedman and Bansal 2006).

An improved understanding of the processes of fibrosis resolution is leading research into anti-fibrotic therapy. The elimination of MFs is thought to be central to fibrosis resolution. During resolution, apoptosis of MFs was thought to be ubiquitous (Gressner 2001). Recent research, using lineage tracing of HSCs, has led to an alternative fate. It is suggested that the majority of aHSCs undergo apoptosis, whilst a sub-population revert to an ‘inactive’ or ‘primed’ phenotype, limiting pathological collagen production. These cells, however, respond very quickly to further insults and promote a more dramatic fibrotic response (Kisseleva et al. 2012).

1.6.1.1 Apoptosis

HSC removal occurs by a variety of immune cells, including Kupffer cells, NK cells, T cells and NKT cells. The ultimate action of all cells is by activation of caspase enzymes (proteases that cleave aspartate residues) leading to apoptosis. This can be regulated through death receptor or mitochondrial pathways (Kong et al. 2012). TNF-related apoptosis inducing ligand (TRAIL), TNF-α and Fas ligand (FasL) are ligands for death receptors. Death receptors, TRAIL receptors 1 and 2, induce
apoptosis by classical caspase activation. NK cells expressing TRAIL promote
apoptosis of MFs and aHSCs but are harmless to hepatocytes and quiescent HSCs
(Malhi and Gores 2008). Janus kinase–signal transducer and activator of
transcription (JAK–STAT) pathway can be activated by various cytokines. Signal
transducers and activator of transcription-1 (STAT-1) has been demonstrated to have
anti-fibrotic and pro-apoptotic effects (Kong et al. 2012). STAT1 knockout mice had
reduced death of HSCs by NK cells, owing to a reduced expression of TRAIL (Jeong
et al. 2006).

Disruption of HSC cell-cell interaction has also been demonstrated as a model of
apoptosis (Hartland et al. 2009). In this study N-cadherin, a cell-cell adhesion
membrane glycoprotein, was demonstrated to increase as HSCs activated and
promote cellular survival. Cleavage of N-cadherin by MMP-2 resulted in apoptosis
of HSCs. As HSCs themselves produce MMP-2, this underlines a potential auto-
regulatory mechanism.

Resistance to apoptosis is an important attribute of activated HSCs and MFs and
therefore, it is a key component of fibrosis resolution. Inhibitors of apoptosis include
canonical activation of the NF-κB system and TIMP-1, through the induction of B-
cell lymphoma 2 (Bcl-2). Activated HSC apoptosis was increased by inhibition of
κB kinase inhibitor (IKκB) and this has been postulated as a potential anti-fibrotic
strategy (Oakley et al. 2005).

1.6.1.2 Inactivation HSCs
Two recent studies have demonstrated that potentially not all activated HSCs
Using different rodent models to trace HSCs through fibrosis and repair, it has been
shown that some activated HSCs revert towards a quiescent-like phenotype. They are
distinct from actual quiescent HSCs by their transcription profile and BAMBI, HSP-
70, and PPAR-γ have all been suggested as putative markers for an inactive
phenotype. BAMBI is a TGF-beta pseudoreceptor with varying transcription in
HSCs. The roles of PPAR-γ have previously been described in Section 1.5.4.7 and,
like BAMBI, it is down-regulated in activated HSCs. Heat shock protein-70 (HSP-
70) is an anti-apoptosis protein and up-regulation in inactivated HSCs may confer
survival advantage from activated HSCs that undergo apoptosis. The inactivated HSC cellular population also demonstrates a more aggressive fibrotic response to further liver injury. This was elegantly demonstrated using wild-type, healthy mice, which were injected with extracted inactivated HSCs. Upon liver injury, these animals went on to have a more florid fibrotic response, compared to controls (Kisseleva et al. 2012).

1.6.2 Liver development and post-injury repair

The liver demonstrates remarkable regenerative qualities. After injury causing a reduction in liver cell mass, repopulation occurs by hepatocyte self-renewal. In chronic injury or a very significant insult, this regenerative capacity is overwhelmed. Liver progenitor cells proliferate from their peri-portal location; a phenomenon known as ductular reaction. In murine models, progenitor cells have been demonstrated to differentiate into functional hepatocytes, suggesting an alternative method for liver repopulation (Espanol-Suner et al. 2012).

This phenomenon mimics developmental processes (Lemaigre 2009). During normal liver development, a selection of hepatoblasts around the forming portal tract begins to express markers of biliary ductular cells. These include SOX9, osteopontin (OPN) and cytokeratin 19 (CK-19) and are referred to as the ductal plate (Antoniou et al. 2009). In murine models ductal plate cells go on to contribute towards biliary epithelial cells and have been shown to contribute towards peri-portal hepatocytes (Carpentier et al. 2011). Taken together, these three studies suggest commonality between the ductal plate and the ductular reaction seen in chronic injury.

The study by Espanol-Suner et al exhibited supply of only peri-portal hepatocytes by progenitor cells in liver injury. Peri-central hepatocyte repopulation was considered to be orchestrated by hepatocytes themselves. This was in conflict to a study Furuyama et al, who have suggested that ductular progenitor cells supply progenitors from portal to central areas, essentially repopulating all liver hepatocytes after injury and partial hepatectomy (Furuyama et al. 2011). This divergence may be explained by differing models of injury used in the studies, with Furuyama et al using a very short acute injury model as opposed to chronic fibrosis induction used by Espanol-Suner et al.
The link between an embryonic ductal plate and ductular reaction after injury has been postulated previously (Santoni-Rugiu et al. 2005). The presence of shared markers, namely SOX9, OPN and cytokeratin, corroborates this hypothesis (Carpentier et al. 2011). In fibrosis the precise functional role of progenitor cells remains elusive and if their role extends beyond cellular repopulation to fibrogenesis itself needs further exploration.

1.7 Diagnosis of liver fibrosis

In the majority of cases liver fibrosis is clinically silent until the latter, end-stages. Chronic hepatitis C (CHC) for example can remain undetected for upwards of 20 years and present with complications of cirrhosis, a point at which therapy is of limited benefit. Understanding the extent of the fibrosis before symptomatic presentation provides clinicians with information, which can be used to tailor therapy. Therefore, assessment of the stage of fibrosis has remained crucial for disease investigation and management.

1.7.1 Liver Biopsy

Presently, the gold standard of assessment is the liver biopsy (Rockey et al. 2009). In the context of chronic liver disease, liver biopsy can be used to establish diagnosis and help with therapeutic decision-making. As an important tool for prognosis and management, the information from a liver biopsy has been refined to provide data on fibrosis stage and inflammatory activity (Knodell et al. 1981; Ishak et al. 1995). This reduces intra-observer variability and provides a comparable, standardised system.

Liver biopsy is not a ideal investigation. Liver biopsy is an invasive procedure with associated morbidity and mortality (Myers et al. 2008). Due to this risk, its use is limited and sequential biopsies are not recommended to monitor disease progression or success of therapeutic intervention (Rockey et al. 2009). The quality of tissue obtained can be variable and dependent on type of needle used, operator and lobe sampled (Bedossa et al. 2003). Interpretation of the samples is also subjective, with variability between two observers and the same observer over a time period (Regev
et al. 2002). Due to these limitations, alternative methods for the assessment of liver disease are a key area of research.

1.7.2 Serum biomarkers

Multiple markers have been proposed to assist in the assessment of fibrosis (Manning and Afdhal 2008). An ideal biomarker should have the capability to provide information regarding the level of fibrosis/cirrhosis and the rate of progression, as well as having liver specificity and being relatively simple to perform (Afdhal and Nunes 2004). It seems unlikely that a single biomarker would be able to provide all this information, resulting in assessment of biomarkers in panels. Individual biomarkers are diametrically divided into direct and indirect groupings. Direct biomarkers are assays for ECM turnover and have been discovered by understanding the mechanism of liver fibrosis. Indirect markers usually assess changes in hepatic function and not necessarily ECM turnover. These are generally identified by statistical analysis to best correlate with fibrosis. Despite being a somewhat simplistic division, it provides a framework and understanding of biomarker derivation.

1.7.2.1 Direct Markers

Several different collagens are implicated in hepatic fibrogenesis and have been tested as potential biomarkers. Most promising amongst these is procollagen type III amino-terminal peptide (P3NP). P3NP has been studied in comparison to other biomarkers, biomarker panels and histology. Compared against biopsy in a number of chronic liver disease, it showed promise at identifying inflammatory activity but not stage of fibrosis (Trinchet et al. 1991; Giannini et al. 2001). P3NP performed favourably in biomarker panels European liver fibrosis panel (ELF) and traffic-light test (see Table 1-5). P3NP used in conjunction with the PGA Index (prothrombin time, γ-GT, apolipoprotein A1) it increased sensitivity and specificity of fibrosis stage compared to PGA Index alone (Teare et al. 1993). Further studies have demonstrated reduced P3NP in serum following therapy in autoimmune hepatitis (AIH) (McCullough et al. 1987) and abstaining from alcohol in alcoholic liver disease (ALD) (Niemela et al. 1990). P3NP has also been shown to be useful in monitoring liver fibrosis following methotrexate therapy in rheumatoid arthritis
(Chalmers et al. 2005; Maurice et al. 2005), but not in methotrexate therapy with psoriatic arthritis (Lindsay et al. 2009).

Collagen I is extensively found in the ECM with progressing fibrosis and is the archetypal collagen of fibrosis. Surprisingly given its crucial role, serological assessment of both collagen I and its precursor have been suboptimal. C-terminal procollagen I peptide was investigated as a potential biomarker. However, it appears to be inferior to P3NP at reflecting improvement after therapy with interferon in HCV and is poorer at identifying fibrosis in mild to moderate disease (Gallorini et al. 1994). Serum levels of type I collagen are increased in patients with chronic liver disease and correlate with fibrosis score but not with the inflammatory activity score (Trinchet et al. 1991).

Collagen IV is a normal constituent of the hepatic sub-endothelial membrane. In fibrosis, collagen IV is increased in biopsy specimens (Hahn et al. 1980). Several different assays for collagen IV are available and they appear to produce good correlation with disease (Murawaki et al. 1996). Serum type IV collagen levels are increased in patients with chronic liver diseases due to hereditary haemochromatosis compared with normal controls (George et al. 1999). When compared against liver biopsy, collagen IV showed 100% sensitivity and 68% specificity for severe (F3-4) fibrosis, particularly peri-sinusoidal fibrosis in alcoholic liver disease (Ueno et al. 1992). In a study of patients with chronic HCV, Collagen IV increased with progression of fibrosis. Used together with P3NP, they showed a sensitivity of 87% and specificity of 97% in detecting fibrosis compared to normal controls (Attallah et al. 2007). In non-alcoholic steatohepatitis (NASH) collagen IV showed superiority to hyaluronan in detecting the presence fibrosis (Yoneda et al. 2007).

Several MMP have been identified as potential biomarkers, but due to the dynamic nature of fibrosis only a few have shown promise as biomarkers. MMP-2 and MMP-9 are increased in hepatocytes of patients with HCV (Nunez et al. 2004). Independently, MMP-2 has been disappointing (Murawaki et al. 1999; Murawaki et al. 2001) but in combination with TIMP-1 in HCV, diagnostic accuracy is improved (Kasahara et al. 1997). In HCV, serum assay and liver biopsy samples show a
reduction of MMP-2 after therapy in responders compared to non-responders, but did not correspond to estimation of fibrosis stage (Marinosci et al. 2005).

TIMP-1 is amongst the widest used biomarkers of liver fibrosis. TIMP-1 can accurately diagnose the presence of fibrosis in HCV and ALD, but was unable to predict fibrosis progression with reasonable accuracy (Rosenberg et al. 2004; Lieber et al. 2008). In studies looking at both TIMP-1 and MMP-1, plasma levels altered appropriately to responders and non-responders with lamivudine therapy in HBV. They also corresponded to histological changes and aminotransferases levels (Flisiak et al. 2004). TIMP-1 is used in conjunction with hyaluronan, P3NP, and age in ELF score to detect cirrhosis in ALD and NAFLD (Rosenberg et al. 2004). MMP-1/TIMP-1 ratio was used to assess improvement in patients on mono-therapy for HCV with positive results (Ninomiya et al. 2001). This observational study, however, was not appropriately designed to assess the MMP-1/TIMP-1 ratio as a biomarker.

Laminin is a non-collagenous glycoprotein and is a common constituent of basement membrane. In fibrosis, laminin is deposited around vessels, peri-sinusoidal spaces, and portal tracts (Hahn et al. 1980). Laminin and its P1 fragment have been highlighted as biomarkers. Laminin has clinically been assessed in comparison to hyaluronan and found to be superior at identifying acute alcoholic hepatitis (Annoni et al. 1989), and at predicting complications in patients with known cirrhosis (Korner et al. 1996).

Hyaluronan (previously hyaluronic acid) is a glycosaminoglycan produced by HSCs and degraded by hepatocytes. Hyaluronan is regarded as one of the better available biomarkers. It has shown to work well in panels (e.g. SHASTA, Hepascore, Fibrospect, ELF) with TIMP, P3NP and age (see Table 1-5) in ALD (Rosenberg et al. 2004) and NAFLD/NASH (Miele et al. 2009). Independently, In ALD, it shows correlation with histological fibrosis and P3NP (Pares et al. 1996) and in HCV could discriminate between Ishak stages on biopsy (Mehta et al. 2008). Comparison with collagen IV in HCV and NASH has produced conflicting results with hyaluronan appearing to be superior in HCV (Murawaki et al. 2001), however in NASH the opposite was noted with hyaluronan unable to discriminate stages of fibrosis after correction for known NASH risk factors (Yoneda et al. 2007). Comparing
hyaluronan to P3NP indicated superiority in primary biliary cirrhosis (Nyberg et al. 1992). Like laminin, hyaluronan can be predictive for complications of chronic liver disease (CLD) in HCV cirrhosis (Guechot et al. 2000).

Chondrex (YKL-40) is a glycoprotein and amongst the newer biomarkers under assessment. It has been used in several models of inflammation but performed poorly at identifying fibrosis in NAFLD (Malik et al. 2009). In HCV, pre-treatment levels are an independent predictor of initial virological response to therapy, with a decrease in assay in responders (Saitou et al. 2005; Fontana et al. 2009). YKL-40 is able to discriminate for the presence of any fibrosis in ALD and correlated with survival in more advanced disease (Nojgaard et al. 2003). The correlation with survival in this study may have reflected advancing fibrosis stage, which also corresponded to survival.

Factors implicated in the inflammatory process are another area of biomarker research. With inflammation driving fibrosis, hypothetically these factors may be assays of fibrogenesis. However, their lack of liver specificity and non-linear relationship with scar quantity has generally resulted in poor performance. Cytokines investigated include tissue growth factor alpha (TGF-α), connective tissue growth factor (CTGF), epidermal growth factor (EGF) and hepatocyte growth factor (HGF) (Manning and Afdhal 2008). TGF-α levels are increased in patients with cirrhosis, correlating with bilirubin and Childs-Pugh classification, suggesting they are related with the severity of liver dysfunction but not necessarily fibrosis (Harada et al. 1999). EGF and HGF are elevated in patients with HCV and decrease during therapy. However, the differences seen in responders and non-responders were not statistically significant (Anatol et al. 2005). Though these studies suggest a relationship with disease process no correlation with histological staging has been demonstrated.

1.7.2.2 Indirect Markers

Indirect markers of hepatic fibrosis are usually identified via statistical analysis of serum/urine of patients with fibrosis staged by a liver biopsy. These parameters are diverse and are rarely derived from a mechanistic understanding of fibrosis. Consequently they are infrequently used as individual markers. More commonly,
they are combined with direct biomarkers to produce panels with ability to provide information regarding disease presence, stage and/or progression (see Table 1-5).

Markers of hepatic function, including prothrombin time, bilirubin and albumin have been routinely used in clinical practice and in classification systems such as the Child’s-Pugh score to provide mortality information. Though this was initially designed to predict mortality in patients undergoing surgery with portal hypertension (Pugh et al. 1973), its use has now been expanded and it is more frequently used as a method for assessing prognosis in adults with cirrhosis (Cholangitas et al. 2006).

Aminotransferases and other hepatic enzymes (e.g. γ-GT) have also been assessed, both independently and in combinational ratios. Comparing the ratio of two aminotransferases, AST and ALT, has been considered a simple method for detecting cirrhosis; however, inconsistent results limit its use. Combining ALT with platelet count in the APRI score has shown greater promise, in particular at diagnosing significant fibrosis in CHC (Shaheen and Myers 2007).

Platelet count, age and leucocyte count have all been proposed in multi-parameter tests. Though not sensitive or specific markers independently, they can in combination be useful in detecting presence/absence of fibrosis and aid in staging disease (Adams et al. 2005; Koda et al. 2007; Vallet-Pichard et al. 2007; Guha et al. 2008).

1.7.2.3 Biomarker Panels
In order to improve sensitivity and specificity, biomarkers have been combined and assessed in their ability to diagnose fibrosis and correlate with fibrosis staging scores. The majority of these panels employ both direct and indirect markers (see Table 1-5). Generally, these panels have been derived in large cohorts using multivariate logistical regression modelling (i.e. ELF, Fibrotest, APRI, Hepascore). However, some have been derived by clinical experience. Examples of this include ALT: AST ratio and the ‘traffic light’ test (Sheron et al. 2012).

Multi-parameter panels themselves have been compared to assess their efficacy. In HCV, FibroTest and FIB-4 appear to provide the highest accuracy at diagnosing
fibrosis with FIB-4 having a superior cost-effectiveness (Adler et al. 2008). The more popular fibrosis biomarker panels are shown in Table 1-5 alongside their diagnostic accuracy and the types of fibrosis in which they have been assessed.
<table>
<thead>
<tr>
<th>Name</th>
<th>Parameters</th>
<th>Disease</th>
<th>AUROC / Sensitivity / Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELF score</td>
<td>P3NP, Hyaluronan, Age, TIMP-1</td>
<td>NAFLD/ALD</td>
<td>AUROC 0.9, AUROC 0.84, sens. 90%, spec. 41%</td>
<td>(Guha et al. 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Rosenberg et al. 2004)</td>
</tr>
<tr>
<td>FibroTest</td>
<td>α2-macroglobulin, γGT, bilirubin, apolipoprotein A, haptoglobin</td>
<td>HCV Mixed (meta-analysis)</td>
<td>Sens. 92%, spec. 29% AUROC 0.84</td>
<td>(Rossi et al. 2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Poynard et al. 2007)</td>
</tr>
<tr>
<td>Traffic-light test</td>
<td>Hyaluronan, P3NP, Platelet count</td>
<td>ALD Mixed (meta-analysis)</td>
<td>AUROC 0.83 any fibrosis and 0.88 significant fibrosis</td>
<td>(Sheron et al. 2012)</td>
</tr>
<tr>
<td>Actitest</td>
<td>Fibrotest + ALT</td>
<td>HCV Mixed</td>
<td>AUROC 0.79</td>
<td>(Halfon et al. 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>meta-analysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APRI</td>
<td>AST, Platelets</td>
<td>HCV</td>
<td>AUROC 0.76 (significant fibrosis). Sens 81%, spec 50%</td>
<td>(Shaheen and Myers 2007)</td>
</tr>
<tr>
<td>PGA</td>
<td>Prothrombin time, γGT, apolipoprotein A1</td>
<td>ALD</td>
<td>Sens. 91%, spec. 81%</td>
<td>(Teare et al. 1993)</td>
</tr>
<tr>
<td>Hepascore</td>
<td>Age, gender, bilirubin, γGT, α2-macroglobulin, hyaluronan</td>
<td>HCV</td>
<td>AUROC 0.85 (sig fibrosis), sens. 92%, spec. 67%</td>
<td>(Adams et al. 2005)</td>
</tr>
<tr>
<td>FIB-4</td>
<td>Platelet count, AST, ALT, age</td>
<td>HCV</td>
<td>AUROC 0.85 (sig fibrosis)</td>
<td>(Vallet-Pichard et al. 2007)</td>
</tr>
<tr>
<td>FibroIndex</td>
<td>Platelet count, AST, γ-globulin</td>
<td>HCV</td>
<td>AUROC 0.83</td>
<td>(Koda et al. 2007)</td>
</tr>
</tbody>
</table>

Table 1-5: Common biomarker panels.

Table showing the current available biomarker panels and their constituent variables. The validated diagnostic ability of panels is also shown.
1.7.3 Non-invasive Assessment

Cross-sectional imaging is often used synergistically to provide anatomical information regarding the presence of portal hypertension or focal lesions. Commonly used imaging, such as ultrasound and computed tomography (CT) will only identify advanced fibrosis (Harbin et al. 1980), usually by noting the changes in liver structure and the presence of complications from portal hypertension. Modifications to standard ultrasonography have been appraised in detecting cirrhosis. In a study comparing caudate to right lobe width, a sensitivity of 84% (ability of the test to detect disease compared to the gold standard, liver biopsy) and specificity of 100% (ability of the test to minimise false positives) were observed for advanced fibrosis. Similar work using multiple sonographic parameters and Doppler measurements has demonstrated equivalent sensitivity (greater than 80%) (Talwalkar et al. 2007; Goyal et al. 2009). Microbubble contrast enhanced ultrasound is a new technique and it has been used to assess the level of fibrosis by measuring hepatic vein transit time as a surrogate marker of resistance and hence fibrosis. This has been shown to be useful in differentiating between mild fibrosis and cirrhosis (Searle et al. 2008). However, availability, technical difficulties and operator expertise are obstacles to routine clinical use.

1.7.3.1 Cross-sectional Fibrosis Assessment

Magnetic resonance imaging has been used to provide detail in excess to simple anatomical information outlined previously. Methods using apparent diffusion coefficient, dynamic contrast-enhanced MRI, and measurement of liver stiffness using MR elastography have been employed to assess level of liver disease. Trial results with the first two techniques have shown inadequate sensitivity and specificity to justify the costs involved (Aube et al. 2004; Yin et al. 2007). MR Elastography uses the principle of increased liver stiffness due to fibrosis with early work suggesting correlation resulting in the ability to distinguish normal from fibrotic liver and mild from severe fibrosis (Yin et al. 2007). The high equipment cost, however, has made clinical use very limited.

1.7.3.2 Transient Elastography

Ultrasound assessment of liver stiffness is becoming increasingly common in routine clinical assessment of liver fibrosis. Commercially available devices such as
Fibroscan™ send a pulsed wave through the liver and detect the returning echo to produce a measurement that relates to the stiffness of the liver (Foucher et al. 2006; Fraquelli et al. 2007; Friedrich-Rust et al. 2008). This simple to use, bedside test can be employed to longitudinally assess liver stiffness as a surrogate marker for fibrosis (Friedrich-Rust et al. 2008). Limitations of this method include difficult and variable measurements in patients with a raised body-mass index, decreased reproducibility due to steatosis and anatomical variation. Longitudinal assessment can also be problematic, as a patient’s body habitus may well change over a period of therapy (Yoshioka et al. 2008).

1.7.3.3 Acoustic radiation force impulse imaging
Acoustic radiation force impulse imaging (ARFI) is a method of non-invasively assessing liver fibrosis by measuring speed of shear wave and has the benefit of being integrated into an ultrasound machine. This method has been employed to detect cirrhosis with very high accuracy (AUROC 0.99) and additionally spleen stiffness can be used to determine the presence of oesophageal varices (Ye et al. 2012).

Despite significant advances in the use of imaging to provide surrogate information regarding liver fibrosis, biopsy remains necessary. This is due to the limitations in reliability and cost. To this end there is a need to develop methods to detect presence and progression of fibrosis.

1.8 Prognostic markers of fibrosis
The biological processes outlined in section 1.5 suggest a progressive clinical disease. The rate of fibrosis progression is variable and several factors have been implicated in predicting advancing disease. Amongst the best studied diseases is chronic hepatitis C (CHC). CHC is the world leading cause of hepatocellular carcinoma (HCC) and liver related death. 150 million people are infected worldwide and 350,000 deaths are attributed to CHC (World Health Organization 2012). This RNA virus was first described in 1989 (Lau et al. 1993). Transmission is predominantly blood-borne and is a particular problem in the injecting drug-user community. 90%
of those infected go on to develop chronic carrier state. Of these, 90% will go on to have progressive fibrotic disease and 20-30%, left untreated, will progress to cirrhosis and its complications (Mohsen and Trent 2001). As stated earlier, the rate of progression is very variable and the time between infection and cirrhosis has been reported to vary between 10 to 40 years (Poynard et al. 2001). Epidemiological risk factors for progression include age at infection, male gender and alcohol consumption. Inflammatory activity, as graded histologically, is only weakly associated with progression (Yano et al. 1996). Whilst genotype and mode of infection did not show any correlation (Poynard et al. 2001). However, in the majority of cases, age of infection is unknown and alcohol consumption can be variable over decades, resulting in these factors having limited clinical use. Because of this, more objective predictors of liver fibrosis progression have been sought.

Fibrotest™ a biomarker panel initially designed to assess fibrosis in CHC and NAFLD has been explored as a predictor of fibrosis progression in large population study, showing again concordance with liver biopsy to suggest male gender and age are important predictors of progression. In addition, HIV co-infection also increased risk (Poynard et al. 2012). This study provided corroboration for other chronic fibrotic conditions, including chronic hepatitis B (CHB), alcoholic liver disease (ALD) and non-alcoholic liver disease (NAFLD). However, a clear weakness of this study is that only a single biopsy is used and birth is used as a surrogate for index biopsy, assuming a non-fibrotic liver at birth.

Genome-wide association studies have also investigated possible single nucleotide polymorphisms (SNPs), which may explain differences in liver fibrosis progression amongst individuals (Patin et al. 2012). This study identified 4 SNPs in genes regulating phagocytosis and apoptosis. 25% of patients who progressed at a faster rate had the higher risk SNPs compared to 5% of those that did not. This study has proven interesting and stimulated further avenues for biomedical research. However, at present it provides limited clinical utility.

The hepatitis C anti-viral treatment against cirrhosis (HALT-C) is a long-term, prospective study following a large cohort of patients infected with CHC. This has allowed assessment of liver fibrosis progression as many patients have had serial
biopsies and are extensively phenotyped. This has led to several publications attempting to discover potential markers of fibrosis progression. Using a multivariate regression model, hyaluronan and platelet count were found to predict progression (Fontana et al. 2010). However, the patient group selected had a minimum of Ishak stage 3 fibrosis and the area under the receiver-operator curve (AUROC) gave a value of only 0.663 (Fontana et al. 2010). The HALT-C trial has also confirmed clinical outcomes and their relation to fibrosis stage and demonstrated that a higher Ishak stage on a liver biopsy predicts an increased risk of a liver related outcome (Everhart et al. 2010) and shown rates of progression between biopsies vary considerably, especially in early stages of fibrosis (i.e. Ishak 0-2) (Hoefs et al. 2011).

Surrogate markers to predict progression have been investigated and including $^{13}$C$\text{C}_2$-aminopyrine breath test ($^{13}$C-ABT) (Rocco et al. 2012). This novel method is one of the very few tests that have shown promise in predicting progression of fibrosis, using sequential biopsy as a marker of progression.

Categorising patients with early fibrosis is becoming of increasing importance. As demonstrated by Hoefs et al. in early fibrosis, rates of progression between individuals are very variable and only weakly associated with initial biochemical parameters (Hoefs et al. 2011). Delineating those more likely to progress is important for therapeutic decision-making and surveillance. There are also implications for research, as sub-grouping these patients provides a fairer platform from which new therapies can be assessed.

1.9 **SOX9**

A multitude of pathways and regulatory mechanisms have been proposed to govern liver fibrosis. In order to improve understanding of the fibrosis process it is imperative to understand the difference between pathways specific to liver fibrosis and those generic to organ fibrosis. This distinction may allow the tailoring of anti-fibrotic strategies towards organ specific pathways and separate out ‘core’ mechanisms of fibrosis (Mehal et al. 2011). Cross-species conservation of fibrotic pathways is more likely to indicate ‘core’ factors of fibrosis. The transcription factor
sex-determining region Y-box 9 (SOX9) is a highly conserved factor involved in ECM regulation during development (Gordon et al. 2009). Recently SOX9 has been implicated in a diverse variety of disease states, including liver fibrosis (Hanley et al. 2008) and glomerulosclerosis (Miura et al. 2007; Sumi et al. 2007).

1.9.1 SOX9 in Development

SOX9 is a member of the SOX family of transcription factors, named after the first member of the gene family identified, SRY (for sex determining region of the Y chromosome). SOX proteins are grouped into the same family as SRY due to their high homology (>50%) to the high mobility group (HMG) DNA binding domain. Most HMG domains of SRY and SOX proteins bind to the core binding sequence AACAAT (Harley and Goodfellow 1994) and related motifs (Sudbeck et al. 1996; Kamachi et al. 1999). Like other members of the SRY family, SOX9 plays a pivotal role in the development of a range of tissues (Foster et al. 1994). Haploinsufficiency of SOX9 causes the skeletal disorder campomelic dysplasia primarily characterised by congenital bowing of the legs and defective cartilage formation (Wagner et al. 1994). Homozygotic SOX9 mutation is fatal intra-uterine in both rodents (Kist et al. 2002) and humans (Foster et al. 1994). Mutations of SOX9 are also associated with autosomal XY sex reversal (Foster et al. 1994), which reflects its role in the development of Sertoli cells in the male gonad (Kent et al. 1996).

SOX9 has been implicated in the development of many organs and tissues (Pritchett et al. 2011) however it is best characterised in chondrogenesis. Endochondral ossification occurs in two stages, chondrogenesis and calcification. In chondrogenesis, mesenchyme tissue differentiates into chondrocytes and begins to secrete the ECM that becomes the cartilaginous template around which bone is formed. SOX9 is necessary for this process and is copiously expressed in mesenchymal cells as they commit to a chondrocyte lineage (Bi et al. 1999). Functionally SOX9 activates transcription of ECM factors, including collagens type-2, 9, 11 and 27 (Akiyama 2008), Aggrecan (Sekiya et al. 2000), Matrilin-1 and Cartilage oligomeric protein (Liu et al. 2007). SOX9 needs to be transcriptionally silenced for the final differentiation of chondrocytes and for ossification to take place (Akiyama 2008). Silencing of SOX9 halts the proliferation of chondrocytes, determining bone size and dimensions.
SOX9 has been implicated in the development of many tissues (Pritchett et al. 2011) as outlined in Table 1-6. SOX9 acts as regulator of ECM in a variety of organs, controlling the peri-cellular environment and cellular differentiation. Notable examples include pancreas, testis, and bile duct development. Mounting evidence also centres on SOX9 in liver development (Carpentier et al. 2011). These concepts were previously discussed in Section 1.6.2.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Relevance to SOX9</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tests</td>
<td>SOX9 / SF1 interaction regulates <em>AMH</em></td>
<td>Tests development</td>
</tr>
<tr>
<td></td>
<td>FGF signalling</td>
<td>Regulation of SOX9</td>
</tr>
<tr>
<td></td>
<td>Regulates <em>Ptgds</em></td>
<td>Promotes Pgd2 synthesis / Sertoli cell differentiation</td>
</tr>
<tr>
<td></td>
<td>Pgd2 synthesis</td>
<td>Promotes nuclear import of SOX9</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Sox9 activates <em>Ngn3</em> expression</td>
<td>Endocrine specification</td>
</tr>
<tr>
<td></td>
<td>Sox9 promotes <em>Hes1</em> expression</td>
<td>Maintains progenitor cell population</td>
</tr>
<tr>
<td>Heart</td>
<td>Sox9 promotes proliferation &amp; regulates ECM deposition</td>
<td>Formation of heart valve &amp; septa</td>
</tr>
<tr>
<td></td>
<td>Sox9 expression is associated with <em>Col2a1</em> expression</td>
<td>Vascular calcification</td>
</tr>
<tr>
<td>Liver</td>
<td>Abnormal expression of <em>Hes1</em>, <em>C/EBPα</em> and <em>TβRII</em> in liver specific Sox9^−/− mice</td>
<td>Bile duct development</td>
</tr>
<tr>
<td></td>
<td>Promotes <em>Col1</em> expression in HSCs <em>in vitro</em></td>
<td>ECM deposition in liver fibrosis</td>
</tr>
<tr>
<td></td>
<td>TGF-beta signalling</td>
<td>Increased Sox9 expression by HSCs <em>in vitro</em></td>
</tr>
<tr>
<td>Cartilage</td>
<td>Regulates cartilage ECM components</td>
<td>Chondrogenesis</td>
</tr>
<tr>
<td></td>
<td>Promotes β-catenin degradation</td>
<td>Inhibits chondrocyte proliferation &amp; differentiation</td>
</tr>
<tr>
<td></td>
<td>TGF-β1 signalling</td>
<td>Increases Sox9 expression during chondrogenesis</td>
</tr>
<tr>
<td></td>
<td>Hh signalling</td>
<td>Sox9-mediated chondrogenesis</td>
</tr>
<tr>
<td></td>
<td>Notch signalling</td>
<td>Downstream targets <em>Hes-1</em> &amp; <em>Hey-1</em> compete for Sox9 binding sites on <em>Col2a1</em> enhancer to inhibit activation</td>
</tr>
<tr>
<td></td>
<td>FGF signalling</td>
<td>FGFs promote Sox9 expression by chondrocytes <em>in vitro</em></td>
</tr>
<tr>
<td>Ovary</td>
<td><em>Pgd2</em></td>
<td>Induces Sox9 / tumour growth inhibition</td>
</tr>
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<td>Prostate</td>
<td>Wnt/β-catenin promotes SOX9 expression in tumour cell lines <em>in vitro</em></td>
<td>SOX9 promotes proliferation and androgen receptor expression in prostate cancer cells <em>in vitro</em></td>
</tr>
<tr>
<td></td>
<td>FGF signalling increased Sox9 expression <em>in vivo</em></td>
<td>Tumour metastasis via an EMT-like process <em>in vivo</em> in a murine model</td>
</tr>
<tr>
<td></td>
<td>Increased SOX9 expression in human tumours</td>
<td>Elevated Sox9 increased proliferation in murine prostate epithelium <em>in vivo</em>. Associated with tumour formation in absence of PTEN <em>Suppression of Sox9 expression &amp; glioma growth</em></td>
</tr>
<tr>
<td>Neural</td>
<td>cGMP-dependent protein kinases (<em>cGKI</em> and <em>cGKII</em> encoded by <em>Prkg2</em>)</td>
<td>EMT in avian neural crest</td>
</tr>
<tr>
<td></td>
<td>Sox9 binds to Snail2 (<em>Slug</em>) and directly activates the Snail2 promoter.</td>
<td>Neural crest specification</td>
</tr>
<tr>
<td></td>
<td>CHD7 mediated SOX9 expression</td>
<td>Oligodendrocyte survival in developing spinal cord</td>
</tr>
<tr>
<td></td>
<td>Sox9 (and Sox10) are required for <em>Pdgf receptor a</em> expression</td>
<td>Sox9 is required for Müller cell gliogenesis in mouse retina</td>
</tr>
<tr>
<td></td>
<td>Notch signalling promotes Sox9 expression</td>
<td><em>In vivo</em> regulation of Sox9 during gliogenesis in xenopus</td>
</tr>
<tr>
<td>Skin</td>
<td>Hedgehog signalling is required for Sox9 expression</td>
<td>Sox9 increases / maintains progenitor proliferation</td>
</tr>
<tr>
<td></td>
<td><em>p2f</em> upregulated by SOX9 <em>in vitro</em></td>
<td>SOX9 decreased proliferation in melanoma xenografts <em>in vivo</em></td>
</tr>
<tr>
<td>Bladder</td>
<td>CpG island methylation of SOX9 promoter</td>
<td>Inhibits proliferation of human breast cancer cells <em>in vitro</em></td>
</tr>
<tr>
<td>Breast</td>
<td>SOX9 mediated RA induced <em>Hes1</em> expression</td>
<td>Induces SOX9 expression / reduces proliferation <em>in vitro</em></td>
</tr>
<tr>
<td>Digestive System</td>
<td>SOX9 inhibits <em>CDX2</em> and <em>MUC2</em> expression</td>
<td>Maintains intestinal crypt progenitor cell population by inhibiting expression of differentiation genes</td>
</tr>
<tr>
<td></td>
<td>Sox9 inhibits claudin7 expression</td>
<td>Disrupts cell polarity &amp; increases tumorigenicity</td>
</tr>
</tbody>
</table>

Table 1-6: The role and regulation of SOX9 in development and disease.

Adapted from Pritchett et al. 2011
1.9.2 Regulation of SOX9

Uncertainty exists in the precise mechanisms regulating $SOX9$. Campomelic dysplasia can occur with chromosomal breakpoints or single point mutations up to 1Mb either side to $SOX9$, suggesting regulation of $SOX9$ can occur through distant regulatory elements (Pfeifer et al. 1999; Gordon et al. 2009).

$SOX9$ is highly conserved between species and homology can be traced throughout vertebrates and by studying the conservation of regions proximal to the $SOX9$ gene has led to improved understanding of the regulatory elements (Bagheri-Fam et al. 2001). Within the $SOX9$ promoter two CCATT boxes are present, ~60 and ~100bp up-stream of the transcription start site (Colter et al. 2005). These CCATT promoter elements have been shown to form complexes with nuclear factor Y (NF-Y) (Piper Hanley et al. 2008) and mutation within these regions reduces $SOX9$ expression in chondrocytes (Colter et al. 2005).

Several pathways have been shown to up-stream regulate $SOX9$ expression. Again, these are predominantly, but no exclusively, described in chondrogenesis. Signalling pathways include hedgehog (Hh) and specifically Sonic hedgehog (Shh), whereby Shh knockout reduced Sox9 expression in tracheal development (Park et al. 2010) and Sox9 mRNA is increased in chondrocytes of mice overexpressing Shh (Pritchett et al. 2011). Other regulatory pathways include canonical and non-canonical Wnt, TGF-β, and notch signalling (Pritchett et al. 2011). Examples of control include Notch inhibiting Sox9 expression in vitro and in vivo (Mead and Yutzey 2009); TGF-β increasing expression Smad3 and Sox9 in vitro (Montero and Hurle 2010); and Wnt5 signalling through non-canonical pathways to increase Sox9 in early chondrogenesis (Akiyama et al. 2004).

1.9.3 SOX9 in Disease

$SOX9$ expression at the right time and place leads to appropriate regulation of ECM. However, growing evidence suggests that $SOX9$ at the wrong time and place results in dysregulation of ECM (Pritchett et al. 2011). $SOX9$ is implicated in the excessive inappropriate deposition of matrix in various diseases at a range of sites. Keloid scaring is an inappropriate dermal reaction and a prime example of $SOX9$ ECM...
dysregulation (Naitoh et al. 2005). In this study, cDNA microarray study identified 
*SOX9* amongst the highest expressed genes and postulated that gene expression 
altered in this condition from a normal dermal pattern to that more closely 
representing chondrocyte lineage. In sclerotic glomeruli of kidney, increases in TGF-
\( \beta1 \) after injury led to substantial increases in SOX9 (Miura et al. 2007). In mesangial 
cells, SOX9 was found to be downstream of TGF-\( \beta1 \) and implicated in the regulation 
of *Col4a2*, the key collagen in glomerulosclerosis (Miura et al. 2007; Sumi et al. 
2007). Calcification of blood vessels is another example of SOX9 causing 
dysregulation of ECM, in this instance COL2A1 (Neven et al. 2007).

Recently Sox9 has been implicated in fibrosis of the liver (Piper Hanley et al. 2008). 
Ectopic expression of Sox9 has been shown both *in vitro* and *in vivo* to cause 
deposition of type I collagen. Furthermore, co-localisation with \( \alpha \)-SMA and Sox9 
suggests activated HSCs ectopically express Sox9 (Piper Hanley et al. 2008). In a 
quiescent state, HSC *in vitro* do not produce type I collagen or SOX9. Activation of 
HSCs, either by treatment with TGF-\( \beta \) or by culture on plastic, resulted in an 
increase in Sox9 and type I collagen. Attenuation of Sox9 in activated HSCs caused 
a commensurate reduction of type I collagen; suggesting type I collagen is regulated 
by Sox9 (Piper Hanley et al. 2008).

Many of the upstream regulatory pathways of SOX9 in development are also 
implicated in liver fibrosis (e.g. TGF-\( \beta \), Hh, Wnt – see section 1.5). Taken together 
with SOX9’s role in ECM dysregulation in liver fibrosis, particularly the primary 
fibrillar collagen implicated in fibrosis, suggests that SOX9 may play a pivotal role. 
Evidence from fibrosis in other organs places SOX9 as a potential ‘core’ factor in 
universal fibrosis. Evolutionarily conservation of SOX9 would also support this 
hypothesis.

A growing body of research insinuates SOX9’s potential role in disease processes, in 
particular fibrosis. Exploration of these mechanisms may provide novel insights into 
the processes of fibrosis and the potential promise of diagnostic and therapeutic 
innovations.
1.10 **Aims**

Understanding the role of SOX9 in liver fibrosis is not only important to understand this condition but may also have implications in other fibrotic conditions. Previous work from within the group has suggested the link with this highly conserved transcription factor and liver fibrosis (Piper Hanley et al. 2008). This project aims to expand upon this work and further delineate the role SOX9 may play in liver fibrosis. Translation of mechanistic understanding to clinically applicable endpoints is a recurrent theme throughout this work. To this end, the chapters of this thesis investigate:

1.10.1 **Osteopontin as novel downstream target of SOX9**

This chapter provides insight into the expression of hepatic SOX9 in healthy liver and fibrosis. OPN is identified as a novel downstream target of SOX9, *in vivo* and *in vitro*. SOX9 and OPN are co-localised in liver fibrosis in CHC and OPN concentration correlated to progressive fibrosis.

1.10.2 **Epimorphin alters SOX9 and MMP13 expression in activated HSCs**

EPIM expression is shown to decrease as HSCs activate, in this chapter. Alteration of the phenotype of activated HSCs is demonstrated after the addition of EPIM; in particular, changes in SOX9 and MMP13 levels. A direct, negatively regulated relationship between SOX9 and MMP13 is established as a mechanism by which ECM breakdown is inhibited in liver fibrosis.

1.10.3 **Downstream targets of SOX9 as potential biomarkers of liver fibrosis**

*In vitro* gene expression profiling is used to identify potential downstream SOX9 targets. These targets are validated and explored as biomarkers of liver fibrosis in CHC, using two separate cohorts. The biomarkers identified are compared to currently available biomarkers.
1.10.4 SOX9 expression as a marker to predict liver fibrosis progression

SOX9 expression in progressive fibrosis secondary to CHC is demonstrated. Categorisation of SOX9 positive cells is achieved with immunofluorescence dual staining. Using paired biopsy samples from the same individuals over a period of time allows assessment of SOX9 expression as a predictor of liver fibrosis progression.
2 MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 Primary Rat Hepatic Stellate Cell Culture

The isolation of rat hepatic stellate cells (rHSCs) was performed using a modified protocol originally described by Freidman and Roll (Friedman and Roll 1987).

2.1.1.1 Extraction of Rat Hepatic Stellate Cells

Adult Sprague-Dawley rats were euthanized by Home Office Schedule 1 methods. All animals were under 500g in body weight. Immediately after sacrifice the abdomen was opened and liver exposed. The portal vein was identified and cannulised with a 22-gauge needle. 1000 units of heparin sulphate (LEO Pharma, Denmark) mixed in 2ml of Hanks Balanced Salt Solution without calcium and magnesium (HBSS-; Invitrogen, Life Technologies) were injected into the portal vein and the cannula left in situ. A perfusion pump primed with HBSS- at 37°C was attached to the cannula and started at approximately 10ml/min. The liver was observed filling and after approximately 5 seconds the inferior vena cava was incised promoting drainage of the HBSS- and effluence. Intermittent pressure was applied above the level of the incision and the liver engorged, enhancing perfusion. After perfusion of 100ml, the HBSS- was exchanged for HBSS containing calcium and magnesium (HBSS+; Invitrogen, Life Technologies) and 120mg Pronase (Roche, UK) in 10ml HBSS+ added into the 500ml bottle. When only 150ml of HBSS+ remained, 20mg Collagenase (Roche, UK) in 10ml HBSS+ was added. After the remaining HBSS+ had perfused through the rat liver, the apparatus was removed and the liver dissected out in one piece and placed into 35mg Pronase in 10ml HBSS+.

2.1.1.2 Stellate Cell Preparation

Working in a dedicated primary cell culture hood and room, the liver was transferred onto a 125μm Nyboldt mesh (John Staniar, UK) and filtered into a 500ml beaker using HBSS+ and DNase I (10mg DNase (Roche, UK) in 10ml HBSS+). The filtrate
was transferred into 4, 50ml tubes and a final total volume of 200ml was made with HBSS+. The samples were centrifuged at 1800rpm for 7 minutes and the supernatant removed. The pellet was re-suspended in 2ml of DNase I per tube and then HBSS+ added to make 50ml. A further 1800rpm centrifugation for 7 minutes was applied and the supernatant discarded. During the centrifugation, an OptiPrep gradient was made using 17ml of Optiprep (Axis-Shield Diagnostics Ltd) and 13ml HBSS+. The pellets were all amalgamated and suspended in 2ml of DNase I and made to a volume of 44ml using HBSS+. This was added to the OptiPrep gradient and gently mixed by swirling. The mixture was divided into two 50ml tubes and 2ml layer of HBSS+ added drop-wise. This was centrifuged at 2115 rpm (1500g) for 23 minutes at 4°C without brake. Due to their high vitamin D content, a cloudy layer of rHSCs formed in the top third of the tube. These were removed using a Pasteur pipette and suspending in 2ml of DNase, making up to a total of 50ml with HBSS+. The suspension was centrifuged at 2000rpm for 7 minutes, the supernatant removed and the cells re-suspended in 5ml of rHSC culture medium. rHSC culture medium contains 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). The cell concentration was calculated using a haemocytometer with non-living cells excluded by the addition of trypan blue (Invitrogen, Life Technologies). The cells were plated out into T75 flask (Corning) at 5 million cells per flask with 14ml of culture medium. The following day the medium was completely exchanged.

2.1.1.3 Culture Conditions

Rat HSCs were cultured over a period of 10 to 14 days to enable activation towards a myofibroblastic phenotype. The cells were kept in an incubator at 37°C with 5% CO₂. Changes towards a myofibroblastic phenotype were observed by microscopic analysis. When cells approached 80-90% confluence, they were passaged.
2.1.2 Cell Lines

2.1.2.1 LX-2
Immortalised LX-2 cells were provided courtesy of Professor SL Friedman and generated by spontaneous immortalisation in low serum conditions (Xu et al. 2005). They express α-smooth muscle actin (αSMA), vimentin, and glial fibrillar acidic protein (GFAP), similar to activated HSCs. Also, they express key proteins regulating hepatic fibrosis, including PDGF, MMPs, TIMPs and collagens. Microarray analysis showed high correlation of LX-2s with primary human HSCs (Xu et al. 2005).

2.1.2.2 Culture Conditions
The culture medium used for LX-2 cells consisted of DMEM with 1% FBS, 2mM glutamine and 1% penicillin/streptomycin (100units/ml penicillin and 100µg/ml streptomycin). The cells were kept in 1% media at 37°C with 5% CO₂. In 1% media the LX-2 cells remain in a quiescent state. To activate LX-2 cells, they were cultured for 24-48 hours in medium containing 10% FBS. When cells approached 90% confluence, usually in 2-4 days, they were divided.

2.1.3 Passage
When cells reached a confluence of approximately 80%, growth medium was removed and replaced with 5ml of 1X trypsin-EDTA (Invitrogen, Paisley, UK) for 5 minutes at 37°C or until cells became non-adherent to the flask surface. The reaction was terminated by the addition of 10ml of growth medium containing 16% FBS for rHSCs or 10% FBS for LX-2 cells. The trypsin inhibitors within FBS inactivate the trypsin, stopping the reaction. The cells were pelleted by centrifugation at either 1500rpm (5 minutes) or 2000rpm (7 minutes), for LX-2 and rHSCs, respectively. The supernatant was aspirated and pellet re-suspended in fresh growth medium, split in a ratio of 1:3 and plated in new T75 flasks, with 14ml medium per flask. The following day half the medium was changed.

2.1.4 Freezing and Thawing
All cells were routinely frozen and stored in liquid nitrogen at -196°C. Cells at low passage were suspended in Dulbecco’s modified eagles medium (DMEM, Sigma, UK) containing 50% FBS and 10% dimethylsulphoxide (DMSO, Sigma, UK). The
DMSO facilitated cryopreservation by decreasing the size of any ice crystals that may form in freezing. A cell pellet was obtained from 80% confluent cells as described in Section 2.1.3 and re-suspended in 3ml of freezing medium. 1ml of suspension was aliquoted per cryotube. The tubes 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 rapidly at 37°C. The suspension was diluted with 10 ml of pre-warmed DMEM and centrifuged at 1500rpm (LX-2 cells) or 2000rpm (rHSC) for 5 minutes. The cell pellet was re-suspended in complete culture medium and transferred to a T75 culture flask.

### 2.2 Transfections

#### 2.2.1 Knockdown of Sox9 with siRNA

The process of inserting nucleic acids into a eukaryotic cell is known as transfection. This can be achieved with several different methods. For the purpose of siRNA transfection, 2 different approaches were used. Electroporation transfection uses a brief pulse of electrical current applied to a mixture of cells and RNA. This results in partial disruption of the cell phospholipid bilayer membrane, temporarily creating aqueous pores and facilitating passage of RNA into the cell. The RNA is driven across the cell membrane due to the rise in electrical potential across the membrane by approximately 0.5-1 V, resulting in charged molecules (i.e. RNA) to move across in a similar fashion to electrophoresis (Xu et al. 2012).

Cationic lipid transfection is the most common method of transfection. A positively charged lipid forms a micelle around the negatively charged RNA. The remaining excess positive charge from the lipids allows binding to sialic acid residues on the cell surface and uptake into the cell by endocytosis pathways.

Short interfering RNA (siRNA) are 20-25 base pair sequences of double-stranded RNA (dsRNA) that can be used for post-transcriptional silencing of specific genes. The transfected dsRNA is converted into siRNA by the RNAse enzyme Dicer. The
siRNA is then incorporated into a RNA-induced silencing complex, which binds the targeted mRNA, resulting in degradation and preventing translation. Non-specific effects from this method can occur due to a host immune response secondary to the transfection and off-target effects, primarily from the siRNA acting as miRNA. To minimise these effects, a cocktail of non-specific, or scrambled siRNA, were used as a control.

2.2.1.1 HSCs

Primary rHSC were activated for 10-14 days and transfected with siRNA. The cells were assessed microscopically to be 70-80% confluent and showing phenotypic changes of myofibroblasts. A cell pellet was obtained by aspirating medium and the addition of 5ml trypsin. After 5 minutes of incubation (or until cells dissociated from flask surface) 10ml of 16% growth medium was added and the solution transferred into a tube, centrifuged at 2000rpm for 7 minutes and the supernatant discarded. The cells were re-suspended in 5ml of 16% medium and the concentration assessed using a haemocytometer. Medium containing 5x10^5 cells was aliquoted into a microcentrifuge tube and centrifuged 2000rpm for 7 minutes. The supernatant was discarded and the pellet was re-suspended in 100µl of Nucleofector Solution (Lonza, UK) per tube and either Sox9 siRNA (Sox9 siRNA1 or Sox9 siRNA3; Qiagen) or scrambled siRNA (All stars negative control; Qiagen) added, in a volume to make a final concentration of 10nM. The solution was transferred into a cuvette provided by the Amaxa® Basic Nucleofector Kit (Lonza, UK) and introduced into Lonza Cell Line Nucleofector Kit T (WCA-1002, Lonza). Electroporation was applied using program U-25. This was repeated for solutions containing siRNA for Sox9 and scrambled siRNA. 500µl of growth medium was added to the solution and plated into a 6-well plate. The final volume was made to 2ml with 16% culture medium. The final concentration of siRNA was 10nM. Cells were harvested after 24 hours for protein and RNA extraction (sections 2.4.1 and 2.5.1.1).

2.2.1.2 LX-2

Transfection was achieved using a cationic lipid transfection with siRNA. Two different siRNAs, each targeting different parts of the SOX9 transcript were used together to ensure efficient knockdown. LX-2 cells were plated at 8x10^4 cells / well
onto 12-well plates in their normal culture medium. 24 hours later, a transfection mix was made up of:

100µl serum free DMEM
0.6µl siRNA (20µM stock) / 0.3µl each of two siRNAs
12µl HiPerFect (Qiagen, UK)

The solution was mixed with a vortex mixer and incubated at room temperature for 5-10 minutes. A control, using scrambled siRNA (using 0.6µl of 20µM stock) was also prepared. Culture medium was removed from the wells and replaced with 2.3 ml fresh DMEM and the HiPerFect mixture added drop-wise to the cells. Plates were incubated at 37°C, and a second siRNA treatment was applied 24 hours after the initial treatment. Cells were incubated for a further 24 hours before harvesting for protein or RNA extraction (sections 2.4.1 and 2.5.1.1). The sequence of siRNA used is shown in Table 1.

<table>
<thead>
<tr>
<th>Species</th>
<th>Target</th>
<th>Sequence</th>
<th>Supplier</th>
<th>Catalogue Number</th>
</tr>
</thead>
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<tr>
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<td>SOX9</td>
<td>ATG GGA GTA AAC AAT AGT CTA</td>
<td>Qiagen</td>
<td>SI00007595</td>
</tr>
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<td>Sox9</td>
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<td>Sox9</td>
<td>CCC ACC AGC GTC AGT GAG GAA</td>
<td>Qiagen</td>
<td>SI02952677</td>
</tr>
</tbody>
</table>

Table 2-1: siRNA used for cellular transfection.

### 2.2.2 Transient Transfection

Plasmid DNA was transfected into LX-2 cells using cationic lipid based transfection for luciferase assay (described section 2.9). LX-2 cells were placed onto 12-well plates at a concentration of 5x10⁴ cells / well. 24 hours later, cells were transfected with Transfast reagent (Promega, UK) and plasmids, using the manufacturer’s protocol, in serum-free DMEM for exactly 1 hour at 37°C. After 1 hour medium was made up to 2ml with 10% FBS containing culture medium. The charge ratio of DNA: Transfast was 1:1 (1µg of DNA / 3µl of Transfast reagent). Cells were
transfected with a total of 1.7 µg total DNA: 1.4 µg of expression vector (0.7 µg each if two vectors used) and 0.3 µg of reporter plasmid.

2.3 Immunostaining

2.3.1 Immunohistochemistry

Immunohistochemistry (IHC) and dual immunofluorescence (IF) were used for detection of proteins in histological and cytological specimens. Wax was removed by immersing in two consecutive xylene containers for three minutes each, then sequentially rehydrated in 100%, 90%, 80% ethanol for 3 minutes each, followed by washing in H2O. Slides for use in IHC were pre-treated with 0.1% (v/v) hydrogen peroxide (Sigma-Aldrich) in phosphate buffered saline (PBS; Sigma-Aldrich) for 20 minutes to quench endogenous peroxidase, as secondary antibody is coupled to a peroxidase. Antigen retrieval was achieved with either heat permeabilisation using 10 mM sodium citrate buffer at 95°C for 10 minutes or pepsin reagent (Sigma-Aldrich) at 37°C for 10 minutes. Slides undergoing antigen retrieval with sodium citrate were allowed to cool for 20 minutes in the buffer. All slides were then washed 3 times in PBS for 5 minutes. If necessary, sections were separated using a wax pen (ImmEdge pen; Vector Laboratories, Peterborough, UK).

The primary antibody was diluted to the appropriate concentration in PBS/0.1% Triton X-100 (Sigma-Aldrich) containing 3% serum (from the species in which the secondary antibody was raised; Vector Laboratories), to reduce background and non-specific binding. Sections were incubated overnight at 4°C in a humidified container. Antibodies and dilutions are shown in Table 2.

The following day sections were washed 3 times in PBS for five minutes and incubated with a species-specific secondary antibody diluted in buffer (PBS/0.1% Triton X-100) for 2 hours at 4°C in a humidified container before 3 washes with PBS. For IHC, a biotin-conjugated secondary antibody was used. Sections were then incubated with streptavidin-horseradish peroxidase (dilution 1:200 in PBS/0.1% Triton X-100; Vector Laboratories) for one hour at 4°C before colour reaction was developed with diaminobenzidine (DAB; Merck) for 3 minutes followed by DAB
containing 0.1% hydrogen peroxidase (Sigma) for 3 minutes. Sections were counterstained with toluidine blue and dehydrated by sequential incubation in 70% (10 seconds), 90% (10 seconds), 100% ethanol (3 minutes) and then twice for 2 minutes in xylene. Finally a coverslip was applied using Entellan (Merck).
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Raised In</th>
<th>Company</th>
<th>Dilution</th>
<th>Application</th>
</tr>
</thead>
<tbody>
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<td>Millipore</td>
<td>1:5000</td>
<td>Western</td>
</tr>
<tr>
<td>SOX9</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>1:800</td>
<td>IHC / ICC</td>
</tr>
<tr>
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<td>Abcam</td>
<td>1:750</td>
<td>Western</td>
</tr>
<tr>
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<td>Mouse</td>
<td>Novocastra</td>
<td>1:50</td>
<td>ICC</td>
</tr>
<tr>
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<td>Goat</td>
<td>R&amp;D Systems (AF808)</td>
<td>1:80</td>
<td>IHC (Pepsin Digest)</td>
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<td>R&amp;D Systems (AF1433)</td>
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<td>Mouse</td>
<td>R&amp;D Systems (MAB14332)</td>
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<td>ELISA capture</td>
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<tr>
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<td>1:100</td>
<td>IHC</td>
</tr>
<tr>
<td>α-SMA FITC</td>
<td>Mouse</td>
<td>Sigma</td>
<td>1:250</td>
<td>ICC / IF</td>
</tr>
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<td>β-actin</td>
<td>Mouse</td>
<td>Sigma</td>
<td>1:100 000</td>
<td>Western</td>
</tr>
<tr>
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<td>Rabbit</td>
<td>Abcam</td>
<td>1:2000</td>
<td>Western</td>
</tr>
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<td></td>
<td></td>
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</tr>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>Western</td>
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<td>Novocastra</td>
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</tr>
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<td></td>
<td></td>
<td></td>
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<td>Western</td>
</tr>
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<tr>
<td></td>
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<td></td>
<td>1:1000</td>
<td>ICC / IF</td>
</tr>
<tr>
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<td>Western</td>
</tr>
</tbody>
</table>
Table 2-2: List of antibodies used with dilutions per application.

| Antibody Description | Species   | Brand       | Dilution  | Application 
|----------------------|-----------|-------------|-----------|------------------
| GPNMB                | Goat      | R&D Systems | 1:200     | ELISA capture   
|                      |           | (AF2550)    |           |                  
| GPNMB                | Mouse     | R&D Systems | 1:500     | ELISA detect    
|                      |           | (MAB25501)  |           |                  
| Anti-mouse Alexafluor 488 | Goat    | Invitrogen  | 1:1000    | ICC / IF        
| Anti-goat Alexafluor 488 | Donkey   | Invitrogen  | 1:1000    | ICC / IF        
| Anti-rabbit Alexafluor 594 | Donkey  | Invitrogen  | 1:1000    | ICC / IF        

2.3.2 Immunofluorescence

For dual IF, secondary antibodies were incubated with directly fluorochrome-conjugated secondary antibodies for 2 hours at room temperature (dilutions shown in Table 2-2). Slides were washed 3 times for 5 minutes in PBS and the process repeated with a second primary and corresponding fluorochrome-conjugated secondary antibody. Slides were subsequently dehydrated to xylene, followed by a 100 % ethanol wash and mounted with 4', 6-diamidino-2- phenylindole (DAPI; VectorShield, Vectorlab).

Images were viewed using Axiovert (Zeiss) imaging system, and captured using Axiovision 4.7 software (Zeiss). Pictures were collated with Photoshop (Adobe Systems, Uxbridge, UK).
Figure 2-1: Schematic representation of protein detection in tissue sections by fluorescence and optical colour development. 
(A) Bright field colour development using biotinylated secondary antibody against primary antibody. (B) Immunofluorescence with fluorofluor directly conjugated to secondary antibody, resulting in fluorescence at a specific wavelength (either 488nm or 594nm).

2.3.3 Picrosirius red staining
All picrosirius red (PSR) staining was done by the pathology department of Queens Medical Centre, Nottingham University Hospital NHS Trust, Nottingham. Slides were de-waxed in xylene and hydrated as described in section 2.3.1 and then washed 3 times in PBS for 5 minutes. Slides were then immersed in PSR for one hour followed by 3 washes for 5 minutes in PBS. Sections were then dehydrated through increasing concentrations of ethanol, cleared in xylene and mounted as described in section 2.3.1.

2.3.4 Quantification of SOX9 in CHC Fibrotic Livers
Fibrotic human liver biopsy specimens stained for SOX9 by IHC were all digitised by scanning into Nanozoomer 2.0-HT slide scanner (Hamamatsu, Japan) and viewed with Nanozoomer Imager (Hamamatsu, Japan). Slides were deemed adequate if there were more than 5 portal tracts present and staining could be clearly assessed by eye. 5 sequential portal tract areas were selected per slide at 20x zoom, giving an area per portal tract of 0.28mm². The images were then viewed in Photoshop CS4 (Adobe Systems, Uxbridge, UK). All SOX9 positive cell nuclei were counted, differentiating
between biliary epithelial cells (BEC) and non-biliary epithelial cells (non-BEC). All
groups of cells in a ring-like structure were deemed to be biliary epithelial cells. The
mean from the 5 areas was taken as the average BEC and non-BEC count for that
slide. All cell counting was carried out blind without prior knowledge of sample (i.e.
disease stage, initial / follow on biopsy or progression).

2.4 Immunoblotting

2.4.1 Preparation of Cell Lysate for Protein Analysis
Cultured cells were removed from the incubator, placed onto ice and the medium
removed. The cells were washed 3 times with ice-cold PBS and scraped into 0.5ml
RIPA lysis buffer (0.15M NaCl, 50mM Tris pH 7.2, 1% sodium deoxycholate, 1%
Triton X, 0.1% sodium dodecyl sulphate (SDS; Sigma), 5% glycerol (Sigma), 1mM
dithiothreitol (DTT; Sigma), 1 x EDTA free Protease Inhibitors (Roche), 1 x
Inhibitor Cocktails 1 and 2 (Sigma) and distilled H₂O to volume). The samples were
centrifuged at 4°C for 10 minutes at 8000rpm and the supernatant assayed for protein
content.

2.4.2 Protein Estimation (Bradford Assay)
Protein quantification was performed using Bradford assay, a colourimetric protein
assay. 2μl of each lysate was diluted 1:50 into 98μl PBS. 1ml of Bradford reagent
(Bradford protein assay, Biorad) at a 1:5 dilution with distilled H₂O was added and
the samples incubated at room temperature for 10 minutes. 100μl of each sample was
loaded onto a flat-bottomed, clear, 96-well plate alongside a standard dilution series
of BSA from 0-0.8 mg/ml and the absorbance measured at 595nm with an automated
plate reader (EL800; BioTek, Potton, UK). Data was exported to an excel file and a
standard curve was derived from the BSA concentrations, from which sample protein
concentration was estimated.

2.4.3 SDS-PAGE Gel
Protein was assayed using sodium dodecyl sulphate polyacrylamide gel
electrophoresis (SDS-PAGE). The gels were manufactured using Bio-Rad minigel
western blot equipment (Bio-Rad; Hemel Hempstead, UK). An upper stacker gel
(1M Tris pH 6.8 625μl, 20% SDS 25μl, 40% acrylamide 525μl, 10% ammonium persulphate (APS) 150μl, tetramethylethylenediamine (TEMED) 12.5μl, 3.66ml H₂O) was included to allow proteins to separate before reaching the lower resolving gel (1MTris pH 8.8 2.5 ml, 20% SDS 50μl, 40% acrylamide 2-3ml (for 8-12.5% gel), 2.89-3.89ml H₂O (for 8-12.5% gel), 10% APS 300μl, TEMED 12.5μl). A gel comb was placed in the stacking gel until hardened to create lanes for the addition of the samples.

### 2.4.4 Protein Electrophoresis

Whole cell extracts (10μg protein) were electrophoresed on 8-12% SDS/acrylamide gels to separate proteins according to their molecular mass. Samples were 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), boiled for 5 minutes and separated with a protein ladder (7 kDa – 175 kDa; Colourplus Prestained Protein Marker, Broad Range; New England Biolabs, UK). The gel was submerged in running buffer (250mM Tris, 2M Glycine, 35mM SDS, to volume with dH2O) and protein separated at 200V for one hour.

### 2.4.5 Transfer to Nitrocellulose Membrane

Protein transfer to nitrocellulose membrane (G.E. Healthcare) was performed using a minigel western blot kit (Bio-Rad). The gel was placed onto a nitrocellulose membrane and both were sandwiched between filter paper and a sponge, before being enclosed in a plastic cartridge. The process took place submerged in deionised H₂O. The closed cartridges were submerged in transfer buffer (25mM Tris, 200mM Glycine, 10 % Methanol) and transferred for 1 hour at 100V.

### 2.4.6 Chemiluminescence of Protein

The nitrocellulose membrane was blocked for 2 hours with 5% powdered milk (Sigma-Aldrich) and 0.1% Tween (Sigma-Aldrich) made with PBS and then incubated overnight in the appropriate antibody diluted in 5% milk/0.1% Tween/PBS. The following morning, the membrane was washed 3 times for 5 minutes in PBS and incubated with the corresponding HRP-conjugated secondary antibody made in PBS/milk/tween. After 3 x 5 minute PBS washes, the membrane was incubated in the dark with a chemiluminescent solution for 5 minutes. For β- actin detection, ECL
Basic Uptilight (Uptima, UK) was used and for all other proteins, ECL Advance (Lumigen, USA) at 1:1 - 1:10 dilution with PBS.

2.4.7 Visualisation and Quantification

The chemiluminescent treated membrane was analysed by an automated detection system (ChemiDoc; Biorad). Densitometry analysis of blots was performed using QuantityOne software from the image acquired by the ChemiDoc imager.

2.5 RNA Purification and Quantification

2.5.1 cDNA Synthesis from Isolated RNA

2.5.1.1 Isolation of Cellular RNA

Total RNA was isolated using RNeasy Mini Kit (Qiagen) following the manufacturer’s instructions. Cells were lysed using 350µl of RNeasy kit buffer RLT containing 1:100 dilution of β-mercaptoethanol (Sigma-Aldrich), at room temperature. The lysate was collected using a cell scraper and homogenized by pipetting through a P200 tip. The sample was mixed with an equal amount of 70% ethanol, placed in an RNeasy Mini Spin Column and centrifuged at 10,000 rpm for 15 seconds. The column was washed twice with the kit buffer RPE to reduce contamination. The RPE buffer was removed by drying the column with centrifugation at 8500rpm for 1 minute and RNA eluted in 30µl RNA free water. Contaminating DNA was removed using a DNase Kit (Sigma Aldrich) according to the manufacturer’s guidelines. This constituted adding 1.75µl of kit DNase1 and 3.5µl of 10x buffer to 30µl of RNA containing solution and leaving at room temperature for 15 minutes. Following this 15µl of stop solution was added and the tube left in a water bath at 70°C for 10 minutes.

2.5.1.2 RNA Quantification

The quantity, purity and concentration of the RNA were assessed using a spectrophotometer, Nanodrop 2000C (Thera Scientific), using 1µl of sample. Concentration was determined using absorbance at 260nm, with a reading of 1 being equivalent to 40µg/ ml RNA. The ratio of absorbance at 260 and 280nm was used to
measure purity, with a ratio of 2.0 indicating the sample to be free from protein, DNA and other contaminants.

2.5.1.3 First Strand cDNA Synthesis

cDNA was generated via reverse transcription using a reverse transcriptase High Capacity RNA-to-cDNA kit (Applied Biosystems). This included creating a positive RT reaction with enzyme mix and a negative RT control without reverse transcriptase. 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). The mix was heated for 60 minutes to 37°C and then the reaction stopped by heating to 95°C for 5 minutes – done with the use of Biorad Tetrad thermal cycler (Biorad).

2.5.2 Quantitative Real-Time PCR (qPCR)

A specific 96-well, quantitative PCR plate and cover (Microamp Fast Optical Reaction plate; Applied Biosystems) were used in the StepOne Plus qPCR machine (Applied Biosystems) and the data analysed with the supplied software.

2.5.2.1 Primer Design

Primers to be used for RT-PCR were either selected from previously published work or downloaded from the Roche Universal Probe Library (Roche). If no suitable primers were identifiable by either method, then primers were designed by uploading gene sequence into NCBI Primer BLAST software for intron-spanning primer sequences. The criteria for primer design were to have product size between 70-120 base pairs, primer size between 18-23 base pairs, intron-spanning sequence (is possible) and the product to be identifiable in UCSC in-silico PCR (http://genome.ucsc.edu/cgi-bin/hgPcr). All primers were ordered from Eurofins MWG Operon (Ebersberg, Germany). A list of all primers used is shown in Appendix 1.

2.5.2.2 Product Amplification by SYBR-Green

Using SYBR Green master mix (Primer Design. Southampton), reactions for gene amplification were set. The mastermix contains 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).

Per single reaction in 96-well Fast Optical Reaction Plate (Applied Biosystems, Warrington, UK):

SYBR Green Mastermix (Primer Design, Southampton, UK) 10µl
Forward Primer 10µM 2µl
Reverse Primer 10µM 2µl
cDNA from first strand synthesis 1µl
DEPC-treated H2O to 20µl

Experimental conditions were performed in triplicate with a negative control (minus-RT reaction), and two reference genes, glucuronidase beta (GUSB) and β-actin, on each plate. The plate was sealed and transferred to a thermocycler (StepOne Plus real-time PCR System; Applied Biosystems). The reaction was performed according to the manufacturer’s instruction including a primer melt-curve step to confirm single amplification product, as outlined below:

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

2.5.2.3 qPCR Data Analysis

Quantification is achieved by measuring fluorescent signal emitted as the SYBR Green preferentially binds the double stranded DNA produced by the reaction. The cycle threshold (Ct) value is calculated at the cycle number at which the fluorescent signal of the amplified DNA exceeds the background and becomes exponential. Relative levels of gene expression were calculated as a fold change from control, as shown below:

Fold change = 2-[(ΔΔCt)∕Ct]
Where, \([\Delta][\Delta]Ct = \Delta Ct_{\text{experimental}} - \Delta Ct_{\text{control}}\)

And \(\Delta Ct_{\text{experimental}}\) and \(\Delta Ct_{\text{control}}\) are normalised to the reference genes \((GUSB\ and\ \beta-\text{actin})\)

\([\Delta][\Delta]Ct\) gives the change in cycle number between the conditions. The fold change is given by \(2^{-[\Delta][\Delta]Ct}\) as this adjusts for the exponential increase between samples.

### 2.5.3 Non-quantitative PCR

End-point PCR was used to non-quantitatively assess template input, using a thermostable DNA polymerase and primers to amplify a product, prior to visualisation using agarose gel electrophoresis (section 2.8.1). A reaction mix was made using *Thermusaquaticus* (Taq) polymerase (GoTaq; Promega). Per 20µl reaction:

- 4µl of 5x reaction buffer (final concentration MgCl₂ 1.5 mM)
- 2µl dNTPs (200 µM)
- 0.4µl Forward Primer (5µM)
- 0.4µl Reverse Primer (5µM)
- 0.3µl GoTaq polymerase (0.5 units)
- <1 µg Template
- Sigma H₂O to 20µl

Reactions were optimised for denaturation followed by amplification and extension. Optimised reaction conditions were:

- 94°C for 4 minutes
- 25-34 cycles of
  - 94°C for 45 seconds
  - 50-64°C for 45 seconds
  - 72°C for 60 seconds
- 72°C for 8 minutes
2.6 **Enzyme Linked Immunosorbent Assay (ELISA)**

Enzyme Linked Immunosorbent Assay (ELISA) is a sensitive way of assessing antigens and antibodies in biological samples and hence was used to assess the levels of 5 different proteins in serum samples. The proteins were Osteopontin (OPN), Epimorphin (EPIM), Glycoprotein NMB (GPNMB, Osteoactivin), Secreted protein and rich in cysteine (SPARC, Osteonectin), Fibronectin (FN1), and Vimentin (VIM). A list of all antibodies, standards, dilution ranges and detection methods is shown in Table 2-3.

Two variations of the ELISA protocol were used due to the availability and applicability of antibodies. Sandwich ELISA, whereby a coating antibody was initially used to capture the antigen prior to a detecting antibody being used to detect the relative concentration by comparing to the optical density at 450nm of known standards. The second or direct ELISA differs in that the sample is coated directly onto the plastic wells and then a detecting antibody is used to assay. The two methods are diagrammatically demonstrated in Figure 2-2.
Figure 2-2: Schematic of ELISA protocols.
(A) Sandwich ELISA schematic with capture antibody is coated onto microplate well bottom and (B) direct ELISA method whereby serum is bound to well bottom and detecting antibody binds to the appropriate antigen.

2.6.1 Sandwich ELISA

A Nunc™ MaxiSorp, flat-bottomed, clear 96-well plate ELISA plate (Thermo-scientific) was coated with 100µl of the appropriate coating antibody, diluted in a coating buffer (0.05M carbonate-bicarbonate, pH 9.6; Sigma) and incubated overnight at 4°C. The plate was vigorously washed 5 times with wash buffer (50mM Tris, 0.14M NaCl, 0.05% (v/v) Tween, pH 8.0) using a plate washer (ELX50; Biotek). The plate was then blocked using the blocking solution (50mM Tris, 0.14M NaCl, 1% BSA, pH 8.0) for 1 hour. After this, the plate was washed 5 times with the wash buffer. A dilution series of recombinant human protein was made using the sample diluent (50mM Tris, 0.14M NaCl, 0.05% (v/v) Tween, 1% BSA, pH 8.0). The dilution range was optimised to produce similar detection levels to serum concentrations of the target protein. The human serum samples were diluted with the sample diluent and 100µl per well was added in duplicate. The plate was incubated for 2 hours at 4°C and then was washed 5 times with wash buffer.
2.6.2 Direct ELISA

100µl human serum diluted in coating buffer was added, in duplicate, into a Nunc™ MaxiSorp microplate. Alongside the serum, a dilution series of the protein to be assayed was coated using an appropriate recombinant human protein. Table 2-3 lists the products used and the corresponding concentrations. The plates were incubated at room temperature for 2 hours and then washed 5 times using a plate washer. 200µl of blocking buffer was added to all wells and incubated for 1 hour followed by plate washing.

<table>
<thead>
<tr>
<th>Recombinant Human Standard (ng/ml)</th>
<th>Osteopontin</th>
<th>Fibronectin</th>
<th>GPNMB</th>
<th>Vimentin</th>
<th>SPARC</th>
<th>Epimorphin</th>
</tr>
</thead>
<tbody>
<tr>
<td>cOPN Mouse 2ug/ml 1:250</td>
<td>16 - 0.25 (1:6250) Stock 100 ug/ml</td>
<td>4096 - 64 (1:1750) Stock 1 mg/ml</td>
<td>8 - 0.3.125 (1:12500) Stock 100 ug/ml</td>
<td>16 - 0.25 (1:6250) Stock 100 ug/ml</td>
<td>64 - 1 (1:1562) Stock 100 ug/ml</td>
<td>4 - 1/16 (1:25000) Stock 250 ug/ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Coating AB</th>
<th>Mouse 1:1000</th>
<th>Goat 1ug/ml 1:200</th>
<th>Mouse 1:1000</th>
<th>Mouse 1ug/ml 1:200</th>
<th>1:25 (100ul)</th>
<th>1:25 (100ul)</th>
<th>1:25 - 1:50 (100ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Dilution</td>
<td>1:25 (100ul)</td>
<td>1:800 (50ul) 1:25 (50-100ul)</td>
<td>1:25 - 1:50 (100ul)</td>
<td>1:25 (100ul)</td>
<td>1:25 - 1:50 (100ul)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Block time</td>
<td>300ul for 2hrs</td>
<td>300ul for 2hrs</td>
<td>300ul for 2hrs</td>
<td>300ul for 2hrs</td>
<td>300ul for 2hrs</td>
<td>300ul for 2hrs</td>
<td></td>
</tr>
<tr>
<td>Detecting AB</td>
<td>Goat Biotinylated. 0.1ug/ml (1:500)</td>
<td>Rabbit Polyclonal. 1:2000</td>
<td>Mouse 1ug/ml 1:500)</td>
<td>Mouse 1:1000</td>
<td>Mouse 1:500 (1ug/ml)</td>
<td>Goat polyclonal 1:200 (0.5-1ug/ml)</td>
<td></td>
</tr>
<tr>
<td>x-HRP or 2'</td>
<td>Rabbit HRP 1:2000</td>
<td>Biotinylated anti-mouse 1:1000</td>
<td>Biotinylated anti-mouse 1:1000</td>
<td>Biotinylated anti-mouse 1:1000</td>
<td>Biotinylated anti-goat 1:2000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strept-HRP(1:200) In PBS-BSA</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2-3: ELISA protocol table.

Table showing reagents (including dilutions) used for each target and the protocol employed.
2.6.3 Detection of Target Protein

The following detection protocol is the same for both sandwich and direct ELISA. After washing plates, 100µl of detection antibody was added to the microplate, diluted to the appropriate concentration in sample diluent. Plates were incubated for 2 hours at room temperature. If a non-biotinylated detection antibody was used, then after 2 hours the plates were washed and incubated for 1 hour in a biotinylated secondary antibody raised against the animal the detecting antibody was made in (see Table 2-3). After 5 plate washes, streptavidin-HRP (R&D Systems) was diluted 1:200 in PBS with 1% BSA. 100µl was inserted per well and incubated for 20 minutes in the dark. The plates were vigorously washed 5 times with the wash buffer. A chromogenic solution, 3,3′,5,5′-Tetramethylbenzidinesolution (TMB; R&D systems) was prepared in the dark, 100µl was added per well and colour developed in the dark for 10-20 minutes. The reaction was then stopped using a stop solution (0.18M H₂SO₄) at 100µl per well. The optical density of each well was measured on a microplate reader (EL800; Biotek) at 450nm and optical correction for plate impurities performed at 540nm. Analysis software used was Gemtek (Biotek) and serum concentrations were determined using the known concentrations of standards to create a curve of best fit.

2.7 Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was used to determine if the transcription factor, SOX9, was binding with specific genomic regions, after finding inter-species conserved binding site information with in silico analysis (section 2.10). To ensure the highest quality of ChIP, all reagents were exclusively used for ChIP assay.

2.7.1 Cell Fixation and Chromatin Isolation

LX-2 cells or activated rHSCs were cultured on 15cm dishes (Corning) until at approximately 80% confluence. When cells were at appropriate density they were transferred from their cell culture incubator onto ice. The media was aspirated and cells washed in ice-cold PBS (Sigma) and then fixed by rocking at 4ºC in 1% formaldehyde in DMEM for 10mins. After a PBS wash, the dishes were incubated in STOP fix (0.125M glycine in PBS) for 5 minutes at room temperature and then
washed in PBS. 2ml Scrape Solution (1.25mM PMSF in 10ml PBS) was added to the dish and cells scraped into a 15ml tube. Following centrifugation (10 minutes, 2400rpm at 4ºC) cell pellets were re-suspended and lysed in cell lysis buffer (10mM HEPES pH6.5, 0.5mM EGTA, 10mM EDTA, 0.25% Triton X-100) for 5mins at 4ºC. If necessary, prior to the addition of the cell lysis buffer, the supernatant was removed and the pellet stores at -80ºC. After further centrifugation, pelleted nuclei were washed in ice-cold nuclei wash buffer (10mM HEPES pH6.5, 0.5mM EGTA, 10mM EDTA, 200mM NaCl), centrifuged and lysed in fresh, ice-cold nuclei lysis buffer (50mM Tris-HCl pH 8.1, 10mM EDTA, 1% SDS).

2.7.2 Chromatin Shearing by Sonication

Chromatin was sheared by sonication to generate 200bp – 1000bp fragments. A Bioruptor® Standard (Diagenode) was used at settings:

- Level - High
- 10 minutes in sonicator
- Alternating on/off sonication in 30 second cycles

The nuclei in lysis buffer were kept at 5-8ºC to prevent SDS from precipitating. 50µl per 500µl of sonication was taken and DNA cleaned up for PCR using QIAquick PCR Purification kit (Qiagen) according to the manufacturer’s protocol. The remaining chromatin was stored on ice for immunoprecipitation, if the sonication was adequate.

Briefly, 250µl (5 volumes) buffer PB was added to 50µl sheared chromatin sample, pipetted into a QIAquick column and centrifuged at 13,000rpm for 60 seconds. The column was washed with 750µl of buffer PE, centrifuged, flow-through discarded and then centrifuged again to remove all buffer traces. The DNA was eluted by the addition of 30µl of deionised H₂O (Sigma). 7.5µl of 5x loading dye (Bioline) was added to the sample and the quality of the sonication was assessed by agarose gel electrophoresis using Hyperladder IV (Section 2.8.1). If the sheared DNA was between 200bp – 1000bp, as desired, the remaining sample was taken for immunoprecipitation.
2.7.3 Immunoprecipitation

Magnetic, ChIP-grade, protein G coated beads (Cell Signalling) were pre-cleared by removing the supernatant from 200µl of beads, using a magnetic separation rack, and washed for 1 hour on a rotor at 4°C in 200µl clearing buffer (1mg/ml sheared salmon sperm and 1mg/ml BSA). Following this, beads were washed twice in 1ml IP dilution buffer (1M Tris-HCl, 100mM EDTA, 1M NaCl, 10%SDS, 1% Triton-X, in H₂O) and then re-suspended in 270µl of IP dilution buffer.

Per immunoprecipitation, 450µl of IP dilution buffer was added to 50µl of sheared chromatin and incubated overnight at 4°C on a rotor with 3µg antibody (see Table 2-2) and 1µl protease inhibitor cocktail (PIC; Roche). Using a magnetic separation rack, antibody-bead complexes were washed 3 times in 1ml of each of the following buffers: buffer 1 (20mM Tris-HCl pH8.1, 2mM EDTA, 50mM EDTA, 0.1% SDS, 1% Triton X-100), buffer 2 (10mM Tris-HCl pH8.1, 1mM EDTA, 250mM LiCl, 1% NP-40, 1% Deoxycholate), and TE buffer (10mM Tris-HCl pH8.1, 1mM EDTA). Protein-DNA complexes were eluted by shaking in 300µl fresh elution buffer (1% SDS, 100mM NaHCO₃) for 45 minutes at room temperature. Protein-DNA cross-links were reversed by incubation for 2.5 hours at 65°C following the addition of 12µl of 200mM NaCl. Protein contamination of DNA was cleared by the addition of 2µl Proteinase K (Roche) for 1 hour at 37°C. 6µl of the protease inhibitor PMSF was added to each sample.

DNA was purified using MiniElute PCR Purification kit (Qiagen) according to manufacturer’s guidelines and analysed by end-point PCR and qPCR (section 2.8.1 and 2.5.2). 5 volumes of buffer PB was added to the 1 volume of sample and transferred to a MiniElute column and centrifuged at 13,000rpm for 60 seconds. This was repeated until the entire volume of the sample had been through the column. Following this, the column was washed with 750µl of buffer PE, centrifuged, flow-through discarded and then centrifuged again to remove all buffer traces. The DNA was eluted by the addition of 12µl of deionised H₂O (Sigma).
2.8 DNA manipulation and purification

2.8.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis is used to separate DNA and RNA molecules by size, as negatively charged nucleic-acids will move at different speeds through an agarose matrix with an electric field, depending on size. 1-2% Agarose (Melford labs, UK) was dissolved in TBE buffer (80mM Tris, 80mM Boric acid, 2mM Na₂EDTA) by boiling. Ethidium Bromide (EtBr, Sigma) was added to give a final concentration of 50ng/ml. 150ml gel solution was poured into a gel tray with a well forming comb. The gel was allowed to set and the comb removed. Gels were submerged in TBE within a gel running tank and DNA samples were loaded after being mixed with 5x loading buffer (Bioline). DNA marker ladders (Hyperladder I and Hyperladder IV; Bioline) were loaded alongside samples to allow determination of size. Electrophoresis was carried out at 90-100V for 30-60 minutes, assessed using the colour markers in the loading dye, to maximise resolution. The gel was removed from the tank and bands visualised and photographed under UV light using Molecular Imager Gel Doc XR+ System with Image Lab Software (Biorad).

2.8.2 Gel Extraction

After agarose gel electrophoresis, DNA was visualised using a UV transilluminator (Uvitec) and the appropriate protective equipment. Bands were excised with a new sharp blade and transferred into a 1.5ml microcentrifuge tube. The DNA was extracted using a Gel Extraction kit (Qiagen), according to manufacturer’s protocol. The gel was dissolved in 3 volumes of buffer QG at 50°C for 10 minutes with vigorous mixing. 1 volume of isopropanol was added, mixed and applied to a QIAquick spin column followed by centrifugation at 13,000rpm for 1 minute. The column was washed with 500μl buffer QG and 750μl of buffer PE. After a further centrifugation at 13,000rpm for 1 minute, to remove all liquid, DNA was eluted in 20-50μl of deionised H₂O (Sigma). If necessary, DNA was quantified with a spectrophotometer (section 2.5.1.2).
2.8.3 DNA Cloning

2.8.3.1 Preparation of Insert DNA

The SOX9 binding site in the \textit{MMP13} gene was cloned into the plasmid vector pGL3-promotor (Promega, UK) for luciferase assay (section 2.9). All plasmids generated were sequenced to confirm insertion (full protocol in Section 2.8.6). The plasmid map is shown in Figure 2-3. DNA for ligation into the vector was generated by PCR from genomic DNA. Primers were designed to contain recognition sequences for restriction endonucleases, Kpn I (New England Biolabs) and Xho I (New England Biolabs), so the ends of the amplified insert DNA contained the sequence for each restriction enzyme. See Appendix 1 for details of primers and product. The restriction enzymes were selected after confirming absence of potential binding sites within the insert sequence (seq-BLAST, NCBI-database). Following amplification, DNA products were separated by agarose gel electrophoresis (section 2.8.1) and gel extracted (section 2.8.2). ‘Sticky ends’ were created by overnight digestion with restriction enzymes at 37ºC. The products were re-purified by agarose gel electrophoresis and extraction. DNA concentration was analysed by Nanodrop (section 2.5.1.2).

![Figure 2-3: Plasmid map of pGL3-Promotor vector.](image)

Ampicillin resistance sequence in red, restriction sites used are in yellow, and luciferase reporter site is in green. Adapted from Promega product information.
2.8.3.2 Preparation of Vector DNA
pGL3-Promoter vector DNA was digested with the complementary restriction enzymes. As restriction produced ‘sticky-ends’, re-ligation of the vector was not an issue. The vector DNA was purified by agarose gel electrophoresis and gel extracted. 1µl of DNA was analysed by Nanodrop to quantify concentration (section 2.3.2).

2.8.3.3 Ligation of Insert and Vector
Ligations were carried out in a 10µl volume containing DNA ligase buffer (50mM Tris-HCl, 10mM MgCl2, 1mM DTT, 1mM ATP, 5% PEG), digested insert DNA and dephosphorylated vector and 1 unit of T4 DNA ligase (Roche). Vector: insert ratios were calculated for efficient ligation. Prior to the addition of ligase and buffer, the cocktail was incubated at 65°C for 10 minutes to linearise vector and insert.

Ligation reaction was set at:

16°C 16 hours (facilitate ligation)
65°C 10 minutes (to heat inactivate ligase)

2.8.3.4 Mutation of Insert DNA
A mutated version of the site was created from the cloned vector, whereby 6 base pairs of the binding site were removed, to use as a negative control. This was achieved using primers missing these base pairs. A series of two PCRs were performed using known pGL3-promotor primers (RVprimer3 and GLprimer2) and primers at the known binding site, with 6bp removed (mut-MMP13 primers; shown in Appendix 1). This created two template strands with an overlap of 15 base pairs (Figure 2-4A). Reaction conditions were as follows:

94°C 3 minutes
30 cycles of
    94°C 45 seconds
    55°C 45 seconds
    72°C 1 minute
72°C 8 minutes
5 units of Klenow fragment (New England Biolabs) were added to each reaction 15 minutes at 37°C. The products were separated on an agarose gel and then gel extracted. A second PCR was performed using alternating ratios of the two products, alongside the previous *MMP13* primers used to create the insert for cloning (Figure 2-4B). Reactions conditions for second synthesis PCR were as follows:

94°C 3 minutes  
72°C 15 minutes  
30 cycles of  
94°C 45 seconds  
55°C 45 seconds  
72°C 1 minute  
72°C 8 minutes  

The product was separated on an agarose gel and then gel extracted. The product was digested with KpnI and XhoI to create a similar product to the original 310bp cloned insert. Crucially however, it was missing a large portion of the site for SOX9 binding and was hence 304bp in length. The mutated insert was ligated into vector DNA as previously described (section 2.8.3.3).
Two-step PCR to create mutant DNA insert.

(A) First round of PCR using pGL3-promoter vector with SOX9 binding site inserted with KpnI and XhoI digestion. Two reactions are setup with this vector, one containing RVprimer3 and reverse MMP13 primer missing 6bp (shown in orange) and a second reaction with forward MMP13 primer missing 6bp and GLprimer2 (shown in green). This creates two separate products that are used together in the second synthesis PCR reaction. (B) Second reaction uses both products from the first reaction with the original MMP13 insert primers (shown in blue). After restriction with KpnI and XhoI, this creates a single product almost identical to the original binding site, however, it is lacking 6bp that would be required by SOX9 to bind to that site. This gives an almost identical negative control.

2.8.4 Plasmid Preparation

2.8.4.1 Transformation of Competent Cells

2-5µl (20-50ng) of plasmid DNA was added to 50µl of chemically competent cells DH5α (Invitrogen) and then incubated on ice 30 minutes. A negative reaction was created using DH5α cells without plasmid. The mix was heat-shocked by incubation in a H₂O waterbath at 42°C for 20 seconds, chilled on ice for a further 2 minutes and rescued with 950µl of LB media (Tryptone 1%, Yeast extract 0.5%, Agar 1.5%, NaCl 8.6mM, Glucose 20mM) at 37°C. The broth was incubated with vigorous
shaking at 200 rpm at 37°C for 1 hour. The ensuing cloudy mixture was centrifuged at 5000rpm for 3 minutes and the cells re-suspended in 150µl of the same broth. The transformed cells were then plated out onto LB agar containing ampicillin (100µg/ml; Sigma), inverted and grown overnight at 37°C.

2.8.4.2 Growth of Bacterial Cultures
After overnight growth, colonies were selected from the agar plates using a sterile pipette tip and inoculated in 5 ml of LB media containing ampicillin. This starter culture was incubated for 6-8 hours at 37°C, shaking at 200 rpm. 1ml of this culture was used for mini-preparation and if this proved successful, 500µl of the starter preparation was added to 500µl of glycerol and stored as stock at -80°C, whilst the remaining culture was inoculated into 200ml of LB containing ampicillin and cultured overnight for maxi-preparation.

2.8.4.3 Plasmid Preparation from Bacterial Cells
Prior to embarking maxi-preparation to produce a useable quantity of plasmid, an alkaline lysis method using QIAprep Spin Miniprep (Qiagen) was used to confirm the viability of the transformation. Following the manufacturer’s instructions, cells were pelleted at 4000rpm for 3 minutes then re-suspended in buffer (P1). Lysis buffer (P2) was added and the microcentrifuge tube shaken vigorously prior to neutralisation with buffer (N3). Precipitate formed by gently inverting the tube 5-10 times and was removed by centrifugation at 7500rpm for 10 minutes. The clear supernatant was applied to a spin column and centrifuged at 7500rpm for 1 minute. The column was rinsed with wash buffer and the plasmid DNA eluted in 50µl distilled water (Sigma). To confirm transformation viability, the same restriction enzymes (KpnI and XhoI) were added to the eluted plasmid in the appropriate buffer. These products were analysed on an agarose gel to confirm two products matching the size of both insert and vector DNA.

For a larger quantity of plasmid DNA, HiSpeed Maxiprep kit (Qiagen) was used. This typically produces 500µg of DNA per 1ml of elute and employs a similar alkaline lysis method as Miniprep. Cells were isolated by centrifugation at 2400rpm for 15 minutes at 4°C and re-suspended in buffer P1, lysed in buffer P2 then neutralised with P3 for 10 minutes. Lysate was added to a HiSpeed MaxiT-tip and
precipitate cleared by flow-through filtration. The HiSpeed Tip was washed with QC buffer and DNA eluted with QF buffer. DNA was precipitated by mixing with 0.7 volumes of isopropanol and incubation for 5 minutes at room temperature. This mixture was filtered through a QIA precipitator and washed with 70% ethanol before final elution with 500µl of distilled water.

2.8.5 DNA Concentration
DNA concentration and quality was determined by measurement in a spectrophotometer (Nanodrop 2000c; Thermo Scientific). 1µl of elute was tested for absorbance at 280nm with high quality DNA assumed with a 260/280 ratio between 1.8 and 2.0. The absorbance was used to give a concentration of DNA within the sample.

2.8.6 Sequencing
For absolute verification of plasmid insert, formal sequencing was used. 500ng plasmid DNA mixed with 4pmol of primer, specific to the insert, was sent to the core facility at The University of Manchester DNA Sequencing Facility and the nucleotide sequence confirmed. 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.

2.9 Luciferase Assay

To assay for gene regulatory region activation and repression by the transcription factor SOX9, a firefly luciferase reporter assay was used. Specifically, plasmids containing the cloned and mutated binding site of SOX9 within the *MMP13* gene were transfected into LX-2 cells with an empty vector or vector containing the *SOX9* gene, as described in section 2.2.2. A plasmid containing Renilla reniformis was transfected concurrently allowing measurement of transfection efficiency and normalisation of values. Further controls were achieved by comparison to transfections using empty reporter vectors.
The luciferase assay was performed 48 hours after transfection. Cells were incubated for 15 minutes in 15µl passive lysis buffer (PLB; Promega). Following lysis, 20µl of the supernatant from each well was added into a 96-well opaque luminometer plate (Greiner). Light production was measured using an Orion L luminometer (Berthold Technologies), programmed to dispense 100µl of luciferin per well and measure cumulative bioluminescence over 10 seconds. After this, Renilla was assayed by the addition of 20µl of Stop & Glo substrate (Promega) per well and measurement of bioluminescence over 1 second. Analysis was undertaken on firefly and luciferase expression by dividing firefly units by renilla units to normalise for transfection efficiency.

2.10 In silico Analysis

2.10.1 Transcription Factor Binding Sites
Transcription factor binding sites (TFBSs) conserved between human and non-human genomes were identified using the online genome browsing software Dcode [www.dcode.org]. Genomic regions of interest were also directly scanned for independent conserved TFBSs using the ECR Browser. Sequence data was entered into the Mulan function, which performs multiple local DNA sequence alignments to identify conserved sequences between human and non-human genomes. Conserved sequences were uploaded to the MultiTF function, which identified TFBSs within the conserved sequence DNA. Hence, any conserved TFBSs, specifically for SOX9, in the original region of interest were identified.

2.10.2 Literature Searches
Using individual gene name and/or symbol, manual searches of published literature for genes of interest were performed using PubMed (NCBI).

2.11 Trent Chronic Hepatitis C Cohort

Paired human liver biopsies and serum samples were obtained from the Trent HCV Cohort Study with informed written consent and multicentre research ethical
approval (Mohsen and Trent 2001; Mohsen 2001). As of January 2010, the Trent Hepatitis C Cohort contains in excess of 3000 liver biopsies. These are stored across multiple centres. The patient selection and data collection criteria have been described elsewhere (Mohsen 2001; Ryder et al. 2004; Williams et al. 2009). Patients with known human immunodeficiency virus (HIV) were excluded from the cohort (Ryder et al. 2004). Liver biopsies were taken within 6 months of serum sampling and assessed blindly by one of three histopathologists (Ryder et al. 2004). The adequacy threshold for histological assessment was the presence of five or more portal tracts. Biopsies were scored according to the Ishak staging (IS) criteria, on a 7 point scale from IS 0 to IS 6; the latter representing the most severe fibrosis/cirrhosis (Ishak et al. 1995).

For the purpose of this project, there was access to all samples in the Nottingham University hospitals NHS Trust, namely the Queens Medical Centre and Nottingham City Hospital. Within the two sites, there were 909 biopsies available. To assess for fibrosis progression over a period of time, only patients with more than one biopsy were selected, leaving a total of 197 patients. To assess potential serum biomarkers, only biopsies with a serum samples taken within 6 months of biopsy were selected. This left a total of 115 patients with paired liver biopsies (Index and follow-up) all with a parallel serum sample. The breakdown is diagrammatically represented in Figure 2-5.

All serum samples were aliquoted into screw-sealed tubes and stored at -80°C until needed. Aliquots were only used once and did not go through repeated freeze-thaw cycles.
Data was analysed using the SPSS 19 (IBM, USA) software package in multiple samples after a minimum of 3 determinations. Where appropriate, data was expressed as mean ± standard error of the mean (SEM). In samples with 2 groups,
comparison was made using an un-paired t-test, assuming unequal variance. In groups with 3 or more ordinal or nominal variables, means were compared by one-way ANOVA, followed by Tukey HSD (highest significant difference) or Dunnett’s post-hoc test, depending on assumption of equal or unequal variance between groups. All box-and-whisker plots show the median value with the box representing 75% confidence interval (CI) and the whiskers representing 95% CI. Progression data was compared with binomial logistical regression modelling, and is shown with 95% CI using a significance probability of p<0.05. Serum markers’ ability to discriminate between stages of fibrosis was achieved by area under the receiver operator analysis.
3 RESULTS: OSTEOPONTIN IS A NOVEL DOWNSTREAM TARGET OF SOX9 WITH DIAGNOSTIC IMPLICATIONS FOR PROGRESSION OF LIVER FIBROSIS IN HUMANS

3.1 Introduction

Fibrosis of the liver is characterised by excessive extracellular matrix (ECM) deposition. One of the major cell types responsible for this is the hepatic stellate cell (HSC) (Iredale 2007; Friedman 2008). In response to injury, HSCs become activated into proliferative myofibroblasts, migrate into the surrounding parenchymal cells and secrete tissue-damaging ECM; the major component of which is collagen type 1 (Col1). In light of this, it is apparent the ECM has a major influence on fibrotic progression. In addition to scar formation, as a complex mix of various components involved in cell proliferation, migration and differentiation, the ECM is central to a cell’s ability to mediate a range of signals and regulatory networks. One ECM component with such varied cellular functions is the matricellular glycoprophosphoprotein osteopontin (OPN).

3.1.1 Osteopontin

Osteopontin (OPN) is a highly phosphorylated acidic matrix protein characterised by polyaspartic acid and arginine-glycine-aspartate (RGD) sequences (Sodek et al. 2000). OPN was initially documented as a key protein in the ECM of bone (Oldberg et al. 1986). Since its discovery, the importance of OPN in bone remodelling has come to the fore and the name reflects the ability to act as a bridge between cells and hydroxyapatite (through RGD and polyaspartic acid). Over the last 25 years OPN has been shown to have a diverse range of functions including an involvement in the
cellular response to inflammation, wound healing, angiogenesis, apoptosis, and
tumour metastasis (Giachelli and Steitz 2000).

Bone and epithelial cells remain the predominant source of OPN (Giachelli and
Steitz 2000) but a growing number of cells including macrophages (Singh et al.
1990), T cells (Ashkar et al. 2000) and smooth muscle cells have been shown to
produce OPN. In healthy tissue OPN is present at low levels in the proximity to
regions of epithelial lining (Xie et al. 2001). In contrast to the limited distribution of
OPN in healthy tissues, during injury, inflammation and repair, OPN is unregulated
in almost all tissues that have been studied (Giachelli and Steitz 2000). Mice lacking
OPN remaining normal and healthy demonstrate this. Indeed, these animals appear to
be protected from autoimmune disease and certain inflammatory conditions;
however, they are more susceptible to infections (Rittling 2011).

In the healthy liver, OPN is only present in biliary epithelial cells and at a low level
in hepatocytes. OPN’s association with inflammation and the wound healing
response has led to interest of its function in liver disease. This link has been
confirmed following carbon tetrachloride (CCl₄) liver injury in rodents, with Kupffer
cells shown to secrete OPN into necrotic areas, whilst expression was also noted in
HSCs and macrophages. (Kawashima et al. 1999). OPN is up regulated in several
liver diseases, which suggests that it may play a role in pathogenesis of these
conditions. For example, studies have implicated OPN as an important chemokine
for neutrophil infiltration in liver injury secondary to alcohol and steatohepatitis
(Apte et al. 2005; Ramaiah and Rittling 2007).

3.1.2 Regulation of OPN expression

The mechanism by which OPN expression is increased during liver injury has not yet
been fully elucidated. In the context of bone remodelling, OPN expression can be
regulated by various mediators of bone development including parathyroid hormone,
vitamin D3, calcium and phosphate (Xie et al. 2001). Many cytokines and growth
factors can also be up-regulated by OPN, including TNF-α and IL-1, which are
known to induce OPN expression through the action of protein kinase C, suggesting
a positive feedback mechanism (David and Masaki 1998).
Potential transcriptional regulatory motifs have been identified in genomic regions upstream of *OPN* including a TATA-like sequence and CCAAAAT-like sequence (Ramaiah and Rittling 2008).

Hedgehog signalling is known to regulate SOX9 expression, as discussed in section 1.9 and has also been shown to be involved in *OPN* control. In melanoma, OPN has been shown to be regulated by the transcription factor Glioblastoma-1 (Gli-1), via sonic hedgehog (Shh) (Das et al. 2009). In liver fibrosis, OPN regulation by Gli-2 and Hh signalling has also been demonstrated (Syn et al. 2011).

### 3.1.3 Osteopontin Receptors

OPN interacts with cells by binding to an array of integrins. These include αv (β1, β3 or β5) and (α4, α5, α8, or α9)β1. Binding is through both the RGD and non-RGD motifs. αvβ3 is amongst the best studied integrin for OPN and binding promotes cell adhesion, migration and proliferation (Panda et al. 1997). OPN also acts as a ligand for the cluster of differentiation-44 (CD44) receptor (non-RGD binding motif), which stimulates macrophage migration (Marcondes et al. 2008). The CD44 receptor is known to interact with multiple ECM proteins, including hyaluronan (HA), MMPs and collagens.

Cleavage of OPN by thrombin produces two active sub-units (see Figure 3-1). The N-terminal fragment, containing the RGD domain binds integrins, whilst the C-terminal unit acts as a CD44 ligand (Ramaiah and Rittling 2008).
Figure 3-1: Diagram demonstrating the C and N terminal fragments produced by thrombin cleavage at the protease hypersensitive region.

Adapted from Ramaiah and Rittling 2008. N-terminal contains the RGD domain for integrin binding and C-terminal contains CD44 binding region.

3.1.4 Osteopontin and Inflammation

Cells from a macrophage-derived lineage (e.g. monocytes, macrophages, osteoclasts) are an important source of OPN (Takahashi et al. 2001) and are also the primary OPN target in inflammation (Giachelli and Steitz 2000). OPN has multiple actions on these cells including macrophage adhesion (Nasu et al. 1995), migration (Singh et al. 1990), generation of ROS (Hwang et al. 1994), cytokine release (Ashkar et al. 2000), differentiation (Crawford et al. 1998) and phagocytosis (Schack et al. 2009). Macrophage chemotaxis is reduced by OPN targeted mutagenesis with neutralising antibody (Giachelli et al. 1998) and is also evident in liver, where OPN depletion reduces macrophage infiltration in response to inflammatory stimuli (Diao et al. 2004; Lorena et al. 2006). Heterogeneity of OPN and its receptors has led to debate about OPN function, especially in vitro (Rittling 2011). For example, experiments into the role of OPN in cytokine production in macrophages have led to inconsistent and conflicting results. The isomeric differences in OPN and the heterogeneity of receptors are reasons attributed to these variations (Rittling 2011). These data underlines the complexities in structure and function of this protean factor.

In liver injury, OPN has an effect on other cells involved in both the innate and adaptive immune responses. These include T cells, especially Th1 cells, NKT cells and neutrophils (Diao et al. 2004; Banerjee et al. 2006). NKT cells appear to be a
major source of Hh ligands in NASH and this drives fibrosis by OPN up-regulation in HSCs (Syn et al. 2012).

3.1.5 Osteopontin and fibrosis

OPN is detected in a wide range of tissues and bodily fluids with normal physiological roles during developmental (e.g. bone, hepatic bile duct formation, vascular remodelling), immune system regulation and wound repair (Rangaswami et al. 2006) and associated with pathological processes relating to cancer and inflammation (Rangaswami et al. 2006; Chiodoni et al. 2010). Furthermore, several lines of evidence implicate OPN in fibrotic mechanisms; for example it contributes to wound scarring in skin (Mori et al. 2008) and has been implicated in lung, kidney, and heart fibrosis (Klingel and Kandolf 2010; Merszei et al. 2010; Sabo-Attwood et al. 2011). The ability of OPN to mediate such diverse cellular functions is likely related to its extensive post-translational modifications and ability to signal through several integrin and CD44 variant receptors (Rangaswami et al. 2006; El-Tanani 2008).

3.1.5.1 Pro-fibrotic effects

OPN is associated with fibrosis in a range of organs. These include cardiac fibrosis (Matsui et al. 2004) bleomycin-induced pulmonary fibrosis (Takahashi et al. 2001), ureteral obstruction (Ophascharoensuk et al. 1999) and scarring (Mori et al. 2008). In skin wound healing, attenuation of OPN resulted in reduction of fibrillar collagen levels and down-regulation of TGF-β (Mori et al. 2008). OPN is implicated in aberrant remodelling following injury in cardio-respiratory conditions and this progresses to fibrosis (Takahashi et al. 2001; Matsui et al. 2004). Furthermore, in models of pulmonary fibrosis, OPN promotes migration, adhesion and activation of fibroblasts (Kohan et al. 2009). Similar evidence has also been established in liver fibrosis with OPN contributing to in vitro proliferation and migration of HSCs (Koh et al. 2005). These studies suggest a common role of OPN in pathological remodelling of ECM.

3.1.5.2 Anti-fibrotic effects

The anti-fibrotic effects of OPN centre on its ability to support cell survival. An increased survival of hypoxic cells in the renal tubule by OPN mediated reduction in
cell peroxide levels has been demonstrated (Denhardt et al. 2001) and OPN deficient mice have increased renal dysfunction in response to renal ischemia (Noiri et al. 1999). The potential mechanisms regulating this effect may be suppression of nitric oxide synthesis through inducible nitric oxide synthase (iNOS) up-regulation (Nagasaki et al. 1999) or decreased apoptosis by increasing NF-κB activity through integrin αVβ3 binding (Scatena et al. 1998).

Promoting cell survival and limiting apoptosis are not necessarily anti-fibrotic effects. As discussed earlier, activated HSCs and myofibroblasts in liver fibrosis are resistant to apoptosis and this results in an accumulation of these cells and hence fibrosis. By promoting this activity, OPN may well be contributing to fibrosis in the liver.

3.1.6 Osteopontin in liver disease

Hh signalling has been shown to regulate OPN in NASH models of liver fibrosis with Patched-deficient mice showing increased OPN and fibrosis (Syn et al. 2011). As Hh cytokines are released by hepatocytes committed to apoptosis, this mechanism may be applicable to other causes of fibrosis. The intermediate step between Hh and OPN up-regulation may be PI3K activation, suggesting other pro-fibrotic cytokines like PDGF and TGF-β may also share this pathway (Machado and Cortez-Pinto 2011). In contrast to this work, OPN deficient mice were noted to have increased fibrosis after CCl4 treatment and bile duct ligation (Lorena et al. 2006). Furthermore, single dose of CCl4 led to a more severe necrosis compared to wild-type animals. The cause of the reduced fibrosis was thought to be decreased macrophage infiltration in the OPN deficient mice and the increased necrosis due to decreased breakdown of nitric oxide in OPN-deficient mice. Further conflicting results have been shown in OPN over-expressing mice. Mice injected with concanavalin A had massive necrosis (Mimura et al. 2004) whilst CCl4 resulted in reduced necrosis in the over-expressing model (Koh et al. 2005).

The protective role of OPN in fibrosis is at odds with other studies in liver fibrosis and other organs (Ophascharoensuk et al. 1999; Takahashi et al. 2001; Matsui et al. 2004). OPN has been shown to induce migration and proliferation of HSCs in vitro. OPN also increases the synthesis of type I collagen by increasing the availability and activity of active TGF-β. (Lee et al. 2004). OPN has also been demonstrated to up-
regulate type I collagen expression in a TGF-β independent manner, as well as down-regulating MMP13, leading to increased fibrosis (Urtasun et al. 2012). More recently, plasma OPN levels have been implicated as a potential biomarker in CHC induced liver disease (Xie et al. 2007; Zhao et al. 2008; Huang et al. 2010) and as a marker to predict survival following transplantation for hepatocellular carcinoma (Sieghart et al. 2011). These data suggest that up regulation of OPN is an important pathway to HSC activation and suggest a role of OPN as a potential biomarker.

### 3.1.7 Summary

OPN has diverse roles in fibrosis due to regulation of inflammation, tissue remodelling and cell survival. This suggests a range of outcomes during wound healing that can both contribute to and protect against fibrosis.

The importance of ECM proteins such as OPN in fibrotic progression suggests factors responsible for maintaining the fibrotic microenvironment through ECM regulation will be core to this process. To explain aspects of this, similar to studies in cancer (Das et al. 2009), OPN is thought to be regulated by Hh signalling during liver fibrosis (Syn et al. 2010). However, evidence from development and disease indicate the transcription factor, Sex-Determining Region Y-box 9 (SOX9), has a key role in mediating ECM.

We have previously shown SOX9 becomes ectopically expressed in activated HSCs where it regulates Col1 expression (Piper Hanley et al. 2008). During development SOX9 has critical roles in deposition of ECM proteins and has also been associated with fibrotic pathologies affecting the skin, kidney and heart (Naitoh et al. 2005; Sumi et al. 2007; Levay et al. 2008; Airik et al. 2010; Peacock et al. 2010; Pritchett et al. 2011). However, the precise mechanisms underlying the role and regulation of SOX9 in these settings have yet to be defined.
3.2 Aims

In this chapter SOX9 was identified in the biliary cells of healthy human and rodent liver and shown to co-localise with fibrillar collagens within the portal tracts. In fibrotic liver, SOX9 was identified in non-biliary cells in regions of fibrosis, as shown by collagen deposition.

OPN was identified as a novel downstream target of SOX9 during activation of HSCs, highlighting both proteins as potential markers of fibrotic progression in human tissue and serum. OPN and SOX9 are markers of biliary cells in normal human and rodent liver. During liver fibrosis SOX9 and OPN were localised to the same regions in fibrotic tissue sections and both are increased during HSC activation where SOX9 binds to an upstream region of the OPN gene. Moreover, in human biopsy samples we demonstrate SOX9 and OPN expression was increased with severity and this was paired with an increase in serum OPN. These data provide further evidence of the importance of SOX9 during progression of liver fibrosis and highlight SOX9 and OPN as novel targets to develop anti-fibrotic therapies, or as diagnostic markers aimed at predicting severity of disease.
3.3 Results

3.3.1 Expression of Sox9 and fibrillar collagens in the liver

Normal liver basement membrane contains laminin, COL4 and various proteoglycans. Portal architectural structure contains a greater concentration of fibrillar collagens, namely COL1 and COL3, whilst biliary epithelial cells and ductal plate cells are known to be SOX9 positive (Si-Tayeb et al. 2010). Figure 3-2 A-D exhibit SOX9 staining in the nuclei of a bile duct within the portal tract. In the consecutive images, COL1 and COL3 are seen in the portal tract and COL4 can be noted in the basement membrane surrounding the portal tract and the sinusoid. A similar pattern is established in the non-fibrotic rat liver, as shown in Figure 3-2 I-L. In the foetal liver (Figure 3-2 E-H), SOX9 staining is apparent in the biliary epithelial cells but also along the forming ductal plate. The collagen staining in these sections is more pronounced and extended outside of the developing portal tract, especially for the fibrillar collagens (Figure 3-2 F and G).

To investigate the expression of SOX9 in relation to collagen expression directly in fibrotic tissue, the established technique of carbon tetrachloride (CCl4) induced fibrosis was used. As noted by the arrow in Figure 3-2M, Sox9 positive nuclei are visible in areas outside of the portal tract and not formed in ring-like structures normally associated with biliary epithelial cells. In control rat tissue, Col1 and 3 are seen within the portal tract only (Figure 3-2; J and K), whilst Col4 is seen to a lesser extent and is mainly found outside the portal tract (Figure 3-2L, shown by arrow). Figure 3-2 N-P reveals the presence of Col1, 3 and to a lesser extent, Col4 in co-localising to areas of Sox9 positive cells. The distribution of these areas is consistent with the portal expansion and bridging fibrosis and suggests the presence of Sox9 positive cells at the junction of fibrosis and the parenchyma.
Figure 3-2: Bright-field images in normal and fibrotic liver.

Consecutive 5µm sections with staining for SOX9 and collagens 1, 3 and 4 (Brown). COL1 and 3 are fibrillar collagens and COL4 is a normal basement membrane constituent. In healthy liver, SOX9 is just seen in the nuclei of biliary epithelial cells within the portal tract (A, E and I). Collagen can be noted in and around the periphery of the portal tract (B, C, D, F, G, and H). In the rat liver after CCl4 treatment, Sox9 can be seen on the periphery of the scar edge (Arrow M). All three collagens co-localise to this area, especially collagens 1 and 3 (N, O and P). Counterstained with toluidine blue and scale bar represents 50µm.

3.3.2 Sox9 and Opn in normal healthy liver

During early liver development in the mouse, Sox9 is expressed in a subset of precursor cells or hepatoblasts that give rise to the biliary duct (Antoniou et al. 2009; Carpentier et al. 2011). Similarly, Opn has also been described as an early biliary duct marker, maintained in mature biliary structures (Antoniou et al. 2009; Carpentier et al. 2011). In line with this, we detected robust expression of SOX9 and OPN in the biliary ducts of adult rodents, during human development at 18 weeks post-conception (wpc) and in mature biliary ducts in the adult (Figure 3-3).
3.3.3 SOX9, OPN and Desmin in fibrotic liver

Previous data have independently identified OPN (Huang et al. 2010; Syn et al. 2011) and SOX9 (Piper Hanley et al. 2008) in areas of liver fibrosis in animal models. Here, in rat and mouse models of liver fibrosis, nuclear Sox9 localised to desmin-positive cells, confirming its presence in HSCs (Figure 3-4A). Opn localised...
with Sox9 to spindle-shaped HSCs with elongated nuclei in areas of fibrosis as well as to biliary cells (Figure 3-4B).

![Figure 3-4: SOX9, OPN and Desmin in fibrotic liver.](image)

(A) Consecutive 5µm sections shown from fibrotic rat and mouse liver staining for Sox9 and Opn (brown) counterstained with toluidine blue. Note similarly located staining for Sox9 and Opn in cells with more spindle shaped nuclei (arrows) as well as in biliary cells (*). (B) Dual IF in fibrotic tissue from rat and mouse showing nuclear Sox9 (red) in biliary cells (*) and in cells with cytoplasmic staining for desmin (green) (white arrowheads). Scale bar represents 100µm. Done in collaboration with Miss Emma Harvey.

### 3.3.4 SOX9 and OPN expression in activated LX-2 cells

Recognising that Opn has recently been described in fibrotic liver disease (Zhao et al. 2008; Huang et al. 2010; Syn et al. 2010) and in combination with our previous data showing Sox9 becomes ectopically expressed in regions of increased α-Sma and Coll1a during liver fibrosis in rat (Piper Hanley et al. 2010), we were interested to see if the two proteins were similarly expressed in fibrosis. Consistently, expression of Opn was increased and associated with Sox9 in tissue sections from fibrotic livers in both rat and mouse. This was not just associated with biliary cells (Figure 3-4B). To place this with one of the major cell types associated with fibrotic mechanism, in the immortalised human HSC cell line, LX-2, Opn was not expressed in α-Sma/Sox9.
negative quiescent cells (Figure 3-5). However, similar to other studies (Lee et al. 2004; Syn et al. 2010), following activation of LX-2 cells by culture in high serum media, Opn protein and mRNA became expressed, paralleling that of Sox9 (Figure 3-5).

Figure 3-5: SOX9 and OPN expression in activated LX-2 cells.
Quantification of SOX9 and OPN in quiescent and activated LX-2 cells by qPCR (A) and immunoblotting (B). SOX9 up-regulated a 0.5-fold and OPN up-regulated 2-fold by immunoblotting. (C) Representative immunoblot. All immunoblotting quantification was normalised to β-actin. *p<0.05.

3.3.5 SOX9 and OPN expression after knockdown of SOX9
To determine whether Sox9 regulates Opn, we carried out Sox9 knockdown using siRNA in activated LX-2 cells. Sox9 abrogation (reduction of 62%) caused a commensurate decrease in Opn protein levels (40%) (Figure 3-6). In silico analysis identified a conserved SOX9-binding site in the enhancer region of OPN (Figure 3-7), containing the CAAT core SOX-binding motif with additional binding affinity for SOX9 conferred by additional flanking nucleotides (Mertin et al. 1999). Taken together, these data suggest that the transcription factor SOX9 may directly regulate OPN transcription.
SOX9 and OPN expression after attenuation of SOX9.
siRNA abrogation of SOX9 in LX-2 cells after activation with high serum. Cells transfected with siRNA at 0 and 24 hours and harvested at 48 hours. Standardised against scrambled siRNA controls. (B) Representative immunoblot. All immunoblotting quantification was normalised to β-actin. *p<0.05; ±p<0.001.

SOX9 conserved binding site in the enhancer region of OPN.
Alignment of the upstream OPN enhancer region with conserved SOX9-binding motif highlighted in black (human sequence shown is -3,886 to -3,842 base pairs relative to transcriptional start site). Conserved nucleotides indicated by asterix (*).

3.3.6 SOX9 and OPN expression in CHC liver fibrosis
The above results demonstrate that in liver fibrosis SOX9 becomes ectopically expressed in HSCs and is capable of instigating downstream expression of OPN. However, as further evidence of the importance of SOX9 and OPN during liver fibrosis in humans we investigated their expression in HCV patients with increasing
severity of fibrosis. In a test cohort of staged biopsy samples from mild (F0; n=5), moderate (F3; n=4) and severe (F6; n=4) fibrosis, expression of SOX9 and OPN was increased (Figure 3-8). In tissue sections from mild fibrosis, SOX9 and OPN expression was mainly restricted to the biliary ducts. Whereas in both moderate and severe forms of the disease, in addition to expression in cholangiocytes, SOX9 and OPN could also be detected in cells surrounding the periphery of the scar. In the case of SOX9, low level expression also appeared to be located within the hepatocytes directly around the forming scar.

Figure 3-8: Expression of α-SMA, SOX9 and OPN in fibrotic human liver.

Consecutive 5 μm sections of human liver biopsy samples from patients with mild, moderate and severe fibrosis. Brown immunohistochemistry staining is shown for α-SMA, SOX9 and OPN. SOX9 and OPN are detected in the biliary ducts in all tissues, however in moderate and severe fibrosis SOX9 and OPN are also present surrounding the scar edge (indicated by α-SMA) and possibly in few hepatocytes. Boxes highlight SOX9 in biliary cells and ectopic regions. Counterstained with toluidine blue. Size bar represents 20μm.
3.3.7 Nuclear SOX9 counting in liver sections

With clear nuclear staining for SOX9 (Figure 3-8), we were able to provide a significant quantitative measurement of increasing SOX9 positive cells related to severity using cell counting (Figure 3-9). Every measure was taken to account for expression relating to biliary epithelial cells, positive cells at the scar edge and ectopic SOX9 expression within the parenchyma (cells morphologically suggestive of hepatocytes). In this small cohort, an increase in SOX9 expressing cells was detected correlating with severity of disease for cells surrounding the scar edge and in hepatocytes, whereas biliary cells only appeared to increase in severe fibrosis possibly relating to ductular reaction observed in severe fibrotic / cirrhotic tissue (Figure 3-9).

![Graph showing SOX9 counting in fibrotic liver sections.](image)

Figure 3-9: SOX9 counting in fibrotic liver sections.

Cell counting for SOX9 positive cells in biopsy samples (mild, n=5, moderate, n=5 and severe, n=4) separated into biliary epithelial cells (BEC), cells surrounding scar edge and in hepatocytes / ectopic expression. Error bar (standard error of the mean) and statistical significance is based on total cells numbers. *p<0.05, between mild and severe fibrosis.
3.3.8 OPN concentration in corresponding serum samples

As a circulating glycoprotein, OPN concentration was also assayed in serum samples (Figure 3-10). In this test case (linked to the biopsy samples but expanded to ~10 per group) OPN concentration was also significantly increased, particularly in the severe samples compared to mild and moderate fibrosis (Figure 3-10).

![Figure 3-10: Serum OPN concentration.](image)

Serum OPN concentration determined in each group of fibrosis (mild, n=13, moderate, n=14 and severe, n=13). Circle (*) indicates single outlier in mild and moderate fibrosis. B, Mild to severe, * p<0.05 (based on total cell counts), C, mild to severe, †, p<0.005 and moderate to severe, ‡, p<0.001.
3.4 Discussion

Ectopic expression of SOX9 is a mechanism to explain aspects of ECM deposition during liver fibrosis (Piper Hanley et al. 2008). Under the influence of TGF-β, SOX9 becomes increased in activated HSCs in vitro where it is responsible for COL1 and COL2 expression (Piper Hanley et al. 2008). More broadly, as a conserved mechanism of ECM deposition in organ fibrosis, SOX9 has also been linked to activation of COL4 in glomerulosclerosis and COL2 in calcified aortas (Neven et al. 2007; Sumi et al. 2007). In line with this, we demonstrate a more diverse role for SOX9 in the expression of other secreted ECM components and have identified OPN as a novel downstream target (Pritchett et al. 2012). Given the importance of OPN in directing cell signalling responses relating to cell proliferation / migration and as an inflammatory mediator potentially involved in macrophage recruitment, these data provide increasing evidence of the importance of SOX9 in orchestrating the pathogenesis of liver fibrosis.

3.4.1 SOX9 in liver fibrosis

SOX9 expression in biliary epithelial cells and with the developing ductal plate has been reported in rodent livers (Antoniou et al. 2009). The ductal plate is believed to give rise to bipotent hepatoblasts able to differentiate towards either a cholangiocyte or hepatocyte fate (Lemaigre 2009). SOX9 positivity is a hallmark of these cells and this is maintained or lost dependent on direction of cell fate. In this chapter, this phenomenon is demonstrated in 18wpc foetal liver and shown alongside the main fibrillar and basement membrane collagens. The loss of the ductal plate is apparent in healthy adult liver, with collagens confined to the portal tract and sub-endothelial space. A similar picture is evident in non-fibrotic rat liver. However, with liver fibrosis, the portal tract expands and this can be noted by the abundance of fibrillar collagens extending from the portal tract towards central veins and other portal tracts. This phenomenon, known as porto-central and porto-portal fibrosis, respectively, is a mark of fibrosis. Interestingly, Sox9 positive nuclei are seen at the periphery of the septa, co-localising with collagen. As previously described, activated HSCs are a key driver of fibrogenesis and Sox9 has been shown to be ectopically expressed in these cells (Piper Hanley et al. 2008), giving rise to the possibility that these cells are Sox9 positive activated HSCs.
3.4.2 Osteopontin and SOX9

Within the normal liver both SOX9 and OPN localise to cholangiocytes. During liver fibrosis, both proteins have been implicated in disease progression (Piper Hanley et al. 2008; Zhao et al. 2008; Huang et al. 2010; Piper Hanley et al. 2010; Syn et al. 2010). As a result we hypothesised that SOX9 may regulate OPN, raising the question whether other biliary components may be SOX9 targets relevant to fibrosis. This would be particularly relevant in the case of cholangiopathies (e.g. primary biliary cirrhosis, primary sclerosing cholangitis) where SOX9 is required for normal biliary formation and function, whereas our studies indicate excessive SOX9 would result in a fibrosis.

In fixed tissue sections from rodent models of fibrosis Sox9 and Opn were located in the same fibrotic regions. To place these data with activation of HSCs as part of the fibrotic response, the timing of Opn expression in HSCs in vitro was consistent with the presence of Sox9 and its reduction in activated HSCs following siRNA treatment, demonstrating a reliance on Sox9 for Opn expression. The presence of a conserved SOX9 binding motif with the enhancer region of OPN suggested a direct regulation by SOX9. This was confirmed by ChIP analysis in both HSCs and LX-2 cells by work within the group (Pritchett et al. 2012).

The possibility of hedgehog signalling being upstream of SOX9 was also suggested by work within the group (Pritchett et al. 2012). This would be consistent with the known regulation of OPN in fibrosis (Syn et al. 2011) and SOX9 in development (Pritchett et al. 2011). In support of this and indicative of feedback mechanisms, Opn has also been shown to enhance expression of the TGF-β receptor (TβRII) in HSCs (Lee et al. 2004). Unravelling signalling pathways relevant to core mechanisms is likely to be complex but will ultimately provide important mechanistic insight into liver fibrosis progression.

3.4.3 Osteopontin as a biomarker of liver fibrosis

The value of investigating mechanisms of liver fibrosis impacts on novel development of targeted anti-fibrotic therapies and non-invasive biomarkers to predict clinical progression of the disease. Recent studies on OPN have already highlighted its potential use as a diagnostic tool in liver disease (Xie et al. 2007;
Zhao et al. 2008; Huang et al. 2010). In particular two studies investigating serum OPN concentrations in patients with viral hepatitis B and C (HBV and HCV) indicated that OPN levels significantly correlated to severity of liver damage (Zhao et al. 2008; Huang et al. 2010). Here further validation of these studies is provided through investigating fibrosis severity in HCV patient samples but provide additional data correlating SOX9 cell numbers in biopsy samples with its novel downstream target, OPN, in serum. In biopsy samples, every effort was taken to distinguish between the cholangiocytes and myofibroblastic cell phenotypes associated with fibrosis and shown SOX9 cell number is increased in both cell types, suggesting both biliary hyperplasia and myofibroblastic mediated fibrotic mechanisms are initiated as disease progresses. These data provide additional evidence to support a robust role for OPN in liver fibrosis and as a potential marker of disease progression. In addition, our studies on SOX9 provide novel insight and opportunity to correlate SOX9 cell counting as a potential diagnostic marker in biopsy samples (explored further in Chapter 6) to assess fibrotic status in the liver either as a standalone strategy or in conjunction with its secreted targets (e.g. OPN; see Chapter 5).

3.4.4 Summary

The emerging evidence of the role of SOX9 in fibrotic mechanisms associated with skin, kidney, heart, and liver imply an unappreciated conserved mechanism for this transcription factor in these and related diseases (Pritchett et al. 2011). Combined with its well defined role in regulating components of ECM during development (Pritchett et al. 2011), our data describing COL1 and OPN as downstream targets of SOX9 during liver fibrosis leave unanswered questions relating to whether other SOX9 targets may define circulating proteins amenable to assay as novel biomarkers of fibrosis (see Chapter 5).
4 RESULTS: EPIMORPHIN ALTERS THE DIRECT INHIBITORY EFFECTS OF SOX9 ON MMP13 IN ACTIVATED HSCs

4.1 Introduction

Liver fibrosis is caused by repetitive rounds of injury and repair resulting in excessive extracellular matrix (ECM) deposition. As a bidirectional process, subtle alterations in cellular processes can determine a propensity towards on-going fibrosis or repair (Ramachandran and Iredale 2012). One of the key cells mediating fibrosis is the hepatic stellate cell (HSC). Following injury to the liver, HSCs become activated into proliferative myofibroblasts (MFs), secrete damaging ECM and infiltrate the surrounding tissue causing fibrosis. Although multiple pathways and factors regulate this process, the transcription factor SOX9 (sex determining region Y-box 9) appears very important. In response to transforming growth factor-beta (TGF-β), SOX9 becomes expressed in activated HSCs (Piper Hanley et al. 2008) where it regulates production of Collagen type 1 (Col1), the major collagen associated with the fibrotic scar (Piper Hanley et al. 2008), and Osteopontin (OPN) (Pritchett et al. 2012).

We hypothesised that reduction in SOX9 alters the fibrogenic process towards fibrosis resolution. Work within the group has previously identified epimorphin (EPIM) as interacting with SOX9 in a yeast-2-hybrid study (Piper Hanley, unpublished). This morphogenic protein is implicated in wound healing and liver regeneration and we postulated that it might interact with SOX9, leading to alteration in the phenotype of the SOX9 expressing, activated, HSC. This is especially relevant with recent publications suggesting that not all activated HSCs undergo apoptosis, as was previously believed, but may revert to an ‘inactive’ phenotype, where they are not potentiating fibrosis but are primed to do so in the event of further injury (Kisseleva et al. 2012; Troeger et al. 2012).
The ECM in liver fibrosis is a complex, dynamic region with simultaneous degradation and accumulation of matrix. Whilst in normal physiology these are kept in a fine balance, in disease net change in either direction can result in dramatic alteration in matrix quantity and quality. Key factors capable of degrading matrix are matrix metalloproteinases (MMPs), and their inhibitors (tissue inhibitors of matrix metalloproteinases; TIMPs). In fibrosis, these favour ECM accumulation, with up-regulation of TIMPs, especially TIMP-1, and down regulation of key collagenases, especially MMP13 (Iredale 2001). A broader overview of ECM regulation is given in Section 1.5.5. We identified Mmp13 as potentially negatively regulated by Sox9 in a microarray study of activated HSCs after knockdown with siRNA against SOX9. Given known roles of SOX9 in ECM regulation in development and dysregulation in disease, the hypothesis that SOX9 is involved in MMP and TIMP regulation is explored in this chapter.

4.1.1 Epimorphin

Epimorphin (EPIM) is a highly conserved, extracellular protein known to induce morphogenesis of hepatocytes (Hirose et al. 1996) and has a role in liver repair (Hirai et al. 1992; Daisuke et al. 2005). Originally identified as a mesenchymal cell-surface associated protein (Hirai et al. 1992), EPIM (also known as syntaxin 2) plays significant morphogenic roles in several tissues including skin, hair follicle, mammary gland, lung, kidney, intestine, pancreas and liver (Radisky et al. 2003; Jia et al. 2011; Zhou et al. 2011). The protean functions of Epim during development were demonstrated in Epim-null mice, whereby changes in Epim were noted in several different tissues, with the greatest phenotypic changes in gastrointestinal epithelial tissues (Wang et al. 2006). These animals displayed impaired testicular development, spermatogenesis and intestinal growth. Interestingly, liver development was abnormal with increased liver weight in Epim−/− mice.

Because of its important role in directing morphogenesis during development, interest has grown around EPIM in tissue repair. Studies have highlighted EPIM as a regulator of tissue repair in lung (Terasaki et al. 2000), kidney (Yamada et al.) and liver (Daisuke et al. 2005).
4.1.2 Localisation of epimorphin

4.1.2.1 Extracellular epimorphin

EPIM is also known as syntaxin-2, dependent on its topography. As an extracellular protein it is known as EPIM and can be cell membrane bound or secreted (Hirai et al. 1992). When intracellular it is referred to as syntaxin-2. With no signalling peptide at the N-terminal, EPIM is secreted by non-classical externalisation, avoiding post-translational modifications by Golgi apparatus and endoplasmic reticulum (Hirai et al. 2007). A hydrophobic region on the C-terminus anchors EPIM to the cell membrane through phosphatidylinositol (Hirai et al. 1992). Release of EPIM can occur by Phospholipase C or deletion of the C-terminus transmembrane domain (Yohei 1994), shown in Figure 4-1. Cell stress and calcium signalling mechanisms also are triggers for the secretion of EPIM. Sub-toxic levels of toxins or calcium have been shown to release EPIM in vitro (Hirai et al. 2007).

4.1.2.2 Intracellular epimorphin

EPIM is member of t-SNARE (Soluble NSF Attachment Protein Receptor) syntaxin proteins. Syntaxin-1 and EPIM/syntaxin-2 share 60% amino acid sequence (Bennett et al. 1993). These membrane-bound, vesicle docking proteins are present in a broad group of tissues (Bennett et al. 1993). Syntaxin-2 has been shown to be attached to the cytoplasmic surface of the cell membrane where it mediates the docking/fusion of synaptic vesicles as part of the SNARE machinery (Band and Kuismanen 2005).

Therefore, dependent on topography, EPIM/syntaxin-2 has dual functionality. As an intracellular protein it is involved in vesicular transport and, as an extracellular protein, acts as a morphogen capable of binding to the epidermal growth factor receptor (EGFR) and integrins to mediate its effects (Radisky et al. 2009).
Figure 4-1: Functional domains of Epimorphin/Syntaxin-2.
Protein is anchored to the cell membrane by the transmembrane (TM) domain. EPIM is membrane bound by the TM domain and cleavage of TM, or TM and SNARE helices, releases EPIM into the ECM. Deletion analysis has shown that N-terminal helices (yellow) are necessary for morphogenetic activity but deletion of SNARE helix and C-terminal transmembrane (TM) domain (both in red) are non-essential. Conversely, the SNARE domain is needed for vesicular functioning. Adapted from Radisky et al, 2003.

4.1.3 Epimorphin regulation
The parallels between embryonic morphogenesis and adult tissue repair are becoming increasingly apparent (Jacinto et al. 2001; Martin and Parkhurst 2004) with numerous pathways common to both (Wei et al. 2009). Hedgehog (Hh) signalling and SOX9 are both critical regulators of developmental processes (Cristin M et al. 1998; Piper et al. 2002). Regulation of EPIM by SOX9 and sonic Hh has been shown in vitro where cells transfected to over-express SOX9 or sonic Hh went on to markedly increase EPIM expression and suggested sonic Hh as initiating SOX9, which in turn up-regulated EPIM (Oka et al. 2006). Data from Chapter 3 and from within the group has also postulated Hh signalling regulating SOX9 in liver fibrosis (Pritchett et al. 2012).

4.1.4 Epimorphin receptors
EPIM is a ligand for EGF receptor (Iizuka et al. 2007) and $\alpha_v$-integrins (Hirai et al. 2007). Focal-adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK) cascades are downstream effectors of both EGF receptor and $\alpha_v$-integrins. Both are implicated in development (Eliceiri and Cheresh 2000) and tissue repair (Jia et al. 2011).
4.1.5 Epimorphin in the liver

4.1.5.1 Epimorphin expression in the liver
EPIM is a mesenchyme/myofibroblast protein and in the liver expression is limited to HSCs and myofibroblasts (Hirose et al. 1996). In the adult liver, EPIM is expressed in the connective tissue surrounding blood vessels, along the sinusoidal lining where HSCs reside (Segawa et al. 2005) and in mesenchyme surrounding the bile duct where it is thought to play a role in duct formation (Zhou et al. 2010; Jia et al. 2011). In vivo, there is a transient reduction in EPIM expression following liver injury which appears to increase in HSCs during late recovery phase (Yoshino et al. 2006), highlighting a potential role in tissue repair. These findings suggest that activation of HSCs has an inhibitory effect on EPIM expression.

4.1.5.2 Functions of epimorphin in the liver
EPIM has important roles in liver development. Culture models show its ability to induce the morphogenesis of hepatocytes into a mature hepatocyte phenotype (Hirose et al. 1996) and acquire polarity which is a crucial property for development and differentiation (Abu-Absi et al. 2002). Rodent models also indicate the importance of EPIM in bile duct formation, a function regulated through FAK and ERK pathways (Zhou et al. 2010; Jia et al. 2011).

The role of EPIM in tissue repair has been explored in liver regeneration. Post CCl₄ induced liver injury; there is a dramatic rise in EPIM whilst the liver regenerates. These levels normalise after the after return of normal liver architexture (Segawa et al. 2005). Similar expression of EPIM has also been shown after partial hepatectomy (Yoshino et al. 2006).

One of the reoccurring features of EPIM is its ability to induce protease expression by activating MMPs to allow re-epithelisation (Daisuke et al. 2005). EPIM induces expression of MMP3, MMP9, MMP13 and uroplasminogen (uPA) in primary cultured hepatocytes (Miura et al. 2007). These proteases all have roles in ECM remodelling (see section 1.5.5) and could orchestrate repair after injury. This theory is supported by a study showing EPIM is necessary for repair in renal fibrosis; whereby EPIM was associated with the increased activity of MMP2 and MMP9 and the breakdown of type I collagen (Yamada et al. 2010). More recently, Epim has
been reported to promote hepatocellular carcinoma (HCC) invasion and metastasis (Jia et al. 2011), in part, through activation of Mmp9. Although this provides clues of EPIMs role in modulating ECM degradation during fibrosis regression the mechanisms linked to this are less clear.

4.1.6 MMP13

Matrix metalloproteinase-13 (MMP13) is a collagenase (also known as collagenase-3) with activity against collagens type 1, 2, and 3. Similar to other members of the MMP family, MMP13 is secreted as a zymogen and cleaved into its active form by extra-cellular proteinases. *MMP13* gene is found on chromosome 11q within a gene locus consisting of many other MMPs (MMPs 1, 3, 7, 8, 10, 12 and 20) and in an evolutionary conserved region (Leeman et al. 2002). Structurally, MMP13 is similar to fellow collagenase MMP1, containing a propetide domain, zinc containing catalytically active region and C-terminal domain, which is needed for catalytic activity (shown in Figure 4-2). Activation of MMP13 from its proenzyme form can be achieved by several peptidases, including autoproteolysis. Other MMPs, especially MMP3 and MT-MMP1 are central to activation, with MT-MMP1 activation being catalysed in the presence of MMP2 (Knauper et al. 1996). Non-MMP activation of MMP13 has been demonstrated by plasmin and uroplasminogen (Leeman et al. 2002).

Besides its direct collagenase role, MMP13 affects fellow metalloproteases, activating proMMP2, 3 and 9. Crucially, active MMP9 is only found in the presence of both MMP2 and MMP13 (Knauper et al. 1997), suggesting both are needed for its activation.
4.1.6.1 Roles of MMP13
MMP13 is known to disrupt the triple helix structure of collagens, this unravelling presenting the fibres to further degradation. This helicase role of MMP13 requires both catalytic domain and the C-terminal domain. Inhibitors of MMPs, TIMPs, can bind to C-terminal domain and prevent helicase activity (Knauper et al. 1997) (Leeman et al. 2002). Uniquely amongst collagenases, MMP13 can still perform other catalytic activity without the C-terminal domain. Non-collagenase action of MMP13 include gelatinase activity, degradation of laminar collagen fibrils, aggrecan, heparin sulphate proteoglycans and certain serpins (Leeman et al. 2002). MMP1, a similarly structured collagenase is unable to carry out any further catalytic activity in the presence of TIMPs bound to its C-terminal domain.

4.1.6.2 MMP13 in liver fibrosis
MMP13 is known to be produced by HSCs and shown to be responsible for the degradation of matrix (Watanabe et al. 2000). Mmp13 deficient mice displayed decreased fibrosis resolution owing to reduced HSC and scar associated macrophage derived Mmp13 (Fallowfield et al. 2007). In conflict to this, Mmp13 has been shown to be up-regulated in early stages of injury from bile duct ligation (BDL) and responsible for the initial inflammatory response (Uchinami et al. 2006). In this study, Mmp13 deficient mice demonstrated reduced cholestasis induced inflammation and fibrogenesis compared to wild-type littermates. Mmp13−/− mice also had reduced expression of Mmp2 and 9. This would be consistent with previous work, whereby Mmp13 is known to induce the initial cleavage of type-1 collagen and partially degraded collagen then activates Mmp2 and 9, resulting in positive feedback and
further collagen degradation (Benyon et al. 1999; Theret et al. 1999). This function has also been suggested as a mechanism promoting the migratory and proliferative capacity of activated HSCs (Benyon et al. 1999).
4.2 Aims

In this chapter, Epim protein is shown to decrease during activation of HSCs, in contrast to pro-fibrotic genes \(\alpha\)-Sma, Sox9 and Col1. Treatment of activated HSCs with recombinant EPIM (rhEPIM) caused a reduction in fibrotic genes (e.g. Sox9, \(\alpha\)-Sma, Col1 and Opn) and altered protease expression by increasing the collagenase Mmp13. Similarly, abrogation of pro-fibrotic Sox9 in activated HSCs resulted in increased Epim and Mmp13. Moreover, Sox9 was capable of binding to the Mmp13 gene to inhibit its expression. As a secreted factor, EPIM levels were detected in human serum in patients with liver fibrosis secondary to hepatitis C infection and were significantly raised in cirrhosis. These data provide further evidence for Sox9 as a core fibrotic gene in HSCs with important roles regulating the balance of ECM production and degradation.
4.3 Results

4.3.1 Epimorphin expression in during activation of HSCs

Epim expression was investigated in quiescent and activated rHSCs (Figure 4-3). In contrast to increased expression of Sox9 and Col1 proteins, we detected an 84% decrease in full-length 34kDa Epim in activated rHSCs (Figure 4-3A and B). Similarly, a time course activation of rHSCs indicated high levels of Epim in quiescent HSCs, which was significantly decreased by 3 days of activation, in contrast to increasing levels of α-Sma (Figure 4-3C and D). These data are consistent with our previous data showing an increase in Sox9, Col1 and Opn expression associated with activated HSCs from 3 days (Piper Hanley et al. 2008; Pritchett et al. 2012).
4.3.2 EPIM alters the profile of pro-fibrotic genes in HSCs

Epim appears to be associated with a quiescent HSC phenotype (Segawa et al. 2005; Yoshino et al. 2006). As a secreted extracellular morphogenic agent known to have a role in liver regeneration, it was theorised that Epim may revert activated HSCs to a quiescent phenotype. To determine this, activated HSCs were treated with recombinant human Epim (rhEPIM). Treatment of activated rHSCs with rhEPIM resulted in a dose responsive decrease in Sox9, Col1 and α-Sma proteins (Figure 4-4A). In particular, 2 µg/ml rhEPIM was capable of reducing pro-fibrotic protein

Figure 4-3: Epim expression in activated HSCs.

(A) Relative protein estimation of quiescent and activated HSCs. Markers of activation and myofibroblasts (α-Sma and Col1) rising with activation alongside Sox9. Epim levels demonstrate a decrease. HSCs activated by tissue culture plastic reveal progressively increasing α-Sma levels and decreasing Epim. Representative immunoblot shown in (B) and (D). All immunoblotting quantification was normalised to β-actin. *p<0.05; **p<0.01.
expression by > 60% (Figure 4-4A), including a 65% decrease in Opn expression (Figure 4-4B); likely as a result of reduced Sox9 (Pritchett et al. 2012).

These data are remarkably similar to culturing activated HSCs on Matrigel, known to contain Epim and believed to resemble the complex ECM environment surrounding native cells. Previous studies have indicated activated HSCs cultured on Matrigel can be deactivated to a more ‘quiescent-like’ state with greatly reduced $\alpha$-Sma and Col1 expression. In this study, we found Matrigel similarly decreased $\alpha$-Sma and Col1 proteins in activated HSCs but also caused a 48% and 71% reduction in Sox9 and Opn expression respectively (Figure 4-4C and D). These data suggested secreted Epim was capable of ‘switching off’ pro-fibrotic genes in HSCs similar to culturing activated HSCs on a basement membrane-like matrix (Matrigel) to mimic the ‘natural’ cellular environment.

**Figure 4-4: Epim alters the pro-fibrotic profile of activated HSCs.** Activated HSCs treated with varying concentrations of recombinant human EPIM had a reduction in Sox9, $\alpha$-Sma, Col1 and Opn (A) and (B). (C) These results mimicked the effects of matrigel culture. Representative immunoblots shown in (D). All immunoblotting quantification was normalised to $\beta$-actin. *p<0.05; **p<0.01.
4.3.3 EPIM alters protease expression in activated HSCs

To further characterise rhEPIM treated HSCs we investigated the expression of genes associated with an inactive or deactivated HSC phenotype as reported during the recovery phase following liver injury (Kisseleva et al. 2012; Troeger et al. 2012). Although, we detected no change in gene expression characteristic of these cells (e.g. increased Gfap, Pparγ or Bambi; decreased Svep1 or Cyp1B1), EPIM did appear to increase the pro-survival gene Hspa1a/b (Fig. 3A). Further analysis detected no difference in total Hsp70 protein, encompassing Hspa1a/b, or any difference in apoptosis determined by the ratio of full length to cleaved Caspase 3 expression (Figure 4-5B-C).

Studies have suggested Epim plays a protective role in the liver during the recovery phase and has been associated with resolution of fibrosis in both kidney and liver by inducing protease expression associated with ECM degradation (Miura et al. 2007; Yamada et al. 2010). In line with this, we detected altered MMP / TIMP expression in activated rHSCs treated with rhEPIM. Whereas expression of Timp1 and Mmp2 mRNA was decreased following rhEPIM treatment, levels of Mmp9 and Mmp13 mRNA were increased (Fig. 3D). In particular, 2 µg/ml of rhEPIM induced Mmp9 and Mmp13 by 2.7 and 8.2 fold respectively.
Figure 4-5: EPIM causes alteration in proteases.

EPIM treated stellates do not revert to an ‘inactivated’ phenotype suggested by the markers shown in (A) and (B). (D) The addition of EPIM to activated HSCs decreased mRNA levels of Timp-1 and Mmp-2, whilst increasing levels of Mmp-8 and Mmp13 were seen. This was not a result of increased apoptosis, as shown by no increase in cleaved caspase (C). *p<0.05; **p<0.01.

4.3.4 Mmp13 expression is reduced in activated HSCs

In rodents MMP13 (MMP-1 in humans) is the primary interstitial collagenase, thought to be a key enzyme involved in ECM resolution. During liver fibrosis, MMP13 is thought to be transiently increased during initial stages but becomes almost undetectable as the disease progresses, coincident with an increase in TIMPs 1 and 2. In activated HSCs, both pro and active MMP13 were decreased (41% and 34% respectively; Figure 4-6A). Similar to Epim treated activated HSCs, culturing cells on Matrigel also increased both pro and active forms of Mmp13 (Figure 4-6B).

In combination with our results showing decreased expression of pro-fibrotic proteins (α-Sma, Sox9, Col1 and Opn), these data imply secreted Epim alters the fibrotic phenotype of HSCs associated with modulating the ECM environment.
Figure 4-6: Mmp13 expression is reduced in activated HSCs.
(A) Pro-Mmp13 and active cleaved Mmp13 are reduced as HSCs activate. (B) Culturing activated HSCs on matrigel results in an increase in pro and active Mmp13, in line with rhEPIM therapy. All immunoblotting quantification was normalised to β-actin.

4.3.5 Sox9 expression in HSCs is associated with inhibition of Mmp13

Since EPIM has been implicated in biliary development (Zhou et al. 2010; Jia et al. 2011), similar to our studies on OPN (Pritchett et al. 2012) we investigated whether EPIM was a downstream target of SOX9. In line with their contrasting expression profile during activation of HSCs, Sox9 gene silencing resulted in a 1.6 fold induction of Epim (Figure 4-7A & D). Despite this change, a search of a comparative genomics database (dcode) did not locate any conserved SOX9 binding regions surrounding EPIM (~10kb up and downstream) or its intronic regions. In addition, Sox9 siRNA treated activated HSCs demonstrated little change in the profibrotic enzymes Mmp2, Mmp9 and Timp1, however, Mmp13 mRNA was significantly increased by >2 fold. Similarly, pro and active Mmp13 protein was increased >1.2
and 1.5 fold respectively in response to Sox9 knockdown, suggesting Mmp13 may be a direct target of Sox9 (Figure 4-7C and D).

![Figure 4-7: Sox9 knockdown increases Epim and Mmp13.](image)

(A) Attenuation of Sox9 with siRNA resulted in a 63% reduction in Epim protein detection. (B) Protease transcript levels remained unchanged except Mmp13, which doubled with knockdown. (C) Protein levels mimicked transcript change for both pro-Mmp13 and active Mmp13 with immunoblots shown in (D). All immunoblotting quantification was normalised to β-actin. *p<0.05, **p<0.01, ±p<0.001.

### 4.3.6 Sox9 directly binds to Mmp13 in activated HSCs

Using in-silico analysis software (dcode.org), a conserved Sox9 binding motif was identified in intron 7 of Mmp13 (Figure 4-8A) capable of binding Sox9. Binding was demonstrated by 3-fold enrichment in Mmp13 gene expression following Sox9 ChIP, noted by qPCR and apparent on RT-PCR (Figure 4-8B and C). Transcriptional activity was assessed in LX-2 cells in high serum using a luciferase assay after
creation of a vector with cloned SOX9 binding region of MMP13, in the presence or absence of SOX9. Transcriptional activity was reduced in pGL3-promoter SV40 vector after the addition of vector containing SOX9 by 9%. Activity after addition of SOX9 vector in cells transfected with pGL3-promoter vector containing the SOX9 binding site demonstrated a 26% reduction (see Figure 4-8D). The difference between these two values was not statistically significant.

**Figure 4-8: Sox9 directly binds to Mmp13 in rHSCs and decreases Mmp13 transcription in LX-2 cells.**
Conserved Sox9 binding site shown for human and rat within the Mmp13 gene shown in (A). ChIP showing Sox9 binding to this site by (B) qPCR (*p<0.05) and (C) RT-PCR on agarose gel electrophoresis with enrichment for Sox9 shown compared with IgG as negative control and histone H3 as positive control. (D) Transcriptional activity measured by luciferase assay. Values shown as ratio comparing SV40 vector with or without SOX9 binding region in MMP13 to the addition of SOX9. There is 9% reduction in empty vector after addition of SOX9, compared to 25% reduction in vector with MMP13 binding site after addition of SOX9.
4.3.7  EPIM serum concentration increases with cirrhosis

As a secreted protein, we investigated whether EPIM could be detected in serum of a subset of patients with fibrosis due to chronic HCV infection from the Trent HCV cohort. Fibrosis had been assessed in these patients according to Ishak criteria from biopsy samples and given a score from IS 0 to IS 6, the latter representing cirrhosis (Ishak et al. 1995; Mohsen 2001). Derivation and further details of this cohort can be found in methods Section 2.11 and Section 0. Serum concentrations assayed for different Ishak stages are shown in Figure 4-9 and demonstrate that EPIM concentration remain generally unchanged between stages 1-5. In IS 6 there is an increase in serum EPIM concentration compared to IS 1-5 and this is reflected in an AUROC of 0.77 (CI 0.66-0.87), shown in Table 4-1 with the AUROC plot shown in Figure 4-9B. Because of the relatively unchanging serum concentrations up to cirrhosis, discrimination ability of EPIM is poor when not comparing cirrhotics to non-cirrhotics (Table 4-1).

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<table>
<thead>
<tr>
<th>Fibrosis Stage</th>
<th>AUROC</th>
<th>CI (95%)</th>
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<tbody>
<tr>
<td>0 vs. 1-6</td>
<td>0.58</td>
<td>0.48-0.69</td>
</tr>
<tr>
<td>01 vs. 2-6</td>
<td>0.65</td>
<td>0.56-0.74</td>
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<tr>
<td>0-2 vs. 3-6</td>
<td>0.61</td>
<td>0.51-0.70</td>
</tr>
<tr>
<td>0-3 vs. 4-6</td>
<td>0.66</td>
<td>0.55-0.76</td>
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<tr>
<td>0-4 vs. 5,6</td>
<td>0.66</td>
<td>0.55-0.77</td>
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<tr>
<td>0-5 vs. 6</td>
<td>0.77</td>
<td>0.66-0.87</td>
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Table 4-1: Performance of EPIM in distinguishing different stages of fibrosis by AUC values with confidence intervals.

Discriminatory ability of serum EPIM to distinguish between Ishak fibrosis stages. Serum EPIM is best able to categorise IS 6 / cirrhosis from other stages with good accuracy but diagnostic ability between other groups is uncertain.
Figure 4-9: Serum concentration of EPIM is increased in IS 6 / cirrhosis compared to other stages of fibrosis.

(A) Box-and-whisker plot showing serum concentration of EPIM by ELISA against Ishak fibrosis stage. (B) Receiver operator curve demonstrating test's ability to correctly define IS 6 / cirrhosis from other levels of fibrosis (AUC 0.77 CI 0.66-0.87).
4.4 Discussion

EPIM has been implicated in tissue repair mechanisms associated with pathological organ damage in lung, kidney and liver (Terasaki et al. 2000; Segawa et al. 2005; Miura et al. 2007; Yamada et al. 2010). Previously, we have described the transcription factor SOX9 regulates profibrotic COL1 and OPN in activated HSCs (Piper Hanley et al. 2008; Pritchett et al. 2012) suggesting it plays a key role in ECM deposition in liver fibrosis (Pritchett et al. 2011). In this study we show EPIM alters the profibrotic phenotype of HSCs, in part through decreased SOX9 expression. Moreover, we demonstrate SOX9 binds to a conserved region in the collagenase gene, Mmp13, to potentially inhibit its expression in activated HSCs. These data support a broad role for SOX9 in ECM deposition (e.g. reduced Col1 and Opn) and degradation (mediated by proteases) during liver fibrosis.

4.4.1 Epimorphin expression in activated HSCs

This chapter shows expression of Epim decreased with in vitro activation of HSCs. This is in contrast to Sox9; a transcription factor that becomes expressed as HSCs activate, where it regulates the expression of the pro-fibrotic ECM proteins Col1 and Opn (Piper Hanley et al. 2008; Pritchett et al. 2012). Moreover, similar to others (Yoshino et al. 2006), we detected an inverse relationship of α-Sma and Epim expression as HSCs became activated. A recent publication indicated EPIM is increased in activated HSCs and hepatocellular carcinoma (Jia et al. 2011). This study was largely based on expression in the stellate cell line LX-2 rather than primary HSCs and in HCC cell lines. Furthermore, LX-2 cells were activated by culture in HCC cell line medium and divergent results from primary cells activated by culture on plastic is not unexpected.

4.4.2 Sox9 and Epimorphin

The recent interest in EPIM playing a role in bile duct formation (Zhou et al. 2010; Jia et al. 2011) prompted us to investigate whether, similar to OPN (Pritchett et al. 2012), EPIM was a downstream target of SOX9. Although Epim protein increased following Sox9 knockdown in activated HSCs, no conserved SOX9 binding motif was detected in a 10kb region surrounding EPIM. These data suggested EPIM was not a direct target of SOX9, although SOX9 regulation of EPIM could occur through
distant regulatory regions or indirect means; certainly studies elsewhere indicate the
two are linked in signalling cascades. Both proteins appear to be important during
development of ductular structures in liver and pancreas (Tulachan et al. 2006; Zhou
et al. 2010; Jia et al. 2011). However, the influence of EPIM on SOX9 or vice versa
may reflect differing signalling mechanisms occurring under normal physiological
conditions as opposed to disease states. This may include dose response signalling
under the influence of an EPIM gradient similar to studies in skin suggesting EPIM
functions to establish differentiated epidermal structures (Hirai et al. 2007); or the
ability of EPIM to selectively direct branching morphogenesis and luminal formation
depending on its presentation in the mammary gland (Chen et al. 2009). Similarly in
the pancreas, cellular presentation of a 150KDa EPIM tetramer is thought to be a
critical mediator of duct formation (Tulachan et al. 2006). In addition, Epim null
mice have increased intestinal growth and crypt cell proliferation (Wang et al. 2006).
Interestingly, Sox9 is known to be expressed in the crypt cells of the intestine where
it is thought to be involved in maintenance of the stem cell population (Blache et al.
2004). Although teasing apart these signalling mechanisms is likely to be complex,
these data provide some clues to the role of Epim in HSCs, for example reduced
Epim signalling allowing a more proliferative environment for SOX9 positive HSCs.

4.4.3 Epimorphin and ‘inactivated’ HSCs
In HSCs, our data suggested EPIM expression coincided with a more quiescent-like
phenotype. Given EPIM appears to be increased in the recovery phase following
injury (Miura et al. 2007) and, with recent studies suggesting some HSCs escape
apoptosis and revert to a quiescent-like state during this phase (Kisseleva et al. 2012;
Troeger et al. 2012), we investigated the influence of EPIM on activated HSCs.
Treatment of activated HSCs with rhEPIM reduced expression of the profibrotic
genes Sox9, α-Sma, Col1 and Opn, similar to culturing activated HSCs on matrigel,
(Friedman et al. 1989; Sohara et al. 2002; Gaca et al. 2003; Shimada and
Rajagopalan 2012). Although analysis of genes characteristic of inactive or reverted
HSCs (Kisseleva et al. 2012; Troeger et al. 2012) only detected an increase in the
pro-survival gene Hspa1a/b, we detected no change in Hsp70 protein expression in
rhEPIM treated HSCs or any difference in apoptosis by Caspase 3 expression.
However, in line with others (Yoshino et al. 2006; Miura et al. 2007; Yamada et al.
2010), rhEPIM did appear to alter protease expression in HSCs.
During liver fibrosis, the changing environment characterised by excessive ECM deposition results from an imbalance between ECM production and degradation (Iredale 2007). One of the pathways involved in ECM degradation involves MMPs (Iredale 2007) and their inhibitors TIMPs. In particular, fibrotic livers have increased TIMP1 and TIMP2 expression (Iredale et al. 1992; Kossakowska et al. 1998) with limited expression of the fibrillar collagen degrading enzyme MMP13 (Watanabe et al. 2000). With this in mind, rhEPIM treated activated HSCs have reduced Timp1 expression with high level expression of Mmp13. In addition, cells had a reduction in Mmp2, known be increased during fibrosis (Preaux et al. 1999), and increased Mmp9, a gelatinase potentially involved in cytokine regulation (Iredale et al. 2012). These data provided further evidence of EPIMs ability to change the profibrotic characteristics of activated HSCs.

4.4.4 SOX9 and MMP13
Similar to rhEPIM treated HSCs, increased Epim following Sox9 abrogation altered protease expression, in particular through increased Mmp13 expression. Further analysis indicated SOX9 was capable of directly binding to a conserved motif upstream of MMP13; suggesting SOX9 may inhibit MMP13 in activated HSCs. These data are supported by studies in bone formation. Whereas SOX9 plays a key role during early stages of chondrogenesis involved in proliferation and expression of cartilage matrix genes (Pritchett et al. 2011), in later stages of chondrocyte maturation SOX9 is absent concomitant with an increase in MMP13 (Hattori et al. 2010; Nishimura et al. 2012). In combination, these data indicate a conserved mechanism in development and disease for SOX9 inhibition of MMP13, in keeping with SOX9s role in ECM deposition. Given there are currently no approved effective antifibrotic drugs to treat liver fibrosis (Friedman 2003), this study has important clinical implications. Of interest, as a potential antifibrotic target, studies suggest enhanced expression of MMP13 attenuates liver fibrosis with reduced collagen levels (Endo et al. 2011; Kim et al. 2011). However, our previous and present data suggest attenuation of SOX9 represents a more attractive target capable of both reducing scar formation though decreased ECM deposition and increased degradation.
4.4.5 Serum Epimorphin concentration increases in cirrhosis

Collectively, our data indicated Epim was increased in quiescent HSCs and appears to play a role in the induction of a less fibrotic phenotype. As a secreted protein, we were interested in the potential of serum EPIM as a biomarker for the severity of liver damage in patients HCV. The majority of serum biomarker assays under investigation to date have concentrated on proteins that are increased as a result of fibrosis severity. However, equally important to this would be the detection of proteins that decrease during progression of the disease. As expected, serum EPIM levels initially decreased during moderate stages of fibrosis. Whereas, in severe fibrosis, EPIM concentration increased, potentially as a result of regenerating nodules and / or increased expression associated with ductal hyperplasia in cirrhotic samples.

4.4.6 Conclusion

In summary, EPIM is expressed in quiescent HSCs and appears to alter the ECM environment in activated HSCs in part through influencing the expression of profibrotic SOX9. More broadly, this study provides further evidence for SOX9 as a key factor in liver fibrosis, beyond its role in ECM deposition, through inhibition of ECM degrading enzymes such as MMP13. Capitalising on factors capable of manipulating SOX9 such as EPIM may lead to development of novel therapeutic agents in the treatment of liver fibrosis.
5 RESULTS: DOWNSTREAM TARGETS OF SOX9 AS POTENTIAL BIOMARKERS OF LIVER FIBROSIS

5.1 Introduction

In the developed world, chronic hepatitis C (CHC) is the leading cause of liver transplant and annual mortality is in excess of 350,000 (World Health Organisation 2011). CHC infection causes progressive fibrosis, however the rate of advancement is variable and prediction of the stage of disease can be challenging. Rates of progression to cirrhosis and complications vary between 2-4% over 20-30 years (Wiese et al. 2005). Given the heterogeneous nature of disease progression, defining extent of fibrosis in an individual is an important clinical measurement.

Liver Fibrosis is characterised by alteration in the composition of the extracellular matrix resulting in increased collagen deposition, decreased liver elasticity and micro-vascular changes. Microscopic evaluation of these changes by liver biopsy sampling led to qualitative estimation and by using set observational criteria, semi-quantitative methods such as Ishak/modified Knodell score (Ishak et al. 1995) and Scheuer/METAVIR scoring (Bedossa and Poynard 1996) systems were developed. This facilitates an ordinal scale on which to evaluate the extent of fibrosis and also provide limited information about on-going inflammatory activity. This framework has been the bedrock of fibrosis assessment. However, variability in sampling and observer assessment coupled with procedure associated morbidity make liver biopsy a less than perfect tool (Bedossa et al. 2003; Rockey et al. 2009). To this end, alternatives have been sought.

5.1.1 Biomarkers for liver fibrosis

Increased ECM, and specifically collagens, causes mechanical stiffness within the liver and this has been employed to assess fibrosis, showing good correlation (Sandrin et al. 2003). Products like Fibroscan™ and MR elastography provide a real-
time value that can be extrapolated to a fibrosis stage. However, steatosis, raised BMI and hepatic inflammation can all distort the accuracy (Yoshioka et al. 2008), making this a less than ideal method.

The simplicity of a blood test that would correlate with fibrosis stage is an elusive goal in biomarker research. Biomarker discovery has broadly had two approaches, either exploring targets thought to be involved in ECM turnover of fibrosis or those that are thought to be surrogate markers of fibrosis. Both of these have been combined with parametric and clinical indices to create panels. These have been shown to be good at distinguishing early/no fibrosis and cirrhosis (Manning and Afdhal 2008). Classifying patients with intermediate stages of fibrosis has proven more of a challenge with biomarkers and non-invasive tests (Boursier et al. 2009). Fibrogenesis at these intermediate stages can be very variable, with some patients progressing at a far greater rate to cirrhosis than others (Thein et al. 2008).

To address this, several different techniques have been attempted. Genome wide approaches have been employed to more broadly distinguish genetic factors involved in disease susceptibility. This has resulted in identification of SNP and gene loci conferring susceptibility to fibrosis progression, but as yet no clinically available biomarkers (Chambers et al. 2011; Patin et al. 2012). Attempts have been made to use clinical glycomic techniques to identify cirrhotics and non-cirrhotic patients from a variety of chronic liver diseases (Callewaert et al. 2004). This has delivered putative targets, which need assessing in larger cohorts. However, their derivation as predominantly markers of cirrhosis suggests limited use across the spectrum of fibrosis. Proteomic microarrays have also been used to identify potential new targets in serum sample of patients with advancing fibrosis and cirrhosis. This identified six potential targets of fibrosis, with three already having been commercially tested before (White et al. 2007).

5.1.2 Identifying novel biomarkers
Understanding drivers of fibrogenesis may identify potential biomarkers of fibrosis that are more capable of discriminating between intermediate stages of fibrosis. Recent work has shown the transcription factor, sex-determining region Y-box 9 (SOX9), to be ectopically expressed in activated HSCs in liver fibrosis and has been
shown to regulate type I collagen (Hanley et al. 2008). Work from this thesis has demonstrated that SOX9 regulates the inflammatory cytokine, OPN (Chapter 3) and negatively regulate MMP13, a collagenolytic metalloproteinase (Chapter 4). During development, SOX9 has diverse roles regulating the expression of a number of genes encoding ECM proteins (Pritchett et al. 2011) and SOX9 has also been associated with fibrotic pathologies affecting the skin, kidney, and heart (see Section 1.9). Chapter 3 also suggested that serum OPN concentration may be used as a biomarker for liver fibrosis, an observation consistent with recent work, whereby OPN has been suggested as a putative marker for HCC (Shang et al. 2012) and HCV associated liver fibrosis (Huang et al. 2010).
5.2 **Aims**

In this chapter, *in vitro* gene expression profiling was employed to provide large-scale analysis into the mechanistic changes occurring in progressive fibrosis. This provided a broader understanding of the networks involved and regulated by SOX9. Data from a RNA microarray of activated rHSCs after attenuation of Sox9 was used to identify targets with the greatest transcript levels in activated HSCs and assess them as potential targets of Sox9. We demonstrated that 7 of these 20 validated as regulated by SOX9 and determine that 5 can be used as potential biomarkers of fibrosis in CHC. These biomarkers are validated in 2 separate cohorts, using both METAVIR and Ishak staging systems and compared to commercially available biomarkers and biomarker panels.
5.3 Results

5.3.1 RNA microarray of aHSCs after transfection with siRNA Sox9 identified potential targets of Sox9

To identify other novel SOX9 targets expression microarrays have been carried out in activated rat HSCs with Sox9 knockdown (~80% reduction in Sox9). This was achieved using two different siRNAs directed against Sox9, knocked down individually in 10 day cultured HSCs, extracted from separate animals and compared to their scrambled control. To ensure consistency, the entire experiment was performed in duplicate. From these data-set three important groups were extracted. The first two groups identified potential positively and negatively regulated Sox9 targets. The third group is shown in Table 5-1. The transcript levels of these genes were so abundant (i.e. above the level of ribosomal RNAs) that the microarray was unable to perform an assay. This suggested that transcript levels of these genes were the greatest found in activated rHSCs. From this list, 2 targets, Spp1/Opn and Col1a2, have previously been shown to be Sox9 targets (Chapter 3; (Piper Hanley et al. 2008). 18 are known matrix proteins, underlining the function of activated HSCs in ECM deposition in liver fibrosis. Furthermore, 4 proteins (Opn, Fn1, Timp1 and Col-1) encoded by these genes have previously been investigated as potential biomarkers of fibrosis (Trinchet et al. 1991; Rosenberg et al. 2004; Attallah et al. 2007; Huang et al. 2010). Given the role of Sox9 in ECM regulation in development (Section 1.9.1) and the increasing evidence of its role in ECM dysregulation in disease (Pritchett et al. 2011), it was hypothesised that more of these targets may be Sox9 regulated and could possibly be used as biomarkers of fibrosis.
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vim</td>
<td>vimentin</td>
</tr>
<tr>
<td>Gpnmb</td>
<td>glycoprotein transmembrane nmb (osteoactivin)</td>
</tr>
<tr>
<td>Spp1</td>
<td>secreted phosphoprotein 1 (osteopontin)</td>
</tr>
<tr>
<td>Ctgf</td>
<td>connective tissue growth factor</td>
</tr>
<tr>
<td>Ccl2</td>
<td>chemokine (C-C motif) ligand 2</td>
</tr>
<tr>
<td>Pf4</td>
<td>platelet factor 4</td>
</tr>
<tr>
<td>Tnn1</td>
<td>thioredoxin 1</td>
</tr>
<tr>
<td>Ccl7</td>
<td>chemokine (C-C motif) ligand 7</td>
</tr>
<tr>
<td>Tpm2</td>
<td>tropomyosin 2</td>
</tr>
<tr>
<td>Ctsl1</td>
<td>cathepsin L1</td>
</tr>
<tr>
<td>Ctsb</td>
<td>cathepsin B</td>
</tr>
<tr>
<td>Mgp</td>
<td>matrix Gla protein</td>
</tr>
<tr>
<td>Tagin</td>
<td>Transgelin</td>
</tr>
<tr>
<td>Cxcl3</td>
<td>chemokine (C-X-C motif) ligand 3</td>
</tr>
<tr>
<td>Sparc</td>
<td>secreted protein, acidic, cysteine-rich (osteonectin)</td>
</tr>
<tr>
<td>Cd63</td>
<td>Cd63 molecule</td>
</tr>
<tr>
<td>Cox2</td>
<td>cyclooxygenase 2</td>
</tr>
<tr>
<td>Cxcl3</td>
<td>chemokine (C-X-C motif) ligand 3</td>
</tr>
<tr>
<td>Eno1</td>
<td>Enolase 1, (alpha)</td>
</tr>
<tr>
<td>Timp1</td>
<td>TIMP metallopeptidase inhibitor 1</td>
</tr>
<tr>
<td>Cryab</td>
<td>crystallin, alpha B</td>
</tr>
<tr>
<td>Col1a2</td>
<td>collagen, type I, alpha 2</td>
</tr>
<tr>
<td>Tpm4</td>
<td>tropomyosin 4</td>
</tr>
<tr>
<td>Fn1</td>
<td>fibronectin 1</td>
</tr>
</tbody>
</table>

**Table 5-1: Highly expressed targets by RNA microarray.**

List of highly expressed targets, as demonstrated by RNA microarray. List consists of highest expressed targets in culture activated HSCs. Transcript levels of all targets were above the threshold detectable by Affymetrix™ microarray chip. These gene motifs were the highest expressed in activated rHSCs.
5.3.2 Validation of highly expressed targets

Assessment of potential Sox9 targets was undertaken using activated rHSCs after transfection with siRNA against Sox9. mRNA levels were assessed with real-time qPCR. rHSCs extracted from a minimum of 3 different animals were used per target. Cells were harvested and RNA extracted 24 hours after siRNA transfection. The mean reduction in Sox9 was 71%, as shown in Figure 5-1A. 7 of the 24 targets demonstrated a significant commensurate reduction after Sox9 knockdown. These are shown in Figure 5-1A. Figure 5-1B has an example of targets that were not regulated by Sox9, demonstrating no overall difference after attenuation of Sox9. Interestingly, all positively regulated targets, with the exception of Eno1, encode for secreted matrix proteins.
5.3.3 Expression of all positively regulated targets increases with HSC activation

To confirm that the targets were relevant to fibrosis and activated HSCs and not simply involved in HSC turnover, difference in mRNA levels was explored using quiescent and activated rHSCs, shown in Figure 5-2. All targets demonstrated a marked increase with HSC activation (\textit{Vim 8.52, Sparc 4.87, Gpnmb 11.06, Eno1(v1)}).
0.36, Eno1(v2) 3.83, Fn1 44.5, Opn 49.82), with the exception of the full-length transcript variant of Eno1.

**Figure 5-2: mRNA expression of individual targets in rHSC in quiescent and activated rHSCs.**
Quantification of all targets by qPCR. Levels of all targets are increased with activation of rHSCs. The only exception was Eno1, whereby the full-length transcript levels appeared to decrease and the truncated variant, which transcribes for myc-binding protein 1 (Mbp-1) increased, though neither results were significant statistically. *p<0.05.

In order to confirm transcript changes proceeded to an increase in protein levels, immunoblotting was utilised (Figure 5-3A and B). This demonstrated a 26-fold increase in Sox9 detection, by densitometry, comparing activated to quiescent rHSCs. Protein levels of targets revealed consistent increase compared to quiescent rHSCs and demonstrated a similar pattern of increase compared to mRNA changes, with Opn (5.11) and Fn1 (7.89) having the greatest fold increase and Vim (3.78), Sparc (3.35) and Gpnmb (3.25) showing a more modest increase (Figure 5-3A and B). Eno1 (1.63) and Mbp-2 (data not shown), the two proteins encoded by Eno1, did not alter significantly with rHSC activation.
5.3.3.1 Sox9 co-localises with all targets in activated rHSCs

Co-localisation of Sox9 with individual targets was performed by immunocytochemistry on activated rHSCs. Further exploration of Enol1 was abandoned at this stage, as it did not appear to alter with HSC activation and, unlike the others, is not a secreted matrix protein. By immunofluorescence, all targets were identifiable in the cytoplasm of activated HSCs, with Sox9 present in the nuclei, as expected of the transcription factor (Figure 5-3 C). As the antibody used for Sox9 and Fn1 were both raised in rabbit, these could not be used in conjunction with each other and hence only Fn1 is shown in the cytoplasm with DAPI staining confirming the nuclei of culture activated rHSCs.

**Figure 5-3: Protein expression of Sox9 and targets in activated HSCs.**

(A) Quantification by densitometry of targets from immunoblot. Data shown for each target and Sox9 in rHSCs in quiescent and culture activated states. (B) Representative immunoblot image. (C) Co-localisation of individual targets with Sox9 shown for all targets in activated rHSCs beside Fn1, which was limited by anti-body cross-reactivity. Scale bar represents 50µm. All immunoblotting quantification was normalised to β-actin. *p<0.05; **p<0.01; ***p<0.001.
5.3.4 Protein expression of all positively regulated targets is reduced by Sox9 siRNA

Using siRNA abrogation of Sox9, as previously described, similar protein level changes were confirmed (Figure 5-4), corresponding to mRNA changes shown Figure 5-1A. Using this technique, a 70% Sox9 knockdown was seen by densitometric quantification (Figure 5-4) and a commensurate reduction was seen in all targets (Opn – 0.480, Gpnmb – 0.585, Fn1 – 0.540, Sparc – 0.500, Vim – 0.605, Eno1 – 0.681), suggesting positive regulation by Sox9.

**Figure 5-4: Sox9 and targets expression after attenuation of Sox9 in activated rHSCs.**

(A) Quantification by densitometry of individual targets after transfection with siRNA against Sox9 compared to control samples transfected with ‘scrambled’ siRNA. (B) Representative immunoblot. All immunoblotting quantification was normalised to β-actin. *p<0.05; **p<0.01; ***p<0.001.

5.3.5 Proof of principle cohort

Data shown within this chapter suggests Sox9 potentially regulates six novel targets. Five of these targets are secreted matrix proteins and alter with HSC activation. They are also amongst the highest expressed genes in activated HSCs (Table 5-1). Sox9 up-regulates with HSC activation and has been shown to involved in ECM
dysregulation in liver fibrosis. To assess if these targets could be used as biomarkers of liver fibrosis a proof of principle cohort of patients was selected from the Trent HCV cohort. This consisted of 39 patients, divided into 3 groups based on liver biopsy sampling. Patients had Ishak stage (IS) 0, 3, or 6/cirrhosis defined on liver biopsy by an expert liver histopathologist. Alongside each biopsy a serum sample was identified which had been taken within 6 months of biopsy. ELISA was performed on all serum samples for each target and concentration assayed compared to known standards (Figure 5-5). VIM, SPARC, OPN, and GPNMB all showed a significant increase in IS 6 from IS 3 (ANOVA with Tukey HSD post-hoc test; p<0.05). FN1 demonstrated an increase from IS 0 to IS 3, but this was statistically non-significant and no difference was seen between IS 3 and IS 6.

With 4 of the 5 targets showing potential as biomarkers of fibrosis, two further cohorts were established. A Southampton cohort of 50 patients with normal controls, staged by METAVIR system, as an experimental cohort and a much larger Trent HCV cohort of 116 patients with paired biopsies and serum, used as a validation cohort.
Figure 5-5: Box-and-whisker plots showing serum concentrations of all targets at 3 stages of fibrosis.

Ishak stage at time of serum sampling was determined from biopsy taken with 6 months of sample. IS 0 n=12, IS 3 n=14, IS 6 n=13. Concentration plotted on a logarithmic scale. Note: Fn1 concentration is in µg/ml and not ng/ml.

5.3.6 Southampton cohort

A pre-existing cohort of 50 patients that was originally collected in Southampton by Dr Neil Guha, was used as an experimental cohort. The benefits of this cohort were that it was well phenotyped; previous biomarkers have been explored using these samples; there was a broad mix of METAVIR fibrosis stages (Figure 5-6); samples were collected from a different centre to the Trent HCV cohort used for validation and it was staged using an alternative system. A further advantage was the addition of non-CHC infected controls with normal liver on biopsy. Figure 5-6 presents in a bar-graph form the distribution of METAVIR stages in this cohort.
5.3.7 Assessing biomarkers in Southampton cohort

Using previously optimised serum ELISA protocols, serum concentrations for all targets were assayed in the Southampton cohort. These are diagrammatically represented in Figure 5-7. The METAVIR stages have been amalgamated for simplicity into clinically significant groups. The figure shows 3 distinct groups; controls, those with no or minimal fibrosis (F0-1) and those with significant fibrosis (F2-4). Data was analysed using analysis of variance with Tukey HSD post-hoc correction. OPN, GPNMB, VIM, and SPARC all show a difference at differentiating between groups, especially between F0-1 (minimal fibrosis) and F2-4 (significant fibrosis). OPN demonstrated a significant difference between controls and F0-1 (p<0.05) and F2-4 (p=0.001). VIM also had a significant difference between controls and significant fibrosis (F2-4; p<0.05). The other targets, with the exception of FN1, demonstrated a trend towards significance, but did not reach the threshold value set at p<0.05.
Figure 5-7: Serum concentration of individual targets.
Serum levels plotted against grouped METAVIR fibrosis stages assessed on accompanying liver biopsy. To reduce staging bias, the serum samples were divided into 3 clinically significant groups; controls, those with early or no fibrosis (F0-1) and significant fibrosis (F3-4). With the exception of FN1, all targets showed an increase in concentration between F0-1 and F2-4, and Opn, Sparc and Vim also demonstrated a difference between control and F0-1. *p<0.05; **p≤0.001.

5.3.7.1 AUROC of targets in Southampton cohort
Area under the receiver operator curve analysis (AUROC) has become the expected mathematical method of assessing biomarker potential. This binomial method takes into account specificity and sensitivity of a test against a ‘gold standard’, in this case
staging by biopsy. A perfect test would have a maximal value of 1, suggesting all available area under the curve was encompassed by the proposed test.

Figure 5-8A plots an AUROC curve for each of the targets comparing F4 cirrhosis to all other samples. The AUROC values are shown in the bottom row of table in Figure 5-8B. Figure 5-8B gives AUROC values of the targets with biopsies divided into METAVIR stages. OPN performs the best across the groups, with AUROC remaining consistently above 0.75. VIM performed favourably, with AUROC above 0.7, and showed its best discriminatory ability in earlier fibrosis (controls, F1 and F2). SPARC showed similar results, but was poorer than VIM at later stages of fibrosis.
Figure 5-8: Performance of individual targets in distinguishing different METAVIR Fibrosis stages as measured by AUROC values.

(A) Graphical representation of AUROC for all targets comparing F4 fibrosis against controls and F0-3. Comparison between these targets reveals OPN concentration as the best biomarker to distinguish between cirrhotic and non-cirrhotic patients (AUROC: OPN 0.781, VIM 0.705, SPARC 0.69, GPNMB 0.716, FN1 0.429).

(B) Table of AUROC values of targets against different thresholds of METAVIR fibrosis stages.
5.3.8 Trent HCV cohort

The Trent HCV cohort used was collected from the much larger cohort based in Queen’s Medical Centre, Nottingham. The schematic of how this cohort was derived is shown in Figure 2-5 within Chapter 2. The cohort consists of CHC infected patients who have had 2 biopsies separated over a period of time. Each biopsy has a parallel serum sample taken within 6 months of biopsy and in most cases taken at time of biopsy sampling. A total of 115 patients were included in this cohort and Figure 5-9A shows the breakdown of Index and Follow-up biopsies. These data is diagrammatically presented in Figure 5-9B. All biopsies were staged and graded using the Ishak-Knodell system by an expert liver histopathologist. The majority of patients (80 of 115) had early or no fibrosis (IS 0-1) and 25 of these demonstrated progression. Only 17 patients were identified as having significant fibrosis (IS 4-6). 1 patient had regression by greater than two stages of fibrosis.
Figure 5-9: Breakdown of index and follow-up biopsies in Trent HCV cohort.

(A) Table showing number of patients grouped by staging of index and follow-up biopsy. Majority of index biopsies showing no/minimal fibrosis (n=80) with 25 progressing in subsequent biopsy (19 and 6). Total n=115. (B) Index and follow-up biopsy data plotted together against fibrosis stage, showing a decrease in numbers of patients with early fibrosis and an increase in biopsies in the intermediate and significant fibrosis groups.

Figure 5-9B shows that there is a decrease in IS 0-1 fibrosis from index to follow-up biopsy, with an increase in IS 2-3 and IS 4-6 stages in the follow-up biopsies. This would suggest a predictable progression of disease over time for some patients. As CHC is known to be a variable but generally a progressive disease, these data are consistent with known epidemiological data (Poynard et al. 2001).
Necroinflammatory activity (NI) is assessed histologically by Ishak NI grading, consisting of 4 separate parameters, accumulating in a score from 0-18. An NI score of 0 would suggest no inflammatory activity and a score of 18, the greatest amount of necrosis and inflammation. Figure 5-10A and B show how index and follow-up biopsies divide into Ishak stage and grade. It is notable that lower levels of NI (0-2) are only seen in biopsies with minimal or no fibrosis (IS0-1) and the number of these decreases in the follow-up biopsy (45 in index biopsy and 27 in follow-up biopsy).

Figure 5-10: Diagrammatic representation of Ishak stage and necroinflammatory activity of Trent HCV Cohort.
(A) Distribution of index and (B) follow-up biopsies divided into 3 columns for early (F0-1), intermediate (F2-3) and significant (F4-6) fibrosis. Each bar is sub-divided into groups of NI grading, as shown in graph legends.

5.3.9 Validating targets in Trent HCV cohort

Targets were validated by using the Trent HCV cohort. The serum concentrations of targets from the proof of principle cohort were added to this as they are identified in the same way from the same cohort. As shown in Table 5-2, all targets performed generally worse than in Southampton cohort, though once again OPN was the best target and overall (AUROC 0.63-0.76) with a similar trend to as seen in the Southampton cohort. VIM performed far worse in the validation cohort with AUROC 0.509-0.606 Trent cohort compared to 0.705-0.876 in Southampton cohort. SPARC (Trent AUROC 0.544-0.679, Southampton AUROC 0.68-0.834) and GPNMB (Trent AUROC 0.587-0.701, Southampton AUROC 0.457-0.716) were comparable to each other with similar to results in the Southampton cohort. FN1 remained a poor biomarker (AUROC 0.318-0.572), with consistently poor discriminatory ability. AUROC are graphically shown in Figure 5-11 for each
target’s ability to differentiate cirrhosis (IS 6) from all other stages of fibrosis and AUROC are listed for each target against different stages of fibrosis in Table 5-2.

Figure 5-11: AUROC of targets’ ability to distinguish between IS 6 / cirrhosis and IS 0-5.

Area under curve for each curve demonstrates individual targets ability to correctly assign a patient as either IS 6 / cirrhosis or IS 0-5. Values of AUROC analysis are shown in Table 5-2.
### Table 5-2: Performance of all targets in distinguishing different stages of fibrosis as measured by AUROC values.

<table>
<thead>
<tr>
<th>Ishak Stage</th>
<th>OPN</th>
<th>VIM</th>
<th>SPARC</th>
<th>GPNMB</th>
<th>FN1</th>
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<tbody>
<tr>
<td>0 vs. 1-6</td>
<td>0.63</td>
<td>0.509</td>
<td>0.553</td>
<td>0.587</td>
<td>0.572</td>
</tr>
<tr>
<td>0.1 vs. 2-6</td>
<td>0.686</td>
<td>0.575</td>
<td>0.603</td>
<td>0.635</td>
<td>0.509</td>
</tr>
<tr>
<td>0-2 vs. 3-6</td>
<td>0.677</td>
<td>0.59</td>
<td>0.544</td>
<td>0.611</td>
<td>0.439</td>
</tr>
<tr>
<td>0-3 vs. 4-6</td>
<td>0.719</td>
<td>0.506</td>
<td>0.635</td>
<td>0.642</td>
<td>0.413</td>
</tr>
<tr>
<td>0-4 vs. 5,6</td>
<td>0.747</td>
<td>0.512</td>
<td>0.626</td>
<td>0.649</td>
<td>0.377</td>
</tr>
<tr>
<td>0-5 vs. 6</td>
<td>0.76</td>
<td>0.606</td>
<td>0.679</td>
<td>0.701</td>
<td>0.318</td>
</tr>
</tbody>
</table>

5.3.10 Certain targets can identify early and significant fibrosis

AUROC values in the Trent HCV cohort indicated that the targets were less than ideal markers of disease. However, diagnostic ability to differentiate clinically significant stages of fibrosis demonstrated their improved functioning. Table 5-3 divides fibrosis stages into 3 distinct categories: early or no fibrosis (IS 0-1), intermediate fibrosis (IS 2-3) and significant fibrosis (IS 4-6). An analysis of variance was performed on all targets using these three groups followed by Tukey post-hoc analysis, with significance values shown in Table 5-3. Using these groups, OPN and VIM were able to distinguish early and significant fibrosis groups with high accuracy. SPARC and GPNMB trend towards this, but were not statistically significant at the cut-off set at p<0.05. However, none of the targets were able to differentiate patients in the important intermediate (IS 2-3) stages of fibrosis, from early or significant fibrosis.
<table>
<thead>
<tr>
<th>Target</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stage 0-1</td>
</tr>
<tr>
<td>OPN</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>VIM</td>
<td>0.02*</td>
</tr>
<tr>
<td>SPARC</td>
<td>0.09</td>
</tr>
<tr>
<td>GPNMB</td>
<td>0.06</td>
</tr>
<tr>
<td>FN1</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Table 5-3: Certain targets can potentially predict between grouped stages of fibrosis.

Analysis of variance (ANOVA). OPN and VIM are able to discriminate between clinically significant groups with high probability. None of these targets was able to accurately distinguish intermediate (IS2-3) fibrosis. Post-hoc analysis statistically significant values shown with *, with cut-off p<0.05.

5.3.11 Comparison of proposed biomarkers with previously validated individual biomarkers and biomarker panels

One of the advantages of the Southampton cohort is that the samples are accurately phenotyped and have been used to test biomarkers, HA, P3NP and TIMP1, and biomarker panels, ELF and APRI. These are regarded as amongst the better currently available individual biomarkers for liver fibrosis and AUROC values of these are shown in Table 5-4. For the biomarker panels, APRI and ELF, data was consistent with previously published work (Shaheen and Myers 2007; Guha et al. 2008). Direct comparison can be made with the proposed biomarkers in Figure 5-8. It can be demonstrated that OPN and VIM perform better than APRI biomarker panel across the fibrosis stages and, with the exception of TIMP1, are better at distinguishing earlier stages of fibrosis and controls. HA, P3NP, TIMP and ELF have a better diagnostic ability to identify F4/cirrhosis, compared to the targets identified in this chapter. Interestingly, the targets identified, and in particular OPN, VIM, GPNMB and SPARC, appear to be better than all of the previously validated biomarkers at identifying patients with no evidence of fibrosis (controls and F0). The derivation of
these biomarkers as SOX9 targets may explain this phenomenon as they are expressed in the presence of fibrogenesis and may therefore, have low levels in patients with no ongoing scarring.

<table>
<thead>
<tr>
<th>METAVIR Stage</th>
<th>APRI</th>
<th>HA</th>
<th>P3NP</th>
<th>TIMP1</th>
<th>ELF</th>
</tr>
</thead>
<tbody>
<tr>
<td>C vs. 0-4</td>
<td>0.313</td>
<td>0.348</td>
<td>0.256</td>
<td>0.111</td>
<td>0.276</td>
</tr>
<tr>
<td>C, 0 vs. 1-4</td>
<td>0.687</td>
<td>0.652</td>
<td>0.744</td>
<td>0.889</td>
<td>0.724</td>
</tr>
<tr>
<td>C, 0, 1 vs. 2-4</td>
<td>0.695</td>
<td>0.675</td>
<td>0.78</td>
<td>0.768</td>
<td>0.729</td>
</tr>
<tr>
<td>C, 0-2 vs. 3, 4</td>
<td>0.661</td>
<td>0.758</td>
<td>0.929</td>
<td>0.778</td>
<td>0.831</td>
</tr>
<tr>
<td>C, 0-3 vs. 4</td>
<td>0.543</td>
<td>0.897</td>
<td>0.947</td>
<td>0.851</td>
<td>0.958</td>
</tr>
</tbody>
</table>

Table 5-4: AUROC of individual biomarkers and biomarker panels in Southampton cohort.

Biomarker panels (APRI and ELF) and individual markers (HA, P3NP and TIMP1) assessed using the same samples as used to assess targets identified in this chapter. Comparable results of individual biomarkers and APRI to targets OPN, GPNMB, VIM and SPARC. ELF performs better across different stages and P3NP is better at distinguishing significant (F3-4) fibrosis.

5.3.12 Biomarkers are unable to identify patients more likely to have progressive fibrosis

To investigate whether the panel of SOX9 targets were able to predict individuals more likely to have progressive fibrosis, sub-groups of patients from the Trent HCV cohort were investigated.

The cohort extrapolated for this thesis from the larger Trent HCV cohort was selected to allow assessment of liver fibrosis progression. This was achieved by having two biopsies per patient separated over a period of time. The change in Ishak stage from index to follow-up biopsy allows the development of two individual sub-groups of patients, selected from individuals with early or no fibrosis on their index biopsy (IS 0-2). The first group of patients had no change in fibrosis stage between
biopsies (Non-progressors) and the second group had an increase in Ishak fibrosis stage by a minimum of two (Progressors). All patients with an increase in Ishak stage by 1 were excluded to allow for sampling and interpretation errors inherent in liver biopsy assessment (Regev et al. 2002; Bedossa et al. 2003). These sub-groups are further assessed in Chapter 6.

Serum concentration of all targets was assayed in the paired serum sample taken with the index biopsy from both groups. Concentrations were compared between the two groups for all potential biomarkers (shown in Figure 5-12). Unfortunately, none of the targets yielded any significant difference between the two groups, suggesting that these biomarkers were unable to predict disease progression in a CHC infected individual with early fibrosis.
Figure 5-12: Box-and-whisker plots comparing Progressors and Non-progressors.

Serum concentration in index samples of (A) OPN, (B) VIM, (C) SPARC, (D) GPNMB and (E) FN1 plotted against progression from early fibrosis as defined by >2 increase in Ishak stage between biopsies or no progression, as defined by an unchanged Ishak stage. None of the targets showed a significant difference between the two groups.
5.4 Discussion

Biomarker discovery remains an important area of research, as a need exists for better categorisation of patients in order to best aid therapy. Several different approaches have been employed. We used an RNA microarray of activated rHSCs as a starting point. HSCs are intimately involved in the fibrogenic process and we postulated that by identifying dynamic changes occurring within HSCs, especially those involving ECM regulation, potential novel biomarkers could be identified. The microarray itself examined the alteration in transcript after siRNA knockdown of Sox9. However, a number of genes were noted to be above detection threshold. These appeared important for further exploration given their abundance in activated HSCs and the fact that these genes predominantly transcribed for ECM proteins.

5.4.1 Several highly expressed genes in activated HSCs are potentially regulated by Sox9

Examination of the list of array genes (Table 5-1) revealed interesting results. 70% were known matrix proteins and hence secreted from HSCs and could potentially be assayed in serum. *Opn* and *Col1a2* were known Sox9 targets and perhaps most interestingly, there were several previously explored biomarkers within the list. Namely, TIMP-1, FN1, and COL1. OPN has also been identified as a potential biomarker in hepatocellular carcinoma (HCC) (Shang et al. 2012) and as a putative biomarker for fibrosis in alcoholic liver disease (Patouraux et al. 2012) and viral hepatitis (Huang et al. 2010).

The possibility that more of these highly expressed targets were regulated by Sox9 was further strengthened by the known role of Sox9 in ECM regulation in development and dysregulation in disease (Pritchett et al. 2011). This thesis has provided further support for the important role of SOX9 in liver fibrogenesis, especially in the context of ECM alterations (Chapters 3 and 4). For example, OPN was noted in the array list and, as demonstrated in Chapter 3, this is a downstream target of SOX9. The hypothesis was tested using siRNA against Sox9 in activated rHSCs and demonstrated that a total of 8 of these targets were potential downstream targets of Sox9. If Sox9 regulates approximately a third of the genes with the highest
transcript levels, this would suggest a pivotal role of Sox9 in regulating fibrosis associated with activated HSCs.

5.4.2 Sox9 regulated targets

Reassuringly all identified Sox9 targets were increased during HSC activation, with the exception of Eno1, and published data suggested all had known ECM functions. OPN (Syn et al. 2011; Pritchett et al. 2012) and SPARC (also known as osteonectin) (Atorrasagasti et al. 2011) have recently been implicated as drivers of liver fibrosis. FN1 has previously been explored as a biomarker, showing an increase in concentration between IS0 and IS1-3 fibrosis (Attallah et al. 2007). GPNMB has been assessed as a biomarker of renal injury and as a transmembrane protein with a RGD-integrin binding site has been shown to have several ECM roles (Patel-Chamberlin et al. 2011). Collectively these data provide clues to their potential as biomarkers.

5.4.2.1 α-Enolase

Eno1 was the only validated target not found to alter with HSC activation. The gene for Eno1 encodes for two separate proteins depending on transcriptional isoform. These proteins are α-Enolase and myc-binding protein 1 (MBP-1). MBP-1 is located in the nucleus and is a tumour repressor whilst α-Enolase has dual functionality depending on post-translational modifications. It either performs enzymatic actions within the cell or is expressed on the cell surface membrane (Capello et al. 2011). MBP-1 and α-Enolase are relatively ubiquitous throughout mammalian cells. Although the primary roles for the proteins encoded by Eno1 are not directly involved in ECM regulation, the membrane bound α-Enolase is thought to activate extra-cellular plasminogen (Miles et al. 1991). Plasmin is known to regulate MMPs and may be a possible mechanism for matrix degradation. However, given the multiple roles of α-Enolase in cell turnover and its ubiquitous expression, suggests as a biomarker it would be less than ideal and potentially complex to analyse. Furthermore, protein detection revealed a minimal change between quiescent and activated HSCs. It is a possibility that there may be a change in the ratio of cytoplasmic and cell membrane bound α-Enolase resulting in functional changes to the ECM. The detection methods used would not have detected this change and was not explored further and α-Enolase was not investigated as a biomarker in this study.
5.4.2.2 SPARC

SPARC is a matr-ice cellular protein expressed in a variety of tissues in development and notable for binding to basement membrane proteins, including fibrillar collagens (Brekken and Sage 2000). Phenotype changes have been described in Sparc knockout mice, including cataractogenesis with 100% penetrance, improved skin wound healing, and understandably given that Sparc is a major non-collagenous component of bone, osteopenia (Bradshaw and Sage 2001).

SPARC has been shown to reduce fibrosis *in vitro* using siRNA to attenuate its effect (Atorrasagasti et al. 2011) and *in vivo*, using an inducible Sparc knockout mouse (Atorrasagasti et al. 2013). Interestingly, microarray analysis of Sparc knockout mice revealed an increase in Sox9 and pathway analysis suggested TGF-β signalling as an important intermediary (Atorrasagasti et al. 2013). Microarray analysis, however, was performed after BDL on whole liver as opposed to individual cell types (e.g. HSCs). Sparc knockout after BDL may not have an effect on the ductular reaction, resulting in a net increase in Sox9. Another explanation may be that reduced Sparc may lead to induction of signalling up-stream of Sox9, in a negative feedback loop. Nonetheless, given its alteration in fibrosis and secretion into the ECM, SPARC represented a suitable candidate to investigate as a biomarker.

5.4.2.3 GPNMB

Glycoprotein NMB (GPNMB) is also known as osteoactivin and is a transmembrane or secreted protein, dependent on post-translational modification. Secreted GPNMB undergoes greater glycosylation than its transmembrane counterpart. It is abundantly expressed in bone and involved in osteoblast differentiation. Overproduction of GPNMB leads to osteopetrosis, whereby there is excessive thickening of bone (Safadi et al. 2001; Abdelmagid et al. 2008). Multiple functions of GPNMB have been postulated, including cell proliferation, adhesion, differentiation and synthesis of several ECM proteins and processes, in both physiological and pathological conditions (Singh et al. 2010). Taken together, GPNMB was a highly plausible SOX9 target in HSCs. In this study, protein detection of GPNMB was performed on the 65kDa product, corresponding to the non-glycosylated isoform. However, it is plausible that further exploration may indicate greater changes in other isoforms.
This also applies to serum levels of GPNMB, whereby detecting certain isoforms may lead to improved specificity.

In the liver, GPNMB has been shown to up-regulate after single dose CCl₄ induced liver injury (Haralanova-Ilieva et al. 2005). Co-localisation was used in this study to suggest that macrophages and peri-sinusoidal cells were the predominant source of GPNMB upon injury. GPNMB has also been shown to increase in rodent models of cirrhosis, with a further increase with HCC onset (Onaga et al. 2003). Comparing transcript level of GPNMB in tumorous and non-tumorous resected tissue, this study demonstrated increasing ratio of GPNMB in patients’ resected liver dependent on histological grade of their tumour (Onaga et al. 2003). Though not previously identified in activated HSCs, the ECM dysregulation roles of GPNMB in disease add credence to work in this study suggesting GPNMB may be regulated by SOX9. Urinary GPNMB has previously been explored as a biomarker of renal injury in chronic kidney disease (Patel-Chamberlin et al. 2011), suggesting that it may be a suitable as a biomarker in liver fibrosis.

5.4.2.4 Fibronectin

FN1 is a dimeric glycoprotein that exists in two isoforms: a soluble plasma fibronectin and an insoluble cellular fibronectin. FN1 has been shown to control ECM assembly by interacting with latent TGF-β binding protein (LTBP) and regulating bioavailability of active TGF-β (Dallas et al. 2005). FN1 contributes to tissue remodelling following liver injury, however, remodelling can occur by FN1 independent mechanisms (Moriya et al. 2011). FN1 has been found in portal areas and in the fibrous septa in CHC related liver fibrosis (Matsui et al. 1997). These areas correspond to regions containing SOX9 positive cells, as shown in Chapters 3 and 6 and would corroborate the in vitro immunocytochemistry in Figure 5-3.

Total FN1 (Attallah et al. 2007) and individual isoforms (Hackl et al. 2010) have previously been explored as serum biomarkers in liver fibrosis. Interestingly, a difference was shown in HCV infected patients, between those without fibrosis (IS 0) and those with early fibrosis (IS 1-3) (Attallah et al. 2007). No data was shown in more advanced fibrosis. The results are consistent with our own, as demonstrated in Figure 5-7, Figure 5-8 and Table 5-2. Additionally our data reveals that with
advancing fibrosis, FN1 concentration falls back to levels similar to IS 0. These data may be explained by recent in vivo studies. Collagen fibrillogenesis in response to initial liver injury was mediated by FN1 and type IV collagen (Moriya et al. 2011). This would corroborate our data, showing an increase of FN1 in the earlier stages of fibrosis. As FN1 levels fall fibrosis advances and this may be explained by increasing bioavailability of active TGF-β driving collagen deposition (Kawelke et al. 2011).

5.4.2.5 Vimentin
VIM is a type III intermediate filament protein found in mesenchymal cells. Other family members include GFAP and Desmin. VIM has a very high conservation amongst species and this homology suggests that it has an important, evolutionarily conserved role (Herrmann et al. 1989). Originally thought to be solely involved in cellular and nuclear cytoskeletal maintenance, recent work has demonstrated its more diverse roles. VIM is instrumental to cell migration in a variety of ways, including integrin organisation by trafficking integrins to the cell membrane and by vimentin-associated matrix adhesions enabling cell motility (Eriksson et al. 2009).

Increasing protein detection of VIM with HSC activation, as shown in Figure 5-3, is understandable given the mesenchymal properties of HSCs and the fact that other type III intermediate filaments, such as Desmin, also increase with activation. VIM, however, has more diverse roles than its family members and it may be more directly involved in propagating fibrosis by facilitating the migratory nature of aHSCs and MFs, as well as altering their interaction with ECM by altering the integrin profile. Serum ELISAs (and Table 5-2) revealed VIM amongst the better biomarkers in liver fibrosis, with high predictive value in identifying early and advanced fibrosis (Table 5-3). This diagnostic accuracy may reflect VIM’s involvement in multiple mechanisms of fibrogenesis, making it a more dynamic marker of on-going processes.

5.4.2.6 Osteopontin
OPN as a SOX9 regulated target has been discussed in depth in Chapter 3 and it has been previously investigated as a biomarker in both liver fibrosis and HCC. As a secreted ECM protein with multiple known functions (Denhardt et al. 2001), OPN is
a prime candidate as a biomarker. In both the Trent HCV and Southampton cohorts, OPN performed the best, with AUROC values consistently above 0.7. Comparing to known biomarkers (Table 5-4 and Figure 5-8) revealed OPN to be comparable, if not superior, at diagnosing stage of fibrosis. Using groups of fibrosis in the Trent HCV cohort (Table 5-3) further strengthened OPN as a good marker of fibrosis, as it was able to categorise patients into those with early or no fibrosis and those with severe fibrosis, with exceptionally high accuracy (p<0.001 for both).

A potential difficulty of using OPN as a biomarker may stem from its relatively ubiquitous nature during inflammation associated with fibrotic disease. It has been described as a potential biomarker for several conditions including retroperitoneal fibrosis, pulmonary fibrosis, cardiac fibrosis and HCC (Takahashi et al. 2001; Matsui et al. 2004; Binder et al. 2012; Shang et al. 2012; Zhao et al. 2012; Briggs 2013) and this may well make it a far less specific marker for liver fibrosis.

5.4.3 Biomarker exploration

The studies outlined above combined with the validated array data presented in this chapter, suggested a panel of five ECM proteins may be useful as biomarkers of liver fibrosis. After confirming viability of potential biomarkers with a small cohort, biomarker utility of the targets was undertaken in two discreet cohorts. Using two cohorts allowed biomarkers to be explored and validated in independent groups. A further advantage was that specimens from the Southampton cohort were staged using a different system (METAVIR), enabling the biomarkers to be tested against both popular systems. All biomarkers performed better against the METAVIR system (compare Figure 5-8B against Table 5-2). A likely reason for this is the increased number of stages in the Ishak system (7 compared to 5) results in greater spread of data, leading to poorer AUROC values. Grouping the Ishak stages into clinically significant categories (Table 5-3) provided further evidence for this, as diagnostic accuracy improved. OPN and VIM proved able to distinguish early fibrosis and significant fibrosis with the highest precision. Diagnosing intermediate (IS 2-3) fibrosis remains elusive, a problem with many biomarkers (Boursier et al. 2009).
Comparing targets against three of the best currently available biomarkers showed comparable results (Table 5-4). TIMP1 and P3NP were better at identifying cirrhosis, however OPN, VIM and GPNMB showed better results at identifying earlier stages of fibrosis. The derivation of Sox9 targets biomarkers may help explain this. As the targets are altering with Sox9 expression and HSC activation, alteration in serum concentration may be more marked in early fibrosis due to greater relative increases in fibrogenesis, resulting in superior predictive ability at these stages.

To test their ability to predict long-term, on-going fibrogenesis, all 5 targets were assayed to predict a difference between patients who had a quicker progression of fibrosis. None of the targets demonstrated an ability to distinguish between these groups, suggesting fibrogenesis over long time periods may require alternative methods of assessment.

5.4.4 Conclusion
Biomarker discovery remains an important area for hepatology research. The advent of new therapies for CHC will stretch limited resources and better definition of disease states is needed to stratify therapy and assess disease response. In this chapter, gene profiling has been used to identify potential biomarkers. The basis of this has been the transcriptional changes underpinning fibrogenesis, providing targets that can be shown to predict disease. Due to their derivation, these targets appear superior to previously explored biomarkers at predicting earlier stages of fibrosis in CHC.
6 Results: SOX9 predicts progression of liver fibrosis in patients with chronic hepatitis C

6.1 Introduction

Fibrosis of the liver is a major cause of morbidity and mortality characterised by accumulation of extracellular matrix (ECM) proteins that destroy normal tissue architecture. It is a common step in the progression of several liver diseases, ultimately resulting in cirrhosis, liver failure and death. Although potentially reversible during early stages, the process is commonly silent until end-stage disease for which the only treatment is transplantation.

Despite limitations associated with variable sampling and pathology reporting (Bedossa et al. 2003), liver biopsy remains the acceptable standard to assess liver disease with repeated sampling required to determine progression. Progress using serum markers and/or imaging techniques to assess liver fibrosis has been significant (Castera and Pinzani 2010). However, problems remain in stratifying between early stages of the disease and in predicting disease progression or fibrogenesis. To address this, improving current techniques or developing new measures are attractive for diagnostic, prognostic and therapeutic reasons.

6.1.1 Chronic Hepatitis C

One of the most common causes of chronic liver diseases worldwide, with a significant risk of progression to cirrhosis and hepatocellular carcinoma, results from hepatitis C viral (HCV) infection. With careful monitoring already required to manage these patients, the ability to identify ‘at risk’ individuals to develop a liver related complication would have significant benefit on treatment choices, including the directly acting ant-viral agents (DAAs) (Schaefer and Chung 2012). These new agents show therapeutic efficacy but are more costly than conventional treatments. Moreover, the ability to phenotype patients who have active fibrogenesis may enable specific targeting with emerging anti-fibrotic therapy to alter the evolution from fibrosis to cirrhosis when there is continuing liver injury.
6.1.2 SOX9 and fibrosis progression

The transcription factor, Sex determining region Y box 9 (SOX9) has previously been identified as a critical mediator of ECM deposition during liver fibrosis. It is ectopically expressed by the main fibrogenic cell type in the liver, the hepatic stellate cell (HSC), as it becomes activated into a proliferative myofibroblast in response to injury. Sox9 is increased in animal models of fibrosis where it appears responsible for deposition of collagen type 1 (Col1) and osteopontin (Opn), both implicated in fibrotic progression (Piper Hanley et al. 2008; Pritchett et al. 2012) and the latter as a marker of disease severity (Huang et al. 2010). These data would suggest that SOX9 is key to the fibrogenic process. Data from Chapter 3 established the presence of SOX9 positive cells in liver biopsies of patients infected with CHC (Figure 3-8). Of interest in these samples was the increasing SOX9 count as fibrosis progressed. Given the role of SOX9 in HSC activation and ECM deposition, it can be hypothesised that increasing SOX9 within a biopsy may predispose an individual to increased risk of progressive fibrosis.

There has been considerable work aimed at establishing risk factors for progression of fibrosis. This is explored in depth in the introduction Section 1.8. Despite genome-wide association studies, only qualitative markers of fibrosis progression exist. In essence, presently the best predictor of fibrosis progression is age at HCV infection, with older patients progressing quicker (Yano et al. 1996). After this, male gender, concomitant alcohol abuse and persistent raised ALT also increase risk of progression (Poynard et al. 2001). With the advent of newer, more expensive antiviral therapies becoming available, predicting those patients likely to progress at a faster rate is of increasing interest. This is most applicable to patients with early fibrosis, as delaying therapy in anticipation of better, more tolerable therapies will be in the interest of both patient and health care provider.
6.2 Aims

In this chapter, nuclear expression of SOX9 in fibrotic biopsy samples from patients with chronic hepatitis C virus (HCV) is shown. Morphological features of SOX9 positive cells are described in sequential sections using known cellular markers. Further categorisation of the SOX9 cells is achieved using dual-staining with known markers of certain cell types with SOX9 localised to CK19 / CK7 positive biliary ducts and progenitor cells. The ability of SOX9 count to predict progression was achieved using patients from the Trent HCV cohort with index and follow-up biopsies and assessed with multivariate analysis and logistical regression modelling.
6.3 Results

6.3.1 Identification of SOX9 as a marker of severity in liver fibrosis

As work leading up to this study we identified Sox9 in areas of liver fibrosis from two independent animal models of the disease, where it is co-localised with desmin positive cells demarcating activated HSCs (Piper Hanley et al. 2008; Pritchett et al. 2012). In vitro, Sox9 was barely detected in quiescent rat HSCs, however as rat HSCs became activated on plastic over 2 weeks Sox9 was induced, paralleling the HSC activation marker α- Smooth muscle actin (α-Sma) and pro-fibrotic Col1 (Figure 6-1A). Despite previous data describing nuclear detection of SOX9 in human culture activated HSCs and its ability to regulate ECM in the human stellate model LX-2 cells (Piper Hanley et al. 2008; Pritchett et al. 2012), SOX9 has not been described in human fibrotic tissue. To address this we conducted a preliminary analysis of SOX9 in the liver of a subset of patients with fibrosis due to chronic HCV infection. In this test cohort, fibrosis had been assessed according to the Ishak criteria and given a score from IS 0 to IS 6, the latter representing the most severe staging of fibrosis (Ishak et al. 1995; Mohsen 2001). Staining for collagen with picrosirius red demonstrated progression of fibrosis with increasing Ishak stage (Figure 6-1B). Similarly, α-SMA expression was increased with fibrosis stage suggesting greater activation of HSCs with severity of disease (Figure 6-1C). In tissue sections from normal biopsy, SOX9 was mainly restricted to the biliary ducts (Figure 6-1C; asterisk). However, in both moderate and severe fibrosis, SOX9 could also be observed in cells associated with the developing scar (Figure 6-1C; hatched line). In addition, low level SOX9 expression also appeared to be located within the hepatocytes directly around the forming scar, potentially indicative of regenerative cells (Carpentier et al. 2011; Dorrell et al. 2011; Furuyama et al. 2011) (Figure 6-1C; arrows).
Identification of SOX9 as a marker of severity in liver fibrosis. (A) Example immunoblot showing induction of Sox9, Col1 and α-SMA in activated rat HSCs (A; day 10) relative to quiescent (Q; day 0). Loading control is β-actin. (B) Example of Ishak staged whole liver biopsy section with histology staining for collagen using picrosirius red. IS 0 (normal), IS 3 (moderate) and IS 6 (severe) stages of fibrosis. (C) Consecutive 5μm sections of human liver biopsies from patients with mild, moderate and severe fibrosis due to chronic HCV infection. Brightfield immunohistochemistry is shown for α-SMA and SOX9 (brown staining) counterstained with toluidine blue. Right hand panel, higher magnification images of boxed areas showing SOX9 and expression in the bile duct (*), elongated cells surrounding scar edge (hatched line), and hepatocytes (arrows). Size 20μm (left and middle panel) and 200μm (right panel).
6.3.2 SOX9 localisation and cell numbers in liver fibrosis

To further sub-classify cell types expressing SOX9 we carried out co-immunohistochemistry with known liver markers detecting biliary epithelial cells (Cytokeratin 19 and 7; CK19 and CK7), HSCs (α-SMA), hepatocytes (α1-antitrypsin; α1AT), and blood vessels (CD34). As expected, SOX9 was not detected in the vasculature and, consistent with other reports, was co-expressed with CK19 and CK7 in biliary epithelial cells within obvious ducts and cells likely generated from ductular reaction (Figure 6-2A). In line with our previous studies, although less frequent, we also detected SOX9 within α-SMA positive regions with some positive cells displaying an elongated shape characteristic of myofibroblasts (Figure 6-2A). More surprisingly, SOX9 was also localised with α1AT hepatocytes particularly within cells surrounding the scar tissue (Figure 6-2A). Recent publications have suggested in mouse models of fibrosis that transit-amplifying cells (or oval cells) develop from the ductular reaction to repopulate the peri-portal hepatocyte population (Espanol-Suner et al. 2012). Collectively, the data shown in Figure 6-2A suggest SOX9 expression in fibrotic livers may indicate that this is occurring in progenitor cell types, similar to its expression in murine fibrosis models.

Irrespective of its localisation, between biopsy samples from normal (IS 0; n=5), moderate (IS 3; n=5) and severe (IS 6; n=4) fibrosis, the number of SOX9 positive nuclei increased (Figure 6-1C and Figure 6-2B). To explore this further, SOX9-positive cells were counted in two distinct compartments; biliary epithelial cells (BEC) and non-BEC (Figure 6-2B). The majority of the increase was observed in non-biliary cells associated with the scar. Only in severe fibrosis was SOX9 detection increased in the biliary compartment, consistent with bile duct hyperplasia in more advanced disease (Figure 6-2B).
Figure 6-2: SOX9 localisation and cell numbers in liver fibrosis.
(A) Localisation of SOX9 expressing cells (red) with biliary epithelial cells (BEC) marked by CK19 and CK7 immunohistochemistry (green and arrows); in α1AT positive hepatocytes (green and arrow heads in SOX9/ CK19, SOX9/CK7 and SOX9/ α1AT sections); and EPCAM positive hepatobiliary progenitor cells (green). (B) Counting of SOX9-positive cells in biopsy samples (mild, n=5, moderate, n=5 and severe, n=4) separated into biliary epithelial cells (BEC) and non-BEC; cells surrounding the scar edge and in adjacent hepatocytes. * p<0.05.
6.3.3  Assessing liver fibrosis progression

These data suggested SOX9 quantification in liver biopsy samples might correlate with fibrosis stage and progression. To investigate this we extended our analysis to a larger cohort of patients extrapolated from the Trent HCV cohort. From 909 available biopsies, 115 patients were identified with an initial and follow-up biopsy. The mean inter-biopsy interval was 36.88 months (±2.49 SEM) and all samples had a paired serum sample taken within 6 months of the biopsy (shown in Section 2 Methods; Figure 2-5). The breakdown in Ishak stage grouped samples into those with no or early fibrosis (IS 0-1; n=80), moderate (IS 2-3; n=19) and advanced fibrosis (IS 4-6; n=16) according to their initial biopsy, with 60% of patients displaying early fibrosis. Fibrosis staging from these patients in follow-up biopsies showed a trend towards moderate and severe fibrosis, displaying progression of disease (Table 6-1).

<table>
<thead>
<tr>
<th>1st Biopsy</th>
<th>IS 0-1</th>
<th>2nd Biopsy IS 2-3</th>
<th>IS 4-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS 0-1</td>
<td>55</td>
<td>19</td>
<td>6</td>
</tr>
<tr>
<td>IS 2-3</td>
<td>6</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>IS 4-6</td>
<td>1</td>
<td>1</td>
<td>14</td>
</tr>
</tbody>
</table>

Table 6-1: Ishak staging comparing initial and follow-up biopsy samples in patients with HCV. Table demonstrates a trend of progression toward moderate (IS 2-3) and severe fibrosis (IS 4-6).

6.3.4 Validation and distribution of SOX9 according to fibrosis progression

71 initial and 81 follow-up biopsy samples from the 115 patients identified above contained adequate tissue to study SOX9 expression (Figure 2-5). Combined analysis of all biopsies (n=152) for BEC and non-BEC SOX9 cell numbers plotted against Ishak stage displayed a linear increase in non-BEC SOX9 with severity of fibrosis (Figure 6-3A). There was no demonstrable change in BEC component of SOX9
expression. Moreover, analysis of the BEC and non-BEC SOX9 cell numbers in the patients’ initial biopsy against their progression determined by Ishak staging in the follow-up sample displayed a high correlation of non-BEC SOX9 cell count with stage progression (Figure 6-3C; R²=0.988). BEC expression also had high correlation (Figure 6-3B; R²=0.827), however, the gradient of the line was in effect flat.

In combination with our previous data describing SOX9 mediating ECM components in liver fibrosis (Piper Hanley et al. 2008; Pritchett et al. 2012) and its localisation to potential progenitor cell types (Figure 6-2A), we questioned whether the ratio of SOX9 positive cells in non-BEC to BEC differed across the stages of fibrosis. Analysis of all liver biopsies indicated there was an increased ratio in Ishak stages 3 to 5 associated with active ECM deposition and scar formation, whereas in cirrhotic stage of the disease (IS 6) the ratio of SOX9 cells in non-BEC to BEC was decreased in line with ductal hyperplasia (Figure 6-3B).
Figure 6-3: Increased SOX9 cell numbers correlate with progression of disease in liver fibrosis.

(A) Bar graph of BEC and non-BEC SOX9 cell counts determined in each group of fibrosis from IS 0-1 (n=101), IS 2-3 (n=30) and IS 4-6 (n=21). Error bar represents SEM.

(B) Break down in the ratio of SOX9 expressing cells in non-BEC to BEC per Ishak stage of fibrosis. Significant difference between IS 0-2 and IS 3-5 fibrosis and between IS 3-5 and IS 6/cirrhosis.

(C) Correlation between SOX9 cell numbers and progression of liver fibrosis. Data points represent mean SOX9 cell count for patients who do not progress (0) or progress by 1, 2 or 3 stages of disease (as determined by change in Ishak score between biopsies) irrespective of baseline Ishak stage. The line shows best-fit linear regression ($R^2=0.988$).

(D) SOX9-positive cell numbers separated into BEC and non-BEC in biopsy samples from non-progressors (n=25) and progressors (n=13) groups. (E) Example of bright field immunohistochemistry image for SOX9 (brown) counterstained with toluidine blue showing one field of view. BEC and non-BEC are indicated. Total SOX9 cell numbers in each section are non-progressor (53) and progressor (113).

Groups analysed by ANOVA with Tukey HSD post-hoc analysis.
6.3.5 Modelling fibrosis progression as a function of SOX9 risk score

To determine the usefulness of SOX9 as a predictive marker, patients were classified into two sub-groups from those with \( \leq \) IS 2 staged fibrosis, on index liver biopsy. Groups were defined as progressors (n=13) based on progression of \( \geq 2 \) Ishak stages between the initial and follow-up biopsy, whereas non-progressors (n=25) were selected based on no progression in fibrosis. Critically samples with a single stage progression were excluded to correct for variability in histopathological interpretation. Analysis of total SOX9 cell numbers displayed a 1.9 fold increase in progressors versus non-progressors, associated with an increased number of non-BEC expressing SOX9 (~3 fold; Figure 6-3D and E). Moreover, these differences were consistent and irrespective of index Ishak stage (Table 6-2).

<table>
<thead>
<tr>
<th>Fibrosis Stage</th>
<th>SOX9 count (Total average count per high field)</th>
<th>Progressors</th>
<th>Non-progressors</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>64.7</td>
<td>33.55</td>
<td>0.02</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>64.87</td>
<td>32.42</td>
<td>0.04</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>77.63</td>
<td>41.8</td>
<td>NS</td>
</tr>
<tr>
<td>0-1</td>
<td></td>
<td>64.8</td>
<td>33.32</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>0-2</td>
<td></td>
<td>67.76</td>
<td>33.66</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 6-2: Increased SOX9 cell numbers in progressors versus non-progressors in early stages of fibrotic disease.

Total average SOX9 count per biopsy sub-divided into index biopsy stages. Consistent difference in SOX9 count at every stage of fibrosis between progressors and non-progressors.
6.3.6 Area under the receiver operator curve comparison in progressors and non-progressors

To assess average SOX9 count as a method of stratifying patients with early fibrosis into those more likely to progress, area under the receiver operator curve (AUROC) analysis was employed (Figure 6-4). This confirmed an AUROC of 0.913 (CI: 0.824–1.00) suggesting a very high discriminatory ability of SOX9 to categorise patients correctly into those more likely to have liver fibrosis progression in CHC.

![AUROC analysis of SOX9 count as a discriminator of fibrosis progression. AUROC value of 0.913 (95% CI: 0.824 – 1.000).](image)

6.3.7 SOX9 risk score as a predictor of fibrosis progression

Fibrosis progression is known to be associated with male sex, increasing age, ALT and NI (Yano et al. 1996; Poynard et al. 2001) (Table 6-3). Although data indicated a potential difference between the two sub-groups in all of the above variables, only ALT and age were statistically significant (p<0.05 for both). In addition, there was a significant (p<0.001) association of SOX9 with fibrosis progression (Table 6-3). There were no appreciable differences in ethnicity, HCV genotype and reported alcohol consumption between the groups (Table 6-3).
### Table 6-3: Distribution of risk factors for progressors and non-progressors.

<table>
<thead>
<tr>
<th></th>
<th>Non-Progressors</th>
<th>Progressors</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>37.28</td>
<td>44.97</td>
<td>( P &lt; 0.05 )</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>84.6</td>
<td>64.0</td>
<td>NS (( \chi^2 ))</td>
</tr>
<tr>
<td><strong>Alcohol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Teetotal (%)</td>
<td>28.00</td>
<td>23.10</td>
<td>NS</td>
</tr>
<tr>
<td>Current (units/wk.)</td>
<td>17.30</td>
<td>16.70</td>
<td>NS</td>
</tr>
<tr>
<td>Ever &gt;50 units/wk. (%)</td>
<td>32.00</td>
<td>30.80</td>
<td>NS</td>
</tr>
<tr>
<td>Ethnicity (% Caucasian)</td>
<td>88.00</td>
<td>92.30</td>
<td>NS (( \chi^2 ))</td>
</tr>
<tr>
<td>ALT (mmol/L)</td>
<td>67.64</td>
<td>85.55</td>
<td>NS</td>
</tr>
<tr>
<td>SOX9 (nuclei/hfa)</td>
<td>33.66</td>
<td>67.76</td>
<td>( P &lt; 0.001 )</td>
</tr>
<tr>
<td>NI grade</td>
<td>2.29</td>
<td>3.62</td>
<td>NS</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a/b</td>
<td>14</td>
<td>11</td>
<td>NS (( \chi^2 ))</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>1</td>
<td>NS (( \chi^2 ))</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>0</td>
<td>NS (( \chi^2 ))</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>1</td>
<td>NS (( \chi^2 ))</td>
</tr>
</tbody>
</table>

Binary logistic regression analysis identified only SOX9 (OR 1.13) and age (OR 1.08) as significant risk factors in disease progression (Table 6-4). These data suggested each 1-year increase in age was associated with an 8% increase in the risk of fibrosis progression and for every 1-cell count increase in SOX9 expression there was a 13% increase in the risk of fibrotic progression. Using adjusted logistical regression modelling, these differences were unaffected by adjusting for age and time between biopsy sampling (Table 6-4). Male patients were associated with a greater likelihood of progression than females, however this did not reach statistical significance. ALT was not associated with fibrosis progression (Table 6-4).

Sub-dividing total SOX9 into BEC and non-BEC revealed intriguing results. In the unadjusted values there appears to be no difference between BEC and non-BEC counts. However, adjusting for time to progression has a notable impact on the relationship between BEC and non-BEC and the likelihood of progression. BEC
count does not appear to predict progression, whilst non-BEC count remains associated with progression (Table 6-4). These data suggest that the non-BEC component of SOX9 cell count is crucial for predicting liver fibrosis progression.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Unadjusted</th>
<th>Adjusted for age</th>
<th>Adjusted for time to progression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.08 (1.00, 1.17)*</td>
<td>-</td>
<td>1.04 (1.00, 1.08)*</td>
</tr>
<tr>
<td>Age (&gt;55 vs. ≤55)</td>
<td>4.36 (0.36, 53.39)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gender</td>
<td>3.09 (0.56, 17.17)</td>
<td>3.32 (0.50, 21.92)</td>
<td>2.87 (0.44, 18.70)</td>
</tr>
<tr>
<td>Total Sox9</td>
<td>1.13 (1.04, 1.23)*</td>
<td>1.13 (1.03, 1.23)*</td>
<td>1.12 (1.03, 1.22)*</td>
</tr>
<tr>
<td>Sox9 Non-BEC</td>
<td>1.13 (1.04, 1.23)*</td>
<td>1.14 (1.04, 1.25)*</td>
<td>1.12 (1.03, 1.23)*</td>
</tr>
<tr>
<td>Sox9 BEC</td>
<td>1.14 (1.00, 1.29)*</td>
<td>1.14 (0.99, 1.31)</td>
<td>1.09 (0.95, 1.25)</td>
</tr>
<tr>
<td>ALT</td>
<td>1.01 (0.99, 1.02)</td>
<td>1.01 (0.99, 1.02)</td>
<td>1.01 (0.99, 1.02)</td>
</tr>
<tr>
<td>NI</td>
<td>1.63 (0.98, 2.71)*</td>
<td>1.55 (0.90, 2.68)</td>
<td>1.68 (0.94, 3.01)</td>
</tr>
</tbody>
</table>

Table 6-4: Binary logistical regression analysis indicating risk factor association with progression of liver fibrosis. Predictors all per unit/year increase. *p<0.05 (all significant values shown in bold).

6.3.8 SOX9 count in patients that progress to HCC

The data shown in this chapter suggests that SOX9 correlates strongly with progression of fibrosis in early disease. CHC is one of the leading causes of hepatocellular carcinoma (HCC) and the majority of patients who go on to develop HCC already have cirrhosis secondary to CHC infection. (Singal et al. 2012). Additionally, SOX9 expression has previously been described to be increased within regions of HCC compared to adjacent non-cancerous fibrotic liver (Guo et al. 2012). To explore if SOX9 count was increased in liver biopsies of patients who went on to develop HCC compared to those that did not, a second cohort was established from the Trent HCV cohort. Patients with more than one biopsy and with IS 5-6 on index biopsy were selected. Patients were divided into two groups dependent on if they developed HCC (proven by biopsy, tissue resection or definitive imaging) or not.
Mean interval to repeat biopsy was 3.70 years (SEM 0.93 years). A total of 20 patients were included, 10 with progression to HCC (HCC) and 10 without proven progression to HCC (No HCC). SOX9 was counted in biopsy specimens and subdivided as previously into biliary epithelial cells (BEC) and non-BEC. The breakdown is shown in Figure 6-5. The main variation was the greater BEC cells in the ‘No HCC’ group (46.0 compared to 32.9). This was not significantly different and other parameters were relatively similar. This suggested that SOX9 expression does not appear to drive patients towards HCC.

Figure 6-5: SOX9 count in cirrhotic patients who went on to HCC.

Average SOX9 per biopsy sub-divided into BEC and non-BEC in patients with cirrhosis on biopsy and CHC. Count done in Index biopsy and patients categorised into two groups; HCC (n=10) – those that went on to develop confirmed HCC in 5 years and No HCC (n=10) – those that did not. Error bar represents SEM of total count.
6.4 Discussion

6.4.1 SOX9 count increases risk of fibrosis progression

The data from this chapter suggests SOX9 is a novel histological marker in biopsy samples with the ability to predict fibrotic progression in early stages of the disease (≤ IS 2 fibrosis). Specifically, in patients with an index and follow-up biopsy, the data suggested SOX9 was highly correlated with fibrotic progression ($R^2=0.988$). Moreover, when patients were categorised into progressors (n=13) versus non-progressors (n=25), SOX9 cell numbers were 1.9 fold increased in individuals who showed at least 2 stages of advancement in fibrosis. This appeared to be entirely associated with SOX9 positive non-BEC cells (~3 fold). Strikingly, logistical regression analysis and comparison with other known risk factors of fibrotic progression indicated for every one-cell increase in SOX9 cell numbers there was a 13% increased risk of disease progression. Importantly, these data were superior at predicting progression of disease, compared to traditional surrogates of hepatic inflammation, including serum ALT and necroinflammation in the liver biopsy. Finally the data remained significant when age and time taken between initial and follow-up biopsy sampling were considered in the model.

6.4.2 Categorisation of SOX9 positive cells

In severe disease, SOX9 was localised to CK19 and CK7 positive cells characteristic of bile ducts and duct-like cells, indicative of ductular reaction in chronic liver damage. However, we also detected nuclear SOX9 in non-biliary cells expressing α1AT, particularly located around the periphery of the scar area. These data are in-line with the role of SOX9 in ductal plate formation during early liver development in both mice (Antoniou et al. 2009) and humans as previously described (Carpentier et al. 2011) and shown by data in chapter 3 (Figure 3-2). Recent in vivo studies have shown SOX9 in liver progenitor cell derived transit-amplifying cells (or oval cells) as part of the regenerative response to injury (Espanol-Suner et al. 2012).

In contrast to previously described Sox9 expression in rodent fibrosis models (Piper Hanley et al. 2008; Pritchett et al. 2012), although present, fewer SOX9 positive cells were detected in cells expressing α-SMA, used to mark myofibroblasts, potentially
indicating differences associated with *in vitro* versus *in vivo* modelling of a dynamic disease.

### 6.4.3 SOX9 driving fibrogenesis in CHC

The non-BEC SOX9 cell count showed a trend of increase across the broad stages of fibrosis (Figure 6-3A) but was not statistically significant. Interestingly, the ratio of non-BEC to BEC expressing SOX9 differs across the stages of fibrosis (Figure 6-3B) with an increased ratio found in IS 3-5 associated with significant matrix deposition. However, in cirrhosis (IS 6) the ratio of non-BEC to BEC decreases, coincident with biliary hyperplasia in latter stages of the disease. This pattern is replicated in progressors versus non-progressors suggesting hepatobiliary cells may be driving fibrosis progression, at the earliest stages of fibrosis, and remain expressed in higher proportion during more advanced disease. In combination with our previous data describing the role of SOX9 in ECM deposition from activated HSCs and in fibrotic rodent liver, these results may indicate SOX9 is a marker of active fibrogenesis.

### 6.4.4 Clinical implication of SOX9 risk score

The results of this study have important clinical implications. The identification of patients with rapid progression will allow personalisation of treatment. Specifically novel directly acting antiviral (DAA) therapies to treat HCV have shown an improved sustained virological response (SVR) (Jacobson et al. 2011; Poordad et al. 2011; Silva et al. 2013)) but have side effects and are costly. The identification of a phenotype with rapid progression may stratify those who should be prioritised for these treatments. Moreover, patients not achieving SVR (approximately 30%) are potential candidates for emerging antifibrotic therapies (e.g. decreased fibrosis progression following immunomodulation with interferon treatment (Morishima et al. 2012)). Therefore the use of SOX9 to identify individuals at risk of fibrotic progression may provide a step toward personalised therapy.

The ability to phenotype a ‘fibrogenic’ profile will have additional therapeutic benefit. Analysis from the Hepatitis C Antiviral Long-term Treatment against Cirrhosis (HALT-C) study shows evidence that interferon therapy, during the lead in phase, reduced future fibrosis progression and clinical outcomes in those individuals that had significant improvement of their inflammatory index (hepatic activity index
score; HAI) on liver biopsy (Morishima et al. 2012). Importantly, this effect was maintained despite viral recurrence at a later time point and was independent to randomisation to maintenance interferon therapy. This raises the intriguing possibility that even short-term immune modulation can alter long-term fibrosis progression and clinical outcomes. SOX9 may improve the identification of these individuals. Additionally, understanding which cell types are driving the fibrogenic reaction will open opportunities to develop novel therapeutic strategies.

6.4.5 Summary

This study represents the first description of SOX9 as a novel prognostic marker of liver disease. In combination with our previous in vitro studies implicating SOX9 in ECM regulation, it provides an interesting concept whether SOX9 is a marker of active fibrogenesis. Although, the use of SOX9 in a clinical setting would still require biopsy, there are several previously unmet advantages to this. In contrast to recent serum studies to predict disease progression in moderate disease (IS ≥ 3) (Fontana et al. 2010), our data suggest SOX9 has significant prognostic value during early stages of fibrosis (IS ≤ 2). In addition, rather than a complex algorithm from several serum markers, the methodology for SOX9 expression and cell counting represents a simple assay, easily incorporated into current automated histology assessment of liver disease. Finally, its utility as a prognostic marker will have significant impact on defining treatment in at risk individuals, whereas, as a marker of fibrogenesis, determining expression of SOX9 would provide a useful determinant for novel antifibrotic therapies.
7 GENERAL DISCUSSION

7.1 Overview

Understanding the process of liver fibrosis is a priority in hepatology research. The last thirty years have seen significant advances in understanding the molecular mechanisms under-pinning fibrosis; however, there has been limited translation into clinical practice. The overall aim of this research was to investigate fibrosis with a view to producing clinically applicable outcomes.

The transcription factor SOX9 has key developmental roles in regulating ECM and there is growing evidence of its function in fibrotic disease (Pritchett et al. 2011). My supervisor (Dr Piper Hanley) previously discovered ectopic expression of SOX9 as a novel mechanism to underlie aspects of liver fibrosis (Piper Hanley et al. 2008). This discovery allowed me to investigate the molecular genetic network in which SOX9 operates including defining roles for SOX9 mediating ECM through inhibition of collagenases (e.g. MMP13) and identification of the inflammatory glycoprotein Osteopontin (OPN) as a target of SOX9. Additional to OPN, four other novel target genes of SOX9 action were identified, some of which have already been highlighted as candidate biomarkers, for new diagnostic strategies in liver fibrosis. Moreover, investigating the expression of SOX9 directly during liver fibrosis in human biopsy samples highlighted the factor as a prognostic marker of the disease. Taken together, these data place SOX9 as a key mediator of liver fibrosis.

7.2 SOX9 and liver fibrosis

SOX9 has been proposed as a transcriptional regulator of the ECM changes which occur in liver fibrosis (Piper Hanley et al. 2008) and in other fibrotic conditions, such as glomerulosclerosis (Bennett et al. 2007). Data from Chapter 3 further defines Sox9 in liver fibrosis. Using rodent models of fibrosis, Sox9 is co-localised within the fibrotic niche with fibrillar collagens and α-Sma, indicative of activated HSCs. Work within the group has demonstrated nuclear SOX9 expression in the activated
human HSCs, again co-localising with α-SMA. This novel finding corroborates previous rodent specific data. Perhaps more interesting is the expression of SOX9 in fibrotic liver biopsies. Here expression is noted to be increasing with fibrosis and suggested more than one cell type being responsible. This is the first description of SOX9 in human fibrosis and a theme further explored in Chapter 6, where greater classification of the cells responsible was undertaken.

### 7.2.1 SOX9 and HSC activation

During this project, significant publications cast doubt over the presence of SOX9 in activated HSCs (Carpentier et al. 2011; Furuyama et al. 2011). Using GFP-tagged lineage tracing of Sox9 positive cells, Sox9 was found to mark putative adult stem cell population, in response to injury. Functionally, these cells were shown to be involved in cellular repopulation after liver injury, in gastrointestinal organs of the liver, pancreas and gut (Carpentier et al. 2011; Furuyama et al. 2011). Using this model, activated HSCs were not shown to be Sox9 positive (Furuyama et al. 2011) and this outwardly appears at odds with the data presented here. However, there are important methodological differences in the models employed. In both publications, liver injury was induced by a single injection of CCl4, whereas, in our fibrosis models, CCl4 is injected twice weekly for 8-12 weeks. The data presented in Furuyama et al. 2011 is that of acute liver injury and not chronic liver fibrosis, and therefore could not be extrapolated to the results presented in this thesis.

### 7.2.2 OPN

Discovering downstream targets of SOX9 is a key theme to this project and in Chapter 3, OPN is shown to be regulated by SOX9 in liver fibrosis. This observation is in keeping with previous data, whereby OPN and SOX9 have both been shown in biliary epithelial cells in healthy liver tissue (Piper Hanley et al. 2008; Antoniou et al. 2009; Syn et al. 2011). Importantly, cells positive for both OPN and SOX9 are shown in regions and structures not associated with BECs. The distribution in fibrotic tissue and in vitro analysis of isolated HSCs, provides evidence supporting these cells being activated HSCs. Further proof is shown in the microarray analysis of activated rat HSCs, where mRNA levels of Spp1, the gene transcribing for Opn, is above detection thresholds.
This work also provides proof of direct regulatory relationship with SOX9, using siRNA to attenuate Sox9 expression and evidence of a conserved SOX9 binding motif in an enhancer region of \textit{OPN}. Additional work in the group demonstrated SOX9 binding to this region, using ChIP (Pritchett et al. 2012). Data from this thesis sits comfortably with recently published work in non-alcoholic fatty liver disease, where OPN was shown to be pro-fibrotic and regulated by the hedgehog pathway (Syn et al. 2011).

Functionally, the role of OPN in liver fibrosis is more uncertain, with evidence to support pro-fibrotic and anti-fibrotic activity (see Section 3.1.5). The discordancy between these results may be resolved by appreciating the multiple isoforms of OPN, including cleavage of the RGD-domain, having a plethora of functions in response to inflammatory changes within the liver. Our work suggests that OPN is involved in the fibrogenic process, increasing as HSCs activate and decreasing with knockout of SOX9. In this work, only full-length OPN was assayed, and fluctuations in the different isoforms may have more complicated outcomes. This was not included in the scope of this research, but is an area that requires greater understanding, especially as the significance of OPN grows, both functionally and as a biomarker.

OPN is readily assayed in serum and has been investigated as a biomarker in a variety of diseases, including HCC (Shang et al. 2012), CHC induced liver fibrosis (Huang et al. 2010), systemic lupus erythematos (Briggs 2013), retroperitoneal fibrosis (Binder et al. 2012) and bladder urothelial carcinoma (Zhao et al. 2012). The data from Chapter 5 adds to this growing body of work. We show a very high discriminatory ability at identifying early (IS 0-1) and significant (IS \geq 4) fibrosis. From the published work outlined above, OPN particularly appears to increase in concentration in the presence of fibrotic diseases and this may make it a less specific biomarker of organ fibrosis, but a better biomarker indicating the presence of ongoing fibrosis. This would be consistent with these data, placing OPN as a mediator of fibrogenesis.
7.3 **Fibrosis resolution**

Amongst the *in vitro* techniques employed in this thesis was abrogation of Sox9 by siRNA. Using this system in LX-2 cells and activated rHSCs, a commensurate reduction in Col1 and Col2 has been demonstrated (Piper Hanley et al. 2008) and OPN in Chapters 3 and 5. In Chapter 4, using the same system, EPIM levels are shown to go in the opposite direction, increasing as SOX9 is knocked down. EPIM is known to be involved in post-injury hepatic regeneration (Segawa et al. 2005); (Yoshino et al. 2006) and we show Epim to be elevated in quiescent HSCs and decrease upon activation. This posed the question whether Epim may be involved in altering HSCs towards a less fibrogenic phenotype. This hypothesis was tested using recombinant human EPIM on activated rHSCs. Mmps and Timp1 assayed suggested a dose dependent alteration towards ECM degradation, with a reduction in Timp1 and increase in Mmp13 and Mmp9. Apoptosis was unchanged in response to rhEPIM and recently highlighted markers of an ‘inactive’ HSC phenotype in fibrosis (Kisseleva et al. 2012; Troeger et al. 2012) were not altered. EPIM may induce an inactive phenotype in HSCs, however the transient nature of these experiments (i.e. 24 hour rhEPIM treatment *in vivo*) may not be able to reliably distinguish the effect.

MMP13 is a key collagenase and shown to be up-regulated in fibrosis resolution (Watanabe et al. 2000) and in initial inflammatory response resultant in early fibrosis (Uchinami et al. 2006). Beyond its helicase activity, MMP13 also activates other MMPs and is directly involved in collagen degradation. These protean roles place MMP13 central to regulating the ECM environment in matrix degradation. Parallel work within the group highlighted Mmp13 as potentially regulated by Sox9 in an RNA microarray. These data support my studies indicating Mmp13 is reduced in activated HSCs. Moreover, in line with increased Sox9 in activated HSCs, SOX9 appears to bind to a conserved motif in *MMP13*. Although luciferase assay detected no change in MMP13 expression in response to SOX9, siRNA treatment caused an increase in MMP13. These data indicated SOX9 may directly inhibit MMP13. In addition, potential reasons why the luciferase data was unsuccessful include two CAAT binding motifs in close proximity within the binding region and the presence of endogenous SOX9 within the LX-2 cells used for the assay making alteration of
transcription due to transfected SOX9 difficult to detect. Nonetheless, these data provides evidence for the transcriptional regulation of MMP13 in HSCs.

### 7.4 Biomarkers of liver fibrosis

Biomarkers of fibrosis remain an important area of research, especially in CHC, where rapidly emerging anti-viral therapies are driving the need for greater classification of fibrosis stage (Lai and Afdhal 2011). Multiple methods of biomarker discovery have been employed, including genome-wide association studies (Holmes et al. 2011) and proteomics in serum (Gangadharan et al. 2012), cirrhotic liver (Molleken et al. 2009) and HSCs (Kristensen et al. 2000). We were interested in the transcriptional changes that drive fibrosis, particularly in activated HSCs. To this end, we employed an RNA microarray in an effort to expose potential biomarkers. With the working hypothesis that SOX9 is critical to HSC activation and hence the ECM changes of fibrosis, positively regulated ECM proteins were explored as potential biomarkers. Within this five potential targets were found.

Biomarkers were explored in a similar fashion to other studies (Guha et al. 2011), using an exploration and validation cohort. Four of the five biomarkers performed generally well, whilst FN1 was less able, given principally to a bell-shaped curve of concentration to stage. Directly comparing the targets to the presently best available biomarkers revealed exciting findings. The biomarkers had comparable AUCs overall and performed better in early fibrosis and in distinguishing the presence of fibrosis compared to controls. The difference in performance may well reflect the method of biomarker discovery. Our biomarkers are ECM proteins discovered due to the regulation of SOX9. Given SOX9’s regulation of ECM in fibrosis, they more likely reflect the dynamic nature of fibrogenesis and hence are more precise in early to intermediate fibrosis.

Extrapolating from this argument, it could be hypothesised that the biomarkers reflect on-going fibrosis and so should be able to predict patients more likely to have liver fibrosis progression. Dividing the cohort into two groups of those that had ≥2-stage increase of Ishak stage to those that had no change, did not divulge any
difference in concentrations of any of the targets. This disappointing result underlines the complexities and heterogeneous nature of liver fibrosis progression. Data from Chapter 6 demonstrates the difference in SOX9 expression in progressors and non-progressors and the difference in ratio of Non-BEC to BEC SOX9 expression at different stages of fibrosis. Rather than serum biomarkers, the number of non-biliary SOX9 positive cells appears to correlate better to liver fibrosis progression.

7.5 SOX9 positive cells in fibrotic liver

Previous work on SOX9 in liver fibrosis focused on rodent models of disease (Piper Hanley et al. 2008). One of the main aims of this project was to define SOX9 in human liver fibrosis. This was done in the context of CHC using serial sections and dual staining with known cellular markers. Several different cell types exhibited SOX9 positivity, with abundance altering dependent on fibrosis stage.

7.5.1 HSCs

SOX9 positive cells co-localising with α-SMA, a marker of activated HSCs/MFs, was found at all stages of fibrosis. Interestingly, the majority of SOX9 positive cells were not α-SMA positive. This was in contrast to our previous data in rodent models of fibrosis and exemplified the difference in human disease and rodent models. A further explanation may be the mode of fibrosis caused by CHC. With largely a portal-portal fibrosis, which is portal myofibroblast predominant, may produce α-SMA negative MFs not originating from HSCs.

7.5.2 BECs and ductular reaction

Biliary epithelial cells are known to be SOX9 positive, in development and in the normal adult liver (Antoniou et al. 2009). These data was replicated by our data, with co-localisation with biliary markers, CK-7 and CK-19. Additionally, our data shows that cells in the ductular reaction accompanying CHC fibrosis are both SOX9 and CK positive. The precise nature and function of ductular reaction remains obscure and previous work has related this to increased portal fibrosis (Clouston et al. 2005) as opposed to a source of liver progenitors.
7.5.3 Progenitors

The importance of SOX9 in cell replacement has been demonstrated in the liver (Carpentier et al. 2011) and in other organs such as pancreas (Furuyama et al. 2011) and mammary glands (Guo et al. 2012). The exact function and nature of these cells still remains somewhat elusive. There is conflicting data with regards to direction of cell supply, with some papers suggesting SOX9 positive progenitor cells can supply cells of both biliary and hepatocyte lineage (Furuyama et al. 2011), whilst others suggest unidirectional lineage with mature hepatocytes repopulating liver mass after chronic injury (Espanol-Suner et al. 2012). The divergence of these data may reflect the differences in acute and chronic liver injury as well as different mouse models.

Data from this thesis presents SOX9 expression in human liver fibrosis and differences are to be expected from rodent models. In the developing liver, SOX9 positive cells are seen at the portal-parenchyma interface, a region referred to as the ductal plate. In the healthy adult liver this ductal plate is not present, as presented in Section 3.3.1. In CHC related fibrosis, SOX9 positive cells are again seen at the scar edge and are reminiscent of the ductal plate. Dual staining shows some to be CK7 positive, suggesting a progenitor/biliary lineage whilst others are α1-AT positive, suggestive of mature hepatocytes. The presence of these SOX9 positive cells in the areas of fibrogenesis also raises a question as to their exact role. Are these cells actually causing harm by aberrant ECM production in the attempt to regenerate? This possibility would be supported by our data in Chapter 6, where an increase in SOX9 positive cells at the scar edge heralds a markedly greater chance of fibrosis progressing.

7.5.4 Hepatocytes

A subset of SOX9 positive cells was found to be α1AT positive, suggestive of hepatocytes. Dual staining for these two markers was predominantly evident in advanced fibrosis and exclusive to hepatocytes in peri-portal / zone 1 areas of the hepatic lobule. Given the location and SOX9 positivity of these cells, may suggest a dissimilar phenotype to hepatocytes derived from mature hepatocytes and a derivation from a progenitor cell population.
7.6 Liver fibrosis progression

Liver fibrosis progression is an area that is increasingly important, especially in CHC. We are currently in a golden age of therapies for CHC, with anticipated release of interferon free regimes with greater potency and fewer side-effect (Poordad 2011). Unfortunately these therapies are likely to be expensive and with health care worldwide striving for efficiency, categorising patients with the greatest need for therapy and those that may wait for newer, safer regimes is of high importance. This heralds an era of more personalised medicine for CHC, going beyond simply genotype of virus.

In Chapter 6 we present data showing that the amount of SOX9 positive cells in early liver fibrosis predicts rate of progression. The strength of these data was far greater than any other predictor of progression, such as age, ALT and necroinflammation. Other markers of fibrosis progression have been proposed, notably aminopyrine breath test (Rocco et al. 2012) and certain serum biomarkers (Fontana et al. 2010). SOX9 counting performs better than any other proposed tests (based on comparative AUROC values) and, crucially, is able to predict progression from a much earlier disease stage. Practically, SOX9 count also has benefits. It would be relatively simple to implement in a clinical pathology laboratory without a need for additional equipment or resources.

It can be argued that liver biopsy itself has become increasingly redundant in CHC. Present guidelines do not necessitate liver biopsy before treatment or to assess treatment success. However, if progression could be gleaned from biopsy, this would update the clinical need and give more information than a fibrosis stage and grade. Further information can be extrapolated from the ratio of SOX9 expression in BECs and non-BECs. Data from Chapter 6 suggests that the ratio is at its greatest at intermediate stages of fibrosis (IS 3-4) and this is when fibrosis is reported to be at its most progressive (Thein et al. 2008). It is notable that in cirrhosis, this ratio returns to levels near early fibrosis, suggesting alternatives mechanisms take over at this point. The inability of nuclear SOX9 expression to predict those that would go on to HCC may also reflect these mechanistic alterations.
7.7 Summary

In summary, this work builds upon earlier work in our group demonstrating that SOX9 may be a key regulator of liver fibrosis. SOX9 has clear functions in HSC activation and ECM dysregulation as well as roles in other cell types involved in fibrosis. Clinical applications from this thesis include identification of novel biomarkers of liver fibrosis and the quantification of SOX9 expression to correlate strongly with liver fibrosis progression, an observation with potential clinical utility.
7.8 Future work

Data from this thesis stimulated many questions that need to be addressed in future work. These include:

- **SOX9 expression in other fibrotic disease**
  - Does SOX9 expression predict progression in other disease such as ALD, autoimmune hepatitis, NASH, etc.?
  - Does SOX9 count predict progression in a prospective cohort?

- **Define the role of Sox9 in in vivo models of fibrosis**
  - Does conditional knockout of Sox9 in vivo, alter the propensity towards liver fibrosis?
  - Can this effect be identified to individual cells with specific CRE-drivers to knockout Sox9?

- **What exactly are the roles of SOX9 positive cells in fibrosis?**
  - Are liver progenitor cells involved in fibrosis?
  - Are SOX9 derived mature cells functionally similar to mature cells from other origins?
  - Both the above questions could potentially be investigated by laser micro-dissection of SOX9 positive nuclei from fibrotic human liver.
  - The current interest in the ductular reaction (Carpentier et al. 2011; Yoon et al. 2011) as a source of repopulating liver post injury needs exploration. EpCAM, a marker of progenitor cells, could be co-localised with SOX9, CK and α1AT to answer if SOX9 cells of the ductular reaction populate hepatocytes and if these are the same SOX9 / α1AT hepatocytes described in this thesis.

- **Biomarker exploration**
  - The four best biomarkers identified could be further validated in a prospective cohort. This could be done alongside SOX9 count.
Further mathematical analysis of the identified biomarkers with previously known markers may be able to provide a more accurate biomarker panel.

- Osteopontin clearly is important in fibrosis and further assessment of its function is needed.
  - What are the functional consequences of the multiple isoforms of OPN?
  - What are the modes of action of OPN?
  - How is OPN feedback regulated and what is the relationship with macrophages/Kupffer cells?

- Epimorphin appears to alter HSC phenotype
  - Can this be exploited as a potential anti-fibrotic strategy?
  - Do Epim knockout mice exhibit decreased fibrosis?

- Ectopic SOX9 appears important in driving other fibrotic processes
  - Is SOX9 a global driver of fibrosis?
  - Can SOX9 be found in other important fibrotic conditions, such as pulmonary fibrosis?
  - Is there a difference in fibrotic conditions that are determined by fibroblasts as compared to myofibroblasts?
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Nunez, O., A. Fernandez-Martinez, et al. (2004). "Increased intrahepatic cyclooxygenase 2, matrix metalloproteinase 2, and matrix metalloproteinase 9 expression is associated with progressive liver disease in chronic hepatitis..."


Yoneda, M., H. Mawatari, et al. (2007). "Type IV collagen 7s domain is an independent clinical marker of the severity of fibrosis in patients with nonalcoholic steatohepatitis before the cirrhotic stage." J Gastroenterol 42(5): 375-381.
## APPENDIX I: TABLE OF PCR PRIMERS

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Application</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
<th>Product Length</th>
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The transcription factor SOX9 is crucial for multiple aspects of development. Mutations in SOX9 cause campomelic dysplasia, a haploinsufficiency disorder concordant with the expression profile of SOX9 during embryogenesis. The mechanistic understanding of development has revealed roles for SOX9 in regulating cartilage extracellular matrix (ECM) production and cell proliferation, among others. More recently, it transpires that SOX9 becomes expressed and induces destructive ECM components in organ fibrosis and related disorders. Although commonly absent from the parent cell type, SOX9 is expressed in a wide range of cancers, where it regulates cell proliferation. These data have potential diagnostic, prognostic and therapeutic relevance, suggesting that disease mechanisms might result from re-expressing this developmental transcription factor in ectopic locations.

An introduction to SOX9

The formation of organs during normal development requires the precise activation and silencing of gene expression. Inappropriate or reactivated expression of genes can cause disease. Sex-determining Region Y (SRY) box 9 (SOX9) is a member of a highly conserved family of transcription factors defined by their similarity to the high mobility group DNA-binding domain of SRY [1]. Like other family members, SOX9, part of the SOX E subgroup, plays crucial and diverse roles during development [Box 1; Table 1]. In 1994, SOX9 was identified as the gene underlying the haploinsufficiency disorder campomelic dysplasia (CD), a human syndrome characterized by defective chondrogenesis and variable 46,XY sex reversal [2]. However, studies of Sox9-deficient mice and Sox9 function in other species have highlighted the importance of this transcription factor at many sites during development including cells in the testis, pancreas, intestine, brain, kidney, heart valves and derivatives of the neural crest (NC; reviewed in [1]). Recently, SOX9 expression, as transcript or protein, has been reported in several disease states. A causal role for this transcription factor in fibrosis and related disorders is emerging [3–8]. SOX9 is also expressed and implicated in the growth of cancers derived from several different cell types [9–12]. These diverse effects of SOX9 arise from roles controlling the pericellular environment, cell differentiation and proliferation. Here, we review these roles of SOX9 in developmental and disease settings and discuss the potential for SOX9 and its modulation as a strategy for diagnosing, predicting and treating disease.

SOX9 in aspects of development

SOX9 is widely expressed and plays important roles during the development of many organs (Figure 1; Table 1).

Chondrogenesis

Endochondral ossification is the two-stage process whereby the skeleton, other than the cranium, forms as a cartilaginous template that is subsequently turned into bone. SOX9 is essential for the first stage - chondrogenesis [13,14]. SOX9 is abundantly expressed in mesenchymal cells as they commit to being chondroprogenitors and is retained in differentiated chondrocytes, but needs to be transcriptionally silenced for the terminal differentiation of chondrocytes and ossification [13]. Sox9 activates the transcription of many cartilage-specific genes, in part by direct interaction with Sox5 and Sox6. In particular, SOX9 regulates many genes encoding extracellular matrix (ECM) components such as Collagens type-2, 9, 11 and 27 (Col2a1, Col9a1, Col11a2 and Col27a1), Aggrecan (Agc1), Matrillin-1 (Matn-1) [13] and Cartilage oligomeric protein (Comp) [15]. Recent evidence suggests paired SOX9-binding sites are a common feature of regulatory regions for several of these genes [16]. Dimerized SOX9 binds to these elements in the relevant enhancer regions of Col2a1, Col9a1 and Col11a2 with mutations that disrupt dimerization greatly reducing reporter construct activity [16]. Interestingly, this nuance of function seems to be particularly important for chondrogenesis. Similar experiments on SOX9-regulated promoter elements from testis-specific genes encoding Steroidogenic factor-1 (SF-1; an orphan nuclear receptor officially designated NR5A1) and Anti-Müllerian hormone (AMH; also known as Müllerian-inhibiting substance) were unaffected by SOX9 dimerization [16]. In concordance, a missense mutation (A76E) in a 46,XY individual that abrogated dimerization caused the skeletal manifestations of CD but not sex reversal [17].
SOX9 is a crucial gene in mammalian skeletogenesis and testicular determination, with mutations causing the rare and usually lethal syndrome CD (OMIM 114290) [1,2]. The features of CD beyond the skeleton and testis include impaired development of the kidneys, heart, brain and pancreas [1]. Recent data have also associated SOX9 with isolated Pierre Robin syndrome, characterized by craniofacial defects [PRS, OMIM 261800] [1,74]. Mutations have been identified in discrete enhancer elements flanking the gene. Interestingly, the conditional inactivation of Sox9 in mouse cranial NC cells results in a phenotype resembling PRS (e.g. reduced jaw and cleft palate) [1].

The molecular mechanisms governing SOX9 action operate at the levels of transcription, translation, post-translational modification (influencing degradation), nuclear transport and cofactor interaction. The studies of patients with CD highlight the complex transcriptional regulation of SOX9 as mutations and chromosomal rearrangements dispersed up to 1 Mb from the transcriptional start site of SOX9 can result in CD [1]. Within the proximal 5′ flanking region, several conserved promoter elements have been identified including a binding site for the nuclear factor kappa B complex subunit RelA [75], a cAMP response element-binding protein (CREB) consensus motif Sp1 site [76] and two CCAAT elements. The latter are located within the first 100 bp of the 5′ flanking region and bind the trimeric transcription factor complex nuclear factor Y [4].

In the 3′ untranslated region, factors affecting mRNA stability regulate SOX9 function via interaction with conserved AU-rich elements (AREs). Increased Sox9 mRNA stability has been attributed to the activation of the p38 MAPK pathway, and several conserved 3′ AREs have been identified as being capable of binding RNA stability proteins [77]. Other post-transcriptional regulators of SOX9 expression are microRNAs. Several regulate Sox9 levels, including the miR-17-92 cluster during lung development [78], miR-140 in chondrogenesis [79] and miR-124 during neurogenesis [80]. Although these miRNAs largely inhibit SOX9 expression, miRNAs can also enhance gene expression [81].

**Box 1. Overview and molecular properties of SOX9**

SOX9 is a crucial gene in mammalian skeletogenesis and testicular determination, with mutations causing the rare and usually lethal syndrome CD (OMIM 114290) [1,2]. The features of CD beyond the skeleton and testis include impaired development of the kidneys, heart, brain and pancreas [1]. Recent data have also associated SOX9 with isolated Pierre Robin syndrome, characterized by craniofacial defects [PRS, OMIM 261800] [1,74]. Mutations have been identified in discrete enhancer elements flanking the gene. Interestingly, the conditional inactivation of Sox9 in mouse cranial NC cells results in a phenotype resembling PRS (e.g. reduced jaw and cleft palate) [1].

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One of the key determinants of bone growth is the balance between chondrocyte proliferation and terminal differentiation according to the respective presence or absence of SOX9. This context-specific expression of SOX9 is mediated either directly or indirectly by several signaling pathways (Table 2). These same signaling mechanisms also regulate bone repair as a type of wound healing response [18]. However, emerging evidence suggests that some of these pathways might also underlie SOX9 expression in fibrosis and related disorders [3,4,6–8] when many of the SOX9 target genes encoding ECM components become inappropriately and excessively expressed, i.e. wound healing that has gone awry.

**Testis formation**

In mammals, the presence of a Y chromosome causes differentiation of the bipotential gonad to a testis rather than an ovary [19]. Although SRY initiates this program, SOX9 is a key testis-determining gene specifying the Sertoli cell lineage [19]. Two-thirds of 46,XY patients with CD have varying degrees of male-to-female sex reversal (a type of 46,XY disorder of sex development), whereas duplication of SOX9 has been linked to human 46,XX female-to-male sex reversal [20]. In mice, Sox9 expression is initiated by a testis-specific enhancer bound by Sry in cooperation with SF-1 [21]. Sox9 expression is then maintained as levels of Sry recede by Sox9 substituting for Sry in its interaction with SF-1. Thus, an important aspect of SOX9 expression seems to be its ability to positively regulate its own transcription. The latter interaction between SOX9 and SF-1 also regulates transcription of AMH [19]. Experiments in vitro and in silico have shown that SOX9 binds to and
**Table 1. The role and regulation of SOX9 in development and disease.**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Relevance to SOX9</th>
<th>Significance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Testis</strong></td>
<td>SOX8/3F-1 Interaction regulates AMH</td>
<td>Testis development</td>
<td>[19,22]</td>
</tr>
<tr>
<td></td>
<td>FGF signaling</td>
<td>Regulation of SOX9</td>
<td>[19]</td>
</tr>
<tr>
<td></td>
<td>Regulates Pgd2</td>
<td>Promotes Pgd2 synthesis/Sertoli cell differentiation</td>
<td>[22]</td>
</tr>
<tr>
<td></td>
<td>Pdg2 synthesis</td>
<td>Promotes nuclear import of SOX9</td>
<td>[22]</td>
</tr>
<tr>
<td><strong>Pancreas</strong></td>
<td>Activates Ngn3 expression</td>
<td>Endocrine specification</td>
<td>[31,33]</td>
</tr>
<tr>
<td></td>
<td>Promotes Hep-1 expression</td>
<td>Maintains progenitor cell population</td>
<td>[32]</td>
</tr>
<tr>
<td><strong>Heart</strong></td>
<td>Promotes proliferation and ECM deposition</td>
<td>Formation of heart valve and septa</td>
<td>[37,38]</td>
</tr>
<tr>
<td></td>
<td>Activates Col2a1 expression</td>
<td>Vascular calcification</td>
<td>[8]</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td>Sox9 expression</td>
<td>Bile duct development</td>
<td>[43]</td>
</tr>
<tr>
<td></td>
<td>Increases Col1 expression in HSCs</td>
<td>ECM deposition in liver fibrosis</td>
<td>[4]</td>
</tr>
<tr>
<td><strong>Cartilage</strong></td>
<td>TGF-β signaling</td>
<td>Increases Sox8 expression in HSCs</td>
<td>[4]</td>
</tr>
<tr>
<td></td>
<td>Regulates cartilage ECM components</td>
<td>Chondrogenesis</td>
<td>[13,14]</td>
</tr>
<tr>
<td></td>
<td>Promotes β-catenin degradation</td>
<td>Inhibits chondrocyte proliferation and differentiation</td>
<td>[90,91]</td>
</tr>
<tr>
<td></td>
<td>TGF-β signaling</td>
<td>Increases Sox9 expression during chondrogenesis</td>
<td>[13,92]</td>
</tr>
<tr>
<td></td>
<td>Hh signaling</td>
<td>Sox9-mediated chondrogenesis</td>
<td>[1,93]</td>
</tr>
<tr>
<td></td>
<td>Notch signaling</td>
<td>Downstream targets Hes-1 and Hey-1 compete for Sox9-binding sites on Col2a1 enhancer to inhibit activation</td>
<td>[94]</td>
</tr>
<tr>
<td><strong>Ovary</strong></td>
<td>Pgd2</td>
<td>Induces Sox9/tumor growth inhibition</td>
<td>[53]</td>
</tr>
<tr>
<td><strong>Prostate</strong></td>
<td>SOX9 expression</td>
<td>Essential for prostate development</td>
<td>[44]</td>
</tr>
<tr>
<td></td>
<td>Wnt5β-catenin</td>
<td>Increases Sox9 expression/promotes proliferation and AR expression in prostate cancer cell lines</td>
<td>[55]</td>
</tr>
<tr>
<td><strong>Neural</strong></td>
<td>cGMP-dependent protein kinases</td>
<td>Suppress Sox9 expression and glioma growth</td>
<td>[61]</td>
</tr>
<tr>
<td></td>
<td>cGKI and PdkII encoded by Pkd2</td>
<td>EMT in avian NC</td>
<td>[27]</td>
</tr>
<tr>
<td></td>
<td>Sox9 activates the Snai2 promoter</td>
<td>NC specification</td>
<td>[29]</td>
</tr>
<tr>
<td><strong>Skin</strong></td>
<td>Chd7-mediated SOX9 expression</td>
<td>In vitro upregulation of Sox9 during gliogenesis in Xenopus</td>
<td>[99]</td>
</tr>
<tr>
<td></td>
<td>Required for Pdgf receptor α expression</td>
<td>Oligodendrocyte survival in developing spinal cord</td>
<td>[97]</td>
</tr>
<tr>
<td><strong>Bladder</strong></td>
<td>Pd27 upregulation</td>
<td>Decreased proliferation in melanoma xenografts in vivo</td>
<td>[65]</td>
</tr>
<tr>
<td><strong>Breast</strong></td>
<td>CpG island methylation of SOX9 promoter</td>
<td>Hypermethylation silences SOX9-associated epithelium/associated with tumor formation in absence of PTEN</td>
<td>[100]</td>
</tr>
<tr>
<td><strong>Digestive</strong></td>
<td>Sox9 expression</td>
<td>Inhibits proliferation of human breast cancer cells in vitro</td>
<td>[68]</td>
</tr>
<tr>
<td><strong>FGF</strong></td>
<td>Sox9 expression</td>
<td>Suppress Sox9 expression/reduces proliferation in vitro</td>
<td>[36]</td>
</tr>
<tr>
<td><strong>FGF</strong></td>
<td>Sox9 expression</td>
<td>Maintains intestinal crypt progenitor cell population by inhibiting expression of differentiation genes</td>
<td>[11]</td>
</tr>
</tbody>
</table>

**Table 2. Signaling pathways regulating SOX9 during chondrogenesis.**

<table>
<thead>
<tr>
<th>Key factors</th>
<th>Function</th>
<th>Relevance to SOX9</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nh</td>
<td>Stimulates chondrocyte proliferation and mediates chondrocyte differentiation</td>
<td>Sox9 mRNA is increased in mice overexpressing Shh specifically in chondrocytes and Sox9 is decreased in mouse models of tracheal development lacking Shh Binding sites of the downstream Hh modulators, GLI, are present in the upstream regions of the human SOX9 gene</td>
<td>[1,10,33]</td>
</tr>
<tr>
<td>PTH/PTHrP</td>
<td>Expressed in early proliferating chondrocytes and mediates chondrocyte differentiation</td>
<td>Stimulates the phosphorylation of SOX9 thereby activating it</td>
<td>[13,18]</td>
</tr>
<tr>
<td>Wnt</td>
<td>Combination of canonical and noncanonical Wnt signaling essential for chondrocyte survival, proliferation and bone formation</td>
<td>Dependent on Wnt type, for example Wnt5 can differentially signal through noncanonical pathways to increase Sox9 during early stages of chondrogenesis and inhibit it during chondrocyte maturation Sox9 physically interacts with β-catenin to inhibit its transcriptional activity and targets it for proteosomal degradation</td>
<td>[13,90,91]</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Mediates chondrogenic commitment</td>
<td>Increases expression of Sox9 and Smad3, as a mediator of TGF-β signaling, activates Sox9 in vitro</td>
<td>[13,92]</td>
</tr>
<tr>
<td>Notch</td>
<td>Necessary for proper cartilage progenitor proliferation and chondrocyte differentiation</td>
<td>The downstream Notch transcriptional mediators, Hes-1 and Hey-1, compete for the Sox9 binding of the Col2a1 enhancer to prevent Sox9-mediated activation</td>
<td>[84,101]</td>
</tr>
<tr>
<td>FGF</td>
<td>Required throughout skeletogenesis</td>
<td>Uregulates the expression of Sox9 in vitro</td>
<td>[95]</td>
</tr>
</tbody>
</table>
activates the promoter of Prostaglandin D synthase (Ptgds) [22], encoding an enzyme required for the synthesis of prostaglandin D2 (Pgd2), which is an important paracrine mediator of mouse Sertoli cell differentiation. Interestingly, PGD2 increases nuclear import of cytoplasmic SOX9 in human NT2/D1 cells, presumably facilitating SOX9 action as a transcription factor. This provides another mechanism whereby, once expressed, SOX9 can maintain its own production – a mechanism probably important during development and potentially harmful in disease settings.

Epithelial-to-mesenchyme transition (EMT) during NC cell migration

Sox9 plays a significant role in development of the vertebrate nervous system [23–25]. It is required for both the formation and maintenance of neural stem cells during mouse embryogenesis [25] and specification of the glial lineage in the central nervous system [24]. During early embryogenesis, multipotent NC cells become migratory and, in a process involving EMT (Figure 2, Box 2), contribute to diverse tissues including cartilage, adrenal medulla, skin pigment cells and cardiac tissue [23]. From studying NC cells, Sox9 seems to be important for EMT. SOX9 is expressed in premigratory and migratory NC cells in humans (Figure 1f), mice and chicks [26]; loss of function experiments in Xenopus imply Sox9 is required for NC formation [23]. In cell lines and primary chick neural plate culture models, Sox9 binds to the promoter of the gene encoding EMT-related transcription factor Snail2 to enhance its expression [27]. Moreover, in chick neural tube cotransfection of Sox9 and Snail2 was sufficient to induce ectopic EMT [28]. Both factors seem dependent on the chromdomain helicase DNA-binding domain protein Chd7, a protein underlying CHARGE syndrome that is characterized by malformations in craniofacial structures as well as the peripheral nervous system (PNS), eyes, ears and heart [29]. Using knockdown in Xenopus, loss of Chd7 greatly diminished Sox9 and Snail2 expression and perturbed cell migration [29]. In addition, using an in vitro model of NC migration and differentiation from human embryonic stem cells, Chd7 was shown to regulate Sox9 via a previously identified conserved NC upstream enhancer region [1,29]. Pertinent to this review, EMT is important at multiple steps during development (Box 2), but has also been implicated in fibrosis and cancer [30], permitting speculation that SOX9’s role in EMT of NC cells will also be relevant to disease.

Progenitor cells in the pancreas and intestine

The major cell types of the adult pancreas are endocrine, exocrine and duct cells. The role of SOX9 in the progenitor cell population underlying these mature cell types was first intimated in human fetal and campomelic specimens [31]. SOX9 is expressed in all early human fetal pancreatic epithelial cells (Figure 1e) with subsequent downregulation at endocrine commitment. Analyses of autopsy pancreas from patients with CD illustrated hypoplastic pancreata, consistent with its role in progenitor cell proliferation [31]. Subsequent specific inactivation of Sox9 in the mouse pancreas and duodenum confirmed a requirement for the transcription factor in proliferation; the pancreata of Sox9 null mice were similarly hypoplastic [32]. SOX9 is also important for differentiation along the endocrine lineage. In cancer cell lines, Sox9 binds to and transactivates the Neurogenin 3 (NGN3) gene [33], which encodes a transcription factor necessary for endocrine commitment. In patients with CD and in mice heterozygous for Sox9, islets are small and/or lack maturity markers, which is indicative of glucose sensing [31,32].

During intestinal development, SOX9 is expressed in the stomach epithelium (Figure 1e) and in the progenitor cells towards the base of the intestinal crypts [4]. Sox9 is required for Paneth cell differentiation [34]. In adult small intestine, complete epithelial renewal occurs every 3 to 14 days driven by stem cells and transit amplifying cell populations located in the lower regions of the adult crypts. By lineage tracing experiments in mice, it seems that low levels of Sox9 maintain the transit-amplifying cells by suppressing expression of the prodifferentiation gene Caudal-type homeobox 2 (Cdx2) and Mucin 2 (Muc2), whereas higher levels promote differentiation of the enteroendocrine lineage [35]. This repression of Cdx2 and MUC2 transcription, also apparent in human cancer cell lines producing SOX9 and in a xenograft tumor model aberrantly expressing SOX9, has led to speculation that the transcription factor is causal in intestinal tumorigenesis [36].

Other tissues

Additional roles for Sox9 have been uncovered from lineage tracing and tissue-specific gene inactivation studies in mice. In the heart, Sox9 is required for the normal development of cardiac valves [37], playing a role in the early

**Box 2. Epithelial-to-mesenchyme transition**

EMT is the process whereby cell morphology and behavior changes from that of an epithelial cell to a mesenchymal one (Figure 2). EMT occurs repeatedly during normal development and is increasingly recognized as a disease mechanism relevant to both fibrosis [82] and metastasis of epithelial tumors [83]. It is characterized by the loss of the cell surface protein E-cadherin, the upregulation of E-cadherin repressor molecules such as Snail and Slug proteins, the expression of VE-cadherin, the acquisition of a motile phenotype and significant remodeling of the surrounding ECM via the expression of extracellular proteases and direct regulation of ECM proteins [82,83].

SOX9 regulates EMT during development. In NC cells, Sox9, under the influence of fibroblast growth factor (FGF) signaling [84] and bone morphogenetic protein-2, activates the transcription of Slug to elicit EMT [27,85]. Sox9 protein binds to the zinc finger protein Snail2 (encoded by the Slug gene) to activate further Slug transcription [27]. This process also requires the Rho GTPase Rhoc [86]. EMT occurs during development of other cell types and tissues involving Sox9, such as astrocytes, cardiac valves, pancreatic progenitors and cartilage [83]. Interestingly, SOX9 is highly expressed in the cardiac cushions that undergo EMT to form valve structures and all pancreatic progenitor cells prior to differentiation (Figures 1d and e). Similar roles for SOX9 are probable in disease settings. EMT is one of the major processes to generate myofibroblasts that promote fibrosis affecting the lungs, heart and kidneys. It is driven by a range of pathways, known to influence or be influenced by SOX9, such as PDGF, FGF and especially TGF-β1 signaling [4,87].

In prostate cancer and colon cancer, PTEN loss associated with increased SOX9 expression enhances EMT and this could initiate metastasis [88]. In breast cancer cells, SOX9 mediates the effect of RA on VE-cadherin expression [89], again implicating a role for SOX9 in EMT because a switch from E-cadherin to VE-cadherin expression is a classic step during EMT.
Loss of epithelial markers: e.g. E-Cadherin, Cytokeratin, Laminin-1

Gain of mesenchymal markers: e.g. N- & VE-Cadherin, Vimentin, Snail, α-sma

(a) Epithelial cells → Cells undergoing EMT → Mesenchymal cells

(b) Development

Human embryo (~4-5 weeks)

Ventral

Dorsal (D)

Migratory Neural crest cells

(c) Cancer

Blood vessel

Blood cells

Epithelial cancer cells

Invasive cancer cells

(d) Fibrosis

Increased ECM deposition

'Activated' fibroblasts

Human embryo 32 dpc

NT

Migratory Neural crest cells

Human embryo 32 dpc

Sox9/α-sma

Activated hepatic stellate cell

Figure 2. (a) During EMT, epithelial cells progressively lose their epithelial characteristics, including specific markers and cell-cell junctions to acquire a mesenchymal phenotype with increased cell mobility and altered ECM secretion. (b) An example of EMT during development: delamination and migration of NC cells. The plane of sectioning (upper panel) is shown through a human embryo at 4–5 weeks of development. Within this plane, migratory NC cells are shown schematically (middle panel) and stained for SOX9 immunoreactivity (brown, lower panel). Dpc = days post-conception. SOX9 is also apparent in the neural tube (NT) and condensing vertebra (not marked). (c) EMT contributes to cancer metastasis. Epithelial cancer cells invade and metastasize following EMT, entering the circulation (lower image) or lymphatic system. (d) In fibrosis, EMT is thought to be one of the processes that generate activated migratory fibroblasts responsible for ECM deposition and tissue destruction. The lower image highlights immunofluorescent staining of nuclear Sox9 (red) surrounded by α-Sma (green) in activated primary HSCs.
expansion of precursor cells and in organizing the assembly of valvular ECM proteins [38]. SOX9 specifies hair follicle stem cells and contributes to the differentiation of all skin epithelial cell lineages [39,40]. Developmental roles for SOX9 have also been described in the otic placode [41], tooth [42], kidney [31], biliary duct [43], prostate [44] and pituitary gland [45], where, similar to many other tissues, SOX9 is expressed in early cell types prior to terminal differentiation.

**SOX9 in acquired disease**
SOX9 seems to be required at the appropriate time and place, and in the right amount (Box 1) during development; therefore, it seems logical that inappropriate or ectopic expression of SOX9 could result in disease. Recent findings in several fibrosis-related disorders and carcinomas support this hypothesis (Table 1).

**Fibrosis, sclerosis and related disorders**
Fibrosis, sclerosis and related disorders can affect virtually all tissues and organs in the body and, as a result, are a prominent feature of several chronic diseases. They are characterized by excessive, inappropriate ECM deposition, resulting in the destruction of tissue architecture and function. Recent and emerging data suggest SOX9 plays a pivotal role in this ECM deposition [3,4,6,7,46]. In certain fibrotic or related disorders, the data are associative or are derived from in silico prediction and pathway analysis: for instance, increased expression of SOX9 and potential SOX9 target genes were detected by expression microarray in skin keloids, a disorder characterized by excessive scarring of the skin [6]. In other tissues, data have begun to demonstrate mechanistic roles for SOX9.

In liver fibrosis, the primary cell type responsible for ECM deposition is the hepatic stellate cell (HSC) [47]. In response to liver injury, transforming growth factor-β1 (TGF-β1), known to regulate SOX9 in cartilage and biliary duct [43], and platelet-derived growth factor (PDGF) activate HSCs into a proliferative myofibroblast phenotype [47]. The activated HSCs migrate into the liver parenchyma and secrete the damaging ECM that defines fibrosis. In rat HSCs activated in culture, Sox9, under the influence of TGF-β1, becomes ectopically expressed (Figure 2d) and is significantly responsible for Col1 production; short interfering RNA (siRNA) diminution of Sox9 expression by ~60% reduces Col1 production by a similar degree [4]. In fibrotic rat livers, Sox9 was coexpressed with Col1 [4]. Interestingly, a recent study reporting that Sox9-expressing cells in the mouse biliary duct are the source for hepatocyte regeneration after injury by green fluorescent protein (GFP) lineage tracing experiments has questioned the presence of Sox9 in HSCs [48]. However, the transcription factor would not be expected in quiescent HSCs in uninjured livers [4], and it is difficult to exclude colocalization in cells expressing GFP with the activated HSC marker, namely α-smooth muscle actin (α-Sma), in injured livers [48]. Furthermore, the injury models used (for instance, a single injection of carbon tetrachloride) did not induce significant fibrosis with follow-up analysis at less than two weeks. Although these studies give important insights into certain circumstances of hepatocyte regeneration, it remains important to study the role of Sox9 in fibrosis by testing its inactivation in different liver cell populations.

In the kidney, SOX9 has also been implicated in glomerulosclerosis where, similar to the liver, TGF-β1 is greatly increased following injury, resulting in compromised podocyte function and reduced glomerular filtration rate [5]. In microarray experiments from human sclerotic glomeruli captured by laser microdissection, Sox9 was greatly increased, as was COL1A1 and COL1A2 [5]. Increased Sox9 and Col1a2 mRNA have also been detected in models of nephritis in mice [7]. In mesangial cells, Sox9 functions downstream of TGF-β1 to activate Col1a2 transcription via an enhancer element upstream of exon 1 [7]. Collectively, these data provide a potential Sox9-dependent mechanism for the collagen-rich thickening of the glomerular basement membrane that defines glomerulosclerosis. More recently, Sox9 has been linked to the urinary obstrusive disorder hydronephrosis, a cause of renal injury and scarring that can ultimately require kidney transplantation. Inactivation of Sox9 in the uterine mesenchyme of mice compromised ECM deposition and smooth muscle cell differentiation, leading to hydroureter and associated hydronephrosis [49]. Defective ECM has also been linked to hydroureter in humans [49].

Vascular calcification is a further example of abnormal ECM deposition, in this instance mimicking endochondral ossification. Sox9 is expressed in calcified aortas along with its target gene COL2A1 [8], and the transcription factor has been implicated in valvular calcification in mice [3,46]. Consistent with this, TGF-β1, which would presumably induce Sox9, enhances cartilaginous metaplasia in the arterial endothelium in rats [50]. Collectively, these studies begin to place Sox9 as a key mediator of TGF-β1 in fibrosis and related disorders characterized by excessive ECM disposition. At present, much of the data arise from observational studies of pathological tissue or from the use of cell lines. To extend this understanding it will be important to study Sox9 directly in vivo, for instance examining whether cell-specific inactivation of Sox9 in the liver or in mesangial cells protects against hepatic fibrosis or glomerulosclerosis.

**Cell proliferation and cancer**
SOX9 has been implicated in the formation and growth of tumors in the bladder [51], ovary [52,53], prostate [54,55], intestine [56], brain and PNS [57]. It seems logical that SOX9’s role in controlling progenitor cell proliferation during development, when dysregulated, could promote tumor formation and growth.

In humans, SOX9 is predominantly expressed in the basal epithelial cells of the adult prostate [58]. However, in prostate cancer SOX9 is induced in the luminal cells with some evidence that SOX9 levels are increased in more advanced lesions [54,55]. The transcription factor is also expressed in a range of prostate cancer cell lines [55,59], whereas in the hair follicle and intestinal crypt its expression is increased by wingless-type MMTV integration factor (WNT) signaling [55] (Table 1). Lowering endogenous SOX9 levels in the human prostate cancer cell line CWR22Rv1 inhibited proliferation [55] and decreased tumor growth following their transplantation into immuno-
compromised mice [12]. Consistent with the interpretation that SOX9 advances prostate cancer growth, SOX9 is capable of binding to and transactivating the androgen receptor potentially relevant to the androgen dependence seen in certain types of prostate cancer [12,55]. In addition, xenographs of LnCAP cells overexpressing SOX9 demonstrated markedly increased tumor growth [12]. However, not all data are concordant with this paradigm; in M12 prostate cancer cells, stably transfected SOX9 expressed at high levels suppressed growth and tumorigenicity [59]. It is not possible to reconcile these differences at present beyond the obvious factor that individual cancer cell lines differ markedly even when modeling diseases from the same organ. It is perhaps noteworthy that SOX9-transfected M12 cells not only stopped proliferating but also displayed markedly altered cell morphology, perhaps indicative of more fundamental phenotypic changes [59].

SOX9 is also implicated in tumor growth in the brain [60] and nervous system [57,61]. In glioma cell lines, SOX9 knockdown by siRNA reduced cell proliferation [61]. In vivo, SOX9 production is increased in malignant nerve sheath tumors [57]. As occurred in glioma cells, repressing this expression in culture (by small hairpin RNA) caused cell death, implying a causal relationship between the presence of SOX9 and tumor cell survival and growth.

In colon cancer patients, elevated SOX9 expression has been associated with lower survival rates [56]. The transcription factor potentially acts downstream of insulin growth factor and insulin signaling [62]. Insulin receptor substrate 1 (Irs1) null mice had fewer Sox9-expressing crypt cells and developed fewer, smaller tumors when crossed with a mouse model of adenomatous polyposis coli, an autosomal dominant tumor predisposition syndrome of the colon [62].

In the skin, Sox9 is expressed in basal cell carcinomas [63]. By extrapolation from hair follicle development, it is predicted to lie downstream of Sonic hedgehog (Shh) and the transcription factor Gli2, both of which cause skin tumors [64]. Data in melanomas are inconsistent. An initial report demonstrated that SOX9 was absent from nearly all examined melanomas, but vector-derived SOX9 either in melanoma cell lines or in xenographs decreased cell proliferation and tumor growth by directly upregulating the cell cycle arrest gene p21 [65]. These data imply that SOX9 restricts cancer growth in melanomas. However, these data warrant caution as others have detected nuclear SOX9 in over 80% of melanomas [66]. Furthermore, in lung adenocarcinoma, which also highly produce SOX9, the transcription factor inhibited p21 expression and enhanced tumor cell proliferation [67].

Taken together, these data present opposing roles for SOX9 in tumors, either inducing or potentially inhibiting cell proliferation. To reconcile these differences, it is important to consider cellular context. For instance, SOX9 is expressed in breast cancer tissue and in several breast cancer cell lines such as T-47D and MCF7 [68]. In the presence of components of the retinoic acid (RA) signaling pathway, SOX9 mediates the inhibitory effect of RA by upregulating the expression of Hairy/Enhancer of Split-1 (Hes-1) [68,69]. More systematic analysis of the potential interplay between RA signaling and SOX9 might be warranted in a range of tumor types and cell lines. Similarly, models in which levels of SOX9 can be altered might help reconcile whether and when SOX9 is tumor promoting or repressing.

Concluding remarks

SOX9 potentially carries prognostic value in a range of tumors including neurofibromatoma [57], medulloblastoma [10], colon cancer [56] and prostate cancer [9]. Although the identification, validation and application of biomarkers is complex [70], immunostaining for SOX9 in biopsy samples and pathology resections could aid diagnosis and prognostication for patients. For example, SOX9 staining could be incorporated into liver biopsy analysis alongside that of its targets COL1 and COL3, which are currently part of some staging strategies for liver fibrosis [71]. The relevance of SOX9 needs to be addressed clinically by correlating and scoring its presence over serial organ biopsies to outcomes from fibrosis and related disorders.

In fibrosis, unless the precipitating stimulus (frequently inflammation) can be removed, deterioration is common until organ transplantation is the only option. Although potentially reversible during early stages of the disease, effective antifibrotic therapies have been elusive. Similarly, despite increasing knowledge of the mechanisms underlying cancer, intervention with novel therapies for many tumor types has been disappointing. As a transcriptional regulator of genes involved in cell proliferation, EMT and fibrotic ECM deposition, SOX9 could be a potential target for therapeutic intervention in fibrosis and several cancers. However, modulating transcription factor levels is difficult and ensuring cell-specific effects to minimize potential side effects is challenging. For instance, targeting SOX9 in the liver as a potential antifibrotic measure would need to avoid disrupting normal SOX9 expression in biliary epithelial cells.

Several studies have established the use of RNAi to treat disease, and recent advances are exciting in terms of specificity, toxicology and clinically relevant dosage [72]. Targeted delivery of siRNA using lipid-like materials coated with antibodies has successfully been used to deliver siRNA against the human epidermal growth factor receptor-2 in pancreatic and breast cancer to resensitize tumor cells to chemotherapeutic drugs [72]. Similar methods have been used to target cyclin D1 to reduce leukocyte proliferation in a mouse model of gut inflammation [72] and others are in clinical trials (e.g. Phase I trials of siRNA treatment against p53 in acute renal failure) [72]. Perhaps siRNA is one avenue that could be explored as a mechanism of targeting SOX9.

Other approaches that could reduce SOX9 production and/or action include structure-based modulation whereby small peptides or molecules modify function or expression, or the use of neutralizing antibodies [73]. The manipulation of SOX9 levels might also be possible indirectly via manipulation of its upstream regulatory pathways such as TGF-β1, WNT and HH signaling, all three of which have been linked to cancer and fibrosis.

In summary, whereas much has been learned, gaps remain, especially in how signaling pathways regulate SOX9 and in identifying the complete repertoire of target
genes downstream of the transcription factor. Applying technology such as ChIP-seq combined with next generation sequencing (ChIP-seq) with direct studies of Sox9 in vivo in mouse models of disease are required.

Acknowledgements
The authors receive support from the Manchester NHIR Biomedical Research Centre. NH is a Wellcome Trust Senior Fellow in Clinical Science and receives funding from BBSRC, EPSRC and SC4SM. KPH receives funding from BBSRC. NR holds a studentship awarded to KPH by the Gerald Kerkut Trust. Robin Deller is acknowledged for his graphics.

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Osteopontin Is a Novel Downstream Target of SOX9 With Diagnostic Implications for Progression of Liver Fibrosis in Humans

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Osteopontin (OPN) is an important component of the extracellular matrix (ECM), which promotes liver fibrosis and has been described as a biomarker for its severity. Previously, we have demonstrated that Sex-determining region Y-box 9 (SOX9) is ectopically expressed during activation of hepatic stellate cells (HSC) when it is responsible for the production of type 1 collagen, which causes scar formation in liver fibrosis. Here, we demonstrate that SOX9 regulates OPN. During normal development and in the mature liver, SOX9 and OPN are coexpressed in the biliary duct. In rodent and human models of fibrosis, both proteins were increased and colocalized to fibrotic regions in vivo and in culture-activated HSCs. SOX9 bound a conserved upstream region of the OPN gene, and abrogation of Sox9 in HSCs significantly decreased OPN production. Hedgehog (Hh) signaling has previously been shown to regulate OPN expression directly by glioblastoma (GLI) 1. Our data indicate that in models of liver fibrosis, Hh signaling more likely acts through SOX9 to modulate OPN. In contrast to GLI2 and GLI3, GLI1 is sparse in HSCs and is not increased upon activation. Furthermore, reduction of GLI2, but not GLI3, decreased the expression of both SOX9 and OPN, whereas overexpressing SOX9 or constitutively active GLI2 could rescue the antagonistic effects of cyclopamine on OPN expression. Conclusion: These data reinforce SOX9, downstream of Hh signaling, as a core factor mediating the expression of ECM components involved in liver fibrosis. Understanding the role and regulation of SOX9 during liver fibrosis will provide insight into its potential modulation as an antifibrotic therapy or as a means of identifying potential ECM targets, similar to OPN, as biomarkers of fibrosis. (Hepatology 2012;56:1108-1116)

Fibrosis of the liver is characterized by excessive extracellular matrix (ECM) deposition. One of the major cell types responsible for this is the hepatic stellate cell (HSC).1,2 In response to injury, HSCs become activated into proliferative myofibroblasts, migrate into the surrounding parenchymal cells, and secrete tissue-damaging ECM, the major component of which is type 1 collagen (COL1). In addition, the ECM contains a complex mix of proteins that promote cell proliferation, migration, and differentiation. One ECM component with such roles is the matricellular glycoporphosphoprotein, osteopontin (OPN), also known as secreted phosphoprotein 1.
OPN is detected in a wide range of tissues and body fluids, with physiological roles during development (e.g., in bone, bile duct formation, and during vascular remodeling), immune system regulation, and wound repair. However, it is also associated with pathological processes relating to cancer and inflammation.\textsuperscript{3,4} The ability of OPN to mediate such diverse cellular functions is likely related to its extensive post-translational modifications and ability to signal through several integrin and CD44 variant receptors.\textsuperscript{3,5}

OPN contributes to wound scarring in skin\textsuperscript{6} and has been implicated in lung, kidney, and heart fibrosis.\textsuperscript{7-9} It has previously been detected in activated HSCs, where it is thought to mediate cell migration.\textsuperscript{10} More recently, OPN levels have been highlighted as a potential biomarker of liver disease, levels correlating with the severity of disease,\textsuperscript{11-15} and has been reported to promote the progression of fibrosis in nonalcoholic steatohepatitis.\textsuperscript{14} The latter study, and others,\textsuperscript{15} has demonstrated regulation of OPN expression by Hedgehog (Hh) signaling, mediated by the member of the glioblastoma (GLI) family of transcription factors, GLI1, binding to an upstream element of the OPN promoter.\textsuperscript{15} There are three GLI transcription factors, with different activator and repressor forms of GLI2 and GLI3 generated by alternative splicing of the parent transcripts.\textsuperscript{15-16}

Previously, we have shown that the transcription factor, sex-determining region Y-box 9 (SOX9), becomes ectopically expressed in activated HSCs, where it is responsible for COL1 production.\textsuperscript{17} During development, SOX9 has diverse roles regulating the expression of a number of genes encoding ECM proteins.\textsuperscript{18} SOX9 has also been associated with fibrotic pathologies affecting the skin, kidney, and heart.\textsuperscript{18-23}

In this present study, we show that OPN and SOX9 colocalize to biliary cells in the healthy liver and to the same regions of fibrotic tissue. Both are markedly increased during HSC activation, when it appears unlikely that GLI1 regulates OPN. Instead, we demonstrate that SOX9 lies downstream of GLI2 and is responsible for OPN expression. These data support a role for SOX9 during the progression of liver fibrosis as a regulator of key fibrotic ECM components, and suggest that the manipulation of SOX9 or its downstream targets may be a means of developing antifibrotic therapies. Furthermore, the identification of other ECM targets of SOX9 may have additional utility as biomarkers of fibrotic severity in liver disease similar to recent studies on OPN.\textsuperscript{11,12}

**Materials and Methods**

**Human Tissue and Serum Collection.** Human fetal material was collected under guidelines issued by the Polkinghorne Committee, as described previously.\textsuperscript{17,24} Ethical approval was granted by the North West Regional Ethics Committee. Freshly isolated adult liver was purchased after resection (Invitrogen Ltd., Warrington, UK) and processed as previously described.\textsuperscript{17,24}

**Animal Models of Liver Fibrosis.** Liver fibrosis was induced by 5-week treatment of adult male Sprague-Dawley rats with CCl\textsubscript{4} or in C57Bl/6 mice fed a methionine- and choline-deficient diet for 7 weeks.

**Immortalized and Primary Cell Culture.** Primary rat hepatic stellate cells (rHSCs) were isolated as described previously.\textsuperscript{17,25} Human LX2 cells were a gift from Prof. Scott Friedman (Mount Sinai School of Medicine, New York, NY).\textsuperscript{26} Primary human HSCs (hHSCs) were isolated after liver resection (see Supporting Materials and Methods) under ethical approval from the Nottingham Research Ethics Committee, activated in culture, and fixed for immunocytochemistry (ICC).\textsuperscript{17} All cells were cultured in monolayer at 5% CO\textsubscript{2} and 37°C in Dulbecco’s modified Eagle’s medium plus L-glutamine, Na-pyruvate, and antibiotics supplemented with fetal bovine serum: 1% (low serum) or 10% (high serum) for LX2 cells, as indicated, or 16% for rHSCs and 10% for hHSCs.\textsuperscript{17} Gene silencing was carried out transiently using short interfering RNA (siRNA) (see Supporting Table 1) with HiPerFect (LX2 cells) or Nucleofection for HSCs (Amara Biosystems GmbH, Cologne, Germany), as described previously.\textsuperscript{17} To interrogate Hh signaling, additional supporting information may be found in the online version of this article.

K.P.H. receives support from the Medical Research Council (MRC). K.P.H. and N.H. receive support from the Manchester National Institutes of Health Research Biomedical Research Center and Stem Cells for Safer Medicine. N.H. is a Wellcome Trust Senior Fellow in Clinical Science. A.D.S. receives support from a Royal Society-Wolfson research merit award. V.A. is an MRC Clinical Training Fellow, and E.H. holds a Biotechnology and Biological Sciences Research Council doctoral account Ph.D. studentship.

Additional Supporting Information may be found in the online version of this article.
LX2 cells and rHSCs were treated with 5 μM of 3-Keto-N-(aminoethyl-aminocaproyl-dihydrocinnamoyl)/cyclopamine (CYC) (Merck Chemicals Ltd., Nottingham, UK) or 100 and 50 nM of smoothened agonist (SAG; Merck Chemicals Ltd.) for LX2 cells and HSCs, respectively. SAG treatments were performed in serum-free conditions. Overexpression experiments were carried out in LX2 cells. Plasmid transient transfections were achieved using Transfast reagent (Promega, Madison, WI), as described previously,17 in the presence or absence of CYC (described above). Briefly, 0.5 μg of expression plasmids (see Supporting Table 2) containing SOX9 or myc-tagged constitutively active GLI2 (GLI2D)27 or active GLI3 (GLI3A-myc)28,29 were transiently transfected into LX2 cells. After 24 hours, cells were then treated with CYC or vehicle control for an additional 24 hours and assayed for protein expression. All experiments were carried out with the appropriate empty vector (EV) control.

Gene Expression, Protein Analysis, and Chromatin Immunoprecipitation Assays. Antibodies used are listed in Supporting Table 3. Tissue preparation, immunohistochemistry (IHC), ICC, immunoblotting, and quantification were performed as described previously.17 For quantitative reverse-transcription polymerase chain reaction (qPCR), RNA was isolated from cells using the RNeasy kit (Qiagen Ltd., West Sussex, UK). Complementary DNA (cDNA) was synthesized from 1 μg of RNA with a RNA-to-cDNA kit (Applied Biosystems Ltd., Cheshire, UK). qPCR reactions were carried out on a StepOnePlus Real-Time PCR system (Applied Biosystems Ltd) using 1 μL of cDNA, intron-spanning primers, wherever possible (Supporting Table 4), and SYBR green (PrimerDesign Ltd., Southampton, UK). GusB was used as a housekeeper control for gene expression, as described previously.30 Changes in messenger RNA (mRNA) expression were calculated using ΔΔCt methodology. Chromatin immunoprecipitation (ChIP) assays were performed as described previously.31,32 Further details are described in the Supporting Materials and Methods.

Statistical Analysis. Statistical significance was determined by the two-tailed Student t test. All experiments were carried out three times or more (n = 3). For rHSCs, experimental data arise from three different preparations of stellate cells from different animals. Error bars in graphs show the standard error for each experiment.

Results

Expression of SOX9 and OPN in Biliary Duct and Liver Fibrosis in Humans and Rodents. SOX9 was detected in the round nuclei of biliary epithelial cells in fetal and adult livers, where it demonstrated cellular colocalization with OPN (Fig. 1 and Supporting Fig. 1). Previous data have independently identified OPN11,12,14 and SOX917 in areas of liver fibrosis in animal models. Here, in rat and mouse models of liver fibrosis, nuclear Sox9 localized to desmin-positive cells, confirming its presence in HSCs (Fig. 2A). Opn localized with Sox9 to spindle-shaped HSCs with elongated nuclei in areas of fibrosis as well as to biliary cells (Fig. 2B). In vitro, Opn was barely detected in quiescent rHSCs that lacked Sox9 (Fig. 3A,B and Supporting Fig. 2A,B). However, as reported by others,10,14 Opn expression was induced ~60-fold and its protein increased as rHSCs became activated on tissue culture plastic over 2 weeks, paralleling the induction of Sox9 and the sequential increase in Col1 (Fig. 3A,B). Similar results were gained using the human cell line, LX2, an in vitro model of stellate cells.26 In high-serum conditions, which mimic stellate cell activation, OPN was increased along with SOX9 (Fig. 3C-E). Final confirmation of OPN cellular colocalization with SOX9 in both activated rHSCs and hHSCs was demonstrated in vitro using immunofluorescence (IF). Nuclear SOX9 is shown surrounded by OPN or α-smooth muscle actin (α-SMA) (Fig. 4). These data led us to question whether SOX9 was capable of regulating OPN.

Sox9 Is Responsible for Opn Expression in Activated HSCs. To determine whether Sox9 regulates Opn expression, we abrogated Sox9 using siRNA in...
activated rHSCs. Reducing Sox9 by 70%-80% caused a commensurate 50%-70% decrease in Opn transcript and its encoded protein (Fig. 5A,B). Similar results were detected in the LX2 HSC line (Fig. 5C). In silico analysis of the OPN 5’ flanking region identified a conserved SOX9 binding motif ∼3 kilobase pairs...
upstream of the transcriptional start site (Fig. 5D). ChIP demonstrated that Sox9 was enriched at this site in both activated rHSCs and human LX2 cells (Fig. 5E; negative control data for GAPDH shown in Supporting Fig. 3). These data indicate that OPN is a novel downstream target of SOX9. Because others have implicated the Hh pathway in liver fibrosis and as a regulator of OPN expression, and because in

**Fig. 4.** SOX9 and OPN expression in activated hHSCs and rHSCs. IF showing nuclear SOX9 (red) and cytoplasmic α-SMA (green; left panel) or OPN (green; right panel) in rat and human activated HSCs (aHSCs). Size bar represents 20 μm.

**Fig. 5.** SOX9 regulation of OPN in HSCs. (A-C) siRNA abrogation of Sox9 in activated rHSCs (A and B) and LX2 cells (C) standardized against scrambled siRNA control for mRNA (A) and protein (B and C). Example immunoblotting is shown as inset in (B) and (C). *p < 0.05; **p < 0.01; †p < 0.005; ‡p < 0.001, compared to control. (D) Alignment of the upstream OPN enhancer region with conserved SOX9-binding motif highlighted in black (human sequence shown is −3,886 to −3,842 base pairs relative to transcriptional start site). Conserved nucleotides indicated by asterisk (*). The core SOX-binding motif is CAAT with increased binding affinity for SOX9 conferred by additional flanking nucleotides. (E) ChIP assay for SOX9-binding element in conserved upstream OPN enhancer element in LX2 cells cultured in high serum and activated rHSCs. Negative control is immunoglobulin G (IgG), and positive control is input (diluted 10-fold).
different circumstances SOX9 has been reported downstream of Hh signaling, we were curious to investigate whether SOX9 might be regulated by Hh in stellate cells as a means of determining OPN production.

**Hh Signaling Regulates SOX9 and Its Downstream Target, OPN.** Serum-activated LX2 cells and rHSCs activated in culture for 10 days were incubated with the Hh antagonist, CYC, or the Hh agonist, SAG (Fig. 6A-D and Supporting Fig. 4A,B). Both SOX9 and OPN proteins were significantly decreased by 45%-60% in response to CYC and increased 2- to 3-fold after SAG treatment in both stellate cell models. These data demonstrate that both OPN and SOX9 are positively regulated by Hh signaling in stellate cells. To intimate a role for SOX9 as the mediator of Hh’s effect on OPN production, we used siRNA in LX2 cells after SAG augmentation of Hh signaling (Fig. 6E and Supporting Fig. 4C). SAG induced increases in both SOX9 and OPN protein, compared to dimethyl sulfoxide (DMSO) control, which were unaffected by control siRNA. However, siRNA abrogation of SOX9 prevented the Hh agonist from increasing OPN levels above DMSO control values. To perform the converse experiment, transient transfection of an expression vector containing the human SOX9 coding sequence was carried out to overexpress SOX9 in LX2 cells (Supporting Fig. 4D). Overexpression of SOX9 rescued the inhibitory effect of CYC on OPN production (Fig. 6F). Collectively, these data implicate SOX9 as a positive regulator of OPN production downstream of Hh signaling in stellate cells.

**The Hh Mediator, GLI2, Regulates SOX9 Expression.** The GLI family of transcription factors is known to mediate the effects of Hh signaling. To determine which GLI factor might be responsible for Hh’s effect on SOX9 expression, we first investigated the expression of family members in quiescent and activated rHSCs. By qPCR, Gli1 was poorly detected in quiescent HSCs and unaltered upon activation (Fig. 7A).

In contrast, Gli2 and Gli3 mRNAs were increased...
~6- and ~50-fold, respectively, in activated cells. Although, by this analysis, GLI3 appears the more likely candidate for the regulation of SOX9 in stellate cells, detection of mRNA is not indicative of protein levels, especially given the potential for both repressor or activator forms of GLI2 and GLI3. Several commercial and published antibodies were available to us; however, we found them unhelpful in detecting or distinguishing the different forms by immunoblotting. Therefore, we investigated the potential contribution of GLI2 and GLI3 to SOX9 and OPN expression by using siRNA in LX2 cells (Fig. 7B, C). Diminution of GLI2 transcripts by ~67% significantly reduced SOX9 and OPN expression by ~43% and ~64%, respectively (Fig. 7B). In comparison, although achieving more robust reduction in GLI3 expression (~86%) with siRNA, SOX9 expression was less affected and OPN was unaltered (Fig. 7C). Moreover, overexpression of constitutively active GLI2 (GLI2D\textsuperscript{N}) was able to rescue, at least partially, the antagonistic effects of CYC on SOX9 and OPN production (Fig. 8A, B and Supporting Fig. 5A). In contrast, overexpressing the activator form of GLI3 (GLI3A) in the presence of CYC had little or no positive effect on SOX9 or OPN production (Fig. 8C, D and Supporting Fig. 5B). These data imply that GLI2 is the predominant regulator of SOX9 expression in HSCs. In keeping with these results, nuclear immunoreactivity against Gli2 was detected in activated rHSCs \textit{in vitro} and in regions of fibrosis after CCl\textsubscript{4} treatment \textit{in vivo} (Fig. 8E). Interestingly, Gli2 was also detected in the round nuclei of biliary epithelial cells similar to Sox9 and Opn (Fig. 8E, arrows). In contrast, despite detecting nuclear immunoreactivity for Gli3 in control brain tissue during human fetal development, such staining was not apparent in fibrotic rat tissue (data not shown).

Fig. 7. Gli2 mediates the expression of Sox9 and Opn in HSCs. (A) Expression of Gli factors in quiescent and activated rHSCs by qPCR. (B and C) siRNA for GLI2 (B, 67% knockdown) and GLI3 (C, 86% knockdown) or scrambled control in LX2 cells, followed by qPCR for GLI2 (B) or GLI3 (C), SOX9, and OPN. *P < 0.05; †P < 0.005; ‡P < 0.001, compared to scrambled siRNA treatment.

Fig. 8. Gli2 overexpression rescues antagonistic effects of CYC on the expression of Sox9 and Opn in HSCs. (A-D) Quantification of SOX9 and OPN protein after overexpression of constitutively active GLI2 (GLI2\textsuperscript{D\textsuperscript{N}}; A and B) or active GLI3 (GLI3A; C and D) in LX2 cells in the presence or absence of CYC. (E) IF showing nuclear Gli2 (red) and cytoplasmic α-Sma (green) in activated rHSCs (aHSCs) and bright-field IHC showing nuclear Gli2 (brown staining) in CCl\textsubscript{4}-treated fibrotic rat liver. Arrows indicate Gli2 expression in a bile duct. *P < 0.05; **P < 0.01; †P < 0.005; ‡P < 0.001, compared to EV transfection. Size bar represents 20 μm.
Discussion

OPN has been implicated as an important mediator, by which the inflammatory response ultimately leads to scarring and fibrosis in various organs, with the potential that its presence in the circulation can be used as a biomarker of disease progression.11-13 Previously, we demonstrated a novel role for the transcription factor, SOX9, in models of liver fibrosis. Under the influence of transforming growth factor-beta (TGF-β) signaling, SOX9 became expressed in activated HSCs, where it was responsible for the production of the profibrotic collagen, COL1.17 In this study, we have demonstrated a more diverse role for SOX9 by regulating OPN expression. In the liver, SOX9 and OPN colocalized in the regions of fibrosis. The onset of OPN production during HSC activation, its reduction in activated HSCs after Sox9 abrogation, and the binding of SOX9 to an upstream OPN enhancer element infers that the transcription factor is required for OPN expression during liver fibrosis.

SOX9 was responsive to Hh signaling in our models of liver fibrosis. Although Hh’s precise role in vivo during liver fibrosis remains incompletely understood,36,37 the signaling pathway is reactivated after injury in adult tissue,38 and HSCs can produce and respond to Hh ligands.39 Several lines of evidence place SOX9 downstream of Hh signaling. SOX9 is up-regulated by Hh ligands during chondrogenesis and, in neural stem cells, skin and intestine.18 The GLI family of zinc-finger transcription factors mediates Hh signaling in mammals.16 GLI1 is generally thought to be a transcriptional activator, whereas GLI2 and GLI3 have additional potential N-terminal repressor functions after proteolytic cleavage. There are several conserved GLI1-binding motifs important for SOX9 expression in its extended 5' flanking region (up to 1.1 Mb).39,40 Whereas GLI1 seems important for Sox9 expression during chondrogenesis, and for a SOX9-independent effect on OPN expression in malignant melanoma,15 the transcription factor was poorly detected in our models of liver fibrosis. In contrast, GLI2 increased Sox9 during mouse pancreatic β-cell dedifferentiation.42 Here, we demonstrate a role for GLI2 in regulating SOX9 and OPN in models of liver fibrosis. This is in line with the detection of hepatic GLI2 by others.43 However, compared to a direct effect of GLI2 on OPN, our collective data indicate that GLI2 functions significantly through SOX9 in its regulation of OPN production. This mechanism may also extend to the up-regulation of SOX9 by TGF-β, because GLI2 is induced by TGF-β in several cell types, including fibroblasts, keratinocytes, and cancer cells.43

In the healthy liver, both SOX9 and OPN localize to the bile ducts. SOX9 is required for normal biliary formation and function.44,45 From our data, it seems likely that SOX9 would also be responsible for OPN production by healthy cholangiocytes. Furthermore, based on SOX9’s additional roles in regulating both COL117 and collagen type 4 (COL4),28 there is the potential that SOX9 could be important in chronic cholestatic liver injury by regulating all these ECM components as part of the pathology of primary biliary cirrhosis (PBC) and primary sclerosing cholangitis. Interestingly, TGF-β and Hh signaling (including Gli2), both of which would up-regulate SOX9, have been implicated in the fibrotic response of PBC.46,47 where COL1, COL4, and OPN are all increased.48

In summary, these data expand the role for SOX9 in regulating components of the ECM and begin to provide insight into its regulation by signaling pathways linked to fibrosis and related pathologies in the liver and other sites, such as the skin, kidney, lung, and major blood vessels.18 Finally, given the potential use of serum OPN as a biomarker for the severity of liver damage in patients with HBV or HCV11,12 it is possible that additional downstream ECM targets of SOX9 action may be useful in helping to stage and predict the severity of liver fibrosis.

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