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Original Article

AMPK-related kinase 5 (ARK5) enhances gemcitabine resistance in pancreatic carcinoma by inducing epithelial-mesenchymal transition

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Abstract: AMPK-related kinase 5 (ARK5) is a member of the human AMP-activated protein kinase (AMPK) family, which is associated with increased tumor survival and drug resistance in many cancers. However, the function of ARK5 in pancreatic carcinoma (PC) is unclear. Our study investigated the role of ARK5 in the chemo-resistance of PC and its underlying mechanism. PC cell lines that displayed high expression levels of ARK5 had low sensitivity to gemcitabine (GEM). Suppression of ARK5 increased sensitivity to GEM in PC cell lines. Western blotting and immunofluorescence showed that suppression of ARK5 upregulated expression of E-cadherin and downregulated vimentin expression. Suppression of ARK5 also inhibited the epithelial-mesenchymal transition (EMT) efficiency associated with GEM in PC cell lines and upregulation of ARK5 expression enhanced GEM resistance in PC cell lines by inducing Twist-mediated EMT. In addition, we found that suppression of ARK5 increased GEM sensitivity in PC cell lines under hypoxic conditions. ARK5 increases GEM resistance in PC cell lines via EMT, and suppression of ARK5 increases sensitivity to GEM under both normoxic and hypoxic conditions.

Keywords: ARK5, pancreatic carcinoma (PC), GEM, chemo-resistance, epithelial-mesenchymal transition (EMT)

Introduction

Pancreatic carcinoma (PC) is the thirteenth most common solid tumor and the eighth leading cause of cancer-related deaths worldwide [1]. Considering the characteristics of early locoregional spread and distant metastases, patients diagnosed with this disease have few opportunities to undergo surgical operations, leading to poor prognoses and limited treatment options [2]. Many studies have reported that systemic chemotherapy for advanced PC is largely ineffective. Only GEM is still widely considered to be the first-line treatment for advanced PC [3-7]. However, clinical trials have shown that gemcitabine (GEM) can only extend patient survival by a few months. Even the combination of GEM and nab-paclitaxel can only improve overall survival by 1.8 months over GEM-only therapies. Furthermore, the safety profiles are less favorable than for GEM-only treatments [8]. Thus, investigations regarding the mechanism of GEM resistance are necessary for improving advanced PC therapies.

ARK5 is a member of the novel human AMP-activated protein kinase (AMPK) family. The gene encodes 661 amino acids with a molecular mass of 74 kDa [9]. ARK5 was found to be overexpressed in many solid tumors, such as breast cancers, colorectal carcinomas, and hepatocellular carcinomas. Patients with high levels of expression of ARK5 tend to have a poor prognosis [10-12]. Many researchers have demonstrated that the activation of ARK5 can induce tumor cell survival during nutrient starvation [13, 14]. ARK5 can also inhibit cell apoptosis under nutrient starvation conditions by

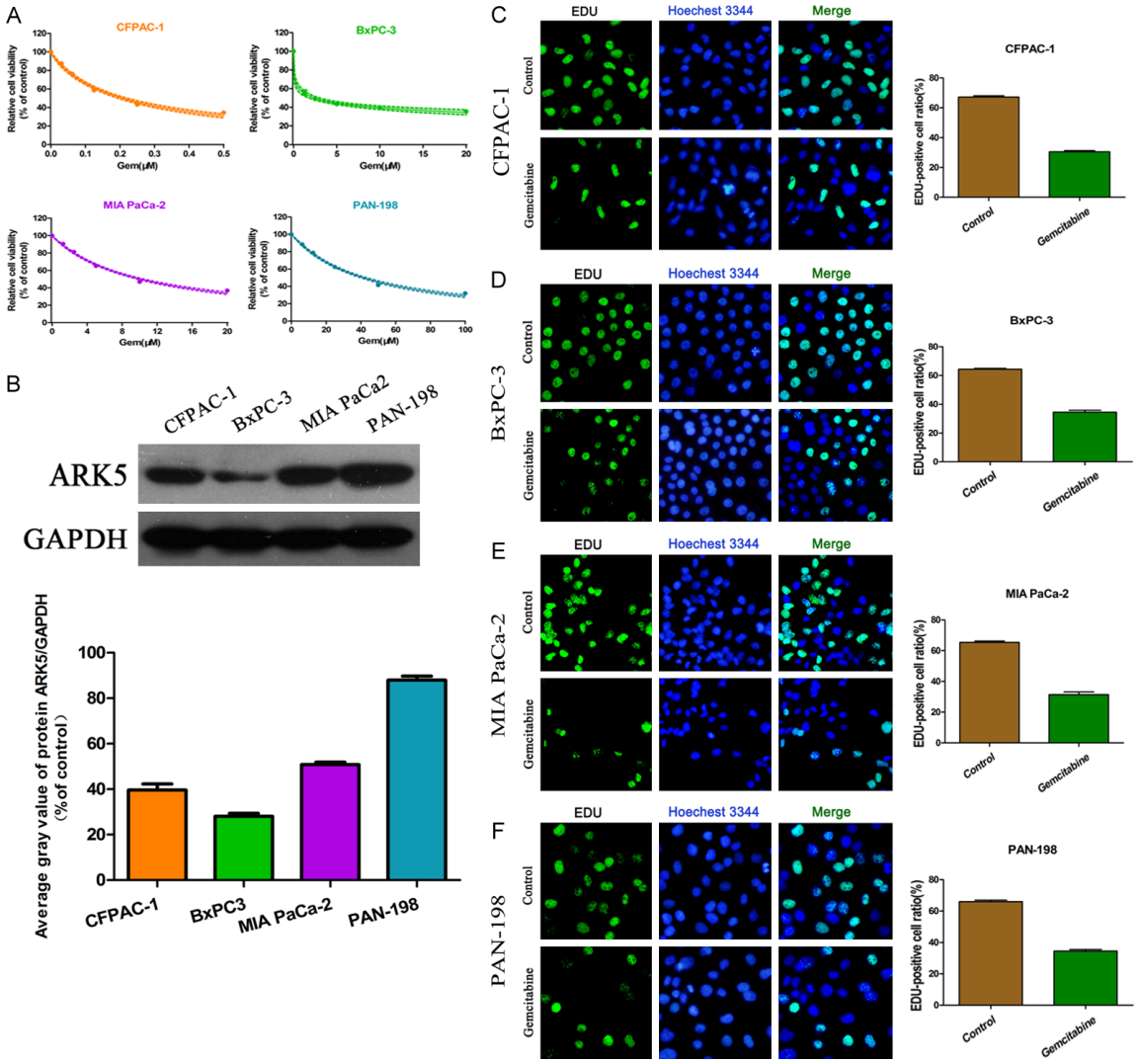

Figure 1. Expression of ARK5 is associated with increased GEM sensitivity in PC cells. A. Cell viabilities of PC cells (CFPAC-1, BxPC-3, MIA, PaCa-2, and PAN-198) were determined under different concentrations of GEM. B. Expression of ARK5 in PC cells was detected and quantified by comparing the expression levels to the internal control, GAPDH. C-F. Cell proliferation rates of PC cells were determined using the EdU assay under an IC_{50} concentration of GEM.

Table 1. IC_{50} values and statistical analyses of gemcitabine (Gem) treatments in PDAC cell lines

IC_{50} (μM)	0.208 (0.1946 to 0.2215)	2.393 (1.779 to 3.006)	10.12 (9.632 to 10.61)	41.45 (39.32 to 43.58)

IC_{50} values show gemcitabine concentration [μM. mean (95% confidence intervals)].

inhibiting activation of caspase-8 and the caspase-6-associated FasL/Fas system [15, 16]. Recent studies showed that the activation of ARK5 is associated with tumor drug resistance, including doxorubicin resistance in hepatocellular carcinoma and cisplatin resistance in non-small cell lung cancer [17, 18]. These findings showed that ARK5 may play an essential role in cancer progression and chemotherapy resistance. However, little is known about the role of ARK5 in PC drug resistance.

In the present study, we investigated the role of ARK5 in PC GEM resistance and explored the potential mechanisms underlying GEM resistance. We found that ARK5 expression in PC cell lines inversely correlated with GEM sensitivity. The mechanism at work involved the

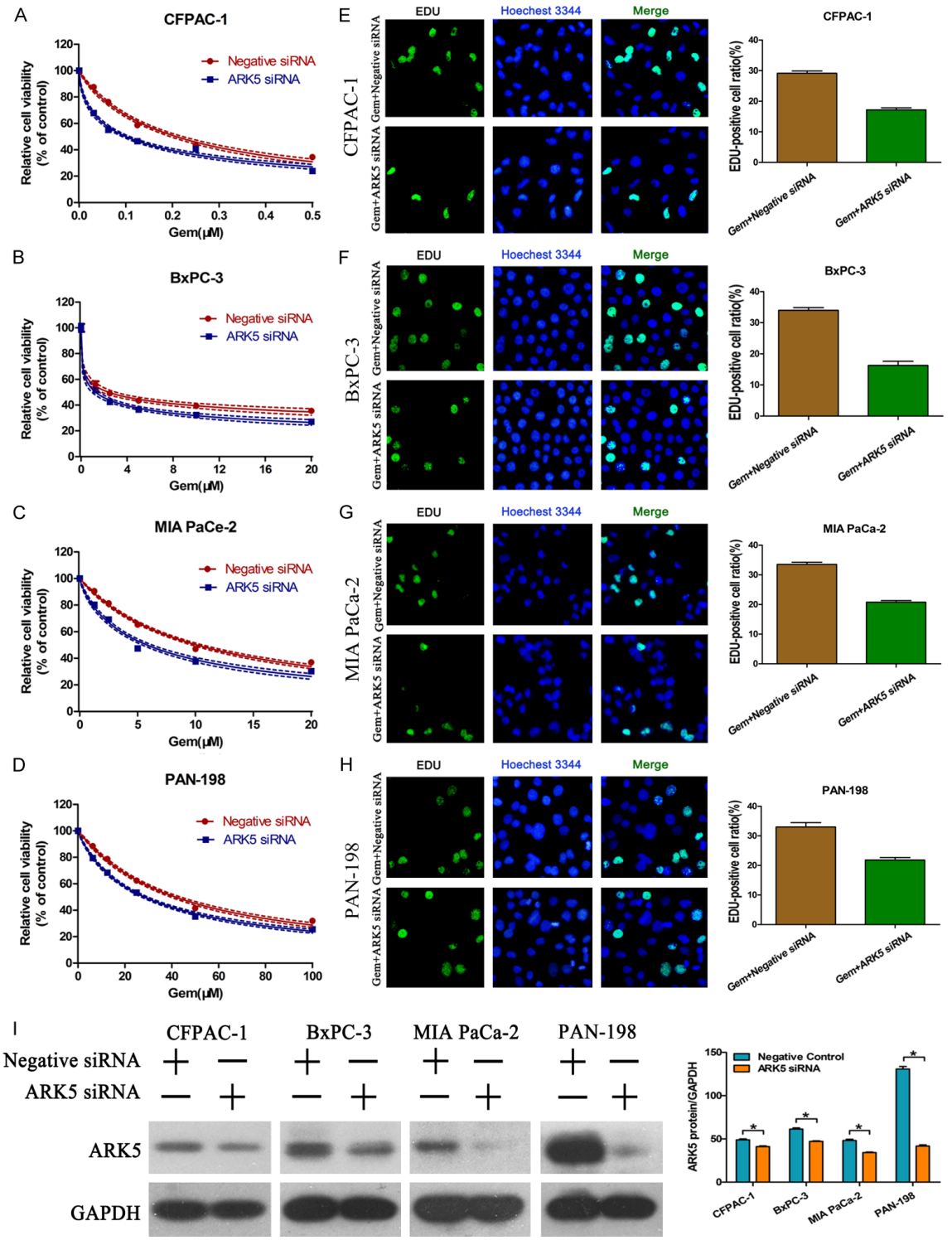

Figure 2. Suppression of ARK5 increases GEM sensitivity in PC cells. A-D. Cell viabilities of PC cells were determined under different concentrations of GEM with or without ARK5 knockdown. E-H. Cell proliferation rates of PC cells were determined using the EdU assay under an IC_{50} concentration of GEM with or without ARK5 knockdown. I. The efficiency of *ARK5* siRNA knockdown was verified by Western blotting using GAPDH as an internal control. *P < 0.05.

ARK5-mediated induction of epithelial-mesenchymal transition (EMT) in PC cell lines under GEM conditions. The same result was observed under hypoxic conditions. Cumulatively, we report that ARK5 conferred GEM resistance in PC by inducing EMT.

Table 2. The viability of PDAC cells in which ARK5 was knocked down cultured in different concentrations of gemcitabine (Gem)

Cell lines	IC_{50} (μM)	
	Negative siRNA+Gem	ARK5 siRNA+Gem
CFPAC-1	0.2135 (0.1953 to 0.2349)	1.269 (0.9429 to 1.594)
BxPC-3	2.40 (2.113 to 2.687)	1.269 (0.9429 to 1.594)
MIA PaCa-2	11.84 (11.13 to 12.56)	6.852 (6.235 to 7.469)
PAN-198	45.63 (42.43 to 48.83)	28.33 (26.95 to 29.70)

IC_{50} values show gemcitabine concentration [μM. mean (95% confidence intervals)].

Materials and methods

Cell culture

Pancreatic carcinoma cell lines CFPAC-1, BxPC-3, MIA, PaCa-2, and PAN-198 were purchased from the Chinese Academy of Science Cell Bank (Shanghai, China). CFPAC-1 cells were maintained in Dulbecco's modification of Eagle's medium (DMEM) (Gibco-Invitrogen, Carlsbad, CA, USA). BxPC-3, MIA, PaCa-2, and PAN-198 cells were maintained in RPMI1640. All culture media were supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA). At 37°C under a humidified atmosphere of 5% CO_2. GEM was obtained from Sigma (San Francisco, USA) and dissolved in ddH_2O.

CCK-8 assay

Pancreatic carcinoma cell lines (CFPAC-1, BxPC-3, MIA, PaCa-2, and PAN-198) were seeded at a density of 3000 cells/well in 96-well plates. After the cells completely adhered to the wells, culture medium was replaced with medium containing 1% FBS. After 24 h, the medium was replaced with 10% FBS supplemented with the indicated concentrations of GEM and incubated at 37°C under 5% CO_2 for 48 h. Cell viability was subsequently measured using a Cell Counting Kit-8 (Dojindo Laboratories, Japan) at 1, 2, and 3 h of culture according to the manufacturer's instructions. An MRX II microplate reader (Dynex, Chantilly, VA, USA) was used to measure the optical density (OD) value at 450 nm.

Western blotting

PC cell lines (CFPAC-1, BxPC-3, MIA, PaCa-2, and PAN-198) were seeded at a density of 2.0 × 10^5 cells/well in 6-well plates. After the cells completely adhered to the wells, the medium was replaced with 10% FBS and the IC_{50} concentration of GEM was added. Protein extraction and SDS-PAGE were performed according to the instructions from the manufacturer's website. Primary antibodies for E-cadherin, vimentin, Twist, and ARK5 (Abcam, Cambridge, MA, USA) were used at a dilution of 1:1000, the secondary antibody was used at a dilution of 1:2000.

Ethynyl deoxyuridine (EdU) assay

Quantifications of the proliferation of PC cell lines (CFPAC-1, BxPC-3, MIA, PaCa-2, and PAN-198) were determined using the Click-iTEdU Imaging Kit (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, cells were incubated with an IC_{50} concentration of GEM for 24 h, followed by 10 μM EdU for 2 h before fixation, permeabilization, and EdU staining. Cell nuclei were stained with Hoechst 33342 (Invitrogen) at a concentration of 5 μg/mL for 30 min.

Immunofluorescence

Immunofluorescence was used to assess E-cadherin and vimentin expression. PC cell lines (CFPAC-1, BxPC-3, MIA, PaCa-2, and PAN-198) were seeded into confocal dishes containing a sterile coverslip on the bottom. After transfection with *ARK5* or *Twist* siRNAs for 48 h in the presence of GEM or hypoxic conditions, cells were washed with PBS, fixed using 2% paraformaldehyde for 5 min, permeabilized using Triton X-100 0.1% for 10 min, and blocked in 10% FBS for 1 h at room temperature. Next, the cells were incubated with a 1:200 dilution of anti-E-cadherin monoclonal antibody or anti-vimentin monoclonal antibody (Abcam, Cambridge, MA, USA) overnight at 4°C. All samples were washed twice in PBS and incubated with goat-anti-rabbit CY3 at a 1:200 dilution as the secondary antibody (Beyotime Institute of Biotechnology, China). Finally, samples were incubated with DAPI for 5 min, washed twice in PBS, and examined by confocal microscopy (Olympus, Tokyo, Japan) to produce a merged image.

Statistical analysis

SPSS17.0 software and GraphPad Prism 5 softwar (GraphPad Software, Inc., La Jolla, CA, USA)

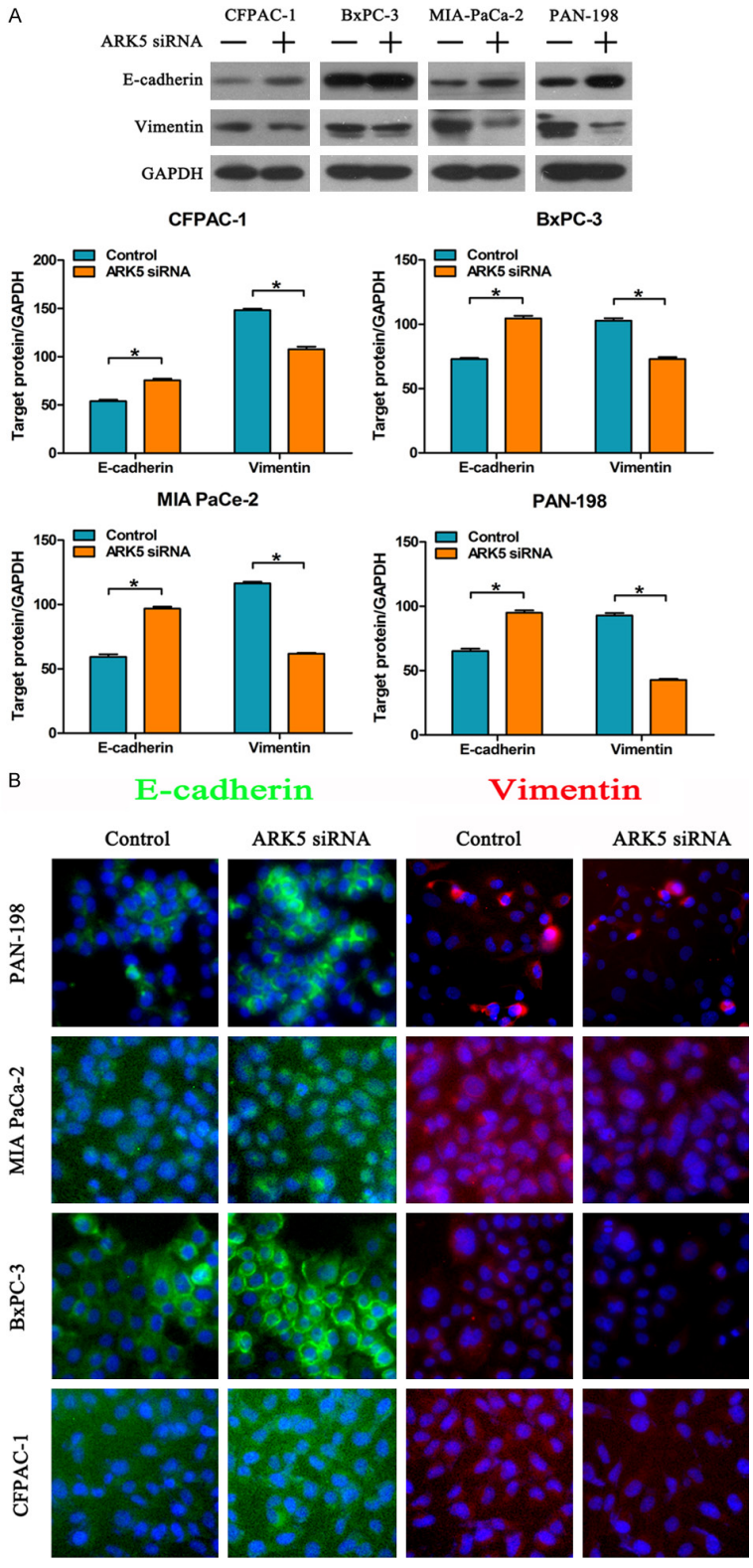

Figure 3. ARK5 regulates EMT. A. Expression of E-cadherin and vimentin were detected in PC cells treated with *ARK5* siRNAs or negative control PC cells, and the results were quantified by comparing expression levels to GAPDH. *P < 0.05. B. The expression change of E-cadherin and vimentin in PC cells treated with *ARK5* siRNAs or negative control PC cells detected by immunofluorescence.

was used for statistical analyses. The experimental data were expressed as the means ± SD, and assessed using a two-tailed Student's t-test to compare two groups and one-way analysis of variance (ANOVA) to compare multiple groups. Statistical significance was considered to be $P < 0.05$.

Results

Expression of ARK5 was associated with GEM sensitivity in PC cells

We first detected GEM sensitivity in four PC cell lines (CFPAC-1, BxPC-3, MIA, PaCa-2, and PAN-198) using the CCK-8 assay, and found that CFPAC-1 was the most sensitive to GEM while PAN-198 was the least sensitive (**Figure 1A**; **Table 1**). The IC_{50} of these four cell lines to GEM is shown in **Table 1**. We detected ARK5 expression in these four cell lines and found that PAN-198 had the highest expression of ARK5, which was associated with highest IC_{50} value for GEM (**Figure 1B**). In addition, we used the EdU assay to verify that GEM inhibited the proliferation of PC cells (**Figure 1C-F**).

Suppression of ARK5 increased the sensitivity of PC cells to GEM

To investigate the function of ARK5 in regulating GEM sensitivity in PC cells, we knocked down ARK5 using siRNAs and quantified cell viabilities in the presence of different concentrations of GEM in four PC cell lines (CFPAC-1, BxPC-3, MIA, PaCa-2, and PAN-198). We found that suppression of ARK5 increased GEM sensitivity in four PC cell lines (**Figure 2A-D**). The IC_{50} of these four cell lines to GEM after tranfected

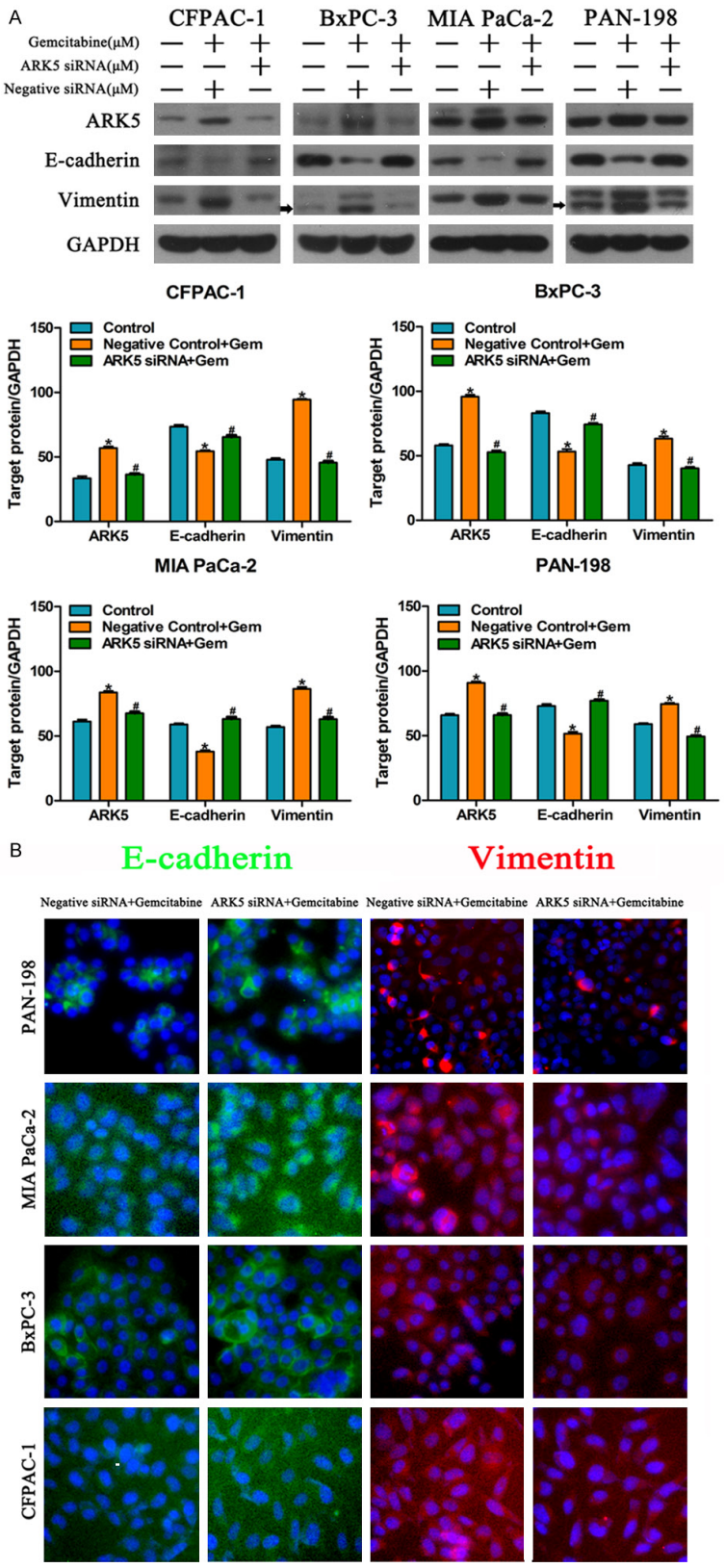

Figure 4. ARK5 regulates GEM sensitivity in PC cells via EMT. A. The expression change of E-cadherin and vimentin after GEM treatment with or without suppression of ARK5 detected by Western blotting. *P < 0.05. B. Immunofluorescence was used to verify the results of the Western blots.

with Negative siRNA or AKR5 siRNA is shown in **Table 2**. The EdU assay in these four PC cell lines confirmed these results (**Figure 2E-H**). The efficiency of knockdown of the ARK5 siRNAs was verified by Western blotting in the four PC cell lines (**Figure 2I**).

ARK5 regulates GEM resistance via EMT in PC cells

Since EMT is an important process mediating drug resistance in many solid tumor cells, we hypothesized that ARK5 was involved in EMT regulation in PC cells. We detected expression of EMT-related proteins following ARK5 suppression, and found that E-cadherin was up-regulated and vimentin was downregulated (**Figure 3A**). Interestingly, these observations were also verified by immunofluorescence (**Figure 3B**). Because we found that treatment with GEM increased E-cadherin expression and decreased vimentin expression in PC cells, we examined PC cells in the presence of GEM and *ARK5* siRNA, and the results showed that the expression dynamics of E-cadherin and vimentin were reversed with respect to the GEM group (**Figure 4A**). Similarly, these dynamics were verified by immunofluorescence analysis (**Figure 4B**).

ARK5 regulates EMT via the Twist pathway in PC cells

Since Twist is one of the major transcription factors regulating EMT, we examined whether Twist played a role in GEM resistance in PC cells. We first compared cell viability in PC cells under different concentrations of GEM with or without Twist suppression. The results showed that PC cells were

Table 3. The viability of PDAC cells in which Twist was knocked down cultured in different concentrations of gemcitabine (Gem)

Cell lines	IC_{50} (μM)	
	Negative siRNA+Gem	Twist siRNA+Gem
CFPAC-1	0.1893 (0.1734 to 0.2051)	0.09746 (0.08870 to 0.1062)
BxPC-3	4.220 (3.895 to 4.545)	1.995 (1.853 to 2.136)
MIA PaCa-2	12.13 (11.12 to 13.13)	6.689 (6.129 to 7.249)
PAN-198	44.83 (42.06 to 47.60)	25.92 (24.00 to 27.84)

IC_{50} values show gemcitabine concentration [μM. mean (95% confidence intervals)].

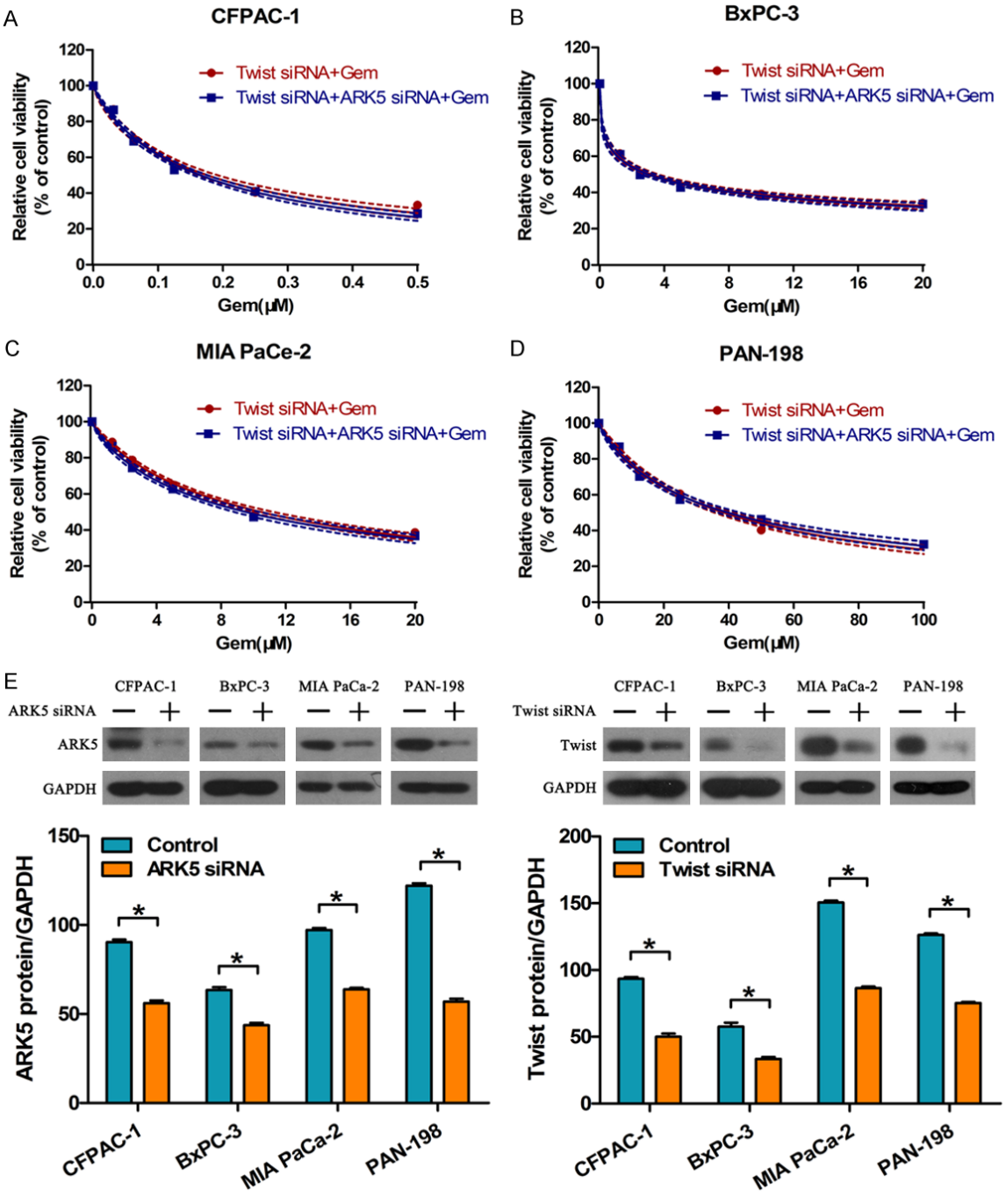

Figure 5. ARK5 regulates GEM sensitivity in PC cells via the Twist pathway. A-D. Cell viabilities of PC cells after GEM treatment coupled with *Twist* siRNA alone or both *Twist* siRNA and *ARK5* siRNA detected by the CCK-8 assay. E. The efficiencies of *ARK5* siRNAs and *Twist* siRNAs were verified by Western blotting, and the results were quantified by comparing the levels to GAPDH. *P < 0.05.

Table 4. The viability of PDAC cells in which Twist was knocked down, then knocked down the ARK5 cultured in different concentrations of gemcitabine (Gem)

Cell lines	IC_{50} (μM)	
	Twist siRNA+Gem	Twist siRNA+ARK5 siRNA+Gem
CFPAC-1	0.1760 (0.1613 to 0.1907)	0.1650 (0.1542 to 0.1757)
BxPC-3	3.167 (2.735 to 3.600)	2.982 (2.545 to 3.419)
MIA PaCa-2	10.47 (9.760 to 11.19)	9.538 (8.763 to 10.31)
PAN-198	38.48 (35.56 to 41.40)	39.81 (36.44 to 43.18)

IC_{50} values show gemcitabine concentration [μM. mean (95% confidence intervals)].

more sensitive to GEM following Twist suppression compared to the control group (Figure S1A-D). The IC_{50} of these four cell lines treated with GEM following tranfected with Negative siRNA or Twist siRNA is shown in **Table 3**. We also found that the suppression of Twist upregulated E-cadherin expression and downregulated vimentin expression in PC cells. These results were confirmed by both Western blotting and immunofluorescence analysis (Figure S1E, S1F). Furthermore, we detected cell viability in PC cells under different concentrations of GEM with Twist suppression alone or Twist and ARK5 double knockdown. We found that these two treatment conditions were not significantly different (**Figure 5A-D**). The IC_{50} of these four cell lines treated with GEM following tranfected with Twist siRNA or AKR5 siRNA is shown in **Table 4**. The suppression efficiency of Twist and ARK5 in PC cells was verified by Western blotting (**Figure 5E**, **5F**).

Suppression of ARK5 increased the sensitivity of PC cells to GEM under hypoxic conditions

Because hypoxia commonly occurs in the microenvironment of PC cells *in vivo* and it plays a crucial role in drug resistance and EMT regulation, we examined whether ARK5 contributes to drug resistance under hypoxic conditions. We first compared the cell viabilities of the BxPC-3 and MIA PaCa-2 cell lines under hypoxic versus normoxic conditions, and found that hypoxia increased the GEM resistance of PC cells. Next, we examined the cell viabilities of the BxPC-3 and MIA PaCa-2 cell lines under hypoxic conditions with or without ARK5 suppression and found that ARK5 suppression significantly increased GEM sensitivity during hypoxia (**Figure 6A**, **6B**). The IC_{50} of these four cell lines treated with GEM exposed to normoxia alone, hypoxia alone or hypoxia+ ARK5 siRNA is shown in **Table 5**. Furthermore, we examined the expression of E-cadherin and vimentin by Western blotting and immunofluorescence under hypoxic conditions with or without ARK knockdown and found that suppression of ARK5 reversed the influence of hypoxia on EMT regulation (**Figure 6C**, **6D**).

Discussion

PC is typically characterized by a high desmoplastic reaction, which plays a crucial role in inducing cross-talk between stromal cancer cells and inhibits the delivery and efficacy of chemotherapy. This unique microenvironment leads to chemotherapy drug resistance [19]. Because only 15-20% of patients are eligible for resection as a result of the presence of a locally advanced or metastatic tumor at the time of diagnosis, and GEM is the only first-line drug for treating advanced PC, investigating the mechanisms of GEM resistance is necessary for improving PC treatments [2-4]. In addition, recent studies have shown that ARK5 is required for drug resistance in solid tumors [17, 18]. Therefore, we suspected that ARK5 may play a vital role in GEM resistance in PC cells.

In this study, we found that expression of ARK5 was associated with GEM sensitivity in PC cell lines, and high levels of expression of ARK5 led to low GEM sensitivity. Furthermore, suppres-

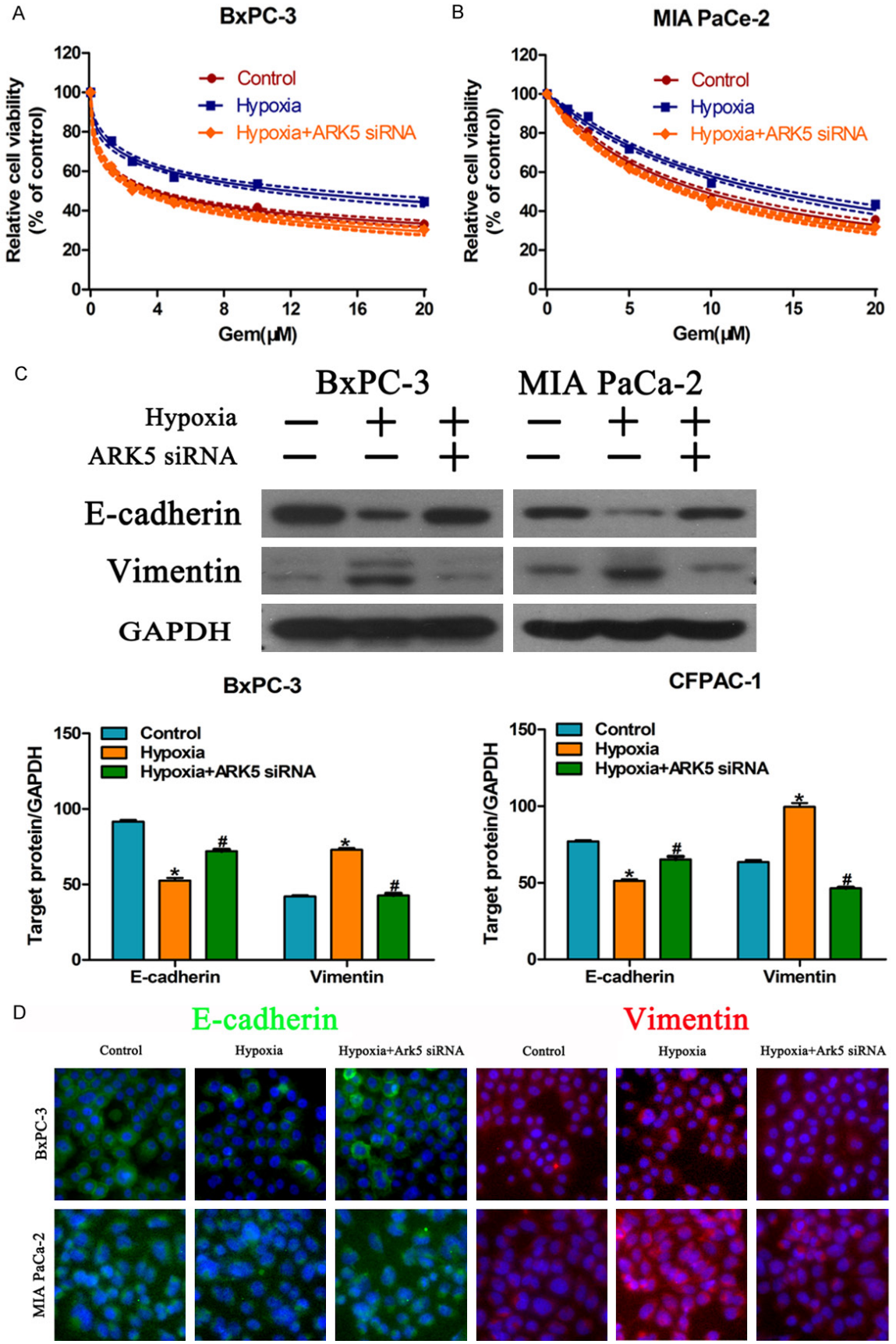
A
BxPC-3
Relative cell viability (% of control)
Control
Hypoxia
Hypoxia+ARK5 siRNA
Gem(μM)
B
MIA PaCe-2
C
BxPC-3
MIA PaCa-2
Hypoxia
ARK5 siRNA
E-cadherin
Vimentin
GAPDH
BxPC-3
CFPAC-1
Target protein/GAPDH
E-cadherin
Vimentin
D
E-cadherin
Vimentin
Control
Hypoxia
Hypoxia+Ark5 siRNA
BxPC-3
MIA PaCa-2

Figure 6. Suppression of ARK5 increases GEM sensitivity in PC cells under hypoxic conditions. A, B. Cell viabilities of the BxPC-3 and MIA PaCa-2 cell lines in the presence of different concentrations of GEM were determined under hypoxic conditions with or without *ARK5* siRNA. The negative control was examined under normoxic conditions. C. Expression of E-cadherin and vimentin were quantified under hypoxic conditions, following *ARK5* siRNA, or both. The negative control underwent none of these treatments. D. Immunofluorescence was used to verify the results of the Western blotting.

Table 5. The viability of PDAC cells in which ARK5 was knocked down with different concentrations of gemcitabine (Gem) under hypoxic or normoxic conditions

Cell lines	IC_{50} (μM)		
	Control	Hypoxia	Hypoxia+ARK5 siRNA
BxPC-3	3.736 (3.366 to 4.107)	10.04 (10.04 to 13.82)	3.116 (2.769 to 3.463)
MIA PaCa-2	9.547 (8.907 to 10.19)	13.73 (12.72 to 14.73)	8.321 (7.910 to 8.731)

IC_{50} values show gemcitabine concentration [μM. mean (95% confidence intervals)].

sion of ARK5 in PC cell lines significantly increased the efficiency of GEM treatment. Thus, our study preliminarily demonstrated that ARK5 was associated with drug resistance in PC cells.

It is known that EMT is required for tumor cells to acquire drug resistance, and recent studies have demonstrated that ARK5 can regulate the progression of EMT in many solid tumors [17, 18, 20-22]. Thus, we hypothesized that ARK5 may regulate drug resistance in PC cells via EMT. To test this, we first knocked down the expression of ARK5 in PC cells and found that this upregulated E-cadherin expression and downregulated vimentin expression. Next, we conducted the same experiment after exposing the cells to GEM. As expected, suppression of ARK5 after GEM exposure reversed the expression dynamics of E-cadherin and vimentin. Since Xu T [17] and his colleagues found that ARK5 regulates EMT in HCC cells via the Twist pathway, we confirmed the expression of Twist following ARK5 suppression and found that ARK5 also regulated EMT in PC cells via the Twist pathway. Thus, our study found that ARK5 increased GEM resistance in PC cells via Twist-mediated EMT.

Hypoxia is characteristic of the microenvironment of most solid tumors, and it helps control cell proliferation, angiogenesis, and apoptosis by affecting many signaling pathways. It has been shown to be involved in resistance to chemotherapy and radiation therapy and, hence, poorer patient prognoses [23]. Because it has been showed that hypoxia can increase the resistance of PC cells to apoptosis induced by GEM [24], we examined whether ARK5 played a role in the drug resistance of PC cells under hypoxic conditions. As expected, we found that suppression of ARK5 significantly increased GEM sensitivity in PC cells under hypoxic conditions. We also found that suppression of ARK5 reversed the effects of hypoxia on the expression of E-cadherin and vimentin. Thus, ARK5 clearly regulates GEM resistance under hypoxic conditions via EMT.

In conclusion, our study found that ARK5 increases GEM resistance via EMT under both normoxic and hypoxic conditions. These findings may inform new therapeutic targets for increasing the effectiveness of PC chemotherapy, and identify new molecular biomarkers for predicting chemotherapy drug resistance. However, further clinical trials are needed to verify this.

Acknowledgements

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Disclosure of conflict of interest

None.

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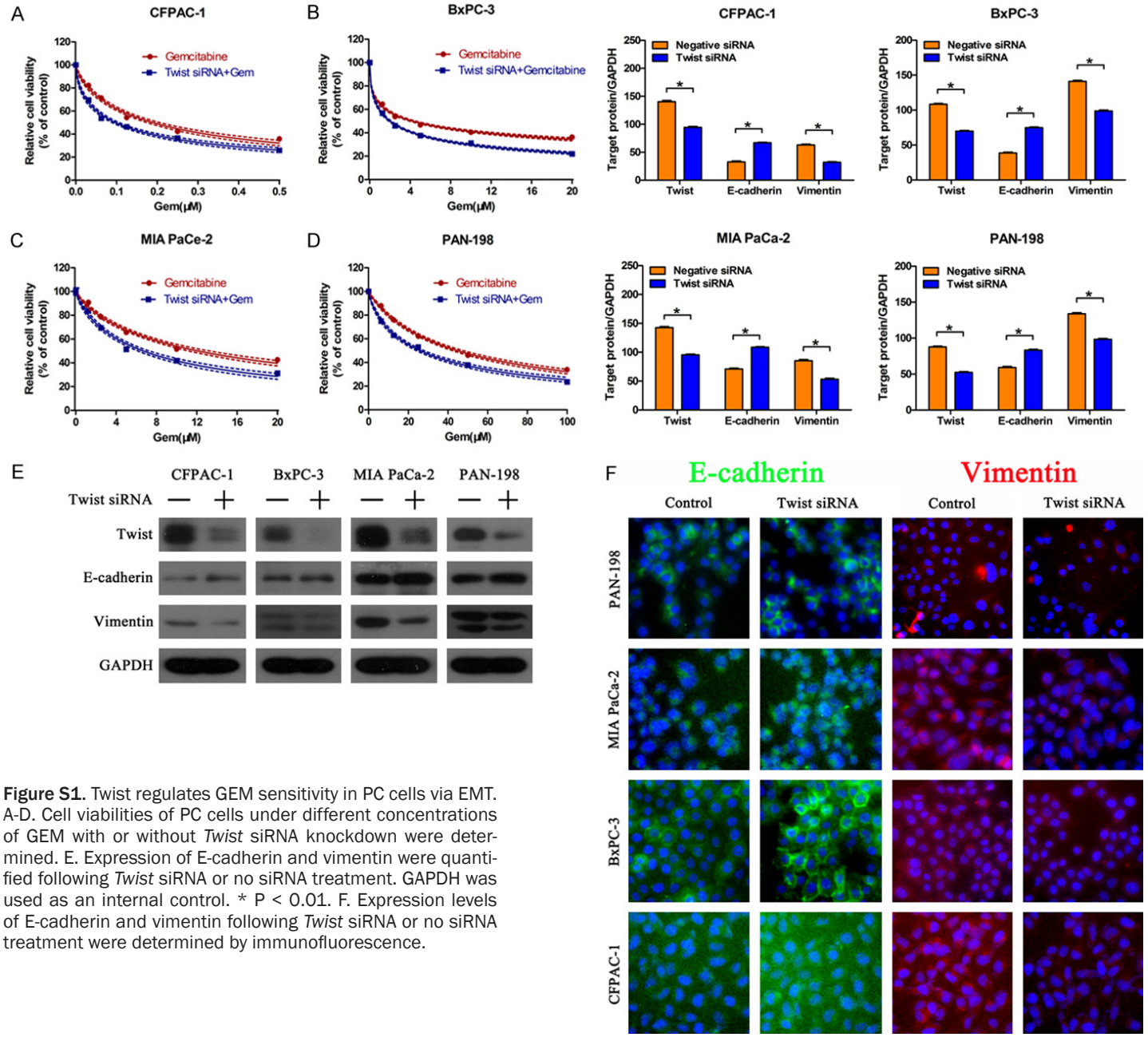

Figure S1. Twist regulates GEM sensitivity in PC cells via EMT. A-D. Cell viabilities of PC cells under different concentrations of GEM with or without *Twist* siRNA knockdown were determined. E. Expression of E-cadherin and vimentin were quantified following *Twist* siRNA or no siRNA treatment. GAPDH was used as an internal control. * $P < 0.01$. F. Expression levels of E-cadherin and vimentin following *Twist* siRNA or no siRNA treatment were determined by immunofluorescence.