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Discipline: Aspects moléculaires et cellulaires de la Biologie

ROLE OF CALCIUM-PERMEABLE CHANNELS IN PANCREATIC DUCTAL ADENOCARCINOMA RESISTANCE TO CHEMOTHERAPY

Thèse dirigée par le Professeur Roman Skryma

Présentée et soutenue publiquement par

Kateryna Kondratska (nom de jeune fille Klymenko)

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Membres du Jury:

Prof. Michel Salzet, Université Lille1 (FR)	Président
Prof. Albrecht Schwab, Université de Münster (D)	Rapporteur
Prof. Halima Ouadid-Ahidouch, Université de Picardie Jules Verne (FR)	Rapporteur
Prof. Ildikò Szabò, Université Padova (IT)	Examinateur
Prof. Roman Skryma, Université Lille1 (FR)	Examinateur
Prof. Natalia Prevarskaya, Université Lille1 (FR)	Examinateur

Thèse réalisée au sein du Laboratoire de Physiologie Cellulaire INSERM U1003,
Université Lille1, Villeneuve d'Ascq France
Directrice Prof. Natalia Prevarskaya

I would like

to dedicate this thesis

to my husband and kids

Acknowledgments

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Contributions by the author

Publications:

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2. **Kondratska K.**, Kondratskyi A., Yassine M., Lemonnier L., Lepage G., Morabito A., Skryma R., Prevarskaya N. Orai1 and STIM1 mediate SOCE and contribute to apoptotic resistance of pancreatic adenocarcinoma. *Biochim Biophys Acta.* 2014 Oct;1843(10):2263-9.

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11. Nielsen N., **Kondratska K.**, Prevarskaya N., Schwab A. "The Role of TRPC1 and TRPC6 Channels in Hypoxia-Mediated Activation of Pancreatic Stellate Cells". PhD meeting of the Italian Society of Immunology Clinical Immunology and Allergology (SIICA), Bari, Italy, October 9-11, 2014.

12. Nielsen N., **Kondratska K.**, Schimmelpfennig S., Welzig J., Prevarskaya N., Schwab A. "TRPC6 channels are required for hypoxia-mediated stimulated of pancreatic stellate cells". 4th International meeting on Ion channels, transporters and cancer ("INCA2015") September 9-10, 2015, London, UK.

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Other activities:

1.1st IonTraC Workshop 7-9 September 2012 Benediktushöhe Zellingen – Retzbach, Germany.

2. Course Français Langue Étrangère CUEEP Octobre 2012 - Juin 2013. Level A2.

3. International meeting on ion transport and cancer September 9 - 12, 2012 Würzburg, Germany.

4. 1st IonTraC Winter School. Experimental methods in cancer research. 23-25 February, 2013, Hamburg, Germany.

5. IonTraC Mid-Term Review 29-30 May 2013 Sandbjerg Denmark.

6. Course Français Langue Étrangère Maison de la Langue Octobre 2013 - December 2013. Level B1.

7. IonTraC Oxford Workshop on scientific writing, bioethics and grant application. 21-23 November 2013, Oxford, England.

8. Course Français Langue Étrangère Maison de la Langue Fevrier 2014 - Juin 2014. Level B2+.

9. Exchange PhD-student at the Westphalian-Wilhelms University Münster. February-March , 2014 Münster, Germany.

10. IonTraC Summer School and Italian-French Erasmus Intensive Course in Oncology, 26-28 March, 2014, Florence, Italy.

Teaching experience:

1) November 2014 – lecture “Ion channels” for master students, Université de Lille 1

2) December 2014 – practical course on “Calcium imaging approach” for master students, Université de Lille 1

IonTraC Project

With over 3 million new cases and 1.5 million arising deaths each year in Europe, cancer is a major public health problem with an urgent need for new therapies. IonTrac Project builds upon mounting evidence that ion channels and transporters underlie many of the hallmarks of cancer. Thus, proteins involved in membrane transport, long known as important drug targets in other pathologies (channelopathies), are a new class of therapeutic and/or diagnostic targets in oncology. IonTraC is first to propose a systematic analysis of the expression, function, as well as therapeutic and diagnostic potential of proteins involved in ion transport (the "transportome") in cancer. This paradigm will be implemented in pancreatic ductal adenocarcinoma (PDAC) which has one of the worst prognoses of all cancers, with an overall 5-year survival rate of less than 5%.

IonTraC thereby provides the framework for an inter- and supra-disciplinary training for early stage researchers in a highly innovative, exponentially growing field in oncology which will have a major impact on other disciplines such as immunology and angiology as well.

The main objectives of IonTraC are:

- to provide a scientific and methodological platform for supra-disciplinary training of early stage researchers in and beyond the fields of ion transport and oncology
- to provide an inter-sectoral training programme with special focus on career development of young researchers
- to determine the concerted expression and function of ion channels and transporters required for the progression of PDAC, and
- to provide validated therapeutic and diagnostic concepts and tools that are based on transport proteins serving as novel drug targets and/or biomarkers.

IonTraC is a Marie Curie Initial Training Network comprising 10 partners-laboratories as well as 2 associated partners with long-standing, complementary expertises in ion transport and carcinogenesis. Every partner is studying the role of a particular type of ion channels in PDAC and/or pancreatic stellate cells (PSCs). In the framework of IonTrac project our laboratory is focused on the studying of the expression/function and role of calcium-permeable channels (in particular TRP and ORAI channels) in PDAC and PSCs.

The homepage for IonTraC is: http://www.iontrac.uni-muenster.de/home.html

English abstract

Pancreatic cancer is one of the leading causes of cancer-related deaths in the world. The exceptionally high mortality rate of pancreatic cancer is a consequence of asymptomatic course of the disease till the late incurable stages, as well as of a high resistance of this cancer to existing chemotherapy and radiotherapy treatments. The mechanisms that contribute to high chemotherapy resistance of pancreatic cancer include gene mutations and aberrant gene expression, deregulation of many signaling pathways and others. Moreover, the prominent desmoplastic stroma (which is mainly produced by pancreatic stellate cells (PSCs)) essentially contributes to the chemotherapy resistance of pancreatic cancer. Thus, understanding the physiology of pancreatic cancer cells as well as PSCs is of great importance as it can reveal novel approaches for treating pancreatic cancer.

Pancreatic cancer cells and cancer associated PSCs have been shown to exhibit the increased proliferation, migration and resistance to programmed cell death (apoptosis). Many of these processes are known to be typically controlled and/or modified by calcium and calcium-permeable ion channels. However, the information on the expression/function/role of calcium-permeable ion channels in pancreatic cancer cells as well as PSCs is very limited.

In this thesis we demonstrate that store-operated calcium channels (SOCs), which represent one of the major calcium-entry pathways in non-excitable cells, are expressed and functional in pancreatic cancer cell lines as well as in PSCs. We showed that the classical major components of SOCs, namely ORAI1 and STIM1 mediate store-operated calcium entry (SOCE) in pancreatic cancer cell lines as well as in PSCs. We show that both ORAI1 and STIM1 play pro-survival antiapoptotic role in pancreatic adenocarcinoma cell lines, as siRNA mediated knockdown of ORAI1 and/or STIM1 increase apoptosis induced by chemotherapy drugs 5-fluorouracil (5-FU) or gemcitabine. We also demonstrate that both 5-FU and gemcitabine treatments increase SOCE in Panc1 pancreatic adenocarcinoma cell line via upregulation of ORAI1 and STIM1. We also revealed the prosurvival role of another ORAI family member, ORAI3 in MiaPaca2 cell line. We demonstrate the role of ORAI3 in proliferation, cell cycle and viability of MiaPaca2 cells. We showed that silencing of ORAI3 impairs pancreatic tumor growth *in vivo*, suggesting that ORAI3 could represent a potential therapeutic target in PDAC treatment.

Altogether our results revealed the expression, function and role of SOCs in pancreatic adenocarcinoma cell lines, and emphasize the antiapoptotic role of ORAI1, ORAI3 and STIM1 in pancreatic adenocarcinoma cells.

French abstract

Le cancer du pancréas est l'une des principales causes de décès liés au cancer dans le monde. Le taux de mortalité exceptionnellement élevé de ce cancer est une conséquence de son développement qui reste asymptomatique jusqu'à des stades tardifs incurables, et de sa grande résistance à la radiothérapie et aux chimiothérapies. Les mécanismes qui contribuent à la résistance à la chimiothérapie du cancer du pancréas comprennent des mutations géniques, l'expression aberrante des gènes, la dérégulation de nombreuses voies de signalisation, etc. De plus, le stroma desmoplastique important (produit principalement par les cellules pancréatiques stellaires (PSCs)) contribue à la résistance à la chimiothérapie du cancer du pancréas. Ainsi, la compréhension de la physiologie des cellules du cancer du pancréas et des PSCs est d'une grande importance car elle pourrait conduire à de nouvelles approches pour le traitement de ce cancer. Il a été montré que les cellules du cancer du pancréas ainsi que PSCs présentent plusieurs mutations génétiques qui conduisent à la prolifération et à la migration incontrôlée des cellules, ainsi qu'à la résistance à la mort cellulaire programmée (apoptose). La plupart de ces processus sont connus pour être contrôlés et/ou modifiés par le calcium et les canaux calciques. Cependant, les informations sur l'expression/fonction ainsi que sur le rôle des canaux calciques dans les cellules du cancer du pancréas et les PSCs sont encore limitées.

Dans cette thèse, nous démontrons que les canaux calciques de type SOC, qui représentent l'une des principales voies d'entrée du calcium dans les cellules non-excitables, sont exprimés et fonctionnels dans des lignées cellulaires du cancer du pancréas ainsi que dans les PSCs. Nous avons montré que ORAI1 et STIM1 sont le support moléculaire de l'activité du canal SOC dans des lignées cellulaires du cancer du pancréas ainsi que dans les PSCs. Ces deux protéines jouent un rôle anti-apoptotique dans les cellules du cancer du pancréas, car la régulation négative de ORAI1 et/ou de STIM1 augmente les niveaux d'apoptose induite par les agents chimiothérapeutiques 5-FU et gemcitabine. Nous démontrons également que 5-FU et gemcitabine peuvent influer sur les niveaux de calcium cytosolique via l'augmentation de l'expression de ORAI1 et STIM1. Nous avons également mis en évidence le rôle « pro-survie » d'un autre membre de la famille ORAI, à savoir ORAI3, dans la lignée cellulaire MiaPaca2. Nous démontrons le rôle de ORAI3 sur la prolifération, le cycle cellulaire et la viabilité des cellules MiaPaca2. Enfin, nous avons montré que la régulation négative de ORAI3 altère la croissance de tumeurs pancréatiques in vivo, suggérant que ORAI3 pourrait représenter une cible thérapeutique potentielle dans le traitement de ce cancer.

En conclusion, nos résultats ont révélé l'expression, la fonction, ainsi que le rôle des canaux calciques de type SOC dans les cellules du cancer du pancréas, soulignant ainsi le rôle anti-apoptotique de ORAI1, ORAI3 et STIM1 dans ce cancer.

Table of Contents

Glossary

AA	Arachidonic acid
ABC	ATP-binding cassette
AIF	Apoptosis-inducing factor
ADP-ribose	Adenosine diphosphate ribose
AKT	Serine/threonine kinase
Apaf-1	Apoptotic protease activating factor 1
ATCC	American Type Culture Collection
ATP	Adenosine 5'-triphosphate
BAK	BCL-2 antagonist
BAX	BCL-2-assosiated protein
BCL-2	B cell lymphoma 2
BCL-XL	B cell lymphoma 2 – like protein 1
BID	BH3-interacting domain death agonist
Ca^{2+}	Calcium ion
CaN	Calcineurin
Cyt C	Cytochrome C
CDK	Cyclin-dependent kinase
CEA	Carcinoembryonic antigen
CFTR	Cystic fibrosis transmembrane conductance regulator
CICR	Calcium-induced calcium release
DAG	Diacylglycerol
dATP	Deoxiadenosine 5'-triphosphate
DISC	Death-inducing signalling complex
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
EndoG	Endonuclease G
ER	Endoplasmic reticulum
FdUMP	Fluorodeoxyuridine monophosphate

FdUTP	Fluorodeoxyuridine triphosphate
FGF	Fibroblast growth factor
FOXOs	The forkhead family of transcription factors
FUTP	Fluoridine triphosphate
GB	Gemcitabine
GPCR	G protein coupled receptor
GRP75	Glucose regulated protein
GSK3	Glycogen synthase kinase 3
IAP	Inhibitors of apoptosis proteins
IGF-I	Insulin-like growth factor I
InsP3	Inositol 1,4,5-triphosphate
IP3R	Inositol 1,4,5-triphosphate receptor
IPMN	Intraductal papillary mucinous neoplasms
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LVA	Low voltage-activated
hCTR1	Human copper transporter 1
hENT1	Human equilibrative nucleoside transporter-1
HTRA2	High-temperature requirement protein A2
HVA	High voltage-activated
MAMs	Mitochondria-assosiated membranes
MAPK	Mitogen-activated protein kinaise
MCN	Mucinous cystic neoplasms
MCU	Mitochondrial calcium uniporter
MOMP	Mitochondrial outer membrane permeabilization
MDR	Multidrug resistance
MPTP	Mitochondrial permeability transition pore
mTOR	Mammalian target of rapamycin
NCX	$Na+/Ca^{2+}$ exchanger
NFAT	Nuclear factor of activated T cells
NF-kB	Nuclear factor kB
OMM	Outer mitochondria membrane
PanIN	Pancreatic Intraepitelial Neoplasia
PDAC	Pancreatic ductal adenocarcinoma
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B

PLC	Phospholipase C
PM	Plasma membrane
PSCs	Pancreatic Stellate Cells
PTEN	Phosphatase and tensin homolog deleted from chromosome 10
RT-PCR	Reverse transcription polymerase chain reaction
RYR	Ryanodine receptor
SERCA	Sarcoendoplasmic reticular Ca^{2+} ATPases
SES	Standart external solution
siRNA	Small interfering ribonucleic acid
SOCs	Store operated channels
SOCE	Store operated calcium entry
SR	Sarcoplasmic reticulum
STIM1	Stromal interaction molecule 1
tBID	Truncated BH3-interacting domain death agonist
TET	Tetracycline
TG	Thapsigargin
TNF	Tumor necrosis factor
TPC	Two-pore channels
TRAIL	TNF-related apoptosis inducing ligand
TRP	Transient receptor potential
TRPA	Transient receptor potential ankyrin-like
TRPC	Transient Receptor potential canonical
TRPM	Transient receptor potential melastatin
TRPML	Transient receptor potential mucolipin
TRPP	Transient receptor potential polycystin
TRPV	Transient receptor potential valinoid
TS	Thymidylate synthase
VDACs	Voltage-dependent anion channels
VEGF	Vascular endothelial growth factor
VGCC	Voltage-gated calcium channels
VOCs	Voltage operated channels
5FU	5-fluorouracil

Introduction

1.1 Pathophysiology of the pancreas

1.1.1 Anatomy and physiology of the pancreas

The pancreas (from the Greek pan (all) and kreas (flesh)) is an essential part of the digestive system and an important regulator of blood sugar levels. The pancreas represents a lobulated, tapered organ located behind the stomach in the back of the abdomen. Adult pancreas measures 12-20 cm long, 2-9 cm wide and weighs 75-100g. The pancreas is anatomically divided into four regions (head, neck, body, and tail) with the head lying near the duodenum and the tail extending to the spleen (Figure 1.1).

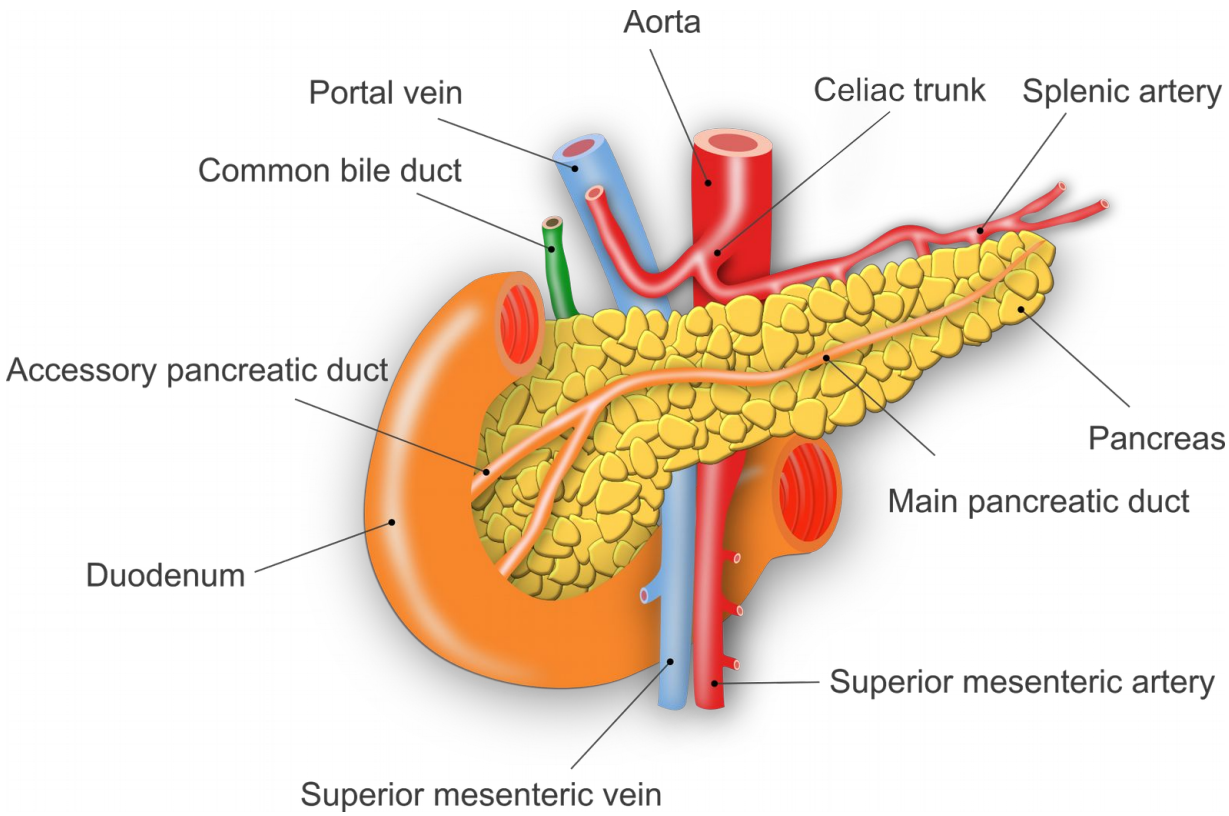

Figure 1.1. The anatomy of the pancreas.

The main pancreatic duct (duct of Wirsung) begins in the tail, runs through the body, the neck and the head, and joins the common bile duct (Figure 1.1). The two ducts then empty into the duodenal lumen. In some cases, an accessory pancreatic duct (duct of Santorini) could drain pancreatic enzymes into the duodenum (Bockman, 1993).

The innervation of the pancreas comprises both intrinsic and extrinsic components. The

intrinsic component is represented by the intrapancreatic ganglia, the aggregates of neural cell bodies randomly distributed throughout the pancreatic parenchyma. The extrinsic nerve fibers include efferent as well as afferent nerve fibers. The afferent sensory nerve fibers conduct sensory information (i.e. pain) from pancreas to the central nervous system. The efferent fibers belong to the sympathetic and parasympathetic systems. These nerve fibers are essential to regulate pancreatic secretory function and microcirculation. Sympathetic nerves inhibit pancreatic secretion, whereas parasympathetic nerves are stimulatory (Bockman, 1993; Salvioli et al., 2002).

The pancreas receives its blood supply from the celiac trunk and the superior mesenteric artery, the two large arterial trunks that arise from the aorta. The head of the pancreas is supplied by the superior mesenteric artery and the pancreaticoduodenal arteries (originating from superior mesenteric artery) whereas the neck, body, and the tail receive their blood supply from the branches of splenic artery (one of the major branches of celiac trunk) (Figure 1.1).

The venous drainage of the pancreas is carried out by the branches of superior mesenteric and splenic veins which drain into the portal vein (Bockman, 1993).

The pancreas has an extensive lymphatic drainage. The lymphatic vessels and nodes generally follow the major pancreatic arteries. The pancreaticosplenic nodes (situated along the splenic artery) mainly collect lymph from the body and tail of the pancreas. Other chains of lymph nodes, including pancreaticoduodenal, superior mesonteric and hepatic are responsible for drainage of the head and neck of the pancreas (Bockman, 1993; Cesmebasi et al., 2015).

The pancreas is both an exocrine and endocrine gland and has two main functions – digestion and blood sugar regulation. The exocrine pancreas comprises about 95% of the total pancreas mass. The exocrine pancreas makes and secretes pancreatic juice into the duodenum. The exocrine pancreas consists of acini (composed of acinar and centroacinar cells) connected to ducts (lined by ductal cells). The acinar cells synthesize, store, and secrete digestive enzymes into a network of small ducts (including intercalated, intralobular and interlobular ducts) that lead to the main pancreatic duct (Figure1.2). These enzymes include trypsinogen and chymotrypsinogen (inactive forms of trypsin and chymotrypsin, respectively) (for protein digestion), pancreatic lipase (for lipid digestion), pancreatic amylase (for carbohydrate digestion) and others. The centroacinar and duct cells secrete bicarbonate-rich fluid to neutralize acid gastric juice, thus ensuring neutral/alkaline environment for pancreatic enzymes. The secretion of exocrine pancreas is mainly regulated by hormones cholecystokinin and secretin released by duodenal endocrine cells. Cholecystokinin is released to the blood in

response to the presence of proteins and fats in the small intestine, and stimulates acinar cells to secrete digestive enzymes. Secretin, which is released in response to acid in duodenum, stimulates ductal cells to secrete bicarbonate-rich fluid (Pandol, 2010).

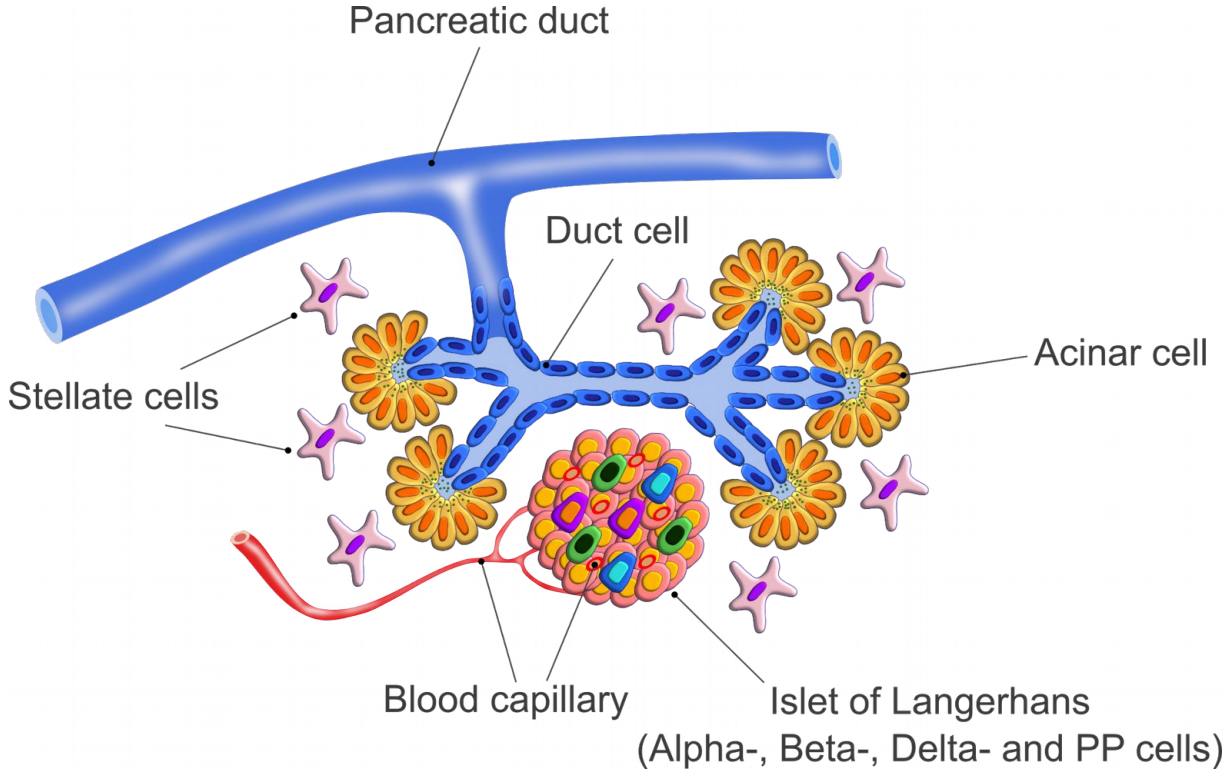

Figure 1.2. Cellular components of the pancreas.

The endocrine pancreas comprises about 2% of pancreatic mass. The endocrine pancreas makes and releases hormones into the blood stream. The endocrine pancreas is composed of small clusters of cells, called the islets of Langerhans (Figure 1.2). The islets of Langerhans are highly vascularized that ensures direct and easy release of hormones into the blood stream. Four major types of cells make up the islets of Langerhans. These are alpha-cells, beta-cells, delta-cells and PP-cells. The alpha-cells produce glucagon, a catabolic hormone which promotes gluconeogenesis and glycogenolysis and raises the concentration of glucose in the blood. The beta-cells produce insulin, an anabolic hormone which reduces the amount of glucose in the blood and increases the storage of glucose, fatty acids and amino acids in cells and tissues. The delta-cells produce somatostatin which inhibits both glucagon and insulin secretions as well as suppresses the exocrine secretions of the pancreas. The PP-cells produce pancreatic polypeptide. The function of pancreatic polypeptide is still uncertain although it has been shown to influence gastrointestinal function (Longnecker, 2014).

Along with the exocrine and endocrine cells some other cell types are present in the pancreas. Among them, pancreatic stellate cells (PSCs) predominantly located in the periacinar, periductal and perivascular spaces of the pancreas. PSCs comprise about 4-7% of pancreatic cells. In healthy pancreas PSCs are in their quiescent state with abundant vitamin A containing lipid droplets in their cytoplasm. It has been proposed that PSCs play a role in normal extracellular matrix (ECM) remodeling in the pancreas via the synthesis and secretion of ECM proteins (collagen, laminin and fibronectin), ECM degrading enzymes (matrix metalloproteinases 2, 9 and 13) and their inhibitors (tissue inhibitors of metalloproteinases 1 and 2) (Xu et al., 2014).

1.1.2 Pancreatic cancer

Pancreatic cancers are a group of diseases characterized by out-of-control growth and dissemination of abnormal pancreatic cells. Although pancreatic cancer is relatively rare (twelfth most common cancer in the world accounting for about 2% of all cancer cases), it is one of the leading causes of cancer-related death in men and women (Hariharan et al., 2008; Siegel et al., 2015). The current five-year relative survival rate is about 5-7% whereas most of patients die within the one year following cancer detection (Jemal et al., 2009; Siegel et al., 2015). One of the reasons of this is that early stage pancreatic cancer usually has no symptoms and thus the majority of cases are diagnosed at the late metastatic or invasive stages which are not suitable for surgery. Common late stage symptoms may include weight loss, jaundice, loss of appetite, abdominal and back pain, itching, nausea and others (Holly et al., 2004; Porta et al., 2005).

1.1.2.1 Risk factors

The causes of pancreatic cancer remain poorly understood, however, several factors have been reported to increase the risk. Among them advanced age, obesity, smoking, chronic pancreatitis, diabetes mellitus, and others. A number of studies have also linked family history to an increased risk of pancreatic cancer. Thus, about 5 to 10% of pancreatic cancer patients have a family history of this disease. Some studies have shown an increased incidence of pancreatic cancer among patients with genetic mutations in *BRCA2, CDKN2A, PRSS1* and some other genes (Hidalgo, 2010).

1.1.2.2 Pancreatic tumor types

Tumors that develop within the pancreas fall into two major categories: exocrine tumors and endocrine tumors. Endocrine (also known as neuroendocrine or “islet cell”) tumors are rare (account for less than 5% of all pancreatic tumors) and typically grow slowly compared to exocrine tumors. They develop from the abnormal growth of islet cells. These tumors could be either functional (produce and release hormones into the blood stream) or nonfunctional. Thus, according to hormones produced by the tumors the pancreatic endocrine tumors are classified to the following subtypes: insulinoma, gastrinoma, VIPoma, glucagonoma, somatostatinoma and nonfunctional tumors (Halfdanarson et al., 2008).

Exocrine tumors represent the most prevalent pancreatic neoplasms accounting for more than 95% of all pancreatic tumors. These tumors develop from exocrine cells. About 90% of cancers of the exocrine pancreas are adenocarcinomas that develop from ductal cells (pancreatic ductal adenocarcinoma, PDAC). Less common types of exocrine pancreatic cancers include acinar cell carcinoma, adenosquamous carcinoma, serous cystadenocarcinoma, pancreatoblastoma, solid pseudopapillary neoplasm, signet ring cell carcinoma, undifferentiated carcinoma, undifferentiated carcinoma with giant cells and others (Bosman et al., 2010; Hezel et al., 2006).

1.1.3 Pancreatic ductal adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer accounting for about 90% of all pancreatic malignancies. PDAC commonly arises in the head of the pancreas with infiltration into surrounding tissues including spleen, lymphatics, and peritoneal cavity, and with metastasis to the liver and lungs (Hezel et al., 2006). PDAC primarily exhibits a glandular pattern with duct-like structures. Unlike other cancers, the metastatic spread of PDAC begins when the primary tumor is approximately 10 mm in size (Chari, 2007).

1.1.3.1 Precursor lesions of PDAC

PDAC arises from a ductal cell lineage and evolves from precursor lesions to fully invasive cancer. The three precursor lesions mainly responsible for the development of PDAC include pancreatic intraepithelial neoplasms (PanINs), mucinous cystic neoplasms (MCN), and intraductal papillary mucinous neoplasms (IPMN) (Lennon and Wolfgang, 2013; Maitra et al.,

2005). PanINs are the most common and the best-characterized precursors to PDAC. PanINs represent microscopic flat or papillary lesions arising in the intralobular pancreatic ducts. PanINs are classified into three grades depending on the severity of cytological and architectural atypia (Figure 1.3). The lowest grade is characterized by the minimal cytological and architectural atypia and is subclassified into PanIN-1A (flat) and PanIN-1B (papillary). PanIN-2 lesions show moderate cytological and architectural atypia (including nuclear abnormalities) with frequent papillae. High-grade PanINs (PanIN-3) are characterized by severe cytological and architectural atypia and ultimately transform into invasive PDAC (Distler et al., 2014; Hidalgo, 2010).

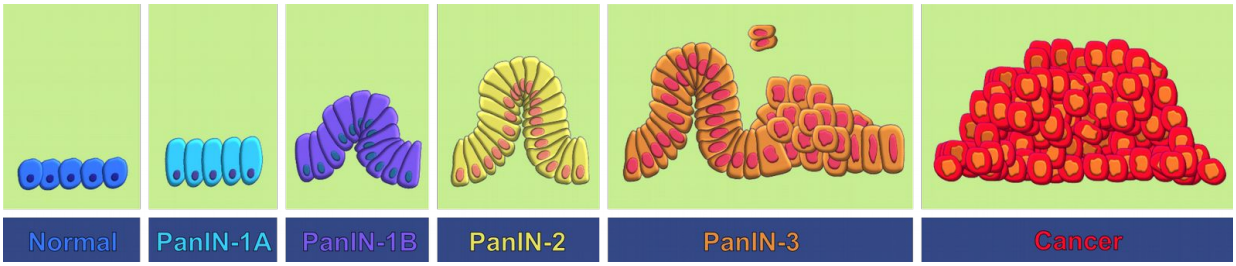

Figure 1.3. Pancreatic ductal adenocarcinoma progression.

1.1.3.2 Molecular genetics of PDAC

The histological progression from low-grade PanINs to PDAC is paralleled by the accumulation of genetic changes such as telomere shortening, gene mutations, oncogene activation, inhibition of tumor suppressor genes as well as alterations in gene expression (Hezel et al., 2006; Hidalgo, 2010).

Telomere shortening has been show to be one of the first alterations that can be found in displastic pancreatic epithelium. Telomeres are distinctive structures at the ends of chromosomes that function to protect chromosomes from breakage or aberrant fusion (van Heek et al., 2002). A critical shortening of telomere length in PanINs may predispose these noninvasive ductal lesions to accumulate progressive chromosomal abnormalities and to develop toward the stage of invasive carcinoma (van Heek et al., 2002). However, chromosomal instability could be counterproductive to tumor growth, which explains the fact that telomerase (a DNA polymerase that synthesizes telomeric DNA and prevents telomeres from shortening during cell division) is reactivated at late stages of tumor develoment to

elongate the telomeres of cancer cells (Koorstra et al., 2008).

Along with telomere shortening, PDAC progression is commonly accompanied by the inhibition of tumor suppressor genes. The p16INK4A/CDKN2A gene is one of the most frequently inactivated tumor suppressor gene in PDAC (Caldas et al., 1994; Kanda et al., 2012). Virtually all pancreatic carcinomas have loss of p16INK4A/CDKN2A function. The protein p16 belongs to the cyclin-dependent kinase (CDK) inhibitor family and functions to inhibit progression of the cell cycle through the G1/S transition. Therefore, inactivation of p16 results in inappropriate progression from G1 phase to S phase of cell cycle, thus contributing to PDAC progression (Koorstra et al., 2008).

Another tumor suppressor gene which is inactivated in 50-75% of PDAC cases is p53 gene. The p53 is the most commonly mutated gene in human cancers (Kandoth et al., 2013). Alterations have been found in virtually every region of the p53 protein (Leroy et al., 2013). The p53 protein plays a central role in modulating cellular responses to diverse cellular stress signals (such as DNA damage, hypoxia and oncogenes activation) and contribute to cell-cycle arrest, senescence and apoptosis and other processes (Bieging et al., 2014). Therefore, mutational loss of p53 function during carcinogenesis can lead to inappropriate cell growth, increased cell survival, and genetic instability. However, it is becoming clear that at least some of mutant p53 proteins give rise to a more aggressive tumor profile, indicating that they have acquired novel functions in promoting tumorigenesis (Muller and Vousden, 2014).

Loss of p21 (CDK inhibitor) activity has been observed in approximately 30–60% of pancreatic cancer cases (Garcea et al., 2005). p21 is a major player in cell cycle control. p21 is a downstream target of p53 activation and allows time for repairing damaged DNA via G1 arrest. Inhibition of p21 has been proposed to promote cell growth and tumorigenesis (Gartel and Radhakrishnan, 2005). However, most studies thus far, have not found a convincing relationship between p21 and survival from pancreatic cancer (Garcea et al., 2005).

SMAD4/DPC4 represents another tumor suppressor gene frequently inactivated in PDAC (in about 55% of the cases) (Hahn et al., 1996). The transcription factor SMAD4/DPC4 is an important regulator of the transforming growth factor-β (TGF-β) signaling pathway and its loss is thought to facilitate the progression of PDAC (Biankin, 2002; Massagué et al., 2000). SMAD4/DPC4 controls the expression of several genes related to proliferation or differentiation and loss/inactivation of SMAD4/DPC4 results in loss of proapoptotic signaling, inappropriate G1/S transition and decreased growth inhibition (Bardeesy and DePinho, 2002; Massagué et al., 2000). Preoperative assessment of SMAD4/DPC4 expression has been

proposed as a potential prognostic indicator in patients with PDAC (Biankin, 2002).

Multiple other tumor suppressor genes were observed to be mutated/inactivated/deleted in PDAC but at lower frequency. These include LKB1/STK11, BRCA1, BRCA2, p27, BCLAF1, CHEK2 and others (Witkiewicz et al., 2015).

In contrast to tumor suppressor genes, a number of oncogenes are activated/overexpressed during pancreatic cancer development. Among them V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) represents the most frequently mutated oncogene in PDAC (about 92%) (Witkiewicz et al., 2015). KRAS is a member of the RAS family of GTP-binding proteins that mediate a wide variety of cellular functions including proliferation, differentiation, and survival (Hezel et al., 2006). KRAS mutations are one of the earliest genetic alterations in the progression model for pancreatic cancer. 98% of KRAS point mutations in PDAC occur at codon 12. In PDAC, KRAS activating mutation is an early and initiating event as it has been shown that over 90% of low-grade PanINs harbor oncogenic KRAS mutations (Kanda et al., 2012). Mutation of KRAS results in a constitutive gain of function, which leads to proliferation, suppressed apoptosis and cell survival (Koorstra et al., 2008). KRAS mutation is thought to be necessary, but not sufficient, to drive PanINs to PDAC.

A small subset of PDAC cases harbor wild-type KRAS. In these cases cancer cells exhibit mutations in other known oncogenes, including BRAF and PIK3CA. The BRAF gene encodes a serine/threonine kinase implicated in the regulation of ERK MAP kinase signalling cascade and controlling cell proliferation, differentiation, migration and survival. BRAF is a frequent mutational target in several cancer types including melanoma (66%) and colorectal carcinomas (10%). KRAS and BRAF mutations are mutually exclusive (Holderfield et al., 2014; Witkiewicz et al., 2015).

Other oncogenes amplified in PDAC include AKT2, AIB1, MYC, MYB, CCND1 and others (Koorstra et al., 2008; Witkiewicz et al., 2015).

Various growth factors and their receptors have been also reported to be overexpressed in PDAC. Among them epidermal growth factor (EGF) and EGF receptors, insulin-like growth factor I (IGF-I) and IGF-I receptor, fibroblast growth factor (FGF) and FGF receptors, and vascular endothelial growth factor (VEGF) and its receptors. Alterations in growth-controlling genes together with abundance of growth-promoting factors give cancer cells a distinct growth advantage, which clinically results in rapid tumor progression and poor survival prognosis (Ozawa et al., 2001).

1.1.3.3 Core signaling pathways deregulated in PDAC

A recent study reported that PDACs contain an average of 63 genetic alterations, the majority of which are point mutations. These alterations affect a number of cellular signaling pathways and processes (Jones et al., 2008). Activated KRAS engages multiple signaling pathways, including RAS/mitogen- activated kinase (MAPK) pathway, phosphoinositide-3-kinase (PI3K) pathway and RalGDS pathway. The RAS/MAPK pathway plays an important role in the regulation of cell cycle, growth, differentiation, migration, apoptosis and senescence. This pathway is triggered by growth factors or activating mutations of oncogenic kinases (Neuzillet et al., 2014). Ras GTPases act as molecular switches that control the activity of many signaling pathways. Activated Ras oncogenes bind and activate their effectors including Raf kinases. Activated Raf phosphorylates and activates MEK1 and MEK2 kinases which in turn activate ERK MAP kinases. Active ERKs phosphorylate numerous cytoplasmic and nuclear targets, including kinases, phosphatases, transcription factors and cytoskeletal proteins, and in this way regulate numerous processes such as proliferation, differentiation, survival, migration, angiogenesis and chromatin remodeling (Dhillon et al., 2007; Yoon and Seger, 2006). Pancreatic cancer is characterized by constitutive activation of the MAPK pathway due to activating mutations of KRAS and BRAF genes. It has been reported that pharmacologic inhibition of RAS/MAPK signaling elicits pancreatic cancer cell cycle arrest through induced expression of p27Kip1 (Gysin et al., 2005). Therefore, the development of drugs targeting genes downstream of MAPK may provide a novel therapeutic option for pancreatic cancer (Furukawa, 2015).

The phosphatidylinositol 3-kinase (PI3K) represents another pathway which can be activated by RAS (Vivanco and Sawyers, 2002). PI3K pathway regulates cell survival, proliferation, growth, cell cycle and other important cellular processes (Cantley, 2002). PI3Ks are major effectors downstream of receptor tyrosine kinases and G protein coupled receptors (GPCRs). PI3Ks transduce signals from various growth factors and cytokines into intracellular messages by generating phospholipids, which in turn activate the serine/threonine kinase AKT and other downstream effector pathways (Liu et al., 2009). Once activated, AKT kinase, also known as protein kinase B (PKB), phosphorylates or indirectly activates many other proteins, including mTOR (mammalian target of rapamycin, also known as FRAP1), GSK3 (glycogen synthase kinase 3), FOXOs (the forkhead family of transcription factors), and others, thereby regulating numerous cellular processes involved in protein synthesis, cell survival, proliferation, and

metabolism (Manning and Cantley, 2007). The tumor suppressor PTEN (phosphatase and tensin homolog deleted from chromosome 10) is the most important negative regulator of the PI3K signaling pathway (Cully et al., 2006).

Recent comprehensive cancer genomic analyses have revealed that multiple components of the PI3K pathway are frequently mutated or altered in common human cancers, underscoring the importance of this pathway in cancer (Liu et al., 2009). In PDAC PI3K pathway has been reported to be constitutively activated, however the mutation in this pathway that are common for other cancer types are not commonly observed in PDAC. For example, PTEN is mutated or deleted in many tumors but not in PDAC. Nevertheless, the aberrant expression of PTEN in PDAC has been reported to contribute to the activation of the PI3K pathway and its downstream mediators, thereby giving pancreatic cancer cells an additional growth advantage (Asano et al., 2004; Ebert et al., 2002). Moreover, the amplification and overexpression of the AKT2 kinase has been previously demonstrated in a subset of human pancreatic carcinomas, thus supporting the importance of this pathway in PDAC (Altomare et al., 2002).

The nuclear factor kB (NF-κB) represents another important downstream mediator of mutated KRAS signaling in PDAC. NF-κB is a transcriptional factor that controls and regulates expression of many genes involved in inflammation, proliferation and apoptosis. NF-kB is constitutively activated in a number of solid tumors, including pancreatic cancer, and its activation is thought to contribute to cancer cell survival, angiogenesis, and invasion (Carbone and Melisi, 2012). NF-κB has been also reported to contribute to chemoresistance of PDAC through the suppression of proapoptotic signaling pathways (Baldwin, 2001; Godwin et al., 2013).

The Notch signaling pathway is another pathway which has been shown to contribute to the development of PDAC. The Notch signaling pathway is a highly evolutionarily conserved pathway that mediates cell-to-cell communication and regulates cell differentiation, proliferation and apoptosis during embryonic development. The Notch pathway comprises Notch receptors (Notch1-4) and Notch ligands as well as intracellular proteins that function to transmit the Notch signal to the cell's nucleus (Bi and Kuang, 2015). Interactions between receptor and ligand induce proteolysis of the Notch receptor and subsequent nuclear translocation of the Notch intracellular domain, which mediates the transcriptional activation of a series of target genes (Hezel et al., 2006; Miele et al., 2006). Notch signaling is frequently deregulated in human cancers with up-regulated expression of Notch receptors and their ligands in many cancer types including pancreatic cancer (Wang et al., 2006). Notch receptors have

been identified as oncogenes in multiple tumors, including leukaemia, breast, colorectal, cervical, lung, and oral squamous cell carcinoma. In contrast, an antiproliferative role of Notch has been reported in human hepatocellular carcinoma and small cell lung cancer (Ranganathan et al., 2011). In pancreatic cancer both oncogenic and tumor suppressive roles for Notch signaling have been demonstrated (Avila and Kissil, 2013). Overall, additional studies are needed to clarify the role of the various Notch receptors in PDAC development.

The Hedgehog (Hh) pathway is another signaling pathway important in PDAC progression. Hh pathway is involved in the development of many organs and tissues in numerous animal species (Evangelista et al., 2006; Scott, 2003). The Hh pathway comprises the secreted Hh protein, the cell-surface receptor (Patched, PTC) and an intracellular protein (Smoothened, SMO) that activates genes in response to the Hh signal. In the absence of Hh signal, PTC inhibits the activity of SMO. Hh signal disrupts the inhibition of SMO by PTC, thus enabling the signal transduction to transcriptional regulators and activation of growth promoting genes (Ingham and McMahon, 2001; Scott, 2003). Loss of PTC, activating mutations in SMO or overexpression of Hh proteins have been associated with a number of cancers. Activation of the Hedgehog pathway has been implicated in both the initiation of PanINs and in the maintenance of advanced cancers. It has been shown, that a secreted Hh protein is abnormally expressed in PDAC and its precursor lesions, thus activating Hh pathway (Evangelista et al., 2006; Thayer et al., 2003). Moreover, it has been reported that blockade of Hh signaling inhibits pancreatic cancer invasion and metastases suggesting pharmacologic Hh pathway inhibition as a potential therapeutic strategy for pancreatic cancer (Feldmann et al., 2007).

1.1.4 Pancreatic cancer staging

The prognosis of pancreatic cancer is mainly determined by the stage of disease at diagnosis. The most widely used staging system for pancreatic cancer is the American Joint Committee on Cancer (AJCC) TNM system. The TNM system (T for tumor, N for node, and M for metastasis) is an anatomically based system that describes the primary tumor, regional nodal extent of the tumor and the absence or presence of metastases. A number is added to each letter to indicate the size and/or extent of the primary tumor and the degree of cancer spread.

The TNM classification for pancreatic cancer is provided below (Greene et al., 2013).

Stage	T	N	M	Description
0	Tis	N0	M0	Carcinoma in situ. Also includes PanINs-3. A tumor has not yet invaded outside the duct in which it started.
I-A	T1	N0	M0	A tumor is limited to the pancreas and is 2cm or less in greatest dimension. No spread to regional lymph nodes or distant sites.
I-B	T2	N0	M0	A tumor is limited to the pancreas and is more than 2cm in greatest dimension. No metastasis.
II-A	T3	N0	M0	A tumor extends beyond the pancreas, without spread to nearby arteries or veins. No metastasis.
II-B	T1	N1	M0	A tumor is either limited to the pancreas or extends beyond the pancreas, without spread to nearby arteries or veins. Regional lymph node metastasis are present. No distant metastasis.
	T2	N1	M0	
	T3	N1	M0	
III	T4	Any N	M0	Unresectable primary tumor. A tumor has spread to nearby arteries, veins. It may or may not have spread to regional lymph nodes. No distant metastasis.
IV	Any T	Any N	M1	A tumor has spread to distant sites.

Along with TNM classification, a simpler staging system could be utilized. This system divides tumors based on whether or not they can be resected with surgery. Thus, all pancreatic tumors can be divided to: resectable, borderline resectable, and unresectable (locally advanced or metastatic) (Garrido-Laguna and Hidalgo, 2015).

1.1.5 Pancreatic cancer treatment options

The stage of a pancreatic cancer is the most important factor in choosing treatment options and predicting a patient's outlook. Other factors that determine the type of treatment include a type of pancreatic cancer and its localization, age and general health of a patient.

Several treatment options for pancreatic cancer exist to date. These are surgery, radiation therapy, chemotherapy, chemoradiation therapy, targeted therapy, immune therapy, palliative therapy and personalized therapy.

Surgery remains the only treatment that offers a chance of cure for patients with resectable

tumors (Shaib et al., 2007). Depending on the tumor location, the operative approaches may involve cephalic pancreatoduodenectomy (the Whipple procedure), distal pancreatectomy, or total pancreatectomy. Along with complex open surgeries, pancreatic cancer is also being increasingly approached laparoscopically. Sometimes, palliative surgery (or bypass surgery) is performed in order to relieve or prevent symptoms. An emerging strategy in patients with resectable pancreatic cancer is the use of preoperative (neoadjuvant) treatment, which may decrease the rate of local failures and positive resection margins after surgery (Garrido-Laguna and Hidalgo, 2015; Hidalgo, 2010). For patients with surgically resected ductal adenocarcinoma of the pancreatic head, the 5-year overall survival is about 20–25% (Vincent et al., 2011).

Unfortunately, only 10-20% of patients are considered as candidates for surgical treatment, because the majority of cases are diagnosed at the late metastatic or invasive stages which are not suitable for surgery. Thus, in most of the cases other therapeutic approaches are implemented.

Radiation therapy is the use of high-energy radiation to destroy cancer cells. Radiotherapy is not very often used to treat pancreatic cancer because it damage healthy cells and cause severe side effects. External beam radiation therapy is the most commonly used type of anticancer radiation therapy, where radiation is delivered from a machine outside the body. Some other ways to deliver radiation to pancreatic tumors that are less common include intraoperative radiation therapy (when a concentrated dose of radiation therapy is delivered to a tumor during surgery) and proton beam radiation (a special type of radiation that is less damaging to normal cells) (Hajj and Goodman, 2015; Roeder et al., 2012). Usually, radiotherapy is used in combination with chemotherapy (chemoradiation therapy) to treat patients with unresectable advanced pancreatic cancer or to relieve painful symptoms of cancer. Radiation therapy can be also used as neoadjuvant/adjuvant therapy before/after surgical resection. Radiosensitizing agents such as 5-fluorouracil, capesitabine and gemcitabine are frequently utilized to increase the efficacy of radiotherapy (Ben-Josef and Lawrence, 2012; Vincent et al., 2011).

Another treatment option for pancreatic cancer is targeted therapy. Targeted therapy attacks unique aspects of cancer cells, thus causing little harm to healthy cells. It includes therapies targeting against different kinases, EGFR, RAS/MAPK pathway, IGFR, PI3K pathway, DNA repair genes, histone deacetylases, microRNA, stroma and others. A number of ongoing clinical trials address the efficacy of targeted therapies in combination with radiochemotherapies against pancreatic cancer (Seicean et al., 2015).

Immune therapies might represent an alternative treatment modality for PDAC with

significantly less toxicity (Gunturu et al., 2013). Immune therapies attempt to boost a person's immune system to attack cancer cells. Immune therapies could include vaccine therapies (which stimulate the body's natural ability to protect itself), tumor antigen-specific monoclonal antibodies-based therapies, and targeting immune system checkpoints (Gunturu et al., 2013; Schnurr et al., 2015; Wang et al., 2015). The use of immunotherapy for PDAC treatment is promising, though its immunotolerant environment continues to be a major obstacle. Combinations immunologic treatments conventional chemoradiotherapies represent promising strategies for increasing efficacy of pancreatic cancer treatment (Seicean et al., 2015).

The genetic landscape of PDAC is extremely heterogeneous. Recent advances in human genomics and genetics provided the basis for the development of personalized therapy of PDAC (Fang et al., 2013; Yee, 2013). The personalized therapy aims to improve outcomes for patients with PDAC by using molecular analysis of tumor tissue for prediction of therapeutic responses and selection of treatment options. Thus, many attempts has been already made to incorporate tumor profiling into the treatment of many cancers including PDAC. Specific gene profiles in blood, pancreas tissue, and pancreas juice can potentially be used as new biomarkers for diagnosis, prognosis, and to assess the response to treatment (Chantrill et al., 2015; Sjoquist et al., 2014).

Given that the majority of patients with pancreatic cancer are not resectable for cure at the time of diagnosis, the palliative therapy is of primary importance. Palliative therapy is intended to relieve suffering and improve quality of patient's life. Palliative therapy could include surgery or nonoperative approaches, such as radiotherapy, pharmacological pain-control treatments, neurolytic celiac plexus blockage, emotional and spiritual care (Habermehl et al., 2014; House and Choti, 2005; Lillemoe, 1998).

1.1.5.1 Chemotherapy

Chemotherapy is the main treatment for metastatic unresectable pancreatic tumors. Chemotherapy could be used at any state of pancreatic cancer: as a neoadjuvant (before surgery), as a adjuvant (following surgery), and at advanced stages when surgery is not an option anymore. Chemotherapy could be given alone or along with radiation therapy (chemoradiotherapy). A number of chemotherapeutic drugs, including Gemcitabine (Gemzar®), 5-fluorouracil (5-FU), Irinotecan (Camptosar®), Oxaliplatin (Eloxatin®), Capecitabine (Xeloda®), Cisplatin, Paclitaxel (Taxol®), Docetaxel (Taxotere®), FOLFIRINOX

(combination of 5-FU, irinotecan, folinic acid and oxaliplatin), and albumin-bound paclitaxel (Abraxane®) has been tried against pancreatic cancer (Garrido-Laguna and Hidalgo, 2015). Since the 1990s, gemcitabine has been considered the standard treatment of choice. In recent years multiple different agents have been evaluated in combination with gemcitabine or alone, however few of them have demonstrated positive impact on survival in patients with advanced disease (Mohammed et al., 2014). In patients with advanced-stage pancreatic cancer, modest improvements in survival have recently been attained with FOLFIRINOX or albumin-bound paclitaxel (nab-paclitaxel) plus gemcitabine chemotherapy (Conroy et al., 2011; Von Hoff et al., 2013). However, despite the fact that FOLFIRINOX has been promising, studies report its significant toxicity constraining its utility in patients of older age and with poor performance status (Mohammed et al., 2014).

Chemotherapeutic agents can be classified into cell-cycle-phase-specific (agents with major activity in a particular phase of cell cycle) or cell-cycle-phase-nonspecific. Moreover, according to their mechanism of action, chemotherapy drugs could be classified into alkylating agents, antimetabolites, antitumor antibiotics, taxanes, topoisomerase inhibitors, spindle poisons, platinum complexes and others (Malhotra and Perry). Gemcitabine and 5FU, the two most widely used chemotherapeutic drugs in PDAC treatment, belong to antimetabolites group. Antimetabolites exert their cytotoxic activity either by competing with normal metabolites for the catalytic or regulatory site of a key enzyme or by substituting for a metabolite that is normally incorporated into DNA and RNA. Thus, antimetabolites interfere with DNA and RNA synthesis, and are most effective during S phase of cell cycle. Folinic acid is often used in cancer chemotherapy in combination with 5-FU to enhance it's effect by inhibiting thymidylate synthase (Neoptolemos et al., 2010).

Irinotecan (Camptosar®) belongs to topoisomerase inhibitors group. Topoisomerase inhibitors affect the activity of topoisomerases (enzymes involved in DNA replication and transcription) and thus prevent DNA replication (Yi et al., 2009).

Platinum complexes (including Cisplatin and Oxaliplatin (Eloxatin®)) have been reported to form both inter- and intra-strand cross links in DNA, which prevent DNA replication and transcription, thus causing cell death (Saif and Kim, 2007).

The taxanes (Paclitaxel (Taxol®), Docetaxel (Taxotere®)) promote microtubular assembly and stability, therefore blocking the cell cycle in the M phase (Belli et al., 2012).

To increase the treatment efficacy chamotherapeutic drugs are often administered in combinations to simultaneously target cancer cells at different phases of cell cycle. For

example, FOLFIRINOX which is the combination of 5-FU, irinotecan, folinic acid and oxaliplatin showed encouraging results. However, combining several drugs usually results in increased toxicity limiting the clinical application of this approach (Papadatos-Pastos et al., 2014).

1.1.6 Pancreatic cancer drug resistance

Over the past decades many efforts have been made to improve the clinical efficacy of chemotherapy. Different chemotherapeutic drugs and their combinations have been tested in PDAC treatment. However, up to date, gemcitabine remains the standard treatment for pancreatic cancer patients. None of the combinational trials with gemcitabine have proven to be significantly more effective than gemcitabine alone as the first-line therapy (Gresham et al., 2014; Mohammed et al., 2014).

Drug resistance (intrinsic and acquired) has been proposed to be one of the main reasons of ineffective treatment of pancreatic cancer. Various mechanisms of drug resistance in pancreatic cancer have been described, including gene mutations and aberrant gene expression, impaired drug delivery, deregulation of many signaling pathways (both prosurvival and proapoptotic), the influence of the tumor microenvironment (presence of stroma cells), epithelial-mesenchymal transistion (EMT) and the presence of highly resistant stem cells (Long et al., 2011).

Multidrug resistance (MDR) is the principal mechanism by which many cancers develop resistance to chemotherapy drugs. MDR has been correlated to the presence of special pumps in tumor-cell membranes that actively expel chemotherapy drugs from the interior, thus preventing drug accumulation (Gillet and Gottesman, 2010). The members of ATP-binding cassette (ABC) transporters superfamily are recognized to play essential role in MDR development in cancer cells. ABC transporters mediate transport of different substrates across plasma membrane (Gottesman et al., 2002). Different members of ABC transporters family (including ABCC1, C3, C4, C5, P-glycoprotein and others) have been shown to be implicated in pancreatic cancer resistance to chemotherapy including gemcitabine and 5-FU (König et al., 2005; Nambaru et al., 2011).

In contrast to ABC transporters which exclude drugs from the cell without altering drug uptake, aberrant expression of some transporters can affect uptake of a chemotherapeutic drug. For example, human equilibrative nucleoside transporter-1 (hENT1) mediate gemcitabine uptake by

the cells. Thus decreased hENT1 expression could favor gemcitabine-resistance through its limited uptake (Mackey et al., 1998; Nordh et al., 2014). Another rtransporter - copper transporter 1 (hCTR1), has been shown to regulate cisplatin resistance by controlling its uptake (Holzer et al., 2006).

In addition to aberrant expression of different transporters, deregulated signaling pathways (including Notch, PI3K/AKT, NF-kB and others) have been also connected to pancreatic cancer drug resistance. The nuclear factor κB (NF-κB) has been shown to be constitutively activated in pancreatic cancer (in contrast to normal pancreatic cells), and its activation is thought to contribute to cancer cell survival, angiogenesis, and invasion (Carbone and Melisi, 2012). NF-κB has been also reported to contribute to chemoresistance of PDAC through up-regulation of BCL-xL, X-linked inhibitor of apoptosis protein (XIAP), inhibitor of apoptosis protein homolog c (cIAP), survivin and other downstream target genes (Bharti and Aggarwal, 2002). Another constitutively activated pathway in PDAC is prosurvival PI3K/AKT pathway. Several papers have proposed PI3K/AKT pathway to be one of the mediators of chemoresistance to gemcitabine in pancreatic cancer. Inhibition of PI3K/AKT pathway has been shown to enhance gemcitabine-induced apoptosis in human pancreatic cancer cells as well as promote gemcitabine antitumor activity in orthotopic human pancreatic cancer xenografts (Kim and Gallick, 2008; Ng SSW et al., 2000; Ng et al., 2001). Along with PI3K and AKT several other kinases (including integrin-linked kinase, focal adhesion kinase, c-Src kinase and others) have been implicated in pancreatic cancer cells resistance to gemcitabine (Kim and Gallick, 2008).

Epithelial-mesenchymal transition (EMT) (a process by which epithelial cells become mesenchymal stem cells with fibroblast-like properties) has been also implicated in drug resistance (Arumugam et al., 2009). Following EMT pancreatic cancer cells usually become gemcitabine resistant. In different cancers EMT has been reported to be tightly connected with other drug-resistance mechanisms such as signaling pathway deregulation, aberrant gene expression, hypoxia and others. Several signaling pathways, including TGF-b, Hedgehog, NF-kB and Notch were shown to control EMT (Arumugam et al., 2009; Biddle and Mackenzie, 2012). For example, inhibition of Notch pathway resulted in reversal of EMT and has been suggested as a potential therapy for overcoming drug resistance in pancreatic cancer (Wang et al., 2009). It has been also shown that expression of zinc finger E-box binding homeobox 1 (ZEB1 – transcriptional factor required for EMT) is strongly increased in pancreatic ductal adenocarcinoma, while there is no ZEB1 in normal pancreatic tissue (Grosse-Steffen et al., 2012). Thus, targeting EMT-related pathways is a promising strategy against pancreatic cancer.

Pancreatic cancer stem cells (CSCs) have been also associated with chemotherapy resistance and high recurrence rate of pancreatic cancer (Zhu and Yuan, 2015). CSCs are believed to be responsible for tumor initiation, progression and metastasis. These cells could be identified by the expression of special markers, like CD24, CD44, CD133, CXCR4, ESA an others. Cells expressing these markers have been shown to be highly tumorigenic (Rao and Mohammed, 2015). Moreover, increased expression of stem cell markers have been detected in gemcitabine-resistant pancreatic cancer cells (Shah et al., 2007).

Along with above mentioned drug resistance mechanisms, pancreatic tumor stroma has been shown to essentially contribute to the chemotherapy resistance of pancreatic cancer (Xu et al., 2014). One of the characteristic features of PDAC is the presence of abundant desmoplastic stroma in which cancer cells are embedded. This stromal reaction is comprised of abundant extracellular matrix (composed of type I collagen, fibronectin, laminin as well as proteoglycans), stromal cells, blood vessels/endothelial cells, immune cells, nerves/neurons and other soluble proteins, e.g., cytokines, growth factors (Wilson et al., 2014). The present of extensive stroma results in exclusive microenvironment where cross-talk between stromal cells and cancer cells promotes local tumor progression, metastasis and chemoresistance (Long et al., 2011; McCarroll et al., 2014). Moreover, the desmoplastic stroma significantly affects the penetration and delivery of chemotherapeutic drugs to cancer cells. Indeed, depletion of stroma (through Hedgehog pathway inhibition) enhanced delivery of chemotherapy in a mouse model of pancreatic cancer (Olive et al., 2009). There is now unequivocal evidence that fibrosis of the pancreas is produced by pancreatic stellate cells (PSCs) (McCarroll et al., 2014).

1.1.7 Pancreatic stellate cells

Pancreatic stellate cells (PSC) is a cellular component of the pancreas whose function under normal conditions is not entirely clear. PSCs comprise about 4%-7% of total parenchymal cells in the gland. It has been proposed that PSCs play a role in normal extracellular matrix remodelling in the pancreas via the synthesis and secretion of matrix degrading enzymes and their inhibitors. In health, the cells are in their quiescent phase, with abundant vitamin A containing lipid droplets in their cytoplasm. During pancreatic injury, the cells are activated by several factors such as cytokines, growth factors and oxidant stress. Activated PSCs lose their cytoplasmic lipid droplets, transform into a myofibroblast-like phenotype, proliferate and synthesise excessive amounts of extracellular matrix proteins that comprise fibrous tissue (Apte

et al., 2012; McCarroll et al., 2014; Wilson et al., 2014). Activated PSCs become the most important drivers of desmoplasia (the growth of fibrous or connective tissue) and thereby contribute to therapy refractoriness of pancreatic cancer (Xu et al., 2014). PSCs become activated by and remain under the influence of the tumour microenvironment, of which hypoxia and paracrine growth factor signalling are important elements. PSC activation results among others in proliferation, migration, resistance to apoptosis (which accounts for radio- and chemo-resistance) and cross-communication with the cancer cells (Apte et al., 2012).

Recently the prominent desmoplastic reaction, characteristic of pancreatic cancer, has attracted attention of scientists as a potential anticancer therapeutic target (Xu et al., 2014). Pancreatic stellate cells (PSCs) have been identified as essential regulators of pancreatic cancer development and resistance to chemotherapy (Apte et al., 2012). Accordingly, new therapeutic concepts are being developed that are directed against the PDAC stroma/PSCs (Figure 1.4). Several preclinical studies have reported encouraging results with combinatorial treatments targeting both the stroma and the cancer cells (Apte et al., 2013). Thus, it's clear that controlling activation of PSCs in cancer could represent a very beneficial strategy to overcome chemotherapy resistance of pancreatic cancer.

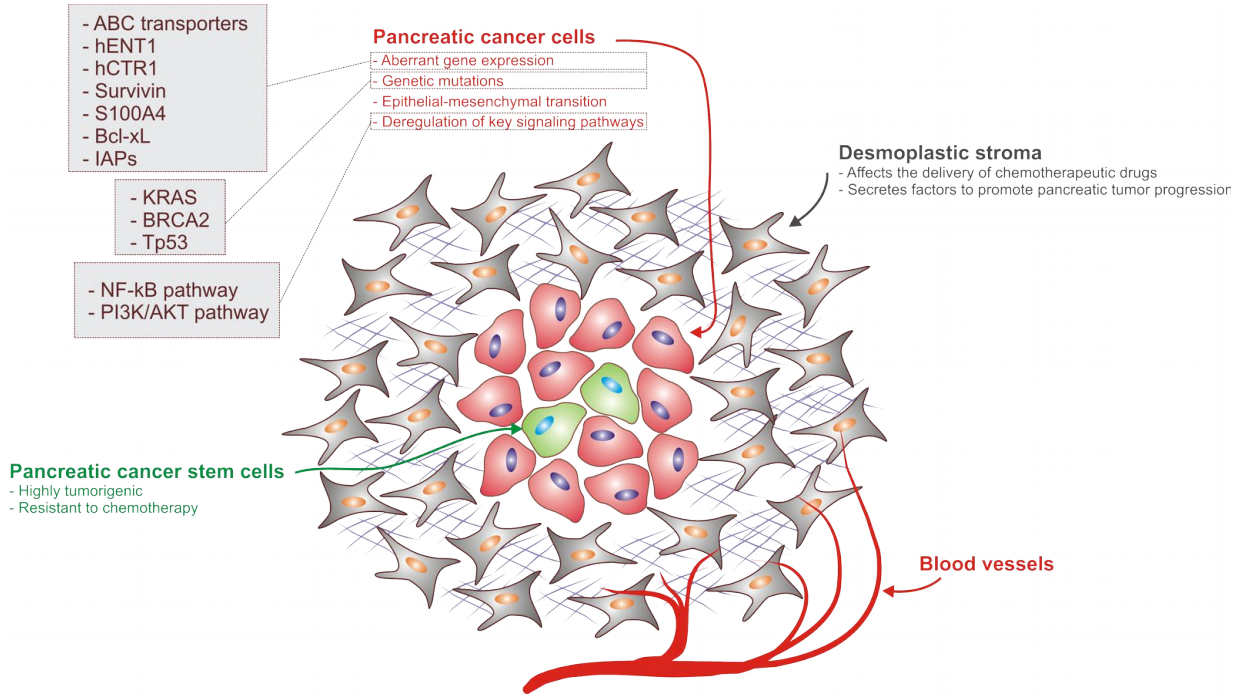

Figure 1.4. Pancreatic cancer drug resistance mechanisms.

It is now increasingly clear that targeting tumor cells alone is insufficient to improve pancreatic cancer clinical outcome. The successful treatment should target both tumor cells and tumor

microenvironment.

Therefore, identification of the molecular nature as well as functions of ion channels and in particular calcium-permeable channels in PDAC cells and PSCs is of great importance as it can reveal novel approaches for treating pancreatic cancer through targeting calcium-dependent processes.

1.1.8 Specific PDAC hallmarks

As it is mentioned above, a number of physiological processes are altered in pancreatic cancer cells. These include proliferation, growth, cell death, migration and others. Interestingly, Hanahan and Weinberg have proposed several general hallmarks, characteristic to most forms of cancer, including pancreatic cancer. These hallmarks include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, avoiding immune destruction, deregulating cellular energetics, tumor-promoting inflammation as well as genome instability and mutation (Hanahan and Weinberg, 2000, 2011). At the same time, different types of cancer differ from each other, so it's almost impossible to extrapolate the mechanisms existing in a given cancer type to another one.

Thus, there are several hallmarks that distinguish pancreatic cancer from other common cancer types. At first, pancreatic cancer is one of the deadliest cancer types with incidence almost equaling mortality. Further, unlike many other cancers, the metastatic spread of pancreatic cancer is thought to begin very early in its development (when the primary tumor is about 10 mm in size), when results of diagnosis are often negative (Chari, 2007).

Perhaps, one of the most characteristic hallmarks of pancreatic cancer is the presence of extensive and dense desmoplastic/fibrotic stroma in which cancer cells are embedded (Xu et al., 2014). Apparently, the interaction between the stroma and pancreatic cancer cells supports tumor growth, invasion and metastasis. Moreover abundant stroma creates the barrier for delivery of chemotherapy drugs. Therefore, the successful treatment should target both tumor cells and tumor microenvironment.

Thus, it's clear that with all the similarities and general hallmarks, pancreatic cancer stands apart from other cancer types with its specific hallmarks, highlighting the need in focused experimental research on pancreatic cancer.

1.2 Calcium signalling

1.2.1 Calcium signalling and calcium signalling toolkit

Changes in the cytosolic free Ca^{2+} concentration play a central role in many fundamental cellular processes including muscle contraction, transmitter release, cell proliferation, differentiation, gene transcription and cell death (Berridge et al., 2000). Given that Ca^{2+} controls so many vital processes, disturbance of the Ca^{2+} homeostasis regulatory mechanisms leads to a vast variety of severe pathologies, including cancer. Indeed, the role of Ca^{2+} is well-established in many cell signaling pathways involved in carcinogenesis (Monteith et al., 2007, 2012; Prevarskaya et al., 2011).

Increases of cytosolic Ca^{2+} concentration organized in space, time and amplitude have long been known to control different cellular processes. Rapid and highly localised Ca^{2+} signals regulate fast physiological responses such as secretion and contraction, whereas global Ca^{2+} transient or intracellular Ca^{2+} waves control slower responses such as mitosis and fertilization. For instance, cytosolic Ca^{2+} oscillations stimulate cell proliferation via activation of the Ca^{2+}-dependent transcription factor, NFAT (Nuclear Factor of Activated T cells), and a sustained elevation in cytosolic Ca^{2+} concentration induces apoptosis of cancer cells (Flourakis and Prevarskaya, 2009).

Increase in cytosolic calcium can occur as a result of Ca^{2+} influx from the extracellular space across the plasma membrane and Ca^{2+} release from intracellular sources. Both Ca^{2+} influx and Ca^{2+} release are tightly controlled by numerous regulatory systems that provide the specific spatial and temporal characteristics of an intracellular calcium signal that are required for sustaining certain cellular functions (Berridge et al., 2000). All these regulatory systems could be united into a comprehensive "**calcium signalling toolkit**" containing many components and ensuring tight regulation of all intracellular calcium signals including calcium mobilizing signals, calcium entry, calcium binding, and calcium efflux. Thus, **calcium mobilizing signals** are triggered following activation of cell-surface receptors (G-protein-coupled receptors (GPCRs), Tyrosine kinase-linked receptors (RTKs)) and include such Ca^{2+}-mobilizing second messengers as inositol 1,4,5-trisphosphate (IP3), nicotinic acid dinucleotide phosphate (NAADP), sphingosine 1-phosphate (S1P) and others. Calcium entry to the cell depends on **calcium-permeable ion channels** located in the plasma membrane or in the internal stores (i.e. ER-residents IP3 and ryanodine receptors). Following calcium elevation in the cytosol, this

signal acts through a variety of **Ca^{2+}-binding proteins** (Ca^{2+} buffers and Ca^{2+} sensors) and triggers various **Ca^{2+}-sensitive processes**. Once Ca^{2+} has finished its signalling functions, it is removed from the cytosol by numerous **exchangers** and **pumps**, including plasma membrane Na^{+}/Ca^{2+} exchangers (NCX) and Ca^{2+}-ATPase pumps (PMCA) as well as sarco-endoplasmic reticulum ATPase (SERCA) pumps (Figure 1.5). Alternatively, calcium can be sequestered by mitochondria through mitochondrial calcium uniporter (MCU) (Berridge et al., 2000).

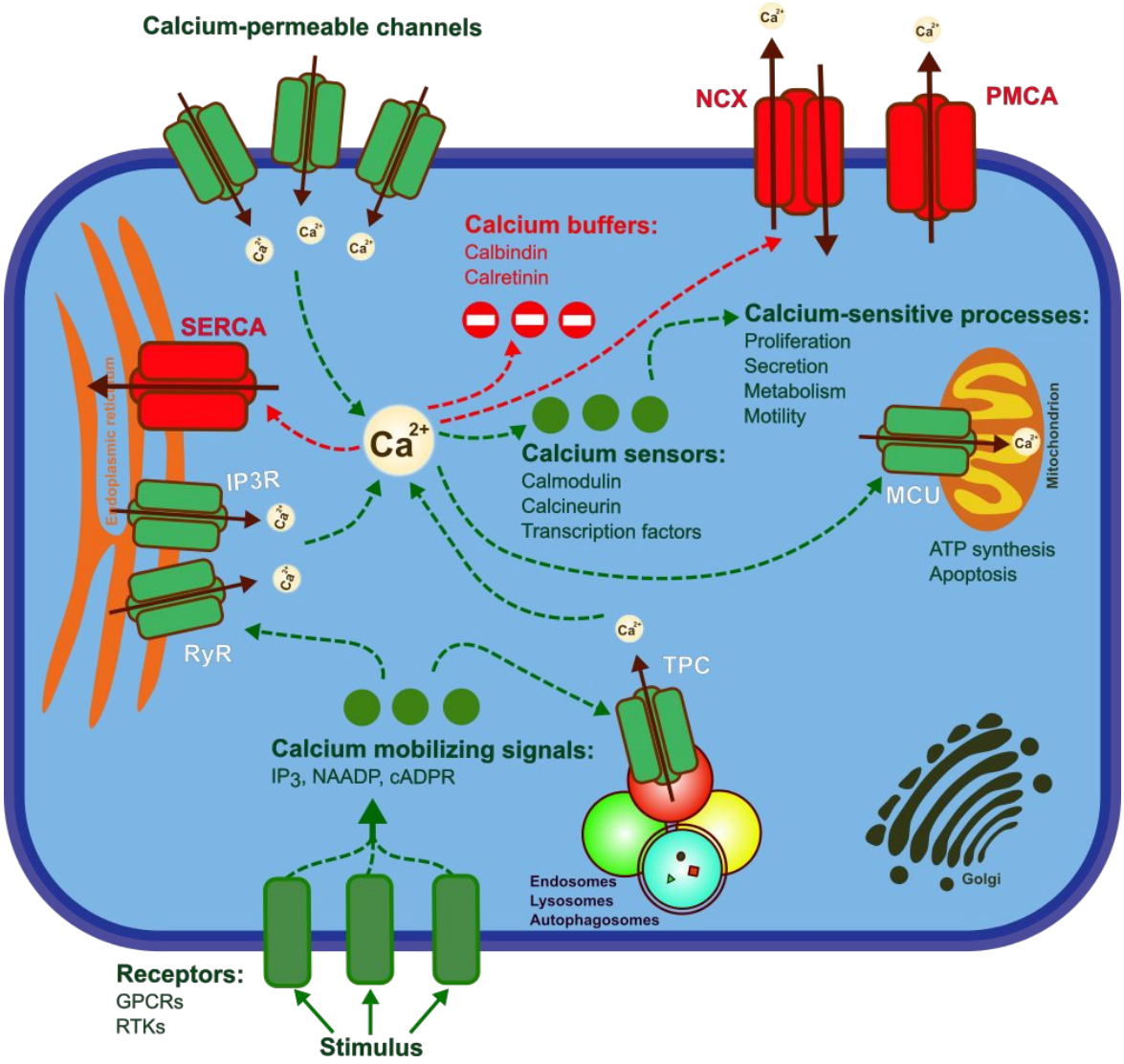

Figure 1.5. Calcium signalling toolkit.

Thus, it's clear that "**calcium signalling toolkit**" is a very complex set of regulatory elements to control intracellular calcium signals. In this set each element is important to ensure proper calcium signal management. Considering that our laboratory has long been studying the role of ion channels in cancer, in this thesis we were focused on calcium-permeable ion channels.

1.2.2 Calcium-permeable ion channels

Mitochondrial, ER, lysosomal and cytosolic calcium levels are regulated by calcium permeable ion channels localized either on the membranes of the intracellular organelles or on the plasma membrane (Berridge et al., 2003; Rizzuto et al., 2012). Ion channels are integral membrane proteins that mediate the influx/efflux of essential signaling ions into/from the cell or intracellular organelles thereby controlling cytoplasmic/intraorganellar ion concentrations, membrane potential and cell volume. The calcium permeable ion channels, including families of transient receptor potential (TRP) channels, store-operated channels (SOCs), arachidonate-regulated Ca^{2+} (ARC) entry channels, voltage-gated calcium channels, two-pore channels, mitochondrial permeability transition pore (MPTP), mitochondrial calcium uniporter (MCU), IP3 and ryanodine receptors and others (Figure 1.6) contribute to changes in $[Ca^{2+}]i$ by providing Ca^{2+} entry pathways, by modulating the driving force for the Ca^{2+} entry, and also by providing intracellular pathways for Ca^{2+} uptake/release into/from cellular organelles (Bernardi and von Stockum, 2012; Berridge et al., 2003; Pedersen et al., 2005; Rizzuto et al., 2012).
The calcium-permeable channels are highly heterogenous, are differentially expressed in various cell types and mediate calcium entry in response to various stimuli.

Voltage-gated calcium channels are mostly expressed in excitable cells and are activated by depolarizing membrane potentials. Voltage-gated calcium channels are divided into two major categories: high voltage-activated (HVA) channels that open in response to large voltage changes and low voltage-activated (LVA) channels that are activated by smaller changes in membrane potential (Catterall, 2000).

Ligand-gated channels (including members of the P2X, glutamate and Cys-loop receptor families) are mainly involved in fast chemical synaptic transmission in the nervous system, but can also be found extrasynaptically, as well as outside the nervous system (particularly, P2X channels) (Collingridge et al., 2009).

Transient receptor potential channels (TRP) channels are a large superfamily of 28 mammalian cation channels with diverse physiological functions and cellular distributions. This superfamily is divided into six subfamilies (TRPC (canonical), TRPM (melastatin), TRPV (vanilloid), TRPA (ankyrin-like), TRPP (polycystin), TRPML (mucolipin)) based on structural

homology. Most of TRPs are non-selective Ca^{2+}-permeable cation channels, with the exception of TRPM4 and TRPM5, which are not permeable for Ca^{2+}. The members of TRP family are activated by a plethora of different stimuli (including temperature changes, mechanical force, extra- and intracellular messengers, osmotic stress and many others) and function as important regulators of various cellular processes (Pedersen et al., 2005).

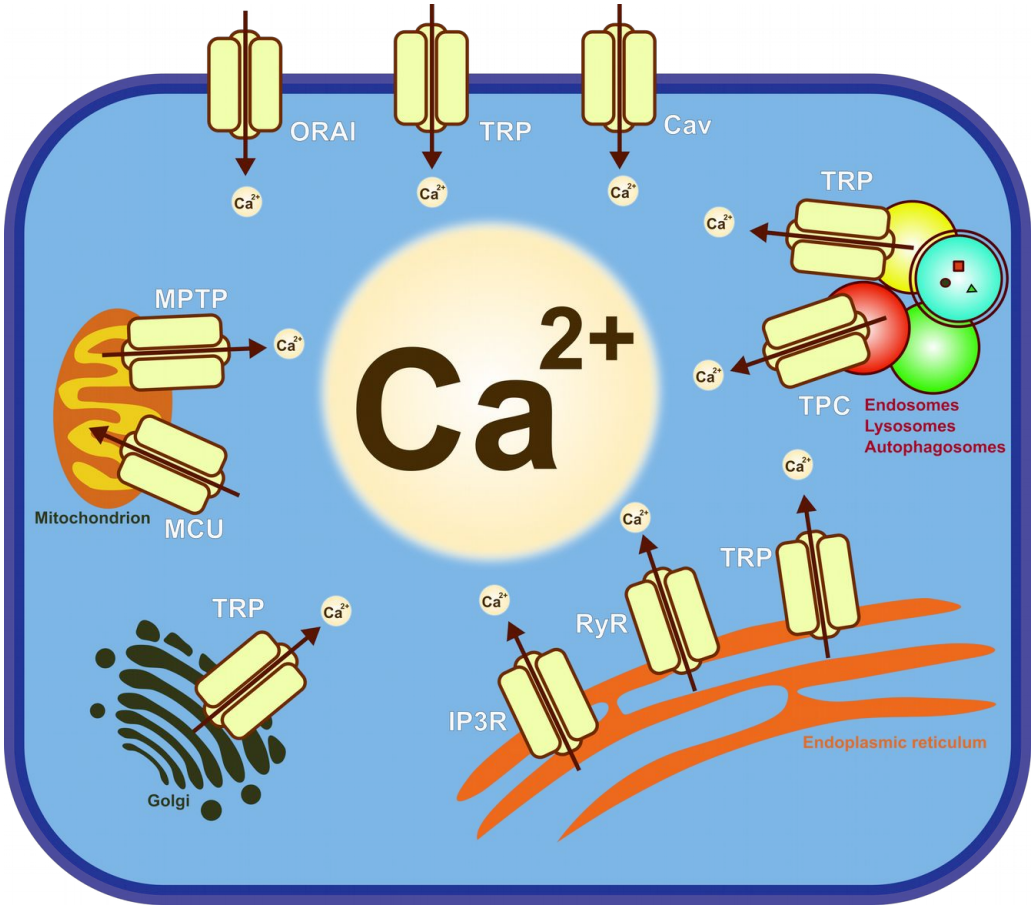

Figure 1.6. Calcium-permeable ion channels.

The inositol 1,4,5-trisphosphate receptor (IP3R) represents primary calcium release channel on ER (the major Ca^{2+} store in most cells) membranes. Under normal conditions this channel is responsible for Ca^{2+} release into the cytoplasm, in response to inositol 1,4,5-trisphosphate (IP3) generation stimulated by numerous stimuli, to maintain normal calcium homeostasis. Agonist-mediated stimulation of G protein-coupled receptors (GPCRs) induces inositol phospholipid breakdown by phospholipase C (PLC), which in turn generates two second messengers, diacylglycerol (DAG) and IP3. DAG can regulate protein kinase C (PKC) or some TRP channels, whereas IP3 activates IP3Rs, thus releasing calcium from ER into the cytosol (Foskett et al., 2007; Joseph and Hajnóczky, 2007).

Ryanodine receptor (RyR) represents another major ER Ca^{2+}-release channel. RyRs mediate the rapid release of calcium from intracellular stores into the cytosol, which is essential for numerous cellular functions including excitation-contraction coupling in muscle, synaptic transmission and pancreatic beta cell function. RyRs are activated by the inward flow of Ca^{2+} via plasma-membrane Ca^{2+} channels, resulting in a massive and rapid release of Ca^{2+} from intracellular stores (a process known as Ca^{2+}-induced Ca^{2+} release (CICR)) (Zalk et al., 2007, 2014).

Mitochondrial calcium uniporter (MCU) represents the major calcium-permeable channel of the inner mitochondrial membrane responsible for Ca^{2+} uptake into the matrix and thus plays a crucial role in the control of metabolism and apoptosis (Murgia and Rizzuto, 2015; De Stefani et al., 2015).

Two-pore channels (TPC) family comprise two members TPC1 and TPC2, widely expressed in humans and localized intracellularly on endolysosomes, with TPC2 being specifically targeted to lysosomes. Several groups proposed TPC as a mediator of endolysosomal calcium release in response to the elevation of the second messenger, nicotinic acid adenine dinucleotide phosphate (NAADP). It has been proposed that calcium that is locally released from endolysosomes can further be coupled to the Ca^{2+} release from the ER through CICR, thereby inducing global calcium signals (Calcraft et al., 2009).

Among all the types of calcium-permeable ion channels, ORAI calcium channels represent the best studied group of channels in regard to cancer. This could be explained by the ubiquitous expression of these channels as well as by the fact that these channels represent one of the major calcium-entry pathways in non-excitable cells (e.g. cancer cells).
Given that in this thesis we were mostly studied ORAI channels, the information on ORAI family of channels is represented in more detail (see below) compared to other types of calcium-permeable channels.

1.2.2.1 Family of ORAI calcium channels

The most common mechanism of Ca^{2+} signal generation results from the activation of plasma membrane G protein-coupled receptors (GPCRs) followed by PLC-mediated cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) into DAG and IP3 (Smyth et al., 2010). DAG is

known as PKC activator, but can also play a PKC-independent role in regulating Ca^{2+} signal generation. IP3 production results in cytoplasmic Ca^{2+} elevation that can be separated into two distinct phases. At first, IP3 activates ER-localized IP3Rs, thus releasing calcium from ER into the cytosol. At second, the decrease in ER Ca^{2+} content (following IP3R activation) stimulates influx of extracellular Ca^{2+} via plasma membrane Ca^{2+} channels in a process known as capacitative or store-operated Ca^{2+} entry (SOCE) (Parekh and Putney, 2005).

Numerous studies demonstrate the important role of SOCE in a plethora of cellular processes and functions in different cell types, including endothelial cell proliferation (Abdullaev et al., 2008), smooth muscle migration (Bisaillon et al., 2010; Potier et al., 2009) and skeletal muscle development and contraction (Stiber et al., 2008). Moreover, SOCE has been implicated in a number of pathological processes typical for cancer, such as breast tumor cell migration and metastasis (Yang et al., 2009), human glioblastoma invasion (Motiani et al., 2013) and apoptosis in a variety of cell types (Henke et al., 2013; Khadra et al., 2011; Liu et al., 2011).

Store-operated calcium channels (SOCs) represent one of the major calcium-entry pathways in non-excitable cells and are widely distributed in various cell types. SOCs are plasma membrane ion channels activated in response to ER Ca^{2+} store depletion and thereby provide Ca^{2+} for ER store refilling as well as for signaling purposes (Parekh and Putney, 2005; Smyth et al., 2010). The major molecular components of SOC are stromal interaction molecule 1 (STIM1) and ORAI1 proteins, where ORAI1 constitutes plasma membrane calcium channel and STIM1 represents mostly ER-localized single-transmembrane domain protein, functioning as a sensor of ER calcium. Following ER Ca^{2+}-depletion STIM1 translocates to the plasma membrane, where it interacts with and activates ORAI1 channel, thereby mediating store-operated calcium entry (SOCE) (Prakriya et al., 2006; Zhang et al., 2005).

In fact, two human STIM proteins exist, STIM1 and STIM2. Both are predominantly located in the ER, though a minor amount of STIM1 is expressed at the plasma membrane (Soboloff et al., 2012). Both STIMs have similar architecture, with an N-terminal domain in the ER lumen, a single transmembrane segment, and a C-terminal cytoplasmic domain (Hooper et al., 2013). In vertebrates, STIM1 and STIM2 are expressed ubiquitously throughout cell types are thought to function as ER calcium sensors (Williams et al., 2001). In contrast to STIM1, STIM2 exclusively localizes in the ER. STIM2 has been reported to be considerably weaker activator of ORAI1 than STIM1 whereas it represents more sensitive sensor of ER luminal Ca^{2+}. The Kd of STIM2 for Ca^{2+} (~400 μM) is 2-fold higher than that of STIM1. Thus, it is assumed that the physiological role of STIM2 consists in stabilization of basal cytosolic and ER calcium levels

(Brandman et al., 2007). The role of STIM2 in the regulation of SOCE is complex. It has been reported that STIM2 protein mediates distinct store-dependent and store-independent modes of SOC channel activation (Parvez et al., 2008). However, overexpression of STIM2 inhibited STIM1-mediated SOCE (Soboloff et al., 2006). Moreover, different splice variants of STIM2 has been shown to differentially regulate SOCE (Miederer et al., 2015). Recently, it has been proposed that STIM2 enhances agonist-mediated activation of SOCE by promoting STIM1 clustering in ER-PM junctions at low stimulus intensities, when ER Ca^{2+} stores are mildly depleted, thus increasing the sensitivity of Ca^{2+} signaling to agonists (Ong et al., 2015).

ORAI1 is the founding member of ORAI family of Ca^{2+} channels that are phylogenetically distinct from other calcium-permeable channels. ORAI family includes three members (ORAI1, 2, and 3) consisting of four transmembrane domains with cytosolic N and C termini (Hogan and Rao, 2015; Prakriya, 2013). Although the first recordings of calcium-release activated calcium (CRAC) currents have been reported in 1980s, ORAI1 has been linked to these currents just in 2006 (Feske et al., 2006; Lewis and Cahalan, 1989; Prakriya et al., 2006). ORAI1, is a widely expressed 33-kDa cell surface protein, the missense mutation of which has been associated with abrogated CRAC channel activity and human severe combined immune deficiency (SCID) syndrome (Feske et al., 2006). ORAI1 is localized on the plasma membrane and forms the Ca^{2+}-selective pore of the CRAC channel. The functional CRAC channel is believed to be a tetramer of four ORAI1 subunits, however several studies suggested higher order of assembly (i.e. hexameric) (Hou et al., 2012; Mignen et al., 2008; Penna et al., 2008; Zhou et al., 2010).

In a classical model of SOCE, activation of ORAI1 involves direct binding of STIM1 and ORAI1 (Park et al., 2009). Ca^{2+} store depletion causes STIM1 to accumulate in ER regions closely associated with the plasma membrane, where it binds to and activate ORAI1 (Hogan and Rao, 2015).

ORAI2 and ORAI3 represent two highly conserved paralogues of ORAI1. Like ORAI1, both ORAI2 and ORAI3 are highly calcium selective in physiological conditions, and both channels have been reported to be activated by calcium store depletion (DeHaven et al., 2007; Lis et al., 2007). Similarly to ORAI1, ORAI2 and ORAI3 appear to have broad expression pattern (Gross et al., 2007; Gwack et al., 2007a; Takahashi et al., 2007). At the moment, functional implications for ORAI2 are sparse. Several publications suggested that ORAI2 mediates SOCE in immune cells with silenced ORAI1 (Gwack et al., 2008; Vig et al., 2008). However, no effect of siRNA against ORAI2 on SOCE has been reported by other groups (Baryshnikov et al., 2009; Takahashi et al., 2007).

In contrast to ORAI1 and ORAI2, ORAI3 is an exclusively mammalian protein. In estrogen receptor -positive breast cancer cells ORAI3 (but not ORAI1) has been shown to mediate SOCE (Motiani et al., 2010). However, in HEK 293 and human fibroblasts silenced for ORAI1, ectopically expressed Orai3 only partially restored SOCE, suggesting the primary role for ORAI1 in this process (Gwack et al., 2007a). ORAI3 was also reported to mediate decreased SOCE sensitivity to reactive oxygen species (ROS) in human T helper lymphocytes (Bogeski et al., 2010).

Along with ORAI1, ORAI3 contributes to store-independent calcium entry. Thus, Orai3 has been reported to be an important component of store-independent arachidonate-regulated Ca^{2+} (ARC) entry in HEK293 cells, as well as of a store-independent leukotrieneC4-regulated Ca^{2+} (LRC) entry in vascular smooth muscle cells (González-Cobos et al., 2013; Shuttleworth, 2012).

ARC channels are activated in response to receptor-mediated derivation of arachidonic acid (AA), a polyunsaturated fatty acid which has multiple actions on living cells. As it was discussed above, the activation of plasma membrane GPCRs stimulates PLC-mediated cleavage of PIP2 into DAG and IP3. Along with PKC activation, DAG can also generate AA by the enzyme DAG lipase. Alternatively, AA can be generated by the enzyme phospholipase A2 (PLA2). AA has been known to exert its effects either by a direct action or by action of the AA metabolites. AA has been reported to regulate the activity of different types of ion channels including calcium-permeable channels (Meves, 2008).

The ARC channel is a small conductance, highly Ca^{2+}-selective ion channel whose activation is specifically dependent on low concentrations of AA acting at an intracellular site. Like the SOC channels, the ARC channels are widely expressed in a variety of different cell types (Shuttleworth, 2012). These channels are primarily involved in the generation and modulation of agonist-induced oscillatory calcium signals (Mignen and Shuttleworth, 2000). Recent findings suggest that, the same proteins that form SOC channels are also integral components of ARC channels; however, there are mechanistic differences between these channels. Activation of ARC channels depends on a pool of STIM1 that is constitutively present in the plasma membrane (but not ER-localized STIM1) (Mignen et al., 2007; Thompson and Shuttleworth, 2012), whereas the pore of the ARC channels is thought to be formed by the heteromeric assembly (pentameric) of ORAI1 with its homologue ORAI3 (Mignen et al., 2009). How arachidonic acid activates the channels remains unclear (Shuttleworth, 2009).

Recently it has been reported, that a store-independent ARC-like channel (called leukotriene

C4-regulated calcium (LRC) channel) could be activated by leukotrieneC4 (LTC4) in primary vascular smooth muscle cells (González-Cobos et al., 2013). LTC4 is produced through the catalytic activity of leukotrieneC4 synthase, which is the key enzyme responsible for the synthesis of cysteinyl leukotrienes through metabolism of arachidonic acid downstream of the 5-lipoxygenase pathway. LRC channel requires STIM1 for its activation. However, it's not clear which STIM1 pool (ER or PM) is important here. Both ORAI1 and ORAI3 have been shown to contribute to LRC channel-mediated calcium entry, thus confirming that ORAI proteins could be activated by various agonists/pathways and are important players in store-independent calcium entry (Zhang et al., 2015).

1.2.3 Calcium and calcium-permeable channels in cancer

Cancer is caused by defects in the mechanisms controlling cell proliferation and cell death. Calcium ions are central to these phenomena and thus are inextricably linked to cancer initiation (regulation of autophagy, senecense etc.) and progression (regulation of proliferation, migration and apoptosis resistance). The malignant transformation of a cell is generally associated with a major rearrangement of Ca^{2+} signalling toolkit, leading to enhanced proliferation, invasion, apoptosis resistance and consequently cancer progression. However, it should be noted that calcium signalling can also contribute to the suppression of tumorigenesis at both early and late stages by regulating such important processes as autophagy, senescence and apoptosis (Prevarskaya et al., 2013, 2014).

Modulation of calcium permeable ion channel's expression/function affects intracellular Ca^{2+} concentrations and consequently calcium dependent processes, such as proliferation, apoptosis and autophagy (Decuypere et al., 2011; Dubois et al., 2013; Flourakis and Prevarskaya, 2009). Indeed, defects in Ca^{2+} channels expression/function are involved in a number of pathologies, including tumorigenesis, since increased expression of Ca^{2+} channels could lead to elevated cytosolic Ca^{2+} levels and promotion of Ca^{2+}-dependent proliferative pathways (Nilius, 2007; Prevarskaya et al., 2010). As an example, several members of the TRP family of ion channels, namely TRPC1, TRPC3, TRPC6, TRPV1, TRPV6, TRPM1, TRPM4, TRPM5, TRPM7 and TRPM8, show altered expression in cancer cells. The involvement of SOCs, MPTP, MCU, IP3 receptors and ryanodine receptors in the regulation of cell death has also been described (Curry et al., 2013; Dubois et al., 2013; Flourakis et al., 2010; Hajnóczky et al., 2000; Prevarskaya et al., 2010).

1.2.4 Calcium and calcium-permeable channels in cell proliferation

Numerous studies emphasized the important role of calcium in the regulation of cellular proliferation (Roderick and Cook, 2008). Indeed, lowering of extracellular calcium was reported to inhibit cell proliferation (Kahl and Means, 2003). Ca^{2+} has been shown to have an important role in cell cycle control and is especially important in G1 phase as well as at the G1/S and G2/M transitions. Calcium controls the expression and activation of early genes, including c-Myc, c-Jun, c-Fos, nuclear factor of activated T cells (NFAT), and cAMP-responsive element binding protein 1 (CREB1) that in turn coordinate the expression of important cell-cycle regulators cyclins D and E as well as cyclin-dependent kinases CDK4 and CDK2. Calcineurin also plays a major role in the progression through G1 and S phases by regulating NFAT and CREB1 (Roderick and Cook, 2008). In basal conditions, inactive phosphorylated NFAT is localized within the cytoplasm. However, following cytosolic calcium increase, activated calcineurin dephosphorylates NFAT, thus triggering NFAT nuclear translocation, where it regulates expression of the target genes involved in cell proliferation (Hogan et al., 2003).

Many calcium-permeable channels have been implicated in proliferation of both normal and cancer cells. Among them various members of TRP family (including TRPV6, TRPC1, TRPC3, TRPC6, TRPM2, TRPM7, and TRPM8), SOC channels, IP3- and ryanodine receptors, voltage-gated calcium channels as well as numerous calcium pumps. Commonly, these calcium-permeable channels show higher expression levels in cancer cells comparing to normal cells, resulting in the increased cytosolic calcium levels, activation of pro-proliferative genes and consequently in enhanced proliferation (Prevarskaya et al., 2014). For example, TRPV6 channel (which is strongly expressed in advanced prostate cancer) has been reported to control prostate cancer cell proliferation via Ca^{2+}/NFAT-dependent pathways (Lehen'kyi et al., 2007). The same mechanism has been implicated in TRPC6-mediated proliferation of primary human prostate cancer epithelial cells (Thebault et al., 2006). The store-operated channel ORAI1 has been also suggested to be responsible for engaging the NFAT pathway and inducing changes in gene expression in lymphosytes (Gwack et al., 2007b). Moreover, SOCs have been shown to regulate proliferation of different types of cancer cells. ORAI1-mediated calcium entry has been proposed to regulate melanoma cell proliferation via the CaMKII/Raf-1/ERK signaling pathway (Umemura et al., 2014). The crucial role of ORAI1 in cell proliferation has been also demonstrated in HEK293 cells (El Boustany et al., 2010). ORAI1 was reported to drive

proliferation of the ER^- breast cancer cell line, MDA-MB231 (Yang et al., 2009). Moreover, store-independent activation of Orai1 by secretory pathway Ca^{2+}-ATPase (SPCA2) has been shown to be essential for proliferation of breast cancer cells (Feng et al., 2010). ORAI3 has been suggested as a native SOCE pathway, that controls proliferation and cell cycle progression via Akt pathway in non small cell lung adenocarcinoma cells (Ay et al., 2013). Moreover, ORAI3 silencing alters cell proliferation and cell cycle progression via c-myc pathway in breast cancer cells (Faouzi et al., 2011, 2013).

Recently, ARC channels have been implicated in prostate cancer cell proliferation. The increased expression of ORAI3 in prostate cancer has been demonstrated to promote cell proliferation by increasing ARC-mediated calcium entry, as well as confer apoptosis resistance by decreasing SOCE (Dubois et al., 2014).

In conclusion, Ca^{2+} plays important role in the regulation of proliferation by regulating the activity of transcription factors that further control expression of cell-cycle regulators. Apparently, the specific activation/inhibition of a given transcription factor by calcium is achieved through the formation of local signalling complexes comprising stimulus receptor, calcium channel and calcium sensitive transcription factor.

1.2.5 Calcium and calcium-permeable channels in the regulation of apoptosis

Apoptosis, a type of genetically controlled or programmed cell death, is a fundamental cellular mechanism utilized by multicellular organisms for disposal of cells that are no longer needed or potentially detrimental (Kerr et al., 1972). The apoptotic machinery is highly complex and sophisticated, involving a variety of molecular players. Depending on the mechanism of initiation, signaling pathways that lead to apoptosis can be divided to two core pathways: the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway. However, it should be noted that despite the differences in mechanisms of initiation and molecules involved, these two pathways are closely interrelated (Elmore, 2007). Most of the morphological and biochemical changes observed in apoptotic cells are primarily caused by the activity of a family of cysteine proteases called caspases, which normally exist in healthy cells as inactive proenzymes and become activated upon apoptosis initiation (Hengartner, 2000).

Most of the known caspases have the role in apoptosis and can be divided into two groups: initiator caspases (e.g. caspase-8, caspase-9 and caspase-10) and effector caspases (e.g. caspase-

3, caspase-6 and caspase-7). Initiator caspases activate effector caspases, which in turn realize much of the proteolisis and DNAses activation seen in apoptosis (Hengartner, 2000). However, although pronounced caspase activation is frequently found in apoptosis, the cases of caspase-independent apoptosis have been reported as well (Galluzzi et al., 2012).

Accumulated evidence suggests that apoptosis plays an integral part in tumor development and progression (Cotter, 2009; Dubois et al., 2013; Fulda, 2014). Impairment in apoptosis breaks the balance between cell death and cell proliferation, leads to accumulation of "undead" cells and therefore supports cancerogenesis. Given that acquired resistance to apoptosis represents the common feature of most and perhaps all types of cancer, "evading apoptosis" has been defined by Hanahan and Weinberg as one of cancer hallmarks (Hanahan and Weinberg, 2000). Resistance to apoptosis has been also implicated in moderate efficiency or failure of a number of anticancer treatments. Thus, targeting apoptosis represents a promising strategy to fight cancer (Dubois et al., 2013; Fulda, 2014).

Numerous studies indicate that calcium is an important regulator of apoptosis at all stages, from initiation to final phagocytosis of apoptotic bodies (Orrenius et al., 2003; Pinton et al., 2008). Cytoplasmic calcium overload has been shown to promote apoptosis via different pathways: mitochondrial, cytosolic and ER. For example, excessive elevation of calcium in the cytosol stimulates increase in mitochondrial calcium uptake, which in turn can induce opening of MPTP resulting in triggering of intrinsic apoptotic pathway (Giorgi et al., 2012; Kroemer et al., 2007). Another mechanism involves Ca^{2+}-dependent cysteine proteases, called calpains, which mediate cleavage of several members of BCL-2 family (including pro-apoptotic BID, as well as anti-apoptotic BCL-2 and BCL-2-like protein 1 (BCL-XL)), and promote MOMP and Cyt C release (Chen et al., 2001; Gil-Parrado et al., 2002). Cytosolic calcium overload (following treatment with calcium-mobilizing agents: ionomycin, A23187 or thapsigargin (TG)) has been also connected to apoptosis through the activation of Ca^{2+}-activated protein phosphatase calcineurin (CaN). Calcineurin dephosphorylates BCL-2-associated agonist of cell death (BAD), a pro-apoptotic member of the BCL-2 family, thus enhancing BAD heterodimerization with BCL-XL and promoting apoptosis (Wang et al., 1999).

Alternatively, ER-stress induction following alterations in ER-calcium homeostasis can activate specific ER-localized caspase-12 and thus can trigger apoptosis in a mitochondria-independent manner (Morishima et al., 2002; Nakagawa et al., 2000). In addition, calcium-dependent cleavage and activation of caspase-12 by m-calpain has been suggested as a mechanism underlying apoptotic cell death induced by oxygen and glucose deprivation (Nakagawa and

Yuan, 2000). Increase in cytosolic calcium has been also connected to the activation of several DNA-degrading endonucleases (Robertson et al., 2000).

Malignant transformation of cells, which is facilitated by apoptosis impairment, is often accompanied by alterations in ion channels' expression/function (Leanza et al., 2013; Prevarskaya et al., 2010). Below we discuss the involvement of some types of calcium-permeable channels in apoptosis regulation in normal and cancer cells.

Inositol 1,4,5-trisphosphate receptor. Apoptotic stimuli often provoke increased calcium release through IP3R, which in turn causes augmented mitochondrial calcium uptake and apoptosis induction. In line with this silencing of different IP3R isoforms significantly diminished apoptosis in lymphocytes in response to different stimuli (Joseph and Hajnoczky, 2007). Several mechanisms underlying a pro-apoptotic role of IP3R have been proposed, including direct transfer of calcium from ER to mitochondria through IP3R (Rizzuto et al., 2009), cleavage of IP3R by caspase-3 or calpains, which has been suggested to provide an enhanced ER calcium leak pathway promoting cell death (Hirota et al., 1999; Kopil et al., 2011), direct binding of Cyt C to IP3R, which amplifies calcium-dependent apoptosis (Boehning et al., 2003), and others.

In cancer, current evidence suggests the importance of IP3R in control of tumor growth, resistance to chemotherapy and aggressiveness. Thus, the anti-apoptotic role for IP3R type III has been proposed in colorectal carcinoma, where knockdown of this receptor enhanced apoptosis, whereas high levels of IP3R type III have been associated with increased aggressiveness (Shibao et al., 2010). The increased levels of IP3R type III have been also found in glioblastoma cells (Kang et al., 2010). In contrast, markedly reduced expression of IP3R type I in cisplatin-resistant bladder cancer cells compared to parental cells has been demonstrated. Further, knockdown of IP3R type I in these cells prevented cisplatin-induced apoptosis (Tsunoda et al., 2005). It seems that the contribution of IP3R to cancer-related processes could vary depending on cancer type, IP3 isoform involved, expression levels as well as regulation by proto-oncogenes and tumor suppressors (Akl and Bultynck, 2013).

Mitochondrial Calcium Uniporter. The excessive mitochondrial calcium uptake can lead to the opening of MPTP resulting in triggering of intrinsic apoptotic pathway. Mitochondrial Calcium Uniporter (MCU) represents the calcium-permeable channel of the inner mitochondrial membrane responsible for Ca^{2+} uptake into the matrix and thus plays a crucial role in the control of metabolism and apoptosis (Baughman et al., 2011; De Stefani et al., 2011). Recent data have shown that microRNA miR-25 decreases mitochondrial Ca^{2+} uptake through selective MCU

downregulation and in this way protects cells from Ca^{2+}-dependent apoptosis. MCU appears to be downregulated in human colon and prostate cancers and this correlates well with upregulation of miR-25 (Marchi et al., 2013). Expression of anti-miR-25 in PC3 prostate cancer cells or in HCT116 colon cancer cells increased mitochondrial Ca^{2+} levels and re-sensitized cells to apoptosis, confirming the key role of mitochondrial Ca^{2+} accumulation in the mitochondria-dependent apoptotic pathways (Marchi et al., 2013). In contrast, elevated levels of MCU have been detected in estrogen receptor negative and basal-like breast cancers (Curry et al., 2013). Interestingly, MCU silencing did not alter caspase-dependent cell death initiated by BCL-2 inhibitor ABT-263, while ionomycin-induced caspase-independent cell death was potentiated by MCU knockdown independently of changes in cytosolic Ca^{2+} levels. Thus, the authors concluded that MCU overexpression may offer a survival advantage against some cell death pathways and inhibition of MCU was proposed as a therapeutic strategy to treat breast cancers (Curry et al., 2013).

Transient receptor potential channels. Current evidence supports that TRP channels are implicated in the regulation of cellular processes altered in cancer, including apoptosis (Shapovalov et al., 2011). TRPM2, a widely expressed ion channel-enzyme, contains an enzymatic region with ADP-ribose (ADPR)-hydrolase activity (Perraud et al., 2001). TRPM2 knockdown in rat insulinoma RIN-5F cells has been demonstrated to significantly suppress Ca^{2+} influx and cell death induced by H_2O_2 and TNFα, whereas the heterologous overexpression of this channel enhanced H2O2-induced apoptosis (Hara et al., 2002). In malignant melanoma and prostate cancer TRPM2 function has been shown to be downregulated due to the upregulation of antisense TRPM2 transcripts. Functional knockdown of these antisense transcripts or over-expression of wild-type TRPM2 increases melanoma and prostate cancer cells susceptibility to apoptosis (Orfanelli et al., 2014).

TRPM7, another ubiquitously expressed Ca^{2+}-permeable nonselective cation channel with enzyme activity, contains an atypical serine/threonine protein kinase within the C-terminal domain (Runnels et al., 2001). It is involved in the regulation of cellular magnesium homeostasis (Ng et al., 2012). In rat basophilic leukemia mast cell line RBL-2H3 siRNA-mediated knockdown of TRPM7 has been reported to significantly increase apoptotic cell death (Ng et al., 2012). In contrast, TRPM7 has been shown to positively regulate Fas-induced apoptosis in T-lymphocytes (Desai et al., 2012).

TRPM8, a cold receptor in sensory neurons (McKemy et al., 2002), was first cloned from the human prostate as prostate-specific gene upregulated in cancer (Tsavaler et al., 2001). TRPM8

has been proposed to form a calcium-permeable channel at both plasma membrane and ER membranes in prostate cancer cells (Bidaux et al., 2007). TRPM8 has been suggested to be required for the survival of prostate cancer cells, as pharmacological inhibition by capsazepine or knockdown of TRPM8 in prostate cancer LNCaP cells induced apoptosis (Zhang and Barritt, 2004). However, in the same experimental conditions, activation of TRPM8 by menthol also induced apoptosis in LNCaP cells. The authors concluded that the normal function of TRPM8 is required for LNCaP cell survival, whereas menthol-induced cell death is mediated at least in part by the sustained increase in cytosolic calcium (Zhang and Barritt, 2004). In line with this, TRPM8 activation by menthol was reported to decrease the viability of melanoma cells, presumably by calcium-dependent mechanism (Yamamura et al., 2008). Further, in human bladder cancer cells menthol induces cell death via TRPM8-mediated mitochondrial membrane depolarization (Li et al., 2009). Consistent with this, menthol has been demonstrated to induce TRPM8-mediated apoptosis in rat synoviocytes via mitochondrial membrane depolarization and caspases activation (Zhu et al., 2014). In contrast, knockdown of TRPM8 has been reported to enhance epirubicin-induced apoptosis in human osteosarcoma cells (Wang et al., 2013).

Several members of the vanilloid subfamily of TRP channels have been also implicated in the regulation of apoptosis. TRPV1 was originally identified in sensory neurons as a heat-activated ion channel, which functions as a transducer of painful thermal stimuli in vivo (Caterina et al., 1997). The tumor suppressor function has been suggested for TRPV1, as its expression inversely correlates with tumor grade and growth in many cancers (Amantini et al., 2007; Bode et al., 2009; Miao et al., 2008). Capsaicin, the TRPV1 agonist, has been reported to induce TRPV1-dependent apoptosis in glioma cells. The authors demonstrated that capsaicin induced TRPV1-mediated Ca^{2+} influx, p38 mitogen-activated protein kinase activation, phosphatidylserine exposure, mitochondrial permeability transition pore opening and mitochondrial transmembrane potential dissipation as well as caspase-3 activation (Amantini et al., 2007). In urothelial cancer cells activation of TRPV1 by capsaicin has been suggested to induce apoptosis by both extrinsic Fas/CD95-dependent and intrinsic mitochondrial pathway (Amantini et al., 2009).

However, in human small cell lung cancer capsaicin induces apoptosis via the TRPV6 channel and the calpain pathway (Lau et al., 2014). The role for TRPV6 in capsaicin-induced apoptosis was also confirmed in gastric cancer cells, however the mechanisms are nor well understood (Chow et al., 2007).

TRPV2, another heat-activated TRPV channel, has been reported to be overexpressed in several

cancers (Lehen'kyi and Prevarskaya, 2012). In glioma cells, TRPV2 knockdown increased cell survival via ERK-dependent increase in BCL-XL expression, Akt/PKB phosphorylation and decrease in Fas expression (Nabissi et al., 2010). Accordingly, TRPV2 overexpression increased spontaneous apoptosis and sensitised glioma cells to Fas- and chemotherapy-induced apoptosis (Nabissi et al., 2010). Further, triggering of the TRPV2 by cannabidiol was demonstrated to sensitize glioblastoma cells to chemotherapy-induced apoptosis (Nabissi et al., 2013). Activation of TRPV2 by cannabidiol has been also linked to apoptotic cell death in human T24 bladder cancer cells. It has been proposed that T24 cell death occurred via apoptosis caused by continuous influx of calcium through TRPV2 (Yamada et al., 2010). In prostate cancer, TRPV2 has been suggested to contribute to apoptotic resistance of androgen-independent prostate cancer cell lines, likely by augmenting Ca^{2+} influx into these cells (Monet et al., 2010).

TRPV6, a channel mediating intestinal calcium absorption in duodendum, has been also found to be overexpressed in many cancers (Lehen'kyi et al., 2012). In prostate cancer TRPV6 expression correlates with tumor grade and TRPV6-mediated Ca^{2+} entry has been suggested to be involved in apoptosis resistance of LNCaP cells (Lehen'kyi et al., 2012). An elevated expression of TRPV6 was also detected in colon carcinoma cells, where siRNA-mediated TRPV6 knockdown inhibited proliferation and induced apoptosis (Peleg et al., 2010).

The members of TRPC subfamily also contribute to apoptosis regulation. For example, TRPC6 overexpression induces calcium-dependent apoptosis in cancer cells (Madan et al., 2013). TRPC6-mediated calcium entry has been demonstrated to be involved in high glucose-induced podocyte apoptosis through the RhoA/ROCK pathway (Yang et al., 2013).

TRPC1-mediated Ca^{2+} influx has been implicated in oxidized low-density lipoprotein-induced apoptosis in vascular smooth muscle cells (Ingueneau et al., 2009). Further, TRPC1 overexpression has been found to sensitize intestinal epithelial cells to apoptosis by increasing activity of protein phosphatase 2A followed by the NF-kappaB inhibition (Marasa et al., 2008). In contrast, TRPC1 was shown to protect human neuroblastoma cells against salsolinol-induced apoptosis by inhibiting cytochrome c release and BAX downregulation (Bollimuntha et al., 2006).

Accumulated evidence strongly suggests the involvement TRP channels in the regulation of apoptosis. However, in many cases the precise mechanisms of such regulation are elusive, suggesting the need in future research in this area.

ORAI family of channels. The role of SOCs in apoptosis seems to be dependent on multiple factors such as cell type, apoptotic stimuli as well as intracellular signaling pathway involved.

Thus, several studies suggest that SOCE, ORAI1 as well as STIM1 contribute to apoptosis induced by various stress stimuli (Flourakis et al., 2010; Henke et al., 2013), whereas others demonstrate their pro-survival antiapoptotic role (Khadra et al., 2011; Li et al., 2013). Indeed, ORAI1 has been demonstrated to contribute to the establishment of an apoptosis-resistant phenotype in prostate cancer cells and ORAI1 knockdown protected LNCaP cells against TG- or oxaliplatin/cisplatin-induced apoptosis (Flourakis et al., 2010). The authors proposed that ORAI1 constitutes the principal source of Ca^{2+} influx used by prostate cancer cells to trigger apoptosis via mitochondrial and cytosolic mechanisms (Flourakis et al., 2010). Consistent with this, pharmacological SOCE inhibition or STIM1 knockdown have been shown to inhibit hydrogen peroxide-induced apoptosis in HT22 cells via alleviation of intracellular Ca^{2+} overload, restoration of the mitochondrial membrane potential and decrease of cytochrome C release (Rao et al., 2013). In contrast, pharmacological inhibition of SOCE or downregulation of STIM1 have been shown to enhance apoptosis induced by cisplatin in non-small cell lung cancer cells (Li et al., 2013). Similar results were obtained on ovary carcinoma cells, where cisplatin-induced apoptosis was significantly lower in cisplatin-resistant cells (characterized by the increased expression of both ORAI1 and STIM1) than in parental cells. Pharmacological SOC inhibition by 2-APB or Akt inhibition by SH-6 restored cisplatin sensitivity of resistant cells. The authors concluded, that ORAI1/STIM1 play protective anti-apoptotic role in these cells and proposed that enhanced Akt activity could be responsible for this (Schmidt et al., 2014). Further, it was reported that Orai1-driven Ca^{2+}-entry delays the induction of the CD95-mediated apoptotic signal in leukemic T-cell lines through the translocation of the Ca^{2+}-dependent protein kinase C (PKC) β2 to the DISC and its subsequent inactivation in T-cells. This prevented CD95-mediated caspase activation and delayed delivery of the apoptotic signal (Khadra et al., 2011). When analyzing the involvement of SOCs in apoptosis regulation in different cell models, one should take in mind that ORAI protein family comprises three members ORAI1, ORAI2 and ORAI3, whereas STIM family is represented by two isoforms STIM1 and STIM2. All of these proteins participate in SOCE in different ways as well as have SOCE-independent functions (DeHaven et al., 2007; Hoth and Niemeyer, 2013). Therefore, future research is indispensable to better understand the specific mechanisms of apoptosis regulation by SOCE to finally conclude if their modulators could be effective in cancer treatment in each particular case. The general mechanisms of apoptosis regulation by calcium and calcium-permeable channels are represented in Figure. 1.7.

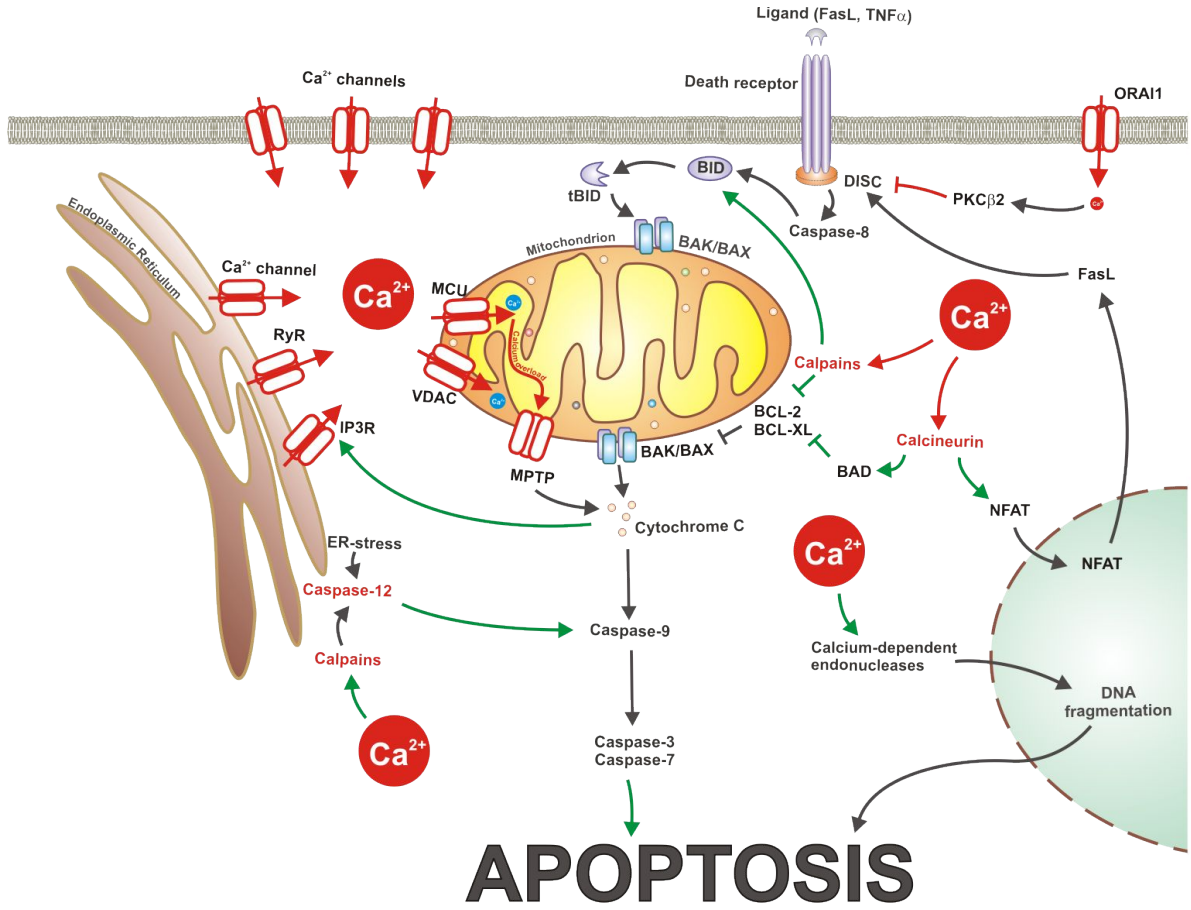

Figure. 1.7. Calcium and calcium-permeable channels in apoptosis regulation.

The involvement of other types of calcium-permeable channels in apoptosis has been also reported. These types include voltage-gated calcium channels, ryanodine receptors, voltage-dependent anion channel and others. The role of ion channels in apoptosis is reviewed in our recent review article (Kondratskyi et al., 2014).

1.2.6 Calcium-permeable channels in PDAC

Despite the suggested important role of calcium and calcium-permeable channels in the regulation of apoptosis, proliferation, metastasis and invasion in different cancers, the information on their expression and role in PDAC is rather scarce. Thus, the expression and function of TRPC1 and Na^+/Ca^{2+} exchanger NCX1 has been demonstrated in pancreatic cancer cell line BxPC3 (Dong et al., 2010). The authors suggested the role of TRPC1/NCX1-mediated calcium entry in pancreatic cancer cell motility and invasion. Moreover, the expression of TRPC4 and TRPC6 in BxPC3 cells has been also demonstrated in this study (Dong et al., 2010). TRPM8 channel has been reported to be upregulated in multiple PDAC cell lines

compared to “normal” human pancreatic ductal epithelial cell line H6C7 (Yee et al., 2010). TRPM8 has been suggested to be required for PDAC cells proliferation and invasion (Yee et al., 2010, 2014). Further, TRPM8 has been shown to be required for preventing replicative senescence in pancreatic adenocarcinoma cells and has been proposed as a potential clinical biomarker/therapeutic target in PDAC treatment (Yee et al., 2012a). Moreover, PDAC cells were reported to express functional plasma membrane TRPM8 channels, as revealed by patch-clamp studies. These plasma membrane TRPM8 channels were shown to inhibit PDAC cells motility (Cucu et al., 2014). The expression of another temperature-sensitive channel TRPV1 has been also shown to be upregulated in human pancreatic cancer and chronic pancreatitis. Interestingly, TRPV1 expression correlated well with the intensity of pain reported by cancer patients, suggesting its role in nociception (Hartel et al., 2006). Resiniferatoxin (a potent agonist of TRPV1) induced apoptosis by targeting mitochondrial respiration and decreased cell growth in pancreatic cancer cells (Hartel et al., 2006). TRPM7 channel was found to be upregulated in a proportion of the pre-malignant lesions and malignant tumors of the pancreas, and has been suggested to be necessary for cancer cell invasion as well as tumor growth and metastasis (Yee et al., 2015). Moreover, targeted silencing of TRPM7 induced replicative senescence and produced enhanced cytotoxicity with gemcitabine in PDAC cell lines (Yee et al., 2012b). Thus, it's clear that calcium-permeable channels are important players in PDAC cells, however the information on their expression/function/roles in these cells is very limited.

1.3 Conclusions

In conclusion, the information presented above is intended to show that despite significant scientific progress in understanding the biology of pancreatic cancer major clinical advances, however, have not occurred. Apparently, several specific hallmarks of pancreatic cancer are primarily responsible for the lack of progress in treatment. These hallmarks include lack of symptoms, late diagnosis, anatomical location of the pancreas, very early metastatic spread, presence of dense fibrotic stroma, high chemotherapy resistance and high mortality rate. These factors shows that special sophisticated approaches should be implemented to treat pancreatic cancer. Thus, a thorough understanding of physiology of pancreatic cancer is a must to design effective therapeutic strategies. In this context, it is important to study and understand the essential cellular processes, alterations of which have been linked to cancer initiation and progression. These processes include proliferation, differentiation, autophagy, migration, senescence, apoptosis and others. Surprisingly, despite the suggested crucial role of calcium and

calcium-permeable channels in the regulation of these processes in different cancers, the information on this subject in PDAC is very limited, highlighting the need in further research in this direction.

Purpose of the thesis

The role of calcium in physiology could be hardly overestimated. As a ubiquitous secondary messenger, calcium regulates plethora of physiological processes such as proliferation, differentiation and apoptosis and thus could determine cell fate. Therefore the intracellular concentration of calcium is tightly regulated by numerous calcium-permeable channels and pumps. Given such a crucial role of calcium in physiology it's not surprising that disturbance in calcium homeostasis has been implicated in a variety of pathologies, including cancer. Indeed, numerous studies emphasize the critical role of calcium and calcium-permeable channels in the development, progression and resistance to treatment of many cancers, including that of prostate, breast, colon and others. In contrast, the information on the expression/function and role of calcium-permeable channels in PDAC is scarce. Indeed, PDAC represents one of the worst studied types of cancer with regard to calcium. There is a lack of data regarding calcium-dependent regulation of such important processes as proliferation, differentiation and apoptosis in PDAC. Considering that different types of cancer differ greatly from each other, it's almost impossible to extrapolate the mechanisms existing in a given cancer type to another one, highlighting the need in direct experimental research on each cancer case.
This thesis represents one of the pioneering studies on the expression, function and role of some calcium-permeable channels in PDAC.

Main goal:

The main goal of this thesis is to study the expression of calcium-permeable channels in pancreatic ductal adenocarcinoma as well as to establish the involvement of calcium-permeable channels in the regulation of proliferation, apoptosis and chemotherapy resistance of pancreatic ductal adenocarcinoma cells.

Given that store-operated calcium (SOC) channels represent one of the major calcium-entry pathways in non-excitable cells and have also been reported to regulate the balance between cell death and survival in a number of human cancers, we decided to focus mainly on SOC channels.

Thus, our specific aims were the following:

Specific aims:

1. To characterize the functional expression of SOC channels in PDAC as well as PSC cells.
2. To uncover the molecular nature of SOCE in PDAC as well as PSC cells.
3. To study the role of SOC channels in proliferation and apoptosis regulation in PDAC cells.
4. To investigate the mechanism of SOC-dependent regulation of chemotherapy resistance of PDAC cells.

Materials and methods

3.1 Cellular culture

3.1.1 Cell lines

In this study we have used different cancer models:
Human Pancreatic cancer cell lines - AsPC1, BxPC3, Capan1, MiaPaCa2, Panc1;
Human Pancreatic Ductal epithelial Cell Line HPDE (HPDE6-E6E7 (H6C7));
Primary murine pancreatic stellate cells (mPSCs);
Immortalized human pancreatic stellate cell line (RLT-PSCs).

AsPC1 from the American Type Culture Collection (ATCC) is a human pancreatic tumor cell line that was delivered from the metastatic site – ascites of a patient with adenocarcinoma in the head of the pancreas. Despite of the fact that these cells are adherently differentiated, they are of great interest, because they represent the novel model of the pancreatic cancer. Nude mice tumor model of AsPC1 shows the similar characteristics of human PDAC with abundant mucin production and granular differentiation.

BxPC3 from the ATCC is a primary human pancreatic tumor cells line was delivered from the body of the pancreas of a patient with adenocarcinoma. The differentiation of these cells is moderate to poor, they don't give metastasis and also do not express the cystic fibrosis transmembrane conductance regulator (CFTR). Nude mice tumor model of BxPC3 shows the similar characteristics of human PDAC with mucin production and displaying moderately well to poorly differentiated adenocarcinomas with occasional lymphocytic infiltrations at the tumor peripheries.

Capan1 from the ATCC is a human pancreatic ductal adenocarcinoma cell line which was initially delivered from the liver metastasis. Different experiments had confirmed the high resistance of Capan1 to different chemotherapeutic drugs, in particular to 5-FU. Tumor model with Capan1 shows a well-differentiated adenocarcinoma with abundant mucin production.

MiaPaCa2 (from the ATCC) cell line was initially delivered from the body and tail of the pancreas from the 65-year-old Caucasian male with pancreatic ductal adenocarcinoma. This cells show adherent growth.

Panc1 (from the ATCC) cell line was initially delivered from the head of the pancreas which invaded duodenal wall from the 56-year old Caucasian male. This cells show adherent growth, could give metastases.

HPDE6-E6E7 (**H6C7**) is a "normal" human pancreatic duct epithelial cell line, generated by transformation of cultures of normal pancreatic duct epithelial cells with human papilloma virus 16 (HPV-16). Cultured HPDE cells express low levels of epidermal growth factor receptor (EGFR), erbB2, transforming growth factor (TGF)-alpha, Met/hepatocyte growth factor receptor (HGFR), vascular endothelial growth factor (VEGF), and keratinocyte growth factor (KGF). Immortalized human pancreatic ductal epithelial cells H6C7 were obtained from Dr. Ming-Sound Tsao.

3.1.2 Primary murine pancreatic stellate cells (mPSCs)

The procedure of mPSC isolation was adapted from Nikolaj Nielsen (University of Munster, Germany) and also described in (Haanes et al., 2012). Quiescent mouse pancreatic stellate cells were isolated from eight- to twelve-week-old BL-6 mice. Mice were killed by cervical dislocation. Pancreas was dissected and incubated at 37°C for 30 min in Gey's balanced salt solution (GBSS) supplemented with 1 mg/ml collagenase type IV (Wortington). The digested tissue was then washed three times with DMEM/F12 medium supplemented with 10% FCS Gold and 1% penicillin/streptomycin, centrifuged at 1000 rpm for 4 min, and resuspended in DEMEM/F12. Dissociated cells were obtained by pipetting. Cell suspension was transferred to pre-coated (30 min with FCS Gold) tissue culture dishes (100 mm diameter). In 1h the medium was removed and cells were washed several times with medium to remove floating as well as poorly attached cells. Following this step, the majority of attached cells were PSCs. Cells were grown at 37°C in a humidified atmosphere containing 5% CO2.
Culture media: DMEM/F12 medium (31330, Gibco-Life Technologies) supplemented with 10% FCS (PAA Gold) and 1% penicillin/streptomycin.

3.1.3 Culture conditions

Pancreatic adenocarcinoma cell line Panc1 from the American Type Culture Collection (ATCC)

was cultured in Dulbecco's minimal essential medium DMEM+GlutaMAX (31966, Invitrogen, Life Technologies Inc.) supplemented with 10% FCS (PAA Gold). Pancreatic adenocarcinoma cell lines ASPC1 and BxPC3 from the ATCC were cultured in RPMI 1640 medium (31870, Gibco-Life Technologies) supplemented with 5 mM L-glutamine (25030, Gibco) and 10% FCS (PAA Gold). Pancreatic adenocarcinoma cell line MiaPaca2 from the ATCC was cultured in DMEM/F12 medium (31330, Gibco-Life Technologies) supplemented with 2,5% Horse Serum (S 9135, Biochrom) and 10% FCS (PAA Gold). Pancreatic adenocarcinoma cell line Capan1 from the ATCC was cultured in IMDM medium (SH 30229.01 HyClone, Thermoscientific) supplemented with 20% FCS (PAA Gold). Immortalized human pancreatic ductal epithelial cells H6C7 were obtained from Dr. Ming-Sound Tsao and cultured in KBM medium (CC-3101, Lonza) supplemented with KGM SingleQuots (CC-4131, Lonza).

Human immortalized pancreatic stellate cells RLT-PSCs and mouse pancreatic stellate cells were cultured in DMEM/F12 medium (31330, Gibco-Life Technologies) supplemented with 10% FCS (PAA Gold) and 1% penicillin/streptomycin.

3.1.4 Storage conditions

The cells were trypsinized, resuspended in fresh medium, counted and centrifuged in 1000 rpm for 5 minutes. Cells were resuspended in a culture freezing medium (Recovery Cell Culture Freezing Medium, Gibco) and stored in cryotubes at -80°C.

To defrost the cells cryotubes were placed for several minutes in the water bath 37°C and then were transferred to T25 or T75 flasks with culture medium. Cells were grown at 37°C in a humidified atmosphere containing 5% CO2. The medium was changed next day and subsequently every 3 days.

3.2 Calcium imaging

Pancreatic cancer cells were grown on glass coverslips to carry out calcium imaging experiments. Ratiometric dye Fura-2/AM (F1221, Invitrogen) was used as a Ca^{2+} indicator. Cells were loaded with 2 µM Fura-2/AM for 45 min at 37°C and 5%CO2 in corresponding medium and subsequently washed three times with external solution containing (in mM): 140 NaCl, 5KCl, 1 MgCl2, 2 CaCl2, 5 Glucose, 10 Hepes (pH 7.4). The coverslip was then transferred in a perfusion chamber on the stage of Nikon Eclipse Ti microscope. Fluorescence

was alternatively excited at 340 and 380 nm with a monochromator (Polychrome IV, TILL Photonics Gmbh) and captured at 510 nm by a QImaging CCD camera (QImaging). Acquisition and analysis was performed with the MetaFluor 7.7.5.0 software (Molecular Devices Corp.).

3.3 Cell transfection

Cells were transfected with 40 nM of siRNA against ORAI1, ORAI2, ORAI3 or STIM1 (Eurogentec, France, or Dharmacon Inc., Fremont, CA, USA) using Hyperfect transfection reagent (Qiagen Inc.), following the manufacturer's instructions. siRNA sequences are present in the following table.

Table. List of siRNA used for transfection.

siRNA 1

No	Name	Sequence
1	siCT	5'-CUUACGCCUGAGUACUUCGA(dTdT)-3'
2	siOrai1	5′-UGAGCAACGUGCACAAUCU (dTdT)-3′
3	siOrai2	5′-AUGUGGAGGCCGUGAGCAA (dTdT)-3′
4	siOrai3	5′-UUGAAGCUGUGAGCAACAUA (dTdT)-3′
6	siSTIM1	5′-GGCUCUGGAUACAGUGCUC (dTdT)-3′

siRNA 2

No	Name	Reference number
1	siCT	D-001830-10-20
2	siOrai1	L-014998-00-0020
3	siOrai2	L-015012-00-0020
4	siOrai3	L-015896-00-0020
6	siSTIM1	L-011785-00-0020

3.4 RT-PCR

Total RNA was extracted using TRI reagent (Sigma) and treated with DNAse (Ambion). cDNA was synthesized by reverse transcription. qRT–PCR was performed in a real-time thermal cycler Cfx C1000 (Biorad) using EvaGreen Supermix (Biorad). Primers are listed in Table 1.

Table 3.1. List of primers used for qPCR

No.	Name	Forward (5'-…-3')	Backward (5'-…-3')
1	hSTIM1	TGTGGAGCTGCCTCAGTAT	CTTCAGCACAGTCCCTGTCA
2	hOrai1	ATGGTGGCAATGGTGGAG	CTGATCATGAGCGCAAACAG
3	hGAPDH	TTCGTCATGGCTGTGAACCA	CAGTGATGCGCATGGACTGT
4	hHPRT	GGCGTCGTGATTAGTGATGAT	CGAGCAAGACGTTCAGTCCT
5	hOrai3	GACCGCTACAAGCAGGAACT	ATCCTTCAACTGAGGCCAGC
6	hTRPC1	ATCCTTCAACTGAGGCCAGC	ATGCACATTGTGTTCGGCAA
7	mβ2m	GCTATCCAGAAAACCCCTCAA	CATGTCTCGATCCCAGTAGACGGT
8	mTRPC1_1	TGGGCCCACTGCAGATTTCAA	AAGATGGCCACGTGCGCTAAGGAG
9	mTRPC1_2	GCAACCTTTGCCCTCAAAGTG	GGAGGAACATTCCCAGAAATTTCC
10	mTRPC1_3	CATGGAGCATCGTATTTCAC	GAGTCGAAGGTAACTCAGAA
11	mTRPC2	ACTTCACTACATATGATCTGGGTCAC	CACGTCCAGGAAGTTCCAC
12	mTRPC3	AGCCGAGCCCCTGGAAAGACAC	CCGATGGCGAGGAATGGAAGAC
13	mTRPC4	GGGCGGCGTGCTGCTGAT	CCGCGTTGGCTGACTGTATTGTAG
14	mTRPC5	GCTGAAGGTGGCAATCAAAT	AAGCCATCGTACCACAAGGT
15	mTRPC6	GACCGTTCATGAAGTTTGTAGCAC	AGTATTCTTTGGGGCCTTGAGTCC
16	mTRPC7	CCCAAACAGATCTTCAGAGTGA	TGCATTCGGACCAGATCAT
17	hORAI2	CCTGTCGTGGCGGAAGCTCT	CGGGTACTGGTACTGCGTCT
18	hSTIM2	TATGCAAAGGAGGAGGCT	ATGCGAACCTGTTCCAATTC

Table 3.2. List of primers used for PCR

No.	Name	Forward (5'-…-3')	Backward (5'-…-3')
1	mβ-actin	TGGCTACAGCTTCACCACC	ACTCCTGCTTGCTGATCCAC
2	mOrai1	GGCCAGAGTTACTCCGAGGTGATG	GGCAGGATGCAGGTGCTGATC
3	mOrai2	TGGCCAGGCACATCTGTAAGGTAA	ATACACAGATGGCTGTCCCATGCT
4	mOrai3	AGTTGTTTGCTCCTCCAGGAAGGT	GTGGTGGTGGTGCATGCCTTTAAT
5	mSTIM1	CAATGGTGATGTGGATGTGGAAGA	AGTAACGGTTCTGGATATAGGCAAACC
6	mSTIM2	CATCAGAAGGTAAAACTGTGCAGTGCTC	GGATGTCCTGGACTCACTCTGTAGACCA
7	mTRPC1	GCTGAGGATGACGTGAGGAGA	GAAAACGGGTGAGTCGTTCC
8	mTRPV6	AAGCTACCTCGTTGCCTGTG	AGTAGAGGCCATCTTGTTGC

3.5 Apoptosis assays

3.5.1 Hoechst staining

The level of apoptosis was determined by Hoechst staining. Cells were grown on 6-well plates and transfected with siCT, siORAI1 or siSTIM1. In 24 h cell were subjected to chemotherapy treatments for another 72 h. At the end of the treatments, both floating and attached cells were collected by trypsinization, centrifuged and resuspended in 1 ml of phosphate-buffered saline (PBS). Following cytospin cells were fixed with ice-cold methanol for 10 min, washed with PBS and stained with 5 μg/ml Hoescht 33258 for 10 min at room temperature. Cells were then washed twice with PBS, mounted in glycerogel (DAKO) and subjected to fluorescence microscopy analysis. Nuclear morphology was displayed on Zeiss Axio Imager A1 fluorescence microscope (405–435 nm). The percentage of apoptotic cells (with condensed/fragmented nuclei) was determined by counting at least 500 cells in random fields.

3.5.2 Annexin V/Propidium iodide

Alternatively, apoptotic cells were detected by Alexa Fluor® 488 Annexin V/Propidium iodide double staining. At the end of the treatments, both floating and attached cells were collected by trypsinization, centrifuged, washed with PBS and stained with Alexa Fluor® 488 Annexin V and Propidium iodide according to the manufacturer instructions (Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit, Life Technologies). Cells were examined by fluorescence microscopy on Zeiss Axio Imager A1 microscope. The percentage of Alexa Fluor® 488 Annexin V-positive cells was determined by counting at least 500 cells in random fields.

3.6 Western blotting

Cells were washed with cold PBS and lysed in ice-cold buffer containing: 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1% Sodium deoxycholate, 10 mM PO4Na2/K buffer, a protease inhibitor cocktail (Sigma-Aldrich) and a phosphatase inhibitor cocktail PhosSTOP (Roche). The lysates were centrifuged at 15,000×g at 4°C for 15 minutes to remove cell debris and supernatant protein concentration was determined by the BCA protein assay kit (Pierce Biotechnology). 30 μg of total protein were subjected to SDS-PAGE followed by transfer to PVDF membranes using the Trans-Blot® SD semi-dry transfer cell (Bio-Rad). The membranes

were blocked in a 5% fat-free milk containing TNT buffer (Tris-HCl, pH 7.5, 140 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature. The membranes were next incubated overnight at 4°C with primary antibodies, and then for 1 h at room temperature with secondary antibodies conjugated to horseradish peroxidase. After washing, the membranes were processed for chemiluminescence detection using Luminata Western HRP substrate (Milipore). Image J software was employed for quantitative analysis.

3.7 Cell viability assay

Cells were seeded at 10000 cells/well on 96-well plates in normal medium. The cells were treated either with DMSO or siRNA for 48 h in full media. Cell viability was monitored using the CellTiter 96 Aqueous One Solution cell proliferation assay (Promega), on the basis of the cellular conversion of the colorimetric reagent MTS [3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt] into soluble formazan by dehydrogenase enzymes found only in metabolically active cells. Following treatment the cells were incubated with reagent solution and absorbance was recorded at 490 nm wavelength using an ELISA plate reader (Molecular Devices).

3.8 Electrophysiology

Whole-cell patch-clamp experiments were performed using PC-9 amplifier and analyzed using pClamp (Molecular Devices) and Origin software (OriginLab Corporation). Patch pipettes for the whole-cell recordings were fabricated from borosilicate glass capillaries (World Precision Instr., Inc., Sarasota, FL) on horizontal puller (Sutter Instruments Co., Novato, CA) and had resistance of 3–5 MΩ when filled with intracellular solutions. The high Ca^{2+} extracellular solution used for SOC current recordings contained: 140mM NaCl, 10mM CaCl2, 10mM HEPES, 10mM Glucose, pH 7.4. The patch pipettes were filled with the following intracellular solution: 150 mM CsCl, 10 mM 1.2-bis (2-aminophenoxy)ethane N,N,N',N' – tetraacetic acid (BAPTA), 6mM MgCl2, 10mM HEPES, pH 7.4. IP3 was added directly to the intracellular solution from the stock solution.

3.9 Clonogenic assay

The MiaPaCa2 cells were seeded in a 6-well plate in normal medium (500 cells per well) and

transfected with siCT or siORAI3. The next day the medium was changed and cell were left to recover for 10 days. In 10 days cells were washed with PBS and fixed with methanol:acetic acid (3:1) for 5 min at room temperature. The cells were then stained with 0.5% crystal violet (in methanol) for 2h, washed with tap water and dried. The cell colonies were then photographed and counted.

3.10 Cell cycle analysis

The MiaPaCa2 cells were seeded in a 6-cm dishes in normal medium and transfected with siCT or siORAI3. In 96 hours cells were collected by trypsinization, centrifuged and washed with PBS and fixed overnight with 70% ice-cold ethanol at − 20 °C. The fixed cells were treated with RNase A (100 µg/ml) for 15 min at room temperature, and stained with a PBS-based solution containing Propidium Iodide (50 µg/ml). Cell cycle was analyzed with Cyan LX9 cytometer (Beckman Coulter, France) and data were processed by a Summit 4.5 software (Beckmann Coulter, France).

3.11 Immunocytochemistry

Pancreatic stellate cells were grown on glass coverslips. For identification of lipid droplets cells were incubated for 5 min in Nile Red staining solution (1microg/ml in PBS) at room temperature. The cells were then washed with PBS and analyzed using confocal laser scanning microscope (LSM 700, Carl Zeiss MicroImaging GmbH) with a Plan Apochromat 40×/1.3 numerical aperture oil immersion objective. The images were analyzed in Zeiss LSM Image Browser software.

For immunodetection of alphaSMA, PSC cells were rinsed with PBS, fixed with 4% paraformaldehyde-1X PBS for 5 min, washed three times with PBS and incubated for 30 min in permeabilizing solution (0.05% Tween, 0.2 M Glycine). The cells were next incubated with primary antibodies overnight at 4oC. Following three washes in PBS, cells were incubated with Alexa Fluor 488-labeled anti-mouse IgG secondary antibodies for 1 h at room temperature. The cells were then washed in PBS, stained with DAPI for 15 min, washed three times and slides were mounted with Mowiol® on glass slides and subjected to subsequent fluorescence analysis using confocal laser scanning microscope (LSM 700, Carl Zeiss MicroImaging GmbH).

3.12 Confocal microscopy

Live cell images were obtained using confocal laser scanning microscope (LSM 700, Carl Zeiss MicroImaging GmbH) with a Plan Apochromat 40×/1.3 numerical aperture oil immersion objective and equipped with a CO_2 and thermocontrolled chamber. The images were analyzed in Zeiss LSM Image Browser software and prepared for publication in Adobe Photoshop.

3.13 Animal experiments

Studies involving animals, including housing and care, method of euthanasia, and experimental protocols, were conducted in accordance with the local animal ethical committee in the animal house (C59-00913; protocol CEEA 202012) of the University Lille1. MiaPaCa cells were plated in T75 flasks. After reaching 80% confluency, the cells were washed with PBS, trypsinized, washed and centrifuged at 1000pm for 5 min. Cells were resuspended in Matrigel (100microl) and were injected subcutaneously into 6- to 8-week-old female nude mice (2x10^6/mouse). After tumor formation, mice were injected i.p.with siRNA diluted in PBS on a daily basis. Tumors were measured every 4 days and tumour volume was calculated as volume= (length x width^2)/2.

3.14 Data analysis

Data were analyzed using Origin 7.0 (Microcal Software). Statistical analysis was performed using Student's t-test, and $p<0.05$ was considered as significant. Asterisks denote: * - $p<0.05$, ** - $p<0.01$ and *** - $p<0.001$.

Results

4.1 ORAI1 and STIM1 in chemotherapy resistance of PDAC cell lines.

4.1.1 Calcium-permeable channels are differentially expressed in pancreatic cancer cell lines.

Although different calcium-permeable channels have been previously shown to be expressed in a variety of cell types, the information about their expression in PDAC cell lines is very limited. Therefore, we first sought to examine if they are present in several available PDAC cell lines, namely Capan1, ASPC1, Panc1, MiaPaca2 and BxPC3 as well as in "normal" immortalized human pancreatic ductal epithelial cells H6C7. The channels of interest included proteins potentially involved in SOCE, namely ORAI1, ORAI2, ORAI3, STIM1 and STIM2, TRPC1 and TRPV6. By using qRT-PCR technique we found that ion channels examined are differentially expressed in all the cell lines tested (Figure 4.1).

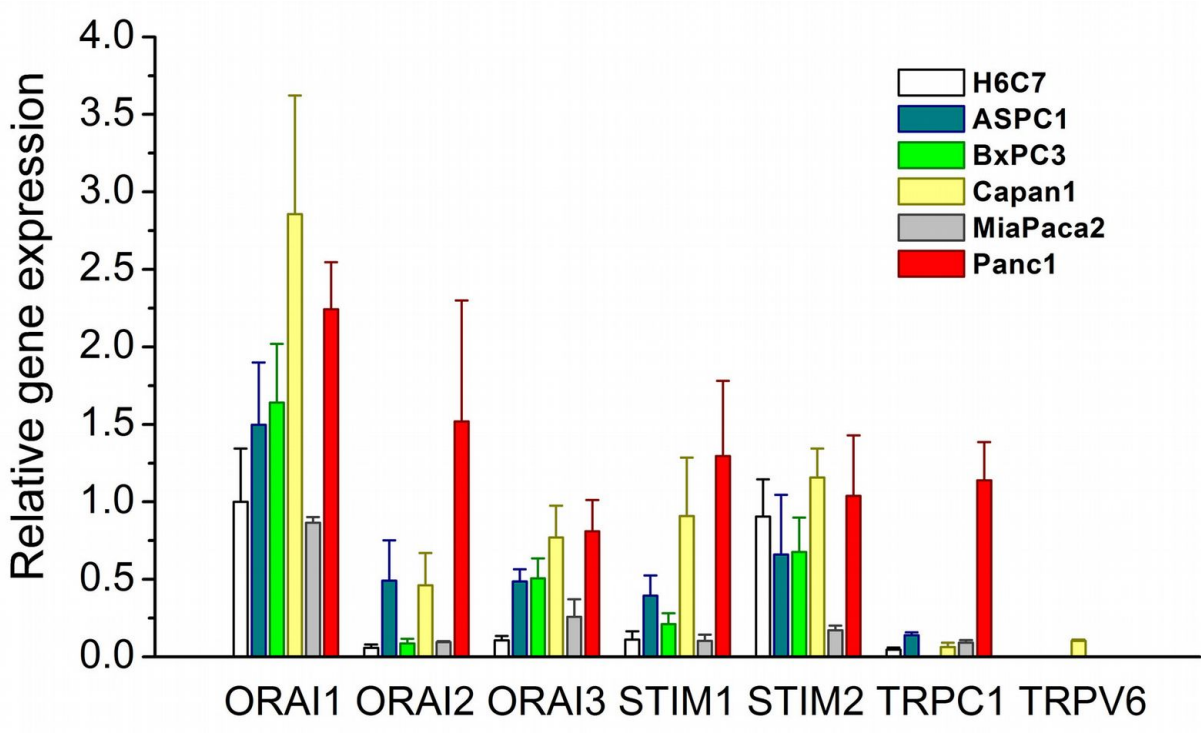

Figure 4.1. Calcium-permeable channels are differentially expressed in pancreatic cancer cells. *qRT-PCR detection of expression of ORAI1, ORAI2, ORAI3, STIM1, STIM2, TRPC1 and TRPV6 in H6C7, Capan1, ASPC1, Panc1, MiaPaca2 and BxPC3 cell lines. Gene expression levels relative to GAPDH for each cell line were normalized to ORAI1 level in "normal" H6C7 cell line. Data presented as means±S.D. n=3.*

Given that SOC channels represent one of the major calcium-entry pathways in non-excitable cells and have also been reported to regulate many processes in a number of human cancers, we decided to focus on SOC channels first. Therefore, we next characterized the expression of the SOCs' major components ORAI1 and STIM1 on the protein level by western blotting. We found that both ORAI1 and STIM1 are expressed on the protein level in all the cell lines tested and the levels of their expression differ between the cell lines, which correlates well with qRT-PCR data (Figure 4.2).

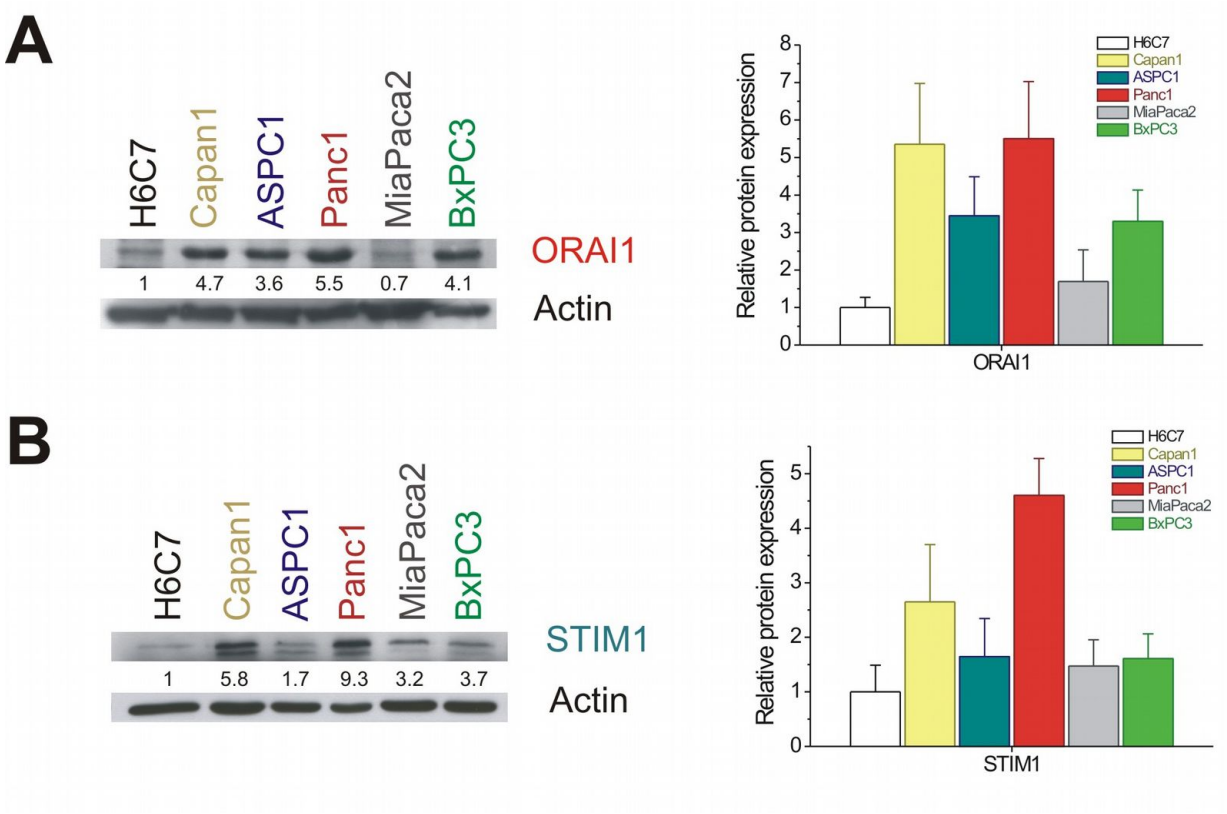

Figure 4.2. ORAI1 and STIM1 are differentially expressed in pancreatic cancer cells. *(A) Western blot showing the expression of ORAI1 in H6C7, Capan1, ASPC1, Panc1, MiaPaca2 and BxPC3 cell lines. Densitometric quantitations for normalized ORAI1 relative to Actin are shown. Bar plot shows the quantification of the protein expression levels of ORAI1 relative to Actin for each cell line normalized to ORAI1 level in H6C7 cell line. Data presented as means±S.D. n=3. (B) Western blot showing the expression of STIM1 in H6C7, Capan1, ASPC1, Panc1, MiaPaca2 and BxPC3 cell lines. Densitometric quantitations for normalized STIM1*

relative to Actin are shown. Bar plot shows the quantification of the protein expression levels of STIM1 relative to Actin for each cell line normalized to STIM1 level in H6C7 cell line. Data is represented as means±S.D. n=3.

In line with qRT-PCR and immunoblotting data, SOCE induced by thapsigargin (TG) (an inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)) was lower in normal H6C7 cells than in cancer cells (Figure 4.3).

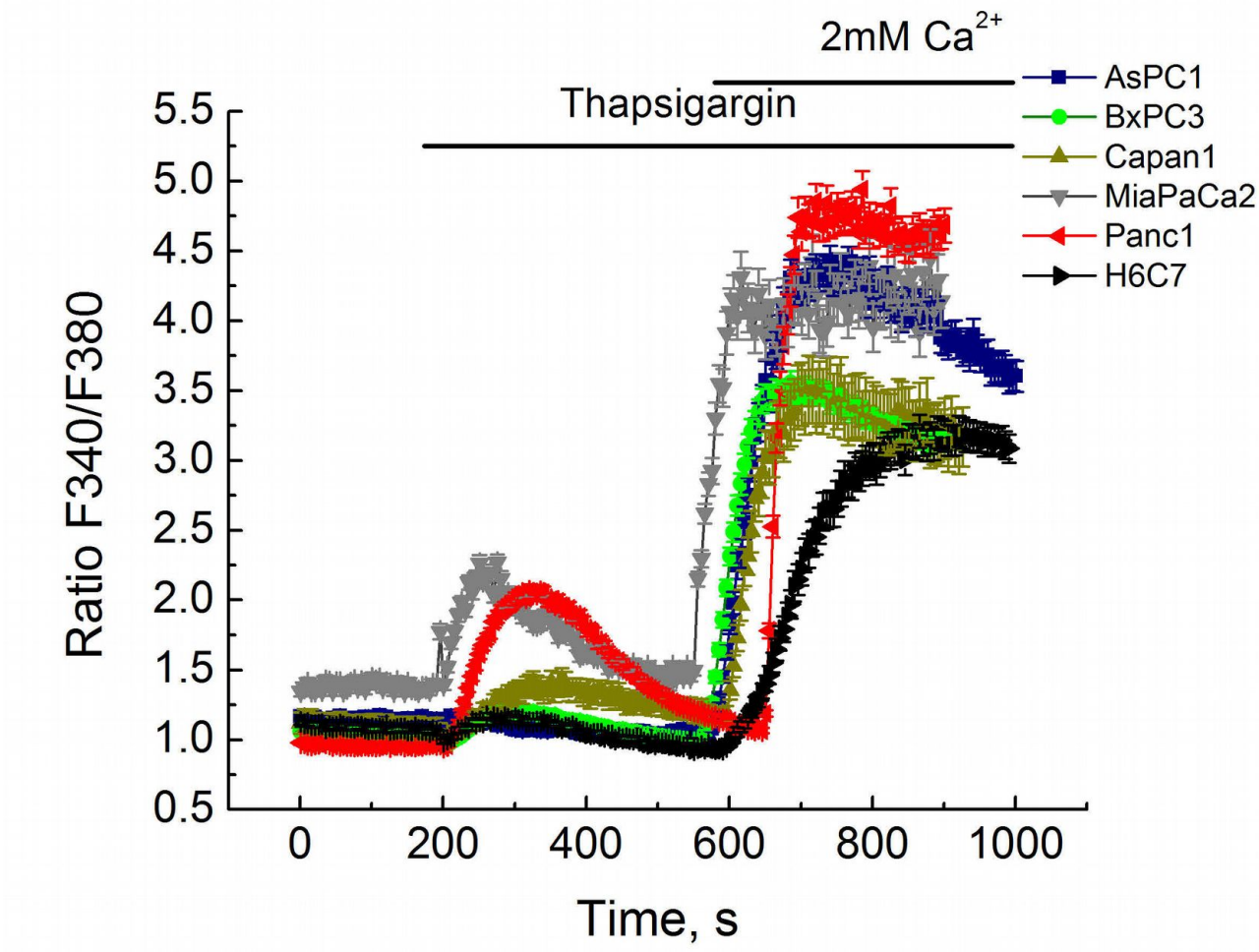

Figure 4.3. SOCE in PDAC and normal cells. SOCE were induced by TG in PDAC and H6C7 cells. *Cells were loaded with Fura2/AM probe and subjected to calcium imaging experiment. Data is represented as means±S.E.M. n=30-100.*

Moreover, the density of store-operated current (stimulated by intracellular IP3) was significantly lower in H6C7 cells, compared to Panc1 cells (Figure 4.4).

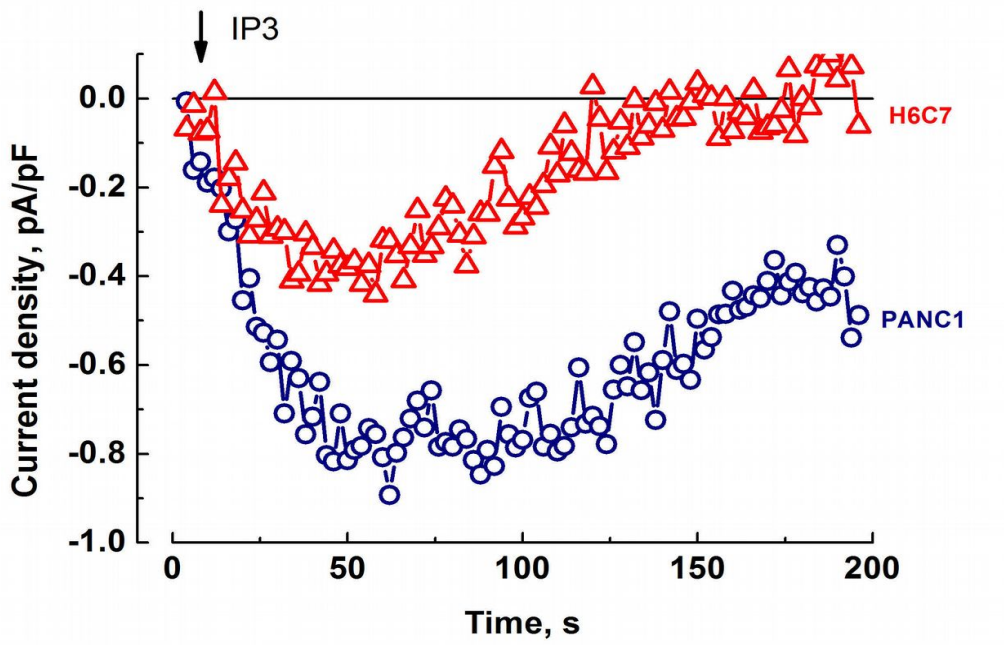

Figure 4.4. Store-operated currents in H6C7 and Panc1 cells. *SOC currents were stimulated by intracellular IP3. Representative traces are shown.*

4.1.2 ORAI1 and STIM1 are involved in SOCE in PDAC and H6C7 cells

Numerous reports demonstrated that ORAI1 and STIM1 mediate calcium release-activated channel (CRAC) activity and thus SOCE in a variety of cell types. Given that this function of ORAI1 and STIM1 has never been demonstrated in PDAC as well as normal pancreatic ductal epithelial cells, we next investigated if ORAI1 and STIM1 are involved in SOCE in these cells by using siRNA-approach. H6C7, AsPC1, MiaPaCa2, Capan1 and Panc1 cell lines were transfected with siCT, siORAI1 or siSTIM1. We determined the efficiency of siRNAs transfections in 48 h on mRNA by using qRT-PCR (Figure 4.5). Alternatively, siRNAs transfection efficiency was determined by western blotting (data not shown). 48 h following siRNAs transfection cells were loaded with Fura2/AM probe and subjected to calcium imaging experiment. We checked whether siRNA-mediated knockdown of ORAI1 or STIM1 influence cytosolic calcium levels in PDAC and H6C7 cells using thapsigargin (TG). More specifically, intracellular stores were depleted by TG in nominally calcium free extracellular saline followed by addition of 2mM Ca^{2+} to the cells to initiate the influx via store-operated calcium channels. When this assay was performed on siORAI1- or siSTIM1-transfected H6C7, AsPC1, MiaPaCa2, Capan1, Panc1 cells, calcium entry was significantly reduced (Figure 4.5A, B, C, D, E).

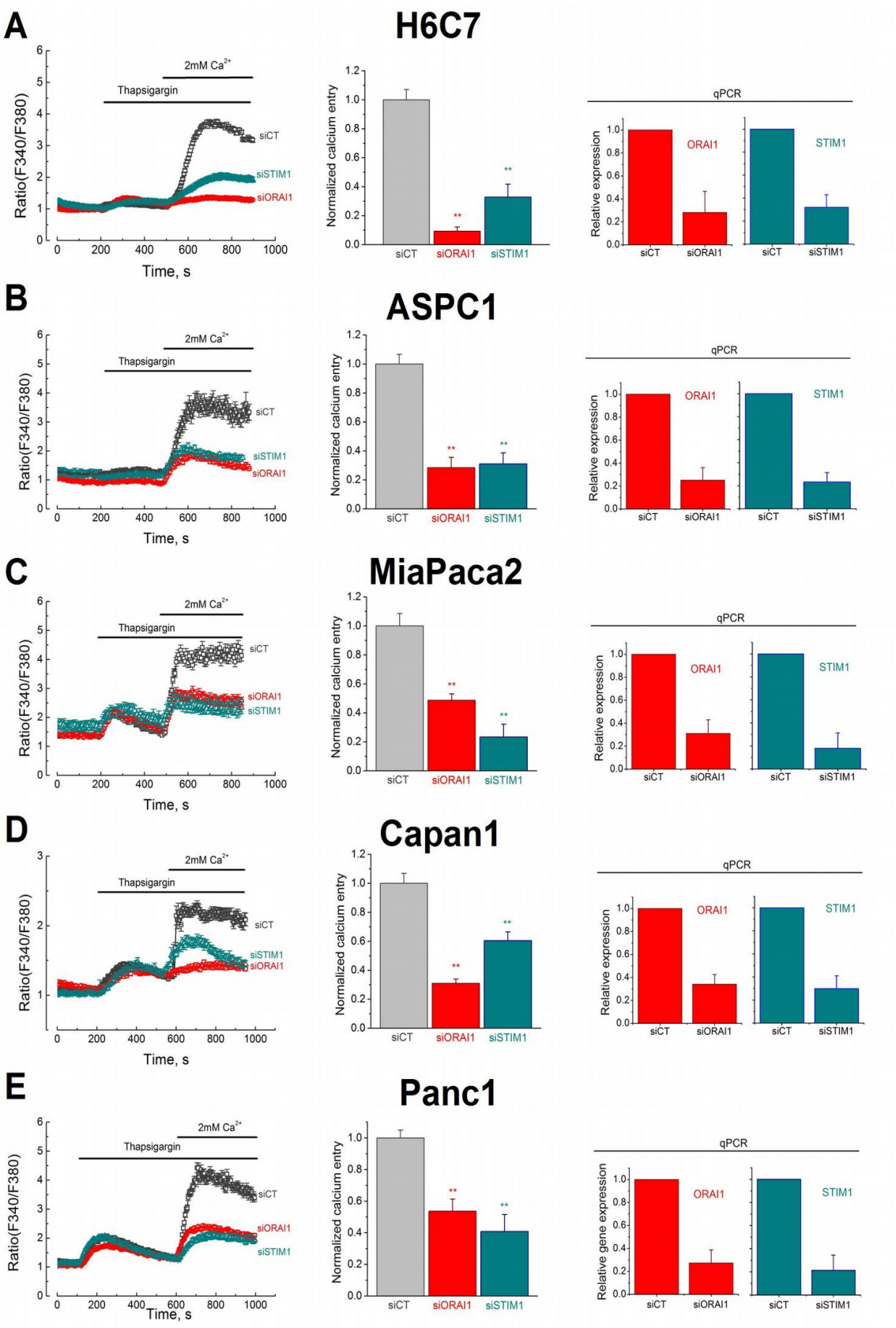

Figure 4.5. ORAI1 and STIM1 are involved in SOCE in PDAC and H6C7 cells. *(A) Knockdown of ORAI1 or STIM1 inhibits SOCE in H6C7 (A), AsPC1 (B), MiaPaCa2 (C),*

Capan1 (D) and Panc1 (E) cell lines. Cells were transfected with siCT, siORAI1 or siSTIM1. In 48 h cells were loaded with Fura2/AM probe and subjected to calcium imaging experiment. TG-induced SOCE was analyzed (n=30-100). Quantification of the TG-induced SOCE amplitude in cells transfected with siCT, siORAI1 or siSTIM1 is represented. qRT-PCR detection of expression of ORAI1 and STIM1 in PDAC and H6C7 cells following siRNA transfection is also represented. Data is represented as means±S.E.M.

Alternatively, calcium stores were depleted by preincubation in 0 Ca^{2+} extracellular solution for 30 min. SOCE was stimulated by the addition of 10 mM Ca^{2+} to the cells. This protocol was performed on MiaPaca2, Capan1 and Panc1 cell lines. When this assay was performed on siORAI1- or siSTIM1-transfected cells, calcium entry was significantly reduced (Figure 4.6).

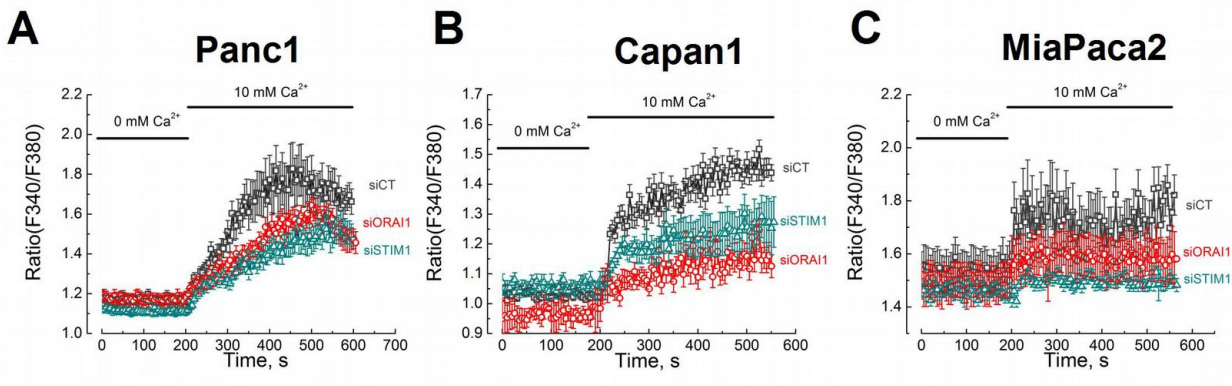

Figure 4.6. ORAI1 and STIM1 are involved in SOCE in PDAC cells. *Knockdown of ORAI1 or STIM1 inhibits SOCE in Panc1 (A), Capan1 (B), MiaPaCa2 (C) cell lines. Cells were transfected with siCT, siORAI1 or siSTIM1. In 48 h cells were loaded with Fura2/AM probe and subjected to calcium imaging experiment. Data is represented as means±S.E.M. n=30-100.*

These results suggested that ORAI1 and STIM1 mediate SOCE in PDAC and H6C7 cell lines. In addition, TG-stimulated calcium entry was completely inhibited by La^{3+} (10 microM), Gd^{3+} (10 microM) or BTP2 (10 microM) in Panc1 cells, exhibiting pharmacological properties characteristic of SOCE (Figure 4.7A and B).

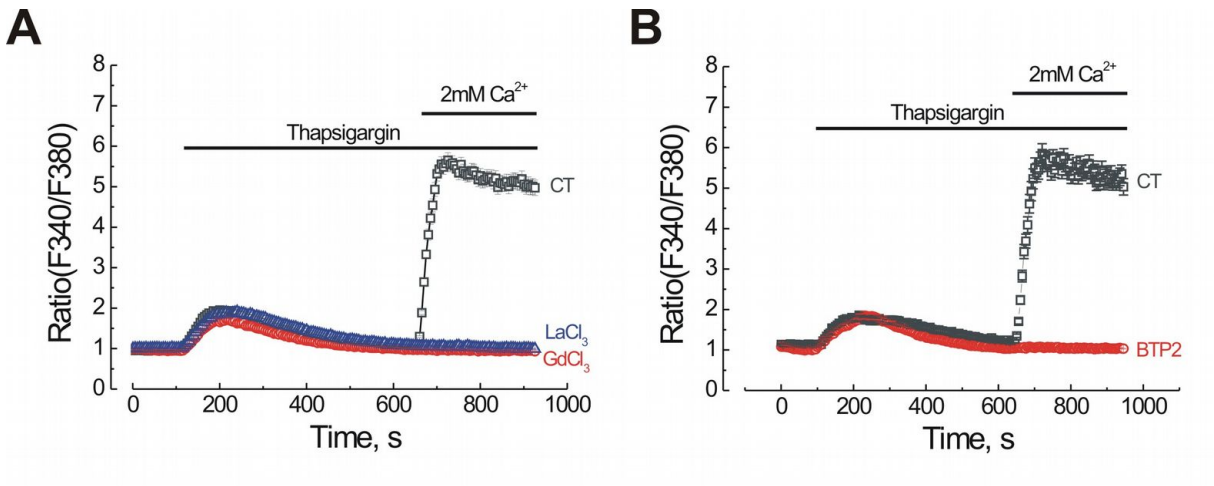

Figure 4.7. Pharmacology of SOCE in Panc1 cells. *Cells were loaded with Fura2/AM probe and subjected to calcium imaging experiment. TG-induced SOCE was analyzed. (A) La^{3+} and Gd^{3+} inhibit SOCE in Panc1 cells. $LaCl_3$ (10 microM) or $GdCl_3$ (10 microM) were added to the cells at the beginning of calcium imaging experiment. (B) BTP2 inhibits SOCE in Panc1 cells. BTP2 (10 microM) was added to the cells at the beginning of calcium imaging experiment. Data is represented as means±S.E.M. n=30-100.*

4.1.3 ORAI1 and STIM1 influence proliferation of PDAC cells.

Previous reports indicated that ORAI1, STIM1 as well as SOCE play an important role in cell physiology and pathology. Therefore we next examined how downregulation of ORAI1 or STIM1 will affect PDAC cells proliferation. Panc1, Capan1 or MiaPaca2 cells were plated on 96-well plate, and were left untransfected or transfected with siCT, siORAI1 or siSTIM1. Cell proliferation was monitored 6 days later using the CellTiter 96 Aqueous One Solution cell proliferation assay (MTS assay) (Figure 4.8).

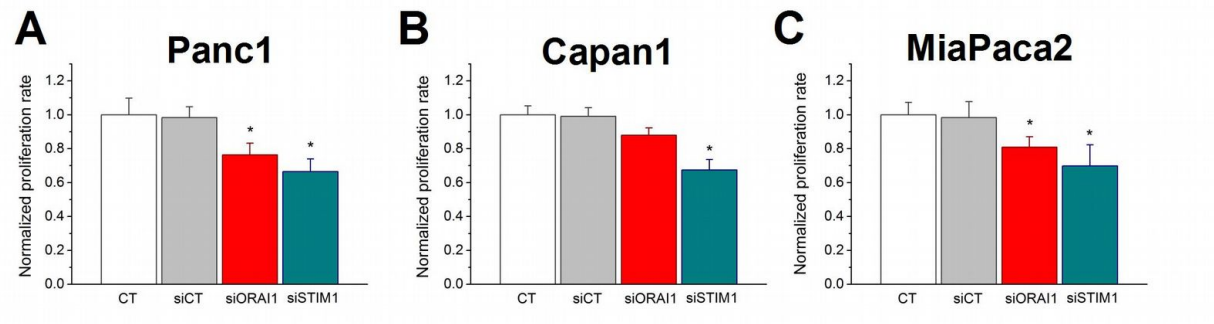

Figure 4.8. ORAI1 and STIM1 influence proliferation of PDAC cells. *Knockdown of ORAI1 or STIM1 inhibits proliferation of Panc1 (A), Capan1 (B), and MiaPaCa2 (C) cell lines. Cells were transfected with siCT, siORAI1 or siSTIM1. In 6 days cells cell proliferation was*

monitored using the CellTiter 96 Aqueous One Solution cell proliferation assay (MTS assay). Data is represented as means±S.E.M. n=3.

Silencing of ORAI1 or STIM1 reduced the proliferation rate of all the cell lines tested, suggesting that both ORAI1 and STIM1 (and apparently SOCE) regulate proliferation in PDAC cells (Figure 4.8).

For the subsequent experiments, we decided to focus on Panc1 cell line, as it is characterized by relatively high levels of ORAI1 and STIM1 expression.

4.1.4 Downregulation of ORAI1 and STIM1 sensitizes Panc1 cells to chemotherapy treatments.

The role of ORAI1, STIM1 and SOCE in apoptosis regulation has been extensively studied in different cell types. Therefore, we next investigated whether ORAI1 and STIM1 are involved in apoptosis regulation in Panc1 cells. Cells were transfected with siCT, siORAI1, siSTIM1 or siORAI1+siSTIM1. In 24 h cells were left untreated or treated with chemotherapy drugs 5-FU (50microM) or gemcitabine (50microM) for 72 h to induce apoptosis. At the end of treatments cells were collected, stained with Hoechst 33258 dye and subjected to fluorescence microscopy analysis of nuclear morphology. Alternatively, apoptotic cells were detected by Annexin V/PI staining. Downregulation of ORAI1 or/and STIM1 did not induce significant changes to the basal level of apoptosis in untreated cells (Figure 4.9A and B).

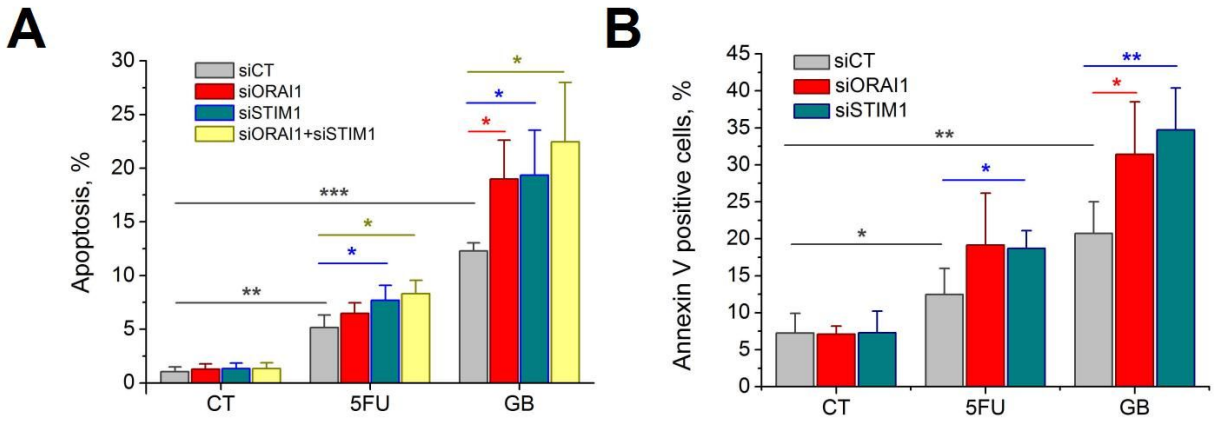

Figure 4.9. Downregulation of ORAI1 and STIM1 sensitizes Panc1 cells to chemotherapy treatments. *(A) Cells were transfected with siCT, siORAI1, siSTIM1 or siORAI1+siSTIM1. In 24 h cells were treated with chemotherapy drugs 5-FU or gemcitabine (GB) for 72 h. At the end*

of the treatment cells were collected, stained with Hoechst and subjected to fluorescence microscopy analysis of nuclear morphology. Data is represented as means±S.D. n=3. (B) Cells were transfected with siCT, siORAI1 or siSTIM1. In 24 h cells were treated with chemotherapy drugs 5-FU or gemcitabine for 72 h. At the end of the treatment cells were collected, stained with Annexin V/PI and subjected to fluorescence microscopy analysis. Data is represented as means±S.D. n=3.

However, when cells were treated with 5-FU or gemcitabine, downregulation of ORAI1 or/and STIM1 increased the levels of apoptosis induced by these drugs (Figure 4.9A and B). These results suggested that ORAI1 and STIM1 play pro-survival antiapoptotic role in Panc1 cells.

4.1.5 Chemotherapy drugs 5-FU and gemcitabine increase SOCE in Panc1 cells.

The regulation of chemotherapy-induced apoptosis by ORAI1 and STIM1 suggests that apparently there is a link between chemotherapy drugs and Ca^{2+}-homeostasis. To test this assumption we incubated Panc1 cells in basal medium with or without chemotherapy agents (5-FU (50microM) or gemcitabine (50microM)) for 24 h. At the end of the treatment cells were loaded with Fura2/AM probe and subjected to calcium imaging experiment. We tested whether chemotherapy treatments influence cytosolic calcium levels in Panc1 cells using the same TG-based approach as described above. Interestingly, we have found that both 5-FU and gemcitabine treatments significantly increased SOCE in these cells (Figure 4.10A and B). Furthermore, gemcitabine significantly increased store-operated current in Panc1 cells (Figure 4.10B inset).

We hypothesized that this effect can be the consequence of the increase in expression levels of SOCs by chemotherapy agents. To test this hypothesis, we next assessed the expression levels of ORAI1 and STIM1 following 24 h treatment with 5-FU or gemcitabine. 5-FU and gemcitabine significantly increased the expression of ORAI1 and STIM1 on both mRNA and protein levels (Figure 4.10C and D).

These results correlate well with calcium imaging data (Figures 4.10A and B), suggesting that chemotherapy agents 5-FU and gemcitabine can influence cytosolic calcium levels by upregulation of ORAI1 and STIM1.

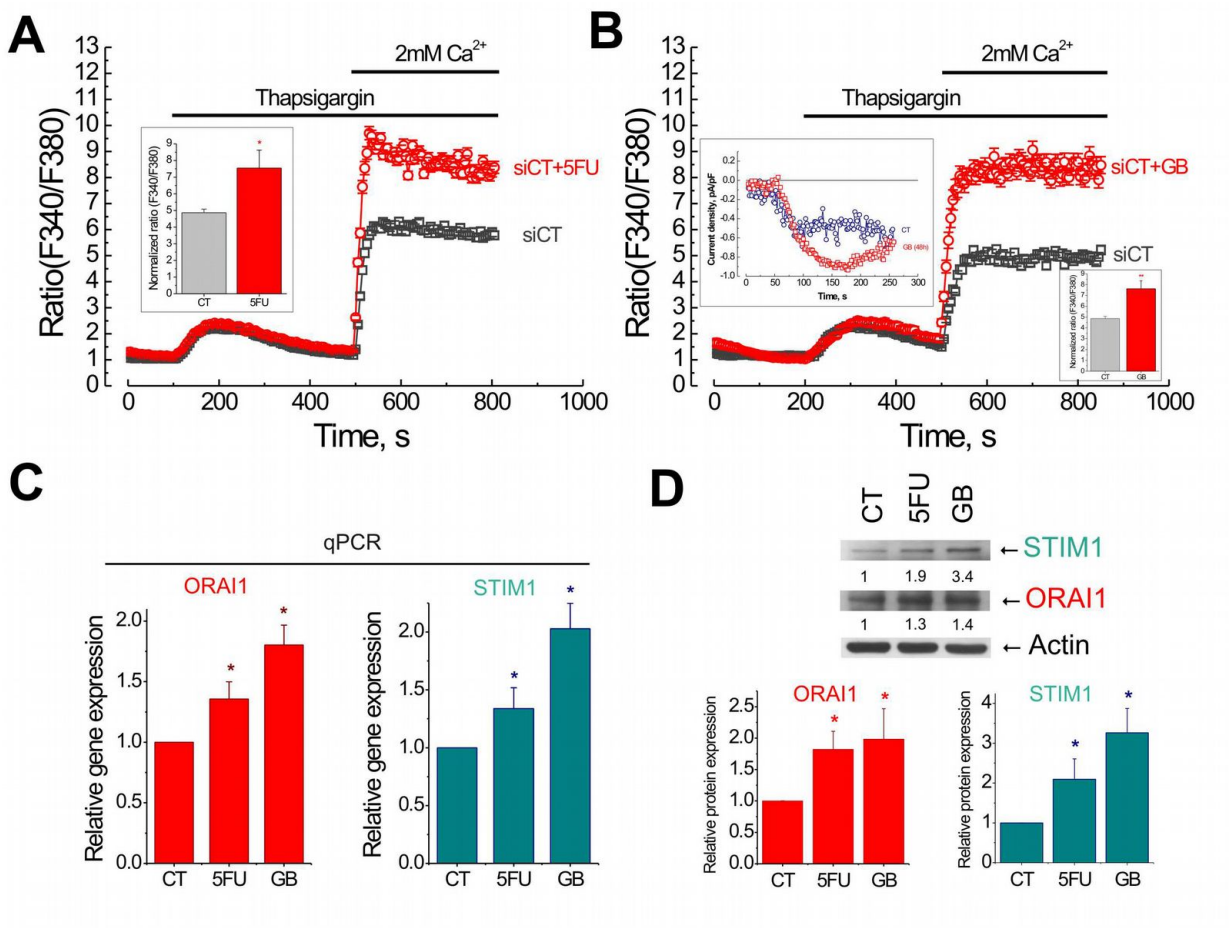

Figure 4.10. Chemotherapy drugs 5-FU and gemcitabine (GB) increase SOCE in Panc1 cells. *(A) 5-FU increase SOCE in Panc1 cells. Panc1 cells were incubated in basal medium with or without 5-FU for 24 h. At the end of the treatment cells were loaded with Fura2/AM probe and subjected to calcium imaging experiment. TG-induced SOCE was analyzed. Inset shows quantification of the TG-induced SOCE amplitude. means±S.E.M. n=4. (B) Gemcitabine increases SOCE and SOC current in Panc1 cells. Panc1 cells were incubated in basal medium with or without gemcitabine for 24 h. At the end of the treatment cells were loaded with Fura2/AM probe and subjected to calcium imaging experiment. TG-induced SOCE was analyzed. Inset shows quantification of the TG-induced SOCE amplitude. means±S.E.M. n=4. Another inset shows SOC current registrations in Panc1 cells untreated or treated with gemcitabine. (C) 5-FU and gemcitabine upregulate ORAI1 and STIM1 mRNAs in Panc1 cells. qRT-PCR detection of expression of ORAI1 and STIM1 in Panc1 cells following 48 h treatment with 5-FU or gemcitabine. (D) 5-FU and gemcitabine upregulate ORAI1 and STIM1 on the protein level in Panc1 cells. Western blot showing the expression of ORAI1 and STIM1 in Panc1 cells following 48 h treatment with 5-FU or gemcitabine. Densitometric quantitations*

for normalized ORAI1 and STIM1 relative to Actin are shown. Bar plots show the quantification of the normalized expression levels of ORAI1 and STIM1 relative to Actin. Data presented as means±S.D. n=3.

In conclusion, we demonstrated that SOC channels, which represent one of the major calcium-entry pathways in non-excitable cells, are expressed and functional in pancreatic cancer cell lines. We showed that the classical major components of SOCs, namely ORAI1 and STIM1 mediate store-operated calcium entry in pancreatic cancer cell lines. We show that both ORAI1 and STIM1 play pro-survival antiapoptotic role in Panc1 cell line, as siRNA mediated knockdown of ORAI1 and/or STIM1 increase apoptosis induced by chemotherapy drugs 5-fluorouracil (5-FU) or gemcitabine. We also demonstrate that both 5-FU and gemcitabine treatments increase SOCE in Panc1 pancreatic adenocarcinoma cell line via upregulation of ORAI1 and STIM1 (Figure 4.11).

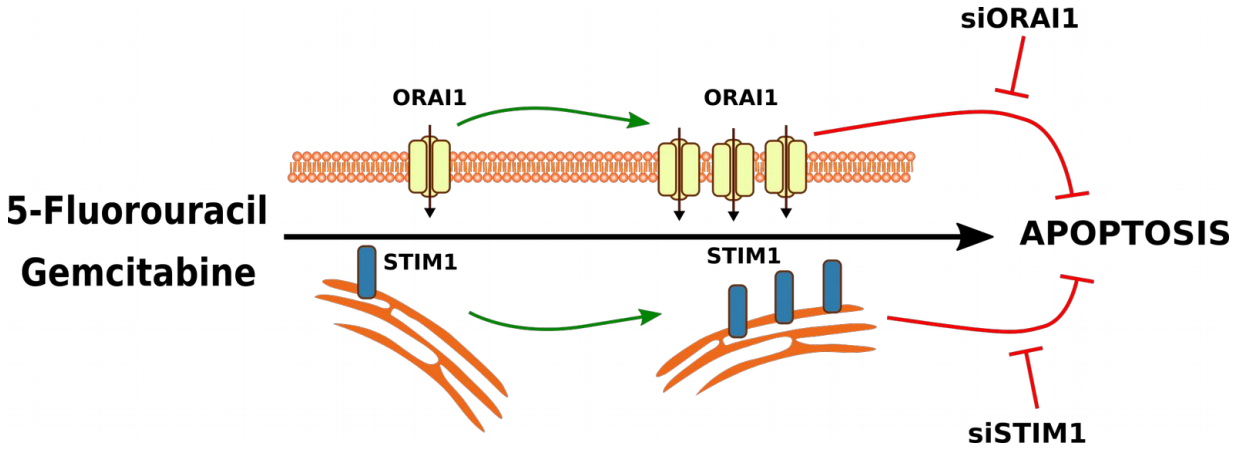

Figure 4.11. ORAI1 and STIM1 in chemotherapy resistance of Panc1 cell line.

Along with ORAI1, other members of ORAI family, namely ORAI2 and ORAI3, have been also reported to mediate SOCE in some cell types. Therefore, our next goal was to examine the expression/ function and role of these channels in PDAC and H6C7 cells.

4.2 ORAI2 and ORAI3 in survival of PDAC and H6C7 cell lines.

4.2.1 ORAI2 and ORAI3 are differentially expressed in PDAC and H6C7 cell lines.

We analyzed the expression of ORAI2 and ORAI3 at the mRNA level in Capan1, ASPC1, Panc1, MiaPaca2, BxPC3 and H6C7 cells by using qRT-PCR technique. We found that both ORAI2 and ORAI3 are expressed in all the cell lines tested, and the level of this expression differs between the cell lines (Figure 4.1).

4.2.2 Involvement of ORAI2 and ORAI3 in SOCE in PDAC and H6C7 cell lines.

Our next goal was to investigate if ORAI2 and ORAI3 contribute to SOCE in PDAC and H6C7 cells. H6C7, AsPC1, BxPC3, MiaPaCa2, Capan1 and Panc1 cell lines were transfected with siCT, siORAI2 or siORAI3. We determined the efficiency of siRNAs transfections in 48 h on mRNA by using qRT-PCR (Figure 4.12).
48 h following siRNAs transfection cells were loaded with Fura2/AM probe and subjected to calcium imaging experiment. We checked whether siRNA-mediated knockdown of ORAI2 or ORAI3 influence cytosolic calcium levels in PDAC and H6C7 cells using thapsigargin (TG).

More specifically, intracellular stores were depleted by TG in nominally calcium free extracellular saline followed by addition of 2mM Ca^{2+} to the cells to initiate the influx via store-operated calcium channels. Surprisingly, when this assay was performed on siORAI2 or siORAI3-transfected PDAC cells (AsPC1, BxPC3, MiaPaCa2, Capan1 and Panc1 cells) calcium entry was significantly increased (Figure 4.12). Moreover, in H6C7 cells siORAI2 treatment also resulted in increased SOCE.

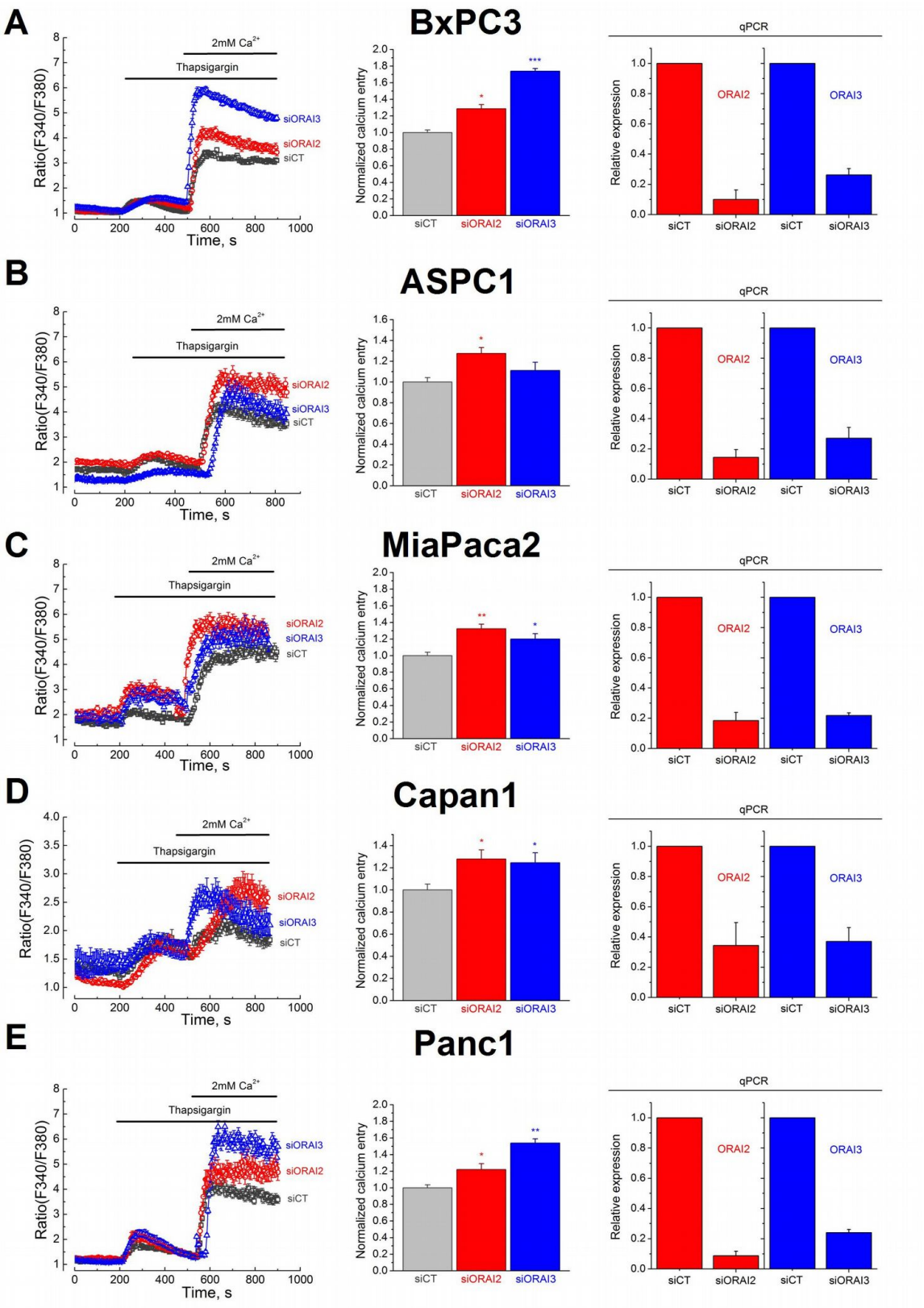

Figure 4.12. The involvement of ORAI2 and ORAI3 in SOCE in PDAC cells. *(A) Knockdown of ORAI2 or ORAI3 increases SOCE in BxPC3 (A), AsPC1 (B), MiaPaCa2 (C), Capan1 (D)*

and Panc1 (E) cell lines. Cells were transfected with siCT, siORAI2 or siORAI3. In 48 h cells were loaded with Fura2/AM probe and subjected to calcium imaging experiment. TG-induced SOCE was analyzed. Quantification of the TG-induced SOCE amplitude in cells transfected with siCT, siORAI2 or siORAI3 is represented. qRT-PCR detection of expression of ORAI2 and ORAI3 in PDAC cells following siRNA transfection is also represented. Data is represented as means±S.E.M.

Alternatively, calcium stores were depleted by preincubation in 0 Ca^{2+} extracellular solution for 30 min. SOCE was stimulated by the addition of 10 mM Ca^{2+} to the cells. This protocol was performed on MiaPaca2, Capan1 and Panc1 cell lines. When this assay was performed on siORAI2- or siORAI3-transfected cells, calcium entry was significantly increased (Figure 4.13).

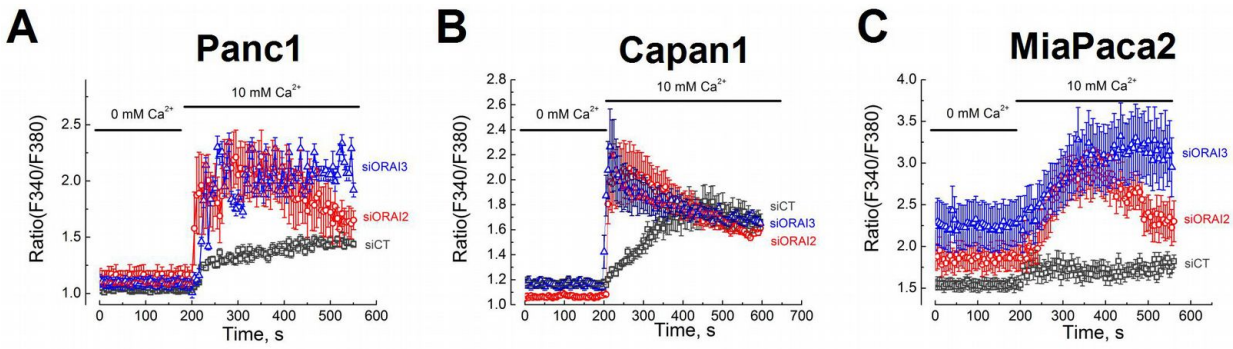

Figure 4.13. The involvement of ORAI2 and ORAI3 in SOCE in PDAC cells. *Knockdown of ORAI2 or ORAI3 increases SOCE in Panc1 (A), Capan1 (B), MiaPaCa2 (C) cell lines. Cells were transfected with siCT, siORAI2 or siORAI3. In 48 h cells were loaded with Fura2/AM probe and subjected to calcium imaging experiment. Data is represented as means±S.E.M. n=30-100.*

In contrast, silencing of ORAI3 in H6C7 cells resulted in slightly decreased SOCE (Figure 4.14).

These experiments show that both ORAI2 and ORAI3 are expressed in PDAC as well as H6C7 cells and are functional proteins, as their knockdown affected SOCE.

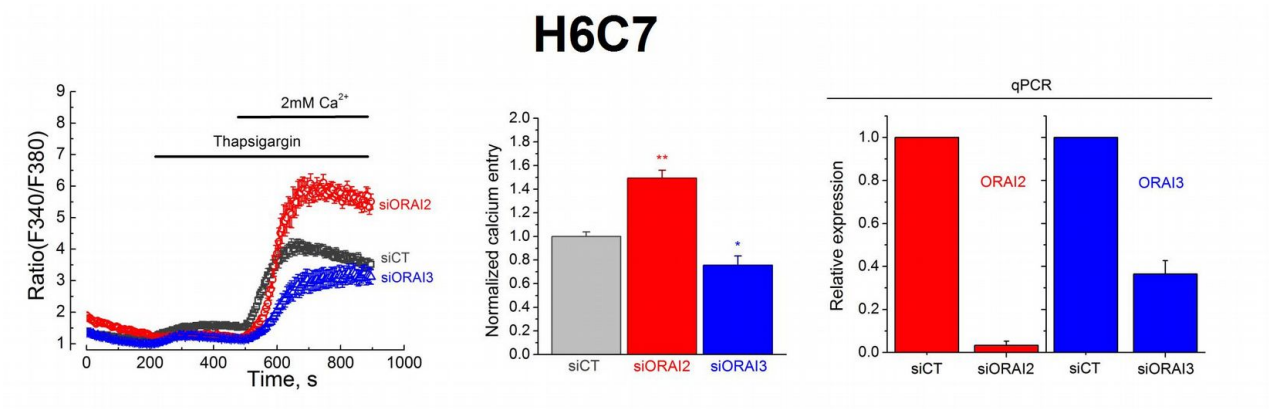

Figure 4.14. The involvement of ORAI2 and ORAI3 in SOCE in H6C7 cells. *(A) Knockdown of ORAI2 increases, whereas knockdown of ORAI3 decreases SOCE in H6C7 cell line. Cells were transfected with siCT, siORAI2 or siORAI3. In 48 h cells were loaded with Fura2/AM probe and subjected to calcium imaging experiment. TG-induced SOCE was analyzed. Quantification of the TG-induced SOCE amplitude in cells transfected with siCT, siORAI2 or siORAI3 is represented. qRT-PCR detection of expression of ORAI2 and ORAI3 in H6C7 cells following siRNA transfection is also represented. Data is represented as means±S.E.M. n=4.*

4.2.3 ORAI2 and ORAI3 influence proliferation of PDAC cells.

Our next goal was to examine how silencing of ORAI2 or ORAI3 will affect PDAC cells proliferation. Panc1, Capan1 or MiaPaca2 cells were plated on 96-well plate, and were left untransfected or transfected with siCT, siORAI2 or siORAI3. Cell proliferation was monitored 96 hours later using the CellTiter 96 Aqueous One Solution cell proliferation assay (MTS assay). Silencing of ORAI2 or ORAI3 reduced the proliferation rate of all three cell lines tested, suggesting that both ORAI2 and ORAI3 regulate proliferation in PDAC cells (Figure 4.15).

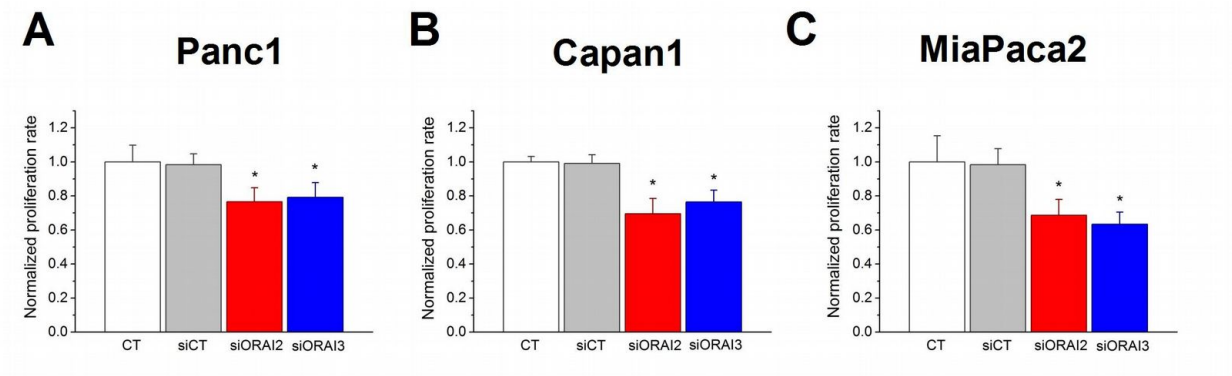

Figure 4.15. ORAI2 and ORAI3 influence proliferation of PDAC cells. *Knockdown of ORAI2 or ORAI3 reduces the proliferation of Panc1 (A), Capan1 (B), and MiaPaCa2 (C) cell lines. Cells were transfected with siCT, siORAI2 or siORAI3. In 96 hours cells cell proliferation was*

monitored using the CellTiter 96 Aqueous One Solution cell proliferation assay. Data is represented as means±S.E.M. n=3.

4.2.4 Silencing of ORAI3 induces morphological changes in PDAC cells.

Interestingly, starting from 2-3 days following siORAI3 transfection several PDAC cell lines showed remarkable changes in their cell morphology (Figure 4.16). Observation of cell morphology by light microscopy revealed the significant increase in cell size, an unusual cell shape as well as cell flattening in siORAI3-transfected MiaPaca2, BxPC3 and Panc1 cells, but not in siCT-transfected cells (Figure 4.16).

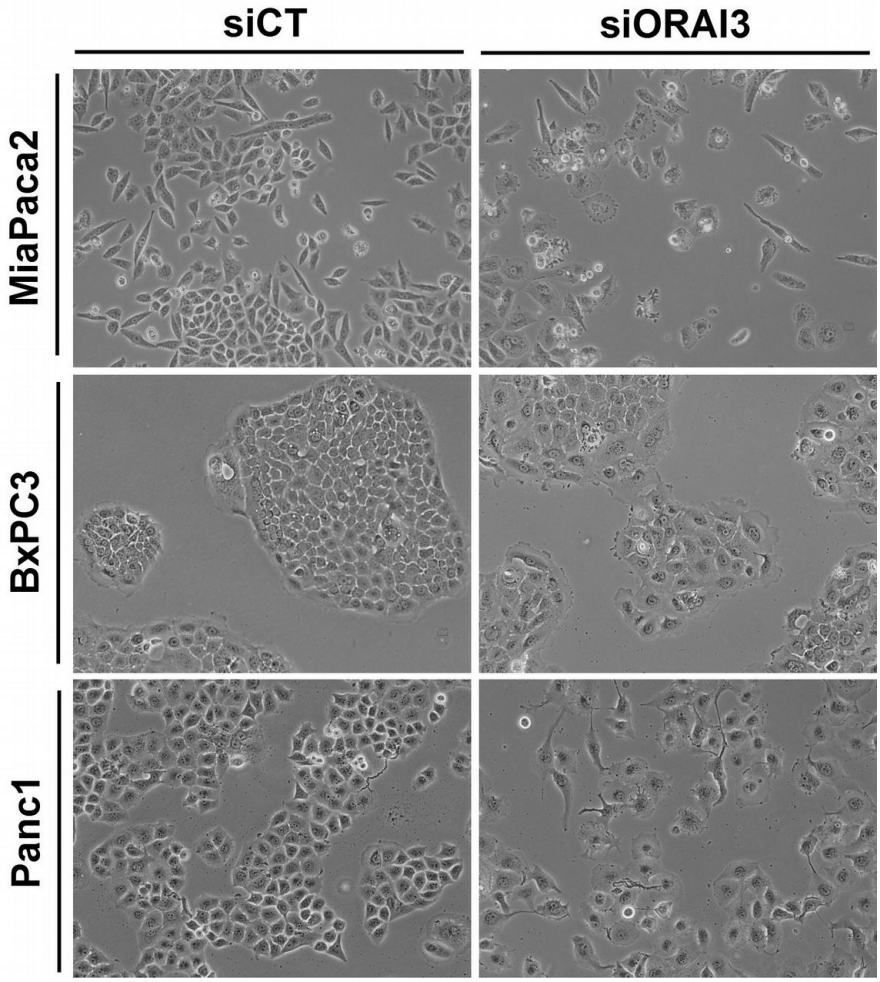

Figure 4.16. ORAI3 induces morphological changes in PDAC cells. *Light microscopy photographs of MiaPaca2, BxPC3 and Panc1 cell lines transfected either with siCT or siORAI3 are represented. The significant increase in cell size, an unusual cell shape as well as cell flattening is clearly visible.*

For the subsequent experiments, we decided to focus on the role of ORAI3 protein in MiaPaca2 cell line, as knockdown of ORAI3 in these cells induced the most significant changes in both morphology and cell number.

4.2.5 ORAI3 controls cell proliferation, cell cycle progression and viability of MiaPaca2 cells.

First, to confirm the role of ORAI3 in MiaPaca2 cell proliferation, we complemented MTS experiments (see Figure 4.15) with simple cell counting experiments as well as immunodetection of a widely used proliferation marker PCNA (proliferating cell nuclear antigen). Consistent with our previous experiments, knockdown of ORAI3 reduced the number of MiaPaca2 cells as well as decreased the expression of PCNA (Figure 4.17).

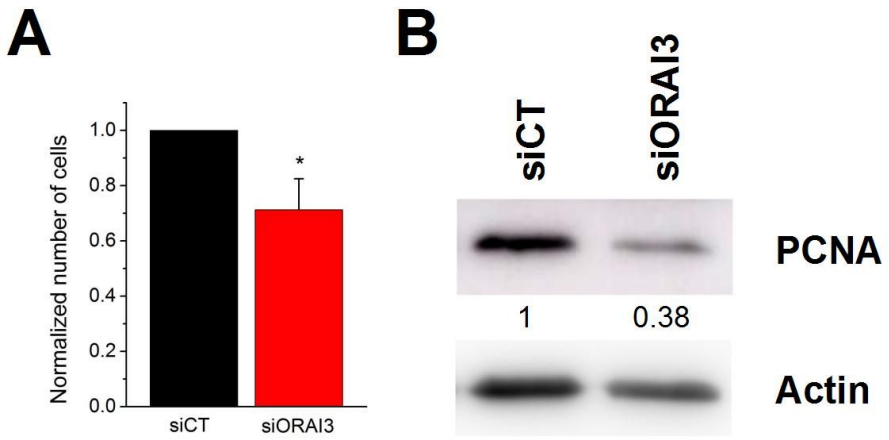

Figure 4.17. ORAI3 controls proliferation of MiaPaca2 cells. *(A) Knockdown of ORAI3 reduces the number of MiaPaCa2 cells, as revealed by simple cell counting 96 hours following siORAI3/siCT transfection. means±S.E.M. n=3. (B) Knockdown of ORAI3 decreases the expression of proliferation marker PCNA (96 hours after siRNA transfection).*

Given that ORAI3 controls MiaPaca2 cell proliferation, we next investigated if ORAI3 regulate cell cycle progression of these cells. Flow cytometry analysis revealed that knockdown of ORAI3 imposed significant alterations in cell cycle distribution. More specifically, the fraction of cells in G2/M phase significantly increased in siORAI3-transfected cells compared to siCT-transfected cells. This effect was accompanied by the concomitant decrease in G0/G1-phase-cell population (Figure 4.18 A and B).

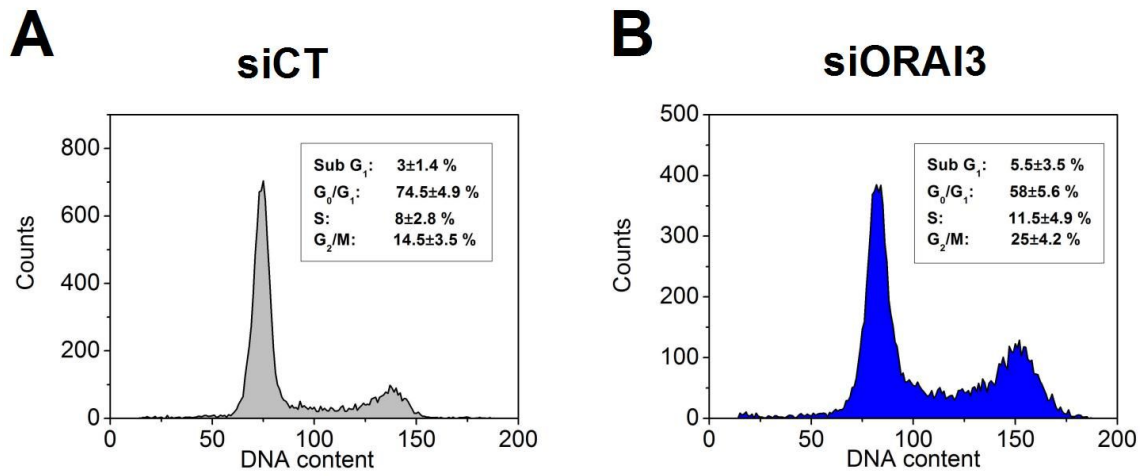

Figure 4.18. ORAI3 controls cell cycle progression in MiaPaca2 cells. *96 hours following siRNA transfection cells were fixed with ethanol and cell cycle distribution was assessed by FACS analysis. Representative histograms of two independent experiments are shown. Values represent the percentages of Sub G1-, G0/G1-, S-, and G2/M-phase cells. Data are represented as means±S.D.*

These data suggest that knockdown of ORAI3 in MiaPaca2 cells induces proliferative blockade via cell cycle arrest in G2/M-phase. Therefore, we next studied the effect of ORAI3 silencing on MiaPaca2 cell viability. To assure the effective knockdown of ORAI3 the cells were retransfected with siORAI3 on the 5th day. Interestingly, starting from 6th-7th days following initial siORAI3 transfection the increased cell death rate became evident (Figure 4.19).

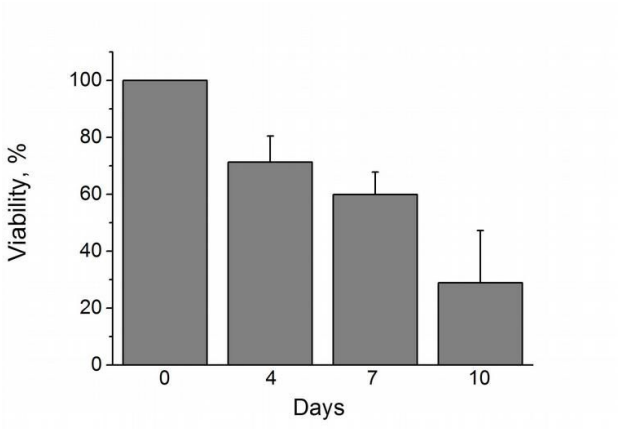

Figure 4.19. Silencing of ORAI3 induces MiaPaca2 cell death. *Cell viability was assessed by MTS assay at different time points. The graph shows mean of two independent experiments. Data are represented as means±S.E.M.*

The number of detached floating cells increased progressively. Apparently, ORAI3 knockdown

induced cell death which progressed slowly, beginning after 6-7 days and continuing until day 13-16, when almost no viable cells were present (Figure 4.19).

We therefore next assessed whether ORAI3 knockdown induces apoptosis in MiaPaca2 cells. Cells were transfected with siORAI3. In 10 days cells were collected, stained with Hoechst 33258 dye and subjected to fluorescence microscopy analysis of nuclear morphology (Figure 4.20).

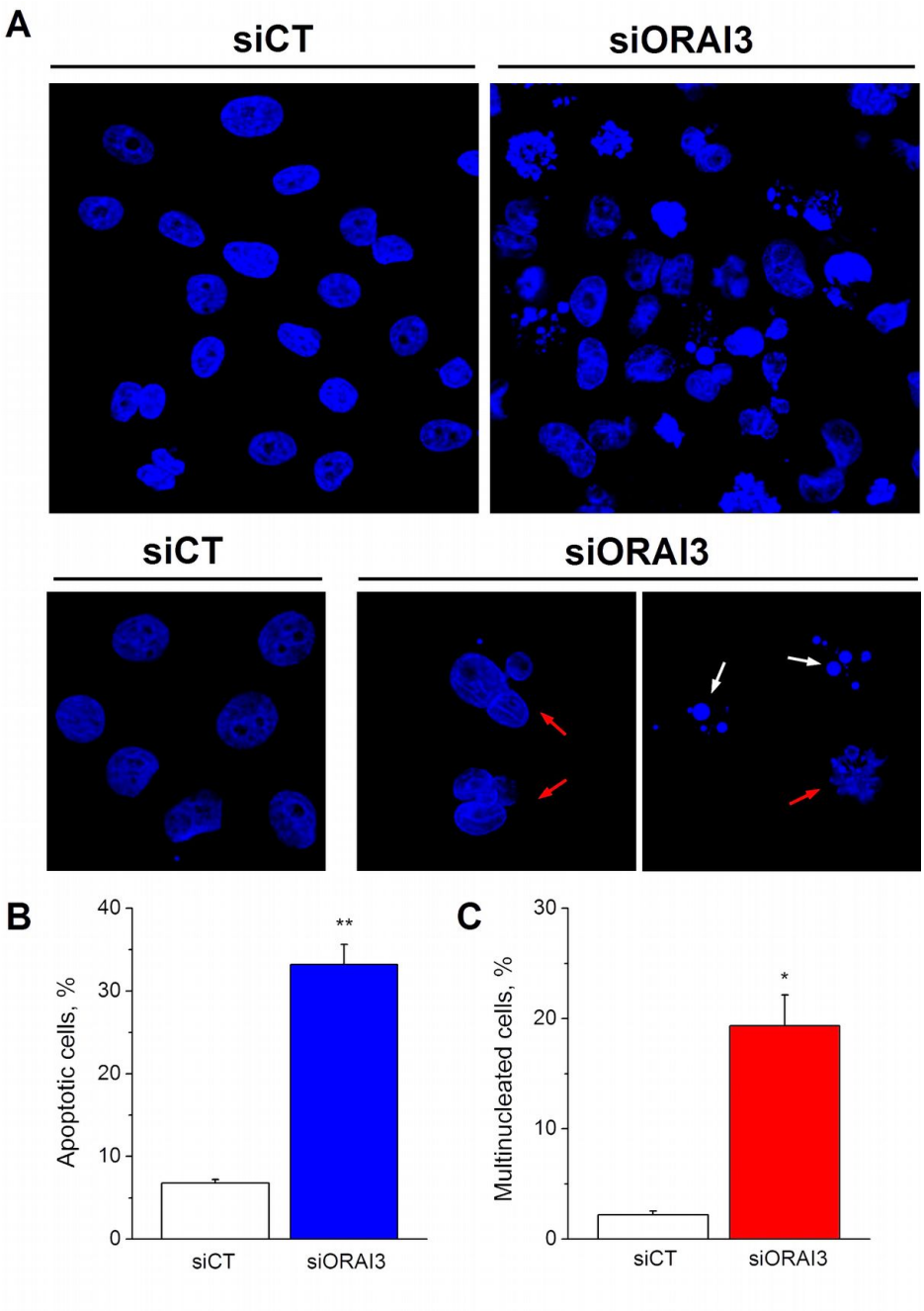

Figure 4.20. Silencing of ORAI3 induces apoptosis and mitotic catastrophe in MiaPaca2 cells. *(A) Nuclear morphology of MiaPaca2 cells 10 days after transfection with siCT or*

siORAI3. Cells undergoing apoptosis (white arrows) or mitotic catastrophe (red arrows) are represented. (B) Quantification of apoptosis in siCT- and siORAI3-treated cells. (C) Percentage of multinucleated cells (undergoing mitotic catastrophe) in siCT- or siORAI3 treated cell populations. Data are represented as means±S.E.M.

Knockdown of ORAI3 resulted in significant changes in nuclear morphology. Interestingly, along with increase in number of "classical" apoptotic condensed/fragmented nuclei we have also observed many cells with multiple nuclei/micronuclei (the morphological hallmark of mitotic catastrophe) (Figure 4.20).

Thus, we conclude that siRNA mediated knockdown of ORAI3 in MiaPaca2 cells results in decreased proliferation and cell cycle arrest (in G2/M phase) at early stages, as well as in cell death by mitotic catastrophe and apoptosis at later stages.

4.2.6 Knockdown of ORAI3 reduces clonogenic survival of MiaPaca2 cells.

We next checked if ORAI3 is important for cell clonogenic survival. The cells were plated on a 6-well plate at a density of 500 cells/well and transfected with siORAI3 or siCT. The next day the medium was changed and cell were left to recover for 10 days. The cell colonies were then photographed and counted. Knockdown of ORAI3 resulted in a significant reduction in clonogenic survival (Figure 4.21), again suggesting the prosurvival role for ORAI3 in MiaPaca2 cells.

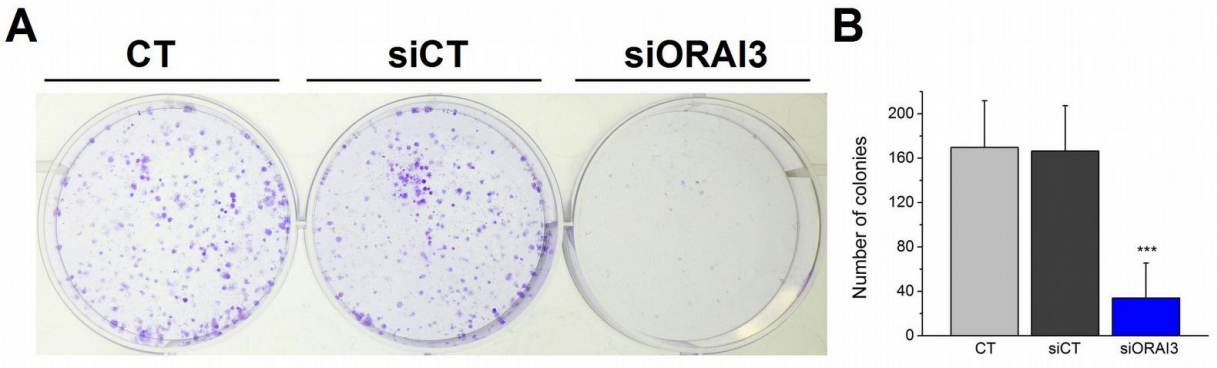

Figure 4.21. Knockdown of ORAI3 reduces clonogenic survival of MiaPaca2 cells. *(A) Clonogenic survival assay showing that knockdown of ORAI3 impairs long term survival of MiaPaca2 cells. (B) Graph representing the average number of colonies for each condition (means±S.D.).*

4.2.7 Knockdown of ORAI3 inhibits tumor xenograft growth *in vivo*.

To test whether silencing of ORAI3 could impair tumor growth *in vivo*, we conducted *in vivo* experiments with nude mice bearing s.c. MiaPaca2-derived xenografts. To achieve ORAI3 knockdown in tumors, siORAI3 was injected i.p. on a daily basis. Animals from control group were injected with siCT. Tumor growth was significantly reduced in siORAI3-treated animals compared to control (Figure 4.22). This impairment in tumor growth correlated with downregulation of ORAI3 mRNA expression in tumors from siORAI3-treated mice (Figure 4.22).

This result suggests, that targeting ORAI3 could potentially represent a beneficial strategy in pancreatic cancer treatment.

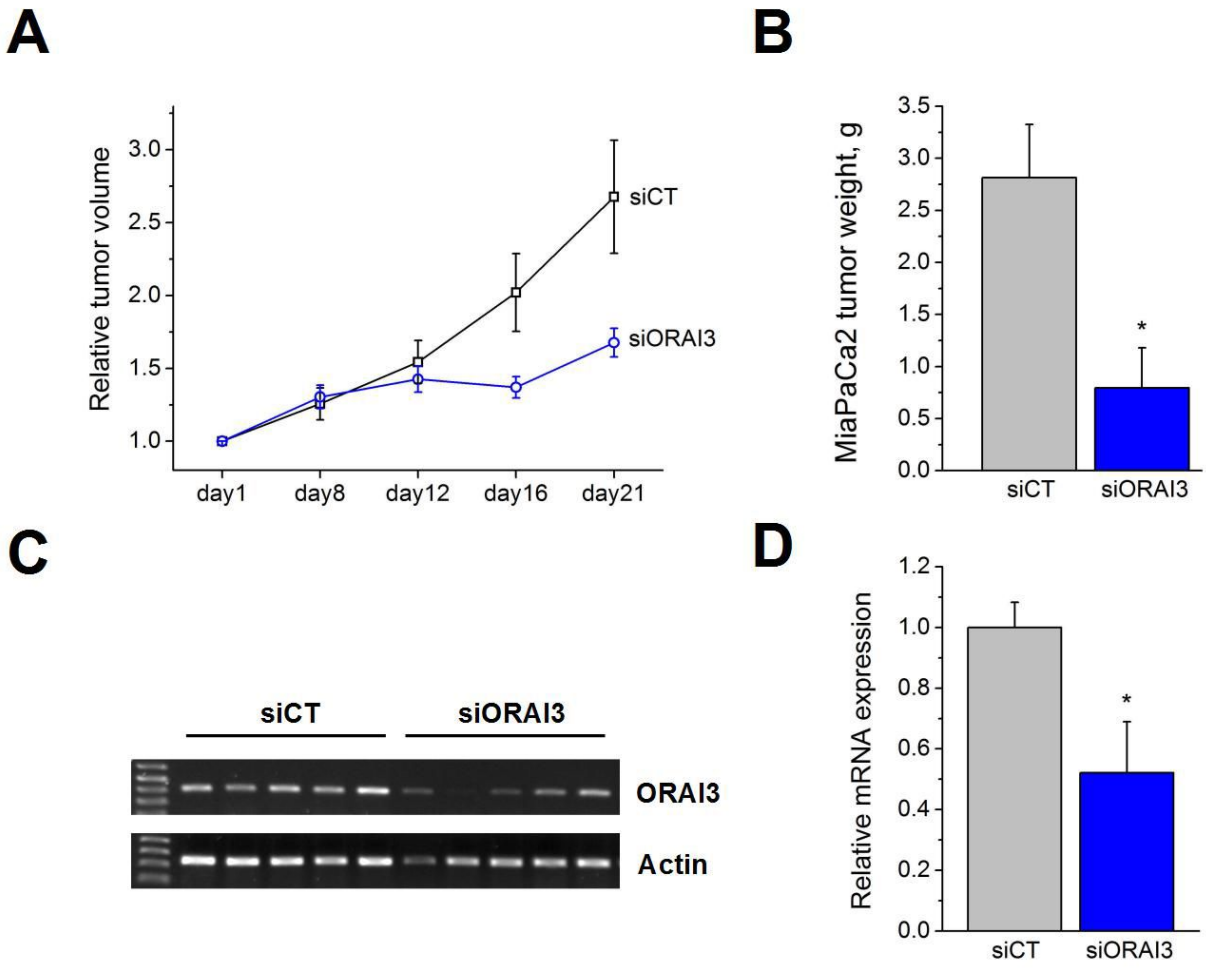

Figure 4.22. Knockdown of ORAI3 inhibits tumor xenograft growth in vivo. *(A) Tumor growth curves for both siCT- and siORAI3-treated mice are represented. (B) Weight of excised tumors (means±S.D.). (C) RT-PCR experiment showing ORAI3/Actin expression in siCT- and*

siORAI3-treated mice. (D) Relative expression of ORAI3 mRNA normalized to Actin is shown (means±S.E.M).

In conclusion, we demonstrated that ORAI2 and ORAI3 channels are differentially expressed in PDAC as well as H6C7 cell lines and are functional proteins, as their knockdown affected SOCE. More spesifically, knockdown of ORAI3 increased SOCE in PDAC cell lines, while decreasing SOCE in H6C7 cell line. Silencing of ORAI2 increased amplitude of SOCE in all cancer and normal cell lines. We also showed the role of ORAI3 in proliferation, cell cycle and viability of MiaPaca2 cells. We further demonstrated that silencing of ORAI3 impairs pancreatic tumor growth in vivo, suggesting that ORAI3 could represent a potential therapeutic target in PDAC treatment (Figure 4.23).

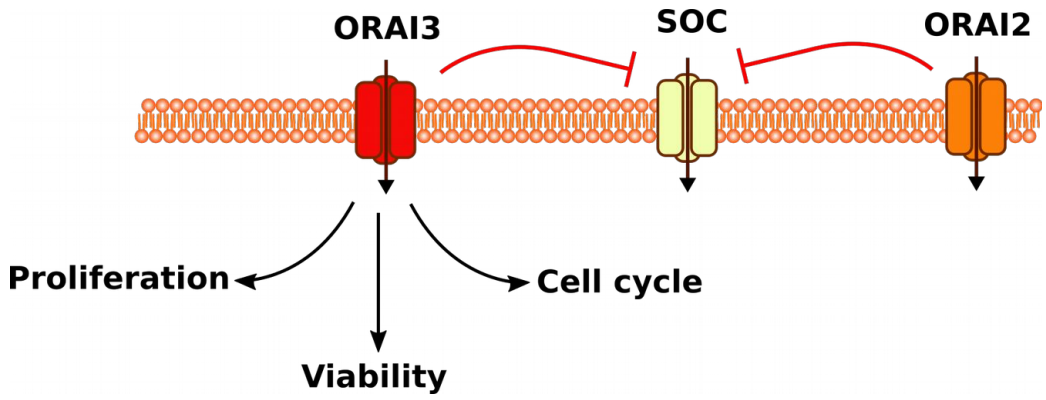

Figure 4.23. ORAI3 controls cell proliferation, cell cycle progression and viability of MiaPaca2 cells.

4.3 Calcium signalling in pancreatic stellate cells

4.3.1 Calcium-permeable channels in pancreatic stellate cells.

As it is discussed in the "Introduction" part of this thesis, pancreatic stellate cells (PSC) represent the most important drivers of desmoplasia in pancreatic cancer. In health, the cells are in their quiescent phase, with abundant vitamin A containing lipid droplets in their cytoplasm, whereas during injury PSCs become activated, lose their cytoplasmic lipid droplets, transform into a myofibroblast-like phenotype, proliferate and synthesise excessive amounts of extracellular matrix proteins that comprise fibrous tissue.

Thus, it's clear that PSCs activation could potentially contribute to pancreatic cancer progression as well as resistance. Therefore, it's important to understand the physiology of PSCs and study the cellular mechanisms that control PSCs activation.

PSC activation results among others in proliferation, migration, resistance to apoptosis (which accounts for radio- and chemo-resistance) and cross-communication with the cancer cells. Many of these processes are known to be typically controlled and/or modified by calcium and calcium-permeable ion channels. However, the information on the expression/function as well as role of calcium-permeable channels in PSCs is still very limited.

We hypothesized that calcium as well as calcium-permeable channels could be involved in the regulation of PSCs activation. Further, calcium-permeable channels could be implicated in proliferation, migration and resistance to apoptosis of activated PSCs.

To study these hypotheses, we have chosen human immortalized PSCs (RLT-PSCs) as well as mouse primary PSCs.

As revealed by Nile Red staining, freshly isolated mouse PSCs exhibit abundant lipid droplets in the cytoplasm upon isolation and in early culture (Figure 4.24). However, all PSCs lose their lipid droplets after trypsinisation at the first passage and transform into a myofibroblast-like phenotype (as revealed by alpha-smooth muscle actin immunostaining) (Figure 4.24). Thus, freshly isolated PSCs as well as cells in early culture represent quiescent PSCs, whereas alpha-smooth muscle actin-expressing cells represent activated PSCs.

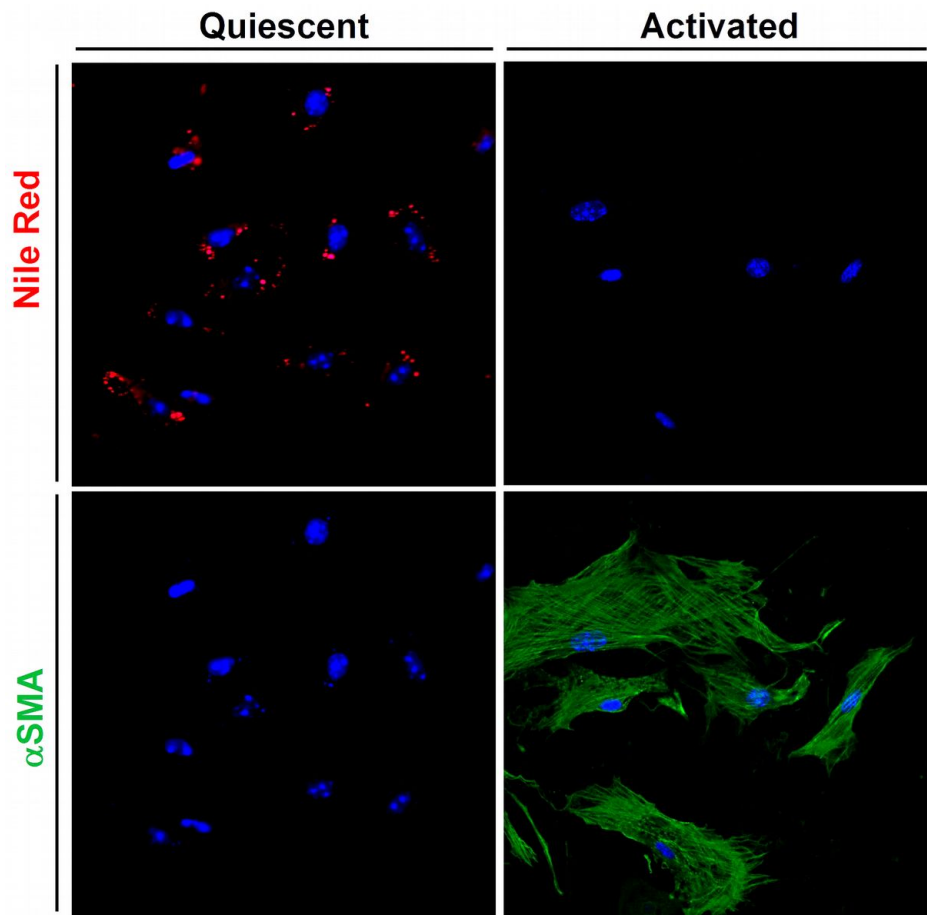

Figure 4.24. Mouse PSCs. *Activated and quiescent mouse PSCs are shown. PSCs in early culture (24 hours following extraction) exhibit Nile Red-positive lipid droplets, while PSCs in late culture (following trypsinization) loose lipid droplets and are positive to alpha-SMA.*

Given that store-operated calcium channels (SOCs) represent one of the major calcium-entry pathways in non-excitable cells, we first sought to examine if ORAIs, STIMs, TRPCs and TRPV6 are present in quiescent and activated primary mouse PSCs as well as human immortalized RLT-PSCs.

By using RT-PCR and qRT-PCR techniques we found that ORAI1,2,3, and STIM1 are differentially expressed in quiescent and activated mouse pancreatic stellate cells (Figure 4.25A). More specifically, activated PSCs are characterized by the increased expression of ORAI1,2,3, and STIM1 compared to quiescent PSCs. We have also detected mRNAs of TRPC1, TRPC2, TRPC3, TRPC4 and TRPC6 in activated mouse PSCs (these experiments were performed in collaboration with Nikolaj Nielsen, University of Munster, Germany). Similar to mouse PSCs, human RLT-PSCs also showed expression of ORAIs, STIMs, and TRPC1 (Figure 4.25B). None of the cell lines showed detectable expression levels of TRPV6 channel.

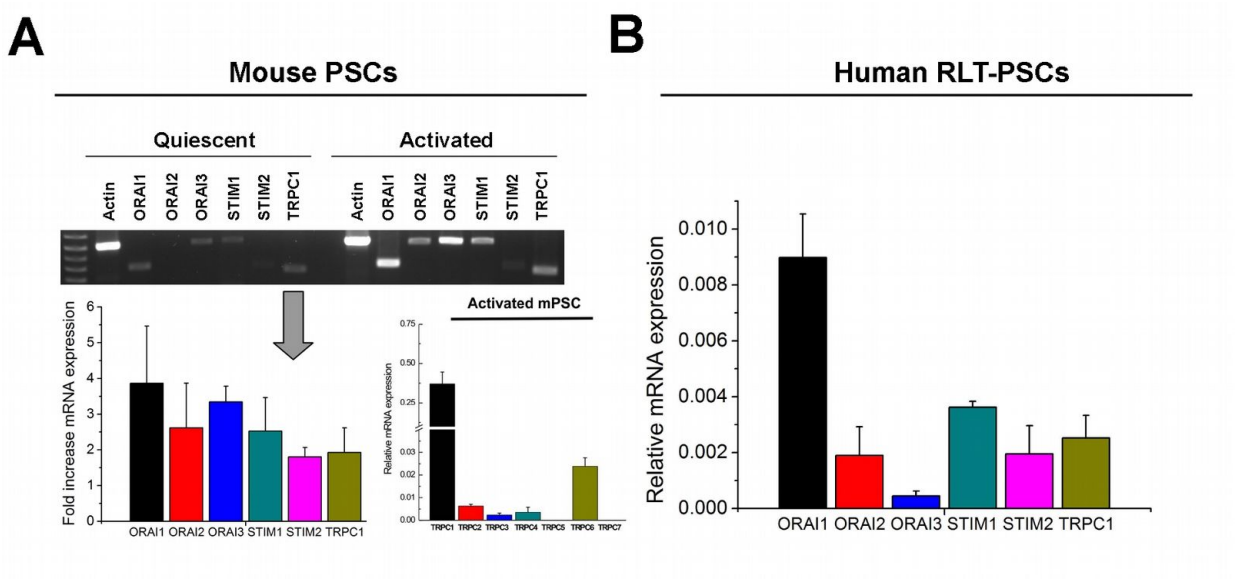

Figure 4.25. Expression of ORAIs, STIMs and TRPCs in mouse and human PSCs. *(A) Expression of ORAIs, STIMs and TRPCs in quiescent and activated mouse PSCs. (B) Expression of ORAIs, STIMs and TRPC1 in human RTL-PSCs. Data are represented as means±S.E.M. n=3.*

4.3.2 Store-operated Ca^{2+}-entry in mouse and human PSCs.

In line with PCR data, SOCE induced by TG was significantly lower in quiescent than in activated mouse PSCs (Figure 4.26A). Human RLT-PSCs were characterized by a prominent SOCE in response to TG (Figure 4.26B).

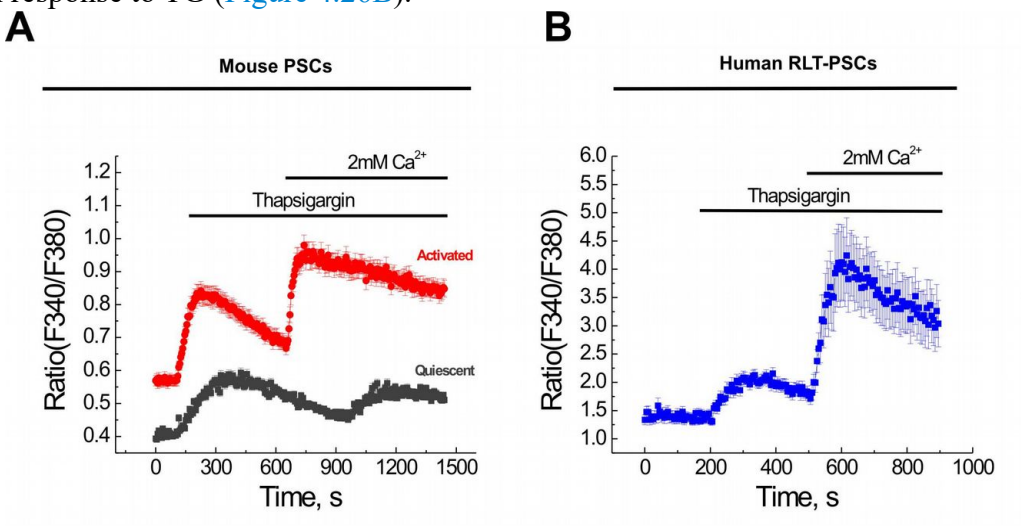

Figure 4.26. SOCE in mouse and human PSCs. *(A) SOCE induced by TG in quiescent and activated mouse PSCs. (B) SOCE induced by TG in human RTL-PSCs. Data are represented as means±S.E.M. n=15-50.*

Next, we investigated if ORAI1 and STIM1 are involved in SOCE in PSCs by using siRNA-approach (Figure 4.27A and B).

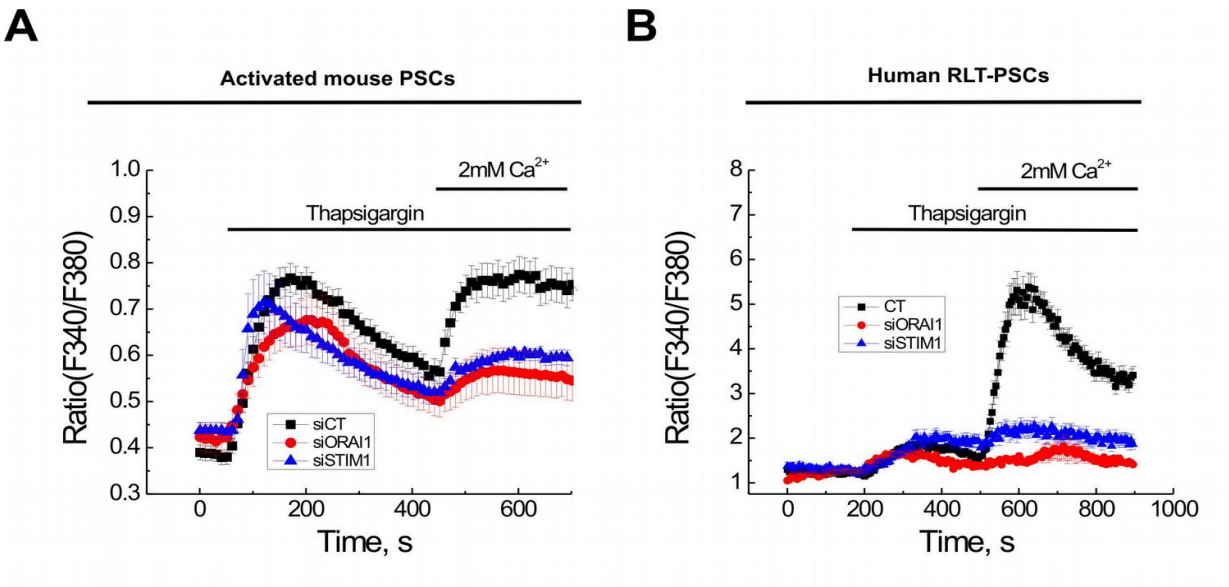

Figure 4.27. ORAI1 and STIM1 are involved in SOCE in mouse and human PSCs. *(A) Knockdown of ORAI1 or STIM1 inhibits SOCE in activated mouse PSCs. (B) Knockdown of ORAI1 or STIM1 inhibits SOCE in human RLT-PSCs. Data are represented as means±S.E.M. n=15-50.*

Activated mouse PSCs or human RLT-PSCs were transfected with siCT, siORAI1 or siSTIM1. 48 h following siRNAs transfection cells were loaded with Fura2/AM probe and subjected to calcium imaging experiment. We checked whether siRNA-mediated knockdown of ORAI1 or STIM1 influence cytosolic calcium levels in PSCs using thapsigargin (TG). More specifically, intracellular stores were depleted by TG in nominally calcium free extracellular saline followed by addition of 2mM Ca^{2+} to the cells to initiate the influx via store-operated calcium channels. When this assay was performed on siORAI1- or siSTIM1-transfected PSCs cells, calcium entry was significantly reduced (Figure 4.27A and B). These results suggest that ORAI1 and STIM1 mediate SOCE in activated mouse PSCs and human RLT-PSCs.

4.3.3 Calcium homeostasis in quiescent and activated mouse PSCs.

We next analyzed cytosolic calcium levels in quiescent and activated mouse PSCs. The cells were loaded with Fura2/AM probe and subjected to calcium imaging experiment. Basal level of

cytosolic calcium in the presence of 2mM Ca^{2+} in extracellular medium was analyzed. Quiescent mouse PSCs showed significantly lower level of cytosolic calcium compared to activated PSCs (Figure 4.28A).

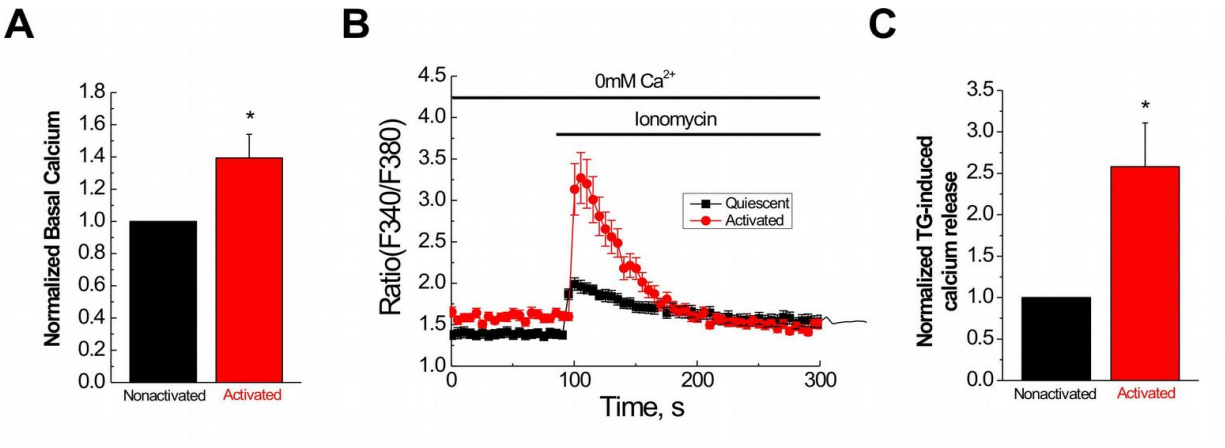

Figure 4.28. Intracellular calcium in quiescent and activated mouse PSCs. *(A) Cyosolic calcium in quiescent and activated mouse PSCs. (B) Ionomycin-induced calcium release from intracellular stores in quiescent and activated mouse PSCs. (C) Calcium release from TG-sensitive intracellular stores in quiescent and activated mouse PSCs. Data are represented as means±S.E.M.*

Further, we analyzed the level of calcium in intracellular stores in both quiescent and activated PSCs using Ca^{2+} ionophore ionomycin as well as SERCA inhibitor thapsigargin (TG). Application of 5 μM ionomycin in the absence of extracellular Ca^{2+} caused a transient increase in cytosolic calcium which was significantly less in quiescent compared to activated PSCs (Figure 4.28B). Consistent with this, quiescent PSCs showed significantly lower TG-induced calcium release compared to activated PSCs (Figure 4.28C).

In conclusion, we demonstrated that quiescent PSCs are characterized by significantly lower levels of both basal cytosolic calcium and calcium in intracellular stores compared to activated PSCs. Moreover, activated PSCs show higher expression levels of major components of store-operated calcium channels, namely ORAI 1,2,3 proteins as well as STIM1 protein (Figure 4.29). This elevated expression correlates well with increased amplitude of SOCE in activated cells compared to quiescent PSCs. Our data clearly

demonstrate that ORAI1 and STIM1 mediate SOCE in activated PSCs, as knockdown of either of these proteins significantly reduces SOCE.

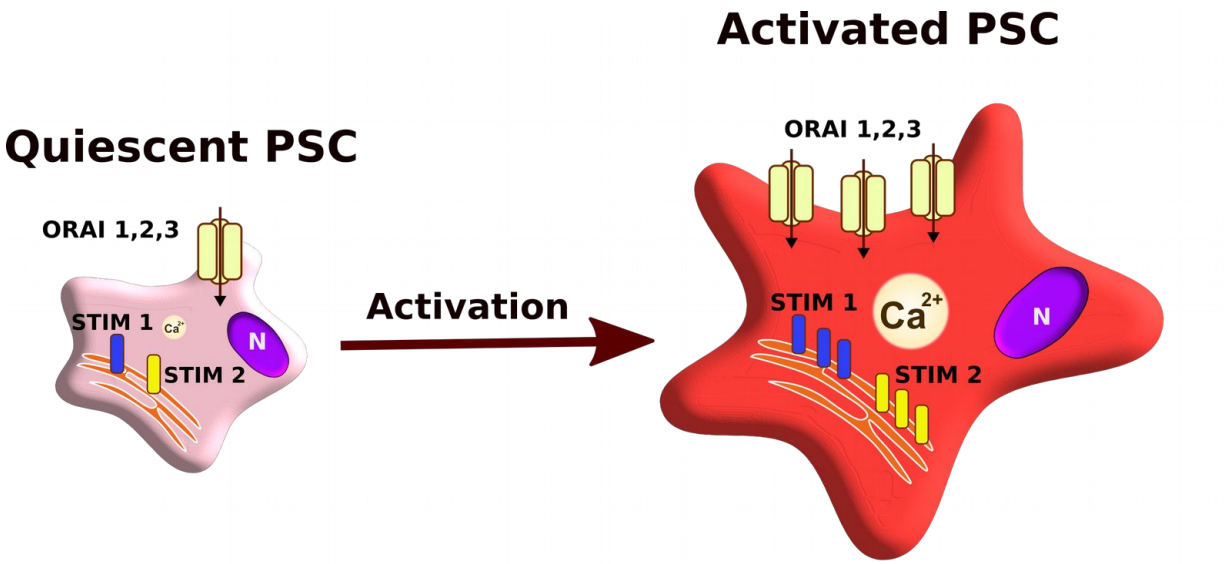

Figure 4.29. Calcium signalling in quiescent and activated mouse PSCs.

Discussion

5.1 ORAI1 and STIM1 in chemotherapy resistance of PDAC cell lines.

In this study, we provide evidence that ORAI1 and STIM1 mediate SOCE in pancreatic adenocarcinoma cell lines. We show that both ORAI1 and STIM1 play pro-survival antiapoptotic role in pancreatic adenocarcinoma cell lines, as siRNA mediated knockdown of ORAI1 and/or STIM1 increase apoptosis induced by chemotherapy drugs 5-FU or gemcitabine. We also demonstrate that both 5-FU and gemcitabine treatments increase SOCE in Panc1 pancreatic adenocarcinoma cell line apparently via upregulation of ORAI1 and STIM1.

Numerous studies demonstrate the important role of SOCE in a plethora of cellular processes and functions in different cell types, including endothelial cell proliferation (Abdullaev et al., 2008), smooth muscle migration (Bisaillon et al., 2010; Potier et al., 2009) and skeletal muscle development and contraction (Stiber et al., 2008). Moreover, SOCE has been implicated in a number of pathological processes typical for cancer, such as breast tumor cell migration and metastasis (Yang et al., 2009), human glioblastoma invasion (Motiani et al., 2013) and apoptosis in a variety of cell types (Flourakis et al., 2010; Henke et al., 2013; Khadra et al., 2011; Liu et al., 2011). Therefore, the identification of the molecular nature of SOCs in PDAC is of great importance as it can reveal novel approaches for treating pancreatic cancer through targeting SOCE-dependent processes.

The evidence presented here clearly demonstrates that ORAI1 and STIM1 are expressed in five PDAC cell line tested namely Capan1, ASPC1, Panc1, MiaPaca2 and BxPC3 as well as in “normal” human pancreatic ductal epithelial cells H6C7. It should be noted, that “normal” H6C7 cells show relatively low levels of ORAI1 and STIM1 expression compared to several cancer cell lines, in particular Capan1 and Panc1. This result indirectly suggests that these cancer cells upregulate ORAI1 and STIM1 to protect themselves against apoptosis.

Interestingly, despite the suggested important role of SOCs in the regulation of apoptosis, metastasis and invasion in different cancers (Flourakis et al., 2010; Henke et al., 2013; Khadra et al., 2011; Liu et al., 2011; Motiani et al., 2013; Yang et al., 2009), their role in PDAC cells have never been studied.

Our results revealed the antiapoptotic role of ORAI1 and STIM1 in Panc1 cells. We showed that siRNA-mediated knockdown of ORAI1 and/or STIM1 increase apoptosis induced by 5-FU or gemcitabine in Panc1 cells.

Previous studies reported conflicting results in regard to the role of SOCE, ORAI1 and STIM1 in apoptosis regulation. Some reports suggest that SOCE, ORAI1 as well as STIM1 contribute

to apoptosis induced by various stress stimuli (Flourakis et al., 2010; Henke et al., 2013), while others demonstrate their pro-survival antiapoptotic role (Khadra et al., 2011; Li et al., 2013; Liu et al., 2011). Indeed, ORAI1 was reported to contribute to the establishment of an apoptosis-resistant phenotype in prostate cancer cells and ORAI1 knockdown protected LNCaP cells against TG- or oxaliplatin/cisplatin-induced apoptosis (Flourakis et al., 2010). In line with this, pharmacological SOCE inhibition or STIM1 knockdown was demonstrated to inhibit hydrogen peroxide-induced apoptosis in HT22 cells via alleviation of intracellular Ca^{2+} overload, restoration of the mitochondrial membrane potential and decrease of cytochrome C release (Rao et al., 2013). In contrast, pharmacological inhibition of SOCE or STIM1 downregulation was shown to enhance apoptosis induced by cisplatin in non-small cell lung cancer cells (Li et al., 2013). Further, it was reported that Orai1-driven Ca^{2+}-entry delays the induction of the CD95-mediated apoptotic signal in leukemic T-cell lines through the translocation of the Ca^{2+}-dependent protein kinase C (PKC) β2 to the death-inducing signaling complex and its subsequent inactivation in T-cells. This prevented CD95-mediated caspase activation and delayed delivery of the apoptotic signal (Khadra et al., 2011).

Although it seems that these studies represent contradictory results, there are several clues which can explain this discrepancy. First, it is well known that different cells are characterized by different set and level of expressed genes, in particular ORAI1 and STIM1 (Kiviluoto et al., 2011). Thus, the contradiction could arise from the different cell types used in these studies. Second, both ORAI1 and STIM1 were shown to regulate the activity of a number of intracellular effectors including PKC β2 (Khadra et al., 2011), PKC δ (Limnander et al., 2011), extracellular signal-related kinases 1 and 2 (ERK 1/2) (Soltoff and Lannon, 2013), calpains as well as cytoplasmic kinase Pyk2 (Chen et al., 2011). Further, the expression/function of ORAI1 and STIM1 were reported to be regulated by several protein kinases, such as serum and glucocorticoid-inducible kinase 1 (SGK1) and AMP activated kinase (AMPK) (Lang et al., 2012), by transcription factor nuclear factor κB (NF-κB) (Eylenstein et al., 2012) as well as by cytoskeleton reorganization (Vanoverberghe et al., 2012). Moreover, both ORAI1 and STIM1 were shown to have SOCE independent functions. It was reported that ORAI1 could stimulate mammary tumorigenesis by store- and STIM1-independent pathway involving secretory pathway Ca^{2+}-ATPase, SPCA2 (Feng et al., 2010). Likewise, STIM1 was suggested to be a general stress sensor (Soboloff et al., 2011) capable of interacting with multiple molecular targets, including ORAI1 (Park et al., 2009), TRPC1(Yuan et al., 2007) and Cav1.2 channels

(Park et al., 2010). Considering such a complex regulatory network and the proven role for many of ORAI1/STIM1 targets in both pro-survival and pro-apoptotic processes, the role of SOCE, ORAI1 and STIM1 in each particular cell type could differ depending on the signaling pathway activated.

We noticed that STIM1-knockdown increased chemotherapy-induced apoptosis more effectively than ORAI1-knockdown, although the difference did not reach statistical significance. This result points to the potential ORAI1-independent role of STIM1 in apoptosis regulation in Panc1 cells.

Further, the effect of ORAI1- and/or STIM1-knockdown on apoptosis in Panc1 cells was more pronounced in the case of treatment with gemcitabine compared to 5-FU. This result could point to increased calcium-dependency of gemcitabine mechanisms of action compared to 5-FU.

Interestingly, our results also suggest that both 5-FU and gemcitabine could affect intracellular calcium homeostasis. Indeed, both 5-FU and gemcitabine treatments increase SOCE in Panc1 cells and upregulate ORAI1 and STIM1. This result suggests that chemotherapy treatments could have calcium-dependent effects, which are unrelated to the primary DNA-targeting mechanisms of their action. Moreover, these calcium dependent effects could potentially contribute to the final efficacy of the drugs in cancer therapy. Further experiments are needed to understand the mechanism of this effect. We hypothesize that during chemotherapy treatments cells upregulate SOCs to resist apoptosis. One of the possible pathways could involve activation of transcription factor NF-kB, and subsequent inhibition of apoptosis. Downregulation of SOCs in these conditions will decrease the level of NF-kB activation and will make cells more sensitive to apoptosis induction.

In conclusion, given the important role of ORAI1 and STIM1 in a number of cellular processes and functions in different cell types, they could be potentially considered as a promising target in anticancer therapy. However, the vast heterogeneity in the reported roles for ORAI1 and STIM1 in different cell types confirms that their final effect on apoptosis could depend on multiple factors, such as cell type, nutrient and growth factor availability, pathology (cancer, inflammation, etc.) as well as intracellular signaling pathways involved. Therefore, future research is indispensable to better understand the specific mechanisms of apoptosis regulation by SOCE, ORAI1 and STIM1 to finally conclude if their modulators could be effective in cancer treatment in each particular case.

5.2 ORAI3 in survival of PDAC cell lines.

In our study we also demonstrate the expression of two highly conserved paralogues of ORAI1, namely ORAI2 and ORAI3 in PDAC as well as H6C7 cell lines. Both ORAI2 and ORAI3 are differentially expressed in all the cell lines tested. The levels of ORAI3 are elevated in cancer cell lines compared to H6C7 cell line. The levels of ORAI2 are elevated in 3 out of 5 cancer cell lines. These expression patterns indirectly suggest that both ORAI2 and ORAI3 could have prosurvival role in cancer cells.

Considering that ORAI2 and ORAI3 have been suggested to mediate SOCE in different cell types, we tested if these channels are implicated in SOCE in PDAC cells. Interestingly, knockdown of ORAI3 increased SOCE in PDAC cell lines, while decreasing SOCE in H6C7 cell line. Silencing of ORAI2 increased amplitude of SOCE in all cancer and normal cell lines. These results suggest that both ORAI2 and ORAI3 act as SOCE inhibitors in cancer cells, whereas in normal cells ORAI3 (in contrast to ORAI2) could potentially contribute to SOCE. Although it seems that these results conflict with previously published data, we can propose a possible explanation of the effects observed. Indeed, ORAI2 has been suggested to mediate SOCE in immune cells lacking ORAI1 (Gwack et al., 2008; Vig et al., 2008). However, no effect of ORAI2 silencing on SOCE has been detected by other groups (Baryshnikov et al., 2009; Takahashi et al., 2007). ORAI3 has been shown to mediate SOCE in estrogen receptor -positive breast cancer cells as well as in non small cell lung adenocarcinoma cells (Ay et al., 2013; Motiani et al., 2010). However, along with ORAI1, ORAI3 has been reported to be an important component of store-independent arachidonate-regulated Ca^{2+} (ARC) entry, as well as of a store-independent leukotrieneC4-regulated Ca^{2+} (LRC) entry (González-Cobos et al., 2013; Shuttleworth, 2012). Recently, the increased expression of ORAI3 in prostate cancer has been demonstrated to promote cell proliferation by increasing ARC-mediated calcium entry, as well as confer apoptosis resistance by decreasing SOCE (Dubois et al., 2014). It has been proposed that ARC channel represents heteromultimeric complex of ORAI1 and ORAI3 proteins, whereas SOC channel represents homomultimeric complex of ORAI1 proteins. Therefore, increase in ORAI3 expression favors the increased formation of heteromeric ORAI1/ORAI3 complexes, and at the same time impedes the formation of homomeric ORAI1 complexes. Thus, it's possible that in PDAC cells ORAI3 could form heteromeric complexes with ORAI1, and in this way decreases SOCE. Silencing of ORAI3 “liberates” ORAI1 from heteromeric complexes, so more homomeric ORAI1 complexes could be formed, leading to SOCE increase.

To confirm this hypothesis future research on ARC channels in PDAC is needed.

Interestingly, in "normal" H6C7 cells ORAI3 contributes to SOCE in contrast to cancer cells. This result suggests that ORAI3 could change the "specialization" during malignant transformation. Thus, cancer and normal cells could utilize ORAI3 in different ways and possibly for different purposes.

Further, we have shown that silencing of ORAI3 in MiaPaca2 cancer cells results in decreased proliferation and cell cycle arrest (in G2/M phase) at early stages, as well as in cell death by mitotic catastrophe and apoptosis at later stages. These results strongly suggest that ORAI3 plays a prosurvival and proproliferative role in PDAC cells, consistent with previously published data (Ay et al., 2013; Faouzi et al., 2011, 2013).

Moreover, we demonstrated that silencing of ORAI3 inhibited xenograft tumor growth in vivo, suggesting that ORAI3 could potentially be a useful anticancer therapeutic target. Indeed, ORAI3 has been already proposed as a potential target in prostate cancer treatment (Dubois et al., 2014).

5.3 Calcium signalling in pancreatic stellate cells.

As it was stated above, pancreatic cancer is characterized by a prominent fibrosis, and PSCs have been identified to be mainly responsible for this phenomenon. Pancreatic cancer cells were reported to stimulate the activation of PSCs, leading to excessive synthesis of extracellular matrix proteins that comprise fibrous tissue. Activated PSCs become the most important drivers of desmoplasia and thereby contribute to therapy refractoriness of pancreatic cancer. Thus, not only pancreatic cancer cells but also stellate cells are involved in cancer progression, suggesting that combined targeting of the stroma and cancer cells should be a goal for therapies.

Therefore, taking into account the important role of PSCs in pancreatic cancer progression as well as the lack of data on calcium signaling pathways in these cells, we studied calcium homeostasis in quiescent and activated PSCs. We showed that quiescent PSCs are characterized by significantly lower levels of both basal cytosolic calcium and calcium in intracellular stores compared to activated PSCs. Moreover, activated PSCs show higher expression levels of major components of store-operated channels, namely ORAI 1,2,3 proteins as well as STIM1 protein. This elevated expression correlates well with increased amplitude of SOCE in activated cells compared to quiescent PSCs. Our data clearly demonstrate that ORAI1 and STIM1 mediate SOCE in activated PSCs, as knockdown of either of these proteins

significantly reduces SOCE.

Thus, based on our results, we hypothesize that in healthy conditions quiescent PSCs are in calcium-"dormant" state with low levels of both cytosolic and stored calcium, as well as with low expression levels of calcium-permeable channels. Following stimulation with activating-factors, PSCs trigger the expression of calcium-permeable channels (i.e. SOCs), accumulate calcium in intracellular stores and increase the level of cytosolic calcium. We assume that increase in intracellular calcium could be an important factor for full activation and function of PSCs, and preventing of intracellular calcium increase via inhibition of calcium-permeable channels on plasma membrane could potentially be a useful strategy to decrease fibrosis in pancreatic cancer. Further studies are needed to elaborate this hypothesis.

Conclusions and perspectives

The role of calcium in physiology could be hardly overestimated. As a ubiquitous secondary messenger, calcium regulates plethora of physiological processes such as proliferation, differentiation and apoptosis and thus could determine cell fate. Therefore the intracellular concentration of calcium is tightly regulated by numerous calcium-permeable channels and pumps. Considering such a crucial role of calcium in physiology it's not surprising that disturbance in calcium homeostasis has been implicated in a variety of pathologies, including cancer. Indeed, numerous studies emphasize the critical role of calcium and calcium-permeable channels in the development, progression and resistance to treatment of many cancers, including that of prostate, breast, colon and others. In contrast, the information on the expression/function and role of calcium-permeable channels in PDAC is scarce. Indeed, PDAC represents one of the worst studied types of cancer with regard to calcium. There is a lack of data regarding calcium-dependent regulation of such important processes as proliferation, differentiation and apoptosis in PDAC. Considering that different types of cancer differ greatly from each other, it's almost impossible to extrapolate the mechanisms existing in a given cancer type to another one, highlighting the need in direct experimental research of each cancer case.

This thesis represents one of the pioneering studies of the expression, function and role of some calcium-permeable channels in PDAC as well as in pancreatic stellate cells.

Here we demonstrate that store-operated calcium channels (SOCs), which represent one of the major calcium-entry pathways in non-excitable cells, are expressed and functional in pancreatic cancer cell lines as well as in pancreatic stellate cells (PSCs). We showed that the classical major components of SOCs, namely ORAI1 and STIM1 mediate store-operated calcium entry (SOCE) in pancreatic cancer cell lines as well as in PSCs. We show that both ORAI1 and STIM1 play pro-survival antiapoptotic role in pancreatic adenocarcinoma cell lines, as siRNA mediated knockdown of ORAI1 and/or STIM1 increase apoptosis induced by chemotherapy drugs 5-fluorouracil (5-FU) or gemcitabine. We also demonstrate that both 5-FU and gemcitabine treatments increase SOCE in Panc1 pancreatic adenocarcinoma cell line via upregulation of ORAI1 and STIM1. We also revealed the prosurvival role of another ORAI family member, ORAI3 in MiaPaca2 cell line. We demonstrate the role of ORAI3 in proliferation, cell cycle and viability of MiaPaca2 cells. We showed that silencing of ORAI3 impairs pancreatic tumor growth *in vivo*, suggesting that ORAI3 could represent a potential therapeutic target in PDAC treatment.

Altogether our results revealed the expression, function and role of SOCs in pancreatic adenocarcinoma cell lines, and emphasize the antiapoptotic role of ORAI1, ORAI3 and STIM1

in pancreatic adenocarcinoma cells.

The results presented in this thesis for the first time demonstrate that SOC channels (or their components) are important regulators of essential physiological processes in PDAC cells. Therefore, these channels could be regarded as potential therapeutic targets in PDAC treatment. Indeed, considering the exceptionally high resistance of PDAC to chemotherapy treatments, there is a crucial need in novel approaches to overcome drug resistance of PDAC. Ion channels could provide some advantages, when targeting apoptosis *in vivo* for cancer treatment. Given that a number of ion channels are localized on cell surface, they represent an "easily"-accessible target, compared to intracellular components. For example, they can be subjected to antibody-based targeting – an approach, that can be particularly useful in the case of channel upregulation in cancer. Moreover, anti-channel antibodies could be used as carriers for radionuclides, toxic molecules or nanoparticles, which can themselves affect essential cellular processes and as such influence cell fate.

Although, we have identified potential candidates for targeting (ORAI1, STIM1, ORAI3), further studies are needed to understand the mechanisms and pathways involved. Indeed, it's not clear by which mechanism knockdown of ORAI1/STIM1 increased gemcitabine-induced apoptosis, what is the cause of this effect – inhibition of Ca^{2+}-entry or Ca^{2+}-independent functions of ORAI1/STIM1.

Interestingly, we have shown that gemcitabine treatment increases SOCE in Panc1 cells. This result suggests that chemotherapy treatments could have calcium-dependent effects, which are unrelated to the primary DNA-targeting mechanisms of their action. Moreover, these calcium dependent effects could potentially contribute to the final efficacy of the drugs in cancer therapy. Therefore it's important to study the role of gemcitabine-stimulated Ca^{2+}-entry in apoptosis, whether this role is pro-survival or pro-death. The expression and translocation of different transcription factors (including NFAT and NF-κB) following treatment with gemcitabine in the presence or absence of Ca^{2+}-entry inhibitors should be assessed to better understand how can we control calcium-dependent mechanisms to overcome PDAC resistance to gemcitabine.

We have also identified ORAI3 as a potential therapeutic target in PDAC treatment. Further studies should reveal the mechanism by which ORAI3 knockdown induces pancreatic cancer cell death. The involvement of ARC channels, caspases and ER-stress should be assessed.

It is now increasingly clear that targeting tumor cells alone is insufficient to improve pancreatic cancer clinical outcome. The successful treatment should target both tumor cells and tumor

microenvironment.

Therefore, identification of the molecular nature as well as functions of ion channels and in particular calcium-permeable channels in PDAC cells and PSCs is of great importance as it can reveal novel approaches for treating pancreatic cancer through targeting calcium-dependent processes.

Our results on pancreatic stellate cells demonstrate that calcium signalling toolkit is significantly rearranged following (or together with) PSC activation. And now it is very important to understand what is first – calcium or activation. So, the next step will be to answer the question: are calcium and calcium channels important for PSC activation/function? Is it possible to prevent/control the process of activation by changing calcium concentration or by controlling the expression of calcium channels in PSCs? Answering these questions could potentially help to elaborate the tools to control PSCs activation in cancer, and thus could lead to the development of a new therapeutic approach for treating pancreatic cancer.

Résumé détaillé français

ROLE DES CANAUX CALCIQUES DANS LA RÉSISTANCE DE L'ADÉNOCARCINOME PANCRÉATIQUE A LA CHIMIOTHÉRAPIE

Introduction

Parmi les différents types de cancer du pancréas, l'adénocarcinome pancréatique (PDAC) est la forme la plus fréquente de néoplasme de cet organe puisqu'il représente environ 90% de toutes les tumeurs pancréatiques (Hezel et al., 2006) et constitue l'une des principales causes de décès par cancer chez l'Homme (Siegel et al., 2015). Le taux de survie à cinq ans n'est que de 5-7%, la plupart des patients meurant environ un an après la détection du cancer (Jemal et al., 2009; Siegel et al., 2015). L'une des raisons à cela est que, au début du développement du cancer du pancréas, il n'y a généralement pas de symptômes, et donc la majorité des cas sont diagnostiqués à des stades tardifs métastatiques ou invasifs pour lesquels une intervention chirurgicale n'est plus possible. L'absence de marqueurs des stades précoces et de techniques de diagnostique aggravent encore plus la situation.

La grande résistance du PDAC à la radiothérapie conventionnelle et aux chimiothérapies contribue au pronostic pessimiste de cette maladie (Erkan et al., 2012; Kindler et al., 2010, 2012). Il a été montré que les cellules du cancer du pancréas présentent plusieurs mutations génétiques qui conduisent à la prolifération incontrôlée des cellules, à l'acquisition d'un potentiel métastatique, ainsi qu'à l'évasion de la mort cellulaire programmée (apoptose) (Jones et al., 2008).

L'apoptose est un processus physiologique qui est essentiel pour l'homéostasie tissulaire normale et la dérégulation de la machinerie apoptotique contribue à la transformation maligne. La prévention de l'apoptose représente l'une des caractéristiques du cancer initialement définies par Hanahan et Weinberg (Hanahan and Weinberg, 2000, 2011).

Effectivement, la résistance à l'apoptose a été impliquée dans la défaillance d'un certain nombre de traitements anticancéreux. Donc, le ciblage de l'apoptose représente une stratégie prometteuse dans le traitement du cancer (Dubois et al., 2013).

Le cancer du pancréas est caractérisé par une forte réaction desmoplasique/stromale (Xu et al., 2014). Il est maintenant connu que les cellules pancréatiques stellaires (PSCs) sont la principale

source de la fibrose dans le stroma et interagissent étroitement avec les cellules cancéreuses pour créer un environnement qui stimule la croissance tumorale locale et les métastases à distance (Apte et al., 2012, 2013). Il a été suggéré que les PSCs sont impliquées dans la résistance des tumeurs à la chimiothérapie et à la radiothérapie (McCarroll et al., 2014). Il est intéressant de noter que les PSCs et les cellules du cancer du pancréas interagissent d'une manière bidirectionnelle. Via la production de médiateurs mitogéniques et de fibrogènes, les cellules cancéreuses pancréatiques attirent et promeuvent l'activation, la prolifération et la motilité des PSCs (Wilson et al., 2014; Xu et al., 2014).

Vu que les cellules du cancer du pancréas et les cellules pancréatiques stellaires sont impliquées dans la progression du cancer, il est clair que le ciblage des cellules du stroma et du cancer doit être un objectif pour les thérapies.

Les changements de concentration du Ca^{2+} cytosolique jouent un rôle central dans de nombreux processus cellulaires fondamentaux, et la perturbation des mécanismes de régulation de l'homéostasie du Ca^{2+} conduit à une grande variété de pathologies graves, dont le cancer. En effet, le rôle du Ca^{2+} est bien établi dans plusieurs voies de signalisation cellulaire impliquées dans la carcinogenèse (Monteith et al., 2007; Prevarskaya et al., 2014).

Les niveaux de calcium cytosolique sont régis par les canaux calciques localisés soit sur les membranes des organelles intracellulaires, soit sur la membrane plasmique. Ainsi, la modulation de l'expression et/ou de la fonction des canaux ioniques perméables au calcium affecte la concentration intracellulaire de Ca^{2+} et par conséquent, les processus dépendants du calcium tels que la prolifération, l'apoptose et l'autophagie (Berridge et al., 2003; Rizzuto et al., 2012).

Un certain nombre d'études suggèrent que la transformation maligne est souvent accompagnée par des modifications de l'expression et/ou de la fonction des canaux ioniques. C'est notamment le cas pour les canaux calciques de type SOC, composés des protéines ORAI1 et STIM1 (Park et al., 2009), qui régulent une variété de processus cellulaires dépendants du calcium tels que l'apoptose, la prolifération, la migration et l'invasion (Flourakis et al., 2010; Khadra et al., 2011; Liu et al., 2011; Prevarskaya et al., 2013, 2014).

Cependant, bien que le rôle du Ca^{2+} et des canaux calciques soit bien établi dans de nombreuses voies de signalisation de différents types cellulaires, les informations sur l'expression/fonction ainsi que sur le rôle des canaux calciques dans le PDAC et les PSCs sont encore limitées.

Donc, l'identification de la nature moléculaire ainsi que des fonctions des canaux calciques (en particulier SOC) revêt une grande importance dans ces cellules car elle pourrait à termes fournir

de nouvelles approches relatives au traitement du cancer du pancréas par le ciblage des processus dépendants du calcium.

Mes objectifs lors de ce projet ont donc été de :

1. Étudier l'expression fonctionnelle des canaux calciques de type SOC dans les cellules du PDAC ainsi que dans les cellules PSCs (quiescentes et activées).
2. Analyser l'implication de ORAI1 et STIM1 dans le SOCE dans les cellules du PDAC et les PSCs.
3. Étudier le rôle de ORAI1 et STIM1 dans la régulation de l'apoptose dans les cellules du PDAC.
4. Étudier le mécanisme de la régulation ORAI1 / STIM1-dépendante de l'apoptose induite par différents traitements chimiothérapeutiques.

Matériels et Méthodes

Objets d'étude:

Pour la réalisation de ces travaux, j'ai travaillé sur:
5 lignées cellulaires de cancer du pancréas – AsPC1, BxPC3, Capan1, MiaPaCa2, Panc1;
La lignée cellulaire épithéliale immortalisée du pancréas humain– H6C7;
Les cellules pancréatiques stellaires primaires de souris – mPSCs;
Les cellules pancréatiques stellaires humaines immortalisées – RLT-PSCs .

Culture cellulaire et transfection

La lignée cellulaire d'adénocarcinome pancréatique Panc1 de l'American Type Culture Collection (ATCC) a été cultivée dans le milieu DMEM+ GlutaMAX (31966, Invitrogen, Life Technologies Inc.) contenant du sérum FCS Gold décomplémenté (10% PAA Gold). Les lignées cellulaires d'adénocarcinome pancréatique ASPC1 et BxPC3 de l'ATCC sont cultivées dans le milieu RPMI 1640 (31870, Gibco-Life Technologies) supplémenté en L-glutamine (5mM, 25030, Gibco) et en sérum FCS Gold décomplémenté (10% PAA Gold). La lignée cellulaire d'adénocarcinome pancréatique MiaPaCa2 de l'ATCC a été cultivée dans le milieu DMEM/F12

(31330, Gibco-Life Technologies) supplémenté en Horse Serum (2,5%, S 9135, Biochrom) et en sérum FCS Gold décomplémenté (10% PAA Gold). La lignée cellulaire d'adénocarcinome pancréatique Capan1 de l'ATCC a été cultivée dans le milieu IMDM (SH 30229.01 HyClone, Thermoscientific) supplémenté en sérum FCS Gold décomplémenté (20% PAA Gold). La lignée cellulaire épithéliale immortalisée de pancréas humain – H6C7 a été obtenue auprès du Dr Ming-Sound Tsao et a été cultivée dans le milieu KBM (CC-3101, Lonza) supplémenté en KGM SingleQuots (CC-4131, Lonza).

Les cellules pancréatiques stellaires humaines – RLT-PSCs ont été cultivées dans le milieu DMEM/F12 (31330, Gibco-Life Technologies) supplémenté en pénicilline/streptomycine 1% et en sérum FCS Gold décomplémenté (10% PAA Gold).

Les cellules stellaires pancréatiques de souris ont été isolées chez les souris âgées de huit à douze semaines. Les souris ont été tuées par dislocation cervicale. Le pancréas a été disséqué et incubé à 37 °C pendant 30 min dans une solution de Gey (GBSS) supplémenté en 1mg/ml de collagénase de type IV (Wortington). Le tissu digéré a été ensuite lavé trois fois avec du milieu DMEM/F12 supplémenté en pénicilline/streptomycine 1% et en sérum FCS Gold 10%, centrifugé à 1000 tpm pendant 4 minutes et remis en suspension dans le milieu DMEM/F12. La dissociation des cellules est obtenue mécaniquement par pipetage. Les cellules ont été cultivées à 37 ° C dans une atmosphère humidifiée contenant 5% de CO2.

Au besoin, ces cellules ont été transfectées avec 40 nM de siRNA contre ORAI1, STIM1 ou les deux (Dharmacon Inc., Fremont, CA, USA) en utilisant le réactif de transfection Hyperfect (Qiagen Inc.), et en suivant les instructions du fabricant.

Les séquences des siARN utilisés sont les suivantes :

CT: 5’-CUUACGCCUGAGUACUUCGA(dTdT)-3’,

hORAI1 : 5′-UGAGCAACGUGCACAAUCU (dTdT)-3′,

hSTIM1 : 5′-GGCUCUGGAUACAGUGCUC (dTdT)-3′.

QRT-PCR

L'ARN total a été extrait en utilisant le réactif TRI (Sigma) et traité avec de la DNAse (Ambion).

L'ADNc a été synthétisé par transcription inverse. La qRT-PCR a été réalisée dans un thermocycleur Cfx C1000 (Biorad), et la réaction suivie à l'aide de l’EvaGreen Supermix (Biorad). Les primers sont présentés dans le tableau 1.

Tableau 1

No.	Nom	Forward (5'-…-3')	Backward (5'-…-3')
1	hSTIM1	TGTGGAGCTGCCTCAGTAT	CTTCAGCACAGTCCCTGTCA
2	hOrai1	ATGGTGGCAATGGTGGAG	CTGATCATGAGCGCAAACAG
3	hGAPDH	TTCGTCATGGCTGTGAACCA	CAGTGATGCGCATGGACTGT
4	hHPRT	GGCGTCGTGATTAGTGATGAT	CGAGCAAGACGTTCAGTCCT

Western blotting

Les cellules ont été lavées avec du PBS froid et lysées dans un tampon glacé contenant 1% de Triton X-100, NaCl 150 mM, EDTA 5 mM, 1% de désoxycholate de sodium, 10 mM de tampon PO4Na2 / K, un cocktail d'inhibiteurs de protéase (Sigma-Aldrich) et un inhibiteur de phosphatase PhosSTOP (Roche). Les lysats ont été centrifugés à 15000 g à 4 ° C pendant 15 minutes pour éliminer les débris cellulaires et la concentration en protéines du surnageant a été déterminée par le kit de dosage de protéine BCA (Pierce Biotechnology). 30 µg de protéines totales ont été soumises à un SDS-PAGE, suivi d'un transfert sur des membranes de PVDF en utilisant une cellule de transfert SD Trans-Blot® semi-sèche (cellule de transfert) (Bio-Rad). Les membranes ont été bloquées dans un lait sans matières grasses de 5% contenant du tampon TNT (Tris-HCl, pH 7,5, 140 mM de NaCl et 0,05% de Tween 20) pendant 1 h à température ambiante. Les membranes ont ensuite été incubées durant la nuit à 4°C avec des anticorps primaires, puis pendant 1h à température ambiante avec des anticorps secondaires conjugués avec des péroxydase de raifort. Après le lavage, les membranes ont été traitées pour la détection à la chimioluminescence à l'aide du Luminata Western substrat HRP (Millipore). Le logiciel Image J a été utilisé pour l'analyse quantitative.

L'imagerie calcique

Les cellules cancéreuses pancréatiques sont cultivées sur des lamelles en verre en vue des expériences d'imagerie calcique. La sonde ratiométrique Fura-2 / AM (F1221, Invitrogen) a été utilisée comme indicateur calcique. Les cellules ont été chargées avec 2 µM de Fura-2 / AM pendant 45 min à 37 ° C dans leur milieu de culture respectif et ensuite lavées trois fois avec

une solution externe contenant (en mM) : 140 NaCl, 5KCl, 1 MgCl2, 2 CaCl2, 5 Glucose, 10 Hepes (pH 7,4). La lamelle est ensuite transférée dans une chambre de perfusion placée sur un microscope Nikon Eclipse Ti. La préparation est alternativement excitée à 340 nm et 380 nm avec un monochromateur (Polychrome IV, TILL Photonics GmbH) et la fluorescence émise est capturée à 510 nm par une caméra CCD QImaging. L'acquisition et l'analyse ont été réalisées avec le logiciel MetaFluor 7.7.5.0 (Molecular Devices Corp.).

Détection de l'apoptose

Le niveau d'apoptose a été déterminé par marquage au Hoechst. Les cellules ont été cultivées sur des plaques de 6 puits et transfectées avec des siARN contrôles (siCT) ou dirigés contre ORAI1 (siORAI1) ou STIM1 (siSTIM1). Après 24 h, les cellules ont été soumises à des traitements (chimiothérapies) pendant 72 h. À la fin des traitements, les cellules flottantes ont été recueillies et fixées, centrifugées et remises en suspension dans 1 ml de solution saline tamponnée au phosphate (PBS). Suite au passage au cytospin, les cellules sont fixées avec du méthanol glacé pendant 10 minutes, lavées avec du PBS et colorées avec 5 µg/ml de Hoechst 33258 pendant 10 min à température ambiante. Les cellules sont ensuite lavées deux fois avec du PBS, placées dans du glycergel (DAKO) et soumises à une analyse par microscopie à fluorescence. La morphologie nucléaire est visualisée au microscope à fluorescence Zeiss Axio Imager A1 (405-435 nm). Le pourcentage de cellules apoptotiques (à noyaux condensés/fragmentés) a été déterminé en comptant au moins 500 cellules dans des champs aléatoires.

Alternativement, les cellules apoptotiques ont été détectées par un double marquage Alexa Fluor® 488 Annexin V/iodure de propidium. À la fin des traitements, les cellules flottantes et les cellules attachées sont récupérées par trypsinisation puis centrifugées, lavées avec du PBS et marquées avec de l'Alexa Fluor® 488 Annexin V et de l'iodure de propidium selon les instructions du fabricant (Alexa Fluor® 488 Annexin V / Dead Cell apoptose Kit, Life Technologies). Les cellules sont ensuite examinées au microscope à fluorescence Zeiss Axio Imager A1. Le pourcentage de cellules positives à l'Alexa Fluor ® V-488 Annexine a été déterminé en comptant au moins 500 cellules en champs aléatoires.

Immunocytochimie et microscopie confocale

Pour ces expériences, les cellules PSCs (quiescentes et activées) de souris ont été préalablement

cultivées sur des lamelles en verre.

Les cellules ont été rincées avec du PBS, fixées avec du paraformaldehyde à 4% pendant 15 min, lavées avec du PBS, puis perméabilisées dans du PBS-gélatine complété avec 0,01% de Tween 20 et 100 mM de glycine pendant 30 min à 37°C.

Ensuite les cellules ont été incubées avec des anticorps primaires dirigés contre alphaSMA à 4 ° C pendant une nuit. Après des lavages approfondies, les cellules sont incubées avec des anticorps secondaires Alexa Fluor 488-anticorps anti-lapin (Jackson Immuno Research) dilués dans du PBS-gélatine pendant 1 heure à température ambiante. Les cellules sont ensuite lavées trois fois et les lames sont montées avec du Mowiol (81381, Sigma-Aldrich) sur des lames de verre et soumises à une analyse par fluorescence en utilisant un microscope confocal (LSM 700, Carl Zeiss GmbH, Jena, Allemagne) avec un objectif numérique Plan Apochromat 40×/1.3. Les images sont analysées à l'aide du logiciel Zeiss LSM Image Browser (Carl Zeiss GmbH).

Résultats

Partie 1. Adénocarcinome pancréatique

1.1 ORAI1 et STIM1 sont différemment exprimés dans des cellules de cancer du pancréas.

Il a déjà été montré que ORAI1 et STIM1 sont exprimés dans une grande variété de types cellulaires, mais il n'existe pas d'informations relatives à leur expression dans les lignées cellulaires du PDAC. Nous avons donc dans un premier temps examiné leur présence dans plusieurs lignées cellulaires de PDAC représentant différents stades de ce cancer, à savoir les Capan1, ASPC1, Panc1, MiaPaca2 et BxPC3 ainsi que dans les cellules épithéliales pancréatiques « normales » de la lignée cellulaire humaine H6C7. En utilisant la technique de qRT-PCR, nous avons trouvé que ORAI1 et STIM1 sont exprimés de manières différentes dans toutes les lignées cellulaires testées (Figure 1.1A).

Deuxièmement, nous avons caractérisé l'expression de ORAI1 et de STIM1 au niveau protéique par Western Blotting. Nous avons constaté que ORAI1 et STIM1 sont tous deux exprimés dans toutes nos lignées cellulaires, et que leurs niveaux d'expression diffèrent entre lignées cellulaires, ce qui est bien corrélé avec les données du qRT-PCR (Figure 1.1B et 1.1C).

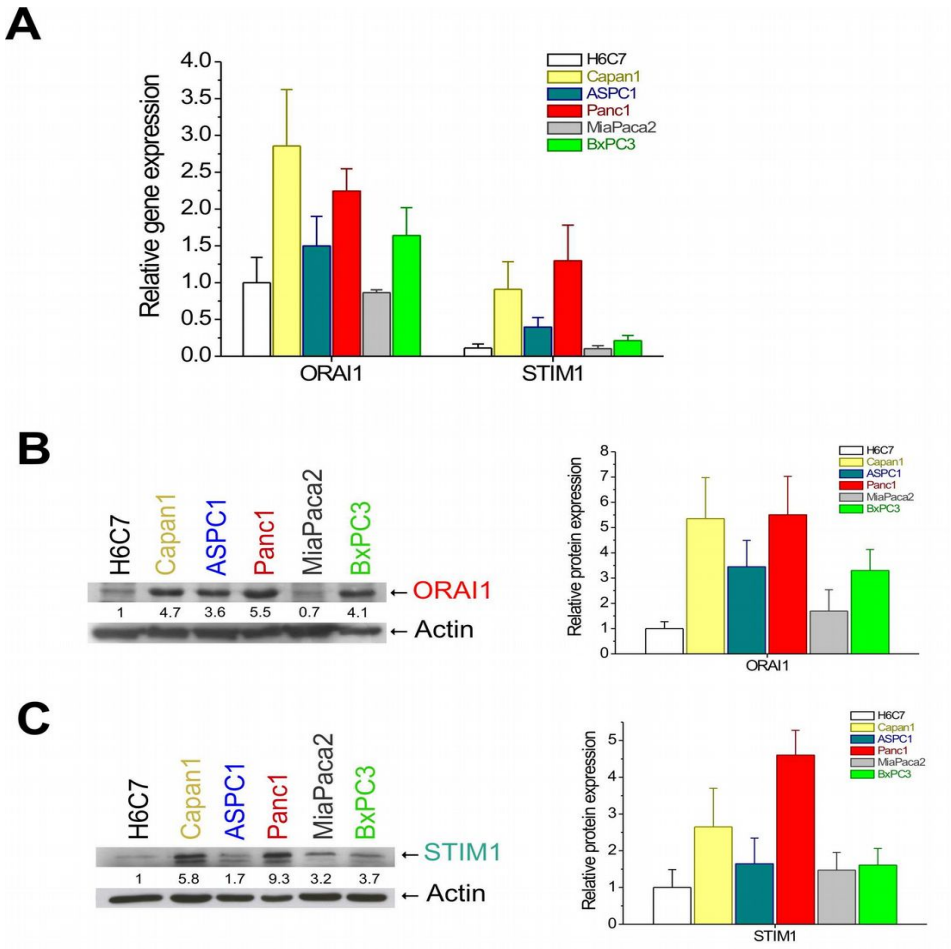

Figure 1.1. ORAI1 et STIM1 sont différemment exprimés dans les cellules du cancer du pancréas. *(A) Détection par qRT-PCR de l'expression de ORAI1 et STIM1 dans les lignées cellulaires H6C7, Capan1, ASPC1, Panc1, BxPC3 et MiaPaca2. Les niveaux d'expression de ORAI1 et STIM1 par rapport à GAPDH pour chaque lignée cellulaire ont été normalisés par rapport à leur niveau d'expression dans la lignée cellulaire "normale" - H6C7. Les données sont présentées comme moyenne ± écart-type. N = 3. (B) Western blot montrant l'expression de ORAI1 dans les H6C7, Capan1, ASPC1, Panc1, MiaPaca2 et BxPC3. L'histogramme montre la quantification des niveaux d'expression de ORAI1 par rapport à l'actine pour chaque lignée cellulaire normalisée par le niveau de ORAI1 dans la lignée cellulaire H6C7. Les données sont présentées comme moyenne ± écart-type. N = 3. (C) Western blot montrant l'expression de STIM1 dans les lignées H6C7, Capan1, ASPC1, Panc1, MiaPaca2 et BxPC3. L'histogramme montre la quantification des niveaux d'expression de STIM1 par rapport à l'actine pour chaque lignée cellulaire normalisée par le niveau de STIM1 dans la lignée H6C7. Les données sont représentées en tant que moyenne ± écart-type. n= 3.*

En concordance avec les résultats de qRT-PCR et de l'immunoblotting, l'entrée capacitive de

calcium (SOCE) induite par la thapsigargine (TG, un inhibiteur de sarco/endoplasmique réticulum Ca2 + ATPase (SERCA)) était plus faible dans les cellules normales H6C7 que dans les cellules cancéreuses (Figure 1.2A). Ce résultat a été confirmé par la technique de patch-clamp : la densité de courant du SOC (stimulé du côté intracellulaire par 100 µM d'IP3) était significativement plus faible dans des cellules H6C7 que dans les cellules Panc1 (Figure 1.2B).

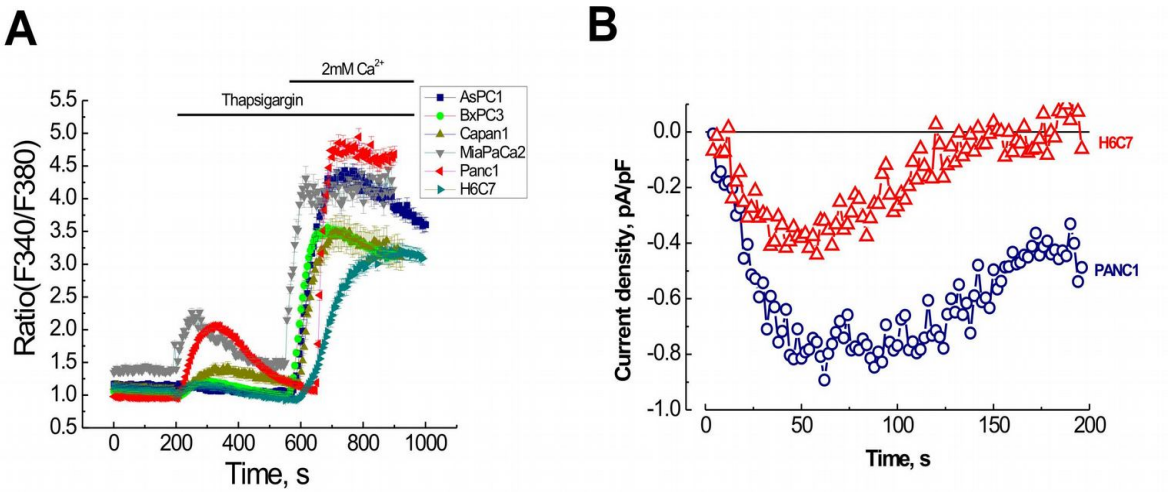

Figure 1.2. Le SOCE et le courant SOC dans les cellules cancéreuses et normales. *(A) SOCE induite par TG dans les cellules PDAC et H6C7. Les cellules ont été chargées avec la sonde Fura2/AM et leur activité est suivie grâce à la technique de l'imagerie calcique. (B) Courants SOC activés par l'IP3 et enregistrés à -100mV dans les cellules H6C7 et Panc1.*

Pour les expériences suivantes, nous avons décidé de mettre l'accent sur la lignée cellulaire Panc1, celle-ci étant caractérisée par des niveaux d'expression relativement élevés de ORAI1 et STIM1.

1.2 ORAI1 et STIM1 sont impliqués dans le SOCE dans les cellules du cancer du pancréas.

De nombreux rapports ont démontré que ORAI1 et STIM1 sont le support moléculaire de l'activité du canal SOC (Store-Operated Channel) et par conséquent de la SOCE dans une variété de types cellulaires. Sachant que cette fonction de ORAI1 et STIM1 n'a jamais été démontrée dans le PDAC, nous avons ensuite étudié si ORAI1 et STIM1 étaient impliqués dans la SOCE dans ces cellules en utilisant une approche basée sur l'usage de siRNA. Les cellules Panc1 ont donc été transfectées avec des siCT, siORAI1 ou siSTIM1. Nous avons déterminé l'efficacité de la transfection par ces siRNA à 48 h en regardant les niveaux d'ARN et de

protéines grâce aux techniques de qRT-PCR et de Western Blotting (Figure 1.3C et 1.3D).

48 h après la transfection avec ces siRNA, les cellules ont été chargées avec la sonde Fura2/AM et observées en imagerie calcique. Nous avons vérifié si le knockdown par des siRNA dirigés contre ORAI1 ou STIM1 influençait les niveaux de calcium cytosolique dans les cellules Panc1 exposées à la thapsigargine (TG). Plus précisément, dans notre protocole, les réserves calciques intraréticulaires ont été épuisées par la TG dans une solution extracellulaire sans calcium, puis l'addition de 2 mM de Ca^{2+} sur les cellules nous a permis de visualiser l'influx de calcium via des canaux de type SOC.

Lorsque ce test a été réalisé sur des cellules Panc1 transfectées avec les siORAI1 et siSTIM1, nous avons observé que l'entrée de calcium par les SOC été réduite de manière significative (Figure 1.3A et 1.3B).

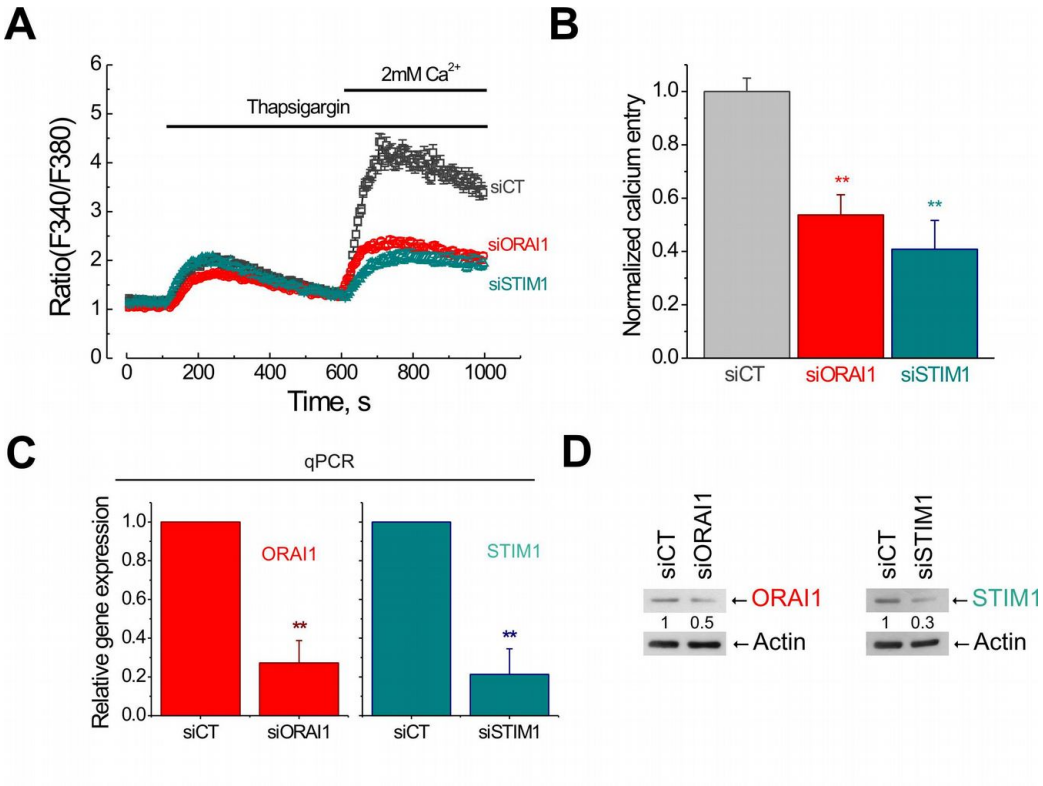

Figure 1.3. ORAI1 et STIM1 sont impliqués dans la SOCE des cellules PDAC. *(A) Le knockdown de ORAI1 ou de STIM1 inhibe la SOCE dans les cellules Panc1. Les cellules Panc1 ont été transfectées avec les siCT, siORAI1 ou siSTIM1. Après 48 h, les cellules sont chargées avec la sonde Fura2/AM, observées en imagerie calcique. (B) Quantification de l'amplitude de la SOCE induite par la TG dans les cellules transfectées avec les siCT, siORAI1 ou siSTIM1. N = 5 (C) Détection par qRT-PCR de l'expression de ORAI1 et STIM1 dans les cellules Panc1 48 h après transfection avec siORAI1 ou siSTIM1. (D) Western blot montrant l'expression de*

ORAI1 et STIM1 dans les cellules Panc1 transfectées avec siCT-, siORAI1- ou siSTIM1, 48 h après transfection. Quantifications densitométriques pour ORAI1 et STIM1 normalisés par rapport à l'actine sont présentées.

1.3 La régulation négative de ORAI1 et STIM1 sensibilise les cellules Panc1 à la chimiothérapie.

Les précédentes études ont montré que ORAI1, STIM1 ainsi que la SOCE jouent un rôle important dans la physiologie cellulaire et dans la pathologie. Leur rôle dans la régulation de l'apoptose a ainsi été étudié dans différents types cellulaires. C'est pourquoi nous avons ensuite cherché à savoir si ORAI1 et STIM1 étaient impliqués dans la régulation de l'apoptose dans les cellules Panc1. Ces cellules ont été transfectées avec les siCT, siORAI1, siSTIM1 ou siORAI1 + siSTIM1. 24h après transfection, les cellules transfectées et non-transfectées sont traitées avec les agents chimiothérapeutiques 5-FU (50µM) ou la gemcitabine (50µM) pendant 72 heures pour induire l'apoptose. À la fin des traitements, ces cellules ont été recueillies, colorées avec le réactif Hoechst 33258 et soumises à une analyse de la morphologie nucléaire par microscopie de fluorescence. Alternativement, les cellules apoptotiques ont été détectées par marquage à l'Annexin V/PI. La régulation négative de ORAI1 et / ou de STIM1 n'a pas induit de changements importants sur le niveau de base de l'apoptose dans les cellules non transfectées (Figure 1.4A et 1.4B).

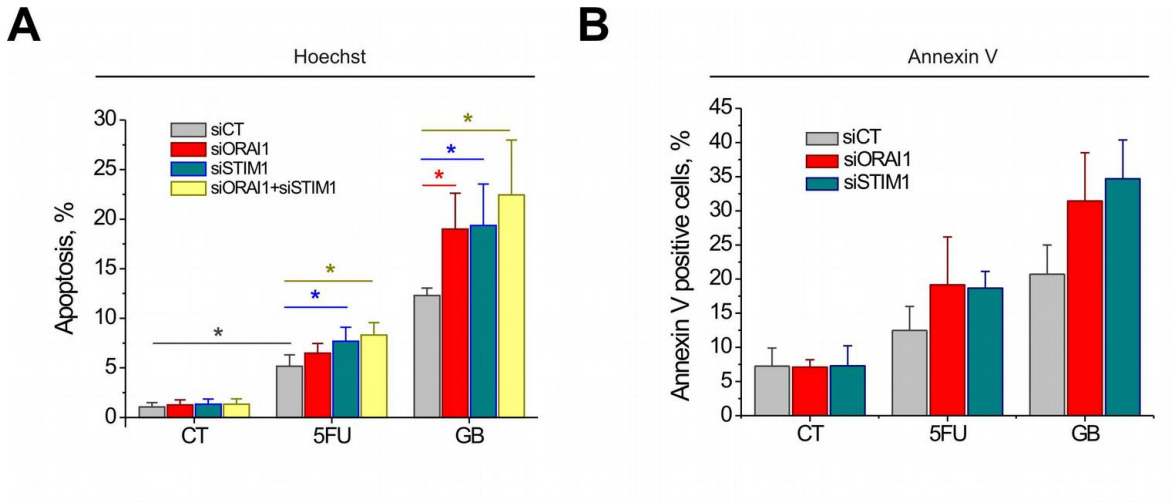

Figure 1.4. La régulation négative de ORAI1 et STIM1 sensibilise les cellules Panc1 à des traitements de chimiothérapie. *(A)Les cellules ont été transfectées avec les siCT, siORAI1, siSTIM1 ou siORAI1 + siSTIM1. Après 24 h, les cellules ont été traitées avec des drogues chimiothérapeutiques comme le 5-FU ou la gemcitabine (GB) pendant 72 h. À la fin du*

traitement, les cellules ont été recueillies, colorées avec du Hoechst et soumises à une analyse par microscopie de fluorescence de la morphologie nucléaire. Les données sont représentées en tant que moyenne ± écart-type, N = 3. (B) Des cellules ont été transfectées avec les siCT, siORAI1 ou siSTIM1. Après 24 h, ces cellules ont été traitées avec le 5-FU ou la gemcitabine pendant 72 h. À la fin des traitements, les cellules ont été recueillies, colorées avec l'annexine V/PI et soumises à une analyse par microscopie de fluorescence. Les données sont représentées comme moyenne ± écart-type, n = 3.

Cependant, lorsque les cellules ont été traitées avec le 5-FU ou la gemcitabine, la régulation négative de ORAI1 ou/et STIM1 a augmenté les niveaux d'apoptose induite par ces drogues (Figure 1.4A et B). Ces résultats suggèrent que ORAI1 et STIM1 jouent un rôle anti-apoptotique dans les cellules Panc1.

1.4 Les drogues chimiothérapeutiques 5-FU et gemcitabine augmentent la SOCE dans les cellules Panc1.

La régulation par ORAI1 et STIM1 de l'apoptose induite par la chimiothérapie suggère qu'il y aurait un lien entre agents chimiothérapeutiques et l'homéostasie calcique. Pour tester cette hypothèse, nous avons incubé des cellules Panc1 dans un milieu de base avec ou sans agents de chimiothérapie (50µM 5-FU ou 50µM gemcitabine) pendant 24 h. À la fin du traitement, les cellules ont été chargées avec la sonde Fura2/AM et observées par imagerie calcique. Nous avons vérifié si des traitements chimiothérapeutiques influencent les taux de calcium cytosolique dans les cellules Panc1 en utilisant TG comme décrit ci-dessus.

De manière intéressante, nous avons constaté que les traitements avec le 5-FU et la gemcitabine augmentent considérablement la SOCE dans ces cellules (Figure 1.5A et B). Ce résultat a pu être confirmé par le technique de patch-clamp, la gemcitabine augmentant de manière significative le courant SOC dans les cellules Panc1 (Figure 1.5B, en encart).

Cet effet pourrait être la conséquence de l'augmentation des niveaux d'expression du SOC par les agents chimiothérapeutiques. Pour tester cette hypothèse, nous avons ensuite évalué les niveaux d'expression de STIM1 et ORAI1 24 h après le traitement avec le 5-FU ou la gemcitabine. Nos résultats montrent que ces deux substances augmentent de façon significative l'expression de STIM1 et ORAI1 –aussi bien au niveau protéinque que de l'ARNm (Figure 1.5C et D).

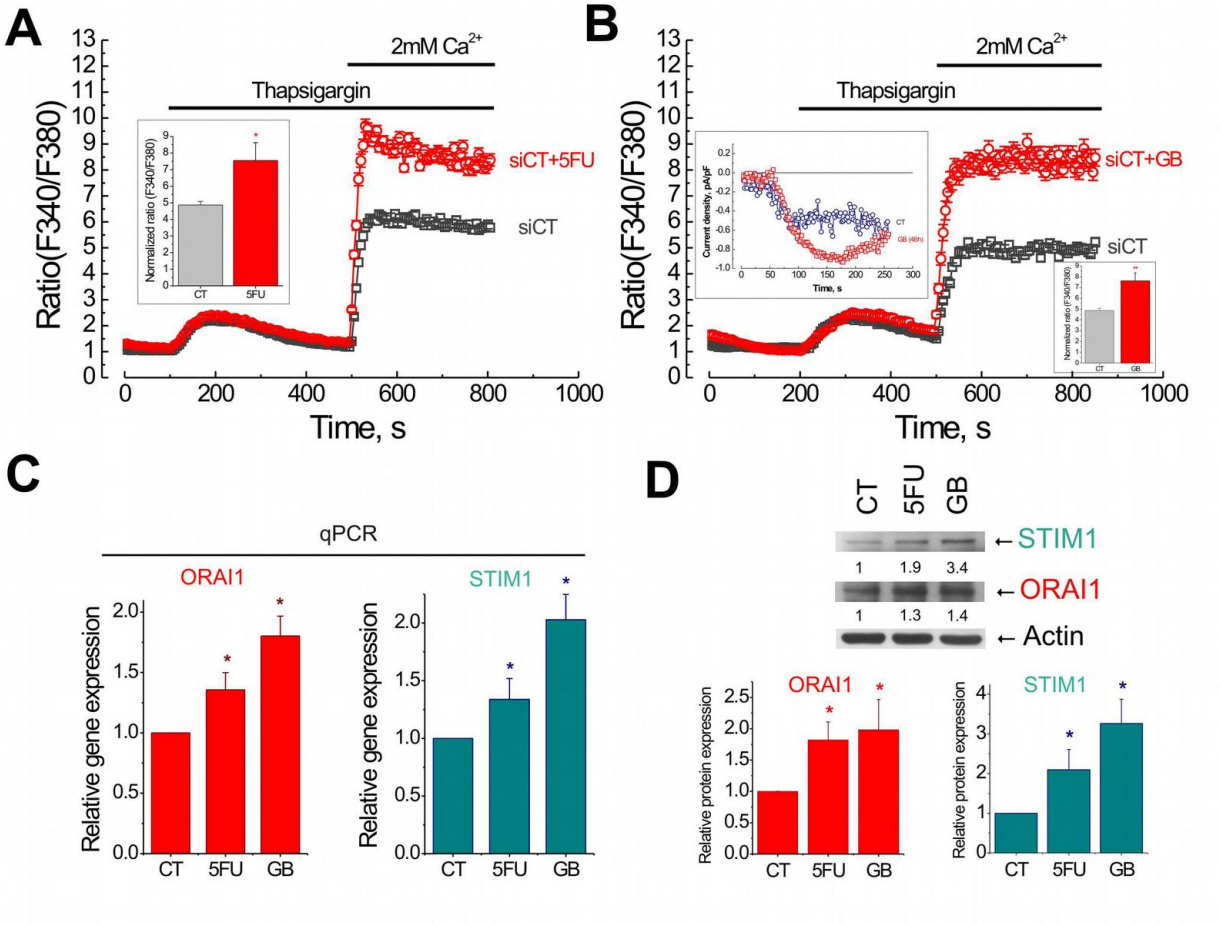

Figure 1.5. Les drogues chimiothérapeutiques 5-FU et gemcitabine (GB) augmentent la SOCE dans les cellules Panc1. *(A) Les cellules Panc1 ont été incubées dans un milieu de base avec ou sans 5-FU pendant 24 h. À la fin du traitement, les cellules ont été chargées avec la sonde Fura2/AM et observées en imagerie calcique. La SOCE induite par la TG a été analysée, et elle est présentée en encart, N = 4. (B) Les cellules Panc1 ont été incubées dans un milieu de base avec ou sans gemcitabine pendant 24 h. À la fin du traitement, les cellules ont été observées par imagerie calcique. La SOCE induite par la TG a été analysée, et est ici présentée en encart, N = 4. (C) Détection par qRT-PCR des taux d'expression des ARNm de ORAI1 et STIM1 dans les cellules Panc1, 48 h après traitement par le 5-FU ou la gemcitabine. (D) Western blot montrant l'expression de ORAI1 et STIM1 dans les cellules Panc1, 48h après traitement avec le 5-FU ou la gemcitabine. Les histogrammes montrent la quantification des niveaux d'expression normalisés de ORAI1 et STIM1 relatifs à l'actine. Les données sont présentées comme moyenne ± écart-type, n = 3.*

Ces résultats sont bien corrélés avec nos résultats d'imagerie calcique (Figure 1.5A et B),

suggérant que les agents de chimiothérapie 5-FU et gemcitabine peuvent influer sur les niveaux de calcium cytosolique par la régulation positive de ORAI1 et STIM1.

Perspectives

Comme nous avons déjà montré que la gemcitabine augmente significativement la SOCE, nous allons par la suite étudier le rôle de cette entrée de Ca^{2+} dans l'apoptose induite par la gemcitabine pour savoir si elle favorise la survie ou la mort cellulaire. Nous allons utiliser des inhibiteurs d'entrée du Ca^{2+} avec la gemcitabine pour voir le rôle de cette entrée de Ca^{2+} dans la régulation de l'apoptose. Aussi, nous allons vérifier l'expression et la translocation du facteur de transcription du facteur nucléaire kB (NF-kB) après le traitement par la gemcitabine en présence ou en absence d'inhibiteurs d'entrée de Ca^{2+}.

Sachant que la combinaison des siSTIM1/siORAI1 avec la gemcitabine a augmenté le niveau d'apoptose induite par la chimiothérapie, notre prochain objectif est d'identifier l'origine de cet effet – à savoir l'inhibition de l'entrée du Ca^{2+} ou les fonctions indépendantes du Ca^{2+} de ORAI1 / STIM1. Sachant que le stress réticulaire a été montré comme l'un des stimuli apoptotiques, nous étudierons l'effet du traitement à la gemcitabine seule ou avec des siRNA dirigés contre ORAI1 / STIM1 sur l'induction du stress réticulaire dans les cellules Panc 1. Pour cela, nous allons vérifier la présence de marqueurs de stress réticulaire bien connus tels que PERK et GRP78.

Il a été démontré que l'autophagie joue un rôle important dans la survie des cellules cancéreuses au cours du traitement par la chimiothérapie. Nous allons donc vérifier si l'autophagie est impliquée dans la régulation de ORAI1/STIM1 dans l'apoptose induite par la gemcitabine. Nous allons vérifier les niveaux d'expression de marqueurs autophagiques dans les cellules Panc1 contrôles et des cellules traitées avec la gemcitabine seule ou en combinaison avec les siORAI1 ou siSTIM1. Nous allons également utiliser des inhibiteurs de l'autophagie en présence de gemcitabine pour voir l'implication de l'autophagie dans l'apoptose induite par cette substance.

Nous allons également vérifier quelles voies d'apoptose sont déclenchées par la gemcitabine (intrinsèque ou extrinsèque), si cette apoptose est caspase-dépendante ou caspase-indépendante, s'il y a accumulation de Ca^{2+} dans les mitochondries après le traitement par la gemcitabine et s'il sera changé par les siORAI1 / siSTIM1. Nous allons également vérifier la possible régulation de la voie de la mort du récepteur par le SOCE.

Partie 2. Les cellules pancréatiques stellaires (PSC)

2.1 Les canaux calciques dans les cellules pancréatiques stellaires.

Les cellules pancréatiques stellaires (PSC) sont un composant cellulaire du pancréas dont la fonction dans les conditions normales n'est pas entièrement claire. Il a été proposé qu'elles jouent un rôle dans le remodelage de la matrice extracellulaire normale de la glande par la synthèse et la sécrétion d'enzymes dégradant la matrice et leurs inhibiteurs. Dans le pancréas sain, les cellules sont dans leur phase quiescente, contenant des gouttelettes lipidiques avec la vitamine A dans leur cytoplasme. En cas de lésion du pancréas, les cellules sont activées par plusieurs facteurs tels que les cytokines, les facteurs de croissance et le stress oxydant. Les PSCs activées perdent leurs gouttelettes lipidiques cytoplasmiques, se transforment (phénotype myofibroblastes-like), prolifèrent et synthétisent des quantités excessives de protéines de la matrice extracellulaire qui composent le tissu fibreux. Les PSCs activées deviennent les facteurs les plus importants de la desmoplasie (la croissance du tissu fibreux ou conjonctif) et contribuent à l'aspect réfractaire aux traitements du cancer du pancréas. En conséquence, de nouveaux concepts thérapeutiques, qui sont dirigés contre les interactions PDACs/stroma/PSCs, sont en cours de développement. Les PSCs sont activées et restent sous l'influence du microenvironnement de la tumeur, dont l'hypoxie et les facteurs de croissance paracrines sont des éléments importants.

L'activation des cellules pancréatiques stellaires se distingue de celle des autres dans la prolifération, la migration, la résistance à l'apoptose (qui représente la radio et chimio-résistance) et dans la communication croisée avec les cellules cancéreuses. Par conséquent, il est clair que le contrôle de l'activation des PSCs dans le cancer pourrait être très bénéfique.

Grand nombre de processus, caractéristiques des cellules pancréatiques stellaires activées, sont connus pour être typiquement contrôlés et / ou modifiés par des canaux ioniques. Toutefois, les informations sur l'expression/fonction ainsi que sur le rôle des canaux calciques dans les PSCs sont encore limitées.

Nous faisons l'hypothèse que le calcium ainsi que les canaux calciques pourraient être impliqués dans la régulation de l'activation des PSCs. En outre, des canaux calciques pourraient être impliqués dans la prolifération, la migration et la résistance à l'apoptose des PSCs activées.

Pour étudier ces hypothèses, nous avons choisi les cellules stellaire humaines immortalisées RLT-PSC ainsi que les PSCs primaires de la souris. Comme révélé par marquage au Nile Red,

les PSCs de souris fraîchement isolées présentent d'abondantes gouttelettes lipidiques dans leur cytoplasme au cours de l'isolement et durant leur mise en culture (Figure 2.1).

Cependant, toutes les PSCs perdent leurs gouttelettes lipidiques après la trypsinisation au premier passage et se transforment en un phénotype de myofibroblastes-like (comme révélé par immunomarquage de l'alpha-actine musculaire lisse) (Figure 2.1). Par conséquent, les PSCs fraîchement isolées ainsi que les cellules récemment mises en culture primaire représentent les PSCs quiescentes, alors que les cellules exprimant l'alpha-actine musculaire lisse représentent les PSCs activées.

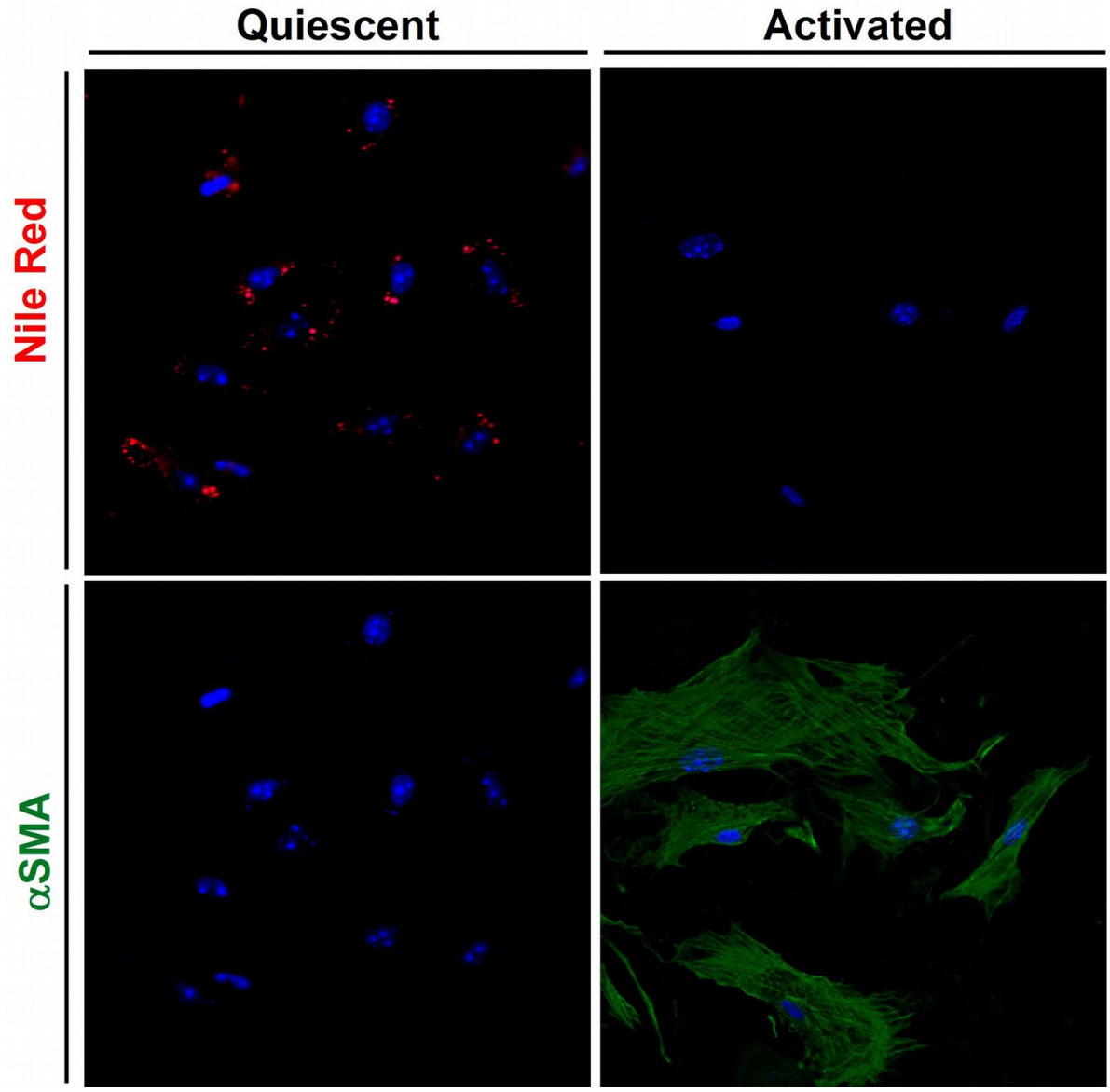

Figure 2.1. Les cellules pancréatiques stellaires de la souris. *Les PSCs activées et quiescentes de la souris sont présentées. Les PSCs en culture primaire (24 heures après l'extraction) présentent des gouttelettes lipidiques positives au Nile-Red, tandis que les PSCs issues d'une*

culture plus ancienne (après trypsinisation) relâchent leurs gouttelettes lipidiques et sont positives à l'alpha-SMA.

Sachant que les canaux calciques de type SOC représentent l'une des principales voies d'entrée de calcium dans les cellules non-excitables, nous avons d'abord examiné si les protéines ORAIs, STIMs, TRPCs et TRPV6s sont présents dans les PSCs primaires quiescentes et activées de la souris ainsi que dans les cellules humaines immortalisées RLT-PSC. En utilisant des techniques de PCR et de qRT-PCR, nous avons trouvé que ORAI1,2,3, et STIM1 sont exprimés de manière différente dans les cellules PSCs quiescentes et activées chez la souris (Figure 2.2A).

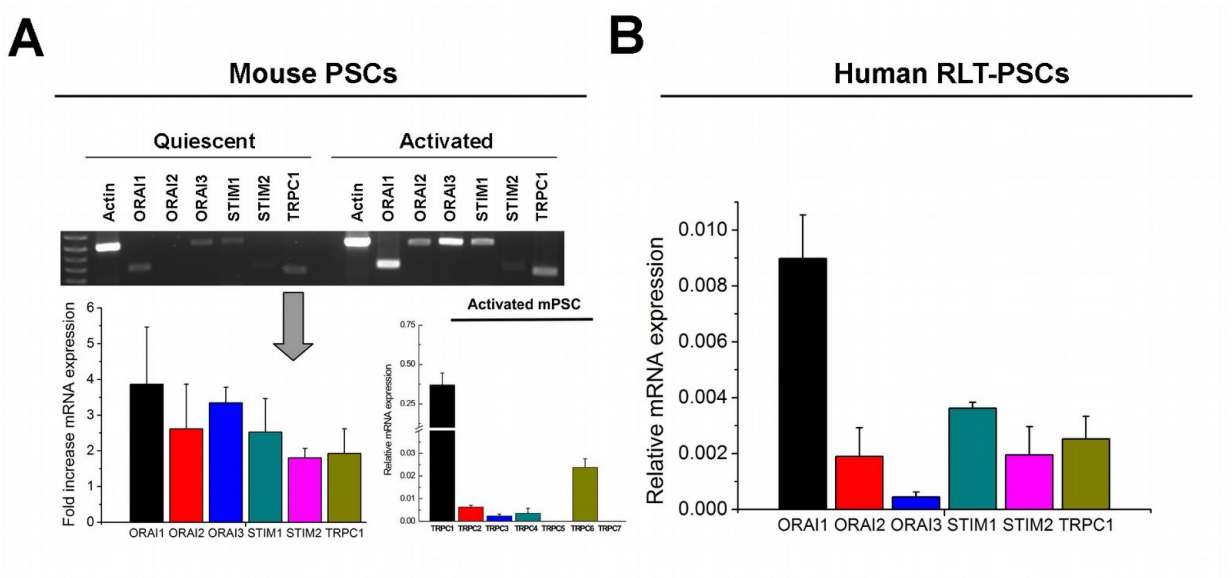

Figure 2.2. Expression de ORAIs, STIMs et TRPCs dans les PSCs de la souris et les PSCs humaines. *(A) Expression de ORAIs, STIMs et TRPCs dans les PSCs quiescentes et activées de la souris. (B) Expression de ORAIs, STIMs et TRPC1 dans les RTL-PSCs humaines.*

Plus précisément, les PSCs activées sont caractérisées par l'expression accrue de ORAI1,2,3, et STIM1 par rapport aux PSCs quiescentes. Nous avons également détecté de l'ARNm de TRPC1, TRPC2, TRPC3, TRPC4 et TRPC6 dans les PSCs activées de la souris. De manière similaire aux PSCs de la souris, les RLT-PSCs humaines expriment également ORAIs, STIMs et TRPC1 (Figure 2.2B). Aucune lignée cellulaire n'a montré des niveaux d'expression détectables du canal TRPV6 (Figures 2.2A et B).

2.2 Store-Operated Ca^{2+} Entry dans les PSCs de la souris et de l'homme.

En concordance avec les données de PCR, la SOCE induite par la TG est significativement plus

faible dans les PSCs quiescentes par rapport à celle des PSCs activées de la souris (Figure 2.3A). Les RLT-PSCs humaines sont elles caractérisées par une importante SOCE en réponse à la TG (Figure 2.3B).

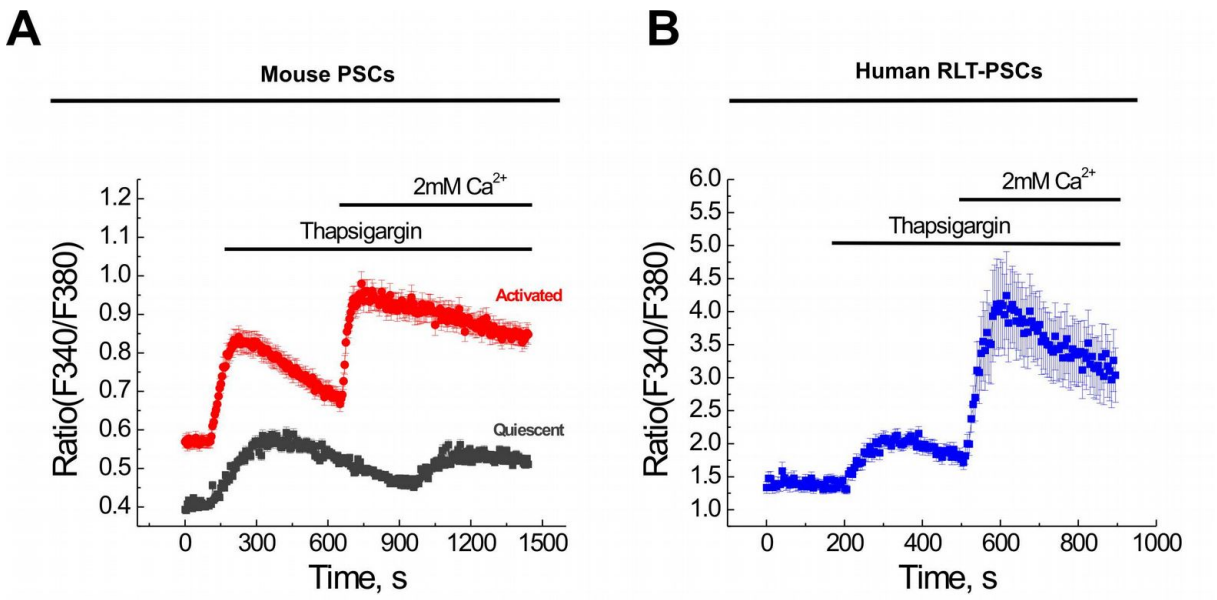

Figure 2.3. SOCE dans les PSCs de la souris et de l'homme. *(A) SOCE induite par la TG dans les PSCs quiescentes et activées de la souris. (B) SOCE induite par la TG dans les RTL-PSCs humaines.*

Ensuite, nous avons cherché à savoir si ORAI1 et STIM1 étaient impliqués dans la SOCE dans les PSCs en utilisant des siRNA. Les PSCs activées de la souris ou les RLT-PSCs humaines ont été transfectées avec les siCT, siORAI1 ou siSTIM1. 48 h après transfection, les cellules sont chargées avec la sonde Fura2/AM et observées en imagerie calcique. Nous avons vérifié si le knockdown de ORAI1 ou de STIM1 influençait les niveaux de calcium cytosolique dans les PSCs en utilisant la thapsigargine (TG).

Plus précisément, les stocks intracellulaires ont été épuisés par la TG nominalement dans une solution extracellulaire sans calcium, puis 2 mM de Ca^{2+} sont ajoutés sur les cellules pour initier un influx de calcium via les canaux de type SOC. Lorsque ce test a été effectué sur des PSCs transfectées avec les siORAI1 ou siSTIM1, l'entrée de calcium a été réduite de manière significative (Figures 2.4A et 2.4B).

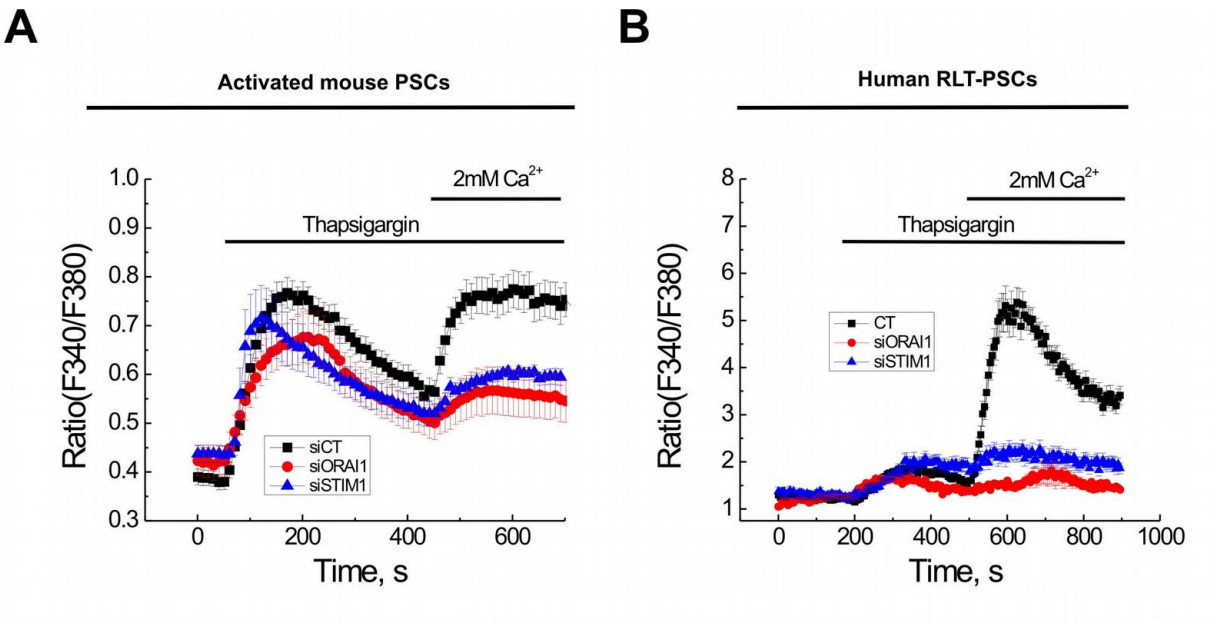

Figure 2.4. ORAI1 et STIM1 sont impliqués dans la SOCE dans les PSCs de la souris et les PSCs de l'Homme. *(A) Le knockdown fonctionnel de ORAI1 ou STIM1 inhibe la SOCE dans les PSCs activées de la souris. (B) Le knockdown de ORAI1 ou STIM1 inhibe la SOCE dans les RLT-PSCs humaines.*

Ces résultats suggèrent que ORAI1 et STIM1 constituent les bases moléculaires de la SOCE dans les PSCs activées de la souris et les RLT-PSCs humaines.

2.3 L'homéostasie calcique dans les PSCs quiescentes et activées de la souris.

Nous avons ensuite analysé les niveaux de calcium cytosolique dans les PSCs quiescentes et activées de la souris. Les cellules ont été chargées avec la sonde Fura2/AM et observées en imagerie calcique. Le niveau de base du calcium cytosolique en présence de 2 mM de Ca^{2+} dans le milieu extracellulaire a été analysé. Les PSCs quiescentes de la souris ont montré un niveau significativement plus faible de calcium cytosolique que celui des PSCs activées (Figure 2.5A). En outre, nous avons analysé le niveau de calcium dans les réserves intracellulaires pour les deux types de PSCs - quiescentes et activées en utilisant un ionophore calcique, la ionomycine, ainsi que la thapsigargine (TG), un inhibiteur de SERCA.

L'application de 5 µM de ionomycine en absence de calcium extracellulaire provoque une augmentation transitoire du calcium cytosolique, qui est significativement plus faible dans les PSCs quiescentes que dans les PSCs activées (Figure 2.5B). Conformément à cela, les PSCs quiescentes ont montré une libération de calcium induite par la TG significativement plus faible

par rapport à celle des PSCs activées (Figure 2.5C).

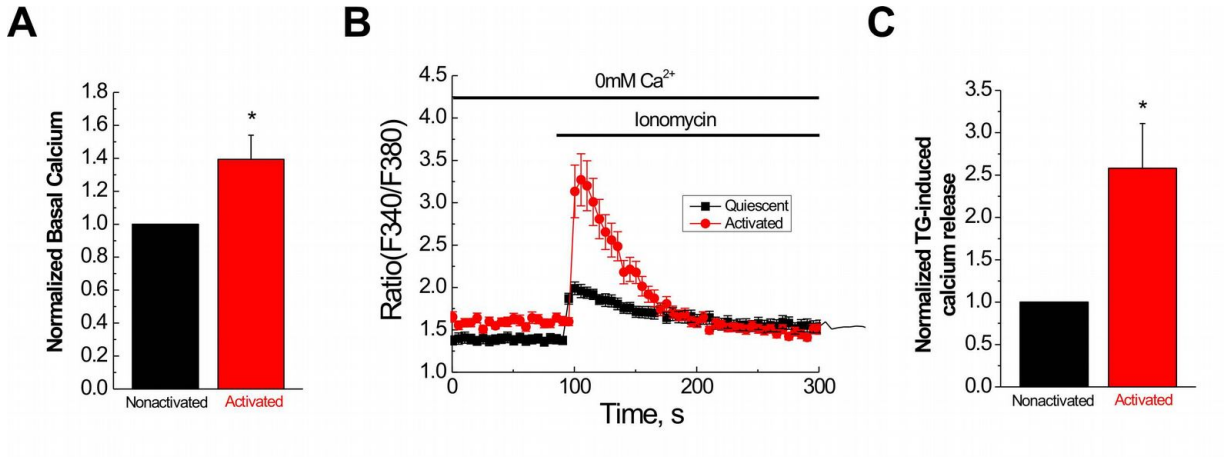

Figure 2.5. Calcium intracellulaire dans les PSCs quiescentes et activées de la souris. *(A) Calcium cytosolique dans les PSCs quiescentes et activées de la souris. (B) La ionomycine induit la libération de calcium des réserves intracellulaires dans les PSCs quiescentes et activées de la souris. (C) Libération de calcium des réserves intracellulaires sensibles à la TG dans les PSCs quiescentes et activées de la souris.*

Perspectives

Nos résultats montrent que la signalisation calcique est augmentée dans les PSCs activées du pancréas de souris par rapport aux PSCs quiescentes. En conséquence, nous allons étudier le rôle de ces canaux calciques et de l'augmentation de la concentration en Ca^{2+} intracellulaire dans le processus d'activation et la fonction des PSCs. Pour cela, nous allons utiliser les inhibiteurs pharmacologiques de la SOCE ainsi que des siRNA et vérifier les marqueurs d'activation des cellules pancréatiques stellaires. (α-actine du muscle lisse, la sécrétion, la migration, la croissance...).

Nous allons également voir comment la fonction des PSCs activées sera modifiée suite à des altérations de l'homéostasie calcique (par inhibition de l'entrée de Ca^{2+}), et plus spécifiquement la sécrétion, la migration et la croissance des cellules pancréatiques stellaires activées seront analysées suite à un traitement avec des inhibiteurs de la SOCE ou ORAI1/STIM1 knockdown.

Conclusions

Notre travail prouve que ORAI1 et STIM1 supportent la SOCE dans des lignées cellulaires de l'adénocarcinome du pancréas. Nous avons montré que ORAI1 et STIM1 jouent tous deux un rôle pro-survie anti-apoptotique dans des lignées cellulaires d'adénocarcinome du pancréas, et

que le knockdown médié par les siRNA de ORAI1 et/ou STIM1 augmente l'apoptose induite par les drogues chimiothérapiques 5-FU ou la gemcitabine. Nous avons également démontré que les traitements avec le 5-FU et la gemcitabine augmentent la SOCE dans la lignée cellulaire Panc1 de l'adénocarcinome pancréatique, apparemment via une régulation positive de l'expression de ORAI1 et STIM1.

Nous démontrons que ORAI1 et STIM1 sont exprimés dans cinq lignées cellulaires testées de PDAC (Capan1, ASPC1, Panc1, MiaPaca2 et BxPC3) ainsi que dans les cellules "normales" épithéliales pancréatiques humaines H6C7. Il est à noter que les cellules "normales" H6C7 révèlent des niveaux relativement faibles d'expression de ORAI1 et de STIM1 par rapport à plusieurs lignées cellulaires de cancer, en particulier Capan1 et Panc1. Ce résultat suggère indirectement que ces cellules cancéreuses régulent positivement ORAI1 et STIM1 pour se protéger contre l'apoptose.

Nos résultats ont révélé le rôle anti-apoptotique de ORAI1 et STIM1 dans les cellules Panc1. Nous avons montré que les siRNA de ORAI1 et/ou de STIM1 augmentent l'apoptose induite par le 5-FU ou la gemcitabine dans les cellules Panc1.

En outre, l'effet du knockdown de ORAI1 et / ou de STIM1 sur l'apoptose dans les cellules Panc1 a été plus prononcé dans le cas du traitement à la gemcitabine par rapport au 5-FU. Ce résultat pourrait indiquer une dépendance accrue au calcium des mécanismes d'action de la gemcitabine par rapport au 5-FU.

De manière intéressante, nos résultats suggèrent également que le 5-FU et la gemcitabine pourraient tous deux affecter l'homéostasie calcique intracellulaire. En effet, ces deux traitements augmentent la SOCE dans les cellules Panc1 et régulent positivement ORAI1 et STIM1. Ce résultat suggère que les traitements (chimiothérapies) peuvent avoir des effets calcium-dépendants.

De plus, ces effets dépendant du calcium pourraient contribuer à l'efficacité des drogues dans la thérapie du cancer. D'autres expériences sont cependant nécessaires pour comprendre ce mécanisme. Nous faisons l'hypothèse que pendant la chimiothérapie des cellules régulent positivement les SOCs pour résister à l'apoptose. Par conséquent, la régulation négative des SOCs dans ces conditions rend les cellules plus sensibles à l'induction de l'apoptose.

Fait important, le cancer du pancréas est caractérisé par une importante fibrose et des PSCs ont été identifiées comme étant principalement responsables de ce phénomène. Il a été signalé que des cellules du cancer du pancréas peuvent stimuler l'activation des PSCs, conduisant à un excès de synthèse des protéines de la matrice extracellulaire qui composent le tissu fibreux.

Donc les PSCs activées deviennent les facteurs les plus importants de desmoplasie et ainsi contribuent à l'aspect réfractaire à la thérapie du cancer du pancréas. Ainsi, non seulement les cellules cancéreuses pancréatiques mais aussi les cellules stellaires sont impliquées dans la progression du cancer, ce qui suggère que le ciblage combiné des cellules du stroma et du cancer devrait être un objectif pour de futures thérapies innovantes.

Par conséquent, compte tenu du rôle important des PSCs dans la progression du cancer du pancréas ainsi que le manque de données sur les voies de signalisation calciques dans ces cellules, nous avons étudié l'homéostasie calcique dans les PSCs quiescentes et activées. Nous avons montré que les PSCs quiescentes sont caractérisées par des niveaux de base de calcium cytosolique et de calcium dans les réserves intracellulaires significativement plus faibles par rapport aux PSCs activées. De plus, les PSCs activées montrent des niveaux d'expression plus élevés des principaux composants des canaux de type SOC correspondants aux protéines ORAI 1,2,3 ainsi qu'à la protéine STIM1. Cette expression élevée est bien corrélée à une augmentation de la SOCE induite par la TG dans les cellules activées par rapport aux PSCs quiescentes. Nos données démontrent clairement que ORAI1 et STIM1 supportent la SOCE dans les PSCs activées, puisque le knockdown de l'une de ces protéines réduit significativement la SOCE.

Donc, en nous basant sur nos résultats, nous faisons l'hypothèse que dans des conditions saines, les PSCs sont dans état "dormant", avec de faibles niveaux de calcium cytosolique et stocké, ainsi qu'avec de faibles niveaux d'expression des canaux calciques. Suite à la stimulation par des facteurs d'activation restant à déterminer, les PSCs déclenchent l'expression des canaux calciques (SOC), accumulent le calcium dans les réserves intracellulaires et augmentent leur niveau de calcium cytosolique. Nous supposons que l'augmentation du calcium intracellulaire pourrait être un facteur important pour l'activation complète et la fonction des PSCs, et la prévention de l'augmentation du calcium intracellulaire par l'inhibition des canaux calciques sur la membrane plasmique pourrait être une stratégie utile pour diminuer la fibrose dans le cancer du pancréas.

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