

# Estrogen-Related Receptor- $\alpha$ Promotes Pancreatic Cancer Progression by Enhancing the Transcription of PAI1 and Activating the MEK/ERK Signaling Pathway

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## Research

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# Abstract

**Background:** Estrogen-related receptor alpha (ERR $\alpha$ ), an orphan nuclear receptor, was reported to be highly associated with the progression and tumorigenesis of several human malignancies. However, the biological role and underlying molecular mechanisms of ERR $\alpha$  in pancreatic cancer (PC) remain unknown.

**Methods:** The expression of ERR $\alpha$  in PC tissues was determined by qRT-PCR and immunohistochemistry. A series of in vitro and in vivo assays were performed to investigate the function of ERR $\alpha$  and Plasminogen activator inhibitor 1 (PAI1) in tumorigenesis in PC cells. The relationship between ERR $\alpha$  and PAI1 was identified by RNA sequencing, Chromatin immunoprecipitation and dual-luciferase reporter assays. The effects of ERR $\alpha$  on the MEK/ERK signaling pathway were determined by western blotting and rescue assays using ERK inhibitor GDC-0994.

**Results:** ERR $\alpha$  was significantly overexpressed in PC tissues and cell lines. Its high expression was correlated with tumor size, distant metastasis, TNM stage, tumor differentiation and poor prognosis of PC. Subsequent functional assays showed that ERR $\alpha$  promoted PC cell proliferation, tumor growth, as well as migration and invasion via activating the epithelial-mesenchymal transition. In addition, knockdown of ERR $\alpha$  induced apoptosis and G0/G1 cell cycle arrest in PC cells. PAI1 was identified by RNA sequencing, knockdown of which could suppress the cell proliferation, migration and invasion that promoted by ERR $\alpha$  overexpression. Further mechanistic investigation using chromatin immunoprecipitation and dual-luciferase reporter assays revealed that ERR $\alpha$  could bind to the PAI1 promoter region and transcriptionally enhance PAI1 expression. Moreover, our data indicated that ERR $\alpha$  played its oncogenic role in PC via activating the MEK/ERK pathway.

**Conclusions:** Our study demonstrates that ERR $\alpha$  promotes PC progression by enhancing the transcription of PAI1 and activation of the MEK/ERK pathway, pointing to ERR $\alpha$  as a novel diagnostic and therapeutic target for PC.

## Background

Pancreatic cancer (PC) is one of the most lethal malignant tumors and the fourth leading cause of cancer-related death worldwide (1)(Siegel et al., 2016). As opposed to the steady increase in survival rates for most cancers, the 5-year survival rates of PC have not improved significantly in the past decades and remain less than 5% (2–4)(G and M, 2017; R et al., 2020; Tempero and Margaret, 2017). The dismal prognosis of PC is mainly due to its rapid disease progression and highly metastatic potential (5, 6)(H et al., 2018; Uccello et al., 2018). To date, the diagnosis and treatment of PC relies mostly on imaging methods and curative resection, which are limited and insufficient in many cases (7)(Zhang et al., 2018). It is reported that most patients with PC are diagnosed at an advanced stage when radical surgery becomes impossible (8, 9)(Alatise et al., 2012; Vincent et al., 2011). Therefore, uncovering the molecular

mechanism governing the process and metastasis of PC as well as identifying novel diagnostic and therapeutic biomarkers are of utmost clinical importance for patients with PC.

Estrogen-related receptor alpha (ERR $\alpha$ ) is an orphan nuclear receptor that structurally similar to estrogen receptors, which could interact with estrogen-responsive elements but not with natural estrogens (10, 11) (Felicity, 2014; Welboren et al., 2009). Accumulating evidence indicate that ERR $\alpha$  acts as an oncogenic regulator in the tumorigenesis of a variety of human cancers (12)(Chang and McDonnell, 2012). For example, the expression of ERR $\alpha$  was aberrantly higher in gallbladder cancer and correlated with poor prognosis (13)(L et al., 2020). In prostate cancer studies, ERR $\alpha$  has been identified as an important negative prognostic factor (14, 15)(Fujimura et al., 2007; Z et al., 2020). However, nothing is currently known regarding the expression and role of ERR $\alpha$  in PC.

In the current study, we performed a series of in vitro and in vivo experiments and revealed that, ERR $\alpha$  was overexpressed in PC tissues and negatively correlated with the prognosis of PC patients. ERR $\alpha$  facilitates PC cell proliferation both in vitro and in vivo, and promotes cell migration and invasion via inducing epithelial-mesenchymal transition (EMT) process. In addition, the knockdown of ERR $\alpha$  induces cell apoptosis and cycle arrest in PC cells. Further mechanistic analysis demonstrated that ERR $\alpha$  plays its oncogenic role in PC by enhancing the transcription of plasminogen activator inhibitor 1 (PAI1, also known as SERPINE1) and activating the MEK/ERK signaling pathway. Our study provides the first data that ERR $\alpha$  might be a novel diagnostic and therapeutic target in PC.

## Methods

### Patient samples and clinicopathologic data

A total of 50 pairs of pancreatic cancer and adjacent non-tumor tissues were obtained from the Department of General Surgery in Xinhua Hospital affiliated with Shanghai Jiao Tong University (Shanghai, China) between 2012 and 2016. All cases hadn't received any radiotherapy or chemotherapy before surgery, and were histologically confirmed and staged based on the American Joint Committee on Cancer Staging Manual (8th edition). The informed consent was obtained from all enrolled patients and the study was approved by Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine (Approval No. XHEC-D-2020-076).

### Immunohistochemistry

Immunohistochemistry (IHC) staining was conducted following the standard staining procedure (16)(X et al., 2019). The ERR $\alpha$  expression level was scored based on the percentage of immunoreactive cells: Negative, < 5% immunoreactive cells; Weak, 5%-34% immunoreactive cells; Moderate, 35%-64% immunoreactive cells; Strong,  $\geq$  65% immunoreactive cells. The total staining score was based on the sum of the extent and intensity, and samples were classified as negative (0–1), weak (2–3), moderate (4–5) and strong (6–7) staining. Among all the PC tissues, 34 were identified as ERR $\alpha$ -high tissues

(moderate and strong staining) and 16 were identified as ERR $\alpha$ -low tissues (negative and weak staining) according to the IHC score.

## Cell culture and reagents

Pancreatic cancer cell lines (Mia PaCa-2, PaTu8988, PANC1), normal human pancreatic ductal epithelial cell line (HPNE) and 293T cell line were obtained from the Shanghai Key Laboratory of Biliary Tract Disease Research, Shanghai, China. All cell lines were cultured in high-glucose DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), and incubated in a humidified incubator with 5% CO<sub>2</sub> at 37°C.

GDC-0994 (HY-15947) was purchased from MedChemExpress and dissolved in dimethylsulfoxide (DMSO). The final DMSO concentration in culture medium was less than 0.1%. Mia PaCa-2 cells were pretreated with 5  $\mu$ M or 10  $\mu$ M GDC-0994 for 24 hours before further assays.

## Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from patient samples and cells using Trizol reagent (Invitrogen). cDNA was synthesized using PrimeScript RT reagent kit with gDNA Eraser (Takara), and the levels of transcripts were detected using SYBR-Green method (Takara) by the StepOnePlus Real-Time thermocycler (Applied Biosystems) following the manufacturer's instructions. The primers sequences are as follows: ERR $\alpha$  forward, 5'-CACTATGGTGTGGCATCCTG-3' and ERR $\alpha$  reverse, 5'-CGCTTGGTGATCTCACACTC-3'; PAI1 forward, 5'-ACCGCAACGTGGTTTTCTCA-3' and PAI1 reverse, 5'-TTGAATCCCATAGCTGCTTGAAT-3'; GAPDH forward, 5'-GGAGCGAGATCCCTCCAAAAT-3' and GAPDH reverse, 5'-GGCTGTTGTCATACTTCTCATGG-3'.

## Cell transfection

ERR $\alpha$  was silenced with small interfering RNAs (siRNAs) using Rfect reagent (BAIDAI) according to the manufacturer's protocol. The sense sequence are as follows: si-ERR $\alpha$ -1 (sense, GCGAGAGGAGUAUGUUCUA; antisense, UAGAACAUAUACUCCUCUCGC); si-ERR $\alpha$ -2 (sense, GAGAGGAGUAUGUUCUACUAA; antisense, UUAGUAGAACAUAUACUCCUCUC); si-PAI1 (sense, UCUCUGCCCUCACCAACAUUC; antisense, GAAUGUUGGUGAGGGCAGAGA). The shRNA targeting ERR $\alpha$  was synthesized using the si-ERR $\alpha$ -1 sequence and inserted into PGMLV-SC5 vector, and the full-length sequence of ERR $\alpha$  was cloned into pCDNA3.1 vector (Genomeditech, Shanghai). Empty vectors were used as control. Cells were infected by concentrated lentivirus at a multiplicity of infection (MOI) of 90 for 48 hours. Cell lines were selected by puromycin (1  $\mu$ g/ml) for 1 week to construct stable transfected cell lines, transfection efficiency was verified by qRT-PCR and western blotting.

## Immunofluorescence assay

Cells were fixed with 4% paraformaldehyde, and immunofluorescence (IF) assay was conducted using Immunol Fluorence Staining Kit with Cy3 (Beyotime, China) following the manufacturer's instructions. Briefly, cells were permeabilized in 0.1% Triton-X-100, blocked by 3% BSA, incubated with primary antibody at 4°C overnight and then incubated with Labeled Goat Anti-Rabbit IgG at room temperature away from light. DAPI was used for cell counterstaining. A fluorescence microscope (Leica) was used to capture photos.

## Western blot analysis

Protein extraction and western blot was performed according to the standard protocol as previously described (17)(S et al., 2019). In brief, equal amounts of proteins were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% skim milk, incubated with specific primary antibodies at 4°C overnight and then with HRP-conjugated secondary antibody at room temperature for 1 h. Protein signals were detected using a Gel Doc 2000 (Bio-Rad, USA). All primary antibodies were purchased from Cell Signaling Technology.

## Cell proliferation assays

Cell Counting Kit-8 (CCK-8) assay and 5-Ethynyl-2'-deoxyuridine (EdU)-488 proliferation assay were performed to evaluate cell proliferation state. For CCK-8 assay, cells were seeded into a 96-well plate at the density of 2000/well with 100  $\mu$ L complete medium overnight. Each well was then replaced with 10  $\mu$ L CCK-8 reagent with 90  $\mu$ L medium, incubated in dark at 37 °C for 2 hours and then using SpectraMax 190 Microplate Reader to measure the optical density at 450 nm.

EdU Cell Proliferation Kit (Beyotime) was used to detect cell proliferation according to the manufacturer's instructions. Cells were incubated with 10  $\mu$ M EdU in complete medium at 37 °C for 2 hours. Azide 488 and Hoechst reagent was used for detection of EdU-positive cells and cell counting, respectively. Images were taken under a fluorescence microscope (Leica).

## Colony formation assay

Transfected cells were plated onto 6-well plates (1000 cells/well) and cultured for 7 days. Then cells were fixed with 4% paraformaldehyde for 20 minutes and stained with 0.1% crystal violet stain solution for 15 minutes. Images of the stained plates were photographed and the colonies were counted.

## Nude mice xenograft models

Female BALB/c nude mice (4 weeks old, weighted 18–22 g) were purchased from Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. The mice were randomly divided into 4 groups (ERR $\alpha$ , vector, shERR $\alpha$ , vector-shRNA) of 5 mice each and housed under appropriate condition.  $2 \times 10^6$  PaTu8988 cells with stable transfection of LV-ERR $\alpha$ , LV-shERR $\alpha$  or their empty vectors were injected into the left axilla of each mouse. The volume of tumor was measured with calipers weekly using the following formula:  $0.5 \times \text{width}^2 \times \text{length}$ . 4 weeks after the cells injection, all mice were sacrificed by cervical dislocation, and the xenograft tumors were carefully dissected and weighed. This animal study was approved by the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine.

## Transwell assays

Transwell migration and invasion assays were applied using chamber inserts (Corning) and BioCoat Matrigel Invasion chamber inserts (Corning), respectively. Treated cells were suspended in 200  $\mu\text{L}$  serum-free medium and seeded in the upper chamber. For both assays, the lower chamber was added with 750  $\mu\text{L}$  complete medium. After 24 hours incubation, cells in the lower surface were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet stain solution. 5 random fields of views were photographed under a microscope.

## Wound healing assay

Cells were seeded in 6-well plates and cultured to more than 90% cell confluence. Wounds were scratched with same strength by use a 200- $\mu\text{L}$  pipette tip. After washing with fresh medium and incubating for 24 hours, images were taken by a microscope at 5 random fields of views. Wound closure percentage was evaluated by comparing the changes before and after 24 hours.

## Apoptosis assay

Annexin V-FITC Apoptosis Detection Kit (Beyotime) was used to detect cell apoptotic ratio following the manufacturer's instructions. Transfected cells were harvested and resuspended with binding buffer, and stained with 5  $\mu\text{L}$  Annexin V-FITC and 10  $\mu\text{L}$  propidium iodide (PI). After 20 minutes incubation at room temperature away from light, stained cells were analyzed by flow cytometry (BD Biosciences).

## Cell cycle analysis

Cell Cycle Analysis Kit (Beyotime) was used for analysis of cell cycle distribution. Transfected cells were collected and fixed with ice-cold 70% ethanol at 4  $^{\circ}\text{C}$  overnight. The next day, cells were incubated with RNase A and PI for 30 minutes. DNA content was measured by flow cytometry (BD Biosciences) and analyzed by FlowJo software.

## RNA sequencing

Total RNA was extracted from PaTu8988 and PANC1 cells after transfection with si-NC or si-ERR $\alpha$ -1, and then subjected to mRNA sequencing on the BGISEQ-500 platform (Beijing Genomics Institute, China). Of all the differentially expressed genes, 40 most up-regulated genes and 40 most down-regulated genes were selected and analyzed using the BGI online analysis system.

## Chromatin immunoprecipitation

The promoter sequence of PAI1 was obtained from NCBI (<https://www.ncbi.nlm.nih.gov/>), the potential binding sites of ERR $\alpha$  in the promoter region of PAI1 were identified by JASPAR (<http://jaspar.genereg.net/>). Chromatin immunoprecipitation (ChIP) was performed using the PaTu8988 cells and a ChIP Assay Kit (Beyotime) according to the manufacturer's protocol. Briefly, cross-linked chromatin was sonicated into 200- to 1000-bp fragments, and then immunoprecipitated with normal rabbit IgG antibody (Cell Signaling Technology) or anti-ERR $\alpha$  antibody (Cell Signaling Technology). Precipitated ChIP samples were analyzed by 2% agarose gel DNA electrophoresis and quantitative PCR (qPCR). Primers for the 2 binding sites are as follows: site 1 forward, 5'-CTCCAACCTCAGCCAGACAA-3' and site 1 reverse, 5'-CCTCCGATGATACACGGCTG-3'; site 2 forward, 5'-CTCCACAGTGACCTGGTTCG-3' and site 2 reverse, 5'-CGGGTGACCCAAAAAGCCTA-3'.

## Dual-luciferase reporter gene assay

293T cells transfected with full-length ERR $\alpha$ , shERR $\alpha$  or their empty vectors were seeded onto 24-well plates and co-transfected with indicated luciferase reporters. pGL3-PAI1-WT-luc (containing the -2000 bp to -1 bp promoter sequence of PAI1) and pGL3-PAI1-MUT-luc (the 2 binding sites of ERR $\alpha$  in the promoter region of PAI1 were mutated) were synthesized by Genomeditech. At 48 hours post-transfection, luciferase activities were determined by a Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instructions. The relative luciferase activities were presented after normalization to Renilla luciferase activity.

## Statistical analysis

All experiments were repeated at least three times. Data were analyzed using Prism 8 software and presented as the mean  $\pm$  standard deviations (SD). The Student's test was performed for the comparisons between two groups, and Pearson chi-square test was used to analyze the correlation between ERR $\alpha$  expression and clinicopathologic variables. Survival analysis was applied by Kaplan-Meier method and log-rank test.  $P < 0.05$  was considered statistically significant.

## Results

# Up-regulated expression of ERR $\alpha$ is associated with poor prognosis in human PC

To identify the biological role of ERR $\alpha$  in PC, we first performed qRT-PCR to detect the mRNA levels of ERR $\alpha$  in 50 pairs of PC tissues and adjacent non-tumor tissues. As shown in Fig. 1A-B, ERR $\alpha$  mRNA expression was significantly higher in PC tissues than in adjacent non-tumor tissues. We also conducted IHC assay to compare the protein expression of ERR $\alpha$  in these samples (Fig. 1D). The results of IHC were consistent with that of qRT-PCR to suggest an up-regulation of ERR $\alpha$  in PC tissues at the protein level (Fig. 1E). Then we determined the relationship between ERR $\alpha$  and clinical features in PC patients. As shown in Table 1, high ERR $\alpha$  expression was associated with tumor size ( $P=0.0065$ ), distant metastasis ( $P=0.0026$ ), TNM stage ( $P=0.0021$ ) and tumor differentiation ( $P=0.0045$ ). Moreover, Kaplan-Meier analysis of overall survival (OS) revealed that upregulated expression of ERR $\alpha$  was correlated with poor prognosis in PC ( $P<0.0001$ , Fig. 1C).

Table 1  
Correlation between ERR $\alpha$  expression and clinicopathological characteristics in 50 PC patients.

Parameters	Number of cases	ERR $\alpha$ expression		p-value ( $\chi^2$ -test)
		Low	High	
Gender				NS
Male	29	9	20	
Female	21	7	14	
Age (years)				NS
< 60	28	9	19	
$\geq$ 60	22	7	15	
Location				NS
Head, urinate	22	8	14	
Body, tail	28	8	20	
Tumor Size (cm)				<b>0.0065**</b>
< 2	30	14	16	
$\geq$ 2	20	2	18	
Lymphatic invasion				NS
Positive	21	6	15	
Negative	29	10	19	
Distant metastasis				<b>0.0026**</b>
Positive	18	1	17	
Negative	32	15	17	
TNM Stage				<b>0.0021**</b>
I-II	28	14	14	
III-IV	22	2	20	
Tumor differentiation				<b>0.0045**</b>
High/ Moderate	26	13	13	
Poor	24	3	21	

## **ERRα expression in PC cell lines**

We performed IF assay, qRT-PCR and western blot assay to detect the expression of ERRα in PC cell lines (PaTu8988, PANC1 and Mia PaCa-2) and HPNE. The results revealed that ERRα was expressed in the nuclei of cells and overexpressed in PC cell lines compared with HPNE (Fig. 2A-C). To investigate further, we knocked down ERRα in PaTu8988 and PANC1 cells using siRNAs and overexpressed ERRα in Mia PaCa-2 cells. The transfection efficiency was validated by qRT-PCR and western blot analysis (Fig. 2D).

## **ERRα promotes PC proliferation in vitro and in vivo.**

To explore the effects of ERRα on PC cell proliferation in vitro, we conducted CCK-8 assay, EdU-488 proliferation assay and colony formation assay. As shown in Fig. 3A-C, the proliferation ability of PC cells was markedly suppressed after ERRα depletion, whereas was enhanced after ERRα overexpression. We also investigated the effect of ERRα on PC tumor growth in vivo. We used PaTu8988 cells with stable transfection of LV-ERRα, LV-shERRα or their empty vectors (Fig. 3D) to construct xenograft models. As indicated by the volume, weight and growth curves of the tumors, ERRα overexpression significantly promoted the growth of PaTu8988 cells in xenograft models, whereas ERRα silencing did the opposite (Fig. 3E and F). These results indicated a growth-promoting role for ERRα on PC in vitro and in vivo.

## **ERRα facilitates PC cell migration and invasion through EMT**

To determine the effect of ERRα on PC metastasis, we assessed PC cell migration and invasion abilities in vitro using wound healing assay as well as transwell migration and invasion assays. As shown in Fig. 4A-D, ERRα depletion notably suppressed the migration and invasion ability of PaTu8988 and PANC1 cells compared with negative controls. Conversely, ERRα overexpression remarkably enhanced the cell migration and invasion in Mia PaCa-2 cells. As the EMT process played a vital role in the cancer metastasis (18)(I and C, 2019), we explored whether EMT was involved in the effect of ERRα on migration and invasion. As shown in Fig. 4E and F, knockdown of ERRα significantly increased the expression levels of epithelial marker E-cadherin, but decreased the expression levels of mesenchymal markers N-cadherin, Vimentin and Snail. What's more, the overexpression of ERRα presented exactly the opposite results. Collectively, these results indicated that ERRα promotes the ability of migration and invasion via inducing EMT in PC cells.

## **Knockdown of ERRα induces cell apoptosis and cycle arrest in PC cells**

To assess whether the proliferation defects observed in ERR $\alpha$ -depleted PC cells were due to the cell apoptosis and arrest of cell cycle progression, we performed flow cytometry assays to evaluate the effects of ERR $\alpha$  knockdown on PC cell apoptosis and cell cycle regulation. The results demonstrated that ERR $\alpha$  silencing significantly increased the apoptosis rate in PC cells, and the analysis of cell cycle distribution showed that the proportion of G0/G1 phase cells was remarkably increased and that of S-phase cells was decreased after ERR $\alpha$  knockdown (Fig. 5A and C). In addition, we detected the expression of apoptosis-related and cell cycle-related markers by western blotting and IF assay (Fig. 5B, D and E). The data revealed that protein expression levels of cleaved-PARP, cleaved-caspase 3, cytochrome c, p21 Waf1/Cip1 and p27 Kip1 were significantly increased while that of bcl-2, CDK2, CDK4 and cyclin D1 were decreased after ERR $\alpha$  knockdown. Moreover, ERR $\alpha$  overexpression led to diametrically opposite results. Taken together, the above results provide more insight into the mechanism and indicate that knockdown of ERR $\alpha$  inhibits PC cell proliferation by inducing apoptosis and arrest of the G0/G1 phase.

## **PAI is upregulated and correlated with poor prognosis in PC**

RNA-sequencing was performed to further explore the potential mechanism by which ERR $\alpha$  affects pancreatic cancer progression. Compared with the negative control cells, we selected the top 40 down-regulated and top 40 up-regulated genes in PaTu8988 and PANC1 cells after ERR $\alpha$  knockdown, among which the plasminogen activator inhibitor 1 (fold change= -1.600,  $p < 0.001$ ) attracted our attention (Fig. 6A). PAI1, a serine protease inhibitor, has been reported to be involved in the development and progression of several cancers (19–21)(BA et al., 2019; I et al., 2019; JD et al., 2019), however, little is known about the role of PAI1 during tumorigenesis of PC. Based on the GEPIA database, we discovered that the expression level of PAI1 was significantly higher in PC tissues compared with normal tissues (Figure, 6B). What's more, the survival analysis indicated that PC patients with higher PAI1 expression had shorter OS ( $p = 0.017$ , Fig. 6C) and disease free survival (DFS,  $p = 0.00028$ , Fig. 6D). Then the expression levels of PAI1 in PC cell lines and HPNE cell line were determined by qRT-PCR and western blotting. The results demonstrated that PAI1 was upregulated in PC cell lines compared with HPNE cells (Fig. 6E). In addition, the expression levels of PAI1 could be decreased by transfection with si-ERR $\alpha$ -1 or si-PAI1 and increased by ERR $\alpha$  overexpression (Fig. 6F).

## **PAI1 mediates the oncogenic behavior of ERR $\alpha$ in PC cells.**

We then investigated whether PAI1 is involved in ERR $\alpha$ -mediated oncogenic effects on PC cells. As shown in Fig. 7A-E, the knockdown of PAI1 remarkably inhibited the cell proliferation, migration and invasion in PaTu8988 and PANC1 cells. More importantly, PAI1 depletion could rescue the cell proliferation, migration and invasion enhanced by ERR $\alpha$  overexpression in Mia PaCa-2 cells. Therefore, it is hypothesized that PAI1 participates in the ERR $\alpha$ -induced oncogenic effects in PC.

# ERR $\alpha$ enhances the transcription of PAI1 via directly binding to its promoter

We then investigated the mechanisms responsible for ERR $\alpha$  regulated PAI1. Bioinformatic analysis was performed using JASPAR database and 2 high-scoring predicted binding sites of ERR $\alpha$  in the PAI1 promoter sequence were found (Fig. 8A and B). ChIP assays were conducted in PaTu8988 cells to confirm the bounding relationship between ERR $\alpha$  protein and PAI1 promoter. ChIP samples were further analyzed via DNA electrophoresis and qPCR. The results of DNA electrophoresis showed the predicted DNA band in Input and ERR $\alpha$  groups but not in the IgG group (Fig. 8C), and the results of qPCR confirmed the enrichment of PAI1 promoter in ERR $\alpha$  precipitates (Fig. 8D).

Subsequent dual luciferase assays demonstrated that the luciferase activity of WT PAI1 promoter reporter was notably increased by ERR $\alpha$  overexpression and decreased by ERR $\alpha$  inhibition. What's more, the luciferase activity of MUT PAI1 promoter was unaffected by ERR $\alpha$  (Fig. 8E). Together, these data indicated that ERR $\alpha$  could bind to the promoter region of PAI1 and transcriptionally enhance the expression of PAI1.

## ERR $\alpha$ promotes PC progression through the MEK/ERK signaling pathway

Previous studies have reported that ERR $\alpha$  is associated with the PI3K/AKT pathway (22, 23)(HS, 2015; LD et al., 2015), and the MEK/ERK pathway has crosstalk with PI3K/AKT pathway and plays a vital role in PC (24, 25)(CC, 2018; YW et al., 2018). Therefore, we investigated whether MEK/ERK signaling pathway was responsible for the oncogenic effects of ERR $\alpha$  in PC. As shown in Fig. 8F, the expression levels of p-MEK1/2 and p-ERK1/2 were significantly decreased by ERR $\alpha$  knockdown and increased by ERR $\alpha$  overexpression. What's more, silencing of PAI1 could rescue the effects of ERR $\alpha$  overexpression on p-MEK1/2 and p-ERK1/2. To further determine whether MEK/ERK signaling pathway is critical for ERR $\alpha$  function, rescue experiments were conducted using the ERK inhibitor GDC-0994. The CCK-8 and transwell assays revealed that GDC-0994 attenuated the cell proliferation, migration and invasion enhanced by ERR $\alpha$  overexpression (Fig. 8G and H). These results indicate that ERR $\alpha$  promotes PC progression via activating the MEK/ERK signaling pathway.

## Discussion

Accumulating evidence has confirmed the aberrant expression of ERR $\alpha$  in human cancers, and increasing attention has been focused on the vital role of ERR $\alpha$  in the processes related to cancer progression, such as tumor growth, metastasis and chemoresistance (14, 16, 26, 27)(G et al., 2019; S et al., 2018; X et al., 2019; Z et al., 2020). In the present study, we demonstrated that ERR $\alpha$  was extensively upregulated in PC tissues, and its high expression was associated with poor prognosis and several clinicopathological characteristics, such as tumor size, distant metastasis, TNM stage and tumor differentiation in PC

patients. Subsequently, a series of in vitro and in vivo assays were conducted and the results showed that, ERR $\alpha$  promoted PC cell proliferation, migration, invasion as well as induced cell apoptosis and G0/G1 cycle arrest via enhancing the transcription of PAI1 and activating the MEK/ERK signaling pathway. To our knowledge, this is the first study to identify the critical role of ERR $\alpha$  in PC progression, suggesting that ERR $\alpha$  may be a novel diagnostic and therapeutic target for PC.

The present study revealed that ERR $\alpha$  was highly expressed in PC cell lines and knockdown of ERR $\alpha$  could suppress the proliferation and colony formation of PC cells. In addition, EdU proliferation assays demonstrated that ERR $\alpha$  inhibition notably reduced the percentage of EdU-positive cells, suggesting a decreased in DNA replication. Consistent with the in vitro assays, our in vivo tumor growth assay showed that ERR $\alpha$  overexpression increased the PC growth whereas ERR $\alpha$  silencing exerted the opposite effect. Furthermore, flow cytometry analysis indicated that the suppressive effects of ERR $\alpha$  knockdown on the proliferation of PC may be due to induction of cell apoptosis and cell cycle arrest. Apoptosis is a crucial gene-directed program that is engaged to regulate normal cellular growth and development (28)(I and AB, 2019). The apoptosis signaling cascade can be divided into 2 major pathways: the death receptor pathway (also known as extrinsic pathway) and the mitochondrial pathway (also known as intrinsic pathway) (29) (GM et al., 2012). In the intrinsic pathway, cytochrome c is induced by apoptotic stimuli and released from mitochondria into cytosol, interacts with Apaf-1 and activates downstream effector caspases including caspase 3 (29, 30)(DR and G, 2004; GM et al., 2012). Bcl-2 proteins function as gatekeepers for cytochrome c and caspase 3 acts as a vital executioner to cleave the downstream cellular substrates, such as PARP (31, 32)(R et al., 2014; Thornberry and Lazebnik, 1998). In our study, it was found that ERR $\alpha$  knockdown upregulated protein expression of cytochrome c, cleaved-caspase 3, cleaved-PARP while downregulated that of bcl-2, suggesting that intrinsic pathway was involved in ERR $\alpha$  knockdown induced cell apoptosis. Aberrant function of cell cycle regulators lead to deregulation of cell cycle progression that result in uncontrolled cell proliferation. The cell cycle analysis demonstrated that ERR $\alpha$  knockdown significantly decreased the proportion of S phase cells and increased the G0/G1 phase cells. Consistent with the cell cycle analysis, the western blot results showed that the expression of CDK2, CDK4, cyclin D1 was downregulated while p21 Waf1/Cip1 and p27 Kip1 was upregulated after ERR $\alpha$  knockdown, indicating the cell cycle arrest at G0/G1 phase. These data validate a crucial role for ERR $\alpha$  in PC growth.

Pancreatic cancer is extremely aggressive and has a high metastatic potential (33)(M and IIC, 2019). Increasing evidence supports the notion that EMT is a vital step in the progression and metastasis of pancreatic cancer (34)(M et al., 2015). Our present study showed that the silencing of ERR $\alpha$  notably inhibited the migration and invasion of PC cells while the overexpression of ERR $\alpha$  enhanced that of PC cells. More important, the best characterized alteration of EMT process, such as the loss of E-cadherin, and the upregulation of N-cadherin, vimentin and snail was enhanced by ERR $\alpha$  overexpression while suppressed by ERR $\alpha$  knockdown. These data suggested that the oncogenic effects of ERR $\alpha$  on PC cell migration and invasion was associated with the activation of EMT process, indicating the potential benefits of anti-ERR $\alpha$  therapy for PC patients with established metastases.

Plasminogen activator inhibitor 1 (PAI1) belongs to the serpin superfamily and is a unique type of serine protease inhibitor (19)(BA et al., 2019). The deregulation of PAI1 expression has been involved in various types of cancers (35)(C and DJ, 2005), however, the specific functions and roles of PAI depend on the type of cancer. PAI1 overexpression increased cell migration and invasion in rectal cancer and triple-breast cancer (36, 37)(S et al., 2020; W et al., 2018), whereas it inhibits proliferation in prostate cancer (38)(SC et al., 2008). In this work, we found that PAI1 is significantly overexpressed in PC tissues compare with normal tissues, and its high expression leads to shorter OS and DFS in PC patients. Subsequent in vitro assays demonstrated that knockdown of PAI1 could attenuate cell proliferation, migration and invasion enhanced by ERR $\alpha$  overexpression. Mechanically studies indicated that ERR $\alpha$  can bind to the promoter region of PAI1 and then increase its transcription.

To further explore the potential molecular mechanism by which ERR $\alpha$ /PAI1 promoted PC proliferation, migration and invasion, we focused our attention on the MEK/ERK pathway which had been reported to play a vital role in PC progression (24, 25, 39)(CC, 2018; YW et al., 2018; ZH et al., 2018). The western blot analysis showed that the expression levels of p-MEK1/2 and p-ERK1/2 were decreased by ERR $\alpha$  inhibition, and knockdown of PAI1 can reverse the increasing levels of p-MEK1/2 and p-ERK1/2 induced by ERR $\alpha$  overexpression. In the subsequent rescue experiments, it was noted that ERK inhibitor GDC-0994 suppressed the cell proliferation, migration and invasion induced by overexpressed ERR $\alpha$ , supporting the hypothesis that ERR $\alpha$ /PAI1 promotes PC progression through the MEK/ERK signaling pathway.

## Conclusions

In conclusion, we determined that ERR $\alpha$  is significantly upregulated in PC tissues and results in poor prognosis of PC patients. It can promote cell proliferation, migration and invasion via promoting the transcription of PAI1 and activating the MEK/ERK pathway. Our study provides the first evidence that ERR $\alpha$  might be a potential diagnostic and therapeutic target for PC.

## List Of Abbreviations

PC, pancreatic cancer; ERR $\alpha$ , Estrogen-related receptor alpha; EMT, epithelial-mesenchymal transition; PAI1, plasminogen activator inhibitor 1; IHC, immunohistochemistry; DMSO, dimethylsulfoxide; qRT-PCR, quantitative real-time PCR; MOI, multiplicity of infection; IF, immunofluorescence; CCK-8, Cell Counting Kit-8; EdU, 5-Ethynyl-2'-deoxyuridine; PI, propidium iodide; ChIP, chromatin immunoprecipitation; qPCR, quantitative PCR; SD, standard deviations; OS, overall survival; DFS, disease free survival.

## Declarations

## Ethics approval and consent to participate

This study was approved by the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine.

## Consent for publication

Not applicable.

## Availability of data and materials

The data of the current study are available from the corresponding author upon reasonable request.

## Competing interests

The authors declare that they have no competing interests.

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## Author's contributions

SL, XS, WG and XW conceived and designed the research. SL and XS performed the experiments. FL, WY, ZW and PD partially participated in the experiments. SL, XS, FL and PD analyzed the data and interpreted the data. SL, XS, WG and WX drafted, reviewed, and revised the manuscript.

## Acknowledgements

Not applicable.

## References

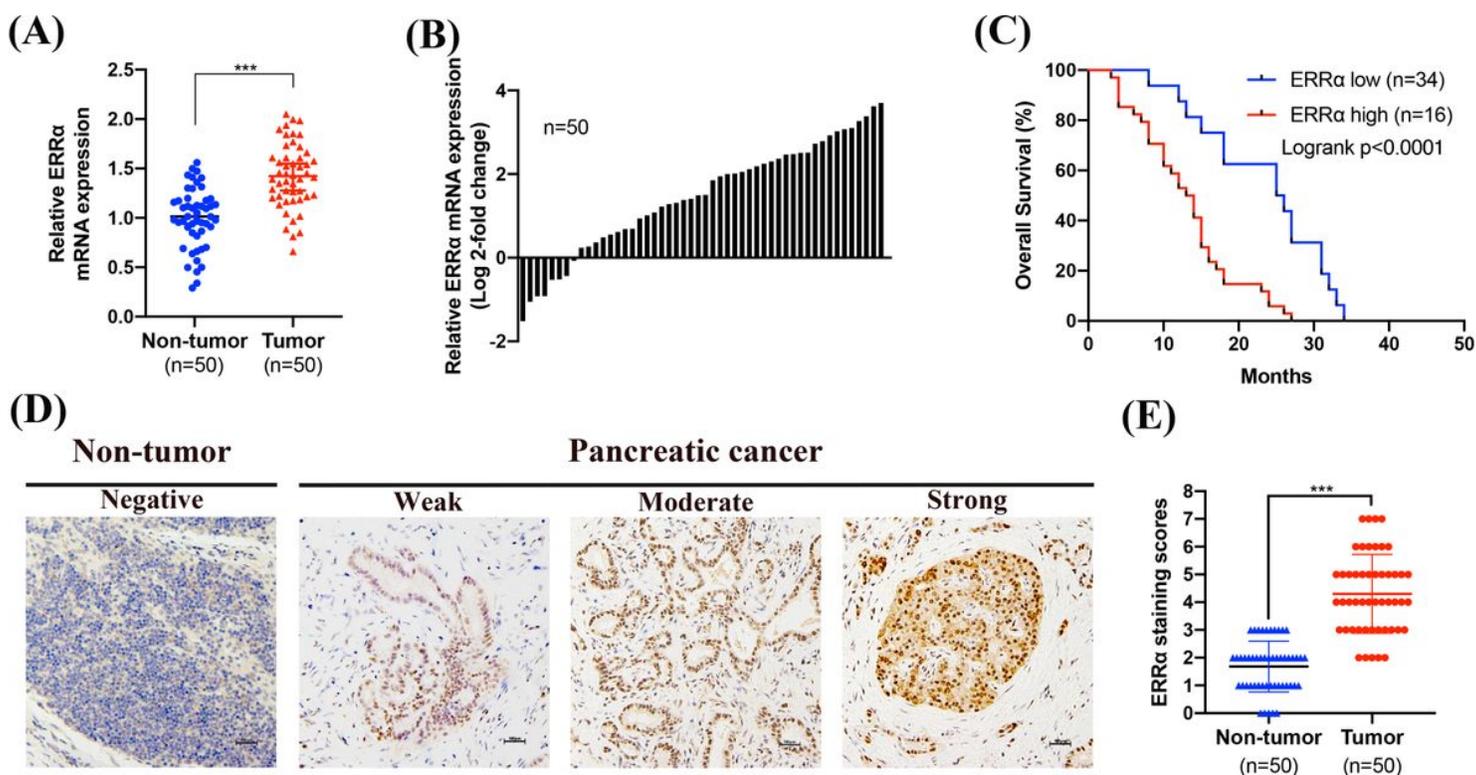
1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA: A Cancer Journal for Clinicians*. 2016;66(1).
2. G G, M O. Systemic Combination Chemotherapy in Elderly Pancreatic Cancer: a Review. *Journal of gastrointestinal cancer*. 2017;48(2):121-8.
3. R O, E Y, S I, D K, H I, K T, et al. High expression of olfactomedin-4 is correlated with chemoresistance and poor prognosis in pancreatic cancer. *PloS one*. 2020;15(1):e0226707.
4. Tempero, Margaret. Active Systemic Treatment of Pancreatic Cancer. *Journal of the National Comprehensive Cancer Network Jncn*. 2017;15(5S):723-5.

5. H J, P L, Y W, X M, M W, J H, et al. Exosomal zinc transporter ZIP4 promotes cancer growth and is a novel diagnostic biomarker for pancreatic cancer. *Cancer science*. 2018;109(9):2946-56.
6. Uccello M, Moschetta M, Mak G, Alam T, Murias HC, H.-T. A. Towards an optimal treatment algorithm for metastatic pancreatic ductal adenocarcinoma (PDA). *Current Oncology*. 2018;25(1):90-.
7. Zhang L, Sanagapalli S, Stoita A. Challenges in diagnosis of pancreatic cancer. *World Journal of Gastroenterology*. 2018;v.24(19):4-17.
8. Alatise OI, Ndububa DA, Ojo OS, Agbakwuru EA, Adekanle O, Arowolo OA. Pancreatic cancer in Nigeria: Past, present and future. *Nigerian Journal of Gastroenterology & Hepatology*. 2012;1(2).
9. Vincent A, Herman J, Schulick R, Hruban RH, Goggins M. Pancreatic Cancer. *Lancet*. 2011;378(9791):607-20.
10. Felicity M. Novel drugs that target the estrogen-related receptor alpha: their therapeutic potential in breast cancer. *Cancer Management & Research*. 2014:225-.
11. Welboren WJ, Sweep FCGJ, Span PN, Stunnenberg HG. Genomic actions of estrogen receptor?: what are the targets and how are they regulated? *Endocr Relat Cancer*. 2009;16(4):1073-89.
12. Chang CY, McDonnell DP. Molecular Pathways: The Metabolic Regulator Estrogen- Related Receptor a as a Therapeutic Target in Cancer. *Clinical Cancer Research An Official Journal of the American Association for Cancer Research*. 2012;18(22).
13. L W, M Y, X G, Z Y, S L, Y J, et al. Estrogen-related receptor- $\alpha$  promotes gallbladder cancer development by enhancing the transcription of Nectin-4. *Cancer science*. 2020.
14. Z X, T M, J Z, W G, Y L, S Y, et al. Nuclear receptor ERR $\alpha$  contributes to castration-resistant growth of prostate cancer via its regulation of intratumoral androgen biosynthesis. *Theranostics*. 2020;10(9):4201-16.
15. Fujimura T, Takahashi S, Urano T, Kumagai J, Inoue S. Increased expression of estrogen-related receptor  $\alpha$  (ERR $\alpha$ ) is a negative prognostic predictor in human prostate cancer. *International Journal of Cancer*. 2007;120(11):2325-30.
16. X Y, J G, H Z, Q M, Y M, R L, et al. The enhanced expression of estrogen-related receptor  $\alpha$  in human bladder cancer tissues and the effects of estrogen-related receptor  $\alpha$  knockdown on bladder cancer cells. *Journal of cellular biochemistry*. 2019;120(8):13841-52.
17. S L, F L, L P, Z Y, Y S, W L, et al. BRD4 inhibitor and histone deacetylase inhibitor synergistically inhibit the proliferation of gallbladder cancer in vitro and in vivo. *Cancer science*. 2019;110(8):2493-506.
18. I P, C B. EMT Transition States during Tumor Progression and Metastasis. *Trends in cell biology*. 2019;29(3):212-26.
19. BA H, JM B, YC C, HR H, T Q, B C, et al. Plasminogen Activator Inhibitor 1 (PAI1) Promotes Actin Cytoskeleton Reorganization and Glycolytic Metabolism in Triple-Negative Breast Cancer. *Molecular cancer research : MCR*. 2019;17(5):1142-54.
20. JD Y, L M, Z Z. SERPINE1 as a cancer-promoting gene in gastric adenocarcinoma: facilitates tumour cell proliferation, migration, and invasion by regulating EMT. *Journal of chemotherapy (Florence,*

- Italy). 2019;31:408-18.
21. I A-S, MÁ P, X L, M L, A G, MV C, et al. Effect of serpinE1 overexpression on the primary tumor and lymph node, and lung metastases in head and neck squamous cell carcinoma. *Head & neck*. 2019;41(2):429-39.
  22. LD Z, L C, M Z, HJ Q, L C, HF C, et al. Downregulation of ERR $\alpha$  inhibits angiogenesis in human umbilical vein endothelial cells through regulating VEGF production and PI3K/Akt/STAT3 signaling pathway. *European journal of pharmacology*. 2015;769:167-76.
  23. HS R. Estrogen-related receptor alpha and mitochondria: tale of the titans. *Journal of receptor and signal transduction research*. 2015;35(5):386-90.
  24. CC S. Tanshinone IIA can inhibit MiaPaCa-2 human pancreatic cancer cells by dual blockade of the Ras/Raf/MEK/ERK and PI3K/AKT/mTOR pathways. *Oncology reports*. 2018;40(5):3102-11.
  25. YW K, JE L, KH J, MK S, SM S, SJ K, et al. KRAS targeting antibody synergizes anti-cancer activity of gemcitabine against pancreatic cancer. *Cancer letters*. 2018;438:174-86.
  26. G V, M B, L B, P R, C B, M G, et al. ERR $\alpha$  promotes breast cancer cell dissemination to bone by increasing RANK expression in primary breast tumors. *Oncogene*. 2019;38(7):950-64.
  27. S Z, H X, H X, Q T, Y N, QY G, et al. ERR $\alpha$  suppression enhances the cytotoxicity of the MEK inhibitor trametinib against colon cancer cells. *Journal of experimental & clinical cancer research : CR*. 2018;37(1):218.
  28. I M, AB A. Involvement of the uridine cytidine kinase 2 enzyme in cancer cell death: A molecular crosstalk between the enzyme and cellular apoptosis induction. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie*. 2019;109:1506-10.
  29. GM M, A N, RW J. Intrinsic and extrinsic apoptotic pathway signaling as determinants of histone deacetylase inhibitor antitumor activity. *Advances in cancer research*. 2012;116:165-97.
  30. DR G, G K. The pathophysiology of mitochondrial cell death. *Science (New York, NY)*. 2004;305(5684):626-9.
  31. Thornberry NA, Lazebnik Y. Caspases: Enemies Within. *Science*. 1998;281(5381):p.1312-6.
  32. R B, Y S, X W, H W, Q D, Y C, et al. Oridonin induces apoptosis and cell cycle arrest of gallbladder cancer cells via the mitochondrial pathway. *BMC cancer*. 2014;14:217.
  33. M AP, IIC C. Metastasis in Pancreatic Ductal Adenocarcinoma: Current Standing and Methodologies. *Genes*. 2019;11(1).
  34. M B, I N, S P, AD I, C B, RI N, et al. The epithelial to mesenchymal transition in pancreatic cancer: A systematic review. *Pancreatology : official journal of the International Association of Pancreatology (IAP) [et al]*. 2015;15(3):217-25.
  35. C D, DJ L. Historical analysis of PAI-1 from its discovery to its potential role in cell motility and disease. *Thrombosis and haemostasis*. 2005;93(4):631-40.
  36. S M-G, M R, J P-S, J M-P, MC F-F, MJ O, et al. PAI1 is a Marker of Bad Prognosis in Rectal Cancer but Predicts a Better Response to Treatment with PIM Inhibitor AZD1208. *Cells*. 2020;9(5).

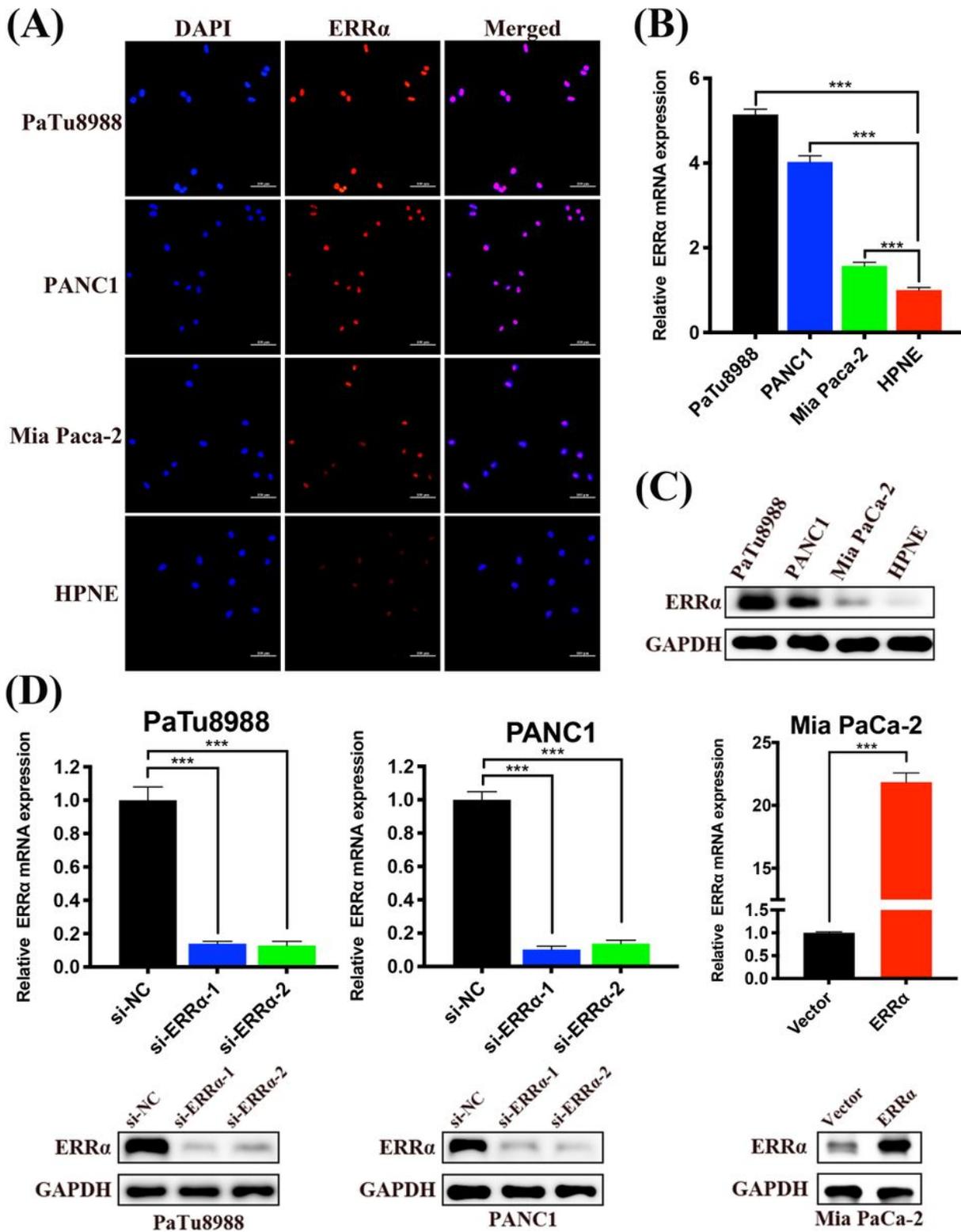
37. W Z, J X, H F, L T, W C, Q S, et al. Endothelial cells promote triple-negative breast cancer cell metastasis PAI-1 and CCL5 signaling. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2018;32(1):276-88.
38. SC C, DO H, PR R, MK W. Plasminogen activator inhibitor-1 inhibits prostate tumor growth through endothelial apoptosis. *Molecular cancer therapeutics*. 2008;7(5):1227-36.
39. ZH Q, HX X, SR Z, JZ X, S L, HL G, et al. RIPK4/PEBP1 axis promotes pancreatic cancer cell migration and invasion by activating RAF1/MEK/ERK signaling. *International journal of oncology*. 2018;52(4):1105-16.

## Figures



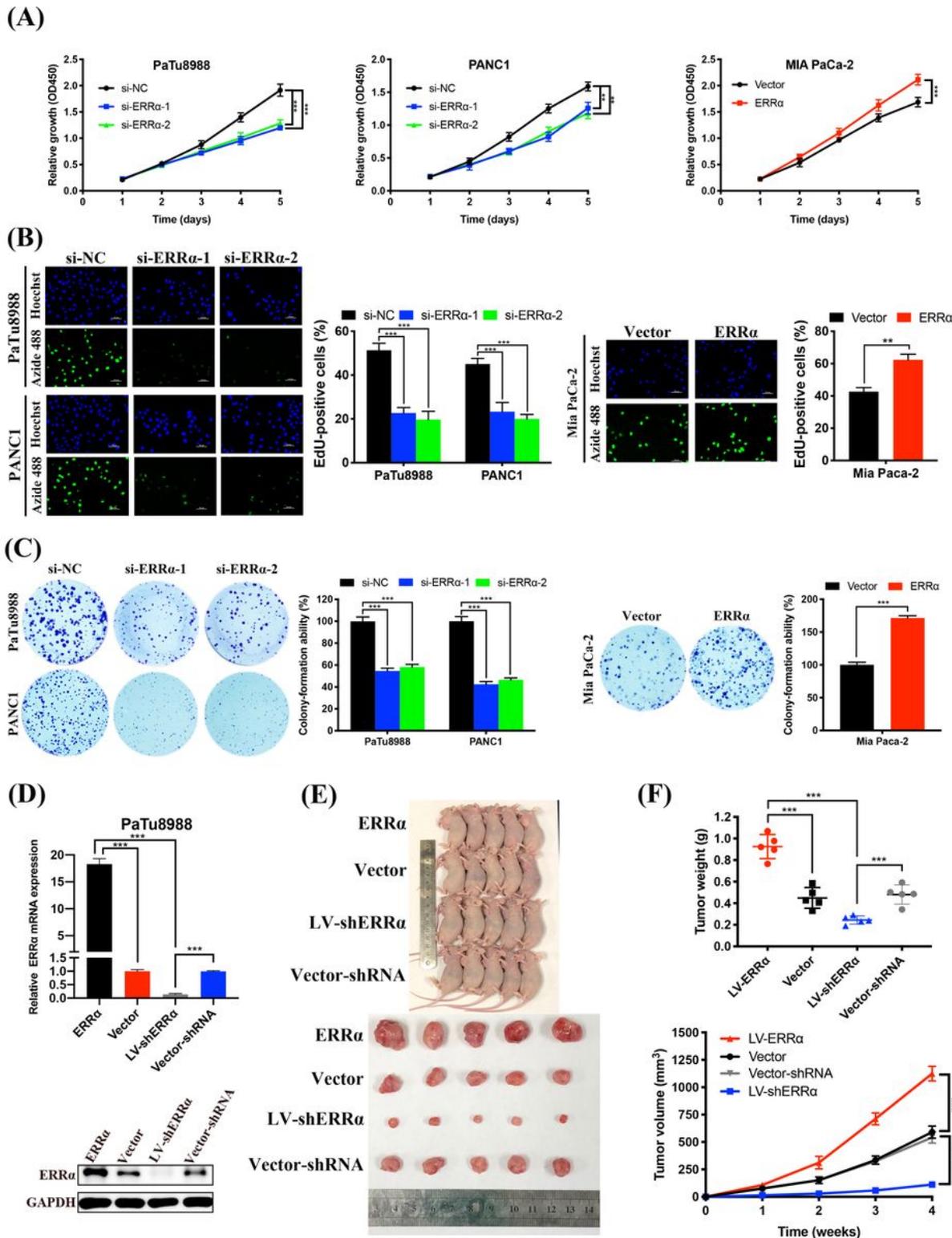
**Figure 1**

ERRα is overexpressed and correlates with poor prognosis in PC. A, ERRα expression was determined by qRT-PCR in 50 paired PC tissues and adjacent non-tumor tissues. B, The comparisons of ERRα mRNA expression level between PC tissues and adjacent non-tumor tissues. The results were presented as log 2-fold change of tumor tissues relative to non-tumor tissues. C, Overall survival curves of PC patients stratified by ERRα expression. D, Representative images of ERRα expression in PC tissues and adjacent non-tumor tissues using IHC staining. E, The IHC staining scores of ERRα were higher in PC tissues than in adjacent non-tumor tissues. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



**Figure 2**

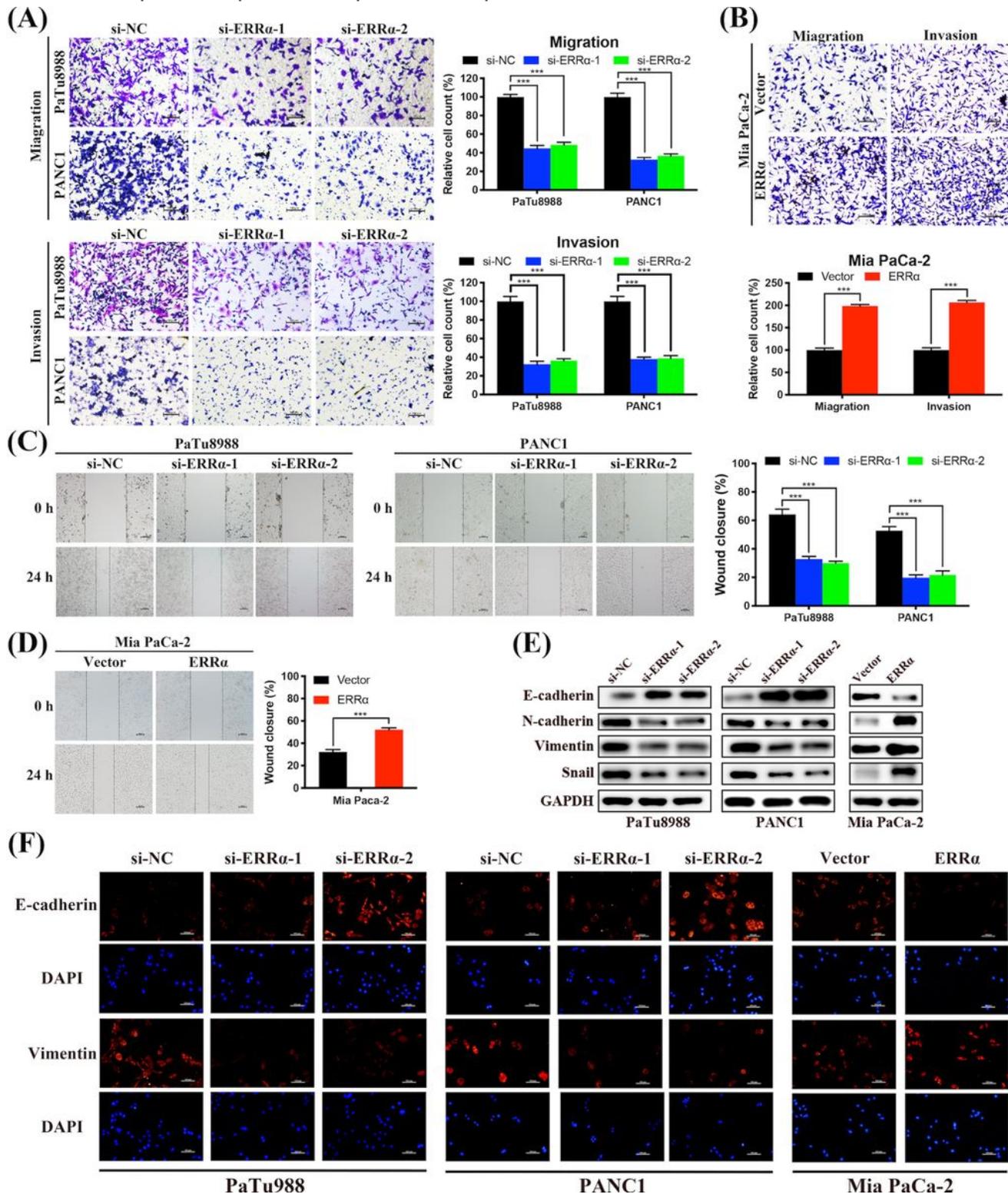
The expression of ERRα in PC cell lines. A, Immunofluorescence staining of ERRα (red) in PC cell lines and normal human pancreatic ductal epithelial cell line (HPNE). Nucleus was stained by DAPI (blue). B and C, The mRNA and protein expression levels of ERRα in PC cell lines and HPNE. D, Knockdown of ERRα in PaTu8988 and PANC1 cells, and overexpression of ERRα in Mia PaCa-2 cells was confirmed at the mRNA and protein level by qRT-PCR and western blotting. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



**Figure 3**

ERR $\alpha$  promotes PC cell proliferation and tumor growth. A-C, CCK-8, EdU and colony formation assays were applied to determine the proliferation of PaTu8988 and PANC1 cells after ERR $\alpha$  depletion, and that of Mia PaCa-2 cells after ERR $\alpha$  overexpression. D, The mRNA and protein expression level of ERR $\alpha$  in PaTu8988 cells transfected with LV-ERR $\alpha$ , LV-shERR $\alpha$  or their empty vectors. E, Compared with empty vector groups, overexpression of ERR $\alpha$  promoted while knockdown of ERR $\alpha$  suppressed the tumor growth

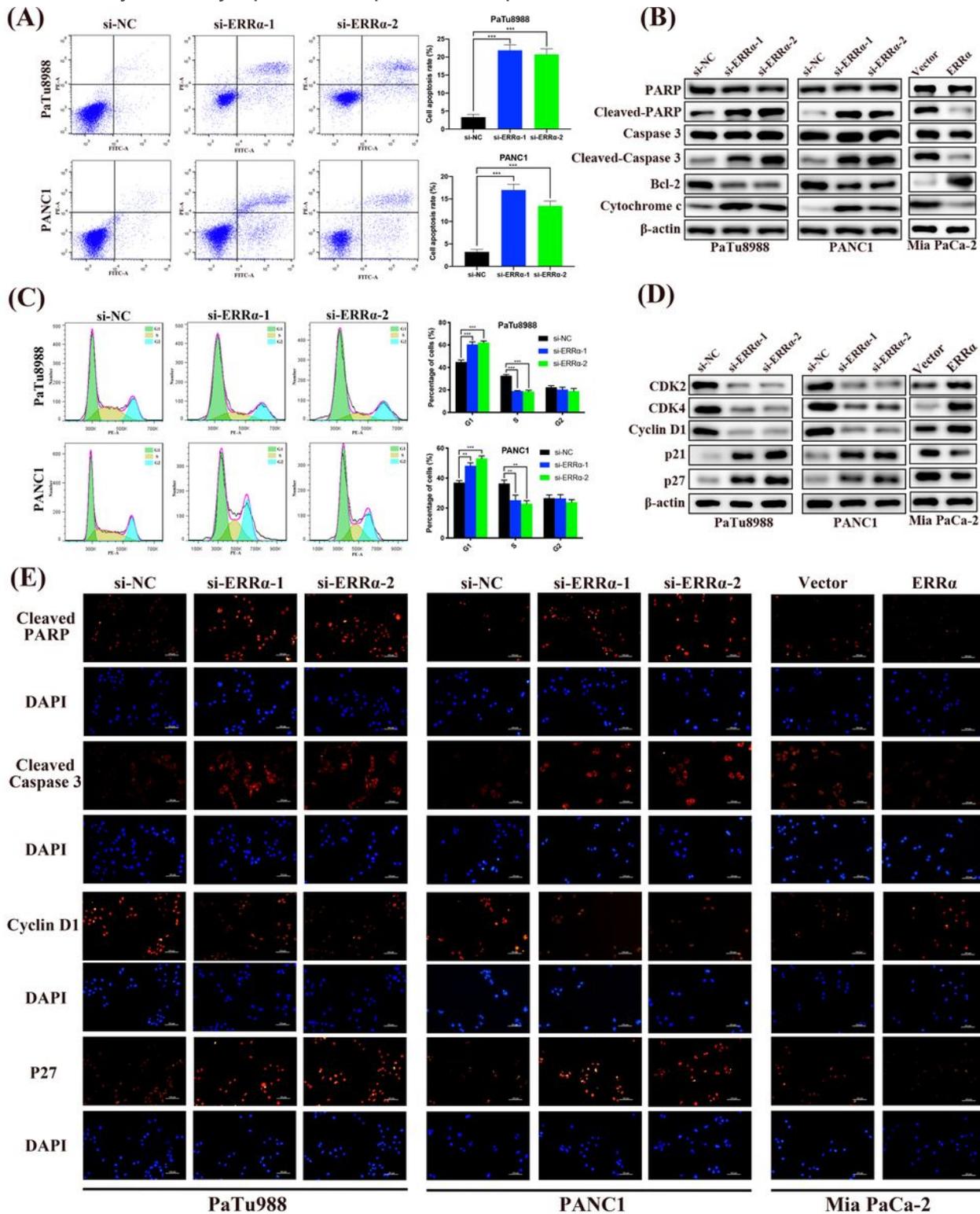
in nude mice. F, Tumor weight was measured after mice sacrificed and tumor resection; Tumor growth curves were plotted. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



**Figure 4**

ERR $\alpha$  enhances the migration and invasion of PC cells. A and B, Transwell assays were conducted to measure the migration and invasion capabilities of PC cells. C and D, Wound healing assays were used to further determine the migration abilities of PC cells. E, The protein expression levels of EMT related

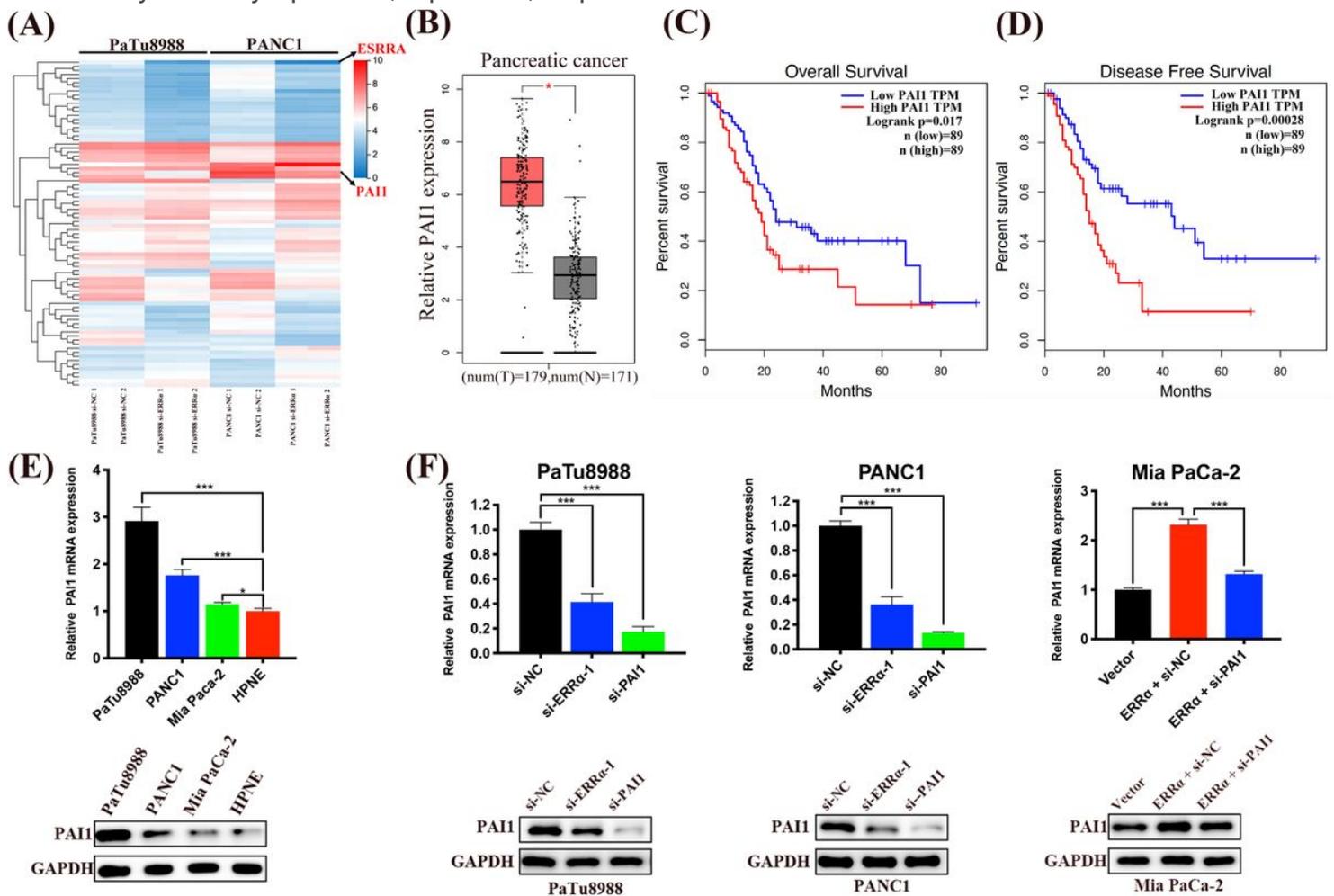
molecules were detected by western blotting. F, The expression levels of E-cadherin and vimentin were assessed by IF assay. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



**Figure 5**

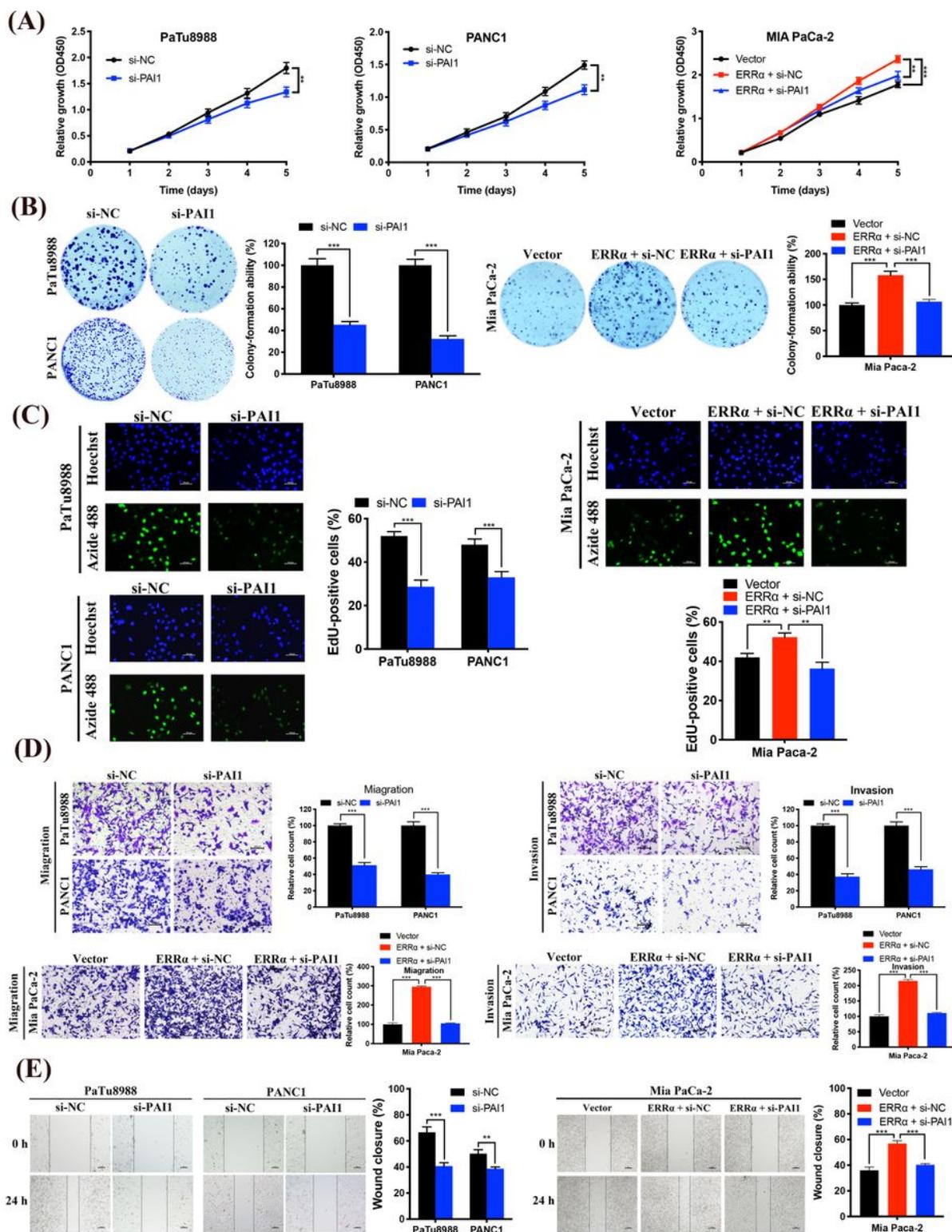
Knockdown of ERR $\alpha$  induces cell apoptosis and G0/G1 arrest in PC cells. A, Flow cytometric analysis of apoptosis in PaTu8988 and PANC1 cell after ERR $\alpha$  knockdown. B, Western blot analysis of apoptosis related markers. C, After silencing of ERR $\alpha$ , flow cytometry showed that the cell cycles of PaTu8988 and

PANC1 cells were arrested at G0/G1 phase. D, Cell cycle related proteins were analyzed by western blotting. E, The expression levels of cleaved-PARP, cleaved-caspase 3, cyclin D1 and p27 Kip1 were detected by IF assay. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



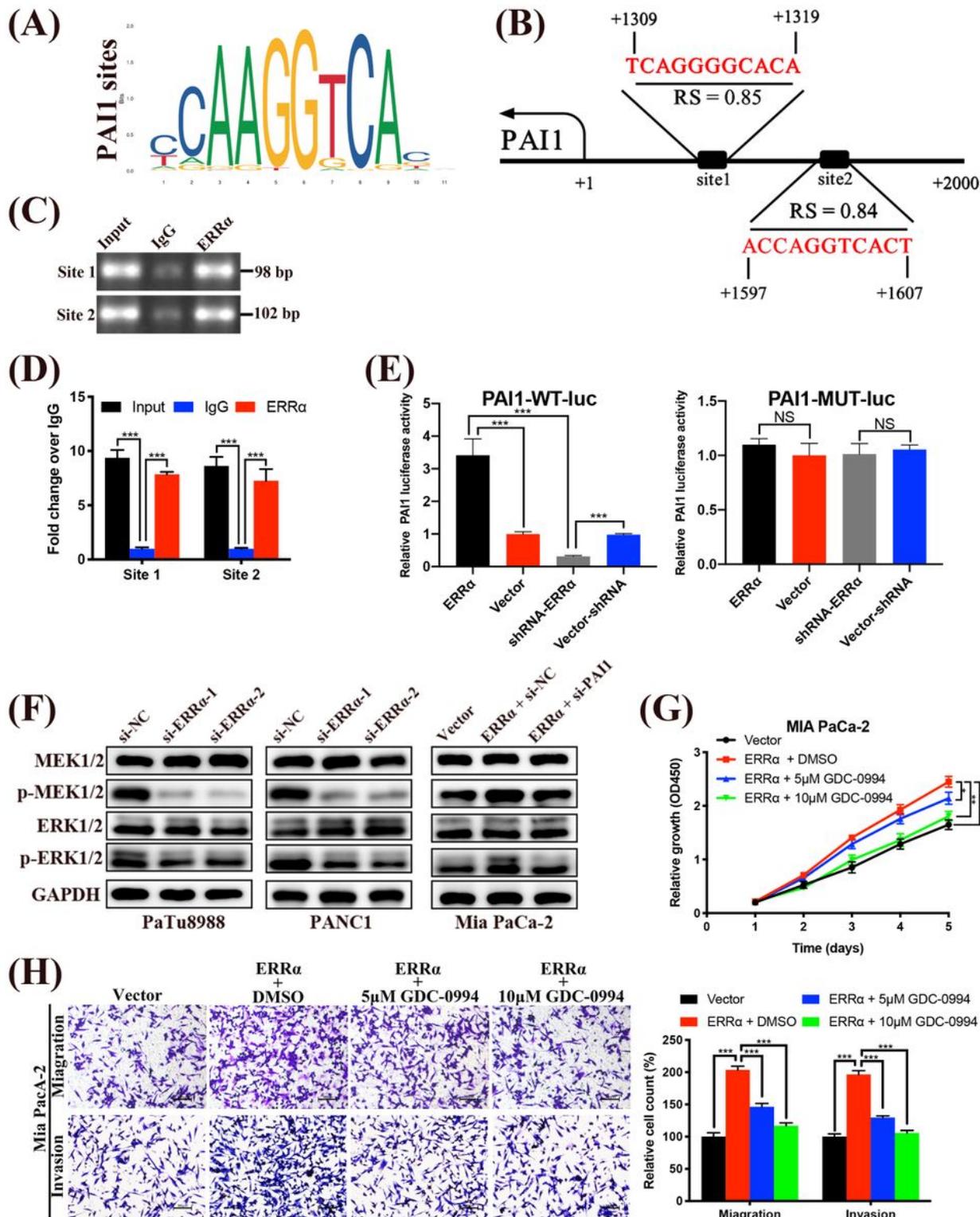
**Figure 6**

Upregulated PAI1 correlates with poor prognosis in PC. A, Expression heatmap of top 40 down-regulated and top 40 up-regulated genes after the RNA sequencing analysis following ERR $\alpha$  knockdown in PaTu8988 and PANC1 cells. B, The mRNA expression levels of PAI1 in PC tissues and normal tissues according to GEPIA database. C and D, OS and DFS rate was analyzed in PC patients using GEPIA tool based on PAI1 expression. E, The mRNA and protein levels of PAI1 in PC cell lines and HPNE. F, Expression of PAI1 was decreased in PaTu8988 and PANC1 cells after transfection with si-ERR $\alpha$ -1 or si-PAI1, whereas was increased by ERR $\alpha$  overexpression and could be reversed by PAI1 knockdown. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



**Figure 7**

PAI1 is involved in ERR $\alpha$  regulated cell proliferation, migration and invasion of PC cells. A-C, CCK-8, colony formation and EdU assays were performed to evaluate the cell proliferation of PC cells with PAI1 knockdown and/or ERR $\alpha$  overexpression. D and E, Knockdown of PAI1 significantly inhibited the migration and invasion abilities of PC cells, and abolished those abilities enhanced by ERR $\alpha$  overexpression. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



**Figure 8**

ERRα promotes the transcription of PAI1 and activates the MEK/ERK pathway. A and B, ERRα binding motif and the predicted ERRα binding sites within the promoter region of PAI1 according to the JASPAR database. C and D, ChIP-DNA electrophoresis and ChIP-qPCR using primers designed for the 2 predicted sites validated the binding capacity of ERRα to the PAI1 promoter. E, Dual-luciferase reporter assay showed that ERRα overexpression significantly increased while ERRα knockdown suppressed the activity

of the wild-type PAI1 promoter luciferase reporter. Moreover, ERR $\alpha$  had no effect on the activity of mutant PAI1 promoter luciferase reporter. F, The expression levels of p-MEK1/2 and p-ERK1/2 were suppressed by ERR $\alpha$  knockdown; ERR $\alpha$  overexpression enhanced the expression of p-MEK1/2 and p-ERK1/2, which was abolished by knockdown of PAI1. G and H, The cell proliferation, migration and invasion promoted by ERR $\alpha$  overexpression was significantly antagonized by ERK1/2 inhibitor GDC-0994.