CMLS, Cell. Mol. Life Sci. 60 (2003) 2516–2525
1420-682X/03/112516-10
DOI 10.1007/s00018-003-3299-x

CMLS Cellular and Molecular Life Sciences

Research Article

Suppression of TGF-β signaling by conophylline via upregulation of c-Jun expression

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Received 25 July 2003; received after revision 25 August 2003; accepted 1 September 2003

Abstract. In the course of screening for inhibitors of transforming-growth factor-β (TGF-β) functions we found that conophylline, a vinca alkaloid, inhibited TGF-β-induced apoptosis in rat hepatoma cells. Because conophylline also inhibited TGF-β-induced promoter activity in mink lung cells, we studied the mechanism of the inhibition in this cell line. Conophylline did not inhibit nuclear translocation of Smad2. Instead, we found that conophylline increased the expression of c-Jun, which had been earlier shown to interact with the corepressor TGIF to suppress the transcriptional activity dependent on Smad2. Conophylline attenuated the interaction between the Smad2 complex and p300 but enhanced that between the Smad2 complex and TGIF. In cells overexpressing c-Jun, suppression of promoter activity induced by TGF-β and the enhancement of the association of the Smad2 complex with TGIF were also observed. Thus, our data suggest that inhibition of TGF-β-induced promoter activity by conophylline can be attributed to its potency in modulating the interaction of downstream transcriptional factors via upregulation of c-Jun expression.

Key words. TGF-β; c-Jun; TGIF; Smad; conophylline; p300; FAST; Mv1Lu.

Transforming growth factor-β TGF-β has been shown to regulate broad aspects of cellular functions including cell proliferation, differentiation, and apoptosis [1, 2]. TGF-β promotes the accumulation of extracellular matrix and inhibits matrix degradation [3, 4]. Induction of fibrosis by TGF-β in lung, kidney [5], and liver [6] has been widely studied, and deregulation of TGF-β function has been implicated in the generation of human fibrotic disorders [3, 4]. Overexpression of TGF-β is observed in scirrhous carcinoma of the stomach [7] characterized by pronounced proliferation of the interstitium, which is associated with excess collagen deposition in the tissue [8]. TGF-β overexpression may correlate with decreased survival in this disease [9]. TGF-β also induces apoptosis in liver both in vitro and in vivo [10]. Transgenic mice overexpressing TGF-β showed continuing apoptotic death of hepatocytes, as well as hepatic fibrosis [11]. Inhibitors of TGF-β functions are therefore expected to be antifibrotic and cytoprotective agents [12].

TGF-β binds to cell surface transmembrane serine/threonine kinases, known as type I and type II receptors [13–15] and induces receptor-mediated phosphorylation of Smad proteins, which are central mediators of TGF-β signals. Once phosphorylated, Smad proteins become oligomerized, and the oligomers are translocated into the nucleus, and bind to target promoters in association with DNA-binding cofactors (FAST1 or FAST2) [16]. In the nucleus, Fast-bound Smads positively or negatively regu-

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late the transcription of target genes, depending on the physiological context [17]. Transcriptional activation by its Smad complex has been shown to occur by its ability to recruit p300 and CBP [18–20], which act as coactivators by modifying the chromatin structure through histone acetylation. On the other hand, Smad2 or Smad3 can also interact with the corepressor TGIF instead of the coactivators p300/CBP in response to TGF-β, thereby forming a transcriptional repressor complex [17]. The repression of Smad-dependent transcription by the corepressor TGIF correlates with the recruitment of a histone deacetylase into the Smad complex. A similar mechanism has also been advocated to explain the action of the corepressor Ski, which interacts with Smads and represses TGF-β-induced transcription [21, 22].

Induction of transcription by TGF-β was decreased in cells overexpressing c-Jun, which directly binds to TGIF and enhances the association of Smad with TGIF. c-Jun is presumed to be required for TGIF-mediated repression of Smad2 transcriptional activity [23]. c-Jun has also been suggested to interact with the corepressor Ski, preventing the recruitment of p300/CBP in response to TGF-β signaling [24]. However, details of the modulation of Smad complex transcriptional activity by c-Jun have remained unclear.

In the course of screening for inhibitors of TGF-β functions, we found that the vinca alkaloid, conophylline (fig. 1) [25] inhibited TGF-β-induced apoptosis and PAI-1 promoter activity. We attributed its potency of inhibition to the upregulated expression of c-Jun.

Materials and methods

Materials

Rat hepatoma McA-RH8994 and mink lung Mv1Lu cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 200 µg/ml kanamycin under an atmosphere of 5% CO_2 at 37 °C. p3TP-Lux [26] was kindly supplied by Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center). ARE-Lux and FAST2 were gifts from Dr. Wrana (Mount Sinai Hospital). HA-c-Jun, HA-c-JunbZip and myc-Smad DNA were generous gifts of Dr. A. Atfi (Hôspital Saint-Antonie). Conophylline was isolated from the leaves of *Ervatamia micrrophylla* collected in Khou Kaen, Thailand [25]. Control pGL2 vector was purchased from Promega. Polyclonal c-Jun, phospho-c-Jun (Ser63), SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185), p38, phospho-p38 (Thr180/Tyr182), MAPK/Erk1/2, and phospho-MAPK/Erk1/2 (Thr202/Tyr204) antibodies were purchased from Cell Signaling Technology. The monoclonal p300 antibody and polyclonal Smad2/Smad3 and phospho-Smad2 antibodies were purchased from Upstate Biotechnology. Polyclonal TGIF antibody came from Santa Cruz Biotechnology.

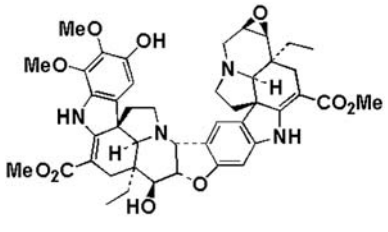

Figure 1. Structure of conophylline.

Apoptosis assay using McA-RH8994 cells

The cells (1.25×10^4) were plated in each well of 24-well plates. After cultivation for 24 h, the medium was exchanged for serum-free medium containing TGF-β. After incubation with TGF-β, surviving cells were counted after staining with trypan blue. For the detection of apoptosis, the cell nuclei were stained with Hoechst 33258 after fixation with 3% paraformaldehyde, and observed under a fluorescence microscope. The DNA was extracted and analyzed by 1.5% agarose gel electrophoresis as a DNA fragmentation assay.

Promoter reporter assays

Mv1Lu cells (1.5×10^4) were plated in each well of 24-well plates. After cultivation for 18–24 h, the cells were transfected with 200 ng of reporters and/or other plasmids using the FuGENE transfection reagent (Roche). After transfection, the cells were cultivated in DMEM with 10% FCS for 10 h, and the medium was then changed to DMEM with 0.2% FCS. Twelve hours after transfection, TGF-β and/or conophylline were added, and then 16 h later, luciferase assays were performed using a luciferase assay system (Promega) and a luminometer Lumat LB9507 (Berthold). All transfections were normalized to the WST-1 assay (Roche).

Immunofluorescence assay

Subcellular localization of Smad2 in Mv1Lu cells was determined as previously reported [27]. Mv1Lu cells (2.5×10^4) were plated in each well of an eight-chamber slide (Falcon). After cultivation for 16 h, the chamber devices were removed and the cells were washed with phosphate-buffered saline (PBS) and then fixed by acetone treatment for 2 min at –20 °C. After the slide had been air-dried, the cells were incubated for 60 min at room temperature with rabbit antiserum against Smad2 diluted 50- to 100-fold in PBS with 5% FCS. Then the cells were washed three times with PBS-0.05% Tween 20 and subsequently reacted for 60 min with the secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) in PBS with 5% FCS. Detection was then performed using a TSA-direct kit (NEN

Life Science Products) following three washings with PBS-0.05% Tween 20.

Protein analysis

For Western blots, Mv1Lu cells (2×10^5 cells) were plated in each well of six-well plates. After cultivation in DMEM with 10% FCS for 16 h, the medium was changed to DMEM with 0.2% FCS, and the cells were cultured for several hours. Then TGF-β and/or conophylline were added, and the cells were incubated for the desired period. The cells were then lysed in RIPA buffer [25 mM HEPES (pH 8.0), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 5 mM EDTA, 50 mM NaF, 100 mM sodium vanadate with complete protease inhibitor cocktail (Roche)] buffer after a wash with PBS. The cell lysates were subjected to SDS-PAGE gel electrophoresis and then transferred onto a nitrocellulose membrane, which was incubated in TBST buffer [100 mM Tris-HCl (pH 7.5), 0.9% NaCl] containing 5% nonfat milk. The proteins were visualized by reaction with specific primary antibodies followed by horseradish peroxidase-conjugated second antibodies. Immunoreactivity was detected using an enhanced chemiluminescence reagent, 'Western Lightning' (Perkin Elmer), following the manufacturer's instructions.

For coimmunoprecipitation, Mv1Lu cells (3.2×10^5 cells) were plated in 6-cm dishes. After transfection, the cells were cultivated in DMEM with 10% FCS for 16 h, and then the medium was changed to DMEM with 0.2% FCS, and the cells were incubated for several hours. They were then treated with TGF-β and/or conophylline for 3 h and thereafter, the cells were solubilized in RIPA buffer. The samples were centrifuged at 10,000 g for 10 min, and the supernatant was precleared with normal IgG (Sigma) from the same species as the immunoprecipitating antibody and Protein G PLUS/Protein A-agarose beads (Oncogene Research Products) for 30 min at 4°C, and then incubated with 1 µg of anti-myc or anti-Smad2 antibody for 1 h at 4°C followed by 25 µl of Protein G PLUS/Protein A-agarose and incubation overnight at 4°C. The beads were washed four times in PBS and resuspended in 40 µl of electrophoresis sample buffer (125 mM Tris-HCl, 1% SDS, 15% glycerol, 5% 2-mercaptoethanol, 0.005% bromo-phenol-blue). The samples were loaded for SDS-PAGE electrophoresis and subsequent Western blot analysis.

RT-PCR

Approximately 8×10^5 Mv1Lu cells were plated in 10-cm dishes and cultivated for 24 h before conophylline and/or TGF-β treatment for the specified times. Total RNA was extracted from cells using a MasterPure RNA purification kit (Epicentre). For RT-PCR experiments, we used an Access RT-PCR System (Promega). For oligonucleotide primers, we chose the sequences 5′-CGGCTACAGTAACCCCAAGA-3′ (forward) 5′-CCTGCTCATCTGTCACGTTC-3′ (reverse) and 5′-TGCGTGACATCAAGGAGAAG-3′ (forward); 5′-AGGAAGGAAGGCTGGAAGAG-3′ (reverse) in high-homology regions of bovine c-Jun and cat actin, respectively. Amplifications were performed for 40 cycles with an annealing temperature of 55°C. PCR products were visualized on ethidium bromide-stained 2% agarose gels.

Results

Inhibition of TGF-β-induced apoptosis by conophylline

TGF-β is known to induce apoptosis in rat Morris hepatoma McA-RH8994 cells [28]. As shown in fig. 2A, TGF-β killed McA-RH8994 cells in a dose-dependent manner. TGF-β at 3 ng/ml induced approximately 50% and 90% cell death in 48 and 72 h, respectively; whereas the cell death in the control was less than 20%. We confirmed that the cell death was due to apoptosis, as evidenced by chromatin condensation, nuclear fragmentation (fig. 2C), and DNA fragmentation in the nuclear extract (fig. 2D). We used TGF-β-induced apoptosis in McA-RH8994 cells as an indicator for screening for anti-TGF-β substances from natural sources. As a result, conophylline, a vinca alkaloid, was found to inhibit TGF-β-inducible apoptosis in a dose-dependent manner (fig. 2B). Conophylline at 30–100 ng/ml inhibited TGF-β-induced cell death. In the presence of conophylline at 100 ng/ml, only 10% cell death occurred in 48 h in serum-free medium.

Inhibition of TGF-β-induced transcription by conophylline

To demonstrate that conophylline inhibits TGF-β-induced signal transduction, we transiently transfected Mv1Lu and McA-RH8994 cells with p3TP-Lux containing a luciferase reporter gene under the control of the PAI-1 promoter and TPA-responsive element (TRE) corresponding to a region of the collagenase promoter, which are responsive to TGF-β [26]. We observed marked p3TP-Lux reporter activity in response to TGF-β in mink Mv1Lu cells but only a feeble response in McA-RH8994 cells. As shown in figure 3A, the concurrent addition of conophylline at 30 ng/ml inhibited 80% of the luciferase reporter activity triggered by TGF-β in Mv1Lu cells.

Xenopus FAST1 (also known as FoxH1) in association with Smad2 and Smad4 is required for activation of the Mix.2 gene via a TGF-β/activin response element (ARE) [29–31]. Binding to ARE is jointly mediated by the DNA-binding activities of FAST1 and Smad4 [29–31], whereas transcriptional activation is mediated by p300/CBP bound to Smads in the complex [18–20]. FAST1 and its mouse homologue, FAST2, can mediate activation of an ARE re-

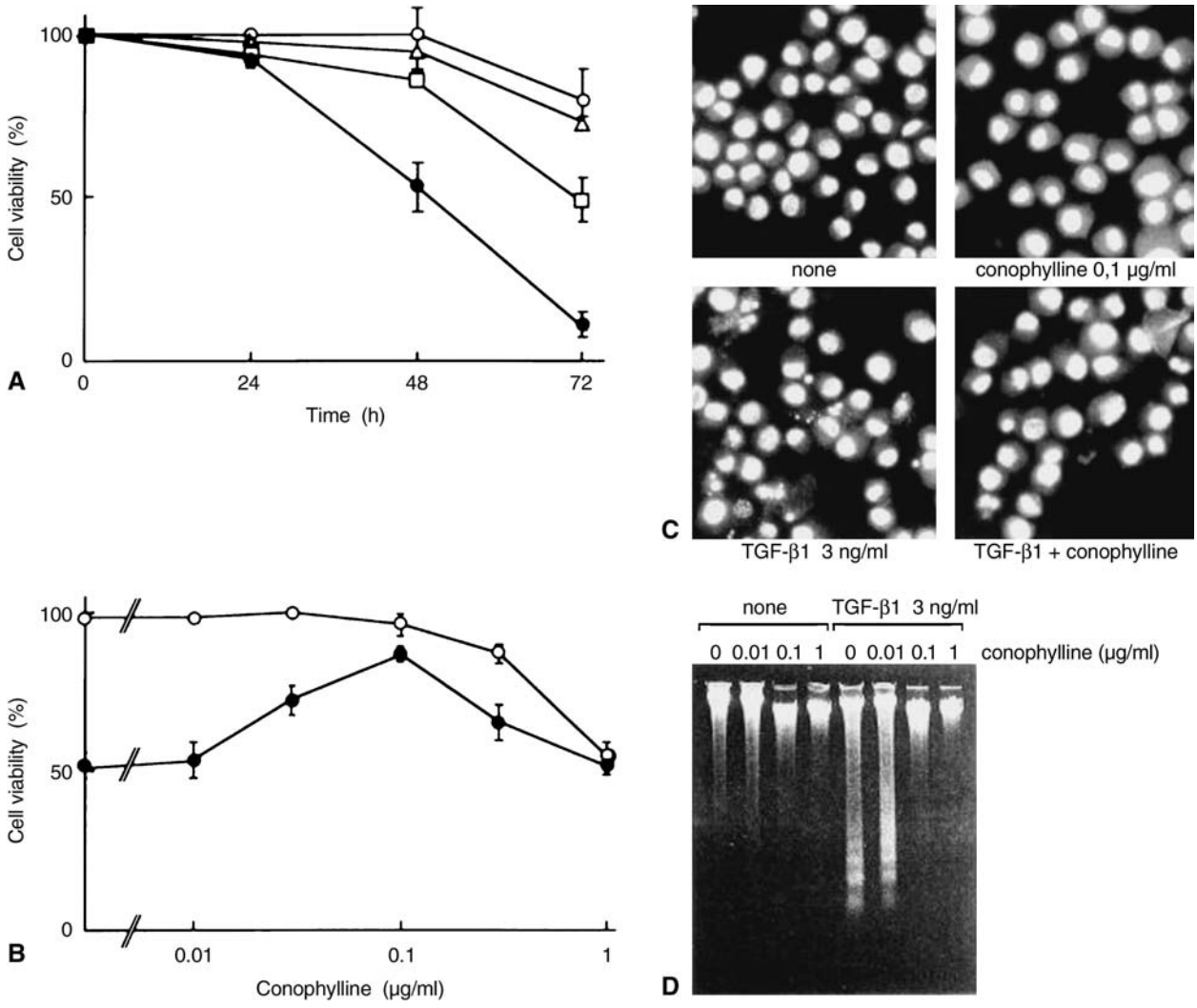

Figure 2. Conophylline-mediated inhibition of cell death induced by TGF-β in McA-RH8994 cells. (*A, B*) Induction of cell death by TGF-β in rat hepatoma McA-RH8994 cells (*A*). The cells were cultured in medium containing 0 (open circle), 0.03 (open triangle), 0.3 (open square), or 3 (closed circle) ng/ml of TGF-β. Inhibition of TGF-β-induced cell death by conophylline (*B*). The cells were cultured with conophylline for 1 h, and then 0 (○) or 3 (●) ng/ml of TGF-β was added. After 48 h, cell viability was assayed by trypan blue dye exclusion. The values are means ± SD of triplicate determinations. (*C, D*) Chromatin condensation, nuclear fragmentation (*C*), and DNA fragmentation in the nuclear extract (*D*) were promoted by conophylline in the cells.

porter construct (ARE-Lux) [32, 33]. Since the molecular mechanism of the TGF-β response of the ARE-Lux reporter is more clearly understood than that of p3TP-Lux, we examined the effect of conophylline on the ARE-Lux reporter. As shown in figure 3B, expression of the ARE-Lux construct in Mv1Lu cells was minimal, but a strong TGF-β-dependent increase of transcriptional activity was detected in cells cotransfected with FAST2 (fig. 3B). Conophylline also inhibited ARE-Lux reporter activity induced by TGF-β in the cells coexpressing FAST2. On the other hand, luciferase activity derived from the SV40 promoter-enhancer in the pGL2-control vector was not affected by conophylline (fig. 3C). No cytotoxicity was detected in these cells treated with 5 ng/ml TGF-β and/or 100 ng/ml conophylline for 24 h.

Effect of conophylline on TGF-β-induced Smad2 translocation

To specify the molecular target of conophylline in TGF-β signaling, we studied the effect of the drug on TGF-β-induced Smad2 translocation from the cytoplasm to the nucleus. As shown in figure 4, Smad2 translocation was observed 60 min after treatment of Mv1Lu cells with TGF-β. This translocation induced by TGF-β was not affected by the presence of conophylline, even at 300 ng/ml. Therefore, the target of conophylline in TGF-β signal transduction is considered to be downstream of the nuclear translocation of Smad2.

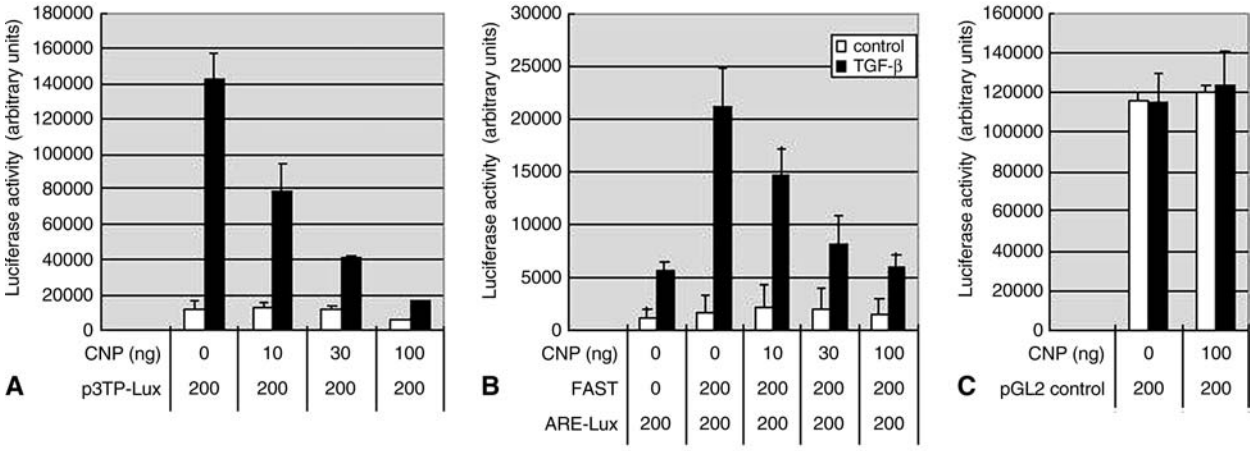

Figure 3. Inhibition of the TGF-β-responsive luciferase reporter by conophylline in mink lung Mv1Lu cells. The cells transfected with p3TP-Lux (*A*) or ARE-Lux (*B*) and pGL2 (*C*) were cultured with conophylline in the absence (control) or presence (TGF-β) of 5 ng/ml TGF-β for 16 h. The luciferase activity in the cell lysates was then detected. Data represent the means ± SD of triplicate determinations.

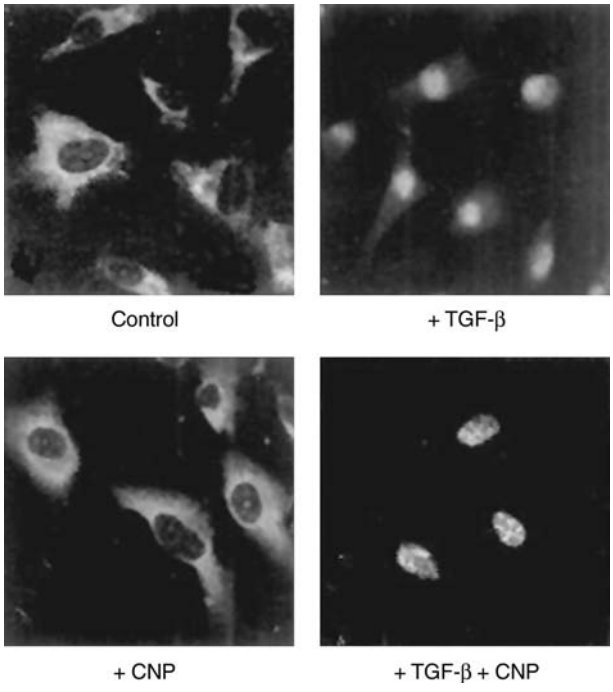

Figure 4. Effect of conophylline on nuclear translocation of Smad2 induced by TGF-β in Mv1Lu cells. Mv1Lu cells were treated with TGF-β (5 ng/ml) and/or 300 ng/ml of conophylline (CNP) for 1 h. Localization of Smad2/Smad3 was detected by immunofluorescent antisera.

Inhibition of TGF-β signaling in Mv1Lu cells overexpressing c-Jun

c-Jun has previously been demonstrated to interact with the corepressor TGIF to suppress Smad2 transcriptional activity in transiently transfected COS-7 cells, and TGF-β-dependent transcriptional activation is decreased by overexpression of c-Jun in HepG2 and COS-7 cells [23]. We examined the effect of c-Jun overexpression on TGF-β signaling using ARE-Lux in Mv1Lu cells. As shown in figure 5, the TGF-β-dependent activation of ARE-Lux was markedly decreased by cotransfection with c-Jun in Mv1Lu cells.

Induction of c-Jun expression by conophylline

Modulation of Jun expression by TGF-β is a cell type-specific phenomenon, as TGF-β activates c-Jun expression only in epithelial cells, whereas it induces JunB expression in mesenchymal cells [34, 35]. In Mv1Lu cells, TGF-β has been observed to activate JunB transiently [36]. To examine the possible effect of conophylline and TGF-β on expression of c-Jun in Mv1Lu cells, we performed a Western blot analysis on cell lysates after treatment of the cells with TGF-β and/or conophylline. After the cells had been treated with conophylline for 4 h, their c-Jun expression was markedly increased (fig. 6A). On the other hand, TGIF, JNK/SAPK, and actin expression were not affected. TGF-β alone had no remarkable effect on c-Jun in our conditions in Mv1Lu cells (fig. 6A). As shown in figure 6B, c-Jun expression was increased by the addition of conophylline in a dose-dependent manner at 10–100 ng/ml, conditions in which the expression of actin was unaffected. We also performed RT-PCR with total RNA prepared from the cells after conophylline treatment for 1–20 h. c-Jun expression at the transcriptional level was increased by conophylline (fig. 6C), in parallel with that at the protein level shown by the Western blot analysis.

Stimulation of SAPK/JNK by conophylline

To determine further the effect of conophylline on c-Jun, we examined the effect of conophylline on the phosphorylation of c-Jun by Western blot analysis using anti-

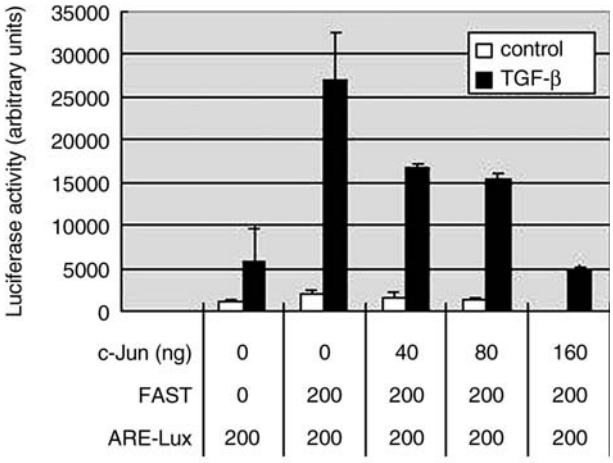

Figure 5. Inhibition of TGF-β-responsive luciferase reporter in cells overexpressing c-Jun. Mv1Lu cells cotransfected with c-Jun and pARE-Lux were cultured in the absence (control) or the presence of 5 ng/ml of TGF-β for 16 h. The luciferase activity in the cells was then detected. Data represent the means ± SD of triplicate determinations.

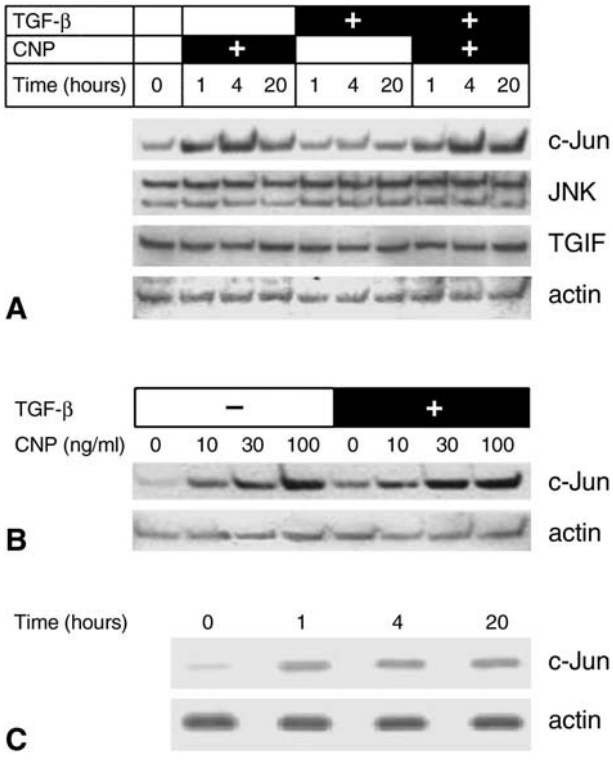

Figure 6. Upregulation of c-Jun expression by conophylline. (*A*) Conophylline (100 ng/ml) (CNP) was added to the culture medium and Mv1Lu cells were then incubated for the indicated times, lysed and subjected to gel electrophoresis and immunoblotting with anti-c-Jun, JNK, TGIF, and actin antibodies. (*B*) Mv1Lu cells were incubated with the indicated doses of conophylline for 4 h, and then harvested. The lysates were subjected to gel electrophoresis and immunoblotted with anti-c-Jun and anti-actin. (*C*) RT-PCR was performed using 50 ng of total RNA extracted from the cells treated with 100 ng/ml conophylline for the indicated periods. The primers described in Materials and methods were used.

phospho-SAPK/JNK (Thr183/Tyr185) or anti-phospho-c-Jun (Ser63). The Western blot was performed on cell lysates after treatment of the cells for a short period (5–30 min). An increase in c-Jun protein expression after conophylline and/or TGF-β was not observed in this short period. As shown in figure 7A, conophylline stimulated SAPK/JNK activity, the stimulation being accompanied by activation of its downstream substrate c-Jun. We also examined the effect of conophylline on the stimulation of other mitogen-activated protein kinases, p38 and MAPK/Erk1/2, which were earlier reported to be stimulated by TGF-β and to contribute partly to the regulation of TGF-β signaling [37–39]. The stimulation of p38 was also observed, along with its phosphorylation detected by anti-phospho-p38 (Thr180/Tyr182). Interestingly, MAPK/

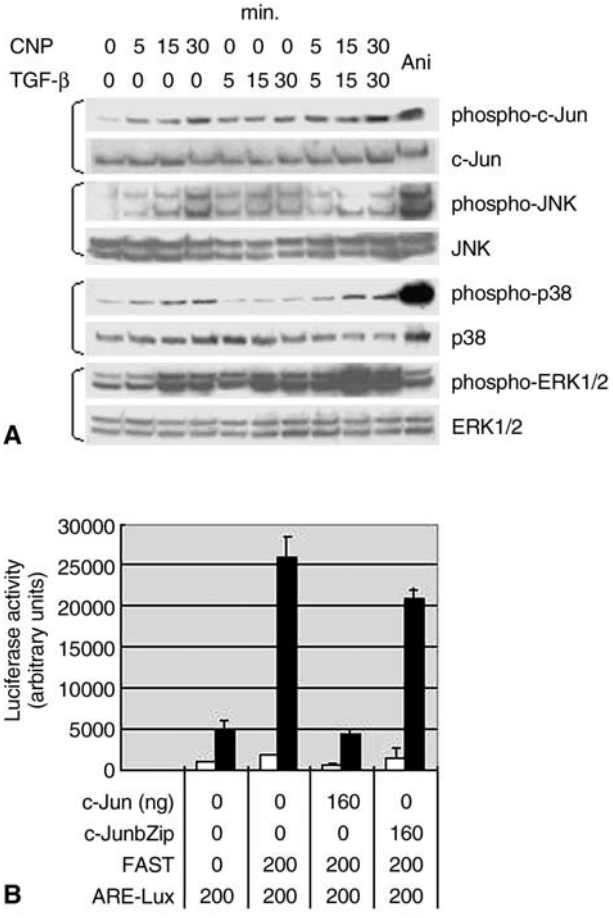

Figure 7. Stimulation of the SAPK/JNK cascade by conophylline. (*A*) Mv1Lu cells were treated with 100 ng/ml conophylline in the presence or absence of TGF-β for the indicated times, and then Western blotting was performed on the cell lysates. The identity of the investigated proteins is indicated to the right. Anisomycin (Ani) is an activator of SAPK/JNK and p38 signal cascades. (*B*) Effect of cotransfection with c-Jun or c-JunbZip on pARE-Lux reporter activity induced by TGF-β. Mv1Lu cells were treated with (filled bars) or without (open bars) TGF-β for 16 h. Then the luciferase activity in the cell lysates was assayed. The data represent the means ± SD of triplicate determinations.

Erk1/2 was markedly stimulated by conophylline, with a significant rise in phosphorylation at the sites detected with anti-phospho-MAPK/Erk1/2 (Thr202/ Tyr204). We also found that TGF-β stimulated SAPK/ JNK and MAPK/Erk1/2 in the short term. An additive effect was observed in the stimulation of the mitogen-activated protein kinases when cells were treated with both conophylline and TGF-β.

Next, to investigate whether JNK could be needed for the repression of TGF-β signaling through c-Jun, we examined the effect of overexpression of a c-Jun mutant version or c-JunbZip, which harbors a deletion in the N-terminus that includes the binding sites of JNK [40]. In contrast to wild-type c-Jun, the inhibition of TGF-β-inducible ARE-Lux activity was not observed in the cells overexpressing c-JunbZip (fig. 7B).

Enhancement of an association between Smad2 and TGIF by conophylline through c-Jun induction

Upon entering the nucleus, the Smad2 complex has been shown to interact with the coactivator, p300, forming a transcriptional activation complex, or with the corepressor TGIF, forming a transcriptional repressor complex, in response to TGF-β signaling [17]. c-Jun has been prosposed to enhance the association of Smad2 with TGIF [23]. Thus, the effect of conophylline on the interaction of the Smad2 complex with p300, TGIF, and c-Jun was investigated. For this purpose coimmunoprecipitation was performed with cells overexpressing myc-tagged Smad2 protein. As shown in figure 8A, association of the Smad2 complex with p300 was enhanced by TGF-β. This enhancement induced by TGF-β was attenuated by the addition of conophylline. Inversely, the association of the Smad2 complex with TGIF induced by TGF-β was enhanced by the treatment with conophylline. Furthermore, the association of the Smad2 complex with c-Jun stimulated by TGF-β was enhanced by the addition of conophylline (fig. 8A). On the other hand, the phosphorylation of Smad2 induced by TGF-β was not affected by conophylline. To test if increments in c-Jun expression would elevate the association of the endogenous Smad2 complex with c-Jun, we performed coimmunoprecipitation with cells overexpressing c-Jun. In such cells, the association of the Smad2 complex with c-Jun was increased, and was accompanied by an increased tendency for Smad2 to associate with TGIF in response to TGF-β (fig. 8B). There was no effect of overexpressed c-Jun on the phosphorylation of Smad2.

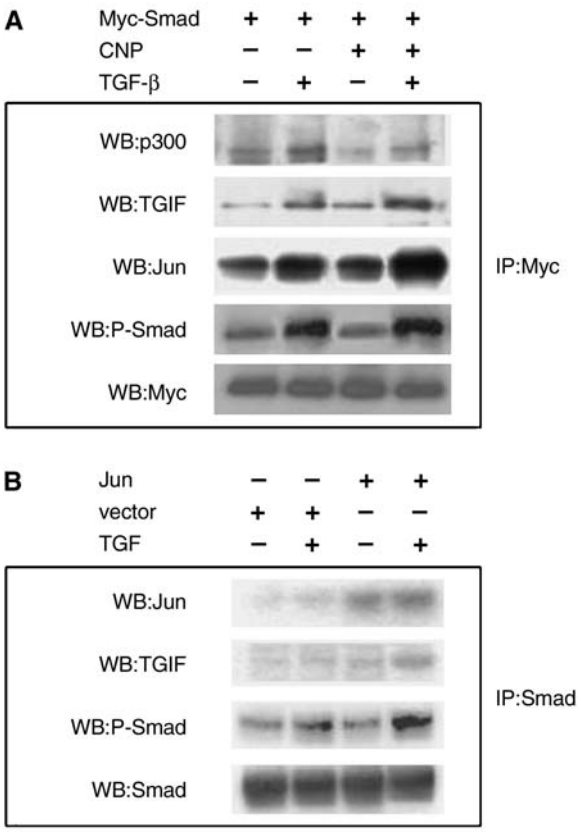

Figure 8. (*A*) Enhancement of an association between Smad2 and TGIF by conophylline. Mv1Lu cells were transfected with myc-Smad2, and cell lysates were subjected to immunoprecipitation with anti-myc antibody that recognized myc-Smad2. Expression levels of proteins were determined by immunoblotting aliquots of total cell lysates with the antibodies against the proteins indicated. WB means, Western blot. (*B*) Elevation of association of endogenous Smad2 with c-Jun and TGIF in Mv1Lu cells overexpressing c-Jun. Lysates of cells transfected with HA-c-Jun or control vector were subjected to immunoprecipitation with anti-Smad2 antibody. Expression levels of proteins were determined by immunoblotting aliquots of total cell lysates with the antibodies indicated.

Reduction of TGF-β signaling by Smad2 in cells overexpressing c-Jun or treated with conophylline

Smad2 complex was postulated to play an inductive role in the TGF-β-inducible transcription without excessive c-Jun, becoming a suppressor when c-Jun is expressed abundantly. In cells treated with conophylline which induces overexpression of c-Jun, Smad2 was presumed to behave as a suppressor. To test this hypothesis, we investigated the effect of c-Jun or conophylline on Smad2 regulation.

As shown in figure 9, the transcriptional activity in response to TGF-β was stimulated by transfection of Smad2 without excessive c-Jun. Inversely, the reporter activity was suppressed by abundant Smad2 in cells overexpressing c-Jun. The suppression by Smad2 expressed abundantly was also observed in cells treated with conophylline.

Discussion

Conophylline was shown to inhibit TGF-β-induced apoptosis in McA-RH8994 cells (fig. 2). Caspases are known to be involved in TGF-β-mediated apoptosis in hepatoma

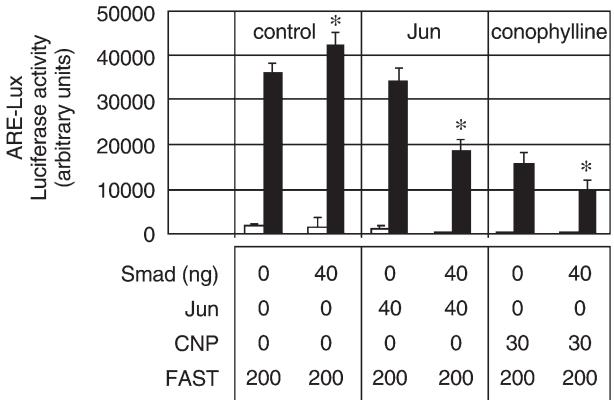

Figure 9. The suppression of TGF-β signaling by Smad2 in cells overexpressing c-Jun or treated with conophylline. The ARE-Lux reporter assay was performed on the cells transfected with c-Jun or treated with conophylline. Cells cotransfected with myc-Smad2 or vehicle only were treated with TGF-β for 16 h, and then harvested and assayed for luciferase activity. The data represent the means ± SD of triplicate determinations. *$p < 0.05$ vs control.

cells [41–43], and a broad-spectrum caspase inhibitor completely blocked TGF-β-induced apoptosis [44]. If conophylline is a broad-spectrum caspase inhibitor, it might inhibit anticancer agent-induced apoptosis; however, conophylline did not inhibit adriamycin-induced apoptosis in McA-RH8994 cells (data not shown).

We employed Mv1Lu cells for the mechanistic study because these cells are appropriate for the reporter assay. We have shown that conophylline also inhibited not only p3TP-Lux reporter, containing the PAI-1 promoter and TPA-responsive element corresponding to a region of the collagenase promoter, but also ARE-Lux. We chose to focus our analysis on the *Xenopus* Mix.2 promoter as a target of Smad2 because activation of Mix.2 by TGF-β requires the formation of a Smad2-Smad3 (Smad4)-FAST complex that binds to a sequence promoter known as ARE. Furthermore, FAST-bound Smads have to be shown to recruit p300/CBP histone acetyl transferase, activating transcription, or TGIF and a histone deacetylase (HDAC), repressing transcription [17]. The upstream molecular mechanism of TGF-β signaling of the ARE-Lux reporter is understood more clearly than p3TP-Lux. In addition ARE does not have any known c-Jun- or AP-1-binding site. Therefore, ARE-Lux is adequate to study the relationship between c-Jun expression promoted by conophylline and repression of TGF-β signaling.

We showed that c-Jun expression and stimulation were markedly increased by the addition of conophylline to Mv1Lu cell cultures (figs. 6, 7). Conophylline suppressed the association of the Smad2 complex with p300, whereas it enhanced the association of this complex with TGIF, perhaps due to the enhancement of the Smad2 complex-c-Jun interaction in the presence of TGF-β (fig. 8A). This upregulation of the Smad2 complex-c-Jun interaction is probably attributable to the elevation of c-Jun expression by conophylline, since the interaction of the Smad2 complex with c-Jun was increased in c-Jun-overexpressing Mv1Lu cells (fig. 8B). In c-Jun-overexpressing cells, the reduction in the TGF-β-induced transcriptional level has been attributed to stabilization of the Smad2 complex/TGIF by c-Jun [23]. This observation agrees with our results that c-Jun overexpression (fig. 8B) and conophylline addition (fig. 8A) had similar effects on the tendency of Smad2 to associate with TGIF. As shown in figure 9, Smad2 plays a positive role in TGF-β signaling without excessive c-Jun. However, when the expression of c-Jun was enhanced, Smad2 became a negative transcriptional regulator in response to the TGF-β signal. This positive-to-negative change in Smad2 was also observed in the cells treated with conophylline, which enhanced c-Jun expression. Thus, these results suggest that suppression of TGF-β signaling by conophylline can be attributed to the ability of this drug to upregulate c-Jun expression.

Conophylline enhanced c-Jun expression (fig. 6) with stimulating JNK (fig. 7). Once activated, JNK phosphorylates c-Jun, and in turn, phosphorylated c-Jun homodimerized with members of the Jun family or heterodimerizes with members of the Fos family. All these complexes, named activating-protein-1 (AP-1), bind to AP-1 sites and can control the expression of a number of genes, including c-Jun itself [45, 46]. The underlying mechanism of activation of JNK by conophylline is now being studied in detail. c-Jun has been described as interacting with Ski, another corepressor in TGF-β signaling, and enhancing suppression of transcriptional activity [21, 22]. Ski protein was not detectable in our Western blot analysis in Mv1Lu cells. Presumably, the Ski expression level is extremely low in this cell line. c-Jun may be able to recruit different transcriptional corepressors depending on cell type or physiological context. Therefore, conophylline may suppress TGF-β-induced transcriptional activity in a wide range of cell types, and may be a candidate therapeutic agent for diseases caused by deregulation of TGF-β signaling.

Acknowledgements. We wish to thank Dr. J. Massagué, Memorial Sloan-Kettering Cancer Center, for providing the 3TP-Lux plasmids, Dr. J. Wrana, Mount Sinai Hospital, for ARE-Lux and FAST2 plasmids, and Dr. A. Atfi, Hôspital Saint-Antonie for Ha-Jun, Tam67 and Myc-Smad2 plasmids.

1 Lyons R. M. and Moses H. L. (1990) Transforming growth factors and the regulation of cell proliferation. Eur. J. Biochem. **187:** 467–473
2 Massagué J. (1990) The transforming growth factor-β family. Annu. Rev. Cell Biol. **6:** 597–641
3 Kovalszky I., Nagy P., Szenda B., Lapis K., Szalay F., Jeney A. et al. (1998) Experimental and human liver fibrogenesis. Scand. J. Gastroenterol. Suppl. **228:** 51–55
4 Zhang K. and Phan S. H. (1996) Cytokines and pulmonary fibrosis. Bio. Signals **5:** 232–239

5 Bitzer M., Sterzel R. B. and Bottinger E. P. (1998) Transforming growth factor-β in renal disease. Kidney Blood Press. Res. **21:** 1–12

6 Qi Z., Atsuchi N., Ooshima A., Takeshita A. and Ueno H. (1999) Blockade of type beta transforming growth factor signaling prevents liver fibrosis and dysfunction in rat. Proc. Natl. Acad. Sci. USA **96:** 2345–2349

7 Yoshida K., Yokozaki H., Nimoto M., Ito H., Ito M. and Tahara E. (1989) Expression of TGF-β and procollagen type I and type III in human gastric carcinomas. Int. J. Cancer **44:** 394–398

8 Mahara K., Kato J., Terui T., Takimoto R., Horimoto M., Murakami T. et al. (1994) Transforming growth factor-β1 secreted from scirrhous gastric cancer cells is associated with excess collagen deposition in the tissue. Br. J. Cancer **69:** 777–783

9 Kinugasa S., Abe S., Tachibana M., Hishikawa Y., Yoshimura H., Monden N. et al. (1998) Overexpression of transforming growth factor-β 1 in scirrhous carcinoma of stomach correlates with decreased survival. Oncology **55:** 582–587

10 Oberhammer F. A., Pavelka M., Sharma S., Tiefenbacher R., Purchio A. F., Bursch W. et al. (1992) Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor-β 1. Proc. Natl. Acad. Sci. USA **89:** 5408–5412

11 Sanderson N., Factor V., Nagy P., Kopp J., Kondaiah P., Wakefield L. et al. (1995) Hepatic expression of mature transforming growth factor-β 1 in transgenic mice results in multiple tissue lesions. Proc. Natl. Acad. Sci. USA **92:** 2572–2576

12 Flanklin T. J. (1997) Therapeutic approaches to organ fibrosis. Int. J. Cell Biol. **29:** 79–89

13 Heldin C. H., Miyazono K. and Dijke P. ten **(**1997) TGF-β signaling from cell membrane to nucleus through SMAD proteins. Nature **390:** 465–471

14 Attisano L. and Wrana J. L. (1998) Mads and Smads in TGF-β signaling. Curr. Opin. Cell Biol. **10:** 188–194

15 Kretzschmar M. and Massagué J. (1998) Smads: mediators and regulators of TGF-β signaling. Curr. Opin. Genet. Dev. **8:** 103–111

16 Kawabata M., Inoue H., Hanyu A., Imamura T. and Miyazono K. (1998) Smad proteins exist as monomers in vivo and undergo homo- and hetero-oligomerization upon activation by serine/threonine kinase receptor. EMBO J. **17:** 4056–4065

17 Wotton D., Lo R. S., Lee S. and Massagué J. (1999) A Smad transcriptional corepressor. Cell **97**: 29–39

18 Janknecht R., Wells N. J. and Hunter T. (1998) TGF-β-stimulated cooperation of Smad proteins with the coactivators CBP/p300. Genes Dev. **12:** 2114–2119

19 Topper J. N., Dichiara M. R., Brown J. D., Williams A. J., Falb D., Collins T. et al. (1998) CREB binding protein is a required coactivator for Smad-dependent, transforming growth factor-β transcriptional responses in endothelial cells. Proc. Natl. Acad. Sci. USA **95:** 9506–9511

20 Pouponnot C., Jayaraman L. and Massagué J. (1998) Physical and functional interaction of Smads and p300/CBP. J. Biol. Chem. **273:** 22865–22868

21 Akiyoshi S., Inoue H., Hanai J., Kusanagi K., Nemoto N., Miyazono K. et al. (1999) c-Ski acts as a transcriptional co-repressor in transforming growth factor-β signaling through interaction with Smads. J. Biol. Chem. **274:** 35269–35277

22 Luo K., Stroschein S. L., Wang W., Chen D., Martens E., Zhou S. et al. (1999) The Ski oncoprotein interacts with the Smad proteins to repress TGF-β signaling. Genes Dev. **13:** 2196–2206

23 Pessah M., Prunier C., Marais J., Ferrand N., Mazars A., Lallemand F. et al. (2001) c-Jun interacts with the corepressor TG-interacting factor (TGIF) to suppress Smad2 transcriptional activity. Proc. Natl. Acad. Sci. USA **98:** 6198–6203

24 Pessah M., Marais J., Prunier C., Ferrand N., Lallemand F., Mauviel A. et al. (2002) c-Jun associates with the oncoprotein Ski and suppresses Smad2 transcriptional activity. J. Biol. Chem. **277:** 29094–29100

25 Umezawa K., Ohse T., Yamamoto T., Koyano T. and Takahashi Y**.** (1994) Isolation of a new vinca alkaloid from the leaves of *Ervatamia microphylla* as an inhibitor of ras functions. Anticancer Res. **14:** 2413–2417

26 Wrana J. L., Attisano L., Carcamo J., Zentella A., Doody J., Laiho M. et al. (1992) TGF-β signals through a heteromeric protein kinase receptor complex. Cell **71:** 1003–1014

27 Nakao A., Imamura T., Souchelnytskyi S., Kawabata M., Ishisaki A., Oeda E. et al. (1997) TGF-β receptor-mediated signaling through Smad2, Smad3 and Smad4. EMBO J. **16:** 5353–5362

28 Yamamoto M., Ogawa K., Morita M., Fukuda K. and Komatsu Y. (1995) The herbal medicine Inchi-Ko-To inhibits liver cell apoptosis induced by transforming growth factor-β1. Hepatology **23:** 552–559

29 Chen X., Rubock M. J. and Whiteman M. (1996) A transcriptional partner of MAD proteins in TGF-β signaling. Nature **383**: 691–696

30 Chen X., Weisberg E., Fridmacher V., Watanabe M., Naco G. and Whitman M. (1997) Smad4 and FAST-1 in the assembly of activin responsive factor. Nature **389:** 85–89

31 Liu F., Pouponnot C. and Massagué J. (1997) Dual role of the Smad4/DPC4 tumor suppressor in TGF-β-inducible transcriptional responses. Genes Dev. **11:** 3157–3167

32 Labbé E., Silvestri C., Hoodless P. A., Wrana J. L. and Attisano L. (1998) Smad2 and Smad3 positively and negatively regulate TGF-β dependent transcription through the forkhead DNA-binding protein FAST2. Mol. Cell **2:** 109–120

33 Liu B., Dou C. L., Prabhu L. and Lai E. (1999) FAST-2 is a mammalian winged-helix protein which mediates transforming growth factor beta signals. Mol. Cell. Biol. **19:** 424–430

34 Mauveil A., Chen Y. Q., Dong W., Evans C. H. and Uitto J. (1993) Transcriptional interactions of transforming growth-factor-β with pro-inflammatory cytokines. Curr. Biol. **3:** 822–831

35 Mauviel A., Chung K. Y., Agarwal A., Tamai K. and Uitto J. (1996) Cell-specific induction of distinct oncogenes of the Jun family is responsible for differential regulation of collagenase gene expression by transforming growth factor-β in fibroblasts and keratinocytes. J. Biol. Chem. **271:** 10917–10923

36 Zentella A., Weis F. M., Ralph D. A., Laiho M. and Massagué J. (1991) Early gene responses to transforming growth factor-β in cells lacking growth-suppressive RB function. Mol. Cell Biol. **11:** 4952–4958

37 Schiemann W. P., Blobe G. C., Kalume D. E., Pandey A. and Lodish H. F. (2002) Context-specific effects of fibulin-5 (DANCE/EVEC) on cell proliferation, motility, and invasion: fibulin-5 is induced by transforming growth factor-β and affects protein kinase cascades. J. Biol. Chem. **277:** 27367–27377

38 Sowa H., Kaji H., Yamaguchi T., Sugimoto T. and Chihara K. (2002) Activations of ERK1/2 and JNK by transforming growth factor-β negatively regulate Smad3-induced alkaline phosphatase activity and mineralization in mouse osteoblastic cells. J. Biol. Chem. **277:** 36024–36031

39 Watanabe H., de Caestecker M. P. and Yamada Y. (2001) Transcriptional cross-talk between Smad, ERK1/2, and p38 mitogen-activated protein kinase pathways regulates transforming growth factor-β-induced aggrecan gene expression in chondrogenic ATDC5 cells. J. Biol. Chem. **276:** 14466–14473

40 Leppa S., Saffrich R., Ansorge W. and Bohmann D. (1998) Differential regulation of c-Jun by ERK and JNK during PC12 cell differentiation. EMBO J. **17:** 4404–4413

41 Chen R. H. and Chang T. Y. (1997) Involvement of caspase family proteases in transforming growth factor-β-induced apoptosis. Cell Growth Differ. **8:** 821–827

42 Choi K. S., Lim I. K., Brady J. N. and Kim S. J. (1998) ICE-like protease (caspase) is involved in transforming growth factor-mediated apoptosis in FaO rat hepatoma cell line. Hepatology **27:** 415–421

43 Shima Y., Nakao K., Nakashima T., Kawakami A., Nakata K., Hamasaki K. et al. (1999). Activation of caspase-8 in transforming growth factor-β-induced apoptosis of human hepatoma cells. Hepatology **5:** 1215–1222
44 Brown T. L., Patil S., Basnett R. K. and Howe P. H. (1998) Caspase inhibitor BD-fmk distinguishes transforming growth factor-β-induced apoptosis from growth inhibition. Cell Growth Differ. **9:** 869–875
45 Angel P., Hattori K., Smeal T. and Karin M. (1988) The Jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. Cell **55:** 875–885
46 Derijard B., Hibi M., Wu I. H., Barrett T., Su B., Deng T. et al. (1994) JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell **76:** 1025–1037