ZFP36L2 promotes cancer cell aggressiveness and is regulated by antitumor microRNA-375 in pancreatic ductal adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies; despite recently developed treatments, the 5-year survival rate after diagnosis is only 5%.

Using miRNA expression signatures, we have previously identified tumor-suppressive miRNA and the novel cancer networks controlled by these miRNA.

We recently used the same method, miRNA expression signature, to identify aberrantly expressed miRNA in PDAC cells. According to these miRNA signatures, microRNA-375 (miR-375) was significantly reduced in miRNA expression signatures of several types of cancers, including PDAC. The aim of the present study was to investigate the functional roles of miR-375 in PDAC cells and to identify miR-375-regulated molecular networks involved in PDAC aggressiveness. The expression levels of miR-375 were markedly downregulated in PDAC clinical specimens and cell lines (PANC-1 and SW1990). Ectopic expression of miR-375 significantly suppressed cancer cell proliferation, migration and invasion. Our in silico and gene expression analyses and luciferase reporter assay showed that zinc finger protein 36 ring finger protein-like 2 (ZFP36L2) was a direct target of miR-375 in PDAC cells. Silencing ZFP36L2 inhibited cancer cell aggressiveness in PDAC cell lines, and overexpression of ZFP36L2 was confirmed in PDAC clinical specimens. Interestingly, Kaplan–Meier survival curves showed that high expression of ZFP36L2 predicted shorter survival in patients with PDAC. Moreover, we investigated the downstream molecular networks of the miR-375/ZFP36L2 axis in PDAC cells. Elucidation of tumor-suppressive miR-375-mediated PDAC molecular networks may provide new insights into the potential mechanisms of PDAC pathogenesis.
analysis showed that high expression of ZFP36L2 predicted a significantly shorter survival of patients with PDAC. Elucidation of miR-375-mediated molecular networks in PDAC may provide new insights into the potential mechanisms of PDAC pathogenesis.

Materials and Methods

Clinical specimens and cell lines. Clinical tissue specimens (n = 27) and formalin-fixed, paraffin-embedded blocks (n = 37) were collected from patients with PDAC who underwent curative surgical resection at Kagoshima University Hospital between 1991 and 2014. Normal pancreatic tissue specimens (n = 14) were obtained from noncancerous tumor-adjacent tissue. Each surgical specimen was histologically classified according to the TNM classification system. All patients in this study provided informed consent and the study protocol was approved by the Institutional Review Board of Kagoshima University. Two human PDAC cell lines were investigated in this study. PANC-1 cells were obtained from RIKEN Cell Bank (Tsukuba, Ibaraki, Japan) and SW 1990 cells were obtained from the ATCC (Manassas, VA, USA).

Total RNA, including miRNA, was isolated using ISOGEN (NIPPON GENE, Toyama, Japan) according to the manufacturer’s protocol.

Quantitative RT-PCR. Quantification of miRNA was performed using quantitative RT-PCR (qRT-PCR) as previously described. Briefly, miRNA were quantified using stem-loop RT-PCR, TaqMan MicroRNA Assays and Assay-on-Demand Gene Expression TaqMan probes and primers as directed by the manufacturer. Probes and primers for miR-375 (product ID: 000564; Thermo Fisher Scientific, Kanagawa, Japan), ZFP36L2 (product ID: Hs00272828_m1; Thermo Fisher Scientific), CADM1 (product ID: Hs00942508_m1; Thermo Fisher Scientific), TSPYL5 (product ID: Hs00603217_s1; Thermo Fisher Scientific), ELF2N (product ID: Hs00287464_s1; Thermo Fisher Scientific), TOSCA (product ID: Hs00999908_m1; Thermo Fisher Scientific) and HOMER1 (product ID: Hs01029333_m1; Thermo Fisher Scientific) were used. Human GUSB (product ID: Hs99999908_m1; Thermo Fisher Scientific) and RNU48 (product ID: 001006; Thermo Fisher Scientific) were used as internal controls. Expression fold-changes were determined using the ΔΔCt method.

Transfection of miRNA mimic, inhibitor and siRNA into pancreatic ductal adenocarcinoma cell lines. Pancreatic ductal adenocarcinoma cell lines were transfected with a miRNA mimic for gain-of-function experiments, miRNA inhibitors for loss-of-function experiments, and siRNA for loss-of-function experiments. Pre-miR miRNA precursors for miR-375 (product ID: PMi0327), negative control miRNA (product ID: AM 17111), two ZFP36L2 siRNA (product IDs: HSS101105 and HSS101106) and negative control siRNA (product ID: D-001810-10) were purchased from Thermo Fisher Scientific. Two types of miR-375 inhibitors (product ID: AM10327 and IH-300682-07-0005) were used: Thermo Fisher Scientific and GE Healthcare JAPAN (Tokyo, Japan). The transfection efficiencies of miRNA in PANC-1 and SW 1990 cells were calculated as described in previous studies.

Cell proliferation, migration and invasion assays. Pancreatic ductal adenocarcinoma cells were transfected with 10 nmol/L miRNA or siRNA by reverse transfection and seeded in 96-well plates at 5 x 10^3 cells per well. After 72 h, cell proliferation was evaluated by the XTT assay using a Cell Proliferation Kit II (Roche Molecular Biochemicals, Mannheim, Germany). Cell migration assays were performed with BD Falcon Cell Culture Inserts (BD Biosciences, Franklin Lakes, NJ, USA) that contained uncoated Transwell polycarbonate membrane filters with 8-μm pores in 24-well tissue culture plates. Cells were transfected with 10 nm miRNA or siRNA by reverse transfection and seeded in 6-cm dishes at 2 x 10^5 cells. After 48 h, the cells were collected and 1 x 10^5 cells were added to the upper chamber of each migration well and were allowed to migrate for 48 h. After gentle removal of the nonmigratory cells from the filter surface of the upper chamber, the cells that migrated to the lower side were fixed and stained with Diff-Quick (Sysmex Corporation, Kobe, Japan). The number of cells that migrated to the lower surface was determined microscopically by counting eight areas of constant size per well.

Cell invasion assays were performed using modified Boyden chambers containing Transwell membrane filter inserts precoated with Matrigel with 8-μm pores in 24-well tissue culture plates (BD Biosciences, Bedford, MA, USA). All experiments were carried out in triplicate.

Western blot analyses. Protein lysates were collected 72 h after transfection and 20 μg of protein was separated using gel electrophoresis on e-PAGEL 5-20% gels (ATTO, Tokyo, Japan) before transfection to polyvinylidene fluoride membranes. Rabbit anti-ZFP36L2 antibodies (product ID: A1978; Sigma Aldrich) were used as an internal loading control. A detailed description of the western blotting procedure is published elsewhere.

Immunohistochemistry. Tissue sections were incubated overnight at room temperature with ZFP36L2 antibodies diluted 1:50 (product ID: HPA047428; Atlas Antibodies AB, Stockholm, Sweden). Following incubation, antibodies were visualized using an avidin–biotin complex (ABC) detection kit (Vector Laboratories, Burlingame, CA, USA) and a diaminobenzidine substrate system according to the manufacturer’s protocol. Cytoplasmic staining of ZFP36L2 in at least 1% of cancer cells was classified as high. If no cancer cells were stained, specimens were classified as low for ZFP36L2 staining. The expression of ZFP36L2 was evaluated in 10 fields of 100 cells each using high-power microscopy (400x).

Genome-wide gene expression and in silico analyses. To identify miR-375 target genes, a combination of genome-wide gene expression and in silico analyses was conducted as described previously. The microarray data were deposited into the GEO repository under the accession numbers GSE77790 and GSE82108. Next, we selected putative miRNA target genes using the microRNA.org (August 2010 release, http://www.micorna.org/). Figure 1 shows the methodology for selecting target genes.

Plasmid construction and dual luciferase reporter assays. Partial wild-type sequences of the 3' UTR of ZFP36L2 containing the miR-375 target site (positions 269–275 of ZFP36L2 3' UTR, and positions 308–314 of ZFP36L2 3' UTR for miR-375) or sequences with a deleted miR-375 target site were inserted between the XhoI and Pmel restriction sites in the 3' UTR of the hRluc gene in the psiCHECK-2 vector (product ID: C8021; Promega, Madison, WI, USA). PANC-1 and SW 1990 cell lines were transfected with 50 ng of the vector and 10 nM miR-375 using Lipofectamine 2000 (Thermo Fisher Scientific) in Opti-MEM (Thermo Fisher Scientific). The activities of firefly and Renilla luciferases were determined in lysates of

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transfected cells using a dual luciferase reporter assay system according to the manufacturer’s recommendations (product ID: E1960, Promega, Madison, WI, USA). Data were normalized to firefly luciferase activity (ratio of Renilla/firefly luciferase activities).

**Statistical analysis.** Using expression values and the Mann–Whitney U-test or Bonferroni-adjusted Mann–Whitney U-test, relationships between two conditions or variables were analyzed. The correlation between expression of miR-375 and ZFP36L2 was evaluated using Spearman’s rank test. Associations between different categories were assessed using Fisher’s exact test and the \( \chi^2 \)-test. Overall survival (OS) after surgery was gauged using Kaplan–Meier curves. Patients were divided into two groups based on ZFP36L2 expression, and differences in survival were estimated using the log-rank test. Univariate and multivariate analyses were performed using the proportional hazards model. We used Expert StatView software (version 5.0 SAS Institute, Cary, NC, USA) for these analyses.

**Results**

**Expression levels of miR-375 in pancreatic ductal adenocarcinoma specimens and cell lines.** We evaluated expression levels of miR-375 in PDAC tissues (n = 27), normal pancreas tissues (n = 14) and two PDAC cell lines (PANC-1 and SW 1990). Patient backgrounds and clinicopathological characteristics are summarized in Table 1. The expression levels of miR-375 were significantly lower in tumor tissues and PDAC cell lines compared with normal pancreas tissues (Fig. 2a). However, there were no significant relationships between any of the clinicopathological parameters (i.e. TNM stage, metastasis or survival rate) and the expression of miR-375 (Fig. S1).

**Effect of miR-375 expression on cell growth, migration and invasion in pancreatic ductal adenocarcinoma cell lines.** To investigate the functional roles of miR-375, we performed gain-of-function studies using transfection. XTT, cell migration and cell invasion assays demonstrated that cell proliferation, migration and invasion were significantly inhibited in miR-375 transfectants compared with mock or miR-control transfectants (each \( P < 0.0001 \), Fig. 2b–d). Furthermore, we performed loss-of-function assays using miR-375 inhibitors in PDAC cells. Our present data showed that cancer cell proliferation, migration and invasion were significantly enhanced by suppression of miR-375 in PDAC cells (Fig. S2). These results suggested that miR-375 could have a tumor-suppressive function in PDAC cells.

**Identification of genes regulated by miR-375 in pancreatic ductal adenocarcinoma cells.** To gain further insight into the molecular mechanisms and pathways regulated by tumor-suppressive miR-375 in PDAC cells, we used a combination of in silico and gene expression analyses. Figure 1 presents the strategy for narrowing down the target genes of miR-375. In gene expression analyses, 45 and 52 downregulated genes were identified in PANC-1 and SW 1990 miR-375 transfectants, respectively, in comparison with control transfectants (GEO accession number GSE77790). Next, we pared down the list of genes using the microRNA.org database. We found that 9 and 14 genes were putatively targeted by miR-375 in PANC-1 and SW 1990 miR-375 transfectants, respectively. Of those genes, 6 were common to both miR-375 transfectants. We validated the changes of 6 genes by miR-375 regulation using miR-375 transfectant cells (Fig. S3). Among them, we determined that ZFP36L2 was upregulated in clinical PDAC samples using qRT-PCR (Table 3, Fig. 3a,c). No negative correlations between miR-375 expression and ZFP36L2 mRNA expression were found using Spearman’s rank test (\( r = -0.240, P = 0.1042 \), Fig. 3b).

ZFP36L2 is a direct target of miR-375 in pancreatic ductal adenocarcinoma cells. We performed qRT-PCR to validate miR-375 repression of ZFP36L2 mRNA expression in PDAC cell lines. Our studies revealed that ZFP36L2 mRNA was significantly reduced in miR-375 transfectants in comparison with mock or miR-control transfectants (\( P < 0.0001 \) and \( P = 0.0036 \), Fig. 4a). Protein expression of ZFP36L2 was also repressed in the miR-375 transfectants (Fig. 4b).

Target prediction databases indicated two putative target sites in the 3’-UTR of ZFP36L2 (Fig. 4c). To determine whether ZFP36L2 mRNA had a functional target site, we performed a luciferase reporter assay. Compared with the miR-control, luminescence intensity was significantly reduced by transfection with miR-375 at the miR-375 target site, position 308–314 in the 3’-UTR of ZFP36L2 (Fig. 4c, lower).

**Effects of silencing ZFP36L2 on PDAC cell lines.** To investigate the functional role of ZFP36L2 in PDAC cells, we carried out loss-of-function studies using si-ZFP36L2 transfectants. First, we evaluated the knockdown efficiency of si-ZFP36L2 transfection in PDAC cell lines. In the present study, we used two types of si-ZFP36L2 (si-ZFP36L2-1 and si-ZFP36L2-2). According to qRT-PCR and western blot analyses, both siRNA effectively downregulated ZFP36L2 expression in both cell lines (Fig. 5a,b). XTT, cell migration and cell invasion assays demonstrated that cell proliferation, migration and invasion
Fig. 2. Expression levels of miR-375 and its effects on pancreatic ductal adenocarcinoma (PDAC) cells. (a) Expression levels of miR-375 in clinical specimens and PDAC cell lines were determined using quantitative RT-PCR (qRT-PCR). Data were normalized to RNU48 expression. (b) Cell growth was determined using XTT assay 72 h after transfection with 10 nM miR-375. *P < 0.0001. (c) Cell migration activity was determined using BD Falcon Cell Culture Inserts. *P < 0.0001. (d) Cell invasion activity was determined using Matrigel invasion assays. *P < 0.0001.

Table 1. Characteristics of the patients

| Pancreatic ductal adenocarcinoma |  |
|----------------------------------|---|
| Total number                     | 27 |
| Median age (range), years        | 67.1 (42–85) |
| Gender                           |  |
| Male                             | 12 |
| Female                           | 15 |
| T category                       |  |
| pTis                             | 1 |
| pT1                              | 2 |
| pT2                              | 0 |
| pT3                              | 22 |
| pT4                              | 2 |
| N category                       |  |
| 0                                | 14 |
| 1                                | 13 |
| M category                       |  |
| 0                                | 25 |
| 1                                | 2 |
| Neoadjuvant chemotherapy         |  |
| (−)                              | 12 |
| (+)                              | 15 |
| Recurrence                       |  |
| (−)                              | 11 |
| (+)                              | 16 |
| Normal pancreas tissue           | 14 |

Table 2. Characteristics of patients included in the immunohistochemistry

| Pancreatic ductal adenocarcinoma |  |
|----------------------------------|---|
| Total number                     | 37 |
| Median age (range), years        | 66.9 (44–85) |
| Gender                           |  |
| Male                             | 21 |
| Female                           | 16 |
| T category                       |  |
| pTis                             | 0 |
| pT1                              | 0 |
| pT2                              | 2 |
| pT3                              | 26 |
| pT4                              | 9 |
| N category                       |  |
| 0                                | 14 |
| 1                                | 23 |
| M category                       |  |
| 0                                | 37 |
| 1                                | 0 |
| Neoadjuvant chemotherapy         |  |
| (−)                              | 37 |
| (+)                              | 0 |
| Recurrence                       |  |
| (−)                              | 8 |
| (+)                              | 29 |
were inhibited in si-ZFP36L2 transfectants compared with mock-control or siRNA-control-transfected cells (Fig. 5c–e).

Expression of ZFP36L2 in pancreatic ductal adenocarcinoma clinical specimens. We confirmed the expression of ZFP36L2 in PDAC clinical specimens using immunohistochemistry. A total of 37 specimens were evaluated, and 13 samples were classified as having high expression of ZFP36L2 (Fig. 6a–c). Clinicopathological characteristics are summarized in Table 2.

Table 3. Candidate target genes regulated by miR-375 in pancreatic ductal adenocarcinoma

| Entrez gene ID | Gene symbol | Description | Microarray (Log2 ratio) miR-375 | Target site (miRanda) |
|----------------|-------------|-------------|-------------------------------|-----------------------|
| 23705          | CADM1       | Cell adhesion molecule 1 | Panc-1: -3.86, SW1990: -5.17, Average: -4.51 | (-) |
| 9456           | HOMER1      | Homer scaffolding protein 1 | Panc-1: -1.76, SW1990: -1.77, Average: -1.76 | (-) |
| 8140           | SLC7A5      | Solute carrier family 7 member 5 | Panc-1: -1.51, SW1990: -1.52, Average: -1.52 | (-) |
| 85453          | TSPYL5      | TSPY-like 5 | Panc-1: -1.38, SW1990: -1.38, Average: -1.38 | (-) |
| 678            | ZFP36L2     | ZFP36 ring finger protein-like 2 | Panc-1: -1.20, SW1990: -1.04, Average: -1.12 | (-) |
| 114794         | ELFN2       | Extracellular leucine-rich repeat and fibronectin type III domain containing 2 | Panc-1: -1.00, SW1990: -1.13, Average: -1.07 | (+) |

Fig. 3. Expression of putative miR-375 target genes in pancreatic ductal adenocarcinoma (PDAC) clinical specimens and cell lines. (a) Expression levels of ZFP36L2 in clinical specimens and PDAC cell lines were determined using quantitative RT-PCR (qRT-PCR). Data were normalized to GUSB expression. (b) Correlation between miR-375 and ZFP36L2 expression. (c) Expression levels of other putative miR375 target genes in clinical specimens and PDAC cell lines were determined using qRT-PCR. Data were normalized to GUSB expression.
Table 4 shows the correlation between ZFP3L2 expression and various clinicopathological factors. High ZFP3L2 expression was significantly associated with increased lymph node metastasis. Furthermore, patients with high ZFP3L2 expression had significantly shorter OS than those with low ZFP3L2 expression \( (P = 0.0167) \) (Fig. 6d). In addition, univariate and multivariate analysis showed that ZFP3L2 served as an independent prognostic factor for PDAC (Table 5).

**Investigation of downstream genes regulated by ZFP3L2 in pancreatic ductal adenocarcinoma cells.** To identify the
downstream genes regulated by ZFP36L2, genome-wide gene expression and in silico analyses were performed in a PDAC cell line (PANC-1) transfected with si-ZFP36L2. A total of 1287 genes were commonly downregulated (log2 ratio < -1.0) in si-ZFP36L2-transfected PANC-1 cells. We also assigned the downregulated genes to KEGG pathways using the GENECODIS program and identified 60 pathways as significantly enriched. The 5 most enriched pathways are presented in Table 6a. Of these 5 pathways, we focused on genes in the ‘cell cycle’ and ‘pathways in cancer’ pathways. The genes composing these 2 pathways are listed in Table 6b,c. Furthermore, we checked the expression status of these genes and
pathologic relations of the PDAC by using TCGA-based large cohort study data (Table 6b,c). We applied the OncoLnc database using 87 pancreatic adenocarcinoma samples (http://www.oncolnc.org/). Clinical outcome for patients with high expression of \textit{CCNB1} and \textit{CCNB2} or low expression of these genes are displayed as Kaplan–Meier plots with log-rank tests (Fig. S4).\cite{27}

**Discussion**

Most patients with PDAC already have advanced or metastasized cancer at the time of first diagnosis. The prognosis for patients with advanced stage PDAC is extremely poor, and there are few effective treatments to date.\cite{28} An oncogenic \textit{KRAS} mutation is frequently observed in patients with PDAC and leads to constitutive activation of \textit{KRAS} downstream signaling pathways.\cite{29} Many studies have failed to directly

**Table 4. Correlation between the expression of ZFP36L2 and clinicopathological factors in pancreatic ductal adenocarcinoma (n = 37)**

| Characteristic                  | ZFP36L2                 | P       |
|--------------------------------|-------------------------|---------|
|                                | Low (n = 24)            | High (n = 13) | |
| Age (<60)                      | 15                      | 11      | NS     |
| Gender (Male)                  | 13                      | 8       |        |
| Tumor size (>40 mm)            | 4                       | 5       | NS     |
| Lymph node metastasis (No)     | 12                      | 2       | 0.0382 |
| TNM Stage (I/II)               | 17                      | 10      | NS     |
| Recurrence (No)                | 7                       | 1       | NS     |
| ZFP36L2                        | 0.383                   | 0.186–0.788 | 0.0091 |

**Table 5. Univariate analysis and multivariate analysis in pancreatic ductal adenocarcinoma**

| Characteristic                  | Hazard ratio | 95% CI       | P-value |
|--------------------------------|--------------|--------------|---------|
| Age                            | 0.971        | 0.490–1.926  | 0.9333  |
| Gender                         | 0.405        | 0.206–0.795  | 0.0086  |
| Tumor size                     | 1.544        | 0.737–3.236  | 0.2499  |
| Lymph node metastasis (No)     | 0.584        | 0.294–1.161  | 0.1248  |
| TNM stage                      | 2.321        | 1.174–4.587  | 0.0154  |
| ZFP36L2                        | 0.383        | 0.186–0.788  | 0.0091  |

**Discussion**

Most patients with PDAC already have advanced or metastasized cancer at the time of first diagnosis. The prognosis for patients with advanced stage PDAC is extremely poor, and there are few effective treatments to date.\cite{28} An oncogenic \textit{KRAS} mutation is frequently observed in patients with PDAC and leads to constitutive activation of \textit{KRAS} downstream signaling pathways.\cite{29} Many studies have failed to directly
Table 6. (a) Top 5 enriched KEGG pathways downregulated by si-ZFP36L2; (b) Regulation of genes related to the cell cycle; (c) Regulation of genes related to the pathways in cancer

(a) Number of genes Pathway name P-value
26 Cell cycle 2.25E-16
20 Oocyte meiosis 1.23E-11
30 Pathways in cancer 4.32E-09
14 Small cell lung cancer 4.67E-08
14 Progesterone-mediated oocyte maturation 6.36E-08

(b) Cell cycle

| Entrez gene ID | Gene symbol | Gene name | PANC-1 log2 ratio | GEO data (GSE 15471) | OncoLnc P-value |
|---------------|-------------|-----------|-------------------|-----------------------|----------------|
| 8318          | CDC45       | Cell division cycle 45 | -2.02             | 2.97                  | 0.00269        |
| 8243          | SMC1A       | Structural maintenance of chromosomes 1A | -1.59             | 11.23                 | NS             |
| 7029          | TFD2        | Transcription factor Dp-2 (E2F dimerization partner 2) | -1.57             | No data               | 0.00226        |
| 5933          | RBL1        | Retinoblastoma-like 1 | -1.49             | No data               | NS             |
| 5347          | PLK1        | Polo-like kinase 1     | -1.38             | No data               | 0.00729        |
| 1021          | CDK6        | Cyclin-dependent kinase 6 | -1.36             | 2.67                  | <0.001         |
| 4087          | SMAD2       | SMAD family member 2   | -1.36             | No data               | NS             |
| 10744         | PTTG2       | Pituitary tumor-transforming 2 | -1.35             | No data               | No data        |
| 9133          | CNB2        | Cyclin B2              | -1.35             | 6.78                  | <0.001         |
| 25847         | ANAPC13     | Anaphase promoting complex subunit 13 | -1.28             | No data               | NS             |
| 7042          | TGFB2       | Transforming growth factor, beta 2 | -1.25             | 2.62                  | NS             |
| 996           | CDC27       | Cell division cycle 27 | -1.21             | 2.96                  | NS             |
| 4193          | MDM2        | MDM2 proto-oncogene, E3 ubiquitin protein ligase | -1.20             | 2.35                  | NS             |
| 9700          | ESPL1       | Extra spindle pole bodies homolog 1 (S. cerevisiae) | -1.19             | No data               | NS             |
| 1869          | E2F1        | E2F transcription factor 1 | -1.17             | No data               | 0.00152        |
| 9232          | PTTG1       | Pituitary tumor-transforming 1 | -1.15             | No data               | NS             |
| 983           | CDK1        | Cyclin-dependent kinase 1 | -1.14             | 2.11                  | 0.00137        |
| 10735         | STAG2       | Stromal antigen 2      | -1.13             | No data               | NS             |
| 699           | BUB1        | BUB1 mitotic checkpoint serine/threonine kinase | -1.13             | 2.61                  | 0.00515        |
| 891           | CCNB1       | Cyclin B1              | -1.11             | 4.77                  | 0.00710        |
| 4174          | MCM5        | Minichromosome maintenance complex component 5 | -1.10             | No data               | NS             |
| 995           | CDC25C      | Cell division cycle 25C | -1.08             | No data               | 0.00457        |
| 51434         | ANAPC7      | Anaphase promoting complex subunit 7 | -1.06             | No data               | NS             |
| 6502          | SKP2        | S-phase kinase-associated protein 2, E3 ubiquitin Protein ligase | -1.05             | No data               | NS             |
| 10971         | YWHAQ       | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta | -1.04             | 2.51                  | NS             |
| 10274         | STAG1       | Stromal antigen 1      | -1.01             | No data               | NS             |

(c) Pathways in cancer

| Entrez gene ID | Gene symbol | Gene name | PANC-1 log2 ratio | GEO data (GSE 15471) | OncoLnc P-value |
|---------------|-------------|-----------|-------------------|-----------------------|----------------|
| 4312          | MMP1        | Matrix metalloproteinase 1 (interstitial collagenase) | -3.08             | 11.23                 | NS             |
| 6513          | SLC2A1      | Solute carrier family 2 (facilitated glucose transporter), member 1 | -2.26             | 2.67                  | 0.02410        |
| 1956          | EGF8        | Epidermal growth factor receptor | -2.06             | No data               | 0.00674        |
| 7422          | VEGFA       | Vascular endothelial growth factor A | -1.97             | No data               | NS             |
| 5290          | PIK3CA      | Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha | -1.82             | No data               | NS             |
| 1499          | CTNNB1      | Catenin (cadherin-associated protein), beta 1, 88 kDa | -1.66             | 2.35                  | NS             |
| 3918          | LAMC2       | Laminin, gamma 2 | -1.65             | 6.78                  | NS             |
| 3655          | ITGA6       | Integrin, alpha 6 | -1.56             | No data               | 0.0388         |
| 3675          | ITGA3       | Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor) | -1.42             | 2.51                  | <0.001         |
| 5291          | PIK3CB      | Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit beta | -1.40             | No data               | 0.00681        |
| 207           | AKT1        | v-akt murine thymoma viral oncogene homolog 1 | -1.40             | No data               | NS             |
| 3910          | LAMA4       | Laminin, alpha 4 | -1.37             | 4.77                  | NS             |
| 1021          | CDK6        | Cyclin-dependent kinase 6 | -1.36             | No data               | <0.001         |
| 4087          | SMAD2       | SMAD family member 2 | -1.36             | No data               | NS             |
| 6772          | STAT1       | Signal transducer and activator of transcription 1 | -1.33             | 2.96                  | 0.01520        |
| 8453          | CUL2        | Cullin 2 | -1.29             | No data               | NS             |
inhibit activation of KRAS, suggesting that KRAS is a non-druggable target in human cancers.\(^{29}\) To develop new treatment strategies for the disease, it is necessary to elucidate the molecular pathogenesis of PDAC aggressiveness using current genomic approaches.

Substantial evidence has demonstrated that aberrant expression of miRNA is deeply involved in human cancer pathogenesis, including that of PDAC.\(^{14,30}\) Functionally, miRNA fine-tune expression of protein-coding and non-coding RNA in human cells.\(^{31}\) Therefore, aberrantly expressed miRNA lead to the collapse of tightly regulated RNA networks in cancer cells. Recently, we summarized the aberrant expression of miRNA in PDAC cells.\(^{32}\) Several miRNA were significantly downregulated in PDAC cells, such as miR-200c, miR-141, miR-148a, miR-375 and miR-29c. Recent studies have also demonstrated that these miRNA function as antitumor miRNA in PDAC.\(^{32,34}\) For example, expression of miR-217 was significantly reduced in PDAC tissues and cell lines and directly targeted KRAS mRNA.\(^{32}\) It is well known that miR-200c/miR-141 form a miRNA cluster and inhibit epithelial–mesenchymal transition (EMT) in cancer cells.\(^{35,36}\) In PDAC, miR-200c and miR-141 directly bind to the 3'-UTR of ZEB1 mRNA and TGF\(_B\) mRNA, respectively, and inhibit cell invasion and migration.\(^{33}\) MiR-148a has been reported to be associated with DNA methylation in malignant tumors, including PDAC.\(^{34}\)

Our previous studies showed that expression of miR-375 was markedly reduced in several types of cancers and functions as an antitumor miRNA.\(^{37-39}\) Other studies confirm the antitumor function of miR-375 in cancer.\(^{40-42}\) In contrast to these antitumor activities, expression of miR-375 was upregulated in pediatric acute myeloid leukemia and prostate cancer, suggesting that miR-375 acts as an oncogenic miRNA in these diseases.\(^{42,43}\) The dual function of miR-375 is very unique; thus, it is important to identify miR-375-regulated pathways in various cancer types. In this study, we focused on miR-375 and miR-375-mediated oncogenic pathways in PDAC. Previous studies of miR-375 in PDAC indicated that pyruvate dehydrogenase kinase, isozyme 1 (PDK1) is a regulatory target of miR-375. PDK1 is a key component in the phosphatidylinositol 3-kinase-Akt-mammalian target of rapamycin (PI3K-Akt-mTOR) signaling pathway and has been shown to inhibit proliferation and promote apoptosis in PDAC cells.\(^{19,44}\)

In this study, we showed that ectopic expression of miR-375 significantly suppressed cancer cell aggressiveness and confirmed the antitumor function of miR-375 in PDAC cells. Moreover, we identified that ZFP36L2 was directly regulated by antitumor miR-375 in PDAC cells. ZFP36L2 is zinc finger protein 36, CSH type-like 2 (also known as Efz2, Efz2 and Tis11D).\(^{22}\) ZFP36L2 directly binds to the AU-rich element (ARE) in the 3'-UTR of the target mRNA and regulates the expression of target mRNA.\(^{21,22}\)

According to the previous studies, ZFP36 has antitumor function in several types of cancers.\(^{22,45}\) Past studies have shown that deletion of ZFP36L2 induced Notch1-dependent T cell acute lymphoblastic leukemia in mice, and a frameshift mutation in the ZFP36L2 gene was identified in several types of leukemic cells.\(^{46}\) In leukemic cells, a heterozygous frameshift mutation of ZFP36L2/TIS11D gene was detected in a patient with acute myeloid leukemia.\(^{47}\) Moreover, overexpression of wild-type of ZFP36L2/TIS11D gene inhibited growth of HeLa cells.\(^{47}\) In colorectal cancer, expression of ZFP36 was significantly reduced in cancer tissues and restored ZFP36 expression inhibited epithelial-to-mesenchymal transition and induces a higher susceptibility to anoikis.\(^{48}\) These studies indicate that ZFP36L2 acts as a tumor suppressor in human cancers.

In contrast to previous studies, we showed that overexpression of ZFP36L2 was detected in PDAC clinical specimens and knockdown of ZFP36L2 significantly inhibited cancer cell aggressiveness in PDAC cell lines. Furthermore, high expression of ZFP36L2 is significantly associated with lymph node metastasis and poor prognosis of patients with PDAC. Our data demonstrated that ZFP36L2 functions as an

### Table 6 (Continued)

| Entrez gene ID | Gene symbol | Gene name | PANCl-1 log2 ratio | GEO data (GSE 15471) | OncoLnc P-value |
|---------------|-------------|-----------|-------------------|----------------------|----------------|
| 7042          | TGFβ2       | Transforming growth factor, beta 2 | -1.25 | 2.62 | NS |
| 5899          | RALB        | v-ral simian leukemia viral oncogene homolog B | -1.24 | No data | -0.001 |
| 4193          | MDM2        | MDM2 proto-oncogene, E3 ubiquitin protein ligase | -1.20 | No data | NS |
| 7477          | WNT7B       | Wingless-type MMTV integration site family, member 7B | -1.19 | No data | NS |
| 208           | AKT2        | v-akt murine thymoma viral oncogene homolog 2 | -1.18 | No data | NS |
| 1869          | E2F1        | E2F transcription factor 1 | -1.17 | No data | 0.00152 |
| 355           | FAS         | Fas cell surface death receptor | -1.13 | 2.97 | 0.03640 |
| 330           | BIRC3       | Baculoviral IAP repeat containing 3 | -1.13 | 2.61 | NS |
| 4824          | NKK3-1      | NK3 homeobox 1 | -1.11 | No data | NS |
| 3091          | HIF1A       | Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor) | -1.10 | 2.11 | NS |
| 6502          | SKP2        | S-phase kinase-associated protein 2, E3 ubiquitin protein ligase | -1.05 | No data | NS |
| 5728          | PTEN        | Phosphatase and tensin homolog | -1.05 | No data | NS |
| 2250          | FGFR5       | Fibroblast growth factor 5 | -1.02 | No data | 0.04710 |
| 7186          | TRAF2       | TNF receptor-associated factor 2 | -1.01 | No data | NS |

NS, not significant. OncoLnc, OncoLnc database.
oncogene in PDAC cells and is deeply involved in PDAC pathogenesis.

To investigate the oncogenic function of ZFP36L2 in PDAC cells, we identified ZFP36L2-regulated PDAC pathways using genome-wide gene expression analysis of si-ZFP36L2-transfected cells. Downstream genes modulated by ZFP36L2 were categorized by KEGG pathways. Our data showed that “cell cycle” and “pathways in cancer” pathways were downregulated by ZFP36L2. Recent study showed that ZFP36L2 and SLC2A1 were critical roles for developing B lymphocytes regulating cell cycle pathways. Moreover, overexpression of EGFR, LAMC2, ITGA6 and ITGA3 was observed in several cancers, including PDAC, and these genes were involved in enhancing EMT and cancer cell migration and invasion. Furthermore, SLC2A1 is also known as GLUT-1 and regulates the entry of glucose into cells. High expression of SLC2A1 is associated with higher histological grade and larger tumor size. Overexpression of SLC2A1 increases MMP-2 expression and enhances cancer cell invasion. These studies have supported our present data of knockdown of ZFP36L2 in PDAC cells.

The primary finding of the present study is the overexpression of ZFP36L2 and several ZFP36L2-regulated genes that are involved in the pathogenesis of PDAC. A TCGA-based large cohort study and gene expression data indicated that high expression of 10 genes (CDC45, CDK6, CCNB2, CDK1, BUB1, CCNB1, SLC2A1, ITGA3, STAT1 and FAS) predicted poorer survival of PDAC patients. These findings showed that ZFP36L2-mediated pathways are deeply involved in PDAC pathogenesis. The identification of the downstream genes regulated by the miR-375/ZFP36L2 axis may lead to a better understanding of PDAC aggressiveness.

In conclusion, downregulation of miR-375 was validated in PDAC clinical specimens, and miR-375 was shown to function as an antitumor miRNA in PDAC cells. To the best of our knowledge, this is the first report demonstrating that ZFP36L2 is directly regulated by antitumor miR-375 and acts to regulate several oncogenic genes. Expression of ZFP36L2 might be a useful prognostic marker for survival of PDAC patients. The identification of novel molecular pathways and targets regulated by the miR-375/ZFP36L2 axis may lead to a better understanding of PDAC progression and aggressiveness.

Disclosure Statement
The authors have no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. The association between the expression levels of miR-375 with clinicopathological parameters.

Fig. S2. Effects of miR-375 inhibition on pancreatic ductal adenocarcinoma (PDAC) cell lines.

Fig. S3. Regulation of putative target genes by miR-375 in pancreatic ductal adenocarcinoma (PDAC) cells.

Fig. S4. The association between the expression level of CCNB1 or CCNB2 and overall survival.