Impact of fibroblastic FGFBP2 mutation on pancreatic carcinogenesis

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Referat

Bauchspeicheldrüsenkrebs ist eine der aggressivsten Formen bösartiger Erkrankungen mit einer extrem schlechten Prognose. Die Mikroumgebung von Tumoren ist ein komplexes zelluläres Netzwerk, in dem krebsassoziierte Fibroblasten (CAFs) eine zentrale Rolle bei der Auslösung und dem Fortschreiten der Krankheit spielen. In dieser Studie wird die Rolle des Fibroblasten-Wachstumsfaktor-Bindeproteins 2 (FGFBP2) und seiner Mutation in Fibroblasten bei Bauchspeicheldrüsenkrebs untersucht. Die Analyse der Expression von Aktivierungsmarkern und Zytokinen ergab, dass die FGFBP2-Mutation die Fibroblastenaktivierung fördert. Außerdem zeigte die Untersuchung der mutierten Fibroblasten, dass sie eine erhöhte Proliferation und Migration aufweisen. Behandlung mit konditioniertem Medium (CM) aus mutierten Fibroblasten konnte das Wachstum der Pankreaskarzinom Zelllinie PANC-1 steigern. Die RNA-Sequenzierungsanalyse (RNA-Seq) von PANC-1-Zellen, die mit konditioniertem Medium aus mutierten Fibroblasten behandelt wurden, ergab u.a. eine Hochregulierung des Gens ETS homologous factor (EHF) und die Förderung der Zellzyklusprogression. Die Analyse der Einzelzell-RNA-Sequenzierung (scRNA-seq) zeigte, dass EHF vor allem in duktalen Zellen exprimiert ist und die Fibroblasten über den PDGF-Signalweg (Platelet-Derived Growth Factor) reguliert. Die Expression von FGF2 ist in mutierten Fibroblasten vermindert. Darüber hinaus verstärkt die Behandlung von PANC-1-Zellen mit CM oder CM, das mit rekombinantem humanem Fibroblasten-Wachstumsfaktor 2 (rhFGF2) ergänzt wurde, die Aktivierung des kanonischen ERK1/2-Signalwegs durch Förderung der Phosphorylierung. Die Phosphorylierung in konditioniertem Medium aus FGFBP2-mutierten Fibroblasten führt zu einer erhöhten Aktivierung des ERK1/2-Signalwegs im Vergleich zum Wildtyp. Zusammenfassend zeigt unsere Studie das FGFBP2-Punktmutation eine Fibroblastenaktivierung, und verstärkte Tumorprogression zum größten Teil über ERK1/2-Signalweg in Pankreaskarzinomzellen fördert.

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Abstract

Pancreatic cancer (PDAC) is one of the most aggressive forms of malignant diseases, with an extremely poor prognosis. The tumor microenvironment is a complex cellular network, with cancer-associated fibroblasts (CAFs) playing a pivotal role in disease initiation and progression. Somatic CAF mutations identified by our group may have functional consequences and affect PDAC. One of them is replacement of guanine (G) to cytidine (C) in Fibroblast Growth Factor Binding Protein 2 (FGFBP2) causing an amino acid change from threonine to serine. Fibroblast Growth Factors (FGFs), a family of more than 20 proteins, are involved in tumor growth, progression and angiogenesis. The central question of my thesis is to investigate how this somatic mutation in FGFBP2 affects fibroblast functions in the pancreatic cancer microenvironment and how these interactions contribute to tumor progression. The analysis of the expression of activation markers and cytokines revealed that the FGFBP2 mutation promotes fibroblast activation. Furthermore, the investigation of mutant fibroblasts showed that they exhibit increased fibroblast proliferation and migration. Moreover, conditioned medium (CM) derived from mutant fibroblasts promoted the growth of pancreatic cancer cells. RNA sequencing (RNA-Seq) analysis of PANC-1 cells treated with conditioned media derived from mutant fibroblasts revealed the upregulation of the ETS homologous factor (EHF) gene and the promotion of cell cycle progression. Single-cell RNA sequencing (scRNA-seq) analysis showed that EHF is primarily expressed in tumor cells, and it regulated fibroblasts through the platelet-derived growth factor (PDGF) signaling pathway. The expression of FGF2 is decreased in mutant fibroblasts. Additionally, treatment of PANC-1 cells with CM or CM supplemented with recombinant human fibroblast growth factor 2 (rhFGF2) enhanced the activation of the canonical ERK1/2 signaling pathway by promoting phosphorylation. Phosphorylation in conditioned medium derived from FGFBP2 mutant fibroblasts resulted in increased activation of the ERK1/2 signaling pathway as compared to wildtype. In conclusion, our study shows that FGFBP2 point mutation promotes fibroblast activation which trigger the enhancement of the tumor progression, partly by activating the ERK1/2 signaling pathway in pancreatic cancer cells.

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Abbreviations

AKT	Serine/Threonine Kinase
ANXA1	Annexin A1
apCAFs	antigen-presenting CAFs
APS	Ammonium Persulfate
α-SMA	Alpha-Smooth Muscle Actin
CCR7	C-C motif chemokine receptor 7
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A
cDNA	Complementary DNA
CDX1	Caudal type homeobox 1
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CHAPS	3-Cholamidopropyldimethylammonio-1-propanesulfonate
CM	Conditioned medium
CNV	Copy Number Variation
COL1A1	Collagen type I alpha 1 chain
COL11A1	Collagen type XI alpha 1 chain
CXCR2	C-X-C Motif Chemokine Receptor 2
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
ECM	Extracellular matrix
EGFR	Epidermal Growth Factor Receptor
EHF	ETS Homologous Factor
EMT	Epithelial-mesenchymal transition
EPA	Eicosatetraenoic acid
EREG	Epiregulin
ERK1/2	Extracellular signal-regulated kinase 1/2
PDGF	Platelet-derived growth factor

ETS1	ETS Proto-Oncogene 1, Transcription Factor
FAP	Fibroblast Activation Protein
FBS	Fetal Bovine Serum
FDR	False Discovery Rate
FGF2	Fibroblast Growth Factor 2
FGFBP2	Fibroblast Growth Factor Binding Protein 2
FGFRs	Fibroblast Growth Factor Receptors
FOLFIRINOX	fluorouracil, leucovorin, irinotecan, and oxaliplatin
FOXP3	Forkhead box P3
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
Gal-1	Galectin-1
GEPIA	Gene Expression Profiling Interactive Analysis
GNLY	Granulysin
GO	Gene Ontology
GTEx	Genotype-Tissue Expression
GZMA	Granzyme A
GZMH	Granzyme H
GZMK	Granzyme K
HER	Human epidermal growth factor receptor
iCAFs	Inflammatory CAFs
IGF1R	Insulin-like growth factor 1 receptor
IGF2	Insulin-like growth factor2
IL-1	Interleukin-1
IL-1α	Interleukin 1 Alpha
IL-6	Interleukin 6
IL-1β	Interleukin 1 Beta
IPF	Idiopathic pulmonary fibrosis
JUK	c-Jun N-terminal kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes

KRAS	KRAS Proto-Oncogene, GTPase
KRT19	Keratin 19
LAM	Lymphangioleiomyomatosis
Lin-28b	Lin-28 homolog b
MAPK	Mitogen-activated protein kinase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MVs	Macrovesicles
myCAFs	myofibroblastic CAFs
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCAM1	Neural Cell Adhesion Molecule 1
p38 MAPK	p38 mitogen-activated protein kinase
PAAD	Pancreatic Adenocarcinoma
PanIN	Pancreatic intraepithelial neoplasia
PBS	Phosphate-Buffered Saline
Pcsk9	Proprotein convertase subtilisin/kexin type 9
PDAC	Pancreatic ductal adenocarcinoma
CAFs	Cancer-associated fibroblasts
PDGFA	Platelet-derived growth factor alpha
PDGFB	Platelet-derived growth factor beta
PDGFRα	platelet-derived growth factor receptor alpha
PDGFRβ	Platelet-derived growth factor receptor beta
PFA	Paraformaldehyde
POSTN	Periostin
PSC	Pancreatic stellate cell
qPCR	Quantitative Polymerase Chain Reaction
RGS5	Regulator of G-protein signaling 5
rhEGF	Recombinant human epidermal growth factor
rhFGF2	Recombinant human fibroblast growth factor 2

SDS	Sodium Dodecyl Sulfate
SMAD4	SMAD Family Member 4
SOX9	SRY-box transcription factor 9
TBS	Tris-Buffered Saline
TCGA	The Cancer Genome Atlas
TEMED	Tetramethylethylenediamine
TGF-β	Transforming Growth Factor Beta
TME	The tumor microenvironment
TNF-α	Tumor Necrosis Factor
TP53	Tumor Protein P53
TRIS	Trishydroxymethylaminomethane
UMAP	Uniform Manifold Approximation and Projection
Wnt5a	Wnt family member 5a
YAP	Yes-associated protein
ZEB	Zinc Finger E-Box Binding Homeobox 1

1. Introduction

1.1 Pancreatic ductal adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) is a malignant tumor, which is the fourth leading cause of cancer-related death in the USA[1]. It remains one of the most aggressive solid tumors, which is predicted to become the second leading cause of cancer-related death in the Western world by 2030[2]. Despite advances, current treatment options - including surgery, chemotherapy regimens such as fluorouracil, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX), radiotherapy for local control, and novel approaches such as targeted therapy and immunotherapy - have shown limited success[3, 4]. The stroma in PDAC, which consists of a dense extracellular matrix, plays a key role in shielding tumor cells from therapeutic intervention[5, 6]. In addition, the pancreatic tumor microenvironment contains several immune cells, including regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs) and macrophages, all of which contribute to immune evasion and therapy resistance[7]. The presence of these cells and the cross-talk between tumor cells and stromal components create a barrier to effective treatment[8]. Thus, this dismal outcome is largely attributed to the highly complex tumor microenvironment, which plays a pivotal role in tumor progression and mediating resistance to therapy[9]. PDAC develops from the pancreatic ductal epithelium and is often preceded by precursor lesions such as pancreatic intraepithelial neoplasia (PanIN)[10]. Molecularly, PDAC is driven by frequent mutation in genes such as KRAS, TP53, CDKN2A, and SMAD4, which contribute to its unique biological behavior[11]. Fibroblasts have been identified as playing a role in the pancreatic cancer tumor microenvironment, through complex interactions between immune cells and cancer cells. These interactions foster an immunosuppressive environment, promote angiogenesis, and facilitate invasion and metastasis.

1.2 The Tumor Microenvironment

The tumor microenvironment (TME) is a complex and dynamic system made up of various cell types, signaling molecules, and interactions that influence tumor growth, progression, and metastasis. It includes fibroblasts, immune cells, endothelial cells, pericytes, and other stromal cells, as well as the extracellular matrix (ECM) and secreted factors like growth factors and cytokines, which play key roles in regulating communication between tumor and surrounding cells (Figure 1). Pancreatic cancer is characterized by massive proliferation of fibrous tissue that produces excessive amounts of ECM[12]. The ECM remodeling, particularly through collagen cross-linking, promotes tumor invasion, and growth factor signaling by enhancing focal adhesions and activating PI3K activity[13]. Among the various cellular components of the tumor microenvironment, cancer-associated fibroblasts (CAFs) are key contributors to the initiation and progression of pancreatic cancer[14]. ECM is produced primarily by fibroblasts in the tumor microenvironment, particularly by activated myofibroblasts expressing αSMA, which remodel the ECM and support tumor growth[15-17]. A deeper understanding of the mechanisms by which CAFs influence tumor biology is essential for the identification of novel therapeutic targets.

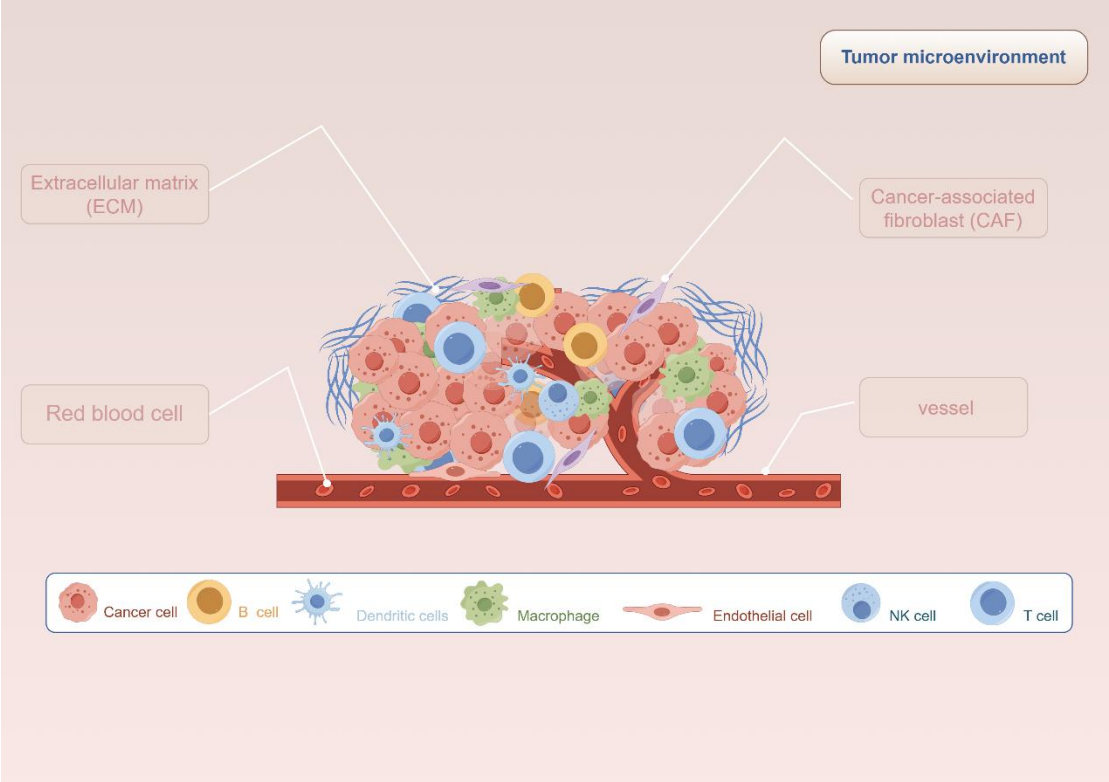


Figure 1. Composition of the tumor microenvironment. The tumor microenvironment is composed of cancer cells, various immune cells, cancer-associated fibroblasts, the extracellular matrix, and blood vessels, all interacting within this complex environment. Image created by Figdraw(https://www.figdraw.com).

1.3 Fibroblasts

Fibroblasts are spindle-shaped non-epithelial, non-immune cells of most likely mesenchymal origin[18]. Fibroblast infiltration and activation in the proximity of early precursor lesions is a long-known phenomenon. In pancreatic cancer, two distinct subtypes of cancer-associated fibroblasts (CAFs) are identified: inflammatory CAFs (iCAFs), which produce cytokines and chemokines, and myofibroblastic CAFs (myCAFs), which exhibit high levels of α-SMA and are predominantly located near neoplastic cells[16]. In addition, Elyada and colleagues identified a novel subtype of CAFs, termed antigen-presenting CAFs (apCAFs), characterized by MHC class II expression and the ability to interact with CD4+ T cells, potentially influencing immune

regulation[19]. The molecular networks that contribute to fibroblast activation have been defined in many studies[20, 21]. Recent research efforts have focused on understanding the molecular and cellular mechanisms underlying fibroblast activation in PDAC carcinogenesis and development[22, 23]. We hypothesize that several cytokines and chemokines induce fibroblast activation in pancreatic cancer.

1.4 Biomarkers of Cancer-associated fibroblasts

1.4.1 αSMA

α-SMA (Alpha-Smooth Muscle Actin) is a cytoskeletal protein and a specific marker of myofibroblasts and is most widely utilized marker for CAFs[24]. An elevated expression of alpha-SMA is sufficient to promote enhanced contractile activity in fibroblasts[25]. CAFs with high αSMA expression, often referred to as myCAFs, play a critical role in wound contraction and extracellular matrix (ECM) deposition, thereby facilitating cancer cell migration[26].Additionally, αSMA-expressing cells contribute to an immunosuppressive tumor microenvironment (TME) through various mechanisms. NCAM1+αSMA+ CAFs, which express TGF-β, contribute to T cell inactivation and an immunosuppressive tumor microenvironment[27].

1.4.2 FAP

FAP (Fibroblast Activation Protein) is a type II integral serine protease[28] and is a protein found in activated fibroblasts, involved in tissue repair, fibrosis, and supporting tumor growth by remodeling the extracellular matrix[29, 30]. FAP expression is upregulated in activated fibroblasts and is also detected in mesenchymal stem cells derived from bone marrow[31]. In pancreatic cancer, FAP-expressing CAFs have a role in immunity through upregulation of pro-inflammatory genes and contribution to tumor immune evasion[32].

1.5 Activation of Fibroblasts and its functions

Activated fibroblasts show rapid proliferation, increased migration and promote tumor formation and development (Figure 2). Activated CAFs express a variety of markers, including alpha-smooth muscle actin (α-SMA). Numerous factors, including signals from tumor cells, the surrounding microenvironment, and inflammatory mediators, have been shown to contribute to CAF activation[33-35].Once activated, fibroblasts transition into a more dynamic state, characterized by the secretion of a diverse array of cytokines. These cytokines, transforming growth factor beta (TGF-β), tumor necrosis factor (TNF-α), interleukin 6 (IL-6), including interleukin 1 beta (IL-1β) and interleukin 1 alpha (IL-1α), play pivotal roles in modulating inflammation, promoting tissue remodeling, and contributing to pathological processes such as fibrosis and tumor progression[36-38]. Activated cancer-associated fibroblasts (CAFs) also secrete angiogenic molecules and extracellular matrix (ECM) components, which play crucial roles in promoting tumor metastasis[39, 40]. In pancreatic cancer, studies have shown that EGFR-activated myofibroblasts facilitate metastasis by promoting tumor progression, invasion and remodeling of the pro-metastatic tumor microenvironment[41].

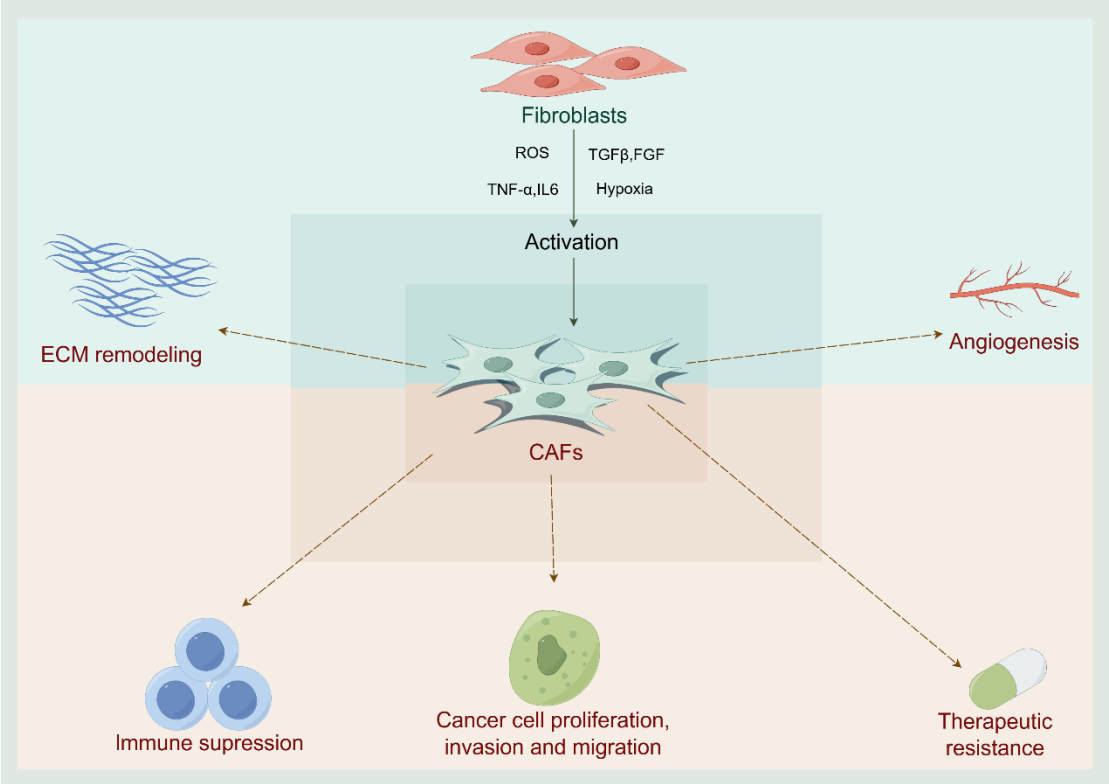


Figure 2. Fibroblast activation and its functions. Fibroblasts are activated by factors such as ROS, TGFβ and hypoxia, leading to their transformation into cancer-associated fibroblasts (CAFs). CAFs play a key role in the tumor microenvironment by promoting ECM remodeling, angiogenesis, immune suppression, cancer cell growth and therapy resistance. Image created by Figdraw(https://www.figdraw.com).

1.6 FGFBP2

FGFBP2 is a member of the fibroblast growth factor binding protein family. Growth factor signaling is known to play an important role in tumorigenesis and development. Among the growth factors and receptors involved in the malignant process, fibroblast growth factors (FGF), a family of more than 20 proteins, have been described to enhance tumor growth, angiogenesis, and progression[42, 43]. FGFBP2 protein is constitutively secreted by Th1-type CD4-positive lymphocytes and lymphocytes with cytotoxic potential, and may be involved in an essential process of cytotoxic lymphocyte-mediated immunity[44]. Yamanaka et al. found that high

FGFBP2 expression in high-grade gliomas was positively correlated with survival[45]. In ovarian cancer, FGFBP2 showed higher levels in clear cell carcinoma stage I compared with the more advanced staged carcinoma, and correlated positively with an improved clinical outcome[46]. FGFBP2 stabilizes and enhances the bioavailability of FGF2, facilitating its interaction with FGF receptors (FGFRs)[47, 48]. Activation of FGFRs triggers downstream signaling cascades, including the ERK and AKT pathways, which regulate cell proliferation, and migration[49]. Previous next-generation sequencing (NGS) studies performed on PCSs originating from pancreatic cancer patients revealed that FGFBP2 bears a point guanine (G) to cytidine (C) mutation. This mutation was found particularly in pancreatic stellate cells (PSCs) and not in the peripheral blood DNA of pancreatic cancer patients[50].

1.7 FGF2 and pathway

1.7.1 FGF2

FGF2, also known as basic FGF (bFGF), is an important regulator of cell growth and differentiation under physiological and pathological conditions. FGF2 is an extracellular mitogen secreted by a variety of cell types[51]. In tumors, cancer cells release FGF2 to drive tumorigenesis and protect themselves from apoptosis through autocrine signalling[52, 53]. To initiate signaling, FGF2 binds to the fibroblast growth factor receptor (FGFR) in a ternary complex, triggering a cascade of downstream signaling events through the Ras-Raf-MAPK-ERK pathway[52].

1.7.2 ERK1/2

Extracellular signal-related kinase (ERK1/2) is a serine/threonine kinase and a key protein in the MAPK pathway, which is involved in the transmission of signals that regulate various cellular processes such as growth and differentiation. ERK1/2 are activated by phosphorylation

at specific residues (Thr202 and Tyr204 in ERK1; Thr185 and Tyr187 in ERK2) by the upstream kinase MEK1/2 in response to signals such as growth factors and cytokines[54]. Their phosphorylated forms, p-ERK1/2, represent the active state that drives cell proliferation and survival[55]. Overactivation of ERK1/2, often caused by mutation in upstream regulators such as RAS or RAF, is commonly observed in cancer. This dysregulation drives uncontrolled cell growth and survival, making the ERK1/2 signaling pathway a critical therapeutic target in cancer treatment[56].

1.7.3 AKT

AKT, also known as protein kinase B (PKB), is a serine/threonine kinase that plays a central role in cell signaling pathways. It is activated downstream of phosphatidylinositol 3-kinase (PI3K) and regulates processes such as cell growth, survival, metabolism and proliferation[57]. Phosphorylation of S473 in the hydrophobic motif is essential for maximal AKT activation as it stabilizes the active state of AKT, whereas its absence significantly reduces AKT activity[58]. AKT plays a critical role in cancer through amplification, overexpression or mutation, driving tumorigenesis, enhancing cancer cell survival, promoting uncontrolled proliferation, facilitating metastasis and contributing to therapy resistance[59].

1.8 EHF

EHF (ETS Homologous Factor) is a member of the transformation-specific (ETS) transcription factor family, a group of proteins defined by a highly conserved DNA-binding domain (the ETS domain) which controls critical cellular processes such as proliferation and differentiation. EHF is associated with epithelial cell characteristics, as it is primarily expressed in epithelial tissues and involved in regulating genes important for maintaining epithelial integrity[60]. EHF plays a dual role in cancer, exhibiting both tumor-suppressive and tumor-promoting functions in varying conditions[61]. EHF inhibits cancer progression by downregulating ETS Proto-Oncogene

1, Transcription Factor (ETS1)-driven Zinc Finger E-Box Binding Homeobox 1(ZEB) expression, effectively preventing epithelial-mesenchymal transition (EMT) and limiting metastatic potential[62]. Increased EHF expression, driven by gene amplification, promoted HER family signaling, accelerated tumor growth and enhanced proliferative and metastatic processes, which correlated with poorer survival in gastric cancer and highlighted EHF as a potential therapeutic target[63].

2. Aim

Somatic CAF mutations identified by our group may have functional consequences and affect PDAC. One of them is replacement of guanine (G) to cytidine (C) in Fibroblast Growth Factor Binding Protein 2 (FGFBP2) causing an amino acid change from threonine to serine (FGFBP2 p.T186S).

The central question of my thesis is to investigate how this somatic mutation in FGFBP2 affects fibroblast functions in the pancreatic cancer microenvironment and how these interactions contribute to tumor progression.

This project will investigate the following aspects:

a. The function of FGFBP2 mutant fibroblasts and particular molecular alterations.

b. The effect of FGFBP2 mutant fibroblasts on the function and signaling pathway of pancreatic cancer cells.

c. The molecular changes and mechanisms of pancreatic cancer cells treated with conditioned medium from FGFBP2 mutant fibroblasts investigated with RNA-Seq.

3. Materials and Methods

3.1 Materials

Table 1. Chemicals and Reagents

Chemicals and Reagents	Company, Location, Country
Tryptone	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Yeast extract	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
NaCl	Sigma-Aldrich GmbH, Steinheim, Germany
NaOH	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
DEPC-H2O	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Ethanol ≥99,8%	Thermo Fisher Scientific GmbH, Dreieich, Germany
QIAzol® Lysis Reagent	QIAGEN GmbH, Hilden, Germany
HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX), 5x	Solis BioDyne OÜ, Tartu, Estonia
PCR-grade water	Jena Bioscience GmbH, Jena, Germany
Urea	Amersham Bioscience AB, Uppsala, Sweden
Thiourea	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
CHAPS	EMD Biosciences, Inc., La Jolla, USA
DTT	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Complete™, Mini, EDTA-free Protease-Inhibitor-Cocktail	Roche Diagnostics GmbH, Mannheim, Germany
Rotiphorese®NF-Acrylamid/Bis-	Carl Roth GmbH + Co. KG, Karlsruhe, Germany

Lösung 30% (29:1)	
TRIS	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
SDS	Amersham Bioscience AB, Uppsala, Sweden
APS	Merck KGaA, Darmstadt, Germany
TEMED	Bio-Rad Laboratories GmbH, Feldkirchen, Germany
Glycine	SERVA Electrophoresis GmbH, Heidelberg, Germany
Methanol ≥99,8%	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
PBS buffer	AppliChem GmbH, Darmstadt, Germany
TBS buffer	AppliChem GmbH, Darmstadt, Germany
Tween 20	Amersham Bioscience AB, Uppsala, Sweden
Amersham™ ECL™ Rainbow™ Marker	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
MTT	Sigma-Aldrich, Munich, Germany
DMSO	Merck KGaA, Darmstadt, Germany
Paraformaldehyde (PFA)	Merck KGaA, Darmstadt, Germany
Hematoxylin	Merck KGaA, Darmstadt, Germany
HBSS (Hank's Balanced Salt Solution)	Thermo Fisher Scientific GmbH, Dreieich, Germany
FBS Superior, S0615	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Penicillin-Streptomycin	Thermo Fisher Scientific GmbH, Dreieich, Germany
0,05% Trypsin-EDTA	Thermo Fisher Scientific GmbH, Dreieich, Germany
DMEM high glucose, D5796	Sigma-Aldrich Chemie GmbH, Steinheim, Germany

Geneticin disulphate (G418) solution	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Lipofectamine™ 3000 Transfection Reagent	Thermo Fisher Scientific GmbH, Dreieich, Germany
Opti-MEM® I (1X) Reduced Serum Medium	Thermo Fisher Scientific GmbH, Dreieich, Germany
pCMV6-AC-GFP Mammalian Expression Vector, PS100010	OriGene Technologies, Inc., Rockville, USA
FGFBP2 (NM_031950) Human Tagged ORF Clone, RG206392	OriGene Technologies, Inc., Rockville, USA
Mutate the FGFBP2 (NM_031950) Human Tagged ORF Clone, CW307647	OriGene Technologies, Inc., Rockville, USA
dam－/dcm－ Competent E. coli, C2925H	New England Biolabs, Inc., Ipswich, USA
Corning® Matrigel® Growth Factor Reduced (GFR)，# 354230	Merck KGaA, Darmstadt, Germany

Table 2. Instruments

Instruments	Company, Location, Country
Absorptions-Mikroplatten-Reader Sunrise	Tecan Group AG, Männedorf, Switzerland
Biometra® T3 Thermocycler	Analytik Jena AG, Jena, Germany
QuantStudio3 Real-Time-PCR-System	Thermo Fisher Scientific GmbH, Dreieich, Germany
NanoDrop™ One	Thermo Fisher Scientific GmbH, Dreieich, Germany
Mini-PROTEAN Tetra Vertical Electrophoresis for Handcast Gels (1.0 mm)	Bio-Rad Laboratories GmbH, Feldkirchen, Germany
PowerPac 200 Power Supply	Bio-Rad Laboratories GmbH, Feldkirchen,

	Germany
PowerPac 300 Power Supply	Bio-Rad Laboratories GmbH, Feldkirchen, Germany
Trans-Blot® transfer cell Tank-Blot-System	Bio-Rad Laboratories GmbH, Feldkirchen, Germany
ChemiDoc™Touch Imaging System	Bio-Rad Laboratories GmbH, Feldkirchen, Germany
Heracell 150i CO2 cell incubator	Thermo Fisher Scientific GmbH, Dreieich, Germany
Inverted fluorescence phase contrast microscope, BZ-X810	Keyence GmbH, Neu-Isenburg, Germany
Mikroskop Axiovert 25	Carl Zeiss Microscopy GmbH, Jena, Germany

Table 3. Consumables

Consumables	Company, Location, Country
Pipette tips Sapphire filter tips	Greiner Bio-One GmbH, Frickenhausen, Germany
Cell culture flask $25cm^2$,$75cm^2$	Greiner Bio-One GmbH, Frickenhausen, Germany
6-well plate	Greiner Bio-One GmbH, Frickenhausen, Germany
12-well plate	Greiner Bio-One GmbH, Frickenhausen, Germany
24-well plate	Greiner Bio-One GmbH, Frickenhausen, Germany
96-well plate	Greiner Bio-One GmbH, Frickenhausen, Germany
96-well PCR plate	NIPPON Genetics EUROPE GmbH, Düren, Germany

ThinCert® cell culture set 24-well, 8 µm pores	Greiner Bio-One GmbH, Frickenhausen, Germany
Amersham Hybond PVDF Blotting Membrane	GE Healthcare GmbH, Solingen, Germany
Eppendorf Safe-Lock Tubes	Eppendorf AG, Hamburg, Germany
CELLSTAR® tubes	Greiner Bio-One GmbH, Frickenhausen, Germany
Ampliseal™, transparent microplate cover film	Greiner Bio-One GmbH, Frickenhausen, Germany
CRYO.S™ cryotubes	Greiner Bio-One GmbH, Frickenhausen, Germany

Table 4. Kits

Kits	Company, Location, Country
ZymoPURE - Express™ Plasmid Midiprep Kit, D4213	ZYMO RESEARCH EUROPE GMBH, Freiburg, Germany
Direct-zol RNA Miniprep Kit, R2050	ZYMO RESEARCH EUROPE GMBH, Freiburg, Germany
High-Capacity cDNA Reverse Transcription Kit, 4368814	Thermo Fisher Scientific GmbH, Dreieich, Germany
Bio-Rad Protein Assay Kit II	Bio-Rad Laboratories GmbH, Feldkirchen, Germany
WesternBright™ ECL Western blotting detection Kit	Biozym Scientific GmbH, Hess. Oldendorf, Germany

Table 5. Human Primers

gene	Forward primer (5' → 3')	Reverse primer (5' → 3')	Company, Location, Country

FGFBP2	CTG CTG ACC CCA AAC CTT ACT	CCT TGA GGC TGG AAG TCA CC	Metabion international AG, Planegg, Germany
α-SMA	CTA TGC CTC TGG ACG CAC AAC T	CAG ATC CAG ACG CAT GAT GGC A	Metabion international AG, Planegg, Germany
TGF-β	TAC CTG AAC CCG TGT TGC TCT C	GTT GCT GAG GTA TCG CCA GGA A	Metabion international AG, Planegg, Germany
IL-1β	CCA CAG ACC TTC CAG GAG AA	GTG CAG TTC AGT GAT CGT ACA GG	Metabion international AG, Planegg, Germany
IL-6	AGA CAG CCA CTC ACC TCT TCA G	TTC TGC CAG TGC CTC TTT GCT G	Metabion international AG, Planegg, Germany
TNF-α	CTC TTC TGC CTG CTG CAC TTT G	ATG GGC TAC AGG CTT GTC ACT C	Metabion international AG, Planegg, Germany
FGF2	AGC GGC TGT ACT GCA AAA ACG G	CCT TTG ATA GAC ACA ACT CCT CTC	Metabion international AG, Planegg, Germany
EHF	ATC AGA GGC AGT GGC TCA GCT A	ACC AGT CTT CGT CCA TCC ACA C	Metabion international AG, Planegg, Germany
β-Actin	AGG CAC CAG GGC GTG AT	GCC CACA TA GGA ATC CTT CTG AC	Metabion international AG, Planegg, Germany

Table 6. Antibodies

Antibodies	Host	Dilution	Catalog number	Company, Location, Country
FGFBP2	Rabbit	1:1000	PA5-113480	Thermo Fisher Scientific Inc., Waltham, USA
ERK1/2	Rabbit	1:2000	4695	Cell Signaling Technology, Inc., Danvers, USA
P -ERK1/2	Rabbit	1:2000	4370	Cell Signaling Technology, Inc., Danvers, USA
AKT	Rabbit	1:1000	4685	Cell Signaling Technology, Inc., Danvers, USA

P-Akt	Rabbit	1:1000	4060	Cell Signaling Technology
β-Actin	Mouse	1:10000	A5441	Merck KGaA, Darmstadt, Germany
α-Tubulin	Mouse	1: 5000	T5168	Merck KGaA, Darmstadt, Germany
Goat-anti-mouse IgG-HRP	Mouse	1:20000	sc-2004	Santa Cruz Biotechnology, Heidelberg, Germany
Goat-anti-rabbit IgG-HRP	Rabbit	1:20000	sc-2005	Santa Cruz Biotechnology, Heidelberg, Germany

Table 7. Human recombinant proteins

Recombinant proteins	Catalog number	Company, Location, Country
FGF2	100-18B	Thermo Fisher Scientific Inc., Waltham, USA
EGF	AF-100-15	Thermo Fisher Scientific Inc., Waltham, USA

3.2 Methods

3.2.1 Cell Culture

In this study, the SV80 fibroblast cell line and the PANC-1 pancreatic cancer cell line were used. The SV80 cells, which were immortalized by the SV40 virus, were derived from healthy adult female skin fibroblasts. SV80 cells were purchased from CLS Cell Lines Service GmbH (Eppelheim, Germany) and PANC-1 cells were purchased from American Type Culture Collection (Manassas, USA). Both SV80 and PANC-1 cells were cultured in an incubator at 37°C with 5% CO_2 and maintained in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Germany) supplemented with 10% fetal bovine serum (FBS), 100

U/mL penicillin, and 100 mg/mL streptomycin.

Ethics vote: all experiments in this study will not involve any patient material and any written consents. Animal testing approval: no animals will be used in this study.

3.2.2 Cell transfection

3.2.2.1 SV80 cell death curve

All plasmids used in this study contained a neomycin resistance gene. Selection of transfected cells was performed with the proper concentration of neomycin according to a cell death curve. SV80 cells were incubated in growth medium with different concentrations of neomycin (as detailed in Table 8) for 10 days. By day 10, almost all SV80 cells had died when exposed to 0.6 mg/ml neomycin. It was determined that 0.6 mg/ml was the lowest concentration that caused complete cell death, making it the optimal selection concentration. To prepare the selection medium for transfected SV80 cells, 0.6 mg/ml neomycin was added to the standard SV80 growth medium.

Table 8. Neomycin concentration gradient

0mg/ml	0.2mg/ml	0.4mg/ml	0.5 mg/ml	0.6 mg/ml	0.7 mg/ml
0mg/ml	0.2mg/ml	0.4mg/ml	0.5 mg/ml	0.6 mg/ml	0.7 mg/ml
0.8mg/ml	1.0mg/ml	1.5 mg/ml	2.0mg/ml	4.0 mg/ml	
0.8 mg/ml	1.0mg/ml	1.5 mg/ml	2.0mg/ml	4.0 mg/ml	

3.2.2.2 Plasmid transformation and extraction

Plasmid DNA was prepared by transformation using dam-/dcm- competent E. coli cells and plasmid extraction using the ZymoPURE - Express™ Plasmid Midiprep Kit. A tube of dam-/dcm- competent E. coli cells was removed from storage and placed on ice to thaw. Once thawed, 1 μl of plasmid DNA (containing 100 ng) was added to the cells and mixed well before returning

the tube to ice for 30 minutes. The mixture was then subjected to a heat shock at exactly 42°C for 30 seconds and returned to ice for a further 5 minutes.

To amplify the target bacterial population and express the plasmid-encoded resistance gene, the plasmid-cell mixture was transferred into LB medium (as detailed in Table 9) and ampicillin was added. The culture was incubated on a shaker at 37°C and 200 rpm for 16-18 hours. Plasmid extraction was performed according to the manufacturer's protocol using the ZymoPURE - Express™ Plasmid Midiprep Kit and plasmid DNA was stored at -80°C.

NanoDrop™ One was used for concentration determination. For pure plasmid DNA samples, the A260/A280 ratio was approximately 1.8-2.0 and the A260/A230 ratio was not less than 2.0; only samples meeting these quality criteria were used for further analysis.

Table 9. LB Medium

Substance	Required amount
Tryptone	10 g
Yeast extract	24 g
NaCl	5 g
NaOH	1 mL

3.2.2.3 Cell transfection procedure

To construct wild-type and mutant FGFBP2 gene expression in SV80 cells, Lipofectamine® 3000 reagent was used to transfect plasmid DNA into the cells via a liposome-mediated method. The empty vector was used as a control group. This technique relies on the formation of complexes between liposomes and DNA, allowing the DNA to efficiently cross the cell membrane and express the target gene.

The day before transfection, SV80 cells were seeded in a 24-well plate at a density of 6×10^5 cells per well. Prior to transfection, the medium was replaced with serum-free medium. Lipofectamine® 3000 and plasmid DNA (with pT3000 reagent) were each diluted in Opti-MEM®, then combined and incubated for 15 minutes at room temperature to allow DNA-lipid

complex formation. The mixture was then added to each well. After 8 hours, the medium was replaced with fresh growth medium and the cells were monitored daily by fluorescence microscopy. After 72 hours, the cells were passaged at a ratio of 1:10 and selection medium was added to maintain selective pressure. Finally, monoclonal colonies were isolated and expanded, and FGFBP2 expression was confirmed by qPCR and Western blot analysis.

3.2.3 RNA isolation, cDNA synthesis and qPCR

For RNA extraction and purification, cells were lysed with TRIZOL reagent and the resulting solution was diluted 1:1 with 96% ethanol and mixed thoroughly by vortexing. RNA was extracted using the Direkt-zol™ RNA Miniprep Kit according to the manufacturer's protocol. The purified RNA was then stored at -80°C. Its concentration was measured using NanoDrop™ One and only samples with an A260/A280 ratio between 1.8 and 2.0 and an A260/A230 ratio of at least 2.0 were selected for further analysis.

For cDNA synthesis, the High-Capacity cDNA Reverse Transcription Kit was used. The reaction mixture was prepared as described in Table 10. 1 µg RNA was added to a tube and the volume was adjusted to 10 µL with DEPC-treated water. After adding another 10 µL of the prepared cDNA synthesis mixture, the tube was placed in a thermocycler according to the conditions given in Table 11. cDNA was then stored at -20°C.

Table 10. Complete cDNA synthesis Mixture

Substance	Required amount
1 µg RNA	10 µl
10X RT Buffer	2 µl
25X dNTP Mix 100mM	0.8 µl
10X RT Random Primer	2 µl
MultiScribe™ Reverse Transcriptase	1 µl
DEPC-H_2O	4.2 µl

Table 11. cDNA Synthesis thermocycler Profile

Temperature	Time
25°C	10 mins
37°C	120 mins
85°C	5 mins
4°C	final hold

Gene expression analyses were performed with qPCR by employment of HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) and the QuantStudio 3 Real-Time PCR system. A mixture was prepared by combining 4 µl of qPCR ROX, 0.5 µl each of forward and reverse primers, and 13 µl of PCR-grade water. A total of 18 µl of this mixture was dispensed into each well of a 96-well plate, followed by the addition of 2 µl of cDNA. The housekeeping gene β-actin was used as an internal control and reference. Each sample was tested in duplicate, with PCR-grade water serving as a negative control. The plate was centrifuged at 2,000 x g for 10 minutes and placed into the QuantStudio 3 system. The initial activation step was carried out at 95°C for 15 minutes, followed by 41 amplification cycles consisting of 95°C for 15 seconds, 60°C for 20 seconds, and 72°C for 20 seconds.

3.2.4 Protein extraction and Western blot

3.2.4.1 Protein extraction

After aspirating the culture medium, cells were immediately placed on ice and washed with cold PBS. Protein Lysis Buffer (see Table 12) was added, and the protein was rapidly transferred to the pre-cooled tube. The sample was placed on ice and lysed for 15 minutes. The mixture was centrifuged for 15 minutes at 4°C and transferred to a new pre-cooled tube. Protein was stored at -20°C. All procedures were performed on ice. To measure protein concentration for subsequent studies, the Bio-Rad Protein Assay Kit II was used according to the manufacturer's

protocol. The measurement was carried out at a wavelength of 595 nm. BSA was used as the standard protein for calibration, and the protein concentration in the samples was calculated based on this standard.

Table 12. Protein Lysis Buffer

Substance	Required amount
Urea	4.2 g
Thiourea	1.524 g
CHAPS	0.4 g
DTT	0.0616 g
Proteinase inhibitor	1 tablet
Double-distilled water	fill to 10 mL

3.2.4.2 Western blot

Western blot integrates electrophoretic separation, membrane transfer and antibody detection to allow both qualitative and semi-quantitative analysis of protein expression. The separating and stacking gels were prepared according to the formulations given in Tables 13 and 14. Protein samples were thawed, mixed, briefly centrifuged and heated to 95°C before loading onto the gel using running buffer (Table 15). Electrophoresis was performed at 10 mA per gel, increasing to 20 mA as bands aligned. Proteins were transferred to a PVDF membrane for 120 minutes at 6°C using the Trans-Blot® transfer cell tank blot system filled with transfer buffer (Table 16). The membrane was then blocked with 5% milk in TBS/T (Table 17) for one hour and incubated with the primary antibody overnight at 4°C. After washing with TBS/T, a compatible secondary antibody was applied for one hour at room temperature and the protein bands were visualized by enhanced chemiluminescence. The membrane was stored in TBS at 4°C to maintain moisture.

Table 13. Separating Gel (12%)

Component	Volume (mL)
Double-distilled water	3.3
30% Acrylamide mix	4.0
1.5M Tris (pH 8.8)	2.5
10% SDS	0.1
10% APS	0.1
TEMED	0.004

Table 14. Stacking Gel (4%)

Component	Volume (mL)
Double-distilled water	1.4
30% Acrylamide mix	0.33
1.5M Tris (pH 6.8)	0.25
10% SDS	0.02
10% APS	0.02
TEMED	0.002

Table 15. Running buffer

Substance	Required amount
TRIS	144 g
Glycine	30 g
SDS	6 g
Double-distilled water	Fill to 1 liter

Table 16. Transferring buffer

Substance	Required amount
Glycine	42 g
TRIS	9 g
Methanol	600 mL
Double-distilled water	Fill to 3 liters

Table 17. TBS/T buffer

Substance	Required amount
TBS (10X)	100 mL
Tween 20	1 mL
Double-distilled water	Fill to 1 liter

3.2.5 MTT -TEST

This study evaluated the effects of FGFBP2 mutation in SV80 cells and the effects of conditioned medium on proliferation of PANC-1 cells using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. In the experiment, a control group, three FGFBP2 wild-type groups and three FGFBP2 mutant groups were analyzed.

Cells were seeded in a 96-well plate at a density of 3×10^3 cells/ml and incubated at 37°C with 5% CO_2 for 24 hours. After incubation, the medium was replaced with either fresh growth medium or conditioned medium. At 24, 48 and 72 hours, 20 µl of MTT solution (prepared by dissolving 25 mg MTT in 5 ml HBSS) was added to each well. The plate was then returned to the incubator for a further 4 hours, after which 100 µl of DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured at 570 nm using a Sunrise Absorbance Microplate Reader.

3.2.6 Transwell assay

To investigate the migration and invasion of FGFBP2 mutant SV80 cells, a transwell assay was performed. In the experiment, a control group, three FGFBP2 wild type groups and three FGFBP2 mutant groups were analyzed. For the invasive experiment, an appropriate amount of Matrigel was applied to the upper chamber in advance. Cells (1×10^4 in 200 μL serum-free medium) were seeded onto each transwell insert, which was then placed in a 24-well plate containing 500 μL of medium with 10% FBS and incubated at 37°C with 5% CO_2 for 24 hours. After incubation, the cells were fixed with 4% paraformaldehyde and stained with hematoxylin to visualize migrated and invaded cells. The upper surface of the insert was gently cleaned and the inserts were then examined under a microscope to assess the number of migrated cells. Images were captured from five random areas using inverted fluorescence phase contrast microscopy, and the number of cells per field was counted and analyzed using ImageJ (v.1.5.3).

3.2.7 Conditioned medium

This experiment was designed to investigate the effects of conditioned medium derived from transfected cells on PANC-1 cells. To prepare the conditioned medium, 3×10^5 cells were passaged into a culture medium containing 10% FBS and maintained under standard conditions. After 24 hours, the cells were washed with PBS and provided with serum-free medium (0% FBS). The cells were then cultured for a further 48 hours, after which the culture medium was collected. This medium was then centrifuged to remove any cell debris and stored at -80°C until further use.

3.2.8 FGF2 and EGF treatment

To investigate the effects of conditioned medium (CM) and CM combined with FGF2 or EGF on ERK1/2 and AKT phosphorylation, PANC-1 cells were seeded in a medium containing 10% FBS and incubated for 24 hours. The medium was then replaced with serum-free medium, and

the cells were cultured for an additional 12 hours. Recombinant FGF2 or EGF was added directly to the culture to achieve a final concentration of 10 ng/ml, and the cells were treated for 0, 5, and 20 minutes.

3.2.9 RNA-Seq

The sequencing of RNA samples from the PANC-1 cells treated with conditioned medium from transfected SV80 cells was carried out by Novogene. In the experiment, PANC-1 cells treated with conditioned medium were analyzed, including a control group, three FGFBP2 wild type groups, and three FGFBP2 mutant groups, with untreated PANC-1 cells serving as the negative control. At least 200 ng per sample was sent in, which was subjected to quality control before further processing. The integrity and purity of the RNA were ensured using agarose gel electrophoresis, NanoDrop™ and the Agilent 2100 Bioanalyzer. This was followed by library preparation and further quality control before the actual paired-end sequencing of the 150 bp fragments was carried out using Illumina PE150 technology. At least 20 million reads were read out per sample. This also allowed the transcriptome of cells to be compared under different conditions. The abundance of transcript reflected the gene expression level directly.

In the RNA-seq experiment, gene expression levels were estimated by the abundance of transcripts (count of sequencing) that mapped to the genome or exon. Reads count was proportional to gene expression level, gene length and sequencing depth. FPKM (Fragments Per Kilobase of transcript sequence per Million base pairs sequenced) was the most common method of estimating gene expression levels, which took the effects of both sequencing depth and gene length on the counting of fragments into consideration. To avoid errors, the raw reads were filtered to obtain clean reads. The procedure for raw reads filtering was as follows: (1) Reads containing adapters were removed; (2) Reads containing N > 10% (N represents base that could not be determined) were removed; (3) Low-quality reads were removed, where the Qscore (Quality value) of over 50% of the bases was $\leqslant$ 5. Mapping the clean reads to the reference genome or the transcriptome was the basis for the following analysis. HISAT2 was used to accomplish the mapping. To avoid losing a lot of effective junction reads, HISAT2

software was used for RNA-seq sequencing data analysis, and the junction reads were positioned precisely.

The input data for differential gene expression analysis consisted of read counts from gene expression level analysis. Differential gene expression analysis generally included three steps: 1) Read counts normalization; 2) Model-dependent p-value estimation; 3) FDR value estimation based on multiple hypothesis testing. Through the enrichment analysis of the differentially expressed genes, biological functions or pathways significantly associated with the differentially expressed genes were identified. Several popular gene functional enrichment analyses were provided to help researchers infer the biological processes of RNA-seq experiments. A common way for searching shared functions among genes was to incorporate the biological knowledge provided by biological ontologies. Gene Ontology (GO) annotates genes to biological processes, molecular functions, and cellular components in a directed acyclic graph structure, and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotates genes at the pathway level.

3.2.10 Expression and Survival analysis

GEPIA (http://gepia.cancer-pku.cn/index.html) was used to analyze the expression of EHF in The Cancer Genome Atlas Program (TCGA) and The Genotype-Tissue Expression (GTEx) datasets[64]. The prognostic significance of EHF in PDAC was assessed through Kaplan-Meier survival analysis. Kaplan-Meier curves, generated using the survival package, were utilized to compare overall survival between groups with high and low EHF expression. The samples were divided into high and low expression groups according to the median EHF expression level.

3.2.11 scRNA-seq data integration and cluster annotation analysis

The filtered count matrices were transformed into sparse matrices using the Seurat package (v5.1.0)[65]. Single-cell data integration was carried out using Seurat's canonical correlation analysis (CCA) method. Cells were filtered based on the following criteria: fewer than 500

genes per cell, more than 25% mitochondrial genes, or fewer than 1000 transcripts per cell. Additionally, genes expressed in fewer than 3 cells were removed from downstream analysis. Raw expression data were normalized using the NormalizeData function and scaled using the ScaleData function. Principal component analysis (PCA) was performed using the RunPCA function with default parameters, and dimensionality reduction was performed using the RunUMAP function. Cell clustering was performed using the FindClusters function with a resolution of 0.7. The FindAllMarkers function was used to identify specific genes for each cell subset, and gene marker signatures were used to define cell identities.

3.2.12 KEGG analysis

Differentially expressed genes (DEGs) were identified using the 'FindAllMarkers' function in Seurat, with a |log2 FC| > 1 and an adjusted p-value < 0.05 as the threshold for significance. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment pathway analysis was performed using the R-package "clusterProfiler"[66].

3.2.13 Copy Number Variation (CNV) estimation Tumor and pancreatic ductal cells

To detect large-scale chromosomal CNVs, the InferCNV package (version 1.22.0) was used to infer CNVs in ductal cells and identify tumor cells with the default parameters[67]. Macrophages and NK cells served as the reference normal set.

3.2.14 Cell-cell communication analysis

Cell–cell communication network inference and analysis were performed using CellChat (version 2.1.2)[68] and visualized intercellular communication interactions between cell subsets at the single-cell level.

3.2.15 Statistical Analysis

All bioinformatics analyses and visualizations were conducted using R (v4.4.2). Statistical analyses were performed with Prism (v10), using one-way ANOVA with Tukey's test for multiple group comparisons and two-tailed unpaired Student's t-tests for two-group comparisons. A P-value < 0.05 was statistically significant.

4. Results

4.1 FGFBP2 mutation regulates fibroblast activation

To investigate the role of FGFBP2 p.T186S mutation in SV80 fibroblasts, we transfected control group with empty plasmid (Control), wild type group (WT1, WT2, WT3) with plasmid overexpressing native FGFBP2 and mutant group (MUT1, MUT2, MUT3) with plasmid overexpressing FGFBP2 carrying the p. T186S mutation. Monoclonal cells were selected based on their morphological characteristics (Fig. 3A). QPCR was used to confirm overexpression of FGFBP2 in WTs and MUTs groups as compared to the control group (Fig. 3B). The transfection effect was further verified at the protein level (Fig. 3C, D).

Investigations of α-SMA which is a key marker of fibroblast activation, revealed its upregulation in fibroblasts carrying FGFBP2-mutation (Fig. 4A). Activated fibroblasts secreted pancreatic inflammatory cytokines, such as IL-1β, IL-6, TNF-α, and TGF-β[69]. Importantly, fibroblasts carrying FGFBP2-mutation had upregulated IL-6, TNF-α and TGF-β levels (Fig. 4B-E), which are critical in shaping the inflammatory tumor microenvironment and driving tumor initiation and progression[70, 71]. In the next steps we focused on cell proliferation and migration which are key characteristics of fibroblast activation. We conducted MTT and migration assays to assess the effects of FGFBP2 mutation on fibroblast function. The MTT assays showed that FGFBP2 mutation enhanced cell proliferation, and consistent with this, the migration assay confirmed that the mutation also increased fibroblast motility (Fig. 5A, B). Taken together, these results indicated that FGFBP2 mutation contributed to fibroblast activation.

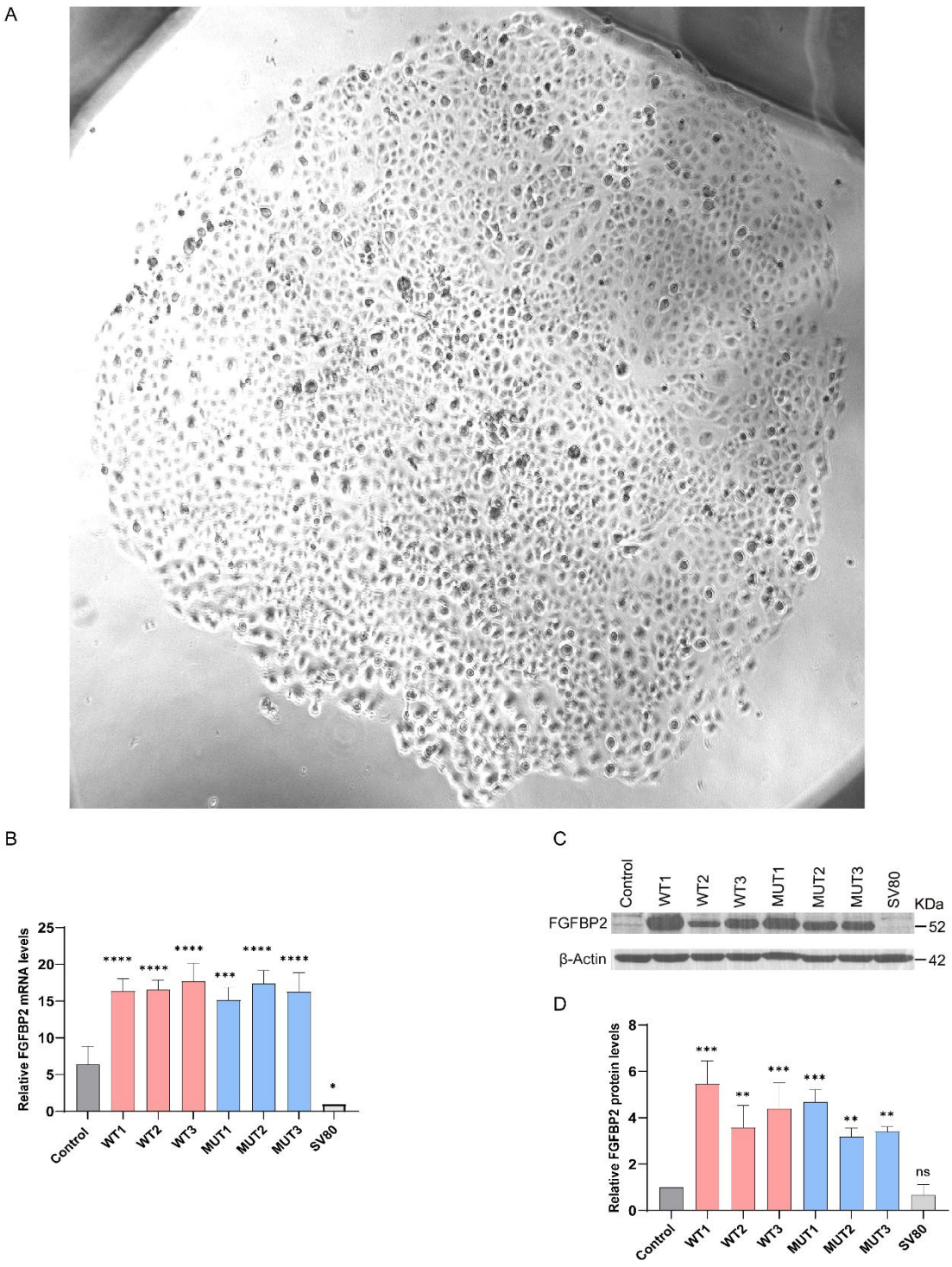


Figure 3. Cell transfection and verification. (A)The morphological characteristics of the monoclonal cells. (B) SV80 were transfected with empty (Control), wild type FGFBP2 (WT1, WT2, WT3) and FGFBP2-Mutation (MUT1, MUT2, MUT3) plasmid and verified by qPCR. The qPCR results showed that the FGFBP2 wild type and mutant groups are significantly higher than the FGFBP2 control. (C, D) Western blot images and quantifications of FGFBP2

expression in control, wild type and mutant groups. The Western blot results showed that the FGFBP2 wild type and mutant groups were significantly higher than the FGFBP2 control; means with SD;(*p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant)

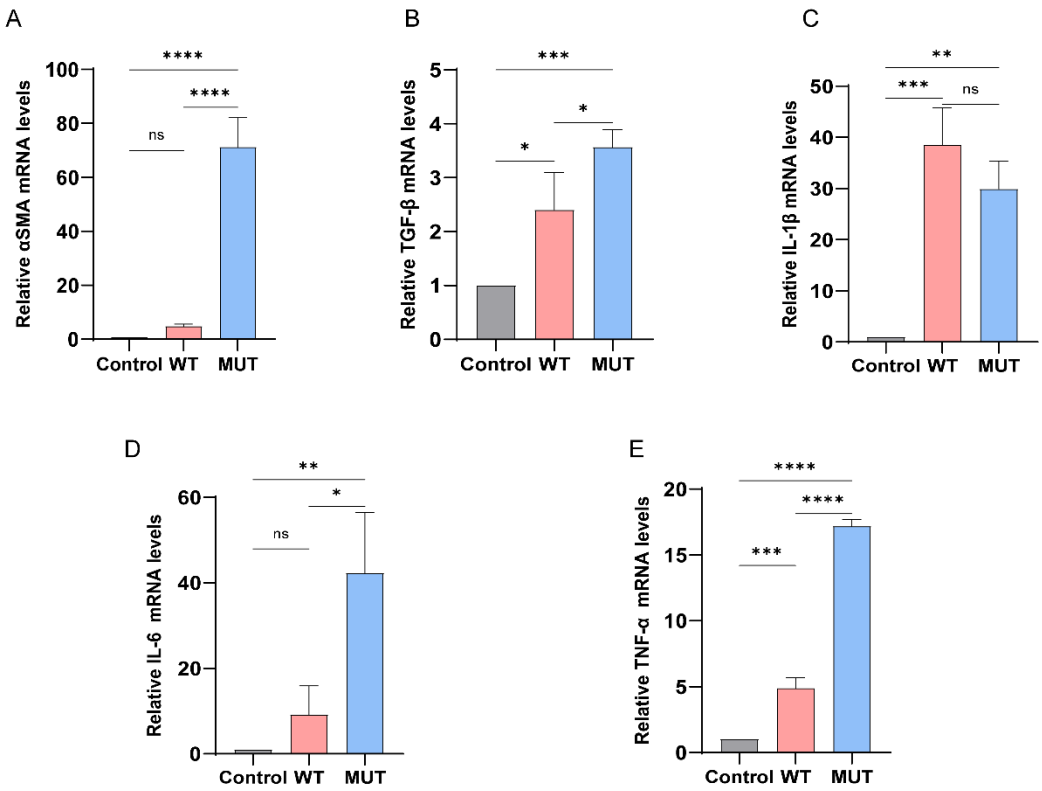


Figure 4. The expression of fibroblast activation markers and cytokines. (A) α-SMA expression levels were verified by qPCR in FGFBP2 control, wild type (WT), and mutant (MUT) groups; (B)TGF-β expression levels were verified by qPCR in FGFBP2 control, wild-type, and mutant groups; (C) IL-1β expression levels were verified by qPCR in FGFBP2 control, wild-type, and mutant groups; (D) IL-6 expression levels were verified by qPCR in FGFBP2 control, wild-type, and mutant groups; (E)TNF-α expression levels were verified by qPCR in FGFBP2 control, wild-type, and mutant groups; The results showed that the expression of α-SMA, TGF-β, IL-6 and TNF-α was significantly higher in the mutant groups than in the wild-type groups; results of WT and MUT are presented as means. (*p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant)

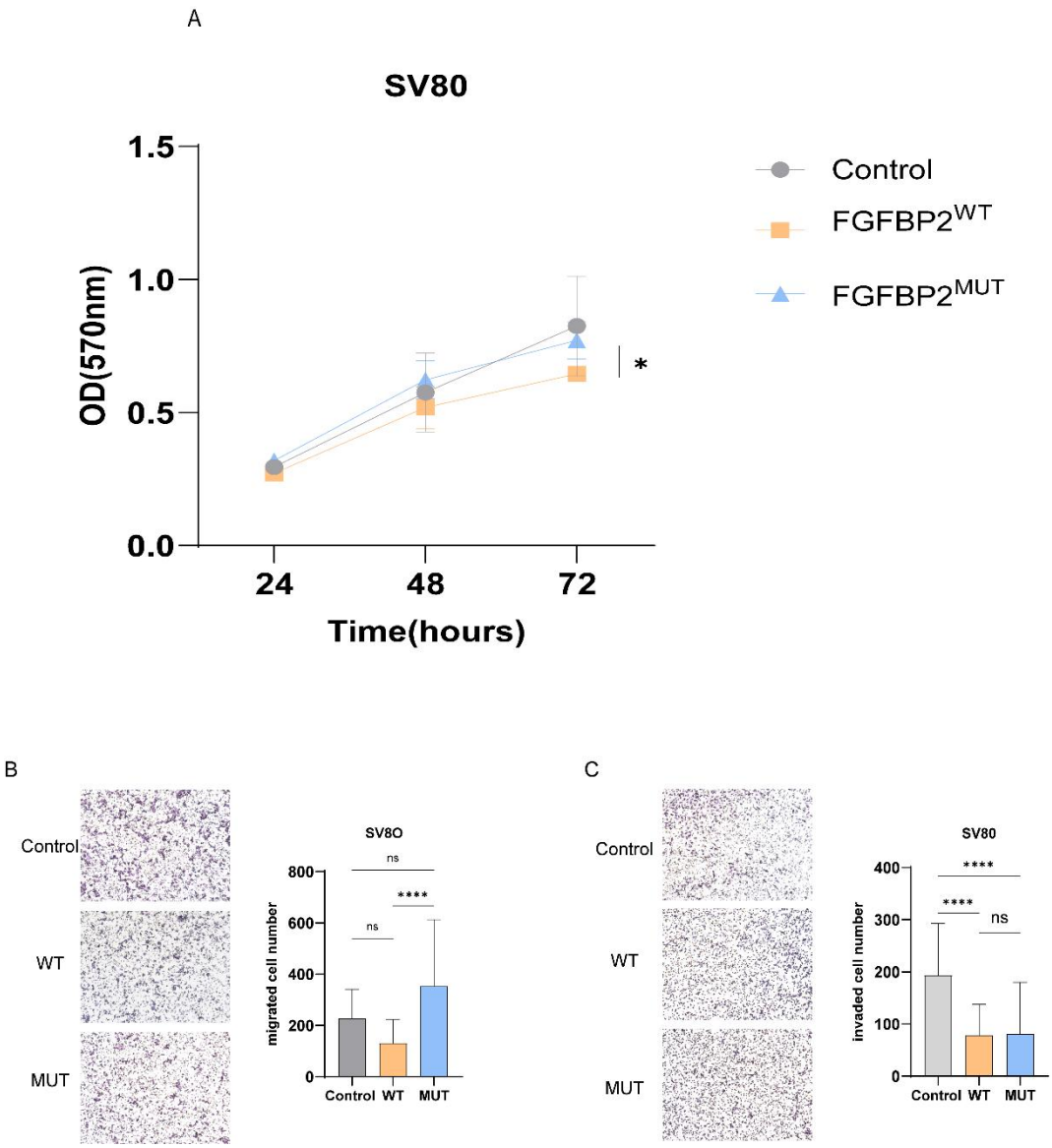


Figure 5. Functional experiments on transfected cells. (A) Proliferation of FGFBP2 control, wild type (FGFBP2^{WT}) and mutant (FGFBP2^{MUT}) groups. MTT assay showed that the cells with FGFBP2 mutation have increased proliferation ability compared to the wild type cells. (B) Cell migration ability of FGFBP2 control, wild type and mutated cells. The transwell migration assay showed that the cells with FGFBP2 mutation have an increased ability to migrate compared to the wild type cells. (C) Cell invasion ability of FGFBP2 control, wild type and mutated cells. The transwell invasion assay showed that the FGFBP2 control cells had an increased ability to invade compared to the wild type and mutated cells; results of WT and MUT are presented as means. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant)

4.2 FGFBP2 mutation promotes the proliferation and regulates cell cycle of PDAC cancer cells

It was acknowledged that activated fibroblasts promoted the initiation and progression of tumor cells. We performed MTT assay on PANC-1 cells treated with conditioned media, revealing that medium derived from the FGFBP2-mutated fibroblasts enhanced the proliferation of pancreatic cancer cells (Fig.6C). For further mechanistic insights, RNA-Seq analysis was conducted on PANC-1 cells treated with conditioned media. Differential analysis revealed that EHF was upregulated in PANC-1 cells treated with conditioned medium from FGFBP2mutated fibroblasts (Fig.6A and Table 18). ETS homologous factor (EHF) plays a pivotal and diverse role in the regulation of epithelial cell differentiation, influencing several molecular pathways that controlled cell proliferation and the maintenance of epithelial homeostasis[60, 72]. The results were validated by measuring EHF expression levels using qPCR (Fig.6B). Comparison of EHF expression between pancreatic cancer tumors and normal tissues showed significantly higher expression of EHF in tumor tissues, which was associated with poorer prognosis (Fig.7A, B). In addition, KEGG analysis revealed that FGFBP2 mutation promoted cell cycle in pancreatic cancer cells (Fig.8). Effective cell cycle control is crucial for maintaining cellular homeostasis and preventing cancer, as dysregulation of cell cycle proteins could lead to uncontrolled cell proliferation by enabling cells to bypass critical checkpoints[73]. Thus, FGFBP2 mutation in fibroblasts affected the progression of pancreatic cancer cells by modulating the cell cycle pathway and enhancing EHF expression.

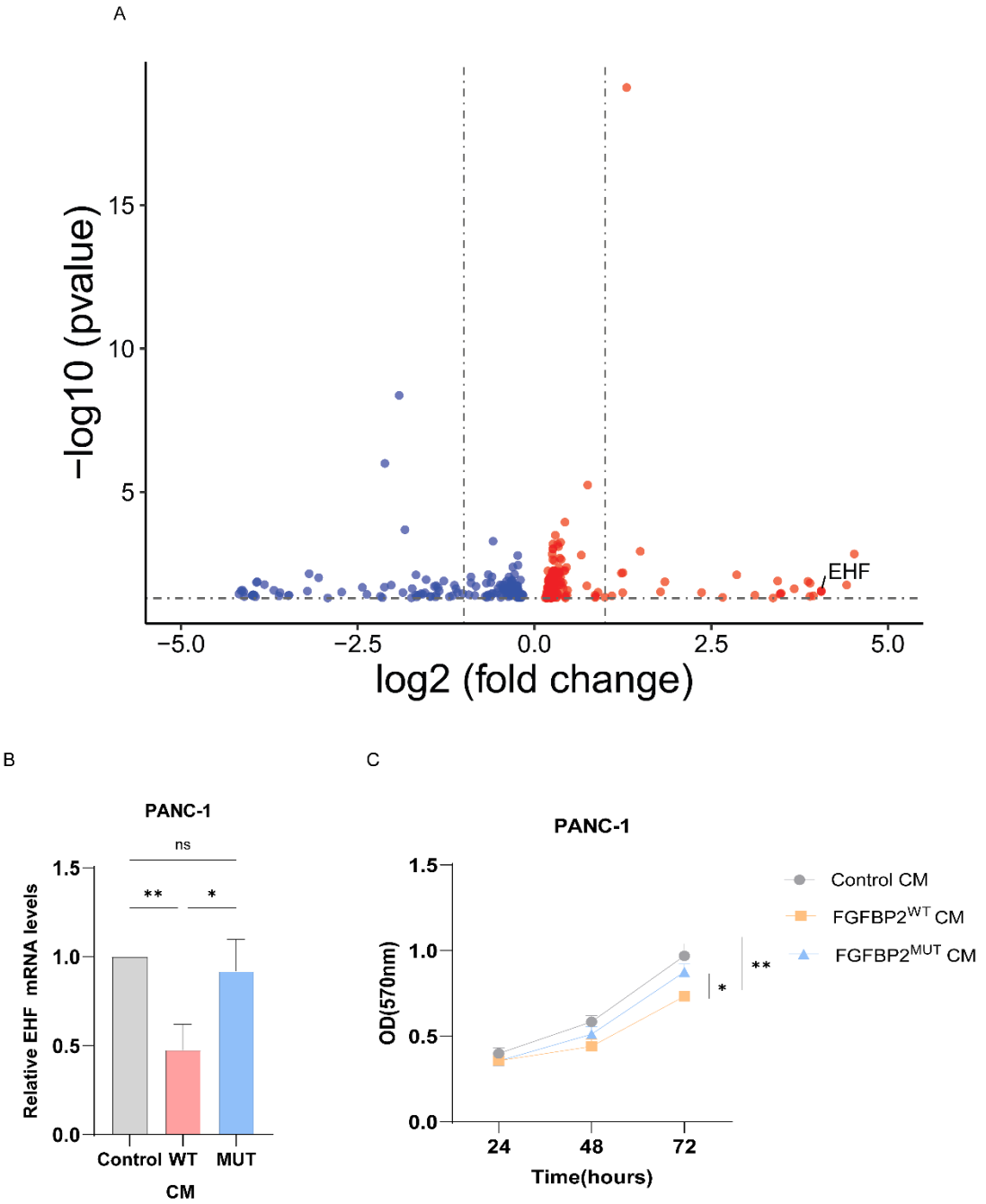


Figure 6. Differential gene expression and functional experiments on transfected cells impact on PANC-1 cells. (A) Volcano plot showing significantly altered genes (upregulation or downregulation) in PANC-1 cells treated with conditioned medium from FGFBP2 mutated and wild type cells. (B) qPCR analysis of EHF expression in PANC-1 cells treated with conditioned medium from FGFBP2 mutated and wild-type cells. (C) Cell proliferation of PANC-1 cells treated with conditioned medium from FGFBP2 control, wild type and mutated cells. MTT assay showed that the PANC-1 cells treated with conditioned medium from

FGFBP2 control and mutated cells have increased proliferation ability compared to PANC-1 cells treated with conditioned medium from FGFBP2 wild type cells. results of WT and MUT are presented as means. (*p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant)

Table 18. List of the top 25 differentially expressed genes in PANC-1 cells treated with conditioned medium from FGFBP2 mutated cells (in comparison to PANC-1 cells treated with conditioned medium from FGFBP2 wild type cells)

Genes	Log2 FC	P value
BOLA2B	4.524257	0.001452
AC073592.6	4.416684	0.017535
SOX7	4.057016	0.027718
AC008073.1	4.055854	0.029652
EHF	4.055854	0.029652
AL121829.1	3.945054	0.041583
BX255925.2	3.89824	0.015112
RPL10P19	3.897264	0.043446
C9orf131	3.868507	0.013141
MT-TK	3.674913	0.023737
RN7SL473P	3.489197	0.033895
AC008758.4	3.476591	0.035348
C4orf54	3.47574	0.036683
MT1JP	3.440079	0.012687
TATDN1P1	3.375421	0.049534
AC007881.3	3.118752	0.039749
AC007216.2	2.861564	0.007761
AL627230.3	2.661553	0.047256
HRAT5	2.36468	0.032021
RBMS2P1	1.844506	0.013563
FOS	1.783995	0.030258
FAM157C	1.496892	0.001174
EGR1	1.303457	7.96E-20
CKMT1B	1.251069	0.006557
HINT2	1.248283	0.032309

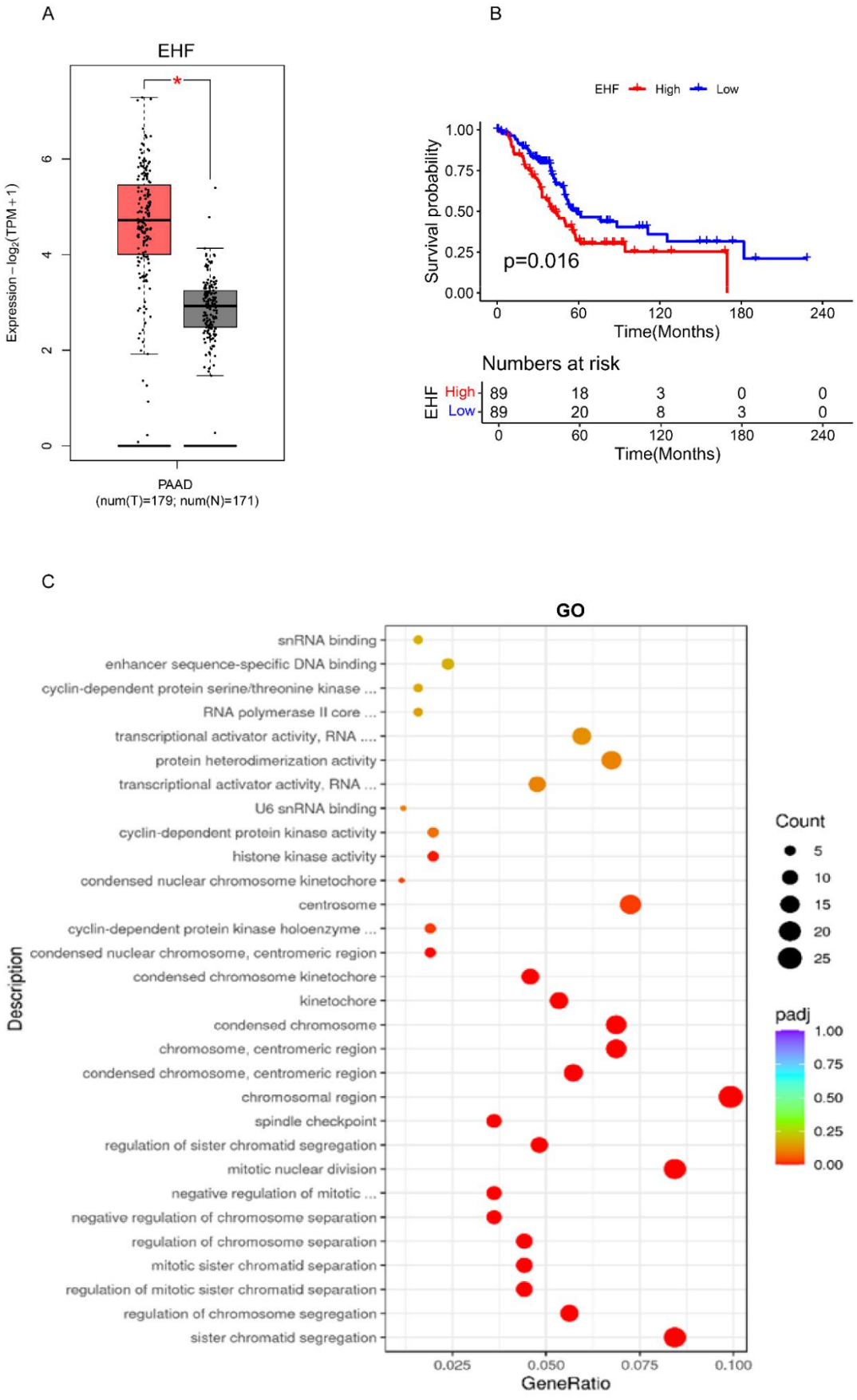

A
EHF
Expression – log2(TPM+1)
PAAD
(num(T)=179; num(N)=171)
B
EHF High Low
Survival probability
p=0.016
Time(Months)
Numbers at risk
EHF High 89 18 3 0 0
Low 89 20 8 3 0
Time(Months)
C
GO
snRNA binding
enhancer sequence-specific DNA binding
cyclin-dependent protein serine/threonine kinase ...
RNA polymerase II core ...
transcriptional activator activity, RNA ....
protein heterodimerization activity
transcriptional activator activity, RNA ...
U6 snRNA binding
cyclin-dependent protein kinase activity
histone kinase activity
condensed nuclear chromosome kinetochore
centrosome
cyclin-dependent protein kinase holoenzyme ...
condensed nuclear chromosome, centromeric region
condensed chromosome kinetochore
kinetochore
condensed chromosome
chromosome, centromeric region
condensed chromosome, centromeric region
chromosomal region
spindle checkpoint
regulation of sister chromatid segregation
mitotic nuclear division
negative regulation of mitotic ...
negative regulation of chromosome separation
regulation of chromosome separation
mitotic sister chromatid separation
regulation of mitotic sister chromatid separation
regulation of chromosome segregation
sister chromatid segregation
Description
GeneRatio
Count
padj

Figure 7. Expression and prognostic analysis of EHF in pancreatic cancer and GO analysis. (A) Correlation of EHF expression with pancreatic tumor and normal tissue. EHF was expressed at higher levels in pancreatic tumor tissue than in normal tissue. (B) The overall survival analysis of the expressed EHF. (C) Gene Ontology (GO) analysis revealed significant associations with key biological processes in PANC-1 cells treated with conditioned medium from FGFBP2 mutant and wild-type groups. The high expression of EHF was associated with a worse prognosis.

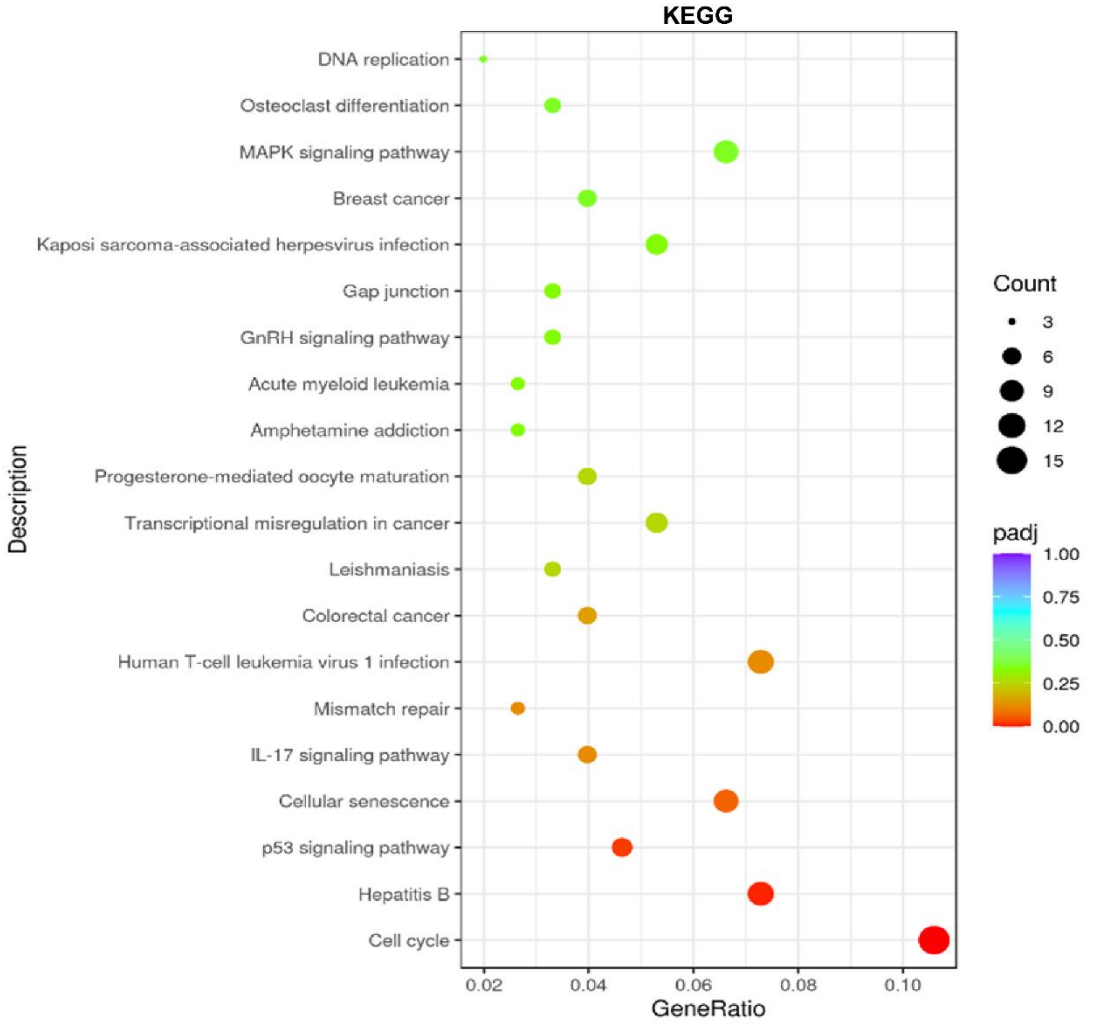


Figure 8. KEGG enrichment analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis PANC-1 cells treated with conditioned medium from FGFBP2 mutated and wild-type cells. The results showed a positive correlation with cell cycle progression.

4.3 Expression of EHF in pancreatic cancer cell clusters and its role in regulation of signaling pathways

To investigate the expression and function of EHF in different cell types, the public single-cell dataset GSE2122966 was analyzed[74]. This analysis identified 27 cell clusters (Fig.9A). The clusters were then annotated using cell cluster-specific marker genes, assigning them to 15 major cell clusters (Fig.9B). The common marker genes included: Fibroblasts (PDGFRA, COL11A1, FAP, COL1A1), Stellate cells (RGS5), CD4+ T cells (CCR7, FOXP3), CD8+ T cells (GZMK, GZMH, GZMA), NK cells (GNLY), Ductal cells (CFTR, KRT19, SOX9), Macrophages (CD163, CD68), Dendritic cells (CD1A, CD1C, CD86), B cells (CD79A, MS4A1), Endothelial cells (KDR, CDH5), Mast cells (CPA3, KIT), Endocrine cells (SCG3, PCSK1), Neutrophils (S100A8, S100A9), and Schwann cells (PLP1) (Fig.10A). To identify different cell types within ductal clusters, KRT19 and SOX9 were used to represent ductal cells, while FXYD3 and MUC1 were used to represent cancer cells[75-77]. Furthermore, CNV analysis was used to assess the malignancy of ductal cell clusters, with macrophages and NK cells serving as the reference normal set. The expression of FXYD3 and MUC1 was predominantly observed in clusters 5, 8, 17, and 18 (Fig.10B). Similarly, CNV analysis revealed that these clusters exhibited higher CNV scores compared to normal cells, leading to their classification as tumor cells (Fig.11A, B). EHF was primarily expressed in tumor cells and ductal cells, whereas its expression was almost absent in immune and stromal cells (Fig.12A, B). To analyze EHF expression in tumor cell clusters, tumor cell clusters were extracted and divided into 7 clusters. Based on the expression levels of the EHF gene, clusters 0, 1, 2, 3, 4, 5, and 6 were defined as EHF+ tumor cells, whereas cluster 7, which shows almost no expression, was defined as EHF- tumor cells (Fig.13A, B). KEGG analysis of EHF revealed significant enrichment in the PI3K-AKT signaling pathway, ECM-receptor interactions and cell adhesion molecules (Fig.14). The PI3K-AKT pathway was essential for cell growth, while ECM-receptor interactions were critical for cell adhesion and migration[78]. These results suggested that EHF might have played a role in regulating these pathways involved in pancreatic cell proliferation

and tumor progression.

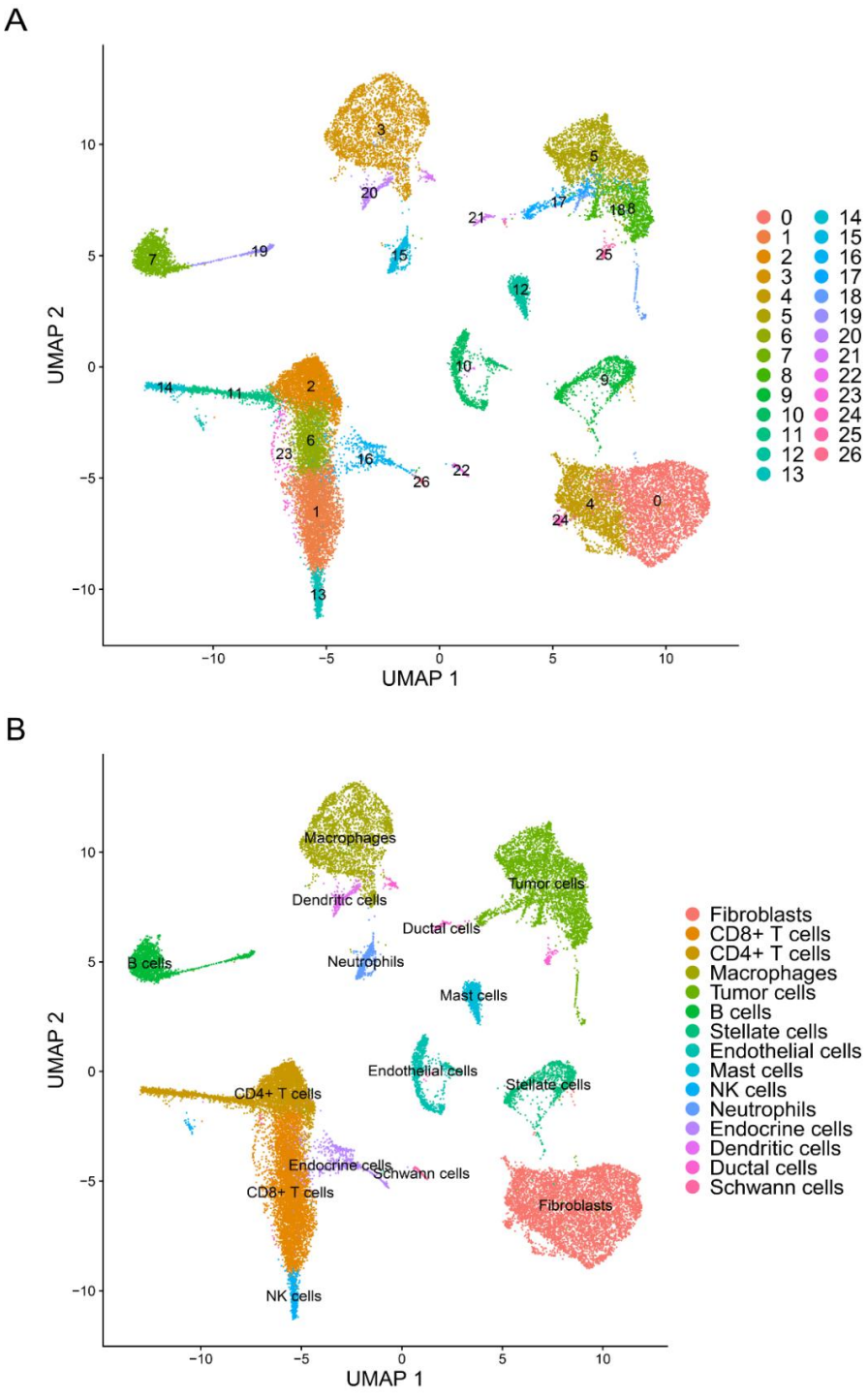


Figure 9. Single-cell RNA sequencing integration and annotation. (A) Uniform Manifold Approximation and Projection (UMAP) plot displays an integrated cellular map composed of 27 cell clusters. (B) UMAP plot displays an integrated cellular map composed of 15 annotated cell types.

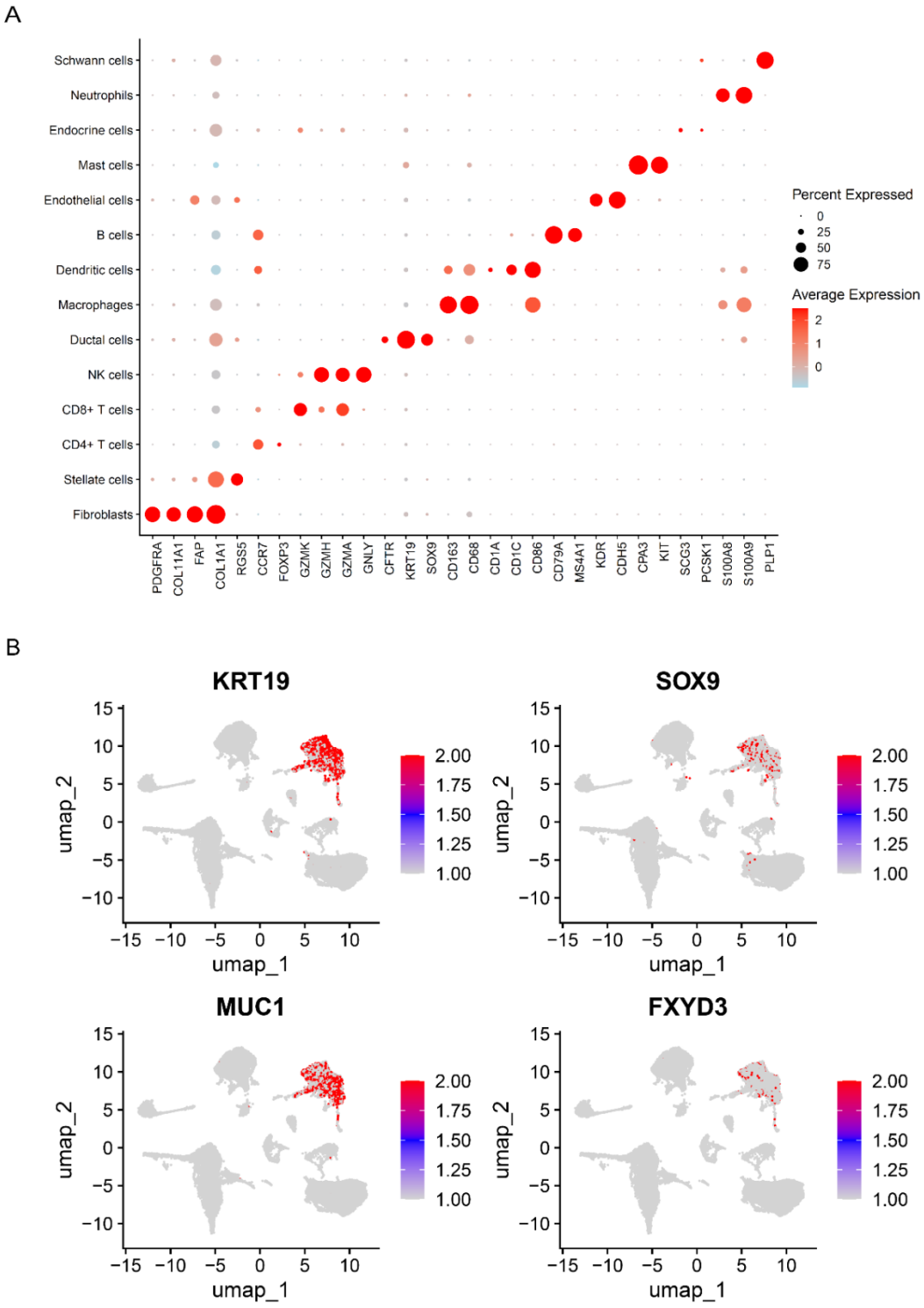
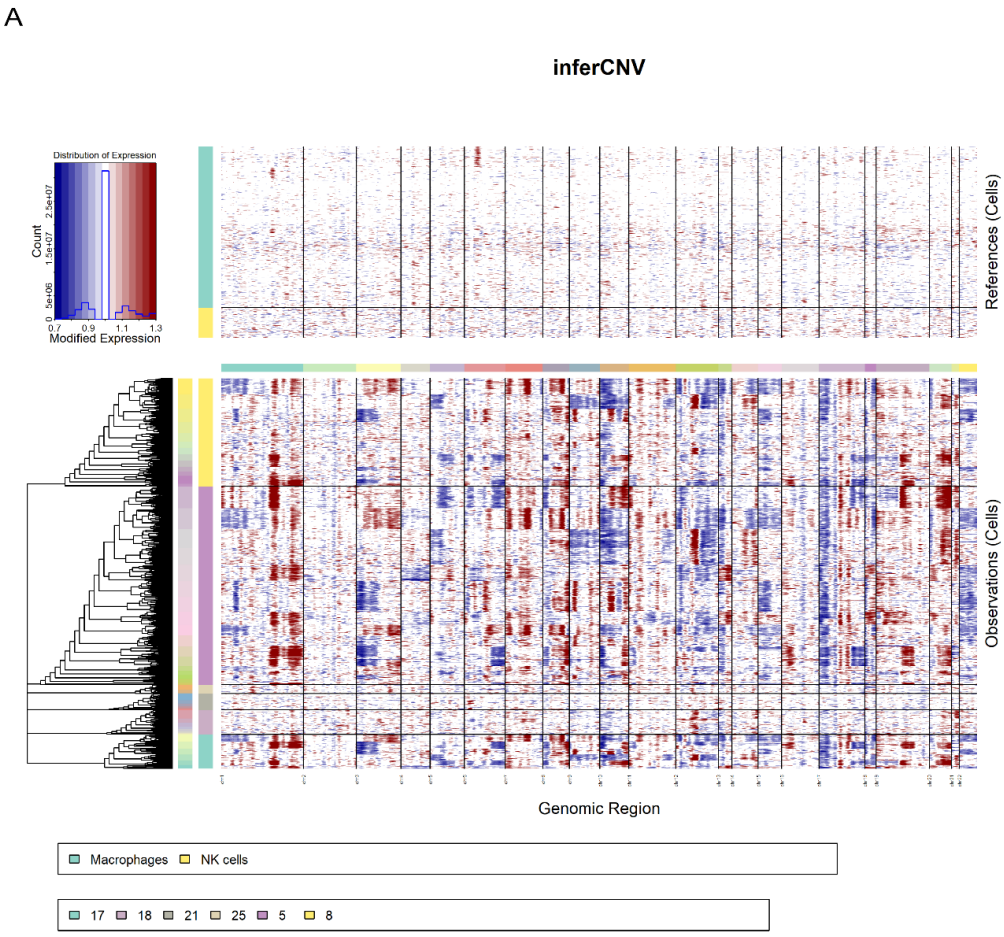


Figure 10. Single-cell RNA sequencing marker gene annotation. (A) Dot plot showing representative common marker genes in cell types. (B) UMAP shows ductal cell markers and cancer cell markers. The results showed that the expression of FXYD3 and MUC1 was predominantly observed in clusters 5, 8, 17, and 18.



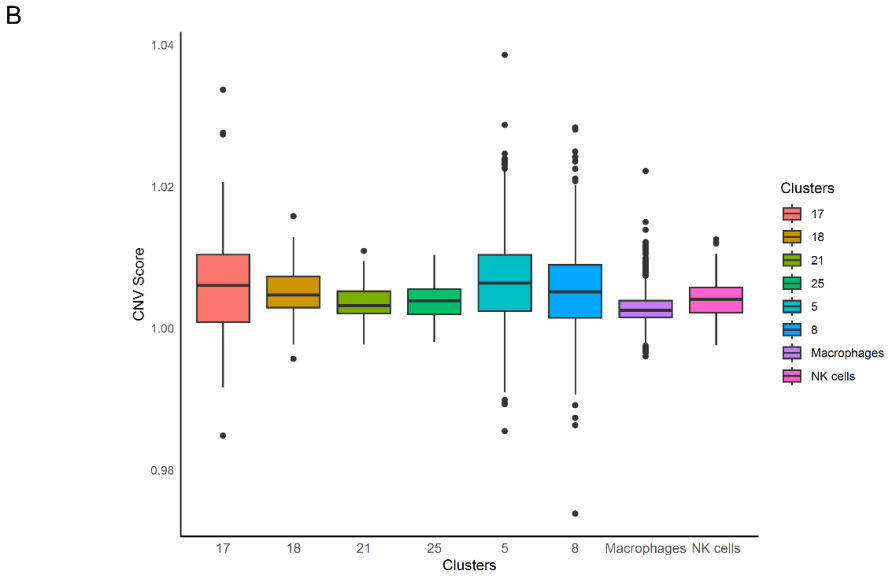


Figure 11. Assessment of CNV in ductal cells. (A) Heatmap showing ductal cells with different CNV levels. (B) Box Plot showing ductal cells with CNV scores. The ductal cells in clusters 5, 8, 17, and 18 had higher CNV scores compared to the macrophages and NK cells that served as the reference normal set.

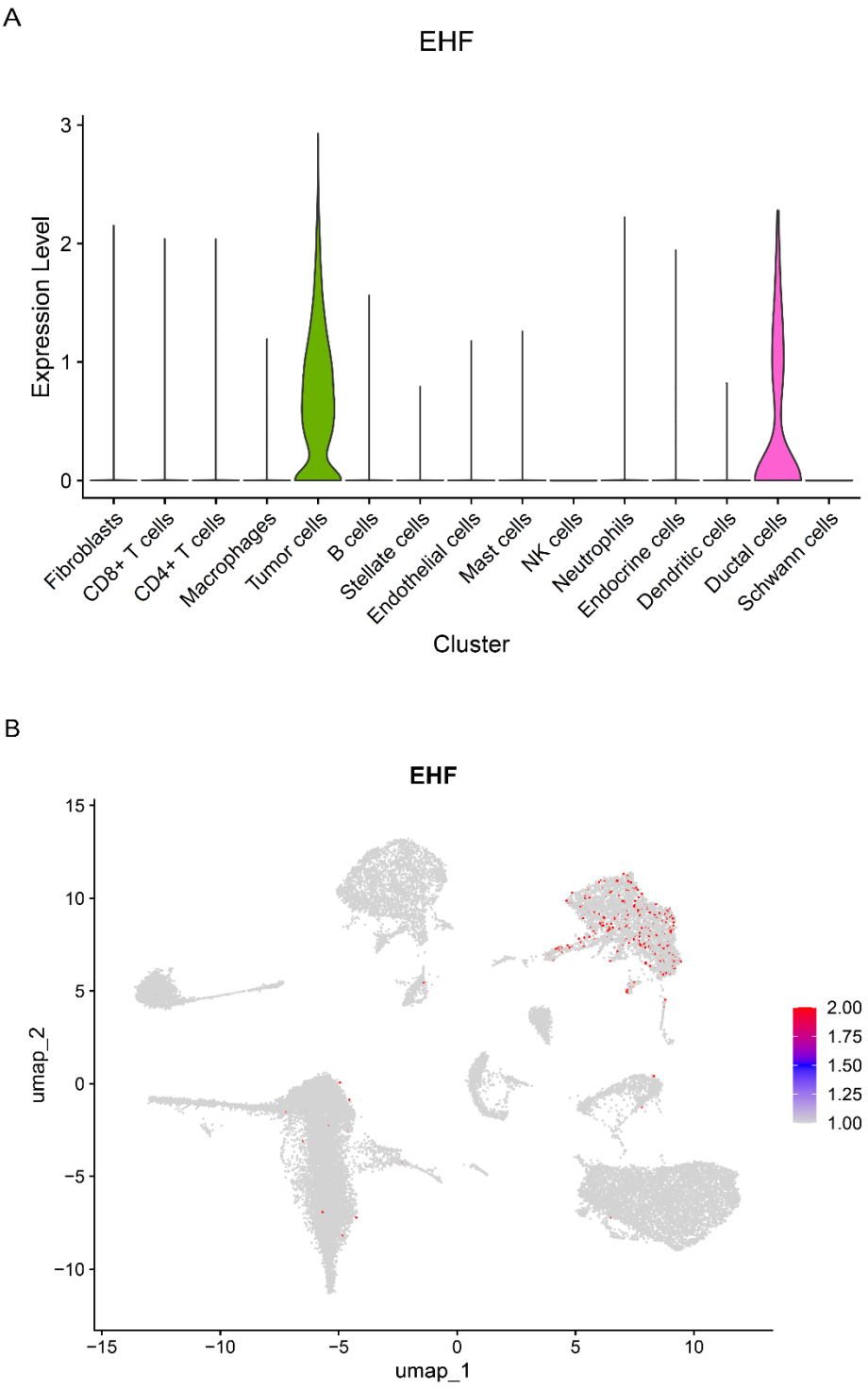


Figure 12. Expression of EHF in different cell types. (A) VInPlot showing EHF expression in each cell type. (B)UMAP showing EHF expression in each cell type. EHF was primarily expressed in tumor cells and ductal cells.

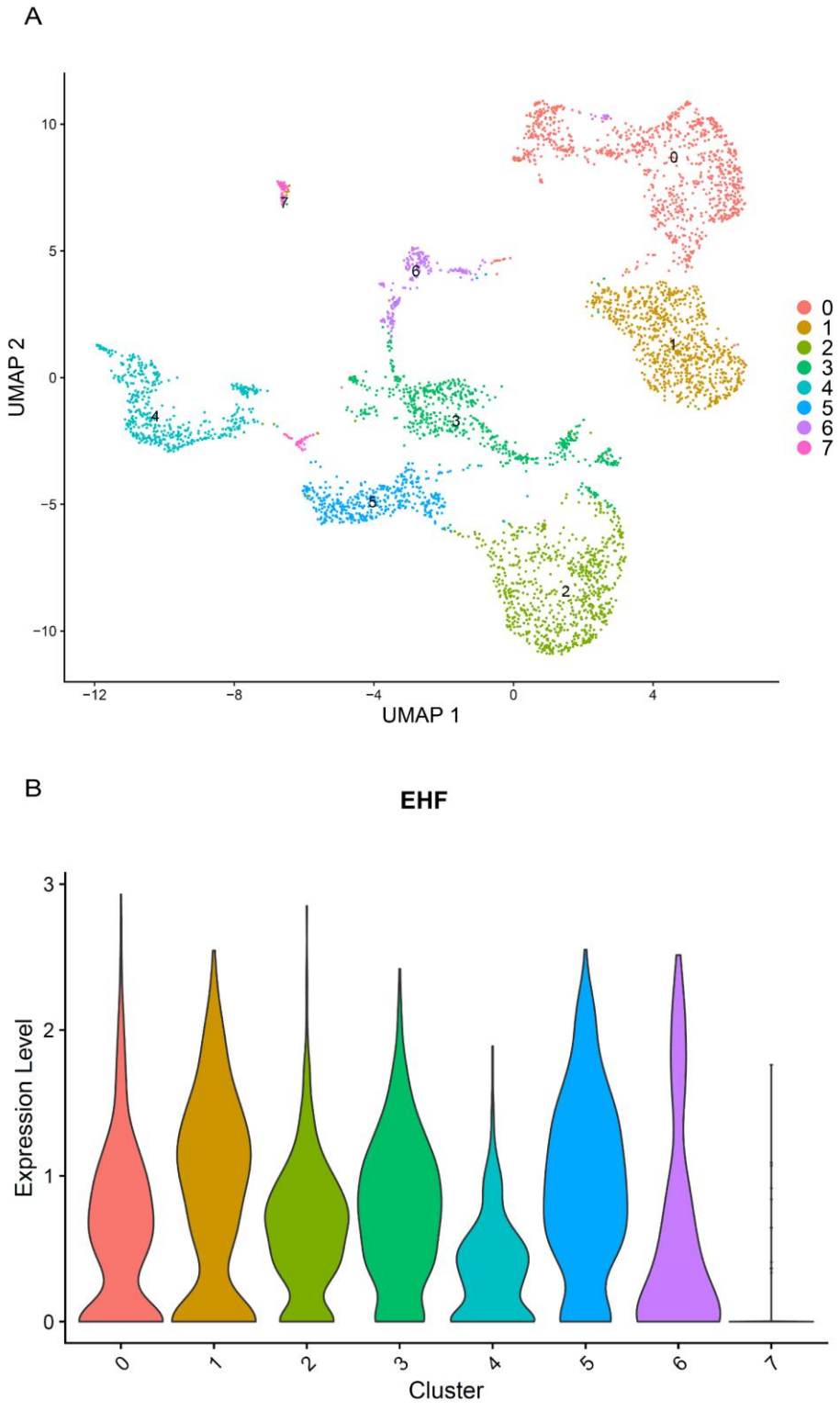


Figure 13. Tumor cell clustering and EHF expression. (A) UMAP showing seven tumor cell clusters. (B) VlnPlot showing EHF expression in tumor cell clusters.

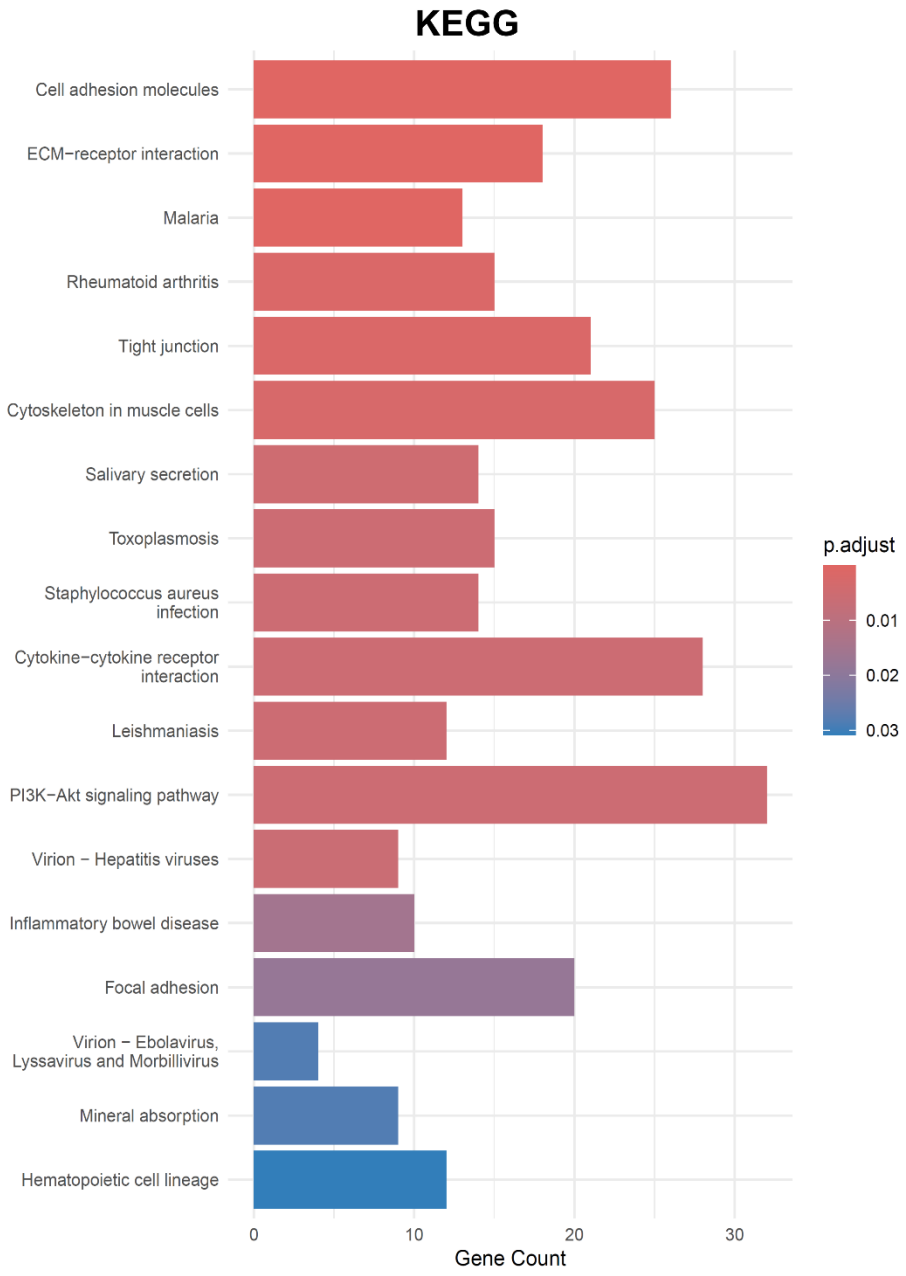


Figure 14. KEGG enrichment analysis. Barplot showing the KEGG enrichment analysis of EHF+ tumor cells and EHF- tumor cells. The results showed a positive correlation with PI3K-AKT signaling pathway, ECM-receptor interactions and cell adhesion molecules.

4.4 EHF regulates fibroblast signaling through the PDGF pathway

To further explore the role of EHF in pancreatic cancer and fibroblasts, CellChat was used to

analyze the intercellular signaling pathways and ligand–receptor interactions (Fig.15A, B). In the PDGF signaling pathway, EHF+ tumor cells regulated fibroblasts through PDGF/PDGFR interactions (Fig.15A, B and Fig.16A-D). EHF primarily drove its effects through interactions between platelet-derived growth factor alpha (PDGFA)/platelet-derived growth factor beta (PDGFB) ligands in tumor cells and PDGFRB receptors in fibroblasts (Fig.16B, D). PDGFA/B are expressed in a broad range of cell types and utilize autocrine or paracrine mechanisms to perform their biological functions[79, 80]. Tumor cells released paracrine signals, including transforming growth factor (TGF-β) and platelet-derived growth factor (PDGF), which activated fibroblasts[41, 81]. Platelet-derived growth factor receptor alpha (PDGFRα) and platelet-derived growth factor receptor beta (PDGFRβ), predominantly expressed in fibroblasts, serve as key markers for these cells[82]. Moreover, PDGFRα controlled the fibroblast-to-myofibroblast transition during wound healing[83]. These results suggested that EHF might have driven fibroblast and remodeling of the tumor microenvironment by modulating PDGF signaling.

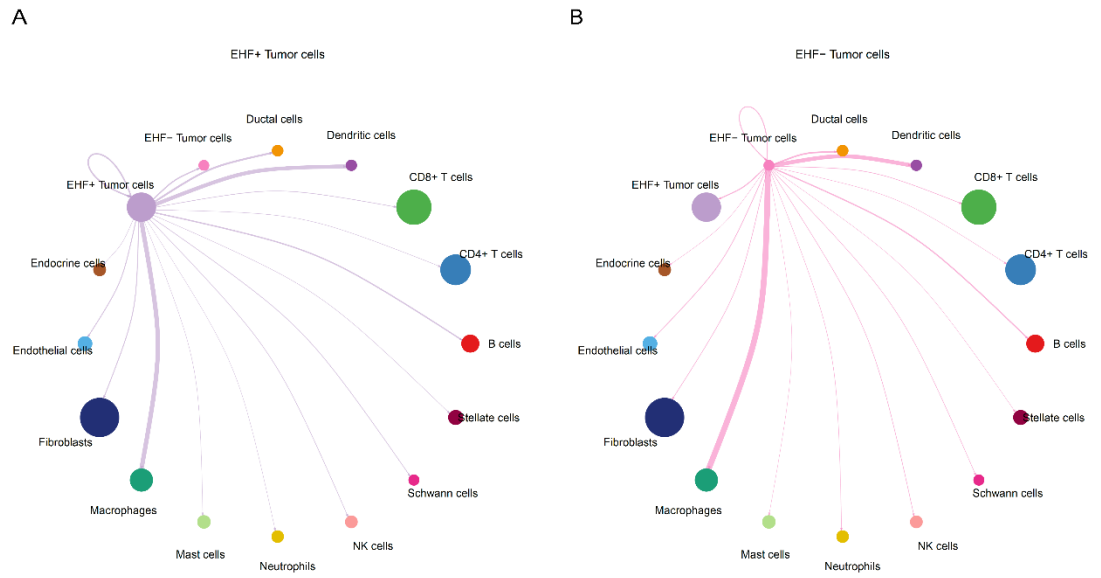


Figure 15. Interaction between EHF + tumor cells and fibroblasts. (A) Circle plot of the cell–cell communication network among EHF+ tumor cells and other cell types, with edge widths representing the strength of the interactions. (B) Circle plot of the cell–cell communication network among EHF- tumor cells, and other cell types, with edge widths representing the strength of the interactions.

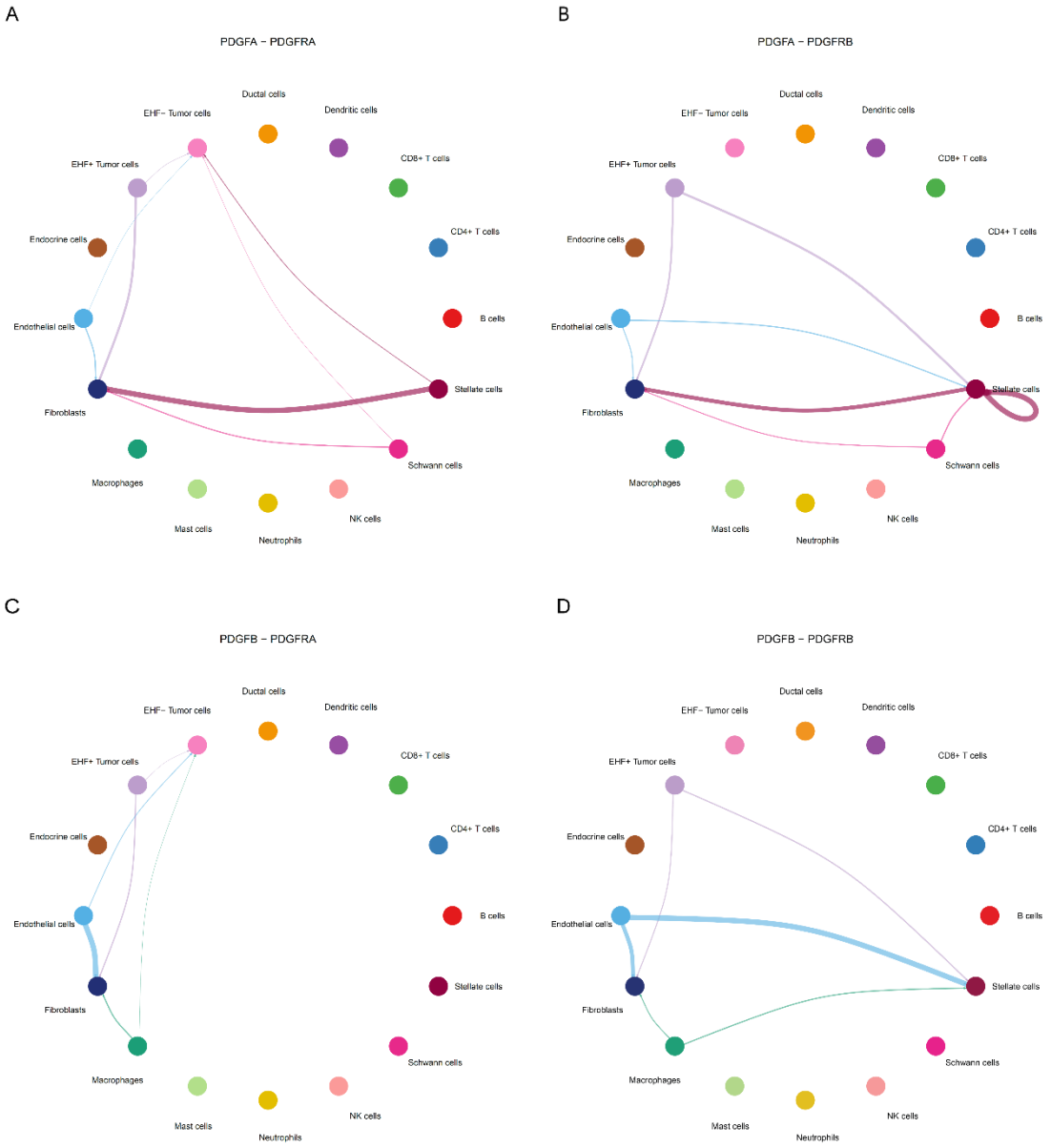


Figure 16. Interaction between the ligands and receptors of the PDGF pathway. (A) Circle plot of the cell–cell communication network among PDGF signaling pathway ligand–receptor pairs: PDGFA/PDGFRA. (B) Circle plot of the cell–cell communication network among PDGF signaling pathway ligand–receptor pairs: PDGFA/PDGFRB. (C) Circle plot of the cell–cell communication network among PDGF signaling pathway ligand–receptor pairs: PDGFB/PDGFRA. (D) Circle plot of the cell–cell communication network among PDGF signaling pathway ligand–receptor pairs: PDGFB/PDGFRB. Interaction of the PDGF signaling pathway was observed in EHF+ tumor cells and fibroblasts, whereas no interaction occurred in EHF- tumor cells.

4.5 The expression of FGF2 is suppressed in fibroblasts carrying FGFBP2-mutation

FGF2 plays a critical role in cell proliferation and cancer development, and studies showed that FGFBP2 interacts with FGF2 to enhance its biological activity[47]. Using the STRING database, we identified interactions involving FGFBP2, but its function following mutation remains unclear (Fig.17A). Interestingly, FGF2 expression was also downregulated in fibroblasts with mutated FGFBP2 (Fig.17B). A mutation in the gene altered the DNA binding capacity, impairing the capacity of FGFBP2 to bind DNA and suggesting that FGFBP2 might regulate FGF2 expression[84]. This suggested that the mutation of FGFBP2 might lead to a reduction in FGF2 expression, indicating a potential regulatory relationship between the two proteins.

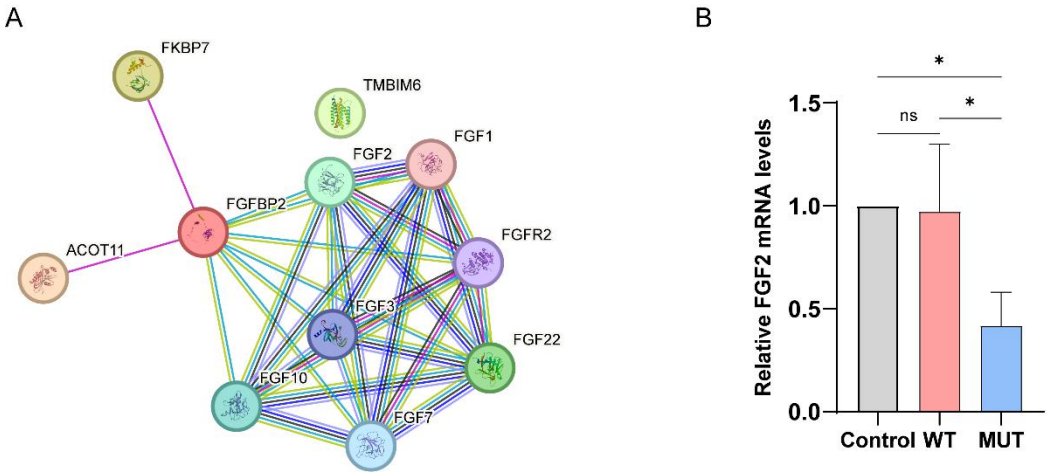


Figure 17. FGFBP2 mutation downregulates FGF2. (A) STRING Analysis of the interaction between FGFBP2 and FGF2. (B) FGF2 expression levels were verified by qPCR in FGFBP2 control (Control), wild-type (WT), and mutant groups (MUT); means with SD; (*p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant)

4.6 Pancreatic cancer cells are affected by fibroblastic FGFBP2 mutation in ERK1/2 signaling manner

FGF2 regulated key biological processes such as cell proliferation and migration by activating FGFR and downstream pathways, including Ras-MAPK (ERK1/2, p38 MAPK, JNK) and PI3K-Akt[49]. To investigate the effect of FGF2 on cancer cells, we cultured PANC-1 cells using conditioned medium (CM) supplemented with rhFGF2 or CM obtained from three groups of FGFBP2-transfected cells, with EGF as a control. Either CM or rhFGF2-CM and rhEGF-CM increased the activation of the canonical ERK1/2 signaling cascade by increasing phosphorylation (Fig.18A, B, Fig.19A, B and Fig.20A, B). In addition, rhEGF-CM promoted the activation of the canonical AKT signaling cascade through increased phosphorylation (Fig.20A, C), whereas CM or rhFGF2-CM did not significantly enhance AKT signaling activation through phosphorylation (Fig.18A, C and Fig.19A, C). Moreover, conditioned medium derived from FGFBP2-mutant cells enhanced ERK1/2 signaling activation via phosphorylation more significantly than that derived from wild-type cells (Fig.18A, B). It is known that inhibition of ERK1/2 suppressed EMT, upregulated cellular senescence markers and activated autophagy in cancer-associated PSCs, which in turn reduced pancreatic cancer cell proliferation[85]. These results suggested that FGFBP2 mutation promoted pancreatic cancer cell progression through increased activation of ERK1/2 signaling through phosphorylation.

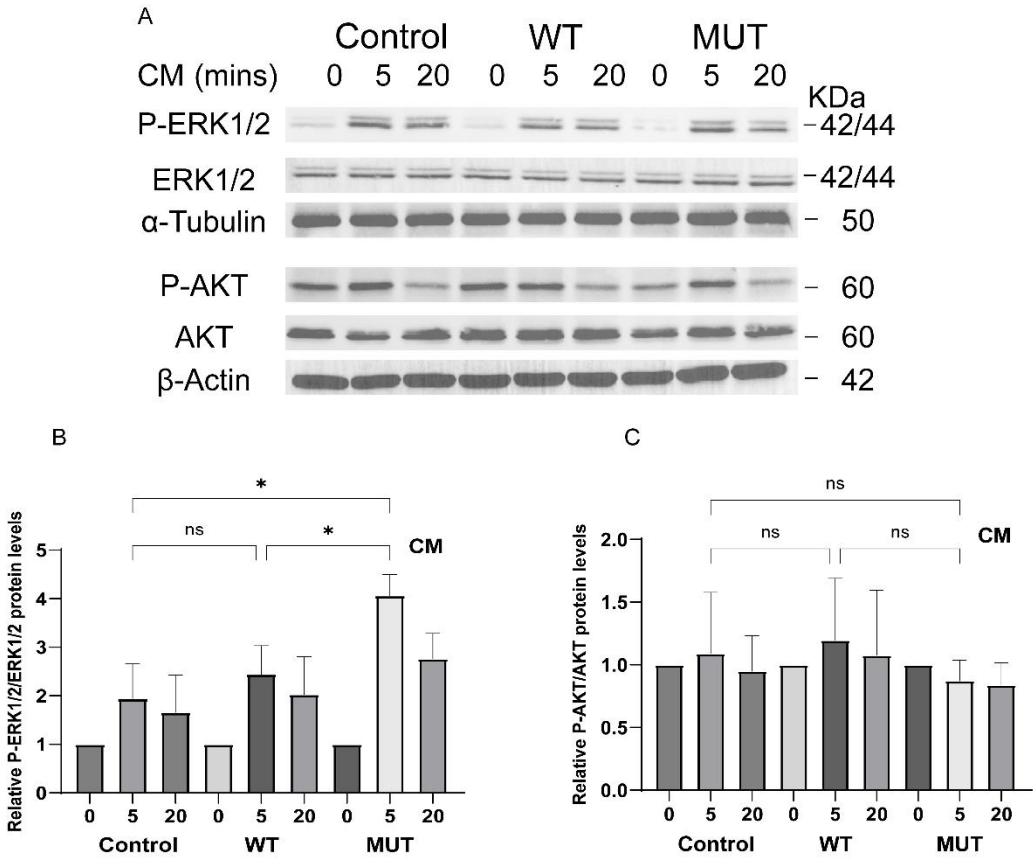


Figure 18. ERK1/2 and AKT signaling on PANC-1 treated with conditioned media derived from transfected fibroblasts. (A-C) Western blot for ERK1/2, P-ERK1/2, AKT, and P-AKT. in PANC-1 cells treated with conditioned medium of FGFBP2 control (Control), wild type (WT), and mutant groups (MUT). Phosphorylation upon treatment with conditioned medium derived from FGFBP2 mutant fibroblasts results in increased activation of the ERK1/2 signaling pathway compared to wild-type and control.; means with SD of three independent experiments; results are presented as ratios of phosphorylated and unphosphorylated forms of the proteins; (*p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant)

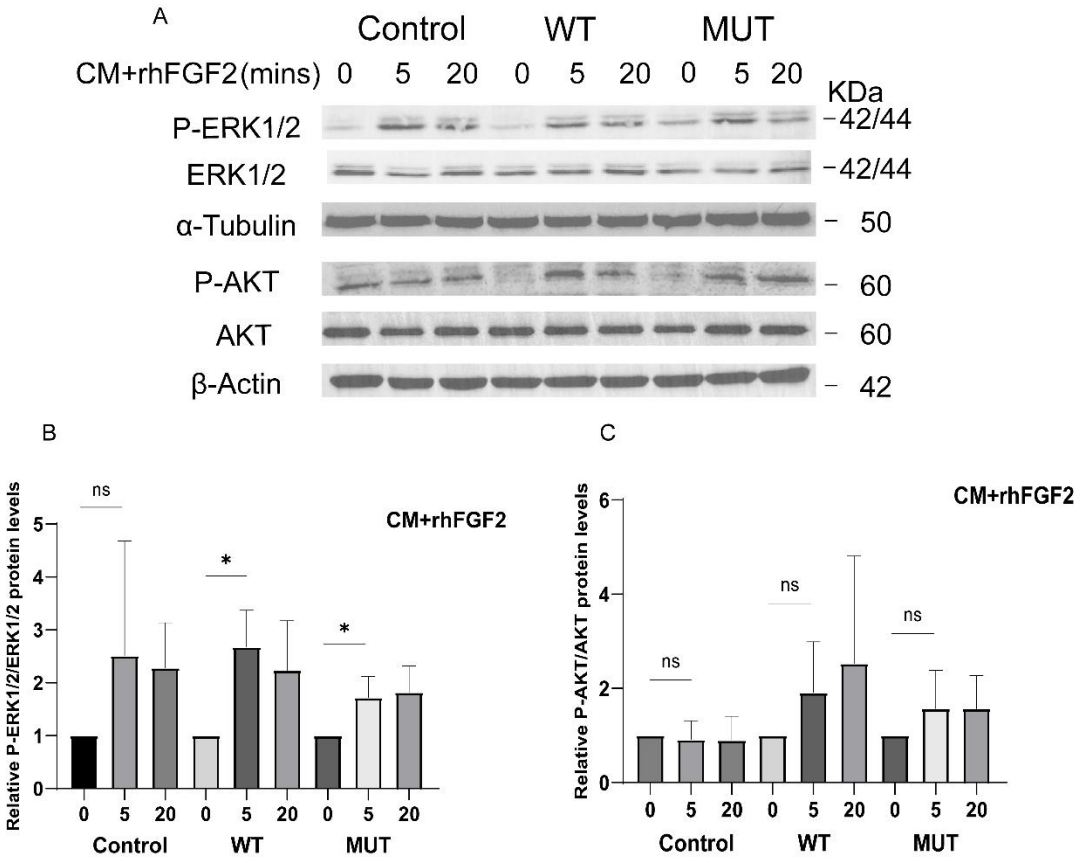


Figure 19. ERK1/2 and AKT signaling on PANC-1 treated with conditioned media derived from transfected fibroblasts supplemented with rhFGF2. (A-C) Western blot for ERK1/2, P-ERK1/2, AKT, and P-AKT. in PANC-1 cells treated with conditioned medium of FGFBP2 control (Control), wild type (WT), and mutant groups (MUT) supplemented with rhFGF2. The results showed that rhFGF2 activated the ERK1/2 pathway by promoting the phosphorylation of ERK1/2.; means with SD of three independent experiments; results are presented as ratios of phosphorylated and unphosphorylated forms of the proteins; (*p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant)

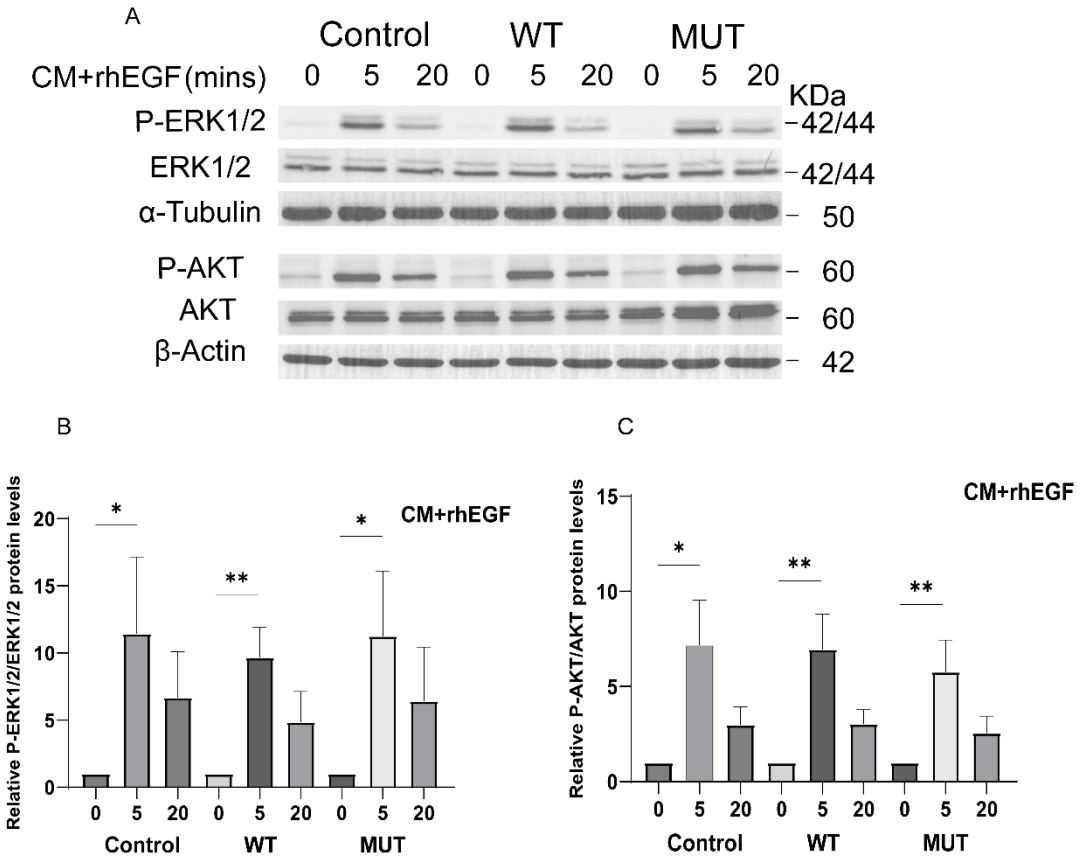


Figure 20. ERK1/2 and AKT signaling on PANC-1 treated with conditioned media derived from transfected fibroblasts supplemented with rhEGF. (A-C) Western blot for ERK1/2, P-ERK1/2, AKT, and P-AKT. in PANC-1 cells treated with conditioned medium of FGFBP2 control (Control), wild type (WT), and mutant groups (MUT) supplemented with rhEGF. The results showed that rhEGF activated the ERK1/2 and AKT pathways by promoting the phosphorylation of ERK1/2 and AKT.; means with SD of three independent experiments; results are presented as ratios of phosphorylated and unphosphorylated forms of the proteins; (*p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant)

5. Discussion

The poor prognosis of PDAC can be largely attributed to our limited understanding of the intricate interactions between molecular and cellular components within the TME. Cancer-associated fibroblasts (CAFs) play a critical role in the tumor microenvironment by modulating pro-tumorigenic processes and secreting various cytokines. Here we report the role of FGFBP2 mutation in facilitating fibroblast activation and promoting tumor progression.

5.1 Mutation in fibroblastic FGFBP2 and activation of the cells

Fibroblast activation is influenced by a variety of factors, including mechanical stress, inflammatory signals, and interactions with other cell types in the microenvironment[86]. Transforming growth factor β (TGF-β) signaling is widely recognized as central of the activation of cancer-associated fibroblasts (CAFs) by promoting factors that enhanced cancer invasiveness[87, 88]. In addition, TGF-β1 could induce resident normal fibroblasts (NFs) to transform into CAFs in various tumor types, such as pancreatic cancer, underscoring that TGF-β1-driven CAF generation is a common event during cancer development[89]. Cancer-associated fibroblasts (CAFs) promote tumor growth by promoting angiogenesis, proliferation, invasion, and inflammation, a pro-inflammatory state that cancer cells can also induce in normal fibroblasts, while inflammatory modulators such as interleukin-1 (IL-1) activated CAFs via NF-κB[90]. In breast cancer progression, mechanical factors such as matrix stiffening activated Yes-associated protein (YAP) in cancer-associated fibroblasts. This activation then drove actomyosin contractility and extracellular matrix remodeling to enhance cancer cell invasion and angiogenesis. Meanwhile, transient Rho-associated coiled-coil containing protein kinase (ROCK) inhibition could disrupt this self-reinforcing loop and reverse the CAF phenotype[91]. In prostate cancer, overexpression of Yes-associated protein 1 (YAP1) in stromal cells converted normal fibroblasts into CAFs, promoting tumor progression and poor prognosis, whereas silencing YAP1 effectively inhibited tumor growth[92].

This study demonstrated that FGFBP2 mutation promoted fibroblast activation through both direct mechanisms and the upregulation of various inflammatory factors. In previous pancreatic cancer studies, quiescent pancreatic stellate cells (PSCs) showed increased expression of the CAF-like surface marker α-SMA upon activation by TGF-β, resulting in their conversion to activated CAFs[93]. Similarly, FGFBP2 mutation in fibroblasts led to upregulated expression of α-SMA, further supporting their transition to activated CAFs. Additionally, activated fibroblasts produce extracellular matrix (ECM) components and secrete a wide range of cytokines, including TGF-β, TNF-α, IL-6, and IL-1β[36, 37, 40]. Our results indicated that FGFBP2 mutation in fibroblasts upregulates the production of TGF-β, TNF-α, and IL-6. Previous studies showed that activated fibroblasts exemplified the paracrine function of fibroblasts by secreting cytokines that created an IL-1β-enriched microenvironment, which promoted ER^+ breast cancer cell proliferation and reduced tamoxifen efficacy[94]. The crosstalk between CAFs and tumor cells mainly takes place through paracrine signaling, where CAFs secrete various growth factors and cytokines, such as FGF2, EGF, and TGF-β, which effectively promote tumor cell proliferation in vitro[82].

5.2 Functional consequences of FGFBP2 mutation in the fibroblasts

Activated fibroblasts acquired enhanced proliferative, migratory, and secretory capabilities that not only drove extensive extracellular matrix remodeling and immunomodulatory functions, but also facilitated the development of drug resistance, collectively establishing a tumor microenvironment that impeded therapeutic efficacy. Within the tumor microenvironment, the reactive stroma was largely defined by cancer-associated fibroblasts (CAFs), also known as activated fibroblasts. These cells communicated directly with cancer cells through cell-cell adhesion, while also indirectly shaping tumor progression and modulating immune cell infiltration[95, 96]. FGFBP2 mutation in fibroblasts led to enhanced proliferation and migration, reflecting characteristics consistent with those of activated fibroblasts. Cancer-associated fibroblasts (CAFs) influence cancer cell function by secreting excessive factors and activating

signaling pathways that regulate proliferation, migration and immune modulation, revealing the complex interactions between CAFs and cancer cells within the tumor microenvironment. In colorectal cancer, increased insulin-like growth factor 2 (IGF2) secretion by CAFs activated an IGF1R-YAP1 signaling cascade in cancer cells that drove tumor growth, migration, and invasion, while targeting insulin-like growth factor 1 receptor (IGF1R) and YAP1 enhanced anti-tumor efficacy[97]. A study demonstrated that tumor-activated fibroblasts overexpressed Galectin-1 (Gal-1) and released macrovesicles (MVs) enriched with this protein, which, upon uptake by tumor cells, enhanced cancer cell migration[98]. The secretory function of CAFs plays a critical regulatory role in tumor cells. A study showed that the lin-28b/let-7 signaling pathway regulated the expression of Wnt family member 5a (Wnt5a) in tumor epithelium. This in turn, upregulated lin-28 homolog b (lin-28b) in cancer-associated fibroblasts (CAFs) and promoted PDAC growth by inducing the production of the cytokine pro-protein convertase subtilisin/kexin type 9 (PCSK9)[99].

We treated PANC-1 cells with conditioned media and observed that medium derived from FGFBP2 mutant fibroblasts enhanced PANC-1 cell proliferation, suggesting that this mutation promoted tumor cell growth through paracrine signaling and intercellular crosstalk. Furthermore, RNA-Seq analysis of PANC-1 cells treated with conditioned medium revealed that FGFBP2-mutant fibroblasts regulated tumor cell proliferation by modulating cell cycle signaling pathways. The cell cycle signaling pathway involved a balance between cyclins and cyclin-dependent kinases, which regulated cell cycle progression and maintained cellular integrity. Dysregulation of these pathways leads to uncontrolled cell proliferation and tumor growth[100, 101].

5.3 Relations between fibroblastic FGFBP2 and EHF in pancreatic cancer

Fibroblasts secret various cytokines that regulated changes in tumor cell genes, thereby modulating tumor cell functions. These secreted factors could influence tumor cell proliferation,

play a critical role in the tumor microenvironment, and contribute to tumor progression. Feldmann et al.[102] showed that the Prrx1 transcription factor was critical for tuning CAF activation, allowing a dynamic switch between dormant and activated states. Its regulation of CAF plasticity significantly impacted tumor differentiation, gemcitabine resistance, and metastasis in pancreatic ductal adenocarcinoma (PDAC). More importantly, we found that EHF expression was upregulated in cancer cells treated with conditioned medium from FGFBP2-mutant fibroblasts. EHF is a member of the ETS family of transcription factors, which regulate key processes such as differentiation, proliferation and epithelial homeostasis, and play diverse roles in cancer progression and the tumor microenvironment. In colorectal cancer, EHF activated TGF‐β signaling, leading to tumor progression[103]. Analysis of the TCGA data showed that EHF was highly expressed in pancreatic cancer tissue and was associated with a better prognosis. Analysis of single cell data further supported that EHF was predominantly highly expressed in pancreatic cancer cells. KEGG analysis indicated that EHF regulated the PI3K-AKT signaling pathway, ECM-receptor interactions and cell adhesion molecules. Therefore, FGFBP2 mutation in fibroblasts may lead to the upregulation of EHF in tumor cells, contributing to cancer cell progression. Tumor cells may further promote fibroblast activation. Chen et al.[104] reported that the loss of Annexin A1 (ANXA1) expression in epithelial cells during the progression of esophageal squamous cell carcinoma (ESCC) disrupted fibroblast homeostasis by activating the ANXA1/FPR2 signaling pathway. This led to the transformation of normal fibroblasts into cancer-associated fibroblasts (CAFs), thereby promoting tumor progression. Similar studies reported that EHF expression in pancreatic stellate cells (PSCs) influenced PDAC chemoresistance and tumor growth by activating PSCs through the transcription of α-SMA, collagen-I, and IL-1β. IL-1β further upregulated EHF in PSCs, and this IL-1β/EHF feedback loop contributed to tumor progression[105]. Moreover, our results showed that EHF regulated fibroblast signaling through the PDGF pathway, which influenced key cellular processes such as fibroblast proliferation and migration[106]. The PDGF signaling pathway was essential for regulating fibroblast development and long-term homeostasis by supporting the self-renewal, proliferation and migration of fibroblast stem cells[18]. By interacting with PDGF receptors, EHF may regulate fibroblast activation and ECM production, promoting tumor cell progression[107].

5.4 Association with FGF signaling

Mutations generally resulted in significant changes in DNA, leading to alterations in numerous downstream molecules[84]. FGFBP2 enhances FGF2 signaling by releasing FGF2 from the extracellular matrix, thereby promoting its biological activity[47]. Through STRING data analysis, the direct connection between FGFBP2 and FGF2 suggested a physical or functional interaction, with FGFBP2 binding to FGF2 and facilitating its release from the extracellular matrix, thereby enhancing its signaling activity. However, the binding between these both factors after FGFBP2 mutation remains uncertain. Our results show that FGF2 expression was also downregulated in fibroblasts with mutated FGFBP2. Fibroblast growth factor (FGF) signaling plays a crucial role in the activation of fibroblasts[18, 48]. Inoue et al. [108] found that mast cell-derived basic fibroblast growth factor (bFGF) promoted fibrogenic and proliferative effects on smooth muscle cell/myofibroblast-like cells in idiopathic pulmonary fibrosis (IPF) and lymphangioleiomyomatosis (LAM) through its receptors. Strutz et al[109] showed that FGF-2 expression correlated with interstitial scarring in the kidney and promoted fibroblast growth and proliferation, particularly in interstitial and tubular cells, which may have contributed to renal fibrosis. These results were opposite to ours, as FGF2 was downregulated in fibroblasts with FGFBP2 mutation. However, Ishiguro et al.[110] identified that bFGF was a potent inhibitor of alpha-SMA expression in myofibroblasts, both in vitro and in vivo, likely through the activation of extracellular signal-regulated kinase 1/2 and the promotion of apoptosis, which led to a reduction in the myofibroblastic area. Dolivo et al.[111] reported that FGF2 inhibited TGFβ-mediated fibroblast activation and myofibroblast transition, reduced the expression of myofibroblast markers and promoted more rapid cell proliferation. Interestingly, these results were consistent with ours. Bordignon et al.[112] demonstrated that FGF and TGF-β signaling opposed regulation of CAF effector genes in human dermal fibroblasts, with differential effects on cancer cell proliferation, invasion, epithelial-mesenchymal transition and macrophage infiltration, indicating distinct roles for these signaling pathways in cancer development. This

may explain why FGF2 has different effects on the formation of fibroblasts with different roles.

5.5 Relations to ERK1/2 signaling

CAFs secreted cytokines that are taken up by cancer cells may promote effective proliferation in vitro by triggering critical signaling pathways, such as MAPK, through factors like IL-6, FGF and TGF-β[82]. FGF2 bound to its receptor (FGFR), leads to the activation of the MAPK/ERK cascade, which promotes gene expression changes essential for growth and development[49]. The ERK signaling pathway plays a critical and complex role in mediating interactions between cancer-associated fibroblasts (CAFs) and tumor cells. Neufert et al.[113] showed that epiregulin (EREG) expression was upregulated in colitis-associated colorectal cancer, with tumor-associated fibroblasts being a major source of EREG, which promoted tumor growth through ERK activation and epithelial cell proliferation. Kikuchi et al.[114] found that periostin (POSTN) produced by cancer-associated fibroblasts promoted gastric cancer growth by increasing cancer cell proliferation and activating the ERK pathway. Our investigations with conditioned medium revealed that ERK1/2 phosphorylation was significantly enhanced in PANC-1 cells treated medium derived from FGFBP2 mutant group. This suggested that cytokines secreted by FGFBP2-mutant fibroblasts altered cancer cell behavior through increased ERK1/2 phosphorylation. Although FGF2 expression was downregulated in the FGFBP2-mutant group, other cytokines could promote the observed changes in ERK1/2 phosphorylation. Ando et al.[115] showed that eicosatetraenoic acid (EPA) reduced cancer angiogenesis in colon cancer-associated fibroblasts (CAFs) by inhibiting IL-6 and VEGF secretion and ERK phosphorylation. Further detailed studies are required to clarify these complex interactions and identify the key factors involved.

In conclusion, our results demonstrate that the analyzed FGFBP2 mutation promotes fibroblast activation and enhances pancreatic cancer cell proliferation. Mechanistically, this effect may be mediated through upregulation of EHF and activation of the cell cycle pathway in pancreatic cells. Additionally, fibroblastic FGFBP2 mutation appears to influence pancreatic cancer cells

by modulating ERK1/2 phosphorylation levels. Our study reveals a novel molecular mutation that drives the crosstalk between tumor cells and fibroblasts, promoting pancreatic cancer cells and supporting the development of more effective therapeutic strategies.

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7. Theses

1. Somatic point mutation p.T186S (replacement of guanine to cytidine) in Fibroblast Growth Factor Binding Protein 2 (FGFBP2) is related to increased expression of fibroblast activation marker α-SMA as well as cytokines IL-6, TNF-α, and TGF-β, indicating the activated state of mutated fibroblasts.

2. Fibroblasts carrying somatic point mutation p.T186S in FGFBP2 reveal augmented proliferation rates and increased motility.

3. Treatment of pancreatic cancer cells PANC-1 with conditioned medium derived from mutated fibroblasts leads to elevated proliferation rates, upregulation of cancer-associated ETS homologous factor (EHF) and alterations in cell cycle progression.

4. Somatic point mutation p.T186S in FGFBP2 leads to decreased expression of Fibroblast Growth Factor 2 (FGF2).

5. Treatment of pancreatic cancer epithelial cells PANC-1 with conditioned medium derived from mutated fibroblasts enhances ERK1/2 signaling activation through protein phosphorylation.

6. Bioinformatic analyses of intercellular signaling pathways and ligand–receptor interactions reveal that fibroblasts are affected by EHF in a Platelet Derived Growth Factor (PDGF) signaling manner.

7. Somatic point mutation p.T186S in FGFBP2 is related with altered cytokine expression which in turn may affect the tumor microenvironment and promote invasive potential of pancreatic cancer epithelial cells.

Affidavit

I hereby declare, the submitted thesis entitled "**Impact of fibroblastic FGFBP2 mutation on pancreatic carcinogenesis**" is my work. The work presented in this thesis was carried out between 11/2021 and 11/2024 under the supervision of Prof. Dr. Jörg Kleeff, PD Dr. Bogusz Trojanowicz and Dr. Yoshiaki Sunami, Department of Visceral, Vascular and Endocrine Surgery, University Hospital Halle (Saale).

The data presented in this thesis is entirely original, collected by me, and has not been previously submitted or presented as part of any university examination. Furthermore, I declare that neither the thesis nor any part of it has been used for obtaining a degree at any other university. All data and materials from external sources have been properly referenced, with appropriate attribution provided wherever material has been cited or utilized.

Halle (Saale),03.02.2025 Xing Wu _____

Place, date Signature of the applicant

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