Transforming Growth Factor β Mediates Drug Resistance by Regulating Expression of Pyruvate Dehydrogenase Kinase 4 in Colorectal Cancer*

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ABSTRACT

Drug resistance is one of the main causes of colon cancer recurrence. However, our understanding of the underlying mechanisms and availability of therapeutic options remain limited. Here we show that expression of PDK4 is positively correlated with drug resistance of colon cancer cells and induced by 5-fluorouracil (5-FU) treatment in drug-resistant but not -sensitive cells. Knockdown of PDK4 expression sensitizes colon cancer cells to 5-FU or oxaliplatin-induced apoptosis in vitro and increases the effectiveness of 5-FU in the inhibition of tumor growth in a mouse xenograft model in vivo. In addition, we demonstrate for the first time that TGFβ mediates drug resistance by regulating PDK4 expression and 5-FU induces PDK4 expression in a TGFβ signaling-dependent manner. Mechanistically, knockdown or inhibition of PDK4 significantly increases the inhibitory effect of 5-FU on expression of the anti-apoptotic factors Bcl-2 and survivin. Importantly, studies of patient samples indicate that expression of PDK4 and phosphorylation of Smad2, an indicator of TGFβ pathway activation, show a strong correlation and that both positively associate with chemoresistance in colorectal cancer. These findings indicate that the TGFβ/PDK4 signaling axis plays an important role in the response of colorectal cancer to chemotherapy. A major implication of our studies is that inhibition of PDK4 may have considerable therapeutic potential to overcome drug resistance in colorectal cancer patients, which warrants the development of PDK4-specific inhibitors.

INTRODUCTION

Colorectal cancer is the third most common cancer diagnosed and the second leading cause of cancer mortality in the United States. In addition to surgery, treatment for colorectal cancer patients,
especially for patients with advanced disease, primarily relies on chemo- and radiation therapy. Fluorouracil (5-FU) is one of the most commonly used chemotherapeutic agents for colorectal cancer treatment. It induces cell cycle arrest and apoptosis in cancer cells (1). Although adjuvant 5-FU treatment has a good success rate, the high recurrence is still a major hurdle of treating colorectal cancer due to the resistance to chemotherapeutic drugs. Therefore, it is important to understand the mechanisms of drug resistance and identify new targets to increase the effectiveness of chemotherapy in colorectal cancer.

TGFβ plays an important role in cancer development and progression. Upon ligand binding, TGFβ type II receptor (RII) recruits and activates TGFβ type I receptor (RI), which then activates Smad2 and Smad3. Activated Smad2 and Smad3 form complexes with Smad4 and translocate to the nucleus, where they regulate gene expression (2). We and others have demonstrated that TGFβ suppresses tumorigenecity in a variety of cancers including colorectal cancer, and that loss of TGFβ signaling leads to malignancy (3-6). Though many studies have shown that TGFβ promotes metastasis (7), others have demonstrated that TGFβ suppresses metastasis (8,9). Recently, studies indicate that TGFβ signaling is an emerging player in cancer drug resistance (10-12). Although TGFβ signaling may act as a tumor suppressor, it could have adverse effect in chemotherapy. More studies are needed to better understand the underlying mechanisms.

Metabolic abnormality is one of the hallmarks of cancer (13). Aberrant glucose metabolism is widespread in cancer cells and has been shown to play a role in drug resistance (14,15). Otto Warburg first reported that cancer cells use glycolysis rather than oxidative phosphorylation for energy, which is referred to as aerobic glycolysis or the “Warburg effect” (16). Pyruvate dehydrogenase (PDH) complex, which converts pyruvate to acetyl-CoA, is one of the important mediators of glucose oxidative metabolism. It interconnects glycolysis and the TCA cycle, therefore representing a key regulatory step in glucose metabolism. The activity of PDH is tightly controlled by reversible phosphorylation mediated by PDH kinases (PDKs) and PDH phosphatases (PDPs) (17). Phosphorylation and inactivation of PDH by PDKs favors the glycolytic phenotype, which confers resistance to apoptosis (18,19). Inhibition of PDKs switches glucose metabolism to aerobic oxidation, which is disadvantageous to tumor growth (20).

There are four different human PDKs (PDKs 1-4) (17). These different PDKs respond to various stimuli, including hypoxia and nutrient deprivation, to regulate glucose metabolism (21,22). Among the four PDKs, PDK4 is a principal regulator responsible for the modulation of PDH activity and fuel selection between glucose and fatty acids for energy utilization (23). Expression of PDK4 is selectively induced in most tissues and organs in response to metabolic stress (23). In this study, we have identified a novel function of PDK4 that mediates the response of colon cancer cells to 5-FU treatment. We show that PDK4, but not PDK 1-3, is differentially expressed in colon cancer cells, and that its expression positively correlates with the resistance to 5-FU treatment. Knockdown of PDK4 sensitizes colon cancer cells to 5-FU- or oxaliplatin-induced apoptosis. Remarkably, experiments in tumor xenograft mouse models demonstrate that knockdown of PDK4 significantly increases the effectiveness of 5-FU-mediated inhibition of tumor growth in vivo. Furthermore, we have discovered a novel crosstalk between TGFβ signaling and PDK4: TGFβ signaling enhances PDK4 expression and elevated PDK4 expression contributes to TGFβ-mediated drug resistance in colon cancer cells. Studies of patient specimens show that PDK4 expression and TGFβ activation positively correlate with each other and with chemoresistance in colorectal cancer, indicating the clinical relevance of our studies. Collectively, our studies unveil a novel function of TGFβ/PDK4 in mediating drug resistance in colorectal cancer.
EXPERIMENTAL PROCEDURES

Cell lines and reagents The immortalized human colon epithelial cells (HECE) were provided by Dr. Jerry Shay and were cultured as described previously (24). The human colon carcinoma RKO, HCT116, FET and CBS cells were cultured in McCoy’s 5A serum-free medium (Sigma, St. Louis, MO) supplemented with 10 ng/ml epidermal growth factor, 20 µg/ml insulin, and 4 µg/ml transferrin(25). Cells were maintained at 37°C in a humidified incubator with 5% CO₂. 5-FU, oxaliplatin and DCA were purchased from Sigma (St. Louis, MO). TGFβ was obtained from R&D Systems (Minneapolis, MN). Antibodies for western blot analyses were purchased as indicated: anti-PARP and anti-cleaved PARP, Cell Signaling Technology (Beverly, MA), anti-Bcl-2 and anti-survivin, Santa Cruz Biotechnology, Inc (Santa Cruz, CA), anti-PDK4, Abgent, Inc. (San Diego, CA), and for actin and anti-GAPDH, Thermo Scientific (Waltham, MA).

Western blot analysis, RT-PCR, Q-PCR and apoptosis assays Whole cell lysates were prepared in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris–HCl (pH 7.5), 5 mM EDTA and a protease inhibitor cocktail from Sigma-Aldrich). Equivalent amounts of protein were separated by SDS–PAGE and transferred to a PVDF membrane (Millipore, Billerica, MA). Proteins were detected using an enhanced chemiluminescence system (Amersham Biosciences).

For RT-PCR, 2 µg of RNA was reverse-transcribed with M-MLV reverse transcriptase (Promega, Madison, WI) using a random primer. 2ul of cDNA product was used to amplify human PDK1, PDK2, PDK3, PDK4, actin and GAPDH. Primer sequences for PDK1 were 5'-GGCAAGAGTTGCCCTGAGCT-3' (forward) and 5'-GCATCTTTACCACATCCGC-3' (reverse), for PDK2 were 5'-ACTGCAACGTCGAGGTTG-3' (forward) and 5'-TACGTGACCGTTCTGTTGG-3' (reverse), for PDK3 were 5'-AAGCAGATCGACCGCTTACTC-3' (forward) and 5'-GCATCTTTACCACATCCGC-3' (reverse), for PDK4 were 5'-CCTTGAGACTGCAACAT-3' (forward) and 5'-GTTCAACTGTTGCGGCTATT-3' (reverse), and for actin were 5'-TGACGCGGTCTACCCACATCCGG-3'(forward) and 5'-CTAGAGCATTGGTGAGCATGGAGG-3' (reverse), for GAPDH were 5'-CTCCTGCTCTCCTGTTGACG-3' (forward) and 5'-AAATGAGCCCACTGCTCCC-3' (reverse).

For Q-PCR, reverse transcription was performed using iScriptTM Reverse Transcription Supermix for RT-qPCR (Bio-Rad) with 1 µg of total RNA per 20 µl reaction. RT-qPCR was performed using the PDK4 primers : 5'-CATCTGGGTTTTTCTCTGGA-3' (forward) and 5'-TCCCGACCCAATAGAATACC-3' (reverse). All targets were amplified using SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad) with 40 cycles of a 2-step program (95°C x 5 sec, Tm x 45 sec) on a Mx3000P.

Plasmids construction and lentiviral infection ShRNAs targeting PDK4 were constructed by cloning annealed oligos into the FSIPPP lentiviral vector. The targeting sequences of PDK4 shRNA-2, shRNA-3, Smad2 shRNA and Smad3 shRNA are 5'-ACTGCAACGTCGAGGTTG-3', 5'-AAGCAGATCGACCGCTTACTC-3', 5'-GCACTTGGCTGAAATTTG-3' and 5'-GGATTGAGCTGACCTGAAGT-3' respectively. A scrambled shRNA was used as a control. 293 packaging cells were co-transfected with pPackH1 packaging plasmid mix (SBI) and the lentiviral vectors using Fugene HD (Promega, Madison, WI). Viruses were harvested 48 hours later and used to infect target cells. Expression vectors of DNRII and RII were described previously (5,6). Expression vectors of Bcl-2 and survivin were provided by Dr. Michael Brattain.
PDK4 expression vector was obtained from Harvard Medical School (Boston, MA).

**Knockdown of expression of PDKs 1-3 by siRNAs** Individual pools of siRNAs against PDK1, PDK2 or PDK3 were purchased from Dharmacon, GE Healthcare Inc. (Lafayette, CO) and transfected into the cells using Dharmaconfect-2 (Dharmacon, GE Healthcare Inc.) following the manufacturer’s protocol.

**Apoptosis assays** Apoptosis was detected using a DNA fragmentation ELISA kit (Roche, Indianapolis, IN) or Annexin V staining.

DNA fragmentation ELISA assays were performed according to manufacturer’s protocol. Briefly, cells were seeded in 96-well plates and treated with different concentrations of 5-FU for 72 hours. The cells were stained with MTT to determine cell numbers or lysed for the ELISA assays to determine apoptosis. The relative apoptosis was determined by dividing ELISA values by MTT values of each sample. Fold changes were calculated by dividing relative apoptosis of 5-FU treated samples by that of the controls.

For Annexin V staining, cells were seeded and treated with 2.5 μg/ml 5-FU for 72 hours. Cells were trypsinized and washed with PBS before staining with a Cell Apoptosis Annexin V FITC/PI Kit (Fisher Scientific). Cells were then analyzed by flow cytometry. Percentage of Annexin V-positive and PI-negative cells was calculated.

**In vivo xenograft model** Experiments involving animals were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. HCT116 cells (2.5 x 10⁶) infected with a lentiviral vector encoding PDK4 sh-3 or a scrambled shRNA were injected into the flank of male athymic nude mice (4-5 weeks old). One week after injection, 5-FU (25 mg/kg/day) or carrier was administered by i.p. injection for 5 consecutive days per week for 2 weeks(26,27). Tumor volumes were measured at the beginning of the treatment and every other day afterwards till the mice were euthanized. The estimated tumor volumes (V) were calculated by the formula $V = W^2 \times L \times 0.5$, where W represents the largest tumor diameter and L represents the next largest tumor diameter. The relative tumor volumes (RTV) were calculated by $RTV = V_x/V_0$ where $V_x$ is the volume at a given time and $V_0$ is the volume at the beginning of the treatment (26).

**TUNEL and Ki67 staining** Formalin fixed paraffin embedded (FFPE) tissue blocks of xenograft tumors were stained for TUNEL and Ki67 using the procedure described previously(28). Three xenograft tumors from each group were analyzed. Ten histologically similar fields were randomly selected from each slide for analysis. Apoptosis and proliferation of tumor cells were determined quantitatively by calculating the percentage of positively stained cells for TUNEL and Ki67 at 20x magnification respectively.

**Determination of PDK4 expression and Smad2 phosphorylation in human tissue samples** FFPE blocks of human colorectal adenocarcinomas were obtained from files of Department of Pathology and Microbiology at University of Nebraska Medical Center. The ages of all patients (including both men and women) were between 55-85 years. The cancer patients received neoadjuvant chemoradiotherapy prior to surgical removal of the tumors. Moderate responders refer to patients who have residual cancer with evident tumor regression, but with more than single cells or rare small groups of cancer cells remaining; poor or non-responders refer to those who have extensive residual cancer with no evident tumor regression after neoadjuvant therapy. The study was performed with the approval of the ethics committee (Institutional Review Board).

Immunohistochemistry (IHC) staining was performed to examine PDK4 expression and Smad2 phosphorylation in tumor samples following Novolink™ Min Polymer Detection System kit protocol (Leica). Briefly, slides were subjected to antigen retrieval using Novocastra Epitope Retrieval Solutions, pH6, followed by incubation with an anti-PDK4 or anti-p-Smad2 antibody for
overnight at 4 °C. On the next day, slides were developed with DAB after incubation with Novolink polymer for 30 minutes. Finally, the sections were counterstained with hematoxylin. For each slide, ten randomly chosen tumor fields were captured at 40 × magnification. The staining intensity was quantified with Imagescope Software (Aperio Technologies, Inc.) as described previously (29).

Statistical analysis Statistical analyses were performed using two-way ANOVA or Student’s t-test.

RESULTS

Expression of PDK4 positively correlates with 5-FU resistance in colon cancer cells. To understand the mechanisms and identify the determinants underlying drug resistance, we examined a panel of colon cancer cell lines for their response to 5-FU treatment. Among the cell lines tested, RKO and HCT116 cells were much more sensitive to 5-FU-induced apoptosis than FET and CBS cells. DNA fragmentation assays revealed that the induction of apoptosis by 5-FU treatment was much higher in RKO and HCT116 cells than in FET and CBS cells (Fig. 1A). Annexin V/PI staining was performed to determine the percentage of early apoptotic cells after 5-FU treatment. 5-FU-mediated increase of early apoptotic cells represented by Annexin V+/PI- cell population was markedly higher in RKO and HCT116 cells than in FET and CBS cells (Fig. 1A). Consistent with these results, 5-FU induced a significant increase of poly ADP ribose polymerase (PARP) cleavage in RKO and HCT116 cells whereas FET and CBS cells were relatively resistant to this induction (Fig. 1C).

Aberrant glucose metabolism has been shown to play a role in drug resistance (14,15). To identify the determining factors that mediate 5-FU response, we examined expression of some key regulators of glucose metabolism. We found that PDK4 was differentially expressed in those cell lines. As shown in Figure 1D & 1E, 5-FU resistant FET and CBS cells expressed higher levels of PDK4 than 5-FU sensitive RKO and HCT116 cells at both the mRNA and protein levels, indicating a positive correlation between PDK4 expression and drug resistance. However, expression of other PDKs, PDKs 1-3, was similar among those cell lines (Fig. 1D, right). Of note, PDK4 expression was much lower in immortalized human colon epithelial cells (HEC) than in colon cancer cell lines (Fig. 1D, left). In addition, 5-FU treatment led to increased PDK4 mRNA and protein expression in drug-resistant FET and CBS cells but not in sensitive RKO and HCT116 cells (Fig. 1F, left & 1G). Expression of PDKs 1-3 was not affected in either resistant or sensitive cells (Fig. 1F, right). These results suggest that elevated PDK4 expression may contribute to 5-FU resistance in colon cancer cells.

Genetic or pharmacological inhibition of PDK4 sensitizes colon cancer cells to 5-FU- or oxaliplatin-induced apoptosis in vitro. To further investigate the possibility that PDK4 plays an important role in drug resistance, expression of PDK4 was knocked down in HCT116 and CBS cells. Two shRNAs against PDK4 (sh-2 and sh-3) significantly reduced PDK4 expression in both cell lines (Fig. 2A). Expression of PDKs 1-3 was not affected by PDK4 knockdown (data not shown). Knockdown of PDK4 expression sensitized CBS cells to 5-FU-induced apoptosis as reflected by a 2-3-fold increase of apoptosis induction in PDK4 knockdown cells as compared to the control cells (Fig. 2B, lower). Consistently, the increase of PARP cleavage was enhanced in PDK4 knockdown cells (Fig. 2C, lower). Although HCT116 cells were sensitive to 5-FU treatment, knockdown of PDK4 further enhanced its sensitivity to the treatment (Fig. 2B & 2C, upper). These results suggested that down-regulation of PDK4 expression, even in sensitive cells, could increase their response to 5-FU treatment. In contrast, knockdown of expression of PDK1, PDK2 or PDK3 individually had little effect on 5-FU sensitivity (Fig. 2D). As a complementary approach, PDK4 cDNA was introduced into HCT116 cells. Consequently,
PDK4 expression was markedly increased (Fig. 2E, left). When treated with 5-FU, PDK4-overexpressing cells displayed enhanced resistance to 5-FU-induced apoptosis as compared to the control cells (Fig. 2E, right). Taken together, these results indicate that PDK4 mediates 5-FU response of colon cancer cells.

To determine whether knockdown of PDK4 sensitizes colon cancer cells to other chemotherapeutic drugs, HCT116 cells were treated with oxaliplatin, an alkylating agent commonly used in combination with 5-FU for treating advanced colon cancer (30,31). Similar to 5-FU, oxaliplatin treatment induced more apoptosis in PDK4 knockdown cells than in the control cells as shown by DNA fragmentation assays and PARP cleavage (Fig. 3A & 3B). Although 5-FU and oxaliplatin function through different mechanisms, these data suggest that PDK4 up-regulation may be a common mechanism for their resistance in colon cancer cells.

Dichloroacetate (DCA) is a non-specific pharmacological inhibitor of mitochondria PDK isoforms (32). DCA has been shown to attenuate 5-FU resistance in gastric cancer cells (14). In preclinical studies, different cancer cells showed different responses to DCA-induced apoptosis (32-34). We next investigated whether DCA would increase the effectiveness of 5-FU against colon cancer cells. Low concentrations of DCA or 5-FU alone showed slight increase of apoptosis in FET and CBS cells respectively. However, combined treatment with both significantly increased apoptosis as compared to that with either one alone (Fig. 3C). The effect on PARP cleavage further confirmed the results (Fig. 3D). These data indicated that pharmacological inhibition of PDK4 potentiated the apoptotic effect of 5-FU in colon cancer cells. Taken together, these findings suggest that PDK4 is a promising target for overcoming drug resistance of colon cancer cells.

In order to further understand the mechanisms by which knockdown of PDK4 expression or treatment with DCA sensitized colon cancer cells to 5-FU-induced apoptosis, we examined expression of effectors involved in apoptosis of colon cancer cells. Among the effectors tested, expression of both Bcl-2 and survivin was reduced by PDK4 knockdown, which was further decreased by 5-FU treatment (Fig. 2C). Similarly, combined treatment by 5-FU and DCA significantly decreased expression of Bcl-2 and survivin as compared to either single treatment alone (Fig. 3D). In addition, overexpression of PDK4 increased Bcl-2 and survivin expression in HCT116 cells (Fig. 2E, middle). These results indicate that PDK4 regulates expression of Bcl-2 and survivin. It has been shown that Bcl-2 and survivin protect colon cancer cells from stress-induced apoptosis (35,36) and that 5-FU induces apoptosis in colon cancer cell lines with modulation of Bcl-2 family proteins (37). To demonstrate the potential role of Bcl-2 and survivin in 5-FU-induced apoptosis in PDK4 knockdown cells, Bcl-2 and survivin were ectopically expressed individually in CBS/PDK4 sh-2 cells. Increased expression of Bcl-2 or survivin reduced 5-FU-induced PARP cleavage in PDK4 sh-2 cells whereas it had little effect in the control cells (Fig. 3E). DNA fragmentation assays confirmed those results (Fig. 3F). Therefore, inhibition of PDK4 expression or activity increases 5-FU-induced apoptosis by suppressing the expression of anti-apoptotic proteins such as Bcl-2 and survivin.

**Knockdown of PDK4 expression increases the efficiency of 5-FU-induced inhibition of tumor growth in vivo.** To test whether increased sensitivity to 5-FU-induced apoptosis by knockdown of PDK4 expression translates to enhanced drug efficacy in vivo, we utilized a colon tumor xenograft model. Since high concentrations of 5-FU can be toxic to mice, we chose to use HCT116 cells that are relatively sensitive to 5-FU so that a low dose of 5-FU could be used for the study which would have limited adverse effect on mice. Knockdown of PDK4 in HCT116 cells has been shown to further sensitize the cells to 5-FU-induced apoptosis (Fig. 2B & 2C). Therefore,
athymic nude mice were subcutaneously inoculated with 2.5 million control HCT116 cells or cells expressing a PDK4 shRNA. After 7 days, mice were randomly divided into two groups. One group was treated with 5-FU administered by i.p. injection and the other with the carrier. A 5-FU dose of 25 mg/kg/day was used because the control tumors showed little response to this dose, which would provide a good window to observe enhanced drug efficacy in vivo by knockdown of PDK4 expression. The treatment was for 5 consecutive days per week for 2 weeks (26,27). Throughout the treatment, the weight of the mice remained stable. Tumor growth and therapeutic sensitivity were monitored during the course of 5-FU treatment.

Xenograft tumor growth curves showed that tumors with control cells (designated as control tumors) and those with PDK4 shRNA-expressing cells (designated as PDK4 KD tumors) grew at similar rates (Fig. 4A). However, there was a marked difference in the response of the tumors to 5-FU treatment. PDK4 KD tumors did not grow under drug treatment whereas control tumors continued to grow at a steady rate (Fig. 4A). The difference in the degree of inhibition by 5-FU treatment between control and PDK4 KD tumors was significant (**P < 0.001). These results indicate that 5-FU treatment was more effective in inhibiting the growth of PDK4 KD tumors than that of control tumors.

To examine whether PDK4 knockdown-mediated sensitization to apoptosis in vitro was associated with increased 5-FU effect in vivo, TUNEL assays were performed to determine the apoptotic index of the tumors. TUNEL staining showed that, although the percentage of apoptotic cells was similar in control and PDK4 KD tumors, the increase of apoptotic cells induced by 5-FU treatment was significantly higher in PDK4 KD tumors than in control tumors (8.4 fold vs. 2.6 fold, Fig. 4B). In addition, Ki67 staining showed that 5-FU treatment decreased the percentage of proliferative cells in PDK4 KD tumors by 75% and in control tumors by 38% (Fig. 4C). These studies indicated that the effect of PDK4 knockdown on drug efficacy was a combined result of both increased sensitivity to 5-FU-induced apoptosis and inhibition of proliferation. Therefore, these in vitro and in vivo results demonstrate an important role for PDK4 in mediating drug resistance of colon cancer cells.

**TGFβ signaling mediates drug resistance by regulating PDK4 expression.** Based on the in vitro and in vivo studies described above, PDK4 contributes to drug resistance of colon cancer cells. Therefore, it is critical to elucidate how its expression is regulated, which would provide important information to increase the efficacy of drug treatment. One important difference between 5-FU-sensitive and -resistant cells is TGFβ signaling. While 5-FU sensitive RKO and HCT116 cells are defective of TGFβ signaling due to the mutations in TGFβ RII (3), 5-FU resistant FET and CBS cells are responsive or partially responsive to TGFβ signaling respectively (36,38). This suggests that the TGFβ signaling pathway may play a role in 5-FU response. To determine whether this is the case, a dominant negative RII (DNRII) construct was transfected into FET cells to inactivate TGFβ signaling (6,36). Complementarily, wild type RII cDNA was introduced into HCT116 cells to restore TGFβ signaling (5). As shown in Figure 5A, FET-DNRII cells displayed higher sensitivity to 5-FU than FET-Vec control cells as indicated by increased apoptosis induced by 5-FU treatment in DNA Fragmentation assays. Consistently, 5-FU induced a significant increase of PARP cleavage in FET-DNRII but not in FET-Vec cells (Fig. 5A, right). In contrast, expression of RII in HCT116 cells conferred them increased resistance to 5-FU treatment as reflected by reduced apoptosis in DNA fragmentation assays and decreased PARP cleavage in response to 5-FU treatment (Fig. 5B). These results indicate that TGFβ signaling contributes to 5-FU resistance in colon cancer cells.

Given that FET and CBS cells with active TGFβ signaling express higher levels of PDK4 than RKO and HCT116 cells with defective TGFβ
signaling (Fig. 1D & 1E), we next investigated whether TGFβ regulates PDK4 expression. Treatment with TGFβ increased PDK4 expression at both protein and mRNA levels in FET-Vc and HCT116-RII cells with active TGFβ signaling whereas it had no effect in FET-DNRII and HCT116-Vc cells with inactivated TGFβ pathway (Fig. 5C). Next, we determined whether Smad2 and Smad3 play a role in TGFβ-mediated PDK4 expression. Expression of Smad2 and Smad3 was knocked down individually by shRNAs in FET cells (Fig. 5D, left). Reduced expression of Smad2 or Smad3 attenuated or abrogated TGFβ-induced PDK4 expression (Fig. 5D, right), indicating that Smad2/3 contributes to TGFβ-mediated PDK4 expression.

As shown in Figure 1F & 1G, 5-FU treatment led to increased PDK4 expression in FET and CBS cells, but not in RKO and HCT116 cells. To determine whether TGFβ signaling contributes to 5-FU-induced PDK4 expression, the effect of 5-FU treatment on PDK4 expression was examined in FET-Vc, FET-DNRII, HCT116-Vc and HCT116-RII cells. While inactivation of TGFβ signaling by DNRII in FET cells abrogated 5-FU-induced PDK4 expression, restoration of TGFβ signaling by expressing TGFβ RII in HCT116 cells led to increased PDK4 expression by 5-FU treatment (Fig. 5E). These results indicate that 5-FU-induced PDK4 expression is dependent upon the active TGFβ signaling pathway. Taken together, it suggests that 5-FU may increase PDK4 expression by activating TGFβ signaling. To test whether this is the case, FET cells were treated with 5-FU and Smad2 phosphorylation, an indicator of TGFβ activity, was determined. TGFβ treatment was used as a control. As shown in Figure 5F, 5-FU increased p-Smad2 to a similar level as TGFβ treatment, indicating that 5-FU activates the TGFβ signaling pathway.

To determine whether PDK4 is responsible for the resistance of colon cancer cells with active TGFβ signaling to 5-FU-induced apoptosis, one of the PDK4 shRNAs, sh-2, was infected into FET-Vc (designated Vec-sh-2) and FET-DNRII (designated DNRII-sh-2) cells. As a result, expression of PDK4 in FET-Vc cells was significantly reduced and 5-FU failed to increase PDK4 expression in Vec-sh-2 cells (Fig. 5G, upper). Vec-sh-2 cells became more sensitive to 5-FU-induced apoptosis as compared to control cells (Vec-Ctr, Fig. 5G). In contrast, PDK4 shRNA had little effect in FET-DNRII cells (Fig. 5G). These results indicate that one of the mechanisms by which TGFβ mediates 5-FU resistance is through the induction of PDK4 expression and that elevated PDK4 expression contributes to 5-FU resistance in colon cancer cells with active TGFβ signaling.

**PDK4 expression and TGFβ signaling positively correlate with chemoresistance in colorectal cancer specimens.** To determine the clinical relevance of PDK4 expression in human cancer, we extended our analyses by assaying PDK4 expression in human colorectal adenocarcinoma specimens using immunohistochemistry (IHC) staining. We first verified an anti-PDK4 antibody used for IHC staining. IHC analysis of PDK4 expression was performed in control tumors and PDK4 KD tumors described in Figure 4. The intensity of PDK4 staining was much lower in PDK4 KD tumors than in control tumors, indicating the specificity of the anti-PDK4 antibody (Fig. 4D). IHC staining was then performed on paraffin sections prepared from 18 patients who had received neoadjuvant chemoradiotherapy. Four patients had sigmoid colon cancer and the rest rectal cancer. The patients were at different stages and none of them had microsatellite instability (MSI). Among them, 8 patients had moderate response and the other 10 had no or poor response. PDK4 staining varied significantly in samples from moderate responders and non- or poor response groups (Fig. 6A, upper). Quantification of the staining showed that the average intensity of PDK4 staining is approximately 5.0-fold higher in non- or poor responders than in moderate responders (Fig. 6B, upper, *** P < 0.001). These results indicate that
expression of PDK4 positively correlates with chemoresistance in colorectal cancer patients.

Since TGFβ signaling enhances 5-FU resistance in colon cancer cells (Fig. 5A & 5B), Smad2 phosphorylation (p-Smad2), was determined by IHC staining in the same set of tissue samples. P-Smad2 staining was significantly higher in non- or poor responders than in moderate responders (Fig. 6A, lower). Quantification of the staining showed that the average intensity of p-Smad2 staining is approximately 2.7-fold higher in non- or poor responder group than in moderate responder group (Fig. 6B, lower, *** P < 0.001), indicating that the activation of the TGFβ pathway is associated with chemotherapy resistance in colorectal cancer.

Given that TGFβ increases PDK4 expression in 5-FU resistant colon cancer cells (Fig. 5C), we next determined the relationship between PDK4 expression and Smad2 phosphorylation in patient samples. The study revealed a strong and positive correlation between PDK4 and p-Smad2 staining intensity (Fig. 6C, Pearson r = 0.8545, *** P < 0.001). These results indicate that TGFβ-mediated up-regulation of PDK4 expression is relevant to chemoresistance of colorectal tumors. Taken together with the in vitro and in vivo results in colon cancer cells, our studies demonstrate that the TGFβ/PDK4 signaling axis plays an important role in drug resistance of colorectal cancer.

DISCUSSION

The switch of glucose metabolism for energy production from oxidative phosphorylation to aerobic glycolysis is a hallmark of cancer (13,16). This metabolic switch is an important step to acquire aberrant survival capacity under stress conditions such as starvation or hypoxia (18,19). Although this switch has also been implicated in drug resistance, the molecular mechanisms are not well understood. PDKs 1-4 are a group of enzymes that control this metabolic switch by phosphorylating and thus inactivating PDH. In this study, we have made novel findings that PDK4 mediates the response of colon cancer cells to the chemotherapeutic agent 5-FU and that TGFβ signaling confers drug resistance through up-regulation of PDK4 expression. Our studies indicate that PDK4 is expressed at higher levels in 5-FU-resistant cells than in 5-FU-sensitive cells. 5-FU induces PDK4 expression in a TGFβ signaling-dependent manner. Knockdown of PDK4 expression increases the sensitivity to 5-FU-induced apoptosis in vitro and to 5-FU-mediated inhibition of tumor growth in vivo. Importantly, studies of patient samples indicate that PDK4 expression and TGFβ activation are positively correlated with each other and with chemoresistance in colorectal cancer. Since resistance to 5-FU treatment is one of the major causes for the failure of chemotherapy in treating advanced colorectal cancer (39-41), the discovery of the TGFβ/PDK4 signaling axis as a contributing factor of drug resistance would identify TGFβ signaling and PDK4 as potential targets of cancer therapy and warrant the development of novel PDK4-specific inhibitors to overcome chemoresistance and increase efficacy of chemotherapeutic treatment of colorectal cancer.

It has been reported that metabolic stress such as starvation, fasting and glucose deprivation markedly upregulate PDK4 levels and that expression of PDK4 is induced by dietary lipids and hormones, and is inhibited by insulin (23). Transcription of PDK4 is regulated by many transcription factors including FOXOs, ERRs, PPARs and the coactivator PGC1 (42). Therefore, PDK4 expression is regulated by distinct pathways in different cells, highlighting cell type differences in the control of this key metabolic enzyme. In this study, we show, for the first time, that TGFβ increases PDK4 mRNA and protein expression in colon cancer cells and that expression of PDK4 is induced by 5-FU in a TGFβ-dependent manner. Our study thus unveils another mechanism by which PDK4 expression is regulated.

Previous studies reported that DCA effectively reduced tumor growth of lung and breast
cancer cells both in vitro and in vivo (32,33). It was suggested that DCA could rapidly translate to cancer clinical trials since it has been used to treat lactic acidosis and found to be relatively safe in humans (43,44). However, the doses of DCA required to induce apoptosis in cancer cells are very high and unlikely to be achieved clinically without causing significant side effects (45). When combined with 5-FU, low doses of DCA and 5-FU could significantly induce apoptosis in colon cancer cells. Therefore, DCA may not be an appropriate single agent for colon cancer treatment but could be used to potentiate the effect of 5-FU. Our studies provide proof-of-concept that the combination of DCA and 5-FU could be a potentially effective therapy for colorectal cancer treatment. Moreover, since PDKs 1-3 appear to be not involved in drug resistance of colon cancer cells, development of PDK4-specific inhibitors would further reduce cytotoxicity and increase efficacy of chemotherapy in colorectal cancer treatment.

It has been shown that TGFβ signaling is associated with chemotherapy resistance in different types of cancer (10-12). One of the proposed mechanisms is through the induction of EMT by TGFβ (11). In addition, loss or inactivation of Smad4 has been shown to be a predictive marker for resistance to 5-FU-based chemotherapy in colorectal cancer (46,47). TGFβ activates non-canonical signaling (i.e. PI3K, ERK, JNK, etc.) in Smad4-deficient cells (48), which could contribute to drug resistance. In our studies, the colon cancer cells used express wild type Smad4 and TGFβ does not induce EMT in those cells (data not shown). Instead, TGFβ increases PDK4 expression, which confers resistance to drug treatment.

PDK4 has been reported to suppress cell proliferation by inhibiting PDH-mediated TCA cycle (49). In lung cancer cells, inhibition of PDK4 promotes EMT associated with erlotinib resistance (50). On the other hand, PDK4 has been shown to contribute to anoikis resistance in cancer cells (51). A recently study reported that PDK4 promote tumorigenesis through activation of CREB-RHEB-mTORC1 signaling cascade (52). Therefore, like TGFβ, the function of PDK4 is cell context-dependent and PDK4 may also function as a double-edged sword in cancer. While it could inhibit cell proliferation or sensitize cancer cells to drug treatment, it could also confer drug resistance or promote anoikis and tumorigenesis. We have shown in this study that PDK4 inhibits 5-FU-induced apoptosis by increasing expression of Bcl-2 and survivin. Future studies will determine the mechanisms by which PDK4 regulates their expression.

In summary, our discovery of the TGFβ/PDK4 axis as a mediator of drug response in colorectal cancer provides a potential therapeutic target against drug resistance. Inhibition of TGFβ signaling or PDK4 expression/activity could increase chemosensitivity in colorectal cancer treatment. Therefore, development of novel PDK4-specific inhibitors would greatly increase anti-tumor specificity and enhance efficacy of chemotherapy in colorectal cancer treatment.

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CONFLICT OF INTEREST
TGFβ/PDK4 confers drug resistance

The authors declare that they have no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

J.W. conceived and designed the experiments. Yang Z. performed most of the experiments. Yi Z., L.G., H.Y. and W.H. performed part of the experiments. G.T. provided and helped analyzed the human samples. Y.C.K. and S.M.W. helped with bioinformatics analysis. J.W. and Yang Z. analyzed and discussed the data. J.W. wrote the manuscript with the help of Yang Z.

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FOOTNOTES

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1 The abbreviations used are: CoA, coenzyme A; DNA, deoxyribonucleic acid or deoxyribonucleate; ERK,
extracellular-signal-regulated kinase; EDTA, ethylenediaminetetraacetate; ELISA, enzyme-linked
immunosorbent assay; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; FITC,
fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JNK, c-Jun N-terminal
kinase; mRNA, messenger RNA; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered
saline; PCR, polymerase chain reaction; PI3K, phosphatidylinositol 3-kinase PVDF, poly (vinylidene
difluoride); RNA, ribonucleic acid or ribonucleate; RT-PCR, reverse transcription-polymerase chain
reaction; SDS, sodium dodecyl sulfate; shRNA, short hairpin RNA; siRNA, small/short interfering RNA;
Tris, Tris(hydroxymethyl)aminomethane; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP
nick end labeling; TCA cycle, tricarboxylic acid cycle and TGFβ, Transforming Growth Factor β.
FIGURE LEGENDS

Figure 1  Expression of PDK4 is positively correlated with the resistance to 5-FU-induced apoptosis in colon cancer cells. A, RKO, HCT116, FET and CBS cells were treated with different concentrations of 5-FU for 72 hrs. DNA Fragmentation assays were performed. B, Annexin V/PI staining was performed after 2.5 µg/ml of 5-FU treatment for 72 hrs. C, Western blot analysis of cleaved PARP was performed after 5.0 µg/ml of 5-FU treatment for 72 hrs. D, Q-PCR assays show PDK4 expression in HCEC and colon cancer cells (left). RT-PCR assays were performed to examine expression of PDKs 1-3 in colon cancer cells (right). E, Western blot analysis of PDK4 expression was performed in colon cancer cells. F, Q-PCR assays of PDK4 expression were performed in colon cancer cells treated with 5.0 µg/ml of 5-FU for 72 hrs (left). RT-PCR assays were performed to examine expression of PDKs 1-3 in colon cancer cells treated with 5.0 µg/ml of 5-FU for 72 hrs (right). G, Western blot analysis of PDK4 expression was performed in colon cancer cells treated with 5.0 µg/ml of 5-FU for 72 hrs. The data are presented as the mean ± SD of triplicate experiments. *** P < 0.001.

Figure 2  PDK4 mediates the response of colon cancer cells to 5-FU induced apoptosis. A, Knockdown of PDK4 expression by shRNAs was confirmed in HCT116 and CBS cells by western blot. B, PDK4 knockdown and control cells were treated with different concentrations of 5-FU for 72 hrs. DNA fragmentation assays were performed. C, Western blot analysis of cleaved PARP, Bcl-2 and survivin were performed in PDK4 knockdown and control cells after 2.5 µg/mL (for HCT1116) or 5.0 µg/mL (for CBS) of 5-FU treatment for 72 hrs. D, Expression of PDK1, PDK2 and PDK3 was knocked down individually by their respective siRNA pools in HCT116 cells (upper). The knockdown and control cells were treated with different concentrations of 5-FU for 72 hrs. DNA fragmentation assays were performed (lower). E, PDK4 was overexpressed in HCT116 cells (left). Expression of Bcl-2 and survivin was determined in those cells (middle). Cells were treated with different concentrations of 5-FU for 72 hrs. DNA fragmentation assays were performed (right). The data are presented as the mean ± SD of triplicate experiments. ** P < 0.01, *** P < 0.001.

Figure 3  Inhibition of PDK4 expression or activity increases 5-FU-induced apoptosis by suppressing Bcl-2 and survivin expression. A&B, PDK4 knockdown and control cells were treated with different concentrations of oxaliplatin for 72 hrs. DNA fragmentation assays (A) and western blot analysis of cleaved PARP (B, oxaliplatin concentration, 10 µM) were performed. C&D, FET and CBS were treated with 1.0 µg/ml 5-FU, 15 mM DCA or both for 72 hrs. DNA fragmentation assays (C) and western blot analysis of cleaved PARP, Bcl-2 and survivin (D) were performed. E,F, Bcl-2 or survivin was ectopically expressed in CBS control (ctr) and PDK4 knockdown cells (sh2). Cells were treated with 5.0 µg/mL 5-FU for 72 hrs. Western blot analysis of cleaved PARP, Bcl-2 or survivin were performed (E). Cells were treated with 1.0 µg/mL 5-FU for 72 hrs. DNA fragmentation assays were performed (F). The data are presented as the mean ± SD of triplicate experiments. ** P < 0.01, *** P < 0.001.

Figure 4  Knockdown PDK4 expression increases the effectiveness of 5-FU in the inhibition of tumor growth in vivo. A, Xenograft tumor growth curves is shown. B & C, Images of TUNEL (B) and Ki67 (C) staining of tumors are shown in the left panels. The images are representative of multiple fields of tumor sections from each group. Scale bars, 25 µm. Percentage of positive TUNEL (B) and Ki67 (C) staining cells were determined as described in Materials and Methods. The data are presented as the mean 

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± SE (right panels). D, IHC analysis of PDK4 expression was performed in the xenograft tumors of the control and PDK4 knockdown cells. Representative images were shown in the left panel. Scale bars, 25 µm. Quantification of staining intensity of PDK4 was performed (right). The data are presented as the mean ± SD. *P < 0.05, **P < 0.01.

Figure 5 TGFβ mediates 5-FU resistance through regulation of PDK4 expression. A, FET-Vec and FET-DNRII cells were treated with different concentrations of 5-FU for 72 hrs. DNA Fragmentation assays were performed (left). Western blot analysis of cleaved PARP was performed after 2.5 µg/ml of 5-FU treatment for 72 hrs (right). B, HCT116-Vec and HCT116-RII cells were treated with different concentrations of 5-FU for 72 hrs. DNA Fragmentation assays were performed (left). Western blot analysis of cleaved PARP was performed after 2.5 µg/ml of 5-FU treatment for 72 hrs (right). C, FET-Vec, FET-DNRII, HCT116-Vec and HCT116-RII cells were treated with 4.0 ng/ml of TGFβ for 48 hrs. Western blot (upper) and RT-PCR (lower) analysis were performed to detect PDK4 expression. D, Expression of Smad2 or Smad3 was knocked down individually in FET cells (left). Cells were treated with 4.0 ng/ml of TGFβ for 48 hrs. Western blot (upper) and RT-PCR (lower) analysis were performed to detect PDK4 expression (right). E, FET-Vec, FET-DNRII, HCT116-Vec and HCT116-RII cells were treated with 5.0 µg/ml of 5-FU for 72 hrs. Western blot (upper) and RT-PCR (lower) analysis were performed to detect PDK4 expression. F, FET cells were treated with 5.0 µg/ml of 5-FU or 4.0 ng/ml of TGFβ for 1 hr. Western blot analysis was performed to examine p-Smad2. G, A scrambled shRNA or PDK4 shRNA (sh-2) was infected into FET-Vec and FET-DNRII cells. The cells were treated with 2.5 µg/ml 5-FU (upper) or different concentration of 5-FU (lower) for 72 hrs. Western blot analysis of PDK4 and cleaved PARP (upper) and DNA fragmentation assays (lower) were performed. The data are presented as the mean ± SD of triplicate experiments. * P < 0.05, ** P < 0.01.

Figure 6 PDK4 expression and Smad2 phosphorylation positively correlate with chemoresistance in colorectal cancer specimens. IHC staining of PDK4 and p-Smad2 was performed in sections prepared from 8 moderately and 10 non- or poorly responding colorectal tumors. A, Representative images of IHC staining of PDK4 and p-Smad2 in each group are shown. Scale bars, 100 µm. B, Quantification of staining intensity of PDK4 and p-Smad2 was performed. Error bars indicate SEM of the values in each group. *** P < 0.001. C, Correlation of PDK4 expression and pSmad2 was determined using Pearson's test (r = 0.8545, *** P < 0.001). The slope was generated by lineage regression analysis.
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Fig. 1

A

B

C

D

E

F

G
Fig. 2

TGFβ/PDK4 confers drug resistance
Fig. 3

A

B

C

D

E

F

TGFβ/PDK4 confers drug resistance
Fig. 4

A

B

C

D

TGFβ/PDK4 confers drug resistance
TGFβ/PDK4 confers drug resistance
**Fig. 6**

A. **Non/poor** vs **Moderate**

B. **Relative PDK4 intensity**
- Non/poor (n=10)
- Moderate (n=6)

C. **Relative P-Smad2 intensity**
- Pearson r=0.8545
- ***P<0.001
- Non/poor
- Moderate

**TGFβ/PDK4 confers drug resistance**
Transforming Growth Factor β Mediates Drug Resistance by Regulating Expression of Pyruvate Dehydrogenase Kinase 4 in Colorectal Cancer
Yang Zhang, Yi Zhang, Liying Geng, Haowei Yi, Wei Huo, Geoffrey Talmon, Yeong C. Kim, San Ming Wang and Jing Wang

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