

Expression of connective tissue growth factor in pancreatic cancer cell lines

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Abstract. Connective tissue growth factor (CTGF/CCN2) is thought to play a role in normal wound repair and bone remodeling, but also promotes fibrosis in several disease processes including diabetic nephropathy, scleroderma and pancreatitis. A contribution to desmoplasia associated with pancreatic cancer progression has also been proposed. CTGF is induced by TGF β in diverse cell types, but TGF β receptor mediated signaling is impaired in pancreatic cancers and cell lines, usually due to DPC4/Smad4 mutations which arise during the later stages of intraepithelial neoplastic progression. Therefore, in order to define signaling pathways that mediate basal and TGF β -induced CTGF expression in normal and transformed cells, we compared CTGF gene regulation in pancreatic cancer cells and fibroblasts by measuring the effects of small molecule inhibitors and dominant negative mutants of signaling proteins on CTGF promoter reporter activity, message, and protein expression. We determined that the previously identified TEF-1 *cis* element is essential for CTGF promoter reporter activity in pancreatic cancer cell lines. Whereas p38 mediated CTGF induction by TGF β in fibroblasts, MEK/ERK signaling mediated TGF β -induced CTGF expression in pancreatic cancer cells and was also responsible for basal CTGF expression in pancreatic cancer cell lines with defective Smad signaling. Since activating Ras mutations occur in the earliest stages of pancreatic cancer, CTGF may be induced independent of Smad4 in pancreatic cancer cells.

Introduction

The high mortality rate associated with pancreatic cancer has been attributed to its aggressive nature and advanced stage at the time of diagnosis (1). Some of the molecular changes associated with the earliest stages of the disease have been identified and include mutations of Ras, overexpression of p21CIP/WAF, cyclin D1, p53 and HER2/neu, mutation of p16INK4A and DPC4 (deleted in pancreatic cancer locus 4)/Smad4 (2). A pathologic characteristic of pancreatic cancers is their extensive desmoplasia, and there is evidence that TGF β plays an essential role in pro-fibrogenic processes by the induction of CTGF and extracellular matrix proteins, including collagen and fibronectin (3-5).

Human CTGF is a 349-amino acid polypeptide, comprised of four distinct domains: an insulin-like growth factor binding domain, a von Willebrand Factor type C domain, a thrombospondin-1 domain, and a C-terminal cystine knot (4). Increased expression of CTGF has been found in inflammatory bowel disease (6), skin lesions from scleroderma and systemic sclerosis (7), renal fibrosis (8), liver fibrosis (9), pancreatitis (10,11) and pancreatic cancer (12,13).

TGF β is well known to stimulate CTGF expression in different cell types. In general, TGF β induces gene transcription by binding to the TGF β type II receptor (TGF β RII), which associates with and phosphorylates TGF β type I receptor (TGF β RI). This receptor kinase in turn phosphorylates receptor-associated Smad proteins (Smad2 or 3), which causes them to associate with co-Smad4 and translocate to the nucleus. In the nucleus, Smads are known to associate with diverse transcription factors and transcriptional co-activators, which enhance and modulate both their binding to DNA and their transcriptional activity (reviewed in refs. 14,15). However, Smad-independent TGF β signaling has also been extensively described, and can involve the mitogen-activated protein kinase (MAPK) pathways, including Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 mitogen-activated protein kinase. Phosphatidylinositol 3-kinase-Akt (PI3K-Akt) and RhoA pathways have also been reported to be activated by TGF β signaling (14,16).

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TGF β -mediated signaling pathways may be of particular relevance in pancreatic cancers and cell lines where TGF β signaling is often impaired by DPC4/Smad4 mutation or by decreased expression or mutation of TGF β RII (17,18). Mutation and deletion of CTGF promoter elements have demonstrated that several different signaling pathways mediate the effects of TGF β on CTGF transcription in a cell-type specific manner. In NIH 3T3 fibroblasts, an -805/+17 CTGF promoter reporter [expressing secreted enhanced alkaline phosphatase (SEAP)] was induced by TGF β in a protein kinase C- and Ras/MEK/ERK-dependent manner, and this was antagonized by c-Jun and by MEKK1 activities (19). Additional investigations using the same reporter showed that while activation by TGF β required the Smad binding element (SBE; CAGACGGA) at -174 (numbered according to ref. 20), a different basal control element (BCE) located at -156 was responsible for high basal promoter activity in scleroderma fibroblasts, independent of TGF β (21). Another NIH 3T3 study using a -742/+133 pGL3 luciferase reporter found that ERK and p38 inhibitors were both able to reduce TGF β -induced CTGF promoter reporter activity (22). Transfection of a series of heterologous thymidylate kinase (TK) promoter reporter deletion constructs into human foreskin fibroblasts (HFF) identified a pair of transcription enhancer factor-1 (TEF-1; GAGGAATG) consensus sequences at -102 of the CTGF promoter (23,24). These elements were required for basal and TGF β -induced promoter activity. In human chondrocytes, a series of promoter reporter deletion constructs within a pGL3 vector also showed that the BCE at -156 is important for basal and TGF β -stimulated activity (25). Similar effects were observed using fibroblasts (26). In addition, an Sp1 site located downstream of the TATA box was found to be required for basal activity in normal and scleroderma fibroblasts, and reporter activity was not impaired by mutation of the SBE in these cells (27).

On the other hand, TGF β up-regulation of CTGF expression in human lung fibroblasts clearly required Smad2 and JNK signaling (28). In airways smooth muscle cells it was found that TGF β induction required ERK and JNK signaling, which also correlated with Smad2/3 phosphorylation (29). Induction of a CTGF -2065/+72-pGL3 reporter by TGF β in aortic smooth muscle cells was also potentially inhibited by a peroxisome proliferator-activated receptor (PPAR) γ ligand, and mediated by Smad3 (30). TGF β activation of a -805/+17 SEAP CTGF promoter reporter in rat mesangial cells required the SBE, whereas mutation of the BCE inhibited both basal and TGF β -induced promoter reporter activity. This study also found that MEK inhibitor U0126 completely inhibited reporter activity, as well as CTGF protein synthesis, and that PKC inhibitors and dominant negative Ras were also inhibitory. These authors hypothesized that Smad signaling is necessary but not sufficient for CTGF induction by TGF β , and that there is an additional requirement for Ras/MEK/ERK signaling (31). Several studies have implicated the small Rho kinase signaling pathway in CTGF up-regulation by TGF β (32), including one utilizing a -2065/+73 pGL3 luciferase reporter in human lung fibroblasts (33 and refs. therein).

Due to the TGF β -dependent and -independent pathways described for CTGF expression, we sought to identify the signaling pathways and transcription factors involved in basal

and TGF β -mediated CTGF up-regulation in pancreatic cancer cells, where TGF β signaling pathway is frequently defective or non-functional. Specific inhibitors of several different signaling pathways were used to assess their roles in regulating CTGF transcription. We determined that CTGF expression in pancreatic cancer cells is regulated by the MEK/ERK signaling pathway, and that the TEF-1 *cis* element is essential for both basal and TGF β -induced promoter activity.

Materials and methods

Reagents. SP600125 (JNK inhibitor) and SB202190 (p38 inhibitor) were obtained from BioMol (Plymouth Meeting, PA); U0126 (MEK inhibitor) from Cell Signaling (Beverly, MA); PD98059 (MEK inhibitor) was obtained from EMD Biosciences (La Jolla, CA). SB431542 (TGF β RII kinase inhibitor) was obtained from Tocris Biosciences (Ellisville, MO). Antibodies against ERKp44/42 Thr202/Tyr204 (#9101) and phospho-p38Thr180/Tyr204 (#9211) were obtained from Cell Signaling Technology. Oligonucleotides were synthesized by Operon (Alameda, CA).

Cell lines and plasmids. Cell lines were originally obtained from ATCC and maintained at 37°C in a 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, penicillin, and streptomycin. Human pancreatic ductal adenocarcinoma cell lines used were as follows: AsPC-1 [from ascites of a grade 2 primary tumor; DPC4 negative by Western blotting (34)]; BxPC3 (grade 2, containing a homozygous deletion of DPC4); Panc-1 (grade 3 primary tumor; wild-type with respect to DPC4); MIA PaCa-2 [grade 3 tumor; wild-type DPC4 but with low TGF β RII (18)]; SW1990 (splenic metastasis; DPC4 status not previously reported); and the COLO-357 pancreatic cancer cell line (originally from lymph node metastases; DPC4-expressing subline kindly provided by Professor Murray Korc of Dartmouth Medical School). NIH 3T3 is a mouse embryonic fibroblast cell line.

CTGF promoter reporter and an SBE-mutated version of the same were prepared from the corresponding SEAP reporters described previously (27), by sub-cloning the *KpnI*/*HindIII* fragment into pGL2 (Promega, Madison, WI). The human CTGF promoter in this reporter construct contains base pairs -775 to +73 (numbering according to ref. 20). Plasmids were prepared using the EndoFree Plasmid MAXI prep kit (Qiagen, Valencia, CA). The TEF-1 mutated version of this reporter was made by PCR using the megaprimer method (35), with forward primer at -123 bp (5'-GATGAGG CAGGAAGGTGGGGATCCGGATGAGGAATGTCCCTG TTTGTGTAGGACTCCATTCAGCTC-3'; altered sequence underlined) paired with pGL2 primer (Promega) to amplify and mutate the first of the TEF-1 sequences. The resulting PCR fragment was used as the reverse primer in touchdown PCR, with an upstream primer at -654 bp (5'-CTGAGTCAC ACGCGTCTTTGTTC-3'). The resulting PCR product was cut with *MluI*/*NotI*, ligated into the same site of the parental vector, and its sequence verified.

RNA isolation and RT-PCR. Cells were serum-starved overnight in DMEM containing insulin-transferrin-selenium

(ITS; Invitrogen, Carlsbad, CA) before treatment with TGF β (10 ng/ml) for indicated times. Total RNA was harvested using TriReagent (Molecular Research Center Inc., Cincinnati, OH). Total RNA was reverse transcribed using Superscript II (Invitrogen) primed with random hexamers. The sequence from 803-1126 bp was amplified using HotStar Taq polymerase with the following primers: (forward, human) 5'-CGGGTTA CCAATGACAACGC-3'; (forward, mouse) 5'-CGAGTTAC CAATGACAATAC-3'; and (reverse) 5'-TAATGGCAGGCA CAGGTCTTG-3' (annealing temperature 58°C). 18 S rRNA was simultaneously amplified as an internal standard, using primers from the Ambion Quantum RNA 18S Internal Standards kit. PCR products were separated using agarose gel electrophoresis, with detection using ethidium bromide. Bands were quantitated by densitometry, using NIH Image software.

Transient transfection and inhibitor assays. Cells were transfected with reporter vectors, using Superfect as a transfection reagent and including pRLO renilla luciferase expression vector as internal control to correct for transfection efficiency (36). Cells were serum-starved in ITS media on the day before treatment with 10 ng/ml TGF β for certain times. Cells were harvested using Passive Lysis buffer (Promega), and reporter activities were measured using the Dual-Luciferase™ reporter assay system (Promega). Promoter activities are expressed as relative light units (RLU), calculated as the ratio of pGL2 firefly:pRLO renilla luciferase activities. Standard deviations are indicated by error bars, with statistical differences analyzed by t-test and indicated with asterisks (*P<0.05 and **P<0.01).

Cell staining. Cells on coverslips were fixed for 2 h with 10% buffered formalin, rinsed with phosphate buffered saline (PBS), blocked for 5 min with 10 mM NH₄Cl/PBS, and then blocked and stained using the Vectastain Elite Goat IgG kit (Vector Labs, Burlingame, CA), with a 1:500 dilution of goat anti-CTGF L20 (sc-14939, Santa Cruz Biotechnology, Santa Cruz, CA) in 1% BSA/TBST for 1 h. AEC chromogen (red; Zymed) was used for the final color development step, and nuclei were counterstained using Meyer's hematoxylin.

Western blotting. Cells were serum-starved in DMEM/ITS media and treated with TGF β (10 ng/ml) overnight or for indicated times. Heparin (50 μ g/ml) was added at the same time, in order to enhance recovery of secreted CTGF from the culture medium. Conditioned medium was analyzed directly by SDS-PAGE and Western blotted using a 1:500 dilution of goat anti-CTGF (L20; Santa Cruz Biotechnology), with bands detected using rabbit anti-goat horseradish peroxidase conjugate (Zymed, South San Francisco, CA) and chemiluminescent detection (ECL Blotting System, Amersham).

Electrophoretic mobility shift assay and supershift assay. Cells were serum-starved in ITS/DMEM and treated with TGF β for 1 h. Nuclear extracts were prepared as previously described (37), with protein concentrations determined using the Bradford assay method (BioRad, Hercules, CA).

TEF probes. A labeled, double-stranded probe encompassing base pairs -104 to -65 (5'-GGGAGGAATGCGAGGAATG

TCCCTGTTTGTGTAGGACTCC-3'; TEF-1 consensus sequences underlined) was prepared by annealing forward and reverse oligonucleotides to give 3' overhangs, which were filled in using Klenow fragment and ³²P- α -dCTP.

Mutated TEF-1 oligonucleotides had the following sequences: 5'TEF, 5'-GGGAGGggcaCGAGGAATGTCCCT GTTTGTGTAGGACTCC-3'; 3'TEF, 5'-GGGAGGAATGC GAGGggcaTCCCTGTTTGTGTAGGACTCC-3'; and 5' and 3'TEF, 5'-GGGAGGggcaCGAGGggcaTCCCTGTTTGTGT AGGACTCC-3'; with mutations shown in lower case.

Sp1 probes. Oligonucleotides for Sp1 EMSA were similarly radiolabeled. 5'-Sp1 encompassed base pairs -50 to -20 (5'-GAGCCGCGGCCCGCCCGGAGCGTATAAAAGCCTC-3' (putative Sp1 site underlined). 5'Sp1-poly was an oligonucleotide probe designed to contain the polymorphic sequence of the same region (20) (5'-GAG-CGC-GCCGCCCGGAGC GTATAAAAGCCTC-3'). 3'-Sp1 (from base pair -32 to +3) had the sequence 5'-GCGTATAAAAGCCTCGGGCCGCCGCCCAAATC-3'.

Nuclear extracts (5 μ g protein) were incubated with radiolabeled probes at room temperature for 20 min in a solution containing 1 μ g of poly(dI-dC) in 10 mM Tris-HCl at pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 0.5 mM dithiothreitol, 0.05% NP-40 and 10% glycerol. For supershift assays, antibody specific for Sp1 (PEP2, Santa Cruz Biotech) was added to the reaction for 30 min prior to addition of the probe. For competition experiments a 100-fold excess of unlabeled oligonucleotide was added. Samples were applied to 4% polyacrylamide gels in Tris-acetate-EDTA buffer at 130 V for 3 h; the gel was dried and exposed to X-ray film.

Results

TGF β up-regulates CTGF expression in Panc-1 cells via TGF β IR and Smad3. Basal and TGF β -induced expression of CTGF in HFF and Panc-1 cells was readily demonstrated by immunohistochemical staining of cultured cells (Fig. 1A-D). Consistent with the cell staining experiments, Panc-1 cells typically exhibited some basal expression of CTGF message (Fig. 2A), and this was rapidly (<1 h) increased and maximally sustained upon treatment with TGF β concentrations as low as 1 ng/ml. Similar expression kinetics, i.e. rapid induction by low levels of TGF β within 1 h and sustained expression up to 24 h, were exhibited by HFF cells (not shown). Unlike Panc-1 cells, NIH 3T3 exhibited no basal CTGF expression and showed a delayed response to TGF β that was not sustained beyond 18 h (Fig. 2B). In these experiments CTGF mRNA was transiently induced by simple media change in both HFF and Panc-1. CTGF protein synthesis, as measured by Western blotting of medium harvested from cultured cells treated with TGF β , also showed profound up-regulation of CTGF in all three cell lines (Fig. 3 and NIH 3T3 not shown). Under non-reducing conditions bands for human CTGF appeared as a heterogeneous mixture of glycoforms between 30 and 35 kDa. A minor proteolytic fragment (~21 kDa) was also faintly visible in media harvested from HFF; similar fragments were also observed for Panc-1 in other experiments.

Preliminary experiments showed that the endogenous alkaline phosphatase activity of pancreatic cancer cell lines

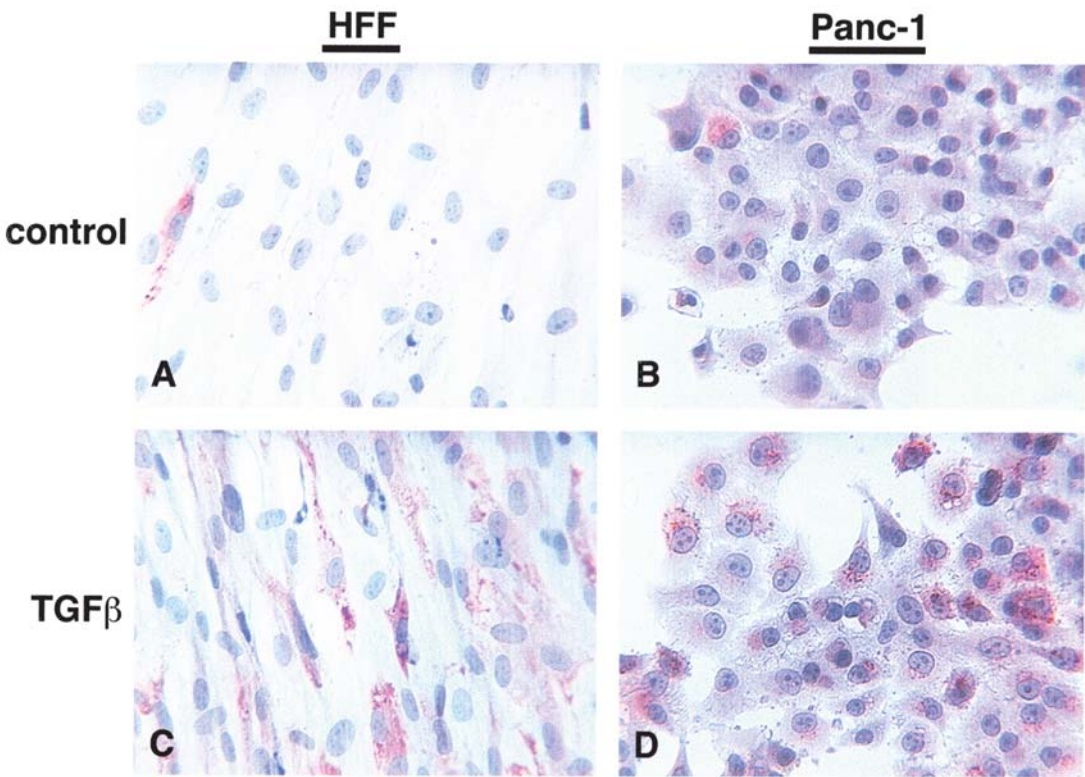


Figure 1. TGFβ up-regulated CTGF protein expression. HFF (A and C) and Panc-1 (B and D) cells grown on glass coverslips were serum-starved in DMEM-ITS media and then treated (C and D) with TGFβ overnight. HFF cells were simultaneously treated with brefeldin A (3.6 uM) to ensure retention of CTGF within the cells. Cells were stained immunohistochemically using goat anti-CTGF, with AEC (red) detection and hematoxylin (blue) nuclear counterstain.

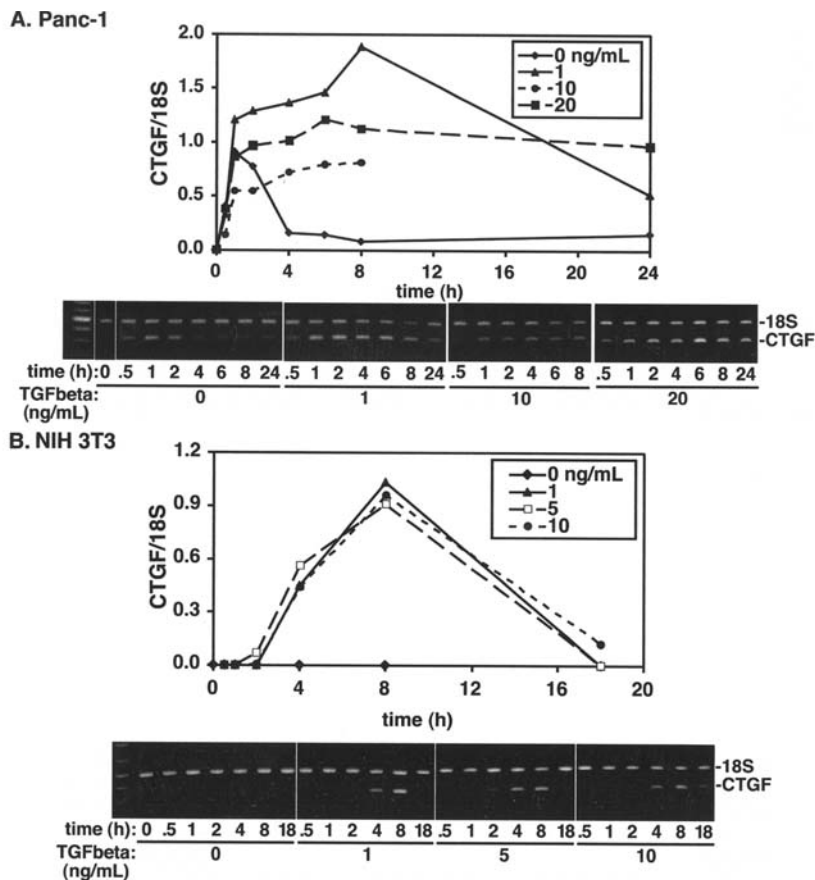


Figure 2. CTGF induction in Panc-1 cells was more rapid and sustained than in NIH 3T3 cells. The time- and dose-dependence of CTGF message induction by TGFβ in (A) Panc-1 and (B) NIH 3T3 cells was analyzed by semi-quantitative RT-PCR. Values expressed as a ratio of CTGF:18S band intensities from densitometry (NIH Image software) of ethidium bromide-stained bands are plotted versus time above photos of the gels.

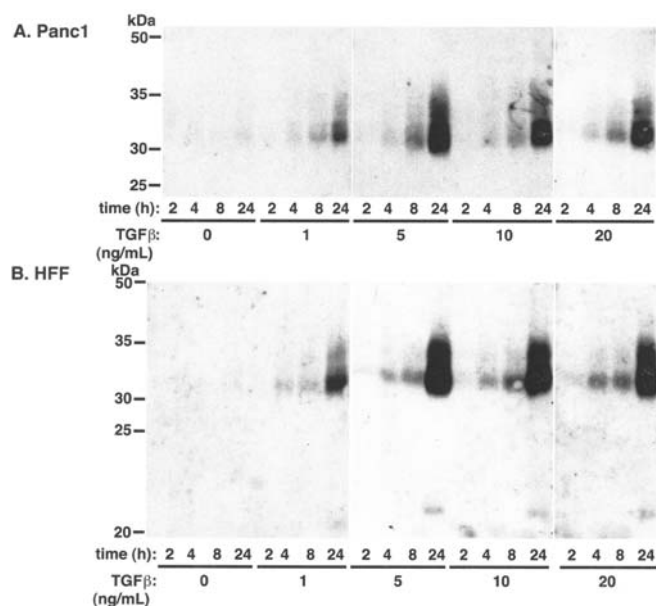


Figure 3. Secretion of CTGF into culture media followed similar kinetics for Panc-1 and NIH 3T3. Media harvested from serum-starved cells treated with TGF β for the indicated times were directly analyzed by non-reducing SDS-PAGE and Western blotting as described in Materials and methods.

was too high and variable to permit the use of SEAP reporter vectors. Therefore a pGL2 firefly luciferase vector containing base pairs -775/+73 of the CTGF promoter was used to establish a CTGF promoter reporter assay. This reporter was readily activated by TGF β in NIH 3T3 cells (Fig. 4A), demonstrating that all sequence elements essential for activation by TGF β in this cell line are included within the CTGF 5'-flanking sequence from -775 to +73. In contrast, we observed only modest, and often statistically insignificant, activation in Panc-1 cells (Fig. 4B), much lower than the ~7-fold increase in endogenous CTGF mRNA observed after TGF β stimulation (Fig. 2A). The weaker response of the -775 promoter to TGF β stimulation in Panc-1 cells was also shown by the pancreatic cancer cell line COLO-357, which possesses an intact Smad signaling pathway (Fig. 4C).

SBE-TK promoter reporter assays demonstrated 7.6-, 2.5- and 6.6-fold activation by TGF β in COLO-357, Panc-1 and NIH 3T3, respectively, indicating that the failure of TGF β to induce the -775 promoter reporter in Panc-1 and COLO-357 cells is not due to these cells' inability to respond to TGF β signaling (Fig. 4D). Thus, in pancreatic cancer cells the stimulatory effects of TGF β on endogenous CTGF gene and protein expression are not fully reflected in the -775 CTGF-promoter reporter assay. However, a comparison of the SBE-TK reporter activity with the -775 CTGF promoter reporter activity shows that the latter is generally half as responsive to TGF β stimulation, such that in NIH-3T3 cells the -775 promoter reporter exhibits approximately 3-fold induction, which is approximately half of the >6-fold induction of the SBE-TK reporter in this cell line. The lower CTGF reporter responsiveness provides a simple explanation for why the -775 CTGF promoter shows activation in NIH 3T3 but not Panc-1, where only a 1.25-fold activation would be predicted from the 2.5-fold activation exhibited by the SBE-TK reporter.

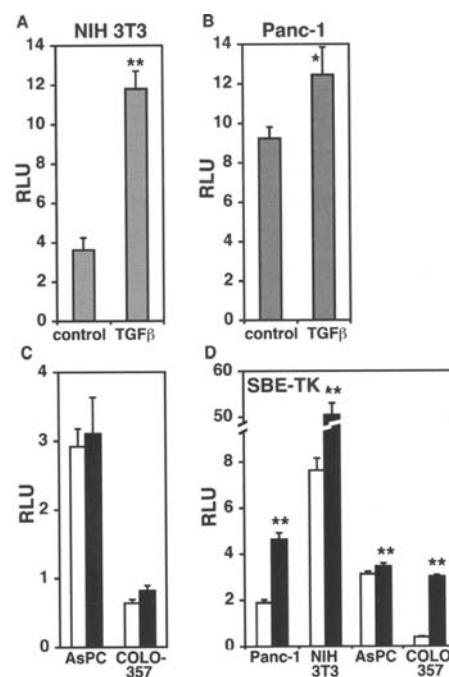


Figure 4. CTGF promoter reporter was responsive to TGF β induction in NIH 3T3 but not Panc-1. CTGF promoter reporter assays in (A) NIH 3T3, (B) Panc-1, and (C) COLO-357, with AsPC included as a negative control. (D) SBE-TK reporter assays in Panc-1, COLO-357 and NIH-3T3, which contain intact Smad signaling pathways, and in AsPC, which do not. TGF β treatments were carried out for 16 h. Open bars are controls; closed bars represent TGF β -treated cells (C and D). Standard deviations are indicated by error bars, with statistical differences between values for TGF β -treated and untreated cells analyzed by t-test (* P <0.05 and ** P <0.01).

The weak response of the -775 CTGF promoter in Panc-1 and COLO-357 cells may also indicate that another *cis*-acting element outside of the -775 to +73 promoter region is required for maximal TGF β induction in pancreatic cancer cells. The pronounced up-regulation of endogenous CTGF mRNA and protein but not the -775 promoter reporter by TGF β could also indicate that endogenous CTGF gene activation by TGF β involves epigenetic changes that are not required and do not occur during transcription of the reporter gene from exogenous plasmid DNA. Conversely, it is possible that the promoter reporter is susceptible to some transcriptionally repressive events that do not affect the endogenous gene. It is also possible that TGF β has a post-transcriptional effect on CTGF mRNA, affecting pre-mRNA splicing or message stabilization, as has been reported (25). However, RT-PCR did not detect any pre-mRNA species or splice variants, and experiments using actinomycin D did not reveal any changes in CTGF mRNA half-life in Panc-1 cells after TGF β stimulation (not shown).

To confirm that CTGF induction by TGF β required the TGF β receptor type I (TGF β RI), kinase inhibitor SB431542 (38,39) was used to block TGF β -mediated phosphorylation of Smads2 and 3, which served to inhibit the induction of endogenous CTGF mRNA and protein in cells expressing Smad4, as well as induction of the -775 promoter in NIH 3T3 cells. This demonstrated that induction of endogenous CTGF and of the -775 promoter is mediated by TGF β RI and depends completely on TGF β RI kinase activity (Fig. 5). Unlike the robust induction of CTGF by TGF β , the low basal levels of

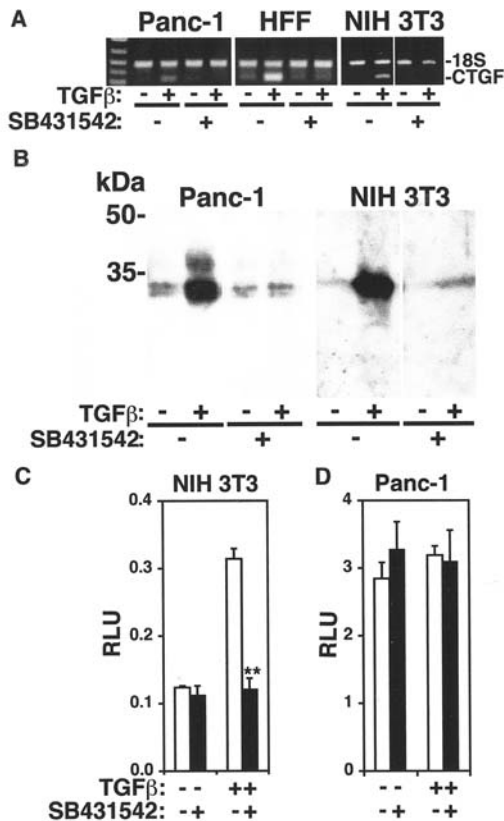


Figure 5. SB431542 small molecule inhibitor of TGF β IR kinase inhibited TGF β -induced, but not basal, CTGF expression, as determined using (A) RT-PCR and (B) SDS-PAGE/Western blotting (reducing conditions). Promoter reporter assays showed inhibition of reporter activity in NIH 3T3 (C) but not Panc-1 (D). Open bars are controls; closed bars represent SB431542-treated cells. TGF β treatments were carried out for 8 h (NIH 3T3) or 4 h (Panc-1). Statistical differences are indicated for SB431542-treated compared with corresponding untreated cells, analyzed by t-test (* $P < 0.05$ and ** $P < 0.01$).

CTGF mRNA (Fig. 5A) and protein (Fig. 5B) in untreated Panc-1 and HFF cells were unaffected by SB431542, indicating that basal expression in Panc-1 cells is not attributable to autocrine TGF β signaling. SB431542 likewise failed to inhibit the basal activity of the -775 CTGF promoter reporter in both Panc-1 and NIH 3T3 cells (Fig. 5C and D), indicating that basal expression in normal and transformed cells is not due to autocrine TGF β signaling, and also indicating that the low response of the reporter in Panc-1 cells cannot be attributed to endogenous TGF β expression.

The activity of CTGF promoter-reporter constructs containing mutations within the SBE was determined. The SBE mutation did significantly impair the responsiveness of the reporter to TGF β in NIH 3T3 cells and in Panc-1 cells, despite the low TGF β responsiveness of the CTGF promoter reporter in the latter. Mutation of the SBE did not reduce the basal activity of the -775 CTGF promoter reporter in either Panc-1 or NIH 3T3 cells, indicating that basal reporter activity is not mediated by this site (Fig. 6). This result provides further evidence that low reporter response in Panc-1 cells is not due to constitutive, autocrine activation by endogenous TGF β . The SBE has been shown to be required for TGF β induction of the CTGF reporter in NIH 3T3 (24) and normal skin fibroblasts, but not in scleroderma fibroblasts which constitutively

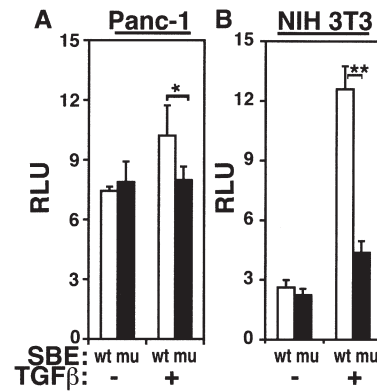


Figure 6. The SBE-mediated promoter reporter activity in NIH 3T3, but a role in Panc-1 cells was less clearly demonstrated. (A) Mutation of the SBE within the CTGF promoter reporter did not reduce basal promoter activity in Panc-1 cells, indicating that basal promoter activity was independent of the SBE. Six-hour TGF β treatment of Panc-1 cells gave a small increase in reporter activity, which was not demonstrated for the SBE mutated reporter. (B) NIH 3T3 positive control shows that mutation of the SBE abrogated all promoter response to TGF β in this cell line. Cells were TGF β -treated for 16 h. Open bars represent the activity of the wild-type reporter; closed bars represent the activity of the SBE mutated reporter. Statistical differences are indicated for the wild-type reporter compared with the SBE mutated reporter, analyzed by t-test (* $P < 0.05$ and ** $P < 0.01$).

express high basal levels of CTGF, independent of TGF β stimulation (21). Other promoter reporter assays using human chondrocytic cell line HCS-2/8 had indicated that essential *cis* elements lie downstream of the SBE, between -103 and -89, at a TGF β responsive element centered near -156 (25). The function of this site in pancreatic cancer cells remains to be evaluated.

Taken together these results demonstrate that basal promoter reporter activity and basal CTGF expression in Panc-1 and NIH3T3 are independent of both TGF β IR kinase activity and the SBE at -174 bp. In contrast, TGF-induced CTGF depends completely on TGF β IRI kinase activity in Panc-1 and NIH3T3. Furthermore, in NIH3T3, TGF β induction of the CTGF promoter reporter is mediated by the SBE, whereas the role of the SBE in Panc-1 cells remains uncertain, due to the low responsiveness of the reporter in this cell line.

The TEF-1 cis element is essential for reporter activity. Several functional *cis* elements within the CTGF promoter have been identified and characterized in different cell types, including the SBE at -174, the BCE at -156, tandem TEF-1 sites at -102 and -95, and Sp1 sites at -41 (5'-Sp1) and -14 (3'-Sp1).

Sp1 has been reported to be a downstream target of MEK/ERK signaling (reviewed in ref. 40) and has also been reported to mediate TGF β -dependent/Smad4-independent up-regulation of p21/WAF1 (24). EMSAs performed using double-stranded oligonucleotides designed from the sequence encompassing the 3' Sp1 site demonstrated binding by Sp1 in nuclear extracts from Panc-1 and NIH 3T3; this binding was enhanced only very slightly for cells treated with TGF β (data not shown). Pre-treatment of cells with p38 or MEK inhibitors did not discernibly alter Sp1 binding (not shown). Our findings are consistent with studies by Holmes *et al* (27) which demonstrated that the 3'Sp1 site is bound by transcription

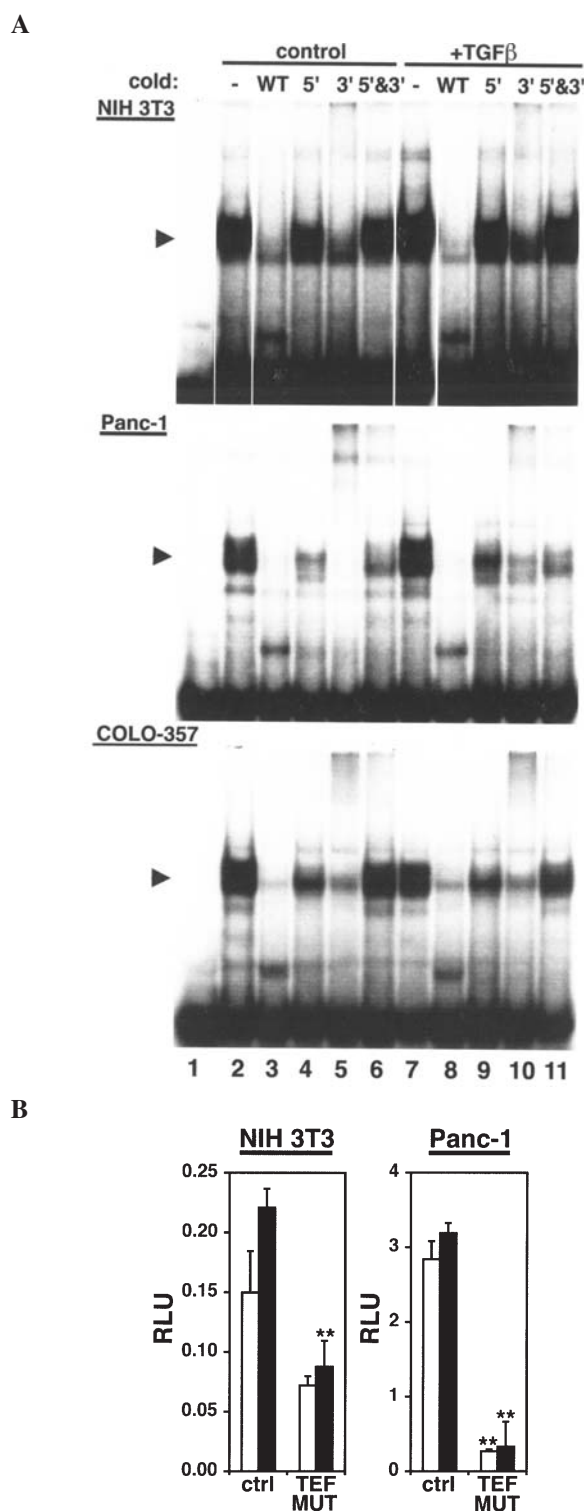


Figure 7. EMSA analysis shows that nuclear proteins from TGF β -treated and untreated Panc-1 and NIH 3T3 cells bound to the tandem TEF-1 sites. (A) Nuclear proteins from NIH 3T3, Panc-1 and COLO-357 cells bound to the TEF-1 elements. Shifted complexes are indicated with an arrow head. Addition of unlabeled (cold) competitor oligonucleotide with identical wild-type (WT) sequence confirmed the binding specificity (lane 3), whereas addition of unlabeled oligonucleotides containing mutations within the 5' or 3' TEF-1 elements (lanes 4 and 5), or both (5'&3'; lane 6), competed less effectively. Lane 1 contains no nuclear protein. (B) A CTGF promoter reporter containing a mutation of the 5' TEF-1 element (TEF-MUT) exhibited reduced reporter activity when transfected into NIH 3T3 and Panc-1 cells, compared to wild-type control (ctrl). Open bars are controls; closed bars represent TGF β -treated (4 h) cells. Statistical differences are indicated for wild-type reporter compared with corresponding assays using TEF-MUT reporter, analyzed by t-test (* P <0.05 and ** P <0.01).

factors; they also demonstrated that the 3'Sp1 is required for promoter activity in NIH 3T3. This remains to be determined for Panc-1 and other pancreatic cancer cell lines.

EMSA's were also carried out using an oligonucleotide designed from the region from -106 to -67, which contains two TEF-1 consensus sequences previously reported to be essential for CTGF reporter activity in NIH 3T3 but not in MvLu epithelial cells (23). Nuclear proteins from TGF β -treated and untreated NIH 3T3, Panc-1 and COLO-357 bound to this probe, but little change in band intensity was observed for nuclear extracts from TGF β -treated cells (Fig. 7A, lanes 2 and 7). The lack of suitable TEF-1 antibodies precluded the use of supershift experiments for identification of the bound protein(s). However, the addition of a 100-fold excess of cold competing oligonucleotides containing 4-bp mutations within either the 5' and/or the 3' TEF-1 consensus sequences competed less effectively than oligonucleotide with wild-type sequence (lanes 4, 5 and 6 compared with lane 3). Sequence alterations to the 5' TEF-1 site provided less effective competition with the probe than the 3' TEF-1 mutant probe for all three cell lines, suggesting that the 5' site is more important for transcription factor binding (Fig. 7A, lanes 4 and 9). Mutated oligonucleotides competed more effectively in EMSA of Panc-1 and COLO-357 nuclear extracts, but with similar trends. Alterations outside of the TEF-1 consensus sequences, including mutations to the NF- κ B consensus sequence, did not impair transcription factor binding (not shown). The double-mutated oligonucleotides were included in this analysis in case transcription factor binding only required one out of the two TEF sites. In the case of COLO-357, for example, double mutation of the competing oligonucleotide appears to be most effective for preventing competition with the labeled oligonucleotide, whereas only the 5' TEF mutation appears to be required to prevent effective competition when using nuclear extracts from NIH 3T3.

The functionality of the TEF-1 site was demonstrated by mutating the 5' TEF-1 site within the -775/+73 CTGF promoter reporter (TEF-MUT). As was previously reported by others using a chimeric thymidylate kinase version of the same CTGF promoter in NIH 3T3 cells (23), mutation of the 5' TEF-1 sequence completely abrogated reporter activity in both Panc-1 and NIH 3T3 cells (Fig. 7B).

MEK/ERK signaling mediates CTGF expression in pancreatic cancer cell lines, whereas expression in fibroblasts is p38-dependent. Small molecule inhibitors were used to identify MAPK pathways regulating basal and TGF β -inducible CTGF expression in cells with intact Smad signaling pathways. RT-PCR and Western blot analysis of Panc-1 and NIH 3T3 treated with MEK inhibitors (U0126, PD98059), p38 inhibitor (SB202190) or JNK inhibitor (SP600125) indicated that MEK mediated basal and TGF β -inducible CTGF expression in Panc-1 cells (Fig. 8A). On the other hand, both p38 and to a lesser extent MEK, were required for CTGF expression in NIH 3T3 cells (Fig. 8B), as has been reported previously (22). Inhibition of CTGF expression in rat mesangial cells has been reported using U0126 (6). Our inhibitor results were supported by Western blot analysis of CTGF protein secreted into media (Fig. 8C), and by promoter-reporter assays (Fig. 8D). CTGF secretion from HFF cells was also inhibited by the p38 inhibitor

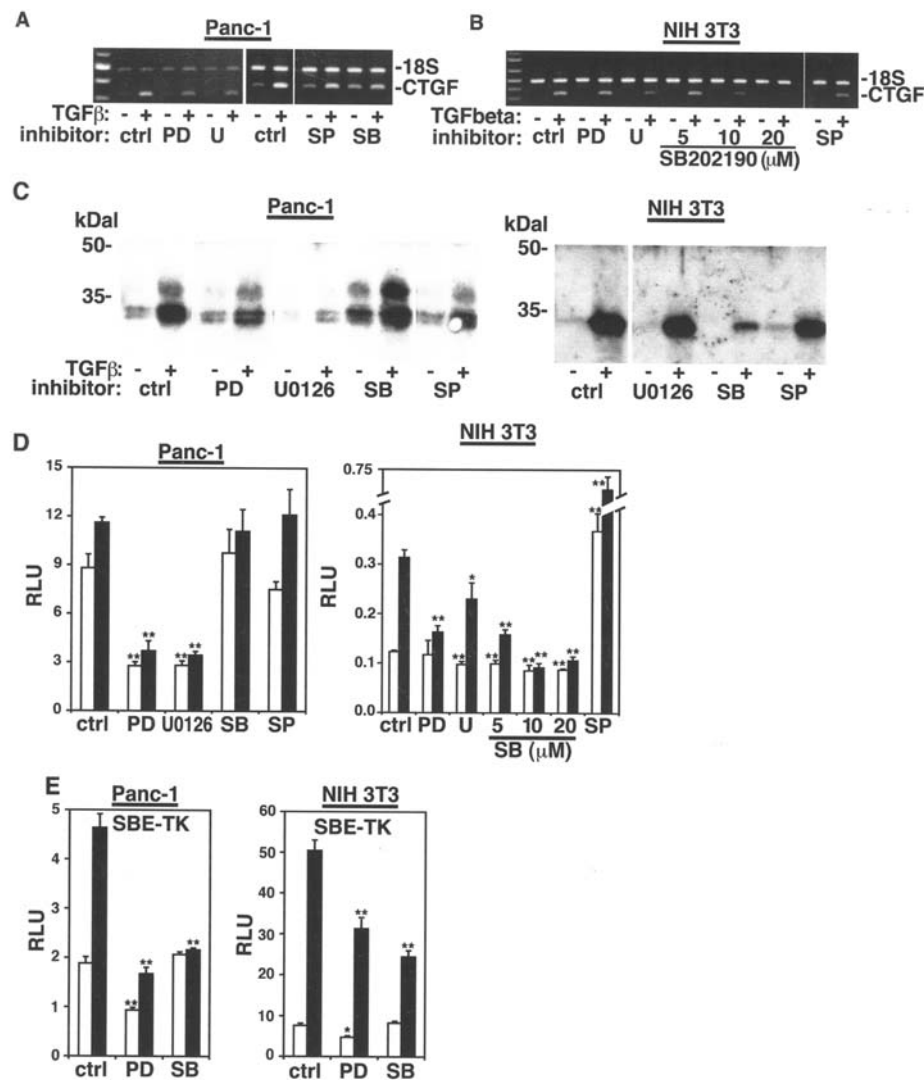


Figure 8. MEK signaling pathway mediated CTGF expression in pancreatic cancer cell lines, whereas p38 was required for CTGF expression in fibroblasts. RT-PCR analysis was carried out using RNA isolated from Panc-1 (A) and NIH 3T3 (B) cells treated with the indicated MEK (PD98059, U0126), p38 (SB202190) or JNK (SP600125) inhibitors. These results were confirmed by reducing SDS-PAGE/Western blotting (C) and by reporter assays (D) for Panc-1 and NIH 3T3. Unlike the CTGF promoter reporter, the SBE-TK reporter activity was effectively inhibited by both MEK and p38 inhibitors in Panc-1 and NIH 3T3 (E). PD, 50 μ M PD98059; U, 10 μ M U0126; SB, 10 μ M SB202190 except where indicated otherwise. Statistical differences are indicated for assays containing inhibitors compared with their respective controls, analyzed by t-test (* P <0.05 and ** P <0.01).

(data not shown), indicating that this may be a fibroblast-specific pathway. The JNK inhibitor SP600125 activated the reporter in NIH 3T3 cells by approximately 1-fold, but did not affect the expression of CTGF, as determined by RT-PCR and Western blotting. Thus, results with this inhibitor in this cell line are unclear.

Western blotting of cell lysates using antibodies recognizing phosphorylated kinases showed that ERK and p38 are constitutively phosphorylated in Panc-1 cells, and this phosphorylation is not discernibly increased upon treatment with TGFβ (data not shown). The ability of the MEK inhibitors to repress not only CTGF promoter reporter activity but also expression of CTGF protein and message, indicate that this constitutively active, phosphorylated ERK plays a role in CTGF expression in pancreatic cancer cell lines. This result indicates that MEK/ERK signaling is necessary but not sufficient for CTGF expression, and that some additional signal is provided by TGFβ-receptor signaling. In contrast, p38 became phosphorylated in NIH 3T3 cells only in response to

treatment with TGFβ, indicating that this pathway is activated by TGFβ, and providing an explanation for the inhibition of CTGF promoter reporter, message and protein effected by p38 inhibitors. ERK2 is constitutively phosphorylated in NIH 3T3, so the failure of MEK inhibitors to reduce CTGF expression in this cell line indicates that this pathway is activated but does not play a major role in CTGF expression in NIH 3T3.

The effects of p38 inhibitor SB202190 and MEK inhibitor PD98059 were also tested on SBE-TK transfectants (Fig. 8E). Unlike the -775 CTGF promoter reporter, SBE-TK reporter activity in Panc-1 cells was completely inhibited by SB202190. This significant difference between the behavior of the CTGF and SBE-TK promoter reporters provides further evidence that CTGF transcription is less SBE-dependent in pancreatic cancer cells, and/or that the Smad complexes which assemble on these two promoter reporters are different.

Constitutive ERK phosphorylation in Panc-1 cells may be attributable to an activating K-Ras mutation, which has been reported in this and most other pancreatic cancer cell lines

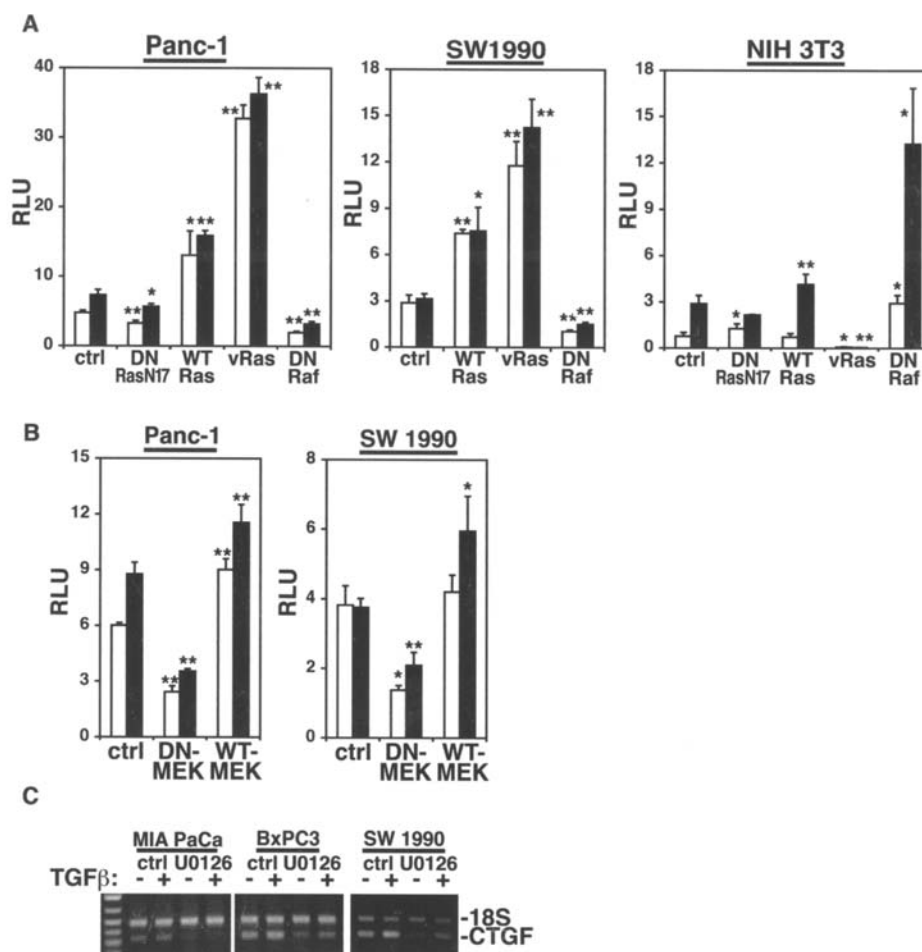


Figure 9. Ras/Raf/MEK regulates CTGF expression in pancreatic cancer cell lines. (A) Co-transfection of CTGF promoter reporter with expression vectors encoding active and dominant negative Ras and Raf indicate that these proteins can activate CTGF promoter reporter activity in Panc-1 and SW1990 cells but have an inhibitory effect on transcription in NIH 3T3 cells. (B) Promoter reporter was co-transfected with expression vectors encoding either wild-type (WT-MEK) or dominant negative (DN-MEK) MEK, and TGFβ treated for 16 h. Statistical differences are indicated for expression vectors compared with their corresponding pcDNA3-transfected controls, analyzed by t-test (* $P < 0.05$ and ** $P < 0.01$). (C) A positive role for MEK was confirmed by RT-PCR analysis of RNA isolated from different pancreatic cancer cell lines treated with MEK inhibitor U0126.

(17). Co-transfection of the CTGF promoter reporter vector with different active and dominant negative Ras and Raf mutants indicated that this pathway plays a role in CTGF expression in Panc-1 cells (Fig. 9A). Co-transfection of the Ras expression vectors into pancreatic cancer cell line SW1990 gave similar results, indicating that this effect is not unique to Panc-1 cells. It has also been reported that DN-Ras can suppress CTGF promoter reporter activity in rat mesangial cells (31). By contrast, co-transfection of the same expression vectors into NIH 3T3 gave very much the opposite result, indicating an antagonistic role for Ras in this cell type, and confirming that CTGF is regulated differently in this fibroblast cell line compared to Panc-1.

In addition, co-transfection of both Panc-1 cell lines and SW1990 with dominant-negative MEK (DN-MEK) reduced CTGF promoter reporter activity, whereas wild-type MEK (WT-MEK) activated it (Fig. 9B). Further evidence for the role of MEK/ERK in CTGF expression was demonstrated by the ability of MEK inhibitor U0126 to reduce CTGF message levels in other pancreatic cancer cell lines SW1990, MIA PaCa-2, and BxPC-3 (Fig. 9C). These pancreatic cancer cell lines express relatively low levels of CTGF message and lack intact Smad signaling pathways.

Discussion

A summary and comparison of the above findings regarding signaling pathways mediating the effects of TGFβ on CTGF expression in NIH 3T3 fibroblasts and PANC-1 are shown in Fig. 10. The failure of TGFβ to induce CTGF up-regulation in cell lines lacking an intact Smad signaling pathway has indicated that CTGF up-regulation by TGFβ is Smad-dependent (33). Smad translocation to the nucleus and/or its transcriptional activation has also been reported to be Smad4-independent (38,41). In our studies, CTGF message was detectable in untreated pancreatic cancer cells and was rapidly induced by TGFβ in Panc-1 cells; whereas CTGF message was not detected in untreated NIH 3T3 cells and induction by TGFβ in this cell line exhibited a two-hour delay. Although the CTGF promoter reporter was readily activated by TGFβ in NIH 3T3 cells, it showed very little activation by TGFβ in Panc-1 or COLO-357 cells. This was not due to autocrine activation of the TGFβ receptor in these cells, since TGFβIR kinase inhibitor SB 431542, which effectively inhibited induction of CTGF message and protein, was unable to inhibit basal reporter activity in Panc-1 cells. Likewise, mutation of the SBE did not lower basal promoter activity in Panc-1 or NIH 3T3, whereas

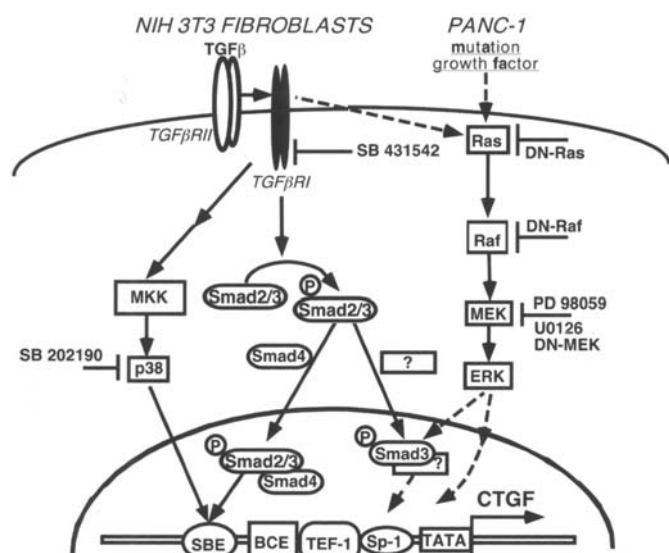


Figure 10. Summary and comparison of effects of small molecule inhibitors and dominant negative mutants on CTGF expression in NIH 3T3 (left) and Panc-1 (right). Arrows indicate activating pathways. Broken arrows indicate tentative assignments. Blocked lines indicate inhibitory processes. *Cis* elements downstream of MEK/ERK signaling remain to be identified (6,14,18,21,22,28,29,43).

it did abrogate the promoter response to TGF β by Panc-1 and NIH 3T3 cells.

The limited promoter reporter activation by TGF β in pancreatic cancer cells suggests that essential *cis* elements may lie outside of the -775/+73 bp 5' flanking region. As demonstrated by others using NIH 3T3 cells (23,24), the tandem TEF-1 sites were bound by nuclear protein(s) from Panc-1 and COLO-357 cells, and reporter assays confirmed that at least the 5' TEF-1 *cis* element was absolutely required for both basal and induced promoter reporter activity. The transcription factors binding to this site remain to be identified.

Since non-Smad-mediated signaling by TGF β has been extensively reported (reviewed in ref. 16), small molecule and dominant negative inhibitors of MAP kinase signaling pathways were tested for their ability to inhibit expression of CTGF message, protein and reporter activity in Panc-1 and NIH 3T3 cells. In pancreatic cancer epithelial cells, basal CTGF expression required MEK/ERK signaling, possibly mediated by active Ras. These results are similar to reports of CTGF regulation by Ras and MEK previously described for rat mesangial cells (31). In contrast, CTGF expression was p38-dependent and was antagonized by active Ras in NIH 3T3 cells.

In summary, this study delineated pathways involved in the cell type-specific regulation of CTGF expression in pancreatic cancer cells. While a positive role for the SBE *cis* element was confirmed for NIH 3T3 cells, this element appeared to play less of a role in Panc-1. Furthermore, we found CTGF up-regulation to be TGF β RI kinase-dependent in all cell lines studied but our data shows basal CTGF transcription in pancreatic cancer cell lines to be Smad4-independent. This would permit expression in pancreatic cancers that typically contain DPC4 mutations. Also, we determined that TEF elements are essential for basal and TGF β -induced reporter activity in both NIH 3T3 and Panc-1. However, we determined

that MEK/ERK signaling mediates basal CTGF expression in pancreatic cell lines regardless of Smad mutational status, whereas p38 mediates expression in fibroblasts. Finally, consistent with the role of MEK/ERK signaling in pancreatic cancer cell lines, Ras activation can induce CTGF in pancreatic cancer cell lines, whereas Ras has a suppressive effect in NIH 3T3. These results suggest a mechanism whereby CTGF could become up-regulated in pancreatic ductal epithelial cells, independent of Smad4, as a consequence of Ras mutations.

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