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Fibulin-3 negatively regulates ALDH1 via c-MET suppression and increases γ-radiation-induced sensitivity in some pancreatic cancer cell lines

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ABSTRACT

Fibulin-3 (FBLN-3) has been postulated to be either a tumor suppressor or promoter depending on the cell type, and hypermethylation of the *FBLN-3* promoter is often associated with human disease, especially cancer. We report that the promoter region of the *FBLN-3* was significantly methylated (>95%) in some pancreatic cancer cell lines and thus *FBLN-3* was poorly expressed in pancreatic cancer cell lines such as AsPC-1 and MiaPaCa-2. *FBLN-3* overexpression significantly down-regulated the cellular level of c-MET and inhibited hepatocyte growth factor-induced c-MET activation, which were closely associated with γ-radiation resistance of cancer cells. Moreover, we also showed that c-MET suppression or inactivation decreased the cellular level of ALDH1 isozymes (ALDH1A1 or ALDH1A3), which serve as cancer stem cell markers, and subsequently induced inhibition of cell growth in pancreatic cancer cells. Therefore, forced overexpression of *FBLN-3* sensitized cells to cytotoxic agents such as γ-radiation and strongly inhibited the stemness and epithelial to mesenchymal transition (EMT) property of pancreatic cancer cells. On the other hand, if *FBLN3* was suppressed in *FBLN-3*-expressing BxPC3 cells, the results were opposite. This study provides the first demonstration that the FBLN-3/c-MET/ALDH1 axis in pancreatic cancer cells partially modulates stemness and EMT as well as sensitization of cells to the detrimental effects of γ-radiation.

1. Introduction

Recent studies have suggested that increased aldehyde dehydrogenase (ALDH), which represents a family of evolutionarily conserved enzymes localized in the nucleus, cytoplasm, and mitochondria, is a key feature of cancer stem cells (CSCs) that is measurable by the ALDEFLUOR assay [1,2]. The assay was originally used for the isolation of hematopoietic stem cells and is now successfully and universally used to isolate CSCs of many cancers, including those of the lung, prostate, colon and pancreatic cancer [3–5]. Importantly, identifying specific ALDH isoforms prevalent in certain cancer types may have critical prognostic applicability. Among 19 ALDH isoforms expressed in human, ALDH1A1 was reported to be responsible for the ALDH activity of pancreatic CSCs [6]. A more recent experiment with human breast CSCs indicated that other ALDH isoforms, particularly ALDH1A3, significantly contribute to ALDEFLUOR positivity, which may be tissue and cancer specific [7]. In addition, ALDH4A1 or ALDH7A1 is prevalent in other breast cancer cell types or in prostate cancer cells [7,8]. Therefore, quantification of prevalent ALDH isoforms and identification of their controlling signal pathway are required for potential prognostic or therapeutic applications.

Fibulin-3(FBLN-3) is a secreted extracellular matrix glycoprotein, which is characterized by repeated epidermal growth factor-like domains. Recent studies have yielded conflicting results regarding the role of FBLN-3 in cancer biology, showing either tumor-suppressive or oncogenic behavior depending on the cancer type. In glioma and pancreatic cancer, *FBLN-3* overexpression was associated with poor prognosis because of malignant cell growth [9–11]. On the other hand, reduced FBLN-3 caused by *FBLN-3*

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promoter methylation occur in hepatocellular carcinoma (HCC) and lung, breast, and prostate cancers and are associated with worse prognosis [12–16].

In pancreatic cancer cells, chemo- and radioresistance are more prevalent compared with other cancer cell types; these characteristics are contributed by CSCs. Some studies have explored the functional role of ALDH1 or c-MET as a novel marker for pancreatic CSCs [17,18]. In this study, in spite of previous reports that *FBLN-3* overexpression promotes cancer metastasis, angiogenesis, and tumor growth [10,11], we first investigated whether *FBLN-3* gene silencing is associated with poor prognosis in some pancreatic cancer cell lines, which are highly methylated in the *FBLN-3* promoter region. Moreover, we also showed that FBLN-3 inhibited c-MET activation and gene expression, and this FBLN-3/c-MET axis also negatively regulated cellular levels of ALDH1 isoforms, ALDH1A1 and ALDH1A3, which have been linked to the "stemness" characteristics of CSCs.

2. Materials and methods

2.1. Cell culture and irradiation

Human pancreatic cancer cell lines (AsPC1, BxPC3 and MiaPaCa2) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cell lines were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Hyclone Lab, Logan, UT, USA) and penicillin/streptomycin. The MiaPaCa2 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and penicillin/streptomycin. Cells were incubated at 37 °C in a humidified atmosphere with 95% air/5% CO_2. Cells were inoculated at a density of 1×10^5 - cells in a T-25 flask, incubated for 1 day, and then irradiated with a total dose of 6 Gy from a ^{60}Co γ-ray at a rate of 0.5 Gy/min.

2.2. cDNA synthesis and PCR amplification (FBLN-3, ALDH1A1, ALDH1A3, c-MET)

Total RNA was isolated from cancer cells using RNA extraction TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and first-strand complementary DNA (cDNA) was synthesized from the total RNA (1 μg) using a cDNA synthesis kit (Intron Biotechnology, Gyungki-do, Korea). Resultant cDNAs served as templates for polymerase chain reaction (PCR) amplification with the following forward and reverse primers: FBLN-3-forward, 5′-ATGTTGAAAGCCCTTTTCC-3′; FBLN-3-reverse, 5′-CTAAAATGAAAATGGCCCC-3′; ALDH1A1-forward, 5′-ATATAAGCTTATGTCATCCTCAGGCACGCC A-3′; ALDH1A1-reverse, 5′-ATATGAATTCTTATGAGTTCTTCTGAGAGAT; ALDH1A3-forward, 5′-GCCCTGGAGACGATGGATAC-3′; ALHD1A3-reverse, 5′-TCCACTGC CAAGTCCAAGTC-3′; c-MET-forward, 5′-GCCCGTTCCTTAGATCCTAT-3′; c-MET-reverse, 5′-ATGGTCGAATTGTCCCAATG-3′; GAPDH-forward, 5′-ATGGGGAAGGTGAAGG-3′; GAPDH-reverse, 5′-TTACTCCTTG-GAGGCC-3′; β-actin-forward, 5′-ATGTG CAAGGCCCGCTTCG-3′; β-actin-reverse, 5′-TTAATGTCACGCACGATTTCC. The PCR conditions were as follows: denaturating at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, and then at 58 °C for 1 min and 72 °C for 1 min 30 s, with a final extension at 72 °C for 10 min. The amplified PCR products were analyzed by agarose gel (1%; Intron Biotechnology) electrophoresis. *FBNL3* expression vector was constructed according to the previous methods [13].

2.3. Western blot analysis

Western blot analysis was performed with primary antibodies specific for human FBLN-3, p-c-MET, c-MET and Twist (Santa Cruz Biotechnology, Dallas, TX, USA); Oct4 (Millipore, Billerica, MA, USA); E-cadherin, Sox2 and β-actin (Cell Signaling Technology, Beverly, MA, USA); N-cadherin (BD Biosciences, San Jose, CA, USA); and Snail, ALDH1A1 and ALDH1A3 (Abcam, Cambridge, UK). Protein concentration was determined with a protein assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were separated on a 10–15% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond; Amersham Pharmacia, Pittsburgh, PA, USA). The blots were blocked for 1 h at room temperature with blocking buffer (10% nonfat milk in phosphate-buffered saline [PBS] containing 0.1% Tween 20 [TBS]). The membrane was incubated overnight in a cold chamber with specific antibodies. After being washed with TBS, the membrane was incubated with a horseradish peroxidase-labeled secondary antibody and visualized with the Westzol enhanced chemiluminescence detection kit (Intron Biotechnology).

2.4. Silencing RNA targeting of FBLN-3 and c-MET

Cells were transfected with Stealth™ RNA targeting *FBLN-3* and *c-MET* (Invitrogen; primer sequences: FBLN-3; 5′-CCUUGUCAAG-AUCCCUACAUUCUAA-3′/5′-UUAG AAUGUAGGGAUCUUGACAAGG-3′, c-MET; 5′-CCAUUUCAACUGAGUUUGCU GUUAA-3′/5′-UUAACAGC-AAACUCAGUUGAAAUGG-3′) or with Stealth™ RNAi Negative Control Medium GC (Invitrogen) at a concentration of 80 nM of Lipofectamine™ RNAi MAX reagent (Invitrogen). The cells were incubated for 72 h after transfection, and then *FBLN-3* and *c-MET* expression was determined by RT-PCR.

2.5. Flow cytometric analysis

Cells were stained with the ALDEFLUOR reagent system (STEMCELL Technologies, Vancouver, BC, Canada) to characterize populations of ALDH-positive cells according to the manufacturer's instructions. Briefly, Cells (1×10^6) were resuspended in ALDEFLUOR assay buffer containing the ALDH substrate in the presence or absence of the ALDH inhibitor, diethylaminobenzaldehyde. Following 30 min incubation at 37 °C, the cells were washed. The ALDH content was measured with a FACScan (Cytomics FC 500; Beckman Coulter Counter, Fullerton, CA, USA). A minimum of 50,000 cells were counted for each sample.

2.6. Colony-forming assay

For the colony-forming assay, cells were plated in 35-mm culture dishes at a density of 1000 cells per plate and allowed to attach overnight. Cells were incubated for 10–14 days post-irradiation and stained with 0.5% crystal violet. Colonies, defined as groups with $\geqslant$50 cells, were counted. Clonogenic survival was expressed as a percentage relative to the untreated controls.

2.7. Sodium bisulfite modification

Bisulfite-modified genomic DNA (gDNA) was prepared with the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA). The bisulfite reaction was carried out on 400 ng of gDNA, the reaction volume was adjusted to 20 μL with sterile water, and 130 μL of CT Conversion Reagent was added. The sample tubes were placed in a thermal cycler (MJ Research, Waltham, MA, USA) and the following steps were performed: 10 min at 98 °C and 2 h 30 min at 64 °C, and then storage at 4 °C. The resultant DNA was purified with the reagent provided in the EZ DNA Methylation-Gold Kit. The converted samples were added to a Zymo-Spin IC™ column containing 600 μL of Zymo M-Binding Buffer and mixed by inverting the column several times. The column was centrifuged for 30 s and the flow discarded. The column was washed by adding 200 μL of Zymo M-Wash Buffer and centrifuged at full speed; then

200 μL of Zymo M-Desulphonation Buffer was added to the column, followed by incubation at room temperature for 15–20 min. After incubation, the column was centrifuged at full speed for 30 s. The column was washed by adding 200 μL M-Wash Buffer and centrifuged at full speed. The converted gDNA was eluted by adding 20 μL of Zymo M-Elution Buffer into the column. The DNA samples were finally stored at −20 °C until further use.

2.8. Pyrosequencing analysis

PCR was performed in a volume of 50 μL with ⩽20 ng of converted gDNA, 5 μL of 10× *Taq* buffer, 5 U of Hot/Start *Taq* polymerase (Enzynomics, Daejeon, Korea), 4 μL of each 2.5 mM deoxynucleoside triphosphate mixture, 2 μL of 10 pmol/μL Primer-S, and 2 μL of 10 pmol/μL biotinylated Primer-As. The amplification was performed according to the general guidelines suggested for pyrosequencing: denaturing at 95 °C for 15 min, followed by 45 cycles at 95 °C for 40 s, and then at 55 °C for 40 s and 72 °C for 40 s, followed by a final extension at 72 °C for 10 min. PCR (5 μL) was confirmed by electrophoresis in a 3% agarose gel and visualized by ethidium bromide staining. A single-strand DNA template was prepared from 20–25 μL of the biotinylated PCR product with the use of streptavidin Sepharose® HP beads (Amersham Biosciences, Uppsala, Sweden) following the PSQ 96-sample preparation guide (Biotage, Charlottesville, VA, USA) using multichannel pipettes. Then, 15 pmol of the respective sequencing primer set was added for analysis. Sequencing was performed on a PyroMark ID system using the Pyro Gold reagents kit (Biotage) according to the manufacturer's instructions without further optimization. The methylation percentage was calculated as the average of the degree of methylation at four CpG sites formulated during pyrosequencing [19].

3. Results

3.1. Methylation analysis of CpG islands in the promoter region of *FBLN-3* in pancreatic cancer cell lines

Previous investigations have shown that many pancreatic cancer cells are notorious for their resistance to chemotherapy and radiotherapy [20,21]. This characteristic results in poor therapeutic efficiency. In the current study, we investigated whether FBLN-3 is involved in cell resistance and stamens in the pancreatic cancer cell lines. First, we showed by RT-PCR and Western blot analysis that expression of the *FBLN-3* scarcely occurred in the pancreatic cancer cell lines AsPC1 and MiaPaCa2 compared with *FBLN-3*–expressing H460 cells, in which significant cellular levels of FBLN-3 were detected (Fig. 1A). To investigate the involvement of epigenetic modification on *FBLN-3* expression, we analyzed the methylation pattern of *FBLN-3* using the bisulfite pyrosequencing method. Each primer was designed with the PSQ assay design program (Biotage); the sequences of primers are listed in Fig. 1B. We selected four CpG sites (positions 1 to 4) as shown in Fig. 1B and prepared bisulfite-modified gDNA using the EZ DNA Methylation-Gold Kit as noted in Section 2. The Y sequence (depicted as boxes) shows the methylated sites in the pancreatic cancer cell lines in

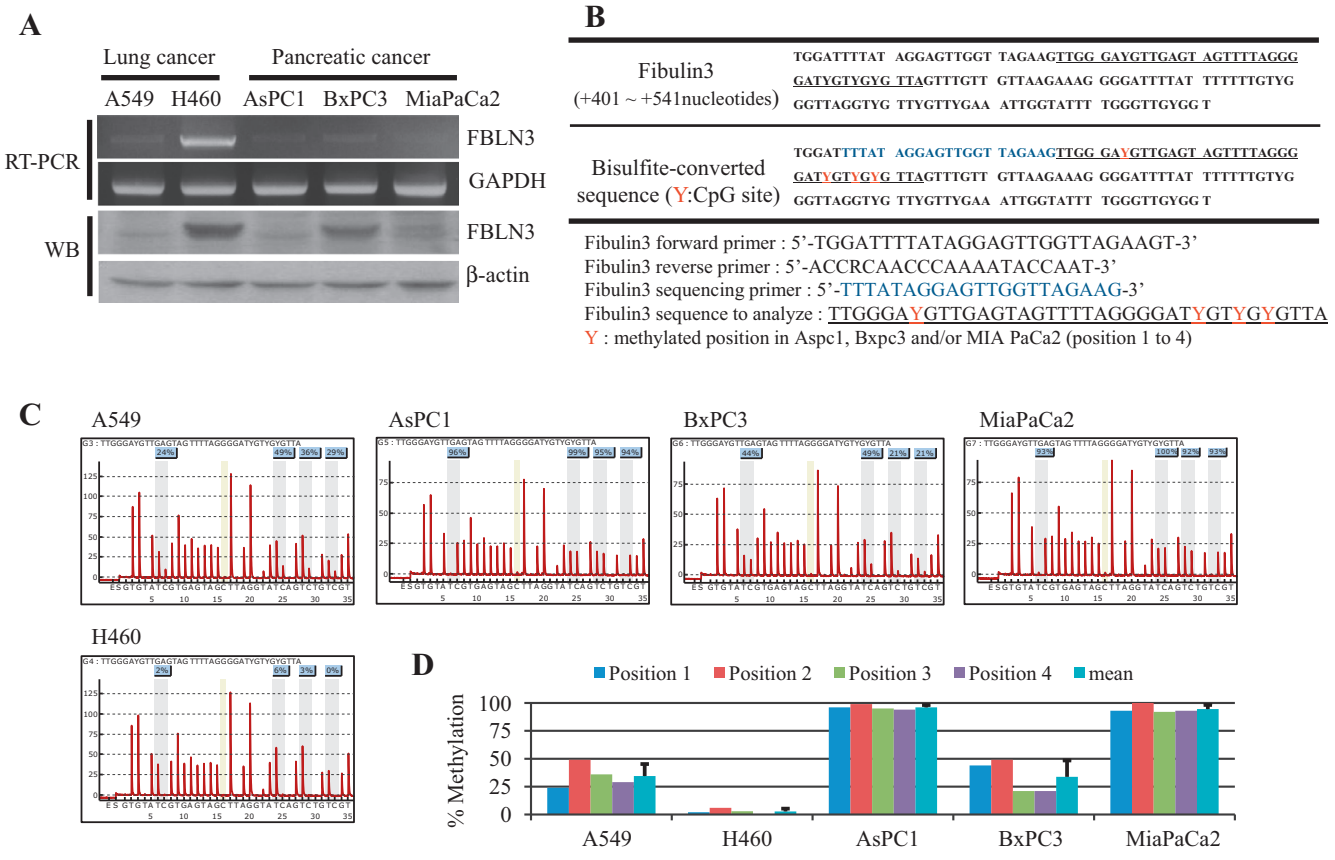

Fig. 1. Pyrosequencing of four CpG islands on the *FBLN-3* gene. (A) RT-PCR and Western blot analysis of *FBLN-3* gene expression in pancreatic cancer cell lines. (B) Original sequence of *FBLN-3* gene and bisulfite-converted sequences. Each Y shown in the red character indicates the methylated positions in each cell line. (C) Diagram of pyrosequencing in each cell lines. Each gray-colored box indicates the position of the four Y's shown in panel (B). (D) Comparison of fractions at the methylated CpG positions. The set of last bars indicates the means of all the methylated positions, 1–4.

positions 1 to 4. After PCR amplification was performed, the methylation percentage was calculated as the average degree of methylation at four CpG sites formulated during pyrosequencing. As shown in Fig. 1C, methylation of CpG sites at all positions tested was higher in pancreatic cancer cells in comparison with *FBLN3*-expressing H460 cells, and the average extent of methylation was 95% or more in AsPC1 and MiaPaCA cell lines and approximately 30% in BxPC3 cell line (Fig. 1D).

3.2. The FBLN-3 gene is associated with cell resistance and stemness regulation in pancreatic cancer cells

In the fast-growing variant cell line of the COLO375 human pancreatic cancer cell, a few reports have shown that expressing FBLN-3 promotes cell growth and metastasis [10,11]. Moreover, the resistance of pancreatic cancer cells is contributed by CSCs [21,22]. Therefore, in this study, to investigate whether the FBLN-3 is involved in the signaling pathway for γ-radiation resistance and cell stemness in some pancreatic cancer cells, *FBLN-3* was overexpressed by expression vectors, and cell stemness and resistance were evaluated by the colony-forming and spheroid formation assay. As shown in Fig. 2A, overexpression of the *FBLN-3* strongly induced the inhibition of cell growth in pancreatic cancer cells. We also found that forced overexpression of the *FBLN-3* significantly suppressed the expression of stemness markers such as Sox2 and Oct4 and thus inhibited spheroid formation, which implies that FBLN-3 suppresses the self-renewal activity of the CSC subpopulation in pancreatic cancer cells (Fig. 2B). Moreover, *FBLN-3* overexpression inhibited invasion and migration, with decreases of representative EMT markers such as snail and N-cadherin (Fig. 2C). The colony-forming assay also showed that forced expression of the *FBLN-3* also significantly sensitized the cellular response caused by a single dose of 6 Gy γ-radiation (Fig. 2D). On the other hand, *FBLN-3* suppression with siRNA in BxPC3 cell strengthened the cell growth, EMT property and γ-radiation resistance. This result indicates that resistance of pancreatic adenocarcinoma cells against γ-radiation may partly result from reinforcement of stemness by the very low expression level of *FBLN-3* via a high percentage of methylation at a CpG island.

3.3. FBLN-3 partially regulated c-MET activation and cellular level of c-MET and ALDH1

To identify downstream targets that mediated cell stemness and cell resistance functions of the *FBLN-3* gene in pancreatic cancer cells, we used RT-PCR analysis to investigate expression levels of *ALDH1* and *c-MET*, which are representative genes related to cell stemness and resistance in pancreatic cancer cells. *ALDH1* and

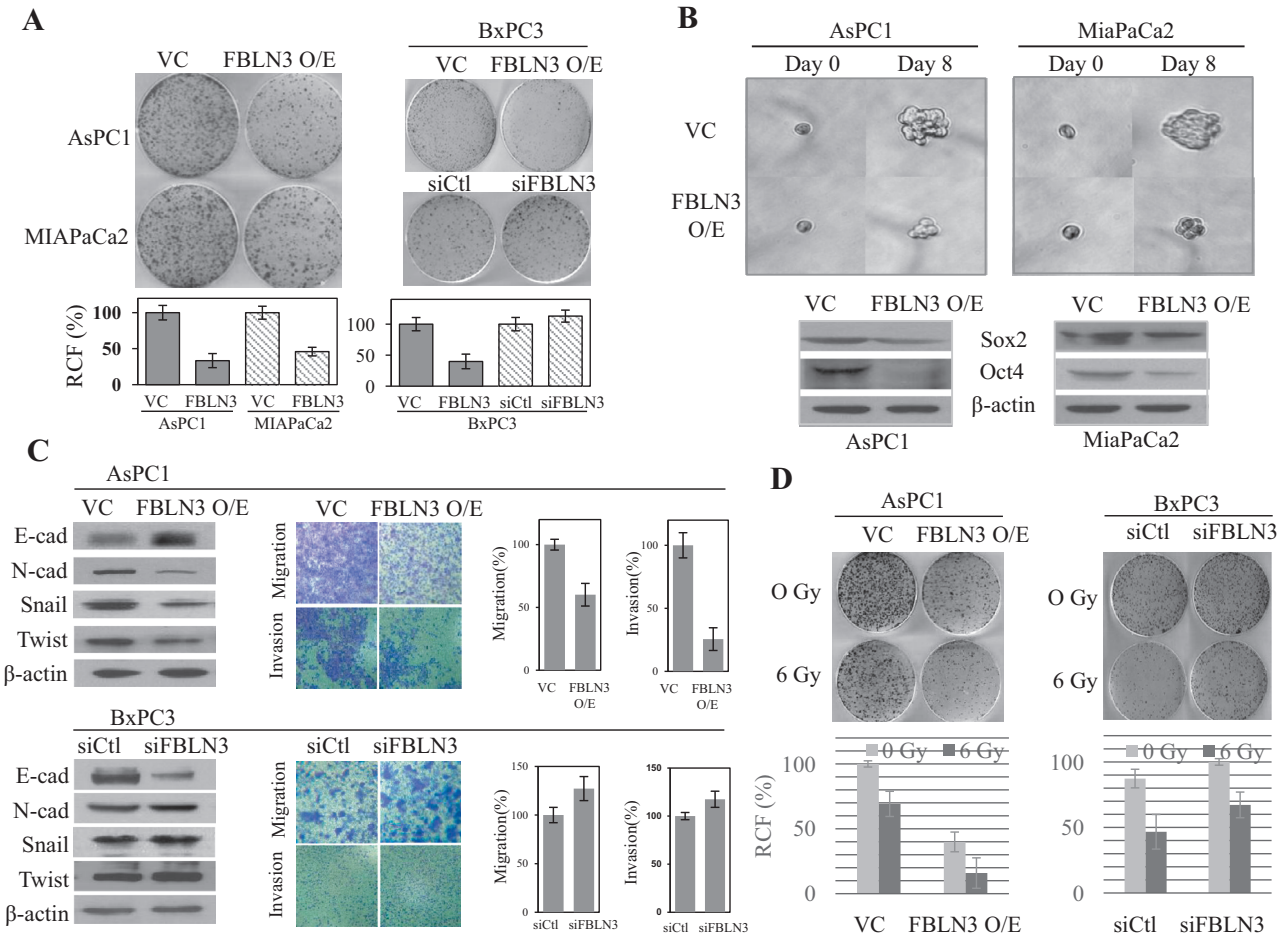

Fig. 2. Comparison of cell stemness, cell cytotoxicity, and metastatic property in *FBLN-3* overexpressing pancreatic cancer cells. (A) Cell growth of *FBLN-3*-overexpressing or suppressing pancreatic cancer cells. (B) Inhibition of cancer cell stemness by *FBLN-3* overexpression. Spheroid-formation and the expression of stemness markers (Sox2 and Oct4) were decreased by *FBLN-3* overexpression in AsPC1 and MaiPaCa2 pancreatic cancer cells. (C) Comparison of invasion and migration properties in *FBLN3*-overexpressing and suppressing cells using Matrigel analysis and Western blot assay of EMT markers. (D) Colony-forming assay of *FBLN-3*-overexpressing and suppressing cells after γ-irradiation (single dose of 6 Gy).

c-MET were highly expressed in some pancreatic cancer cell lines (Fig. 3A). The level of c-MET in AsPC 1 cell was much higher in pancreatic cancer cells than in A549 cells, which is a representative γ-radiation-resistant non-small cell lung cancer cell line. Moreover, AsPC1 cells showed a high expression level of ALDH1 compared with A549 cells through RT-PCR. In AsPC-1 and BxPC3 cell lines, ALDH1A3 is the dominant isoform, and in MiaPaCa cells, both ALDH1A1 and ALDH1A3 exist. We investigated whether FBLN-3 regulates the cellular level of ALDH1 isozymes and c-MET. *FBLN-3* overexpression in pancreatic cancer cells significantly suppressed the cellular level of c-MET (Fig. 3B). Moreover using RT-PCR analysis and the ALDEFLUOR assay, we found that FBLN-3 significantly regulated the expression levels of ALDH1 isozymes, consequently its activity. On the other hand *FBLN-3* suppression in BxPC3 cells increased cellular level of c-Met and ALDH activity (Fig. 3B and C). Moreover, we first showed that c-MET is associated with the cellular ALDH1 level in pancreatic cancer cells. Blockage of *c-MET* expression with siRNA or c-MET inactivation with inhibitor (SU11274) resulted in a significant reduction of Aldefluor staining and the expression of ALDH isoforms (ALDH1A1 and ALDH1A3) in the c-MET–rich AsPC1 cell lines (Fig. 4A). In pancreatic cells, c-MET activation (phosphorylation) by hepatocyte growth factor (HGF) significantly induced the expression of ALDH1 isoforms and thus increased cellular ALDH1 activity (Fig. 4A). On the other hand *FBLN-3* overexpression suppressed an increase of cellular ALDH1 activity by inhibiting the HGF/c-MET activation axis, indicating that FBLN-3 may function as inhibitor of HGF action (Fig. 4B and C). This means that the chemo- and radioresistance of some pancreatic cancer cell lines partially results from the increased level of c-MET and ALDH1 arising from a relatively scant level of *FBLN-3* expression caused by a relatively high percentage of DNA methylation at a CpG island. Taken together, these data suggest that the FBLN-3/c-MET/ALDH1 axis is partially associated with the modulation of stemness and EMT in pancreatic cancer cells and the sensitization of cells to the detrimental effects of damaging agents.

4. Discussion

FBLN-3 is a member of the fibulin family of an extracellular glycoprotein that is associated with the extracellular matrix. Recent findings indicated that FBLN-3 is closely involved in tumor aggressiveness, functioning as a component of the tumor microenvironment. There have been several reports about positive regulation of cancer cell signaling by FBLN-3. FBLN-3 activated the Notch signaling pathway in glioma and the EGFR-AKT pathway in pancreatic carcinoma cells [9,10]. In contrast, the *FBLN-3* gene is expressed at a very low level in invasive lung adenocarcinoma and HCC, which is related to a poor prognosis. In lung adenocarcinoma and HCC, low expression of the *FBLN-3* gene is closely related to hypermethylation of the promoter region [12,15]. Our study showed that forced FBLN-3 overexpression in lung cancer cells exerts these effects by inhibiting the PI3K/AKT/GSK3β pathway through IGF1R [13]. However, until now the involvement of low expression of FBLN-3 in tumor aggressiveness has not been reported in pancreatic cancer cells. Based on these, we investigated whether FBLN-3 is a negative regulator in some pancreatic cancer cell lines. We confirmed that forced expression of *FBLN-3* effectively inhibited the stemness and EMT-related properties of pancreatic cancer cells. Furthermore, we identified the signaling pathways influenced by FBLN-3.

The CSC model postulates that a small population of undifferentiated tumorigenic cells is responsible for tumor initiation, propagation, and spread to distant sites. The first evidence of a role for CSCs emerged from a study of human acute myeloid leukemia. In this study, isolated $CD34^{+}/CD38^{-}$ cells were identified as an acute myeloma leukemia-initiating cell population and characterized as CSCs; the hierarchy of CSCs mimicked the normal hierarchy of hematopoietic stem cells [23]. In a stochastic model, cancer therapies kill differentiated cells with limited replication potential and thus shrink the bulk of the tumor. However, a small population of cancer cells survives and acquires metastatic characteristics. These metastatic cells are highly resistant to chemo- and radiotherapy. Therefore, current chemotherapy regimens are usually not effective against CSCs, and thus a number of therapies targeting drug-resistant CSCs are currently under investigation. It has been reported that the number of CSCs can increase after cancer therapy. In pancreatic cancer cells, gemcitabine treatment led to an increase in the number of $CD133^{+}$ cancer stem-like cells [24]. Treatment of unsorted HCC cells with doxorubicin or fluorouracil in vitro significantly enriched the number of CSCs characterized by the $CD133^{+}$ phenotype [25]. Doxorubicin and

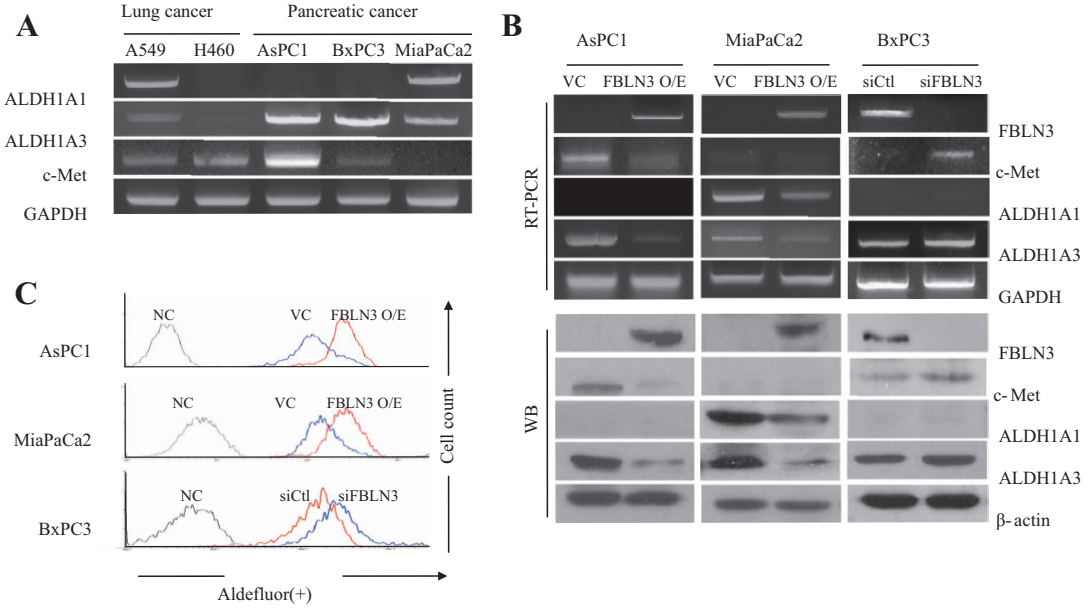

Fig. 3. FBLN-3 negatively regulates ALDH and c-MET in pancreatic cancer cell lines. (A) RT-PCR analysis of ALDH isozymes (ALDH1A1 and ALDH1A3) and c-MET in pancreatic cancer cell lines. (B) RT-PCR and Western blot analysis of ALDH isozymes and c-MET in *FBLN-3*-overexpressing AsPC1 and MiaPaCa2 cells or in *FBLN-3*-suppressing BxPC3 cells. (C) ALDEFLUOR assay of ALDH1 activity in *FBLN-3* overexpressing or suppressing pancreatic cancer cell lines.

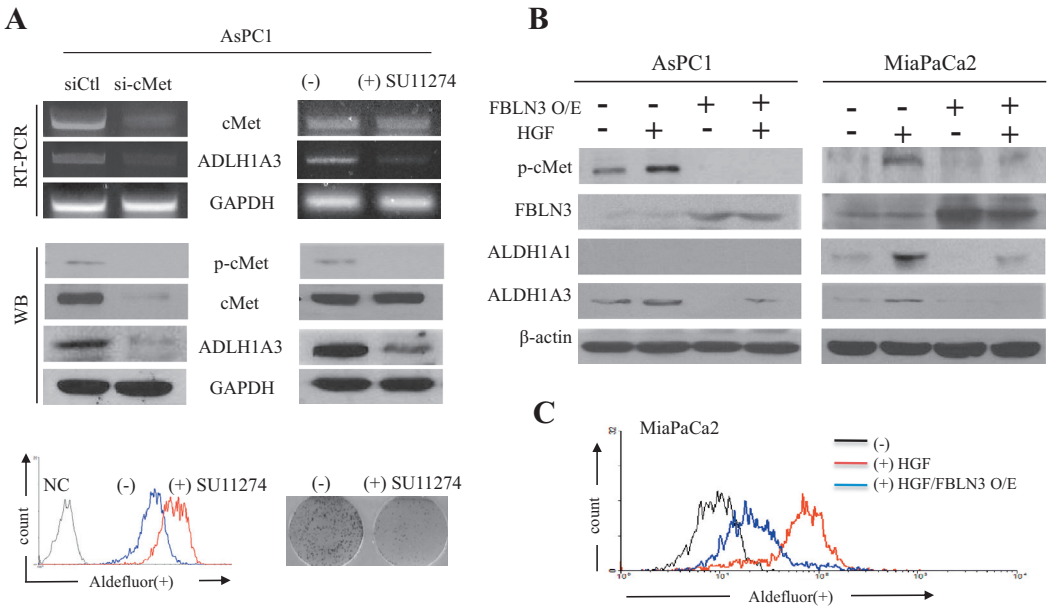

Fig. 4. FBLN-3 regulates HGF-induced c-MET activation and ALDH level in pancreatic cancer cell lines. (A) RT-PCR, Western blot analysis, ALDEFLUOR assay and colony forming analysis of c-MET inactivated AsPC1 cells using SU11274 inhibitor or siRNA. (B) c-MET activation and ALDH1 induction caused by HGF in pancreatic cancer cells were suppressed by *FBLN-3* overexpression. (C) ALDOFLUOR assay of MiaPaCa2 and *FBLN-3*-overexpressing MiaPaCa2 cells treated with HGF. HGF was treated about 2 h.

cyclophosphamide treatment of breast cancer also resulted in the selection of CSCs characterized by an increased percentage of $CD44^{+}/CD24^{-/low}$ cells [26].

Cancer stem cells with ALDH enzyme activity in colorectal cancer were also enriched following treatment with anticancer drugs, such as cyclophosphamide [27]. ALDH1 has been widely used as a marker for the identification and isolation of CSCs. When 50,000 $ALDH1^{-}$ breast cancer cells were transplanted into NOD/SCID mice, no tumor formed, but 500 $ALDH1^{+}$ cells were able to form a tumor [28]. This finding strongly indicated that $ALDH1^{+}$ cells have the characteristic of tumor initiation (cancer stem cell like) and thus are highly tumorigenic. It has been shown that breast cancer patients with $ALDH1^{+}$ tumors have a lower overall survival after cancer therapy because $ALDH1^{+}$ cells are more resistant to therapeutic agents such as γ-radiation. c-MET is also important for cell survival, resistance, and metastatic ability. After c-MET was first identified in the early 1980s, it was proposed that MET amplification leads to gefitinib (EGFR inhibitor) resistance in lung cancer by activating ERBB3 signaling, suggesting c-MET as a potential therapeutic target and biomarker [29]. Furthermore, a recent study showed that MET amplification is a closely related characteristic of CSCs. Some studies showed that c-MET activation in glioblastoma is a functional requisite for the CSC phenotype and a promising therapeutic target [30]. In pancreatic cancer cells, c-MET inhibition reduced the population of CSCs and thus blocked the self-renewal capacity, along with preventing the development of metastases [31]. Focusing on the expression of *FBLN-3*, we concluded that the extent of methylation of a CpG island in the *FBLN-3* promoter, thus the expression level of the protein, partially determined the stemness of pancreatic cancer cells through c-MET/ALDH1 regulation. This study provides the first demonstration that the FBLN-3/c-MET/ALDH1 axis modulates cell stemness, metastasis, and sensitization of cells to the detrimental effects of damaging agents.

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