

Regulation of CCN2 mRNA expression and promoter activity in activated hepatic stellate cells

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Abstract The matricellular protein connective tissue growth factor (CCN2) is considered a faithful marker of fibroblast activation in wound healing and in fibrosis. CCN2 is induced during activation of hepatic stellate cells (HSC). Here, we investigate the molecular basis of CCN2 gene expression in HSC. Fluorescence activated cell sorting was used to investigate CCN2 expression in HSC *in vivo* in mice treated with CCl₄. CCN2 and TGF- β mRNA expression were assessed by polymerase chain reaction as a function of culture-induced activation of HSC. CCN2 promoter/reporter constructs were used to map cis-acting elements required for basal and TGF β -induced CCN2 promoter activity. Real-time polymerase chain reaction analysis was used to further clarify signaling pathways required for CCN2 expression in HSC. CCl₄ administration *in vivo* increased CCN2 production by HSC. *In vitro*, expression of CCN2 and TGF- β mRNA were concommi-

tantly increased in mouse HSC between days 0 and 14 of culture. TGF β -induced CCN2 promoter activity required the Smad and Ets-1 elements in the CCN2 promoter and was reduced by TGF β type I receptor (ALK4/5/7) inhibition. CCN2 overexpression in activated HSC was ALK4/5/7-dependent. As CCN2 overexpression is a faithful marker of fibrogenesis, our data are consistent with the notion that signaling through TGF β type I receptors such as ALK5 contributes to the activation of HSC and hence ALK4/5/7 inhibition would be expected to be an appropriate treatment for liver fibrosis.

Keywords Hepatic fibrosis · Transcriptional regulation · CTGF · Transforming growth factor- β · ALK5

Abbreviations

ALK	activin linked kinase
CMV	cytomegalovirus
CTGF	connective tissue growth factor
FACS	fluorescence activated cell sorter
HSC	Hepatic stellate cells
HSPG	heparan sulfate containing proteoglycans
PBS	phosphate buffered saline
PKC	protein kinase C
SEAP	secreted enhanced alkaline phosphatase
TGF β	Transforming growth factor β

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Introduction

Hepatic stellate cells (HSC) are the primary target of fibrogenic stimuli in the diseased liver. During fibrogenesis, HSC transition from a resting, vitamin A-rich phenotype to a myofibroblastic phenotype characterized by loss of vitamin A, expression of α -smooth muscle actin, and

extracellular matrix production (Gressner and Bachem 1994; Pinzani 1995). Connective tissue growth factor (CCN2), a cysteine-rich matricellular protein, regulates cell adhesion, migration, proliferation, survival, and differentiation via integrin- and heparan sulfate proteoglycan-mediated adhesive signaling (Brigstock 1999; Leask and Abraham 2006). CCN2 has fibrogenic properties *in vitro* and is characteristically over-expressed in a wide variety of fibrotic lesions, correlating with disease severity (Blom et al. 2002; Dziadzio et al. 2005). Consistent with these observations, CCN2 production is enhanced during the progressive activation of primary HSC *in vitro*. Moreover, CCN2 is further induced in HSC by the potent pro-fibrotic cytokine TGF- β 1 (Paradis et al. 1999; Williams 2000).

The molecular basis underlying CCN2 expression in HSC is unknown. In other cell types, CCN2 expression is controlled primarily at the transcriptional level; therefore elements in its promoter are likely to contribute to its expression in HSC (Blom et al. 2002). Previously, we have analysed the regions in the CCN2 promoter required for its induction in response to TGF β and endothelin-1 in normal fibroblasts and mesangial cells, and its constitutively elevated activity in fibrotic scleroderma fibroblasts and pancreatic cancer cells (Abraham et al. 2000; Holmes et al. 2001, 2003; Chen et al. 2006; Pickles and Leask 2007). CCN2 induction in response to TGF β in unactivated fibroblasts and mesangial cells requires Smad and Ets response elements in the CCN2 promoter [Holmes et al. 2001; Chen et al. 2002; Leask et al. 2003; Van Beek et al. 2006]. Smads generally regulate TGF β signaling, and Smad3 in particular is required for TGF β to induce CCN2 protein in fibroblasts (Holmes et al. 2001). The Ets family of transcription factors are key regulators of differentiation, hormone responses and tumorigenesis, and Ets-1 in particular is required for TGF β -induced CCN2 protein expression (Van Beek et al. 2006). Intriguingly, CCN2 overexpression in scleroderma cells is independent of TGF β signaling via Smads and ALK5, but is dependent on endothelin-1 by the endothelin A and B receptors (Holmes et al. 2001; Chen et al. 2006; Shi-wen et al. 2007). Moreover, CCN2 overexpression in pancreatic cancer cells is independent of both TGF β and endothelin-1, but is dependent on activated ras/MEK/ERK (Pickles and Leask 2007). Thus although TGF β can mediate the induction of CCN2 in normal mesenchymal cells, overexpression of CCN2 in pathological states has been shown thus far to be independent of TGF β .

Although CCN2 is induced by TGF β and autocrine TGF β signaling is associated with HSC activation (Paradis et al. 1999; Williams 2000), it is unclear whether TGF β signaling mediates the elevated CCN2 expression that occurs during HSC activation. This issue is of particular relevance given that the overexpression of CCN2 in other

activated cells is TGF β independent. To address this question, it is necessary to first identify the mechanism underlying TGF β -induced CCN2 expression in HSC and then address whether this mechanism is of relevance to the overexpression of CCN2 in activated HSC. The studies reported here address these outstanding issues and provide valuable insights into the fundamental molecular mechanism underlying HSC activation, and hence into how to control hepatic fibrogenesis.

Materials and methods

CCN2 production in activated mouse HSC *in vivo* All animal procedures were approved by Institutional Animal Care and Use Committee of The Research Institute at Nationwide Children's Hospital, Columbus OH. Male 5-week old Balb/c mice received daily i.m. injections (1 ml/kg) of olive oil or a 1:1 ratio of carbon tetrachloride (CCl₄): olive oil for 1 week (Paradis et al. 1999). Mice ($n=3$ per group) were sacrificed 2 days later and HSC were isolated as described below. Freshly isolated HSC from CCl₄- or oil-treated mice were individually pooled, washed twice in ice-cold PBS, and resuspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). The samples were incubated at 4°C overnight with a 1:1000 dilution of polyclonal CCN2 antiserum raised in rabbits against full-length recombinant CCN2. The samples were incubated at 4°C overnight with a 1:1000 dilution of polyclonal CCN2 antiserum raised in rabbits against full-length recombinant CCN2 (Ball et al. 2003). This antibody, termed "38B", has been previously used for immunoprecipitation and Western blot and shows absolute specificity for CCN2, with no cross-reactivity with other CCN proteins (Ball et al. 2003 and data not shown). This antibody was selected for cell sorting based on its superior performance in the detection of CCN2-stably transfected Chinese Hamster Ovary cells versus parental cells (Ball et al. 2003), as compared to either non-immune serum or six other CCN2 antibodies produced in the investigators' laboratory (D.R.B., unpublished observations). After subsequent incubation of the HSC with FITC-conjugated anti-rabbit IgG secondary antibody (1:1000; Invitrogen), the cells were analysed on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). 10⁴ cells were tested in triplicate per sample and the data were analysed with FlowJo software (Tree Star Inc., USA).

Isolation and culture of HSC Mouse livers were perfused *in situ* with sequential 37°C treatments of heparin (100 U), 70 ml HBSS (without Ca²⁺/Mg²⁺), 0.5 mg pronase in 40 ml DMEM/F12, and 5 mg collagenase IV in 70 ml DMEM/F12. The livers were then removed, cut into pieces and

agitated in 20 ml DMEM containing 0.2 mg DNase at 37°C for 10 min. The resulting cell suspension was filtered through a metal sieve and HSC were isolated based on their buoyancy using an Optiprep gradient in which the cells were collected in the 11% phase (Iredale 1992). Cell purity (>95%) was determined using phase contrast microscopy and UV excited fluorescence microscopy. Oil-red staining was used to confirm the presence of intracellular lipid droplets. Cell viability (>95%) was determined by trypan blue exclusion. For the oil or CCl₄-treated animals, HSC were isolated from all 3 animals in each group and pooled for FACS analysis. For *in vitro* culture experiments, HSC isolated from ten untreated mice were resuspended and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C. Cells used were activated by 14 days of culturing and were placed (3×10^5 cells/well) in 6-well tissue culture plates (Falcon; Becton Dickinson, Franklin Lakes, NJ, USA) for further analysis.

Reverse-transcriptase polymerase chain reaction Total RNA from cultured mouse HSC was extracted at various points using a TRIzol extraction kit (Gibco/BRL) according to the manufacturer's directions. First-strand cDNA was synthesized using 2 µg of RNA in 20 µl of reaction buffer by reverse transcription using SuperScript™ II RNase H- Reverse (Invitrogen) with oligo-dT(18)-primers. Complementary DNA was amplified in a 50 µl total volume reaction comprising 50 mM KCl, 20 mM Tris-HCl (pH 8.0), 10 mM deoxynucleoside triphosphate (dNTP), 1.5 mM MgCl₂, 1 U Taq polymerase, and 10 pmol of the following polymerase chain reaction (PCR) primer sets: CTGF: 5'-GCAGGGATC CATGCTCGCCTCCGTCGCA-3' and 5'-CCAGCGC CGCGAATCTTACGCCATGTCTCC-3'; TGF-β: 5'-CCGGATCCTGTCCAAACTAAGGCTCGC-3' and 5'-CCTCTAGACCAGTGACGTCAAAGACAGCC; and β-actin: 5'-AGCTTGCTGTATTCCCCTCCATCGTG-3' and 5'-AATTCGGATGGCTACGTACATGGCTG-3'. Amplification products were separated by electrophoresis on 1% agarose gel containing ethidium bromide and quantitated by scanning densitometry. The proportion of specific gene product to β-actin product was used for semiquantitative analysis which was determined using an Alpha Innotech (San Leandro, CA, USA) imaging system.

Transfections and DNA Constructs Cells were allowed to grow for 24 h at 37°C. Cells were then transfected with Fugene (Roche, Indianapolis, IN, USA) at a ratio of 3 µl:2 µg DNA per well. Cells were transfected (1.75 µg/well) with plasmids containing a CCN2 promoter containing DNA spanning from nucleotides -805 to +17, or otherwise identical

constructs containing point mutations in either Smad or Ets sites, fused to a secreted alkaline phosphatase (SEAP) reporter gene (Abraham et al. 2000; Holmes et al. 2001; Leask et al. 2003; Van Beek et al. 2006). To control for transfection efficiency cells were transfected with 0.25 µg of a cytomegalovirus (CMV) promoter-β-galactosidase (CMV-β-gal) reporter gene (Clontech, Palo Alto, CA, USA) construct. Cells were allowed to grow for 48 h in 37°C. Promoter assays were performed with a Phospha-Light kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol to detect SEAP reporter expression and β-galactosidase expression was determined by a Galacto-star kit (Applied Biosystems) according to manufacturer's protocol. After adjusting the SEAP expression values to reflect differences in transfection efficiencies as determined by the β-galactosidase expression values, adjusted data was expressed as average values ± standard deviation of at least three replicates and at least two independent trials. Measurement of SEAP levels were obtained from an LMax II 384 luminometer (Molecular Devices, Sunnyvale, CA, USA) and SoftMax Pro 4.7.1 (Molecular Devices, Sunnyvale CA, USA). Statistical tests were done using one-way analysis of variance (ANOVA) and Tukey's post-hoc test on GraphPad.

Real time RT-PCR Assays were performed essentially as previously described (Shi-wen et al. 2006; Kennedy et al. 2007; Pala et al. 2008). Cells were plated onto a six well plate and allowed to grow for 24 h at 37°C. Cells were then treated for 18 h with p38 MAP Kinase inhibitor SB203580, MEK/ERK inhibitor U0126, TGFβ type I receptor (ALK4/5/7) inhibitor SB431542 or the protein kinase C (PKC) inhibitor bisindolmaleimide I (all Calbiochem, La Jolla, CA, USA) at a concentration of 10 µM, except SB203580 which was used at 30 µM. Alternatively, cells were serum-starved for 18 h prior to addition of actinomycin D (15 µg/ml; Sigma) for 1 h. Cells were then treated in the presence or absence of TGFβ to 6 h. RNA was harvested using the Trozol (Invitrogen) and used for Real-Time RT-PCR. 25 ng of RNA was reverse transcribed and amplified using TaqMan Assays on Demand (Applied Biosystems) in a 15 µl reaction containing primers for TaqMan Human CCN2 (Applied Biosystems) and 6-carboxyfluorescein labeled TaqMan MGB probe. Reverse Transcriptase One-Step qPCR Mastermix (Applied Biosystems) was added to samples and the ABI Prism 7900 HT sequence detector (Perkin-Elmer-Cetus, Vaudreuil, QC) was used according to manufacturer's instructions to detect amplified sequences. Samples were run in triplicate, and expression values were standardized to control values from GAPDH primers using the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001). Statistical analysis was done using one way ANOVA and Tukey's post-hoc test on GraphPad.

Results

CCN2 production in mouse HSC is associated with the activated phenotype

Since CCl_4 causes liver injury and HSC activation *in vivo*, this approach was used to explore CCN2 production by mouse HSC. As shown in Fig. 1A, 92% of the HSC isolated from the livers of CCl_4 -treated mice were CCN2-positive whereas only 12% of the HSC from oil-treated mice were CCN2-positive. Cultured HSC from non-treated mice, contained cytoplasmic oil droplets on days 1–2 that were visible under phase contrast and when stained with oil red (Fig. 1B). By day 7, the droplets were no longer present and the cells had undergone dramatic phenotypic changes, appearing myofibroblastic and positive for αSMA expression (Fig. 1B and data not shown). Whereas freshly isolated HSC exhibited very low expression of CCN2, the amount of CCN2 mRNA increased as a function of time in culture and was correlated with the autonomous transformation of the cultured cells from a quiescent to activated phenotype (Fig. 1C). Culture-induced HSC activation was also associated with a concomitant increase in TGF- β expression (Fig. 1C). Collectively, these data showed that activation of mouse HSC either *in vivo* or *in vitro* is accompanied by elevated CCN2 production.

Basal and TGF β -induced CCN2 expression in HSC depends on transcription

To begin to probe the mechanism underlying the over-expression of CCN2 in 14 day culture-activated HSC, we assessed if autocrine TGF β signaling could contribute to this process. First, we ascertained whether transcriptional regulation was likely to play a role. To perform this assay, we serum-starved culture-activated HSC for 18 h, and cells were treated in the presence or absence of TGF β for 6 h. Prior to addition of TGF β , cells were treated for 1 h in the presence or absence of actinomycin D, which blocks *de*

novo transcription. RNAs were harvested and subjected to real-time PCR analysis to detect CCN2 mRNA. We found that actinomycin D reduced basal CCN2 mRNA expression in HSC (Fig. 2). Moreover, actinomycin D blocked TGF β -

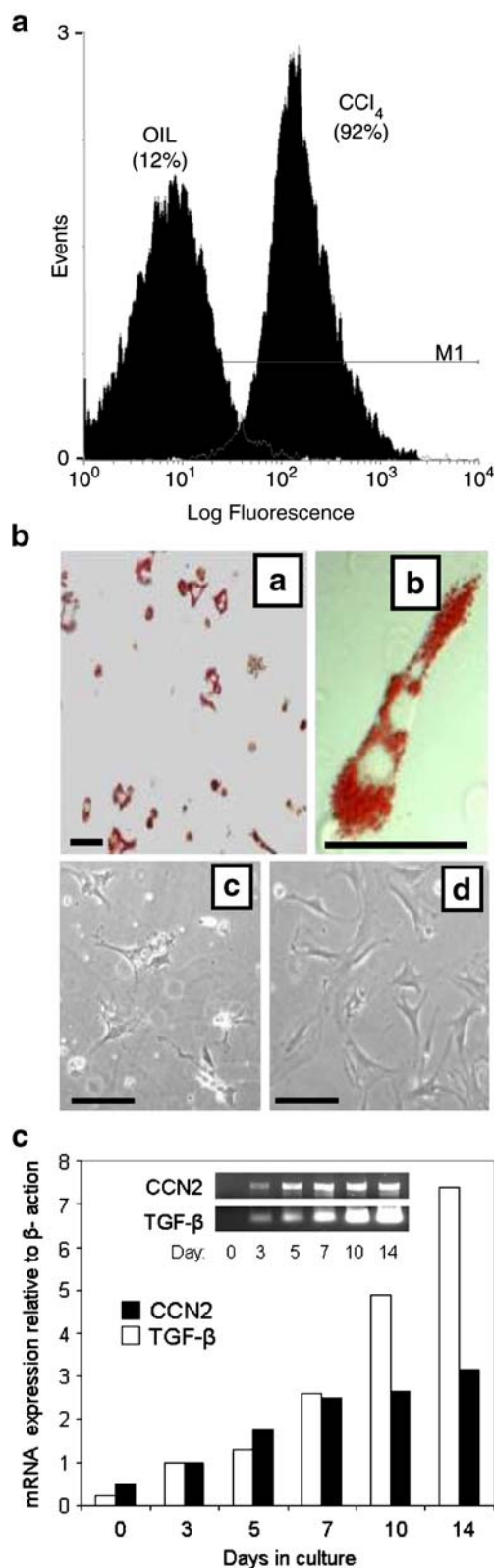


Fig. 1 CCN2 expression in activated mouse HSC. **A** FACS analysis of CCN2-positive HSC from Balb/c mice receiving oil or CCl_4 administration. HSC from each treatment group ($n=3$) were isolated and a total of 10,000 cells were counted. The absolute number of CCN2-positive cells is expressed as a percent of the total number counted. **B** Characterization of cultured mouse HSC. Primary cultures of mouse HSC on day 1 (*a*, *b*) or day 2 (*c*) were poorly spread and contained oil droplets that stained with oil red. By day 7 (*d*), cells had lost their oil droplets, had numerous cytoplasmic extensions, and were αSMA -positive (not shown). *Scale bars*: 100 μm . **C** Reverse-transcriptase PCR analysis of CCN2 or TGF- β mRNA expression in mouse HSC during culture-induced activation. Data from each time point shown in the inset were scanned and the signal intensity was determined relative to that of β -actin. Data shown in A–C were typical of three or more independent analyses

induced CCN2 mRNA expression (Fig. 2). Thus transcriptional regulation contributes to both basal and TGF β -induced CCN2 expression in HSC.

Basal and TGF β -induced CCN2 expression in HSC depends on ALK5 and Smad and Ets elements in the CCN2 promoter

To begin to evaluate the hypothesis that TGF β signaling was responsible for the elevation of CCN2 expression during HSC activation and to begin to determine which promoter elements contributed to CCN2 overexpression in HSC, we first ascertained the elements required for TGF β -induced CCN2 expression. HSC were transfected with CCN2 promoter-SEAP reporter constructs containing regions spanning between -805 to +17, or otherwise identical constructs in which Smad or Ets-1 sites were mutated (Holmes et al. 2001; Leask et al. 2003; Van Beek et al. 2006; Pickles and Leask 2007). Mutation of either the Smad or the Ets-1 site significantly reduced the ability of the CCN2 promoter to respond to TGF β (Fig. 3). Similarly, addition of the TGF β type I receptor (ALK4/5/7) inhibitor SB431542 blocked the ability of TGF β to induce the CCN2 promoter. Thus, the induction of the CCN2 promoter by TGF β in HSC required ALK5, and the Smad and Ets-1 sites.

To further investigate the specific promoter elements involved in CCN2 overexpression in HSC and to probe the involvement of autocrine TGF β signaling in this process, cells were transfected with CCN2 promoter-SEAP reporter constructs containing the wild-type promoter sequence, or point mutations to either the Smad or Ets-1 sites. Point mutation of either the Smad or Ets-1 sites resulted in a significant decrease in CCN2 promoter activity (Fig. 4). Moreover, ALK5 inhibition also reduced activity of the wild-type CCN2 promoter (Fig. 4). These results suggest that activation of CCN2 expression in HSC is due at least in part to activated TGF β signaling.

To confirm these data and to determine which signaling pathways contributed to CCN2 overexpression in HSC, cells were treated with inhibitors to p38 (SB 203580, p38i), MEK/ERK (U0126, ERKi), ALK4/5/7 (ALKi) or PKC (bisindolmaleimide I, PKCi) at concentrations previously demonstrated to selectively block action of the respective targets. Real-time RT-PCR analysis determined that while all inhibitors somewhat reduced CCN2 mRNA expression, inhibition of either ALK5 and PKC resulted in a potent (~70%) reduction of CCN2 mRNA expression in HSC (Fig. 5). Collectively, these data suggest that overexpression of CCN2 in HSC is due, at least in part, to autocrine TGF β signaling acting through ALK5 and the Smad and Ets-1 sites in the CCN2 promoter.

Discussion

CCN2 is over-expressed in fibrotic diseases, including those of the liver; indeed, CCN2 expression is a reliable marker of the severity of fibrosis (Blom et al. 2002; Leask and Abraham 2006). CCN2 appears to be a key contributor to the fibrogenic phenotype of HSC in that CCN2 promotes proliferation, adhesion, and collagen production of this cell type through integrins and HSPGs (Gao et al. 2004; Gao and Brigstock 2004). Through its role as a pro-adhesive matricellular molecule, CCN2 can also amplify adhesive signaling emanating from other pro-fibrotic ligands such as fibronectin and TGF β , and pro-inflammatory ligands such as TNF α (Shi-wen et al. 2006; Chen et al. 2004, 2007). Moreover, CCN2-deficient mouse embryonic fibroblasts show impairment of a myriad of fibrogenic activity, including cell adhesion, matrix contraction, and gene expression (Shi-wen et al. 2006; Kennedy et al. 2007; Pala et al. 2008). Thus CCN2 is a promising marker of fibrosis and also represents an excellent direct anti-fibrotic target (Blom et al. 2002; Leask and Abraham 2006). As such,

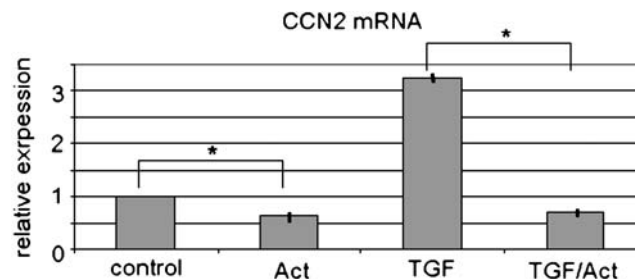


Fig. 2 Transcription is required for basal and TGF β -induced CCN2 mRNA in HSC. Culture-activated HSC were serum-starved for 18 h prior to addition of actinomycin D (15 μ g/ml, Act) for 1 h. Cells were then incubated for 6 h in the presence or absence of TGF β 1 (4 ng/ml; TGF). RNAs were harvested and subjected to real-time PCR analysis with primers recognizing CCN2 or 18S. Relative CCN2 expression was calculated using the $\Delta\Delta C_t$ method. Fold increase relative to

control, untreated samples is shown. Assays were performed in triplicate. Standard deviation was less than 10%. Whereas TGF β increases CCN2 mRNA in HSC, this induction is blocked by actinomycin D (TGF vs. TGF/Act, * p <0.05). Moreover, basal CCN2 expression is reduced in the presence of actinomycin D (control vs. Act, * p <0.05)

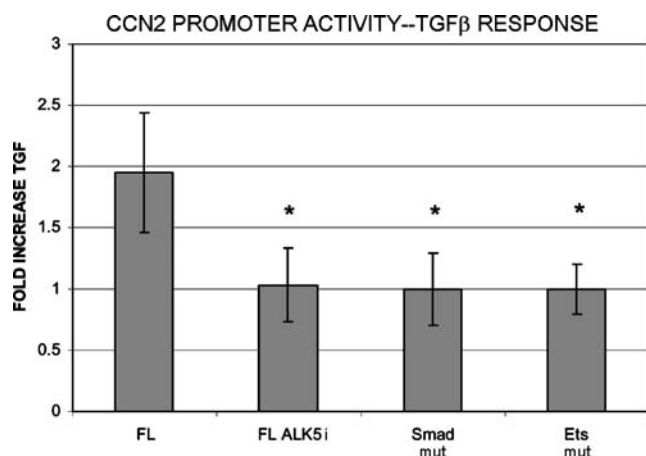


Fig. 3 Activity of CCN2 promoter constructs in HSC: elements required for TGF β response. HSC (14 days in culture) were seeded into 12 well plates and transfected with CCN2 promoter/SEAP reporter constructs, as described in Methods. Experiments were performed in quadruplicate, at least twice. SEAP reporter activity (after adjustment for differences among samples in transfection efficiency as revealed by cotransfected CMV- β -gal reporter construct) was calculated. Fold increase in response to TGF β for each construct and treatment was then calculated. Average fold increase \pm standard deviation is shown. Post-transfection, cells were serum-starved for 18 h prior to addition of TGF β 1 (4 ng/ml) for an additional 24 h. When indicated, cells were pre-treated for 45 min with 10 μ M ALK4/5/7 inhibitor prior to addition of TGF β 1. Whereas TGF β induced a promoter/reporter construct containing nucleotides spanning -805 to +17 CCN promoter (FL), mutation of the Smad or Ets element within this promoter fragment or addition of ALK4/5/7 inhibitor (ALKi) blocked this induction (* p <0.05)

CCN2 is expressed in activated HSC and is induced by TGF β in this cell type. However, until this report there was no proposed mechanism for CCN2 induction and overexpression in activated HSC. Moreover, although TGF β

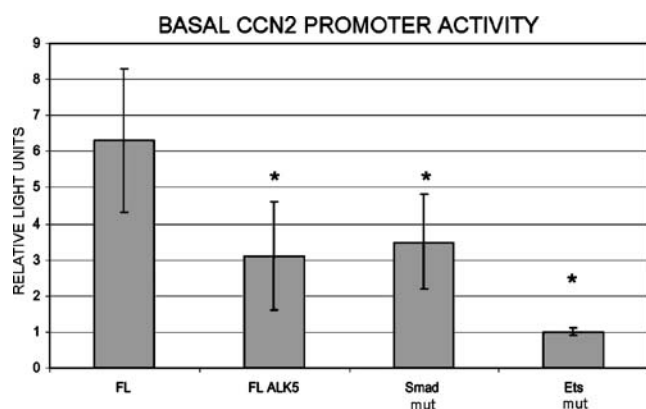


Fig. 4 Activity of CCN2 promoter constructs in HSC: elements required for basal promoter activity HSC (14 days in culture) were seeded into 12 well plates and transfected with CCN2 promoter/SEAP reporter constructs, as described in Methods. SEAP reporter activity (after adjustment for differences among samples in transfection efficiency as revealed by cotransfected CMV- β -gal reporter construct) was calculated. Experiments were performed in quadruplicate, at least twice. Relative light units (averages \pm standard deviation) are shown. Mutation of the Smad or Ets element or addition of ALK4/5/7 inhibitor also reduced basal CCN2 promoter activity (* p <0.05)

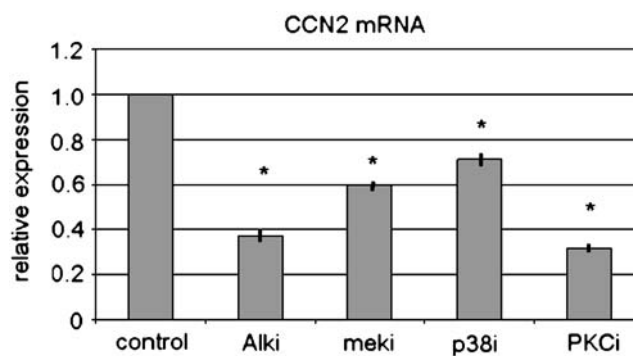


Fig. 5 The effect of signal transduction inhibitors on CCN2 mRNA expression in HSC. HSC (14 days in culture) were incubated for 24 h with: DMSO (control); ALK4/5/7 inhibitor (Alki, 10 μ M); the MEK inhibitor U0126 (meki, 10 μ M); the p38 inhibitor SB 203580 (p38i, 30 μ M) or the PKC inhibitor Bisindolylmaleimide I (PKCi, 10 μ M). RNAs were harvested and subjected to real time PCR analysis with primers recognizing CCN2 and 18S. Relative expression (to 18S) was calculated and adjusted to control (DMSO) alone treated values using the $\Delta\Delta C_t$ method. Average expression values (three replicates) is shown. The standard deviation was less than 5% (* p <0.05). Blocking the TGF β type I ALK4/5/7 receptor (Alki) and PKC (PKCi) potentially reduces basal CCN2 mRNA expression

can mediate the induction of CCN2 in normal mesenchymal cells, TGF β through the Smad/ALK5 pathway does not appear to mediate the overexpression of CCN2 in activated myofibroblasts (Holmes et al. 2001; Chen et al. 2006). As there is currently no sufficient treatment for liver fibrosis, obtaining a greater understanding of the mechanisms underlying HSC activation, such as the mechanism underlying the overexpression of CCN2, is crucial in order to identify novel potential drug targets and to evaluate the possibility of targeting the TGF β receptor ALK5 to combat fibrosis.

In this report we probe the mechanism underlying the regulation of CCN2 expression in HSC. Basal and TGF β -induced expression in activated HSC relied at least on part on gene transcription, as basal CCN2 mRNA expression was reduced and TGF β -induced CCN2 mRNA expression was ablated in the presence of actinomycin D. It has been shown previously in normal fibroblasts and mesangial cells that CCN2 is induced by TGF- β acting through the Smad 3/4 pathway and an Ets element (Leask et al. 2003; Chen et al. 2002; Van Beek et al. 2006). In this report we show that such a mechanism also operates in HSC, in that the increased activity of the CCN2 promoter in response TGF β was diminished by mutation of the Smad and Ets elements. We extend our data in other cell systems to show that, in HSC, ALK4/5/7 inhibition can block the TGF β -induction of the CCN2 promoter and mRNA. For the first time, however, we present data indicating that activated TGF β signaling can contribute to CCN2 overexpression in myofibroblasts by showing that in activated HSC, mutation of the Ets and Smad elements and addition of an ALK4/5/7

inhibitor reduced the activity of the CCN2 promoter in this cell type. Moreover, ALK4/5/7 inhibition reduced CCN2 mRNA expression in this cell type. Collectively, these results support the notion that activated TGF β signaling may contribute to HSC activation. Conversely, in scleroderma fibroblasts in which CCN2 overexpression is TGF β /Smad/ALK5-independent but ET-1 dependent and in pancreatic cancer cells CCN2 expression is independent of both TGF β and ET-1 (Holmes et al. 2001; Chen et al. 2006; Pickles and Leask 2007). Our results indicate that, CCN2 overexpression in HSC can be antagonized by ALK4/5/7 inhibition and support the notion that activated TGF β signaling via the ALK4/5/7 receptor may play a key role HSC activation. Given that CCN2 overexpression is a faithful marker of the fibrogenic phenotype, ALK4/5/7 inhibition may be an appropriate method of attenuating liver fibrosis.

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