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Original Article

Imbalanced expression of Tif1γ inhibits pancreatic ductal epithelial cell growth

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Abstract: Transcriptional intermediary factor 1 gamma (Tif1γ) (Ectodermin/PTC7/RFG7/TRIM33) is a transcriptional cofactor with an important role in the regulation of the TGFβ pathway. It has been suggested that it competes with Smad2/Smad3 for binding to Smad4, or alternatively that it may target Smad4 for degradation, although its role in carcinogenesis is unclear. In this study, we showed that Tif1γ interacts with Smad1/Smad4 complex *in vivo*, using both yeast two-hybrid and coimmunoprecipitation assays. We demonstrated that Tif1γ inhibits transcriptional activity of the Smad1/Smad4 complex through its PHD domain or bromo-domainin pancreatic cells by luciferase assay. Additionally, there is a dynamic inverse relationship between the levels of Tif1γ and Smad4 in benign and malignant pancreatic cell lines. Overexpression of Tif1γ resulted in decreased level of Smad4. Both overexpression and knockdown of Tif1γ resulted in growth inhibition in both benign and cancerous pancreatic cell lines, attributable to a G2-phase cell cycle arrest, but only knockdown of Tif1γ reduces tumor cell invasiveness *in vitro*. Our study demonstrated that imbalanced expression of Tif1γ results in inhibition of pancreatic ductal epithelial cell growth. In addition, knockdown of Tif1γ may inhibit tumor invasion. These data suggest that Tif1γ might serve as a potential therapeutic target for pancreatic cancer.

Keywords: Transcriptional intermediary factor 1 gamma (Tif1γ), Smad, pancreatic cancer

Introduction

Pancreatic cancer is one of the most deadly forms of malignancy [1]. The dismal prognosis is largely due to the difficulty in early diagnosis and lack of effective therapy. The complexity of pancreatic carcinogenesis involves multiple signaling cascades, including transforming growth factor beta (TGFβ), KRAS–MAPK, and p53 pathways, which play important roles in the initiation, promotion, and progression of pancreatic cancer. Among them, dysregulation of TGFβ pathway is implicated in the development of the majority of pancreatic cancers [2, 3]. The TGFβ signaling pathway is activated by ligands such as Bone Morphogenetic Proteins (BMP), Smad transcriptional factors, and downstream intranuclear effectors [4]. Dysregulation of the TGFβ pathway may occur along any step of the pathway, due to various genetic or genomic abnormalities (including mutation/deletions) of the members of the TGFβ pathway family. Inactivation or deletion of *Smad4* leading to major dysregulation of TGFβ pathway, is observed in 50-90% of pancreatic cancers with abnormal TGFβ activity [5-7]. There is evidence suggesting that additional factors other than Smad4 are involved in pancreatic carcinogenesis [8, 9]. One such factor is a recently discovered cofactor of Smad4, the transcriptional intermediary factor 1 gamma (Tif1γ) [10-13].

Tif1γ, also known as Ectodermin/PTC7/RFG7/TRIM33, is one of seventy tripartite motif-containing Trim proteins [10]. Together with Trim24 (Tif1α) and Trim28 (Tif1β), it forms a transcrip-

tion intermediary factor 1 (Tif-1) subfamily of transcriptional regulators [11]. Tif1γ plays a major role in embryonic development, spermatogenesis [10], and hematopoiesis [12, 13]. Although its exact biological functions are unclear, Tif1γ is an important regulator of the TGFβ pathway. However, the regulative role of Tif1γ in the TGF pathway is complicated by its dual effects. Some studies have shown that it may upregulate TGFβ signaling by competing with Smad4, a tumor suppressor, for binding to Smad2 and Smad3, forming Tif1γ-Smad2/3 complex [10]. On the other hand, Tif1γ was shown to promote Smad4 degradation via the ubiquitin-proteasome pathway [14, 15], and relocate the Smad4 protein from the nucleus to the cytoplasm, showing a negative or down-regulative effect in the TGFβ pathway. The dual effects of Tif1γ suggest the complexity of the role of Tif1γ in regulating TGFβ pathway.

Very few studies of Tif1γ have been focused on its role in carcinogenesis. Translocations between *TIF1γ* and *RET* genes were shown to result in a fusion gene encoding a receptor tyrosine kinase that leads to development of childhood papillary thyroid carcinomas [16]. In colorectal cancer, it appears that Tif1γ is involved in early stages of carcinogenesis, with high levels of Tif1γ detected in almost 50% of the early stage cancers [17]. In Smad4-deficient pancreatic cancer cells with intact TGFβ receptors, Smad2/3, and Tif1γ [18], Tif1γ can effectively form a complex with Smad2/3 which participates in the pro-tumorigenic responsiveness of tumor cells to TGFβ [12], whereas Smad4-positive lines do not. It appears that there is a link between inactivation of Smad4 and overexpression of Tif1γ in cancer cell lines, irrespective of cell types. However, it has been found [19] that human pancreatic adenocarcinoma showed a low level of Tif1γ expression compared to the adjacent normal ductal epithelium and that loss of Tif1γ did not impair Smad4 expression. These contradictory results may reflect heterogeneity of pancreatic cancers which may be due in part to the dual functions of Tif1γ in regulating the TGFβ pathway. The exact mechanism of Tif1γ functions therefore needs to be elucidated.

This study was designed to determine the biological roles of Tif1γ in pancreatic carcinogenesis and its relationship with Smad proteins, particularly Smad1 and Smad4. We analyzed the effects of up- and down-regulation of Tif1γ on Smad4 expression, the cell proliferation, and invasive ability in various pancreatic cancer cell lines.

Materials and methods

Cell culture

HPNE cell lines (hTERT-HPNE, hTERT-HPNE-E6/E7, hTERT-HPNE-E6/E7+K-Ras^{G12D}), immortalized acinar-to-ductal intermediary cells isolated from adult pancreas, were kindly provided by Michel Ouellette, University of Nebraska Medical Center, Omaha, NE [20, 21]. The cells were cultured in medium consisting of 3 volumes low glucose DMEM (11885, Gibco), one volume M3 medium (M300A-500, In Cell), 5% fetal bovine serum (FBS, 100-506, Gemini Bio-Products), and 50 μg/ml gentamycin (15750-060, Gibco). The immortalized human pancreatic ductal cell line HPDE6-E6E7-c7 (H6c7) [22, 23], donated by Ming-Sound Tsao, University Health Network, Toronto, Ontario M5G 2C4 Canada, was grown in Keratinocyte-SFM medium (10724, Gibco) containing 1x antibiotic-antimycotic (15240, Gibco). Panc-1 cells (CRL-1469, ATCC) isolated from a pancreatic duct adenocarcinoma, were cultured in DMEM (high glucose) medium (11965, Gibco) supplemented with 10% cosmic calf serum (SH30087.03, HyClone) and 50 μg/ml gentamycin. Capan-1 cells (HTB-79, ATCC), originating from a pancreatic adenocarcinoma [24], were cultivated in Iscove's MDM medium (31980, Gibco) supplemented with 20% FBS and penicillin-streptomycin (15140, Gibco). BxPC-3 cells (CRL-1687, ATCC), isolated from a pancreatic adenocarcinoma [24], were grown in RPMI-1640 medium (22400, Gibco) supplemented with 10% FBS and penicillin-streptomycin.

Western blot analysis

Western blot analysis was used to examine expression of Tif1γ, Smad4, and β-actin (loading control) in pancreatic cell lines. The cells were lysed in an appropriate volume of lysis buffer containing protease cocktail inhibitor (P8340, Sigma) and the extracts were separated using SDS-polyacrylamide gel electrophoresis. The proteins were transferred to a nitrocellulose membrane for western blot analysis and developed with antibodies raised against Tif1γ (ab57172, Abcam), Smad4 (ab40759, Abcam),

and β-actin (A2228, Sigma), and with the appropriate horseradish peroxidase-conjugated secondary antibody (NA931V or NA9340V, GE Healthcare).

Co-immunoprecipitation

Capan-1 was chosen for this experiment due to its high expression of Tif1γ and complete inactivation of Smad4. Batches of Capan-1 cells I (approx. 10^7) were transfected respectively with a vector carrying Smad4 open reading frame (pRK5-hSmad4-flag), or a vector carrying Smad1 (pRK5-hSmad1-flag) or the empty vector in the presence of BMP2 (10 ng/μl) or absence of BMP2. Cells were lysed in cell lysis buffer (K0301, Fermentas) supplemented with protease and phosphatase inhibitors (P8340, Sigma). Tif1γ antibody (ab57172, Abcam) was added to the lysate, followed by Sepharose G slurry. Western blotting was performed as described above using antibodies against Tif1γ (ab57172, Abcam), Smad4 (ab40759, Abcam), and Smad1 (ab66737, Abcam) antibodies. The intensities of western blot bands were measured by densitometry.

RNA isolation and qRT-PCR

Total RNA was extracted from the cell lines using RNAqueous-4PCR kit (AM1914, Ambion). Retro Script kit (AM1710, Ambion) was used for cDNA synthesis with isolated RNA as template, according to manufacturer's instructions. The qPCR reactions were performed using Tif1γ, Smad4, and 18S rRNA primers (Tif1γ-4 forward: AGCACCATGAATCCTTCTCC, reverse: GTACTTGGGGGTCTCACAGG; Smad4 forward: TGGAGCTCATCCTAGTAAATGTG, reverse: AGGAAATCCTTTCCGACCAG; 18S rRNA forward: GGATCCATTGGAGGGCAAGT, 18S rRNA reverse: AATATACGCTATTGGAGCTGGAATTAC1) in the CFX96 qPCR system (360-0037, Biorad). The data were analyzed using CFX Manager, version 1.5 (184-5000, Biorad).

Immunofluorescence

Cells were grown and fixed as described previously [25]. The cells were then incubated with Tif1γ primary antibody followed by fluorescein-conjugated rabbit polyclonal antibody (ab-98458, Abcam). The localization of Tif1γ was then examined using Nikon Digital DXM1200F microscope with Nikon-ACT program.

Plasmid transfection using electroporation

The pBabe-Tif1γ expression vector was constructed by cloning the complete PCR fragment of Tif1γ between NdeI and BamHI restriction sites of pBabe vector and verified by sequencing [26]. Plasmids pBabe-Tif1γ, pRetroSuper-Tif1γ (15728, Addgene), and their corresponding control plasmids, were transfected into the pancreatic cell linesusing the Nucleofector II system (Lonza).

Flow cytometry

Cells were prepared for flow cytometry as described previously [25]. The cell cycle analysis was performed on FACS Calibur flow cytometer (BD Biosciences) and analyzed using Weasel software (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia).

Luciferase assay

The effect of Tif1γ on transcriptional activity of Smad1/Smad4 complex was tested using HPNE cells that were transfected with expression plasmids pBabe-Tif1γ, pRK5-hSmad1-flag [27], pRK5-hSmad4-flag [27], reporter plasmid $(gCCg)_{12}$-luc [28], as well as pRL plasmid carrying the Renilla luciferase internal control and a plasmid carrying a constitutively active allele of BMP-2 receptor ALK6 [29], using electroporation as described above. The final amount of plasmid DNA used for transfection was 200 ng/well for Smad1 and Smad4, and 100, 200, and 400 ng/well for Tif1γ. The electroporated cells were allowed to grow for 24 hours, and in the presence of 10 ng/μl BMP-2 for the last 7 hours. Dual-Luciferase Reporter Assay System (E1910, Promega) was used according to manufacturer's instructions. The luminescence was measured on the Lumat LB 9507 luminometer (81957-52, Berthold Technologies).

Cell proliferation assay

Pancreatic cell lines were maintained as described above (see Cell culture) and a proliferation assay was performed under either one of following conditions: 1) Overexpressing Tif1γ by transfecting plasmid containing Tif1γ (pBabe-Tif1γ) in the absence or presence of BMP2 (10 ng/μl) (GenScript USA Inc., 860 Centennial Ave., Piscataway, NJ); 2) Inhibiting expression of Tif1γ by transfecting Tif1γ shRNA in the absence or presence of BMP2 (10 ng/μl).

Cell lines transfected with pBabe-vector were used as control. Cell proliferation was measured by the colorimetric WST assay (Roche Applied Science).

Matrigel invasion assay

Cells were resuspended in medium without serum at 10^5 cells/ml, and 0.5 ml of cell suspension was loaded into the inserts of Biocoat Matrigel invasion chambers with reduced growth factor levels (#354483, BD Bioscience). The lower chambers were filled with 750 μl of the medium containing 10% fetal bovine serum as the chemoattractant. Invading cells were counted as described previously [25].

Yeast two-hybrid assays

Yeast strain W303-1A [30] was transfected [31] with pSH18-34 reporter vector (Origene Technologies) and the appropriate plasmids carrying constructs to be tested. The two-hybrid tests were performed as described previously [32].

Proteomic pathway array analysis (PPAA)

PPAA was performed as described previously [33]. Antibodies were obtained from a number of sources (Table S2). The blot was hybridized with secondary horseradish peroxidase-conjugated antibodies (Bio-Rad). Chemiluminescence signal was detected using the ChemiDoc XRS System. Differences in protein levels were analyzed by densitometric scanning and normalized using internal standards.

Statistical analysis

The Fisher exact test was used to compare categorical data. Statistical analysis was performed using Statistical Package for Social Sciences software (for Windows 12.0, SPSS Inc.). We used a two-sided significance level of 0.05 for all statistical analyses.

Results

Physical interaction of Tif1γ with Smad1/Smad4 complex in the yeast two-hybrid system and co-immunoprecipitation

It has been shown previously that Tif1γ acts as an inhibitor of Smad2/3 in the TGFβ signaling pathway in human hematopoietic stem/progenitor cells [12]. We wanted to test whether Tif1γ could also interact with other Smad proteins, in particular Smad1, a key factor in the bone morphogenetic protein (BMP) branch of the TGFβ pathway.

To determine the interaction between Tif1γ and Smad proteins, we performed immunoprecipitation of Tif1γ both in the presence and absence of Smad1 using anti-Tif1γ antibody (**Figure 1A**). Capan-1 cells, expressing high levels of endogenous Tif1γ and lacking Smad4, were transfected with either Smad4 and Smad1 expression vectors or a control plasmid, in the presence or absence of BMP2. Immunoprecipitation and subsequent western blotting of the lysate prepared from the cells without overexpression of Smad1/4 revealed the presence of Smad1 coimmunoprecipitating with Tif1γ, suggestive of the interaction of these two proteins with one another in Capan-1 cells. The immunoprecipitation of Smad1 and Smad4 proteins was significantly enhanced in cells transfected with Smad1 and Smad4 expression vectors, suggestive of possible interaction between all three proteins *in vivo*. Furthermore, in response to BMP2 stimulation, the amount of Smad1 and Smad4 that are co-precipitated with Tif1γ is significantly increased, suggestive of a direct interaction among Tif1γ, and Smad1/Smad4 complex in the BMP pathway in pancreatic cancer cells (**Figure 1B**).

We next used the yeast two-hybrid system to determine whether the physical interactions between Tif1γ and Smad proteins are direct (Figure S1). In particular, we sought to determine if Tif1γ interacts with Smad1 and/or Smad4 individually or if the interaction is Smad1/4 complex specific. We constructed a yeast expression vector containing N-terminal fusion of full length Tif1γ to the LexA bacterial DNA-binding domain (Tif1γ-FL-DBD). We built similar constructs containing N-terminal LexA fusion to the N-terminal half of Tif1γ, containing the RING domain as well as the B1, B2, and coiled-coil domains (Tif1γ-N-DBD), and the N-terminal LexA fusion to the C-terminus of Tif1γ, harboring the PHD domain and the bromo-domain (Tif1γ-C-DBD). When we co-transfected each of the Tif1γ yeast vectors together with either Smad1-TA or Smad4-TA vectors, we were unable to detect any interaction by our two-hybrid system in either of these combinations with only a single Smad protein (**Figure**

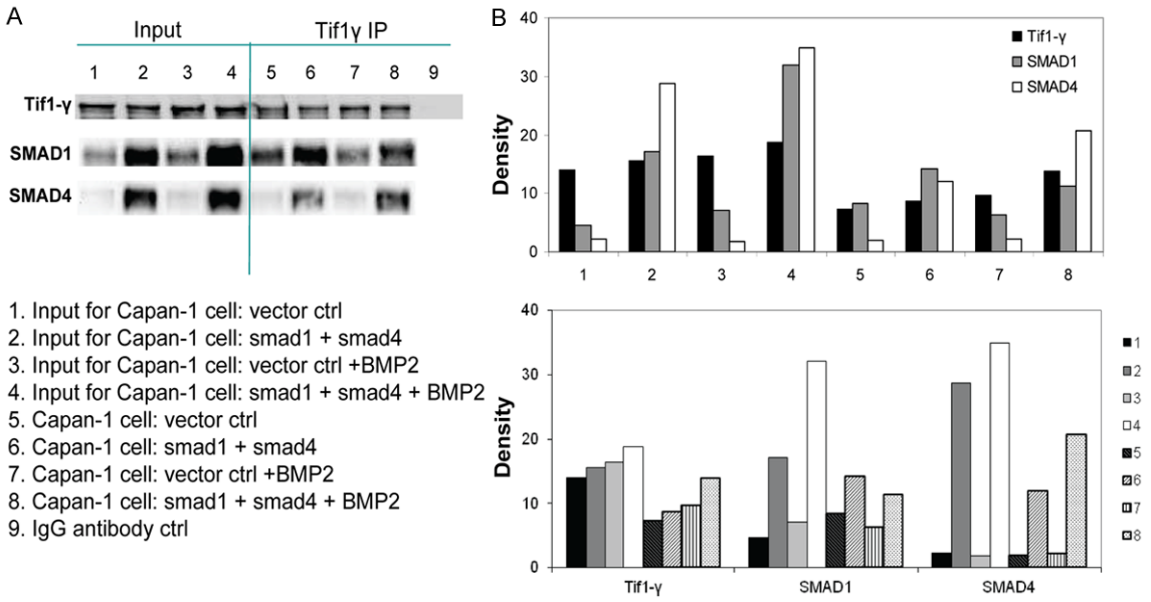

Figure 1. Co-immunoprecipitation of Tif1γ-Smad1/Smad4 protein complexes by anti-Tif1γ antibody from lysates of Capan-1 cells. Cells were transfected with Smad1 expression vector together with either Smad4 expression vector or a control vector in the absence or presence of BMP-2. The presence of Tif1γ, Smad4, and Smad1 was detected by the appropriate antibodies by western blot analysis (A, left panel). As a control, input lysate represented 1/50 of the immunoprecipitation volume. IP result was depicted in (A, right panel). The intensities of western blot and IP bands were measured by densitometry (B).

2A). To test whether Smad1 and Smad4 together as a complex would be able to interact with Tif1γ, we transfected yeast with each of the Tif1γ-DBD fusions, paired with Smad4-TA expression vector and a plasmid expressing full length, unfused Smad1 as a protein bridge. These extracts of yeast cells expressing full length Tif1γ together with both Smad1 and Smad4 were able to cleave the chromogenic substrate at four-fold rate compared to the baseline (**Figure 2B**). These results suggest that Tif1γ interacts with the Smad1-Smad4 complex but does not interact individually with either of these two proteins. When we substituted the full length Tif1γ-DBD fusion with the construct carrying only its C-terminal sequence, we did not observe this interaction. However, expressing the fusion of the C-terminal part of Tif1γ and the DBD domain resulted in interaction almost as strong as that of the full length Tif1γ, suggesting that the PHD domain or the bromo-domain maybe responsible for the interaction of Tif1γ with the Smad1/Smad4 complex. To test whether Smad2 is required for Tif1γ function, we transfected the yeast with the individual Tif1γ-DBD fusions together with Smad4-TA and an expression vector carrying wild-type, unfused Smad2 (analogous to Smad1 above). We could not detect any interaction between these proteins (**Figure 2C**). Therefore, Tif1γ is able to bind to the Smad1/Smad4 complex, but doesn't recognize the Smad2/Smad4 complex in this system [12].

TIF1γ functions as a nuclear transcriptional corepressor, inhibiting the activity of Smad1/4 complex in pancreatic cells

To determine whether Tif1γ functions as a transcription coactivator or corepressor of the TGFβ pathway in pancreatic cells, we performed a transcriptional assay using the dual luciferase reporter system. The HPNE cell line was chosen for this experiment due to its low relative Tif1γ expression among the pancreatic cell lines tested (**Figure 3A**). HPNE cells were transiently transfected with a luciferase reporter plasmid under the control of promoter containingtwelve GCCG Smad1-binding repeats [28]. Cells were cultured in the presence of BMP-2 ligand, specific for the Smad1/4 signaling pathway. As shown in **Figure 2D**, expression of Tif1γ alone had no effect on basal reporter activity. Co-transfection of both Smad1 and Smad4 plasmids increased reporter activity by approximately 70% as compared to the baseline level defined by reporter activity in cells transfected only with control plasmids. Next we introduced

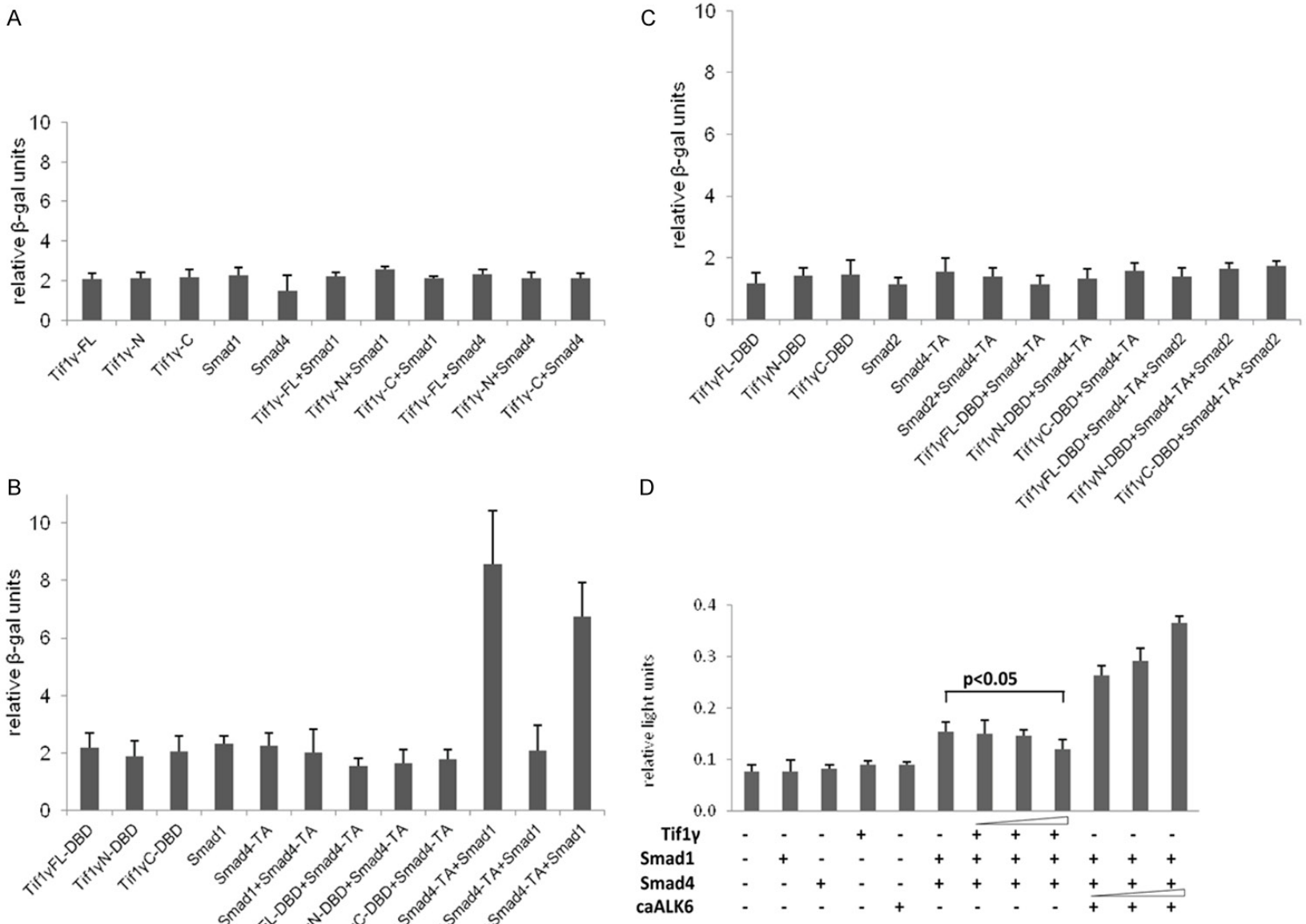
A
relative β-gal units
Tif1γ-FL
Tif1γ-N
Tif1γ-C
Smad1
Smad4
Tif1γ-FL+Smad1
Tif1γ-N+Smad1
Tif1γ-C+Smad1
Tif1γ-FL+Smad4
Tif1γ-N+Smad4
Tif1γ-C+Smad4
B
relative β-gal units
Tif1γFL-DBD
Tif1γN-DBD
Tif1γC-DBD
Smad1
Smad4-TA
Smad1+Smad4-TA
Tif1γFL-DBD+Smad4-TA
Tif1γN-DBD+Smad4-TA
Tif1γC-DBD+Smad4-TA
Tif1γFL-DBD+Smad4-TA+Smad1
Tif1γN-DBD+Smad4-TA+Smad1
Tif1γC-DBD+Smad4-TA+Smad1
C
relative β-gal units
Tif1γFL-DBD
Tif1γN-DBD
Tif1γC-DBD
Smad2
Smad4-TA
Smad2+Smad4-TA
Tif1γFL-DBD+Smad4-TA
Tif1γN-DBD+Smad4-TA
Tif1γC-DBD+Smad4-TA
Tif1γFL-DBD+Smad4-TA+Smad2
Tif1γN-DBD+Smad4-TA+Smad2
Tif1γC-DBD+Smad4-TA+Smad2
D
relative light units
p<0.05
Tif1γ
Smad1
Smad4
caALK6

Figure 2. Interaction between Tif1γ and Smad1 and Smad4 in yeast two-hybrid assay. Yeast cells were transfected with (A) Full length or truncated Tif1γ-DBD fusion, together with either Smad1-B42 or Smad4-B42 fusions. (B) Full length or truncated Tif1γ-DBD fusion, together with both Smad4-B42 fusion and Smad1. (C) Full length or truncated Tif1γ-DBD fusion, together with both Smad4-B42 fusion and Smad2. Expression of constructs was induced by addition of galactose to the growth media and the activity of β-galactosidase was measured spectrophotometrically. (D) Tif1γ inhibits transcriptional activity of Smad1/4 complex. HPNE cells were transfected with Smad1 and Smad4 expression vectors, as well with Tif1γ expression vectors at the final concentration of 100, 200, and 400 ng/well and induced with BMP-2. Cells were lysed and the reporter activity was measured as relative light output units.

increasing amounts of Tif1γ to determine whether it affect the activity of the Smad1/4 complex. We observed that Tif1γ inhibited the transcriptional ability of Smad1/4 complex to facilitate expression from Smad1 promoter in a dose-dependent manner. At the highest amount of transfected Tif1γ tested, its presence resulted in a statistically significant ($p<0.05$) decrease of reporter gene activity of approximately 25% (**Figure 2D**).

Inverse relationship between expression of Tif1γ and Smad4 in pancreatic cancer cell lines

We next examined how the expression of Tif1γ is related to the loss of Smad4 in pancreatic cancer cell lines. Using immunofluorescence, we verified that the Tif1γ protein is primarily localized within the nucleus (Figure S2). We also performed western blot analysis of cell lysates to detect protein levels of Tif1γ and Smad4 in benign and malignant pancreatic cell lines (**Figure 3A**). In HPNE cells, where Tif1γ protein was hardly detectable, Smad4 signal was the strongest. On the other hand, in HPDE, BxPC-3, and Capan-1 cells, relatively high levels of Tif1γ were accompanied by low expression of Smad4. In Panc-1 cells we observed intermediate levels of both Tif1γ and Smad4 (**Figure 3A**). In summary, the levels of Tif1γ were inversely correlated with Smad4 protein levels.

To test if this inverse correlation was present at the transcriptional level, we also quantified mRNA levels of TIF1γ and Smad4 using quantitative PCR. The relative levels of Tif1γ transcripts in most of the cells we tested corresponded to the protein levels detected by western blotting (**Figure 3B**). However, in the HPDE cell line the level of Smad4 transcript was relatively higher than the level of protein (**Figure 3A** and **3B**).

To determine the dynamics of the relationship between overexpression of Tif1γ and reduced level of Smad4, we first transiently overexpressed Tif1γ in the HPNE cell line, expressing undetectable level of endogenous Tif1γ protein and abundant wild type Smad4, and analyzed the Smad4 protein level by western blot. Compared to control cells, the cells overexpressing Tif1γ demonstrated a significant decrease of Smad4 protein level (55% as determined by densitometric analysis of the blots, adjusted for β-actin levels as a loading control) as shown in **Figure 3C**, left side. BxPC-3 cells have high level of Tif1γ and negligible level of wild-type Smad4 protein, and therefore we used this cell line to test the effect of increased Smad4 expression on the amount of Tif1γ. After transiently overexpressing Smad4, we detected no change in the levels of Tif1γ between the Smad4-overexpressing and control cells (**Figure 3C**, right side). This observation suggests that Tif1γ could regulate Smad4 level but not *vice versa.*

The effect of Tif1γ overexpression and knockdown on pancreatic cell growth

We next sought to determine how the transcriptional inhibitory effect of Tif1γ on the Smad1/4 complex affects cell growth. For this purpose, we transfected HPDE, BxPC-3, Capan-1, and Panc-1 cells with either vector carrying Tif1γ overexpression or shRNA for Tif1γ, or control vector. The Tif1γ expression level was followed using western blot and RT-PCR (Figure S4). For the HPNE cells we used transiently transfected Tif1γ plasmid due to its natural resistance to puromycin at all concentrations tested to prevent stable selection. Overexpression of Tif1γ led to reduced proliferation rates in all cells tested. Stable overexpression of Tif1γ in the cancer cell lines (HPDE, Capan-1, BxPC-3 and Panc-1) reduced growth rate by 40-50% compared to control cells, whereas the inhibitory effect on both benign pancreatic cell lines was about 25% (**Figure 4**). BMP-2 stimulation did not change the inhibitory effect of overexpressed Tif1γ on Capan-1 and HPNE cells (data not shown).

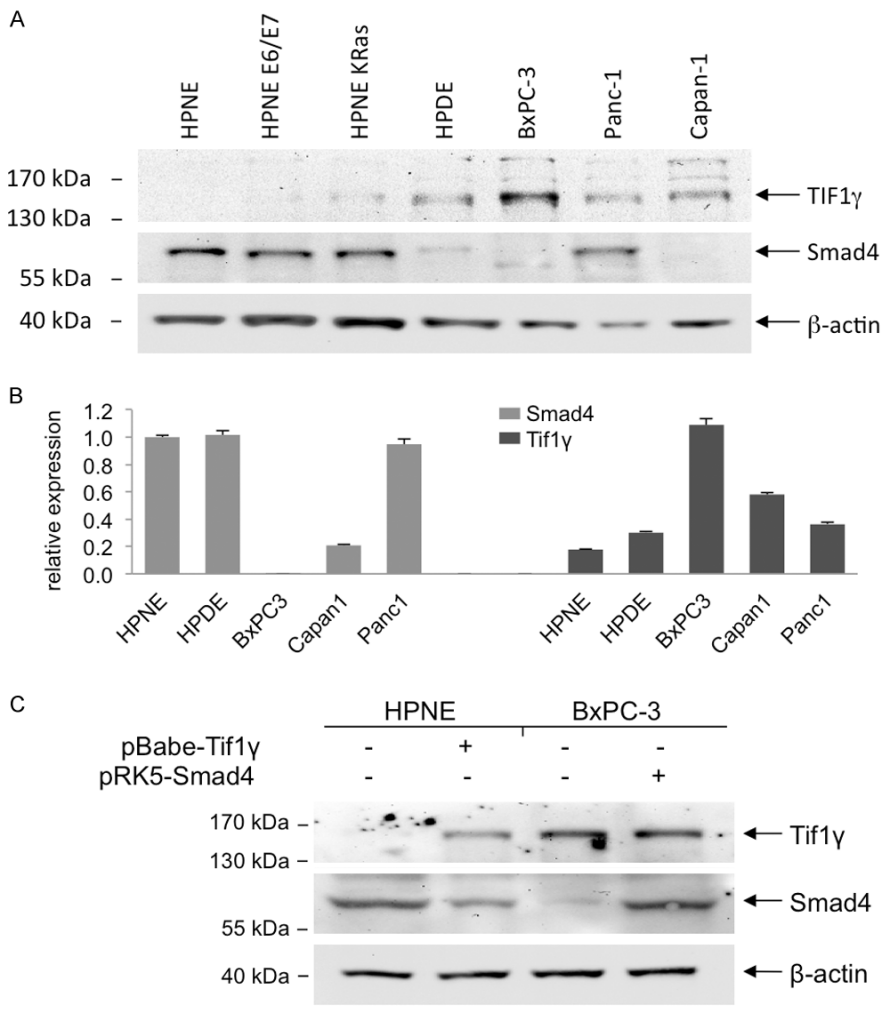

Figure 3. Inverse expression of Tif1γ and Smad4 in pancreatic cell lines. Pancreatic cell lines were grown in their appropriate media and harvested. A. Cell lysates were subjected to SDS-PAGE and western transfer, followed by the detection of Smad4, Tif1γ, and β-actin (loading control) proteins. B. RNA was extracted from the harvested cells and Tif1γ as well as Smad4 transcripts were quantified using qRT-PCR relative to 18S rRNA transcript. C. Overexpression of Tif1γ decreases Smad4 levels. HPNE cells (left side) were transfected with Tif1γ overexpression vector. BxPC-3 cells (right side) were transfected with Smad4 overexpression vector. The effects of Tif1γ overexpression on intrinsic Smad4 levels in HPNE, as well as the effect of overexpression of Smad4 on intrinsic Tif1γ levels in BxPC-3, were detected after western transfer using the appropriate antibodies.

In BxPC-3, Capan-1, and Panc-1 transfected with Tif1γ shRNA, the decrease in Tif1γ protein levels resulted in marked inhibition of cell proliferation. In BxPC-3, Capan-1, and Panc-1, the proliferation rates after knockdown cells were reduced 50% compared to control cells. In HPDE cells this decrease was more pronounced and reached a 65% reduction of proliferation rate (**Figure 5**). In the presence of BMP-2, Capan-1 cell growth was inhibited only at day 7 with Tif1γ knockdown, whereas the effect of BMP-2 on the inhibition of HPNE cell growth by Tif1γ knockdown was not evident (data not shown).

Next, we wanted to determine if the reduced proliferation rates in these transfected cells was due to cell cycle arrest. We used flow cytometry to analyze the cell cycle of HPDE, BxPC-3, Capan-1, and Panc-1 cells after stable Tif1γ overexpression or knockdown. In all four cell lines the knockdown of Tif1γ led to a decreased number of the cells in S-phase, and accumulation of cells in the G2-phase (**Figure 6A-D**, Figure S3A-D). The number of the cells in the G0/G1 phase decreased slightly in all the cell lines. The reduction of the number of the cells in the S-phase ranged from around 30% in HPDE cells, to 40% in Panc-1 cells and 50% in BxPC-3 and Capan-1 cells, corresponding with an increase in G2 phase cells by approximately two fold.

Overexpression of Tif1γ also led to reduced S phase and increased G2 phase cells (**Figure 6E-H**, Figure S3E-H). The decrease of S-phase cells after overexpression of Tif1γ ranged from 25% in HPDE cells, to 40% in Panc-1 cells and 50% in BxPC-3 and Capan-1 cells, corresponding with an increase of G2 phase cells between two to three fold.

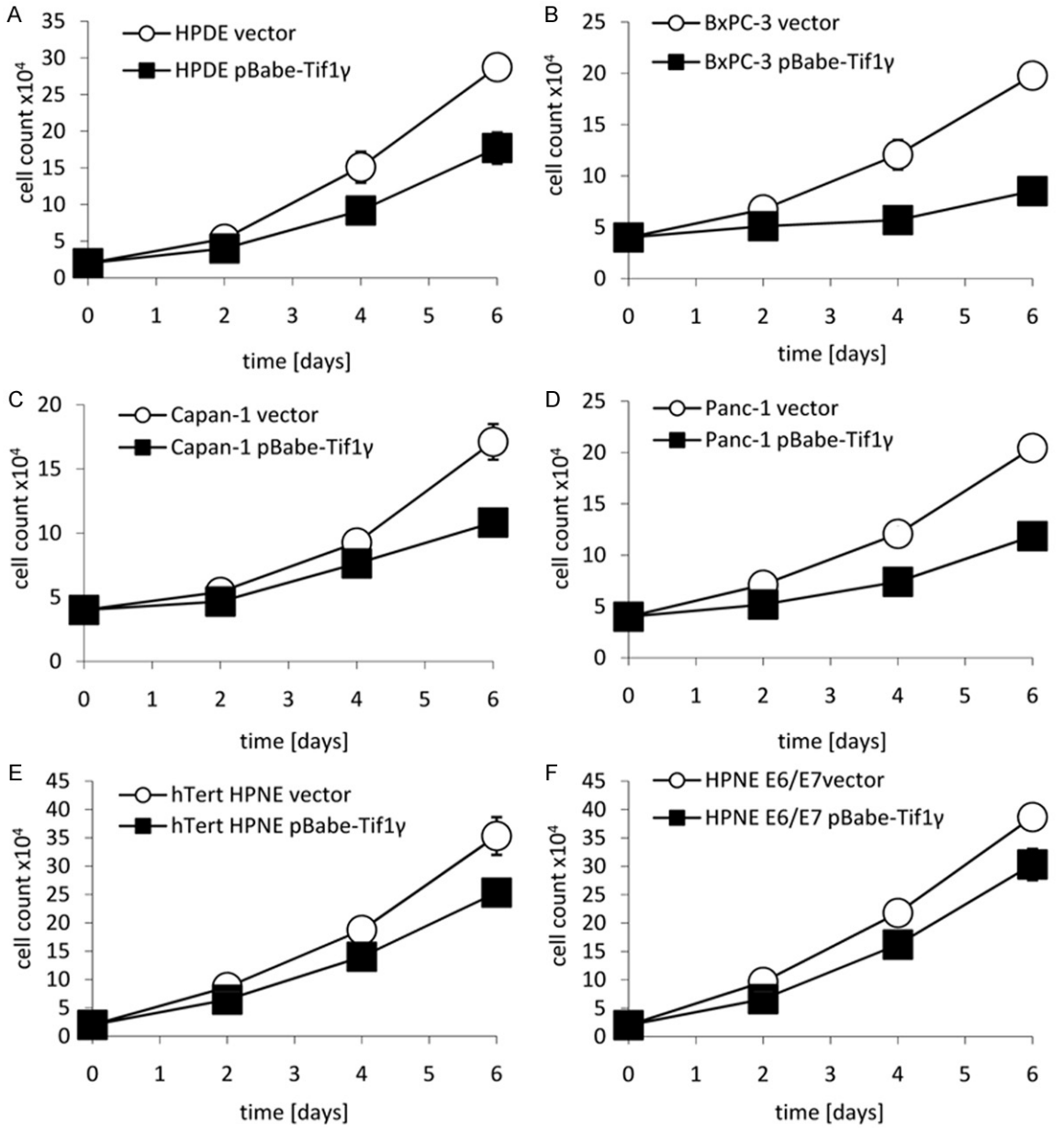

Figure 4. The effect of Tif1γ overexpression on pancreatic cell growth. HPDE (A), BxPC-3 (B), Capan-1 (C), Panc-1 (D), hTert HPNE (E), and HPNE E5/E6 (F) cells were transfected either with Tif1γ overexpression vector (■) or an empty vector (○). Cells were grown for six days and the number of the cells in wells was counted every other day.

The effect of Tif1γ overexpression and knockdown on invasion

Due to a significant impact of Tif1γ on cell proliferation, we next analyze if Tif1γ had an effect on invasive abilities in these cells. We used a Matrigel invasion assay to test the effect of Tif1γ knockdown and overexpression on in pancreatic cell lines. In all cell lines tested with Tif1γ knockdown, invasive ability was greatly reduced (**Figure 7**). HPDE cells have limited invasive ability (only 1×10^5 cells being able to cross the membrane, compared to 1.2×10^6 and 7×10^6 for BxPC3 and Panc-1 cells, respectively). Even this low level of invasiveness was reduced 4.5 fold compared to the control after Tif1γ knockdown. Control Panc-1 and BxPC-3 cells exhibited a strong ability to invade through the Matrigel membrane. Their invasive ability was markedly reduced by Tif1γ knockdown, resulting in 70-85% decrease in the number of cells crossing the membrane. In contrast, over-

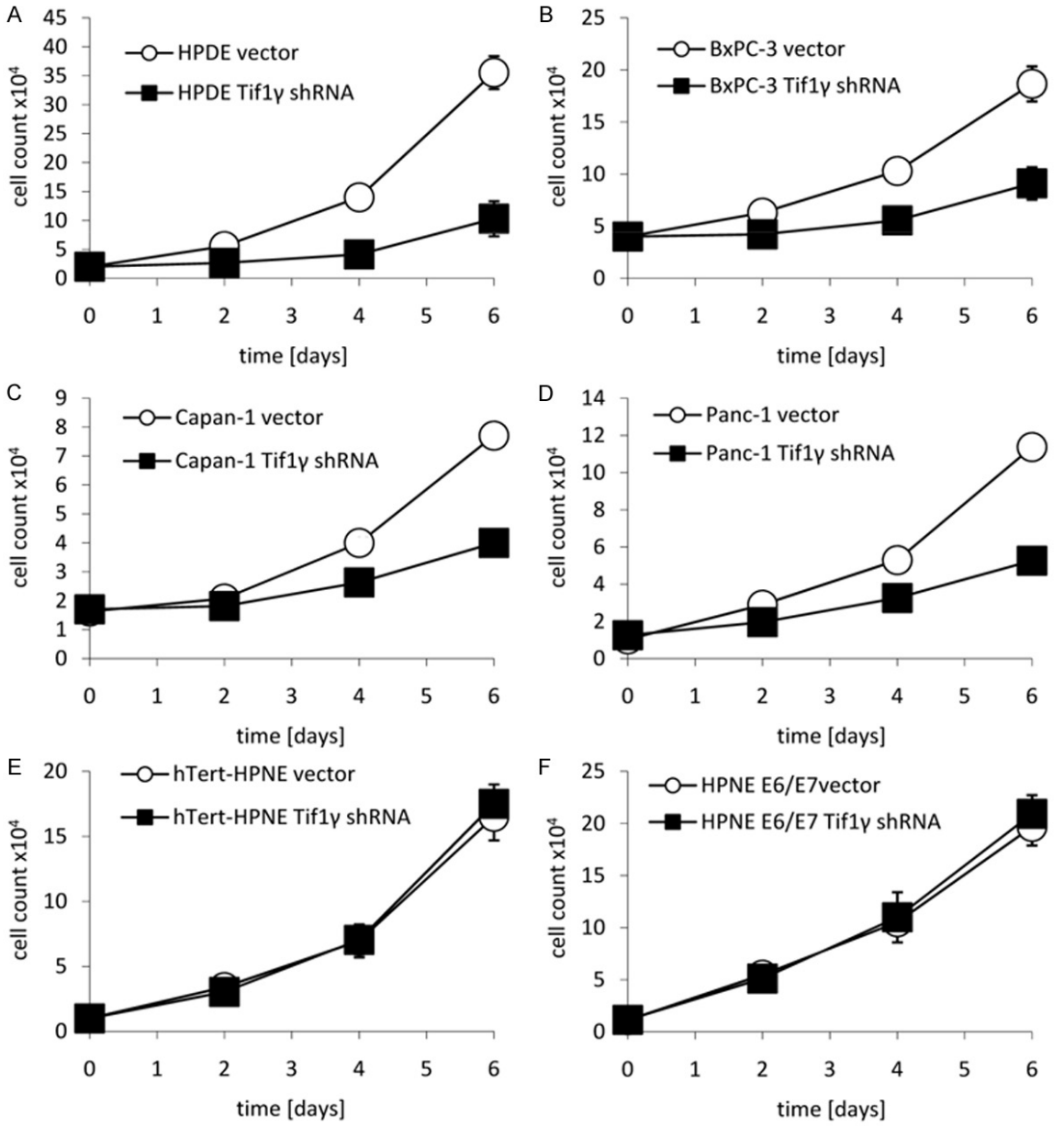

Figure 5. The effect of Tif1γ knockdown on pancreatic cell growth. HPDE (A), BxPC-3 (B), Capan-1 (C), Panc-1 (D), hTert HPNE (E), and HPNE E6/E7 (F) cells were transfected with vector expressing either Tif1γ-specific shRNA (■) or non-specific scrambled shRNA (○). Cells were grown for six days and the number of the cells in wells was counted every other day.

expression of Tif1γ had no effect on the invasive potential of any pancreatic cell lines, irrespective of benign or malignant (**Figure 7**).

Proteomic pathway array analysis of signaling networks affected by Tif1γ overexpression and knock-down

To explore the mechanism of the effect of Tif1γ on pancreatic cancer cells, we performed PPAA [33] on the cells with either overexpression or knock-down of Tif1γ (Table S1). Overexpression of Tif1γ led to significant increase in the levels of three classes of molecules involved in regulating cell proliferation, inflammation and immune response, and cell-to-cell signaling and cell adhesion. There was more than two-fold increase of the amount of cyclin B1 and p53 in the analyzed extracts, and more than

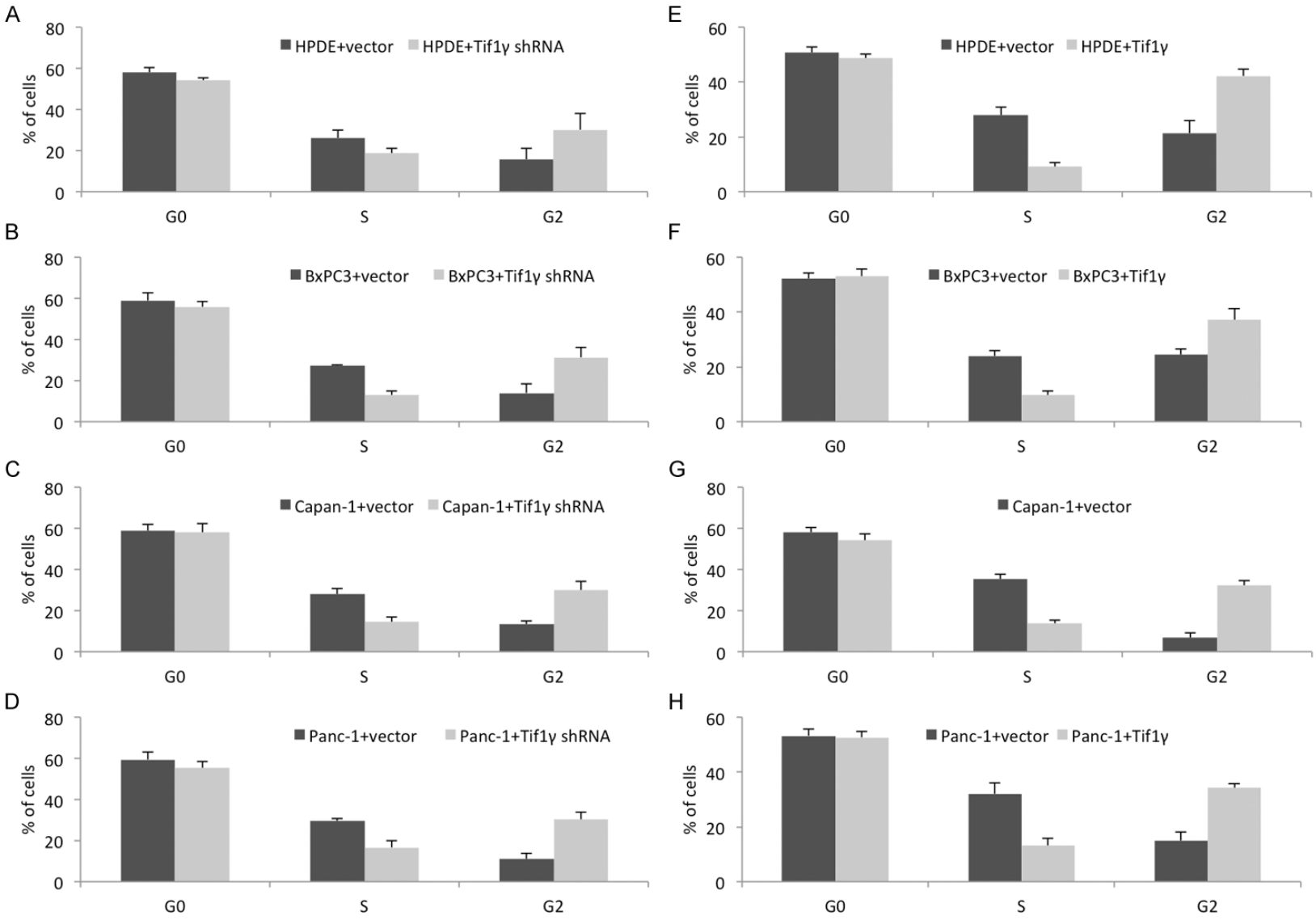

Figure 6. The effect of Tif1γ knockdown and overexpression on cell cycle distribution of pancreatic cells. HPDE (A, E), BxPC-3 (B, F), Capan-1 (C, G), Panc-1 (D, H) cells were transfected either with either vector expressing Tif1γ-specific shRNA (A-D) or Tif1γ overexpression vector (E-H, grey columns), as well as the corresponding control vectors (black columns). Exponentially growing cells were collected and analyzed by flow cytometry.

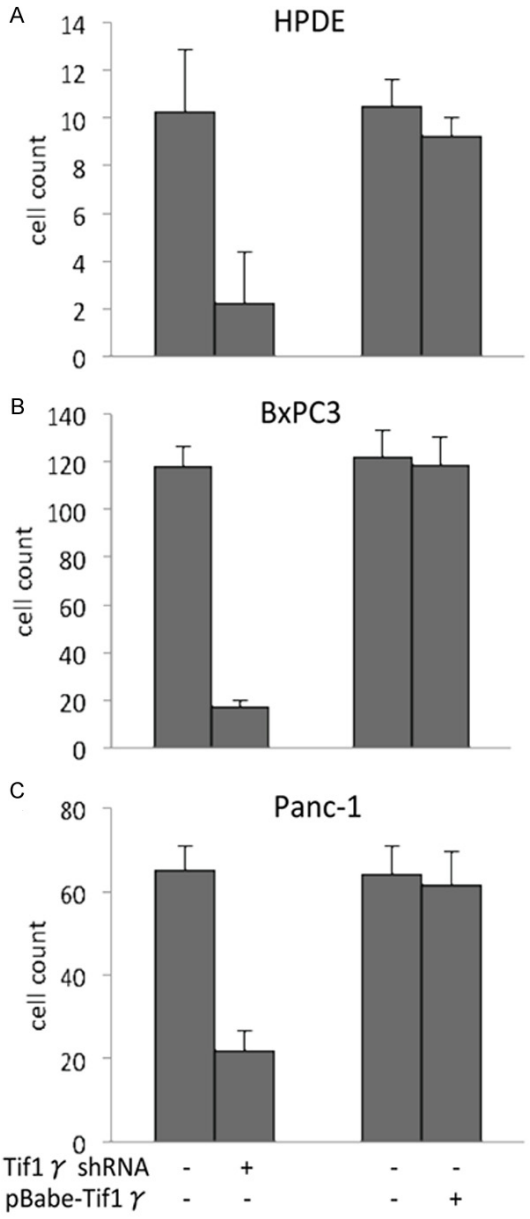

Figure 7. The effect of Tif1γ overexpression and knockdown on invasion. HPDE (A), BxPC-3 (B) and Panc-1 (C) cells were transfected with either Tif1γ overexpression vector or vector expressing Tif1γ-specific shRNA, as well as the appropriate control vectors. Cells were seeded into inserts with membrane covered in Matrigel, and allowed to migrate towards the chemoattractant in the bottom chamber. After 24 hours the number of cells that managed to cross the membrane were counted.

two-fold reduction in the level of Cdc25C, an inhibitor of p53 as well as retinoblastoma protein pRb phosphorylated on serine 780 and serine 807/811. The cell cycle protein expression observed is in agreement with flow-cytometric results which showed that overexpression of Tif1γ leads to inhibition of cell growth and accumulation of cells in the G2 phase.

We also performed PPAA using the cells transfected with a vector expressing shRNA targeted to knock-down Tif1γ levels. We found that shRNA knockdown of Tif1γ resulted in increase in levels of signaling molecules associated with cell cycle control, cancer, immunological and inflammatory disease, and cell development and morphology. As for the molecules whose level decreased as a result of Tif1γ knockdown, they belonged to the same categories as the molecules whose levels increased, and in addition to categories implicated in cell-to-cell signaling and hepatic system disease.

We detected a number of overlaps in the groups of proteins whose levels changed in response to either Tif1γ overexpression or knock-down. A number of proteins showed an inverse pattern, where a particular molecule would be overexpressed under one condition and underexpressed under another one (Table S1).

Discussion

In human hematopoietic stem/progenitor cells, He *et al.* identified that Tif1γ acts as an inhibitor of the TGFβ-Smad2/3 branch of the TGFβ signaling pathway [12]. We observed that Tif1γ performs an analogous function in pancreatic cells, regulating the transcriptional activity of the Smad1/4 complex in the BMP branch of the TGFβ signaling pathway. Our co-immunoprecipitation results suggest that Tif1γ physically interacts with the Smad1/4 complex, and Smad1 in much less extent, but not with Smad4 alone (**Figure 1**). Furthermore, our study using the yeast two-hybrid system found that Tif1γ directly binds to the Smad1/4 complex through its C-terminal functional domain, exerting transcriptional activity in pancreatic cells. In contrast to previous findings in hematopoietic cells, we did not observe the interaction of Tif1γ with Smad2/3 in the pancreatic cell lines we tested. Since Smad1 is a key factor in BMP signaling, these findings suggest that the interaction between Tif1γ and Smad proteins is cell- or tissue specific. Dysregulation of the BMP branch of the TGFβ signaling system has been shown to be involved in cancer development and progression in GI cancer [34-37]. Our findings provide additional evidence that the dysfunction of the BMP signaling by Tif1γ is an important factor in pancreatic carcinogenesis.

Early studies showed that Tif1γ could down-regulate the levels of Smad4 by its ubiquitinase function [14, 15]. Consistent with the monoubiqitination activity of Tif1γ, an inverse relationship between overexpression of Tif1γ and decrease of Smad4 was observed in colonic and pancreatic cell lines [12] and on colonic carcinoma by immunohistochemistry [17], but not in other cancers [12]. Tif1γ could also form a Tif1γ-Smad2/3 complex, competing with Smad4, resulting in upregulation of TGFβ signaling [10]. To reconcile this discrepancy, we compared the expression levels of Tif1γ and Smad4 in three pancreatic cancer cell lines and two immortalized pancreatic ductal or intermediate duct-acinic cell lines. Interestingly, all malignant cell lines that showed high levels of Tif1γ also showed low or intermediateSmad4 protein level, as compared to the immortalized benign pancreatic cell lines. One pancreatic cell line, Panc-1, expressing Smad4 showed no significant difference in TIF1γ expression compared to a normal pancreatic cell line (HPDE). When we overexpressed Tif1γ in these cell lines, both mRNA and protein levels of Smad4 were reduced in all cell lines we tested, except for the benign pancreatic cell line, HPDE, which showed to have a relatively high level of mRNA in contrast to the lower level of its protein. This finding suggests that overexpression of Tif1γ in HPDE cells may possibly lead to a post-translational modification or regulation on Smad4 protein. Overall results suggest that in addition to monoubiquitination of Smad4, Tif1γ also transcriptionally regulates gene expression of Smad4 through an unknown mechanism(s). However, we did not observe effects on levels of Tif1γ upon changes in Smad4 expression. Our observations support the inverse relationship between the overexpression of TIF1γ and decrease in Smad4 protein level, and the down regulation of Smad4 by Tif1γ is unidirectional in these pancreatic cell lines.

The regulative function of Tif1γ in cell growth and proliferation appears to be complex. To our surprise, both overexpression and knockdown of Tif1γ results in reduction in cell growth, and G2 phase arrest in the cell cycle. This is consistent with the notion that Tif1γ functions in the context of a protein complex with Smad proteins and a crucial balance of each protein level is required for its optimal function. Either increased or decreased expression of the Tif1γ component leads to a shift of the stoichiometric balance of Tif1γ in the complex and may derail the function of the complex. Knockdown of Tif1γ would elevate the level or enhance the activity of Smad4, which may explain this inhibitory activity on both tumor cell growth and invasiveness. In the future, it is of interest to determine the mechanism of which overexpression of Tif1γ inhibits cell growth. It is of great interest to determine the levels of expression for Tif1γ and Smad4 at tissue level in large cohorts of benign and malignant pancreatic cancer cases, although the results from our published data on colon cancer is consistent with our finding in pancreatic cells [38]. Our study demonstrated that imbalanced expression (either overexpression or knockdown) of Tif1γ results in abnormal pancreatic ductal epithelial cell growth through regulation of the BMP pathway, mainly by Tif1γ interaction with Smad1/Smad4 complex, resulting in G2 phase cell cycle arrest. Because either knockdown or overexpression of Tif1γ could prevent pancreatic cell growth, our results suggest that Tif1γ might potentially serve as a therapeutic target for pancreatic cancer therapy.

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Disclosure of conflict of interest

None.

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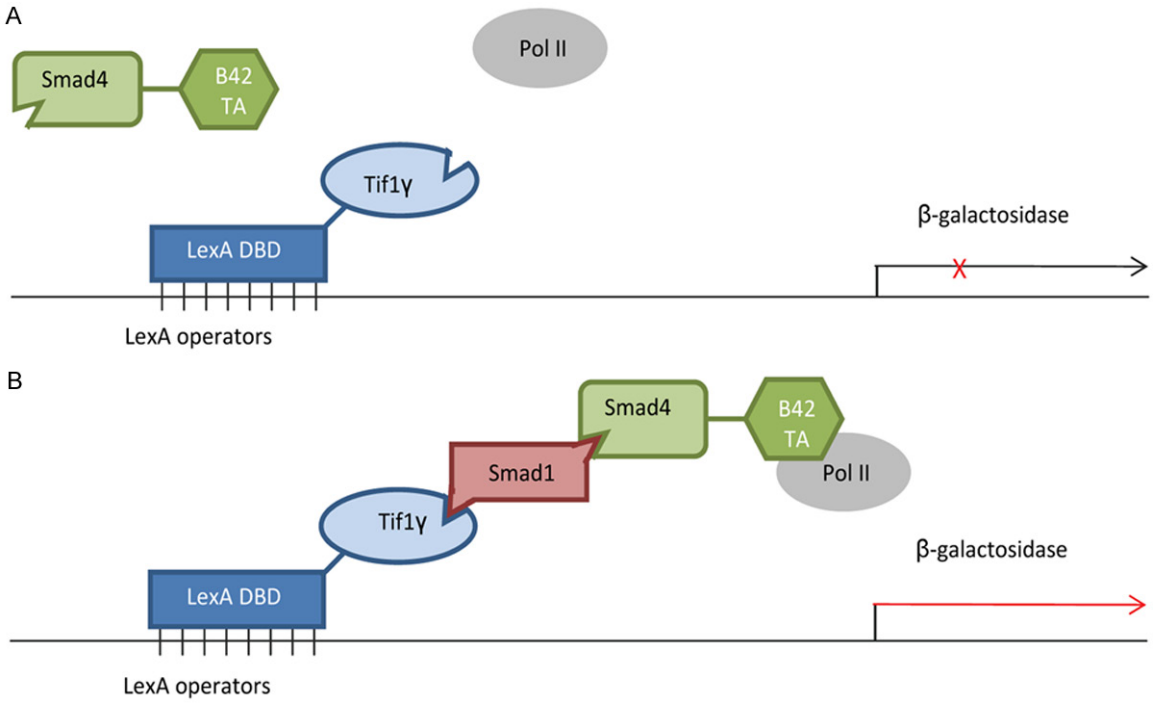
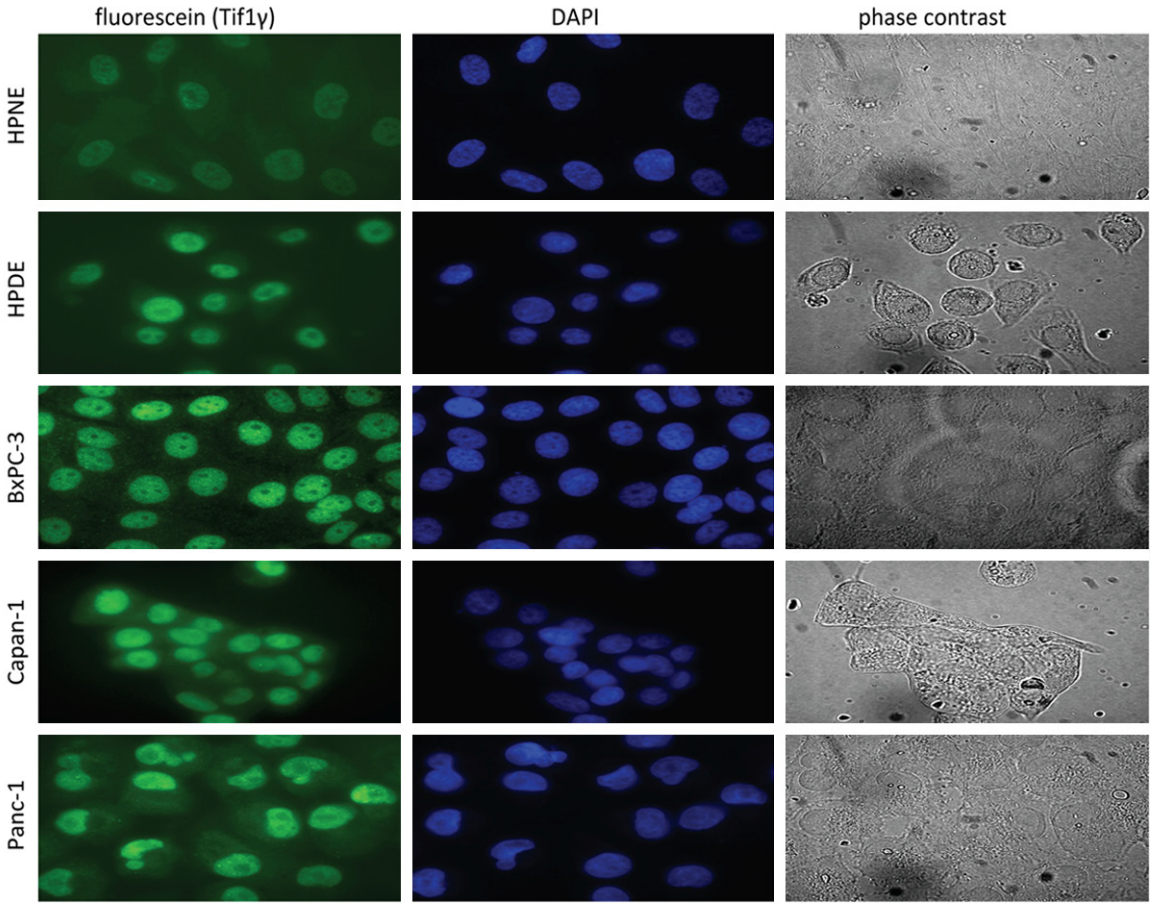
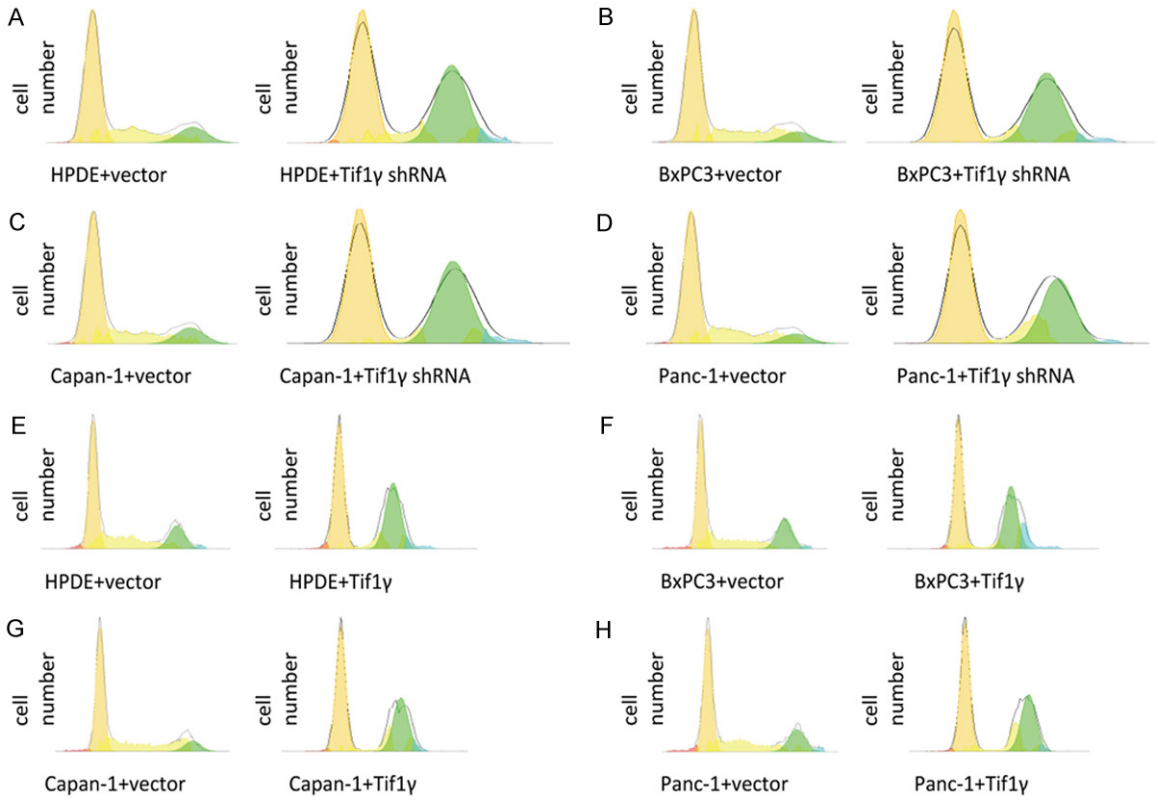


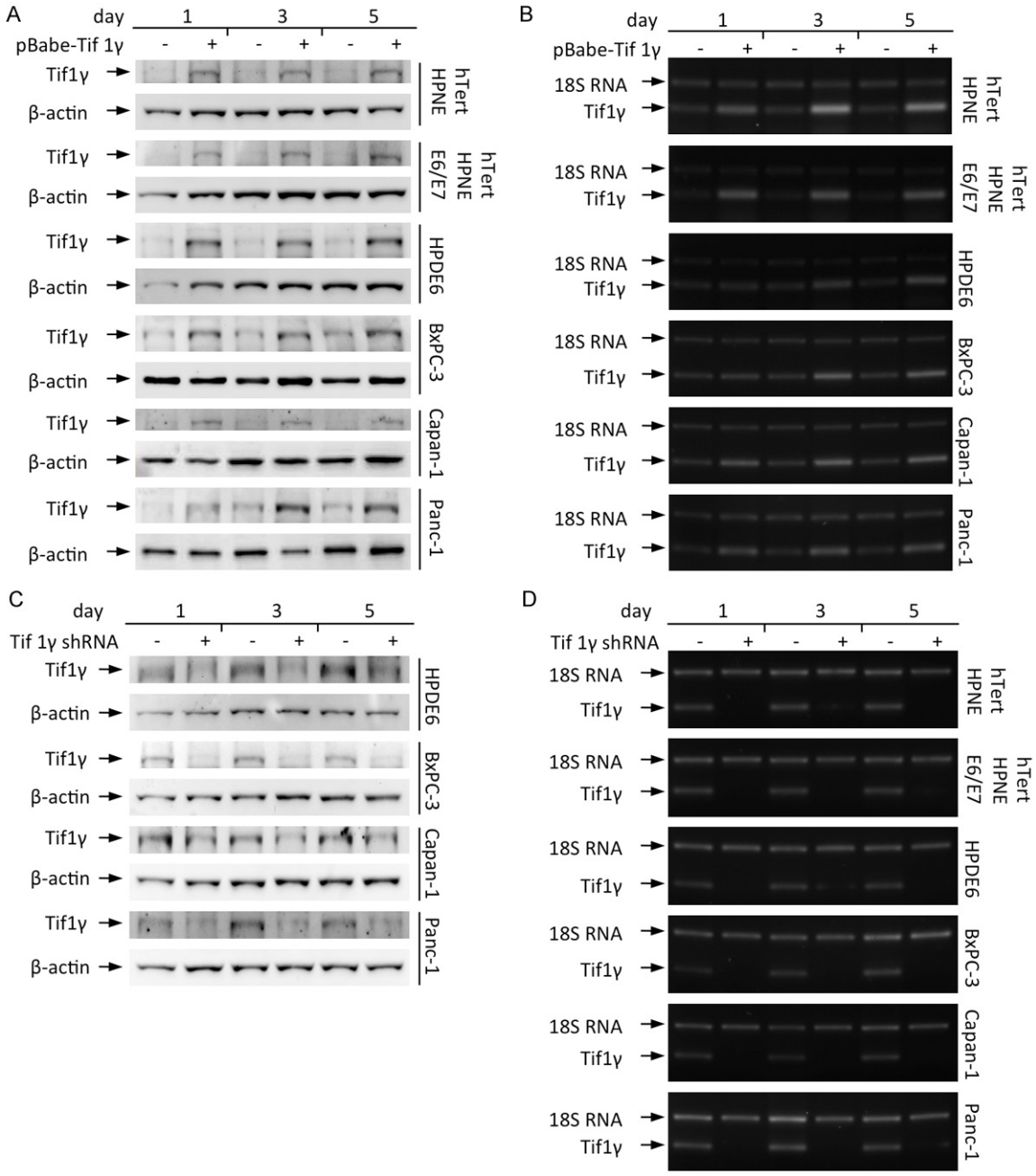
Supplementary Figure S1. Simplified illustration of the experimental design to detect interaction between Tif1γ and Smad1/4. A. Tif1γ fused to the LexA DNA binding domain binds to the promoter of the β-galactosidase reporter containing LexA operators. In the absence of Smad1, both RNA polymerase PolII and Smad4 (fused to B42 transactivation domain) are distant from the promoter and the reporter gene remains silent. B. When Smad1 is present, it brings together Smad4 and Tif1γ fusion proteins. RNA polymerase gets recruited by B42 domain fused to Smad4 and initiates expression from the β-galactosidase reporter. The protein product of the reporter can then convert a chromogenic substrate, thus visualizing the interaction of the proteins.



Supplementary Figure S2. Immunofluorescent localization of Tif1γ in pancreatic cell lines. Green signal in the first column of photographs corresponds to the localization of Tif1γ, detected by fluorescein-labeled immunocomplexes in the fixed cells. The blue signal in the second column represents the nuclei, visualized by DAPI stain. In the third column are the images of the cells in the Nomarski optic.



Supplementary Figure S3. The effect of Tif1γ knockdown and overexpression on cell cycle distribution of pancreatic cells. HPDE (A, E), BxPC-3 (B, F), Capan-1 (C, G), Panc-1 (D, H) cells were transfected with either vector expressing Tif1γ-specific shRNA (A-D) or Tif1γ overexpression vector (E-H), as well as the corresponding control vectors. Exponentially growing cells were collected and analyzed by flow cytometry.



Supplementary Figure S4. The efficiency of overexpression (A, B) and knock-down (C, D) of Tif1γ was followed using western blot (A, C) and RT-PCR (B, D). Western blot for HPNE in panel C is not shown since the Tif1γ was at undetectable levels.

Supplementary Table S1. Results of the proteomic pathway array analysis

Tif1γ overexpression

Network	>2x increase	>2x decrease
Cell Cycle, Cancer, Reproductive System Disease	**cyclin B1**, **p53**, **H-Ras**, Maspin, NKX-3.1	**p-AKT**, **p-RB (Ser780)**, p-Rb (Ser807/811), **Cdc25C**, Notch4, Eg5
Immunological Disease, Inflammatory Disease, Cancer	**EGFR**, α-tubulin	**p-PKC α**/βII, **p-ERK**, p-GSK-3α/β, NFkB50, **Bcl-6**, NFkB p65
Cardiovascular Disease, Cell-To-Cell Signaling and Interaction, Connective Tissue Development and Function	Rap1, Bad, **ICAM-1**, **VCAM-1**, Bak	NFkB50, NFkB p65
Cellular Development, Cellular Movement, Cell Morphology	Rap1, **E-cadherin**	**p-ERK**, p-GSK-3α/β, **Vimentin**
Cancer, Hepatic System Disease, Genetic Disorder	**NEP**	

Tif1γ knock-down

Network	>2x increase	>2x decrease
Cell Cycle, Cancer, Reproductive System Disease	**p-AKT** (Ser473), **cyclin B1**, Akt, PTEN, Hsp90, p38	**p-RB (Ser780)**, ERα, **Cdc25C**, **p53**, VEGF, **H-Ras**
Immunological Disease, Inflammatory Disease, Cancer	cPKCα, **ERK**, Akt	**EGFR**, **Bcl-6**, **p-PKCα**, **EGFR**
Cardiovascular Disease, Cell-To-Cell Signaling and Interaction, Connective Tissue Development and Function		Bax, **ICAM-1**, **VCAM-1**
Cellular Development, Cellular Movement, Cell Morphology	**ERK**, **Vimentin**	p-p70 S6 kinase, **E-cadherin**
Cancer, Hepatic System Disease, Genetic Disorder	FOLH1	**NEP**
Other	Mesothelin, Stat3, Bcl-xL	p-Stat3, β-catenin, HIF-3α, β-catenin

Legend:

bold: increase after overexpression and decrease after knock-down, or *vice versa*

green: increase after both overexpression and knock-down

red: decrease after both overexpression and knock-down

Supplementary Table S2. Antibodies used for proteomic pathway array analysis

Phosphorylation-specific antibodies (total 21)
p-AKT (Ser473), p-β-catenin (Ser33/37/Thr41), p-cdc2 (Tyr15), p-c-Jun (Ser73), p-CREB (Ser133), p-eIF4B (Ser422), p-ERK (Thr202/Tyr204), p-ERK5 (Thr218/Tyr220), p-FAK (Tyr397), p-GSK-3α/β (Ser21/9), p-p53 (Ser392), p-p70 S6 Kinase (Thr389), p-PDK1 (Ser241), p-PKC α/βII (Thr638/641), p-PKCδ (Thr505), p-PTEN (Ser380), p-Rb (Ser780), p-Rb (Ser807/811), p-Smad 1/5 (Ser463/465), p-p53
Antibodies for signal transduction proteins (total 110)
14-3-3β, Akt, α-tubulin, ATF-1, Bad, Bak, Bax, Bcl-2, Bcl-6, Bcl-xL,, BID, Calretinin, CaMKKa, cdc2/p34, Cdc25B, Cdc25C, cdc42, cdk2, Cdk4, Cdk6, c-Flip, CHK1, c-IAP2, cPKCα, CREB, cyclin B1, cyclin D1, cyclin E, DRG1, E2F-1, E-cadherin , Eg5, EGFR, eIF4B, Endoglin, Ep-CAM, Epo, ERa (HC-20), Erb, ERCC1, ERK, E-Selectin, Estrogen Receptor α (62A3), Factor XIII B, FAS, FGF-8, FOXM1, HCAM (DF1485), HCAM (H-300), HER2, HIF-1α, HIF-2α, HIF-3α, H-Ras, Hsp90, ICAM-1, IGFBP5, IL-1β, KAI1, KLF6, K-Ras, Lyn, Maspin, MDM2, Mesothelin, MetRS, N-cadherin, NEP, NFkB p65, NFkB50, NF-κB p52, Nkx-3.1, NM23, Notch4, Osteopontin, p27 (F-8), p27(C-19), p38, p53, p63, patched, PCNA, PDEF, PSCA, PSM, PTEN, Rab 7, Rap 1, RIP, SK3, SLUG, SRC-1, Stat-3, Survivin, Syk, TDP1, TFIIH p89, TGF-β, TNF-α, TTFv -1, Twist, uPA, uPAR, VCAM-1, VEGF, Vimentin, WT1, XIAP
Loading controls
β-actin, GAPDH