

microRNA-26a Inhibits the Malignant Tumor Behavior of Pancreatic Cancer Cells via Targeting E2F7

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Research

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Abstract

Background: Dysregulation of microRNAs (miRNAs) plays important roles in the development of pancreatic cancer (PCa). miR-26a was reported as a tumor suppressor in multiple cancers. The aim of this study was to explore the potential role and molecular mechanism of miR-26a in the progression of PCa.

Methods: The expression of miR-26a in PCa tissues and cells was detected by RT-qPCR. The effects of miR-26a on the growth of PCa cells were determined by the cell counting kit-8 (CCK-8) assay, colony formation and flow cytometry. The targets of miR-26a were predicted by miRDB database and confirmed by luciferase report assay. The protein level was detected by western blot. CHIP assay was performed to detect the effects of miR-26 on the transcription of E2F7 targets.

Results: miR-26a was down-regulated in PCa tissues and cell lines. Ectopic expressed miR-26a suppressed the proliferation, colony formation and the tumor stem cell properties of PCa cells. The transcription factor E2F7 was identified as a target of miR-26a. MiR-26a bound the 3'-untranslated region (UTR) of E2F7 and inhibited the expression of E2F7 in PCa cells. Decreased miR-26a in PCa tissues was inversely correlated with that of E2F7. miR-26a transcriptionally reduced the expression of VEGFA via suppressing the binding of E2F7 with the promoter of VEGFA. Overexpression of E2F7 attenuated the suppressive role of miR-26a in the proliferation of PCa cells. Consistently, knockout of E2F7 further significantly inhibited the growth of PCa cells combined with miR-26a overexpression.

Conclusion: Our results uncovered the novel function of miR-26a/E2F7/VEGFA in the malignancy of PCa, suggesting miR-26a as a potential target for the treatment of patients with PCa.

Introduction

Pancreatic cancer (PCa) is one of the most fatal malignant tumors with extremely low 5-year survival rate(1-3). It has been considered as the seventh leading cause of cancer-related death worldwide. Currently, surgery still remains as the only potential curative treatment for patients with early pancreatic cancer(4-6). However, for those patients who are diagnosed at advanced stage, the prognosis remains unsatisfactory due to lacking of efficient treatment strategy and non-specific symptom for early diagnosis. Therefore, identification of novel targets that accurately detect PCa and the development of novel treatment options would be desirable.

MicroRNAs (miRNAs) are endogenous, small single-stranded non-coding RNA molecules that negatively regulate gene expression via suppressing the translation or promoting the mRNA degradation in a sequence-specific manner(7-9). MiRNAs play important roles in modulating the cell proliferation, differentiation and migration(10, 11). Currently, increasing evidence has indicated that miRNAs are aberrantly expressed in human cancers, and play a virtual role in the initiation and progression of malignancy(12-17). In pancreatic cancer, it has been found that miRNAs affect the development of PCa via modulating the expression of oncogenes or tumor suppressors(18-22). For example, miR-1225

inhibited the apoptosis of pancreatic cancer cells via targeting JAK1(23). MiR-374a inhibited the expression of SRC Kinase Signaling Inhibitor (SRCIN1), which promoted the proliferation and epithelial to mesenchymal transition of pancreatic cancer cells(24). Recent study also showed that miR-137 targeted KLF12 and suppressed the stemness features of pancreatic cancer cells(25). The tumor suppressive role of miR-26a in the progression of cancers has been demonstrated by a growing body of evidence(26-32). Decreased expression of miR-26a was observed in cancer tissues and correlated with the advanced progression of cancer patients. Overexpressed miR-26a suppressed the cell proliferation and sensitized cancer cells to anti-cancer drugs. It was reported that delivery of miR-26a was a possible novel strategy for inhibiting pancreatic cancer by restoring wild-type functions to mutant p53(33). However, the functional mechanism of miR-26a in PCa still remains largely unknown.

The aim of this study was to detect the expression of miR-26a in PCa and evaluate the possible mechanism involved in. Our results showed that miR-26a was significantly down-regulated in PCa tissues and cell lines. miR-26a overexpression inhibited the proliferation, colony formation and induced apoptosis of PCa cells. Furthermore, mechanism study suggested that the tumor suppressive function of miR-26a in PCa was achieved through targeting E2F7. These findings uncovered the novel involvement of miR-26a/E2F7 axis in the progression of PCa, which suggested miR-26a as a promising anti-cancer target for PCa.

Materials And Methods

Patient tissue samples and cell lines

A total of 50 paired pancreatic cancer tissues and adjacent normal tissue samples were obtained from patients that experienced surgery at the First Affiliated Hospital of Jinzhou Medical University from January 2012 to December 2015. Tissues were stored at liquid nitrogen before use. The research was approved by the Ethics Committee of The First Affiliated Hospital of Jinzhou Medical University. Informed consents were obtained from all the patients.

The human PCa lines AsPC-1, PANC-1, Sw1990 and BXPC-3 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS, Gibco, NY, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, NY, USA). Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

RNA preparation and RT-qPCR

Total RNA was extracted from tissues or cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The synthesis of cDNA was performed by reverse transcription using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. RT-qPCR was carried out using the TaqMan Universal PCR Master Mix Kit (Thermo Fisher Scientific, Waltham, MA, USA) with the ABI 7500 Fast Real-Time PCR System (Applied Biosystems). The thermocycling conditions were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec and

60°C for 30 sec. The expression of U6 RNA was detected as the control. The primers were designed as: miR-26a forward, 5'-GACTGTTCAAGTAATCCAGGATA; miR-26a reverse, 5'-GTGCAGGGTCCGAGGTATTC; U6 RNA forward, 5'-CTCGCTTCGGCAGCACA; U6 RNA reverse, 5'-AAACGCTTCACGAATTTG CGT. The relative level of miR-26a was calculated using the $2^{-\Delta\Delta CT}$ method.

Cell counting kit-8 (CCK-8) assay for cell proliferation

The proliferation of PCa cells was assessed by the CCK-8 assay. Cells (1,000 cells/well) transfected with miR-26a mimic or miR-NC were seeded into the 96-well plates and cultured for 1-, 2-, 3-, 4- and 5 day. CCK-8 solution (Beyotime, Shanghai, China) was added into the medium at the indicated time points and incubated at 37°C for 4 h. The absorbance at 450 nm was measured with the microplate reader (Bio-Rad Laboratories, Inc., USA).

Colony formation assay

PCa cells expressing miR-26a mimics or miR-NC were seeded into the 6-well plates with 500 cells per well. Cells were grown in DMEM containing FBS and maintained for 10 days. Cells were stained with 0.1% crystal violet (Beyotime, Shanghai, China) after fixation with methanol at room temperature (RT) for 10 min. The colonies were counted with the light microscopy.

Western blot

Protein samples were prepared using the RIPA lysis buffer containing protease inhibitor (Beyotime, Shanghai, China). Equal amount of proteins were loaded and separated by 15% SDS-PAGE, and then transferred onto the PVDF membrane. After blocking with 5% non-fat milk, the membrane was incubated with the primary antibodies against E2F7 (1:1000 dilution; cat. no ab56022, Abcam) or GAPDH (1:3000 dilution; cat.no ab181602, Abcam) overnight at 4°C. The membrane was then incubated with fluorescently labeled secondary antibodies and the bands were visualized using the Odyssey infrared scanner (Li-Cor Bioscience, Lincoln, NE, USA).

Dual-luciferase reporter assay

The wild-type (WT) or mutant (MT) 3'-UTR sequence of E2F7 was constructed into the pGL3 luciferase vectors (Promega, Madison, USA) and transfected into pancreatic cancer cells with miR-26a mimic or miR-NC. To detect the binding between E2F7 and VEGFA, the promoter sequence of VEGFA was inserted into the backbone of pGL-Basic (Promega, Madison, USA) vector. Cells were co-transfected with pGL-Basic-VEGFA and pcDNA-E2F7 or pcDNA-empty vector. After transfection for 48 h, cells were harvested and the luciferase activity was determined with the Dual-luciferase reporter assay kit (Promega, Madison, USA). The activity of *renilla* luciferase was also determined as the normalization. The experiment was performed in triplicates.

Chromatin Immunoprecipitation (ChIP) assay

The CHIP assay was performed with the protocol as previously described. Briefly, cells transfected with the indicated expression vectors were harvested after cultured for 48 h. Cells were cross-linked with 1% formaldehyde at RT for 10 min and then resuspended in lysis buffer containing protease inhibitor on ice for 10 min. Samples were then sonicated at 4°C at the condition of 20 kHz to generate chromatin fragments of 200-500 bp. DNA fragments were purified using the QIAquick PCR purification kit (Qiagen, USA). Recovered DNA was used for qPCR amplification using the SYBR master mix (Bio-Rad, USA) with the primers of VEGFA (forward, 5'-GCTGTTTGGGAGGTCAGAAATAGG and reverse, 5'-ACGCTGCTCGCTCCATTAC). Antibody against E2F7 (sc-H300, Santa Cruz Biotechnology, USA) and normal rabbit IgG was used as a negative control.

Statistical analysis

Data was presented as the mean \pm standard deviation. Data was analyzed with the SPSS 19.0 (IBM, Armonk, NY, USA). Difference was determined with the Student's *t* test or One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. $P < 0.05$ was considered as significant.

Results

MiR-26a level was decreased in PCa

To investigate the potential involvement of miR-26a in PCa, the expression of miR-26a was detected by RT-qPCR in 50-paired cancerous tissues and adjacent normal tissues. The result revealed that the level of miR-26a was significantly decreased in PCa tissues compared with that of the non-cancer tissues (Fig.1A). Additionally, the expression of miR-26a was also evaluated in PCa cells and the normal cell HPDE6-C7. As indicated in Fig.1B, lower level of miR-26a was observed in PCa cells than that of the normal cell. These results suggested the down-regulation of miR-26a in PCa.

Overexpression of miR-26a inhibited the malignant tumor behaviors of PCa cells

Since miR-26a was markedly down-regulated in PCa, we investigated the biological roles of miR-26a via gain-of-function experiments. Transfection of miR-26a mimics significantly increased the level of miR-26a in PCa cells (Fig.2A). The CCK-8 assay showed that overexpression of miR-26a significantly inhibited the proliferation of PANC-1 and AsPC-1 cells (Fig.2B and 2C). The colony formation assay also demonstrated that transfection of miR-26a resulted into reduced colony number of both PANC-1 and AsPC-1 cells (Fig.2D). Moreover, increased miR-26a expression obviously promoted the apoptosis of PCa cells (Fig.2E). Increasing evidence has showed that tumor stem cells play important roles in regulating the tumorigenesis(34, 35). To identify whether miR-26a affected the growth of PCa cells via modulating the stemness, the relative expressions of stem-cell like markers OCT4, CD133, Nanog and SOX2 were determined. The data showed that compared with the control cells, miR-26a overexpression reduced the expression of these four stem-cell markers in PCa cells (Fig. 2F), which suggested the inhibited stem cell property of PCa cells with overexpressed miR-26a. Collectively, these results indicated the tumor suppressive function of miR-26a in regulating the malignant behaviors of PCa.

E2F7 was a target of miR-26 in PCa

To further understand the molecular mechanism of miR-26a in PCa, the targets of miR-26a were predicted using the online prediction tool. It was found that the 3'-UTR of E2F7 carrying the putative binding sites of miR-26a (Fig.3A). To confirm the prediction, luciferase reporter assay was performed. The data showed that overexpression of miR-26a inhibited the luciferase activity of PCa cells expressing WT-3'-UTR of E2F7 (Fig.3B and 3C). However, the luciferase activity of vector expressing mutated (Mut) 3'-UTR of E2F7 was not significantly changed with the transfection of miR-26a (Fig.3B and 3C). This result suggested the specific binding between miR-26a with the 3'-UTR of E2F7. To detect whether the binding could affect the mRNA abundance of E2F7, RT-qPCR assay was performed to examine the mRNA level of E2F7 in PCa cells. As indicated in Fig.3D, compared with that of the control cells, overexpression of miR-26a significantly reduced the mRNA expression of E2F7. Consistently, the protein expression of E2F7 was also suppressed by overexpressing miR-26a in both PANC-1 and AsPC-1 cells (Fig.3E). The results demonstrated that miR-26a targeted E2F7 and down-regulated the expression of E2F7 in PCa.

E2F7 played a major role in the tumor suppressive function of miR-26a in PCa

To explore the role of E2F7 in miR-26a-mediated growth inhibition of PCa, the expression of E2F7 in PCa tissues and paired adjacent normal tissues was compared by RT-qPCR. The data indicated that the expression of E2F7 was significantly up-regulated in PCa tissues compared with that of the non-cancerous tissues (Fig.4A). Moreover, higher level of E2F7 was also found in PCa cells than the normal cell (Fig.4B). Since miR-26a was found to bind the 3'-UTR of E2F7 and decreased the expression of E2F7 in PCa cells, the correlation between the expression of miR-26a and E2F7 was evaluated by the Spearman test. As shown in Fig.4C, the down-regulated miR-26a in PCa tissues was inversely correlated with that of E2F7.

To further investigate the involvement of E2F7 in PCa, both PANC-1 and AsPC-1 cells were co-transfected with miR-26a mimics and E2F7 vector, the decreased expression of E2F7 induced by miR-26a was recovered by the ectopic E2F7 (Fig.4D). Interestingly, the suppressed proliferation of PCa cells by miR-26a was attenuated with the reintroduction of E2F7 (Fig.4E and 4F). To further investigate the critical function of E2F7, E2F7 was knockout by CRISPR and the confirmed by western blot (Fig.4G). Consistently, combined E2F7 knockout and miR-26a overexpression further inhibited the proliferation of PCa cells compared with E2F7^{-/-} cells (Fig.4H and 4I). These results suggested that miR-26a inhibited the malignant behaviors of PCa cells at least partially via targeting E2F7.

miR-26a transcriptionally reduced the expression of VEGFA via suppressing the binding of E2F7 with the promoter of VEGFA

As a transcription factor, the E2F7 can bind the promoters of target genes and regulate cancer progression. Recent study showed that E2F7 transcriptionally activated the expression of VEGFA, an important factor in angiogenesis(36). To further understand the possible mechanism by which E2F7 involved in PC, we performed CHIP assay and found that E2F7 bound the promoter region of VEGFA in PC

cells (Fig.5A and 5B). However, overexpression of miR-26a reduced the binding of E2F7 to the promoter region of VEGFA (Fig.5A and 5B). Moreover, to provide more evidence for the binding between E2F7 and VEGFA, we constructed the luciferase reporter vector by inserting the promoter sequence of VEGFA into the backbone of pGL3-Basic vector and transfected into the cells. As indicated in Fig. 5C, overexpression of E2F7 significantly increased the luciferase activity of VEGFA, however, co-transfection of miR-26a clearly attenuated the effects of E2F7.

To explore the consequence of transcriptionally inactivation of VEGFA by miR-26a, the mRNA levels of VEGFA in PC cells transfected with miR-26a mimics or E2F7 were detected. The result showed that overexpression of E2F7 increased the mRNA level of VEGFA, which co-transfection of miR-26a significantly abolished the effect of E2F7 (Fig. 5D). These results demonstrated that E2F7 acted as a transcriptionally activator of VEGFA in PC, which could be negatively regulated by miR-26a.

Discussion

PCa is one of the highly lethal malignancies with extremely low 5-year survival rate(37-40). Understanding the molecular mechanism that contributes to the progression of PCa is critical to design new biomarkers and therapeutic options. Notably, the association between miRNAs and the tumorigenesis of PCa has been a hot topic in the field of cancer research(41-45). Increasing amount of miRNAs were aberrantly expressed in PCa and correlated with the poor prognosis of patients with PCa. miR-26a has been reported to inhibited the proliferation, invasion and metastasis of papillary thyroid carcinoma(46). miR-26a suppressed the tumor metastasis of HCC by regulating the EMT process(47). Consistent with previous reports, the present study found that miR-26a was down-regulated in PCa tissues and cell lines. Ectopic expressed miR-26a suppressed the proliferation, colony formation, stemness features and promoted the apoptosis of PCa cells, suggesting the inhibitory effects of miR-26a on the growth of PCa cells. Consistent with the roles of miR-26a in other types of cancer, this study demonstrated the tumor suppressive function of miR-26a in PCa.

E2F7 is a member of the E2F transcription factor family. As an atypical E2F transcription factor, E2F7 functions as key regulator of cell cycle progression and its inactivation lead to spontaneous cancer formation(48). The deregulation of E2F7 has been found in a variety of human cancers. Low level of E2F7 predicted poor survival of patients with glioma and might constitute a potential therapeutic target for glioma(49). Interestingly, it was also reported that E2F7 was a tumor-promoting factor in breast cancer, which induced the cancer cell proliferation, invasion and metastasis(50). Therefore, the distinct oncogenic or tumor suppressive function of E2F7 in the progression of cancers relies on individual cancer types. In this study, our results demonstrated the overexpression of E2F7 in PCa tissues. Highly expressed E2F7 was inversely correlated with the level of miR-26a. Consistent with this observation, our finding also indicated that miR-26a bound the 3'-UTR of E2F7 and down-regulated the expression of E2F7 in PCa cells. These results suggested E2F7 as a direct target of miR-26a in PCa. Reintroduction of E2F7 significantly attenuated the inhibitory effects of miR-26a on the proliferation of PCa cells. Further mechanism study revealed that E2F7 bound the promoter of VEGFA and transcriptionally activated the

expression of VEGFA, which can be negatively regulated by miR-26a. This result provided the possible mechanism by which E2F7 involved in the progression of PC, even other transcriptional targets in addition to VEGFA might be also involved. Based on the aberrant expression of miR-26a and E2F7 in PCa, it would be interesting to evaluate the correlation between the levels of miR-26a/E2F7 with the clinical characteristics of patients with PCa. Importantly, there are different targets of miR-26a as reported so far, the function of other targets as well as how they work together with E2F7 in the development of PCa deserve further investigation.

In summary, our present study demonstrated that miR-26a was down-regulated in PCa tissues and cell lines. Overexpression of miR-26a inhibited the malignant behaviors of PCa cells via targeting E2F7. These findings suggested that miR-26a was a potential target for the treatment of PCa.

Conclusions

Our results uncovered the novel function of miR-26a/E2F7 in the malignancy of PCa, which suggested miR-26a as a potential target for the treatment of patients with PCa.

Declarations

Acknowledgements

Not applicable.

Author contribution

All authors contributed to data analysis, drafting the article and gave final approval of the version to be published.

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Availability of data and materials

The data presented in this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The First Affiliated Hospital of Jinzhou Medical University. Informed consents were obtained from all the patients.

Consent for publication

Not applicable.

Competing interests

These authors declare that they have no conflicts of interests.

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Figures

Figure 1

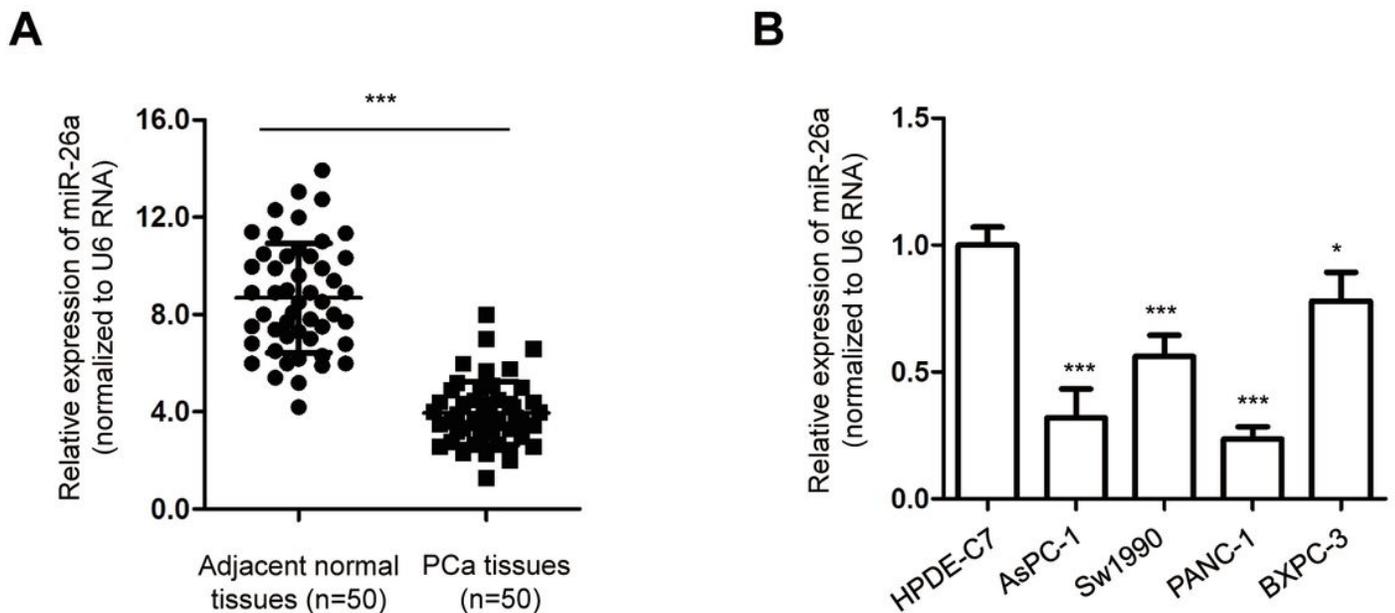


Figure 1

miR-26a expression was down-regulated in PCa tissues and cells. (A) RT-qPCR analysis of miR-26a expression in paired PCa tissues and adjacent normal tissues (n=50). (B) RT-qPCR detection of miR-26a levels in normal cell HPDE-C7 and PCa cell lines AsPC-1, Sw1990, PANC-1 and BXPC-3. *P<0.05; ***P<0.001.

Figure 2

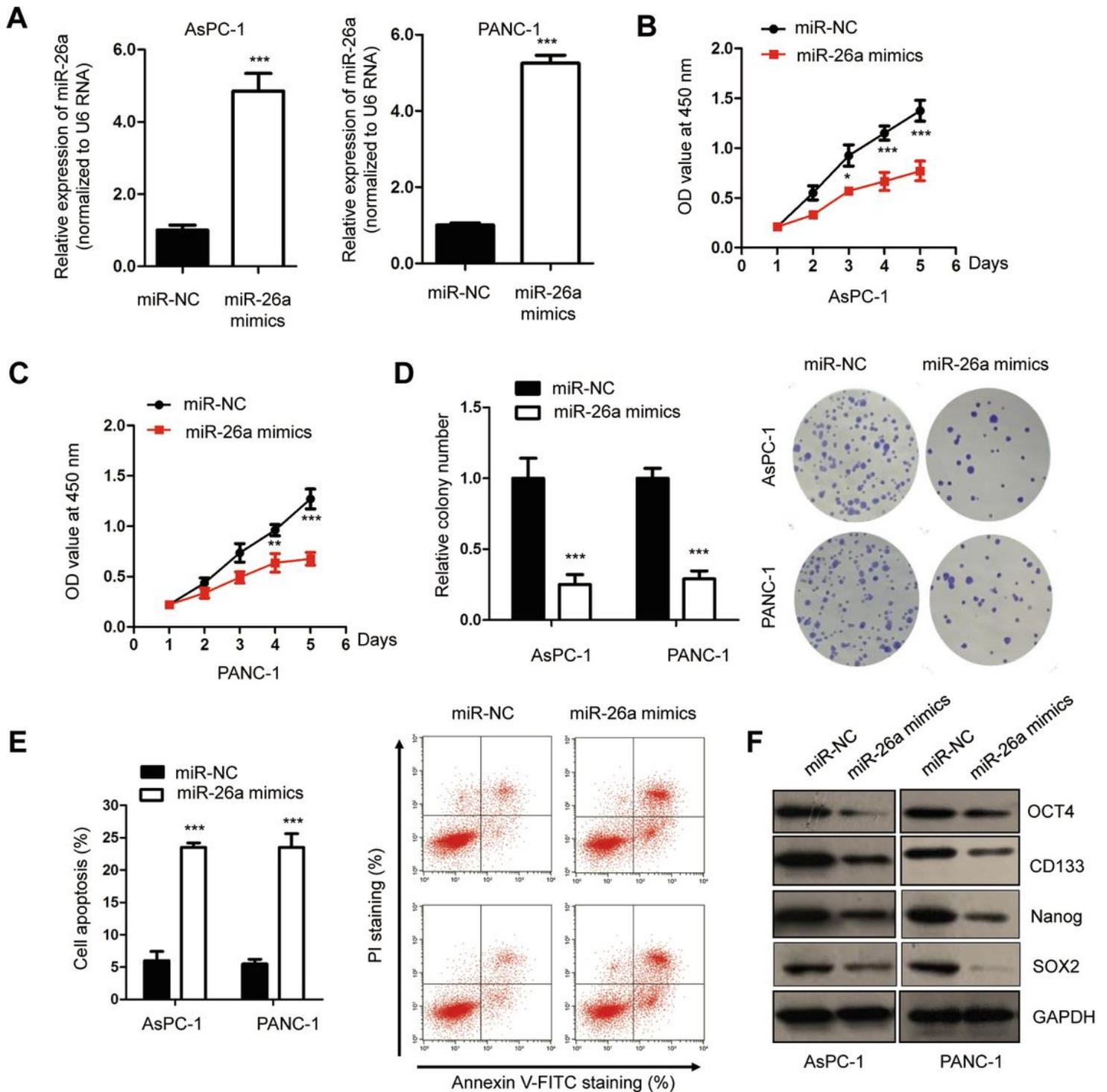


Figure 2

miR-26a overexpression suppressed the proliferative ability of Pca cells. (A) RT-qPCR analysis of miR-26a expression in AsPC-1 and PANC-1 cells following transfection of miR-26a mimics or miR-NC. (B, C) The proliferation of Pca cells with overexpression of miR-26a was significantly inhibited compared with the cells expressing miR-NC. (D) Transfection of miR-26a suppressed the colony number of Pca cells. (E) The apoptosis of both AsPC-1 and PANC-1 cells was notably increased with the overexpression of miR-26a.

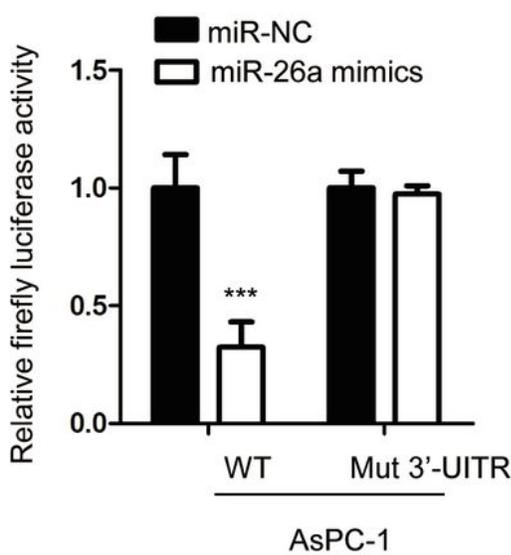
(F) The expression of stemness marker proteins was detected in PCa cells with the transfection of miR-26a mimics or miR-NC. *P<0.05; **P<0.01; ***P<0.001.

Figure 3

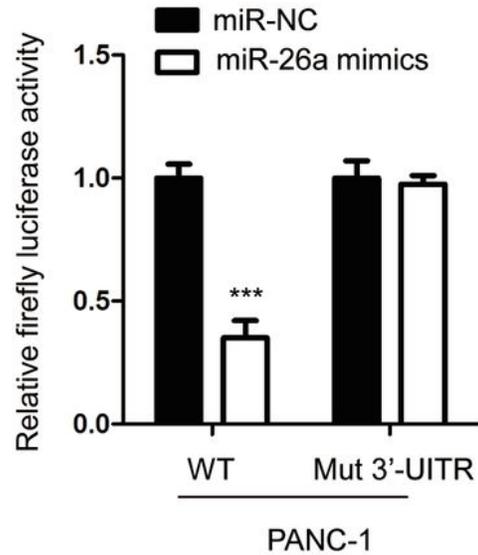
A



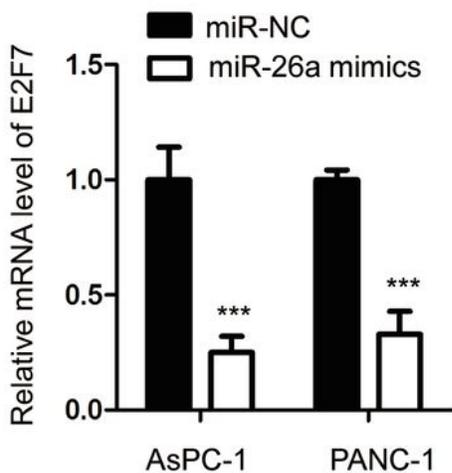
B



C



D



E

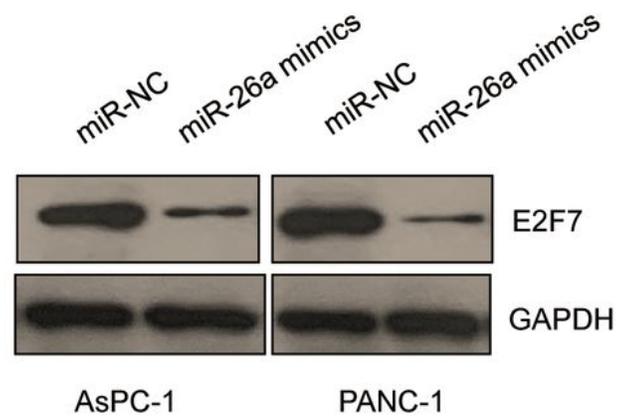


Figure 3

E2F7 was a target of miR-26a in PCa. (A) Predicted miR-26a-binding sites in the 3'-UTR of E2F7 mRNA. (B, C) Luciferase activity was analyzed in AsPC-1 and PANC-1 cells that were co-transfected with miR-26a mimics or miR-NC and either luciferase vector expressing WT or Mut 3'-UTR of E2F7. WT, wild-type; Mut,

mutant. (D, E) miR-26a or miR-NC was introduced into PCa cells. The mRNA and protein levels of E2F7 were measured by RT-qPCR and western blot analysis, respectively. ***P<0.001.

Figure 4

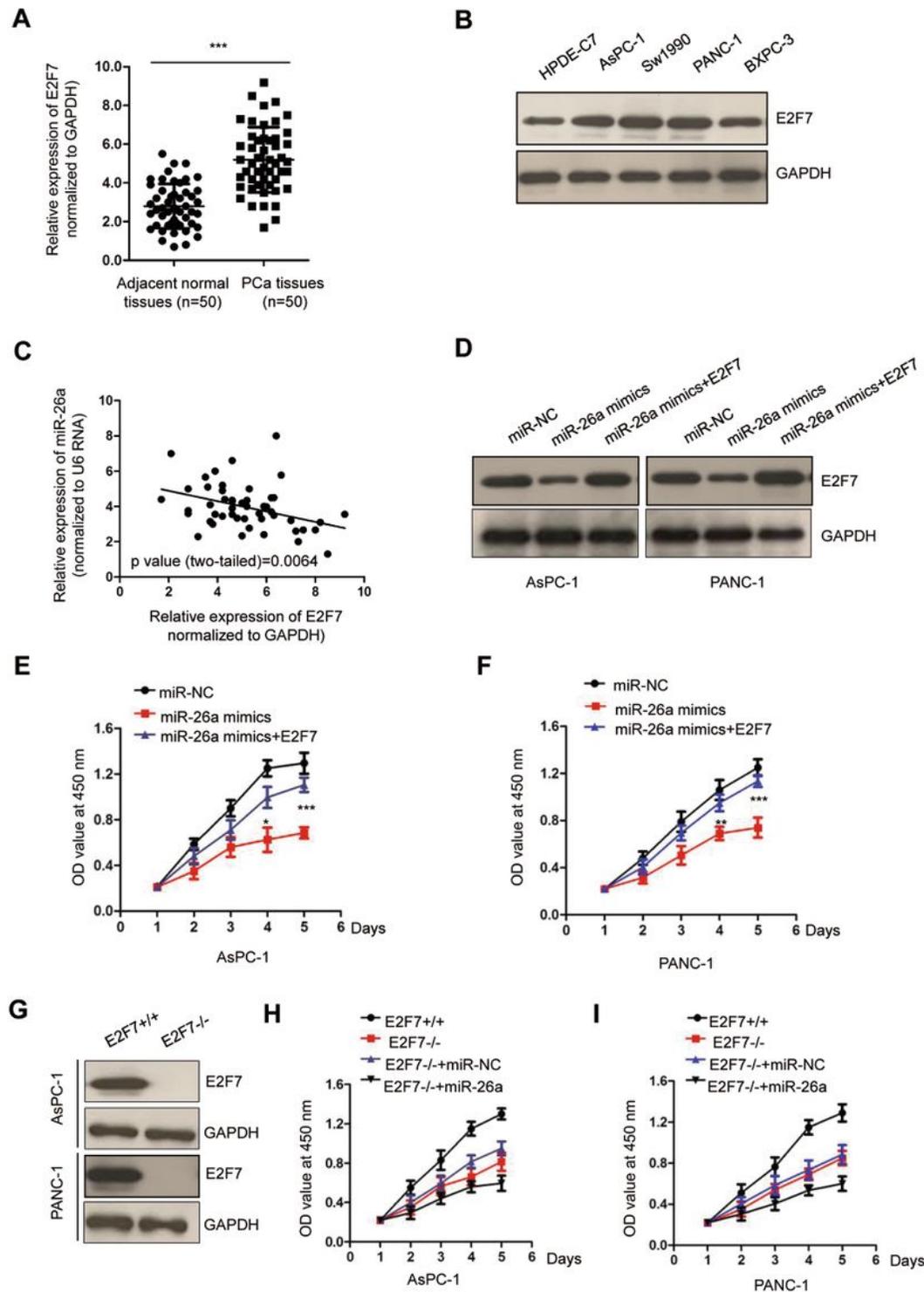


Figure 4

E2F7 was overexpressed in PCa and attenuated the suppressive role of miR-26a in PCa. (A) E2F7 mRNA levels in 50-paired PCa tissues and matched adjacent normal tissues were detected using RT-qPCR. (B) The protein levels of E2F7 in normal cell and PCa cell lines were compared by western blot. (C)

An inverse correlation between the expression levels of miR-26a and E2F7 in PCa tissues was identified by the Spearman correlation test. (D) Both AsPC-1 and PANC-1 cells were transfected with the indicated vectors and the expression of E2F7 was detected. (E, F) Reintroduction of E2F7 significantly reversed the suppressed proliferation induced by miR-26a. (G) The endogenous expression of E2F7 was knocked out by Caspir-cas9 and the silencing efficiency was confirmed via western blotting. (H, I) Silencing of E2F7 expression stimulated the tumor suppressive effects of miR-26a overexpression on the proliferation of PCa cells. *P<0.05; **P<0.01; ***P<0.001.

Figure 5

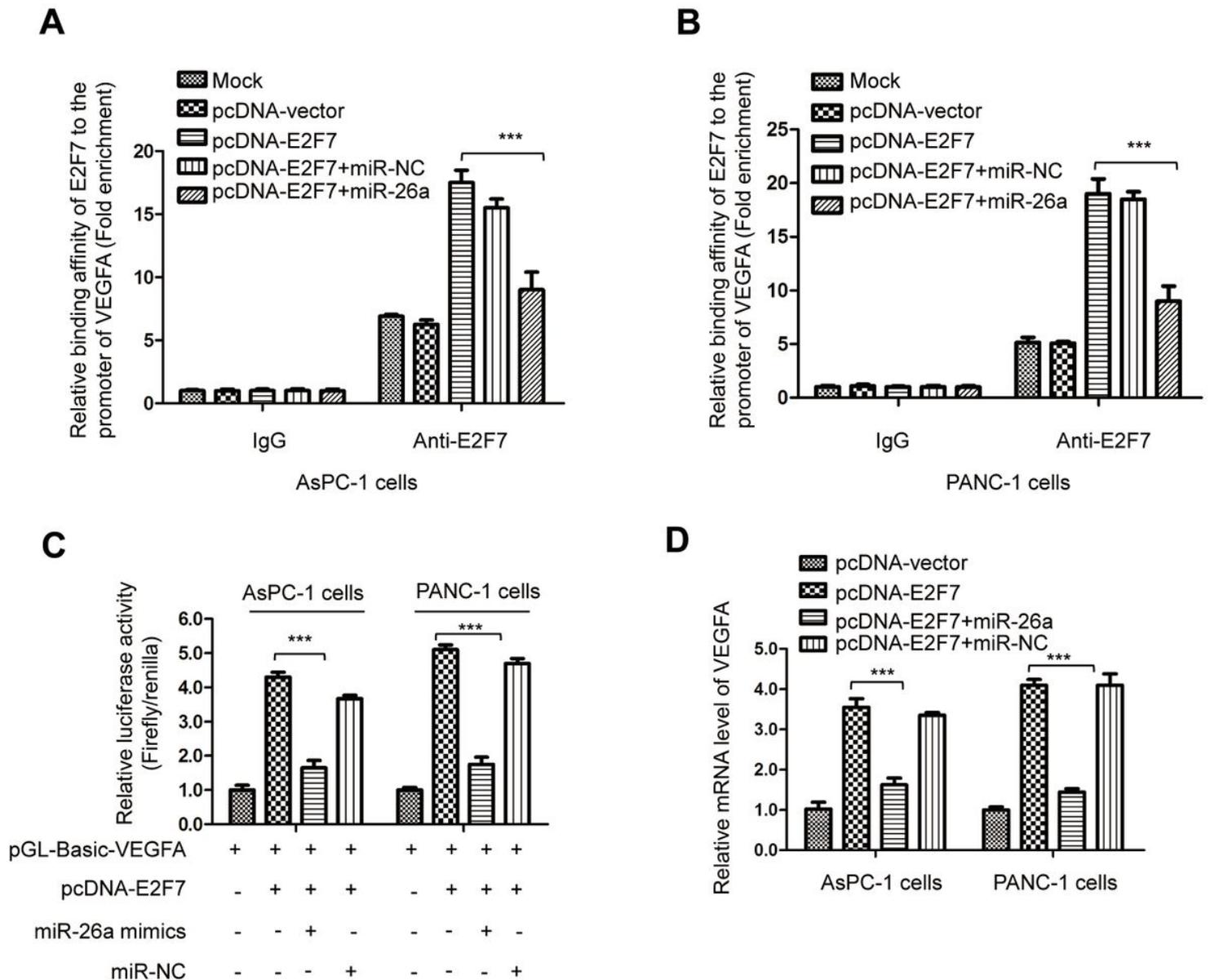


Figure 5

miR-26a negatively regulated E2F7-mediated transcriptional activation of VEGFA. (A, B) Cells were transfected with the indicated expression vector and the binding between E2F7 with the promoter of VEGFA was detected by CHIP. (C) Luciferase reporter assay was performed to evaluate the binding of

E2F7 with the promoter region of VEGFA. (D) PCa cells were transfected with the indicated expression vector and the mRNA levels of VEGFA was detected by RT-qPCR. ***P<0.001.