

Downregulated expression of IL-28RA is involved in the pathogenesis of pancreatic ductal adenocarcinoma

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Abstract. Pancreatic cancer ranks seventh in terms of cancer-related mortality in men and women worldwide, where the most common subtype is pancreatic ductal adenocarcinoma (PDAC). To date, the pathogenesis of PDAC remains incompletely understood and the prognosis of PDAC is poor. In the present study, the expression of interleukin-28 receptor α subunit (IL-28RA) in PDAC tissues was detected using immunofluorescence staining and western blotting. IL-28RA recombinant plasmids and control pCMV6-entry mammalian expression plasmid, short hairpin (sh)IL-28RA plasmids and control pRS scrambled shRNA vector purchased were used to produce stably transfected PANC-1 cells overexpressing IL-28RA or with IL-28RA expression knocked down. MTS assays were used to measure cell viability and wound healing assay was used to assess the cell migratory ability *in vitro*. Flow cytometry analysis was performed to determine the proportion of cells in each phase of the cell cycle whereas total protein and phosphorylated protein levels were assessed using western blotting. Xenograft models of subcutaneous tumors were established by injecting PANC-1 cells hypodermically into nude mice to investigate the effect of IL-28RA on tumorigenesis and tumor growth. The results showed that the

expression of IL-28RA in PDAC tissues was lower compared with that in normal tissues. IL-28RA overexpression *in vitro* resulted in the activation of the IL-28RA pathway, which reduced cell viability and decreased the proportion of cells in the G₂/M phase by reducing cyclin B1 expression. In addition, IL-28RA overexpression inhibited migration of PDAC cells. By contrast, an increased proportion of cells in G₂/M phase, upregulated cyclin B1 expression and enhanced cell viability and migratory ability along with inhibition of the IL-28RA pathway were observed in PANC-1 cells following IL-28RA knockdown. The inhibitory effect of IL-28RA was observed by tumor size in a nude mouse model induced by PANC-1 cells with stable IL-28RA overexpression or knockdown. The tumor size induced by PANC-1 cells with stable IL-28RA overexpression were smaller, whilst larger tumors induced by PANC-1 cells were observed following stable IL-28RA knockdown, when compared to control. Further studies showed that the effect of IL-28RA on PDAC cells was exerted by regulating the phosphorylation levels of STAT1 and AKT. In conclusion, lower IL-28RA expression may contribute to the pathogenesis of PDAC, where results from the present may provide further insights into the progression of PDAC, in addition to highlighting potentially novel therapeutic targets for this disease.

Introduction

Pancreatic cancers ranked seventh in terms of the number of cancer-related mortality in men and women worldwide in 2012, where the most common subtype is pancreatic ductal adenocarcinoma (PDAC) (1). In 2017, an estimated 53,670 individuals were diagnosed with pancreatic cancer and there were 43,090 pancreatic cancer-associated deaths in the United States (2). The mortality rate of pancreatic cancer has continued to increase, particularly in men, where the relative 5-year survival rate is only 8% at present (2). The poor prognosis of patients with PDAC is primarily due to the high degree of invasiveness, difficulties for early diagnosis and high erroneous diagnostic rates in addition

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to intrinsic resistance to chemotherapy, radiotherapy and immunotherapy (3). Moreover, the curative efficacy of specific immunotherapy regimens and molecularly targeted therapies for the management of advanced stage PDAC is poor (4,5). Therefore, there is an urgent need for the development of effective treatments for the management of PDAC.

Interleukin (IL)-28 receptor α subunit (IL-28RA) is a transmembrane protein that serves as a subunit of the type II cytokine receptor and binds to type III interferons (IFNs) or λ IFNs, IL-28A and IL-28B when combined with another IL receptor subunit, such as IL-10 receptor β (6). IL-28RA binds most readily to λ interferon, followed by IL-28A and IL-28B (7). The signaling pathways activated by the type III IFNs exhibit notable similarities with those activated by type I IFNs (8), and exhibit antiviral activity via the Janus kinase (JAK)-STAT pathway (9), especially against hepatitis B and influenza virus (10-13). Similar growth inhibitory functions of IFN- λ and type I IFNs have been reported in several types of cancer cells, including in pancreatic neuroendocrine BON1 tumor cells (14-16), where similar signal transduction pathways are activated by IFN- λ and type I IFNs, although no detectable homology was found in their receptor subunits, IL-28RA and interferon α and β receptor subunit 1 (15). Both of these factors bind to their respective receptor complexes and predominantly modulate STAT1 and STAT2 phosphorylation, but can also regulate STAT3 and STAT5 phosphorylation to a lesser degree (14,17). In addition, signals activated by IFN- λ receptor can regulate the expression of several established type I IFN-responsive target genes, including interferon-stimulated genes and suppressor of cytokine signaling 1 (9). Previous reports suggested that type I IFNs showed antitumor ability against different types of pancreatic cancer cell lines, including PANC-1, MiaPaCa-2 and BxPc-3 cells (18,19). Overexpression of IL-28RA in pancreatic cancer cells exerted inhibitory effects on proliferation and invasion (18). Additionally, IL-28RA increased the apoptotic rate of pancreatic cancer cell and upregulated the expression of the pro-apoptotic protein BAX, whilst decreasing the expression of the anti-apoptotic protein Bcl-2; in a manner that may be associated with inhibition of the JAK2 and STAT3 signaling pathway (18). It was also found that IL-28RA was also involved in breast cancer and colon cancer development, where IL-28RA was positively associated with the prognosis of patients (20,21).

Immunofluorescence and western blotting analysis were used to detect IL-28RA expression levels in PDAC tissues and adjacent normal pancreatic tissues from human samples. To determine the effect of IL-28RA on the development and progression of PDAC with specific focus on the possible molecular mechanisms, stable IL-28RA knockdown and IL-28RA overexpression were achieved using the PDAC cell line PANC-1, in the present study. The effect of IL-28RA on cell proliferation, cell cycle and migration ability *in vitro* and the possible molecular mechanisms were investigated. In addition, xenograft nude mouse models induced by PANC-1 cells with stable IL-28RA overexpression and IL-28RA knockdown were used to detect the effect of IL-28RA on tumorigenesis and progression *in vivo*.

Materials and methods

Clinical specimens. The present study was approved by the Ethics Committee of Anhui Medical University (Hefei, China). A total of eight patients (sex, five males and three females; average age, 56.32 \pm 8.12 years) with stage I-II PDAC based on pathological analysis according to Guideline for the diagnosis and treatment of pancreatic adenocarcinoma (2014 edition) (22), who were diagnosed with PDAC with no portal vein obstruction, intraperitoneal dissemination or colonic ileus and treated with surgical resection at The First Affiliated Hospital of Anhui Medical University between September 2015 and November 2016, were included in the present study. The exclusion criterion was stage IV PDAC according to TNM classification (22) with complications, such as with indication for urgent surgery for symptoms, including acute abdomen. The protocols used were performed in accordance with the ethical guidelines described in the Declaration of Helsinki and written informed consent was obtained from each of the patients diagnosed in the present study prior to surgery. PDAC and paired para-cancerous normal pancreatic tissues were obtained from each individual for immunofluorescence analysis and western blotting.

Cytokines, experimental materials and reagents. Recombinant human IL-29 was purchased from Pepro Tech, Inc. The anti body against IL-28RA (cat. no. ab83865; 1:500) was purchased from Abcam. A JAK inhibitor (JAKI, cat. no. sc-204021) and antibodies against STAT1 (cat. no. sc-464; 1:500), STAT3 (cat. no. sc-8019; 1:500), STAT5 (cat. no. sc-74442; 1:500), AKT (cat. no. sc-5298; 1:500), phosphorylated (p)STAT1 (cat. no. sc-8394; 1:500), pSTAT3 (cat. no. sc-8059; 1:500), pSTAT5 (cat. no. sc-81524; 1:500), p AKT (cat. no. sc-514032; 1:500), CyclinB1 (cat. no. sc-245; 1:500) and β -actin (cat. no. sc-47778; 1:1,000) were acquired from Santa Cruz Biotechnology, Inc. LY294002, a PI3K inhibitor, was purchased from Sigma-Aldrich; Merck KGaA. IL-28RA recombinant plasmid (cat. no. RC221831) constructed in the pCMV6-Entry plasmid carrying Kanamycin resistance in *E. coli* selection and neomycin resistance for cell selection, control plasmid pCMV6-Entry (cat. no. PS100001), IL-28RA short hairpin (sh) RNA (cat. no. TR303938) constructed in pRS retroviral plasmids with ampicillin and puromycin resistance markers and scrambled shRNA plasmid (cat. no. TR20003) were purchased from Ori Gene Technologies, Inc.

Cell culture. The human pancreatic tumor cell line PANC-1 was obtained American Type Culture Collection and cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% Penicillin-Streptomycin at 37°C in a humidified incubator with 5% CO₂.

Establishment of stably transfected PANC-1 cells. PANC-1 cells were seeded at a density of 5 \times 10⁴ cells/well in 24-well plates. PANC-1 cells in the 24-well plates were grown to 80-90% confluence and transfected with the IL-28RA recombinant plasmid (IL-28RA constructed in pCMV6-entry mammalian expression vector), control pCMV6-entry mammalian expression plasmid, shIL-28RA pRS plasmid and shRNA pRS

scrambled shRNA Vector using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Briefly, DNA-lipid complex was prepared by mixing 0.2 µg plasmid with Lipofectamine® 3000 and was added to cells after 15 min incubation at room temperature. IL-28RA recombinant plasmid- and control plasmid pCMV6-entry-transfected cells were selected at 37°C for 2 weeks using G418 (800 µg/ml; Gibco; Thermo Fisher Scientific, Inc.) 24 h after infection, whilst cells transfected with plasmids encoding shIL-28RA and scrambled shRNA were selected at 37°C for 2 weeks using puromycin (1 µg/ml; cat. no. P9620; Sigma-Aldrich; Merck KGaA). Stably transfected cells resistant to G418 or puromycin were grown, following which the expression levels of IL-28RA were screened using western blotting to confirm transfection efficiency.

Hematoxylin and eosin (H&E) and tissue immunofluorescence staining. Formalin-fixed paraffin-embedded sections of PDAC and paired paracancerous normal tissues were used for H&E staining and immunofluorescence analysis to investigate IL28RA expression levels. Briefly, PDAC tissues and paired paracancerous normal tissues were collected, immediately fixed in 10% formalin at room temperature for 24 h. The tissues were dehydrated in an ascending gradient of ethanol followed by xylene and embedded in paraffin. The embedded tissues were sectioned at 4-µm thickness and dewaxed using xylene and hydrated in a descending series of ethanol solutions.

For H&E staining, the sections were stained in hematoxylin for 5 min at room temperature and eosin for 10 sec at room temperature. The stained sections were observed and pictured at x200 magnification using a Nikon light microscope (Nikon TS2-FL; Nikon Corporation) after sealing the slides with neutral balsam.

For immunofluorescence staining, citrate buffer (pH 6.0) was used to heat the sections for antigen recovery at 95°C for 6 min followed by 30 min treatment in H₂O₂ at room temperature after cooling down passively. Following non-specific antigen blocking in 3% BSA (Beyotime Institute of Biotechnology) with 0.2 Triton X-100/PBS at room temperature for 20 min, the specimens were stained with the IL-28RA-specific antibody (1:100) overnight at 4°C. Tissues were then extensively washed and the sections were incubated with goat anti-rabbit IgG secondary antibody with FITC-conjugation (1:100; cat. no. ZF-0311; ZSGB-BIO, Ori Gene Technologies, Inc.) at room temperature for 2 h in a dark box. DAPI working solution at 10 µg/ml (cat. no. C1005; Beyotime Institute of Biotechnology) was used to stain the nuclei for 10 min at room temperature and the sections were mounted using colorless mounting solution. The fluorescence images of the specific stained sections were imaged using a fluorescence microscope (magnification, x200; Olympus Corporation).

Western blotting assay. PDAC and paired paracancerous normal tissues from patients were washed using RIPA buffer (Sigma-Aldrich; Merck KGaA) on ice to obtain the total proteins. PANC-1 cells were cultured, transfected as aforementioned, stimulated with IL-29 (200 ng/ml), JAKI (800 nM) or LY294002 (50 µM) at 37°C for 72 h and harvested. After measuring the total protein concentration using the bicinchoninic

acid protein assay (Beyotime Institute of Biotechnology), equal amounts of total protein (30 µg/lane) lysate from tissues or cells were resolved using 12% SDS-PAGE and transferred onto PVDF membranes. Appropriate dilutions of specific primary antibodies against IL-28RA, cyclinB1, pSTAT1, pSTAT3, pSTAT5, STAT1, STAT3, STAT5, pAKT, AKT (all at 1:500) and β-actin (1:1,000) were used to incubate the membranes at 4°C overnight followed by 2 h incubation at room temperature with the corresponding horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. AP132P; 1:10,000) and goat anti-mouse IgG (cat. no. AP130; 1:10,000) secondary antibodies (Sigma Aldrich, Merck KGaA) diluted in TBS solution. Signals were visualized using a Super Signal West Femto Trial kit (Thermo Fisher Scientific, Inc.), and images of the bands were taken using a Chemi Scope (Clinx Science Instrument Co. Ltd.). The bands on western blots were quantified using the Quantity One software (v4.6.6; Bio-Rad Laboratories, Inc.).

Cell viability assay. A total of 3x10³ stably transfected or control PANC-1 cells in 100 µl culture medium were seeded into 96-well plates, in triplicate and treated with IL-29 (200, 300 and 500 ng/ml), JAKI (800 nM) or LY294002 (50 µM) at 37°C for 48 h. An MTS salt assay (Promega Corporation) was performed to measure cell growth. Briefly, 20 µl MTS reagent was added to each well at the end of cell treatment, followed by 4 h incubation at 37°C. The absorbance value at a wavelength of 490 nm was measured using a PerkinElmer Wallac Victor 2 1420 Multi label Counter (PerkinElmer, Inc.) following color development. The growth inhibition rate was calculated by the following formula: Inhibition rate (%) = [1 - (mean OD_{treated groups} - mean OD_{blank controls}) / (OD_{control groups} - OD_{blank controls})].

Cell cycle analysis. Stably transfected cells, at a density of 1x10⁵ cells/well, were seeded into six-well plates and routinely cultured to 80-90% confluence, followed by treatment with IL-29 (200 ng/ml), JAKI (800 nM) or LY294002 (50 µM) at 37°C for 72 h. Cell cycle distribution was analyzed using a BD FACSV flow cytometer (BD Biosciences) using a Cell Cycle Analysis kit (cat. no. C1052; Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Briefly, the cells were dissociated and resuspended at a density of 5x10⁵ cells/ml, fixed in cold 70% ethanol for 30 min at 4°C and treated with propidium iodide at 50 µg/ml staining buffer (Beijing Biosea Biotechnology Co., Ltd.) in the dark at 4°C for 30 min. The flow cytometry data were analyzed using the Mod Fit program version 3.1 (Verity Software House, Inc.).

In vitro wound healing assay. Stably transfected cells were plated at a density of 4x10⁵ cells/ml in six-well plates following trypsinization and resuspension. Cells were incubated to yield a confluent monolayer before creating the wound. Wounds were created using P1000 pipette tips and incubated with IL-29 (200 ng/ml), JAKI (800 nM) or LY294002 (50 µM) in DMEM supplemented with 2% heat-inactivated FBS at 37°C (23). Images were taken immediately after wounding (0 h), after 24 and 48 h under Nikon light microscope (magnification, x100; Nikon TS2-FL; Nikon Corporation). Quantity on e4.6.6 software (Bio-Rad Laboratories, Inc.) was used to measure the distance migrated by cells after 24 and 48 h. Results are presented as the percentage residual of wound area,

and the percentage of the distance after treatment relative to the distance before treatment. The percentage of residual wound area (%) was calculated by the width of the wound at 24 or 48 h divided by the width of the wound at their corresponding 0 h.

Animal experiments. For *in vivo* oncogenicity experiments, a total 12 male BALB/c nude mice, aged 4 weeks (weight, 16.32±86 g), were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. and adaptively fed for 1 week before experiment. Only males were used in the present study because female mice have estrous cycles which would introduce another variable to negatively influence data analysis (24). The animal experiments were approved by the Anhui Medical University Institutional Animal Care and Treatment Committee (approval no. 20140257) and the mice were raised in the specific-pathogen-free laboratory animal room under human conditions at 22±2°C with 55±5% humidity under a 12-h light/dark cycle with food and water provided *ad libitum* according to guidelines described by the Anhui Medical University Institutional Animal Care and Treatment Committee (25). Animal health and behavior were monitored twice daily by food and water intake, general assessment of animal activity and fur condition. The animal should be excluded from the study and euthanized before the predetermined time point if the size of a subcutaneous tumor was observed to be >2000 mm³ or with decreased food or water intake. No animal reached any of the humane endpoints in the present study. Animal death was verified by the absence of breathing and heartbeat.

PANC-1 Cells stably transfected with pCMV6-IL-28RA, pCMV6,shIL-28RA or scrambled shRNA were inoculated subcutaneously into male BALB/c nude mice 2 weeks after transfection [n=6 for each group, 1×10⁷ cells injection (26) in 0.2 ml PBS] in the hips (pCMV6-IL-28RA and pCMV6 were transfected in the same mice at right and left hips respectively, shIL-28RA and scrambled shRNA were transfected in the same mice at right and left hips respectively). When the tumors reached 0.4-0.5 cm in size (~2 weeks later), PBS or IL-29 (16 mg/kg/50 µl) was injected intratumorally into the mice, two times a week for 3 weeks and tumor growth was monitored in detail. The tumors were allowed to grow for 24 days and the tumor volumes were monitored every 3 days. On day 24 of PBS or IL-29 injection, the mice with administered with pentobarbital (50 mg/kg) by intraperitoneal injection 1 h before surgery and euthanasia by cervical dislocation, where the tumors were carefully resected and weighed. The tumor size was calculated as $(a \times b^2)/2$, where *a* is the length and *b* is the width of the resected tumors.

Statistical analysis. Data are presented as the mean ± standard error of experimental repeats (n=3 for *in vitro* assays; n=6 mice per groups for *in vivo* assays). Data were analyzed using SPSS version 12.0 (SPSS, Inc.). Differences between groups were compared using Tukey test. P<0.05 was considered to indicate a statistically significant difference.

Results

IL-28RA expression is downregulated in PDAC tissues. IL-28RA expression in PDAC tissues and paired adjacent normal pancreatic tissues from eight patients with PDAC was

assessed using immunofluorescence staining. Strong IL-28RA immunoreactivity was observed in the normal pancreatic tissues with little expression in the nucleus, whereas its expression in the tumor tissues was markedly lower (Fig. 1A). H&E staining of PDAC tissue slides showed the ductal glandular structure formed by tumor cells with abundant fibrous stroma (Fig. 1B). Western blotting also showed that expression of IL-28RA in PDAC tissues was lower compared with that in the corresponding paired adjacent normal pancreatic tissue (Fig. 1C and D), confirming the results of the immunofluorescence staining.

Construction of stably transfected cell lines. To elucidate the roles of IL-28RA in PDAC development, stably transfected PANC-1 cell lines in which IL-28RA was overexpressed or downregulated were established. The levels of IL-28RA expression in the transfected PANC-1 cells were determined using western blot analysis. As shown in Fig. 2, IL-28RA expression was markedly increased in the IL-28RA recombinant plasmid-transfected cells compared with that in cells transfected with the empty plasmid pCMV6-entry. By contrast, expression of IL-28RA was markedly decreased in cells transfected with shIL-28RA compared with that in cells transfected with the scrambled shRNA.

IL-29 exerts a cytostatic effect on the PANC-1 cells. Type III IFN interaction with IL-28R has been reported to induce anti-proliferative responses in several types of cancers (23-25). MTS assays were therefore performed to determine the effect of IL-28RA on PANC-1 cell viability. Stimulation of PANC-1 cells with IL-29 (500 ng/ml) for 48 h reduced cell viability by 40.37% (Fig. 3A) when compared with that in control cells (0 ng/ml). Due to almost the same inhibitory effect conferred between the 200 and 300 ng/ml concentrations, whilst cytotoxicity occurred at 500 ng/ml, IL-29 at 200 ng/ml was chosen to treat the cells in the following experiments. In certain types of cells, including the monkey kidney cell line COS-1 and the human colon cancer cell line HT-29, IFN-λ has been reported to induce STAT phosphorylation (17). Consequently, western blotting was used to investigate whether IL-29 treatment induced the phosphorylation of AKT, STAT1, STAT3 and STAT5 in PANC-1 cells. The results showed that IL-29 activated STAT1 when cells were treated from 1 to 24 h, whilst also markedly increasing STAT3 phosphorylation with a peak at 1 h. However, IL-29 conferred no notable effects on STAT5 phosphorylation (Fig. 3B). IL-29 only slightly increased the phosphorylation levels of AKT after 1 h before the levels were lower compared with that at baseline from 6 h onwards (Fig. 3B).

IL-28RA affects PANC-1 cell growth *in vitro* via regulation of the STAT1 and AKT phosphorylation levels. To investigate the importance of IL-28RA on cell growth, shIL-28RA-transfected and IL-28RA-overexpressing PANC-1 cells were used. The effect of IL-29 (200 ng/ml) on the viability of the transfected PANC-1 cells was detected using an MTS assay. The results showed that viability was significantly reduced in the IL-28RA-overexpressing cells compared with that in cells transfected with the pCMV6 vector control (Fig. 4A). By contrast, shIL-28RA transfection significantly

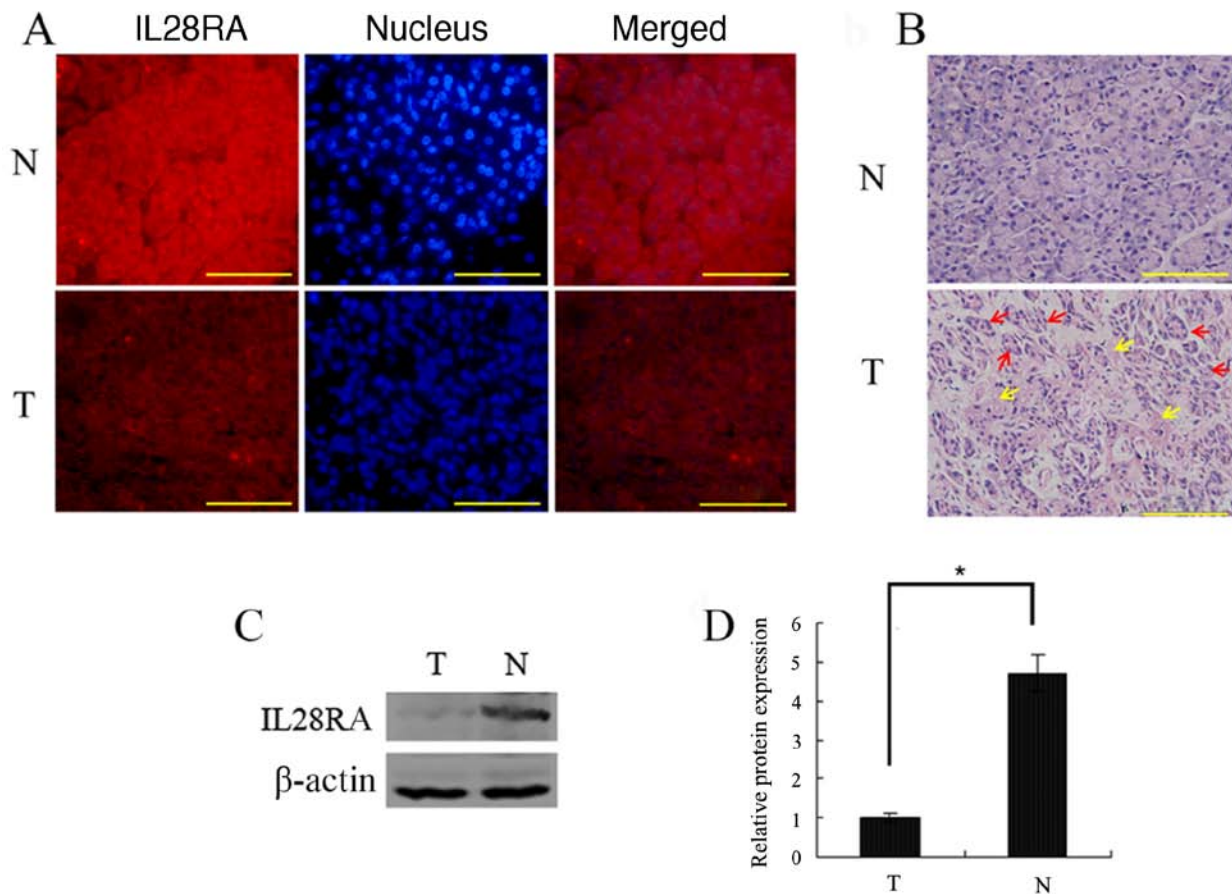


Figure 1. IL-28RA expression levels in the PDAC and paired paracancerous normal pancreatic tissues. (A) Immunofluorescence analysis of IL-28RA expression in PDAC and paired paracancerous normal pancreatic tissues (scale bar, 100 μ m). n=8. (B) Representative hematoxylin and eosin staining of PDAC and paired paracancerous normal pancreatic tissues (scale bar, 100 μ m). Red arrows indicate the ductal glandular structure formed by tumor cells and yellow arrows indicate abundant fibrous stroma. (C) IL-28RA expression was measured in the PDAC and paired paracancerous normal pancreatic tissues, (D) which was quantified. *P<0.05. PDAC, pancreatic ductal adenocarcinoma; T, tumor; N, normal; IL-28RA, interleukin-28 receptor α subunit.

increased cell viability compared with that in cells transfected with the scrambled shRNA (Fig. 4A). Furthermore, the results of western blotting revealed that STAT1 phosphorylation was markedly increased in the PANC-1 cells overexpressing IL-28RA when compared with that transfected with the pCMV6 control plasmid vector, but no obvious change was observed in the IL-28RA-knockdown cells compared with that in the scrambled shRNA control group. The levels of pAKT were upregulated in the IL-28RA-knockdown cells and decreased in the IL-28RA-overexpressing cells compared with those in cells transfected with scrambled shRNA and pCMV6 vector control, respectively (Fig. 4C), following treatment with IL-29.

Next, JAKI (800 nM) or LY294002 (50 μ M) combined with 200 ng/ml IL-29 were used to treat cells to determine which component of the JAK-STAT and PI3K-AKT signaling pathways was involved in mediating the effects of IL-29 and IL-28RA in stably transfected PANC-1 cells. Compared with cells treated with IL-29 alone, JAKI reversed the IL-29/IL-28RA-induced increases in pSTAT1 levels, whereas LY294002 reversed the shIL-28RA-induced increases in p-AKT (Fig. 4D). JAKI significantly reversed the inhibitory effects of IL-29 on the viability of IL-28RA-overexpressing cells, whilst blocking pAKT using LY294002 significantly reduced cell viability in cells transfected with shIL-28RA (Fig. 4B).

IL-28RA regulates cell cycle progression via regulation of cyclin B1 expression. Cell cycle distribution of the transfected PANC-1 cells was next evaluated following treatment with IL-29 (200 ng/ml) for 72 h. The proportion of cells in the G₂/M phases was significantly increased in IL-28RA-knockdown cells when compared with that in the scrambled shRNA control cell group, whilst IL-28RA overexpression resulted in a significant reduction in the proportion of cells in the G₂/M phase compared with cells transfected with the control pCMV6 plasmid (Fig. 5A and B). In addition, compared with their corresponding transfection controls (scrambled shRNA and pCMV6), western blotting showed that cyclin B1 expression was downregulated by the overexpression of IL-28RA, but was upregulated by IL-28RA knockdown (Fig. 5C).

Additionally, the effect of the JAKI and LY294002 treatment on IL-28RA-overexpressing and IL-28RA-knockdown cells, respectively, on cell cycle distribution was investigated. As shown in Fig. 5D, JAKI significantly increased the proportion of cells in the G₂/M phase cells and significant decrease the proportion of cells in the S phase compared with that in untreated JAKI IL-28RA-overexpressing cells. Compared with those in untreated IL-28RA-knockdown cells, significantly decreased proportions of cells in the G₂/M phase were observed in LY294002-treated cells. Western blotting showed that, when compared to the transfected cells or the transfected

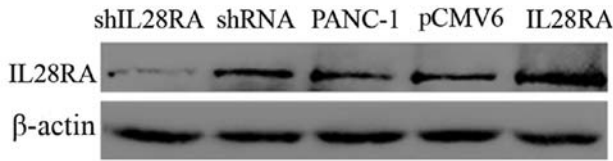


Figure 2. Transfection of IL-28RA targeting shRNA or IL-28RA overexpression vector into PANC-1 cells. shIL-28RA, knockdown of IL-28RA expression using shIL-28RA. shRNA, scrambled negative control for shIL-28RA transfections. IL-28RA, overexpression of IL-28RA using a recombinant plasmid containing the IL-28RA construct. pCMV6, control vector for IL-28RA overexpression. shRNA, short hairpin; IL-28RA, interleukin-28 receptor α subunit.

cells treated with IL-29, JAK1 upregulated cyclin B1 expression in IL-28RA-overexpressing cells, whilst LY294002 downregulated cyclin B1 expression in IL-28RA-knockdown cells, but with no obvious changes were observed in cells treated with only IL-29 (Fig. 5E).

Overexpression of IL-28RA expression results in impaired cell motility. *In vitro* wound healing assays were performed to assess the effects of IL-28RA on PANC-1 cell migration following treatment with IL-29 for every condition. Overexpression of IL-28RA significantly reduced PANC-1 cell migration at 48 h compared with that in cells transfected with the pCMV6 control plasmid (Fig. 6A), as shown by the increased wound area. Opposite trend was observed in IL-28RA-knockdown cells, which exhibited significant increases in migration at both 24 and 48 h, with reduced residual wound areas when compared with those in cells transfected with the non-targeting control shRNA (Fig. 6A). Cell migration in IL-28RA-overexpressing and IL-28RA-knockdown PANC-1 cells was investigated following treatment with JAK1 and LY294002, respectively (Fig. 6B and C). The results showed a reduction in wound area at both 24 and 48 h in the JAK1-treated, IL-28RA-overexpressing cells compared with that in untreated IL-28RA-overexpressing cells, suggesting increased migration in the former. By contrast, there was a significant increase in wound area at 24 and 48 h in LY294002-treated, shIL-28RA-transfected cells compared with that the untreated IL-28RA-silenced cells, suggesting that LY294002 reduced cell migration in this case. These results suggest that IL-28RA may inhibit the migratory potential of PANC-1 *in vitro*. JAK1 and LY294002 partially reversed the inhibitory or enhancing effects on PANC-1 cell migration by IL-28RA-overexpression or IL-28RA knockdown, respectively (Fig. 6B and C).

Knockdown of IL-28RA promotes development of PDAC cells *in vivo*. To further examine the biological effects of IL-28RA in the occurrence and progression of PDAC *in vivo*, pancreatic cancer cells with IL-28RA expression enhanced or knocked down were subcutaneously injected into mice to analyze tumorigenesis *in vivo*. As shown in Fig. 7A-D, the volumes and weights of tumors formed by IL-28RA-overexpressing PANC-1 cells were smaller and lighter compared with those in mice injected with the cells transfected with the pCMV6 control with significant differences after 12 days growth. Conversely, in mice injected with the shIL-28RA-transfected PANC-1 cells, the tumors were larger and heavier compared with those in mice injected with the scrambled shRNA control PANC-1

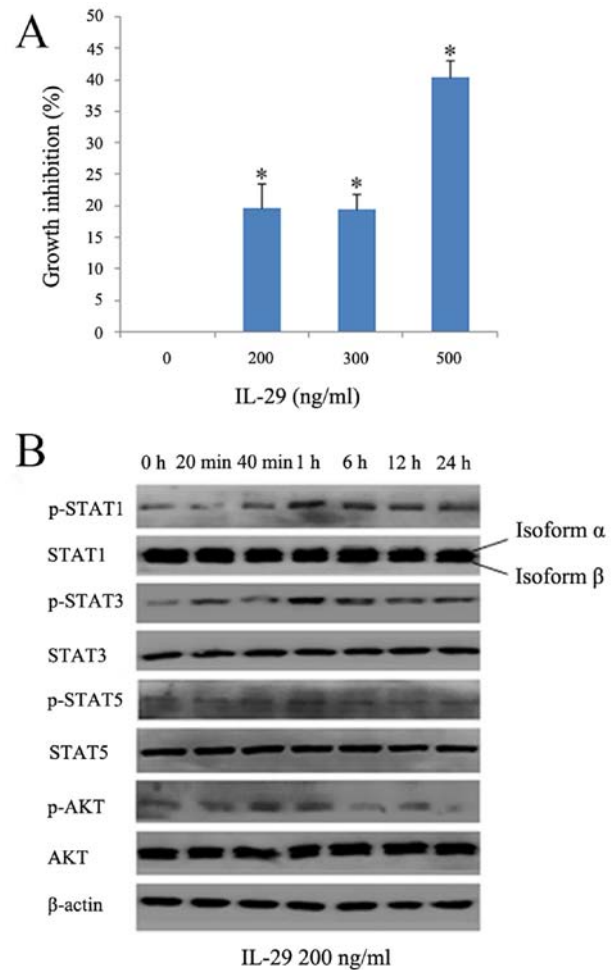


Figure 3. IL-29/IL-28R affects PANC-1 cell growth and phosphorylation levels of signaling proteins in the JAK/STAT and PI3K/AKT pathways. (A) MTS assay analysis of increasing doses of IL-29 on PANC-1 cell growth. * $P < 0.05$ vs. 0 ng/ml. (B) Western blot analysis of members of the JAK/STAT and PI3K/AKT signaling pathways following treatment with 200 ng/ml IL-29 for the indicated time periods in PANC-1 cells. The specific antibody against STAT1 recognizes both isoforms α and β with different molecule weights of 91 and 84 kDa respectively. The specific antibody against AKT recognizes both isoform 1 and isoform 2 with different molecule weights of 56 and 48 kDa, respectively. IL-28RA, interleukin-28 receptor α subunit; p-, phosphorylated; IL, interleukin; JAK, Janus kinase; JAK, Janus kinase.

cells, with significant differences after 6 days. The maximum tumor diameter and the maximum tumor volume observed were 14 mm and 300 mm³, respectively. Together, the *in vitro* and *in vivo* experiments showed that IL-28RA exhibited a suppressive effect on tumor growth in PANC-1 cells.

Discussion

Pancreatic cancer frequently develops in an insidious but rapid manner during the early stages, making early diagnosis difficult (3). However, at latter stages, it exhibits a high degree of invasiveness and metastasis (3). The curative effects of conventional radiotherapy and chemotherapy is unsatisfactory, since the majority of patients are already diagnosed with moderate to advanced stages pancreatic cancer at presentation (27). To overcome the difficulties of early diagnosis and poor treatment efficacy, development of novel diagnostic and therapeutic methods for pancreatic cancer are required.

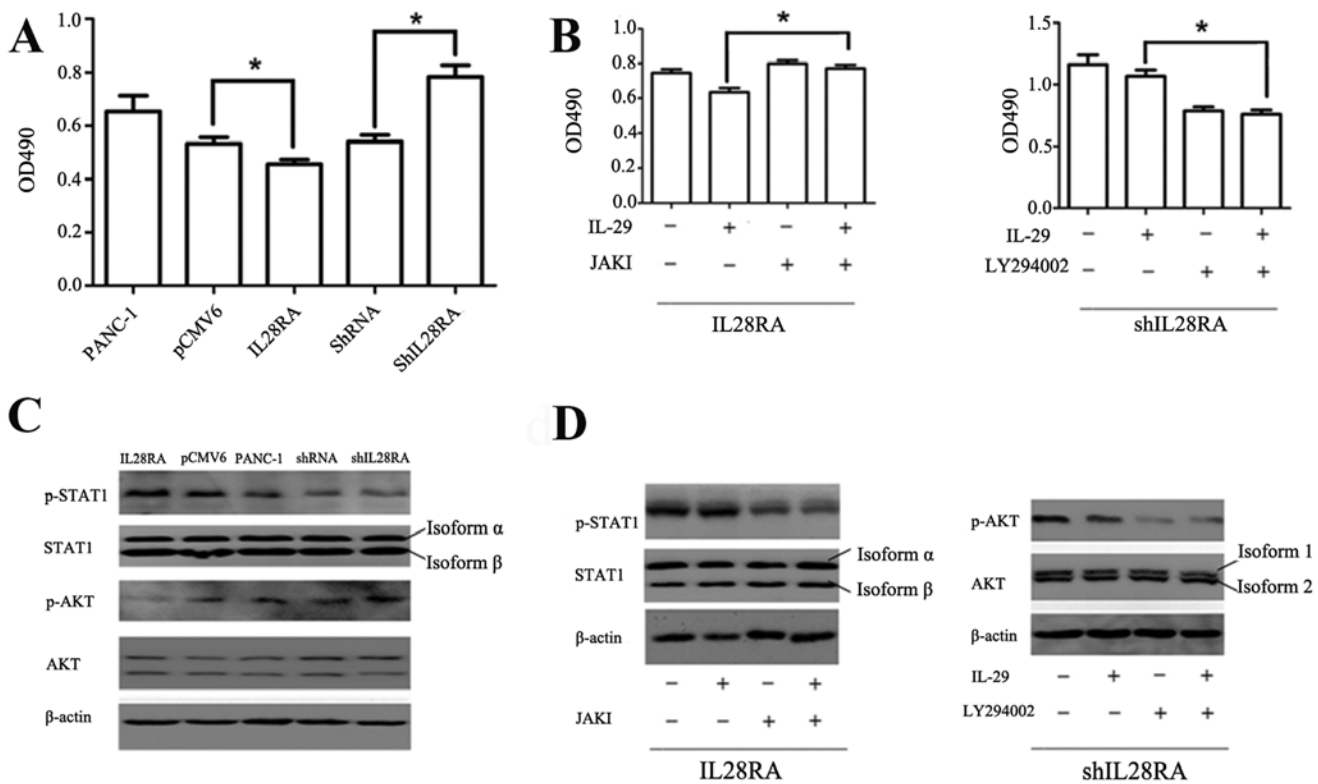


Figure 4. Effects of IL-28RA overexpression or knockdown on PANC-1 cell viability in the presence of JAKI or LY294002, respectively. (A) MTS analysis of the effects of IL-28RA overexpression or IL-28RA knockdown on PANC-1 cell viability. * $P < 0.05$. (B) MTS analysis of the effects of JAKI or LY294002 on cell viability in IL28RA-overexpressing cells or IL-28RA-knockdown cells, respectively, in the presence of IL-29. * $P < 0.05$. (C) Western blotting of STAT1 and AKT phosphorylation levels in the transfected PANC-1 cell lines. (D) Western blot analysis of STAT1 phosphorylation levels in IL-28RA-overexpressing cells in the absence or presence of JAKI in the presence of IL-29, or the phosphorylation levels of AKT in shIL-28RA-cells with or without LY294002 treatment, in the presence of IL-29. The specific antibody against STAT1 recognizes both isoform α and isoform β with different molecule weights of 91 and 84 kDa, respectively. The specific antibody against AKT recognizes both isoform 1 and isoform 2 with different molecule weights of 56 and 48 kDa, respectively. shRNA, short hairpin RNA; p-, phosphorylated; OD, optical density; IL, interleukin; IL-28RA, interleukin-28 receptor α subunit; JAKI, Janus kinase inhibitor.

The present study was performed to determine whether IL-28RA served important roles in the occurrence and development of PDAC, in addition to its potential as a target for the diagnosis and treatment of PDAC. Immunofluorescence staining and western blotting analysis showed that the IL-28RA expression levels in human PDAC tissues were lower compared with that in matched adjacent precancerous tissues. Stimulation of IL-28RA-mediated signaling by IL-29 resulted in notable inhibition of cell viability, suggesting that IL-28RA may be used as a marker for the diagnosis of PDAC. IL-29/IL-28RA may exhibit anti-proliferative effects and act as an inhibitory factor in PDAC. IL-28RA, the receptor of type III IFNs, has been detected in the majority of human tissues (6), such that IL-28RA has been reported to be implicated in tumor cell proliferation (16,28). Reduced IL-28RA expression in tumor tissues was associated with accelerated tumor growth and low survival rates of patients (29). The anti-tumor activity of type III IFNs governed by IL-28RA has been documented to involve the inhibition of cell proliferation and mitosis, induction of cell cycle arrest and cell apoptosis (30). These aforementioned roles may be mediated by increased caspase activity coupled with the induction of p21 and Rb de phosphorylation (31).

To determine the specific role of IL-28RA and underlying molecular mechanisms, stably transfected PANC-1 cells overexpressing IL-28RA or with IL-28RA expression

knocked down were established together with the pCMV6 vector control and scrambled shRNA control. Overexpression of IL-28RA in PANC-1 cells decreased the proportion of G₂/M phase cells and significantly reduced the viability of pancreatic cells, *in vitro* and *in vivo*. Additionally, reduced migration capacity was observed in PANC-1 cells overexpressing IL-28RA. Conversely, IL-28RA knockdown by shRNA resulted in increased cell viability and migration compared with those in cells transfected with the scrambled shRNA control. These results support the notion that IL-28RA served as a suppressor of PDAC. Western blotting revealed increased cyclin B1 expression in shIL-28RA PANC-1 cells, but cyclin B1 protein expression levels in pCMV-IL-28RA PANC-1 cells was decreased, suggesting that regulation of cyclin B1 expression may be a mechanism by which IL-28RA inhibits PDAC progression.

In mouse embryonic fibroblasts and STAT1-deficient human fibroblasts U3A, STAT is necessary for the inhibitory effects of IFN- γ on cell proliferation (32). JAK-STAT signaling pathway is a key pathway involved in the biological functions of IL-28RA, where STAT downstream is a tumor suppressor protein, suggesting that the JAK/STAT signaling pathway also serves an important role in tumorigenesis in addition to regulating inflammatory and immune responses (33). STAT can also be suppressed by the expression of the proto-oncogene c-Myc to promote cell cycle

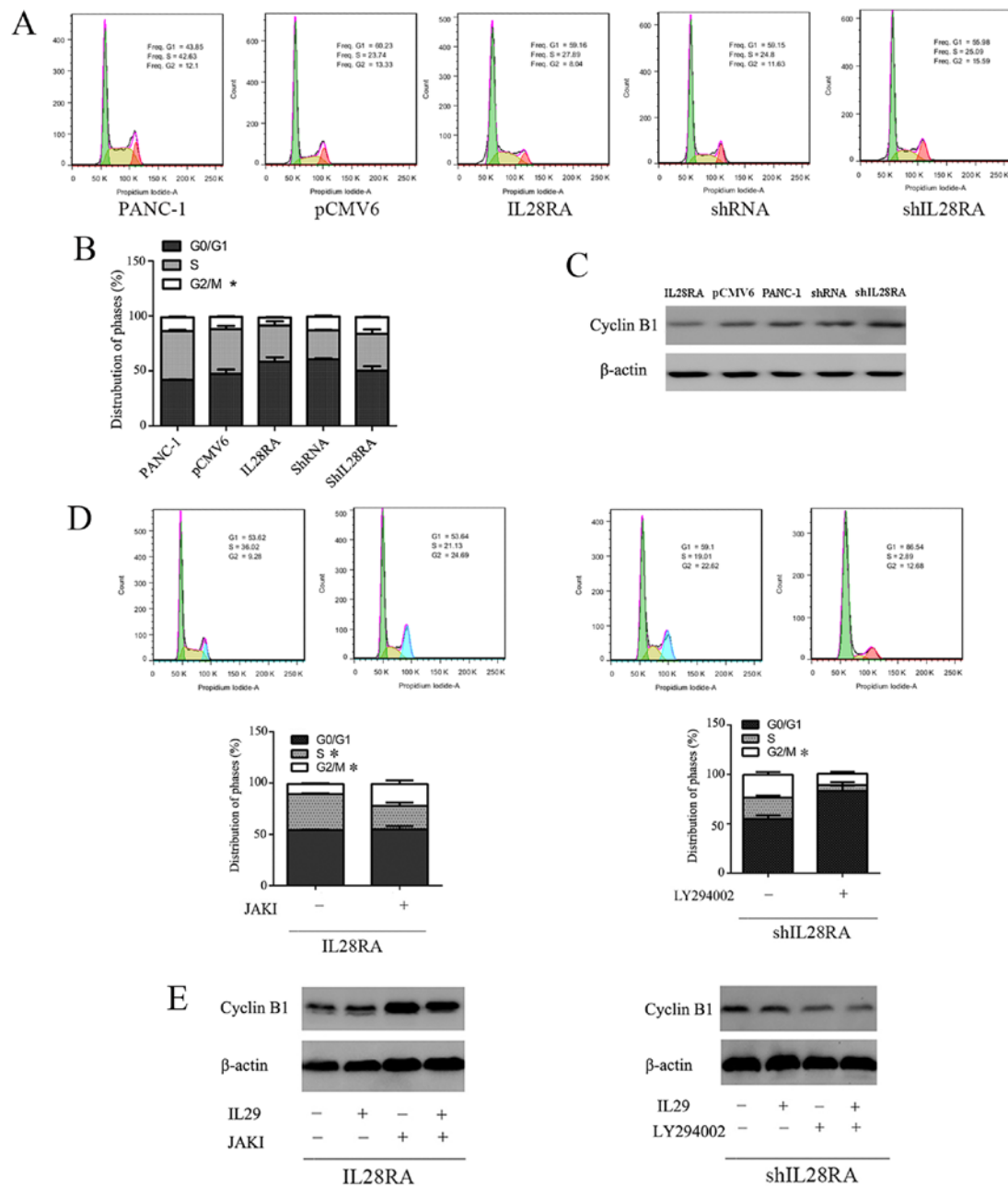


Figure 5. Effects of IL-28RA on cell cycle and cyclin B1 expression, and the effect of JAKI and LY294002 on IL-28RA overexpressing and IL-28RA-knockdown cells. (A) Cell cycle distribution of transfected PANC-1 cells, (B) which was quantified. * $P < 0.05$ IL-28RA vs. pCMV6 and shIL-28RA vs. shRNA. (C) Western blotting of cyclin B1 expression in transfected PANC-1 cells. (D) Effect of JAKI treatment on cell cycle distribution in IL-28RA-overexpressing cells and the effect of LY294002 treatment on cell cycle distribution in IL-28RA-knockdown cells. * $P < 0.05$ vs. cells that were not treated with JAKI or LY294002. (E) Cyclin B1 protein expression in IL-28RA-overexpressing cells after treatment with JAKI and in IL-28RA-knockdown cells after treatment with LY294002, both in the presence of IL-29. shRNA, short hairpin RNA; IL, interleukin; IL-28RA, interleukin-28 receptor α subunit; JAKI, Janus kinase inhibitor.

progression (34). Furthermore, in a previous *in vivo* experiment, fibrosarcoma growth and metastasis was reduced by STAT1-reconstitution in STAT1-deficient fibrosarcoma RAD-105 cells when compared with those in the mice injected with STAT1-deficient fibrosarcoma RAD-105 cells (35).

In addition, previous studies have shown that ~50% of pancreatic cancer types exhibit increased PI3K signaling activity, which can be assessed based on the AKT phosphorylation levels and has been frequently correlated with an undifferentiated state and poor prognosis of the malignant tumor (36,37). It has also been found that the AKT pathway is activated in early precancerous lesions and the initiation of

pancreatic lesions may be associated with KRAS mutations or inflammation (38). LY294002, a specific inhibitor of PI3K, can inhibit pancreatic cancer cell proliferation and G_1 phase progression (39,40). Binding of IL-29 to IL-28RA activates the JAK-STAT and PAKT intracellular signaling pathway and results in anti-proliferative and pro apoptotic functions in human melanoma cells (41).

In studying the signaling mechanism underlying the IL-28RA mediated changes in PDAC cell growth and migration, it was shown that although IL-29 treatment notably activated STAT1, but the effects on phosphorylation levels of AKT were less clear, only increasing slightly after 1 h

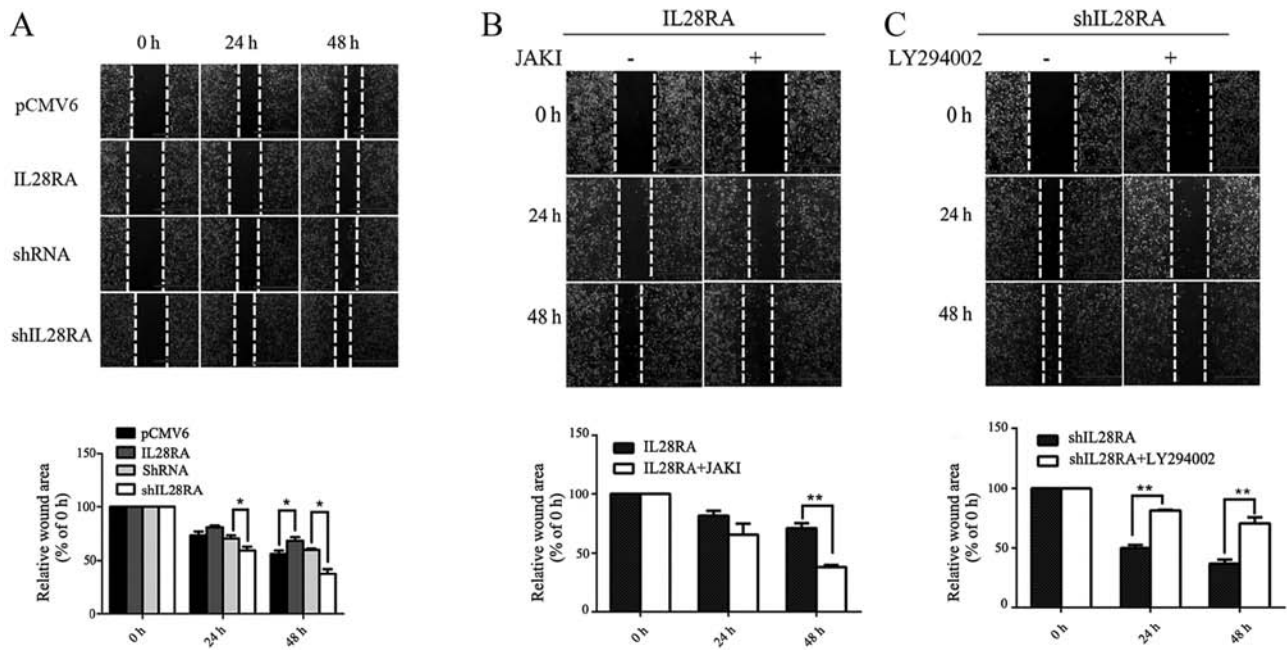


Figure 6. Effects of IL-28RA expression, JAKI and LY294002 on PANC-1 cell migration using wound healing assay. (A-C) The upper panels show representative images of the wound healing assay. Quantitative analysis of the relative wound area (% of 0 h) after 24 and 48 h is shown below. (A) Cell migration in IL-28RA-overexpressing and IL-28RA-knockdown cells. (B) Effect of JAKI on cell migration in IL-28RA-overexpressing cells. (C) Effect of LY294002 on cell migration in shIL-28RA-transfected cells. (Scale bar, 100 μ m). * P <0.05 and ** P <0.01. shRNA, short hairpin; JAKI, Janus kinase inhibitor; IL-28RA, interleukin-28 receptor α subunit.

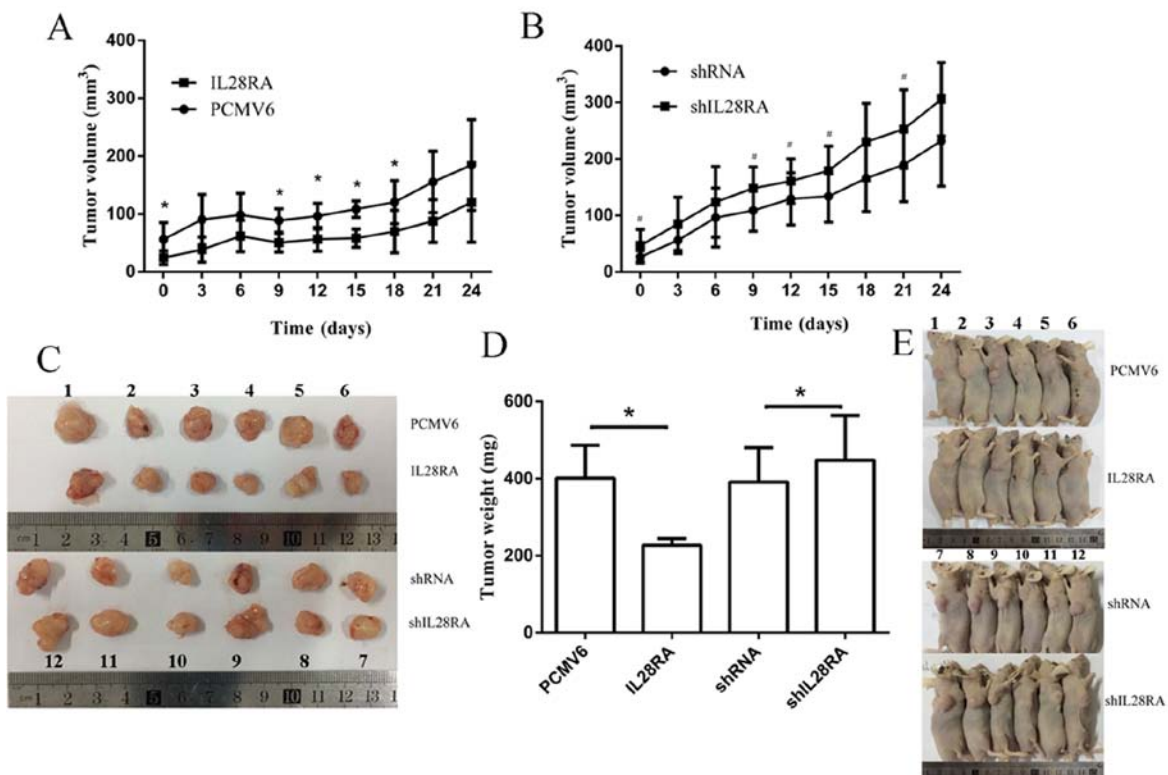


Figure 7. Effects of IL-28RA on the oncogenicity of PANC-1 cells *in vivo*. Tumor volumes in the mice injected with PANC-1 cells (A) overexpressing IL-28RA or (B) with IL28RA knocked down in each group over 24 days. (C) Representative images of the resected tumors from the xenograft mouse model. (D) Tumor weights of each group. (E) Representative images of mice bearing tumors corresponding to those in (B). * P <0.05 vs. pCMV6 group; # P <0.05 vs. shRNA group. ShRNA, short hairpin; IL-28RA, interleukin-28 receptor α subunit.

before decreasing to levels lower than the baseline after 6 h. pSTAT1 levels were also found to be upregulated in the

IL-28RA-overexpressing cells, whilst an increase in p AKT levels was observed in the shIL-28RA-transfected PANC-1

cells. Therefore, it was speculated that IL-28RA activated the JAK-STAT1 pathway to mediate G₁ phase arrest and proliferation inhibition, but in IL-28RA knockdown cells the PI3K-AKT signaling pathway was activated to stimulate cell proliferation. To assess this hypothesis, the JAK inhibitor (JAKI) or PI3K inhibitor (LY294002) were selected to treat the cells overexpressing IL-28RA or with IL-28RA expression knocked down, respectively. JAKI reversed IL-28RA-mediated reduction of cell viability and migration, whilst LY294002 attenuated the increase in cell viability and migration that was stimulated by IL-28RA knockdown.

In summary, IL-28RA was shown to serve as a tumor-inhibiting factor in PDAC both *in vitro* and *in vivo*. Reduced IL-28RA expression was detected in PDAC tissues compared with that in the paracancerous normal tissues. Overexpression of IL-28RA inhibited PANC-1 cell viability and migration and also induced G₂/M phase arrest by decreasing cyclin B1 expression. By contrast, IL-28RA knockdown using shIL-28RA increased cell viability and cell migration by increasing in cyclin B1 expression. JAKI blocked the increase in the phosphorylation levels of STAT1 induced by IL-28RA overexpression and reversed the reductions in cell viability and migration induced by IL-28RA overexpression. In shIL-28RA-transfected cells, AKT was activated and resulted in an increase in cell viability and migration, which was reversed by LY294002. These results suggest that the low expression of IL-28RA in PDAC tissues may contribute to the pathogenesis of PDAC and implicate an important role of IL-28RA in PDAC progression, where sustained IL-29/IL-28RA signaling is partially JAK/STAT and PI3K-AKT dependent. These results may provide insights into the pathogenesis of PDAC and identify novel therapeutic methods for PDAC. However, it should be noted that IL-28RA expression is lower in cancer tissues. Therefore, a method for upregulating its expression followed by examination of its downstream effects is required.

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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

SZ designed the study and revised the manuscript. LL performed the majority of the experiments. DH performed the histological examination. YQ and SZ analyzed and interpreted the data, wrote and revised the manuscript. YX collected the specimen from the patients with PDAC and revised the

manuscript. ZH, YG, ML, BJ and XL analyzed and interpreted the data. SZ and SZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Anhui Medical University (approval no. 20140896, approved on 6 Nov. 2014). The study protocol conformed to the ethical guidelines described in the 1975 Declaration of Helsinki and written informed consent was obtained from each patient included in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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