Impact of CRISPR-Cas9-mediated CD73 knockout in pancreatic cancer

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Der Medizinischen Fakultät der Friedrich-Alexander-Universität Erlangen-Nürnberg zur

Erlangung des Doktorgrades Dr. med.

vorgelegt von

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Als Dissertation genehmigt

von der Medizinischen Fakultät

der Friedrich-Alexander-Universität Erlangen-Nürnberg

Tag der mündlichen Prüfung:25.07.2023

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LIST OF ABBREVIATIONS	
NT5E(CD73)	ecto-5′-nucleotidas (cluster of differentiation 73)
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	breast cancer type 2 susceptibility protein
PALB2	Partner and localizer of BRCA2
TP53	Tumor protein P53
Mlh1	DNA mismatch repair protein Mlh1
Msh2	DNA mismatch repair protein Msh2
STK11	Serine/threonine kinase 11
SPINK1	Pancreatic secretory trypsin inhibitor (PSTI)
SEER	The Surveillance, Epidemiology, and End Results
PDAC	Pancreatic ductal adenocarcinoma
PC	pancreatic cancer
CA199	Carbohydrate antigen 19-9
ATM	Ataxia telangiectasia mutated
K-RAS	Kirsten rat sarcoma virus
IL-6,IL-8,IL-10	Interleukin 6/8/10
EGFR	Epidermal growth factor receptor
PAAD	Pancreatic adenocarcinoma
PARP	Poly ADP ribose polymerase,
BRCA	Bladder urothelial carcinoma
NTRK	Trk receptors
CITN	Cancer Immunotherapy Trials Network
HLA	human leukocyte antigen

PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
OS	Overall survival
KM	Kaplan–Meier
HIRC	Kidney renal clear cell carcinoma
HNSC	Head and Neck squamous cell carcinoma
LUAD	Lung adenocarcinoma
LUSC	Lung squamous cell carcinoma
MESO	Mesothelioma
STAD	Stomach adenocarcinoma
PI	Propidium iodide
D-PBS	Dulbecco's phosphatebuffered saline
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
RPMI-1640	Roswell Park Memorial Institute-1640
BSA	Bovine Serum Albumin
TBST	Tris buffered saline tween

1.ABSTRACT

Background and objectives:

Pancreatic cancer is one of the cancers with the highest mortality rate, because it's early clinical symptoms are not obvious. Most patients are already in advanced stages of cancer when diagnosed, thus losing the chance of surgical treatment. Immunotherapy combined with conventional treatment can be an essential tool to improve patient survival and the identification of immune targets is a top priority for current research. In recent years, tumor immunotherapy has been gaining attention, and more and more immune checkpoints have been discovered, among which CD73 is one of the most popular ones. The aim of this study is to investigate the specific role of CD73 in pancreatic cancer and the genes that may regulate CD73.

Methods:

We used CRISPR/Cas9 technology to knock out CD73 in pancreatic cancer and determined the effect of CD73 deletion on pancreatic cancer by various in vitro cell function assays. Next we identified targets regulating CD73 in pancreatic cancer by CRISPR/Cas9 protein kinase library screening. We also enhanced the screening by fluorescence-activated cell sorting (FACS) to identify populations expressing high and low CD73 expression levels. DNA was extracted for deep sequencing to identify candidate genes.

Results:

We validate that CD73 expression is increased in pancreatic cancer and that knockout of CD73 inhibits cell proliferation and migration and blocks the G1 phase of the cell cycle. We also found that the deletion of CD73 inhibited the ERK/STAT3 pathway and activated the E-cadherin pathway. Loss of Pbk, Fastk, Cdk19, Adck5, Trim28, or Pfkp might be genes regulating CD73 in pancreatic cancer.

Conclusion:

Knockout of CD73 in pancreatic cancer inhibits tumor proliferation and

migration through the ERK/STAT3 pathway, while E-cadherin may also be involved. CRISPR/Cas9 protein kinase library deletion combined with flow cytometry screening can be used to identify critical genes regulating CD73.

2.ZUSAMMENFASSUNG

Hintergrund und Ziele:

Bauchspeicheldrüsenkrebs ist eine der Krebsarten mit der höchsten Sterblichkeitsrate, denn die frühen klinischen Symptome sind nicht offensichtlich. Die meisten Patienten befinden sich bereits in einem fortgeschrittenen Krebsstadium, wenn der Krebs diagnostiziert wird, und haben somit keine Chance auf eine chirurgische Behandlung. Daher kann die Immuntherapie ein wichtiges Instrument zur Verbesserung der Überlebensrate dieser Patienten sein. In den letzten Jahren hat die Tumorimmuntherapie an Aufmerksamkeit gewonnen, und es wurden immer mehr Immun-Checkpoints entdeckt und diskutiert, von denen CD73 einer der bekanntesten ist. In dieser Studie sollen die spezifische Rolle von CD73 bei Bauchspeicheldrüsenkrebs und die Gene, die CD73 regulieren können, untersucht werden.

Methoden:

Wir setzten die CRISPR/Cas9-Technologie ein, um CD73 bei Bauchspeicheldrüsenkrebs auszuschalten, und bestimmten die Auswirkungen der CD73-Deletion auf Bauchspeicheldrüsenkrebs durch verschiedene In-vitro-Zellfunktionstests. Als Nächstes identifizierten wir Gene, die CD73 bei Bauchspeicheldrüsenkrebs regulieren, durch CRISPR/Cas9-Deletionsscreening in einer Proteinkinase-Bibliothek. Darüber hinaus haben wir das Screening durch fluoreszenzaktivierte Zellsortierung (FACS) erweitert, um Populationen mit hoher und niedriger CD73-Expression zu identifizieren. Zur Identifizierung von Genkandidaten wurde DNA für Deep Sequencing extrahiert.

Ergebnisse:

Wir konnten bestätigen, dass die Expression von CD73 bei Bauchspeicheldrüsenkrebs erhöht ist und dass die Ausschaltung von CD73 die Zellproliferation und -migration hemmt und die G1-Phase des Zellzyklus blockiert. Wir fanden auch heraus, dass die Deletion von CD73 den ERK/STAT3-Weg hemmt und den E-Cadherin-Weg aktiviert. Eine Inaktivierung

von Pbk, Fastk, Cdk19, Adck5, Trim28 oder Pfkp könnten die die Expression von CD73 regulieren. Obwohl wir weitere Studien benötigen, um die genaue Regulierung von CD73 zu bestätigen, bietet die Liste der bisher ausgewählten Gene einen guten Ausgangspunkt.

Schlussfolgerung:

Die Ausschaltung von CD73 bei Bauchspeicheldrüsenkrebs hemmt die Tumorproliferation und -migration über den ERK/STAT3-Signalweg, wobei E-Cadherin ebenfalls eine Rolle spielen könnte. Die Deletion von CRISPR/Cas9-Proteinkinase-Bibliotheken in Kombination mit Durchflusszytometrie-Screening kann zur Identifizierung kritischer Gene, die CD73 regulieren, verwendet werden.

3. INTRODUCTION

3.1 Pancreatic cancer

3.1.1 Epidemiology and risk factors

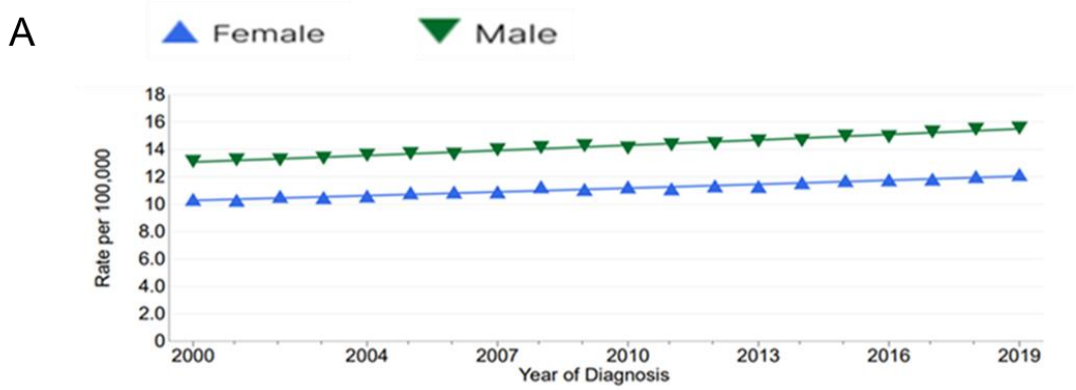
Pancreatic cancer is currently the seventh leading cause of cancer death globally and fourth in the United States and Europe after lung, colorectal, and breast cancer.[1] There will be 60,430 new cases of pancreatic adenocarcinoma in the U.S. in 2021. Pancreatic cancer will kill 48,220 people in 2021, including 25,270 men and 22,950 women.[2] The overall incidence of pancreatic cancer was 9.3 per 100,000 persons from 1975-2016. The incidence of pancreatic cancer from 8.3 per 100,000 persons in 1975 to 11.0 per 100,000 persons in 2016. Expected Pancreatic cancer will surpass colorectal cancer as the second leading cause of cancer-related death and the number one digestive cancer killer in the United States.[3] According to cancer registry data from 185 countries, there were an estimated 495,773 new cases of pancreatic cancer and 466,003 deaths globally in 2020.[4] In addition; pancreatic cancer mortality is projected to continue to rise in the coming decades[5], with more than 800,000 deaths expected by 2040, assuming a stable mortality rate from 2020 to 2040.[6]

In China, from 1990 to 2019, the incidence and mortality of pancreatic cancer increased significantly in the past 30 years, increasing by 82.11% and 79.46%, respectively which is a higher increase than the global level (25.86% and 27.90%).[7] Pancreatic cancer is one of the cancers with the highest mortality rate, so pancreatic cancer is a significant health problem.

The incidence of pancreatic cancer varies widely in different regions, with the lowest incidence in Indian, African, and Southeast Asian populations and relatively high in Europe and North America. But underdiagnosed areas with poor health care may be skewing these estimates.[8]

According to the statistics of NIH(National Cancer Institute), it can be seen that the incidence and mortality of pancreatic cancer have been increasing year by year in recent years. (Figure 1) The cause of pancreatic cancer has not yet been determined, but in many studies, risk factors associated with pancreatic

cancer have been explored. Age, the incidence of pancreatic cancer increases markedly with age, with most cases occurring between the ages of 60 and 80.[8] Gender, the prevalence of males is significantly higher than that of females. Cigarette smoking, smokers are 2.5 to 3.6 times more likely to develop pancreatic cancer than non-smokers; the risk increases with increased tobacco use and prolonged exposure to smoke.[9] Several studies have shown an increased incidence of pancreatic cancer in patients with a history of diabetes or chronic pancreatitis. The proportion of diabetic patients with pancreatic cancer is 30% higher than that of ordinary people.[10] And there is also evidence that chronic cirrhosis, gastrectomy, a high-fat, high-cholesterol diet, and previous cholecystectomy may also increase the risk.[11-14] Family history is also one of the reasons for the incidence of pancreatic cancer. Studies have shown that 4%-16% of patients with sporadic pancreatic cancer have a family history of the disease.[15] Multigene panel testing for pancreatic cancer detected the germline variants,BRCA1,PALB2,BRCA2,TP53,ATM,MLH1,MSH2,STK11 and SPINK1 as high-risk genes.[16,17]



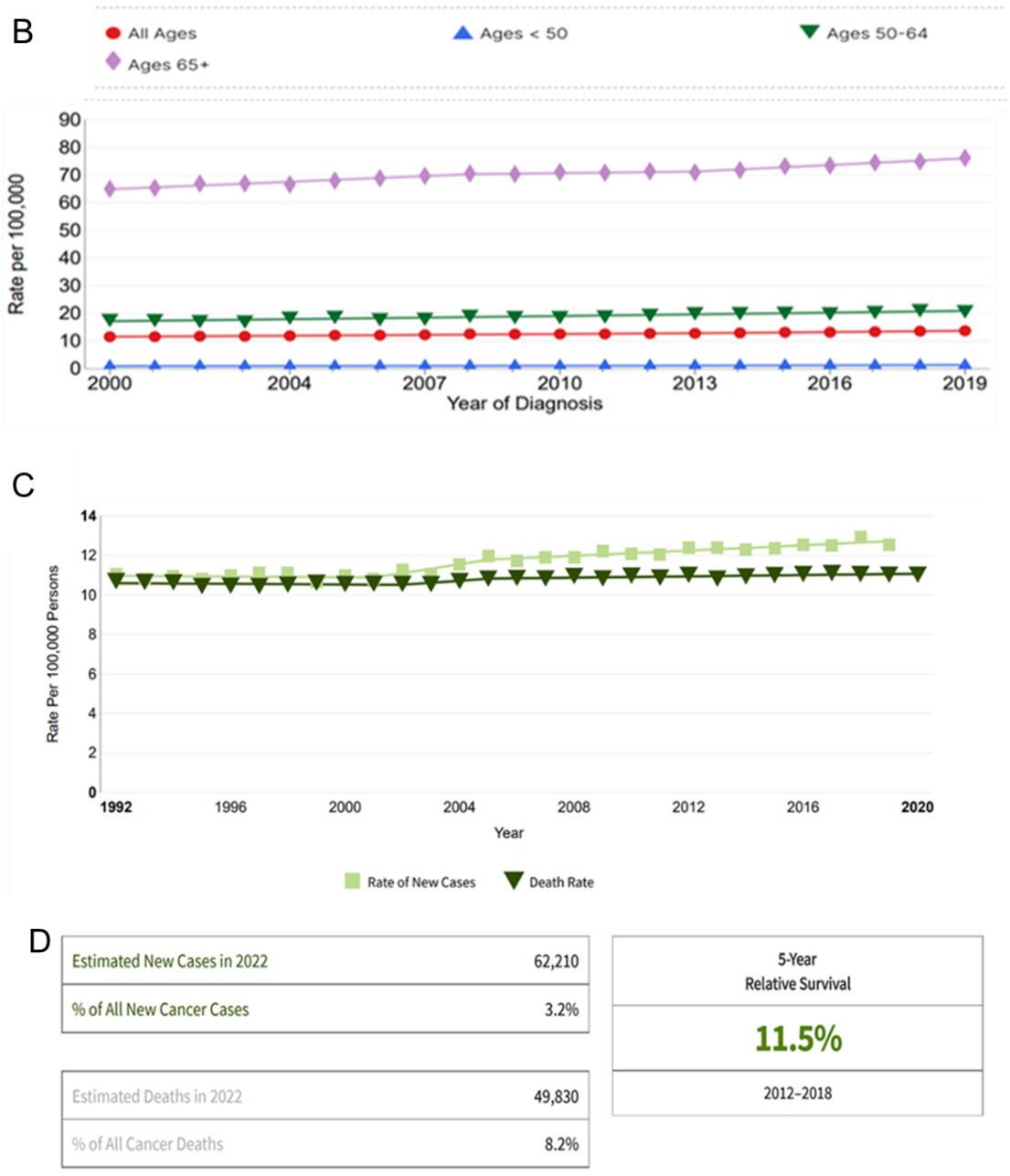


Figure 1 Expression. A.Incidence of pancreatic cancer by gender. B. Incidence of pancreatic cancer by age group. C. New cases come from SEER 12. Deaths come from U.S. Mortality. D.In 2022, and it is estimated that new pancreatic cancers and new pancreatic cancers account for the proportion of all new cancers. Estimated new pancreatic cancer mortality and new mortality as a proportion of all cancer deaths. Pancreatic cancer 5-year survival rate.

3.1.2 Histology and Pathology of Pancreatic Cancer

Histologically, the pancreas comprises the exocrine part (acini secreting pancreatic juice and its transport ducts) and the endocrine part (islets). Pancreatic cancer commonly refers to malignant epithelial tumors that occur in the exocrine part. The exocrine part comprises acinar with small pancreatic lobes as units and gradually enlarged ducts, which merge into the central pancreatic duct (some patients have accessory pancreatic ducts) and open into the duodenum.

The pancreas can be roughly divided into three parts: head, body and tail. Approximately 60%-70% of pancreatic adenocarcinomas occur in the head of the pancreas, with the remainder in the body (15%) and tail (15%)(Figure 2).[18]Exocrine tumors account for 95% of pancreatic cancers, and pancreatic ductal adenocarcinoma (PDAC) is the most common pancreatic tumor.[19] It is an invasive mucin-producing gland-forming neoplasm that elicits an intense stromal desmoplastic reaction.[20]

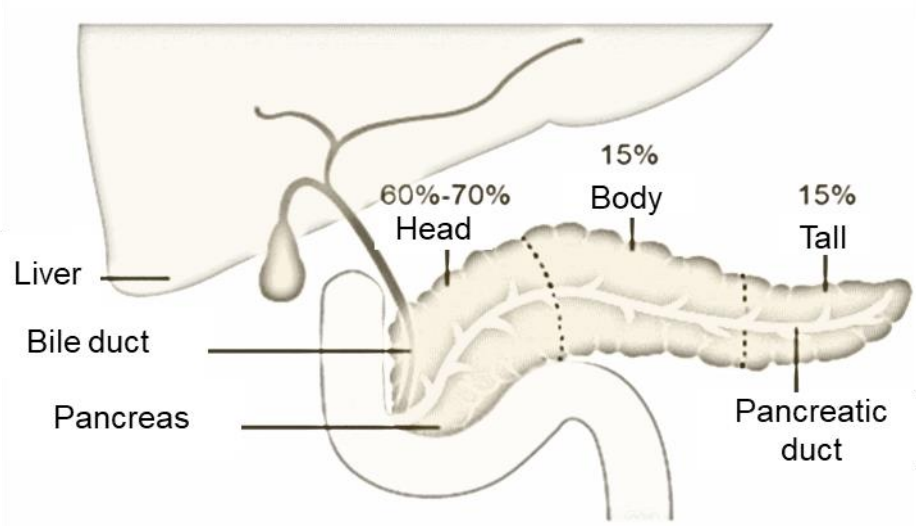


Figure 2 Expression. Pancreatic anatomy and incidence of cancer in different parts of the pancreas

3.1.3 Clinical presentation and diagnostic evaluation

Usually, there are no symptoms in the early stage of pancreatic cancer, and when obvious signs and symptoms appear, it often indicates that the

disease has reached an advanced stage. Most of them are accompanied by distant metastasis.[21,22] The most common signs and symptoms of pancreatic cancer may include yellowing of the skin, abdominal or back pain, unexplained weight loss, light-colored stools, dark urine, and loss of appetite.[23,24]

Commonly used auxiliary diagnosis methods are as follows:1. Histological Examination 2. Tumor Biomarkers 3. Computed Tomography/Positron Emission Tomography 4. Endoscopic Ultrasonography 5. Magnetic Resonance Imaging 6. Endoscopic Retrograde Cholangiopancreatography

The "gold standard" for diagnosing PC is a cytological and/or histopathological examination.[25] All patients, except those undergoing surgical resection, should work toward obtaining a precise pathology diagnosis before developing a therapeutic strategy.[26] Molecular alterations have considerably expanded in recent years, and this has made it possible to find new serum tumor indicators. A combination of tumor markers and imaging methods may be the first choice for early screening of PC. CA199,carcinoembryonic antigen (CEA), CA125, microRNAs, and K-RAS gene alterations are the six most prevalent tumor biomarkers at the moment in PC.[27] CA199 is the most commonly used index to detect PC recurrence and prognosis after surgery. [28,29] In addition, some indicators can be used to evaluate the poor prognosis of PC, such as IL-6, IL-8 and IL-10.[30] Imaging examinations and endoscopy provide a solid basis for diagnosing and treating pancreatic cancer. It has significant reference value in selecting pancreatic cancer surgery methods and postoperative review. [31]

3.1.4 Treatment

With the advancement of modern medical technology, several discoveries in drug research and multidisciplinary comprehensive diagnosis and treatment have given patients with pancreatic cancer more hope and advantages.

Surgery, chemotherapy, radiation therapy, and targeted therapy are the most popular forms of pancreatic cancer treatment.

Surgical resection is the only cure for pancreatic cancer, and adding chemotherapy in an adjuvant setting has improved survival. However, the procedure is complicated, traumatic and has a high rate of complications. When a tumor is found, many patients already have advanced malignancies with distant metastases, which eliminates the possibility of surgical treatment. Additionally, the high prevalence of surgical complications and low survival rates persist even after complete tumor excision.[32] Data studies show that even with "curative" resection and adjuvant therapy, the 5-year survival rate for PDAC with early resection is still only 30%.[33]

Chemotherapy is an essential part of the comprehensive treatment of PADC. Currently, the primary treatment method for metastatic pancreatic cancer is combined with cytotoxic chemotherapy.[34] One study compared complete surgical resection followed by gemcitabine adjuvant therapy with surgery alone. The results showed that the median disease-free survival (13.4 months vs. 6.7 months) and overall survival of patients treated with gemcitabine after surgery were significantly improved, with five-year survival rates of 20.7% and 10.4%, ten-year survival of 12.2% vs 7.7%.[35]

Radiation therapy uses X-rays to destroy or damage cancer cells, making them unable to increase. Patients with locally advanced PC are typically treated with radiotherapy. Numerous studies have demonstrated that receiving chemotherapy was not superior to continuing chemotherapy in individuals with advanced PC and did not increase patient survival.[36,37]

Targeted therapy is a treatment method at the cellular and molecular level that targets a well-defined cancer-causing site (the site can be a protein molecule inside a tumor cell or a gene segment). Corresponding therapeutic drugs can be designed. When the drugs enter the body, they will select the carcinogenic site to combine and act so that the tumor cells die without affecting

the normal tissue cells around the tumor. [38]Currently, there are three main types of targeted drugs for treating pancreatic cancer. 1. Erlotinib (Tarceva) is a drug that targets a protein on cancer cells called EGFR, which normally helps the cells grow.[39] 2. Olaparib (Lynparza) is a type of drug known as a PARP inhibitor. By blocking the PARP pathway, this drug also makes it very hard for tumor cells with a mutated BRCA. gene to repair damaged DNA., which often leads to their death.[40] 3. Larotrectinib (Vitrakvi) and entrectinib (Rozlytrek) target the proteins the NTRK. genes make. These drugs can be used in people with advanced pancreatic cancer that has been found to have an NTRK. gene change, typically when the cancer is still growing despite other treatments.[41]

3.2 Immunological checkpoint molecules

Tumor immunotherapy combined with other traditional therapies can significantly improve patient survival. Tumor immunotherapy mainly includes tumor vaccine, cytokine therapy, adoptive immunotherapy and immunological checkpoint blocking method, among which the immunological checkpoint blocking method has a significant curative effect. The immune checkpoint is a protective molecule in the human immune system responsible for identifying and defending the enemy. The immune system can identify tumor cells and exogenous foreign bodies that have been genetically altered and clear the tumor through a series of immune responses.T cells are the leading performers of antitumor immunity. Activation of T cells requires antigen-presenting cells to provide first signal stimulation, and co-stimulatory molecules are required to provide co-stimulatory signals that enhance immunity; meanwhile, T cell surface The co-inhibitory molecule binds to the corresponding ligand and is capable of transmitting a signal that inhibits the activation of T cells, thereby T cell proliferation and killing of tumor cells.[42]Tumor cells evade the body's immune surveillance and killing through a variety of ways to promote the further growth of the tumor. Immunological checkpoint inhibitors are based on this principle, using anti-suppressor molecules or The monoclonal antibody to block

its signal transmission, relieving the immunosuppression of tumor patients, activating the function of T cells to kill tumors, and then achieving the purpose of treating tumors. Based on this principle, immunological checkpoint inhibitors use monoclonal antibodies against co-suppressor molecules or ligands to block their signaling, relieve immunosuppression in tumor patients, activate T cells to kill tumors and thus achieve tumor treatment. In summary, identifying immune checkpoints is critical in immunotherapy; the most common immune checkpoints are shown below(Table 1). And Many of these immune checkpoints are CD molecules.

The cluster of differentiation (often abbreviated as CD) is a protocol used for identifying and investigating cell surface molecules, providing targets for the immunophenotyping of cells.[43]They are a class of proteins or glycoproteins found on cell membranes. Physiologically, CD molecules have many uses and are often used as essential cell receptors or ligands. It can be used as a surface marker for cell identification and isolation and is also widely involved in cell growth, maturation, differentiation, development, migration, and activation. Cell populations are usually defined using the "+" or "-" symbols to indicate whether a cell fraction expresses or lacks CD molecules.

Table 1: Common immune checkpoints

Receptor	Alternate name	Receptor Expression
CTLA4	CD152	Activated T cells
PD-1/PD-L1	CD279/ B7-H1 or CD274	T cells, B cells
LAG-3	CD223	Activated T cells, B cells, Tregs, NK cells, DCs
BTLA	CD272	Mature B cells, T cells, Tregs, macrophages, DCs
B7-H3/ B7-H4	CD276/ B7S1 or B7X	Activated T cells, N.K. cells, DCs, monocytes, tumor tissue

CTLA4

CTLA-4 (also known as CD152) is the first immunologically clinically targeted therapy and checkpoint receptor, which is a crucial negative regulator of immune response and belongs to the CD28 receptor family.CTLA-4 is a molecule expressed and upregulated on activated CD4+, CD8+ T cells and T-regulatory FOXP3+, CD4+, and CD25+ cells.[44,45]

PD-1/PD-L1

Therapy that targets programmed death 1 or programmed death 1 ligand 1 (PD-1/PD-L1), has recently been rapidly developing as monotherapy for various carcinomas.[46]Programmed cell death protein-1 (PD-1; also known as CD279), is a coinhibitory CD28-family mole cule.PD-1 functions mainly in the late phase, in which PD-1 induces exhaustion or energy in effector T cells.[47]PD-L belongs to the B7 family, including PD-L1 and PD-L2. PD-L1 is expressed on the surface of solid tumor cells, and tumor-infiltrating lymphocytes.[48]PD-L2 is mainly expressed on the surface of dendritic cells, macrophages and B cell subsets.[49]In 2015, the Cancer Immunotherapy Trials Network (CITN.) ranked the immune checkpoint inhibitors targeting programmed death PD-1 or its ligand PD-L1 as the number 1 on the priority list for immunotherapeutic agents.[50]

LAG-3

Lag-3 (CD223) is expressed on activated CD4+ and CD8+ effector T cells, CD4+Foxp3+ Treg, Tr1 cells, B cells, plasmacytoid D.C.s, and a subset of NK cells.[51] LAG-3 is a member of the immunoglobulin class superfamily and is only present in activated immune cells. And it is composed of four extracellular Ig-like domains and a type I transmembrane domain structurally resembling the CD4 co-receptor. It is a critical component of negative feedback for T-cells and is vital in activating regulatory T-cells.[43,52]

BTLA

B and T lymphocyte attenuator (BTLA, also known as CD272) has been identified as a novel co-inhibitory receptor expressed by most lymphocytes, showing structural and functional similarities to CTLA-4 and PD-1.[53] BTLA is a novel checkpoint co-inhibitory receptor belonging to the CD28 superfamily (also known as the Immunoglobulin superfamily), which appears on a wide range of immune cells, including T, B, NK, and other cells.[54]

B7-H3/ B7-H4

B7-H3 (CD276) is a type I transmembrane co-stimulatory molecule of the B7 family. Initially, B7-H3 was thought to co-stimulate the immune response, but recent studies have shown that it has a co-inhibitory role on T-cells, contributing to tumor cell immune evasion.[55] B7-H4 (B7S1 or B7X) is a member of the B7 family and inhibits the T-cell mediated response by inhibiting T-cell proliferation, activation, and cytokine production. B7-H4 also inhibits the innate immune response by suppressing the growth of immunocytes, thereby allowing tumors to avoid immunologic surveillance.[56]

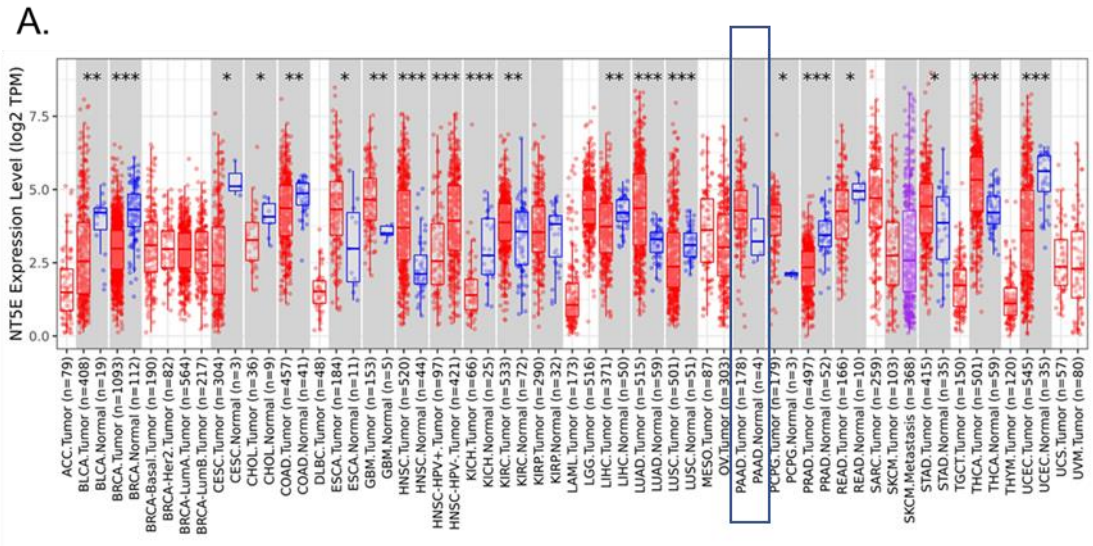
In recent years, tumor immunotherapy has been gaining attention, and more and more immune checkpoints have been discovered and discussed, among which CD73 is one of the most popular ones.

3.3 The function of CD73 in cancer

CD73 also known as ecto-5′-nucleotidase is an enzyme that in humans is encoded by the NT5E gene. CD73 is a surface enzyme expressed on a variety of cells. This enzyme mediates the progressive hydrolysis of autocrine and paracrine danger signals of ATP and ADP to anti-inflammatory adenosine. Immunosuppression mediated by the adenosine pathway is essential for maintaining the immune system's balance. The immunosuppressive function of T regulatory cells also depends on the expression of CD73. Tregs generally suppress the immune response. They affect the proliferation and function of T cells.[57]

CD73 expression is increased in many tumor cells, including esophageal cancer, breast cancer, prostate cancer, etc(Figure 3.A).And its production of free adenosine suppresses the cellular immune response, thus promoting the immune escape of tumor cells.[58] CD73 is an adhesion and signaling molecule that modulates cell signaling of extracellular matrix components such as fibronectin and laminin. This can mediate the metastatic and invasive properties of cancer.[59]

A growing number of reports suggest that CD73 is a key molecule in the regulation of tumor development, that it is involved in the development of a wide range of cancers, and that it affects the survival of patients(Figure 3.B). But there is relatively little discussion on CD73 in pancreatic cancer. In this study, we observed the phenotypic changes of tumor cells after knocking down CD73 in pancreatic cancer cells, and further detected the key molecules affecting CD73 expression in pancreatic cancer by genome-wide CRISPR-Cas9 loss-of-function screening combined with FACS.



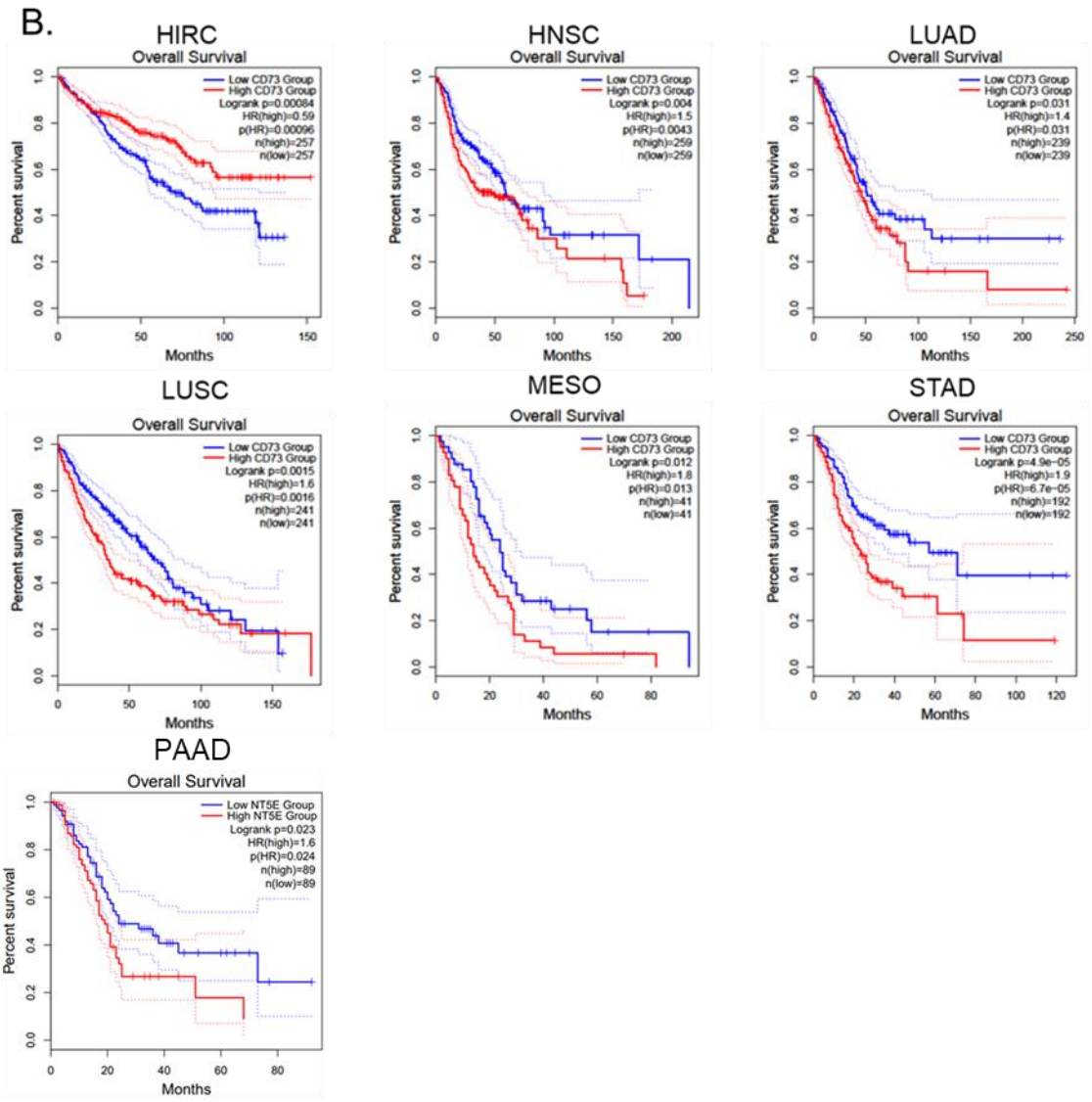


Figure 3 CD73 is highly expressed in a variety of cancers and is associated with prognosis (A).CD73 expression between tumor and adjacent normal tissues across all TCGA tumors. The statistical significance computed by the Wilcoxon test is annotated by the number of stars (*: p-value < 0.05; **: p-value <0.01; ***: p-value <0.001). (B). Overall survival(OS) results of CD73 gene in different cancers were anayzed using TCGA date.

4. Materials and Methods

4.1 Materials

4.1.1 Cell lines and reagents

Table 2: List of cell lines and their cultivation medium

Cell lines	Medium components
PANC-1	RPMI-1640 Medium (Gibco™, #21875034) with 10% FBS (Gibco™, #A3160501)
Aspc1	RPMI-1640 Medium (Gibco™, #21875034) with 10% FBS (Gibco™, #A3160501)
SU86.86	RPMI-1640 Medium (Gibco™, #21875034) with 10% FBS (Gibco™, #A3160501)
SUIT2	Minimum Essential Medium (Gibco™, #11095080) with 10% FBS
Miapaca2	Dulbecco's Modified Eagle's Medium with 10% FBS (Gibco™, #A3160501) and 12.5 mL horse Serum (Gibco™, #16050122)
Mayo 4636	DMEM/F-12 (Thermo Fisher, #11330032) with 10% FBS, 5 mL L-Glutamin (200 mM) and 7.5 mL HEPES (1M)
TKCC10	215 mL Medium 199, Earle's Salts, 215 mL Ham's F-12 Nutrient Mix. 50 mL FBS, 7.5 mL HEPES (1M), 10 µL EGF (Stock: 1mg/mL), 20 µL Hydrocortisone (Stock: 1mg/mL), 5 mL apo-Transferrin Human (Stock: 2,5mg/mL), 1 mL Insulin, human recombinant, zinc solution (Stock: 100 IU/mL), 3 mL Glucose solution (10%), 2.5 mL MEM Vitamin Solution (100X), 2.5 µL 3,3′,5-Triiodo-L-thyronine sodium salt (Stock: 0,1 µg/mL), 50 µL O-Phosphorylethanolamine (Stock: 20 mg/mL), 5 mL 2 mM Glutamine (100x stock)
TB 32047	Dulbecco's Modified Eagle's Medium (Gibco™, #11965092) with 10% FBS
KPC 661	Dulbecco's Modified Eagle's Medium (Gibco™, #11965092) with 10% FBS
HEK293 TN	Dulbecco's Modified Eagle's Medium (Gibco™, #11965092) with

	10% heat-inactivated FBS

4.1.2 Plasmids

Table 3: List of plasmids

Plasmid	Source
lentiCas9-Blast	Addgene, Cat #52962
lentiGuide-Puro	Addgene, Cat #52963
Mouse Brie kinome pooled library	Addgene, Cat #75316
pMDLg/pRRE	Addgene, Cat #12251
pRSV-REV	Addgene, Cat #12253
pMD2.G	Addgene, Cat #12259
pSpCas9(BB)-2A-Puro (PX459) V2.0	Addgene, Cat #62988

4.1.3 Primers of RT-PCR, sgRNAs and single clone check

Table 4: List of RT-PCR primers

Primer target	Direction	Sequences (5'->3')
Ho_CD73	Forward	CCAGTACCAGGGCACTATCTG
Ho_CD73	Reverse	TGGCTCGATCAGTCCTTCCA
Ho_CD80	Forward	GGCCCGAGTACAAGAACCG
Ho_CD80	Reverse	TCGTATGTGCCCTCGTCAGAT
Ho_CD154	Forward	ACATACAACCAAACTTCTCCCCG
Ho_CD154	Reverse	GCAAAAAGTGCTGACCCAATCA
Ho_CD252	Forward	GGTCAGGTCTGTCAACTCCTT
Ho_CD252	Reverse	CATCCAGGGAGGTATTGTCAGT
Ho_CD276	Forward	TCACAGGGCAGCCTATGAC
Ho_CD276	Reverse	TCCTCAGCTCCTGCATTCTC
Mm_CD73	Forward	ATGCCGGAGACCAGTACCA
Mm_CD73	Reverse	CAGTGCCATAGCATCGTAGCC
Mm_CD80	Forward	ACCCCCAACATAACTGAGTCT

Mm_CD80	Reverse	TTCCAACCAAGAGAAGCGAGG
Mm_CD154	Forward	CTTCTGCTCTAATCGGGAGCC
Mm_CD154	Reverse	GCCGCCTTGAGTAAGATTCTC
Mm_CD276	Forward	GGACCTACGTCCAGGGAACAT
Mm_CD276	Reverse	TGGTCACATTGCCAGTCAAGG
Ho_GAPDH	Forward	CTTTGGTATCGTGGAAGGACTC
Ho_GAPDH	Reverse	AGTAGAGGCAGGGATGATGT
Ho_β-Actin	Forward	CACCATTGGCAATGAGCGGTTC
Ho_β-Actin	Reverse	AGGTCTTTGCGGATGTCCACGT
Mm_GAPDH	Forward	AGGTCGGTGTGAACGGATTTG
Mm_GAPDH	Reverse	TGTAGACCATGTAGTTGAGGTCA
Mm_β-Actin	Forward	GTGACGTTGACATCCGTAAAGA
Mm_β-Actin	Reverse	GCCGGACTCATCGTACTC

Table 5: List of sgRNA primers

sgRNA	Direction	Sequences (5'->3')
Ho_CD73_sg1	Forward	CACCGCGCCCTGCGCTACGATGCCA
Ho_CD73_sg1	Reverse	AAACTGGCATCGTAGCGCAGGGCGC
Ho_CD73_sg2	Forward	CACCGGTGTGGACGTCGTGGTGGG
Ho_CD73_sg2	Reverse	AAACCCCACCACGACGTCCACACC
Mm_CD73_sg1	Forward	CACCGCCACTCAGACGTGCCGCTTC
Mm_CD73_sg1	Reverse	AAACGAAGCGGCACGTCTGAGTGGC
Mm_CD73_sg2	Forward	CACCGCCTCTAGCACATCAGATATC
Mm_CD73_sg2	Reverse	AAACGATATCTGATGTGCTAGAGGC

Table 6: List of primers for single clone check

sgRNA	Direction	Sequences (5'->3')
Ho_CD73_sg1_check	Forward	ACCAGCGAGGACTCCAGCAA

Ho_CD73_sg1_check	Reverse	AATCGTCCAAGGGACTTCTATGC
Ho_CD73_sg2_check	Forward	TTTGGTTTTACTGACTCTTGAGC
Ho_CD73_sg2_check	Reverse	ATCCTTTTGAAACAATTACCTGTG
Mm_CD73_sg1_check	Forward	CCTTTCCTCCCTCCCTAGACGC
Mm_CD73_sg1_check	Reverse	GCCACAACCAAAATGCACAGATG
Mm_CD73_sg2_check	Forward	TACTTAGGCACTGGGAAATCAT
Mm_CD73_sg2_check	Reverse	ACAAAGAAGTTCACCGAGCAGA

4.1.4 Antibodies

Table 7: List of antibodies for western blot and FACS

Source	Antibodies	Species	Identifier	Note
Cell signaling	NT5E/CD73	Human, Maus	Cat# 13160 RRID:AB_2716625	70 kDa
	Vinculin	Human, Maus	Cat# 13901 RRID:AB_2728768	124 kDa
	GAPDH	Human, Maus	Cat# 5174 RRID:AB_10622025	37 kDa
	P44/42 MAPK(Erk1/2)	Human, Maus	Cat# 4695 RRID:AB_390779	44/42 kDa
	AKT-pan	Human, Maus	Cat# 4691 RRID:AB_915783	60 kDa
	Stat3	Human, Maus	Cat# 12640 RRID:AB_2629499	86 kDa
	p-AKT	Human, Maus	Cat# 4060 RRID:AB_2315049	60 kDa
	p-Stat3	Human, Maus	Cat# 9145 RRID:AB_2491009	86 kDa
	Phospho-p44/42	Human, Maus	Cat# 4370 RRID:AB_2315112	44/42 kDa

	MAPK(p-Erk1/2)			
	E-Cadherin	Human, Maus	Ca#3195 RRID:AB_2291471	135 kDa
	HRP-linked anti-rabbit IgG	Human, Maus	Cat# 7074 RRID:AB_2099233	/
	HRP-linked anti-mouse IgG	Human, Mouse	Cat# 7076 RRID:AB_330924	/
BD	CD73	Human	Cat# 561014 RRID:AB_2033967	PE
	CD80	Human	Cat# 564158 RRID:AB_2738630	BV650
	CD154	Human	Cat# 566268 RRID:AB_2739646	BV421
	CD252	Human	Cat# 563766 RRID:AB_2738412	BV421
	CD276	Human	Cat# 565829 RRID:AB_2739369	BV421
	CD73	Maus	Cat# 550741 RRID:AB_393860	PE
	CD80	Maus	Cat# 563687 RRID:AB_2738376	BV650
	CD154	Maus	Cat# 561719 RRID:AB_10897018	PE
	CD252	Maus	Cat# 565341 RRID:AB_2739194	BV421
	CD276	Maus	Cat# 563634 RRID:AB_2738336	BV421

4.2 Method

4.2.1 Cell Culture

This study used human and murine pancreatic cancer cells (PANC1 and TB32047). PANC1 (CRL-1469™) PDAC cells were purchased from the ATCC® (American Type Culture Collection). TB32047 cells were obtained courtesy of Prof. Dave Tuveson, Cold Spring Harbor Laboratory. All cell lines were cultured at 37° C in a humidified envi-ronment with 5% CO2. Cells from the HEK293TN cell line were also obtained from the ATCC®. All cells were washed with Dulbecco's Phosphate-Buffered Saline (DPBS) without Cacl2 and Mgcl2 (Cat# 14190094, Gibco™) and 0.25% tryp-sin-ethylenediaminetetraacetic acid (EDTA) (Cat# 25200-072, Gibco™) for collection. Cells were frozen in basal medium with 20% fetal bovine serum (FBS) (Cat# A3160802, Gibco™) and 10% dimethyl sulfoxide (DMSO) (Cat# D2650-5X5ML, Sigma-Aldrich Chemie). We generated the TB32047-Cas9 stable cell line by virally transducing lenti-Cas9-blast (Addgene, Cambridge, MA; cat. # 52962) into the cells and selecting them using 10 µg/mL of blasticidin (InvivoGen, cat. # ant-bl-1) for 3 days. We verified Cas9 expression by Western blot analysis.

4.2.2 Flow Cytometry

The cells were washed twice with DPBS, digested with trypsin, and harvested. The cells were resuspended with FACS buffer to a concentration of 1 x 106 cells/ml (1 mil-lion/100 µL). Transfer of the resuspended cells (1 million/100 µL) into a 5 ml FACS tube was then performed, and fluorescently conjugated antibody (1:100) (see Table S1) was added. We then gently moved a pipette up and down to help disperse the doublet (15-20 times) and incubated it for 20-30 minutes (at 4° C) in the dark. The cells were washed with 500 µL FACS buffer and centrifuged for 5 min (350 × g) at 4° C. The supernatant was then discarded, and the cells were lysed in 200 µL FACS

buffer in a 5 mL FACS tube. We then analyzed the stained cell samples using flow cytometry (BD Biosciences LSRII), and we analyzed the flow cytometry data using FlowJo™ v10.8 software (BD Life Sci-ences).

4.2.3 FACS Sorting

Cells were collected and stained with CD73 antibody, as described in Section 2.2, Flow Cytometry. Cells were transported, on ice, to the FACS facility and sorted with FACS Aria II. After FACS sorting, cells with high levels of CD73 expression were col-lected, and they continued to be cultured for a new round of sorting. Cells were again collected after three rounds of sorting. To identify the sgRNAs integrated into the cells with high levels of CD73 expression, 10 million cells were then collected for genomic DNA isolation.

4.2.4 Lentivirus production

We seeded 7×106 HEK293TN packaging cells in T75 flasks and incubated the cells at 37° C, 5% CO2, for 24 h. We then prepared a mixture of the transfection plasmids (2.8 µg pMDLg/pRRE, 1.4 µg pRSV-REV, 1.4 µg pMD2.G, and 4.3 µg library plasmid) with P3000 and Opti-MEM. (See Table S2 for a list of the plasmids.) The pooled sgRNA library (Mouse Brie kinome pooled library) targeting the murine kinome was a gift from John Doench and David Root [23] (RRID: Addgene_75316, Addgene, Cambridge, MA, USA), and it was modified for the inclusion of pancreatic-cancer-related genes by our lab. (See the Supplementary Materials.) The library contained 3446 sgRNAs for 915 murine genes.Then, we gently added the mixture to the Opti-MEM with lipo3000. The mixture was incubated for 15-20 min at room temperature, and the transfection mix was care-fully transferred to T75 flasks. The cells were then incubated for 6 h, and the media was carefully aspirated. The media was then replaced with 12 mL DMEM complete medium. The virus was harvested 24 h post-transfection, and the viral supernatant was centrifuged at 2000 x g

for 10 minutes to remove the cell pellets. As soon as was possible, the viral supernatant was stored at -80° C after it was filtered through a 0.45 µm PES filter to avoid a loss of titer.

4.2.5 Lentivirus transduction

A quantity of 1.8x106 TB32047 WT or Cas9 cells was seeded into T75 flasks with 12 mL medium on the day before transduction. After 24 h, the medium was removed from the cells and replaced with 0.1% polybrene medium with Cas9 or Mouse Brie kinome pooled library lentivirus. The regular medium was introduced to the flask after the lentivirus had been present for 24 h. The cells were then incubated for another 2 days, and the medium was replaced with 10 µg/mL blasticidin (for the cas9 cells) or puro-mycin (for the library cells) for 72 h. Finally, the cells were trypsinized and counted after puromycin selection. Based on the cell count results, we then calculated the required volume of the virus (MOI = 0.3-0.5).

4.2.6 Deep Sequencing and Data Analysis

Genomic DNA was isolated with a NucleoSpin Blood L Kit (Cat# 740954.20, MACHEREY-NAGEL, Düren, Germany), followed by a PCR procedure to amplify the sgRNAs. A quantity of 20 µg of DNA was amplified using P5 (5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNTCTTGTGGAAAGGACGAAACACCG-3') and P7 (5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCTACTATTCTTTCCCCTGCACTGT-3') primers. For each sample, Q5 Master Mix (Cat# M0494S, Biolabs, Beverly, MA, USA) was used to conduct two independent 100 µL reactions with 10 µg of ge-nomic DNA in each reaction, and the PCR product of the same sample was mixed for deep sequencing in Dresden. The raw data of the deep sequence were analyzed using PinAPL-Py.[60]

4.2.7 CRISPR-Cas9 gene editing

CD73 was knocked out in TB32047 and PANC1 cell lines with the CRISPR-Cas9 gene editing plasmid pSpCas9(BB)-2A-Puro (PX459) and sgRNA designed for CD73 (sgRNA primers of CD73 are listed in Table 4). Ligated vectors were transferred into Endura sterile cells (Cat# 60242-1, Lucigen, Middleton, WI, USA) for plasmid extraction. CRISPR/Cas9 plasmid target CD73 was transfected with lipo3000 for 24 h, followed by selection with puromycin (10 μg/ml) for three days. After growth, Western blotting and qPCR were performed to verify the knockout. Wild-type cells were named WT, and non-targeted control sgRNAs and sgRNAs transfected with CD73 were named NC and KO cells, respectively.

The sgRNAs used in this study were:

mm-CD73-sg1—forward: 5′-CACCgCCCACTCAGACGTGCCGCTTC-3′, reverse: 5′- AAACGAAGCGGCACGTCTGAGTGGC-3′.

mm-CD73-sg2—forward: 5′-CACCgCCTCTAGCACATCAGATATC-3′, reverse: 5′- AAACGATATCTGATGTGCTAGAGGC-3′.

hu-CD73-sg1—forward: 5′-CACCgCGCCCTGCGCTACGATGCCA-3′, reverse: 5′- AAACTGGCATCGTAGCGCAGGGCGC-3′.

hu-CD73-sg2—forward: 5′-CACCgTGTGGACGTCGTGGTGGG-3′, reverse: 5′- AAACCCCACCACGACGTCCACACC-3′.

4.2.8 Confirmation of CRISPR/Cas9-Mediated Knock-out

NucleoSpin® Tissue (MACHEREY-NAGEL, Düren, Germany) was used to isolate DNA from the cell lines. PCR products for sequencing were amplified using primers listed in Table S3. PCR fragments were cloned in pMiniT 2.0 with the NEB® PCR Cloning Kit (New England Biolabs, Frankfurt, Germany, E1202). For each single-cell clone, 10 bacterial colonies were chosen, and plasmid DNA was isolated (GeneJET Plasmid Miniprep Kit, K0503, Thermo Fisher, Langenselbold, Germany) for sequencing in Eurofins Genomics.

4.2.9 Quantitative PCR

Total RNA was extracted from the cells using the NucleoSpin® RNA Plus kit (MACHEREY-NAGEL, Cat# 740984.250). RNA (1000 ng) was reverse-transcribed using a High-Capacity cDNA Reverse-Transcription Kit (Applied BiosystemsTM, Cat# 4368814) according to the manufacturer's protocol. β-Actin and GAPDH (mouse and human) expression levels were used to normalize RNA input levels. Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific) was used for all genes of interest. (qRT-PCR primers are listed in Table S4.) The mRNA expression levels of different genes were quantified using a Light Cycler 480 II system. Gene expression levels were calculated using the 2-ΔΔCT method.

4.2.10 Western Blot

Cells were lysed in RIPA buffer (Cat# 89900, Thermo Fisher Scientific, Waltham, MA, USA) containing 1% protease and phosphatase inhibitors (Cat# 78442, Thermo Fisher Scientific). Protein concentrations were quantified using a BCA Protein Assay Kit (Cat# 23250, Thermo Fisher Scientific). A quantity of 20 µg of protein samples were loaded onto 4-12% NUPAGE Bis-Tris gels (Cat# NP0322BOX; Thermo Fisher Scientific) using a Mini Gel Tank chamber system (Invitrogen), and the proteins were transferred to Amersham™ Protran™ Premium 0.45 µm NC membranes (Cat# GE10600003, Cytiva). After blocking with 5% milk, primary antibodies (listed in Table S6) were used accord-ing to the manufacturer's requirements and incubated overnight at 4° C. HRP-conjugated anti-rabbit or anti-mouse IgG was used as a secondary antibody. Signal quantification was performed with an Amersham Imager 600 (Pittsburgh, PA, USA) and SignalFire™ ECL reagent (Cat# 6883S, CST).

4.2.11 Cell cycle analysis and apoptosis assay

Appropriately harvest the cells and wash them in cold PBS. Add cold 70%

ethanol while shaking the cells. − 20 °C overnight for preservation. Wash 2 times with PBS. Incubate with RNase (100 µg/ml) for 60 min at 37°C, then stained with propidium iodide (PI; 50 µg/mL, Cat. #421,301, BioLegend). Quantitative analysis was performed by flow cytometry on a BD Biosciences LSRII flow cytometer.

Adherent cells and the cells in the culture medium were collected for apoptosis detection and stained with FITC Annexin V Apoptosis Detection Kit I (Cat. #556,547, BD Pharmingen) according to the manufacturer's instructions. FITC-Annexin V uptake was measured on a BD Biosciences LSRII flow cytometer. Flow cytometry results were analyzed using FlowJo™ v10.8 software (BD Life Sciences).

4.2.12 Proliferation assay

1×10^3 cells/well of TB32047 and PANC1 cells were plated in black 96 plates. Cells were cultured for 6 days and stained with DAPI (Hoechst 33,342, Cat. #H3570, Life Technologies) every 24 h. Then take photos of every well using an EVOS FL Auto 2 imaging system (Cat. #AMAFD2000, Invitrogen). Cells in these images were counted by HCS studio cell analysis software V2.0 (Thermo Fisher Scientific, SX000041A, Waltham, MA, USA). Each data point was produced in triplicate and normalized to the counts of Day 1. Each experiment was run three times (n=3).

4.2.13 Colony formation assay

Each cell was seeded in three wells of 6-well plates (100 cells/well). TB32047 cells were cultured for 7 days, and PANC1 cells were cultured for 14 days. The medium was removed, cells were fixed with 4% formaldehyde solution for 10 min, and formaldehyde was discarded. The cells were stained with 0.1% crystal violet for 15 min, washed with water and counted the colonies. Colonies consisting of more than 50 cells were counted visually, and the

average number of colonies was calculated.

4.2.14 Wound-healing assay

TB32047 cells at 3.5×10^5 per well and PANC1 cells at 4×10^5 per well were seeded in 12 wells for wound healing experiments. Then cultured and allowed to grow for 24 hours. After the cells reached 100% confluence, the serum-free medium was replaced and starved the cells for 6 hours. A 100 µL tip was used to create a wound in each well of the plate. Then replace with 1% FBS medium (TB 32047) or 2% serum RPMI medium (PANC-1). Each circular wound within the field of view was photographed with an EVOS microscope at 0 h and 24 h, and the ImageJ software measured the notch area and calculated the percentage of healing.

4.2.15 Immunofluorescence

Cells were grown in 4-well chamber slides, and observed the cell growth status to ensure its uniform distribution and density. After rinsing the slides with PBS 1X (2 times), the slides were fixed with 4% formalin for 15 min. The slides were washed with TBS 1X (filtered) for 5 min and repeated three times, permeabilized with 0,1% Triton (Sigma-Aldrich) for 30 min and washed with TBS 1X (filtered) for 2 x 5 min. Add 10% goat normal serum (GNS) diluted in TBS 1X and incubate for 10 min at RT. Add E-cadherin antibody (1:50-1:100) diluted in 5% GNS and incubate overnight at 4°C. The next day, wash 2 times with TBS 1X for 5 minutes each. Add secondary antibody-rabbit-AlexaFluor(488) diluted 1:500 in 5% GNS and incubate for 1 hour in the dark at RT. Wash 2 times with TBS 1X for 5 min each. Add DAPI diluted 1:5,000 (in water) and incubate for 10 min. Wash 2 times with TBS 1X at RT for 5 min each. Add fluorescent mounting medium (DAKO) and coverslip. Air dry and take pictures by EVOS.

4.2.16 Statistical Analysis

Values are expressed as mean ± SEM or mean ± SD (GraphPad Prism 8.0). Unless indicated, results are from at least two- or three independent experiments. Statistical significance was determined by the Two-way ANOVA or one-way ANOVA (or mixed model). P-values are reported in the graphs. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$. ns. not significant. In all analyses, $p < 0.05$ was considered statistically significant.

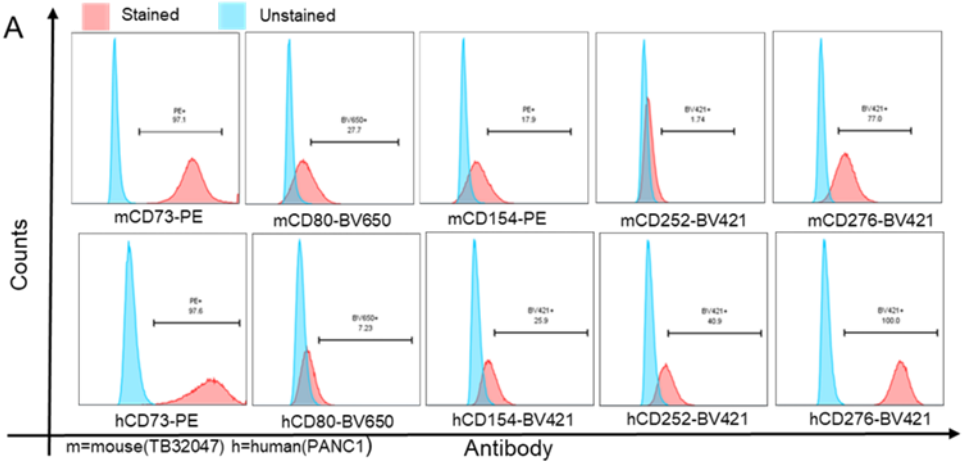
5. RESULT

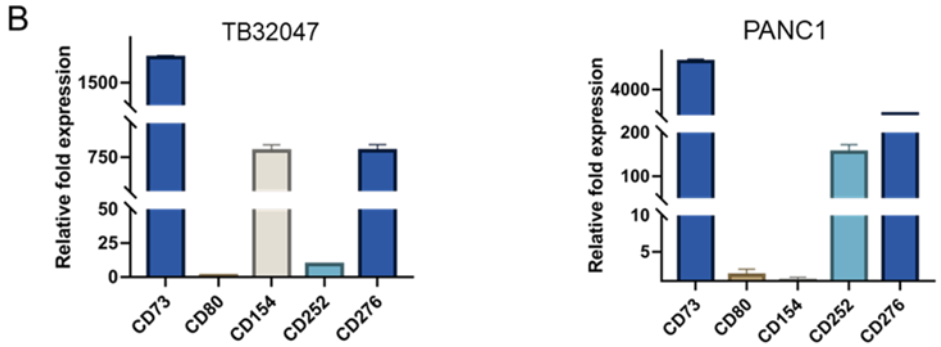
5.1 CD73 is highly expressed in pancreatic cancer and reduces patient survival cycles

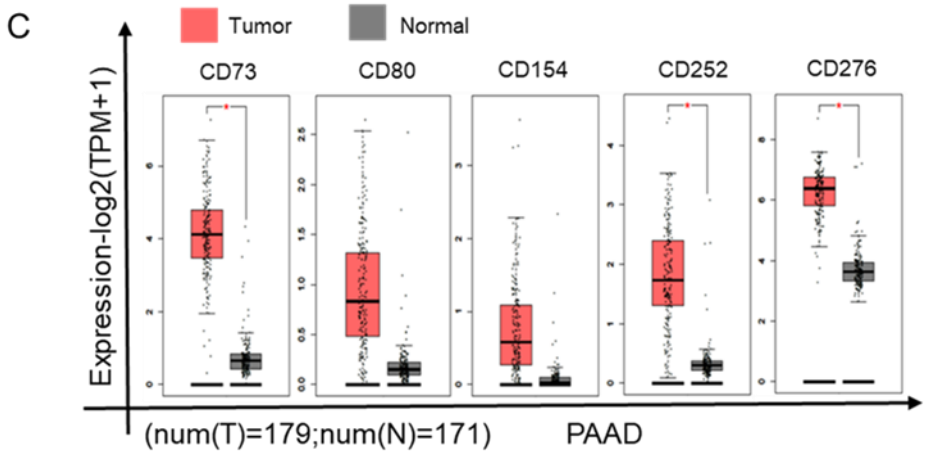
First, I would be Flow cytometry was used to analyzing for CD molecules expression in TB32047 and PANC1 cells, namely CD73 (97.1%), CD80 (27.7%), CD154 (17.9%), CD252 (1.74%) and CD276 (77%) compared with respective unstained controls in TB32047 cells and CD73 (97.6%), CD80 (7.23%), CD154 (25.9%), CD252 (40.9%) and CD276 (100%) compared with respective unstained controls in PANC-1 cells (Figure 4 A). The higher expression level of surface CD73 than the other CD molecules in TB32047 and PANC1 cells. qRT-PCR showed that the mRNA expression of CD73 is the highest in TB32047 cells and PANC1 cells (Figure 4B). TCGA-PAAD indicated that CD73, CD252, and CD276 are upregulated in pancreatic cancer compared to normal tissues (Figure 4C). Therefore, we focused on CD73, which is highly expressed in PDAC, for further analysis.

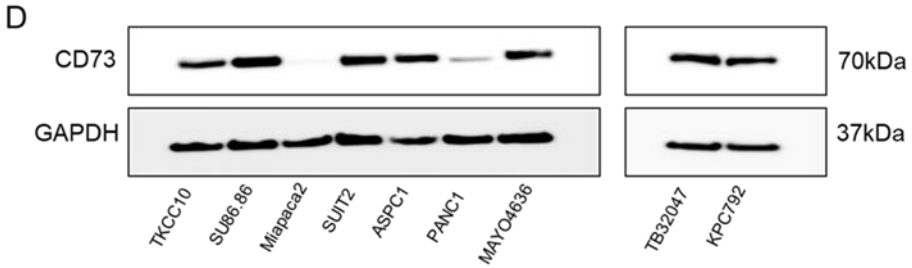
And then,we test expression of CD73 in seven human cell lines (TKCC10, SU86.86, MIAPACA2, SUIT2, ASPC1, PANC1, MAYO4363) and two mouse cell lines(TB32047 and KPC792) by the Western blotting. The results showed that in the human PDAC cell line, the expression of CD73 in SU86.86 cells was the highest, in MIAPACA2 cells was the lowest, in PANC1 and TKCC10 cells were relatively in the middle. In the mouse PDAC cell line, the expression of CD73 in TB32047 cells was higher than in KPC792 cells (Figure 4D). qRT-PCR also showed that the mRNA level of CD73 is highest in the SU86.86 cells and was relatively in the middle in PANC1 cells. The qRT-PCR result of mouse cell lines is consistent with western blotting (Figure 4E). Data from TCGA-PDAC indicated that increased CD73 expression is statistically associated with poor overall survival and disease-free survival in PDAC (Figure 4F). However, there was no difference between the expression of CD73 and different pathological stages of pancreatic cancer by GEPA2 analysis (Figure 4G). Therefore, we

chose the PANC1 and TB32047 cell lines to study the function of CD73 in PDAC.









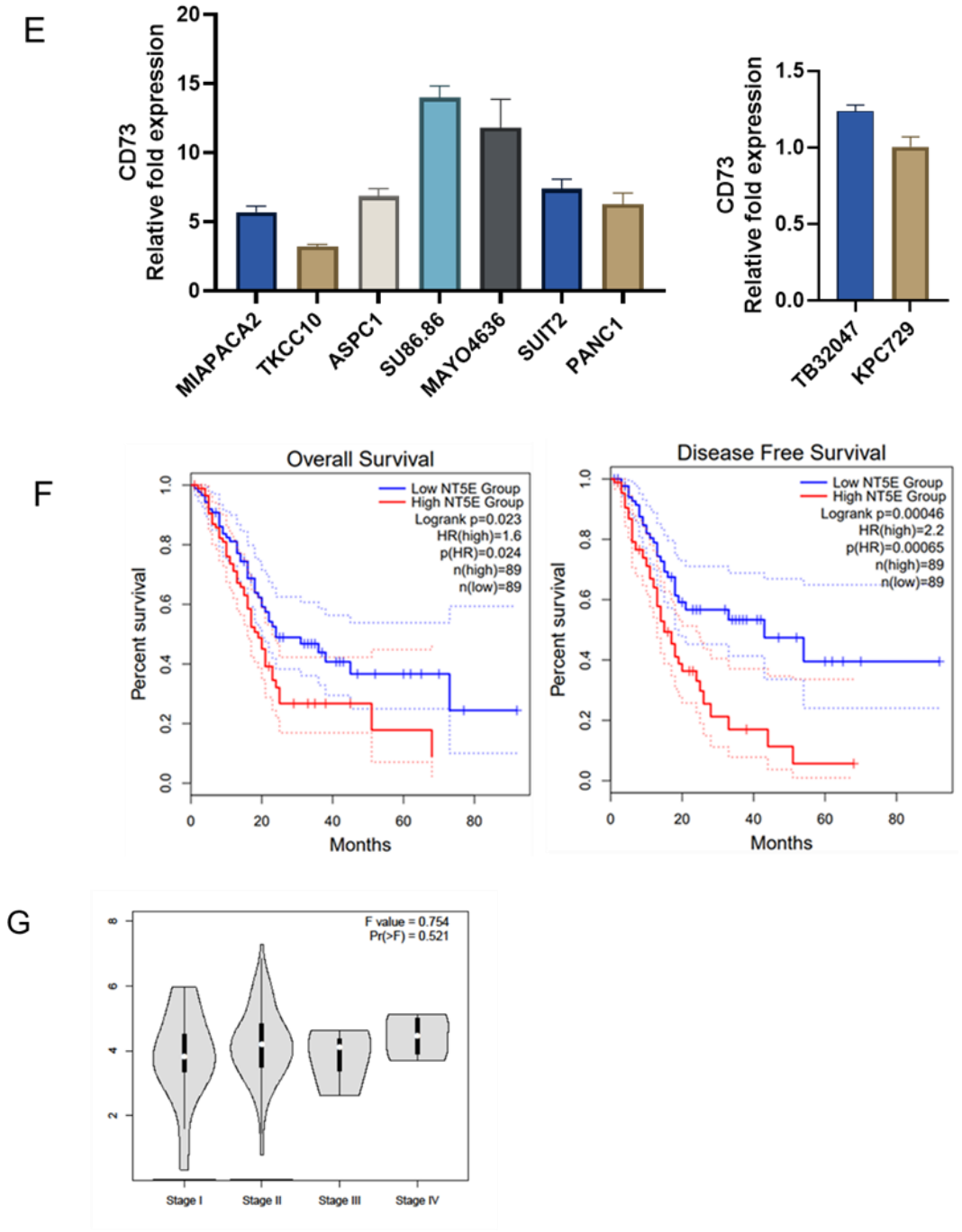


Figure 4. CD73 is highly expressed in pancreatic cancer. (A) Flow cytometry was performed to detect CD73, CD80, CD154, CD252 and CD276 expression on the cell membrane surface of TB32047 cells and PANC1 cells. (B) Expression of CD73, CD80, CD154, CD252 and CD276 mRNAs of TB32047 cells and PANC1 cells. CD154 as the control group in the TB32047 cell. The PANC1 cell control group is CD80. (C) Gene Expression Profiling Interactive Analysis (GEPIA) was performed to validate the CD molecules in PAAD

samples compared with normal samples. The red box was the cancer tissue group, gray was the normal tissue group, and *P< 0.05. (D) Western blot analysis of CD73 expression in human and mouse PDAC cell lines. (E) qRT-PCR showed the expression of CD73 in different PDAC cell lines. (F) Data from TCGA-PDAC was applied for survival analysis. Kaplan–Meier survival analysis shows that higher CD73 expression is associated with poor Overall survival and Disease-free survival. (G) Analysis of CD73 expression in different pathological stages of pancreatic cancer using GEPIA2.

5.2 Expression of CD73 after CRISPR/Cas9-Mediated Knock-Out in TB32047 cell and PANC1 cell

To verify the role of CD73 in pancreatic cancer cell lines, one human cell line (PANC1) and a murine cell line (TB32047) were used to perform a CRISPR/Cas9-based knock-out of CD73. Two sgRNAs targeting different regions of human and murine CD73 exons, respectively, were designed, and CRISPR/Cas9 gene editing was performed to introduce mutations into this region. We verified the knockout of CD73 at the protein level by western blot (Figure 5A). The results showed no CD73 protein level expression in the monoclonal group.The transcription of CD73 mRNAs was inhibited, and these were verified via qRT-PCR (Figure 5B). The expression of CD73 mRNAs in single clones of TB32047 and PANC1 was significantly down-regulated compared to controls.Expression of CD73 on the cell membrane surface verified by flow cytometry (Figure 5C). CD73 knockout resulted in essentially no expression on the cell membrane surface. The mutations of genomic DNA in single clones of both TB32046 cells and PANC1 cells were confirmed via DNA sequencing (Table 8).

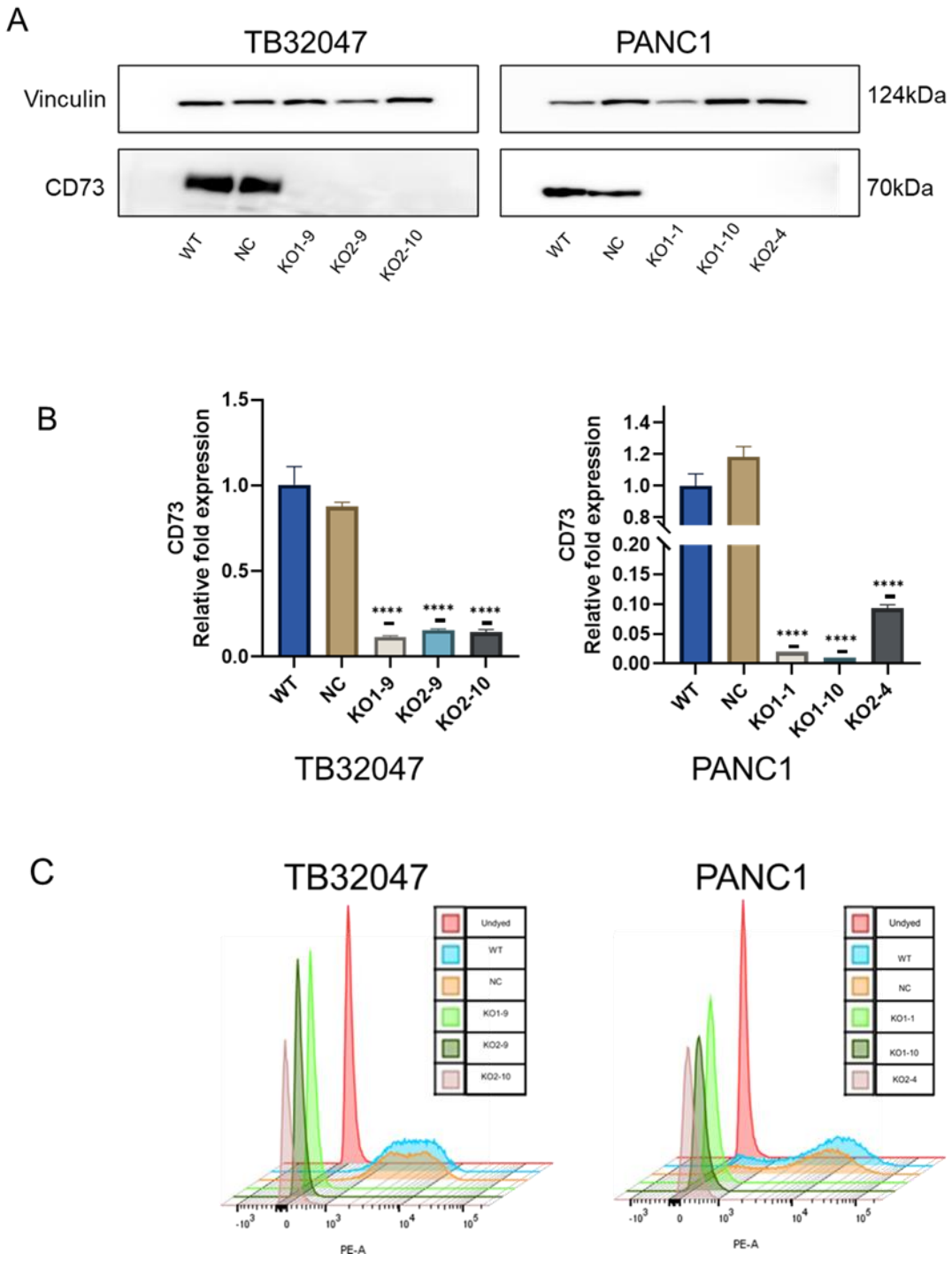


Figure 5. CD73 expression by wildtype cells, negative controls and clones in TB32047 and PANC1 cell lines.(A)Western blot validation of CD73 knockout at the protein level. (B) Expression of CD73 mRNAs in monoclonal cells and control cells(WT); ****P < 0.0001. (C) Stagger offset showing the expression level of CD73 on monoclonal cells and control cells by flow cytometry.

Table 8: Sequencing confirmation of mutations in TB32047 and PANC1 CD73-

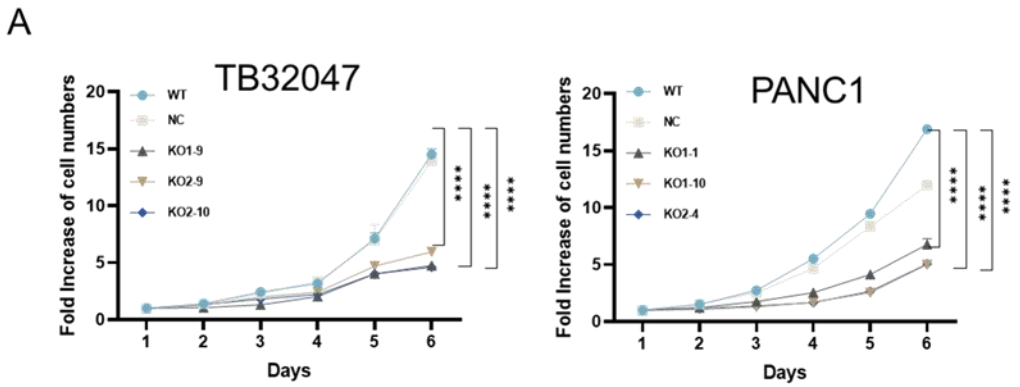
knockout single clones.

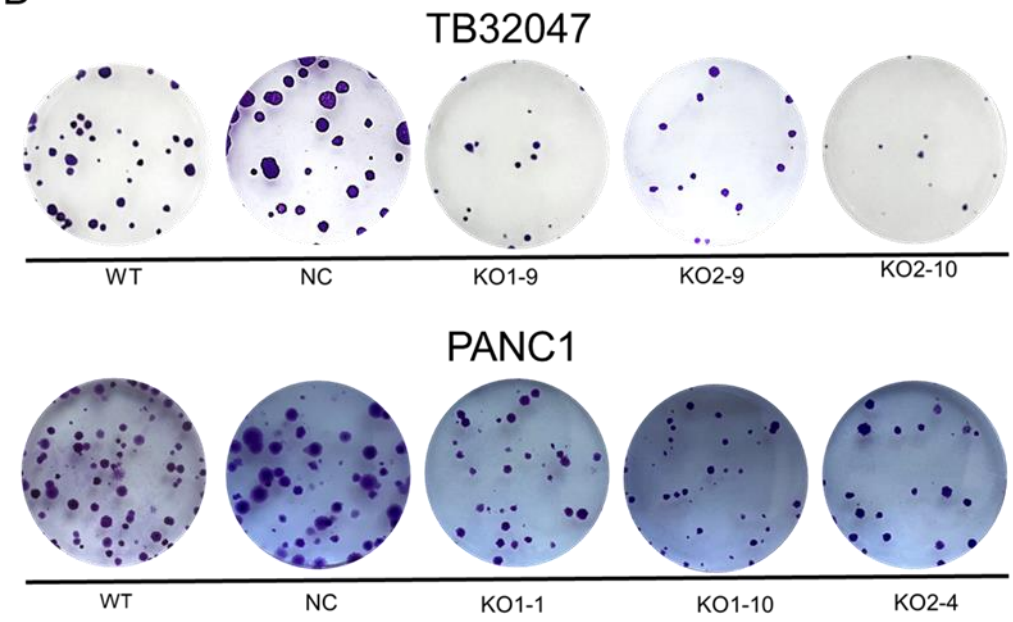
TB32047 Clones	Indel Size	PANC1 Clones	Indel Size
1-9	Delete 1bp	1-1	Delete 5 bp
1-10	Delete 2bp/ Delete 26 bp	1-10	Insert 265bp
2-10	Insert 1bp/ Delete 92bp	2-4	Delete 11 bp

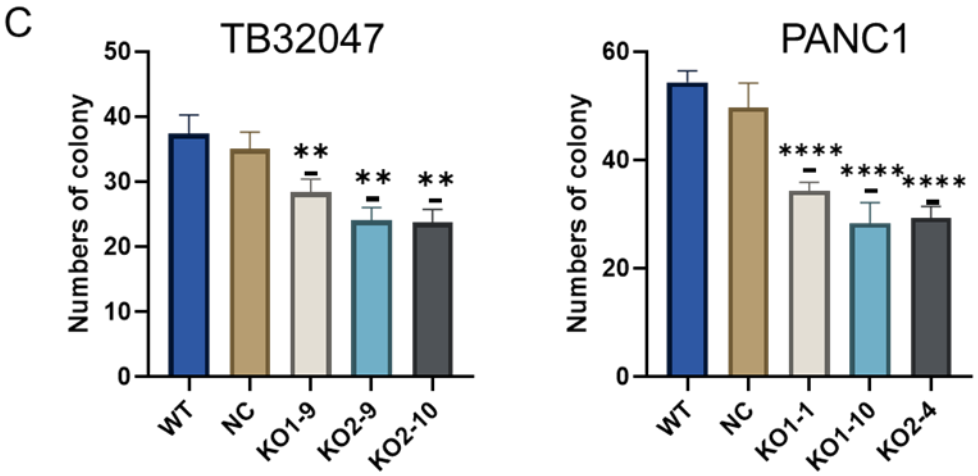
5.3 CD73 knockout inhibits cell proliferation and induces G1 phase arrest, no affect on apoptosis

TB32047 (WT, NC, KO1-9, KO2-9, KO2-10) and PANC1 (WT, NC, KO1-1 KO1-10, KO2-4) cells were seeded 1000 cells per well in black 96-wells and incubated for 6 days. All cells exhibited robust logarithmic growth under these conditions until fully confluent. After the loss of CD73 in TB32047 and PANC1 cells, cell growth was significantly inhibited compared with the wild-type cells (Figure 6A). Clonogenic assays showed a dramatic decrease in the colony number of TB32047 and PANC1 KOs after knockout CD73 compared to WT and NC cells (Figure 6B-C).

Cell cycle detection by Flow cytometry found that knockout of CD73 induced G1 phase arrest (Figure 6D). For the TB32047 cell line, the G1 phase of KO1-9, KO2-9 and KO2-10 cells accounted for 73.0%,72.8% and 75.9% of the entire cell cycle, respectively. At the same time, WT and NC cells' G1 phase is 61.9% and 48.9%. For the PANC1 cell line, the G1 phase of KO1-1, KO1-10, and KO2-4 cells accounted for 79.1%,71.4% and 74.5% of the entire cell cycle, respectively, WT and NC cells' G1 phase is 49.5% and 64.1% (Figure 6E). We also detected the effect on apoptosis after knockout CD73, and the results showed no significant change between the knockout group and the control group (Figure 6F).


A
TB32047
PANC1
Fold Increase of cell numbers
Days
WT
NC
KO1-9
KO2-9
KO2-10
KO1-1
KO1-10
KO2-4
****


B
TB32047
WT
NC
KO1-9
KO2-9
KO2-10
PANC1
WT
NC
KO1-1
KO1-10
KO2-4


C
TB32047
PANC1
Numbers of colony
WT
NC
KO1-9
KO2-9
KO2-10
KO1-1
KO1-10
KO2-4
**
****

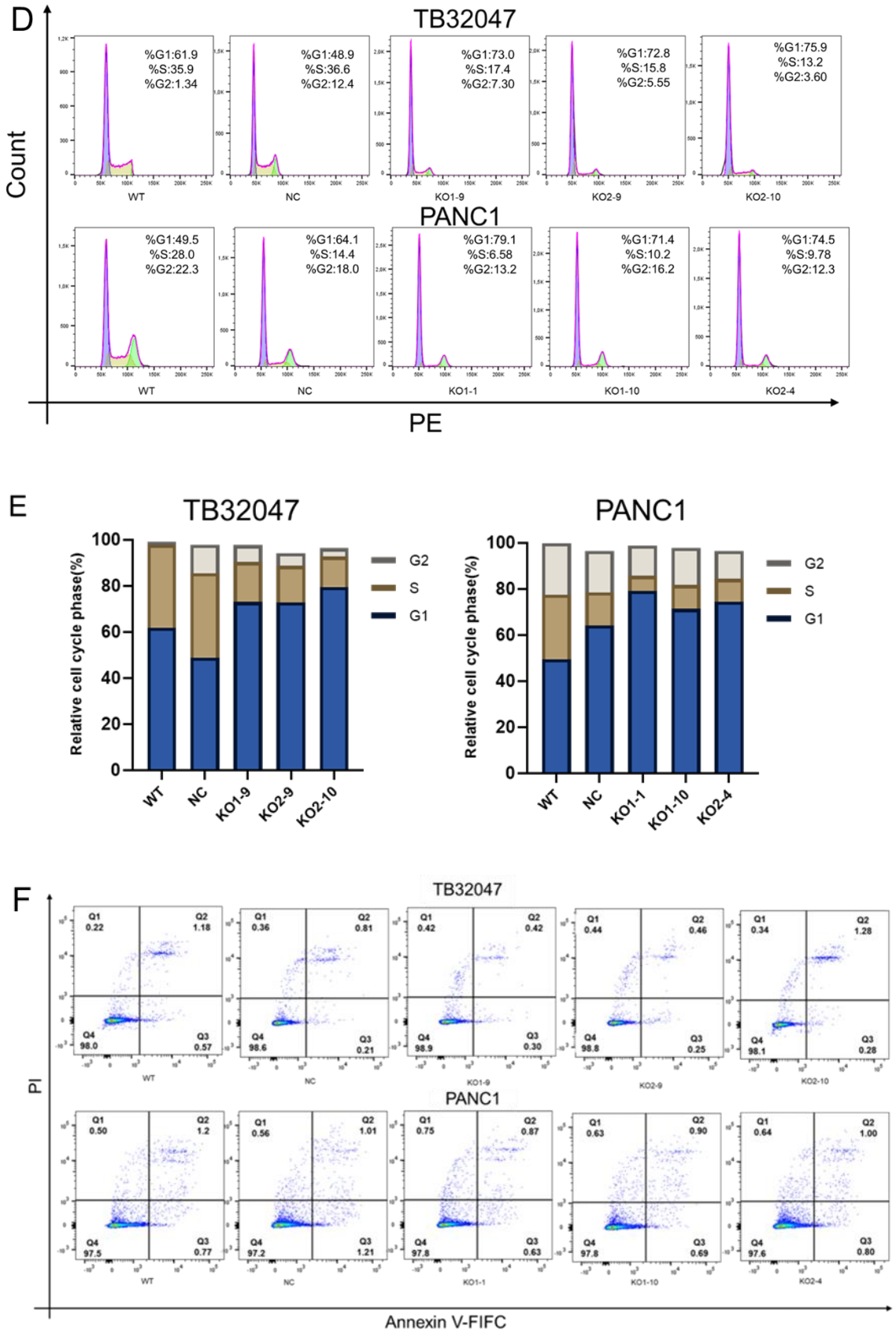
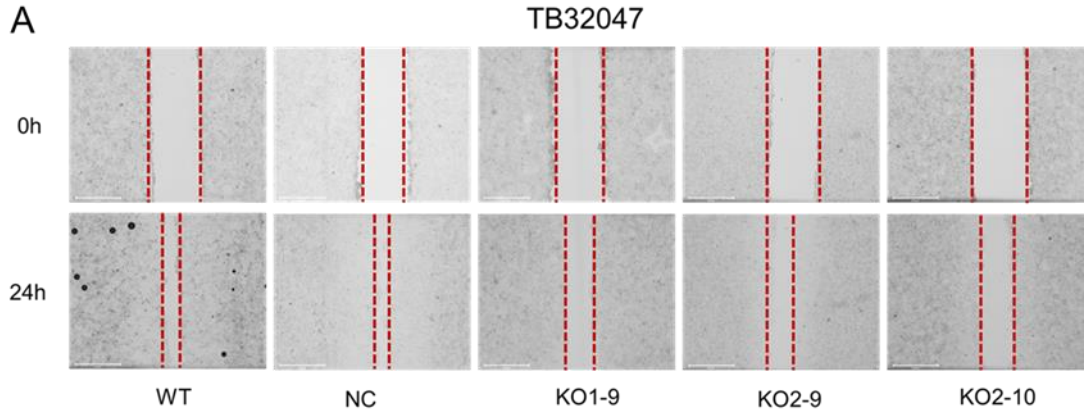


Figure 6. Loss of CD73 inhibited cell growth and blocked the G1 phase of the

cell cycle in pancreatic cancer. (A) Effects of CD73 knockout on the growth of pancreatic cancer cells in vitro. Cell proliferation assays were performed for 6 days. Data are expressed as mean ± SEM of 3 experiments. (B,C) The colony formation assay of TB 32047 and PANC1 cells was performed after knockout CD73. The colony numbers were counted, and data were presented as means of three independent experiments(n= 3), *p < 0.05, **p <0.01. (D) Cell cycle analysis of TB32047 and PANC1 cells. Representative figures from three independent experiments (n=3) are shown. (E) Histogram of the percentage of G1, S and G2 phases in each cell. (F) Apoptosis assay using flow cytometry after staining with annexin V-APC/propidium iodide (PI). Representative scatter plots of PI (y-axis) vs. annexin V (x-axis).

5.4 CD73 knockout inhibits cell migration in vitro

By wound healing assay, we showed that the knockouts of CD73 significantly inhibit the motility of PDAC cells (Figure 7A-B). For the TB32047 cell line, KO1-9, KO2-9 and KO2-10 recovered the wound at 41.98%, 59.95% and 55.18% after 24 h, while WT and NC cells healed more than 75 % of the wound area (Figure 7C). For the PANC1 cell line, KO1-1, KO1-10, and KO2-4 recovered the wound at 37.77%, 33.36% and 32.18% after 24 h, but the control cells healed more than 75 % of the wound area (Figure 7D).



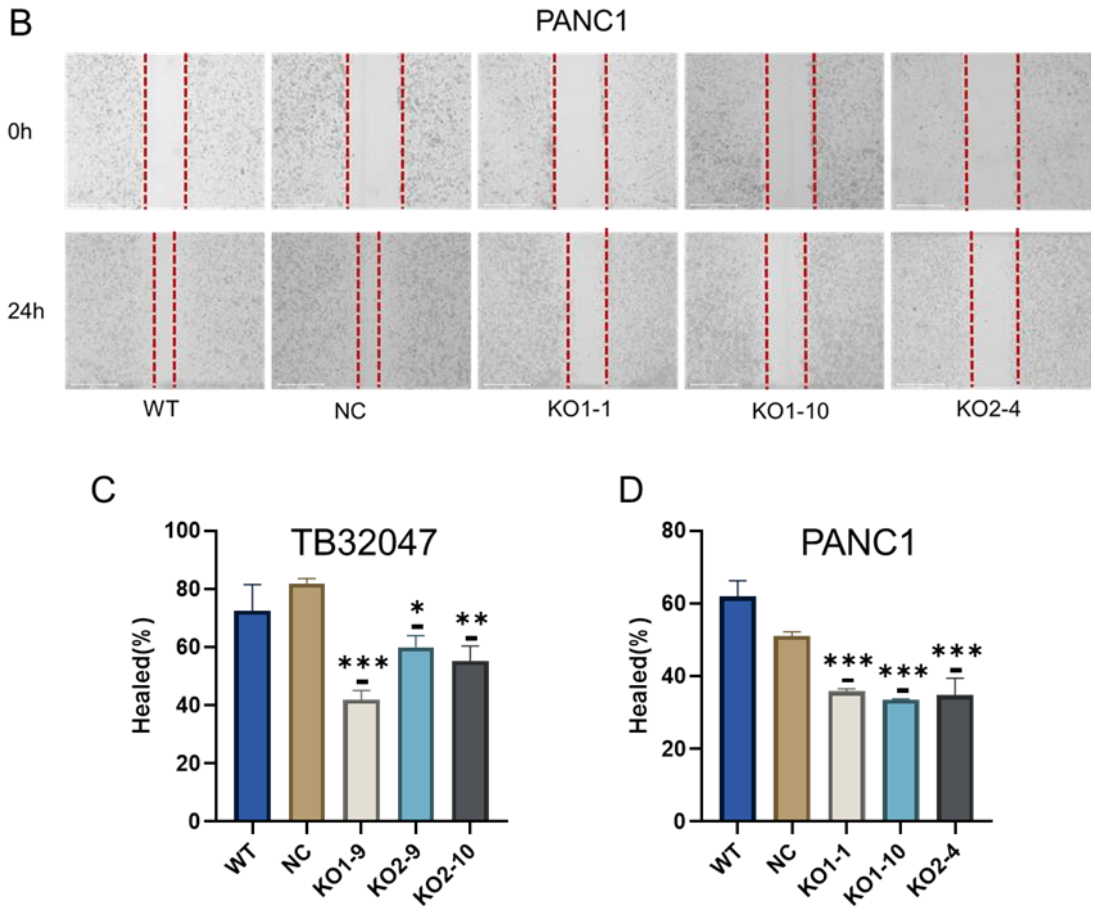
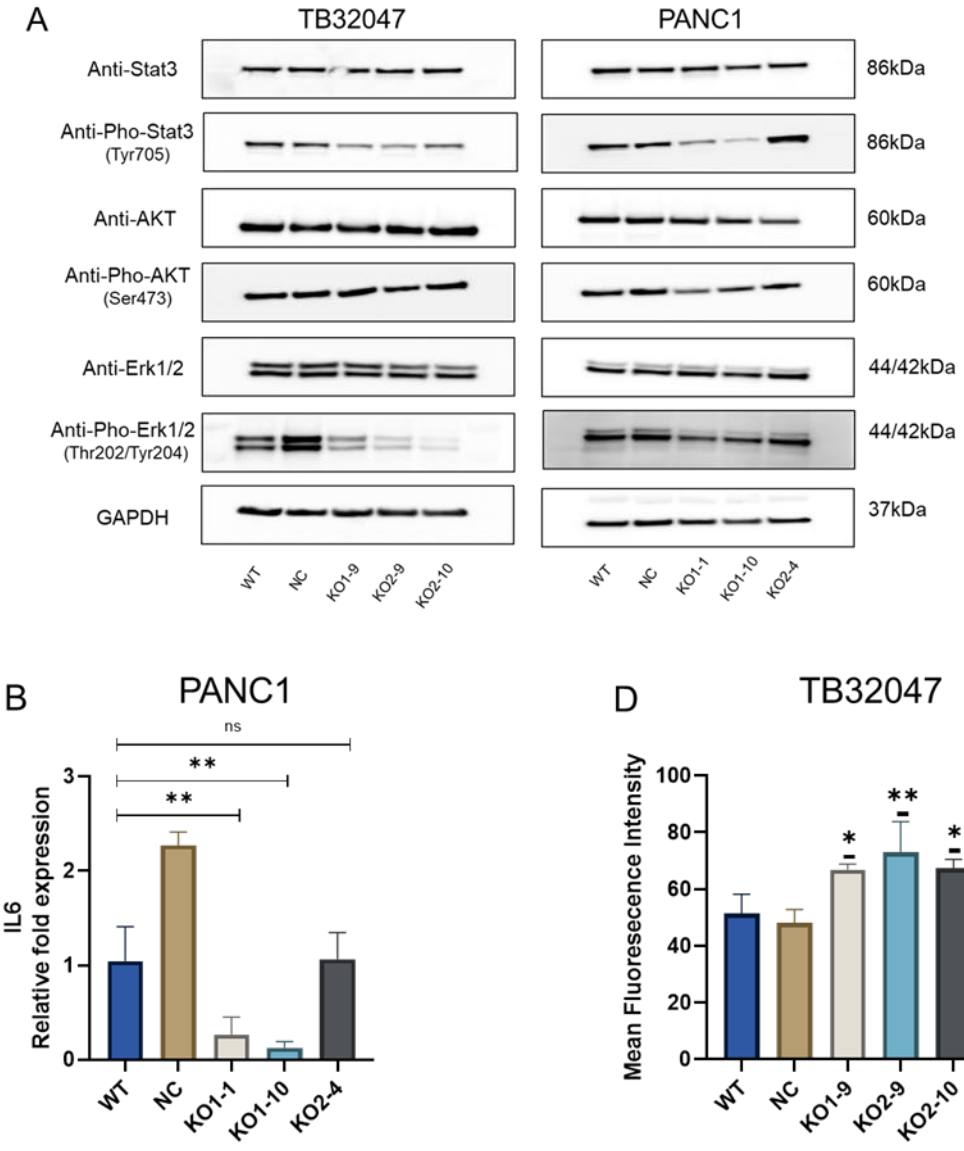


Figure 7. CD73 knockout inhibits pancreatic cancer cell migration. (A, B) Representative images show the area covered by the TB32047 and PANC1 cells at 0h and 24 h after wounding. (C, D) % Cell migration was determined by the rate of cells moving towards the scratched area upon the time using ImageJ ™ software (* P < 0.05 ,** P < 0.01 , *** P < 0.001* **** P < 0.0001).

5.5 CD73 deficiency inhibits the phosphorylation of ERK and Stat3 in pancreatic cancer, promotion of E-cadherin expression in TB32047 cells

After the knockout of CD73 in TB32047 and PANC1 cells, we observed a significant reduction in phosphorylation of ERK (intracellular mononuclear regulatory kinase). Phosphorylation of Stat3 (signal transducer and activator of transcription3) was also significantly reduced in TB32047 cells, while two monoclonal clones (KO1-1, KO1-10) in PANC1 showed low expression.

However, no significant change in Akt was observed (Figure 8A). In addition, positive feedback of CD73 with IL-6 (interleukin 6) has been reported in human breast cancer[61]. Therefore, we verified in PANC1(KO1-1,ko1-10) cells that knockdown of CD73 reduced Pho-Stat3 expression and simultaneously affected IL-6 expression(Figure 8B). We also found that CD73 knockout prompted increased E-cadherin expression in TB32047 cells as detected by immunofluorescence (Figure 8C-D). In contrast, no significant changes were observed in PANC1 cells (Figure 8E).



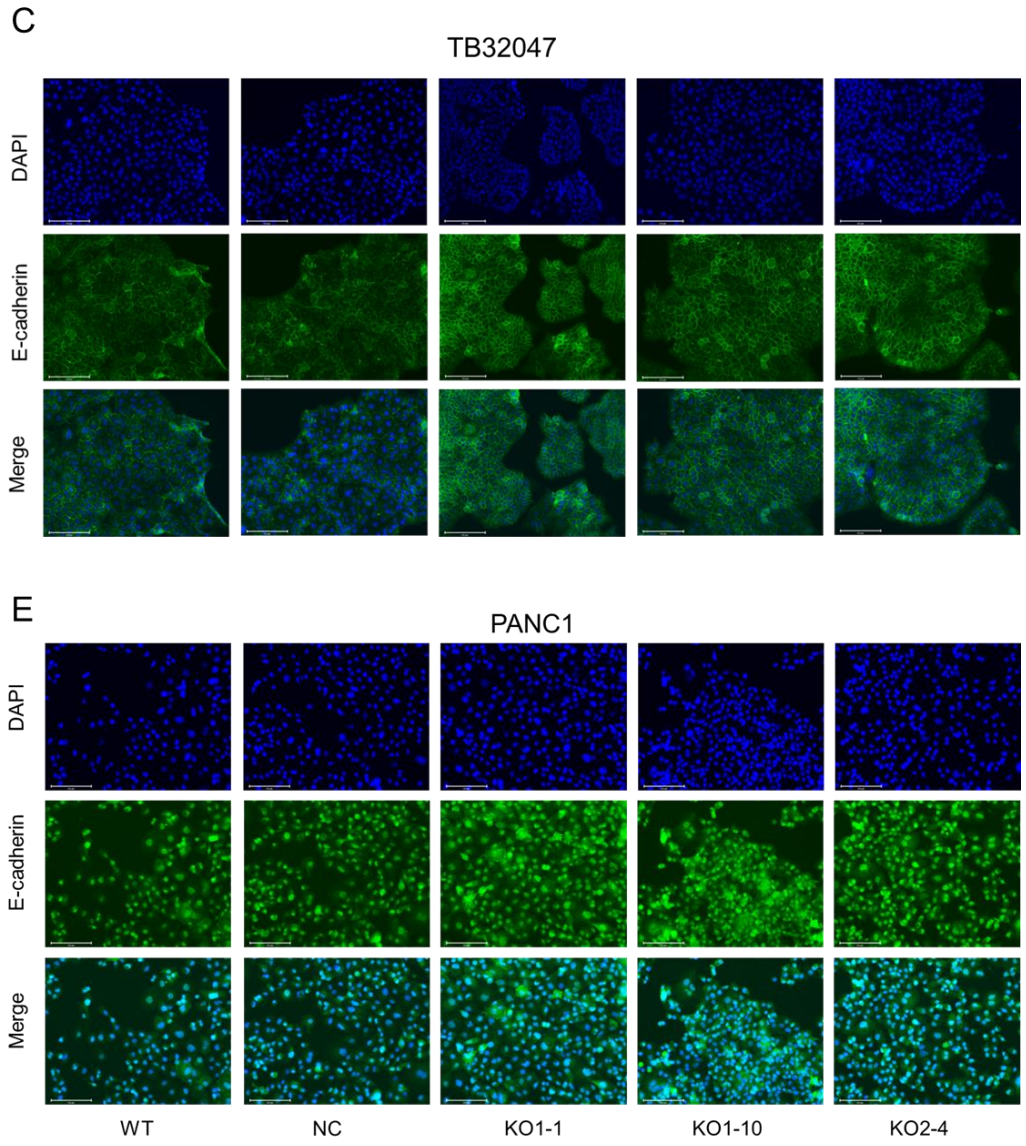


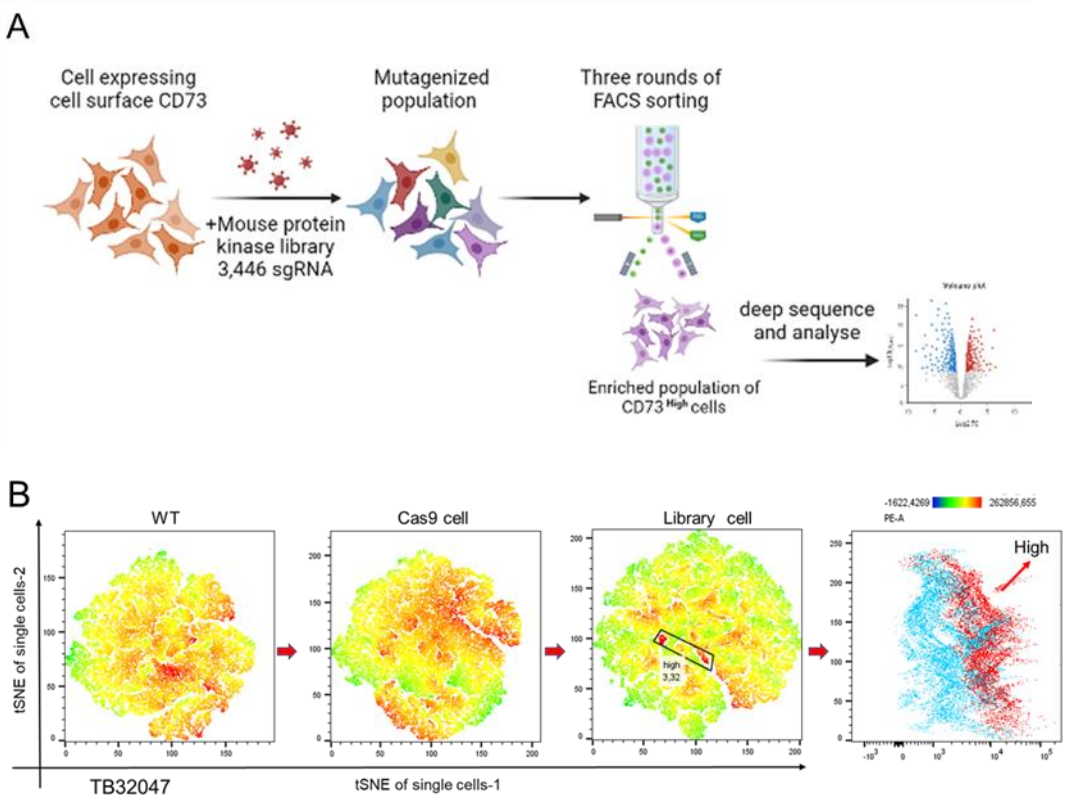
Figure 8. Knockout of CD73 inhibits phosphorylation of Erk and Stat3 and promotes E-cadherin expression. (A) Phosphorylation of Erk and Stat3 was downregulated in TB32047 and PANC1 cells with CD73 knockout. (B) IL-6 expression was decreased in PANC1(KO1-1, KO1-10) cells. (C) Immunofluorescence staining of combined E-cadherin (green), DAPI (blue) and TB32047-KOs and controls. Enhanced expression of monoclonal E-cadherin. (D) Mean immunofluorescence intensity in TB-KOs and controls. Bars represent the mean values and the corresponding standard error of the mean (SEM) of $n = 3$ independent experiments. Significance: $^{*}p < 0.05$, $^{**}p < 0.01$.

(E) Immunofluorescence staining of combined E-cadherin (green), DAPI (blue) and PANC1-KOs and controls. Enhanced expression of monoclonal E-cadherin.

5.6 Protein kinase CRISPR/Cas9 screen of inducible regulators of CD73 expression in pancreatic cancer cells

To identify genes that regulate CD73 expression, we used CRISPR-Cas9 loss-of-function screening combined with FACS technology to establish a screening model in the mouse pancreatic cancer cell line TB32047 (Figure 9A).

We used protein kinase-based screens to find which gene can regulate CD73 expression. 3446 sgRNAs targeting 915 genes were introduced into TB 32047-Cas9 cells at a multiplicity of infection (MOI) of 0.3. Then, the transfected cells were screened for high or low CD73 expression (Figure 9B). As a negative control, the sgRNAs targeting CD73 are present in our protein kinase library. After the first round of sorting, we continued to culture the cells to guarantee enough read-death and coverage for the next round of screening. After three rounds of enrichment, we identified the relevant sgRNAs in the CD73 high- or low-expression group through deep sequencing (Figure 9C).



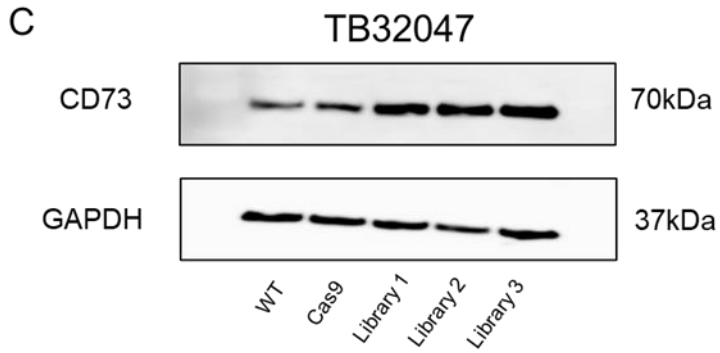


Figure 9. Schematic diagram of flow cytometry sorting and result. (A) Schematic diagram of the timeline and experimental procedure for CRISPR-Cas9 screening using protein kinase library. (B) Schematic diagram of the first cell sorting, by dimensionality reduction analysis. FACS sorted cells, and after cell growth, FACS was repeated for 2 more rounds to extract DNA for deep sequencing. (C) Western blot was performed to verify the protein expression level of CD73 after 3 binning.

PinAPL-Py analyzed the CD73 high express group deep sequencing results. We found 7 sgRNAs (Pbk, Fastk, Cdk19, Adck5, Trim28, and Pfkp) significantly increased in the CD73 high expression group compared to untreated cells(Figure 10A). Among them, Adck5, Trim28 and Cdk19 sgRNA enrichment were relatively high(Figure 10B). It indicates that these sgRNA-targeted genes may be potential regulators of CD73 to increase its expression. In addition, TCGA (PAAD) and GTEx (PAAD) databases obtained from GEPIA2 showed that these genes were expressed higher in pancreatic cancer tumor tissues than in normal tissues, except for Adck5(Figure 10C) To further verify whether these genes are somehow linked to CD73, we examined the expression of the above genes in TB32047 cells after the knockout of CD73. The results showed that the expression of Pbk, Pfkp and Trim28 changed with the knockout of CD73, where the expression of Pbk increased, and Pfkp and Trim28 decreased(Figure 10D).

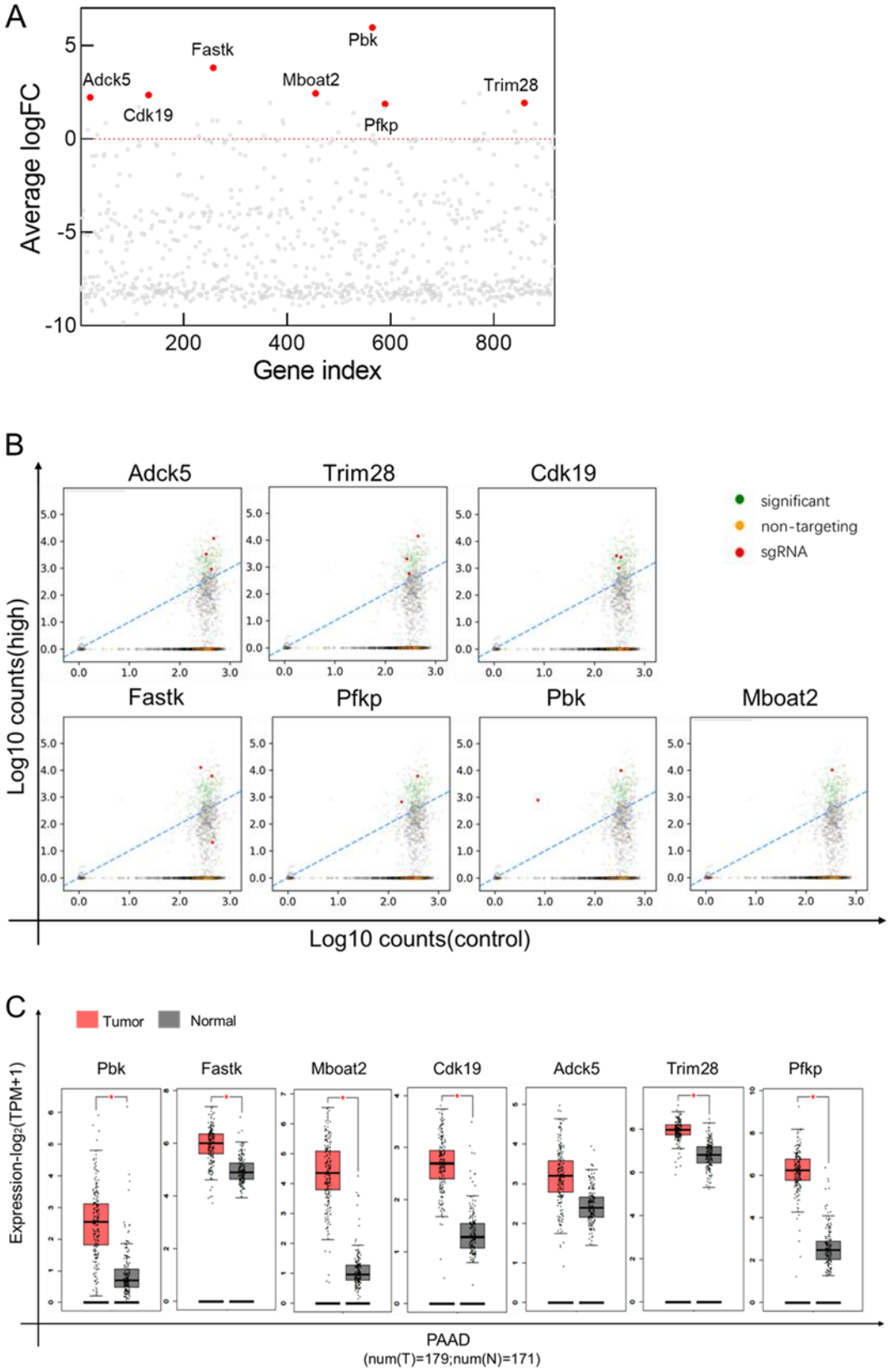


Figure.8 Analysis of the deep sequence results of the CD73 high expression group. (A) The graph shows the enrichment of candidate genes (red dots) in

the CD73 high-expression group identified by Pin-APL versus other genes in the library (gray dots) after selection. (B) PinAPL-Py analysis showed sgRNA enrichment maps for each highly expressed gene. (C) The TCGA (PAAD) and GTEx (PAAD) databases obtained from GEPIA2 showed that these genes were differentially expressed in pancreatic cancer tumor tissue and normal tissue.

Table 9: The deep sequence results show that regulators of CD73 cell surface expression

CD73 high expression group of gene					
gene	**AvgLogFC**	**p_value**	**significant**	**sgRNAs**	**signif. sgRNAs**
Pbk	5.96225	0	True	2	2
Fastk	3.812636	1E-04	True	3	2
Mboat2	2.437256	0.0009	True	2	1
Snai2	2.425797	0.0009	True	2	0
Cdk19	2.350225	0.0011	True	4	3
Adck5	2.2216	0.0012	True	4	3
Scamp5	1.934172	0.0021	True	2	0
Trim28	1.921243	0.0021	True	4	2
Msln	1.903604	0.0021	True	2	0
Pfkp	1.868927	0.0022	True	3	2

6. DISCUSSION

Although some patients with early-stage pancreatic cancer can undergo radical resection, most patients with advanced disease have already lost their chance for surgery.[62,63]Immune checkpoint therapies for pancreatic cancer have received increasing attention in recent years. Many of these immune checkpoints are CD molecules.[64,65,66] These CD molecules are involved in cancer development and play an essential role in the treatment and prognosis of cancer. Flow cytometry tested the expression of various CD molecules in pancreatic cancer cells in this study. We determined that the relative expression of CD73 was high in TB32047 and PANC1 cell membranes. And further validated by qRT-pcr to obtain the qRT-PCR results. And we clarified that CD73 was significantly differentially expressed in pancreatic cancer tissues and normal pancreatic tissues by GEPIA analysis. However, high expression of CD73 in cancer tends to promote tumor progression.[67]Therefore, targeted blockade of CD73 may be a beneficial therapeutic approach for treating cancer patients in the future.

The detailed function of CD73 in pancreatic tumor cells and the genes affecting its expression remain to be determined. In this study, CRISPR/Cas9 gene editing technology was applied to inactivate CD73. The exons of CD73 were edited at the indicated positions by the specific binding of sgRNA-guided Cas9 nuclease and genomic DNA; it's been confirmed by genomic DNA sequencing. The alteration of base sequences between exons decreased mRNA transcription efficiency, and RT-PCR confirmed that CD73 mRNA expression was significantly repressed. Western Blot verified the knockout of CD73 at the protein level. Inhibition of CD73 at the cell membrane surface was verified by flow cytometry.

We found that entirely knockout CD73 significantly inhibited cell proliferation in TB32047 and PANC1 cells, and cell cycle G1 was blocked. This result is consistent with the findings of Zhou, L. et al.[68] However, we did not

observe any effect on apoptosis after the knockout of CD73. We also found that the knockout of CD73 decreased cell migration ability. And these phenotypic changes may be induced through Erk, Stat3, and E-cadherin signaling pathways.

Extracellular signal-regulated kinases (ERK) are widely expressed protein kinase intracellular signaling molecules involved in functions including cell proliferation, cell growth, cell metabolism and cell migration.[69,70] Knockout of CD73 in pancreatic cancer significantly inhibited ERK expression. Some reports have investigated the induction of G1 phase arrest via AKT/ERK/cyclin D signaling pathway after CD73 knockdown.[68] However, our study found no significant changes in AKT.

STAT3 (Signal Transducer And Activator Of Transcription 3) is a protein-coding gene member of the STAT protein family.[71] Stat3 is highly expressed in many different types of cancers.[72] It is highly expressed in breast cancer and promotes cancer proliferation and migration.[73] The key role of STAT3 in promoting the progression of pancreatic cancer is well established, but how to inhibit STAT3 activity remains to be investigated.[74] We found reduced Stat3 expression in B32047 monoclonal cells as well as in PANC1 cells KO1-1 and KO1-10 cells. This suggests that CD73 may be one of the proteins that inhibit Stat3 activity in pancreatic cancer. We also found that PANC1 cells, KO1-1 and KO1-10, inhibited the expression of Interleukin 6(IL-6). The IL-6/JAK/STAT3 pathway is aberrantly over-activated in many types of cancer and drives tumor cell proliferation, survival, invasion and metastasis while strongly suppressing anti-tumor immune responses.[75]Therefore, we suspect that in pancreatic cancer, knockdown of CD73 may lead to inhibition of the IL-6/JAK/STAT3 pathway and thus inhibit tumor cell growth. This result was partially validated in the PANC1 cell line.

E-cadherin is a protein that in humans, is encoded by the CDH1 gene.[76] It is a tumor suppressor gene.[77] E-cadherin has been studied more in pancreatic

cancer and is mainly associated with the invasion and metastasis of pancreatic cancer cells.[78,79]Targeting the expression of E-cadherin with small molecule drugs is a new way to treat GI cancers.[80] While the genes regulating E-cadherin expression in pancreatic cancer are still unclear.We found that the expression of E-cadherin was significantly increased in TB32047 cells when we knocked down CD73. We found that the expression of E-cadherin was significantly increased in TB32047 cells when we knockout CD73. This suggests that the high expression of CD73 in pancreatic cancer may inhibit the E-cadherin pathway and thus promote tumor migration.

We identified candidate genes regulating CD73 using a sgRNA library focused on kinase and PDAC-related genes in the KPC mouse model-derived cell line TB32047 and a systematic approach of reverse selection using CRISPR-Cas9. PinAPL-Py analyzed the CD73 high express group deep sequencing results. We found the sgRNAs of seven genes (Pbk, Fastk, Cdk19, Adck5, Trim28, and Pfkp) significantly increased in the CD73 high expression group compared to untreated cells

Pbk (PDZ Binding Kinase) is a Protein Coding gene.[81,82] Pbk has been shown to be an essential gene in the regulation of mitosis and tumorigenesis, but the role of PBK in various cancers remains unclear.[83]Several studies have shown that Pbk expression is upregulated in tumor tissues such as breast cancer and lung cancer, critical factors in tumor tissue proliferation, invasion and metastasis, and high expression of Pbk often leads to poor prognosis for cancer patients.[84-86] However, its research in pancreatic cancer is currently uncommon.

Fas-activated serine/threonine kinase is an enzyme that in humans is encoded by the FASTK gene.[87]Kras mutations occur in 95% of pancreatic cancer patients, and c-Myc is one of the major effector molecules of the Kras signaling pathway. It has been reported that FASTKD2 promotes cancer cell progression by upregulating Myc expression in pancreatic cancer.[88]

CDK19 (Cyclin Dependent Kinase 19) is a Protein Coding gene. It is involved in the development and progression of many types of cancer, such as stomach, prostate, breast, and liver cancers.[89-92] CDK19 was associated with increased aggressiveness and shorter disease-free survival in primary prostate cancer.[93] However, the specific role of CDK19 in pancreatic cancer is not yet clear.

ADCK5 belongs to the protein kinase superfamily. The function of this protein is not yet clear. It has been shown that ADCK5 regulates lung cancer cell invasion and migration. It regulates the expression of the oncogene human pituitary tumor transforming gene-1 (PTTG1) by phosphorylating the transcription factor SOX9, which enhances the migration and invasion of lung cancer cells.[94]

Trim28(Tripartite motif-containing 28) is a protein that in humans, is encoded by the TRIM28 gene.[95] The complex nature of the TRIM28 protein was determined through extensive studies of TRIM28 in cellular biology.
parties.[96,97] Trim28 has also been increasingly studied in cancer. upregulation of Trim28 expression in gastric cancer leads to poor patient prognosis; increased expression in ovarian cancer promotes tumor migration; and overexpression in liver cancer leads to reduced 5-year survival of patients; High expression in breast cancer leads to enhanced tumor
aggressiveness.[98-101] Other studies have shown that high expression of Trim28 in early-stage lung cancer tends to be accompanied by better survival cycles.[102] In summary, the role of Trim28 in different cancers is complex and variable. The role of Trim28 in pancreatic cancer is currently unknown, and it can only be determined that high levels of TRIM28 are associated with significantly lower survival rates.[103]

Pfkp is an enzyme that in humans, is encoded by the PFKP gene. Pfkp plays a critical role in many steps of cancer initiation and metastasis.[104] One study reported that in lung cancer, low expression of Pfkp significantly reduced

the proliferation of lung cancer cells.[105] Pfkp has been studied relatively little in pancreatic cancer. It has been reported that hyperglycemia promotes glycolysis in pancreatic cancer, and PFKP expression is significantly upregulated under hyperglycemic conditions. This suggests that Pfkp may be involved in glycolysis in pancreatic cancer.[106]

In summary, we identified candidate genes that regulate CD73 using sgRNA libraries focused on kinase and PDAC-related genes in the KPC mouse model-derived cell line TB32047 and a systematic approach of reverse selection using CRISPR-Cas9. Although we need more studies to confirm the precise regulation of CD73, the list of post-selected genes obtained so far provides a good entry point.

In this study, we performed multiple screens with flow cytometry and selected the top 3% of high CD73-expressing cells in each screen. By analyzing the deep sequencing results, we found that since our sgRNA library contained CD73 sgRNA itself, under strict screening conditions, CD73 sgRNA itself was overrepresented in the low expression group of enriched sgRNAs, thus affecting the reads of other possible sgRNAs. Therefore, we suggest that the screening condition be appropriately relaxed to the top 10%. In addition, CRISPR/Cas9, as a gene editing tool, has considerable off-target effects that can affect experimental results. When sgRNA (a short-stranded RNA that matches a target DNA fragment) directs CRISPR/Cas9 to edit the genome, it promotes undesired off-target mutagenesis because sgRNA can tolerate certain mismatches with DNA targets. Several studies have shown that the off-target activity of Cas9 depends on the sgRNA sequence and experimental conditions.[107-109] Therefore, as much as possible, we need to perform multiple transfection sequencing to rule out false positive results due to off-target effects.

Recent studies have shown that genetic and behavioral characteristics vary significantly between cell lines of different origins and subtypes, even within pancreatic cancer cell lines.[110] We also observed that the pathways

affecting CD73 are not identical in murine and human lineage pancreatic cancer cells, so it should be prudent to continue crossover or combinatorial screening between different cell lines and different species in the following experiments to find the critical targets for regulating CD73. Finally, tumorigenesis and progression arise in a relatively perfect internal environment. We next need further studies in vivo to get a more precise picture of the genes that affect CD73 regulation.

7. CONCLUSION

We validate that CD73 expression is increased in pancreatic cancer and that knockout of CD73 inhibits cell proliferation and migration and blocks the G1 phase of the cell cycle. We also found that the deletion of CD73 inhibited the ERK/STAT3 pathway and activated the E-cadherin pathway. Loss of Pbk, Fastk, Cdk19, Adck5, Trim28, or Pfkp might be genes regulating CD73 in pancreatic cancer. Although we need more studies to confirm the precise regulation of CD73, the list of post-selected genes obtained so far provides a good entry point.

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