Am J Transl Res 2019;11(5):2983-2994
www.ajtr.org /ISSN:1943-8141/AJTR0090055

Original Article

miR-23a-5p inhibits cell proliferation and invasion in pancreatic ductal adenocarcinoma by suppressing ECM1 expression

Wenjie Huang[1,2], Yaohuan Huang[1], Jianyou Gu[1], Junfeng Zhang[2], Jiali Yang[2], Songsong Liu[2], Chuanming Xie[2], Yingfang Fan[1], Huaizhi Wang[2]

[1]Department of First Hepatobiliary Surgery, Zhujiang Hospital, Southern Medical University, Guangzhou 510280, Guangdong, China; [2]Institute of Hepatopancreatobiliary Surgery, Southwest Hospital, Army Medical University, Chongqing 400038, China

Received December 18, 2018; Accepted March 20, 2019; Epub May 15, 2019; Published May 30, 2019

Abstract: Pancreatic ductal adenocarcinoma (PDAC) is a genetic disease and a leading cause of cancer-related mortality. However, the molecular mechanism underlying PDAC progression remains unclear. In this study, we first confirmed that ECM1 is significantly upregulated in PDAC tissues and that its high levels of expression are closely associated with an advanced histologic grade and a poor prognosis using The Cancer Genome Atlas (TCGA) dataset and the Gene Expression Omnibus (GEO) database. We then found that miR-23a-5p binds directly to the ECM1 3'-untranslated region (3'-UTR), thereby inhibiting ECM1 expression. Functional studies revealed that the induced expression of ECM1 promoted oncogenic abilities and reversed the suppressive effects induced by miR-23a-5p. Collectively, our findings indicate that ECM1 is a proto-oncogene and show that targeting the miR-23a-5p/ECM1 axis may represent a promising therapeutic strategy for PDAC.

Keywords: miR-23a-5p, ECM1, pancreatic ductal adenocarcinoma (PDAC), proliferation, invasion

Introduction

Pancreatic ductal adenocarcinoma (PDAC) has received widespread attention among oncologists and researchers because of its highly lethal nature. In the past few decades, though the incidence of PDAC has increased, there have been no significant developments toward a cure for this disease [1, 2]. Pancreatic cancer is the fourth leading cause of cancer-related deaths and is expected to be the second deadliest malignancy by 2020 in the USA, paralleling epidemiological trends worldwide [3-5]. For patients with pancreatic cancer, surgery is considered the only curative treatment; however, only up to 20% of patients are eligible for surgical intervention [6]. Early metastasis is a key characteristic of pancreatic cancer, leading to tumor recurrence even after complete resection and resulting in a 5-year survival rate of less than 25% [7]. Promising therapeutic strategies and effective molecular targets are, therefore, urgently needed to improve treatment and increase the survival rate of patients with PDAC.

miRNAs are a class of single-stranded RNAs that are 19-25 nucleotides (nts) long, and they were initially discovered in 1993 [8, 9]. Since their discovery, a large number of miRNAs have been identified in plant and animal species, and more than 2042 mature miRNAs have been recorded in humans [10]. As important post-transcriptional regulators, miRNAs suppress target mRNA translation by binding to the 3'-untranslated region (3'-UTR) of target mRNAs [11]. Through this repressive effect on their target genes, miRNAs play important roles in multiple processes of cancer progression, including tumor growth, invasion, angiogenesis, and immune evasion [12, 13]. Previous studies have demonstrated the clinical value of miRNAs in disease classification and as drug efficacy predictors and non-invasive biomarkers, among

other uses [14]. However, the oncological roles of miRNAs in PDAC remain unclear and require further investigation.

Extracellular matrix protein 1 (ECM1) is a soluble protein that plays important roles in cell proliferation, invasion, angiogenesis, and differentiation [15]. ECM1 is overexpressed in malignant epithelial tumors, especially in those that preferentially undergo metastasis [16]. As metastasis is a hallmark of pancreatic cancer progression, ECM1 may also be an important regulator in this malignant disease. ECM1 has been reported to be aberrantly expressed and play a complex role in multiple types of cancers such as breast cancer, esophageal squamous cell carcinoma, and hepatocellular carcinoma (HCC) [17, 18]. Therefore, there is an urgent need to investigate the expression patterns of ECM1 in PDAC and the underlying functional mechanisms.

In this study, we initially found that ECM1 was aberrantly overexpressed in PDAC tissues and closely correlated with the overall survival of patients with PDAC. Functional investigations indicated that ECM1 enhanced PDAC proliferation and invasion in vitro. Bioinformatics analysis revealed that miR-23a-5p serves as an upstream regulator of ECM1. Mechanistic investigations found that overexpression of ECM1 abrogated the tumor-suppressing effects of miR-23a-5p. To the best of our knowledge, this is the first study to explore the expression patterns and oncological roles of miR-23a-5p and ECM1 in pancreatic cancer.

Materials and methods

Cell culture

The human PDAC cell lines AsPC-1 and CFPAC-1 were purchased from Procell Biotech (Wuhan, China). Dulbecco's modified Eagle's medium (DMEM) with 10% FBS (fetal bovine serum) was used to culture cell lines in a humidified 5% CO_2 atmosphere at 37°C.

Tissue specimens

In this study, 14 fresh-frozen PDAC tissue samples matched with adjacent non-cancerous tissues were used for RNA extraction and RT-qPCR, and another 107 formalin-fixed, paraffin-embedded PDAC tissues and 13 formalin-fixed, paraffin-embedded normal pancreatic tissues were selected to construct the tissue microarray (TMA). The tissues were obtained from the Institute of Hepatopancreatobiliary Surgery, Southwest Hospital, Army Medical University in Chongqing, China. The ethics committee of Southwest Hospital approved the use of the clinical specimens.

GEO and TCGA data collection and analysis

We searched the GEO database to identify genes exhibiting altered expression patterns between PDAC and adjacent non-cancerous tissues. In the GSE28735 dataset, in which the gene-expression profiles of 45 matched pairs of PDAC and adjacent non-tumor tissues from 45 patients with pancreatic ductal adenocarcinoma were examined using microarray analysis, we found that ECM1 was aberrantly upregulated. The TCGA database contains the ECM1 expression profiles for 176 PDAC cases, including detailed clinical information. We downloaded the above data for further analysis.

RNA extraction

TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) was used for RNA extraction according to the manufacturer's instructions; 1 ml of TRIzol™ was used per 30 mg of PDAC tissue or 10^6 PDAC cells. After extraction, RNA concentration was measured, and samples were stored at -80°C to prevent degradation.

Quantitative reverse transcription PCR (RT-qPCR)

Total RNA from tissues was used to assess miR-23a-5p and ECM1 expression levels. For ECM1, RT-qPCR analysis was performed using a PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) and SYBR® Premix Ex Taq™ II (Takara). β-actin was used as an endogenous control. For miR-23a-5p, a Mir-X™ miRNA First-Strand Synthesis Kit (Takara) and SYBR® Premix Ex Taq™ II (Takara) were used for RT-qPCR. RNU6-2 was used as an endogenous control. We used the $2^{-\Delta\Delta CT}$ method to calculate the relative expression levels of miR-23a-5p and ECM1. RiboBio Co. (Guangzhou, China) designed the Bulge-Loop™ miRNA RT-qPCR Primer Sets specific for miR-23a-5p. Sangon Biotech Co, Ltd (Shanghai, China) designed the ECM1 primer set, which

had the following sequences: 5'-GGACAGAGTCAAGTGCAGCC-3' (forward) and 5'-GTTCATTGGGGTGCTGGA GA-3' (reverse).

Cell transfection

miR-23a-5p mimics and NCs (negative controls) were purchased from RiboBio. ECM1-overexpressing plasmids and NC plasmids were purchased from GeneChem Co., Ltd. (Shanghai, China). When cells were cultured to 60-70% confluence, transfection was performed according to the Lipofectamine™ 3000 reagent protocol (Invitrogen).

Western blot assay

Western blot assays were performed as previously described [19]. Antibodies included those targeting E-cadherin (1:1000; Cell Signaling Technology, USA), Vimentin (1:1,000; Proteintech, USA), Cyclin D1 (1:1000; Cell Signaling Technology, USA), CDK4 (1:1000; Cell Signaling Technology, USA), ECM1 (1:500; Proteintech, USA) and β-actin (1:5,000; Proteintech, USA). Horseradishperoxidase-conjugated antibody was used as the secondary antibody (anti-mouse 1:5,000; Cell Signaling Technology, anti-rabbit; 1:5,000; Proteintech).

Transwell® assay

The invasion assay was performed using Matrigel™ Invasion Chambers (MilliporeSigma, Burlington, MA, USA) according to the manufacturer's instructions. After transfection for 24 h, 30,000 cells per well suspended in serum-free DMEM were seeded onto the upper chambers (inserts contained membranes with an 8 μm pore size), and DMEM with 10% FBS was added to the lower chambers. After 48 h, the cells that had migrated through to the bottom of the insert membrane were fixed with 4% paraformaldehyde and then stained with crystal violet.

Clone formation

After transfection, 500 cells were seeded in a six-well pate. After culturing for 2 weeks, the cells were fixed with 4% paraformaldehyde and then stained with crystal violet.

Luciferase reporter assay

The wild-type or mutant 3'-UTR sequences of ECM1 were inserted into the SI-Check2 vector downstream of the SV40 promoter, respectively. Mutations were generated in the binding sites. Then, 0.16 μg plasmid containing ECM1-3'-UTR/ECM1-3'-UTR mutant or 5 pmol hsa-miR-23a-5p/NCs (negative controls) was transfected into 293T cells. After 48 h, firefly luciferase (internal reference) and Renilla luciferase were measured using the Promega Dual-Luciferase system according to the manufacturer's protocol.

Cell cycle

For the cell cycle analysis, no fewer than 10^5 transfected cells were collected and fixed in 70% ethanol overnight at 4°C and stained with propidium iodide (Kaiji, Nanjing, China) in phosphate-buffered saline solution containing RNase. The fixed cells were analyzed using flow cytometry (Beckman FC500, Los Angeles, CA, USA).

Tissue microarray (TMA) and immunohistochemistry

A total of 107 PDAC tissues and 13 normal pancreatic tissues were selected to construct the TMA. Immunohistochemistry was performed following the manufacturer's instructions (Maixin, Fuzhou, China). Briefly, after antigen retrieval, TMA slides were incubated with ECM1 primary antibodies at 4°C overnight. Then, they were incubated with secondary antibodies and further incubated with the streptavidin-biotin complex (Maixin, Fuzhou, China). ECM1-positive cells were defined as those with immunoreactivity in both the cytoplasm and the nucleus. The staining intensity was scored as negative staining (score = 0), weak staining (score = 1), moderate staining (score = 2), or strong staining (score = 3). The staining extent was based on the percentage of positive cells (0, 0%; 1, < 10%; 2, 10-50%; 3, > 50%). Immunoreactivity was quantified by multiplying the staining extent score by the intensity score [20]. For statistical analyses, the tumor sample cohort was grouped into those with low expression (< 5) and high expression (≥ 5).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 software. Quantitative data are presented as the mean ± standard deviation (SD). A paired two tailed t-test or unpaired t-test was used for the quantitative data as appropriate. Kaplan-Meier analysis with a log-

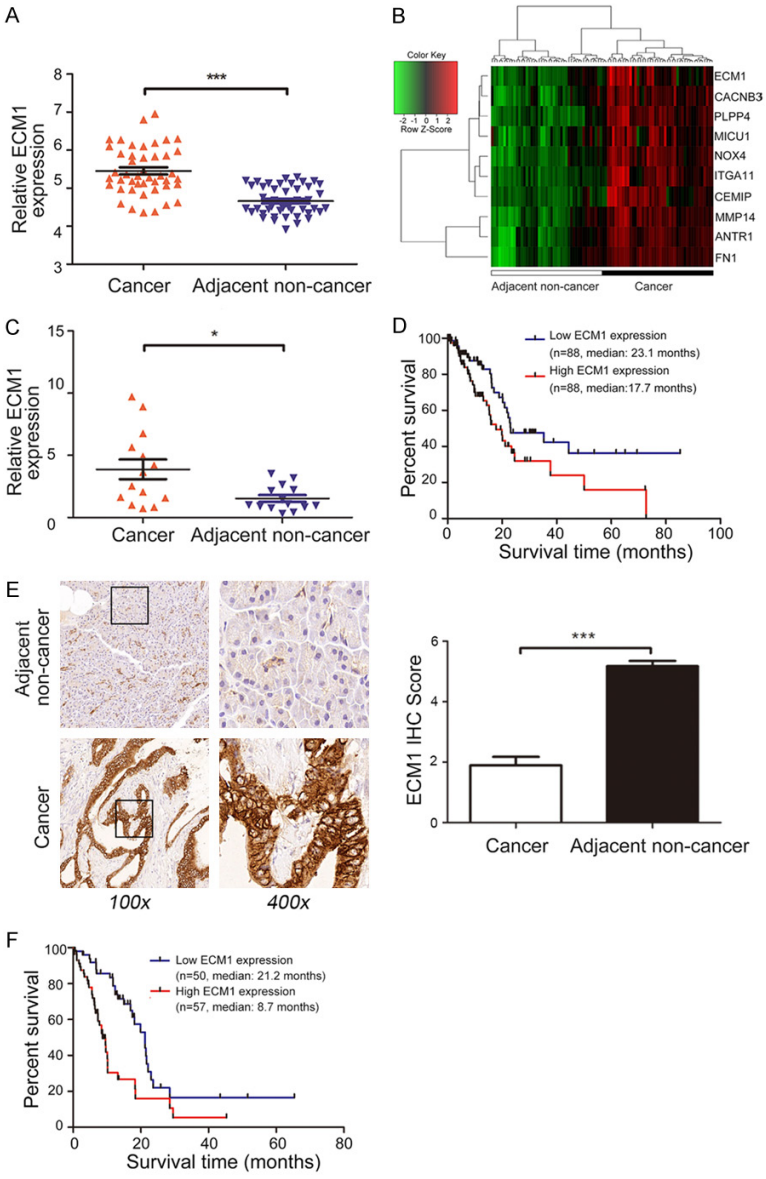

Figure 1. ECM1 is overexpressed in PDAC and ECM1 overexpression is associated with poorer overall survival of PDAC patients. A. GEO dataset (GSE28735) analyses revealed relative ECM1 expression in 45 matched PDAC tissues and adjacent non-tumor tissues by microarray analysis. Paired Student's t-test was applied, the data are represented as the mean ± SD. ***, $P < 0.001$. B. The cluster heat map showed the aberrantly upregulated genes in PDAC tissues with $P < 0.05$. Red color indicates high expression level and green color indicates low expression level. C. Relative expression of ECM1 was detected by RT-qPCR in 14 matched PDAC tissues and adjacent non-tumor tissues. β-actin was used as an endogenous control. Paired Student's t-test was applied, the data are represented as the mean ± SD. *, $P < 0.05$. D. Kaplan-Meier analysis revealed that high ECM1 expression was associated with reduced overall survival time of PDAC patients. The clinical data of 176 PDAC patients was downloaded from the TCGA database. E. IHC staining confirmed that ECM1 was upregulated in pancreatic tissues compared with normal pancreatic tissues. Unpaired Student's t-test was applied, the data are represented as the mean ± SD. ***, $P < 0.001$. F. The overall survival of PDAC patients was evaluated using Kaplan-Meier analysis according to high and low ECM1 expression. The log-rank test was applied, ***$P < 0.001$.

rank test was applied for survival analysis. Chi-square test were used for categorical variables. Differences were considered to be statistically significant when $P < 0.05$ (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Results

Clinical significance of ECM1 expression in PDAC

In order to identify potential oncogenes in PDAC, we searched the Gene Expression Omnibus (GEO) database for aberrantly upregulated genes. Based on the analysis results of the mRNA expression of 45 pairs of PDAC tissue samples and corresponding non-tumor tissues provided in the GSE-28735 dataset, we found that ECM1 was significantly upregulated in pancreatic cancer tissues (**Figure 1A**). The generated cluster heat map graphically illustrated that ECM1 was highly clustered in PDAC tissues (**Figure 1B**). We further explored ECM1 expression in 14 matched PDAC tissues using RT-qPCR. In accordance with the GEO results, elevated ECM1 expression was observed in PDAC tissues compared with that in the matched non-tumorous tissues (**Figure 1C**). Subsequently, ECM1 expression in PDAC was divided into two groups-a high group (n = 88) and low group (n = 88)-based on data derived from The Cancer Genome Atlas (TCGA). Kaplan-Meier survival curve analysis revealed that patients with high ECM1 expression had a lower survival rate than those with low ECM1 expression (**Figure 1D**). To further explore the clinical significance of ECM1 expression in PDAC, a tissue microarray containing 107 PDAC tissues and 13 normal pancreatic tissues

Table 1. Association of ECM1 expression and histologic grade in PDAC

	High group (n = 50)	Low group (n = 57)	
Age			$P = 0.570$
≤ 60	10 (20.0%)	9 (15.8%)	
> 60	40 (80.0%)	48 (84.2%)	
Gender			$P = 0.329$
Female	19 (38.0%)	27 (47.4%)	
Male	31 (62.0%)	30 (52.6%)	
Tumor size (cm)			$P < 0.001$
0-2	4 (8.0%)	27 (47.4%)	
≥ 2, < 5	31 (62.0%)	25 (43.9%)	
> 5	15 (30.0%)	5 (8.8%)	
Differentiation			$P = 0.876$
Grade 1	12 (24.0%)	14 (25.0%)	
Grade 2	37 (74.0%)	40 (71.4%)	
Grade 3	1 (2.0%)	2 (3.6%)	
Pathologic tumor status			$P = 0.016$
T1-T2	33 (66.0%)	47 (82.5%)	
T3-T4	17 (34.0%)	10 (17.5%)	
Clinical stage			$P = 0.002$
I-II	32 (64.0%)	48 (84.2%)	
III-IV	18 (36.0%)	9 (15.8%)	

Chi-squre test was used to test the association between two categorical variables. $P < 0.05$ was considered significant.

was examined by immunohistochemistry (IHC). ECM1 was significantly upregulated in PDAC tissues but seldom detected in normal pancreatic tissues (**Figure 1E**). Therefore, ECM1 expression in PDAC was divided into two groups: a high group (≥ 5, n = 50) and low group (< 5, n = 57). Kaplan-Meier survival analysis revealed that patients with high ECM1 expression had a shorter overall survival time than those with low ECM1 expression (log-rank test, $P < 0.001$, **Figure 1F**). Further analysis revealed that high ECM1 expression was signifcantly associated with tumor size ($P < 0.001$), pathologic features ($P = 0.016$), and advanced clinical stage ($P = 0.001$) (**Table 1**).

In summary, these results indicate that ECM1 is upregulated in PDAC tissues and that its high expression is closely related to PDAC progression and prognosis.

Ectopic overexpression of ECM1 promotes cell proliferation, cell cycle progression, and invasion ability

Because ECM1 is overexpressed in pancreatic cancer tissues, we produced its overexpression status in two pancreatic cell lines (AsPC-1, CFPAC-1) by transfecting them with ECM1-expressing plasmids. An empty vector (EV) was also transfected as a negative control. First, the CCK-8 assay results showed that overexpression of ECM1 promoted cancer cell proliferation (**Figure 2A**). Second, the clone formation assay results indicated that overexpression of ECM1 enhanced clone formation in PDAC cells (**Figure 2B**). Third, flow cytometry analysis revealed that overexpression of ECM1 led to accelerated cell cycle progression (**Figure 2C**). Finally, the Transwell® assay results indicated that overexpression of ECM1 induced an increase in cell invasion ability (**Figure 2D**). Moreover, ectopic ECM1 expression significantly increased the protein levels of cell cycle regulatory proteins (CDK6 and Cyclin D1) and a mesenchymal marker (Vimentin) and decreased the protein levels of an epithelial marker (E-cadherin) (**Figure 2E**). The whole membranes image was showed in Figure S1. Taken together, these results indicate that ECM1 may exert carcinogenic effects by promoting cancer cell proliferation and invasion and accelerating cell cycle progression in vitro.

ECM1 is negatively regulated by miR-23a-5p

It is well established that miRNAs play important roles in cancer progression by inhibiting their target genes. In this study, the miRNAs that interacted with ECM1 were examined. Using TargetScan (http://www.targetscan.org/), miR-23a-5p was selected as a candidate upstream miRNA of ECM1; its potential binding site is presented in **Figure 3A**. Western blot analysis further revealed that upregulated miR-23a-5p expression suppressed ECM1 expression (**Figure 3B**). The whole membranes image was showed in Figure S2. Next, a dual-luciferase reporter system was used to determine whether ECM1 was the direct binding target of miR-23a-5p. miR-23a-5p mimics, NCs, and plasmids containing the wild-type 3'-UTR and mutant 3'-UTR were each constructed, and the characteristics of these vectors are presented in **Figure 3C**. The results indicated that cotransfection of miR-23a-5p mimics and wild-type plasmids significantly decreased luciferase activity compared to that with the transfection of mutant plasmids (**Figure 3D**). These results

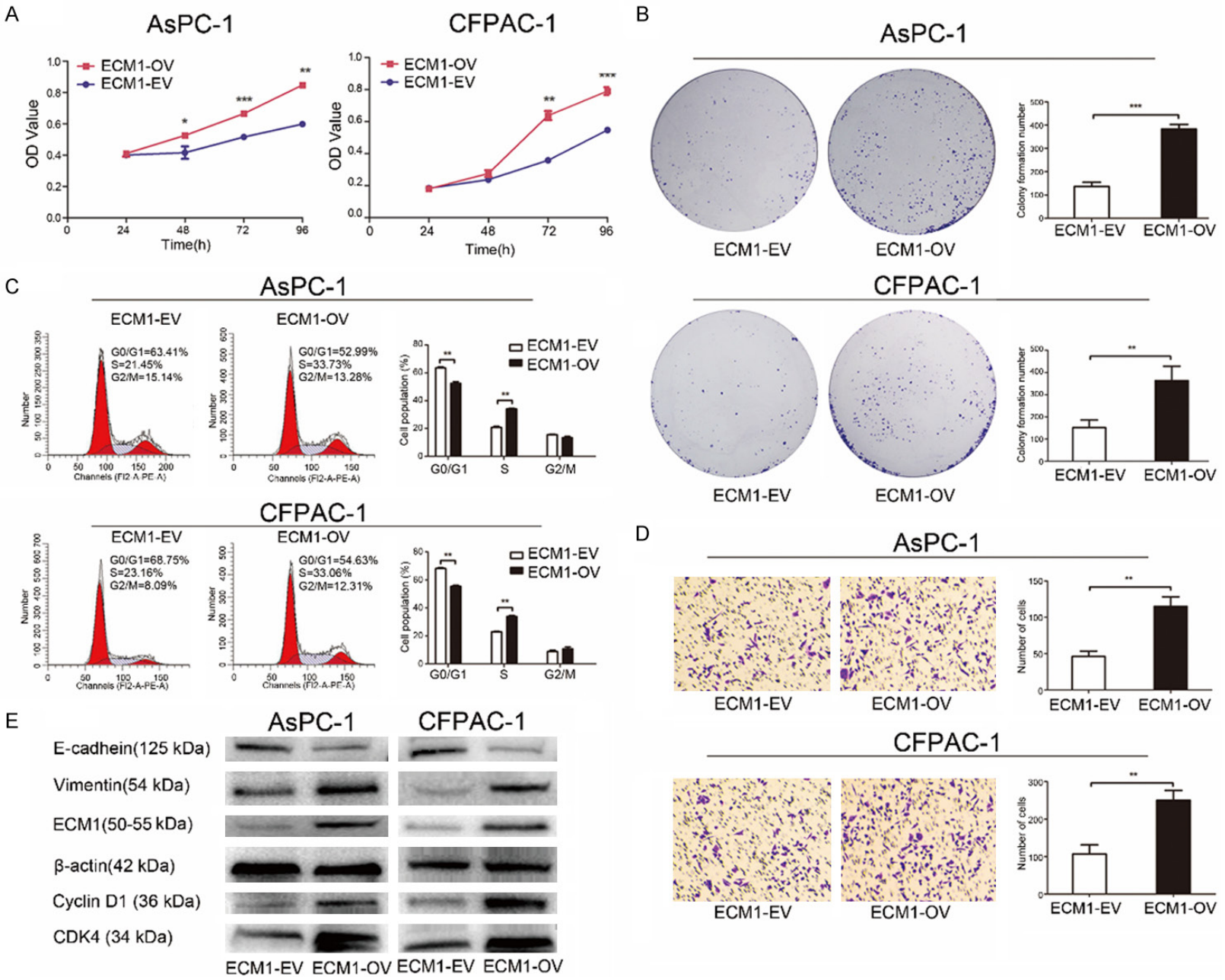
A
AsPC-1
CFPAC-1
ECM1-OV
ECM1-EV
OD Value
Time(h)
B
AsPC-1
CFPAC-1
ECM1-EV
ECM1-OV
Colony formation number
C
AsPC-1
CFPAC-1
ECM1-EV
ECM1-OV
G0/G1=63.41%
S=21.45%
G2/M=15.14%
G0/G1=52.99%
S=33.73%
G2/M=13.28%
G0/G1=68.75%
S=23.16%
G2/M=8.09%
G0/G1=54.63%
S=33.06%
G2/M=12.31%
Number
Channels (FI2-A-PE-A)
Cell population (%)
G0/G1
S
G2/M
D
AsPC-1
CFPAC-1
Number of cells
E
AsPC-1
CFPAC-1
E-cadhein(125 kDa)
Vimentin(54 kDa)
ECM1(50-55 kDa)
β-actin(42 kDa)
Cyclin D1 (36 kDa)
CDK4 (34 kDa)
ECM1-EV ECM1-OV ECM1-EV ECM1-OV

Figure 2. ECM1 overexpression promotes cell proliferation and invasion and accelerated cell cycle in vitro. ECM1 Overexpression vector (OV) was transfected to upregulate ECM1 and empty vector (EV) was transfected as Negative control. A. The effect of ECM1 on pancreatic cancer cell growth was evaluated via CCK-8 assay. B. The effect of ECM1 on colony formation ability of pancreatic cancer cells was evaluated. C. The effect of ECM1 on cell cycle was evaluated via flow cytometric analysis. D. The effect of ECM1 on pancreatic cancer cell invasion ability was measured via Transwell® assay. Data are presented as mean ± SD of three independent experiments; statistical significance was analyzed by unpaired t-test. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$. E. Western blot analysis was used to analyze the protein level of E-cadhein, Vimentin, ECM1, Cyclin D1 and CDK4.

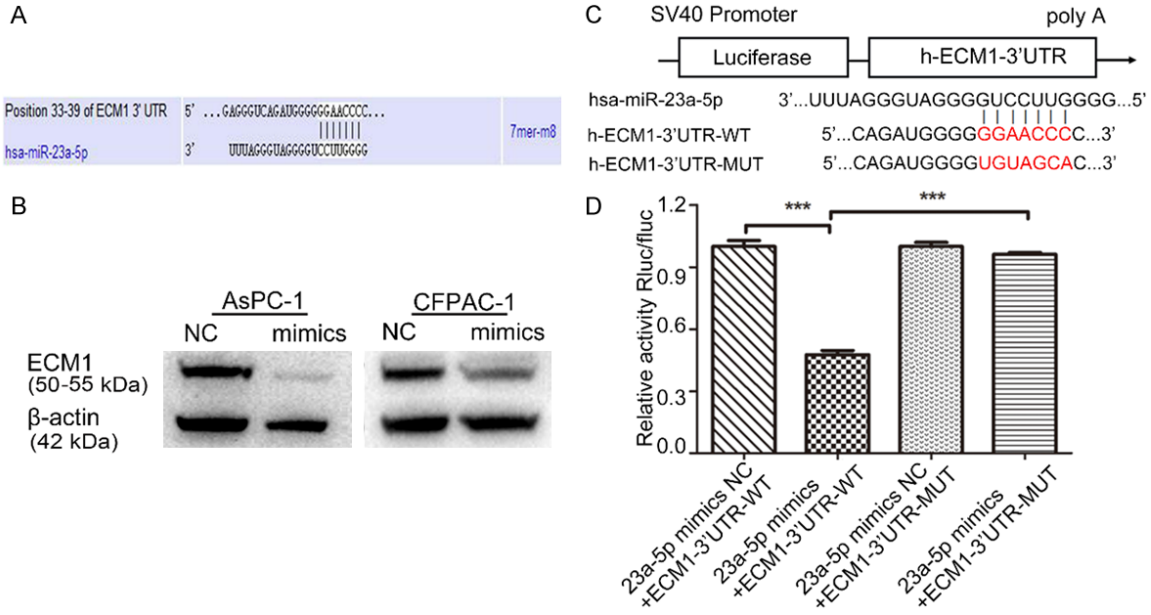

Figure 3. ECM1 is directly targeted and negatively regulated by miR-23a-5p. A. TargetScan identified the potential binding site between miR-23a-5p and the 3'-UTR of ECM1. B. Western blot analysis revealed that overexpression of miR-23a-5p by mimics inhibited ECM1 expression. C. For the dual-luciferase reporter assay, mutations were generated in the ECM1 3'-UTR sequence at the complementary sites for the seed regions in miR-23a-5p. D. Dual-luciferase reporter assay revealed that miR-23a-5p reduced the luciferase activity of the wild type ECM1 3'-UTR compared to the mutant type. The data are represented as the mean ± SD of three independent experiments. Student's t-test was used. ***, $P < 0.001$.

suggest that miR-23a-5p may suppress ECM1 expression by binding directly to its 3'-UTR.

miR-23a-5p is downregulated in pancreatic cancer tissues and associated with a poor prognosis

To clarify the expression level and significance of miR-23a-5p in pancreatic cancer, we first extracted RNA from 14 paired pancreatic cancer and adjacent non-cancer tissues. RT-qPCR results revealed that miR-23a-5p was significantly downregulated in pancreatic tissues (**Figure 4A**). Kaplan-Meier survival analysis 65 PDAC patients showed that patients with lower miR-23a-5p expression levels had significantly reduced overall survival rates (**Figure 4B**). To fully understand its oncological mechanism, Gene Ontology (GO) annotation was used to analyze the putative target genes of miR-23a-5p. The top ten GO terms for biological processes (BP) and significantly enriched pathways are shown in **Figure 4C** and **4D**, respectively.

In summary, miR-23a-5p may have profound influence on the progression of pancreatic cancer, and low miR-23a-5p expression levels may serve as a predictor of poor prognosis.

Overexpression of ECM1 abrogates the anticancer effect of miR-23a-5p in vitro

After clarifying the functional role of ECM1, we then determined whether miR-23a-5p exerts its antitumor effects by targeting ECM1. We constructed an ECM1 overexpression vector (OV)

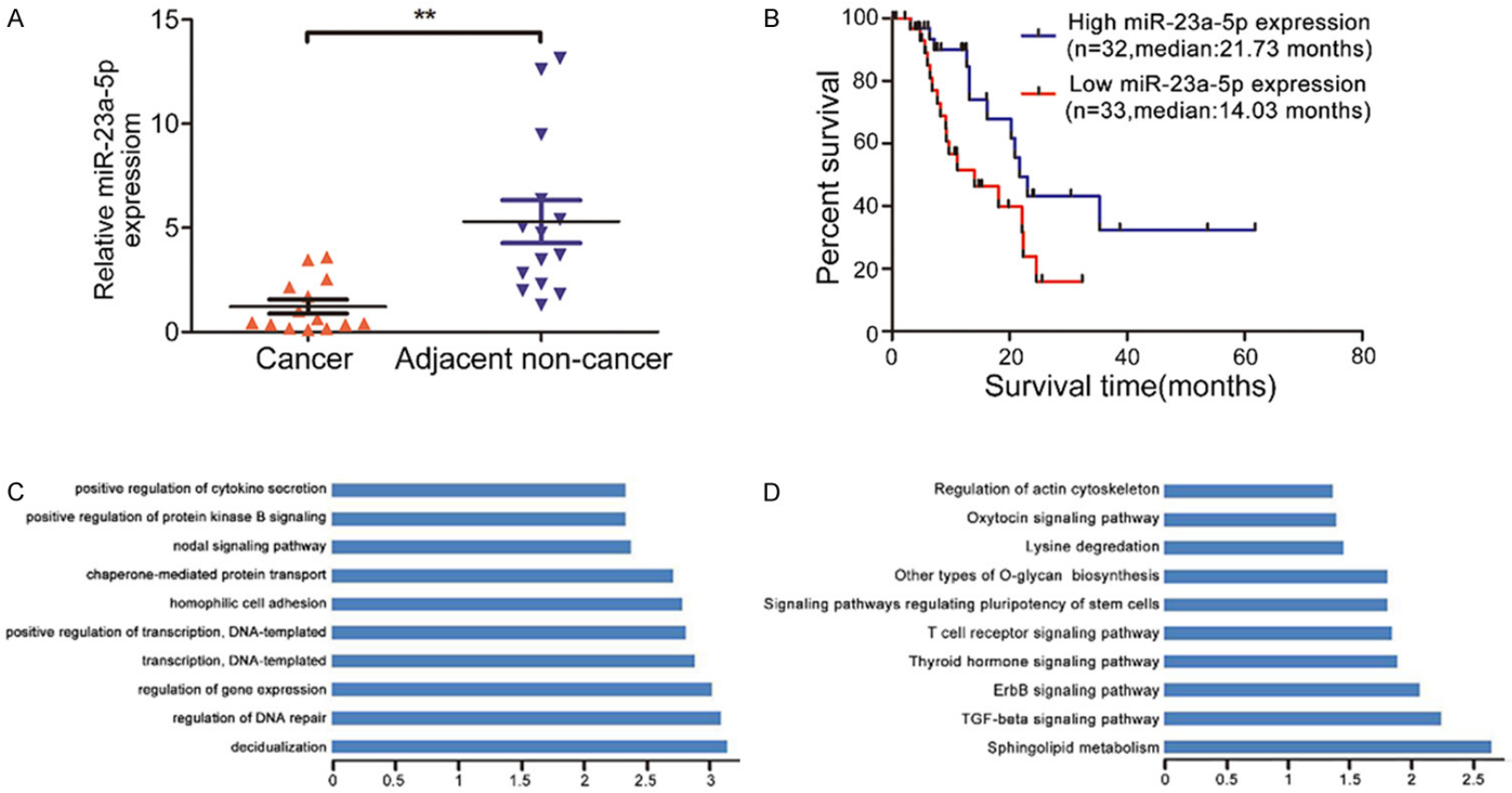

Figure 4. MiR-23a-5p is downregulated in PDAC tissues and closely associated with poorer overall survival of PDAC patients. A. The expression of miR-23a-5p was detected in 14 paired PDAC and adjacent noncancerous tissues. Data are presented as mean ± SD. Paired t-test was applied. ***, $P < 0.01$. B. Kaplan-Meier analysis showed that PDAC patients with low miR-23a-5p expression had lower overall survival rate. Log-rank test was applied, $P = 0.0203$. C. Biological process classification of target genes by GO annotation. D. Significantly enriched pathways of target genes.

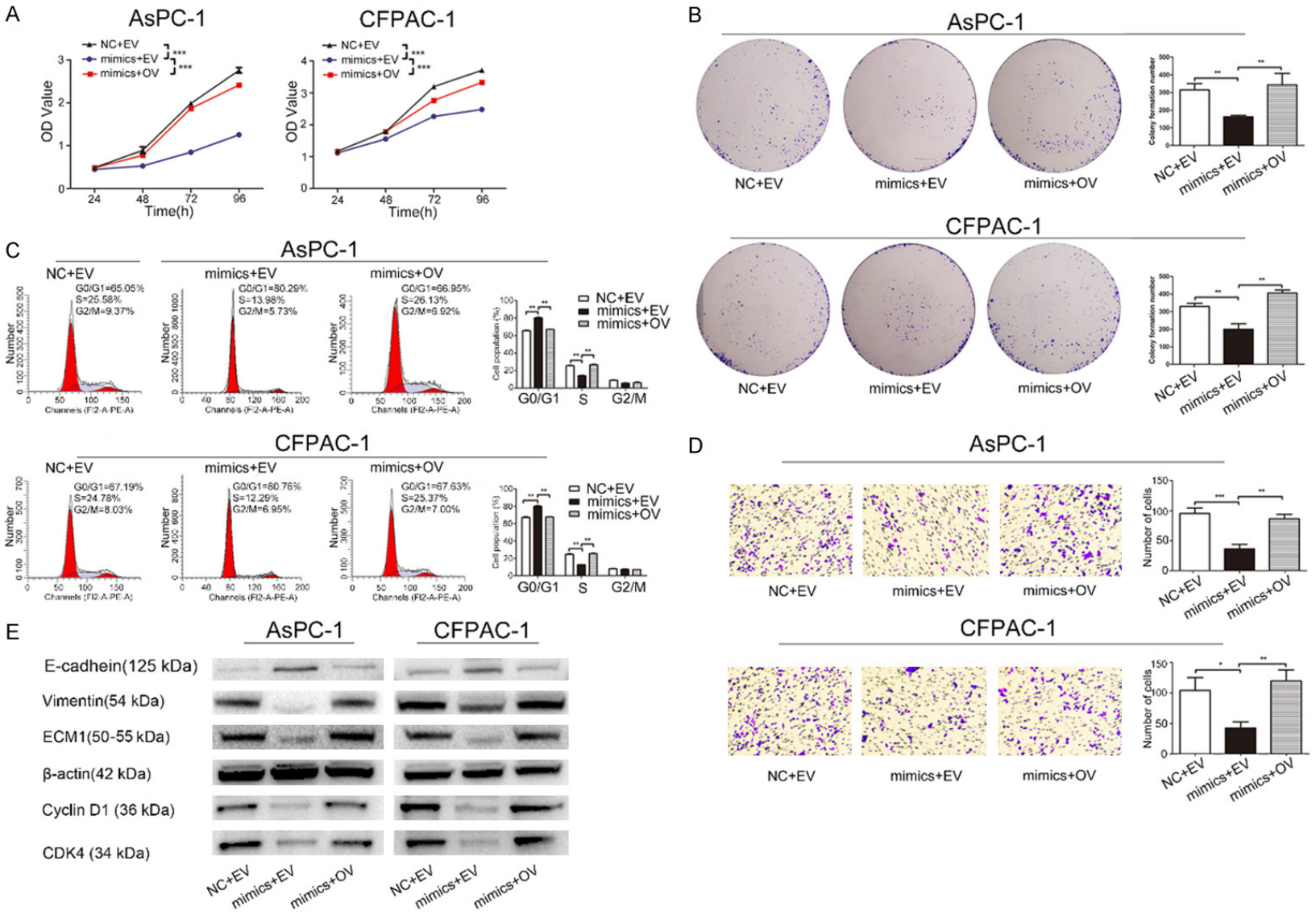
A
AsPC-1
CFPAC-1
NC+EV
mimics+EV
mimics+OV
OD Value
Time(h)
B
AsPC-1
CFPAC-1
NC+EV
mimics+EV
mimics+OV
Colony formation number
C
AsPC-1
CFPAC-1
NC+EV
mimics+EV
mimics+OV
G0/G1=65.05%
S=25.58%
G2/M=9.37%
G0/G1=80.29%
S=13.98%
G2/M=5.73%
G0/G1=66.95%
S=26.13%
G2/M=6.92%
G0/G1=67.19%
S=24.78%
G2/M=8.03%
G0/G1=80.76%
S=12.29%
G2/M=6.95%
G0/G1=67.63%
S=25.37%
G2/M=7.00%
Number
Channels (FI2-A-PE-A)
Cell population (%)
G0/G1
S
G2/M
D
AsPC-1
CFPAC-1
NC+EV
mimics+EV
mimics+OV
Number of cells
E
AsPC-1
CFPAC-1
E-cadhein(125 kDa)
Vimentin(54 kDa)
ECM1(50-55 kDa)
β-actin(42 kDa)
Cyclin D1 (36 kDa)
CDK4 (34 kDa)
NC+EV
mimics+EV
mimics+OV

Figure 5. Overexpression of miR-23a-5p abrogated ECM1 carcinogenic effects in vitro. In this section, miR-23a-5p mimics and ECM1 overexpression vector (mimics + OV) were transfected to investigate the rescue potential of ECM1. Cells transfected with ECM1 mimics NC and empty vector (NC + EV) were used as negative control. A. CCK-8 assay was applied to investigate proliferation ability; B. Colony formation assay was applied to investigate colony formation ability; C. Flow cytometric analysis was used to detect changes in cell cycle; D. Transwell® assay was applied to investigate invasion ability. All experiments were performed in triplicate in two pancreatic cancer cell lines, AsPC-1 and CFPAC-1. E. Western blot analysis was used to analyze the protein levels of E-cadhein, Vimentin, ECM1, Cyclin D1 and CDK4. Data are presented as mean ± SD of three independent experiments. Statistical significance was assessed by unpaired t-test. **, P < 0.05, **, P < 0.01, ***, P < 0.001*.

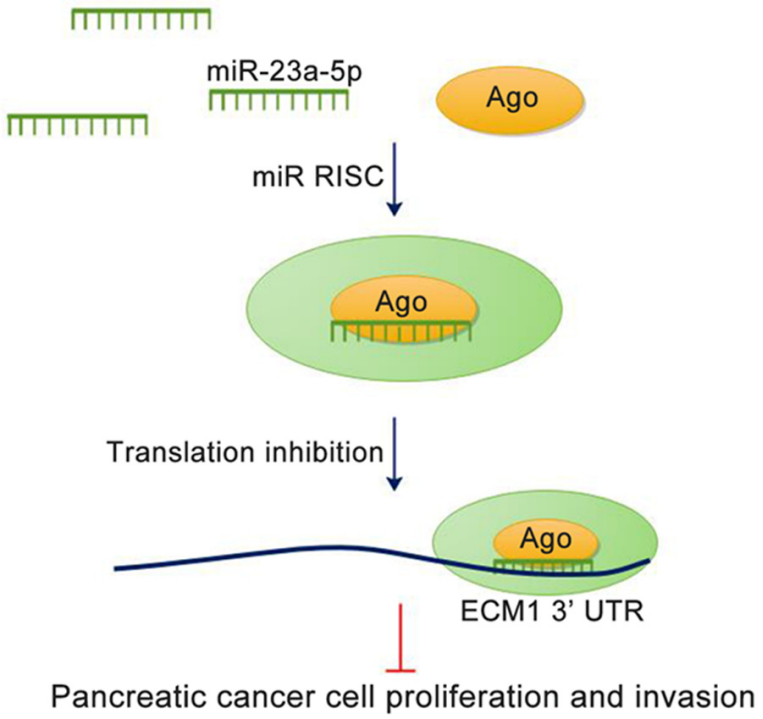

Figure 6. Proposed model for miR-142-3p inhibiting ECM1 expression in PDAC.

without the 3'-UTR, and cells were transfected with miR-23a-5p mimics. Then, we re-introduced ECM1 expression in miR-23a-5p-expressing cells to investigate the antagonizing effect of miR-23a-5p. Re-introduction of ECM1-OV and miR-23a-5p abrogated the effects of reduced cell proliferation (**Figure 5A**), colony formation ability (**Figure 5B**), cell cycle arrest (**Figure 5C**), and invasion ability (**Figure 5D**) induced by miR-23a-5p. In addition, enforced ECM1 overexpression reversed the impaired expression of ECM1, E-cadherin, CDK4, and Cyclin D1 and the enhanced expression of Vimentin, which were induced by miR-23a-5p (**Figure 5E**). The whole membranes image was showed in Figure S3.

Taken together, these results demonstrate that the miR-23a-5p/ECM1 axis plays important roles in PDAC progression. The graphical abstract was shown in **Figure 6**.

Discussion

Based on the associated research, it is widely recognized that pancreatic cancer is a genetic disease [21]. Many gene alterations have been shown to be responsible for PDAC initiation and evolution. In recent years, the indisputable oncogenic roles of ECM1 have attracted the attention of many researchers. For example, recent studies reported that overexpression of ECM1 promotes the metastasis of breast cancer, colon cancer, hepatocellular carcinoma, and laryngeal carcinoma [17, 22-24]. Research conducted by Lee KM *et al.* demonstrated that ECM1 regulates not only cell migration and invasion but also sphere-forming ability, drug resistance, progression of the epithelial to mesenchymal transition (EMT), and cancer stem cell (CSC) maintenance [25]. Other studies reported that ECM1 is negatively regulated by miR-486-3p at the post-transcriptional level, thereby inhibiting its oncogenic function [26]. ECM1 has also been found to regulate glial cell line-derived neurotrophic factor (GDNF)-mediated invasion and metastasis in pancreatic cancer cells [27]. Nevertheless, its expression patterns and biological roles in PDAC remain unclear and hence require further investigation. In our study, we found that ECM1 was upregulated in pancreatic cancer tissues compared with the levels in matched noncancerous tissues. Subsequent clinical analyses revealed that overexpression of ECM1 was closely associated with advanced histologic grade and a poor prognosis in

patients with PDAC. Biological assays further demonstrated that the induced expression of ECM1 promoted cell proliferation, cell cycle progression, and invasion ability.

The dysregulation of miRNAs has been shown to play essential roles in the development and progression of cancers, including pancreatic cancer, by impacting the ability of miRNAs to regulate their target genes involved in cellular processes such as inflammation, cell cycle regulation, stress response, differentiation, apoptosis, and invasion [28, 29]. Previous studies demonstrated that overexpression of microRNA-21 in pancreatic cancer significantly promoted cell proliferation, invasion, and chemoresistance of pancreatic cancer cells [30]. To date, several studies have investigated the effect of miR-23a-5p in cancers. Recent studies have shown that miR-23a-5p was upregulated in renal cell carcinoma (RCC) tissues, promoting the proliferation and invasion of RCC cells [31]. MiR-23a-5p is also involved in the andrographolide-induced inhibition of hepatoma tumor growth [32]. However, its role in pancreatic cancer has not been thoroughly studied. The results of our bioinformatics analysis showed that miR-23a-5p is an upstream miRNA of ECM1. We further found that miR-23a-5p was downregulated in pancreatic cancer tissues. Clinical analysis revealed that patients with low miR-23a-5p expression levels showed poor 5-year survival rates. Furthermore, we demonstrated that miR-23a-5p directly targeted the 3'-UTR of ECM1 and upregulated miR-23a-5p-suppressed ECM1 expression at the post-transcriptional level. Moreover, further functional studies confirmed that the induced expression of ECM1 abrogated the antitumor effect exerted by miR-23a-5p.

To the best of our knowledge, the present study is the first to illustrate the expression patterns and clinical significance of miR-23a-5p and ECM1 in patients with PDAC. Our results provide strong evidence that the absence of miR-23a-5p contributes to the aberrant overexpression of ECM1 to a certain degree. In conclusion, ECM1 and miR-23a-5p may serve as potential prognostic biomarkers as well as effective targets for PDAC therapy. Specifically, understanding the role of miR-23a-5p might aid in the development of a promising therapeutic strategy for PDAC via the suppression of ECM1 expression.

Acknowledgements

This work was supported by the National Key R&D Program of China (No. 2017YFC1308600) for Huaizhi Wang; The National Key Research and Development Program of China (NO. 2016YFC1201802) and The Science and Technology Plan Project of Guangdong Province (No. 2015A020214012 and 2016B090917001 and 2015B020214005) for Yingfang Fan.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yingfang Fan, Department of First Hepatobiliary Surgery, Zhujiang Hospital, Southern Medical University/The Second School of Clinical Medicine, Southern Medical University, Industrial Road No. 253, Guangzhou 510282, Guangdong, China. Tel: 86-20-62782567; E-mail: fanxifan@smu.edu.cn; Dr. Huaizhi Wang, Institute of Hepatopancreatobiliary Surgery, Southwest Hospital, Army Medical University, 30 Gaotanyan Main Street, Shapingba District, Chongqing 400038, China. Tel: 86-23-68765148; E-mail: whuaizhi@gmail.com

References

[1] Siegel RL, Miller KD and Jemal A. Cancer statistics. CA Cancer J Clin 2017; 67: 7-30.

[2] Vaccaro V, Sperduti I, Vari S, Bria E, Melisi D, Garufi C, Nuzzo C, Scarpa A, Tortora G, Cognetti F, Reni M, Milella M. Metastatic pancreatic cancer: is there a light at the end of the tunnel? World J Gastroenterol 2015; 21: 4788-4801.

[3] Malvezzi M, Bertuccio P, Levi F, La Vecchia C, Negri E. European cancer mortality predictions for the year 2014. Ann Oncol 2014; 25: 1650-1656.

[4] Garrido-Laguna I, Hidalgo M. Pancreatic cancer: from state-of-the-art treatments to promising novel therapies. Nat Rev Clin Oncol 2015; 12: 319-334.

[5] Lin QJ, Yang F, Jin C, Fu DL. Current status and progress of pancreatic cancer in China. World J Gastroenterol 2015; 21: 7988-8003.

[6] Gillen S, Schuster T, Meyer Zum Büschenfelde C, Friess H, Kleeff J. Preoperative/neoadjuvant therapy in pancreatic cancer: a systematic review and meta-analysis of response and resection percentages. PLoS Med 2010; 7: e1000267.

[7] Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics. 2014. CA Cancer J Clin 2014; 64: 9-29.

[8] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.
[9] Lee RC, Feinbaum RL and Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 1993; 75: 843-854.
[10] Kozomara A and Griffiths-Jones S. miRBase: integrating microRNA annotation and deep-sequencing data. Nucleic Acids Res 2011; 39: D152-D157.
[11] Krol J, Loedige I and Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet 2010; 11: 597-610.
[12] Kasinski AL and Slack FJ. Epigenetics and genetics. MicroRNAs en route to the clinic: progress in validating and targeting microRNAs for cancer therapy. Nat Rev Cancer 2011; 11: 849-864.
[13] Stahlhut C and Slack FJ. MicroRNAs and the cancer phenotype: profiling, signatures and clinical implications. Genome Med 2013; 5: 111.
[14] Hayes J, Peruzzi PP and Lawler S. MicroRNAs in cancer: biomarkers, functions and therapy. Trends Mol Med 2014; 20: 460-469.
[15] Han Z, Ni J, Smits P, Underhill CB, Xie B, Chen Y, Liu N, Tylzanowski P, Parmelee D, Feng P, Ding I, Gao F, Gentz R, Huylebroeck D, Merregaert J and Zhang L. Extracellular matrix protein 1 (ECM1) has angiogenic properties and is expressed by breast tumor cells. FASEB J 2001; 15: 988-994.
[16] Wang L, Yu J, Ni J, Xu XM, Wang J, Ning H, Pei XF, Chen J, Yang S, Underhill CB, Liu L, Liekens J, Merregaert J, Zhang L. Extracellular matrix protein 1 (ECM1) is over-expressed in malignant epithelial tumors. Cancer Lett 2003; 200: 57-67.
[17] Chen H, Jia W and Li J. ECM1 promotes migration and invasion of hepatocellular carcinoma by inducing epithelial-mesenchymal transition. World J Surg Oncol 2016; 14: 195.
[18] Sercu S, Zhang L and Merregaert J. The extracellular matrix protein 1: its molecular interaction and implication in tumor progression. Cancer Invest 2008; 26: 375-84.
[19] Zhang B, Zhang Z, Li L, Qin YR, Liu H, Jiang C, Zeng TT, Li MQ, Xie D, Li Y, Guan XY, Zhu YH. TSPAN15 interacts with BTRC to promote oesophageal squamous cell carcinoma metastasis via activating NF-κB signaling. Nat Commun 2018; 9: 1423.
[20] Li J, Wu H, Li W, Yin L, Guo S, Xu X, Ouyang Y, Zhao Z, Liu S, Tian Y, Tian Z, Ju J, Ni B, Wang H. Downregulated miR-506 expression facilitates pancreatic cancer progression and chemoresistance via SPHK1/Akt/NF-kappaB signaling. Oncogene 2016; 35: 5501-5514.
[21] Kamisawa T, Wood LD, Itoi T and Takaori K. Pancreatic cancer. Lancet 2016; 388: 73-85.
[22] Gómez-Contreras P, Ramiro-Díaz JM, Sierra A, Stipp C, Domann FE, Weigel RJ, Lal G. Extracellular matrix 1 (ECM1) regulates the actin cytoskeletal architecture of aggressive breast cancer cells in part via S100A4 and Rho-family GTPases. Clin Exp Metastasis 2017; 34: 37-49.
[23] Santasusagna S, Moreno I, Navarro A, Castellano JJ, Martinez F, Hernández R, Muñoz C, Monzo M. Proteomic analysis of liquid biopsy from tumor-draining vein indicates that high expression of exosomal ECM1 is associated with relapse in stage I-III colon cancer. Transl Oncol 2018; 11: 715-721.
[24] Gu M, Guan J, Zhao L, Ni K, Li X, Han Z. Correlation of ECM1 expression level with the pathogenesis and metastasis of laryngeal carcinoma. Int J Clin Exp Pathol 2013; 6: 1132-1137.
[25] Lee KM, Nam K, Oh S, Lim J, Kim RK, Shim D, Choi JH, Lee SJ, Yu JH, Lee JW, Ahn SH, Shin I. ECM1 regulates tumor metastasis and CSC-like property through stabilization of beta-catenin. Oncogene 2015; 34: 6055-6065.
[26] Ye H, Yu X, Xia J, Tang X, Tang L, Chen F. MiR-486-3p targeting ECM1 represses cell proliferation and metastasis in cervical cancer. Biomed Pharmacother 2016; 80: 109-114.
[27] Funahashi H, Okada Y, Sawai H, Takahashi H, Matsuo Y, Takeyama H, Manabe T. The role of glial cell line-derived neurotrophic factor (GDNF) and integrins for invasion and metastasis in human pancreatic cancer cells. J Surg Oncol 2005; 91: 77-83.
[28] Di Leva G, Garofalo M, Croce CM. MicroRNAs in cancer. Annu Rev Pathol 2014; 9: 287-314.
[29] Khan S, Ansarullah, Kumar D, Jaggi M, Chauhan SC. Targeting microRNAs in pancreatic cancer: microplayers in the big game. Cancer Res 2013; 73: 6541-6547.
[30] Moriyama T, Ohuchida K, Mizumoto K, Yu J, Sato N, Nabae T, Takahata S, Toma H, Nagai E, Tanaka M. MicroRNA-21 modulates biological functions of pancreatic cancer cells including their proliferation, invasion, and chemoresistance. Mol Cancer Ther 2009; 8: 1067-1074.
[31] Quan J, Jin L, Pan X, He T, Lai Y, Chen P, Lin C, Yang S, Zeng H, Lai Y. Oncogenic miR-23a-5p is associated with cellular function in RCC. Mol Med Rep 2017; 16: 2309-2317.
[32] Lu B, Sheng Y, Zhang J, Zheng Z, Ji L. The altered microRNA profile in andrographolide-induced inhibition of hepatoma tumor growth. Gene 2016; 588: 124-133.

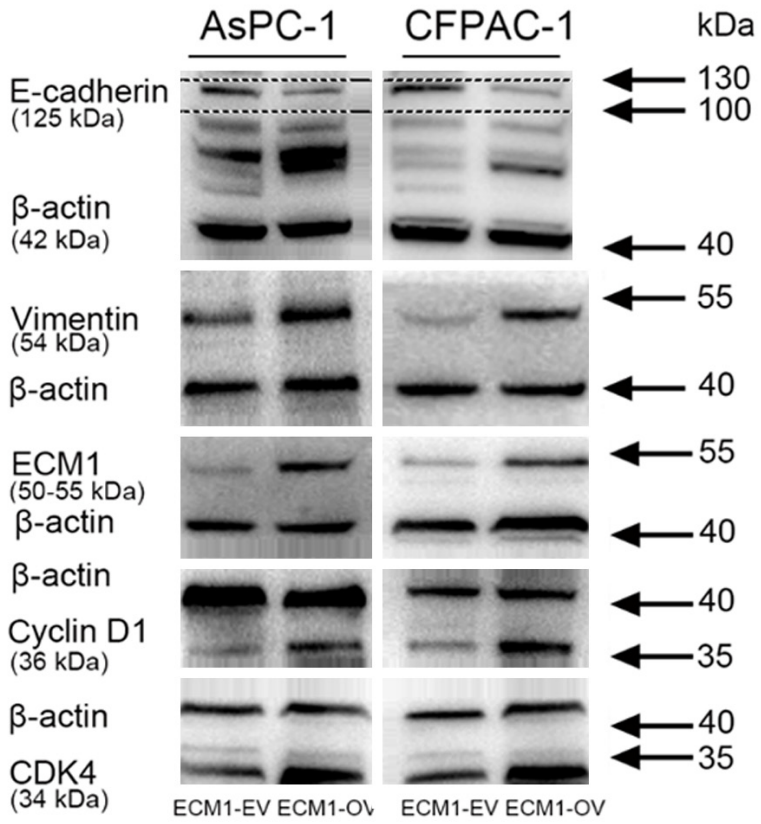

Figure S1. The whole membranes of relevant western blots in **Figure 2**.

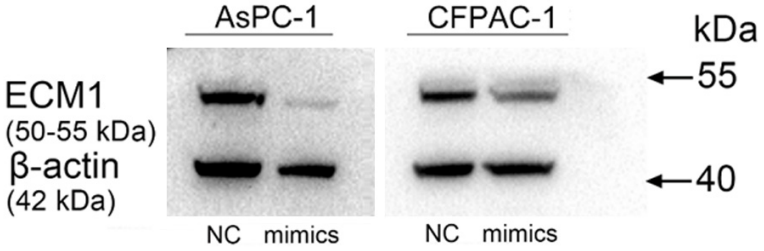

Figure S2. The whole membranes of relevant western blots in **Figure 3**.

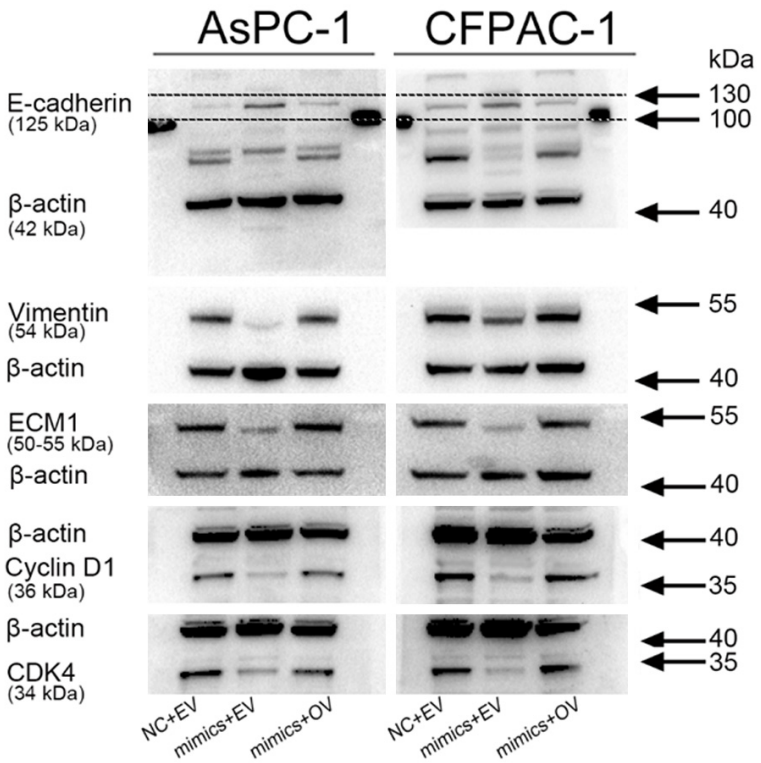

Figure S3. The whole membranes of relevant western blots in **Figure 5**.