Overexpressed Integrin Alpha 2 Inhibits the Activation of the Transforming Growth Factor β Pathway in Pancreatic Cancer via the TFCP2-SMAD2 Axis

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Research Article

Keywords: Pancreatic cancer, Integrin α2, Transcription factor CP2, SMAD2, TGF-β

Posted Date: November 12th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1052794/v1

Version of Record: A version of this preprint was published at Journal of Experimental & Clinical Cancer Research on February 22nd, 2022. See the published version at https://doi.org/10.1186/s13046-022-02286-5.

Abstract

Background

Integrin alpha 2 (ITGA2) has been recently reported to be an oncogene and to play crucial roles in tumor cell proliferation, invasion, metastasis, and angiogenesis. Our previous study showed that ITGA2 was overexpressed in pancreatic cancer and promoted its progression. However, the mechanism of ITGA2 overexpression and other mechanisms for promoting the progression of pancreatic cancer are still unclear.

Methods

The GEPIA database was used to confirm the expression of ITGA2 in pancreatic cancer. To verify the influence of ITGA2 and TGF-β on the morphological changes of pancreatic cancer and tumor cell progression, we conduct CCK8 test, plate cloning, flow cytometry experiments and animal experiments. Then we conduct Western blot, RT-qPCR to explore the relationship between ITGA2 and TGF-β, and then find the key molecules which can regulate them by immunoprecipitation, Western blot, RT-qPCR, CHIP, nuclear and cytoplasmic separation test.

Results

The results of the present study show that the abnormal activation of KRAS induced the overexpression of ITGA2 in pancreatic cancer. Moreover, ITGA2 expression significantly suppressed the activation of the TGF-β pathway. ITGA2 silencing enhanced the anti-pancreatic cancer proliferation and tumor growth effects of TGF-β. Mechanistically, ITGA2 expression suppressed the activation of the TGF-β pathway by inhibiting the SMAD2 expression transcriptionally. In addition, it interacted with and inhibited the nuclear translocation of TFCP2, which induced the SMAD2 expression as a transcription factor. Furthermore, TFCP2 also induced ITGA2 expression as a transcription factor, and the TFCP2 feedback regulated the ITGA2-TFCP2-SMAD2 pathway.

Conclusion

Taken together, our results indicate that ITGA2 expression inhibited the activation of the TGF-β pathway in pancreatic cancer via the TFCP2-SMAD2 axis. Therefore, especially when combined with TGF-β treatment, ITGA2 might be a potential clinical therapeutic target for pancreatic cancer.

Background

Pancreatic ductal adenocarcinoma is the seventh leading cause of cancer death worldwide and one of the deadliest solid tumors, with a 5-year survival rate of <8%, which is further reduced by increased morbidity, late diagnosis, and poor treatment [1–3]. Although the efficacy and survival rates are better with combination therapy with cell inhibitors than with gemcitabine monotherapy, the therapeutic effect is still limited [2]. Elucidating the mechanisms underlying pancreatic cancer progression and identifying

potential therapeutic targets are critical to the improvement of the prognosis of patients with pancreatic cancer.

Integrin is a cell surface receptor that mediates cell-to-cell adhesion and the cell and extracellular matrix [4]. Integrin α2 and β1 subunits form heterodimers of the transmembrane receptors, which are important molecules involved in tumor cell proliferation, migration, survival, and angiogenesis [5, 6]. Integrin α2/β1 is overexpressed in many kinds of cancer cells, but its expression is low or even nonexistent in most normal organs and tissues [6]. Integrin alpha 2 (ITGA2) encodes a subunit of transmembrane receptors for collagen and related proteins [7]. The protein encoded by the gene forms a heterodimer with a β subunit, which mediates the adhesion of platelets and other cell types to the extracellular matrix [6]. ITGA2 is overexpressed in many cancers such as hepatocellular carcinoma [8], ovarian cancer [9], and pancreatic ductal adenocarcinoma [10], and is thought to be related to cell adhesion and cell surface-mediated signal transduction. Although our previous studies showed that overexpressed ITGA2 can upregulate PD-L1 to activate the STAT3 signaling pathway and thereby promote tumor cell progression [10], the carcinogenic mechanism of ITGA2 in pancreatic cancer still needs elucidation in further research.

Our present study shows that overexpressed ITGA2 can inhibit the SMAD2 expression by interacting with and inhibiting the nuclear translocation of TFCP2, the transcription factor of SMAD2. Thus, ITGA2 overexpression can further inhibit the TGF-β pathway to promote the proliferation of pancreatic cancer cells. Therefore, our findings indicate that ITGA2 might become a new therapeutic target for pancreatic cancer, especially when combined with TGF-β treatment.

Materials And Methods

Cell culture

The pancreatic cancer cell lines PANC-1 and AsPC-1 were purchased from ATCC (USA). PANC-1 and AsPC-1 cell lines were cultured in DMEM (Thermo Fisher Scientific, USA) supplemented with 10% FBS (HyClone, USA) at 37°C in a 5% CO incubator.

Antibodies, plasmids and chemicals

Human expression vectors for flag-tagged recombinant proteins were generated using the pcDNA3.1 backbone vector. The ITGA2 antibody (#ab133557, Abcam, USA); GAPDH (#10494–1-AP, Proteintech, USA). TFCP2(#15203-1-AP, Proteintech, USA); SMAD2 (#13684S, Cell Signaling Technology, USA). The TGF-β recombinant protein (#ab50036; Abcam, USA); KRAS(G12D) inhibitor (#S8499, SELLECK, USA),U0126 (#HY-12031A; MedChemExpress, USA).

RNA interference

Lipofectamine 3000 (Invitrogen, USA) and Opti-MEM media (Invitrogen, USA) were used for the transfection reactions; lipofectamine 3000 was used to transfect 293 T cells to shRNA plasmids and viral packaging plasmids (pVSV-G and pEXQV). 24h after transfection, the medium was replaced with fresh

DMEM containing 10% FBS and 1mM sodium pyruvate and 48h post-transfection, the virus culture medium was collected and added to the PANC-1 and AsPC-1 cells supplemented with 12μg/ml of polybrene. 24h after infection, the infected cells were selected with 10μg/ml of puromycin. The sh-RNAs sequence information is provided in the Supplementary table 1.

Immunoprecipitation and Western blot analysis

The radioimmunoprecipitation assay (RIPA) lysis containing 1% protease inhibitor and phosphatase inhibitor was used to cleave the cells on ice, and the cell lytic products were obtained. After centrifugation with 12000rpm at 4 ℃ for 15 minutes, the undissolved impurities were removed and the supernatant was collected. The protein concentration was measured by BCA's experimental method. The protein extract and agarose beads with antibodies were shaken slowly at 4 ℃ and incubated overnight for co-immunoprecipitation test or Western blotting analysis. The precipitated immune complex was electrophoretic by SDS-PAGE, then transferred to a 0.45-μm polyvinylidene fluoride (PVDF) membrane, then blocked with 0.5% bovine serum albumin (BSA) and incubated with a specific first antibody, and then the membrane was then visualized by the electrogenerated chemiluminescence (ECL) method. Bio-rad microscopic imaging system was employed to capture images that were analyzed using Image Lab.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

After total RNA was extracted from cells by Trizol reagent (Invitro-gen, 15,596,026, USA), the concentration of RNA was determined by spectrophotometer. RNA samples (1 μg) were reverse-transcribed using a PrimeScript™ RT reagent Kit (TAKARA, RR047A, JPN). Quantitative real-time PCR was performed using a TB Green™ Fast qPCR Mix kit (TAKARA, RR430A, JPN). Values represent the averages of three technical replicates from at least five independent experiments (biological replicates).

The primer sequences information for RT-qPCR is provided in the Supplementary table 2.

Liquid chromatography-tandem mass spectrometry/mass spectrometry (LC-MS/MS) analysis

293T cells transfected with a Flag-PTEN-expressing plasmid were used to identify novel PTEN-binding proteins. PTEN was immunoprecipitated using an anti-PTEN antibody and protein A+G agarose (#P2012, Beyotime, China) at 4°C. LC-MS/MS analysis was performed using a Thermo Ultimate 3000 liquid phase combined with Q Exactive Plus high-resolution mass spectrometry at Shanghai Applied Protein Technology. The data were retrieved using maxquant (v1.6.6) software and the algorithm Andromeda. The reference database comprised the UniProt human proteome reference database. Proteins and peptides with a false discovery rate (FDR) of 1% were selected.

RNA sequencing

In total, 1 μg of RNA per sample was used as the input material for RNA sequencing (RNA-seq). Sequencing libraries were generated using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA), in accordance with the manufacturer’s instructions. Clustering of the samples was performed on

the cBot Cluster Generation System using the TruSeq PE Cluster Kit v3-cBot-HS (Illumina), in accordance with the manufacturer's instructions. After cluster generation, libraries were sequenced on an Illumina Novaseq platform, and 150-bp paired-end reads were generated. FeatureCounts v1.5.0-p3 was used to count the read numbers mapped to each gene. Differential expression analysis (two biological replicates per condition) was performed using the DESeq2 R package (1.16.1). The clusterProfiler R package was used to test the statistical enrichment of differentially expressed genes (DEGs) in KEGG pathways.

Colony formation assay

Colony formation assay can detect the biological effects of ITGA2 and TGF- β on tumor cell survival. Four stable PANC-1 and AsPC-1 cell lines of sh-Control, sh-ITGA2 and TGF- β-treated sh-Control and TGF- β-treated sh-ITGA2 were inoculated into six-well plates with 500 cells in each well, and repetitive group was set up. After 10-14 days of culture, the colony formation assay was fixed, stained and photographed.

CCK8 cell proliferation assay

Four stable cell lines were used to establish four treatment groups including control groups and experimental groups, and five repetitive groups were set up in each group. 2000 cells were added in each well and 10 microliters of CCK8 solution were added in each well. The cells were incubated in the incubator for 3 hours, and the absorbance was measured by enzyme labeling instrument in 450nm. The data were monitored continuously for five days, and finally the data were processed with GraphPad Prism 7.

Chromatin Immunoprecipitation (ChIP) Assay

The binding sites of ITGA2 and SMAD2 with TFCP2 were verified by CHIP experiment. Formaldehyde was added to the cells to make the target protein cross-linked with the genomic DNA, and the lysate digested the cells. The above samples were treated with ultrasound to break the genomic DNA to 200-100bp. After treatment, the target protein and its bound DNA fragment were pulled down by co-immunoprecipitation, and then purified and amplified by PCR. The primer sequences information for ChIP-qPCR is provided in the Supplementary table 3.

Nuclear-cytoplasmic separation

Two stable transfer cell lines (sh-control and sh-ITGA2) were collected in the centrifuge tube, and the cytoplasmic protein extraction reagent A with pre-added PMSF was added to the centrifuge tube. After shaking and mixing, the above samples were added to the cytoplasmic protein extraction reagent B in the ice bath 10min. Shake and mix well and take an ice bath for 1 minute. Centrifuge 12000g at 4 ℃ for 5 minutes. The supernatant was obtained in a precooled centrifuge tube, and the cytoplasmic protein was obtained. Nuclear protein extraction reagent with PMSF was added to the precipitation of the supernatant, and then 2min was mixed in an ice bath, followed by shaking for 20 seconds every 2 minutes for a total of 30 minutes. The supernatant of the above samples was centrifuged at 4 ℃ for 10

minutes, and the supernatant was obtained as nuclear protein. The concentrations of plasma protein and nuclear protein were measured by BCA method. Finally, the Western blotting analysis was carried out.

Flow cytometry

Four stable pancreatic cancer cell lines in logarithmic growth phase were digested with trypsin and collected in a flow tube with pre-added culture medium. After centrifugation 300g/5min, the supernatant was discarded. After PBS washing, the supernatant was re-suspended with 300 μl of Binding Buffer, 5 μl of Annexin V-FITC was added and incubated in the dark for 10 minutes, then 5 μl of PI was added, mixed and incubated in the absence of light for 5 minutes, and the corresponding channels were detected and observed within 1 hour.

Bioinformatics mining

The correlation between TFCP2 and ITGA2 and SMAD2 was analyzed by GEPIA (http://gepia.cancer-pku.cn/) database, and the binding sites of transcription factors TFCP2 of ITGA2 and SMAD2 were predicted by EPD (https://epd.epfl.ch//index.php). Path enrichment analysis of sequencing results using R language and GSEA analysis. The expression of ITGA2 in KRAS wild type and mutant pancreatic cancer was obtained by cBioPortal database (https://www.cbioportal.org/) query.

Mouse tumor model.

Animal experimental procedures were in accordance with guidelines of the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology. 5×10^6 PANC-1 cells infected with sh-Control or sh-ITGA2 were injected subcutaneously into the left side of purchased BALB/c-nu mice (4-5 weeks old, male). The mice were intra-tumoral injected with TGF-β recombinant protein for 3 times on days 1, 4, and 7 at a dose of 10 mg/kg [11]. The tumor size was evaluated with digital Vernier caliper every two days, and then tumor volumes were calculated using the formula: $(L \times W^2) / 2$. Mice were sacrificed on day 21 or when tumor volume reached 1000 mm^3.

Statistical analysis

All experiments were performed at least three times. Parametric data are shown as means ± standard errors of the mean (SEMs) and nonparametric data as medians and ranges. Two-way ANOVA or one-way ANOVA with Tukey's multiple comparison test was used for multiple group analysis. Unpaired Student's *t*-tests were used to compare data between two groups. Two-tailed *P*-values < 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, Inc, USA).

Results

Abnormal KRAS activation induces ITGA2 overexpression in pancreatic cancer cells

The KRAS-mediated RAS signaling pathway plays a key role in the occurrence and development of diseases and chemotherapeutic drug resistance. KRAS is one of the main driving genes in pancreatic cancer. More than 90% of patients with pancreatic cancer have KRAS carcinogenic point mutations, which result in the constitutive activation of the RAS signaling pathway [12–14]. RAS signal transduction regulates multiple cellular biological processes in pancreatic ductal adenocarcinoma, including cell proliferation, migration, metabolism, and autophagy [15]. In their study, Yao et al. found that the ITGA2 protein was upregulated on the cell surface by oncogenic KRAS [16] (Fig. 1a). Consistent with this finding, by searching the cBioPortal database, we found that the ITGA2 expression level significantly increased in pancreatic cancer with KRAS mutation (Fig. 1b). To verify this finding, we treated pancreatic cancer cells with KRAS (G12D) or ERK1/ERK2 inhibitors. The results showed that ITGA2 expression was significantly inhibited in the pancreatic cancer cells treated with KRAS (G12D) or ERK1/ERK2 inhibitors (Fig. 1c–f). Therefore, our results indicate that abnormal KRAS activation induces ITGA2 overexpression in pancreatic cancer cells.

ITGA2 silencing activates the TGF-β signaling pathway in pancreatic cancer cells

On the basis of previous studies, we verified that ITGA2 overexpression could promote tumor cell proliferation and invasion by regulating the PD-L1 expression [10]. To examine the further mechanism of ITGA2 expression regulating the progression of pancreatic cancer cells, we reanalyzed our previous RNA-sequencing data [10] and identified 427 upregulated and 365 downregulated differentially expressed genes (Fig. 2a, b). In addition, KEGG and gene set enrichment analyses showed that TGF-β was significantly activated after ITGA2 silencing, which indicated that ITGA2 expression could inhibit the activation of the TGF-β signaling pathway (Fig. 2c, d). Moreover, reverse transcriptase polymerase chain reaction (RT-PCR) assay results showed that ITGA2 silencing in pancreatic cancer cells significantly increased the expression levels of CDKN2B, CDKN1A, and SERPINE3, the downstream molecules of the TGF-β signaling pathway (Fig. 2e–g). Furthermore, ITGA2 silencing further increased the downstream molecules of the TGF-β signaling pathway induced by TGF-β recombinant protein (Fig. 2e–g). Thus, the results showed that ITGA2 silencing activated the TGF-β signaling pathway in pancreatic cancer cells.

ITGA2 silencing enhances the anti-pancreatic cancer cell proliferation effect of TGF-β

Considering that ITGA2 silencing activated the TGF-β signaling pathway in pancreatic cancer cells, we hypothesized that ITGA2 silencing could enhance the anti-pancreatic cancer cell proliferation effect of TGF-β. Consistent with our hypothesis, the CCK-8 cell proliferation and colony formation assay results showed that ITGA2 silencing significantly inhibited the proliferation ability of pancreatic cancer cells (Fig. 3a, b). Moreover, ITGA2 silencing further inhibited the proliferation ability of pancreatic cancer cells, which could be inhibited by TGF-β expression (Fig. 3a, b). In addition, we also found that ITGA2 silencing and TGF-β expression could promote the apoptosis of pancreatic cancer cells and that their combination increased the apoptosis rate of tumor cells (Fig. 3f, g). To investigate the biological effects of ITGA2 silencing and TGF-β expression on pancreatic cancer in vivo, PANC-1 cells with or without ITGA2 silencing were subcutaneously injected in nude mice to induce tumorigenesis. As shown in Figure 3c–e, tumors

formed in the ITGA2-silenced PANC-1 cells and the TGF-β-treated PANC-1 cells were smaller and lighter than the controls, whereas the tumors formed in the group with the combined ITGA2 silencing and TGF-β treatment were even smaller and lighter (Fig. 3c, e). Therefore, we can conclude that ITGA2 silencing enhances the anti-pancreatic cancer cell proliferation effect of TGF-β.

ITGA2 silencing induces the SMAD2 expression in pancreatic cancer cells

Activation of the TGF-β signaling pathway requires the entry of the complex formed by SMAD2 and SMAD3 combined with SMAD4 into the nucleus [17]. Therefore, we speculate that ITGA2 silencing may promote the activation of the TGF-β pathway by regulating the SMAD2/SMAD3/SMAD4 expression. The RT-PCR assay revealed that ITGA2 silencing significantly induced the expression of SMAD2 (Fig. 4a), but not those of SMAD3 and SMAD4 (Fig. 4b, c). After that, the relationship between ITGA2 and SMAD2 was verified by western blotting, and the results were consistent with the PCR results (Fig. 4d). Furthermore, ITGA2 overexpression could significantly inhibit the mRNA and protein expressions of SMAD2 in pancreatic cancer cells (Fig. 4e, f). Taken together, these findings indicate that ITGA2 silencing induced the SMAD2 expression in pancreatic cancer cells.

ITGA2 inhibits the activation of the TGF-β pathway via the SMAD2 expression in pancreatic cancer cells

To verify whether SMAD2 is the key molecule of the TGF-β signaling pathway regulated by ITGA2, we detected the downstream genes of the TGF-β signaling pathway, including CDKN2B, CDKN1A, and SERPINE3. The results showed that SMAD2 silencing reversed the upregulation of the CDKN2B, CDKN1A, and SERPINE3 expressions induced by ITGA2 silencing (Fig. 5a, c). By contrast, SMAD2 overexpression reversed the downregulation of the CDKN2B, CDKN1A, and SERPINE3 expressions induced by the ITGA2 overexpression (Fig. 5d, f). Thus, the results showed that ITGA2 inhibited the activation of the TGF-β pathway via the SMAD2 expression in pancreatic cancer cells.

ITGA2 inhibits the SMAD2 expression by interacting with TFCP2 in pancreatic cancer cells

As previously established, ITGA2 can inhibit the TGF-β signaling pathway by regulating the SMAD2 expression, but the specific mechanism remains unclear. By performing a liquid chromatography tandem mass spectrometry assay, we found that TFCP2 is as an interacted protein of ITGA2 by identifying the peptides of TFCP2 (Fig. 6a, b), which had been verified with immunoprecipitation assays in pancreatic cancer cells (Fig. 6c). Thus, we supposed that ITGA2 inhibited the SMAD2 expression via TFCP2 in pancreatic cancer cells. Our results showed that TFCP2 silencing reversed the upregulation of the SMAD2 expression induced by ITGA2 silencing (Fig. 6d, e), whereas TFCP2 overexpression reversed the downregulation of the SMAD2 expression induced by the ITGA2 overexpression (Fig. 6f, g). Taken together, our results indicate that ITGA2 inhibits the SMAD2 expression by interacting with TFCP2 in pancreatic cancer cells.

TFCP2 induces the SMAD2 expression as a transcription factor in pancreatic cancer cells

In our previous study, we found that ITGA2 inhibits the SMAD2 expression by interacting with TFCP2 in pancreatic cancer cells (Fig. 6). However, the specific mechanism is still confusing. By searching the GEPIA database, we found that the mRNA expression of TFCP2 positively correlated with the mRNA expression of SMAD2 in pancreatic cancer (Fig. 7a). The RT-PCR and western blot analyses revealed that TFCP2 silencing significantly inhibited the SMAD2 expression transcriptionally in pancreatic cancer (Fig. 7b, c). In addition, the RT-PCR and western blot analyses also revealed that the TFCP2 overexpression significantly promoted the SMAD2 expression transcriptionally in pancreatic cancer (Fig. 7d, e). Studies have reported that TFCP2 could regulate the expressions of several targeted genes as transcription factors [18]. By searching the Eukaryotic Promoter Database (EPD), we found two potential binding sites of TFCP2 at the promoter of the *SMAD2* gene (Fig. 7f), which had been verified with a chromatin immunoprecipitation (ChIP)-PCR assay (Fig. 7g). ITGA2 inhibits the SMAD2 expression by interacting with TFCP2 but does not change the TFCP2 expression in pancreatic cancer cells (Fig. 6). Thus, we speculated that ITGA2 affected the entry of the transcription factors into the nucleus by interacting with TFCP2, thereby inhibiting the nuclear expression of SMAD2. To verify this conjecture, nuclear and cytoplasmic separation experiments were conducted. As expected, the results showed that ITGA2 silencing significantly increased the nuclear expression and decreased the cytoplasm expression of TFCP2 (Fig. 7h), whereas ITGA2 overexpression significantly decreased the nuclear expression and increased the cytoplasm expression of TFCP2 (Fig. 7i). Taken together, these findings indicate that TFCP2 induces the SMAD2 expression as a transcription factor in pancreatic cancer cells. ITGA2 inhibits the SMAD2 expression by interacting with and inhibiting the nuclear translocation of TFCP2 in pancreatic cancer cells.

TFCP2 as a transcription factor feedback induces the ITGA2 expression in pancreatic cancer cells

With the aforementioned findings, we proved that ITGA2 could interact with TFCP2 at the protein level. However, by searching the GEPIA database, we found that the mRNA expression of TFCP2 positively correlated with the mRNA expression in pancreatic cancer (Fig. 8a). Thus, TFCP2 might transcriptionally induce the ITGA2 expression as a transcription factor in pancreatic cancer cells. TFCP2 silencing significantly inhibited (Fig. 8b, c) but TFCP2 overexpression significantly induced the ITGA2 expression transcriptionally (Fig. 8d, e). In addition, by searching the EPD database, we also identified one potential binding site of TFCP2 at the promoter of the *ITGA2* gene (Fig. 8f) and verified the binding of TFCP2 with a ChIP-PCR assay (Fig. 8g). Therefore, our results indicate that TFCP2 as a transcription factor feedback induced the ITGA2 expression in pancreatic cancer cells.

Discussion

Pancreatic cancer is one of the most invasive malignant tumors, with a 5-year survival rate of <8% [16]. Radiotherapy, chemotherapy, and surgery are the main treatment methods, but the treatment outcome is poor. Therefore, targeted therapy for pancreatic cancer is the current mainstream trend to alleviate the disease and improve the living standards of patients.

Previous studies showed that ITGA2 is overexpressed in tumor cells and related to the poor prognosis of patients with cancer [6, 8–10, 19], especially pancreatic cancer [10]. However, the reason for the high expression level of ITGA2 in pancreatic cancer has not been clarified. Thus, we reviewed previous studies and unexpectedly found that ITGA2 overexpression was associated with the most common KRAS mutation in pancreatic cancer (Fig. 1). The carcinogenic mechanism of pancreatic cancer includes the mutation accumulation of KRAS [20], TP53, CDKN2A, and SMAD4 [21]. The abnormal movement of these molecules is the basis of the histological changes of pancreatic cancer at different stages of its development [21, 22]. Activated KRAS mutations were reported in >90% of all pancreatic cancers [22].

Our previous research and recent local and foreign reports confirmed that the ITGA2 overexpression in pancreatic cancer is caused by the mutation and abnormal activation of KRAS. Previous studies reported that the carcinogenic mechanism of ITGA2 is rarely involved in pancreatic cancer, except that blocking ITGA2 can improve tumor immune response by reducing the phosphorylation level of STAT3 and inhibiting the PD-L1 expression [10]. Therefore, the purpose of this study was to examine other ways thereby ITGA2 regulates the progression of pancreatic cancer. For silenced ITGA2, RNA-seq was used to analyze the pathway enrichment of all genes. We found that the TGF-β signaling pathway was significantly activated when ITGA2 was silenced (Fig. 2). Furthermore, our results also indicated that ITGA2 silencing enhanced the anti-pancreatic cancer cell proliferation effect of TGF-β treatment, and the combined treatment might represent a novel therapeutic strategy for pancreatic cancer.

In the early stage of tumorigenesis, TGF-β expression can inhibit cell proliferation and participate in apoptosis. In the late stage of tumorigenesis, the TGF-β signaling pathway induces tumor invasion and metastasis by promoting angiogenesis, epithelial-mesenchymal transformation, and immune escape [23–25]. The transforming growth factor-β protein can regulate cellular function [26], which is essential for the homeostasis of epithelial cells, matrix compartments, and immune cells in the hepatopancreas and gastrointestinal system [27]. The transforming growth factor-β signaling pathway plays an important role in tumorigenesis by regulating cell proliferation, apoptosis, angiogenesis, immune surveillance, and metastasis [28–30]. The phosphorylation of the receptor-activated Smads (R-Smads) results in the formation of complexes with the common medium Smad (Co-Smad), which are introduced into the nucleus [31]. The TGF-β signaling pathway binds to SMAD4 through a complex composed of SMAD2 and SMAD3 [28]. The nuclear Smad oligomer binds to DNA and transcription factors to regulate the expression of the target gene [32–34]. In our present study, we conclude that ITGA2 affects the activation of the TGF-β pathway by regulating SMAD2, which is the key factor in the activation of the TGF-β pathway. Therefore, SMAD2 upregulation mediated the anti-tumor effect of ITGA2 silencing in pancreatic cancer, which represents treatment by inducing the SMAD2 expression as a novel therapeutic strategy for pancreatic cancer.

For tumor cells, transcription factor CP2 (TFCP2) expression can control the occurrence and regulate the development of tumor cells such as liver cancer [35]. In previous studies, TFCP2 was mostly considered a transcription factor that promotes the development and metastasis of cancer [35–37]. A few studies have reported that TFCP2 not only promotes but also inhibits cancers such as melanoma [18] and has some

important physiological functions that have not yet been discovered [38]. TFCP2 expression participates in epithelial-mesenchymal transformation and enhances angiogenesis [18]. In our study, TFCP2, as a transcription factor of SMAD2, regulated the SMAD2 expression (Fig. 7), and ITGA2 expression influenced the nuclear translocation of TFCP2 by binding to TFCP2. Overexpression of ITGA2 hinders the entry of TFCP2 into the nucleus, resulting in increased and decreased TFCP2 expression levels outside and inside the nucleus, respectively (Fig. 7h, i). An interesting conclusion from this study is that TFCP2 may also be a transcription factor of ITGA2 (Fig. 8a–g), which forms a loop. When the TFCP2 expression level increases, ITGA2 binds to TFCP2 and inhibits its entry into the nucleus (Fig. 7h), resulting in decreases in SMAD2 and ITGA2 expression levels. The entry of TFCP2 into the nucleus increased, and the ITGA2 and SMAD2 expression levels recovered repeatedly, forming a dynamic balance.

Conclusion

In conclusion, our findings show that overexpressed ITGA2 inhibits the SMAD2 expression by competitively binding to the transcription factor TFCP2 to block its entry into the nucleus, thus influencing the activation of the TGF-β signaling pathway, promoting tumor cell proliferation, and inhibiting tumor cell apoptosis in pancreatic cancer (Fig. 8h).

Abbreviations

CCK-8: Cell Counting Kit-8

ChIP: Chromatin immunoprecipitation

qRT-PCR: Quantitative reverse; transcriptase polymerase chain reaction

GEO: Gene Expression Omnibus

TCGA: The Cancer Genome Atlas

GEPIA: Gene Expression Profiling Interactive Analysis

RNA-seq: RNA sequencing

siRNAs: Small interfering RNAs

GSEA: Gene Set Enrichment Analysis

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

BSA: Bovine serum albumin

PBS: Phosphate-buffered saline

DMEM: Dulbecco's Modified Eagle's Medium

ITGA2: Integrin alpha 2

TFCP2: transcription factor CP2

Declarations

Availability of data and materials

Please contact the corresponding author (rendianyun@hust.edu.cn) for data requests.

Acknowledgment

Not applicable.

Funding

This work was supported by grants from National Natural Science Foundation of China (Grant No. 82102789 (D.R.)).

Contributions

HC, FG and SW performed the experiments; XJ collected the data; HW and DR wrote the paper and analyzed the data. All authors read and approved the final manuscript.

Ethics declarations

Ethics approval and consent to participate

All animal studies were carried out under the guidelines of Tongji Medical College of Huazhong University of Science and Technology and approved by the Animal Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology.

Consent for publication

Not applicable.

Competing interests

All authors declare no conflict of interests.

Additional information

Publisher's Note

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Figures

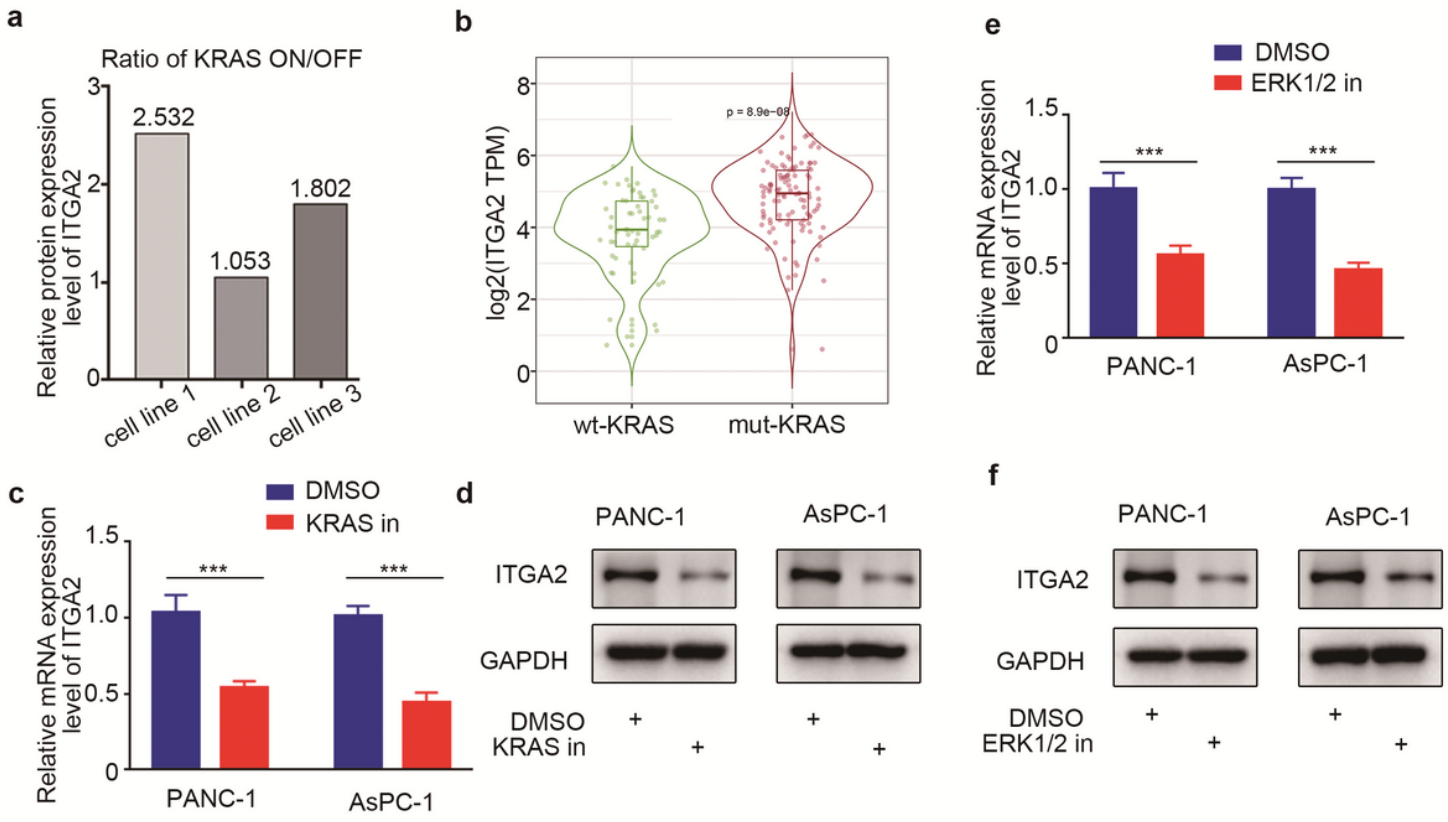

Figure 1

The abnormal KRAS activation induces the overexpression of ITGA2 in pancreatic cancer cells. a. ITGA2 is one of the Kras up-regulated surfaceome genes in different PDAC cell lines (AK10965, AK192, AK196) [16]. b. The cancer molecular expression profile data of cbioportal shows that the expression of ITGA2 in wt-KRAS and mut-KRAS pancreatic cancer. c. RT-PCR analysis to determine the mRNA expression level of ITGA2 in PANC-1 and AsPC-1 cells treated with treated with KRAS inhibitor (KRpep-2d, 10uM). GAPDH served as an internal reference and repeated for three replicates. ***, P < 0.001. d. Western blot analysis to determine the protein expression level of ITGA2 in The PANC-1 or AsPC-1 cells were treated with KRAS inhibitor (KRpep-2d, 10uM). GAPDH served as an internal reference. e. RT-PCR analysis to determine the expression level of ITGA2 in PANC-1 and AsPC-1 cells treated with treated with ERK 1/2 inhibitors (U0126, 10uM). GAPDH served as an internal reference and repeated for three replicates. ***, P < 0.001. f. Western blot analysis to determine the protein expression level of ITGA2 in The PANC-1 or AsPC-1 cells were treated with ERK1/2 inhibitor (U0126, 10uM). GAPDH served as an internal reference.

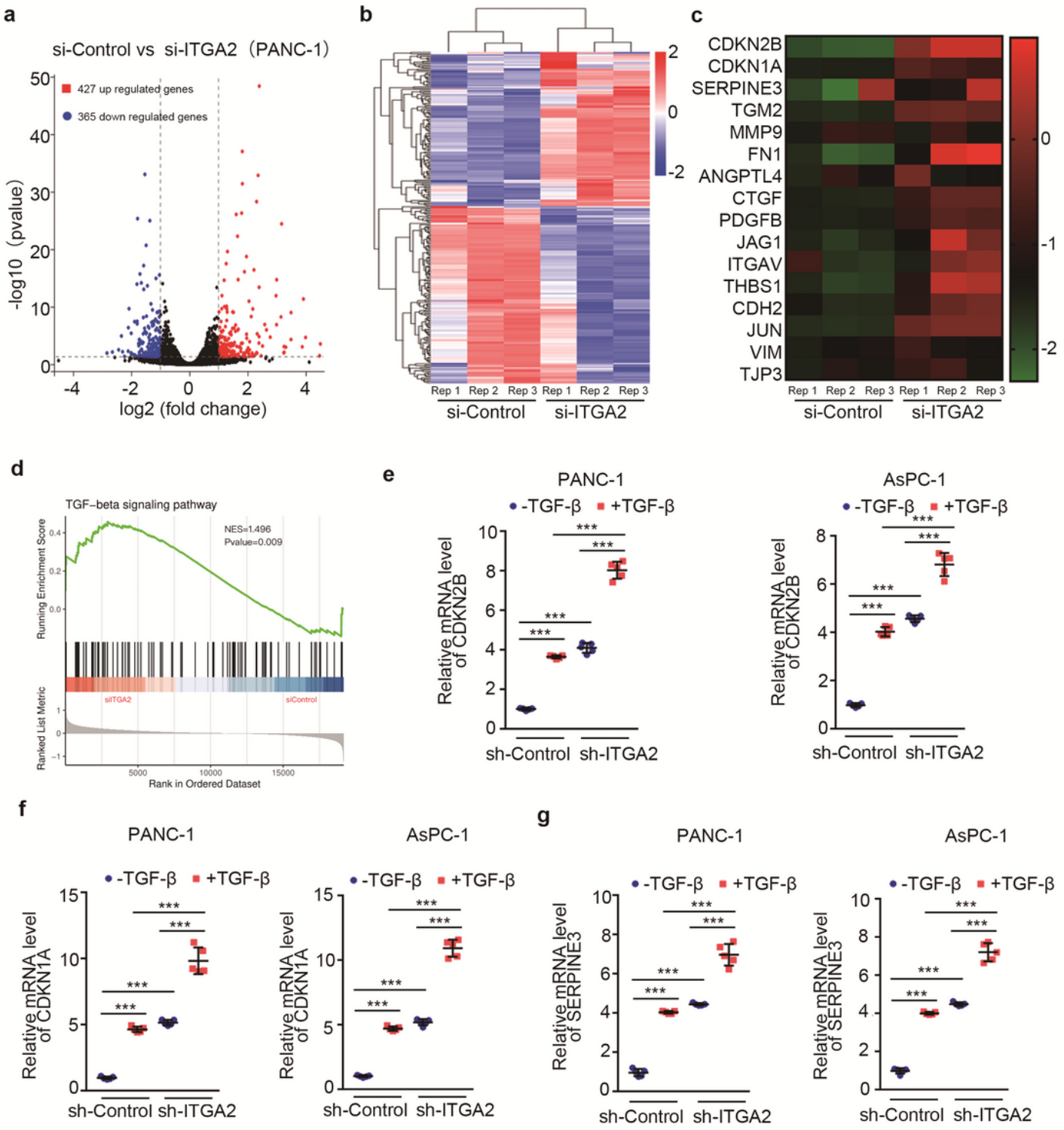

Figure 2

ITGA2 silencing activates TGF-β signaling pathway in pancreatic cancer cells. a and b. Volcano plot (a) and heatmap (b) showing the differentially expressed genes in PANC-1 cells infected with si-Control or si-ITGA2. The blue points represent the downregulated genes (n = 365), while the red points represent the upregulated genes (n = 427). c. Heatmap showing a group of ITGA2 knockdown-regulated genes in TGF-β signaling pathway. d. Gene set enrichment analysis was assessed using the GSEA (R implementation).

Genes regarding TGF-beta signaling pathway in si-ITGA2 group were significantly enriched, compared si-control group. P=0.009. e-g. PANC-1 and AsPC-1 cells infected with sh-ITGA2 or sh-Control were treated with or without TGF-β recombinant protein for 24h (5 ng/ml), and RT-PCR assay was used to detect the mRNA expression level of TGF-β signaling pathway downstream genes, including CDKN2B (e), CDKN1A (f), SERPINE3 (g). GAPDH served as an internal reference and repeated for three replicates. ***, P < 0.001.

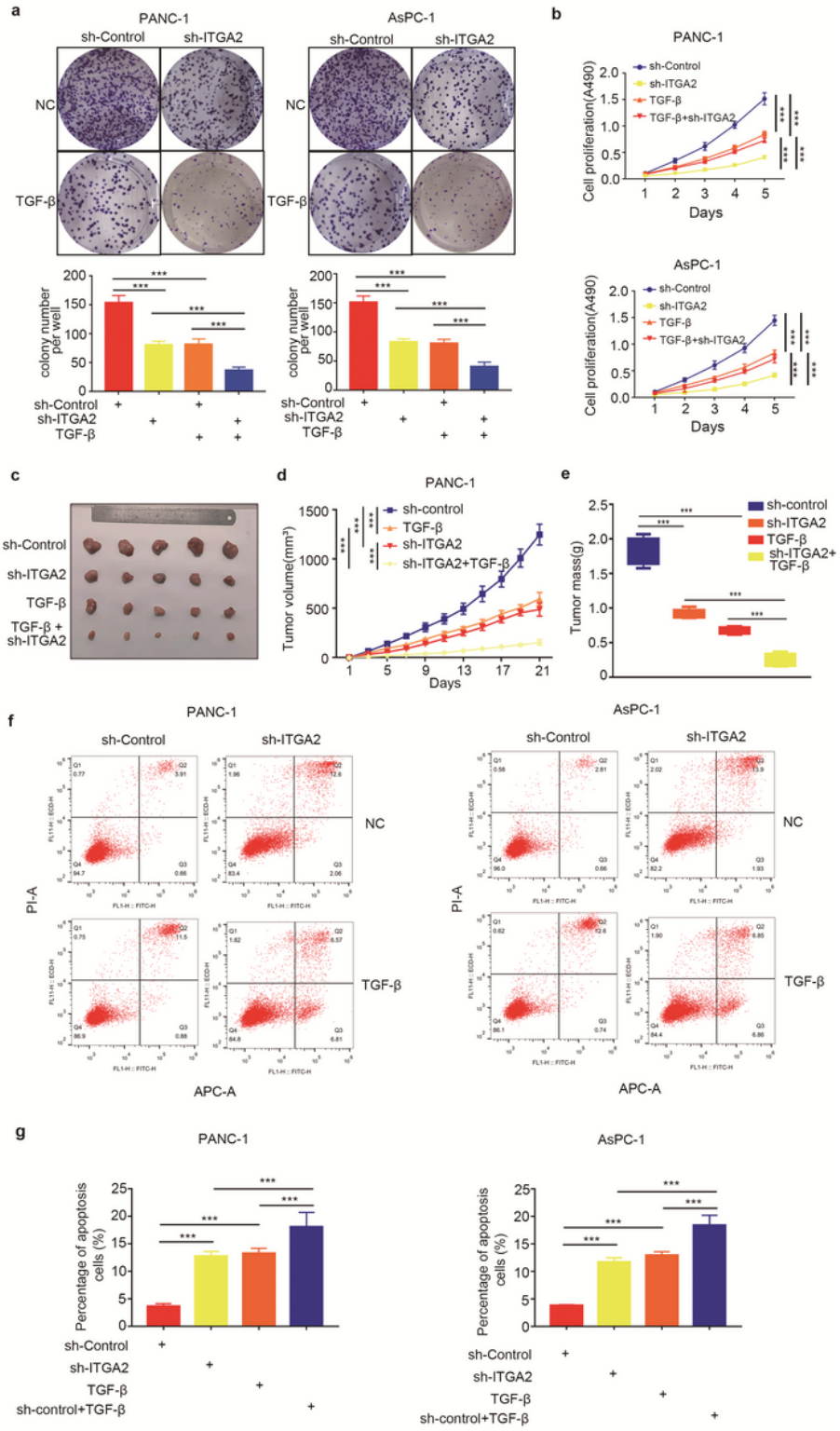

Figure 3

ITGA2 silencing enhances the anti-pancreatic cancer cells proliferation effect of TGF-β. a and b. PANC-1 and AsPC-1 cells infected with sh-ITGA2 or sh-Control were harvested for colony formation assay (c) and CCK-8 cell proliferation assay (d). Each bar represents the mean ± SD of three independent experiments. ***, P < 0.001. c-e. PANC-1 cells infected with infected with sh-ITGA2 or sh-Control were subcutaneously injected into nude mice and treated with or without TGF-β recombinant protein (10 mg/kg). The tumors were harvested and photographed (c) on day 21. Data for tumor volume (e) and tumor mass (f) are shown as the mean ± SD (n = 5). ***, P < 0.001. f and g. PANC-1 and AsPC-1 cells infected with sh-Control or sh-ITGA2 were harvested for Annexin V-FITC/PI dual staining assay using flow cytometry (f) and the percentage of apoptotic cells was qualified (g). Data were shown as means with error bars representing SD (n=3). ***p < 0.001.

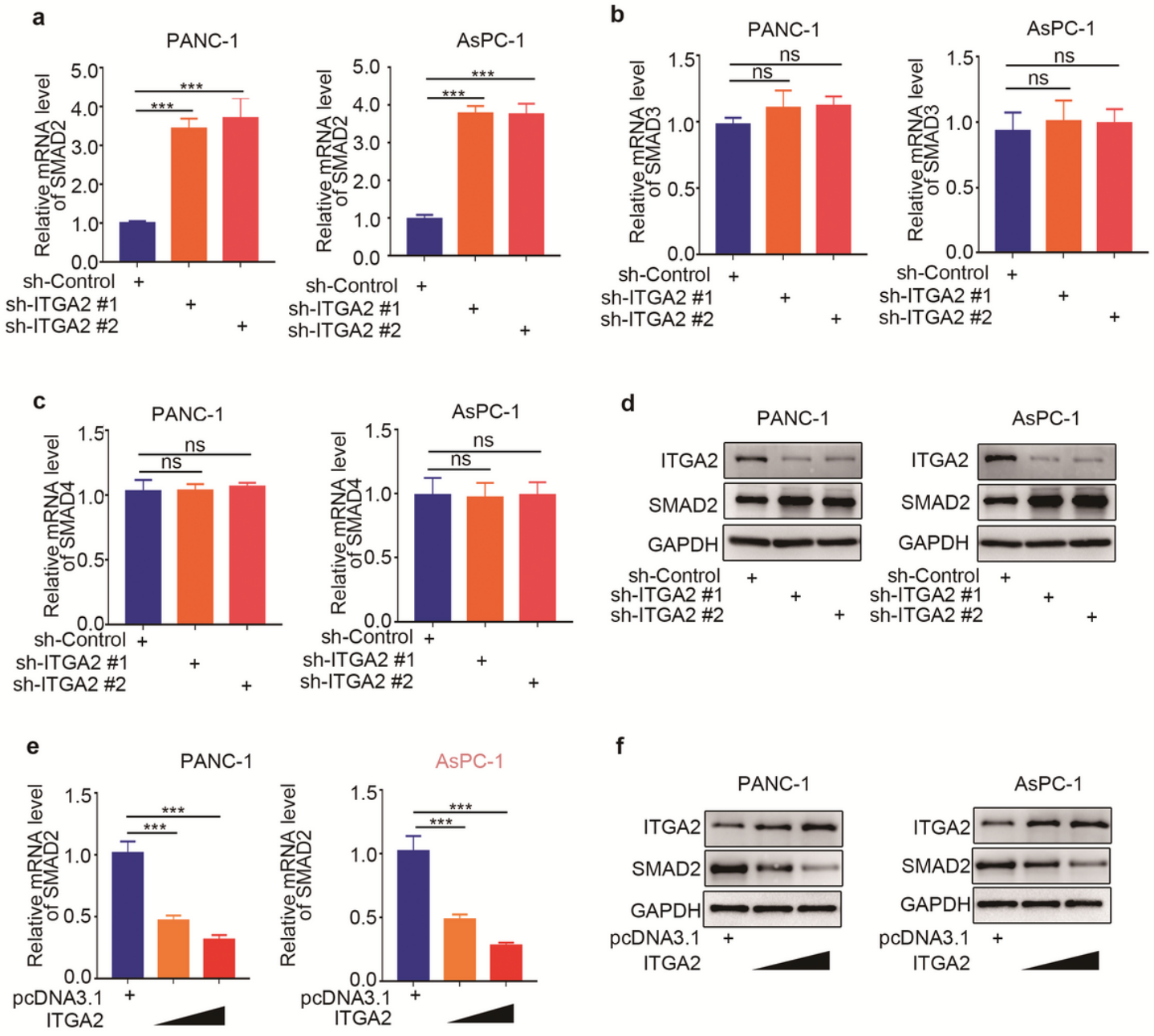

Figure 4

ITGA2 silencing induces the expression of SMAD2 in pancreatic cancer cells. a-c. RT-PCR analysis to determine the mRNA expression level of SMAD2 (a), SMAD3 (b), SMAD4 (c) in PANC-1 and AsPC-1 cells infected with sh-Control or sh-ITGA2s. GAPDH served as an internal reference and repeated for three replicates. ns, not significant; ***, $P < 0.001$. d. Western blot analysis to determine the protein expression level of SMAD2 in PANC-1 and AsPC-1 cells infected with sh-Control or sh-ITGA2s. GAPDH served as an internal reference. e. RT-PCR to determine the mRNA expression level of SMAD2in PANC-1 and AsPC-1 cells infected with or without ITGA2 plasmids. GAPDH served as an internal reference and repeated for three replicates. ***, $P < 0.001$. f. Western blot analysis to determine the protein expression level of SMAD2 in PANC-1 and AsPC-1 cells infected with or without ITGA2 plasmids. GAPDH served as an internal reference.

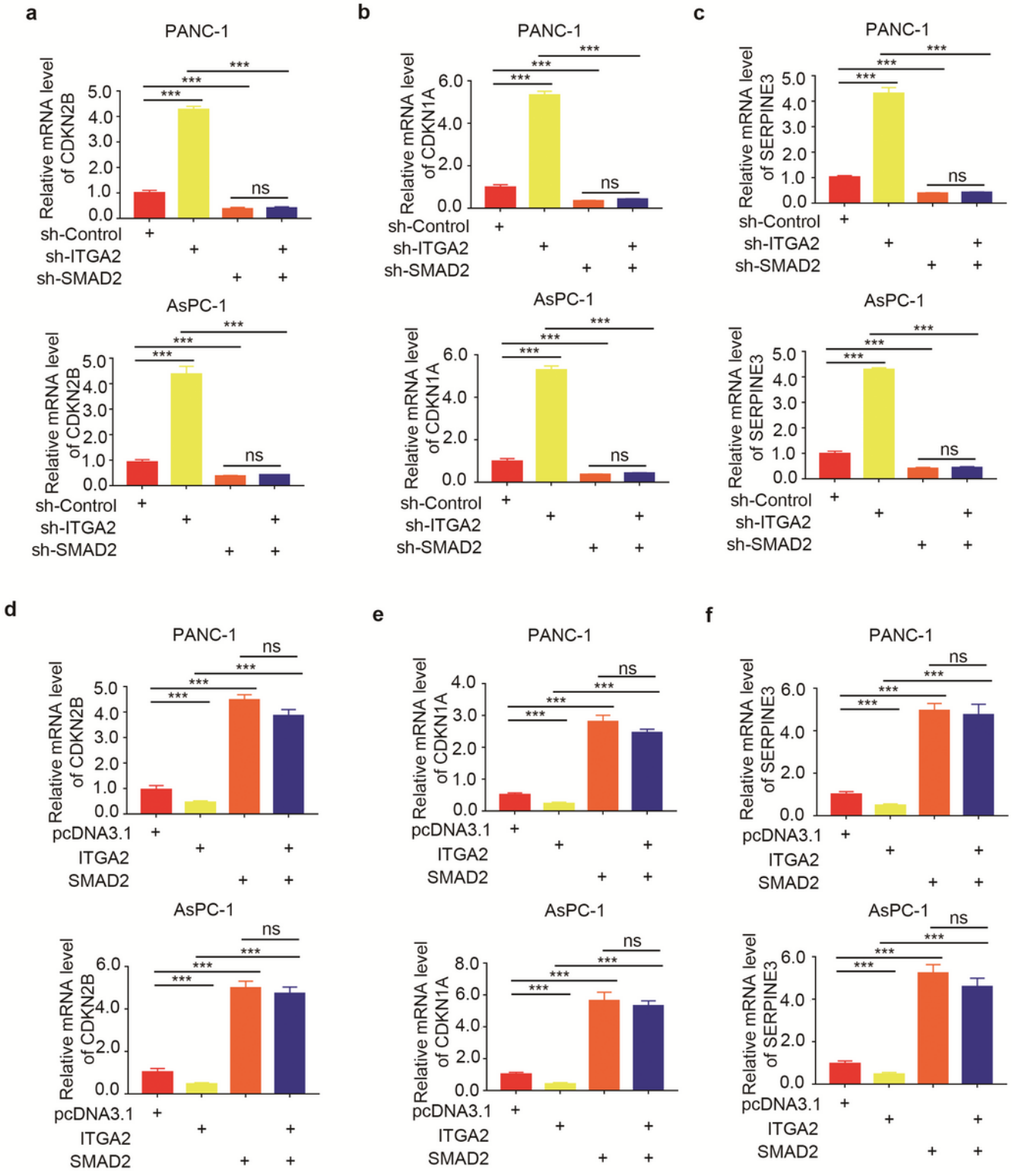

Figure 5

ITGA2 inhibits the activation of TGF- β pathway via SMAD2 in pancreatic cancer cells. a-c. RT-PCR to determine the mRNA expression level of CDKN2B (a), CDKN1A (b) and SERPINE3 (c) in PANC-1 cells infected with sh-ITGA2 and/or sh-SMAD2. GAPDH served as an internal reference and repeated for three replicates. ns, not significant; ***, P < 0.001. d-f. RT-PCR to determine the mRNA expression level of CDKN2B (a), CDKN1A (b) and SERPINE3 (c) in PANC-1 cells infected with ITGA2 plasmids and/or SMAD2

plasmids. GAPDH served as an internal reference and repeated for three replicates. ns, not significant; ***, P < 0.001.

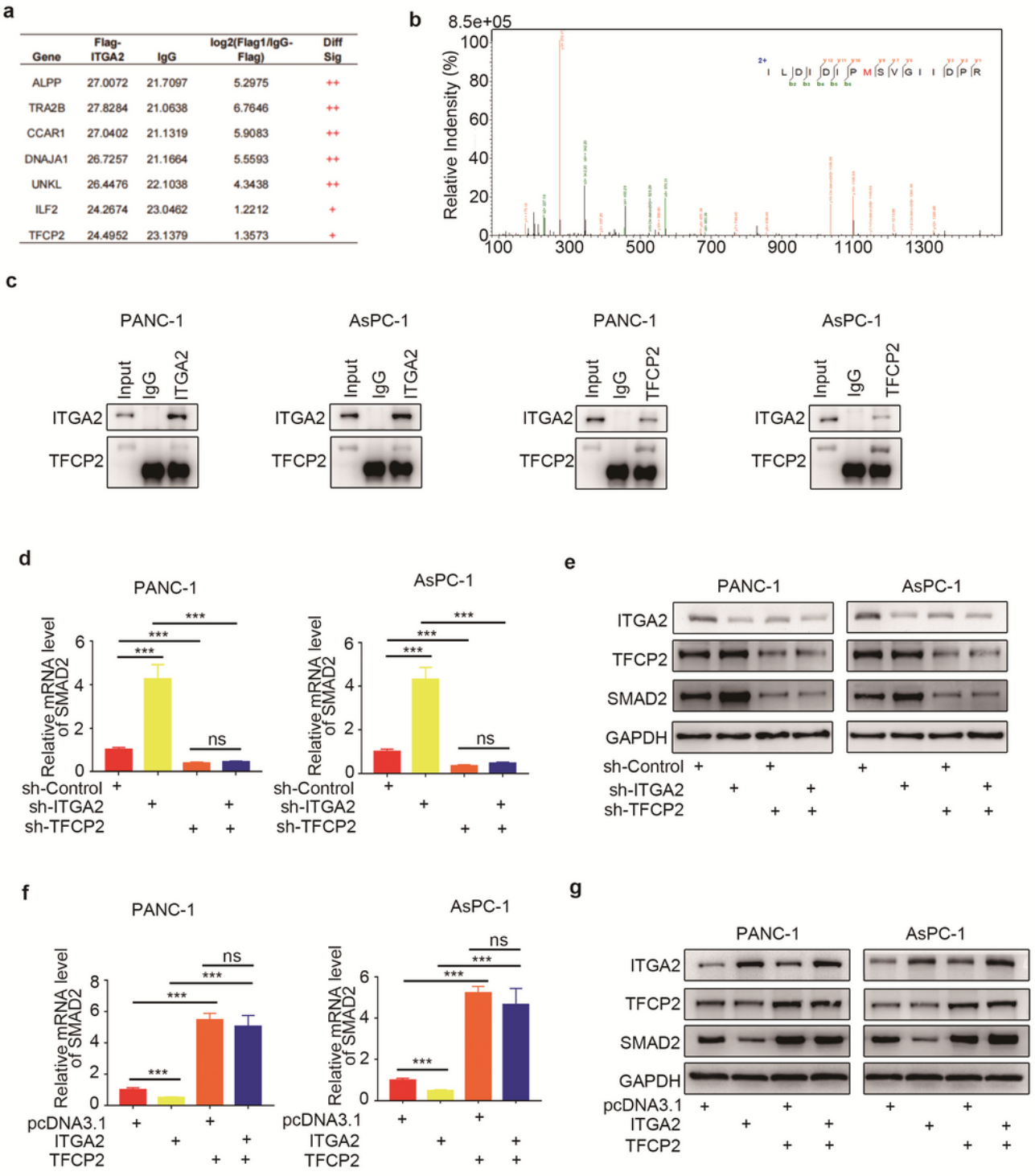

Figure 6

ITGA2 inhibits the expression of SMAD2 by interacting with TFCP2 in pancreatic cancer cells. a-b. LC-MS/MS assay to identify TFCP2 as an interaction protein of ITGA2 (a) by detecting the peptide of TFCP2 (b). c. Immunoprecipitation assay to show the interaction between ITGA2 and TFCP2 in pancreatic cancer

cells. d. RT-PCR and Western Blot assays to determine the mRNA expression level of SMAD2 in PANC-1 and AsPC-1 cells infected with sh-ITGA2 and/or sh-TFCP2. GAPDH served as an internal reference and repeated for three replicates. ns, not significant; ***, P < 0.001. e. Western Blot assays to determine the protein expression level of SMAD2 in PANC-1 and AsPC-1 cells infected with sh-ITGA2 and/or sh-TFCP2. GAPDH served as an internal reference. f. RT-PCR assays to determine the mRNA expression level of SMAD2 in PANC-1 and AsPC-1 cells infected with ITGA2 plasmids and/or TFCP2 plasmids. GAPDH served as an internal reference and repeated for three replicates. ns, not significant; ***, P < 0.001. g. Western Blot assays to determine the protein expression level of SMAD2 in PANC-1 and AsPC-1 cells infected with ITGA2 plasmids and/or TFCP2 plasmids. GAPDH served as an internal reference.

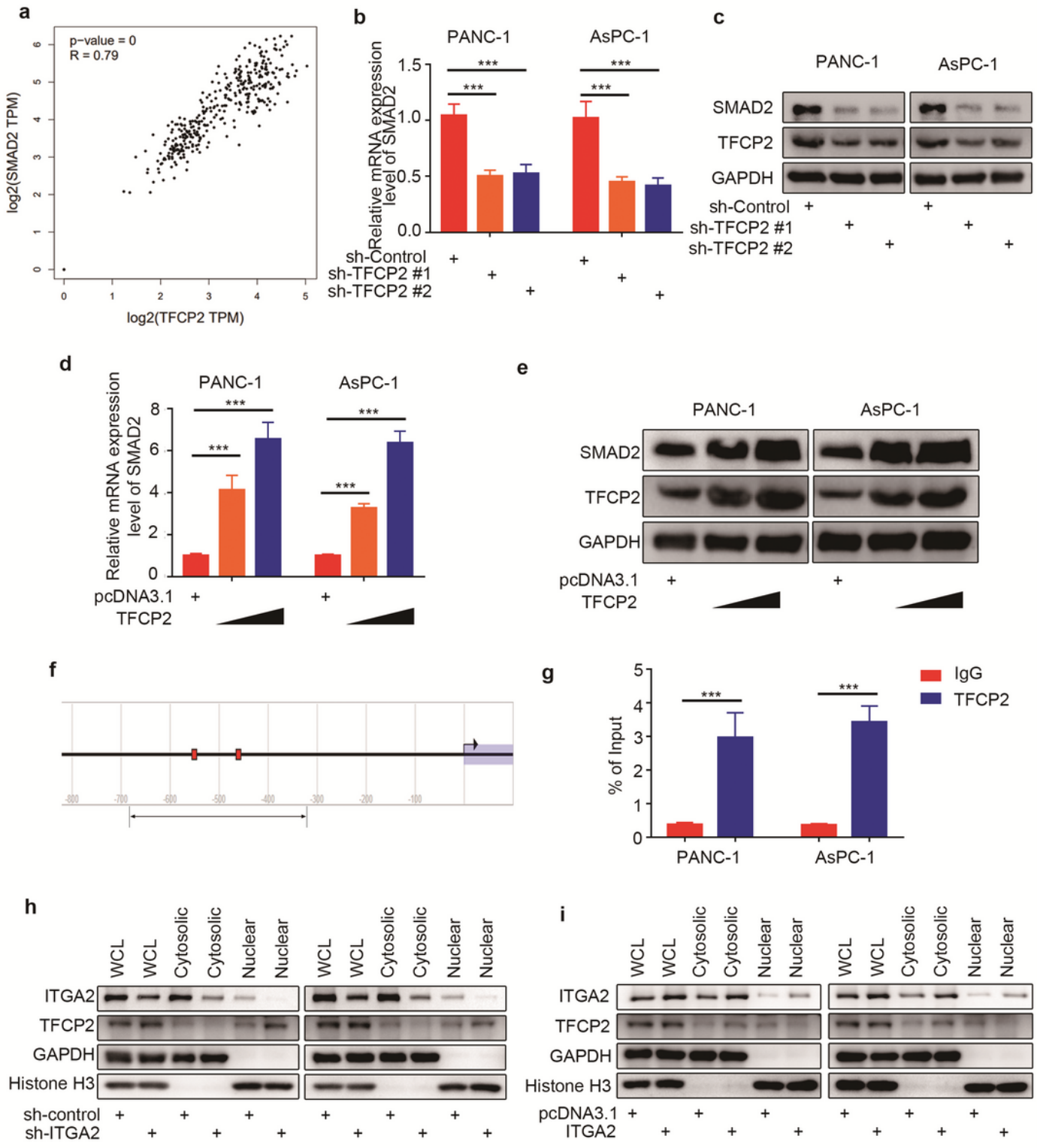

Figure 7

TFCP2 induces the expression of SMAD2 as a transcription factor in pancreatic cancer cells. a. GEPIA database was searched to determine the correlation between the mRNA expression of SMAD2 and TFCP2 in PDAC samples. P values and R values are indicated in the figure. b. RT-PCR assays to determine the mRNA expression level of SMAD2 in PANC-1 and AsPC-1 cells infected with sh-Control or sh-TFCP2s. GAPDH served as an internal reference and repeated for three replicates. ns, not significant; ***, P < 0.001.

c. Western Blot assays to determine the protein expression level of SMAD2 in PANC-1 and AsPC-1 cells infected with sh-Control or sh-TFCP2s. GAPDH served as an internal reference. d. RT-PCR assays to determine the mRNA expression level of SMAD2 in PANC-1 and AsPC-1 cells infected with pcDNA3.1 or TFCP2 plasmids. GAPDH served as an internal reference and repeated for three replicates. ns, not significant; ***, P < 0.001. e. Western Blot assays to determine the protein expression level of SMAD2 in PANC-1 and AsPC-1 cells infected with pcDNA3.1 or TFCP2 plasmids. GAPDH served as an internal reference. f. EPD database was searched to determine the potential binding sites of TFCP2 at the promoter in SMAD2 genes. g. ChIP-qPCR of TFCP2 in PANC-1 and AsPC-1 cells. All data are shown as the mean values ± SD from three replicates. ***, p < 0.001.

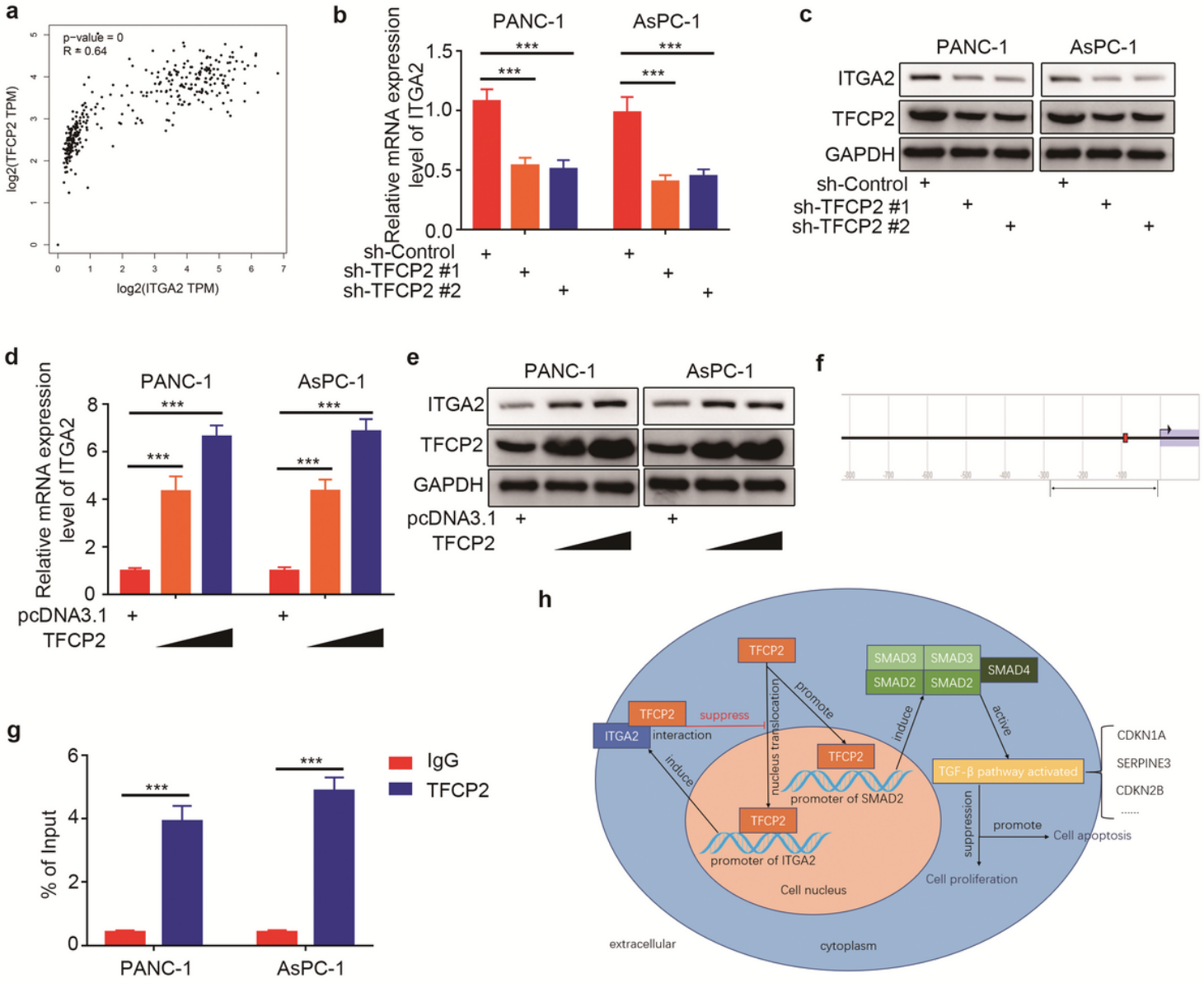

Figure 8

TFCP2 feedback induces the expression of ITGA2 as a transcription factor in pancreatic cancer cells. a. GEPIA database was searched to determine the correlation between the mRNA expression of TFCP2 and ITGA2 in PDAC samples. P values and R values are indicated in the figure. b. RT-PCR assays to determine

the mRNA expression level of ITGA2 in PANC-1 and AsPC-1 cells infected with sh-Control or sh-TFCP2s. GAPDH served as an internal reference and repeated for three replicates. ns, not significant; ***, P < 0.001. c. Western Blot assays to determine the protein expression level of ITGA2 in PANC-1 and AsPC-1 cells infected with sh-Control or sh-TFCP2s. GAPDH served as an internal reference. d. RT-PCR assays to determine the mRNA expression level of ITGA2 in PANC-1 and AsPC-1 cells infected with pcDNA3.1 or TFCP2 plasmids. GAPDH served as an internal reference and repeated for three replicates. ns, not significant; ***, P < 0.001. e. Western Blot assays to determine the protein expression level of ITGA2 in PANC-1 and AsPC-1 cells infected with pcDNA3.1 or TFCP2 plasmids. GAPDH served as an internal reference. f. EPD database was searched to determine the potential binding sites of TFCP2 at the promoter in ITGA2 genes. g. ChIP-qPCR of TFCP2 in PANC-1 and AsPC-1 cells. All data are shown as the mean values ± SD from three replicates. ***, p < 0.001. h. A working model to show that ITGA2 inhibits the activation of TGF-β pathway in pancreatic cancer via TFCP2-SMAD2 axis, and TFCP2 feedback induced the expression of ITGA2 as a transcription factor.

Supplementary Files

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