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LncRNA XIST promotes pancreatic cancer migration, invasion and EMT by sponging miR-429 to modulate ZEB1 expression

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ABSTRACT

Pancreatic cancer (PC) has become a worldwide malignancy accompanied by high metastasis and extremely poor prognosis. The critical roles of long non-coding RNAs (lncRNAs) in PC are generally summarized as molecular sponges of microRNAs (miRNAs). We intended to investigate the biological function and mechanism of lncRNA X-inactive specific transcript (XIST) in PC progression, especially in PC cell migration and invasion. qPCR was applied to detect the expression levels of XIST and miR-429 in PC tissues and cell lines. The roles of XIST and miR-429 on PC cell migration, invasion and epithelial-mesenchymal transition (EMT) were assessed by wound healing, transwell, qPCR and Western blot assays, respectively. The regulating relationship among XIST, miR-429 and zinc finger E-box binding homeobox 1 (ZEB1) was investigated in PC cells. XIST was frequently upregulated while miR-429 was commonly downregulated in PC tissues, especially in metastatic PC tissues. Knockdown of XIST in two PC cell lines caused inhibition of migration, invasion and EMT capacities. Forced expression of miR-429 exerted the similar tumor suppressing effects. XIST repressed miR-429 expression thus upregulated ZEB1, one of the targets of miR-429. ZEB1 mediated the tumor suppressing roles of XIST knockdown in PC cells. We identified the critical axis of XIST/miR-429/ZEB1 in PC cell migration, invasion and EMT, which may aid in developing new therapeutic strategies for PC.

1. Introduction

Pancreatic cancer (PC), especially pancreatic ductal adenocarcinoma (PDAC), represents as one of the most malignant neoplasms with extremely poor overall survival (Raimondi et al., 2009; Egawa et al., 2012; Ryan et al., 2014). It is also a leading contributor of cancer-related lethality worldwide (Fiorino et al., 2018). Due to the shortage of early diagnostic options and effective therapy strategies, most PC patients are newly diagnosed in advanced stages with local infiltration or distant metastasis. This situation limits the treatment efficiency and leads to a poor 5-year survival rate (less than 8%) (Li et al., 2004; Siegel et al., 2017; Walling and Freelove, 2017). Featured by late clinical presentation, early metastasis and dismal prognosis, PC attracts intensive attention recently, and it is quite important to learn more about the underlying mechanisms governing PC progression to develop effective treatment targets for PC. Metastasis is a multi-step process consisting of epithelial-to-mesenchymal transition (EMT), migration and invasion, to escape the primary site and spread into the surrounding or distant tissues (Rhim et al., 2012). The aggressive phenotypes of PC patients are mainly caused by metastasis; even post curative resection, patients usually die of metastasis or recurrence (Walling and Freelove, 2017). Hence, to prevent PC metastasis, it is critical to learn more about the internal mechanisms triggering this process.

Thanks to the human genome project, it is now deeply conscious that the mammalian genome is principally transcribed into non-coding RNAs (ncRNAs), which mainly include long non-coding RNAs (lncRNAs) and short non-coding RNAs like microRNAs (miRNAs) (Esteller, 2011). Briefly, lncRNAs contain over 200 nucleotides in length and possess almost no protein-coding capacities (Wang and Chang, 2011). They function in diverse manners, including as guiders, scaffolds, decoys and tethers of other molecules (Wang and Chang, 2011; Rinn and Chang, 2012). MiRNAs are featured by short length

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(19–25 nucleotides) and can mediate gene silencing through base pairing to the 3′-untranslated region (3′-UTR) of the target genes, thereby leading to mRNA degradation or translational repression (Hutvágner and Zamore, 2002; Winter and Diederichs, 2011). Increasing evidences have revealed that both lncRNAs and miRNAs are implicated in PC development, and might serve as novel therapeutic targets (Moschovis et al., 2017; Guo et al., 2018). Recent studies indicate that lncRNAs could inhibit the expressions and biological roles of specific miRNAs by acting as competitive endogenous RNAs (ceRNAs) (Cesana et al., 2011; Yang et al., 2016). This cognizance establishes a complex internal regulation network in mammalian cells and provide more possibilities to develop cancer therapy options.

LncRNA X inactive-specific transcript (XIST) is imbedded in the XIST gene and a crucial contributor of X chromosome inactivation in female (Brown et al., 1991). Emerging lines of evidences identify that XIST is dysregulated in various cancer types, and exerts either tumor-suppressing or oncogenic roles in different cancers (Yang et al., 2018). In PC, it has been shown that XIST could promote cell proliferation, migration and invasion, and inhibit cell apoptosis (Wei et al., 2017; Sun et al., 2018). However, the role and molecular mechanism of XIST in PC metastasis remain ill-defined. Given that lncRNAs could bind to miRNAs to regulate its expression and activity (Cesana et al., 2011; Yang et al., 2016), we took advantage of the bio-informatic tools (Starbase v2.0, miRcode and RNAhybrid), and predicted miR-429 as a possible binding partner of XIST. MiR-429 is a well characterized anti-tumor factor in PC, mainly through inhibiting PC cell growth (Song et al., 2015). Recently, Liu et al found that miR-429 exerted ant-invasion effects in PC (Liu et al., 2018). The targeting of zinc finger E-box binding homeobox 1 (ZEB1), a major contributor of cancer metastasis, by miR-429 has been mentioned in several human cancers (Kong et al., 2015; Deng et al., 2017). Therefore, the flow from XIST to miR-429 and ZEB1 might be a critical clue to interpret the pro-metastasis effect of XIST in PC cells.

In this study, we intend to investigate the expression, biological roles and molecular mechanism of XIST in PC cells. Our findings revealed that XIST was significantly upregulated in PC tissues, especially in metastatic PC tissues. Knockdown of XIST resulted in decreased migration, invasion and EMT capacities. Further mechanistic exploration demonstrated that XIST could act as a ceRNA for miRNA-429 to modulate ZEB1 expression. Our study identified the oncogenic XIST/miR-429/ZEB1 axis in PC progression, which may be used in the targeted applications in PC therapy.

2. Materials and methods

2.1. Tissue sample collection

Human PC tissues and the matched non-tumor normal tissues were collected from 120 patients who received surgery in Fudan University Shanghai Cancer Center from 2014 to 2017. All these tissues were confirmed by at least two pathologists. The present study was approved by the Ethics Committee of Fudan University Shanghai Cancer Center, and written informed consents were received from all these 120 patients at the initial stage of this study.

2.2. Cell culture

Five PC cell lines (ASPC-1, PANC-1, HPAC, BxPC-3, CFPAC-1) and human pancreatic ductal epithelial (HPDE) cells were obtained from Cell Resource Center of Shanghai Institute of Life Sciences (Shanghai, China). Cells were routinely cultured in DMEM supplemented with 10% FBS (Gibco, USA), 100 U/ml penicillin and 100 μg/ml streptomycin in the 37 °C incubator with 5% CO_2. Cells were authenticated every 1 year by cell morphology survey.

2.3. RNA extraction and quantitative real-time PCR

Total RNAs from PC tissues or cells were extracted with Trizol (Invitrogen, USA) as per the standard protocol. RNAs were then reverse transcribed to complementary DNAs (cDNAs) with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Quantitative real-time PCR (qPCR) was then carried out using the SYBR Green PCR Kit (Takara, Japan) following the manufacturer's instructions. Results were normalized to GAPDH. miR-429 expression was determined by using the All-in-one miRNA qPCR detection kit (GeneCopoeia, USA), and results were normalized against U6 small RNA. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. qPCR primers were listed in Supplemental Table 1.

2.4. Lentiviruses and stable sub-cell lines

Lentiviruses for small hairpin RNAs targeting XIST (sh-XIST) and negative control (sh-NC) were packaged by Genechem (Shanghai, China). PANC-1 and ASPC-1 cells were infected with the indicated lentivirus and selected with puromycin (1 μg/ml) for one week to generate stable sub-cell lines.

2.5. Plasmid, miRNA and cell transfection

ZEB1 expression plasmid was constructed by GeneCopoeia (Guangzhou, China) and validated by DNA sequencing. miR-429 mimic, inhibitor and the negative control oligonucleotides were ordered from Ribobio (Guangzhou, China). Cell transfection was finished with Lipofectamine 2000 (Invitrogen, USA) and cells were harvested for subsequent experiments after 24 h of transfection. All the transfections were repeated at least three times independently.

2.6. Western blot

Total proteins from cells were isolated from cells and separated using SDS-PAGE gels (20 μg proteins were loaded in each lane). Antibodies for E-cadherin, Claudin-1, β-catenin, Snail, ZEB1 and β-actin were all ordered from Cell Signaling Technology (USA). The procedure of Western blot was described as previously (Zhou et al., 2017).

2.7. Wound healing and transwell assays

For wound healing, cells were firstly seeded into 6-well plates. 12 h later, the 200 μl pipette tip was used to create an artificial wound. The wound closure was surveyed and photographed after 48 h. The fraction of cell coverage across the initial wound was monitored to evaluate the migration rate. For transwell assay, the transwell chambers were pre-coated with Matrigel (BD, USA). Cells were then trypsinized and seeded into the upper transwell chamber at the density of 5×10^4 cells in serum-free DMEM medium. Besides, the bottom chamber contains 500 μl DMEM with 10% FBS. After 48 h, none-invaded cells were removed. Cells invading into the bottom chamber were then fixed with methanol and stained with 0.1% crystal violet. Cell invasion was photographed under a microscope and quantitated by counting five random visions. The experiments were performed in triplicate.

2.8. Luciferase reporter assays

The wild-type XIST fragment (5853–6811) containing the putative miR-429 binding sites was inserted into the pmirGLO dual-luciferase vector to generate the reporter XIST-wt. To investigate the binding specificity, the putative miR-429 binding sites in the XIST-wt vector were mutated (5′-AGUAUU-3′ changed to 5′-GACGTT-3′) to create the reporter XIST-mu. PANC-1 cells were then co-transfected with XIST-wt or XIST-mu, and miR-429 mimic or miR-429 inhibitor. After 48 h, luciferase activities were measured with Dual-luciferase Reporter Assay

System (Promega, USA) following the manufacturer's instructions. Besides, the wild-type or mutant ZEB1 3′-UTR containing the putative miR-429 binding sites were inserted into the pmirGLO dual-luciferase vector to construct the ZEB1-3′UTR-wt or ZEB1-3′UTR-mu reporter, respectively. The ZEB1-3′UTR-wt or ZEB1-3′UTR-mu, and miR-429 mimic, sh-XIST or miR-429 mimic, were co-transfected into PANC-1 cells. Luciferase activities were measured as described above.

2.9. Statistics

Data were presented as mean ± SD from at least three independent experiments. Statistical analyses were performed with SPSS 13.0 software. The significance were calculated by Student's *t* test, one way ANOVA with Dunnett's post test, or Pearson correlation analysis. Statistical significance was assigned at $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***).

3. Results

3.1. Upregulation of lncRNA XIST in PC tissues and cell lines

To confirm the expression alteration of lncRNA XIST in PC tissues, we applied qPCR detection in 120 pairs of PC tissues including tumoral and paired normal tissues. In the 120 pairs of PC tissues, our qPCR detection revealed 14 normal samples had a higher level of XIST (fold change of Tumor vs. Normal < 1, only 11.67% of the 120 pairs of PC tissues) than that in paired tumors. The majority of the normal samples had a lower level of XIST than that in paired tumors (fold change > 1, 88.33%). The median expression levels of XIST in normal samples is 1.355, while in tumors is 7.565. Therefore, we validated that XIST was indeedly significantly upregulated in PC tissues in comparison with paired normal tissues ($P < 0.001$; Fig. 1A). Its upregulation was more evident in tissues with distant metastasis than those without distant metastasis ($P < 0.001$; Fig. 1B), implying that XIST might impact on PC cell metastasis. Subsequent expression detection revealed that XIST was also upregulated in several PC cell lines in comparison with that in human pancreatic ductal epithelial (HPDE) cells (Fig. 1C).

3.2. Knockdown of XIST inhibits PC cell migration, invasion and EMT capacities

Based on the XIST expression results in PC cell lines, we firstly established XIST stable knockdown sub-cell lines by infecting the two PC cell lines (PANC-1 and ASPC-1) with sh-XIST lentivirus and selecting with puromycin. qPCR validated the successful knockdown of XIST in the sh-XIST treated cells (Fig. 2A). Significantly, knockdown of XIST resulted in altered cell morphology from the original spindle shape to a rounded or cobblestone-like shape in PANC-1 cells (Fig. 2B). Furthermore, knockdown of XIST markedly suppressed cell migration and invasion in PANC-1 and ASPC-1 cells as guarded by wound healing and transwell assays, respectively ($P < 0.001$; Fig. 2C and D). Since EMT is an essential step of tumor pro-metastasis, we tested whether XIST knockdown affected EMT in PC cells. Interestingly, knockdown of XIST upregulated the expressions of epithelial markers (E-cadherin and Claudin-1) and downregulated the expressions of mesenchymal markers (β-catenin and Snail; Fig. 2E and F, and Supplemental Fig. 1). These data collectively suggest that XIST is a promoter for PC cell migration, invasion and EMT in vitro.

3.3. Reciprocal regulation between XIST and miR-429

Numerous studies have indicated that lncRNAs could bind with miRNAs via shared miRNA binding sites to regulate the expressions and activities of specific miRNAs (Cesana et al., 2011; Yang et al., 2016). We then used the online bio-informatic tools (Starbase v2.0, miRcode and RNAhybrid) to screen the potential miRNAs which could interact with XIST. To our interest, miR-429, a formally characterized anti-tumor factor in PC (Song et al., 2015), was predicted to bind to XIST in three sites (Fig. 3A). We then detected the expression of XIST in miR-429 overexpressing (by miR-429 mimic) and inhibition (by miR-429 inhibitor) cells, and found that XIST was repressed by miR-429 in two PC cell lines (Fig. 3B and C). Mock indicated the group treated with the equal amount of negative control oligonucleotides and transfection reagent as the miR-429 mimic/inhibitor groups. In order to confirm the relationship between XIST and miR-429, we conducted a luciferase reporter assay. Both the wild type reporter containing three miR-429 binding sits (XIST-wt) and the mutant type reporter (XIST-mu) were constructed (Fig. 3D). Co-transfection of miR-429 mimic and XIST-wt obviously reduced the luciferase activity, whereas co-transfection of miR-429 inhibitor and XIST-wt led to a significant elevation of the luciferase activity, which was in consistence with the XIST expression repression by miR-429 as detected above (Fig. 3E). However, XIST-mu reporter showed no change upon co-transfections (Fig. 3E). We then analyzed the expression of miR-429 in PC cells after XIST inhibition by its shRNA, and found that miR-429 was increased by sh-XIST treatment (Fig. 3F). Our findings demonstrate that there is a reciprocal regulation between XIST and miR-429.

3.4. miR-429 inhibits PC cell migration, invasion and EMT capacities

Although studies have shown that miR-429 inhibits cell growth in PC, whether it participates in PC migration, invasion and EMT remains unclear. We then examined its expression in the aforementioned 120 pairs of PC tissues. In the 120 pairs of PC tissues, our qPCR detection revealed 20 normal samples had a lower level of miR-429 (fold change of Tumor vs. Normal > 1, only 16.67% of the 120 pairs of PC tissues)

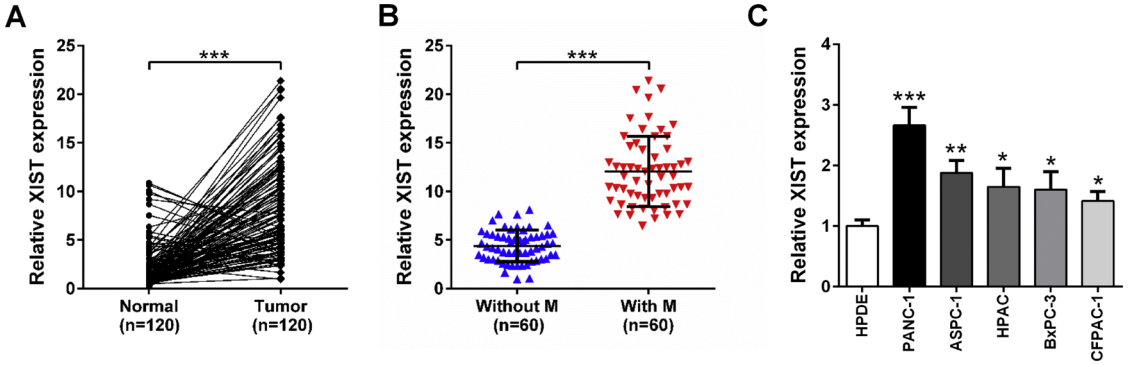

Fig. 1. Upregulation of lncRNA XIST in PC tissues and cell lines. (A) Relative expression of XIST in PC tissues (n = 120; Tumor) and paired normal tissues (n = 120; Normal). (B) Relative expression of XIST in PC tissues without (n = 60) and with (n = 60) distant metastasis. (C) Relative expression of XIST in PC cell lines. Data were presented as mean ± SD from at least three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

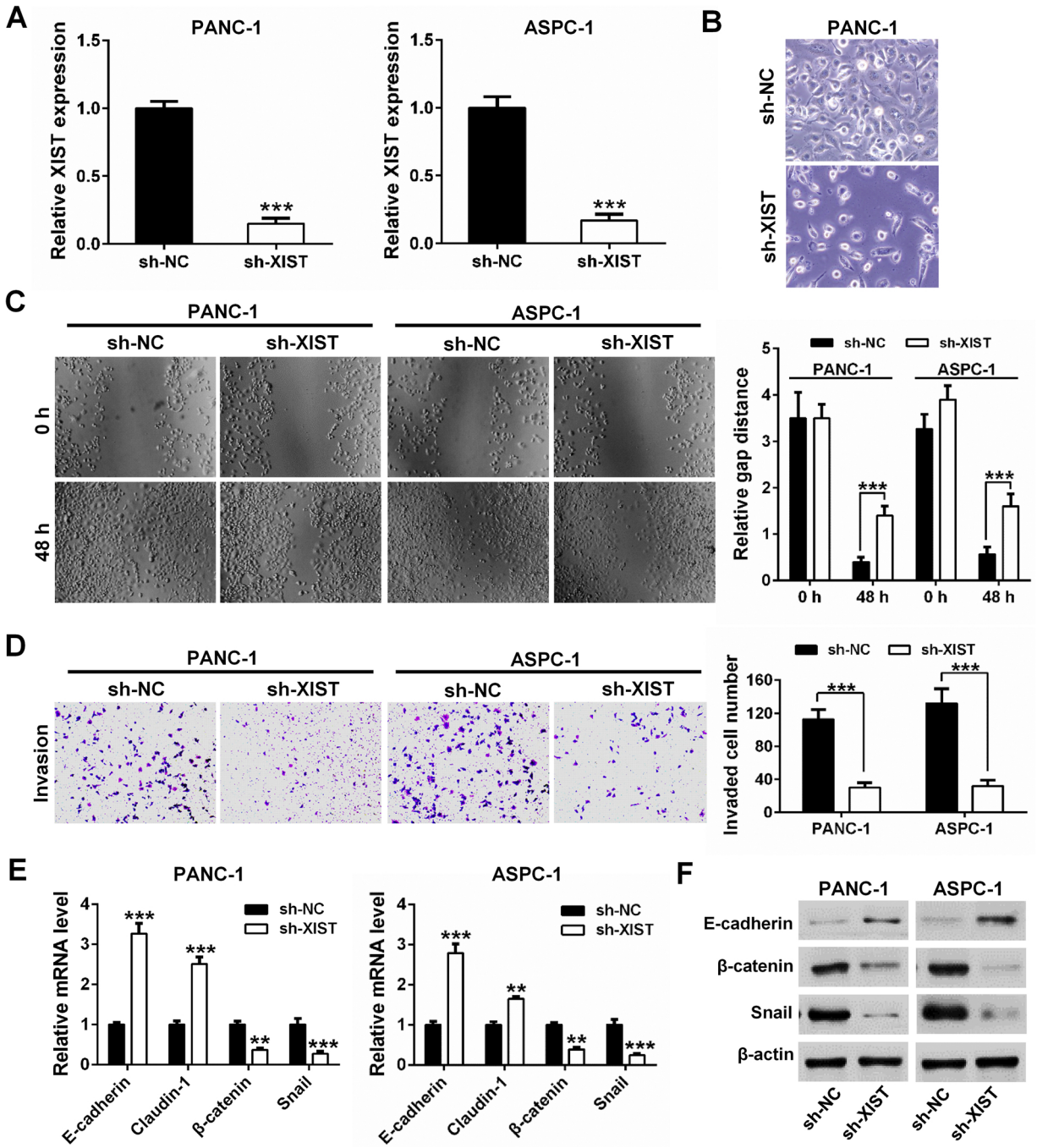

Fig. 2. Knockdown of XIST inhibits PC cell migration, invasion and EMT. (A) qPCR confirmation of the XIST knockdown in two PC cell lines PANC-1 and ASPC-1. (B) PANC-1 cells underwent morphological change from a spindle shape to a rounded or cobblestone-like shape upon XIST knockdown. Magnification: ×400. (C) Knockdown of XIST inhibited PC cell migration as indicated by wound healing assay. (D) Knockdown of XIST inhibited PC cell invasion as indicated by transwell assay. (E) qPCR analysis of the expression of epithelial markers (E-cadherin, Claudin-1) and mesenchymal markers (β-catenin, Snail) upon XIST knockdown. (F) Western blot analysis of the expression of epithelial markers (E-cadherin) and mesenchymal markers (β-catenin, Snail) upon XIST knockdown. Data were presented as mean ± SD from at least three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$.

than that in paired tumors. The majority of the normal samples had a higher level of miR-429 than that in paired tumors (fold change > 1, 83.33%). The median expression levels of miR-429 in normal samples is 5.055, while in tumors is 0.61. Therefore, we verified that miR-429 was decreased in PC tissues ($P < 0.001$; Fig. 4A). The decrease was more evident in tissues with distant metastasis than those without distant metastasis ($P < 0.001$; Supplemental Fig. 2A). Co-expression analysis revealed a notable inverse correlation between XIST and miR-429 levels ($P < 0.001$; Fig. 4B), and it was decreased in PC cell lines (Supplemental Fig. 2B). These results further validated the negative regulation relationship between XIST and miR-429. Similar to XIST inhibition, miR-429 overexpression by transfecting miR-429 mimic resulted in alteration of cell morphology (Fig. 4C), inhibition of cell migration and invasion in PANC-1 and ASPC-1 cells ($P < 0.001$; Fig. 4D and E). Also, miR-429 overexpression increased the expressions of epithelial markers (E-cadherin and Claudin-1) and decreased the expressions of mesenchymal markers (β-catenin and Snail; Fig. 4F and G, and Supplemental Fig. 3). These results verify that miR-429 exerts the opposite effects of XIST in PC cell migration, invasion and EMT.

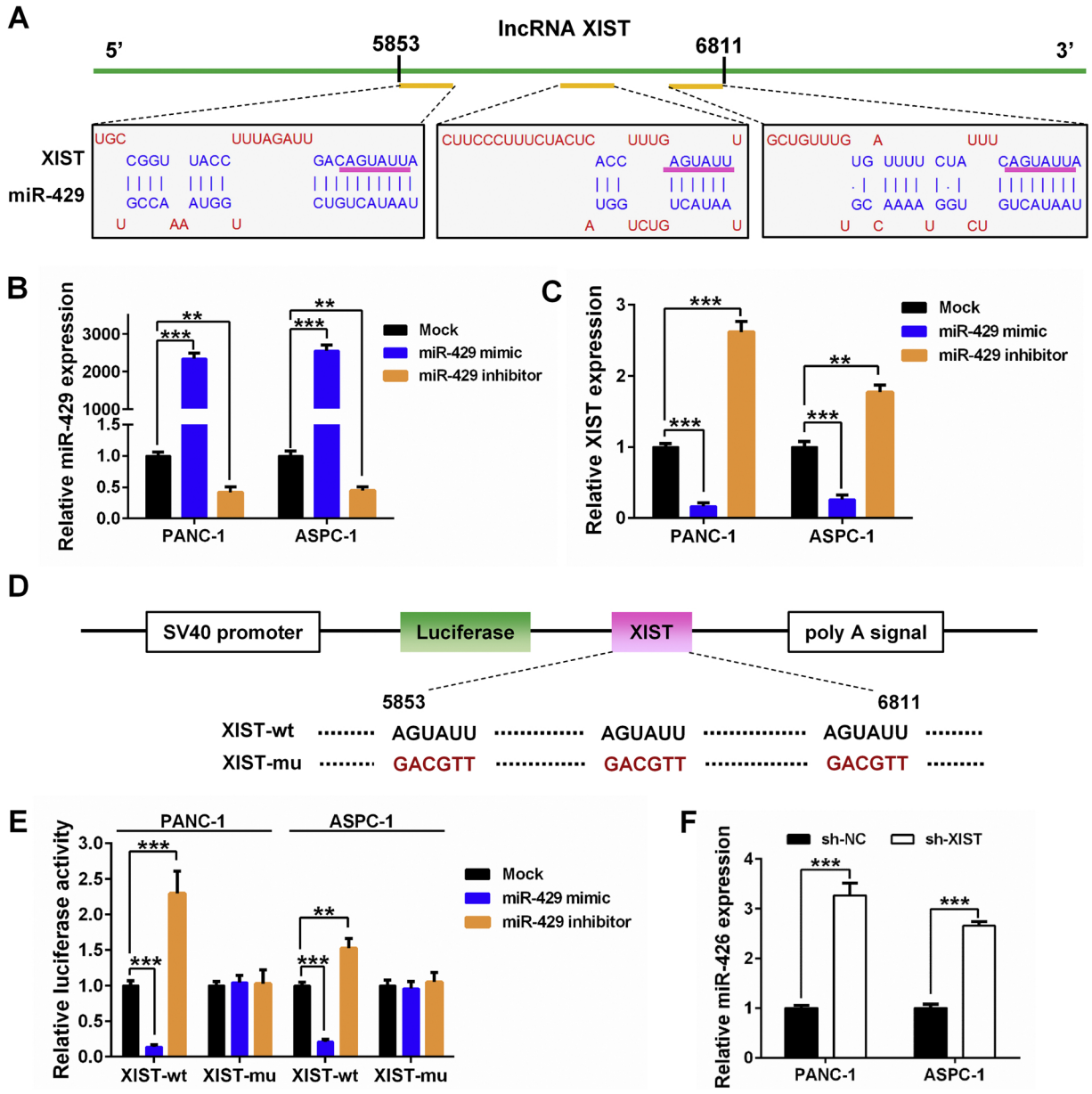

Fig. 3. Reciprocal repression between XIST and miR-429. (A) Schematic representation of the predicted target sites for miR-429 in lncRNA XIST. (B) qPCR confirmation of miR-429 overexpression by its mimic, or downregulation by its inhibitor in PANC-1 and ASPC-1 cells. (C) Ectopic miR-429 expression decreased XIST expression while inhibition of miR-429 increased XIST expression. (D) Construction of XIST luciferase reporters containing either wild type miR-429 binding sequences (XIST-wt) or mutant miR-429 binding sequences (mutation in red; XIST-mu). (E) Luciferase reporter assay in PANC-1 cells co-transfected with the reporter plasmid (XIST-wt or XIST-mu) plus Mock/miR-429 mimic/miR-429 inhibitor. (F) Knockdown of XIST increased miR-429 expression in PANC-1 and ASPC-1 cells. Data were presented as mean ± SD from three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.5. XIST serves as the ceRNA for miR-429 to regulate ZEB1 expression

Having demonstrated that XIST negatively regulates miR-429 in PC cells, we wondered whether XIST impacted on the expression of the target of miR-429. It has been reported that zinc finger E-box binding homeobox 1 (ZEB1) is an important validated target of miR-429 in several human cancers (Kong et al., 2015; Deng et al., 2017). As is shown, there are two miR-429 binding sites in the 3′-UTR of *ZEB1* mRNA (Fig. 5A). Expression detection confirmed the inhibition of ZEB1 expression by miR-429 (Fig. 5B and C). As a molecular sponger of miR-429, lncRNA XIST theoretical induced ZEB1 expression. Quite in line with the hypothesis, sh-XIST downregulated ZEB1 expression at both mRNA and protein levels (Fig. 5D and E). Luciferase reporter assays showed that miR-429 overexpression (by miR-429 mimic) or knockdown of XIST (by sh-XIST) could reduce the luciferase activity of the wild type ZEB1-3′UTR (ZEB1-3′UTR-wt) but not the mutant type ZEB1-3′UTR (ZEB1-3′UTR-mu; Fig. 5F). The reduction resulted from XIST knockdown could be even aggravated by miR-429 overexpression (sh-XIST + miR-429 mimic) and be reversed by miR-429 inhibition (sh-XIST + miR-429 inhibitor; Fig. 5F). Protein expression of ZEB1 exhibited the similar alteration as the luciferase reporter assays (Fig. 5G). These results indicate that XIST could upregulate ZEB1 expression by sponging miR-429 in PC cells.

3.6. ZEB1 rescues the anti-invasion effects exerted by XIST knockdown

Through overexpressing ZEB1 alone into PC cells (Supplemental Fig. 4A and 4B), we found that ZEB1 itself promoted PC cell migration,

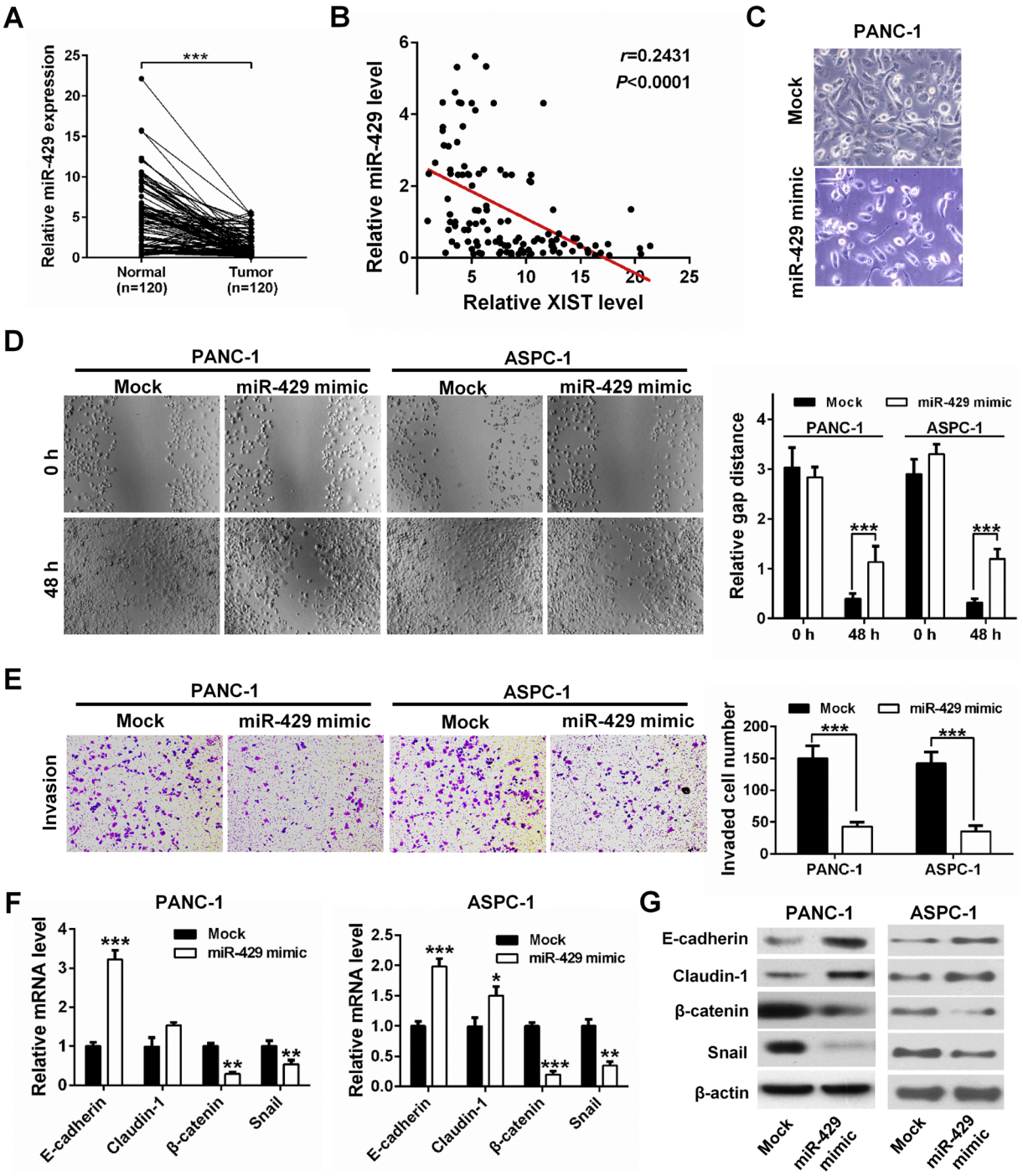

Fig. 4. miR-429 inhibits PC cell migration, invasion and EMT. (A) Relative expression of miR-429 in PC tissues (n = 120; Tumor) and paired normal tissues (n = 120; Normal). (B) XIST expression inversely correlated with the miR-429 expression in PC tissues (n = 120). (C) PANC-1 cells underwent morphological change from a spindle shape to a rounded or cobblestone-like shape upon miR-429 overexpression. Magnification: ×400. (D) Overexpression of miR-429 inhibited PC cell migration as indicated by wound healing assay. (E) Overexpression of miR-429 inhibited PC cell invasion as indicated by transwell assay. (F) qPCR analysis of the expression of epithelial markers (E-cadherin, Claudin-1) and mesenchymal markers (β-catenin, Snail) upon miR-429 overexpression. (E) Western blot analysis of the expression of epithelial markers (E-cadherin, Claudin-1) and mesenchymal markers (β-catenin, Snail) upon miR-429 overexpression. Data were presented as mean ± SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

invasion and EMT (Supplemental Fig. 4C-F and Supplemental Fig. 5A). To clarify the roles of ZEB1 in bridging the XIST's pro-metastasis effects in PC cells, we introduced ZEB1 in XIST-knockdown cells and confirmed its successful restoration (Fig. 6A and B). Moreover, we observed that ZEB1 introduction efficiently rescued the anti-migration, -invasion and -EMT effects caused by XIST knockdown (Fig. 6C-F, and Supplemental Fig. 5B). Therefore, our above results identified that XIST functions as a ceRNA to upregulate ZEB1 expression by competitively binding to miR-429, and ultimately gives rise to the enhancement of PC cell migration, invasion and EMT in vitro.

4. Discussion

Our present work represents a comprehensive study of the role of

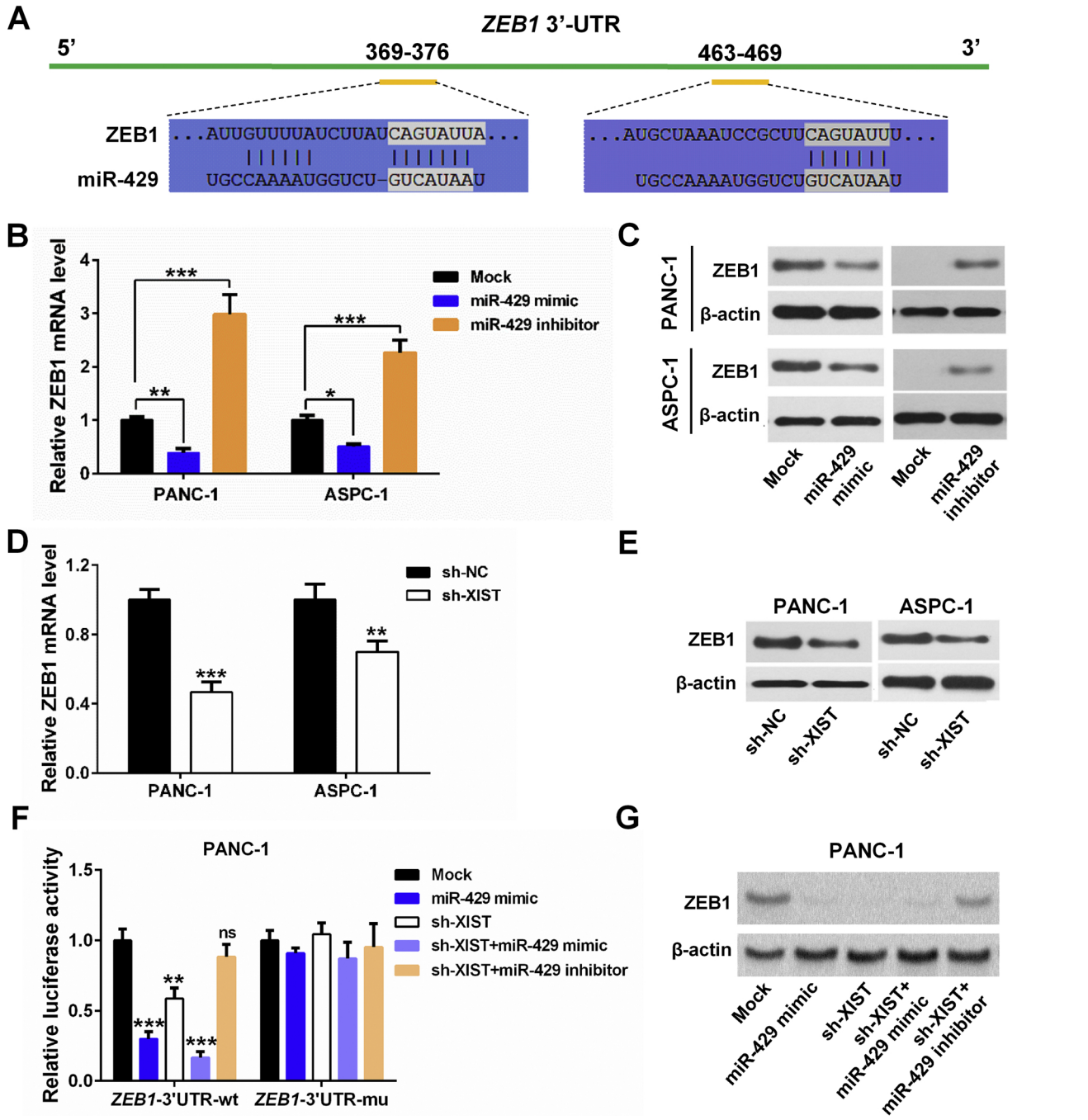

Fig. 5. XIST regulates ZEB1 expression by acting as a molecular sponge. (A) The predicted miR-429 binding sites in ZEB1 mRNA 3′-UTR predicted by Targetscan. (B) qPCR analysis of ZEB1 mRNA expression in PC cells transfected with miR-429 mimic or inhibitor. (C) Western blot confirmation of ZEB1 protein expression in PC cells transfected with miR-429 mimic or inhibitor. (D) qPCR analysis of ZEB1 mRNA expression in XIST-knockdown PC cells. (E) Western blot confirmation of ZEB1 protein expression in XIST-knockdown PC cells. (F) Luciferase reporter assay in PANC-1 cells co-transfected with the ZEB1 reporter (ZEB1-3′UTR-wt or ZEB1-3′UTR-mu) plus indicated shRNA/miRNA. (G) Western blot confirmation of ZEB1 protein expression in PANC-1 cells treated as indicated. Data were presented as mean ± SD from three independent experiments. ns > 0.05, $^{**}P < 0.01$, $^{***}P < 0.001$.

lncRNA XIST and its ceRNA mechanism in PC. During this study, we confirmed the upregulation of XIST and downregulation of miR-429 in PC, and verified their opposite effects on cell migration, invasion and EMT. Mechanically, we identified the critical axis of XIST/miR-429/ZEB1 in PC. Our findings highlight the importance of the downstream ceRNA pathway in mediating the oncogenic role of XIST, which may facilitate our understanding of the complex internal RNA regulatory networks and contribute to the therapeutic progress for PC.

LncRNAs are implicated in carcinogenesis and are potent biomarkers or therapeutic targets for PC (Han et al., 2016). XIST is one of the firstly identified lncRNAs in mammals, and is known to be dysregulated and functions as either an oncogenic factor or suppressor depending on specific tumor types (Yang et al., 2018). Meta-analysis conducted by Zhu et al demonstrated that increased XIST level predicts poor survival, larger tumor size, more commonly distant metastasis and advanced tumor stage in malignant tumors, highlighting its possible usage as a novel biomarker for poor prognosis and metastasis of cancer patients (Zhu et al., 2018). Herein we confirmed that XIST was indeed upregulated in PC tissues, especially increased in the metastatic PCs compared with those non-metastatic ones. This finding on one hand

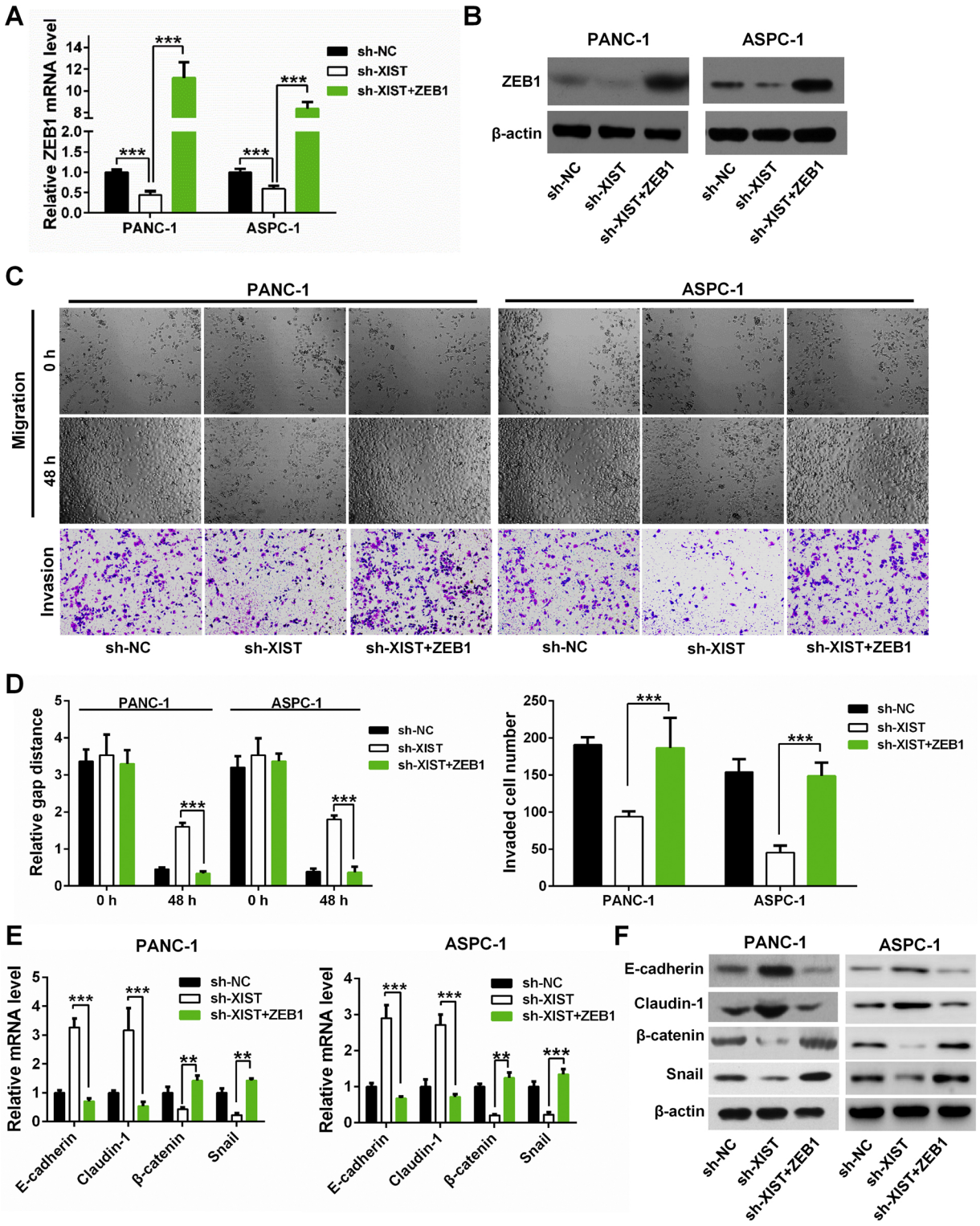

Fig. 6. ZEB1 mediates the anti-metastasis effects of XIST in PC cells. (A) qPCR confirmation of ZEB1 expression in PANC-1 and ASPC-1 cells treated as indicated. (B) Western blot confirmation of ZEB1 expression in PANC-1 and ASPC-1 cells treated as indicated. (C) ZEB1 rescued the inhibition of PC cell migration (upper) and invasion (bottom) caused by sh-XIST treatment in PC cells. (D) Quantification of the migration (left) and invasion (right) abilities in (C). (E) qPCR analysis of the expression of epithelial markers (E-cadherin, Claudin-1) and mesenchymal markers (β-catenin, Snail) upon ZEB1 restoration. (F) Western blot analysis of the expression of epithelial markers (E-cadherin, Claudin-1) and mesenchymal markers (β-catenin, Snail) upon ZEB1 restoration. Data were presented as mean ± SD from three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$.

validates the previous findings by others (Wei et al., 2017; Sun et al., 2018), on the other hand implies that XIST might also contribute to PC metastasis. Moreover, previous studies have reported that XIST possessed oncogenic roles in PC cells, including promoting cell proliferation, migration and invasion, and inhibiting cell apoptosis (Wei et al., 2017; Sun et al., 2018). In the present study, we provided additional in vitro evidences that knockdown of XIST in PC cells altered the cell morphology, inhibited migration, invasion and EMT processes

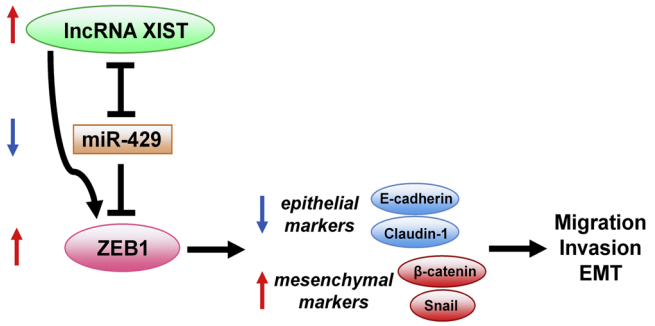

Fig. 7. A functional model underlying the mechanism of lncRNA XIST on PC cell metastasis. By sponging to miR-429, lncRNA XIST acts as a ceRNA to regulate ZEB1 expression and promotes PC cell migration, invasion and EMT in vitro.

Emerging lines of evidences have suggested the existence of an extensive ceRNA interaction network, in which the lncRNAs act as molecular sponges to competitively inhibit miRNAs and tip them off their binding sites on target mRNAs (Cesana et al., 2011; Yang et al., 2016). Herein we predicted several miRNAs that might bind to XIST by using the three online bio-informatic tools (Starbase v2.0, miRcode and RNAhybrid), and surprisingly found that miR-429 was one of the common miRNAs that might interact with XIST predicted by all the three tools. Consistently, miR-429 was downregulated in PC and exhibited inverse correlation with XIST expression. The regulation relationship between XIST and miR-429 was further validated by the following evidence: (1) miR-429 inhibits XIST expression; (2) miR-429 inhibits the luciferase activity of XIST-wt reporter containing 3 binding sites of miR-429; (3) knockdown of XIST increases miR-429 level. Similar to the effects of XIST knockdown, we found that miR-429 itself acted as a potent tumor suppressor by inhibiting migration, invasion and EMT capacities in PC cells. As is known, miR-429 inhibits PC progression through suppressing cell growth (Song et al., 2015) and invasion (Liu et al., 2018). Our data validated the anti-invasion effect of miR-429 in PC cells, and described more about its roles in PC cell migration and EMT. In addition, the linkage of miR-429 with XIST was firstly mentioned in glioma (Cheng et al., 2017), whereas our study provided solid evidence that this linkage also existed in PC and was a major contributor in the aggressiveness of PC cells.

The targeting of ZEB1, a key regulator of EMT and cell invasion (Krebs et al., 2017), by miR-429 has been mentioned in several human cancers (Kong et al., 2015; Deng et al., 2017). In our study, we validated ZEB1 as a direct target of miR-429. ZEB1 overexpression led to enhanced PC cell migration, invasion and EMT. XIST could positively regulate ZEB1 expression through sponging to miR-429, and the anti-metastasis effects caused by XIST knockdown could be effectively rescued by introduction of ZEB1 in PC cells, highlighting the XIST/miR-429/ZEB1 axis in facilitating PC cell migration, invasion and EMT (Fig. 7). According to the current knowledge, EMT is considered a major driver of tumor progression from initiation to metastasis (Nieto et al., 2016). The EMT-inducing transcription factor ZEB1 was shown to be required for tumorigenicity and metastasis of PC, mainly through triggering combined activation of cell motility and stemness properties (Brabletz and Brabletz, 2010; Zhang et al., 2015). In addition, ZEB1 also links EMT-activation and stemness-maintenance in PC via suppressing stemness-inhibiting miRNAs, and thereby is a promoter of tumorigenicity (Wellner et al., 2009). Besides, the core and most recognized mechanism of ZEB1 in EMT induction and metastasis is its transcription repression on E-cadherin gene (Wellner et al., 2010). In terms of this, we observed the consistent alteration of E-cadherin expression by ZEB1 overexpression. Due to the space limitation, we did not examine the transcription activity of ZEB1 in our system. We speculate that the transcription repression or activation on EMT markers by ZEB1 could interpret its positive roles in PC cell migration, invasion and EMT.

5. Conclusion

Taken together, our findings verify that lncRNA XIST may promote PC cell migration, invasion and EMT by acting as a ceRNA for miR-429 to regulate ZEB1 expression. XIST might serve as a promising target for preventing PC metastasis which benefit the outcomes of this lethal disease.

Additional in vivo studies on XIST and the downstream miR-429/ZEB1 pathway in PC tumorigenesis will further strengthen our present findings. Besides, although we consider miR-429 as a vital linkage connecting with XIST and responsible for its oncogenic role in PC, we believe other miRNAs existed as binding partners of XIST. Dissecting the complex ceRNA networks centering on XIST will finely interpret its effects in PC progression and providing new insights into the mechanisms of PC development.

Author contributions

J.S., L.H., D.Y., T.C., contributed to experimental design and performed experiments; J.S., L.H., Z.Z., S.H. analyzed the data; Z.Z., S.H. wrote the manuscript.

Competing financial interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2019.05.021.

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