ZIP4 regulates pancreatic cancer cell growth by activating IL-6/STAT3 pathway through zinc finger transcription factor CREB

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Abstract

Purpose: Recent studies indicate a strong correlation of zinc transporter ZIP4 and pancreatic cancer progression; however, the underlying mechanisms are unclear. We have recently found that ZIP4 is overexpressed in pancreatic cancer. In this study, we investigated the signaling pathway through which ZIP4 regulates pancreatic cancer growth.

Experimental Design: The expression of cyclin D1, interleukin 6 (IL-6), and signal transducer and activator of transcription 3 (STAT3) in pancreatic cancer xenografts and cells were examined by real-time PCR, Bio-Plex cytokine assay, and Western blot, respectively. The activity of cAMP response element–binding protein (CREB) is examined by a promoter activity assay.

Results: Cyclin D1 was significantly increased in the ZIP4 overexpressing MIA PaCa-2 cells (MIA-ZIP4)–injected orthotopic xenografts and was downregulated in the ZIP4-silenced ASPC-1 (ASPC-shZIP4) group. The phosphorylation of STAT3, an upstream activator of cyclin D1, was increased in MIA-ZIP4 cells and decreased in ASPC-shZIP4 cells. IL-6, a known upstream activator for STAT3, was also found to be significantly increased in the MIA-ZIP4 cells and xenografts and decreased in the ASPC-shZIP4 group. Overexpression of ZIP4 led to a 75% increase of IL-6 promoter activity and caused increased phosphorylation of CREB.

Conclusions: Our study suggest that ZIP4 overexpression causes increased IL-6 transcription through CREB, which in turn activates STAT3 and leads to increased cyclin D1 expression, resulting in increased cell proliferation and tumor progression in pancreatic cancer. These results elucidated a novel pathway in ZIP4-mediated pancreatic cancer growth and suggest new therapeutic targets, including ZIP4, IL-6, and STAT3, in pancreatic cancer treatment.

Pancreatic cancer is one of the most devastating diseases, which holds the number one fatality rate of all cancers, with an overall 5-year survival rate of <5% (1–3). Pancreatic cancer is characterized of invasive growth and early metastasis and is also highly resistant to chemotherapy and radiation therapy (4). There are no reliable early diagnostic markers and no effective treatment for pancreatic cancer, which is typically diagnosed at an advanced stage. Although progress has been made in elucidating the biology of pancreatic cancer development, little is known on the mechanisms of pancreatic cancer cell proliferation and signal transduction pathways. Clearly, there is a pressing need to understand more about those cellular and molecular processes of pancreatic cancer and to develop more effective treatments for this deadly disease.

The zinc transporter ZIP4 is overexpressed in most pancreatic cancer and is a malignant factor in cancer progression. Our previous studies have shown that pancreatic cancer cells expressing high levels of ZIP4 exhibit greater in vitro cell proliferation and in vivo tumor progression (5). Silencing of ZIP4 inhibits pancreatic cancer growth and significantly increases the survival rate of nude mice with pancreatic cancer xenografts (6). However, the mechanisms through which ZIP4 promotes cancer progression are largely unknown. Altered expression of zinc transporters is associated with many other cancers. Recently, low levels of Zinc transporter (ZnT1) have been observed in mammary gland tumor cells. The zinc concentration in these cells are also higher than that in normal cells, which suggests that zinc transport is deregulated in these fast proliferating tumor cells (7). In another study, ZIP6 (also known as LIV-1), a breast cancer-associated protein, has been associated with estrogen-positive breast cancer and...
A zinc transporter, ZIP4, is overexpressed in human pancreatic cancer cell lines and contributes to tumor progression. Silencing of ZIP4 inhibits pancreatic cancer cell growth and increases the survival rate. In this study, we have found that ZIP4 overexpression causes increased interleukin 6 (IL-6) transcription through a zinc-dependent transcription factor, CAMPT response element–binding protein, which in turn activates signal transducer and activator of transcription 3 (STAT3) and leads to increased cyclin D1 expression, resulting in increased cell proliferation and tumor progression in pancreatic cancer. These results elucidated a novel pathway through which ZIP4 regulates pancreatic cancer growth and suggest new therapeutic targets, including ZIP4, interleukin 6, and signal transducer and activator of transcription 3, in pancreatic cancer treatment.

**Materials and Methods**

**Cell culture and chemicals.** Human pancreatic cancer cell lines MIA PaCa-2, ASPC-1, and BxPC-3 were purchased from the American Type Culture Collection and were cultured in DMEM and RPMI 1640 medium, respectively, with 10% fetal bovine serum as previously described (11, 12). ZIP4 overexpression and shRNA-silenced stable cells were selected in MIA PaCa-2, ASPC-1, and BxPC-3 cells with retrovirus vectors (OriGene) as previously described (5, 6). Briefly, stable cell lines overexpressing ZIP4 (MIA-ZIP4) or empty vector (MIA-V) were selected with adding 0.5 μg/mL of puromycin into the medium. Stable cell lines expressing ZIP4 shRNA (ASPC-shZIP4 and BxPC-shZIP4) or empty vector (ASPC-shV and BxPC-shV) were selected with adding 1 μg/mL or 0.5 μg/mL of puromycin into the medium. The sequence of the ZIP4 shRNAs used in this study is as follows: 5′ CCTCAGTACITGTCGGACITTTGTTCCA 3′. Cyclin D1 antibody (sc-753) was purchased from Santa Cruz Biotechnology. Phospho-STAT3 (9131), total STAT3 (9132), phospho-CREB (9191), and total CREB (9197) antibodies were purchased from Cell Signaling Technology. Stat3 siRNA smart pool and nontarget siRNA control were purchased from Dharmacon. Other chemicals were from Sigma.

**mRNA detection of cyclin D1 and IL-6.** The mRNA levels of cyclin D1 and IL-6 were analyzed by real-time reverse transcriptase-PCR as previously described (5, 11). Briefly, real-time PCR was done using the iQ SYBR Green Supermix kit (Bio-Rad). PCR included 100 mmol/L each primer, diluted cDNA templates, and iQ SYBR Green supermix and was run 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 triplicates, and the corresponding no–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^ΔC(T(β-actin) - C(T[gene of interest])]. The primer sequences for human cyclin D1 gene are sense 5′ TATTGCGCTGTCACCCGTTGA 3′ and antisense 5′ CCA-TAGGCAAAACATGTGAAA 3′. The primer sequences for human IL-6 gene are sense 5′ TCCAGAACAGATTTGACCA-3′ and antisense 5′ GCAATTTGTTGGTGTCC- 3′.

**Western blot analysis.** MIA-V, MIA-ZIP4, ASPC-shV, ASPC-shZIP4, BxPC-shV, and BxPC-shZIP4 cells were lysed with ice-cold lysis buffer (20 mmol/L Tris-HCl, pH 7.5; 150 mmol/L NaCl, 1 mmol/L Na2EDTA; 1 mmol/L EGTA; 1% Triton; 2.5 mmol/L sodium pyrophosphate; 1 mmol/L β-glycerophosphate; 1 mmol/L Na3VO4; 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 was loaded, and total cellular protein was separated with 15% SDS-PAGE and then transferred overnight at 4°C onto Hybond-P PVDF membrane (Amersham Biosciences). The membrane was probed with anti–pSTAT3 (1:1,000), total STAT3 (1:1,000), pCREB (1:1,000), or anti–β-actin (1:10,000) antibodies at 4°C overnight; washed thrice 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 thrice with 0.1% Tween 20–TBS, and the immunoreactive bands were detected by using enhanced chemiluminescent (ECL) plus reagent kit.

**Promoter activity assay.** For assessing CREB-directed IL-6 promoter activity, IL-6 promoter luciferase reporters were used. The wild-type IL-6 promoter reporter (pGL3-IL6-Luc wt), which contains a 2,120-bp IL-6 promoter sequence from -2,161 to -41 bp upstream of the IL-6 gene, was obtained from Dr. William L. Farrar at NIH (13). The IL-6 promoter reporter was cotransfected with ZIP4 or vector control plasmid into 293 cells, and the IL-6 promoter activity was determined by a chemiluminescence reader. The pRL-SV40 plasmid, a *Renilla reniformis* luciferase reporter vector (Promega), was also cotransfected with all the samples as internal control and normalization of transfection efficiency.

**Statistical analysis.** Quantitative results are shown as means ± SDs. The statistical analysis was done by the Student’s *t* test between control and treatment groups. *P* < 0.05 was considered statistically significant.

**Results**

**ZIP4 upregulates the expression of cyclin D1 in pancreatic cancer.** Our previous results indicate that ZIP4 is overexpressed in most pancreatic cancer specimens and pancreatic cancer cell lines to varying degrees. Forced overexpression of ZIP4 increases pancreatic cancer cell proliferation and tumor progression. Conversely, silencing of ZIP4 by shRNA inhibits pancreatic cancer growth and increases survival rate of nude mice with pancreatic cancer xenografts. To delineate the signaling pathways in ZIP4-mediated pancreatic cancer growth, we did a gene profiling study on the xenografts (orthotopic model) from MIA-V– and MIA-ZIP4–cells–injected nude mice by using the comprehensive human Genome U133 PLUS 2.0 array chips (Affymetrix). We found that the expression of cyclin D1, a key molecule controlling cell proliferation and survival in cancers, was significantly increased by 2.6-fold in MIA-ZIP4 group compared with that in MIA-V group. We confirmed the microarray data with quantitative real-time PCR in the same orthotopic xenograft tissues, and we found a 2.5-fold increase of cyclin D1 in the MIA-ZIP4 group compared with that in the MIA-V group (Fig. 1A). Cyclin D1 was also upregulated by 3.3-fold in the MIA-ZIP4 group in the s. c. xenografts (Fig. 1B). Conversely, cyclin D1 was downregulated by 41% in the ASPC-shZIP4 group of the orthotopic xenografts compared with that in the ASPC-shV group (Fig. 1C). The protein levels of cyclin D1 were also observed in MIA-ZIP4 xenografts and decreased in ASPC-shZIP4 xenografts (data not shown). Similar results were also observed in MIA-ZIP4 and ASPC-shZIP4 cell lines, in which overexpression of ZIP4 upregulated cyclin D1 and silencing of ZIP4 downregulated cyclin D1 expression.

**ZIP4 regulates the phosphorylation of STAT3 in pancreatic cancer cells.** The expression of cyclin D1 can be regulated by activated STAT3, an upstream molecule of cyclin D1. To further understand the molecular mechanisms of ZIP4-induced cell proliferation and tumor growth, we determined the expression and phosphorylation status of STAT3 in ZIP4 overexpressing and silenced pancreatic cancer cells. As shown in Fig. 2, phosphorylation of STAT3 was significantly increased in MIA-ZIP4 cells compared with that in MIA-V cells, whereas the phosphorylation of STAT3 was substantially decreased in ASPC-shZIP4 and

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mediated STAT3 activation in pancreatic cancer. That survivin may not be directly involved in the ZIP4-significant change of survivin after blocking STAT3, suggesting survivin by Western blot, and we did not found any signif-

antigens. We also examined the expression of clinD1 is activated by STAT3 in MIA-ZIP4 cells (Supple-

densities. Our results indicate that, although cell conflu-

effects STAT3 activation (14–18). We repeated the above experiments using Western blot experiment with cultured cells at different densities. These results indicate that ZIP4 upregulation of cyclin D1 in pancreatic cancer with high ZIP4 levels.

Previous studies have shown that cell confluence can affect STAT3 activation (14–18). We repeated the above Western blot experiment with cultured cells at different densities. Our results indicate that, although cell confluence increases the baseline activation of STAT3 in MIA PaCa-2 and ASPC-1 cells at various degrees, the dramatic increase of STAT3 phosphorylation was specifically caused by overexpression of ZIP4 in MIA-ZIP4 cells. Similarly, in ASPC-1 cells, the STAT3 phosphorylation was specifically decreased by ZIP4 shRNA (Supplementary Fig. S1). Therefore, we believe that ZIP4 regulates STAT3 activation in pancreatic cancer cells; silencing of ZIP4 can downregulate STAT3 activation.

To further elucidate that cyclinD1 is the downstream target of STAT3 in ZIP4 overexpressing pancreatic cancer cells, we blocked STAT3 with STAT3 siRNA, and we found that cyclinD1 decreased accordingly, indicating that cyclinD1 is activated by STAT3 in MIA-ZIP4 cells (Supplementary Fig. S2). We also examined the expression of survivin by Western blot, and we did not found any significant change of survivin after blocking STAT3, suggesting that survivin may not be directly involved in the ZIP4-mediated STAT3 activation in pancreatic cancer.

ZIP4 upregulates the expression of IL-6 in pancreatic cancer cells and xenografts. IL-6 is one of the upstream activators for STAT3 and is a known malignant factor in pancreatic cancer pathogenesis. To elucidate whether the activated STAT3 is due to increased IL-6 levels, we examined the expression of IL-6 in MIA-ZIP4 and ASPC-shZIP4 xenografts and cells by quantitative real-time PCR and BioPlex cytokine assay. IL-6 level was significantly increased in the MIA-ZIP4 group of the orthotopic or s.c. xenografts by 23.7- or 8.8-fold, respectively, compared with that in the MIA-V group (Fig. 3A and B). Conversely, IL-6 level decreased in the ASPC-shZIP4 group of the orthotopic xenografts by 51% compared with that in the ASPC-shV group (Fig. 3C). To show the direct evidence that ZIP4 can regulate the expression and secretion of IL-6 in pancreatic cancer cells, the IL-6 protein levels in the conditioned medium of the cultured stable MIA PaCa-2 and ASPC-1 cells were examined using Bio-Plex cytokine assay (Bio-Rad). As shown in Fig. 4A and B, IL-6 protein level was increased by 3.4-fold in MIA-ZIP4 cells compared with that in MIA-V cells, whereas IL-6 was decreased by 52% in ASPC-shZIP4 cells compared with that in ASPC-shV cells. These results indicate that ZIP4 does regulate the expression of IL-6 in pancreatic cancer.

ZIP4 activates the IL-6 promoter activity through CREB. To determine whether the increased expression of IL-6 by ZIP4 is due to increased transcription of IL-6 or other factors, we examined the effect of ZIP4 on the activity of IL-6 promoter. As shown in Fig. 5A, there is a binding site for zinc finger transcriptional factor CREB upstream of the IL-6 promoter region. IL-6 promoter reporter construct was used to cotransfect 293 cells with ZIP4 construct; we found that ZIP4 transfection led to a 75% increase of IL-6 promoter activity compared with the vector control (Fig. 5B). To further determine whether CREB is activated by ZIP4 and consequently initiates the transcription of IL-6, we examined the phosphorylation of CREB in ZIP4 cells. As shown in Fig. 5C, phosphorylation of CREB was significantly increased in MIA-ZIP4 cells compared with that in MIA-V cells. These results indicate that ZIP4 upregulates IL-6 expression and secretion through activating IL-6 promoter by increasing the phosphorylation of CREB, which binds on the CREB-responsive elements upstream of the IL-6 promoter. IL-6 is one of major proinflammatory cytokines and an activator of STAT3 phosphorylation. These data suggest that IL-6 might be an important intermediate signaling molecule connecting ZIP4 and STAT3 pathways in pancreatic cancer (Fig. 6).

Discussion

Our previous studies and other reports have shown that zinc transporters play important roles in cancer growth and progression. The detailed mechanisms of how zinc transporters regulate cancer cell growth and whether zinc transporters interact with other signal pathways in cancer cells remain unknown. Our current study indicate that ZIP4 overexpression causes phosphorylation of CREB...
and increase of IL-6 transcription and secretion, which in turn activates STAT3 and leads to increased cyclin D1 expression, resulting in increased cell proliferation and tumor progression in pancreatic cancer. This study has identified a new role for ZIP4 in pancreatic malignancy that has not been previously characterized, thereby suggesting a new target for pancreatic cancer therapy.

Zinc is an essential trace element and catalytic cofactor used by many metalloenzymes and transcription factors, which contain zinc finger motifs (19, 20). Zinc deficiency in animals leads to growth retardation, decreased food intake, impaired DNA synthesis, immune-system dysfunction, and severe dermatitis (21). Zinc availability is also important for tumor growth and progression because zinc is an essential component for many enzymes such as carbonic anhydrase and matrix metalloproteinases, which are involved in hypoxia, angiogenesis, cell proliferation, and metastasis (22, 23). Previous studies have shown that zinc regulates the activity of many zinc finger transcription factors such as metal-responsive transcription factor-1, CREB, and CREB-binding protein. Depletion of intracellular zinc caused decreased CREB expression and activity, and addition of exogenous zinc restored the expression and activity of CREB in neuron cells and other cell systems (24–27).

Our data indicate that overexpression of ZIP4 caused increased phosphorylation of CREB and upregulated IL-6 promoter activity. These results suggest that ZIP4 provides zinc ions for zinc finger transcriptional factors such as CREB, which turn on the downstream signal pathways in pancreatic cancer cells, indicating a new mechanism of ZIP4-mediated cell growth.
IL-6 is a proinflammatory cytokine that is associated with a variety of malignancies and is a poor prognostic factor in several hematopoietic and solid tumors (28). Pancreatic cancer patients secrete elevated levels of IL-6 compared with healthy control individuals (28–30). Serum levels of IL-6 are tightly associated with decreased performance and increased mortality rates in pancreatic cancer. IL-6 has been shown to be a prominent cachectic factor, affecting tumor-related weight loss, decreasing albumin in cancer patients, increasing pancreatic cancer in vitro growth and worsening prognosis in clinical investigations, and regulating the expression of key molecules in angiogenesis and cancer metastasis, such as vascular endothelial growth factor and neuropilin-1 (28–34). IL-6 has also been shown to play a critical role in resistance to hypoxia, which might involve p38 mitogen-activated protein kinase activation (35, 36). Our previous study found that IL-6 upregulates the expression of several Th2 cytokines that pattern together to create a local environment that favors tumor growth and suppresses the anticancer immune response in pancreatic cancer (32). Other studies have shown that IL-6 might also interact with the zinc transport pathway by responding to plasma zinc concentration (37–39). IL-6 level is controlled by the availability of zinc, whose intracellular transport is regulated by zinc transporters and metallothioneins, the zinc carrier proteins (37). Zinc also stimulated peripheral blood mononuclear cells to release many cytokines, including IL-6 (40–43). Our current data indicate a close correlation between ZIP4 and IL-6 expression levels in pancreatic cancer cells. Therefore, ZIP4 induced IL-6 upregulation represents a new mechanism to activate the downstream signaling pathways in pancreatic cancer.

STAT3 is activated in most pancreatic cancer cells at various levels. The downstream targets of STAT3 include genes that control survival and cell proliferation, such as cyclins (44). Activation of the STAT3 signaling pathway plays an important role in the progression of pancreatic cancer.

**Fig. 5.** ZIP4 upregulates the IL-6 promoter activity through phosphorylating CREB in pancreatic cancer cells. A, schematic diagram of IL-6 promoter luciferase reporter construct. B, IL-6 promoter activity. Wild-type IL-6 promoter reporter was cotransfected with ZIP4 or vector control plasmid into 293 cells, the IL-6 promoter activity was determined by a chemiluminescence reader. C, phosphorylation of CREB in MIA-ZIP4 cells. Stable MIA-V and MIA-ZIP4 cells were examined for the expression and phosphorylation of CREB. Briefly, cell lysates were extracted and probed with anti-pCREB (1:1,000) and anti-CREB (1:1,000) antibodies.

**Fig. 6.** Working model for ZIP4-mediated signal transduction in pancreatic cancer. ZIP4 overexpression increases IL-6 expression and secretion through phosphorylating CREB, which, in turn, activates STAT3 and leads to increased cyclin D1 expression, resulting in increased cell proliferation and tumor progression.
ZIP4 Regulates Pancreatic Cancer Growth

Cancer and blockade of STAT3 signals may provide a novel therapeutic strategy for pancreatic cancer (45). In this study, we have found that overexpression of ZIP4 caused increased phosphorylation of STAT3 in MIA PaCa-2 cells and silencing of ZIP4 led to decreased phosphorylation of STAT3 in ASPC-1 and BxPC-3 cells, indicating a strong correlation of ZIP4 and STAT3 activation. Previous reports have shown that cell confluence and density can affect STAT3 activation (14–18). We found that, although cell confluence increases the baseline activation of STAT3 in MIA PaCa-2 and ASPC-1 cells, the dramatic increase of STAT3 phosphorylation was specifically caused by overexpression of ZIP4 in MIA-ZIP4 cells. Similarly, in ASPC-1 cells, the STAT3 phosphorylation was specifically decreased by ZIP4 shRNA. Therefore, ZIP4 regulates STAT3 activation in pancreatic cancer cells; silencing of ZIP4 can downregulate STAT3 activation. Blocking of STAT3 by STAT3 siRNA can decrease the expression of cyclinD1, but not survivin, indicating that cyclin D1 is the downstream target for STAT3 in ZIP4-overexpressing pancreatic cancer cells, and STAT3/cyclin D1 pathway might be one of the major signaling pathways in ZIP4-mediated pancreatic cancer cell growth. We also examined other STAT3 activators such as Src and did not found significant difference of Src expression in MIA PaCa-2 and ASPC-1 cells with ZIP4 overexpression or knockdown (data not shown), indicating that ZIP4 activates STAT3 maybe independent of Src pathway in pancreatic cancer. Additional studies are also warranted to determine whether there are other molecules and pathways involved in ZIP4-mediated pancreatic cancer growth and whether those molecules interact with the IL-6/STAT3 pathway.

Recent studies indicated that zinc transport and zinc homeostasis play important roles in cancer progression, especially in pancreatic cancer and breast cancer (5, 9, 46). However, the underlying molecular pathways are not clear. Our study strongly indicate that ZIP4 can regulate the activity of CREB, an important zinc finger transcriptional factor, and consequently increases the expression of IL-6, which leads to STAT3 activation and pancreatic cancer growth. In the future studies, it would be interesting to investigate that whether blocking the CREB/IL-6/STAT3 pathway in combination with silencing ZIP4 have any synergistic effect in pancreatic cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. William L. Farrar at NIH for providing the IL-6 promoter reporter constructs.

Grant Support

NIH grant R21CA133604, the Dan L. Duncan Cancer Center pilot grant, the MacDonald Research Fund 09RDM004, and the William and Ella Owens Medical Research Foundation (M. Li).

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Received 09/03/2009; revised 12/30/2009; accepted 01/05/2010; published OnlineFirst 02/16/2010.

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