Zinc is an essential trace element and catalytic/structural component utilized by many enzymes and transcription factors that contain zinc finger motifs (1, 2). Zinc is essential for growth, and zinc deficiency in animals leads to growth retardation, impaired DNA synthesis, and severe dermatitis (3). Recent studies have shown that zinc availability is also important for tumor growth and progression because zinc is a critical component for many enzymes involved in hypoxia, angiogenesis, cell proliferation, and metastasis of cancers (4, 5). On the other hand, high concentrations of zinc are toxic, so cells have evolved a complex system to maintain the balance of zinc uptake, intracellular storage, and efflux (6, 7). Two solute-linked carrier (SLC) gene families were identified as zinc transporters: SLC30, which encodes for ZnT proteins, and SLC39, which encodes for ZIP proteins (7–9). They seem to have opposite roles in cellular zinc homeostasis. ZnT transporters reduce intracellular zinc availability by promoting zinc efflux from cells or into intracellular vesicles, whereas ZIP transporters increase intracellular zinc availability by promoting extracellular zinc uptake and vesicular zinc release into the cytoplasm (10). Altered expression of zinc transporters is associated with many diseases, including cancers. Recently, low levels of ZnT1 have been observed in mammary gland tumor cells. The zinc concentration in these cells is also higher than that in normal cells, which suggests that zinc transport is misregulated in these proliferating tumor cells, and zinc availability might be essential for tumor growth, and zinc deficiency in animals leads to growth retardation. The stable cells were characterized in vitro and in vivo using a nude mouse xenograft model.

Experimental Design: ZIP4 stable silencing was established in pancreatic cancer cell lines ASPC-1 (ASPC-shZIP4) and BxPC-3 (BxPC-shZIP4) by short hairpin RNA using retrovirus vectors. The stable cells were characterized in vitro and in vivo using a nude mouse xenograft model.

Results: Silencing of ZIP4 was associated with decreased cell proliferation, migration, and invasion. Both ASPC-shZIP4 and BxPC-shZIP4 cells showed a significant reduction in tumor volume and weight in the s.c. model, and decreased primary tumor weight in the orthotopic model compared with the vector control cells (ASPC-shV and BxPC-shV). Silencing of ZIP4 also caused reduced incidence of tumor metastasis in the mice and downregulated the tumor grade. More importantly, silencing of ZIP4 significantly increased the survival rate of nude mice with orthotopic xenografts (F = 0.005). All ASPC-shZIP4-injected mice (100%) remained alive up to 32 days after tumor implantation, whereas only 30% of the ASPC-shV mice were alive at the same time point. CyclinD1 expression was decreased in the ASPC-shZIP4 xenografts.

Conclusions: These results identify a previously uncharacterized role of ZIP4 in pancreatic cancer progression, and indicate that knocking down ZIP4 by short hairpin RNA might be a novel treatment strategy for pancreatic cancers with ZIP4 overexpression.

Translational Relevance

We have found that a zinc transporter, ZIP4, is overexpressed in human pancreatic cancer and contributes to tumor progression. In the current study, we found that silencing of ZIP4 using short hairpin RNA is associated with decreased cell proliferation, migration, and invasion in vitro, and with significant reduction in tumor growth in a nude mouse xenograft model. More importantly, silencing of ZIP4 significantly increased the survival rate of nude mice with orthotopic xenografts. These results identify a previously uncharacterized role of ZIP4 in pancreatic cancer progression, and indicate that ZIP4 could be a promising marker for pancreatic cancer. Knocking down ZIP4 by short hairpin RNA might be a novel treatment strategy for pancreatic cancers with ZIP4 overexpression.

cell growth (11). In another study, ZIP6 (also known as LIV-1), a breast cancer–associated protein that belongs to a new subfamily (LZT subfamily) of ZIP transporters, has been associated with estrogen-positive breast cancer and metastasis to lymph nodes (12). Similarly, Kagara et al. found that zinc and the transporter ZIP10 were involved in invasive behavior of breast cancer cells (13). Our previous study also indicated that ZIP4 may play an important role in pancreatic cancer (14). These reports thus suggest a positive correlation among zinc, zinc importers, and cancer progression.

ZIP4, encoded by the SLC39A4 gene, plays an important role in maintaining the cellular zinc level by facilitating uptake of dietary zinc into intestinal epithelial cells, and releasing zinc from vesicular compartments (1, 6, 15). Mutations in the SLC39A4 gene are thought to be the cause of acrodermatitis enteropathica, a genetic disorder of zinc deficiency (2, 16). Our recent study indicated that ZIP4 is overexpressed in human pancreatic cancer, and aberrant expression of ZIP4 contributes to pancreatic cancer growth (14). As the fourth leading cause of cancer-related deaths in North America, pancreatic cancer has the highest fatality rate of all cancers. Survival statistics are poor because there are no reliable tests for early diagnosis and no effective therapies for the metastatic form of pancreatic cancer (17–19). There is a pressing need to understand more about the pathogenesis and to develop effective prevention and treatment of pancreatic cancer. Recently, more attention has been given to diet and nutrition prevention in cancer therapy, including that for pancreatic cancer (20–23). Epidemiology studies on patient diet and nutrition uptake have indicated that pancreatic cancer patients do better on a largely plant-based diet that contains low zinc (20). Tested subjects who ate the most processed meat or most red meat (high-zinc diet) had a 68% or 50% greater risk of having pancreatic cancer, respectively, compared with those who ate the least processed meat and red meat (20). These results provide a diet recommendation for pancreatic cancer patients with a low-zinc diet. Considering the importance of zinc in many enzymes for tumor growth and metastasis, it is of great interest to investigate the role and mechanisms of ZIP4 in pancreatic cancer pathogenesis. In this study, we examined the effect of silencing ZIP4 in human pancreatic cancer both in vitro and in vivo. This study suggests a new target for pancreatic cancer therapy.

Materials and Methods

Chemicals and cell culture. The human pancreatic cancer cell lines ASPC-1 and BxPC-3 were purchased from the American Type Culture Collection, and were cultured in RPMI 1640 medium with 10% fetal bovine serum as previously described (24, 25). The human ZIP4 (hZIP4) antibody was generated in rabbits against a KLH-conjugated 14 aa synthetic peptide and affinity purified basically as described previously (15). Other chemicals were from Sigma.

ZIP4 mRNA detection. The ZIP4 mRNA was analyzed by real time reverse transcriptase-PCR as previously described (14, 24). Briefly, real-time PCR was done using the SYBR supermix kit (Bio-Rad). PCR reaction included the following components: 100 nmol/L each primer, diluted cDNA templates and iQ SYBR Green supermix, and running for 40 cycles at 95°C for 20 s and 60°C for 1 min. PCR efficiency was examined by serially diluting the template cDNA, and the melting curve data were collected to check PCR specificity. Each cDNA sample was run as triplicate and the corresponding reverse transcriptase mRNA sample was included as a negative control. The β-actin primer was included in every plate to avoid sample variations. The relative mRNA level was presented as unit values of 2−ΔΔCt (gene of interest)/ΔCt(β-actin) − Ct(gene of interest). The primer sequences for the human ZIP4 gene (SLC39A4) were: sense 5′ATGTGACGAGCGGCTGTGC3′; and antisense 5′ GCTGCTGTGCTCCTGGAAC 3′.

Stable cell line selection. ZIP4 short hairpin RNA (shRNA) expressing stable cells were selected in ASPC-1 and BxPC-3 cells with retrovirus vectors (Origene), following the manufacturer’s instructions. The sequence of the ZIP4 shRNAs used in this study was as follows: 5′ AACGACACCTGCGACATGGTCAGGATG 3′. Briefly, ZIP4-shRNA constructs were cotransfected into 293T cells with packing plasmids. Viral supernatants were collected and transduced to the target cells. Stable cell lines expressing ZIP4 shRNA (ASPC-shZIP4) and BxPC-shZIP4) or empty vector (ASPC-sh and BxPC-sh) were selected with the addition of 0.5 μg/mL of puromycin into the medium. Three stable cells expressing different shRNAs were selected.

Western blot analysis. ASPC-sh and ASPC-shZIP4 cells were lysed with ice-cold lysis buffer (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L Na,EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L [γ-32P] glycerophosphate, 1 mmol/L Na,VO4, 1 μg/ml leupeptin, and protease inhibitor cocktail) for 30 min in ice. Cell lysates were then collected after centrifugation at 12,000 rpm for 5 min at 4°C. Sixty micrograms of lysate protein were loaded and total cellular protein was separated with 15% SDS-PAGE and then transblotted overnight at 4°C onto Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences). The membrane was probed with anti-ZIP4 (1:500) or anti-β-actin (1:3,000) antibody at room temperature for 1 h and then washed three times with 0.1% Tween 20-TBS and incubated in a horseradish peroxidase–linked secondary antibody (1:2,000) for 1 h at room temperature. The membrane was washed three times with 0.1% Tween 20-TBS and the immunoreactive bands were detected by using enhanced chemiluminescence reagent kit.

Cell proliferation assay. Cell proliferation was analyzed with the MTS assay. Stable ASPC-1 or BxPC-3 cells were seeded in 96-well plates (2 × 103 cells/well) and serum-starved (0% fetal bovine serum) for 24 h. Cell growth was assessed 1, 2, 3, 4, and 5 days after serum starvation. For zinc-dependent assay, ASPC-sh and ASPC-shZIP4, or BxPC-sh and BxPC-shZIP4 cells were treated with a membrane-permeable metal chelator, N,N′,N′,N′-tetakis(2-pyridylmethyl) ethylenediamine (TPEN), at 4 μmol/L for 1 h at 37°C. Cells were washed with PBS to remove excess TPEN because TPEN is toxic to cells with long-time incubation. Cells were then incubated with DMEM in the presence of 0, 1, and
Silencing of ZIP4 Inhibits Pancreatic Cancer Growth

Results

Silencing of ZIP4 inhibits pancreatic cancer cell proliferation, migration, and invasion. Our previous study had indicated that ZIP4 expression was differentially higher in most human pancreatic cancer cell lines compared with that in normal human pancreatic duct epithelial cells (14). We selected ASPC-1 cells to silence ZIP4 by shRNA because parental ASPC-1 cells express more ZIP4 than other pancreatic cancer cell lines. We also selected another pancreatic cancer cell line, BxPC-3, which expresses intermediate levels of ZIP4, to silence ZIP4 by shRNA as comparison. Three stably overexpressing ZIP4 shRNA cell lines that contained different shRNA sequences were established in both ASPC-1 and BxPC-3 cells (ASPC-shZIP4 or BxPC-shZIP4) using a retrovirus vector (Origene). Stable cells containing empty vectors (ASPC-shV or BxPC-shV) were also established in ASPC-1 and BxPC-3 cells as controls. Silencing of ZIP4 in the three ASPC-shZIP4 or BxPC-shZIP4 cell lines was confirmed when compared with the ASPC-shV or BxPC-shV controls by real time PCR, Western blotting, and flow cytometry. ZIP4 silencing in a representative ASPC-shZIP4 stable cell line is shown in Fig. 1A and B (71% reduction in mRNA level; \( P < 0.05 \)). ZIP4 silencing in a representative BxPC-shZIP4 stable

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**In vitro migration and invasion assays.** The cell migration/invasion was determined using a modified Boyden chamber assay. Uncoated polycarbonate inserts (BD Biosciences) of 8-μm pore size were either used uncoated or were coated with 100 μL of 100 μg/mL of Matrigel (BD Biosciences). Cells trypsinized and resuspended in growth media (10^5 cells/200 μL) were added into the upper compartment of an invasion (Matrigel-coated insert) or migration (uncoated insert) chamber, and 600 μL of the same growth media were added into the lower chamber. After 24 h (for migration) and 48 h (for invasion) the cells were incubated in Calcein-AM (Molecular Probes) for 1 h at 37°C before fixation. The fluorescence was read from the bottom at an excitation wavelength of 495 nm and emission wavelength of 520 nm. Cells in the upper chamber were then removed, and cells that had migrated or invaded onto the lower surface of the membrane were quantified. The migration/invasion rate was presented as the ratio of the mean fluorescence reading after scraping of the cells divided by the reading before removing the top cells.

**Cancer mouse models.** Subconfluent ASPC-shV and ASPC-shZIP4, or BxPC-shV and BxPC-shZIP4 cells were harvested by trypsinization, and resuspended in RPMI 1640. Only single-cell suspensions with >95% viability were used. The cells (3 \times 10^6) were inoculated either into the right flank (s.c. tumor model) or the body of the pancreas (orthotopic tumor model) of 5- to 6-week-old male nude mice (NIC-Charles River). All mice were cared for in accordance with the Office for Protection from Research Risks (OPRR) and Animal Welfare Act guidelines under an animal protocol approved by Baylor College of Medicine Institutional Animal Care and Use Committee. For the s.c. tumor model, the tumor size was measured weekly using a digital caliper (VWR international), and the tumor volume was determined with the formula: tumor volume \( [\text{mm}^3] = \text{length } [\text{mm}] \times \text{width } [\text{mm}]^2 \times 0.52 \). For intrapancreatic injections, the mice were anesthetized with 2.5% avertin and a 0.5- to 1-cm incision was made in the left subcostal region. The tumor cells (3 \times 10^6) in a volume of 50 μL were injected into the body of the pancreas. The peritoneum and skin were closed with a 4.0 surgical suture. After 4 wk, all surviving mice were euthanized with an overdose of CO2 exposure and evaluated macroscopically for the presence of orthotopic tumors and the metastases in the abdominal cavity. For both s.c. and orthotopic experiments, the animals were euthanized when their tumor size reached 2 cm in diameter or the animals became moribund during the observation period, and the time of euthanization was recorded as the time of mortality.

**Immunohistochemical staining.** Mouse orthotopic tumors were collected and processed into 5-μm slices. Fixed tissue slides were either H&E-stained, or incubated with anti-Ki67 or cyclin D1 antibodies (Biodesign International) for 30 min at 4°C, before 3,3′-diaminobenzidine visualization, and the sections were then mounted and observed under a phase contrast microscope as described previously (14).

**Statistical analysis.** Quantitative results are shown as means ± SD. The statistical analysis was done by Student’s t test between control and treatment groups. The Kaplan-Meier method was used to plot survival curves for the treatment and control groups. Because the proportional hazards assumption does not hold, the log rank test was not used to compare the survival curves. Instead, the \( P \) value for a two-sample test for proportions was reported at the 32-day time point. A value of \( P < 0.05 \) was considered statistically significant.
cell line is also indicated in Fig. 1C (71% reduction in mRNA level; \( P < 0.05 \)). Because of the low level of the endogenous ZIP4 protein in BxPC-3 cells, we were unable to detect the ZIP4 protein by Western blot. However, we used an alternative method, flow cytometry, to examine the ZIP4 expression in BxPC-3 cells, and we found that the percentage and the mean fluorescence of ZIP4-positive cells were decreased in BxPC-shZIP4 cells compared with those in BxPC-shV cells (data not shown).

To investigate whether silencing of ZIP4 affects the proliferation of pancreatic cancer cells, we compared the proliferation of ASPC-shV and ASPC-shZIP4, or BxPC-shV and BxPC-shZIP4 cells by using a MTS assay. Cells were cultured in zinc suboptimal conditions, in which the culture medium was preincubated with chelx 100 to chelate the zinc ion, and low concentration and high concentration of \( \text{ZnCl}_2 \) were added to the medium, respectively. As shown in Fig. 2A and B, at low concentration of zinc (0.5 \( \mu \text{mol/L} \)), ASPC-shV cells with wild-type ZIP4 showed a growth advantage over ASPC-shZIP4 cells with reduced ZIP4 expression. Silencing of ZIP4 in ASPC-shZIP4 cells was associated with 41% decreased cell proliferation by day 4, compared with that in ASPC-shV cells (\( P < 0.05; n = 5 \)). In high concentration of zinc (5 \( \mu \text{mol/L} \)), silencing of ZIP4 in BxPC-shZIP4 cells was associated with 35% decreased cell proliferation by day 4. These results indicate that zinc plays an important role in ZIP4 shRNA-mediated growth inhibition, and different pancreatic cancer cell lines respond to different ranges of zinc supplement.

To further study the functions of ZIP4 and to determine the effects of silencing of ZIP4 in vitro, we did cell migration and invasion assays. As shown in Fig. 2C and D, silencing of ZIP4 significantly decreased the cell migrative and invasive abilities of ASPC-1 cells by 68% and 81%, respectively, compared with those of ASPC-shV cells (\( P < 0.05 \)). In BxPC-3 cells, silencing of ZIP4 also decreased the cell migrative and invasive abilities of BxPC-3 cells by 14% and 9%, respectively, compared with...
those of BxPC-shV cells. These results indicate that ZIP4 plays an important role in pancreatic cancer cell migration and invasion in vitro, which are essential features of pancreatic cancer progression.

Silencing of ZIP4 inhibits pancreatic cancer growth in the nude mouse model of s.c. xenograft. We further analyzed the role of ZIP4 on tumor growth in vivo using an immunodeficient nude mouse model. ASPC-shZIP4 cells showed a delayed onset of tumor growth and a dramatic decrease (62%) in tumor volume after 4 weeks compared with the ASPC-shV control cells in the s.c. tumor model ($P < 0.001; n = 10$; Fig. 3A). ASPC-shZIP4 also significantly reduced s.c. tumor weight after 4 weeks compared with ASPC-shV control cells ($P < 0.05; n = 10$; Fig. 3B). Mice given injections of ASPC-shV cells had severe skin ulcer (90%) and loss of body weight (50%), whereas only 50% of ASPC-shZIP4 control mice showed mild skin ulcer, but no other symptoms. BxPC-shZIP4 cells also showed a delayed onset of tumor growth and a dramatic decrease (67%) in tumor volume after 4 weeks compared with the BxPC-shV control cells in the s.c. tumor model ($P < 0.05; n = 10$; Fig. 3C). BxPC-shZIP4 also reduced s.c. tumor weight (58%) after 4 weeks compared with BxPC-shV control cells (Fig. 3D).

Silencing of ZIP4 inhibits pancreatic cancer growth and down-sizes the tumor grade in the nude mouse model of orthotopic xenograft. ASPC-shZIP4 cells inhibited orthotopic tumor weight by 34% compared with the ASPC-shV control cells in the orthotopic model (Fig. 4A). Furthermore, mice given injections of ASPC-shV cells showed significant loss of body weight (80%), multiple peritoneal dissemination (100%), jaundice (50%), liver metastasis (40%), lung metastasis (20%), colon/intestine obstruction (40%), and severe abdominal ascetic fluid (40%). On the contrary, only 40% of ASPC-shZIP4 mice showed mild peritoneal dissemination, but no other symptoms (Fig. 4A and Supplementary Table S1). Tumors from orthotopically injected

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**Fig. 3.** Effects of ZIP4 silencing on pancreatic cancer growth in the nude mouse model of s.c. xenograft. A, ASPC-shV and ASPC-shZIP4 cells (3 x 10^6) were s.c. inoculated into the right flank of nude mice (n = 10 per treatment group). Tumor size was measured weekly for 4 wk. Tumor volume was calculated by the formula: tumor volume [mm^3] = length [mm] x width [mm]^2 x 0.52. *, $P < 0.001$. B, S.c. tumor weight. The above mentioned s.c. tumor was dissected, and the tumor weight was recorded. Insets, a representative s.c. tumor mass from each group. *, $P < 0.05$. C, BxPC-shV and BxPC-shZIP4 cells (3 x 10^6) were s.c. inoculated into the right flank of nude mice (n = 10 per treatment group). Tumor size was measured weekly for 4 wk as described above. D, S.c. tumor weight. The above mentioned s.c. tumor was dissected, and the tumor weight was recorded as above.
mice were removed and processed for further histologic and immunohistochemical analysis. As shown in Fig. 4B and Table 1, the majority of the tumors from the ASPC-shV group were poorly differentiated or sarcomatoid, and the tumor area percentage was between 70% and 95% in most tumors, whereas most of the tumors from the ASPC-shZIP4 group were moderately differentiated, and the tumor area percentage was between 20% and 60%. Furthermore, tumors from ASPC-shZIP4-injected mice showed much decreased cell proliferation as indicated by the staining of Ki67, compared with that of the ASPC-shV mice (Fig. 4C). The stability of ZIP4 shRNA in orthotopic tumors from the ASPC-shZIP4 group was confirmed by real-time reverse transcriptase-PCR, and the expression of ZIP4 was decreased by 63% in the ASPC-shZIP4 group compared with that in the ASPC-shV group at 3 weeks after tumor implantation (data not shown). Similarly, silencing of ZIP4 in BxPC-3 cells also inhibited orthotopic tumor growth. The take rate of the orthotopic tumors in the BxPC-shZIP4 group was significantly decreased (from 60% to 20%) compared with the BxPC-shV group. BxPC-shZIP4 cells also inhibited orthotopic tumor weight compared with the BxPC-shV control cells (data not shown). These results indicate that ZIP4 could be a malignant factor that significantly contributes to pancreatic cancer growth in vivo.

Silencing of ZIP4 significantly increases the survival rate of nude mouse with orthotopic xenografts. To study the effect of
ZIP4 silencing in the survival of the mice, 10 nude mice were orthotopically implanted with ASPC-shV or ASPC-shZIP4 cells, and the survival rate was examined everyday up to 32 days. As shown in Fig. 4D, 100% of the ASPC-shZIP4 mice remained alive up to 32 days, whereas only 30% of the ASPC-shV mice were alive at the same point time. The survival rate was significantly higher in the ASPC-shZIP4 group at 32 days ($P = 0.005$). These results indicate that knocking down ZIP4 by shRNA might be an effective method to control pancreatic cancer growth, and represents a novel and potent treatment for ZIP4-overexpressed pancreatic cancer.

**Silencing of ZIP4 inhibits pancreatic cancer growth through decreasing the expression of cyclin D1.** In order to delineate the mechanisms of ZIP4 shRNA-induced tumor growth inhibition, we did a gene profiling study using the orthotopic tumor tissues as mentioned above. We found that cyclin D1 expression was significantly down-regulated in the ASPC-shZIP4 group compared with that in the ASPC-shV group (data not shown). Cyclin D1 is an important molecule controlling cell proliferation and cell cycle progression, and is also found to be overexpressed in pancreatic cancer. To confirm the gene profiling results, we examined the expression of cyclin D1 in the orthotopic xenografts by immunohistochemistry. As shown in Fig. 5A, tumor cells in the ASPC-shZIP4 group had significantly lower levels of cyclin D1 expression as compared with those in the ASPC-shV group. The percentage of cyclin D1–positive cells from 10 random fields in the ASPC-shZIP4 group was decreased by 53% (Fig. 5B). The down-regulation of cyclin D1 was also confirmed in the xenografts by real time reverse transcriptase-PCR and Western blot (41% reduction in cyclin D1 mRNA level; data not shown), and in the cell lines (64% reduction in cyclin D1 level in ASPC-shZIP4 cells, and 22% reduction in cyclin D1 level in BxPC-shZIP4 cells; Supplementary Fig. S2), indicating that the silencing of ZIP4 to inhibit pancreatic cancer growth may be through decreasing the expression of cyclin D1. Cyclin D1 is a downstream transcriptional target of NF-κB, which is overexpressed in pancreatic cancer. We examined the NF-κB expression and activation in ASPC-shZIP4 cells, and we found that the activation of NF-κB is significantly decreased when ZIP4 is silenced (Supplementary Fig. S3), which indicates that NF-κB signaling might be involved in ZIP4 silencing–induced growth inhibition of pancreatic cancer.

**Table 1. Summary of histologic staining of orthotopic tumors**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mouse ID</th>
<th>Tumor differentiation (Grading)</th>
<th>Tumor area percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASPC-shV</td>
<td>1</td>
<td>M/P</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>P/S</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>M/P</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>P/S</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>P</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>P/S</td>
<td>&gt;95</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>P/S</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>M</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>P/S</td>
<td>70</td>
</tr>
<tr>
<td>ASPC-shZIP4</td>
<td>10</td>
<td>M/P</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>M</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>M</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>M/P</td>
<td>50</td>
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<td></td>
<td>14</td>
<td>M</td>
<td>60</td>
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<tr>
<td></td>
<td>16</td>
<td>M</td>
<td>60</td>
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</table>

Abbreviations: M, moderately differentiated; P, poorly differentiated; S, sarcomatoid.

**Discussion**

Recent studies have indicated that zinc transport and zinc homeostasis play important roles in cancer progression, especially in pancreatic cancer and breast cancer (13, 14, 26). However, the molecular mechanism by which zinc transporters regulate cancer growth remains unknown, and it has not been described whether silencing of zinc transporters has any impact on tumor growth. In this study, we showed that silencing of ZIP4 was associated with decreased cell proliferation, migration, and invasion in two different pancreatic cancer cell lines. In the immune deficient mice with pancreatic cancer xenografts, silencing of ZIP4 resulted in a dramatic decrease in tumor volume, weight, and differentiation grade, and significantly increased the survival rate of nude mice with orthotopic xenografts. The inhibition of tumor growth by ZIP4 shRNA may be through decreasing the expression of cyclin D1. These results indicate that ZIP4 plays an important role in pancreatic cancer growth, and knocking down ZIP4 by shRNA might be a novel treatment for ZIP4-overexpressed pancreatic cancer.
Zinc is an essential factor for cell growth. Zinc deficiency is associated with diverse disorders, such as growth retardation, and delayed wound healing (3, 27). Previous studies suggest that zinc plays a critical role in cell proliferation and growth, possibly by influencing the DNA synthesis and cell cycle. However, a high concentration of zinc is toxic to the cells, and causes apoptosis (28, 29). Therefore, cells must have a fine homeostatic mechanism to maintain the intracellular zinc level within a narrow physiologic range through activity of the zinc transporters. In pancreatic cancer cells, zinc stimulates cell proliferation at low concentrations. We recently found a concentration-dependent increase of cell proliferation when <20 μmol/L of exogenous ZnCl₂ were added to Mia-ZIP4 cells, pancreatic cancer cells (Mia PaCa-2) that stably overexpress ZIP4. Higher concentrations of ZnCl₂ (>50 μmol/L) caused a dramatic decrease in cell proliferation (14). In ASPC-1 cells, the zinc concentration that stimulates cell proliferation is even lower. In zinc suboptimal conditions, when a low concentration of ZnCl₂ (0.5 μmol/L) was added, ASPC-shV cells with wild-type ZIP4 had a significant growth advantage over ASPC-shZIP4 cells with reduced ZIP4 expression. When incubated with a high concentration of zinc (5 μmol/L), no significant growth advantage was observed in ASPC-shV cells compared with ASPC-shZIP4 cells, probably because of the toxicity of high zinc to ASPC-1 cells. When the exogenous ZnCl₂ exceeded 5 μmol/L, both ASPC-shV and ASPC-shZIP4 cells died quickly after 2 days. Similarly, in another pancreatic cancer cell line, BxPC-3, exogenous zinc treatment (1 μmol/L and 5 μmol/L) caused BxPC-shV cells to proliferate faster than BxPC-shZIP4 cells. These results indicate that zinc and zinc transporter ZIP4 play important roles in pancreatic cancer cell proliferation, and different pancreatic cancer cell lines respond to different ranges of zinc supplement.

Silencing of ZIP4 was also associated with decreased cell migration and invasion, which strongly suggests that ZIP4 not only regulates pancreatic cancer cell proliferation, but also has an impact on cancer metastasis. The two cell lines we used in this study, ASPC-1 and BxPC-3, responded differently to the ZIP4 silencing. We found that silencing of ZIP4 in BxPC-3 cells inhibited cell proliferation in vitro and suppressed tumor growth in vivo, but only had mild inhibition on cell migration and invasion. We hypothesize that silencing of ZIP4 in BxPC-3 cells inhibits tumor growth mainly through suppressing cell proliferation. This is a little different from what we saw in another pancreatic cancer cell, ASPC-1, in which both cell proliferation and migration/invasion were significantly suppressed by ZIP4 silencing. These results indicate that silencing of ZIP4 inhibits tumor growth through different mechanisms in different pancreatic cancer cell lines. Previous studies have shown that another zinc transporter, ZIP10, is a malignant factor and is associated with metastatic phenotype of breast cancer cells. Depletion of intracellular zinc and silencing of ZIP10 in invasive breast cancer cells caused a decrease in the migratory activity of these cells, suggesting a positive correlation between zinc transport and breast cancer metastasis (13). Similarly, ZIP6 (LIV-1) has been indicated to play an important role in estrogen-positive, metastatic breast cancer (12). ZIP4, ZIP6, and ZIP10 are homologs of the nine-member LZT subfamily of ZIP transporters (10), and these studies suggest a novel function of zinc transporters of the LZT subfamily in cancer metastasis and progression.

Consequently, our study shows that silencing of ZIP4 caused an inhibition of pancreatic cancer growth in both s.c. and orthotopic xenografts of nude mice. Both ASPC-shZIP4 and BxPC-shZIP4 cells showed a delayed onset of tumor growth and a dramatic decrease in tumor volume and tumor weight in the s.c. model. In the orthotopic model, silencing of ZIP4 was associated with significant inhibition of orthotopic primary tumor growth, and reduction of the incidence of loss of body weight, peritoneal dissemination, jaundice, liver metastasis, lung metastasis, colon/intestine obstruction, and abdominal ascites fluid. Further histologic analysis indicated that silencing of ZIP4 led to reduced tumor grades, and thereby silencing of ZIP4 may sensitize the tumors to additional therapies.

Cyclin D1 plays a critical role in promoting cell proliferation by triggering cell cycle progression from G₁ to S phase. Increased expression of cyclin D1 is found in pancreatic cancer and is associated with poor prognosis and survival (30–32). Forced overexpression of cyclin D1 contributes to chemoresistance of pancreatic cancer cells, possibly due to the roles of cyclin D1 in promoting cell proliferation and inhibiting drug-induced apoptosis (32). In the current study, we show that silencing of ZIP4 caused significantly reduced expression of cyclin D1 in the orthotopic xenografts. Only 39% of the tumor cells were cyclin D1-positive in the ASPC-shZIP4 mice, as compared with 82% cyclin D1-positive tumor cells in the ASPC-shV mice (Fig. 5B). These data indicate that silencing of ZIP4 inhibits pancreatic cancer growth, maybe through decreasing the expression of cyclin D1. Cyclin D1 is a downstream transcriptional target of NF-κB, which is often overexpressed in pancreatic cancer. We found that the activation of NF-κB is significantly decreased when ZIP4 is silenced in ASPC-1 cells, which indicates that NF-κB signaling might be involved in ZIP4 silencing-induced growth inhibition of pancreatic cancer. In future studies, it would be interesting to elucidate the more detailed upstream and downstream signaling pathways of cyclin D1 and how ZIP4 regulates the expression of cyclin D1 in pancreatic cancer.

Another exciting finding of our study is that silencing of ZIP4 increased the survival rate in nude mice with orthotopic xenografts. These results indicate that knocking down ZIP4 by shRNA might be an effective method to control pancreatic cancer growth, and represents a novel and potent treatment for ZIP4-overexpressed pancreatic cancer. This strategy may also have a great potential in translating to clinical trials for the treatment of pancreatic cancer. By defining the ZIP4 profile in pancreatic cancer before and after therapy, we can use the ZIP4 profile to guide the choice of initial and sequential ZIP4-based therapies. Individuals with different expressions of ZIP4 may respond to the ZIP4 shRNA therapy differently. Considering the short life span of shRNA, it might be necessary to modify the shRNA or use multiple cycles of shRNA treatment (33). This concept is important, because it will most likely require multiple treatment cycles to have an impact on survival in patients with metastatic pancreatic cancer, and sequential therapy could be guided by the ZIP4 profile. To increase the efficacy of shRNA therapy, combinational therapy (double or triple therapy) may represent a novel strategy for pancreatic cancer treatment. Our studies have shown that silencing of ZIP4 downsized tumor grade, and may sensitize the tumors to subsequent treatment. Therefore, the combinational therapies with ZIP4 shRNA and chemo/radiation therapy may increase the efficacy of individual...
therapy. Further studies are warranted to investigate whether gemcitabine, the standard chemotherapy drug for pancreatic cancer, regulates the expression of ZIP4, and whether silencing of ZIP4 sensitizes pancreatic cancer cells to gemcitabine treatment. In summary, zinc transporter ZIP4 may be a promising marker for pancreatic cancer. New therapies targeting ZIP4 have potential clinical significance in both human pancreatic cancer and other cancers with high expression of ZIP4.

References


Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Down-regulation of ZIP4 by RNA Interference Inhibits Pancreatic Cancer Growth and Increases the Survival of Nude Mice with Pancreatic Cancer Xenografts

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