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Knockdown of TBRG4 suppresses the migration, invasion, and epithelial-to-mesenchymal transition of pancreatic cancer cells via TGF-β/smad3 signaling

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Summary. Introduction. Pancreatic cancer (PC) is one of the deadliest malignancies worldwide, with a low five-year survival rate of less than 10%. Transforming growth factor β regulator 4 (TBRG4) is differentially expressed in PC tissues, but its specific functions and regulatory role in PC have not been clarified.

Methods. TBRG4 mRNA expression in PC cells was measured by qRT-PCR. Protein levels of TBRG4, key markers related to the epithelial-mesenchymal transition (EMT) process, and factors related to the TGF-β/smad3 pathway were quantified by western blot. The migratory and invasive abilities of PC cells were evaluated by wound healing and Transwell assays, respectively. Spearman's correlation analysis was performed to analyze the expression correlation between TBRG4 and TGF-β1 (or SMAD3). Xenograft mouse models were established to explore the *in vivo* role of TBRG4.

Results. The mRNA and protein expression of TBRG4 were elevated in PC cells. TBRG4 knockdown repressed PC cell migration, invasion, and the EMT process. Moreover, TBRG4 activated TGF-β/smad3 signaling in PC cells and positively correlated with TGF-β1 (or SMAD3) expression in PC tissues based on bioinformatics analysis. Furthermore, SRI-011381 (an agonist of TGF-β1) counteracted the inhibitory influence of TBRG4 knockdown on PC cellular behaviors, and SB431542 (an inhibitor of the TGF-β type I receptor) treatment countervailed the promoting influence of TBRG4 overexpression on PC cell invasion, migration, and EMT. Results of *in vivo* assays verified that TBRG4 silencing inhibited tumorigenesis and TGF-β/smad3 signaling.

Conclusion. The silencing of TBRG4 inhibits PC cell invasion, migration, EMT, and tumorigenesis by inactivating TGF-β/smad3 signaling.

Key words: TBRG4, Pancreatic cancer, TGF-β/smad3, Epithelial-mesenchymal transition

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Introduction

Pancreatic cancer (PC) is a fatal malignancy and a leading cause of cancer-related death worldwide, causing an increasing global burden (Klein, 2021). The current five-year survival rate for patients with PC is less than 10% (Yin et al., 2022). According to Global Cancer Statistics, PC accounts for almost as many deaths (466,000) as cases (496,000) in 2020 because of its poor prognosis and is the 7th leading cause of cancer death in both men and women (Sung et al., 2021). Clinical studies have revealed that PC occurrence is associated with tobacco smoking, high-fat diets, alcohol intake, and genetic factors (Korc et al., 2017). To date, the therapeutic approaches for patients with PC are chemotherapy, radiotherapy, and surgical resection (Zhang et al., 2020). Considering the difficulty in reaching the anatomic location of the pancreas, routine examinations for PC patients are impractical (Skandalakis et al., 1993). Additionally, the low survival rate is mainly ascribed to the late diagnosis of metastasis in patients (Blum and Kloog, 2014; Ryan et al., 2014). Therefore, early detection methods and effective prevention strategies are urgently needed to reduce the mortality rate. Recently, molecular-targeted therapies have become novel approaches to treating intractable PC and can improve the prognosis of patients (Lee and Gibbs, 2019). It is imperative to explore the molecular mechanisms in PC progression to improve PC clinical outcomes.

Increasing evidence revealed that transforming growth factor-beta (TGF-β) is a hallmark of many types of cancer, including PC (Cruz-Bermúdez et al., 2019; Huang et al., 2020a,c). The tumor-suppressive or tumor-

promoting role of TGF-β largely depends on the developmental environment of cancer cells (David and Massagué, 2018). For example, miR-193a contributes to PC cell cycle and cell proliferation by suppression of TGF-β2/TGF-βRIII signaling (Fang et al., 2018). MiR-501-3p accelerates the progression of pancreatic ductal adenocarcinoma by activating TGF-β signaling (Yin et al., 2019). Transforming growth factor-β regulator 4 (TBRG4) is recognized for its involvement in regulating TGF-β1 expression during the progression of human osteosarcoma and PC (Huang et al., 2020c; Tao et al., 2024).Hence, we hypothesize that TBRG4 may regulate TGF-β/Smad signaling and thus influence PC cell progression. In addition, TBRG4 has been implicated in the progression of other malignant tumors. TBRG4 silencing repressed osteosarcoma cell proliferation and tumor formation (Huang et al., 2020b,c). Overexpression of TBRG4 accelerates the development of esophageal squamous cell carcinoma by inhibiting the mitochondria-dependent apoptotic pathway (Wang et al., 2020). TBRG4 depletion influences tumor growth in lung cancer by upregulating DNA damage-inducible transcript 3 and downregulating caveolin 1 and ribonucleotide reductase regulatory subunit M2 (Wang et al., 2018). Nonetheless, the biological role and regulatory mechanism of TBRG4 in PC were not reported.

In the current study, it was preliminarily speculated that TBRG4 may modulate TGF-β/Smad signaling to promote the metastasis and invasion of PC cells. Therefore, functional experiments and rescue assays were carried out to explore the role of the TBRG4/TGF-β/Smad axis in PC. This study may strengthen the understanding of TBRG4 in PC tumorigenesis.

Materials and methods

Cell culture

PC cell lines (CFPAC-1, AsPC-1, and PANC-1) and pancreatic ductal epithelial cell line (HPDE) were purchased from the American Type Culture Collection (Manassas, USA). Cells were cultured under 5% CO_2 and 37°C in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) with 10% fetal bovine serum (Invitrogen, USA).

Cell treatment

After 24h of cell culture, PANC-1 and AsPC-1 cells were treated with 5 μM of SRI-011381 hydrochloride (an agonist of TGF-β1) (2070014-88-7, Medchem Express, USA) while CFPAC-1 cells were treated with 5 μM of SB431542 (an inhibitor of TGF-β type I receptor) (S4317, Sigma-Aldrich, USA) for 48h.

Cell transfection

The specific shRNAs against TBRG4 (sh-TBRG4) were synthesized to silence TBRG4 while pcDNA3.1/TBRG4 vectors were constructed for TBRG4 overexpression. Sh-TBRG4#1/2, pcDNA3.1/TBRG4, and the negative controls (empty pcDNA3.1 and sh-NC) were designed by Sangon Biotech Co., Ltd. (Shanghai, China). Cells were seeded in six-well plates and cultured until cell confluence was up to 85%. Then, Lipofectamine 3000 (Invitrogen, USA) was applied to transfect the shRNAs (40 nM) or pcDNA3.1 vectors (20 nM) into PANC-1 or AsPC-1 cells as instructed by the supplier. After 12h, the medium was refreshed for a further 12h of cell incubation, and then quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed to evaluate transfection efficiency.

qRT-PCR

RNA extraction from PC or HPDE cells was performed using TRIzol reagent (Invitrogen). Total RNA (2 μg) was processed with DNase to remove DNA. Then, cDNA was achieved through reverse transcription of RNA using the SuperScript First Strand cDNA System (Invitrogen). Subsequently, the NovoStart SYBR qPCR SuperMix Plus kit (E096-01A, novoprotein, Shanghai, China) was utilized to perform qRT-PCR. TBRG4 expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) with normalization to GAPDH. The primers were synthesized by BGI (Shenzhen, China): TBRG4 forward: 5'-CAACAGTCAGATTGCCTCG-3', reverse: 5'-TTGTACTTGAGCTTCCGCA-3'; GAPDH forward, 5'-TCAAGATCATCAGCAATGCC-3', reverse: 5'-CGATACCAAAGTTGTCATGGA-3'.

Wound healing assay

PC cells (1×10^6/mL) with indicated treatment were inoculated into six-well plates and cultured overnight to form monolayer cells. When the cell confluence reached 80-90%, a sterile pipette tip (200 μl) was utilized to create wounds, and the exfoliated cells were removed with PBS (IPHASE, China) rinsing three times. The initial scratch widths (0h) and the final scratch widths (24h) were imaged and recorded under a microscope. The wound closure rate was measured according to the movement distance of PC cells.

Transwell assay

First, 25 μL of Matrigel glue (356255, Corning) was added to the upper chamber of a Transwell chamber (8-μm, 24-well plates) and cultured at 37°C to ensure complete polymerization of the Matrigel glue. Trypsin was used to digest the transfected cells to collect a single-cell suspension. Cells were plated on the culture plate (2×10^5 cells/well) containing 200 μL serum-free DMEM in the upper chamber. In addition, DMEM (650 μL, Gibco) with 10% fetal bovine saline (Invitrogen) was put in the lower chamber. After cell incubation at

37°C for 2 days, cotton swabs were used to remove the noninvaded cells in the upper chamber, while the invaded cells in the lower chamber were treated with 100% methanol and 0.1% crystal violet for fixing and staining. A microscope (Leica, Germany) was utilized to determine the number of invaded cells. The assay was repeated three times.

Western blot

The PC cells with indicated treatment were lysed using RIRA lysis buffer (E-BC-R327, Elabscience Biotechnology, China). The protein concentration was determined using a diquinoline formic acid kit (MAK059, Sigma Aldrich, USA). Next, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was utilized to separate the proteins. After the transfer of protein to polyvinylidene fluoride membrane (Millipore, Billerica, USA), the membrane was first blocked with 5% non-fat milk for 1h and then incubated with primary antibodies diluted in TBST (Sigma Aldrich, St. Louis, USA) in a 4°C refrigerator overnight. The primary antibodies are against TBRG4 (1/2000, ab180775, Abcam, UK), E-cadherin (1/1000, ab40772), Vimentin (1/1000, ab92547), N-cadherin (1/1000, ab245117), TGF-β1 (1/1000, ab215715), p-smad3 (1/2000, ab52903), p-smad2 (1/1000, ab280888), ZEB1 (1/1000, ab303480), TWIST (1/500, ab50887), SLUG (1/1000, ab51772), and the loading control GAPDH (1/10000, ab181602). Then, the membranes were rinsed with PBS in triplicate and incubated with secondary antibodies at room temperature for 2h. The proteins were visualized using an ECL Western Blot Kit (CWBIO, China), and ImageJ software (NIH, Bethesda, USA) was applied for quantitative analysis (Rha et al., 2015).

In vivo tumorigenicity model and metastasis

A total of 12 male nude mice (6 weeks old) were purchased from Vital River Laboratory Animal

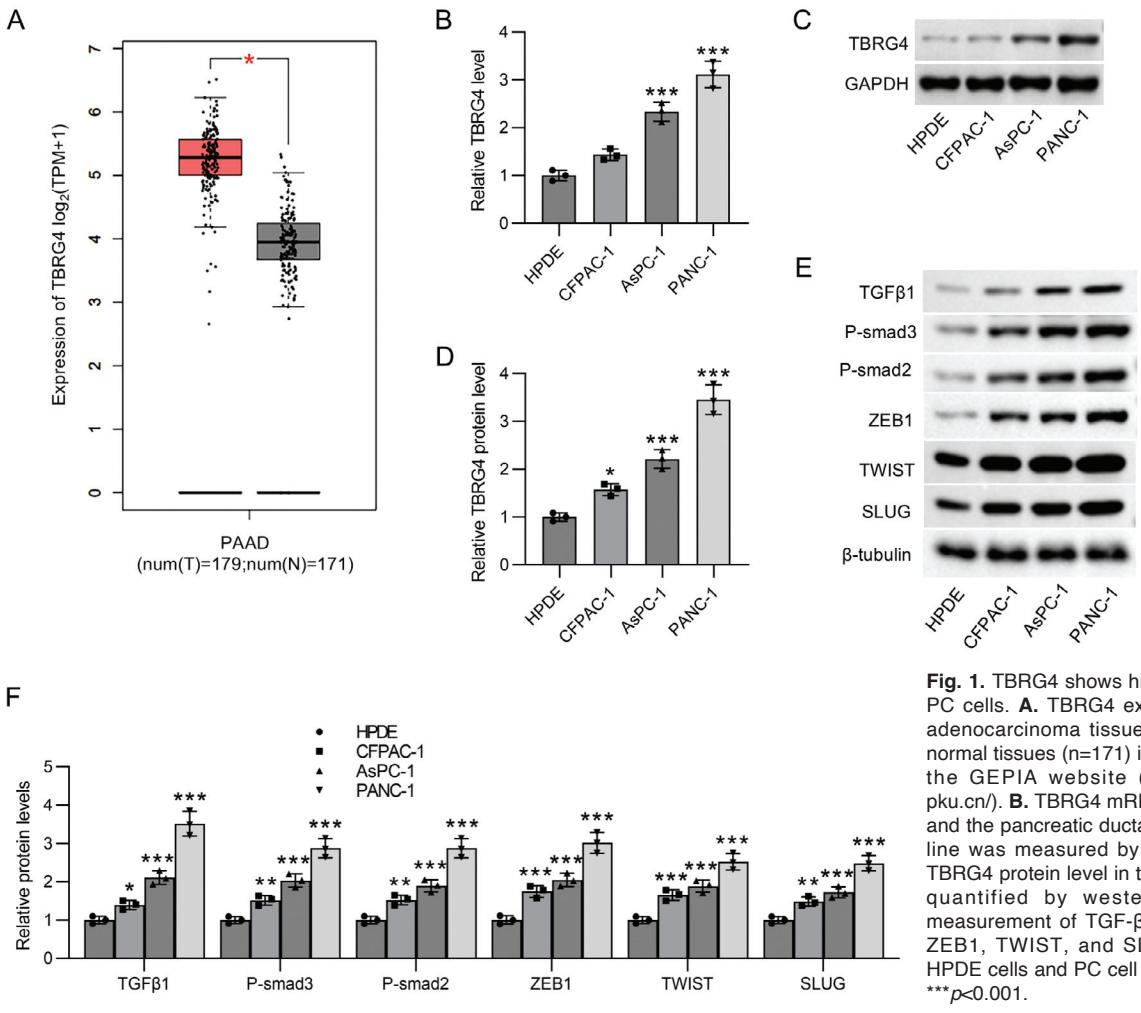

Fig. 1. TBRG4 shows high expression levels in PC cells. **A.** TBRG4 expression in pancreatic adenocarcinoma tissues (PAAD; n=179) and normal tissues (n=171) is shown. Data are from the GEPIA website (http://gepia.cancer-pku.cn/). **B.** TBRG4 mRNA level in PC cell lines and the pancreatic ductal epithelial (HPDE) cell line was measured by qRT-PCR. **C, D.** The TBRG4 protein level in the above cell lines was quantified by western blot. **E, F.** The measurement of TGF-β1, p-smad3, p-smad2, ZEB1, TWIST, and SLUG protein levels in HPDE cells and PC cell lines. *p<0.1, **p<0.01, ***p<0.001.

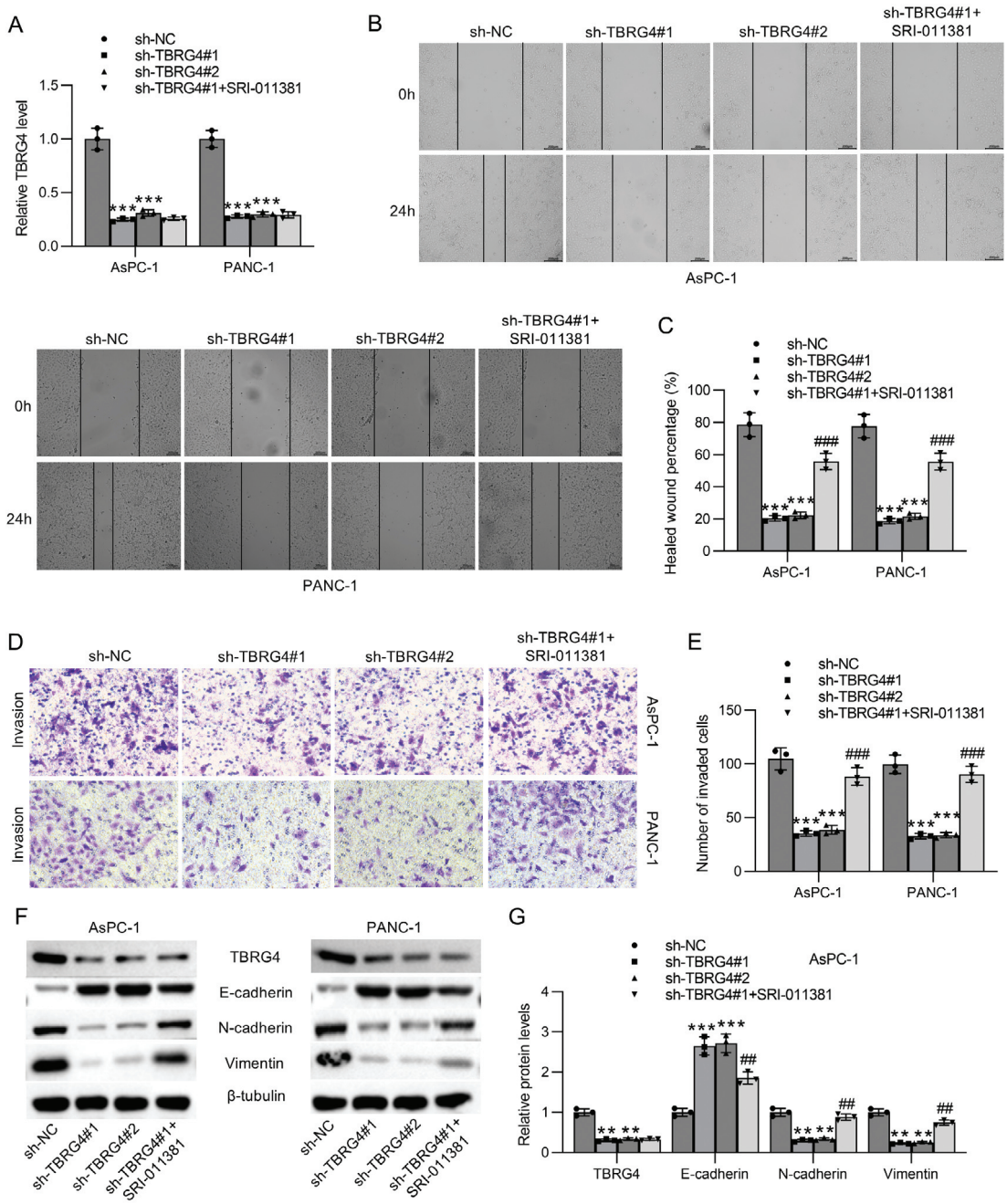

Fig. 2. TBRG4 contributes to the migration, invasion, and EMT of PC cells by regulating TGF-β signaling. **A.** TBRG4 expression in four experimental groups was measured using qRT-PCR. **B, C.** The migratory ability of AsPC-1 and PANC-1 cells transfected with sh-NC, sh-TBRG4#1/2, or sh-TBRG4#1+SRI-011381 was evaluated by wound healing assays. **D, E.** The invasive capacity of transfected PC cells was detected by Transwell assays. **F-H** Western blot analysis was performed to detect the protein levels of TBRG4 and EMT-related markers (E-cadherin, N-cadherin, and Vimentin) in AsPC-1 and PANC-1 cells with the transfection of sh-NC, sh-TBRG4#1/2, or sh-TBRG4#1+SRI-011381. $^{**}p<0.01$, $^{***}p<0.001$ vs. the sh-NC group. $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. the sh-TBRG4#1 group.

Technology (Beijing, China) and randomly divided into CFPAC-1/scramble group (n=5) and CFPAC-1/sh-TBRG4#1 group (n=5). Equal amounts of corresponding cells (2×10^6 cells) were subcutaneously injected into the right armpit of the mice. The diameter and weight of tumors were calculated twice each week after the end of the first week. Tumor volume was calculated according to the formula: (shortest diameter)$^2\times$(longest diameter)/2. After four weeks, mice were finally sacrificed to harvest the tumors.

For tumor metastasis, CFPAC-1 cells (2×10^6 cells) in the scramble or sh-TBRG4#1 group were administered intravenously into the tail vein of nude mice (n=5/group). After 28 days, the mice were euthanized and lung metastases were assessed by quantifying the number of tumor nodules using a microscope.

All experimental protocols were granted approval from Wuhan Myhalic Biotechnology Co., Ltd (approval number: HLK-202307166; approval date: July 12, 2023).

Cell Counting Kit-8 assay

Cell proliferation was evaluated using the Cell Counting Kit-8 (CCK-8) assay from Dojindo, Kumamoto, Japan. The transfected PC cells were plated on 96-well plates (1×10^3 cells/well). CCK-8 reagent was added at 24, 48, 72, and 96h, followed by measurement of the absorbance at 450 nm using a microplate reader after a 2 hour incubation period.

Statistical analyses

SPSS 20.0 software (IBM Corp., USA) and GraphPad Prism (La Jolla, USA) were used to analyze the statistics. Data are shown as the mean ± standard deviation. Experimental data were obtained from at least three independent trials. The expression correlation between TBRG4 and TGF-β1 (or SMAD3) in PC tissues was analyzed using Spearman's correlation analysis. Student's t-test or one-way analysis of variance followed by Tukey's *post-hoc* test was adopted to compare the significance between two or more groups. A *p*-value of less than 0.05 was used to indicate statistical significance.

Results

High TBRG4 levels in PC cells

TBRG4 is found to be differentially expressed in PC tissues by bioinformatics analysis. The bioinformatics tool GEPIA manifested that TBRG4 expression is higher in PC tissues than in normal pancreatic tissues (Fig. 1A). We further detected the expression levels of TBRG4 in PC cell lines and control HPDE cells. Results manifested that TBRG4 mRNA and protein expression were upregulated in PC cell lines (CFPAC-1, AsPC-1, and PANC-1), compared with the expression levels in HPDE cells (Fig. 1B-D). Moreover, EMT-associated proteins, including TGF-β1, p-smad2, p-smad3, ZEB1, TWIST, and SLUG, were upregulated in PC cells compared with HPDE cells (Fig. 1E). Therefore, AsPC-1 and PANC-1 cell lines were randomly selected for the following loss-of-function experiments, and the other cell line, CFPAC-1, was used for subsequent gain-of-function experiments. The results of PCR and western blot revealed that TBRG4 is expressed at a high level in PC cell lines, suggesting the possible involvement of TBRG4 in the malignant behavior of PC cells.

TBRG4 facilitates PC cell migration, invasion, and EMT by regulating TGF-β signaling

To investigate the biological function of TBRG4 in PC cell behaviors, sh-TBRG4#1/2 were transfected into AsPC-1 and PANC-1 cells. The results illustrated that TBRG4 expression was significantly reduced upon TBRG4 silencing, implying the effective knockdown efficiency of sh-TBRG4#1/2 (Fig. 2A). Additionally, SRI-011381 (TGF-β1 activator) treatment exerts no significant impact on TBRG4 expression in the context of TBRG4 knockdown (Fig. 2A). The CCK-8 assay indicated that the knockdown of TBRG4 attenuated cell viability, and SRI-011381 treatment reversed this result (Fig. 3A,B). Through the wound healing assay, the migratory ability of AsPC-1 and PANC-1 cells was repressed by TBRG4 deficiency, and the change mediated by sh-TBRG4 was reversed after activation of TGF-β signaling (Fig. 2B,C). The Transwell assay was conducted to measure PC cell invasion, which

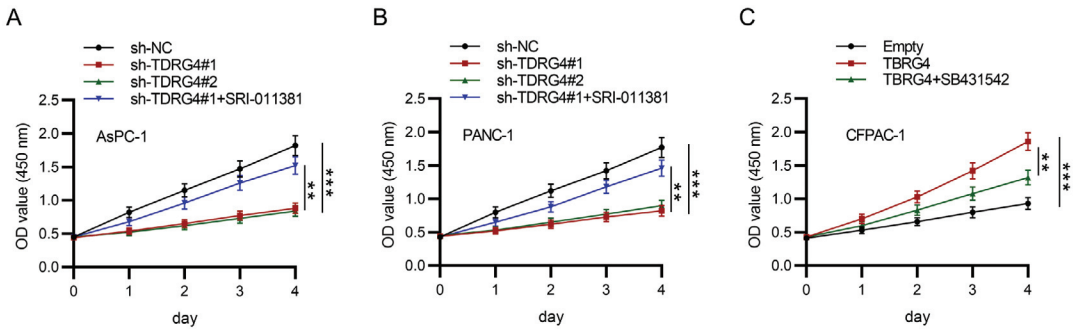

Fig. 3. A-C. The measurement of cell viability of PC cells in indicated groups. **p<0.01, ***p<0.001.

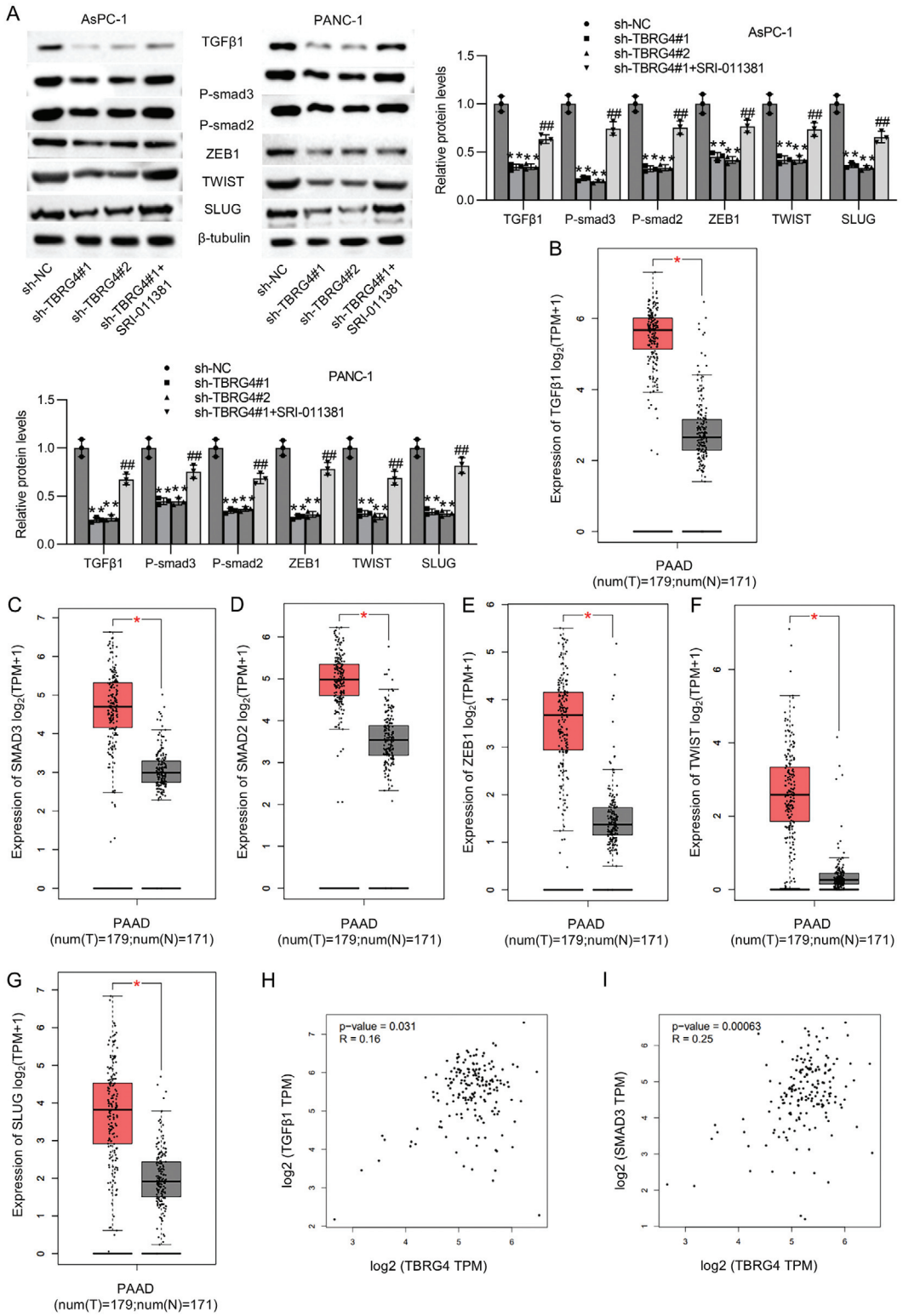

Fig. 4. TBRG4 silencing inactivates TGF-β/smad3 signaling in PC cells. After AsPC-1 and PANC-1 cells were transfected with sh-NC, sh-TBRG4#1/2, or sh-TBRG4#1+SRI-011381. **A.** the protein levels of key factors (TGF-β1, p-smad3, and p-smad2) involved in TGF-β/smad3 signaling and the protein levels of transcription factors (ZEB1, TWIST, and SLUG) associated with EMT were quantitated by western blot analysis. **B-G.** Expression of TGF-β1, SMAD3, SMAD2, ZEB1, TWIST, and SLUG in pancreatic adenocarcinoma tissues (PAAD; n=179) and normal tissues (n=171) are analyzed using the GEPIA website. **H, I.** The expression correlation between TBRG4 and TGF-β1 (or SMAD3) was analyzed by Spearman's correlation analysis. $^{**}p<0.01$ vs. the sh-NC group. $^{\#\#}p<0.01$ vs. the sh-TBRG4#1 group.

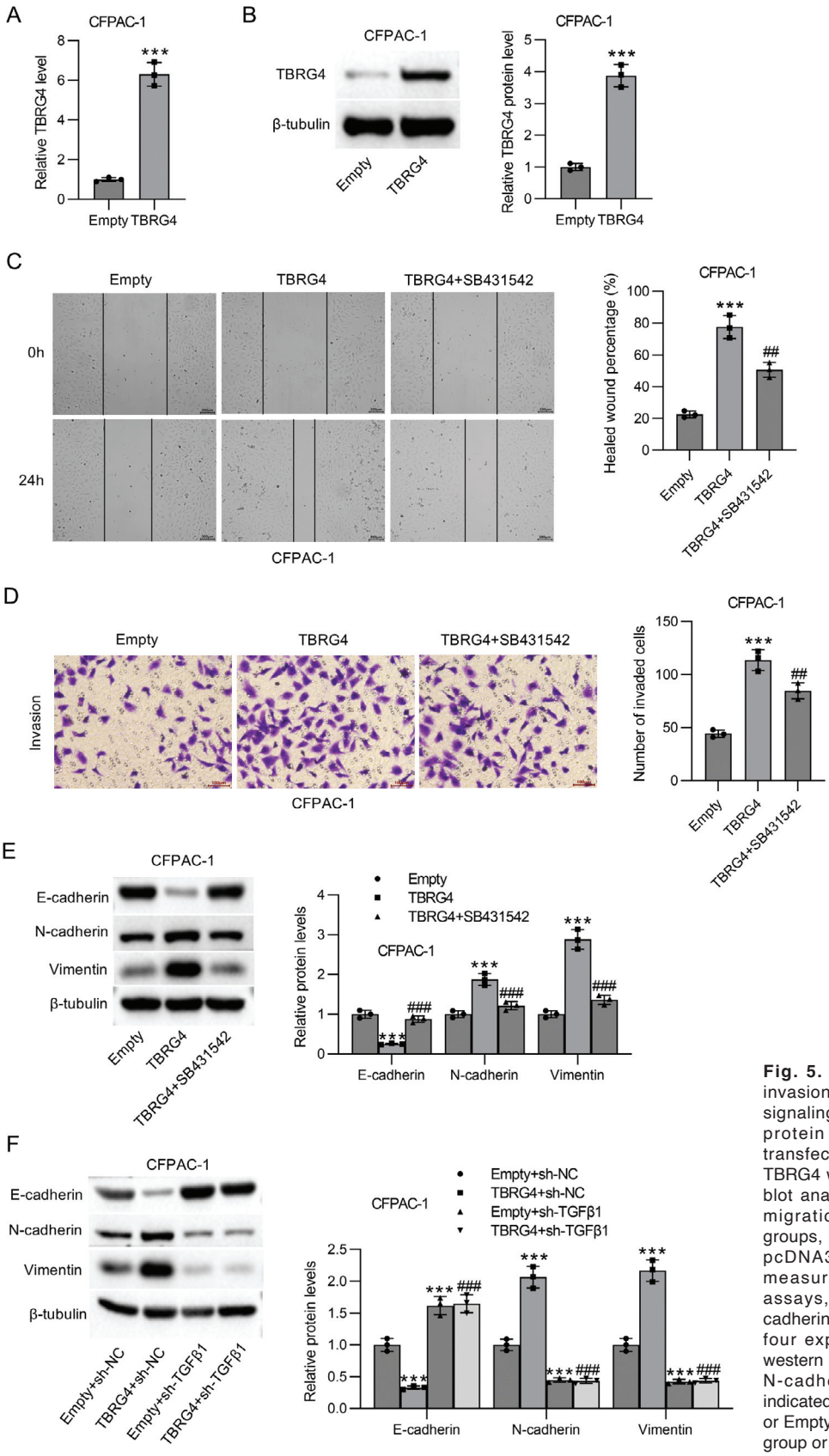

Fig. 5. TBRG4 promotes PC cell migration, invasion, and EMT by activating TGF-β/smad3 signaling. **A, B.** TBRG4 mRNA expression and protein levels in CFPAC-1 cells with the transfection of empty pcDNA3.1 or pcDNA3.1/TBRG4 were measured by qRT-PCR and western blot analysis, respectively. **C, D.** CFPAC-1 cell migration and invasion in four experimental groups, empty pcDNA3.1, pcDNA3.1/TBRG4, or pcDNA3.1/TBRG4+SB431542 (5 μM), were measured by wound healing and Transwell assays, respectively. **E.** Protein levels of E-cadherin, N-cadherin, and Vimentin in the above four experimental groups were quantified by western blot. **F.** The measurement of E-cadherin, N-cadherin, and Vimentin protein levels in indicated groups. ***$p<0.001$ vs. the Empty group or Empty+sh-NC group. ###$p<0.001$ vs. the TBRG4 group or TBRG4+sh-NC group.

manifested that TBRG4 depletion induced a suppressive effect on AsPC-1 and PANC-1 cell invasion, and the influence was offset by activating TGF-β signaling (Fig. 2D,E). Western blot revealed that the protein levels of TBRG4, N-cadherin, and Vimentin were notably downregulated, while E-cadherin was increased after transfection of sh-TBRG4#1/2 into AsPC-1 and PANC-1 cells, indicating that TBRG4 knockdown inhibits the EMT process (Fig. 2F,G). Additionally, the protein levels of EMT markers altered by TBRG4 knockdown were reversed by SRI-011381, indicating that TBRG4 regulates the EMT process by activating TGF-β signaling. In conclusion, TBRG4 depletion hampered the migration, invasion, and EMT of PC cells by inhibiting TGF-β signaling.

TBRG4 activates TGF-β/smad3 signaling in PC cells

TGF-β/Smad signaling is reported to exert a key role in PC, but whether TBRG4 can regulate TGF-β/smad3 signaling in AsPC-1 and PANC-1 cells is unknown. Therefore, we examined the protein levels of key factors (TGF-β1, p-smad3, and p-smad2) involved in TGF-β/smad3 signaling and the protein expression of EMT-related transcription factors, including ZEB1, TWIST, and SLUG, using western blot analysis. Experimental data suggested that the levels of all these proteins were inhibited by silencing TBRG4, suggesting that TBRG4 may activate the TGF-β/smad3 pathway to promote the EMT process (Fig. 4A). Additionally, the decrease in protein levels of these factors induced by TBRG4 deficiency was reversed after SRI-011381 treatment, implying the successful activation of TGF-β/smad3 signaling (Fig. 4A). Subsequently, we explored the expression levels of markers related to TGF-β/smad3 signaling and the EMT process in PC tissues and normal pancreatic tissues through the GEPIA database. As shown in Figures 4B-G, TGF-β1, SMAD3, SMAD2,

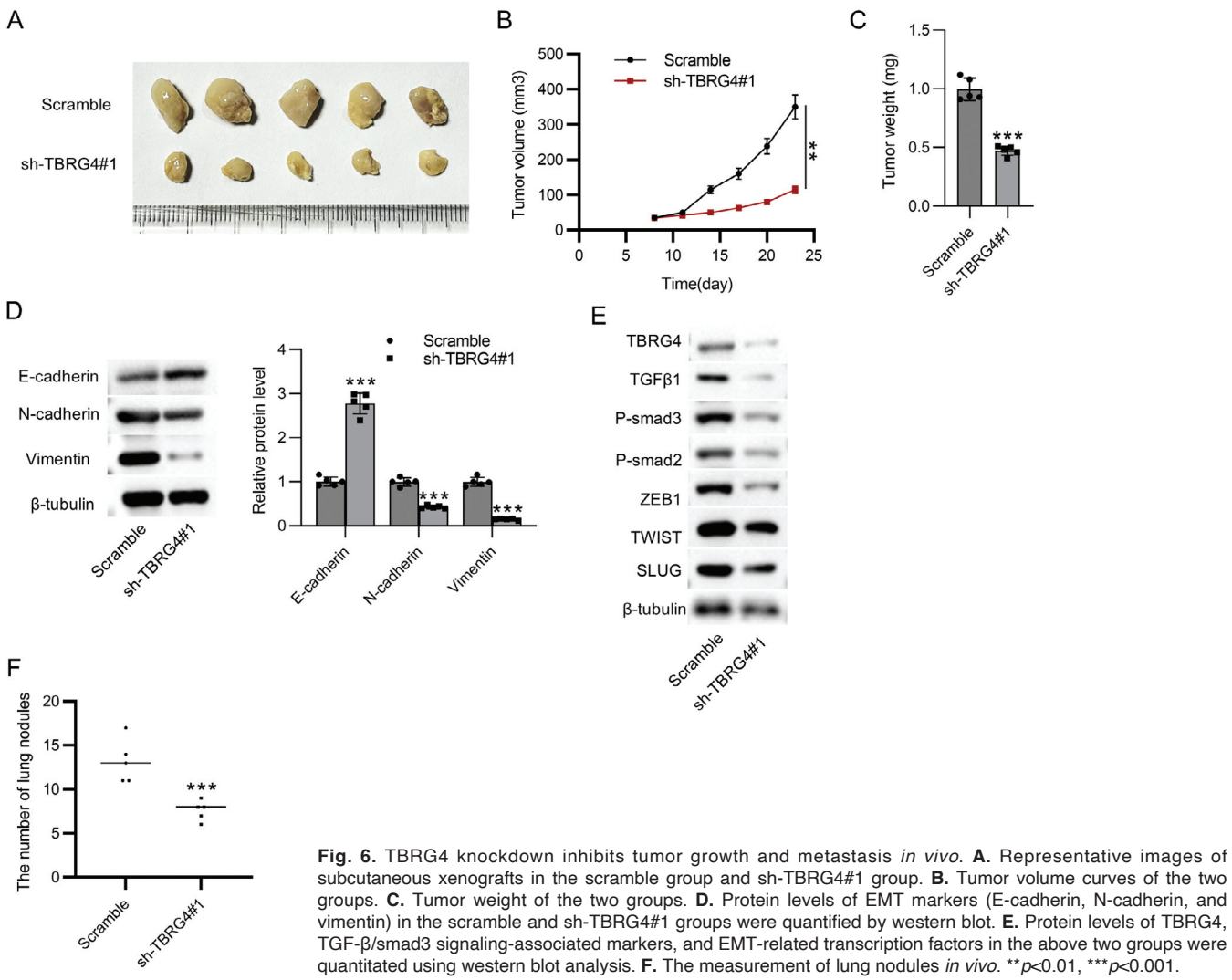

Fig. 6. TBRG4 knockdown inhibits tumor growth and metastasis *in vivo*. **A.** Representative images of subcutaneous xenografts in the scramble group and sh-TBRG4#1 group. **B.** Tumor volume curves of the two groups. **C.** Tumor weight of the two groups. **D.** Protein levels of EMT markers (E-cadherin, N-cadherin, and vimentin) in the scramble and sh-TBRG4#1 groups were quantified by western blot. **E.** Protein levels of TBRG4, TGF-β/smad3 signaling-associated markers, and EMT-related transcription factors in the above two groups were quantitated using western blot analysis. **F.** The measurement of lung nodules *in vivo*. ***p*<0.01, ****p*<0.001.

ZEB1, TWIST, and SLUG displayed significantly higher expression in tumor tissues than in healthy tissues. Further, the positive expression correlations between TBRG4 and TGF-β1 (or SMAD3) were observed through Spearman's correlation analysis (Fig. 4H,I). Hence, we concluded that TBRG4 may activate TGF-β/smad3 signaling to promote EMT in PC cells.

TBRG4 promotes PC cell migration, invasion, and EMT via the activation of TGF-β/smad3 signaling

To conduct the gain-of-function experiments and further explore the relationship of TBRG4 and TGF-β/smad3, TBRG4 was overexpressed through transfection of pcDNA3.1/TBRG4 into CFPAC-1 cells. As displayed in Figures 5A,B, the mRNA and protein levels of TBRG4 were greatly increased in the pcDNA3.1/TBRG4 group. In addition, SB431542 was used to treat CFPAC-1 cells after transfection. The CCK-8 assay displayed that the TGF-β-induced increase in cell viability was inhibited by SB431542 treatment (Fig. 3C). Wound healing and Transwell assays showed that SB431542 treatment reversed the increase in cell migration and invasion caused by TBRG4 overexpression (Fig. 5C,D). Importantly, SB431542 treatment also counteracted the reduction of E-cadherin protein levels and the elevation of N-cadherin and Vimentin levels induced by TBRG4 overexpression (Fig. 5E). Besides, TBRG4 promoted EMT in CFPAC-1 cells, while it had no significant effect in TGF-β knockout CFPAC-1 cells (Fig. 5F).

TBRG4 knockdown inhibits tumor growth and metastasis in vivo

To verify the oncogenic role of TBRG4 *in vivo*, a tumorigenicity model was constructed by subcutaneously injecting CFPAC-1 cells with sh-TBRG4#1 into mice. Compared with the scramble group, the sh-TBRG4#1 group showed decreased tumor volume and weight (Fig. 6A-C). Afterward, we analyzed the protein levels of EMT markers and TGF-β/smad3 signaling-associated factors in tissues of harvested tumors. As suggested in Figure 6D, increased E-cadherin levels and reduced N-cadherin and vimentin levels were discovered in the sh-TBRG4#1 group. In addition, TBRG4 depletion inhibited the protein levels of TGF-β/smad3-related factors and EMT-related transcription factors in xenograft tumor tissues (Fig. 6E). Furthermore, the *in vivo* metastasis assay indicated that knockdown of TBRG1 reduced the number of lung nodules (Fig. 6F). All these findings were in line with those of *in vitro* experiments, indicating the oncogenic role of TBRG4 in PC and confirming the strategy of the TBRG4/TGF-β/smad3 pathway. All these results implied that TBRG4 contributes to PC cell invasion, migration, and the EMT process by activating TGF-β/smad3 signaling. The regulatory relationship between TBRG4 and the TGF-β/smad3 pathway is clearly explained in Figure 7.

Discussion

PC often leads to a poor survival rate with limited therapeutical choices for treatment, as most affected individuals present with advanced disease (Stoffel et al., 2023). To date, molecular-targeted therapies have been gradually applied to improve the prognosis of patients (Lee et al., 2018). Previous studies revealed many abnormally expressed genes in the occurrence and development of PC (Xu et al., 2019; Cao and Zhou, 2020; Luo et al., 2020). TBRG4 has been identified to be differentially expressed in PC tissues by microarray analysis, and TBRG4 is frequently reported to participate in the progression of several types of cancer

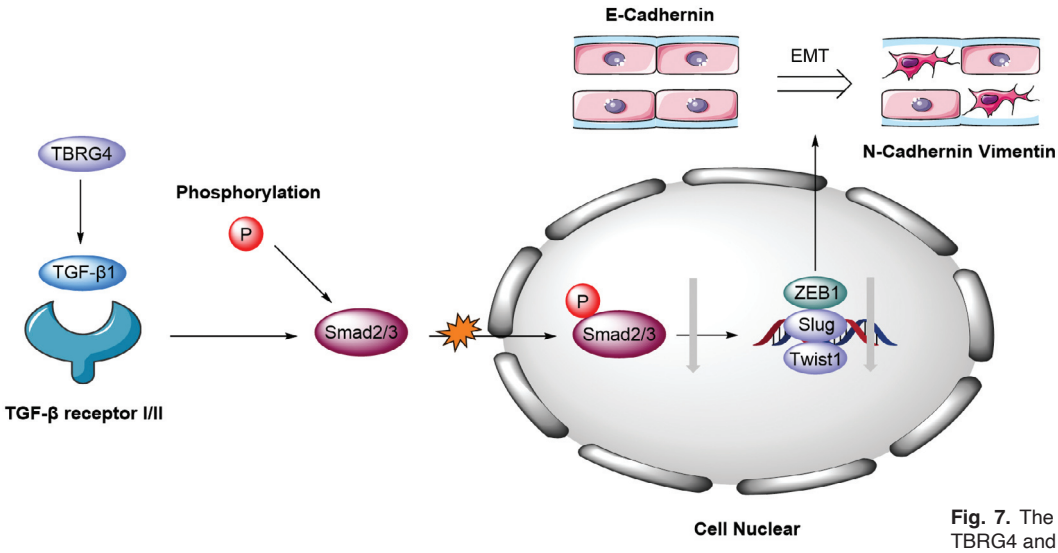

Fig. 7. The regulatory relationship between TBRG4 and the TGF-β/smad3 pathway.

(Liang et al., 2021). For example, the downregulation of TBRG4 hampers osteosarcoma cell proliferation and invasion, and the inhibitory impact of TBRG4 depletion on tumor growth and metastasis has also been verified *in vivo* (Huang et al., 2020b,c). Silencing of TBRG4 contributes to the aggravation of esophageal squamous cell carcinoma through the regulation of caveolin-1 expression and the formation of reactive oxygen species (Wang et al., 2020). In this work, the mRNA and protein levels of TBRG4 were demonstrated to be upregulated in PC cells, and the findings are consistent with the prediction from the GEPIA database, i.e., that TBRG4 is highly expressed in PC tissues. In addition, the knockdown of TBRG4 hampered the invasion, migration, and EMT process of PC cells, while overexpression exerted the opposite effect.

TGF-β is a member of a family of secreted cytokines with vital biological functions in cells, acting as a central player in human conditions, including cancer, fibrosis, and autoimmune disease (Sisto et al., 2021; Mirzaei et al., 2022). TGF-β is a potent inducer of the highly complex EMT (Pallasch and Schumacher, 2020). The EMT process is defined as the transformation of epithelial cells into mesenchymal cells by losing the cell-cell connection but gaining high motility and invasive ability (Lamouille et al., 2014). Through the EMT process, cells can invade through the basement membranes and stromal tissues, leading to cell migration and invasion and thereby contributing to tumor progression (Tan and Weinberg, 2013; Zhang et al., 2018). According to previous studies, RUNX1 modulates the migration and EMT process, which were induced by TGF-β in colorectal cancer (Lu et al., 2020). MiR-190 represses breast cancer metastasis via suppression of TGF-β-induced EMT (Yu et al., 2018). As a mitochondrial mRNA regulator, TBRG4 participated in the regulation of non-canonical junction and non-coding mitochondrial RNA processing (Zhang et al., 2022). However, the effect of TBRG4 on cytoplasmic or nuclear gene expression has been rarely reported. Coincidently, a recent study reported that TBRG4 can upregulate TGF-β expression in osteosarcoma cells (Huang et al., 2020c). Moreover, TGF-β is a key factor in TGF-β/smad signaling. Hence, we postulated that TBRG4 activated TGF-β/smad signaling by upregulating TGF-β1 in PC cells. In this study, we discovered that the protein levels of key markers (TGF-β1, p-smad3, and p-smad2) of TGF-β/smad3 signaling and EMT-associated proteins (ZEB1, TWIST, and SLUG) were significantly reduced by silenced TBRG4. According to bioinformatics analysis, TGF-β1, p-smad3, p-smad2, ZEB1, TWIST, and SLUG, all display high levels in PC tissues. Additionally, the positive expression correlation between TBRG4 and TGF-β1 (or SMAD3) was determined. These findings confirmed that TBRG4 knockdown inactivates the TGF-β/smad3 pathway and inhibits the EMT process in PC cells. In the present study, SB431542, an inhibitor of the TGF-β type I receptor, was found to reverse the promoting impact of TBRG4 upregulation on PC cell invasion, migration, and EMT. In addition, SRI-011381 (an agonist of TGF-β1) offset the suppressive effect of TBRG4 knockdown on PC cell behavior. All these results suggested that TBRG4 activates the TGF-β/smad3 pathway and, therefore, promotes PC cell invasion, migration, and EMT.

In conclusion, this study demonstrated that TBRG4 facilitates the migration, invasion, and EMT process of PC cells and promotes tumor growth *in vivo* by activating the TGF-β/smad3 signaling pathway. The study further clarified the role of TBRG4 in cancer development and may provide a novel strategy for PC-targeted therapy. However, due to the complexity of the molecular mechanisms, there may be other potential upstream molecules or downstream signaling pathways associated with TBRG4/TGF-β/smad3 and future work should be carried out for further exploration.

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