

# TGF- $\beta$ as Multifaceted Orchestrator in HCC Progression: Signaling, EMT, Immune Microenvironment, and Novel Therapeutic Perspectives

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## Abstract

Therapeutic attempts to treat hepatocellular carcinoma (HCC) frequently result in a poor response or treatment failure. The efficacy of approved drugs and survival expectancies is affected by an ample degree of variability that can be explained at least in part by the enormous between-patient cellular and molecular heterogeneity of this neoplasm. Transforming growth factor- $\beta$  (TGF- $\beta$ ) is hyperactivated in a large fraction of HCCs, where it influences complex interactive networks covering multiple cell types and a plethora of other local soluble ligands, ultimately establishing several malignancy traits. This cytokine boosts the invasiveness of cancerous epithelial cells through promoting the epithelial-to-mesenchymal transition program, but also skews the phenotype of immune cells toward a tumor-supporting status. Here, we discuss recent strategies pursued to offset TGF- $\beta$ -dependent processes that promote metastatic progression and immune surveillance escape in solid cancers, including HCC. Moreover, we report findings indicating that TGF- $\beta$  reduces the expression of the proinflammatory factors CCL4 and interleukin-1 $\beta$  (IL-1 $\beta$ ) in human ex vivo treated HCC tissues. While this is consistent with the anti-inflammatory properties of TGF- $\beta$ , whether it is an outright tumor promoter or suppressor is still a matter of some debate. Indeed, IL-1 $\beta$  has also been shown to support angiogenesis and cell invasiveness in some cancers. In addition, we describe an inhibitory effect of TGF- $\beta$  on the secretion of CCL2 and CXCL1 by HCC-derived fibroblasts, which suggests the existence of an indirect stroma-mediated functional link between TGF- $\beta$  and downstream immunity.

## Keywords

- transforming growth factor- $\beta$
- HCC
- galunisertib
- cytokine
- immunity

There are more than 30 transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily ligands in humans, which can be grouped into several subfamilies on the basis of sequence similarity and function; the major subgroups comprise TGF- $\beta$ s, activins, inhibins, bone morphogenetic proteins (BMPs), and growth and differentiation factors.<sup>1,2</sup> Of the three TGF- $\beta$  isoforms expressed in mammals, TGF- $\beta$ 1 is the most abundant and hence well-studied. The bioactive ligands are homo- or heterodimers synthesized as precursor molecules and matured by proteolytic cleavage by endoproteases. Active TGF- $\beta$  dimers mediate signaling through the TGF- $\beta$  type I and

type II receptors (T $\beta$ RI and T $\beta$ RII, respectively), which are active serine/threonine kinases. Due to its dimeric structure, TGF- $\beta$  is able to interact simultaneously with both type I and type II receptors. The binding of the ligand to the extracellular domain of T $\beta$ RII triggers cross-phosphorylation of T $\beta$ RI by T $\beta$ RII, activating its kinase activity, which then propagates signal transduction through phosphorylation of the Smad proteins.<sup>3–5</sup> The Smad proteins are divided into three classes: the receptor-regulated Smads (r-Smad), the common mediator Smad (co-Smad), and the inhibitory Smads (i-Smad). r-Smads include Smad1, Smad2, Smad3, Smad5, and Smad8,

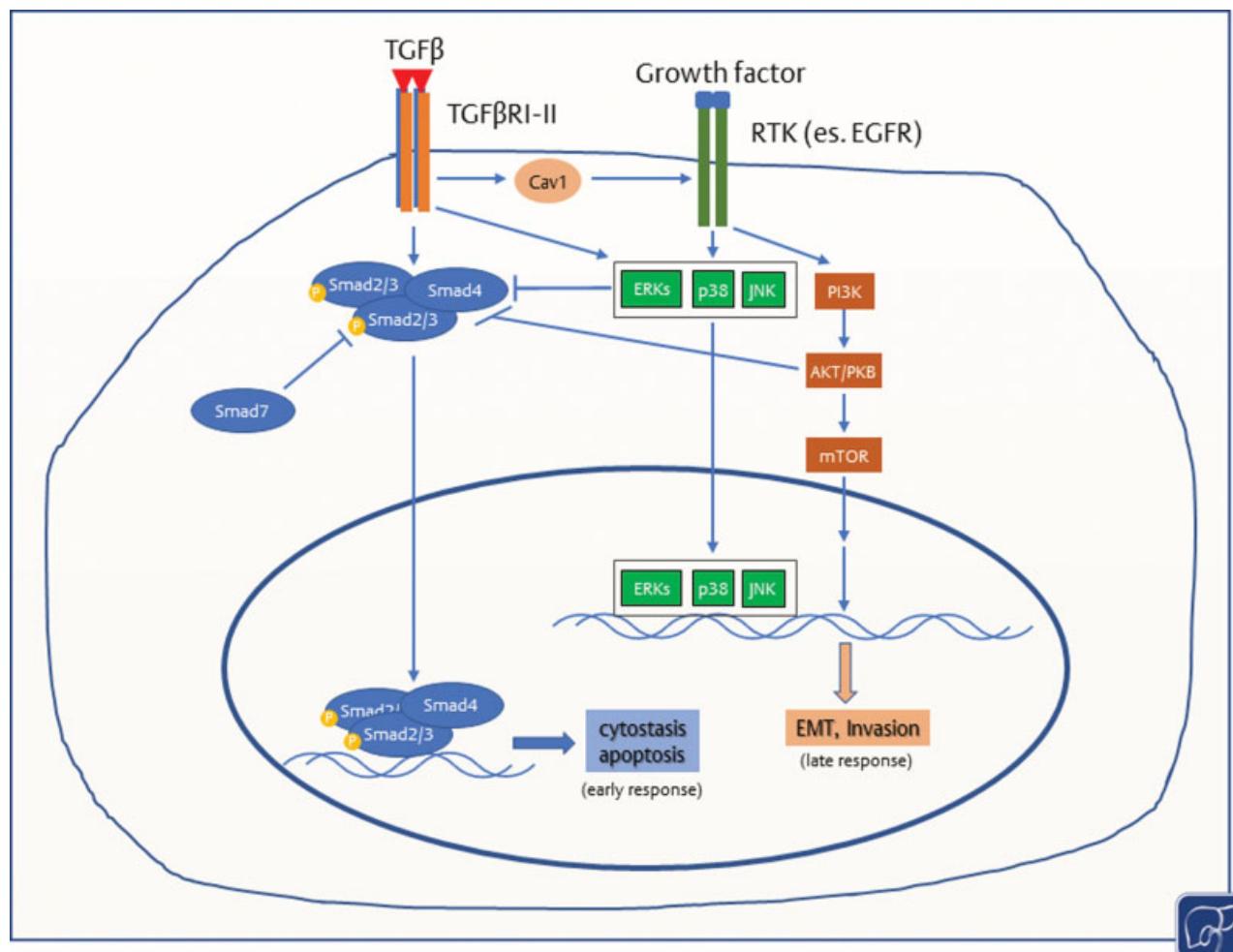
which act as direct substrates of specific type I receptors.<sup>6</sup> While Smad1, Smad5, and Smad8 are targets of BMP receptors, Smad2 and Smad3 are substrates of TGF- $\beta$  receptors.<sup>7-9</sup> Once phosphorylated, r-Smads associate with the common Smad, Smad4, a critical effector of intracellular signaling, mediating nuclear translocation of the heteromeric complex.<sup>10</sup> In the nucleus, Smad complexes then regulate specific genes such as integrins, E-cadherin, collagen, and others through cooperative interactions with DNA and other DNA-binding proteins.<sup>11-15</sup> In fact, to function as transcription factors, SMAD proteins need to interact with other DNA-binding transcription factors. For example, SMAD2 cannot bind directly to DNA, and the affinity of SMAD3 for DNA is weak. The many SMAD interacting transcription factors identified so far explain, at least in part, how TGF- $\beta$  exerts its highly contextual activity on different cell types.<sup>15</sup> In addition, SMADs are regulated by various posttranslational modifications, including phosphorylation, ubiquitination, SUMOylation, and poly(ADP-ribosylation).<sup>16</sup> An added complexity is the presence of various phospho-SMAD isoforms, whose phosphorylation at terminal carboxyl groups, at the intermediate linker region or at both, depends on the sur-

rounding microenvironment and the presence of growth factors, which then mediate differential roles for TGF- $\beta$ , specifically, during acute and chronic liver injury.<sup>17,18</sup>

The TGF- $\beta$  pathway (**Fig. 1**) is an important regulator of liver homeostasis and plays a major role in physiological but also in pathological conditions, modulating all the stages of disease progression, from initial liver injury through inflammation and fibrosis to cirrhosis and hepatocellular carcinoma (HCC). TGF- $\beta$  is highly expressed in HCC and the crosstalk between malignant hepatocytes and the surrounding stroma plays a dominant role in HCC development.<sup>19</sup>

Aberrations in TGF- $\beta$  signaling affect HCC development in different ways: although in early phases, it tends to inhibit the proliferation of premalignant hepatocytes, later it promotes stromal formation, the epithelial-to-mesenchymal transition (EMT), and tumor invasion, indicating a role for this pathway in disease progression and poor outcomes.<sup>20,21</sup>

During HCC progression, TGF- $\beta$  can act as an autocrine or a paracrine growth factor and in this way can induce changes in the microenvironment, via activating stromal fibroblasts, influencing regulatory T cells (Treg), and acting on tumor initiating cells.<sup>22,23</sup>



**Fig. 1** Basic TGF- $\beta$  pathway.

Couloouarn and colleagues, through a comparative functional genomics approach, showed that a temporal TGF- $\beta$  gene expression signature as "early and late," established in mouse primary hepatocytes, successfully discriminated distinct subgroups of HCC. The early response pattern reflects the physiologic response of TGF- $\beta$ , while the late response pattern is associated with prolonged TGF- $\beta$  activation<sup>24</sup>; tumors expressing late TGF- $\beta$  responsive genes displayed an invasive phenotype and increased tumor recurrence. Of interest, these cells expressed high levels of TGF- $\beta$  and Smad7, and showed a significantly reduced Smad3 signaling.<sup>25</sup>

New mechanisms by which TGF- $\beta$  exerts its cellular effects by changing genomic responses keep on being discovered. Like TGF- $\beta$ , SMAD4 has been associated with not only tumor suppression, but also tumor promotion in HCC.<sup>26</sup> TGF- $\beta$  was recently shown to induce genome-wide changes in DNA methylation, thereby enabling stable changes in liver cancer cell subpopulations.<sup>27</sup> Activation of long noncoding RNA-ATB by TGF- $\beta$  in HCC has a powerful effector role in mediating invasion and metastasis.<sup>28</sup> In addition, repression of miR-122 in hepatic stellate cells (HSCs) by TGF- $\beta$  is important for the profibrotic response on these cells.<sup>29</sup>

Smad6 and Smad7 are considered to inhibit ligand-dependent signaling.<sup>30,31</sup> Smad6 binds to receptor-activated Smad1, preventing Smad1 association with Smad4. Smad7 induces Smurf (E3 ligase) inactivation of TGF- $\beta$  and BMP receptors.

Another observation is that the expression of T $\beta$ RII is reduced and the receptor is mutated. This finding is associated with poor prognosis in HCC; in fact, approximately 25% of malignant hepatocytes show low T $\beta$ RII staining when compared with the surrounding nonmalignant hepatocytes.<sup>21</sup>

Cell lines associated to the late TGF- $\beta$  response lack T $\beta$ RI, have low levels of T $\beta$ RII, and are not subject to growth inhibition. These lines show high levels of EMT-associated proteins, suggesting that TGF- $\beta$ -related EMT is independent of the expression of TGF- $\beta$  receptors.<sup>17,21,24,32</sup> One possible mechanism underlying the switch from early (tumor suppression) to late gene response (tumor promotion) is by c-Jun N-terminal kinase (JNK) phosphorylation of the linker region of R-Smad.

Besides SMAD signaling, TGF- $\beta$  receptors are able to induce a non-SMAD response in the liver, through crosstalk with other alternative pathways, including MAP kinases, phosphatidylinositol-3-kinase (PI3K)/AKT, Ras, and Rho-like small GTPases, among others<sup>33,34</sup> (TGF- $\beta$  noncanonical pathways). The crosstalk between TGF- $\beta$  and these other pathways is being actively investigated in liver cells.

Moreover, activin A and B, which are highly expressed in both acute and chronic inflammation, are emerging as important mediators of liver (and other tissues) fibrosis.<sup>35</sup> BMP9, which has a high, selective liver expression, was recently shown to have pro-oncogenic effects on liver tumor cells; BMP9 stimulated the survival of liver cancer cells via the activation of p38MAP kinase.<sup>36,37</sup> A recent work showed that BMP9, a member of the TGF- $\beta$  family of cytokines, is constitutively expressed at low levels by HSCs, maintaining a stable hepatocyte function in healthy liver. Upon HSCs

activation, endogenous BMP-9 levels increase in vitro and in vivo, and high levels of BMP-9 cause enhanced damage following acute or chronic injury, interfering with liver regeneration and promoting fibrosis.<sup>38</sup> However, their functions, and those of many other related family members, are still not entirely clear in chronic liver disease.

The generation of mouse models is fundamental for preclinical and translational studies, but the design of an adequate mouse model is difficult owing to the role of TGF- $\beta$  in modulating all the stages of disease up to HCC development. HCC generally develops in the context of a diseased liver, being the result of progressive genetic and epigenetic changes that accumulate in liver epithelial cells. To design a model that may mimic the human disease as closely as possible, methods have been devised to induce liver disease in mice resembling viral hepatitis, fatty liver disease, fibrosis, alcohol-induced liver disease, and cholestasis.<sup>39</sup>

In this context, Morris et al developed a TP53 knockout mouse model in which TGF- $\beta$  signaling promotes the formation of liver tumors that arise in the setting of TP53 inactivation. Starting from the in vitro evidence that p53 and TGF- $\beta$  can cooperate to regulate several cellular responses, and that p53 physically interacts with SMAD2 and SMAD3, they set out to unravel the importance of the relationship between p53 and TGF- $\beta$  signaling pathways for in vivo HCC formation. The TP53 knockout mouse model showed features seen in human liver cancers, including an increased expression of TGF- $\beta$ 1, Afp, Pai1, and Ctgf. Interestingly, the loss of T $\beta$ RII in the context of the loss of TP53 decreased the incidence of HCCs and CCs and attenuated many of the features seen in tumors with inactive TP53 alone. The data presented clearly demonstrate the cooperation between the two pathways in HCC development and provide a rationale for developing therapies directed against these molecular targets.<sup>40</sup>

Another interesting mouse model was generated by Yang et al in the effort to develop an ideal animal model for the purposes of analyzing the mechanisms of hepatocarcinogenesis, and especially the link between inflammation, fibrosis, and carcinogenesis. Mice carrying a deletion of TGF- $\beta$  associated kinase 1 (Tak1) in hepatocytes spontaneously develop HCC accompanied by liver inflammation and fibrosis, indicating that this gene is a tumor suppressor in the liver. The data presented suggest that TGF- $\beta$ -Smad signaling in hepatocytes promotes liver fibrosis and the formation of liver tumors that develop spontaneously in the setting of TAK1 inactivation. The authors demonstrate the role of TGF- $\beta$ -Smad by generating a double knockout mouse model: Tak1/Tgfb2 and Tak1/Smad4. The additional deletion yielded a decreased spontaneous carcinogenesis, fibrosis, inflammation, and hepatocyte apoptosis mainly with Tak1/Tgfb2 and to a lesser extent with Tak1/Smad4, highlighting the TGF- $\beta$  crosstalk with other pathways in mediating the TAK1 liver phenotype. Specifically, they showed that TGF- $\beta$  promotes the development of HCC in Tak1 mice by inducing hepatocyte apoptosis and compensatory proliferation during early phases of tumorigenesis, and inducing the expression of antiapoptotic, pro-oncogenic, and angiogenic factors during tumor progression.<sup>41</sup>

## TGF- $\beta$ in the Pathogenesis of HCC

Transforming growth factor- $\beta$  signaling molecules act on most cell types of the body and have pleiotropic effects, regulating cell growth, differentiation, apoptosis, motility and invasion, extracellular matrix (ECM) production, angiogenesis, and the immune response. They also play essential roles in early embryonic development and in regulating tissue homeostasis in adults.<sup>42</sup> In many altered states, including fibrosis and cancer, the levels of TGF- $\beta$  are chronically and aberrantly elevated. TGF- $\beta$  signaling pathway alterations are frequent in tumors, and exert their protumorigenic function by directly modulating the tumor cell invasion and metastatic ability, sustaining a cells niche with a tumor-inducing capacity. Furthermore, the protumorigenic TGF- $\beta$  activity influences the tumor microenvironment (TME), resulting in ECM deposition, myofibroblast differentiation, angiogenesis, and the suppression of both the innate and the adaptive immune systems. This triggers a continuous interaction between tumor cells and TME, which further increases progression, and the invasive and metastatic ability of the tumor. Over the last decades, the TGF- $\beta$  signaling pathway has become an emerging strategic focus for cancer therapy as a target for drug development, in relation also to the new field of cancer immunotherapy.<sup>43</sup>

However, the role of TGF- $\beta$  as a tumor promoter or suppressor at the cancer cell level is still a matter of debate. Coherently with the above-described early/late TGF- $\beta$  signature paradigm, this cytokine has been proposed to induce cytostasis and apoptosis of hepatocytes in premalignant lesions and early stages of liver carcinogenesis, while at later HCC stages it might contribute to cancer progression via orchestrating processes such as the EMT, invasion, fibrogenesis, and cancer-stromal cells crosstalk.<sup>44</sup>

Some recent studies support a role as tumor suppressor for TGF- $\beta$  in HCC, regardless of the disease stage. Zhang et al found that TGF- $\beta$ -induced expression of large tumor suppressor 1 (LATS1) and nucleus-cytoplasm translocation of yes association protein 1 (YAP1) resulted in cell growth inhibition in HCC cells.<sup>45</sup> Chen et al screened almost 1,000 HCCs, clustering patients into subsets with mutational loss or gain of TGF- $\beta$  pathway activation. Interestingly, patients with the inactivated TGF- $\beta$  pathway (showing reduced TGFB1, SMAD3, and SMAD4) exhibited a loss of the tumor suppressor genes required for DNA damage repair (ATM, BRCA1, and FANCF), an aberrant expression of pro-oncogenic genes, such as sirtuin and HDAC, and had shorter survival times than those with the activated TGF- $\beta$  pathway status.<sup>46</sup>

Various molecular mechanisms are thought to modulate the actions of TGF- $\beta$  between the early and late phases of liver carcinogenesis. The CXXC-type zinc-finger domain-containing protein CXXC5 was shown to be expressed in HCC cells through a positive feedback loop in response to TGF- $\beta$ . CXXC5 interacts with the histone deacetylase HDAC1, preventing it from inhibiting Smad2/3, and finally impairing the TGF- $\beta$ -mediated cytostasis. Given the observation that CXXC5 is downregulated in HCC compared with normal hepatic tissue, the pro-oncogenic role of TGF- $\beta$  is probably prominent in this scenario.<sup>47</sup> In

addition, Moreno-Càceres et al have shown that Caveolin-1 (CAV1) activity acts as a molecular switch in tuning the balance between the counteractive pro- and antiapoptotic effects of TGF- $\beta$  in HCC cells. While TGF- $\beta$  induces apoptosis through a pathway that requires the activation of the proapoptotic BMF and, ultimately, the upregulation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX4), it also hinders the apoptotic program through transactivating EGFR-dependent signaling. CAV1 was proven to block BMF induction, as well as to boost EGFR signaling, while reducing PI3K/AKT pathway activation. In addition, CAV1 resulted more strongly expressed in HCC tumor than peritumor tissues, consistently with a more proapoptotic attitude of TGF- $\beta$  in a premalignant state of hepatocytes.<sup>48</sup>

Compelling evidence has suggested that failure of liver cancer stem cells (CSCs) to respond to TGF- $\beta$  unleashes their protumorigenic potential. A defective TGF- $\beta$  pathway was proven to be required for the fulfillment of the tumorigenic program by CD133+ tumor-initiating stem-like cells (TICs) in HCC. Importantly, the toll-like receptor 4 (TLR4) expressed by TICs was shown to target the pluripotency marker NANOG that, in turn, activates Yap1 and Igf2bp3, which then subtract phospho-SMAD3 from IGF2BP3/AKT/mTOR pathway-mediated activation, ultimately resulting in chemoresistance and a tumorigenic potential of these cells.<sup>49</sup> Defective TGF- $\beta$  signaling in liver stem cells also results in the development of HCC in experimental animal models. Heterozygous loss of  $\beta$ II-spectrin (which interacts with the downstream mediator of the TGF- $\beta$  pathway, SMAD3) in mice leads to a spontaneous HCC with a phenotypic similarity to human HCC, which develops as a result of Beckwith-Wiedemann syndrome. The TGF- $\beta$ /NRF2/ARE axis has also been involved in inducing the expression of cytoprotective genes, such as heme oxygenase-1 (HO-1), known to protect against the action of carcinogens. This explains how TGF- $\beta$  prevents carcinogen-induced oncogenic transformation. Moreover, SMAD4 depletion is able to convert TGF- $\beta$  from an anti- into a protumorigenic cytokine.<sup>50</sup> While the Smad3/4 adaptor embryonic liver fodrin (ELF) and TGF- $\beta$ RII were found (along with the other stemness markers Stat3, Oct4, and Nanog) in stem cells of regenerating livers, their expression was lost in HCC CSCs, which instead expressed interleukin-6 (IL-6). This observation suggests that TGF- $\beta$  signaling blocks the aberrant proliferation of regenerating liver stem cells, but HCC CSCs, which are unresponsive to TGF- $\beta$ , may exploit the IL-6 pathway to gain their oncogenic capacity.<sup>51</sup> In addition, in vitro and in vivo HCC models, ELF was demonstrated to play a tumor suppressor role via exerting a dual control on both differentiation of endothelial progenitor cells (avoiding aberrant angiogenesis) and HCC cell proliferation.<sup>52</sup> Based on this knowledge, attempts to counteract HCC progression through blocking TGF- $\beta$  signaling may prove detrimental, because they could abrogate the TGF- $\beta$ -induced impairment of cancer cell growth, CSCs expansion, and oncogenic potential, raising concerns about the effective advantage of using this strategy. However, some evidence supports the view that, in HCC, TGF- $\beta$  maintains the stemness status of CSCs. Rani et al reported that blockade of TGF- $\beta$  receptor I with galunisertib (LY2157299) decreases the expression of the stemness markers CD44 and THY1 in invasive

HCC cells.<sup>53</sup> Another study shows that in some HCC cell lines and most tumors, TGF- $\beta$  induces a partial EMT phenotype which, unlike the complete mesenchymal status, is related to a higher stemness potential and CD44 expression.<sup>54</sup> In addition, the balance between pro-oncogenic and tumor-limiting functions of TGF- $\beta$  in HCC does not presumably result only from the arrangement of molecular switches that modulate its signaling status in hepatocytes, but, as will be discussed later, may also depend on the roles this chemokine plays as a mediator in the complex interactive network engaging multiple intratumor cell types, including cancer, stromal, endothelial, and inflammatory cells, which cooperate to support the malignancy. Moreover, although a defective TGF- $\beta$  pathway has been related to poor prognosis in HCC patients,<sup>46</sup> the concept of an early-to-late switch of TGF- $\beta$  has elicited a different view. Coulouarn et al have shown that while the early signature is related to a better prognosis and seems to reflect a responsive status of cells to TGF- $\beta$  that induces cell cycle arrest and apoptosis, the late TGF- $\beta$  signature is related to a more aggressive phenotype, probably due to the acquired ability of cells to escape the Smad-dependent cytostatic effects of TGF- $\beta$ . Several pathways, including EGFR, PI3K/AKT, TACE/ADAM17, and EMT-related, may participate in the noncanonical late signaling arm of TGF- $\beta$  to counteract the proapoptotic effects of this cytokine.<sup>24,55</sup> At the microenvironment level, the TGF- $\beta$  pathway is known to generate a favorable microenvironment for tumor growth and metastasis throughout all the steps of carcinogenesis. Then, targeting the TGF- $\beta$  pathway in cancer may be considered primarily as a microenvironment-targeted strategy.<sup>56</sup>

## Regulation of EMT by TGF- $\beta$ in HCC

Several intriguing studies have demonstrated that HCC cells overcome TGF- $\beta$  tumor-suppressive activities by responding with a complex biological process known as the EMT.<sup>57</sup>

Conventionally, the EMT is a functional reprogramming that attributes phenotypic changes to carcinoma cells. This plasticity process, to which the tumor cells are subjected, involves the loss of many of their epithelial characteristics, including the epithelial cell junctions as tight, adherent, and gap junctions and apical-basal polarity, while, concomitantly, there is a gain of anterior and posterior polarity with the acquisition of mesenchymal traits with a fibroblastic-like morphology.<sup>58–60</sup>

This prominent role of the EMT is strongly induced by overactivation of the TGF- $\beta$  receptor pathway, which leads to a greater invasive and migratory capacity to local or distant regions,<sup>61,62</sup> CSC heterogeneity, and drug resistance.<sup>63,64</sup> Therefore, evidence has shown in human HCC cells that downregulation of the TGF- $\beta$  pathway is not involved in the inhibition of proliferation or in the induction of apoptosis, but does strongly block their migration and invasion, as well as their stemness capacity.<sup>57</sup>

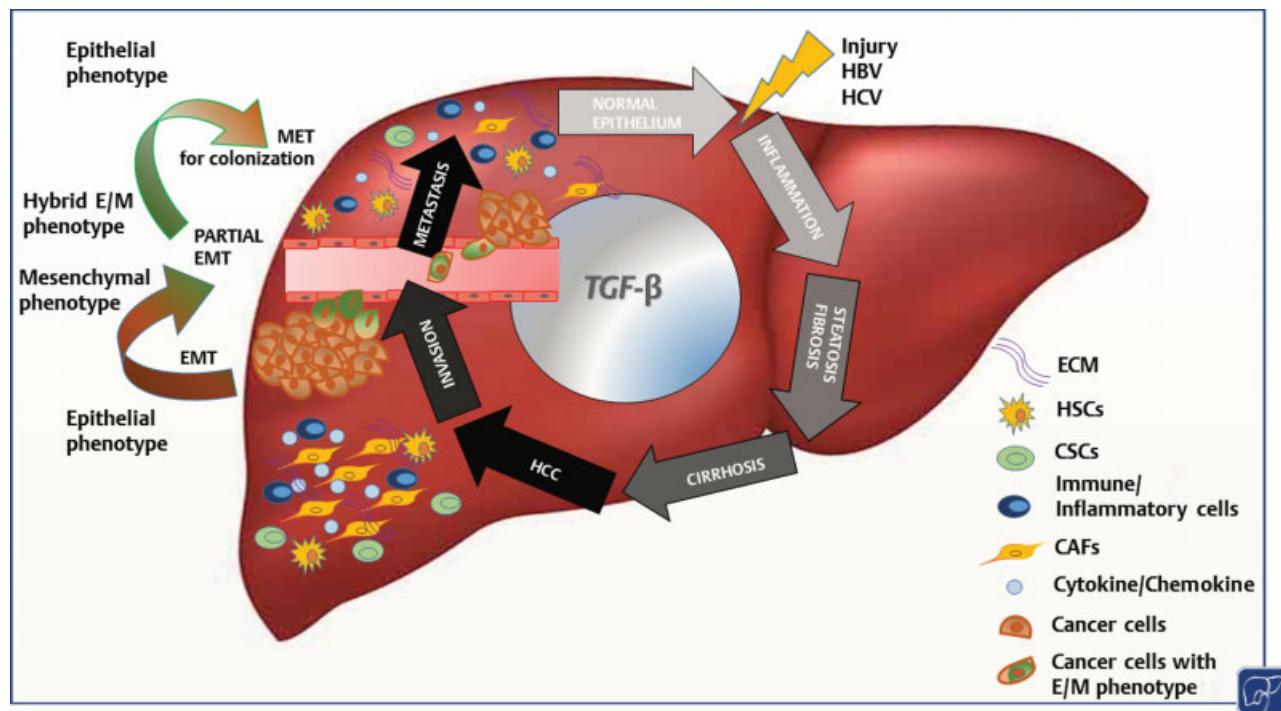
Meanwhile, mesenchymal cells can be redifferentiated to epithelial structures through a reversible dynamic process called the mesenchymal-to-epithelial transition (MET). In pathological situations (i.e., cancer metastasization), the MET drives the migrating mesenchymal-like cells that repopulate secondary sites, recovering cell-cell contacts and

polarity to regain their epithelial phenotype.<sup>65</sup> The dynamic and reversible transitions between multiple phenotypic states require not only the reprogramming of gene expression, but also epigenetic regulation.<sup>66</sup>

The TGF- $\beta$  signaling pathway converges on the activation of pro-EMT inducers that have been identified as key transcriptional factors (EMT-TFs) triggered by basic helix-loop-helix transcription factors (TWIST1, TWIST2, E12, E47, ID, and TCF3), the zinc-finger transcriptional repressors SNAI (SNAI 1) and SLUG (SNAI2), and the zinc-finger E-box binding homeobox (ZEB1 and ZEB2). An overexpression of EMT-TFs was reported in 662 (49.6%) of the 1,334 HCC patients studied. The highest positive expression rate based on immunohistochemistry (IHC) or western blot analysis (WB) was Twist1, accounting for 60.3%, followed by Snail (51.9%), ZEB2 (50.3%), ZEB1 (43.6%), and Slug (29.4%).<sup>67</sup> These major regulators of the EMT program drive the transcription of EMT-associated genes, and activation or suppression of the promoter modified the chromatin structure.<sup>68</sup> EMT-TFs often cooperate to regulate the expression of these common genes of interest.<sup>68,69</sup> More specifically, many studies indicate that the inhibition of these genes is associated with the epithelial cell phenotype, such as E-cadherin, while genes upregulation is associated with the mesenchymal cell phenotype, including N-cadherin, fibronectin, vimentin, and nuclear localization of  $\beta$ -catenin, through the upregulation of TGF- $\beta$ .<sup>68</sup> However, the long-term treatment response of HCC cells to TGF- $\beta$  does not always correlate with a full EMT. Indeed, PLC/PRF/5 cells are observed in response to this cytokine-increased levels of vimentin and N-cadherin, but no loss of the expression of E-cadherin and epithelial structures.<sup>54</sup> This event is understandable if we consider that transitions between the epithelial and mesenchymal cellular phenotype are not a direct passage but, interestingly, there is evidence of a set of multiple and dynamic transitional states in which cells can also attain a hybrid epithelial/mesenchymal phenotype (E/M). These epithelial cells with an intermediate EMT (i.e., partial EMT) phenotype do not completely lose the epithelial morphology (cell-cell adhesion) and do not fully acquire mesenchymal (migration) properties.<sup>70,71</sup> Notably, the potential crosstalk with the TGF- $\beta$ -induced E/M phenotype stage has recently been identified as a crucial driver of the initiation/progression of primary liver tumorigenesis. Furthermore, the effects of TGF- $\beta$  on the activation of a partial EMT can be also attributed to HCC the greatest advantage in acquiring a migratory stemness phenotype<sup>54,72</sup> and a higher intra- and extrahepatic metastatic risk in patients with poor prognoses.<sup>73</sup>

## The HCC Microenvironment: TGF- $\beta$ as Inducer of Cancer-Stromal Cells Interaction

It is worth noting that various EMT phenotypes can contribute to distinct tumor cell subpopulations, increasing the complexity and cellular heterogeneity of HCC in the tumor and the surrounding microenvironment.<sup>44</sup> Importantly, during the development from chronic inflammation to HCC aberrant TGF- $\beta$  activation plays a potent role in organizing



**Fig. 2** Role of TGF- $\beta$  activation during the development from chronic inflammation to HCC.

a favorable microenvironment for liver cancer cells growth (►Fig. 2). It orchestrates a dynamic dialogue between tumor cells and host stroma, stimulating the production of soluble factors such as cytokines and growth factors released by fibroblasts/myofibroblasts, macrophages, and immune cells. This interaction generates massive deposits of ECM proteins (ECM),<sup>74</sup> angiogenesis,<sup>75</sup> immune cell reprogramming,<sup>76</sup> and hypoxic responses.<sup>77</sup>

In this context, HSCs are the main profibrogenic cell type in fibrotic liver.<sup>78</sup> The HSCs, so-called because of their typical stellar morphology, reside in the subendothelial space of Disse, in close association with the sinusoidal endothelial cells.<sup>79</sup> HSCs are quiescent and accumulate numerous lipid drops of vitamin A in healthy liver.<sup>80</sup> HSCs are stimulated by chronic injury of the hepatocytes and TGF- $\beta$  is generally considered the most potent stimulus released by several cell populations in the liver to promote their activation.<sup>79,81</sup> This process is mediated by the activation of NOX4, a fibrotic mediator downstream of TGF- $\beta$ , independently of Smads activation.<sup>82</sup> Thus, liver damage-induced levels of active TGF- $\beta$ 1 mediate HSCs activation, transdifferentiating from quiescent cells to myofibroblasts-like cells. They are characterized by a gradual loss of retinoic acid and lipid stores, express  $\alpha$  smooth muscle actin ( $\alpha$ Sma), enhance the production of ECM components such as fibronectin and collagen,<sup>83</sup> and can contribute directly, or via secreted products, including growth factors and cytokines (e.g., hepatocyte growth factor, IL-6), to the tumor induction and to the progression of HCC.<sup>84</sup> Cellular and molecular approaches demonstrated a bidirectional crosstalk between HSC-derived myofibroblasts and tumor hepatocytes,<sup>79</sup> creating a favorable microenvironment for progression, invasion, and metastasis through the EMT. In this regard, Sancho-Bru et al observed

that, when co-cultivated with Huh7 or HepG2, HSCs are stimulated to migrate and, in turn, can regulate the migration and proliferation of HCC cells through modulating the turnover of TGF- $\beta$  and ECM proteins.<sup>85</sup> In addition, other mechanisms by which HSC may facilitate HCC development and progression are through other biological processes that promote tumor angiogenesis and immunomodulation. Indeed, activated HSCs secrete numerous chemokines, including CCL2, CCL3, CCL5, CXCL1, CXCL8, CXCL9, and CXCL10, thus amplifying the inflammatory response by inducing the activation and infiltration of inflammatory cells at the site of injury.<sup>86,87</sup> In addition, the interaction between HCC and activated HSCs creates a proangiogenic microenvironment through the overexpression of vascular endothelial growth factor  $\alpha$  (VEGF- $\alpha$ ).<sup>88–90</sup> Furthermore, early studies showed that the exposure of HSC to conditioned media derived from HCC tumor cells resulted in HSC activation, migration, and the expression of VEGF- $\alpha$  and angiopoietins by HSC, favoring a proangiogenic microenvironment.<sup>84,86</sup> *In vitro*, VEGF stimulates type I collagen production and proliferation in activated HSCs, while, in *in vivo* models of liver fibrosis, the inhibition of VEGF signaling via blockade of its receptors, VEGFR-1 and VEGFR-2, is associated with a significant decrease in fibrosis.<sup>91</sup> Increased portal pressure underlies many of the clinical complications of liver disease and is related to changes in the intrahepatic resistance to blood flow.<sup>92</sup> Resistance to blood flow through the sinusoids is increased by deposition of fibrotic ECM proteins including collagens, laminins, elastin, and tenacins within the sinusoidal space, along with “capillarization.” The accumulation of ECM proteins and inhibition of the endogenous matrix-degrading activities of various matrix metalloproteinases (MMPs) are important in chronic tissue damage with liver fibrosis. The

mechanical stiffness of the matrix is determined by its components (collagens, proteoglycans, and other matrix proteins), together with their posttranslational modifications, organization, and cross-linking.<sup>93</sup> Deregulation of the ECM collagen cross-link and ECM stiffness is important for integrin signaling. A previous study indicated that TGF- $\beta$ 1 was able to induce a significant increase in the expression level of  $\alpha$ 3 $\beta$ 1 in HCC cells, which consecutively cooperated with TGF- $\beta$ 1 to induce the EMT,<sup>94</sup> while high  $\alpha$ 6 integrin expression has been correlated with a worse clinical outcome, poor survival, and early cancer recurrence.<sup>95</sup> Laminin-332 is produced by HSCs and stimulates the proliferation of HCC cells via interactions with  $\alpha$ 3 $\beta$ 1 and  $\alpha$ 6 $\beta$ 4. In parallel, the level of FAK Y397 phosphorylation, shown to be a necessary step for FAK to become functional after the integration of the ECM proteins, is upregulated with the increase in matrix rigidity, thereby facilitating the formation of focal adhesions and polymerization of intracellular cytoskeletal proteins.<sup>96,97</sup> A previous study has identified the mechanism of resistance to the pharmacological action of sorafenib-induced cell death, identifying the integrin  $\alpha$ 3 $\beta$ 1, but not  $\alpha$ 6 $\beta$ 4, in presence of Ln-332, as responsible for cell survival in the presence of sorafenib, by re-establishing the activation of FAK to the residue Y397.<sup>98</sup> Accordingly, TME is also a factor that mediates EMT-driven drug resistance.

In addition, tumor-activated HSCs, in turn, create a proangiogenic, prometastatic microenvironment by facilitating endothelial proliferation and survival through the release of VEGF, an extremely important proangiogenic factor in the progression of the most aggressive HCC. Koudelkova et al have also demonstrated that malignant hepatocytes that exhibit a mesenchymal-like invasive phenotype are stimulated by TGF- $\beta$ , using a model that mimics vascular invasion. In practice, transendothelial migration using an endothelial barrier constituted by HUVEC cells revealed the proteome profile, with 36 and 559 proteins regulated in hepatocytes and endothelial cells, respectively. These results indicate that significant changes during active transmigration are involved in blood vessel invasion of HCC cells.<sup>99</sup>

Intriguingly, TGF- $\beta$  signaling also induces a high expression of the receptor tyrosine kinase Axl in EMT-transformed hepatoma cells. The overexpression of Axl by its ligand Gas6 induces, through the interaction of 14-3-3  $\zeta$ , metastatic colonization of epithelial hepatoma cells in vivo. Axl/14-3-3  $\zeta$  signaling causes an upregulation of tumor-progressive TGF- $\beta$  target genes such as PAI1, MMP9, and Snail in mesenchymal HCC cells. Accordingly, high Axl expression in HCC patient samples was correlated with elevated vessel invasion by HCC cells, a higher risk of tumor recurrence after liver transplantation, and reduced survival of HCC patients.<sup>100</sup>

Several points of evidence strongly indicate that the hypoxic microenvironment in liver is mediated by the expression of hypoxia-inducible factor 1 (HIF-1), which binds to the promoter region of VEGF and induces its transcription, promoting invasion and metastasis in HCC.<sup>101</sup>

Notably, the TME immune status is altered by hypoxia and the contribution of specific cytokines such as inflammatory soluble mediators of the EMT. TGF- $\beta$  emerges as a potent inducer of the EMT. As well as maintaining tissue home-

ostasis and suppressing inflammation and tumorigenesis, TGF- $\beta$  can also induce and sustain inflammation, favoring tumor progression depending on the cellular context.<sup>102</sup>

Communication between HCC cells and their environment is due also to an aberrant expression of noncoding microRNAs (miRNAs) that contributes to HCC development. The expression and functions of EMT-TFs are controlled by posttranscriptional regulator miRNAs, which regulate the expression of specific proteins by binding to mRNA transcripts with complementary sequences, destabilizing it.<sup>103</sup> Among the best characterized miRNAs regulating the EMT program, *miR-200* family (*miR-141*, *-200a*, *-200b*, *-200c*, and *-429*) is well known to be associated with the progression of HCC,<sup>104,105</sup> while *miR-205* can directly inhibit EMT by targeting EMT-TFs, ZEB1, and ZEB2 proteins.<sup>106,107</sup> Moreover, *miR-200b*-ZEB1 circuit has been suggested to function as a master regulator of stemness in HCC.<sup>104</sup> Intriguingly, the *miR-200*-ZEB1-E-cadherin axis has been demonstrated to be a crucial pathway downstream of TGF- $\beta$  in the EMT, while reciprocal repression between *ZEB1* and the *miR-200* family has recently been reported to promote the EMT and invasion in cancer cells.<sup>108,109</sup> Similarly, members of the *miR-34* family attenuate the expression of SNAIL.<sup>110</sup>

Many other miRNAs can directly target EMT-TFs, such as *miR-429*, that might function as an antimetastatic miRNA to regulate HCC metastasis, decreasing the migratory capacity and reversing the EMT to the MET in HCC cells.<sup>111,112</sup> However, ZEB1 and ZEB2 can also reduce the *miR-200* family expression through a negative feedback loop.<sup>113,114</sup> The expression of *MiR-612* directly targets Akt2, and its expression is in reverse correlation to the EMT and metastasis in HCC patients. Malta et al have shown that negative correlation between EMT gene signature and stemness observed in The Cancer Genome Atlas (TCGA) primary tumors was also found in metastatic samples. Typically, tumor cells in many solid tumors are fundamentally epithelial, but some of them acquire a mesenchymal phenotype due to the accumulation of mutations or epigenetic changes induced by the TME. These mesenchymal cells can cross the underlying tissue, enter the bloodstream, and disseminate in distant places, where they reacquire an epithelial phenotype for metastatic tumor formation.<sup>115</sup> However, the relationship between EMT and stemness remains a debated topic, since other evidence reveals that EMT is necessarily associated with stemness.<sup>116</sup>

Other examples include *miR-216A/217*, which were reported to be correlated with the EMT, CSC phenotype, and poor survival of patients with HCC, via phosphatase PTEN and SMAD7 that activate PI3K/Akt and TGF- $\beta$  signaling.<sup>44</sup> Furthermore, different evidence has indicated that the tumor suppressor p53 could regulate EMT-associated stem cell properties.

Interestingly, treatment of HCC epithelial cells with TGF- $\beta$  is also able to upregulate CD44,<sup>117</sup> a CSC marker which plays an important role in maintaining the mesenchymal phenotype in HCC by inducing the EMT. In line with this result, a CSC-like phenotype has generated great interest in HCC cells because it acquired a more aggressive and chemotherapy-resistant phenotype. Fernando et al have shown in vitro the presence of two groups of HCC cells with different phenotypes, which respond

distinctly to the action of cell-induced sorafenib.<sup>118</sup> HCC epithelial cells expressing EPCAM and PROM-1 (CD133) are sensitive to sorafenib, undergoing an arrested cell cycle in G0/G1 (PLC/PRF/5 and HepG2) or even cell cycle arrest and cell death (Hep3B). By contrast, cells with a mesenchymal phenotype, mediated by an autocrine overactivation of the TGF- $\beta$  receptor pathway,<sup>119</sup> with a high expression of CD44, do not respond after exposure to the maximum dose of sorafenib, in terms of bringing about cell death (Snu449, HLE, and HLF) nor to the inhibitory effect of TGF- $\beta$ . However, a recent study has shown that the inhibition of TGFR1 by galunisertib decreases the expression of CD44 and THY1 in HLE and HLF cells, reducing the clonogenic capacity and 3D-liver spheroid formation as well as the invasive growth ability of HCC cells. Furthermore, studies in ex vivo HCC patient samples confirmed a reduced expression of CD44 and THY1 following treatment with galunisertib in responders but not in nonresponder patients.<sup>53</sup> Overall, these results have suggested that a high expression of CD44 is correlated with the EMT, intrahepatic HCC dissemination, and chemoresistance in liver tumor cells, inducing stemness features.<sup>53,118</sup>

### TGF- $\beta$ , Cancer Immune Microenvironment, and Regulatory T Cells as Potential Therapeutic Targets: Implications for HCC

The setting of *tailor-made* therapies to target solid cancers, such as HCC, poses a major challenge, in particular in view of the extreme biological variability of this neoplasm found among different patients.<sup>120–122</sup> Owing to the intratumor heterogeneity and the drug-induced adaptive plasticity of cancer cells, which critically affect their susceptibility to pharmacological agents, an unpredictable resistance to treatment frequently occurs. Although the search for novel approaches to this problem is ongoing,<sup>123</sup> a further layer of complexity is added by the wide repertoire of infiltrating immune cells, which support the inflammatory status of the preexisting liver disease, while extensively influencing cancer growth.<sup>124</sup>

Indeed, a characterization of immune microenvironment in HCC through histopathological analysis of 196 nodules revealed that 22% exhibit elevated or moderate levels of lymphocyte infiltration with remarkably different immune marker expression between HCC and the adjacent normal tumor. In addition, a gene expression analysis has identified a list of 66 immune markers of different populations of immune cells, thus defining six immune profiles in patients with HCC.<sup>125</sup> Multiple subsets of leukocytes are attracted into primary lesions, as a consequence of events triggered by tumor-associated/specific antigens,<sup>126</sup> but the inconstant patterning they often induce accounts to some extent for the variable prognostic expectancies.<sup>127</sup> TGF- $\beta$  is a master regulator of immunity, as the integrity of its signaling must be preserved to maintain the functional homeostasis between effector and regulatory immune cells, which, in turn, is required to properly control inflammatory processes and prevent autoimmune alterations.<sup>128</sup> The frequent overexpression of TGF- $\beta$  in some cancers profoundly shapes the immunological environment, affecting both the fate of differentiating

lymphoid precursors and the activity of multiple leukocyte subsets within the tumor.<sup>128,129</sup> In the context of cancer immunology, two major groups of immune cells have been designated, based on evidence that they play a role in promoting or restraining cancer progression. A large panel of leukocytes, including natural killer cells, some subsets of effector CD4+ T cells, and CD8+ cytotoxic T lymphocytes, potentially recognize and clear cancer cells.<sup>130</sup> Conversely, a heterogeneous class of CD4+ forkhead/winged helix transcription factor P3+ (FoxP3+) expressing Tregs has been shown to quench antitumor immunity and enhance cancer development, and has thus aroused growing interest as a potential target in cancer immunotherapy. Two general subsets of Tregs, namely natural (nTregs) and induced (iTregs), have been defined, depending on their involvement in different regulatory contexts. While the development of nTregs takes place within the thymus, and does not require TGF- $\beta$ , but rather IL-2 or IL-15,<sup>131–134</sup> iTregs differentiation occurs in peripheral lymphoid organs and is strictly dependent on TGF- $\beta$  and IL-2. Despite some degree of divergence in their functional competences, both Treg subtypes work in healthy subjects to prevent the onset of autoimmunity, immune reactions against food antigens or allergens, and to terminate inflammation after the triggering microbial agent has been cleared.<sup>128</sup> The need for TGF- $\beta$  to achieve a sufficient repertoire of Tregs to prevent some pathological conditions has been documented. Mice with transgenic expression of the BDC2.5 T cell receptor and deletion of TGFR2II in CD4Cre-Tgfb2f/f NOD develop type 1 diabetes (T1D), associated with an accumulation of peripheral Th1 and Th17, but reduced Treg cells.<sup>135</sup> The deregulation of TGF- $\beta$  signaling occurring in some cancers appears to reflect a detrimentally over-reactive Tregs arm of immunity. In animal models of melanoma, this cytokine, alone or in the presence of others (such as VEGF), can induce Tregs, which directly inhibit the activity of killer cells (CTL CD8+, NK), and render anti-CTLA4 or anti-PD1 therapies inefficient.<sup>136,137</sup> Nonepithelial intratumor cell types, such as mesenchymal stem cells, also produce TGF- $\beta$ , which is responsible for defective NK and CTL activities, while increasing Tregs numbers.<sup>138,139</sup> Tregs inhibit tumor-specific cytotoxicity of CD8 T cells, even without affecting their ability to expand or produce IFN $\gamma$ .<sup>140</sup> TGF- $\beta$  derived from Tregs residing in tumor-draining lymph nodes (TDLNs) upregulate the expression of oncogenic IL-17rb in breast tumor cells that have invaded TDLNs, thus enhancing their malignancy.<sup>141</sup> TGF- $\beta$  can also indirectly affect Tregs development. When treated with TGF- $\beta$  and IFN $\gamma$ , mesenchymal stem cells-derived exosomes have been shown to orientate the differentiation of mononuclear cells toward a Treg phenotype.<sup>142</sup> Other than in soluble form, TGF- $\beta$  exposed on Tregs membrane are also used to inhibit tumor-limiting cells. Cell-cell contact between Tregs and antigen-specific CD8 T cells unleash a suppressive activity of Tregs surface-bound TGF- $\beta$ , which blocks the cytotoxicity of CD8 cells against melanoma cells.<sup>143</sup> A rise in the frequency of FoxP3 Tregs belonging to the ICOS+ subset, or a concomitant increase in the number of highly suppressive Tregs subsets and myeloid-derived suppressor cells in neoplasms from HCC patients, has been associated with a dysfunctional T cell-mediated antitumor activity and

hence unfavorable prognosis.<sup>144,145</sup> This evidence has driven attempts to adopt drug-based approaches to counteract the Tregs tumor-supporting activity in preclinical models of HCC. Sunitinib has proven to be successful in eradicating tumors in a CCl<sub>4</sub>-induced HCC mouse model by inducing an impairment of Tregs frequency and their release of TGF- $\beta$  and IL-10 which, in turn, leads to re-enabling the tumor antigen-specific CD8+ T cells killing capacity.<sup>146</sup> Other authors have found that norcantharidin, combined with coix lacryma-jobi seed oil, besides exerting potent cytotoxic and proapoptotic activity on HCC cells, compared with either compound alone, enforces anti-tumor immunity through impairing Tregs development in Hepal-1 hepatoma-bearing mice.<sup>147</sup>

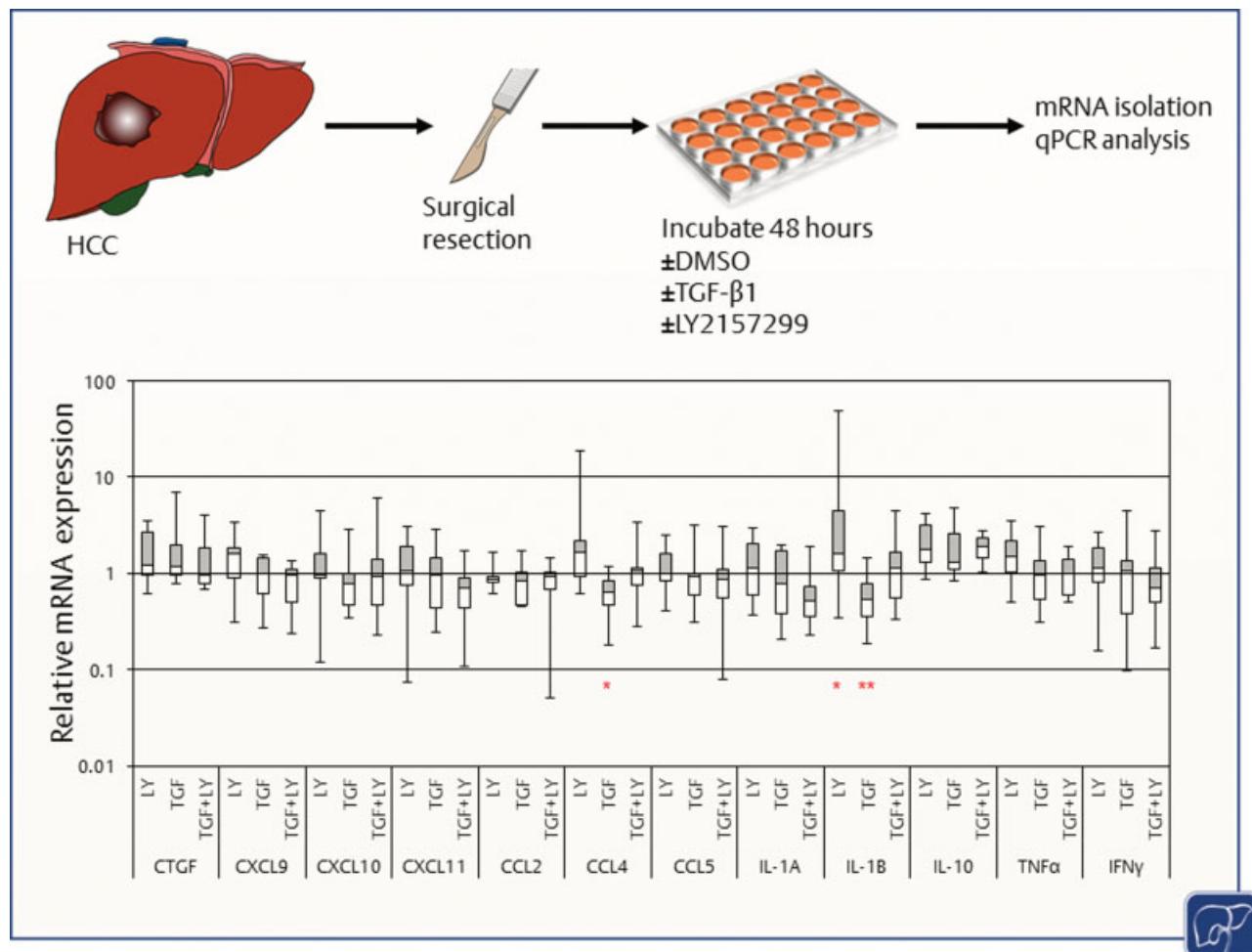
### Control of Cytokine/Chemokine Arrangement by TGF- $\beta$ in HCC and the Role of Stromal Cells

In parallel to Tregs targeting in in vitro or animal settings, some approaches to treat HCC which rely on drugs that block TGF- $\beta$  pathway are being exploited in humans. Galunisertib (LY2157299) is an anti-TGF $\beta$ R1 small molecule which has entered clinical practice, either alone or in association with other drugs<sup>23</sup> (►Table 1).

**Table 1** Clinical trials of TGF- $\beta$  signaling blockade using Galunisertib in solid tumors and HCC

	Drug	Study	Organ site	Clinical trial number
1	Galunisertib	A phase 1 study of galunisertib on the immune system in participants with cancer	Neoplasm	NCT02304419
2	Galunisertib Radiotherapy	A phase 1 study of galunisertib (LY2157299) plus stereotactic body radiotherapy (SBRT) in advanced hepatocellular carcinoma (HCC)	Advanced hepatocellular carcinoma (HCC)	NCT02906397
3	Galunisertib Durvalumab	A phase 1 study of galunisertib (LY2157299) and durvalumab (MEDI4736) in participants with metastatic pancreatic cancer	Metastatic pancreatic cancer	NCT02734160
4	Galunisertib Nivolumab	A phase 1 and 2 study of galunisertib (LY2157299) in combination with nivolumab in advanced refractory solid tumors and in recurrent or refractory non-small-cell lung cancer or hepatocellular carcinoma	Solid tumor Recurrent non-small-cell lung cancer Recurrent hepatocellular carcinoma	NCT02423343
5	Galunisertib Capecitabine	A phase 1 and 2 study of galunisertib and capecitabine in advanced resistant TGF- $\beta$ activated colorectal cancer	Colorectal cancer metastatic	NCT03470350
6	Galunisertib Sorafenib	A phase 1 study of LY2157299 in participants with unresectable hepatocellular cancer (HCC)	Hepatocellular carcinoma	NCT02240433
7	Galunisertib Gemcitabine	A phase 1 study of LY2157299 in participants with pancreatic cancer that is advanced or has spread to another part of the body	Pancreatic neoplasms	NCT02154646
8	Galunisertib Sorafenib Placebo	A phase 2 study of LY2157299 in participants with advanced hepatocellular carcinoma	Hepatocellular carcinoma	NCT02178358
9	Galunisertib Sorafenib Ramucirumab	A phase 2 study of LY2157299 in participants with hepatocellular carcinoma	Hepatocellular carcinoma	NCT01246986
10	Galunisertib Gemcitabine Placebo	A phase 1 and 2 study in metastatic cancer and advanced or metastatic unresectable pancreatic cancer	Neoplasms Neoplasm metastasis Pancreatic cancer	NCT0137316

We here show that IL-1 $\beta$  and CCL4 represent two major targets of TGF- $\beta$  in HCC tissues treated in an ex vivo model. Noteworthily, while exogenously added TGF- $\beta$ 1 significantly downregulated either mRNA, galunisertib was able to offset the effects of both exogenous and residual tumor-produced TGF- $\beta$ . IL-1 $\beta$  is a potent proinflammatory innate cytokine that, like TNF $\alpha$ , activates endothelial cells to ultimately induce leukocytes extravasation from the bloodstream into the sites of inflammation. A role for IL-1 $\beta$  as a promoter of angiogenesis and the invasiveness of malignant cells in different models of solid cancers has also been reported.<sup>148,149</sup> This is in apparent contrast with the inhibitory effect of TGF- $\beta$  on IL-1 $\beta$  expression as we found, which seems to suggest a tumor-limiting activity for TGF- $\beta$  along with blocking inflammation. Unlike IL-1 $\beta$ , a CCL4 mRNA decrease induced by TGF- $\beta$  is consistent with a tumor-promoting activity of TGF- $\beta$ , since CCL4 is a chemokine attracting CD8+ lymphocytes.<sup>150</sup> Instead, the expression of some other immune mediators, such as CCL2, CCL5, or TNF $\alpha$ , other than IL-1 $\beta$  and CCL4, is unaffected by TGF- $\beta$ 1 in this ex vivo model (►Fig. 3). Notably, TGF- $\beta$ 1 may be involved in a mechanism that finely tunes positive and negative regulators of inflammation, to achieve successful malignant progression.



**Fig. 3** Effects of TGF- $\beta$  on mRNA expression of major cytokines, chemokines, and growth factors in ex vivo cultured HCCs. Specimens obtained from primary HCC tumors were treated for 48 hours in serum-free conditions in the presence of TGF- $\beta$ 1 (5 ng/ml), galunisertib (LY2157299, 10  $\mu$ M), or both. The expression of the mRNA of interest was then analyzed by quantitative PCR.

The best-known effects of TGF- $\beta$  on cancer-associated fibroblasts (CAF) consist of upregulation of the activity of genes that promote a profibrotic phenotype.<sup>151</sup> Liu et al have shown that several cytokines and chemokines are also secreted by CAFs.<sup>152</sup> In addition, we here report that TGF- $\beta$  potently dampens the release by CAFs of highly expressed chemokines, CCL2 and CXCL1, but also GM-CSF and the soluble form of ICAM-1, supporting the assumption that TGF- $\beta$  elicits immunosuppression. Albeit slightly increased by TGF- $\beta$ , the level of secreted CXCL12 remains barely detectable (**►Fig. 4**). These data suggest that CAFs may mediate indirect effects of TGF- $\beta$  on the immune environment of HCC.

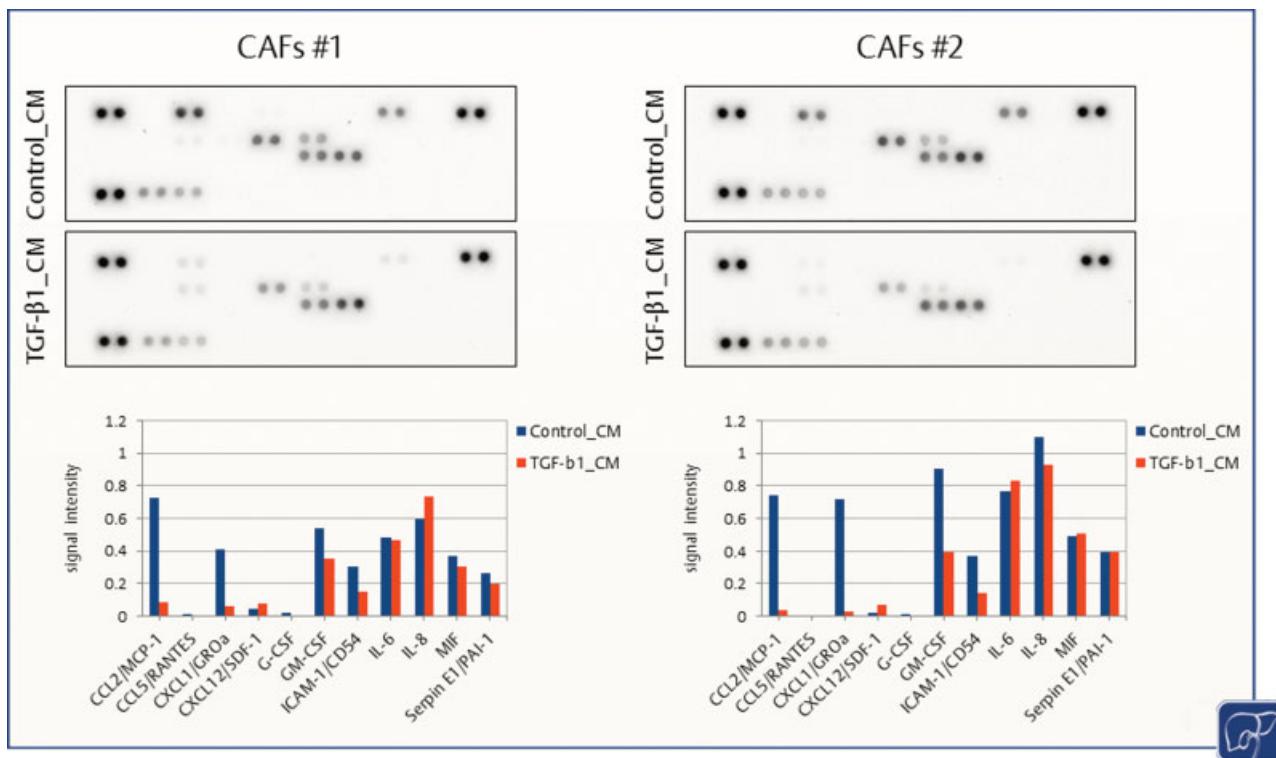
### Inhibiting the TGF- $\beta$ Signaling Pathway

Targeting the TGF- $\beta$  pathway as a therapeutic option for cancer treatment may be a promising research direction but is still a challenging task to pursue. The biggest issue is to discriminate between the negative effects of TGF- $\beta$  and its other physiological roles, and delineate the tumor-suppressive versus tumor-promoting roles of TGF- $\beta$  in each tumor.

Therefore, the timing of treatment and selection of patients should be carefully evaluated before administering

drugs which enhance or decrease TGF- $\beta$  effects. Many TGF- $\beta$  pathway inhibitors have been investigated in the preclinical setting, some of which are now in clinical development. Briefly, TGF- $\beta$  pathway inhibition can be divided into three levels<sup>56,153</sup>:

1. Ligand level: using antisense molecules for the prevention of TGF- $\beta$  synthesis. Single-stranded oligonucleotides operate through direct delivery intravenously or engineered into immune cells that bind complementary sequences on specific mRNA, thereby preventing the translation and accelerating the degradation of target genes.<sup>43,153</sup> Examples of these antisense molecules include Trabedersen (AP12009, Pharma), targeting TGF- $\beta$ 2, and Lucanix (belagenpumatucel-L), a TGF- $\beta$ 2 antisense gene-modified allogeneic cancer cell vaccine. Trabedersen,<sup>154,155</sup> in particular, was successfully used on glioma cells and in a murine model of pancreatic cancer. It was also successfully tested in an open label Phase I/II study in patients with stage III/IV pancreatic cancer, malignant melanoma, and colorectal cancer (CRC).<sup>156</sup> Results showed that the drug was safe and well tolerated even if some patients developed thrombocytopenia. Furthermore, in one pancreatic cancer patient



**Fig. 4** Long-term effects of TGF- $\beta$  on the secretion of cytokines, chemokines, and growth factors by CAFs. CAFs isolated from two HCC primary tumors were left untreated or incubated in the presence of TGF- $\beta$ 1 (5 ng/mL) for 14 days in complete medium. Then cells were serum-starved and conditioned medium was collected, concentrated and then assayed with a panel of 36 cytokines/chemokines. Only detectable factors (11) are shown.

there was a complete response of liver metastases to the treatment and, at that time, he was still alive after 75 months.<sup>156</sup>

2. Ligand–receptor level: using antiligand and antireceptor monoclonal antibodies or soluble receptors, blocking ligand–receptor engagement. Fresolimumab, lerdelimumab, and metelimumab are three fully humanized monoclonal antibodies against TGF- $\beta$  developed by Genzyme and tested in clinical trials. However, none of these were included in clinical trials for gastrointestinal cancers.<sup>153</sup> TR1 or IMC-TR1 (LY3022859) is another fully human anti-T $\beta$ RII monoclonal antibody developed by Eli-Lilly & Co, tested in clinical trials in patients with advanced solid tumors.<sup>157</sup> As regards liver tumors, Dituri et al reported a different response in an HCC preclinical model between IMC-TR1 and galunisertib (LY2157299, Eli-Lilly & Co), suggesting that receptor expression on tumor cells is only one aspect in the patients selection approach and microenvironment, and that immune cell expression for this receptor should be taken into consideration.<sup>158</sup>

3. Intracellular level: signal transduction blockade by receptor kinase inhibitors. Generally, these small-molecule kinase inhibitors lack specificity and, at certain doses, cause off-target effects. Furthermore, each molecule/drug shows distinct advantages and disadvantages that have to be balanced to gain the greatest benefit for use in the clinic. Parameters to be considered are the affinity and specificity for the target, drug stability, clearance, and bioavailability *in vivo*, as well as the mode of drug delivery

(i.e., oral or endovenous). Most of the TGF- $\beta$ -associated receptor kinase inhibitors act by inhibiting the catalytic ATP binding site of T $\beta$ RI.<sup>43,153</sup> Regarding T $\beta$ RI, although these inhibitors potentially block the kinase activity, they will not avoid noncanonical TGF- $\beta$  signaling independently of the kinase activity. In the last decade, many preclinical studies have been attempts to evaluate the whole plethora of receptor kinase inhibitors developed. However, galunisertib is the only TGF- $\beta$  receptor kinase inhibitor currently in use in clinical trials.<sup>159</sup> So far, there are several ongoing clinical trials in which galunisertib is used alone as monotherapy with or without standard of care, namely the alkylating agents, lomustine, or temozolamide in radiochemotherapy for glioblastoma, in combination with the antimetabolite gemcitabine for metastatic pancreatic cancer or sorafenib for HCC.

The role of TGF- $\beta$  signaling in HCC is particularly complex, as it influences different hallmarks of cancer, such as tumor proliferation, angiogenesis, invasion, metastasis, and immune surveillance escape. As regards HCCs, several trials are ongoing in the so-called postsorafenib systemic treatment era, in which, among others, galunisertib was revealed as a promising small-molecule inhibitor, currently under investigation with patients showing highly unmet medical needs. As shown in ►Table 1, there are active clinical trials in which galunisertib in combination with sorafenib are under study in patients with unresectable or advanced HCC. A recent study reveals that a mesenchymal profile and the

expression of CD44, linked to the activation of the TGF- $\beta$  pathway, may predict lack of response to sorafenib in HCC patients. Targeted CD44 knock-down in the mesenchymal-like cells evidenced that CD44 exerts an active role in protecting HCC cells from sorafenib-induced apoptosis.<sup>118</sup> On the other hand, it was demonstrated that galunisertib treatment reduces the expression of stemness-related genes in ex vivo human HCC specimens. Galunisertib overcomes stemness-derived aggressiveness via a decreased expression of CD44 and THY1 (CD90).<sup>53</sup> Finally, an open-label Phase II clinical study is enrolling both naive and previously sorafenib-treated patients to test the combination of galunisertib with sorafenib or ramucirumab (a recombinant IgG1 monoclonal antibody and a VEGFR-2 antagonist) in patients showing increased  $\alpha$ -fetoprotein levels.

Other trials include combination with radiotherapy for breast cancer and also with the checkpoint inhibitor nivolumab (anti-PD1, Bristol-Myers Squibb).<sup>43,153</sup> A Phase Ib/2 clinical trial is currently enrolling HCC patients refractory to sorafenib to evaluate the safety of the combination of galunisertib + nivolumab.

Hepatocellular carcinoma is a malignancy characterized by a great biological heterogeneity. As such, trial enrollment with only few stratification factors runs the risk of failure. Of course, personalized therapy is one of the biggest challenges as a means of successfully overcoming HCC heterogeneity and offering patients the most effective treatment. Tailored treatment according to the individual HCC genetic profile could provide therapeutic choices beyond standard sorafenib regimen for liver cancers.<sup>160,161</sup> From this perspective, as galunisertib is not similarly effective in all patients, several studies have been aimed at identifying new potential diagnostics biomarkers for patient stratification. In one study, next-generation sequencing-based massive analysis of cDNA ends was used to investigate the transcriptome of an invasive HCC cell line responses to TGF- $\beta$ 1 and galunisertib. Then, the identified mRNAs were validated in HCC frozen samples and ex vivo HCC tissues treated in vitro with the drug. The results indicated that mRNA levels of two genes, SKIL and PMEPA1, were positively correlated with TGF- $\beta$ 1 mRNA concentrations in HCC tissues and strongly downregulated by galunisertib.<sup>162</sup> The data presented in the study suggest that SKIL and PMEPA1 mRNA levels, used in combination with TGF- $\beta$ 1 mRNA, could be important biomarkers for selecting patients more likely to respond to treatment with galunisertib, providing a pathway toward personalized medicine thanks to a better patients stratification.<sup>162</sup>

Ki26894 and SB-435 are other T $\beta$ RI inhibitors demonstrating positive effects in in vitro experiments using gastric cell lines<sup>163</sup> and CRC,<sup>164</sup> respectively, but these have not yet been tested in clinical trials. More recently, a first human dose study of a new T $\beta$ RI kinase inhibitor, Vactosertib (TEW-7197, MedPacto), was started as monotherapy in subjects with advanced-stage solid tumors.<sup>165–167</sup> Vactosertib has been shown to cause Smad4 degradation in cytotoxic T cells, resulting in an enhanced cytotoxic T cell activity,<sup>168</sup> as well as reduced breast tumor metastases to the lung in mice.<sup>167</sup>

Enhancing the tumor suppressive role of TGF- $\beta$  could be another strategy to pursue in new therapeutic targets devel-

opment. In this scenario, several tumor cell types show the activation of cell cycle proteins such as CDK4, c-Myc, and  $\beta$ -catenin when TGF- $\beta$  signaling is inactivated. Thus, these molecules could be new functional targets for therapeutics of lethal cancers that evade TGF- $\beta$ .<sup>169</sup> For example, several studies showed that CDK4 activation and high levels of cyclin D1 with inactivated TGF- $\beta$  signaling are common in colon and hepatocellular cancers.<sup>170,171</sup> These studies have led to a series of clinical trials targeting these molecules. Clinical trials of CDK4 inhibitors such as ON123300<sup>172</sup> (currently in Phase I) and palbociclib<sup>173</sup> (PD0332991, currently in Phase I–III) are ongoing.

Other options for cancer treatment are the pathways that control stem cell proliferation. The activation of canonical Wnt signaling in cooperation with TGF- $\beta$  results in rapid cell cycle arrest and differentiation. However, in CRC, TGF- $\beta$  signaling is inactivated and mutation in the Wnt cascade leads to aberrant crypt foci. In addition, components of TGF- $\beta$  signaling (including SMAD) and Wnt cascade were found frequently mutated also in gastrointestinal tract adenocarcinomas.<sup>174</sup> An analog of vitamin D3, seocalcitol, able to block  $\beta$ -catenin, the key protein in Wnt signaling, has been tested and showed encouraging effects in colon, but not in liver cancer, in which the clinical trial failed.<sup>175,176</sup>

Targeting STAT3 has been reported as a favorable option when TGF- $\beta$  signaling is disturbed. Crosstalk between TGF- $\beta$ /Smad and JAK/STAT signaling pathways has been observed, in which TGF- $\beta$  can downregulate IL-6-induced phosphorylation of STAT3.<sup>177</sup> Several inhibitors have been developed to prevent the aberrant activation of STAT3 that occurs in many tumors and in HCC, indicating that IL6/STAT3 could be a novel approach for the treatment of these malignancies.<sup>51,178</sup>

## Conclusion

Due to the dichotomic nature of TGF- $\beta$  signaling in the context of liver carcinogenesis, a well-defined view of its role as pro-oncogenic or tumor suppressor is still widely debated. Although some studies support the pro-apoptotic tumor-limiting functions of TGF- $\beta$ , other authors describe a scenario wherein multiple molecular switches can modulate the ability of this cytokine to support or limit the malignant progression of HCC. In addition, a multitude of mutual interactions, some of which are mediated by TGF- $\beta$ , between cancer and microenvironment cells (stromal, immune, etc.) may contribute to the progression of the disease. A careful evaluation of the molecular signature of each single HCC patient is recommended to choose the most eligible candidates for anti-TGF- $\beta$  therapies, as well as possible “drugable” targets to be exploited in multidrug approaches to achieve a more effective treatment.

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