Metformin inhibits cell proliferation, migration and invasion by attenuating CSC function mediated by deregulating miRNAs in pancreatic cancer cells

Bin Bao¹, Zhiwei Wang¹, Shadan Ali², Aamir Ahmad¹, Asfar S. Azmi¹, Sanila H. Sarkar¹, Sanjeev Banerjee¹, Dejuan Kong¹, Yiwei Li¹, Shivam Thakur¹, and Fazlul H. Sarkar¹

Authors' Affiliations: ¹Department of Pathology, ²Department of Oncology Karmanos Cancer Institute, Wayne State University, Detroit, Michigan

*Corresponding Author: Fazlul H. Sarkar, Department of Pathology, Karmanos Cancer Institute, Wayne State University School of Medicine, 740 Hudson Webber Cancer Research Center, 4100 John R Street, Detroit, MI 48201. Phone: 313-576-8327; Fax: 313-576-8389; Email: fsarkar@med.wayne.edu

Running Title: Targeted killing of CSCs by metformin

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ABSTRACT

Pancreatic cancer (PC) is the fourth leading cause of cancer-related deaths in the United States, which is in part due to intrinsic (de novo) and extrinsic (acquired) resistance to conventional therapeutics, suggesting that innovative treatment strategies are required for overcoming therapeutic resistance in order to improve overall survival of patients. Oral administration of metformin in diabetes mellitus (DM) patients has been reported to be associated with reduced risk of PC and that metformin has been reported to kill cancer stem cells (CSCs); however, the exact molecular mechanism(s) has not been fully elucidated. In the current study, we examined the effect of metformin on cell proliferation, cell migration and invasion, self-renewal capacity of cancer stem-like cells (CSC), and further assessed the expression of CSC marker genes and microRNAs (miRNAs) in human PC cells. We found that metformin significantly decreased cell survival, clonogenicity, wound healing capacity, sphere-forming capacity (pancreatospheres), and increased disintegration of pancreatospheres in both gemcitabine-sensitive and gemcitabine-resistant PC cells. Metformin also decreased the expression of CSC markers, CD44, EpCAM, EZH2, Notch-1, Nanog and Oct4, and caused re-expression of miRNAs (let-7a, b, miR-26a, miR-101, and miR-200b, c) that are typically lost in PC and especially in pancreatospheres. We also found that re-expression of miR-26a by transfection led to decreased expression of EZH2 and EpCAM in PC cells. These results clearly suggest that the biological effects of metformin are mediated through re-expression of miRNAs and decreased expression of CSC-specific genes, suggesting that metformin could be useful for overcoming therapeutic resistance of PC cells.
INTRODUCTION

Pancreatic Cancer (PC) is one of the most lethal malignancies, and annually 37,000 patients are newly diagnosed with PC, resulting in 34,000 deaths annually in the United States, ranking PC as the fourth leading cause of cancer-related deaths (1). Due to the absence of any specific early symptoms and the lack of early detection methods, PC is usually diagnosed at advanced and incurable stage (2), consequently, the 5-year overall survival rate is less than 5% (1, 2). Among many risk-factors, smoking, alcohol consumption, chronic pancreatitis, and family risk factors have been widely recognized as potential risk factors of PC (3, 4). Moreover, epidemiological studies have shown that diabetes, especially type 2 diabetes mellitus (DM) is positively associated with increased risk of PC. Therefore, anti-diabetic drugs have been investigated for their use in the prevention and/or treatment of cancers such as PC for more than a decade although the precise molecular mechanism of action of metformin is still unknown.

The primary systemic effect of metformin for the treatment of DM is to increase insulin sensitivity and to decrease blood glucose levels through reduced hepatic gluconeogenesis and increased glucose uptake in peripheral tissues, including skeletal muscles and adipose tissues (3-5). A large number of epidemiological data have revealed that the oral use of metformin in DM patients elicits a protective effect by decreasing incidence of different tumors and improving prognosis of patients with cancers including PC (6, 7) For example, one recent large case-control clinical trial for the treatment of PC with metformin in DM patients was conducted involving 973 PC patients (including 259 DM patients) and 863 controls (including 109 DM patients) (8) showed that DM patients who received metformin had a significantly lower risk of PC compared to those who did not. In contrast, DM patients administered insulin had a significantly higher risk of PC compared to those without insulin. These recent findings clearly
suggest that the oral administration of metformin is positively associated with decreased risk of PC in DM patients while the use of insulin or other agents is associated with an increased risk. Therefore, metformin appears to exert a protective effect against the development and progression of PC; however, the molecular mechanism by which metformin elicits its biological effects has not been fully established.

Among several proposed mechanism of action of metformin (9-11), recent *in vitro* and *in vivo* studies are compelling showing that metformin can block tumor growth by inactivation of breast cancer stem-like cells (CSCs) and Epithelial-to-Mesenchymal Transition (EMT) phenotypic cells that are believed to be the root cause of tumor recurrence and metastasis (12, 13) especially because CSCs or EMT-phenotypic cells are highly resistant to conventional therapeutics. Moreover, it is clear that metformin could selectively kill CSCs (12, 14); however, the precise molecular mechanism of action of metformin remains to be elucidated. In the current study, we found that metformin inhibited cell proliferation, migration and invasion, and the CSC self-renewal capacity of both gemcitabine-sensitive and gemcitabine-resistant PC cells. These effects of metformin was in part due to decreased expression of CSC marker genes including CD44, EpCAM, EZH2, Notch-1, Nanog, and Oct4, which was associated with re-expression of specific miRNAs (let-7a,b,c, miR-26a, miR-101, and miR-200b,c) that are typically lost in PC and in pancreatospheres of gemcitabine-resistant cells. We have further demonstrated that re-expression of miR-26a by the transfection of its precursor decreased the mRNA and protein expression of EZH2 and EpCAM in PC cells, suggesting that the re-expression of miR-26a and other specific miRNAs results in the down-regulation of CSC-specific genes contributing to the killing of PC cells especially CSCs and EMT-type cells that are highly resistant to conventional therapeutics.
MATERIALS AND METHODS

Cell culture:

Human pancreatic cancer cell lines AsPC-1, AsPC-1-GTR, MiaPaCa-2, and MiaPaCa-2-GTR were used for this study based on their sensitivities to chemotherapeutic drugs, gemcitabine and tarceva (erlotinib). AsPC-1 and MiaPaCa-2 cells were exposed to 200 nmol/L of gemcitabine and 5 μmol/L of tarceva for 3 days every other week for about 6 months to establish gemcitabine and tarceva resistant cell lines, named as AsPC-1-GTR and MiaPaCa-2-GTR, respectively. All the cell lines were maintained in DMEM medium (Invitrogen, Carlsbad, CA), supplemented with 5% fetal bovine serum (FBS), 2 mmol/L glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin. All the cells were maintained in a standard culture condition, as described previously (15). The cell lines have been tested and authenticated in core facility at the Applied Genomics Technology Center of Wayne State University on March 13, 2009. The method used for testing was short tandem repeat (STR) profiling using the PowerPlex® 16 System from Promega (Madison, WI).

Reagents and antibodies:

Metformin was purchased from Sigma Chemicals (St Louis, MO). Difluorinated-Curcumin (CDF), a synthetic derivative of curcumin was synthesized as described in our earlier publication (15). Antibodies against CD44 and EpCAM were purchased from Cell Signaling Technology (Beverly, MA). Antibody against Notch-1 was purchased from Santa Cruz (Santa Cruz, CA). Antibody against β-actin was acquired from Sigma Chemicals (St. Louis, MO). Antibody against EZH2 was obtained from BD Transduction. Alexa Fluor 488 goat anti-mouse IgG for CD44 and EpCAM were purchased from Invitrogen. The miRNA and mRNA reverse
transcription (RT) primers and PCR probes were purchased from Applied Biosystems (Carlsbad, CA). Crystal violet was purchased from Sigma (St Louis, MO).

**Cell survival assay:**

MTT assay was performed using AsPC-1, AsPC-1-GTR, MiaPaCa-2, and MiaPaCa-2-GTR cells as described previously (15). All the cell lines were exposed to different concentrations of metformin (1-20 mmol/L) for 3 days of treatment. After 3 days, MTT assay was performed as described previously (15, 16).

**Clonogenic assay:**

Clonogenic assay was conducted to examine the effect of metformin on cell growth and proliferation in all the cells, as described previously (16). 5 x10⁴ cells were plated in a six-well plate. After 72h of exposure to 20 mmol/L of metformin, the cells were trypsinized, and 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 and invasion assays**

Cell migration (wound healing) assay was conducted to examine the capacity of cell migration and invasion, as described previously (17). Briefly, the wound was generated when the cells reached 90-95% confluent by scratching the surface of the plates with a pipette tip. The cells were then incubated in the absence and presence of metformin (20 mmol/L) for 18h, and
then photographed with a Nikon Eclipse TS100 microscope. Chamber invasion assay was conducted using BD BioCoat Tumor Invasion System, as described previously (16).

**Sphere formation/disintegration assay:**

Sphere formation assay was performed to assess the capacity of CSC self-renewal as described previously (18). Briefly, single cell suspensions of PC cells were plated on ultra low adherent wells of 6-well plates (Corning, Lowell, MA) at 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 (300xg, 5 min), and counted. The proportion of sphere-generating cells was calculated by dividing the number of cells seeded by the number of pancreatospheres. The sphere formation assay of secondary pancreatospheres was conducted by using primary pancreatospheres. Briefly, primary pancreatospheres were harvested and incubated with accutase (Sigma) at 37°C for 5-10 min. Single cell suspensions of pancreatospheres were plated at 500 cells/well in the sphere formation medium. After 1 or 3 weeks of incubation with metformin, secondary pancreatospheres were harvested for counting as described above.

For sphere disintegration assay, 1,000 cells/well on ultra low adherent wells of 6-well plate were incubated for 10 days, following 5 days of drug treatment the cells were harvested as described previously (18). The pancreatospheres were collected by centrifugation and counted under a microscope as described above.

CDF (difluorinated curcumin), a novel synthetic curcumin-derived compound, has been shown a greater bioavailability in mice compared to curcumin, and an anti-tumor activity of human pancreatic cancer in vivo by inactivation of NF-κB and COX2 and deregulation of miR-
21 and miR-200 (15, 16), suggesting that CDF may have an impact on the treatment of human cancers with a better outcome. Thus, our previous publications (15, 16, 19-21) provided the rationale for studying the role of CDF in the current manuscript. In order to examine the combined effect of CDF and metformin on CSC growth, we also conducted sphere formation and disintegration assays by the combination of metformin and CDF in PC cells.

Immunostaining assay and Confocal imaging microscopy:

For this experiment, we used enriched CSCs as described below. Single cell suspensions of secondary sphere cells formed by the cells derived from the tumors of MiaPaCa-2 pancreatospheres developed in a mouse xenograft, were plated at 5,000 cells/well in the sphere formation medium as described above. After 7 days of incubation with 20 mmol/L of metformin, the pancreatospheres were collected by centrifugation, washed with 1xPBS, and fixed with 3.7% paraformaldehyde. Monoclonal CD44 and EpCAM antibodies were used for immunostaining following the manufacturer’s protocol as described previously (22). The CD44 or EpCAM-labeled pancreatospheres were photographed under a Nikon E800. Confocal microscopy (Leica TCS SP5) was conducted in the MIRL Core Facility of Wayne State University School of Medicine.

Transfection of miRNA precursor miR-26a

2 × 10^5 cells/well of MiaPaCa-2 cells were seeded in six-well plates and transfected with pre-miR-26a or miRNA-negative control (Ambion, Austin, TX) at a final concentration of 20 nM using DharmaFECT transfection reagent (Dharmacon), following the manufacturer’s protocol as described previously (22, 23). 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 using standard methods as detailed below.

**Protein extraction and Western blot analysis:**

Western blot analysis was performed using whole cell lysates. Total cell lysates from different experiments were obtained by lysing the cells in the protein lysis buffer containing 50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM Na3VO42, 1 mM EDTA, 1 mM EGTA, and 1 x protease inhibitor cocktail, and Western blot analysis was performed as previously described elsewhere (24).

**TaqMan miRNA Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR):**

To determine the expression of miRNAs (let-7, miR-26a, miR-101, and miRNA-200b, c) in AsPC-1, AsPC-1-GTR, MiaPaCa-2, and MIAPaCa-2-GTR cells, we used TaqMan MicroRNA Assay kit (Applied Biosystems) following manufacturer’s protocol. Five ng of total RNA was reverse transcribed and real-time PCR reactions were carried out in 10 μL of reaction mixture as described earlier (25), using AB StepOnePlus Real-Time PCR System (Applied Biosystems). Data were analyzed using Ct method and were normalized by RNU6B or RNU48 expression in each sample.

**Levels of mRNAs by real time RT-PCR**

To determine the mRNA expression of EZH2, Notch-1, EpCAM, Nanog, Sox2, and Oct4 mRNAs in the cells, two micrograms of total RNAs from each sample were used for RT reaction.
in 20 μL of reaction volume, using a reverse transcription system (Invitrogen) according to the manufacturer's instructions. SYBR Green Assay kit (Applied Biosystems, Carlsbad, CA) was used for real time PCR reaction, following manufacturer's protocol. Sequences of PCR primers were described previously (22, 23). Data were analyzed using Ct method and were normalized by GAPDH expression in each sample.

**Animal experiments:**

The animal protocol was approved by the Animal Investigation Committee, Wayne State University, Detroit, MI. Female CB17 SCID mice (4 weeks old) were purchased from Taconic Farms (Germantown, NY) and fed Lab Diet 5021 (Purina Mills, Inc., Richmond, IN). About 5,000 pancreatospheres of MIAPaCa-2 were implanted in xenograft mouse model (4 weeks) for enriching the cancer stem cell (CSC) population. The tumor was removed and the cells were used in the formation of secondary pancreatospheres. The secondary tumor pancreatospheres were then treated with metformin for 1 or 3 weeks for assessing the effects of metformin on CSCs.

**Statistical analysis**

The data with mean and standard deviation (SD) as presented here were prepared using GraphPad Prism software (version 4.03). Comparisons of treatment outcome were tested for statistical difference by the paired t test. Statistical significance was assumed at a p value of <0.05.
RESULTS

Effect of metformin on cell survival and clonogenicity of gemcitabine-resistant PC cells

In order to investigate the effect of metformin on cell proliferation of PC cells, we conducted MTT and clonogenic assays using gemcitabine-resistant AsPC-1 and MiaPaCa-2 cells (AsPC-1-GTR and MiaPaCa-2-GTR cells). We found that metformin significantly inhibited cell survival by a dose-dependent manner in AsPC-1 and MiaPaCa-2 cells as well as gemcitabine and tarceva-resistant cells (AsPC-1-GTR and MiaPaCa-2-GTR cells) (Fig-1A). We also found that metformin significantly decreased the clonogenicity in both drug-sensitive and drug-resistant PC cells compared to those cells without metformin treatment (Fig-1B). These results suggest that metformin could elicit pronounced cell growth inhibitory effect on both drug-sensitive and drug-resistant PC cells.

Metformin inhibited cell migration and invasion of gemcitabine-resistant PC cells

In order to examine the effect of metformin on cell migration and invasion, we conducted cell migration (wound healing) assay using gemcitabine-sensitive and gemcitabine-resistant AsPC-1 and MiaPaCa-2 cells. We found that metformin treatment significantly inhibited the capacity of wound healing in both gemcitabine-sensitive and gemcitabine-resistant AsPC-1 and MiaPaCa-2 cells compared to cells without metformin treatment (Fig-1C), which suggests that metformin could inhibit the cell migration and invasion in drug-resistant PC cells. We also conducted Chamber Invasion assay in parental MiaPaCa-2 and its tumor sphere cells. The results indicate that MiaPaCa-2 tumor sphere cells had greater capacity of tumor invasion, compared to its parental cells (Fig-1D). Metformin treatment profoundly inhibited the capacity of tumor
invasion in both parental MiaPaCa-2 and its tumor sphere cells, compared to the cells without treatment (Fig-1D).

**Effect of metformin on the formation/disintegration of pancreatospheres in drug-sensitive and drug-resistant PC cells**

We examined the effect of metformin on self-renewal capacity of CSCs or CSC-like cells by conducting sphere-formation assay using both drug-sensitive and drug-resistant PC cells. We found that metformin significantly decreased the formation of pancreatosphere in both drug-sensitive and drug-resistant cells in a dose-dependent manner (Fig-2A). We also examined the effect of metformin and its combination with CDF, a novel curcumin-derived anti-tumor agent, on the formation of secondary pancreatospheres using both gemcitabine-sensitive and gemcitabine-resistant AsPC-1 and MiaPaCa-2 cells (Fig-2B). We found that metformin and its combination with CDF also inhibited the formation of secondary pancreatospheres of gemcitabine-resistant PC cells. In order to further examine the effect of metformin on disintegration of pancreatospheres, we incubated cells in sphere-formation medium for a period of 10 days to establish pancreatospheres, followed by 5 days of metformin treatment and its combination with CDF to assess the disintegration of pancreatospheres that already formed. We found that metformin and its combination with CDF increased the disintegration of pancreatospheres of both gemcitabine-sensitive and gemcitabine-resistant PC cells (Fig-3A). These results suggest that metformin could exert inhibitory effect on self-renewal capacity of both gemcitabine-sensitive and gemcitabine-resistant PC cells.
Effect of long-term metformin treatment on the formation of pancreatospheres of tumor cells derived from mouse xenograft tumors established by MiaPaCa-2 sphere-forming cells

In order to examine the long-term effect of metformin treatment on the formation of pancreatospheres induced by CSC-like cells, we conducted the sphere formation assay in the secondary pancreatospheres of mouse xenograft tumors derived from MiaPaCa-2 sphere-forming cells after 1 or 3 weeks of metformin treatment. The results show that metformin significantly decreased the formation of pancreatospheres in the secondary pancreatospheres assay (Fig-3B), suggesting that long-term metformin treatment can inhibit the capacity of CSC self-renewal.

Metformin decreased the expression of CD44 and EpCAM in pancreatospheres of mouse xenograft tumors derived from MiaPaCa-2 sphere-forming cells

We conducted Confocal imaging microscopy to examine the effect of metformin on the expression of CSC surface biomarkers, CD44 and EpCAM, in the secondary pancreatospheres of mouse xenograft tumors derived from MiaPaCa-2 sphere-forming cells. We found that metformin inhibited the expression of CD44 and EpCAM in pancreatospheres (Fig-3C), suggesting that the inhibition of pancreatospheres may be associated with the down-regulation of CD44 and EpCAM expression, markers of CSCs. These results are consistent with our in vitro findings.

Effect of metformin on the mRNA expression of CSC marker genes in pancreatospheres of drug-resistant PC cells

In order to examine the effect of metformin on the mRNA expression of CSC marker genes in drug-sensitive and drug-resistant pancreatospheres of PC cells, we measured the relative
mRNA levels of CSC marker genes, Nanog, Sox2, Oct4, Notch-1, and EZH2 in AsPC-1, AsPC-1-GTR, MiaPaCa-2, and MiaPaCa-2-GTR cells by qRT-PCR. We found that metformin significantly decreased the expressions of Oct4, Notch-1, and EZH2 mRNAs in pancreatospheres of AsPC-1, AsPC-1-GTR, MiaPaCa-2, and MiaPaCa-2-GTR cells (Fig-4). Metformin only decreased the relative expression of Nanog mRNA in pancreatospheres of MiaPaCa-2, and MiaPaCa-2-GTR cells (Figure 4). These results strongly suggest that metformin elicits pronounced inhibitory effects on the expression of CSC marker genes in pancreatospheres of PC cells.

**Effect of metformin on the expression of miRNAs (let-7, miR-26a, miR-101, and miR-200), in pancreatospheres of PC cells**

We examined the effect of metformin on the expression of miRNAs (let-7, miR-26a, miR-101, and miR-200) in pancreatospheres of PC cells by qRT-PCR. We found that metformin treatment increased the relative expressions of let7a, b, miR-26a, miR-101, miR-200b, c in pancreatospheres of AsPC-1, AsPC-1-GTR (Fig-5A), MiaPaCa-2, MiaPaCa-2-GTR cells (Fig-5B). Metformin increased the relative expressions of let-7c miRNA in pancreatospheres of MiaPaCa-2 and MiaPaCa-2-GTR cells, but not AsPC-1 and AsPC-1-GTR cells. Metformin increased the relative expression of miR-200b in pancreatospheres of AsPC-1, AsPC-1-GTR, and MiaPaCa-2 cells, but not MiaPaCa-2-GTR cells. These results suggest that metformin could cause re-expression of specific miRNAs although there are certain degrees of selectivity among the PC cells used in our study.
The role of miR-26a in the regulation EZH2 and EpCAM expression in PC cells

We investigated the mechanistic role of miR-26a in the regulation of EZH2 and EpCAM in PC cells by transfection of miR-26a precursor into MiaPaCa-2 cells. We found that the transfection of miR-26a precursor increased the relative level of miR-26a and consequently decreased the relative levels of EZH2 and EpCAM proteins and mRNAs in MiaPaCa-2 cells (Fig-6A-B). Re-expression of miR-26a significantly decreased the relative mRNA levels of EZH2, Oct4, Notch-1, and EpCAM in MiaPaCa-2 cells (Fig-6C), which suggest that re-expression of miR-26a is mechanistically associated with the down-regulation of EZH2, Oct4, Notch-1, and EpCAM expression in PC cells, and thus metformin-mediated re-expression of miR-26a is mechanistically associated with its biological effects on CSCs.

Effect of re-expression of let7b, miR-26a, and miR-200b on the formation of pancreatospheres of PC cells

In order to examine the role of let7b, miR-26a, and miR-200b in the growth of pancreatospheres, we conducted the sphere forming assay using PC cells transfected with premiRNA. The results show that re-expression of let7b and miR-26a significantly decreased the formation of pancreatospheres (p<0.05) (Fig-6D), suggesting that the loss of expression of let7b and miR-26a is mechanistically linked with the regulation of the growth of pancreatospheres of PC cells, which is consistent with the acquisition of CSC function of PC cells. These results clearly suggest that the re-expression of miRNAs that are lost in aggressive PC cells could become a novel approach for the treatment of PC, which requires further in vivo pre-clinical studies.
DISCUSSION

Accumulating *in vitro and in vivo* studies have demonstrated that metformin could exert its anti-tumor effect by targeting multiple pathways such as AMPK/mTOR, anti-inflammatory pathway via inactivation of NF-κB, cell cycle/apoptosis, insulin/IGF-1R, and angiogenesis in cancers (9-11, 26) Moreover, recent studies have demonstrated that metformin can block tumor growth by inactivation of breast CSC-like cells and by targeted killing of Epithelial-to-Mesenchymal Transition (EMT) phenotypic cells (12, 27-29). It is now well accepted that CSCs possess the ability to self-renew and give rise to differentiated tumor cells, which contribute to resistance to conventional therapeutics, and it is believed to be the root cause of tumor recurrence and metastasis (12). Therefore targeting CSC self-renewal pathways might provide a new strategy for cancer treatment with better therapeutic outcome.

It has also been shown that metformin can decrease the dose of chemotherapy (doxorubicin) and prolonged tumor remission in mouse xenograft of human breast cancer cells by selectively killing CSCs (30). This effect of metformin is due to inhibition of cell proliferation, migration and invasion, the CSC self-renewal capacity, and the expression of CSC marker genes such as CD44, EpCAM, EZH2, Notch-1, Nanog, and Oct4 in gemcitabine-resistant human PC cells as documented in our current study. Collectively, these results suggest that metformin could inhibit tumor cell growth via targeted killing of CSCs that are highly resistant to conventional therapeutics especially for PC.

Enhancer of Zeste Homologue 2 (EZH2) is a histone methyltransferase enhancer of polycomb group complexes and it is a critical part of the cellular machinery involved in the epigenetic regulation of gene transcription (31, 32). The over-expression of EZH2 has been observed in a variety of tumors such as breast cancer, prostate cancer, and PC (33, 34), and the
over-expression of EZH2 is believed to directly responsible for *de novo* suppression of multiple genes in human cancers (35, 36). Emerging evidence also suggest that the over-expression 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(35, 37). In this study, we have shown for the first time, demonstrating that metformin decreases the gene expression of EZH2 in pancreatospheres of drug-resistant PC cells, suggesting that metformin may exert its anti-tumor activity by targeting EZH2 in PC cells. We further investigated how metformin could down-regulate EZH2 expression as well as the expression of other CSC marker genes. To that end, it is known that microRNAs (miRNAs) are key players which function as endogenous post-transcriptional gene regulators by specific binding to the 3’ untranslated region (3’UTR) of target mRNAs to mediate protein synthesis or mRNA stability (38, 39). A large number of miRNAs have been reported to be associated with tumorigenesis, and are suspected to be mechanistically associated with malignant diseases (40-42); however, their exact roles in the pathogenesis of PC are not fully understood.

In the pathogenesis of cancer, let-7 family of miRNAs has been found to be down-regulated thereby increasing the expression of Ras and c-Myc in malignant cells (40, 42, 43). In addition, the down-regulation of let-7 has been observed in PC, which was associated with the aggressiveness of tumors (44, 45). Recent studies have shown that the increased expression of CSC marker genes has been found to be associated with the down-regulation of let-7 expression (44, 45). In the current study, we have demonstrated that metformin treatment could increase the expression of let-7a, b in pancreatospheres of PC cells, but most importantly in drug-resistant cells, suggesting that inactivation of CSC function would inhibit CSC self-renewal capacity by metformin treatment, which is in part due to re-expression of let-7. Emerging evidence also
suggest that miR-26a and miR-101, potential tumor suppressors, could modulate the cancer epigenome by repressing the polycomb group protein, EZH2 (46-49) whose expression has been linked with tumor angiogenesis, self-renewal capacity and regulation of CSC marker genes associated with tumor invasion and metastasis (31, 50). Altered expression of miR-26a and miR-101 have been found in a wide variety of cancers such as gastric cancer, breast cancer, prostate cancer, nasopharyngeal carcinoma, and PC (41, 49). Re-expression of miR-26a or miR-101 in cancer cells caused down-regulation of EZH2, resulting in the inhibition of tumor invasion and metastasis (46, 49). Therefore, targeting miR-26a and miR-101 provides a novel therapeutic approach for cancer treatment. Our data showed that re-expression of miR-26a results in the down-regulation of EZH2 and EpCAM expression, which was consistent with metformin-mediated re-expression of miR-26a associated with the inhibition of cell proliferation, migration and invasion, and CSC function, all of which was in part due to down-regulation of EZH2 and other CSC marker genes. Collectively, our results suggest that metformin could be useful for reverting CSC function or the killing of CSCs by re-expression of specific miRNAs and down-regulation of CSC-specific genes that are typically over-expressed in PC, and thus will result in better treatment outcome of patients diagnosed with PC, which requires further in-depth pre-clinical experiments using relevant animal models.
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Legends

Figure-1: Metformin decreased cell survival (A), clonogenicity (B), wound healing capacity (C), and invasion (D), in AsPC-1, AsPC-1-GTR, MiaPaCa-2, and MiaPaCa-2-GTR cells. The drug-resistant cell lines AsPC-1-GTR and MiaPaCa-2-GTR were established as described under the Methods section. Wound healing assay was performed to assess the capacity of cell migration and invasion of both drug-sensitive and drug-resistant cells. The cells were incubated with 20 mM of metformin for 18h. Chamber invasion assay was conducted in parental MiaPaCa-2 and its tumor sphere cells for 18h of incubation.

Figure-2: Metformin and/or its combination with CDF inhibited the capacity of CSC-self-renewal in primary (A) and secondary (B) pancreatospheres of AsPC-1, AsPC-1-GTR, MiaPaCa-2, and MiaPaCa-2-GTR cells. The sphere-forming assay was conducted using drug-sensitive and drug-resistant cells incubated with different concentrations of metformin or its combination with CDF (10 mM metformin plus 0.25 μM CDF) for 7 days.

Figure-3: Metformin or/and its combination with CDF increased the disintegration of pancreatospheres (A), decreased CSC self-renewal capacity in the secondary pancreatospheres of mouse xenograft tumor derived from MiaPaCa-2 sphere-forming cells after 1 and 3 weeks of treatment (B), Metformin (20 mM) decreased the expression of CD44 and EpCAM in the secondary pancreatospheres of mouse xenograft tumor derived from MiaPaCa-2 sphere-forming cells after 1 week of treatment (C).
**Figure-4:** Metformin down-regulated the mRNA expression of Oct4, EZH2, and Notch-1 in pancreatospheres of AsPC-1, AsPC-1-GTR, MiaPaCa-2, and MiaPaCa-2-GTR cells as assessed by qRT-PCR.

**Figure-5:** Metformin up-regulated the miRNA expression of let-7a,b,c, miR-26a, miR-101, miR-200b, c in AsPC-1, AsPC-1-GTR, MiaPaCa-2, and MiaPaCa-2-GTR cells as assessed by qRT-PCR.

**Figure-6:** Re-expression of miR-26a decreased the expression of EZH2 and EpCAM proteins (A) and mRNAs (B) in MiaPaCa-2 cells, and inhibited the mRNA expressions of EZH2, Oct4, Notch-1, and EpCAM in MiaPaCa-2 tumor sphere cells (C). Re-expression of let7b and miR-26a decreased the formation of pancreatospheres of MiaPaCa-2 cells (D). The transfection of miR-26a miRNA precursors were conducted in MiaPaCa-2 cells and its tumor sphere cells as described under the Methods section. Western blot analysis and qRT-PCR were performed to measure the relative protein levels and mRNA respectively. The sphere forming assay was conducted as described under the Methods section. P values represent comparison between controls as calculated by the paired t test.
Figure 2

A

B
Figure 5

A

B

[Bar charts showing mRNA expression levels for different conditions and cell lines.]
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