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REVIEW

The rationale for targeting TGF-β in chronic liver diseases

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ABSTRACT

Background Transforming growth factor (TGF)-β is a pluripotent cytokine that displays several tissue-specific biological activities. In the liver, TGF-β is considered a fundamental molecule, controlling organ size and growth by limiting hepatocyte proliferation. It is involved in fibrogenesis and, therefore, in worsening liver damage, as well as in triggering the development of hepatocellular carcinoma (HCC). TGF-β is known to act as an onco-suppressor and also as a tumour promoter in HCC, but its role is still unclear.

Design In this review, we discuss the potential role of TGF-β in regulating the tumoural progression of HCC, and therefore the rationale for targeting this molecule in patients with HCC.

Results A considerable amount of experimental preclinical evidence suggests that TGF-β is a promising drug-gable target in patients with HCC. To support this hypothesis, a phase II clinical trial is currently ongoing using a TGF-β pathway inhibitor, and results will soon be available.

Conclusions The identification of new TGF-β related biomarkers will help to select those patients most likely to benefit from therapy aimed at inhibiting the TGF-β pathway. New formulations that may provide a more controlled and sustained delivery of the drug will improve the therapeutic success of such treatments.

Keywords EMT, galunisertib, HCC, targeting TGF-βRI, TGF-β, tumour progression.

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Introduction

In a significant percentage of patients, that varies according to the aetiology, chronic liver diseases (CLD) lead to cirrhosis and hepatocellular carcinoma (HCC), which are end-stage CLD. The incidence of HCC is also increasing and is expected to rise yet further in the coming years according to the WHO, ranking as the fifth most common neoplasm worldwide but the second largest contributor to cancer-related mortality owing to its very poor prognosis [1–5]. Despite this, the clinical concept of liver fibrosis and cirrhosis has changed over the last years. There is evidence that liver fibrosis, even in advanced stages, as well as cirrhosis, are dynamic processes that can be reversed with the use of appropriate therapies [6–8]. Although the complex hepatic response accompanying CLD is still not well defined, it is now clear that transforming growth factor-beta (TGF-β) plays a central role as from the initial liver injury right up to end-stage HCC [9–11]. Indeed, in response to tissue injury, active TGF-β is released from latent TGF-β complexes and becomes available for binding to surface receptors and triggering signalling [12]. Continuous or upregulated TGF-β signalling in this context controls key cellular events that dictate the progression of the disease. Distinct liver cell populations respond differently to TGF-β. Thus, TGF-β triggers the activation of hepatic stellate cells into myofibroblasts that start to produce extracellular matrix (ECM) components, an event that is considered to be

pivotal in the fibrogenic process. In hepatocytes, TGF-β induces both cell death and the epithelial mesenchymal transition (EMT). However, the cytostatic and apoptotic effects of TGF-β on hepatocytes in early stages of liver damage and regeneration are lost in later stages, marking another critical step in the disease progression. Meanwhile, the EMT conversion allows cells to acquire motility and scattering properties, while facilitating ECM deposition and scar formation [9, 13]. On top of all this, new players enter the scene, namely the hepatic stem/progenitor cells, that are activated in response to chronic liver injury and become targets of TGF-β actions [14,15]. As a result of this altered hepatic microenvironment, profound changes take place in TGF-β-driven signalling, and a specific cross-signalling between different cellular components is established that, together with genetic and epigenetic alterations, contributes to CLD progression and HCC development [16–18]. In this review, we summarize TGF-β signalling and its multiple actions in the liver disease context and discuss the potential of this signalling pathway as a therapeutic target for drug development.

TGF-β signalling

TGF-β1 is a member of a family of structurally and functionally related dimeric proteins, that includes TGF-β1, TGF-β2 and β3, activins and bone morphogenetic proteins (BMPs) [19,20]. Nearly all cells produce TGF-β, including parenchymal and nonparenchymal liver cells. While some doubt exists as to the ability of hepatocytes to synthesize and secrete nascent TGF-β, these cells have been demonstrated to be surrounded by an ECM with a rich content of TGF-β ligands, possibly derived from neighbouring nonparenchymal cells (reviewed in Ref. [21]). The nonparenchymal liver cells that synthesize and secrete TGF-β include hepatic stellate cells (HSC), liver sinusoidal endothelial cells and inflammatory cells (Kupffer cells), as well as adjacent biliary epithelial cells (reviewed in Ref. [21]). Nascent TGF-β is secreted in a latent form consisting of an N-terminal long pro-peptide, the latency associated peptide (LAP) and a C-terminal mature polypeptide. They form covalently linked dimers via disulphide bonds, which are deposited in the ECM via cross-linking of the LAP with the latent TGF-β binding proteins (LTBPs) and other integral proteins of the matrix [22,23]. The latent TGF-β then requires activation prior to its association with its signalling receptors, a process that is mediated by integrin receptor-mediated mechanical stretching and proteolytic processing of the LAP [22,23]. In this manner, nonparenchymal cells of the liver generate TGF-β, which acts upon the same cells in an autocrine manner or acts on the neighbouring hepatocytes in a paracrine manner. Like TGF-β, liver cells synthesize and secrete several ligands of the TGF-β family, including ligands with a more restricted mode of action, such as BMP-9, which is secreted only by liver cells and which regulates vascular homoeostasis and growth or iron metabolism in hepatocytes [24,25].

Signalling across the membrane is accomplished via TGF-β type I and type II dual specificity kinase receptors (i.e. TβRI and TβRII). These receptors can exist in dimeric form on the cell surface or in submembrane vesicles [26] and mainly exhibit a serine/threonine kinase activity, together with a weaker but detectable tyrosine kinase activity [27]. Upon activation from its latent form, TGF-β initially interacts with TβRII, and thereafter TβRI is recruited and becomes phosphorylated on specific serine and threonine residues by the TβRII kinase. This trans-phosphorylation of TβRI elicits conformational changes to TβRI, leading to the release of the receptor from its cytoplasmic chaperon FKBP12 and the generation of a catalytically active form of TβRI [27,28]. The activated TβRI transmits its signals into the cell by phosphorylating downstream effector proteins. In an evolutionarily conserved signalling branch, receptor-regulated (R-) SMADs, that is SMAD2 and SMAD3, are recruited to, and phosphorylated by TβRI, causing their structural alteration and eliciting functional activation [20]. Activated R-SMADs can form heteromeric complexes with a common mediator (Co)-SMAD4, and these complexes accumulate in the nucleus where they act as sequence-specific transcription factors. The affinity of SMADs for DNA is weak and they need to partner with other DNA-binding transcription factors for efficient recruitment to specific gene sequences. By contrast, coactivators and corepressors assemble around the SMAD–cotranscription factor complex to mediate the regulation of gene expression by the RNA polymerases, thus allowing SMADs to function as transcriptional activators or repressors. The TGF-β receptor can be activated via phosphorylation or recruitment and association with its heteromeric receptor core additional signalling effectors. For example, via its weak tyrosine kinase activity, TβRI is phosphorylated and recruits the adaptor protein Shc, which then promotes a cascade of molecular signalling effectors, including the small GTPase Ras, the serine/threonine kinase Raf and its downstream serine/threonine kinases ERK1/2 (extracellular regulated kinase), known as the mitogen-activated protein kinase (MAPK) pathway [29]. In the absence of direct phosphorylation, the activated TβRI can also recruit the ubiquitin ligase tumour necrosis factor α receptor-associated factor (TRAF)4 or TRAF6, which is poly-ubiquitinate, via lysine 63 interlinked ubiquitin chains, serine/threonine kinase TGF-β-activated kinase 1 (TAK1). This causes its catalytic activation and further signalling via the MAPKs p38 or c-Jun N-terminal kinase (JNK) [30,31]. Through these signalling pathways, TGF-β controls the activity of ERKs or JNKs, which mediate the phosphorylation of transcriptional cofactors of the SMAD complex, for example the activation protein 1

(AP-1) complex, eliciting a regulated control of specific groups of target genes (Fig. 1) [32].

TGF-β family members act on all liver cell types. TGF-β may affect cell proliferation, and hepatocytes are especially sensitive to TGF-β exhibiting arrest of their cell cycle at the G1 phase or often reacting through an apoptotic response [25]. In addition, TGF-β may affect the migration and differentiation of various liver cells. A potent effect of TGF-β is to induce the extracellular matrix by promoting the expression of ECM proteins (e.g. collagen and fibronectin), mitigating the expression of proteases that breaks down the ECM [e.g. collagenases and metalloproteases (MMPs)], and by inducing the expression of inhibitors of these proteases [e.g. plasminogen activator inhibitor and tissue inhibitor of MMPs (TIMPs)] [25]. TGF-β effects are highly context-dependent. Differences in the surrounding extracellular matrix, the presence of other cytokines and (homo- or heterotypic) cell density may change the output of TGF-β on liver cells, not only quantitatively but also qualitatively. Being such a pleiotropic molecule, TGF-β's activity needs to be kept carefully in check to maintain tissue homoeostasis. In part, this is achieved through positive and negative regulation of the function of TGF-β ligands, TGF-β receptor and SMAD signalling components. Ligand-binding proteins may sequester TGF-β from receptor interactions or direct it to complementary but distinct receptor complexes. Accessory cell surface coreceptors regulate the presentation of ligands to the signalling TβRII/TβRI receptor complexes. SMADs are recruited or sequestered by anchor proteins such as SARA (SMAD anchor for receptor activation) and TMEPAI, respectively. Receptors and SMADs are subject to regulation by other protein kinases (and phosphatases) and other posttranscriptional modifications, including ubiquitination, sumoylation, acetylation and

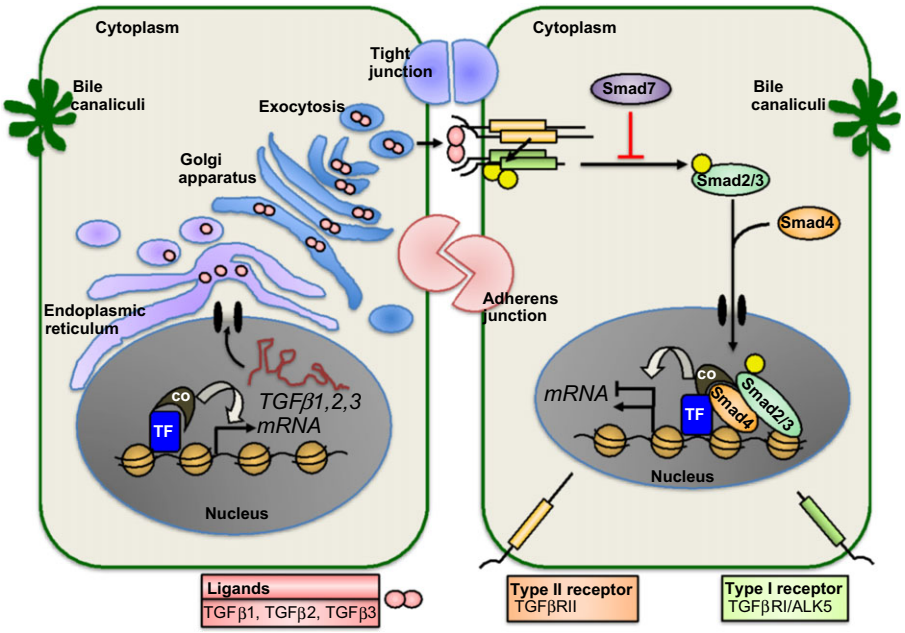

Figure 1 TGFβ signalling. Two adjacent hepatocytes are shown; their bile canaliculi, tight and adherens junctions are highlighted. Via regulated transcription guided by transcription factors (TF) and coactivators (co), the *TGFβ1*, *TGFβ2* and *TGFβ3* genes are transcribed to mRNA, which is exported to the cytoplasm for translation by ribosomes (not shown) on the rough endoplasmic reticulum. Monomeric latent TGFβs are synthesized and traffic towards the Golgi apparatus. Upon protease cleavage and disulfide bond formation, dimeric latent ligands follow the secretory pathway towards exocytic vesicles. Secreted latent ligands (not shown) are activated into their mature signalling forms and meet their signalling receptors on the basolateral membrane of the adjacent cell, possibly in signalling platforms localized between tight and adherens junctions (these details have not yet been proven for hepatocytes). Upon ligand binding, the type II receptor trans-phosphorylates (P in yellow circle) the type I receptor (black arrow). Activated type I receptors phosphorylate (P in yellow circle) the R-Smads, Smad2 and Smad3 (black arrow). For simplicity, the two distinct R-Smads are drawn inside one oval shape. Phosphorylated Smad2 and Smad3 oligomerize with Smad4, enter the nucleus and bind to chromatin (DNA helix wrapped around nucleosomal shapes is drawn). The chromatin-bound Smad complex associates with other transcription factors (TF) and coactivators or corepressors (co), respectively, causing a positive or negative regulation or transcription of target genes. The inhibitory Smad7 is shown to block the activation of R-Smads by the type I receptor. Ligand and receptor names are listed at the bottom of the cells.

ADP-ribosylation to regulate their stability, activity or binding to DNA [20,27,28]. Inhibitory SMADs (I-SMADS, i.e. SMAD6 and SMAD7) antagonize the activation of signal transducing R-SMADs by competing with R-SMADs for type I receptor interaction and by the recruitment of specific E3 ubiquitin ligases (such as SMAD ubiquitin regulatory factor (SMURF1/2) that target the activated type I receptor for degradation [33]. Ubiquitination can be reversed by the activity of deubiquitinating enzymes; ubiquitin specific proteases (USP)4 and USP15 were found to deubiquitinate TβRI, stabilize the TβRI at the plasma membrane, and thereby boost TGF-β signalling [34–36]. I-SMAD7 is transcriptionally induced by TGF-β itself and thus provides a potent negative feedback regulator; I-SMAD7 can also be induced by other cytokines, such as interferon, providing the molecular means for other signalling pathways to cross-talk with the TGF-β pathway and control its activity, duration or spatial expansion within a tissue [33]. I-SMAD stability is also carefully controlled. The RNF12 and Arkadia E3 ubiquitin ligases target SMAD7 for proteasomal degradation and promote or prolong TGF-β signalling [35,37]. These molecular examples illustrate some of the most central regulatory nodes in the TGF-β signalling network that provide targets for control of the biological functions of TGF-β. During liver development, homoeostasis in the adult body, and during waves of injury and repair that occur during the course of various pathological conditions, the function or malfunction of theTGF-β signalling pathway and its molecular checkpoints can generate the essential basis for the progression of liver disease.

Role of TGF-β in liver fibrosis

The principal producers of ECM are 'activated fibroblasts' or myofibroblasts (MFB). Hepatic MFB are transdifferentiated from heterogeneous cell populations in response to different fibrogenic stimuli. According to the most recent studies, the major sources of MFB in experimental models of liver fibrosis are HSC and portal fibroblasts [38]. In the normal liver, sinusoidal endothelial cells and Kupffer cells (macrophages) contain relatively high levels of TGF-β mRNA, whereas HSC express small amounts of the cytokine. However, in response to pro-fibrogenic stimuli, HSC express the three different isoforms of TGF-β, which contribute to the development of fibrosis through both autocrine and paracrine loops [9].

TGF-β is a key player in the transdifferentiation process of HSC to MFB [39,40]. Indeed, for maximal expression of collagen type I in activated HSCs, SMAD3 is required both *in vivo* and in culture [41]. SMAD3-overexpressing HSC, as well as TGF-β-treated cells, show more focal adhesions and an increased α-smooth muscle actin (α-SMA) organization in contractile and thick fibrous bundles, indicating that SMAD3 also regulates cytoskeletal organization in HSC [42]. A cross-talk between SMAD binding elements and the vitamin D receptor (VDR) signalling has recently been proposed for profibrotic target genes [43]. In addition to HSC transdifferentiation to MFB, it is now fully accepted that hepatocyte death is critical for hepatic fibrosis [44,45]. Indeed, it has been proven that apoptosis and phagocytosis of hepatocytes directly induce HSC activation and the initiation of fibrosis [46]. Although the issue is still controversial, it is worth mentioning that EMT from hepatocytes might be another source of MFB [47]. In proliferating hepatocytes, TGF-β is able to induce both pro-apoptotic and survival signals, and it is the balance between these two physiological signals that defines the cell fate. Cells that survive TGF-β's pro-apoptotic effects respond to it by undergoing the EMT, which confers them a mesenchymal-like phenotype and migratory capacity [48,49]. However, whether this effect is relevant during liver fibrosis has not yet been fully elucidated. In fact, recent reports using lineage tracing technology provide evidence against the EMT being the source of MFBs in the liver [50].

Oxidative stress during CLD, due to an increased reactive oxygen species (ROS) production, as well as a decreased activity of antioxidant systems, is not only a consequence of chronic liver injury but may also significantly contribute to the activation of HSC to become MFBs. ROS are mainly produced in the liver mitochondria, as hepatocytes contain hundreds of these organelles, and mitochondrial electron transport is disrupted in a large number of pathophysiological circumstances, resulting in an increased electron leak. However, recent works have highlighted that TGF-β1 induces the expression of a NADPH oxidase (NOX4), which is required for HSC activation as well as for the maintenance of the fibrotic phenotype [51,52]. HSC activation is attenuated either by NOX4 downregulation or against a $Nox4^{-/-}$ genetic background and, importantly, the MFBs activated state could also be reversed by NOX4 downregulation [52,53].

Targeting the TGF-β pathway seems to be a promising strategy in the treatment of a variety of pathological conditions related to liver fibrosis disorders [54]. However, well-designed clinical trials will be needed to evaluate the effectiveness of these new agents prior to implementing their routine use in the clinic.

Role of TGF-β in alcoholic liver diseases

Investigating a cross-talk between TGF-β signalling and ethanol exposure is of relevance, as drinkers with fibrosis or even advanced stages of liver damage principally show increased TGF-β serum/liver tissue levels.

However, contradictory results in terms of TGF-β levels have been reported in different studies, likely because of the limited number of patients investigated and probably of a different disease stage [55,56].

In line with TGF-β overexpression in ALD, there are data from a clinical study of HCV patients in which 26 drinkers of alcohol and 40 nondrinkers were compared in regard to lipid peroxidation, which was estimated as malondialdehyde serum levels. They were found to be significantly higher in patients than controls, thereby showing a significant correlation with TGF-β levels [57]. Similarly, in a study on HCV core protein-expressing transgenic mice additionally fed ethanol, both factors acted synergistically to increase hepatic expression of TGF-β [58].

This is also supported by the fact that lactate dehydrogenase release due to cell damage is increased by cotreatment of cultured hepatocytes with TGF-β and ethanol provoking elevated oxidative stress [59]. Hepatocytes underwent tremendous programmed cell death with ethanol alone, which was mediated by an increased cytochrome C release from mitochondria. This was further supported by the finding that the anti-apoptotic factor BCL-2 blunts TGF-β triggered apoptosis, as well as death caused by costimulation with TGF-β and ethanol. In fact, we found that TGF-β's pro-apoptotic function is massively enhanced by alcohol, leading to exorbitant programmed cell death. Noteworthily, this finding is not explained by increased SMAD signalling but rather by a regulation of other signalling cascades leading to a distinct apoptosis-related gene expression signature.

On the other hand, TGF-β is induced in mouse livers after chronic ethanol insult, and results indicate that in presence of ethanol, TGF-β is pro-steatotic in hepatocytes via a decreasing ADH1 expression. Low ADH1 levels are correlated with enhanced hepatocyte damage upon chronic alcohol consumption by favouring secondary metabolic pathways [60].

In mice fed an ethanol-containing L. De Carli diet, steatosis and TGF-β expression were induced. Furthermore, liver BMP2, but not BMP4 or BMP6 expression was significantly elevated. Despite the increased BMP expression, the BMP receptor, and transcription factors, SMAD1 and SMAD5, were not activated. By contrast, alcohol stimulated SMAD2 phosphorylation. However, SMAD4 DNA-binding activity and the binding of Smad4 to the hepcidin promoter were attenuated, indicating alcohol as a modulator of SMAD signalling in hepatocytes [61]. It has been shown that HCC development arising from the combination of HCV infection and alcohol consumption critically depends on TGF-β. Upon HCV infection, hepatocytes upregulate TLR4, which subsequently sensitizes these for alcohol/LPS [62]. In this setting, LPS triggers the expression of the cancer stem cell factor Nanog. Downstream of Nanog, oncogenes Yap1 and Igfbp2 are induced, which both interfere with cytostatic TGF-β signalling via Smad7 stabilization and AKT/mTOR dependent suppression of Smad3, respectively, thus facilitating cancer stem cell formation [63]. These data indicate that TGF-β signalling in hepatocytes under metabolic stress facilitates hepatocyte death and lipid accumulation through Smad signalling and ROS production, leading to the development of NASH.

There is robust evidence that ethanol is a crucial modulator of TGF-β phenotypes (Fig. 2), and in patients with pre-existing liver fibrosis (with upregulated TGF-β), this may have clinical implications on even moderate drinking.

In summary, there is considerable evidence that TGF-β and alcohol may induce very relevant cross-communication in hepatocytes, and this cross-talk needs more in-depth investigation in the future.

TGF-β regulates the epithelial-to-mesenchymal transition in HCC

EMT reflects a process of de-differentiation, which is consistent with the loss of epithelial cell-to-cell-contacts and the gain of a mesenchymal-like phenotype that endows individual cells with the ability to invade the surrounding tissue and transmigrate into the vasculature [64,65]. At the molecular level, prototypic changes associated with the EMT transformation include the loss of the adherent junction component E-cadherin, along with the upregulation of EMT-inducing transcription factors (EMT-TFs) and mesenchymal proteins such as vimentin, α-smooth muscle actin and type 1 collagen [66]. In principle, the change in epithelial plasticity by EMT is a transient event leading to its reversal, termed the mesenchymal-to-epithelial transition (MET; [67]). Remarkably, the EMT is essentially involved during embryogenesis, wound healing and carcinoma progression, and it is considered to link aspects of hepatic inflammation with liver fibrosis and HCC [68]). In general, TGF-β plays a central role in hepatocellular EMT [69], and is thus of the utmost relevance in a variety of CLDs.

Hepatocytes undergoing EMT activate AKT, that is required for resistance to TGF-β-induced apoptosis, and induce ERK1/2, that is responsible for de-differentiation in a SRC/FAK-dependent manner [70]. Similarly, hepatoblasts undergoing the EMT after long-term administration of TGF-β upregulate EGF-R and Src-dependent Akt, allowing TGF-β-mediated apoptosis to be escaped (Fig. 3) [48,71].

Although the relevance of the EMT in liver fibrogenesis remains controversial, a large body of evidence shows that TGF-β-driven epithelial plasticity of neoplastic hepatocytes is a key event in cell dissemination and intrahepatic metastasis. Importantly, the induction of the EMT by TGF-β signalling essentially occurs in collaboration with other signalling pathways, to abrogate its anti-oncogenic mechanisms. In this context, hepatocellular EMT associated with liver cancer progression was firstly recognized in a murine model of malignant hepatocytes showing an invasive and metastatic phenotype through the collaboration of TGF-β with oncogenic

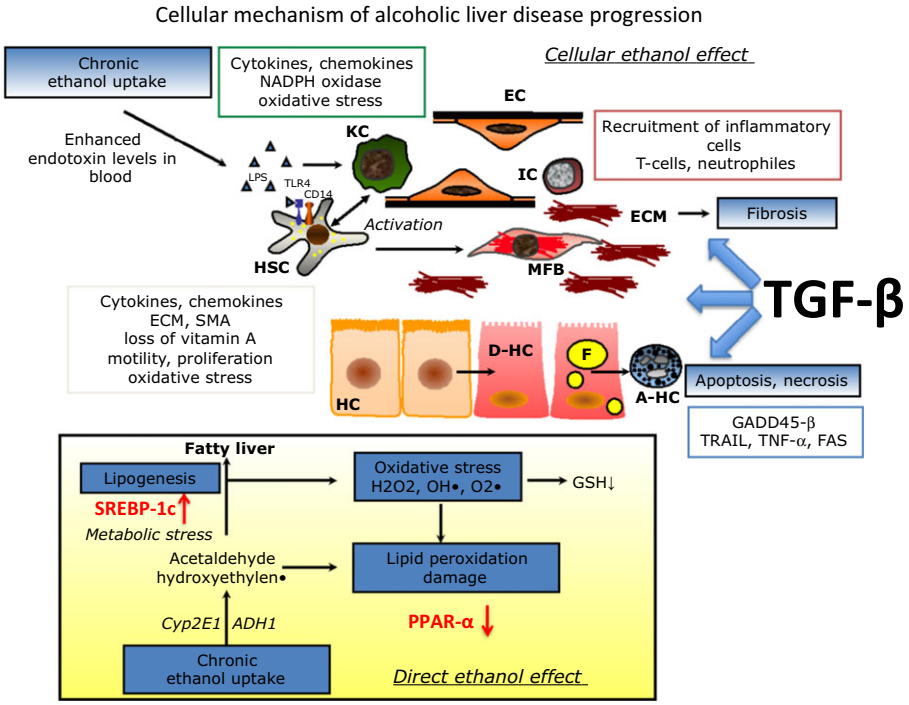

Figure 2 Mechanisms of alcohol-mediated progressive liver damage. Chronic alcohol consumption damages the liver from two angles. Firstly, there is a cellular effect induced by alcohol-mediated upregulation of bacterial endotoxin concentrations (Lipopolysaccharides, LPS) in the blood. LPS binds to cell surface receptors of HSCs, which will be activated to synthesize and release profibrogenic signals (z. B. TGF-β). This then leads on to the activation of macrophages (Kupffer cells), which synthesize and release inflammatory signals. The next step in the cellular reaction is the recruitment of inflammatory cells, which initiates the hepatitis disease stage. The second relevant mechanism is direct damage of the liver parenchyma (hepatocytes) by reactive oxygen species. Alcohol is metabolized in the liver by hepatocytic enzymes (ADH, CYP2E1). Alcohol degradation products cause metabolic stress, which results in an increased fat deposition (fatty liver) and hepatocyte damage (apoptosis or necrosis) or fibroblastoid phenotypical modulation (cellular plasticity). Then signalling molecules will be released, which drive fibrogenesis. TGF-β acts on all the aforementioned cell types and has signalling impacts on many of the processes involved in liver damage, inflammation, repair, fibrosis and even cancer. KC, Kuppfer cells; EC, endothelial cells; IC, inflammatory cells; ECM, extracellular matrix; MFB, Myofibroblast; EMT, Epithelial–Mesenchymal Transition; HC, Hepatocyte; ADH, Alcohol dehydrogenase; Cyp2E1, Cytochrom C P450 Oxidase; GSH, Glutathione.

RAS/MAPK signalling [72,73]. These studies further suggested that the synergy of TGF-β and RAS/MAPK signalling during EMT induces the upregulation and activation of platelet-derived growth factor (PDGF)/PDGFR-alpha. This causes both the nuclear accumulation of β-catenin and the translational upregulation of laminin (Ln)-B1 via the binding of La/SSB to the internal ribosome entry site located in the leader region of Ln-B1 [74–76]. Studies focusing on the tumour–stroma interaction revealed that the secretion of TGF-β by activated hepatic stellate cells (HSCs), as well as MFBs, is responsible for the TGF-β/PDGF-mediated induction of hepatocellular EMT [77,78]. In patients with HCC and human hepatoma cells, the EMT was firstly observed through the high Ln-5 expression which cooperates with TGF-beta to reduce E-cadherin levels and activate beta-catenin in an alpha3 integrin-dependent manner [79]. The migration and proliferation of de-differentiated HCC cells was found to be stimulated by Ln-5, produced and secreted by HSCs [80,81]. Inhibiting TGF-β signalling with the TGF-βR kinase inhibitor (galunisertib) restores E-cadherin expression and diminishes the migratory capacity of hepatoma cells, as well as abolishing the TGF-β1-dependent activation of integrin β1, required for vascular invasion [82,83]. This seems to be important also in patients with HCC, in whom a strong correlation between circulating levels of TGF-β1 and an increased aggressiveness of the HCC phenotype has been shown [79].

A disruption of TGF-β signalling by pharmacological intervention using LY2109761 further reduces the connective tissue growth factor (CTGF)-mediated cross-talk of HCC cells with cancer-associated fibroblasts and decreases the blood vessel

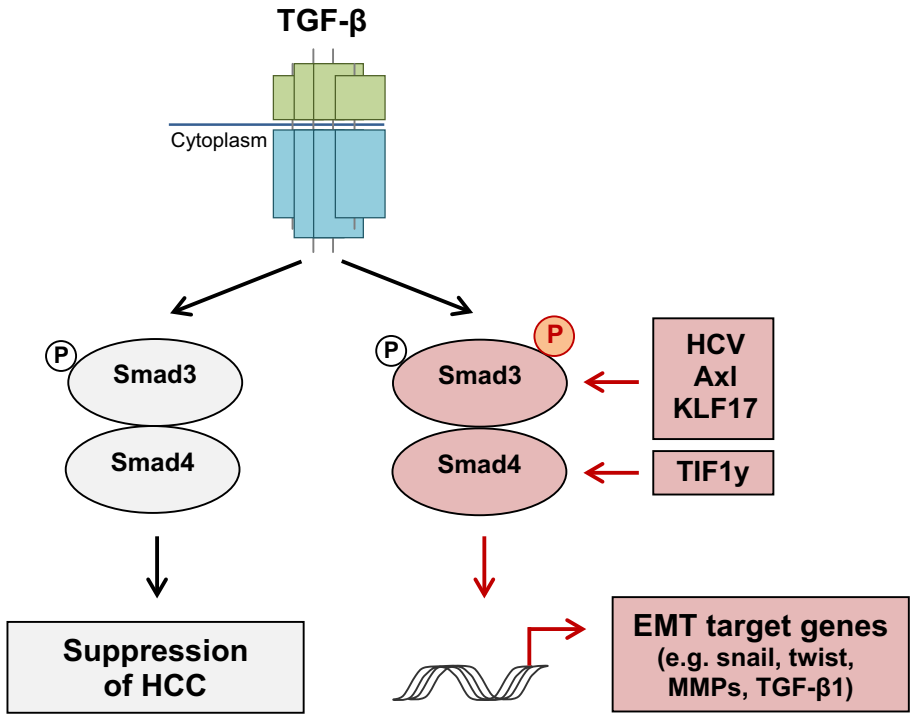

Figure 3 Role of TGF-β on the EMT in HCC. TGF-β/Smad signalling switches from anti-oncogenic functions (left panel) to EMT and HCC progression (right panel). HCV core proteins (HCV), Axl or the ablation of KLF17 impinge on Smad 3 through an aberrant regulation of its abundance, phosphorylation or its transcriptional activation, respectively, leading to EMT signatures. De-repression of Smad4 levels by loss of TIF1y facilitates the EMT.

formation mediated by the vascular endothelial growth factor (VEGF) released from HCC cells [84,85]. VEGR and the related receptor (VEGFR) are important targets, together with EGFR, for HCC progression [86]. Recent studies showed the functional collaboration of TGF-β with the receptor tyrosine kinase AXL in EMT-transformed human hepatoma cells and in patients with HCC, redirecting TGF-β signalling from tumour-suppression to tumour-promoting actions. Activation of AXL by its ligand GAS6 phosphorylates the SMAD3 linker region at Ser213 in a JNK-dependent manner after interaction with the scaffold protein 14-3-3ζ, leading to the activation of pro-tumourigenic TGF-β target genes and autocrine TGF-beta signalling [87,88]. Further studies showed the collaboration of hepatitis C virus (HCV) with TGF-β signalling in HCC progression. Interestingly, HCV core proteins downregulate SMAD3, causing the EMT and an insensitivity of HCC cells to the anti-oncogenic effects of TGF-β [89]. HCV glycoproteins stabilize hypoxia inducible factor 1-α (HIF-1α) that induces the EMT of HCC cells, associated with an upregulation of SNAIL and TWIST, as well as of TGF-β, that is responsible for enhanced cell motility [90]. Overall, TGF-β plays a paramount role in hepatocellular EMT and intrahepatic metastasis, suggesting that TGF-β and particularly its cooperating partners, are promising targets to combat HCC progression.

Targeting TGF-β in patients with HCC

The potential role of TGF-β1 in patients with CLD was firstly underlined in studies reporting the levels of this cytokine in different biological fluids, as well as the expression levels of TGF-βR [91,92]. More recently, an inverse correlation between circulating TGF-β1 and E-cadherin levels has been reported in patients with HCC, that resembles the EMT process, as documented *in vitro* [93]. A large body of evidence indicates that, as previously discussed, TGF-β1 is a key molecule involved in the tumoural progression of HCC either by promoting the EMT or by activating WNT, the best identified oncogenic pathway in HCC. Hoshida *et al.* have recently stratified patients with HCC according to the molecular pathway activation related to the clinical outcome. Patients with the worst survival had TGF-β activation, inducing β-catenin nuclear translocation leading to WNT signalling, responsible for tumoural growth [94]. The finding in this subset of patients is also consistent with the late-TGF-β signature and the consequent EMT cascade [95].

Recently, it has been shown that the TGF-βRI kinase inhibitor galunisertib, but not a monoclonal inhibitor of TGF-βRII (D10), blocks the canonical and noncanonical pathways [96,97]. The inhibition of SMAD2 phosphorylation is responsible for reducing the tumoural progression of HCC in preclinical conditions; this is the scientific rationale for using this compound in clinical trials [16]. A phase II clinical trial H9H-MC-JBAK (NCT01246986, http://clinicaltrials.gov) using galunisertib in patients with advanced HCC to test safety, time to progression and overall survival is currently ongoing, and the results will be available next year. In this study, patients have been enrolled without any TGF-β related selection; however, a large panel of circulating biomarkers is under investigation to assess their clinical role. These data, supported by experimental studies, would be very important to better personalize medicine, in terms of both the therapeutic and management decisions to be made.

Very recently, close attention has been paid to the immunosuppressor properties of TGF-β, which allow surveillance mechanisms to be evaded, facilitating tumour growth and metastatic progression. In this sense, isolated HCC-infiltrating Treg cells directly suppress the cytotoxic function and IFN-γ secretion of γδ T cells in a TGF-β-dependent manner [98]. Indeed, some TGF-β-related strategies are aimed directly at this immune reinforcing concept. Nowadays, LucanixTM (belagenpumatucel-L, a nonviral gene-based allogeneic tumour cell vaccine that demonstrates enhancement of tumour antigen recognition as a result of transforming growth factor β-2 inhibition) has shown improved survival in a phase III study in patients with nonsmall cell lung cancer (NSCL) [99,100]. The TAG vaccine is a novel 'triad vaccine' that involves transfection of autologous tumour with a dual plasmid, TGFβ2 antisense gene and GM-CSF gene, to force antigen presentation and thereby enhance dendritic cell migration to the vaccination site [101,102]. Results obtained in these ongoing studies will indicate the adequacy of the pharmacological inhibitors of TGF-β signalling used in combination with immunotherapeutic strategies in different types of cancer.

Besides intervention with the canonical and noncanonical TGF-β pathway through TGF-β receptors, inhibition of the signalling events collaborating with the TGF-β cascade promise to combat HCC progression. In this context, particular attention should be paid to the switch from tumour-suppressive to pro-oncogenic TGF-β actions via the synergistic cross-talk with RTKs such as EGF-R, HGF/Met or Axl in clinical trials. As EGF-R is necessary for the activation of Akt, which confers resistance to TGF-β mediated apoptosis [71], interference with EGF-R signalling by employing approved targeted drugs in TGF-β/Smad-positive HCC patients might be effective. Similarly, as Axl receptor activation causes the aberrant phosphorylation of Smad3 and induction of EMT-target genes during HCC progression [88], intervention on Axl signalling in TGF-β-responsive HCC patients is suggested to selectively arrest the tumour-progressive TGF-β signalling without affecting the anti-oncogenic actions of TGF-β. Thus, inhibition of the cross-talk between Axl and TGF-β signalling is considered superior to treatment modalities that exclusively target TGF-β signalling. Notably, specific Axl inhibitors are available and already in use in various clinical settings [103]. Clinical trials must further focus on the targeting of miRs that are essentially involved in oncogenic TGF-β signalling in HCC. Importantly, synthetic miR-125b mimics are considered to efficiently suppress EMT and EMT-associated traits of HCC cells by targeting Smad2/Smad4, which reduce the cancer stem cell potential and block HCC progression [104]. Along the same lines, pharmacological inhibition of the EMT-associated miR-216a/217 cluster, which activates PI3K/Akt and TGF-β by targeting PTEN and Smad7 in HCC, is a novel anticancer strategy [105].

A further development of therapies requires a more sophisticated delivery of the drug. Nanotechnology can come to the aid of standard medicine in a new area called 'nanomedicine'. Novel methods for encapsulating drugs in more efficient ways are being developed. One of these methods deals with the use of biodegradable hollow polyelectrolyte capsules as a protective sieve to load and release a cargo in a targeted manner [106,107]. These capsules are produced starting from a template dissoluble core (that can be organic or inorganic) which is layer-by-layer (LbL) assembled with oppositely charged polyelectrolytes layers until a shell with a set layer number is formed. Then, using a dissolving agent, the core is removed and a hollow capsule is obtained, that is ready to be filled with a drug. The surface chemistry and the thickness can be tailored (starting from few nanometres, depending on the number of layers). Several biological and chemical components can be assembled within the layers or in the interior. Hollow capsules have also been shown to achieve slow release with a high apoptotic efficiency at high doses [108]. Using this self-assembly technique, galunisertib was encapsulated in polyelectrolyte capsules, and their uptake was studied in two invasive hepatocellular carcinoma (HCC) cell lines [109]. Two polyelectrolyte pairs (biocompatible but not degradable, and biodegradable cross-linked with glutaraldehyde) were employed as components for LbL coating. Using both confocal laser scanning microscopy and fluorimetric analysis, it was shown that loading galunisertib into polyelectrolyte capsules improves the uptake of this drug in HCC cells. The authors showed that it was possible to improve the wall stability of hollow shells by chemical cross-linking and that there was no increase in cytotoxicity. A very efficient loading was also demonstrated. Furthermore, they observed that, as compared with free drug administration, TGF-βRI kinase inhibitor-loaded capsules were able to more efficiently inhibit tumour cell migration. All these

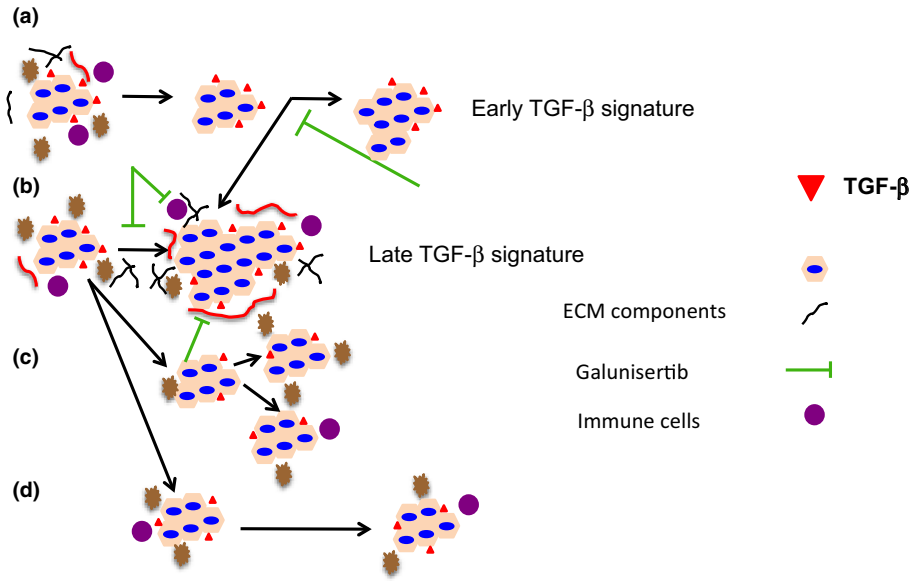

Figure 4 Effects of inhibiting the TGF-β pathway in HCC progression. Early TGF-β signature tumours progress slowly in the presence of TGF-β and are insensitive to the inhibition of this pathway. By contrast, the late-TGF-β signature is a better candidate for such therapy. Preclinical data showed that Galunisertib is able: (i) to reduce desmoplastic reactions, interrupting the cross-talk local stroma/tumour, and thus reducing tumour development; (ii) to reduce neoangiogenesis, affecting tumoral growth; (iii) to increase cell–cell adhesion and to inhibit intravasation, affecting local and distant spread. Based on these data, Galunisertib is the first drug to show an effectiveness against HCC development and progression, targeting the microenvironment and rebalancing tissue homeostasis.

data demonstrated that these polymeric capsules are promising candidates as novel carriers for targeted drug delivery against HCC, in the context of a more personalized therapy (Fig 4).

Breath tests for liver function and HCC

Another important aspect from the perspective of personalized medicine is to be able to rely on tests during treatment to help assess drug effectiveness. In this context, the availability of a test evaluating hepatic clearance by a number of exogenous substances has paved the way to the study of specific metabolic pathways in a more 'dynamic' fashion. Specific breath tests (BTs) have been employed as dynamic tools for studying liver function. The principle of the BT is to measure CO_2 in exhaled air as a marker of the hepatic metabolism of a test compound (substrate). The exhaled concentration of $^{13}CO_2$ measured by mass spectrometry reflects the hepatic clearance of the given test compound, reflecting the mitochondrial, microsomal or cytosolic metabolism [110,111]. Demethylation of methacetin (a cytochrome P-450 mediated process) and the decarboxylation of α-keto isocaproic acid (KICA) (a marker of specific mitochondrial function) have been used as substrates to evaluate the effects on liver function in patients with HCC undergoing different treatments) [112,113]. Consistently, the type of treatment of HCC (i.e. radiofrequency ablation, RFA or transarterial chemoembolization, TACE) influenced the post-therapy liver function and consequently the BTs. In fact, HCC recurred in patients whose KICA BT did not return to baseline within 6 months following RFA. The methacetin metabolism was significantly decreased following TACE but not RFA [114]. However, no data are so far available for patients with HCC undergoing systemic therapy, although using KICA BT as a tool to select and follow patients with HCC in terms of outcomes and disease recurrence seems to be an interesting approach. In particular, the KICA BT test might be relevant to evaluate the efficacy of treatment for HCC but also the residual liver function, in particular in patients receiving galunisertib. This could have a positive effect also on the underlying liver disease. In conclusion, the crucial role of TGF-β discussed in this review offers the rationale for inhibiting this target in patients with HCC. The upcoming data from the clinical trial using galunisertib may shed further light on this topic.

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References

1 Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011;**61**:69–90.
2 Milic S, Stimac D. Nonalcoholic fatty liver disease/steatohepatitis: epidemiology, pathogenesis, clinical presentation and treatment. *Dig Dis* 2012;**30**:158–62.
3 Blachier M, Leleu H, Peck-Radosavljevic M, Valla DC, Roudot-Thoraval F. The burden of liver disease in Europe: a review of available epidemiological data. *J Hepatol* 2013;**58**:593–608.
4 Corey KE, Kaplan LM. Obesity and liver disease: the epidemic of the twenty-first century. *Clin Liver Dis* 2014;**18**:1–18.
5 El-Serag HB. Epidemiology of hepatocellular carcinoma in USA. *Hepatol Res* 2007;**37**(Suppl 2):S88–94.
6 Trautwein C, Friedman SL, Schuppan D, Pinzani M. Hepatic fibrosis: concept to treatment. *J Hepatol* 2015;**62**:S15–24.
7 Tsochatzis EA, Bosch J, Burroughs AK. Liver cirrhosis. *Lancet* 2014;**383**:1749–61.
8 Lee YA, Wallace MC, Friedman SL. Pathobiology of liver fibrosis: a translational success story. *Gut* 2015;**64**:830–41.
9 Dooley S, ten Dijke P. TGF-beta in progression of liver disease. *Cell Tissue Res* 2012;**347**:245–56.
10 Meindl-Beinker NM, Matsuzaki K, Dooley S. TGF-beta signaling in onset and progression of hepatocellular carcinoma. *Dig Dis* 2012;**30**:514–23.
11 Zhang S, Sun WY, Wu JJ, Wei W. TGF-beta signaling pathway as a pharmacological target in liver diseases. *Pharmacol Res* 2014;**85**:15–22.
12 Hayashi H, Sakai T. Biological significance of local TGF-beta activation in liver diseases. *Front Physiol* 2012;**3**:12.
13 Fabregat I, Fernando J, Mainez J, Sancho P. TGF-beta signaling in cancer treatment. *Curr Pharm Des* 2014;**20**:2934–47.
14 Sanchez A, Fabregat I. Growth factor- and cytokine-driven pathways governing liver stemness and differentiation. *World J Gastroenterol* 2010;**16**:5148–61.
15 Williams MJ, Clouston AD, Forbes SJ. Links between hepatic fibrosis, ductular reaction, and progenitor cell expansion. *Gastroenterology* 2014;**146**:349–56.
16 Giannelli G, Villa E, Lahn M. Transforming growth factor-beta as a therapeutic target in hepatocellular carcinoma. *Cancer Res* 2014;**74**:1890–4.
17 Rani B, Cao Y, Malfettone A, Tomuleasa C, Fabregat I, Giannelli G. Role of the tissue microenvironment as a therapeutic target in hepatocellular carcinoma. *World J Gastroenterol* 2014;**20**:4128–40.
18 Yoshida K, Murata M, Yamaguchi T, Matsuzaki K. TGF-beta/Smad signaling during hepatic fibro-carcinogenesis (review). *Int J Oncol* 2014;**45**:1363–71.
19 Meulmeester E, Ten Dijke P. The dynamic roles of TGF-beta in cancer. *J Pathol* 2011;**223**:205–18.
20 Moustakas A, Heldin CH. The regulation of TGFbeta signal transduction. *Development* 2009;**136**:3699–714.
21 Schon HT, Weiskirchen R. Immunomodulatory effects of transforming growth factor-beta in the liver. *Hepatobiliary Surg Nutr* 2014;**3**:386–406.
22 Munger JS, Sheppard D. Cross talk among TGF-beta signaling pathways, integrins, and the extracellular matrix. *Cold Spring Harb Perspect Biol* 2011;**3**:a005017.
23 Robertson IB, Rifkin DB. Unchaining the beast; insights from structural and evolutionary studies on TGFbeta secretion, sequestration, and activation. *Cytokine Growth Factor Rev* 2013;**24**:355–72.
24 Herrera B, Dooley S, Breitkopf-Heinlein K. Potential roles of bone morphogenetic protein (BMP)-9 in human liver diseases. *Int J Mol Sci* 2014;**15**:5199–220.
25 Karkampouna S, Ten Dijke P, Dooley S, Julio MK. TGFbeta signaling in liver regeneration. *Curr Pharm Des* 2012;**18**:4103–13.
26 Ehrlich M, Gutman O, Knaus P, Henis YI. Oligomeric interactions of TGF-beta and BMP receptors. *FEBS Lett* 2012;**586**:1885–96.
27 Xu P, Liu J, Derynck R. Post-translational regulation of TGF-beta receptor and Smad signaling. *FEBS Lett* 2012;**586**:1871–84.
28 Macias MJ, Martin-Malpartida P, Massague J. Structural determinants of Smad function in TGF-beta signaling. *Trends Biochem Sci* 2015;**40**:296–308.
29 Lee MK, Pardoux C, Hall MC, Lee PS, Warburton D, Qing J *et al.* TGF-beta activates Erk MAP kinase signalling through direct phosphorylation of ShcA. *EMBO J* 2007;**26**:3957–67.
30 Sundar R, Gudey SK, Heldin CH, Landstrom M. TRAF6 promotes TGFbeta-induced invasion and cell-cycle regulation via Lys63-linked polyubiquitination of Lys178 in TGFbeta type I receptor. *Cell Cycle* 2015;**14**:554–65.

31 Zhang L, Zhou F, Garcia de Vinuesa A, de Kruijf EM, Mesker WE, Hui L *et al.* TRAF4 promotes TGF-beta receptor signaling and drives breast cancer metastasis. *Mol Cell* 2013;**51**:559–72.
32 Sundqvist A, Zieba A, Vasilaki E, Herrera Hidalgo C, Soderberg O, Koinuma D *et al.* Specific interactions between Smad proteins and AP-1 components determine TGFbeta-induced breast cancer cell invasion. *Oncogene* 2013;**32**:3606–15.
33 Itoh S, Itoh F. Inhibitory machinery for the TGF-beta family signaling pathway. *Growth Factors* 2011;**29**:163–73.
34 Eichhorn PJ, Rodon L, Gonzalez-Junca A, Dirac A, Gili M, Martinez-Saez E *et al.* USP15 stabilizes TGF-beta receptor I and promotes oncogenesis through the activation of TGF-beta signaling in glioblastoma. *Nat Med* 2012;**18**:429–35.
35 Zhang L, Huang H, Zhou F, Schimmel J, Pardo CG, Zhang T *et al.* RNF12 controls embryonic stem cell fate and morphogenesis in zebrafish embryos by targeting Smad7 for degradation. *Mol Cell* 2012;**46**:650–61.
36 Zhang L, Zhou F, Drabsch Y, Gao R, Snaar-Jagalska BE, Mickanin C *et al.* USP4 is regulated by AKT phosphorylation and directly deubiquitylates TGF-beta type I receptor. *Nat Cell Biol* 2012;**14**:717–26.
37 Koinuma D, Shinozaki M, Komuro A, Goto K, Saitoh M, Hanyu A *et al.* Arkadia amplifies TGF-beta superfamily signalling through degradation of Smad7. *EMBO J* 2003;**22**:6458–70.
38 Iwaisako K, Brenner DA, Kisseleva T. What's new in liver fibrosis? The origin of myofibroblasts in liver fibrosis. *J Gastroenterol Hepatol* 2012;**27**(Suppl 2):65–8.
39 Hellerbrand C, Stefanovic B, Giordano F, Burchardt ER, Brenner DA. The role of TGFbeta1 in initiating hepatic stellate cell activation *in vivo*. *J Hepatol* 1999;**30**:77–87.
40 Dooley S, Delvoux B, Lahme B, Mangasser-Stephan K, Gressner AM. Modulation of transforming growth factor beta response and signaling during transdifferentiation of rat hepatic stellate cells to myofibroblasts. *Hepatology* 2000;**31**:1094–106.
41 Schnabl B, Kweon YO, Frederick JP, Wang XF, Rippe RA, Brenner DA. The role of Smad3 in mediating mouse hepatic stellate cell activation. *Hepatology* 2001;**34**:89–100.
42 Uemura M, Swenson ES, Gaca MD, Giordano FJ, Reiss M, Wells RG. Smad2 and Smad3 play different roles in rat hepatic stellate cell function and alpha-smooth muscle actin organization. *Mol Biol Cell* 2005;**16**:4214–24.
43 Ding N, Yu RT, Subramaniam N, Sherman MH, Wilson C, Rao R *et al.* A vitamin D receptor/SMAD genomic circuit gates hepatic fibrotic response. *Cell* 2013;**153**:601–13.
44 Brenner DA. Molecular pathogenesis of liver fibrosis. *Trans Am Clin Climatol Assoc* 2009;**120**:361–8.
45 Nikolaou K, Tsagaratou A, Eftychi C, Kollias G, Mosialos G, Talianidis I. Inactivation of the deubiquitinase CYLD in hepatocytes causes apoptosis, inflammation, fibrosis, and cancer. *Cancer Cell* 2012;**21**:738–50.
46 Jiang JX, Venugopal S, Serizawa N, Chen X, Scott F, Li Y *et al.* Reduced nicotinamide adenine dinucleotide phosphate oxidase 2 plays a key role in stellate cell activation and liver fibrogenesis *in vivo*. *Gastroenterology* 2010;**139**:1375–84.
47 Wells RG. The epithelial-to-mesenchymal transition in liver fibrosis: here today, gone tomorrow? *Hepatology* 2010;**51**: 737–40.
48 Valdes F, Alvarez AM, Locascio A, Vega S, Herrera B, Fernandez M *et al.* The epithelial mesenchymal transition confers resistance to the apoptotic effects of transforming growth factor Beta in fetal rat hepatocytes. *Mol Cancer Res* 2002;**1**:68–78.
49 Caja L, Bertran E, Campbell J, Fausto N, Fabregat I. The transforming growth factor-beta (TGF-beta) mediates acquisition of a mesenchymal stem cell-like phenotype in human liver cells. *J Cell Physiol* 2011;**226**:1214–23.
50 Chu AS, Diaz R, Hui JJ, Yanger K, Zong Y, Alpini G *et al.* Lineage tracing demonstrates no evidence of cholangiocyte epithelial-to-mesenchymal transition in murine models of hepatic fibrosis. *Hepatology* 2011;**53**:1685–95.
51 Proell V, Carmona-Cuenca I, Murillo MM, Huber H, Fabregat I, Mikulits W. TGF-beta dependent regulation of oxygen radicals during transdifferentiation of activated hepatic stellate cells to myofibroblastoid cells. *Comp Hepatol* 2007;**6**:1.
52 Sancho P, Mainez J, Crosas-Molist E, Roncero C, Fernandez-Rodriguez CM, Pinedo F *et al.* NADPH oxidase NOX4 mediates stellate cell activation and hepatocyte cell death during liver fibrosis development. *PLoS ONE* 2012;**7**:e45285.
53 Jiang JX, Chen X, Serizawa N, Szyndralewiez C, Page P, Schroder K *et al.* Liver fibrosis and hepatocyte apoptosis are attenuated by GKT137831, a novel NOX4/NOX1 inhibitor *in vivo*. *Free Radic Biol Med* 2012;**53**:289–96.
54 Breitkopf K, Haas S, Wiercinska E, Singer MV, Dooley S. Anti-TGF-beta strategies for the treatment of chronic liver disease. *Alcohol Clin Exp Res* 2005;**29**:121S–31S.
55 Szuster-Ciesielska A, Daniluk J, Kandefer-Zerszen M. Serum levels of cytokines in alcoholic liver cirrhosis and pancreatitis. *Arch Immunol Ther Exp (Warsz)* 2000;**48**:301–7.
56 Chen WX, Li YM, Yu CH, Cai WM, Zheng M, Chen F. Quantitative analysis of transforming growth factor beta 1 mRNA in patients with alcoholic liver disease. *World J Gastroenterol* 2002;**8**:379–81.
57 Castellano-Higuera A, Gonzalez-Reimers E, Aleman-Valls MR, Abreu-Gonzalez P, Santolaria-Fernandez F, De La Vega-Prieto MJ *et al.* Cytokines and lipid peroxidation in alcoholics with chronic hepatitis C virus infection. *Alcohol Alcohol* 2008;**43**:137–42.
58 Perlemuter G, Letteron P, Carnot F, Zavala F, Pessayre D, Nalpas B *et al.* Alcohol and hepatitis C virus core protein additively increase lipid peroxidation and synergistically trigger hepatic cytokine expression in a transgenic mouse model. *J Hepatol* 2003; **39**:1020–7.
59 Ehnert S, Lukoschek T, Bachmann A, Martinez Sanchez JJ, Damm G, Nussler NC *et al.* The right choice of antihypertensives protects primary human hepatocytes from ethanol- and recombinant human TGF-beta1-induced cellular damage. *Hepat Med* 2013;**5**:31–41.
60 Ciuclan L, Ehnert S, Ilkavets I, Weng HL, Gaitantzi H, Tsukamoto H *et al.* TGF-beta enhances alcohol dependent hepatocyte damage via down-regulation of alcohol dehydrogenase I. *J Hepatol* 2010;**52**:407–16.
61 Gerjevic LN, Liu N, Lu S, Harrison-Findik DD. Alcohol activates TGF-Beta but Inhibits BMP receptor-mediated Smad signaling and Smad4 binding to hepcidin promoter in the liver. *Int J Hepatol* 2012;**2012**:459278.
62 Machida K, Tsukamoto H, Mkrtchyan H, Duan L, Dynnyk A, Liu HM *et al.* Toll-like receptor 4 mediates synergism between alcohol and HCV in hepatic oncogenesis involving stem cell marker Nanog. *Proc Natl Acad Sci USA* 2009;**106**:1548–53.
63 Chen CL, Tsukamoto H, Liu JC, Kashiwabara C, Feldman D, Sher L *et al.* Reciprocal regulation by TLR4 and TGF-beta in tumor-initiating stem-like cells. *J Clin Invest* 2013;**123**:2832–49.
64 Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell* 2009;**139**:871–90.

65 Giannelli G. The epithelial-mesenchymal transition: fact or fiction in cancer? *Hepatology* 2009;**50**:1344–6.
66 Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol* 2014;**15**:178–96.
67 Brabletz T, Jung A, Reu S, Porzner M, Hlubek F, Kunz-Schughart LA *et al.* Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. *Proc Natl Acad Sci USA* 2001;**98**:10356–61.
68 Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* 2009;**119**:1420–8.
69 Reichl P, Haider C, Grubinger M, Mikulits W. TGF-beta in epithelial to mesenchymal transition and metastasis of liver carcinoma. *Curr Pharm Des* 2012;**18**:4135–47.
70 Godoy P, Hengstler JG, Ilkavets I, Meyer C, Bachmann A, Muller A *et al.* Extracellular matrix modulates sensitivity of hepatocytes to fibroblastoid dedifferentiation and transforming growth factor beta-induced apoptosis. *Hepatology* 2009;**49**:2031–43.
71 Murillo MM, del Castillo G, Sanchez A, Fernandez M, Fabregat I. Involvement of EGF receptor and c-Src in the survival signals induced by TGF-beta1 in hepatocytes. *Oncogene* 2005;**24**:4580–7.
72 Fischer AN, Herrera B, Mikula M, Proell V, Fuchs E, Gotzmann J *et al.* Integration of Ras subeffector signaling in TGF-beta mediated late stage hepatocarcinogenesis. *Carcinogenesis* 2005;**26**:931–42.
73 Gotzmann J, Huber H, Thallinger C, Wolschek M, Jansen B, Schulte-Hermann R *et al.* Hepatocytes convert to a fibroblastoid phenotype through the cooperation of TGF-beta1 and Ha-Ras: steps towards invasiveness. *J Cell Sci* 2002;**115**:1189–202.
74 Fischer AN, Fuchs E, Mikula M, Huber H, Beug H, Mikulits W. PDGF essentially links TGF-beta signaling to nuclear beta-catenin accumulation in hepatocellular carcinoma progression. *Oncogene* 2007;**26**:3395–405.
75 Petz M, Them NC, Huber H, Mikulits W. PDGF enhances IRES-mediated translation of Laminin B1 by cytoplasmic accumulation of La during epithelial to mesenchymal transition. *Nucleic Acids Res* 2012;**40**:9738–49.
76 Petz M, Them N, Huber H, Beug H, Mikulits W. La enhances IRES-mediated translation of laminin B1 during malignant epithelial to mesenchymal transition. *Nucleic Acids Res* 2012;**40**:290–302.
77 Mikula M, Proell V, Fischer AN, Mikulits W. Activated hepatic stellate cells induce tumor progression of neoplastic hepatocytes in a TGF-beta dependent fashion. *J Cell Physiol* 2006;**209**:560–7.
78 van Zijl F, Zulehner G, Petz M, Schneller D, Kornauth C, Hau M *et al.* Epithelial-mesenchymal transition in hepatocellular carcinoma. *Future Oncol* 2009;**5**:1169–79.
79 Giannelli G, Bergamini C, Fransvea E, Sgarra C, Antonaci S. Laminin-5 with transforming growth factor-beta1 induces epithelial to mesenchymal transition in hepatocellular carcinoma. *Gastroenterology* 2005;**129**:1375–83.
80 Santamato A, Fransvea E, Dituri F, Caligiuri A, Quaranta M, Niimi T *et al.* Hepatic stellate cells stimulate HCC cell migration via laminin-5 production. *Clin Sci* 2011;**121**:159–68.
81 Bergamini C, Sgarra C, Trerotoli P, Lupo L, Azzariti A, Antonaci S *et al.* Laminin-5 stimulates hepatocellular carcinoma growth through a different function of alpha6beta4 and alpha3beta1 integrins. *Hepatology* 2007;**46**:1801–9.
82 Fransvea E, Angelotti U, Antonaci S, Giannelli G. Blocking transforming growth factor-beta up-regulates E-cadherin and reduces migration and invasion of hepatocellular carcinoma cells. *Hepatology* 2008;**47**:1557–66.
83 Fransvea E, Mazzocca A, Antonaci S, Giannelli G. Targeting transforming growth factor (TGF)-betaRI inhibits activation of beta1 integrin and blocks vascular invasion in hepatocellular carcinoma. *Hepatology* 2009;**49**:839–50.
84 Mazzocca A, Fransvea E, Dituri F, Lupo L, Antonaci S, Giannelli G. Down-regulation of connective tissue growth factor by inhibition of transforming growth factor beta blocks the tumor-stroma cross-talk and tumor progression in hepatocellular carcinoma. *Hepatology* 2010;**51**:523–34.
85 Mazzocca A, Fransvea E, Lavezzari G, Antonaci S, Giannelli G. Inhibition of transforming growth factor beta receptor I kinase blocks hepatocellular carcinoma growth through neo-angiogenesis regulation. *Hepatology* 2009;**50**:1140–51.
86 Giannelli G, Sgarra C, Porcelli L, Azzariti A, Antonaci S, Paradiso A. EGFR and VEGFR as potential target for biological therapies in HCC cells. *Cancer Lett* 2008;**262**:257–64.
87 Calvisi DF. When good transforming growth factor-beta turns bad in hepatocellular carcinoma: Axl takes the stage. *Hepatology* 2015;**61**:759–61.
88 Reichl P, Dengler M, van Zijl F, Huber H, Fuhrlinger G, Reichel C *et al.* Axl activates autocrine transforming growth factor-beta signaling in hepatocellular carcinoma. *Hepatology* 2015;**61**:930–41.
89 Battaglia S, Benzoubir N, Nobilet S, Charneau P, Samuel D, Zignego AL *et al.* Liver cancer-derived hepatitis C virus core proteins shift TGF-beta responses from tumor suppression to epithelial-mesenchymal transition. *PLoS ONE* 2009;**4**:e4355.
90 Wilson GK, Brimacombe CL, Rowe IA, Reynolds GM, Fletcher NF, Stamataki Z *et al.* A dual role for hypoxia inducible factor-1alpha in the hepatitis C virus lifecycle and hepatoma migration. *J Hepatol* 2012;**56**:803–9.
91 Bedossa P, Peltier E, Terris B, Franco D, Poynard T. Transforming growth factor-beta 1 (TGF-beta 1) and TGF-beta 1 receptors in normal, cirrhotic, and neoplastic human livers. *Hepatology* 1995;**21**:760–6.
92 Tsai JF, Jeng JE, Chuang LY, Yang ML, Ho MS, Chang WY *et al.* Elevated urinary transforming growth factor-beta1 level as a tumour marker and predictor of poor survival in cirrhotic hepatocellular carcinoma. *Br J Cancer* 1997;**76**:244–50.
93 Dituri F, Serio G, Filannino D, Mascolo A, Sacco R, Villa E *et al.* Circulating TGF-beta1-related biomarkers in patients with hepatocellular carcinoma and their association with HCC staging scores. *Cancer Lett* 2014;**353**:264–71.
94 Hoshida Y, Nijman SM, Kobayashi M, Chan JA, Brunet JP, Chiang DY *et al.* Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma. *Cancer Res* 2009;**69**:7385–92.
95 Coulouarn C, Factor VM, Thorgeirsson SS. Transforming growth factor-beta gene expression signature in mouse hepatocytes predicts clinical outcome in human cancer. *Hepatology* 2008;**47**:2059–67.
96 Fransvea E, Mazzocca A, Santamato A, Azzariti A, Antonaci S, Giannelli G. Kinase activation profile associated with TGF-beta-dependent migration of HCC cells: a preclinical study. *Cancer Chemother Pharmacol* 2011;**68**:79–86.
97 Dituri F, Mazzocca A, Peidro FJ, Papappicco P, Fabregat I, De Santis F *et al.* Differential inhibition of the TGF-beta signaling pathway in HCC cells using the small molecule inhibitor LY2157299 and the D10 monoclonal antibody against TGF-beta receptor type II. *PLoS ONE* 2013;**8**:e67109.
98 Yi Y, He HW, Wang JX, Cai XY, Li YW, Zhou J *et al.* The functional impairment of HCC-infiltrating gammadelta T cells, partially mediated by regulatory T cells in a TGFbeta- and IL-10-dependent manner. *J Hepatol* 2013;**58**:977–83.

99 Nemunaitis J, Dillman RO, Schwarzenberger PO, Senzer N, Cunningham C, Cutler J *et al.* Phase II study of belagenpumatucel-L, a transforming growth factor beta-2 antisense gene-modified allogeneic tumor cell vaccine in non-small-cell lung cancer. *J Clin Oncol* 2006;**24**:4721–30.

100 Giaccone G, Bazhenova LA, Nemunaitis J, Tan M, Juhasz E, Ramlau R *et al.* A phase III study of belagenpumatucel-L, an allogeneic tumour cell vaccine, as maintenance therapy for non-small cell lung cancer. *Eur J Cancer* 2015;**51**:2321–9.

101 Olivares J, Kumar P, Yu Y, Maples PB, Senzer N, Bedell C *et al.* Phase I trial of TGF-beta 2 antisense GM-CSF gene-modified autologous tumor cell (TAG) vaccine. *Clin Cancer Res* 2011;**17**:183–92.

102 Nemunaitis J, Senzer N, Olivares J, Kumar P, Barve M, Kuhn J *et al.* Immune response and survival of refractory cancer patients who received TGF-beta2 antisense/GM-CSF gene modified autologous tumor cell (TAG) vaccine. *Gene Ther* 2013;**20**:875–9.

103 Feneyrolles C, Spenlinhauer A, Guiet L, Fauvel B, Dayde-Cazals B, Warnault P *et al.* Axl kinase as a key target for oncology: focus on small molecule inhibitors. *Mol Cancer Ther* 2014;**13**:2141–8.

104 Zhou JN, Zeng Q, Wang HY, Zhang B, Li ST, Nan X *et al.* MicroRNA-125b attenuates epithelial-mesenchymal transitions and targets stem-like liver cancer cells through small mothers against decapentaplegic 2 and 4. *Hepatology* 2015;**62**:801–15.

105 Xia H, Ooi LL, Hui KM. MicroRNA-216a/217-induced epithelial-mesenchymal transition targets PTEN and SMAD7 to promote drug resistance and recurrence of liver cancer. *Hepatology* 2013;**58**:629–41.

106 del Mercato LL, Ferraro MM, Baldassarre F, Mancarella S, Greco V, Rinaldi R *et al.* Biological applications of LbL multilayer capsules: from drug delivery to sensing. *Adv Colloid Interface Sci* 2014;**207**:139–54.

107 Vergaro V, Scarlino F, Bellomo C, Rinaldi R, Vergara D, Maffia M *et al.* Drug-loaded polyelectrolyte microcapsules for sustained targeting of cancer cells. *Adv Drug Deliv Rev* 2011;**63**:847–64.

108 Palama IE, Leporatti S, de Luca E, Di Renzo N, Maffia M, Gambacorti-Passerini C *et al.* Imatinib-loaded polyelectrolyte microcapsules for sustained targeting of BCR-ABL+ leukemia stem cells. *Nanomedicine (Lond)* 2010;**5**:419–31.

109 Baldassarre F, Vergaro V, Scarlino F, De Santis F, Lucarelli G, Torre AD *et al.* Polyelectrolyte capsules as carriers for growth factor inhibitor delivery to hepatocellular carcinoma. *Macromol Biosci* 2012;**12**:656–65.

110 Bonfrate L, Grattagliano I, Palasciano G, Portincasa P. Dynamic carbon 13 breath tests for the study of liver function and gastric emptying. *Gastroenterol Rep (Oxf)* 2015;**3**:12–21.

111 Grattagliano I, Lauterburg BH, Palasciano G, Portincasa P. 13C-breath tests for clinical investigation of liver mitochondrial function. *Eur J Clin Invest* 2010;**40**:843–50.

112 Portincasa P, Grattagliano I, Lauterburg BH, Palmieri VO, Palasciano G, Stellaard F. Liver breath tests non-invasively predict higher stages of non-alcoholic steatohepatitis. *Clin Sci* 2006;**111**:135–43.

113 Lauterburg BH, Grattagliano I, Gmur R, Stalder M, Hildebrand P. Noninvasive assessment of the effect of xenobiotics on mitochondrial function in human beings: studies with acetylsalicylic acid and ethanol with the use of the carbon 13-labeled ketoisocaproate breath test. *J Lab Clin Med* 1995;**125**:378–83.

114 Palmieri VO, Grattagliano I, Minerva F, Pollice S, Palasciano G, Portincasa P. Liver function as assessed by breath tests in patients with hepatocellular carcinoma. *J Surg Res* 2009;**157**:199–207.