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Original Article

The lncRNA-HOXA-AS2/EZH2/LSD1 oncogene complex promotes cell proliferation in pancreatic cancer

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Abstract: Emerging evidence have indicated that long non-coding RNAs (lncRNAs) play crucial roles in cancer development and progression. Previous studies have suggested that lncRNA-HOXA cluster antisense RNA 2 (HOXA-AS2) is involved in tumorigenesis of several cancers. However, little is known about the alteration and biological functions of HOXA-AS2 in pancreatic cancer (PC). The purpose of this study is to identify the role of HOXA-AS2 in PC. Here, we provided evidence that lncRNA HOXA-AS2 was up-regulated in PC tissues. In addition, Loss-of-function experiments revealed that HOXA-AS2 knockdown effectively suppressed proliferation by blocking the cell cycle transition and caused apoptosis of PC cells in vitro and in vivo. Mechanistically, we found that HOXA-AS2 directly interacted with enhancer of zeste homolog 2 (EZH2) and lysine specific demethylase 1 (LSD1), which promoted PC cell growth ability. Collectively, our findings demonstrated that lncRNA-HOXA-AS2/EZH2/LSD1 complex may function as an oncogene in PC cell proliferation, and also provides a potential therapy target for PC.

Keywords: lncRNA, HOXA-AS2, pancreatic cancer, cell proliferation

Introduction

Pancreatic cancer (PC), one of the most frequent cancer types in the world, is a deadliest solid malignancies with poor prognosis [1, 2]. Based on the GLOBOCAN 2012 expected numbers, pancreatic cancer causes approximately 330000 deaths per year (accounts for 4.0% of total cancer deaths), ranking as the seventh leading cause of cancer death worldwide [3]. In spite of recent improvements in diagnosis and surgical treatment, the estimated 5-year survival rate for pancreatic cancer continues to be poor [4]. It has been confirmed that a series of risk factors contribute to the development of pancreatic cancer, including cigarette smoking, long-standing type 2 diabetes mellitus, heavy alcohol consumption and inherited risk factors [5]. Although the cause of PC is complex and multifactorial, deeply investigating the cancer-associated molecular events could be the key to understanding the progression of this deadly disease and thus the cornerstone of developing a potential therapy strategy [6, 7].

The Human Genome Project has showed that the whole human genome is pervasively transcribed into non-coding RNA elements [8-10]. Long non-coding RNAs, which are non-coding RNAs with length more than 200 nt, have been demonstrated to be involved in various types of malignancy, including pancreatic cancer [11, 12]. Mounting evidence has indicated that lncRNAs play important roles in the pathogenesis of pancreatic cancer development and have a potential value in the diagnosis, treatment and prognostic prediction of PC [13]. For example, Li et al found that lncRNA MALAT1 could facilitate pancreatic cancer cell proliferation and metastasis via activating autophagy [14]. Huang et al reported that upregulation of the lncRNA PVT1 is associated with poor prognosis in pancreatic cancer patients [15]. Our previous studies showed that the lncRNA IRAIN expression level was significantly increased in PC tissues and overexpression of IRAIN was correlated with tumor size, TNM stage, and lymph node metastasis in a cohort of 37 PC patients [16]. Exploring more PC-related lnc-

Table 1. Summary of the cohorts clinical characteristics

Characteristics	Xiamen cohort	Nanjing cohort
	Number of patients (%)	Number of patients (%)
Total cases	16 (100%)	12 (100%)
Gender		
Male	11 (68.8%)	8 (66.7%)
Female	5 (31.2%)	4 (33.3%)
Age (years)		
< 60	3 (18.8%)	3 (25.0%)
≥ 60	13 (81.2%)	9 (75.0%)
Tumor size		
< 2 cm	7 (43.8%)	3 (25.0%)
≥ 2 cm	9 (56.2%)	9 (75.0%)
Differentiation		
Well/Moderate	4 (25.0%)	2 (16.7%)
Poor	12 (75.0%)	10 (83.3%)
TNM stage		
I/II	7 (43.8%)	5 (41.7%)
III/IV	9 (56.2%)	7 (58.3%)
Lymph-node metastasis		
Negative	8 (50.0%)	5 (41.7%)
Positive	8 (50.0%)	7 (58.3%)

RNAs will help us better understanding of the molecular details about the development of PC, which are required for the design of novel therapeutic targets for the treatment of this deadly disease.

HOXA cluster antisense RNA 2 (HOXA-AS2) is a 1048 bp lncRNA located between the HOXA3 and HOXA4 genes in the HOXA cluster [17]. Several lines of evidence suggested that HOXA-AS2 was implicated and aberrantly expressed in multiple cancers, such as gastric carcinoma, breast cancer, hepatocellular carcinoma, gallbladder carcinoma and colorectal cancer [17-21]. However, the expression and mechanism of action of HOXA-AS2 in pancreatic tumorigenesis have not been well characterized. The aim of the present study was to clarify the expression levels and functional characterization of HOXA-AS2 in pancreatic cancer.

Material and methods

Bioinformatics analysis

Pancreatic cancer gene expression data (GSE15471) were obtained from Gene Expression Omnibus database GEO (http://www.ncbi.nlm.nih.gov/geo). The raw CEL files were downloaded from GEO database and normalized using Robust Multichip Average (RMA). After we downloaded probe sequences from GEO or microarray manufacturers, blast+2.2.30 was used to re-annotates probe on GENCODE Release 21 sequence databases for lncRNA. For multiple probes corresponding to one gene, maximum normalized signal was selected to generate expressions of lncRNA. Two-sample t-test or paired-sample t-test according to experimental design were employed as differential expression calling method, followed by Benjamini & Hochberg (False discovery rate, FDR) adjustment.

Tissue collection and ethics statement

A total of 16 pair patients tissues analyzed in this study underwent resection of the primary pancreatic cancer at Zhongshan Hospital, Xiamen University, Xiamen, Fujian, China. Another 12 pair patients tissues analyzed in this study underwent resection of the primary pancreatic cancer at the Second Affiliated Hospital, Nanjing Medical University, Nanjing, Jiangsu, China. All collected PC tissue samples were immediately snap frozen in liquid nitrogen and stored at -80°C until required. Our study was approved by the Research Ethics Committee of Xiamen University, (Xiamen, Fujian, PR China), and written informed consent was obtained from all patients. The clinical characteristics of the pancreatic cancer patients are summarized in **Table 1**.

Cell lines and culture conditions

Three pancreatic cancer cell lines (AsPC-1, BxPC-3, and PANC-1) were obtained from the American Type Culture Collection (Manassas, VA). All of the cell lines were grown and maintained in RPMI 1640 or DMEM Medium (Invitrogen) and supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, Shanghai, China) at 37°C with 5% CO_2.

RNA extraction and qRT-PCR assays

Total RNA was extracted from tissues or cultured cells using TRIZOL reagent (Invitrogen). For qRT-PCR, RNA was reverse transcribed to

Table 2. Sequences of primers for qRT-PCR and siRNA sequence

Name	Sequences (5' to 3')
Primers for qRT-PCR	
HOXA-AS2 (Forward)	CCCGTAGGAAGAACCGATGA
HOXA-AS2 (Reverse)	TTTAGGCCTTCGCAGACAGC
GAPDH (Forward)	GAAGAGAGAGACCCTCACGCTG
GAPDH (Reverse)	ACTGTGAGGAGGGGAGATTCAGT
LSD1 (Forward)	AGCGTCATGGTCTTATCAA
LSD1 (Reverse)	GAAATGTGGCAACTCGTC
EZH2 (Forward)	TGCACATCCTGACTTCTGTG
EZH2 (Reverse)	AAGGGCATTCACCAACTCC
Interference sequences (siRNA)	
siHOXA-AS2 1#	GAGUUCAGCUCAAGUUGAACAUACA
siHOXA-AS2 2#	AAACCUUGUAGAUAGCUUGAGCUGG
siHOXA-AS2 3#	CAAGCUUGACAAGUUCAGCUCAA
siNC	UUCUCCGAACGUGUCACGUTT
siLSD1	GCCACCCAGAGAUAUUACUTT
siEZH2	GAGGUUCAGACGAGCUGAUUU

cDNA by using a Reverse Transcription Kit (Takara, Dalian, China). Real-time PCR analyses were performed with SYBR Premix Ex Taq (Takara, Dalian China). Results were normalized to the expression of GAPDH. The specific primers used were shown in **Table 2**. The qRT-PCR assays were conducted on an ABI 7500, and data collected with this instrument. Our qRT-PCR results were analyzed and expressed relative to threshold cycle (CT) values, and then converted to fold changes.

Cell transfection

Briefly, pancreatic cancer cells were seeded at six-well plates and then transfected in the next day with specific siRNA (100 nM) or control siRNA (100 nM) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions (Invitrogen). After transfection, the cells were harvested for further studies. The primer sequences and siRNA sequences are summarized in **Table 2**.

MTT and colony formation assay

Cell viability was performed using the Cell Proliferation Reagent Kit I (MTT; Roche Applied Science). The BxPC-3 and PANC-1 cells were transfected with siNC or siHOXA-AS2 (3000 cells/well) and were cultured in 96-well plates with six replicate wells. Cell viability was assessed according to the manufacturer's recommendations. For the colony formation assay, a total of 500 cells were placed in a six-well plate and maintained in media containing 10% FBS. The medium was replaced every 4 days. After 2 weeks, cells were fixed with methanol and stained with 0.1% crystal violet (SigmaAldrich). Visible colonies were manually counted. Triplicate wells were measured in each treatment group.

Flow cytometry

BxPC-3 and PANC-1 cells transfected with siNC or siHOXA-AS2 were harvested after 48 h for apoptosis analysis. The cells were then treated with fluorescein isothiocyanate (FITC) Annexin V and propidium iodide (PI) in the dark at room temperature according to the manufacturer's protocol. Subsequently, the cells were analyzed by FACScan®, and they were identified as viable, dead, early apoptotic, or late apoptotic cells. For cell cycle analysis, cells were stained with propidium oxide using the CycleTEST PLUS DNA Reagent Kit (BD Biosciences) following the manufacturer's recommendations and analyzed by FACScan. The percentages of cells in G0/G1, S, and G2/M phases were counted and compared.

Tumor formation assay in a nude mouse model

Five-week-old athymic BALB/c mice were purchased from the Animal Center of the Xiamen University (Xiamen, China) and maintained under specific pathogen-free conditions and manipulated according to protocols. The BxPC-3 cells were transfected with Empty vector or shHOXA-AS2. After 48 h, the cells were obtained and injected into either side of the posterior flank of the nude mouse. The tumor volumes (length × width × high) were measured every 3 days in mice from the control (six mice) or shHOXA-AS2 (six mice) groups. Fifteen days after injection, the mice were killed and the tumor volumes were measured.

RNA immunoprecipitation

RIP experiments were carried using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer's

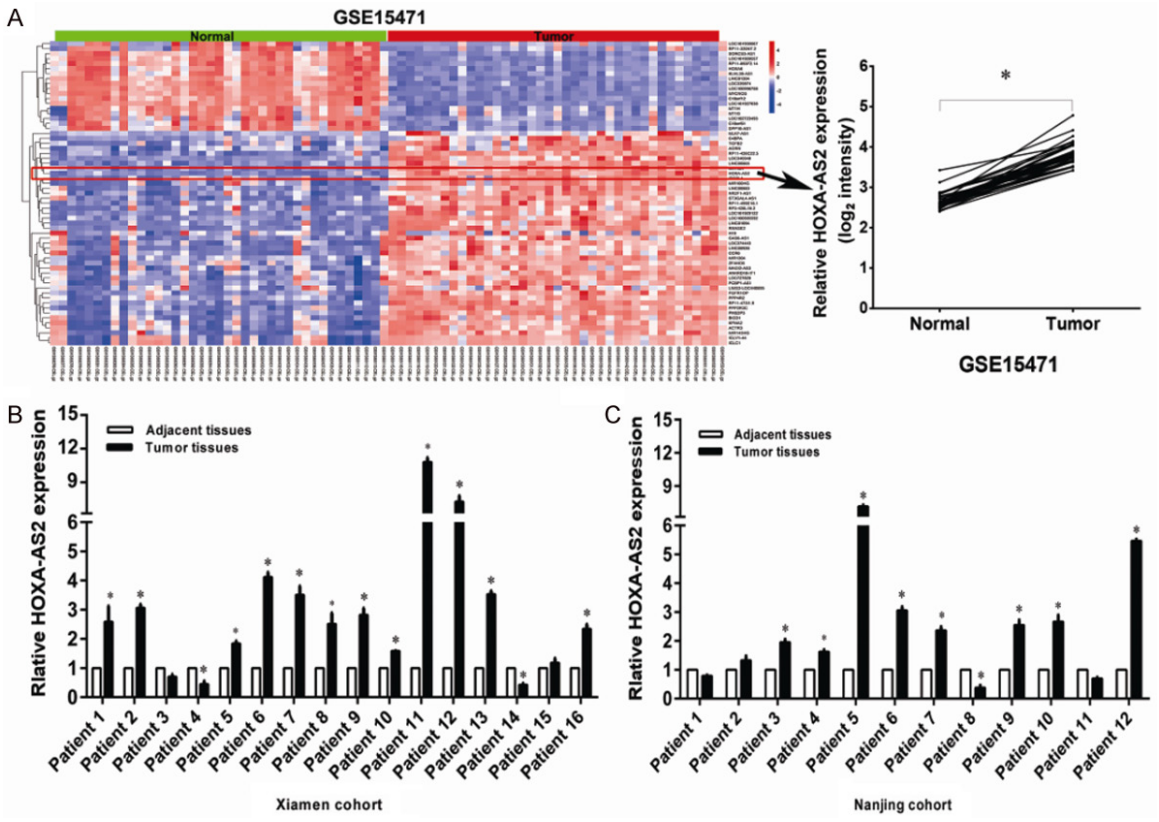

Figure 1. HOXA-AS2 expression is increased in PC tissues. A. Relative expression of HOXA-AS2 in PC tissues compared with normal tissue was analyzed by using Gene Expression Omnibus data sets GSE15471 (n=39). B, C. Relative expression of HOXA-AS2 in PC tissues compared with adjacent normal tissues (Xiamen cohort: n=16, Nanjing cohort: n=12) was tested by qRT-PCR and normalized to GAPDH expression. Results are presented as the fold change in PC tissues relative to adjacent normal tissues. The mean values and SD were calculated from triplicates of a representative experiment, *P < 0.05.

protocol. Antibodies for RIP assays against EZH2 and LSD1 were purchased from Millipore.

Ethynyl deoxyuridine (Edu) analysis

Proliferating cells were assessed using the 5-ethynyl-2-deoxyuridine labeling/detection kit (Ribobio, Guangzhou, China) according to the manufacturer's recommendations. Briefly, Bx-PC-3 cells were cultured in 96-well plates at 5×10^3 cells per well and transfected with siNC or siHOXA-AS2 for 48 h. Then, 50 μM Edu labeling medium was added to the cell culture and incubated for 2 h at 37°C under 5% CO_2. Next, the cultured cells were fixed with 4% paraformaldehyde (pH 7.4) for 30 min and treated with 0.5% Triton X-100 for 20 min at room temperature. After washing with PBS, the cells were stained with anti-Edu working solution at 25°C for 30 min. Subsequently, the samples were incubated with 100 μL DAPI (5 μg/mL) at room temperature for 30 min, followed by observation under a fluorescent microscope. The percentage of Edu-positive cells was calculated from five random fields in three wells.

Statistical analysis

All statistical analyses were performed using SPSS software, version 17.0 (SPSS, Chicago, IL, USA). The Student's t test, or the chi-squared test, was used to evaluate significant differences between groups of data. All data are represented as the means ± SD. Differences were considered significant if P < 0.05. '*' indicates P < 0.05.

Results

HOXA-AS2 is upregulated in pancreatic cancer tissue samples

To identify whether HOXA-AS2 are involved in pancreatic tumorigenesis, we performed an

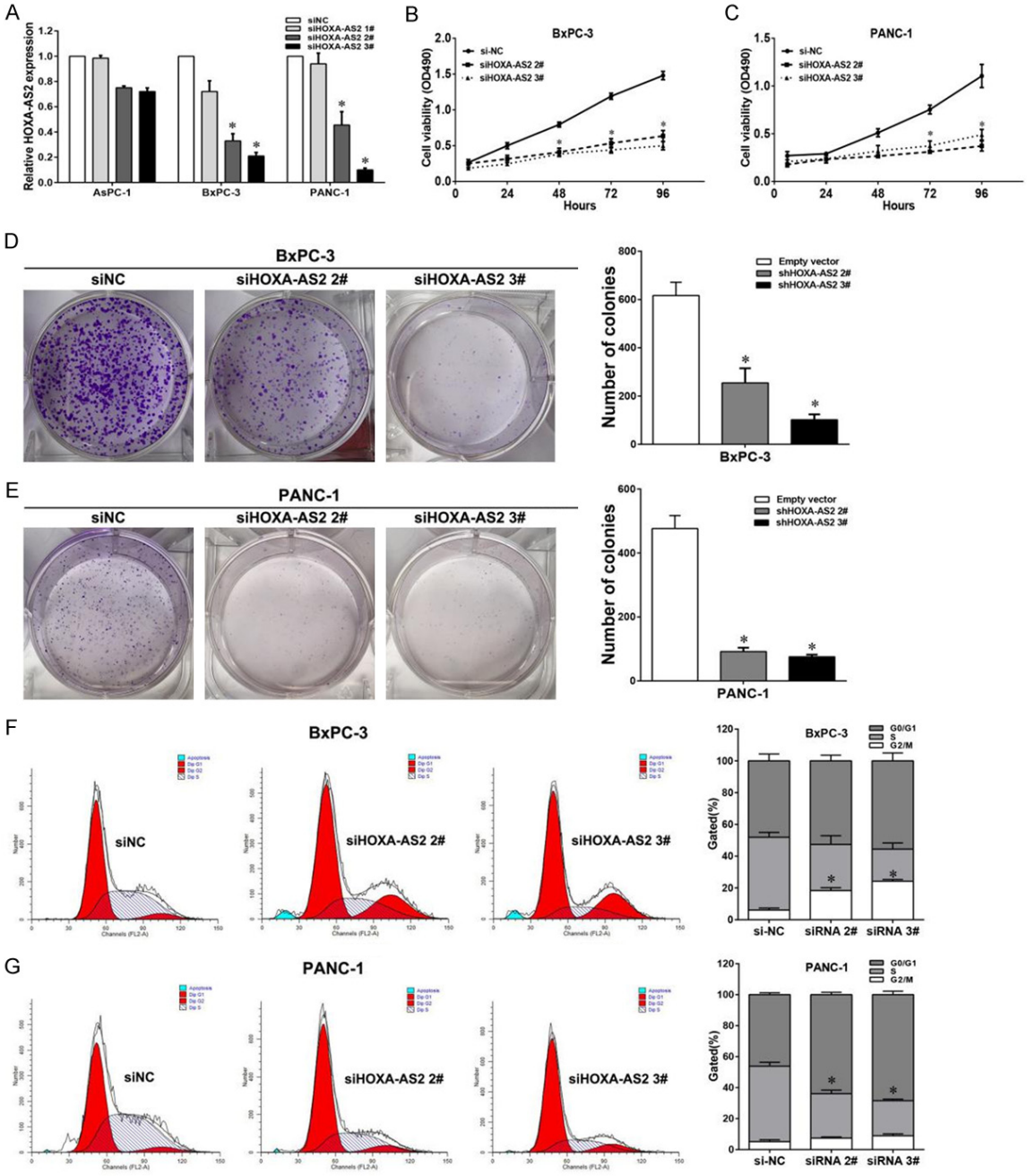

Figure 2. HOXA-AS2 knockdown significantly inhibited PC cell proliferation. A. The relative expression level of HOXA-AS2 in PC cells, transfected with siNC or siHOXA-AS2 (siHOXA-AS2 1 #, 2# and 3#), was detected using qRT-PCR. B, C. MTT assays were used to determine the cell viability for siHOXA-AS2-transfected BxPC-3 and PANC-1cells. D, E. Colony-forming assays were performed to evaluate the colon ability of siHOXA-AS2-transfected BxPC-3 and PANC-1 cells. F, G. At 24 h after transfection, the cell cycle was analyzed by flow cytometry. The bar chart represented the percentage of PC cells in G0/G1, S or G2/M phase, as indicated. The mean values and SD were calculated from triplicates of a representative experiment, *P < 0.05.

integrative analysis of pancreatic cancer microarray profile GSE15471, which described the lncRNAs profiles in 39 pairs of human pancreatic cancer and the corresponding adjacent normal tissues, from GEO datasets. Normalized signal data were then downloaded and z-score-transformed. As show in **Figure 1A**, we found that HOXA-AS2 was remarkably upregulated in

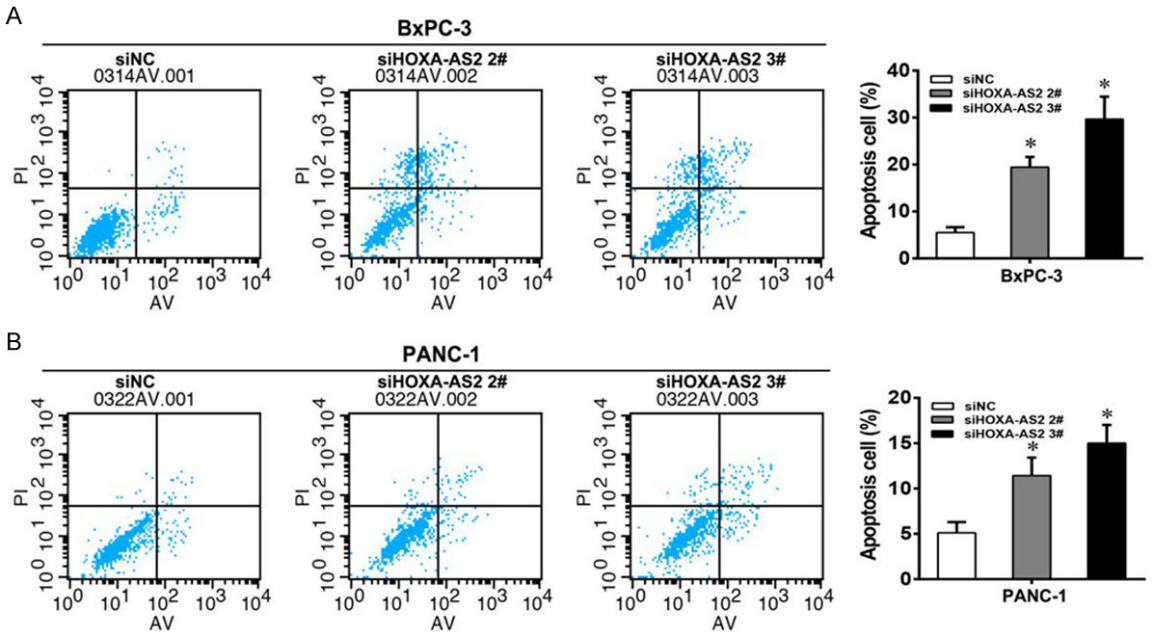

Figure 3. Silencing HOXA-AS2 expression induced PC cell apoptosis. A, B. BxPC-3 and PANC-1 cells were harvested for cell apoptosis analysis by flow cytometry 48 h after transfection. The mean values and SD were calculated from triplicates of a representative experiment, *P < 0.05.

pancreatic cancer tissues (fold change > 2.0, P < 0.05). To validate the expression results from microarray, we measured the expression level of HOXA-AS2 both in 16 paired PC tissues and adjacent normal tissues (Xiamen cohort) and 12 paired PC tissues and adjacent normal tissues (Nanjing cohort) by qRT-PCR. Notably, consistently higher expression level of HOXA-AS2 was found in both Xiamen and Nanjing cohorts (P < 0.05, **Figure 1B**, **1C**). Taken together, it is indicated that overexpression of HOXA-AS2 is a frequent event in human pancreatic cancer.

HOXA-AS2 promotes PC cell proliferation and induced apoptosis in vitro

To determine the role of HOXA-AS2 in pancreatic cancer, we silenced HOXA-AS2 in PC cells (including AsPC-1, BxPC-3 and PANC-1 cell lines) using special short interfering RNAs (siRNAs). A knockdown effect was observed by qRT-PCR, and we found that siHOXA-AS2 2# and siHOXA-AS2 3# achieved more effective knockdown efficiency both in BxPC-3 and PANC-1 cell lines (**Figure 2A**). Therefore these two cell lines were chosen to study the potential biological functions of endogenous HOXA-AS2 through a loss-of-function approach. To elucidate the biological roles of HOXA-AS2 on pancreatic cancer, the MTT assay was first used to evaluate the effect of HOXA-AS2 on cell viability. As depicted in **Figure 2B** and **2C**, cell growth was inhibited following knockdown of HOXA-AS2 in si-HOXA-AS2-transfected PC cells compared with respective controls. Additionally, the results of colony-formation assays showed that clonogenic survival was repressed following the down-regulation of HOXA-AS2 in BxPC-3 and PANC-1 (**Figure 2D** and **2E**). Next, to investigate the mechanism involved in growth suppression, we performed the flow cytometry analysis. However, the results showed that siHOXA-AS2 decreased the proportion of cells in S phase and increased of the proportion of cells in G2/M phase in BxPC-3 cell, while increased the percentage of cells in G0/G1 phase and decreased the percentage of cells in S phase in PANC-1 cell line (**Figure 2F** and **2G**). This difference indicates that the mechanism of HOXA-AS2 in promoting PC cell proliferation and survival is more complex than we imagine. To further determine whether the effect of HOXA-AS2 on PC cells proliferation reflected cell apoptosis, we performed flow cytometry assays. The results showed that BxPC-3 and PANC-1 cells transfected with HOXA-AS2 siRNA had higher apoptotic rate in comparison with control cells (**Figure 3A** and **3B**). These investigations suggest that HOXA-AS2 could acceler-

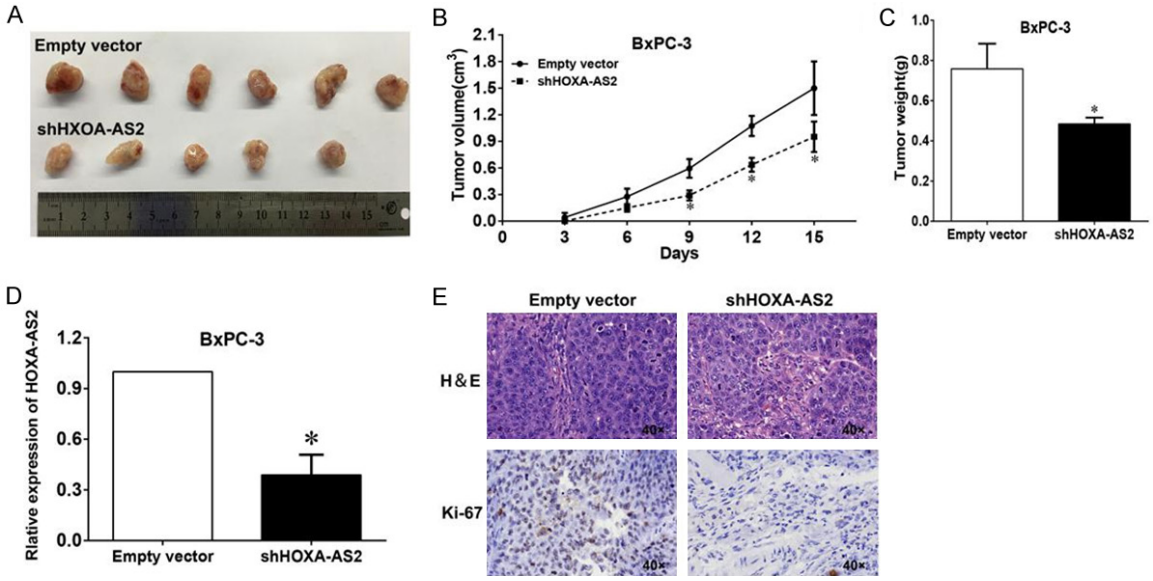

Figure 4. HOXA-AS2 promotes PC cell tumor growth in vivo. A. The stable HOXA-AS2 knockdown BxPC-3 cells were used for the in vivo study. The nude mice carrying tumors from respective groups were shown and tumor growth curves were measured after the injection of BxPC-3 cells. B. Tumor volume was calculated every 3 days. C. qRT-PCR was carried to test the average expression of HOXA-AS2 in xenograft tumors (n=6). D. Tumor weights are represented. E. Ki-67 protein levels in tumor tissues formed from shHOXA-A2 or empty vector-transfected BxPC-3 cells were determined by IHC staining. Left: H&E staining. Right: immunostaining, *P < 0.05.

ate the growth phenotype and suppress apoptosis of pancreatic cancer cells.

Stable downregulation of HOXA-AS2 inhibits PC cells tumorigenesis in vivo

To determine whether HOXA-AS2 affects tumor growth in vivo, we injected BxPC-3 cells transfected with either empty vector or shHOXA-AS2 into male nude mice. At 16 days post-injection, tumor growth in the shHOXA-AS2 group was slower than in the empty vector group (**Figure 4A**). Consistently, the tumor volumes and tumor weigh were remarkably decreased compared with the controls (**Figure 4B** and **4C**). As shown in **Figure 4D**, qRT-PCR confirmed that the level of HOXA-AS2 was lower in the tumor tissues derived from the sh-HOXA-AS2-transfected cells. Furthermore, immunohistochemistry (IHC) analysis confirmed that the tumors formed from BxPC-3/shHOXA-AS2 cells showed weaker Ki-67 staining than those formed from the control cells (**Figure 4E**). These results indicate that HOXA-AS2 is significantly associated with PC cell proliferation in vivo.

HOXA-AS2 exert oncogene function via interacting with LSD1 and EZH2

Recently, it has been reported that lncRNAs could exerted their biologic functions via interacting with specific RNA binding proteins [22-24]. Thus, to further investigate the molecular mechanisms by which HOXA-AS2 contributes to the proliferation phenotype of PC cells, we firstly predicted the interaction probabilities of HOXA-AS2 and RNA binding proteins using RNA-protein interaction prediction (http://pridb.gdcb.iastate.edu/RPISeq/), and found that HOXA-AS2 potentially binds EZH2, LSD1 (**Figure 5A**). To further validate the prediction, we performed RIP assays and the results showed that HOXA-AS2 indeed binds with EZH2 and LSD1 in BxPC-3 cells (**Figure 5B**). Next, to validate whether EZH2, LSD1 are involved in the HOXA-AS2 promoted of PC cells proliferation, we performed EDU (red)/DAPI (blue) immunostaining assay. The results of siHOXA-AS2 2#, siEZH2, siLSD1 or si-NC transfection for 48 h showed that HOXA-AS2, EZH2 or LSD1 knockdown could inhibit the PC cells ability to proliferate comparing with cells transfected with siNC (**Figure 5C** and **5D**). Finally, we explored the correlations between HOXA-AS2 expression levels and EZH2 or LSD1 in GSE15471 pancreatic tissue samples. The results showed that HOXA-AS2 had significantly positive correlation with EZH2 and LSD1 gene expression (**Figure 5E**). These data suggest that lncRNA-HOXA-AS2/EZH2/LSD1 complex promotes cell proliferation in pancreatic cancer cell.

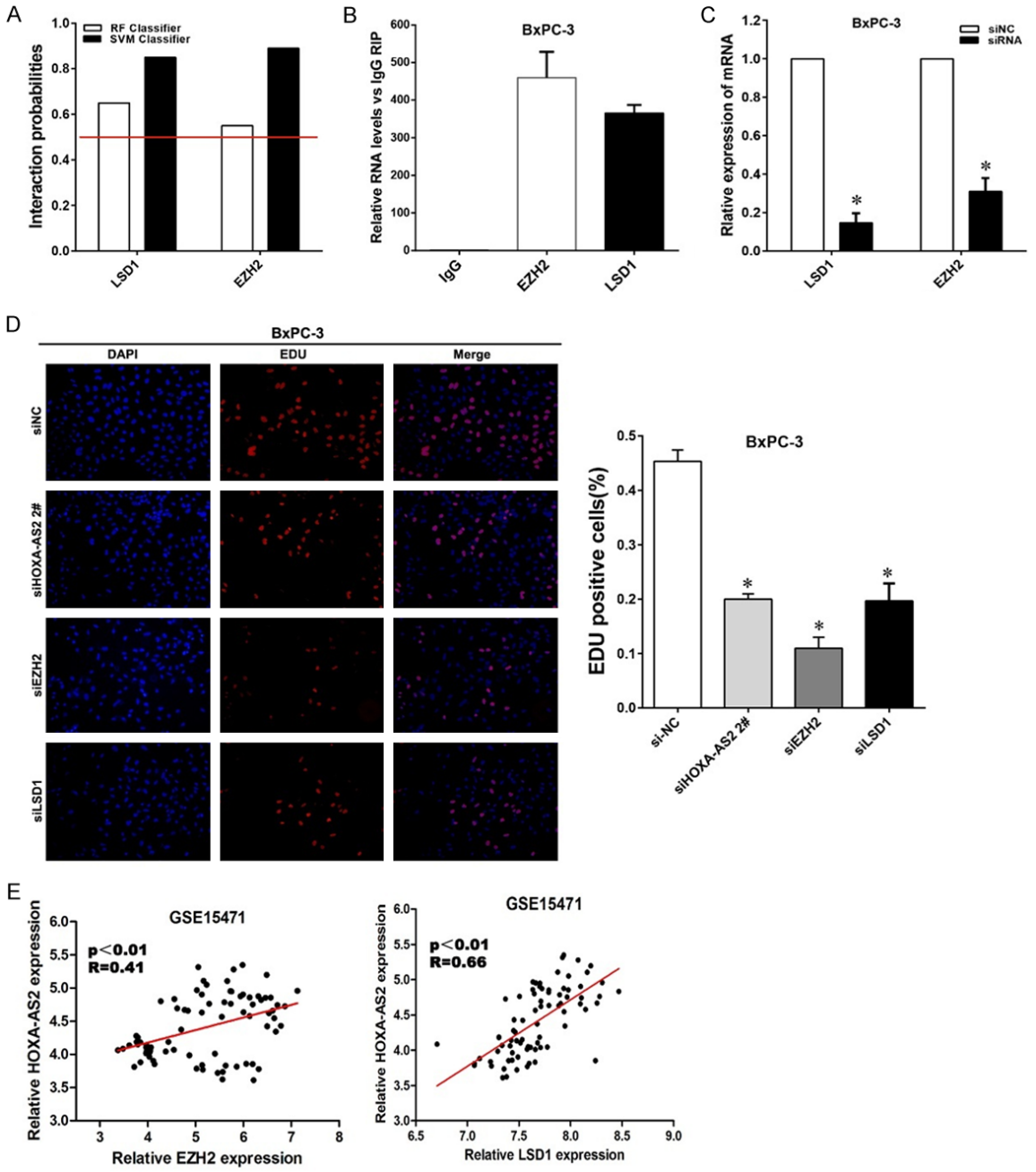

Figure 5. HOXA-AS2 binding with EZH2 and LSD1 to enhance PC cell proliferation. A. Bioinformatics were used to predict this possibility of interaction of HOXA-AS2 and EZH2, LSD1. Predictions with probabilities > 0.5 were considered positive. RPISeq predictions are based on Random Forest (RF) or Support Vector Machine (SVM). B. RIP assays were performed, and the co-precipitated RNA was subjected to qRT-PCR for HOXA-AS2. The fold enrichment of HOXA-AS2 in RIPs is relative to its matching IgG control RIP. C. The mRNA levels of EZH2 or LSD1 was determined after transfected with siNC, siEZH2 or siLSD1 by qRT-PCR. D. EdU staining assays were carried to determine the growth of siNC/siHOXA-AS2/siEZH2/siLSD1-transfected BxPC-3 cells. E. The relationship between HOXA-AS2 expression and EZH2/LSD1 mRNA levels was analyzed in the profile of PC patient tissue from GSE15471. The mean values and SD were calculated from triplicates of a representative experiment, *P < 0.05.

Discussion

Great effort in the past has been made to identify cancer-related lncRNAs and elucidate their biology roles in cancer development [25-27]. In the current study, we found that lncRNA HOXA-AS2 which is upregulation in human pancreatic cancer specimens; In addition, knock-

down of HOXA-AS2 could suppress PC cells proliferation and induce cell apoptosis. Furthermore, mechanism study indicated that HOXA-AS2 could interact with EZH2 and LSD1, then enhancing PC cell proliferation ability.

Compared with normal cells, tumor cells possess unique gene expression programs, enhancing cell cycle progression and cell proliferation, whereas resisting apoptosis [28, 29]. Recently, a number of lncRNAs have been identified as having tumor suppressive or oncogenic functions via affecting cancer cell growth ability [30]. For example, Nie et al reported that up-regulation of lncRNA ANRIL could promoted non small cell lung cancer cells proliferation through silencing of KLF2 and P21 transcription [31]. Yu et al found that elevated expression level of lncRNA CCAT1 is a tumor promoter, which facilitates cell growth in pancreatic cancer [32]. Our results also showed that silencing HOXA-AS2 could inhibit cell proliferation and colony formation, promote cell cycle arrest, induce cell apoptosis. These results provide an explanation for HOXA-AS2 upregulation both in GEO Datasets (GSE15471) and two cohorts of pancreatic cancer samples, which indicated that HOXA-AS2 exhibits oncogenic activity in pancreatic cancer.

Typically, lncRNAs involve in regulation of cancer cells phenotype through activation of oncogenes or inhibition of tumor suppressors via interacting with specific RNA-binding proteins [33, 34]. EZH2, the core subunit of polycomb repressive complex 2 component, functions as an oncogene in various cancer types, including pancreatic cancer [35, 36]. LSD1, also known as KDM1A, is well documented that is necessary for various cancer development and participates in many biological processes, such as cell differentiation and cell cycle progression [37, 38]. In our present study, we found that EZH2 and LSD1 also function as oncogenes in PC cells and HOXA-AS2 plays a crucial role in the pancreatic cancer cell proliferation by binding to EZH2 and LSD1.

In summary, our current study revealed that lncRNA HOXA-AS2 exhibits oncogenic activity in pancreatic cancer. Shedding new light on the understanding of the mechanistic actions of pancreatic cancer progression. Our experiments identified the lncRNA-HOXA-AS2/EZH2/LSD1 oncogene complex that is implicated in PC cell proliferation, providing the pursuit of these molecules as potential targets for pancreatic cancer intervention. However, one limitation of the present study is that other possible mechanisms that with the regulatory behaviors of HOXA-AS2 were not investigated in our study, and these remain incompletely understood and warrant further investigation.

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Disclosure of conflict of interest

None.

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