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Original Paper

Doublecortin-Like Kinase 1 (DCLK1) Regulates B Cell-Specific Moloney Murine Leukemia Virus Insertion Site 1 (Bmi-1) and is Associated with Metastasis and Prognosis in Pancreatic Cancer

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Key Words
DCLK1 • Pancreatic cancer • Prognosis • Metastasis • EMT

Abstract
Background/Aims: Cancer stem cells (CSCs) are largely responsible for tumor relapse and metastatic behavior. Doublecortin-like kinase 1 (DCLK1) was recently reported to be a biomarker for gastrointestinal CSCs and involved in the epithelial-mesenchymal transition (EMT) and tumor progression. B cell-specific Moloney murine leukemia virus insertion site 1 (Bmi-1) is a crucial regulator of CSC self-renewal, malignant transformation and EMT, and a previous study from our group showed that Bmi-1 is upregulated in pancreatic cancer progression and participates in EMT. However, it remains unclear whether DCLK1 is involved in pancreatic cancer or whether DCLK1 is associated with the altered level of Bmi-1 expression. ***Methods:*** The correlation of DCLK1 expression and clinical features of pancreatic cancer was analyzed in 210 paraffin-embedded archived pancreatic cancer specimens by immunohistochemical analysis. The biological effects of DCLK1 siRNA on cells were investigated by examining cell proliferation using a cell counting kit and cell colony assays, cell migration by wound healing assay and cell invasion by Transwell invasion assay. We further investigated the effect of therapeutic siRNA targeting DCLK1 on pancreatic cancer cell growth *in vivo*. Moreover,

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the molecular mechanism by which DCLK1 upregulates Bmi-1 expression was explored using real-time PCR, western blotting and Co-immunoprecipitation assay. ***Results:*** DCLK1 is overexpressed in pancreatic cancer and is related to metastasis and prognosis. Knockdown of DCLK1 markedly suppressed cell growth *in vitro and in vivo* and also inhibited the migration and invasion of pancreatic cancer cells. Furthermore, we found that DCLK1 silencing could inhibit EMT in cancer cells via downregulation of Bmi-1 and the mesenchymal markers Snail and Vimentin and upregulation of the epithelial marker E-cadherin. Moreover, high DCLK1 expression in human pancreatic cancer samples was associated with a mesenchymal phenotype and increased cell proliferation. Further co-immunoprecipitation indicated that DCLK1 did not interact with Bmi-1 directly. ***Conclusion:*** Our data suggest that upregulation of DCLK1 may contribute to pancreatic cancer metastasis and poor prognosis by increasing Bmi-1 expression indirectly. The findings indicate that inhibiting DCLK1 expression might be a novel strategy for pancreatic cancer therapy.

Introduction

Pancreatic cancer is one of the deadliest solid malignancies, with a 5-year overall survival rate of less than 7% [1]. Despite progress in the medical treatment for this disease, surgical resection is the only curative therapy to date. Regardless, only a small subset, approximately 15%-20%, of tumors are resectable at the time of diagnosis, and only approximately 20% of those patients survive to 5 years. Thus, the pancreatic cancer-related death rate remains very high [1, 2]. Such a poor prognosis is due to the late presentation, early metastasis, and unresponsiveness of pancreatic cancer to most treatment options [3], and the development of new diagnostic and treatment strategies for this disease is urgently needed.

Doublecortin-like kinase 1 (DCLK1), a member of the doublecortin family, contains a distinct N-terminus microtubule-binding domain with two doublecortin (DCX) motifs [4]. DCLK1 was initially characterized as a brain-specific transmembrane protein involved in microtubule polymerization and neuronal migration [4, 5] and later reported to be a putative normal intestinal and pancreatic stem cell marker [6, 7]. However, using lineage-tracing experiments, Nakanishi et al. demonstrated that DCLK1 does not mark normal stem cells in the intestine but instead marks cancer stem cells (CSCs) that continuously produce tumor progeny in the polyps of Apc(Min/+) mice [8]. By applying the same strategy, Westphalen et al. defined an intestinal DCLK1-positive tuft cell population that is long lived and reported that these long-lived DCLK1-positive cells could function as colon cancer-initiating cells [9]. Moreover, a recent report showed that in preinvasive pancreatic cancer, DCLK1 distinguishes a morphologically distinct and functionally unique population of cancer-initiating cells with stem cell properties [10]. These and several other studies provide strong evidence that DCLK1 can be regarded as a CSC marker in pancreatic cancer [11], colon cancer [12, 13], breast cancer [14] and clear renal cell carcinoma [15]. CSCs are now considered to be primarily responsible for tumor relapse and can potentially initiate metastatic growth [16]. In addition, Gao et al. recently demonstrated that DCLK1 is overexpressed in colorectal cancer and is associated with metastasis and prognosis [17]. Another study showed using immunochemistry that DCLK1 is upregulated in stage II-III tumors compared to normal kidney and stage I tumors [15]. Furthermore, several recent studies report that DCLK1 knockdown inhibits the epithelial-mesenchymal transition (EMT) and mesenchymal marker expression and also suppresses cell migration [17-19]. These findings indicate that DCLK1 may be correlated with cancer metastasis and can potentially serve as a therapeutic target for cancer treatment.

B cell-specific Moloney murine leukemia virus insertion site 1 (Bmi-1), a key polycomb group protein, has an important function in governing stem self-renewal and is involved in the maintenance of somatic stem cells and in malignant transformation within the same tissues [20, 21]. Indeed, accumulating evidence demonstrates that Bmi-1 is upregulated in malignant tumors and is correlated with disease progression [22-24]. In addition, it has been

shown that Bmi-1 induces EMT, an important mechanism in the initial step of metastasis [25, 26]. A previous study from our group showed that Bmi-1 is overexpressed in pancreatic cancer and promotes EMT via downregulation of E-cadherin [27]. Given the functions of DCLK1 and Bmi-1 in CSCs and in regulating EMT factors, we sought to determine whether DCLK1 regulates Bmi-1 expression and plays a role in pancreatic cancer progression.

To this end, we explored the clinical implication of DCLK1 and the association between DCLK1 and Bmi-1 in pancreatic cancer in this study. We found that DCLK1 expression was not only commonly overexpressed in human pancreatic cancer tissues but that it also correlated well with cancer metastasis and a poor prognosis. We show here that inhibition of DCLK1 expression markedly suppressed cell growth *in vitro* and *in vivo* and inhibited the migration and invasion of pancreatic cancer cells. Furthermore, silencing of DCLK1 expression inhibited EMT in cancer cells via downregulation of Bmi-1 and the mesenchymal markers Snail and Vimentin and upregulation of the epithelial marker E-cadherin. Moreover, high DCLK1 expression was found to be associated with a mesenchymal phenotype as well as increased proliferation in human pancreatic cancer samples. Our data suggest that DCLK1 upregulation may contribute to pancreatic cancer metastasis and poor prognosis through activation of Bmi-1 and EMT.

Materials and Methods

Cell lines

Human pancreatic cancer cell lines AsPC-1, PANC-1, BxPC-3, CFPAC-1 and Hs766t (ATCC, Manassas, VA, USA) were cultured in DMEM medium (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco) in an incubator at 37°C with a humidified atmosphere of 5% CO_2.

Tissue samples

A total of 210 paraffin-embedded pancreatic cancer samples and 10 normal pancreatic tissues were acquired from the archival collections of Southwest Hospital, Third Military Medical University. None of the patients had received radiotherapy or chemotherapy prior to surgery. Normal pancreatic samples were obtained from organ donors. Written informed consent was obtained from all patients prior to the study. The use of human tissues was approved by the ethics committee of Southwest Hospital.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA from tumor cells and tissues was extracted using RNAiso (TaKaRa, Dalian, China). qRT-PCR was performed as previously described [28]. The primer sequences are listed in Table 1.

Western blot analysis

Protein extracts were separated by electrophoresis through sodium dodecyl sulfate (SDS)-polyacrylamide gels (Invitrogen, Camarillo, CA, USA) and then transferred onto polyvinylidene fluoride membranes (Millipore Biotechnology, Billerica, MA, USA) for immunoblotting. The membranes were hybridized overnight at 4°C with a primary antibody and then for one hour at room temperature with a horseradish peroxidase (HRP)-conjugated secondary antibody. Antibodies against the following were used: DCLK1, Snail, Vimentin and E-cadherin (Abcam, Cambridge, UK), NANOG, c-MYC, SOX2 and KLF4 and Bmi-1 (Cell Signaling Technology, MA, USA).

Table 1. Primer sequences for genes of interest

Gene	Primer sequence
miR-200a-F	5'-TAACACTGTCTGGTAACG ATGT-3'
Let-7a-F	5'- CGCUGAGGUAGUAGGUUGUAUAGUU -3'
U6-F	5'-TGGCACCCAGCACAATGAA-3'
U6-R	5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'
DCLK1-F	5'- TAGCCAGCGCCATCAAATAC -3'
DCLK1-R	5'- ACCCAGCTTCAGTGATTTGC -3'
Bmi-1-F	5'-GCCAACAGCCCAGCAGGAGG-3'
Bmi-1-R	5'-ATTGGTGGTTACCGCTGGGGC-3'
Snail-F	5'- AAGGCCTTCTCTAGGCCCT -3'
Snail-R	5'- CGCAGGTTGGAGCGGTCAG -3'
E-cadherin-F	5'- CCTCCCATCAGCTGCCC-3'
E-cadherin-R	5'- GTGATGCTGTAGAAAACCTT-3'
β-actin-F	5'-GACAGGATGCAGAAGGAGATTACT-3'
β-actin-R	5'-TGATCCACATCTGCTGGAAGGT-3'

As a loading control, an anti-GAPDH antibody (Santa Cruz, CA, USA) was used. Bands were visualized using an enhanced chemiluminescence (ECL) kit (Millipore) according to the manufacturer's protocol.

Immunohistochemical staining

Immunohistochemistry was performed as previously described [28]. Ki-67-positive cells were defined as those with brown staining in the nucleus, and expression of Ki-67 was evaluated based on the percentage of positive cells among 1000 tumor cells. DCLK1-, Bmi-1-, Snail-, Vimentin- and E-cadherin-positive cells were defined as those with immunoreactivity in both the cytoplasm and the nucleus. Expression of these markers was quantified using a composite score that was obtained by multiplying the scores for the percentage of positive cells (0, 0%; 1, <10%; 2, 10–50%; 3, >50%) by the scores for the staining intensity (0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining). To statistically analyze the relationship between DCLK1 expression and clinicopathologic characteristics, the tumor sample cohort was divided into high expression (≥6) and low expression (≤4) cohorts.

Cell transfection

DCLK1 siRNA (GGGAGUGAGAACAAUCUACtt) and negative control siRNA were transfected using RNAiMAX (Invitrogen, Camarillo, CA, USA) at a final concentration of 100 nM, as previously described [28].

Co-immunoprecipitation assay

Forty-eight hours after transfection, cells were harvested in immunoprecipitation lysis buffer, and centrifugated for 30 min at 12, 000 rpm. Ten percent of cell lysates were used as input. The remaining proteins were immunoprecipitated by incubating with normal mouse IgG (Santa Cruz) or an anti-Flag antibody (Sigma) in IP washing buffer overnight at 4°C with continuous agitation, and then incubated with precleared protein A/G beads (Santa Cruz) for another 2h at 4°C. The eluted samples were then subjected to western blotting analysis as described above with anti-DCLK1 and Flag antibodies.

Cell viability and colony formation assays

Assays were performed as described in our previous report [28, 29]. Briefly, cell viability was measured using a Cell Counting Kit-8 (Dojinodo, Shanghai, China) according to the manufacturer's instructions. For the colony formation assay, 1×10^3 cells were seeded in six-well plates and allowed to grow for 2 weeks until visible colonies were formed. The colonies were stained using Fast Richie dye (Jiancheng Biotech, Nanjing, China). Colonies were counted and the numbers were normalized as the percentage of colonies formed in the control group.

Migration and invasion assays

For the wound-healing assay, cells (1×10^6/well) were seeded in six-well plates, cultured overnight and transfected with DCLK1 siRNA or the negative control siRNA. Upon reaching the appropriate confluence, the cell layer was scratched, washed, and cultured again for up to 48 h. Cell migration was measured using a transwell chamber (24-well insert, 8-μm pore size, Millipore). For the invasion assay, the chamber was first coated with extracellular matrix gel (BD Biosciences, Sparks, MD, USA).

Animal experiments

BALB/c-nu mice (aged 4–5 weeks, 18–20 g) were purchased from the Peking University Animal Center (Beijing, China). After 5 days of acclimatization, the mice were subcutaneously injected in the right thigh with 5×10^6 AsPC-1 cells. Two weeks after implantation, the mice were randomly divided into different groups and treated by intratumoral injection of cholesterol-conjugated DCLK1 siRNA, negative control siRNA or phosphate-buffered saline (PBS). For delivery of cholesterol-conjugated RNA, 10 nmol RNA in 0.1 ml saline buffer was locally injected into the tumor xenograft once every three days for two weeks, as previously described [30, 31]. Cholesterol-conjugated DCLK1 siRNA for *in vivo* RNA interference and its negative control were purchased from RiboBio (Guangzhou, China). The tumor volume was calculated using the equation $(L\times W^2)/2$, and tumor weights were measured and recorded in grams. All animal studies were approved by the Institutional Animal Care and Use Committee of Third Military Medical University.

Gene set enrichment analysis (GSEA)

GSEA was performed to gain further insight into the biological pathways involved in pancreatic cancer pathogenesis via the DCLK1 pathway. Human whole-genome microarray datasets GSE15471/16515/32676/32688/42952/9599/71989 for pancreatic cancer and normal adjacent tissue were downloaded from Gene Expression Omnibus (GEO). All these datasets, consisting of 146 pancreatic cancer specimens, were analyzed using an Affymetrix U133 plus 2.0 chip. Raw data processing, quality control and normalization were performed as previously described [32, 33]. GSEA was performed using java GSEA Desktop Application (Broad Institute) with the hallmark gene sets (n=50) and microRNA target sets (n=221) implemented in Molecular Signatures Database (MsigDB, http://software.broadinstitute.org/gsea/msigdb); expression and phenotype data were formatted following the user guide.

Statistical analysis

All data were analyzed using SPSS 17.0 software (version 17.0, Chicago, IL, USA).

The results are presented as the means ± standard deviation (SD) of three independent experiments; Student's t-test was used to determine the statistical significance of differences between samples. A value of $P<0.05$ was considered significant.

Results

Upregulation of DCLK1 correlates with poor prognosis in human pancreatic cancer.

To investigate the biological role of DCLK1 in the progression of human pancreatic cancer, we first examined DCLK1 expression in pancreatic cancer cell lines. As shown in Fig. 1A, DCLK1 was markedly overexpressed in pancreatic cancer cell lines at the protein level. To determine the clinical relevance of DCLK1 in pancreatic cancer, DCLK1 expression was examined using immunohistochemistry (IHC) in 210 paraffin-embedded, archived pancreatic cancer tissues and 10 normal pancreatic tissues. DCLK1 was clearly upregulated in the pancreatic cancer compared with the normal tissues (Fig. 1B). In normal specimens, DCLK-1 was observed within islets but not in the intervening stromal cells or epithelium. Conversely, DCLK-1 immunoreactivity was observed in both ductal epithelial cells and intervening stromal elements in pancreatic cancer tissues. In addition, statistical analyses revealed that DCLK1 expression strongly correlated with the clinical stage, pathological stage and distant metastasis in patients with pancreatic cancer (Table 2), suggesting that DCLK1 has potential clinical value as a predictive biomarker for disease outcome in this disease. Kaplan–Meier survival analysis and the log-rank test showed DCLK1 overexpression to be correlated with shorter overall survival (Fig. 1C), and univariate and multivariate analyses revealed DCLK1 expression and clinical stage each as independent prognostic factors in pancreatic cancer (Table 3). Taken together, these data suggest a potential link between DCLK1 overexpression and pancreatic cancer progression.

DCLK1 silencing suppresses pancreatic cancer cell growth.

To evaluate the effects of DCLK1 on tumor cell growth, we used a specific small interfering RNA (siRNA) against DCLK1 to suppress DCLK1 expression in pancreatic cancer AsPC-1 and PANC-1 cell lines, both of which have high levels of DCLK1 (Fig. 2A). In vitro cell proliferation assays revealed that DCLK1 downregulation significantly inhibited proliferation in both AsPC-1 and PANC-1 cells (Fig. 2B and C). Colony formation assays also revealed that DCLK1 knockdown cells formed fewer and smaller colonies than control cells (Fig. 2D). To determine whether DCLK1 knockdown inhibits pancreatic cancer cell growth in vivo, AsPC-1 pancreatic cancer cells were implanted subcutaneously into nude mice. Following intratumoral injection of cholesterol-conjugated DCLK1 siRNA [34], DCLK1 mRNA and protein expression was decreased in tumors (Fig. 2E and F), and tumor growth was inhibited (Fig. 2G). Moreover, after intratumoral injection for two weeks, the weights of the tumors of the DCLK1 siRNA group were significantly lower than those of the control

group (Fig. 2H and I). Taken together, these data indicate a growth-inhibitory role of DCLK1 knockdown in pancreatic cancer.

DCLK1 knockdown significantly inhibits migration and invasion of pancreatic cancer cells.

To determine the role of DCLK1 in pancreatic cancer cell migration, we first performed a wound-healing assay and found that transfection of DCLK1 siRNA (si-DCLK1) significantly decreased the migratory ability of AsPC-1 and PANC-1 cells in a time-dependent manner (Fig. 3A). Consistent with the results of the wound-healing assay, in a transwell migration assay, we observed a fewer cells penetrating the membrane in the si-DCLK1 group in comparison to the control group (Fig. 3B). Similar results were obtained in an invasion assay, whereby DCLK1 silencing significantly decreased the invading cell numbers compared with the control group (Fig. 3C).

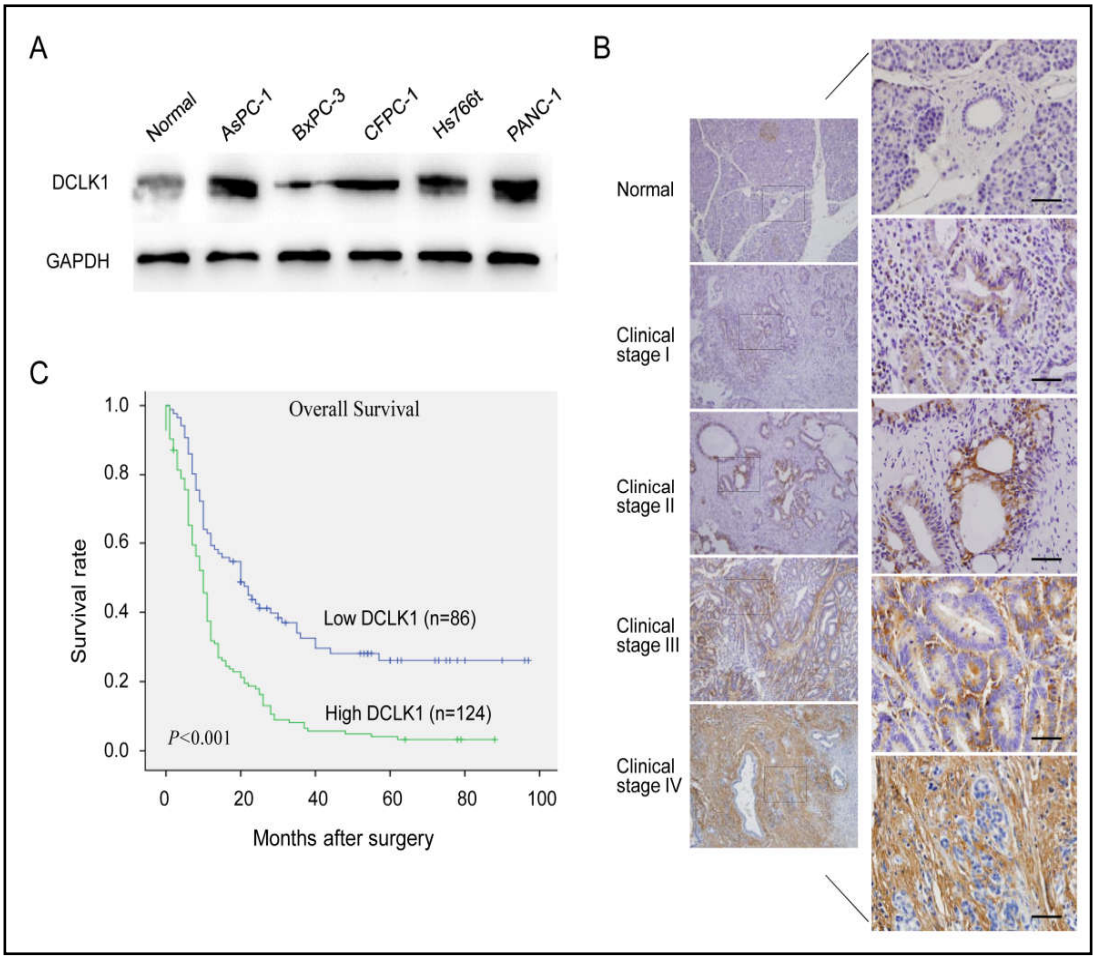

Fig. 1. Upregulation of DCLK1 correlates with poor prognosis in human pancreatic cancer. (A) Western blotting analysis of DCLK1 expression in an immortalized human pancreatic ductal epithelial cell line and cultured pancreatic cancer cell lines. GAPDH was used as a loading control. (B) Immunohistochemistry staining for DCLK1 expression in human pancreatic cancer (clinical stages I–IV) compared with normal pancreatic tissue (left panel: magnification × 100; right panel: magnification × 400; Scale bar: 5μm). (C) Kaplan-Meier curves of pancreatic cancer patients with high vs. low expression of DCLK1 (n=210; P<0.001). P-values were calculated based on the log-rank test.

Downregulated DCLK1 expression inhibits Bmi-1 expression and EMT.

To further elucidate the potential mechanisms by which DCLK1 participates in cancer metastasis, we used gene set enrichment (GSE) datasets and categorized pancreatic cancer specimens according to their relative DCLK1 expression level. Strikingly, subtypes with different levels of DCLK1 expression exhibited markedly distinct pathway profiles. For example, the high DCLK1 expression subtype displayed average increases in EMT pathway genes and strong enrichment for expression of the targets of miR-200 (Fig. 4A), an important inhibitor of EMT [35]. The high DCLK1 subtype was also distinguished by higher levels of let-7 targets and KRAS signaling (Fig. 4B). Moreover, si-DCLK1-transfected AsPC-1 and PANC-1 cells showed epithelial cell features (characterized by aggregated cells) (Fig. 4C), consistent with the reduced expression of Bmi-1, Snail and Vimentin and increased expression of the epithelial marker E-cadherin (Fig. 4D). Accordingly, intratumoral injection of cholesterol-conjugated DCLK1 siRNA led to decreased Bmi-1 and Snail expression but increased E-cadherin, miR-200a and let-7a expression in xenograft tumor tissues (Fig. 4E). Previous studies demonstrated that DCLK1 marks CSC in pancreatic and colorectal cancer and regulates stemness [11, 12]. We therefore examined the expression of pluripotency markers after cells were transfected with si-DCLK1. Western blot analyses showed that siRNA-mediated knockdown of DCLK1 resulted in downregulation of pluripotency factors c-MYC, NANOG, SOX2 and KLF4 in both AsPC-1 and PANC-1 cells (Fig. 4F).

Table 2. Correlation between DCLK1 expression and clinicopathologic characteristics of pancreatic cancer patients. Abbreviations: DCLK1, Doublecortin-like kinase 1. [a]P value from the Chi-square or Fisher exact test. *Statistically significant (P<0.05)

Characteristic	No. (n=210)	DCLK1 expression Low (n=86)	High (n=124)	P [a]
Gender				0.109
Male	136	51 (59.3%)	85 (68.5%)	
Female	74	35 (40.7%)	39 (31.5%)	
Age (years)				0.403
≤60	131	55 (64.0%)	76 (61.3%)	
>60	79	31 (36.0%)	48 (38.7%)	
Tumor location				0.532
Head	138	56 (65.1%)	82 (66.1%)	
Body/tail	72	30 (34.9%)	42 (33.9%)	
Tumor size (cm)				0.629
0-2	51	24 (27.9%)	27 (21.8%)	
2-5	128	50 (58.1%)	78 (62.9%)	
>5	31	12 (14.0%)	19 (15.3%)	
Tumor differentiation				0.102
Well	22	11 (12.8%)	11 (8.9%)	
Moderate	130	57 (66.3%)	73 (58.9%)	
Poor	49	13 (15.1%)	36 (29.0%)	
Others	9	5 (5.8%)	4 (3.2%)	
Clinical stage				0.001*
I-II	150	72 (83.7%)	78 (62.9%)	
III-IV	60	14 (16.3%)	46 (37.1%)	
Pathologic tumor status				0.002*
T1-T2	87	46 (53.5%)	41 (33.1%)	
T3-T4	123	40 (46.5%)	83 (66.9%)	
Lymph node status				0.341
N0	137	58 (67.4%)	79 (63.7%)	
N1	73	28 (32.6%)	45 (36.3%)	
Distant metastasis				0.024*
M0	174	77 (89.5%)	97 (78.2%)	
M1	36	9 (10.5%)	27 (21.8%)	
Perineural invasion				0.018*
Absent	151	69 (80.2%)	82 (66.1%)	
Present	59	17 (19.8%)	42 (33.9%)	
Vascular invasion				0.012*
Absent	172	77 (89.5%)	95 (76.6%)	
Present	38	9 (10.5%)	29 (23.4%)	

Table 3. Univariate and multivariate Cox regression analyses of different prognostic variables in pancreatic cancer patients. Abbreviations: DCLK1, Doublecortin-like kinase 1; HR, hazard ratio; CI, confidence interval. [a]95% Wald Confidence Limits. [b]P value from Cox regression analyses. *Statistically significant (P<0.05)

Variable	Subset	HR (95%) CI [a]	P [b]
Univariate analysis			
DCLK1 expression	High versus Low	2.271 (1.657-3.114)	<0.001*
Gender	Male versus Female	1.054 (0.777-1.431)	0.734
Age (years)	> 60 versus ≤60	1.207 (0.890-1.636)	0.226
Tumor location	Head versus Body/tail	1.805 (0.912-3.421)	0.125
Tumor size (cm)	>2 versus ≤2	1.311 (0.891-2.002)	0.315
Tumor differentiation	Poor/Moderate versus Well	1.230 (0.764-1.979)	0.395
Clinical stage	III + IV versus I + II	2.180 (1.567-3.033)	<0.001*
Pathologic tumor status	T3+T4 versus T1+T2	1.836 (1.350-2.498)	<0.001*
Lymph node status	N1 versus N0	1.601 (1.181-2.170)	0.002*
Distant metastasis	M1 versus M0	1.730 (1.182-2.532)	0.005*
Perineural invasion	Present versus Absent	1.759 (1.275-2.426)	0.001*
Vascular invasion	Present versus Absent	1.684 (1.165-2.435)	0.006*
Multivariate analysis			
DCLK1 expression	High versus Low	1.878 (1.347-2.619)	<0.001*
Clinical stage	III + IV versus I + II	1.709 (1.100-2.654)	0.017*
Pathologic tumor status	T3+T4 versus T1+T2	1.284 (0.907-1.817)	0.159
Distant metastasis	M1 versus M0	1.030 (0.635-1.672)	0.904
Perineural invasion	Present versus Absent	1.371 (0.967-1.944)	0.076
Vascular invasion	Present versus Absent	1.026 (0.673-1.563)	0.906

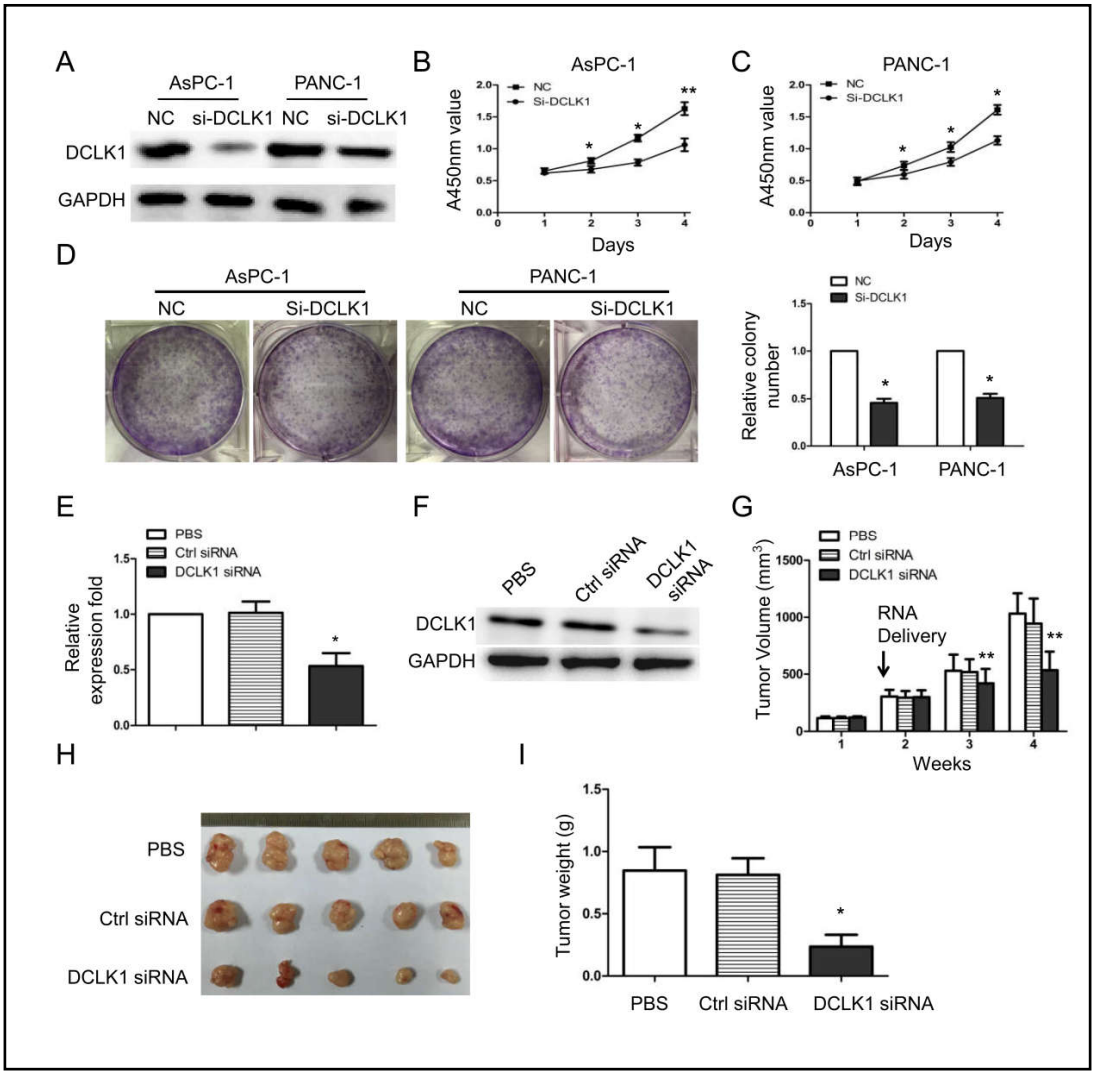

Fig. 2. Downregulation of DCLK1 inhibits tumor cell growth in pancreatic cancer. (A) Western blot analysis of DCLK1 expression in AsPC-1 and PANC-1 cells transfected with negative control (NC) siRNA or DCLK1 siRNA after 48 h; GAPDH was used as a loading control. (B, C) The effects of DCLK1 silencing on pancreatic cancer cell growth were measured using a CCK-8 assay. The results are presented as the means ± S.D. of the values obtained in three independent experiments. Significance was calculated using Student's t-test. (D) Representative images and results of AsPC-1 and PANC-1 cells colony formation assay was summarized as means±S.D. of three independent experiments.*P <0.05. (E, F) qRT-PCR and western blot analysis of DCLK1 expression in pancreatic cancer cell xenograft tumor tissue at 72 h after intratumoral injection of cholesterol-conjugated DCLK1 siRNA or control siRNA. (G) Effect of DCLK1 knockdown on xenograft tumor growth. Two weeks after implantation, mice were randomly divided into different groups and treated with intratumoral injection of cholesterol-conjugated DCLK1 siRNA, negative control siRNA and PBS. Tumor volumes were measured on the indicated days. Data are presented as the mean tumor volumes ± SD. (H, I) Mice were sacrificed two weeks after intratumoral injection of cholesterol-conjugated siRNA. A photograph of a representative xenograft is shown for each group (H). Xenograft weights were measured, and those from mice in the DCLK1 siRNA injection group showed the most significant reduction in tumor weight (I).

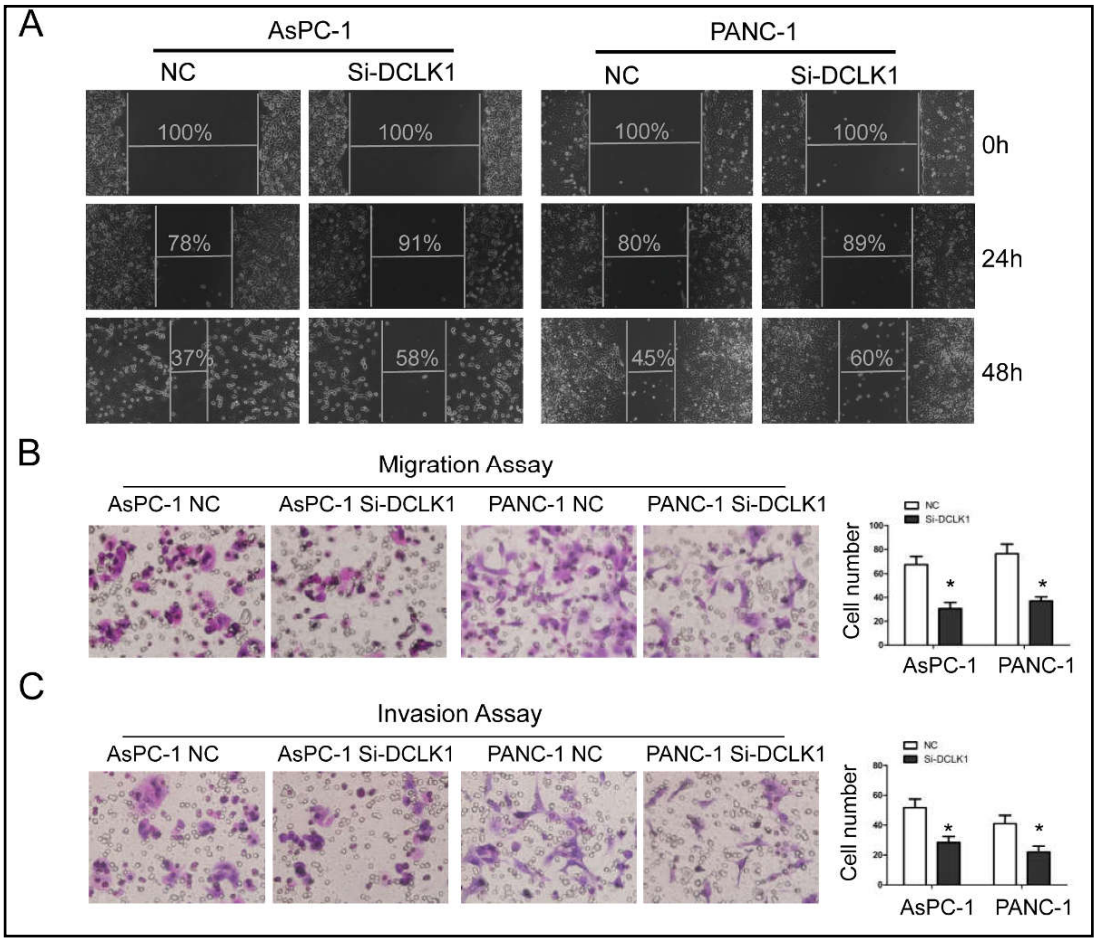

Fig. 3. DCLK1 silencing inhibits migration and invasion in pancreatic cancer cells. (A) DCLK1 knockdown suppresses cell migration in AsPC-1 and PANC-1 cells. Images of wound-healing assays were captured at 0, 24 and 48 h. (B, C) The inhibitory effect of DCLK1 knockdown on in AsPC-1 and PANC-1 cell migration and invasion was confirmed using transwell assays. Data are shown as the mean ± S.D. of three independent experiments. *P<0.05.

High DCLK1 expression is associated with the mesenchymal phenotype and increased proliferation in clinical samples.

To further investigate the clinical role of EMT in DCLK1-mediated tumor progression, we performed IHC for Bmi-1, Snail, Vimentin, E-cadherin and Ki-67 using human pancreatic cancer tissues and analyzed correlations with DCLK1 expression. The results showed a significant correlation between DCLK1 expression and that of Bmi-1 and mesenchymal markers Snail and Vimentin but an inverse association with expression of the epithelial marker E-cadherin in 84 cases of pancreatic cancer tissues (P<0.05 for all) (Fig. 5A and B). These findings were consistent with the results from in vitro and animal model experiments. In addition, high DCLK1 expression was significantly associated with increased Ki-67 expression (P<0.05). To further confirm this result, we analyzed the mRNA expression levels of DCLK1, Bmi-1, Snail, Vimentin and E-cadherin in human whole-genome microarrays datasets (GSE15471/16515/32676/32688/42952/9599/71989) for pancreatic cancer samples (n=146). Consistently, DCLK1 expression levels were positively correlated with those of Bmi-1, Snail and Vimentin and inversely associated with that of E-cadherin (Fig. 5C). Altogether, our results from in vitro, in vivo and human tumor samples showed that DCLK1 enhances EMT and cell proliferation in pancreatic cancer.

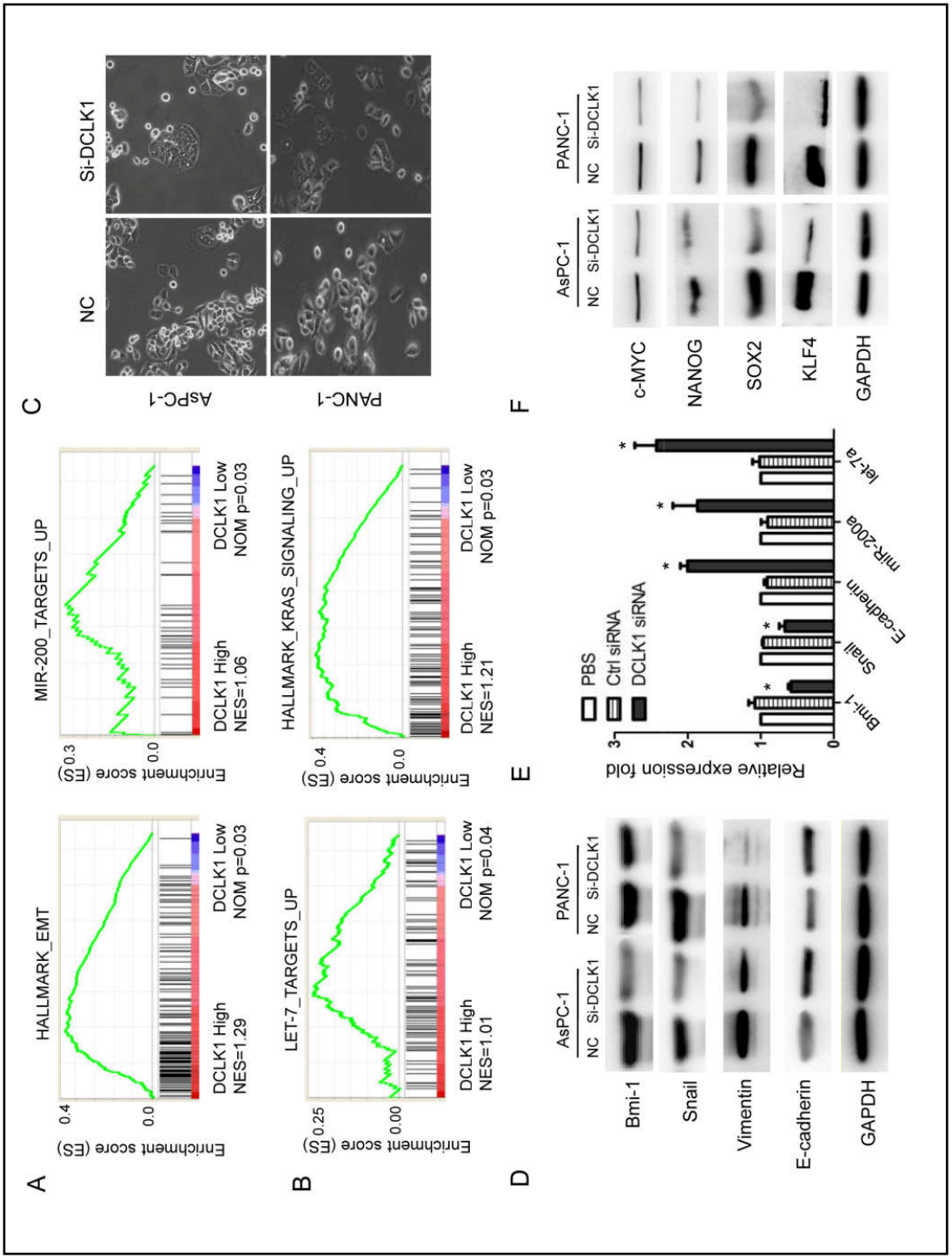

Fig. 4. Downregulation of DCLK1 inhibits EMT. (A, B) GSEA plot of EMT, miR-200 and let-7 targets and KRAS signaling pathways between DCLK1 high and low subgroups. (C) Inverse-phase microscopy of AsPC-1 and PANC-1 cells transfected with si-DCLK1 or negative control siRNA (NC). (D) Western blot analysis of Bmi-1, Snail, Vimentin and E-cadherin expression in AsPC-1 and PANC-1 cells transfected with si-DCLK1 or NC after 48 h. (E) qRT-PCR analysis of Bmi-1, Snail, E-cadherin, miR-200a and let-7a expression in nude mice xenografts after intratumoral injection of cholesterol-conjugated DCLK1 siRNA. (F) Western blot analysis of pluripotency factors c-MYC, NANOG, SOX2 and KLF4 expression in AsPC-1 and PANC-1 cells transfected with si-DCLK1 or NC after 48 h.

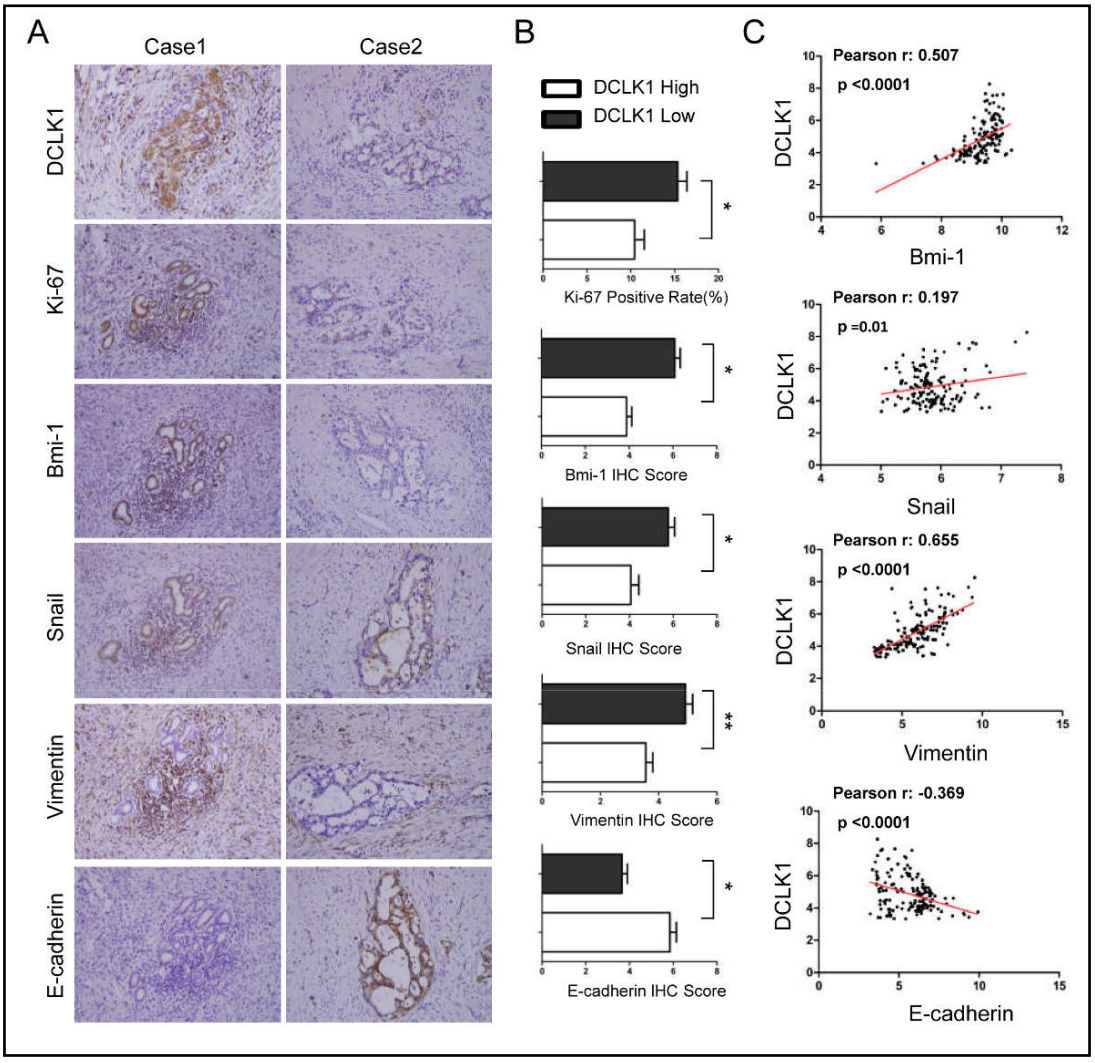

Fig. 5. High DCLK1 expression is associated with the mesenchymal phenotype and increased proliferation in human pancreatic cancer. (A) Representative images (200×) of IHC staining for Ki-67, Bmi-1, Snail, Vimentin and E-cadherin in high or low DCLK1 expression groups are shown. (B) Bar charts show the association between DCLK1 expression and the expression levels of Ki-67, Bmi-1, Snail, Vimentin and E-cadherin. The x-axes represent the relative expression levels of Ki-67, Bmi-1, Snail, Vimentin and E-cadherin, as indicated by IHC. Results are presented as the means ± SEM. *P<0.05; **P<0.01. (C) Co-expression analysis for DCLK1 in pancreatic cancer vs. Bmi-1, Snail, Vimentin and E-cadherin. Plotted data consist of log2 mRNA expression data from GSE datasets.

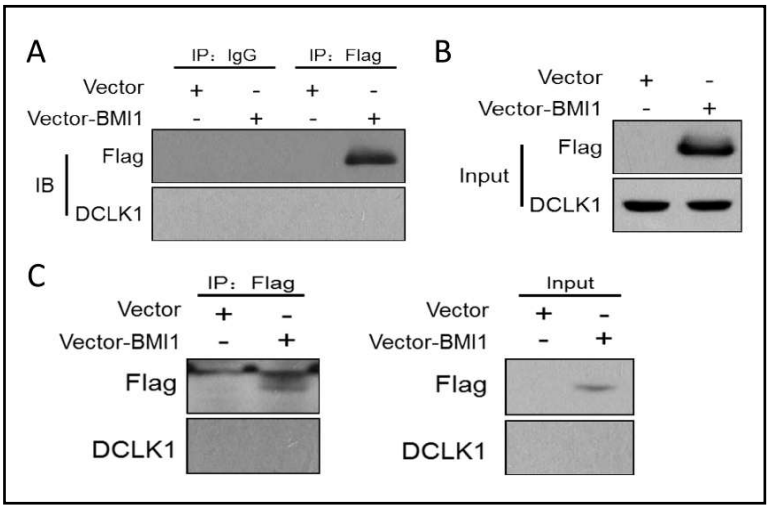

Fig. 6. Validation of the interaction between DCLK1 and Bmi-1 in vitro. (A, B) Recombinant vector and Flag-Bmi-1 vector were transfected into HEK293 cells. After 48 h, the proteins were collected. Input samples represents 10% of total supernatant used in Co-IP assays (A). Remaining extract were purified with anti-Flag M2 affinity gel from the residual protein extracts of HEK293 cells and detected by Western blot with antibody against DCLK1 (B). (C) There was no direct interaction between DCLK1 and Bmi-1 in PANC-1 cells.

To further study whether DCLK1 interacts with Bmi-1 in pancreatic cancer, we performed co-immunoprecipitation assay in HEK293 cell line and pancreatic cancer cell line PANC-1. Result showed that there was no direct interaction between DCLK1 and Bmi-1 (Fig. 6).

Discussion

Recent studies provide strong evidence that DCLK1 is involved in tumor progression and can be regarded as a CSC marker [11-13, 17]. In this study, we found that DCLK1 was overexpressed in pancreatic cancer, correlating with metastasis and prognosis. siRNA-mediated knockdown of DCLK1 markedly suppressed cell growth *in vitro* and *in vivo* and inhibited the migration and invasion of pancreatic cancer cells. In addition, we showed that silencing of DCLK1 expression inhibited EMT in cancer cells via downregulation of Bmi-1, Snail and Vimentin and upregulation of E-cadherin. Moreover, high DCLK1 expression was positively correlated with the mesenchymal phenotype and increased proliferation in clinical samples. These findings suggest that DCLK1 overexpression could activate EMT in pancreatic cancer cells, which is likely one of the mechanisms underlying its involvement in metastasis.

Accumulating evidence suggests that CSCs are responsible for cancer metastasis, recurrence and radio/chemo-resistance [36-38], and previous reports demonstrate that DCLK1 can mark CSCs in gastrointestinal and other cancers [12-14]. A recent study revealed that DCLK1 is essential for the invasive and metastatic properties of CSCs in human pancreatic cancer and that it might be a promising therapeutic target [11]. Indeed, several reports demonstrate that DCLK1 is overexpressed in certain cancers. For example, DCLK1 is upregulated in colorectal cancer and correlated with metastasis and poor prognosis [17, 39]. Expression of DCLK1 is also increased in esophageal adenocarcinoma tissues compared with normal esophageal mucosa [40]. In addition, IHC results showed that DCLK1 is upregulated in stage II-III tumors compared to normal kidney and stage I tumors [15]. Conversely, DCLK1 was found to be associated with favorable clinicopathologic features and a good prognostic factor in breast cancer, particularly in invasive breast cancers with neuroendocrine differentiation (IBC-NED) [14]. These contrasting results with regard to tumor-promoting roles in gastrointestinal cancers suggest different functional roles for DCLK1 in different type of cancers. In this study, DCLK1 was clearly upregulated in pancreatic cancer tissues compared with normal tissues, and DCLK1 overexpression was correlated with shorter overall survival. In addition, univariate and multivariate analyses revealed DCLK1 expression

to be an independent prognostic factor in pancreatic cancer. Moreover, DCLK1 knockdown significantly inhibited tumor cell growth *in vitro* and *in vivo* and suppressed the migration and invasion of pancreatic cancer cells. The results of our study suggest that DCLK1 is involved in pancreatic cancer pathogenesis and progression and that it may serve as a prognostic biomarker for pancreatic cancer.

Activation of the developmental program EMT has been shown to play a crucial role in promoting metastasis in epithelium-derived carcinoma [41, 42]. During EMT, cells undergo a molecular switch from a polarized epithelial phenotype to a highly motile, non-polarized mesenchymal phenotype, endowing tumor cells with the ability to migrate from the primary tumor to distant locations [43, 44]. Loss of E-cadherin (an epithelial cell marker) and gain of mesenchymal markers such as Snail and Vimentin are regarded as characteristics of EMT. Indeed, recent studies illustrate a direct link between EMT and CSCs in promoting tumor metastasis and relapse in advanced cancer [36, 45]. In addition, it has been shown that Bmi-1 is a crucial regulator of CSC self-renewal, malignant transformation and EMT [21]. A recent study demonstrated that DCLK1 could accelerate EMT in human pancreatic cells through a miR-200a-dependent mechanism [19]. In the present study, we showed that DCLK1 knockdown inhibited EMT, decreased Bmi-1, Snail and Vimentin expression, and enhanced E-cadherin expression in both pancreatic cancer cell lines and xenografts in nude mice. We reveal for the first time the key role of DCLK-1 and Bmi-1, a potential stem cell protein, in cancer metastasis and EMT. Our results showed there was no direct interaction between DCLK1 and Bmi-1. Previous studies demonstrated that DCLK1 regulates pluripotency, cancer EMT and metastasis via miRNA-dependent mechanisms in various malignancies [19, 46, 47]. DCLK1 might indirectly regulate Bmi-1 by other mechanism, such as miRNA. Therefore, further investigations into the mechanisms by which DCLK1 regulates Bmi-1 in human cancers are necessary to increase our knowledge of the way cancer progression is regulated. Moreover, we found the high DCLK1 subtype to be strongly enriched for EMT pathway genes as well as miR-200 and let-7 targets, both of which have been reported to be EMT inhibitors [35, 48, 49]. Therefore, the study presented here provides strong evidence that DCLK1 silencing could inhibit Bmi-1 expression and EMT, which may be one of the molecular mechanisms by which DCLK1 facilitates cancer metastasis.

Conclusion

In summary, we demonstrate that DCLK1 expression is clearly upregulated in human pancreatic cancer and that DCLK1 expression might serve as a biomarker for cancer metastasis and prognosis. As knockdown of DCLK1 markedly suppressed Bmi-1 expression and EMT, DCLK1 might be an important target for pancreatic cancer therapy.

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Disclosure Statement

The authors declare no conflicts of interest.

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