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Original Paper

ZFX Promotes Proliferation and Metastasis of Pancreatic Cancer Cells via the MAPK Pathway

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Key Words
Zinc finger X-chromosomal protein • Cell proliferation • Pancreatic cancer • RNA interference •

Abstract
Background/Aims: The role of ZFX in tumourigenesis is unclear. We aimed to study ZFX expression, regulation, and function and the clinical implications of this protein in human pancreatic cancer (PCa). ***Methods:*** One hundred and twenty patients with histologically confirmed PCa who underwent surgery were recruited for this study. Tumour samples and PCa cell lines were used to examine ZFX. Various cell functions related to tumourigenesis were assessed. *In vivo* mouse tumour xenografts were used to confirm the *in vitro* results. ***Results:*** Patients with ZFX-positive tumours had worse overall survival than patients with ZFX-negative tumours. The depletion of ZFX using lentiviral shRNAs significantly inhibited cell proliferation by inducing cell cycle arrest in G0/G1 phase and resulted in increased cell apoptosis and invasive repression. *In vivo* studies confirmed that ZFX promoted tumour growth. Mechanistically, MAPK pathway activation was involved in the oncogenic functions of ZFX. ***Conclusions:*** ZFX acts as a putative oncogene in PCa and could be a novel therapeutic target for this disease.

Xiaoling Song, Minghui Zhu and Fahong Zhang contributed equally to this work.

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Introduction

Pancreatic cancer (PCa) is the most lethal solid tumour and is the fourth leading cause of cancer-related death, with an overall 5-year survival rate of <5%[1, 2]. Due to local invasion, early metastasis, and resistance to standard chemotherapy, the median survival time of PCa patients is only 5-8 months despite recent advances in surgical techniques and medical management [3, 4]. In addition, although surgical resections are commonly performed, the long-term survival of PCa has not improved in two decades [5, 6]. Therefore, identification of effective early markers for the diagnosis and prognosis of the disease as well as novel therapeutic targets will be clinically valuable.

Zinc finger protein X-linked (ZFX), which is encoded on the X chromosome, contains an acidic transcriptional activation domain, a nuclear localization sequence, and a DNA binding domain consisting of 13 C2H2-type zinc fingers [7]. ZFX is highly conserved in vertebrates. Studies have demonstrated that ZFX plays a key role in controlling the self-renewal of embryonic and haematopoietic stem cells [8]. Recently, ZFX was shown to be involved various physiological functions, including proliferation, differentiation, cell cycle and cell death [9, 10]. Consistent with these critical roles, ZFX has been characterized as an oncogene in laryngeal squamous cell carcinoma, hepatocellular carcinoma, gastric carcinoma and colorectal cancer [11-14]. Clinicopathological analysis showed that high ZFX expression was correlated with poor prognosis in a variety of solid cancers, and increased ZFX expression was identified as an independent prognostic biomarker [11, 14, 15]. However, the biological function and molecular mechanisms of ZFX in PCa are unclear.

The present study aimed to elucidate the role of ZFX in PCa. To this end, we examined ZFX protein expression and localization in PCa tissues and investigated the phenotypic effects and mechanisms of ZFX in PCa.

Materials and Methods

Patients and clinicopathological data

The study was approved by the ethics committee of Xinhua Hospital, and all patients provided informed consent. Cancer tissue specimens were obtained from 120 PCa patients who underwent radical pancreaticoduodenectomy (without prior radiotherapy or chemotherapy) between 2014 and 2016 at the Department of General Surgery, Xinhua Hospital Affiliated with Shanghai Jiao Tong University School of Medicine, China. All diagnoses of PCa and lymph node metastasis were confirmed by histopathological examination, and adjacent control samples were confirmed to be free of tumour cells. All tissue samples were fixed in 4% formalin immediately after removal and embedded in paraffin for immunohistochemical staining.

Immunohistochemical analysis and evaluation of ZFX expression

Immunohistochemical staining was performed using a standard immunoperoxidase staining procedure, and ZFX expression in the benign and malignant specimens was evaluated as described by Li et al [16]. The sections were semi-quantitatively scored according to the extent of immunoreactivity as follows: 0, 0% immunoreactive cells; 1, <5% immunoreactive cells; 2, 5–50% immunoreactive cells; and 3, >50% immunoreactive cells. Additionally, the staining intensity was scored semi-quantitatively as follows: 0, negative; 1, weak; 2, intermediate; and 3, strong. The final immunoreaction score was defined as the sum of both parameters (extension and intensity), and the samples were classified as negative (0), weakly stained (1–2), moderately stained (3), and strongly stained (4–6). For statistical purposes, only moderate and strong final immunoreaction scores were considered positive; the other final scores were considered negative.

Cell culture

The PANC-1 and MIAPaca-2 were purchased from the Shanghai Institutes for Biological Sciences (Shanghai, China). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing

10% (v/v) foetal bovine serum (FBS) and routinely cultivated in a humidified incubator at 37°C with 5% CO_2.

Lentivirus-mediated RNA interference

The guiding strand sequence of human small interfering RNA (siRNA) is 5'-GTCGGAAATTGATCCTTGTAA-3'. The negative control siRNA sequence is 5'-TTCTCCGAACGTGTCACGT-3', with no significant sequence similarity to human gene sequences. Short hairpin RNAs (shRNAs) were synthesized and inserted into the pFH1UGW lentivirus vector containing a CMV-driven EGFP reporter gene and an H1 promoter upstream of restriction sites (NheI/PacI). Recombinant lentiviruses expressing ZFX siRNA or control siRNA (Lv-shZFX or Lv-shCon) were produced by co-transfecting HEK293 cells with pVSVG and pCMV⊿R 8.92 using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. The virus was harvested from the culture medium 2 days after transfection, concentrated by centrifugation, and used for subsequent studies.

Quantitative real-time PCR (qPCR)

Total RNA was extracted from cultured cells with TRIzol reagent (Invitrogen). The following primers were used to detect the expression of ZFX and β-actin (internal control):

ZFX (sense): 5'-GGCAGTCCACAGCAAGAAC-3';

ZFX (antisense): 5'- TTGGTATCCGAGAAAGTCAGAAG-3';

β-actin (sense): 5'- GTGGACATCCGCAAAGAC-3';

β-actin (antisense): 5'- AAAGGGTGTAACGCAACTA-3'.

The protocol was performed according to the manufacturer's instructions. The results were normalised to the expression of β-actin.

Western blot analysis

Total cell lysates were separated on a 10% SDS-PAGE gel and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked and then probed with primary antibody against ZFX (1:200 dilution, Abcam, Cambridge, UK) and GAPDH (1:5000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After the membranes were washed, they were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz, CA, USA) and visualised using the enhanced chemiluminescent detection reagent.

Cell proliferation assay

Cell proliferation was assessed with the CCK8 assay following the manufacturer's instructions. In addition, the absorbance at 450 nm was detected using a microplate reader (Bio-Rad, Hercules, CA, USA). A growth curve was prepared according to the absorbance values at 450 nm. The results reflect the average of three replicates under the same conditions.

Colony formation assay

Both non-transfected and transfected PANC-1 and MIAPaca-2 cells (400 cells/well) were seeded in 6-well plates. The culture medium was changed at regular time intervals. After 14 days of culture, adherent cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Colonies were stained with Giemsa solution for 15 min and then washed with water and air-dried. Cell colonies were counted by light microscopy. Experiments were performed in triplicate.

Cell cycle and apoptosis analyses

Forty-eight hours after lv-shRNA transfection, the cells were collected and stained using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA, USA). Cell cycle analysis was performed after staining with propidium iodide. Both apoptosis and cell cycle distribution were quantified using a flow cytometer. The data presented are representative of at least three different experiments.

In vitro cell migration assay

Cell migration was assessed using 6.5-mm transwell chambers with a pore size of 8 μm (Corning). Transwell upper chambers were pre-coated with 50 μg/chamber of solubilized basement membrane.

DMEM/10% FBS was added to the bottom chamber. PCa cells (2.5×10^4 per chamber) were seeded in serum-containing media in the upper well of the transwell chambers and incubated for about 20 h at 37°C and 5% CO_2. The cells in the lower chamber (below the filter surface) were fixed in 70% ethanol, stained with 0.1 mg/ml crystal violet solution, and counted under a microscope (20× magnification). Five random visual fields were counted for each well, and the average was determined.

Wound healing assay

Cells were cultured for 72 h until confluency after the designated treatments in 6-well plates. Cells were then treated with mitomycin C (10 μg/ml) at 37°C in a 5% (v/v) CO_2 incubator for 1 h to inhibit cell division. Three separate wounds through the cells were made by moving a sterile 200 μl pipet tip perpendicular to the demarcation line. Cells were gently rinsed with PBS and incubated with 2 ml serum-free media. Cells were photographed under a phase contrast microscope at 0 h (control) and 48 h. Each sample was assayed in triplicate.

Tumour xenografts in nude mice

Four- to 6-week-old nu/nu nude mice were purchased from the Shanghai Laboratory Animal Centre of the Chinese Academy of Sciences (Shanghai, China). All mice were housed under specific pathogen-free conditions following the guidelines of the Ethics Committee of Xinhua Hospital, School of Medicine, Shanghai Jiaotong University. For induction of subcutaneous PCa tumours, two groups (Lv-shCon group, and Lv-shZFX group) of PCa cells (1×10^6) in 0.1 mL serum-free DMEM were implanted subcutaneously into the left flank of the mice (5 mice/group). Tumour growth was monitored every 3 days and measured in 2 dimensions. Tumour volume was calculated using the formula width^2 (mm^2)×length (mm)/2, where width and length were the shortest and longest diameters, respectively. After approximately 4 weeks, mice were sacrificed, and primary tumours were collected for further immunohistochemical staining and western blot analysis.

Statistical analysis

Statistical analysis was conducted with SPSS software, version 22.0 (SPSS Inc., Chicago, IL, USA). The data are expressed as the mean±S.D. Independent Student's t-tests and χ^2 tests were used to assess the effects of ZFX in the cell lines. The Kaplan-Meier test was used for univariate survival analysis. The Cox proportional hazard model was used for multivariate analysis and for determining the 95% confidence interval. The results were considered to be significant at $P<0.05$.

Results

ZFX is highly expressed in patients with PCa and correlated with poor prognosis

To determine the potential role of ZFX in PCa progression, we evaluated ZFX expression in PCa and adjacent control tissues by immunohistochemistry (Fig. 1A). ZFX was primarily located in the nucleus. Approximately 61.7% (74/120) of the PCa cases had positive ZFX staining in the tumour cells. In contrast, only 38.3% (46/120) of the cases had positive staining in the adjacent control tissues (Fig. 1A-1B, $P<0.001$).

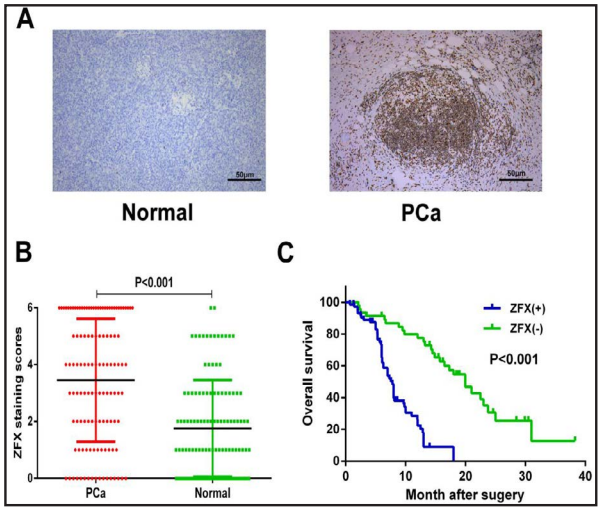

Fig. 1. The enhanced expression of ZFX in PCa and the negative association with prognosis. Immunohistochemistry of ZFX expression (scale bar, 50 μm). The positive staining was predominantly localized to the cell cytoplasm. (A) Immunohistochemistry of ZFX in normal tissues and tumour tissues from PCa patients. (B) Average staining scores for ZFX in PCa tumours and in adjacent tissues ($P<0.001$). (C) Kaplan–Meier plots of the overall survival of PCa patients based on ZFX expression ($P<0.001$).

Next, we assessed the correlation between ZFX expression levels and histopathological parameters in patients with PCa. As shown in Table 1, ZFX overexpression was significantly correlated with TNM stage (P<0.001) and lymph node metastasis (P<0.001) but not with sex, age, histopathological subtypes, tumour size, or tumour location, indicating a potential role of ZFX in promoting aggressive phenotypes in PCa.

The survival information of the 120 PCa patients was obtained through phone calls. Kaplan–Meier survival analysis revealed that lymph node metastasis (P<0.001) and TNM stage (P<0.001) were significantly associated with the average survival time. The average survival time for ZFX-negative patients was significantly higher than that of patients with positive ZFX expression (P<0.001) (Table 2, Fig. 1C). To obtain a more precise estimate of the prognosis, we used the Cox proportional hazards regression model. The results confirmed that ZFX expression, as well as the presence of lymph node metastasis and TNM stage, was negatively correlated with post-operative survival, suggesting that high ZFX expression is a risk factor (Table 3).

Ectopic expression of ZFX promotes tumourigenic properties of PCa cells

As unlimited growth, invasiveness, and metastasis are hallmarks of malignancy, we explored the role of ZFX in PCa progression. We silenced ZFX expression in PANC-1 and MIAPaca-2 cells, and the knockdown efficiency is shown in Fig. 2A. ZFX knockdown suppressed the growth of PANC-1 and MIAPaca-2 cells as shown by CCK8 assays (Fig. 2B). Furthermore, colony formation assays demonstrated that ZFX silencing significantly reduced the number of colonies (Fig. 2C). These results indicated that ZFX promotes cell growth *in vitro*. Next,

Table 1. Association between ZFX expression with the clinicopathological parameters of PCa. Notes: *, Statistically significant

Parameter	Category	No. of cases	ZFX expression No. of positive cases n(%)	χ^2	P value
Age	<60	59	40(67.8)	1.845	0.193
	≥60	61	34(55.7)		
Sex	male	74	45(60.8)	0.06	0.849
	female	46	29(63.0)		
Histopathological Subtypes	high	21	12(57.1)	4.848	0.089
	middle	54	39(72.2)		
	low	45	23(51.1)		
TNM Stage	1-II	61	23(37.7)	30.135	<0.001*
	III-IV	59	51(86.4)		
Tumor Size	<3 cm	80	49(61.3)	0.018	0.894
	≥3 cm	40	25(62.5)		
Tumor Location	Body/tail	50	28(56.0)	1.164	0.342
	Head	70	46(65.7)		
Lymph Node Metastasis	Negative	63	26(41.3)	23.342	<0.001*
	Positive	57	48(84.2)		

Table 2. Univariate log-rank analysis of overall survival (OS). Notes: *, Statistically significant

Parameter	Category	Case number (n)	Median survival time (month) 95% CI	P value
Age	<60	59	12.0(9.155-14.845)	0.774
	≥60	61	9.7(4.293-15.037)	
Sex	male	74	12.0(8.716-15.284)	0.609
	female	46	9.7(2.676-16.664)	
Histopathological subtypes	high	21	12.0(6.522-17.478)	0.389
	middle	54	10.0(6.277-13.723)	
	low	45	13.0(4.735-21.265)	
TNM stage	1-II	61	17.3(12.516-22.084)	<0.001*
	III-IV	59	6.0(5.862-6.174)	
Tumor Size	<3 cm	80	13.0(7.172-18.828)	0.285
	≥3 cm	40	10.0(7.891-12.091)	
Tumor Location	Body/tail	50	12.5(11.647-13.353)	0.132
	Head	70	9.7(6.808-12.532)	
Lymph node Metastasis	Negative	63	17.3(12.519-22.081)	<0.001*
	Positive	57	6.0(5.797-6.023)	
ZFX expression	Negative	46	20.0(15.729-24.211)	<0.001*
	Positive	74	7.8(6.679-8.921)	

to investigate whether ZFX affects the proliferation of PCa cells *in vivo*, we established xenograft mouse models. As shown in Fig. 2D, the tumour volume and weight of ZFX-depleted xenografts were significantly inhibited compared with the negative control group. Immunohistochemical analysis indicated that ZFX and Ki67 levels were substantially decreased in ZFX-depleted implanted tumour tissues (Fig. 2E).

Table 3. Multivariate analysis of overall survival (OS). Notes: *, Statistically significant. [1] HR, hazard ratio

Parameter	Category	HR [1]	95% CI	P value
Age	<60 ≥60	2.096	1.434(0.880-2.338)	0.538
Sex	male female	0.379	1.169(0.711-1.922)	0.538
Histopathological subtypes	high middle low	0.594	0.880(0.637-1.217)	0.441
TNM stage	1-II III-IV	4.034	8.095(1.052-62.300)	0.045*
Tumor Size	<3 cm ≥3 cm	0.635	0.806(0.475-1.369)	0.425
Tumor Location	Body/tail Head	0.376	1.193(0.679-2.095)	0.54
Lymph node Metastasis	Negative Positive	5.588	8.991(1.455-55.536)	0.018*
ZFX expression	Negative Positive	10.074	3.163(1.553-6.439)	0.002*

Metastasis is frequently observed in patients with PCa [4], and therefore, we investigated whether ZFX might be involved in the migration of PANC-1 and MIAPaca-2 cells. In transwell migration assays, ZFX knockdown significantly impaired the migration of PANC-1 and MIAPaca-2 cells (Fig. 2F). Moreover, migration was also determined by wound healing assays. Knockdown of ZFX in PCa cells moderately decreased the migration rate (Fig. 2G). These results suggested that ZFX regulated the proliferation, survival and migration of PCa cells *in vitro* and further explained the aforementioned correlation of ZFX with poor prognosis of PCa patients.

Effects of ZFX silencing on cell cycle progression and apoptosis

To determine the potential mechanism underlying the role of ZFX in PCa cell growth, we assessed the cell cycle profile of the Lv-shCon and Lv-shZFX groups by FACS analysis 48 h after infection. As shown in Fig. 3A, the cell cycle analysis revealed G0/G1 phase arrest in PCa cells (Fig. 3A) after depletion of ZFX, induced cell cycle arrest in the G0/G1 phase. Furthermore, the effect of ZFX knocgkdown on the expression of cell cycle regulators was examined by real-time PCR and western blot analyses. As shown in Fig. 3B-3C, the protein and mRNA levels of CDK2, CDC25A, and cyclin D1 in the Lv-shZFX group were lower than those in the Lv-shCon group. These results indicated that ZFX knockdown induced cell cycle arrest in the G0/G1 phase, possibly by affecting the expression of cell cycle regulators.

To further investigate whether expression of ZFX affects apoptosis in PCa cells, we performed an apoptosis assay by FACS analysis. Transfection of ZFX shRNA resulted in a significant increase in the percentage of apoptotic cells compared with that of the Lv-shCon in both PCa cell lines (Fig. 3D). Moreover, knockdown of ZFX dramatically increased the expression of Bax and Bad and decreased the expression of Bcl-2 (Fig. 3E); these factors play important roles in apoptosis. The above data indicated that ZFX was involved in the regulation of PCa cell apoptosis and cell cycle progression.

ZFX regulates MAPK/ERK signalling in PCa

To elucidate the molecular mechanisms by which ZFX modulates the tumourigenic properties of PCa cells, we investigated the effects of ZFX on cell survival and cell cycle-related signalling molecules in PCa cells after Lv-shZFX infection. Western blot analysis showed that transfection with Lv-shZFX significantly decreased p-MEK and p-ERK in both PANC-1 and MIAPaca-2 cells but had no effect on p-STAT3 and p-JNK (Fig. 4A). Encouraged by the above results, we investigated whether targeting MAPK/ERK could attenuate the oncogenic function of ZFX. We treated PCa cells with the ERK inhibitor SCH772984 and found that this compound inhibited ZFX overexpression-induced cell proliferation and invasion (Fig. 4B-4C). As expected, SCH772984 also promoted the ZFX overexpression-inhibited apoptosis and G0/G1 phase cell accumulation (Fig. 4D-4E). Moreover, the downstream genes

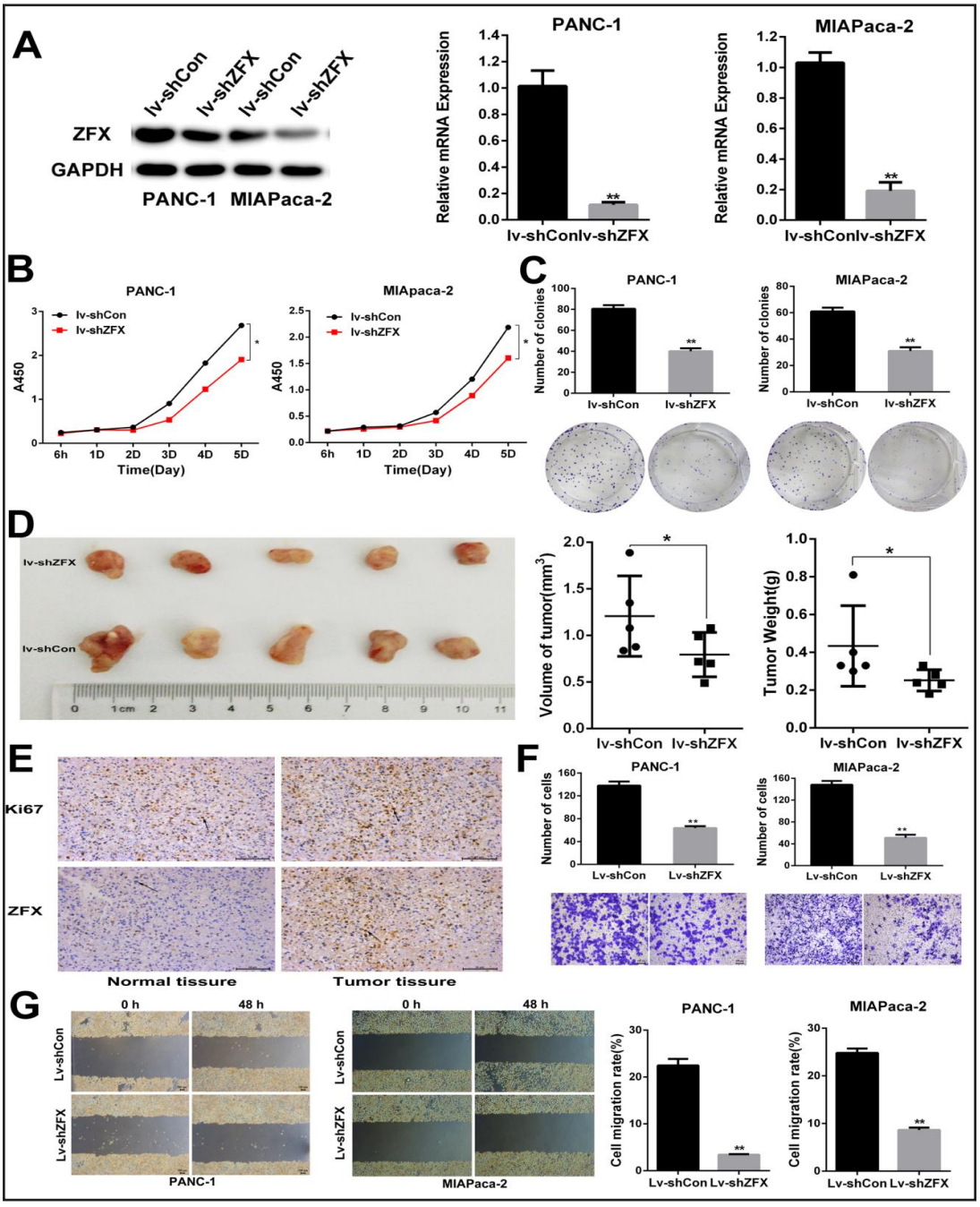

Fig. 2. ZFX exhibiting oncogenic properties in PCa cell lines. (A) The transfection efficiency was determined 2 days after incubation with lentivirus at an MOI of 40. Total protein and RNA were extracted 2 days after infection, and relative ZFX expression was determined using western blot analysis and RT-PCR. Data represent the mean±S.D. of three independent experiments. ***P<0.001, compared with Lv-shCon. (B) Cellular proliferation of untransfected or transfected PANC-1 and MIAPaca-2 cells was measured using a CCK8 assay daily for 5 days. (C) PANC-1 cells and MIAPaca-2 cells were seeded at 500 cells/well, and the cells were allowed to form colonies. Colony numbers were counted and recorded. (D) Mice were treated with Lv-shCon and Lv-shZFX PCa cells for 4 weeks. Tumour volumes and weights were measured. (E) Immunohistochemical analysis shows a decrease in Ki67 and ZFX expression in Lv-shZFX cells compared with Lv-shCon cells. (F) Migration in PCa cell lines measured by transwell assays decreased after transfection. The number of migrating cells was calculated and is depicted in the bar chart (P<0.01). (G) Wound closure was delayed in Lv-shZFX cells compared to Lv-shCon cells after 48 h (P<0.01).

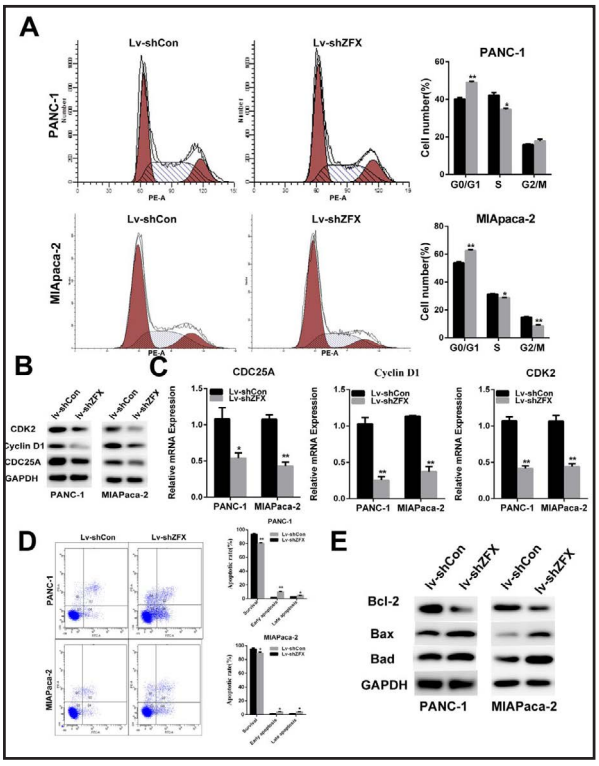

Fig. 3. Effects of ZFX silencing on cell cycle progression and apoptosis. (A) The cell cycle phases of treated cells were evaluated by flow cytometry after transfection for 48 h. The x-axis represents the periodic distribution, and the y- axis represents the number of cells. (B) The protein levels of cell cycle regulators, such as CDK2, CDC25A and cyclinD1, were examined by western blotting analysis. (C) The mRNA levels of cell cycle regulators, such as CDK2, CDC25A and cyclinD1, were examined by RT-PCR. (D) Untransfected and transfected PANC-1 and MIAPaca-2 cells were analysed by flow cytometry with annexin V–FITC/propidium iodide (PI) staining. The x- axis represents annexinV-FITC, and the y- axis represents propidium iodide (PI) staining. Annexin V versus PI plots from the gated cells show the populations corresponding to viable (annexin V/PI), necrotic (annexin V/PI+), early apoptotic (annexin V+/PI), and late apoptotic (annexin V+/PI+) cells. (E) The protein levels of bad, bax and bcl-2 were examined by western blotting analysis.

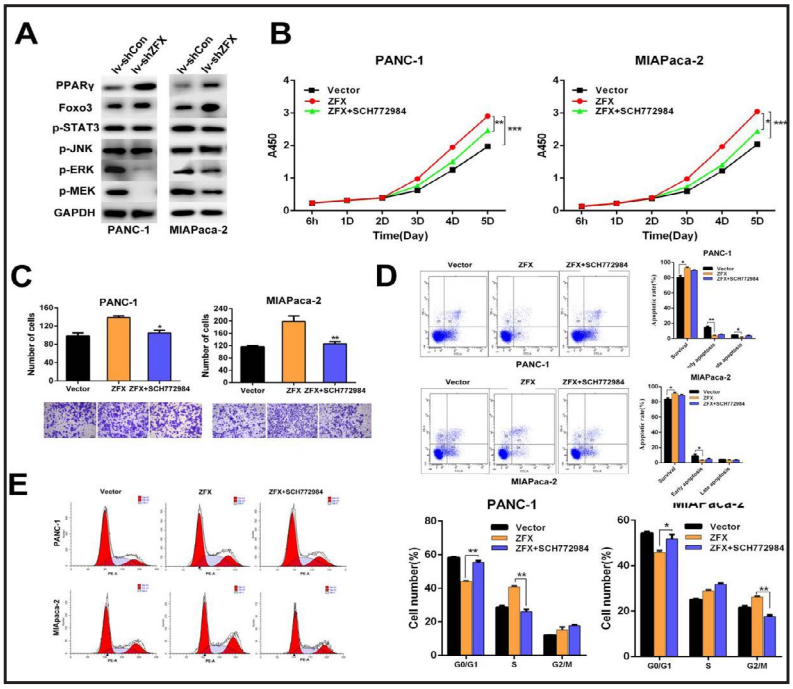

Fig. 4. ZFX regulates MAPK/ERK signalling in PCa. (A) Western blotting analysis of MAPK/ERK signalling-related proteins in both cell lines. GAPDH was used as a loading control. (B) Cellular proliferation of PANC-1 and MIAPaca-2 cells treated with or without the ERK inhibitor SCH772984 were measured using a CCK8 assay daily for 5 days. (C) Migration in PCa cell lines PANC-1 and MIAPaca-2 cells treated with or without the ERK inhibitor SCH772984 were measured by transwell migration assays. The number of migrating cells was calculated and is depicted in the bar chart. (D) PANC-1 and MIAPaca-2 cells treated with or without the ERK inhibitor SCH772984 were analysed by flow cytometry with annexin V–FITC/propidium iodide (PI) staining. The x- axis represents annexinV-FITC, and the y- axis represents propidium iodide (PI) staining. Annexin V versus PI plots from the gated cells show the populations corresponding to viable (annexin V/PI), necrotic (annexin V/PI+), early apoptotic (annexin V+/PI), and late apoptotic (annexin V+/PI+) cells. (E) The cell cycle phases of treated cells were evaluated by flow cytometry. The x- axis represents the periodic distribution, and the y- axis represents the number of cells.

of the MAPK signalling pathway, including $Foxo_3$ and PPARγ, were upregulated, which also supports activation of the MAPK pathway in PCa cells (Fig. 4A). Thus, the data indicated that the MAPK/ERK pathway might participate in the oncogenic function of ZFX.

Discussion

PCa remains a lethal malignancy despite the progress made in elucidating its molecular mechanisms [17, 18]. Therefore, identification of novel methods that can effectively inhibit PCa cell growth and metastasis is urgently needed. Targeted therapies have shown promise in achieving these goals [19-21]. ZFX, also known as ZNF926, is a sex chromosome gene [21]. This protein is a novel member of the Kruppel C2H2-type ZNF protein family and encodes a zinc finger transcription factor that contains 13 C2H2 type ZNF motifs at the carboxyl terminus [22]. ZFX was originally identified as a factor that determines whether an embryo develops as a male or female. Further studies confirmed that ZFX was required for the self-renewal of haematopoietic and embryonic stem cells and could regulate cell survival [15, 22]. Furthermore, ZFX forms a unique transcription network with Cnot3, Trim28 and c-Myc that is required for self-renewal in embryonic stem cells, suggesting a potential role of ZFX in tumourigenesis [10, 11]. Recent studies have also linked ZFX upregulation to cancer development and progression in various types of cancer. ZFX facilitates the development of experimental basal cell carcinoma and medulloblastoma in mice initiated by deletion of the Hedgehog (Hh) inhibitory receptor Ptch1. These results indicate that ZFX is a common cell-intrinsic regulator of diverse Hh-induced tumours and also suggest new therapeutic targets for these malignancies [23]. Furthermore, knocking down ZFX dramatically inhibited the proliferation and metastasis of glioma cells. Based on these findings, ZFX plays an important role in carcinogenesis. However, to date, ZFX has never been linked to PCa.

In the present study, we found for the first time that significantly increased ZFX expression was detected in the vast majority of PCa tissues compared with adjacent control samples. Moreover, increased ZFX protein expression correlated not only with advanced tumour status but also with poor patient survival, suggesting the significance of ZFX in PCa progression. Notably, Cox multivariate analysis indicated that a high nuclear ZFX level was an independent factor for poor prognosis in PCa patients. Thus, ZFX could be a potential therapeutic target in PCa.

Additionally, we found that ZFX may play an important role in PCa cell apoptosis, cell cycle, metastasis and proliferation *in vitro* and *in vivo*. Knockdown of ZFX significantly impaired PCa cell growth *in vitro* and *in vivo* and increased the number of apoptotic PANC-1 and MIAPaca-2 cells; these results were further confirmed by enhanced expression of Bad and Bax and reduced expression of Bcl-2, key apoptosis-related factors. Notably, progression through the cell cycle depends on the activation of cyclin-dependent kinases (CDKs) and their regulatory subunits, the cyclins. In this study, we observed that ZFX knockdown inhibited PCa cell proliferation by inducing cell cycle arrest in G0/G1 phase while down-regulating CDK2, CDC25A, and Cyclin D1. Thus, ZFX may regulate the expression of cell cycle regulators. Additionally, consistent with the results of a previous study [24, 25], knockdown of ZFX substantially inhibited cell migration and invasion in PCa cells. These properties might contribute to the ZFX-associated aggressive biological behaviours of PCa.

We further elucidated the downstream signalling pathway responsible for ZFX oncogenic function in PCa and found that the ZFX-mediated tumour growth and metastasis could be, at least partly, attributed to the activation of MAPK/ERK signalling, which is critical for the initiation and progression of PCa. The ZFX-induced MAPK/ERK signalling in PANC-1 and MIAPaca-2 cells was confirmed because targeting MAPK/ERK could attenuate the oncogenic function of ZFX and the upregulation of MAPK/ERK target gene expression. However, the direct link between ZFX and the MAPK/ERK pathway should be further investigated in future studies.

In summary, the present study showed that ZFX might be involved in PCa progression and aggressiveness. The classification of patients according to ZFX expression levels provides

a valuable tool with which to identify PCa patients with poor prognoses. Furthermore, we demonstrated that knockdown of ZFX inhibited PCa cell growth via the MAPK/ERK pathway. These findings will help elucidate the biological progression of PCa and could provide a new therapeutic target for PCa.

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Disclosure Statement

No conflict of interests to disclose.

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