Correlation of glypican-1 expression with TGF-ß, BMP, and activin receptors in pancreatic ductal adenocarcinoma

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Abstract. Glypican1 (GPC1) is a cell surface heparan sulfate proteoglycan that acts as a co-receptor for heparin-binding growth factors as well as for members of the TGF-ß family. GPC1 plays a role in pancreatic cancer by regulating growth factor responsiveness. In view of the importance of members of the TGF-ß family in pancreatic cancer, in the present study, the role of GPC1 in TGF-ß, BMP and activin signaling was analyzed. Quantitative RT-PCR and immunohistochemistry were utilized to analyze GPC1 and TGF-ß, BMP and activin receptor expression levels. Panc-1 and T3M4 pancreatic cancer cells were transfected in a stable manner with a GPC1 antisense expression construct. Anchorage-dependent and -independent growth was determined by MTT and soft agar assays. TGF-ß1, activin-A and BMP-2 responsiveness was determined by MTT assays and immunoblotting with p21, p-Smad1, and p-Smad2 antibodies. QRT-PCR demonstrated increased GPC1 mRNA levels in pancreatic ductal adenocarcinoma (PDAC) compared to normal pancreatic tissues (NPT), as described previously. There was a significant correlation between GPC1 mRNA levels and TßRII, act-R1a, act-R1b, act-R2a, BMP-R1a, and BMP-R2 mRNA expression in NPT. In contrast, GPC1 mRNA expression correlated directly with act-R1a and BMP-R1a in N0 PDAC cases and with act-R2a and BMP-R1a in lymph node positive cases. Down-regulation of GPC1 resulted in increased doubling time in Panc-1 but not in T3M4 cells, and decreased anchorage-independent growth in both cell lines. GPC1 down-regulation resulted in a slightly altered response towards TGF-ß1, activin-A and BMP-2 in terms of growth, p21 induction and Smad2 phosphorylation. In conclusion, enhanced GPC1 expression correlates with BMP and activin receptors in pancreatic cancer. GPC1 down-regulation suppresses pancreatic cancer cell growth and slightly modifies signaling of members of the TGF-ß family of growth factors.

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Introduction

Glypican1 (GPC1) is a member of the family of heparan sulfate proteoglycans (HSPG), which are ubiquitous proteins that are attached to the extracytoplasmic surface of the cell membrane. GPC1 is anchored by its C-terminal to the cell surface through a glycosyl-phosphatidylinositol (GPI) anchor (1,2). Several growth factors require HSPGs as co-receptors for efficient signaling. These include heparin-binding EGF-like growth factor (HB-EGF) (3) hepatocyte growth factor (HGF) (4), vascular endothelial growth factor (VEGF) (5), platelet-derived growth factor (PDGF) (3) and the Wnt family of secreted glycoproteins (6). Other growth factors, such as transforming growth factors (TGF-ßs) (7), bone morphogenetic proteins (BMPs) (8), and activin (9), are also known to bind heparin and heparan sulfate, but the physiological consequence of this binding is still unclear (10-12).

Members of the TGF-ß superfamily have growth stimulatory or inhibitory effects in different types of tumors (13-16). TGF-ßs, BMPs and activins exert their effects by signaling via serine/threonine kinase receptors, phosphorylation and nuclear translocation of Smad signaling molecules, and subsequent transcription of target genes (15,17-24). Enhanced expression of TGF-ß and its type I and type II receptors (TßRI and TßRII) (25,26) is correlated with decreased survival and advanced stages in pancreatic ductal adenocarcinoma (PDAC) (24,27,28). In pancreatic cancer cells, the growth inhibitory effects of TGF-ßs are abolished by several mechanisms, including the presence of Smad4 mutations (29), the overexpression of Smad6 and Smad7 (30,31) which act as inhibitors of the TGF-ß pathway, and the low to absent expression of TGF-ß receptors in a subgroup of human pancreatic cancers (24,32,33). In addition, BMP-2, BMP receptors IA and II are overexpressed in PDAC, and this overexpression has been associated with decreased patient survival (23). Furthermore,

activin-A and its type I and type II receptors (act-RI/Ib, act-RII/IIb) are also overexpressed in PDAC and have the potential to participate in autocrine activation of pancreatic cancer cells (22).

GPC1 is expressed at increased levels in pancreatic cancer cells and the adjacent fibroblasts (34), and downregulation of GPC1 by stable transfection of a full-length GPC1 antisense construct decreases the tumorigenicity of pancreatic cancer cells (35), reduces anchorage-dependent and anchorage-independent growth, and attenuates TGF-ß1 signaling in Colo-357 pancreatic cancer cells (36). Therefore, in the current study we sought to determine whether there is a correlation between the expression of GPC1 and TGF-ß signaling receptors. We also analyzed the effects of GPC1 downregulation on the responses of pancreatic cancer cells towards BMP-2, activin-A and TGF-ß1.

Materials and methods

Tissue samples. Pancreatic cancer specimens were obtained from 28 patients with a median age of 71 years (range 43-82 years) in whom pancreatic resections were performed. Normal human pancreatic tissue samples were obtained through an organ donor program from previously healthy individuals. All samples were confirmed histologically. Freshly removed tissues (within 5 min after surgical excision) were fixed in paraformaldehyde solution for 12-24 h and then paraffin-embedded for histological analysis. In addition, a portion of the tissue samples was kept in RNAlater (Ambion Ltd., Huntingdon, Cambridgeshire, UK) or snap-frozen in liquid nitrogen immediately upon surgical removal and maintained at -80˚C until use. The Human Subjects Committee of the University of Heidelberg, Germany, and the University of Bern, Switzerland, approved all studies, and written informed consent was obtained from all the patients.

Cell culture. Pancreatic cancer cell lines were grown routinely in RPMI medium (Aspc-1, BxPc-3, Capan-1, Colo-357, SU8686 and T3M4) or DMEM medium (MiaPaCa-2 and Panc-1), supplemented with 10% FCS and 100 U/ml penicillin (complete medium), and incubated in a 5% CO_2 humidified atmosphere.

Real-time quantitative polymerase chain reaction (QRT-PCR). All reagents and equipment for mRNA/cDNA preparation were supplied by Roche Applied Science (Mannheim, Germany). mRNA was prepared by automated isolation using the MagNA pure LC instrument and isolation kit I (for cells) and kit II (for tissues). cDNA was prepared using the first strand cDNA synthesis kit for RT-PCR (AMV) according to the manufacturer's instructions. Real-time PCR was performed using the LightCycler FastStart DNA SYBR Green kit. The primer sequences for GPC1, TßRI, TßRII, act-R1a, act-R1b, act-R2a, BMP-R1a BMP-R2, and were obtained from Search-LC (Heidelberg, Germany). The number of specific transcripts was normalized to housekeeping genes (cyclophilin B and HPRT). The transcript number was calculated from a virtual standard curve, obtained by plotting a known input concentration of a plasmid to the PCR-cycle number at which the detected fluorescence intensity reaches a fixed value. The data of the two independent analyses for each sample and parameter were averaged and presented as adjusted transcripts/μl cDNA (37,38).

Antibody production. Mouse monoclonal IgM antibodies (mAbs) for protein GPC1 were obtained by immunization with the synthetic peptide CGNPKVNPQGPGPEEKRR (22) representing the amino acid sequence (aa 343-360) deduced from the cDNA of GPC1 (NCBI, accession no. NM002081). The peptide was conjugated to keyhole limpet hemocyanin (KLH). For screening, the carrier protein conjugated to the peptide was bovine serum albumin (BSA). The specificity of the antibodies were tested against a specific GPC1 peptide sequence by ELISA in comparison to non-specific peptide sequences, and further confirmed by immunoblotting using the GPC1 epitope coupled with albumin, following standard protocols. The mAbs were raised essentially according to the method of Köhler and Milstein (39).

Immunohistochemistry. Paraffin-embedded tissue sections (2-3 μm thick) were immunostained using the Dako Envision TM + system (Dako Corp., Carpinteria, CA, USA). Tissue sections were deparaffinized in xylene and rehydrated in progressively decreasing concentrations of ethanol. Thereafter, the slides were placed in washing buffer (10 mM Tris-HCl, 0.85% NaCl, 0.1% bovine serum albumin, pH 7.4) and subjected to immunostaining. After antigens were retrieved by boiling the tissue sections in 10 mM citrate buffer for 10 min in the microwave oven, sections were incubated with normal goat serum (Dako) for 45 min to block non-specific binding sites. Next, consecutive sections were incubated with a mouse monoclonal anti-GPC1 IgM antibody or mouse IgM as a negative control, diluted 1:2, at 4˚C overnight. The slides were then rinsed with washing buffer and incubated with anti-mouse IgM-avidin/biotin (KPL, Gaithersburg, MD, USA) conjugated for 45 min at room temperature. Tissue sections were then washed in washing buffer and each section was subjected to 100 μl streptavidin peroxidase (KPL) for 35 min at room temperature. DAB-chromogen substrate mixture (Dako) was applied to tissue sections and then counter-stained with Mayer's hematoxylin. Sections were washed, dehydrated in progressively increasing concentrations of ethanol, and mounted with xylene-based mounting medium (27,37,38). Slides were analyzed using the Axioplan 2 imaging microscope (Carl Zeiss Light Microscope, Göttingen, Germany).

Construction of GPC1 antisense vector and stable transfection. The glypican-1 antisense construct was prepared as described previously (34). Panc-1 and T3M4 pancreatic cancer cells were stably transfected with the glypican-1 antisense construct pMH6/G1-AS-1750 and with the empty control vector using the lipofectamine method. Briefly, after reaching confluence, cells were split 1:10 into selection medium (complete medium supplemented with 0.5 mg/ml G418) and single clones were isolated after 2-4 weeks. After expansion, cells from each individual clone were screened for the expression of GPC1 antisense RNA by Northern blot analysis. Parental Panc-1 and T3M4 cells were also transfected with an empty pEGFPN1 expression vector carrying the neomycin resistance gene as a

Table I. mRNA expression of TGF-ß1 receptors and co-receptors in normal human pancreas and PDAC tissues by QRT-PCR in copies/μl cDNA.

Tissue	NPT		N0 (Stage I/II)		N1 (Stage III/IV)	
Gene	Median	Range	Median	Range	Median	Range
GPC1	238	79-414	453	63-1338	300	114-1033
act-R1a	180	12-466	621	271-767	574	88-1500
act-R1b	103	0-251	148	25-217	83	31-263
act-R2a	153	30-270	320	159-597	244	79-398
BMP-R2	241	54-523	477	61-958	399	107-1388
BMP-R1a	240	5-564	353	304-566	263	68-827
TßRII	908	79-3088	1376	458-2218	793	95-5511
TßRI	197	8-920	349	271-856	405	62-2793

control (40). Positive clones were routinely grown in selection medium.

Immunofluorescence. Cells were washed 3 times with PBS and fixed in 4% paraformaldehyde for 20 min, followed by 5-min incubation in 30 mM glycine/PBS to quench the effect of paraformaldehyde. Cells were washed in PBS-T (0.05% Tween-20 in PBS pH 7.4) and incubated with normal goat serum (KPL, Gaithersburg, MD, USA) for 1 h, followed by incubation with monoclonal mouse anti-GPC1 IgM antibodies for 1 h. Cells were washed in washing buffer and incubated with fluorolink, cy™5 goat anti-mouse IgM (Chemicon Europe Co., Hampshire, UK) for 1 h. All incubation steps were carried out at room temperature. Next, cells were washed and their nuclei were counterstained with propidium iodide (50 mg/ml PBS, pH 7.4). Then, confocal microscopic analysis was carried out using the Spectral Confocal Microscope Leica TCS SL (Leica Microsystems GmbH, Heidelberg, Germany).

Cell proliferation assay. The 3-(4,5-dimethyl-thiazol-2-yl)-2.5-diphenyltetrazolium-bromide (MTT) (Sigma Aldrich, St. Louis, MO, USA) assay was used to assess cell proliferation (30). Cells were seeded in 96-well plates at a density of $2x10^3$ cells/well in 100 μl of complete medium, and doubling time was calculated over 5 days. For induction, cells were seeded at a density of $5x10^3$ cells/well in 100 μl of complete medium, then TGF-ß1, activin-A, and BMP-2 (R&D systems, Wiesbaden-Nordenstadt, Germany) were added in 100 μl of serum-free medium at the indicated doses for 48 h. MTT (5 mg/ml PBS pH 7.4) was added to a final concentration of 0.5 mg/ml. After 4 h of incubation, the formazan products were solubilized with acidic isopropanol and the optical density was measured at 570 nm. The absorbance was corrected for blank readings. The experiments were carried out in triplicate.

Colony formation in soft agar. Cells (2000) were suspended in 0.35% low-melting agarose dissolved in 1.5 ml of 20% FCS/RPMI or DMEM medium and plated on top of 1.5 ml of 0.6% agarose in the same medium in 12-well culture plates. Every 3 days, 0.5 ml of complete medium containing 0.35% agarose was added. After 2 weeks of incubation, colonies were visualized by MTT staining (300 μl/well). Experiments were carried out in triplicate (30).

Immunoblotting. Cells were washed twice with ice-cold PBS and lysed with lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA,1% SDS) containing one tablet of complete mini-EDTA-free protease inhibitor cocktail (in 10 ml buffer). Protein concentration was determined by the BCA protein assay (Pierce Chemical Co., Rockford, IL, USA). Cell lysates (20 μg/lane) were separated on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. Membranes were then incubated in blocking solution (5% non-fat milk in 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20), followed by incubation with goat polyclonal anti-p21 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit polyclonal anti-p-Smad2 (Ser465/467) (Cell Signaling Technology Inc., Beverly, MA, USA), rabbit polyclonal anti-p-Smad1 (Ser463/465) (Upstate, Lake Placid, NY, USA) or rabbit polyclonal anti-active mitogen-activated protein kinase (MAPK) (pTEpY) (Promega, Madison, WI, USA) at 4˚C overnight. The membranes were then washed in blocking solution and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Antibody detection was performed by an enhanced chemiluminescence reaction.

Statistical analysis. Results are expressed as the mean ± standard error of the mean (SEM) unless indicated otherwise. For statistical analysis the Student's t-test was used, and significance was defined as $p<0.05$. The Spearman r-test was used for correlation analysis. Graphs were generated using GraphPad prism software (GraphPad Software, San Diego, CA, USA).

Results

Previously it has been shown by Northern blot analysis that GPC1 mRNA expression is increased in PDAC tissues compared to normal pancreatic tissues (NPT) (34). In the

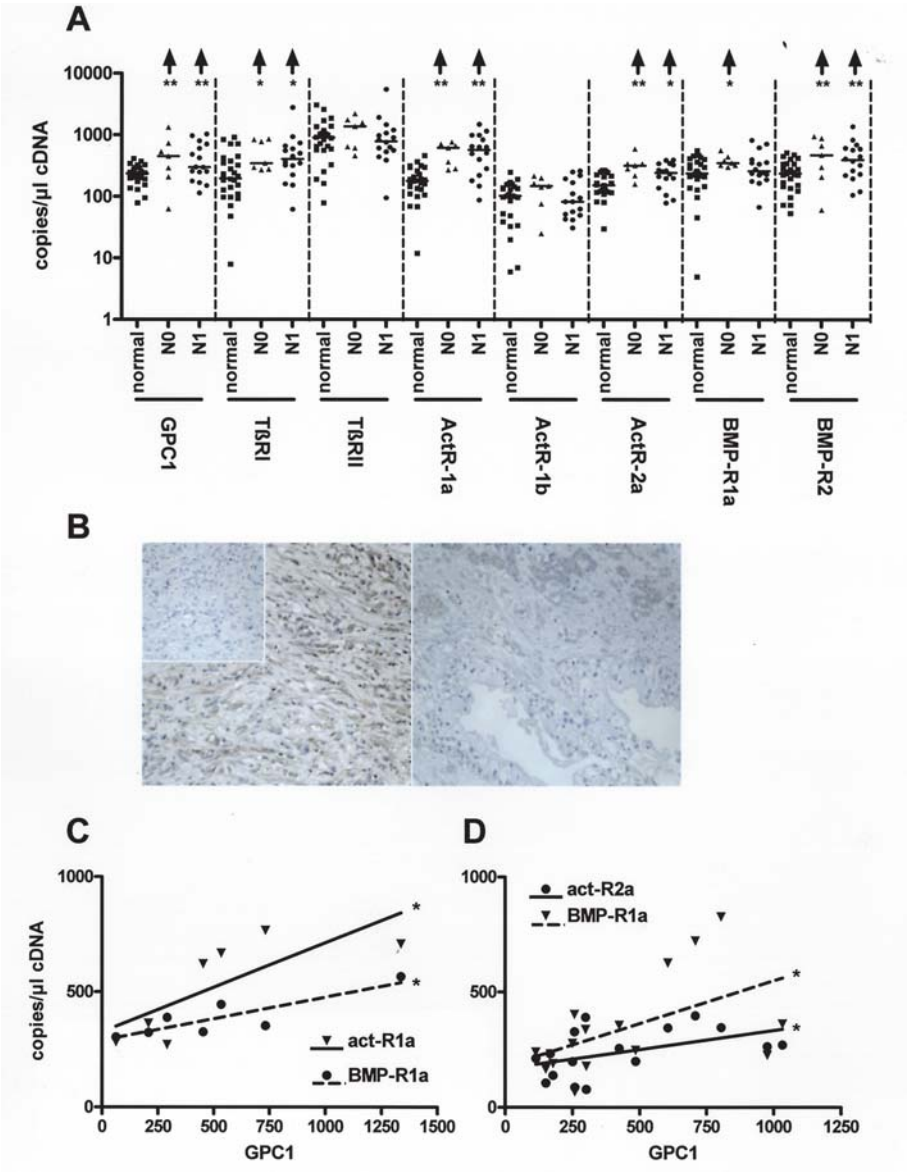

Figure 1. (A) mRNA expression levels of GPC1 and receptors of the TGF-ß family in pancreatic tissues. Real-time quantitative RT-PCR analysis of mRNA levels for GPC1 and TGF-ß receptors in normal (n=27) (squares), N0 (n=7) (triangles) and N1 (n=16) (dots) PDAC tissue samples was performed as described in Materials and methods. Horizontal lines represent the median mRNA levels. The direction of the arrows represents the change of expression between normal and both N0 and N1 cases for each parameter. RNA input was normalized to the average expression of the two housekeeping genes HPRT and cyclophilin B, and is presented as copy number/μl cDNA ($^{*}p<0.05$; $^{**}p<0.01$). (B) GPC1 localization in human pancreatic tissues. Immunohistochemistry was performed as described in Materials and methods. Moderate to strong cytoplasmic GPC1 staining is present in the fibroblasts (left) and in pancreatic cancer cells (right) of PDAC tissues. Note the absent staining in a consecutive tissue section incubated with mouse IgM as a negative control (left inset). Correlation between GPC1 and receptors of the TGF-ß family in N0 (C) and N1 (D) PDAC tissues. Real-time quantitative RT-PCR was performed as described in Materials and methods. GPC1 mRNA expression levels directly correlate with the mRNA levels of act-R1a and BMP-R1a in N0 PDAC cases, and with act-R2a and BMP-R1a in N1 PDAC cases. Values on x and y axes represent mRNA values in copy number/μl cDNA ($^{*}p<0.05$; $^{**}p<0.01$).

present study, we quantitatively confirmed these data in a wider range of both normal pancreatic tissues (n=27) and PDAC tissue samples (n=23) by QRT-PCR. PDAC cases were classified into two groups: cases associated with lymph node metastasis (N1) (n=16) and cases without lymph node metastasis (N0) (n=7). In N1 cases the median GPC1 mRNA levels were 300 copies/μl cDNA (range of 114-1033 copies/μl cDNA), whereas N0 PDAC cases exhibited higher GPC1 mRNA levels (453 copies/μl cDNA with a range of 63-1338 copies/μl cDNA) (Fig. 1A and Table I). The differences between the two groups were not statistically significant. In contrast, NPT displayed lower median GPC1 mRNA levels (238 copies/μl cDNA with a range of 79-414 copies/μl cDNA). Comparing all the values obtained in PDAC samples (N0 + N1) with the values obtained in NPT, revealed that there was a statistically significant increase in GPC1 in the cancer samples by comparison with NPT samples ($p<0.05$). GPC1 was localized in the fibroblasts and pancreatic cancer cells of PDAC tissues (n=5) (Fig. 1B), as has been shown previously (34).

GPC1 might act as a co-receptor for members of the TGF-ß1 family (41,42). Therefore, we next measured mRNA levels encoding TGF-ß receptors (TßRI and TßRII), activin receptors (act-R1a, act-R1b and act-R2a), and BMP receptors (BMP-R1a and BMP-R2), and correlated these mRNA levels

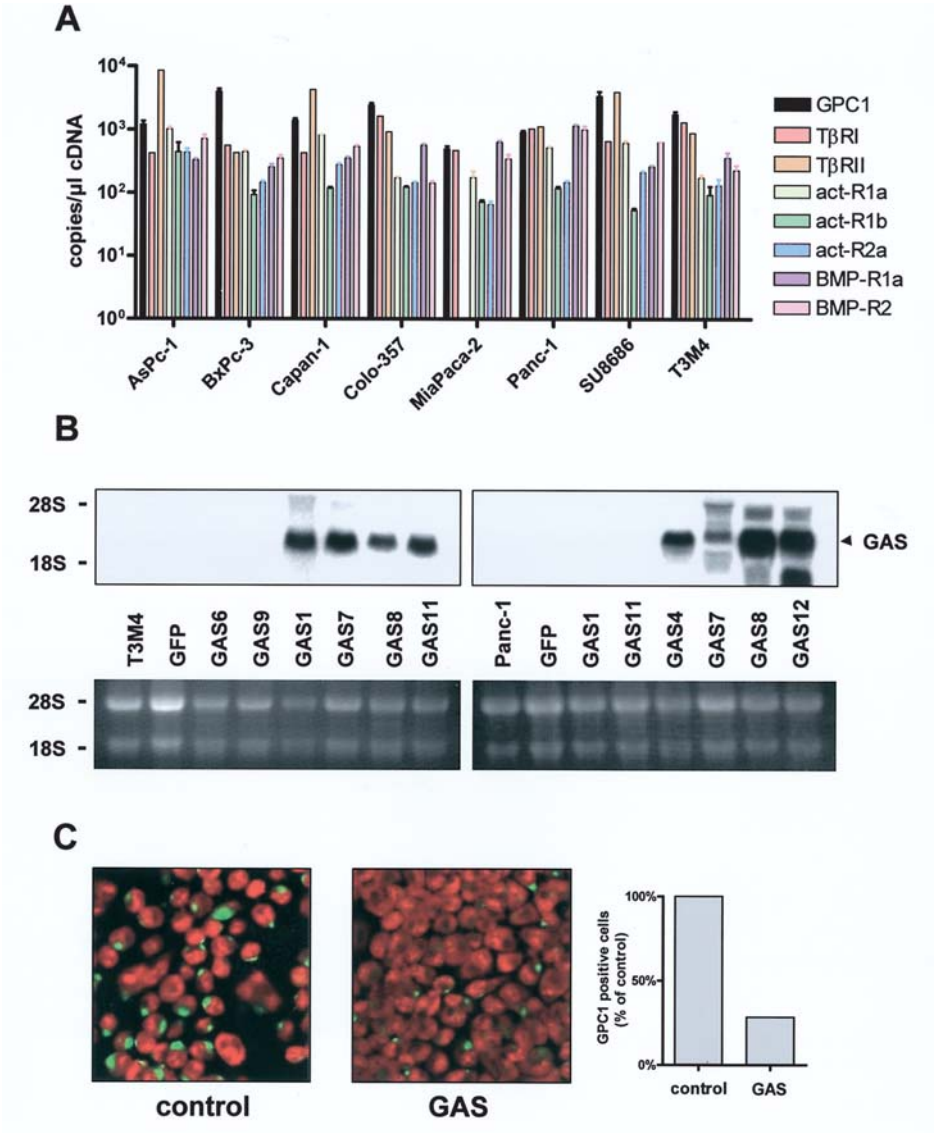

Figure 2. (A) mRNA expression levels of GPC1 and receptors of the TGF-ß family in pancreatic cancer cell lines. Real-time quantitative RT-PCR analysis of GPC1 and TGF-ß receptor mRNA levels in pancreatic cancer cell lines was performed as described in Materials and methods. RNA input was normalized to the average expression of the two housekeeping genes HPRT and cyclophilin B, and is presented as copy number/μl cDNA. Bars represent the mRNA expression values for each parameter as indicated. (B) Stable transfection of a GPC1 antisense construct in Panc-1 and T3M4 cells. Northern blot analysis, and RNA gel electrophoresis of GPC1 antisense (GAS) RNA expression was performed in controls and in GAS-expressing Panc-1 and T3M4 pancreatic cancer cells, as described in Materials and methods. (C) Confocal immunofluorescence microscopy of GPC1 staining in control (left) and GAS-expressing (right) cells. Note the specific membranous and cytoplasmic staining in control cells compared to the absent immunoreactivity using the same antibody in GAS-expressing cells. Graphs represent the percentage of GPC1 downregulation in GAS-expressing cells compared to control cells as detected by confocal immunofluorescence.

with GPC1 mRNA levels in NPT, N0 and N1 PDAC tissues. All receptors were significantly overexpressed in both N0 and N1 PDAC cases compared to NPT except for act-R1b and TßRII. There was also no significant difference in BMP-R1a mRNA expression between N1 PDAC cases and NPT. Interestingly, N1 PDAC cases exhibited lower median mRNA levels of act-R1a, act-R1b, act-R2a, BMP-R1a, BMP-R2 and TßRII, but higher median TßRI mRNA levels compared to N0 PDAC cases (Fig. 1A and Table I).

Next, correlation analysis between GPC1 mRNA levels and the mRNA levels of receptors of the TGF-ß family was performed. There was a significant direct correlation between the expression of GPC1 mRNA and mRNA of all tested receptors in NPT except for TßRI. There was, however, a direct correlation between GPC1 and act-R1a and BMP-R1a mRNA expression in N0 PDAC cases (Fig. 1C), whereas N1 PDAC cases exhibited a direct correlation between GPC1 and act-R2a and BMP-R1a mRNA expression (Fig. 1D). In contrast, there was no significant correlation between GPC1 mRNA expression and expression of the other tested receptors in either N0 or N1 PDAC cases.

To analyze whether GPC1 would interfere with signaling by members of the TGF-ß family, we quantitatively analyzed the GPC1 mRNA expression levels in 8 pancreatic cancer cell lines. GPC1 and receptor mRNA moieties were present in all of the investigated pancreatic cancer cell lines except for MiaPaCa-2 cells, which failed to express detectable levels of TßRII mRNA (Fig. 2A). Previously, it has been

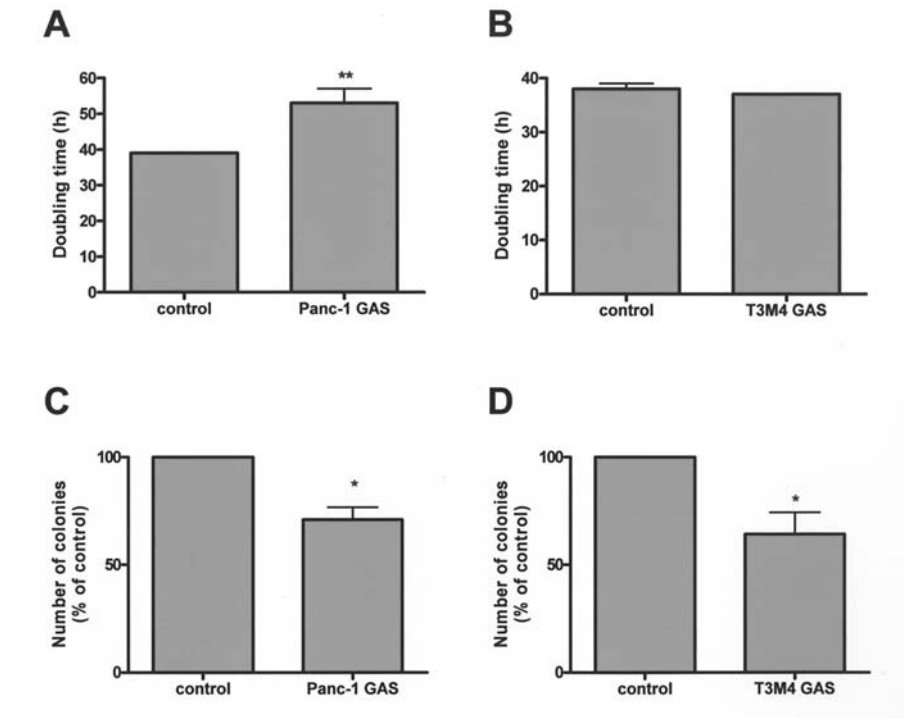

Figure 3. Anchorage-dependent and anchorage-independent growth. (A and B) Basal cell growth was determined by the MTT assay as described in Materials and methods. Data are expressed as mean ± SEM of 3 independent experiments. (C and D) Anchorage-independent cell growth was determined by the soft agar assay as described in Materials and methods. Data are presented as mean ± SEM. Data are presented for control Panc-1 and T3M4 cells as well as for all Panc-1 and T3M4 GAS cells combined ($^{*}p<0.05$).

shown that GPC1 is required for efficient TGF-ß1 signaling in pancreatic cancer cells. Thus, down-regulation of GPC1 expression resulted in attenuated TGF-ß1-induced cell growth inhibition and Smad2 phosphorylation but no significant difference in TGF-ß1-induced p21 expression in Colo-357 pancreatic cancer cells (36). In this study we performed stable transfection of the full-length GPC-1 antisense construct in two other pancreatic cancer cell lines, Panc-1 and T3M4, which express GPC1 and all receptors of TGF-ß, activin and BMP. The expression of GPC1 antisense (GAS) RNA was confirmed by Northern blotting (Fig. 2B). In addition, reduced expression of GPC1 was confirmed by confocal immunofluoresence using a specific GPC1 antibody (34) (Fig. 2C). Two control Panc-1 clones (Panc-1 transfected with GFP vector, and clone #11) and T3M4 clones (T3M4 transfected with GFP vector, and clone #9) as well as two Panc-1 GAS clones (clones #7 and #8) and two T3M4 GAS clones (clones #7 and #11) were used in subsequent experiments. The results are presented for both GAS clones compared to both control clones combined (Fig. 2B). MTT assays were used to determine the difference in proliferation between GAS transfected Panc-1 and T3M4 cells compared to the corresponding control cells. Panc-1 control cells exhibited a shorter doubling time of 39.4±0.1 h compared to Panc-1 GAS cells (53.0 ±3.61 h) ($p<0.05$). T3M4 control and GAS cells exhibited similar doubling time of 38.2±1.0 h and 37.1±0.0 h, respectively (Fig. 3A and B). Anchorage-independent assays revealed that both Panc-1 GAS and T3M4 GAS cells exhibited a significant reduction of colony formation by -29±5.8% and -35.7±10.1%, respectively, compared to the corresponding control cells ($p<0.05$) (Fig. 3C and D).

Previously, it has been shown that the growth inhibitory effects of TGF-ß1 on Colo-357 cells are dependent on GPC1 levels (36). Therefore, in the next set of experiments, the effects of TGF-ß1, activin-A and BMP-2 were tested at the indicated doses for 48 h in both Panc-1 GAS cells and T3M4 GAS cells compared to the corresponding control cells. As expected, TGF-ß1 exerted slight dose-dependent growth inhibitory effects on Panc-1 cells (-12.5±10% at a dose of 500 pM). Surprisingly, there was a slightly more pronounced effect on Panc-1 GAS cells (-18.6±8.4% at 500 pM) (Fig. 4A). In line with this observation, there was a significant increase (2.4-fold) in the TGF-ß1-induced p21 expression in Panc-1 GAS cells compared to control cells (Fig. 4B). In contrast, there was no difference in TGF-ß1-induced Smad2 phosphorylation between Panc-1 GAS and control cells (Fig. 4C). There was no MAPK p42/44 phosphorylation in response to TGF-ß1 in either Panc-1 GAS or control cells. In contrast, the rate of growth of T3M4 control cells increased slightly (maximum effects of +18.9±2.9% in response to 50 pM TGF-ß1), whereas these effects were not present in T3M4 GAS cells (Fig. 4D). There was no p21 induction, Smad1 or 2 phosphorylation or p42/44 MAPK phosphorylation in either control or GAS transfected cells in response to TGF-ß1.

There were no significant effects for activin-A on the growth of Panc-1 control cells. In contrast, activin-A exerted slight growth inhibitory effects (-7.8±8.7% at 10 ng/ml) in Panc-1 GAS cells (Fig. 5A). There was no difference in activin-A-induced p21 expression between Panc-1 GAS and control cells (Fig. 5B). In addition, there was no phosphorylation of either Smad2 (Fig. 5C) or MAPK p42/44. The growth of T3M4 control cells was not affected by activin-A,

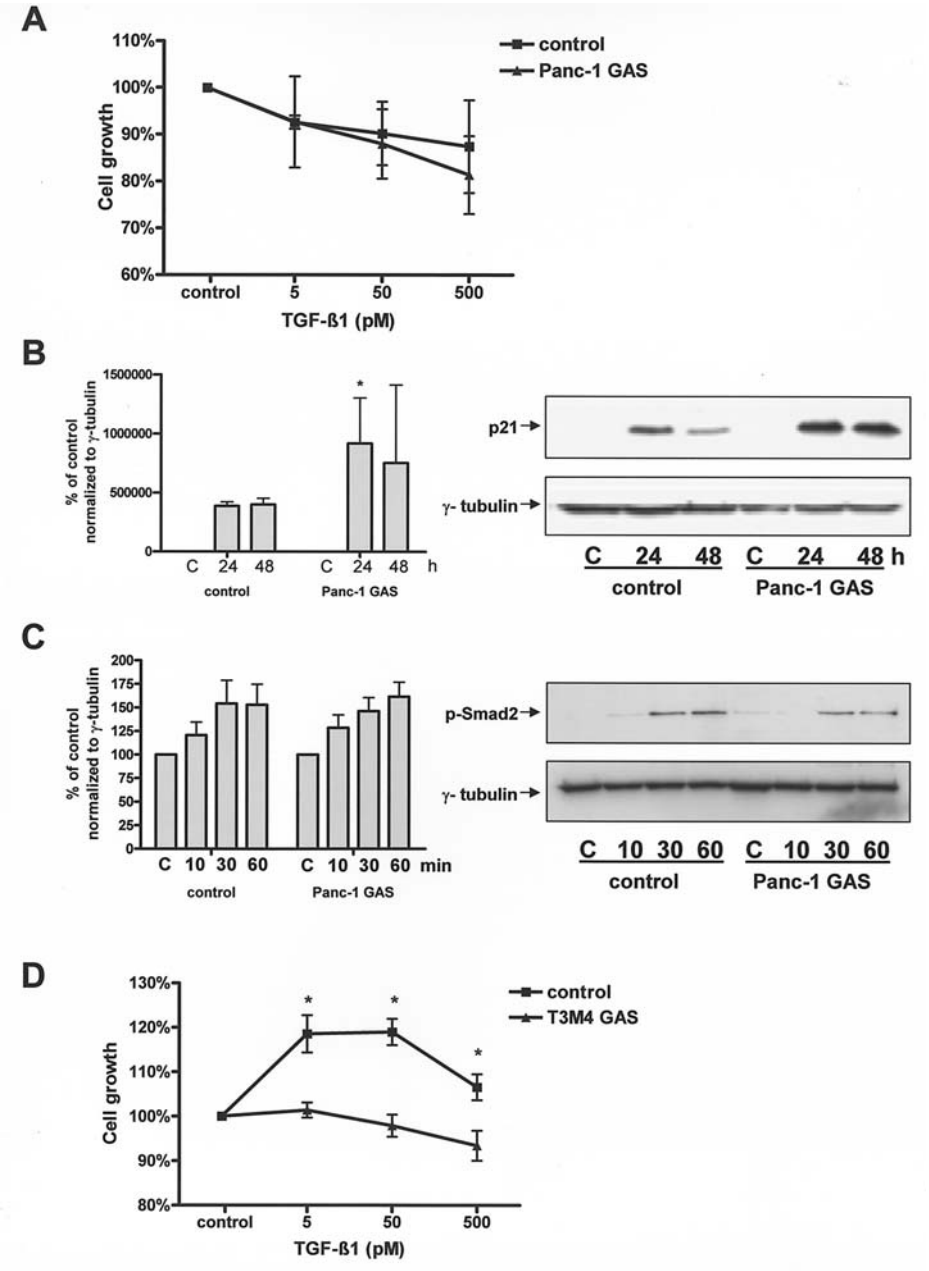

Figure 4. The effects of GPC1 on TGF-ß1 signaling. (A) Control Panc-1 and T3M4 cells (squares) as well as Panc-1 and T3M4 GAS cells (triangles) were incubated with TGF-ß1 at the indicated doses for 48 h before analysis. Data are expressed as percent change compared to controls, and are plotted as mean ± SEM of three independent experiments ($^*p<0.05$). (B and C) Panc-1 control and Panc-1 GAS cells were incubated with 500 pM TGF-ß1, for the indicated time. Protein was extracted and immunoblotting was carried out using anti-p21 (A) and anti-pSmad2 (B) antibodies as described in Materials and methods. Membranes were reprobed with anti γ-tubulin antibody to verify equal loading and transfer. Densitometry data are presented as mean ± SEM ($^*p<0.05$).

whereas T3M4 GAS cells exhibited a +16.2±3.7% increase of growth in response to 10 ng/ml of activin-A (Fig. 5D). Activin-A did not induce p21 expression, and did not phosphorylate Smad2, Smad1 or MAPK p42/44 in T3M4 control and GAS cells.

BMP2 exerted slight growth inhibitory effects on control Panc-1 cells (-7.8±2.5% at 100 ng/ml), and these effects were more pronounced in Panc-1 GAS cells (-15.2±6.3% at 100 ng/ml) (Fig. 6A). In contrast, there was no difference in BMP2-induced p21 (Fig. 6B) and Smad1 phosphorylation (Fig. 6C) between Panc-1 GAS and control cells. In contrast, the growth of T3M4 control cells increased by +12.1±4.5% in response to 10 ng/ml BMP2, and these effects were reduced in T3M4 GAS cells, which exhibited a +4.5±6.9% increase of growth in response to the same dose of BMP2 (Fig. 6D). There was no p21 induction, Smad1 or 2 phosphorylation or p42/44 MAPK phosphorylation in either control or GAS transfected cells in response to BMP2.

Discussion

GPC1 plays an important role in PDAC by acting as a co-receptor for heparin-binding growth factors, thereby contributing to the aggressive phenotype of the PDAC. It has been

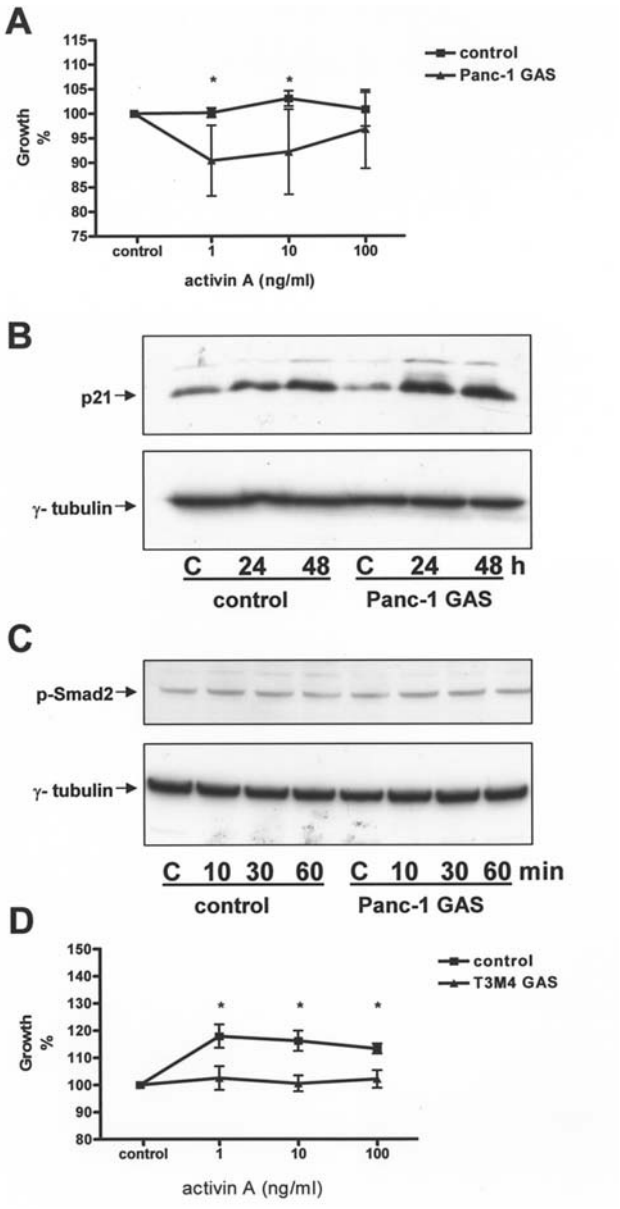

Figure 5. The effects of GPC1 on activin-A signaling. Control Panc-1 (A) and T3M4 (D) (squares) as well as Panc-1 and T3M4 GAS cells (triangles) were incubated with activin-A at the indicated doses for 48 h before analysis. Data are expressed as percent change compared to controls, and are plotted as mean ± SEM of three independent experiments (*p<0.05). (B and C) Panc-1 control and Panc-1 GAS cells were incubated with 100 ng/ml activin-A, for the indicated time. Protein was extracted and immunoblotting was carried out using anti-p21 (A) and anti-pSmad2 (B) antibodies as described in Materials and methods. Membranes were reprobed with anti-γ-tubulin antibody to verify equal loading and transfer.

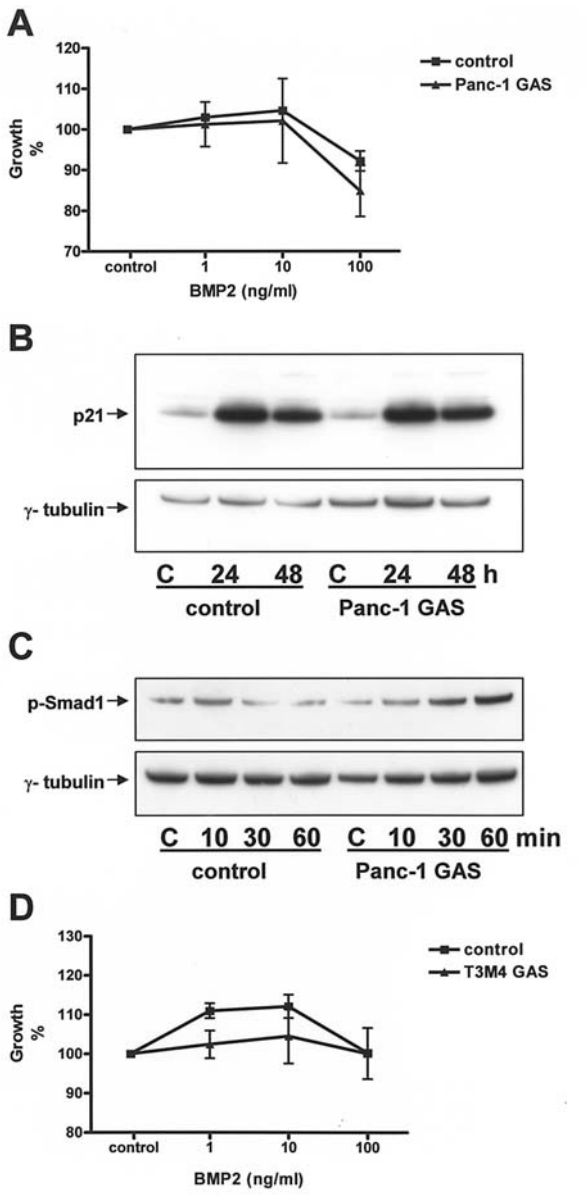

Figure 6. The effects of GPC1 on BMP2 signaling. Control Panc-1 (A) and T3M4 (D) (squares) as well as Panc-1 and T3M4 GAS cells (triangles) were incubated with BMP2 at the indicated doses for 48 h before analysis. Data are expressed as percent change compared to controls, and are plotted as mean ± SEM of three independent experiments (*p<0.05). (B and C) Panc-1 control and Panc-1 GAS cells were incubated with 100 ng/ml BMP2, for the indicated time. Next, protein was extracted and immunoblotting was carried out using anti-p21 (A) and anti-pSmad1 (B) antibodies, as described in Materials and methods. Membranes were reprobed with anti-γ-tubulin antibody to verify equal loading and transfer.

shown that downregulation of GPC1 in pancreatic cancer cells results in diminished responsiveness towards heparin-binding growth factors as well as in reduced tumorigenicity in nude mouse tumor models (34,35).

The importance of HSPGs such as GPC1 in human cancers has been demonstrated in a number of studies. For example, GPC1 is overexpressed in breast cancer and modulates the effects of heparin-binding growth factors in cultured breast cancer cells (43). GPC1 has also been observed in ovarian cancer, where high expression correlates with poor survival (44). In addition, GPC1 overexpression has been observed in gliomas, where it potentially contributes to angiogenesis (45). Other members of the GPC family also exhibit increased expression in certain tumors, such as GPC3 in hepatocellular carcinoma (HCC) (46), gastric tumors (47) and melanoma (48), suggesting a tumor-type specific pattern of expression.

While the function of GPC1 as a co-receptor for heparin-binding growth factors is well established, its role in signaling of members of the TGF-ß family is less clear. For example, Dally, a *Drosophila* member of the GPC family, is required for normal Dpp (a *Drosophila* member of the TGF-ß family) signaling (41). In addition, GPC3 modulates BMP7 signaling in HCC cells (41). GPC3 also interferes with BMP7 signaling during renal branching morphogenesis (49) and BMP4

signaling in limb patterning and skeletal development (8). However, the exact mechanisms by which GPC influences TGF-ß/activin/BMP signaling are currently not known.

We have previously shown that GPC1 is required for efficient TGF-ß signaling in Colo-357 pancreatic cancer cells (36). In the present study, we determined that GPC1 interferes with TGF-ß, BMP and activin signaling in Panc-1 and T3M4 cells, although to a very modest extent. It seems, therefore, that GPC1 is not a major modulator of these signaling pathways. Nonetheless, we demonstrated that there is at least the potential for interaction between GPC1 and TGF-ß signaling, which might be important since pancreatic tumors frequently overexpress not only GPC1 but also receptors of the TGF-ß family (22,23,26,28).

In conclusion, GPC1 expression correlates with a subset of receptors of the TGF-ß family in PDAC and has the potential to interfere with TGF-ß, BMP and activin signaling.

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