Smad4/DPC4-dependent Regulation of Biglycan Gene Expression by Transforming Growth Factor-β in Pancreatic Tumor Cells

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The abbreviations used are: ATCC, American Type Culture Collection; BGN, biglycan; bp, base pair(s); CHX, cycloheximide; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; kb, kilobase(s); nt, nucleotide(s); PAI-1, plasminogen activator inhibitor-1; R-Smad, receptor-regulated Smad; RT-PCR, reverse transcription-polymerase chain reaction; SBE, Smad-binding element; SLRP, small leucine-rich proteoglycan; TGF-β; transforming growth factor-β
**Summary**

Overexpression of the small leucine-rich proteoglycan biglycan (BGN) in fibrosis and desmoplasia results from enhanced activity of transforming growth factor-β (TGF-β). In pancreatic adenocarcinoma, the tumor cells themselves may contribute to BGN synthesis *in vivo* since 8/18 different pancreatic carcinoma cell lines constitutively expressed BGN mRNA as shown by RT-PCR analysis. In PANC-1 cells, TGF-β1 dramatically stimulated BGN mRNA accumulation through a BGN transcription-indepenent, cycloheximide-sensitive mechanism and strongly increased the synthesis and release of the proteoglycan form of BGN. The ability of TGF-β1 to induce BGN mRNA was critically dependent on Smad signaling since 1) the upregulation of BGN mRNA was preceded by a marked increase in Smad2 phosphorylation in TGF-β1-treated PANC-1 cells, 2) TGF-β1 was unable to induce BGN mRNA in pancreatic carcinoma cell lines that carry homozygous deletions of the Smad4/DPC4 gene, 3) inhibition of the Smad pathway in PANC-1 cells by transfection with a dominant negative Smad4/DPC4 mutant significantly reduced TGF-β1-induced BGN mRNA expression, 4) stable reintroduction of wild type Smad4/DPC4 into Smad4-null CFPAC-1 cells restored the TGF-β1 effect, and 5) overexpression of Smad2 and Smad3 in PANC-1 cells augmented TGF-β1 induction of BGN mRNA, whereas forced expression of Smad7, an inhibitory Smad, effectively blocked it. These results clearly show that a functional Smad pathway is crucial for TGF-β regulation of BGN mRNA expression. Since BGN has been shown to inhibit growth of pancreatic cancer cells, the Smad4/DPC4-mediation of the TGF-β effect may represent a novel tumor suppressor function for Smad4/DPC4: antiproliferation via expression of autoinhibitory BGN.
Introduction

The transforming growth factor-β (TGF-β) family comprises a large group of multifunctional cytokines with widespread distribution. They participate in a wide array of biological activities such as cell growth, differentiation, wound healing, apoptosis, and immunomodulation (1, 2). TGF-β1, one of three mammalian TGF-β isoforms, (TGF-β1-3), is a potent inducer of extracellular matrix formation and has been implicated as the key mediator of fibrogenesis and desmoplasia in a variety of tissues (3). TGF-β1 promotes extracellular matrix accumulation primarily by inducing the synthesis of matrix proteins, such as collagens, fibronectin and proteoglycans. Among the proteoglycans that are upregulated by TGF-β in vitro is biglycan (BGN), a prototype member of the small leucine-rich proteoglycan (SLRP) family (reviewed in Refs. 4-6). BGN can be considered a marker gene for TGF-β activity which is reflected in vivo by the close spatial and temporal association of both proteins under physiological and various pathophysiological conditions (7-10).

Due to its widespread, albeit cell type specific expression in the mammalian body, data on BGN function have been gathered from different organs and tissues and include regulation of matrix assembly, cellular adhesion (11), migration (12) and growth factor, e. g. TGF-β activity (13). The ability of BGN to bind TGF-β with high affinity (14) has been proposed to control the bioavailability of this growth factor. Because of its pericellular localization BGN may function as a TGF-β binding protein that increases the probability of an interaction of TGF-β with its specific surface receptors. This scenario may have important implications for early progression of carcinomatous tumors, such as pancreatic carcinoma (10), since at this stage carcinoma cells are usually growth inhibited by TGF-β. Besides this indirect antiproliferative function, BGN may also directly inhibit growth of cancer cells in a TGF-β-independent manner, as Weber et al. (10) showed that exogenously administered recombinant BGN induced pancreatic cancer cells to arrest in the G1-phase of the cell cycle.
The intracellular signaling mechanisms by which TGF-β controls the expression of BGN and other matrix-associated proteins remain poorly understood. Signaling by TGF-β ligands requires two transmembrane serine-threonine kinase receptors (type II and type I). The ligand brings the two receptors together in a complex, and the constitutively active type II receptor kinase phosphorylates and activates the type I receptor kinase, which in turn activates downstream signaling pathways (15). Several signaling pathways are originating from the type I receptor, the most prominent one being the Smad pathway (16, 17). This pathway is initiated when the type I receptor phosphorylates the receptor-regulated (R-)Smads, Smad2 or Smad3. Subsequently, the R-Smads heterodimerize with a co-Smad, Smad4. The Smad2/3-Smad4 heterodimer is then translocated to the nucleus where it binds directly or via other DNA binding proteins to the promoters of TGF-β-responsive genes to stimulate or repress their transcription (16, 17). The antagonistic Smads, Smad6 and Smad7, on the other hand, inhibit the phosphorylation of R-Smads by the type I receptor and prevent the association of the R-Smads with Smad4/DPC4 (20-22).

Smad4 (also termed DPC4, for deleted in pancreatic carcinoma locus 4) has the characteristics of classical tumor suppressor gene, being mutated or deleted in ~50% of pancreatic carcinomas and 15% of colorectal cancers (18, 19). Since the discovery as a tumor suppressor much interest has focused on the role of Smad4/DPC4 as a mediator of TGF-β anti-proliferative signals which were originally thought to account for most, if not all, of its anti-oncogenic activity. However, several recent studies have confirmed that suppression of tumor formation and metastasis through this protein is more complex involving inhibition of tumor angiogenesis (23) and changes in the expression and activity of genes implicated in the control of cell adhesion and invasion (24). While all these effects may be directly or indirectly controlled by TGF-β, there are likely to be tumor suppressive activities that are unrelated to TGF-β signaling as inferred from the observation that inactivating DPC4 mutations occur together with mutations in the genes encoding TGF-β type II or type
I receptor (25).

TGF-β regulation of several matrix-associated proteins has been demonstrated to depend entirely or partially on a functional Smad pathway, e. g. PAI-1 (26, 27), TIMP-1 (28), collagens type α2(I) (28-31), α1(III), α1(VI), α3(VI), α2(V) (28) and VII (32), laminin (33), and aggrecan (34), yet other matrix proteins are controlled in a Smad-independent fashion, e. g. fibronectin (35) and pro-a1(I) collagen (36). Notably, except for aggrecan equivalent data for other TGF-β-targeted proteoglycans including SLRPs are not available. We, therefore, sought to shed light on the intracellular signaling events that are initiated by TGF-β to upregulate BGN expression. Cell lines established from pancreatic carcinoma were used as a cellular model as these comprise TGF-β-responsive and non-responsive cells that had been well characterized with respect to alterations in genes involved in TGF-β signaling and cell cycle control (18, 37, 38).

Our data indicate that TGF-β1 upregulation of BGN expression occurs through activation of Smad proteins and is critically dependent on a functional Smad4/DPC4. This is the first report demonstrating the involvement of Smad proteins in the TGF-β control of BGN and SLRP gene expression.
Experimental Procedures

Cell Lines and Cell Culture - The human pancreatic cancer cell lines PANC-1 and BxPC-3 and the osteoblastic osteosarcoma cell line MG-63 were purchased from the American Type Culture Collection (Rockville, MD). The pancreatic carcinoma cell lines CFPAC-1 and Hs766T were a kind gift of Dr. W. von Bernstorff (University of Kiel). The pancreatic carcinoma cell line COLO-357 and its supplier has been described previously (39). The COLO-357 cells used in this study harbor a wild type DPC4 gene (38) and are genetically distinct from COLO-357 cells obtained from another source, which have a homozygous deletion of the Smad4/DPC4 gene (40). All pancreatic cell lines were routinely maintained in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, and 1 mM sodium pyruvate (all from Life Technologies, Inc.). MG-63 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% FCS, 2 mM L-glutamine, and nonessential amino acids. PANC-1 and CFPAC-1 cells stably transduced with retroviral expression vectors were cultured in the presence of 700 and 250 µg/ml geneticin (biologically active concentration, Life Technologies, Inc.), respectively.

Antibodies - The Smad4 (B-8) antibody was purchased from Santa Cruz Biotechnology (Heidelberg, Germany), the anti-phospho-Smad2 (Ser 465/467) antibody was supplied by Upstate Biotechnology/Biomol (Hamburg, Germany) and the anti-total Smad2 antibody from Zymed Laboratories Inc. (Berlin, Germany). An antibody raised against a peptide within the mature form of human BGN (LF-51) was a kind gift of Dr. L.W. Fisher (NIDCR, National Institutes of Health). The anti-β-actin (AC-15) antibody used was obtained from Sigma (Taufkirchen, Germany).

RNA Isolation and Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) - Total RNA was isolated from cells with RNA Clean (AGS, Heidelberg, Germany) according to the manufacturer’s instructions. The general RT-
PCR protocol was described in detail earlier (41). The following oligonucleotide primers were used: BGN-sense (nt 124-143): 5’-CCCTCTCCAGGTCCATCCGC-3’, BGN-antisense (nt 623-604): 5’-GAGCTGGGTAGGTTGGGCGGG-3’, PAI-1-sense (nt 357-378, GenBank accession: X04744): 5’-CTTCTTCAGGCTGTTCCGGAGC-3’, PAI-1-antisense (nt 1164-1143): 5’-GGGTCAGGGTTCCATCACCTTGG-3’, GAPDH-sense: 5’-GGCGTCTTCACCACCACATGGAG-3’, GAPDH-antisense: 5’-AAGTTGTCATGGATGACCTTGGC-3’. For semiquantification of BGN, PAI-1 and GAPDH mRNAs we carried out a competitive approach using an internal standard. The internal standard DNA for BGN of 386-bp was constructed by excision from the cDNA of an internal 114-bp BglII fragment, the standard DNA for PAI-1 was constructed by PCR using the PAI-1 antisense primer and a hybrid sense primer that contained nucleotides 357-378 followed by nucleotides 588-610 (5’-CTTCTTCAGGCTGTTCCGGAGCCACAAATCAGACGGCAGCAGACTG-3’) resulting in a 599-bp product upon amplification with PAI-1-sense and PAI-1-antisense primers. Multiple reactions were run in parallel containing identical amounts of cDNA (corresponding to 100 or 200 ng of total RNA) but different concentrations of internal standard DNA. For this purpose, the standard DNA was serially diluted (0.9, 0.8, …, 0.09, 0.08, … and so forth). In order to keep reactions in the exponential phase, the number of cycles with an annealing temperature of 59°C was in the case of BGN adjusted to 16 cycles (PANC-1), 20 cycles (CFPAC-1), and 30 cycles (COLO-357) and for PAI-1 to 10 cycles, respectively. Following electrophoretic separation of PCR products on agarose gels and staining with ethidiumbromide photographs were taken and densitometrically scanned using the NIH Image software (version 1.62). TGF-β induction of BGN mRNA was assessed from those reactions that showed an equimolar concentration of target and internal standard. The corresponding amount of target mRNA in these reactions was considered to be accurately determined when this ratio and the target to standard ratios of at least two neighboring reactions plotted against the corresponding standard dilutions on a semilogarithmic scale formed a linear relationship. To control for differences in cDNA synthesis the same cDNA was
subjected to competitive PCR for GAPDH mRNA using primers GAPDH-sense: 5’-GGCGTCTTCACCACCATGGGAG-3’ and GAPDH-antisense: 5’-AAGTTGTCATGACGACCTTGGC-3’ resulting in a fragment of 206-bp (nt 358-563 of the human GAPDH cDNA). The standard was constructed by PCR using the same antisense primer and a hybrid sense primer that contained nts 358-378 followed by nts 439-457 yielding a standard fragment of 146-bp. Relative values for BGN and PAI-1 RNA concentrations were normalized to GAPDH mRNA levels. For each stimulation experiment at least two independent competitive RT-PCR assays were performed yielding the same results.

For detection of Smad4/DPC4 mRNA the entire coding region of human Smad4 (GenBank accession: NM_005359) was amplified with primers Smad4-sense: 5’-AAATGGACAATATGTCTATTACGAATAC-3’ (nt 127-154, start codon underlined) and Smad4-antisense: 5’-TCAGTCTAAAGGTTGTGGGTCTGC-3’ (nt 1787-1764, stop codon underlined) resulting in a specific PCR product of 1661-bp. Amplification of Smad7 mRNA was carried out with primers Smad7-sense: 5’-CATGTTCAGGACCAAACGATCTG-3’ (nt 295-317, GenBank accession: NM_005904, start codon underlined) and Smad7-antisense: 5’-GCTACCGGCTGTTGAAGATGAC-3’ (nt 1577-1556, stop codon underlined).

Construction of Retroviral Expression Vectors and Generation of Stable Transductants of the PANC-1 and CFPAC-1 Cell Lines - For retroviral transduction of Smad4/DPC4, the cDNA was excised from the pBK-CMV-DPC4 vector (a gift from S. A. Hahn, Bochum, Germany) using NheI and SmaI and subcloned after filling-in the 5’ overhang of NheI with Klenow in sense orientation into the PmeI site of the retroviral vector TJBA5bMoLink-neo (42). A cDNA fragment of a C-terminally truncated mutant of Smad4/DPC4, Smad4(1-514), was generated by RT-PCR using primers Smad4-mut-sense (nt 126-154, with a 126 C to A mutation to create a Kozak consensus): 5’-AAAATGGACAATATGTCTATTACGAATAC-3’ and Smad4(514)-antisense (nt 1670-1650, stop codon introduced behind codon 514):
5'-TCATGGGTAATCCGGTCCCCAGCC-3' and Pfu polymerase (Stratagene, Heidelberg, Germany) and was subcloned directly in sense orientation into the Pmel site of TJBA5bMoLink-neo. This cDNA was fully sequenced and found to be identical with the published Smad4 sequence. Positive clones (evaluated by PCR, restriction analysis and sequencing of the plasmid-cDNA junctions) were cotransfected into 293T producer cells along with retroviral packaging vectors as described previously (42). Retroviral particles released by 293T cells were used to infect PANC-1 (Smad4(1-514)), and CFPAC-1 cells (Smad4 wild type). Pools of productively infected cells were obtained by selection with geneticin (PANC-1: 700 µg/ml; CFPAC-1: 350 µg/ml, active concentration, Life Technologies, Inc.) and were analyzed for expression of the desired proteins by RT-PCR analysis and immunoblotting.

Transfections and Reporter Gene Assays - For transient transfections PANC-1 cells were seeded in 3.5-cm well plates at a density of 4x10^5 cells/well. For detection of luciferase activity PANC-1 cells were seeded in 96-well plates at 1x10^4 cells/well. On the next day cells were transfected by serum-free lipofection using LipofectAMINE Plus (Life Technologies, Inc.) according to the manufacturer’s instructions. Following removal of the transfection mixture, cells were incubated in normal growth medium for 24 h to allow expression of proteins from the transfected plasmids. All cultures within an experiment were transfected with the same total amount of plasmid; empty parental expression vector was added as needed to equalize cotransfection of Smad expression vectors. The mean and standard deviations for each sample and treatment were determined from 6-8 wells processed in parallel. Since control experiments showed that the overall results were not affected by unequal transfection efficiencies, normalization to β-galactosidase activity was omitted. For immunoblot detection of proteins, cells were lysed at this stage in RIPA buffer (see below). For analysis of TGF-β effects on BGN mRNA, TGF-β1 (5 ng/ml, R&D Systems, Wiesbaden, Germany) was added to normal growth medium and cells were
incubated for 24 h followed by lysis in RNA Clean (for mRNA isolation, see above) or „Glo Lysis Buffer“ (for determination of luciferase activities with the „Bright Glo Luciferase Assay System“, Promega). Luciferase activities were measured with a MicroBeta TriLux 1450 system (Wallac Inc., Gaithersburg, MD) for 2 s. Pilot experiments indicated that (pre)incubation of cells in serum-reduced medium (0.5% FCS) before and during TGF-β treatment only marginally affected the overall TGF-β effect. For transient expression of various Smad proteins the following plasmids were used: FLAG-Smad2 and FLAG-Smad3, kindly supplied by K. Miyazono (The Cancer Institute, Tokyo, Japan), and Smad7 in pcDNA3, kindly supplied by C.-H. Heldin (Institute for Cancer Research, Uppsala, Sweden). For measurement of reporter gene activity we used either p3TP-lux (kindly provided by Dr. J. Massagué, Memorial Sloan-Kettering Cancer Center, N.Y.), or p6SBE-Luc and p6MBE-Luc (kindly supplied by S. E. Kern, The Johns Hopkins Medical Institutions, Baltimore, MD).

Preparation of Proteinaceous Extracts, Partial Purification of Proteoglycans and Chondroitinase ABC Digestion - Preparation of protein extracts was carried out as described previously (43). Briefly, pancreatic tumor cells were rinsed in PBS and lysed in RIPA buffer (0.1% SDS, 1% NP40, 0.5% sodium deoxycholate containing Complete™ protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)) for 20 min on ice followed by one freeze-thaw cycle. For detection of (phospho-)
Smad2 cells were lysed directly in 2x Laemmli-buffer (125 mM Tris-HCl, pH 6.8; 100 mM dithiothreitol; 20% glycerol; 4% SDS). For separation of proteoglycans by SDS-PAGE and subsequent immunoblotting, samples of proteoglycans in conditioned medium were partially purified on DEAE Trisacryl M (Sigma) and concentrated as described in detail earlier (44). Briefly, 5 ml of conditioned or non-conditioned medium was applied to an 0.4 ml DEAE Trisacryl M column in 8 M urea with 0.5% Triton X-100, 0.1 M Tris-HCl, pH 7.5, and 0.25 M NaCl and washed four times with urea buffer. The proteoglycan fraction was eluted with 3 M NaCl in urea buffer and precipitated at –20°C (1-1.5 h) by the addition of 3.5 volumes of ethanol and 1.3%
potassium acetate with the addition of chondroitin sulfate (Sigma) as carrier. The resulting pellet was dissolved in water and the precipitation was repeated once without further addition of carrier. Finally, the pellet was dissolved in TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl). To remove glycosaminoglycan chains from the BGN core protein equal volumes of DEAE-purified proteoglycans, equal amounts of cellular proteins (previously dialysed into TBS) or BGN purified from bovine articular cartilage (Sigma, dissolved in TBS) were digested with chondroitinase ABC (Sigma) (0.4 U/ml or 1.0 mU/µg, respectively) for 4 h at 37°C.

Immunoblot Analysis – 20-40 µg of total cellular protein or equal volumes of Laemmli lysates and partially purified proteoglycans, respectively, were separated by SDS-PAGE on 12.5% gels or precasted 4-20% gradient gels and transferred to a PVDF membrane (Immobilon-P, Millipore, Eschborn, Germany). Membranes were blocked with PBS containing 5% non-fat dry milk overnight at 4°C, washed several times with PBS containing 0.1% Tween 20 (PBST) and then incubated with the primary antibody. For detection of BGN and phosphorylated Smad2, TBST plus 5% bovine serum albumin was used for membrane blocking and TBST for washing. After washing, blots were incubated with the appropriate peroxidase-conjugated secondary antibodies and developed with the chemiluminescent detection kit (ECL or ECL+Plus, Amersham Pharmacia Biotech, Buckinghamshire, UK) following the manufacturer's protocol. Some blots were reprobed with an antibody against β-actin to confirm equal loading.

Proliferation Assays - Growth inhibition by TGF-β was measured by [³H]-thymidine incorporation and essentially carried out as described previously (37). Briefly, 1x10⁴ cells/well were seeded in 96-well microtiter plates in 100 µl of culture medium containing 10% FCS. After 24 h cells were treated with various concentrations of TGF-β1 for 24 h. During the last 4 h, cells were pulsed with 0.2 µCi of [³H]-thymidine (1.74 TBq/mmol, Amersham, UK) without changing the medium. At the end of the incubation period, cells were detached from the bottom by addition
of 100 µl of 10x trypsin and transferred on glass filters using a cell harvester. The radioactivity incorporated into the DNA was measured by liquid scintillation counting.
Results

*BGN is Expressed in Pancreatic Cancer Cells and is Strongly Upregulated by TGF-β1 in PANC-1 Cells* - Initially, we screened a panel of pancreatic tumor cell lines for the presence of BGN mRNA by RT-PCR analysis. As shown in Fig. 1A, 8/18 cell lines were found to be positive in this assay. Next we analysed in BGN mRNA positive PANC-1 cells whether BGN proteoglycan synthesis is sensitive to upregulation by TGF-β1 (Fig. 1B). For this purpose immunoblots were prepared from cellular extracts and the proteoglycan fraction extracted from conditioned medium of TGF-β1-treated PANC-1 cells. BGN antigenic sites were detected with an antibody against mature human BGN (45). However, this antibody cannot or only weakly detect fully glycanated BGN which is likely due to steric hindrance (46) but recognizes the deglycanated ~50 kDa form of mature BGN that is generated upon digestion of the proteoglycan form with chondroitinase ABC (arrow). This species is much more abundant in TGF-β treated PANC-1 (lanes 2 and 17) and MG-63 cells (lane 9) and likely represents a nonglycanated but Asn-glycosylated form of the BGN core protein (47) because it is larger than the *M*ₐ 42,510 predicted for prepro-BGN by the cDNA sequence (48). In chondroitinase ABC treated samples with higher BGN content (lanes 2, 9, 17, and 18) several high molecular species were apparent (upper arrowhead). They may represent partial degradation products of the proteoglycan form in which the Gly corresponding to the first amino acid of the antigenic peptide (48) and contained in one of the two Ser-Gly glycosaminoglycan attachment sites became accessible to the antibody. In addition, this antibody detected a band of ~35 kDa in cell extracts (left panel, lower arrowhead) which may represent a proteolytic fragment of BGN (46). From these data we conclude that BGN proteoglycan is strongly upregulated by TGF-β1 and properly secreted as a fully glycanated (and glycosylated) proteoglycan.
BGN mRNA is Upregulated by TGF-β1 in Cell Lines that Harbor a Wild Type DPC4 Gene – Since results shown in Fig. 1B indicated that BGN (core protein) synthesis in PANC-1 cells was strongly enhanced by TGF-β1, we tested by applying semiquantitative RT-PCR whether this was due to concomitant changes in BGN steady-state mRNA levels (Fig. 2A). As expected, PANC-1 cells responded to TGF-β1 (5 ng/ml, 24 h) with a dramatic, ~50-fold upregulation of BGN mRNA. An induction, albeit much smaller than in PANC-1, was also seen in COLO-357 cells. In contrast, in three other BGN expressing cell lines (BxPC-3, CFPAC-1, and Hs766T) treated under the same conditions BGN mRNA levels remained unchanged. TGF-β regulation of gene expression may involve activation of intracellular Smad proteins including the common mediator Smad, Smad4/DPC4. Interestingly, in the cell lines analyzed BGN mRNA induction by TGF-β1 correlated well with the mutational status of DPC4: whereas PANC-1 and COLO-357 cells express a wild type Smad4/DPC4 protein, CFPAC-1, BxPC-3 and Hs766T cells all lack Smad4/DPC4 protein due to a homozygous deletion of DPC4 (Fig. 2B). Absence of Smad4/DPC4 expression in these cell lines was confirmed by RT-PCR using primers that span the entire coding region (1659-bp) (Fig. 2B). In two other cell lines known to carry loss-of-function mutations in DPC4 (AsPC-1, Capan-1) the TGF-β effect on BGN mRNA could not be evaluated since these cells lack detectable BGN mRNA expression (Fig. 1).

TGF-β Induction of BGN mRNA in PANC-1 Cells is Cycloheximide-sensitive and Does not Involve an Increase in BGN Promoter Activity – An earlier study has shown that the TGF-β-induced rise in BGN mRNA was first detectable at 8h and peaked between 12 and 24 h after TGF-β addition to PANC-1 cells (10). To obtain some clues as to the underlying mechanism, we performed TGF-β stimulation experiments in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX) (Fig. 3A). At both concentrations tested CHX effectively blocked the TGF-β effect on BGN mRNA indicating that de novo protein synthesis was required. To investigate whether the TGF-β-induced increase in BGN mRNA is
the result of increased transcriptional activity from the BGN gene promoter, we performed transient transfections with the full-length and minimal human BGN promoter (1218-bp and 78-bp, respectively) fused to the luciferase reporter gene. As shown in Fig. 3B, neither the full-length nor the minimal BGN promoter responded to TGF-β stimulation with significantly enhanced activity. Under the same conditions, TGF-β was capable of increasing 3.3-fold the transcription from p3TP-lux, a plasmid containing well characterized TGF-β-responsive elements from the PAI-1 and collagenase promoters (49). However, the 3.3-fold stimulation of p3TP-lux activity by TGF-β1 contrasts with the ~50-fold induction of BGN mRNA, further arguing in favor of the assumption that transcriptional activation of the BGN promoter cannot account for the TGF-β effect. For this reason, in all subsequent functional studies the effects of the potential TGF-β signaling intermediates could not be assessed in transient transfection assays with BGN promoter constructs but instead it was necessary to measure endogenous mRNA levels.

**TGF-β Induces a Marked Increase in Smad2 Phosphorylation in PANC-1 Cells** – TGF-β signaling from the cell surface to the nucleus via Smad proteins is initiated by activation (by phosphorylation) of R-Smads, Smad2 and Smad3. To analyze whether the Smad pathway is activated in PANC-1 cells in response to TGF-β, cell extracts from these cells, treated with TGF-β1 (5 ng/ml) at various time points, were analyzed by immunoblotting for phosphorylated Smad2 (upper panel) and total Smad2 (lower panel) using an anti-phospho-Smad2 and an anti-Smad2 antibody, respectively (Fig. 4). The ~58-kDa Smad2 protein was present in both control and TGF-β-treated PANC-1 cells, and an increase in phosphorylated Smad2 was noticed within 30 min of TGF-β treatment. This increase in Smad2 phosphorylation peaked at 2 h and remained high up to 8 h. Thus, Smad2 phosphorylation in PANC-1 cells is increased shortly after TGF-β1 treatment, indicating that these cells can respond to the ligand through a Smad-dependent pathway.
Inhibition of the Smad Pathway by a Dominant Negative Smad4/DPC4 Mutant

Inhibits TGF-β-induced Expression of BGN - The data presented in Figs. 2 and 4 raised the possibility that Smad4/DPC4 is necessary for TGF-β induction of BGN. We therefore asked whether inhibition of Smad4/DPC4 function would compromise the TGF-β effect. PANC-1 cells were stably transduced with Smad4(1-514), a C-terminally truncated, naturally occurring Smad4 mutant (18), that acts in a dominant negative fashion to suppress Smad4/DPC4 function (50, 51). Immunoblot analysis revealed that Smad4(1-514) protein was expressed (Fig. 5A), albeit at levels not exceeding those of the wild type protein. The comparatively low expression may be due to the unstability of this mutant that has been shown to be rapidly degraded through the ubiquitin-proteasome pathway (52). To verify that expression of the 1-514 mutant inhibits wild type Smad4/DPC4 function, we determined its effect on TGF-β induced transcription of PAI-1, a gene known to be induced by TGF-β via Smad4/DPC4 (26, 27). As shown in Fig. 5B, PANC-1 cells stably expressing Smad4(1-514) had greatly decreased PAI-1 mRNA levels after a 24-h exposure to TGF-β1 when compared with control cells that contained only the empty vector and, hence, did not express this C-terminal Smad4/DPC4 deletion mutant (see Fig. 5A). Next, we evaluated the effect of the Smad4(1-514) mutant on endogenous BGN mRNA levels under the same conditions. In cells expressing Smad4(1-514) BGN mRNA induction was inhibited by 80% relative to vector controls (Fig. 5C).

Restoration of Smad4/DPC-4 Expression Renders CFPAC-1 Cells Sensitive to TGF-β Induction of BGN mRNA Expression – To further confirm the crucial role of Smad4/DPC4 for TGF-β-induction of BGN mRNA we tested whether reconstitution of wild type Smad4/DPC4 protein expression in Smad4/DPC4-null cells would render these cells responsive to TGF-β with respect to matrix protein regulation. CFPAC-1 cells which lack Smad4/DPC4 mRNA and protein due to a homozygous genomic deletion of DPC4 (compare Fig. 2) were retrovirally transduced with a full-length wild type Smad4/DPC4 cDNA. Successful restoration of Smad4 expression was
verified by immunoblotting (Fig. 6A); the pool of transduced cells expressed nearly physiological level of this protein when compared to other cell lines with functional Smad4/DPC4, e. g. PANC-1 (Fig. 6A). Notably, when this pool of Smad4-reconstituted CFPAC-1 cells was challenged with TGF-β1, BGN and PAI-1 mRNA levels markedly increased (Fig. 6B and C). To test whether other known functions of Smad4/DPC4, e. g. mediation of the TGF-β antiproliferative effect, have been restored in these cells, we measured [³H]-thymidine incorporation in TGF-β-treated CFPAC-1-DPC4 cells. As depicted in Fig. 6D, CFPAC-1-Smad4/DPC4 cells were not growth inhibited under conditions that efficiently arrested growth of PANC-1 cells. Together, these data clearly show that Smad4/DPC4 is involved in the induction of BGN and PAI-1 mRNA expression by TGF-β in pancreatic carcinoma cells.

*The TGF-β effect on BGN mRNA Expression in PANC-1 Cells is Enhanced by Overexpression of Smad2 or Smad3 and Inhibited by Overexpression of Smad7.*

Since functional Smad4/DPC4 protein appeared to be necessary for TGF-β action on BGN mRNA, we reasoned that overexpression of R-Smads Smad2 and/or Smad3 should potentiate the TGF-β effect, whereas the inhibitory Smad Smad7 should interfere with it. The effects of Smad2, Smad3, and Smad7 were analyzed following their transient transfection into PANC-1 cells. Initially, functionality of the various Smad-encoding expression vectors was evaluated in a p6SBE reporter assay (Fig. 7A). The p6SBE-lux plasmid contains 6 tandem repeats of the Smad-binding element (SBE) cloned in front of the luciferase reporter and has been used to specifically confer Smad4/DPC4-dependent transcriptional activation to a minimal promoter (40). The TGF-β-induced reporter gene activity was strongly enhanced upon cotransfection with Smad2 or Smad3 but was repressed upon cotransfection with Smad7. The negative control plasmid p6MBE-lux containing 6 mutated SBEs was without any activity (data not shown). We then assessed the effect of these Smad proteins on BGN mRNA expression. Transient transfection of both Smad2 and Smad3 resulted in an 1.75-fold increase in TGF-β-induced BGN mRNA levels (Fig. 7B), whereas Smad1
under the same conditions had no effect (data not shown). In contrast, Smad7 potently inhibited the TGF-β effect on BGN mRNA (75% inhibition relative to vector-transfected controls, Fig. 7B). It should be mentioned that the transfection efficiency in these assays was ~30% as determined by cotransfection with plasmid encoding enhanced green fluorescent protein. Taken together, these results further confirm the participation of the Smad pathway in TGF-β induction of BGN mRNA in pancreatic carcinoma cells and, with respect to Smad7, suggest an inhibitory role for this protein in TGF-β regulation of BGN.

TGF-β Induces Expression of Smad7 in PANC-1 Cells - TGF-β-induced upregulation of Smad7 occurs via transcriptional activation of the Smad7 gene promoter and is thought to be part of a negative feed-back loop terminating TGF-β-induced Smad signaling (20, 21). As shown in Fig. 8, Smad7 mRNA is strongly induced by TGF-β1 (~11-fold after 24-h of stimulation). Besides the demonstration that negative Smad signaling is operating in these cells, this result together with the data from the Smad7 overexpression (see Fig. 7B) lend support to the contention that Smad7 is involved in antagonizing the TGF-β-induced and Smad4/DPC4-mediated rise of BGN expression.
Discussion

While aberrant BGN expression in fibrosis is well documented (7, 9, 53, 54), enhanced BGN expression in the tumor stroma of desmoplastic carcinomas has so far not been reported. Given its role as a TGF-β response gene, the recent observation that BGN is also overexpressed in the tumor stroma of pancreatic carcinoma (10) came thus not unexpected since most pancreatic tumor cells overexpress biologically active TGF-β1 and -2 and the type II receptor in vitro and in vivo (55, 56, and our own unpublished observation). Weber et al. (10) suggested that the bulk of BGN is secreted by normal stromal cells in response to TGF-β - secreted by cancer cells in a paracrine fashion – but is also released by the tumor cells themselves via autocrine stimulation provided they possess a functional TGF-β pathway. However, these authors did not present evidence for protein production of this proteoglycan by pancreatic tumor cells in vitro. Using PANC-1 cells we initially showed that pancreatic carcinoma cells synthesize and secrete BGN in its proteoglycan form and that both synthesis and secretion was strongly enhanced by TGF-β1. A survey of 18 different pancreatic carcinoma cell lines by RT-PCR revealed that 8 expressed BGN under in vitro culture conditions. This is a higher percentage than that found by Weber et al. (10) but this discrepancy may be explained by the fact that our RT-PCR assay was more sensitive than Northern blotting which allowed us to identify also cell lines with low level expression. However, even this larger fraction of ~50% may underestimate the expression in vivo as suggested by comparative data from Weber and coworkers (10) who showed that SUIT-2 cells strongly expressed BGN transcripts when cultured as xenografts in nu/nu mice whereas the same cells lack expression when cultured in vitro.

Recently, BGN has been shown to inhibit pancreatic tumor cell proliferation in vitro by inducing (a TGF-β-independent) arrest in the G1-phase of the cell cycle (10). This had led to the hypothesis that BGN synthesis by host stromal cells is part of a matrix based host defense mechanism against tumor progression. Given such a
scenario, it is evident that tumor cells that manage to inactivate the TGF-pathway responsible for BGN induction (which is presumably chronically active due to high intratumoral concentrations of TGF-β) would gain a survival advantage. Many pancreatic carcinomas have inactivating mutations in the TGF-β pathway, the most characteristic one being Smad4/DPC4. This mutation is thought to account for the loss of TGF-β-mediated antiproliferation as the major tumor suppressive effect (see „Introduction“ and below). We were intrigued by the idea that induction of selfinhibitory BGN via Smad4/DPC4 may yet represent another mechanism of tumor suppression resembling the stress-induced secretion of growth inhibitors induced by wild type p53 (57). Based on this idea we asked whether Smad4/DPC4 functions as an signaling intermediate in BGN induction by TGF-β in pancreatic tumor cells. A positive correlation of wild type Smad4/DPC4 protein expression with TGF-β-inducibility of BGN mRNA in a set of pancreatic cancer cell lines strongly hinted to a critical role for Smad4/DPC4 that was subsequently confirmed by functional inhibition and reconstitution experiments. Forced expression of the C-terminal deletion mutant Smad4(1-514) that acts in a dominant negative fashion to suppress wild type Smad4/DPC4 activity (50, 51), also repressed the TGF-β effect on BGN mRNA in PANC-1 cells. Notably, de novo expression of wild type Smad4/DPC4 in a Smad4-null cell line rendered it TGF-β-sensitive with respect to BGN (and PAI-1) mRNA induction. However, another TGF-β biological response, growth inhibition, was not restored confirming similar observations in other pancreatic (58) and colon carcinoma cell lines (24) and lending support to the emerging concept that tumor suppressor activities other than mediation of direct effects of TGF-β on the cell cycle account for the high frequency of functional inactivation of this gene in pancreatic carcinoma.

The extent of TGF-β induction of BGN expression was extremely high in PANC-1 but comparatively low in COLO-357 and Smad4/DPC4-transduced CFPAC-1 cells, the latter exhibiting response rates previously found in other cell types (41). This raised the possibility that the TGF-β/Smad pathway in COLO-357 and CFPAC-1
cells was not fully active which could be due to either downregulation of TGF-β receptors and/or overexpression of inhibitory Smads (59, 60). Alternatively, in PANC-1, additional signaling pathway(s) may be stimulated by TGF-β that function to amplify the Smad-mediated effect on BGN mRNA. We have recently obtained preliminary evidence for an involvement of the p38 MAPK pathway in TGF-β regulation of BGN expression in this cell line (unpublished observation).

An interesting issue concerns the mechanism of Smad4/DPC4-mediated induction of BGN expression. In contrast to PAI-1 (26, 27) and various collagens (61), the BGN gene is likely not to be a direct Smad target as 1) no SBE (the consensus sequence is the 8-bp palindrome, GTCTAGAC (38)) is present within the available 1218 bp of human BGN promoter sequence previously reported by us (41) and others (62, 63), 2) transfected BGN promoter-reporter fusion genes were essentially unresponsive to TGF-β, 3) the increase in BGN mRNA in PANC-1 by far exceeded the overall TGF-β-mediated transcriptional activity on the TGF-β-responsive reporter plasmids p3TP-lux and p6SBE, and 4) the rise in BGN mRNA levels occurred relatively late (10). Stabilization of cytoplasmic mRNA would represent another potential mechanism through which TGF-β could increase BGN mRNA. However, inducing a 50-fold increase within 24 h would require a high turnover rate of the mRNA. In a previous study in MG-63 cells we showed that basal BGN transcription was low and that the half-life of cytoplasmic BGN mRNA was long (2.5 days), irrespective of whether the cells were treated with TGF-β or not (41). We therefore consider it more likely that TGF-β1 exerts its effect in PANC-1 cells at a nuclear post-transcriptional level, e. g. pre-mRNA processing and/or nuclear export rather than on mature mRNA in the cytoplasm (41). Regarding the lack of transcriptional induction of the BGN gene Smad4/DPC4 may initially induce another gene(s) whose protein product(s) then translocates to the nucleus to mediate the BGN mRNA increase. This possibility is supported by our observation that TGF-β-induced accumulation of BGN mRNA in PANC-1 was effectively blocked by CHX. This results are in sharp contrast to equivalent data from another study where CHX had no
effect (10). Further studies are required to elucidate the molecular mechanism of BGN mRNA accumulation and the immediate cellular targets of Smad4/DPC4.

Transient transfection of both Smad2 and Smad3 potentiated the TGF-β effect on BGN expression while overexpression of Smad7 blunted it further arguing for the participation of the entire Smad signaling cascade in TGF-β regulation of BGN expression. Smad3-mediated gene expression has been implicated in TGF-β-mediated immunosuppression and enhanced deposition of extracellular matrix (61, 64) both TGF-β responses that provide an advantage for tumor development (64). Smad2 has a crucial role in TGF-β-induced expression of p21^{Waf1/CIP1} or p15^{INK4B} CDK inhibitors, of which the former may be responsible for growth inhibition by TGF-β in pancreatic carcinoma cells (37). Though DPC4 and MADH2 are both considered tumor suppressor genes, the mechanistic basis of their anti-oncogenic function(s) are still not clear. As discussed above the (direct) growth inhibitory response of pancreatic cancer cells to TGF-β seems to rely on an intact p21^{ras} effector pathway (58) or ERK activation (65) rather than on functional Smad4/DPC4 expression. Based on the data presented here and the discovery of the growth inhibitory effect of BGN on pancreatic tumor cells (10), we reasoned that Smad4/DPC4 could still be capable of mediating an indirect effect of TGF-β on the cell cycle; the question therefore arises if tumor cells with non-functional mutations in DPC4 would have a selective advantage by switching off TGF-β-induced BGN expression. Local production of BGN in tumor cell aggregates would certainly inhibit tumor cell growth, hence loss of Smad4/DPC4 function is supposed to relieve this inhibition. However, it is not known if a functional TGF-β-Smad pathway would indeed result in higher production of BGN in the tumor cell vicinity in vivo. To answer this question we are currently analyzing tumors derived from Smad4/DPC4-reconstituted pancreatic cancer cell lines and growing as xenografts in nude mice (23) to see whether BGN is enriched in the (mouse) stromal tissue adjacent to the human cancer cells.

Pancreatic carcinoma cells have evolved multiple mechanisms of TGF-β resistance (64). Besides mutational inactivation of DPC4, these include
downregulation of receptors or overexpression of inhibitory Smads. Interestingly, enforced expression of Smad7 in COLO-357 cells has been found to inhibit the growth inhibitory response to TGF-β1, without affecting the TGF-β1-mediated induction of PAI-1 (59). This has been interpreted to mean that Smad7 overexpression in pancreatic cancer cells in vivo may lead to enhanced tumor growth by blocking growth inhibition by TGF-β but at the same time allowing for TGF-β-induced expression of genes that promote tumor spread and metastasis. Assuming that Smad7 is indeed capable of selectively blocking the anti-oncogenic TGF-β responses in the cancer cells, then BGN expression by pancreatic tumor cells could be considered „harmful“ to these cells.

In conclusion, we have demonstrated for the first time for a SLRP that TGF-β regulation occurs through the Smad pathway involving Smad4/DPC4. Since BGN is a major matrix-organizing component of fibrotic and desmoplastic tissues, this knowledge may be exploited to inhibit their formation by application of specific inhibitors of Smad4/DPC4 (61). Based on our data and recent results from Weber et al. (10), we also proposed a novel tumor suppressor function of Smad4/DPC4: (indirect) antiproliferation via expression of autoinhibitory BGN.
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Figure legends

Fig. 1. **Expression of biglycan (BGN) in pancreatic carcinoma cells.** A, RT-PCR analysis for BGN expression in various pancreatic carcinoma cell lines. The human osteosarcoma cell line MG-63 served as positive control. Total RNA of the indicated cell lines was reverse-transcribed and equal amounts of first-strand cDNA subjected to PCR amplification with BGN and GAPDH-specific primers. Amplification products were loaded on an agarose gel and visualized by ethidium bromide staining. 

B, Immunoblot analysis of BGN in PANC-1 cellular extracts (left panel) and in the proteoglycan (PG) fraction from conditioned medium of PANC-1 cells (right panel). In both cases MG-63 cells and purified bovine BGN were used as positive control. PANC-1 and MG-63 cells were stimulated with TGF-β1 (5 ng/ml) in normal growth medium for 24 h, except for the sample in lane 8 where stimulation was for 12 h. Subsequently, cells were lysed in RIPA buffer, dialysed against TBS and digested with chondroitinase ABC (Ch. ABC). For the generation of conditioned medium cells were grown to confluence in normal growth medium containing 10% FCS. Cells were then incubated in medium with 0.1% FCS for 24 h and stimulated with TGF-β1 (5 ng/ml) in the same medium for another 24 h. Secreted proteoglycans were partially purified on DEAE cellulose and digested with chondroitinase ABC. To allow for quantitative comparison, PANC-1 cells were seeded at equal density and equal volumes of conditioned medium were harvested and processed throughout the purification and digestion procedure. Equal amounts of cellular proteins (Prot.,) and purified bovine BGN (bBGN), and equal volumes of proteoglycan samples (each with and without chondroitinase ABC treatment) were then fractionated by SDS-PAGE and BGN core protein was detected by immunoblotting using the LF-51 antibody. Numbers on the right of each panel indicate the migratory positions of marker bands (in kDa).
Fig. 2. The inducibility of BGN mRNA by TGF-β in pancreatic carcinoma cells correlates with the mutational status of DPC4. A, quantification of BGN transcript levels using competitive RT-PCR. BGN mRNA expressing cell lines BxPC-3, CFPAC-1, Hs776T, PANC-1 and COLO-357 cells were stimulated with TGF-β1 [5 ng/ml] for 24 h and subjected to RNA isolation and first-strand cDNA synthesis. Equal amounts of cDNA were amplified along with different concentrations (as indicated by Rel. Std.-dil. = Relative Standard-Dilution) of internal standard for BGN and GAPDH (data not shown). Following agarose gel fractionation and ethidium bromide staining, relative concentrations of BGN and GAPDH mRNAs were determined from those reactions in which the ratio of target to standard amplification products approximated 1 and was equal between the TGF-β-treated sample and the control (marked by an arrowhead). Induction of BGN mRNA by TGF-β (right) was calculated as the ratio of BGN transcripts in TGF-β stimulated over control cells after normalization to GAPDH mRNA. B, Immunoblot (IB) analysis for Smad4/DPC4 expression in the indicated BGN expressing pancreatic carcinoma cell lines. Subconfluent cultures of the indicated cell lines were lysed in RIPA buffer (for IB) or RNA Clean (for RT-PCR) and equal amounts of protein and RNA subjected to SDS-PAGE and RT-PCR, respectively. Following transfer of fractionated proteins to PVDF membrane, Smad4/DPC4 protein was detected with anti-Smad4 antibody and visualized by enhanced chemiluminescence.

Fig. 3. The TGF-β effect on BGN mRNA is blocked by cycloheximide and does not involve enhanced transcription from the BGN promoter. A, PANC-1 cells were treated with TGF-β1 (5 ng/ml) for 24 h in the absence or presence of the indicated concentrations of the protein synthesis inhibitor cycloheximide (CHX). CHX was given to the cells 0.5 h prior to the addition of TGF-β. Subsequently, BGN mRNA levels were quantified by competitive RT-PCR. B, PANC-1 cells were transiently transfected by lipofection with either the full-length (BGNSac-Luc) or minimal (BGN-78-Luc) human BGN promoter fused to the luciferase reporter in the
plasmid pGL2-E. Control cells received the empty vector or the TGF-β-responsive reporter plasmid p3TP-lux. Following transfection, cells were cultured in normal growth medium for 24 h and stimulated with TGF-β1 (5 ng/ml) for another 24 h. Cell extracts were then assayed for luciferase activity. One representative experiment out of three experiments performed in total is shown. Results are the mean ± S.D. of 6 wells processed in parallel and are expressed relative to the value in pGL2-E-transfected control cells set arbitrarily at 1.

Fig. 4. **Phosphorylation of Smad2 by TGF-β in PANC-1 cells.** PANC-1 cells were treated with 5 ng/ml TGF-β1 for the indicated time periods. Cell lysates were analyzed by immunoblotting with anti-phospho-Smad2 antibody (upper panel) for phosphorylated Smad2 (p-Smad2) or anti-Smad2 antibody (lower panel) for total Smad2 protein (t-Smad2). Smad2 protein (~58 kDa) was detected in both control and TGF-β1-stimulated cells. A marked increase in Smad2 phosphorylation was noticed starting 0.5 h after TGF-β1 addition.

Fig. 5. **Decreased BGN expression by inhibition of the Smad pathway with a dominant negative Smad4/DPC4 mutant, Smad4(1-514).** A, Immunoblot analysis of PANC-1 cells stably overexpressing a C-terminally truncated Smad4/DPC4 construct, Smad4(1-514), a mutant full-length control construct (Smad4-C442R), or the empty vector. B, inhibition of PAI-1 mRNA expression by a dominant negative Smad4/DPC4 mutant. PANC-1 cells stably transduced with Smad4(1-514) were treated with TGF-β1 (5 ng/ml) for 24 h and subjected to semiquantitative RT-PCR for PAI-1 as described in the legend to Fig. 2A and under „Experimental Procedures.“ PAI-1 mRNA concentrations were expressed relative to unstimulated vector controls set arbitrarily at 1. C, inhibition of BGN mRNA expression by a dominant negative Smad4/DPC4 mutant. PANC-1 cells stably expressing the Smad4(1-514) mutant were stimulated with TGF-β1 (5 ng/ml) for 24 h and analyzed by semiquantitative RT-PCR for BGN expression as described in the legend to Fig. 2A and under „Experimental
Procedures.” BGN mRNA concentrations were expressed relative to unstimulated vector controls set arbitrarily at 1.

Fig. 6. Effect of de novo expression of wild type Smad4/DPC4 protein in Smad4-null CFPAC-1 cells on the TGF-β effect on BGN mRNA. A, Immunoblot analysis of CFPAC-1 cells re-expressing wild type Smad4/DPC4. CFPAC-1 cells were retrovirally transduced with either Smad4/DPC4 or the empty vector and stably expressing pools selected with G418. Cell extracts were prepared and subjected to SDS-PAGE and immunoblotting using anti-Smad4 antibody and chemiluminescent detection. Note that expression levels of exogenous Smad4/DPC4 in the pool are similar to endogenous Smad4/DPC4 levels in PANC-1. B, C, Analysis of the TGF-β response of BGN and PAI-1 mRNA levels in CFPAC-1 cells re-expressing Smad4/DPC4. TGF-β1-treated CFPAC-1-vector and CFPAC-1-Smad4 cells were subjected to semiquantitative RT-PCR analysis as described in the legend to Fig. 2A and under „Experimental Procedures.“ D, Analysis of the proliferative response of CFPAC-1-Smad4 cells to TGF-β stimulation measured by [3H]-thymidine incorporation assay. Two independent experiments were performed with very similar results. Data shown are the mean ± S.D. of 8 wells processed in parallel. Standard deviations were all below 10% and were omitted from the graph for reasons of clarity.

Fig. 7. Effect of Smad2, Smad3 and the inhibitory Smad7 on TGF-β induction of BGN transcript levels. A, Effect of transfected Smad proteins on p6SBE reporter gene expression. PANC-1 cells (1x10^4) were seeded in 96-wells on day 1 and were cotransfected on day 2 with 20 ng p6SBE reporter plasmid and 180 ng of expression vectors for either Smad2, Smad3, Smad7, or empty vector using LipofectAMINE Plus. After a 3-h transfection period, cells were incubated for 24 h in normal growth medium to allow for sufficient protein synthesis from transfected plasmids followed by another 24-h incubation period in the presence or absence of TGF-β1 (5 ng/ml). Subsequently, cells were lysed in „Glo Lysis Buffer“ and subjected to luciferase
assay. Three independent experiments were performed with similar results. Data shown are the mean ± S.D. of 8 wells processed in parallel. B. Analysis of the TGF-β response of BGN mRNA in transiently transfected PANC-1 cells. Cells (4x10^5) were seeded in 3.5-cm well plates on day 1 and were transfected on day 2 with 2 µg of expression vectors for either Smad2, Smad3, Smad7, or empty vector using LipofectAMINE Plus. The subsequent processing was as described under A, except that cells were lysed in RNA Clean and subjected to RT-PCR analysis.

Fig. 8. Expression of Smad7 is upregulated in response to TGF-β. PANC-1 cells were treated with TGF-β1 for 24 h in normal growth medium and subjected to RT-PCR analysis with specific primers for Smad7. PCR conditions were the same as for BGN except that the reactions were terminated after 24 cycles with an annealing temperature of 59°C. GAPDH reactions were run in parallel to control for equal cDNA input. Ethidiumbromide-stained bands for Smad7 and GAPDH were photographed and densitometrically scanned using the NIH image 1.62 software. Data represent the normalized mean ± S.D. of three PCR reactions processed in parallel.
Figure 1

A

Figure 1 A shows a gel electrophoresis analysis of BGN and GAP expression in different cell lines. The gel is labeled with the following cell lines: H2O, AsPC-1, A87984, BxPC-3, Capan-1, Capan-2, CFPAC-1, Colo3857, H5765, MIA PaCa-2, MPANC-96, PaCa-3, Panc-1, Panc-99, PancTu1, P1-45P1, QGP-1, T3M4, MG-63. The bands are indicated as 500 bp and 206 bp.

B

Figure 1 B presents a protein expression analysis in PANC-1, bBGN, and MG-63 cell lines. The table includes the following columns: Prot. [µg], Ch. ABC, TGF-β, and BGN. The protein expression is visualized in a Western blot analysis with markers at 245, 123, 77, 42, and 30 kDa.
### Figure 2

#### A

| Cell Line       | Rel. Std.-Dil. [x10^-6] | -TGF-β | +TGF-β | -fold induction |
|-----------------|-------------------------|--------|--------|----------------|
| BxPC-3          | 400                     |        |        | 1              |
| Hs766T          | 50                      |        |        | 1              |
| CFPAC-1         | 3                       |        |        | 1              |
| PANC-1          | 60                      |        | 3000   | 50             |
| COLO-357        | 1                       |        | 4      | 4              |

#### B

- **IB**
  - Smad4/DPC4
  - β-actin

- **RT-PCR**
  - Smad4/DPC4

DPC4 Status: wt null null null wt
Figure 3

**Panel A**

Relative Luciferase Activity

- BGNSac-Luc
- BGN-78-Luc
- pGL2-E
- p3TP-lux

- TGF-β1
- + TGF-β1

**Panel B**

Relative BGN mRNA Conc.

- TGF-β1
  - -
  - +

- CHX [μg/ml]
  - 0
  - 25
  - 250
Figure 4

| Time [h] | 0.25 | 0.5  | 1    | 2    | 4    | 8    |
|---------|------|------|------|------|------|------|
| TGF-β1  | -    | +    | -    | +    | -    | +    |

- **p-Smad2**
- **t-Smad2**
Figure 5

A

vector Smad4-C442R Smad4 (1-514)

FL

1-514 Smad4/DPC4

β-actin

B

Relative PAI-1 mRNA Conc.

- TGF-β1

+ TGF-β1

vector Smad4(1-514)

C

Relative BGN mRNA Conc.

- TGF-β1

+ TGF-β1

vector Smad4(1-514)
Figure 6

A

[Image showing a Western blot with bands labeled as Smad4/DPC4 and β-actin]

B

[Bar graph showing relative BGN mRNA concentration for vector and Smad4/DPC4 with -TGF-β1 and +TGF-β1 conditions]

C

[Bar graph showing relative PAI-1 mRNA concentration for vector and Smad4/DPC4 with -TGF-β1 and +TGF-β1 conditions]

D

[Graph showing 3H-Thymidine Incorporation % for CFPAC1-wt, CFPAC1-vector, CFPAC1-Smad4, and PANC-1 with varying TGF-β1 concentrations (0.1, 0.5, 1, 5, 10 ng/ml)]
Figure 7

A

Relative Luciferase Activity.

- TGF-β1
+ TGF-β1

vector Smad2 Smad3 Smad7

B

Relative BGN mRNA Conc.

- TGF-β1
+ TGF-β1

vector Smad2 Smad3 Smad7
Figure 8

- TGF-β  |  + TGF-β

Smad7

GAPDH

Smad7 mRNA Conc. (normalized to GAPDH)

- TGF-β1
- + TGF-β1