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ORIGINAL ARTICLE

Desumoylating Isopeptidase 2 (DESI2) Inhibits Proliferation and Promotes Apoptosis of Pancreatic Cancer Cells through Regulating PI3K/AKT/mTOR Signaling Pathway

Xi Ou[1] · Guang-tao Zhang[1] · Zhe Xu[2] · Jing-sen Chen[3] · Yong Xie[1] · Ji-Kui Liu[1] · Xiao-Ping Liu[1]

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Abstract

This study aimed to investigate the effects of desumoylating isopeptidase 2 (DESI2) on tumor cell proliferation, apoptosis and invasion of pancreatic cancer, and to assess the signaling pathway involved. Overexpression and silence of DESI2 were designed and the experiments were divided into 5 groups: a normal control group, an interference control group (shRNA-NC); an interference group (sh-DESI2); an overexpression control group (NC), an overexpression group (DESI2). Quantitative real time polymerase chain reaction (qRT-PCR) was used to screen the appropriate interference sequence. The silencing and overexpression of DESI2 were confirmed by qRT-PCR and western blotting. Cell cycling, apoptosis, invasion, and the expression of phosphatidylinositol-3-kinase (PI3K)-protein kinase B (AKT)-mammalian target of rapamycin (mTOR) pathway and caspase 3 were measured. Overexpression and silence of DESI2 were successfully designed in two pancreatic cancer cells, and the interference effect of sh-DESI2–3 showed the best silencing effects. The biological activities of DESI2 were detected in both ASPC-1 and PANCE-1 cells. Our results showed that cell proliferation was significantly increased in the sh-DESI2 group, while decreased in DESI2 group compared with the control group in both cell lines. In ASPC-1 cells, the events in G1 phase decreased and in S phase increased obviously in the sh-DESI2 group, compared with control group. An opposite result was found when DESI2 was overexpressed. In PANCE-1 cells, the events in G2 phase were higher in the sh-DESI2 group, while in the DESI2 group was significantly lower than that in control group. In ASPC-1 and PANCE-1 cells, sh-DESI2 group showed decreased apoptosis, increased cell invasion and increased expression of AKT, p-Akt, PI3K, p-PI3K, p-mTOR and mTOR and decreased caspase 3 expression compared with the control group, while overexpression of DESI2 leaded to increased apoptosis, decreased cell invasion and reduced expression of AKT, p-Akt, PI3K, p-PI3K, p-mTOR and mTOR and increased expression of caspase 3. DESI2 regulates the proliferation and apoptosis of pancreatic cancer cells through PI3K/AKT/mTOR signaling pathway.

Keywords Desumoylating isopeptidase 2 · Pancreatic cancer · Phosphatidylinositide 3-kinases · AKT · mTOR

Introduction

Pancreatic cancer is a highly malignant digestive tract tumor. The abnormal activation of a number of oncogenes and the inactivation of tumor suppressor genes are associated with the development of pancreatic cancer and the malignant phenotype. It is difficult to diagnose and treat this type of malignant tumors, about 90% of which are derived from the adenocarcinoma of the ductal epithelium of the adenocarcinoma [1].

Desumoylating isopeptidase 2 (DESI2) gene locates on the long arm of chromosome 1 (1q44), encoding a protein containing 194 amino acid, with a theoretical molecular weight of 21.4 kD and an isoelectric point of 4.86. The protein contains a conserved DUF862 domain [2]. The amino acid sequence of DESI2 is conservative from lower plants to higher animals, suggesting its critical function in biological evolution [3]. Recent studies have shown that DESI2 has a close relationship with the development of a variety of malignant tumors, especially with pancreatic cancer [4].

✉ Ji-Kui Liu
liu89201@sina.com

[1] Department of Hepatopancreatobiliary Surgery, Peking University Shenzhen Hospital, Lian Hua Road 1120, Shenzhen 518036, China

[2] The Third Ward of Liver Disease, The Third People's Hospital of Shenzhen, Shenzhen 518112, China

[3] Breast Department, Shenzhen Maternity and Child Healthcare Hospital, Shenzhen 518028, China

Phosphatidylinositol-3-kinase (PI3K)-protein kinase B (AKT)-mammalian target of rapamycin (mTOR) pathway is one of the classic signaling pathways involved in cell growth, survival, differentiation and neuronal plasticity [5–7]. This signaling pathway plays an important role in the occurrence and development of tumors through inhibition of cell apoptosis; promoting cell cycle; promoting tumor angiogenesis; promoting tumor invasion and metastasis [8]. The activation state of the signaling pathway was related to the progression and prognosis of pancreatic cancer, suggesting the important role of PI3K/AKT/mTOR in the development of pancreatic cancer, and also for the targeted treatment of pancreatic cancer [9].

DESI2 and PI3K/AKT/mTOR are important molecules and signaling pathways in the development of pancreatic cancer, and whether there is a link between them and whether they interact to affect the development of pancreatic cancer has not been reported. Although studies have shown that DESI2 has a potential antitumor activity [9] and has a significant negative correlation with the expression of AKT and mTOR, it is also necessary to further study and explore whether DESI2 inhibits the growth of pancreatic cancer by inhibiting the activity of AKT/mTOR signaling pathway. In this study, we aimed to investigate the biological activities of DESI2 in pancreatic cancer. This study would provide early prediction for the recurrence and metastasis of pancreatic cancer, and even provide a potential target for the treatment of the disease and the new idea of drug development.

Materials and Methods

Cell Lines

PANC-1 and ASPC-1 cell lines were purchased from cell bank of Shanghai Academy of Sciences and cultured in RPMI1640 (KGM31800S-500, KeyGEN BioTECH, Nanjing, China). The culture was supplemented with 10% fetal bovine serum (FBS; SKU: 04–007-1A, BI) and 100 U/ml penicillin-streptomycin (P1400, Solarbio) in 5% CO_2 at 37 °C. The cells at 70% confluence were used in the following experiments. This study was approved by the ethics committee of Peking University Shenzhen Hospital.

The experiments were divided into five groups: a normal control group (control); an interfering control group (shRNA-NC); an interference group (sh-DESI2); an overexpression control group (NC); a DESI2 overexpression group (DESI2).

DESI2 Overexpression and Silence

The sequence of DESI2 gene was searched in NCBI. Enzyme cut site (XhoI/XbaI) was introduced and gene fragment was cloned into pCDNA3.1 vector and to construct DESI2 overexpression vector DESI2-pcDNA3.1, which was then transformed to receptive cells and the extract plasmids were verified by enzyme digestion. According to the sequence coding for amino acids in protein sequence, the shRNA target sequence was designed, the sense chain was introduced into the BamHI enzyme cut site, the antisense chain was introduced into the EcoRI enzyme cut site, the annealing was used to form double chains and integrated into a carrier pGreenpuro to construct the lentivirus recombinant vector sh-DESI2–1, sh-DESI2–2 and sh-DESI2–3. The lenti-viruses encoding sh-DESI2 were transformed to the receptive cells. The sequences of sh-DESI2 were listed in Table 1.

Cell Counting Kit Assay

The cells (3×10^3/mL) were seeded in 96-well plates. After indicated transfections, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay was applied to detect cell proliferation as previously described [10]. The optical density (OD) was determined by Microplate Reader (Bio-Tek, USA) at 550 nm and represented the cell viability.

Cell Cycle Analysis

After the cells were transfected for 48 h, the medium was discarded and the cells were washed by PBS and digested by trypsin and RPMI1640 containing 10% FBS. After that, the cells ($1\sim5 \times 10^5$) were fixed in 70% pre-cooled ethanol for at least 2 h. The cells were stained by propidium (PI) and detected by flow cytometry (NovoCyte 2060R, ACEA Biosciences, Inc. Hangzhou, China).

Apoptosis Analysis

After transfections, the supernatant was discarded and cells were washed by PBS and digested by trypsin solution containing EDTA at 37 °C. The digestion was terminated with the complete medium containing serum, and the cell suspension was collected after centrifuge (2500 rpm, 4 °C, 10 min). Each tube added 5 μL Annexin V-FITC and 5 μL PI. After 10 min incubation at dark at room temperature, the apoptosis was detected by flow cytometry.

Cell Invasion

The cells were starved for 12 h, and collected by routine digestion and centrifugation, and the single cell suspension with a density of 5×10^5/ml was suspended with a low serum RPMI1640 culture (including 0.2% FBS). A transwell culture pool was put into the 24-well cultured plates, and 100 μL cell suspension (about 5×10^4 cells) was added. 500 μL RPMI1640 medium (containing 10% FBS) was added in the lower chamber. 24 h later, the cells in the lower chamber were fixed in 4% polyformaldehyde for 20–30 min and stained by

Table 1 Interference sequences of sh-DESI2

	Sequence (5′-3′)
sh-DESI2-F1	GATCCCCGGGCTTTATCAGAGATTCTTTGTCTCGAGACAAAGAATCTCTGATAAAGCTTTTTTG
sh-DESI2-R1	AATTCAAAAACCGGGCTTTATCAGAGATTCTTTGTCTCGAGACAAAGAATCTCTGATAAAGCTG
sh-DESI2-F2	GATCCCCGGCGGACTTCCTAGAAGATGATACTCGAGTATCATCTTCTAGGAAGTCCGTTTTTTG
sh-DESI2-R2	AATTCAAAAACCGGCGGACTTCCTAGAAGATGATACTCGAGTATCATCTTCTAGGAAGTCCGTG
sh-DESI2-F3	GATCCCCGGGAAAGAGATTCCTCGCTGGATCTCGAGATCCAGCGAGGAATCTCTTTCTTTTTTG
sh-DESI2-R3	AATTCAAAAACCGGGAAAGAGATTCCTCGCTGGATCTCGAGATCCAGCGAGGAATCTCTTTCTG

0.1% crystal violet dyeing for 30 min. The images in four fields were taken.

Western Blot

After indicated transfections, the cells were collected. Proteins were extracted using a protein isolation kit (28–9425-44, ReadyPrep; GE Healthcare Life Sciences) and quantified with a bicinchoninic acid protein assay kit, run via 12% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked in 5% skim milk for 2 h at room temperature and incubated with the following primary antibodies overnight at 4 °C: rabbit polyclonal anti-AKT (bs-2720R, Bioss, 1/1500); rabbit polyclonal anti-p-AKT (bs-0876R, Bioss, 1/1500); rabbit monoclonal anti-mTOR (ab32028, Abcam, 1/1000); rabbit polyclonal anti-p-Mtor (Ser2448, Cell Signaling, 1/1000); rabbit polyclonal anti-PI3K (bs-2067R, Bioss, 1/1500); rabbit polyclonal anti-p-PI3K (Tyr458, Cell Signaling, 1/1000); mouse monoclonal anti-caspase 3 (bsm-33,199 M, Bioss, 1/1000); rabbit polyclonal anti-DESI2 (bs-19976R, Bioss, 1/1000) and mouse monoclonal anti-GAPDH (TA-08, ZSJQ Bio. Inc., 1/2000). The secondary antibody (1: 2000; cat. Nos. ZB-2305; ZSJQ Bio. Inc. or 1:2000; cat. Nos. ZB-2301; ZSJQ Bio. Inc.) was added and co-incubated for 2 h at room temperature. Enhanced chemiluminescence exposure liquid droplet (cat. no. RPN2133; GE Healthcare Life Sciences, Chalfont, UK) was added to the membranes. The membranes were visualized using a gel imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and quantified with Quantity One version 1.4.6 (Bio-Rad Laboratories, Inc.).

Real Time PCR

The expression of each gene in mRNA level was detected by real time PCR. mRNA was extracted from each group. After extracting RNA, cDNA was synthesized according to the reverse transcriptase kit (00081405, CWBIO, Taizhou, China). cDNA was used as the template. GAPDH was used as the internal reference. The relative expression of DESI2, AKT, caspase 3, mTOR and PI3K was calculated. The sequence of primers was listed in Table 2.

Statistical Analysis

Statistical analysis was carried out by SPSS19.0 statistical software package, and the result of measurement data was expressed as mean ± SD. Multivariate analysis was performed by one-way ANOVA, and *t* test was used for comparison between groups. $P < 0.05$ indicated statistical significance for the difference.

Results

Efficacy of the Overexpression and Silencing of DESI2

After XhoI/XbaI enzyme digestion, the DESI2-pcDNA3.1 of DESI2 overexpression and interference plasmids should have 5428 bp and 597 bp bands, respectively. As shown in Fig. 1a, the results of electrophoretic detection were consistent with the theoretical values, proving that DESI2-pcDNA3.1 was the correct plasmid. The plasmid pGreenPuro should have a 570-bp band in theory, and the size of the sh-DESI2–1 gene fragment was 59 bp. It would theoretically have 629-bp band (Fig. 1b). The results showed that the electrophoretic results were in conformity with the theoretical values and had positive points.

Table 2 The primers of the genes

Genes	Sequence (5′-3′)	Product length (bp)
DESI2-F	CCATCCTTACCCCTTTTCTG	363
DESI2-R	TCCTGGAGTTCTTGGCTGAC	
AKT-F	CGAGGTGCTGGAGGACAAT	284
AKT-R	ACACGATACCGGCAAAGAA	
Caspase3-F	AGCGAATCAATGGACTCTGG	382
Caspase3-R	GACTTCTACAACGATCCCCTCT	
mTOR-F	TCCAGGGCTTCTTCCGTT	306
mTOR-R	GGGCTGTCGTGGTAGACTTAG	
PI3K-F	CTTTTGGAGTCCTATTGTCGTG	238
PI3K-R	GCCTGAGGTTTCCTAGTTGAT	
GAPDH-F	GAAGGTCGGAGTCAACGGAT	221
GAPDH-R	CCTGGAAGATGGTGATGGG	

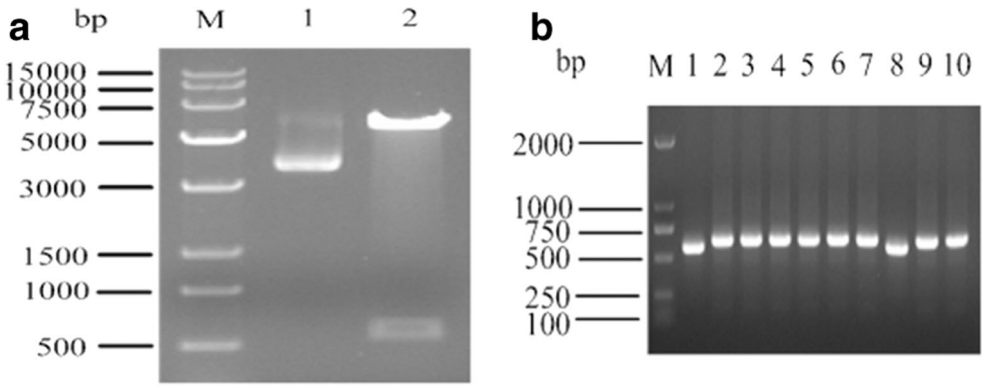

Fig. 1 Confirmation of DESI2 overexpression and interference plasmids using electrophoresis. **a** M:DL15000 maker; 1: DESI2-pcDNA3.1(+) plasmid; 2: DESI2-pcDNA3.1(XhoI/XbaI); **b** M:DL15000 maker; 1: pGreenpuro; 2–4:sh-DESI2–1-pGreenpuro; 5–7: sh-DESI2–2-pGreenpuro; 8–10: sh-DESI2–3-pGreenpuro

The efficacies of overexpression and silencing were verified. As shown in Fig. 2a, sh-DESI2–3 had the optimal interference effect in the three interference sequences. Therefore, sh-DESI2–3 was selected in the following experiments. As shown in Fig. 2b, c, the expression of DESI2 in DESI2 group was significantly higher than that of the control group, and the sh-DESI2 group decreased significantly, the difference was statistically significant compared with control ($P < 0.05$).

Silencing of DESI2 Increased, while Overexpression of DESI2 Reduced Cell Proliferation

In APC-1 cells, OD values in sh-DESI2 group after 48 h, 72 h and 96 h transfection were significantly higher than those in control group. By contrast, DESI2 overexpression had a significant lower OD value compared with control ($P < 0.05$) (Fig. 3a). The PANCE-1 cells showed consistent results that OD values were increased in sh-DESI2 group, but decreased in DESI2 overexpression group compared with control 24 h, 48 h, 72 h after transfection ($P < 0.05$) (Fig. 3b).

Silencing of DESI2 Increased, while Overexpression of DESI2 Reduced Cell Cycling

In ASPC-1 cells, the events in G1 phase were decreased and in S phase were increased in sh-DESI2 group compared with the control group ($P < 0.05$). By contrast, the events in G1 phase

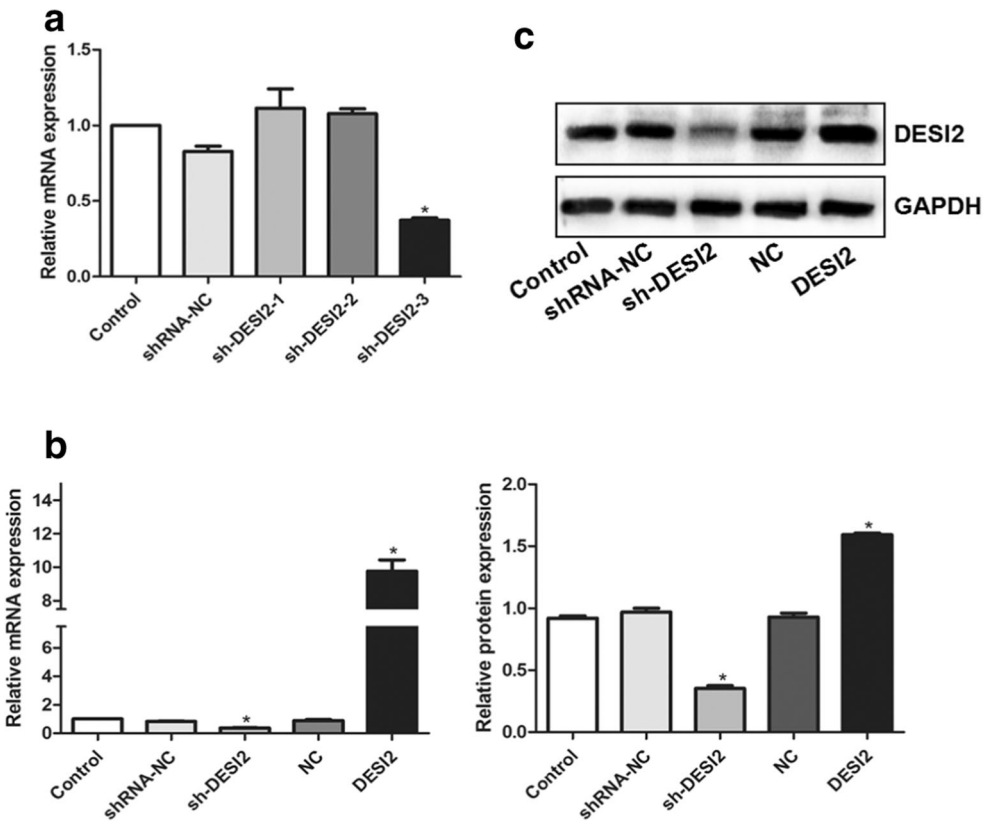

Fig. 2 Efficacy of the overexpression and silencing of DESI2. **a** mRNA expression of DESI2 after transfection of virus encoding three different sh-DESI2; **b** mRNA expression of DESI2 after transfection of virus encoding sh-DESI2 and DESI2 plasmids. **c** Protein expression of DESI2 after transfection of virus encoding sh-DESI2 and DESI2 plasmids. Upper panel: representative blots; Down panel: quantified data. *P < 0.05 compared with control

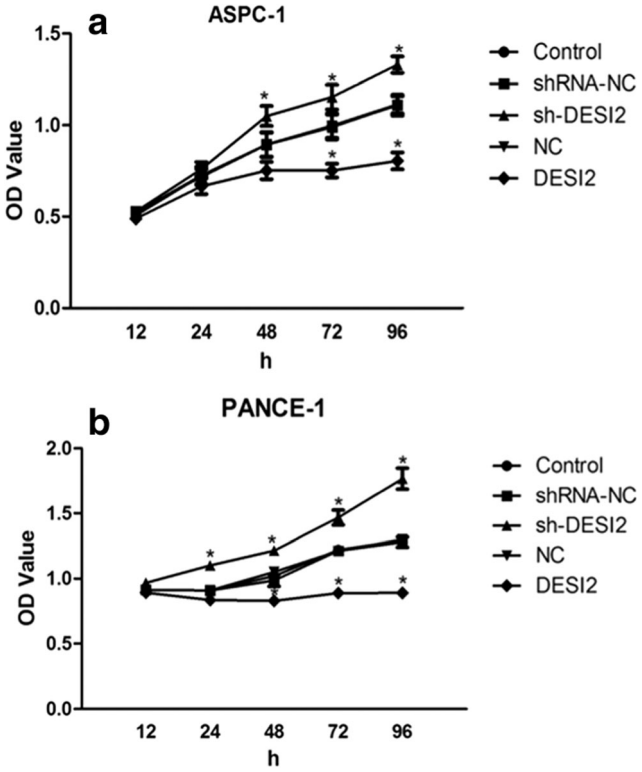

Fig. 3 Silencing of DESI2 increased, while overexpression of DESI2 reduced cell proliferation of pancreatic cancer cells. a APC-1 cells; **b** PANCE-1. *$P < 0.05$ compared with control

of the DESI2 group was significantly higher and in S phase decreased obviously compared with control ($P < 0.05$) (Fig. 4a). In PANCE-1 cells, the difference was statistically significant ($P < 0.05$). The events in G2 phase of sh-DESI2 group increased significantly, and in the G2 phase of DESI2 group decreased significantly compared with the control group ($P < 0.05$) (Fig. 4b).

Silencing of DESI2 Decreased, while Overexpression of DESI2 Promoted Apoptosis

In both ASPC-1 and PANCE-1 cells, the apoptosis in the sh-DESI2 group was significantly decreased, and the apoptosis in the DESI2 group was significantly increased compared with the control group ($P < 0.05$) (Fig. 5).

Silencing of DESI2 Increased, while Overexpression of DESI2 Reduced Cell Invasion

In ASPC-1 and PANCE-1 cells, the cell invasion in sh-DESI2 group was significantly higher than that in the control group, and the invasion of the DESI2 group was significantly lower than that in control group ($P < 0.05$) (Fig. 6).

Silencing of DESI2 Promoted, while Overexpression of DESI2 Reduced AKT/mTor

The expressions of AKT, p-AKT, PI3K, p-PI3k, p-mTOR, mTOR and caspase 3 in ASPC-1 and PANCE-1 cells were shown in Figs. 7 and 8. DESI2 silencing promoted AKT, PI3K, mTOR, while reduced caspase 3 expression at mRNA level and protein level. By contrast, DESI2 overexpression reduced AKT, PI3K, mTOR, while promoted caspase 3 expressions at mRNA level and protein level. Additionally, the expression of p-PI3K and p-mTOR was also promoted after DESI2 silencing, and reduced after DESI2 overexpression compared with control ($P < 0.05$). The expression of caspase 3 was also reduced after DESI2 silencing, and promoted after DESI2 overexpression compared with control ($P < 0.05$).

Discussion

In this study, we found that overexpression of DESI2 gene could significantly decrease the proliferation, cell cycling and invasion, and increase the apoptosis. By contrast, silencing of DESI2 increased the proliferation, cell cycling and invasion, and reduced the apoptosis. Potential mechanisms were related to PI3K/AKT/mTOR pathway, as DESI2 overexpression inhibited, while silencing promoted the signaling pathway. This study promoted a candidate therapeutic or diagnosis target for pancreatic cancer.

DESI2 gene was firstly identified by the National Institutes of health in a large-scale sequencing. DESI2 locates at the human chromosome 1q44, and has 5 exons, which can encode a protein consisting of 194 amino acids [11]. DESI2 is a kind of apoptosis-related protein, which is not only required for normal physiological activities, but also participates in the development of many tumors [12]. The expression of DESI2 in colorectal cancer, lung cancer and other tumors is obviously reduced, and the DESI2 may play a role in inhibiting the occurrence of tumor in many kinds of tumors [2]. Some studies have shown that overexpression of DESI2 in human lung adenocarcinoma cells arrested the cells at S phase, block the cell cycling from the S phase to the G2/M phase, and then lead to apoptosis. DESI2 may also function through binding to IP10 by activating the T lymphocyte infiltration and activating the immune stimulating function of the NK cells [13]. DESI2 inhibits cell proliferation by blocking apoptosis and arresting cell cycle at the S phase [14–16]. DESI2 overexpression in mice can inhibit the growth of CT26 xenografts in colon cancer cells in mice and LL2 xenografts in lung cancer cells, and can improve the anti-tumor effect of cisplatin, a chemotherapeutic drug [17, 18]. Overexpression of DESI2 combined with

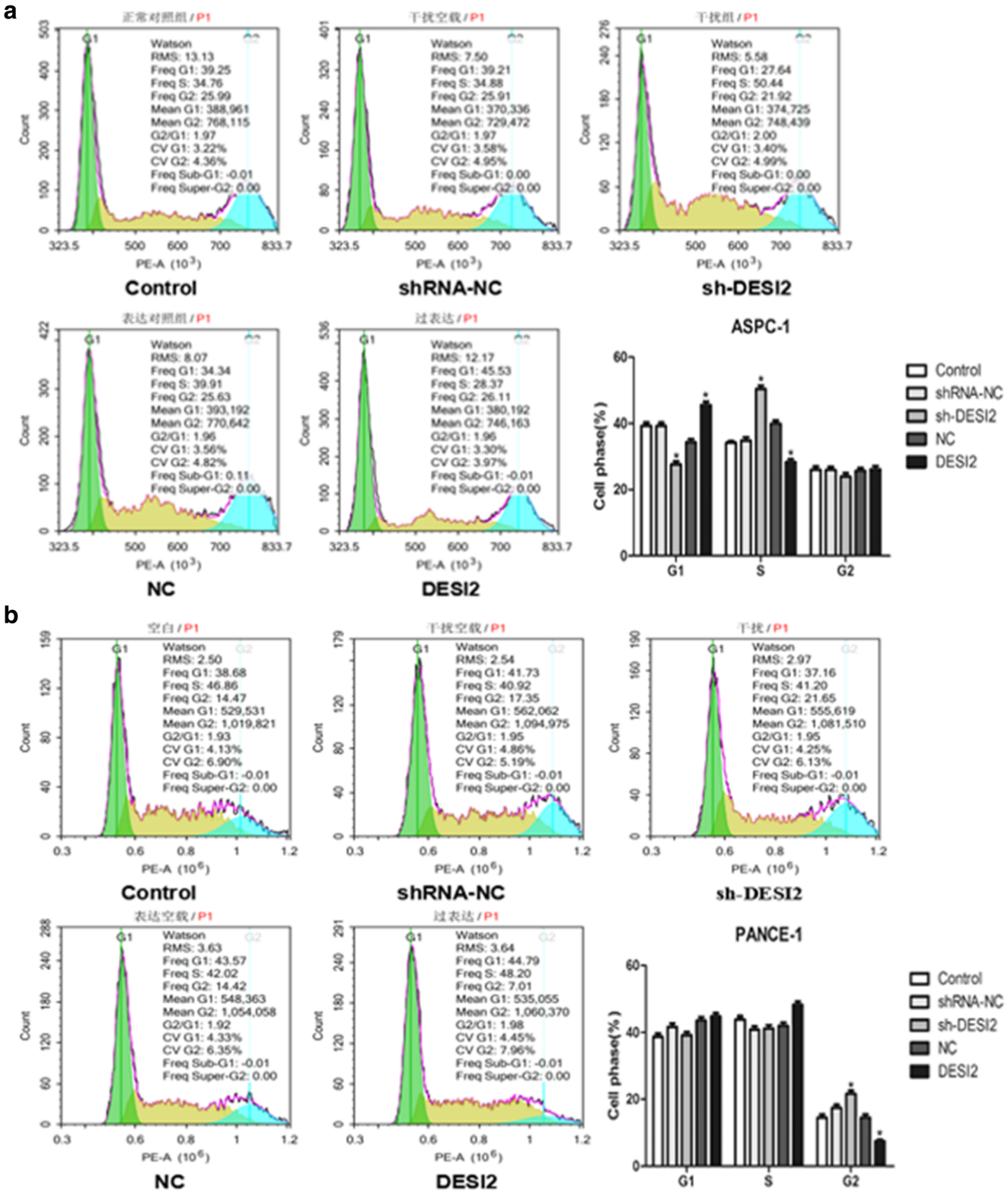

Fig. 4 Silencing of DESI2 increased, while overexpression of DESI2 reduced cell cycling of pancreatic cancer cells. a APC-1 cells; **b** PANCE-1. *$P < 0.05$ compared with control

gemcitabine or honokiol can increase the apoptosis of tumor cells in vitro and in vivo [19, 20]. These results are in agreement with the results of this study.

In addition, it was found that the invasion ability of the cells decreased significantly after the overexpression of DESI2 gene. These results may indicate that DESI2

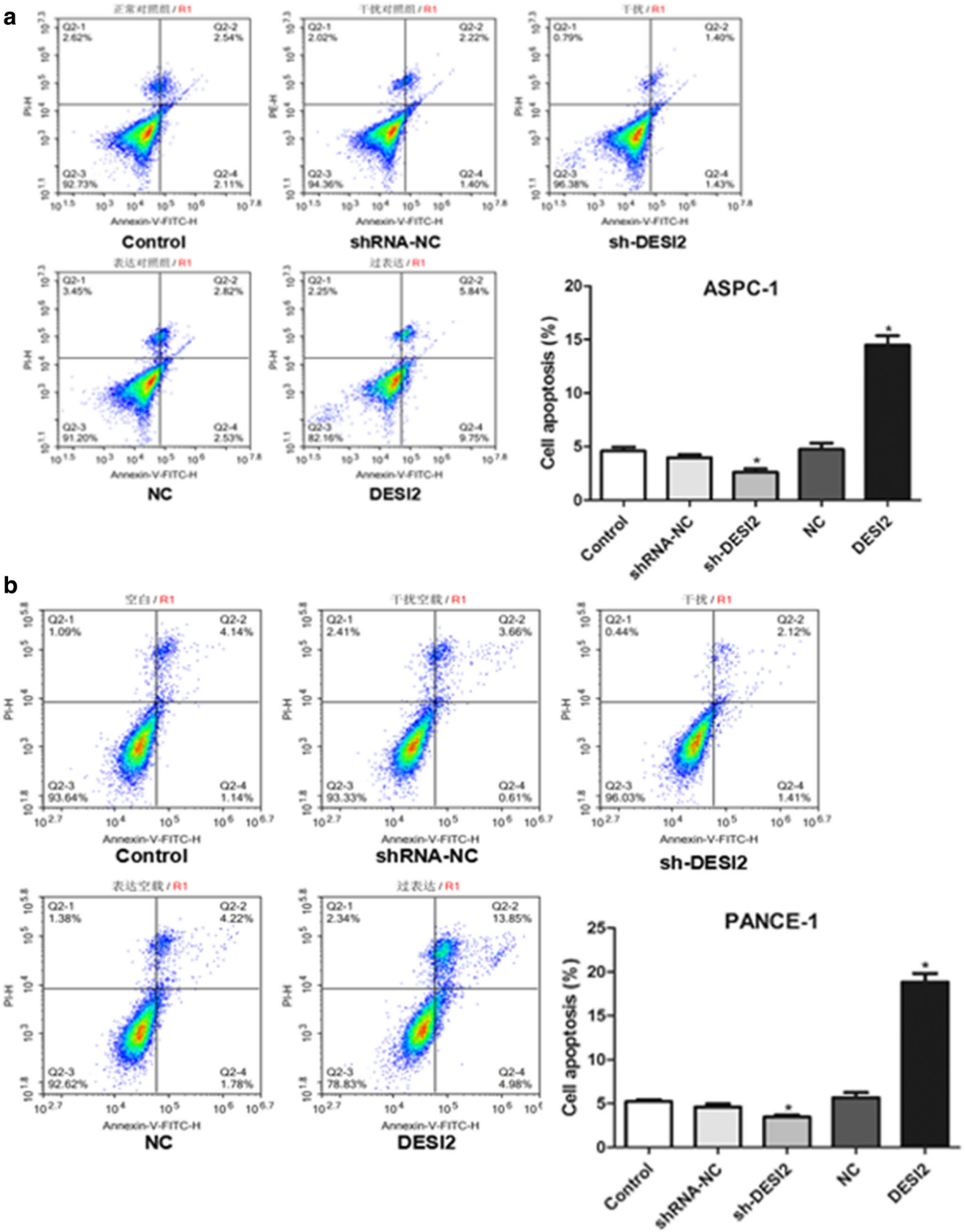

Fig. 5 Silencing of DESI2 decreased, while overexpression of DESI2 promoted apoptosis of pancreatic cancer cells. a APC-1 cells; **b** PANCE-1. *P < 0.05 compared with control

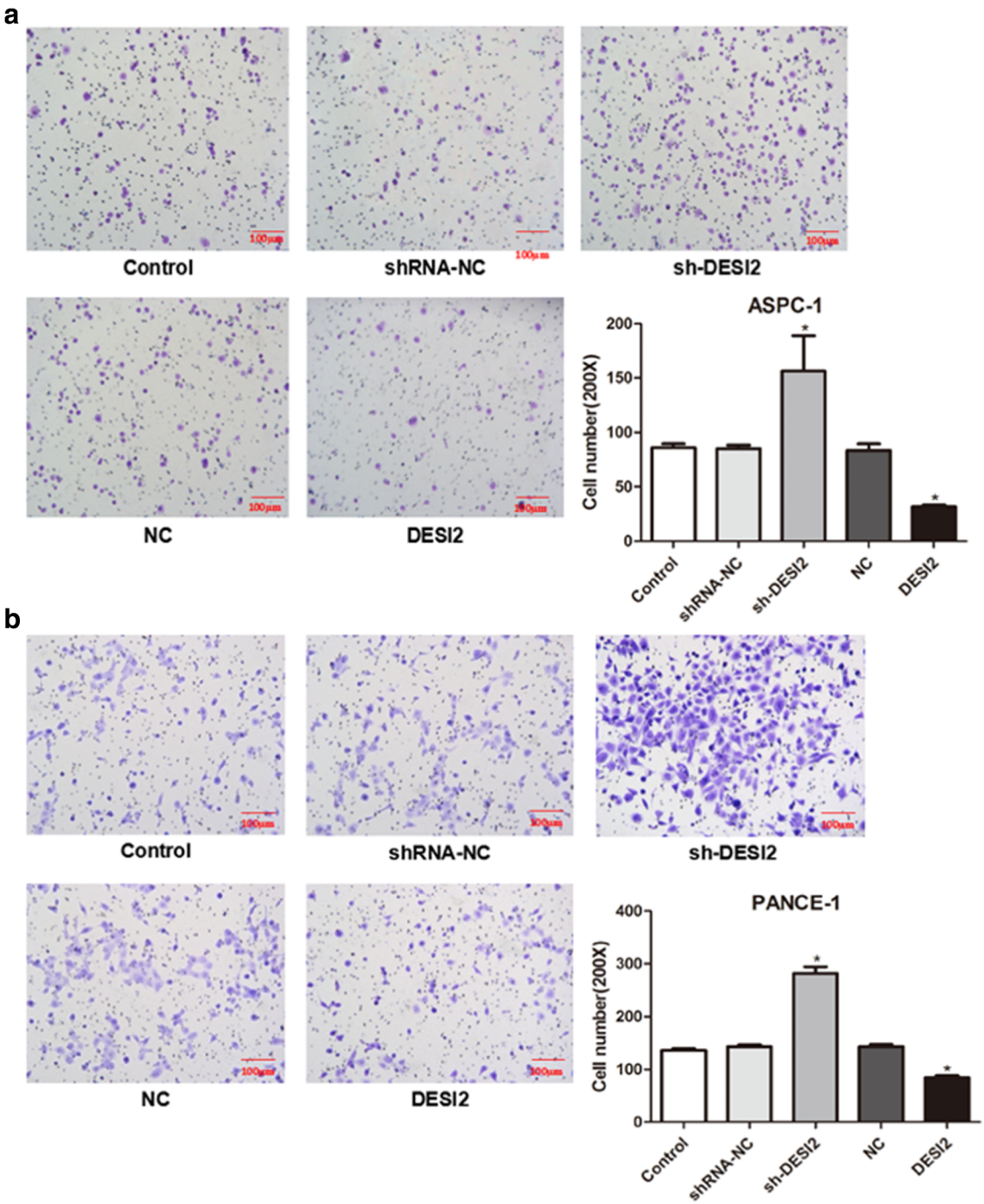

Fig. 6 Silencing of DESI2 increased, while overexpression of DESI2 reduced cell invasion of pancreatic cancer cells. a APC-1 cells; **b** PANCE-1. *P < 0.05 compared with control

affects cell invasion. Caspase-3 plays an irreplaceable role in apoptosis. One of the most important events in the process of apoptosis is the activation of caspase-3. The activation of caspase-3 and the upstream signaling pathway causing cell death depends on the specific combination of ligand and specific cell types [21]. Studies have shown that oncogenesis is closely related to the lack of apoptosis, while apoptotic protein caspase-3 plays an important role in the process of apoptosis [22]. Caspase 3 is the key executor of apoptosis by activation of extracellular apoptotic signaling pathway [23]. Some studies have shown that caspase-3 is closely related to the apoptosis of pancreatic cancer cells, and inhibits the proliferation of pancreatic cancer cells [24–26]. The results of this study found that the expression of caspase-3 was significantly increased after DESI2 overexpression, and the

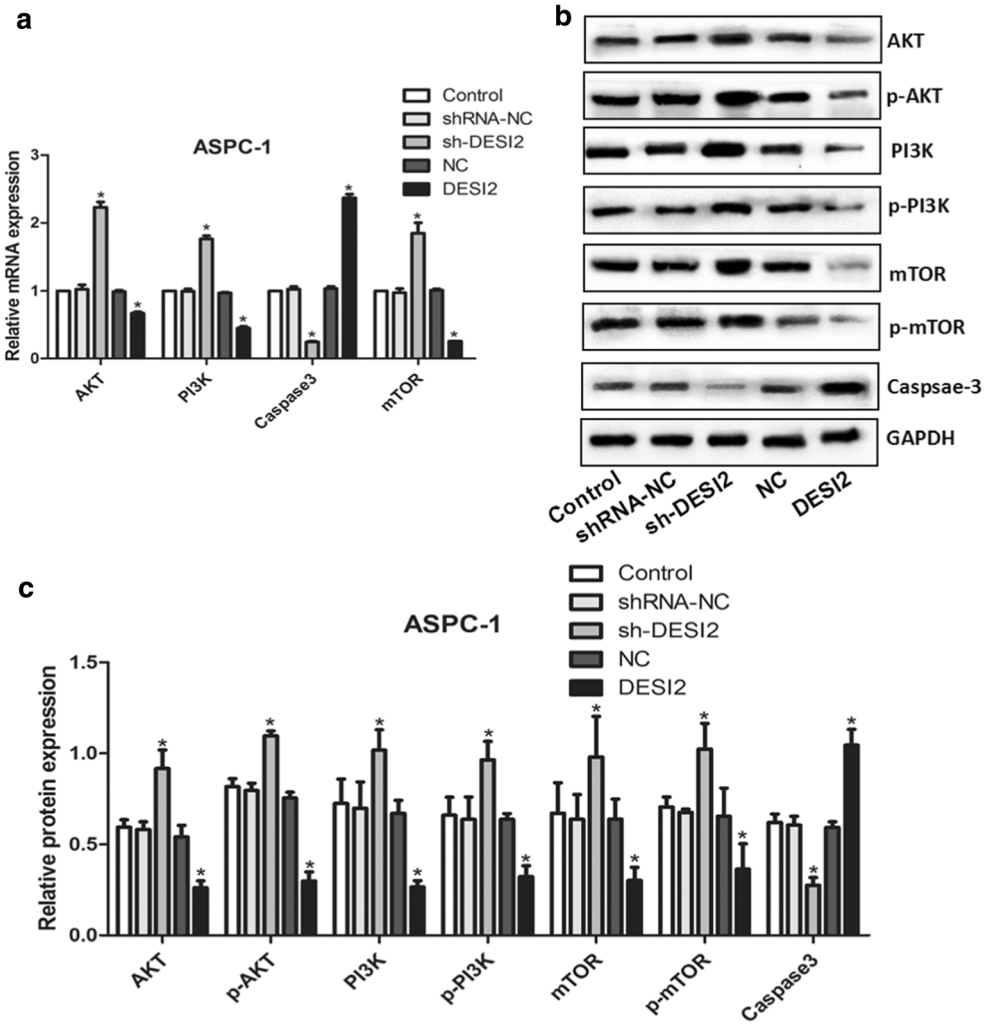

Fig. 7 Silencing of DESI2 promoted, while overexpression of DESI2 reduced AKT/mTor in APC-1 cells. a mRNA expression of AKT, PI3K, caspase 3, mTOR; **b** representative blots of p-PI3K, p-AKT, p-mTOR and caspase 3; **c** Quantified data of protein expression. *P < 0.05 compared with control

expression of caspase-3 protein decreased significantly after the silence of DESI2. The results showed that DESI2 could promote apoptosis and increase the expression of apoptosis-related proteins.

PI3K/AKT/mTOR signaling pathway is crucial in the development of tumors, including transcription, translation, metabolism, angiogenesis, apoptosis, proliferation, cell cycling, migration and invasion [27]. Abnormal expression and overactivation of AKT can promote the growth of pancreatic cancer, antagonizing apoptosis and increasing the viability of tumor cells [28]. PI3K is an upstream molecule of AKT/mTOR signaling pathway. PI3K regulates activation of AKT/mTOR signaling pathway [29]. AKT is an important downstream target kinase in the PI3K signaling pathway, which can be activated by the production of phosphorylated AKT under the action of PI3K, and p-AKT further activates the downstream factor to exert its functions [30]. p-AKT can phosphorylate the downstream tyrosine and tryptophan residues and activate the downstream factors to inhibit apoptosis, promote cell proliferation, growth, migration and invasion, and promote tumor angiogenesis [31]. PI3K/AKT/mTOR signaling pathway is an intracellular regulator, which plays an important role in regulating apoptosis, metabolism and cell proliferation [32], especially pancreatic cancer cells [16, 33]. Studies have shown that Longikaurin E can induce apoptosis of pancreatic cancer cells through promoting reactive oxygen species, thereby regulating p38 and PI3K/AKT/mTOR pathway [34]. This study showed that when the DESI2 gene was overexpressed, the expression of AKT, p-Akt, PI3K, p-PI3K, p-mTOR and mTOR was significantly decreased, which reveals that DESI2 affects the apoptosis of pancreatic cancer cells through PI3K/AKT/mTOR pathway. Some studies have shown that the expression level of DESI2 protein is negatively correlated with AKT, PI3K and mTOR. When DESI2 is highly expressed in normal pancreatic tissue, the latter two expressions are relatively low. By contrast, when DESI2 is lowly expressed in the tumor tissues of pancreatic

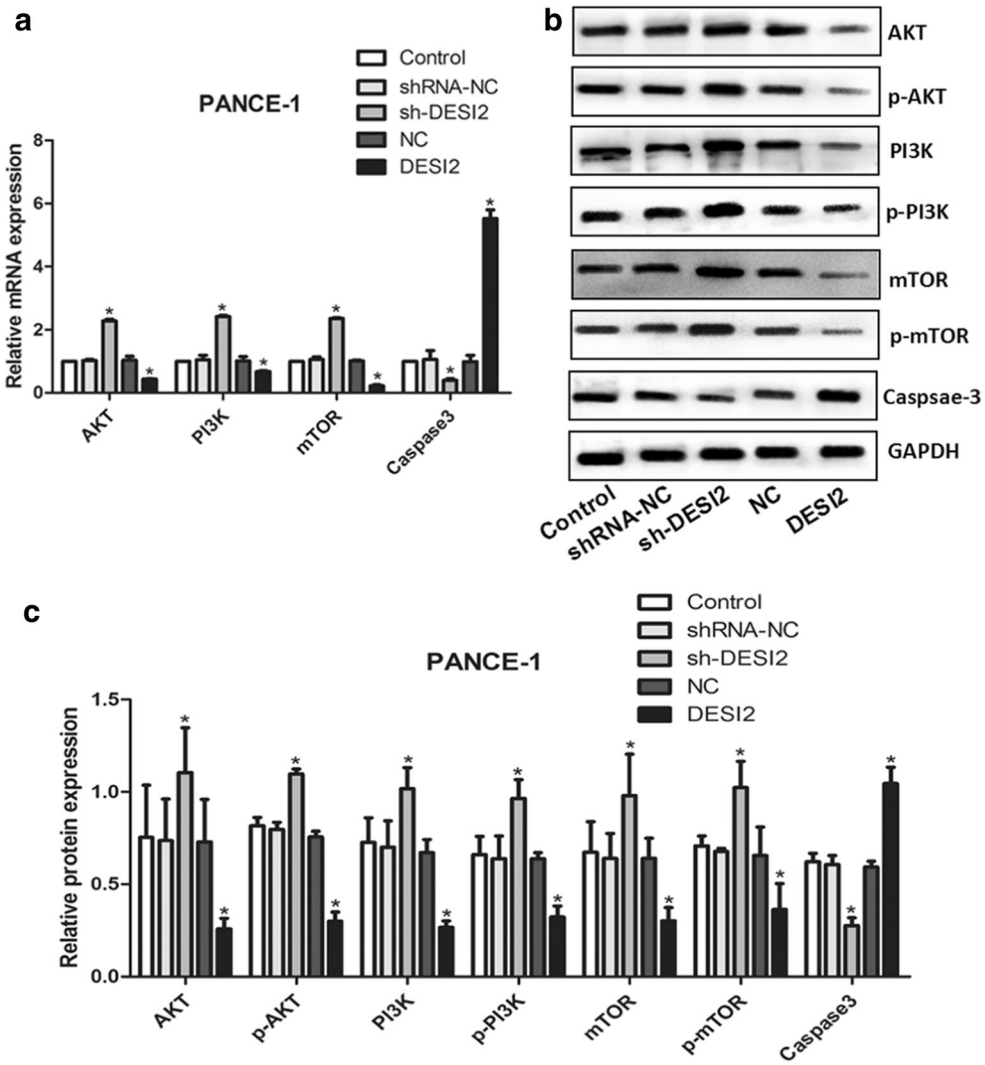

Fig. 8 Silencing of DESI2 promoted, while overexpression of DESI2 reduced AKT/mTor in PANCE-1 cells. a mRNA expression of AKT, PI3K, caspase 3, mTOR; **b** representative blots of p-PI3K, p-AKT, p-mTOR and caspase 3; **c** Quantified data of protein expression. *P < 0.05 compared with control

cancer AKT, PI3K and mTOR are relatively high expressed [3]. The results of this study are in agreement with the results of those studies. Therefore, this study further prompts the potential antitumor activity of DESI2 and its potential association with PI3K/AKT/mTOR signaling pathway in pancreatic cancer.

Conclusion

DESI2 is an important molecule in the development of pancreatic cancer. DESI2 likely regulates the proliferation, apoptosis and biological activity of pancreatic cancer cells through the PI3K/AKT/mTOR signaling pathway.

Author Contributions XO, GZ, ZX, JC, YX and JL did the experiments and analyzed the data. X Liu YC and XO designed the study and wrote the manuscript.

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