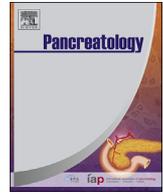




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MicroRNA let-7d targets thrombospondin-1 and inhibits the activation of human pancreatic stellate cells

Hiroyuki Asama^a, Rei Suzuki^{a,*}, Takuto Hikichi^b, Tadayuki Takagi^a, Atsushi Masamune^c, Hiromasa Ohira^a

^a Department of Gastroenterology, Fukushima Medical University School of Medicine, Fukushima, Japan

^b Department of Endoscopy, Fukushima Medical University Hospital, Fukushima, Japan

^c Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Japan



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ABSTRACT

Objectives: The microRNA (miRNA) let-7d is linked to the formation of pancreatic cancer-related fibrosis. In this study, the mechanism by which let-7d regulates the activation of the human pancreatic stellate cell (hPSC) was evaluated.

Methods: The transient transfection of a let-7d mimic in the hPSCs was performed, and the altered thrombospondin 1 (THBS1) expression was confirmed by western blotting and real-time qPCR. Targeting of the 3'-untranslated region (UTR) of *THBS1* by let-7d was investigated by the luciferase assays. After hPSC transfection using *THBS1* siRNA, the fibrosis markers (α -SMA and collagen 1A1) were evaluated by western blotting and real-time qPCR. The correlation between tumor fibrosis and let-7d or THBS1 was estimated using the data from The Cancer Genome Atlas project. Finally, the effects of genistein on the hPSCs were evaluated.

Results: We found that a let-7d mimic inhibits THBS1 expression by targeting its 3'-UTR. THBS1 inhibition by siRNA inhibited hPSC activation. An *in silico* analysis revealed that let-7d and THBS1 expression are negatively correlated. Additionally, let-7d was negatively correlated with the stromal score, while THBS1 was positively correlated with this score. Genistein substantially induced let-7d and decreased the expression of fibrosis marker along with the inhibition of THBS1.

Conclusions: Let-7d inhibited hPSC activation by targeting THBS1. Genistein induced the expression of let-7d and might modulate pancreatic fibrosis.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) involves the desmoplastic reaction, which is characterized by a dense concentration of extracellular matrix proteins, activated pancreatic stellate cells (PSCs), and immune cells surrounding tumor cells [1]. The desmoplastic reaction inhibits drug penetration and uptake in PDAC, which eventually induces drug resistance [2,3]. Understanding the mechanism underlying desmoplastic reaction formation in PDACs, especially the mechanism underlying PSC activation, may facilitate the development of new therapeutic methods to address the issue of drug resistance.

MicroRNAs (miRNAs) are small, noncoding RNAs (18–25 nucleotides in length) that regulate gene expression at the post-transcriptional level by promoting the degradation of mRNAs or by blocking mRNA translation. They have essential roles in various biological processes. In a previous study, we reported that the miRNA let-7d-5p (let-7d) was uniquely dysregulated in the human pancreatic stellate cells (hPSCs) co-cultured with PDAC cell lines. Furthermore, the transient transfection of the hPSCs with a let-7d inhibitor activated the hPSCs and resulted in an increased expression of fibrosis markers (α -SMA and COL1A1) [4]. Additionally, a computational analysis suggested that let-7d targets thrombospondin 1 (THBS1), which is important for the control of latent TGF- β activation in fibrotic diseases, such as diabetic complications and liver fibrosis [5,6]. Considering these results, we speculated that the induction of let-7d might inhibit hPSC activation by targeting THBS1.

In this study, we first clarified whether let-7d targets THBS1

* Corresponding author. Department of Gastroenterology, Fukushima Medical University School of Medicine, 1 Hikarigaoka, Fukushima, 960-1295, Japan.

E-mail address: subaru@fmu.ac.jp (R. Suzuki).

in vitro. We then evaluated the relationship between THBS1 and hPSC activation using *THBS1* siRNA and validated the results by an *in silico* analysis. Finally, the altered let-7d expression by a natural compound, genistein, was confirmed.

Materials and methods

Cell lines and reagents

Two commercially available pancreatic ductal adenocarcinoma (PDAC) cell lines (Panc-1 and BxPC-3) were obtained from the American Type Culture Collection (Manassas, VA, USA). The immortalized human pancreatic stellate cell (hPSC) line, hPSC21-S/T, was established by the introduction of the simian virus 40 T antigen and human telomerase reverse transcriptase into human primary pancreatic stellate cells (PSCs) [7]. The hPSCs expressed typical stellate cell markers, including α -SMA, vimentin, COL1A1, and glial fibrillary acidic protein [8]. The hPSCs were cultured in DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 4.5 g/L glucose, 10% fetal bovine serum, and 1% penicillin-streptomycin. The other cell lines were grown in RPMI-1640 containing the same supplements. All the cell lines were cultured in a humidified atmosphere containing 5% CO₂ at 37 °C and were grown to 70%–80% confluence in 10-cm culture dishes prior to experiments.

Extraction of total RNA

Total RNA was extracted from the cultured cells using the RNeasy Mini Kit (Qiagen, Hilden, NRW, Germany), and total RNA (including microRNAs (miRNAs)) was extracted using the miRNeasy Mini Kit (Qiagen, Hilden, NRW, Germany) according to the manufacturer's protocols. The cDNA was prepared from the 500 ng of total RNA using the iScript™ Advanced cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) and a TaqMan miRNA Reverse Transcription Kit (Thermo Fisher, Waltham, MA, USA) with the primers targeting mir-let-7d-5p and mir-RNU48. The purity of RNA was determined by calculating the A260/A280 ratio. The A260/A280 values for all total RNA samples were >1.8.

Quantitative real-time PCR

One microliter of cDNA was used as a template in a 20 μ L PCR reaction. The PCR products were amplified using specific primers (TaqMan MiRNA Assay) and the TaqMan Universal PCR Master Mix II (Applied Biosystems, Foster City, CA, USA). PCR products were detected using the StepONE Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each sample was run in triplicate. The following TaqMan probes for miRNA detection were obtained from the Applied Biosystems: α -SMA (Hs00426835), COL1A1 (Hs00164004), and GAPDH (Hs02786624) for the cultured cells and miR-let-7d (assay ID: 002283) and miR-RNU48 (assay ID: 001006) for the miRNA analysis. The results were analyzed using the comparative cycle threshold ($\Delta\Delta C_t$) method.

Transfection of hPSCs

For the transient transfection of a miRNA mimic and thrombospondin 1 (THBS1) siRNA, 5×10^5 hPSCs were cultured in 6-well plates for 24 h. A total of 3 μ L of 10 μ M miRNA mimics (mirVana miRNA mimic for mir-let-7d-5p; Applied Biosystems, Foster City, CA, USA) or negative controls were diluted in 150 μ L of serum-free media and then mixed with 9 μ L of Lipofectamine 2000 (Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in 150 μ L of serum-free media for 5 min at room temperature. A total of 25 μ L or 50 μ L of

20 μ M THBS1 siRNA or negative control siRNA was diluted in 250 μ L of serum-free media for 15 min. These mimic or siRNA/lipid complexes were added to the cells and incubated for 48 h. Real-time PCR with TaqMan probes was performed to validate the miRNA and mRNA profiling results.

Western blot analysis

The cells were washed twice with PBS and scraped from the plates in RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing a Protease Inhibitor Cocktail (Thermo Fisher, Waltham, MA, USA). The proteins were electrophoresed in sample buffer on acrylamide gels and then transferred to polyvinylidene difluoride membranes (Clear Blot Membrane-P; ATTO Co., Ltd., Tokyo, Japan). The membranes were blocked with 0.5% TBST containing 3% non-fat milk, incubated with the primary antibodies (1:1000) overnight at 4 °C, and then incubated with horseradish peroxidase-conjugated anti-rabbit antibodies (1:3000; Sigma-Aldrich, St. Louis, MO, USA). The blots were visualized using the ECL Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK). β -Actin was used as a loading control. The antibodies against THBS1, α -SMA, COL1A1, and β -actin were purchased from Cell Signaling Technology (Boston, MA, USA). The analysis was repeated three times, and the representative images are shown. Densitometry analysis was conducted using ImageJ from NIH. The protein levels reported in the figures were obtained as a ratio between the band intensity for the protein of interest and the band intensity of β -actin, used as a loading control. Data were shown presented as mean \pm standard error of the mean (SEM).

MTT cell proliferation assay and viability staining

The hPSCs were seeded at a density of 3000 cells in 200 μ L media with 10% FBS in a 96-well plate. The cells in each well were treated with either 0.5 μ L let-7d mimic (10 μ M) or negative control, and 0.8 μ L THBS1 siRNA (20 μ M) or negative control for 48 h. After treatment, MTT (5 mg/mL) was added to each well, and the optical density of each well was measured at 570 nm by using a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). For the viability staining, a LIVE/DEAD® Cell Imaging Kit (488/570, Thermo Fisher, Waltham, MA, USA) was used to assess the cell viability according to the manufacturer's protocol. After 48 h incubation, culture media was removed from the wells and replaced by PBS. Then, the cells were incubated with dead/live imaging kit for 15 min. Fluorescence images were obtained using a fluorescence microscope (model BZ-9000; Keyence, Osaka, Japan). The recorded images of the cells (live cell: green, dead cell: red) were analyzed with an imaging process software ImageJ. Images were taken in three different parts randomly at magnification of 100 \times . Percent of dead cell counts (red) to live cell counts (green) were calculated and data were shown presented as mean \pm SEM.

Luciferase 3'-UTR reporter assay in hPSCs

The hPSCs were plated in 6-well plates (5×10^5 cells/well) and transiently transfected with 50 ng of THBS1 3'-untranslated region (UTR) or negative control vector (cat. no. HmiT018068-MT06 and cat. no. CmiT000001-MT06; GeneCopoeia Inc., Rockville, MD, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. At the same time, the let-7d mimic or negative control was co-transfected with the reporter vector at a final concentration of 50 nM. The cells were harvested at 48 h after transfection using the reporter lysis buffer (GeneCopoeia Inc., Rockville, MD, USA). Firefly and *Renilla* luciferase activities were analyzed at room temperature using a Glomax 20/20 luminometer

(Promega, Madison, WI, USA) and the Luc-Pair™ Duo-Luciferase Assay Kit 2.0 (GeneCopoeia Inc., Rockville, MD, USA). The relative luciferase activity was defined as the mean value of the Firefly/*Renilla* normalized ratio [9].

Analysis of RNA-Seq and miRNA-Seq data

The mRNA-Seq data for the pancreatic tissues from 178 patients and miRNA-Seq data for the 183 patients were obtained from TCGA (<https://tcga-data.nci.nih.gov/tcga/>). Both mRNA and miRNA data were available for 172 patients. Normalized mRNA and miRNA expression data (the calculated expression for all reads aligning to a particular mRNA per sample) were collected from TCGA Data Portal using the Subio platform version 1.21 (Kagoshima, Japan). The stromal scores were estimated utilizing the DNA copy number-based tumor purity across the samples from 25 different tumor types, profiled on Agilent, Affymetrix platforms, or based on RNA sequencing and available in TCGA [10]. Data were accessed through the MD Anderson Bioinformatics Portal on May 1, 2018 (<http://bioinformatics.mdanderson.org/estimate/>). Briefly, Yoshihara et al. firstly selected 1147 up-regulated genes in the stromal of various tumors (e.g., ovarian cancer) and further picked up 173 genes which are commonly up-regulated in the other tumors. Finally, 141 genes were extracted. For a given sample, gene expression values were rank-normalized and rank-ordered. The empirical cumulative

distribution functions of the signature genes and the remaining genes were calculated. A value of statistical significance was calculated by integrating the difference between the empirical cumulative distribution function, which is similar to the one used in GSEA but is based on absolute expression rather than differential expression.

cDNA microarray

To determine how THBS1 deficiency altered fibrosis-related cell signaling pathway, we conducted the cDNA microarray between the hPSCs treated with THBS1 siRNA and the cells treated with siRNA negative control. A total of 50 μL of 20 μM THBS1 siRNA or negative control siRNA was diluted in 250 μL of serum-free media for 15 min. This siRNA or negative control/lipid complexes were added to the cells and incubated for 48 h. After extracting total RNA, the cDNA was created. Differences in the transcript levels of fibrosis-related genes were analyzed using an RT2 Profiler PCR Array (PAHS-120Z; SABiosciences, Frederick, MD, USA). Quantitative PCR was performed with the StepOne Plus (Applied Biosystems, Frederick, MD, USA) using RT2 Real-Time SYBR Green PCR Master Mix according to the manufacturer's protocol. We chose glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control and hPSC treated with siRNA negative control as a control group.

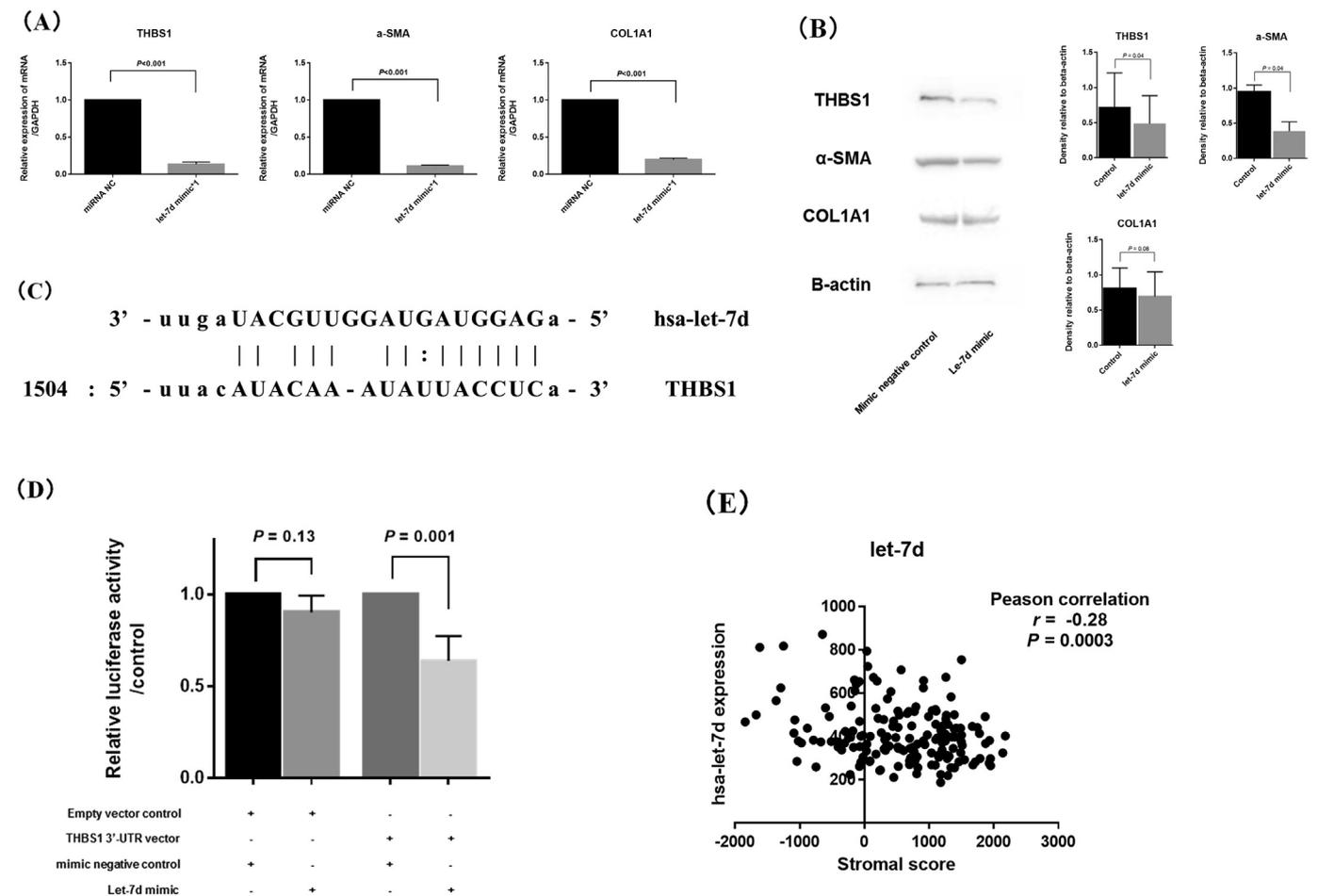


Fig. 1. The relationship between let-7d and human pancreatic stellate cell (hPSC) activation. (A) (B) Transient transfection of a let-7d mimic decreased the expression of THBS1 and fibrotic markers. (C) (D) A luciferase assay revealed that let-7d directly modulated THBS1 expression by binding to the 3'-UTR of THBS1 mRNA. (E) (F) An *in silico* analysis using TCGA revealed that let-7d expression was negatively correlated with both the stromal score and THBS1 expression ($r = -0.25$, $P = 0.0003$ and $r = -0.15$, $P = 0.04$, respectively).

Statistical analysis

All the results are presented as raw data or as means \pm SD. All statistical analyses were implemented in GraphPad Prism 7.0 (GraphPad, San Diego, CA, USA) using the unpaired Student's *t*-test (two-tailed). $P < 0.05$ was considered statistically significant. Correlations between miRNA, mRNA, and stromal score were evaluated using the Pearson's correlation analysis.

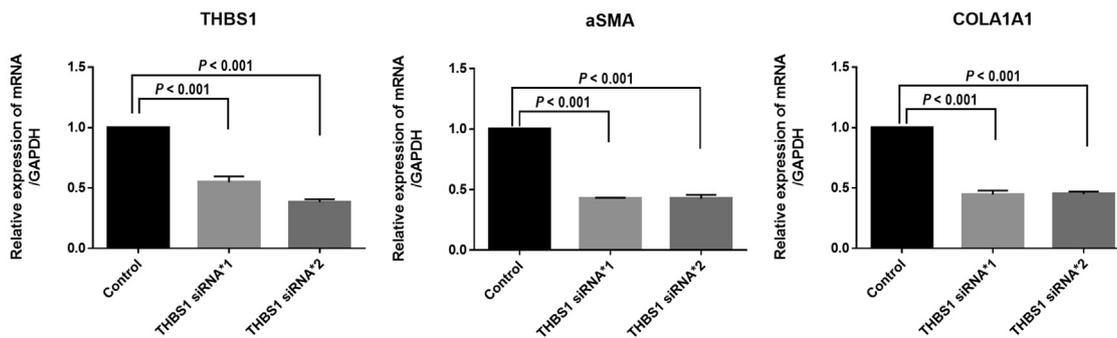
Results

Let-7d targeted THBS1 and decreased the expression of THBS1 as well as other fibrotic markers in vitro

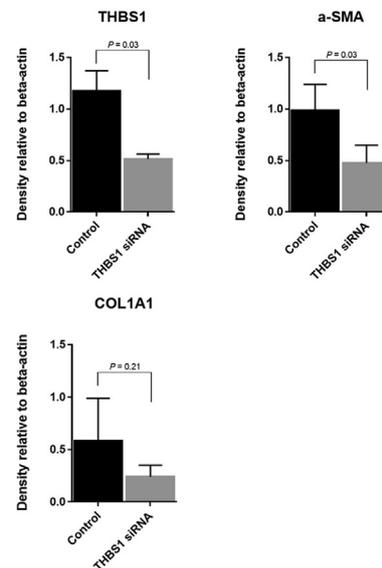
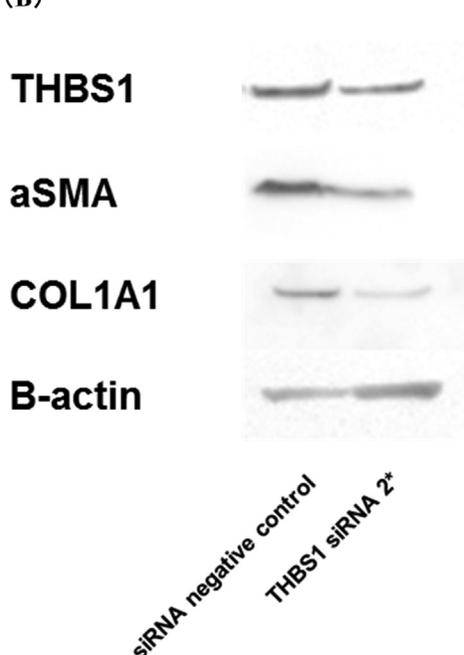
As we reported previously, thrombospondin 1 (THBS1) is a predicted target of let-7d. To clarify the relationship between let-7d and THBS1 expression, we performed the transient transfection of a let-7d mimic in the human pancreatic stellate cells (hPSCs). The results showed that let-7d significantly decreased the expression of

THBS1 mRNA ($P < 0.001$) along with other fibrotic markers, such as α -SMA and COL1A1 ($P < 0.001$ and $P < 0.001$, respectively) (Fig. 1A). Similarly, the western blot analysis showed the decreased expression of THBS1, α -SMA, and COL1A1 (Fig. 1B). Densitometry analysis also showed a significant difference in the expression of THBS1, α -SMA ($P = 0.04$ and $P = 0.04$, respectively), but not in COL1A1 ($P = 0.08$). The computational prediction of miRNA targets (www.microrna.org) revealed a potential target site of let-7d in the 3'-untranslated region (UTR) of THBS1 (Fig. 1C). To further verify whether THBS1 was a direct target of let-7d, we constructed a THBS1 plasmid containing the 3'-UTR of THBS1 mRNA and conducted a reporter gene assay. As shown in Fig. 1D, the induction of let-7d by the miRNA mimic led to a remarkable decrease in the luciferase activity in pEZXT-THBS1, whereas no change in the luciferase activity was found in the negative control-transfected cells. These findings indicated that let-7d directly modulated the THBS1 expression by binding to its 3'-UTR. Additionally, an *in silico* analysis using TCGA revealed that let-7d expression was negatively correlated with both the stromal score and THBS1 expression

(A)



(B)



(C)

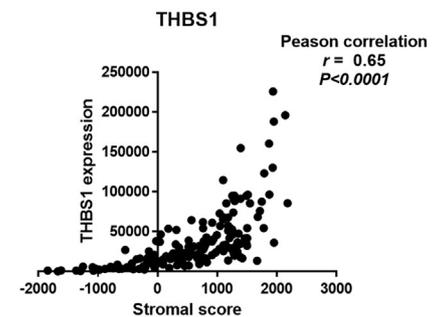


Fig. 2. Engagement of THBS1 in the hPSC activation. (A) (B) Transient transfection of THBS1 siRNA in the hPSCs indicated a direct relationship between THBS1 expression and the hPSC activation. (C) The stromal score showed a significant correlation with the THBS1 expression ($r = 0.65$, $P < 0.001$).

($r = -0.25$, $P = 0.0003$ and $r = -0.15$, $P = 0.04$, respectively), further supporting the results of the *in vitro* experiments (Fig. 1E and F).

THBS1 expression was directly correlated with the activation of hPSCs

After the transient transfection of *THBS1* siRNA in the hPSCs, we investigated the direct relationship between the *THBS1* expression and hPSC activation. *THBS1* siRNA successfully decreased the expression of *THBS1* in a dose-dependent manner (Fig. 2A and B). Additionally, *THBS1* siRNA also decreased the expression of fibrotic markers, i.e., α -SMA and COL1A1. Densitometry analysis also

showed a significant difference in the expression of *THBS1*, α -SMA ($P = 0.03$ and $P = 0.03$, respectively), but not in COL1A1 ($P = 0.21$). As shown in Fig. 2C, the stromal score showed a significant correlation with *THBS1* expression ($r = 0.65$, $P < 0.001$). Therefore, our results demonstrated that *THBS1* mediated the effect of let-7d on hPSC activation.

Let-7d/THBS1 axis controlled the cell proliferation and viability by modulating the TGF- β pathway

We conducted the cell proliferation and viability assay to clarify how let-7d and *THBS1* affect cell proliferation and viability. Both

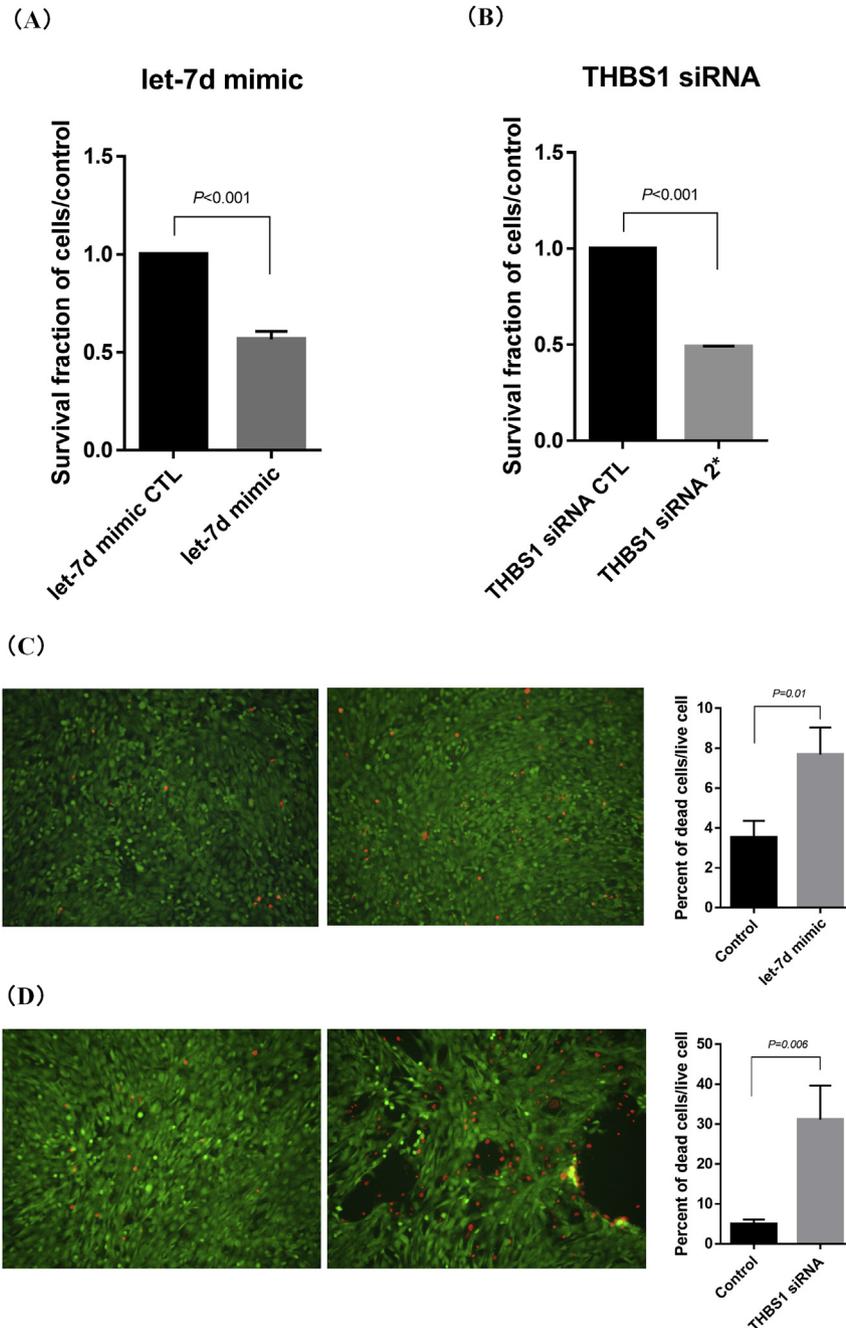


Fig. 3. Let-7d/THBS-1 axis inhibited the cell proliferation and viability. (A) (B) Let-7d and THBS1 siRNA inhibited the cell proliferation compared to the control (100% vs. 56%, $P < 0.0001$ and 100% vs. 48%, $P < 0.0001$, respectively). (C) (D) Let-7d and THBS1 siRNA induced the cell death compared to the control (3.53% vs. 7.68%, $P = 0.01$ and 4.89% vs. 31.1%, $P = 0.006$, respectively).

let-7d mimic and THBS1 siRNA inhibited the cell proliferation significantly compared to the controls (100% vs. 56%, $P < 0.0001$ and 100% vs. 48%, $P < 0.0001$, respectively) (Fig. 3A and B). Additionally, the cell viability using fluorescent microscopy revealed an increased ratio of dead cells to live cells in the cells treated with either let-7c mimic or THBS1 siRNA compared to the controls (3.53% vs. 7.68%, $P = 0.01$ and 4.89% vs. 31.1%, $P = 0.006$, respectively) (Fig. 3C and D). To further elucidate the molecular mechanism of these results, we conducted microarray targeting 84 fibrosis-related genes in THBS1 siRNA-treated hPSC. The result showed that the down-regulation of THBS1 altered various genes in the TGF- β pathway (Table 1).

Genistein induced let-7d expression and inhibited the activation of hPSCs by down-regulating THBS1

Our results suggested the potential application of let-7d for the regulation of hPSC activation by targeting THBS1. However, microRNAs (miRNAs) could be easily degenerated in human body fluid and might be difficult to utilize without chemical modification. Genistein, a soy-derived isoflavone, exhibits multiple biological effects against various cancers based on *in vitro* and *in vivo* models [22–26]. It also induces the expression of miRNAs, including those in the let-7 family, in some malignancies. To verify whether genistein could induce let-7d expression in the hPSCs,

Table 1

Alteration of 57 fibrosis-related gene expression (>2-fold change) in THBS1-treated cells compared to the control cells.

Gene Symbol	Description	Fold Up- or Down- Regulation (THBS1 siRNA/Control)
VEGFA	Vascular endothelial growth factor A	-127.28
IL1B	Interleukin 1, beta	-125.07
TGFB1	Transforming growth factor, beta receptor 1	-64.01
TGFB2	Transforming growth factor, beta receptor II (70/80 kDa)	-33.63
INHBE	Inhibin, beta E	-32.66
SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	-16.45
AGT	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	-16.09
TGFB1	Transforming growth factor, beta 1	-16.06
AKT1	V-akt murine thymoma viral oncogene homolog 1	-15.96
IL13RA2	Interleukin 13 receptor, alpha 2	-15.73
TGFB2	Transforming growth factor, beta 2	-8.25
THBS1	Thrombospondin 1	-8.2
COL3A1	Collagen, type III, alpha 1	-8.05
COL1A2	Collagen, type I, alpha 2	-7.99
CCL11	Chemokine (C-C motif) ligand 11	-7.89
SMAD3	SMAD family member 3	-4.09
IL10	Interleukin 10	-4.04
SMAD4	SMAD family member 4	-4.04
BCL2	B-cell CLL/lymphoma 2	-3.99
CAV1	Caveolin 1, caveolae protein, 22 kDa	-3.97
MMP13	Matrix metalloproteinase 13 (collagenase 3)	-3.95
IL13	Interleukin 13	-3.94
SMAD7	SMAD family member 7	-3.94
TGIF1	TGFB-induced factor homeobox 1	-3.94
TGFB3	Transforming growth factor, beta 3	-3.89
SMAD6	SMAD family member 6	-2.08
ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	-2.05
ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	-2.05
ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	-2.05
CTGF	Connective tissue growth factor	-2.04
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	-2.03
CCR2	Chemokine (C-C motif) receptor 2	-2.01
HGF	Hepatocyte growth factor (hepatoietin A; scatter factor)	1.98
ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	2.01
IL1A	Interleukin 1, alpha	2.02
SERPINH1	Serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	2.02
ILK	Integrin-linked kinase	2.03
MMP3	Matrix metalloproteinase 3 (stromelysin 1, progelatinase)	2.03
SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	2.03
STAT6	Signal transducer and activator of transcription 6, interleukin-4 induced	2.03
MMP8	Matrix metalloproteinase 8 (neutrophil collagenase)	3.81
MMP14	Matrix metalloproteinase 14 (membrane-inserted)	3.85
PDGFA	Platelet-derived growth factor alpha polypeptide	3.85
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	3.89
TIMP3	TIMP metalloproteinase inhibitor 3	3.95
GREM1	Gremlin 1	3.99
JUN	Jun proto-oncogene	4.04
ITGB5	Integrin, beta 5	4.05
LOX	Lysyl oxidase	4.05
TIMP2	TIMP metalloproteinase inhibitor 2	4.13
SP1	Sp1 transcription factor	7.95
LTBP1	Latent transforming growth factor beta binding protein 1	8.07
PLG	Plasminogen	15.8
STAT1	Signal transducer and activator of transcription 1, 91 kDa	16.03
MMP1	Matrix metalloproteinase 1 (interstitial collagenase)	16.13
TIMP4	TIMP metalloproteinase inhibitor 4	32.08
TNF	Tumor necrosis factor	63.58

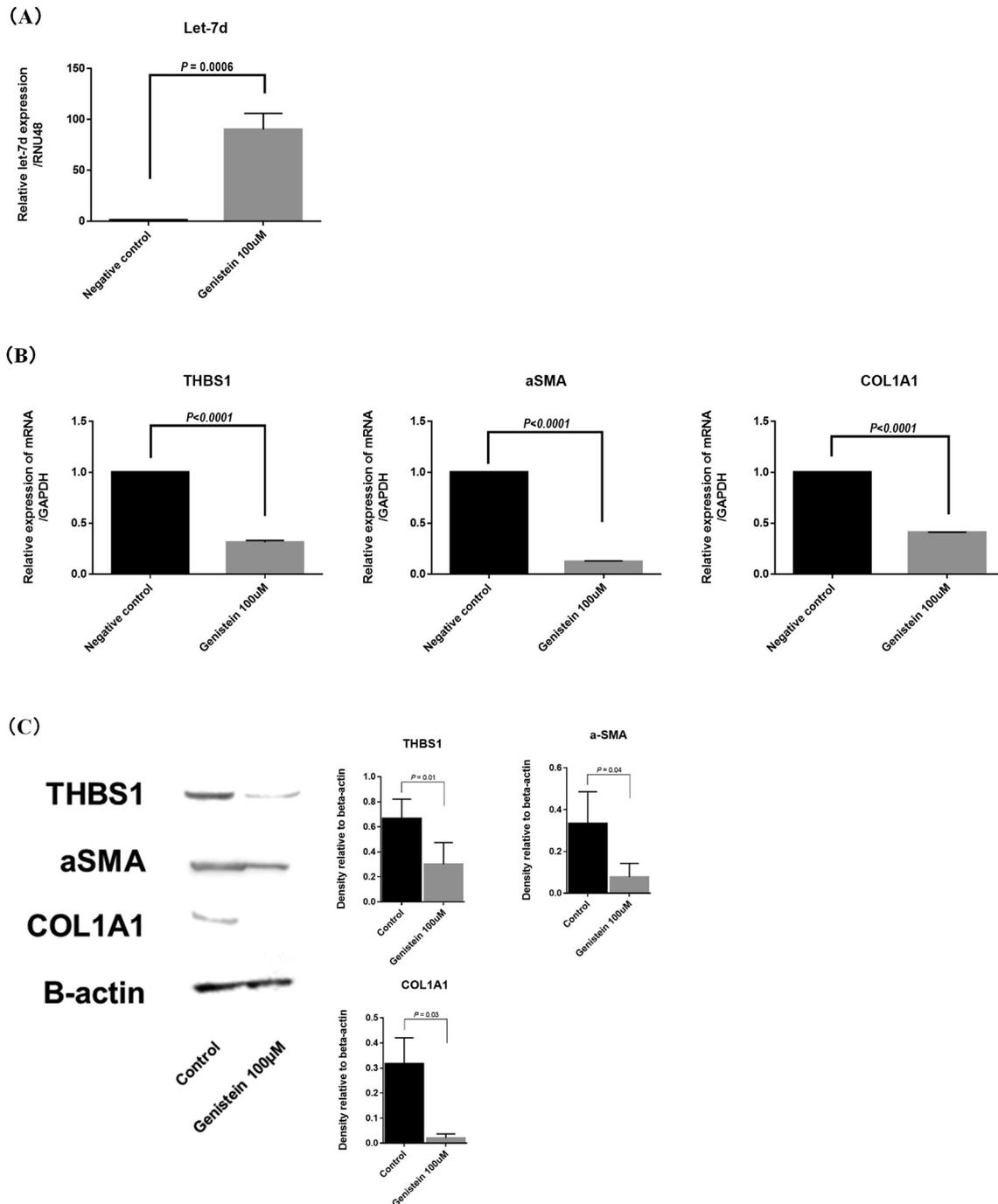


Fig. 4. Induction of let-7d by genistein. (A) Genistein induced let-7d expression and (B and C) subsequently inhibited the expression of THBS1, α -SMA, and COL1A1.

we added 100 μ M genistein to the hPSCs harvested in 6-well plates (5×10^5 cell/well), followed by the incubation for 48 h. As shown in Fig. 4A, let-7d expression in genistein-treated hPSCs was approximately 100-fold higher than that in the hPSCs without genistein treatment. This drastic induction of let-7d subsequently inhibited the expression of THBS1, α -SMA, and COL1A1 (Fig. 4B and C). Densitometry analysis also showed a significant difference in the expression of THBS1, α -SMA, and COL1A1 ($P = 0.01$, $P = 0.04$, and $P = 0.03$, respectively). These results suggested that genistein-induced let-7d up-regulation could be utilized, instead of the let-7d mimic, to inhibit the hPSC activation by down-regulating THBS1.

Discussion

We found that let-7d inhibited the activation of human pancreatic stellate cells (hPSCs) by targeting thrombospondin 1 (THBS1) and downstream TGF- β pathway. Additionally, our results also revealed that a natural soy-derived compound, genistein, could induce let-7d. To the best of our knowledge, this study provides the first evidence for the potential application of let-7d induction to modulate pancreatic fibrosis.

The Let-7 family has known functions in tumor suppression in various cancers [11–14]. In the pancreatic ductal adenocarcinomas (PDACs), let-7d promotes tumor cell proliferation, migration, and

chemo-agent resistance [15–17]. With respect to fibrosis, the role of the let-7 family in cancers is unclear; only a few studies have reported a relationship between the let-7 family and fibrosis in the kidney and lung [18–20]. In our previous study, let-7d was predicted to target various genes, including 18 genes related to the TGF- β pathway. Among these genes, only *THBS1* showed a significant negative relationship with let-7d based on RNA-Seq and miRNA-Seq analyses using TCGA [4]. Consistent with our expectations, a let-7d mimic targeted the 3'-untranslated region (UTR) of *THBS1* mRNA and decreased the relative luciferase activity compared with the control vector or negative mimic control. Furthermore, both the let-7d mimic and *THBS1* siRNA itself could deactivate the hPSCs. These results suggested that targeted therapy against the let-7d/*THBS1* axis might contribute to the inhibition of PDAC-related fibrosis. Alternatively, silencing targeted miRNAs or mRNAs is challenging, since the stability and efficacy of miRNA mimics and siRNAs are often inconsistent [21].

Natural compounds from dietary sources often have multi-functional effects and are the first agents to be tested against a novel therapeutic target. We selected genistein, a soy-derived isoflavone, in this study, since it exhibits multiple biological effects against various cancers using *in vitro* and *in vivo* models [22–26]. Several studies have shown that genistein can potentiate the anti-tumor effects of chemotherapeutic agents (e.g., gemcitabine, 5-FU, and cisplatin) by modulating the vital cell signaling pathways [24,26,27]. Moreover, a recent study has shown that isoflavone mixtures containing genistein could increase the expression of miRNAs [17]. In the current study, we found that let-7d expression in genistein-treated hPSCs was approximately 100-fold higher than that in the hPSCs without genistein treatment. As predicted, the expression of *THBS1* and fibrotic markers decreased in the genistein-treated hPSCs.

In summary, let-7d inhibited the activation of hPSCs by targeting *THBS1*. Genistein could induce the expression of let-7d and might modulate pancreatic fibrosis. Further studies are needed to investigate whether targeted therapy against let-7d/*THBS1* can inhibit the formation of cancer-related fibrosis and eventually ameliorate drug resistance.

Conflict of interest and source of funding

None declared.

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References

- [1] Erkan M, Adler G, Apte MV, et al. StellaTUM: current consensus and discussion on pancreatic stellate cell research. *Gut* 2012;61:172–8.
- [2] Apte MV, Park S, Phillips PA, et al. Desmoplastic reaction in pancreatic cancer: role of pancreatic stellate cells. *Pancreas* 2004;29:179–87.
- [3] Walter K, Omura N, Hong SM, et al. Overexpression of smoothed activates the sonic hedgehog signaling pathway in pancreatic cancer-associated fibroblasts. *Clin Canc Res* 2010;16:1781–9.
- [4] Suzuki R, Asama H, Waragai Y, et al. Fibrosis-related miRNAs as serum biomarkers for pancreatic ductal adenocarcinoma. *Oncotarget* 2018;9:4451–60.
- [5] Sun D, Ma Y, Han H, et al. Thrombospondin-1 short hairpin RNA suppresses tubulointerstitial fibrosis in the kidney of ureteral obstruction by ameliorating peritubular capillary injury. *Kidney Blood Press Res* 2012;35:35–47.
- [6] Murphy-Ullrich JE, Suto MJ. Thrombospondin-1 regulation of latent TGF-beta activation: a therapeutic target for fibrotic disease. *Matrix Biol* August 2018;68–69:28–43.
- [7] Hamada S, Masamune A, Takikawa T, et al. Pancreatic stellate cells enhance stem cell-like phenotypes in pancreatic cancer cells. *Biochem Biophys Res Commun* 2012;421:349–54.
- [8] Hamada S, Masamune A, Yoshida N, et al. IL-6/STAT3 plays a regulatory role in the interaction between pancreatic stellate cells and cancer cells. *Dig Dis Sci* 2016;61:1561–71.
- [9] Kikulska A, Rausch T, Krzywinska E, et al. Coordinated expression and genetic polymorphisms in Grainyhead-like genes in human non-melanoma skin cancers. *BMC Canc* 2018;18:23.
- [10] Yoshihara K, Shahmoradgoli M, Martinez E, et al. Inferring tumour purity and stromal and immune cell admixture from expression data. *Nat Commun* 2013;4:2612.
- [11] Yu F, Yao H, Zhu P, et al. let-7 regulates self renewal and tumorigenicity of breast cancer cells. *Cell* 2007;131:1109–23.
- [12] Ohshima K, Inoue K, Fujiwara A, et al. Let-7 microRNA family is selectively secreted into the extracellular environment via exosomes in a metastatic gastric cancer cell line. *PLoS One* 2010;5, e13247.
- [13] Esqueda-Kerscher A, Trang P, Wiggins JF, et al. The let-7 microRNA reduces tumor growth in mouse models of lung cancer. *Cell Cycle* 2008;7:759–64.
- [14] Boyerinas B, Park SM, Murmann AE, et al. Let-7 modulates acquired resistance of ovarian cancer to Taxanes via IMP-1-mediated stabilization of multidrug resistance 1. *Int J Canc* 2012;130:1787–97.
- [15] Torrisani J, Bournet B, du Rieu MC, et al. let-7 MicroRNA transfer in pancreatic cancer-derived cells inhibits *in vitro* cell proliferation but fails to alter tumor progression. *Hum Gene Ther* 2009;20:831–44.
- [16] Patel K, Kollory A, Takashima A, et al. MicroRNA let-7 downregulates STAT3 phosphorylation in pancreatic cancer cells by increasing SOCS3 expression. *Cancer Lett* 2014;347:54–64.
- [17] Li Y, VandenBoom 2nd TG, Kong D, et al. Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells. *Cancer Res* 2009;69:6704–12.
- [18] Brennan EP, Nolan KA, Borgeson E, et al. Lipoxins attenuate renal fibrosis by inducing let-7c and suppressing TGFbetaR1. *J Am Soc Nephrol* Mar 2013;24:627–37.
- [19] Wang B, Jha JC, Hagiwara S, et al. Transforming growth factor-beta1-mediated renal fibrosis is dependent on the regulation of transforming growth factor receptor 1 expression by let-7b. *Kidney Int* 2014;85:352–61.
- [20] Pandit KV, Corcoran D, Yousef H, et al. Inhibition and role of let-7d in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2010;182:220–9.
- [21] Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat Rev Drug Discov* 2017;16:203–22.
- [22] Hwang KA, Park MA, Kang NH, et al. Anticancer effect of genistein on BG-1 ovarian cancer growth induced by 17 beta-estradiol or bisphenol A via the suppression of the crosstalk between estrogen receptor alpha and insulin-like growth factor-1 receptor signaling pathways. *Toxicol Appl Pharmacol* 2013;272:637–46.
- [23] Hwang KA, Kang NH, Yi BR, et al. Genistein, a soy phytoestrogen, prevents the growth of BG-1 ovarian cancer cells induced by 17beta-estradiol or bisphenol A via the inhibition of cell cycle progression. *Int J Oncol* 2013;42:733–40.
- [24] Banerjee S, Zhang Y, Wang Z, et al. *In vitro* and *in vivo* molecular evidence of genistein action in augmenting the efficacy of cisplatin in pancreatic cancer. *Int J Canc* 2007;120:906–17.
- [25] Ouyang G, Yao L, Ruan K, et al. Genistein induces G2/M cell cycle arrest and apoptosis of human ovarian cancer cells via activation of DNA damage checkpoint pathways. *Cell Biol Int* 2009;33:1237–44.
- [26] Banerjee S, Zhang Y, Ali S, et al. Molecular evidence for increased antitumor activity of gemcitabine by genistein *in vitro* and *in vivo* using an orthotopic model of pancreatic cancer. *Cancer Res* 2005;65:9064–72.
- [27] Suzuki R, Kang Y, Li X, et al. Genistein potentiates the antitumor effect of 5-Fluorouracil by inducing apoptosis and autophagy in human pancreatic cancer cells. *Anticancer Res* 2014;34:4685–92.

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