

Curcumin Analogue CDF Inhibits Pancreatic Tumor Growth by Switching on Suppressor microRNAs and Attenuating EZH2 Expression

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Abstract

The histone methyltransferase EZH2 is a central epigenetic regulator of cell survival, proliferation, and cancer stem cell (CSC) function. EZH2 expression is increased in various human cancers, including highly aggressive pancreatic cancers, but the mechanisms underlying for its biologic effects are not yet well understood. In this study, we probed EZH2 function in pancreatic cancer using diflourinated-curcumin (CDF), a novel analogue of the turmeric spice component curcumin that has antioxidant properties. CDF decreased pancreatic cancer cell survival, clonogenicity, formation of pancreatospheres, invasive cell migration, and CSC function in human pancreatic cancer cells. These effects were associated with decreased expression of EZH2 and increased expression of a panel of tumor-suppressive microRNAs (miRNA), including *let-7a,b,c,d*, *miR-26a*, *miR-101*, *miR-146a*, and *miR-200b,c* that are typically lost in pancreatic cancer. Mechanistic investigations revealed that reexpression of *miR-101* was sufficient to limit the expression of *EZH2* and the proinvasive cell surface adhesion molecule EpCAM. In an orthotopic xenograft model of human pancreatic cancer, administration of CDF inhibited tumor growth in a manner associated with reduced expression of *EZH2*, *Notch-1*, *CD44*, *EpCAM*, and *Nanog* and increased expression of *let-7*, *miR-26a*, and *miR-101*. Taken together, our results indicated that CDF inhibited pancreatic cancer tumor growth and aggressiveness by targeting an EZH2-miRNA regulatory circuit for epigenetically controlled gene expression. *Cancer Res*; 72(1); 335–45. ©2011 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies with worst prognosis. Annually, 37,000 patients are diagnosed with pancreatic cancer, and accounting for 34,000 deaths, which is the fourth leading cause of cancer-related deaths in the United States (1). Over the past 2 decades, numerous efforts have been made in the treatment strategies including chemotherapy, radiation therapy, gene therapy, and immunotherapy to improve the survival of patients diagnosed with pancreatic cancer; however, the outcome has been very disappointing. Because of the absence of specific early symptoms and the lack of early detection

methods, pancreatic cancer is usually diagnosed at an advanced incurable stage (2, 3). Thus, the median overall survival is only 5 to 6 months after conventional therapies, resulting in less than 5 % overall 5-year survival rate (3). This disappointing survival outcome is, in part, due to late diagnosis as well as intrinsic (*de novo*) and extrinsic (acquired) therapeutic resistance of pancreatic cancer to conventional therapeutics.

A large number of studies have shown that the resistance to chemoradiation therapy and other currently available targeted therapies are, in part, due to the survival of tumor-initiating cells or cancer stem cells (CSC), a minor population of cells in the tumor mass (3–6), and their further enrichment after therapy. CSCs possess the ability of self-renewal and have the potential to regenerate into all types of differentiated cells within the tumor cell populations, which contribute to drive continued expansion of the population of malignant cells, which leads to increased invasion and metastasis (3–6). Thus, the failure to eliminate these special cells is considered to be one of the underlying causes of treatment failure, which is, in part, due to tumor cell resistance to conventional therapeutics, resulting in tumor recurrence and metastasis, suggesting that the identification of novel targets and targeted therapies are urgently needed for overcoming therapeutic resistance, which indeed could be achieved by targeted killing of CSCs.

To that end, enhancer of Zeste homologue 2 (EZH2) received increased attention in recent years. EZH2 is a histone methyltransferase enhancer of polycomb group complexes and it is a

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critical part of the cellular machinery involved in the epigenetic regulation of gene transcription (7). Polycomb group proteins are a class of chromatin-modifying enzymes that are capable of methylation of both DNA and core histones to regulate gene expression. Emerging evidence suggest that polycomb group proteins play important role in stem cell maintenance, X-inactivation, imprinting, development, differentiation, and proliferation. The altered expressions of many polycomb group proteins have been found to be associated with the development of cancers (8). Overexpression of EZH2 has been found in a variety of tumors such as breast cancer, prostate cancer, and PDAC (9–12) and found to be directly responsible for the *de novo* suppression in the expression of multiple genes involved in human cancers (13–15); however, the exact molecular mechanism(s) by which EZH2 plays a role in tumorigenesis is not clear. Emerging evidence suggest that the overexpression of EZH2 might cause normal cells to dedifferentiate back to the stem cell-like state by epigenetically repressing cell fate-regulating genes and tumor suppressor genes, leading to the development of tumor (8, 14, 16), suggesting that further mechanistic understanding of EZH2 regulation and finding novel agents that could inactivate EZH2 would become a novel strategy for the treatment of pancreatic cancer.

In this study, we examined the effect of diflourinated-curcumin (CDF), a novel synthetic derivative of curcumin and a known natural antitumor agent, on cell survival, clonogenicity, sphere-forming capacity, cell migration and invasion, CSC self-renewal capacity, and the expression regulation of EZH2 by miRNAs in pancreatic cancer cells *in vitro*. We also examined the role of EZH2 in the regulation of cell growth, clonogenicity, cell migration and invasion, and miRNA expression in pancreatic cancer cells and mechanistically investigated the regulation of EZH2 expression by siRNA transfection approach. Most importantly, we examined the role of miR-101 in the expression of *EZH2*, *EpCAM*, *Notch-1*, and *Oct4* in pancreatic cancer cells *in vitro* and also in an orthotopic animal model of pancreatic cancer *in vivo* and their mechanistic correlation with antitumor activity of CDF.

Materials and Methods

Cell culture

Human pancreatic cancer cell lines AsPC-1 and MiaPaCa-2 were used for this study on the basis of their sensitivities to chemotherapeutic drug gemcitabine, as described previously (17, 18). Both cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen), supplemented with 5% FBS, 2 mmol/L glutamine, 50 units/mL penicillin, and 50 µg/mL streptomycin in a standard culture condition, as described previously (17, 18). The cell lines have been tested and authenticated in the core facility "Applied Genomics Technology Center" at Wayne State University, Detroit, MI, on March 13, 2009. The method used for testing was short tandem repeat (STR) profiling using the PowerPlex 16 System from Promega.

Reagents and antibodies

CDF, a synthetic derivative of curcumin, was synthesized as described in our earlier publications (17, 19). Antibodies

against CD44, EpCAM, cleaved Notch-1, and SHH were purchased from Cell Signaling Technology. Antibodies against Notch-1, ABCG2, matrix metalloproteinase (MMP)-9, and Hes-1 were purchased from Santa Cruz. Antibodies against β-actin and EZH2 were acquired from Sigma Chemicals and BD Transduction.

Cell growth and survival assay

MTT assay was conducted to assess cell survival and growth. Both cell lines were exposed to different concentrations of CDF (0.1–1 µmol/L) for 3 days of treatment, and after 3 days, MTT assay was conducted as described previously (17).

Clonogenic assay

Clonogenic assay was conducted to examine the effect of CDF (0.5 µmol/L) on cell growth and proliferation of pancreatic cancer cells as described previously (17, 20). Briefly, 5×10^4 cells were plated in a 6-well plate and after 72 hours of exposure to 0.5 µmol/L of CDF, 1,000 single viable cells were plated in 100-mm Petri dishes. The cells were then incubated for 10 to 12 days at 37°C in a 5% CO₂/5% O₂/90% N₂ incubator. Colonies were stained with 2% crystal violet, washed with water, and counted.

Cell migration (wound-healing) assay

Cell migration (wound-healing) assay was conducted to assess the capacity of cell migration and invasion as described previously (21). Briefly, when the cells reached 90% to 95% confluency, the wound was generated by scratching the surface of the plates with a pipette tip. The cells were then incubated in the absence and presence of CDF (0.5 µmol/L) for 18 hours and then photographed with a Nikon Eclipse TS100 microscope.

Sphere formation assay

Sphere formation assay was conducted to assess the capacity of CSC self-renewal, as described previously (18). Briefly, single-cell suspensions of cells were plated on ultra-low-adherent wells of 6-well plate (Corning) at 500 to 1,000 cells/well in sphere formation medium (1:1 DMEM/F12 medium supplemented with B-27 and N-2; Invitrogen). After 7 days, the spheres termed as "pancreatospheres" were collected by centrifugation (300 × g, 5 minutes) and counted. The proportion of sphere-generating cells was calculated by dividing the number of cells seeded by the number of pancreatospheres with the diameter greater than 50 µm.

Protein extraction and Western blot analysis

Western blot analysis was conducted using whole-cell protein lysates of pancreatic cancer cells or tumor tissues. Total cell lysates from different experiments were obtained by lysing the cells in protein lysis buffer and 1× protease inhibitor cocktail, and Western blotting was conducted as described previously (17, 18).

TaqMan miRNA real-time reverse transcriptase PCR

To determine the expression of miRNAs (*let-7* family, *miR-26a*, *miR-101*, and *miR-200* family) in the cells or tumor samples, we used TaqMan MicroRNA Assay kit (Applied

Biosystems) following manufacturer's protocol. Five nanograms of total RNA was reverse transcribed and real-time PCR reactions were carried out in 10 μ L of reaction mixture as described previously (17, 22) using AB StepOnePlus Real-Time PCR System (Applied Biosystems). Data were analyzed using C_t method and were normalized by *RNU6B* or *RNU48* expression in each sample.

Levels of mRNAs by real-time reverse transcriptase PCR

To determine the mRNA expression of *EZH2*, *Notch-1*, *EpCAM*, *Nanog*, *Sox2*, and *Oct4*, 2 μ g of total RNAs extracted from each sample (pancreatic cancer cells of xenograft tumor sample) were used for reverse transcription (RT) reaction in 20 μ L of reaction volume using a reverse transcription system (Invitrogen) according to the manufacturer's instructions. SYBR Green Assay kit (Applied Biosystems) was used for real-time PCR reaction, following manufacturer's protocol. Sequences of PCR primers were described previously (17, 22). Data were analyzed using C_t method and were normalized by *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) expression in each sample.

Transfection of EZH2 siRNA

MiaPaCa-2 cells were plated in 6-well plate overnight and transfected with 10 μ g of EZH2 siRNA or scramble control siRNA (Applied Biosystems) by ExGen 500 (Fermentas) following the manufacturer's protocol. After 48 hours of transfection, the cells were collected for the cell growth, clonogenic assay, wound-healing assay, Western blot analysis, or RT-PCR of miRNAs as described above.

Transfection of miRNA precursor *miR-101*

A total of 2×10^5 cells per well of MiaPaCa-2 cells were seeded in 6-well plates and transfected with pre-*miR-101* or miRNA-negative control (Ambion) at a final concentration of 20 nmol/L using DharmaFECT transfection reagent (Dharmacon), following the manufacturer's protocol, and as described previously (21). After 3 days of transfection, the transfected cells were harvested for total RNA isolation or protein extraction. The relative levels of miRNA, mRNAs, and proteins were measured as described above.

Animal experiments

The animal protocol was approved by the Animal Investigation Committee, Wayne State University. Female CB17 severe combined immunodeficient (SCID) mice (4 weeks old) were purchased from Taconic Farms and fed Lab Diet 5021 (Purina Mills, Inc.). Initially, 1×10^6 MiaPaCa-2 cells or 5,000 pancreatospheres of MiaPaCa-2 were implanted in xenograft mouse model for comparison of protein expression between cells, xenograft tumor, and xenograft pancreatospheres. The tumors were removed and protein was extracted. For the actual therapeutic experiment, 1×10^6 MiaPaCa-2 cells were orthotopically implanted into the pancreas of the mice. One mouse was sacrificed after 2 weeks to confirm the growth of the tumor and were then randomly divided into 3 groups with 7 animals in each group: (i) untreated control; (ii) CDF (2.5 mg/mouse/d), intragastric once daily for 3 weeks; and (iii)

CDF (5 mg/mouse/d), intragastric once daily for 3 weeks. The tumor tissue from all groups were rapidly frozen in liquid nitrogen and stored at -70°C for later use for RNA, protein extraction, and another portion was fixed in formalin for histopathologic and immunohistochemical studies.

Immunohistochemistry

Formalin-fixed tumor tissue sections were evaluated by hematoxylin and eosin staining and immunohistochemistry was carried out through the core facility of Department of Pathology, Karmanos Cancer Institute, Detroit, MI, as described previously (23). Briefly, immunohistochemical staining was carried out for the expression of Ki-67 and CD44 using specific antibodies followed by staining with appropriate horseradish peroxidase-conjugated secondary antibodies. The slides were developed in diaminobenzidine and counterstained with hematoxylin. The stained slides were dehydrated and mounted in Permount solution and visualized using an Olympus microscope. Images were captured with an attached camera linked to the computer.

Statistical analysis

The data with mean and SD in this study were calculated using GraphPad Prism software (version 4.03). Comparisons of each treatment outcome were carried out for statistical difference by the paired *t* test. Statistical significance was assumed at a value of $P < 0.05$.

Results

Effect of CDF on cell survival of pancreatic cancer cells

To investigate the effect of CDF on cell survival of pancreatic cancer cells, we conducted MTT assays using AsPC-1, MiaPaCa-2 cells, and MiaPaCa-2 tumor sphere cells. The results indicate that CDF significantly inhibited cell survival in a dose-dependent manner in AsPC-1, MiaPaCa-2 cells, and MiaPaCa-2 tumor sphere cells (Fig. 1A). These results suggests that CDF could exert a pronounced cell growth-inhibitory effect on pancreatic cancer cell growth and proliferation, which is consistent with our previously published reports (17, 18).

Characterization of parental MiaPaCa-2 cells and tumors derived from either MiaPaCa-2 parental cells or MiaPaCa-2 sphere cells

We assessed the mRNA expressions of CSC markers such as *EZH2*, *Lin28B*, *Nanog*, and *EpCAM* in the parental MiaPaCa-2 cells, the MiaPaCa-2 sphere cells, and the MiaPaCa-2 tumor sphere cells. We found that the relative mRNA expressions of *EZH2*, *Lin28B*, *Nanog*, and *EpCAM* were significantly higher in the tumor sphere cells (Fig. 1B). We also assessed the protein expression of CSC markers such as EpCAM, Lin28B, Nanog, and EZH2 in the parental MiaPaCa-2 cells and tumors derived from either the MiaPaCa-2 parental cells or MiaPaCa-2 sphere cells (also called as tumor sphere cells). We found that the relative expressions of EpCAM, Lin28B, EZH2, and Nanog were significantly lower in the parental MiaPaCa-2 cells and tumors derived from MiaPaCa-2 parental cells (Fig. 1C), whereas the

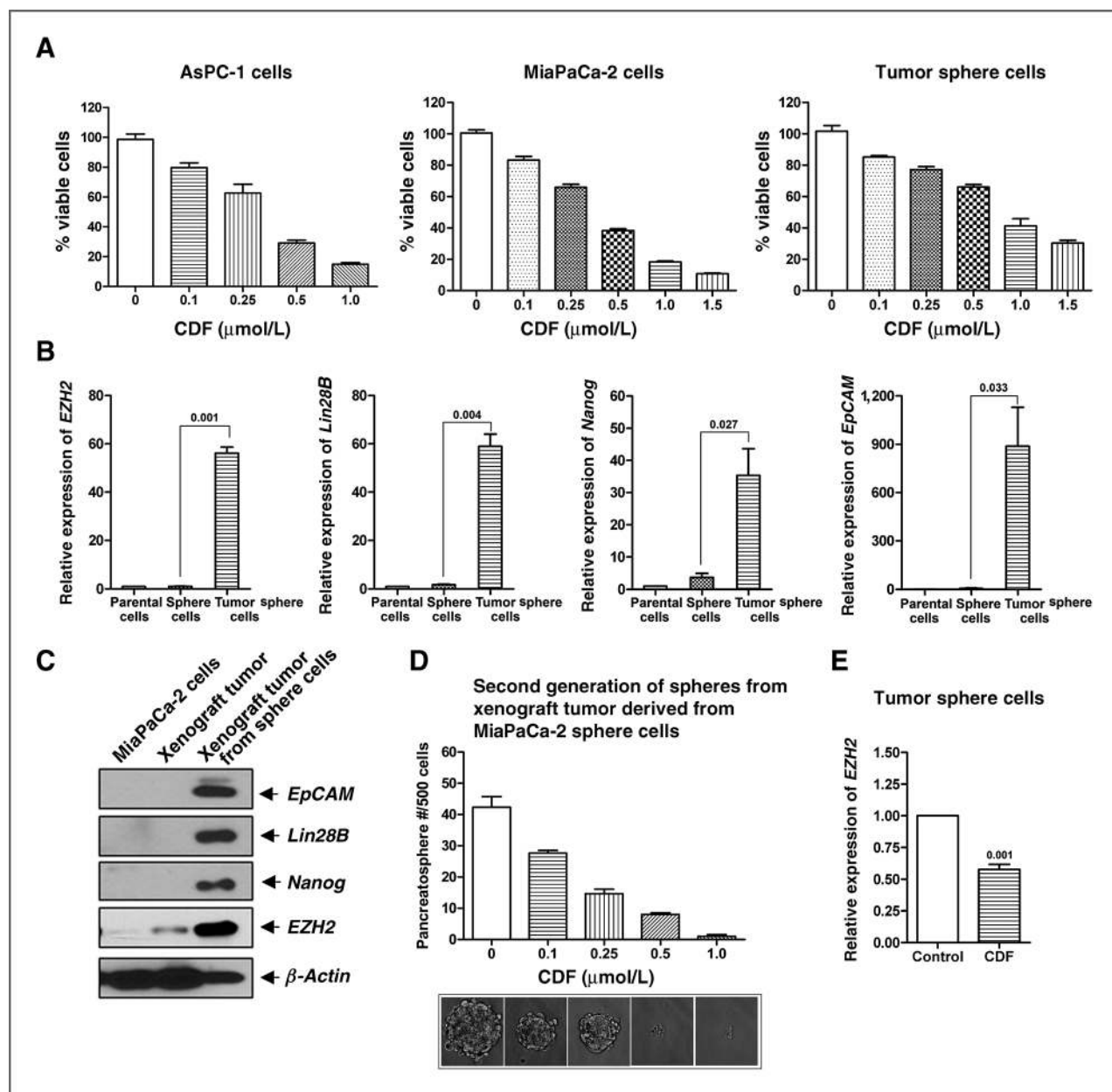


Figure 1. A, CDF decreased cell survival of AsPC-1, MiaPaCa-2 cells, and MiaPaCa-2 tumor sphere cells as assessed by MTT assay. B, differential mRNA expression of CSC marker genes in the parental MiaPaCa-2 cells, sphere cells, and tumor sphere cells. C, differential protein expression of CSC marker genes in MiaPaCa-2 cells, xenograft tumor from MiaPaCa-2 cells, and xenograft tumor derived from MiaPaCa-2 sphere cells. D, CDF inhibited the formation of second generation of spheres from mouse xenograft tumors derived from MiaPaCa-2 sphere cells. E, CDF decreased the expression of *EZH2* mRNA in MiaPaCa-2 sphere cells.

expression of all CSC makers was found to be highly expressed in the tumors derived from the MiaPaCa-2 sphere cells (Fig. 1C), suggesting CSC enrichment in the tumors derived from the pancreatospheres of the MiaPaCa-2 cells.

Effect of CDF on the sphere formation and *EZH2* mRNA in MiaPaCa-2 tumor sphere cells

In our previous studies (18), we have shown that CDF was effective in the inhibition of pancreatospheres formation

and as shown above that CSCs are highly enriched in the tumors derived from the MiaPaCa-2 pancreatospheres. Therefore, to examine the effect of CDF on self-renewal capacity of CSCs or CSC-like cells, we conducted sphere formation assay of the cells derived from the xenograft tumors established using pancreatospheres of MiaPaCa-2 cells (second generation of pancreatospheres or tumor sphere cells). We found significant inhibition of secondary pancreatospheres formation by CDF treatment in a

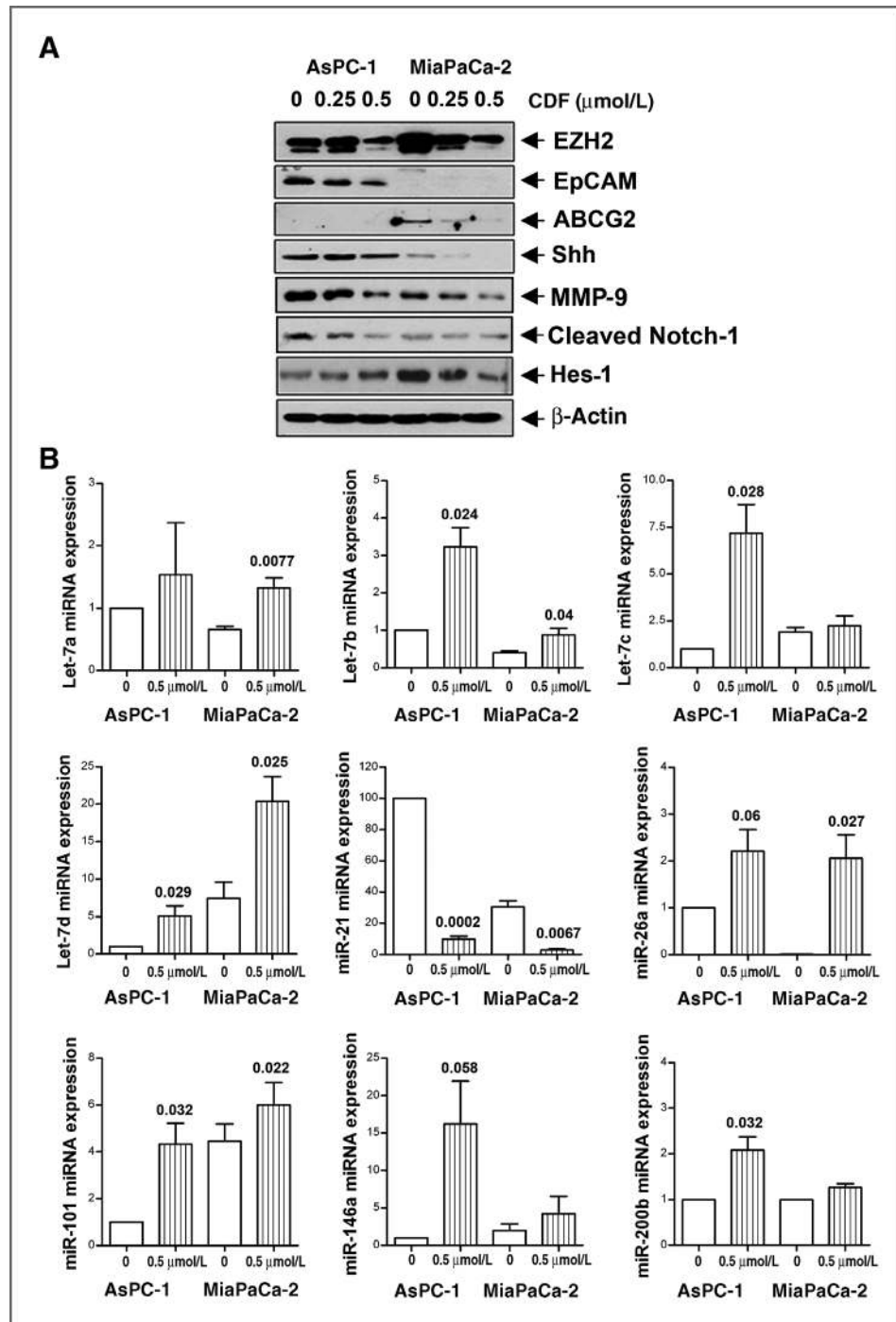


Figure 2. CDF decreased the expression of EZH2, EpCAM, ABCG2, Shh, MMP-9, cleaved Notch-1, and Hes-1 (A) and increased the miRNA expressions of *let-7* family, *miR-26a*, *miR-101*, *miR-146a*, and *miR-200* (B) in pancreatic cancer cells. Western blot analysis and real-time RT-PCR of miRNAs were carried out as described under the Materials and Methods section.

dose-dependent manner (Fig. 1D). These results clearly documents that CDF can inhibit the formation of pancreato-spheres, suggesting that CDF could indeed attenuate CSC function. We also examined the effect of CDF on *EZH2* mRNA which is overexpressed in CSCs such as MiaPaCa-2 tumor sphere cells. The results showed that CDF could significantly inhibit the expression of *EZH2* mRNA in tumor sphere cells (Fig. 1E).

Effect of CDF on EZH2, EpCAM, ABCG2, Shh, Hes-1, and MMP-9 in AsPC-1 and MiaPaCa-2 cells

We assessed the expression of CSC markers (EZH2, EpCAM, Shh, and Hes-1) as well as a marker (ABCG2) for drug resistance. Figure 2A shows that CDF was effective in inhibiting the expression of EZH2, Shh, and cleaved Notch-1 in AsPC-1 and MiaPaCa-2 cells, and CDF remarkably decreased the expression of EpCAM in AsPC-1 cells. CDF treatment also

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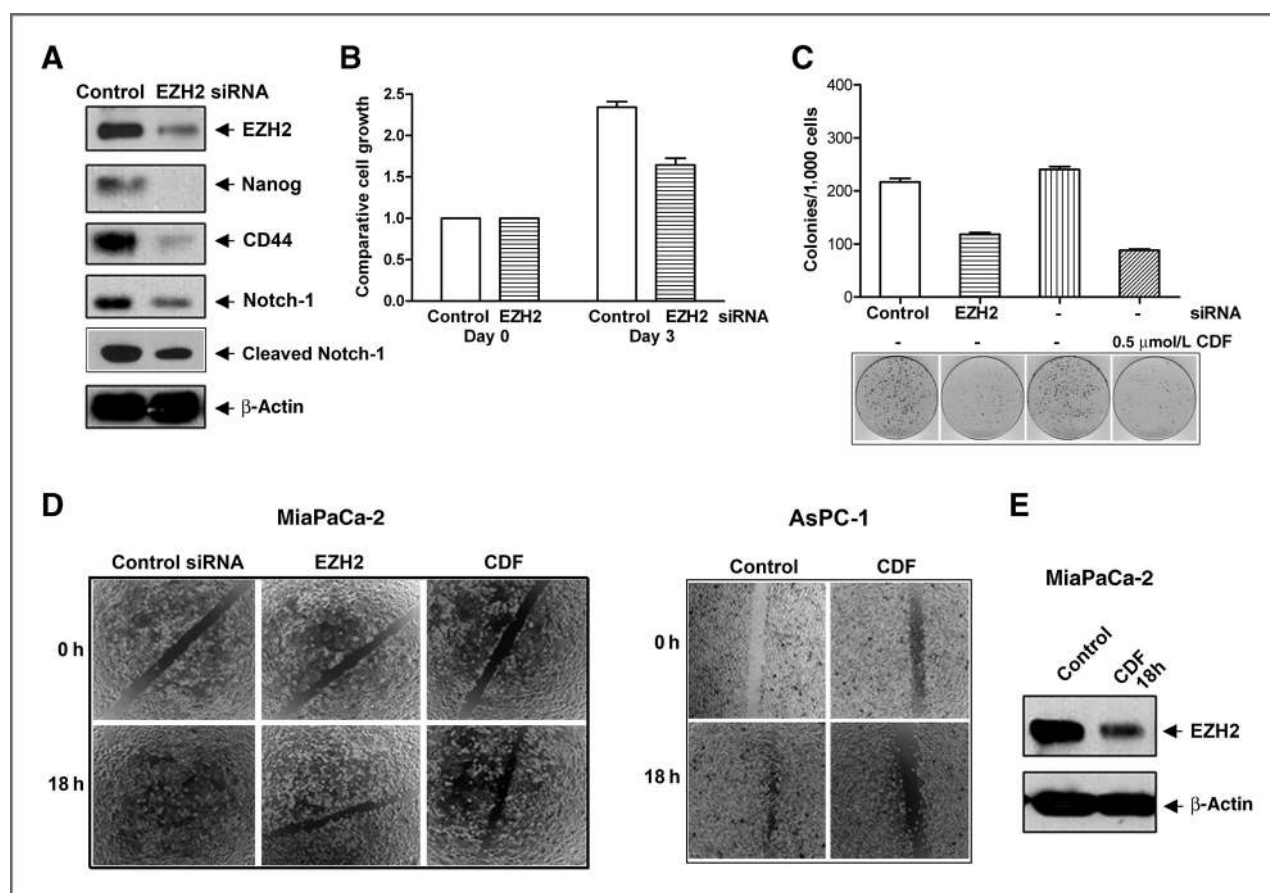


Figure 3. EZH2 siRNA decreased the relative protein levels of EZH2, Nanog, CD44, and Notch-1 expression (A), cell growth (B), clonogenicity (C), in MiaPaCa-2 cells and the wound-healing capacity (D) of MiaPaCa-2 and AsPC-1 cells. The transfection of EZH2 siRNA was conducted in MiaPaCa-2 cells. The transfected cells were then collected for cell growth assay, Western blot analysis, clonogenic assay, and wound-healing assay as described under the Materials and Methods section. CDF decreased the wound-healing capacity (D) and EZH2 expression (E) in pancreatic cancer cells after 18 hours of treatment.

remarkably decreased the expression of ABCG2 and Hes-1 in MiaPaCa-2 cells. We also found that CDF was very effective in inhibiting the expression of MMP-9, a biomarker for tumor metastasis, in AsPC-1 and MiaPaCa-2 cells (Fig. 2A).

Effect of CDF on the expression of miRNAs in AsPC-1 and MiaPaCa-2 cells by real-time RT-PCR

We examined the effect of CDF on the expression of miRNAs (*let-7* family, *miR-21*, *miR-26a*, *miR-101*, *miR-146a*, and *miR-200b*) in pancreatic cancer cells. The results revealed that CDF treatment caused reexpression of the miRNAs such as *let-7a,b,c,d*, *miR-26a*, *miR-101*, *miR-146a*, and *miR-200b* that are lost in AsPC-1 and MiaPaCa-2 cells (Fig. 2B). Interestingly, *miR-21* expression was very high in the AsPC-1 and MiaPaCa-2 cells and CDF was able to downregulate its expression. These data suggest that CDF differentially regulate the expression of these tumor-associated miRNAs in pancreatic cancer cells.

Effect of EZH2 deficiency on cell growth, clonogenicity, and cell migration of pancreatic cancer cells

To investigate the role of EZH2 in the regulation of cell growth, clonogenicity, and cell migration of pancreatic cancer cells, we carried out the EZH2 siRNA transfection experiments

using MiaPaCa-2 cells. We found that EZH2 siRNA transfection resulted in decreased expression of EZH2, but most interestingly, the knockdown of EZH2 led to decreased expression of Nanog, CD44, Notch-1, and cleaved Notch-1 expression (Fig. 3A). The downregulation of EZH2 resulted in decreased cell growth (Fig. 3B), decreased clonogenicity (Fig. 3C), and decreased cell migration (Fig. 3D). CDF decreased cell migration in MiaPaCa-2 and AsPC-1 cells after 18 hours of treatment (Fig. 3D). The Western blot analysis shows that CDF also decreased the expression of EZH2 in MiaPaCa-2 cells after 18 hours of treatment (Fig. 3E). These results suggest that EZH2 mediates cell growth, clonogenicity, and cell migration of pancreatic cancer cells and that the inhibition of cell growth/survival, clonogenicity, cell migration by CDF treatment is indeed mediated through the downregulation of EZH2 in pancreatic cancer cells.

Effect of EZH2 siRNA on the expression of miRNAs (*let-7* family, *miR-26a*, *miR-101*, and *miR-200b*) in MiaPaCa-2 cells

We examined the effect of EZH2 inactivation by EZH2 siRNA on the expression of miRNAs (*let-7* family, *miR-26a*, *miR-101*, and *miR-200b,c*) in MiaPaCa-2 cells by real-time RT-PCR.

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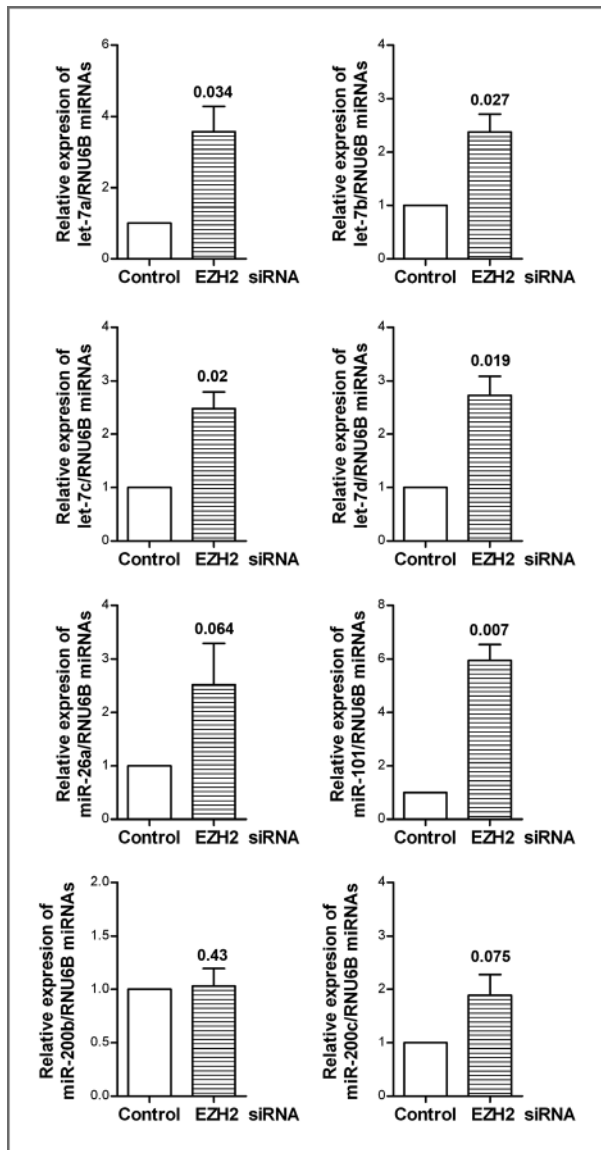


Figure 4. Effect of EZH2 siRNA on the expression of miRNAs in MiaPaCa-2 cells. After 72 hours of the transfection with EZH2 siRNA, the transfected cells were collected for real-time RT-PCR for assessing the expression of miRNAs as described under the Materials and Methods section.

We found that the inactivation of EZH2 led to reexpression of *let-7* family, *miR-26a*, *miR-101*, and *miR-200c* which are typically lost in pancreatic cancer cells (Fig. 4), and these results are consistent with CDF treatment results as presented above in Fig. 2B.

The role of *miR-101* in the regulation of *EZH2*, *EpCAM*, *Sox2*, *Oct4*, and *Notch-1* in MiaPaCa-2 cells

To investigate the role of *miR-101* in the regulation of CSC marker genes, we carried out transfection experiment using *miR-101* precursor. We found that the transfection of *miR-101* precursor led to increased expression of *miR-101* as expected, which led to decreased expression of EZH2 and EpCAM

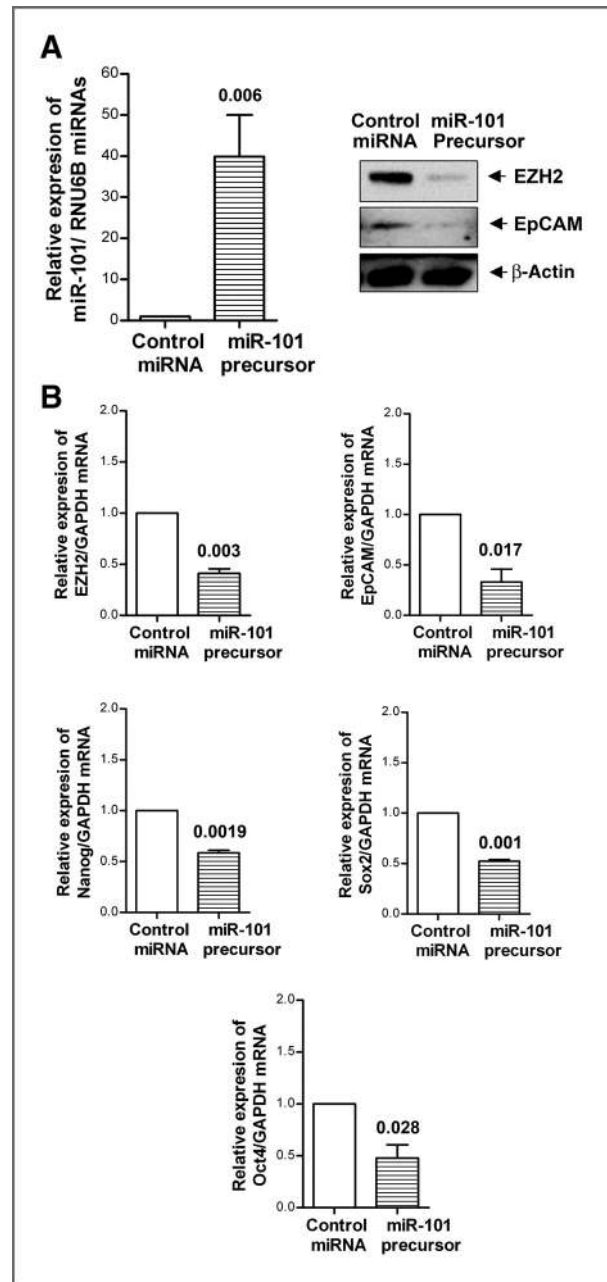


Figure 5. Reexpression of miR-101 decreased the expression of EZH2 and EpCAM proteins (A) and mRNAs of EZH2, EpCAM, Nanog, Sox2, and Oct4 (B) in MiaPaCa-2 cells. The transfection of miR-101 precursor was conducted in MiaPaCa-2 cells as described under the Materials and Methods section. Real-time RT-PCR and Western blot analysis were conducted to measure the relative mRNA and protein levels, respectively.

proteins in MiaPaCa-2 cells (Fig. 5A). Moreover, we found that the reexpression of miR-101 also led to decreased mRNA expression of *EZH2*, *EpCAM*, *Nanog*, *Sox2*, and *Oct4* mRNAs in MiaPaCa-2 cells (Fig. 5B), which suggests that *miR-101* plays a key role in the regulation of CSC marker genes such as *EZH2*, *EpCAM*, *Nanog*, *Sox2*, and *Oct4*.

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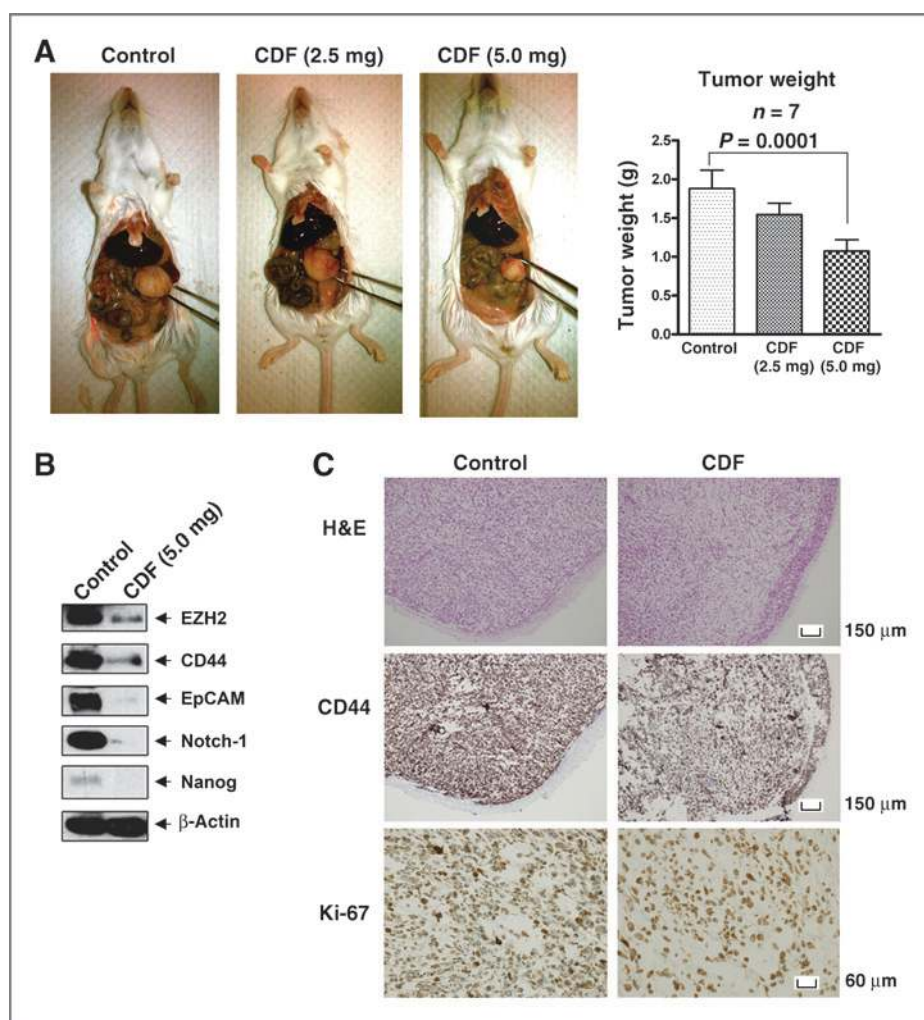


Figure 6. CDF decreased tumor growth (A), the relative protein levels of EZH2, CD44, EpCAM, Notch-1, and Nanog by Western blot analysis (B), the cell proliferation, Ki-67, and CD44 (C) immunohistochemistry using pancreatic tumor tissues orthotopically derived from human pancreatic cancer MiaPaCa-2 cells showing reduced expression of Ki-67 and CD44. H&E, hematoxylin and eosin.

Effect of CDF on tumor growth and on the expression of EZH2, CD44, EpCAM, Notch-1, and Nanog in tumor remnants *in vivo*

We carried out animal experiment using orthotopic mouse model of pancreatic cancer in which the tumors were derived from MiaPaCa-2 cells injected into the pancreas. We found that group with 5 mg CDF per mouse could significantly decrease tumor size and weight, compared with the control group or group with 2.5 mg CDF per mouse (Fig. 6A), which is consistent with our previous findings (17). Interestingly, we found that CDF treatment could decrease the protein expression of EZH2, CD44, EpCAM, Notch-1, and Nanog in pancreatic tumor remnant (Fig. 6B). Immunohistochemical study of pancreatic tumors revealed that the intensity scores of Ki-67 and CD44 from the control and CDF groups were 3 and 2, respectively, indicating that CDF treatment reduced the expression of Ki-67 and CD44, in pancreatic tumor tissue remnants (Fig. 6C).

Effect of CDF on gene expression and miRNA expression *in vivo*

We examined the effect of CDF treatment of orthotopic tumors on both the mRNA and miRNA expression in tumor

remnants. We found that CDF treatment significantly decreased the relative mRNA levels of *EZH2*, *Notch-1*, *Nanog*, and *Oct4* in pancreatic tumor tissues (Fig. 7A), suggesting that CDF could inhibit the mRNA expression of these CSC marker genes *in vivo*. Similarly, we also found that CDF treatment led to increased expression of *let-7* family, *miR-26a*, and *miR-101* in tumor tissue remnants (Fig. 7B) and these *in vivo* results are consistent with our *in vitro* findings.

Discussion

Emerging evidence suggests that EZH2 plays a key role in tumorigenesis by maintaining stem cell function including CSCs through epigenetic repression of cell fate-regulating genes and tumor suppressor genes (8, 14, 16). Increased expression of EZH2 has been observed in variety of tumors. For example, in prostate cancer, the overexpression of EZH2 was associated with aggressive and metastatic disease (11, 24). Similarly, in breast cancer, the expression of EZH2 was found to be elevated in invasive and metastatic tumor, and its increased expression was positively associated with poor prognosis (25, 26). The increased expression of EZH2 has also been found in

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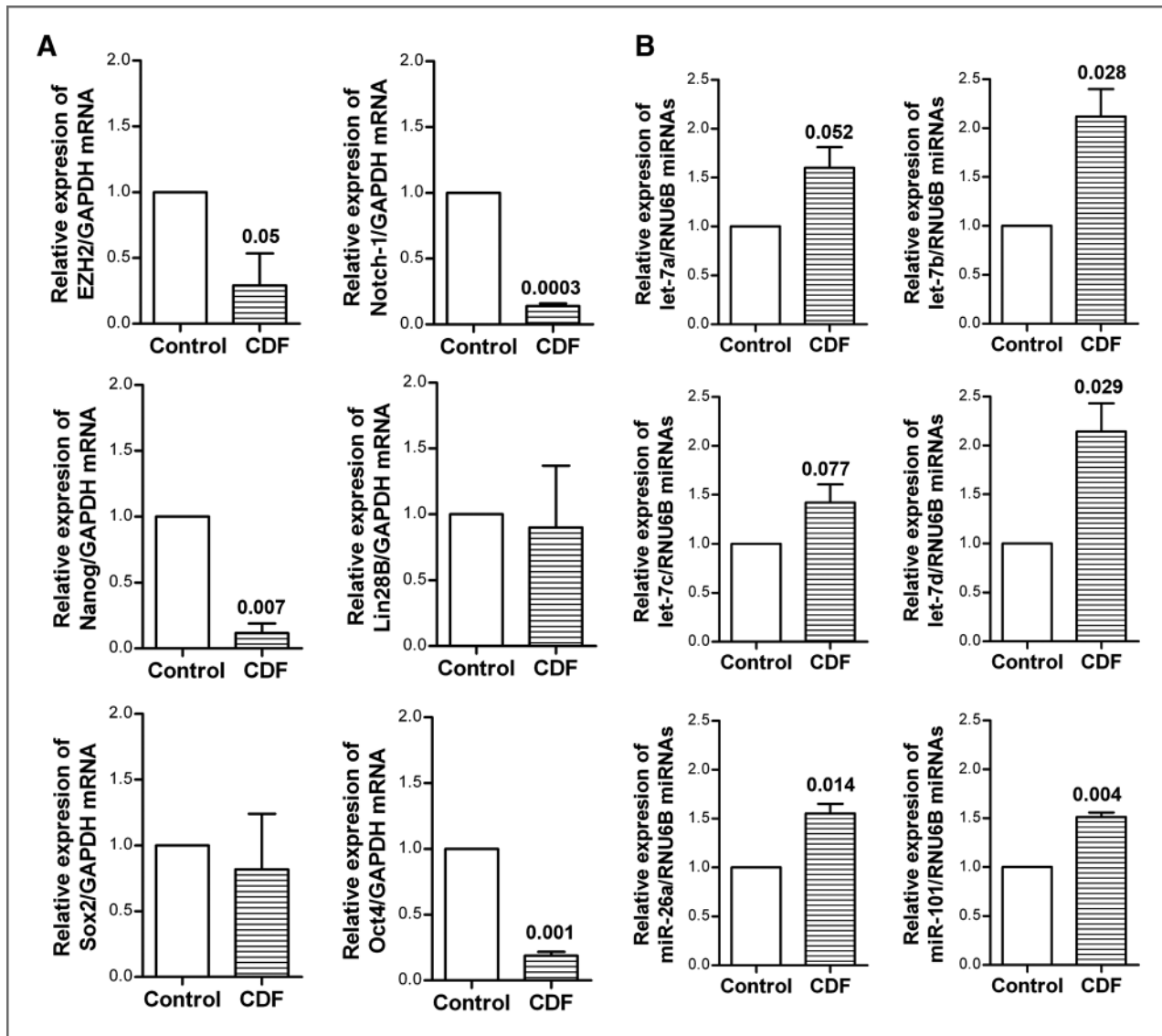


Figure 7. CDF decreased the mRNA expression of *EZH2*, *Notch-1*, *Nanog*, and *Oct4* in pancreatic tumor tissues (A), and increased miRNA expression of *let-7a,b,c*, *miR-26a*, and *miR-101* in pancreatic tumors (B) derived from human pancreatic cancer MiaPaCa-2 cell-induced tumor implanted orthotopically in the pancreas of severe combined immunodeficient (SCID) mice, and real-time RT-PCR was carried out as described under the Materials and Methods section.

other tumors including pancreatic cancer (26, 27). Recently, EZH2 has been identified to be associated with tumor angiogenesis, the self-renewal capacity, and the gene expression of CSC markers associated with tumor aggressiveness (12, 26, 28–31). In our current study, we have shown that the decreased EZH2 expression using EZH2 siRNA transfection led to decreased cell proliferation, migration, and the protein expressions of CSC markers such as Nanog, CD44, and Notch-1 in pancreatic cancer cells. Moreover, EZH2 deficiency led to increased expression of miRNAs such as *let-7a,b,c,d*, *miR-26a*, *miR-101*, and *miR-200* in pancreatic cancer cells, suggesting that these miRNAs are involved in the regulation of EZH2 and that inactivation of EZH2 could contribute to inhibit tumor aggressiveness. Our results on the effects of CDF on pancreatospheres formation of tumor cells derived from the

tumors induced by the pancreatospheres of MiaPaCa-2 cells, clearly suggest that CSCs could be eliminated by CDF by attenuating EZH2 and other CSC-specific genes through reexpression of miRNAs.

As documented earlier that miRNAs function as endogenous posttranscriptional gene regulators by specific binding to the 3' untranslated region (3'-UTR) of target mRNAs to mediate protein synthesis or mRNA stability (32, 33). The miRNAs are currently recognized as important and key regulators of the expression of most genes, and consequently, they play critical roles in wide variety of biologic processes, including cell differentiation, proliferation, death, metabolism, and energy homeostasis (33, 34). A large number of miRNAs have been reported to be associated with tumorigenesis by regulation of the expression/transcription of many tumor-related genes

(35). Emerging evidence suggests that miRNAs may play an important role in the regulation of stem cell function in tumors including pancreatic cancer (36). However, their exact roles in the pathogenesis of pancreatic cancer are not fully understood. Moreover, it has been reported that the *let-7* family function as a potential tumor suppressor and has been found to be downregulated in tumors, resulting in enhanced expression of Ras and c-Myc in tumor cells (35, 37, 38). In addition, the downregulation of *let-7* has been observed in pancreatic cancer (35, 38). A recent study has shown that the expression of CSC marker genes could be regulated bidirectionally and associated with the downregulation of *let-7* expression (39), suggesting that *let-7* may play a role in the regulation of CSC function. Our findings clearly show that inactivation of EZH2 led to increased *let-7* expression, and thus CDF-mediated reexpression of *let-7* family of miRNAs could be responsible for the biologic effects of CDF in pancreatic cancer cells and tumors.

Emerging evidence also suggests that *miR-200* serve as a potential tumor suppressor, primarily by repressing the acquisition of epithelial-to-mesenchymal transition (EMT) phenotype during the development and progression of tumors (17, 22, 35, 40, 41). Decreased expression of *miR-200* has been observed in many tumors including pancreatic cancer, which is associated with tumor invasion and metastasis (35, 42, 43). The *in vitro* studies revealed that the downregulation of *miR-200* enhances the acquisition of EMT phenotype in the cancer cells and that reexpression of *miR-200* results in the reversal of EMT phenotype (44), which is consistent with our previous findings in pancreatic cancer cells as summarized in our recent review article (36). A recent study has shown that loss of *miR-200b* increases breast CSC growth and invasive capacity by targeting CSC gene *Suz12*, suggesting a key role of *miR-200* in the deregulation of CSC function (45). Our results on the EZH2 siRNA transfection study showed that the inhibition of EZH2 led to increased expression of *let-7* and *miR-200* in pancreatic cancer cells, suggesting that EZH2 could be involved in the regulation of *let-7* and *miR-200* in cancer cells and that CDF could lead to the reexpression of *let-7* and *miR-200*, which would be highly valuable in reverting tumor cell aggressiveness or in the killing of CSCs.

Furthermore, *miR-26a* and *miR-101* are known potential tumor suppressors and have been reported to modulate the cancer epigenome by repressing the polycomb group protein, EZH2 (9, 10, 30, 46–48). Decreased expression of *miR-26a* and

miR-101 has been found in a wide variety of cancers such as gastric cancer, breast cancer, prostate cancer, nasopharyngeal carcinoma, and pancreatic cancer (49). Reexpression of *miR-26a* or *miR-101* in cancer cells leads to the downregulation of EZH2, resulting in the inhibition of tumor metastasis and invasion (9, 30, 47, 50). Our findings showed that reexpression of miR-101 results in the downregulation in the expression of EZH2 and EpCAM and that inactivation of EZH2 results in the upregulation of *miR-101* in pancreatic cancer cells, showing a negative mutual feedback loop of EZH2 and *miR-101*. These findings are also consistent with treatment of cells with CDF, suggesting that miRNAs that are lost in pancreatic cancer cells could be reexpressed using our novel agent CDF (18, 19). In our previous reports, we showed that CDF inhibited tumor growth by attenuating multiple targets such as NF- κ B, Akt, COX-2 signaling pathways, EMT phenotype and CSC function, PTEN, *miR-21*, and *miR-200* *in vitro* and *in vivo* (17, 18), which is consistent with our current findings showing that CDF could inhibit the expression of EZH2, Notch-1, and Oct4 through reexpression of *let-7a,b,c,d*, *miR-26a*, and *miR-101* in the pancreatic cancer cells as well as in tumor remnants. Collectively, our results together with our published reports clearly suggest that CDF could be a useful agent for the prevention of tumor progression and/or treatment of pancreatic cancer which would be accomplished by attenuating CSC function and overcoming therapeutic resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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