JCMM
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Invited Review - Stellate Cells

J. Cell. Mol. Med. Vol 10, No 1, 2006 pp. 76-99

Modern pathogenetic concepts of liver fibrosis suggest stellate cells and TGF-β as major players and therapeutic targets

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Received: November 28, 2005; Accepted: February 20, 2006

Abstract

Hepatic fibrosis is a scarring process that is associated with an increased and altered deposition of extracellular matrix in liver. At the cellular and molecular level, this progressive process is mainly characterized by cellular activation of hepatic stellate cells and aberrant activity of transforming growth factor-β1 and its downstream cellular mediators. Although the cellular responses to this cytokine are complex, the signalling pathways of this pivotal cytokine during the fibrogenic response and its connection to other signal cascades are now understood in some detail. Based on the current advances in understanding the pleiotropic reactions during fibrogenesis, various inhibitors of transforming growth factor-β were developed and are now being investigated as potential drug candidates in experimental models of hepatic injury. Although it is too early to favour one of these antagonists for the treatment of hepatic fibrogenesis in human, the experimental results obtained yet provide stimulatory impulses for the development of an effective treatment of choice in the not too distant future. The present review summarises the actual knowledge on the pathogenesis of hepatic fibrogenesis, the role of transforming growth factor-β and its signalling pathways in promoting the fibrogenic response, and the therapeutic modalities that are presently in the spotlight of many investigations and are already on the way to take the plunge into clinical studies.

Keywords: liver fibrosis - hepatic stellate cells - antifibrotic therapy - transforming growth factor- β - Smad - endoglin - betaglycan - extracellular matrix - signal transduction - α-smooth muscle actin - collagen

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Introduction

Fibrosis is a well-known histological and biochemical hallmark of cirrhosis, but fibrosis is not necessarily combined with cirrhosis. Originally fibrosis was defined by a WHO expert group in 1978 "as the presence of excess collagen due to new fibre formation" [1]. At that time this definition of fibrosis was based on a modern pathophysiological concept, because collagen was identified as the most prevalent connective tissue component in fibrotic liver and the pathogenesis of fibrosis, *i.e.* fibrogenesis was considered as an active biosynthetic process leading to excess deposition of extracellular matrix (ECM). Previously, the development of fibrosis in chronically injured liver was hypothesized to be a "passive" mechanism resulting from collapse of damaged parenchyma leading to septa-forming condensation of pre-existing stroma [2]. During the past 20 years of intensive experimental research it became evident that fibrogenesis in liver is an active wound-healing process. Accordingly, specialized perisinusoidal cells, *i.e.* liver specific pericytes are primed to generate a broad spectrum of matrix proteins. Their expression is regulated not only by cytokines and chemokines but also by non-peptide signals such as reactive oxygen species, lipid mediators and prostaglandins [3–6]. The cell types participating in fibrogenesis, the fibrogenic mediators and, hence, the potential targets of antifibrotic trials are almost identified and promise therapeutic success in the not too distant future [7, 8].

Fibrosis, fibrogenesis, and fibrolysis

Fibrosis, a histologically based diagnosis, describes a several fold elevation of the matrix proteins collagens and elastin, of structural (basement) glycoproteins, proteoglycans (core protein-glycosaminoglycan macromolecules) and pure carbohydrates, *i.e.* hyaluronan (formerly termed hyaluronic acid) (Fig. 1). Deposition of ECM starts frequently (*e.g.* in ethanol-induced fibrosis) in the perisinusoidal space of Disse, preferentially in the most vulnerable metabolic zone 3 of the liver azinus (perivenous) leading to initial pericentral fibrosis [9]. Fibrosis of other etiologies, *e. g.* in primary biliary cirrhosis and HCV-induced cirrhosis starts from periportal fields. Subendothelial matrix deposition (in the space of Disse) leads to incomplete basement membranes ("capillarization") [10, 11], which hinder bidirectional exchange processes between hepatocytes and sinusoidal blood stream, and, thus, impairs the clearance function and the biosynthetic delivery function of the parenchymal tissue. Furthermore, narrowing of the sinusoidal lumen by perisinusoidal fibrosis is considered to be a contributing factor to intraparenchymal hemodynamic resistance (portal hypertension). Beside this topographic redistribution of matrix and elevation of its total concentration, fibrosis includes also changes of the matrix profile (*e.g.* preferential elevation of the chondroitin sulfate/heparan sulfate ratio, of the type I/type III collagen ratio) and changes of the molecular structure of some of these molecules, *e. g.* hydroxylation of collagen α-helices and the degree of sulfatization of glycosaminoglycan-like heparan sulfate, chondroitin and dermatan sulfate (Fig. 2).

Formal pathogenesis of fibrosis is initiated by parenchymal cell destruction (necrosis rather than apoptosis) due to multiple injurious agents and mechanisms followed by inflammation, which in turn activates "resting" hepatic stellate cells (HSC), formerly called fat or vitamin A or retinoid storing cells, Ito cells, perisinusoidal lipocytes [12] to express and secrete matrix molecules, cyto- and chemokines, matrix metalloproteinases (MMPs) and their respective inhibitors (TIMPs) [13, 14]. Thus, pluripotent HSC participate pathophysiologically both in fibrogenesis and fibrolysis, *i.e.* enzymatic dissolution of ECM and, thus, in tissue remodelling (Fig. 3).

Hepatic stellate cells as major but not exclusive fibrogenic liver cell type

The pioneering work of Leeuw *et al*. [15] and Friedman *et al.* [16, 17] in isolating animal and human HSC, respectively enabled systematic *in vitro* studies of this highly versatile type of non-parenchymal liver cells [18]. In normal liver they display a low mitotic activity, comprise only about 1.4% of total liver volume, and are present in a ratio of about 3.6–6 cells/100 hepatocytes (PC) (Ito cell index: PC: HSC ~ 20:1). The cell body (700 μm^3) is located in the recess of adjacent hepatocytes having star-like dendritic cytoplasmic processes of

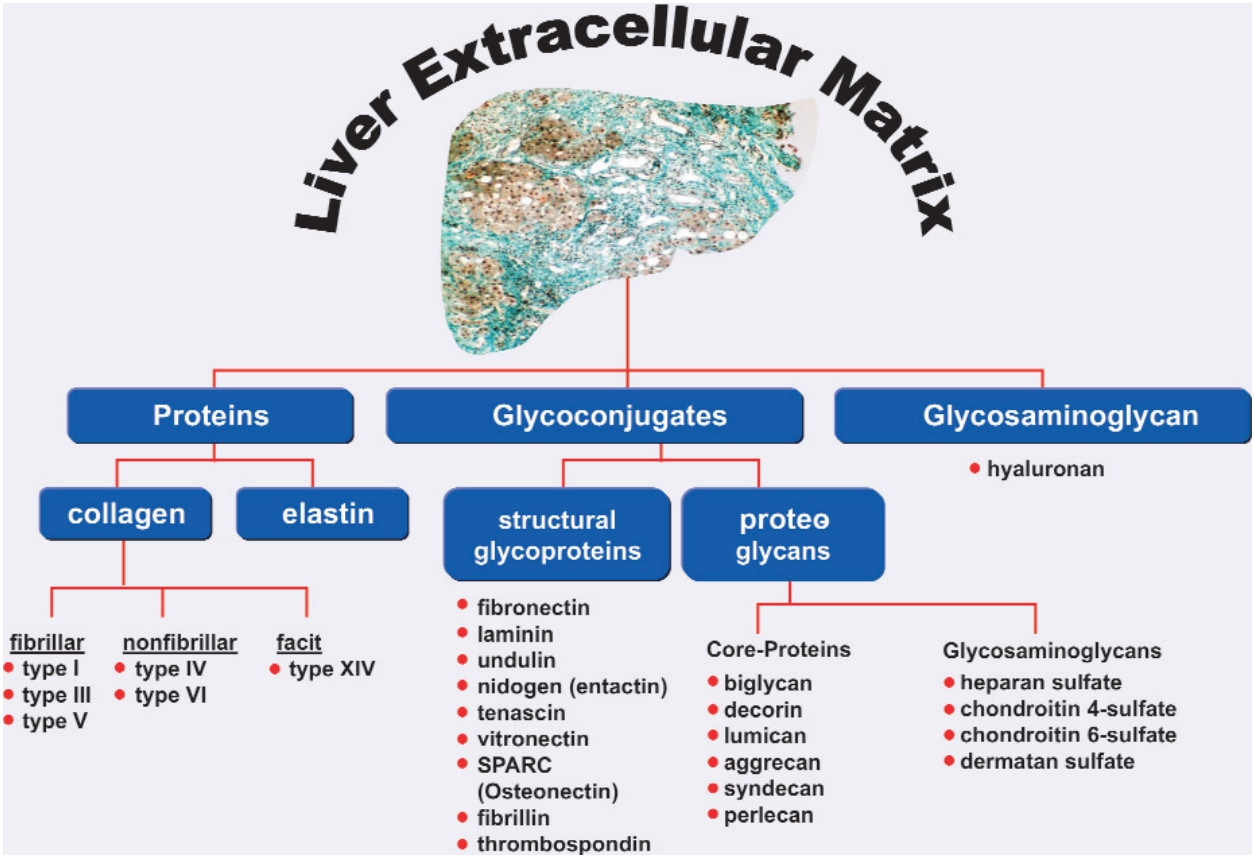


Fig. 1 Major components of ECM in human fibrotic liver.

20–30 µm ("stellate cells") embracing the endothelial boundary of the sinusoid (Fig. 4). In large, triacylglycerol-rich droplets more than 80% of total liver vitamin A (retinoids) is stored. Despite their much smaller cell volume HSC store about 50 times more retinol (30 nmol/106 cells) than hepatocytes (0.5–0.8 nmol/106 cells). Both, in injured liver and in culture on plastic surfaces, HSC undergo a gradual phenotypic change from non-proliferating, retinoid-storing cells to a proliferating, fat and retinoid loosing phenotype, which increasingly expresses α-smooth-muscle actin (α-SMA) as a characteristic cytoskeletal protein or receive some novel characteristics (Fig. 5). This process, termed transdifferentiation, is considered to be the key event in the pathobiology of fibrogenesis [19] since the resulting phenotype, *i.e.* the myofibroblast (MFB) not only produces almost all of the ECM components described above for fibrotic connective tissue but also a broad array of cytokines and chemokines (Fig. 6) and, furthermore, acquires contractility in response to ligands such as endothelin and NO [20, 21]. Transdifferentiation of HSC *in situ* is a tissue phenomenon, *i.e.* it is mediated by paracrine acting mediators released from genuine liver cells (hepatocytes, Kupffer cells, sinusoidal endothelial cells) and from invading, non-hepatic cells such as platelets, lymphocytes and leukocyte subtypes (Fig. 7). Once the transdifferentiation process is advanced *via* the intermediate cell type (transitional cells), the fully transformed MFB are able of autocrine stimulation due to the broad array of mediators secreted and the expression of the respective receptors [22]. Among the numerous pro-fibrogenic mediators a functional hierarchy might exist, which points to transforming growth factor-β1 (TGF-β1) [23] and platelet-derived growth factor (PDGF) [24, 25] as the most effective ones. However, non-peptide mediators also are involved in activation and transdifferentiation of HSC since reactive oxygen species (ROS) [26–28], acetaldehyde [29, 30] and lipid mediators [31] can be fibrogenic. HSC and/or MFB express a certain structural and functional heterogeneity [32, 33],

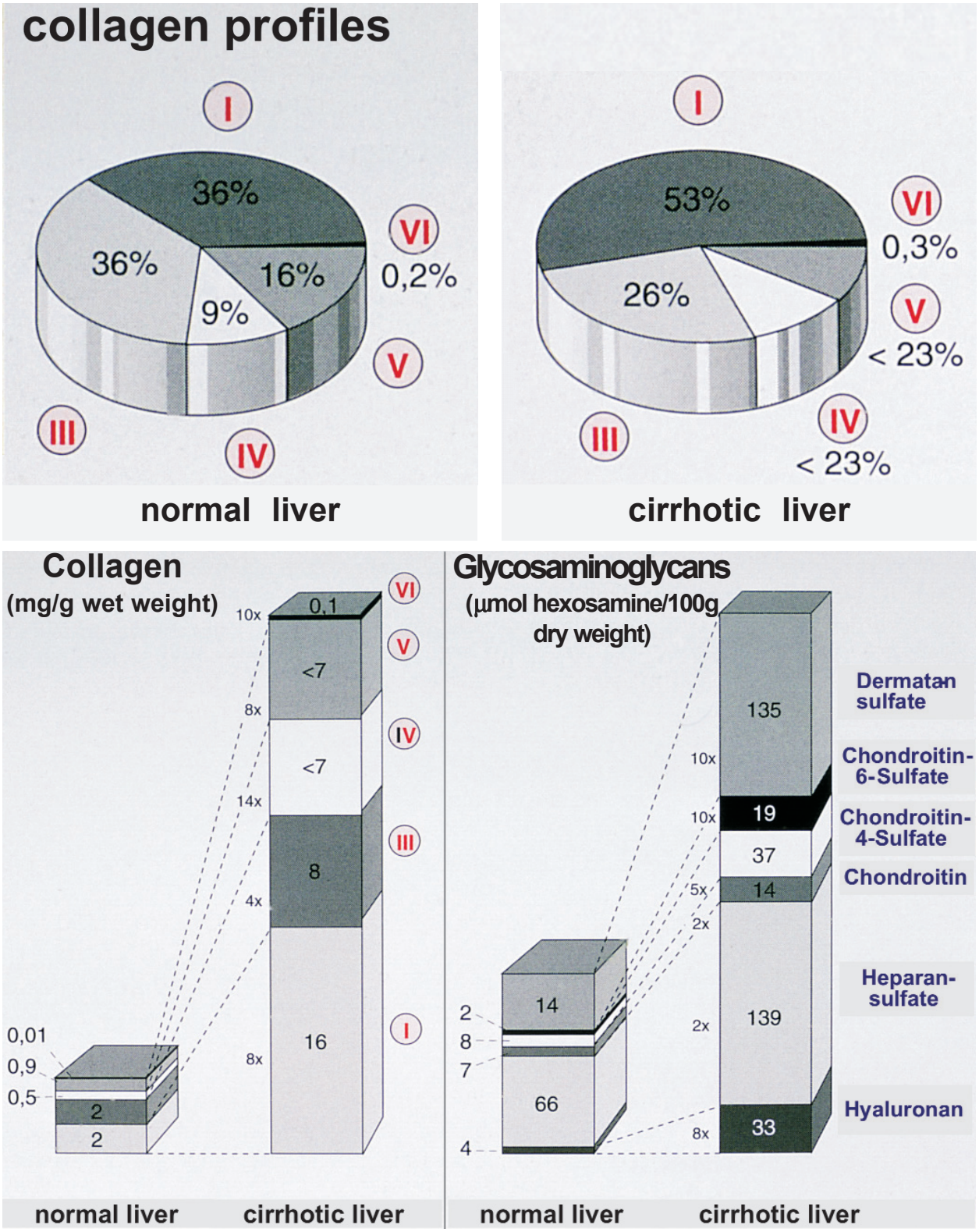


Fig. 2 Changes of the composition of collagen types I - IV and of glycosaminoglycans from normal to cirrhotic human liver. Numbers give the concentrations for collagen in mg/g wet weight and for glycosaminoglycans in mol hexosamine/100g dry weight. In addition, the proportions (%) of specific types of collagens in normal and cirrhotic liver are presented. Data are compiled from literature. The x-fold increases in cirrhotic liver are given.

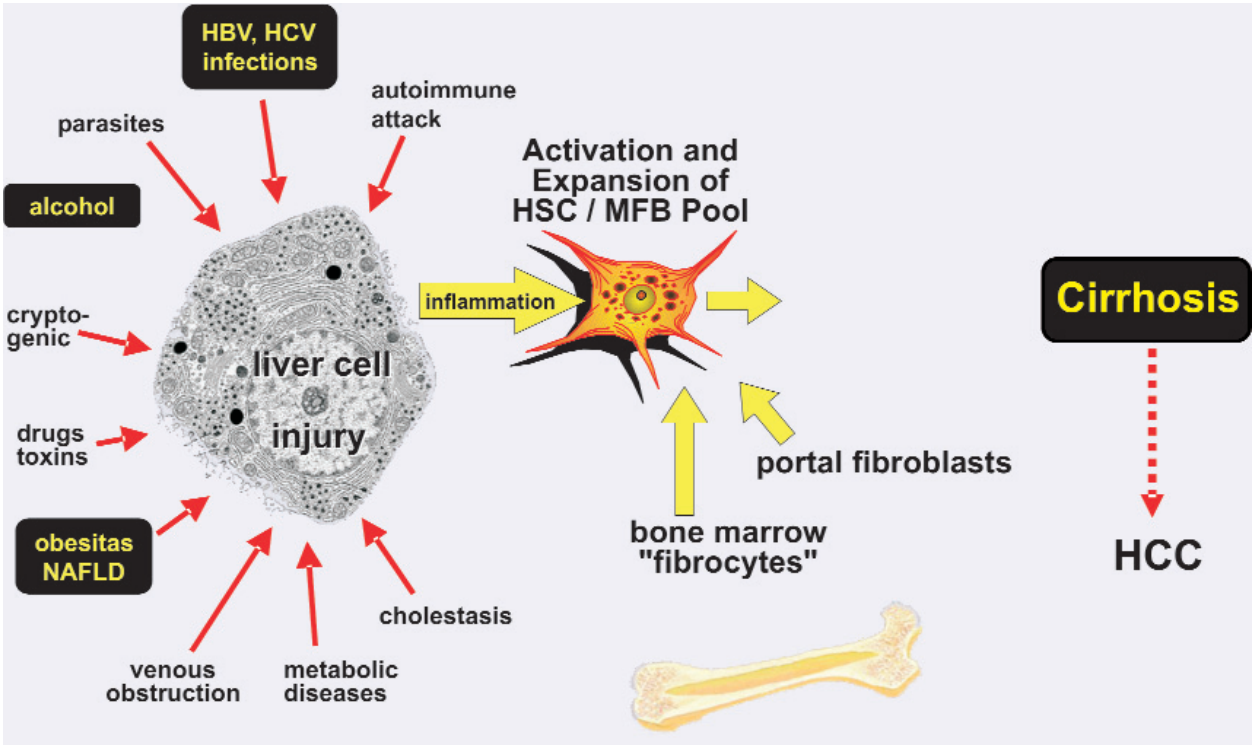


Fig. 3 Formal pathogenetic sequence of fibrogenic activation of HSC leading to fibrosis and cirrhosis. The potential contribution of bone marrow-derived fibrocytes to the extension of the pool of MFB in damaged liver is illustrated. Abbreviations used are: HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease.

which led to the observation that a potentially significant proportion (up to 20%) originate as fibrocytes or similar cell types from bone marrow and reach the inflamed liver tissue *via* the systemic circulation [34, 35]. Beside HSC/MFB, portal fibroblasts and bile duct epithelial cells might participate in fibrogenesis albeit their fractional contribution is not strictly assessed and might be of minor importance. Recently, epithelial to mesenchymal transition (EMT) has been suggested in some fibrotic organs as an important pathogenetic pathway to increase the number of fibrogenic cells [36, 37]. EMT occurs in embryonic development and morphogenesis, cancer progression and metastasis, and in chronic degenerative, fibrotic disorders of mature organs. In particular, in kidney EMT is suggested as an important pathway, which converts epithelial cells, showing a high degree of plasticity, to mesenchymal matrix-producing cell types. Interestingly, *in vitro* and *in vivo* evidence suggests a promoting role for TGF-β in EMT but bone morphogenetic protein (BMP), another member of the TGF-β super-family [38], counteracts this process. Up to now a role of EMT (*e.g.* from bile duct epithelial cells to fibroblastic cells) in liver fibrogenesis (if at all) has not been established.

Fibrogenic signalling of TGF-β

As outlined above, TGF-β is among other polypeptide mediators involved in hepatic fibrogenesis influencing the plasticity of HSC [23, 39]. The TGF-β family contains three closely related isoforms (*i.e.* TGF-β1, TGF-β2, TGF-β3) that are active as secreted peptides that often have similar biological activities *in vitro*, while eliciting more distinct biological responses *in vivo*. They are synthesized as precursors containing an N-terminal located secretory signal sequence, a long precursor segment, *i.e.* the latency-associated peptide (LAP), and a C-terminal part corresponding to the mature TGF-β. The biologically active form is a dimer of 25kd in which the two subunits are linked by a disulfide-bridge. In this complex each monomer

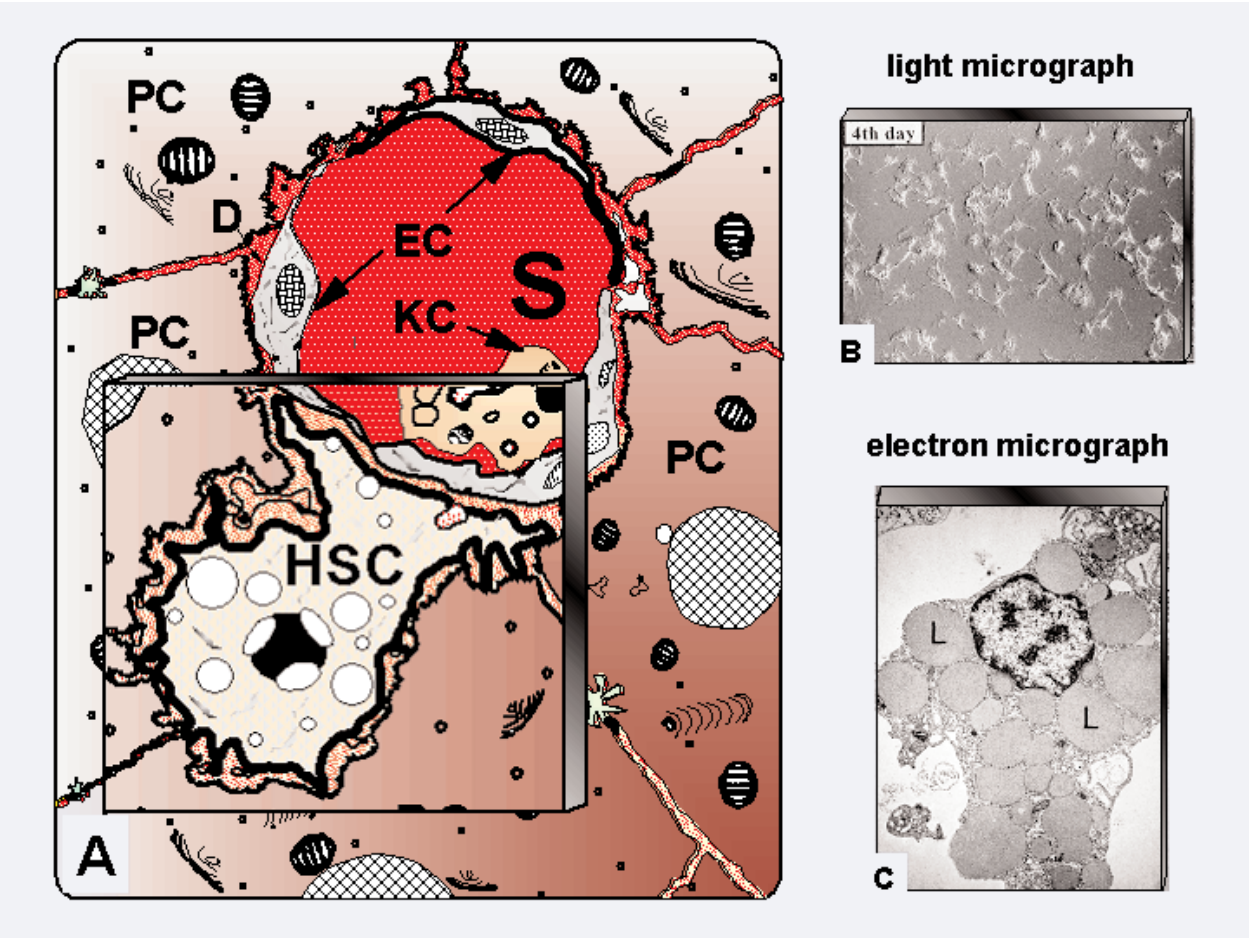


Fig. 4 Diagrammatic presentation of the histological localisation of HSC in recessus of adjacent parenchymal cells (PC) in the perisinusoidal space of Disse in the liver. (**A**) The sinusoid (S), endothelial lining (EC) and Kupffer cells (KC) are shown. HSC location is enlarged. In the upper right (**B**) a light micrograph of primary cultures of rat HSC, in the lower right side (**C**) an electron micrograph showing numerous lipid droplets (L) indenting the nucleus are shown.

contains two anti-parallel pairs of β-strands and a α-helix stretch (Fig. 8).

Generally, TGF-β acts as potent growth inhibitor for many different (preferentially epithelial) cell types, plays a key role in the control of parenchymal apoptosis, and stimulates the production of ECM components. During hepatic fibrogenesis, TGF-β has a pivotal role in initiating, promoting, and progression of transdifferention HSC into MFB. Concomitant with increased activity of TGF-β during fibrogenesis, HSC increase the production and deposition of collagen leading to progressive scarring and loss of organ function. Thus, overexpression of TGF-β in the liver induces severe liver fibrosis [40]. Conditionally regulated expression of TGF-β further reveals that the induction of fibrogenesis is directly linked to the concentration of this causative agent [41]. Conversely, the blockade of TGF-β synthesis or signalling using different experimental strategies prevents ongoing liver fibrosis in various animal models [42–45].

The different isoforms of TGF-β exert their biological effects through a distinct network of TGF-β type I (TβRI), type II (TβRII), and type III (TβRIII) cell-surface receptors as well as several intracellular signalling mediators commonly known as Smad proteins [46, 47]. TβRI and TβRII are structurally similar serine/threonine kinases containing a cysteine-rich extracellular domain, a short hydrophobic transmembrane region, and a cytoplasmic region harbouring the kinase motif (Fig. 9). The typical scenario in TGF-β signalling is the follow-

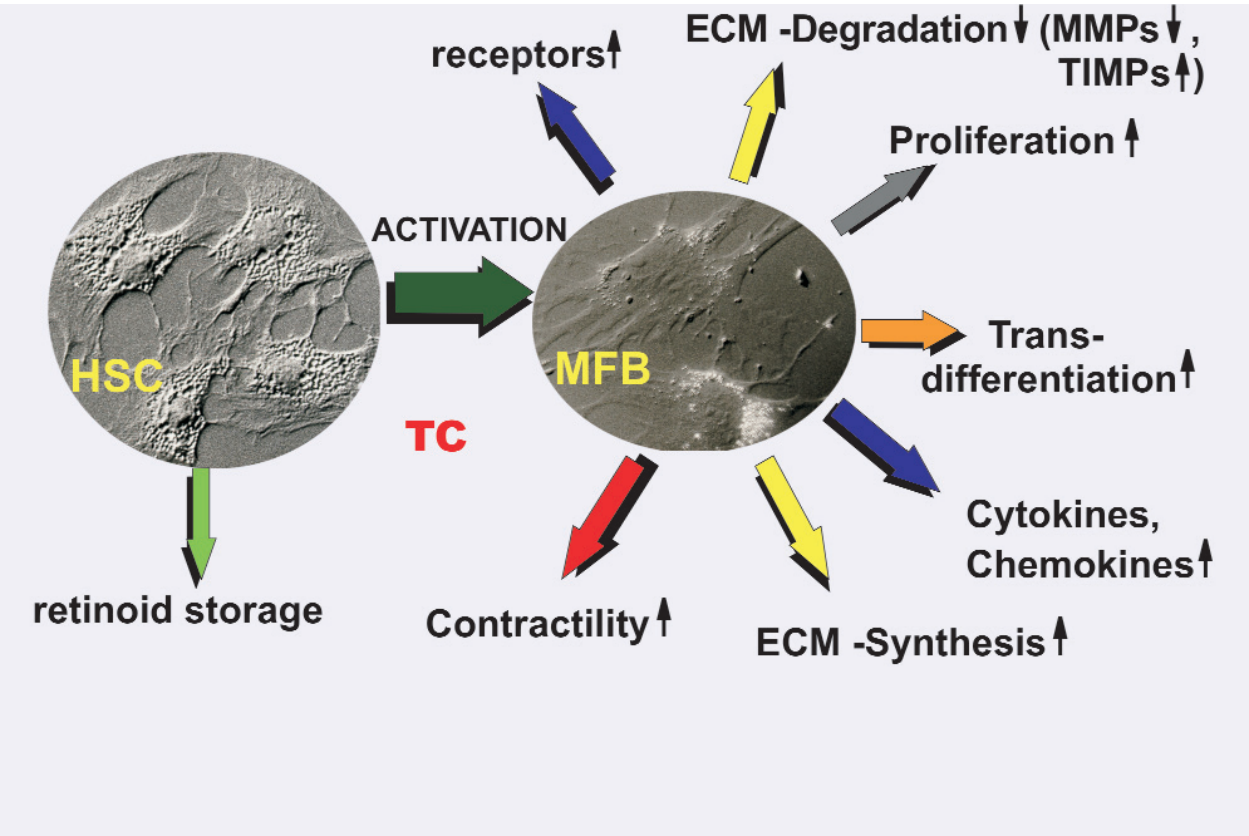


Fig. 5 Transdifferentiation of vitamin A (retinoid)-storing HSC *via* transitional cells (TC) to α-SMA-positive MFB. The pathogenetic key event in fibrosis leads to a dramatic phenotypic change of HSC to a potent fibrogenic cell type (MFB), which is capable of paracrine activation of resting HSC and of autocrine stimulation.

ing: in the absence of ligands, both TβRI and TβRII are present as homodimers (Fig. 10A). Following ligand binding to TβRII, hetero-tetrameric complexes composed of two molecules each of TβRII and TβRI are formed, in which the TβRII kinases phosphorylate TβRI. This phosphorylation activates TβRI kinases inducing autophosphorylation and phosphorylation of Smads (Fig. 10B). Currently, eight different Smads have been isolated from mammalians. Based on their structural and functional properties, Smads are classified into receptor-mediated Smads (R-Smads), common mediator Smads (co-Smads), and inhibitory or anti-Smads (I-Smads). The R-Smads (Smad2 and Smad3) bind to the TβRI and are phosphorylated at their carboxyl-termini by the activated TβRI kinase. Subsequently, they form a complex with the co-Smad (Smad4), and translocate into the nucleus to regulate expression of specific target genes like collagen type I and potentially stimulate of the I-Smads (*e.g.* Smad7). In the following, the I-Smads (Smad6, Smad7) can associate with activated TβRI and inhibit R-Smad phosphorylation (Fig. 10C). This complex signalling cascade is further modulated by proteins binding to individual receptors or Smads leading to a network of fine tuned reactions. Further import modulators of TGF-β action are the TβRIII, *i.e.* betaglycan and endoglin (also known as CD105). They have a more indirect role in TGF-β signal transduction and are involved in signalling through the modulation of ligand-binding affinity (specificity). They share an overall similar structure (Fig. 11) with a large N-terminal extracellular domain, a single hydrophobic transmembrane domain, and a cytoplasmic tail with no obvious signalling motif [48, 49]. The transmembrane region and the short cytoplasmic tail of both, betaglycan and endoglin, are very similar and both proteins bind the different TGF-β isoforms with different affinities and are able to present them to the TGF-β

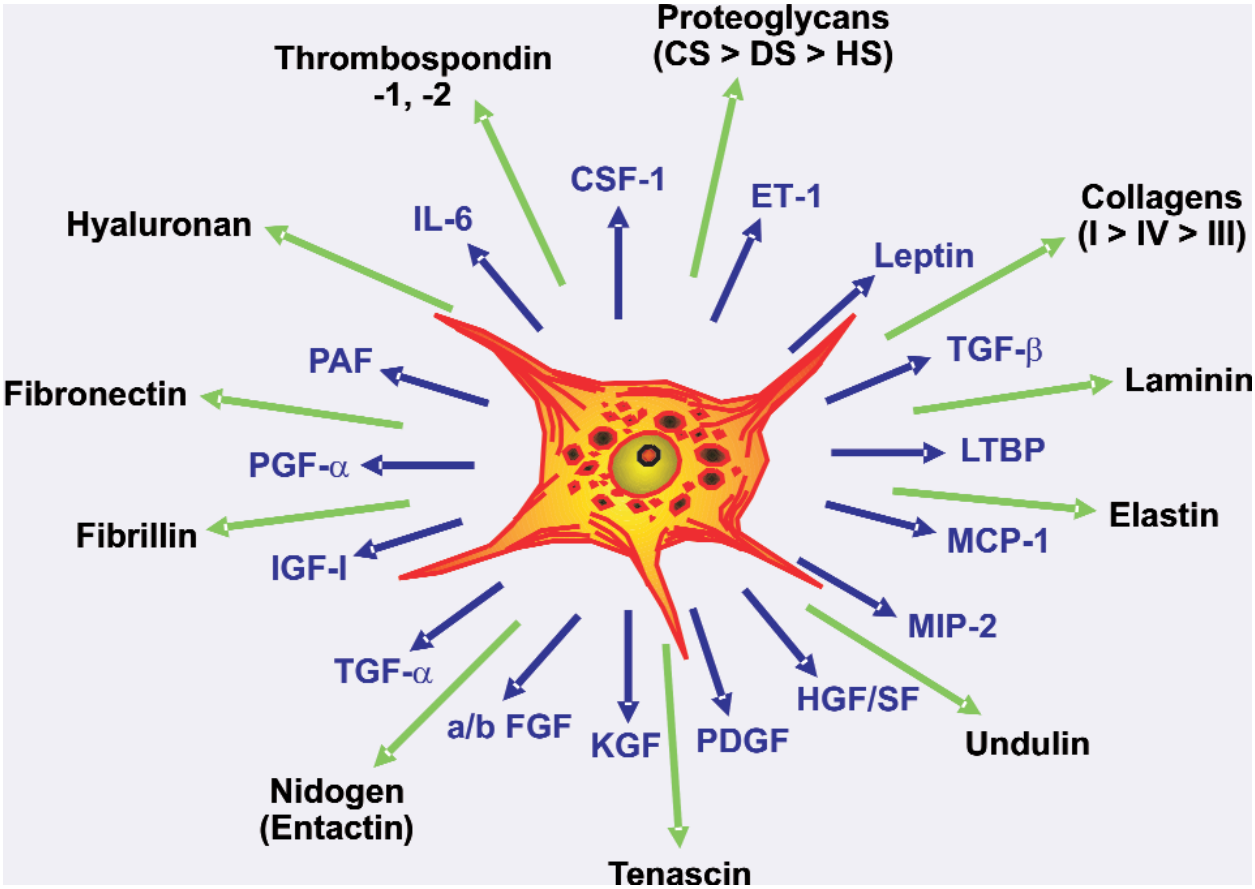


Fig. 6 Matrix molecules and cyto-/chemokines secreted by activated HSC and MFB. Abbreviations used are: ET-1, endothelin-1; FGF, fibroblast growth factor; IGF, insulin like growth factor; IL-6, interleukin-6; KGF, keratinocyte growth factor; MCP, monocyte chemoattractant protein; MIP-2, macrophage inflammatory protein-2; PAF, platelet activating factor; PGF, prostaglandin F; CSF-1, colony stimulating factor-1; LTBP, latent transforming growth factor-β binding protein

signalling receptor [49, 50]. Both TβRIII are expressed in HSC suggesting that they might act as important determinants of binding of TGF-β to TβRI and TβRII or allowing the fine tuning of TGF-β signalling [51, 52].

In primary cultured HSC undergoing transdifferentiation the effects of TGF-β on intracellular signalling is dependent on cellular activation; TGF-β causes phosphorylation and nuclear translocation of Smad2 primarily in quiescent HSC and Smad3 in transdifferentiated HSC [53]. Furthermore, the cytokine is able to inhibit proliferation of quiescent but not transdifferentiated HSC or MFB [53, 54]. In addition, phosphorylated nuclear Smad2 but not Smad3 is present constitutively in activated HSC [53] suggesting that the two R-Smads have distinct roles in the process of cellular activation. A more recent study demonstrated that the overexpression of Smad3 was sufficient to exhibit increased deposition of fibronectin and type 1 collagen and increased α-SMA organization in stress fibers [55]. These characteristics were not noticed when Smad2 was overexpressed supporting the notion that Smad3 plays the more important role in transmitting the morphological and functional maturation of hepatic MFB and that Smad3 is a direct mediator of matrix production in HSC [55]. In line with the hypothesis that Smad3 is a main "fibrogenic mediator" in HSC is the demonstration that hepatic collagen expression in response to an acute fibrogenic stimulus is decreased in mice lacking Smad3 [56]. Hence, given the prominent role of Smad3 in mediating the pathobiology of fibrotic diseases, inhibition of Smad3 signalling could be a prime target for intervention in fibrotic conditions (for review see [57]).

The most investigated I-Smad in HSC is Smad7. Compared to the R-Smad it lacks the typical phos-

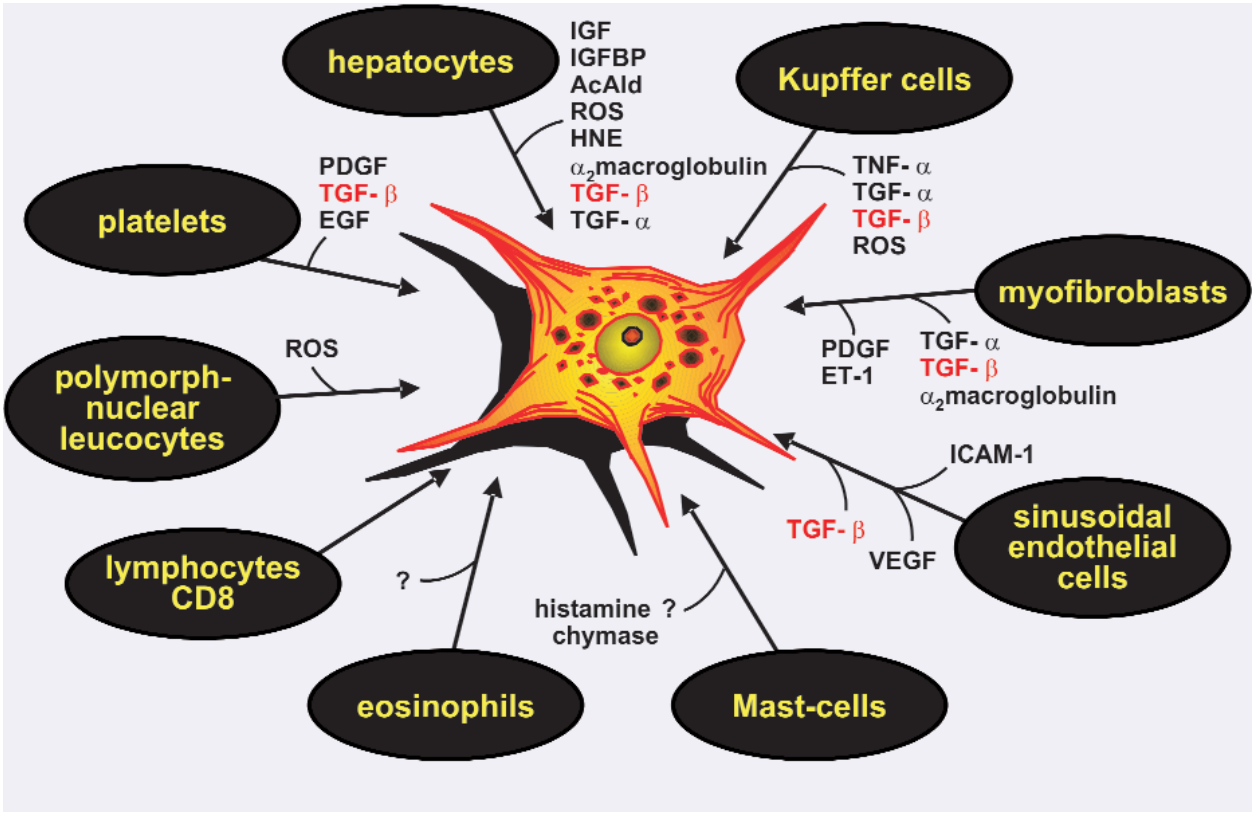


Fig. 7 Synopsis of cellular interactions involved in activation and transdifferentiation of HSC to MFB and the major peptide and non-peptide mediators involved. Abbreviations used are: AcAld, acetaldehyde; EGF, epidermal growth factor; HNE, 4-hydroxynonenal; ET-1, endothelin-1; ICAM-1 intercellular adhesion molecule-1; VEGF, vascular endothelial growth factor.

phorylation motif SSXS at the C-terminus, and the MH1 domain is not able to bind DNA (Fig. 12). Upon TGF-β stimulation, this feedback inhibitor of TGF-β signalling is transcriptionally activated [58] and, therefore, it is conceivable that the transient overexpression of Smad7 results in inhibition of HSC transdifferentiation and attenuation of experimental fibrosis *in vivo* [59]. However, the blocking of TGF-β signalling by Smad7 does not result in decreased α-SMA expression in cultured HSC [59], although it was clearly found that the blockade of TGF-β synthesis reduces α-SMA expression in culture-activated HSC [60].

Despite our remarkable progress in unravelling the TGF-β signalling in HSC and MFB, many important issues are still unknown. Most importantly, the activities and functions of BMPs belonging to the TGF-β superfamily are not systematically analysed. From studies in other cell types, it is well established that another subset of R-Smads, *i.e.* Smad1, 5, and 8, are activated by BMP receptors. Since the TGF-β type III receptor endoglin that is expressed in HSC can also interact with type I receptor that activates Smad1, 5 and 8 further studies are needed to examine the relevance of the BMP signalling pathways in HSC and its impact for hepatic fibrogenesis. In this regard it should be mentioned that BMP-7 is a strong antagonist of TGF-β action in other organs, *i.e.* the administration of BMP-7 in pharmacological doses attenuates the process of fibrosis and accelerates its reversal in kidney [61, 62].

Moreover, the antagonistic and synergistic effects of other polypeptide factors influencing the TGF-β-induced transdifferentiation, cellular activation, and pathogenesis of hepatic fibrogenesis must be addressed in future work. In this regard, PDGF-BB and other PDGF isoforms are of particular relevance. PDGF-BB is a homodimer of disulfide-bonded polypeptide chains that is one of the most potent mitogen for cultured HSC isolated

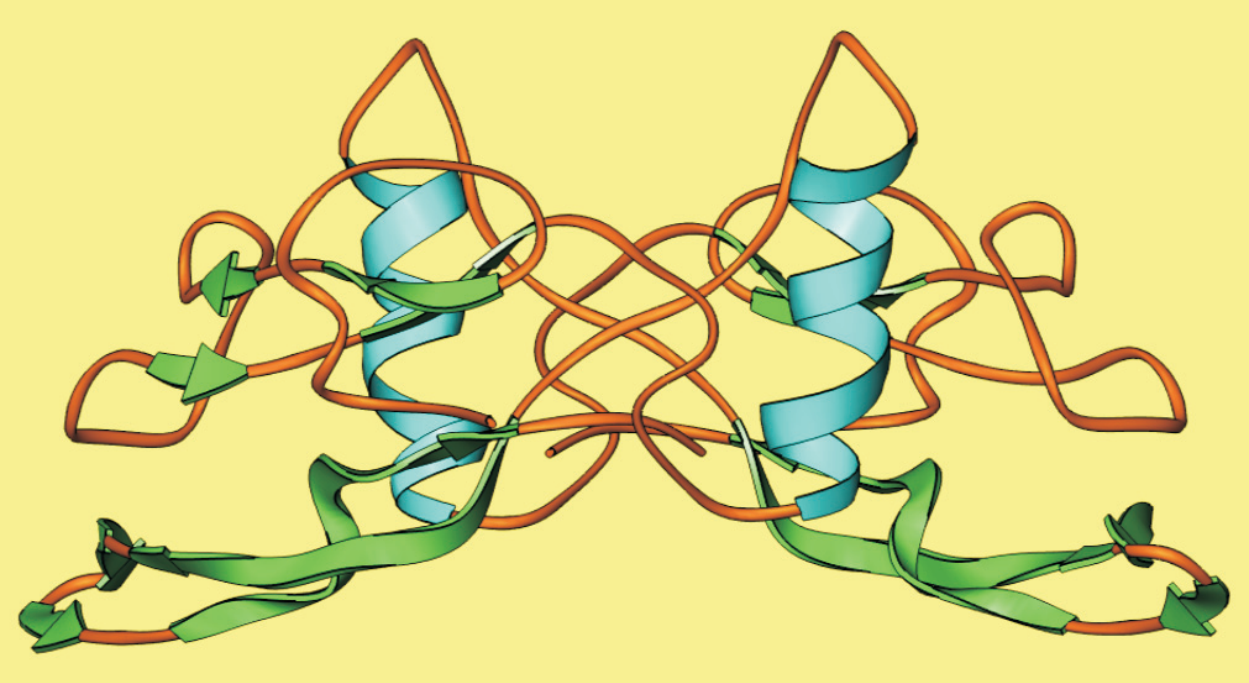


Fig. 8 Ribbon drawing of the X-ray-determined structure of human TGF-β1. The diagram was drawn using the Ribbon 2.0 software [128] and the coordinates deposited under accession no. 1KLC in the Brookhaven Protein Database (PDB) [129]. Mature TGF-β is a 25-kDa dimer in which the two subunits are joined by a disulfide bond. The fold of each subunit contains two antiparallel pairs of β-strands (regions in green) and a α-helix stretch (region in blue).

from rat, mouse, or human livers (for review see [63]). The solving of the three dimensional structure has revealed that each subunit is folded into two highly twisted antiparallel pairs of β-strands and that the two chains in the disulfide-linked dimer have an antiparallel arrangement (Fig. 13)

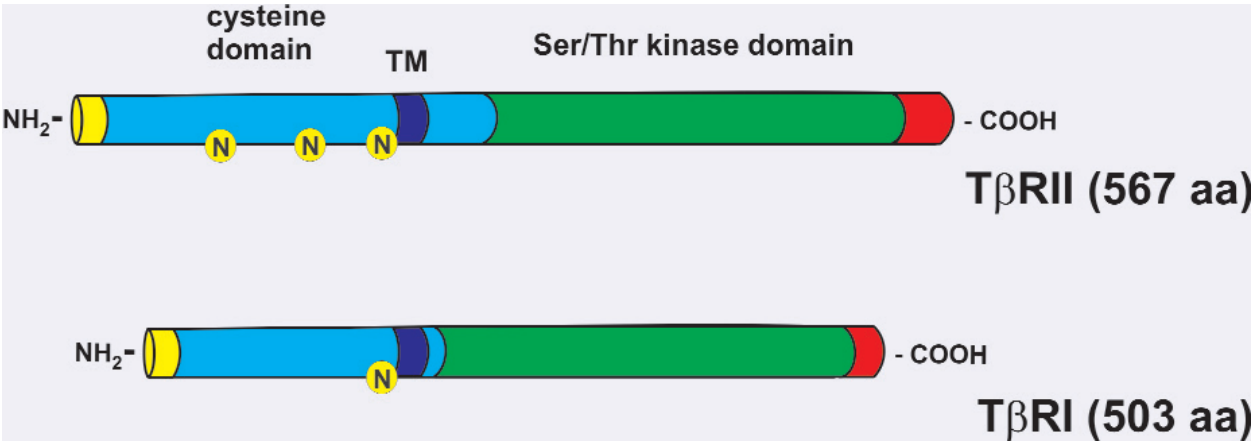


Fig. 9 Schematic structure of human TGF-β receptors type II and type I found on hepatic stellate cells. The overall architecture of TβRII and TβRI receptors is very similar. Each receptor contains an N-terminal signal sequence (in yellow), an extracellular cysteine domain (in light blue), a short transmembrane region (TM, in dark blue), an intracellular serine/threonine kinase domain, and a short C-terminus (in red). Potential N-glycosylation sites (N) are located in the extracellular regions of the receptors. Human TβRII (Genbank accession no. M85079) and the activin receptor-like kinase 5 (Genbank accession no. P36897), a typical TβRI, are proteins of 567 and 503 amino acids in size, respectively.

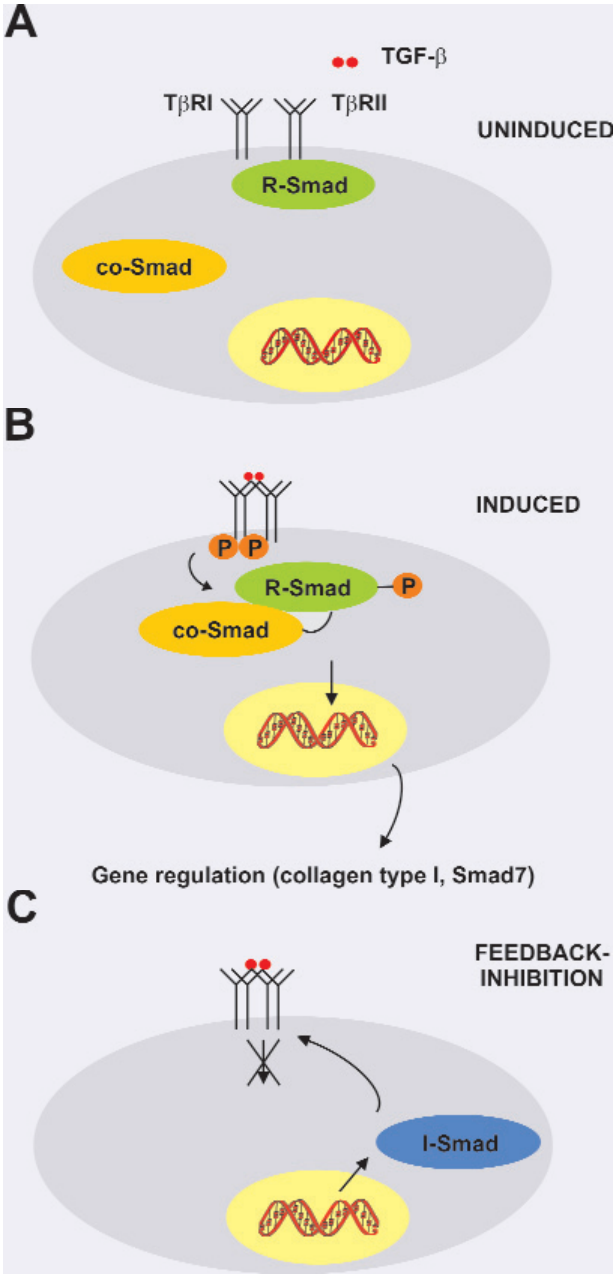


Fig. 10 TGF-β signalling in hepatic stellate cells. (**A**) Under uninduced conditions, receptor-regulated Smads (R-Smads) are associated with transforming growth factor-β receptors. (**B**) On ligand stimulation, the TGF-β type II receptor kinases phosphorylate TβRI and subsequently the R-Smads are phosphorylated by their cognate receptors, triggering the heterodimerization with co-Smads. The R-Smad/co-Smad complexes translocate into the nucleus, bind to DNA, and either activate or repress transcription of their target genes (*e.g.* collagen type I). (**C**) Simultaneous to the intermediation of the signal, Smad7 is transcriptionally induced by TGF-β. This functions as negative feedback inhibitor by association with activated TβRI resulting in prevention of R-Smad phosphorylation and modulation of downstream target genes.

[64]. Albeit much effort has been made to study the effects of PDGF-BB and TGF-β on HSC, their interaction is still unclear, but it is most likely that both cytokines have differential and synergistic effects in the course of fibrogenesis. A recent study investigating the potential crosstalk between PDGF-BB and TGF-β1 in activated pancreatic stellate cells, which are similar to HSC, found that PDGF-BB augments the effects of TGF-β1 presumably because of an elevated expression of TGF-β1 and a common use of signalling pathways and probably because of up-regulation of TGF-β1 synthesis and subsequent enhanced auto-stimulation of pancreatic stellate cells [65]. Although the linkage of PDGF signalling and TGF-β-triggered cascades are not understood in HSC, there is emerging evidence indicating that both cytokines activate HSC by transmitting their signals through the c-Jun N-terminal kinase-dependent Smad2/3 phosphorylation, both *in vivo* and *in vitro* [66]. In addition, both, the stimulation of HSC with PDGF-BB and TGF-β1 resulted in an increase of their migratory capacity and up-regulated MMP activity [67]. Likewise, a cumulative effect of TGF-β1 and PDGF in chemotaxis and invasion was observed in human HSC, collectively suggesting that PDGF and TGF-β have at least some similar biological effects on HSC [68]. However, there are also some hints pointing to antagonistic function of PDGF and TGF-β in the control of cell proliferation when administered sequentially possibly while TGF-β treatment decreased the abundance of PDGF receptors [69] again demonstrating the considerable versatility in TGF-β signalling and cellular response. Of future interest will be the role of extracellular modifier proteins of TGF-β such as connective tissue growth factor (CTGF), nephroblastoma overexpressed protein (Nov) and Kielin/Chordin, which are known in other cell types to affect ligand binding and signal generation. Their expression in HSC was recently established.

Nonfibrogenic roles of HSC and TGF-β

Work of the last decades has shown that HSC present a highly dynamic phenotype during liver damage with key functions in the development of human and experimental fibrosis [23, 39]. In nor-

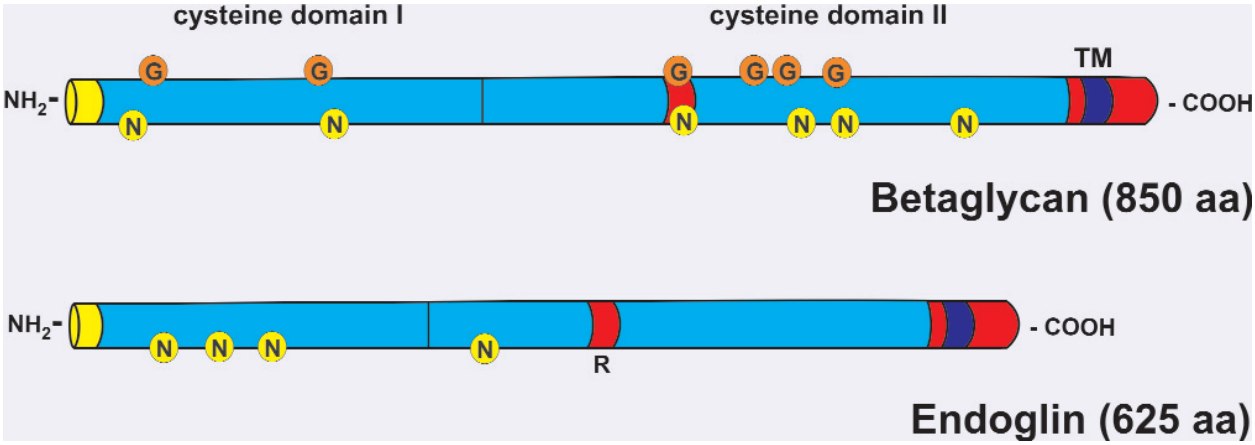


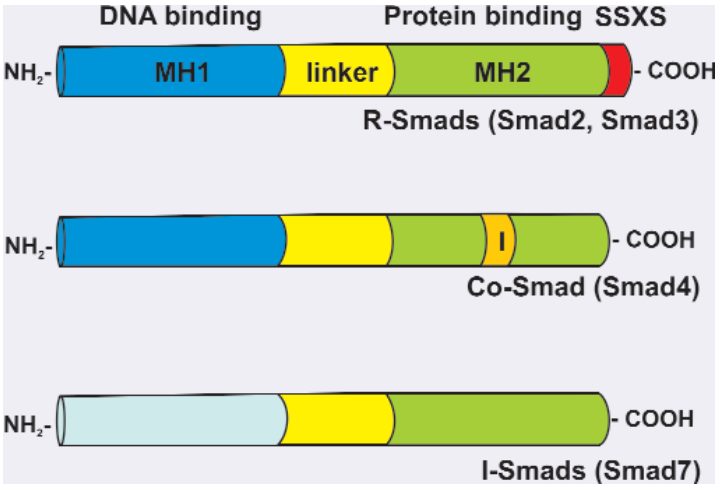
Fig. 11 Schematic structure of the TGF-β type III receptors betaglycan and endoglin. Each TβRIII contains an N-terminal signal sequence (in yellow), two long extracellular cysteine domains (in light blue), a short transmembrane region (TM, in dark blue), and a short C-terminus. Both receptors are highly conserved at their C-terminal parts. Potential N-glycosylation sites (N), glycosaminoglycan-attachment sites (G), and a conserved RGD sequence motif (R) are marked. Human betaglycan and endoglin (Genbank accession nos. Q03167, CAA50891) are proteins of 850 and 625 amino acids in size, respectively.

mal liver this cellular subpopulation is characterised by abundant lipid droplets, low proliferative rate, and low synthetic capacity. In the activated phenotype (*i.e.* MFB) the amount of lipid is decreased and cell proliferation and synthesis of ECM components is increased. However, the role of HSC under normal conditions is far less understood than in the pathogenesis of hepatic fibrosis.

To date, many investigations have shown that in normal liver, HSC have diverse functions. They are involved in the control of body retinoid homeostasis, modulation of the sinusoidal blood flow, synthesis of ECM components, intercellular communication through the synthesis of polypeptide mediators, synthesis of erythropoietin, production of components of the plasminogen activation system, and potentially act as antigen-presenting cells (APC) in the liver (Fig. 14).

Surely, the storage of retinoids points to a prominent function in metabolism and homeostasis of vitamin A. As already mentioned, the majority of the total body vitamin A in rats is stored in the liver, and 80–90% of the retinoid in the liver are stored in the lipid vacuoles of HSC [70]. In line, HSC express specific receptors for retinol-binding protein (RBP), a binding protein specific for retinol, on their cell surface, and take up the complex of retinol and RBP by receptor-mediated

Fig. 12 Structural characteristics of Smad proteins. The Smads share an overall similar structure containing two so called Mad homology domains, MH1 and MH2, that are separated by a proline-rich linker region. The MH1 domain (in blue) within the R-Smads (*e.g.* Smad2, Smad3) and co-Smads (Smad4) harbours a DNA binding motif, which is missing in the I-Smads (*e.g.* Smad7). The MH2 domain (in green) mediates Smad oligomerization, association with TβR, protein interaction, and is involved in transcriptional regulation. R-Smad can be activated by phosphorylation of the SSXS motif within their C-termini. The MH2 domain of the co-Smad (Smad4) contains an insertion (I) not found in R- and I-Smads.



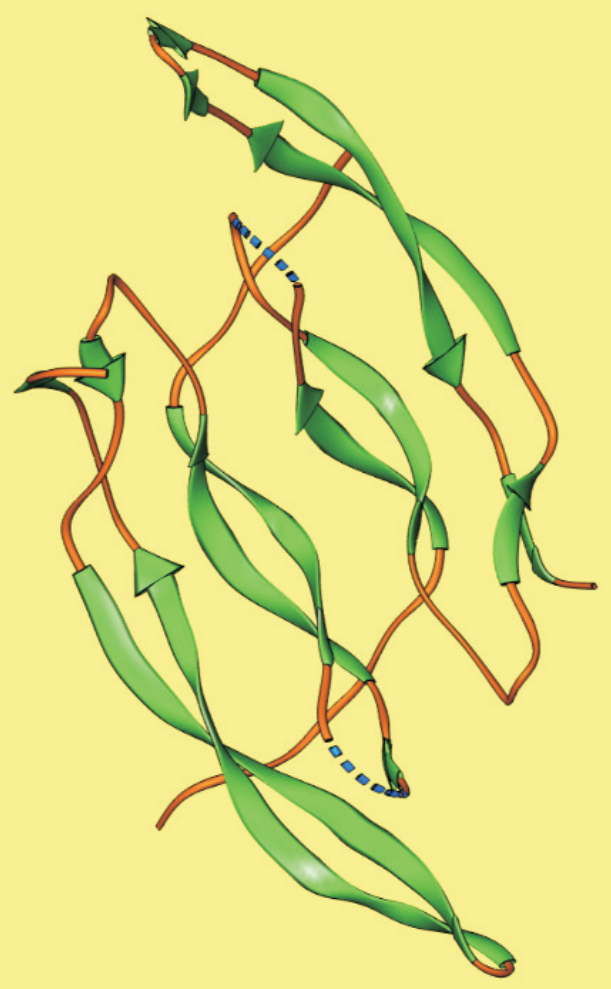


Fig. 13 Illustration of the three-dimensional structure of mature PDGF-BB. The schematic structure was generated with the Ribbon 2.0 software and coordinates deposited under accession no. 1PDG in the Brookhaven Protein Database [64]. The two homodimers in the dimer are arranged in an antiparallel manner and joined by two intermolecular disulfide bonds.

endocytosis [71]. In addition, these cells are enriched in cellular retinal-binding protein, retinyl palmitate hydrolase, and cellular retinoic acid-binding protein, and thus have the metabolic capabilities for hydrolysis of stored retinyl esters [72].

The demonstration that HSC express hormonal receptors controlling contractile properties, secrete respective effectors, and mediate a contractile response after exposure to endothelin has raised the hypothesis that HSC are a major regulator of the sinusoidal blood flow [73–75]. HSC express functional receptors for endothelins as demonstrated by ligand-binding assay [73] that are the most important modulators of hepatic microcirculation and sinusoidal perfusion [76]. Interestingly, the release of endothelin-1 in sinusoidal endothelial cells (SEC) is triggered by TGF-β [77]. Since both HSC and SEC are located in the perisinusoidal space it is possible that their common strategic position allows them to cooperate in controlling the sinusoidal blood flow under normal and pathological conditions.

HSC are also important for the synthesis of several soluble factors that are necessary for the establishment of paracrine and autocrine loops within the liver. They synthesize a broad spectrum of cytokines and growth factor-binding proteins including epidermal growth factor, insulin-like growth factor-I and –II, insulin-like-growth factor binding proteins, interleukins, macrophage colony-stimulating factor, latent TGF-β-binding proteins, CTGF, chemokines, and many substances or proteins controlling the activity of these factors and allowing them to interact with all hepatic and non hepatic cell types (for review see [78] and references therein). Furthermore, erythropoietin and different components of the plasminogen activation system were identified in HSC [79, 80], again demonstrating that the biological functions of this hepatic cell fraction are yet only partially understood and are not solely restricted to the liver.

Preliminary studies demonstrate that cultured human HSC express membrane proteins involved in antigen presentation, including members of the HLA family, lipid-presenting molecules, and factors involved in T-cell activation [81]. Importantly, the exposure to proinflammatory cytokines markedly increases these molecules and activated HSC are able to internalise low- and high-molecular-weight components, indicating that they can perform fluid-phase and receptor-mediated endocytosis. Moreover, mouse HSC were also able to take up large latex beads of 1.0 μM in size through a process of phagocytosis involving pseudopodiae [82]. Based on these data, it is reasonable to assume that HSC play a role in the immune function of the liver. The finding that HSC can serve as an APC is also underscored when human tumor cells are grown in murine livers. In this xenograft model, it was shown that the growing human tumors are encapsulated by activated murine HSC possibly reflecting immunogenic responses at the invasion front [83].

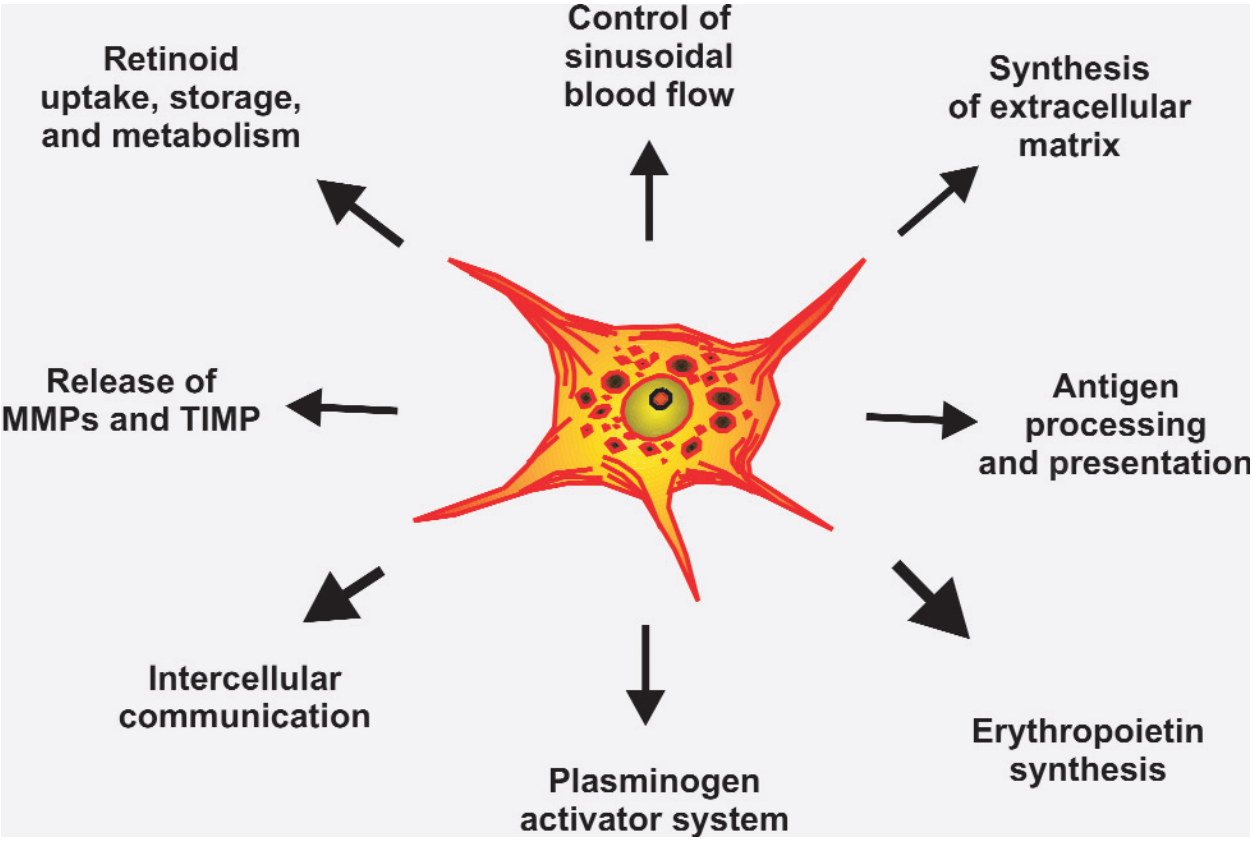


Fig. 14 Nonfibrogenic roles of hepatic stellate cells. The following functions can be attributed to hepatic stellate cells: (*i*) retinoid uptake, storage and metabolism, (*ii*) modulation and control of sinusoidal blood flow, (*iii*) synthesis/turnover of ECM components, (*iv*) intercellular communication through the synthesis and secretion of cytokines and growth factors, (*v*) erythropoietin synthesis, (*vi*) the expression of components of the plasminogen activation system, (*vii*) release of matrix metalloproteinases and their inhibitors, and (*viii*) antigen processing and presentation.

The understanding of HSC functionality is further complicated by reports demonstrating that HSC constitute a heterogeneous population of cells that differ in regard to their gene expression profile, their retinoid content, their proliferation, and their function in hepatic tissue repair [32, 33, 84–86]. Moreover, the amounts of individual subfractions are variable during acute or chronic liver injury suggesting distinct roles of these subpopulations [87]. Using a dual murine reporter gene transgenic, it was further demonstrated that typical marker genes of fibrogenesis [collagen α1(I), α-SMA] were not uniformly expressed but differentially regulated in peribiliary, parenchymal and vascular fibrogenic cells following bile duct ligation [32]. Some cells are positive for α-SMA, some for collagen α1(I), while the activity of both genes was increased in others.

Also the functionality of TGF-β on apoptosis is potentially dependent on the culture conditions [88, 89]. Saile *et al.* reported that TGF-β has anti-apoptotic effect on activated HSC that is paralleled by proliferation inhibition and G_1-arrest when fully activated cells were cultured under serum-reduced conditions. In contrast, apoptosis was increased when cells were stimulated with TGF-β1 in serum free medium. Based on this plasticity in response, caution is mandatory when describing general effects of TGF-β functionality on HSC. This is also true for the *in vivo* situation, in which TGF-β has dual, sometimes opposing effects in pathological conditions. It is well accepted that TGF-β, usually acting as a tumour suppressor, can switch his activities and become a tumour promoter in cancer and is associated with several oncogenic activities [90]. A clear tumour-suppressive effects of TGF-β has been demonstrated in transgenic mouse models

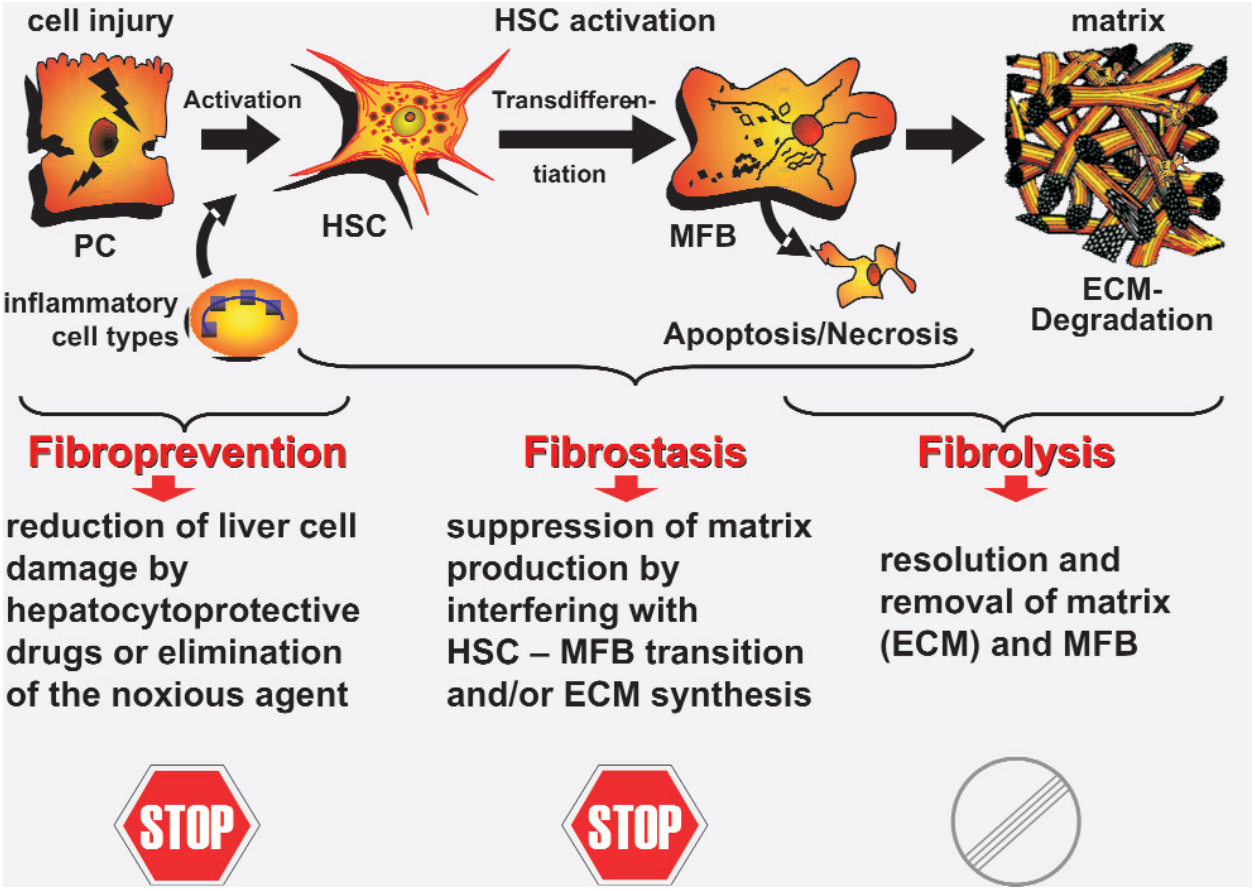


Fig. 15 Summary of the three overlapping levels of pharmacological intervention with hepatic fibrogenesis which are based on the pathogenetic sequence.

in which heterozygous or homozygous TGF-β1-nulls show an increased incidence of chemically or spontaneously induced tumours, respectively [91–93]. In line, other investigations demonstrated that the lifetime exposure to a soluble TGF-β antagonist protects mice against metastasis revealing that the role of TGF-β, particularly TGF-β1, in tumorigenesis, invasion, and metastasis upon environmental challenge is complex [94]. Further, it is linked to EMT, which is a cellular hallmark observed during early tumour stages and invasiveness, when the inhibitory effects of TGF-β are lost [36].

Present status of antifibrotic strategies

Based on the above described, sequential concept of fibrogenesis, pharmacological intervention can be effective on three different levels (Fig. 15): fibroprevention = hepato(cyto)protection, fibrostasis = inhibition of HSC transdifferentiation and/or matrix expression, fibrolysis = resolution of matrix and targeted necrosis or apoptosis of MFB [95]. Numerous compounds with antifibrogenic effects have been identified in experimental models of fibrosis [96, 97]. Clinically most relevant will be approaches, which stimulate fibrolysis either by eradication of MFB (*e.g.* by targeted induction of apoptosis or necrosis) or dissolution of fibrotic ECM [95, 98] (Fig. 16). Under certain conditions reversibility of fibrosis can be reached [6, 99–101]. Drugs leading to a fibrostasis (Fig. 17) would be of clinical value if valid non-invasive parameters are available, which allow to monitor the dynamic process of fibrogenesis and, hence, therapeutic control of fibrogenesis. The currently most relevant problem inherent to the various modalities of antifibrotic trials is the limited organ (tissue) specificity and disease related activity of the drugs. Thus, unexpected severe adverse effects cannot be excluded dur-

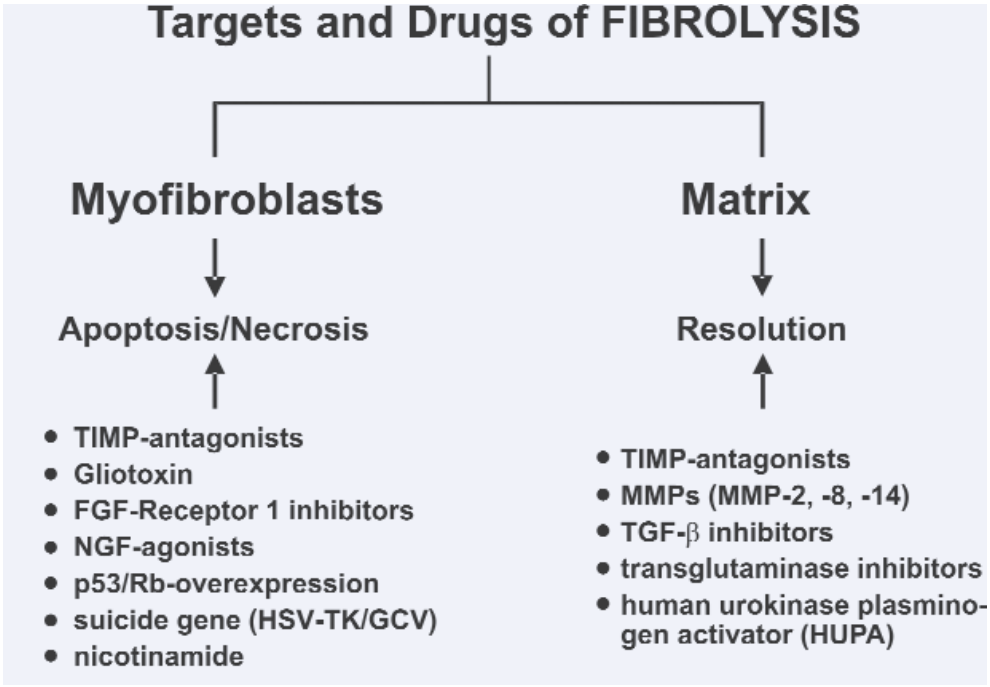


Fig. 16 Compilation of targets and respective drugs used for experimental therapeutic fibrolysis. Abbreviations used are: GCV, ganciclovir; HSV, Herpes simplex virus; Rb, retinoblastoma protein; TK, thymidine kinase.

ing long-term administration of some of these components, in particular, if gene therapeutic approaches are considered [102]. Cytoprotection is achieved by effective antiviral treatments of hepatitis B and C and the availability of immunisation procedures.

Suspected adverse effects of HSC eradication and of TGF-β knock-down

Eradication of HSC as the prevalent fibrogenic cell type in liver is not yet feasible although approaches of suicide gene therapy of HSC were made experimentally [103]. At present, we do not know the complete set of functions of HSC in normal liver since it might not be justified to consider HSC as a purely vitamin A-storing cell type (see above). Newly recognized functions, *e.g.* as an antigen-presenting cell (APC) [81] indicate additional functional properties, which could be relevant in many physiological aspects. Quite more successful might be experiments investigating elimination and/or local inhibition of TGF-β and TGF-β signalling as antifibrotic principle [104, 105]. Considering the pluripotency of this almost ubiquitously distributed cytokine systemic inhibition could be potentially hazardous if the many physiological roles of TGF-β are considered, *e.g.* immunosuppressive, anti-inflammatory, tumour-suppressive and antiproliferative effects. Thus, long-term experiments are needed to exclude adverse effects of TGF-β neutralization or inhibition such as autoimmunopathies, asthma, infections, and tumour development.

Proposals for antifibrotic fine tuning using stellate cells and TGF-β as targets

Based on the plasticity within the TGF-β-controlled transdifferentiation process, there are many ancient and more recent proposals for useful antagonists to TGF-β overactivity or fibrogenesis. The list of potential strategies is increasing daily and the involvement of TGF-β in various diseases makes them clinically useful.

A recent comprehensive proposal was made to classify a number of pharmacological agents directing hepatic fibrogenesis [96]. Based on the mode of pharmacological actions, the authors divided them into five different groups: (*i*) compounds with a "direct" anti-fibrogenic potential modulating gene expression and synthesis of ECM components or inducing their regulation, (*ii*) those acting as "indirect" anti-fibrogenic activity affecting the deposition of fibrillar ECM through the inhibition of other

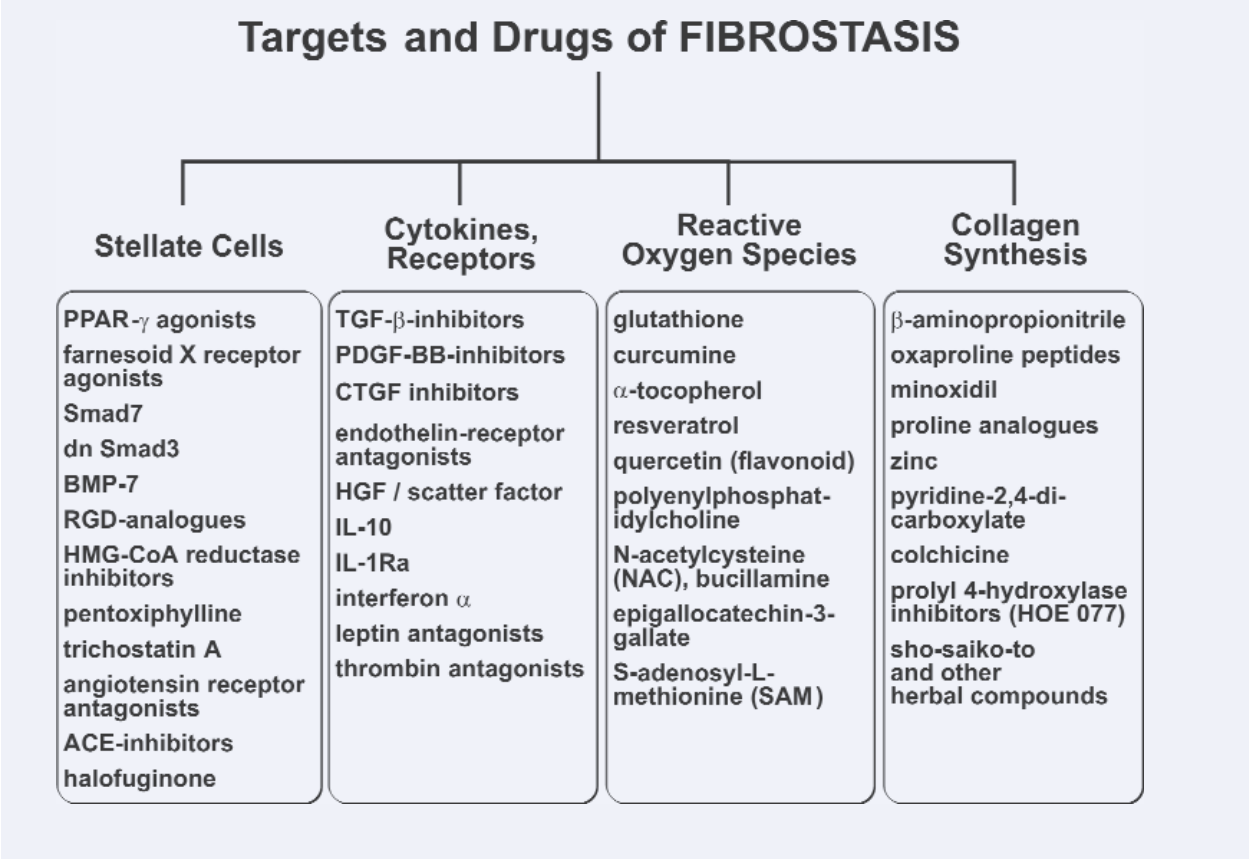


Fig. 17 Compilation of targets and respective drugs used for experimental therapeutic fibrostasis. Abbreviations used are: ACE, angiotensin-converting enzyme; dn, dominant negative; HGF, hepatocyte growth factor; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IL-10, interleukin-10; IL-1Ra, interleukin-1 receptor antagonist; PPAR-γ, proliferator-activated receptor; RGD, Arg-Gly-Asp.

pro-fibrogenic features of HSC such as proliferation and motility or through the induction of HSC apoptosis, (*iii*) "anti-oxidants" reducing the pro-fibrogenic effects of ROS and intermediates, (*iv*) "biotechnological devices" aiming to sequester TGF-β or its synthesis as well as other mediators involved in the establishment of fibrogenesis, and (v) miscellaneous substances with divers actions on HSC contraction, proliferation, and motility.

Some of the "direct" antifibrogenic components like colchicine, 6-ethyl chenodeoxycholic acid (6-ECDCA), pentoxifylline, hepatocyte growth factor (HGF), or the serine protease inhibitor camostat mesilate (CMM, FOY305) are known to reduce TGF-β1 expression or its proteolytic activation by suppression of plasmin activity in HSC or during hepatic injury [106–110]. Consequently, administration of these compounds results in a decrease of HSC transdifferentiation, hepatocyte apoptosis, stimulated hepatocyte mitosis, leading to resolution of fibrosis in experimental models [111]. The spectrum of "indirectly" acting anti-fibrogenic substances includes cyclooxygenase 2 inhibitors, gliotoxin, ACE inhibitors, and inhibitors of proton exchangers. Their mode of action is versatile and only indirectly linked to TGF-β functionality.

"Antioxidative" components attack ROS that play a crucial role in the induction and progression of different liver diseases and evidence of oxidative stress has been detected in almost all the clinical and experimental conditions of liver diseases of different etiology and progression rate of fibrosis [27]. The rationale for the use of antioxidants is the finding that oxidative stress is associated with increased activity of TGF-β1 (and NFκβ) and *vice versa* [112, 113]. TGF-β-mediated accumulation of hydrogen

peroxide was shown to activate and bind CCAAT/enhancer-binding protein-containing transcriptional complex to the α1(I) collagen gene promoter activating its activity. Thus, antioxidants such as resveratrol, quercetin, *N*-acetylcysteine, glutathione, α-tocopherol and epigallocatechin gallate interfere with fibrogenesis [112, 114]. In line with these findings is the demonstration that some "antioxidants", *e.g.* a standardized extract of the milk thistle *Silybum marianum* (silymarin), suppress the expression of TGF-β1 and decrease deposition of fibrillar ECM [115]. The last group including "biotechnological devices" is presently of more academic interest. Many of these strategies were tested in culture-activated HSC or in experimental rat or mouse models of liver fibrogenesis induced by ligation of the common bile-duct or induced by toxic substances, *e.g.* carbon tetrachloride (CCl_4), thioacetamide (TAA), dimethylnitrosamine (DMN), porcine serum, or ethanol. One of these therapeutic options is the application of soluble or dominant negative receptors against TGF-β. Since TβRII is the primary binding receptor for bioactive TGF-β, overexpression of an inactive TβRII allows competitive binding (sequestering) of ligand while the signal transduction into the cell is abrupted. The efficacy of these bio-engineered receptors counteracting TGF-β actions in the course of hepatic fibrosis was shown in different rat models of hepatic injury when either a truncated human TβRII or a soluble, artificial human TβR consisting of the ectodomain of human TβRII and the Fc portion of human immunoglobulin G were applied [42, 43]. Often these devices were transferred using adenoviral expression systems known to have high affinity for the hepatic tissue [116]. Furthermore, hepatic fibrogenesis was also inhibited when a traditional expression vector was injected into the muscle or when respective transgenes were given systemically as purified proteins [42, 117, 118]. Interestingly, also the application of a soluble PDGF type β receptor in the BDL model significantly attenuates ongoing fibrosis as assessed by reduced expression of α-SMA and collagen [118]. However, compared to soluble TβRII, the PDGF antagonist exerts an overall weaker antifibrotic effect supporting the notion that TGF-β plays a more pivotal role in the fibroproliferative changes occurring during hepatic injury. Interestingly, the expression of the soluble PDGF receptor in culture-activated HSC results in inhibition of PDGF signalling and PDGF-BB mRNA expression [119]. Moreover, the synthesis of thrombospondin-1 (TSP-1) was markedly reduced [119] which may influence the activation of TGF-β [120] and subsequently in a partial decrease in fibrogenesis.

Noteworthy, most of the bio-engineered-driven approaches were up to now only tested in culture-activated HSC undergoing transdifferentiation or in experimental models of ongoing hepatic fibrosis. However, this prophylactic treatment does not reflect the clinical situation. Therefore, it is mandatory that future experiments will be performed focussing on treatments allowing the reversal of an already established fibrosis.

The observed differential gene expression during the transdifferentiation process including the activation if transcription factors, ECM proteins, cell adhesion molecules, smooth muscle specific genes, and proteins involved in matrix remodelling, or cytoskeletal organization has established investigations aiming to answer the question if the regulatory element of such genes allow to express antifibrotic bio-engineered drugs in a cell type- and/or transdifferentiation-dependent manner. For this purpose we have tested several promoters for their ability to mediate cell-specific expression in our laboratory demonstrating that some of them indeed allowed selective gene expression in HSC/MFB *in vitro* [121]. Although the specificity was lost when applied *in vivo*, these studies will be the basis for further characterisation and isolation of respective controlling elements. Once characterised, these elements will serve to develop new strategies for the selective targeting of activated HSC and treatment of hepatic fibrosis. Alternatively, to these investigations, specific receptors located at the surface of HSC were tested as docking sites for drug targeting to HSC. Using liver tissue slices or competition studies in activated HSC, it was shown that albumin coupled to mannose 6-phosphate or a small peptide that recognises the PDGF receptor type β is applicable as HSC-selective carriers [122, 123].

A critical issue of these artificial drugs is that they create immunogenic epitopes that may prevent long-term application. Therefore, some approaches were initiated trying to reduce the overall concentration of TGF-β by blocking its synthesis by antisense technology. In a study of our laboratory we have demonstrated that the expression of an anti-sense

(single-stranded) RNA, complementary to the coding region of TGF-β1, is able to inhibit TGF-β synthesis at the translational level [60]. Consequently, we observed an increase in cell proliferation, suppression of fibrogenic marker proteins (*e.g.* α-SMA, collagen type I, LTBP-1, TβRI, TβRII), and alterations in TGF-β1-sensitive genes in cultured HSC [60]. In bile-duct-ligated rat liver, the transgene abrogates TGF-β-enhanced production of collagen and α-SMA [45]. Comparable, antisense oligonucleotides or small interfering RNAs (SiRNAs, RNAi) targeting individual TGF-β mRNAs isoforms are under close investigation. Although these were not tested yet for treatment of hepatic fibrogenesis they have given promising effects for the treatment of glioblastoma, pancreatic as well as colon cancer (for recent review see [105]).

Other possibilities to block TGF-β activity are the usage of mono- or polyclonal neutralizing antibodies (mAbs, pAbs), the inhibition of proteins necessary to release biological TGF-β from its precursor or latent complexes, the utilization of TGF-β sequestering proteins such as α_2-macroglobulin, decorin or LAP, or the usage of CTGF inhibitors (see above, Fig. 17). The latter ones belong to a group of "trap proteins" that act as accessory co-receptors, or circulate in interstitial spaces as soluble moieties to block receptor activation by free ligand [124, 125]. Since some of these proteins, *e.g.* kielin/chordin-like protein (KCP), were found to modify the activities of different members of the TGF-β superfamily displaying antagonistic functions [126], these proteins will definitely open a new avenue for the development of novel antifibrotic therapies.

In one study, TSP-1 involved in the conversion of TGF-β from its latent precursor to the active form was targeted using a peptide antagonist [127]. The rationale of this approach is the finding that the activation of TGF-β is dependent on the interaction of the tetrapeptide KRFK in TSP-1 with a LSKL peptide within the TGF-β precursor. The injection of the LSKL peptide in the course of DMN-induced hepatic fibrogenesis was able to reduce the amount of active TGF-β1, phosphorylation of Smad2, and hence improved liver function.

More recently, small molecules acting as potent inhibitors of TβRI subspecies reached scientific and therapeutic interest. Some of them (*e.g.* SB-431542, SB-505124, SD-208, A-83-01) show a remarkable activity against TGF-β overactivity in several cellular and *in vivo* models. They inhibit the TβRI kinase activity, the downstream Smad phosphorylation and the transcriptional activation or silencing of respective target genes. However, amongst their general functionality, the individual compounds differ in specificity, inhibition capacity (IC_{50}), and safety profile [105]. Based on the tremendous efforts in understanding the molecular effects induced by application of these drugs, it is most likely that some of them will be evaluated in clinical trials in near future.

In summary, the development of TGF-β inhibitors is an important and exciting field for scientists and clinicians. Although, the master antagonistic strategy is not established yet, the first initiation of different clinical studies aimed to test antifibrogenic drugs in various diseases of lung, kidney, heart, and liver will be hopeful for those patients suffering from organ and tissue fibrosis.

Conclusions and future perspectives

The rapid scientific progress within the past 20 years has shown that fibrogenesis in liver is a highly active process leading to excess deposition of ECM. During hepatic fibrogenesis, TGF-β and other soluble peptide mediators and non-peptide signals have a pivotal role in initiating, promoting, and progression of transdifferention of HSC into MFB. TGF-β exerts its biological effects through a distinct network of TGF-β cell-surface receptors and several intracellular signalling mediators commonly known as Smad proteins. Several reports have suggested that the cellular mediator Smad3 plays an important role in transmitting the morphological and functional maturation of hepatic MFB and is a direct mediator of matrix production in HSC. Based on the greatly augmented knowledge underlying the profibrogenic response of HSC we will gain further insight into disease-specific molecular aberrations and simultaneously enlarge our repertoire to antagonize excess production of TGF-β with drugs and gene therapeutic devices. Although the general sequestering of active TGF-β by neutralizing antibodies, soluble receptors, and other binding proteins, as well as the blockade of TGF-β synthesis has raised very promising experi-

mental data, it is obvious that a long-term treatment with these agents will produce severe adverse effects. Therefore, more differentiated forms of pharmacologic agents such as TGF-β receptor kinase inhibitors have been developed. Initial experimental results are clearly encouraging and it is an intriguing task to investigate if these antagonists are effective in clinical trials and will allow the beneficial treatment of the millions of patient's worldwide suffering from chronic liver diseases.

Acknowledgements

The authors are supported by grants from the Deutsche Forschungsgemeinschaft (SFB-542, TP A9) and the Federal Ministry of Education and Research of Germany (Network of Excellence in Viral Hepatitis *Hep-Net*).

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