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Original Research

LncRNA CRNDE promotes the progression and angiogenesis of pancreatic cancer via *miR-451a/CDKN2D* axis

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ABSTRACT

Background: The lncRNA colorectal neoplasia differentially expressed (lncRNA CRNDE) has been reported to play a pivotal role in various cancers. However, the expression and function of CRNDE in pancreatic cancer remain unclear. The objective of this study was to investigate the effects of CRNDE on pancreatic cancer and the underlying mechanisms.

Methods: The expression of CRNDE in pancreatic cancer tissues and cell lines was determined by RT-qPCR. Proliferation and angiogenesis were detected by MTT, colony formation, transwell and tube formation assays *in vitro* and *in vivo*. ELISA assay was used to detect the secretion of VEGFA. IHC was performed to test the expression levels of Ki67 and CD31. The binding sites between CRNDE, CDKN2D and miR-451a were predicted by bioinformatics analysis. Dual luciferase reporter and RNA immunoprecipitation assays were conducted to confirm the interaction with each other.

Results: The results showed that CRNDE was significantly up-regulated in pancreatic cancer tissues as well as cell lines. CRNDE overexpression promoted the progression and angiogenesis of pancreatic cancer cells *in vitro* and *in vivo*. Moreover, we identified that CRNDE functioned as a sponge for miR-451a and CRNDE overexpression inhibited the expression of miR-451a. Furthermore, we confirmed that miR-451a directly interacted with CDKN2D and negatively regulated CDKN2D expression. In addition, CRNDE was found to positively regulate CDKN2D expression and mediate pancreatic cancer cell proliferation and angiogenesis through miR-451a/CDKN2D axis.

Conclusion: CRNDE modulates cell proliferation and angiogenesis via miR-451a/CDKN2D axis in pancreatic cancer, which provides a potential therapeutic target for pancreatic cancer treatment.

Introduction

Pancreatic cancer is one of the most frequent gastrointestinal malignancies in the world, with a 5-year survival rate of less than 5% [22]. Recently, its mortality rate has been increasing year by year [31]. Pancreatic cancer is generally asymptomatic in the early stage, but highly aggressive in the later stage [18]. Nowadays, although surgical resection remains the main treatment strategy for pancreatic cancer, the prognosis of the patients is still poor [7]. Most patients have lost the opportunity for surgery because of early diagnosis difficulties. Besides, the molecular mechanism underlying pancreatic cancer progression remains largely unknown. Thus, it is urgent to understand the detailed molecular basis and identify the potential biomarkers for the effective diagnosis and novel treatment strategies of pancreatic cancer [26].

Long non-coding RNAs (lncRNAs) refer to a class of non-coding RNAs with a length of more than 200 nucleotides that can regulate gene expression [6]. Recent studies have shown that lncRNAs play an important role in the occurrence and development of cancer [15]. Some of lncRNAs were reported to control pancreatic cancer progression [8,35,37]. The lncRNA colorectal neoplasia differentially expressed (lncRNA CRNDE) is a crucial lncRNA that is located on chromosome 16 and acts as an oncogene in various malignant diseases [5,34,38]. Studies have found that lncRNA CRNDE is up-regulated in pancreatic cancer and promotes the proliferation and metastasis of pancreatic cancer cells [28]. Nev-

Abbreviations: Long non-coding RNAs lncRNAs, LncRNA colorectal neoplasia differentially expressed lncRNA CRNDE; MicroRNAs miRNAs, RNA immunoprecipitation RIP; Enzyme linked immunosorbent assay ELISA, Immunohistochemistry IHC.

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ertheless, the regulatory mechanisms of lncRNA CRNDE in pancreatic cancer have not been elucidated.

MicroRNAs (miRNAs) are a group of single-stranded RNAs, with a length of about 22 nucleotides, which are used as gene regulator in many diseases [14] [2]. Accumulating evidence suggested that miRNAs can regulate cancer progression [25,36]. Recently, miR-451a has been confirmed to be down-regulated in various cancers such as breast cancer, thyroid cancer and prostate cancer [17,19,3]. Moreover, reduced miR-451 level was observed in eutopic endometrium [9]. However, miR-451a has not yet been studied in pancreatic cancer. Therefore, we speculate a role of miR-451a in pancreatic cancer progression.

CDKN2D belongs to the INK4 family CKIs [27]. The INK4 family is capable to control the G1-to-S phase transition through suppressing the activity of CDK4 and CDK6 [13]. CDKN2D acts as an inhibitor of cyclin D-dependent kinases [21]. In addition, CDKN2D plays a critical role in the regulation of tumor growth [20]. For example, CDKN2D regulates both cell proliferation and granulocytic differentiation in acute promyelocytic leukemia cells [30]. However, it is unclear whether CDKN2D is involved in pancreatic cancer cell proliferation and angiogenesis.

In the present study, we investigated that lncRNA CRNDE was up-regulated in pancreatic cancer cell lines and tissue samples. Moreover, lncRNA CRNDE overexpression significantly promoted cell proliferation and angiogenesis in pancreatic cancer cells. In addition, we found that lncRNA CRNDE exerted its function via miR-451a/CDKN2D axis. Our findings indicated that CRNDE could serve as a novel prognostic marker and therapeutic target for pancreatic cancer.

Materials and methods

Clinical samples

Paired pancreatic cancer tissue specimens and adjacent non-tumor tissue specimens were obtained from 38 patients who were recruited for pancreatic cancer surgery at Suqian First Hospital from October 2019 to October 2020. The tissues were collected and stored in –80 ˚C freezer. The research procedures were approved by the Clinical Research Ethics Committee of Suqian First Hospital. The ethical approval code is 2020-SL-0087.

Cell culture

Five human pancreatic cancer cell lines (MIAPaCa-2, PANC-1, SW1990, CAPAN-1 and JF305), pancreatic duct epithelial cell line (HPDE6-C7) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human umbilical vein endothelial cells (HUVECs) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 1% Penicillin-Streptomycin (Sigma, St. Louis, MO, USA) and 1% L-glutamine (Invitrogen) at 37°C and 5% CO_2 for cell passage.

Oligonucleotides and cell transfection

The small interference RNA targeting lncRNA CRNDE (si-CRNDE 5'-GUCACGCAGAAGAAGGUUATT-3') and CDKN2D (si-CDKN2D 5'-GCCGTTGGTCTTTGAAATTTC-3') were constructed by GenePharma (Shanghai, China). PcDNA-3.1 vector containing CRNDE or CDKN2D was used for producing overexpressing cells (GenePharma).The miR-451a mimics and miR-451a inhibitor were prepared by RiboBio (Guangzhou, China). Transfection was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol.

Total RNA extraction and real-time quantitative PCR

The extraction of total RNA from paired tissues and cells was carried out using Trizol reagent (Invitrogen). LncRNA and mRNA were reverse transcribed into cDNA using a PrimeScript^{TM} RT Reagent Kit (Takara, Kyoto, Japan). The reverse transcription of miRNA into cDNA was made with the use of a Mir-X^{TM} miRNA First Strand Synthesis Kit (Takara). Real-time quantitative PCR was performed with SYBR Green PCR Kit (Takara) on ABI 7500 PCR System (Applied Biosystems, Foster City, CA, USA). The sequences of the primers were: lncRNA CRNDE forward: 5'-AAATTCATCCCAAGGCTGGT-3', lncRNA CRNDE reverse: 5'-AAACCACTCGAGCACTTTGA-3'; miR-451a forward 5'-GCCCGAAAC CGTTACCATTAC-3', miR-451a reverse 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC AACTCA-3'; CDKN2D forward: 5'-GCTGCAGGTCATGATGTTTG-3', CDKN2D reverse: 5'-CTGCCAGATGGATTGGAAGT-3'; GAPDH forward: 5'-CCAGGTGGTCTCCTCTGA-3', GAPDH reverse: 5'-GCTGTAGCCAAATCGTTGT-3'; U6 forward: 5'-CGCTTCGGCAGCACATATAC-3', reverse: 5'-TTCACGAATTTGCGTGTCATC-3'. Both GAPDH and U6 were used as internal control. Relative quantity of gene expression was analyzed by the standard $2^{-\Delta\Delta Ct}$ method.

Western blot

Cells were lysed in RIPA lysis buffer to extract total protein. Furthermore, protein concentrations were detected using the BCA method. Protein was then separated by 10% SDS-PAGE gels, followed by transferring to PVDF membrane (Millipore, Boston, MA, USA). After blocking with 5% non-fat milk in TBST for 1 h, the membranes were incubated with following primary antibodies at 4°C overnight: anti-CDKN2D (1:1000, Abcam, MA, USA). After washing in TBST, the membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibodies (1:20000, BOSTER, Beijing, China) at room temperature for 1 hour. The protein bands were determined with enhanced chemiluminescence (ECL) detection (Pierce Biotechnology, Rockford, IL, USA). GAPDH was used as an internal loading.

MTT assay

MTT assay was used to determine the cell viability. Cells were seeded in 96-well plates and then transfected with plasmids for 24 h. Then, 5 mg/ml MTT solution (Sigma) was added to the wells at 37 ˚C for 4 h. Afterwards, 100 μl of dimethyl sulfoxide (DMSO) was added to each well and the absorbance was measured at a wavelength of 490 nm.

Transwell assay

For cell migration assay, the transfected cells were plated in Transwell upper chambers (Corning, NY, USA) and the lower chambers were added with DMEM medium containing 10% serum for 24 h incubation. After fixing with 4% formaldehyde and staining with 0.1% crystal violet, the migrated cells on the lower surface were observed and counted under a microscope.

Colony formation assay

Cells (1.0×10^3/well) were seeded in six-well plates and cultured for 10 days after transfection. Colonies were then preset with 10% formaldehyde for 10 min and stained using 0.5% crystal violet solution (Sigma). The colonies were observed and calculated using Olympus microscope (Olympus, Tokyo, Japan).

Tube formation assay

For tube formation assay, pancreatic cancer cells and HUVECs were co-cultured. Subsequently, HUVECs were collected and seeded into the

polymerized Matrigel (BD Biosciences, San Jose, CA, USA) coated wells at a density of 1×10^4 cells/well. After 8 hours, the cells were fixed with 4% formaldehyde and the images of tube formation were photographed and then analyzed using the ImageJ software (National Institutes of Health, USA).

Dual luciferase reporter assay

LncRNA CRNDE or the 3'-UTR of CDKN2D containing the wild-type or mutant that had the predicted miR-451a binding site was constructed into psi-CHECK2 vector (Promega, Madison, WI, USA) following the manufacturer's instructions. Wild or mutant type constructs were co-transfected with the miR-451a mimic or negative control (mimic NC) into MIAPaCa-2 cells with the use of Lipofectamine 2000. After 48 h transfection, the luciferase activities were detected using Dual-Luciferase reporter assay (Promega) system according to the manufacture's protocol.

RNA immunoprecipitation (RIP) assay

The transfected cells were collected according to the protocol of Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore). Briefly, cells were lysed in RNA lysis buffer containing protease and RNase inhibitors. Then, a portion of the cell extract was isolated as the input. The other portion was incubated with RIP immunoprecipitation buffer containing magnetic beads conjugated with anti-Argonaute protein 2 (Ago-2) antibody (Millipore) or control anti-IgG antibody (Millipore). The samples were digested with proteinase K to extract RNA. The beads were then washed twice with RIP washing buffer. Subsequently, the level of extracted RNA was determination by qRT-PCR.

Enzyme linked immunosorbent assay (ELISA)

The serum was collected to analyze the protein concentration of VEGFA with an ELISA kit (Abcam) according to the manufacturer's protocol. The intensity was measured using colorimetric analysis at 450 nm wavelength.

Nude mice tumorigenesis

Four-week-old male BALB/c nude mice were purchased from Shanghai laboratory Animal Center of Chinese Academy of Sciences (Shanghai, China). PANC-1 and MIAPaCa-2 cells were stably transfected with vector, pcDNA-CRNDE, si-NC or si-CRNDE. The cells were then subcutaneously injected into the nude mice at the right axilla, with 5 mice in each group. The tumor nodules were then examined every four days. Tumor volume = (Width^2 x Length)/2. At the end of the 32th day after injection, the mice were sacrificed. The tumor was excised and weighed. All of the animal experimental procedures were permitted by the Animal Ethics Committee of The First Affiliated Hospital of Soochow University.

Immunohistochemistry (IHC)

Paraffin embedded tumor tissues from nude mice were sectioned. The sections were deparaffinized, rehydrated and incubated with 0.3% hydrogen peroxide for 10 min at room temperature to inhibit endogenous peroxidase activity, followed by blocking with 10% normal goat serum for 30 min at room temperature. Next, the sections were incubated with primary antibodies against Ki67 (1:200, Abcam), CD31 (1:200, Abcam) at 4 °C overnight. Following washing with PBS, the sections were incubated with a second antibody coupled with horseradish peroxidase (HRP) for 1 h at room temperature, and washed with PBS. Slides were then assessed using a light microscope (Olympus, Tokyo, Japan).

Table 1
Correlation between CRNDE expression and clinicopathological factors of pancreatic cancer patients (tissue samples).

Characteristic	Expression of CRNDE		*P* value
	Low	High	
Age (yr)			
≥ 60	11	9	0.7459
< 60	8	10	
Gender			
Male	6	12	0.1031
Female	13	7	
Tumor diameter (cm)			
≤ 4	9	14	0.1837
> 4	10	5	
TNM stage			
I-II	12	3	0.0069
III-IV	7	16	
Lymph node metastasis			
No	13	4	0.0081
Yes	6	15	

Statistical analysis

All experimental data were reported as mean ± standard deviation (SD) from three independent experiments. Statistical analysis was conducted with Graphpad Prism 7 (Graphpad, La Jolla, CA, USA). Paired Two-tailed Student's t test was used when two cases were compared. One-way-ANOVA was performed for multi-group comparison. *P* values <0.05 were statistically significant.

Results

LncRNA CRNDE promotes proliferation and angiogenesis of pancreatic cancer cells in vitro

To investigate the expression pattern of lncRNA CRNDE in pancreatic cancer, we first measured the expression level of CRNDE in paired human primary pancreatic cancer tissues and adjacent non-tumor tissues by RT-qPCR and found that the level of CRNDE increased obviously in pancreatic cancer tissues compared to the normal tissues (Fig.1A). In addition, the clinicopathological characteristics of the pancreatic cancer patients indicated that CRNDE expression has no significant correlation with the age, gender or tumor size of pancreatic cancer patients, but it was remarkably related to TNM stage and lymph node metastasis (Table 1). Moreover, the levels of CRNDE in pancreatic cancer cell lines were also determined by RT-qPCR. The results showed that CRNDE expression levels were greatly up-regulated in five pancreatic cancer cell lines compared with that in normal pancreatic cells (Fig.1B), suggesting that CRNDE may play an important role in pancreatic cancer. Next, we selected pancreatic cancer cell lines (namely MIAPaCa-2 and PANC-1) with significantly high expression of CRNDE for the follow-up experiments.

To assess the effects of CRNDE in pancreatic cancer, the CRNDE gene was knocked down using si-CRNDE or overexpressed with oe-CRNDE. The expression of CRNDE was verified by RT-qPCR. The decreased expression of CRNDE was observed in cells with si-CRNDE, while increased expression of CRNDE was detected in cells with pcDNA-CRNDE (Fig. 1C). Additionally, MTT and colony formation assays were used to test the cell growth of MIAPaCa-2 and PANC-1 cells. CRNDE overexpression significantly promoted the cell viability and proliferation of pancreatic cancer cells. However, when CRNDE was knockdown, these cell functions were suppressed (Fig. 1D and 1E). Furthermore,we detected the secretion of VEGFA in MIAPaCa-2 and PANC-1 cells using ELISA assay, and the migration of these cells with transwell assay. As a result, CRNDE overexpression elevated VEGFA expression and enhanced the migration ability in these cells (Fig. 1F and 1G). Simi-

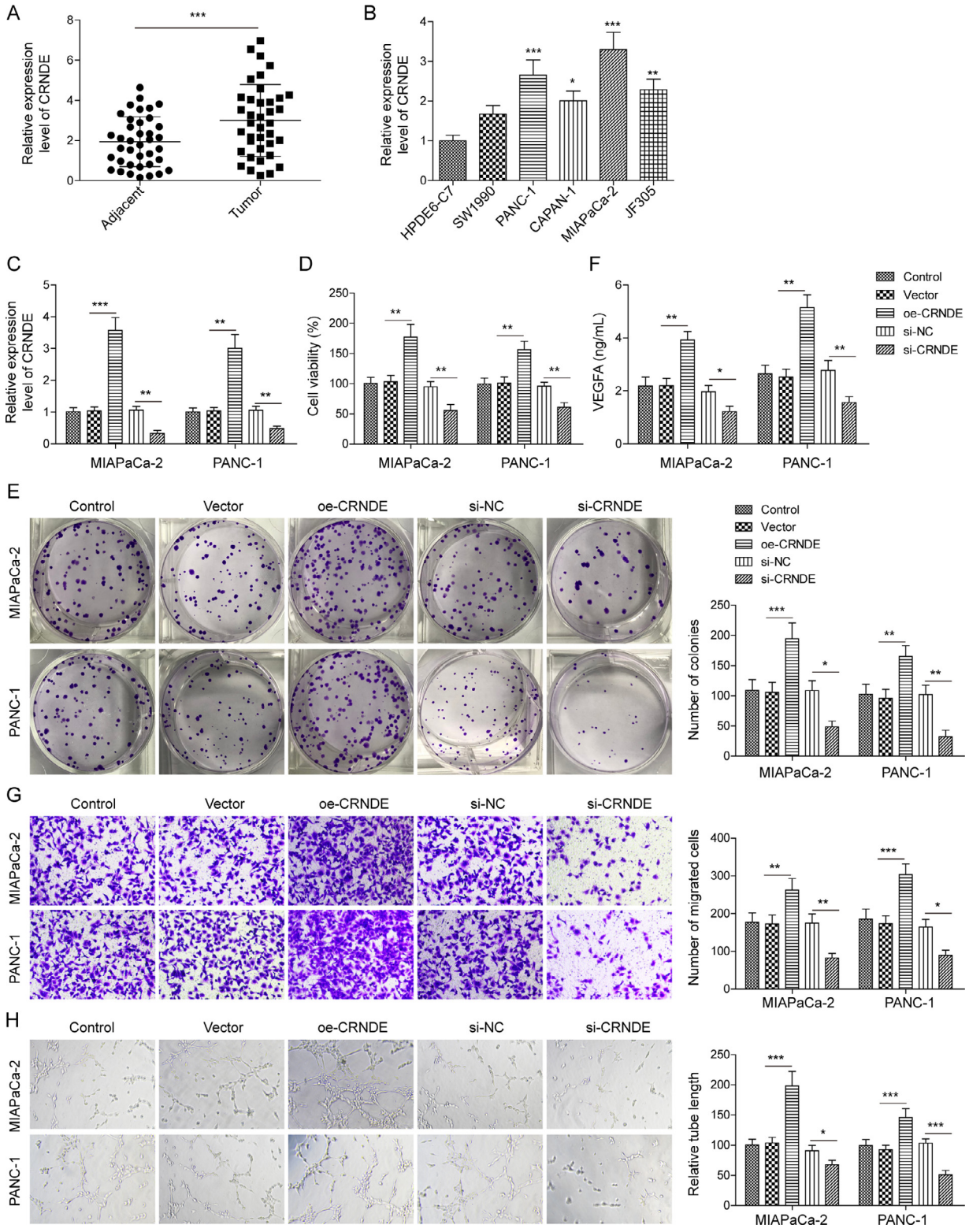

Fig. 1. Effect of CRNDE on proliferation and angiogenesis of pancreatic cancer cells *in vitro*. (A) RT-qPCR was used to measure CRNDE expression in paired human primary pancreatic cancer tissues and adjacent non-tumor tissues. Difference between two groups was measured using the paired t test. (B) The expression of CRNDE in five human pancreatic cancer cell lines (MIAPaCa-2, PANC-1, SW1990, CAPAN-1 and JF305) and pancreatic duct epithelial cells (HPDE6-C7) was determined by RT-qPCR. MIAPaCa-2 and PANC-1 cells were transfected with vector, pcDNA-CRNDE, si-NC or si-CRNDE. (C) The transfection efficiencies were verified by RT-qPCR. (D) MTT assay was performed to measure the cell viability. (E) Colony formation assay was performed to test the cell proliferation. (F) ELISA assay was used to detect the secretion of VEGFA in each group of cells. (G) Transwell assay was used to determine the cell migration ability in each group. (H) The tube formation ability of HUVECs was showed as the relative tube length in each group. $^{*}P < 0.05$,$^{**}P < 0.01$,$^{***}P < 0.001$. Data are the means ± SD from three independent experiments.

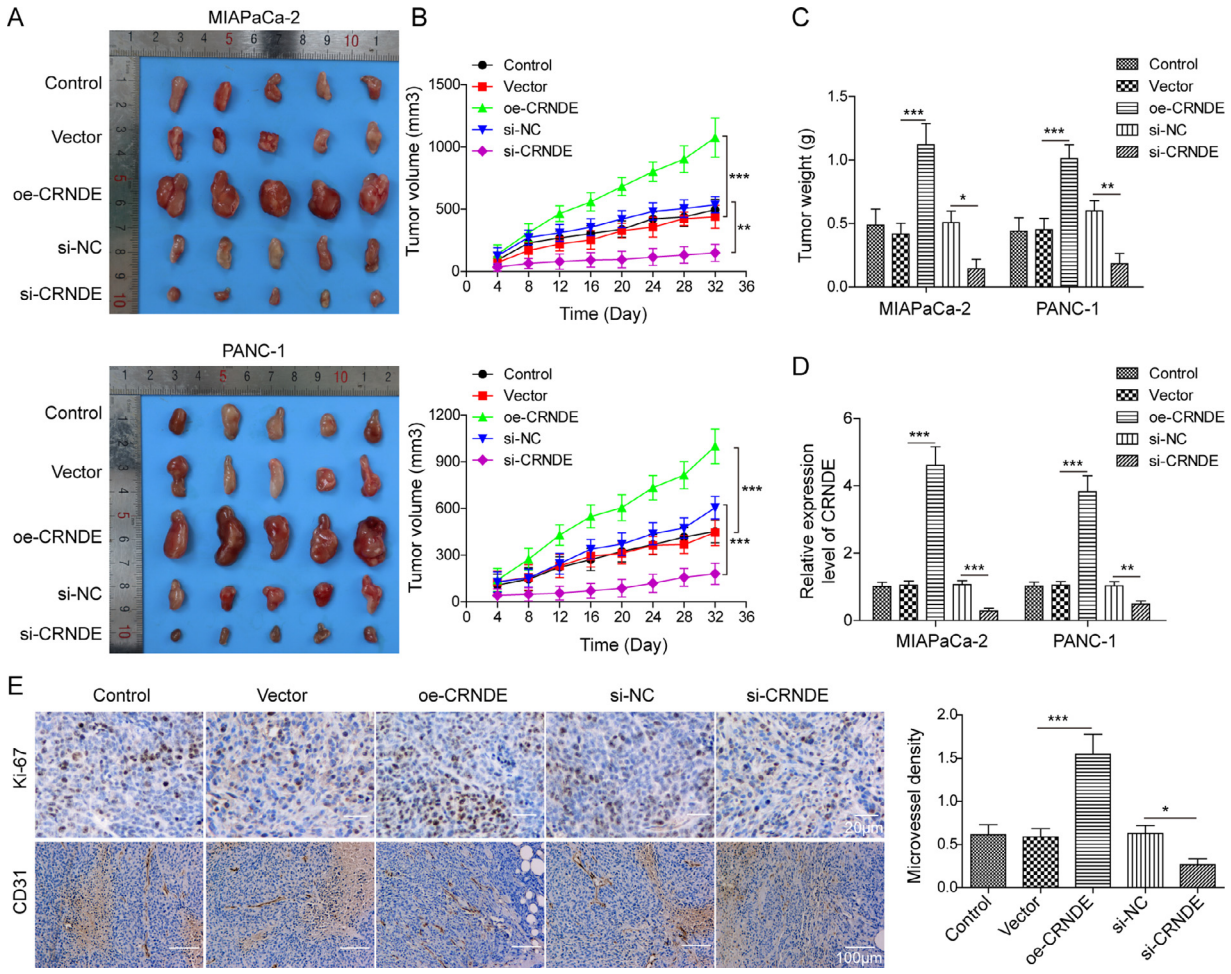

Fig. 2. CRNDE promotes pancreatic cancer progression *in vivo*. The nude mice were injected subcutaneously with table MIAPaCa-2 and PANC-1 cells transfected with vector, pcDNA-CRNDE, si-NC or si-CRNDE. (A) On the 32th day after transfection, nude mice and their tumor tissues with vector, pcDNA-CRNDE, si-NC or si-CRNDE. (B) The tumor volume in each group was measured at 1, 2, 3, 4, and 5 weeks. (C) The tumor weight in each group was detected at the end of the fifth week after injection. (D) RT-qPCR was used to measure CRNDE expression in the nude mouse tissues. (E) IHC analysis was performed to test the proliferation-related factor Ki67 and angiogenesis-related factor CD31 in each group. $^{*}P < 0.05$,$^{**}P < 0.01$,$^{***}P < 0.001$. Data are the means ± SD from three independent experiments.

larly, tube formation assay implied that the tube formation ability of HUVECs was effectively induced by CRNDE overexpression (Fig. 1H). Taken together, these above data indicate that up-regulation of CRNDE can promote proliferation and angiogenesis of pancreatic cancer cells *in vitro*.

LncRNA CRNDE promotes pancreatic cancer progression in vivo

To explore the function of CRNDE in pancreatic cancer progression *in vivo*, we injected into the nude mice subcutaneously with the stable MIAPaCa-2 and PANC-1 cells transfected with pcDNA-CRNDE, si-CRNDE and the negative control. We then measured the tumor volume in each group every week for five weeks and the tumor weight at the end of the fifth week after injection. As shown in Fig. 2A-2C, CRNDE promoted the growth of pancreatic cancer xenografts, while CRNDE knockdown suppressed tumor growth. Furthermore, the results of RT-qPCR verified the up-regulation of CRNDE expression in the nude mouse tissues with CRNDE overexpression, while silencing of CRNDE caused a significant decrease in its expression (Fig. 2D). In addition, IHC analysis was used to detect the proliferation-related factor Ki67 and angiogenesis-related factor CD31 in each group. It showed that the expression levels of Ki67 and CD31 in tumor tissues with CRNDE overexpression were greatly up-regulated compared with the control group. However, these effects were reversed in tumor tissues with CRNDE knockdown (Fig. 2E). The above results indicate that CRNDE can promote pancreatic cancer tumor growth and angiogenesis *in vivo*.

LncRNA CRNDE functions as a sponge for miR-451a in pancreatic cancer cells

To further reveal the potential mechanism of CRNDE on pancreatic cancer progression, miR-451a was predicted to be a potential target of CRNDE through bioinformatics tool StarBase (Fig. 3A). To verify that CRNDE can directly bind to miR-451a, luciferase reporter assays were conducted. The fragment of CRNDE containing a miR-451a binding site (CRNDE-WT) or mutant fragment (CRNDE-MUT) was synthesized and inserted into plasmids. The plasmids were co-transfected with miR-451a mimic or miR-NC into MIAPaCa-2 cells. The results demonstrated that overexpression of miR-451a suppressed the luciferase activity in MIAPaCa-2 cells with CRNDE-WT, while no effects were observed in MIAPaCa-2 cells with CRNDE-MUT (Fig. 3B). Furthermore, RIP results indicated that the enrichment of CRNDE was increased in Ago2 immunoprecipitation complex by miR-451a overexpression compared to MIAPaCa-2 cells with miR-NC transfection, which further confirmed the binding relationship between CRNDE and miR-451a (Fig. 3C). In addition, the expression of miR-451a decreased obviously in pancreatic cancer compared to the normal samples (Fig. 3D). Moreover, pearson analysis showed that the expression of miR-451a and CRNDE was neg-

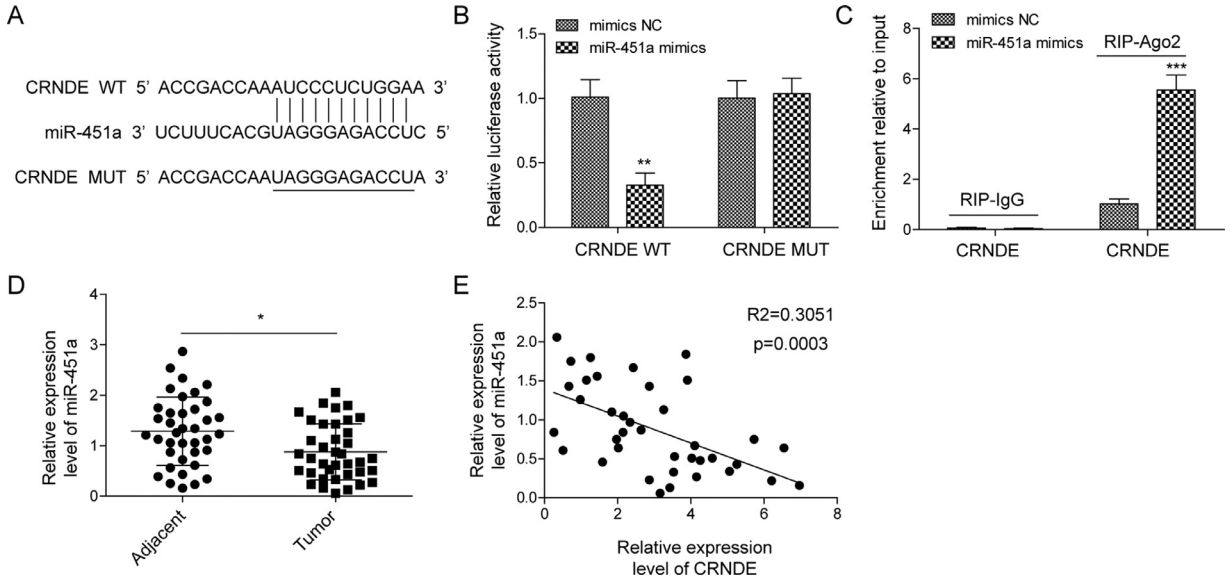

Fig. 3. CRNDE specifically binds to miR-451a in pancreatic cancer cells. (A) The binding site between CRNDE and miR-451a predicted by bioinformatics analysis. (B) The luciferase activity of CRNDE-WT and CRNDE-MUT in MIAPaCa-2 cells co-transfected with miR-451a mimic or miR-NC. (C) CRNDE and miR-451a co-immunoprecipitated with Ago2 revealed by RIP assay. (D) RT-qPCR was used to measure the expression of miR-451a in pancreatic cancer and normal samples. Difference between two groups was measured using the paired t test. (E) Pearson analysis showed the correlation between the expression of miR-451a and CRNDE was negatively correlated in pancreatic cancer tissures. $^{*}P<0.05$, $^{**}P < 0.01$,$^{***}P < 0.001$. Data are the means ± SD from three independent experiments.

atively correlated in pancreatic cancer tissures (Fig. 3E). Therefore, the above results indicate that CRNDE functions as a sponge for miR-451a in pancreatic cancer cells.

LncRNA CRNDE regulates pancreatic cancer cell growth and angiogenesis through miR-451a

To further investigate the interaction between CRNDE and miR-451a, MIAPaCa-2 and PANC-1 cells were transfected with pcDNA-CRNDE and miR-451a mimic. RT-qPCR results showed that CRNDE overexpression inhibited the expression of miR-451a, while miR-451a mimic overturned this inhibitory effect (Fig. 4A). In addition, MTT and colony formation assays demonstrated that the viability and proliferation of pancreatic cancer cells were greatly enhanced in cells with CRNDE overexpression. However, when miR-451a was also overexpressed, these effects were suppressed (Fig. 4B and 4C). Besides, we detected that CRNDE overexpression elevated VEGFA expression and enhanced the migration ability in these cells, whereas these effects were abolished by miR-451a mimic co-transfection (Fig. 4D and 4E). Moreover, the tube formation ability of HUVECs was obviously induced by CRNDE overexpression, while co-transfection with miR-451a mimic had the opposite effect (Fig. 4F). These data further indicate that CRNDE mediates pancreatic cancer cell proliferation, migration, and angiogenesis through miR-451a.

MiR-451a directly interacted with CDKN2D and negatively regulated CDKN2D expression in pancreatic cancer cells

We further discovered the direct target of miR-451a using the online bioinformatics tool targetscan, which predicted CDKN2D (Fig. 5A). Luciferase reporter analysis then verified that co-transfection of CDKN2D-WT and miR-451a mimic reduced luciferase reporter activity in MIAPaCa-2 cells when compared with these cells transfected with CDKN2D-MUT (Fig. 5B). Moreover, the detection of CDKN2D in MIAPaCa-2 cells by Western blot showed that overexpression of miR-451a significantly inhibited the expression of CDKN2D, while miR-451a inhibitor induced the expression of CDKN2D (Fig. 5C). In addition, the expression of CDKN2D increased obviously in pancreatic cancer compared to the normal samples (Fig. 5D). Moreover, pearson analysis showed that the expression of miR-451a and CDKN2D was negatively correlated in pancreatic cancer tissures (Fig. 5E), while the expression of CRNDE and CDKN2D was positively correlated (Fig. 5F). Therefore, these results indicate that CDKN2D is a direct target of miR-451a in pancreatic cancer cells.

Inhibition of miR-451a on CDKN2D expression affects pancreatic cancer cell proliferation and angiogenesis

We then investigated whether miR-451a affects the proliferation and angiogenesis of pancreatic cancer cells by regulation of CDKN2D. MIAPaCa-2 and PANC-1 cells were transfected with pcDNA-CDKN2D and miR-451a mimic. The results revealed that the expression of CDKN2D was notably repressed after overexpression of miR-451a in MIAPaCa-2 and PANC-1 cells, while CDKN2D overexpression could restore CDKN2D expression (Fig. 6A and 6B). Moreover, the up-regulated miR-451a significantly inhibited pancreatic cancer cell proliferation, migration, and angiogenesis. On the other hand, CDKN2D overexpression reversed these effects (Fig. 6C-6G). These results suggest that miR-451a mediates pancreatic cancer progression through CDKN2D.

LncRNA CRNDE induces CDKN2D expression and affects pancreatic cancer cell proliferation and angiogenesis via miR-451a

To explore whether CRNDE affects pancreatic cancer growth and metastasis via miR-451a/CDKN2D axis, we transfected MIAPaCa-2 and PANC-1 cells with pcDNA-CRNDE and si-CDKN2D. Western blot results indicated that overexpression of CRNDE increased CDKN2D level and this was suppressed by miRNA-451a overexpression (Fig. 7A). Moreover, CRNDE overexpression promoted the expression of CDKN2D. However, knockdown of CDKN2D reversed the effect of CRNDE on CDKN2D (Fig. 7B), suggesting that CRNDE up-regulates CDKN2D through miR-451a. In addition, CRNDE overexpression significantly promoted pancreatic cancer cell proliferation, migration, and angiogenesis. Nevertheless, down-regulated CDKN2D reversed these effects (Fig. 7C-7G). The above data demonstrate that CRNDE affects pancreatic cancer cell proliferation and angiogenesis through miR-451a up-regulation of CDKN2D.

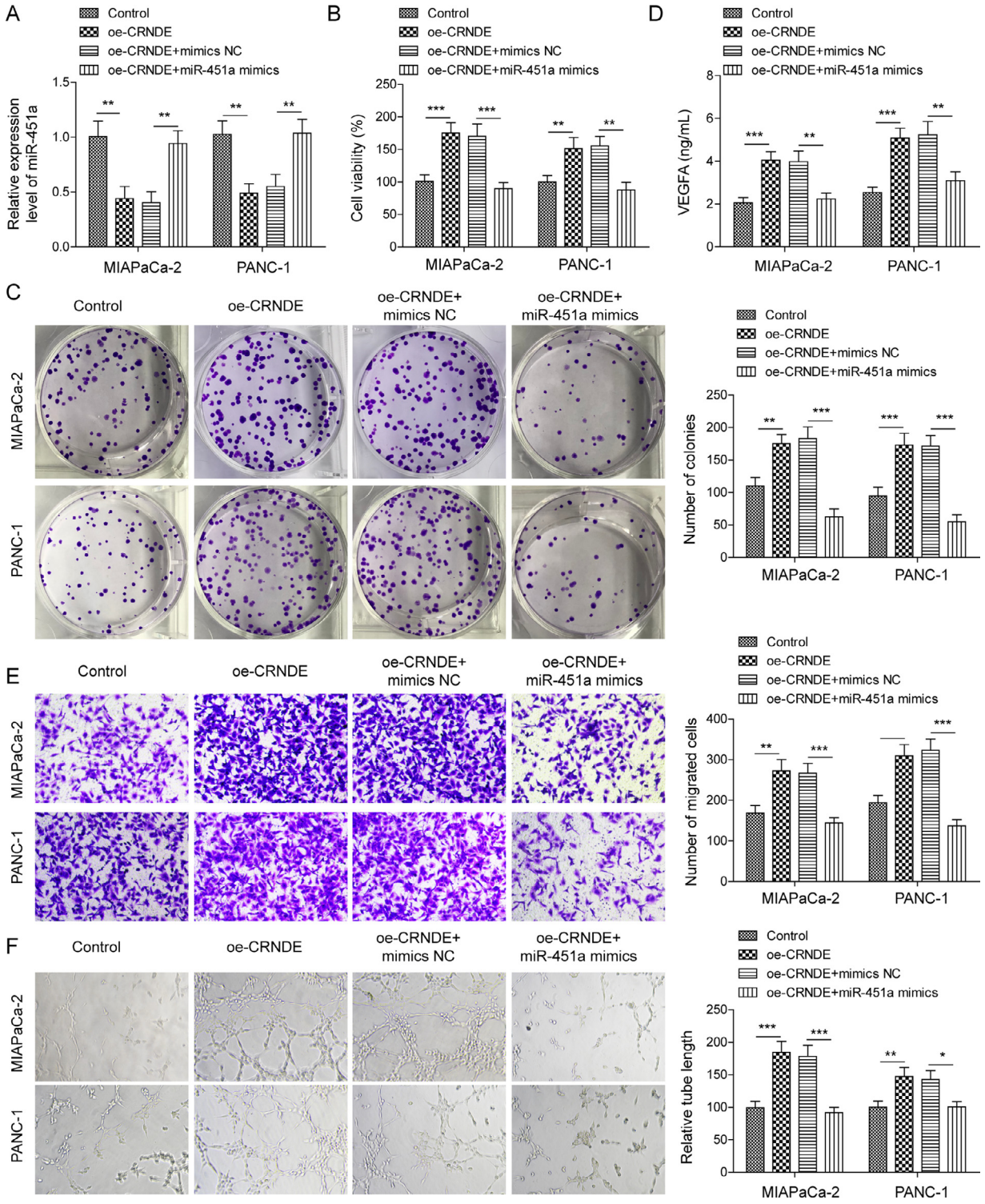

Fig. 4. CRNDE regulates pancreatic cancer cell growth and angiogenesis through miR-451a. MIAPaCa-2 and PANC-1 cells were transfected with pcDNA-CRNDE, miR-451a mimic and negative control. (A) MiR-451a expression in each group was measured by RT-qPCR. (B, C) The cell viability and proliferation in each group were determined by MTT and colony formation assay. (D) ELISA assay was used to detect the secretion of VEGFA in each group of cells. (E) Transwell assay was used to determine the cell migration ability in each group. (F) The tube formation ability of HUVECs was showed as the relative tube length in each group. $^{*}P < 0.05$,$^{**}P < 0.01$,$^{***}P < 0.001$. Data are the means ± SD from three independent experiments.

Discussion

Pancreatic cancer has been regarded as a highly malignant tumor with poor prognosis [16]. It is difficult to diagnose pancreatic cancer in the early stage and it is easily resistant to radiotherapy and chemotherapy [1]. Thus, it is necessary to fully clarify the molecular mechanisms related to the tumorigenesis of pancreatic cancer. Previous evidence suggests that lncRNAs may be involved in the growth and progression of pancreatic cancer [23]. Therefore, exploring the function roles and the underlying mechanisms of lncRNAs in pancreatic cancer

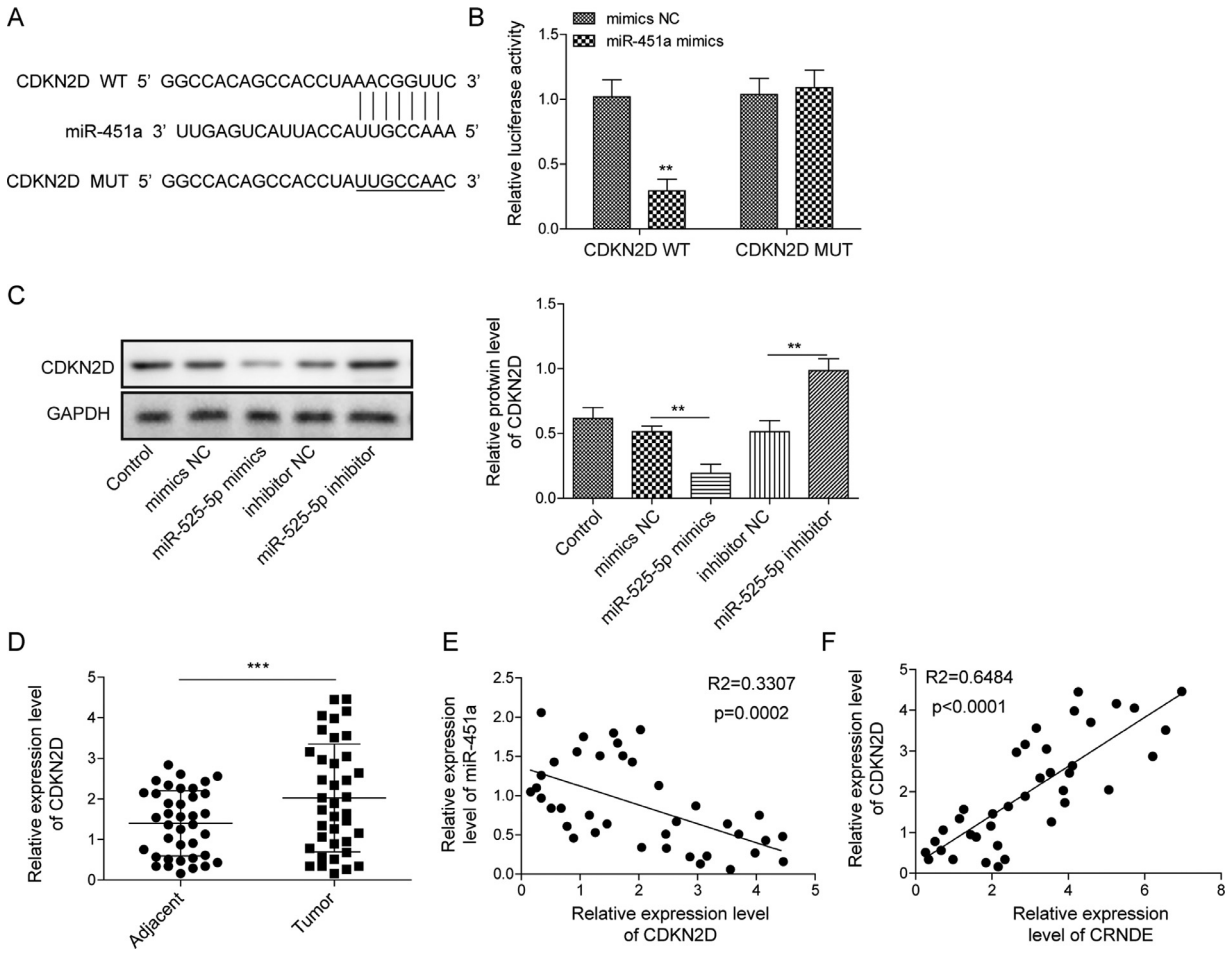

Fig. 5. MiR-451a directly binds to CDKN2D and regulates CDKN2D expression in pancreatic cancer cells. (A) The predicted binding site between miR-451a and CDKN2D by bioinformatics analysis. (B) The luciferase activity of CDKN2D-WT and CDKN2D-MUT in MIAPaCa-2 cells co-transfected with miR-451a mimic or miR-NC. (C) The protein level of CDKN2D in each group was tested by Western blot. (D) RT-qPCR was used to measure the expression of CDKN2D in pancreatic cancer and normal samples. Difference between two groups was measured using the paired t test. Pearson analysis showed the correlation between the expression of miR-451a and CDKN2D was negatively correlated in pancreatic cancer tissues (E), while the correlation between the expression of CRNDE and CDKN2D was negatively correlated (F). $^{*}P < 0.05$, $^{**}P < 0.01$,$^{***}P < 0.001$. Data are the means ± SD from three independent experiments.

progression will provide novel insights for diagnosis and treatment. In this study, we firstly demonstrated that lncRNA CRNDE overexpression could promote pancreatic cancer cell proliferation and angiogenesis via miR-451a/CDKN2D axis.

Many studies have demonstrated the wide involvement of lncRNA CRNDE in the proliferation and angiogenesis of cancers [4,29]. For example, the up-regulation of lncRNA CRNDE is associated with renal cell carcinoma progression [24]. Ji et al. demonstrated that lncRNA CRNDE overexpression accelerates hepatocellular carcinoma cell proliferation, invasion, and migration by sponging miR-203 [10]. Moreover, lncRNA CRNDE was found to promote the proliferation and metastasis of pancreatic cancer cells via miR-384/IRS1 pathway [28]. Consistent with these findings, we showed that lncRNA CRNDE expression was elevated in pancreatic cancer tissues and cell lines. Meanwhile, lncRNA CRNDE overexpression could promote the proliferation and angiogenesis of pancreatic cancer cells *in vitro* and *in vivo*. Therefore, lncRNA CRNDE might be a potential oncogene in pancreatic cancer.

Increasing evidences have reported that miR-451a can function as a tumor suppressor in various cancers. For instance, Xu et al. reported that miR-451a could inhibit the proliferation of colerectal cancer and increase its apoptosis [32]. In non-small cell lung cancer, miR-451a was considered as a noninvasive biomarker for early prediction of cancer recurrence and prognosis [11]. In our study, bioinformatics prediction and luciferase reporter assay identified that miR-451a was a target of CRNDE in pancreatic cancer cells. In accordance with the previous researches, we found that CRNDE overexpression inhibited the expression of miR-451a. In addition, miR-451a mediated the impacts of CRNDE in pancreatic cancer growth as well as angiogenesis.

Furthermore, we verified that miR-451a directly interacted with CDKN2D and found that miR-451a negatively regulated CDKN2D expression in pancreatic cancer. Similarly, increased expression of CDKN2D was observed in human lung cancer cells [39]. It was also reported that CDKN2D was correlated with high-grade serous ovarian carcinoma [12]. Functional testing further revealed that miR-451a could exert its tumor suppressive effect through CDKN2D in pancreatic cancer. In addition, our data demonstrated that miR-451a/CDKN2D mediated the effects of CRNDE on pancreatic cancer progression. Consistent with our results, Zang et al. reported that miR-451 could suppress the proliferation of esophageal carcinoma cells through targeting CDKN2D [33]. We report for the first time that CDKN2D regulates pancreatic cancer angiogenesis. In the present study, we found that CDKN2D promoted angiogenesis by promoting the secretion of VEGFA from pancreatic cancer cells. CDKN2D has been reported to function as a cell growth regulator that controls cell cycle G1 progression [21]. Thus, the mechanism of CDKN2D modulating the proliferation of pancreatic cancer may be related to its regulation of cell cycle. Further study should be performed to explore the specific molecular networks in this context.

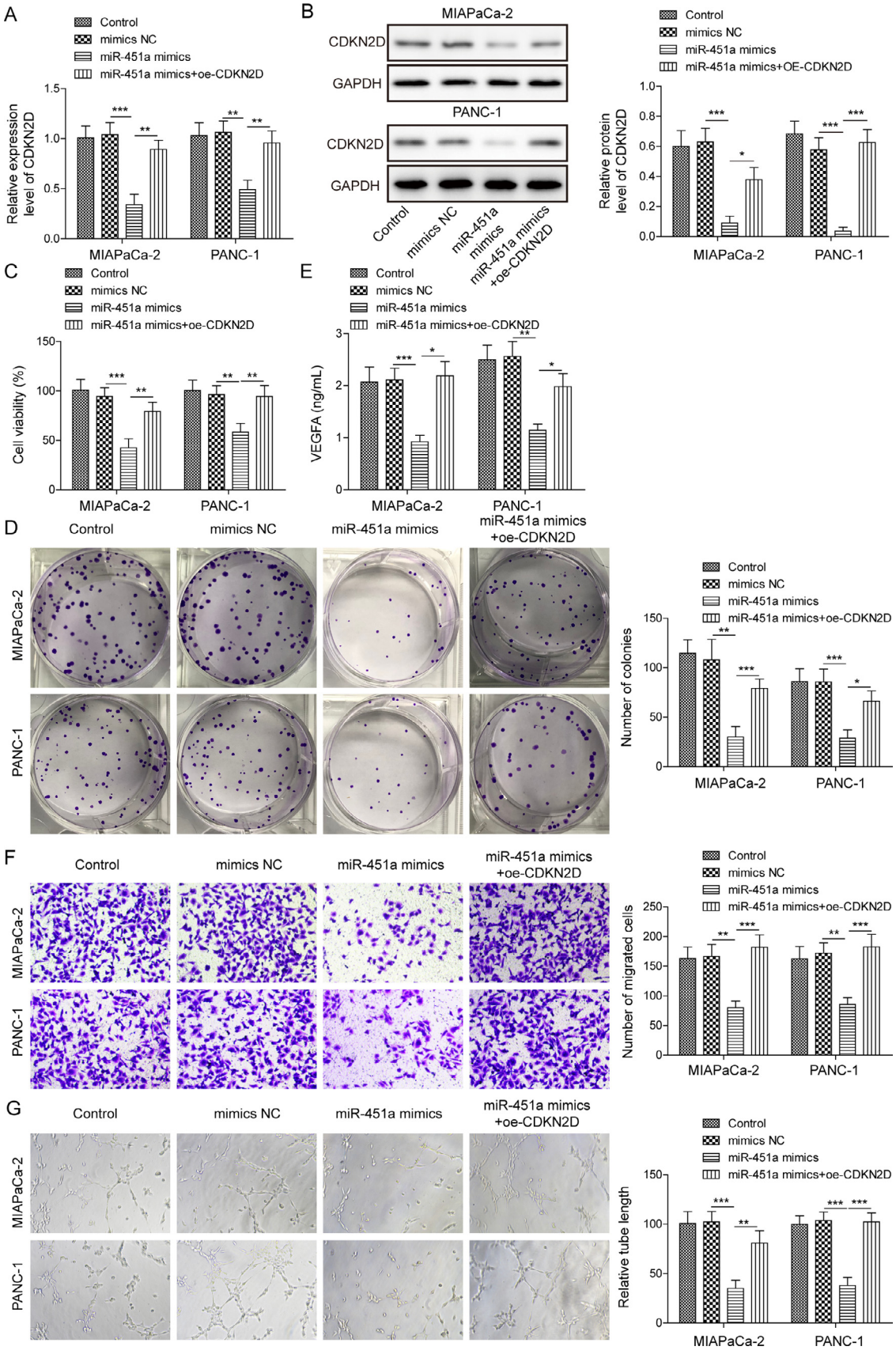

Fig. 6. MiR-451a mediates pancreatic cancer cell proliferation and angiogenesis via CDKN2D. MIAPaCa-2 and PANC-1 cells were transfected with pcDNA-CDKN2D, miR-451a mimic and negative control. (A, B) CDKN2D expression in each group was measured by RT-qPCR and Western blot. (C, D) The cell viability and proliferation in each group were determined by MTT and colony formation assay. (E) ELISA assay was used to detect the secretion of VEGFA in each group of cells. (F) Transwell assay was used to determine the cell migration ability in each group. (G) The tube formation ability of HUVECs was showed as the relative tube length in each group. $^{*}P < 0.05$,$^{**}P < 0.01$,$^{***}P < 0.001$. Data are the means ± SD from three independent experiments.

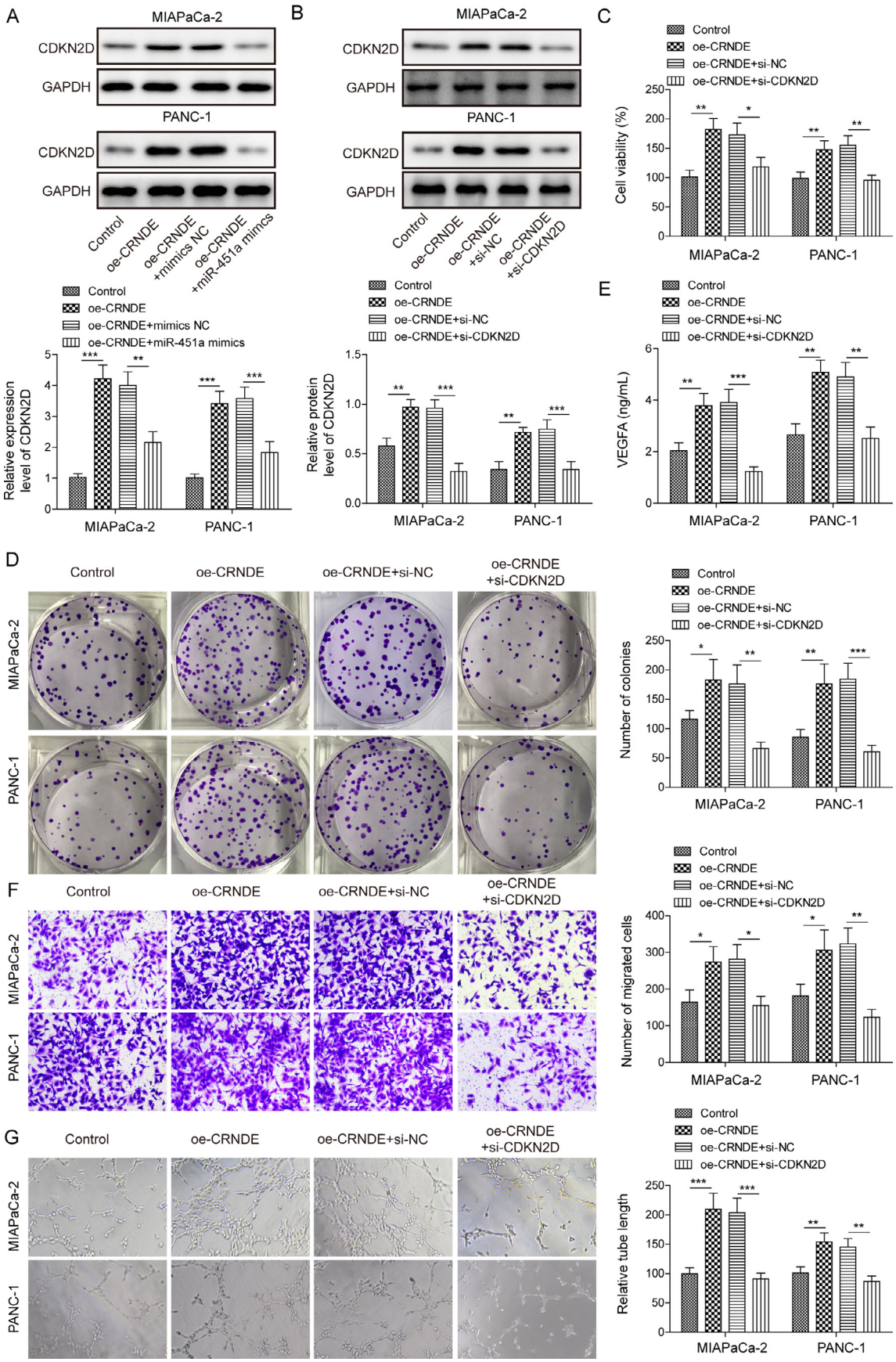

Fig. 7. CRNDE induces CDKN2D expression and affects pancreatic cancer cell proliferation and angiogenesis via miR-451a. (A, B) The protein level of CDKN2D in each group was tested by Western blot. (C, D) The cell viability and proliferation in each group were determined by MTT and colony formation assay. (E) ELISA assay was used to detect the secretion of VEGFA by cells in each group. (F) Transwell assay was used to determine the cell migration ability in each group. (G) The tube formation ability of HUVECs was showed as the relative tube length in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data are the means ± SD from three independent experiments.

In summary, it was discovered in this study that the expression lever of lncRNA CRNDE was significantly up-regulated in pancreatic cancer *in vitro* and *in vivo*. LncRNA CRNDE overexpression promoted the proliferation and angiogenesis of pancreatic cancer cells. Specifically, lncRNA CRNDE could positively regulate CDKN2D expression and promote pancreatic cancer progression through modulating miR-451a. Future researches are needed to identify the specific signaling pathways mediated by lncRNA CRNDE/miR-451a/CDKN2D in pancreatic cancer. Our study indicates that lncRNA CRNDE might become a novel biomarker and therapeutic target for pancreatic cancer treatment in the future.

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Ethics approval

The study procedures were approved by the Clinical Research Ethics Committee of the First Affiliated Hospital of Soochow University. All patients were informed of the study and signed the written consent.

All the animal experimental procedures were permitted by the Animal Ethics Committee of The First Affiliated Hospital of Soochow University.

Author contributions statement

Hong-Yan Zhu: design, concepts, Writing- Original draft preparation; data acquisition, data analysis; Yu-Jie Gao: experimental studies; Yong Wang: experimental studies; Chi Liang: data acquisition, data analysis; Yu Chen: Writing- Original draft preparation, Writing- Reviewing and Editing; Zi-Xiang Zhang: design, concepts, Supervision, Writing- Reviewing and Editing; All the authors approved for the final version.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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