DISSERTATION

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The Functions of EP300 in Activated Pancreatic Stellate Cells and the Drug Resistance Problem in Pancreatic Cancer

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DECLARATION

I hereby declare that the work described in this thesis has been done and written only by the undersigned. I confirm that no other materials or sources have been used unless those expressly indicated in this thesis and proper accreditation is given when other people's work is described. The work has not been presented elsewhere for any kind of certificate.

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Related publication

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DEDICATION

I would like to dedicate my work to my parents. Thanks my father Xiaogong Liu and my mother Baoqin Li for giving me a life, for always supporting me and encouraging me, for believing that their little girl is good enough, smart enough, capable and competent. Without their love and concern, I could not have gone this far.

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SUMMARY

Pancreatic stellate cells (PSCs) are generally quiescent in normal conditions, but during inflammation or cancer these cells are activated, differentiate to myofibroblast-like cells, proliferate, migrate and start secreting extracellular matrix protein, which are the main contributors to the stromal formation during the process of cancer. EP300 is an important transcription coactivator and plays an important role in the process of cell proliferation and differentiation. Thus, we hypothesize that targeting EP300 will affect the activation of PSCs and may influence the process of pancreatic cancer, especially for pancreatic ductal adenocarcinoma (PDAC). Transient specific small interfering RNA (SiRNA) knockdown of EP300 resulted in reduced expression of fibronectin (FN) and collagen I (Col-I) in activated PSCs. Stable knockdown of EP300 by CRISPR/Cas9 gRNA plasmid had the same effects. However, the migration of PSCs was increased. And we firstly showed that EP300 manipulated cell migration through ERK pathway. Furthermore, EP300 down regulation in PSCs increased the proliferation effect PSCs had on pancreatic cancer cells and PSCs protected tumor cells from chemotherapy more. Together, the evidences draw the conclusion that EP300 is a tumor suppressor gene, its downregulation increases the migration of PSCs and PSCs becomes more supportive for pancreatic cancer cells, but that reduces the extra cellular matrix production of PSCs.

High resistance to chemotherapy is a frustrating issue in treating pancreatic ductal adenocarcinoma. It is one reason for a 5-year survival rate of PDAC patients lower than 5%. In recent years, researcher showed that the tumor microenvironment might make a great contribution to the drug resistance of pancreatic cancer. PSCs are important cells that exist in the tumor stroma of pancreatic cancer. Gemcitabine is a nucleoside analog, which is currently used as the best standard treatment for pancreatic cancer patients. In the present study, I analyzed how PSCs will affect the drug resistance of different drug sensitive pancreatic cancer cell lines. My results for the first time showed that conditioned medium from PSCs promotes chemo-resistance of Bxpc-3 cells by up regulating RRM1 and RRM2, but has no influence on the drug sensitivity of Panc-1 and Miapaca-2 cells. In addition, I could show that factors that are <100kDa and produced by pancreatic stellate cells are responsible for the effects. These factors are heat insensitive, trypsin and proteinase K insensitive and cannot be degraded by nucleases either, but the exact factor has yet to be determined.

ZUSAMMENFASSUNG

Pankreas Sternzellen (PSCs) befinden sich üblicherweise in einem ruhenden Zustand. Während einer Entzündung des Pankreas oder bei Auftreten von Krebs werden diese Zellen jedoch aktiviert. Sie differenzieren dann in Zellen, die sich durch myofibroblastäre Eigenschaften auszeichnen, proliferieren, Migration zeigen und beginnen, extrazelluläre Matrix-Proteine zu sezernieren. Sie leisten einen wesentlichen Beitrag zur Bildung des Stroma, das bei Pankreaskrebs und speziell dem duktalen Adenokarzinom des Pankreas (PDAC) stark ausgeprägt ist. Das Protein EP300 ist ein zentraler Co-Aktivator der Transkription und spielt eine wichtige Rolle im Prozess der Zellproliferation und Zelldifferenzierung. Deshalb hatten wir die Hypothese aufgestellt, dass eine Beeinflussung von EP300 auf die Aktivierung von PSCs und damit auf Pankreaskrebs einwirkt. Eine transiente Herunterregulierung von EP300 mittels spezifischer, kleiner, interferierender RNA (*small interfering RNA*; siRNA) Moleküle reduzierte die Expression von Fibronektin (FN) und Collagen I (Col-I) in aktivierten PSCs. Eine stabile Reduzierung der EP300 Expression mittels des CRISPR-Cas9 Systems zeigte den gleichen Effekt. Die Zellmigration wurde dagegen verstärkt. Ich konnte zeigen, dass der Mechanismus, mit dem EP300 die Migration beeinflusst, durch den ERK Stoffwechselweg erfolgt. Außerdem steigerte die Reduzierung der EP300 Menge in PSCs den Effekt auf die Zellproliferation in Pankreaskrebs, die von den PSCs ausgeht. Gleichzeitig schützten solch modifizierten PSCs den Tumor stärker vor Chemotherapie. In Kombination erlauben die Ergebnisse die Schlussfolgerung, dass *EP300* ein Tumorsuppressor-Gen darstellt. Eine reduzierte Expression führt zu mehr PSC Migration. Gleichzeitig unterstützen diese PSCs die Tumorzellen, während die Produktion der extrazellulären Matrix reduziert wird.

Der hohe Grad an Resistenz gegenüber Chemotherapie ist eine der frustrierenden Facetten bei der Behandlung von duktalem Adenokarzinom des Pankreas (PDAC). Sie ist ein Grund, warum die 5-Jahres Überlebensrate mit etwa 5% der Patienten so gering ist. In den letzten Jahren konnte gezeigt werden, dass die Tumor-Mikroumgebung (*tumor microenvironment*) einen großen Beitrag zu dieser Resistenzausbildung leistet. PSCs sind eine wichtige Zellkomponente im Stroma von PDAC Tumorgeweben. Gemcitabin ist eine Nukleosid-Analog, das zurzeit immer noch die Grundlage der Standardbehandlung von PDAC darstellt. In meiner Arbeit habe ich analysiert, wie PSCs für verschiedenen PDAC Zelllinien die Resistenz auf Chemotherapie beeinflussen. Meine Ergebnisse zeigen, dass Medium mit den sezernierten Molekülen (*conditioned medium*) von PSCs die Chemo-Resistenz der PDAC Zelllinie Bxpc-3 durch eine Regulation von RRM1 und RRM2 stärken. Im Gegensatz dazu hat es keinen Einfluss auf die Sensitivität der Zelllinien Panc-1 und Miapaca-2. Zusätzlich konnte ich zeigen, dass Faktoren einer Größe kleiner 100 kDa für den Effekt verantwortlich sind. Diese Faktoren konnten weder durch Hitze noch Verdau mit Trypsin und Proteinase K oder Nukleasen inaktiviert werden.

Part I: Exploring the Functions of EP300 in Activated Pancreatic Stellate Cells

1 Introduction

1.1 Pancreas

Pancreas is a glandular organ, located across the back of the abdomen, behind the stomach. It contains two types of glands: (1) Exocrine. The exocrine gland excretes various enzymes to digest different substances in food. (2) Endocrine. The endocrine gland secretes hormones into the blood, which controls the blood sugar levels throughout the day. These two functions are vital to the body's survival [1].

1.1.1 Anatomy of the pancreas

In humans, the pancreas weighs on average 80g and is about 15 to 20 cm long, which extends laterally and superiorly across the abdomen from the curve of the duodenum to the spleen. It composes of three regions. The head of the pancreas connects to the duodenum, which is the widest region of the organ. The body of the pancreas extends laterally toward the left. The tapered left side of the pancreas is referred as the tail region, which is near the spleen. As showed in Figure 1.

The exocrine of the pancreas is composed of grape like cell clusters, which are called acini. When acinar cells are stimulated, they release enzyme-rich pancreatic juice into the ducts. Scattered through the sea of exocrine acini are small islands of endocrine cells, the islets of Langerhans. The hormones secreted by endocrine cells are important in glucose homeostasis. There are two main types of endocrine cells, alpha cells, which raise blood glucose levels, and beta cells, which lower blood glucose levels [2].

1.1.2 Regulation of the pancreas

There are two systems which can regulate the function of the pancreas: the autonomic nervous system (ANS) and the endocrine system. The sympathetic and the parasympathetic division in the ANS control the glucose levels in the blood. Sympathetic division stimulates alpha cells of the pancreas to release glucagon, which increases the glucose level in the blood. Parasympathetic division stimulates the release of insulin and pancreatic juice by the pancreas, to digest food and store glucose, which reduces the glucose level in the blood. The endocrines

system uses two hormones to regulate the digestive function of the pancreas. Secretin helps to maintain a neutral pH in the stomach. Cholecystokinin contributes to the digestion of large protein and lipid molecules that are difficult to break down [3].
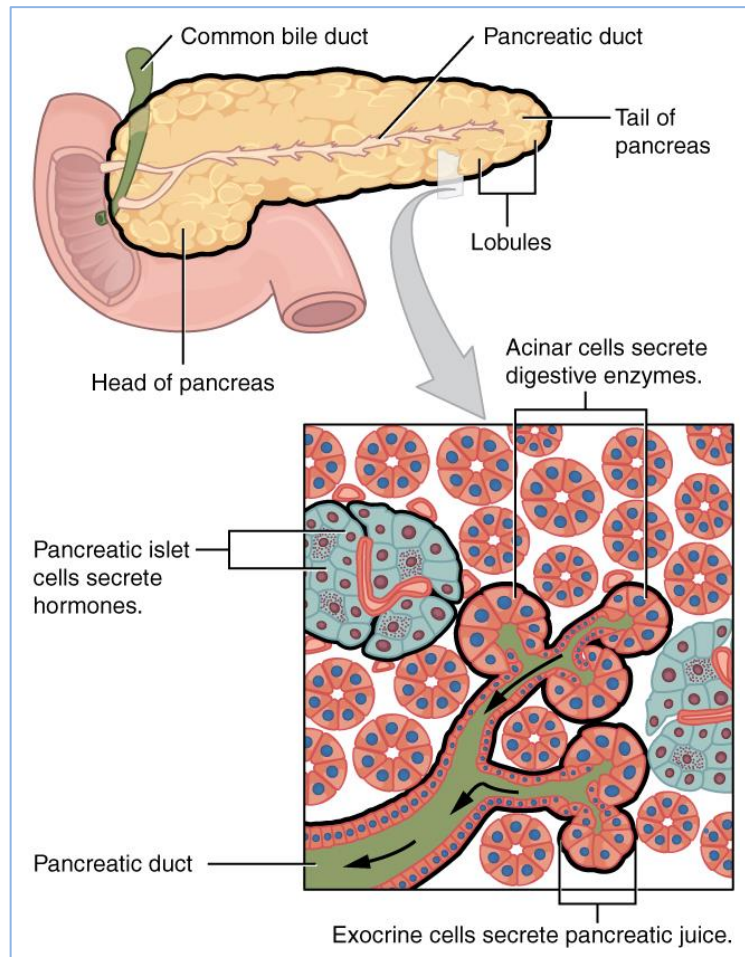
Figure 1. The exocrine and the endocrine of pancreas. The pancreas has a head, a body and a tail. It delivers pancreatic juice to the duodenum through the pancreatic duct [4].

1.1.3 Common pancreatic problems

Diabetes: Diabetes is a condition where the amount of sugar in the blood is too high, which is caused by the malfunction of the pancreas. The pancreas loses the ability to produce and release insulin, so the sugar level can't be lowered in the blood. Diabetes patient will feel very thirsty, pass more urine than normal, lose weight and feel tired [5].

Pancreatitis: The pancreas becomes inflamed and damaged by its own digestive chemicals. It can occur as acute painful attacks lasting a matter of days, or maybe a chronic condition that progresses over a period of years. Sometimes it will be life-threatening. Alcohol or gallstones can contribute to it, but the real cause of the most pancreatitis is unknown [6].

1.2 Cancer

Cancer is a disease caused by abnormal cell growth and it has the potential to spread to other parts of the body. It is one of the leading causes of death worldwide. For example, in 2014, cancer is responsible for 8.2 million deaths around the world [7]. The earliest written record in the history of cancer is from approximately 1600BC in Egyptian, which describes breast cancer [8]. However, till now, there is still no cure for most cancer.

Actually, cancer is mainly a genetic disease, which is caused by changes in genes that control our cells functions, especially those related to cell growth and division. In general, they are three types of genes highly responsible for cancer, which are: proto-oncogenes, tumor suppressor genes and DNA repair genes. When proto-oncogenes are altered, they will become cancer-causing genes, which allow cells to grow and survive when they shouldn't. When tumor suppressor genes are mutated, they will allow cells to divide without control. If DNA repair genes are changed, that will make cells become cancerous. In a word, some gene mutations cause cancer.

They are many types of cancer, since it can start almost everywhere, such as leukemia, lymphoma, melanoma, carcinoma, brain cancer and so on. Many cancers form solid tumors, but some are not, for example, leukemia. For those that can form tumors, there are generally two types: malignant tumors and benign tumors. Benign tumors don't invade or spread to nearby tissues, once removed, they usually don't grow back. However, unlike benign tumor, malignant tumors can spread into or invade nearby tissues. By travelling through the blood or the lymph system, new tumors can be formed far from the original ones in benign tumor, which is life threatening.

1.3 Pancreatic cancer

Pancreatic cancer is the fourth leading cause of cancer-related death in both Europe and USA [9]. Despite many efforts have been put on it, the survive rate has not been improved in the past 30 years. Patients who diagnosed with pancreatic cancer will die within 6 months and the 5 years survival rate is less than 5% [10]. This because it is often diagnosed at a late stage and it's highly resistant to chemo and radiation therapy [11]. The most effective treatment for pancreatic cancer is surgery, however, only 20% of patients are suitable for surgery because when diagnosed it has already spread and 80% of patients after surgery suffer a relapse of the cancer [12]. For 2017, the American Cancer Society estimates that in the United States about

53,670 people will be diagnosed with pancreatic cancer and about 43,090 people will die of pancreatic cancer. Hence, it is very urgent and important to study and research in the field of pancreatic cancer, hoping that it will improve the conditions of the patients and provide new insights to fight against it.

1.3.1 Molecular biology of pancreatic cancer

Currently, it's still unknown what the exact causes of pancreatic cancer, but risk factors have been identified. Cigarette smoking, family history of pancreatic cancer, diabetes mellitus, heavy alcohol consumption (>60 mL ethanol/day) and history of pancreatitis are considered to be the most significant risk factors for pancreatic cancer [13, 14]. Scientists reported that 25% of pancreatic cancer cases are related to smoking and pancreatic cancer developed 20 years earlier in smokers than in nonsmokers [15, 16]. Researchers showed that inherited genetic variants contribute to at least 5%-10% of all pancreatic cancer cases [17-19]. The following genes with variants have been considered can increase the risk for pancreatic cancer in PDAC familial cases: BRCA1, BRCA2, PALB2, ATM, CDKN2A, APC, MLH1, PMS2, PRSS1 and STK11 [20]. Diabetes patients are also more likely to be diagnosed with pancreatic cancer [21].

As mentioned before cancer is a gene related disease, so does pancreatic cancer. Scientists analyzed pancreatic tumor tissues and found that on average there are 63 genetic alterations relevant to tumor progression per sample [22]. Some gene mutations are present in almost all pancreatic samples. These genes include: KRAS, INK4A/ARF, SMAD4 and p53 [23-25].

KRAS is a GTPase that encoded by KRAS gene, it functions as a second messenger in growth factor receptor signaling pathways that stimulate the transition through the G1 phase of the cell division cycle. Approximately 90% of identified pancreatic cases have KRAS mutations [26]. When KRAS is mutated, it will impair the intrinsic GTPase activity resulting in a protein that is constitutively active in signal transduction, which will alter cell proliferation, survival and migration [27]. KRAS is considered as an oncogene in pancreatic cancer, and its mutation appears early during the process of pancreatic carcinoma [28]. Researchers have shown that KRAS is required for both the initiation and maintenance of pancreatic cancer in mice [29]. So KRAS could be the potential target for the therapeutic treatment of pancreatic cancer.

INK4A and ARF are two genes that are encoded in an overlapping region of the chromosome 9. INK4A functions as an inhibitor of G1 cyclin-dependent kinase. The name of ARF implies that an alternate reading frame from INK4A encodes it. ARF family members encode small guanine nucleotide proteins and play a role in vesicular trafficking. These two genes are tumor suppressors and about 85% of pancreatic cancers are mutated in them [30]. Researches found that activated KRAS and INK4A/ARF deficiency cooperate to promote the development of pancreatic cancer [31]. ARF is found to be an activator of the p53 pathway, but it has p53-independent functions, such as inhibition of NF-κB activity, degradation of E2F and reducing the synthesis of ribosomal RNA [32]. Hence, when INK4A/ARF loss their function, many pathways will be influenced, cancer may begin to develop.

SMAD4 is the number 4 protein of SMAD family, which functions as a signal transduction protein. This family plays a core role in the transforming growth factor-β (TGF-β) pathway, as shown in Figure 2. SMAD4 gene is found to be inactivated in about 55% of pancreatic cancers [33]. It is a tumor suppressor gene, its inactivation related to the development of pancreatic tumors. Normally, when TGF-β binds to their transmembrane receptors, after a series of phosphorylation, a SMAD4/SMAD complex transmit into the nucleus, binds to specific DNA sequence and activates gene transcription [34]. However, when SMAD4 is inactivated, many functions of TGF-β, such as growth suppression and apoptosis are no longer existed. Evidence showed that SMAD4 deficiency accelerates KRAS mediated pancreatic tumor development [35].

p53 is a transcriptional activator, it plays an important role in cell cycle control and apoptosis. In a healthy cell, p53 protein level is low, however, when there is stress, such as DNA damage or hypoxia, it will be activated. p53 mainly has three functions: growth arrest, DNA repair and apoptosis. Over 50% of pancreatic tumors have p53 mutations [36, 37]. And in pancreatic cancer, p53 is often mutated in its DNA-binding domain, which will damage a lot of gene transcription, thus cells with abnormal DNAs remain growing. It's a star molecule in cancer, over 50% of all human tumors have p53 mutations [38]. Unlike other tumor suppressors, research found that most of p53 mutations are missense mutations, but the reason for that remains unknown.

Despite these most frequently mutated genes in pancreatic cancer, there are also other gene mutations found in PDAC, such as oncogenes: BRAF, AKT2, MYB and AIBI; tumor

suppressor genes: p21, p16/CDKN2A; genome maintenance genes: MLH, MSH2, BRCA2 [27, 39], which we are not going to be discussed in details in this thesis.
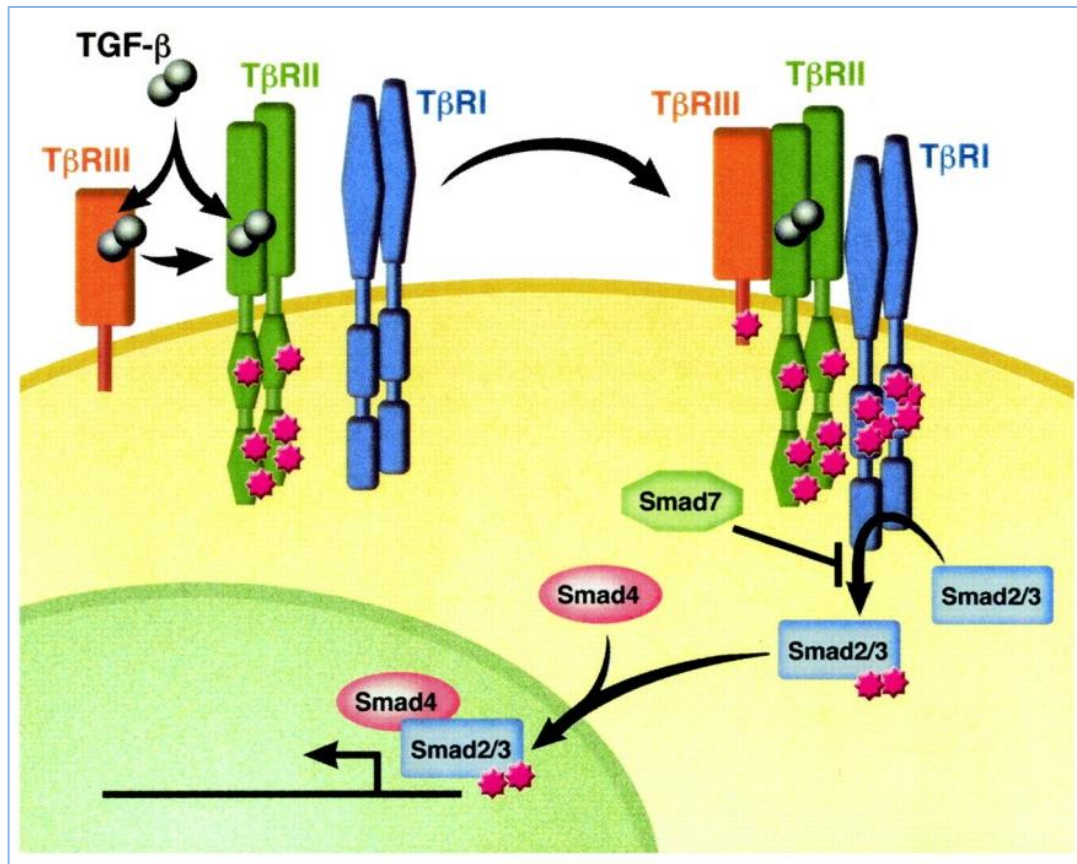
Figure 2. The transforming growth beta (TGF-β) signaling pathway. TGF-β binds to type II TGF-β receptor (TβRII), inducing the association of TβRII and TβRI, which activate TβRI. TβRI then phosphorylates Smad2 or Smad3. Activated Smad2 or Smad3 associates with Smad4 and then translocate to the nucleus to influence the target gene expression [40].

Besides genetic abnormalities, epigenetic aberrations have also been found in PDAC. There are mainly three epigenetic modifications that affect gene expression, which are DNA methylation, histone modification and microRNA expression. Studies showed that more than 90% of pancreatic cancers have aberrantly methylated PENK. Other genes that are found highly methylated in pancreatic cancer are: SPARC, CDKN2A/p16 and CDH1 [41]. Mucins, which play important roles in carcinogenesis, found undergo histone alterations in pancreatic cancer [42]. MicroRNAs are some non-coding RNA molecules, which negatively regulate the expression of target genes. In PDAC, several miRNAs have been shown over expressed, such as miR-155, miR-222, miR-221 and miR-21 [43].

The core signaling pathways that are highly related pancreatic cancer have also been studied. Besides the commonly mentioned Hedgehog and Notch pathway, the Wnt/Notch signaling pathway, small GTPase-dependent signaling pathway and integrin signaling pathway also involved in pancreatic cancer [22].

1.3.2 Pancreatic desmoplasia

Solid tumors are organ-like structures, they are not only consist of tumor cells but also contain immune cells, fibroblasts, lymphocytes, macrophages, bone marrow-derived inflammatory cells, blood vessels and extracellular matrix (ECM), which form a cellular environment called tumor microenvironment [44]. During cancer, the tumor and its microenvironment constantly interact with each other to promote the process of cancer. Researchers have showed that tumor microenvironment maybe the leading player in the initiation of carcinomas. Such as mutations in stromal cells that specifically regulate paracrine growth factor expression have been found initiated epithelial cancer [45, 46]. Cancer cells' ability to invade and metastasize has also been shown influenced by tumor microenvironment [47, 48]. In addition, studies found that cancer cells promoted the form of the tumor microenvironment by releasing various extracellular signals, such as cytokines, hormones, growth factors and so on [49, 50].

Another problem caused by tumor environment is hypoxia. Most solid tumors contain some regions of hypoxia. These regions are deprived of oxygen and are likely to have a decreased supply of nutrients such as glucose and essential amino acids. Tumor cells in these regions have to undergo oxidative metabolism, which will lead to low interstitial pH or acidosis inside the tumor. The lower pH in the tumor microenvironment will influence the cytotoxicity of anticancer drugs [51]. Tumor hypoxia also activates angiogenesis and cell survival related genes, which may lead to a more aggressive tumor type [52, 53]. Such as, hypoxia stimulates the transcription of vascular endothelial growth factor (VEGF), transforming growth factor-β (TNF-β), platelet-derived growth factor-β (PDGF-β) and insulin-like growth factor, which promotes tumor growth [54]. In a word, tumor hypoxia in the microenvironment is strongly associated with tumor propagation and influences cancer treatment.

Scientists mainly focused on cancer cells to fight against tumors and they achieved significant advances in colorectal cancer, lung cancer and melanoma [55]. Unfortunately, the same method wasn't successful in pancreatic cancer. A peculiar hallmark of pancreatic cancer is the presence of high percent of reactive stroma that can cumulate up to 90% of the tumor mass, as

showed in Figure 3 [56, 57]. And in recent years, an accumulating body of evidence suggests that the highly reactive stroma of pancreatic microenvironment is one of the prime reasons of the tumor aggressiveness and resistance to therapy. Researcher found that this massive stroma contributes to an increase interstitial fluid pressure inside of the tumor [58] and causes hypoxia in the tumor [59], which makes it more difficult to find a good therapy for PDAC. Therefore, targeting stroma could be a new way to fight against pancreatic cancer.
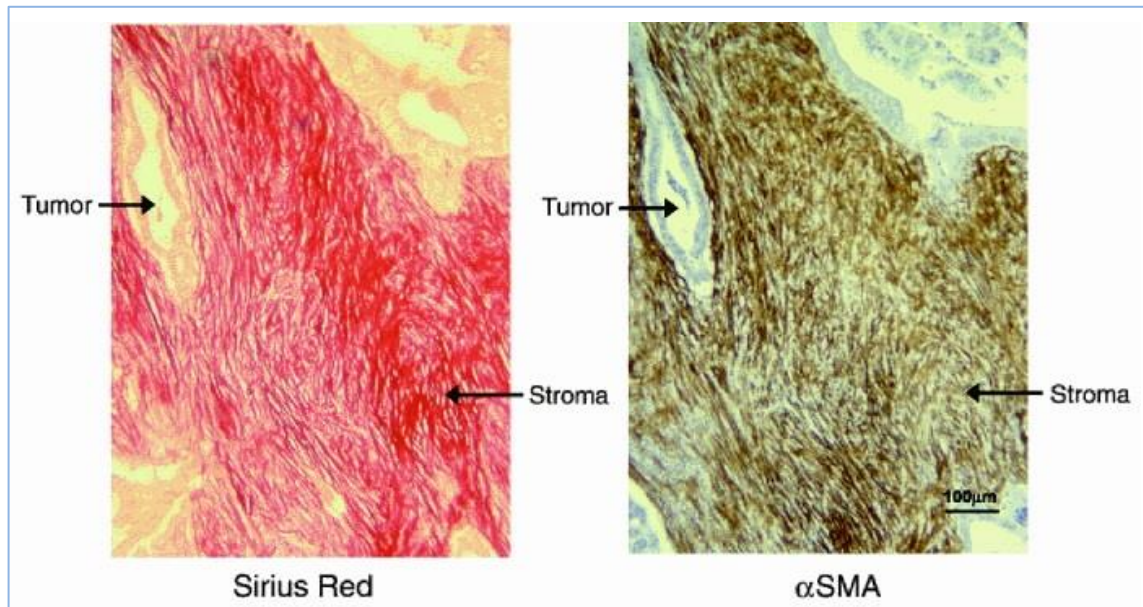
Figure 3. Colocalization of collagen and αSMA staining in pancreatic cancer [60]. Stroma exists positive stain for collagen I and αSMA.

The dense desmoplasia of pancreatic cancer is also formed by many kinds of cells, such as endothelial cells, leukocyte, macrophages, inflammatory cells, nerve fibers and marrow-derived stem cells. Among them, there is one type of cell we just can't ignore, pancreatic stellate cells (PSCs). PSCs are generally quiescent during normal physiology, but when in the event of inflammation or cancer these cells are activated, differentiate to myofibroblast-like cells, proliferate, migrate and start secreting extracellular matrix (ECM) proteins, which are the main contributors to pancreatic fibrosis during the course of pancreatitis and pancreatic cancer. The details of pancreatic stellate cells will be discussed below.

1.3.2.1 Pancreatic stellate cells

Pancreatic stellate cells were first observed in 1982 by using autofluorescence and electron microscopy [61]. Then Apte [62] and Bachem [63] isolated stellate cells from rat and human pancreas, the study of PSCs began to develop. Early studies of PSCs based on the knowledge

and experience gained from hepatic stellate cell, which were first described in 1876 by Karl von Kupffer [64]. Stellate cells have a star like shape and they are also present in other tissues, including the kidney and lung [65, 66].

PSCs are specific stroma cells of pancreatic cancer, they generally have two states: quiescence and activation. In health pancreas, stellate cells are quiescent, they are located at the basolateral aspect of acinar cells and constitute approximately 4% to 7% of pancreatic cells [67]. They are round shape and fat storing cells, can be identified by the presence of abundant vitamin A and the expression of cytoskeletal proteins such as glial acidic fibrillary protein (GAFP) and desmin [62, 63, 68, 69]. By secreting matrix degrading enzymes and inhibitors of these enzymes, PSCs play a crucial role in maintaining the regular ECM turnover during health [70]. A study in 2010 also demonstrated that PSCs might play a role in regulating enzyme secretion from acinar cells [71].

During inflammatory or cancer, PSCs undergo various changes. They loss the vitamin A droplets, become myofibroblast-like cells, proliferate, migrate and produce extracellular matrix proteins such as collagen I, fibronectin, laminin, which make great contributions to the stoma formation in pancreatic cancer [72-76]. In addition, activated PSCs secrete cytokines, chemokines, which work as feedback loops making PSCs more activated [77-79]. What's more, other neighboring cells in the microenvironment such as acini, tumor, immune cells and platelets, work in a paracrine manner, stimulating the activation of PSCs, which promotes desmoplasia further [80]. A lot of factors have been shown involved in the activation of PSCs, such as transforming growth factor (TGF)-β1, tumor necrosis factor (TNF) α, platelet-derived growth factor (PDGF), vascular endothelial growth (VEGF) factors, interleukin (IL)-1, IL-6, IL8, IL-10 [63, 76]. The most potent activators of PSCs are believed to be TGF-β1 and PDGF. TGF-β1 is a fibrogenic mediator that stimulates the ECM synthesis of activated PSCs [81, 82] and IL-1 and IL-6 were found to affect the activation of PSCs through the production of TGF-β1 [83]. PDGF induces the proliferation and migration of PSCs [84, 85]. Besides factors mentioned above, there are other potential sources related to the activation of PSCs, such as pressure, oxidative stress, ethanol and its metabolites, as well as the composition changes in the ECM [82, 86, 87]. Figure 4 showed the mechanisms of pancreatic stellate cells activation.

In recent years, several signaling pathways and molecules that are important in the process of PSCs activation have been identified, which are peroxisome proliferator activated receptor

gamma (PPAR$_\gamma$), protein kinase C (PKC), the JAK-STAT pathway, the PI3K-AKT pathway, Rho kinases and transcription factor nuclear factor-kappa B (NF-κB) and so on [88].
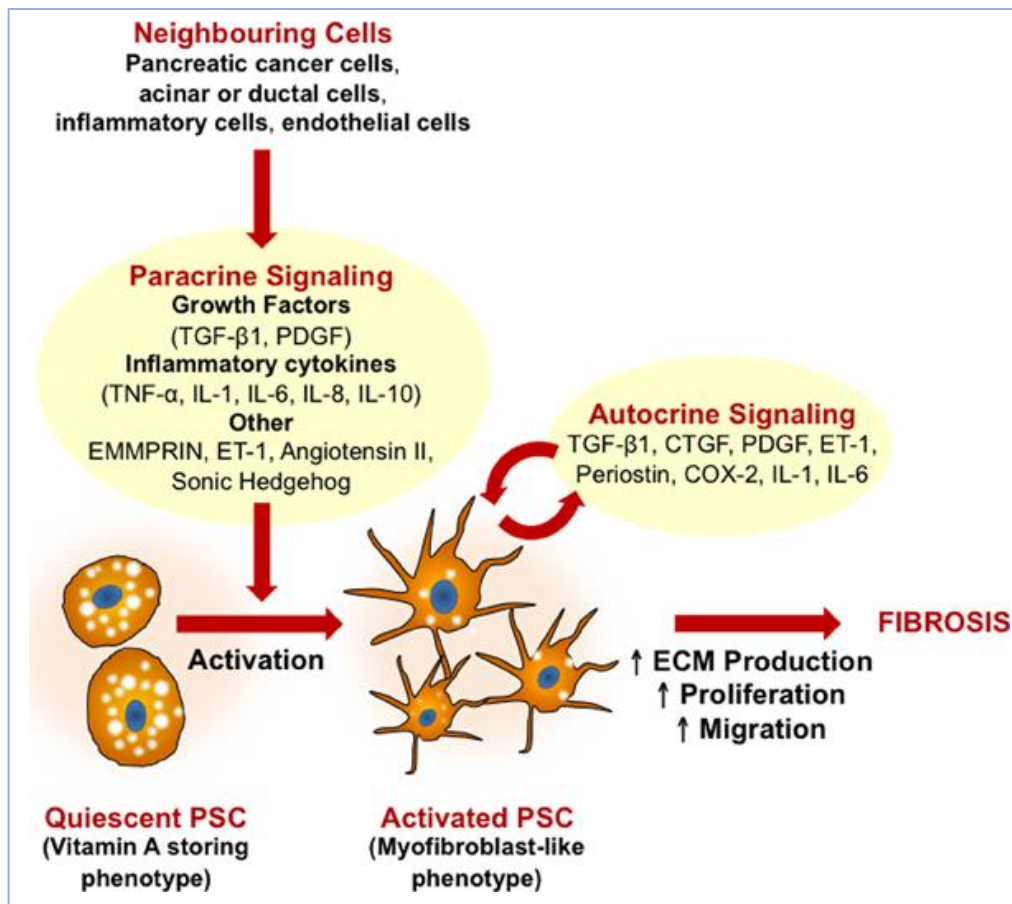
Figure 4. Mechanisms of pancreatic stellate cells activation. Growth factors and pro-inflammatory cytokines released by PSCs and its neighboring cells all induce PSCs activation [89].

PPAR$_\gamma$, also known as the glitazone receptor, is mainly present in adipose tissue. It can regulate fatty acid storage and glucose metabolism [89-91]. Researchers showed that overexpression of PPAR$_\gamma$ blocks the activation of pancreatic stellate cells and down regulation of PPAR$_\gamma$ is associated with PSCs activation [92]. Protein kinase C is a family of protein kinase enzymes. They are known for their long-term activation: they remain activated after the original activation signal is gone. Angiotensin II has been found to be able to promote the proliferation of activated PSCs through a protein kinase C pathway [93]. The JAK-STAT signaling pathway is a pathway that can transmit information from extracellular chemical signals to the nucleus. The activation of JAK-STAT is related to the activation of PSCs. PDGF was found stimulated the proliferation of PSCs via JAK-STAT pathway [94]. The PI3K-AKT pathway is also involved in the regulation of PSCs. PDGF promotes the migration

of PSCs through PI3K-AKT pathway [95]. Rho kinases play a role in regulating the shape and movement of cells. Treating PSCs with Rho kinase inhibitors blocks the activation of freshly isolated PSCs in culture [81]. NF-κB is a protein complex that controls transcription of DNA, cytokine production and cell survival. Researchers found that activated PSCs express a variety of NF-κB responsive genes [96]. There are other pathway proteins relate to the activation of PSCs, such as activator protein-1 (AP-1), Smad proteins, Hypoxia-inducible factors (HIF-1), Reactive oxygen species and Indian hedgehog (IHH), we are not going to explain them in details here [97-101].

After activation, PSCs have two fates, if the injury is not that severe, PSCs will lose their active phenotype and become quiescent again. If the injury is severe and continuous, PSCs will remain active and pancreatic fibrosis will develop. Irreversible activation of PSCs will cause the composition changes of the extra cellular matrix, which means that collagen I will deposit and fibrosis begins. The origin of PSCs has also been studied. Researcher showed that bone marrow-derived progenitor cells contribute around 5% to the PSCs population [102]. Some studies proved that PSCs are derived from pancreas precursor [103]. The contribution of endothelial cells to the myofibroblast cell population in pancreatic cancer has also been reported [104].

1.3.2.2 Tumor stroma interactions

Considering the large amount of stroma in pancreatic adenocarcinoma, the role it plays in the process of cancer just can't be ignored. Researchers found that the interactions between PSCs and pancreatic cancer cells can influence the progression of pancreatic cancer. On the one hand, pancreatic cancer cells not only secrete different kinds of growth factors such as transforming growth factor-β1 (TGF-β1), platelet-derived growth factor (PDGF), Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which can activate PSCs and thus stimulate proliferation, migration and matrix synthesis of cultured PSCs [63, 74, 76, 80, 105], but also they can produce MMPs, which digest stroma and release stored growth factors in the stroma, aiding in the desmoplastic reaction in PDAC [106]. Besides these factors mentioned above, two secretory proteins: cyclo-oxygenase-2 and trefoil factor 1, which is up regulated by pancreatic cancer cells, have also been reported can promote the proliferation of PSCs [60, 107].

On the other hand, scientists noticed that pancreatic stellate cells are important in promoting pancreatic cancer cell proliferation, invasion and metastasis [80]. An in vitro study showed that pancreatic stellate cells promote proliferation and invasiveness of human pancreatic cancer cells via galectin-3 [108]. A three dimension in vitro research proved that pancreatic stellate cells increase the invasion and epithelial-mesenchymal transition of pancreatic cancer cells [109]. In a subcutaneous mouse model of pancreatic cancer, it has been shown that animals injected with both PSCs and pancreatic cancer cells grew much bigger tumor than animals injected with cancer cells alone [110]. In another orthotopic model of pancreatic cancer, injection of pancreatic cancer cells and PSCs together into the pancreas of mice, histology experiments verified that activated PSCs are related to fibrosis and co-injection experiment mouse demonstrated larger tumors and more local and distant metastases than mouse only injected with tumor cells alone [111]. In pancreatic cancer patients, researchers found that extensive fibroblastic cell proliferation correlates with poor disease outcome [112].

PSCs also have been found to play a role in regulating epithelial-mesenchymal transition (EMT) and stemness of cancer cells. EMT is a well-known hallmark of highly invasive cancer cells. When cells go epithelial mesenchymal transition, they will lose their cell polarity and cell-cell connections, and begin to migrate and invade. Researchers showed that PSCs promote EMT in pancreatic cancer cells. Cancer cells grow with PSCs gaining a fibroblast-like appearance and begin express mesenchymal markers, such as vimentin and zeb [113]. Stemness is the ability to self-renew and differentiates. Cancer stem cells have the ability to move to distant sites and retain their stemness properties and thus grow new tumors at these sites. Researchers found that PSCs enhance stem cell-like phenotypes in pancreatic cancer cells. Hamada [114] showed that when co-cultured pancreatic cancer cells with PSCs, the spheroid-forming ability of pancreatic cancer cells was increased and some stem cell related genes were expressed in cancer cells. Al-Assar [115] demonstrated that PSCs enhanced cancer stem cell phenotype and radio resistance of pancreatic cancer cells.

1.3.2.3 Macrophages

Macrophages are a type of white blood cell that engulfs and digests unwanted particles, such as cell debris, foreign substances, microbes, and so on, which is an important part of our immune system. Generally, macrophages can divide into two types: (1) classically activated macrophages or called M1 macrophages. It encourages inflammation and during acute infectious diseases it provides host protection against bacteria and viruses [116, 117]; (2)

alternatively activated macrophages or called M2 macrophages. It plays a key role in dampening inflammation, promotes wounding healing, fibrosis and tumorigenesis [118, 119]. Both M1 and M2 macrophages are existed in the pancreatic tumor microenvironment. These macrophages have been postulated as being involved in the process of cancer [120, 121]. Liu demonstrated that the migration and proliferation of pancreatic cancer cells were increased when co-culture of tumor associated macrophages with pancreatic cancer cells [122]. Macrophages also interact with pancreatic stellate cells. Shi showed that quiescent PSCs were activated when co-culture with macrophage cell lines and PSCs in turn increased the cytokine production of macrophages [123].

1.4 Pancreatic cancer models

In order to simulate the in vivo environment of pancreatic cancer, models have been built for a better understanding of the biology of pancreatic cancer. These models include: three-dimensional in vitro models and in vivo mouse models.

Three dimension (3D) models often consist of a matrix, which is composed of extracellular proteins such as collagen and basement membrane proteins, with the cells or tissue cultured on top or within the matrix [124]. It allows the study of cell-cell and cell-ECM interactions, in addition to the influence of the microenvironment on cells. At present time, the most widely used three dimension system is multicellular tumor spheroids [125]. Spheroids are aggregates of cells grown in suspension or embedded in a 3D matrix using 3D culture methods [126]. They can be used to study tumor growth and proliferation, invasion, matrix remodeling, immune interactions and drug screening [127]. Compared to 2D models, 3D models have many advantages. They make it possible to capture and quantify invasion, which is not possible in 2D culture. Also, in cancer, they provide a very good method to study how tumor microenvironment interacts with cancer cells. Besides, they resemble more closely the in vivo situation [124]. However, they also have their limitation, the matrix composition and stiffness will alter cellular response and the thickness of the matrix will affect the nutritional status of cells [128].

For in vivo mouse models, there are mainly three kinds: xenograft mouse models, carcinogen induced mouse models and genetically engineered mouse models, showed in Figure 5. Xenograft mouse model of pancreatic cancer is created by transplanting human pancreatic cancer cell lines under the skin of immune compromised nude mice. They can be used to

study cancer cell/host cell interactions and the efficacy of new anticancer drugs [129]. For these models, it is easy to measure tumor dimensions after resection. However, it's impossible to study metastasis by using these models and they ignore the contribution of the host immune system in the tumor progress [130]. Orthotopic mouse models are a little bit more complicated. They generated by injecting cancer cell into the mouse pancreas. They can help to study the tumor in its native position but they are expensive and technically difficult. Carcinogen induced models are generated by treating mice with certain chemicals that will lead to pancreatic cancer. Such as intraperitoneal injection of N-nitrosobis(2-oxopropyl)amine in hamsters [131]. Since about 70% of human tumors are induced by carcinogens, chemically induced models are of particular value. These models can be used to assess risk factors and find possible preventive and therapeutic methods for cancer [132]. However, these carcinogens also affect other organs, so the usage is limited. As mentioned above, pancreatic cancer is a gene related disease, so using genetically engineered mouse models to mimic relevant genetic mutations in pancreatic cancer is an invaluable tool to study cancer. And compared to xenograft tumors, genetically engineered mouse models are considered as an even closer approximation of human disease conditions [133].
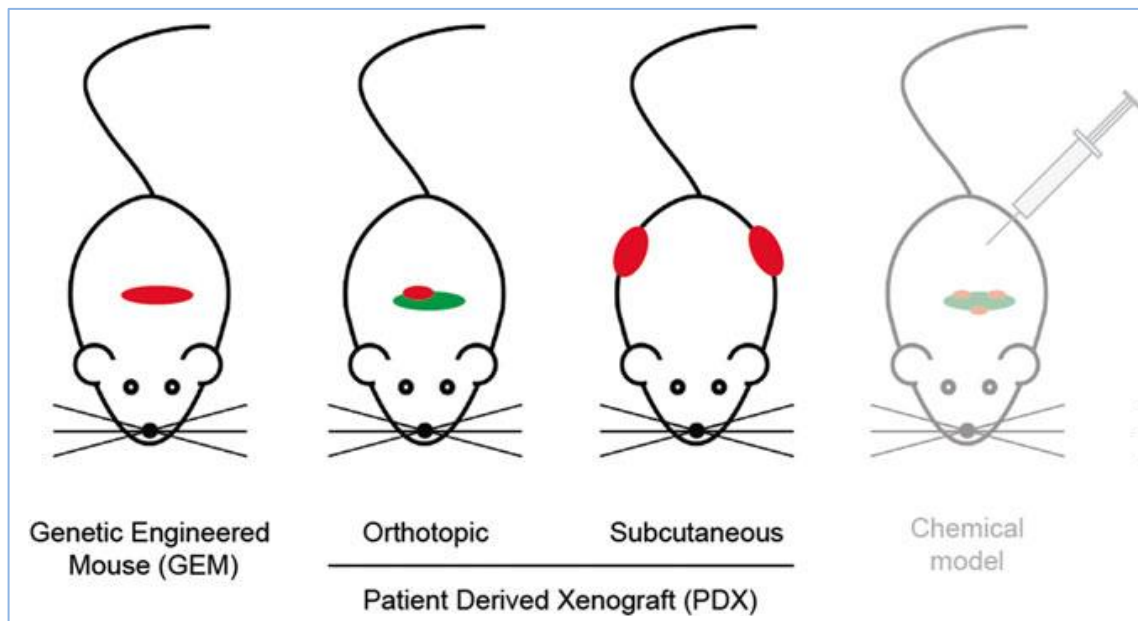

Figure 5. Mouse models of pancreatic cancer: genetically engineered models and xenograft models are currently considered to best recapitulate the human pancreatic adenocarcinoma [134].

1.5 EP300

EP300 is short for E1A-associated protein p300. It is a large size protein and has a molecular weight of about 300 kDa. This protein is commonly expressed in human tissues and highly evolutionary conserved and present in many organisms including flies, worms and plants. It is a nuclear protein and mainly has three different functions: (1) Acetylation of histones tails. EP300 can acetylate promoter nucleosomal histones resulting in chromatin remodeling and relaxation, thus increase accessibility of the DNA to regulators. (2) Acetylation of other target proteins. EP300 can acetylate transcriptional factors such as E2F, HMGI and HNF4, modulating their activity and causing either positive or negative effect on transcription. (3) RNA Polymerase II stabilization. EP300 can work as a bridge to link the DNA-bound transcription factors to the basal transcription machinery [135].

Besides the functions mentioned above, EP300 also involves in a lot of biological processes, such as proliferation, cell cycle regulation, apoptosis and differentiation [136-138]. Evidences showed that EP300 activity is required for G1/S transition [139, 140]. Down regulation of EP300 inhibits apoptosis, which is possible by damaging the p53-mediated response to DNA damage [141]. Furthermore, EP300 is often found mutated or in a truncated form in various human tumors, such as colorectal cancer, gastric cancer, ovarian cancer, breast cancer and pancreatic cancer [142-144]. Research showed that lower expression of EP300 in colon carcinoma cells induces these cells to go epithelial mesenchymal transition [145]. And EP300 proved to be a tumor suppressor gene in metaplastic breast cancer [146]. EP300 has also been implicated in embryonic development. It is showed that EP300 and CBP knockouts are early embryonic lethal and these two genes are essential for mammalian cell proliferation and development [147].

The crystal structure of human EP300 has been well studied. It mainly has three catalytic cores: bromodomain, CH2 region and HAT domain. The CH2 region includes a PHD domain and a RING domain, showed in Figure 6 [148]. Mutations that inactivate the HAT domain are found in various cancers [149], mutations in the PHD domain are found in Rubinstein-Taybi syndrome [150]. Cancer-related mutations in the RING domain has been found lead to an increase in EP300 histone acetyltransferase activity [148]. Studying the core structure of EP300 and understanding the difference between different disease-related EP300 mutations may have important implications for pharmacological targeting.
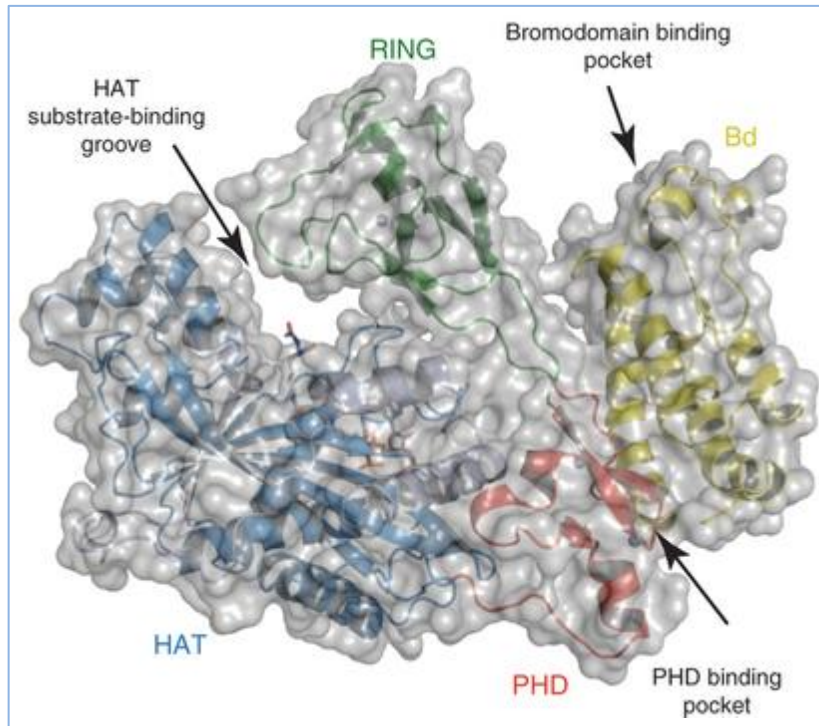
Figure 6. Domain architecture of EP300. The bromodomain, RING and PHD domains are shown in yellow, green and red, respectively. The N and C subdomains of HAT domain are shown in blue and gray, respectively [148]. The author of this thesis modified the picture.

1.6 **Aim of the study**

As mentioned above, EP300 is an important transcription coactivator and participates in regulating cell proliferation, differentiation and apoptosis. Moreover, it has been found mutated in pancreatic cancer. Additionally, in PDAC, PSCs change from quiescent cells to active cells, they differentiate to myofibroblast-like cells, begin to proliferate and migrate. Therefore, we hypothesized that targeting EP300 may affect the activation of PSCs and influence the communications between PSCs and pancreatic cancer cells. Hence, we are going to explore the gene functions of EP300 in PSCs in the current study.

2 Materials and Methods

2.1 Materials

Table 1 Cell lines

Cell lines	Resources
Immortalized PSCs	A gift from Ralf Jesnowski [69], Mannheim Univerisity Hospital
Bxpc-3	Authentificated by DKFZ, Heidelberg, Germany
Panc-1	Authentificated by DKFZ, Heidelberg, Germany

Table 2 Antibodies

Product	Company	Catalogue Number
AKT antibody	Cell signaling	9272
Anti-mouse IgG(H+L) Peroxidase	Biozol	VEC-PI-2000
Anti-rabbit IgG(H+L) Peroxidase	Biozol	VEC-PI-1000
Col-I antibody	Abcam	Ab34710
EP300 antibody	Abcam	Ab3164
ERK1/2 antibody	Cell Signaling	9102
Fibronectin antibody	Sigma Aldrich	F3648
GAPDH	Sigma-Aldrich	G9295
pAKT antibody	Abcam	Ab81283
Phopho-ERK 1/2 antibody	Cell Signaling	4307
pSTAT3 antibody	Abcam	Ab76315
STAT3 antibody	Cell signaling	8768
α smooth muscle actin antibody	Acris	14395-1-AP

Table 3 Kits

Product	Company	Catalogue Number
BCA Protein Assay Kit	Thermo Scientific	23225
Fast SYBR® Green Master Mix	Thermo Scientific	4385612
Go Taq Green Master Mix	Promega	M7122
Immobilon western chemiluminescent HRP substrate	Millipore	WBKLS0500
RevertAid First strand cDNA synthesis kit	Life technology	K1622
QIAquick® Gel extraction kit	Qiagen	28704
DNeasy Blood & Tissue Kit	Qiagen	69504

Table 4 Reagents

Product	Company	Catalogue Number
1,2-Bis (dimethylamino) ethane(TEMED)	Roth	2367
12-Maltoside	Sigma-Aldrich	D4641
Accutase	Sigma-Aldrich	A6964
Acrylamid-stammlösung 30%	Rotiphor	12623
Agarose	Sigma-Aldrich	A9539
Albumin from bovine serum	Sigma-Aldrich	A2153
Ammoniumpersulfate (APS)	Sigma-Aldrich	A3678
ASB-14	Sigma-Aldrich	A1346
Benzonase nuclease	Merck	70746-4
Bicine	Sigma-Aldrich	B3876
C646>98%(HPLC), 5mg	The Geyer	SML0002
Chloroform	Sigma-Aldrich	288306
DMEM	Life Technologies	41965062
DMSO	Genaxxon Bioscience	M6323.0100
DNA Gel loading dye(6×)	Thermo Sicentific	R0611
DPBS	Life Technologies	14040174
Ethanol, absolute	Sigma-Aldrich	24102

Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA.2Na)	Sigma-Aldrich	E5134
FBS	Life Technologies	10500064
Gemcitabine	Biomol	Cay11690-10
Generuler Low range DNA Ladder, ready to use	Life Technology	SM1193
Glycerol	Sigma-Aldrich	G5516
Glycine for electrophoresis	Sigma-Aldrich	G8898
Glycogen, RNA grade	Fisher Scientific	R0511
Halt^{TM} Protease and Phosphatase inhibitor	Thermo Sceintific	78443
Hydrochloric acid (HCl), 37%	VWR international	85848.290
IMDM (with phenol red)	Life Technologies	21980065
IMDM (without phenol red)	Life Technologies	21056023
Isopropanol	Sigma-Aldrich	W292907
Laemmli Sample Buffer 4×	Bio-Rad Laboratories	161-0747
LightCycler 480 Multiwell Plate 384,white	Roche	04729749001
Methanol	Sigma-Aldrich	322415
Millex-GP, 0.22µm filter	EMD Millipore	SLGP033RS
Mission predesigned siRNA	Sigma-Aldrich	PDSIRNA2D
Na-cholate	Sigma-Aldrich	C6445
Nonfat dry milk	Bio-Rad Laboratories	170-6404
NP-40	Sigma	74385
Nuclease-free water	Life Technologies	AM9939
PBS	Life Technologies	10010056
PepGREEN DNA/RNA dye	VWR	37-5010
Pen/Strep	Life Technologies	15140122
PMSF	Cell Signaling	8553
Ponceau S solution	Serva	33427.01
Positive control siRNA	Sigma-Aldrich	PDsiRNAPC2D
Prosie Quadcolor protein marker4.6-300kda	Biozym	830537
Resazurin	Fisher Scientific	10684882
Restore Plus western blot stripping buffer	Life Technologies	46430
RNase ZAP^{TM}	Sigma	R2020-250ml

RNaseOUT^{TM} Ribonuclease inhibitor	Invitrogen	10777-019
siRNA transfection reagent, X-treme	Roche	04476093001
Sodium Acetate Solutaion 3M	Life Technologies	R1181
Sodium Chloride (NaCl)	Sigma-Aldrich	S9888
Sodium dodecyl sulfate(SDS)	Sigma-Aldrich	71725
Sodium hydroxide	Fisher Scientific	11958484
Spectra Multicolor Broad Range Protein Ladder	Life Technologies	26634
T7 Endonuclease 1	NEB	M0302S
TMB Liquid substrate system for ELISA	Sigma-Aldrich	T0440
Triton X-100	Sigma-Aldrich	T8787
Trizma® Base	Sigma-Aldrich	T1503
Trizma® HCL	Sigma-Aldrich	T3253
Trizol Reagent	Invitrogen	15596-0108
Trypsin (0.05%)	Life Technologies	25300062
Tween® 20	Sigma-Aldrich	P2287
U0126	Abcam	Ab120241
X-treme GENE HP DNA transfection reagent	Roche	06366244001

Table 5 Buffers and Solutions

Name	Composition
1×TBST	100ml 10×TBS, 1ml Tween 20, dilute it in 900 H_2O
10%APS	1gAPS, 10ml H_2O
10%SDS	10g SDS, 100ml H_2O
10× Laemmli running buffer	30g Tris base, 10g SDS, 144g Glycin , 1L H_2O
10×TBS	31.52g Tris HCl, 80g NaCl, add 900ml H_2O, adjust pH to 7.6, then fill the bottle to 1L
4×Loading buffer for western (10ml)	2.0ml 1M Tris-HCl, 4.0ml 100% glycerol, 1.0ml 0.5M EDTA, 8mg bromophenol blue 0.8g SDS, 0.4ml 14.7 M β-mercaptoethanol

5% Milk	10g fat skim milk powder and solve it in 200ml TBST
Anode I buffer	36.4g Tris base, 200ml Methanol, fill it up to 1L with H_2O
Anode II buffer	3g Tris base, 200ml Methanol, fill it up to 1L with H_2O
Cathode buffer	3g Tris base, 5.2g 6-aminocaproic acid, 200ml Methanol, fill it up to 1L with H_2O
Lysis buffer (10ml)	NP-40(20%) 500μl, Na-cholate (10%) 1000μl, ASB-14 (5%) 1000μl, 12-Maltoside(2.5%) 1000μl, Glycerol(99%) 2000μl, Bicine (0.5M, pH 8.5) 1000μl NaCl(1.50M) 1000μl, EDTA.2Na(0.02M) 1000μl PMSF(200mM) 50μl, Pro&Phosph inbihitor 100μl Benzonase 4μl, dH$_2$O 1346μl
PBST 1×(1L)	8g NaCl, 0.2g KCl, 1.44g NaHPO$_4$, 0.24g KH$_2$PO$_4$ 1ml Tween 20, adjust pH to 7.4
Sammel Buffer	47.28g TrisHCl in 200ml dH$_2$O, adjust pH to 6.6 with NaOH
TBE Buffer 10×(1 L)	108g Tris, 55g Boric acid, 40 ml 0.5M Na$_2$EDTA, pH 8
Trenn Buffer	36.33g Tris.Base in 200ml dH$_2$O, adjust pH to 8.8 with HCL
Western wet transfer buffer	3g Tris Base, 14.4g Glycine, 1gSDS, 800ml H$_2$O, 200ml methanol

Table 6 Materials

Product	Company	Catalogue Number
8 strip PCR tubes (0.2ml)	Life Technologies	AM12230
Adhesive PCR seal	Biozyme	600208
Amicon® Ultra-0.5ml Centrifugal Filters Ultracel® -3K	Merck Millipore	UFC500396
Cell culture flasks 175cm	DKFZ Lager	12649
Cell culture flasks 25cm	DKFZ Lager	13640
Cell culture flasks 75cm	DKFZ Lager	12667
Cell culture plates-6 well	DKFZ Lager	657160
Cell culture plates-96 well	DKFZ Lager	655180

Cell Scraper, 39cm	Neolab	100128121
Cell Scraper, small 24cm	Neolab	100128120
Cover slips, square 0.22×0.22 mm	Carlroth	H87
Cryovials, 1ml	Greiner	123263
Cryovials, 2ml	Greiner	121261
Eppendorf safe lock micro centrifuge tubes (0.5ml, 1.5ml and 2ml)	Eppendorf	0030121594/ 0030121597/ 0030121570
Filter tips 1000µl	Biozym	701281
Filter tips 20 µl	Biozym	701221
Filter tips 200 µl	Biozym	701261
Fisherbrand™ Graduated Cylinders 100,250ml, 1000ml	Fisher scientific	S63458 S63459 S63461
Fisherbrand™ Reusable Galss Media Bottles with Cap, 100ml, 250ml, 1000ml	Fisher scientific	FB800100 FB800250 FB8001000
Flacon tube 15ml	DKFZ Lager	14258
Flacon tube 50ml	DKFZ Lager	12633
GE Healthcare 3mm CHR blotting paper sheets 46×57 cm	GE Healthcare	3030917
Gloves, Latex medical examination	BM11228-PF-AV	Blossom
Gloves, Nitril Freeform SE	FFS-700	Microflex
HTS Transwell-96 system, 8µm	Sigma-Aldrich	CLS3374
Light Cycler® 480 Multiwell plate 384, white	Roche	04729749001
Millex GS Filter, steril, 0.22µm	Millipore	SLGS033SS
Mycoplasma ExS Spray	Promo Cell	PK-CC91-5051
Nitrocellulose membrane 0.45µm	GE Healthcare	GE10600007
Pasteur pipettes 230mm	DKFZ Lager	12908
PVDF membrane 0.45µm	Merck Milipore	IPFL10100
Reservors,25ml	Fisher Scientific	11475748
Serological Pipettes 10ml	DKFZ Lager	14301

| Serological Pipettes 25ml | DKFZ Lager | 14302 |
| Serological Pipettes 5ml | DKFZ Lager | 14300 |

Table 7 Equipments

Name	Manufacturer
CO_2 Water Jacketed incubator	Thermo Life Science
Electronic Balances, Kern 434,440-45	Kern & Sohn GmbH
Fluostar Galaxy plate reader	MTX Lab System
Gilson Pipetman P1000 single channel pipette	Gilson
Gilson Pipetman P2 single channel pipette	Gilson
Gilson Pipetman P20 single channel pipette	Gilson
Gilson Pipetman P200 single channel pipette	Gilson
Ice maker	Scotsman
LAS-4000 mini	Fujifilm Corporation
Light Cycler system	Roche
Microwave oven	Bosch
Mini-PROTEAN Tetra Vertical Electrophoresis cell for Mini precast gels	Biorad
Nanodrop Spectrophotometer N1000	Thermo Scientific, USA
PCR Thermocycler PTC200 MJ research	BioRad, USA
PH-Meter MP230 Mettler Toledo	Mettler Toledo, Germany
Power scanner	Tecan
Spectrafuge 3-1810 Centrifuge	NeoLab
Sterilgrad Hood Class II Type A/B s	The Baker Company
TE 70 Semi-dry transfer unit	Amersham Bioscience
TKA MilliQ water supply	Millipore
Vi cell XR cell viability analyzer, cell counter	Beckman Coulter
Vortex Mixer	Neolad
Water Bath	Grant Instruments
WILOVERT 30, Microscope	Helmut Hund GmbH
Heating Block	Grant Instrument
Microcomputer electrophoresis power supply	Renner GmbH

Orbital Shaker	Fröbel Instruments
Mr. Frosty™ Freezing Containers	Thermo Fisher scientific

2.2 Method

2.2.1 Cell culture

Immortalized human pancreatic stellate cells were a gift from Ralf Jesnowski[69]. Bxpc-3 and Panc-1 used in this article were authentificated by DKFZ internal service. All cell lines were cultured on 175 cm^2 flasks in IMDM medium containing 10% fatal bovine serum, 50 units/mL penicillin and 50μg/mL streptomycin at 37 °C with 5% CO_2. Cells were separated every two days at a ratio of 1:5 and tested for mycoplasma contamination every month.

For sub-culturing of these cells, when cells reached 80%-90% confluence, removed the old medium and washed the flask twice with PBS, then added 1ml of 0.05% trypsin per flask. Incubating them at 37 °C for 5-10min, once the cells were detached, medium containing 10% FBS was used to inactivate trypsin. Then cells were separated at the ratio mentioned before.

For storage of cells, when cells reached 80-90% confluence, cells were detached as mentioned above. The cell suspension was centrifuged at 1500 rpm for 5 minutes and cell pellet was collected. The pellets were subsequently re-suspended in a cryoprotectant containing 60% FBS, 30% complete medium and 10% DMSO. One ml of cell suspension (around 1×10^6 cells) was added to each cryovial. The vials were placed in a Mr. Frosty and stored at -80 °C for 1-2 days, prior to being transferred to liquid nitrogen tank for long term storage. When recovering cells from liquid nitrogen tank, cells were thawed in a 37 °C water bath as quickly as possible. Then cell suspensions were transferred to a 15ml falcon tube containing pre-warmed complete medium, centrifuged and the supernatant was removed. Complete medium was used to resuspend the cells and cells were transferred into a culture flask for recovering.

For counting of cells, cells were detached and well mixed. Then 500μl of cell suspension was put into the 4ml sample cup and counted by the Vi Cell counter.

2.2.2 siRNA transfection

PSCs were seeding on a 6-well plate 24 hours before transfection (1×10^5/well), making sure that the cell confluence would reach 50%-60% at the time of transfection. Cells were treated with a mixture of 100nM EP300 siRNA (SASI_Hs01_00052818, sigma aldrich) and 20 μL X-

tremeGENE siRNA transfection reagent (Roche Diagnostics) in a volume of 2 mL according to the manufacturer's protocol. Scramble siRNA control was purchased from Santa Cruz. Seven hours after transfection, medium was changed to full growth medium. Cells were harvested 24 hours post transfection for real time quantitative PCR and 48 hours post transfection for western blot. For supernatant collection, cells were grown for 48 hours after transfection, serum free for 48 hours and then the media were collected.

2.2.3 CRISPR/Cas9 gRNA transfection

A commercial CRISPR plasmid pGS-gRNA-Cas9-Puro with gRNA sequence: TTTGCCGGGGTACAATAGG specifically targeting EP300 was bought from the company GenScript. The same plasmid with scramble gRNA sequence was served as control. Cells were seeded at 6-well plate 24 hours before transfection, making sure that they would reach 80%-90% confluence at the time of transfection. X-tremeGene HP DNA transfection reagent were used according to the manufacturer's instruction. Briefly 2µg plasmid and 8µL transfection reagent in a total volume of 2 mL were added in each well. 72 hours post transfection, cells were selected with 1µg/mL puromycin for approximately 14 days. Every 3 days, fresh medium with puromycin was added. Surviving cells were pooled. T7 endonuclease I assay (T7E1) was used to detect Cas9 induced mutations, western blot was used to check the protein expression. For supernatant collection, cells were seeding in a 75cm^2 flask for 24 hours, so they could reach 80-90% confluence, then serum free for 48 hours and the media were collected.

2.2.4 T7E1 assay

Genomic DNA was extract from the stable knockdown cell lines by using a DNA extraction kit. A fragment of approximately 900bp was amplified from genomic DNA with the primer mentioned below. The PCR products were then purified on a 1.5% agarose gel and extracted by using a gel extraction kit. After that, 400ng purified DNA was denatured at 95 °C for 5 minutes and slowly reannealed. Last, 1µL (10U) T7 endo I (NEB) enzyme was added and incubated at 37°C for 15min. The reaction was stopped by adding EDTA, and the digestion product was immediately run on a 1.5% agarose gel.

2.2.5 C646 treatment

Normal PSCs were grown in a T75 flask (2.5×10^5/flask), 24 hours later when the confluence of the cell would reach 80%-90%, serum free overnight. Then cells were treated with 20μM C646 in serum free medium for 24 hours or 48 hours. Serum free medium was used since C646 was inhibited by serum. No longer treating time was done, because when cells were treating with C646 in serum free medium for 72h, they lose the viability. Cells treated with 20μM DMSO were served as control, since C646 was dissolved in DMSO.

2.2.6 Quantitative real time PCR (qRT-PCR)

24 hours after siRNA transfection, the knockdown efficiency and gene expression of αSMA, FN and Col-I by PSCs were quantified with RT-PCR. Total RNA was extracted using Trizol reagent (Invitrogen). Briefly, 1ml of Trizol reagent was added to each well of the 6-well plate to lysis cells. Subsequently, RNA was separated with chloroform and precipitated out of the aqueous fraction with isopropanol and glycogen. 70% ethanol was used to wash the pellet twice. Then the pellet was dried and resuspended in water. RNA concentration was measured with Nano drop and 500 ng of RNA was used for the reverse transcription. cDNA synthesis was performed with a kit and following the instructor's protocol. Quantitative real time PCR was performed using Light Cycler system (Roche) and Fast Sybr green (Life technology). All things were done according to the manufacturer's protocol. HPRT1 was served as the control gene. The primer, reaction system and program used for real time PCR were as follows:

Table 8 Primer

Gene	Primer
EP300	Forward primer: 5'-GCAGTGTGCCAAACCAGATG-3' Reverse primer: 5'-GGGTTTGCCGGGGTACAATA-3' (105bp)
αSMA	Forward primer:5'- GAGGGAAGGTCCTAACAGCC-3' Reverse primer:5'- TAGTCCCGGGGATAGGCAAA-3'
FN	Forward primer:5'- GTCGGAGAAACGTGGGAGAA-3' Reverse primer:5- GAAGTGCAAGTGATGCGTCC-3'
Col-I	Forward primer: 5'-GCTCTTGCAACATCTCCCCT-3' Reverse primer: 5'-CCTTCCTGACTCTCCTCCGA-3'
EP300	Forward primer: 5'- CTGCTACTGTGAATGAGACAGA-3' Reverse primer: 5'- AGAACCAGGCAAAAACGCAC-3' (867bp)

Hprt1	Bought from Qiagen
	Product: Hs_HPRT1_1_SG QuantiTect Primer Assay
	Product no.249900
	Cat.no. QT00059066

Table 9 Reaction system used for realtime PCR

Gene		1× run (μL)
αSMA FN Col-I EP300	cDNA	1
	Forward primer (10μM)	0.2
	Reverse primer(10μM)	0.2
	Sybr Green Master Mix 2×	5
	Nuclease-free water	3.6
	Total Volume	10

Gene		1× run (μL)
Hprt1	cDNA	1
	Primer (10μM)	1
	Sybr Green Master Mix 2×	5
	Nuclease-free water	3
	Total Volume	10

Table10 Program used for real time PCR

Step	Temperature (°C)	Duration	Cycles
Polymerase activation	95	20 second	Hold
Denature	95	3 second	
Anneal/Extend	60	30 second	40

2.2.7 Western blot

For isolation of protein, cells in culture were washed three times with ice-cold PBS and lysed on ice with lysis buffer prepared by ourselves with freshly added PMSF for 30min. Cells were subsequently collected in a 1.5ml Eppendorf tube by scraping with a cell Scraper. Then the

liquid was resuspended with a syringe for 20 times and centrifuged at 15,000 g, 4 °C for 20 minutes. The supernatant was transferred to a second labled Eppendorf tube and protein concentration was determined with a BCA kit according to the manufacturer's instructions. If the proteins were not to be used immediately, samples were stored at -80 °C.

For western analysis, certain amounts of proteins (5-10μg) with loading dye were boiled at 95 °C for 5 minutes and loaded onto SDS-PAGE gels. Samples were run in the running buffer for 10min, 75V constant, then 90min, 135V constant (12% gel). The transfer of proteins from the gel to a Nitrocellulose membrane was carried out by a semidry transfer system. A sandwich model was made by soaking CHR blotting paper in Anode buffers I, Anode buffer II and Cathode buffer with membrane and gel. The semidry electrophoretic transfer was carried out for 60 minutes at 35V, 500mA. Then Membranes were blocked in 5% non-fat milk in Tris-buffered saline (TBS, 10 mM Tris, 10 mM NaCl) for 1h at room temperature. Subsequently washed and incubated with first antibody overnight at 4°C. Immunodetections were done with the corresponding secondary antibodies. ECL (Roche) and ImageQuant LAS 4000 mini was used for visualization. The densitometric analysis was done by using Image J software.

For large molecular weight proteins, such as FN, Col-I and EP300, 6% SDS-PAGE gel and PVDF membrane were used. The transfer of these proteins to PVDF membrane was done by using a wet-transfer system. And the wet electrophoretic transfer was carried out at room temperature for 4 hours at 150mA, 45V. For Col-I, the western was done at native condition. All the other steps were the same as mentioned above.

For stripping, membranes were put into the stripping buffer for 10 minutes at room temperature, washed with PBS three times and blocked with 5% milk for 60 minutes before incubating with another primary antibody.

The primary antibodies and the dilution ratios used were GAPDH (Sigma, 1:5000), EP300 (Abcam, 1:500), αSMA (Acris, 1:1000), FN (Sigma, 1:500), α-tubulin (Sigma, 1:5000), pAKT (Abcam, 1:5000) , AKT(Cell signaling, 1:1000), pSTAT3 (Abcam, 1:100000), STAT3α (Cell signaling, 1:1000), pERK1/2(Cell signaling, 1:3000), ERK (Cell singaling,1:3000). The secondary antibodies used were HRP-conjugated goat-anti-rabbit (Vector, 1:5000), horse-anti-mouse (Vector, 1:5000).

2.2.8 ELISA Assay

Col-I and FN are secreted protein, in order to analyze the secretion of them, the supernatant was collected as showed before. Subsequently, the media was condensed with Amicon Ultra-10 centrifugal filter (Merck Millipore, MA, USA) and ELISA assay was performed to test the secretion of Col-I and FN. Results was normalized to cell numbers. Briefly, condensed media were coated in a 96 well microtiter plate (Immunoplates MaxiSorp C 96, invritogen,) at 4°C overnight, then wells were blocked with 3% BSA for 1 hours, after washing three times with PBST, first antibody was incubated, then HRP conjugated secondary antibody was incubated. At Last TMB liquid were added to each well and signal was detected at 370 nm with a plate reader.

2.2.9 Cells cultured on coverslips

The 0.22×0.22 mm glass coverslips were soaked in 70% ethanol for at least 2 hours before using. Then one coverslip was placed over the bottom of each well of the 6-well plate with clean tweezer. After that, open the lid of the 6-well plate and put it into the cell culture hood, air dry for 30 minutes with the UV light on. Subsequently, stable EP300 down regulation cells and control group cells were seeded onto the coverslips at a concentration of $5×10^4$ for 48 hours before the morphology was checked.

2.2.10 Proliferation assay

Stable EP300 knockdown PSCs and corresponding control PSCs were seeded in 96-well plates at $4×10^3$ per well. 24 hours later, serum free overnight, and fresh complete medium was added. Then at the time point 24 h, 48 h, 72h, the proliferation of the cells was tested by resazurin assay. For conditioned medium treatment, $8×10^3$ Bxpc-3 or Panc-1 cells were seeding in 96 well plates, 24 hours later, serum free overnight, and conditioned medium were added. Then at the time point of 72h, the proliferation of the cells was tested by resazurin assay. Briefly, resazurin solution was added to each well, and make sure the final concentration of resazurin is 20μg/mL. Then the plates were incubated at 37°C for 2 hours and the fluorescence was recorded using FLUOstar Galaxy system. Ex=544 nm, Em=590 nm.

2.2.11 Drug cytotoxicity assay

The same number of cells was seeded and the same treatments were done as proliferation assay. It's just that after serum free overnight, different concentrations of gemcitabine were

added to the cells in complete medium. 72 hours later, cell numbers were tested by resazurin assay as described above. For medium treatment drug sensitivity assay, complete medium was changed to conditioned medium during the experiment. All the other steps were the same.

2.2.12 Migration assay

HTS Transwell-96 well plate (Corning) with a pore size of 8 μm was used to do the migration assay. Briefly, 1.2×10^4 PSCs were added in serum-free medium in the upper layer. The lower layer was filled with complete medium, 48 hours later, cells on the bottom surface of the upper layer were detached and counted using resazurin assay. In the inhibitor treatment experiment, both the upper layer and the lower layer contains 20 μM DMSO or 20 μM U0126 during the whole experiment.

2.2.13 Conditioned media collection

Control PSCs and stable EP300 knockdown PSCs were grown to 70% to 80% confluence in 175 cm^2 flasks in IMDM/10%FBS. Then the media were changed to serum free IMDM and cells were cultured for 48h. Media were collected, centrifuged at 3,000 for 15min, condensed by Amicon Ultra centrifugal filter and protein concentration was tested by BCA kit. Then they were aliquoted and store at -80 °C until use. The same protein concentration media from control PSCs and stable EP300 knockdown PSCs were used for medium treatment assay.

2.3 Statistic analysis

All experiments were done three or more times. Data were shown as mean ± standard error mean. Two-tailed Student's t test was used to make comparisons between different groups. Significant difference was defined differently based on different experiments. Statistical analysis was done using Excel.

3 Results

3.1 siRNA transient knockdown of EP300

To study the gene functions of EP300, siRNA was used knockdown EP300 in PSCs. 24 hours post transfection, quantitative RT-PCR results showed that the mRNA expression of EP300 was reduced by 77.7%±0.04% compared to control (Fig. 7A). Western result verified that the protein expression of EP300 was reduced correspondently 48 hours after transfection (Fig. 7B).
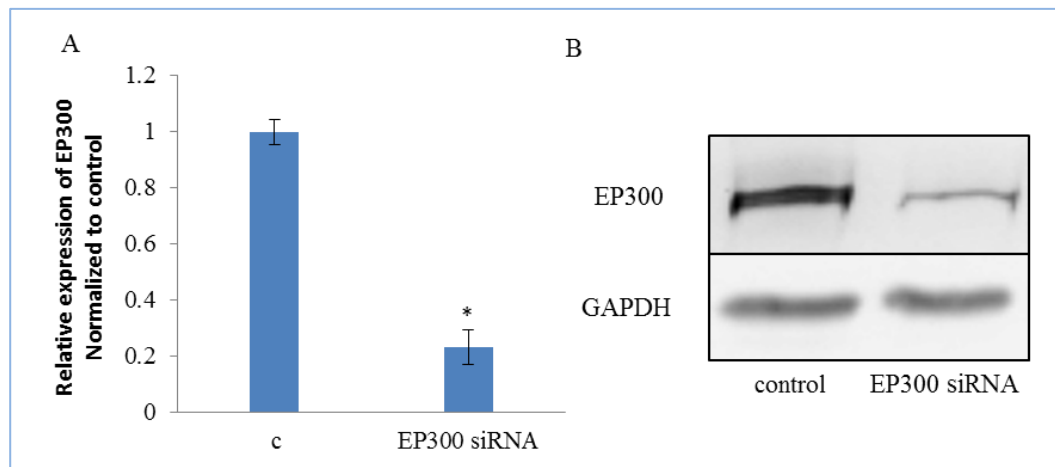
Figure 7. siRNA transient knockdown of EP300. A.24 hours after knockdown, cells were collected and qRT-PCR was used to analyze EP300 gene expression. Hprt1 was used as control. The data represented the mean and SD of three independent experiments,*, $P<0.05$; B. 48 hours after knockdown, EP300 protein expression of PSCs was analyzed by western blot, GAPDH was served as loading control.

3.2 Transient knockdown of EP300 affects the expression of PSCs' activation markers

To determine the activation status of PSCs after EP300 knockdown, the expression of α SMA, FN and Col-I, which were activation markers of PSCs, were studied in both mRNA and protein levels. FBS activates PSCs and PSCs were cultured in complete medium during the whole experiment, therefore PSCs were in an activation stimulation environment during the whole process. And, mRNA was collected 24 hours post knockdown and protein was collected 48 hours post knockdown. The expression of αSMA didn't change in both mRNA and protein levels. FN was down regulated in both levels (Fig. 8A, Fig. 8B). However, Col-I expression was increased at mRNA level, but reduced at the protein level. For the western results of Col-I, the antibody showed two major bands, the lower bands were 170 kDa for pro-collagen and the upper bands were 270 kDa for the dimer.
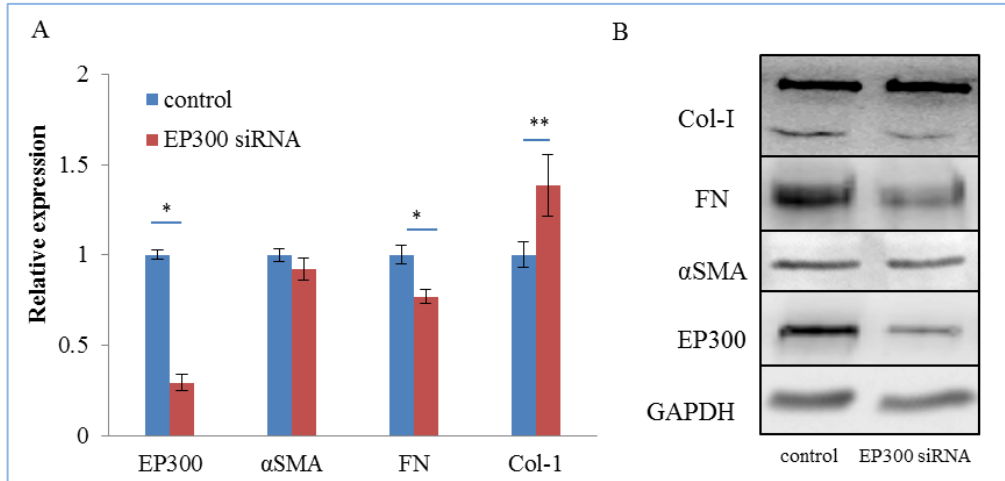
Figure 8. Effects of EP300 down regulation on the expression of activation markers of PSCs. A.24 hours after knockdown, cells were collected and qRT-PCR was used to analyze specific gene expression. Hprt1 was used as control. The data represented the mean and SD of three independent experiments,*, P<0.05; **, P<0.01. B. 48 hours after knockdown, selected protein expression of PSCs was analyzed by western blot, GAPDH was served as loading control.

3.3 Transient knockdown of EP300 reduces the secretion of FN and Col-I by PSCs

Since that FN and Col-I are extracellular matrix proteins secreted by activated PSCs, Elisa was used to analyze the protien expression levels of them in the conditioned medium. And the results showed that there were less amount of Col-I and FN in the experiment group's conditioned medium (CM) than in the control group's CM. Statistically, Col-I secreation was decreased by 50% ±0.05% (Fig. 9A), FN secreation was reduced by 32%±0.03% (Fig. 9B)
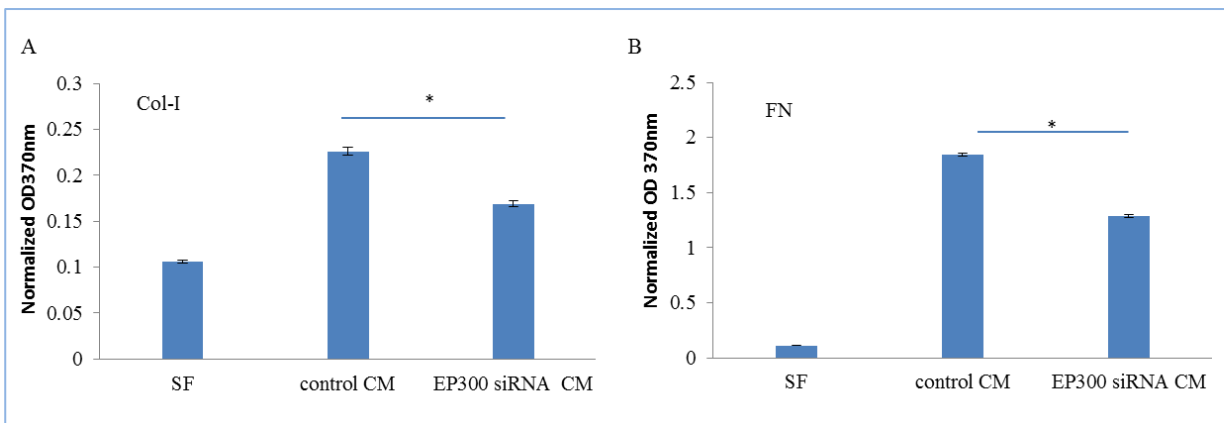
Figure 9. Transient knockdown of EP300 reduces the secretion of FN and Col-I by PSCs. 48 hours after knockdown, cells were cultured in serum-free medium for another 48 hours and the condioned medium was collected and condensed, subsequently, ELISA was used to analyze the secretion of Col-I and FN by PSCs. Results was normalized to cell numbers. Figure A and B respectivey showed the ELISA assay results of Col-I and FN.*,P<0.05.

3.4 Generation of EP300 stable knockdown cell lines

CRISPR/Cas9 gRNA was used to generate stable EP300 knockdown cell lines and scramble gRNA plasmid transfected cells were served as control. After antibiotic selection, stable knockdown cell line and corresponding control cell line were generated. Figure 10A was the result of T7 endonuclease I (T7E1) assay, which showed that there was cleavage in the DNA sequence in gRNA treated cells, so our targeting was successful. Figure 10B showed that EP300 protein expression was highly down regulated in gRNA treated group.
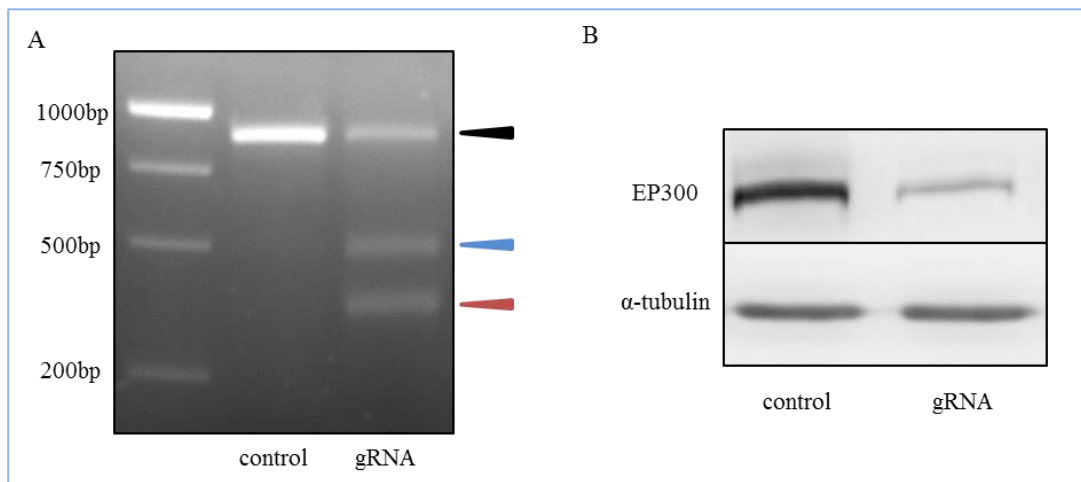
Figure 10. Generation of EP300 stable knockdown cell lines. A. After antibiotic selection, cells were cultured for 24h, then the genome DNA was extracted for T7E1 assay. PCR products of target regions are shown by black arrowhead. Colored arrowheads indicate cleaved products by Cas9. B. Cells were cultured for 48h, then lysis buffer was used to collect protein from them, western results showed that EP300 was downregulated.

3.5 Stable EP300 knockdown inhibits FN and Col-I synthesis by PSCs

After obtaining EP300 stable knockdown cell lines, cell lysis and conditioned medium were collected to test FN and Col-I expression inside and outside the cells. Same results were obtained as transient knockdown, compared to the control, both the synthesis and the secretion of FN and Col-I was reduced in the EP300 down regulation group. Figure 11A showed the western results of cell lysis, FN and Col-I expression was declined significantly. For the western results of Col-I, as mentioned before, the lower bands were for pro-collagen and the upper bands were for the dimer. Figure 11B showed the ELISA results of the conditioned media, Col-I secretion was decreased by 47%±0.07% and FN secretion was reduced by 25%±0.03% statistically compared to the control.
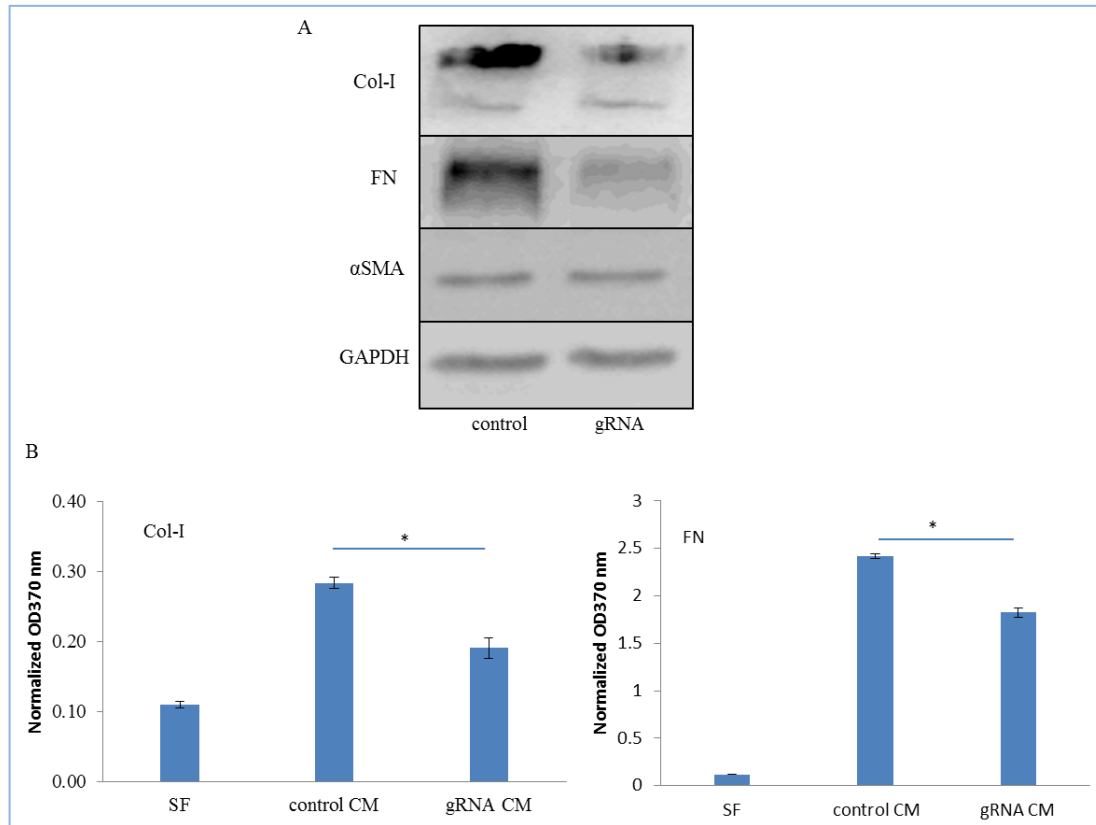
Figure 11. Stable EP300 knock down inhibits FN and Col-I synthesis by PSCs. A. EP300 downregulation PSCs and corresponding control PSCs were grown for 48 hours, then cell lysis was collected, subsequently, specific protein expression of PSCs were tested by western blot. The results showed that FN and Col-I expression was downregulation when EP300 is knockdown. B. For medium collection, cells were seeding in a 6-well plate for 24 hours, so they could reach 80%-90% confluence, then serum free for 48 hours before media collection. Media was condensed by Ultra filter from Merck Millipore before used for ELISA assay and results were normalized to cell number. *, P<0.05.

3.6 EP300 downregulation induces phenotype changes in PSCs

After seeding stable EP300 downregulation cells and corresponding control cells on glass cover slips for 48 hours, cell morphology was observed. A number of cells in the EP300 down regulation PSCs showed exactly fibroblast cell morphology, thin, long and spindle like with expanded cytoplasm, as indicated in Figure 12B with red arrow. However, in the control group, most of the PSCs were round shape like and tend to grow together, as showed in Figure 12A with blue arrow. We randomly selected 10 different fields of visions in both groups, took pictures and counted cells in them. The EP300 downregulation group has 16.7%±3.3% of cells possess a myofibroblast-like morphology, but in the control group only 5.4%±0.3% of cells were fibroblast-like (Fig. 12C).
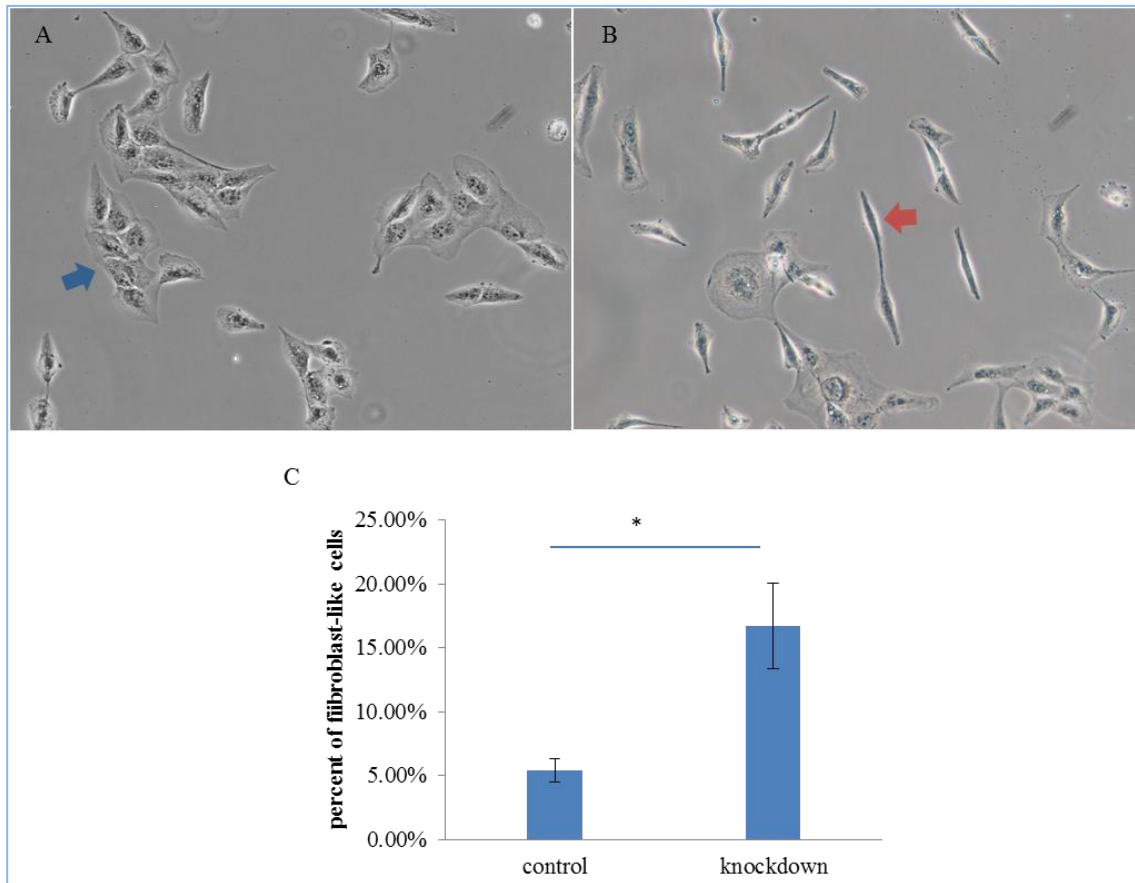
Figure 12. The morphology changes of pancreatic stellate cells. Control PSCs and stable EP300 knockdown PSCs were grew separately on glass slides for 48 hours, then cell morphology were observed. A.The morphology of control PSCs. Cells were round shape like and tend to grow together, as showed with blue arrow. B. The morphology of EP300 knockdown PSCs. Some cells showed fibroblast-like shape, thin, long with expanded cytoplasm, as indicated with red arrow. Original magnification 200×. C. Ten different fields of visions were randomly selected in both groups and cells were calculated. The percent of cells that possess myofibroblast-like phenotype in each group were showed. *, P<0.01.

3.7 EP300 down regulation doesn't affect the proliferation of PSCs

To determine whether EP300 knockdown will influence the proliferation of PSCs. The proliferation of stable EP300 knockdown cells and corresponding control group cells were tested at 24h, 48h and 72h by resazurin assay. Figure 13 showed that EP300 knockdown had no influence on the proliferation of PSCs.
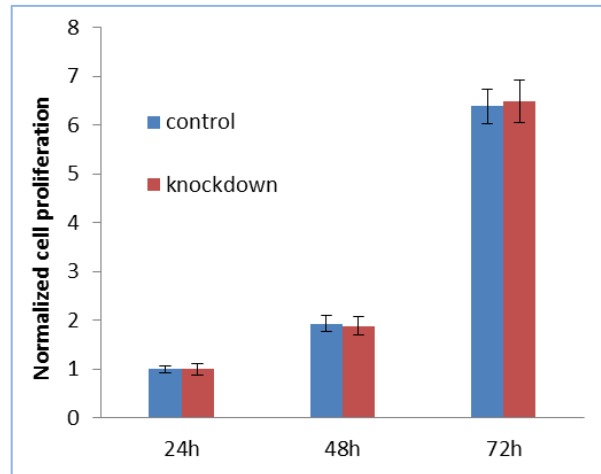
Figure 13. EP300 knockdown doesn't affect the proliferation of PSCs. The stable EP300 down regulation cell line and corresponding control cell line were cultured in 96 well plates. Cell proliferation was tested at time point 24h, 48h and 72h by resazurin assay. The data represented the mean and SD of three independent experiments performed in 8 replicates.

3.8 EP300 down regulation increases the drug sensitivity of PSCs

To study the drug sensitivity of cells after knockdown, stable EP300 down regulation cells and control group cells were treated with different concentrations of gemcitabine for 72 hours. As showed in Figure 14: at the concentration of 0.01μM, 60.1%±0.02% of the cells were still alive in the control group after drug treatment. However, only 34.3%±0.02% cells were still alive in the knockdown group. At the concentration of 0.1μM, 43.2%±0.01% of the cells were still alive in the control group, but only 28.8%±0.01% of the cells were alive in the experiment group.
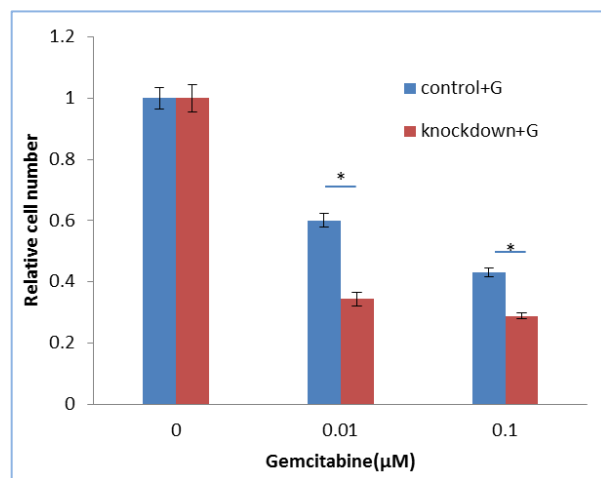
Figure 14. EP300 knockdown increases the drug sensitivity of PSCs. Control group and knockdown group cells were treated with different concentrations of gemcitabine for 72h in complete medium, and then the cell

numbers were tested by resazurin assay. These data represented the mean and SD of three independent experiments performed in 8 replicates.*, P<0.01.

3.9 EP300 down regulation promotes PSCs migration

When PSCs are activated, they will begin to migrate and promote the migration of pancreatic cancer cells simultaneously [75, 151]. Hence, after obtaining the stable knockdown PSCs, to investigate the modulation of metastasis by EP300, the migration of the PSCs was studied using trans-well plates. Results showed that EP300 down regulation increases PSCs migration significantly. Around a 3.5 fold increment in migration was observed.
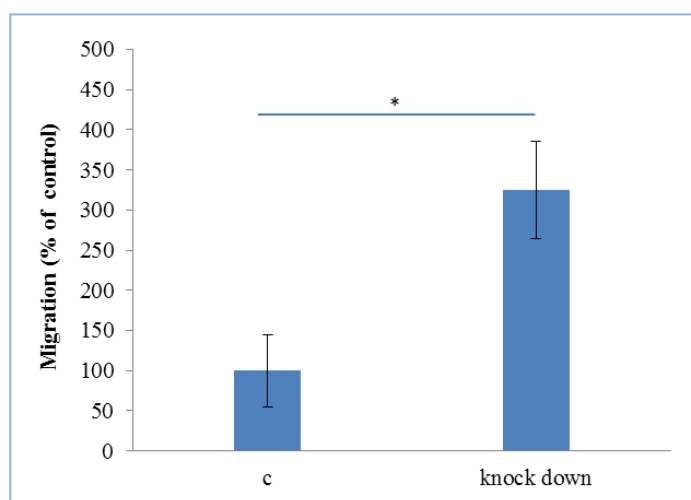
Figure 15. EP300 down regulation promotes cell migration significantly. Cells were seeded in a 96 trans-well plates according to the protocol, 48 hours later, cells that migrated through the membrane were counted. As shown above, EP300 down regulation increased the migration of PSCs more than 3.5 fold compared to the control. *, P< 0.05.

3.10 EP300 promotes the migration of PSCs through activation of ERK pathway

Totally, there are three pathways that are highly related to cell migration, which are PI3K pathway, JAK-STAT pathway and ERK pathway. To find out which pathway is related to EP300 induced migration, the activation status of the three pathways were tested. The expression of functional protein phospho-STAT (p-STAT) of JAK-STAT pathway was reduced (Figure 16A) compared to the control group and the expression of functional protein phospho-AKT (p-AKT) of PI3K-AKT pathway was not changed compared to the control (Figure 16B). Activation of ERK pathway is the result of phosphorylation of ERK1 and EKR2 (ERK1/2) on their serine and threonine residues by MAP kinase kinase. Figure 16C showed that EP300 down regulation led to phosphorylation of ERK1/2 even to 48 hours. EP300 chemical inhibitor C646 got the same effects (Fig.16D). However, 48h treatment with

C646 made the cells express less total ERK1/2, which means that the inhibitor is toxic to the cells in long time treatment.
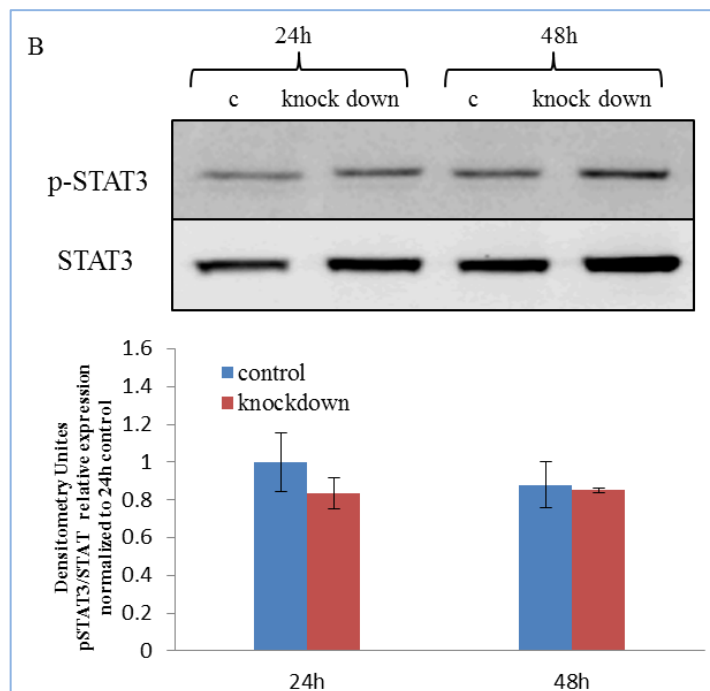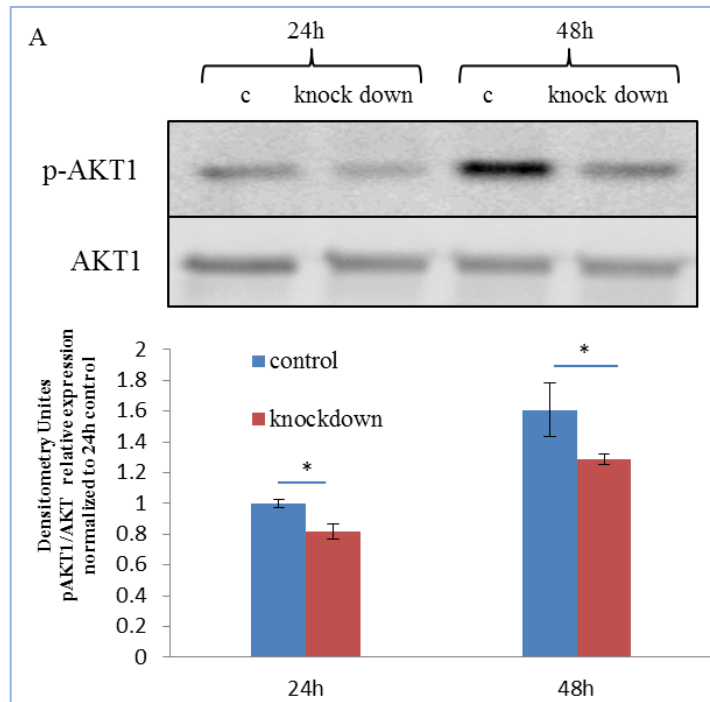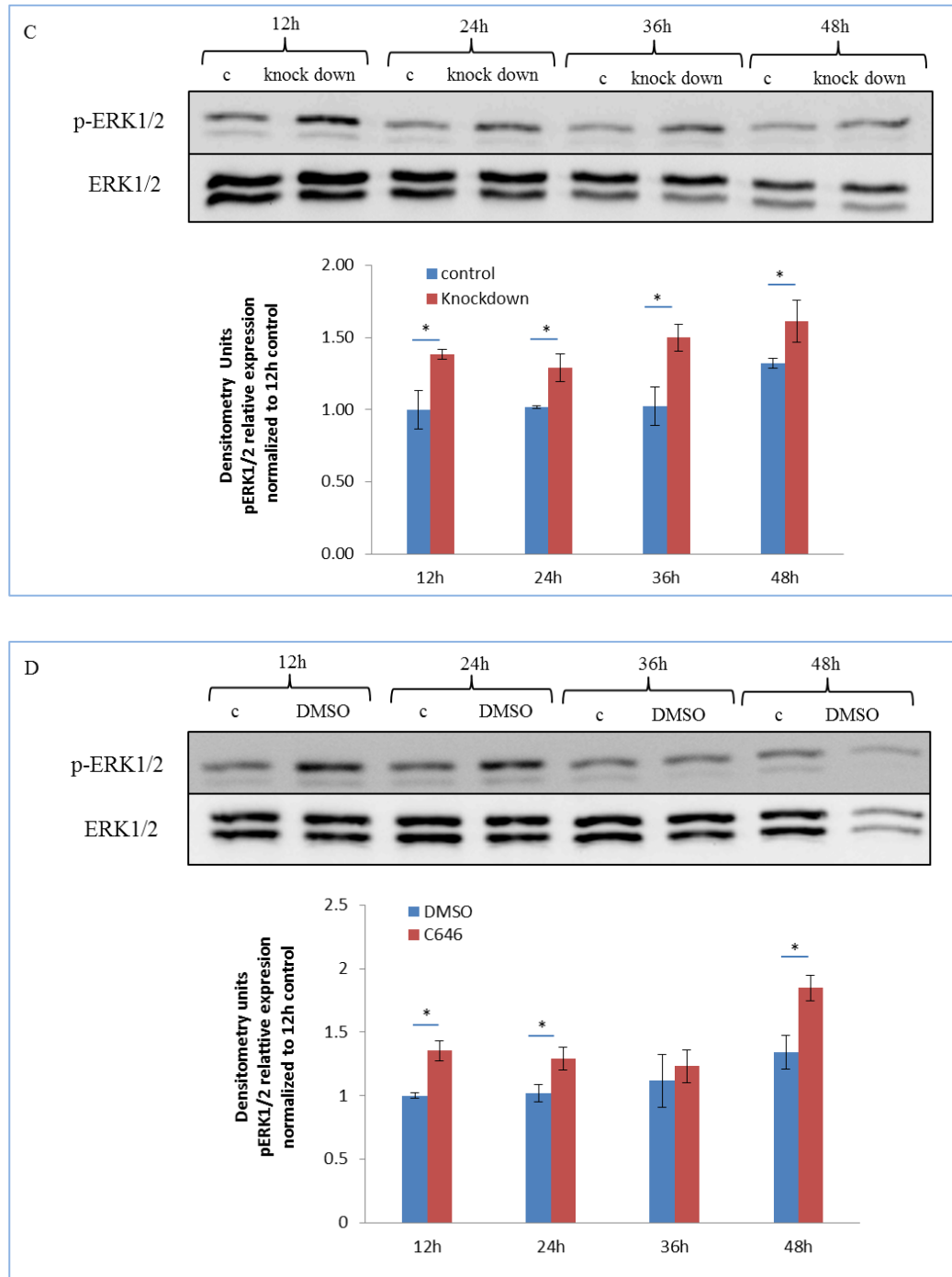
Figure 16. EP300 downregulation induces migration of PSCs through ERK pathway. A. Stable EP300 knockdown cell line and control group cell line were cultured for 24h or 48h. Then total cell lysates were collected. The functional protein expression of JAK-STAT pathway was checked by western blot and the result were quantified by densitometry. B. Same experiment was done as in Figure 16A, except that the functional protein expression of PI3K-AKT pathway was checked. C. Stable EP300 knockdown cell line and control group cell line were cultured for 12h, 24h, 36h or 48h. Then total cell lysates were collected. The activation of ERK1/2 was determined by western blotting. And densitometry was used to quantify the relative expression of pERK1/2. D. Results from EP300 down regulation were also verified by treating normal PSCs with inhibitor C646 at the concentration of 20μM for different time points. The relative expression of pERK1/2 was quantified by densitometry. *, P< 0.05, n≥3.

3.11 EKR pathway is required for EP300 induced migration

In order to find out whether EKR pathway is essential for EP300 induced migration, a chemical inhibitor-U0126 was used in the current experiment. U0126, a highly selective inhibitor for both EKR1 and ERK2, can block the activation of EKR pathway. As showed in Figure 17, when cells were treated with U0126, the migration of EP300 down regulated group was decreased dramatically to the control level.
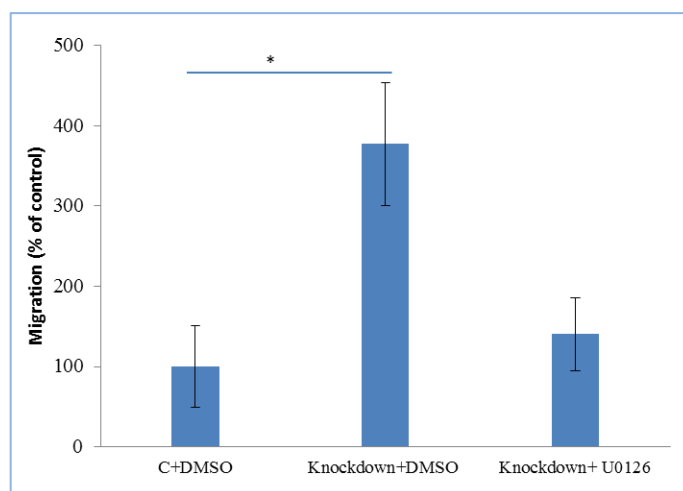
Figure 17. ERK pathway is required for EP300 induced migration of PSCs. U0126 blocks activation of ERK1/2. Cells were treated with 20μM DMSO or 20μM U0126 during the migration assay, 48 hours later, cells that migrated through the membrane were counted. *, P<0.01, n≥3.

3.12 EP300 down regulation increases the proliferation effect PSCs have on pancreatic cancer cells

To determine whether reduced expression of EP300 in PSCs will affect the proliferation effect PSCs have on pancreatic cancer cells, conditioned medium (CM) was collected from control group PSCs and EP300 knockdown PSCs to treat pancreatic cancer cells. At 72h, CM from control group PSCs increased the proliferation of Bxpc-3 cells by 33%±8% compared to serum free (SF) medium, however, CM from EP300 knockdown PSCs increased the proliferation of Bxpc-3 cells by 57%±6% compared to SF medium (Figure 18A). For Panc-1 cells, the increment in proliferation by CM from control group is 62%±6%, by CM from EP300 knockdown group is 89%±5% compared to SF medium (Figure 18B).
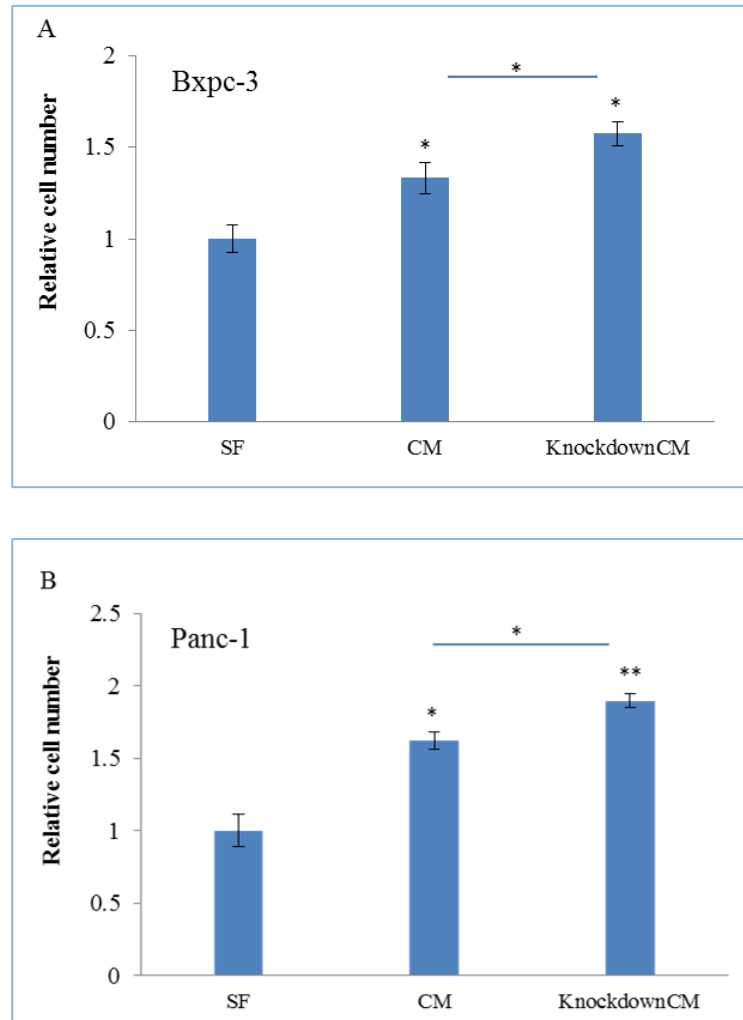
Figure 18. EP300 down regulation increases the proliferation effect PSCs have on pancreatic cancer cells. Conditioned medium from control group PSCs (CM) and EP300 knockdown PSCs were collected and used for the treatment for pancreatic cancer cells. After 72 hours treatment with CM, the proliferation of pancreatic cancer cells was tested by resazurin test. A, showed the results of Bxpc-3 cells, B showed the results of Panc-1 cells. *, $P<0.01$; **, $P<0.001$; $n \geq 3$.

3.13 EP300 down regulation in PSCs inhibits effects of chemotherapy on tumor cells

In order to study whether EP300 down regulation in PSCs will influence the chemotherapy on tumor cells, Bxpc-3 and Panc-1 cells were treated with different concentration of gemcitabine for 72 hours, together with serum free medium, or conditioned medium (CM) from control PSCs or conditioned medium from EP300 knockdown PSCs. For Bxpc-3, cells treated with knockdown CM were more drug resistant than cells treated with control group CM. For Panc-1, there was no significant difference between different treatment groups.
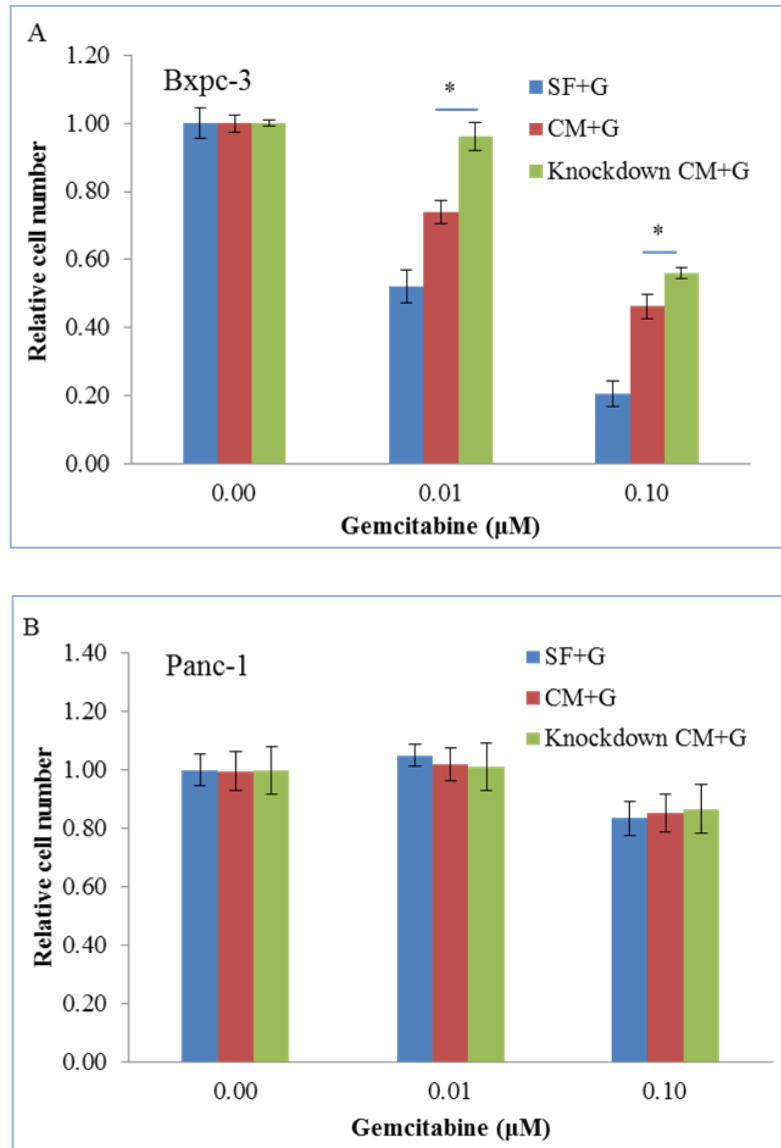
Figure 19. EP300 down regulation in PSCs inhibits effects of chemotherapy on tumor cells. Bxpc-3 and panc-1 were treated with different concentration of gemcitabine for 72 hours, together with serum free medium (SF) or conditioned medium (CM) from control group PSCs or conditioned medium from knockdown PSCs, then cell proliferation was tested. *, $P<0.05$, n≥3.

4 Discussions

The activation of PSCs is a phenomenon that can't be ignored in pancreatic ductal adenocarcinoma, which makes a great contribution to the stroma formation in this cancer. Researchers have found that growth factors, cytokines, such as TNFα, TGFβ, PDGF, interleukin 1, interleukin 6 [63, 76, 84, 152-154], ethanol and oxidant stress [86] can activate pancreatic stellate cells. However, little research has been done on how a gene will influence the activation of PSCs. PSCs' activation process involves proliferation, migration, enhanced production of extracellular matrix proteins and a phenotypic transition towards myfibroblasts. EP300, as mentioned above, is a histone acetyltransferases and plays a very important role in regulating cell proliferation and differentiation and it has been implicated in cancer. So in this article, we explored how EP300 down regulation will affect the activation of PSCs and how that will influence the communications between PSCs and pancreatic cancer cells.

As mentioned before, activated PSCs are the main contributor to the stroma formation in pancreatic cancer. Since when PSCs were activated, it began to excrete ECM proteins, including FN, Col-I and so on [152]. These ECM proteins were also the activation makers of PSCs. Another important activation maker of PSCs is α SMA [153]. Transient knockdown of EP300 resulted in less synthesis of FN and Col-I in PSCs, but α SMA expression was not changing. And the expression of FN reduced in both mRNA and protein levels, however, Col-I expression increased in mRNA level, decreased in protein level, it is possible that some miRNA working in the translation process of Col-I. Since EP300 lower expression has no influence on the expression of α SMA, it is unreasonable to say that EP300 knockdown deactivate PSCs. The conclusion we could draw from the results is that EP300 down regulation reduces the ECM synthesis of PSCs. And further ELISA experiments verified that the secretion of FN and Col-1 were also decreased in the conditioned medium when EP300 is knockdown.

CRISPR/Cas9 is a gene editing technique that can target and modify DNA with extremely high accuracy. It can be used to generate knock-out cells or animals. To better understand the role of EP300 in activated PSCs, this technique was used to intervene in the expression of EP300. After antibiotic selection, T7E1 assay result showed that there was cleavage on the genome DNA, so targeting was successful. However, western results showed that there was still some protein left in the experiment group. It is possible that this is a heterozygous

knockout. Similarly, Wang [155] used CRISPR/Cas9 to mediate heterozygous knockout of the gene CHD8, there were still CHD8 proteins left in the knockout groups. Shetty [156] revealed the same western result for heterozygous knockout of the gene CDH8. It is also possible that EP300 is essential for the survival of cells, so EP300 knockout cells couldn't be obtained. As showed by previous study, EP300 and CBP knockouts are early embryonic lethal [147]. Since down regulation of EP300 is enough to study of the gene functions of EP300, and during all the following experiments in this article, the lower expression of EP300 was always existed in the knockdown group, so no further experiment was done to clarify this problem. The reduced ECM synthesis was also proved in stable EP300 down regulation cell lines.

We accidentally seeded control group PSCs and EP300 knockdown PSCs on glass cover slips and find that EP300 knockdown PSCs possess more percent of fibroblast-like cells than the control group. It has long been known that the behavior of Hepatic stellate cells (HSCs) is influenced by the interaction between HSCs and matrix components [157]. For example, Sohara [158] showed that hepatic stellate cells were de-activated by growth on matrigel. PSCs and HSCs have a lot in common. Jesnowski [69] found that immortalized PSCs could be deactivated by matrigel and N-acetylcysteine. In the current study, cells were seeded on glass, an unfavorable basement substrate, which will also interact with the cells. Current results of morphological changes indirectly proved that EP300 knockdown PSCs were more active. Absence of EP300 induces cellular phenotypic changes has been shown before, Krubasik [159] found that colon carcinoma cell lines loss of EP300 obtained aggressive cancer phenotypes.

The expression of EP300 influences cell proliferation has been studied before. For example, researchers found that lower expression of EP300 reduced the proliferation of dental pulp cells [160], acute myeloid leukemia cells [161] and prostate cancer cells[162]. Despite that, it is also found EP300 knockdown inhibits apoptosis in human breast cancer cells [141]. In the present study, we showed that EP300 down regulation has no effect on the proliferation of PSCs. Scientists have proved that same gene can play very different roles in the cell [163]. PSCs are not regular cells, they changes from a quiescent fat storing cells to a highly proliferate cells during cancer, so EP300 down regulation doesn't affect the proliferation of PSCs is reasonable.

Another observation in the current study was that EP300 lower expression increased the drug sensitivity of PSCs. Similarly, Bourguignon [164] found that up regulation of EP300 was related to chemo-resistance in breast cancer. And Ono[165] clarified that EP300 inhibition enhanced the effect of gemcitabine through E2F1 activation in pancreatic cancer.

What's more, how EP300 down regulation affectes the migration of PSCs was studied. In previous studies, Mees [166] investigated genetic and epigenetic data found that EP300 is a miRNA regulated metastasis suppressor gene in pancreatic ductal adenocarcinoma. Zhou [167] studied breast cancer and verified that by targeting EP300, miR-106b ~25 cluster increased motility and invasion of these cancer cells. Krubasik [159] found that down regulation of EP300 in colon carcinoma cell lines increased their migration. In the present study, our results showed that lower expression of EP300 significantly increased the migration of PSCs. And to elucidate the mechanisms involved, three pathways were studied: PI3K pathway, JAK-STAT pathway and ERK pathway, which were verified by formal studies highly related to cell migration [168-172]. The results showed that EP300 lower expression activates ERK pathway. And treating PSCs with C646, a chemical inhibitor of EP300, has the same effects within 48h. Except that at the time point of 48 hours, C646 has begun to show toxic effects on cells. Furthermore, inhibition of ERK with U0126 abolished EP300-induced migration. These evidences for the first time showed that EP300 could manipulate cell migration through ERK pathway.

Finally, how EP300 down regulation in PSCs will affect the communications between PSCs and pancreatic cancer cells was analyzed. In the formal study, Hwang [110] found that conditioned medium from PSCs increased the proliferation, migration and invasion of pancreatic cancer cells. Vonlaufen [111] proved that a significant interaction between PSCs and pancreatic stellate cell is existed and pancreatic cancer cells recruit stromal cells to build an environment that promotes cancer progression. In this article, we showed that lower the expression of EP300 in PSCs increases the proliferation effect PSCs have on pancreatic cancer cells, which means that EP300 down regulation makes PSCs more active and more supportive for pancreatic cancer cells.

At last, how lower expression of EP300 in PSCs will influence the effects of chemotherapy on pancreatic cancer cells were examined. Conditioned media from PSCs have been proved to be able to reduce pancreatic cancer cell sensitivity to gemcitabine and radiation therapy [111]. And in an orthotopic model of pancreatic cancer, it has been shown that animals injected with

both PSC and PDAC cells were more resistant to radiation and gemcitabine treatment than animals injected with PDAC cells alone [110]. Our study showed that conditioned media from PSCs can protect Bxpc-3 cells from chemotherapy, and when EP300 is knockdown in PSCs the effect is much higher. However, for Panc-1 cells, conditioned media from control PSCs and EP300 knockdown PSCs have no influence on the drug sensitivity of Panc-1 cells. Why there is a difference between different pancreatic cancer cell lines need further study.

In conclusion, this study demonstrated evidence that down regulation of EP300 increases the activation of PSCs and makes PSCs are more supportive for pancreatic cancer cells, but it reduces the ECM synthesis by PSCs. Moreover, we firstly showed that EP300 manipulated cell migration through ERK pathway. And our results support the concept that targeting stromal cells can influence the interactions between stromal cells and pancreatic cancer cells, which may become an important therapeutic approach in pancreatic cancer.

References

1. Kleitsch W P. Anatomy of the pancreas; a study with special reference to the duct system. *AMA Arch Surg*. 1955;71(6):795-802.

2. Lenriot J P. [The pancreas: anatomy and radiologic exploration]. *Soins*. 1965;10(8):297-302.

3. Chandra R and Liddle R A. Neural and hormonal regulation of pancreatic secretion. *Curr Opin Gastroenterol*. 2009;25(5):441-446.

4. Openstax, *in Anatomy & Physiology*2016, OpenStax: OpenStax. p. 159.

5. Muller-Wieland D, Petermann A, Nauck M, et al. Definition, Classification and Diagnosis of Diabetes mellitus. *Diabetologie Und Stoffwechsel*. 2016;11:S78-S81.

6. Bradley E L, 3rd. A clinically based classification system for acute pancreatitis. Summary of the International Symposium on Acute Pancreatitis, Atlanta, Ga, September 11 through 13, 1992. *Arch Surg*. 1993;128(5):586-590.

7. Mcguire S. World Cancer Report 2014. Geneva, Switzerland: World Health Organization, International Agency for Research on Cancer, WHO Press, 2015. *Adv Nutr*. 2016;7(2):418-419.

8. Hajdu S I. A note from history: landmarks in history of cancer, part 2. *Cancer*. 2011;117(12):2811-2820.

9. aHariharan D, Saied A, and Kocher H M. Analysis of mortality rates for pancreatic cancer across the world. *HPB (Oxford)*. 2008;10(1):58-62.

10. Li D, Xie K, Wolff R, and Abbruzzese J L. Pancreatic cancer. *Lancet*. 2004;363(9414):1049-1057.

11. Ryan D P, Hong T S, and Bardeesy N. Pancreatic adenocarcinoma. *N Engl J Med*. 2014;371(11):1039-1049.

12. Gudjonsson B. Pancreatic Cancer: 80 Years of Surgery-Percentage and Repetitions. *HPB Surg*. 2016;2016:6839687.

13. Hassan M M, Bondy M L, Wolff R A, et al. Risk factors for pancreatic cancer: case-control study. *Am J Gastroenterol*. 2007;102(12):2696-2707.

14. Midha S, Chawla S, and Garg P K. Modifiable and non-modifiable risk factors for pancreatic cancer: A review. *Cancer Lett*. 2016;381(1):269-277.

15. Lowenfels A B, Maisonneuve P, Whitcomb D C, Lerch M M, and Dimagno E P. Cigarette smoking as a risk factor for pancreatic cancer in patients with hereditary pancreatitis. *Jama-Journal of the American Medical Association*. 2001;286(2):169-170.

16. Wolfgang C L, Herman J M, Laheru D A, et al. Recent progress in pancreatic cancer. *CA Cancer J Clin*. 2013;63(5):318-348.

17. Brand R E, Lerch M M, Rubinstein W S, et al. Advances in counselling and surveillance of patients at risk for pancreatic cancer. *Gut*. 2007;56(10):1460-1469.

18. Klein A P, Beaty T H, Bailey-Wilson J E, Brune K A, Hruban R H, and Petersen G M. Evidence for a major gene influencing risk of pancreatic cancer. *Genet Epidemiol*. 2002;23(2):133-149.

19. Connor A A and Gallinger S. Hereditary Pancreatic Cancer Syndromes. *Surg Oncol Clin N Am*. 2015;24(4):733-764.

20. Solomon S, Das S, Brand R, and Whitcomb D C. Inherited pancreatic cancer syndromes. *Cancer J*. 2012;18(6):485-491.

21. Klein A P. Identifying people at a high risk of developing pancreatic cancer. *Nature Reviews Cancer*. 2013;13(1):66-74.

22. Jones S, Zhang X, Parsons D W, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science*. 2008;321(5897):1801-1806.

23. Buchholz M and Gress T M. Molecular changes in pancreatic cancer. *Expert Rev Anticancer Ther*. 2009;9(10):1487-1497.

24. Haddad D, Socci N, Chen C H, et al. Molecular network, pathway, and functional analysis of time-dependent gene changes associated with pancreatic cancer susceptibility to oncolytic vaccinia virotherapy. *Mol Ther Oncolytics*. 2016;3:16008.

25. Aguirre A J, Bardeesy N, Sinha M, et al. Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma. *Genes & Development*. 2003;17(24):3112-3126.

26.   Bryant K L, Mancias J D, Kimmelman A C, and Der C J. KRAS: feeding pancreatic cancer proliferation. *Trends Biochem Sci*. 2014;39(2):91-100.

27.   Sakorafas G H, Tsiotou A G, and Tsiotos G G. Molecular biology of pancreatic cancer; oncogenes, tumour suppressor genes, growth factors, and their receptors from a clinical perspective. *Cancer Treat Rev*. 2000;26(1):29-52.

28.   Hingorani S R, Petricoin E F, Maitra A, et al. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse (vol 4, pg 434, 2003). *Cancer Cell*. 2004;5(1):103-103.

29.   Collins M A, Bednar F, Zhang Y Q, et al. Oncogenic Kras is required for both the initiation and maintenance of pancreatic cancer in mice. *Journal of Clinical Investigation*. 2012;122(2):639-653.

30.   Goldstein A M, Fraser M C, Struewing J P, et al. Increased Risk of Pancreatic-Cancer in Melanoma-Prone Kindreds with P16(Ink4) Mutations. *New England Journal of Medicine*. 1995;333(15):970-974.

31.   Wang Z, Banerjee S, Ahmad A, et al. Activated K-ras and INK4a/Arf deficiency cooperate during the development of pancreatic cancer by activation of Notch and NF-kappaB signaling pathways. *PLoS One*. 2011;6(6):e20537.

32.   Rocha S, Campbell K J, and Perkins N D. p53- and Mdm2-independent repression of NF-kappa B transactivation by the ARF tumor suppressor. *Mol Cell*. 2003;12(1):15-25.

33.   Tascilar M, Skinner H G, Rosty C, et al. The SMAD4 protein and prognosis of pancreatic ductal adenocarcinoma. *Clinical Cancer Research*. 2001;7(12):4115-4121.

34.   Heldin C H, Miyazono K, and Tendijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature*. 1997;390(6659):465-471.

35.   Bardeesy N, Cheng K H, Berger J H, et al. Smad4 is dispensable for normal pancreas development yet critical in progression and tumor biology of pancreas cancer. *Genes & Development*. 2006;20(22):3130-3146.

36.   Sato Y, Nio Y, Song M M, et al. p53 protein expression as prognostic factor in human pancreatic cancer. *Anticancer Res*. 1997;17(4A):2779-2788.

37.   Barton C M, Staddon S L, Hughes C M, et al. Abnormalities of the P53 Tumor

Suppressor Gene in Human Pancreatic-Cancer. *Br J Cancer*. 1991;64(6):1076-1082.

38.   Hainaut P, Soussi T, Shomer B, et al. Database of p53 gene somatic mutations in human tumors and cell lines: updated compilation and future prospects. *Nucleic Acids Res*. 1997;25(1):151-157.

39.   Belda-Iniesta C, De Caceres I I, Barriuso J, Carpeno J D, Baron M G, and Feliu J. Molecular biology of pancreatic cancer. *Clinical & Translational Oncology*. 2008;10(9):530-537.

40.   Elliott R L and Blobe G C. Role of transforming growth factor Beta in human cancer. *J Clin Oncol*. 2005;23(9):2078-2093.

41.   Hong S M, Park J Y, Hruban R H, and Goggins M. Molecular signatures of pancreatic cancer. *Arch Pathol Lab Med*. 2011;135(6):716-727.

42.   Yamada N, Hamada T, Goto M, et al. MUC2 expression is regulated by histone H3 modification and DNA methylation in pancreatic cancer. *Int J Cancer*. 2006;119(8):1850-1857.

43.   Lee E J, Gusev Y, Jiang J, et al. Expression profiling identifies microRNA signature in pancreatic cancer. *Int J Cancer*. 2007;120(5):1046-1054.

44.   Joyce J A and Fearon D T. T cell exclusion, immune privilege, and the tumor microenvironment. *Science*. 2015;348(6230):74-80.

45.   Bhowmick N A, Neilson E G, and Moses H L. Stromal fibroblasts in cancer initiation and progression. *Nature*. 2004;432(7015):332-337.

46.   Bhowmick N A and Moses H L. Tumor-stroma interactions. *Curr Opin Genet Dev*. 2005;15(1):97-101.

47.   Bissell M J and Radisky D. Putting tumours in context. *Nat Rev Cancer*. 2001;1(1):46-54.

48.   Roskelley C D and Bissell M J. The dominance of the microenvironment in breast and ovarian cancer. *Semin Cancer Biol*. 2002;12(2):97-104.

49.   Korneev K V, Atretkhany K S N, Drutskaya M S, Grivennikov S I, Kuprash D V, and Nedospasov S A. TLR-signaling and proinflammatory cytokines as drivers of

tumorigenesis. *Cytokine*. 2017;89:127-135.

50. Tredan O, Galmarini C M, Patel K, and Tannock I F. Drug resistance and the solid tumor microenvironment. *J Natl Cancer Inst*. 2007;99(19):1441-1454.

51. Gerweck L E, Vijayappa S, and Kozin S. Tumor pH controls the in vivo efficacy of weak acid and base chemotherapeutics. *Mol Cancer Ther*. 2006;5(5):1275-1279.

52. Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, and Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Research*. 1996;56(19):4509-4515.

53. Maxwell P H, Dachs G U, Gleadle J M, et al. Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. *Proceedings of the National Academy of Sciences of the United States of America*. 1997;94(15):8104-8109.

54. Hockel M and Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer Inst*. 2001;93(4):266-276.

55. Yauch R L and Settleman J. Recent advances in pathway-targeted cancer drug therapies emerging from cancer genome analysis. *Curr Opin Genet Dev*. 2012;22(1):45-49.

56. Korc M. Pancreatic cancer-associated stroma production. *Am J Surg*. 2007;194(4 Suppl):S84-86.

57. Mantoni T S, Schendel R R E, Rodel F, et al. Stromal SPARC expression and patient survival after chemoradiation for non-resectable pancreatic adenocarcinoma. *Cancer Biology & Therapy*. 2008;7(11):1806-1815.

58. Provenzano P P and Hingorani S R. Hyaluronan, fluid pressure, and stromal resistance in pancreas cancer. *Br J Cancer*. 2013;108(1):1-8.

59. Finger E C and Giaccia A J. Hypoxia, inflammation, and the tumor microenvironment in metastatic disease. *Cancer Metastasis Rev*. 2010;29(2):285-293.

60. Apte M V, Park S, Phillips P A, et al. Desmoplastic reaction in pancreatic cancer: role of pancreatic stellate cells. *Pancreas*. 2004;29(3):179-187.

61. Watari N, Hotta Y, and Mabuchi Y. Morphological studies on a vitamin A-storing cell and its complex with macrophage observed in mouse pancreatic tissues following excess vitamin A administration. *Okajimas Folia Anat Jpn*. 1982;58(4-6):837-858.

62. Apte M V, Haber P S, Applegate T L, et al. Periacinar stellate shaped cells in rat pancreas: identification, isolation, and culture. *Gut*. 1998;43(1):128-133.

63. Bachem M G, Schneider E, Gross H, et al. Identification, culture, and characterization of pancreatic stellate cells in rats and humans. *Gastroenterology*. 1998;115(2):421-432.

64. Geerts A. History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. *Seminars in Liver Disease*. 2001;21(3):311-335.

65. Liu Y H. Renal fibrosis: New insights into the pathogenesis and therapeutics. *Kidney International*. 2006;69(2):213-217.

66. Keane M P, Strieter R M, and Belperio J A. Mechanisms and mediators of pulmonary fibrosis. *Crit Rev Immunol*. 2005;25(6):429-463.

67. Erkan M, Reiser-Erkan C, Michalski C W, et al. The impact of the activated stroma on pancreatic ductal adenocarcinoma biology and therapy resistance. *Curr Mol Med*. 2012;12(3):288-303.

68. Jaster R. Molecular regulation of pancreatic stellate cell function. *Mol Cancer*. 2004;3:26.

69. Jesnowski R, Furst D, Ringel J, et al. Immortalization of pancreatic stellate cells as an in vitro model of pancreatic fibrosis: deactivation is induced by matrigel and N-acetylcysteine. *Laboratory Investigation*. 2005;85(10):1276-1291.

70. Phillips P A, Mccarroll J A, Park S, et al. Rat pancreatic stellate cells secrete matrix metalloproteinases: implications for extracellular matrix turnover. *Gut*. 2003;52(2):275-282.

71. Phillips P A, Yang L, Shulkes A, et al. Pancreatic stellate cells produce acetylcholine and may play a role in pancreatic exocrine secretion. *Proceedings of the National Academy of Sciences of the United States of America*. 2010;107(40):17397-17402.

72. Wehr A Y, Furth E E, Sangar V, Blair I A, and Yu K H. Analysis of the Human Pancreatic Stellate Cell Secreted Proteome. *Pancreas*. 2011;40(4):557-566.

73. Omary M B, Lugea A, Lowe A W, and Pandol S J. The pancreatic stellate cell: a star on the rise in pancreatic diseases. *J Clin Invest*. 2007;117(1):50-59.

74. Apte M V, Haber P S, Darby S J, et al. Pancreatic stellate cells are activated by proinflammatory cytokines: implications for pancreatic fibrogenesis. *Gut*. 1999;44(4):534-541.

75. Phillips P A, Wu M J, Kumar R K, et al. Cell migration: a novel aspect of pancreatic stellate cell biology. *Gut*. 2003;52(5):677-682.

76. Schneider E, Schmid-Kotsas A, Zhao J S, et al. Identification of mediators stimulating proliferation and matrix synthesis of rat pancreatic stellate cells. *American Journal of Physiology-Cell Physiology*. 2001;281(2):C532-C543.

77. Aoki H, Ohnishi H, Hama K, et al. Autocrine loop between TGF-beta(1) and IL-1 beta through Smad3- and ERK-dependent pathways in rat pancreatic stellate cells. *American Journal of Physiology-Cell Physiology*. 2006;290(4):C1100-C1108.

78. Ohnishi N, Miyata T, Ohnishi H, et al. Activin A is an autocrine activator of rat pancreatic stellate cells: potential therapeutic role of follistatin for pancreatic fibrosis. *Gut*. 2003;52(10):1487-1493.

79. Jiang X, Abiatari I, Kong B, et al. Pancreatic islet and stellate cells are the main sources of endocrine gland-derived vascular endothelial growth factor/prokineticin-1 in pancreatic cancer. *Pancreatology*. 2009;9(1-2):165-172.

80. Bachem M G, Schunemann M, Ramadani M, et al. Pancreatic carcinoma cells induce fibrosis by stimulating proliferation and matrix synthesis of stellate cells. *Gastroenterology*. 2005;128(4):907-921.

81. Masamune A, Kikuta K, Satoh M, Kume K, and Shimosegawa T. Differential roles of signaling pathways for proliferation and migration of rat pancreatic stellate cells. *Tohoku Journal of Experimental Medicine*. 2003;199(2):69-84.

82. Watanabe S, Nagashio Y, Asaumi H, et al. Pressure activates rat pancreatic stellate cells. *Am J Physiol Gastrointest Liver Physiol*. 2004;287(6):G1175-1181.

83. Aoki H, Ohnishi H, Hama K, et al. Cyclooxygenase-2 is required for activated pancreatic stellate cells to respond to proinflammatory cytokines. *Am J Physiol Cell*

Physiol. 2007;292(1):C259-268.

84. Luttenberger T, Schmid-Kotsas A, Menke A, et al. Platelet-derived growth factors stimulate proliferation and extracellular matrix synthesis of pancreatic stellate cells: implications in pathogenesis of pancreas fibrosis. *Lab Invest*. 2000;80(1):47-55.

85. Mews P, Phillips P, Fahmy R, et al. Pancreatic stellate cells respond to inflammatory cytokines: potential role in chronic pancreatitis. *Gut*. 2002;50(4):535-541.

86. Apte M V, Phillips P A, Fahmy R G, et al. Does alcohol directly stimulate pancreatic fibrogenesis? Studies with rat pancreatic stellate cells. *Gastroenterology*. 2000;118(4):780-794.

87. Erkan M, Kleeff J, Gorbachevski A, et al. Periostin creates a tumor-supportive microenvironment in the pancreas by sustaining fibrogenic stellate cell activity. *Gastroenterology*. 2007;132(4):1447-1464.

88. Masamune A and Shimosegawa T. Signal transduction in pancreatic stellate cells. *J Gastroenterol*. 2009;44(4):249-260.

89. Mccarroll J A, Naim S, Sharbeen G, et al. Role of pancreatic stellate cells in chemoresistance in pancreatic cancer. *Front Physiol*. 2014;5:141.

90. Jones J R, Barrick C, Kim K A, et al. Deletion of PPAR gamma in adipose tissues of mice protects against high fat diet-induced obesity and insulin resistance. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102(17):6207-6212.

91. Tontonoz P and Spiegelman B M. Fat and beyond: the diverse biology of PPARgamma. *Annu Rev Biochem*. 2008;77:289-312.

92. Masamune A, Kikuta K, Satoh M, Sakai Y, Satoh A, and Shimosegawa T. Ligands of peroxisome proliferator-activated receptor-gamma block activation of pancreatic stellate cells. *J Biol Chem*. 2002;277(1):141-147.

93. Hama K, Ohnishi H, Aoki H, et al. Angiotensin II promotes the proliferation of activated pancreatic stellate cells by Smad7 induction through a protein kinase C pathway. *Biochemical and Biophysical Research Communications*. 2006;340(3):742-750.

94. Masamune A, Satoh M, Kikuta K, Suzuki N, and Shimosegawa T. Activation of JAK-STAT pathway is required for platelet-derived growth factor-induced proliferation of pancreatic stellate cells. *World Journal of Gastroenterology*. 2005;11(22):3385-3391.

95. Mccarroll J A, Phillips P A, Kumar R K, et al. Pancreatic stellate cell migration: role of the phosphatidylinositol 3-kinase (PI3-kinase) pathway. *Biochemical Pharmacology*. 2004;67(6):1215-1225.

96. Masamune A, Sakai Y, Kikuta K, Satoh M, Satoh A, and Shimosegawa T. Activated rat pancreatic stellate cells express intercellular adhesion molecule-1 (ICAM-1) in vitro. *Pancreas*. 2002;25(1):78-85.

97. Karin M, Liu Z, and Zandi E. AP-1 function and regulation. *Curr Opin Cell Biol*. 1997;9(2):240-246.

98. Lee H, Lim C, Lee J, et al. TGF-beta signaling preserves RECK expression in activated pancreatic stellate cells. *J Cell Biochem*. 2008;104(3):1065-1074.

99. Semenza G L. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J Appl Physiol (1985)*. 2000;88(4):1474-1480.

100. Masamune A, Watanabe T, Kikuta K, Satoh K, and Shimosegawa T. NADPH oxidase plays a crucial role in the activation of pancreatic stellate cells. *Am J Physiol Gastrointest Liver Physiol*. 2008;294(1):G99-G108.

101. Shinozaki S, Ohnishi H, Hama K, et al. Indian hedgehog promotes the migration of rat activated pancreatic stellate cells by increasing membrane type-1 matrix metalloproteinase on the plasma membrane. *J Cell Physiol*. 2008;216(1):38-46.

102. Marrache F, Pendyala S, Bhagat G, Betz K S, Song Z, and Wang T C. Role of bone marrow-derived cells in experimental chronic pancreatitis. *Gut*. 2008;57(8):1113-1120.

103. Seaberg R M, Smukler S R, Kieffer T J, et al. Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. *Nature Biotechnology*. 2004;22(9):1115-1124.

104. Zeisberg E M, Potenta S, Xie L, Zeisberg M, and Kalluri R. Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts. *Cancer Research*. 2007;67(21):10123-10128.

105. Korc M, Chandrasekar B, and Shah G N. Differential binding and biological activities of epidermal growth factor and transforming growth factor alpha in a human pancreatic cancer cell line. *Cancer Res*. 1991;51(23 Pt 1):6243-6249.

106. Ellenrieder V, Alber B, Lacher U, et al. Role of MT-MMPs and MMP-2 in pancreatic cancer progression. *International Journal of Cancer*. 2000;85(1):14-20.

107. Arumugam T, Brandt W, Ramachandran V, et al. Trefoil Factor 1 Stimulates Both Pancreatic Cancer and Stellate Cells and Increases Metastasis. *Pancreas*. 2011;40(6):815-822.

108. Jiang H B, Xu M, and Wang X P. Pancreatic stellate cells promote proliferation and invasiveness of human pancreatic cancer cells via galectin-3. *World J Gastroenterol*. 2008;14(13):2023-2028.

109. Karnevi E, Rosendahl A H, Hilmersson K S, Saleem M A, and Andersson R. Impact by pancreatic stellate cells on epithelial-mesenchymal transition and pancreatic cancer cell invasion: Adding a third dimension in vitro. *Exp Cell Res*. 2016;346(2):206-215.

110. Hwang R F, Moore T, Arumugam T, et al. Cancer-associated stromal fibroblasts promote pancreatic tumor progression. *Cancer Res*. 2008;68(3):918-926.

111. Vonlaufen A, Joshi S, Qu C, et al. Pancreatic stellate cells: partners in crime with pancreatic cancer cells. *Cancer Res*. 2008;68(7):2085-2093.

112. Watanabe I, Hasebe T, Sasaki S, et al. Advanced pancreatic ductal cancer: fibrotic focus and beta-catenin expression correlate with outcome. *Pancreas*. 2003;26(4):326-333.

113. Kikuta K, Masamune A, Watanabe T, et al. Pancreatic stellate cells promote epithelial-mesenchymal transition in pancreatic cancer cells. *Biochem Biophys Res Commun*. 2010;403(3-4):380-384.

114. Hamada S, Masamune A, Takikawa T, et al. Pancreatic stellate cells enhance stem cell-like phenotypes in pancreatic cancer cells. *Biochem Biophys Res Commun*. 2012;421(2):349-354.

115. Al-Assar O, Demiciorglu F, Lunardi S, et al. Contextual regulation of pancreatic cancer stem cell phenotype and radioresistance by pancreatic stellate cells. *Radiother*

Oncol. 2014;111(2):243-251.

116. Martinez F O and Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep*. 2014;6:13.

117. Sica A, Larghi P, Mancino A, et al. Macrophage polarization in tumour progression. *Semin Cancer Biol*. 2008;18(5):349-355.

118. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol*. 2003;3(1):23-35.

119. Wynn T A and Barron L. Macrophages: master regulators of inflammation and fibrosis. *Semin Liver Dis*. 2010;30(3):245-257.

120. Jinushi M, Chiba S, Yoshiyama H, et al. Tumor-associated macrophages regulate tumorigenicity and anticancer drug responses of cancer stem/initiating cells. *Proc Natl Acad Sci U S A*. 2011;108(30):12425-12430.

121. Esposito I, Menicagli M, Funel N, et al. Inflammatory cells contribute to the generation of an angiogenic phenotype in pancreatic ductal adenocarcinoma. *J Clin Pathol*. 2004;57(6):630-636.

122. Liu C Y, Xu J Y, Shi X Y, et al. M2-polarized tumor-associated macrophages promoted epithelial-mesenchymal transition in pancreatic cancer cells, partially through TLR4/IL-10 signaling pathway. *Laboratory Investigation*. 2013;93(7):844-854.

123. Shi C J, Washington M K, Chaturvedi R, et al. Fibrogenesis in pancreatic cancer is a dynamic process regulated by macrophage-stellate cell interaction. *Laboratory Investigation*. 2014;94(4):409-421.

124. Kim J B. Three-dimensional tissue culture models in cancer biology. *Semin Cancer Biol*. 2005;15(5):365-377.

125. Sutherland R M. Cell and Environment Interactions in Tumor Microregions - the Multicell Spheroid Model. *Science*. 1988;240(4849):177-184.

126. Labarbera D V, Reid B G, and Yoo B H. The multicellular tumor spheroid model for high-throughput cancer drug discovery. *Expert Opin Drug Discov*. 2012;7(9):819-830.

127. Hirschhaeuser F, Menne H, Dittfeld C, West J, Mueller-Klieser W, and Kunz-

Schughart L A. Multicellular tumor spheroids: An underestimated tool is catching up again. *Journal of Biotechnology*. 2010;148(1):3-15.

128. Katt M E, Placone A L, Wong A D, Xu Z S, and Searson P C. In Vitro Tumor Models: Advantages, Disadvantages, Variables, and Selecting the Right Platform. *Front Bioeng Biotechnol*. 2016;4:12.

129. Schneiderhan W, Diaz F, Fundel M, et al. Pancreatic stellate cells are an important source of MMP-2 in human pancreatic cancer and accelerate tumor progression in a murine xenograft model and CAM assay. *J Cell Sci*. 2007;120(Pt 3):512-519.

130. Richmond A and Su Y. Mouse xenograft models vs GEM models for human cancer therapeutics. *Dis Model Mech*. 2008;1(2-3):78-82.

131. Mizumoto K, Kitazawa S, Ito S, et al. Cycles of repeated augmentation pressure in rapid production of pancreatic and cholangiocellular carcinomas in hamsters initiated with N-nitrosobis(2-oxopropyl)amine. *Carcinogenesis*. 1989;10(8):1457-1459.

132. Standop J, Schneider M B, Ulrich A, and Pour P M. Experimental animal models in pancreatic carcinogenesis: Lessons for humane pancreatic cancer. *Digestive Diseases*. 2001;19(1):24-31.

133. Ijichi H. Genetically-engineered mouse models for pancreatic cancer: Advances and current limitations. *World J Clin Oncol*. 2011;2(5):195-202.

134. Mazur P K, Herner A, Neff F, and Siveke J T. Current methods in mouse models of pancreatic cancer. *Methods Mol Biol*. 2015;1267:185-215.

135. Ahmad M, Arsaban M, Delabrousse H, et al. Atlas of Genetics and Cytogenetics in Oncology and Haematology: an Internet information source with free access. *Oncologie*. 2010;12(11-12):685-686.

136. Giordano A and Avantaggiati M L. p300 and CBP: Partners for life and death. *Journal of Cellular Physiology*. 1999;181(2):218-230.

137. Goodman R H and Smolik S. CBP/p300 in cell growth, transformation, and development. *Genes & Development*. 2000;14(13):1553-1577.

138. Chan H M and La Thangue N B. p300/CBP proteins: HATs for transcriptional bridges and scaffolds. *Journal of Cell Science*. 2001;114(Pt 13):2363-2373.

139. Ait-Si-Ali S, Polesskaya A, Filleur S, et al. CBP/p300 histone acetyl-transferase activity is important for the G1/S transition. *Oncogene*. 2000;19(20):2430-2437.

140. Iyer N G, Xian J, Chin S F, et al. p300 is required for orderly G1/S transition in human cancer cells. *Oncogene*. 2007;26(1):21-29.

141. Yuan Z M, Huang Y, Ishiko T, et al. Role for p300 in stabilization of p53 in the response to DNA damage. *J Biol Chem*. 1999;274(4):1883-1886.

142. Gayther S A, Russell P, Ponder B a J, Kouzarides T, and Caldas C. Truncating mutations of the EP300 acetylase in human cancers. *British Journal of Cancer*. 2000;83:23-23.

143. Shigeno K, Yoshida H, Ling P, et al. Disease-related potential of mutations in transcriptional cofactors CREB-binding protein and p300 in leukemias. *Cancer Lett*. 2004;213(1):11-20.

144. Chim C S, Wong A S Y, and Kwong Y L. Absence of p300 gene promoter methylation in acute leukemia. *Cancer Genetics and Cytogenetics*. 2004;150(2):164-167.

145. Krubasik D, Iyer N G, English W R, et al. Absence of p300 induces cellular phenotypic changes characteristic of epithelial to mesenchyme transition (vol 94, pg 1326, 2006). *British Journal of Cancer*. 2006;95(2):245-245.

146. Asaduzzaman M, Constantinou S, Min H, et al. Tumour suppressor EP300, a modulator of paclitaxel resistance and stemness, is downregulated in metaplastic breast cancer. *Breast Cancer Res Treat*. 2017.

147. Yao T P, Oh S P, Fuchs M, et al. Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. *Cell*. 1998;93(3):361-372.

148. Delvecchio M, Gaucher J, Aguilar-Gurrieri C, Ortega E, and Panne D. Structure of the p300 catalytic core and implications for chromatin targeting and HAT regulation. *Nature Structural & Molecular Biology*. 2013;20(9):1040-+.

149. Kalkhoven E. CBP and p300: HATs for different occasions. *Biochem Pharmacol*. 2004;68(6):1145-1155.

150. Bordoli L, Husser S, Luthi U, Netsch M, Osmani H, and Eckner R. Functional analysis of the p300 acetyltransferase domain: the PHD finger of p300 but not of CBP is dispensable for enzymatic activity. *Nucleic Acids Res*. 2001;29(21):4462-4471.

151. Lu J, Zhou S, Siech M, Habisch H, Seufferlein T, and Bachem M G. Pancreatic stellate cells promote hapto-migration of cancer cells through collagen I-mediated signalling pathway. *British Journal of Cancer*. 2014;110(2):409-420.

152. Bachem M G, Schunemann M, Ramadani M, et al. Pancreatic carcinoma cells induce fibrosis by stimulating proliferation and matrix synthesis of stellate cells. *Gastroenterology*. 2005;128(4):907-921.

153. Haber P S, Keogh G W, Apte M V, et al. Activation of pancreatic stellate cells in human and experimental pancreatic fibrosis. *Am J Pathol*. 1999;155(4):1087-1095.

154. Yu J H, Lim J W, Namkung W, Kim H, and Kim K H. Suppression of cerulein-induced cytokine expression by antioxidants in pancreatic acinar cells. *Lab Invest*. 2002;82(10):1359-1368.

155. Wang P, Lin M, Pedrosa E, et al. CRISPR/Cas9-mediated heterozygous knockout of the autism gene CHD8 and characterization of its transcriptional networks in neurodevelopment. *Molecular Autism*. 2015;6(1):1-18.

156. Shetty D K and Inamdar M S. Generation of a heterozygous knockout human embryonic stem cell line for the OCIAD1 locus using CRISPR/CAS9 mediated targeting: BJNhem20-OCIAD1-CRISPR-20. *Stem Cell Research*. 2016;16(2):207-209.

157. Preaux A M, Mallat A, Nhieu J T, D'ortho M P, Hembry R M, and Mavier P. Matrix metalloproteinase-2 activation in human hepatic fibrosis regulation by cell-matrix interactions. *Hepatology*. 1999;30(4):944-950.

158. Sohara N, Znoyko I, Levy M T, Trojanowska M, and Reuben A. Reversal of activation of human myofibroblast-like cells by culture on a basement membrane-like substrate. *J Hepatol*. 2002;37(2):214-221.

159. Krubasik D, Iyer N G, English W R, et al. Absence of p300 induces cellular phenotypic changes characteristic of epithelial to mesenchyme transition. *Br J Cancer*. 2006;94(9):1326-1332.

160. Liu H J, Wang T, Li Q M, Guan X Y, and Xu Q. Knock-down of p300 decreases the proliferation and odontogenic differentiation potentiality of HDPCs. *Int Endod J*. 2015;48(10):976-985.

161. Gao X N, Lin J, Ning Q Y, et al. A histone acetyltransferase p300 inhibitor C646 induces cell cycle arrest and apoptosis selectively in AML1-ETO-positive AML cells. *PLoS One*. 2013;8(2):e55481.

162. Santer F R, Hoschele P P S, Oh S J, et al. Inhibition of the Acetyltransferases p300 and CBP Reveals a Targetable Function for p300 in the Survival and Invasion Pathways of Prostate Cancer Cell Lines. *Molecular Cancer Therapeutics*. 2011;10(9):1644-1655.

163. Yang X, Coulombe-Huntington J, Kang S, et al. Widespread Expansion of Protein Interaction Capabilities by Alternative Splicing. *Cell*. 2016;164(4):805-817.

164. Bourguignon L Y, Xia W, and Wong G. Hyaluronan-mediated CD44 interaction with p300 and SIRT1 regulates beta-catenin signaling and NFkappaB-specific transcription activity leading to MDR1 and Bcl-xL gene expression and chemoresistance in breast tumor cells. *J Biol Chem*. 2009;284(5):2657-2671.

165. Ono H, Basson M D, and Ito H. P300 inhibition enhances cytotoxic effect of Gemcitabine through E2F1 activation in pancreatic cancer. *Cancer Res*. 2015;75.

166. Mees S T, Mardin W A, Wendel C, et al. EP300--a miRNA-regulated metastasis suppressor gene in ductal adenocarcinomas of the pancreas. *Int J Cancer*. 2010;126(1):114-124.

167. Zhou Y, Hu Y, Yang M, et al. The miR-106b similar to 25 cluster promotes bypass of doxorubicin-induced senescence and increase in motility and invasion by targeting the E-cadherin transcriptional activator EP300. *Cell Death and Differentiation*. 2014;21(3):462-474.

168. Saxena N K, Sharma D, Ding X K, et al. Concomitant activation of the JAK/STAT, PI3K/AKT, and ERK signaling is involved in leptin-mediated promotion of invasion and migration of hepatocellular carcinoma cells. *Cancer Res*. 2007;67(6):2497-2507.

169. Bousquet E, Mazieres J, Privat M, et al. Loss of RhoB Expression Promotes Migration and Invasion of Human Bronchial Cells Via Activation of AKT1. *Cancer Res*.

2009;69(15):6092-6099.

170. Devreotes P and Horwitz A R. Signaling Networks that Regulate Cell Migration. *Cold Spring Harbor Perspectives in Biology*. 2015;7(8).

171. Beggs J E, Tian S Y, Jones G G, et al. The MAP kinase-interacting kinases regulate cell migration, vimentin expression and eIF4E/CYFIP1 binding. *Biochemical Journal*. 2015;467:63-76.

172. Huang C, Jacobson K, and Schaller M D. MAP kinases and cell migration. *Journal of Cell Science*. 2004;117(20):4619-4628.

Part II: Pancreatic Stellate Cells and Drug Resistance in Pancreatic Cancer

1 Introduction

1.1 Pancreatic cancer

Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal malignancy and ranked the fourth leading cause of cancer-related death in both Europe and USA [1]. It is associated with its rapid tumor progression and metastasis [2, 3], it's highly resistant to both chemo and radiation therapy [4, 5]. For 2017, it is predicted that 87,400 people will dye of pancreatic cancer in the European countries and 44,090 people will dye of pancreatic cancer in the USA [6, 7]. Despite so many years have passed, the survival rate of pancreatic cancer has not improved and no cure treatment has been found. As an increasing number of people in the Europe have been diagnosed with pancreatic cancer (showed in Figure 1) and most of them die within 6 months, it is very crucial to find efficient ways to fight against it.
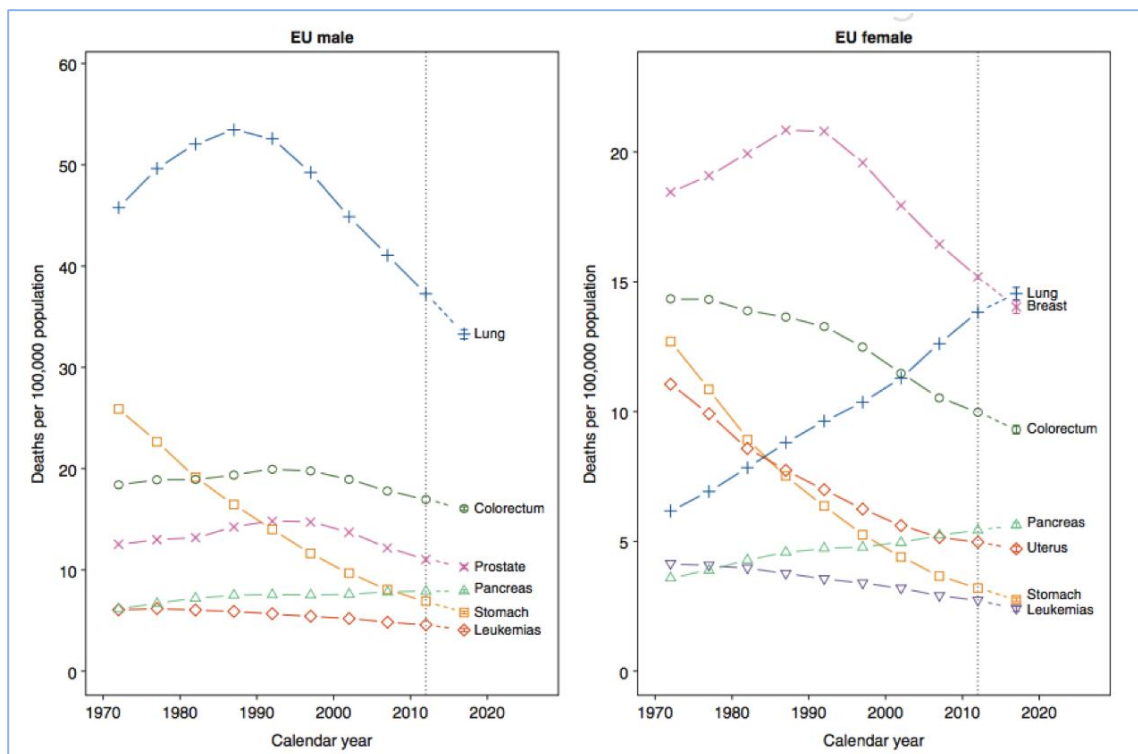

Figure 1. Age-standardized EU male and female cancer mortality rate trends in quinquennia from 1970- 1974 to 2005-2009 plus the year 2012 and predicted rates for 2017 with 95% prediction intervals. Pancreas (triangles) in both men and women [6].

1.2 Treatments for cancer

Cancer treatments vary between different hospitals and different patients, which is largely based on experience. Generally, there are three types of treatment: surgery, radiotherapy and drugs. These treatments may be used alone or in combination. Surgery treatment usually applies for early stage patients and specific cancer types. For example, it is the mainstream treatment for primary stage liver cancer patients and the survival rate has improved a lot after surgery, sometimes ever curable [8]. Radiotherapy is the most utilized treatment for cancer, and there exist a suggestion that nearly 50% of all cancer patients should do radiation [9]. Indeed, almost all types of cancer could receive radiation therapy, such as breast cancer, lung cancer, melanoma, lymphoma, stomach cancer, colon cancer and so on. Drug treatment contains chemotherapy, which intend to use drugs to destroy cancer cells but not to damage healthy cells. These used drugs usually intervene the DNA synthesis, replication or transcription process of the cancer cells, such as Mercaptopurine, Melphalan and Cispaltin.

Then how do the treatments mentioned above work on PDAC? In the past ten years, pancreatic surgery has improved a lot. The surgery process is safe and after surgery the morbidity and mortality rates are the same as other gastrointestinal cancer [10]. However, since it is hard to diagnosed pancreatic cancer in the early stage, only 20% of the patients are suitable for surgery, and the prognosis of pancreatic cancer has not changed for decades, the overall survival rate of PDAC has not improved. Chemoradiation therapy is commonly chosen for cancer, when the tumor is unresectable. However, pancreatic cancer is highly resistant to traditional chemo and radiation therapy, which makes it more difficult to cure. Right now, the standard treatment for pancreatic cancer patients is drug treatment, the use of gemcitabine, which has modest benefit and the overall survival rate has not improved much after the treatment [11, 12]. Therefore, it is urgent to overcome the drug resistant problem in pancreatic cancer and find new therapies for it.

1.3 Mechanisms of drug resistance in cancer

The concept of drug resistance comes from antibiotics. After scientist Alexander Fleming discovered penicillin in 1928, penicillin was used to treated infections [13]. But as years pass by, the effect of penicillin was reduced on some patients, and scientist found out that it is because bacteria developed drug resistance to penicillin. Since then the same drug resistance problem began to occur in other diseases, such as cancer. Resistance to treatment is a

frustration issue and a lot of factors contribute to that. In cancer, patients can become drug resistant for general two reasons：(1) Host factors, such as poor absorption, low tolerance, or rapid metabolism of a drug; (2) Gene mutations in cancer cells, some gene alterations may cause cells insensitive to drugs [14]. In the following paragraph, we will discuss the biological mechanisms of drug resistance in cancer.

1.3.1 Multidrug resistance proteins and drug resistance in cancer

In the field of drug resistance, multidrug resistance proteins are famous, which play important roles in transport drugs out of the cell. Generally, there are two subfamilies of them: (1) Multidrug resistance protein (MDR) family, it consists of MDR1 and MDR2 or alternative names ABCB1 and ABCB2; (2) Multidrug resistance-associated protein (MRP) family, it has 6 members: MRP1, MRP2, MRP3, MRP4, MRP5 and MRP6 or alternative names ABCC1, ABCC2, ABCC3, ABCC4, ABCC5 and ABCC6 [15]. They are all the members of human ATP-binding cassette (ABC) transporters and expressed in the epithelial cells of the liver and the intestine [16]. Normally, they pump harmful molecules out of cell to protect the body. However, when treating cancer patients with drugs, the expression of them will cause poor clinical outcome. For example, MDR1 and MRP1 have been found highly expressed in many drug resistance cancers [17, 18]. And treatment with doxorubicin in lung cancer has been discovered highly activated MDR1 expression [19]. In addition, high-level expression of multidrug resistance-associated protein 1 has been found associated with poor clinical outcome in neuroblastoma [20].
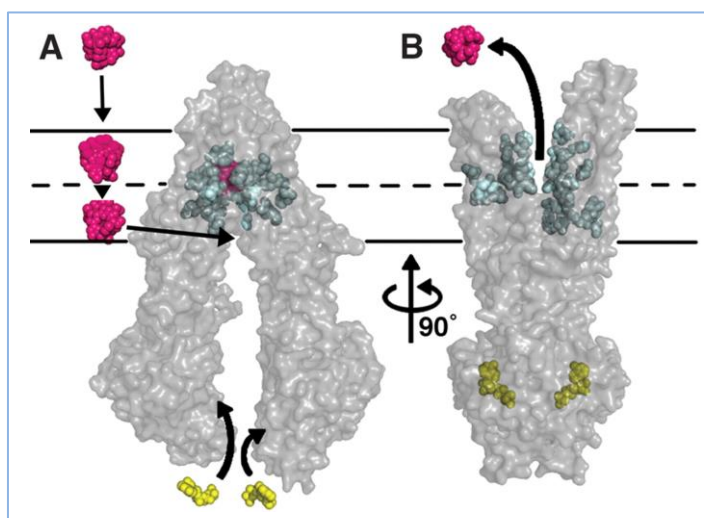

Figure 2. Model of substrate transported by multidrug resistance protein. A. The magenta substrate enters the membrane and moves in the transporter. B. The yellow ATP binding to the transporter and causes a structure

change of it. The magenta substrate was pumped out of the membrane by the transporter [21].

1.3.2 Gene mutations and drug resistance in cancer

Drug resistance is still a major problem existed in cancer chemotherapy. Scientist found that certain gene mutations are related to chemo-resistance in cancer. For instance, a clinical research showed that leukemia patients with p53 gene mutations are more resistant to chemotherapy than those without p53 mutations [22]. In leukemia, clinical resistance to drug therapy was found to be caused by BCR-ABL gene mutation or amplification [23]. In breast cancer, specific p53 mutations were found related to resistance to doxorubicin [24]. In lung cancer, researcher showed that T790M mutation in EGFR kinase can cause drug resistance [25] and mutations in KRAS are related to drug resistance to gefitinib and erbotinib [26].

1.3.3 Epigenetic modifications and drug resistance in cancer

There are mainly two types of epigenetic modifications: DNA methylation and histone modification. DNA methylation is important for the development of human beings. It often happens at the GC rich area by adding methyl group to cytosine. When the CpG-rich promoter is highly methylated, the transcriptional initiation of the gene will be stopped. DNA methylation plays an important role in tissue-specific gene expression and this epigenetic methylation patters on DNA are inheritable [27-29]. Histone modification includes acetylation and methylation. It can regulate the expression of genes by changing the structure of chromatin [30]. During cancer, these normal epigenetic modification patters mentioned above are disrupted, which will cause the highly expression of oncogenes by low methylation or the silence of tumor suppress genes by high methylation. Furthermore, it is found that these epigenetic changes are associated with drug resistance in cancer. For example, Kantharidis [31] showed that the acquired drug resistance in leukemia is related to altered methylation of human MDR1 gene. Chen [32] discovered that chemo-sensitivity to temozolomide is regulated by DNA methylation and histone acetylation in melanoma. A study of Steele [33] found that drug sensitivity was improved by inhibiting DNA methylation and histone acetylation together in ovarian cancer.

1.3.4 Epithelial-mesenchymal transition (EMT) and drug resistance in cancer

Epithelial-mesenchymal transition is an important process during embrogenesis. In cancer, it plays a crucial role in tumor invasion and metastasis. When cancer cells go through EMT, they will lose their cell-cell contacts and begin to migrate and invade, their morphology may

also change. In lung cancer, scientist showed that the acquired gefitinib resistance in cancer cells is highly associated with EMT process and when EMT process is reversed, the sensitivity to gefitinib is restored [34]. In liver cancer, researcher found that microRNA216a/217 induces EMT of cancer cells, which promote drug resistance of these cells [35]. In bladder cancer, EMT was showed to regulate drug resistance and muscle invasion /metastasis in this cancer [36]. Furthermore, Saxena [37] found that EMT contributes to drug resistance by up regulating ABC transporters.

1.3.5 Cancer stem cells and drug resistance in cancer

Stem cells are cells that can produce more stem cells and generate mature cells of certain types [38]. In human beings, they generally have two types: embryonic stem cells and adult stem cells, which function as a repair system. The concept of cancer stem cells started in the 1990s [39] and gradually accepted worldwide and began to influence the research area of cancer. In tumor tissue, cancer stem cells only possess a very small part, around 0.1%-1%, and it varies among different tumors [40, 41]. There exists a hypothesis that it's cancer stem cells that make cancer difficult to cure and it is believed that chemotherapy only kills most of the tumor cells, but leaves cancer stem cells along. Since stem cells have the ability to self-renew and differentiate, cancer relapses [42]. As normal tissue stem cells are drug resistant, which is related to MDR transporters and detoxifying enzymes [43], it is reasonable to think that cancer stem cells are drug resistant and maybe with the same mechanisms. Liu [44] showed that cancer stem cells in glioblastoma are chemo-resistance. Ma [45] found that by expression of survival pathways, hepatocellular carcinoma cancer stem cells are drug resistant. Fillmore [46] discovered that breast cancer stem cells are resistant to chemotherapy. So, in the future, maybe targeting cancer stem cells could be a new therapy to fight against cancer.

1.4 Strategies to fight against drug resistance in cancer

There are generally two ways to overcome MDR caused drug resistance problem in cancer: First, develop anticancer drugs that don't bind to ABC transporter, such as antimetabolites (5-fluorouracil) [47]; Second, find nontoxic ABC transporter inhibitors. Till now, three generations of MDR inhibitors have been developed. The first generation inhibitor, such as verapamil, has unacceptable toxicity. The second-generation inhibitor, such as valspodar, has unwanted interactions with other proteins. The most promising inhibitor right now is the

third-generation inhibitor, such as tariquidar XR9576, which has high specificity and has shown promise in clinical trials [48].

Another common strategy to overcome drug resistance problem in cancer is the using of combined therapy. Different drugs may have different metabolisms and mechanisms of action, therefore using two different drugs together may result in synergistic effect. For example, in colorectal cancer, combined treatment of irinotecan and fluorouracil increased the survival of the patients [49]. In breast cancer, trastuzumab and docetaxel combined treatment has better effect in terms of survival rate, response rate and response duration compared to docetaxel treatment alone [50]. Combine treatment also has been shown to have superior effect in ovarian cancer [51]. However, sometimes, using two drugs simultaneously may result in antagonism, so it is important to choose the combination of the drugs.

1.5 Mechanisms of drug resistance in pancreatic cancer

In pancreatic cancer, the drug resistance problem can also impute to multidrug resistance proteins, gene mutations, EMT and pancreatic stem cells. For example, multidrug resistance proteins have been found expressed in pancreatic cancer [52]. The BRCA2 gene mutation has been shown related to drug resistance in pancreatic adenocarcinoma [53]. EMT has been proved made a contribution to drug resistance in pancreatic cancer [54]. Pancreatic stem cells have verified play a role in the acquisition of drug resistance in pancreatic adenocarcinoma [55]. Despite these common mechanisms, there are other mechanisms exist in pancreatic cancer that are related to drug resistance.

1.5.1 Signaling pathways and drug resistance in pancreatic cancer

Scientist found that abnormal regulation of certain signaling pathways is related to drug resistance problem in pancreatic cancer. For example, Arlt [56] showed that activation of NF-κB confers resistance against gemcitabine in pancreatic cancer. NF-κB is an important regulator in control cell proliferation and cell survival in healthy cells. In cancer, the activation of NF-κB will stop cancer cells from apoptosis. PI3K/AKT pathway also has been found associated with drug resistance problem in pancreatic cancer [57]. And inhibition the activation of PI3K/AKT pathway increased the drug sensitivity of pancreatic carcinoma cells [58].

1.5.2 Pancreatic stellate cells and drug resistance in pancreatic cancer

In pancreatic cancer, for many years, researches have been focused on cancer cells to deal with the problem, which have largely failed. In recent decades, there has been a growing number of data suggest that tumor microenvironment plays an important role in the process of pancreatic cancer, and PDAC is especially stroma rich [59-63]. Pancreatic stellate cells (PSCs), which are activated during chronic pancreatitis and cancer, were found to produce extracellular matrix (ECM) proteins that comprise the pancreatic tumor stroma [64]. Both in vitro and in vivo evidence proved that pancreatic stellate cells play an important role in the process of the development of pancreatic cancer [65].
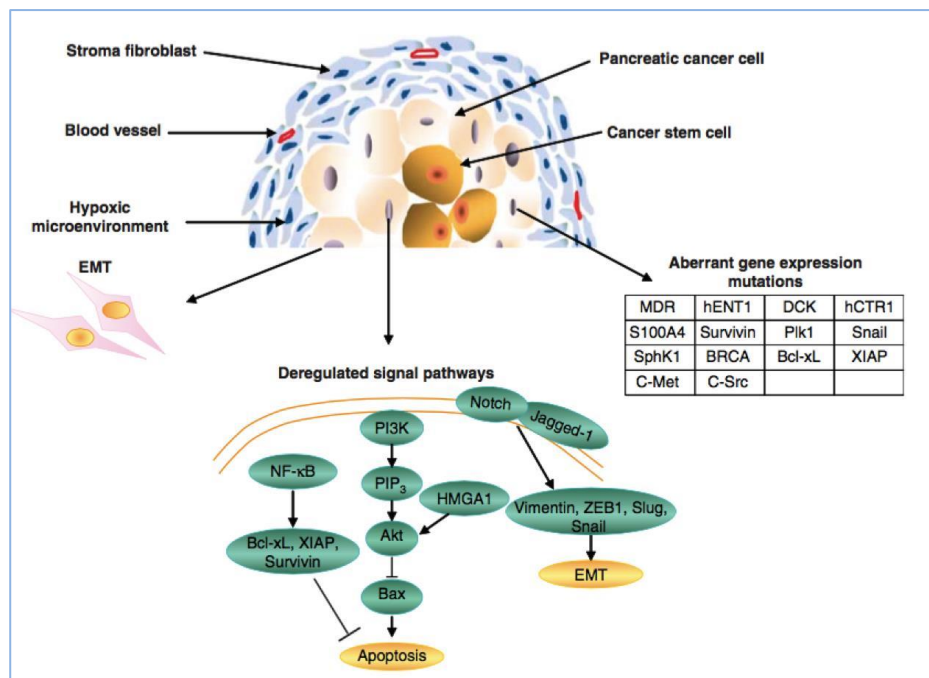

Figure 3. Drug resistance pathways in pancreatic cancer [66].

Furthermore, studies have shown that pancreatic stellate cells are highly related to the chemo-resistance of pancreatic cancer. In an orthotopic model of pancreatic cancer, it has been shown that animals injected with both PSC and PDAC cells were more resistant to radiation and gemcitabine treatment than animals injected with PDAC cells alone [67]. And there is a hypothesis indicate that the role of PSC is to function as a barrier preventing chemo-drugs to be delivered to the tumor core [66]. Researchers found that the extensive fibrosis produced by PSCs result in significant intratumoural hypoxia and a self-perpetuating hypoxia-fibrosis cycle, which limits the drug delivery to tumor cells [68-71]. Conditioned media from PSCs have been proved to reduce pancreatic cancer cell sensitivity to gemcitabine and radiation

therapy [65]. The secretions of PSCs also have been shown to be able to decrease H_2O_2-induced apoptosis and increase survival of pancreatic cancer cells, which makes cancer cells more drug resistant [72]. However, no study has ever been done on how PSCs will affect the drug sensitivity of different drug sensitive cancer cell lines and the mechanisms of how PSCs protect tumor cells from chemotherapy.

1.6 Gemcitabine and pancreatic cancer

The high resistance to chemotherapy of pancreatic cancer is really a frustration issue, which makes it more difficult to deal with. Right now, gemcitabine is the most effective drug that works on pancreatic adenocarcinoma and it is used as a standard treatment. As an analogue to cytosine, gemcitabine is incorporated into the DNA to block it from replication, which results in cell death. It was first synthesized by Eli Lilly Company in 1980s and approved by the FDA for the treatment for patients in 1996 [73]. However, the life quality of the patients and the survival rate of the patients have not improved much even after gemcitabine treatment, it has some success but the response rates are still low [74].
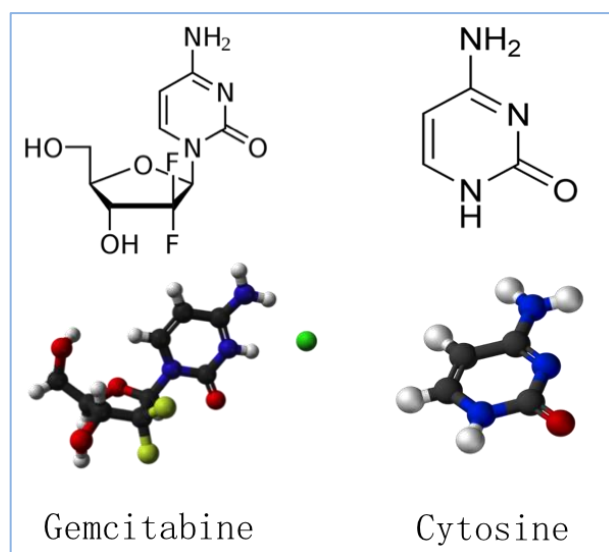
Figure 4. Structure of gemcitabine and cytosine

The mechanisms of gemcitabine resistance have been well studied since it is the only effective drug for pancreatic adenocarcinoma. To understand the mechanism of gemcitabine resistance, we need to know the metabolism of gemcitabine. Generally, gemcitabine needs to go through 10 steps to work inside of the cell. The first step is transporting across the membrane, the second step is phosphorylating by enzyme and so on and the last step is imbed into DNA or RNA. Each step can influence the efficiency of gemcitabine. For example, the

first step, there are mainly two types of nucleoside transporters that are related to gemcitabine transport: the sodium-dependent type or concentrative type (CNT) and the sodium-independent type or equilibrative type (ENT). The expression of human ENT1 has been demonstrated to associate with the survival time of the patients under gemcitabine treatment [75, 76]. And overexpression of human ENT1 increases gemcitabine response in pancreatic cancer [77].

Besides the nucleoside transporters mentioned above, some enzyme activities in between have also been proved highly related to gemcitabine resistance: (1) Downregulation of deoxycytidine kinase (dCK). dCK plays a pivotal role in gemcitabine activation, after entering of the cells, dCK phosphorylates gemcitabine to its monophosphate. (2) Up regulation of cytidine deaminase (CDA). Gemcitabine can be effectively inactivated by activation of CDA. (3) Up regulation of ribonucleotide reductase, ribonucleotide reductase plays a role in the synthesis of DNA. Ribonucleotide reductase consists of two subunits: M1 and M2, together these two subunits form an active heterodimer [78-80].
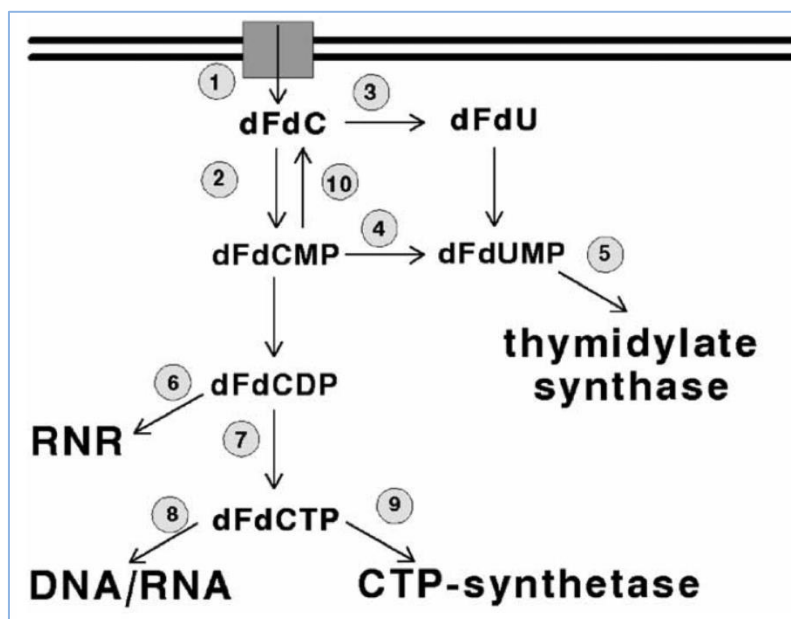
Figure 5. Metabolism and mechanisms of action of gemcitabine; 1: transprot across the cell membrane, 2: phosphorylation of gemcitabine by dCK and TK2, 3: deamination of gemcitabine by dCDA, 4: deamination of gemcitabine by dCMP-deaminase, 5: inhibition of thymidylate synthase by dFdUMP, 6:inhibition of ribonucleotide reductase by dFdCDP, 7: accumulation of the triphophate dFdCTP, 8: incorporation into DNA and RNA, 9: inhibition of CTP-synthase by dFdCTP, and 10: dephosphorylaton by 5'-nucleotidase [81] .

1.7 Aim of the study

As mentioned above, PSC plays a very import role in pancreatic cancer's drug resistance. However, no study has ever been done on how PSCs will affect the drug sensitivity of different pancreatic cancer cell lines and the exact mechanisms of how PSCs protect tumor cells from chemotherapy. That's the problem what we are going to solve in the current study.

2 Materials and Methods

2.1 Materials

Table 1 Cell lines

Cell lines	Resources
Immortalized PSCs	A gift from Ralf Jesnowski [82] , Mannheim Univerisity Hospital
Bxpc-3	Authentificated by DKFZ, Heidelberg, Germany
Panc-1	Authentificated by DKFZ, Heidelberg, Germany
Miapaca-2	Authentificated by DKFZ, Heidelberg, Germany

Table 2 Antibodies

Product	Company	Catalogue Number
Anti-mouse IgG(H+L) Peroxidase	Biozol	VEC-PI-2000
Anti-rabbit IgG(H+L) Peroxidase	Biozol	VEC-PI-1000
GAPDH	Sigma-Aldrich	G9295
RRM1	Abcam	Ab137114
RRM2	Abcam	Ab57673

Table 3 Kits

Product	Company	Catalogue Number
Caspase-Glo 3/7® Assay	Promega	G8091
Fast SYBR® Green Master Mix	Thermo Scientific	4385612
Immobilon western chemiluminescent HRP substrate	Millipore	WBKLS0500
RevertAid First strand cDNA synthesis kit	Life Technology	K1622
BCA Protein Assay Kit	Thermo Scientific	23225

Table 4 Reagents

Product	Company	Catalogue Number
Chloroform	Sigma-Aldrich	288306
DNase	Thermo scientific	EN0523
Gemcitabine	Biomol	Cay11690-10
Isopropanol	Sigma-Aldrich	W292907
Lipofectamine 2000 Transfection Reagent	Life Technologies	11668027
Nuclease-free Water	Life Technologies	AM9939
Proteinase K	Life Technologies	EO0419
Resazurin	Fisher Scientific	10684882
RNase A	Life Technologies	EN0531
Spectra Multicolor Broad Range Protein Ladder	Life Technologies	26634
TRIzol Reagent	Invitrogen	15596-018
Trypsin type I from soybean	Sigma	T8003
Trypsin inhibitor type I from soybean	Sigma	T6522
RIPA buffer	The Geyer	89900

Table 5 Materials

Product	Company	Catalogue Number
96 well plate, white (LumiNunc), F96	Fisher Scientific	10072151
Amicon$^®$ Ultra-0.5ml Centrifugal Filters Ultracel$^®$ -100K	Merck Millipore	UFC510024
Amicon$^®$ Ultra-0.5ml Centrifugal Filters Ultracel$^®$ -10K	Merck Millipore	UFC505096
Amicon$^®$ Ultra-0.5ml Centrifugal Filters Ultracel$^®$ -3K	Merck Millipore	UFC500396
Amicon$^®$ Ultra-0.5ml Centrifugal Filters Ultracel$^®$ -50K	Merck Millipore	UFC505024
Cell culture plates- 96 well (transparent)	DKFZ Lager	655180
Light Cycler$^®$ 480 Multiwell plate 384, white	Roche	04729749001

Cell culture plates-6 well (transparent)	DKFZ Lager	657160
Nitrocellulose membrane 0.45µm	GE Healthcare	GE10600007
Open-Top Polyallomer centrifuge tubes	Scientific service	S5030

Table 6 Equipments

Name	Manufacturer
NeoBlock1	NEOLAB
Fluostar Galaxy Plate Reader	MTX Lab System
Mithras LB 940 Multimode Microplate Reader	Berthold Technologies
Sigma 2K15 Micro-centrifuge	M&S Labor GERATZ GMBH
Eppendorf centrifuge 5810R	Eppendorf
Infinite® M200 Microplate Reader	Tecan
Roller mixers, RS-TR05	Phoenix Instrument
Beckman L8-M Ultracentriguge	Beckman
Beckmann Rotor SW41Ti	Beckman

2.2 Method

2.2.1 Cell lines and culture conditions

Human pancreatic cancer cell lines Panc-1, Miapaca-2 and Bxpc-3 were obtained from commercial providers prior to this study. They were all authenticated by DKFZ in-house service and tested mycoplasma free before and after the experiments. Immortalized human pancreatic stellate cells (PSCs) were a gift from Ralf Jesnowski [82]. They were all routinely cultured in IMDM complete medium containing 10% fetal bovine serum, 50 units/mL penicillin and 50µg/mL streptomycin at 37 °C with 5% CO_2, except Miapaca-2 cultured in DMEM complete medium.

2.2.2 Gemcitabine cytotoxicity assay

Analysis of cell growth was used to determine sensitivity of different cell lines to gemcitabine in vitro. Briefly, cells were seeded in 96 well plates at 5×10^3 per well, 24 hours later, serum free overnight and then different concentrations of gemcitabine was added. Cells were treated with gemcitabine in complete medium for 72h. After that, cell viability was assessed using resazurin test. According to the protocol, resazurin solution was added to each well and make sure the final concentration of resazurin is 20μg/ml. Then the plates were incubated at 37°C for 2 hours and the fluorescence was recorded using FLUOstar Galaxy system. Ex=544 nm, Em=590 nm. The relative viable cells were defined as gemcitabine treated group divided by control group.

2.2.3 Conditioned medium collection

PSCs were grown in a 175cm^2 flask to 70%-80% confluence, then the medium were changed to serum free IMDM and cells were cultured for another 48h. Medium were collected, centrifuged at 3,000×g for 15min and then the supernatant was collected, aliquoted and stored at -80 °C until use.

2.2.3 Apoptosis assay

Apoptosis of pancreatic cancer cells were tested by commercial Caspase-Glo3/7 Assay kit according to the manufacturer's protocol. Briefly, Bxpc-3, Miapaca-2 and Panc-1 cells were seeded in 96-well plates at 8×10^3 per well, 24 hours later, serum free overnight. Then the cells were treated with the following four conditions for 72 hours: a. serum free (SF); b. serum free and gemcitabine (SF+G); c. conditioned medium (CM); d. conditioned medium and gemcitabine (CM+G). After that, 100μl of Caspase-Glo3/7 Reagent was added to each well. The plates were gently mixed on a plate shaker for 30min at room temperature, and a Mithras LB940 plate reader was used to measure the luminescence of each sample.

2.2.4 siRNA transfection

Two RRM1 siRNAs and two RRM2 siRNAs were bought from Qiagen. Scramble siRNA control was purchased from Santa Cruz. Transfection of siRNA was carried out with Lipofectamine 2000 according to the manufacturer's protocol. Briefly, Bxpc-3 cells were seeded in a 6 well plate for 24 hours before the transfection, so that they would reach 60%-70% confluence when do the transfection. Then 50nM siRNA and 5 μL Lipofectamine 2000 were

added to each well, 7 hours later, medium was changed to normal medium. mRNA was collected 24 hours post transfection, protein was collected 48 hours after transfection. For drug sensitivity assay, cells were collected 48 hours later and seeded in a 96 well plate for gemcitabine treatment. The information about the siRNAs used were listed below.

Table 7 siRNA Information

Product name	Target sequence	Catalog No.	Company
Hs_RRM1_7	CAGGGCCCATACGAAACCTAT	SI03071355	Qiagen
Hs_RRM1_6	CAGCTACATTGCTGGGACTAA	SI03067904	Qiagen
Hs_RRM2_4	CGGGATTAAACAGTCCTTTAA	SI00020790	Qiagen
Hs_RRM2_3	CACACCATGAATTGTCCGTAA	SI00020783	Qiagen

2.2.5 Real time PCR

Total RNA was extracted from the cells using Trizol reagent, then 1µg RNA was reverse transcribed to cDNA using a commercial reverse transcription kit. Quantitative real time PCR were done by using Fast Sybr green and Light Cycler systems, according to the manufacturer's instruction. The primer used were as follows:

Table 8 Primers

Gene	Primer
RRM1	Forward primer: 5'-CCACTAGCTGCGATGCATGT-3' Reverse primer: 5'-TAGTTCCACTGTGGTGACCC-3'
RRM2	Forward primer: 5'-CCCTGACTATGCTATCCTGGC-3' Reverse primer: 5'-CCAATGTTGACTTGGCCACC-3'
Hprt1	Bought from Qiagen Product: Hs_HPRT1_1_SG QuantiTect Primer Assay Product no.249900 Cat.no. QT00059066

2.2.6 Western blot

Whole cell lysates were harvested by using RIPA buffer containing protease and phosphatase inhibitors. Protein concentration was quantified by BCA kit. 10μg protein was denatured and loaded in 12% SDS-PAGE gel and transferred to nitrocellulose membrane. Subsequently, membranes were blocked in 5% non-fat milk in Tris-buffered saline (TBS, 10 mM Tris, 10 mM NaCl) for 1h at room temperature. Then washed and incubated with first antibody overnight at 4°C. Secondary antibody conjugated with horseradish peroxidase was incubated with the membrane for 1 hour at room temperature. Immunodetections were done with ECL (Millipore, US) detection reagent and visualizations were performed by ImageQuant LAS 4000 mini (Fujifilm Corporation, Japan).

The primary antibodies used were GAPDH (Sigma, 1:5000), RRM1 (Abcam, 1:10000), RRM2 (Abcam, 1:1000). The secondary antibodies used were HRP-conjugated goat-anti-rabbit (Vector, 1:5000), horse-anti-mouse (Vector, 1:5000).

2.2.7 Conditioned medium treatment

In order to find out what in the conditioned medium (CM) is responsible for PSCs-induced drug resistance in Bxpc-3 cells, different enzymes were used to digest certain substances in the CM. The enzymes and treatment conditions were listed below. For proteinase K treatment, CM was incubated with 200μg/ml proteinase K at 37°C for 1 hour. Then certain inhibitor was added to neutralize the enzyme. For trypsin treatment, CM was incubated with 200μg/ml trypsin at 37°C for 1 hour. Thereafter, it was treated with 400 μg/ml soybean trypsin inhibitor at 37°C for 30 min to eliminate the enzyme activity. For heat inactivation, CM was boiled at 100°C for 2 hour. To eliminate RNAs or DNAs, CM was treated with 100 μg/ml RNase A at 37°C for 4 hour or 2 U/μL DNase I at 37°C for 4 hour.

To get rid of exosomes, conditioned media were centrifuged for several steps. First, CM were transferred to 50 ml polypropylene centrifuge tubes, centrifuged at 3000 ×g, 4°C for 30min. Then the supernatant was transferred to ultracentrifugation tubes, centrifuged at 10,000 ×g (7,500 rpm at SW-28), 4°C for 60 min. Thereafter, the supernatant was transferred to new ultracentrifugation tubes, centrifuged at 100,000 ×g (28,000 rpm at SW-41), 4°C for 90 min and the supernatant was collected.

Finally, to obtain different fractions of the conditioned medium, the aliquots of the CM were

filtered by using Amicon® Ultra-0.5ml Centrifugal filter-3K, Amicon® Ultra-0.5ml Centrifugal filter-10K, Amicon® Ultra-0.5ml Centrifugal filter-30K, Amicon® Ultra-0.5ml Centrifugal filter-50K and Amicon® Ultra-0.5ml Centrifugal filter-100K. Briefly, according to the manufacturer's instruction, Amicon® Ultra-0.5 devices were inserted into the micro centrifuge tubes. Then 500 μl of the conditioned medium was added into each filter. The capped filters were spin at 14,000 ×g for 5 min. Subsequently, the Amicon® Ultra-0.5 devices were reverse inserted into new micro centrifuge tubes, centrifuged at 1,000 ×g for 2 min. Both the media in the filter and out of the filter were collected. Finally, the fractions were reconstituted in their original volume-0.5ml by adding serum free media to make sure that they have same concentration of active factors. In this way, the fractions we finally got were: fractions containing low molecular weight substances (< 3kDa, < 10kDa, < 30kDa, <50kDa, <100kDa) and fractions containing high molecular weight substances (> 3kDa, >10kDa, > 30kDa, >50kDa, >100kDa). After treatment or fraction, all the media were stored at -80°C until use.

3 Results

3.1 Drug sensitivity of different cell lines

To determine the drug sensitivity, different human pancreatic carcinoma cell lines Bxpc-3, Panc-1, Miapaca-2 and PSCs were treated with various concentrations (0.01-100μM) of gemcitabine for 72h in complete medium. As shown in Figure 6, for Panc-1, after gemcitabine treatment around 65% of the cells was still alive even at the highest concentration of gemcitabine. For PSCs, around 48% of the cells were still alive after high concentration of gemcitabine treatment. For Miapaca-2, around 35% of the cells were still alive after treatment with gemcitabine at the concentration of 100μM. However, with the cell line Bxpc-3, only around 10% of the cells were still alive after drug treatment.
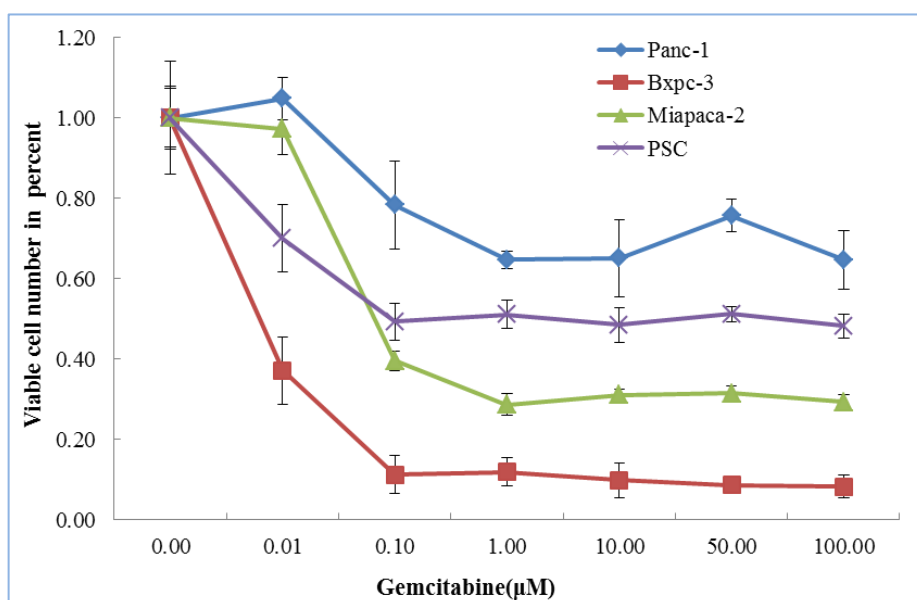

Figure 6. Dose-dependent effects of gemcitabine. PSCs and different pancreatic cancer cell lines were treated with gemcitabine for 72h in complete medium. Then the viability of cells was detected by resazurin test. Data expresses the mean percentage of viable cells of 3 independent experiments.

3.2 Conditioned medium from PSCs induces drug resistance in Bxpc-3 cells

In order to find out how PSCs will affect the drug sensitivity of different pancreatic cancer cell lines, pancreatic cancer cells were treated with conditioned medium from PSCs together with gemcitabine. Results from drug treatment showed that PSCs induce resistance to gemcitabine in Bxpc-3 cells, but not in Miapaca-2 cells or Panc-1 cells. As showed in Figure 7, when Bxpc-3 cells were treated with conditioned medium (CM) and gemcitabine together,

more percent of cells were alive compared to the control. For Miapaca-2 and Panc-1, there was no difference between control group and conditioned medium treatment group.
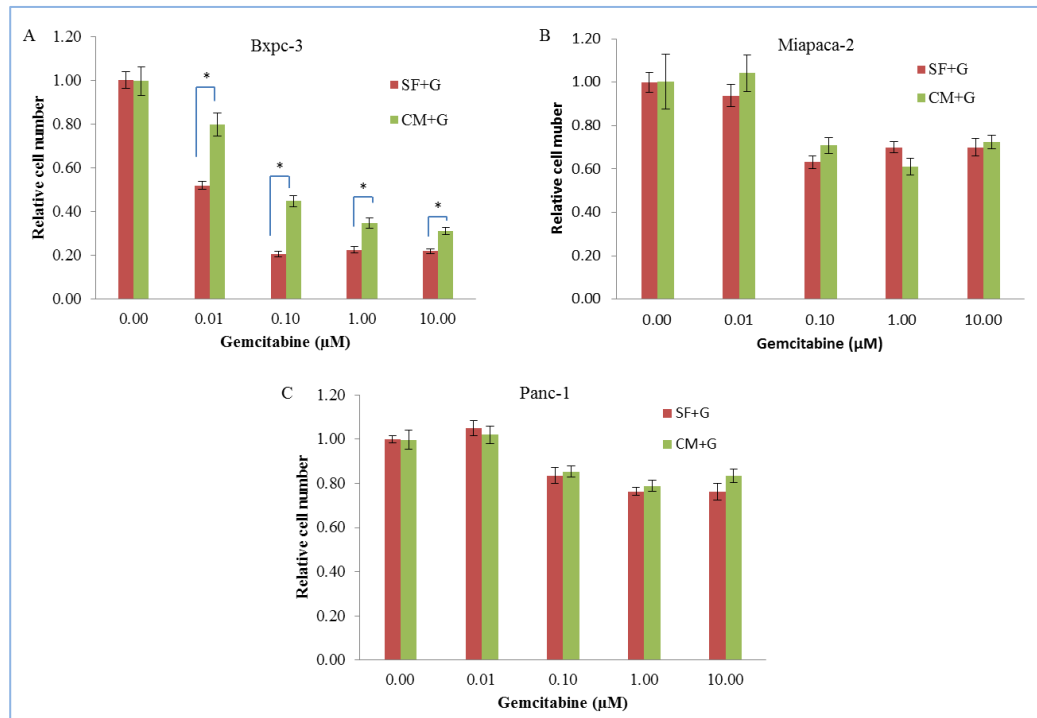

Figure 7. Effects of PSCs-CM on the drug sensitivity of different cancer cell lines. The results showed the sensitivity of Bxpc-3, Panc-1 and Miapaca-2 cells to gemcitabine in two different conditions: serum free medium (SF) and conditioned medium (CM). Cells were treated for 72h. The data represented the mean and SD of three independent experiments performed in 8 replicates. *, $P<0.001$.

3.3 PSCs secretions don't reduce gemcitabine-induced apoptosis in pancreatic cancer cells

The apoptosis of pancreatic cancer cells were tested under different treatments by Caspase-Glo3/7 assay. As shown in Figure 8A and Figure 8C, no apoptosis differences were observed between serum free medium plus gemcitabine (SF+G) treatment groups and conditioned medium plus gemcitabine treatments (CM+G) groups both in Bxpc-3 and Panc-1 cells. For Miapaca-2 cells, the apoptosis was even increased in CM+ G group (Figure 8B).
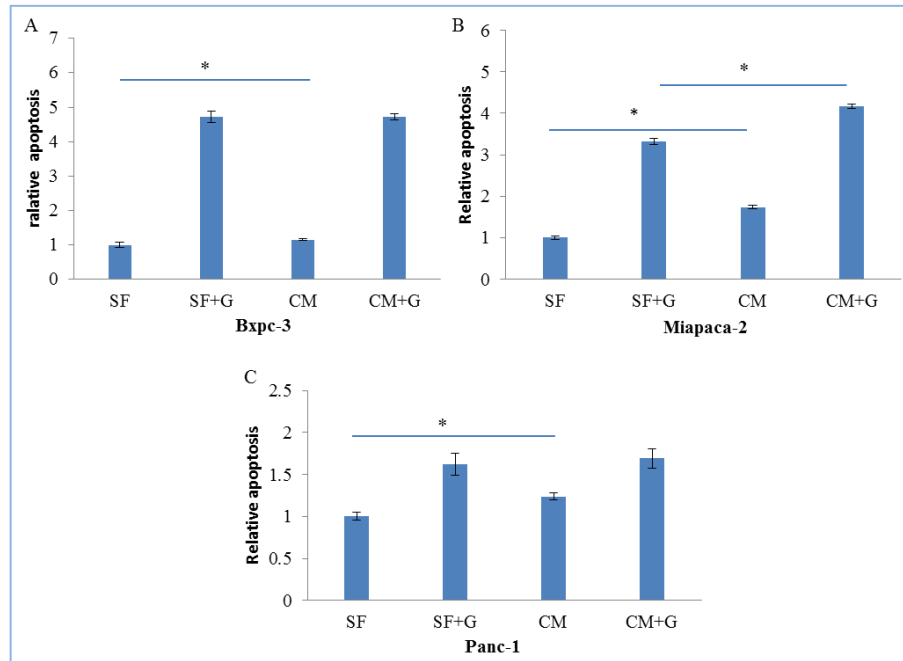
Figure 8. PSCs secretions don't reduce gemcitabine-induced apoptosis in pancreatic cancer cells. Pancreatic cancer cells were treated with the following conditions for 72 hours: a. serum free (SF); b. serum free and gemcitabine (SF+G); c. conditioned medium (CM); d. conditioned medium and gemcitabine (CM+G). Then the apoptosis of cells was analyzed by caspase3/7 activity kit. Relative apoptosis of Bxpc-3, Miapaca-2, and Panc1 treated with the upper conditions were shown separated in A, B, C. The data represented the mean and SD of three independent experiments performed in 8 replicates. *, P<0.05.

3.4 Conditioned medium from PSCs increases RRM1 and RRM2 expression in Bxpc-3 cells

As mentioned before, several mechanisms are responsible for drug resistance to gemcitabine, such as (1) downregulation of deoxycytidine kinase (dCK), (2) upregulation of cytidine deaminase (CDA), (3) upregulation of ribonucleotide reductases, including RRM1 and RRM2. Our results showed that conditioned medium from pancreatic stellate cells increased RRM1 and RRM2 protein expression in Bxpc-3 cells under gemcitabine treatment.
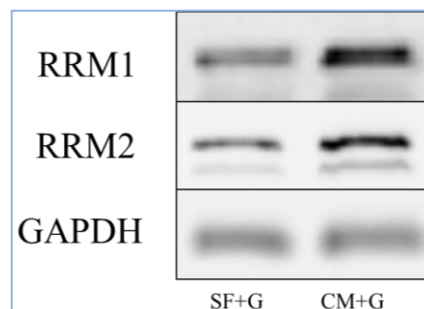
Figure 9. Conditioned medium from PSCs increases RRM1 and RRM2 expression in Bxpc3 cells.72 hours after the treatment, cell lysate was collected and western blot was used to test the RRM1 and RRM2 protein expression in SF+G treated group and CM+G treated group.

3.5 RMM1 overexpression is required for PSCs-induced drug resistance in Bxpc-3 cells

To determine whether PSCs-induced drug resistance in Bxpc-3 cells requires the overexpression of RRM1, two different siRNAs were used to knock down RRM1 in Bxpc-3 cells. These two siRNAs reduced RRM1 mRNA expression to 16% and 19% in Bxpc-3 cells, respectively compared to control (Figure 10A). The results subsequently led to reduce protein expression in Bxpc-3 (Figure 10B). Moreover, drug sensitivity assay results showed that PSCs-induced drug resistance in Bxpc-3 cells was no longer existed after RRM1 knock down in Bxpc-3 (Figure 10C).

Figure 10. RMM1 overexpression is required for PSCs-induced drug resistance in Bxpc-3 cells. Bxpc-3 cells were transfected with control or two different RRM1 siRNAs (50nM) for 24h before determine mRNA expression of RRM1 (A), and 48h before determine protein expression of RRM1 (B). C.48 hours after Bxpc-3 cells transfected with siRNA, cells were serum free overnight and treated with serum free medium or conditioned medium for 72h with or without gemcitabine. Then cell viability was tested by resazurin assay. *, $P < 0.05$.

3.6 RMM2 overexpression plays a role in PSCs-induced drug resistance in Bxpc-3 cells

To determine whether RRM2 also plays a part in PSCs-induced drug resistance in Bxpc-3 cells, two different siRNAs were used to knock down RRM2 in Bxpc-3 cells. After transfection, the mRNA expressions of RRM2 were reduced to 22% and 25% in Bxpc-3, compared to control (Figure 11A). As a consequence, RRM2 protein expression levels were decreased (Figure 11B). However, the drug treatment assay results showed that PSCs-induced drug resistance in Bxpc-3 was only partially affected by down-regulation of RRM2 in Bxpc-3 (Figure 11C).
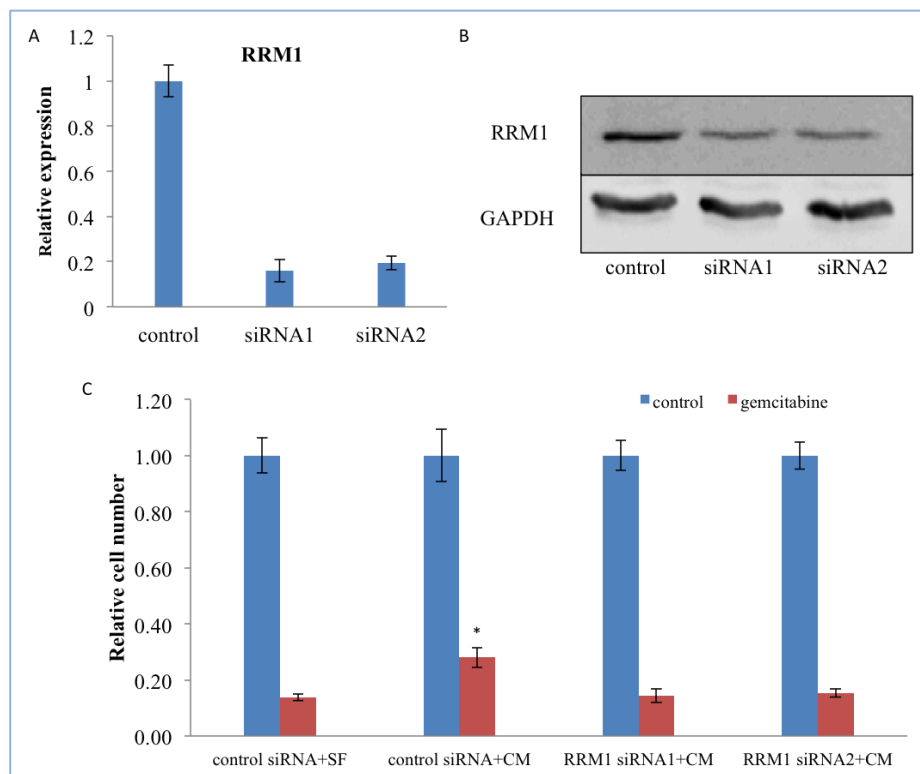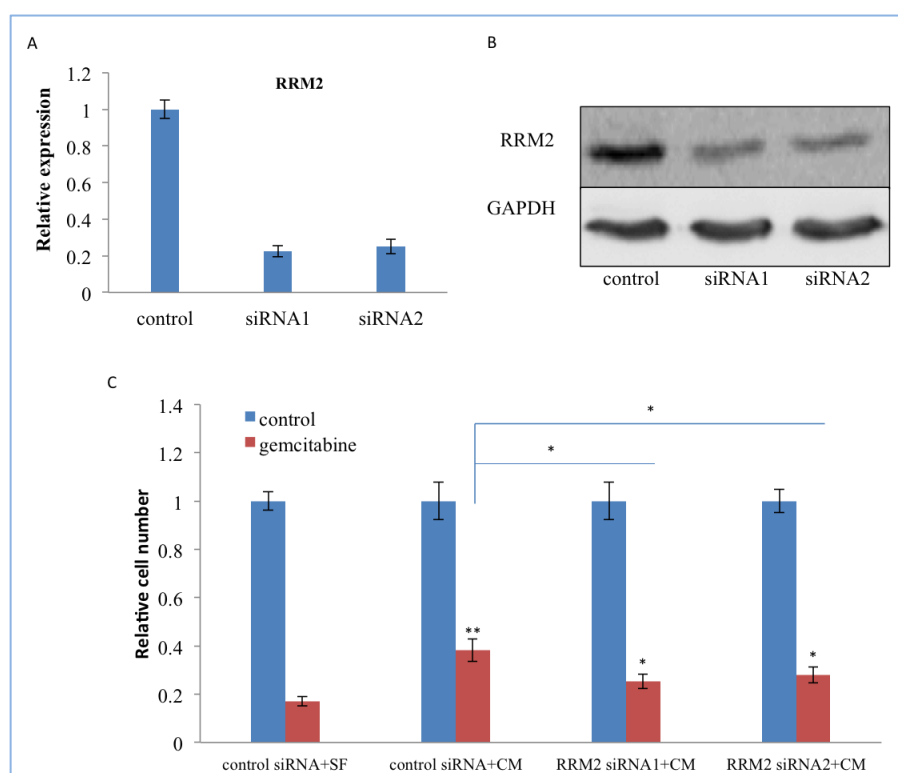

Figure 11. RRM2 overexpression plays a role in PSCs-induced drug resistance in Bxpc-3 cells. Bxpc-3 cells were transfected with control or two different RRM2 siRNAs (50nM) for 24h before determine mRNA expression of RRM1 (A), and 48h before determine protein expression of RRM1 (B). C.48 hours after Bxpc-3 cells transfected with siRNA, cells were serum free overnight and treated with serum free medium or conditioned medium for 72h with or without gemcitabine. Then cell viability was tested by resazurin assay. All experiment were performed in triplicates, and data expressed as mean±SD. **, $P<0.001$, *, $P<0.05$.

3.7 Factor in the conditioned medium is insensitive to enzyme treatments and heat inactivation

In order to find out what in the conditioned medium is responsible for PSCs-induced drug resistance in Bxpc-3 cells, conditioned medium was treated with proteinase K, Trypsin, Rnase A, or Dnase I before the treatment for Bxpc-3 cells. However, the induced drug resistance

effects were still there even after enzyme treatments. And, when conditioned medium was boiled at 100°C for 2 hours, the effect was even much higher. Furthermore, when using ultracentrifugation to get rid of exosomes, the effect was still there.
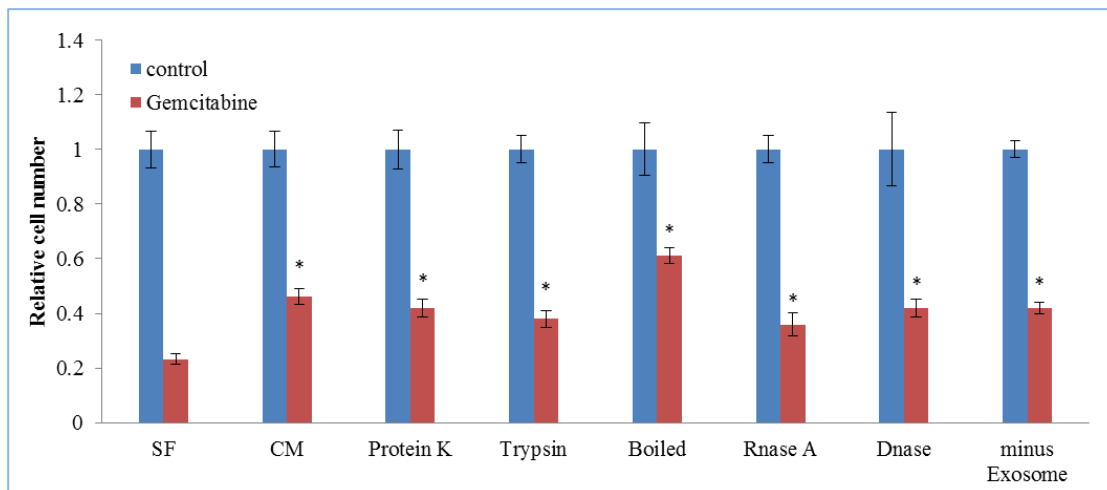

Figure 12. Factor in the conditioned medium is insensitive to enzyme treatments and heat inactivation. Conditioned media from PSCs were incubated with proteinase K (200μg/mL, 1h, 37°C), trypsin (200μg/mL, 1h, 37°C), RnaseA (100μg/mL, 4h, 37°C), Dnase I (2U/μl, 4h, 37°C) or heat inactivation (100°C, 2h), the induced drug resistance effect of conditioned medium was not reversed. And, when using ultracentrifugation to get rid of the exosomes, the effect was still existed.

3.8 Proteins that have a molecular weight smaller than 100 kDa in the conditioned medium are responsible for PSCs-induced drug resistance in Bxpc-3 cells

To study which fraction in the conditioned medium makes contribution to PSCs-induced drug resistance in Bxpc-3 cells, CM was fractioned using Amicon® Ultra-0.5ml Centrifugal filter by their molecular weight difference. Totally, 10 different fractions were obtained. These fractions were reconstituted in their original volume and used to treat Bxpc-3 cells under gemcitabine treatment. As showed in Figure 13, when Bxpc-3 cells were grown in SF medium, 0.1μM gemcitabine can kill around 80% of the cells. However, when treated Bxpc-3 with CM from PSCs, the same concentration of the drug can only kill around 57% of the cells. When Bxpc-3 cells were treated with < 3kDa, >3kDa, < 10kDa, >10kDa, < 30kDa, > 30kDa, <50kDa and >50kDa fractions, conditioned medium induced drug resistance effects were still existed. But, when treated by >100kDa fraction, the CM induced drug resistance effect in Bxpc-3 cells was disappeared. And <100kDa fraction still has the effect.
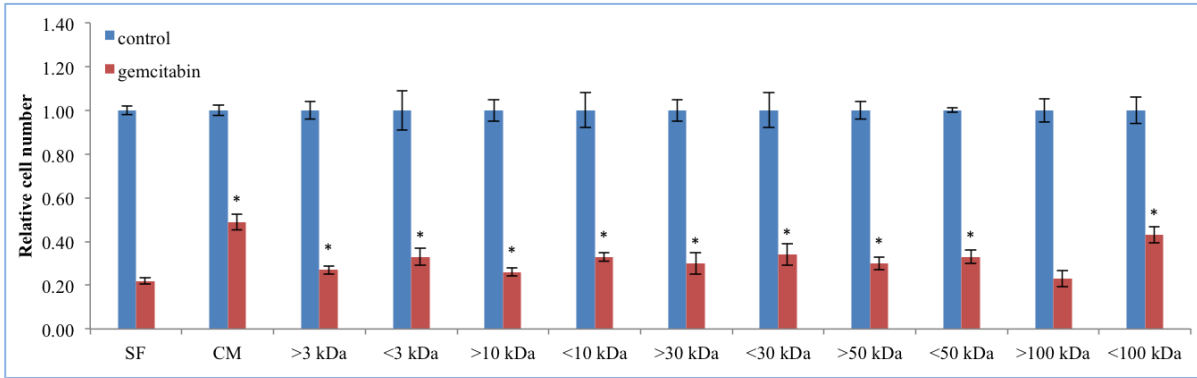
Figure 13. Proteins that have a molecular weight smaller than 100kDa in the conditioned medium are responsible for PSCs-induced drug resistance in Bxpc-3. Conditioned medium was fractioned by their molecular difference and then used to treat Bxpc-3 cells. Among all the 10 fractions, >100kDa fraction loss the ability to induce drug resistance in Bxpc-3 cells. *, $P<0.05$.

4 Discussion

As mentioned above, patients diagnosed with pancreatic cancer will die within 6 months and the survival time of them has not been improved for nearly 40 years. This is due to that pancreatic cancer is highly resistant to chemo and radiation therapy. Currently, gemcitabine seems to be the most effective drug for pancreatic cancer patients, however, its efficacy is limited [73]. The importance of the tumor microenvironment to tumor progression has been recognized in recent years and the extensive tumor stromal has been postulated by researchers influences tumor response to chemotherapy in pancreatic cancer [60, 83]. However, the precise mechanism involved, particularly, how pancreatic stellate cells influence the drug sensitivity of pancreatic cancer cells has not been elucidated. In the current study, we chose three different drug sensitive pancreatic cancer cell lines, and tested how PSCs will influence the chemo-resistance of them.

There are plenty of pancreatic cancer cell lines exist in the research field of PDAC, Panc-1, Miapaca-2 and Bxpc-3 were chose in the current study due to their different drug sensitivity. Previous studies have verified that these three cell lines response differently to gemcitabine treatment. Such as, Pan [84] proved that Panc-1 and Miapaca-2 are gemcitabine resistant cell lines and Bxpc-3 is gemcitabine sensitive cell line. Moreover, Duxbury [85] found that Panc-1 and Miapaca-2 cells are more drug resistant than Bxpc-3 cells since their higher expression of RRM2. In the current study, same results were obtained, we showed that Panc-1 is the most gemcitabine resistant cell line, Miapaca-2 cells are gemcitabine resistant and Bxpc-3 cells are gemcitabine sensitive. Besides that, by treating highly activated PSCs with different concentration of gemcitabine, we found that activated PSCs are also gemcitabine resistant.

Stroma influences the drug resistance of pancreatic cancer cells has been shown before. For example, Miyamoto [61] found that extracelluar matrix proteins in the stroma are responsible for acquired drug resistance of pancreatic cancer cells. Olive [68] discovered that depletion of tumor stroma increases the drug response in pancreatic cancer. Hwang [66] confirmed that PSCs protect pancreatic cancer cells from chemo and radiation therapy. In our study, we observed that PSCs reduce the drug sensitivity of Bxpc-3 cells, but not Panc-1 and Miapaca-2 cells. That explains why even drug sensitive pancreatic cancer cell lines exist, still no pancreatic cancer is curable by drug treatment. It also gives a clue for future personalized medicine in the field of pancreatic cancer, targeting PSCs may not work on all patients.

Numerous studies have proved that PSC play a crucial role in promoting pancreatic cancer cell proliferation [86, 87]. However, no study has shown how PSCs influence the apoptosis of pancreatic cancer cells. From our results, we found that conditioned medium from PSCs surprisingly increases the apoptosis of all three pancreatic cancer cell lines. And, when treated with gemcitabine, CM from PSCs showed on influence on gemcitabine induced apoptosis in Panc-1 and Bxpc-3 cells, but it promote the apoptosis of Miapaca-2 cells. Interactions between cells are complicate. Cells may support each other and oppose each other at the same time. In the current study, for Miapaca-2 cells, even though PSC enhances the apoptosis of them, it has no influence on the drug sensitivity of them. It is possible that the induced proliferation effect of PSCs neutralized the apoptosis effect it has on Miapaca-2 cells.

Since PSCs induce drug resistance in Bxpc-3 cells, the potential mechanism was studied. Multiple studies have shown that RRM1 and RRM2 are highly associated with gemcitabine resistance. Such as, Fujita [88] found that mRNA expression levels of RRM1 and RRM2 are related to gemcitabine sensitivity of patients with pancreatic cancer. Akita [89] discovered that after total pancreas resection, patients who can benefit from gemcitabine treatment have low expression levels of RRM1. By knocking down RRM2 in several pancreatic cancer cell lines, Duxbury [90] found that RRM2 lower expression reduces the invasiveness and gemcitabine chemoresistance of pancreatic cancer cells. In the current study, our results for the first time showed that conditioned medium from PSCs promotes the drug resistance of Bxpc-3 cells through up regulating RRM1 and RRM2.

Substances exist in the conditioned medium are diverse and plenty, such as, growth factors, cytokines, RNA, DNA, biological molecules, exosomes and so on. In an effort to identify what in the conditioned medium causes PSCs-induced drug resistance in Bxpc-3 cells, conditioned medium was treated with various enzymes. However, the results showed that these factors, which we are interested in, are not sensitive to enzyme treatments or heat inactivation. Similarly, Jandu [91] studied factors from the conditioned medium of epithelial cells and found that those factors that have influences were resistant to proteinase K, trypsin and heat treatment. As explained in the article, "proteins can be heat inactivation resistant and protease treatment may leave smaller protein behind, the exact molecular identity of the factor requires further experiments". But, Collins's [92] work about neurite outgrowth verified that the influences of heart-cell conditioned medium on neuron is due to some trypsin sensitive

factors which bound to the culture substratum. In the current study, we also get rid of the influence of the exosomes, but the effect was still there.

In order to further identify the characteristics of these factors in the conditioned medium, we separated the medium according to molecular weight. Totally 10 fractions were obtained, this is the first study showed that <100kDa factors are responsible for the PSCs-induced drug resistance effect in Bxpc-3 cells. The influence of PSCs' conditioned medium on the drug resistance of Bxpc3-cells maybe is a combined effect, multiple factors make contributions to that, so it is difficult to identify one specific substance causative for that. Likewise, Liu [93] found that high molecular fractions (>100kDa) from the conditioned medium of human oviductal cells improve the development of mouse embryo, but no specific substance was identified. Watanabe [94] discovered that >50kDa fractions in the conditioned medium of rat epithelial cells increase the growth of neurons, still no particular factor was mentioned.

In conclusion, our experimental results firstly demonstrated that conditioned medium from pancreatic stellate cells promote the drug resistance of Bxpc-3 cells through up-regulating RRM1 and RRM2 expression in Bxpc-3, but have no influence on the drug resistance of Miapaca-2 cells and Panc-1 cells. Furthermore, we showed the <100kDa factors produced by pancreatic stellate cells are responsible for the effects. And these factors are heat insensitive, trypsin and proteinase K insensitive, but the exact factor is yet to be determined.

References

1. Hariharan D, Saied A, and Kocher H M. Analysis of mortality rates for pancreatic cancer across the world. *HPB (Oxford)*. 2008;10(1):58-62.

2. Vincent A, Herman J, Schulick R, Hruban R H, and Goggins M. Pancreatic cancer. *The Lancet*. 2011;378(9791):607-620.

3. Bardeesy N and Depinho R A. Pancreatic cancer biology and genetics. *Nature Reviews Cancer*. 2002;2(12):897-909.

4. Wang Z, Li Y, Ahmad A, et al. Pancreatic cancer: understanding and overcoming chemoresistance. *Nat Rev Gastroenterol Hepatol*. 2011;8(1):27-33.

5. Staley C A, Lee J E, Cleary K R, et al. Preoperative chemoradiation, pancreaticoduodenectomy, and intraoperative radiation therapy for adenocarcinoma of the pancreatic head. *American Journal of Surgery*. 1996;171(1):118-124; discussion 124-115.

6. Malvezzi M, Carioli G, Bertuccio P, et al. European cancer mortality predictions for the year 2017, with focus on lung cancer. *Annals of Oncology: Official Journal of the European Society for Medical Oncology*. 2017;28(5):1117-1123.

7. Siegel R L, Miller K D, and Jemal A. Cancer statistics, 2017. *CA Cancer J Clin*. 2017;67(1):7-30.

8. Tsim N C, Frampton A E, Habib N A, and Jiao L R. Surgical treatment for liver cancer. *World Journal of Gastroenterology : WJG*. 2010;16(8):927-933.

9. Delaney G, Jacob S, Featherstone C, and Barton M. The role of radiotherapy in cancer treatment. *Cancer*. 2005;104(6):1129-1137.

10. Loos M, Kleeff J, Friess H, and Büchler M W. Surgical Treatment of Pancreatic Cancer. *Annals of the New York Academy of Sciences*. 2008;1138(1):169-180.

11. Garrido-Laguna I and Hidalgo M. Pancreatic cancer: from state-of-the-art treatments to promising novel therapies. *Nature Reviews Clinical Oncology*. 2015;12(6):319-334.

12. P P, M Y, and D A. [Gemcitabine and pancreatic cancer]. *Bulletin du cancer*. 2002;89 Spec No:S91-95.

13. Alexander Fleming Discovery and Development of Penicillin - Landmark - American Chemical Society.

14. Housman G, Byler S, Heerboth S, et al. Drug Resistance in Cancer: An Overview. *Cancers*. 2014;6(3):1769-1792.

15. Borst P, Evers R, Kool M, and Wijnholds J. A Family of Drug Transporters: the Multidrug Resistance-Associated Proteins. *JNCI: Journal of the National Cancer Institute*. 2000;92(16):1295-1302.

16. Borst P and Elferink R O. Mammalian ABC Transporters in Health and Disease. *Annu Rev Biochem*. 2002;71(1):537-592.

17. Fojo A T, Ueda K, Slamon D J, Poplack D G, Gottesman M M, and Pastan I. Expression of a multidrug-resistance gene in human tumors and tissues. *Proceedings of the National Academy of Sciences*. 1987;84(1):265-269.

18. Gottesman M M, Fojo T, and Bates S E. Multidrug resistance in cancer: role of ATP–dependent transporters. *Nature Reviews Cancer*. 2002;2(1):48-58.

19. Abolhoda A, Wilson A E, Ross H, Danenberg P V, Burt M, and Scotto K W. Rapid Activation of MDR1 Gene Expression in Human Metastatic Sarcoma after in Vivo Exposure to Doxorubicin. *Clinical Cancer Research*. 1999;5(11):3352-3356.

20. Haber M, Smith J, Bordow S B, et al. Association of High-Level MRP1 Expression With Poor Clinical Outcome in a Large Prospective Study of Primary Neuroblastoma. *Journal of Clinical Oncology*. 2006;24(10):1546-1553.

21. Aller S G, Yu J, Ward A, et al. Structure of P-Glycoprotein Reveals a Molecular Basis for Poly-Specific Drug Binding. *Science*. 2009;323(5922):1718-1722.

22. Rouby S E, Thomas A, Costin D, et al. p53 gene mutation in B-cell chronic lymphocytic leukemia is associated with drug resistance and is independent of MDR1/MDR3 gene expression. *Blood*. 1993;82(11):3452-3459.

23. Gorre M E, Mohammed M, Ellwood K, et al. Clinical Resistance to STI-571 Cancer

Therapy Caused by BCR-ABL Gene Mutation or Amplification. *Science*. 2001;293(5531):876-880.

24. Aas T, Børresen A L, Geisler S, et al. Specific P53 mutations are associated with de novo resistance to doxorubicin in breast cancer patients. *Nature Medicine*. 1996;2(7):811-814.

25. Yun C-H, Mengwasser K E, Toms A V, et al. The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. *Proceedings of the National Academy of Sciences*. 2008;105(6):2070-2075.

26. Pao W, Wang T Y, Riely G J, et al. KRAS Mutations and Primary Resistance of Lung Adenocarcinomas to Gefitinib or Erlotinib. *PLOS Medicine*. 2005;2(1):e17.

27. Bird A. DNA methylation patterns and epigenetic memory. *Genes & Development*. 2002;16(1):6-21.

28. Jones P A and Takai D. The Role of DNA Methylation in Mammalian Epigenetics. *Science*. 2001;293(5532):1068-1070.

29. Okano M, Bell D W, Haber D A, and Li E. DNA Methyltransferases Dnmt3a and Dnmt3b Are Essential for De Novo Methylation and Mammalian Development. *Cell*. 1999;99(3):247-257.

30. Karlić R, Chung H-R, Lasserre J, Vlahoviček K, and Vingron M. Histone modification levels are predictive for gene expression. *Proceedings of the National Academy of Sciences*. 2010;107(7):2926-2931.

31. Kantharidis P, El-Osta A, Desilva M, et al. Altered methylation of the human MDR1 promoter is associated with acquired multidrug resistance. *Clinical Cancer Research*. 1997;3(11):2025-2032.

32. Chen Y-P, Hou X-Y, Yang C-S, et al. DNA methylation and histone acetylation regulate the expression of MGMT and chemosensitivity to temozolomide in malignant melanoma cell lines. *Tumor Biology*. 2016;37(8):11209-11218.

33. Steele N, Finn P, Brown R, and Plumb J A. Combined inhibition of DNA methylation and histone acetylation enhances gene re-expression and drug sensitivity in vivo. *Br J*

Cancer. 2009;100(5):758-763.

34. Xie M, Zhang L, He C-S, et al. Activation of notch-1 enhances epithelial–mesenchymal transition in gefitinib-acquired resistant lung cancer cells. *Journal of Cellular Biochemistry*. 2012;113(5):1501-1513.

35. Xia H, Ooi L L P J, and Hui K M. MicroRNA-216a/217-induced epithelial-mesenchymal transition targets PTEN and SMAD7 to promote drug resistance and recurrence of liver cancer. *Hepatology*. 2013;58(2):629-641.

36. Mcconkey D J, Choi W, Marquis L, et al. Role of epithelial-to-mesenchymal transition (EMT) in drug sensitivity and metastasis in bladder cancer. *Cancer and Metastasis Reviews*. 2009;28(3-4):335-344.

37. Saxena M, Stephens M A, Pathak H, and Rangarajan A. Transcription factors that mediate epithelial–mesenchymal transition lead to multidrug resistance by upregulating ABC transporters. *Cell Death & Disease*. 2011;2(7):e179.

38. Reya T, Morrison S J, Clarke M F, and Weissman I L. Stem cells, cancer, and cancer stem cells. *Nature*. 2001;414(6859):105-111.

39. S F. The cancer cell as a stem cell unable to differentiate. A theory of carcinogenesis. *Neoplasma*. 1968;15(6):607-622.

40. Adams J M and Strasser A. Is Tumor Growth Sustained by Rare Cancer Stem Cells or Dominant Clones? *Cancer Research*. 2008;68(11):4018-4021.

41. Visvader J E and Lindeman G J. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nature Reviews Cancer*. 2008;8(10):755-768.

42. Donnenberg V S and Donnenberg A D. Multiple Drug Resistance in Cancer Revisited: The Cancer Stem Cell Hypothesis. *The Journal of Clinical Pharmacology*. 2005;45(8):872-877.

43. Dean M, Fojo T, and Bates S. Tumour stem cells and drug resistance. *Nature Reviews Cancer*. 2005;5(4):275-284.

44. Liu G, Yuan X, Zeng Z, et al. Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. *Mol Cancer*. 2006;5:67.

45.   Ma S, Lee T K, Zheng B J, Chan K W, and Guan X Y. CD133+ HCC cancer stem cells confer chemoresistance by preferential expression of the Akt/PKB survival pathway. *Oncogene*. 2007;27(12):1749-1758.

58.   Bondar V M, Sweeney-Gotsch B, Andreeff M, Mills G B, and Mcconkey D J. Inhibition of the Phosphatidylinositol 3′-Kinase-AKT Pathway Induces Apoptosis in Pancreatic Carcinoma Cells in Vivo and in vitro. *Molecular Cancer Therapeutics*. 2002;1(12):989-997.

59.   Neesse A, Michl P, Frese K K, et al. Stromal biology and therapy in pancreatic cancer. *Gut*. 2011;60(6):861-868.

60.   Müerköster S, Wegehenkel K, Arlt A, et al. Tumor Stroma Interactions Induce Chemoresistance in Pancreatic Ductal Carcinoma Cells Involving Increased Secretion and Paracrine Effects of Nitric Oxide and Interleukin-1β. *Cancer Research*. 2004;64(4):1331-1337.

61.   Miyamoto H, Murakami T, Tsuchida K, Sugino H, Miyake H, and Tashiro S. Tumor-stroma interaction of human pancreatic cancer: acquired resistance to anticancer drugs and proliferation regulation is dependent on extracellular matrix proteins. *Pancreas*. 2004;28(1):38-44.

62.   Farrow B, Albo D, and Berger D H. The Role of the Tumor Microenvironment in the Progression of Pancreatic Cancer. *Journal of Surgical Research*. 2008;149(2):319-328.

63.   Kleeff J, Beckhove P, Esposito I, et al. Pancreatic cancer microenvironment. *International Journal of Cancer*. 2007;121(4):699-705.

64.   Apte M V, Park S, Phillips P A, et al. Desmoplastic reaction in pancreatic cancer: role of pancreatic stellate cells. *Pancreas*. 2004;29(3):179-187.

65.   Apte M V, Wilson J S, Lugea A, and Pandol S J. A starring role for stellate cells in the pancreatic cancer microenvironment. *Gastroenterology*. 2013;144(6):1210-1219.

66.   Long J, Zhang Y, Yu X, et al. Overcoming drug resistance in pancreatic cancer. *Expert Opinion on Therapeutic Targets*. 2011;15(7):817-828.

67. Hwang R F, Moore T, Arumugam T, et al. Cancer-Associated Stromal Fibroblasts Promote Pancreatic Tumor Progression. *Cancer Research*. 2008;68(3):918-926

68. Olive K P, Jacobetz M A, Davidson C J, et al. Inhibition of Hedgehog Signaling Enhances Delivery of Chemotherapy in a Mouse Model of Pancreatic Cancer. *Science*. 2009;324(5933):1457-1461.

69. Koong A C, Mehta V K, Le Q T, et al. Pancreatic tumors show high levels of hypoxia. *International Journal of Radiation Oncology Biology Physics*. 2000;48(4):919-922.

70. Evans S M and Koch C J. Prognostic significance of tumor oxygenation in humans. *Cancer Lett*. 2003;195(1):1-16.

71. Erkan M, Reiser-Erkan C, Michalski C W, et al. Cancer-Stellate Cell Interactions Perpetuate the Hypoxia-Fibrosis Cycle in Pancreatic Ductal Adenocarcinoma. *Neoplasia*. 2009;11(5):497-508.

72. Vonlaufen A, Joshi S, Qu C, et al. Pancreatic Stellate Cells: Partners in Crime with Pancreatic Cancer Cells. *Cancer Research*. 2008;68(7):2085-2093.

73. Burris H A, Moore M J, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *Journal of Clinical Oncology*. 1997;15(6):2403-2413.

74. Ishii H, Furuse J, Nagase M, and Yoshino M. Impact of gemcitabine on the treatment of metastatic pancreatic cancer. *Journal of Gastroenterology and Hepatology*. 2005;20(1):62-66.

75. Giovannetti E, Tacca M D, Mey V, et al. Transcription Analysis of Human Equilibrative Nucleoside Transporter-1 Predicts Survival in Pancreas Cancer Patients Treated with Gemcitabine. *Cancer Research*. 2006;66(7):3928-3935.

76. Tanaka M, Javle M, Dong X, Eng C, Abbruzzese J L, and Li D. Gemcitabine metabolic and transporter gene polymorphisms are associated with drug toxicity and efficacy in patients with locally advanced pancreatic cancer. *Cancer*. 2010;116(22):5325-5335.

77. Pérez-Torras S, García-Manteiga J, Mercadé E, et al. Adenoviral-mediated

overexpression of human equilibrative nucleoside transporter 1 (hENT1) enhances gemcitabine response in human pancreatic cancer. *Biochemical Pharmacology*. 2008;76(3):322-329.

78. Weizman N, Krelin Y, Shabtay-Orbach A, et al. Macrophages mediate gemcitabine resistance of pancreatic adenocarcinoma by upregulating cytidine deaminase. *Oncogene*. 2014;33(29):3812-3819.

79. Nakahira S, Nakamori S, Tsujie M, et al. Involvement of ribonucleotide reductase M1 subunit overexpression in gemcitabine resistance of human pancreatic cancer. *International Journal of Cancer*. 2007;120(6):1355-1363.

80. Kroep J R, Loves W J P, Wilt C L V D, et al. Pretreatment Deoxycytidine Kinase Levels Predict in Vivo. Gemcitabine Sensitivity. *Molecular Cancer Therapeutics*. 2002;1(6):371-376.

81. Bergman A M, Pinedo H M, and Peters G J. Determinants of resistance to 2',2'-difluorodeoxycytidine (gemcitabine). *Drug Resist Updat*. 2002;5(1):19-33.

82. Jesnowski R, Fürst D, Ringel J, et al. Immortalization of pancreatic stellate cells as an in vitro model of pancreatic fibrosis: deactivation is induced by matrigel and N-acetylcysteine. *Laboratory Investigation*. 2005;85(10):1276-1291.

83. Kalluri R and Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer*. 2006;6(5):392-401.

84. Pan X, Arumugam T, Yamamoto T, et al. Nuclear Factor-κB p65/relA Silencing Induces Apoptosis and Increases Gemcitabine Effectiveness in a Subset of Pancreatic Cancer Cells. *Clinical Cancer Research*. 2008;14(24):8143-8151.

85. Duxbury M S, Ito H, Zinner M J, Ashley S W, and Whang E E. RNA interference targeting the M2 subunit of ribonucleotide reductase enhances pancreatic adenocarcinoma chemosensitivity to gemcitabine. *Oncogene*. 2004;23(8):1539-1548.

86. Vonlaufen A, Phillips P A, Xu Z, et al. Pancreatic Stellate Cells and Pancreatic Cancer Cells: An Unholy Alliance. *Cancer Research*. 2008;68(19):7707-7710.

87. Haqq J, Howells L M, Garcea G, Metcalfe M S, Steward W P, and Dennison A R. Pancreatic stellate cells and pancreas cancer: Current perspectives and future

strategies. *European Journal of Cancer*. 2014;50(15):2570-2582.

88. Fujita H, Ohuchida K, Mizumoto K, et al. Gene expression levels as predictive markers of outcome in pancreatic cancer after gemcitabine-based adjuvant chemotherapy. *Neoplasia (New York, N.Y.)*. 2010;12(10):807-817.

89. Akita H, Zheng Z, Takeda Y, et al. Significance of RRM1 and ERCC1 expression in resectable pancreatic adenocarcinoma. *Oncogene*. 2009;28(32):2903-2909.

90. Duxbury M S, Ito H, Benoit E, Zinner M J, Ashley S W, and Whang E E. Retrovirally mediated RNA interference targeting the M2 subunit of ribonucleotide reductase: A novel therapeutic strategy in pancreatic cancer. *Surgery*. 2004;136(2):261-269.

91. Jandu N, Ceponis P J M, Kato S, Riff J D, Mckay D M, and Sherman P M. Conditioned Medium from Enterohemorrhagic Escherichia coli-Infected T84 Cells Inhibits Signal Transducer and Activator of Transcription 1 Activation by Gamma Interferon. *Infection and Immunity*. 2006;74(3):1809-1818.

92. Collins F. Induction of neurite outgrowth by a conditioned-medium factor bound to the culture substratum. *Proceedings of the National Academy of Sciences*. 1978;75(10):5210-5213.

93. Liu L P S, Chan S T H, Ho P C, and Yeung W S B. Human oviductal cells produce high molecular weight factor(s) that improves the development of mouse embryo. *Human Reproduction*. 1995;10(10):2781-2786.

94. Watanabe Y, Matsumoto N, Dezawa M, Itokazu Y, Yoshihara T, and Ide C. Conditioned medium of the primary culture of rat choroid plexus epithelial (modified ependymal) cells enhances neurite outgrowth and survival of hippocampal neurons. *Neuroscience Letters*. 2005;379(3):158-163.