EMERGING INSIGHTS INTO TRANSFORMING GROWTH FACTOR β Smad SIGNAL IN HEPATIC FIBROGENESIS

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Irrespective of initial stimuli, increased production of type I collagen is a common hallmark of fibrotic diseases in various organs including the liver. A dynamic balance between the production and degradation of collagen is seen, which is rigorously controlled by several growth factors and cytokines. Of these, transforming growth factor β (TGFβ) is the most potent factor in stimulating type I collagen gene transcription. It also regulates expression of matrix metalloproteinases and their inhibitors, and modulates inflammatory reactions by influencing T cell functions. Therefore, TGFβ is considered to be the major factor accelerating liver fibrosis. Identification and characterisation of Smad proteins, intracellular mediators of the signal transduction of TGFβ, have led to a better understanding of the precise mechanisms of TGFβ functions from the viewpoint of its intracellular signalling pathway and crosstalk with other factors. Several studies have focused on the suppression of TGFβ activation and intervention of the TGFβ/Smad signalling pathways to treat liver fibrosis. However, as generalised blockade of TGFβ activity may result in the promotion of carcinogenesis and excessive immune reactions, much attention has to be paid to selective intervention of the TGFβ/Smad signal specifically in collagen-producing cells in the fibrotic tissue. TGFβ also affects the growth and differentiation of stem and progenitor cells. From this point of view, a new concept of the treatment for liver fibrosis may arise from the discipline of stem cell biology by modulating TGFβ and Smad signalling in stem/progenitor cells.

BACKGROUND

TGFβ is a member of a large family of pleiotropic cytokines that includes bone morphogenetic proteins (BMPs), activins and other related factors. Mammals have three different forms of TGFβ (β1, β2 and β3). Although the three isoforms of TGFβ are encoded by distinct genes located on different chromosomes, they have approximately 80% homology at the level of amino acid sequence. Of these, TGFβ1 has been most extensively studied for its roles in various pathophysiological conditions.

TGFβ1 (henceforth referred to as TGFβ) is a homodimeric polypeptide with a molecular weight of 25 kDa. It was first identified in the culture medium conditioned by transformed cells, and was named for its ability to induce anchorage-independent growth of rodent fibroblasts.[1] However, subsequent studies have shown that it is a potent growth inhibitor for most epithelial types of cells including parenchymal hepatocytes. In addition, TGFβ is multifunctional and regulates cell survival, differentiation, migration, adhesion and synthesis of extracellular matrix (ECM) components. It is therefore involved in many biological and pathological processes including embryonic development, tissue remodelling, inflammation, angiogenesis, atherosclerosis, fibrosis and carcinogenesis. Through these functions, TGFβ plays an important part in both normal and diseased conditions in the liver[2 3] and in other organs (fig 1). During the past decade, identification and characterisation of Smad proteins, intracellular mediators of the signal transduction of TGFβ superfamily members, have led to a better understanding of the precise mechanisms of TGFβ functions from the viewpoint of its intracellular signalling pathway and crosstalk with other factors.[4]

This review summarises recent advances in our understanding of the pathological roles of TGF-β and Smad signalling in hepatic fibrogenesis, with an emphasis on the transcriptional regulation of type I collagen gene expression. It also highlights the current attempts to treat liver fibrosis by targeting TGFβ/Smad signalling.

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OVERVIEW OF THE TGFB/Smad SIGNALLING PATHWAY

TGFβ protein is synthesised and secreted as a precursor that includes a latency-associated peptide (LAP). This complex is often bound by one of the several latent TGFβ-binding proteins, which act as an important safeguard against inadvertent activation of TGFβ. In addition, they assemble with several ECM components such as type IV collagen, fibronectin and fibrillin. Thus, ECM represents a

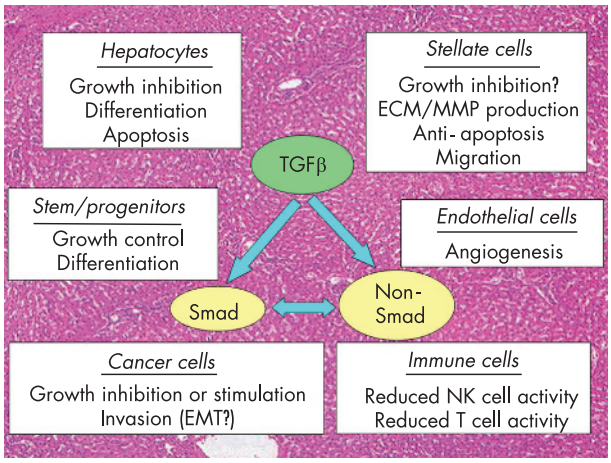

Figure 1 Diverse effects of transforming growth factor β (TGFβ) and Smad signal in liver biology and pathobiology. TGFβ and Smad play important parts in both normal and diseased situations in the liver by exerting differential effects on each cell population. Non-Smad signalling pathways of TGFβ have also been implicated in various pathophysiological conditions either independently of or through crosstalk with Smad signals. ECM, extracellular matrix; MMP, matrix metalloproteinase; EMT, epithelial-to-mesenchymal transition, NK, natural killer.

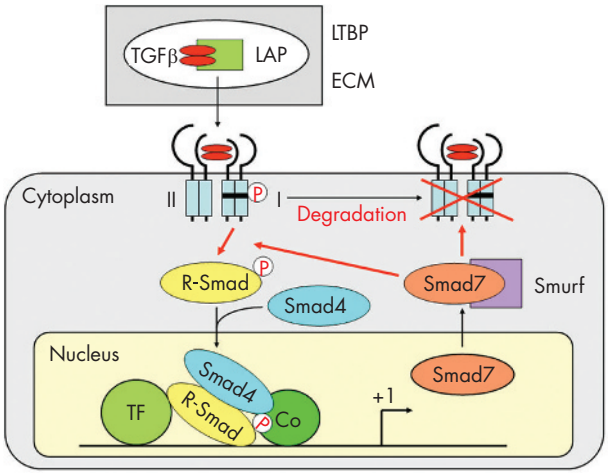

Figure 2 Signal transduction pathways of transforming growth factor β (TGFβ) and its intracellular mediators, Smad proteins. Active TGFβ released from the latent TGFβ-binding proteins (LTBP) and latency-associated peptide (LAP) binds to the specific receptors on the cell surface. Receptor-activated Smad (R-Smad) and Smad4 transduce the signal from the cytoplasm to the nucleus, where they regulate target gene transcription usually in association with DNA-binding transcription factors (TF) and coactivators (Co). By contrast, inhibitory Smad7 is strongly and rapidly induced by TGFβ itself, and acts in an autoregulatory negative feedback loop of the TGFβ/Smad signal. ECM, extracellular matrix.

reservoir from which active TGFβ can readily be recruited without de novo synthesis (fig 2). Several factors including matrix metalloproteinases (MMPs), plasmin, plasminogen activators, αvβ6 integrin and thrombospondins have been reported to accelerate the release of active TGFβ from the latent complex. Of these, thrombospondin 1 expression in hepatic stellate cells (HSC) is induced by platelet-derived growth factor (PDGF) and promotes the effects of TGFβ.[5] A peptide derived from the amino terminus of LAP blocks the activation of TGFβ by thrombospondin 1, and suppresses the progression of experimental liver fibrosis in rats.[6] On the other hand, a protease inhibitor, camostat mesilate, prevents progression of rat liver fibrosis by reducing plasmin activity and suppressing TGFβ activation.[7]

Activated TGFβ first binds to the TGFβ type II receptor on the cell surface. It subsequently recruits the TGFβ type I receptor, thus forming a heterotetrameric complex of these two types of receptors (fig 2). The type II receptor kinase phosphorylates the type I receptor in the GS region, which is located immediately upstream of the kinase domain. Type I receptor in turn phosphorylates Smad2 and Smad3, so-called receptor-activated Smad (R-Smad), which form hetero-oligomers with a common mediator, Smad4 (Co-Smad). They translocate from the cytoplasm to the nucleus, where they regulate the transcription of target genes.[4] In addition to the weak DNA-binding activity of Smad3 and Smad4 (but not Smad2), they usually associate with other DNA-binding transcription factors (fig 2), which specify the promoter binding and transcriptional regulation of target genes. Smad2 and Smad3 are also linked to the general transcriptional machinery through a direct interaction with transcriptional coactivators p300 or CBP (fig 2).

In contrast with those signal-transducing R-Smads and Co-Smads, Smad7 is known as an inhibitory Smad (I-Smad): it inhibits TGFβ signalling by interfering with the phosphorylation of Smad2 and Smad3 by the type I TGFβ receptor kinase.[8] Smad7 expression is strongly and rapidly induced by TGFβ itself. In addition, Smad7 interacts with a group of ubiquitin ligases, termed Smurf. After recruitment of Smad7–Smurf1 complex to the activated TGFβ receptors, Smurf1 degrades the receptors through proteasomal and lysosomal pathways.[9] Thus, Smad7 acts in an autoregulatory negative feedback loop of the TGFβ/Smad signal (fig 2). In addition, several corepressors have been reported, including c-Ski, SnoN, transforming growth inhibiting factor and smad nuclear-interacting protein 1. They interact with Smad2 or Smad3 and inhibit TGFβ responses.

TGFB/Smad SIGNAL IN HEPATIC FIBROGENESIS

Type I collagen, the major ECM component of the fibrotic tissue, is a heterotrimer composed of two α1 chains and one α2 chain. They are encoded by two distinct genes, *COL1A1* and *COL1A2*, respectively. Increased production of type I collagen is a common hallmark of fibrotic diseases in various organs including the liver. A dynamic balance between the production and degradation of collagen is rigorously controlled by several growth factors and cytokines, and a disruption of this equilibrium results in organ fibrosis. TGFβ is the most potent factor in stimulating type I collagen gene transcription. It also regulates expression of MMPs and their inhibitors, and modulates inflammatory reactions by influencing T cell functions. Therefore, TGFβ is considered to be a major factor accelerating the progression of organ fibrosis.

In the normal liver, sinusoidal endothelial cells and Kupffer cells contain relatively high levels of TGFβ mRNA, whereas HSC express little amounts of TGFβ. However, once stimulated by fibrogenic stimuli, HSC are the only cells that respond by expressing increased amounts of all three different isoforms of TGFβ. As activated HSC are the principal cells to produce type I

collagen in fibrotic liver, they contribute to the development of liver fibrosis through autocrine and paracrine loops of TGFβ-stimulated collagen production.

During the past decade, intensive studies have been focusing on transcriptional regulation of type I collagen expression, especially on the molecular mechanisms responsible for the TGFβ-elicited *COL1A2* stimulation and its pathological roles during the fibrogenic process in liver.[10]

Transcriptional regulation of collagen gene expression by transforming growth factor β and Smad proteins

Our initial studies using primary cultures of skin fibroblasts have shown that the −313 to −183 upstream sequence of the *COL1A2* promoter is essential for basal and TGFβ-stimulated transcription.[11] Within this segment are three DNA–protein binding sites, Box 5A, Box 3A and Box B (fig 3). Among them, Box 3A is the binding site for a ubiquitous trans-activator Sp1, whereas unknown cofactors bind to the downstream Box B.[11 12] Treatment with TGFβ modified the interaction between Sp1 and the cofactors and subsequently enhanced their binding to the cognate DNA sequences. We therefore designated the 3A plus B region the TGFβ-responsive element, TbRE.[11] Subsequently, Smad3 was shown to bind to Box B,[13] and its interaction with Sp1 has been implicated in TGFβ-elicited *COL1A2* stimulation in embryonic fibroblasts (fig 3).[14] TGFβ also enhances functional cooperation between Smad3 and p300/CBP coactivators.[15] It is therefore suggested that Sp1, Smad proteins and p300/CBP coactivators form a multimeric complex to mediate the stimulatory effect of TGFβ on *COL1A2* transcription (fig 3).

Synergistic cooperation between Sp1 and Smad3 also mediates the stimulatory effect of TGFβ on *COL1A2* transcription in activated HSC.[16 17] However, the intracellular localisation of R-Smads and their responses to exogenous TGFβ differ depending on the activation status of HSC. TGFβ induces phosphorylation and nuclear translocation of Smad2 in quiescent HSC and those of Smad3 in transdifferentiated HSC.[18] More importantly, ligand-independent nuclear localisation of Smad2, but not Smad3, was observed in culture-activated HSC[18] and in myofibroblasts obtained from chronically CCl_4-treated fibrotic liver.[19] By contrast, constitutive activation of the TbRE due to ligand-independent phosphorylation and nuclear localisation of Smad3 has been shown in an activated HSC clone established from a carbon tetrachloride (CCl_4)-induced cirrhotic liver.[20]

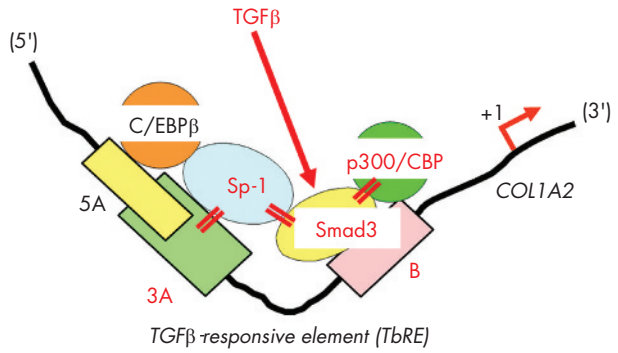

Figure 3 Schematic representation of the transforming growth factor β-responsive element (TbRE) located on the α2(I) collagen (*COL1A2*) gene promoter. TbRE consists of Box 3A and Box B, which are separated 14 bases from each other. TGFβ treatment enhances the interaction between Box 3A-bound Sp1, Box B-bound Smad3 and p300/CBP coactivators, and subsequently stimulates *COL1A2* transcription. On the other hand, Box 5A, located immediately upstream of and partly overlapping Box 3A, is a binding site for a *COL1A2* repressor, CAAT/enhancer binding protein β (C/EBPβ).

In contrast with several studies on the molecular mechanisms regulating *COL1A2* transcription, our knowledge regarding the regulation of coordinately expressed *COL1A1* transcription is limited. Previously, it has been shown that hydrogen peroxide and TGFβ act on the same upstream sequence of *COL1A1* promoter to stimulate gene transcription.[21] More recently, both Smad and p38 mitogen-activated protein kinase (MAPK) pathways have been implicated in transcriptional and post-transcriptional regulation of *COL1A1* expression.[22]

Molecular mechanisms responsible for cell type-specific *COL1A2* transcription

Type I collagen is produced predominantly in mesenchymal cells, but molecular mechanisms responsible for this cell lineage-specific expression are not fully understood. A *COL1A2* upstream sequence containing the TbRE was activated during the development of liver fibrosis.[23] More importantly, the *COL1A2* promoter was used only in non-parenchymal cells but not in parenchymal hepatocytes. We therefore compared *COL1A2* transcription and response to TGFβ between activated HSC and primary cultures of hepatocytes.[17] Parenchymal hepatocytes exhibited low transcriptional activity of the TbRE and no response to TGFβ. In those cells, another GC box binding factor Sp3, rather than Sp1, bound to the TbRE. Transfection of HSC with an Sp3 expression plasmid abolished the *COL1A2* response to TGFβ, whereas overexpression of Sp1 in parenchymal hepatocytes increased basal *COL1A2* transcription and conferred TGFβ responsiveness.[17] In addition, functional and physical interactions were observed between Sp1 and Smad3, but not between Sp3 and Smad3.[17] These results indicate that interaction between GC box binding factors and Smad proteins modulates cell lineage-specific *COL1A2* transcription in the liver.

Differential roles of Smad2 and Smad3 in HSC activation and collagen expression

Despite their high degree of homology, Smad2 and Smad3 have distinct roles in various biological and pathological situations. This has been clearly shown by the comparison of genetically engineered Smad2-null and Smad3-null mice. Smad2-null mice are embryonic lethal, indicating its critical role in prenatal development.[24] By contrast, mice lacking Smad3 are viable, although they exhibit small stature and impaired mucosal immunity.[25] A previous study using embryonic fibroblasts from Smad2-null and Smad3-null mice has shown that Smad2 plays an important part in TGFβ-induced MMP2 expression, whereas Smad3 is required for the induction of c-fos, Smad7 and TGFβ.[26] On the other hand, lack of either Smad2 or Smad3 results in markedly suppressed plasminogen activator inhibitor 1 (PAI) expression, indicating that both Smad2 and Smad3 are necessary to mediate TGFβ-elicited *PAI-1* gene transcription.[26] Another study using HSC isolated from Smad3-null mice has clearly shown that Smad3 plays an important part in stimulating type I collagen expression in activated HSC.[27] However, because of the lack of adult Smad2-null mouse, the relative

levels and functions of Smad2 and Smad3 in specific liver cell types and their relevance to pathophysiological conditions in the liver remained unknown. Recently, differential roles of Smad2 and Smad3 in HSC function have been reported by infecting cultured rat HSC with adenoviruses expressing wild-type and dominant-negative Smad2 or Smad3.[28] Smad3-over-expressing cells exhibited increased expression of type I collagen and fibronectin, increased chemotaxis and decreased proliferation. It was also shown that Smad3, but not Smad2, increased α-smooth-muscle actin organisation in stress fibres. From these findings, it is suggested that Smad3 plays a more important part than Smad2 in the morphological and functional maturation of myofibroblasts, and thus in the development of liver fibrosis.[28] Several small molecules inhibiting kinase activity of the TGFβ/activin type I receptors have been developed for their possible therapeutic application.[29] However, they suppress phosphorylation of both Smad2 and Smad3. Development of specific inhibitors for each of Smad2 and Smad3 not only shows the differential roles of these two R-Smads, but is also useful for more specific regulation of TGFβ target gene expression.

CROSSTALK BETWEEN TGFβ/Smad AND OTHER SIGNALLING PATHWAYS

Signal crosstalk with TGFβ/Smad pathway

Recent studies have shown that several factors interact with the TGFβ/Smad signal. Physical interactions and functional cooperation of Smad proteins with transcriptional factors allow signal crosstalk with other signalling pathways. Crosstalk with the Smad signal may also result from the ability of TGFβ to activate signalling pathways independently of Smad. Among many factors interacting with the TGFβ/Smad signal, signal crosstalk with MAPK and BMP7 has been shown, owing to its relevance to the regulation of type I collagen gene expression.

MAPK

Several mitogenic or stress stimuli activate members of the MAPK family of proteins and induce diverse cellular responses, including proliferation, differentiation and regulation of specific metabolic pathways. TGFβ can induce the activation of all three known MAPK pathways, extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK). One of the first demonstrated signal crosstalk between Smad and MAPK pathways was the ERK-elicited inhibition of nuclear translocation of Smad1.[30] ERK phosphorylates the specific serine residues in the linker domain of Smad1 and counter-represses BMP/Smad1-induced transcription. Similarly, negative regulation of TGFβ/Smad signal by the activated ERK pathway has been reported.[31] On the other hand, p38 MAPK usually acts as an alternative pathway to mediate TGFβ signal,[32] and it has been recently shown that p38 MAPK promotes the effects of Smad3 by enhancing its association with p300/CBP coactivators.[33] TGFβ-stimulated JNK can also phosphorylate Smad3 and induce its nuclear translocation.[34] However, recent studies using phosphorylation site-specific anti-Smad2 and anti-Smad3 antibodies have shown that p38 MAPK[35] and JNK[36] phosphorylate Smad3 at the linker region in activated HSC. Apart from the fact that each experiment used different types of cells, the reason why the same phosphorylation at the liker region of R-Smads by ERK and p38 MAPK/JNK either stimulates or suppresses their nuclear localisation remains an interesting open question. In addition, the effects of MAPK signals on type I collagen gene expression have also been a matter of controversy. Although most studies have shown that specific inhibition of the p38 MAPK pathway results in a decrease in collagen gene expression,[22 35 37] ERK signal either increases[38] or decreases[39] collagen gene expression depending on the cell types examined. Although there is no doubt that MAPK pathways greatly influence the TGFβ/Smad signals, the precise mechanisms of their crosstalk need to be elucidated in each cellular or pathophysiological context.

BMP-7

BMPs, as members of the TGFβ superfamily, are structurally related to the prototype molecule TGFβ. However, they bind to distinct type II receptors and subsequently activate specific type I serine–threonine kinase receptors.[4] While TGFβ receptors phosphorylate Smad2 and Smad3, the BMP signal is transmitted by phosphorylation of Smad1, Smad5 and Smad8. Recent studies have shown that BMP7 opposes the fibrogenic activity of TGFβ by suppressing the nuclear translocation of Smad3 in glomerular mesangial cells[40] and pulmonary myofibroblasts.[41] In the latter study on pulmonary myofibroblasts using transgenic *COL1A2* reporter mice, BMP7 increased the expression of inhibitors of differentiation (Id)2 and 3, and ectopic expression of Id2 and Id3 was found to suppress *COL1A2* transcription.[41] These results therefore indicate that BMP7 antagonises TGFβ-dependent fibrogenic activity of mouse pulmonary myofibroblasts not only by inhibiting the TGFβ/Smad3 signal but also by inducing Id2 and Id3. It is worth examining whether overexpression of BMP7 suppresses hepatic fibrogenesis through the same mechanisms. BMP7 and TGFβ are also reported to antagonise each other in epithelial-to-mesenchymal transition (EMT),[42] which plays an important part in fibrogenesis[43] as well as in carcinogenesis. To date, the presence and functional roles of EMT have not been established in the liver.

Antagonistic factors suppressing TGFβ/Smad-stimulated *COL1A2* transcription

As TGF-β is a key player in stimulating collagen gene transcription during the progression of liver fibrosis, counter-repression of the TGFβ-stimulated collagen expression would be a potent therapeutic means for preventing organ fibrosis by suppressing excessive collagen deposition in the liver.[3 44] Among several growth factors and cytokines, tumour necrosis factor α (TNFα) and interferon (IFN)γ are well known to suppress TGFβ-elicited *COL1A2* stimulation. More recently, hepatocyte growth factor (HGF) has also been reported to suppress experimental liver fibrosis. Most of these antagonistic actions are exerted via crosstalk between TGFβ/Smad and other signalling pathways.

TNFα

TGFβ and TNFα exert opposing effects on both synthesis and degradation of type I collagen. Our initial study has revealed that the counter-repression of TGFβ-stimulated *COL1A2* transcription by TNFα is mediated through the same TbRE as well as by increasing the amount of a repressor protein bound to the immediately upstream sequence, Box 5A (fig 3).[45] It has also been shown that CAAT/enhancer-binding protein β is the major component of the Box 5A-bound complex and mediates the

TNFα-elicited *COL1A2* repression.[46] A subsequent study showed that TNFα-activated nuclear factor κB induced inhibitory Smad7 and suppressed TGFβ/Smad signalling (fig 4).[47] On the other hand, others have proposed that TNFα prevents TGFβ/Smad-induced gene transcription through the induction of c-Jun/Jun B interacting with Smad3 and p300/CBP coactivators.[48] The latter study showed a good example of a crosstalk between TGFβ/Smad and JNK/Jun signals.

IFNγ

There have been several studies showing the inhibitory effects of IFNγ on collagen expression in both in vitro[49 50] and in vivo[51 52] experimental systems. With regard to the molecular mechanisms responsible for the inhibitory effects of IFNγ, several studies have shown crosstalk between the TGFβ and IFNγ signalling pathways (fig 4). The IFNγ-responsive element (IgRE) has been mapped to the −161 to −150 sequence of the *COL1A2* promoter,[53] downstream of the TbRE (fig 4). A Y box binding protein, YB-1, binds to the IgRE and mediates the inhibitory effects of IFNγ on *COL1A2* transcription.[54] Although IFNγ-induced Smad7 expression via Jak1/Stat1 activation was initially proposed,[55] others have reported that Stat1 activated by IFNγ interacts with p300/CBP coactivators and suppresses Smad3/p300-stimulated *COL1A2* transcription.[56] We have shown that YB-1 counter-represses TGFβ-stimulated *COL1A2* transcription by interfering with the Smad3–p300 interaction through its preferential binding to p300.[57] More recently, it has been shown that YB-1 activates *Smad7* gene transcription.[58] Taken together, it is suggested that IFNγ-induced YB-1 inhibits *COL1A2* transcription through several different mechanisms and plays a critical role in counter-repressing TGFβ/Smad-stimulated *COL1A2* transcription (fig 4).

IFNα

IFNα is now widely used for the treatment of chronic hepatitis C. It has been reported that IFNα treatment results in an improvement in the serum levels of fibrotic markers, not only in the patients who responded to the treatment but also in those who did not respond.[59 60] In addition, quantitative histopathological analyses of paired liver biopsy specimens have shown some improvement in the degree of fibrosis after IFNα treatment irrespective of the initial virological response.[61 62] These results may suggest that IFNα has direct antifibrotic effects in addition to its antiviral activity. It has been shown that IFNα, as well as IFNγ, inhibits proliferation and collagen synthesis of cultured HSC,[50] and that they suppress activation of the *COL1A2* promoter induced by repeated CCl_4 injections in vivo.[63] IFNα and IFNγ inhibit *COL1A2* transcription through the same mechanism that interferes with the interaction between phosphorylated Stat1 and p300 (fig 4).[63]

HGF

HGF was originally identified as a potent mitogen for adult rat hepatocytes, but subsequent studies have shown that it exhibits mitogenic, motogenic and morphogenic activities for a variety of cells. HGF administration not only stimulates liver regeneration but also prevents the occurrence of liver fibrosis and accelerates the recovery from the pre-existing fibrosis.[64 65] Moreover, HGF gene treatment has been shown to suppress TGFβ expression, inhibit hepatocyte apoptosis, and produce the complete resolution of fibrosis in rat cirrhotic liver.[66] In addition to its inhibitory effect on TGFβ expression, other possible mechanisms responsible for the antifibrotic actions of HGF are stimulation of collagenase expression[64 67] and its effect on growth inhibition and apoptosis of activated HSC.[68]

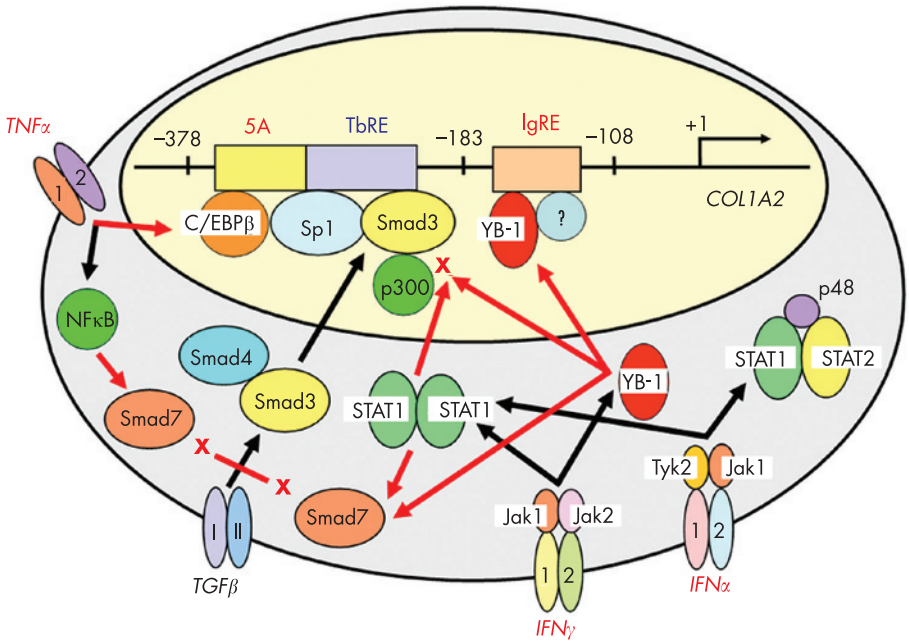

Figure 4 Antagonistic actions of tumour necrosis factor α (TNFα) and interferons (IFNs) on transforming growth factor β (TGFβ)-stimulated ×2(I) collagen gene transcription. Several antagonistic factors including TNFα and IFNs (IFNγ and IFNα) counter-repress TGFβ-stimulated *COL1A2* transcription via crosstalk between TGFβ/Smad and other signalling pathways. IgRE, IFNγ-responsive element; NF-κB, nuclear factor κB; Stat, signal transducer and activator of transcription.

CELL TYPE-SPECIFIC INTERVENTION OF TGFβ/Smad SIGNAL TO TREAT LIVER FIBROSIS

On the basis of the significant roles of TGFβ and Smad proteins in regulating collagen expression, there have been several studies attempting to block the TGFβ/Smad signal and suppress organ fibrosis by using the adenoviral gene transfer techniques. One of them blocked the TGFβ signalling by expressing a dominant-negative TGFβ type II receptor, and prevented liver fibrosis and dysfunction in dimethylnitrosamine-treated rats.[69] It also prevented the progression of pre-existing liver fibrosis and enhanced hepatocyte regeneration.[70] Similarly, a soluble form of TGFβ type II receptor consisting of only the extracellular domain suppressed experimental liver fibrosis induced by common bile duct ligation[71] or dimethylnitrosamine administration.[72] Others injected a recombinant adenovirus carrying inhibitory Smad7 cDNA into rats and succeeded in treating liver fibrosis induced by common bile duct ligation.[73]

All of these adenoviral gene treatments have a great potential in treating liver fibrosis in humans. However, there are several concerns arising before their clinical application. Firstly, it should be seriously considered that blocking of the TGFβ/Smad signal may result in the promotion of carcinogenesis. As already described, TGFβ has an antiproliferative effect on most epithelial types of cells including parenchymal hepatocytes, and Smad4 was originally identified as the product of a tumour suppressor gene. It is especially important in the case of advanced liver fibrosis in humans, as most hepatocellular carcinomas originate from the underlying cirrhotic liver tissue. Owing to its critical roles in immune suppression, generalised blockade of TGFβ activity may also lead to excessive immune reactions and extensive inflammation. In relation to the first problem, the second concern is the limitation in tissue specificity and cell type specificity of gene delivery. All of the above experiments used strong enhancer/promoter elements such as the one derived from cytomegalovirus. These strong expression units exhibit no tissue specificity, which may cause severe adverse effects in the non-target organs. Considering that collagens play critical parts in the maintenance of organ architecture and tissue integrity as well as in various physiological processes, it is more logical to use a tissue-specific enhancer/promoter sequence for the fibrotic tissue-specific regulation of collagen metabolism.

Several promoter sequences have been tested for their ability to mediate cell-specific expression. One of them, the promoter of tissue inhibitor of metalloproteinase 1 gene, which is activated during hepatic fibrogenesis, induced cell death in culture activated HSC by expressing the herpes simplex virus thymidine kinase.[74] We have recently shown in vivo the cell type-specific gene expression by using a recombinant adenovirus carrying the −17 kilobase tissue-specific *COL1A2* enhancer sequence.[75] Under the control of this *COL1A2* enhancer, enhanced green fluorescent protein was expressed only in CCl_4-treated liver but not in untreated normal liver. It was expressed mainly in activated HSC in the fibrotic liver, but not in any other organs including kidney, lung and skin (fig 5). Furthermore, adenovirus-mediated overexpression of YB-1 driven by the *COL1A2* enhancer inhibited *COL1A2* promoter activation after CCl_4 injections, and subsequently suppressed the progression of liver fibrosis.[75] These results validate a new concept of the treatment for liver fibrosis by suppressing excessive collagen deposition in fibrotic tissue without affecting non-target normal organs.

On the other hand, the adenoviral system still has several limitations and problems, despite the cell type-specific gene expression described above. Firstly, adenovirus is immunogenic and rapidly induces neutralising antibodies. Thus, it is not suitable for repeated injections in vivo. Secondly, the virus has dose-related cytotoxicity, which raises safety concerns on clinical application. From this point of view, development of better delivery systems is needed to treat chronic diseases such as organ fibrosis more effectively. Another attempt for cell type-specific fibrosis treatment uses modified human albumin as a selective carrier of antifibrotic reagents. For example, mannose-6-phosphate[76] and a PDGF receptor-recognising macromolecule[77] have been tested for their binding capacity, specifically to activated HSC.

PROBLEMS AND FUTURE PERSPECTIVES

Despite the great advances in understanding the significant roles of TGFβ/Smad signals in the liver, there still remain several important issues to be dealt with. One of them is the differential roles of the three TGFβ isoforms in liver biology and pathobiology. These three isoforms exhibit essentially the same biological activities in vitro, but exert different biological effects in vivo. Most of the findings described above are observed with TGFβ1, and it is not clear whether they are also true for TGFβ2 and TGFβ3. It should be noted that there are two TGFβ type III receptor proteins, betaglycan and endoglin, both of which are expressed in HSC.[78 79] As they bind to the different isoforms of TGFβ with different affinities and modulate the ligand binding to the type I and II receptors, they may play a critical role in modifying the TGFβ/Smad signals in various pathophysiological conditions in the liver.

The second issue is related to the heterogeneity of HSC and other collagen-producing cells in the liver.[80 81] In addition to HSC and myofibroblasts, portal fibroblasts have been implicated in the pathogenesis of biliary fibrosis. It has been shown that portal fibroblasts express a considerable amount of TGFβ2 and, unlike activated HSC, their growth is inhibited by TGFβ1 and TGFβ2, but not stimulated by PDGF treatment.[82] Thus, TGFβ functions may be different depending on its isoforms, cellular context and the causes of liver injury. Another population of collagen-producing cells in the liver is derived from bone marrow, which is described below.

In contrast with the expanding knowledge of molecular mechanisms regulating collagen gene transcription, the role of the TGFβ/Smad signal in the regulation of *MMP* gene expression has been much less understood. Most *MMP* genes possess a TGFβ inhibitory element in their promoter sequences. It has been shown that TGFβ modulates MMP13 expression in HSC by complex mechanisms involving p38 MAPK and other signalling pathways.[83] In addition, reciprocal regulation of collagen and MMP expression by TGFβ is obviously an important but poorly understood area of investigation.[84]

With regard to the treatment of liver fibrosis, we may consider using endogenous factors or cells that can reverse fibrosis, rather than introducing exogenous genes and reagents. One such candidate is bone marrow-derived cells expressing MMPs. It has recently been reported that infusion of bone marrow cells from syngeneic mice into cirrhotic animals

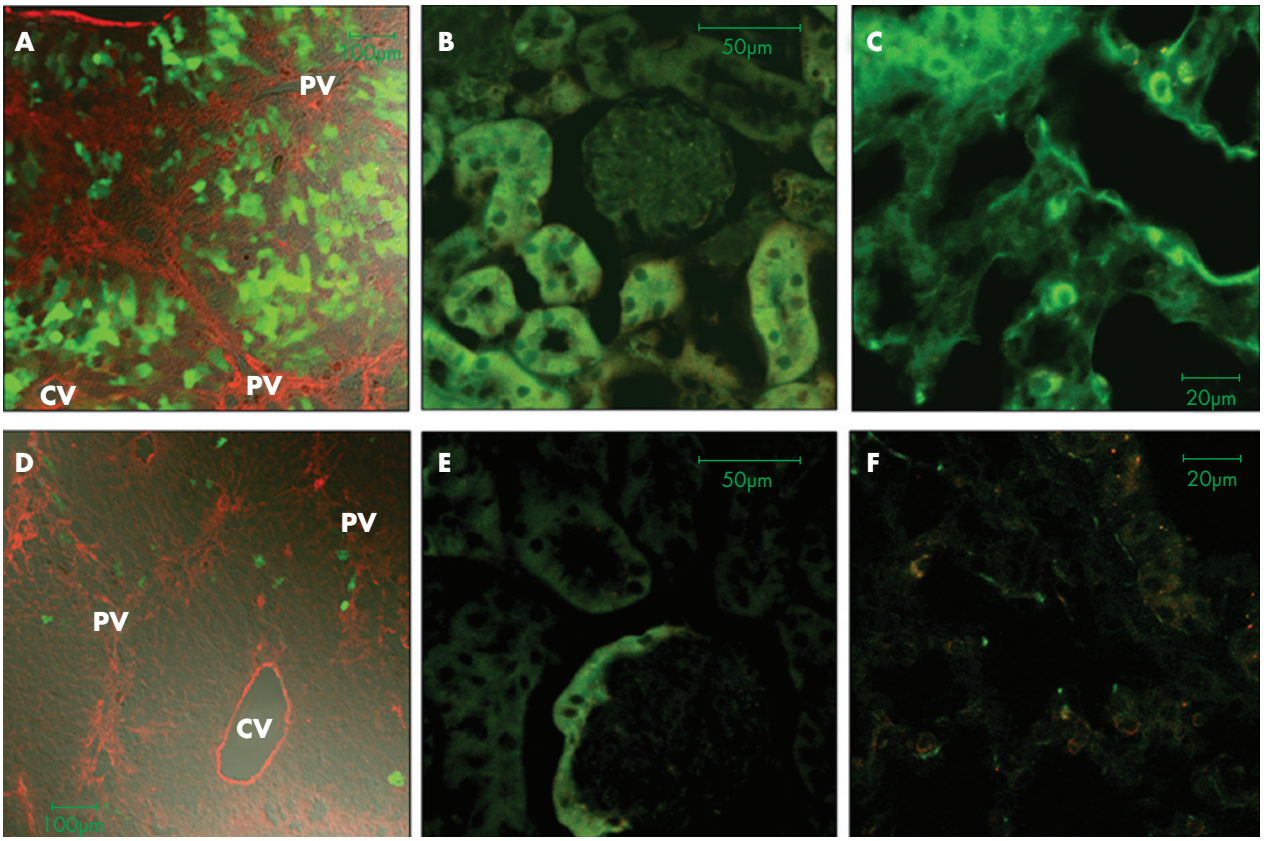

Figure 5 Cell type-specific gene expression in the fibrotic liver tissue by using a tissue-specific ×2(I) collagen gene enhancer sequence.[75] When using the CAG expression unit as a control, enhanced green fluorescent protein (EGFP) fluorescence (green) was expressed in several parenchymal hepatocytes within the regenerating nodules (A) as well as in renal tubules (B) and pulmonary epithelium (C). By contrast, EGFP expression driven by the *COL1A2* enhancer was observed mainly in activated hepatic stellate cells (HSCs) along the fibrotic septa but seldom in parenchymal hepatocytes (D). It was not expressed in kidney (E) or lung tissue (F) either. In panels A and D, expression of α-smooth-muscle actin (red), a marker for activated HSC, was examined by immunofluorescence staining. Note the weak autofluorescence signals observed in the kidney (panels B and E). CV, central vein; PV, portal vein.

resulted in migration of those cells into fibrotic liver.[85] They not only stimulated liver regeneration by differentiating into parenchymal hepatocytes[85] but also suppressed the progression of fibrosis by expressing MMP9.[86] These results indicate that bone marrow transplantation could be a choice of treatment for advanced liver cirrhosis in humans. However, if the therapeutic derivation of autologous bone marrow cells and their differentiation into MMP-expressing cells can be effectively achieved, it will be obviously a safer and less invasive approach for the treatment of liver fibrosis. On the other hand, others have reported that bone marrow-derived cells contribute to the progression of liver fibrosis by expressing type I collagen in human[87] and murine liver.[88] As TGFβ, as well as other cytokines, is also involved in growth control and differentiation of stem/progenitor cells,[89 90] a new concept of the treatment for liver fibrosis may arise from the discipline of stem cell biology by modulating TGFβ and Smad signalling in stem/progenitor cells.

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Emerging insights into Transforming growth factor β Smad signal in hepatic fibrogenesis

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