Aus der Klinik für Allgemeine-, Viszeral-, Transplantations-,
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(Direktor: Prof. Dr. med. Jens Werner)

Verapamil can inhibit the tumorigenicity of chemotherapy resistant side population cells in pancreatic cancer

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To Cure Sometimes

To Relieve Often

To Comfort Always

Declaration

I hereby declare that the thesis is my original work.

The work and results presented in the thesis were performed independently.

The side population analysis on LSR II FACs machine was performed by technical support from Dr. Myslliwitz (Helmholtz center, Munich, Germany). Dr. Ellwart helped to carry out the isolation of side population cells with the Moflo flow cytometer (Helmholtz center, Munich, Germany).

Parts of the results have been included in the following manuscripts (in preparation):

Verapamil can inhibit the tumorigenicity of chemoresistant side population in pancreatic cancer.

No unauthorized data were included.

Information from the literature was cited and listed in the reference.

All the data presented in the thesis will not be used in any other thesis for scientific degree application.

The work for the thesis began from Apr. 2012 with the supervision from Prof. Dr. med. Christiane J. Bruns in Chirurgische Klinik und Poliklinik, Klinikum Großhadern, Ludwig-Maximilians University Munich, Germany.

07.2014, München

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I Contents

II Abstract

Pancreatic cancer is one of the leading causes of cancer death and one of the most challenging solid organ malignancies, due to its aggressiveness, late presentation as advanced disease, high rates of operative morbidity and chemoresistance during oncological therapy. Gemcitabine, the standard chemotherapy agent for advanced pancreatic cancer patients. Most patients respond to the initial treatment, but gradually they become resistant to chemotherapy with disease recurrences even after surgery.

Patients with cancer relapses commonly show resistance to multiple anti-cancer agents of different structures and functions within a single class after treated with a member of the class, this phenomenon is defined as multidrug resistance (MDR). An important and well-known mechanism of tumor MDR is increased drug efflux mediated by several transporters of the ATP-binding cassette (ABC) superfamily, especially BCRP (ABCG2), MDR1/P-glycoprotein (ABCB1) and members of the MRP (ABCC) family.

Recent studies have shown that the calcium channel blocker, verapamil, when combined with standard anticancer chemotherapy drugs, help to overcome resistance through inhibiting the transport function of P-glycoprotein, the product of MDR gene, MDR1 or stimulating glutathione (GSH) transport by MRP1.

The side population (SP) represents a small subtype of tumor cells with stem-like properties, which can be identified by the flow cytometry-based SP technique, and could be substantially eliminated by verapamil during Hoechst 33342 staining. However, little is known about the direct effects of verapamil itself on SP cells.

In this study, we focused on the therapeutic potentiality of verapamil on stem-like SP pancreatic cancer cells and investigated if it can act as the chemosensitizer on pancreatic tumor. We successfully builded up the orthotopic pancreatic cancer model which was induced by the SP subtype sorted cells from the human pancreatic cancer cell line, and most importantly, our results suggested that verapamil had an effective anti-tumorigenesis effect on pancreatic cancer either in *vitro* or in *vivo*.

III Introduction

3.1 Background of pancreatic cancer

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer among different primary tumors arising from the pancreas, arising within the exocrine component of the pancreas, the term pancreatic cancer is often referred to PDAC. Currently it is the fourth to fifth most common cause of cancer-related death in most western industrialized countries, with a poor prognosis of 5-year survival < 5%[1, 2]. Unfortunately, this poor survival rate has not improved in the past decades, in spite of developed dignostic imaging of the pancreas and aggressive therapeutic approaches. The poor prognosis is associated with late diagnosis, high rates of operative morbidity and resistance to chemotherapy.

The risk factors leading to pancreatic cancer may include family history of this disease, smoking, obesity, alcoholism, chronic pancreatitis and diabetes mellitus, especially when presented as new-onset diabetes mellitus[3]. Strolzenberg-Solomon *et al* in their nested case-control study of more than 500 participants, demonstrated adiponectin concentrations as a risk factor in male smokers[4].

3.2 Treatment of pancreatic cancer

Treatment for pancreatic cancer depends on the stage and location of the cancer as well as on patients'age, overall health and personal preferences. The pancreatectomy is the first aid in treating cancer that is confined to the pancreas: the Whipple procedure(pancreatoduodenectomy) is applied for the tumors located in the head of the pancreas, while the distal pancreatectomy is for tumors in the pancreatic tail and body[5]. However, most patients have advanced diseases at the time of diagnosis, less than 20% of pancreatic cancer patients are diagnosed with resectable diseases[6]. Furthermore, in unresectable cases, neoadjuvant and postoperative adjuvant therapy cases, palliation of symptoms and improvement of survival for patients must be pursued by chemotherapies or radiotherapies.

Over the last decade, pancreatic cancer clinical trials have had few successes. Nucleoside analogue, gemcitabine, which remains the standard systemic treatment in unresectable and

advanced cases, can prolong survival by 5-6 months[7]. A pivotal paper from 1997 demonstrated that a 23.8% clinical benefit and modest improvement with gemcitabine in overall survival (OS) of 1.24 months over 5-fluorouracil[8]. In a first-line phase III randomized trial for erlotinib, a biologic agent combined with gemcitabine, the 0.33 month advantage in median OS and 6% benefit in one-year survival comparing to gemcitabine alone is not generally accepted to be clinically relevant[9]. Most recently, the superiority of the combination of gembitabine with nab-paclitaxel, or a combination of 5-fluorouracil, irinotecan and oxaliplatin (FOLFIRINOX) over gemcitabine monotherapy has been demonstrated[10, 11]. Despite the value of gemcitabine in improving clinical benifit and medial survival, to date, satisfactory outcomes have not been achieved with gemcitabine alone or in combination with other cytotoxic drugs[7, 12, 13]. The chemoresistant acquisition further limits the chemotherapy efficiency, even with the latest generation regimens, the median OS of advanced pancreatic cancer patients is still less than 12 months[14]. Predicting and overcoming resistance of tumor cells to chemotherapy are the major challenges in cancer treatment.

3.3 Gemcitabine metabolism

Gemcitabine (2', 2'-Difluorodeoxycytidine) is a nucleoside analogue with anticancer activity in a variety of solid tumors, particularly pancreatic, bladder and non-small cell lung cancers, as well as refractory low-grade non-Hodgkin's lymphoma and myeloid malignancies[15-18]. Two general classifications of nucleoside transporters have been identified for gemcitabine uptaking into the cells: equilibrative nucleoside transporter (ENT) and sodium-dependent concentrative mechanisms (concentrative nucleoside transporter, CNT)[19-21], besides, in pancreatic tumor cells, among human ENT1 (hENT1), hENT2, hCNT1, and hCNT3, the major routes for transporting gemcitabine into the cells, hENT1 is expressed at high levels, whereas members of the CNT family are present only at negligible or at a low functional level[19, 20, 22, 23].

Once inside the cell, gemcitabine is phosphorylated by deoxycytidine kinase to its mononucleotide in a rate-limiting step, and then subsequently to its active metabolites, gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP). The cytotoxicity of gemcitabine is associated with the incorporation of dFdCTP into DNA and sequential inhibition of DNA synthesis, as well as the inhibitor role of dFdCDP on ribonucleotide reductase, to induce

depletion of cellular dNTP pools, which ultimately incude the cell apoptosis[22, 24]. The whole scheme of gemcitabine transport and metabolism is showed as below:

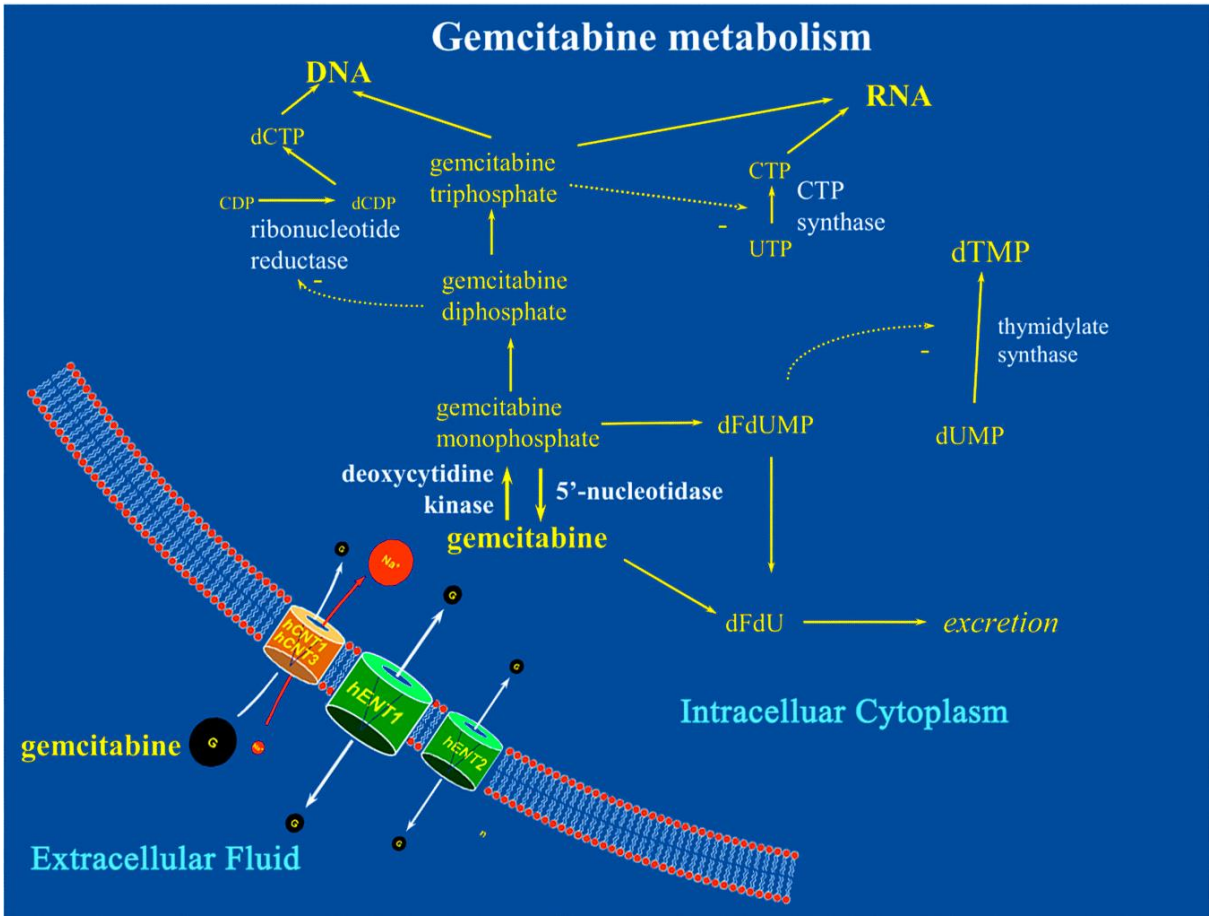


Figure III.1 Gemcitabine Metabolism.

Revised from 'Human Equilibrative Nucleoside Transporter 1(hENT1) in pancreatic adenocarcinoma'[25].

3.4 Gemcitabine induced chemoresistance in pancreatic cancer

The development of chemoresistance in human cancers is a major problem in the long-time benifited effective chemotherapy. Many forms of pancreatic cancer exhibit initial sensitivity to gemcitabine treatment followed by a rapid development of resistance. The mechanism of refractoriness to chemotherapy of pancreatic cancers is not fully understood, such resistance can be the result of various mechanisms and cellular targets, all of which have been identified to influence, as single factors or in combination, resistance to gemcitabine[26-29]. One of the most

common mechanisms of drug resistance is the decreased accumulation of drugs within cancer cells because of an increased drug efflux (pumping drugs out of cells) or reduced drug importation into the cancer cells[14].

3.4.1 Multidrug resistance phenomenon in pancreatic cancer

One form of drug resistance induced by drug efflux pumps, termed Multidrug Resistance (MDR), is a phenomenon characterized by the development of broad cross-resistance to functionally and structurally unrelated drugs within a single class after patients are treated with a member of the class, which due to enhanced outward transport of drugs mediated by a large super family of membrane glycoprotein "ATP-binding cassette (ABC) transporter"[30, 31]. The super family of human ABC transporters has 49 members, which can be divided into 7 subfamilies, named ABCA-ABCG based on sequence similarities[32]. Especially members of the ABCB family include a 170-KD typical transporter protein (ABCB1), also termed P-glycoprotein (P-gp), the product of the human MDR1 gene, and multidrug resistance-associated protein (MRP)[33, 34] were found to correlate with both decreased accumulation of drugs and degree of chemoresistance in many different human cancer cell lines[35-37]. In addition, the BCRP (ABCG2) protein, a member of the ABCG family has manifested the ability to mediate resistance against several anticancer drugs[38].

P-gp, presents little in most tissues, nevertheless, it is expressed in some limited normal tissues with barrier function, including bile canaliculi of the liver, the proximal tubules of the kidey, the capillary endothelial cells in brain, the luminal surface cells of the small and large bowel, adrenal gland and pancreatic ductules[39]. In many different cancer types, nearly 40-50% of the patients diagnosed with cancer have P-gp up to 100-fold over-expression in the malignant tissues[40-42]. The clinical significance of MDR1/P-gp expression in human pancreatic cancer is controversial. Sugawara *et al*. and Bernard research group both failed to demonstrate P-gp expression in pancreatic cancer tissues[43, 44], whereas other studies showed that the expression of MDR1/P-gp is common in pancreatic tumors and could potentially contribute the chemoresistant biology of pancreatic cancer[7, 13, 45].

What's more, previous studies have confirmed that gemcitabine is a substrate for MRP5 (ABCC5) efflux pump[46], further involved in gemcitabine resistance of pancreatic tumors and may contribute to predict the benefit of chemotherapy in patients with pancreatic cancer[29, 47].

3.4.2 Absence of ENT-1 is associated with gemcitabine resistance

On the other hand, another large family of transporters, the solute carrier (SLC) transporter, mediates the transport of a wide spectrum of substrates, including anticancer drugs, into the cell[14]. Nucleoside analogue (including gemcitabine) uptake is mediated by two subfamilies of SLC transporters, i.e. SCL28 and SCL29, which genes respectively encode CNT proteins and ENT proteins. Another point is, although gemcitabine is a substrate of the ATP-dependent effulx pump[29], MRP5 (ABCC5), it is predominantly transported into the cell across cell membranes via hENT1, as we mentioned before (in 3.3 Gemcitabine Metabolism).

Increasing evidences suggested that hENT-1 deficient cells are resistant to cytotoxic nucleosides *in vitro*[48-50]. In pre-clinical studies, hENT1 mRNA levels evaluated by quantitative reverse transcription polymerase chain reaction(RT-PCR), were proved to be correlated with the ability of gemcitabine to inhibit cells growth as determined by inhibitory concentration 50(IC_{50}) values, indicating higher expression levels of hENT1 is directly associated with chemosensitivity[26, 51]. To date, hENT1 immunostaining of pancreatic tumor cells, and its RNA expression levels have been manifested to serve as a prognostic marker for survival after gemcitabine treatment of pancreatic cancer[52, 53].

3.5 Verapamil as a chemosensitizer in cancer chemotherapy

Since the drug resistance is the major obstacle in treating pancreatic cancer, a means to overcome gemcitabine induced chemoresistance is urgently needed. Verapamil is a calcium channel blocker clinically utilized to treat cardiac arrhythmias. It has been reported that, verapamil, when combined with chemotherapeutic agents, can increase intracellular drug accumulation[54] and retention of chemotherapeutic agents in many human cell lines, including non-small cell lung cancer, colorectal carcinoma, leukemia, and neuroblastoma, and the antitumor activity has been achieved without enhancing myeloid toxicity[55-58]. More specifically, for example, resistance to

vinblastine was partially reversed by verapamil in vinblastine-resistant human leukemic lymphoma CCRF-CEM cells and in doxorubicin-resistant B16 murine melanoma cells[59, 60], as measured by short-term cell growth inhibition. A similar potentiation of anthracycline efficacy was demonstrated in P388 leukemia cells resistant to daunomycin, in which verapamil substantially reduced the concentration of the drug required to eliminate colony formation in soft agar[61].

The mechanisms involved include verapamil competitively binding to P-gp or stimulating glutathione (GSH) extrusion by MRP1 *in vitro*, which both resulted in an increase in the accumulation of drugs by tumor cells[54, 62]. Since MRP1 can transport organic anions, many of which are conjugated to the reduced GSH[63], the transportation of GSH conjugates of chemotherapeutic reagents out of cancer cells contributes to the chemotherapy insensitivity[64]. Furthermore, phosphorylation of P-gp by PKC (protein kinase C) is necessary for its pumping function. Verapamil reverses MDR phenotype by inhibiting the PKC activity. It can also sensitize anticancer drugs to tumor cells by increased affinity of the drugs to tumor cells. Verapamil reverses MDR by changing subcellular distribution of chemotherapeutic drugs in tumor cells; inhibiting P-gp expression by decreasing its mRNA level[65]. All of these advantages can help to overcome tumor cell resistance and enhance the efficacy of chemotherapy.

3.6 Cancer stem cells in pancreatic cancer

Emerging evidences have shown that cancer stem cells (CSCs), a small subset of undifferentiated cells within a tumor, with the ability to (i) duplicate themselves and self-renew, (ii) to regenerate a phenocopy of the original tumor and drive neoplastic proliferation when transplanted into the nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice, and (iii) to differentiate to some degree into more mature non-stem cell cancer lineages, are responsible for tumorigenicity, metastasis and malignancy[66] , which lead them to be the novel therapy target for cancer therapy. The existence of CSCs has been proven in many types of solid tumors including acute myelogenous leukemia, breast, brain, gastric cancers, as well as pancreatic cancers[67-71]. CSCs markers, such as CD133+, CD133+CXCR4+, CD24+CD44+ESA+, have been applied to identify pancreatic CSCs[71, 72].

Side population (SP) is a small subtype of tumor cells with CSCs' properties and can rapidly efflux lipophilic fluorescent dyes to produce a characteristic profile based on fluorescence-activated flow cytometric analysis[73]. This is usually measured by efflux of the fluorescent DNA binding dye Hoechst 33342 (H33342), with the main, non-SP cells separating according to DNA content, and hence cell cycle, using the differential emission spectra of H33342 bound to chromatin[73]. The pumps responsible for H33342 efflux, can be attributed to the expressions of several ABC superfamily transporters, including MDR1/P-glycoprotein, ABCG2 MRP1 and ABCA2[74-77], these pumps also transport other compounds out of the cell, including chemotoxic drugs, such as vinblastine, doxorubicin, daunorubicin, paclitaxel etc[78], that's why SP cells can exhibit increased chemoresistance after *in vitro* exposure to gemcitabine[79, 80]. Originally, SP with the vital dye, Hoechst 33342, was discovered for revealing the multipotential hematopoietic stem cells (HSC)[73], and now SP has been identified in many normal and tumor tissues, moreover, the assay has been proposed as a functional method to identify and isolate CSCs[81-84].

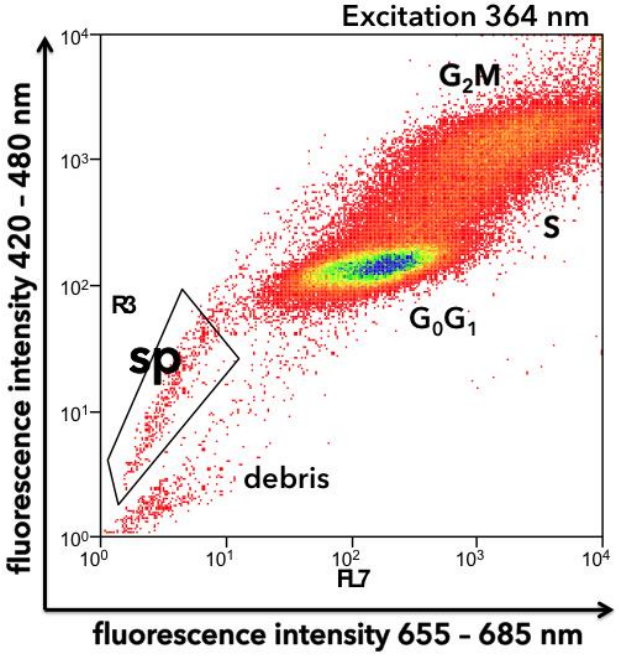


Figure III.2 Murine bone marrow cells stained with Hoechst 33342 and propidium iodide.

SP cells are known to have highly efficient pumps for the dye Hoechst 33342. The emission wavelength shifts to the blue end of the spectrum when the dye concentration is low in the DNA. According to different DNA content, cells in specific phase of the cell cycle(G_0/G_1, S, and G_2/M)

can be differentiate, since Hoechst dye is able to bind with DNA.(Original graph provided by Dr. J. Ellwart).

Goodell *et al*. has shown that in the presence of the drug verapamil, the SP cells was specifically eliminated during Hoechst 33342 staining in hematopoietic stem cells[73], then verapamil presence in the Hoechst 33342 dye as a control technique has been applied in other stem cell lines, such as mouse mammary epithelial progenitor cells, spermatogonial stem cells, pediatric tumor cell line, prostate, breast, colon, melanoma, esophageal and pancreatic cancer cell lines[85-90]. The mechanism involved in the inhibition of SP cells with verapamil, perhaps due to the enriched expression of ABC superfamily transporters in SP cells, which can be blocked by verapamil as we stated before.

3.7 Aim of the study

Increasing evidences suggested that verapamil can reverse the chemoresistance of many cancer cells and keeps a potentiality to generate a synergistic inhibition effect when combined with traditional chemotherapeutic reagents. Previously, our laboratory has identified the evidence for SP cells in human pancreatic cancer cell line (L3.6pl) as CSCs, which are responsible for resistance and metastasis. As little is known about the direct relationship between verapamil and SP on human pancreatic cancer, in our study, we focused on the therapeutic potential of verapamil on stem-like SP cells both *in vitro* and *in vivo* and investigated if verapamil can act as a chemosensitizer with gemcitabine in human pancreatic cancer cells *in vitro*.

IV Materials and methods

4.1 Materials

4.1.1 Cell lines

4.1.1.1 Human pancreatic cancer cell line L3.6pl and L3.6pl gemcitabine resistant variant cell line-L3.6pl$_{Gres}$

The human pancreatic adenocarcinoma cell line L3.6pl was yielded from the parental fast-growing (FG) variant line of the COLO 375 human pancreatic cancer cell lines, which were injected into the pancreas of nude mice, harvested hepatic metastases and reinjected into pancreas, repeated this cycle for three times. Detailed scheme was showed in the graph below[91](Figure IV.1). L3.6pl was attributed with increased metastatic potential, which was associated with increased expression (mRNA and protein) of the proangiogenic molecules basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and interleukin-8 (IL-8). L3.6pl cell line maintained the ability of increased motility and invasiveness, which were related with increased expression of collagenase type IV (MMP-9) and decreased expression of E-cadherin[91]. L3.6pl were cultured in medium with increasing concentrations of gemcitabine, starting at 0.5 ng/ml up to 100 ng/ml over 28-month period to develop gemcitabine-resistant cell line (L3.6pl$_{Gres}$).

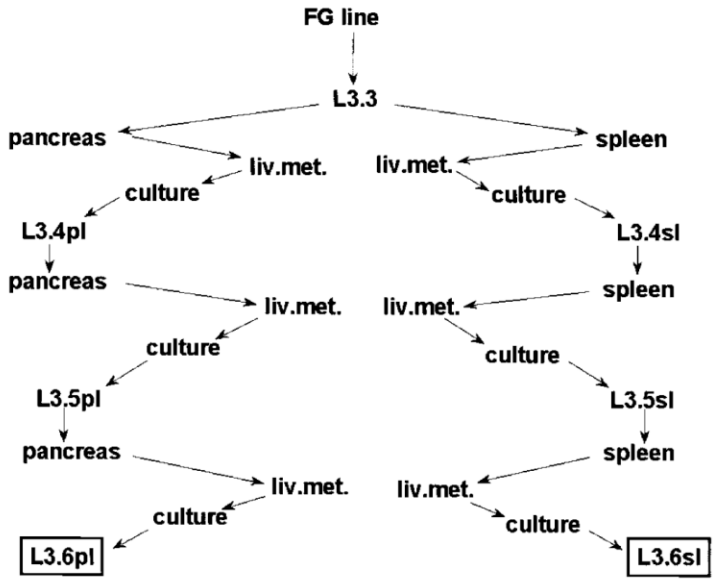


Figure IV.1 *In vivo* selection of highly metastatic human pancreatic cancer cells
(Original graph from Bruns *et al.*[91])

L3.3 cells originated from FG cells were injected into the pancreas or spleen of nude mice. Liver metastases were harvested and treated with DNase and collagenase, established in culture. Primary cells passaged for 2 or 3 times then injected into the speen of pancreas of another set of nude mice. After this cycle repeated for three times, highly metastatic cell line L3.6sl (spleen to liver selection) and L3.6pl (pancrea to liver selection) were generated.

4.1.1.2 Human pancreatic cancer cell line AsPC-1

The human pancreatic adenocarcinoma cell line AsPC-1 was derived from nude mouse xenografts initiated with cells from the ascites of a 62-year-old Caucasian female patient with cancer of the pancreas in 1981. The cell line was established by MH Tan[92], can produce carcinoembryonic antigen (CEA), human pancreas associated antigen, human pancreas specific antigen and mucin[92].

4.1.2 Reagents

4.1.2.1 Medium, buffers, solutions for cell culture

DMEM (Dulbecco's Minimal Essential Medium) 500 mL	Invitrogen GmbH, Karlsruhe, Germany
DMSO (Dimethylsulphoxide)	Sigma-Aldrich, Steinheim, Germany
DPBS-buffer 500 mL	Biochrom AG, Berlin, Germany
Fetal bovine serum 500 mL	Biochrom AG, Berlin, Germany
MEM vitamin mixture 100 mL	PAN Biotech, Aidenbach, Germany

MEM NEAA 100 mL	PAN Biotech, Aidenbach, Germany
Normocin 20 mL	InvivoGen, San Diego, USA
Penicillin/Streptomycin 100 ml (10.000 Units Penicillin/mL, 10 mg Streptomycin/ml)	PAN Biotech, Aidenbach, Germany
Trypsin0.05%/EDTA0.02 % in PBS without Ca^{2+} and Mg^{2+} 100 mL	PAN Biotech, Aidenbach, Germany
Trypan blue (0.4%)	Sigma-Aldrich, Steinheim, Germany

4.1.2.2 Cell culture medium

DMEM (500 ml)	plus	10%FCS 100 IU/ml Penicillin 100 µg/mL Streptomycin 2% MEM vitamin mixture 2% MEM NEAA

1 ml Normocin was added to the above mixed culture media as a "routine addition" to prevent mycoplasma, bacterial and fungal contaminations in small or large scale of animal cell cultures.

4.1.2.3 Cell storage medium

90% FCS	plus	10%DMSO

4.1.3 Cell culture materials

5 ml coster stripette	Corning Inc, New York, USA
10 ml coster stripette	Corning Inc, New York, USA
25 ml coster stripette	Corning Inc, New York, USA
25 cm^2 nunc sterile tissue culture flasks	Thermo Fisher Scientific Inc, Denmark
75 cm^2 nunc sterile tissue culture flasks	Thermo Fisher Scientific Inc, Denmark

150 cm^2 nunc sterile tissue culture flasks	Thermo Fisher Scientific Inc, Denmark
15 ml Centrifuge tubes	TPP, Switzerland
50 ml polypropylene conical tubes	BD Bioscience Europe, Belgium
6, 12, 24 and 96-well nunc delta surface culture plates	Thermo Fisher Scientific Inc, Denmark
12 and 24-well companion plate notched for use with cell culture insert	BD Dicknson Labware, USA
Nunc cryotube (2.0 ml)	Thermo Fisher Scientific Inc, Germany
Eppendorf safe-lock tubes (0.6 ml, 1.5 ml, and 2.0 ml)	Eppendorf AG, Hamburg, Germany
Hemacytometer and cover-slip (Cell counting chambers)	Bürker-Türk, Germany

4.1.4 Technical equipments

Automatic Tissue Processors Model 2065/2	MDS Group GmbH, Buseck, Germany
Automatic pipettes	Gilson, Middleton, WI, USA
Axioskop 40, AxioCam MRc5 Digital fluorescence	Carl Zeiss AG, Oberkochen, Germany
Casting stand	Bio-Rad Laboratories GmbH, Munich, Germany
Centrifuge 5417R	Eppendorf AG, Hamburg, Germany
Centrifuge Rotina 420R	Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany
CO_2 incubators	Heraeus, Rodenbach, Germany
Digital precision scale	KERN & Sohn GmbH, Germany
Dryer cabinet	Heraeus Instruments, Hanau, Germany
FACS Calibur	BD, Biosciences, USA
Freezer -20°C	Siemens AG, Germany

Freezer -80°C	Heraeus, Hanau, Germany
Fridge 4°C	Siemens AG, Germany
Hand tally counter	Carl Roth GmbH, Karlsruhe, Germany
Herasafe EN12469 2000 Class II safety cabinet	Thermo Fisher Scientific Inc, Germany
Histomat 2065/2LZ/Di	Bavimed Laborgeräte GmbH, Birkenau, Germany
Leica RM2255, Fully Motorized Rotary Microtome	Leica Microsystems, Germany
LSR II flow cytometry	BD, Biosciences, USA
Liquid nitrogen tank	MVE, New Prague, MN, USA
Microwave oven	Siemens, Germany
Millipore-Anlage MilliQ A10	Millipore Corporation, Bilerica, USA
MoFlo high speed sorter	DAKO Cytomation, Glostrup, Denmark
Phase contrast microscopy	Carl Zeiss GmbH, Germany
Philips Infrared Lamp	Philips Consumer Lifestyle, Drachten, Netherlands
PH -Meter	Hanna Instruments Ltd, Bedfordshire, UK
Thermo Scientific Heraeus incubator	Thermo Fisher Scientific Inc, Germany
TECAN GENios Plus ELISA reader	TECAN, Salzburg, Austria
Trans-Blot SD Semi-Dry Transfer chamber	Bio-Rad Laboratories GmbH, Munich, Germany
Vortex	IKA Works, Wilmington, NC, USA
Warmer incubator 37 °C	Binder GmbH, Tuttlingen, Germany
Warmer incubator 60 °C	Memmert GmbH & Co. KG, Schwabach, Germany
Waschmaschine Professional G7883	Miele& Cie. KG, Gütersloh, Germany
Water bath	GFL, Burgwedel, Germany

4.1.5 Materials for cell proliferation and cytotoxity assay

Gemcitabine (Gemzar)	Lilly Deutschland GmbH, Germany
Verapamilhydrochloride(verapamil)	Sigma-Aldrich GmbH, Steinheim, Germany
Cell counting kit-8 (CCK-8)	Dojindo Laboratories, Japan

4.1.6 Materials for colony formation assay, western blot, flow cytometry (including apoptosis assay), and immunohistochemistry

2-Mercaptoethanol	Sigma-Aldrich GmbH, Steinheim, Germany
4% paraformaldehyde	Pathology LMU, Germany
7-AAD	BD phamingen, USA
Acryl-bisacrylamide 30%	Bio-Rad Laboratories GmbH, Hercules, USA
Albumin from bovine serum (BSA)	Sigma-Aldrich GmbH,Steinheim, Germany
Ammoniumpersulfat (APS)	Carl Roth GmbH und Co. KG, Karlsruhe, Germany
Annexin V-FITC kit	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Avidin/Biotin blocking kit	Vector Laboratories, CA, USA
BCA protein assay reagent kit	Pierce, Rockford, USA
Biotinylated secondary antibody	Vector Laboratories, CA, USA
Bromphenolblue	ICN Biomedicals GmbH, Frankfurt, Germany
Citrat acid	Merck, Darmstadt, Deutschland
Complete Lysis-M	Roche applied science, Mannheim, Germany
Crystal violet	Sigma-Aldrich GmbH, Steinheim, Germany
DAPI in mounting medium	Vector Laboratories, CA, USA
ECL western blotting detection system	Amersham Biosciences, Germany
Ethanol 70%, 80%, 96%, 100%	CLN GmbH, Niederhummel, Germany
Eosin	Merck, Darmstadt, Germany

FCR blocking reagent (human)	Miltenyi Biotec GmbH, Germany
Hoechst 33342	Sigma-Aldrich, Steinheim, Germany
Hydrochloride	Merck, Darmstadt, Germany
Hydrogen peroxide 30% (H_2O_2)	Merck, Darmstadt, Germany
In situ cell death detection kit (TUNEL assay)	Roche, Penzberg, Germany
IgG1-FITC mouse isotype	Miltenyi Biotech, Bergisch Gladbach, Germany
Isopropanol	Merck, Darmstadt, Germany
Kaiser's glycerolgelatine	Merck, Darmstadt, Germany
Liquid DAB+ substrate chromogen system	Dako, CA, USA
Low fat milk powder	Carl Roth GmbH and Co. KG, Karlsruhe, Germany
Mayer's hemalum solution	Merck, Darmstadt, Germany
Methanol	Merck, Darmstadt, Germany
Monoclonal mouse anti-β- actin antibody	Sigma-Aldrich GmbH, Germany
Monoclonal mouse anti-P-glycoprotein antibody (C219)	Calbiochem, Darmstadt, Germany
Monoclonal rabbit anti-Ki67 antibody (ab16667)	Abcam, UK
Neo-Clear® (Xylene substitute)	Merck, Darmstadt, Germany
Normal rabbit serum	Vector Laboratories, CA, USA
Normal goat serum	Vector Laboratories, CA, USA
Polyclonal goat anti-mouse/ rabbit immunogluobulins HRP	Dako, Glostrup, Denmark
Polyclonal rabbit anti-h-ENT1 antibody (ab48607)	Abcam, Cambridge, UK
Polyclonal rabbit anti-CD31 antibody (ab28364)	Abcam, Cambridge, UK
Polyvinylidene difluoride membranes (PVDF)	Amersham, Braunschweig, Germany
Propidium iodide	BD phamingen, USA
Protease/phosphatase inhibitors	Roche, Mannheim, Germany
Protein-Leiter	Genscript corporation, Picataway, USA
Restore western blot stripping buffer	Pierce, Rockford, USA
Sodium chloride	Merck, Darmstadt, Germany

Sodiumdodecylsulfat(SDS)	Sigma-Aldrich, Steinheim, Germany
Target retrieval solution 10×	Dako, CA, USA
Tetramethylethylendiamin(TEMED)	Sigma-Aldrich, Steinheim, Germany
TRIZMA Base	Sigma-Aldrich, Steinheim, Germany
TRIZMA hydrochloride	Sigma-Aldrich, Steinheim, Germany
Triton® X-100	Sigma-Aldrich, Steinheim, Germany
Tri-sodium-citrat-dihydrat	Merck, Darmstadt, Deutschland
Tween 20	Serva Elektrophoresis GmbH, Heidelberg, Germany
Vectastain® ABC kit	Vector Laboratories, CA, USA
Verapamilhydrochloride (verapamil)	Sigma-Aldrich, Steinheim, Germany
Flow cytometry tubes	BD Bioscience Europe, Belgium

BD Falcon 5 ml polystyrene round-bottom tubes (REF 352052)

BD Falcon 5 ml polystyrene round-bottom tubes with cell strainer cap (REF 352235)

BD Falcon 5 ml polypropylene round-bottom tubes with cap (REF 352063)

4.1.7 Solution and Buffer

4.1.7.1 Solution for cell culture

Verapamil solution

Dissolved in H_2O (dest.) to 5 mM stock, kept in 4°C for six months.

Gemcitabine solution

Dissolved in PBS buffer to 10 mg/ml stock, and kept in 4°C for one week.

Hoechst 33342 solution

Dissolved in H_2O (dest.) to 1 mg/ml stock, aliquot, kept in – 20°C.

1% crystal violet solution

0.5 g crystal violet dissoved in 50 ml 100% ethanol, kept in the room temperature, avoiding light.

4.1.7.2 Solution and Buffer for SDS-PAGE and Westernblot

Separating buffer

TRIZMA Base		90.8 g
SDS		1 g
H_2O (dest.)	ad	500 ml

Adjust pH to 8.8, kept at 4 °C.

Tris-HCl-Buffer

Tris		1 M
pH 6.8		

Tris		1.5 M
pH 8.8		

SDS Running buffer 10 ×

TRIZMA Base		30.3 g
Glycin		144.0 g
SDS		10 g
H_2O (dest.)	ad	1000 ml

kept at 4 °C, before application, diluted to 1 × SDS Running buffer as following:

100 ml 10 × SDS Running buffer + 1000 ml H_2O (dest.)

Transfer-buffer

1× SDS Running buffer	400 ml
Methanol	100 ml

10× TBS-buffer

TRIZMA Base		24.2 g
NaCl		80 g
H_2O (dest.)	ad	1000 ml

Adjust pH to 7.6

TBST 1× (0.1% Tween)

10× TBS-buffer		100 ml
H_2O (dest.)	ad	1000 ml
Tween 20		1 ml

5 × Lade-Buffer

0.5 M Tris HCl pH 6.8	2 ml
Glycerol	3.2 ml
20 % SDS	1.6 ml
2-Mercaptoethanol	0.8 ml
1 % Bromphenolblue	1.6 ml
H_2O (dest.)	6.8 ml

aliquot, kept in – 20°C.

10 % APS

Dissolved in H_2O (dest.), aliquot, kept in – 20°C.

Strip-buffer

TRIZMA hydrochloride		10.32 g
SDS		20 g
2-Mercaptoethanol		7 ml
H_2O (dest.)	ad	1000 ml

Adjust pH to 6.7

4.1.7.3 Solution and Buffer for Immunohistochemistry

Tris-HCl-buffer

TRIZMA Base		6.85 g
TRIZMA Hydrochloride		0.9 g
Sodium chloride		8.75 g
H_2O (dest.)	ad	1000 ml

Adjust pH to 7.5

Tris buffered saline (TBS) buffer, 10×

Tris-HCl	1 M
NaCl	1.5 M

Adjust pH to 7.4

PBS wash buffer 1×

NaCl	140 mM
KCl	2.7 mM
Na_2HPO_4	10 mM
KH_2PO_4	1.8 mM

High purity dH_2O, adjust pH to 7.4

Citrat-Buffer

Solution A: Citrat acid ($C_6H_6O_7*H_2O$) 10.5 g dissolved in 250 ml ddH_2O

Solution B: Tri-Sodium-Citrat-Dihydrat ($C_6H_5O_7Na_3$) 29.41 g dissolved in 500ml ddH_2O

18 ml Solution A + 82 ml Solution B, add ddH_2O to 1 L.

Kept at 4°C for longer time.

4.1.8 Materials for animal experiments

4.1.8.1 Animals

Bagg-albino/c (Balb/c) nu/nu male mice (8-10 week old, 20-22 g)	Charles River, Sulzfeld, Germany

4.1.8.2 Surgical materials

BODE Cutasept® F	Bode Chemie, Hamburg, Germany
Disposable scalpels	Feather Safety Razor Co., Japan
Forceps	Dosch GmbH, Heidelberg, Germany
Hypodermic needle (30G)	B-Braun, Melsungen, Germany
Needle holder	Dosch GmbH, Heidelberg, Germany
Normal saline (0,9%)	B-Braun, Melsungen, Germany
Q-tips (cotton applicator)	NOBA, Wetter, Germany
Rotilabo®-embedding cassettes	Carl Roth GmbH, Karlsruhe, Germany
Scissors, sharp / blunt	Dosch GmbH, Heidelberg, Germany
Syringe (1 ml, 5 ml)	BD Plastipak™, Madrid, Spain
Thread with needle USP 4/0 Seralon®	Serag-Wiessner AG, Naila, Germany

4.1.8.3 Medicine

Growth Factor Reduced (GFR) BD Matrigel™ Matrix10 mL	354230, BD Biosciences, USA
Ketaminhydrochlorid (Ketavet®) 100 mg/mL	Pfizer Pharmacia GmbH, Berlin Germany
Xylazinhydrochlorid, Xylazin (Rompun®) 2% 25 mL	Bayer Healthcare, Leverkusen, Germany

4.1.9 Software

Adobe Acrobat 7.0 Professional	Adobe Systems Inc., USA
Axio Vision 4.4	Carl Zeiss GmbH, Germany
EndNote X7 (MAC Version X7)	Thomson Reuter, CA, USA
FlowJo software	Treestar Inc., Ashland, USA
Graphpad Prism 5.0	GraphPad Software, Inc., USA
Image-Pro Plus 5.0	Media Cybernetics, Inc., USA
Microsoft Office 2010 (Word, Excel, Powerpoint)	Microsoft Corporation, USA
SPSS Statistics 19	SPSS STATISTICS Inc., USA
SoftMax® Pro	Molecular Devices Corp., USA

4.2 Methods

4.2.1 Cell culture conditions

All kinds of cell lines used in this study were incubated at 37°C under 5% CO_2. The humidity of the incubator was 95%. Cells were keeping cultivated with the medium changed twice a week.

4.2.2 Determination of cell number

100 μL of single cell suspension was gently mixed with an equal volume of 0.4% trypan blue. 10 μL of the above mixture cell suspension was aspirated into the space between the glass cover-lip and hemacytometer chamber. Then the number of viable (transparent unstained) cells were counted in an area of 16 squares using a hand tally counter. After summing up all 4 sets of 16 corner squares, the total number was divided by 4 to calculate the average number, and then multipled by 2 (to adjust for the 1:2 dilution factor with trypan blue). Finally, the concentration of cells per mL was obtained as multiplying by 1×10^4 (the formula listed below).

Cell number per mL = (four blue-region unstained total cell number / 4) × 2 × 10^4

4.2.3 Storage and recultivation of the cells

4.2.3.1 Storage of the cells

The cells determined by the above mentioned method were stored as one to four million cell portions in 1.0 mL cell storage medium per cryotube. The 1.0 mL cell storage medium is composed of 900 µL FCS and 100 µL DMSO. DMSO is a cryprotective agent which can minimize the cellular injury by freezing and thawing procedures such as intracellular ice crystals and osmotic effects. The tubes are moved to a gradually temperature-decreasing tank and then kept in -80°C freezer. After 72 hours, the tubes were moved to a liquid nitrogen tank for long-time storage.

4.2.3.2 Recultivation of the cells

Before recultivation of the cells, the culture medium was first aspirated into the cell culture flask and kept in the incubator. The frozen tubes were taken out of the liquid nitrogen tank and immediately put into the 37°C prewarmed water bath for 1-2 minutes until complete thawing of the cells. The cells were then added into the cell culture flask with medium. After 24 hours culturing in the incubator, the medium was changed to avoid toxic effects of the remaining DMSO.

4.2.4 Isolation of SP- and non-SP-cell fractions from L3.6pl$_{Gres}$ and AsPC-1 cell lines

L3.6pl$_{Gres}$ and AsPC-1 cell lines were prepared for SP cells isolation when they reach 60-70% confluence. SP- and non-SP-cell fractions were identified and isolated using a modified protocol described by Goodell *et al.*[73]. Briefly, 10^6/ml cells were re-suspended in DMEM containing 2% fetal bovine serum and labeled with Hoechst 33342 at a concentration of 2.5 µg/ml for 60 minutes at 37 °C water bath, either alone or with 225 µM verapamilhydrochloride (verapamil). After 60min-staining, the cells were centrifuged at 4°C and 300g for 5min and resuspended in ice cold

PBS, containing 2% fetal bovine serum, passed through a 40um mesh filter and maintained at 4 °C in the dark until flow cytometry analysis for sorting or further cell surface marker staining. Cells were counterstained with 10 μg/ml propidium iodide to label dead cells, then analyzed by BD-LSRII flow cytometer and FlowJo software or sorted by MoFlo with the Summit 4.3 software. Hoechst dye was excited at 355nm (UV), and the fluorescence was measured at two wavelengths using a 450/50 nm (blue) band-pass filter and a 670/30 nm (red) long-pass edge filter. Isolated SP cells from $L3.6pl_{Gres}$ and AsPC-1 were kept on ice for further application. After isolation, SP- and non-SP-cell fractions were used for the following in *vitro* assays and in *vivo* orthotopic pancreatic cancer mice model.

4.2.5 Cell viability, proliferation assay and IC50 determination

The trypan blue staining was used for the cell viability measurement. The dye Trypan Blue stains dead cells with membrane defects, thus, living and dead cells can be distinguished by their ability to exclude the blue dye under the phase contrast microscopy. The cell viability was calculated followed the formula displayed below:

Cell viability = unstained cells/(unstained + trypan blue stained cells) × 100%

Cell proliferation was measured using the Cell Counting Kit-8. According to the manufacture's instructions, 5,000 - 8,000 cells/well plated in the 96-well plate were grown over night, treated for 24 hours with specific chemotherapeutics (gemcitabine or verapamil) and read out afterwards using VersaMax tunable microplate reader and Softmaxpro 5.2 for data analysis.

4.2.6 Apoptosis assay

Cell apoptosis assay were applied with the Annexin V-FITC kit following its specification. After determination of the cell number, wash 10^6 cells in 1 mL of 1× binding buffer and centrifuge at 300 g for 10 minutes. Aspirate supernatant completely. Resuspend cell pellet in 100 uL of 1× binding buffer per 10^4 cells. And then add 10 uL of Annexin V-FITC per 10^6 cells, mix well and incubate for 15 minutes in the dark at room temperature. Afterwards, we wash cells with 1× binding buffer, centrifuge and aspirate supernatant completely. Resuspend cell pellet in 500 uL

of 1× binding buffer per 10^6 cells. Add 5 uL of PI solution immediately prior to analysis with by BD-LSRII flow cytometer and FlowJo software. Experiments were repeated three times.

4.2.7 Colony formation assay

We seeded 500 cells/well with 2 ml required DMEM medium into 6-well plates ensuring that all the cells seeded are single cells, changed medium twice per week, stopped the assay when the colonies are clearly visible even without looking under the microscope (each single colony should composed of at least 50 cells). At the end we stained the colonies with 0.1% crystal violet and counted them.

4.2.8 Western Blot assay

4.2.8.1 Preparation of proteinlysates

After wash the cells with cold PBS for three times, they were directly lysed in Complete Lysis-M buffer supplemented with protease/phosphatase inhibitors for 10 min on ice, centrifuged at 14000 g at 4°C for another 10 min, then the supernatant fluid were collected and kept at -20°C.

4.2.8.2 Determination of the protein concentration

Protein concentrations were measured using the BCA protein assay. Mix 5 µl preprepared proteinlysates or 5 µl standard protein samples in 45 µl ddH_2O in a 96-well plate, then add 50 µl BCA working reagent (1 µl solution B + 49 µl solution A) to each well, mix properly, incubate for 30 min at 37°C, cool down to room temperature and measure OD of all samples at 562 nm within 10 minutes, using TECAN GENios plus ELISA reader, and analyse the data with SoftMax Pro.

4.2.8.3 SDS-Polyacrylamidgelelectrophorese PAGE

The cell lysates were mixed with 5 × loading buffer, boiled at 95 °C for 5 min and centrifuged at 16,000 g at 4 °C for 5 min, the samples for monoclonal anti-P-gp mouse antibody detection were not boiled based on the specifications. The different protein samples (up to 20 µg) were loaded onto sodium dodecyl sulfate-polyacrylamide gels for electrophoresis. 6µl protein ladder was loaded as molecular weight standard. The electrophoresis condition was 20 mA per gel for 120-150 min at RT. Different concentration of separating gels and 5% stacking gels were prepared as following:

12% separating gels (for detecting polyclonal rabbit anti-hENT1 antibody)

Components	Volume (ml)
Separating buffer	2.95
Acryl-bisacrylamide 30%	2.0
APS 10%	0.05
TEMED	0.002

8% separating gels (for detecting monoclonal mouse anti-P-gp antibody)

Components	Volume (ml)
Separating buffer	3.65
Acryl-bisacrylamide 30%	1.3
APS 10%	0.05
TEMED	0.003

5% stacking gels for denaturing SDS-PAGE

Components	Volume (ml)
H_2O (dest.)	0.68
Acryl-bisacrylamide 30%	0.17
1M Tris (pH 6.8)	0.13
SDS 10%	0.01
APS 10%	0.01

TEMED	0.001

4.2.8.4 Transfer the protein to PVDF membrane

After separation, proteins were transferred to a PVDF membrane in a semidry transfer chamber. Before transfer, the membrane was immersed in 100% methanol for several minutes, and the equal size of blotting paper were immersed thoroughly in transfer buffer. The transfer took place in a semidry transfer unit at a constant flow of 150 mA per gel (maximum voltage controlled below 25 V) for 60 min at RT.

Transfer structure:

Cover plate (cathode plate)
Three layers of gel blotting paper
SDS-polyacrylamide gel
PVDF membrane
Three layers of gel blotting paper
Bottom plate (anode plate)

4.2.8.5 Detection of protein expression

Sequentially after transferring the membranes were blocked with 5% milk in TBST for 2 hours at RT, then incubated with respective first antibody according to the manufacturer's instructions at 4°C overnight on a rotating platform, washed 3 times with TBST 15min each time, then incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibody at RT for 2 hours, following another washing procedure again and finally detection using an enhanced chemiluminescense system. Afterwards the membranes were used for ß-actin to ensure equal protein amounts. For detection of ENT1 protein, the anti-ENT1 antibody was used at 1:500 dilution in 5% BSA-TBST buffer. For P-gp detection, the monoclonal mouse C219 antibody was

used at 1:100 dilution in 7.5% skim dried milk-PBST buffer. This antibody recognizes the two renal P-gp isoforms[93].

4.2.9 Orthotopic pancreatic cancer mouse model

4.2.9.1 Animals

The 8 to 10-week old, 20-22g body weight, male athymic Bagg-albino/c (Balb/c) nu/nu mice were obtained from Charles River, Inc (Sulzfeld, Germany). All animal experiments were conducted according to german legislation for the protection of animals.

4.2.9.2 Animal's housing conditions

Animals were housed and maintained in laminar flow cabinets under specific pathogen-free conditions with free access to food and water.

4.2.9.3 Anesthesia

Mice were anesthetized with Ketaminhydrochlorid (Ketavet® 100mg/kg mouse body weight) and Xylazinhydrochlorid (Rompun® 5mg/kg mouse body weight). Normal saline, Ketavet and Rompun were mixed in a ratio of 1:1:1 (1 mL of each). The mixed anesthesia reagent was injected 50 uL intraperitoneally for each mouse.

4.2.9.4 Orthotopic pancreatic tumor model and experimental treatment

The operation was carried out in a sterile manner. A 1-cm left abdominal flank incision was made, the spleen was exteriorized, then 10^5 isolated SP- or non-SP L3.6pl$_{Gres}$ cells were injected with a 1 ml syringe, 30G needle and a calibrated pushbutton-controlled device into the subcapsular region of the pancreas. There should be a fluid bleb without intraperitoneal leakage after a successful sub capsular intra-pancreatic injection. To avoid the cell suspension leaking

into the peritoneal cavity, a Q-tip was pressed lightly on the injection site for 30 seconds after the needle was pulled out of the liver. After injection of the tumor cells, the peritoneum and skin were closed with interrupted sutures of USP 4/0 Seralon. The procedure is shown in Figure IV.2. Mice in each group were blindedly categorized (Table IV.1). After orthotopic injection, cell viability was measured with the trypan blue staining in order to control the therapeutic efficiency. Orthotopic tumor growth was monitored twice a week. Four weeks after the orthotopic implantation, therapy started: one treatment group was daily (every workday) intraperitoneal injection of low concentration of verapamil (200 uM, 0.5 mg/kg mice weight), another group was a relatively higher concentration of verapamil (10 mM, 25 mg/kg mice weight). On day 63 after the injection all of the mice were sacrificed and examined for orthotopic tumor growth, lymph node and hepatic metastasis. The pancreas including the tumors and other organs were isolated, weighed and then used for the following Hematoxylin and Eosin (H&E) staining and immunohistochemistry. Detailed experimental time scheme is shown as Figure IV.3.

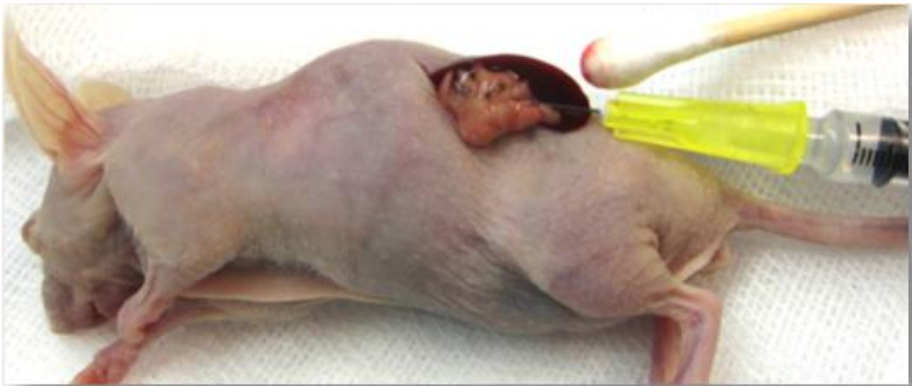


Figure IV.2 Mice orthotopic pancreatic cancer model establishment

A 0.8-1.0 cm left flank incision was conducted to expose the spleen, exteriorized the spleen softly with Q-tips, assisting further exposion of the pancrea. Tumor cell suspension was injected with a 1 ml disposable syringe, 30-gauge needle and a calibrated pushbutton-controlled dispensing device, into the head part of pancrea carefully, avoid of the intraperitoneal leakage. There is a transparent bleb after a successful sub capsular intrapancreatic injection.

Table IV.1 Experimental design for verapamil inhibition of SP tumorgenicity of pancreatic cancer

Group	Group name	Cell name	Cells per mouse	Treatment	Mice number
1	L3.6pl$_{Gres}$-SP	L3.6pl$_{Gres}$-SP	1×10^5	None	5
2	L3.6pl$_{Gres}$-NSP	L3.6pl$_{Gres}$-NSP	1×10^5	None	3
3	L3.6pl$_{Gres}$-SP Vera high con	L3.6pl$_{Gres}$-SP	1×10^5	verapamil(25mg/Kg)	3
4	L3.6pl$_{Gres}$-SP Vera low con	L3.6pl$_{Gres}$-SP	1×10^5	verapamil(0.5mg/Kg)	4

Group 1: Balb/c nu/nu mice injected with SP cells isolated from L3.6pl$_{Gres}$ cells (n=5)

Group 2: Balb/c nu/nu mice injected with NSP cells isolated from L3.6pl$_{Gres}$ cells (n=3)

Group 3: Balb/c nu/nu mice injected with SP cells isolated from L3.6pl$_{Gres}$ cells and treated with relatively higher concentration of verapamil (10mM, 25mg/kg mice weight) (n=3, one died in the orthotopic operation)

Group 4: Balb/c nu/nu mice injected with SP cells isolated from L3.6pl$_{Gres}$ cells and treated with relatively lower concentration of verapamil (200 uM, 0.5mg/kg mice weight) (n=4)

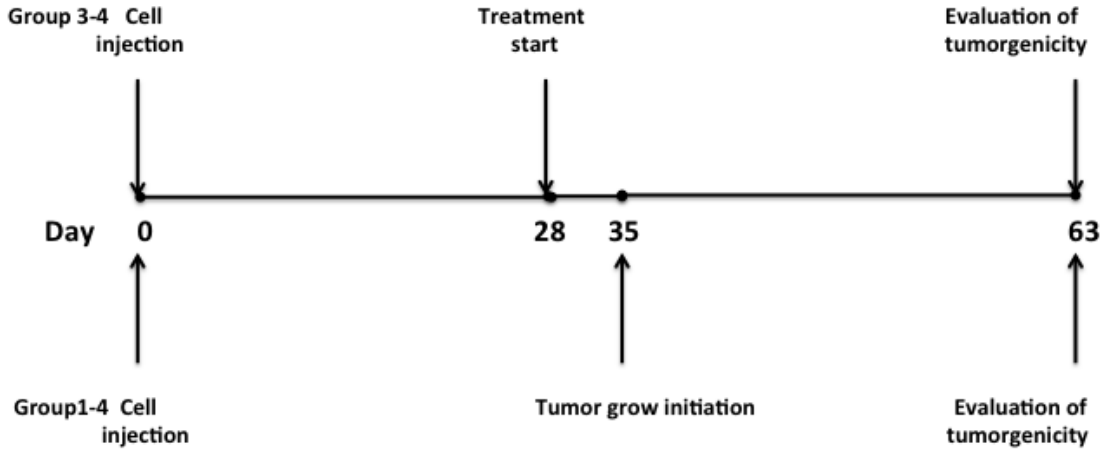


Figure IV.3 Experimental schedule of the tumor model and the experimental treatment.

FACS sorting for Group 1-4 was sequentially carried out during the first 1-3 days. L3.6pl$_{Gres}$-SP cells induced palpable tumors one month after injection. Daily verapamil treatment was started on day 28 sequentially follow the injection. All the mice were sacrificed on day 63.

4.2.10 Histology

4.2.10.1 Hematoxylin Eosin (H&E) staining

All tumors from the *in vivo* experiments were formaldehyde-fixed and embedded in paraffin wax. Then 3-4 μm serial sections were generated. Tissues were deparaffinized in xylene, and rehydrated in a graded series of ethanol. After 5-8 minutes in Mayers Haematoxylin immersion, the sections were washed for 8 minutes with warm running water. Then the sections were immersed several seconds in Millipore water and stained with 0.1% Eosin solution for 2 minutes. After dehydration with graded series of ethatnol, the sections were mounted with Kaiser's glycero gelatine and sealed with coverslips.

4.2.10.2 Immunohistochemistry (staining for *Ki*67, CD31 and TUNEL)

Formaldehyde-fixed and paraffin-embedded tissues were serially sectioned at 3 μm and allowed to dry overnight in 37℃ incubator. Sections were deparaffinized in xylene followed by a graded series of ethanol (100%, 95%, 80%) and rehydrated in phosphate-buffered solution, pH7.5. Paraffin-embedded tissues were used for Ki67 proliferation index assay, microvascular density analysis (CD 31 antibody) and TUNEL assay. TUNEL assay was carried out with in situ cell death detection kit, Fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) and closely followed its instruction, with a DAPI nuclear counterstaining. The antibodies used for the other paraffin-embedded tissues included monoclonal rabbit anti-Ki67 antibody (ab 16667, Abcam, UK) and polyclonal rabbit anti-CD31 (ab 28364, Abcam, UK), the primary antibodies were diluted in PBS containing 3% bovine serum albumin (BSA). In addition, the slides were treated for 20 minutes with blocking solution (8% goat serum or rabbit serum in PBS with 3% BSA) before the primary antibody was applied. Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide (H_2O_2). Endogenous avidin and biotin were blocked using the Avidin/Biotin Blocking Kit (Vector, United States).

Overnight incubation with the primary antibodies was followed by incubation with the respective biotinylated secondary antibodies (goat anti-rabbit, BA-1000, Vector, United States), and the

ABC reagent for signal amplification (Vectastain ABC-Peroxidase Kits, PK-4000, Vector, United States). Between incubation steps, the slides were washed in TBS. 3,3'-diaminobenzidine (DAB, Dako, United States) was used to develop color. Slides were counter-stained with hematoxylin, and mounted in Kaisers Glycerinegelatine and coverlsips.

For quantification of the staining intensity each index (*Ki*67 index, microvascular density CD31 index and TUNEL apoptotic index) were evaluated in a blinded manner. Slides were observed under high/low magnification (200×/100×), areas presenting the highest density of Ki67 positive cells (nuclear with brown color) or nuclear TUNEL signals (nuclear with green fluorescence) were chosen and captured as photographs. These photographs were analyzed by Image-J program. Each index in all groups was evaluated with 3 fields and data analyzed as mean positive signal (Ki67-positive cells, microvessels numbers, cells with strong FITC-fluorenscence) of these 3 fields. Necrotic tumor cells were excluded from the cell count.

4.2.11 Statistical analysis

Statistical evaluation was performed using the paired student's t-test or ANOVA test (Microcal Origin) with $p < 0.05$ considered to be statistically significant. ($p<0.05$ marked as '&'; $p<0.01$ marked as '#'; $p<0.001$ marked as '*'; $p<0.0001$ marked as '**'). GraphPad Prism® 5.0 or Microsoft excel 2010 softwares were used to generate graphs and tables.

V Results

5.1 *In vitro* part

5.1.1 Gemcitabine induced morphological change of L3.6pl cells

We cultivated the human pancreatic adenocarcinoma cell line L3.6pl continuously with gemcitabine, starting at 0.5 ng/ml up to 100ng/ml to develop a gemcitabine-resistant cell line ($L3.6pl_{Gres}$). The 24h IC_{50} significantly increased from 6.1ng/ml ±0.9 (L3.6pl) to 498.8ng/ml ±3.2 ($L3.6pl_{Gres}$) (L3.6pl vs. $L3.6pl_{Gres}$ $p<1E^{-9}$). Besides, the morphology of $L3.6pl_{Gres}$ cells gradually changed into large, fibroblastoid tumor cells (Figure V.1), while L3.6pl cells without treatment kept its original major round shape.

A) L3.6pl (100×)

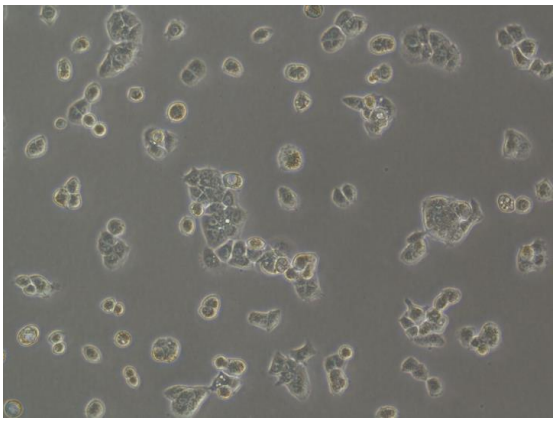


Figure V.1 Cell image of L3.6pl and $L3.6pl_{Gres}$ following gemcitabine treatment.

A) parental L3.6pl cells: small and round cells with some irregular relatively larger cells;

B) L3.6pl$_{Gres}$ (30days after gemcitabine treatment, 100×)

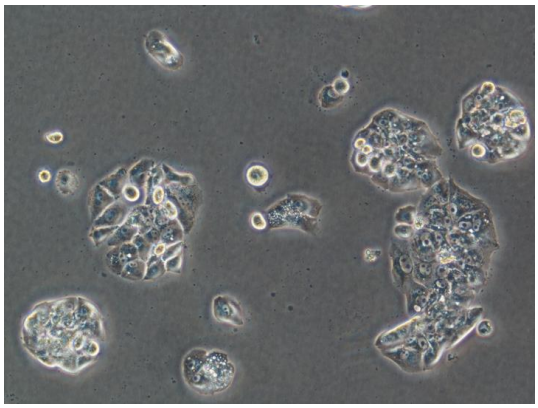


Figure V.1.
B) L3.6pl$_{Gres}$ cells after treatment with gemcitabine over 30 days: enlarged cell shape;

C) L3.6pl$_{Gres}$ (60days after gemcitabine treatment, 100 ×)

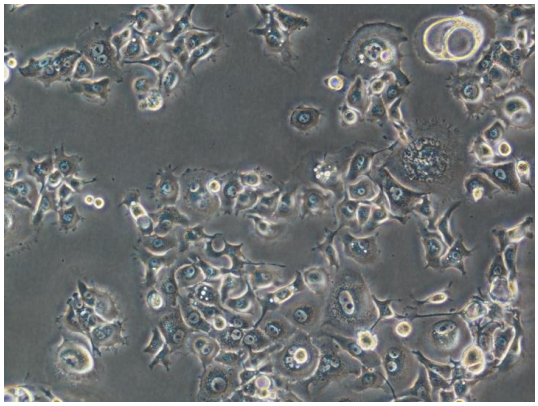


Figure V.1.
C) L3.6pl$_{Gres}$ cells after treatment with gemcitabine over 60 days: the majority of L3.6pl$_{Gres}$ cells have changed their morphology into a fibroblastoid type with loss of polarity, increased intercellular separation and pseudopodia, while an extreme minority of small round cells interspersed inside.

5.1.2 Identification and characterization of SP cells in pancreatic cell lines

5.1.2.1 Identification of SP cells using the Hoechst 33342 staining

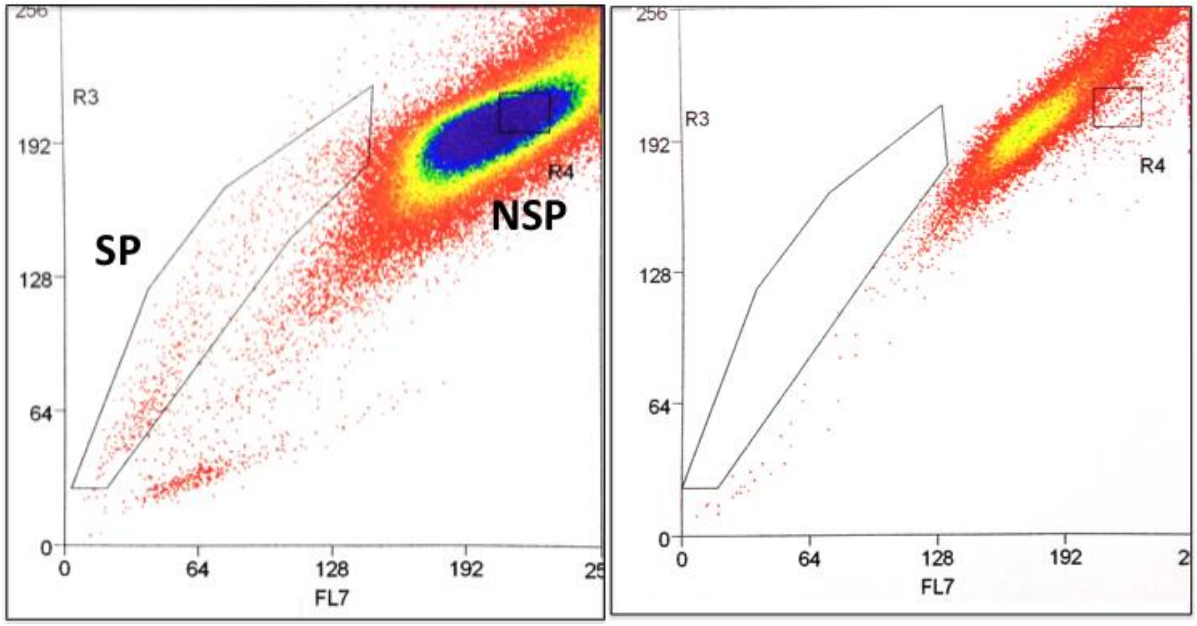
Both, L3.6pl and AsPC-1 cell lines were examined for the existence of SP cells. Verapamil hydrochloride, which blocks transporters of the ABC family and abrogates the ability to efflux the dye, served as control. These two cell lines contained a distinct proportion of SP cells. The percentage of SP cells increased from 0.9% ± 0.22 in L3.6pl to 5.38% ±0.99 in L3.6pl$_{Gres}$ cells following continuous gemcitabine treatment. The proportion of SP cells was diminished by

verapamil hydrochloride (Figure V.2). For each re-analysis of all the cell lines by FACS, the proportions of SP and NSP cells (Non-SP cells) were comparable to the intial distribution ratio of both subpopulations. Isolated NSP cells of L3.6pl or AsPC-1 rarely regenerate SP and NSP subpopulations.

Figure V.2 Identification of SP cells using the Hoechst 33342 staining

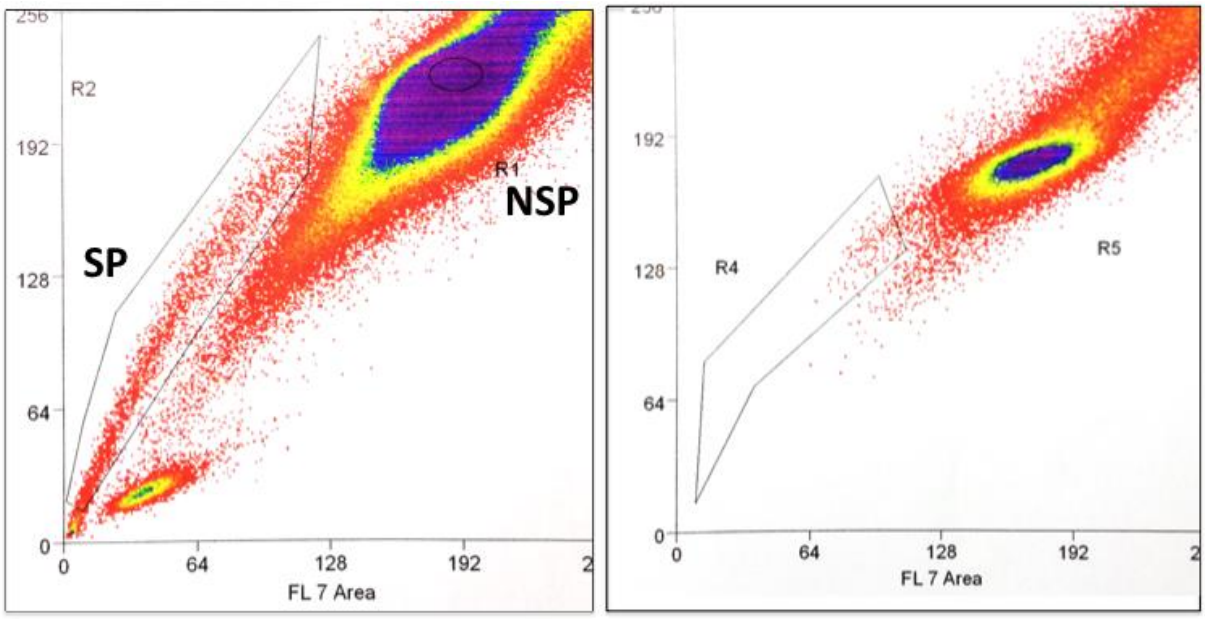
A. L3.6pl & L3.6pl$_{Gres}$

L3.6pl



Verapamil - +

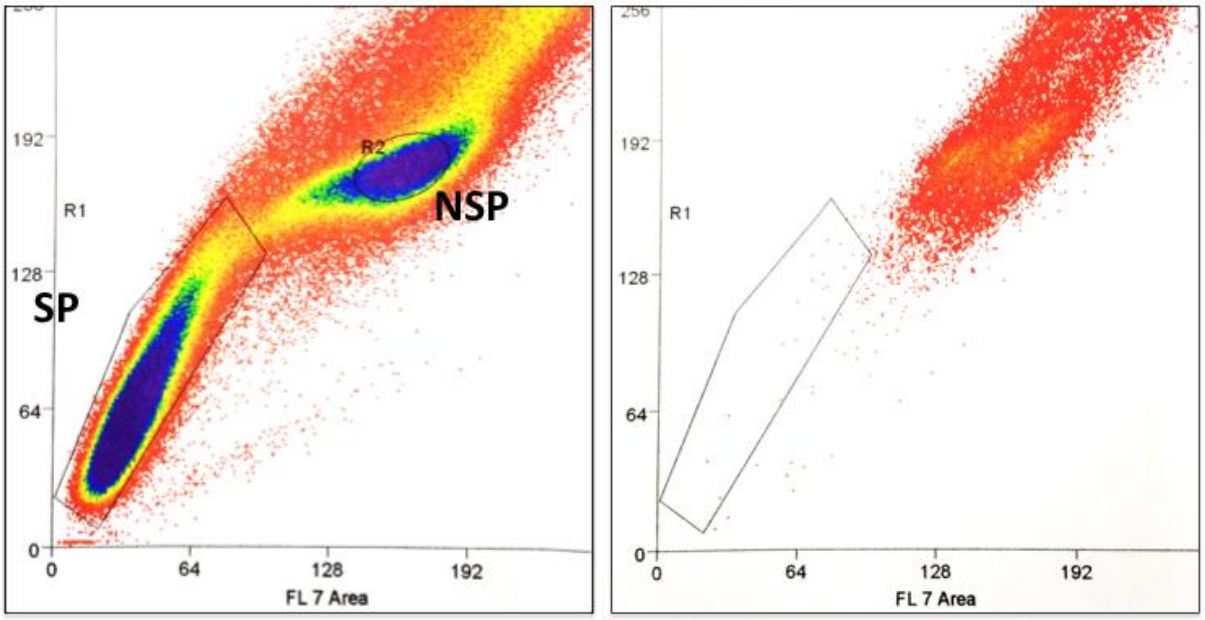
L3.6pl$_{Gres}$



Verapamil **-** **+**

B. AsPC-1

AsPC-1



Verapamil **-** **+**

A). Proportion of SP cells in L3.6pl (0.9% ± 0.22) compared to L3.6pl$_{Gres}$ cells (5.38% ± 0.99). Using verapamil, an ABC family drug-efflux blocker, the percentage of SP cells was significantly reduced to 0.16% ± 0.11(L3.6pl versus L3.6pl + verapamil, $p < 5E^{-10}$); B). SP cells were identified in AsPC-1 cell line with 21.35% ± 3.48. Verapamil again induced a significant reduction of SP cells down to 3.56% ± 0.87(AsPC-1 versus AsPC-1 + verapamil, $p < 5E^{-10}$).

Note: Data are presented as mean ± standard deviation.

5.1.2.2 L3.6pl$_{Gres}$-SP cells were more resistant to gemcitabine than L3.6pl$_{Gres}$-NSP cells

We tested the effect of gemcitabine treatment on L3.6pl$_{Gres}$ cells. SP and NSP cells were sorted out of L3.6pl$_{Gres}$ and treated with different concentrations of gemcitabine for 24 hours demonstrating that L3.6pl$_{Gres}$ SP cells were more resistant to gemcitabine than NSP cells (Figure V.3).

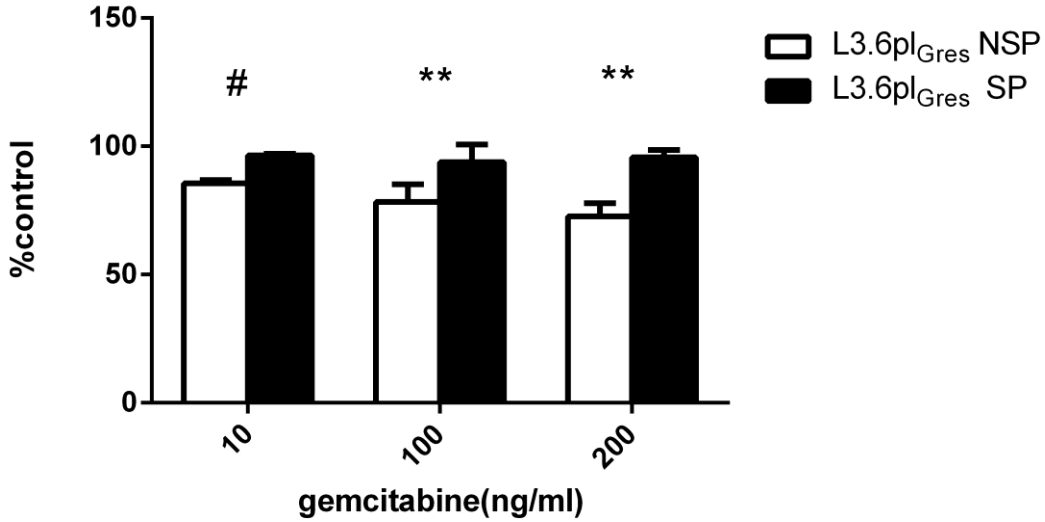


Figure V.3 L3.6plGres-SP cells were more resistant to gemcitabine than L3.6plGres-NSP cells

Immediately after FACS sorting, SP as well as NSP cells from L3.6pl$_{Gres}$ were seeded into 96-well plates to cultivate overnight. Increasing concentrations of gemcitabine were applied for 24

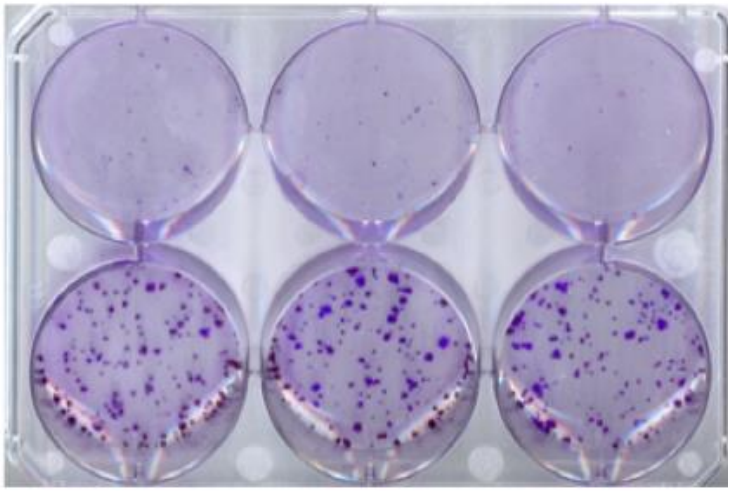
hours. L3.6pl$_{Gres}$-SP cells maintained their gemcitabine resistance compared to NSP cells with increasing doses of gemcitabine (p # < 0.01, p ** < 0.0001).

5.1.2.3 L3.6pl$_{Gres}$-SP cells exhibit stronger colony formation ability

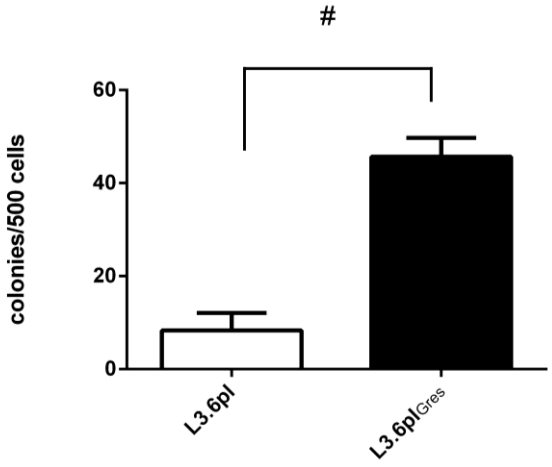
Since the percentage of L3.6pl SP cells is low, it was difficult to harvest enough cell samples by FACS for further analyses, so in part we took L3.6pl$_{Gres}$ cells with enriched SP cells as compared to the original L3.6pl cell line with less SP cells. Under this condition, we compared the colony formation ability between L3.6pl$_{Gres}$ and L3.6pl to support our hypothesis that SP cells have stem cell characteristics. The colony formation assay showed that L3.6pl$_{Gres}$ cells have significant higher colony formation ability than L3.6pl cell lines (Figure V. 4).

Figure V.4 L3.6plGres-SP cells exhibit stronger colony formation ability

A).



B).



A). 500 single cells/well were seeded evenly into the 6-well plates with normal D-MEM culture medium and cultivated up to be macroscopic visible colony(each colony composed of at least 50 cells). The colonies were stained with crystal violet and counted. The upper three wells were seeded with L3.6pl cells, while the lower three wells were seeded with L3.6pl$_{Gres}$ cells.
B). L3.6pl$_{Gres}$ can form more colonies per 500 single cells than L3.6pl (p #< 0.01).

5.1.3 Effects of verapamil on pancreatic cancer cells

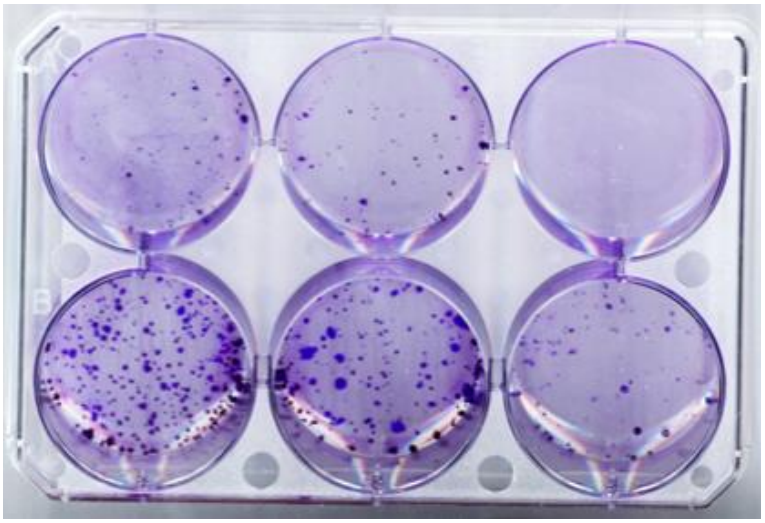
As we stated above for the Hoechst 33342 staining assay, verapamil acts as a negative control since it can eliminate SP cells by effluxing the dye (shown in Figure V.2.A & B). To investigate if verapamil alone can act as a potential treatment for pancreatic cancer cells, especially pancreatic cancer SP cells, we analyzed the colony formation and cell viability with verapamil intervention on pancreatic cancer cells.

5.1.3.1 Verapamil can inhibit the colony formation ability of L3.6pl and L3.6pl$_{Gres}$

24h verapamil treatment was carried out after the cells have already firmly attached to the plate and formed small cell masses (less than 50 single cells), which were visible under the

microscope. In consistence with Figure V.4.A, L3.6pl$_{Gres}$ maintained stonger colony formation ability than L3.6pl even with the treatment of verapamil. The inhibitory effect of verapamil on colony formation was dose independent on both cell lines: L3.6pl and L3.6pl$_{Gres}$ (Figure V.5).

Figure V.5 Verapamil can inhibit the colony formation ability of L3.6pl and L3.6pl$_{Gres}$



Two different concentrations of verapamil were used for both cell lines L3.6pl and L3.6pl$_{Gres}$. L3.6pl$_{Gres}$ cells formed much more colonies than L3.6pl under each equal treatment condition (verapamil 0, 50 uM, 100 uM). At the concentration 100 uM, both cell lines formed less colonies than with 50 uM, than the controls without treatment.

5.1.3.2 Verapamil alone can effectively inhibit viability of L3.6pl$_{Gres}$ and AsPC-1 SP cells *in vitro*

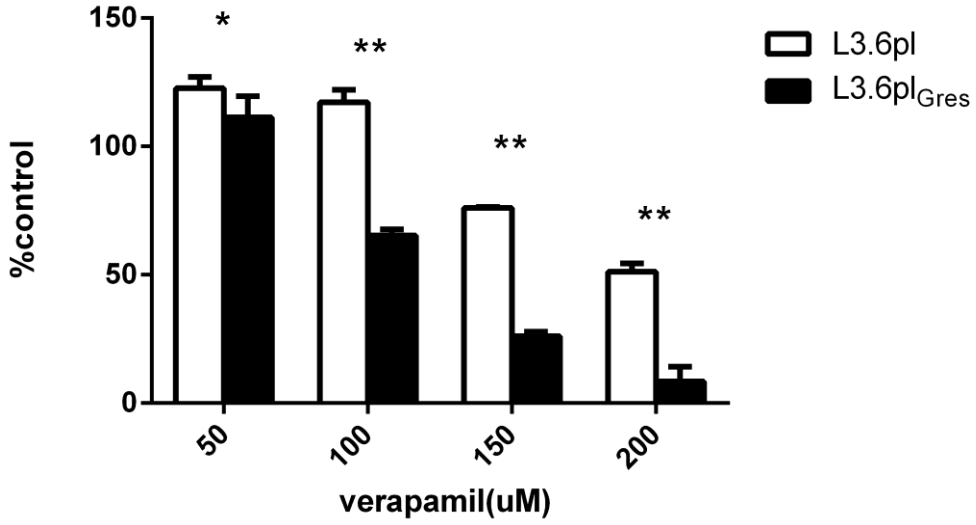
We compared the anti-proliferative effect of verapamil on L3.6pl- and L3.6pl$_{Gres}$ cells as well as AsPC-1-SP and -NSP cells.

All the above-mentioned cell lines demonstrated concentration dependent effects on cell viability following increasing doses of verapamil. Interestingly, under the same concentration of 50 uM,

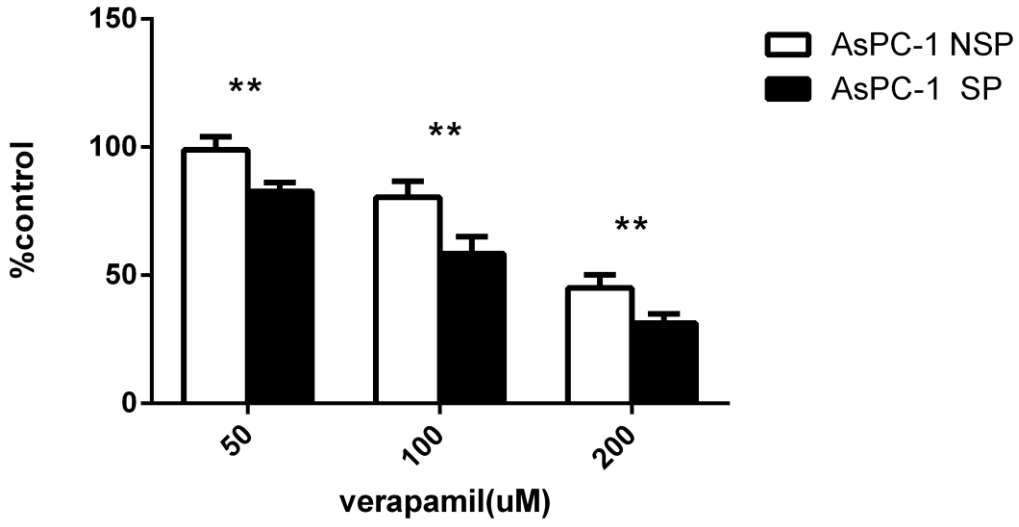
100 uM, 150 uM, 200 uM, the L3.6pl$_{Gres}$- and AsPC-1-SP cells were much more sensitive to verapamil than L3.6pl- and AsPC-1-NSP cells (Figure V.6).

Figure V.6 Verapamil alone can effectively inhibit viability of L3.6pl$_{Gres}$ and AsPC-1 SP cells *in vitro*

A)



B)



A). Treatment of L3.6pl and L3.6pl$_{Gres}$ cells for 24 hours with increasing concentrations of verapamil led to a dose dependent significant reduction of cell viability in L3.6pl$_{Gres}$ cells, which was much more sensitive to verapamil than L3.6pl cells(p * < 0.001, p ** < 0.0001).

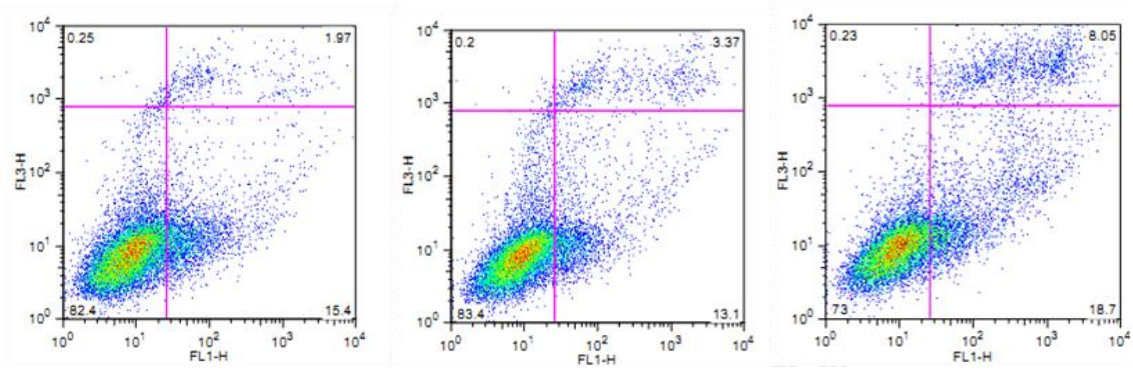
B). FACS sorted SP and NSP AsPC-1 cells were seeded into 96-wells plates evenly overnight, then treated with increasing concentrations of verapamil. Cell viability was significantly reduced in AsPC-1-SP cells than AsPC-1-NSP cells (p ** < 0.0001).

5.1.4 Pro-apoptotic effect of verapamil plus gemcitabine in both L3.6pl and L3.6pl$_{Gres}$ cells

Many studies reported that calcium channel blockers and calmodulin inhibitors are thought to act by blocking efflux of standard anticancer drug from the cell[94, 95]. To investigate an additive or synergistic anti-tumor effect of verapamil and gemcitabine on pancreatic cancer cell lines, apoptotic cells were analysed by FACS.

L3.6pl$_{Gres}$-SP cells were treated seperately with verapamil (50 uM), gemcitabine (10 ng/ml) and verapamil (50 uM) combined with gemcitabine (10 ng/ml). The amount of apoptotic cells after combined treatment for 24 hours increased as well as dead cells, in contrast to treatment with verapamil and gemcitabine alone (Figure V.7).

Figure V.7 The amount of apoptotic cells after combined treatment for 24 hours increased as well as dead cells



Annexin FITC verapamil(50 uM) | Annexin FITC gemcitabine(10 ng/ml) | Annexin FITC verapamil(50uM)+gemcitabine(10 ng/ml)

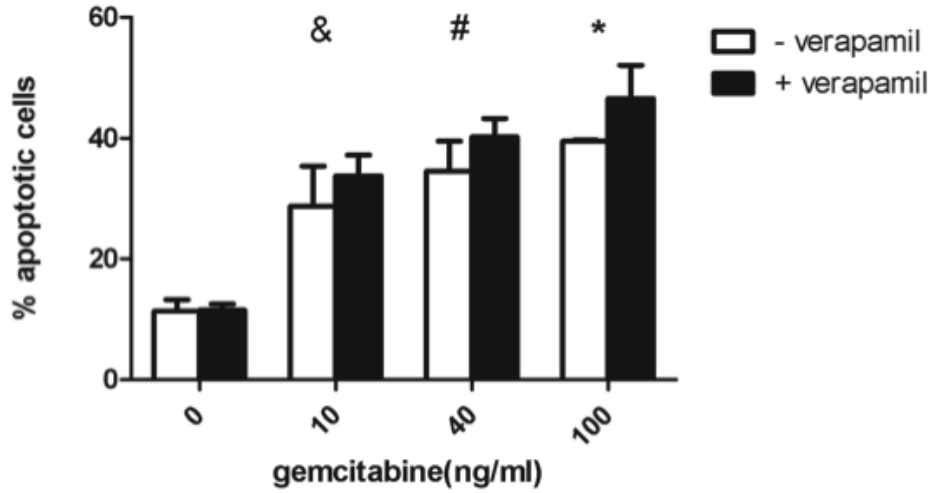
Sorted L3.6pl$_{Gres}$-SP cells were treated with verapamil (50 uM), gemcitabine (10 ng/ml), or verapamil (50 uM) combined with gemcitabine (10 ng/ml) for 24 hours, then stained with Annexin FITC, and analyzed with BD-LSRII flow cytometer and FlowJo software.

Compared to verapamil and gemcitabine treatment alone the proportion of apoptotic cells as well as dead cells was substantially higher after combined treatment.

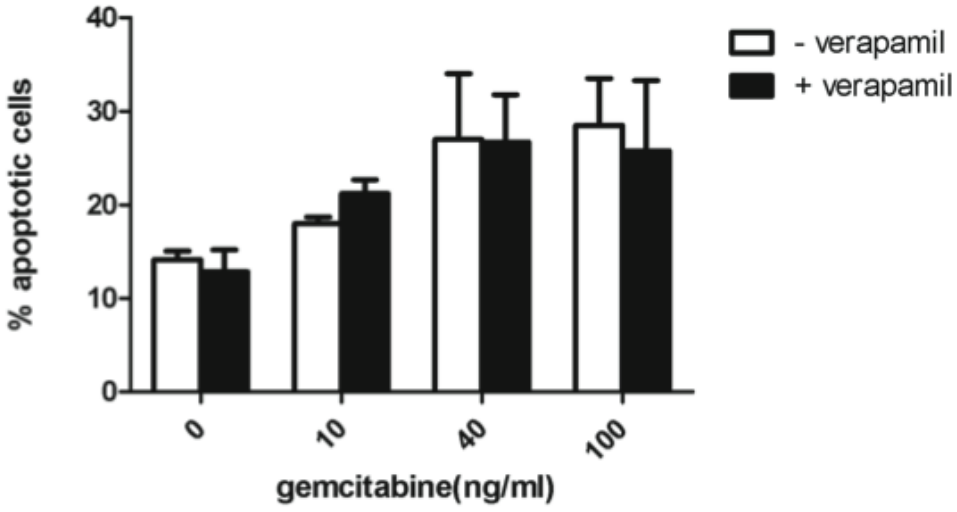
Afterwards, we analyzed the amount of apoptotic cells in L3.6pl and L3.6pl$_{Gres}$ using further detailed concentrations of gemcitabine while keeping the concentration of verapamil at 50uM. An additive effect regarding the amount of apoptotic cells could only be observed in L3.6pl cells, but not in the L3.6pl$_{Gres}$ cells (Figure V.8).

Figure V.8 An additive effect regarding the amount of apoptotic cells could only be observed in L3.6pl cells, but not in the L3.6pl$_{Gres}$ cells

A)



B)

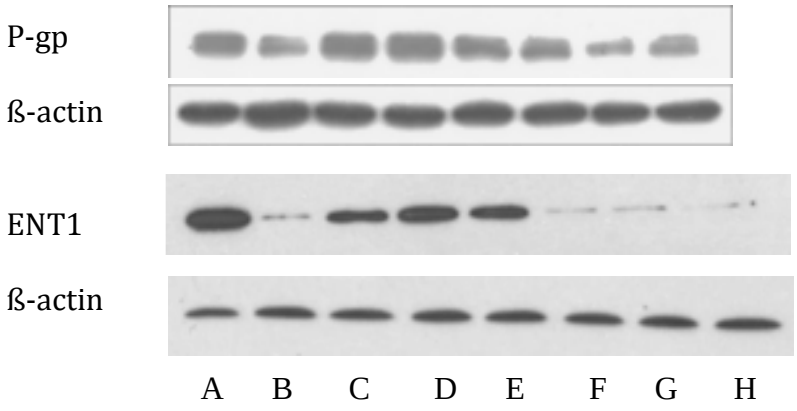


L3.6pl and L3.6pl$_{Gres}$ cells were treated over 24 hours with different concentrations of gemcitabine (10 ng/ml, 40 ng/ml, 100 ng/ml) alone, or in combination with verapamil (50 uM).
A) Under combination therapy, the proportion of apoptotic L3.6pl cells increased in a dose dependent manner (p & < 0.05, p # < 0.01, p * < 0.001);
B) The percentage of apoptotic L3.6pl$_{Gres}$ was only slightly increasing following combination therapy.

5.1.5 Expression levels of drug transporter proteins on L3.6pl and L3.6pl$_{Gres}$

Since we could not observe an additive effect of gemicatbine and verapamil in particular in L3.6pl$_{Gres}$ cells, we analyze the expression levels of the two main drug transporter proteins P-gp and ENT1 by Western Blotting. Our results revealed that the expression of P-gp and ENT1 was substantially lower in L3.6pl$_{Gres}$ as compared to L3.6pl cells. After 24 hours treatment with different concentrations of verapamil, the expression of P-gp and ENT1 on both cell lines was almost the same as without treatment (Figure V.9).

Figure V.9 Expression levels of drug transporter proteins on L3.6pl and L3.6pl$_{Gres}$



The protein lysates from:
A) L3.6pl;
B) L3.6pl$_{Gres}$;
C) L3.6pl treated with verapamil (50 uM) for 24 hours;

D) L3.6pl treated with verapamil (100 uM) for 24 hours;

E) L3.6pl treated with verapamil (200 uM) for 24 hours;

F) L3.6pl$_{Gres}$ treated with verapamil (50 uM) for 24 hours;

G) L3.6pl$_{Gres}$ treated with verapamil (100 uM) for 24 hours;

H) L3.6pl$_{Gres}$ treated with verapamil (200 uM) for 24 hours.

The results indicate that the expression of P-gp in L3.6pl$_{Gres}$ cell line was lower than in L3.6pl cells.

5.2 *In vivo* part

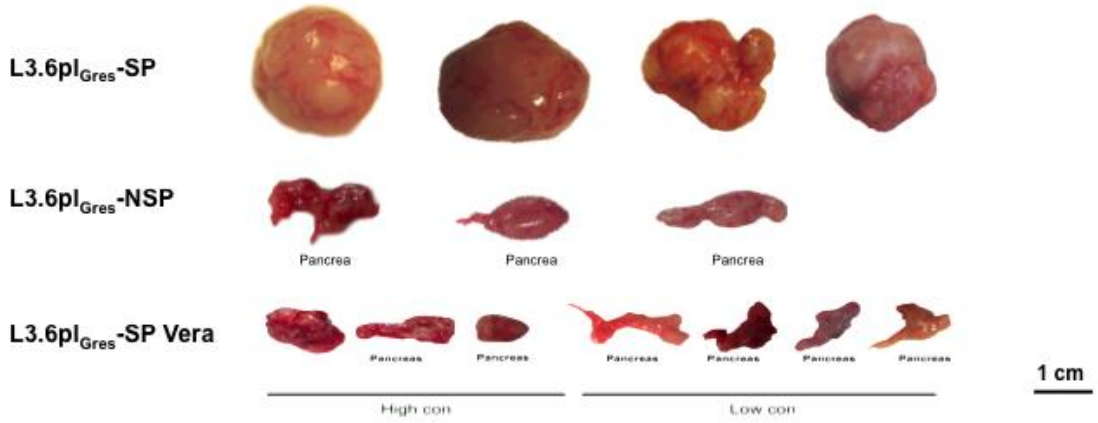
5.2.1 Verapamil can effectively inhibit tumor growth induced by L3.6pl$_{Gres}$-SP cells in *vivo*

L3.6pl$_{Gres}$-SP and NSP cells were injected into the pancreas of athymic Balb/c nu/nu mice and subdivided in the following four groups: 1) L3.6pl$_{Gres}$-SP group (5 mice), 2) L3.6pl$_{Gres}$-NSP control group (3 mice); 3) L3.6pl$_{Gres}$-SP treatment group with high concentration of verapamil (25mg/kg, 10mM, 3 mice, one mouse died in the operation); 4) L3.6pl$_{Gres}$-SP treatment group with low concentration of verapamil (0.5mg/kg, 200uM, 4 mice). Verapamil treatment was started 4 weeks after the orthotopic pancreatic cancer cell injection. 9 weeks after the cell injection, all of the mice were sacrificed. Tumors and other organs were harvested for immunohistochemistry assays.

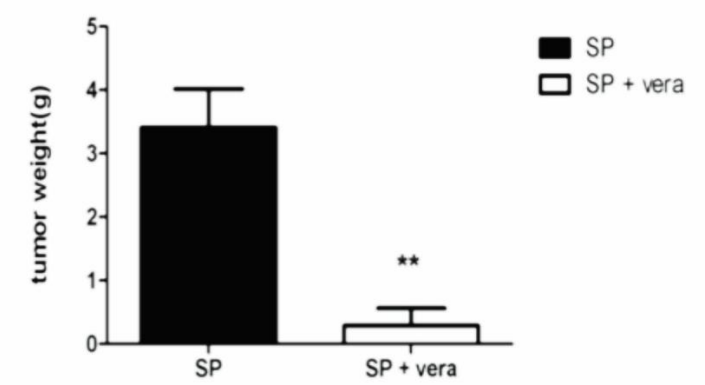
The macroscopic results demonstrated that L3.6pl$_{Gres}$-SP cells have much stronger tumorigenic capacity than L3.6pl$_{Gres}$-NSP cells. Verapamil treatment (low/high concentration) substantially inhibited tumor growth and metastasis formation as compared to the SP control group (Figure V.10). No side effects were observed in the verapamil treatment group, group 3&4.

Figure V.10 Verapamil can effectively inhibit tumor growth induced by L3.6plGres-SP cells in vivo

A)



B)



C)

Group	L3.6plGres-SP	L3.6plGres-SP+Verapamil		L3.6plGres-NSP
		0.5mg/kg	25mg/kg	
Primary Tumor	4/5	0/4	1/3	0/3
Liver metastasis	2/5	2/4	0/3	0/3

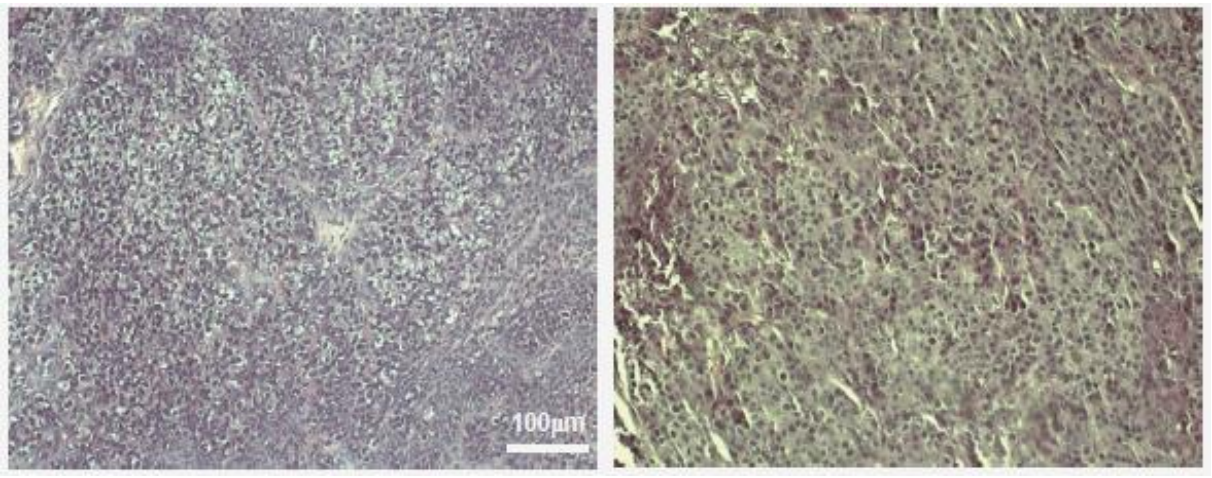
A), B), C) Verapamil treatment significantly inhibited tumor growth in a dose independent way. In the low concentration treatment group, there were no macroscopic tumors in four mice, however, and two mice had liver metastasis. In the high concentration treatment group, there were no metastasis in all three mice, but one mouse presented with a small pancreatic tumor.

5.2.2 Immunohistochemical analysis of L3.6pl$_{Gres}$-SP following verapamil treatment

Immunohistochemical stainings were carried out measuring the proliferation index, microvascular density and apoptosis of the pancreatic tumors in the L3.6pl$_{Gres}$-SP control group and verapamil treatment groups (Figure V.11). The median percentage of *Ki*67+ proliferating cells within the tumors of the verapamil treatment group did not differ significantly from that of the L3.6pl$_{Gres}$-SP group, however, verapamil treatment can limit the *Ki*67 index to a relatively constant range. The amount of apoptotic cells was much more pronounced in the verapamil treatment group as compared to the L3.6pl$_{Gres}$-SP group. The microvascular density as measured by immunohistochemical staining against CD31 revealed increased angiogenesis in tumors of the L3.6pl$_{Gres}$-SP group. H&E staining revealed large, pleomorph cells with hyperchromatic nuclei in the L3.6pl$_{Gres}$-SP group.

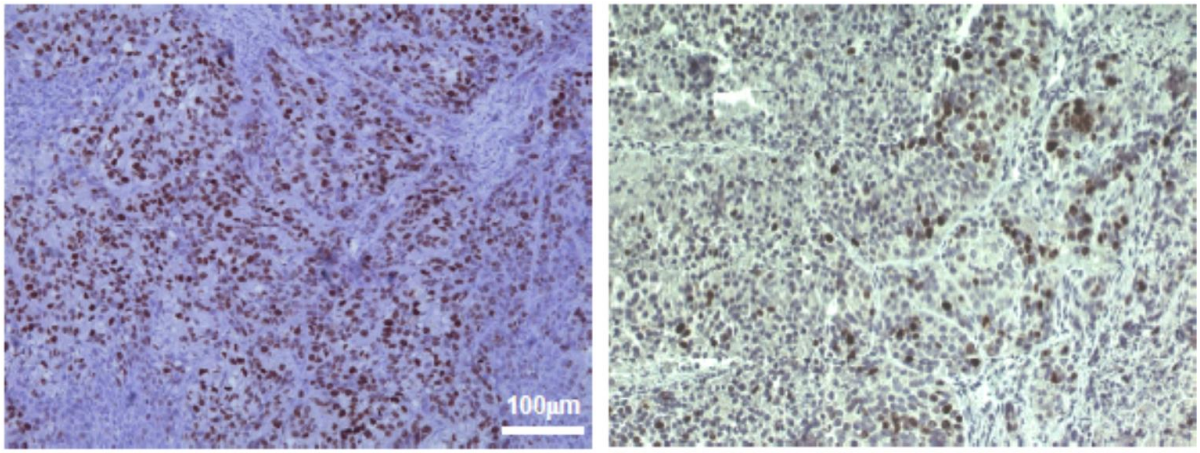
Figure V.11 Immunohistochemical analysis of L3.6pl$_{Gres}$-SP following verapamil treatment

A) H&E staining

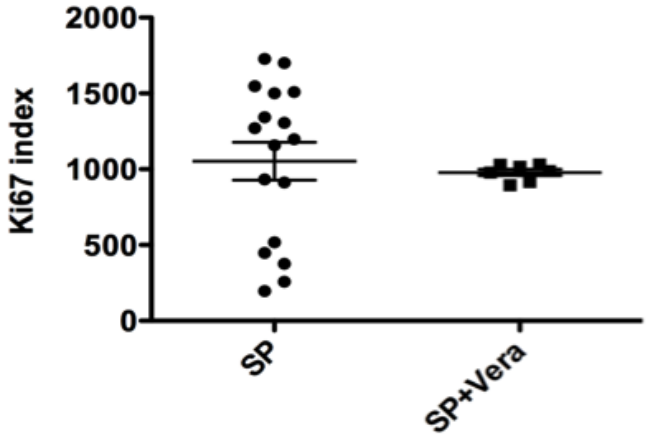


SP **SP + Verapamil**

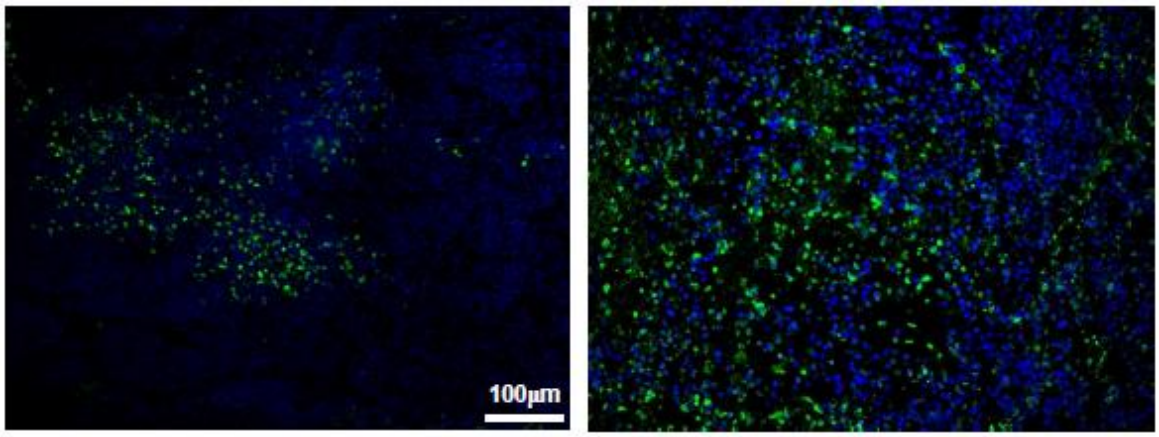
B) *Ki*67 index



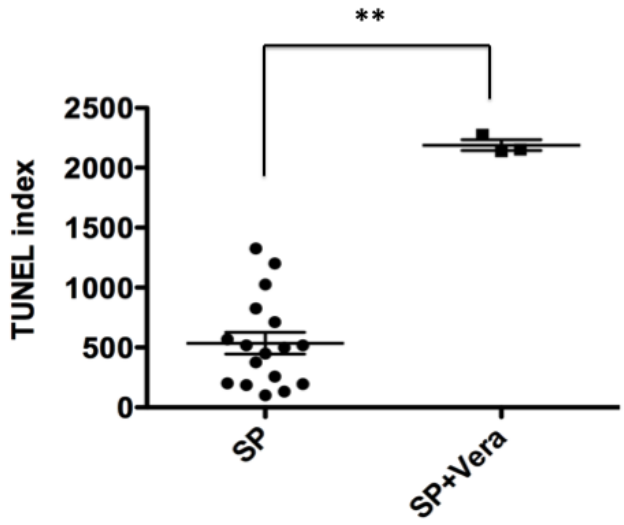
SP **SP + Verapamil**



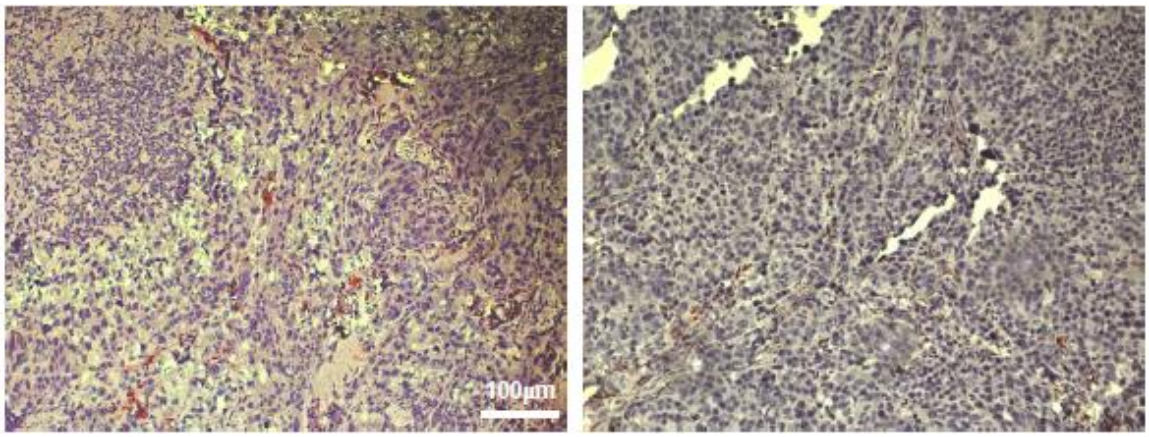
C) TUNEL index



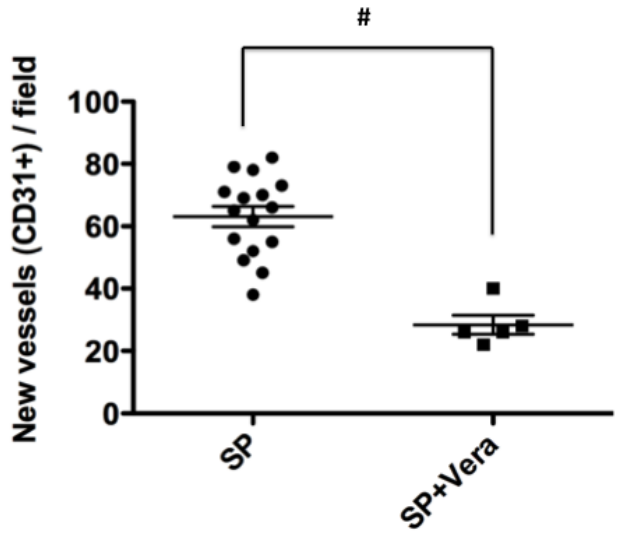
SP **SP + Verapamil**



D) Microvascular density



SP **SP + Verapamil**



Tumors from L3.6pl$_{Gres}$-SP group and L3.6pl$_{Gres}$-SP verapamil treatment groups were paraffin-embedded and sectioned for different immunohistochemical and H&E staining.

A) In H&E staining images large, pleomorph cells with hyperchromatic nuclei can be observed in the L3.6pl$_{Gres}$-SP group, even some cancer nests were interspersed, while the normal, round, light nuclei composed most of the tumor tissue in verapamil treatment group.

B) Immunohistochemical staining against *Ki*67 are shown. *Ki*67+ signal are manifested by dark brown stained cells. The scatter plot of *Ki*67 index shows that the median percentage of *Ki*67+ proliferating cells within the tumors of verapamil treatment group did not differ significantly from that of the L3.6pl$_{Gres}$-SP group, while the standard deviation of *Ki*67 index in L3.6pl$_{Gres}$-SP group is wider than the verapamil treatment group.

C) TUNEL apoptosis staining is shown. Apoptic cells are stained with fluorescencent green. The scatter plot of the TUNEL index shows that the number of apoptotic cells in the verapamil treatment group is significantly higher than in L3.6pl$_{Gres}$-SP group (*p* **<0.0001).

D) CD31 staining is shown. The organge stained portions in the images are endothelial cells lining the vessels following anti-CD31 labeling. The scatter plot of microvessel density

shows that the average microvessel density in the verapamil treatment group is significantly reduced compared to the L3.6pl$_{Gres}$-SP group ($p\#<0.01$).

Both, the macroscopic and microscopic results demonstrate that L3.6pl$_{Gres}$-SP cells have indeed stem cell capacity and that verapamil alone can effectively inhibit tumor growth which was induced by L3.6pl$_{Gres}$-SP cells.

VI Discussion

The existence of CSCs is receiving increasing interest particularly due to its potential use in the clinical routine. These cells have stem-like self-renewal and tumor initiation capacity and are believed to be responsible for recurrence due to their resistance to therapy. CSCs have been enriched by several techniques such as growth in serum-free defined media to induce sphere formation. The isolation was performed by expression of certain surface marker combinations, i.e. CD44+/CD24-/lin- for human breast cancers[68], $EpCAM^{high}$/CD44+/CD166+ for colorectal cancer[96], CD34+/CD38- for acute myeloid leukemia, broadly used as a target for chemotherapy[97], and Stro1+/CD105+/CD44+, a surface marker for bone sarcoma[98]. Another approach to identify the CSC subpopulation is isolation of SP cells because of their inherent ability to efflux a fluorescent dye as well as chemotherapy reagents[99]. In the last decades, SP cells have already been widely applied in various tumors and cancer cell lines as a CSC subpopulation, e.g. acute myeloid leukemia[100], neuroblastoma[76], melanoma[101], ovarian cancer[102], the C6 glioma cell line[103], human retinoblastoma cell line[104], human neuroblastoma cell lines[76], various human gastrointestinal cancer cell lines[105] and pancreatic cancer cell lines, such as MIA PaCa2, PANC-1, Capan-2, KP-1 NL and SW1990[80, 89, 106, 107]. However, litte information has been reported about SP cells in the human pancreatic cancer cell lines L3.6pl and AsPC-1.

Here, we successfully identified the SP subtype cells in the three cell lines with the aid of Hoechst 33342 staining (Figure V.2). We were able to isolate and characterize SP cells from the highly metastatic pancreatic adenocarcinoma cell line L3.6pl, its chemotherapy resistant variant $L3.6pl_{Gres}$ and AsPC-1 cell line.

In vitro, the SP-enriched $L3.6pl_{Gres}$ cell line was endowed with much stronger colony formation ability than its parental cell line L3.6pl (Figure V.4&V.5). Figure V.3 showed $L3.6pl_{Gres}$-SP cells exhibiting higher gemcitabine chemotherapy resistance ability than $L3.6pl_{Gres}$-NSP cells, which was another manifestion their stem cell property.

In vivo, we obtained significantly larger primary pancreatic tumors and higher incidence of liver metastasis following orthotopic injection of $L3.6pl_{Gres}$ SP cells as compared to $L3.6pl_{Gres}$ NSP cells, which was another compelling evidence of the stem cell capacity of SP cells (Figure V.10.A & C).

During the acquisition of gemcitabine resistance some morphologic changes were gradually induced. Fibroblastoid cells with loss of polarity, increased intercellular separation and pseudopodia were in large quantity present in L3.6pl$_{Gres}$ (Figure V.1), which was consistent with the common phenomenon of Epithelial-to-Mesenchymal (EMT) transition. Emerging evidences demonstrates that molecular and phenotypic associations exist between chemotherapy resistance and the acquisition of an EMT-like cancer cell phenotypes[108-111].

The SP subtype cells play a major role in metastasis and chemotherapy resistance. SP cells might be related to chemotherapy resistance induced EMT. Some studies on pancreatic and breast cancer have already analyzed a potential relationship between chemoresistance induced EMT and CSCs[112, 113, 114], however, this hypothesis needs to be directly confirmed by further studies.

Goodell *et al*[73] have demonstrated that the pumps responsible for Hoechst33342 efflux, which differentiates SP from NSP cells, were mainly contributed by transporter proteins of the ABC superfamily including MDR1/P-gp, ABCG2, and MRP, which are the key factors contributing to the development of tumor drug resistance[35-38]. Verapamil, the first generation P-gp inhibitor, has been reported to be able to block the dye efflux activity and reverse partially the resistance caused by P-gp[31, 54, 115], which results in an increase of drug uptake by tumor cells[54]. We also conducted Hoechst 33342 staining following the Goodell's protocol where verapamil usually acts as a negative control since it can eliminate the SP cells in L3.6pl, L3.6pl$_{Gres}$, and AsPC-1 cells shown in Figure V.2.

Our hypothesis was that verapamil could be a specific SP inhibitor and therefore a novel therapy against pancreatic cancer stem cells. Our studies revealed that verapamil alone can inhibit cell proliferation *in vitro* in a dose-dependent manner. Interestingly, verapamil inhibited SP cells more than NSP cells in both human pancreatic cell lines: L3.6pl$_{Gres}$ and AsPC-1 (Figure V.6), while *in vivo* verapamil can effectively prevent tumor growth induced by L3.6pl$_{Gres}$-SP cells (Figure V.10).

Our demonstration of the inhibitiory effect of verapamil on pancreatic cells is in agreement with a few earlier reports that verapamil inhibits proliferation and promotes differentiation in human promyelocytic HL-60 cells[116], produces a growth inhibiting effect on human colonic tumor cells[117] and has an antiproliferative effect alone on the human brain tumor cells *in vitro*[118]. Among the human brain tumor cells, William Schmidt *et al.* showed a reversible,

antiproliferative action of verapamil on human medulloblastoma, pinealoblastoma, glioma, and neuroblastoma tumor lines from pediatric patients[118].

We found that SP cells in L3.6pl$_{Gres}$ and AsPC-1 cell lines are more sensitive to verapamil than NSP cells, which was consistent with our hypothesis. However, as we compared the protein expression level of P-gp between L3.6pl and L3.6pl$_{Gres}$, L3.6pl contained a higher expression level of P-gp than L3.6pl$_{Gres}$, and verapamil treatment did not affect the expression level at all (Figure V.9). This result was in accordance with Jensen *et al*[119] who observed that some small-cell lung cancer (SCLC) cells with P-gp high expression were more sensitive to gemcitabine and the structurally related deoxycytidine analogue 1-β-D-arabinofuranosylcytidine (cytarabine, ara-C). Sheng Zhou *et al.* demonstrated that P-gp is not required for the SP phenotype in hemotopoietic stem cells[75], other scientists showed that the liver and the bone marrow of MDR1 knockout mice present normal numbers of SP cells in primitive hematopoietic cells[120]. Besides, Sugawara *et al*. and Bernard *et al.* both failed to demonstrate P-gp expression in pancreatic cancer tissues[43, 44], therefore, clinical significance of MDR1/P-gp expression in human pancreatic cancer stayed controversial. Obviously, the low protein expression level of P-gp in L3.6pl$_{Gres}$ cells is not the explanation for the high sensitivity of SP cells to verapamil. Some researchers explained this effect with the anti-proliferative and P-gp inhibitiory mechanisms of verapamil. One of these controversial mechanisms may include the phospholipid/Ca^{++}-dependent PKC (protein kinase C) in MDR[121], several studies reported an increase in PKC activity in cells with an overexpression of P-gp or MRP[122, 123], but whether phosphorylation of P-gp modulates the pump function is still debatable[123-126].

Another important issue is that gemcitabine is a deoxycitidine analogue that is activated by deoxycytidine kinase (dCK) to its monophosphate and subsequently to its triphophate dFdCTP, which is incorporated into both RNA and DNA, leading to DNA damage. Since dCK may be phosphorylated by PKC and exhibits a higher activity in the phosphorylated state[127], PKC activation might play a role in the collateral sensitivity to gemcitabine in P-gp high expressing cells.

Both of our *in vitro* apoptosis assay results (Figure V.7 & V.8.) and *in vivo* immunohistochemistry analysis (Figure V.11.C) showed pro-apoptotic effects of verapamil. The enhancement of apoptotic tumor cells under therapy with verapamil may be explained by the fact that verapamil as a calcium channel antagonist, interferes with intracellular signaling pathways[117,

[128-130]. Calcium ions (Ca^{2+}), a cellular messanger that control aspect of cell and tissue physiology, can be turned into death signals when delivered at the wrong time and place[131, 132]. The use of calcium antagonists may break the mobile equilibrium between intracellular and extracellular Ca^{2+}, which causes the Ca^{2+} that is stored in the cell to be released into the cytoplasm, resulting in an increase of the intracellular concentration of Ca^{2+}[117]. Since Ca^{2+} is toxic at high concentrations a low Ca^{2+} concentration must be maintained in the cytoplasm[133], therefore verapamil might induce cell apoptosis though destroying this Ca^{2+} balance.

Other members of the ABC superfamily may contribute to the SP induced chemotherapy resistance and trigger apoptosis in pancreatic cancer as well. Recently, investigators found verapamil and its derivative can trigger apoptosis though glutathione (GSH) extrusion by MRP1[62], while many substrates of MRP1 are conjugated to reduced glutathione. Extrusion of GSH out of the cells will cause oxidative stress, which is a universal well-recognized trigger for apoptosis[134].

We applied two different doses of verapmil for intraperitoneal treatment of SP induced pancreatic tumors, one is 0.5 mg/kg/d, 200 uM, this concentration design was based on the *in vitro* proliferation assay (Figure 5 A.), which showed that the inhibition rate of L3.6pl and $L3.6pl_{Gres}$ can be controlled above 50%; the other concentration is relatively high, 25 mg/kg/d, 10 mM, which referred to clinical combination treatment doses for pancreatic cancer patients (20 mg/d for a 70 KG BW patient[65]) and former publications of verapamil treatment in mouse tumors[135, 136].

The last point needed to elucidate is that, the additive anti-cancer effect of verapamil and gemcitabine in pancreatic cancer. Although this calcium channel blocker is well known for its ability to enhance cytotoxicity when used in combination with the other chemotherapy reagents, such as anthracyclines[56, 137], paclitaxel[42], epipodophyllotoxins[138] and melphalan[94], little is known about the effects of verapamil used alone and in combination with the tranditional pancreatic cancer chemotherapy reagent gemcitabine. In our study, we identified combined pro-apoptotic effects of verapamil and gemcitabine on L3.6pl rather than $L3.6pl_{Gres}$ using the same treatment conditions. In Figure V.9 we demonstrated that the expression level of ENT1, which is the major uptake transporter of gemcitabine was extremely low in $L3.6pl_{Gres}$ cells as compared to L3.6pl cells. The absence of ENT1 has already been clinically associated with reduced survival in patients with gemcitabine treated pancreatic adenocarcinoma[52].

In conclusion, our study revealed that verapamil acts on pancreatic cancer cells *in vitro* most likely by inducing apoptosis of stem like SP cells in L3.6pl, L3.6pl$_{Gres}$ and AsPC-1 cells. Verapamil can significantly inhibit pancreatic cancer tumor growth *in vivo* most likely by targeting stem like side population cells, which could provide evidences for a new clinical feature of this 'old' reagent. However, further investigation should be carried out to make clear the exact mechanisms of verapamil on SP cells.

VII Summary

- SP subtype cells can be clearly identified by Hoechst 33342 assay.
- SP subtype cells were enriched in the gemcitabine induced resistant cell line $L3.6pl_{Gres.}$
- $L3.6pl_{Gres}$-SP cells were characterized as CSCs by exhibiting stronger colony formation capacity, chemotherapy resistance, and higher tumorigenicity.
- Verapamil alone can effectively inhibit $L3.6pl_{Gres}$-SP and AsPC-1-SP *in vitro*, and prevent tumor growth induced by $L3.6pl_{Gres}$-SP *in vivo*.
- Verapamil can induce apoptosis effect in pancreatic cancer cells, however, when combined with gemcitabine, an additive or synergistic pro-apoptotic effect cannot be generated in all the cell lines in our study.

VIII Zusammenfassung

- Side population (SP) Zellen können eindeutig durch die Verwendung des Hoechst 33342 Farbstoffs durch FACS Sorting identifiziert und analysiert warden.
- Side population (SP) Zellen werden durch Induktion einer Gemcitabine-Resistenz in L3.6pl$_{Gres}$ angereichert.
- L3.6pl$_{Gres}$-SP Zellen können als eine Subpopulation von Tumorstammzellen charakterisiert werden, da sie eine deutliche Fähigkeit zur Clone-Formierung *in vitro*, Chemotherapie-Resistenz und eine deutlich erhöhte Tumorigenität *in vivo* haben.
- Verapamil allein hemmt das Tumorzellwachstum von L3.6pl$_{Gres}$-SP und AsPC-1-SP Zellen *in vitro*. Des Weiteren führt es zu einer signifikanten Abnahme des orthotopen Tumorwachstums von L3.6pl$_{Gres}$-SP Zellen *in vivo*.
- Verapamil führt zur Apoptose von Pankreastumorzellen, allerdings ist ein additive oder synergistischer pro-apoptotischer Effekt in Kombination mit Gemcitabine in vitro nicht für alle Zellinien nachweisbar.

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X Abbreviation List

ABC	avitin biotin complex
ABC	ATP-binding cassette
Balb/C nu/nu	bagg-albino/c nude/nude
bFGF	basic fibroblast growth factor
CEA	carcinoembryonic antigen
CNT	concentrative nucleoside transporter
CSCs	cancer stem cells
DAPI	4’, 6-Diamidin-2-phenyl-Indol
DMEM	dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
dTK	deoxycytidine kinase
FACS	fluorescence activated cell scan
FCS	fetal calf serum
FG	fast-growing
ELISA	enzyme-linked immunosorbent assay
ENT	equilibrative nucleoside transporter
EMT	epithelial-to-mesenchymal transition
GSH	glutathione
H33342	Hoechst 33342
HE	hematoxylin and eosin
hENT1	human ENT1
HRP	horseradish peroxidase

ABBREVIATION LIST

HSC	hematopoietic stem cells
IC 50	inhibitory concentration 50
IL	interleukin
MDR	multidrug resistance
mRNA	messenger ribonucleic acid
MRP	multidrug resistance-associated protein
NOD/SCID	nonobese diabetic/severe combined immunodeficient
NSP	non-side poplulation
OS	overall survival
PDAC	pancreatic ductal adenocarcinoma
PKC	protein kinase C
P-gp	p-glycoprotein
RNA	ribonucleic acid
RT-PCR	quantitative reverse transcription polymerase chain reaction
SCLC	small-cell lung cancer
SLC	solute carrier
SP	side population
VEGF	vascular endothelial growth factor

XI Table of figures and tables

XII Curriculum Vitae

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National and international conferences and educational meetings

1. CSC-LMU program auditing seminar, LMU
   10-11. June, 2013, Munich, Germany. Oral presentation
2. 130th. Kongress der Deutschen Gesellschaft für Chirurgie
   30. April - 3. May, 2013, Munich, Germany. Poster presentation
3. Doktorandenkolloqium & Dienstagskolloqium in Surgery Department, LMU
   9. April, 2013, Munich, Germany. Oral presentation
4. 2nd International Meeting on Molecular-Based Treatment of GI Cancer

01-02. March, 2013, Göttingen, Germany.	Poster presentation
5. 16th. Chirurgische Forschungstag	
04-06. October, 2012, Regensburg, Germany.	Oral presentation
6. DGAV-Jahrestagung - Viszeralmedizin 2012	
09-22. September, 2012, Hamburg, Germany.	Oral presentation
7. 3rd EUNE Gastric Cancer International Workshop	
08-09. June, 2012, Köln, Germany.	
8. 30th. Deutscher Krebskongress 2012	
22-25. February, 2012, Berlin, Germany.	

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1. **Lu Zhao**, Yue Zhao, Bao Q, Niess H, Jauch KW, Bruns CJ. Clinical Implication of Targeting of Cancer Stem Cells. Eur Surg Res. 2012 Jul; 49 (1):8-15.
2. Gamba S, Zhao Y, **Zhao L**, Wang Y, Schwarz B, Primo S, Jauch KW, Nelson PJ, Modest DP, Nieß H, Bruns C. Significance of Mesenchymal Stem Cells in Gastrointestinal Disorders. Zentralbl Chir. 2013 Jul 8. German.
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