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Long non-coding RNA XIST promotes cell proliferation of pancreatic cancer through miR-137 and Notch1 pathway

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Abstract. – OBJECTIVE: **Long non-coding ribonucleic acids X-inactive specific transcript (lncRNA XIST) is one lncRNAs which involved in multiple human cancers. However, the functions and potential molecular regulatory mechanisms of XIST/microRNA-137 (miR-137) in pancreatic cancer (PC) still need to explore.**

PATIENTS AND METHODS: PC tissues and cell lines were analyzed for XIST, miR-137 and Notch1 expressions through quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot. Nude mouse xenograft tumor assay was used to detect XIST effects on pancreatic tumorigenesis in vivo. Cell Counting Kit (CCK-8) assay was performed to detect PC cell proliferation. Dual-Luciferase reporter assay, qRT-PCR, RNA immunoprecipitation (RIP) and Western blot assays were applied to validate the target relationship of XIST, miR-137 and Notch1.

RESULTS: Results demonstrated that XIST expression was increased in PC tissues and cells. XIST knockdown inhibited PC cell proliferation in vitro and also repressed the tumor growth in vivo. XIST directly interacted with miR-137 and negatively regulated its expression. Notch1 was identified as a target gene of miR-137 and XIST acted as a competitive endogenous RNA (ceRNA) to positively regulate Notch1 expression by suppressing miR-137. In addition, we detected miR-137 was negatively correlated with XIST and Notch1 respectively, and a positive correlation between Notch1 and XIST expression in PC tissues. Furthermore, Notch1 overexpression could offset the suppressing effect of XIST knockdown or miR-137 overexpression on cell proliferation. Therefore, XIST may play an important role in promoting cell proliferation through miR-137 and Notch1 pathway in PC.

CONCLUSIONS: To sum up, these results proposed that XIST functioned as an endogenous sponge in promoting PC cell proliferation through competing for miR-137 to regulate Notch1 expression, and may provide more therapeutic targets for the patients with PC.

Key Words:
XIST, MiR-137, Notch1 pathway, Pancreatic cancer.

Introduction

Pancreatic cancer (PC) is a malignant tumor with difficult diagnosis and treatment because of its poor prognosis and lower 5-year survival rate[1,2]. The etiology of PC is complex and still unclear, which may be closely related to lifestyle factors, chronic disease, environmental pollution and genetic factors[3]. The early diagnosis of PC is low, along with extensive metastasis and complication, resulting in difficulties for clinical therapy[4]. In recent years, surgery, chemotherapy and radiation therapy are commonly used in clinical treatment of PC[1]; however, the cure rate of these therapies is very low. In addition, it is easy to miss the best time for treatment because of the earlier symptom was atypical in the clinical. Therefore, the underlying mechanisms for PC development and progression need to be investigated to provide new treatments for patients.

LncRNAs are a kind of RNA which more than 200 nucleotides in length and involved in the regulation of various processes in cells, including gene and diverse biological processes[5]. LncRNAs dysregulation closely related to tumorigenesis, tumor progression and metastasis[5,6]. XIST, one of the first found lncRNAs in mammals and associ-

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ated with X chromosome inactivation[7], has been observed to be dysregulated in many type human cancers, including PC[8]. XIST was upregulated in PC, but the potential mechanisms were not thoroughly unclear[8].

MiRNAs are involved in malignancies[9], and interaction between lncRNAs and miRNAs has become a central issue in studying the role of lncRNAs in human cancers[10]. LncRNAs act as miRNA sponges or decoys to compete with miRNAs, thereby interaction with mRNAs[11]. In prostate cancer, XIST suppressed it's progression by sponging miR-23a to regulate Raf kinase inhibitor protein (RKIP) expression[12]. XIST act as an oncogene through regulating miR-132-3p in colorectal cancer[13]. XIST promoted cell proliferation by modulating miR-133a/EGFR in PC[14]. In PC, miR-137 could exert a tumor suppressor through regulating its target genes expression[15,16]; nevertheless, whether XIST could target miR-137 to effect PC cell proliferation was unclear.

In the present study, XIST was highly expressed in PC and XIST knockdown could inhibit cell proliferation. Further studies validated XIST could act as a ceRNA of miR-137 to regulate Notch1 expression in PC cells. Furthermore, we detected XIST expression has a negative correlation with miR-137 but a positive correlation with Notch1, and negative correlation was observed between miR-137 and Notch1 expression in PC tissues. In addition, XIST promoted PC cell proliferation by regulating miR-137 and Notch1 expression. This finding provided new sights in understanding the role of lncRNAs mechanism in PC progression.

Patients and Methods

Tissue Specimens and Cell Culture

40 paired PC tissues and non-tumor adjacent tissues (AN tissues) samples were collected from the Yantai Yuhuangding Hospital Affiliated to Qingdao University. Inclusion criteria: (1) there was no adjuvant therapy before surgery, such as chemotherapy and radiotherapy. (2) Pathology was confirmed as PC by two pathologists after surgery. (3) No other organ metastasis. (4) The patient could tolerate the operation without evident heart, brain and lung diseases. Exclusion criteria: (1) preoperative chemotherapy, radiotherapy and other related adjuvant therapies. (2) Combined with distant organ metastasis before surgery. (3) Patients could not tolerate surgery. This investigation was approved by the Ethics Committee of Yantai Yuhuangding Hospital Affiliated to Qingdao University. All patients provided written informed consent. This investigation was conducted in accordance with the Declaration of Helsinki.

Human pancreatic duct epithelial cell (H6c7) and human PC cell lines (Patu8988, SW1990, MIA PaCa-2, AsPC-1, CFPAC-1, BxPC-3 and Panc-1) were obtained from ATCC (Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and incubated in a humidified atmosphere with 5% CO_2 at 37°C.

Cell Transfection

The pcDNA3.1 vector was used to construct plasmids that overexpressed XIST and Notch1. XIST small interfering RNA (siXIST), miR-137 mimics and NC were obtained from GenePharma (Shanghai, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was applied to cell transfection follow the instructions.

Cell Proliferation Assays

PC cells proliferation was assessed by Cell Counting Kit (CCK-8) assay at 1, 2, 3, 4 days *in vitro*. 3×10^3 cells per well were cultured in 96 well plates. CCK-8 reagents (Dojindo Molecular Technologies, Kumamoto, Japan) were added into each well. The absorbance was detected at 450 nm with a microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA) after 2 hours incubation at 37°C.

Dual-Luciferase Activity Assay

The association between XIST and miR-137 was predicted by Starbase3.0 software (http://starbase.sysu.edu.cn/). TargetScan 7.2 (http://www.targetscan.org/) was used to predict the potential targets of miR-137. The XIST wild type (XIST-wt) and mutant type (XIST-mut) sequences, as well as Notch1 wild type (Notch1-wt) and mutant type (Notch1-mut) sequences were constructed into pMIR-REPORT Luciferase vector (Wuhan, China), then transfected along with miRNA-137 mimic or NC into PC cells. After 48 h transfection, the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was used to analyze the Luciferase activity.

RNA Immunoprecipitation (RIP) Assay

A RIP kit (Millipore, Billerica, MA, USA) was purchased for performing the RIP assay. PC cells were collected, and lysed by RIP lysis buffer. Then, the cell lysates were incubated with anti-Argonaute2 (Ago2) or immunoglobulin G (IgG; negative control) antibody in RIP buffer at 4°C overnight. Co-precipitated RNA was isolated after digestion with 150 µL protease K. The expression of XIST and miR-137 was detected.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA) reagent and cDNA was synthesized using reverse transcription reaction kit (TaKaRa, Komatsu, Japan) following the instruction. QRT-PCR was performed by SYBR Premix DimerEraser (TaKaRa, Komatsu, Japan) on a LightCycler 480 PCR System (Roche, Rotkreuz, Switzerland). Relative expression of RNAs was calculated by $2^{-\Delta\Delta CT}$ method and normalized to U6 or glyceraldehyde phosphate dehydrogenase (GAPDH). Primers are exhibited in Table I.

Western Blot

Total proteins of PC tissues and cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) containing 1 nM phenylmethanesulfonylfluoride fluoride (PMSF; Biotool, Houston, TX, USA) and were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and were then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocked with 5% skim milk, membranes were incubated with GAPDH and Notch1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. All membranes then were probed with horseradish peroxidase (HRP)-labeled secondary antibody (Santa Cruz, CA, USA). Protein bands were detected with enhanced chemiluminescent (ECL) detection reagent Kit (Cwbiotech, Beijing, China).

In Vivo Tumorigenic Assay

Four-week old nude mice were purchased from the Chinese Science Academy (Shanghai, China). Cells stably transfected with siXIST or control were suspended at 5×10^6 cells/mL. The mice were randomly divided into two groups (n=5 mice each group). 200 µl cell suspension were subcutaneously injected into the left hip of mice. Tumor volumes were measured once a week for 5 weeks and calculated by the formula: volume (mm^3) = length × width^2/2. After 5 weeks, tumors were excised for the subsequent experiments. All animal procedures were approved by the Ethics Committee of the Yantai Yuhuangding Hospital Affiliated to Qingdao University.

Statistical Analysis

All data were represented as mean ± SD and analyzed with GraphPad Prism 5.0 software (GraphPad Prism, San Diego, CA, USA). The Student's *t*-test was performed to compare the difference between two groups. The correlation was assessed among XIST, miR-137 and Notch1 using Spearman's method. $p<0.05$ indicated statistical significance.

Results

XIST Expression Increased In PC Tissues and Cells

We detected XIST expression in tissues and cell lines by qRT-PCR. It is s shown that XIST expression was markedly increased in PC tis-

Table I. Primer sequences for real-time fluorescence quantification PCR.

Gene	Primer sequences
GAPDH	Forward primer: 5′-ACGCTGCATGTGTCCTTAG-3′ Reverse primer: 5′-GAGCCTCTTATAGCTGTTTG-3′
U6	Forward primer: 5′-CTCGCTTCGGCAGCACA-3′ Reverse primer: 5′-AACGCTTCACGAATTTGCGT-3′
XIST	Forward primer: 5′-AATGACTGACCACTGCTGGG-3′ Reverse primer: 5′-GTGTAGGTGGTTCCCCAAGG-3′
miR-137	Forward primer: 5′-GCAGCAAGAGTTCTGGTGGC-3′ Reverse primer: 5′-TGGAACCAGTGCGAAAACAC-3′
Notch1	Forward primer: 5′-CGGGTCCACCAGTTTGAATG-3′ Reverse primer: 5′-GTTGTATTGGTTCGGCACCAT-3′

sues and cells *vs.* control group (Figure 1A). Among these cells, XIST exhibited high expression level in Panc-1 and AsPC-1 cells and thus they were selected for subsequent analyses (Figure 1B).

XIST Knockdown Inhibited PC Cell Proliferation

To explore the effects of XIST on PC cell proliferation, cells were transfected with siXIST or siNC respectively. XIST expression was notably reduced in cells after transfected with siXIST (Figure 2A). Cell proliferation was significantly inhibited in siXIST group compared with the siNC group according to CCK-8 assay (Figure 2B and 2C). Thus, we proposed XIST knockdown could inhibit PC cell proliferation *in vitro*.

To further confirm the effects of XIST on PC tumorigenesis, *in vivo* tumorigenic assay was performed. Stable Panc-1 cells transfected with siXIST were selected to inject into the mice. After 5 weeks, we found the tumor growth was significantly inhibited in siXIST group compared to siNC group (Figure 2D). At the same time, tumor volume and weight were conspicuously decreased in siXIST group *vs.* siNC group (Figure 2E and 2F). Moreover, expressions of miR-137 and Notch1 in xenograft tumors tissues were detected by qRT-PCR. MiR-137 expression was notably upregulated in siXIST group, while expression of Notch1 was exhibited the opposite trend (Figure 2G and 2H). These data proposed that XIST knockdown could significantly inhibit cell proliferation capacity of PC *in vivo*, and this function might be achieved by regulating miR-137 and Notch1 pathway.

XIST Inhibited MiR-137 Expression by Directly Interaction

We proposed that XIST might function as a sponge of miR-137, thus regulating target genes of miR-137. To test the hypothesis, we predicted the target relationship and binding site between XIST and miR-137 by bioinformatics analysis (Figure 3A). To confirm the prediction, Dual-Luciferase reporter assay was performed in PC cells. Luciferase reporter assays revealed co-transfection of XIST-wt and miR-137 mimics caused a significant reduction of Luciferase activity compared with other groups in PC cells (Figure 3B and 3C). To verify the direct connection of XIST and miR-137, RIP assay was carried out in PC cells to pull down the XIST using anti-Ago2 or IgG antibody. As expected, RIP assay results showed that miR-137 and XIST levels were significantly higher in Ago2 group than IgG group, suggesting that XIST directly targeted miR-137 (Figure 3D and 3E). Furthermore, miR-137 expression was decreased in PC cells *vs.* H6c7 cells by qRT-PCR analysis (Figure 3F). We also observed that miR-137 expression significantly increased in the case of XIST knockdown in PC cells (Figure 3G). Moreover, Spearman's correlation analysis showed that XIST expression was inversely correlated with miR-137 expression in PC tissues (Figure 3H). Taken together, XIST directly interacted with miR-137 and negatively regulated its expression in PC.

XIST-MiR-137 Axis Regulated Activation of the Notch1 Signaling Pathway

We validated that XIST as a ceRNA directly interacted with miR-137. Notch1 was predicted as a potential target gene of miR-137 through Tar-

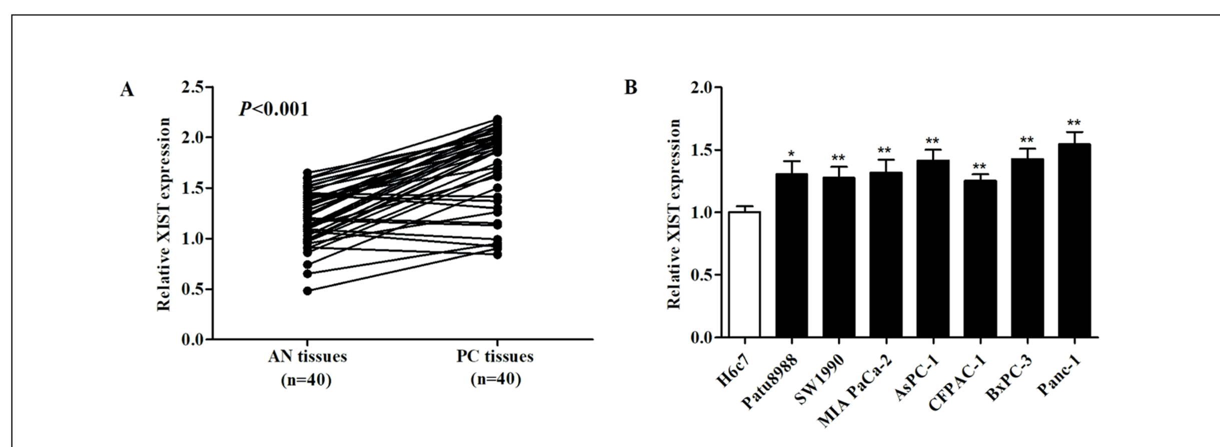

Figure 1. Expression of XIST was examined by qRT-PCR in PC tissues and cell lines. **A,** The expression of XIST in tissue samples. **B,** The expression of XIST in PC cell lines. *$p < 0.05$, **$p < 0.01$, compared with AN tissues or H6c7 group.

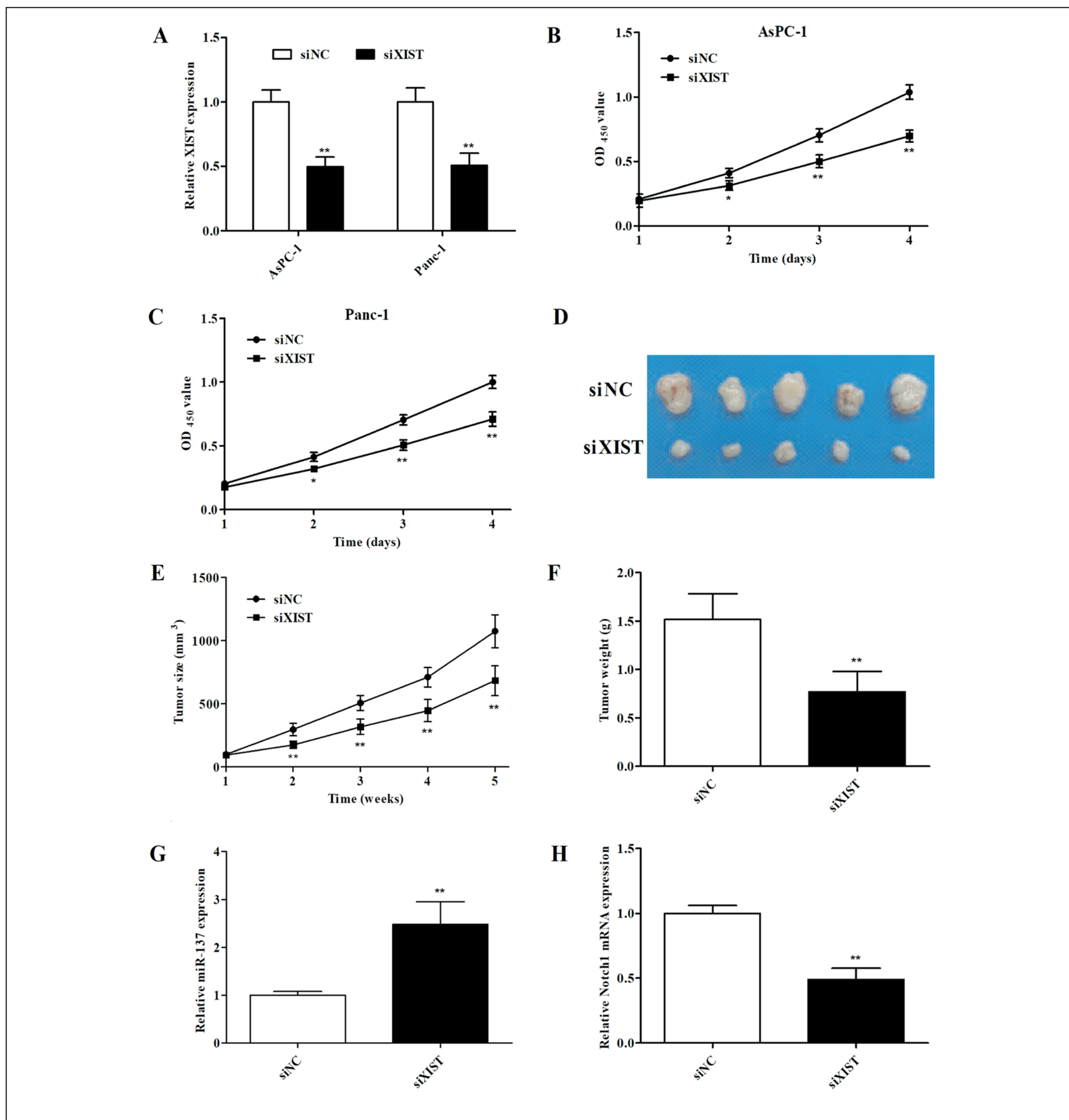

Figure 2. XIST knockdown inhibited PC cell proliferation and tumor growth. **A,** XIST expression in cells after siXIST or siNC transfected. **B, C,** Cell proliferation of AsPC-1 and Panc-1 cells after XIST knockdown. **D,** Photographs of xenograft tumors. **E, F,** The volumes and weights of transplanted tumors. **G, H,** The expression of miR-137 and Notch1 in xenograft tumors. $*p < 0.05$, $**p < 0.01$, compared with siNC group.

getScan software and the binding site was shown in Figure 4A. However, whether XIST could modulate Notch1 expression by competing with miR-137. Thereby, we constructed Notch1-wt and Notch1-mut plasmids containing the complementary sequence of miR-137, and co-transfected these plasmids with miR-137 mimics or opXIST into PC cells. We found co-transfection of Notch1-wt and miR-137 mimics caused a significant reduction of luciferase activity, suggesting that miR-137 could directly bind Notch1 (Figure 4B and 4C). Moreover, XIST overexpression could significantly offset the effect of miR-137 mimics on luciferase activity (Figure 4B and 4C). In addition, expression of Notch1 was detected in PC cells. We found that Notch1 expression was

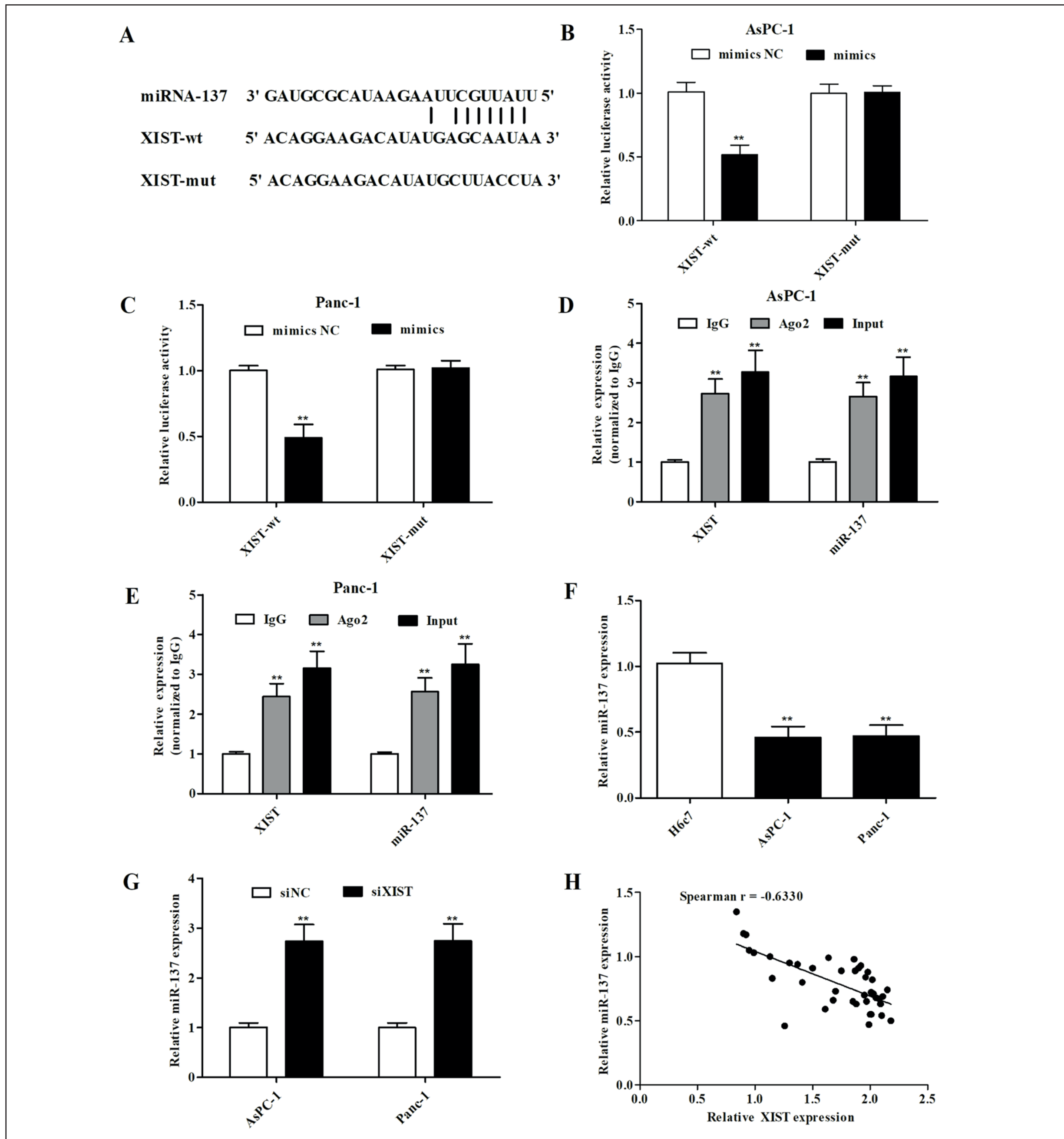

Figure 3. XIST functioned as an endogenous sponge to downregulate miR-137 by competitively binding to miR-137. **A,** Putative binding site between miR-137 and XIST. **B, C,** The Luciferase activity was measured by dual-luciferase reporter assay. **D, E,** MiR-137 and XIST expressions in RIP assay. **F,** MiR-137 expression in AsPC-1 and Panc-1 cells. **G,** MiR-137 expression in AsPC-1 and Panc-1 cells transfected with siXIST or NC. **H,** The correlation between XIST and miR-137 in tissues. $^{**}p < 0.01$, compared with siNC, mimics NC, H6c7 or IgG group.

enhanced in PC cells (Figure 4D and 4E). We also observed that miR-137 mimics significantly decreased Notch1 mRNA and protein levels in PC cells while this tendency was reversed by opXIST (Figure 4F and 4G). Moreover, Spearman's correlation analysis showed that Notch1 levels were inversely correlated with miR-137 expression and while was positively correlated with XIST expression in PC tissues (Figure 4H and 4I). As aforementioned, we suggested that XIST may modulate the activation of Notch1 signaling pathway by sponging miR-137 in PC.

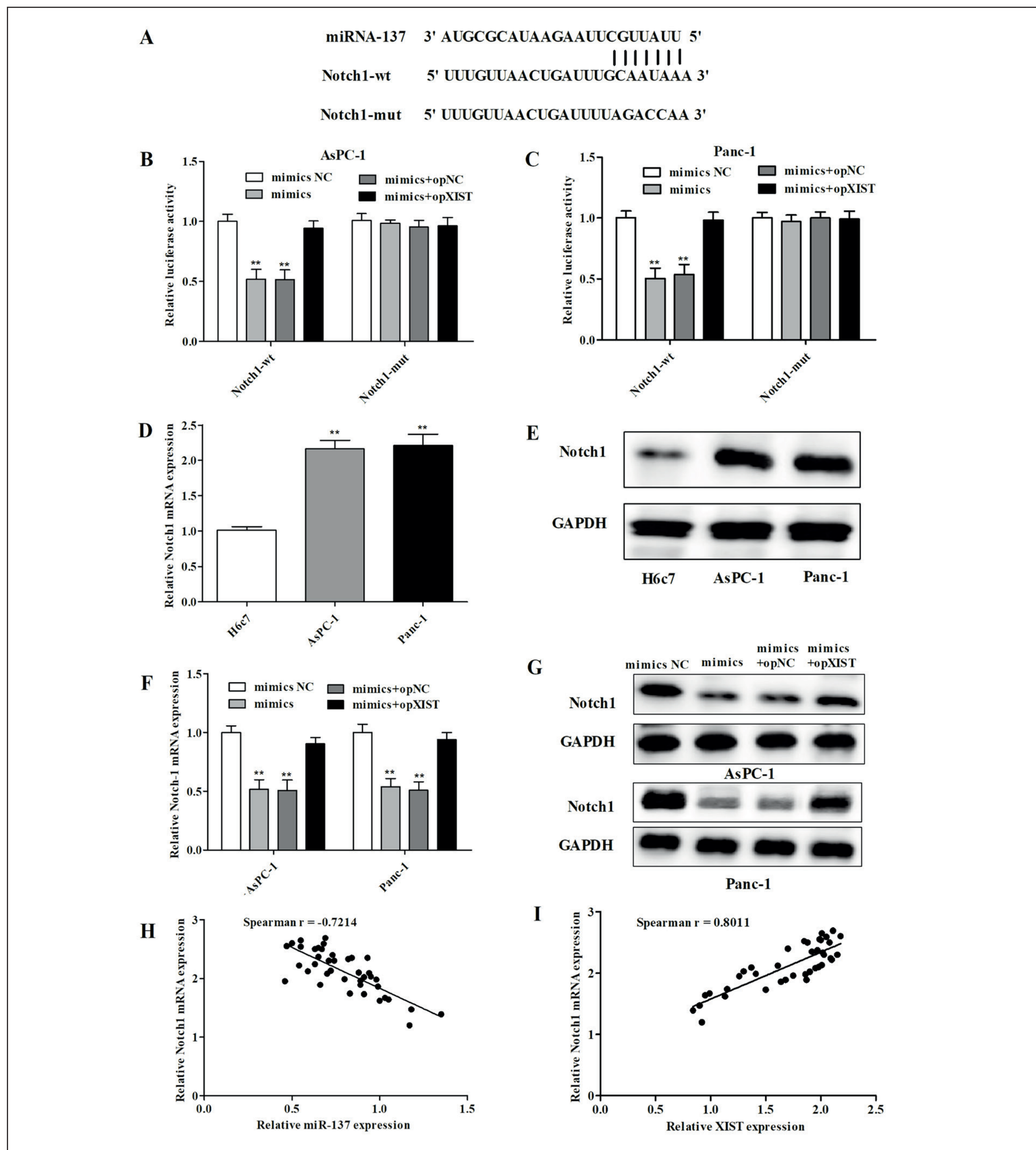

Figure 4. XIST regulated Notch1 expression by competitively binding to miR-137. **A,** Putative binding sites between miR-137 and Notch1. **B, C,** The relative Luciferase activity of in cells co-transfected with Notch1-wt, Notch1-wt and miR-137, or in combination with opNC or opXIST. **D, E,** Notch1 expression in PC cells. **F, G,** The expression of Notch1 in AsPC-1 and Panc-1 cells transfected with mimics NC, mimics, mimics NC and opNC, or mimics NC and opXIST. **H, I,** The correlation between miR-137 and Notch1, between XIST and Notch1 in tissues. $^{**}p < 0.01$, compared with mimics NC or H6c7 group.

XIST/MiR-137/Notch1 Axis Regulated Cell Proliferation In PC Cells

Collectively, we speculated that XIST/miR-137/Notch1 regulatory network might be related to PC progression. Therefore, CCK-8 assay was applied for exploring whether XIST affected cell proliferation by mediating miR-137 and Notch1. The result showed that transfection of siXIST or miR-137 mimics significantly suppressed cell proliferation in AsPC-1 cells, while the effect

could be counteracted with opNotch1 co-transfection (Figure 5A, 5B). It suggested that XIST promoted cell proliferation by competitively binding to and inhibiting miR-137 expression to upregulate Notch1, and then benefiting to the progression of PC.

Discussion

Pancreatic cancer (PC) is a serious malignant tumor, and its clinical characteristics are short course, rapid development and deterioration[2]. The main clinical manifestations of early stage were abdominal distension, dyspepsia, pain and other symptoms, but early pain is not serious, resulting in a high rate of misdiagnosis[17]. The morbidity and mortality of PC are significantly increased in recent years[1]. There are no effective methods of diagnosis and treatment for PC patients. Nevertheless, lncRNAs are detected to involve in many processes in PC progression, including cell invasion, proliferation, apoptosis and angiogenesis[18-21]. However, the function and detailed regulator mechanisms of numerous lncRNA associated with PC have not been completely characterized and need further research.

XIST exerts oncologic functions in PC[14,22]; in the current study, XIST expression increased in PC and its knockdown markedly suppressed cell proliferation *in vitro* and tumors growth *in vivo*. The regulator mechanisms of XIST in cancer progression was extremely intricate, and might exert as a sponge or ceRNA to regulate miRNA target genes expression by competitively binding to miRNA. Sun et al[23] indicated that XIST served as a ceRNA of miR-106b-5p to mediate P21 in suppressing prostate cancer progression. Moreover, Chang et al[24] suggested XIST promotes hepatocellular carcinoma progression by sponging miR-181a to regulate PTEN expression. MiR-137 was demonstrated as a tumor suppressive miRNA in cancer development[25] and it was observed as one target of XIST. This work indicated that XIST negatively regulated the expression of miR-137 and was negatively correlated with miR-137 expression in PC tissues. Moreover, miR-137 expression was increased in PC cell lines and xenograft tumor by XIST knockdown. Consistently, RIP assay suggested that XIST directly interacted with miR-137. Thus, we speculated XIST promoted PC cell proliferation by sponging miR-137 to modulate its target genes.

To demonstrate these suggestions, miR-137 target gene Notch1 became the focus in further exploration. Notch1 is one of Notch receptors in Notch signaling pathway, and its downregulation could inhibit cell growth and promote apoptosis in PC cells[26,27]. In the present work, an inverse correlation between Notch1 and miR-137 expression was exhibited and XIST was positively correlated with Notch1 expression in PC tissues. The Luciferase activity assay confirmed Notch1 was a target of miR-137. Besides, XIST overexpression could counteract the inhibiting effect of miR-137 overexpression on Notch1 expression. Of note, overexpression of Notch1 counteracted the suppression on cell proliferation induced by XIST knockdown or miR-137 overexpression, suggesting that XIST/miR-137/Notch1 axis could involve in PC tumorigenesis by regulating cell proliferation.

In summary, above experimental results revealed that XIST may promote cell proliferation

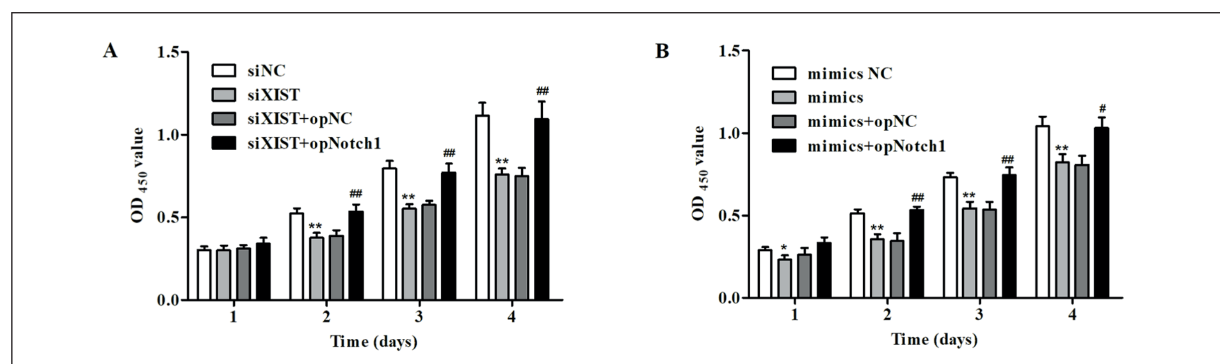

Figure 5. Notch1 overexpression overturned XIST knockdown or miR-137 overexpression induced suppressive role on cell proliferation. **A,** AsPC-1 cell proliferation after siNC, siXIST, siXIST and opNC, or siXIST and opNotch1 transfected. **B,** AsPC-1 cell proliferation after mimics NC, miR-137 mimics, miR-137 mimics and opNC, or miR-137 mimics and opNotch1 transfected. $*p < 0.05$, $**p < 0.01$, $\#p < 0.05$, $\#\#p < 0.01$, compared with NC or mimics+opNC group.

via regulating miR-137 and Notch1 in PC. XIST could be regarded as an oncogene in PC and this was a novel mechanism for XIST in PC. We first identified the regulatory network of XIST/miR-137/Notch1 axis in PC progression. This broadens our understanding of the lncRNA-miRNA-mRNA regulator networks in PC. However, whether XIST/miR-137/Notch1 axis could regulate PC cell migration and invasion was not explored in this study. Furthermore, the expressions of downstream target genes of Notch1 signaling pathway were not detected and the other possible related miRNAs or target genes of XIST were not indicated. Therefore, mechanisms of XIST in PC still need to further explore.

Conclusions

Taken together, our results demonstrated that XIST could regulate Notch1 expression by sponging and negatively regulating miR-137. XIST may promote PC cell proliferation may through miR-137 and Notch1 pathway, and this provided new ideas for the treatment and diagnosis of PC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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