From the Department of Medicine at
Karolinska University Hospital, Solna
Karolinska Institutet, Stockholm, Sweden

ROLE OF MEMBRANE ASSOCIATED MOLECULES IN LIVER FIBROSIS

Peter Rosenberg

M.D.

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To my family

ABSTRACT

The aim of the study was to evaluate the role of membrane associated proteins in tissue formation and remodeling, in normal embryogenesis and in liver disease.

Molecules associated with cellular membranes provide a fundamental basis for cellular interactions during development, homeostasis and disease. Similar basic mechanisms play a crucial role in liver disease such as in tissue modeling during embryogenesis.

In the first study we investigated the expression of the cell adhesion molecule R-cadherin during formation of muscle and parenchymatous organs in the developing mouse embryo. R-cadherin was expressed during myogenesis in a pattern suggesting that it plays a role in myoblast cell-cell interactions. R-cadherin was also expressed by epithelia. In kidney, the expression pattern was associated with mesenchymal-epithelial interactions. R-cadherin exclusively rescued formation of epithelia and muscle in teratomas derived from E-cadherin null embryonic stem cells. From the results of this study we concluded that R-cadherin plays a role in formation of striated muscle and possibly also of epithelia.

In the second study, we evaluated the role of neural cell adhesion molecule (N-CAM) in biliary type fibrosis and liver fibrosis due to parenchymatous disease. N-CAM knock-out mice had attenuated liver fibrosis after bile duct ligation but not after carbon tetrachloride injections. Furthermore, hepatic stellate cells isolated from N-CAM knock-outs had impaired activation. These results suggest a role of N-CAM in biliary type liver fibrosis.

In the third study we studied the distribution of EBP50 in patients with biliary liver disease. EBP50 is a scaffolding protein involved in stabilization of transport proteins at the plasma membrane. EBP50 was normally expressed in hepatocytes and native bile ducts of patients with cystic fibrosis carrying the CFTR mutation ΔF508, primary biliary cirrhosis and primary sclerosing cholangitis. However, EBP50 was delocalized to the basolateral membranes, cytoplasm and nucleus in proliferating cells of the ductular reactions. This finding, together with suppressed proliferation of cultured cholangiocytes when EBP50 is down- regulated, suggests that it has a role the in proliferation of biliary epithelial cells in biliary liver disease.

Liver fibrosis due to long-term treatment with methotrexate is related to continuous low-grade hepatocellular damage. In the fourth study we evaluated the impact of the risk factors diabetes, over-weight, alcohol and viral hepatitis on development of liver fibrosis in methotrexate treated psoriasis patients. The results showed that patients with over-weight and/or diabetes had significantly higher risk of developing liver fibrosis and cirrhosis, at a lower accumulative dose methotrexate.

Key words: adhesion molecules, liver fibrosis, cholestasis, R-cadherin, N-CAM, EBP50, CFTR, cystic fibrosis, methotrexate, diabetes, over-weight, alcohol.

LIST OF PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the following text by their roman numerals:

I. **Rosenberg P**, Esni F, Sjödin A, Larue L, Carlsson L, Gullberg D, Takeichi M, Kemler R, Semb H.
A Potential Role of R-cadherin in Striated Muscle Formation.
Developmental Biology 187, 55-70, 1997.

II. **Rosenberg P**, Söderberg C, Sjöström M, Kinnman N, Stål P, Hultcrantz R
Attenuated liver fibrosis after bile duct ligation and defect hepatic stellate cell activation in N-CAM knockout mice.
In Manuscript

III. Fouassier L**, Rosenberg P**, Martine M, Saubaméa B, Kinnman N, Chignard N, Strandvik B, Barbu V, Hultcrantz R, Housset C
EBP50, a CFTR-binding protein, with cellular redistribution in biliary diseases, controls cholangiocyte proliferation
In Manuscript

IV. **Rosenberg P**, Urwitz H, Johannesson A, Ros AM, Lindholm J, Kinnman N, Hultcrantz R.
Psoriasis patients with diabetes type 2 are at high risk of developing liver fibrosis during methotrexate treatment.
J Hepatol. 2007 Jun;46(6):1111-8

CONTENTS

LIST OF ABBREVIATIONS

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BDL	Bile Duct Ligation
cAMP	3', 5'-cyclic monophosphate
CCl4	Carbon Tetra Chloride
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CNS	Central Nervous System
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dpc	days post coitum
EBP50	Ezrin Binding Protein 50
ECM	Extracellular matrix
ES cells	Embryonic Stem cells
ET-1	Endothelin-1
FAK	Focal Adhesion Kinase
FCS	Fetal Calf Serum
FGFR	Fibroblast Growth Factor Receptor
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDNF	Glial cell line-Derived Neurotrophic Factor
GT	Gamma-glutamyl transferase
HCC	Hepatocellular Cancer
HGF	Hepatocyte Growth factor
HSC	Hepatic Stellate Cell
IL-1	Interleukin 1
MCP-1	Monocyte Chemotactit Protein 1
MTX	Methotrexate
NAFLD	Non Alcohol Fatty Liver Disease
NASH	Non Alcohol Steatohepatitis
N-CAM	Neural cell Adhesion Molecule
NFκB	Nuclear Factor kappa B
PBC	Primary Biliary Cirrhosis
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PDZ	postsynaptic density 95/disc-large/zona occludens
PGK-1	Phosphoglycerate kinase 1
PKC	Protein Kinase C
PSC	Primary Sclerosing Cholangitis
RNA	Ribonucleic acid
RT-PCR	Real-Time Polymerase chain reaction
TBS	Tris Buffered Saline
TGF-β	Tumor Growth factor beta
TIMP	Tissue Inhibitor of Metalloproteinase
TNF-α	Tumor Necrosis Factor alpha
UDCA	Ursodeoxycholic Acid

1 INTRODUCTION

1.1 LIVER FIBROSIS

1.1.1 Epidemiology, Risk Factors and Clinical presentation

Liver cirrhosis, a condition by the general public mostly associated with excessive alcohol consumption, marks the end-point of a number of chronic liver diseases of diverse etiology[1, 2]. Morbidity and death due to complications of liver cirrhosis represents a major health issue globally [3]. The prevalence of liver cirrhosis follows the prevalence of the liver diseases causing it. As an example, there are in the world approximately 360 million individuals with chronic hepatitis B infection and 123 million with chronic hepatitis C infection and death related to these infections have been estimated to over 900 000 per year[4-6].
Common risk factors for liver fibrosis are alcohol over-consumption, diabetes, chronic viral hepatitis and obesity. Hereditary diseases such as hemochromatosis, α1-anti-trypsine deficiency and cystic fibrosis cause liver cirrhosis as well as autoimmune disorders such as primary biliary cirrhosis, primary sclerosing cholangitis and autoimmune hepatitis[2]. In many cases development of liver fibrosis proceed without any symptoms for the patient until it has progressed to severe fibrosis or cirrhosis and complications appear. Common clinical presentations that lead to the diagnosis of liver cirrhosis are ascites, palpable enlargement of the liver, esophageal varices and itching. Once that cirrhosis has occurred the prognosis is poor and those who have decompensated liver cirrhosis have a one-year survival rate of only 60 %, which is less than many malignancies. Liver cirrhosis also increases the risk of primary hepatocellular cancer (HCC) to 1.4-3.3% per year[7]. Other complications of liver cirrhosis are spontaneous bacterial peritonitis and hepato-renal syndrome, both conditions of which have a poor prognosis[8]. At present the only available curative treatment for liver cirrhosis with liver failure is transplantation, a treatment that can only be offered in a minority of cases. Understanding of the basic mechanisms of liver fibrosis will provide ground for the development of new treatments. It is also important to study the impacts of risk factors for development of cirrhosis to identify those patients at higher risk, thereby making an earlier diagnosis possible.

1.1.2 Histopathological features of liver cirrhosis

Liver cirrhosis (Figure 1), is the result of continuous or repeated liver injury that triggers a wound healing process, which transforms the normal liver parenchyma to a dysfunctional state[1]. Typically liver fibrosis starts with an expansion of the portal areas caused by increased deposition of extracellullar matrix (ECM). The fibrosis then expands to form bands of connective tissue that spans between adjacent portal areas. Finally, the histology and composition of ECM has been completely altered with massive depositions of extracellular matrix including fibrillar collagen and bands of fibrillar collagen surround regenerating hepatocytes and bile ducts [9, 10]. These regenerating nodules are the typical histological finding in liver cirrhosis. Ductular reaction with tortous cell aggregates positive for cholangiocyte markers is also a typical finding in advanced liver fibrosis and cirrhosis (Figure 1).

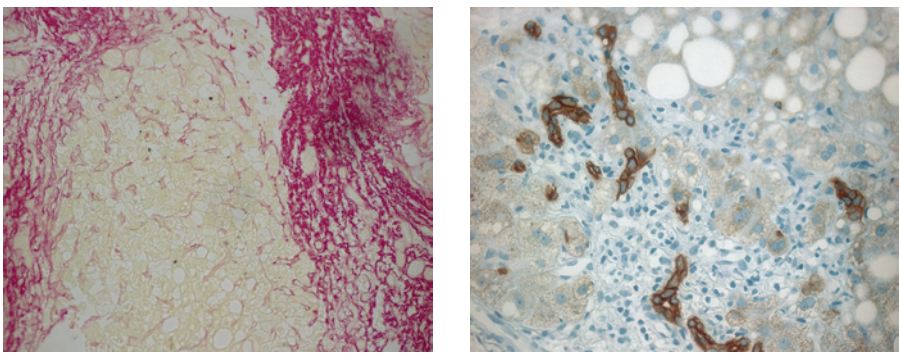
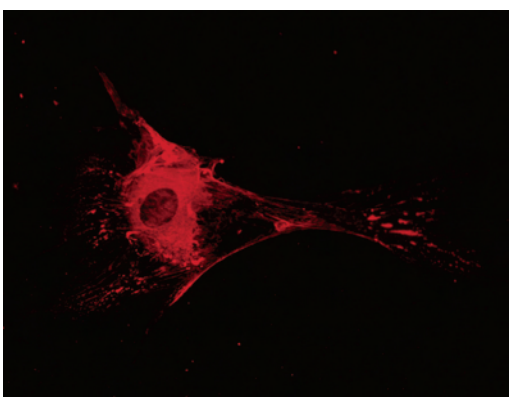


Figure 1: Left; *Hematoxylin-Eosin stained liver biopsy showing liver cirrhosis with nodule of regenerating hepatocytes (yellow) surrounded by fibrotic tissue (red).* **Right;** *Ductular reaction in liver biopsy from a patient with non-alcohol steatohepatitis and advanced fibrosis. Biliary cells are stained brown with monoclonal anti-CK19. Counterstaining with methylene-blue shows nuclei of myofibroblasts and inflammatory cells surrounding the ductular reactions. Original magnification 200x.*

Figure 2: *Hepatic stellate cell isolated from mouse liver and stained with a polyclonal rabbit anti-fibronectin antibody. Original magnification 630x.*



1.1.3 The Hepatic Stellate Cell

The hepatic stellate cell (HSC)(Figure 2), first discribed by von Kupfer 1876 as "Sternzellen" and in 1951 described by Ito as "Fettspeicherungzellen" (fat storing cells), are located in the perisinusoidal space between the endothel cells and the hepatocytes[11, 12]. In 1971, almost 100 years after the original discovery, Wake rediscovered the HSC and showed that the two celltypes described by von Kupfer and Ito were identical[13]. In normal liver, HSC store and metabolize retioids such as vitamin-A[14]. As HSC are in direct contact with the sinusoidal endothelial cells and also have contractile ability, it has been implicated that HSC can regulate sinusoidal blood flow[15]. Thus HSC are sometimes referred to as "hepatic pericytes".

Over the last decade HSC has been considered as the major contributing cell for liver fibrosis[16-18]. This has been based on the findings that HSC both *in vitro* and *in vitro* can transdifferentiate into myofibroblastic matrix producing cells. In response to liver injury, the HSC are activated and change their gene expression pattern and morphology. HSC activation is initiated by interactions with adjacent cell types and ECM[10, 19]. Cytokines excreted by adjacent cells, e.g TGFbeta, PDGF, endothelin-1 TNFalpha, take part in modulation of HSC behavior[20-25]. Also oxidative stress from hepatocytes and inflammatory cells and signaling by fibronectin from endothelial cells contribute to HSC activation[26, 27]. During the early stage of activation, HSC aquire a matrix degrading phenotype[28]. If the injury is stopped, activated HSC undergo apoptosis as regeneration of the injured liver tissue is completed[29]. If the injuring stimuli persist, HSC progress to a myofibroblast-like phenotype with a gene expression pattern that promotes deposition of fibrillar collagen[19, 28]. In this later stage of activation, in addition to activation by paracrine stimuli, HSC are also activated in an autocrine manner, a phenomena often denoted as perpetuation[30]. This involves HSC release of cytokines such as TGF-β1, which promote fibrogenesis and PDGF-BB that promote HSC proliferation. Activated HSC also produce fibronectin that contributes to the perpetuation of the activated state.
Activated HSC aquire increased ability to migrate towards areas of injury as has been observed in cholestatic liver injury[31]. Desmin and αSMA have been used widely as markers of myofibroblastic cells in the liver [32-34]

In recent years increasing evidence for contribution in liver fibrogenesis by several other cell types than HSC has emerged. Cholangiocytes and hepatocytes have been shown able to undergo epithelial mesenchymal transition and transdifferentiate into myofibroblastic cells[35-39]. Bone marrow stem cells have been shown to contribute to the fibroblastic cell population in injured liver and in biliary type fibrosis, portal fibroblasts are major contributors [33, 40-42]. Hence the paradigm of the HSC as the major contributor in liver fibrogenesis has been challenged, as it has become evident that the myofibroblastic cell population in the injured liver is heterogenous both in terms of origin and gene expression[43].

1.1.4 Biliary type fibrosis

Biliary type fibrosis develops in response to bile duct injury by cholestasis. Examples of conditions that may result in biliary fibrosis are obstruction of large bile ducts by atresia or stone impaction and diseases affecting the smaller bile ducts such as primary sclerosing cholangitis, cystic fibrosis and primary biliary cirrhosis. Characteristic histological findings in biliary liver fibrosis are proliferation of small bile ductules, ie ductular reaction, and surrounding the ductular reactions and bile ducts, fibrosis with an increased number of myofibroblastic cells[44].

Biliary epithelial cells contribute to biliary fibrosis by several mechanisms; both directly by synthesis of matrix components[45, 46] and indirectly by regulating matrix degradation, transdifferentiation into matrix producing myofibroblasts[38] and cross talk with other celltypes[47]. In recent years more focus has come upon cholangiocytes as orchestrators of the fibrogenic response. Reactive ductules and damaged cholangiocytes release several cytokines such as TGF-β1[48, 49], ET-1[50, 51], PDGF-BB[50, 52], MCP-1[53], Interleukin-6[54, 55] and TNF-α[55], which modulate both cholangiocytes and other cell-types in the liver[56].

1.1.5 Ductular reaction

Cholangiocyte proliferation has been considered to be a process aiming to compensate for the loss of bile ducts in the injured liver[57, 58]. In acute cholestasis with complete obstruction of the extrahepatic bile duct, such as after bile duct ligation in rat or stone impaction in human, cholangiocytes react with an intense proliferation thereby forming new branches of the biliary tree[59, 60]. The term ductular reaction was proposed by Popper to describe the expanded population of epithelial cells at the interface of the biliary tree and the hepatocytes[61]. The terms ductular reaction or ductular proliferation have also been used to denote the population of epithelial cells positive for cholangiocyte markers found in periportal areas and liver parenkyma in many liver diseases of different etiology. There has been some controversy regarding the nomenclature of the ductular reaction as it has been divided into “typical” ductular reactions referring to bile duct branching in response to acute cholestasis, and “atypical” ductular reactions referring to the bile duct like structures seen in chronic liver diseases without cholestasis, such as steatohepatitis and chronic viral hepatitis. However, the present consensus definition of ductular reaction is “a reaction of ductular phenotype, possibly but not necessarily of ductular origin, in acute and chronic liver disease”[62]. Ductular reactions are tortuous, often without a well-defined lumen and are typically surrounded by fibrosis and myofibroblasts.

Ductular reactions are heterogenous as they consist of stem cells and intermediate cells of both biliary and hepatocyte lineage[63].
The presence of liver stem cells in human liver disease was for many years subject to a long and intense debate[64]. A central issue in this discussion was whether the “oval cells” observed in rodent models of liver injury were true stem cells or if they were proliferating cholangiocytes. In fact they turned out to be both. As more data emerged, the “oval cells”, denoted “hepatic progenitor cells “ in humans, were shown to both take part in the processing and conduction of bile and serve as progenitors for

cholangiocytes and hepatocytes[65]. In light of this, their localization in the canal of Hering at the junction between hepatocyte columns and the finest branches of the biliary tree, seem self-evident[66].

Based on the finding that in chronic liver diseases without cholestasis, e.g. chronic hepatitis C infection[67] , methotrexate induced liver injury[68] and non-alcohol steatohepatitis[69], the extent of the ductular reaction correlates with disease and fibrosis progression, it has also been suggested that the DR conditions the course of these diseases. Modulation of the DR has therefore been proposed as a possible means of treatment for these diseases.

1.1.6 Extracellular matrix and Matrix metalloproteinases

Proteolytic matrix metalloproteinases (MMP) and their inhibitors TIMPs, precisely regulate the continuous turnover of ECM in the adult normal liver. In liver fibrogenesis this balance is disrupted and ECM proteins, predominantly fibrillar collagen accumulates.

Remodeling of the ECM is a crucial event in regeneration and repair of the liver. After liver injury, degradation of ECM by MMPs is rapidly started. This leads to release of several cytokines that are bound to the ECM. Among these are mitogenic factors such as hepatocyte growth factor (HGF) and epithelial growth factor (EGF) and TGFbeta that promote fibrosis. MMPs are a family consisting of at least 22 zink-dependent enzymes that are subdivided according to their main substrate although most MMPs have a broad proteolytic activity[70]. MMPs implicated in liver disease and liver tissue remodeling are MMP-1/MMP-13, MMP-2, MMP-3, MMP-9 and MMP-14(MT1-MMP)[71]. MMP-1 and MMP-13 are interstitial collagenases capable of degrading collagens type I-III and X The gelatinases MMP-2 and MMP-9 degrade collagen type IV which is a component of the basement membrane. They also cleave fibronectin, laminin and elastin and further degrade interstitial collagens that have been initially cleaved by a collagenase. MT1-MMP is a member of the membrane-type MMPs that apart from having a broad proteolytic activity is important in activating MMP-2 and MMP-13. The stromelysin MMP-3 has a broad spectrum of substrates including both collagens and non-collagenous matrix proteins. MMP-3 is also important in activating MMP-1[72]. The MMPs are syntetized as zymogens that are activated by proteolytic cleavage after being secreted and the regulation of their activation is complex as different MMPs may activate each other[28].

Expression of MMPs are induced by several cytokines such as TGF-β, IL-1, TNF-α and EGF[28, 71]. Also several studies have shown that integrin signaling affects MMP expression. Ligation of integrins with specific antibodies results in up-regulation of MMPs[73-75] whereas stimulated integrin signaling causes down regulation [76]. This illustrates well the interplay between the ECM and cells.

MMP-3 and MMP-13 are expressed in the early phase after toxic liver injury and are associated with degradation of the normal liver matrix. Hepatic stellate cells express both MMP-3 and MMP-13 in early activation[77, 78]. During later activation and in

cirrhosis, HSCs express MMP-2 that also promote their migration and proliferation[77, 79, 80]. The main collagenase in human MMP-1, and its mouse homologue MMP-13, has on the basis of experimental data been proposed to mediate resolution of fibrosis after cessation of the injuring stimulus[81, 82]. During recovery of CCl_4 induced liver fibrosis in rat, the collagenase activity increased five fold as levels of TIMP-1 and TIMP-2 decreased. Despite observed apoptosis of HSC, MMP-13 levels were unchanged. This prolonged expression of MMP-13 has been attributed to Kupfer cells[83] and/or mesenchymal cells at the interface between fibrotic septae and the hepatocyte parenkyma[78].

The activities of MMPs are balanced by their specific inhibitors, tissue inhibitors of metalloproteinases (TIMP). During persisting liver injury, large amounts of TIMPs, especially TIMP-1, are produced by myofibroblasts thereby contributing to accumulation of ECM proteins[10]. TIMP-1 is considered as the most important TIMP in liver fibrogenesis as it is strongly upregulated by inflammation and it's expression associated with TGFbeta[71, 84].

1.2 CELL ADHESION MOLECULES

Membrane integral cell-adhesion molecules (CAMs) provide basis for specific adhesion and interaction between individual cells and also between cells and the ECM. During development, injury and tissue repair, CAMs are expressed in a restricted temporo-spatial manner that promotes tissue formation and cellular function. CAMs participate in the formation of specialized cellular junctions that are classified into four groups; adherence junctions, gap junctions, cell-matrix junctions and cell-cell junctions[85]. In addition to providing adhesion between cells, CAMs are also directly involved in cellular signaling thereby modulating the behavior of the cell. An example of this is that hepatocytes alter their phenotype depending on the composition of the matrix they are in contact with[86].

1.2.1 Classification

Cell-adhesion molecules are divided into five classes 1. Cadherins, 2. Selectins, which are dependent on Ca^{2+} for adhesion. 3. Integrins, 4. Immunoglobulin-like cells adhesion molecules and 5. Mucins, which are Ca^{2+} independent. Selectins are lectins, molecules that recognize and bind specific oligosaccharide sequences, and have their main function in extravasation of leukocytes by mediating adhesion between endothelial cells and leukocytes[87]. Mucin-like CAMs have a carbohydrate chain on the extracellular region and act as ligand for the lectin domain of selectins[85].

1.2.2 Cadherins

Classical cadherins are Ca^{2+} dependent CAMs that are part of the adherence junction complex. Cadherins mediate mainly homophilic interactions and can thus provide specificity in the interaction between cells that express the same type of cadherin[88, 89]. It was first assumed that cadherins only could mediate homophilic binding but

heterophilic binding between different cadherins also occur. Cadherins are during embryogenesis expressed in a restricted temporo-spatial manner and serve as regulators for morphogenesis including the formation of polarized epithelia[90]. The classical cadherins are 720-750 aa long membrane-spanning molecules that consists of an extracellular region, a transmembrane region and a cytoplasmatic region that through adapter proteins, catenins, is connected to the actin cytoskeleton[91].

The epithelial cadherin, E-cadherin was fully cloned in human 1995 and had before that in the late eighties been characterized mice and chicken, then named as uvomorulin or L-CAM respectively[92, 93]. E-cadherin is expressed by most polarized epithelial cells, and functions to hold epithelial sheets together. Loss of E-cadherin expression by cancer cells originated from polarized epithelia, eg adenocarcinoma, is a phenomena strongly associated with a metastatic phenotype[94].

Neuronal cadherin, N-cadherin , is during development expressed by neuronal and mesenchymal cells, and also in the adult organism by several cell-types such as nerve, muscle and fibroblasts[95-98]. Upregulation of N-cadherin is often seen in adenocaricinomas as they aquire a more aggressive mesenchymal phenotype[94]. Interferring with N-Cadherin mediated adhesion has therefore been proposed as a possible way of treating cancer[99, 100]. Interestingly human hepatocytes, in addition to E-cadherin, also express N-cadherin however the functional significance of this is unknown[101, 102]. Hepatic stellate cells also express N-cadherin [103, 104] and since N-cadherin is implicated in signaling it could possibly be important in interaction between hepatocytes and HSC or other cells[105].

Retinal-cadherin, R-cadherin, was first discovered in the retina but is also expressed in development by the neural system, myotome, kidney and thymus [I] [95, 106-110]. R-cadherin is not expressed in the liver.

The classical cadherins are involved in several cellular signaling pathways. A classical example of this is when E-cadherin expression is down-regulated and β-catenin, which has been bound to E-cadherin is released, enters the nucleus and takes part in initiation of cell proliferation[111-113]. As the expression patterns of cadherins were found to be dynamic and differentiated during embryogenesis, it was early suspected that they had a central role in cell differentiation and tissue morphogenesis[114]. Indeed it was also shown that abladation of cadherin expression severely interfered with the normal embryo and organogenesis[114-118]

Thus, there were strong indications that cadherins could direct tissue formation. Larue and collegues had shown that teratomas derived from E-cadherin -/- ES cells formed no epithelial tissues and also that transfection of these cells with N-cadherin rescued formation of epithelia, cartilage and bone[119]. Encouraged by the findings that R-cadherin was expressed by developing epithelia and muscle, we wanted to evaluate the morphogenic effect of R-cadherin with the same teratoma model. At the time of our study, R-cadherin was known to be expressed by early developing muscle in chicken[95] albeit expression of the mouse homologue had been reported only in the developing nervous system[120], and the gastrointestinal tract[108]. As professor

Takeichi and collegues were investigating the role of R-cadherin in the developing brain, we focused on investigating its expression in developing muscle and epithelia.

1.2.3 Integrins

Integrins, the main matrix binding molecules, are a large family of homologous cell-matrix receptors that are crucial for basic cellular functions such as migration and proliferation. As an example, many cells are dependent on integrin mediated binding to ECM for proliferation and survival in that they undergo apoptosis if they loose contact with ECM[121]. Each integrin molecule consists of two non-covalently bound subunits; α and β. There are in human nine types of β subunits and 24 types of α subunits. Integrins of the β_1 family are receptors that specifically bind ECM components such as fibronectin, collagen and laminin. Examples of ubiquitous integrins are the fibronectin receptor $\alpha_5\beta_1$ and $\alpha_6\beta_1$ that is a laminin receptor. Integrins link the ECM to the cytoskeleton via intracellular anchor proteins, eg talin, α-actinin, and filamin. This linkage of ECM to the cytoskeleton in turn leads to clustering of integrins that thereby form a more robust focal adhesion. Integrins of the β_2 family mainly mediate cell-cell interaction through binding to CAMs of the immunoglobulin-like family. An important function of integrins is activation of intracellular signaling pathways. The most well characterized mediator of integrin signalling is the thyrosine kinase focal adhesion kinase (FAK). FAK molecules are recruited to the focal adhesions where they mutually phophorylate each other at a specific thyrosine residue. This leads to recruitment of thyrosine-kinases of the src-family and subsequently recruitment and phosphorylation of various other signalling molecules that further propagate the signalling cascade[122]. Integrin signalling often modulate cellular response to other extracellular signals. An example of this is the HGF/c-met-MEK-ERK pathway that induces hepatocyte proliferation where FAK enhances the signal through phosphorylation of ERK[123, 124][125]. Cells also regulate their integrin signalling activity and in this way modulate their response to the extracellular context. Integrins have also been implicated as mechanosensors by communicating changes in tension of the extracellular matrix into the cellular signaling system[126].

1.2.4 Neural Cell Adhesion Molecule

Neural cell adhesion molecule is a member of the large superfamily of immunoglobulin-like adhesion molecules. These CAMs are not dependent on Ca^{2+} for binding activity and all have one or more domain with homology to immunoglobulin. N-CAM was the first neural adhesion molecule to be discovered and is the most extensively studied. Examples of N-CAM function are regulation of cell sorting, migration and proliferation. The N-CAM molecule is encoded by a single gene but is subject to extensive alternative splicing and post-translational modifications. Thus there are more than 20 isoforms of N-CAM. All N-CAM isoforms have five immunoglobulin domains in their extracellular region. The three most predominant isoforms of N-CAM are illustrated in figure 3.

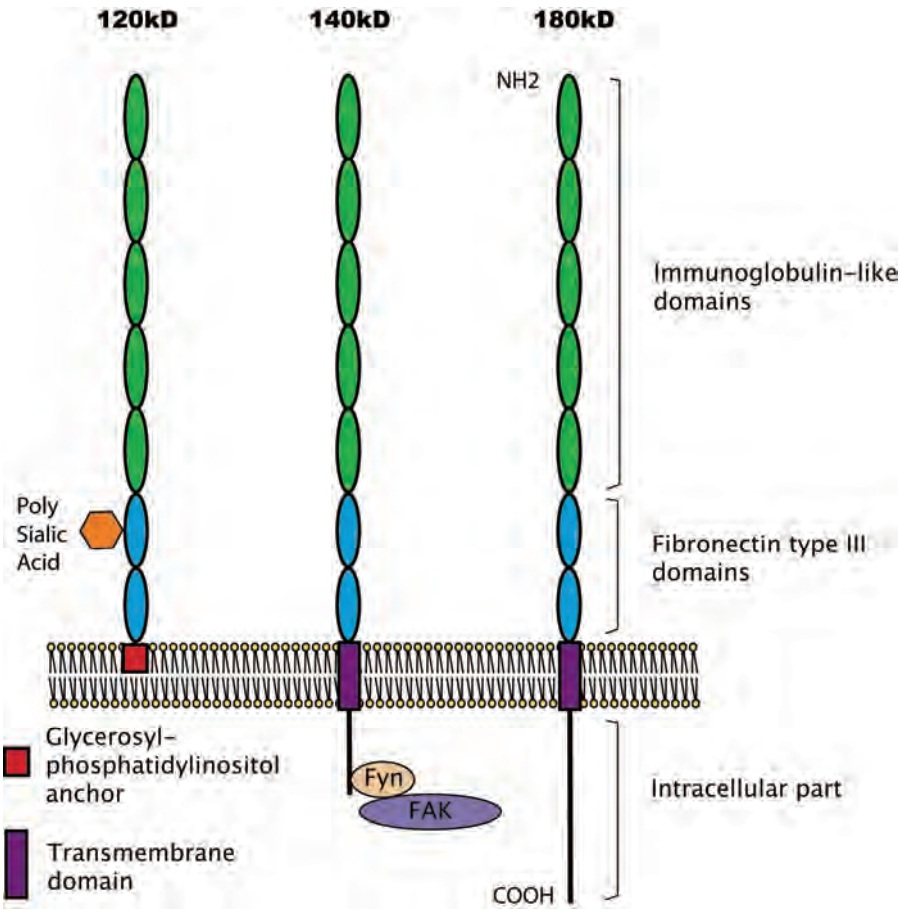


Figure 3: *Schematic and simplyfied figure of the three most predominant isoforms of N-CAM. The 140 kD isoform is expressed in injured liver.*

N-CAM mediates adhesion via homophilic binding but also heterophilic interactions with other molecules such as FGFR, L1, GDNF, N-cadherin and ECM components such as agrin and proteoglycans[127]. A special feature of N-CAM is that some isoforms, especially the 120kD isoform, can have large chains of polysialic acid bound to the extracellular region which functions as an inhibitor of adhesion. Isoforms of N-CAM that have an intracellular region can mediate intracellular signaling through intracellular mediators such as FAK, PKC and NFκB. It has also been reported that N-CAM can activate the FGF receptor [128]. During development cells of all three germlayers express N-CAM but in adult it is restricted to mainly neuronal and neuroendocrine cells. NK lymphocytes express N-CAM that then is denoted as CD56. CD56 positive lymphocytes have been suggested to contribute to hepatocellular damage in chronic hepatitis C infection [129]. N-CAM is also expressed in many tissues in response to injury and it is associated with fibrosis in liver, heart and kidney[34, 130, 131]. N-CAM mediated adhesion is weaker than cadherin adhesion and the effect of N-CAM inactivation is less dramatic than what is the case of many cadherins. N-CAM deficient mice show brain defects presenting as increased anxiety

and cognitive dysfunction. They also have disorganization of cells in the islets of Langerhans and impaired development of the olphactory bulb[132-134]. Despite these defects, N-CAM mice appear relatively normal and are fully viable and fertile. In the adult human normal liver N-CAM is restricted to a few nerve fibers. However in the injured liver cholangiocytes, activated HSC and periportal fibroblasts as well as intermediate cells of the ductular reaction express N-CAM[34, 135-139]. The functional role of N-CAM in liver injury or fibrogenesis has not been studied.

1.3 LIVER DISEASE IN CYSTIC FIBROSIS

1.3.1 Cystic Fibrosis

Cystic fibrosis is caused by mutations in the gene encoding transmembrane conductance regulator (CFTR). It is a fairly common inherited disease in the caucasian population with an approximate incidence of 1/3000 born[125]. Cystic fibrosis was first described 1938 and was until effective treatment became available, in most cases fatal in early childhood due to respiratory infections. Patients with mutations in the gene encoding CFTR may have pathological changes in organs that express CFTR such as the respiratory tract, pancreas, liver and reproductive tract. As life expectancy has improved with effective treatment of the respiratory and pancreatic manifestations, liver manifestations of CF have become an increasingly important clinical problem[140].

CF-associated liver disease usually presents at childhood to mid-adolescence as enlargement of the liver and in most cases it has a slow progress to fibrosis. Only 27-35% of the CF patients develop liver disease and there is also a large variability in the severity of liver disease among those affected[141, 142]. About 10% of the CF patients with liver disease progress to cirrhosis[141]. In addition to the risk for complications directly related to impaired liver function and portal hypertension, CF patients with cirrhosis are also at high risk of other complications such as malnutrition and infections.

The histological features described in CF liver disease are bile plugs in the biliary ducts, steatosis, ductular reaction and portal fibrosis[143-145]. The simplest explanation to the liver injury found in CF was that bile with high viscosity is trapped in the intra-hepatic biliary tree, thereby causing cholestasis and subsequently focal biliary fibrosis[145, 146]. In contrast to this, several studies on liver samples from CF patients have shown only very few signs of cholestasis[143, 144]. Instead, these studies revealed ultrastructural changes in the bile duct epithelium and periductular fibrosis, something that suggests that injury to the bile duct epithelium may be the primary event and not necessarily secondary to cholestasis.

The question why some CF patients get liver disease and some don’t, and also why there is such a large variability, has remained unanswered. No association between any particular CFTR mutation and liver disease has been found[147, 148]. Recognized risk factors for CF liver disease are pancreatic insufficiency, genotype with complete loss of CFTR function, history of meconium ileus, later age at diagnosis of CF and male

sex[140]. Genetic factors independent of the CFTR gene has also been proposed as contributing to CF liver disease[149].

At present the only available treatment for CF liver disease is orally administered ursodeoxycholic acid (UDCA). The positive effects of UDCA treatment in CF liver disease are considered related to increased secretion of chloride and decreased secretion of mucins[150, 151].

1.3.2 CFTR

CFTR, Cystic Fibrosis Transmembrane Conductance Regulator, is a chloride channel protein composed of 1480 amino acid residues. CFTR is regulated by 3', 5'-cyclic monophosphate (cAMP) and is expressed on the apical membrane of polarized epithelia[152]. The gene encoding CFTR was discovered 1989, mapped to chromosome 7 and the entire CFTR gene was cloned 1990[153-155]. CFTR participates in secretion of sweat, mucus, bile and pancreatic juice and dysfunction of CFTR leads to hyperviscosity, which causes stagnation of mucus.
More than 1000 mutations of the CFTR gene have been described. The CFTR mutations are divided into six classes (tabell) [156]. The most common CFTR mutation worldwide is ΔF508 that results in deletion of a phenylanaline residue at position 508. The ΔF508 mutation is a class 2 mutation as it results in defective processing of the precursor in the endoplasmatic reticulum (ER). Indeed, CFTR is subjected to a very stringent quality control. Approximately 60-75% of wildtype CFTR precursor protein and 99% of ΔF508 CFTR is trapped and degraded by proteasomes in the ER[157].

CFTR is in the liver exclusively expressed on the apical membrane of bile duct epithelium[158, 159]. Primary bile produced by the hepatocytes is, as it is further transported through the biliary system, modified by re-absorption and secretion[160]. Central in this process is the secretion of chloride and bicarbonate ions into bile as this regulates the bile flow by creating an osmotic gradient that pulls water through aquaporins into the bile. In biliary epithelia stimulation by secretin increases intracellular cAMP, which then activates CFTR[160]. The main mediator of chloride ion efflux into the bile is CFTR although there are other chloride channel proteins that, at least in part, may compensate for loss CFTR function. CFTR also regulate transport of bicarbonate. The influx of chloride ions into the bile creates an apical gradient that facilitate transport of bicarbonate into the bile by an apical bicarbonate-chloride exchange protein[161]. CFTR is also involved in regulation of other membrane-bound transport proteins[162, 163]. Dysfunction of CFTR causes impaired transport of bicarbonate, which in turn leads to defect alkalinization of bile. The alkalinization is required for proper function of the bile in the digestive system.

Despite the high degree of degradation of ΔF508 CFTR, it has been found properly localized at the apical membrane of gallbladder epithelia in patients homozygous for the mutation that had a normally appearing gallbladder. However, in ΔF508 homozygous patients that had microgallbladder, CFTR was mislocalized to the cytoplasm and the lateral membranes of the cholangiocytes. This suggested a correlation between the degree of structural abnormality and CFTR localization[164].

In the intrahepatic bile ducts of ΔF508 homozygous patients, CFTR is mislocalized to the cytoplasm and the lateral plasma membranes. Furthermore, very few signs of inflammation were seen. These findings were interpreted as further support for the hypothesis that abnormality of the intrahepatic bile ducts is the primary event in CF liver disease[159].

1.3.3 EBP50 and Ezrin

In the search for genetic modifiers that may contribute to CF liver disease, attention has been given proteins that are able to bind CFTR and possibly regulate its function and subcellular localization.

Ezrin-Radixin-Moesin (ERM) binding phosphoprotein 50 (EBP50) is a scaffolding protein that normally is localized at the apical plasmamembrane of epithelial cells [165]. EBP50 has two postsynaptic density 95/disc-large/zona occludens (PDZ) domains, which can bind other proteins containg a PDZ binding domain. There is also an ERM-binding domain in EBP50 that interacts with ezrin, another adapter protein that is capable of binding actin and protein kinase A. EBP50 assembles multi-protein complexes that anchor transporters to the apical actin cytoskeleton[166]. In the liver, EBP50 is expressed at the canalicular membrane of hepatocytes and at the apical membrane of cholangiocytes [167, 168]. CFTR contains a PDZ binding motif that interact with EBP50 and deletion of this leads to increased mobility of CFTR in the plasma membrane[169]. Interaction of CFTR with the PDZ1 domain of EBP50 is required for cAMP-dependent chloride secretion by cholangiocytes, thus suggesting a role of EBP50 in the secretion of bile[167]. A simplified model of CFTR-EBP50-Ezrin interaction is shown in figure 4.

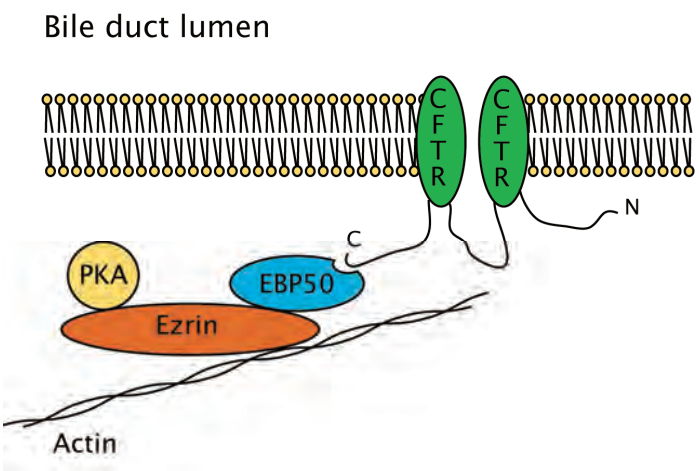


Figure 4: *A simplified model of CFTR-EBP50-ezrin interaction. A PDZ binding motif on CFTR binds to a PDZ domain of EBP50. Ezrin is bound to the ezrin binding domain of EBP50. Ezrin binds to the actin filaments. Protein kinase A (PKA) can bind ezrin.*

Recently a role of EBP50 in cell proliferation has been suggested[170]. Over-expression and intracellular redistribution of EBP50 to the cytoplasm and/or nucleus has been shown in hepatocellular and breast carcinoma, as well as in proliferating endometrium, [171-174]. EBP50 also bind proteins that are involved in cell proliferation, such as platelet-derived growth factor (PDGF) or epidermal growth factor (EGF) receptors [172, 175-179] Experimental studies on cell lines have suggested an anti-proliferative role of EBP50 [178, 180]. However, the impact of EBP50 on cell proliferation is still unclear.

1.4 LIVER FIBROSIS IN LOW-DOSE MTX TREATMENT OF PSORIASIS

1.4.1 Psoriasis and methotexate treatment

Psoriasis is an inflammatory disease affecting primarily the skin. Approximately 5–8% of psoriasis patients also have seronegative arthritis. The prevalence of psoriasis has been estimated to 2% albeit there are considerable variations between different countries and ethnical groups. There is also a large variation in severity of psoriasis between individuals [181]. Psoriasis often has, even in cases with mild skin lesions, a profoundly negative impact on the quality of life. The psoriatic skin lesions are characterized by increased proliferation and altered differentiation of keratinocytes together with infiltration of the dermis with T-lymphocytes[182, 183].

Methotrexate has since the 1970s been widely used and considered as an effective treatment for severe psoriasis[184]. Methotrexate is a folic acid antagonist that inhibits an enzyme that is necessary for normal synthesis of nucleic acids thereby blocking cell replication. In psoriasis, methotrexate blocks the proliferation of epidermal cells and also suppresses inflammation [184, 185]. Despite its effectiveness, methotrexate is also well known for its many, potentially severe, side effects. Development of liver fibrosis and cirrhosis has been a feared complication of long-term treatment with low-dose methotrexate. Studies performed in the 1960s and 1970s had indicated that up to 26% of patients on long-term methotrexate treatment developed liver cirrhosis, resulting in guidelines for monitoring these patients [186-188]. In order to diagnose developing liver fibrosis, the use of repeatedly performed liver biopsies were included in the guidelines. This strategy was chosen since blood chemistry lab tests not reliably can predict or diagnose liver fibrosis[188-190]. A special grading system, the Roenigk classification, was developed for use in the evaluation of these surveillance liver biopsies on methotrexate-treated patients [191]. The Roenigk score shows good interobserver reliability but has been shown as less accurate in staging fibrosis [189]. The current guidelines issued by the American Academy of Dermatology, recommend liver biopsy to be performed after each 1.5 g accumulated dose of methotrexate. For the majority of patients, this strategy results in a liver biopsy every other year. Although generally considered as a safe procedure, percutaneous liver biopsy carries its own morbidity and mortality with fatality rates from 0.03-0.32% and hospitalization rates due to complications at 1-3%[192]. With this in mind, together with more recent studies indicating lower incidence of liver fibrosis, the use of repeated liver biopsies has been questioned[189, 193, 194].

2 AIMS

- (Study I) In light of the previously proven importance of other cadherins in development; we wanted to elucidate the role of R-cadherin in embryogenesis. For this we applied a combined descriptive and experimental approach. First we sought to map the temporo-spatial expression of R-cadherin protein during embryogenesis. Secondly, we wanted to address the question whether expression of R-cadherin could direct the formation of tissue from embryonic stem cells.

- (Study II) Given the induction of N-CAM in the injured liver and its association with fibrosis, we wanted to address if loss of N-CAM would affect fibrosis development in biliary type liver fibrosis and/or fibrosis induced by injury of the liver parenchyma.

- (Study III) This study had two aims: Primarily to investigate if the delocalization of CFTR in bile ducts of patients with the ΔF508 mutation was associated with aberant EBP50 expression. Secondly to address if EBP50 was involved in the regulation of cholangiocyte proliferation.

- (Study IV) In this study we addressed the following question: What is the impact of common risk factors for liver cirrhosis on development of liver fibrosis in psoriasis patients treated long-term with low-dose methotrexate.

3 MATERIALS AND METHODS

3.1 PATIENTS

3.1.1 Study III

Liver samples were collected at the time of the biopsy, from ten CF patients, five patients with primary biliary cirrhosis (PBC), three patients with Primary Sclerosing Cholangitis (PSC) and one patient with bile stone impaction. None of the patients had a history of variceal bleeding or other liver-related events.
Normal liver samples were obtained from 3 patients, who underwent routine cholecystectomy. These patients had neither previous nor current clinical or biochemical signs of liver disease, and the liver specimens had normal histology.
Clinical and laboratory data on the patients in this study is shown in table 1.

Patient number (CFTR genotype)	Gender/Age (yr)	Serum level (U/L)		UDCA treatment	Histological Stage	
		ALT	γGT		Fibrosis	PBC
CF1 (dF508/dF508)	M/17	0,78	0,59	No	I	N/A
CF2 (dF508/112delT)	M/3,6	0,85	0,73	No	III	N/A
CF3 (dF508/dF508)	F/9	0,55	0,2	Yes	II	N/A
CF4(dF508/E640)	F/9,9	0,52	0,39	No	0	N/A
CF5(dF508/dF508)	M13	0,54	0,41	No	II	N/A
CF6(dF508/394delTT)	F/8	0,44	0,2	Yes	0	N/A
CF7(dF508/3659delC)	M/37	0,49	1,1	No	I	N/A
CF8(dF508/dF508)	M10	0,44	0,22	Yes	I	N/A
CF9(dF508/V603F)	F/8	0,56	0,17	Yes	I	N/A
CF10(dF508/R560T)	M/38	0,44	0,64	No	I	N/A
PBC1	F/68	1,11	4,4	No	IV	IV
PBC2	F/67	0,6	6,0	No	II	III
PBC3	F/45	0,94	2,9	No	II	III
PBC4	F/60	1,5	4,3	No	0	II
PBC5	F/64	9,18	7,5	No	0	I
PSC1	F/58	2,28	5,2	No	IV	N/A
PSC2	F/42	0,29	0,4	No	I	N/A
PSC3	F/54	9,79	7,2	No	0	N/A
BDS	M/29	0,31	0,9	No	II	N/A

Table 1: *Clinical data on patients that were included in study III.*

3.1.2 Study IV

Psoriasis patients treated with low-dose methotrexate at the Department of Dermatology at the Karolinska Hospital, the dermatology outpatient clinic in Vällingby or the Södertälje and Enskede psoriasis treatment facilities were identified. Eight of these patients were excluded due to unsufficient available clinical data. The remaining seventy-one patients (35 females and 36 males), that had undergone one or more liver biopsies 1975 and 2003 were included in the study. Twenty-six of these patients (11 females and 15 males) had one or more recognized risk factors for liver injury.
Median age at first biopsy was 52 (range 35–72) years for patients with risk factor and 48 (range 26–77) for those without.
One hundred and sixty-nine of the total 191 liver biopsies performed on the included patients in the study could be retrieved for evaluation.
Data on age, gender, start of methotrexate treatment, weekly and cumulative dose methotrexate, history of drug and alcohol use, presence of diabetes, presence or history of liver disease, overweight (BMI > 25), liver blood chemistry tests (ALT, AST and GT) and concomitant medication, was obtained from the medical charts.

3.2 MORPHOLOGY

3.2.1 Histopathological stainings

Sections of paraffin embedded, formalin fixed, mouse tissue and human liver biopsies were stained with hematoxylin-eosin for general histological evaluation and picro-sirius red for evaluation of fibrosis.

3.2.2 Immunolabelling on human liver tissue

The liver specimen was snap-frozen in liquid nitrogen and thereafter embedded in OCT compound (Tissue Tek). Cryostat sections of 6 µm were fixed in 4% paraformaldehyde in phosphate buffered saline for 20 minutes at 4° C. The sections were incubated for 45 minutes with serum corresponding to the secondary antibody and subsequently incubated with avidin and biotin blocking reagents (Vector labs) for 15 minutes each. The sections were then incubated with primary antibody over night at 4°C. The antibodies used were anti-EBP50 rabbit polyclonal antibody (Calbiochem cat #324620) at a dilution of 1:300, anti-EBP50 mouse monoclonal antibody (RD Biosciences, cat #611160) at 1:50, anti-ezrin mouse monoclonal antibody (NeoMarker, cat #MS-661-P1)) at a dilution of 1:40, anti-CFTR mouse monoclonal antibody (RD Biosciences, cat # MAB-25031) at a dilution of 1:100 or or mouse monoclonal anti-Ki67 (Dianova, cat #dia607) at 1:100. Following incubation with primary antibody, liver sections were incubated with biotinylated secondary antibody (Vector Labs) for 30 minutes at room temperature and finally with Cy3-streptavidin (Sigma, cat #S6402) for 60 minutes at room temperature. For Ezrin immunohistochemistry the APAAP/Fast Red protocol (Vector Laboratories) was used according to the manufacturer's instructions. For double immuno-fluorescence, antibodies direct-conjugated to TRITC (Jackson) or Alexa 488 (Molecular Probes) were used. Secondary antibodies and Cy3-streptavidin

were diluted in Tris buffered saline pH 7.6 containing 3% of their corresponding sera. Between each step, the slides were washed three times in Tris buffered saline, pH 7.6. To the second-last wash, DAPI (Sigma) at a concentration of 1:10000 or SYTO16 (Molecular Probes) at 1:25000 was added for nuclear staining. Finally, the slides were mounted in Vectashield mounting medium (Vector Labs).

3.2.3 Immunolabelling on mouse tissues

Mouse embryos and teratomas were fixed in 4% paraformaldehyde in HBS (10 m*M* Hepes, pH 7.4, 150 m*M* NaCl). They were subsequently dehydrated in a sucrose gradient consisting of ice-cold sucrose/HBS–Ca2/ (HBS with 1 m*M* CaCl2) solutions (12–15–18%), for 2–3 hr in each solution. Finally the specimens were embedded in OCT compound and frozen in liquid nitrogen. Ten µm cryosections were after wash in HBS, postfixed in -20°C methanol for 20 minutes, subsequently blocked in TBS-Ca^{2+} (10 m*M* Tris, pH 7.6, 150m*M*NaCl, 1m*M*CaCl2) with 5% skim milk for 20 minutes at room temperature. Sections to be stained with R-cadherin or N-cadherin antibodies [120, 195] were before post-fixation covered with HBS and incubated for 15 seconds at 750 W in a microwave oven for antigen retrieval. After blocking, the sections were incubated with primary antibodies over-night at 4°C. The slides were after triple wash in TBS-Ca^{2+} incubated with secondary antibodies for 60 minutes at room temperature. Stainings with biotinylated secondary antibody was after washing visualized by incubation with Cy3-streptavidin. All antibodies were diluted in TBS-Ca^{2+} supplemented with 5% skim milk.

Mouse liver was immediately after retrieval put in ice-cold Histocon (HistoLab Products), cut in 5-7 mm pieces, embedded in OCT compound and snap frozen in liquid nitrogen. Six µm cryosections were fixed in 2% paraformaldehyde in phosphate buffered saline containing 0.1% Triton X100 (SigmaAldrich) for 10 minutes at room temperature. The sections were immunostained with rabbit anti-N-CAM (dilution 1:250 Chemicon #5032) or rabbit anti-fibronectin (dilution 1:800 SigmaAldrich #F3648) as described above (see section 3.2.2) except for that PBS with 0.1% Tween 20 (SigmaAldrich) was used in the washing step before applying the secondary antibody. Immunostainings for αSMA was performed as described above except for that the secondary antibody and visualization steps were omitted.

3.2.4 Microsopy

The slides were examined with a Nikon Eclipse E800 microscope. Laser Confocal Microscopy on human liver samples and mouse embryos was performed with a Leica TCS SP microscope or Multiprobe 2001 respectively.

3.2.5 Morphometry on mouse liver tissue

Samples of three different liver lobes from each animal were fixed in 4% buffered formalin and then embedded in paraffin. Paraffin sections from the liver samples were then stained with hematoxylin-eosin and picro-sirius according to standard methods in

routine pathology. The amount of collagen deposition was quantitated by measuring the proportion of Sirius stained area using color thresholding and measurement of area fraction with ImageJ software (NIH Public Domain). Images taken from twenty random 200x fields from three different liver lobes in each animal were measured [196, 197]. Analysis of the proportion of liver area occupied by portal zones was performed on twenty 200x fields in each animal with point-counting[198] morphometry using a grid with 81 line crossings[199]. The number of crossings in each field localized within a portal area was counted.

3.2.6 Histopathological scoring of human liver biopsies

The hematoxylin-eosin and sirius stained liver biopsies from methotrexate treated psoriasis patients biopsies, were evaluated by three persons who did not have knowledge of clinical data on the patient or previous assessment of the biopsy. The degree of inflammation, steatosis, hepatocyte ballooning and fibrosis was scored according Kleiner and Brunt. The non-alcohol steatohepatits score issued by the same authors was calculated as the unweighted sum of scores for steatosis, lobular inflammation and hepatocyte ballooning[200].

Steatosis		**Score**
Proportion of parenchyma involved by steatosis.	<5%	0
	5%-33%	1
	>33%-66%	2
	>66%	3
Fibrosis stage		
	None	0
	Perisinusoidal or periportal	1
	Perisinusoidal and portal/periportal	2
	Bridging fibrosis	3
	Cirrhosis	4
Lobular inflammation		
	No foci	0
	<2 foci per 200× field	1
	2-4 foci per 200× field	2
	>4 foci per 200× field	
Hepatocyte ballooning		
	None	0
	Few balloon cells	1
	Many cells/prominent ballooning	2

3.3 PROCEDURES

3.3.1 Human liver samples

Liver biopsies were performed in routine clinical practice through the intercostal route as described by Menghini[201]. Samples of normal liver were obtained directly after opening of the abdomen in patients undergoing cholecystectomy. The liver specimens were immediately snap frozen in liquid nitrogen.

3.3.2 Embryonic stem cells

Embryonal stem cell line D3[202] and ES cell lines isolated from E-cadherin knockout mice[203] were cultured on inactivated feeder cell layer or in in 60% BRL conditioned medium respectively. Five ES-celliles were used: One wild-type derived, one derived from the E-cadherin knock-out and three derived from E-cadherin knock-out cells that were stably transfected with R-cadherin cDNA driven by the constituvely active phosphoglycerate kinase (PGK-1) promoter.

3.3.3 Teratoma model in mice

Five of ES-cell lines were used to derive teratomas. ES cells (2×10^6) were suspended in Dulbecco's modified Eagle's medium (DMEM) and injected subcutaneously into the back of 8- to 10-week-old male 129/Sv mice. Mice were sacrificed by cervical dislocation when the teratoma had reached a size of 1.5 cm in diameter [119].

3.3.4 Generation of embryonic bodies

ES cells were maintained in DMEM with Glutamax, leukaemia inhibitory factor and 15% fetal calf serum (FCS). ES cells were trypsinized, thoroughly dissociated and thereafter plated, 5×10^3 cells, on 60 mm Petri dishes in liquid medium (Iscove's modified Dulbecco's medium) supplemented with FCS, L-glutamine, monothiolglycerol and ascorbic acid. When the embryonic bodies had grown for 6-10 days, they were removed by pipetting for subsequent analysis. E-cadherin -/- ES cells did not form embryonic bodies.

3.3.5 Bile duct ligation in mice

Under deep anesthesia with Isoflurane, the abdomen was opened by a mid-line incision. The hepatoduodenal ligament was identified and the common bile duct carefully mobilized by blunt dissection. The common bile duct was then ligated with non-resorbable thread and cut between ligatures. Controls were sham operated with laparotomy and identification, but not ligation, of the common bile duct[40, 204]. Mice were given buprenorphine subcutaneously before and after surgery for analgesia. At two weeks after surgery, mice were were sacrificed by exsanguination under deep anesthesia with isoflurane followed by cervical dislocation.

3.3.6 Carbon tetrachloride injections in mice

Carbon tetrachloride (2 mg/kg bodyweight) was mixed 2:5 with sterile mineral oil and given intraperitoneally twice weekly for three weeks. Controls received injections with mineral oil alone. Mice were given buprenorphine subcutaneously before and after injection for analgesia. After three weeks mice were sacrificed as described above.

3.3.7 Isolation and *in vitro* activation of hepatic stellate cells

Isolation of HSC was performed as previously described [205]. C57BL/6J and C57BL/6J N-CAM$^{-/-}$ mice were deeply anesthetized with Isoflurane. Perfusion solutions were administered via the left atrium of the heart and let to flow out through the caval vein that was cut below the liver. First Krebs-Ringer buffer supplemented with 0.01 M Hepes and 0.01 % EGTA was administered to remove blood. Subsequently, the liver was perfused with pronase (2.5 mg/ml) and 35 ml collagenase (0.074 mg/ml) whereafter it was removed, minced and transferred to a solution with pronase (0.6 mg/ml) and DNase (0.16 mg/ml). The mixture was then incubated at 39°C with vigorous shaking for 30 min. The hepatocytes were removed from the cellsuspension by low-speed centrifugation and subsequent washing. HSC were isolated by ultracentrifugation through a Larcoll (SigmaAldrich) gradient [205]. The HSC were plated on Petri dishes in DMEM-F12 supplemented with 10 % FBS, penicillin and streptomycin (100 U/ml and 100 μg/ml respectively). The HSC were cultured for 20 days at 37°C and 5% CO_2 where after they were analyzed or used in experiments. At the first passage, 95-99% purity of the isolated HSC was confirmed by morphological appearance, vitamin A-specific fluorescence in polarized light and immunofluorescence for α-smooth-muscle actin.

After a gentle wash with PBS, the medium was replaced with new medium containing 10ng/ml of recombinant human TGF-β1(R&D Systems). The HSC were stimulated for 0h (control), 4h or 72h before analysis.

3.3.8 Immunoblot and immunoprecipitation

Immunoblots were performed with standard techniques and are described in detail in manuscripts I and II. Briefly, equal amounts of protein extracts from cells or tissues were loaded on SDS-PAGE gels and subsequently electrophoretically blotted to a nitrocellulose membrane. To evaluate equal loading of proteins, the membranes were stained with Ponceu solution before applying antibodies. After blocking with 5% skim milk the membranes were incubated with primary antibody diluted in skim milk over night at 4°C. After washing the antibody complexes were visualized by chemoluminescence using the ECL detection kit (Amersham).

For immunoprecipitations, cells were solubilized in ice-cold lysis buffer containing protease inhibitors and the resulting solution centrifuged. Protein concentrations of the supernatants were determined by Bradford assay[206]. Samples diluted in lysis buffer containing equal amounts of protein were incubated with primary antibody at 4°C

overnight. The antigen-antibody complexes were precipitated by Pansorbin cells (Amersham) and subsequently analyzed with immunoblotting.

The primary antibodies used were; rabbit anti-pancadherin pAb (Sjödin et al. 1995)[108], rat monoclonal anti-R-cadherin (Matsunami and Takeichi 1995)[120], rabbit polyclonal anti-N-CAM (Chemicon#5032), anti-desmin (SigmaAldrich #D8281), anti-fibronectin (SigmaAldrich #F3648) anti-TGF-β1 (SigmaAldrich#T0438) and anti-αSMA (DAKO# M0851).

3.3.9 Reverse transcription-Polymerase chain reaction

Procedures for RT-PCR are described in detail in manuscripts I-II. Briefly, RNA was extracted from cells and tissues with the Ultraspec RNA isolation system (Biotecx). Complemetary DNA (cDNA) was synthesized from total RNA by incubation at 42°C with reverse transcriptase, random primers and nucleotides. Amplification the genes of interest from cDNA was performed with polymerase chain reaction using a thermocycler. The PCR products were separated by electrophoresis on agarose gel and visualized by staining with ethidium bromide.

3.3.10 Real-time Polymerase chain reaction

Real-Time PCR was performed, using SYBR Green PCR Master Mix (Applied Biosystems) and detection of PCR products with the ABI PRISM 7000 sequence-detection system according to the manufacturer's instructions. The primerpairs used had been previously described[207]. Expression levels of genes of interest were normalized to expression levels of the housekeeping gene GAPDH. The Comparative CT method was used for quantification of the amounts of mRNA in N-CAM -/- livers relative to wild-type

SYBR Green is a dye that non-covalently binds double stranded DNA and emits fluorescence when exposed to light of a specific wavelength. In real-time PCR with SYBR Green, the amount of fluorescence light emitted from the PCR sample, which is proportional to the amount of double stranded DNA, is measured after each thermal cycle. The number of thermal cycles it takes for the amplification product of a specific gene to reach a set threshold level is proportional to the expression level, ie amount of mRNA, for that gene. In the Comparative CT method the expression level of the gene of interest is normalized to a house-keeping gene and then compared between two samples. The house-keeping gene must be expressed at equal levels in both samples.

3.4 STATISTICAL ANALYSIS

Single comparisons were made using the Mann-Whitney U-test or two-tailed Student's t-test as indicated in each paper. Multiple comparisons were made using two way analysis of variance (ANOVA) with repeated measures followed by pairwise comparison. To compare categorical variables, Fisher's exact test was used.
To analyze the difference in accumulated dose methotrexate-to-fibrosis between subgroup of risk factors for liver fibrosis the Log-rank test was used.
Values of $p < 0.05$ were considered statistically significant.

3.5 ETHICAL APPROVALS

Experimental studies on mice were performed after *a priori* approval by the regional ethics committee for the humane use of research animals in Umeå or Northern Stockholm respectively.

Studies on human liver samples were performed after *a priori* approval by the local ethics committee at Karolinska hospital or the regional boards for ethical approval of research involving humans in Göteborg or Stockholm respectively.

Carbon tetrachloride (CCl_4) was used with specific permission from the Swedish Environmental Protection Agency.

Studies on cells isolated from human liver were performed in France in accordance with French legislation.

4 RESULTS AND DISCUSSION

4.1 STUDY I

4.1.1 Expression of R-cadherin during mouse embrogenesis

R-cadherin was first detected in the somites, that later will form the limbs, at 10.5-11.5 days post coitum (dpc). By co-localization studies at using markers for skeletal and smooth muscle, desmin and α-SMA respectively, R-cadherin at 11.5 dpc was found more widely expressed in the somites than myotomal cells. In developing skeletal muscle, R-cadherin was first expressed in the limbs at 12.5 pdc. During primary and secondary myogenesis, R-cadherin was distributed att cell-cell contacts of myoblasts and at the interface of primaty myotubes and myoblasts respectively. Furthermore was R-cadherin expressed by vascular and visceral smooth muscle cells at 11.5-12.5 dpc.

Interactions between epithelial and mesenchymal cells are critical in formation of parenchymatous organs. R-cadherin was found to be expressed in the developing kidney, lung and thymus. In kidney, R-cadherin was first expressed at 10.5 dpc by mesonephric epithelial cells. Double immunofluorescence for R-cadherin and e-cadherin on 12.5 dpc kidney revealed that R-cadherin was expressed in pretubular aggregates whereas the ureter bud derived ducts expressed only E-cadherin. In some tubular structures coexpression of these molecules was seen. In later kidney development, at 17.5, R-cadherin, but not E-cadherin, was expressed in the pretubular aggregates, proximal parts of the S-shaped bodies, and in the epithelium that will form the Bowman's capsule. E-cadherin, but not R-cadherin was expressed in the distal tubules. In the proximal tubules and collecting ducts E- and R-cadherin expression co localized, which was confirmed by confocal microscopy analysis.
In the developing thymus at 12.5 dpc, R-cadherin was expessed by epithelial cells as confirmed by the epithelial markers E-cadherin and cytokeratin-8.
In the lung, expression of R-cadherin was seen in smooth muscle from 12.5 dpc to birth.

4.1.2 Histiogenetic activity of R-cadherin in teratomas in mice

Teratomas formed by the R-cadherin expressing E-cadherin -/- ES-cells contained epithelia and striated muscle formed as myotubes. The epithelial cells were confirmed as polarized by stainings with the apical marker actin and the basolateral marker Na/K/ ATPase. Teratomas from E-cadherin -/- ES cells were amorphous without epithelia or striated muscle. Teratomas derived from all cell-lines contained smooth muscle cells in apperantly equal amounts.

4.1.3 Rescuing of embryonic body formation by R-cadherin

When cultured in liquid medium, wild-type embryonic stem cells form so called embryonic bodies, that have a cystic form with striated muscle in the interior covered

by a single layer of epithelia. I contrast to N-cadherin transfected E-cadherin -/- ES-cells[119], transfection with R-cadherin rescued formation of embryonic bodies.

4.1.4 Summary and conclusions

In this paper we investigated the expression of R-cadherin during embryogenesis and experimentally tested its morphogenic activity. The expression pattern of R-cadherin in cell-cell contacts of myoblasts and at the interface between myoblasts and primary myotubes, suggested a role of this molecule in primary and secondary myogenesis. This was also supported by the finding that R-cadherin expressing ES-cells formed striated muscle. In addition our study of R-cadherin, the function of M-cadherin and M-cadherin in myogenesis has been studied. M-cadherin has been shown important for myoblast fusion [208]. N-cadherin has been shown dispensible in myoblast fusion[209] but appears to play an important role in formation of cardiac muscle[210].

The presence of R-cadherin in aggregating mesenchymal cells in the kidney correlated with the transformation of these cells into epithelia. This suggested that R-cadherin could be of importance in this process of differentiation.
The finding that R-cadherin could rescue formation of polarized epithelia may be interpreted as that it could replace the function of E-cadherin.

4.2 STUDY II

4.2.1 N-CAM expression in mouse liver

At 72 hours after BDL, cholangiocytes and mesenchymal cells in the portal areas had positive staining for N-CAM.
Two weeks after bile-duct-ligation, mesenchymal cells in the portal areas and small bile ducts were immunopositive for N-CAM. However, the larger bile ducts were N-CAM negative. In sham-operated or mineral oil alone injected controls, only a few N-CAM positive cells were seen. These were located close to the margins of the portal areas. By immunoblot on whole liver protein extracts, the 140kD isoform of N-CAM could be detected in of 2-week BDL wild-type mice but not in controls.

The 140 kD isoform of N-CAM was first detected at 48h after a single CCl_4 injection followed by a peak level at 72 hours. At 168 hours after injection, N-CAM was still detectable but had declined considerably. Immunostainings showed N-CAM expressed by all bile ducts and also by mesenchymal cells surrounding blood vessels and bile ducts.
In wild-type mice that had been injected with CCl_4 twice weekly for three weeks, N-CAM was expressed in myofibroblastic cells located in portal areas and surrounding necrotic areas. In contrast to at 72h after a single injection the bile ducts were N-CAM negative after three weeks of repeated injections.

4.2.2 Experimentally induced liver fibrosis in N-CAM -/- mice

Two weeks after BDL, the wild-type mice had developed an extensive liver fibrosis with expanded portal tracts and fibrous septae. Compared to wild-type mice, 2-week BDL N-CAM knock-outs had significantly less fibrous bridging and less expansion of the portal areas, as confirmed with image analysis and morphometry.
Mice of both genotypes that received CCl_4 injections repeatedly for three weeks, had a moderate, partially bridging, fibrosis without any significant difference in the proportion of picro-sirius stained area.
Immunoblotting revealed that 2-week BDL N-CAM knock-outs, as compared with wild-type mice, had lower levels of the non-collagen matrix protein fibronectin whereas no difference was seen in CCl_4 induced liver fibrosis. The basal membrane component laminin was found at equal levels in CCl_4 treated, BDL and control mice of both genotypes.

4.2.3 Myofibroblast markers in CCl4 and BDL mice

In opposite to what we expected, higher levels of desmin were found in both BDL and sham operated N-CAM knockouts compared to wild-type mice. The levels of α-SMA were also higher in BDL N-CAM -/- mice. This could be interpreted in two ways; there could be an increased number of myofibroblasts in N-CAM -/- liver or an increased expression of these proteins by the myofibroblasts. Immunostaining showed α-SMA positive cells in the portal areas surrounding bile ducts and also in fibrotic septae. There was no difference in the distribution of α-SMA positive cells between groups.

In mice with CCl_4 induced liver fibrosis, the individual variations of α-SMA and desmin were too large to allow comparison of the expression levels between groups. In CCl_4 treated mice, α-SMA positive cells were equally distributed in mice of both genotypes at the interlobular margins as well as in regeneration areas.

4.2.4 Expression of genes related to liver fibrosis

MMP-13 mRNA levels were significantly higher in N-CAM knock-out mice as compared to wild-type, whereas Collagen-1a, fibronectin, TIMP-1, MMP-2, MMP-3 and TGFß1 mRNA levels were not significantly different. There was however in the BDL N-CAM -/- mice a tendency of higher relative mRNA levels of all these genes, except for fibronectin.

In mice with CCl4 induced fibrosis, mean relative expression of mRNA for Collagen-1a, MMP-3, MMP-13 and TGFß1 appeared higher in N-CAM -/- mice but this was not statistically significant for any individual gene.

*4.2.5 A*ctivation of N-CAM -/- hepatic stellate cells *in vitro*

N-CAM -/- HSC, had decreasing α-SMA levels after 4 and 72 hours stimulation with TGFß1 compared to wild-type HSC were a moderate increase was seen at the same time points. Desmin had in N-CAM -/- HSC after 4 hours decreased significantly and

was at 72h almost undetectable in these cells. On the contrary, a marked temporal increase of desmin was seen in TGFß1-stimulated N-CAM positive HSC.
HSC of both genotypes had a temporal increase in fibronectin levels after stimulation with TGFß1 but quite surprisingly the increase of fibronectin was markedly higher in N-CAM -/- HSC.

4.2.6 Summary and conclusions

The main finding in this study was that N-CAM knockout mice had attenuated liver fibrosis after bile duct ligation. This could not be explained by less synthesis of collagen as the level of mRNA for collagen-1a rather was increased in N-CAM -/- mice. Another possibility is that there was an increased turnover of matrix in N-CAM -/- mice liver due to increased activity of matrix metalloproteinases. As this was not anticipated when the study was planned, we had not retained urin samples or liver tissue for zymography to evaluate MMP activity[211, 212]. However, the increased MMP-13 mRNA levels in BDL N-CAM -/- mice and the increased levels of desmin suggest that the population of partially activated HSC is increased in these mice. HSC are in the early stages of activation capable of degrading ECM and an increased number of such partially activated HSC could counteract expansion of fibrosis from the portal areas and establishment of fibrous septae. A third possibility is that activated HSC in the N-CAM knockouts have impaired ability to migrate to the portal areas as has been previously described[213]. Several studies have shown that portal myofibroblasts, possibly derived from proliferating bile duct epithelia by EMT, are important contributors of biliary fibrosis. N-CAM is expressed by cholangiocytes in acute cholestasis and also by proliferating bile duct epithelia. A speculative hypothesis could therefore be that the loss of N-CAM causes less efficient EMT, which in turn leads to less formation of portal myofibroblastic cells.

4.3 STUDY III

4.3.1 EBP50 and ezrin expression in human liver

In normal controls and patients with PBC, PSC or stone impaction, double stainings showed overlapping localization of EBP50 with CFTR at the apical membrane region of cholangiocytes. This was consistent with the view that EBP50 is a linker between ezrin and CFTR in these cells. In liver specimens from patients carrying the ΔF508 CFTR mutation, EBP50 was detected normally localized in hepatocytes and native bile ducts. In addition to this, EBP50 was also expressed by cells in the ductular reaction, in these cells aberrantly localized at the basolateral membranes and intracellularly. The same expression pattern was found in patients with PBC, PSC or stone impaction.

In all liver samples, ezrin was confined to the apical membrane of native bile duct epithelia and, when present, ductular reactions.

4.3.2 Subcellular distribution of EBP50 in the ductular reaction

EBP50 protein was detected both in the cytoplasm and in the nucleus where it co-localized with the nuclear marker SYTO16. The same subcellular distribution of EBP50 in ductular reactions was seen in patients with primary biliary fibrosis, primary sclerosing and a patient with bile stone impaction.

4.3.3 Summary and conclusions

EBP50 is normally distributed in native bile ducts of CF patients with the ΔF508 CFTR mutation but aberrantly localized to the basolateral membrane and nucleus in cells of the ductular reaction. This delocalization is interpreted as unrelated to CFTR trafficking since it was also seen in patients with other biliary liver diseases such as PBC, PSC and bile duct obstruction by stone impaction in the common bile duct.
Studies on malignant cells had suggested a role of EBP50 in proliferation. Indeed, the proliferation marker Ki67 was found in cells of the ductular reaction that had aberrant localization of EBP50. The possible role of EBP50 in cholangiocyte proliferation has been further studied in cell-lines and bile duct ligated rats by our French collaborating group. The results of these studies show that cholestasis in rat induced increased expression and a similar redistribution of EBP50 as we had observed in human biliary liver disease. Furthermore, down-regulation of EBP50 in a cholangiocyte cell-line resulted in dramatically decreased proliferation of these cells.

4.4 STUDY IV

4.4.1 Liver fibrosis

Of the patients with one or more risk factor, Twenty-five (96%) (mean cumulative dose MTX 1500 mg) developed liver fibrosis, while of the 45 patients without any risk factors, 26 (58%) ($p = 0.012$) developed fibrosis (median cumulative dose methotrexate 2100 mg). In total, 51 (71%), of the patients developed liver fibrosis of any degree.
Ten (38%) of the patients with risk factor had severe fibrosis (median cumulative dose MTX 1600 mg), while of those without risk factors four (9%) ($p = 0.0012$) had severe fibrosis.
The three patients in this study that developed cirrhosis had all one or more of the risk factors. Two of these three patients had no fibrosis in their first liver biopsy.
All nine patients that had alcohol over-consumption developed fibrosis, as compared to 41(66%), of the 62 patients without this risk factor. Two (22%) of the patients with alcohol over-consumption developed severe liver fibrosis compared to 11 (18%) of the patients without alcohol over-consumption ($p = 0.599$).
Seven (100%) of the patients with diabetes mellitus developed liver fibrosis compared to 37 (52%) of those without. Four (57%) of the seven patients with diabetes had severe fibrosis compared to nine (14%) of those without ($p = 0.003$).
Fourteen (93%) of the 15 patients with overweight (BMI > 25) had fibrosis which was severe in five of these patients. Overweight was significantly associated with severe fibrosis ($p = 0.0132$)
All three patients with viral hepatitis developed fibrosis, one of those stage III.

4.4.2 Liver blood chemistry tests

We found no correlation between serum levels of ALT, AST, cGT in blood samples and degree of fibrosis. Neither could these blood tests predict if a patient were to develop fibrosis. These results are in concert with previous studies and illustrate the limited value of these tests in monitoring of these patients except for detecting acute hepatotoxicity.

4.4.3 NAFLD activity score

Ten (38.5%) of the 26 patients with risk factor developed an NAFLD activity score ≥5 which has been shown highly indicative of steato-hepatitis[200]. Five (11%) ($p = 0.0055$) of the patients without any risk factor had a NAFLD activity score ≥5.

4.4.4 Summary and conclusions

It is difficult to perform prospective studies on the development of liver cirrhosis, since fibrogenesis is a slow process. In the present study, the patients had been treated and monitored at the same clinic and in many cases by the same physician for several years. Clinical data and liver biopsies were available which made it possible to perform this follow up study. One difficulty in this setting was the limited number of patients, thus making statistical analysis of each risk factor difficult. We therefore primarily looked at the combined impact of the risk factors, of which many patients have more than one. The results clearly indicate that presence of one or more risk factor makes it more likely for a psoriasis patient to develop liver fibrosis during methotrexate treatment. Diabetes and over-weight were the strongest risk factors associated with increased risk of fibrosis.

Interestingly, liver fibrosis has been found to be more common in psoriasis patients treated long-term with methotrexate than in patients with rheumatoid arthritis receiving the same treatment[190, 193, 214, 215]. In an editorial comment on the article it was noted that we did not have any control group with psoriasis patients that not had received methotrexate and that the increased occurrence of liver fibrosis merely could be attributed to the risk factors and not to methotrexate. The proportion of patients with risk factor in this study that developed severe fibrosis was however so high that it seems unlikely that methotrexate would not have contributed. It would have been optimal if we have had a control group of psoriatic patients that were not treated with methotrexate. Psoriasis patients without methotrexate treatment who underwent liver biopsied, most likely had clinical signs of liver disease or elevated liver transaminases. Unfortunately transaminases, as several others and we have shown, do not predict or correlate with liver fibrosis.

It would also be interesting to address the question whether psoriasis alone, apart from any association with risk factors of steatohepatitis, increases the risk of liver cirrhosis. It is though for ethical reasons questionable to perform repeated liver biopsies on patients without clinical or laborary signs of liver disease. However, the recently developed non-invasive methods for diagnosis of liver fibrosis, such as transient elastography or serum tests, may help answering this question.

To follow a well characterized cohort of psoriatic patients for many years would also give more data on liver disease in this group of patients.

5 GENERAL DISCUSSION

Formation and remodeling of tissues provide a fundamental basis of all multicellular organisms during development, homeostasis and in disease. In these processes cells must interact with their surrounding environment in a coordinated way simultaneously maintaining their functions and integrity. Molecules associated to the cellular membrane play a central role in these interactions.

Tissue formation involves a very large number of genes and proteins, which directly and indirectly influence each other's expression and function. The first study attempted to evaluate the role of R-cadherin in embryonal morphogenesis with a combined descriptive and experimental approach. Indeed, the results from the teratoma model where R-cadherin induced formation of striated muscle and epithelia corresponded to its expression in epithelia and muscle. However, as was later shown in the R-cadherin knockout mice, R-cadherin was not crucial for proper development of epithelia or muscle as these mice only showed minor defects in the kidney. This illustrates the high degree of redundancy between these molecules, which expression patterns often overlap. Indeed, also double knock-outs with R-cadherin and P-cadherin -/-, N-cadherin +/- or Cadherin-6 -/- respectivey did not have additional defects in kidney development[109]. There are several other examples of cadherin knockouts with only minor phenotypic changes such as cadherin-6, P-cadherin and cadherin-11[216-218]. In contrast to this is E-cadherin null mutants that fail to form a blastocyst and N-cadherin knockout mice that die with severe cardiac and neuronal defects ten days after gestation[219]. The functional role of R-cadherin has during the last ten years mainly been studied in the central nervous system (CNS) and there are only a few published studies on R-cadherin function outside the CNS. R-cadherin has in tumor cells been implicated in regulation of cell motility and downregulation of E- and P-cadherin [220, 221]. An interesting observation was that R-cadherin expression was reduced after balloon injury of the carotid artery and that inhibition of R-cadherin in vascular smooth muscle cells increased their proliferation[222]. This illustrates how the behaivor of cells and their expression of adhesion molecules are closely related also in pathological conditions.

Cell-cell adhesion molecules have during the last decade extensively been studied in developmental and tumor biology. However, in context of liver pathology, they have mostly been used as markers for different cell populations and their functional role in these diseases to a great extent remained elusive. The expression of the immuno-globuline-like cell-cell adhesion molecule N-CAM had been well characterized in various liver diseases as it had been found as a good marker for hepatic stellate cells. In the second study we applied two models of different types of liver fibrosis on N-CAM knockout mice; bile duct ligation as a model of biliary type fibrosis and repeated CCl_4 injections as a model of fibrosis induced by hepatocellular injury[223]. The results indicated a role of N-CAM in biliary type fibrosis as fibrosis induced by bile duct ligation was exclusively attenuated in N-CAM deficient mice. In contrast to this, BDL N-CAM -/- mice had higher levels of myofibroblast markers desmin and αSMA than BDL wild-type mice. We therefore decided to evaluate if loss of N-CAM would affect

activation of hepatic stellate cells. To address this, we applied a model system where isolated HSC were activated *in vitro* with TGF-β1. HSC isolated from N-CAM null mice were found that to have impaired ability to activate *in vitro* as assessed with desmin and αSMA, but produced *higher* levels of fibronectin. At this point, these findings just did not make sense. However, portal myofibroblasts had been suggested to play an important role in biliary fibrosis [40]. This was further supported by recently published experimental studies in rat showing that portal mesenchymal cells prominently contributed to fibrosis in cholestatic liver injury whereas HSC, although activated and expressing desmin, only contributed to a minor extent[33]. Since portal mesenchymal cells express N-CAM, there is a possibility that loss of N-CAM reduces the fibrogenic potential of these cells. Another very recent and interesting finding was that blockade of the integrin αVβ6, that is concordantly with N-CAM expressed in cholangiocytes during acute cholestasis, resulted in attenuation of BDL but not CCl_4 induced fibrosis[224]. Indeed, there are some studies implicating a role of N-CAM in integrin signaling which suggest that there is a connection in function of these molecules[128, 225, 226]. Our finding that the collagenase MMP-13 was upregulated in BDL N-CAM knockouts also provide an indication of this since MMPs often have been found upregulated by perturbation of integrin function. The role of N-CAM in the ductular reaction needs to be studied and it would also be interesting to investigate the effect on biliary fibrosis by antagonistic peptides or antibodies directed at N-CAM.

The third study was initiated primarily to test the hypothesis that the delocalization of CFTR in bile ducts of cystic fibrosis patients with the ΔF508 mutation was attributed to delocalization the scaffolding protein EBP50. It was recently published that EBP50 knockout mice have 35% reduction of apical CFTR in enterocytes. We found that EBP50 was normally localized in native bile ducts of these patients, thus a result that did not support our hypothesis. Interestingly enough though, EBP50 was found delocalized in cells of the ductular reactions. To further study EBP50 in the ductular reaction, its subcellular distribution was evaluated with laser confocal microscopy. This confirmed our observation of its aberrant localization. Subsequent experiments performed by our collaborating group in Paris France, lead by professor Chantal Housset, suggested a role of EBP50 in the regulation of proliferation in biliary epithelial cells. This is an important finding as it has been recently shown that biliary cells contribute to the fibrogenic cell population by undergoing epithelial mesenchymal transition[36, 38, 39]. From the clinical perspective, there is accumulating data emphasizing the importance of the ductular reaction in liver fibrosis[67, 69]. Studies on EBP50 in parenchymal liver diseases, such as hepatits C and NASH, should be performed to evaluate if it play a role in these diseases.

In the fourth study we evaluated the impact of alcohol over-consumption, diabetes, over-weight and viral hepatitis on development of liver fibrosis in psoriatic patients treated long-term with methotrexate. We used two thresholds for fibrosis; any fibrosis defined as stage I-IV and severe fibrosis defined as stage III or IV. The cut-off for severe fibrosis was set at stage III since it according to the current guidelines for methotrexate treatment, is recommended that this medication is stopped if the patient has liver fibrosis stage III.
Methotrexate induced liver fibrosis can be looked upon as a model of parenchymal liver injury. The histopathological appearance of methotrexate induced liver disease has

several similarities with steatohepatitis (Figure 5). This raised the question of to what extent risk factors for parenchymal liver injury contributed to liver fibrosis in methotrexate treated patients. The results showed that diabetes and overweight, both conditions that have been associated with increased oxidative stress and are regarded as the main contributors to non-alcohol fatty liver disease (NAFLD)[227, 228], clearly increased the risk of developing liver fibrosis. It has been suggested by results of *in vitro* experiments that increased oxidative stress contributes to methotrexate hepatotoxicity, both through increased reactive oxygen species activity and impaired anti-oxidative defence [229-231]. This is interesting as it implies that methotrexate treated patients could be more vulnerable to the fibrogenic effects of diabetes and obesity on the liver. It has also been noted that psoriasis is associated with metabolic diseases such as diabetes and the metabolic syndrome[232]. Recently it was shown that psoriasis patients had abnormal blood lipid profiles compared with non-psoriatics and suggested that these abnormalities were of genetic origin rather than aquired[233]. This may possibly explain why methotrexate induced fibrosis is more common in psoriasis patients than in patients with rheumatoid arthritis.

Recent studies on have suggested that epithelial mesenchymal transition induced by reactive oxygen species contribute to interstitial fibrosis in the kidney [234, 235]. The connection between EMT and oxidative stress will probably be an interesting field of research in studies of liver pathophysiology.

With this, we are back where this long journey started, where cells change their guise in response to their environment.

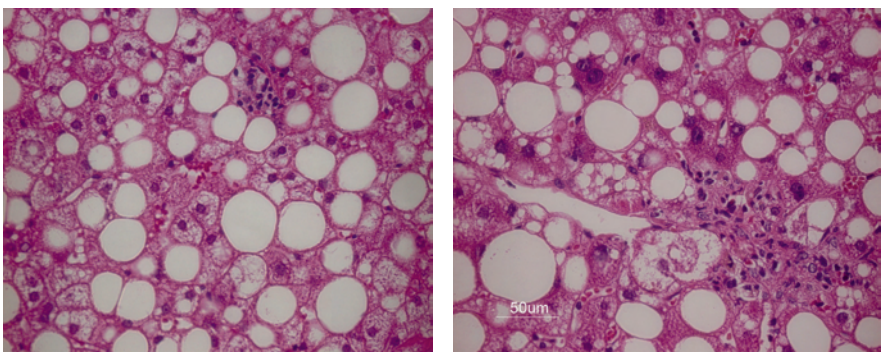


Figure 5: *Hematoxylin-eosin stained liver biopsies from a patient with non-alcohol steatohepatitis (left) and a patient with methotrexate induced liver injury (right). These pictures illustrate the very similar histological appearance with fatty infiltration, aggregates of inflammatory cells and ballooning degeneration of hepatocytes. Original magnification 200x.*

6 GENERAL CONCLUSIONS

(Study I) R-cadherin is expressed at critical stages of muscle and epithelia during embryonic development in mice. The expression of R-cadherin is associated with myoblasts fusion and mesenchymal-epithelial transition. R-cadherin rescued formation of epithelia and striated muscle in teratomas from E-cadherin -/- ES-cells. From this is concluded that R-cadherin can compensate for loss of E-cadherin function in formation of these tissues.

(Study II) Liver fibrosis induced by obstruction of the main bile duct, but not fibrosis induced by CCl4 parenchymal liver injury, is attenuated in N-CAM deficient mice. Hepatic stellate cells isolated from N-CAM deficient mice have impaired activation *in vitro.* From this is concluded that N-CAM contribute to fibrogenesis induced by obstruction of bile.

(Study III) EBP50 is normally localized in native bile ducts of patients with the ΔF508 mutation. From this is concluded that the aberrant subcellular localization of CFTR in native bile ducts of these patients not is dependant on EBP50.
EBP50 is delocalized to intracellular regions in proliferating cells of the ductular reaction. Suppression of EBP50 expression leads to decreased proliferation of cholangiocytes *in vitro*. This implicate that EBP50 is important in proliferation of biliary epithelial cells.

(Study IV) Presence of one or more of the risk factors; diabetes, over-weight, viral hepatitis and/or alcohol overconsumption, significantely increases the risk of developing liver fibrosis in patients treated long-term with low-dose methotrexate.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Kroniska leversjukdomar kan obehandlade i många fall leda till skrumplever vilket är ett tillstånd med hög risk för komplikationer och död. Skrumplever bildas genom långsam inlagring av bindväv i levern, i många fall utan att ge några symtom förrän leverfunktionen blivit avsevärt försämrad.
Adhesionsmolekyler är äggviteämnen (proteiner) i det yttre cellmembranet som ger cellen möjlighet att fästa till andra celler och stora molekyler i sin omgivning. Adhesionsmolekyler medverkar också vid överföringen av signaler till och från cellerna och är helt nödvändiga för bildandet av vävnader under fosterutvecklingen. Kunskap om vilken betydelse adhesionsmolekyler har för fibrosbildning i levern saknas till stor del. Studier av dessa basala mekanismer ger viktig kunskap som grund för utvecklingen av nya behandlingar.

I det första arbetet studerades förekomsten av adhesionsmolekylen R-cadherin under fosterutvecklingen i mus. R-cadherin kunde påvisas hos celler som senare bildade muskler och epitel. Vi kunde även visa att R-cadherin kunde få embryonala stamceller av en typ som normalt inte kan bilda vävnader att bilda muskel och epitel. Vår slutsats av dessa studier är att R-cadherin har betydelse för bildandet av dessa vävnader.

Adhesionmolekylen N-CAM uppträder vid leverskada hos celler som medverkar i läkningsprocessen och bildandet av bindväv. I det andra arbetet studerades om N-CAM har betydelse för bildandet av bindväv vid två olika typer av leverskada. Vi fann att möss som saknade N-CAM jämfört med vanliga möss hade mindre bindväv i levern efter gallgångsskada men lika mycket bindväv efter skada som främst drabbade levercellerna. Vi kunde även visa att så kallade leverstellatceller som saknade N-CAM hade försämrad förmåga att omvandlas till bindvävsbildande celler.

EBP50 binder till CFTR, vilket är det protein som är felaktigt vid cystisk fibros, och kopplar det till nätverket av proteiner i cellens inre. I det tredje arbetet studerade vi om EBP50 kunde bidra till att CFTR är felaktigt lokaliserat i gallgångscellerna hos patienter med CFTRΔ508F, den vanligaste mutationen vid cystisk fibros. Vi kunde dock inte finna stöd för detta. Däremot sågs EBP50 i kärnan och cytoplasman hos nybildade gallgångsliknande celler hos patienter med cystisk fibros men även vid flera andra gallvägssjukdomar. Cellförsök visade att inaktivering av EBP50 ledde till minskad tillväxt av gallgångsceller. Dessa resultat tyder på att EBP50 har betydelse vid bildandet av gallgångsceller.

I det fjärde arbetet studerades om diabetes, övervikt, alkohol-överkonsumtion och virushepatit ökade risken för skrumplever hos patienter med psoriasis som behandlades med metotrexat. De patienter i studien som hade dessa riskfaktorer visade sig ha klart ökad risk för skrumplever. Diabetes och övervikt verkade ha särskilt stor betydelse. Slutsatsen av denna studie är att metotrexatbehandlade psoriasispatienter med dessa riskfaktorer bör kontrolleras extra noga för fibrosutveckling i levern.

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9 REFERENCES

1. Friedman, S.L., *Mechanisms of disease: Mechanisms of hepatic fibrosis and therapeutic implications.* Nat Clin Pract Gastroenterol Hepatol, 2004. **1**(2): p. 98-105.
2. Friedman, S.L., *Liver fibrosis -- from bench to bedside.* J Hepatol, 2003. **38 Suppl 1**: p. S38-53.
3. WHO, *The World Health Report 2003: shaping the future. Geneva: World Health Organization.* 2003.
4. Shepard, C.W., L. Finelli, and M.J. Alter, *Global epidemiology of hepatitis C virus infection.* Lancet Infect Dis, 2005. **5**(9): p. 558-67.
5. Shepard, C.W., et al., *Hepatitis B virus infection: epidemiology and vaccination.* Epidemiol Rev, 2006. **28**: p. 112-25.
6. Perz, J.F., et al., *The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide.* J Hepatol, 2006. **45**(4): p. 529-38.
7. El-Serag, H.B. and K.L. Rudolph, *Hepatocellular carcinoma: epidemiology and molecular carcinogenesis.* Gastroenterology, 2007. **132**(7): p. 2557-76.
8. Kuiper, J.J., R.A. de Man, and H.R. van Buuren, *Review article: Management of ascites and associated complications in patients with cirrhosis.* Aliment Pharmacol Ther, 2007. **26 Suppl 2**: p. 183-93.
9. Martinez-Hernandez, A., *The hepatic extracellular matrix. II. Electron immunohistochemical studies in rats with CCl4-induced cirrhosis.* Lab Invest, 1985. **53**(2): p. 166-86.
10. Schuppan, D., et al., *Matrix as a modulator of hepatic fibrogenesis.* Semin Liver Dis, 2001. **21**(3): p. 351-72.
11. Kupfer, C., *Ueber Sternzellen der Leber. Briefliche Mitteilung an Prof. Waldeyer.* Arch Mikr Anat, 1876(12): p. 535-358.
12. Ito, T., *Cytological studies on stellate cells of Kupfer and fat storing cells in the capillary wall of the human liver.* Acta Anat Nippon, 1951. **26**(2).
13. Wake, K., *"Sternzellen" in the liver: perisinusoidal cells with special reference to storage of vitamin A.* Am J Anat, 1971. **132**(4): p. 429-62.
14. Wake, K., *Perisinusoidal stellate cells (fat-storing cells, interstitial cells, lipocytes), their related structure in and around the liver sinusoids, and vitamin A-storing cells in extrahepatic organs.* Int Rev Cytol, 1980. **66**: p. 303-53.
15. Rockey, D.C., *Hepatic blood flow regulation by stellate cells in normal and injured liver.* Semin Liver Dis, 2001. **21**(3): p. 337-49.
16. Pinzani, M., *Novel insights into the biology and physiology of the Ito cell.* Pharmacol Ther, 1995. **66**(2): p. 387-412.
17. Gressner, A.M., *Perisinusoidal lipocytes and fibrogenesis.* Gut, 1994. **35**(10): p. 1331-3.
18. Olaso, E. and S.L. Friedman, *Molecular regulation of hepatic fibrogenesis.* J Hepatol, 1998. **29**(5): p. 836-47.
19. Friedman, S.L., *Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury.* J Biol Chem, 2000. **275**(4): p. 2247-50.
20. Rockey, D.C., et al., *Cellular localization of endothelin-1 and increased production in liver injury in the rat: potential for autocrine and paracrine effects on stellate cells.* Hepatology, 1998. **27**(2): p. 472-80.
21. Pinzani, M., et al., *Effects of platelet-derived growth factor and other polypeptide mitogens on DNA synthesis and growth of cultured rat liver fat-storing cells.* J Clin Invest, 1989. **84**(6): p. 1786-93.
22. Bachem, M.G., et al., *Tumor necrosis factor alpha (TNF alpha) and transforming growth factor beta 1 (TGF beta 1) stimulate fibronectin synthesis and the transdifferentiation of fat-storing cells in the rat liver into myofibroblasts.* Virchows Arch B Cell Pathol Incl Mol Pathol, 1993. **63**(2): p. 123-30.

23. Matsuoka, M. and H. Tsukamoto, *Stimulation of hepatic lipocyte collagen production by Kupffer cell-derived transforming growth factor beta: implication for a pathogenetic role in alcoholic liver fibrogenesis.* Hepatology, 1990. **11**(4): p. 599-605.
24. Casini, A., et al., *Regulation of extracellular matrix synthesis by transforming growth factor beta 1 in human fat-storing cells.* Gastroenterology, 1993. **105**(1): p. 245-53.
25. Hernandez-Munoz, I., et al., *Tumor necrosis factor alpha inhibits collagen alpha 1(I) gene expression in rat hepatic stellate cells through a G protein.* Gastroenterology, 1997. **113**(2): p. 625-40.
26. Nieto, N., *Oxidative-stress and IL-6 mediate the fibrogenic effects of [corrected] Kupffer cells on stellate cells.* Hepatology, 2006. **44**(6): p. 1487-501.
27. Jarnagin, W.R., et al., *Expression of variant fibronectins in wound healing: cellular source and biological activity of the EIIIA segment in rat hepatic fibrogenesis.* J Cell Biol, 1994. **127**(6 Pt 2): p. 2037-48.
28. Benyon, R.C. and M.J. Arthur, *Extracellular matrix degradation and the role of hepatic stellate cells.* Semin Liver Dis, 2001. **21**(3): p. 373-84.
29. Issa, R., et al., *Apoptosis of hepatic stellate cells: involvement in resolution of biliary fibrosis and regulation by soluble growth factors.* Gut, 2001. **48**(4): p. 548-57.
30. Friedman, S.L., *Seminars in medicine of the Beth Israel Hospital, Boston. The cellular basis of hepatic fibrosis. Mechanisms and treatment strategies.* N Engl J Med, 1993. **328**(25): p. 1828-35.
31. Kinnman, N. and C. Housset, *Peribiliary myofibroblasts in biliary type liver fibrosis.* Front Biosci, 2002. **7**: p. d496-503.
32. Tuchweber, B., et al., *Proliferation and phenotypic modulation of portal fibroblasts in the early stages of cholestatic fibrosis in the rat.* Lab Invest, 1996. **74**(1): p. 265-78.
33. Beaussier, M., et al., *Prominent contribution of portal mesenchymal cells to liver fibrosis in ischemic and obstructive cholestatic injuries.* Lab Invest, 2007. **87**(3): p. 292-303.
34. Cassiman, D., et al., *Hepatic stellate cell/myofibroblast subpopulations in fibrotic human and rat livers.* J Hepatol, 2002. **36**(2): p. 200-9.
35. Kaimori, A., et al., *Transforming growth factor-beta1 induces an epithelial-to-mesenchymal transition state in mouse hepatocytes in vitro.* J Biol Chem, 2007. **282**(30): p. 22089-101.
36. Zeisberg, M., et al., *Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition.* J Biol Chem, 2007. **282**(32): p. 23337-47.
37. Cicchini, C., et al., *TGFbeta-induced EMT requires focal adhesion kinase (FAK) signaling.* Exp Cell Res, 2008. **314**(1): p. 143-52.
38. Rygiel, K.A., et al., *Epithelial-mesenchymal transition contributes to portal tract fibrogenesis during human chronic liver disease.* Lab Invest, 2008. **88**(2): p. 112-23.
39. Robertson, H., et al., *Biliary epithelial-mesenchymal transition in posttransplantation recurrence of primary biliary cirrhosis.* Hepatology, 2007. **45**(4): p. 977-81.
40. Kinnman, N., et al., *The myofibroblastic conversion of peribiliary fibrogenic cells distinct from hepatic stellate cells is stimulated by platelet-derived growth factor during liver fibrogenesis.* Lab Invest, 2003. **83**(2): p. 163-73.
41. Forbes, S.J., et al., *A significant proportion of myofibroblasts are of bone marrow origin in human liver fibrosis.* Gastroenterology, 2004. **126**(4): p. 955-63.
42. Russo, F.P., et al., *The bone marrow functionally contributes to liver fibrosis.* Gastroenterology, 2006. **130**(6): p. 1807-21.
43. Magness, S.T., et al., *A dual reporter gene transgenic mouse demonstrates heterogeneity in hepatic fibrogenic cell populations.* Hepatology, 2004. **40**(5): p. 1151-9.
44. Desmet, V., T. Roskams, and P. Van Eyken, *Ductular reaction in the liver.* Pathol Res Pract, 1995. **191**(6): p. 513-24.

45. Abdel-Aziz, G., et al., *Cellular sources of matrix proteins in experimentally induced cholestatic rat liver.* J Pathol, 1991. **164**(2): p. 167-74.
46. Milani, S., et al., *Cellular localization of type I III and IV procollagen gene transcripts in normal and fibrotic human liver.* Am J Pathol, 1990. **137**(1): p. 59-70.
47. Strazzabosco, M., L. Fabris, and C. Spirli, *Pathophysiology of cholangiopathies.* J Clin Gastroenterol, 2005. **39**(4 Suppl 2): p. S90-S102.
48. Milani, S., et al., *Transforming growth factors beta 1 and beta 2 are differentially expressed in fibrotic liver disease.* Am J Pathol, 1991. **139**(6): p. 1221-9.
49. Nagy, P., Z. Schaff, and K. Lapis, *Immunohistochemical detection of transforming growth factor-beta 1 in fibrotic liver diseases.* Hepatology, 1991. **14**(2): p. 269-73.
50. Pinzani, M., et al., *Expression of platelet-derived growth factor and its receptors in normal human liver and during active hepatic fibrogenesis.* Am J Pathol, 1996. **148**(3): p. 785-800.
51. Housset, C., et al., *Endothelin-1 secretion by human gallbladder epithelial cells in primary culture.* Lab Invest, 1993. **69**(6): p. 750-5.
52. Pinzani, M., et al., *Expression of platelet-derived growth factor in a model of acute liver injury.* Hepatology, 1994. **19**(3): p. 701-7.
53. Marra, F., et al., *Increased expression of monocyte chemotactic protein-1 during active hepatic fibrogenesis: correlation with monocyte infiltration.* Am J Pathol, 1998. **152**(2): p. 423-30.
54. Liu, Z., et al., *Interleukin-6, hepatocyte growth factor, and their receptors in biliary epithelial cells during a type I ductular reaction in mice: interactions between the periductal inflammatory and stromal cells and the biliary epithelium.* Hepatology, 1998. **28**(5): p. 1260-8.
55. Yasoshima, M., et al., *Increased expression of interleukin-6 and tumor necrosis factor-alpha in pathologic biliary epithelial cells: in situ and culture study.* Lab Invest, 1998. **78**(1): p. 89-100.
56. Alvaro, D., et al., *Proliferating cholangiocytes: a neuroendocrine compartment in the diseased liver.* Gastroenterology, 2007. **132**(1): p. 415-31.
57. Lazaridis, K.N., M. Strazzabosco, and N.F. Larusso, *The cholangiopathies: disorders of biliary epithelia.* Gastroenterology, 2004. **127**(5): p. 1565-77.
58. Alpini, G., P. RT., and N. LaRusso, *The liver: biology and pathobiology*. 4th ed, ed. I. Arias, et al. 2001: Lippincott Williams & Wilkins.
59. James, J., et al., *Application of keratin immunocytochemistry and sirius red staining in evaluating intrahepatic changes with acute extrahepatic cholestasis due to hepatic duct carcinoma.* Hepatogastroenterology, 1989. **36**(3): p. 151-5.
60. Alpini, G., et al., *Biliary physiology in rats with bile ductular cell hyperplasia. Evidence for a secretory function of proliferated bile ductules.* J Clin Invest, 1988. **81**(2): p. 569-78.
61. Popper, H.K., G Stein, R, *Ductular reaction in the liver in hepatic injury.* J Mt Sinai Hosp, 1957. **24**: p. 551-556.
62. Roskams, T.A., et al., *Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers.* Hepatology, 2004. **39**(6): p. 1739-45.
63. Zhou, H., et al., *Identification of hepatocytic and bile ductular cell lineages and candidate stem cells in bipolar ductular reactions in cirrhotic human liver.* Hepatology, 2007. **45**(3): p. 716-24.
64. Roskams, T., *Progenitor cell involvement in cirrhotic human liver diseases: from controversy to consensus.* J Hepatol, 2003. **39**(3): p. 431-4.
65. Theise, N.D., *Gastrointestinal stem cells. III. Emergent themes of liver stem cell biology: niche, quiescence, self-renewal, and plasticity.* Am J Physiol Gastrointest Liver Physiol, 2006. **290**(2): p. G189-93.
66. Theise, N.D., et al., *The canals of Hering and hepatic stem cells in humans.* Hepatology, 1999. **30**(6): p. 1425-33.
67. Clouston, A.D., et al., *Fibrosis correlates with a ductular reaction in hepatitis C: roles of impaired replication, progenitor cells and steatosis.* Hepatology, 2005. **41**(4): p. 809-18.

68. Hytiroglou, P., et al., *The canals of hering might represent a target of methotrexate hepatic toxicity.* Am J Clin Pathol, 2004. **121**(3): p. 324-9.
69. Richardson, M.M., et al., *Progressive fibrosis in nonalcoholic steatohepatitis: association with altered regeneration and a ductular reaction.* Gastroenterology, 2007. **133**(1): p. 80-90.
70. Somerville, R.P., S.A. Oblander, and S.S. Apte, *Matrix metalloproteinases: old dogs with new tricks.* Genome Biol, 2003. **4**(6): p. 216.
71. Hemmann, S., et al., *Expression of MMPs and TIMPs in liver fibrosis - a systematic review with special emphasis on anti-fibrotic strategies.* J Hepatol, 2007. **46**(5): p. 955-75.
72. Suzuki, K., et al., *Mechanisms of activation of tissue procollagenase by matrix metalloproteinase 3 (stromelysin).* Biochemistry, 1990. **29**(44): p. 10261-70.
73. Patsenker, E., et al., *Pharmacological inhibition of the vitronectin receptor abrogates PDGF-BB-induced hepatic stellate cell migration and activation in vitro.* J Hepatol, 2007. **46**(5): p. 878-87.
74. Riikonen, T., et al., *Integrin alpha 2 beta 1 is a positive regulator of collagenase (MMP-1) and collagen alpha 1(I) gene expression.* J Biol Chem, 1995. **270**(22): p. 13548-52.
75. Zhou, X., et al., *Engagement of alphavbeta3 integrin regulates proliferation and apoptosis of hepatic stellate cells.* J Biol Chem, 2004. **279**(23): p. 23996-4006.
76. Ylipalosaari, M., et al., *Alpha v beta 6 integrin down-regulates the MMP-13 expression in oral squamous cell carcinoma cells.* Exp Cell Res, 2005. **309**(2): p. 273-83.
77. Knittel, T., et al., *Expression patterns of matrix metalloproteinases and their inhibitors in parenchymal and non-parenchymal cells of rat liver: regulation by TNF-alpha and TGF-beta1.* J Hepatol, 1999. **30**(1): p. 48-60.
78. Watanabe, T., et al., *Gene expression of interstitial collagenase in both progressive and recovery phase of rat liver fibrosis induced by carbon tetrachloride.* J Hepatol, 2000. **33**(2): p. 224-35.
79. Ikeda, K., et al., *In vitro migratory potential of rat quiescent hepatic stellate cells and its augmentation by cell activation.* Hepatology, 1999. **29**(6): p. 1760-7.
80. Takahara, T., et al., *Dual expression of matrix metalloproteinase-2 and membrane-type 1-matrix metalloproteinase in fibrotic human livers.* Hepatology, 1997. **26**(6): p. 1521-9.
81. Iimuro, Y., et al., *Delivery of matrix metalloproteinase-1 attenuates established liver fibrosis in the rat.* Gastroenterology, 2003. **124**(2): p. 445-58.
82. Uchinami, H., et al., *Loss of MMP 13 attenuates murine hepatic injury and fibrosis during cholestasis.* Hepatology, 2006. **44**(2): p. 420-9.
83. Hironaka, K., et al., *Enhanced interstitial collagenase (matrix metalloproteinase-13) production of Kupffer cell by gadolinium chloride prevents pig serum-induced rat liver fibrosis.* Biochem Biophys Res Commun, 2000. **267**(1): p. 290-5.
84. Iredale, J.P., *Tissue inhibitors of metalloproteinases in liver fibrosis.* Int J Biochem Cell Biol, 1997. **29**(1): p. 43-54.
85. *19. Cell Junctions, Cell Adhesion, and the Extracellular Matrix*, in *Molecular Biology of the Cell - Fourth Edition*, A.J. Bruce Alberts, Julian Lewis, Martin Raff, Keith Roberts, Peter Walter, Editor. 2002, Garland Science.
86. Bissell, D.M., et al., *Support of cultured hepatocytes by a laminin-rich gel. Evidence for a functionally significant subendothelial matrix in normal rat liver.* J Clin Invest, 1987. **79**(3): p. 801-12.
87. Sperandio, M., *Selectins and glycosyltransferases in leukocyte rolling in vivo.* Febs J, 2006. **273**(19): p. 4377-89.
88. Yap, A.S., W.M. Brieher, and B.M. Gumbiner, *Molecular and functional analysis of cadherin-based adherens junctions.* Annu Rev Cell Dev Biol, 1997. **13**: p. 119-46.
89. Koch, A.W., et al., *Homophilic adhesion by cadherins.* Curr Opin Struct Biol, 1999. **9**(2): p. 275-81.

90. Halbleib, J.M. and W.J. Nelson, *Cadherins in development: cell adhesion, sorting, and tissue morphogenesis.* Genes Dev, 2006. **20**(23): p. 3199-214.
91. Kemler, R., *Classical cadherins.* Semin Cell Biol, 1992. **3**(3): p. 149-55.
92. Berx, G., et al., *Cloning and characterization of the human invasion suppressor gene E-cadherin (CDH1).* Genomics, 1995. **26**(2): p. 281-9.
93. Gallin, W.J., et al., *Sequence analysis of a cDNA clone encoding the liver cell adhesion molecule, L-CAM.* Proc Natl Acad Sci U S A, 1987. **84**(9): p. 2808-12.
94. Hazan, R.B., et al., *Cadherin switch in tumor progression.* Ann N Y Acad Sci, 2004. **1014**: p. 155-63.
95. Inuzuka, H., C. Redies, and M. Takeichi, *Differential expression of R- and N-cadherin in neural and mesodermal tissues during early chicken development.* Development, 1991. **113**(3): p. 959-67.
96. Takeichi, M., *The cadherin superfamily in neuronal connections and interactions.* Nat Rev Neurosci, 2007. **8**(1): p. 11-20.
97. Cifuentes-Diaz, C., et al., *N-cadherin expression in developing, adult and denervated chicken neuromuscular system: accumulations at both the neuromuscular junction and the node of Ranvier.* Development, 1994. **120**(1): p. 1-11.
98. Van Hoorde, L., K. Braet, and M. Mareel, *The N-cadherin/catenin complex in colon fibroblasts and myofibroblasts.* Cell Adhes Commun, 1999. **7**(2): p. 139-50.
99. Cavallaro, U., *N-cadherin as an invasion promoter: a novel target for antitumor therapy?* Curr Opin Investig Drugs, 2004. **5**(12): p. 1274-8.
100. Mariotti, A., et al., *N-cadherin as a therapeutic target in cancer.* Expert Opin Investig Drugs, 2007. **16**(4): p. 451-65.
101. Doi, Y., et al., *Development of complementary expression patterns of E- and N-cadherin in the mouse liver.* Hepatol Res, 2007. **37**(3): p. 230-7.
102. Kozyraki, R., et al., *Expression of cadherins and alpha-catenin in primary epithelial tumors of the liver.* Gastroenterology, 1996. **110**(4): p. 1137-49.
103. Murphy, F., et al., *N-Cadherin cleavage during activated hepatic stellate cell apoptosis is inhibited by tissue inhibitor of metalloproteinase-1.* Comp Hepatol, 2004. **3 Suppl 1**: p. S8.
104. Lim, Y.S., H.C. Lee, and H.S. Lee, *Switch of cadherin expression from E- to N-type during the activation of rat hepatic stellate cells.* Histochem Cell Biol, 2007. **127**(2): p. 149-60.
105. Khetani, S.R., et al., *Exploring interactions between rat hepatocytes and nonparenchymal cells using gene expression profiling.* Hepatology, 2004. **40**(3): p. 545-54.
106. Redies, C., H. Inuzuka, and M. Takeichi, *Restricted expression of N- and R-cadherin on neurites of the developing chicken CNS.* J Neurosci, 1992. **12**(9): p. 3525-34.
107. Inuzuka, H., S. Miyatani, and M. Takeichi, *R-cadherin: a novel Ca(2+)-dependent cell-cell adhesion molecule expressed in the retina.* Neuron, 1991. **7**(1): p. 69-79.
108. Sjodin, A., U. Dahl, and H. Semb, *Mouse R-cadherin: expression during the organogenesis of pancreas and gastrointestinal tract.* Exp Cell Res, 1995. **221**(2): p. 413-25.
109. Dahl, U., et al., *Genetic dissection of cadherin function during nephrogenesis.* Mol Cell Biol, 2002. **22**(5): p. 1474-87.
110. Fagman, H., et al., *Expression of classical cadherins in thyroid development: maintenance of an epithelial phenotype throughout organogenesis.* Endocrinology, 2003. **144**(8): p. 3618-24.
111. Huber, O., et al., *Nuclear localization of beta-catenin by interaction with transcription factor LEF-1.* Mech Dev, 1996. **59**(1): p. 3-10.
112. Orsulic, S., et al., *E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation.* J Cell Sci, 1999. **112 (Pt 8)**: p. 1237-45.
113. Behrens, J., et al., *Functional interaction of beta-catenin with the transcription factor LEF-1.* Nature, 1996. **382**(6592): p. 638-42.

114. Takeichi, M., *Morphogenetic roles of classic cadherins.* Curr Opin Cell Biol, 1995. **7**(5): p. 619-27.
115. Kintner, C., *Regulation of embryonic cell adhesion by the cadherin cytoplasmic domain.* Cell, 1992. **69**(2): p. 225-36.
116. Fujimori, T. and M. Takeichi, *Disruption of epithelial cell-cell adhesion by exogenous expression of a mutated nonfunctional N-cadherin.* Mol Biol Cell, 1993. **4**(1): p. 37-47.
117. Hermiston, M.L. and J.I. Gordon, *In vivo analysis of cadherin function in the mouse intestinal epithelium: essential roles in adhesion, maintenance of differentiation, and regulation of programmed cell death.* J Cell Biol, 1995. **129**(2): p. 489-506.
118. Dahl, U., A. Sjodin, and H. Semb, *Cadherins regulate aggregation of pancreatic beta-cells in vivo.* Development, 1996. **122**(9): p. 2895-902.
119. Larue, L., et al., *A role for cadherins in tissue formation.* Development, 1996. **122**(10): p. 3185-94.
120. Matsunami, H. and M. Takeichi, *Fetal brain subdivisions defined by R- and E-cadherin expressions: evidence for the role of cadherin activity in region-specific, cell-cell adhesion.* Dev Biol, 1995. **172**(2): p. 466-78.
121. Frisch, S.M. and H. Francis, *Disruption of epithelial cell-matrix interactions induces apoptosis.* J Cell Biol, 1994. **124**(4): p. 619-26.
122. Mitra, S.K. and D.D. Schlaepfer, *Integrin-regulated FAK-Src signaling in normal and cancer cells.* Curr Opin Cell Biol, 2006. **18**(5): p. 516-23.
123. Schuppan, D. and M. Ocker, *Integrin-mediated control of cell growth.* Hepatology, 2003. **38**(2): p. 289-91.
124. Zhang, H., et al., *Mechanism of beta 1-integrin-mediated hepatoma cell growth involves p27 and S-phase kinase-associated protein 2.* Hepatology, 2003. **38**(2): p. 305-13.
125. Rowe, S.M., S. Miller, and E.J. Sorscher, *Cystic fibrosis.* N Engl J Med, 2005. **352**(19): p. 1992-2001.
126. Katsumi, A., et al., *Integrins in mechanotransduction.* J Biol Chem, 2004. **279**(13): p. 12001-4.
127. Ditlevsen, D.K., et al., *NCAM-induced intracellular signaling revisited.* J Neurosci Res, 2007.
128. Ditlevsen, D.K., et al., *NCAM-induced intracellular signaling revisited.* J Neurosci Res, 2007. **86**(4): p. 727-743.
129. Yonekura, K., et al., *Liver-infiltrating CD56 positive T lymphocytes in hepatitis C virus infection.* Liver, 2000. **20**(5): p. 357-65.
130. Markovic-Lipkovski, J., et al., *Neural cell adhesion molecule expression on renal interstitial cells.* Nephrol Dial Transplant, 2007. **22**(6): p. 1558-66.
131. Gattenlohner, S., et al., *NCAM(CD56) and RUNX1(AML1) are up-regulated in human ischemic cardiomyopathy and a rat model of chronic cardiac ischemia.* Am J Pathol, 2003. **163**(3): p. 1081-90.
132. Esni, F., et al., *Neural cell adhesion molecule (N-CAM) is required for cell type segregation and normal ultrastructure in pancreatic islets.* J Cell Biol, 1999. **144**(2): p. 325-37.
133. Cremer, H., et al., *Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning.* Nature, 1994. **367**(6462): p. 455-9.
134. Stork, O., et al., *Anxiety and increased 5-HT1A receptor response in NCAM null mutant mice.* J Neurobiol, 1999. **40**(3): p. 343-55.
135. Roskams, T., et al., *Neuroendocrine features of reactive bile ductules in cholestatic liver disease.* Am J Pathol, 1990. **137**(5): p. 1019-25.
136. Torbenson, M., et al., *Bile ducts and ductules are positive for CD56 (N-CAM) in most cases of extrahepatic biliary atresia.* Am J Surg Pathol, 2003. **27**(11): p. 1454-7.
137. Knittel, T., et al., *Cell-type-specific expression of neural cell adhesion molecule (N-CAM) in Ito cells of rat liver. Up-regulation during in vitro activation and in hepatic tissue repair.* Am J Pathol, 1996. **149**(2): p. 449-62.
138. Knittel, T., et al., *Expression and regulation of cell adhesion molecules by hepatic stellate cells (HSC) of rat liver: involvement of HSC in recruitment of*

inflammatory cells during hepatic tissue repair. Am J Pathol, 1999. **154**(1): p. 153-67.
139. Van Den Heuvel, M.C., et al., *Expression of anti-OV6 antibody and anti-N-CAM antibody along the biliary line of normal and diseased human livers.* Hepatology, 2001. **33**(6): p. 1387-93.
140. Colombo, C., *Liver disease in cystic fibrosis.* Curr Opin Pulm Med, 2007. **13**(6): p. 529-36.
141. Lindblad, A., H. Glaumann, and B. Strandvik, *Natural history of liver disease in cystic fibrosis.* Hepatology, 1999. **30**(5): p. 1151-8.
142. Colombo, C., et al., *Liver disease in cystic fibrosis: A prospective study on incidence, risk factors, and outcome.* Hepatology, 2002. **36**(6): p. 1374-82.
143. Hultcrantz, R., S. Mengarelli, and B. Strandvik, *Morphological findings in the liver of children with cystic fibrosis: a light and electron microscopical study.* Hepatology, 1986. **6**(5): p. 881-9.
144. Lindblad, A., R. Hultcrantz, and B. Strandvik, *Bile-duct destruction and collagen deposition: a prominent ultrastructural feature of the liver in cystic fibrosis.* Hepatology, 1992. **16**(2): p. 372-81.
145. Blanc, W.A. and P.A. Di Sant'Agnese, *A distinctive type of biliary cirrhosis of the liver associated with cystic fibrosis of the pancreas; recognition through signs of portal hypertension.* Pediatrics, 1956. **18**(3): p. 387-409.
146. Oppenheimer, E.H. and J.R. Esterly, *Hepatic changes in young infants with cystic fibrosis: possible relation to focal biliary cirrhosis.* J Pediatr, 1975. **86**(5): p. 683-9.
147. Colombo, C., et al., *Analysis of risk factors for the development of liver disease associated with cystic fibrosis.* J Pediatr, 1994. **124**(3): p. 393-9.
148. Wilschanski, M., et al., *Clinical and genetic risk factors for cystic fibrosis-related liver disease.* Pediatrics, 1999. **103**(1): p. 52-7.
149. Castaldo, G., et al., *Liver expression in cystic fibrosis could be modulated by genetic factors different from the cystic fibrosis transmembrane regulator genotype.* Am J Med Genet, 2001. **98**(4): p. 294-7.
150. Chinet, T., et al., *Regulation of electrogenic anion secretion in normal and cystic fibrosis gallbladder mucosa.* Hepatology, 1999. **29**(1): p. 5-13.
151. Paumgartner, G. and U. Beuers, *Ursodeoxycholic acid in cholestatic liver disease: mechanisms of action and therapeutic use revisited.* Hepatology, 2002. **36**(3): p. 525-31.
152. Anderson, M.P., et al., *Demonstration that CFTR is a chloride channel by alteration of its anion selectivity.* Science, 1991. **253**(5016): p. 202-5.
153. Rommens, J.M., et al., *Identification of the cystic fibrosis gene: chromosome walking and jumping.* Science, 1989. **245**(4922): p. 1059-65.
154. Kerem, B., et al., *Identification of the cystic fibrosis gene: genetic analysis.* Science, 1989. **245**(4922): p. 1073-80.
155. Riordan, J.R., et al., *Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA.* Science, 1989. **245**(4922): p. 1066-73.
156. Ratjen, F. and G. Doring, *Cystic fibrosis.* Lancet, 2003. **361**(9358): p. 681-9.
157. Younger, J.M., et al., *Sequential quality-control checkpoints triage misfolded cystic fibrosis transmembrane conductance regulator.* Cell, 2006. **126**(3): p. 571-82.
158. Cohn, J.A., et al., *Localization of the cystic fibrosis transmembrane conductance regulator in human bile duct epithelial cells.* Gastroenterology, 1993. **105**(6): p. 1857-64.
159. Kinnman, N., et al., *Expression of cystic fibrosis transmembrane conductance regulator in liver tissue from patients with cystic fibrosis.* Hepatology, 2000. **32**(2): p. 334-40.
160. Strazzabosco, M., A. Zsembery, and L. Fabris, *Electrolyte transport in bile ductular epithelial cells.* J Hepatol, 1996. **24 Suppl 1**: p. 78-87.
161. Banales, J.M., J. Prieto, and J.F. Medina, *Cholangiocyte anion exchange and biliary bicarbonate excretion.* World J Gastroenterol, 2006. **12**(22): p. 3496-511.

162. Egan, M., et al., *Defective regulation of outwardly rectifying Cl- channels by protein kinase A corrected by insertion of CFTR.* Nature, 1992. **358**(6387): p. 581-4.
163. Stutts, M.J., et al., *CFTR as a cAMP-dependent regulator of sodium channels.* Science, 1995. **269**(5225): p. 847-50.
164. Dray-Charier, N., et al., *Expression of delta F508 cystic fibrosis transmembrane conductance regulator protein and related chloride transport properties in the gallbladder epithelium from cystic fibrosis patients.* Hepatology, 1999. **29**(6): p. 1624-34.
165. Weinman, E.J., D. Steplock, and S. Shenolikar, *CAMP-mediated inhibition of the renal brush border membrane Na+-H+ exchanger requires a dissociable phosphoprotein cofactor.* J Clin Invest, 1993. **92**(4): p. 1781-6.
166. Bretscher, A., et al., *ERM-Merlin and EBP50 protein families in plasma membrane organization and function.* Annu Rev Cell Dev Biol, 2000. **16**: p. 113-43.
167. Fouassier, L., et al., *Ezrin-radixin-moesin-binding phosphoprotein 50 is expressed at the apical membrane of rat liver epithelia.* Hepatology, 2001. **33**(1): p. 166-76.
168. Ingraffea, J., D. Reczek, and A. Bretscher, *Distinct cell type-specific expression of scaffolding proteins EBP50 and E3KARP: EBP50 is generally expressed with ezrin in specific epithelia, whereas E3KARP is not.* Eur J Cell Biol, 2002. **81**(2): p. 61-8.
169. Haggie, P.M., B.A. Stanton, and A.S. Verkman, *Increased diffusional mobility of CFTR at the plasma membrane after deletion of its C-terminal PDZ binding motif.* J Biol Chem, 2004. **279**(7): p. 5494-500.
170. Voltz, J.W., E.J. Weinman, and S. Shenolikar, *Expanding the role of NHERF, a PDZ-domain containing protein adapter, to growth regulation.* Oncogene, 2001. **20**(44): p. 6309-14.
171. Stemmer-Rachamimov, A.O., et al., *NHE-RF, a merlin-interacting protein, is primarily expressed in luminal epithelia, proliferative endometrium, and estrogen receptor-positive breast carcinomas.* Am J Pathol, 2001. **158**(1): p. 57-62.
172. Shibata, T., et al., *EBP50, a beta-catenin-associating protein, enhances Wnt signaling and is over-expressed in hepatocellular carcinoma.* Hepatology, 2003. **38**(1): p. 178-86.
173. Cardone, R.A., et al., *The NHERF1 PDZ2 domain regulates PKA-RhoA-p38-mediated NHE1 activation and invasion in breast tumor cells.* Mol Biol Cell, 2007. **18**(5): p. 1768-80.
174. Song, J., et al., *Expression and clinicopathological significance of oestrogen-responsive ezrin-radixin-moesin-binding phosphoprotein 50 in breast cancer.* Histopathology, 2007. **51**(1): p. 40-53.
175. Maudsley, S., et al., *Platelet-derived growth factor receptor association with Na(+)/H(+) exchanger regulatory factor potentiates receptor activity.* Mol Cell Biol, 2000. **20**(22): p. 8352-63.
176. He, J., et al., *Phosphorylation and cell cycle-dependent regulation of Na+/H+ exchanger regulatory factor-1 by Cdc2 kinase.* J Biol Chem, 2001. **276**(45): p. 41559-65.
177. Lazar, C.S., et al., *The Na+/H+ exchanger regulatory factor stabilizes epidermal growth factor receptors at the cell surface.* Mol Biol Cell, 2004. **15**(12): p. 5470-80.
178. Kreimann, E.L., et al., *Cortical stabilization of beta-catenin contributes to NHERF1/EBP50 tumor suppressor function.* Oncogene, 2007. **26**(36): p. 5290-9.
179. Pan, Y., E.J. Weinman, and J. Dai, *NHERF1 (Na+/H+ exchanger regulatory factor 1) inhibits platelet-derived growth factor signaling in breast cancer cells.* Breast Cancer Res, 2008. **10**(1): p. R5.
180. Pan, Y., L. Wang, and J.L. Dai, *Suppression of breast cancer cell growth by Na+/H+ exchanger regulatory factor 1 (NHERF1).* Breast Cancer Res, 2006. **8**(6): p. R63.

181. Christophers, E., *Psoriasis--epidemiology and clinical spectrum.* Clin Exp Dermatol, 2001. **26**(4): p. 314-20.
182. Krueger, G. and C.N. Ellis, *Psoriasis--recent advances in understanding its pathogenesis and treatment.* J Am Acad Dermatol, 2005. **53**(1 Suppl 1): p. S94-100.
183. Griffiths, C.E. and J.N. Barker, *Pathogenesis and clinical features of psoriasis.* Lancet, 2007. **370**(9583): p. 263-71.
184. Menter, A. and C.E. Griffiths, *Current and future management of psoriasis.* Lancet, 2007. **370**(9583): p. 272-84.
185. Bertino, J.R., *The Mechanism of Action of the Folate Antagonists in Man.* Cancer Res, 1963. **23**: p. 1286-306.
186. Roenigk, H.H., Jr., H.I. Maibach, and G.D. Weinstein, *Use of methotrexate in psoriasis.* Arch Dermatol, 1972. **105**(3): p. 363-5.
187. Roenigk, H., *Methotrexate*, in *Psoriasis*, D. L, Editor. 1994, ISED Publishing Co: Brescia, Italy. p. 162–173.
188. Roenigk, H.H., Jr., et al., *Methotrexate in psoriasis: consensus conference.* J Am Acad Dermatol, 1998. **38**(3): p. 478-85.
189. Aithal, G.P., et al., *Monitoring methotrexate-induced hepatic fibrosis in patients with psoriasis: are serial liver biopsies justified?* Aliment Pharmacol Ther, 2004. **19**(4): p. 391-9.
190. Ahern, M.J., M.D. Smith, and P.J. Roberts-Thomson, *Methotrexate hepatotoxicity: what is the evidence?* Inflamm Res, 1998. **47**(4): p. 148-51.
191. Berends, M.A., et al., *Reliability of the Roenigk classification of liver damage after methotrexate treatment for psoriasis: a clinicopathologic study of 160 liver biopsy specimens.* Arch Dermatol, 2007. **143**(12): p. 1515-9.
192. Bravo, A.A., S.G. Sheth, and S. Chopra, *Liver biopsy.* N Engl J Med, 2001. **344**(7): p. 495-500.
193. Whiting-O'Keefe, Q.E., K.H. Fye, and K.D. Sack, *Methotrexate and histologic hepatic abnormalities: a meta-analysis.* Am J Med, 1991. **90**(6): p. 711-6.
194. Zachariae, H., *Liver biopsies and methotrexate: a time for reconsideration?* J Am Acad Dermatol, 2000. **42**(3): p. 531-4.
195. Hatta, K. and M. Takeichi, *Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development.* Nature, 1986. **320**(6061): p. 447-9.
196. Pape, L., et al., *Computer-assisted quantification of fibrosis in chronic allograft nephropaty by picosirius red-staining: a new tool for predicting long-term graft function.* Transplantation, 2003. **76**(6): p. 955-8.
197. Bergheim, I., et al., *Critical role of plasminogen activator inhibitor-1 in cholestatic liver injury and fibrosis.* J Pharmacol Exp Ther, 2006. **316**(2): p. 592-600.
198. Glagoleff, A., *On the geometrical methods of quantitative mineralogic analysis of rockes.* Trans Inst Econ Min Moscow, 1933.
199. Volmer, J. and C.J. Luders, *Morphometric investigations on the portal tracts of the liver, the differentiation of variable progression in chronic persistent hepatitis.* Virchows Arch A Pathol Anat Histol, 1981. **392**(3): p. 321-37.
200. Kleiner, D.E., et al., *Design and validation of a histological scoring system for nonalcoholic fatty liver disease.* Hepatology, 2005. **41**(6): p. 1313-21.
201. Menghini, G., *One-second needle biopsy of the liver.* Gastroenterology, 1958. **35**(2): p. 190-9.
202. Doetschman, T.C., et al., *The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium.* J Embryol Exp Morphol, 1985. **87**: p. 27-45.
203. Larue, L., et al., *E-cadherin null mutant embryos fail to form a trophectoderm epithelium.* Proc Natl Acad Sci U S A, 1994. **91**(17): p. 8263-7.
204. Mencin, A., et al., *Alpha-1 antitrypsin Z protein (PiZ) increases hepatic fibrosis in a murine model of cholestasis.* Hepatology, 2007. **46**(5): p. 1443-52.
205. Miyahara, T., et al., *Peroxisome proliferator-activated receptors and hepatic stellate cell activation.* J Biol Chem, 2000. **275**(46): p. 35715-22.

206. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Anal Biochem, 1976. **72**: p. 248-54.
207. Wandzioch, E., et al., *Lhx2-/- mice develop liver fibrosis.* Proc Natl Acad Sci U S A, 2004. **101**(47): p. 16549-54.
208. Zeschnigk, M., et al., *Involvement of M-cadherin in terminal differentiation of skeletal muscle cells.* J Cell Sci, 1995. **108 (Pt 9)**: p. 2973-81.
209. Charlton, C.A., et al., *Fusion competence of myoblasts rendered genetically null for N-cadherin in culture.* J Cell Biol, 1997. **138**(2): p. 331-6.
210. Kaufmann, U., et al., *M-cadherin and its sisters in development of striated muscle.* Cell Tissue Res, 1999. **296**(1): p. 191-8.
211. Alwayn, I.P., et al., *A Critical Role for Matrix Metalloproteinases in Liver Regeneration.* J Surg Res, 2008.
212. Pagenstecher, A., et al., *Regulation of matrix metalloproteinases and their inhibitor genes in lipopolysaccharide-induced endotoxemia in mice.* Am J Pathol, 2000. **157**(1): p. 197-210.
213. Kinnman, N., et al., *PDGF-mediated chemoattraction of hepatic stellate cells by bile duct segments in cholestatic liver injury.* Lab Invest, 2000. **80**(5): p. 697-707.
214. Kevat, S., M. Ahern, and P. Hall, *Hepatotoxicity of methotrexate in rheumatic diseases.* Med Toxicol Adverse Drug Exp, 1988. **3**(3): p. 197-208.
215. Tilling, L., S. Townsend, and J. David, *Methotrexate and hepatic toxicity in rheumatoid arthritis and psoriatic arthritis.* Clin Drug Investig, 2006. **26**(2): p. 55-62.
216. Mah, S.P., et al., *Kidney development in cadherin-6 mutants: delayed mesenchyme-to-epithelial conversion and loss of nephrons.* Dev Biol, 2000. **223**(1): p. 38-53.
217. Radice, G.L., et al., *Precocious mammary gland development in P-cadherin-deficient mice.* J Cell Biol, 1997. **139**(4): p. 1025-32.
218. Horikawa, K., et al., *Adhesive subdivisions intrinsic to the epithelial somites.* Dev Biol, 1999. **215**(2): p. 182-9.
219. Radice, G.L., et al., *Developmental defects in mouse embryos lacking N-cadherin.* Dev Biol, 1997. **181**(1): p. 64-78.
220. Johnson, E., et al., *R-cadherin influences cell motility via Rho family GTPases.* J Biol Chem, 2004. **279**(30): p. 31041-9.
221. Maeda, M., et al., *Expression of inappropriate cadherins by epithelial tumor cells promotes endocytosis and degradation of E-cadherin via competition for p120(ctn).* Oncogene, 2006. **25**(33): p. 4595-604.
222. Slater, S.C., et al., *R-cadherin:beta-catenin complex and its association with vascular smooth muscle cell proliferation.* Arterioscler Thromb Vasc Biol, 2004. **24**(7): p. 1204-10.
223. Iredale, J.P., *Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ.* J Clin Invest, 2007. **117**(3): p. 539-48.
224. Wang, B., et al., *Role of alphavbeta6 integrin in acute biliary fibrosis.* Hepatology, 2007. **46**(5): p. 1404-12.
225. Cavallaro, U., et al., *N-CAM modulates tumour-cell adhesion to matrix by inducing FGF-receptor signalling.* Nat Cell Biol, 2001. **3**(7): p. 650-7.
226. Beggs, H.E., et al., *NCAM140 interacts with the focal adhesion kinase p125(fak) and the SRC-related tyrosine kinase p59(fyn).* J Biol Chem, 1997. **272**(13): p. 8310-9.
227. Eriksson, J.W., *Metabolic stress in insulin's target cells leads to ROS accumulation - a hypothetical common pathway causing insulin resistance.* FEBS Lett, 2007. **581**(19): p. 3734-42.
228. Angulo, P., *Obesity and nonalcoholic fatty liver disease.* Nutr Rev, 2007. **65**(6 Pt 2): p. S57-63.
229. Jahovic, N., et al., *Melatonin prevents methotrexate-induced hepatorenal oxidative injury in rats.* J Pineal Res, 2003. **34**(4): p. 282-7.
230. Neuman, M.G., et al., *Inducers of cytochrome P450 2E1 enhance methotrexate-induced hepatocytoxicity.* Clin Biochem, 1999. **32**(7): p. 519-36.

231. Cetiner, M., et al., *Taurine protects against methotrexate-induced toxicity and inhibits leukocyte death.* Toxicol Appl Pharmacol, 2005. **209**(1): p. 39-50.
232. Mallbris, L., C.T. Ritchlin, and M. Stahle, *Metabolic disorders in patients with psoriasis and psoriatic arthritis.* Curr Rheumatol Rep, 2006. **8**(5): p. 355-63.
233. Mallbris, L., et al., *Psoriasis is associated with lipid abnormalities at the onset of skin disease.* J Am Acad Dermatol, 2006. **54**(4): p. 614-21.
234. Djamali, A., *Oxidative stress as a common pathway to chronic tubulointerstitial injury in kidney allografts.* Am J Physiol Renal Physiol, 2007. **293**(2): p. F445-55.
235. Zhang, A., et al., *Aldosterone induces epithelial-mesenchymal transition via ROS of mitochondrial origin.* Am J Physiol Renal Physiol, 2007. **293**(3): p. F723-31.