

## New Insights in the Roadmap of Liver Fibrosis Pathogenesis

Eman El-Ahwany<sup>1</sup> and Mona Zoheiry<sup>1</sup>

<sup>1</sup>Immunology Department, Theodor Bilharz Research Institute

### Abstract

Liver fibrosis is a wound healing scar response following acute and chronic liver diseases including chronic hepatitis B and C, autoimmune hepatitis, non-alcoholic steatohepatitis and alcoholic liver disease. The patho-histological findings of liver cirrhosis, the end-stage of liver fibrosis, show hepatocellular death, a lobular inflammatory cell infiltrate, excessive deposition of extracellular matrix (ECM) proteins, and the appearance of regenerative nodules that may result in liver failure, portal hypertension and hepatocellular carcinoma.

The pathophysiology of liver injury has attracted the interest of experimentalists and clinicians over many centuries. With the discovery of liver- HSC, the insight into the cellular and molecular pathobiology of liver fibrosis has evolved and the pivotal role of Hepatic Stellate cell (HSC) as a precursor cell-type for ECM-producing myofibroblasts has been established.. Although activation and trans-differentiation of HSC to myofibroblasts (MFs) is still regarded as the pathogenetic key mechanism of fibrogenesis, recent studies point to a prominent heterogeneity of the origin of myofibroblasts .

The newly discovered pathways supplement the concept of HSC activation to myofibroblasts (MFs), point to fibrosis as a systemic response involving extrahepatic organs (lung and kidney) and reactions, and offer innovative approaches for the development of non-invasive biomarkers and anti-fibrotic agents.

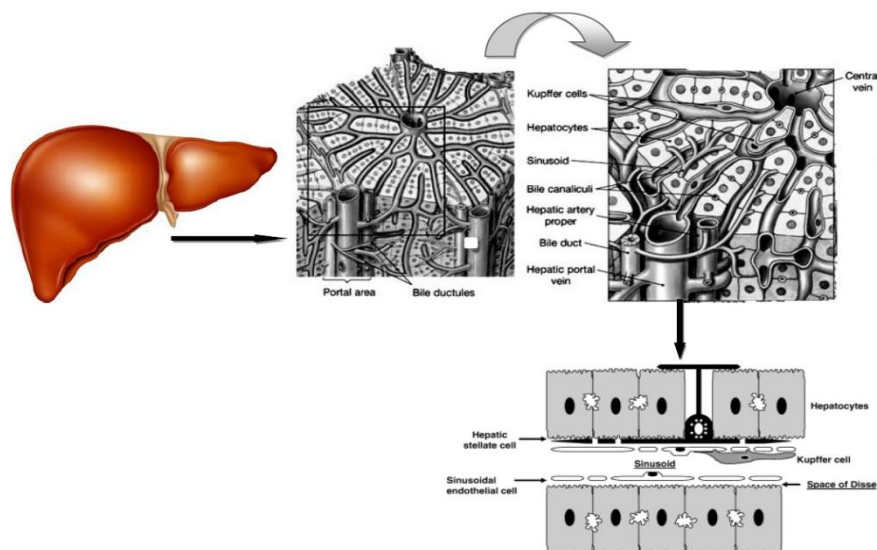
**Key words: Liver Fibrosis – Myofibroblasts - Epithelial Mesenchymal Transition**

### Introduction

The liver holds a unique position with regard to the blood circulation and it receives venous blood draining from almost the entire gastrointestinal tract via the hepatic artery. It is a meeting point for antigens and leukocytes circulating in the blood <sup>[1]</sup>. Among the many functions of the liver, clearance of the blood from macromolecules and its mobilization are important for the understanding of the liver as an immune-regulatory organ <sup>[2]</sup>.

The liver lobule is divided into both hexagonal form of parenchymal cells which are the hepatocytes and non-parenchymal cells <sup>[3]</sup>. In contrast to hepatocytes that occupy almost 80% of the total liver volume and perform the majority of numerous liver functions, non-parenchymal liver cells,

contribute only to 6.5% of the liver volume, and 40% of the total number of liver cells, are localized in the sinusoidal compartment of the hepatic tissue <sup>[1]</sup>. The walls of hepatic sinusoid are lined by three different cell types: Sinusoidal endothelial cells (SECs), Kupffer cells (KCs), and hepatic stellate cells (HSCs), formerly known as fat-storing cells, Ito cells, lipocytes, peri-sinusoidal cells, or vitamin A-rich cells <sup>[4]</sup>. Additionally, intrahepatic lymphocytes (IHL), including pit cells, i.e., liver-specific natural killer cells, are often present in the sinusoidal lumen <sup>[5]</sup>. It has been increasingly recognized that both under normal and pathological conditions, many hepatocyte functions are regulated by substances released from neighboring non-parenchymal cells <sup>[2]</sup> (Fig.1).



**Figure (1):** A diagrammatic view of liver structure showing a typical liver lobule has a hexagonal shape in cross section. The hepatocytes in a liver lobule form a series of irregular plates formed of one cell thick, and exposed hepatocyte surfaces are covered with short microvilli. Hepatic Stellate Cells are located in the subendothelial space (Disse's space which is separated from the lumen by the fenestrated endothelium Kupffer cells). During liver fibrosis Stellate Cells deposit large amounts of fibril forming (scar) matrix. This deposition leads to loss of hepatocytic microvilli and sinusoidal fenestrae which results in deterioration of hepatic function.

Hepatic stellate cells approximately account for 5-8% of total cells in normal liver. Normally, HSCs are characterized by storing vitamin A, controlling the turnover of extracellular matrix (ECM), and the regulation of the contractility of sinusoids<sup>[6, 7, 8]</sup>. Acute damage to hepatocytes induces transformation of the quiescent HSCs into activated myofibroblast (MF)-like cells that play a key role in the development of inflammatory responses<sup>[1]</sup>. Activated HSCs trans-differentiate into proliferative, fibrogenic, and contractile myofibroblasts that initiate further cell proliferation and increase the deposition of ECM components in the process of wound healing<sup>[2, 9]</sup>.

The wound healing scar response in the liver represents a harmful response rather than a beneficial response in liver regeneration<sup>[10, 11]</sup>. Liver fibrosis is highly associated with chronic hepatocellular injury and subsequent inflammatory response that produces inflammatory cytokines and recruits inflammatory leukocytes into the injured site<sup>[5]</sup>. This inflammatory circumstance in the liver drives the activation of hepatic stellate cells (HSCs) through various fibrogenic mediators

including transforming growth factor-beta (TGF- $\beta$ ) and platelet-derived growth factor (PDGF)<sup>[6, 8]</sup>.

Currently, the generation of matrix-synthesizing fibroblasts by epithelial-mesenchymal transition (EMT), by influx of bone marrow-derived fibrocytes into damaged liver tissue, and by differentiation of circulating monocytes to fibroblasts after homing in the injured liver is discussed as important complementary mechanisms to enlarge the pool of MFs in the fibrosing liver<sup>[6, 12]</sup>.

## 1- THE PATHOGENETIC ROADMAP OF LIVER FIBROSIS

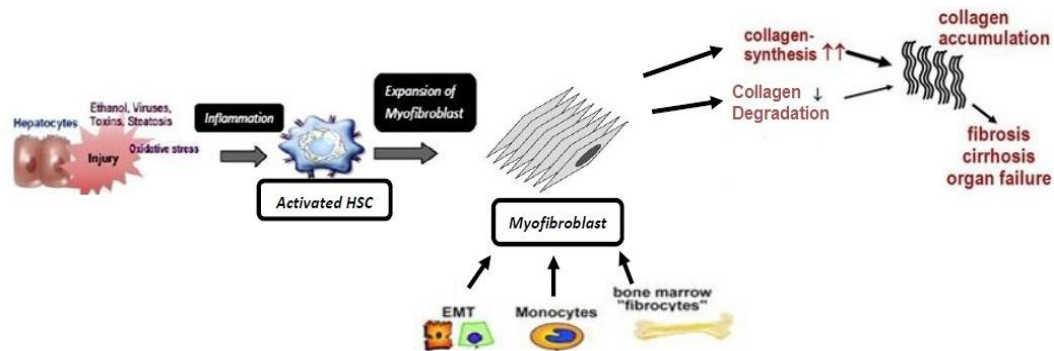
### “Established and New Insights”

#### 1.1. The Canonical Principle of Liver Fibrogenesis

Fibrosis is characterized by a several-fold increase of the ECM that comprises collagens, structural glycoproteins, sulphated proteoglycans and hyaluronan<sup>[1]</sup>. In addition, a histological redistribution with preferred initial matrix deposition in the subendothelial space of Disse, leads to the formation of an incomplete subendothelial basement membrane, creating additional diffusion barriers between hepatocytes

and the liver sinusoid ("capillarization of sinusoids")<sup>[6]</sup>. This is followed by changes in the microstructure of collagens (e.g., degree of hydroxylation of prolin and lysin), glycoproteins (variations of the carbohydrate structure) and proteoglycans (changes of the degree of sulfation

of the glycosaminoglycan side chains)<sup>[5]</sup>. It has been known for a long time that the increase of ECM in the parenchyma is not a passive process caused by condensation of pre-existing septa of connective tissue due to necrotic and apoptotic collapse of the parenchyma<sup>[13]</sup> (Fig. 2).



**Figure (2): Liver fibrosis (Fibrogenesis)** starts with injury, necrosis or apoptosis of hepatocytes and inflammation-connected activation of hepatic stellate cells (HSC) triggering their trans-differentiation to myofibroblasts. New pathogenetic mechanisms concern the influx of bone marrow-derived cells (Fibrocytes) and of circulating monocytes and their TGF- $\beta$  driven differentiation to fibroblasts in the damaged liver tissue. A further new mechanism is epithelial-mesenchymal transition (EMT) of bile duct epithelial cells and potentially of hepatocytes. All of these three complementary mechanisms enlarge the pool of matrix-synthesizing myofibroblasts in the damaged liver. The myofibroblasts increase collagen synthesis and decreased its degradation. This leads to disturbance of the balance between synthesis & degradation leading to extracellular accumulation, liver fibrosis, necrosis and finally organ failure.

The development of MFs is the result of a multi-step sequence, which originates from liver cell necrosis induced by various noxious agents (toxic/immunologic)<sup>[6]</sup>.

Experimental and clinical studies of the past twenty years provide a detailed knowledge of structure and composition of ECM in normal and fibrotic liver tissue, and of the cellular origin of the various matrix components<sup>[12]</sup>.

Studies, also, focus on the cytokine- and growth factor-regulated stimulation of ECM synthesis (fibrogenesis) and regulation of matrix degradation (fibrolysis), in several conditions predisposing for fibrogenesis<sup>[14]</sup>. However, the clinical benefit derived from basic research is scarce with regard to an effective, harmless and site-directed anti-fibrotic therapy and approved non-invasive diagnostic measures of the activity of fibrogenesis ("grading") and/or of the extent of the fibrotic organ transition ("staging") using serum parameters<sup>[5]</sup>.

The failure of clinical success boosts current research on fibrosis and fibrogenesis not only of the liver, but also of the lung, kidney, pancreas,

heart, skin, bone marrow, and other organs. During the last four to five years, very important new insights into the pathogenesis of fibrosis and of related diagnostic and therapeutic options have been made<sup>[11]</sup>. Evolving patho-genetic concepts supplement the so called "canonical principle" of liver fibrogenesis, which is based on the activation of hepatic stellate cells (HSCs)<sup>[6, 12]</sup>.

## 1.2. Role of Hepatic Stellate Cells (HSCs) in Liver Fibrogenesis

HSCs are liver pericytes, which embrace with thorn like micro-projections the endothelial cell layer of the sinusoids providing physical contact not only to sinusoidal endothelial cells, but also with the cell body to the hepatocytes<sup>[15]</sup>. The spindle-like cell body of HSC contains multiple triglyceride-rich vacuoles, in which vitamin A metabolites (retinoids) are dissolved and stored. About 85% of the vitamin A of the liver is found in HSCs<sup>[16]</sup>.

HSC activation can be conceptually divided into two phases: Initiation and perpetuation<sup>[15]</sup>.

Initiation, also known as the pre-inflammatory stage, refers to early changes in gene expression and phenotype. It is the result of primarily paracrine stimulation from damaged parenchymal cells. Maintenance of these stimuli leads to a perpetuation phase regulated by autocrine and paracrine stimuli. Perpetuation involves at least six distinct changes in HSC behavior, including proliferation, chemotaxis, fibrogenesis, HSCs contractility, matrix degradation and retinoid loss<sup>[17]</sup>.

The activation of HSC leads to the expression of  $\alpha$ -smooth-muscle actin and a loss of fat vacuoles combined with a decrease of retinoids<sup>[1]</sup>. However, this increases HSC contractility and strengthens their capacity to express and secrete a broad spectrum of matrix components<sup>[18]</sup>. The activation process includes proliferation and phenotypic trans-differentiation of HSC to MF, but both processes are not causally related<sup>[19]</sup>.

In the "canonical principle" of fibrogenesis., HSCs-derived MFs have the core competency not only for matrix synthesis, but also for the expression and secretion of numerous pro- and anti-inflammatory cytokines and growth factors<sup>[20]</sup>.

HSCs also have a highly synthetic phenotype, characterized by a hypertrophic rough endoplasmic reticulum containing ribosomes necessary for the synthesis of export proteins<sup>[6]</sup>. The mechanism of fibrogenic activation and trans-differentiation of HSC to MFs can be summarized in a three-step cascade model<sup>[5]</sup>, which is initiated by the **pre-inflammatory phase** due to direct paracrine activation of HSCs by necrotic hepatocytes with release of activating cytokines supplemented by a loss of mitoinhibitory cell surface heparan sulfate. The growth promoting activity of hepatocytes, partially due to IGF-1 and respective IGF-binding protein release and parallels the elevation of lactate dehydrogenase and aspartate aminotransferase<sup>[6]</sup>.

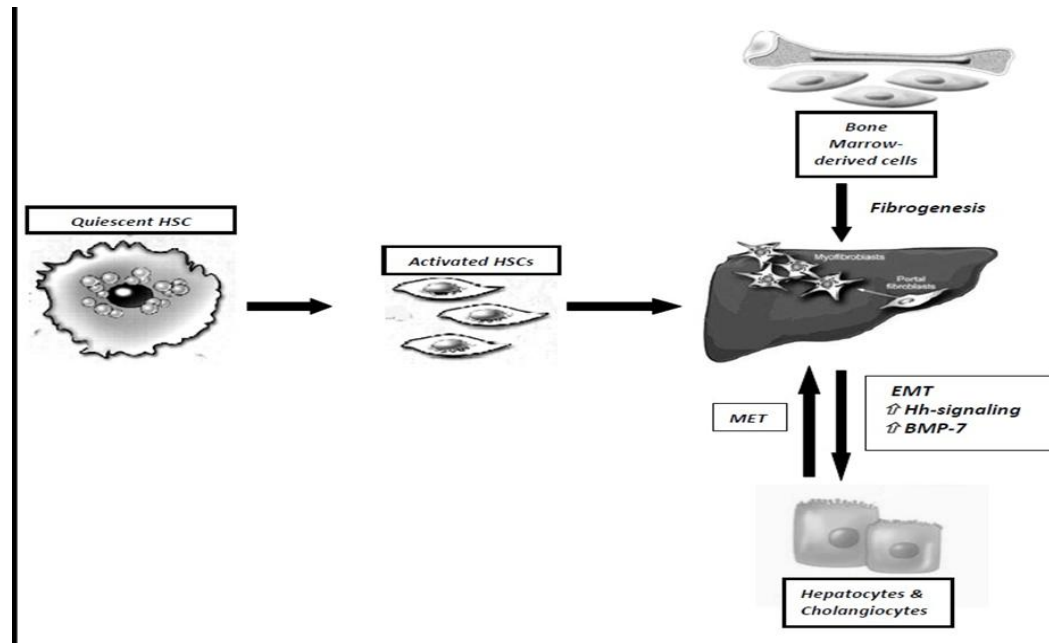
In the following **inflammatory phase**, the pre-activated HSCs are further stimulated in a paracrine mode by invaded leukocytes and thrombocytes, by activated KCs<sup>[21]</sup>, SECs and hepatocytes<sup>[5]</sup> to trans-differentiate to MFs. The consecutive **post-inflammatory phase** is characterized by the secretion of stimulating cytokines from MF and interacting matrix components<sup>[6]</sup>. Some of these cytokines can stimulate in an autocrine way MFs and in a paracrine mode resting HSC<sup>[8]</sup>.

The post-inflammatory phase contributes significantly to the perpetuation of the fibrogenic process, even after elimination or reduction of the pre-inflammatory and inflammatory phases. Most relevant cellular mediators are reactive oxygen species (hydroxyl radicals, oxygen radicals, superoxide anions, hydrogen peroxide) produced by activated KCs<sup>[22]</sup>, the stimulated nicotinamide adenine dinucleotide phosphate (NAD (P) H) oxidase activity of HSCs<sup>[23]</sup>, phagocytosing apoptotic bodies<sup>[24]</sup>, the cytochrome pathway of ethanol-metabolizing hepatocytes], and leukocytes<sup>[25]</sup>. In addition, tissue hypoxia promotes the activation of HSC<sup>[26]</sup>.

Additional functions of HSCs have recently been discovered. They seem to play a role as APCs<sup>[27]</sup>, as CD133<sup>+</sup> progenitor cells with the ability to differentiate to progenitor endothelial cells and hepatocytes suggesting important roles in liver regeneration and repair<sup>[28]</sup>.

## 2. EMERGING CONCEPTS FOR THE MECHANISMS OF LIVER FIBROSIS

Myofibroblasts (MFs) are the prototypical mesenchymal cell type regulating repair following injury in a range of tissues, including liver, kidney, skin, lung, and bone marrow, as well as the central nervous system<sup>[25]</sup>. MFs are defined primarily by their ability to produce ECM and exhibit contractile activity. Although, HSCs are the primary source of this fibrogenic population in the liver<sup>[12, 26]</sup>, contributions from other cells are increasingly being appreciated (Fig. 3).



**Figure 3:** Sources of extracellular matrix: Liver fibrosis is characterized by the proliferation of contractile and fibrogenic myofibroblasts. The primary and best-characterized source of MFs is activated hepatic stellate cells; other cells may also transdifferentiate into MFs, although their exact contribution to human disease remains unclear. These cells include bone marrow–derived cells, portal fibroblasts, and epithelial-to mesenchymal transition from hepatocytes and cholangiocytes. Abbreviations: BMP-7, bone morphogenetic protein 7; EMT, epithelial-to mesenchymal transition; Hh, hedgehog; HSCs, hepatic stellate cells; MET, mesenchymal epithelial transition and MFs, myofibroblasts.

## 2.1. Fibrocytes (Bone Marrow Derived Cells)

### 2.1.1. Original description

Fibrocytes were originally described in 1994 in an experimental model of wound repair, 10% of the cells in the wound chamber were spindle-shaped and expressed collagen, pro-collagen, and CD34<sup>+</sup> within one day following injury<sup>[27]</sup>. Since their appearance was much faster than would be expected by entry of fibroblasts from the surrounding skin, it was thought that they must have originated from the circulation. These cells were named fibrocytes, a term combining fibroblast with leukocyte<sup>[28]</sup>.

Fibrocytes are a unique CD45<sup>+</sup> cell population that are distinct from monocytes, DCs, T lymphocytes, fibroblasts, epithelial cells, endothelial cells, and Langerhans cells. They exhibit prominent cell surface projections on scanning electron microscopy making them morphologically distinct from

leukocytes<sup>[27]</sup>. Fibrocytes comprise 0.1–1% of the nucleated cells in the peripheral blood in healthy hosts<sup>[29]</sup>.

### 2.1.2. Bone marrow (BM) origin

There is substantial information available supporting the hypothesis that fibrocytes derived from the bone marrow<sup>[29]</sup>. Fibrocytes are characterized by the expression of collagen types I and III, fibronectin, major histocompatibility complex II, CD11b, CD13, CD34, and CD45<sup>[30]</sup>.

The co-expression of collagen and the other hematologic markers such as CD45 are used to identify fibrocytes<sup>[28]</sup>. Early in culture, fibrocytes express CD34, CD45, collagen type I, and vimentin<sup>[31]</sup>. Fibrocytes, also, express a number of chemokine receptors on their surface including CCR3, CCR5, CCR7, and CXCR4, and can migrate to wound sites in response to specific chemokine gradients<sup>[32]</sup>. They do not, however, express T cell markers (CD3, CD4, and CD8), B cell markers

(CD19), IL-2 receptor chain (CD25), the low affinity of crystallizable fragment of Ig molecule (Fc), gamma receptor III (CD16), and myeloid markers (CD14 and non-specific esterase) [33].

Fibrocytes can differentiate from CD14<sup>+</sup> peripheral blood monocytes that express the receptors for the Fc portion of IgG, CD64, and CD32 [30, 34]. Circulating fibrocytes may be present in a subset of CD14<sup>+</sup> CD16<sup>-</sup> monocytes that carry CCR2, on their surface [35]. At the time of tissue injury this monocyte subset is released from the bone marrow into the peripheral blood and migrates to inflamed sites via a CCR2-mediated pathway [36]. Human fibrocytes may represent an intermediate stage of differentiation of this monocyte subset into mature fibroblasts and MFs in tissue [37].

### 2.1.3. Differentiation and trafficking

The differentiation of fibrocytes into MFs is augmented in the presence of TGF- $\beta$  or ET-1, and results in cells that produce fibronectin and collagen, and express the myofibroblast marker  $\alpha$ -smooth muscle actin [33].

Fibrocytes spontaneously gain expression of  $\alpha$ -smooth muscle actin in culture, and gradually lose the expression of CD34 and CD45 over time depending on the inflammatory milieu [27]. This response can be augmented by exposure of the fibrocytes to TGF- $\beta$  or ET, resulting in differentiation into myofibroblast-type cells [33]. It has been shown that the pro-fibrotic cytokines IL-4 and IL-13 promote fibrocyte differentiation from CD14<sup>+</sup> peripheral blood monocytes without inducing proliferation, whereas the anti-fibrotic cytokines IL-12 and IFN- $\gamma$  inhibit fibrocyte differentiation [38]. IL-4, IL-13 and IFN- $\gamma$  were found to regulate fibrocyte differentiation through a direct effect on monocytes, whereas IL-12 was found to have an indirect effect possibly through CD16<sup>+</sup> NK cells [39].

Fibrocyte differentiation appears to be influenced by a complex profile of cytokines, chemokines and plasma proteins within the area of tissue injury. Human fibrocytes

express several chemokine receptors including CCR3, CCR5, CCR7, and CXCR4 [40, 41]. They can use different chemokine ligand-receptor pairs for tissue homing. The CXCR4-CXCL12 axis plays an important role in the homing of bone marrow-derived progenitor cells [39]. CXCR4 is an important chemokine receptor in stem cell trafficking, and the differential expression of CXCL12 in tissues creates the gradient required for trafficking of CXCR4<sup>+</sup> cells [41].

### 2.1.4. Contribution to liver fibrogenesis

Fibrocytes have been implicated in the fibrosis of the liver. Regardless of the etiology, chronic liver disease can eventually lead to the excess accumulation of type I collagen and fibrosis [42].

In the liver immature, multi-potent bone marrow cells have the capacity to differentiate to hepatocytes, cholangiocytes, SECs and KCs, if the adequate microenvironment is present [8]. This was recently extended to HSC and MFs under experimental and clinical conditions [43]. By transplantation of genetically tagged bone marrow or of male bone marrow (Y-chromosome) to female mice, it was estimated that up to 30% of HSC in the liver originate from the bone marrow and acquire the MFs phenotype under injurious conditions [33]. Another study indicates that up to 68% of HSC and 70% of MFs in CCl<sub>4</sub>-cirrhotic mice liver are derived from the bone marrow [44]. Even in human liver fibrosis, a significant contribution of bone marrow cells to the population of MFs has been proven [39].

Myelogenic fibrocytes are present in the liver and can be differentiated by TGF- $\beta$  to collagen producing MFs [45]. They are a subpopulation of circulating leukocytes displaying a unique surface phenotype with positivity for CD45 (hematopoietic origin), CD34 (progenitor cell), and type I collagen (capability of matrix synthesis) [46]. They also have potent immuno-stimulatory activities [33].

Mobilization of bone marrow cells and their recruitment into the damaged tissue

is a central mechanism of tissue fibrosis and wound healing <sup>[5]</sup>. This was most likely regulated by colony-stimulating factors (CSFs), such as granulocyte-CSF (G-CSF) <sup>[46]</sup> and CXCR4, CCR2, and CCR7 <sup>[47]</sup>. Thus, activated HSC probably play an important role in liver fibrosis since they secrete a broad spectrum of inflammatory mediators (chemokines M-CSFs) and leukocyte adhesion molecules (ICAM-1, VCAM-1, NCAM) required for recruitment, activation, and maturation of blood-born cells at the site of injury <sup>[48]</sup>.

The homing of myelogenic cells in the damaged liver was claimed to also have a positive effect on the resolution of liver fibrosis, since these cells express MMPs, which augment the degradation of fibrotic ECM <sup>[47]</sup>. Furthermore, a striking relationship between increasing hepatic fibrosis and periportal ductular reaction has been demonstrated <sup>[49]</sup>.

## 2.2. Contribution of Peripheral Blood Cells

Recent studies indicate a highly developed multi-differentiation potential of a subgroup of circulating blood monocytes, which can be recruited quickly to sites of tissue injury <sup>[50, 51]</sup>.

Blood monocytes have been recognized for a long time as heterogenous precursor cells giving rise to both tissue macrophages as well as dendritic cells. It has become increasingly clear over the last years that monocytes not only migrate into tissues during inflammatory response <sup>[52]</sup>, but also contribute to the constitutive turnover of antigen presenting and tissue resident phagocytic cells to various degrees depending on the type of tissue <sup>[53]</sup>.

Blood monocytes consist of two different, distinct subsets, which can be discriminated by their expression levels of Gr1 as well as the chemokine receptors (CX3CR1 and CCR2) <sup>[35]</sup>. Gr1 monocytes have been described as classical monocytes, which patrol the blood and quickly respond to inflammatory signals, infiltrating inflamed tissues and differentiating into effector cells such as

inflammatory DCs and “TNF- $\alpha$ /inducible nitric oxide synthase (iNOS)-producing DC” <sup>[54]</sup>.

Two distinct subsets also exist in humans, which display a differential expression of the surface markers CD14 and CD16. CD14<sup>+</sup> CD16<sup>-</sup> monocytes have been termed ‘classical’ monocytes, which represent about 95% of all human blood monocytes in healthy individuals. These cells express high levels of CCR2, CD62L, Fc $\gamma$ RI and therefore phenotypically resemble Gr1 monocytes from murine system <sup>[51]</sup>.

Some investigations have shown that peripheral blood monocytes in vitro can differentiate into hepatocyte-like cells if they are exposed to M-CSF and specific interleukins (monocyte-derived neo-hepatocytes) <sup>[54]</sup>.

Subgroups of monocytes may also differentiate into fibroblast-like cells (fibrocytes) after entering the damaged tissue where they participate in fibrotic processes <sup>[51]</sup>. The differentiation is positively influenced by G-CSF, M-CSF, MCP-1, and other chemokines and hematopoietic growth and differentiation factors, which are also expressed and secreted by activated HSCs and other liver cell types <sup>[5]</sup>.

Recently an inhibitory effect of the acute-phase protein serum amyloid P (SAP) on the process of differentiation of monocytes to fibrocytes could be established <sup>[51, 52]</sup>. Since SAP is synthesized in hepatocytes, severe liver injury might facilitate the monocyte–fibrocyte differentiation process due to reduction of the inhibitory SAP <sup>[5]</sup>.

The question arises as to whether there are distinct monocyte-macrophage populations that have differing roles in liver fibrosis and repair or whether the same population of cells behaves differently depending upon the local cellular context <sup>[55, 56]</sup>. It has been observed that the fractalkine receptor CX3CR1 is down-regulated in the livers of patients with advanced liver fibrosis <sup>[57]</sup>. This factor has been shown in mice to control the survival and behavior of intrahepatic monocytes and thereby promote an anti-fibrotic phenotype <sup>[51]</sup>.

Circulating stem cells, which are positive for CD34 and CXCR4, can be considered as source of fibroblasts in the fibrotic liver<sup>[50]</sup>. G-CSF and the stromal derived factor (SDF)-1 are probably the most important regulators of stem-cell mobilization from the BM and their integration into the damaged tissue followed by differentiation to fibroblasts and other cells<sup>[6]</sup>.

### 2.3. Contribution of Hepatic Macrophages

Recent studies suggested that the pathogenesis of liver fibrosis is tightly regulated by distinct macrophage populations that exert unique functional activities throughout the initiation, maintenance, and resolution phases of fibrosis<sup>[57]</sup>. Like myofibroblasts, these cells are derived from either resident tissue populations, like KCs<sup>[58]</sup>, or from BM immigrants<sup>[64]</sup>. Through the means of labeled BM transplants in mice it has been shown that tissue macrophages rapidly renew from the BM<sup>[59]</sup>.

Macrophages are almost always found in close proximity with collagen-producing myofibroblasts<sup>[60, 61]</sup>, and there is strong evidence that macrophage/myofibroblast interaction is reciprocal<sup>[8]</sup>.

Activated HSCs attract and stimulate macrophages with multiple chemokines and M-CSF. Once stimulated, macrophages produce pro-fibrotic mediators (TGF- $\beta$ 1 and PDGF) that directly activate fibroblasts<sup>[62]</sup>. Several studies have identified macrophages as a critical source of TGF- $\beta$ 1 and PDGF in fibrosis<sup>[60]</sup>. Imatinibmesylate, a PDGF receptor tyrosine kinase inhibitor, is currently in preclinical trials as a possible therapy for chronic hepatic fibrosis<sup>[39]</sup>.

In addition to producing TGF- $\beta$ 1, activated macrophages and KCs regulate fibrosis by secreting a variety of chemokines, cytokines, and growth factors. Macrophages, like other innate immune cell types, are activated by pathogen-associated molecular patterns (PAMPs) that engage an array of pattern-recognition receptors (PRRs)<sup>[63]</sup>.

Macrophages also regulate fibrosis independently of direct interactions with

myofibroblasts<sup>[64]</sup>. They present antigens to propagate antigen-specific T-cell responses; produce their own MMPs and TIMPs that control ECM turnover and secrete chemokines, cytokines and growth factors that recruit fibroblasts and other inflammatory cells to sites of tissue injury<sup>[65, 66]</sup>. They, also, remove dead cells and debris by phagocytosis, which can otherwise trigger pro-inflammatory and pro-fibrotic signals<sup>[62]</sup>.

They express immune-regulatory mediators like Relm- $\alpha$ /Fizz1/Retnla, chitinase-like proteins, and nitric oxide synthase (NOS)-2 or arginase-1 (Arg-1) that alters the magnitude and duration of the immune response<sup>[67]</sup>.

A growing body of evidence suggests that macrophages and other mediators regulate matrix deposition independently of TGF- $\beta$ 1<sup>[68, 69]</sup>.

Early studies showed the importance of MCP-1 in recruiting CCR2-expressing monocytes<sup>[55]</sup>. Activated monocytes in turn produce MCP-2 and -3, which have been shown to participate in the development of primary biliary cirrhosis (PBC) and liver fibrosis by recruiting and activating myofibroblasts and by regulating the production of macrophage-derived MMPs, including MMP2 and MMP9<sup>[70]</sup>.

The CC chemokines, MIP-1 $\alpha$ , MIP-1 $\beta$ , are regulated upon activation. RANTES, together with their corresponding receptors CCR1 and CCR5 are also up-regulated in models of liver fibrogenesis<sup>[67]</sup>. Furthermore, neutralization studies identified important roles for both CCR1- and CCR5-expressing KCs in the development of fibrosis. MIP-2 also participates in fibrogenesis by regulating angiogenesis<sup>[70]</sup>.

Osteopontin (OPN) is another notable chemoattractant<sup>[57, 71]</sup>. Studies have shown that OPN recruits macrophages to injured tissues<sup>[72]</sup>.

Macrophages, also, produce IGF-1, which stimulates the proliferation and survival of myofibroblasts and promotes collagen synthesis by these cells<sup>[73]</sup>. Under



circumstances, macrophages and KCs secrete IL-4 and IL-13, which are believed to function as potent profibrotic cytokines [57].

### 3.4. Portal Fibroblasts and Portal Myofibroblasts

Increasing attention is being focused on the identification, purification, and analysis of this fibrogenic population, whose contribution to fibrosis is especially important in biliary diseases [72]. Portal fibroblasts were located within the connective tissue of portal areas; the recruitment and activation of resident fibroblasts into MFs are especially relevant in diseases associated with cholestasis [20].

In the normal liver, portal fibroblasts (PFs) are characterized by a morphological aspect and an antigen repertoire which is similar to the one of other fibroblasts. Unlike HSCs, PFs express the highly specific fibroblast marker TE7 [62], as well as other specific markers like elastin, IL-6, fibulin 2 and the ecto-ATPase nucleoside triphosphate diphosphohydrolase-2 (NTPD2). The origin of PFs, as for HSCs, is still debated and two major hypotheses have been raised: (a) PFs as well as vascular smooth muscle cells may originate from  $\alpha$ -SMA positive cells of the ductal plate during human embryogenesis and (b) HSCs and PFs may be derived from a common putative precursor in the early embryo [73].

PFs, similarly to what was described for HSCs, are believed to differentiate into  $\alpha$ -SMA positive MFs (P/MFs) under conditions of chronic liver injury. Although the prevalent hypothesis indicates a direct origin of P/MFs from PFs, it should be noted that P/MFs may theoretically originate also from other sources, including vascular smooth muscle cells of the wall of hepatic artery or portal vein [39].

A major pro-fibrogenic role for both PFs and P/MFs is now sustained by convincing evidence [39]. These pro-fibrogenic cells have an unequivocal primary role in biliary fibrosis although most of the knowledge mainly comes from experimental models of cholestatic liver injury [73].

According to published data, the primary role of PFs and P/MFs in biliary fibrosis is supported by the knowledge that the injury to bile duct epithelial cells (BDEC) is a prerequisite for the differentiation of PFs into P/MFs. The proposed hypothesis suggests that, once damaged, BDEC become able to express TGF- $\beta$ 2 and to release a number of growth factors and pro-inflammatory mediators including PDGF, IL-6 and MCP-1. These factors are believed to be responsible for the initiation of the myofibroblastic differentiation of PFs. This activation is believed to be followed by an autocrine perpetuation by P/MFs similarly to what is described for HSC/MFs [39].

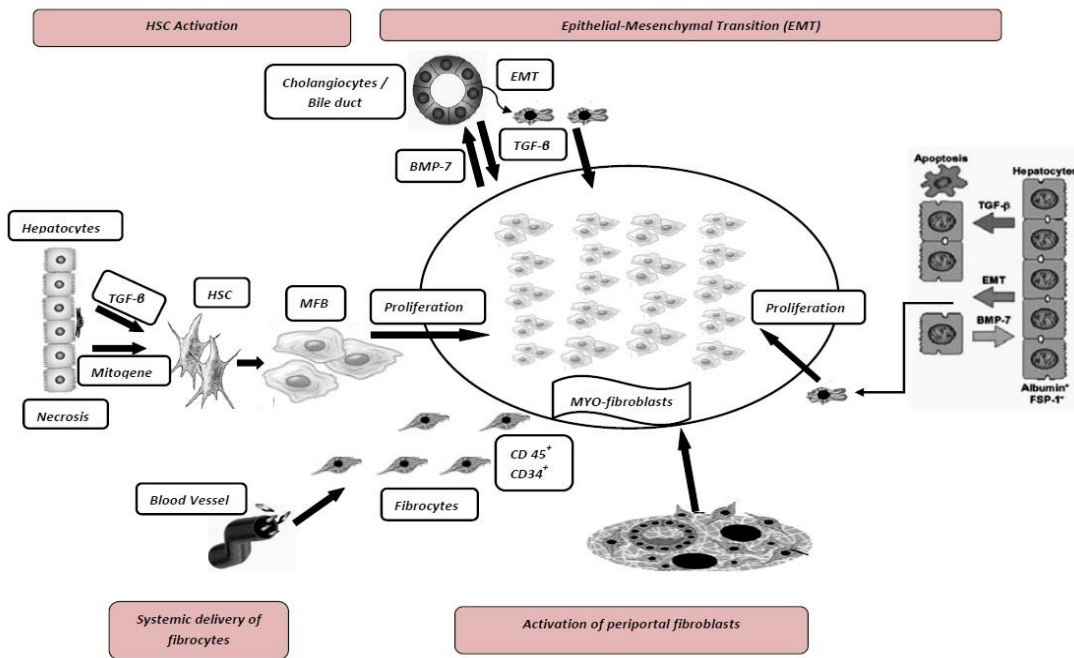
Apart from biliary fibrosis, several studies indicate that P/MFs may also significantly contribute to chronic liver diseases progression in other clinical conditions of different aetiology characterized by bridging fibrosis. Indeed, different laboratories have outlined the existence of a direct relationship between the intensity of the so called ductular reaction (a peculiar form of hyperplastic response of BDEC in pathological conditions) and the severity of fibrosis in either animal models as well as human liver diseases of different aetiologies, including chronic HCV and non-alcoholic fatty liver disease/non-alcoholic-steatohepatitis (NAFLD/NASH) [74]. Moreover, it has been recently proposed that PFs may be as multifunctional as HSC/MFs, including a role in the hepatic progenitor cell (HPC) niche and in HPC expansion and differentiation [62].

### 2.5. Epithelial–Mesenchymal Transition (EMT)

In recent years, the understanding of fibrosis has been strikingly enhanced by the characterization of cellular effectors, key inflammatory/pro-fibrogenic cytokines and molecular mediators and by the emergence of new pathogenetic scenarios. A major determinant of fibrosis is the continuous expansion of the fibroblasts/myofibroblasts that prompts the question of how this cellular pool can be fed. EMT, by generating new mesenchymal cells, may contribute

substantially to the increase of interstitial fibroblasts/myofibroblasts and have an important role in the pathogenesis of liver fibrosis. The traditional “fibrocentric” paradigm of progression in chronic fibrosing liver disease assumes that fibrosis per se is the primary pathogenetic means responsible for parenchymal dysfunction and hepatic failure. However, it provides little explanation to understand the mechanisms leading to atrophy and loss of epithelial unities [75].

Studies point to a potentially important mechanism for the enlargement of the resident pool of fibroblasts during the fibrotic reaction of the damaged organ. This process, designated as EMT, is well known in the context of embryonic development, but is now recognized as an important mechanism in the generation of fibroblasts during fibrogenesis in adult tissues (Fig. 4) [76].



**Figure 4:** Currently known mechanisms of fibrogenesis. Hepatic stellate cells are activated by liver cell necrosis, EMT, influx of fibrocytes from the blood, and activation of periportal fibroblasts at sites of injury. Expression of typical cellular markers is indicated. Abbreviations: BMP-7, bone morphogenetic protein-7; FSP, fibroblast specific protein; HSCs, hepatic stellate cells and TGF-β., transforming growth factor-beta.

Epithelial cells are adherent cells that closely attach to each other, forming coherent layers in which cells exhibit apical-basal polarity. Mesenchymal cells, in contrast, are non-polarized cells, capable of moving as individual cells because they lack intercellular connections. EMT describes the process by which cells gradually lose typical epithelial characteristics and acquire mesenchymal traits. MET refers to the reverse process. It is important to emphasize that EMT/MET refer

to changes in cell shape and adhesive properties [75].

The key epithelial features that are eventually lost during EMT include typical epithelial expression and distribution of proteins that mediate cell-cell and cell-matrix contacts, as well as the cytoskeletal organization that is responsible for normal epithelial polarity [76]. The key mesenchymal characteristics that are ultimately gained during EMT include the ability to migrate and

invade the surrounding matrix. This migratory/invasive phenotype requires induction of mesenchymal filaments, cytoskeletal rearrangements, and increased production of factors that degrade ECM, as well as new matrix molecules<sup>[77]</sup>.

The alterations in cellular phenotype do not occur simultaneously. Completion of EMT (or its reversal) requires a carefully-orchestrated series of events that eventually lead to wide-spread changes in gene expression. This is regulated both at the level of gene transcription and via various post-transcriptional mechanisms<sup>[78]</sup>.

### 2.5.1. Hallmarks and mechanism of EMT

EMT was first recognized in the early 1980s as an important mechanism of embryogenesis and organ development aimed at creating cells with the ability to move and produce matrix. Indeed, the formation of the mesoderm [80] and the neural crest [81], somitogenesis[82], and palatogenesis are developmental processes in which EMT is known to have a prominent role. The two key changes occurring in epithelial cells undergoing EMT are detachment from neighboring epithelial cells and the migration into the interstitium where they may start producing matrix<sup>[79]</sup>.

The molecular hallmarks of EMT include E-cadherin down-regulation, responsible for the loss of cell-cell adhesion and the subsequent detachment from the parent epithelium; the up-regulation of matrix-degrading proteases that digest the epithelial basement membrane; the up-regulation and/or nuclear translocation of transcription factors underlying the specific gene program of EMT such as  $\beta$ -catenin, Smads and members of the Snail family; denovo expression of mesenchymal proteins such as fibroblast specific protein 1 (FSP1) and  $\alpha$ -SMA; Ras (renin angiotensin system) homologous (Rho) guanosinetriphosphatase (GTPase)-mediated cytoskeletal reorganization to favor cell shape changes and to activate motility; loss of cytokeratin and other epithelial-associated markers; and finally, the production of

interstitial-type matrix components such as collagen types I and III and fibronectin<sup>[75]</sup>.

The factors acting on cell surface receptors seem to play important roles in accomplishing EMT<sup>[83]</sup>. Matrix-degrading proteases, including MMPs, released from damaged epithelial cells, inflammatory cells, or activated myofibroblasts possibly initiate the process by dismantling the epithelial basement membrane<sup>[84]</sup>. Among its pleiotropic actions, TGF- $\beta$  is also known to up-regulate matrix-degrading MMPs, and thus, it could participate in basement membrane breakdown<sup>[85]</sup>.

The dissolution of the basement membrane may result in direct contact between epithelial cells and type I collagen-rich stromal microenvironment, which under normal conditions is prevented by an intact basement membrane. Exposure to interstitial type I collagen, which epithelial cells do not come into contact with in normal conditions, as well as to high concentrations of TGF- $\beta$  and other growth factors present in the interstitial stroma destabilize the epithelial phenotype and encourage EMT<sup>[43]</sup>.

Stimulation of integrin-linked kinase (ILK) or  $\beta$ -catenin pathway and activation of the Rho GTPases could provide additional signals, eliciting the completion of cell dissociation and inducing the cytoskeleton remodeling needed for cell reshaping and movement<sup>[76]</sup>. Transitioning cells liberated from contact with neighbors and fully equipped for migration, can leave the epithelium and translocate into the surrounding interstitium where they are supported by local stimuli. Looking like true mesenchymal cells, they can be initiated to produce new matrix<sup>[75]</sup>.

### 2.5.5. EMT in liver fibrosis

Recent studies further provide evidence for the importance of EMT in liver fibrogenesis, as evidenced by transition of albumin-positive hepatocytes to fibroblast specific protein-1 (FSP-1) positive and albumin-negative fibroblasts. It is claimed that up to 45% of hepatic fibroblasts are derived

from hepatocytes, and up to 60% of FSP-1-positive hepatocytes are co-labeled with albumin indicating an intermediate transitional stage of EMT of hepatocytes<sup>[44]</sup>.

The EMT-state was indicated by strong up-regulation of collagen mRNA expression and type I collagen deposition. Thus, hepatocytes are capable of EMT changes and type I collagen synthesis and might be a source of a substantial population of myofibroblasts in fibrogenesis<sup>[86]</sup>. A further target for EMT is cholangiocytes (bile duct epithelial cells). In primary biliary cirrhosis, it has been proven that bile duct epithelial cells express FSP-1/S100A4 and vimentin as early markers of fibroblasts<sup>[87]</sup>. The bidirectional consequence of EMT for cholangiocytes is ductopenia (reduction of bile ducts) and enlargement of the pool of portal fibroblasts, which significantly contribute to portal fibrosis<sup>[88]</sup>. In addition, activation and proliferation of portal/periportal mesenchymal cells to peribiliary MFs, which are stimulated in a paracrine manner by bile duct epithelial cells via TGF- $\beta$ , PDGF-BB, and ET-1<sup>[89]</sup>, are important pathogenetic mechanisms of portal fibrosis and septa formation in cholestatic liver diseases<sup>[90]</sup>. Indeed, only a minority of ECM-producing MFs in obstructive cholestatic injuries are derived from HSCs<sup>[90]</sup>. This underlines the heterogeneous origin of MFs in fibrogenesis and emphasizes the importance of the underlying fibrogenic liver disease<sup>[12]</sup>.

EMT of hepatocytes is dependent on the balance between apoptotic and survival mechanisms. The process of EMT requires the action of MMPs and a TGF- $\beta$  dependent snail-mediated down-regulation of E-cadherin both contributing to the release of epithelial cells from cell-cell and cell-basement membrane binding<sup>[87]</sup>.

The BMP-7, a member of TGF- $\beta$  superfamily, does not only inhibit EMT, but can even induce a mesenchymal-epithelial (retro-) transition (reverse EMT = MET)<sup>[91]</sup>. BMP-7 has been shown to inhibit TGF- $\beta$  dependent EMT of hepatocytes and the progression of experimental fibrosis in mice<sup>[47]</sup>. It has, also, anti-apoptotic properties, anti-

inflammatory, and proliferation-stimulating effects<sup>[92]</sup>. BMP-7 inhibits TGF- $\beta$  signaling via Smads<sup>[93, 94]</sup>, which transduce the effect of the latter cytokine from its receptor, a serine/threonine kinase, to the Smad-binding element of respective target genes in the nucleus<sup>[94]</sup>. In addition, several trapping proteins such as the small proteoglycans decorin and biglycan, latency associated peptide (LAP), Bambi, KCP (kielin-chordin-like protein), gremlin, and alpha-2-macroglobulin change the balance between TGF- $\beta$  and BMP-7 in favor of an anti-EMT effect by binding and neutralization of TGF- $\beta$ <sup>[95]</sup>.

Similarly, the important downstream-modulator protein connective tissue growth factor (CTGF/CCN2)<sup>[95]</sup>, which is expressed in hepatocytes, HSC, portal fibroblasts, and cholangiocytes<sup>[93]</sup> changes the functional TGF- $\beta$ /BMP-7 ratio<sup>[8]</sup>. CTGF is over-expressed in experimental and human liver cirrhosis<sup>[98]</sup> which is mediated mainly by TGF- $\beta$ , but also by ET-1, TNF- $\alpha$ , VEGF, nitrogen oxide (NO), prostaglandin E2, thrombin, high glucose, and hypoxia<sup>[6]</sup>, CTGF inhibits BMP, but activates TGF- $\beta$  signaling by modulation of the receptor-binding of these ligands<sup>[87]</sup>. The prominent functional role of CTGF is supported by recent data, which show sustained anti-fibrotic effects if CTGF expression is reduced by siRNA<sup>[12]</sup>.

Liver tissue sections from patients with a variety of pathologic conditions associated with hepatic fibrosis have shown co-localization of cytokeratin 19 and several mesenchymal markers including FSP1/S100A4, vimentin, heat shock protein-47, Snail, and LOX in biliary epithelial cells engaged in ductular proliferation<sup>[94]</sup>. Epithelial cells of bile ducts and ductular reaction from patients with primary biliary cirrhosis, primary sclerosing cholangitis, and alcoholic liver disease have shown strong expression of TGF- $\beta$ ; nuclear accumulation of phosphorylated Smad2/3; and co-expression of cytokeratins, E-cadherin, FSP1/S100A4, vimentin, and MMP-2, consistent with TGF- $\beta$ /Smad-mediated EMT<sup>[90]</sup>.

Taken together, EMT, MET and in special conditions, even MMT (mesenchymal–mesenchymal transition, e.g. of vascular endothelial cells to fibroblasts), and the fine tuning of the bioactive TGF- $\beta$ /BMP-7 ratio and of their adaptor and trapping proteins, offer multiple regulatory possibilities of influencing fibrogenesis<sup>[5]</sup>.

### 3. FUTURE VIEW

The elucidation of the cellular and molecular mechanisms regulating fibrogenesis in CLDs has provided a relevant clinical advance in the identification of diagnostic and therapeutic strategies<sup>[12, 96]</sup>. Several biomarkers, related to the biology of fibrogenesis, are currently validated for their use as diagnostic and prognostic indicators of disease progression in different CLDs. Some of these markers are likely to replace or integrate the use of repeated liver biopsies in the follow-up of patients undergoing treatment. In addition, a large effort is currently directed at identifying gene polymorphisms conditioning the rate of fibrosis progression in CLDs.

The application of omics platforms (e.g. genomics, proteomics and metabolomics) have already generated an enormous amount of data that now need to be analyzed, framed in the clinical features of the disease and validated in larger cohorts. It is still unclear how these findings will be translated into application with clinical utility in everyday practice<sup>[10]</sup>. From the therapeutic point of view, several potentially effective anti-fibrotic compounds have been identified and are waiting clinical testing in large clinical trials. Importantly, since data obtained *in vitro* and in animal models do not faithfully reflect the situation in human liver disease; definite conclusions can only be drawn by clinical trials performed in humans. It is likely that in a 5-year time frame more and more evidence will be provided for an effective translation of these insights in clinical practice<sup>[12]</sup>.

### SUMMARY AND CONCLUSIONS

The fibrotic response to chronic liver injury depends on both resident and recruited

cell types. Characterization of the fibrogenic cell populations, evidence of their plasticity and pluripotency, and characterization of their cross talk with inflammatory cells will lead to important progress in the understanding of the disease. There have been major advances in characterizing the cellular and molecular biology, fibrogenic pathways and genetic determinants of fibrosis progression. Given such substantial progress in elucidating the underlying mechanisms, the current task is to translate these findings into the development of effective and targeted anti-fibrotic therapies that will modify the natural history of chronic fibrosing disease.

Despite intensive experimental studies, the clinical opportunities for patients with fibrosing liver diseases have not yet significantly improved. It is expected that increasing knowledge of new pathogenetic mechanisms, which complement the ‘canonical principle’ of fibrogenesis, will have a beneficial effect on the translation to clinical medicine.

Based on published data, a change has been made in the pathogenetic roadmap of liver fibrosis. The newly discovered information now establishes a complex network of interacting pathways radiating to systemic response.

Newly recognized pathogenetic mechanisms point to the influx of bone marrow-derived fibrocytes to the damaged liver tissue. Fibrocytes are circulating progenitor cells CD34<sup>+</sup> of haematopoietic origin CD45<sup>+</sup> capable of differentiating into diverse mesenchymal cell types. The additional marker of fibrocytes activation, as the positivity of the CXCR-4 chemokine receptor expression can be used to quantitate these cells.

For early disease stages, it may be promising to interfere with monocyte/macrophage or to other pro-inflammatory immune cell recruitment. Blockage of cell recruiting signals such as chemokines (CCR2 or CCR5) at early stages may be beneficial to suppress a developing

immune response and therefore limiting the amount of organ damage.

Epithelial-mesenchymal transition (EMT), by generating new mesenchymal cells, would seem to contribute substantially to the increase of interstitial fibroblasts/myofibroblasts and could have an important role in the pathogenesis of organ fibrosis. Future effort to understand EMT and its underlying molecular mechanisms and the characterization of pathways suitable for molecular targeting could thus offer novel approaches to diagnose, prevent, or delay the progression of chronic fibrosing diseases.

It is now evident that the heterogeneous pool of myofibroblasts originates from the EMT of hepatocytes, from the influx of bone marrow-derived fibrocytes into the damaged liver tissue, and from differentiation of a subgroup of circulating monocytes to fibroblasts after homing in the damaged tissue. These processes offer innovative diagnostic and therapeutic options.

Among the molecular mediators, transforming growth factor-beta (TGF- $\beta$ ) plays a central role, which is controlled by the bone morphogenetic protein (BMP)-7, an important antagonist of TGF-beta action. The newly discovered pathways supplement the concept of hepatic stellate cell (HSC) activation to myofibroblasts, point to fibrosis as a systemic response involving extrahepatic organs and reactions, and offer innovative approaches for the development of non-invasive biomarkers and anti-fibrotic agents. For example, over-expression of BMP-7 or application of recombinant BMP-7 has a sustained anti-fibrotic effect. In addition, the determination of connective tissue growth factor (CTGF) in serum may provide further information on fibrogenic activity.

Activation of HSC is associated with up-regulation of various signaling pathways. Overexpression of miR-27a and miR-27b resulted in reversal of the activated phenotype of stellate cells to a more quiescent phenotype with increased fat accumulation and decreased proliferation.

The new pathogenetic insights justify strong optimism since the spectrum of potential approaches to interfere with the fibrogenic pathway is greatly broadened.

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