Upregulating sirtuin 6 ameliorates glycolysis, EMT and distant metastasis of pancreatic adenocarcinoma with krüppel-like factor 10 deficiency

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INTRODUCTION

Deregulation of transforming growth factor (TGF)-β signaling is a common feature of pancreatic adenocarcinoma (PDAC)1. TGFβ/Smad signaling suppresses malignant growth in epithelial tumors and promotes metastasis in later stages. This complexity and intratumor genetic heterogeneity make the resulting effects of TGFβ inhibition difficult to predict2.

Krüppel-like factor 10 (KLF10) is an early response gene of TGFβ signaling that provides positive feedback toward the antiproliferative effect of TGFβ3,4. Recent studies, including ours, have revealed the downregulation of KLF10 in several advanced cancers, including breast, lung, and pancreas cancers, which results in the development of cancer metastasis5-7. KLF10 limits epithelial-mesenchymal transition (EMT) by suppressing Slug transcriptionally and activating stromal cell-derived factor-1/CXCR4 signals in lung and pancreatic cancers8,9. Distinguishing the antiproliferative and promotastatic functions of TGFβ makes KLF10 a potential target for the development of therapies for cancers with aberrant TGFβ pathways, such as PDAC.

Additional KLF10 depletion in Kras-mutated (KC) mice revealed the accelerated progression of PDAC10, whereas KLF10 knockout mice did not develop any pancreatic malignancies11. Studies of the livers of KLF10-deficient mice have indicated that KLF10 has a significant role in regulating genes that are involved in glucose metabolism12. Our preliminary study used the chromatin immunoprecipitation (ChIP)-chip assay and found that 550 genes from eight networks were physically associated with the KLF10 deficiency with rapid metastasis, elevated EMT, and distant metastasis of PDAC. Glycolysis activity and the EMT phenotype of PDAC cells were demonstrated in resected PDAC tissues, in vitro assays, and murine models. We identified sirtuin 6 (SIRT6) as an essential mediator of KLF10 that modulates EMT and glucose homeostasis. Overexpressing SIRT6 reversed the migratory and glycolytic phenotypes of PDACshKLF10 cells. Linoleic acid, a polyunsaturated essential fatty acid, upregulated SIRT6 and prolonged the survival of mice injected with PDACshKLF10. Modulating HIF1α and Nfkβ revealed that EMT and glycolysis in PDAC cells were coordinately regulated upstream by KLF10/SIRT6 signaling. Our study demonstrated a novel KLF10/SIRT6 pathway that modulated EMT and glycolysis coordinately via Nfkβ and HIF1α. Activation of KLF10/SIRT6 signaling ameliorated the distant progression of PDAC.

Clinical Trial Registration: ClinicalTrials.gov. identifier: NCT01666184.

Experimental & Molecular Medicine (2021) 53:1623–1635; https://doi.org/10.1038/s12276-021-00687-8

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Received: 18 January 2021 Revised: 27 April 2021 Accepted: 11 May 2021
Published online: 26 October 2021
MATERIALS AND METHODS
Cell culture and chemicals
The human pancreatic cancer cell lines Panc-1 (BCRC Cat# 60284, RRID: CVCL_0480), ASPC-1 (BCRC Cat# 60494, RRID:CVCL_0152), and MiaPaCa (BCRC Cat# 60139, RRID:CVCL_0428) were purchased from the Bioresource Collection and Research Center, Taiwan. All the cell lines were authenticated by DNA fingerprinting every three years. Panc-1, ASPC-1, and MiaPaCa cells were labeled with firefly luciferase plasmid vectors (Panc-1-Luc, ASPC-1-Luc, and MiaPaCa-Luc), which were provided by Dr. Kelvin K.C. Tsai of Taipei Medical University, Taiwan. Linoleic acid (LA) was purchased from Sigma-Aldrich (Cat: 36663S-5G; St. Louis, MO, USA). In addition, 1,4-dihydrophenoxyrin-4-one-3-carboxylic acid (DPCA) and phorbol ester (PMA) were purchased from Cayman (No. 712220; Ann Arbor, MI, USA) and Merck (P1585; Kenilworth, NJ, USA), respectively.

Cell migration, invasion, and trajectory analysis
To assay cell migration and invasion, 5 × 104 cancer cells were suspended in 10% fetal bovine serum (FBS) that contained culture medium and were seeded into the top chambers of a Transwell insert (Corning Costar, Corning, NY, USA) with or without Matrigel coating (Corning Costar). The previously reported14. LA dissolved in 0.1% EtOH was added to the daily water of the mice from injection site for 1 min before the spleen was excised. The wound was authenticated by DNA fingerprinting every three years. Panc-1, ASPC-1, and MiaPaCa cells were labeled with firefly luciferase plasmid vectors.

Glucose uptake, lactate production, and mitochondrial metabolism by Seahorse
Glucose uptake was measured using the Glucose Uptake Assay Kit (BioVision, Milpitas, CA, USA) according to the manufacturer’s instructions. Cell lysates were collected and assayed for lactate levels using a lactate assay kit (BioVision, Milpitas, CA, USA) according to the manufacturer’s instructions. The optical density values of the cell lysates were analyzed for 24 h. Lactate production was calculated from the standard curve and normalized to the cell lysate concentration. The cells were seeded at an optimized density and incubated for 24 h. Metabolic profiles were generated using the Seahorse XF96 extracellular flux analyzer (Seahorse Biosciences, Santa Clara, CA, USA) as previously described15. The baseline oxygen consumption rate and extracellular rate of acidification were determined after replacing the growth medium with assay medium containing the inhibitors or vehicle, according to the manufacturer’s protocol16.

Animals and the murine liver metastasis model
Mice were housed at the animal core facility of the National Health Research Institutes (NHRI), Taiwan. The facility was approved by the National Association for Accreditation of Laboratory Animal Care, Taiwan, and is maintained in accordance with the regulations and standards of the NHRI Animal Council’s procedural and ethical guidelines. Nonobese diabetic (NOD)/severe combined immunodeficient (SCID) mice (NOD. NODSCID, Prkdcscid) were purchased from the National Laboratory Animal Center. Linoleic acid (LA) was made, and the spleen was exteriorized. A 1-mL syringe with a 29-gauge black silk (Chromic, CC125). For LA treatment, 0% to 1.5% supplementary experiments (Supplementary Fig. 1a). The KLF10 cDNA cloned in the pcDNA3.1+ plasmid vector (RRID: Addgene 117727) was retrieved through BamHI and EcoRI restriction enzyme digestion and subcloned into BamHI and EcoRI enzyme-digested pLVX-TetOne vector plasmids (Takara Bio USA, Inc, Mountain View, CA, USA). The plasmid was transfected with appropriate inserts were verified by using polymerase chain reaction (PCR), restriction enzyme digestion, and signature enzyme digestion. MiaPaCa cells were transiently transfected with pLVX-KLF10 plasmids or empty vectors and tested for gene expression by western blotting. The expression was confirmed by western blotting. We obtained at least five stable clones of Panc-1 or ASPC-1 cells by using the most efficient KLF10-targeting shRNA constructs. Clones with optimal KLF10 protein reduction and migratory ability were chosen to perform the following experiments (Supplementary Fig. 1a). The KLF10 expression vectors has been described previously17.

Immunohistochemical staining
Paraffin-embedded tissue sections were deparaffinized in xylene, rehydrated through a series of ethanol dilutions, and boiled for 15 min in 10 mM citrate buffer at pH 6.0. Endogenous peroxidase activity was suppressed using a peroxidase block (Novolink, Chino, CA, USA, RE7148) for 5 min. The tissue sections were then blocked using protein blocks (Novolink, RE-7159) and incubated overnight at 4 °C with antibodies against KLF10 (1:400, mouse monoclonal antibody, LTK BioLaboratories, Taoyuan, Taiwan), SIRT6 (1:200, Abcam Cat# ab62738, RRID:AB_1904096), and PKM2 (1:1000, Abcam Cat# ab120468, RRID:AB_562190), and PKM2 (1:400, Cell Signaling Technology Cat# 4053, RRID:AB_1904096). Antibody detection was performed using the Novolink Poly Mix Detection System (RE7280-K, Leica Biosystems Newcastle Ltd, Newcastle upon Tyne, UK).

Transfection and transduction
The construction of KLF10 expression vectors has been described previously17. The stable downregulation of scramble and KLF10 plasmids (TRCNO0000318921) in Panc-1 or ASPC-1 cells was achieved using a retrovirus-mediated RNA interference system (pSuperRetro.puro, VEC-PRT-0001, OligoEngine, Seattle, WA, USA) and shRNAs purchased from the National Core Facility (Academic Sinica (Taipei, Taiwan); S‘GAAACCTCTCAAGTGTCAAAT-3‘). Cell populations were screened using 2 μg/mL puromycin. The efficacy was confirmed through western blotting. We obtained at least five stable clones of Panc-1 or ASPC-1 cells by using the most efficient KLF10-targeting shRNA constructs. Clones with optimal KLF10 protein reduction and migratory ability were chosen to perform the following experiments (Supplementary Fig. 1a). The KLF10 expression vectors has been described previously17.

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Immunoblot analysis
Cell extracts were prepared in lysis buffer (RIPA Cell Lysis Buffer 5×, RPPS-100) that contained a 1× protease inhibitor mixture (Complete, Roche, Basel, Switzerland) and 1 mM phenylmethylsulfonyl fluoride. For western blot analysis, cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The electrotransferred membrane (Amersham, Darmstadt, Germany; Hybond-PVDF NO 106002) was then incubated with the secondary antibody (IRDye 680RD Donkey anti-Mouse IgG, RRID AB_2716622; IRDye 800CW Donkey anti-Rabbit IgG, RRID AB_2715510) and developed with a LI-COR Odyssey system (LI-COR, Bad Homburg, Germany). Integrated optical density quantification was performed using Image Studio v 5.2. We used E-cadherin (1:1000, Cat# ab77791, RRID:AB_267696) and E-cadherin (1:500, Cat# 9585, RRID:AB_2239535; Cell Signaling Technology, Danvers, MA), vimentin (1:1000, Abcam Cat# ab92547, RRID:AB_10562134), pyruvate dehydrogenase kinase isozyme 1 (PDK1; 1:1000, Abcam Cat# 1624-1 ab202468, RRID:AB_562190), pyruvate kinase isozyme M2 (PKM2; 1:2000, Cell Signaling Technology Cat# 4053, RRID:AB_1904096), and glucose transporter 1 (Glut1; 1:1000, Abcam Cat# ab23551, RRID:AB_732605) to detect EMT and glycolysis protein markers. The KLF10 antibody was purchased from Abcam (1:1000, Abcam Cat# ab73537, RRID:AB_1640621). A β-actin antibody (Sigma-Aldrich Cat# A5454, RRID:AB_476744) at a 1:5000 dilution was used as the control. SIRT6 (1:1000, Cell Signaling Technology Cat# 12486, RRID:AB_2636969), HIF1α (1:1000, Cat# GTX127309, RRID:AB_2616089; Gene Tex, Irvine, CA, USA), and NFKB (1:1000, Abcam Cat# ab12096, RRID:AB_10866623) antibodies were used to study the downstream signaling of KLF10.

Immunohistochemical staining
Paraffin-embedded tissue sections were deparaffinized in xylene, rehydrated through a series of ethanol dilutions, and boiled for 15 min in 10 mM citrate buffer at pH 6.0. Endogenous peroxidase activity was suppressed using a peroxidase block (Novolink, Chino, CA, USA, RE7148) for 5 min. The tissue sections were then blocked using protein blocks (Novolink, RE-7159) and incubated overnight at 4 °C with antibodies against KLF10 (1:400, mouse monoclonal antibody, LTK BioLaboratories, Taoyuan, Taiwan), SIRT6 (1:200, Abcam Cat# ab62738, RRID:AB_1904096), and PKM2 (1:400, Cell Signaling Technology Cat# 4053, RRID:AB_1904096). Antibody detection was performed using the Novolink Poly Mix Detection System (RE7280-K, Leica Biosystems Newcastle Ltd, Newcastle upon Tyne, UK).
by transient transduction of pcDNA3.1-HA-KLF10 by Lipofectamine 3000 (Thermo Fisher Scientific, MA, USA) for 48 h. To determine whether SIRT6 is a major downstream mediator of KLF10, a stable KLF10 mRNA silencing clone was transiently transduced with SIRT6-Flag plasmids (pcDNA3.1-FLAG-SIRT6, Addgene #13817, Watertown, MA, USA) using Lipofectamine 3000 (L3000015, Thermo Fisher Scientific) for 48 h.

**Chip-PCR and reverse transcription PCR analysis**

The EZ ChIP chromatin immunoprecipitation kit (#17–371 Sigma-Aldrich, St. Louis, MO, USA) was used according to the manufacturer’s protocol. Briefly, the cells were transfected with scrambled plasmid or SIRT6 plasmid for 48 h and treated with 1% formaldehyde to cross-link proteins to DNA.

The cells were lysed with protease inhibitors, sonicated to shear DNA into fragments and incubated with antibodies against KLF10 or anti-rabbit IgG overnight. The purified DNA and input genomic DNA were analyzed using real-time PCR (RT-PCR). The primer sequences for Sirt6 were as follows: forward: 5’-TGTTGTGTCAGAGTGAGG, reverse: 5’-TGCAAGCCCTCTACT-GATCCC. RNA was extracted using the RNeasy plus kit (Qiagen, Hilden, Germany) after transfection. For RT-PCR, an ImPromII reverse transcription kit (A3800 Promega, Madison, WI, USA) and GoTaq Mix (M 7122, Promega) were used as recommended by the manufacturer. The primers used were SIRT6 left: TGTGTTGTCCAGAGTGAGG, SIRT 6 right: TGCAAGCCCTCTACT-GATCCC; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) left: ACCACTCTTCCACCTTTGA, GAPDH right: TGTGCTGTAGCCAAATTCGTT.

**Results and Discussion**

A detailed analysis of the results and discussion is provided in the text, including graphical representations of the experiments. The images depict the experimental setup and results, with histograms and bar charts showing the relative migratory and invasive abilities of cells with and without KLF10 expression.
Loss of KLF10 was correlated with enhanced migratory ability and distant metastasis of PDAC

From the pancreatic tumor specimens of 105 patients who received curative intent surgery, 66 (62.9%) patients presented a low expression of KLF10, according to the definition described in the "Materials and methods" section (Fig. 1a, right panel). Distant metastasis-free survival was shorter in patients with low KLF10 expression (p = 0.083, Fig. 1b). To evaluate the role of KLF10 in pancreatic cancer metastasis, we established stable clones of Panc-1 and ASPC-1 cells with KLF10 mRNA silencing (Panc-1shKLF10 [Supplementary Fig. 1a, lower panel], and ASPC-1shKLF10) and MiaPaCa cells with inducible KLF10 overexpression given their inherent expression of KLF10 (Supplementary Fig. 1a, upper panel). A cell trajectory study revealed that both the accumulated and oriented migration distances of Panc-1shKLF10 cells increased compared to those of Panc-1pLKO cells (Fig. 1c, upper panel and Supplementary Fig. 1b, left panel). Elevated migratory ability was detected in both Panc-1shKLF10 and ASPC-1shKLF10 cells (Fig. 1d, left panel; Supplementary Fig. 1b, right panel).

To avoid signal interference of the primary tumor and incidental peritoneal contamination during orthotopic tumor implants, we adopted a liver metastatic model through splenic tumor injection followed by splenectomy, as described previously19. The murine model of liver metastasis presented significantly enhanced luminescence signals and metastatic nodules in the liver of mice with KLF10 mRNA silencing versus the control with both ASPC-1 and Panc-1 cells (Fig. 1e, f; Supplementary Fig. 1c, d). Forced expression of KLF10 in Panc-1shKLF10 cells suppressed the migratory ability (Fig. 1g). In parallel, overexpressing KLF10 in MiaPaCa cells suppressed the migratory ability of PDAC cells (Supplementary Fig. 1e).

KLF10 deficiency correlated with increased migration and distant metastasis of pancreatic cancer cells

KLF10 regulated epithelial-mesenchymal transition

Cell morphology became elongated after KLF10 mRNA silencing (Fig. 1c, lower panel), with enhanced Transwell invasion ability in Panc-1 cells (Fig. 1d, right panel). Immunoblots revealed that the E-cadherin level was significantly reduced and that the mesenchymal protein expression levels were elevated in response to KLF10 mRNA silencing (Fig. 1h). Similar observations were noted in ASPC-1shKLF10 (Supplementary Fig. 1f). Forced expression of KLF10 in
Panc-1shKLF10 cells and overexpression of KLF10 in MiaPaCa elevated E-cadherin levels and downregulated mesenchymal proteins (Fig. 1h and Supplementary Fig. 1g).

Loss of KLF10 is correlated with enhanced EMT phenotypes of PDAC.

KLF10 loss was correlated with glycolysis in pancreatic cancer cells
Given the close association between KLF10 and metabolic disease as reported previously8,9, we measured the glycolysis activity of PDAC cells with genetically manipulated KLF10. Glucose uptake, lactate production and glycolytic activity were enhanced over twofold in Panc-1shKLF10 cells (Fig. 2a; Supplementary Fig. 2a, b). Similar observations were noted in ASPC-1shKLF10. Reduced mitochondrial oxidative phosphorylation, basal respiration, and maximal respiration capacity were noted in Panc-1shKLF10 cells compared with Panc-1pLKO cells (Fig. 2b, c). KLF10 deficiency in Panc-1 cells correlated with increased glycolytic enzymes, including PKM2, PDK1, and Glut1 (Fig. 2d). Glycolytic enzymes and lactate production in Panc-1shKLF10 cells were downregulated

| Database   | Number of sample | Correlation of KLF10 vs PKM2 |
|------------|------------------|-----------------------------|
| TCGC       | 131              | -0.316                      |
| Pei        | 52               | -0.36                       |
| Logsdon    | 27               | -0.52                       |
| Collision  | 20               | -0.2                        |
| Grutzmann  | 25               | -0.22                       |
| Ishikawa   | 49               | -0.38                       |
with forced expression of KLF10 (Fig. 2d, e). In MiaPaCa cells overexpressing KLF10, lactate production and glycolytic enzyme levels were reduced compared with those of the control cells (Supplementary Fig. 2c, d). Pancreatic tumor tissues from KC mice revealed a loss of KLF10 immunolabeling and elevated PKM2 and PDK1 expression (Supplementary Fig. 2e). An immunohistochemical study of KLF10, PKM2, and PDK1 in the PDAC tissue of ten patients showed a negative correlation between KLF10 and glycolytic enzymes (Fig. 2f, g; Supplementary Fig. 2f). Data from Oncomine indicated a moderate inverse correlation of KLF10 with glycolytic enzymes, including PKM2 and PDK1 (Fig. 2h; Supplementary Fig. 2g).

In summary, loss of KLF10 leads to enhanced glycolysis, which parallels the metastatic progression of PDAC.

**KLF10 transcriptionally upregulated SIRT6**

By using the ChIP-chip assay, we discovered that SIRT6, which governs metabolism and tumorigenesis, is a candidate gene that is regulated by KLF10. By using ChIP-PCR and qPCR, we noted that KLF10 physically bound to the promoter of SIRT6 (Fig. 3a, upper panel, and 3b). Furthermore, the luciferase reporter assay showed that KLF10 positively regulated SIRT6 transcriptionally (Fig. 3a, middle and lower panels, and 3c). SIRT6 transcripts were reduced in Panc-1shKLF10 cells (Fig. 3d, right lower panel). The expression of SIRT6 paralleled that of KLF10 in genetically manipulated PDAC cell lines, including Panc-1, BXPC3, and MiaPaCa cells (Fig. 3d). In the PDAC tissue of 29 patients, the KLF10 and SIRT6 levels were reduced in parallel compared with the levels in the normal pancreas, with a correlation coefficient \( r = 0.54, p = 0.003 \) (Fig. 3e). There was a moderately positive correlation between KLF10 and SIRT6 from the Oncomine data (Fig. 3g).

**SIRT6 is an essential mediator of the regulatory effects of KLF10 on EMT, glycolysis, and the metastasis of pancreatic cancer**

Overexpressing SIRT6 in Panc-1shKLF10 cells reversed the migratory capacity induced by KLF10 deficiency (Fig. 4a, b; Supplementary Fig. 3a). Similar observations were noted in ASPC-1shKLF10 cells (Supplementary Fig. 3b). The murine metastatic model revealed the suppressed metastatic luminescent signal caused by SIRT6 overexpression in ASPC-1shKLF10 cells (Fig. 4c, d). The results were recapitulated in Panc-1 cells (Supplementary Fig. 3d, e). The mesenchymal protein level was reduced, and E-cadherin expression was increased by overexpressing SIRT6 in Panc-1shKLF10 cells (Fig. 4e). Glycolysis activity was reduced by the forced expression of SIRT6 in Panc-1shKLF10 or ASPC-1shKLF10 cells with reduced lactate production and downregulation of PDK1, PKM2, and Glut1 (Fig. 4e, f; Supplementary Fig. 3c). We concluded that SIRT6 was an essential downstream mediator of KLF10 and that it contributed to EMT and the glycolysis phenotypes of PDAC.

**Linoleic acid (LA) increased SIRT6 expression and reversed the malignant phenotype of PDAC**

LA, which is a polyunsaturated omega-6 fatty acid, is one of two essential fatty acids. A recent investigation reported that LA at physiological concentrations induces a conformational change in SIRT6 but not in other sirtuins and increases catalytic efficiency. We unexpectedly found that LA induced a dose-dependent increase in SIRT6 expression levels but not other sirtuin expression levels in PDAC cells (Supplementary Fig. 4a, b). The migratory ability and mesenchymal protein expression of Panc-1shKLF10 were suppressed by LA (Fig. 5a, b). The luminescent signals of the murine metastatic model with ASPC-1shKLF10 or Panc-1shKLF10 were suppressed by LA treatment (Fig. 5c; Supplementary Fig. 4c, d). LA treatment in Panc-1shKLF10 cells revealed downregulated lactate production and glycolytic enzyme expression (Fig. 5b, d). SIRT6 expression was elevated by LA in murine tumor tissues (Supplementary Fig. 4e). The survival of mice implanted with Panc-1shKLF10 was prolonged significantly with LA treatment in daily water (median survival time from 37 to 50 d, \( p = 0.038 \); Fig. 5e).

**Interaction of EMT and glycolysis in pancreatic cancer with KLF10 loss**

HIF1α and NFKB were two candidate mediators downstream of the KLF10/SIRT6 signal. From the Oncomine database, we noted a moderate inverse correlation between KLF10/SIRT6 and HIF1α and NFKB transcripts (Fig. 6a). Moderate correlations of HIF1α or NFKB with glycolytic enzymes or EMT molecules, respectively, were also noted (Supplementary Fig. 5a, b). In our study, the expression levels of NFKB and, to a lesser extent, HIF1α, were upregulated in Panc-1shKLF10 cells with low levels of SIRT6 (Fig. 6b). Forced expression of KLF10 reduced the expression of HIF1α and NFKB and upregulated SIRT6 (Fig. 6b). Using DPCA and PMA to activate HIF1α and NFKB, respectively (Fig. 6c, e), the ameliorating effects on migration and lactate production by overexpressing SIRT6 in Panc-1shKLF10 cells were abolished (Fig. 6d, f).

In summary, KLF10 coordinated EMT and glycolysis activity in PDAC through the use of SIRT6. In our model system, NFKB and possibly HIF1α were responsible for the interaction between glycolysis activity and EMT phenotype downstream of the KLF10/SIRT6 signal.

**DISCUSSION**

Distant metastasis is the major cause of death in PDAC, which is known for its aberrant TGFβ signaling. However, the role of TGFβ in cancer proliferation and metastasis is complex and elusive. KLF10, a TGFβ signal early responder, has antiproliferative and antimetastatic effects in contrast to TGFβ and is a potential target for developing therapeutic targets for PDAC. Our previous study revealed that elevating KLF10 genetically or pharmacologically by metformin may reverse the radioresistance of PDAC. In this
study, we discovered that KLF10 transcriptionally activated SIRT6 to coordinate EMT and glucose metabolism in PDAC. Genetic overexpression of SIRT6 or pharmacological use of LA reversed the EMT and glycolysis phenotypes and suppressed the distant metastasis of PDAC. In contrast to targeting TGFβ, the therapeutic strategy of upregulating KLF10/SIRT6 signaling in PDAC may reduce distant metastasis without enhancing tumor proliferation.

The KLF family consists of 18 identified members with diverse regulatory roles in glycolysis, EMT, and metastasis. Redundant subgroups within the KLF family are emerging. Despite a significant role of KLF10 in modulating EMT and glycolysis in PDAC, the interplay among various KLF transcripts that balances opposing outcomes of epithelial homeostasis and glucose metabolism remains unclear. A previous study identified KLF10 as a rheostat that controls TGFβ signaling and EMT activation by transcriptionally regulating Slug. In our study, SIRT6 overexpression in KLF10-deficient PDAC cells reduced Slug and other EMT transcription proteins. The results indicated that in addition to directly modulating Slug transcriptionally, KLF10 coordinated EMT transcription, including Slug, and glucose homeostasis using SIRT6.

In contrast to most studies, KLF10, a transcriptional repressor, was found to transcriptionally activate SIRT6 in pancreatic cancer cells. Sp1-like/KLF transcription factors function as activators or repressors depending on which promoter they bind and the...
Correlators with which they interact25. Class III Sp1-like/KLF family members, including KLF10, share a conserved repression motif that is sufficient to mediate transcriptional repression by interacting with the histone deacetylase corepressor complex mSin3A. However, this function can be modified by cell signaling events. Phosphorylation of four residues in a region adjacent to the SId-like domain involving extracellular regulator kinase 2 disrupts interaction with mSin3A and results in a significant loss of repressor function26. Recent evidence also suggests that histone acetylation at the deacetylase may serve as a switch for Sp1-like/KLF proteins to function as activators or repressors27. The molecular mechanism by which KLF10 switches between transcriptional activation and repression warrants further study.

SIRT6 belongs to the sirtuin family of NAD+-dependent deacetylases involved in stress resistance and metabolic homeostasis28. Recent studies have reported that SIRT6 functions as a key regulator of glucose homeostasis and as a tumor suppressor29. SIRT6 has been reported to inhibit Twist 1 and EMT in lung cancer and idiopathic pulmonary fibrosis, respectively30,31. On the other hand, studies have also shown SIRT6 to enhance cancer metastasis by promoting EMT in lung, thyroid, and liver cancer32,33. The controversial role of SIRT6 in the pathogenesis of cancers may be tissue- and context-dependent. Tian K et al.34 demonstrated that SIRT6 activates TGFβ1/Smad3 signaling and inhibits EMT in bleomycin-injured mice. In our PDAC model, KLF10, a TGFβ downstream mediator, transcriptionally activated SIRT6 and inhibited EMT transcription factors, probably by using Nfkb and Hif1alpha. Overexpressing SIRT6 genetically or pharmacologically did not significantly change the KLF10 expression level in our model system. Whether feedback signals occur between KLF10 and SIRT6 merits exploration. A recent study revealed that nitrated fatty acids with a Michael adduct resulted in 20-fold stronger activation of SIRT634. An unexpected finding was that the SIRT6 protein level was elevated by LA treatment in our model. A possible explanation is that free fatty acids may inhibit cellular proteasome activity and stabilize LA protein levels35,36. In our vector control pancreatic cancer cells, the levels of glycolytic enzymes were significantly elevated by LA but were suppressed by genetically overexpressing SIRT6. A possible explanation is that SIRT6 overexpression is selectively toxic to multiple cancer cells, and there are multiple ways to fine-tune SIRT6 levels in cancer cells in response to stress conditions11,42. In KLF10-deficient pancreatic cancer cells with low SIRT6 levels, LA elevated SIRT6 expression and activity, which led to upregulated glycolytic enzymes. However, in wild-type pancreatic cancer cells with sufficient amounts of SIRT6, the effect of LA in elevating SIRT6 was less efficient. On the other hand, LA may induce PGC-1α to increase glucose transport and lipolysis43, which leads to elevated glycolytic enzymes.

Replacing saturated fat with polyunsaturated fat reduced blood cholesterol concentrations and the risk of coronary artery disease. However, some animal studies have indicated that LA may promote tumor growth in murine models. Current evidence, including meta-analysis and systematic review, suggests an increased risk of cancer by LA44,45. In our in vitro study, the inconsistent effect of overexpressing SIRT6 and LA on glycolytic enzyme levels in Panc-1pLKO cells (Figs. 4e and 5b) suggests a complicated effect of LA on glycolytic activity in PDAC cells without affecting the KLF10/SIRT6 balance. Benefits from the long-term consumption of LA in preventing PDAC progression warrant further investigation.

In our study, significant upregulation of Nfkb and, to a lesser extent, Hif1alpha expression, was noted by interfacing with the KLF10/SIRT6 signal. A previous study revealed that SIRT6 deacetylates histone H3 lysine 9 on promoters of Nfkb target genes to destabilize Nfkb46. SIRT6 was reported to be a co-repressor of the transcription factor HIF1α. SIRT6-deficient cells exhibited increased HIF1α activity and protein stability with upregulation of glycolysis47. Our in vitro studies using pharmacological manipulation of HIF1α and Nfkb revealed an interaction between the EMT and glycolysis phenotypes of PDAC cells without affecting the KLF10/SIRT6 expression levels. The results suggested that Nfkb and HIF1α are two candidate downstream mediators of KLF10/SIRT6 signaling that regulate EMT and glycolysis in PDAC (Fig. 6e).

Distinguishing between the antiproliferative and prometastatic functions of TGFβ makes KLF10 a potential target for the development of therapies for PDAC. We demonstrated the antitumorigenic effect of KLF10 and discovered a novel pathway by which KLF10 coordinates EMT and glycolysis by transcriptionally regulating SIRT6 in PDAC. KLF10/SIRT6 signaling modulates EMT.
Fig. 4 Overexpression of SIRT6 ameliorated the migration, EMT phenotype, glycolysis activity, and metastasis of PDAC. a Cumulated data of the Transwell migratory assay of Panc-1pLKO and Panc-1shKLF10 cells with and without SIRT6 overexpression. Data are presented as the mean ± SE (** signifies \( p < 0.01 \)). The experiments were repeated three times. b Cumulated data of directional migration over time (or. dist.) in Panc-1pLKO and Panc-1shKLF10 cells with and without SIRT6 overexpression. Data are presented as the mean ± SE (** signifies \( p < 0.01 \)). The cell trajectory experiments were repeated three times. c Representative IVIS images of mice at 1–4 weeks after injection with ASPC-1pLKO and ASPC-1shKLF10 cells with and without SIRT6 overexpression as indicated. d Cumulated IVIS signal of at least six mice in each experimental group injected with ASPC-1pLKO and ASPC-1shKLF10 with and without SIRT6 overexpression. Data are presented as the mean ± SE (* represents \( p < 0.05 \)). e Representative immunoblots of E-cadherin and mesenchymal and glycolytic protein expression in Panc-1pLKO and Panc-1shKLF10 cells with and without SIRT6 overexpression. \( \beta \)-Actin was used as the internal control. Quantitative analysis of at least three experiments is shown below the immunoblots. f Cumulated lactate production assay data of Panc-1pLKO and Panc-1shKLF10 cells with and without SIRT6 overexpression. Data are presented as the mean ± SE (** and *** signify \( p < 0.01 \) and \( p < 0.005 \), respectively). The experiments were repeated three times.
Fig. 5  Linoleic acid (LA) enhanced SIRT6 expression and reversed migration, EMT, and the glycolytic phenotypes of PDAC with KLF10 deficiency. **a** Cumulative migratory assay of Panc-1pLKO and Panc-1shKLF10 cells with and without 50 μM LA treatment for 16 h. Data are presented as the mean ± SE (∗ and ** represent *p* < 0.05 and *p* < 0.01, respectively). The experiments were repeated three times. **b** Representative immunoblots of E-cadherin and mesenchymal and glycolytic protein expression in Panc-1pLKO and Panc-1shKLF10 cells with and without 50 μM LA treatment for 16 h. β-Actin was used as the internal control. Quantitative analysis of at least three experiments is shown below the immunoblots. **c** Upper panel: Representative IVIS image of mice at 1–4 weeks after injection with ASPC-1pLKO and ASPC-1shKLF10 with and without 1% LA treatment in daily water for 8 weeks as described in the “Materials and methods.” Lower panel: Cumulated IVIS signal of at least six mice in each experimental group that were injected with ASPC-1pLKO and ASPC-1shKLF10 and received 1% LA treatment in daily water for 8 weeks or did not, as indicated. Each point represents the mean ± SE at least six mice (∗ signifies *p* < 0.05). **d** Cumulated lactate production assay of Panc-1pLKO and Panc-1shKLF10 cells with and without LA treatment at 50 μM for 16 h. Data are presented as the mean ± SE (∗ represents *p* < 0.05). The experiments were repeated three times. **e** The survival curves of mice injected with Panc-1pLKO and Panc-1shKLF10 that received 1% LA treatment in daily water for 8 weeks or did not, as indicated. Each experimental group contained at least six mice. The median survival of mice injected with Panc-1shKLF10 without and with LA treatment was 37 and 50 days (p = 0.038; * signifies *p* < 0.05).
Fig. 6 HIF1α and NFκB are two downstream mediators of KLF10/SIRT6 signaling in PDAC cells. a Representative databases from Oncomine display the correlation between KLF10 (upper panel)/SIRT6 (lower panel) and HIF1α or NFκB transcripts. b Representative immunoblots of HIF1α and phospho-NFκBp65 in Panc-1pLKO and Panc-1shKLF10 cells with and without forced expression of KLF10. Quantitative analysis of cumulative data from at least three experiments is shown in addition to immunoblots. β-Actin was used as the internal control. c Representative immunoblots of HIF1α in Panc-1 cells treated with various dosages of 1,4-dihydrophenonthrolin-4-one-3-carboxylic acid (DPCA) for 8 h. Quantitative analysis from at least two experiments is shown below the immunoblots. d Left panel: Cumulated migratory assay of Panc-1pLKO and Panc-1shKLF10 cells with or without SIRT6 overexpression treated with and without 400 μM DPCA for 16 h. Right panel: Cumulated lactate production assay of Panc-1pLKO and Panc-1shKLF10 cells with and without SIRT6 overexpression that were treated with and without 400 μM DPCA for 16 h. Data are presented as the mean ± SE (n = 3; *, **, and *** indicate p < 0.05, p < 0.01 and p < 0.005, respectively). The experiments were repeated three times. e Graphic summary of the role of the KLF10/SIRT6 signaling pathway in modulating EMT and glycolysis in PDAC.
proteins and glycolysis enzymes using Nfkb and probably Hif1a. Upregulating SirT6 genetically or pharmacologically using LA may mitigate EMT, glycolysis, and distant metastasis of PDAC due to KLF10 deficiency.

DATA AVAILABILITY

The datasets supporting the conclusions of this article are included within the article and its Supplementary files.

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ACKNOWLEDGEMENTS

The authors thank Dr. Chin-Fu Hsiao, Mr. Chao-Tung Lee, Ms. Suwen Nieh, and all research nurses and participating hospitals of the Taiwan Cooperative Oncology Group, NHRI, Taiwan, for helping collect clinical specimens and data and conducting statistical analyses. We thank Dr. Kung-Hung Cheng of the Institute of Biomedical Sciences, National Sun Yat-Sen University, Kaohsiung, Taiwan, and Dr. Shuang-En Chuang, Shih Sheng Jiang, and Dr. Tze-Sing Huang of the National Institute of Cancer Research, NHRI, for helpful discussions and suggestions. We are grateful to Dr. Cheng Chang of National Cheng Kung University Hospital for the pathology consultation.

AUTHOR CONTRIBUTIONS

H.J.C. conceived the project and designed the study. H.J.C. and Y.C.T. designed the experiments. Y.C.T. and S.L.C. generated KLF10 mRNA-silenced and SirT6-overexpressing cell lines. Y.L.T. and Z.M.C. generated inducible KLF10 mRNA-overexpressing cell lines. Y.C.T. performed the immunoblots, immunohistochemistry and in vitro studies. S.L.C. did the metabolic profiling using a Seahorse XF96 extracellular flux analyzer. Y.C.T. and S.L.C. conducted the murine experiments. S.L.P. performed and supervised the pathologic interpretation and analysis. V.H.C. discussed the results and provided scientific suggestions for the project. H.J.C. and Y.C.T. managed the study and wrote the paper. The authors read and approved the final manuscript.
FUNDING
This work was supported by the grants MOST 107-2314B-400-018 and 104-2314-B-400-017-MY3 and NHRI CA-108-PP-14.

COMPETING INTERESTS
The authors declare no competing interests.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE
Tissue specimens were collected from pancreatic cancer patients recruited for clinical trials (ClinicalTrials.gov identifier: NCT01666184), and the study was approved by the Institutional Review Board of the National Health Research Institutes in Taiwan (approval number: EC1010502). The study was performed in accordance with the Declaration of Helsinki.

Consent for publication
Not applicable.

ADDITIONAL INFORMATION
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s12276-021-00687-8.

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