YB-1 IS THE CRUCIAL MEDIATOR OF ANTIFIBROTIC IFN-γ EFFECTS

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INTRODUCTION

Y-box protein-1 (YB-1) is a known negative regulator of collagen (Col) expression by two different mechanisms, acting directly through binding to an IFN-γ response element within the Col1A2 promoter and/or by physically interacting with p300/Smad3, thereby abrogating the stimulatory effect of TGF-β. Here, we report that YB-1 activation via the Jak1 signaling pathway is required and sufficient to confer IFN-γ-dependent activation of the Smad7 gene. By binding to a bona fide recognition site within the Smad7 promoter, YB-1 upregulates Smad7 transcription, which was additively enhanced by autoinhibitory TGF-β signaling. Importantly, the anti-TGF-β effect was not only supplied by induced Smad7 expression but was recapitulated in the context of the Col1A2 promoter, where YB-1 overexpression abolished the trans-stimulatory TGF-β effect in a dominant manner. In conclusion, YB-1 is the main target of IFN-γ signaling via Jak1 that exerts antifibrotic action by both, interference with TGF-β signaling and direct downregulation of collagen expression.

Y-box proteins belong to a family of DNA and RNA binding factors, also named cold shock proteins, that are highly conserved during evolution and have been shown to function as regulators of gene transcription and translation (1,2). A wide range of nucleic acid structures are reported to be specifically bound by Y-box proteins, most of which harbor an inverted CCAAT-box (ATTGG) as the core binding site. YB-1 was originally identified by an expression cloning strategy using the Y-box of the major histocompatibility complex class II and epidermal growth factor receptor promoters as probes (3,4). It is an ubiquitously expressed early response gene that is induced amongst others following IL-2 stimulation of cloned T helper lymphocytes (5) and after partial hepatectomy (6). YB-1 has been implicated in the regulation of proliferation-associated genes, e.g. thymidine kinase, proliferating cellular nuclear antigen and epidermal growth factor receptor (7) and most recently a direct effect on p53 protein expression and interaction with p53 has been described (8). In regard to ECM synthesis and deposition, YB-1 was identified as a negative regulator in glomerular mesangial cells where it trans-activates transcription of the MMP-2 gene via combinatorial interactions with p53 and AP-2, which may be antagonized by non metastasizing protein 23 (Nm23) (9). In human embryonic kidney cells and dermal fibroblasts, a repressive function of YB-1 on the Col1A2 gene promoter was reported, which is mediated by IFN-γ (10). Screening of a human fibroblast cDNA expression library with a radiolabeled Col1A2 IFN-γ response element (IgRE) probe exclusively yielded clones with a sequence identical to YB-1 (10). Studies with a mutated IgRE and expression vectors containing partially deleted YB-1 cDNAs that were GFP-tagged for detection by immunofluorescence microscopy indicated that IFN-γ facilitates
nuclear translocation of YB-1, which thereafter binds to the Coll1-specific IgRE. Similarly, a functional Y-box element was identified within the proximal Coll1A1 gene promoter (CYE; collagen Y-box element), which is conserved between human, rat and mouse (11). Altogether these results indicate that YB-1 is a key regulator of ECM components involved in scarring processes and that YB-1 itself is activated by an IFN-γ-dependent signaling pathway.

The role of TGF-β as the principal factor inducing collagen expression and leading to tissue fibrosis has been suggested by studies showing increased TGF-β and Coll1 gene expression occurring in parallel (12,13). A direct link between TGF-β expression and tissue fibrosis has been established in different models of experimental fibrosis and human diseases of liver and kidney, and various TGF-β antagonizing strategies, e.g. by overexpression of soluble TGF-β type II receptors or Smad7, significantly reduce collagen accumulation in experimental fibrosis (14,15).

In normal and cirrhotic liver, HSC are the main producers of ECM, including Coll1. More detailed studies with primary cultures of in vitro activated HSC and an activated HSC clone derived from cirrhotic rat liver, CFSC-2G, have delineated a TGF-β-dependent pathway leading to Coll1A2 gene transcription and revealed a cooperation between transcription factors Sp1 and Smad3/4 (16). Similarly, TGF-β responsive sequences have been mapped within the Coll1A1 gene (17). Given the enormous medical relevance of progressive fibrotic processes, several groups have sought to identify signaling events that inhibit Coll1 expression. One successful approach is tumor necrosis factor-α (TNF-α), which antagonizes the effects of TGF-β on collagen expression through induction of inhibitory Smad7 or AP-1 components in dermal fibroblasts (18).

Given the antagonistic action of IFN-γ on TGF-β signaling (19) and the previous finding of YB-1 activation by IFN-γ, a direct link between these two signaling events converging on transcription factor YB-1 was hypothesized in the present study. Specifically, we addressed the question, whether YB-1 confers the IFN-γ inhibitory effect on TGF-β activity by upregulating Smad7 gene expression.

MATERIALS AND METHODS

Cell cultures

The clonal cell line CFSC-2G derives from activated HSC harvested from CCl4 induced cirrhotic rat liver (20). CFSC-2G, U4A and stably transfected U4A-Jak1 (21), HEK293 cells and HepG2 cells were maintained in DMEM supplemented with 10% fetal calf serum, 1% penicillin G/streptomycin sulfate and 1% L-glutamine in a humidified 5% CO2 incubator at 37°C. Rat mesangial cells were isolated and cultured as previously described (22).

Plasmids

pSG5-YB-1 contains the complete human YB-1 open reading frame cloned into expression vector pSG5 (Stratagene, Heidelberg, Germany) as previously described (23). CMV driven expression vectors for Flag-tagged murine Smads2, 3, 4 and 7 in pcDNA3 (Invitrogen, Karlsruhe, Germany) were kindly provided by S. Friedman (Mount Sinai School of Medicine). p3TP-lux contains the S-terminal truncated dominant negative murine Smad2 was kindly donated by Zhang et al. (24). Plasmids encoding dominant negative Smad3 (pcDNA3-Flag-dnSmad3) and Smad4 (pCMV5-Flag-dnSmad4) were kindly provided by T. Imamura (Cancer Inst., Tokyo).

The 1321 bp rat Smad7 promoter region (-1276 to +41, AJ236598) and promoter deletions were constructed as reported (25). pSmad7(-230) was produced via cloning of a blunted Stul-HindIII fragment from pSmad7(-1276) into the Smal site of pGL3-basic (Promega, Mannheim, Germany). pGCol2 containing 2.200 bps of the proximal murine Coll1A1 gene promoter was kindly provided by S. Friedman (Mount Sinai School of Medicine). p3TP-lux contains the -740/-636 region of the human PAI-1 gene promoter including the SBE at -730 bps (26).

pGL2P-S7-Y1 harbors the sequence 5’-GCGCGCCTGGGGCGGGGAGGAAGGAA GGGTGAGGGGAGGAGGAGGAAAGG-3’, that was subcloned into the Kpn I and Bgl II restriction sites of the empty vector pGL2P (Promega). Bloc mutations, that is exchange of 4 subsequent nucleotides each, were performed for the Y-box element, as depicted in Figure 3B, and constructs were denoted pGL2P-S7-Y1mut1 and pGL2P-S7-Y1mut2.
Transgenic transfections

Plasmid DNA for transfections was prepared with Endofree Plasmid Maxi kit (Qiagen, Hilden, Germany). Transient transfections were performed using Fugene6 transfection reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions. 100 ng/ml plasmid DNA per 6 well culture plate was co-introduced with pSV-40-β-gal (Promega) for transfection efficiency normalization (27). Cell lysis and luciferase assays were carried out using a luciferase kit (Promega). Each transfection was performed in quadruplicate and repeated at least three times. All data from transient transfections are presented as mean ± standard deviation.

RNA extraction and Northern blot analysis

Northern blotting was performed with 20 µg total RNA (28). The CollAI probe is a human 1.4 kb EcoRI cDNA fragment (29). A 1.3 kb murine Smad7 fragment was generated as probe from pcDNA3-Flag-Smad7 by digestion with EcoRI/XhoI. A GAPDH-specific probe was isolated from pKS321 as described (30). Smad7 mRNA was detected by use of poly-A+ mRNA (Oligotex mRNA Midi kit, Qiagen). Bands were detected with a Molecular Imager® FX scanner (BioRad, München, Germany) and quantified densitometrically with Lumi Imager (Roche). Signals were measured in Boehringer Light Units (BLU) and related to GAPDH.

Preparation of nuclear extracts

According to previous protocols (31) with minor modifications, 5x10⁵ cells/ml were seeded and subconfluent cells were scratched from petri dishes with 10 ml PBS and a pellet was obtained by centrifugation (Beckman CS-6R, 4 min, 800 rpm). After two PBS washes, cells were resuspended in 1 ml PBS and centrifuged at 14,000 rpm for 45 sec. The cell pellet was resuspended in 400 µl ice cold buffer A (10 mM Hepes [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 10 µl complete protease inhibitor cocktail (Roche), 1 mM DTT) and incubated on ice for 15 minutes. For cell lysis 25 µl of 10 % NP-40 was added and cells were homogenized with 10 strokes in a Dounce homogenizer at 4°C, followed by centrifugation for 1 min at 14,000 rpm for nuclei sedimentation. Supernatants were carefully removed and regarded as cytoplasmic fractions. Extraction of nuclear proteins was achieved by adding 50 µl of buffer C (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 0.1 µl complete protease inhibitor cocktail).

Electrophoretic mobility shift analysis (EMSA)

Double-stranded probes were generated by heating complementary synthetic oligonucleotides for 10 minutes at 95°C with subsequent cooling to room temperature over 6 h. The S7-Y1 sequence was: (−248) 5'-GGCGGCTTTGGGGAGGGGAGGGAAGG GGTAGAGGCGAGGGGAGHGGGAAAGG (−199). All probes were radiolabeled by T4-polynucleotide kinase using [γ-³²P]ATP and purified on 14% polyacrylamide gels, eluted, and 6 x 10⁶ cpm of labeled probe was included per binding reaction. Binding reactions and supershift studies were performed as described (32). Recombinant YB-1 was prepared with the pRSSET vector (Invitrogen) containing an insert coding for hexahistidine/-T7-epitope taggedYB-1 fusion protein (22).

Preparation of cell lysates and immunoblotting

Cell lysates were prepared with RIPA buffer (1x TBS, 1% NP-40 (Amresco, Solon, OH, USA), 0.5 % sodium deoxycholate, 0.1 % SDS) as described (33). Western blotting was performed with affinity purified rabbit anti-YB-1 antibody (1:1.000) (32). Protein concentrations of cell lysates and nuclear extracts were determined with Bio-Rad DC protein assay (BioRad).

Southwestern blot analysis

These experiments were performed using oligonucleotides that correspond to the sense, antisense and double-stranded 48 bp Smad7 promoter sequence denoted S7-Y1. Probes were prepared as described for EMSA. Nuclear extracts (30 µg protein/lane) were electrophoresed on 10 % SDS polyacrylamide gels and transferred onto nitrocellulose membranes by electroblotting. Membranes were blocked in 25 mM HEPES [pH 8.0], 10% glycerol, 50 mM NaCl, 1 mM EDTA, 2.5% dry milk powder for 12 hours at 4°C, washed for 5 minutes in TNE-50 buffer (10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1 mM EDTA, 1 mM DTT) and probed for 4 hours at 30°C in TNE-50 containing 10 µg/ml poly(dI-dC) and radiolabeled oligonucleotide probes (10⁶ cpm/ml). Membranes were washed three times...
in excess TNE-50 buffer for 30 seconds each at 4°C and autoradiographed.

**Tet-off system for induced expression of YB-1 protein**

The Tet-off system (34) was set up by stable double transfection of rat mesangial cells with regulatory, response and selection plasmids, that are pTet-Off, pTRE-HA-YB-1 and pTK-Hyg, in the presence of selection media containing G418 (Calbiochem, Schwabach, Germany) and hygromycin (Invitrogen). Transfections were performed using effectene (Qiagen) according to the manufacturers’ recommendations. Single cell colonies were grown in the presence of 1 µg/ml doxycyclin (ICN, Aurora, OH) in order to keep transcription of expression plasmid turned off. Media supplemented with fresh doxycyclin was replaced every other day. Expression of HA-YB-1 at reduced doxycyclin-concentrations (10², 10⁴, 10⁶ mol/l) was detected by Western blotting using anti-HA tag antibody. All plasmids were kindly donated by Bujard et al. (University Heidelberg, Germany) except for pTRE-HA (BD Bioscience, Heidelberg, Germany). In frame cloning of the complete YB-1 coding sequence into pTRE-HA was ascertained by automated sequencing.

**Knock down of endogenous YB-1 by small interfering RNA**

Embryonic mouse fibroblasts (NIH3T3 cells) were grown to 50% confluency on 10 cm plates in complete medium (RPMI 1640 medium with 10% fetal calf serum, 100 µg/ml of streptomycin and 100 U/ml penicillin). Cells were transfected with the empty vector pSuperDuper (OligoEngine, Seattle WA, USA, kindly donated by B. Lüscher, Institute of Biochemistry, University Hospital Aachen, Germany) or the pSuperDuper vector harboring a 63 bp sequence 5'-GGTCATCGCAACGAAGTTTT-3', which is a tail to tail tandem repeat of bp 285-305 of the human YB-1 coding sequence (J03827). Stable transfections with liposomal preparation Fugene were performed in conjunction with G418 resistance plasmid pUHD15-1neo (BD-Clontech, Heidelberg, Germany) according to the manufacturers’ instructions (Roche, Mannheim, Germany). Briefly, 5 µg total plasmid DNA and 15 µl Fugene solution were mixed in 500 µl serum-free medium, incubated for 15 min at room temperature and added dropwise to culture medium (10 ml/plate). After 24 hours the medium was exchanged and selection with G418 at a concentration of 400µg/ml was started. Screening for the presence of pSuperDuper plasmid DNA in single cell clones was performed and reduction of YB-1 mRNA and protein levels performed by Taqman analysis and immunoblotting using anti-YB-1 antibody.

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**RESULTS**

**YB-1 induces expression of Smad7 in HSC**

Our initial hypothesis of a YB-1-dependent regulation of Smad7 was analyzed by Northern blotting using total RNA collected from activated HSC, CFSC, that were manipulated to increase YB-1 expression or treated by cytokines TGF-β and IFN-γ. Transfection efficiency was quantified at 20 percent by performing cotransfection with a GFP-expression plasmid. YB-1 overexpression in HSC resulted in a 4.5-fold stimulation of Smad7 mRNA expression (Figure 1A, cp lanes 1 and 2, quantitated by densitometry in 1B). TGF-β₁ alone (5ng/ml, lane 3) or ectopic expression of constitutively active Alk5 (lane 4) similarly lead to a 2 to 4.5-fold increase of Smad7 mRNA expression. A 5-fold upregulation of Smad7 mRNA was observed in HSC incubated with IFN-γ at 500 U/ml (lane 6). A “superinduction” of Smad7 expression by 19.5-fold compared to non-treated cells (lane 1) was detected when YB-1 overexpressing cells were exposed to TGF-β₁ (lane 5). These stimulatory effects on Smad7 mRNA expression were confirmed in three independent experiments.

Furthermore, a Tet-off model system was set up, which allows for regulated induction of YB-1 expression in mesangial cells. These cells play a similar role as HSC in regard to ECM synthesis and degradation in the kidney and also exhibit a myofibroblastic phenotype in vitro. Decreasing tetracycline
concentration in medium yielded upregulated YB-1 protein expression (Figure 2A, lanes 1 through 4). With elevated YB-1 expression, Smad7 protein was also upregulated (Figure 2B), at most 50-fold (cp. lanes 1 and 4). The inducing effect of YB-1 was not directly correlated with the amount of expressed protein, since the lower concentration of YB-1 at $10^6$ µg/ml compared to $10^4$ µg/ml tetracyclin was associated with the highest Smad7 protein concentration. As positive control, lysates from HSC overexpressing Smad7 were loaded (lane 5).

Identification of a YB-1 responsive element within the Smad7 promoter region

To test for the YB-1 trans-regulatory effect on the rat Smad7 promoter, transient transfection studies with promoter-luciferase reporter constructs harbouring 1276 bps of the immediate 5' regulatory sequence were performed. YB-1 overexpression lead to a 3-fold induction of reporter gene expression (Figure 3A). By introducing constructs with serial deletions of the promoter sequence, the YB-1 responsive site was mapped at -343 to -190 bps relative to the transcription start site, which is 3' to the Smad binding element (SBE) at -346/-341 bps. In construct pSmad7(-343), the SBE is disrupted. Sequence analysis of the Smad7 promoter revealed a putative YB-1 binding site located at -246/-239 that overlaps with an Sp1 site at -240/-232 (Figure 3B). This sequence element exhibits significant similarities with known YB-1-responsive elements, that is 7 out of 8 consecutive nucleotides match with the YB-1 binding motif of the rat MMP-2 gene (Mertens et al., 1998) and in 6 out of 8 nucleotides with a YB-1 responsive element in the human DNA polymerase-α gene (35). This sequence element at -248/-201 bp, including the Y-box and immediate adjacent 3'-sequence was designated S7-Y1.

Sizing of S7-Y1 binding activities

Southwestern blotting of nuclear proteins was used to size S7-Y1 binding activities (Figure 3C). Double-stranded (DS, lanes 1 and 2) and single-stranded (sense strand, SS1, lanes 3 and 4; antisense strand, SS2, lanes 5 and 6) oligonucleotides were used as probes with HSC nuclear extract. The nucleoprotein binding pattern with DS and SS2 were similar, that is complexes with molecular sizes of approximately 97, 52, 35, 30, 25 and 20 kDa were detected, however the band intensities with SS2 were several-fold more intense than with DS probe. Incubation with the SS1 probe only yielded weak bands at 97 and 20 kDa. Binding specificity was confirmed by inclusion of excess amount of homologous competitor DNA, resulting in diminished bands (lanes 2, 4 and 6 in Figure 3C). Western blotting of the same membrane with anti-YB-1 antibody demonstrates a mobility of YB-1 at 52 kDa (lane 7), which underscores the possibility of YB-1 being an S7-Y1 binding activity.

YB-1 binding to the S7-Y1 element

Next, DNA binding studies were performed with recombinant YB-1 (rYB-1) protein and nuclear extracts from HSC, using oligonucleotide probes that harbour the S7-Y1. As YB-1 may recognize and bind to both single- as well as double-stranded DNA templates and the Southwestern blotting results indicated predominant binding to the double stranded and antisense strand, probes were prepared for these conformations (antisense strand, SS2; double-strand; DS). rYB-1 formed distinct complexes with both, double stranded (DS) as well as antisense strand (SS2) S7-Y1 templates, indicated by >1 in Figure 4A (lanes 2 and 6). Specificity of complex formation was ascertained by inclusion of homologous competitor DNA in 500-fold molar excess (lanes 3 and 7) leading to diminished bands, whereas heterologous competitor DNA had no effect on complex formation (lanes 4 and 8). Given the observation of recombinant YB-1 binding to the S7-Y1 element, we next set out to explore binding of endogenous nuclear proteins prepared from different cellular proveniences to this element. As can be seen in Figure 4B at least 6 distinct complexes could be observed with nuclear proteins from HSC. Inclusion of homologous competitor DNA at increasing concentrations (50- and 500-fold molar excess) resulted in loss of complex formation (lanes 2 and 3). On the other hand, heterologous competitor DNA at 500-fold molar excess only marginally affected complexes >1, >2, >5 and >6, whereas complexes >3 and >4 were diminished and are most likely non-specific. These results suggest formation of multiple distinct nucleoprotein complexes within the S7-Y1 sequence context. To elucidate participation of endogenous YB-1 in the binding to this element, specific anti-YB-1 antibody was included in the binding.
reaction (32). This resulted in supershifts (indicated by “*”, in lane 5) and diminished bands >5 and >6. As the supershift coincided with the mobilities of complexes >1 and >2, it remains enigmatic whether these are also supershifted by the antibody. Addition of a non-related IgG antibody had no effect on the binding pattern (data not shown).

An unchanged nuclear protein binding pattern to the S7-Y1 was detected after treatment with TGF-β1, at 5 ng/ml (lane 6). Furthermore, a similar binding pattern was obtained with nuclear extracts from U4A and U4A-Jak1 cells (lanes 7 and 8).

We also set out to explore the pattern of nucleocomplex formation with the antisense strand S7-Y1 template. As can be seen in Figure 4C three distinct complexes were detected with SS2, that are denoted >1 through >3. All of these bands are abrogated after inclusion of excess homologous competitor DNA (lanes 2 and 3), whereas only complex >1 was diminished with inclusion of heterologous competitor DNA at 500-fold molar excess and most likely represents non-specific protein binding. Again, inclusion of anti-YB-1 antibody confirmed participation of YB-1 in complex formation >2 and >3, as they were diminished and, at the same time, a ladder of supershifted bands appeared (“*”, lane 5). DNA binding studies with nuclear proteins from HSC stimulated with TGF-β1 did not reveal a difference in binding to S7-Y1 (lane 6), as did the analysis of other model systems, U4A and U4A-Jak1 cells (lanes 7 and 8).

Taken together, the DNA binding studies with recombinant as well as endogenous YB-1 protein confirm participation of YB-1 in nucleocomplex formation with the S7-Y1, both in the double- as well as single-stranded conformation, and suggest that YB-1 performs partnering with yet unidentified proteins. From the unchanged binding pattern after cell incubation with TGF-β1 it is concluded that there is no alteration of binding affinities and relative amounts of nuclear S7-Y1 binding proteins after TGF-β1 exposure.

YB-1 and TGF-β induce Smad7 expression independently and additively

TGF-β is a known inducer of Smad7 expression, which by itself functions as down-regulator of TGF-β signal transduction. In transient transfection experiments, the trans-regulatory effects of YB-1 and TGF-β on Smad7-promoter constructs were tested (Figure 5A). YB-1 as well as TGF-β1 stimulated pS7(-1276)-reporter gene expression about 2.5-fold in HSC when compared to constitutive Smad7 promoter activity. This induction is in the range of increased mRNA expression detected by Northern analysis (cp Figure 1A) and supports the notion of a primarily transcription-dependent effect. Incubation of YB-1 overexpressing HSC with TGF-β1 at a concentration of 5 ng/ml led to a 4.8-fold increase of reporter gene activity. This finding indicates an additive effect of YB-1 and TGF-β1, which is in contrast to the synergistic stimulatory effect observed in Northern blots for endogenous Smad7. In similar experiments with a Smad7 promoter-reporter construct that contains a mutated Smad binding site (SBE*), the stimulatory TGF-β1 effect was abrogated, whereas the YB-1 effect was unchanged, indicating independent regulatory pathways.

By using dominant negative (dn) constructs for Smads2, 3 and 4, this independent action was further confirmed (Figure 5B). Here, the stimulatory effect of YB-1 was not affected by co-introduced dnSmads, whereas TGF-β1 inducible reporter gene stimulation [(CAGA)-MLP-Luc] was abrogated (not shown).

Coexpression of pathway restricted Smads in various combinations (Smad2/4, Smad3/4, Smad2/3/4) increased luciferase activity in YB-1 expressing and TGF-β treated cells to a similar extent (Figure 5C-E). YB-1 expression as well as treatment with 5 ng/ml TGF-β1 led to a 2.5 fold increase of Smad7 promoter activity, which was further upregulated to about 3 fold in both samples after ectopic expression of Smads2/3/4. TGF-β1 and YB-1 together enhanced luciferase activity about 4.5 fold, which was also slightly increased by Smad 2/3/4 overexpression. These findings were the same for all combinations of Smads, indicating that the expression of endogenous Smads is sufficient for the effects in HSC and overexpression of Smad2/3/4 does not enhance these significantly. The observed stimulatory effect of Smads on the Smad7 promoter in unstimulated cells is due to ligand independent intrinsic activation of Smad complexes after ectopic expression, which has previously been reported (36). In contrast to recent data.
regarding the Col1A2 promoter, where YB-1 interfered with Smad3/CBP/p300 protein interactions (10) resulting in reduced Col1A2 gene transcription, TGF-β/Smad2/3/4 mediated stimulation of the Smad7 promoter in CFSC (25) was not inhibited by YB-1. These results further confirm a Smad-independent action of YB-1 on the Smad7 promoter.

To definitely confirm that the sequence element S7-Y1 confers YB-1 responsiveness to the Smad7 promoter, this sequence element was tested in isolation in the context of a heterologous SV40 promoter construct, denoted pGL2P-S7-Y1. As depicted in Figure 5F, forced YB-1 overexpression resulted in a similar 2-fold induction of reporter gene activity. Notably, IFN-γ was able to induce reporter gene transcription to a similar extent, whereas TGF-β increased reporter gene activity by 5-fold. These observations underscore the relevance of the S7-Y1 element for the YB-1-dependent Smad7 gene regulation. In an attempt to further define the sequence requirements for gene regulation, mutations were introduced in this element (depicted in Figure 3B, pGL2P-S7-Y1mut1 and mut2). With both constructs, that have 4 nucleotide exchanges within the Y-box, the YB-1-responsiveness was completely abrogated (data not shown). However, at the same time basal transcription rates were 100-fold higher than with the wild-type sequence, indicating that novel binding sites were created or the Y-box also confers silencer activities over adjacent binding sites.

**YB-1-dependent Smad7 expression is present in different cell types**

To clarify whether the YB-1-dependent Smad7 regulation is a cell-type specific effect, cells from different origins were tested. In addition to HSC, human hepatocellular carcinoma cells (HepG2; Figure 6A) and human fibrosarcoma cells U4A/Jak1 (compare Figure 7B) exhibited the described additive response to YB-1 overexpression and TGF-β incubation. The presence of YB-1-dependent regulation of the Smad7 gene in a variety of cell types implicates a general mechanism. In contrast, Smad7 promoter activity in HEK293 human embryonic kidney cells was not regulated by TGF-β, whereas the YB-1 stimulatory effect was present (Figure 6B). Additionally, the data with HEK293 cells confirm independency of YB-1 and TGF-β effects in regard to Smad7 regulation.

**JAK1-deficiency leads to abrogated YB-1 regulation of the Smad7 promoter**

As our initial hypothesis of a YB-1-dependent gene regulation of the Smad7 promoter was derived from its IFN-γ responsiveness, and IFN-γ-signal transduction is mediated by janus kinase 1 (Jak1) and signal transducers and activators of transcription factor 1 (STAT1), the Jak1-deficient U4A cell line was chosen as model system and tested for Smad7 promoter regulation. As can be seen in Figure 7A, loss of Jak1 in U4A cells abrogates YB-1-dependent regulation of the Smad7 promoter. This result indicates participation of IFN-γ-and Jak1 signaling in the observed YB-1 effect. To further explore this assumption, U4A/Jak1 cells were tested similarly by overexpressing YB-1 and incubation with TGF-β. Here, a similar result as with other cell systems was observed, that is an independent and additive stimulation of Smad7 reporter construct activity (Figure 7B).

To test the importance of YB-1 for the stimulatory effect of IFN-γ on Smad7 expression, U4A and U4A/Jak1 cells were pretreated as previously described by overexpressing YB-1 and introducing the reporter construct pSmad7(-1276) (Figure 7C). With U4A cells, YB-1 overexpression only marginally stimulated the Smad7 promoter and IFN-γ-treatment did not change reporter gene activity. In the U4A/Jak1 cell system, there was an approximately 2.8 fold YB-1-dependent induction of Smad7 promoter activity. Furthermore, in these cells the Smad7 promoter was IFN-γ responsive. Taken together, the results obtained with the Jak1 deficient model system provide evidence for a link between IFN-γ/Jak1 and YB-1.

Compared to control cells the stimulatory effect of IFN-γ was increased in YB-1 overexpressing cells. Although this increase was only 20% of the total stimulatory effect, it was significant and confirmed in three independent experiments. These results suggest that the stimulatory effect of IFN-γ on the Smad7 promoter (19), as well as the repressive effect on the Col1A2 promoter (10) is mediated by transcription factor YB-1.

**RNAi dependent knock down of YB-1 expression abrogates IFN-γ effects on the Smad7 promoter**

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To conversely examine the effect of depleted YB-1 expression on the IFN-γ-dependent stimulatory effect on Smad7 expression, a model system was set up, where small interfering RNA specifically designed for YB-1 knock down was stably overexpressed in NIH3T3 fibroblasts. In this cell system IFN-γ induces pSmad7(-1276) reporter construct activity about 2-fold (Figure 7D, left panel). In NIH3T3 cells, where the endogenous YB-1 levels were decreased by 90 percent, as determined by Western blotting (Figure 7D, upper part), the IFN-γ stimulatory effect on pSmad7(-1276) reporter activity was completely abrogated (Figure 7D, right panel).

**YB-1 regulation of TGF-β-dependent promoters**

Previous reports describe a suppressive effect of YB-1 both on Col1A1 as well as Col1A2 gene transcription. For TGF-β, a stimulatory effect on Col1 gene transcription is known. In the following, it was tested how these antagonistic effects interfere when both gene regulation pathways are activated. As can be seen in Figure 8A, the Col1A1 promoter construct harbouring 2200 bps of the regulatory sequence was down-regulated by more than 50% when YB-1 was overexpressed. Contrary to this, TGF-β incubation led to a 2.5-fold stimulation of reporter gene transcription. However, in YB-1 overexpressing cells this stimulatory effect by TGF-β was nearly completely lost, indicating that YB-1 is dominant over the stimulatory effect of TGF-β. Given the aforementioned findings with upregulated Smad7 expression in YB-1 overexpressing cells, it is therefore possible that YB-1 prevents TGF-β from signaling in these cells. This was confirmed in a Northern blot analysis of activated HSC, that were transfected with YB-1 or Smad7 expression constructs. Overexpression of Smad7 itself reduced collagen expression to 40% compared to MOCK transfected cells. YB-1 was also able to significantly repress Col1A1 mRNA in HSC to 60%, indicating a fibrogenesis antagonizing effect of YB-1 (Figure 8B).

To further test regulation of collagen gene transcription after stimulation with IFN-γ the U4A / U4A/Jak1 model system was utilized. In these cells the reporter construct for Col1 was introduced in the presence or absence of YB-1 expression plasmid and cells were stimulated with IFN-γ. As can be seen in Figure 8C, there was a minimal suppressive effect of IFN-γ on Col1A1 reporter gene activity in U4A/Jak1 cells that amounted to approximately 20% of total promoter activity. YB-1 overexpression led to a dramatic 70% reduction in reporter gene activity, which was slightly increased by incubation with IFN-γ. As positive control for IFN-γ-dependent gene transcription, a construct harbouring an interferon response element (IRE) was also introduced into U4A/Jak1 cells. Here, a 2-fold stimulatory effect after incubation with IFN-γ was detected. Contrary to these findings with U4A/Jak1 cells, there was no response of Col1 gene transcription in U4A cells, neither to IFN-γ incubation nor to YB-1 overexpression. These results furthermore underscore the importance of the Jak signaling cascade for the YB-1 action on Col1 gene transcription.

**DISCUSSION**

**IFN-γ and TGF-β have opposing effects on ECM deposition**

The orchestration of ECM synthesis and degradation is critical for injured or intoxicated tissues to finally achieve wound healing and tissue repair instead of fibrosis and loss of function. Cytokines of the TGF-β family influence a wide spectrum of cellular processes including differentiation, proliferation, apoptosis and migration (37). In most tissues TGF-β has profibrogenic activity by inducing amongst others collagen and TIMP-1 gene expression and strategies targeting inhibition of ECM-production, especially by abrogating TGF-β signaling, provide significant antifibrotic potential.

Earlier work by Kahari et al. (38) has outlined opposing effects of IFN-γ on TGF-β-dependent type I procollagen expression, both at the transcriptional and posttranscriptional level. A similar antagonizing effect of both cytokines was demonstrated for the regulation of adhesion molecule expression (39). However, it is not completely understood how the two cytokine signaling cascades interfere. Ulloa et al. reported on a direct link between IFN-γ and Smad7, as in U4A cells IFN-γ induces Jak1 mediated STAT-1 phosphorylation, transcriptional induction of Smad7 and subsequent inhibition of TGF-β signal transduction effects (19). Clinical data
suggest that IFN-\(\gamma\) induces the expression of Smad7, which then prevents endogenous TGF-\(\beta\) from downregulating the ongoing tissue damaging Th1 response (40). Our results highlight the role of transcription factor YB-1 as effector of IFN-\(\gamma\)-signaling and provide evidence for YB-1 \(\text{trans}\)-regulating Smad7 expression. The importance of the IFN-\(\gamma\) signaling cascade for YB-1 activation was substantiated in the model system U4A / U4A/Jak1, where YB-1 was only effective to induce Smad7 transcription in the presence of the Jak1 signaling cascade. Furthermore, in a knock down system for YB-1, IFN-\(\gamma\) signaling targeting the Smad7 promoter was completely abrogated. Until now it is elusive how Jak1 activates YB-1. From studies outlined by Fukada et al. (41) with rat fibroblast cells, gp130 mediated signaling and Jak1 activation depends on YB-1 protein levels, that is Jak1 phosphorylation after stimulation with granulocyte colony stimulating factor is threefold increased with reduced YB-1 levels. It is conceivable that by controlling Jak1 phosphorylation levels via the prototypic protein tyrosine phosphatase PTP1B, YB-1 may protect itself from overshooting activation.

**Mapping of a YB-1 responsive element within the Smad7 promoter**

Signaling pathways that rapidly and transiently induce Smad7 expression include EGF, TNF-\(\alpha\), IFN-\(\gamma\) (19,42,43) and TGF-\(\beta\) itself (25), all acting within a region of the Smad7 promoter sequence located at position -417 to -275. This sequence is evolutionarily conserved and identical in man, rat and mouse. Functional transcription factor binding sites of the proximal promoter include the SBE, an E-box, AP-1 and Sp1 site and, as shown in this manuscript, a functional Y-box element. The prediction of functional Y-box motifs, e.g. by \textit{in silico} analysis, has to take into consideration complex binding requirements for YB-1 that may include inverted repeat sequences in conjunction with an inverted CCAAT-box (11,22).

On the protein level, an impressive 50-fold induction of Smad7 expression was found with YB-1 overexpressing cell clones. At the transcription level, a 4-fold stimulation of Smad7 mRNA in YB-1 overexpressing cells was observed, which was synergistically increased to 20-fold after coincubation of these cells with TGF-\(\beta\). However, one has to take into consideration that transfection efficiency in this system was about 30%. These findings clearly demonstrate a transcriptional regulation of the Smad7 gene by YB-1. A YB-1 responsive element within the Smad7 proximal promoter between -343/-190 bps relative to the transcription start site was mapped by reporter gene constructs. Both, YB-1 and TGF-\(\beta\) independently increased Smad7 promoter activity about 3-fold each with additive effects when both components acted at the same time. Co-transfection experiments with dnSmads 2/3/4 and usage of Smad7 promoter constructs bearing a mutated SBE further confirm the independency of TGF-\(\beta\) and YB-1 in gene regulation. The discrepancy of an additive effect with artificial promoter constructs and a synergistic effect on endogenous Smad7 mRNA synthesis may have different reasons, either additional, upstream located regulatory elements or posttranscriptional regulatory events. Posttranscriptional effects have been described for granulocyte-macrophage colony-stimulating factor, ferritin and interleukin-2 genes as YB-1 may specifically bind to the UTR of mRNA and markedly prolong half lives (44-46).

Also noteworthy is the observed course of Smad7 protein expression with increased YB-1 concentrations in the inducible TET-off model system. There is no direct correlation between YB-1 and Smad7 expression and at a submaximal YB-1 concentration the Smad7 protein expression peaked.

**TGF-\(\beta\) and YB-1 target genes: modular regulation**

The general concept of modular gene regulation (47), that is the contiguous alignment of DNA binding motifs involved in the transcriptional control of genes, may be exploited by \textit{in silico} sequence analysis. Our findings of an additive transcriptional effect of YB-1 and TGF-\(\beta\) signaling on the Smad7 promoter is (i) in accord with the modular concept of independent regulatory events that exist in parallel. (ii) This co-regulation is cell-type specific as similar results were obtained with HSC, MC, U4a-Jak1 and HepG2 cells, whereas HEK293 cells in fact displayed the above mentioned YB-1 effect but were refractory for TGF-\(\beta\) dependent Smad7 promoter activation. (iii) In the quest to identify a similar coregulation in other TGF-
responsive genes we tested for the YB-1-responsiveness of the PAI-1 gene. Here, a yet unreported YB-1 trans-regulation and similar modular composition was found and it is expected that other TGF-β responsive genes adhere to this concept. The independence of the responsive DNA binding sites is (iv) exemplified by the collagen I gene. Here, opposing effects were detected after YB-1 overexpression and TGF-β incubation, rendering a model of antagonistic action. How can these results be reconciled? According to current knowledge, YB-1 may act as trans-activator or -repressor of gene transcription, even of the same gene in different cell-types, as it has been demonstrated for gelatinase A (Mertens et al., 1997) and its function is critically determined by co-factors and interacting proteins, e.g. AP-2, multivalent zinc finger factor CTCF, DNA-binding protein A (DbpA), p53, p300/CBP, proliferating cellular nuclear antigen, Pur alpha, Smad3, Sp1 and YY1 (1).

The interaction of YB-1 with Sp1 may be of great importance in this regard, as demonstrated for several genes. There is a coordinate regulation of the human multidrug resistance (MDR) gene promoter by Y-box-binding protein family members and Sp1, with recognition by both required for optimal promoter activity (48). The human immunodeficiency virus type I promoter is similarly regulated by the combined binding of YB-1 and Sp1 (49). In the PTP1B promoter two regulatory elements were identified, an enhancer that is recognized and bound by YB-1 and the PRS motif that is a recognition site for Sp family transcription factors. The presence of both elements is required for maximum promoter activity (41). The immediate neighbourhood/overlapping of S7-Y1 and a Smad7 specific Sp1 site suggests a similar situation for Smad7 promoter regulation. Whether there is such an interaction of YB-1 and Sp1 required for YB-1 dependent Smad7 activation is the focus of ongoing studies.

Role of YB-1 in collagen gene regulation

Recently, we and others have shown that YB-1 is able to repress Col1A1 and Col1A2 gene transcription in various cell types (10,11). Additionally, it has been reported that the direct effect of YB-1 on Col1A2 promoter regulation is IFN-γ-dependent in primary dermal fibroblasts and human embryonic kidney cells (10), that is IFN-γ promotes nuclear YB-1 translocation, IgRE binding and inhibition of Col1A2 promoter activity. The molecular mechanism of IFN-γ-dependent signal transducers interacting with YB-1 has not been characterized. The same group recently described another YB-1/IFN-γ-dependent pathway counteracting Col1A2 expression that involves inhibition of positive regulatory TGF-β effects (10). IFN-γ-dependent nuclear translocation of YB-1 results in physical interaction of YB-1 with both Smad3 and p300, thereby preventing a functional Smad3/p300 complex at the Col1A2 gene promoter and leading to inhibition of Col1A2 gene transcription. The present report adds a third mode of action by which YB-1 may reduce collagen expression (see model, Figure 9). An “indirect” pathway was detected that includes YB-1-dependent activation of the Smad7 gene by binding to a defined Y-box element, denoted S7-Y1. Increased Smad7 activity subsequently results in reduced collagen gene expression by abrogating TGF-β signaling. Compared to the Col1A2 promoter, where YB-1 directly interacts with Smad3 and p300, its biochemical behaviour is different in the context of the Smad7 promoter. TGF-β-mediated binding of activated Smad complexes, including Smad3, with subsequent promoter activation is not abrogated but stimulated additively by activated YB-1 (depicted in model, Figure 9). Furthermore, overexpression of dnSmads2/3/4 or Smad2/3/4 did not influence YB-1 mediated Smad7 promoter activation.

Subcellular YB-1 shuttling in stimulated cells

It is reported that cytokines IFN-γ and TGF-β influence the subcellular YB-1 localization, that is lead to a nuclear shuttling. In our hands endogenous YB-1 was detected by immunohistochemistry in the cytoplasm in cultured HSC and mesangial cells. TGF-β had no effect on the subcellular YB-1 localization (not shown) at concentrations ranging from 1 to 5 ng/ml. Higashi et al. reported on a nuclear translocation of YB-1 after IFN-γ stimulation (10). It is noteworthy that the precise mechanisms leading to subcellular YB-1 shuttling are incompletely understood. YB-1 has been reported to be activated by proteolytic cleavage to result into a 234 aa long fragment after stimulation of endothelial cells with thrombin (50) with subsequent nuclear
localization. On the other hand point mutations within the RNA binding motif RNP1 also resulted in nuclear localization. Apart from this, a concentration-dependent nuclear shuttling of YB-1 by wt p53 and splicing factor SRp30c has been described (51,52).

**YB-1 has antifibrotic activity**

Our findings may have pathobiocemical consequences for fibrogenesis. TGF-β is produced in increasing amounts during transdifferentiation of HSC. HSC cultivated in vitro are initially strongly responsive to exogenous TGF-β and display Smad2/3 phosphorylation, TGF-β reporter gene activation, as well as induction of Smad7 and collagen expression. In contrast, completely transdifferentiated HSC are devoid of a stimulatory effect of TGF-β on collagen and Smad7 expression (33). Further, Takashi et al. (53) have shown that, during the acute phase of liver injury after a single CCl₄ administration, Smad7 expression is rapidly elevated in the liver in response to TGF-β and can inhibit fibrotic signals mediated by receptor Smads (R-Smads), representing a tightly controlled TGF-β effect during wound healing. In chronic liver injury, endogenous TGF-β cannot induce antagonistic Smad7 in transdifferentiated myofibroblasts, further indicating that the TGF-β signaling pathway targeting Smad7 is shut off in HSC during fibrogenesis, thereby providing an important impact on disease progression. In regard to this, it is of particular interest to define alternative signaling pathways besides TGF-β, which stimulate Smad7 expression in HSC and thereby have the potential to inhibit profibrogenic effects of TGF-β. The results presented indicate that YB-1 is a potent inducer of Smad7 expression in activated HSC and myofibroblastic mesangial cells, which could be used to antagonize TGF-β in chronic stages of the fibroproliferative diseases in liver, kidney and other tissues. Experimental studies have shown that IFN-γ is a potent inhibitor of collagen synthesis in tissue fibrosis by interfering with profibrogenic TGF-β effects (54). As IFN-γ lacks major toxic effects in experimental models and in humans, it is regarded a valuable compound for the therapy of fibroproliferative diseases. Similarly, ectopic expression of Smad7 has been used to inhibit tissue fibrosis in animal models (14,55). The presented data and recent findings by Higashi et al. indicate that the beneficial effects of IFN-γ on fibroproliferative disorders are mediated by YB-1, which amongst others counteracts TGF-β signaling. Finally, Inagaki and coworkers reported that adenoviral overexpression of YB-1 under the control of the Col1A2 promoter blunted liver fibrosis in mice treated with CCl₄ (56).

**FIGURE LEGENDS**

Fig. 1 A-B. YB-1 and TGF-β superinduce Smad7 expression in activated HSC. A. Northern blot analysis of Smad7 expression in CFSC-2G transfected for 24 hours with either pSG5-YB-1 (lanes 2 and 5), constitutively active Alk5 (pcDNA-CA-TBRI, lane 4), and/or treated with 1 ng/ml TGF-β (lanes 3 and 5) or IFN-γ (500 units/ml, lane 6) for 2 hours or left untreated, as indicated. The same blot was probed with a GAPDH-specific probe for normalization of loaded RNA. B. Quantification of band intensities was performed and calculated with control transfected cells set as 1.

Fig. 2 A-B. YB-1 overexpression leads to concentration-dependent upregulation of Smad7 expression. Western blot detection of YB-1 and Smad7 expression in mesangial cells was performed in a cell system with tetracycline-dependent conditional induction of YB-1 synthesis (Tet-off system, see Materials and Methods). A. Reduction of tetracycline concentration in the medium resulted in the induction of HA-YB-1 expression that was detected by anti-haemagglutinin antibody. B. The same cell extracts were analyzed for Smad7 protein expression. Lysate from CFSC-2G that overexpress Smad7 was used as positive control (S7+). Quantification of relative protein amounts was performed and illustrated in the lower graphs. Results were confirmed in two independent experiments.

Fig. 3 A-C. YB-1 induces the Smad7 promoter via a novel Y-box located between -343 and -190. HSC were transiently co-transfected with a proximal promoter construct, pSmad7(-1276) or various deletion variants and a YB-1 expression construct (pSG5-YB1) as indicated. Basal and stimulation-dependent promoter activity was related to pGL3basic. 5 ng/ml TGF-β was used for treatment of HSC.
A. Left part: schematic representation of the different reporter constructs used to locate the functional binding site. The numbers in brackets define nucleotide positions relative to the transcription start site, each construct ending at 3'-position +41; SBE, Smad binding element. Right part: basal (white bar) and YB-1-dependent induction of luciferase activity. B. Sequence comparison between S7-Y1, the YB-1 responsive motif within the Smad7 promoter, and known YB-1 regulated elements within the matrix metalloproteinase-2 (MMP-2) and DNA polymerase α promoters. Furthermore, sequences for the constructs pGL2P-S7-Y1mut1 and mut2 are depicted, that both harbour 4 nucleotide exchanges within the Y-box. C. Southwestern blot analysis was performed with lysates from HSC and radiolabeled S7-Y1 double-strand (DS), sense (SS1) and antisense strand (SS2) oligonucleotides as probes. Binding activities were detected with DS and SS2 probes (lanes 1 and 5). Incubation with the SS1 probe yielded only weak bands (lane 3). Binding specificity was confirmed by inclusion of excess amount of homologous competitor DNA (lanes 2, 4 and 6). Western blotting of the same membrane with anti-YB-1 antibody (lane 7).

Fig. 4 A-C. YB-1 participates in nucleocomplex formation with the S7-Y1 element. A. DNA binding studies were performed with recombinant YB-1 (rYB-1) protein and S7-Y1 probe in different conformations. B. Endogenous nuclear proteins prepared from different cellular proveniences were tested for binding to the S7-Y1 double stranded (DS) probe. C. Endogenous nuclear proteins prepared from different cellular proveniences were tested for binding to the S7-Y1 antisense strand (SS2) probe.

Fig. 5 A-F. YB-1 induces the Smad7 promoter independent of TGF-β signaling. A. Reporter plasmid pSmad7(-1276) and a variant construct with mutated SBE, pSmad7(-1276)-SBE*, were used to investigate YB-1- and/or TGF-β1-dependent gene activation. B-E. Various combinations of Smads and dominant negative Smads were coexpressed with reporter gene pSmad7(-1276) to explore the interrelationship between TGF-β- and YB-1-dependent Smad7 promoter activation. F. The sequence element denoted S7-Y1 was tested in isolation in conjunction with a heterologous SV40 promoter. Upon forced overexpression of YB-1, reporter gene transcription was stimulated 2-fold. A similar stimulatory effect was observed after incubation with IFN-γ, whereas TGF-β stimulated transcription via this element 5-fold.

Fig. 6 A-B. YB-1-dependent Smad7 induction is found in various cell types

A-B. Human hepatocellular carcinoma cells (HepG2) (A) and human embryonic kidney cells (HEK293) (B) were transiently co-transfected with promoter reporter construct pSmad7(-1276) and YB-1 expression plasmid and/or incubated with TGF-β.
**Fig. 7** A-B. YB-1 and Jak1 are necessary for optimal IFN-γ-dependent upregulation of the Smad7 promoter. A-B. Human fibrosarcoma cells U4A (A) and U4A/Jak1 (B) were transiently transfected with promoter reporter construct pSmad7(-1276) and YB-1 expression plasmid in the absence or presence of TGF-β. C. U4A and U4A/Jak1 cells were tested for pSmad7(-1276) reporter plasmid activation by overexpressed YB-1 in the absence or presence of IFN-γ. D. NIH3T3 cells harbouring an RNAi-based knock down for YB-1 (NIH-3T3(YB-1 siRNA) and control cells (NIH3T3) were transiently transfected with pSmad7(-1276). Efficacy of knock down was assessed by Western blotting for YB-1 (inserted Figure) which resulted in a 90% reduction of endogenous YB-1 levels. In control cells, IFN-γ incubation led to a 2-fold increase of reporter gene activity, whereas this IFN-γ-responsiveness was completely lost in NIH-3T3(YB-1 siRNA) cells.

**Fig. 8** A-D. YB-1 counteracts TGF-β-dependent regulation of the Col1 gene. A. HSC were transiently transfected with Col1A1 promoter reporter construct and the effect of YB-1 expression and/or TGF-β on reporter gene activity was measured. B. Northern blot analysis of Col1A1 mRNA expression in HSC, transfected with either pcDNA3.1 (MOCK control, C), pSG5-YB-1 or pcDNA Smad7 for 24 hours. One representative of three independent blots is shown. Band intensities from three experiments were equalized to GAPDH expression by densitometry and are presented as means (± SD). C. U4A / U4A/Jak1 cells were transiently transfected with Col1A1 promoter reporter constructs and the effect of YB-1 expression and/or IFN-γ on reporter gene activity was measured. IRE indicates a luciferase construct bearing an artificial IFN-γ response element and served as positive control.

**Fig. 9** A and B. Model of IFN-γ and TGF-β signaling converging on YB-1 and subsequent target gene regulation. IFN-γ signaling results in Jak1-dependent activation and nuclear translocation of YB-1. Elevated YB-1 levels stimulate prototypic protein tyrosine phosphatase (PTP1B) expression and thereby negatively regulates gp130 receptor mediated Jak1 phosphorylation (Fukada et al., EMBO J.), which constitutes an autoinhibitory loop. Antifibrogenic effects of YB-1 are threefold and include: (i) a direct suppressive effect on collagen type I (Col1A2) expression by binding to an interferone response element (IgRE), (ii) interference with Smad3/p300 interactions at the TGF-β response element (TbRE) within the Col1A2 promoter and (iii) inhibition of TGF-β signal transduction mediated at the receptor level by enhancing de novo expression of antagonistic Smad7, which is conferred through direct binding to a bona fide recognition motif within the Smad7 promoter (S7-Y1).
REFERENCES

Figure 3

A

HSC

- YB-1
+ YB-1

Relative luciferase activity

pGL3 basic

pSmad7(-1276)

pSmad7(-625)

pSmad7(-469)

pSmad7(-343)

pSmad7(-190)

B

rat Smad7 Y-box element (S7-Y1)

rat MMP-2 response element-1 (RE-1)

human DNA polymerase-a response element-1

Y-box

rat Smad7 Y-box element (S7-Y1mut1)

rat Smad7 Y-box element (S7-Y1mut2)
Figure 3

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DS | SS1 | SS2

Figure 4

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free probe

S7-Y1  rYB-1  comp. (500x)

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### Figure 4

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Figure 5 E

- Smad2/3/4
+ Smad2/3/4

HSC

Relative luciferase activity

pGL3  pSmad7(-1276)

Figure 5 F

Relative luciferase activity

pGL2P  pGL2P-S7-Y1
Figure 7

C

Relative luciferase activity

- IFN-γ
+ IFN-γ

pGL3  pSmad7(-1276)
---  ---
U4A

YB-1

pGL3  pSmad7(-1276)
---  ---
U4A-Jak1

Figure 7

D

Relative luciferase activity

- IFN-γ
+ IFN-γ

pGL3  pSmad7(-1276)
---  ---
NIH-3T3 (control)

YB-1
CON siRNA

pGL3  pSmad7(-1276)
---  ---
NIH-3T3 (YB-1 siRNA)
Figure 8

A

Relative luciferase activity

HSC

pGL3

pGL3-Col1A1

YB-1

TGF-β

YB-1

TGF-β

Figure 8

B

Col 1A1

GAPDH

CON

YB-1

Smad7

fold Col1A1 mRNA expression

HSC

CON

YB-1

Smad7
YB-1 is the crucial mediator of antifibrotic IFN-γ effects
Steven Dooley, Harun M. Said, Axel M. Gressner, Juergen Floege, Abdelaziz En-Nia
and Peter R. Mertens

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