

# WBSCR22 and TRMT112 synergistically suppress cell proliferation, invasion and tumorigenesis in pancreatic cancer via transcriptional regulation of ISG15

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**Abstract.** Pancreatic cancer (PC) is one of the most aggressive and devastating types of cancer owing to its poor prognosis and deadly characteristics. It is well established that aberrations in the expression of key regulatory genes, namely tumor suppressors and oncogenes, predispose patients to progression and metastasis of PC. Upregulation of Williams-Beuren syndrome chromosomal region 22 (WBSCR22) expression, a ribosomal biogenesis factor, has been reported in multiple types of human cancer. However, the role of WBSCR22 and its underlying mechanism in PC have not been well investigated. In the present study, the tumor suppressive role of WBSCR22 was reported in PC for the first time; the results indicated that WBSCR22 overexpression (OE) significantly suppressed cellular proliferation, migration, invasion and tumorigenesis *in vivo* and *in vitro*. RNA-sequencing analysis revealed that WBSCR22 negatively regulated the transcription of interferon-stimulated gene 15 (ISG15) downstream, which is a ubiquitin-like modifier protein involved in metabolic and

proteasome degradation pathways, while the antitumor function of WBSCR22-OE could be rescued by ISG15 OE. In addition, the oncogenic role of ISG15 was further confirmed in PC; its upregulation promoted the proliferation, migration, invasion and tumorigenesis of PC. Furthermore, WBSCR22 and its cofactor tRNA methyltransferase activator subunit 11-2 (TRMT112) functioned synergistically in PC, and concurrent ectopic OE of WBSCR22 and TRMT112 further promoted the tumor suppressive potential of WBSCR22 in PC. Collectively, the findings indicated that WBSCR22 played an important role in PC development and that the WBSCR22/ISG15 axis may provide a novel therapeutic strategy for PC treatment.

## Introduction

Pancreatic cancer (PC) is one of the most aggressive and devastating types of cancer owing to its poor prognosis and deadly characteristics. It is the 4th leading cause of cancer-led mortality in the USA, with only 8% chance of surviving longer than 5 years (1), and it will be ranked second due to its high mortality by 2030 (2). PC has the most dismal diagnosis among all digestive malignancies due to its late clinical appearance, lack of an effective cure and a constantly increasing global incidence ratio (3,4). Therefore, it is particularly important to reveal the molecular mechanisms that lead to PC.

Human Williams-Beuren syndrome chromosomal region 22 (WBSCR22) was initially identified as one of the 26 contiguous genes that are deleted in Williams-Beuren syndrome (WBS). WBS is a neurological disorder that displays clinically unique features, such as mental retardation, dysmorphic facial appearances, sunken chest, vascular and congenital heart disease, hypertension and infantile hypocalcemia. The etiology of this disease is associated with the induction of oxidative stress. The main contributing genes, including superoxide dismutase-1 and neutrophil cytosolic factor-1, are primarily involved in the regulation of the redox-state (5-7). WBSCR22 is localized on chromosome 7 (7q11.23) and constitutes an S-adenosyl-L-methionine (SAM) motif and nuclear localization signal with well-preserved features of methyltransferase family proteins (8,9). Subsequently, the human WBSCR22

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**Abbreviations:** PC, pancreatic cancer; WBSCR22, Williams-Beuren syndrome chromosomal region 22; TRMT112, tRNA methyltransferase subunit 11-2; PDAC, pancreatic ductal adenocarcinoma; SAM, S-adenosyl-L-methionine; DEGs, differentially expressed genes; WBS, Williams-Beuren syndrome; ISG15, interferon-stimulated gene 15

**Key words:** WBSCR22, ISG15, pancreatic cancer, TRMT112, tumor suppression, PDAC, genetic mutation

protein was identified as a methyltransferase for 18S rRNA m<sup>7</sup>G, which was involved in pre-rRNA processing and 40S ribosome subunit biogenesis (10-12). In addition, WBSCR22 has been revealed to modulate histone methylation (13).

Previous studies have revealed that WBSCR22 expression is upregulated in several types of cancer, including invasive breast cancer (13), multiple myeloma, plasma cells (14), colorectal cancer (15), lung cancer (16) and hepatocellular carcinoma (17). In addition, upregulation of WBSCR22 expression promotes invasion, proliferation and migration, while knockdown of this gene exhibits the opposite effects in glioma cells (18). WBSCR22 exhibits tumor-promoting potential in several types of cancer, whereas WBSCR22 loss has also been reported in specific inflammatory-type human lung pathologies (16). However, the function of WBSCR22 in PC remains unknown. In the present study, it was unexpectedly revealed that WBSCR22 played a tumor suppressor role in PC.

Interferon-stimulated gene 15 (ISG15) is a 17-kDa protein consisting of 165 amino acids, which is induced by interferon (INF-Type I) treatment and is extensively recognized as an antiviral protein (19-21). ISG15 is a ubiquitin-like protein that covalently binds to several nuclear and cytoplasmic proteins, commonly referred to as the ISGylation process (22,23). Similar to ubiquitination, ISGylation requires a three-step enzymatic system, including activating E1 enzyme (UBE1L), conjugating E2 enzyme (UBCH8) and ligating E3 (24-26). It has been widely accepted that the function of ISG15 or the effects of ISGylation are tissue specific (27). Certain studies have suggested its role in multiple cellular functions, including protein turnover (28), protein stability (27), interferon-induced immune responses and in the production of type II IFN, which enhances the proliferation and activity of natural killer cells, particularly neutrophils (29-31). Elevated expression levels of ISG15 have been detected in various human cancer cells, including pancreatic adenocarcinoma, and breast, colorectal, ovarian and prostate carcinomas (32). Additionally, the oncogenic capacities of ISG15, including the promotion of migration, proliferation and tumorigenesis, have been well established in multiple types of cancer (33-35). Conversely, a tumor suppressive function of ISG15 has also been reported in hepatocellular, cervical and breast cancer (36-39). ISG15 exhibits inconsistent biological functions that are explained by its ability to act both as a tumor promoter and suppressor, depending on the cancer type. To the best of our knowledge, the role of ISG15 in PC remains unclear and requires further investigation.

Several methyltransferases require accessory proteins for their activity and stability. The human protein tRNA methyltransferase activator subunit 11-2 (TRMT112) shares homology with the TRMT112 protein of *Saccharomyces cerevisiae*. The TRMT112 protein is highly conserved, and is expressed at high levels in multiple tissues and organs, particularly during early mouse development (40,41). TRMT112 acts as a cofactor for various methyltransferases, including methylated rRNA, tRNA and proteins (42-44). It has been reported that TRMT112 is the interaction partner of WBSCR22, and enhances the stability of WBSCR22 (45). The interaction between WBSCR22 and TRMT112 was also reported to be involved in the processing of pre-rRNA, leading to the generation of 18S-rRNA (10).

However, the biological function of the WBSCR22-TRMT112 interaction in pancreatic tumor development remains unclear.

In the present study, the tumor suppressor role of WBSCR22 was reported for the first time in PC. Overexpression of WBSCR22 significantly suppressed the proliferation, migration, invasion and tumorigenesis of PANC-1 cells *in vivo* and *in vitro*, while knockdown of its expression exhibited the opposite effects. In addition, RNA-sequencing (RNA-seq) analysis revealed that ISG15 is a downstream target of WBSCR22, as its mRNA expression and protein levels were markedly reduced in WBSCR22-overexpressing cells. Additionally, ISG15 plays an oncogenic role in PC, as upregulated expression of ISG15 promoted the proliferation, migration, invasion and tumorigenesis of PC. Furthermore, it was suggested that TRMT112 and WBSCR22 may function synergistically in PC and that ectopic OE of WBSCR22 and TRMT112 may simultaneously promote the tumor suppressive potential of WBSCR22 in PC. WBSCR22 is a clinically important gene in PC and the newly identified WBSCR22/ISG15 axis may represent an innovative approach for therapeutic purposes.

## Materials and methods

*Analysis of the cancer genome atlas (TCGA) and human protein atlas.* The genes of interest, which were differentially expressed in PC cells were investigated in comparison with adjacent normal non-neoplastic tissues using TCGA (<http://gepia.cancer-pku.cn/> and <http://tcga-data.nci.nih.gov/tcga/>). The WBSCR22 prognostic value, clinical significance, overall survival and expression profiles were examined in the TCGA cohort.

*Cell culture.* The human PC cell lines PANC-1 (resource no. 1101HUM-PUMC000023), BXPC3 (resource no. 1101HUM-PUMC000274) and ASPC1 (resource no. 1101HUM-PUMC000214), a normal human pancreatic ductal epithelial cell line, HPDE6-C7 (resource no. C1248; <https://www.whelab.com/pro/580.html>), and the human embryonic kidney cell line, 293T (resource no. 4201HUM-CCTCC00187) were purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences. All cell lines were authenticated at the beginning of the present study by species-specific PCR evaluation (Chinese Academy of Sciences). The cell line authentication profile is provided in Table SI. All the cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.) high glucose, supplemented with 10% fetal bovine serum (FBS; cat. no. 04-007-1A; Biological Industries). In order to inhibit residual bacterial activity two potential antibiotics were supplemented with a concentration of 100 U/ml penicillin and streptomycin (100 ng/ml) reagent (both from Hyclone; Cytiva). The cell culture was performed at 37°C in a humidified incubator with 5% CO<sub>2</sub>.

*Generation of stable cell lines.* The PC cell line PANC-1 was cultured and incubated in a humidified incubator at 37°C with 5% CO<sub>2</sub>. WBSCR22-OE and ISG15-OE stably transfected cells were generated and the CDS portion of WBSCR22 was subcloned into the lentiviral vector pCDH-EF1α-MCS-T2A-Puro (cat. no. CD527A-1; System

Biosciences). Small hairpin (sh)WBSCR22 stable cell line generation was achieved by subcloning the shRNA sequence of WBSCR22 into the pLVX-shRNA2-BSD lentiviral vector upgraded from pLVX-shRNA2 (cat. no. 632179; Clontech laboratories Inc.). The 293T cells were transfected with the aforementioned lentiviral plasmids (0.5  $\mu\text{g}/\mu\text{l}$ ) and the second-generation lentivirus packaging system (0.5  $\mu\text{g}/\mu\text{l}$  pMD2.0 and 0.5  $\mu\text{g}/\mu\text{l}$  pspAX2; mixed ratio was pMD2.0: pspAX2: lentivirus, 1:3:4) using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) for 72 h at 37°C. Subsequently, the supernatant containing the infectious lentivirus was collected and transduced to PANC-1 cells for 72 h, at a multiplicity of infection (MOI) of 10. Following 36 h of incubation after transfection, puromycin (2  $\mu\text{g}/\text{ml}$ ) or Blasticidin S (5  $\mu\text{g}/\text{ml}$ ) was added to the cultured cells to select for overexpressing and knockdown cells, respectively. The selection was performed for 14 days, and overexpression/knockdown in all positive viable cells was further confirmed by reverse transcription-quantitative PCR (RT-qPCR) and western blotting.

**Wound healing assay.** The cells cultured previously were harvested during the logarithmic growth phase and seeded in a six-well plate to create a confluent monolayer. When the confluency reached 70-80%, a horizontal scratch was made using a sterile 200- $\mu\text{l}$  microliter pipette tip. The medium was removed and the cells were washed twice with cold PBS to remove the cells detached during wound scratching. Fresh DMEM supplemented with 2% FBS was added to the cells. Images of the selected areas were obtained at 0 h and subsequently the culture was incubated overnight at 37°C with 5% CO<sub>2</sub>. The selected wound area was imaged following 24 and 48 h of culture using a fluorescence microscope (Leica Microsystems, Inc.), and the wound area was assessed using ImageJ software (version 1.53e). Wound healing was quantified by calculating the percent of wound closure: The % of wound closure = 1 - (wound surface area at the indicated time-point/initial wound surface area). The assay was performed in triplicate.

**Colony formation assay.** To evaluate the reproductive potential of the cells, the colony formation assay was performed. The cells that were cultured previously were harvested at 70-90% confluency and resuspended in 1-2 ml DMEM. Cells were counted, and ~200 cells/well were seeded into a 6-well plate. Fresh DMEM with 10% FBS was added and incubated in a humidified incubator for 14 days at 37°C supplied with 5% CO<sub>2</sub>. Following the incubation period, the media was removed and the cells were washed with cold PBS twice to remove excess media. Cells were fixed with 1% formaldehyde for 15 min followed by staining with 0.5% crystal violet in 25% methanol for 30 min at room temperature. Cell differentiation was performed by washing the cells with tap water and subsequent drying. The purple dots (representing a colony grown during the incubation period, containing >50 cells) were counted manually. The assay was performed in triplicate.

**Transwell invasion assay.** The cell lines cultured previously were harvested and resuspended in serum-free DMEM. The Transwell apparatus consisted of two chambers. The upper chamber of the insert was a Matrigel-coated Millicell chamber (BD Biosciences) with an 8- $\mu\text{m}$  pore size membrane,

to which cells were added at a density of 6x10<sup>3</sup> cells/well for all Transwell assays in this study. Except for one set of experiments involving three cell lines (Ctrl, WBSCR22-OE and WBSCR22-OE + TRMT112OE cells), the cell seeding density in this set was 1x10<sup>4</sup> cells/well. A total of ~200  $\mu\text{l}$  serum-free DMEM was added to the upper chamber. The lower chamber contained ~500  $\mu\text{l}$  DMEM supplemented with 10% FBS. The 24-well plate was incubated for 24 h in a humidified incubator at 37°C with 5% CO<sub>2</sub>. Following incubation, the media were removed and washed with cold PBS twice. The upper chamber was fixed with 1% formaldehyde for 15 min and subsequently stained with 0.5% crystal violet for 15 min at room temperature. The inner side of the upper insert was cleaned with a cotton swab to remove the excess stain and cells. The chamber was air-dried and the cells were counted in three different fields of view using a light microscopy at a magnification of x100 (Leica Microsystems, Inc.).

**Cell proliferation assay.** The cell proliferative potential was analyzed using a Cell Counting Kit-8 (CCK-8) assay. The cells were seeded in a 96-well plate at a concentration of 2x10<sup>3</sup> cells/well. The plate was incubated in a humidified incubator at 37°C with 5% CO<sub>2</sub> and 10 ml CCK-8 (Beijing Solarbio Science & Technology Co., Ltd.) was added to each well. Following incubation for 2 h, the optical density was measured at 450 nm using a Microplate reader (Bio-Rad Laboratories, Inc.). The experiment was performed in triplicate for a single cell line and the optical density was expressed as a percentage of viable cells.

**Promoter luciferase reporter assay.** A promoter sequence 2001 bp in length, ranging from the -2000 base site to the +77 base site of the ISG15 gene, was copied into the modified pGL4.1[luc2P] (Promega Corporation) vector. The *Renilla* luciferase gene sequence was added to the vector for the normalization of transfection efficiency. Cells were cotransfected with the pcDNA3.1-WBSCR22 plasmid or pcDNA3.1 empty vector and 50 ng of the pGL-ISG15-promoter vector using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Firefly luciferase activities and *Renilla* signals were measured 48 h after transfection using a Dual-luciferase reporter assay kit (Promega Corporation) according to the manufacturer's instructions.

**RT-qPCR.** Total RNA was isolated using an RNA purification kit (cat. no. DP430; Tiangen Biotech Co., Ltd.) according to the manufacturer's protocol. The isolated RNA was reverse transcribed to cDNA using a HiScript III 1st Strand cDNA Synthesis kit (Vazyme Biotech Co., Ltd.) according to the manufacturer's protocol. qPCR was performed using ChamQ SYBR Color qPCR MasterMix (Vazyme Biotech Co., Ltd.) on an ABI StepOne system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following thermocycling conditions: Initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 15 sec and extension at 72°C for 30 sec. The 2<sup>- $\Delta\Delta\text{Ct}$</sup>  method was calculated to analyze the relative changes in gene expression according to the previous reported method (46). Normalization of the expression levels was performed using  $\beta$ -actin as the internal control. The sequences for the corresponding forward

and reverse primers used for WBSR22 and  $\beta$ -actin were as follows: human WBSR22 forward, 5'-ATGAGAGGGAAG GTGGAGCA-3' and reverse, 5'-AGAACCGCGTGGTGACTT AG-3'; and human  $\beta$ -actin forward, 5'-TGACGTGGACAT CCGCAAAG-3' and reverse, 5'-CTGGAAGGTGGACAG CGAGG-3'.

**Western blot analysis.** Total protein was extracted using cell lysis buffer (Beyotime Institute of Biotechnology) supplemented with a protease and phosphatase inhibitor cocktail (Beyotime Institute of Biotechnology). The protein concentration was quantified using a bicinchoninic acid protein estimation kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A total of 20  $\mu$ g protein/per lane was loaded on 4-20% SDS-gels and resolved using SDS-PAGE, and subsequently transferred to a PVDF membrane (MilliporeSigma). The membranes were rinsed with Tris-buffered saline with 0.1% Tween-20 (TBST) for 5 min and blocked by 5% skimmed milk powder at room temperature for 2 h. The membranes were then subjected to immunoblotting with the appropriate primary (WBSR22; cat. no. A7317; 1:1,000; ABclonal Biotech Co. and ISG15; cat. no. P05161; 1:1,000; Cusabio technology LLC.) at 4°C overnight and goat anti-rabbit (HRP) secondary antibodies (cat. no. orb43514; 1:5,000; Biorbyt) for 2 h at room temperature. The GAPDH antibody (cat. no. orb555879; 1:5,000; Biorbyt) was used as a control. Signals were visualized using the BeyoECL Moon super sensitivity detection kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol, and the blots were quantified using Image Pro Plus v6.0 software (Media Cybernetics, Inc.).

**RNA isolation, cDNA library preparation and sequencing.** Cells were harvested and total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Assessment of RNA quality was performed using a Qubit Fluorometer (Agilent 2100 Bioanalyzer; Agilent Technologies, Inc.). Total RNA samples, which exhibited optimal quality, were used in subsequent experiments. The following criteria were used: RNA integrity number >7.0 and 28S to 18S ratio >1.8. RNA-seq libraries were generated and sequenced by CapitalBio Technology, Inc. All assays were run in triplicate and an independent library was constructed in order to perform sequencing. The construction of the library for sequencing was performed using the NEB Next Ultra RNA Library Prep for Illumina, Inc. (New England Biolabs, Inc.). A total of 1  $\mu$ g total RNA was used. The mRNA Poly (A) tailed enrichment was performed using the NEB Next Poly (A) mRNA Magnetic Isolation Module kit (New England Biolabs, Inc.), and fragments of ~200 base pairs were generated. The first-strand and second-strand cDNAs were generated from mRNA fragments using random hexamer primers, reverse transcriptase and DNA polymerase-I and RNase H, respectively. A single adenine base was added to the ends of the cDNA fragments followed by adapter ligation. The end products were first purified and subsequently enriched via PCR to amplify the DNA library. The quantification of the final product of the DNA libraries was performed using Agilent 2100 Bioanalyzer and the KAPA Library quantification kit (Kapa Biosystems; Roche Diagnostics GmbH) according to the manufacturer's protocol.

All the RT-qPCR validated libraries were processed by paired-end sequencing with a pair end of 150-base pair length reading on the Illumina Novaseq sequencer (Illumina, Inc.).

**RNA-seq and data analysis.** The hg38 human genome version was used as a reference (Homo\_sapiens.GRCh38.92.gtf). The assessment of the quality of the sequencing was performed using FastQC (v0.11.5; <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and the filtration of the low quality data was achieved through NGSQC (v2.3.3; [http://hpc.ilri.cgiar.org/\\_export/xhtml/ngsqt toolkit-software#installation](http://hpc.ilri.cgiar.org/_export/xhtml/ngsqt toolkit-software#installation)). The alignment of the clear and clean reads was performed using HISAT2 (v2.2.0; <https://daehwankimlab.github.io/hisat2/>). Each sample was aligned from the processed reads against the reference genome using HISAT2. The differentially expressed genes (DEGs) in the samples were analyzed using DESeq9 (v1.28.0) (<https://www.huber.embl.de/users/anders/DESeq/>). Multiple independent statistical hypotheses were separately performed on the DEGs. The obtained P-value was corrected using the FDR method. The BH method was used to calculate the corrected P-value (q-value). Significance analysis was performed using the P-value. The different parameters used for categorizing the significant DEGs were based on  $\geq 2$ -fold differences ( $|\log 2FC| \geq 1$ ; where FC is the fold change in expression) in the transcript abundance and with  $P \leq 0.05$ . Annotation of DEGs was performed on the basis of the information obtained from Uniprot, Gene Ontology (GO), NCBI, ENSEMBL and Kyoto Encyclopedia of Genes and Genomes (KEGG). The RNA-seq data were submitted to the Gene Expression Omnibus (GEO; GSE186154).

**In vivo tumorigenicity assays.** A total of 12 female nude mice (6 to 8 weeks old; 20-25 g) were purchased from Weitong Lihua Experimental Animal Technology Co., Ltd. The mice were allowed to grow in a specific pathogen-free facility (12-h light/dark cycle; 18-23°C; and humidity 50-60%) and were provided with *ad libitum* access to all nutritional supplements. A total of  $2 \times 10^6$  WBSR22-OE cells or the corresponding control cells in 200  $\mu$ l PBS were subcutaneously injected into the left and right flanks of the mice. Following 4-5 weeks of injection, the mice were anesthetized with 1% pentobarbital sodium (45 mg/kg, intraperitoneal) and subsequently euthanized by cervical dislocation. The tumor volume and weight were assessed. The experiments were executed in triplicate. All animal experiments performed in the present study were approved (approval no. IRB-1507) by the Ethics Committee of the Beijing University of Technology (Beijing, China). The maximum tumor volume allowed by the Ethics Committee of the Beijing University of Technology was 300 mm<sup>3</sup> per tumor (or 1.5 cm in diameter).

**Statistical analysis.** Data are presented as the mean  $\pm$  standard deviation of at least three independent experiments. Quantitative results were compared using GraphPad Prism version 5.0 (GraphPad Software, Inc.). Data normality tests were performed using the Shapiro-Wilk and Kolmogorov-Smirnov tests. A two-tailed paired Student's t-test was used to assess the significance between two groups. The differences between multiple groups were compared using a one-way or two-way

ANOVA with a Tukey's post-hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**WBSCR22 gene expression is downregulated in PC cells.** According to data obtained from TCGA, the expression levels of WBSCR22 are associated with the overall survival rate of patients with PC. High expression levels of WBSCR22 were significantly associated with an improved 5-year survival rate (40%) (PDAC,  $n=103$ ), while low expression levels were associated with a reduced 5-year survival rate (9%) (PDAC,  $n=73$ ; Fig. 1A). In addition, analysis of results from the Human Protein Atlas indicated WBSCR22 protein levels were downregulated in PC tissues compared with those of the surrounding normal non-neoplastic healthy tissues. Reduced staining was observed for WBSCR22 in PC tissues, whereas high WBSCR22 staining was observed in normal pancreatic tissues (Fig. 1B). Furthermore, western blot analysis of the three PC cell lines (PANC-1, BXP3 and ASPC1) and one pancreatic ductal epithelial cell line (HPDE6-C7) confirmed the decreased WBSCR22 expression levels in PDAC cells (Fig. 1C). These analyses suggested that high WBSCR22 expression levels may have a direct tumor suppressor role in PC.

**WBSCR22 functions as a tumor suppressor in PC.** To investigate the oncogenic or tumor suppressive behavior of WBSCR22, stably transfected PANC-1 cells were established, using WBSCR22 knockdown (KD) (PANC-1-shWBSCR22) and WBSCR22-OE (PANC-1-WBSCR22-OE) models and their corresponding controls. The transfection efficiency was confirmed at the mRNA and protein level (Fig. 1D and E). OE of WBSCR22 significantly suppressed the proliferation and colony formation capacity of PANC-1 cells, while knockdown of WBSCR22 expression promoted these processes *in vitro* (Fig. 1F and G). In addition, a subcutaneous xenograft mouse model ( $n=3/\text{group}$ ) was established to further verify the role of WBSCR22 in tumorigenesis *in vivo*. WBSCR22-OE significantly suppressed the tumor weight and volume compared with the corresponding control xenografts (Fig. 1H). In addition, the Transwell assays indicated that WBSCR22-OE significantly suppressed the migratory and invasive capacities of PANC-1 cells, whereas WBSCR22 knockdown exhibited the opposite effects (Fig. 1I and J). Furthermore, the tumor suppressor function of WBSCR22 was confirmed in BXP3 cells (Fig. S1). Collectively, these data demonstrated that WBSCR22-OE suppressed proliferation, migration, invasion and tumorigenesis *in vivo* and *in vitro*. The results revealed for the first time the tumor suppressor function of the WBSCR22 gene in PC.

**ISG15 is a downstream target of WBSCR22 in PC.** To explore the downstream pathways of WBSCR22 in PC, RNA-seq was performed using shWBSCR22 and the corresponding control cells. A total of 329 upregulated and 264 downregulated genes were identified in response to WBSCR22 knockdown ( $FC \geq 2$ ,  $P < 0.05$ ; Fig. 2A). ISG15 was one of the most significantly upregulated differentially expressed genes (DEGs) in response to WBSCR22 knockdown, and its role in PC has

not been studied, to the best of our knowledge (Fig. 2B). ISG15 exerts a vital role in protein turnover, protein stability and most importantly, ubiquitin-like modification of several nuclear and cytoplasmic proteins. ISG15 targets  $>300$  proteins, including p53, by altering the cellular metabolic pathway and ISGylation as well as the ubiquitin proteasome degradation pathway (32). Therefore, it was hypothesized that the tumor suppressor function of WBSCR22 in PC may be connected to cellular metabolic, ubiquitin proteasome or RNA degradation pathways via the regulation of ISG15-mediated ISGylation.

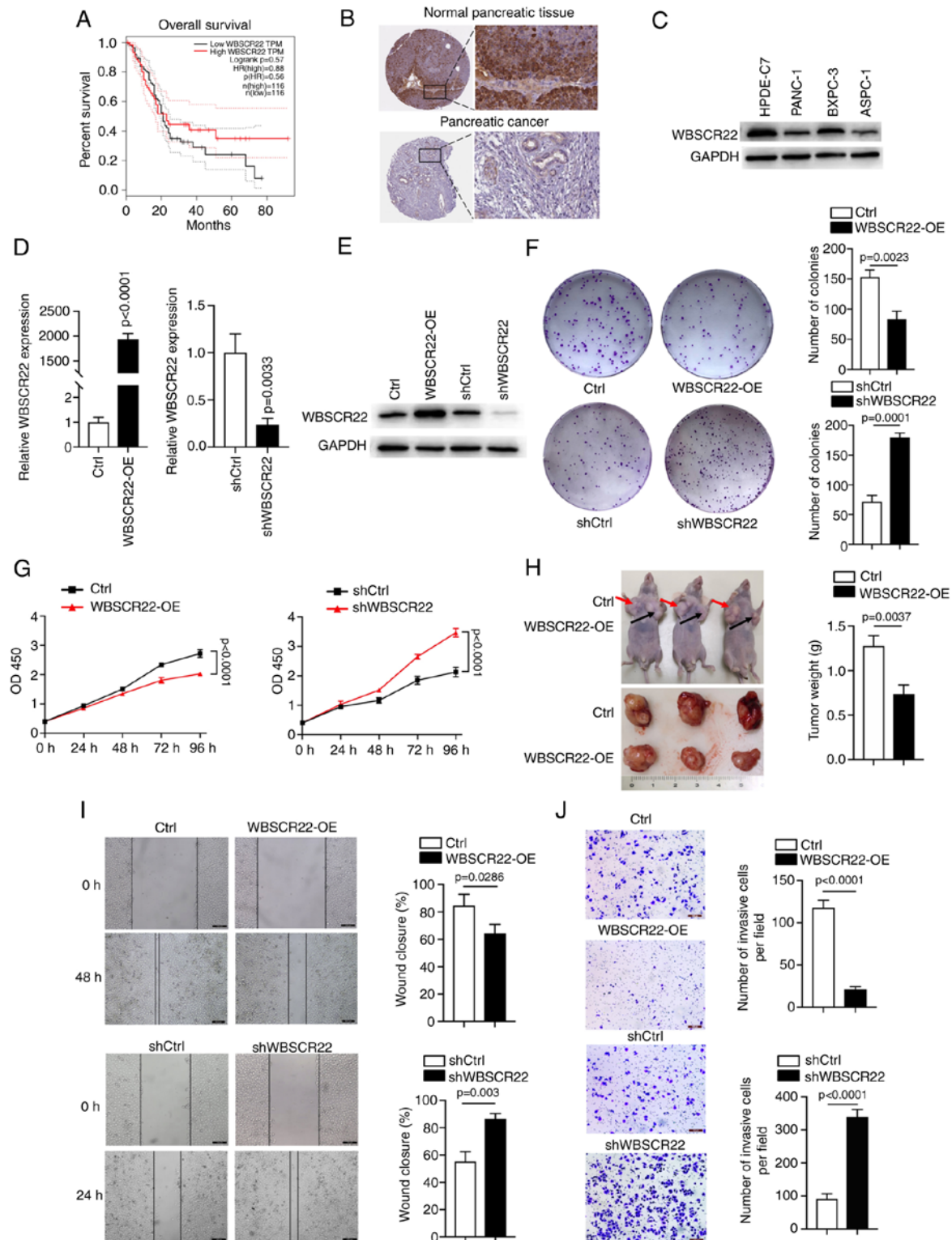
Subsequently, KEGG enrichment pathway analysis revealed that the 'metabolic' and 'RNA degradation pathways' were among the top 5 most enriched pathways in which ISG15 was involved (Fig. 2C). Additionally, several pathways closely related to tumor development, such as the 'JAK-STAT', 'Wnt' and 'TNF signaling pathways', were among the top 15 most enriched pathways, suggesting that WBSCR22 may play a critical role in cancer development/progression. RT-qPCR and western blot assays confirmed the reduced levels of ISG15 in WBSCR22-OE cells (Fig. 2D). The transcriptional regulatory role of WBSCR22 on ISG15 was evaluated by a luciferase promoter reporter assay using the ISG15 promoter. WBSCR22-OE significantly reduced the activity of the ISG15 promoter (Fig. 2E). Collectively, these results suggested that WBSCR22 is an upstream regulator of ISG15 in PC.

**ISG15 promotes tumorigenesis in PC.** Elevated expression levels of ISG15 have been previously reported in multiple types of cancer, including PC (32-35). According to TCGA database analysis, the ISG15 gene is preferentially upregulated in PC specimens (PDAC;  $n=179$ ) compared with adjacent normal tissues ( $n=171$ ; Fig. 3A). In addition, high expression levels of ISG15 in PC were associated with poor survival (Fig. 3B). To further determine the possible oncogenic function of ISG15 in PC, ISG15 was overexpressed in PANC-1 cells. OE of ISG15 significantly promoted the proliferation and migration of PANC-1 cells compared with those of the corresponding controls (Fig. 3C and D). Furthermore, the Transwell assay confirmed that ISG15 OE promoted the invasion of PANC-1 cells compared with that of the corresponding controls (Fig. 3E).

In addition, rescue experiments were performed to further confirm the oncogenic role of ISG15 and its functional association with WBSCR22. Ectopic OE and knockdown ISG15 models were established in WBSCR22-OE and shWBSCR22 PANC-1 cells, respectively. The data indicated that the tumor-inhibitory capacity of WBSCR22-OE could be significantly rescued by ISG15 OE in PC (Fig. 3F and G). In contrast to these observations, the tumor-promoting function caused by WBSCR22 knockdown (shWBSCR22) was significantly abolished by shISG15 (Fig. 3H and I). Collectively, the data indicated that ISG15 functioned as an oncogene in PC cells, and that the antitumor function of WBSCR22 in PC was closely associated with its negative regulatory effect on ISG15.

**TRMT112 promotes the tumor suppressive function of WBSCR22.** The interaction of WBSCR22 with TRMT112 was verified; the stability of WBSCR22 affected its expression. The present study examined further whether this interaction could influence the function of WBSCR22 in PC. To test this





**Figure 1.** Upregulation of WBSCR22 expression suppresses PC progression. (A) Survival plot indicating that upregulation of WBSCR22 is associated with an increased overall survival rate. (B) Data analysis results from the Human Protein Atlas indicating downregulation of WBSCR22 protein in PC compared with the corresponding expression noted in normal pancreatic tissues. Decreased staining was observed for WBSCR22 in PC tissues, whereas increased WBSCR22 staining was observed in normal pancreatic tissues. (C) The protein levels of WBSCR22 were assessed by western blot analysis. Consistent data were obtained from three independent experiments. (D) mRNA expression levels of WBSCR22 in WBSCR22-OE, WBSCR22-KD (shWBSCR22) and the corresponding control cell lines. (E) WBSCR22 protein levels were confirmed in WBSCR22-OE, WBSCR22-KD (shWBSCR22) and in the corresponding control cell lines. Consistent data were obtained from three independent experiments. (F) Colony formation assay of the WBSCR22-OE, WBSCR22-KD (shWBSCR22) and corresponding control cells. (G) Cell proliferation assays were performed in WBSCR22-OE, WBSCR22-KD (shWBSCR22) and in the corresponding control cells. (H) A tumor xenograft model was used to investigate the *in vivo* effect of WBSCR22 ( $n=3$  independent samples for each group). (I) Wound healing and (J) Transwell assays were performed to evaluate the migratory and invasive capacities of WBSCR22-overexpressing and WBSCR22-KD (shWBSCR22) cells relative to their corresponding control cell lines. The data are presented as the mean value  $\pm$  standard deviation;  $n=3$  biologically independent repeats. The data in D, F, H-J were analyzed using a two-tailed, unpaired t-test. The data in G were analyzed using a two-way ANOVA, followed by Bonferroni corrections. P-values are indicated. WBSCR22, Williams-Beuren syndrome chromosomal region 22; PC, pancreatic cancer; OE, overexpression; KD, knockdown; ANOVA, analysis of variance.

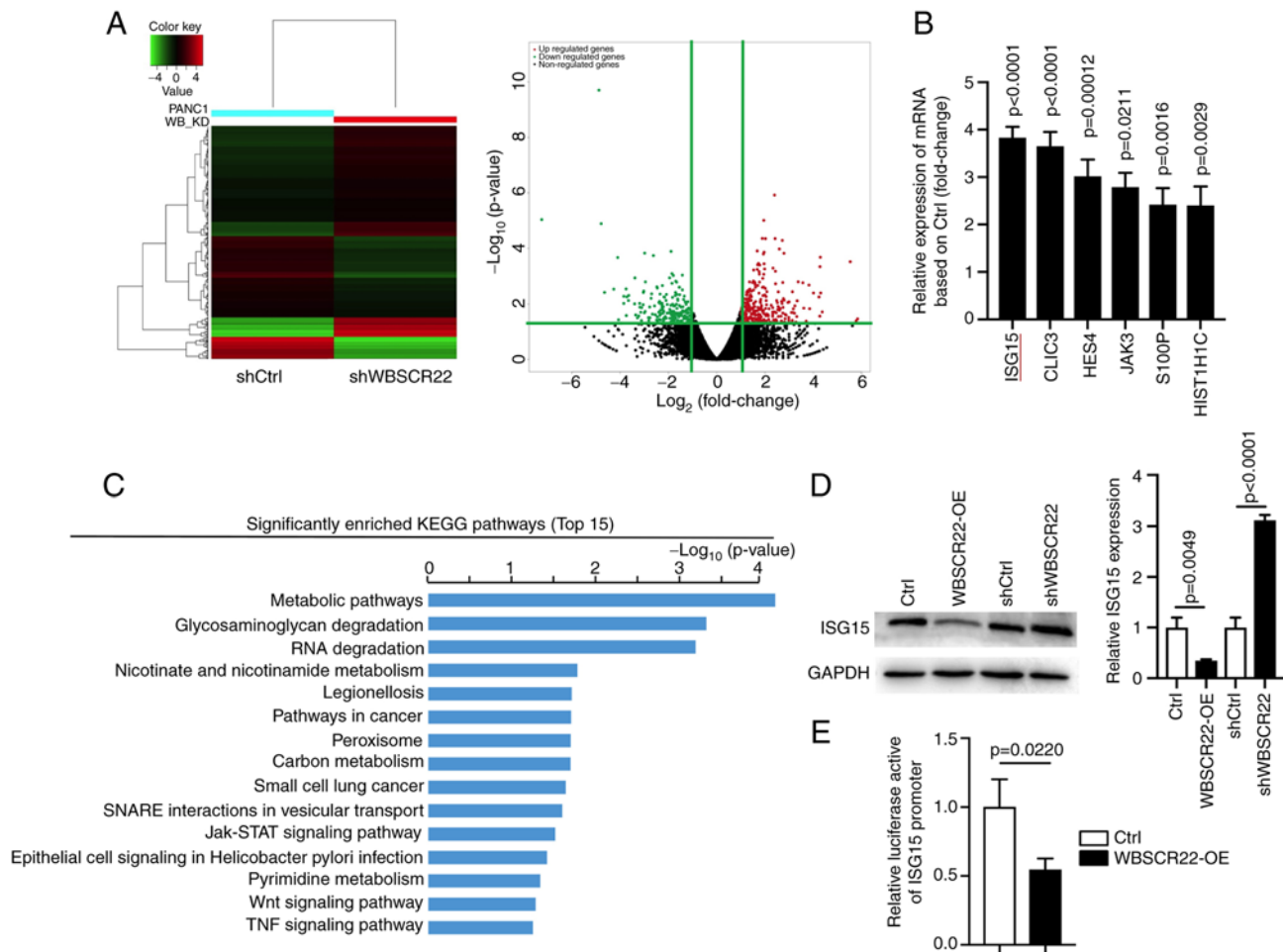


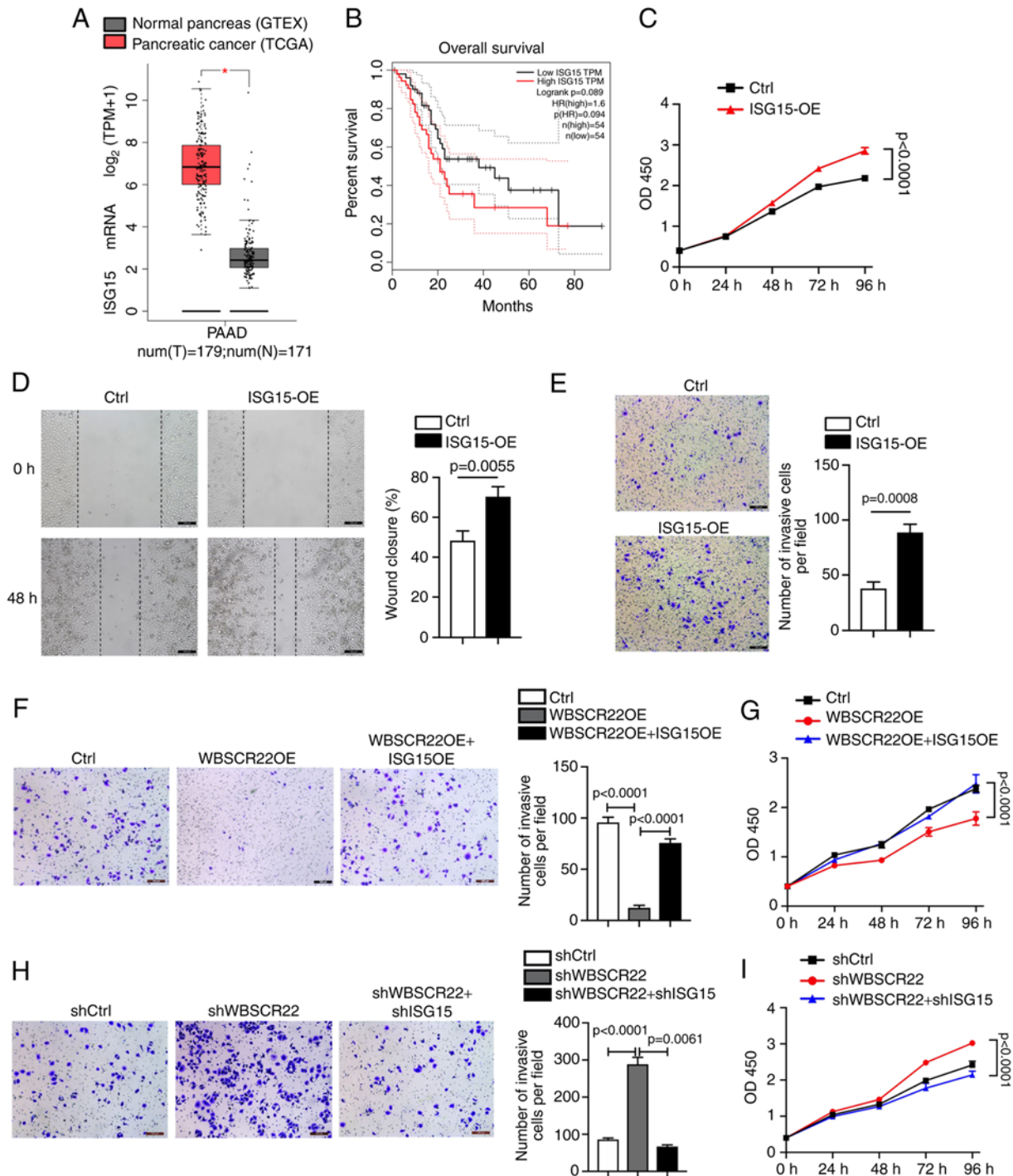
Figure 2. WBSR22 regulates the downstream expression of ISG15. (A) Hierarchical clustering plots and volcano plots were used to identify the DEGs (fold change >2,  $P < 0.05$ ) between wild-type and WBSR22-knockdown PANC-1 cells. (B) Relative mRNA expression levels of genes in WBSR22-knockdown PANC-1 cells as determined by RNA-seq. (C) KEGG pathway enrichment analysis of DEGs in WBSR22-knockdown PANC-1 cells. The graphs indicating the top 15 signaling pathways were listed based on the log<sub>10</sub> (P-value). (D) Protein and mRNA levels of ISG15 in WBSR22-overexpressing, shWBSR22 and control cells, respectively. (E) A luciferase reporter assay was performed to investigate the possible effect of WBSR22-OE on the transcriptional activity of ISG15 in PANC-1 cells. The data indicated the relative ratio of firefly luciferase activity and *Renilla* luciferase activity. The data are presented as the mean value  $\pm$  standard deviation;  $n=3$  biologically independent repeats. The data in B, D and E were analyzed using a two-tailed, unpaired t-test. P-values are indicated. WBSR22, Williams-Beuren syndrome chromosomal region 22; ISG15, interferon-stimulated gene 15; DEGs, differentially expressed genes; RNA-seq, RNA sequencing; KEGG, Kyoto Encyclopedia of Genes and Genomes; OE, overexpression.

hypothesis, the ability of WBSR22 to suppress PC progression was investigated using PANC-1 cells transfected with WBSR22-OE alone or co-transfected with WBSR22-OE and TRMT112-OE. As expected, the tumor suppressive capacity of WBSR22 in PC was significantly enhanced when TRMT112 was concurrently overexpressed with WBSR22. The synergistic ectopic OE of WBSR22-OE + TRMT112-OE significantly decreased cellular proliferation and colony formation of PANC-1 cells compared with that noted in WBSR22-OE alone and in the corresponding control cells (Fig. 4A and B). Wound healing and Transwell assays further confirmed that WBSR22-OE + TRMT112-OE suppressed the cellular migration and invasive capacities of PANC-1 cells compared with those of WBSR22-OE alone and the corresponding controls (Fig. 4C and D). Furthermore, as aforementioned, the tumor suppressive role of WBSR22 in PC was revealed to be associated with its negative regulatory effect on ISG15. It was hypothesized that TRMT112 may also be involved in regulating the expression of ISG15. As expected,

TRMT112 further enhanced the inhibitory effect of WBSR22 on ISG15 expression (Fig. 4E). It was also revealed that the tumor-inhibitory capacity of WBSR22-OE + TRMT112-OE could be partially rescued by ISG15-OE in PC (Fig. S2). Collectively, these results demonstrated that WBSR22 and TRMT112 may function together in PC. Simultaneous targeting of WBSR22 and TRMT112 may represent a novel therapeutic strategy for the treatment of PC (Fig. 4F).

## Discussion

The human WBSR22 protein has been identified as a methyltransferase for 18S rRNA m<sup>7</sup>G and is involved in pre-rRNA processing and 40S ribosome subunit biogenesis. The elevated expression levels and the tumor-promoting potential of WBSR22 have been observed in several types of cancer (10-18). However, the role of WBSR22 in PC remains unknown. In the present study, WBSR22 was revealed for the first time to the best of our knowledge, to act as a tumor



**Figure 3.** ISG15 upregulation promotes PC tumorigenesis. (A) Relative expression levels of ISG15 in PC specimens (n=179) compared with those of adjacent normal non-neoplastic tissues (n=171) according to TCGA database analysis. \* $P<0.05$ . (B) Survival plot indicating the association of patient survival rate (n=89) with ISG15 expression levels. (C) Cell proliferation assay indicating the proliferative potential of ISG15-OE cells compared with that of the control cells. (D) Wound healing and (E) Transwell assays were performed to investigate the migratory and invasive potential of the ISG15-overexpressing cell line relative to its corresponding control in PANC-1 cells. (F) Transwell and (G) proliferation assays indicating that the tumor-inhibitory capacity of WBSCR22 could be markedly rescued by ISG15-OE in PANC-1 cells. (H) Transwell and (I) proliferation assay indicating that the tumor-promoting function induced by WBSCR22 knockdown (shWBSCR22) was significantly abolished by shISG15 in PANC-1 cells. The data are presented as the mean value  $\pm$  standard deviation; n=3 biologically independent repeats. The data in D-F and H were analyzed using a two-tailed, unpaired t-test. The data in C, G, I were analyzed using a two-way ANOVA, followed by Bonferroni corrections. P-values are indicated. ISG15, interferon-stimulated gene 15; PC, pancreatic cancer; OE, over-expression; WBSCR22, Williams-Beuren syndrome chromosomal region 22; sh, small hairpin.

suppressor by attenuating cellular proliferation, migration, invasion and tumorigenesis of PC. The effects mediated by WBSCR22 in PC may involve the downstream regulation of ISG15. In addition, ectopic expression of TRMT112 further

promoted the tumor suppressive potential of WBSCR22 in PC. These results propose a tumor suppressive role of the TRMT112/WBSCR22/ISG15 axis in PC that may represent a novel therapeutic strategy for the treatment of this disease.



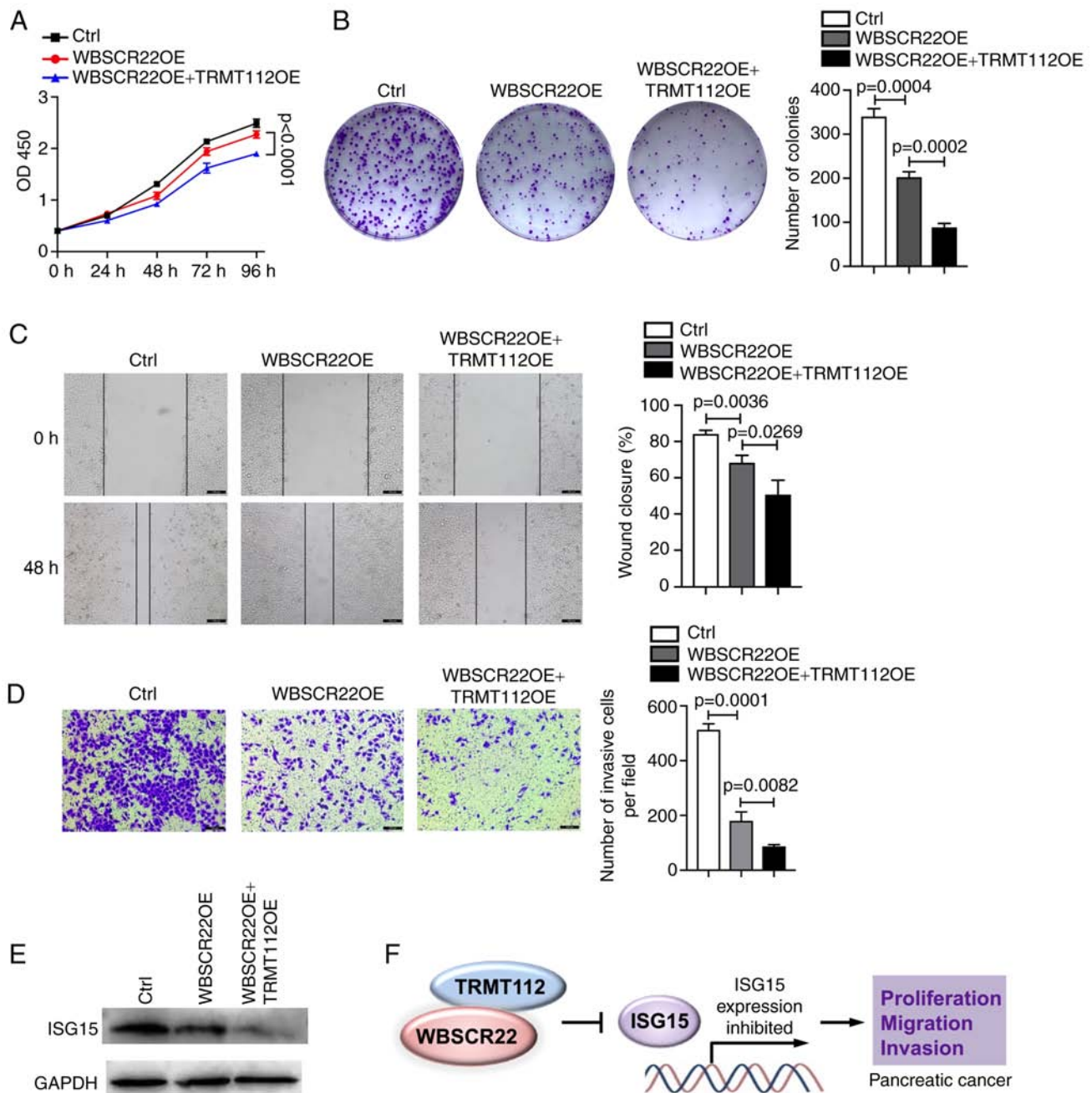


Figure 4. TRMT112 promotes the tumor suppressive function of WBSCR22. (A) Cell proliferation and (B) colony formation assays were performed to determine the proliferative and colony formation activities of PANC-1 cells with WBSCR22 overexpression alone and WBSCR22 + TRMT112 dual overexpression effects compared with those of the corresponding controls. (C) Wound healing and (D) Transwell migration assays were performed to evaluate the migratory and invasive potential of WBSCR22-OE alone and of the WBSCR22 + TRMT112 OE synergistic effect in PANC-1 cells compared with that of the corresponding controls. (E) The protein levels of ISG15 in WBSCR22-OE, WBSCR22-OE + TRMT112-OE and control cells. (F) Schematic summary of the mechanism by which TRMT112 assists WBSCR22 in inhibiting transcription of ISG15, which in turn accelerates development of PC. The data are presented as the mean value  $\pm$  standard deviation;  $n=3$  biologically independent repeats. The data in B-D were analyzed using a two-tailed, unpaired t-test. The data in A were analyzed using a two-way ANOVA, followed by Bonferroni corrections. P-values are indicated. TRMT112, tRNA methyltransferase activator subunit 11-2; WBSCR22, Williams-Beuren syndrome chromosomal region 22; OE, overexpression; ISG15, interferon-stimulated gene 15; PC, pancreatic cancer.

According to RNA-seq using shWBSCR22 and control cells, the metabolic pathway was among the top five enriched KEGG pathways. In addition, the carbon and pyrimidine metabolic pathways and Wnt and Janus kinase-STAT signaling pathways were among the top 15 enriched KEGG pathways. ISG15, a crucial member of the metabolic pathway, plays a vital role in protein turnover, protein stability and ISGylation (27,28). The present study demonstrated that ISG15

was transcriptionally regulated by WBSCR22. Therefore, it was hypothesized that WBSCR22 suppressed the progression and metastasis of PC by regulating ISG15. The oncogenic role of ISG15 and its functional association with WBSCR22 were also confirmed. ISG15 functions as an oncogene in PC cells and its antitumor function in PC is closely related to its negative regulatory effect on ISG15. However, the mechanism by which WBSCR22 regulates ISG15 is not fully known.

In addition, the WBSCR22 protein has been identified as a methyltransferase for 18S rRNA m<sup>7</sup>G involved in pre-rRNA processing and ribosome 40S subunit biogenesis. It would be interesting to investigate whether the regulation of ISG15 by WBSCR22 is associated with its catalytic activity in future studies. In addition, the Human Protein Atlas database indicated that the WBSCR22 protein was localized in the nucleoli and nucleoplasm of cells. Therefore, it is worth investigating whether WBSCR22 exerts a direct transcriptional regulatory role on ISG15 via an interaction with transcription-related proteins.

Numerous studies have established the oncogenic role of WBSCR22 in multiple malignancies. WBSCR22 has been reported as an oncogene in several carcinomas, including invasive breast cancer, multiple myeloma, plasma cell carcinoma, colorectal cancer, lung cancer and hepatocellular carcinoma (13-17). In glioma cells, upregulation of WBSCR22 promotes proliferation, invasion, tumorigenesis and migration, while its knockdown exerts the opposite effects (18). In contrast to these findings, WBSCR22 loss has also been reported in certain neoplastic and inflammatory types of human lung pathologies, which indicates that the role of WBSCR22 in different cancer types is tissue specific (16). It will be interesting to investigate the mechanism and the diverse roles of WBSCR22 in other cancer types.

Aberrant cellular metabolism is the primary effect by which WBSCR22 promotes tumor initiation, progression and cancer cell metastatic dissemination. Cancer cells undergo substantial metabolic rewiring to attain metastatic traits and survive in varying cellular environmental conditions, including oxygen concentration, nutrient availability and extracellular signals (47,48). ISG15 functions as a ubiquitin-like modifier of various nuclear and cytoplasmic proteins by targeting >300 proteins (including p53) through ubiquitination of a cellular metabolic pathway (32). ISG15 primarily targets proteins that play a role in altering cellular metabolic processes (49). However, the biological function of ISG15 is not consistent across different types of cancer. In the present study, the oncogenic role of ISG15 was demonstrated in PC, and that the upregulated ISG15 expression promoted the proliferation, migration, invasion and tumorigenesis of PC. A recent study indicated that ISG15 and ISGylation were essential for maintaining PC stem cell metabolic plasticity and mitophagy (23). An additional study reported that ISG15 was secreted into the tumor microenvironment and that extracellular-free ISG15 played an important role in the maintenance of cancer stem cell-like features of PDACs (50). These data are consistent with our investigation indicating that ISG15 is preferentially upregulated in PC cells in order to promote PC progression. However, the mechanism of ISG15 in PC is still not fully understood. Whether the role of ISG15 in PC depends on the ISGylation of key proteins involved in cellular metabolism and cancer progression or involves completely different regulatory mechanisms is worthy of further investigation.

TRMT112 has been validated as the interaction partner of WBSCR22, and enhances the stability of WBSCR22. The present study examined further whether this interaction could influence the function of WBSCR22 in PC. To test this hypothesis, the ability of WBSCR22 to suppress PC progression was investigated using PANC-1 cells transfected with

WBSCR22-OE alone or co-transfected with WBSCR22-OE and TRMT112-OE. As expected, the tumor suppressive capacity of WBSCR22 in PC was significantly enhanced when TRMT112 was concurrently overexpressed with WBSCR22. A previous study has demonstrated that the stability of WBSCR22 is regulated by interaction with TRMT112 through the ubiquitin-proteasome degradation pathway, resulting in the tight control of WBSCR22 in cells (45). The present study investigated the biological function and significance of the WBSCR22-TRMT112 interaction in the development of PC.

In conclusion, the present study described a novel regulatory network for WBSCR22 in PC. The data verified for the first time that WBSCR22 functions as a tumor suppressor in PC by significantly suppressing cellular proliferation, migration, invasion and tumorigenesis *in vivo* and *in vitro*. In addition, it was confirmed that WBSCR22 regulated the downstream transcriptional activity of ISG15, which acted as an oncogene in PC by promoting the proliferation, migration, invasion and tumorigenesis of PC. Furthermore, the data confirmed that TRMT112 and WBSCR22 functioned cooperatively in PC. Simultaneous ectopic OE of WBSCR22 and TRMT112 further promoted the tumor suppressive potential of WBSCR22 in PC. WBSCR22 is a clinically important gene in PC and the newly identified WBSCR22/ISG15 axis may represent an innovative approach for therapeutic purposes.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

AAK, HH and XL conceived the idea and designed the experiments of the present study. SW, HL, YZ and RP performed the experiments. AAK and XL confirm the authenticity of all the raw data. HH, AAK and XL were involved in writing the manuscript. XL and HH supervised the overall project. All authors read and approved the final version of the manuscript.

## Ethics approval and consent to participate

The present study was approved (approval no. IRB-1507) by the Ethics Committee of Beijing University of Technology (Beijing, China).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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