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Characterization and Targeted Therapy of Stem Cell-Like Side Population Cells in Pancreatic Cancer and Esophageal Cancer

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Declaration

I hereby declare that the thesis is my original work.

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

Part of the *in vitro* target therapy data in pancreatic cancer cell lines was generated in cooperation with Dr. Peter Camaj and Dr. Ivan Ischenko. 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:

1. Antisense inhibition of microRNA-21 and -221 in tumor-initiating stem-like cells modulates biological functions of pancreatic cancer including tumorigenesis, metastasis, and chemoresistance

2. Characterization of stem cell-like side populations in esophageal cancer: a potential source of chemotherapy resistance and metastases. *Stem Cells Dev. 2013 Sep 10. Epub ahead of print.*

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 September 2010 with the supervision from Prof. Dr. Peter J. Nelson and Prof. Dr. med. Christiane J. Bruns in Ludwig-Maximilians University Munich, Germany.

09.2013, München

Yue Zhao

Um Krankheiten zu behandeln und zu heilen, muss man nach ihren Ursprüngen suchen.

- Buch des Gelben Kaisers zur Inneren Medizin[1]

治病必求於本

- 黄帝内经

[1] Ubersetzt aus: Muhammad W Schmidt, 2004, Der klassiker des gelben Kaisers zur Inneren Medizin, viademica.verlag, Berlin

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I. ABSTRACT

1.1 Pancreatic cancer

Introduction: Previous studies from our group have identified a small subpopulation in pancreatic cancer cells, which are referred to as side population (SP) cells showing stem-like properties. These cells were found to induce fast and aggressive tumor formation in nude mice. Cultured SP cells were shown to differentiate into daughter SP or non-SP cells and to be highly chemoresistant. Furthermore, transcriptomic profiling showed a significant difference in the expression of more than 1300 genes in SP cells vs. non-SP cells. This included differences in the expression of a series of microRNAs including miR-21 and miR-221. The potential role of these microRNAs in SP biology was then investigated.

Methods: Pancreatic cancer stem cells from the highly metastatic cell line L3.6pl were identified and characterized by flow cytometry using Hoechst 33342 dye staining. Gene expression was assessed by Affymetrix array analysis and further confirmed by quantitative RT-PCR. Antagomir transfection was performed using microRNA-21 and -221 antisense oligonucleotides. Tumor cell apoptosis, cell cycle progression, chemoresistance, and metastatic potential were quantitated using propidium iodide staining, cytotoxicity assays and Boyden chamber assays, respectively. For *in vivo* studies, SP cells were sorted from L3.6pl gemcitabine resistant cells and implanted orthotopically in nude mice with or without microRNA-21 and -221 antisense mono- and combination therapies.

Results: A series of microRNAs including: miR-21, miR-221, miR-211, and miR-30c-2 were found significantly up regulated in stem-like SP from L3.6pl cells. In these cells miR-21 and miR-221 were shown to be involved in the modulation of expression of more than 200 genes. The administration of antagomir-21 and -221 significantly reduced the SP fraction, affected L3.6pl cell proliferation, invasion, and chemoresistance against gemcitabine and 5-Fluorouracil. Combination of antagomir-21 and -221 therapy showed a better inhibitory effect on tumor growth than single antagomir treatment, especially, in gemcitabine resistant SP induced pancreatic cancer *in vivo*.

Conclusion: The results demonstrate the significance of both miR-21 and -221 in the biology of stem-like tumor cells in pancreatic cancer. Both microRNAs contribute to biological functions of pancreatic cancer including apoptosis, metastasis, and chemoresistance.

Antagomir-21 and -221 treatments may be beneficial in overcoming gemcitabine-associated chemoresistance in pancreatic cancer in the future.

1.2 Esophageal cancer

Introduction: Dye-effluxing side population (SP) cells are resistant to chemotherapy and are thought to resemble cancer stem cells (CSCs). In this study, the relevance of the SP subpopulation as potential cancer stem cells in esophageal cancer cell lines and their relation to chemotherapy resistance and metastasis were investigated.

Methods: The SP subpopulation was detected using Hoechst 33342 staining in five esophageal cancer cell lines: OE19, OE21, OE33, PT1590, and LN1590. CTx-resistant cell lines were then developed after long-term *in vitro* exposure to 5-FU and cisplatin and validated by analysis of resistance markers thymidylate synthase and ERCC1. SP cells isolated from OE19 and OE19/5-FUres resistant cells were then analyzed by epithelial-to-mesenchymal transition (EMT) PCR array for their steady state expression of EMT related genes.

Results: The LN1590 and PT1590 cell lines did not show detectable SP cells, while OE19, OE21, and OE33 cells were found to have varying levels of SP cells about 17.1%, 0.8%, and 8.8%, respectively. Colony formation assays showed significantly higher clonogenic capability of the respective SP populations in OE19, OE21, and OE33 cell lines ($p< 0.01$). *In vivo* subcutaneous injection of the cells showed higher tumorigenicity of SP cells as compared to NSP cells from the OE19 cell line. With increasing duration of 5-FU or cisplatin therapy, the SP subpopulation increased in PT1590 and LN1590 cell lines. The SP fraction of OE19/5-FUres showed an increase in EMT related genes as compared to the SP fraction of OE19. These included: SNAI2, CALD1, WNT11, MSN, ZEB1, SERPINE1, VCAN, COL3A1, ERBB3, TMEFF1, TCF4, ITGA5, TIMP1, GSK3B, ITGAV, BMP1, MMP9, COL5A2, FOXC2, MMP3 and NOTCH1 (>4 fold change) while TSPAN13 and IL1RN were significantly decreased.

Conclusion: These results provide evidence that different proportions of SP cells exist in esophageal cancer and this subpopulation of cells exhibit stem cell properties. SP cells are associated with chemotherapy resistance. Long-term CTx selects for SP cells with an up-regulated EMT gene profile that might be the source of systemic disease relapse.

II. INTRODUCTION

2.1 Pancreatic cancer

2.1.1 Background of pancreatic cancer

In pancreatic cancer 95% of cases are represented by pancreatic ductal adenocarcinoma arising from the exocrine ductal system. It is the fourth most common cause of cancer death across the world (Hariharan et al 2008), and continues to be a major medical challenge in the western countries and arises rapidly in developing countries.

The high mortality rate of this cancer is linked to its propensity for early metastatic spread and the intrinsic or 'acquired' resistance to radiation and chemotherapy. Approximately 50% of patients with pancreatic carcinoma present at the time of diagnosis with liver or lymph node metastases at the time of diagnosis and show a median survival time of 6 months. Another 30-35% of patients present with local regional disease that is considered unresectable, usually because of local vascular invasion. Only 15-20% of patients present with resectable disease. However, at least 80% of these patients will develop local or distant relapse within 2 years of surgery (Hidalgo 2010).

The etiology of pancreatic cancer remains unknown. Some risk factors such as tobacco use, a history of diabetes or chronic pancreatitis and family history of the disease are implicated (Li et al 2004). However, the evidence of moderate intake of alcohol, intake of coffee and use of aspirin as contributors is limited. Recently, a study on blood type observed an increased risk in type A, B or AB as compared with blood type O (Wolpin et al 2009).

2.1.2 Management of pancreatic cancer

Pancreatic cancer is staged according to the most recent edition of the American Joint Committee on Cancer tumor–node–metastasis classification (Edge and Compton 2010).To date, surgical resection is still the only potentially curative treatment for early stage patients. Depending on the location of the tumor, the operative procedures may involve cephalic pancreatoduodenectomy (the Whipple procedure), distal pancreatectomy, or total pancreatectomy (Hidalgo 2010). However, even if the tumor is fully resected, the prognosis in patients with early pancreatic cancer is disappointing. Some randomized clinical trials show

that preoperative (neoadjuvant) treatment and postoperative administration of chemotherapy with gemcitabine, fluorouracil or leucovorin improves progression-free and overall survival (Evans et al 2008). 85% of PDAC patients are detected at advanced stages, characterized by infiltration of proximal lymph nodes and vascular structures, as well as distant metastasis to liver or peritoneum. Adjuvant treatment after surgery with both chemotherapy and radiation therapy demonstrated improvements in disease-free survival and overall survival rates. Whereas gemcitabine or 5-fluorouracil chemotherapy without radiation are the most common treatments outside North America, chemoradiation plus systemic chemotherapy is still widely used in the Unites States of America (Stathis and Moore 2010). But due to high intrinsic resistance of pancreatic cancer to currently available agents, clinical trials have shown that gemcitabine alone and gemcitabine-based combination chemotherapy is not likely to achieve great success (Heinemann et al 2008, Li et al 2004, Sultana et al 2007). A means to overcome gemcitabine-induced chemoresistance is urgently needed.

2.1.3 Gemcitabine induced chemoresistance in pancreatic cancer

Gemcitabine ($C_9H_{11}F_2N_3O_4$, 2', 2'-difluoro-2'-deoxycytidine, dFdC) is a pyrimidine analog with a wide spectrum of antitumor activity (Abbruzzese 1996) (shown in Figure II.1) that is applied as a standard drug for cytotoxic therapy of advanced pancreatic cancer.

Previous studies on the metabolism of gemcitabine have demonstrated that deoxycytidine kinases metabolize this agent intracellularly to the active species gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP). Incorporation of dFdCTP into DNA is responsible for the cytotoxic effects of gemcitabine, via inhibition of DNA synthesis, DNA repair and ultimately via induction of apoptosis (Bergman et al 2005). Another target of gemcitabine is the enzyme ribonucleotide reductase (RNR). The diphosphate analogue binds to RNR active site and inactivates the enzyme irreversibly. Once RNR is inhibited, the cell cannot produce the deoxyribonucleotides required for DNA replication and repair, and cell apoptosis is induced (Cerqueira et al 2007). Moreover, gemcitabine is a radio-sensitizing agent, which acts specifically in the S and G1/S phase of the cell cycle.

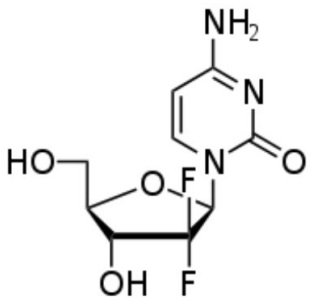

Figure II.1 Structure of gemcitabine

Many forms of pancreatic cancer show initial sensitivity to gemcitabine therapy followed by the rapid development of resistance. The tumor's initial vulnerability and subsequent resistance strongly suggests either the preexistence of resistant cell subpopulation or the rapid development of resistant cells from the tumor itself or from tumor/stromal alterations (Kim and Gallick 2008). Several genetic and/or epigenetic alterations associated with gemcitabine transport and metabolism contributed to gemcitabine resistance. For example, alterations in the nucleoside transporter-1 (hENT1) as well as deoxycytidine kinase and ribonucleoside reductases M1 and M2 have been linked to gemcitabine resistance (Kim and Gallick 2008). Transcriptional enhanceosome-HMGA1, tyrosine kinases focal adhesion kinase, c-Src and c-Met have been all implicated in gemcitabine resistance (Kim and Gallick 2008). In addition, the phosphatidylinositol 3-kinase/Akt/mTOR pathway has also been involved in gemcitabine resistance (Kagawa et al 2012).

2.1.4 The biology of pancreatic cancer

Cancer is considered as multistep of successive accumulation of genetic and epigenetic mutations. This is also generally accepted in pancreatic cancer. Pancreatic carcinogenesis studies have revealed an activation of the KRAS oncogene and inactivation of the tumor suppressor genes CDKN2A and SMAD4 in the development of pancreatic cancer (Hidalgo 2010). Based on microarray technologies, genetic analysis showed identical gene signatures involved 12 cancer-related pathways including apoptosis, DNA damage repair, cell cycle control, RAS, TGF-β, cell adhesion, Hedgehog, Intergrin, JNK, Wnt/β- catenin, invasion and small GTPases (Jones et al 2008). These pathways orchestrate complex gene networks and contribute to tumor growth, metastasis and drug resistance. Thus, the genetic basis of pancreatic cancer is very complex and heterogeneous.

There are at least two models that have emerged to describe the heterogeneity and inherent difference in tumor regenerating capacity: The clonal selection model is based on the theory which states that the tumor cells acquired mutations which support growth advantages and promote selection during tumorigenesis (Nowell 1976). The second, cancer stem cell model, suggests that, heterogeneity within primary and metastatic tumors derive from a subgroup of cancer cells with stem-cell properties.

These two models are not mutually exclusive. Cancer stem cells can undergo clonal evolution, especially the CSC model displayed good implications for drug resistance and tumor relapse.

2.1.5 Cancer stem cells in pancreatic cancer

CSCs generally comprise only 1-5% of the total tumor mass and display three defining characteristics: 1) they are able to self-renew; 2) they are capable of asymmetric /symmetric cell division; 3) they can give rise to more-differentiated cells. These subpopulation cells have been identified in different solid malignancies including pancreatic cancer, which represent $CD44^{+}CD24^{+}ESA^{+}$, $CD133^{+}$, $CD133^{+}CXCR4^{+}$, c-$Met^{high}CD44^{+}$, $ALDH1^{+}$ sub population within pancreatic cancer (Bao et al 2010).

In addition to these makers previously used for the characterization of CSCs, side population (SP) cells also express ATP-binding cassette (ABC) transporters, and are thus able to efflux the Hoechst 33342 DNA dye can be detected by flow cytometry in many tumor cell lines (Goodell et al 1996) (Figure II.2). SP cells often display a complex profile of multidrug resistance such as toptecan, doxorubicin, mitoxantrone, daunorubicin and so on. On the other hand, SP cells are frequently quiescent without cycling, which may also contribute to their resistance to some cell-cycle-specific drugs (Moserle et al 2010).

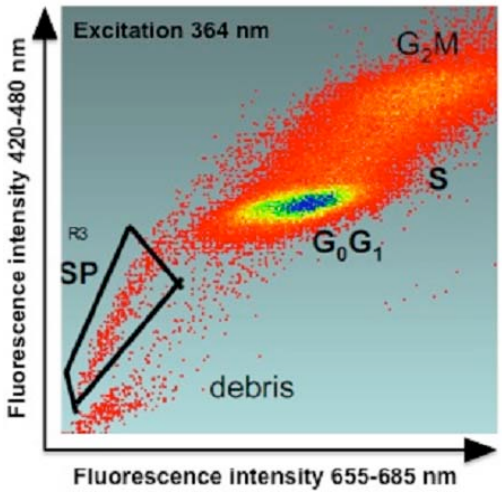

Figure II.2 The SP assay was performed using an established SP detection protocol. Tissue or cell line samples were incubated with Hoechst 33342 for 90 min to 120 min at 37 °C with agitation in the dark. Debris was excluded. Due to its function of DNA binding, the Hoechst dye fluorescence signal can be used to visualize cells in a specific phase of the cell cycle (G0/G1, S, and G2/M) by indicating the DNA content per cell. SP cells are recognized as a dim tail extending first on the left side of G0/G1 cells toward the lower 'Hoechst Blue' signal. (Original graph provided by Dr J. Ellwart).

SP cells are thought to model CSC phenotypes in pancreatic cancer (Haraguchi et al 2006b, Yao et al 2010, Zhou et al 2008). Side population cells described in different pancreatic cancer cell lines have been examined with respect to epithelial to mesenchymal transition (EMT), invasion, metastasis and gemcitabine resistance (Kabashima et al 2009, Zhou et al 2008). In other solid tumor systems it has been shown that small numbers of SP cells are capable of inducing tumor formation, whereas a large number of non-SP cells are generally needed to achieve the same tumor growth in xenografts (Chiba et al 2006, Ho et al 2007). Some groups have linked SP to CSCs in various pancreatic cell lines including: SW1990, Capan-2, CFPAC-1 and BxPC-3. They generally found that SP cells contained more cells in the G1 phase and fewer cells in the S phase when compared with the non-SP cells and exhibited increased tumorigenic ability following *in vivo* transplantation and increased chemoresistance following *in vitro* exposure to gemcitabine (Yao et al 2010, Zhang et al 2010).

2.1.6 miRNAs in pancreatic cancer

MicroRNAs (miRNAs) are, a class of small regulatory noncoding RNAs initially described in

Caenorhabditis elegans in 1993 by Lee et al. and Wightman et al. (Lee et al 1993, Wightman et al 1993). Increasing evidence has implicated a link between miRNAs and cancer (Calin et al 2004).

MiRNAs usually bind to the 3' untranslated region (UTR) of target mRNAs through an imperfect match to repress the translation and stability of the mRNA, but they have also been observed to occasionally switch from repression to the activation of translation (Vasudevan et al 2007). The process of miRNAs biogenesis in animal cells is started in the nucleus by RNA polymerase II enzyme transcription of a long primary-miRNA (Pri-miRNA) sequence with hairpin stem-loop structure. Then Drosha and DGCR8 (DiGeorge syndrome critical region gene 8) enzymes process the RNA into an approximately 70-nucleotide hairpin pre-miRNA that is then transported into cytoplasm by Exportin-5 and Ran-GTP (Gangaraju and Lin 2009). In the cytoplasm, the pre-miRNAs are further processed into a short imperfect double stranded miRNA duplex and then unwound into a mature miRNA by helicase. The mature miRNA will then form complexes with RNA-induced silencing complex (RISC) and subsequently regulate gene expression by mRNA degradation or translation repression (Gangaraju and Lin 2009). One miRNA may target dozens of genes, while one mRNA can also be regulated by multiple miRNAs (Figure II.3).

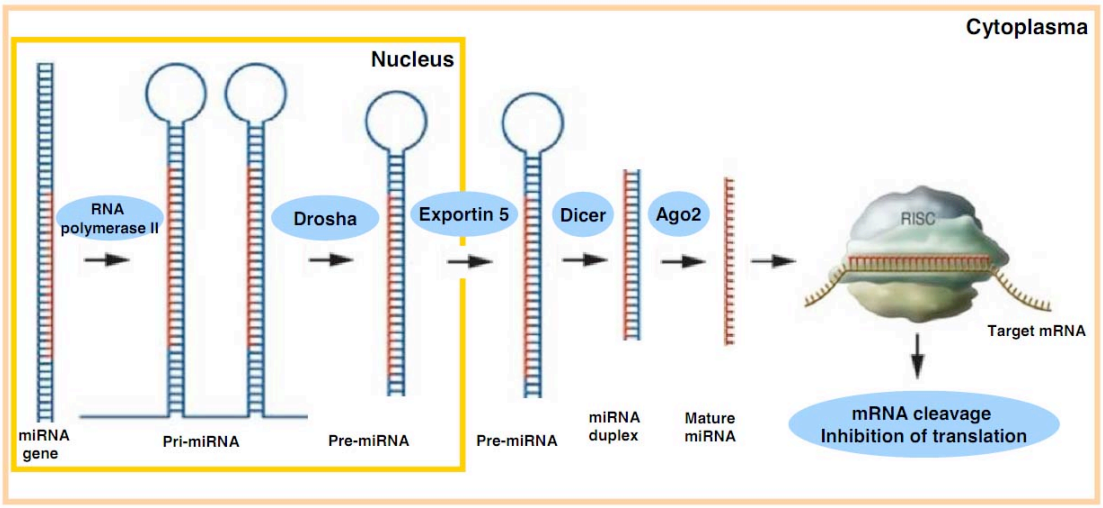

Figure II.3 Biogenesis and function of miRNAs. Revised from 'The magic and mystery of miR-21' (Morrisey 2010) and 'MicroRNAs, cancer and cancer stem cells' (Zimmerman and Wu 2011).

2.1.7 miRNAs regulated cancer stem cells

MiRNAs are important in regulating normal stem cell and cancer stem cell function

(Gangaraju and Lin 2009, Zimmerman and Wu 2011). The earliest study connecting miRNAs and cancer stem cells was described in models of breast cancer. The authors enriched $CD44^{+}CD24^{-/lo}$ breast CSCs by chemotherapy in nude mice with implanted SKBR3 tumors. The authors found that these CSCs also expressed lower levels of let-7, miR-16, miR-107, miR-128 and miR-20b as compared to the parental or differentiated cells (Yu et al 2007). In addition, representatives of miR-200 family were significantly decreased in both breast CSCs and normal mammary stem cells, and the stem cell factor BMI-1was directly modulated by miR-200c (Shimono et al 2009). In a brain CSC study, tumor suppressor miR-451 was reported to be decreased in $CD133^{+}$ GBM (glioblastoma) cells, and that miR-128, miR-34, miR-199-5p were all involved in brain CSC regulation (Gal et al 2008). In gastrointestinal cancers, HCC CSCs identified by an $EpCAM^{+}AFP^{+}$ marker profile overexpressed the miR-181 family and several miR-17-92 cluster miRNAs (Ji et al 2009). Another important miRNA - miR34a, a transcriptional target of p53, not only regulates the progress of several cancer types, but also restrains the biological properties of those CSCs, such as GBM CSCs, prostate CSCs, liver CSCs, gastric CSCs and pancreatic CSCs (Liu and Tang 2011). These 'stem cell miRNAs' interact with CSCs by regulating self-renewal and apoptosis via targeting oncogene or tumor suppressors controlled cancer stem cell pathways (DeSano and Xu 2009). Those overexpressed miRNAs in CSCs inhibiting tumor suppressor genes and acting as oncogenes, are oncogenic miRNAs (oncomiRs); the miRNAs down regulated in CSCs suppress cell aggressive process by nature, function as tumor suppressors, are suppressor miRNAs.

Aberrant miRNAs expression has been implicated in pancreatic cancer. Bloomston et al. and Szafranska et al. compared the expression of numerous miRNAs between pancreatic adenocarcinoma and pancreatitis, and found overexpression of miR-221 in tumor tissues. In addition, miR-103, miR-107, miR-34a, miR-145 were found dysregulated in this disease setting (Bloomston et al 2007, Szafranska et al 2007).

2.1.8 miRNA-based therapeutics

Several critical steps are required in the evaluation of miRNA-based therapeutics: 1) the expression level of target miRNAs must be first evaluated in cancerous vs. healthy tissues; 2) the functional analysis of the candidate miRNAs must be determined in the specific biological setting; 3) an *in vivo* model should be applied to evaluate the functional significance of the specific miRNAs (overexpressed or knockdown) (Kasinski and Slack 2011).

2.1.8.1 Antagomirs and mimics

For oncogenic miRNAs, their overexpression can be reduced using a class of chemically engineered, cholesterol-conjugated single-stranded RNA oligonucleotides called antagomiRNAs (antogomirs) (Esau 2008). Antagomirs irreversibly bind specific miRNAs and inhibit their activity. MiRNAs, down regulated in cancer, can be replaced though the use of miRNA mimics, a group of chemically modified small double-stranded RNAs that mimic their endogenous miRNAs. In addition, lentiviruses and other similar vector based strategy have been used to replace tumor suppressor miRNAs (Nicoloso et al 2009).

2.1.9 Aim of the study

Previous work from our laboratory identified some miRNAs significantly overexpressed in stem-like side population cells as compared to non-side population cells in L3.6pl. Accumulating evidence has suggested that these miRNAs are involved in the regulation of tumorigenesis in pancreatic cancer but little data concerning the therapeutic efficacy of inhibition of about the therapeutic efficacy of inhibition of these oncomirs as cancer stem cell regulators is available. To help address this, we applied SP assay as a model to mimic the cancer stem cells in pancreatic cancer cell lines. The aim of this study was to evaluate the therapeutic potential of inhibiting specific miRNA using antisense oligonucleotides (antagomirs) both *in vitro* and *in vivo*.

2.2 Esophageal cancer

2.2.1 Background of esophageal cancer

Cancers arising from the esophagus, including the gastroesophageal junction are defined as esophageal cancer. More than 90% of esophageal cancer was classified as squamous cell carcinoma (ESCC) and adenocarcinoma (EAC), which represent some of the most aggressive digestive tract malignancies showing bad prognosis (Jemal et al 2007) (Figure II.4). On rare occasions, melanomas, leiomyosarcomas, carcinoids, and lymphomas may develop in the esophagus as well. Approximately 75% of all adenocarcinomas are found in the distal esophagus, whereas squamous cell carcinomas are more evenly distributed between the middle and lower third. The cervical esophagus is an uncommon site of disease. Although ESCC has been responsible for most esophageal cancer worldwide, the incidence of EAC has remarkably increased over the past two decades and has supplanted ESCC as the dominant phenotype in western countries (Devesa et al 1998).

The etiologic factors of esophageal cancer are complex (Enzinger and Mayer 2003). Smoking is associated with an increased risk of both ESCC and EAC mainly due to the tobacco carcinogens, particularly nitrosamines (Wu et al 2001). Chronic irritation and inflammation of the esophageal mucosa appears to increase the incidence of ESCC. Substantial alcohol intake together with smoking may account for more than 90% of ESCC (Brown et al 2001, Wu et al 2001). Other irritation factors include achalasia and esophageal diverticula, and frequent consumption of extremely hot beverages (Garidou et al 1996). For EAC patients, most of them have Barrett' esophagus or a history of gastroesophageal reflux disease, which leads to a high risk of neoplastic transformation(Lagergren et al 1999). Obesity has been implicated recently in increased risk for EAC while helicobacter pylori is thought to provide a degree of protection against the development of EAC (Zhang et al 2009). In addition, a history of radiotherapy to the mediastinum (breast cancer, lymphoma, and other neoplasms) may also predisposes patients to both histologic types of esophageal cancer (Ahsan and Neugut 1998).

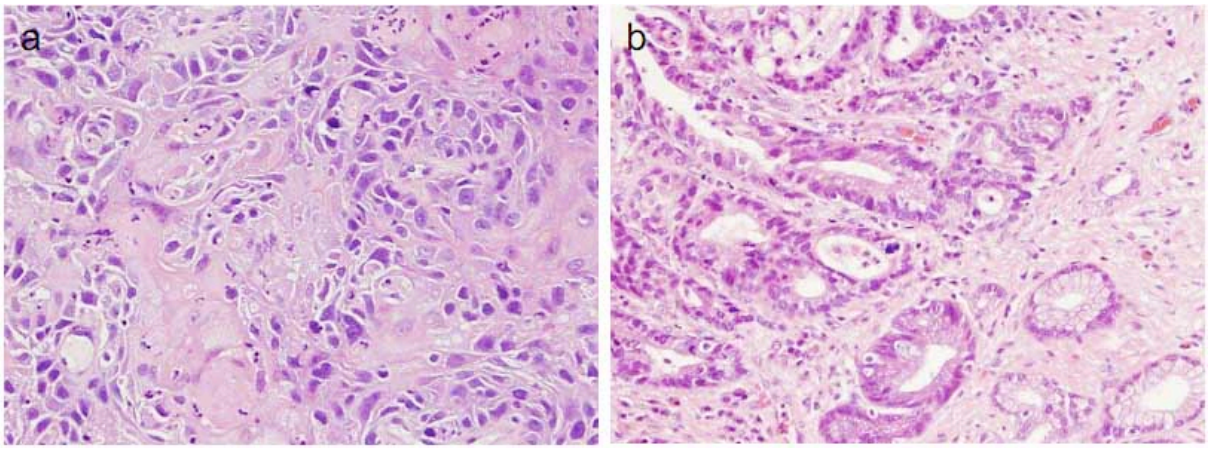

Figure II.4 Histology of esophageal cancer. a) Invasive esophageal squamous cell carcinoma. This carcinoma shows features of squamous differentiation, including keratinization and intercellular bridges b) Processing esophageal adenocarcinoma, showing junction of benign glands in the lower right, Barrett's columnar cell metaplasia with a large goblet cell containing blue mucin in the lower center and adenocarcinoma on the left. (According to http://emedicine.medscape.com/article/277930-overview#a0101)

2.2.2 Management of esophageal cancer

Management of patients with esophageal cancer is complex and requires a multidisciplinary approach (Enzinger and Mayer 2003). Esophageal resection (esophagectomy) remains a crucial part of the treatment for esophageal cancer. Only a minority of patients present with an early stage of localized esophageal cancer which is most commonly resected with the use of either a right transthoracic or a trans hiatal approach by endoscopic therapies (such as endoscopic mucosal resection, radiofrequency ablation, or cryotherapy). The majority of patients undergo surgery when lymph node metastases are already present, the 5-year survival rate for this disease is quite low (Enzinger and Mayer 2003). Chemotherapy alone or combined with radiotherapy has been used before or after surgery in the attempt to improve survival. Monochemotherapy has a response rate of 20-30% while combination chemotherapy works with response rates of 44-55% (Enzinger et al 1999). The internal and acquired drug resistance is main obstacles of current chemotherapy, Alternative forms of systemic treatment or a targeted form of treatment (e.g., an antagonist of the epidermal growth factor receptor or a cyclooxygenase-2 inhibitor) may merit exploration in overcoming resistance.

2.2.3 Chemotherapy and chemoresistance in esophageal cancer

2.2.3.1 5-fluorouracil (5-FU)

5-FU remains a widely applied anticancer drug. 5-FU is a heterocyclic aromatic organic compound ($C_4H_3FN_2O_2$) with a structure similar to pyrimidine molecules of DNA and RNA; it is an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen (Figure II.5). 5-FU interferes with nucleoside metabolism and can be incorporated into RNA and DNA, leading to cytotoxicity and cell death (Parker and Cheng 1990). Research has indicated that 5-FU exerts its anticancer effects mainly through inhibition of thymidylate synthase (TS), which is an essential enzyme for catalyzing the biosynthesis of thymidylate, implicating in the regulation of protein synthesis and apoptotic processes (Longley et al 2003). Dihydropyrimidine dehydrogenase (DPD)-mediated conversion of 5-FU to dihydrofluorouracil (DHFU) is the rate-limiting step of 5-FU catabolism in normal and tumor cells (He et al 2008, Zhang et al 2008a).

However, the overall response rate for 5-FU based chemotherapy remains poor, mainly due to drug resistance resulting from various causes including alterations in drug influx and efflux, enhancement of drug inactivation and mutation of the drug target (Longley and Johnston 2005). ATP-binding cassette (ABC) transporters are promiscuous transporters of both hydrophobic and hydrophilic compounds. ABCC11 referred to as multidrug-resistance-associated protein 8 plays a key role in 5-FU efflux and is dramatically up regulated in 5-FU resistant cancer cell lines. Other ABC transporters such as ABCC3, ABCC4, ABCC5 and ABCG2 are also associated with 5-FU efflux induced drug resistance (Zhang et al 2008a).

Overexpression of TS, increased activity of DPD, methylation of the MLH1 gene, and up-regulation of anti-apoptotic proteins such as Bcl-2, Bcl-XL have all been reported to lead to resistance to 5-FU (Zhang et al 2008a). These reports suggest that multiple factors appear to contribute to 5-FU resistance.

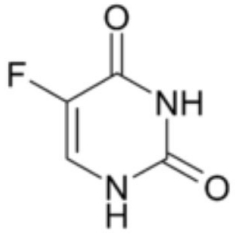

Figure II.5 Structure of 5-FU

2.2.3.2 Cisplatin

Cis-Diamminedichloroplatinum (Cisplatin or cis-DDP, cis-$PtCl_2$ $(NH_3)_2$) is a widely used chemotherapeutic reagent. The structure of cisplatin is shown in Figure II.6, The ligands of cisplatin are replaced by water molecules after cisplatin enters the cell and generates positively charged aquated species which react with nucleophilic sites on intracellular macromolecules to form protein, RNA and DNA adducts. Cisplatin forms approximately 65% 1,2-d(GpG), 25% 1,2-d(ApG) and 5-10% 1,3-d(GpNpG) intrastrand crosslinks, and a reduced percentage of interstrand crosslinks and monofunctional adducts, which result in inhibition of DNA replication, RNA transcription, cell cycle arrest at G2 phase, or programmed cell death of apoptosis (Kartalou and Essigmann 2001).

Due to the significant antitumor effect, cisplatin mono or combination therapy have been tested in various of cancers, and in particular in treatment of testicular cancer (Einhorn 1990). However, the efficacy was still limited because of acquired or intrinsic resistance. The mechanism of resistance to cisplatin is not fully understood. It is known that reduced cisplatin intracellular accumulation due to decreased uptake or increased efflux, or sulfhydryl molecules induced inactivation of cisplatin can cause resistance. Multidrug-resistant-associated protein 2 (MRP2/ABCC2) has been identified as a cisplatin efflux transporter and is highly expressed in cisplatin resistant cells (Niu et al 2012). The dysregulation of oncogenes or tumor suppressor genes have also been implicated in cisplatin-associated resistance. The overexpression of c-fos, ERCC1, c-myc and the mutation of H-ras and P53 were found to be involved in the development of cisplatin resistance (Kartalou and Essigmann 2001).

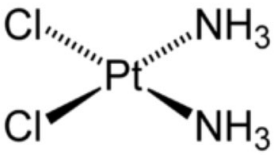

Figure II.6 Structure of cisplatin

2.2.4 The biology of esophageal cancer

As described for pancreatic cancer, esophageal cancer also requires genetic or epigenetic changes in the development of the carcinogenesis. Recently, a genome-wide association study identified seven susceptibility loci on chromosomes 5q11, 6p21, 10q23, 12q24, and 21q22 for

ESCC in Chinese populations. This further implicated the involvement of multiple genetic loci and gene-environment interaction in the development of esophageal cancer (Wu et al 2011). At the genetic level, Cyclooxygenase 2, Bcl-2, p53, p16, p27, cyclin D, retinoblastoma protein, epidermal growth factor and its receptor, transforming growth factor-α (TGF-α), tumor necrosis- α (TNF-α), erb-b2, E-cadherin, α-catenin and β-catenin are thought to play a role in the development of the disease (Enzinger and Mayer 2003).

COX-2 (also known as PTGS2) is an inducible enzyme that catalyzes prostaglandin synthesis and carcinogenesis through several pathways, including those of apoptosis, angiogenesis, inflammation, and immune surveillance (Altorki 2004). COX-2 has been shown to sequentially increase in metaplastic-dysplastic sequence leading to esophageal adenocarcinoma (Brabender et al 2004). Variants in the promoter region of COX-2 have been observed to significantly increase the risk of esophageal adenocarcinoma (Reid et al 2010). Increased COX-2 expression in esophageal squamous cancer patients was found to be significantly correlated at tumor invasion and poor survival (Takatori et al 2008). Accumulating evidence has suggested that the use of aspirin and other nonsteroidal anti-inflammatory drugs to non-selectively inhibit COX-2 may be associated with a lower esophageal cancer rate (Bosetti et al 2003, Farrow et al 1998).

2.2.4.1 EMT (epithelial mesenchymal transition) in esophageal cancer

Promising evidence indicates a crucial role for epithelial mesenchymal transition (EMT) in tumor progression, in particular, metastasis (Kalluri and Weinberg 2009). Esophageal cancer belongs to the family of epithelial carcinomas, which comprises cohesive epithelial cells polarized and tightly connected by E-cadherin-dependent cell-cell junctions, and initially separated from the stroma by the basement membrane. Local invasion through the epithelial basement membrane is the first stage of metastasis. Epithelial cells lose intercellular junctions causing dissociation from surrounding cells, acquire migratory mesenchymal-like characteristics and enable to migrate away from the original tissue (Guarino 2007). Once established in a new environment, metastatic cells may revert back to a non-metastatic phenotype, via a mesenchymal-epithelial transition (Kalluri and Weinberg 2009). Lymph node metastasis is common in esophageal cancer since the esophagus characteristically receives lymphatic supply networks; also the invasion to liver or other organs are often in advanced stage of this disease (Liu et al 2005).

The dynamic switch between epithelial and mesenchymal cells can be distinguished by expression of a number of classical markers (LaGamba et al 2005). For instance, epithelial markers include adherens and tight junction proteins such as E-cadherin and ZO-1, whereas mesenchymal markers include the extracellular matrix component-Fibronectin and the intermediate filament protein-Vimentin (Zeisberg and Neilson 2009). The mechanisms for loss of E-cadherin function include promoter CpG hypermethylation, histone modifications, and direct inhibition by zinc finger transcriptional repressors ZEB1, ZEB2, Snail1, and Twist (Tellez et al 2011). Repression of E-cadherin has also been reported for late stage of human esophageal cancers with particularly aggressive prognosis (Washington et al 1998). Analysis of tissue samples from patients with esophageal SCC suggests that Snail is associated with repressed E-cadherin expression in primary tumors (Natsugoe et al 2007). By contrast, ESCC patients with Vimentin expression can show a significantly higher incidence of lymph node metastasis, and is associated with stronger expression of α-SMA expression (Jin et al 2010). In addition, evaluation of Twist in esophageal SCC has revealed significantly higher Twist expression when compared to non-neoplastic tissue (Yuen et al 2007). Similarly, Slug has been shown to be overexpressed in primary esophageal SCC, correlating with depth of tumor invasion, lymph node metastasis and poorer clinical outcome (Uchikado et al 2005). EMT biology may be linked to the progress of esophageal cancer development.

2.2.4.2 Wnt signaling pathway

There are several important stem cell-associated signaling systems involved in cancer development, including sonic hedgehog, mTOR, TGF-β, Notch, BMP and Wnt signaling. The Wnt signaling pathway is a highly conserved ancient pathway controlling proliferation, differentiation, motility and apoptosis. Mutations or dysregulated expression of components in this pathway have been linked to diverse disease states, in particular cancer (Klaus and Birchmeier 2008). There are three well defined Wnt signaling pathways, the canonical pathway (Wnt/β-catenin pathway) and two noncanonical pathway (the planar cell polarity pathway, the Wnt/Ca^{2+} pathway and the protein kinase A pathway) (Takahashi-Yanaga and Kahn 2010). The Wnt/β-catenin pathway is dependent on the status of β-catenin, a protein kept under low cytoplasmic concentration by the destruction complex, which was scaffolded by APC (Adenomatous polyposis coli), CK1 (Casein kinase 1), GSK3- β (Glycogen synthase kinase 3 β) and Axin2 (Axis inhibition protein 2). In the absence of Wnt signals, the membrane receptor complex Fzd (Frizzled) and LRP5/6 (low density lipoprotein receptor

related protein 5/6) is not engaged and CK1 and GSK3-β phosphorylate β-catenin, are ubiquitinated and targeted for rapid proteasomal degradation (Takahashi-Yanaga and Kahn 2010). Once Wnt ligands bind to LRP5/6 receptor, the destruction complex is dissolved and β-catenin is stabilized in the cytosol and further translocated into nucleus where it converted TCF (T cell factor) into a transcriptional activator that will trigger a complex transcriptional program. Important Wnt regulators or components that become dysregulated (e.g., by mutation, loss of heterozygosity and hypermethylation) may lately be associates with the development of malignancies including colorectal cancer, liver cancer, pancreatic cancer, mammary cancer and esophageal cancer (Klaus and Birchmeier 2008).

Dysregulation of Wnt pathway elements have been described in both ESCC and EAC. Several histopathology studies have shown accumulated localization of β-catenin in the nucleus in adenocarcinoma cells is associated with Barrett**'**s metaplasia (Moyes et al 2012). Unlike colon cancer, mutations of β-catenin, Axin or APC are not frequently in patients with esophageal adenocarcinoma. However, loss of heterozygosity in the APC gene was noted in 60% of adenocarcinoma arising from Barrett**'**s metaplasia (Gonzalez et al 1997).

2.2.5 Cancer stem cells in esophageal cancer (side population model)

As discussed in earlier section on pancreatic cancer, cancer stem cells have been confirmed in many types of cancer. However, comparatively little is known about the existence of CSCs in esophageal cancer. Haraguchi et al. originally identified CSCs in various gastrointestinal cancer cell lines, including 3 squamous cell esophageal cancer lines (TE1, TE2, and TE13), using Hoechst 33342 to isolate small fractions of SP cells (about 0.3%-1.4%) (Haraguchi et al 2006a). Huang et al. detected a SP fraction in SCC cells (EC9706 and EC109). SP cells with the strongest dye efflux activity termed 'Tip'-SP cells showed markedly higher tumorigenicity than non-SP cells *in vitro* and *in vivo* (Huang et al 2009a). Other groups proposed the existence of radio-resistant stem-like cells in esophageal cancer. Che et al. developed a radio-resistant subtype-Eca109R50Gy cells which show some properties of CSCs (Che et al 2011). Zhang et al. found an enrichment of side population cells in radio-resistant esophageal cancer cell lines following fractionated irradiation (Zhang et al 2008b). There is conflicting data regarding the existence of common CSC surface markers on esophageal cancer cells. Grotenhuis et al tested a group of common cancer stem cell markers in Barrett's esophageal adenocarcinoma including CD24, CD29, CD34, CD44, CD133, CD166, EpCAM, β-catenin

and ALDH1 by FACs staining, *in vivo* tumorigenicity and IHC staining in patients tissues. And they found that these common CSC markers do not enrich for EA initiating cells (Grotenhuis 2010).

2.2.6 Aspirin as Wnt signaling inhibitor in esophageal cancer

Aspirin, a non- steroidal anti-inflammatory drug (NSAID) is the leading chemoprevention candidate for esophageal cancer. NSAIDs inhibit cell growth and induce apoptosis at various disease stages, from initiation to progression. Numerous molecular targets have been implicated but the antitumor activity of aspirin cannot be attributed wholly to a single target. It is likely that aspirin influences several molecular pathways and that the nonspecific nature of the effect may be key to cancer prevention (Chan 2012). Aspirin and other NSAIDs inhibit the activity of cyclooxygenase, and were further reported that the inhibition of the Wnt/β-catenin signaling pathway is one of their potential mechanisms of preventive or therapeutic action (Boon et al 2004, Dihlmann et al 2001). In colon cancer, increased COX-generated PGE2 was shown to suppress β-catenin degradation, resulting in activation of Wnt/β-catenin signaling. Therefore, aspirin might provide an easy and effective therapeutic strategy to suppression Wnt pathway activated cancer stem cells or stem like subpopulations.

2.2.7 Aim of the study

My goal was to detect and characterize side population cells in different esophageal cancer cell lines. Previous study found that induced resistance to chemo- or radiotherapy could increase resistant SP cells. To study this phenomenon, I will isolated the SP subpopulation cells from both sensitive and chemotherapy resistant esophageal cancer cell lines and investigated their capacity for self-renewal, differentiation, and tumorigenicity as well as their potential role in chemotherapy resistance and metastasis. Finally I sought to identify a dynamic profile of EMT associated genes in side population cells induced chemoresistance.

III. MATERIAL AND METHODS

3.1 Materials

3.1.1 Cell lines

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

L3.6pl is a highly metastatic cell line variant originally derived from fast- growing variant (FG) of human pancreatic cancer cell line COLO375 under several *in vivo* selection with increasing metastatic potential, displayed an aggressive primary tumor growth in nude mice with spontaneous liver metastases and lymph nodes metastases. L3.6pl cells produced pro-angiogenic factors such as VEGF, bFGF and IL-8 and showed a significantly decreased expression of E-cadherin and increased collagenase type IV expression in contrast to the parental FG cells (Bruns et al 1999).

L3.6pl was cultured in medium with increasing concentration of gemcitabine, starting at 0.5 ng/ml and increasing to over 24-month period. The gemcitabine resistant sub-line was established and displayed different morphology (Figure III.1)

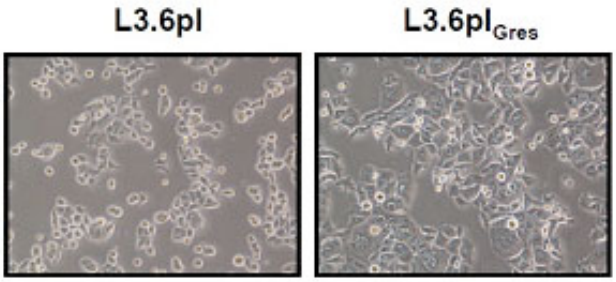

Figure III.1 Morphology of L3.6pl and L3.6pl$_{Gres}$

3.1.1.2 Human esophageal cancer cell lines

Five different esophageal cell lines were used in this study (Figure III.2). The corresponding 5-FU or cisplatin resistant cell lines were established after long-term chemotherapy.

OE19

The cell line OE19 (also known as JROECL19, ECACC number: 96071721) was established

in 1993 from an adenocarcinoma of gastric cardia/esophageal gastric junction of a 72-year-old male patient. The tumor was identified as pathological stage III (UICC) and showed moderate differentiation.

OE21

The cell line OE21 (also known as JROECL21, ECACC number: 96062201) was established in 1993 from a squamous carcinoma of mid esophagus of a 74-year-old male patient. The tumor was identified as pathological stage IIA (UICC) and showed moderate differentiation.

OE33

The cell line OE33 (also known as JROECL33, ECACC number: 96070808) was established from the adenocarcinoma of the lower esophagus (Barrett′s metaplasia) of a 73-year-old female patient. The tumor was identified as pathological stage IIA (UICC) and showed poor differentiation.

All three cell lines express HLA-A, -B and -C antigens (MHC class I) and ICAM-1 constitutively. Expression of HLA-DR (MHC class II) can be induced by treatment with interferon-gamma. The cells express epithelial cytokeratins and are tumorigenic in nude mice (Rockett et al 1997).

PT1590 and LN1590

PT1590 and LN1590 are esophageal cancer cell lines established from primary tumors (PT) and a lymph node with micro metastasis (LN) from a patient who identified as stage IIB and had undergone radical esophagectomy for a poorly differentiated adenocarcinoma of the esophagus at the University Medical Center of Hamburg-Eppendorf. LN1590 was generated from one of Ber-EP4-positive nodes (later known as EpCAM). Both cell lines are tumorigenic in nude mice (Hosch et al 2000).

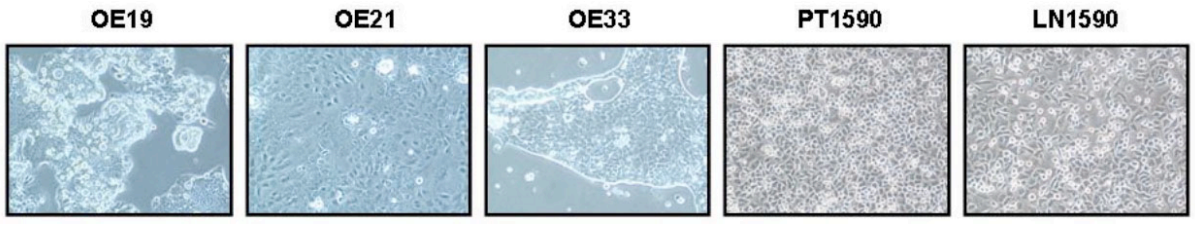

Figure III.2 Morphology of different esophageal cancer cell lines

3.1.2 Reagents

Medium, buffers, solutions for cell culture

DMEM (Dulbecco's Minimal Essential Medium)	Invitrogen GmbH, Karlsruhe, Germany
DMEM/F12	Invitrogen GmbH, Karlsruhe, Germany
DMSO (Dimethylsulphoxide)	Sigma-Aldrich, Steinheim, Germany
DPBS-buffer	Biochrom AG, Berlin, Germany
EGF (Recombinant human EGF)	PeproTech EC, UK
Fetal bovine serum	Biochrom AG, Berlin, Germany
FGF (Recombinant human basic FGF)	PeproTech EC, UK
Insulin solution (human)	Sigma-Aldrich, Steinheim, Germany
MEM vitamin mixture	PAN Biotech, Aidenbach, Germany
MEM NEAA	PAN Biotech, Aidenbach, Germany
Normocin	InvivoGen, San Diego, USA
Penicillin/Streptomycin 100 ml (10.000 Units Penicillin/mL, 10 mg Streptomycin/ml)	PAN Biotech, Aidenbach, Germany
RPMI 1640 + Glutamax-1	Gibco Invitrogen, Germany
Trypsin0.05%/EDTA0.02 % in PBS without Ca^{2+} and Mg^{2+}	PAN Biotech, Aidenbach, Germany
Trypan blue (0.4%)	Sigma-Aldrich, Steinheim, Germany
Transferrin	Sigma-Aldrich, Steinheim, Germany

3.1.3 Technical equipements

ABI StepOnePlus™	Applied Biosystems, Foster City, USA
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
Centrifuges	Eppendorf, Germany
CO_2 incubators	Heraeus, Rodenbach, Germany
Digital precision scale	KERN & Sohn GmbH, 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
Leica RM2255, Fully Motorized Rotary Microtome	Leica Microsystems, Germany
LSR II flow cytometry	BD, Biosciences, USA
Liquid nitrogen tank	MVE, New Prague, MN, USA
Phase contrast microscopy	Carl Zeiss GmbH, Germany
Microwave oven	Siemens, Germany
MoFlo high speed sorter	DAKO Cytomation, Glostrup, Denmark
Thermo Scientific Heraeus incubator	Thermo Fisher Scientific Inc, Germany
TECAN GENios Plus ELISA reader	TECAN, Salzburg, Austria
RNA/DNA calculator	GeneQuant Pro, GE, USA
Vortex	IKA Works, Wilmington, NC, USA
Water bath	GFL, Burgwedel, Germany

3.1.4 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
Cell culture insert 8.0 µm	BD Bioscience, NJ, USA
Lab-Tek™ chamber slides	Thermo Fisher Scientific Inc, 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

3.1.5 Cell culture and frozen medium

3.1.5.1 Pancreatic cancer cell lines

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

3.1.5.2 Esophageal cancer cell lines

OE19, OE21 and OE33

RPMI 1640+ Glutamax-1	plus	10% FCS
		100 IU/ml Penicillin
		100 µg/ml Streptomycin

PT1590 and LN1590

RPMI 1640+ Glutamax-1	plus	10% FCS
		100 IU/ml Penicillin
		100 µg/ml Streptomycin
		10 ng/ml EGF
		10 ng/ml FGF
		10 ng /ml Insulin
		4 µg/ml Transferrin

1ml Normocin was added to 500 ml normal cell culture media as a ‘routine addition’ to cell culture media to prevent mycoplasma, bacterial and fungal contaminations in small or large-scale animal cell cultures.

3.1.5.3 Cell storage medium

90% FCS	plus	10% DMSO

3.1.5.4 Storage and re-cultivation of the cells

One to four million cells were stored in cell storage medium per cryotube. The tubes are moved to a gradually temperature-decreasing box and then kept in a -80°C freezer. After 72 hours, the tubes were moved to a liquid nitrogen tank for long-term storage. Before re-cultivation of the cells, the culture medium was first aspirated into the cell culture flask. The frozen tubes were taken out of the liquid nitrogen tank and put into the 37°C water bath immediately until complete thawing of the cells. The cells were then added into the cell culture flask. After 24 hours culturing in the incubator, the medium was changed to avoid toxic effects of the remaining DMSO.

3.1.6 Materials for cell proliferation and cytotoxity assay

5-FU (Fluorouracil-GRY)	GRY-Pharma GmbH, Germany
Cisplatin (Cis-GRY)	GRY-Pharma GmbH, Germany
Gemcitabine (Gemzar)	Lilly Deutschland GmbH, Germany
Cell counting kit-8 (CCK-8)	Dojindo Laboratories, Japan
TACS MTT cell proliferation/viability assay kit	R&D systems, Minneapolis, USA

3.1.7 Materials for transfection and real time PCR

All stars negative control siRNA with or without Alexa fluor 488 modification	Qiagen, USA
Anti-hsa-miR-21-5p (phosphorotioate)	Qiagen, USA
Anti-hsa-miR-221-3p (phosphorotioate)	Qiagen, USA
HiperFect transfection reagent	Qiagen, USA
Hs_Mir-21 miscript primer assay	Qiagen, USA
Hs_Mir-221 miscript primer assay	Qiagen, USA
Hs_RNU6B miscript primer assay	Qiagen, USA
miRNeasy mini kit	Qiagen, USA
miScript reverse transcription kit	Qiagen, USA
miScript SYBR Green PCR kit	Qiagen, USA
QuantiFast SYBR Green PCR kit	Qiagen, USA
RT^2 First start kit	SuperArray Bioscience, Qiagen, USA
RT^2 Profiler PCR array system	SuperArray Bioscience, Qiagen, USA

3.1.1 Materials for western blot, flow cytometry, Immunofluorescence, and immunohistochemistry

4% paraformaldehyde	Pathology LMU, Germany
7-AAD	BD phamingen, USA
Albumin from bovine serum (BSA)	Sigma-Aldrich, Steinheim, Germany
Avidin/Biotin blocking kit	Vector Laboratories, CA, USA
BCA protein assay reagent kit	Pierce, Rockford, USA
Biotinylated secondary antibody	Vector Laboratories, CA, USA
DAPI in mounting medium	Vector Laboratories, CA, USA
DyLight-594- Donkey anti mouse	Dianova, Pinole, CA, USA
ECL western blotting detection system	Amersham Biosciences, Germany
Ethanol 70%, 80%, 96%, 100%	CLN GmbH, Niederhummel, Germany
FCR blocking reagent (human)	Miltenyi Biotec GmbH, Germany
FITC-Donkey anti rabbit	Dianova, Pinole, CA, USA
FITC-Donkey anti mouse	Dianova, Pinole, CA, USA
Hoechst 33342	Sigma-Aldrich, Steinheim, Germany
Hydrogen peroxide 30% (H_2O_2)	Merck, Darmstadt, Germany
In situ cell death detection kit (TUNEL assay)	Roche, Penzberg, Germany
Kaiser's glycerol gelatine	Merck, Darmstadt, Germany
Liquid DAB+ substrate chromogen system	Dako, CA, USA
Mayer's hemalum solution	Merck, Darmstadt, Germany
Neo-Clear (Xylene substitute)	Merck, Darmstadt, Germany
Normal rabbit serum	Vector Laboratories, CA, USA
Normal goat serum	Vector Laboratories, CA, USA
Propidium iodide	BD phamingen, USA
Restore western blot stripping buffer	Pierce, Rockford, USA
Sodium chloride	Merck, Darmstadt, Germany
Target retrieval solution 10×	Dako, CA, USA
TRIZMA base	Sigma-Aldrich, Steinheim, Germany
TRIZMA hydrochloride	Sigma-Aldrich, Steinheim, Germany
Triton X-100	Sigma-Aldrich, Steinheim, 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)

Table III.1 Antibodies

Antibody	Company	Specificity	Host	Catalog	Molecular weight	Isotype
ABCG2	Abcam	Human	Mouse	ab3380	72 kD	IgG2a
ABCG2-APC	R&D Systems	Human	Mouse	FAB995A	72 kD	IgG2b
Active ß-catenin	Millipore	Human	Mouse	05-665	92 kDa	IgG2
CD105	Abcam	Human	Mouse	ab44967	95 kDa	IgG1
CD133	Abcam	Human	Rabbit	ab19898	97 (110 kDa)	IgG
CD24-PE	BD pharmingen	Human	Mouse	555428	35-45 kDa	IgG2a
CD29	Abcam	Human	Rabbit	ab52971	88 (140 kDa)	IgG
CD31	Abcam	Human	Rabbit	ab28364	130 kDa	IgG
CD44	Abcam	Human	Mouse	ab6124	82 kDa	IgG2a
CD44	Abcam	Human	Rabbit	ab51037	82 kDa	IgG
CD44-FITC	Macs miltenyi	Human	Mouse	130-095-195	82 kDa	IgG1
CD90	Abcam	Human	Mouse	ab23894	18 (26 kDa)	IgG1
C-myc	abcam	Human	Mouse	ab32	41 kDa	IgG1
CXCR4	Abcam	Human	Rabbit	ab2074	39 (43 kDa)	IgG
CXCR4-FE	R&D Systems	Human	Mouse	FAB170P	39 (43 kDa)	IgG2a
E-cadherin (G-10)	Santa Cruz	Human	Mouse	SC 8426	120 (135 kDa)	IgG1
EpCAM	Abcam	Human	Rabbit	ab71916	35 kDa	IgG
EpCAM-FITC	Macs miltenyi	Human	Mouse	130-080-301	36 kDa	IgG1
ERCC1	Abcam	Human	Mouse	ab2356	33-36 kDa	IgG2b
ki67	Abcam	Human	Rabbit	ab16667	345 and 395 (359 kDa)	IgG
Snail1 (H-130)	Santa Cruz	Human	Rabbit	SC 28199	29 kDa	IgG
Snail2	Sigma	Human	Mouse	WH0006591M5	29 (68 kDa)	IgG3κ
ß-actin	Sigma	Human	Mouse	A5316	42 kDa	IgG2a
ß-catenin	BD bioscience	Human	Mouse	610154	92 kDa	IgG1
TS	Abcam	Human	Mouse	ab3145	35 (36 kDa)	IgG1
Vimentin (C-20)	Santa Cruz	Human	Rabbit	SC 7557-R	57 kDa	IgG
ZEB1(H-102)	Santa Cruz	Human	Rabbit	SC 25388	124 kDa	IgG

Table III.2 Flow cytometry isotypes

Isotype	Company	Specificity	Host	Catalog
Mouse IgG1-FITC	Macs miltenyi	Human	Mouse	130-092-213
Mouse IgG2a-PE	BD pharmingen	Human	Mouse	555574
Mouse IgG2b-APC	Macs miltenyi	Human	Mouse	130-092-217

3.1.2 Materials for animal experiments

3.1.2.1 Animals

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

3.1.2.2 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	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^{TM}, Madrid, Spain

3.1.2.3 Medicine

Growth Factor Reduced (GFR) BD Matrigel^{TM} Matrix, 10ml	BD Biosciences, USA
Ketaminhydrochlorid (Ketavet) 100 mg/ml	Pfizer Pharmacia GmbH, Germany
Xylazinhydrochlorid, Xylazin (Rompun) 2% 25 ml	Bayer Healthcare, Leverkusen, Germany

3.1.1 Software

Adobe Acrobat 7.0 Professional	Adobe Systems Inc., USA
Axio Vision 4.4	Carl Zeiss GmbH, Germany
EndNote X5 (Windows Version X5)	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 2003 (Word, Excel, PowerPoint)	Microsoft Corporation, USA
SoftMax Pro	Molecular Devices Corp., USA

SPSS Statistics 19	SPSS STATISTICS Inc., USA
Summit 4.3 software	Beckmann coulter GmbH, Germany
Windows XP Professional	Microsoft Corporation, USA

3.2 Methods

3.2.1 Methods of pancreatic cancer

3.2.1.1 Cell lines and culture

The highly metastatic human pancreatic adenocarcinoma cell line L3.6pl was used to develop gemcitabine resistant cell lines ($L3.6pl_{Gres}$). L3.6pl was cultured in medium with increasing concentrations of gemcitabine, starting at 0.5ng/ml up to 7.5 ng/ml. The media was exchanged twice a week. Cells were incubated in a humidified incubator (37ºC, 5% CO2). For *in vivo* experiment, $L3.6pl_{Gres}$ cell lines were prepared on reaching 60-70% confluence for SP population isolation.

3.2.1.2 Analysis of SP- and NSP-cell fractions of L3.6pl or $L3.6pl_{Gres}$

SP- and NSP-cell fractions of L3.6pl or $L3.6pl_{Gres}$ with or without antisense treatment were identified or isolated using a modification of the protocol described (Goodell et al 1996). Briefly, cells were re-suspended at 37ºC in DMEM containing 2% fetal bovine serum and labeled with Hoechst at a concentration of 2.6 µg/ml for 1 hour at 37ºC, 225 µM verapamil was applied as a specific SP inhibitor. After staining, the cells were maintained at 4ºC in the dark until flow cytometry analysis. Cells were counterstained with 10 µg/ml propidium iodide to label dead cells, analyzed or sorted by MoFlo with the Summit 4.3 software. Hoechst dye was excited at 355 nm (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 and $L3.6pl_{Gres}$ were kept on ice for further application.

3.2.1.3 Antagomirs of miR-21 and miR-221

Antisense oligonucleotides (ASOs) of miR-21 and miR-221 were purchased as phosphorotioate miScript miRNA Inhibitor (Anti-hsa-miR-21-5p or Anti-hsa-miR-221-3p)

and transfected into L3.6pl or isolated SP cells by using HiperFect transfection reagent refer to the manufacturer's instructions. All stars negative control siRNA was used as control. Transfection efficiency was measured using all stars negative control siRNA with Alexa fluor 488 modifications. The knockdown effect of ASOs was detected by real time PCR.

3.2.1.4 RNA isolation and miRNA or target gene quantification

RNA including miRNAs was isolated from in L3.6pl or L3.6pl$_{Gres}$ or sorted SP and NSP fraction as manufacturer described by using miRNeasy kit, 100ng of total RNA were used to prepare cDNA fragments for further quantification. The expression of miR-21 and miR-221 was quantified using the miScript SYBR® Green PCR Kit as the manufacturer's instructions. MiRNA expression was related to the RNU6B internal control, the expression of miRNA putative target genes CDK6, IRAK3, NRP1, SMAD7, SOCS6, C5ORF41, KLF12, MAPK10, EFNA1 and ZBTB41 were predicted by online prediction tools (miRanda, Target Scan and PicTar), scored by mirSVR (Table III.3). And they were further detected by QuantiFast SYBR Green PCR Kit and normalized by 18S ribosomal RNA. Data were calculated using the comparative CT method.

Table III.3 miRNAs target prediction tools.

Method	Type of method	Method availability	Resource
miRanda & mirSVR	Complementary	Download	http://www.microrna.org
Target Scan	Seed Complementary	Online search	http://www.targetscan.org
PicTar	Thermodynamics	N/A	http://pictar.mdc-berlin.de/

3.2.1.5 Cell apoptosis analysis

To determine cell cycle distribution, cells with or without ASOs transfection were collected and fixed with cold ethanol at 4^{o}C for 1 hour. Fixed cells were washed and suspended in 1 ml of PBS containing 50 μg/ml RNase A and 10 μg/ml propidium iodide. After incubating for 20 minutes at 37^{o}C, cells were analyzed for DNA content by flow cytometry FACS Calibur. Cell cycle distributions were determined using FlowJo software. Sub-G0/G1 was then quantified and used as an estimate of the amount of the cells undergoing apoptosis. The cellular debris was excluded from the analysis. Experiments were repeated three times.

3.2.1.6 Cell viability, proliferation and cytotoxicity assay

Cell viability can be distinguished by trypan blue staining. The dead cells with membrane defects that are not able to exclude the blue dye will display blue under the phase contrast microscopy. 10-100 µl of single cell suspension was gently mixed with an equal volume of 0.4% trypan blue. The calculation is as below:

Cell number per ml = equality of four blue-regions unstained cell number × 2 × 10^4

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

Cell proliferation was measured using the CCK-8 kit or TACS MTT cell proliferation and viability assay kit used according to the manufacturer's instructions. Briefly, 8000 cells per well plated on a 96-well plate were grown over night, with or without pre-treatment of ASOs and further treated for 24 hours with chemotherapeutics (gemcitabine or 5-FU) and analyzed afterwards using VersaMax tunable microplate reader and Softmaxpro for data analysis.

3.2.1.7 Transmigration assay

After transfection with antagomirs, L3.6pl cells were cultured in serum-free medium 24 h before the start of the migration assay. 2.5×10^4 cells/well cells were seeded to the upper chamber of the migration assay set. The bottom chamber was filled with 10% FBS medium. After 24 hours of incubation, the cells on the upper surface of the filters were completely removed by wiping with a cotton swab. The chambers were washed with PBS and fixed with 4% Paraformaldehyde, then stained and measured by OD450 nm on VersaMax tunable microplate reader and further analyzed by Softmaxpro software.

3.2.1.8 Human pancreatic cancer specimens and normal adjunct tissue samples

All human pancreatic samples were obtained from the department of surgery, Klinikum Großhadern, Ludwig-Maximilians-University of Munich. Before surgery, all patients provided a written informed consent. Samples were snap-frozen in optimal cutting temperature and stored at −80 °C until use. Total RNA including miRNA was isolated as described before. The pathological status and follow up of the patients was provided by the hospital. The quantitative examination of miRNA expression in those samples follows as described above.

3.2.1.9 Tumorigenicity assay and Antisense therapy *in vivo*

8-10 week-old male athymic BALB/c nu/nu mice were housed and maintained as described before. Mice were anesthetized with ketavet (100 mg/kg mouse body weight) and xylazin (5 mg/kg mouse body weight) followed premedication with atropine sulfate. 10^6 L3.6pl$_{Gres}$ cells or 10^5 isolated SP or NSP cells from L3.6pl$_{Gres}$ (in 40 µl PBS) with or without antisense therapy were injected into the sub-capsular region of the pancreas (group detail shown in Table III.4, procedure detail shown in Figure III.4).

After transfection and injection, cell viability was determined the with trypan blue staining in order to control the therapeutic efficiency. Orthotopic tumor growth was monitored twice a week. On day 35 or 63 after the injection, animals were sacrificed and examined for orthotopic tumors, lymph node and hepatic metastases. The tumor volume and weight was measured. The presence of tumor was further confirmed by Hematoxylin and Eosin (H&E) staining.

3.2.1.10 Immunohistochemistry

Hematoxylin and Eosin staining

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

Staining for Ki67, CD31 and TUNEL (The assessment of in situ cell proliferation, angiogenesis and apoptosis)

Tissue sections (4 µm) were deparaffinized in xylene and rehydrated in a graded series of ethanol. Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide followed by antigen retrieval using antigen retrieval solution. Endogenous avidin and biotin were blocked using the Avidin/Biotin blocking kit. The slides were treated for 20 minutes with blocking solution followed by overnight application of anti-Ki67 antibody and anti-CD31 antibody as primary antibodies. Then biotinylated secondary antibody as well as the

ABC reagent for signal amplification was applied. Slides were washed in TBS buffer, stained with DAB and counter-stained with hematoxylin and then mounted in Kaisers Glycerinegelatine. Apoptotic cells can be detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL). Fluorescent staining for apoptotic cell death was done on paraffin-embedded tissue sections by using the In Situ Cell Death Detection kit according to the manufacturer's protocol, with a DAPI nuclear counterstaining.

After immunohistochemical staining for Ki67 and TUNEL, slides were analyzed at 200x magnification under the microscope or fluorescent microscope. Areas presenting the highest density of Ki67 positive cells or nuclear TUNEL signals were chosen and captured as photographs. These photographs were analyzed by Image-J program. The Ki67 or TUNEL index were evaluated in a blinded manner and calculated as Ki67 positive cells (nuclear with brown color) or apoptotic cells (nuclear with green fluorescence) divided by all tumor cells in one field. Necrotic tumor cells were excluded from the cell count. MVD (Micro vascular density) was evaluated by CD31 positive structures that were identified for vessel counts.

3.2.1.11 Statistical analysis

Statistical evaluation was performed on SPSS 19.0 (Chicago, IL) using the paired student's t-test or ANOVA test. Data were expressed as means ± standard error. Differences were considered statistically significant at $p < 0.05$ ($p<0.05$ marked as '*'; $p<0.005$ marked as '**'; $p<0.0005$ marked as '***'). GraphPad Prism® 5.0, SPSS 19.0 or Microsoft excel 2007 softwares were used to generate graphs and tables.

3.2.2 Methods of esophageal cancer

3.2.2.1 Esophageal cancer cell lines and resistant subtypes

The human esophageal cancer cells lines OE19, OE21, OE33, PT1590 and LN1590 were kept at 37^{o}C under an atmosphere of 5% CO2 and harvested with trypsin-EDTA in their exponential growing phase. IC_{50} of 5-FU and cisplatin were determined by cell cytotoxic assay. Resistant cells were cultured in medium with increasing concentrations of 5-FU or cisplatin according to the IC_{50} values (from 10% IC_{50} up to 5 folds of IC_{50}), respectively. Media were changed twice a week.

3.2.2.2 Flow cytometry analysis and sorting

SP cells in esophageal cell lines were stained as described earlier. Cells with or without treatment were analyzed with a LSR II flow cytometer equipped with 20-mW, 355 nm UV laser. Hoechst fluorescent blue was measured with a 450/50BP and Hoechst fluorescent red with a 670/30BP filter. The accordant SP subpopulation was sorted with MoFlo high speed sorter. Sorted cells were then used for the following experiments.

For further analysis cells were kept in the dark and cold blocked with FCR blocking reagent 15 min and stained with cancer stem cell surface markers 45 min on ice. FITC, PE and APC isotype controls were used as negative control. FITC and PE were excited at 488 nm by an octagon blue laser, and fluorescence was detected using 530/30 and 675/20 filters, respectively. FL4 excitation of APC was at 633 nm, emission was at 660 nm. Dead cells were excluded by gating on forward and side scatter and eliminating PI or 7-AAD positive population cells. All the FACs data were analyzed on FlowJo software.

3.2.2.3 Cell cytotoxicity assay

Cell growth curves or inhibition rates following treatment with chemotherapy were determined in three separate experiments using the CCK-8 and were expressed as percentage of control absorbance. Esophageal cancer cells were seeded as 10^4 per well in a 96-well plate, the accordant chemotherapy was added after 24 hours incubation; the cytotoxicity was measured after 48 hours after treatment. For sorting SP and non-SP cells were plated in 96-well plates at a density of 3000 cells per well and allowed to grow for additional 48h or 72h in either drug-free medium or under treatment. The 50% inhibitory concentration (IC_{50}) of cell growth for each cell line was then analyzed using VersaMax tunable microplate reader and Softmaxpro.

3.2.2.4 Colony formation assay and soft agar assay

500 or 1000 sorted SP and non-SP cells were seeded in 500ul 10%FCS RPMI-1640 on 24-well plate. Media were changed once weekly, after 3-5 weeks colonies (>50 cells) were counted. Colonies were fixed in 100% cold methanol and stained with 0.1% crystal violet. For soft agar assays a 6-well culture plate was coated with 2 ml bottom agar mixture (DMEM/F12 with 10% FBS, 0.6% agar). After the bottom layer was solidified 2 ml top agar-medium mixture (DMEM/F12 with 10% FBS, 0.3% agar) containing 2000 sorted cells was added, and

the plate was incubated at 37°C for 3-5 weeks. Plates were then stained with 0.05% crystal violet and the colonies were counted.

3.2.2.5 Immunofluorescence analysis

After sorting both SP and non-SP cells were plated on Lab-Tek™ chamber slides in normal cell culture media at 37°C with 5% CO2. Cells were fixed with 4% paraformaldehyde for 30 min at room temperature, permeabilized with 0.1% Triton X-100 in blocking serum for 30 minutes at room temperature, and finally incubated with anti-ABCG2 (dilution 1:200), anti-CD133 (dilution 1:200), anti-CD44 (dilution 1:200), anti-CD45 (dilution 1:200), anti-B-catenin (dilution 1:200) at 4°C overnight. After the cells were stained with a matched FITC or DyLight-594 conjugated secondary antibody (dilution 1:200) for 1 hour at room temperature, the chamber slides were counterstained with DAPI in mounting medium and visualized by digital fluorescence microscopy using the AxioVision Rel. 4.6 software. For further image processing and analysis the software Image-pro was used.

3.2.2.6 RT^2 Profiler PCR Array System

The expression of EMT-related genes were examined using the RT^2 Profiler EMT PCR array (Cat.no. 330231 PAHS-090C, SuperArray Bioscience) including cell surface receptor, extracellular matrix, cytoskeletal genes mediating cell adhesion, migration, motility, and morphogenesis, genes controlling cell differentiation, development, growth, and proliferation as well as signal transduction and transcription factor genes that cause EMT and associated processes (Figure III.3). Total RNA of $5x10^5$ sorted SP cells from OE19 or OE19/$5FU_{res}$ was isolated by using miRNeasy kit. cDNA was synthesized from 250 ng of RNA using a RT^2 First start kit. PCR was performed with the RT^2 profiler PCR array system according to the manufacturer's instructions using ABI StepOnePlus™. The expression levels of different mRNAs were normalized using housekeeping genes expression of B2M, HPRT1, GAPDH and ACTB. The expression of Wnt target genes were examined using the RT^2 profiler Wnt signaling targets PCR array (Cat.no. 330231 PAHS-243ZC, SuperArray Bioscience) on 84 key genes responsive to Wnt signal transduction (Figure III.3). Total RNA of $5x10^5$ cells with and without 48h 5 mM aspirin treatment OE19/$5FU_{res}$ were isolated by using miRNeasy kit. Transcription and PCR was performed as EMT array. ACTB B2M, GAPDH, HPRT1 and RPLPO were housekeeping genes in this panel Data was analyzed by Web-Based PCR Array

Data Analysis software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php)

EMT	1	2	3	4	5	6	7	8	9	10	11	12
A	AHNAK	AKT1	BMP1	BMP7	CALD1	CAMK2N1	CAV2	CDH1	CDH2	COL1A2	COL3A1	COL5A2
B	CTNNB1	DSC2	DSP	EGFR	ERBB3	ESR1	F11R	FGFBP1	FN1	FOXC2	FZD7	GNG11
C	GSC	GSK3B	IGFBP4	IL1RN	ILK	ITGA5	ITGAV	ITGB1	JAG1	KRT14	KRT19	KRT7
D	MAP1B	MITF	MMP2	MMP3	MMP9	MSN	MST1R	NODAL	NOTCH1	NUDT13	OCLN	PDGFRB
E	PLEK2	PPPDE2	PTK2	PTP4A1	RAC1	RGS2	SERPINE1	SIP1	SMAD2	SNAI1	SNAI2	SNAI3
F	SOX10	SPARC	SPP1	STAT3	STEAP1	TCF3	TCF4	TFPI2	TGFB1	TGFB2	TGFB3	TIMP1
G	TMEFF1	TMEM132A	TSPAN13	TWIST1	VCAN	VIM	VPS13A	WNT11	WNT5A	WNT5B	ZEB1	ZEB2
H	B2M	HPRT	RPL13A	GAPDH	ACTB	HGDC	RTC	RTC	RTC	PPC	PPC	PPC

WNT	1	2	3	4	5	6	7	8	9	10	11	12
A	ABCB1	AHR	ANGPTL4	ANTXR1	AXIN2	BGLAP	BIRC5	BMP4	BTRC	CACNA2D3	CCND1	CCND2
B	CD44	CDH1	CDKN2A	CDON	CEBPD	CTGF	CUBN	DAB2	DKK1	DLK1	DPP10	EFNB1
C	EGFR	EGR1	ETS2	FGF20	FGF4	FGF7	FGF9	FN1	FOSL1	FST	FZD7	GDF5
D	GDNF	GJA1	ID2	IGF1	IGF2	IL6	IRS1	JAG1	KLF5	LEF1	LRP1	MET
E	MMP2	MMP7	MMP9	MYC	NANOG	NRCAM	NRP1	NTRK2	PDGFRA	PITX2	PLAUR	POU5F1
F	PPAP2B	PPARD	PTCH1	PTGS2	RUNX2	SFRP2	SIX1	SMO	SOX2	SOX9	T	TCF4
G	TCF7	TCF7L1	TCF7L2	TGFB3	TLE1	TWIST1	VEGFA	WISP1	WISP2	WNT3A	WNT5A	WNT9A
H	ACTB	B2M	GAPDH	HPRT1	RPLP0	HGDC	RTC	RTC	RTC	PPC	PPC	PPC

Figure III.3 Panel of genes of EMT and Wnt target PCR array

3.2.2.7 Western blot analysis

Cells were directly lysed in RIPA buffer supplemented with protease/phosphatase inhibitors or sorted into PBS containing 2% fetal bovine serum on ice re-suspended with lysis buffer. Cell lysates were incubated on ice for 10 min and centrifuged at 14000 g at 4°C for 10 min. Protein concentrations were measured using the BCA protein assay. Proteins were then denatured by boiling for 10 minutes. The different proteins (up to 20 µg) were loaded onto sodium dodecyl sulfate-polyacrylamide gels for electrophoresis and then transferred onto nitrocellulose membranes. After blocking with 5% milk in TBST the membranes were incubated with the respective primary antibody according to the manufacturer's instructions at 4°C overnight. After washing with TBST 3 times the membranes were incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 hour. Following another washing procedure with TBST 3 times the detection was performed using the enhanced chemiluminescense system. β-actin was used to ensure equal protein loading.

3.2.2.8 Statistical analysis

Statistical evaluation was referred to the statistical analysis in the part of pancreatic cancer study.

3.3 Experimental setting

3.3.1 Pancreatic cancer

All mice were randomized into the respective experimental groups (as shown in Table III.) and carried out according to the schedule of Figure III.4. Orthotopic model of pancreatic cancer was established as shown in Figure III.5.

Table III.4 Group design of antagomirs therapy of gemcitabine resistant stem like side population cells *in vivo.*

Group	Group name	Cells	Therapy	Cells per Mouse
Group 1	**L3.6pl$_{Gres}$**	**L3.6pl$_{Gres}$**	**No**	**1x10^6**
Group 2	**L3.6pl$_{Gres}$-SP**	**L3.6pl$_{Gres}$-SP**	**No**	**1x10^5**
Group 3	**L3.6pl$_{Gres}$-NSP**	**L3.6pl$_{Gres}$-NSP**	**No**	**1x10^5**
Group 4	**all stars**	**L3.6pl$_{Gres}$-SP**	**All stars**	**1x10^5**
Group 5	**miR-21**	**L3.6pl$_{Gres}$-SP**	**Anti-miR-21**	**1x10^5**
Group 6	**miR-221**	**L3.6pl$_{Gres}$-SP**	**Anti-miR-221**	**1x10^5**
Group 7	**miR-21+221**	**L3.6pl$_{Gres}$-SP**	**Anti-miR-21+221**	**1x10^5**

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

Group 2: Balb/c nu/nu mice injected with SP cells isolated from L3.6pl$_{Gres}$ cells (n=5)

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

Group 4: Balb/c nu/nu mice injected with SP cells isolated from L3.6pl$_{Gres}$ cells and transfected with all stars (n=5)

Group 5: Balb/c nu/nu mice injected with SP cells isolated from L3.6pl$_{Gres}$ cells and transfected with anti-miR-21 (n=5) (one mouse died after operation procedure)

Group 6: Balb/c nu/nu mice injected with SP cells isolated from L3.6pl$_{Gres}$ cells and transfected with anti-miR-221 (n=5)

Group 7: Balb/c nu/nu mice injected with SP cells isolated from L3.6pl$_{Gres}$ cells and transfected with anti-miR-21+221 (n=5)

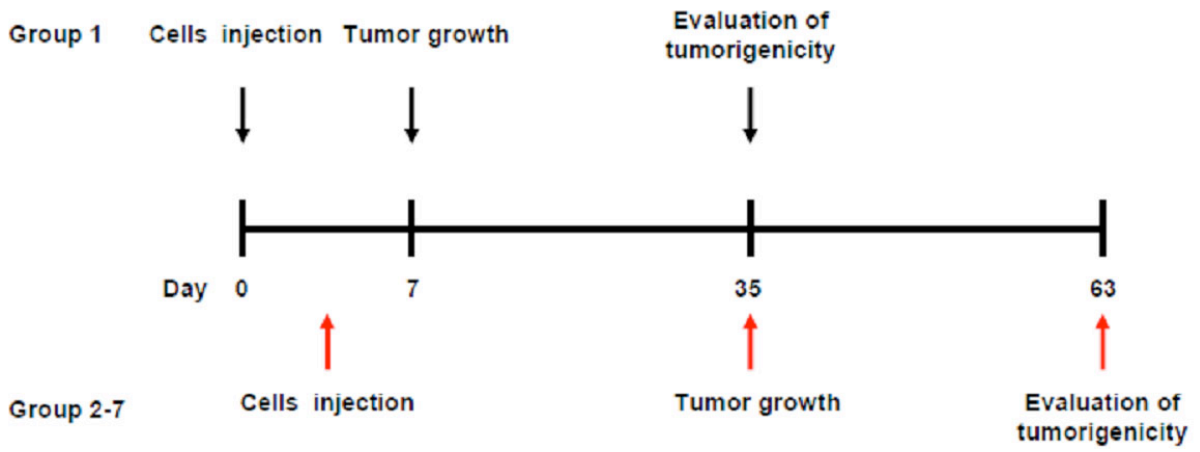

Figure III.4 Experimental schedule for the study of antagomirs therapy. Unsorted L3.6pl$_{Gres}$ grows rapidly after one week of orthotopic injection. Sorting work was sequentially done and transfected during first 3-5 days. Group 1 L3.6pl$_{Gres}$ cells induced tumor growth after 7 days of cell implantation. SP cells induced palpable tumors one month after injection. Tumor growth was monitored twice a week. On day 35, group 1 mice were sacrificed while other mice were sacrificed at 63 days after the injection due to moribund control animals.

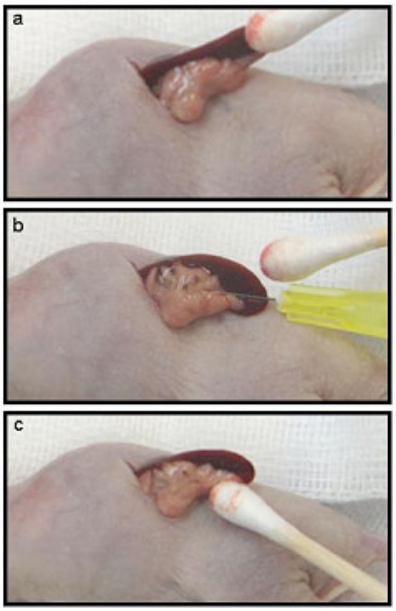

Figure III.5 Orthotopic model of intra-pancreas injection. a) A small left abdominal flank incision was made and the spleen was exteriorized. b) 30-gauge needle, 1 ml disposable syringe, and a calibrated pushbutton-controlled dispensing device were used to inject the tumor cell suspension. c) There should be a fluid bleb without intraperitoneal leakage after a successful sub capsular intrapancreatic injection.

3.3.2 Esophageal cancer

All mice were randomized into the respective experimental groups (as shown below) and the corresponding subcutaneous model of esophageal cancer was established as shown in Figure III.6.

Group design of sorted subpopulation cells from esophageal cancer cell lines

Group 1: Balb/c nu/nu mice injected with SP cells isolated from OE19 (n=3)

Group 2: Balb/c nu/nu mice injected with non-SP cells isolated from OE19 (n=3)

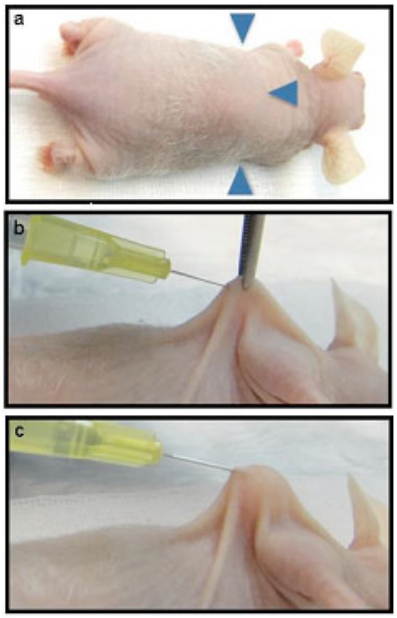

Figure III.6 Subcutaneous model of subpopulation cells. a) A point of right or left or middle in back on the skin of mice was chosen for injection. b) 30-gauge needle, 1 ml disposable syringe were used to inject cell suspension within 100 µl volume. c) There should be a fluid bleb under the skin after a successful subcutaneous injection.

IV. RESULTS

4.1 Cancer stem cell target therapy in pancreatic cancer

4.1.1 Microarray data validation

4.1.1.1 miR-21 and miR-221 are significantly up regulated in SP cells from the pancreatic cancer cell line L3.6pl

MiR-21 is one of the first oncomiRs identified. It is encoded on the human chromosome 17q23.2. MiR-21 has been shown to be overexpressed in hematologic and solid tumors of the lung, breast, stomach, prostate, colon, brain, head and neck, esophagus, and pancreas (Lujambio and Lowe 2012). MiR-221 is highly homologous with miR-222 and is encoded on Xp11.3 of human chromosome. A series of studies have demonstrated the actions of miR-221 as an oncomiR in various human cancers including: glioblastoma, breast, colon, lung, liver, pancreas, prostate, stomach and thyroid papillary cancer (Garofalo et al 2012).

Our previous analysis found a distinct proportion of SP in L3.6pl cells. In an orthotopic xenograft model, L3.6pl-SP cells presented as highly tumorigenic and metastatic compared to non-SP cells (NSP). By microarray expression profiling we identified several dysregulated miRNAs in L3.6pl-SP as compared to NSP cells: Let-7g was 5.2 fold down regulated while miR-221, miR-21, miR-30c-2, and miR-211 were significantly up regulated in L3.6pl-SP cells, in particular, miR-21 and miR-221 displayed a fold change of 6.4 and 10.2, respectively (Table IV.1). Due to the significant fluctuations in levels of miRNAs seen in the cancer samples studied, we choose ultimately to study the role of miR-21 and miR-221 in more advanced models of tumor growth.

RT-PCR results demonstrated significant increased expression of miR-21 with 2.0- and miR-221 at 7.7-fold in L3.6pl-SP as compared to NSP cells ($p<0.05$) (Figure IV.1).

Table IV.1 Mature sequence of human miR-21 and miR-221.

Gene Symbol	Name	Mature Sequence	Microarray Fold Change
MIRN21	MI0000077 Homo sapiens miR-21 stem-loop	5' UAGCUUAUCAGACUGAUGUUGA	6.41
MIRN221	MI0000298 Homo sapiens miR-221 stem-loop	5' AGCUACAUUGUCUGCUGGGUUUC	10.22

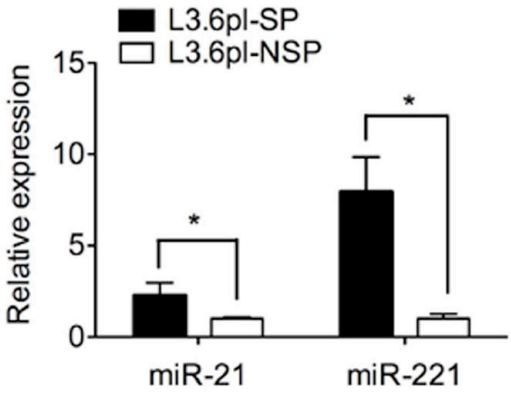

Figure IV.1 Relative expression of miR-21 and miR-221 in SP and NSP from L3.6pl. SP and NSP cells from pancreatic cancer cell line L3.6pl were isolated. RNU6B was used for normalization of expression. miR-21 and -221 are significantly up regulated in SP cells of L3.6pl as compared to NSP cells, *$p<0.05$.

4.1.1.2 miR-21 and miR-221 overexpression is related to gemcitabine resistance

L3.6pl acquired gemcitabine resistance through step-wise exposure of 0.5 ng/ml to 7.5 ng/ml gemcitabine. The IC_{50} increased from 6.1 ng/ml ± 0.9 to 119.8 ng/ml ± 5.1 together with a significant enrichment of SP in $L3.6pl_{Gres}$ (Figure IV.2). A significant enhancement of SP cells was found in $L3.6pl_{Gres}$ cells (0.9% ± 0.2 vs. 5.4% ± 0.8, $p<0.0001$) associated with a 1.8-fold and 2.7-fold increase in the expression of miR-21 and miR-221 in $L3.6pl_{Gres}$ compared to parental L3.6pl cells (both miRNAs, $L3.6pl_{Gres}$ vs. L3.6pl cells, $p<0.05$) (Figure IV.3)

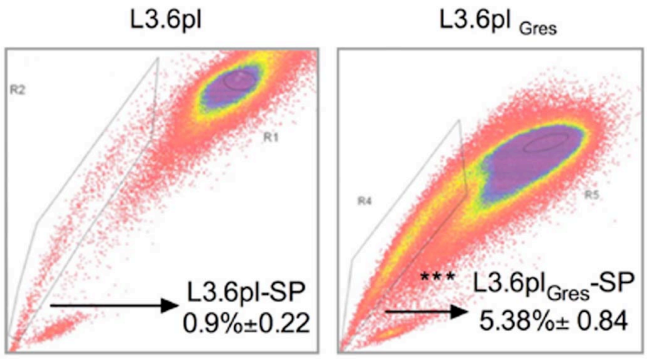

Figure IV.2 Side population proportions in L3.6pl and L3.6pl$_{Gres}$. SP levels were increased from 0.9% ± 0.2 to 5.4% ± 0.8 (***p<0.0001) when L3.6pl acquired enhanced gemcitabine resistance.

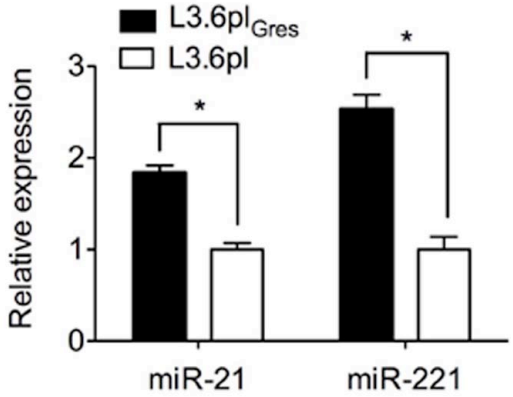

Figure IV.3 miR-21 and -221 relative expressions in L3.6pl and L3.6pl$_{Gres}$. Both miRNAs increased 1.8-fold and 2.7-fold in gemcitabine resistant cells- L3.6pl$_{Gres}$ as compared to parental L3.6pl cells, *p<0.05.

4.1.2 miR-21 and -221 antisense therapies *in vitro*

4.1.2.1 miR-21 and -221 antisense therapies reduced the amount of SP L3.6pl cells significantly

The inhibitory effects of ASOs on their respective target miRNAs were then analyzed. Following transfection of L3.6pl cells with ASOs directed against miR-21, miR-221 or both together, the relative expression of miRNA-21 or -221 was specifically reduced by its corresponding antagomir (antagomirs vs. all stars control, p<0.0005). Combined transfection with ASOs against miR-21 and miR-221 displayed a more than additive effect on miR-221 expression than that seen with monotherapy (combined antagomir therapy vs. each monotherapy, p<0.0001) (Figure IV.4).

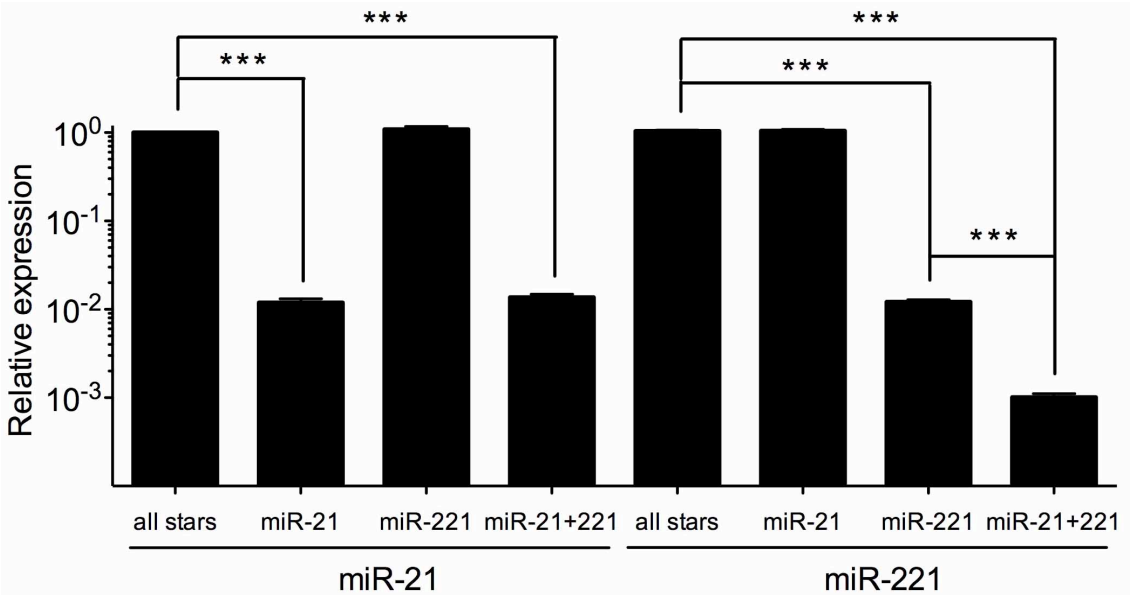

Figure IV.4 The effect of antagomirs on miR-21 and miR-221 expression in L3.6pl. The expression levels of the target miRNA were assessed after transfection. The combination therapy displayed a dramatic synergistic effect on miR-221 expression (antagomirs vs. all stars control, ***$p<0.0005$; combined antagomir therapy vs. each monotherapy, ***$p<0.0001$).

L3.6pl cells were transfected with different antagomirs as described above, followed by Hoechst33342 staining for detection of the SP proportion. Antagomirs against miR-21 slightly reduced the SP content from 5.0% ± 1.8 to 4.7% ± 1.1 while miR-221 substantially decreased to 3.0% ± 0.5, respectively (Figure IV.5). The combination therapy of both antagomirs showed a significant reduction of the SP population (2.1% ± 0.9), as compared to the control group (all stars control vs. anti-miR-21+221, $p=0.034$).

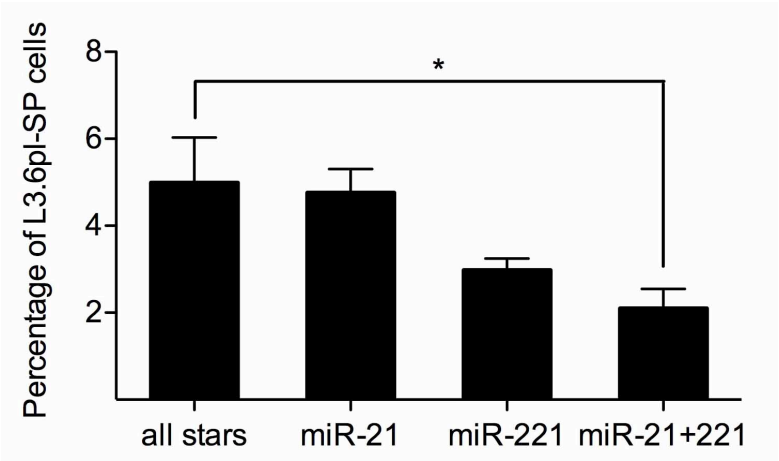

Figure IV.5 Hoechst 33342 based flow cytometric analysis of SP fraction. Cells were treated with control oligos and antisense to miR-21 and -221 with a concentration of 50 nM. Antagomirs against miR-21 or miR-221 reduced the SP content from 5.0% ± 1.8 to 4.7% ±

1.1 and 3.0% ± 0.5, respectively. The combination therapy of both antagomirs showed a significant reduction of the SP proportion (2.1% ± 0.9) compared to the control group (all stars control vs. anti-miR-21+221, *p<0.05).

4.1.2.2 The miR-21 and miR-221 antisense therapies inhibited L3.6pl proliferation

Cytotoxicity of the antagomirs was studied by MTT assay with 48 hours continuous exposure of miR-21 and miR-221 ASOs treatment. Monotherapy with anti-miR-21 did not show a significant reduction in cell viability compared to control oligos in L3.6pl cells while treatment with anti-miR-221 partially inhibited cell viability down to 85.5% ± 21.2. Only the combined ASOs therapy had a significant impact on cell viability, which is from 114.2% ± 31.5 to 53.3% ± 15.2 (all stars control vs. anti-miR-21 + 221, p=0.013) (Figure IV.6).

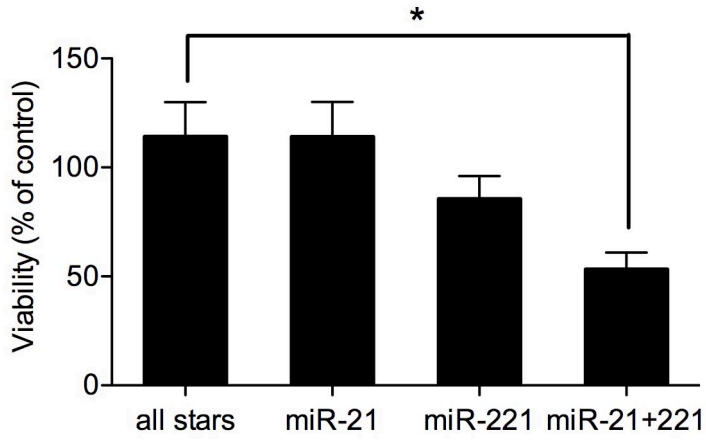

Figure IV.6 Effect of inhibition of miR-21 and miR-221 on cell proliferation. Cells were treated with all stars control oligos and antagomirs against miR-21 and -221 at a concentration of 50nM for the duration of 48 hours. Cell viability was detected by MTT assay. Only the combined ASOs therapy led to a significant reduction of cell viability (53.3% ± 15.2) as compared to all stars control oligos (114.2% ± 31.5) (all stars control vs. anti-miR-21 + 221, *p<0.05).

4.1.2.3 Effect of miRNA inhibition on apoptosis

The effect of anti-miR-21 and/or anti-miR-221 therapy on apoptosis of L3.6pl cells in the subG0/G1 phase of cell cycle was examined. The apoptosis rate under all stars, anti-miR-21, anti-miR-221 and anti-miR-21+221 are 6.4% ± 2.3, 9.1% ± 5.8, 18.5% ± 8.4 and 36.4% ± 13.7, respectively. Treatment with anti-miR-221 alone or in combination with anti-miR-21 resulted in a significantly higher amount of cells in subG0/G1 phase (all stars vs. anti-miR-

221, p=0.015; all stars vs. anti-miR-21+221, p=0.0018) (Figure IV.7).

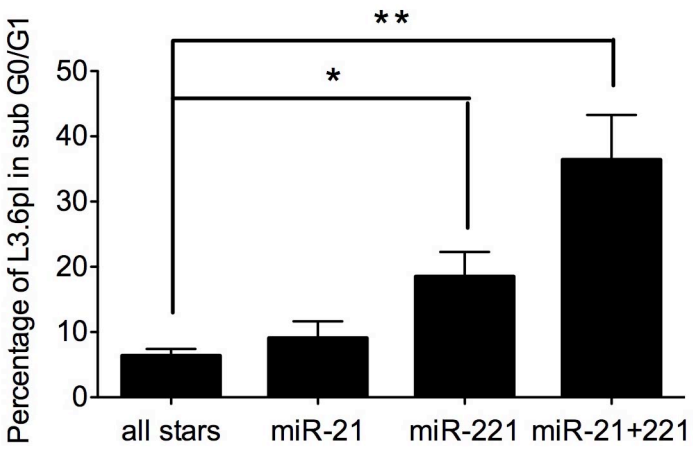

Figure IV.7 Effect of inhibition of miR.21 and miR-221 on cell apoptosis. The percentage of apoptotic cells following transfection with all stars control, anti-miR-21, anti-miR-221, and anti-miR-21+221 are 6.4% ± 2.3, 9.1% ± 5.8, 18.5% ± 8.4 and 36.4% ± 13.7, respectively. Treatment with anti-miR-221 alone or in combination with anti-miR-21 resulted in a significantly higher amount of cells in sub G0/G1 phase (all stars control vs. anti-miR-221, *p<0.05; all stars control vs. anti-miR-21+221, **p<0.005).

4.1.2.4 The miR-21 and miR-221 antisense therapy reduced the migration of L3.6pl cells in a Boyden chamber model

Our previous data indicated a higher metastatic potential of L3.6pl-SP cells as compared to NSP cells in an orthotopic nude mouse model (data submitted). The general migratory potential of treated cells was then evaluated. Transfection of L3.6pl cells with anti-miR-221 and anti-miR-21 ASOs therapy led to a strong reduction of cell migration in a Boyden chamber assay with OD value from 1.7 ± 0.3 to 0.2 ± 0.3 and 1.0 ± 0.5, respectively, as compared to transfection with all stars control oligos. Notably, the combined transfection of anti-miR-21 and anti-miR-221 ASOs completely abolished cell migration (OD=0.02 ± 0.02). In addition, anti-miR-21 had a significantly stronger inhibiting effect on cell migration than anti-miR-221 (Figure IV.8).

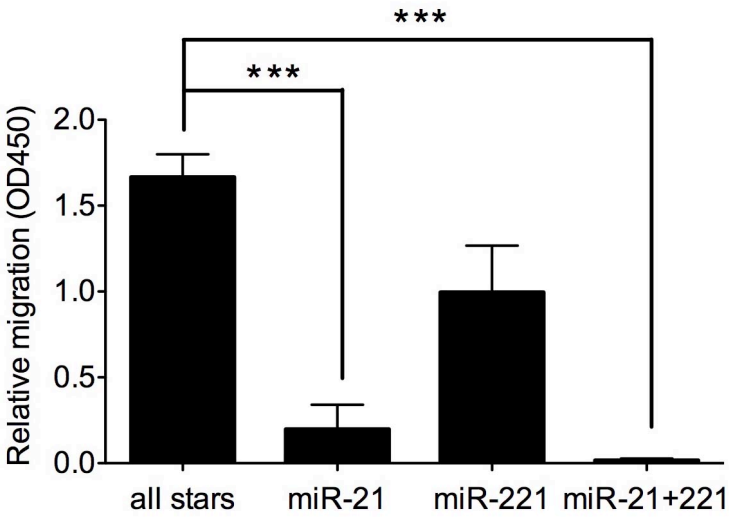

Figure IV.8 Anti-miR-21 and -221 suppressed the transmigration of L3.6pl by Boyden chamber assay. Transfection with anti-miR-21 and anti-miR-221 ASOs led to a reduction of cell migration in a Boyden chamber assay from 1.7 ± 0.3 to 0.2 ± 0.3 and 1.0 ± 0.5, respectively, as compared to transfection with all stars control oligos. Notably, the combined transfection with anti-miR-21 and anti-miR-221 ASOs completely abolished cell migration (OD=0.02 ± 0.02) (all stars control vs. anti-miR-21, ***$p<0.0005$; all stars control vs. anti-miR-21+221, ***$p< 0.0001$). In addition, anti-miR-21 had a significantly stronger inhibiting effect on cell migration than anti-miR-221.

4.1.2.5 The miR-21 and miR-221 antisense therapy sensitized L3.6pl cells towards gemcitabine or 5-FU

To examine the effects of ASOs on chemotherapy resistance, L3.6pl cells were again transfected with ASOs against miR-21 and miR-221 as pre-treatment and followed by administration of gemcitabine or 5-FU at the IC_{50} concentration for 48 hours. The OD value following all stars control oligos transfection decreased from 1.4 ± 0.2 to 0.8 ± 0.1 or 0.7 ± 0.1 with gemcitabine (IC_{50} =6.1 ng/ml ± 0.9) or 5-FU (IC_{50} =10 μg/ml ± 2) treatment. When combined with anti-miR-21+221, relative cell proliferation after gemcitabine treatment was 0.5 ± 0.2 ($p=0.037$), while after 5-FU treatment was 0.2 ± 0.3 ($p=0.030$) (Figure IV.9). These results suggest that suppression of miR-21 and miR-221 combination can sensitize tumor cells to anticancer agents.

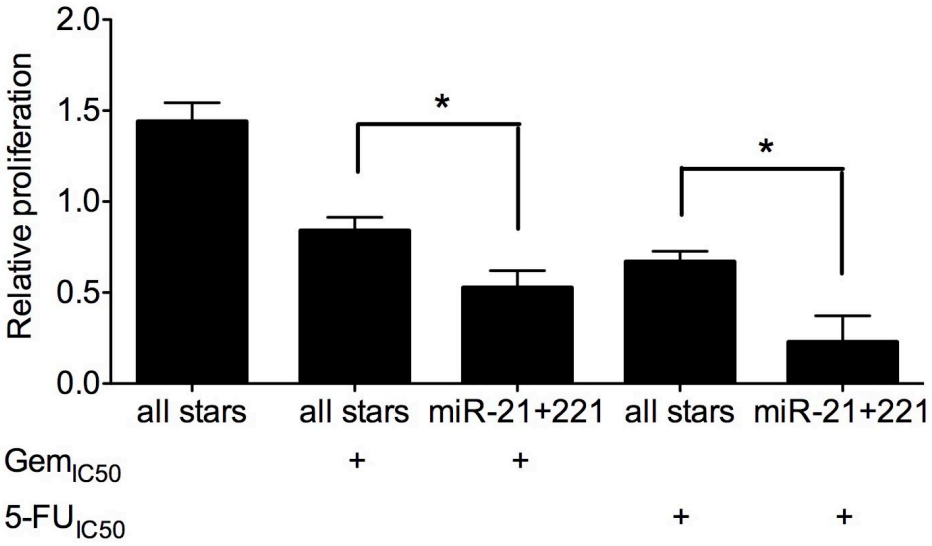

Figure IV.9 Antisense pre-treatment sensitized the anti-proliferative effect of gemcitabine and 5-FU. The anti-proliferative effect was measured 48 hours after application of chemotherapy. The OD value after transfection with all stars control oligos decreased from 1.4 ± 0.2 to 0.8 ± 0.1 or 0.7 ± 0.1 following gemcitabine or 5-FU treatment, respectively. When combined with anti-miR-21+221 ASOs therapy, the measured OD values after treatment with gemcitabine and 5-FU were 0.5 ± 0.2 and 0.2 ± 0.3, *$p<0.05$, respectively.

4.1.2.6 MiR-21 and miR-221 regulate a cohort of putative tumorigenic genes

To further validate the molecular effects of miR-21 and -221, we analyzed the expression of putative target genes of these miRNAs. Both miR-21 and 221 are involved in the expression of more than 200 genes, including CDK6, C5ORF41, EFNA1, IRAK3, KLF12, MAPK10, NRP1, SMAD7, SOCS6 and ZBTB41. To determine whether down regulation of both oncomirs could affect putative target genes L3.6pl cells were transfected with antagomirs. The expression of CDK6, KLF12, MAPK10, and C5ORF41 was significantly increased at the mRNA level after transfection (Figure IV.10). There is no statistic significance in the expression of NRP1 and SMAD7 after either mono- or combined antagomirs transfection. However, EFNA1 and ZBTB41 displayed a relatively down regulation under antisense therapy. Interestingly, mono-therapy with each antagomir did not lead to a re-expression of the target gene IRAK3, however, the transfection with both antagomirs together displayed a dramatic overexpression of IRAK3. All genes are putative targets of miR-21 or miR-221 and play an important role in cancer related biological processes according to their functions in the NCBI gene bank.

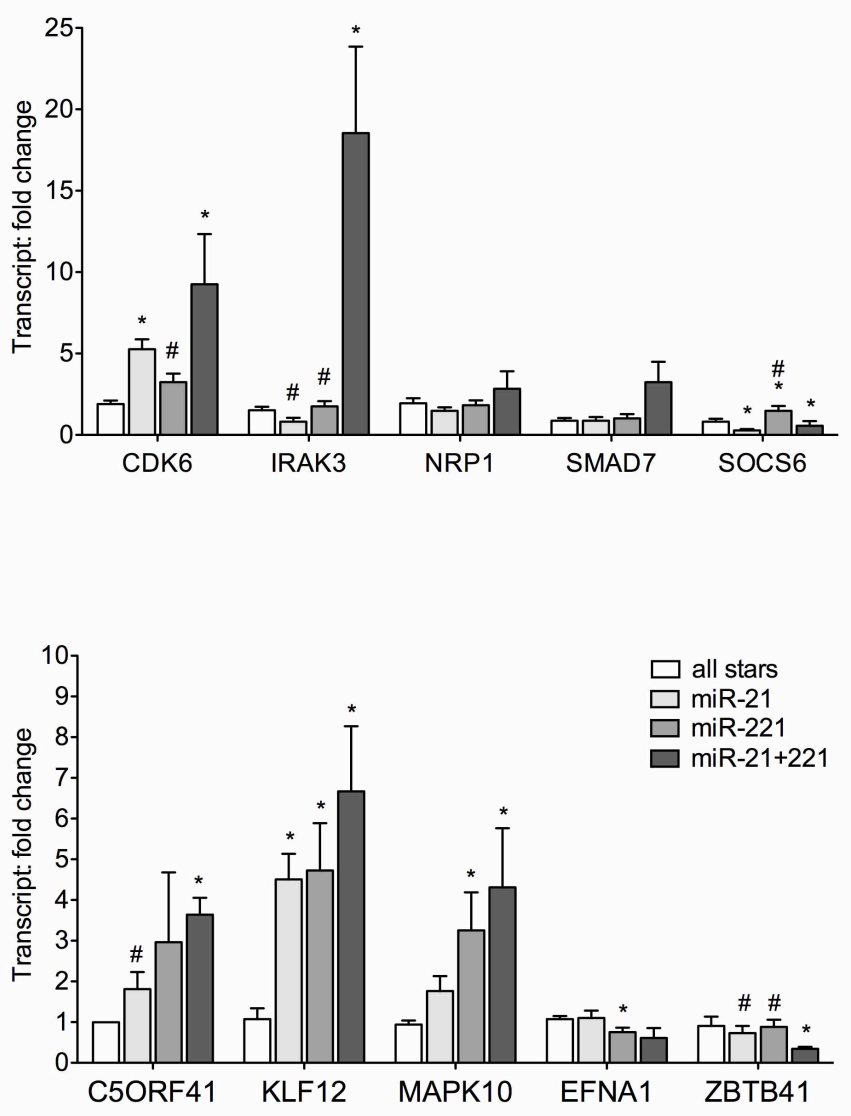

Figure IV.10 Quantification analysis of the putative target gene expression with antagomirs transfection. L3.6pl cells were transfected by antagomirs and detected by SYBR® Green PCR methods. GAPDH was used as housekeeping gene control. The expression of CDK6, KLF12, MAPK10, and C5ORF41 was significantly increased at the mRNA level after transfection. Interestingly, mono-therapy with each antagomir did not lead to a re-expression of the target gene IRAK3, while the combined treatment with both antagomirs displayed a dramatic overexpression of IRAK3 (antagomirs vs. all stars control, *$p<0.05$; combined antagomir therapy vs. all stars control, #$p<0.05$).

4.1.3 Overexpression of miR-21 and -221 in pancreatic adenocarcinoma tumors

To further demonstrate the clinical relevance of miR-21 and -221 in pancreatic cancer, the relative expression of both miRNAs was detected by qRT-PCR in paired human tumor and adjacent normal tissue samples. The expression of both miRNAs was further correlated to demographic and histopathological parameters of the patient collective (Higher amount of

miRNA in tumor tissue was defined as high level of this miRNA while lower amount in tumor tissue was defined as low level, as compared to adjacent normal pancreas, Table IV.2). The expression of miR-21 and -221 was significantly higher in tumor tissues compared to corresponding adjacent normal tissues in all paired tissue samples (miR-21, p=0.012; miR-221, p=0.017) (Figure IV.11). Due to the small sample size, there was no statistically significant correlation between the tumor staging, differentiation, metastasis and miRNA levels in patients.

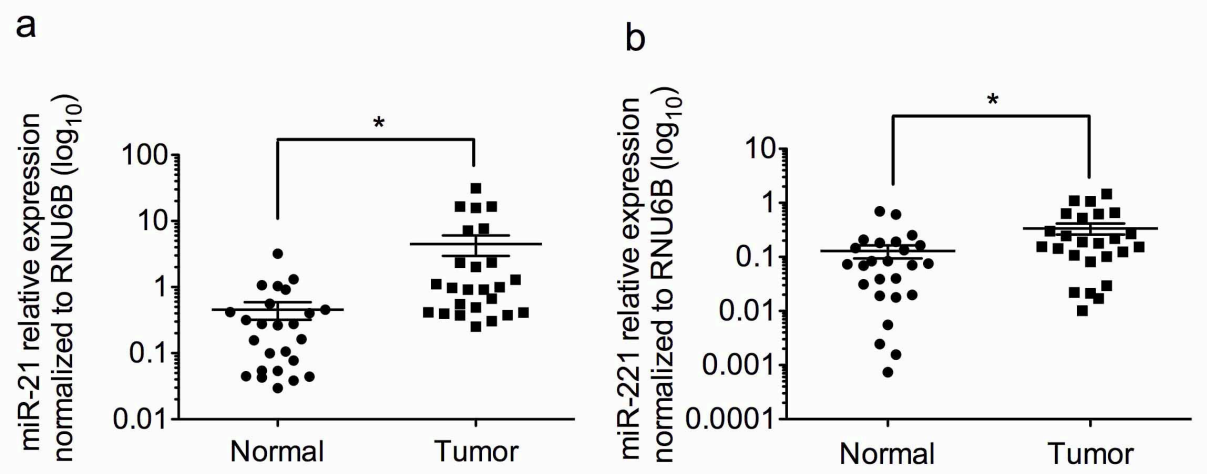

Figure IV.11 Comparison of miR-21 and miR-221 expression in 28 paired tumor and normal tissues by qRT-PCR. RNU6B as control of normalization of expression. miR-21 and miR-221 was significantly up-regulated in tumor tissues comparing to the corresponding normal tissues of pancreatic adenocarcinoma patients, *p< 0.05.

Table IV.2 Association of miR-21 and miR-221 expression with clinic pathologic covariates

Characteristics	Total		miR-21 expression				P value	miR-221 expression				P value
			High level		Low level			High level		Low level		
	N=28		N=20	71.4%	N=8	28.6%		N=20	71.4%	N=8	28.6%	
Age												
> 60 years	22	78.6%	16	80.0%	6	75.0%		17	85.0%	5	62.5%	
< 60 years	6	21.4%	4	20.0%	2	25.0%	1.000	3	15.0%	3	37.5%	0.311
Gender												
Male	17	60.7%	11	55.0%	6	75.0%		11	55.0%	6	75.0%	
Female	11	39.3%	9	45.0%	2	25.0%	0.419	9	45.0%	2	25.0%	0.419
Histological Differentiation												
G1-G2	10	35.7%	8	40.0%	2	25.0%		7	35.0%	3	37.5%	
G3-G4	18	64.3%	12	60.0%	6	75.0%	0.669	13	65.0%	5	62.5%	1.000
TNM stage												
pT												
pT1	1	3.6%	0	0.0%	1	12.5%		0	0.0%	1	12.5%	
pT2	2	7.1%	2	10.0%	0	0.0%		1	5.0%	1	12.5%	
pT3	20	71.4%	15	75.0%	5	62.5%		15	75.0%	5	62.5%	
pT4	5	17.9%	3	15.0%	2	25.0%	0.290	4	20.0%	1	12.5%	0.354
pN												
pN0	13	46.4%	10	50.0%	3	37.5%		9	45.0%	4	50.0%	
pN1	15	53.6%	10	50.0%	5	62.5%	0.686	11	55.0%	4	50.0%	1.000
pM												
pM0	26	92.9%	19	95.0%	7	87.5%		18	90.0%	8	100.0%	
pM1	2	7.1%	1	5.0%	1	12.5%	0.497	2	10.0%	0	0.0%	1.000
Lymphatic invasion												
Negative	13	46.4%	10	50.0%	3	37.5%		9	45.0%	4	50.0%	
Positive(<20%)	8	28.6%	5	25.0%	3	37.5%		5	25.0%	3	37.5%	
Positive(>20%)	7	25.0%	5	25.0%	2	25.0%	0.777	6	30.0%	1	12.5%	0.594

*** Fisher's Exact Test**
Pearson Chi-Square

4.1.4 The miR-21 and -221 antisense mono and combined therapies *in vivo*

The anti-tumor effect of antagomir therapy has been previously evaluated in several cancer models including breast cancer and liver cancer, but as of yet, not extensively in pancreatic cancer. Our results suggest that miR-21 and miR-221 expression may represent a potential target for pancreatic tumor therapy *in vitro*. We then sought to extend our observations to *in vivo* models. To this aim, we transiently transfected resistant side population cells isolated from $L3.6pl_{Gres}$ with anti-miR-21 or anti-miR-221, either alone or in combination or with control negative all stars. The viability of the cells after sorting and transfection was examined by trypan blue staining. The transfection efficiency was found to range from 85% to 95%. $L3.6pl_{Gres}$ whole population, non-transfected SP and NSP sub population cells, or the four test groups of transfected SP cells were orthotopically injected into the pancreas of nude mice.

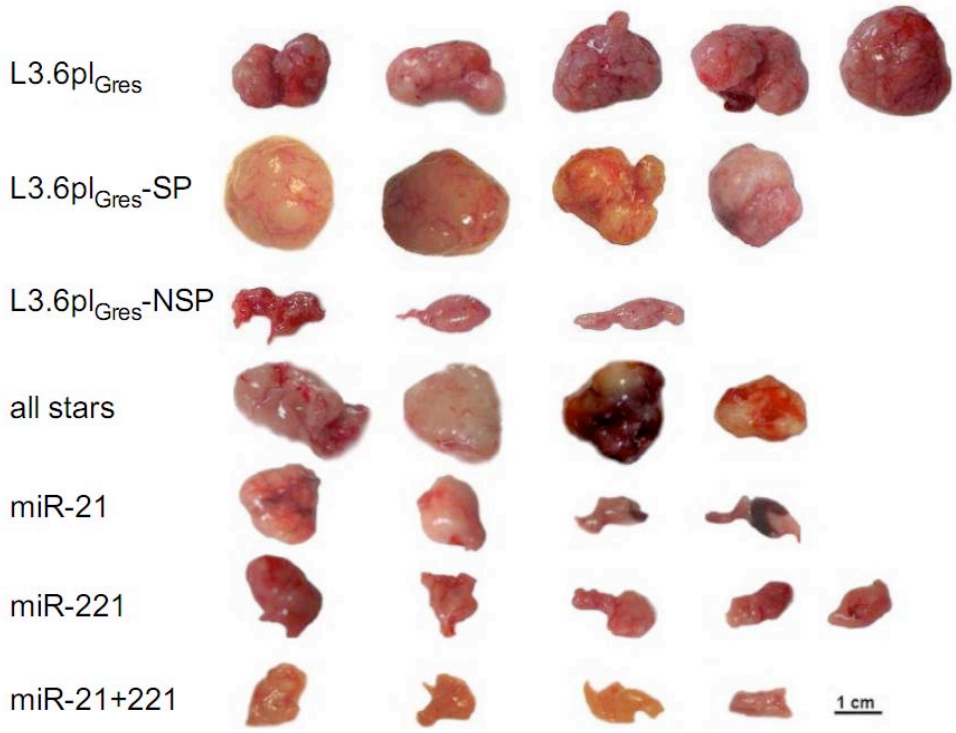

Figure IV.12 Antagomirs therapy suppresses tumorigenicity of stem like SP cells from L3.6pl$_{Gres}$ *in vivo*. Effect of antisense inhibition on tumor formation ability in pre-treated xenograft model. Tumor size was documented twice a week. All stars displayed no significant difference on tumorigenicity as compared to no transfected control. Anti-miR221 showed better effect and the combination therapy show a best inhibition on tumor growth.

Table IV.3 Tumor weight of mice under different antagomirs therapies

Group	L3.6pl$_{Gres}$	L3.6pl$_{Gres}$-SP	all stars	miR-21	miR-221	miR-21+221	L3.6pl$_{Gres}$-NSP
Tumor weight (g)	3.0 ± 1.5	3.4 ± 0.6	2.3 ± 0.6	1.0 ± 1.0	0.3 ± 0.2	0.2 ± 0.02	0.2 ± 0.08
p value	0.67[a]	-	0.034[a]	0.088[b]	0.0002[b]	<0.0001[b]	0.0003[a]

Note: Tumor weight = Mean ± SD; [a] compared with SP group; [b] compared with all stars group

As shown in Table IV.3, there was no significant difference in tumor weight of primary tumors generated from 1x10^6 L3.6pl$_{Gres}$ whole population cell compared to 1x10^5 L3.6pl$_{Gres}$-SP cells (3.0 g ± 1.5 vs. 3.4 g ± 0.6, p=0.67), demonstrating high tumor initiating capacity of SP cells *in vivo*. Pancreatic tissues (including tumors) from 1x10^5 L3.6pl$_{Gres}$-NSP cells were significantly smaller than tumors generated from 1x10^6 L3.6pl$_{Gres}$ whole population cells or 1x10^5 L3.6pl$_{Gres}$-SP cells (L3.6pl$_{Gres}$-SP vs. L3.6pl$_{Gres}$-NSP: 3.4 g ± 0.6 vs. 0.2 g ± 0.1, p=0.0003). In addition, the metastatic activity of 1x10^5 L3.6pl$_{Gres}$-SP cells was found to be relatively equivalent to 1x10^6 L3.6pl$_{Gres}$ whole population cells. Figure IV.12 and Figure IV.13 displayed a significant effect of antisense therapy against miR-21 and miR-221 in

L3.6pl$_{Gres}$-SP cells, in particular, when used as combined treatment, resulting in a significant reduction of primary pancreatic tumor growth in a nude mouse model (all stars control vs. anti-miR-21, p=0.088; all stars control vs. anti-miR-221, p=0.0002; all stars control vs. anti-miR-21+221, p<0.0001). Furthermore, anti-miR-221 as well as the combined therapy led to abolishment of detectable liver and lymph node metastases (Table IV.4).

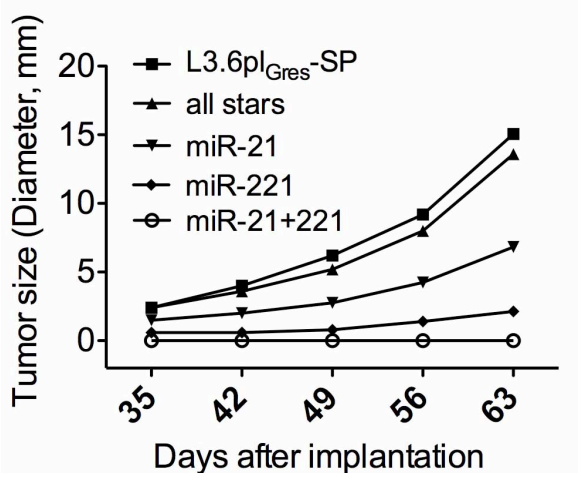

Figure IV.13 Tumor growth curves measured after injection of SP cells transfected with either all stars control or antagomirs. The tumor size was detected as the longer diameter.

Table IV.4 The incidence of primary pancreatic tumors as well as metastatic spreads.

Group	L3.6pl$_{Gres}$	L3.6pl$_{Gres}$-SP	all stars	miR-21	miR-221	miR-21+221	L3.6pl$_{Gres}$-NSP
Primary Tumor	5/5	4/5	4/5	2/4	1/5	0/5	0/3
Metastasis	3/5	3/5	2/5	1/4	0/5	0/5	0/3

4.1.5 *Ex vivo* analysis of tumor proliferation, apoptosis and angiogenesis under target therapy *in vivo*

In addition to histomorphological alterations, we also evaluated the effects of antagomir therapy using immunohistochemical staining for anti-Ki67, TUNEL, and anti-CD31 to assess effects on tumor cell proliferation, apoptosis, and angiogenesis *ex vivo*. Representative fields are shown in Figure IV.14. Pancreatic tumors generated from L3.6pl$_{Gres}$-SP cells or L3.6pl$_{Gres}$-SP cells transfected with all stars control oligos displayed comparably higher Ki67 positivity in the tumor tissue (equivalent to pancreatic tumors generated from L3.6pl$_{Gres}$ whole population cells) while tumors following ASOs therapy showed a significant reduced cell

proliferation. Data were shown in Figure IV.15. In addition, TUNEL assay showed increased apoptosis in pancreatic tumors treated with anti-miR-21, or anti-miR-221, as compared to all stars control oligos (Figure IV.16). Finally, CD31 positive structures indicating MVD were found to be decreased following ASOs therapy, in particular after anti-miR-221 treatment (Figure IV.17). Our results demonstrated that ASOs therapy of pancreatic tumors generated from L3.6pl$_{Gres}$-SP cells showed reduced tumor growth by inhibiting cell proliferation and angiogenesis and by promoting tumor cell apoptosis *in vivo*. Histomorphological and immunohistochemical tissue analysis of tumors generated from L3.6pl$_{Gres}$ whole population versus L3.6pl$_{Gres}$-SP cells showed no significant difference in the Ki67 and TUNEL index or MVD (Ki67: L3.6pl$_{Gres}$ vs. L3.6pl$_{Gres}$-SP, p=0.35; TUNEL: L3.6pl$_{Gres}$ vs. L3.6pl$_{Gres}$-SP, p=0.21; L3.6pl$_{Gres}$ vs. L3.6pl$_{Gres}$-SP, p=0.12).

These results demonstrate that treatment of gemcitabine resistant SP cells from L3.6pl$_{Gres}$ with antagomirs, especially in combined therapy, abolished tumor growth by inhibiting proliferation, angiogenesis and promoted apoptosis.

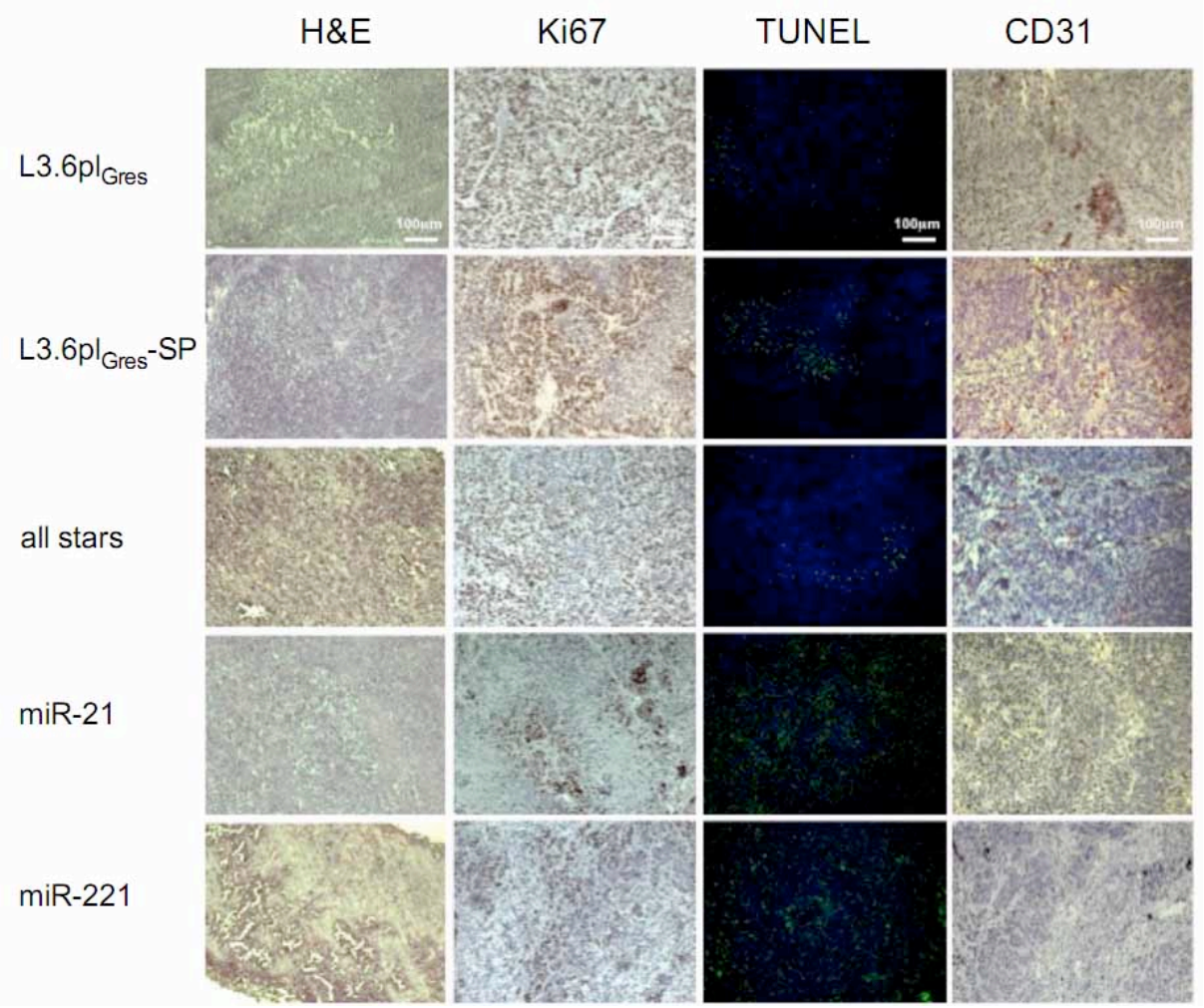

Figure IV.14 Histomorphological and immunohistochemical tissues analysis of cell proliferation, apoptosis and angiogenesis using H& E, Ki67, TUNEL and CD31 staining.

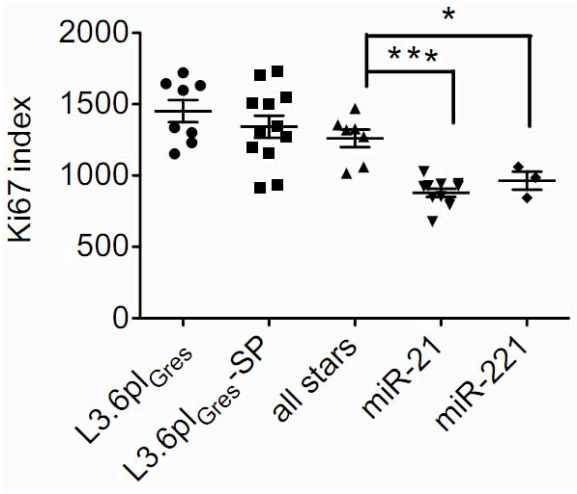

Figure IV.15 Antisense therapy caused inhibition of L3.6pl$_{Gres}$-SP tumor cells proliferation *in vivo*. L3.6pl$_{Gres}$-SP vs. all stars control, p=0.47; all stars control vs. miR-21, ***p<0.0001; all stars control vs. miR-221, *p<0.05.

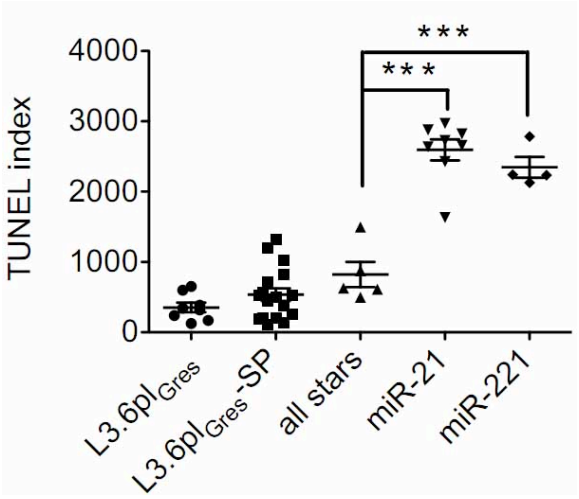

Figure IV.16 Antisense therapy caused apoptosis of L3.6pl$_{Gres}$-SP tumor cells *in vivo*. L3.6pl$_{Gres}$-SP vs. all stars control, p=0.15; all stars control vs. miR-21, ***p<0.0001; all stars control vs. miR-221, ***p<0.0005.

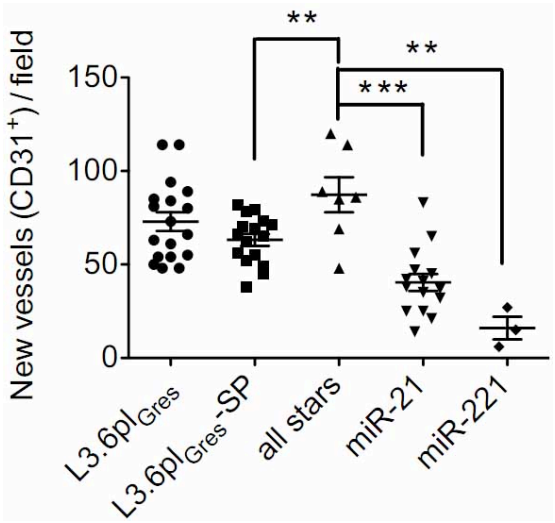

Figure IV.17 Antisense therapy caused reduction of MVD in L3.6pl$_{Gres}$-SP tumor cells *in vivo*, in particular following therapy with anti-miR-221. All stars control vs. miR-21, ***p<0.0001; all stars control vs. miR-221, **p<0.005; L3.6pl$_{Gres}$-SP vs. all stars control, **p<0.005.

4.2 Characterization and target therapy of cancer stem cells in esophageal cancer

4.2.1 Identification of stem like side population in esophageal cancer cell lines

In parallel with the pancreatic cancer studies detailed above, a general analysis of the biology of SP cells in human esophageal cancer was initiated.

4.2.1.1 Identification of side population cells in human esophageal carcinoma cell lines

Hoechst 33342 dye staining of esophageal cell lines showed varying levels of SP cells in the different esophageal cancer cell lines examined. These were 17.1% ± 3.0, 0.6% ± 0.3 and 8.8% ± 2.7 in OE19, OE21, and OE33, respectively. The proportion of SP cells was significantly diminished to 0.34% ± 0.36, 0.10% ± 0.14, and 0.51% ± 0.43 in responses to verapamil treatment (p=0.013, 0.041, and 0.038) (Figure IV.18). Interestingly, PT1590 and LN1590 cells showed no detectable amount of SP cells based on the standard protocol.

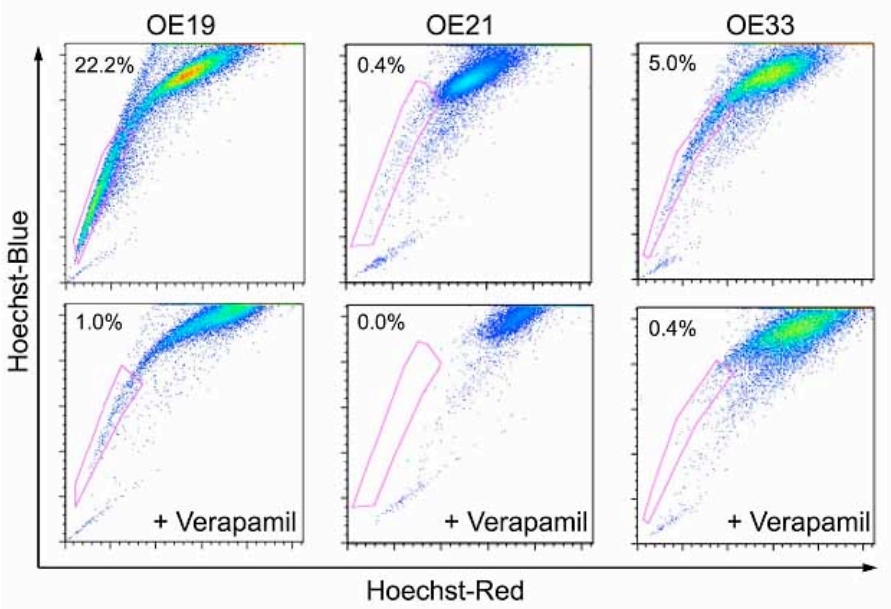

Figure IV.18 Side population cells exist in different esophageal cancer cell lines. OE19, OE21, and OE33 cells were stained with 2.5 µg/ml Hoechst 33342 and analyzed by FACS. After blocking the ABC transporters with 225 µM verapamil, the SP fraction was significant reduced in OE19 and OE33 and vanished totally in OE21.

4.2.1.2 Side population cells are able to self-renew and differentiate *in vitro*

To explore the clonogenic ability of SP and non-SP cells *in vitro*, colony formation assays

were performed. SP cells from OE19, OE21, and OE33 formed more colonies than non-SP cells (Figure IV.19). In soft agar assays, we found that OE19-SP cells were found to generate visible spheres while OE19-non-SP did not (Figure IV.20).

Isolated SP and non-SP cells from OE19 were cultured separately under the same conditions for 1-3 weeks followed by Hoechst 33342-dye re-analysis. SP cells were able to generate extremely higher amount of SP (50.9 % of whole cell population) and lower amount of non-SP cells. The proportion of the second-generation SP cells was reduced under verapamil treatment but displayed a relative resistant to verapamil as compared to first sorting SP cells. By contrast, OE19-NSP cells generated only a proportion of 0.86% SP cells and completely blocked by verapamil (shown in Figure IV.21).

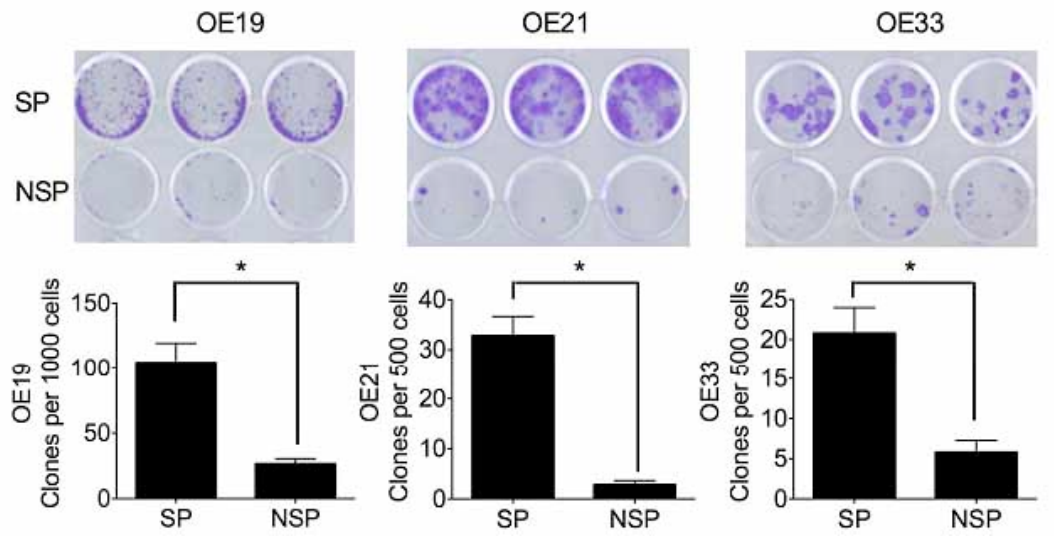

Figure IV.19 Side population cells are able to self-renew *in vitro*. SP cells in OE19, OE21, and OE33 possessed significantly higher clonogenic capacities compared to non-SP cells *in vitro* (*p<0.05). Exemplary pictures of each group are shown in the upper row.

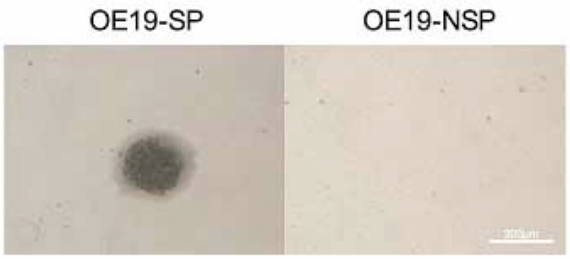

Figure IV.20 Side population cells are able to self-renew in an anchorage independent way. OE19-SP cells were able to grow in an anchorage independent manner (right) in a soft agar assay (100× magnification; scale bar=200 µm).

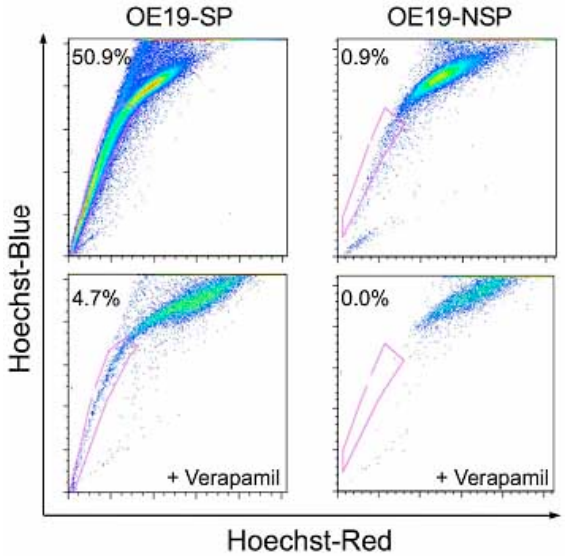

Figure IV.21 Side population cells are able to differentiate *in vitro*. OE19-SP and non-SP cells were isolated and cultured separately for 1 week in RPMI medium with 2% FCS. Cells

derived from the SP proportion showed re-population with SP content of 50.9%. Cells derived from non-SP cells showed a markedly lower re-population rate with a substantially lower content of SP (0.86%), which could be totally diminished after verapamil treatment. In OE33 cells, similar results were obtained (data not shown).

4.2.1.3 Side population cells show higher tumorigenicity *in vivo*

OE19 tumor xenografts were established by subcutaneous injection of 1.0×10^5 SP or non-SP cells on the back of Balb/c nu/nu mice. At 63 days after tumor cell injection, all animals were sacrificed. The weight of the subcutaneous tumors originating from SP cells was significantly higher compared to subcutaneous tumors from non-SP cells (1.0 g ± 0.4 vs. 0.2 g ± 0.3, p=0.04), Figure IV.22).

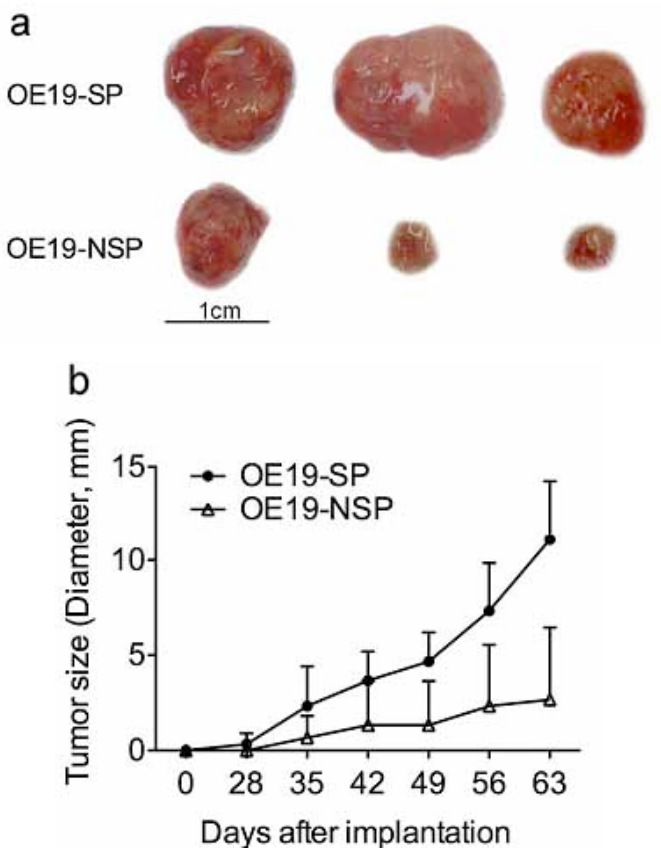

Figure IV.22 Side population cells are more tumorigenic *in vivo*. a) SP or NSP cells were injected into the right flank of the nude mice. Tumor growth was monitored until in 2 months after implantation. SP cells generated significantly larger tumors than NSP cells. SP cell tumor measured 12.0, 13.7, and 7.0 mm (mean of length, width and height) in diameter while NSP cell tumors measured 7.0, 1.0, and 0.0 mm, respectively. SP tumors displayed significantly higher tumor weight than NSP tumors (1.0 g ± 0.4 vs. 0.2 g ± 0.3, *p<0.05) b) Following subcutaneous injection of SP cells *in vivo* tumor growth started 4 weeks later, the diameter was measured and the tumor growth curve was calculated every week. Animals were

sacrificed when the largest tumors reached about 15 mm in diameter.

H&E staining was conducted to ensure that each mass was a tumor and not due to a connective tissue artifacts. Pathology results confirmed that the tumors formed by SP cells were typical human EAC cells similar to those seen using unsorted OE19 cells. The tumors grew as a neoplastic columnar-type epithelium with multiple mitotic figures and mucus production (Figure IV.23).

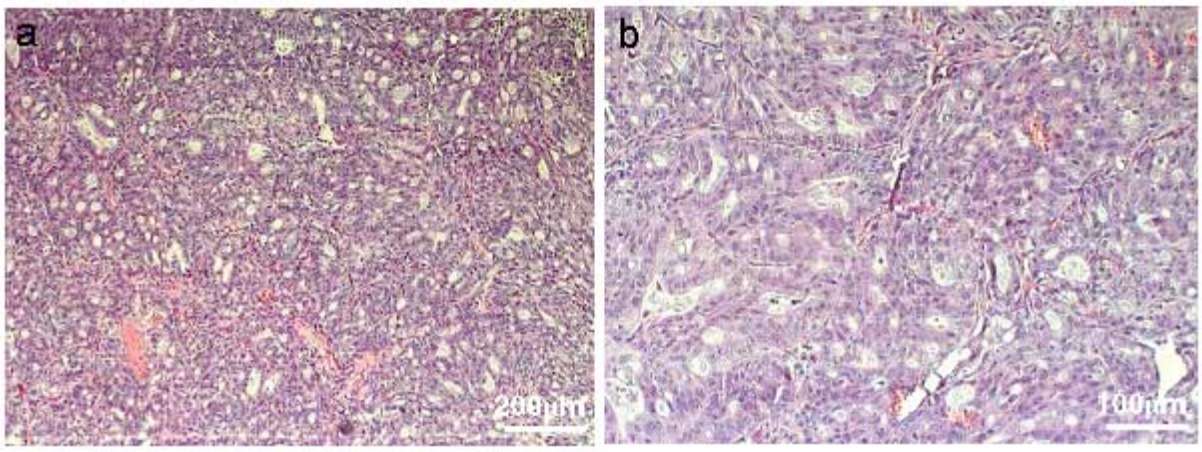

Figure IV.23 H&E staining showed the histological characteristics of OE19-SP formed esophageal adenocarcinoma. a) 100× magnification, scale bar=200 μm. b) 200× magnification, scale bar=100 μm.

4.2.1.4 Intrinsic chemotherapy resistance of SP cells

As discussed above, SP cells often possess resistance to chemotherapy. After sorting, OE19-SP and non-SP cells were plated for 24 hours in tissue culture flasks. Following primary attachment, the cells were harvested and re-plated in 96-well plates for treatment with 5-FU and cisplatin over 48 hours. Following treatment with 5ug/ml 5-FU the cell viability was 45.1% ± 5.1 of SP versus 33.0% ± 2.5 for non-SP cells. Treatment with 5ug/ml cisplatin led to a cell viability of 44.4% ± 6.3 of SP versus 30.8% ± 5.5 of non-SP cells (Figure IV.24).

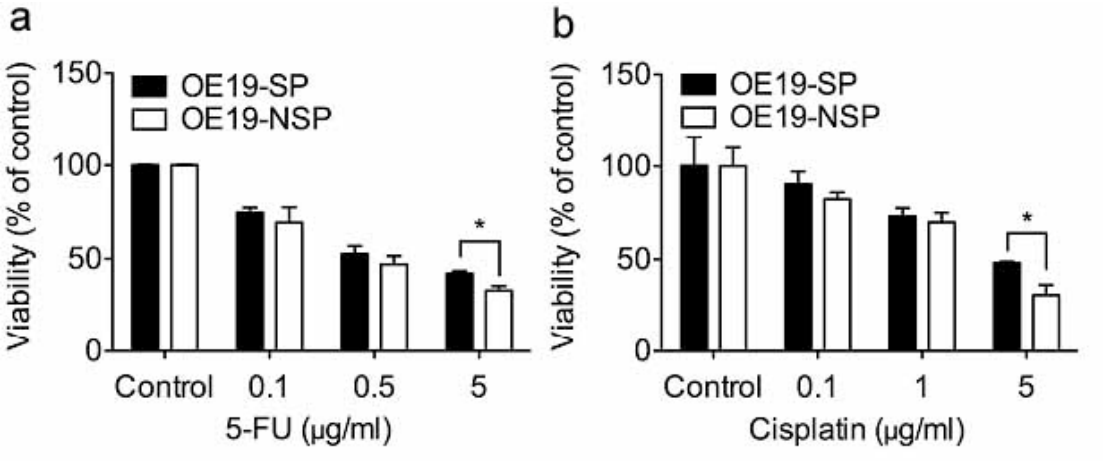

Figure IV.24 Side population cells are chemoresistant. OE19 SP cells were more resistant to chemotherapeutic drugs such as a) 5-Fluorouracil and b) cisplatin than corresponding non-SP cells. Following treatment with 5 µg/ml 5-FU cell viability was 42.3% ± 1.3 and 33% ± 2.5 in SP and non-SP cells, respectively. Treatment with 5 µg/ml cisplatin led to a viability of 48% ± 0.81 and 30.8% ± 5.5 in SP and non-SP cells, respectively (*$p<0.05$).

4.2.1.5 Side population cells show higher expression of cancer stem cell associated markers

OE19-SP cells demonstrated significantly higher expression of ABCG2 and CD44 than that seen in OE19-non-SP cells (Figure IV.25a). In addition, FACS results displayed higher expression of active β-catenin displayed by SP cells (24.4%) than seen in NSP cells (6.6%) (Figure IV.25b). Interestingly, we could not detect a clear $CD133^{+}$ subpopulation either in OE19-SP cells, OE19-non-SP cells or in the whole cell population of OE19 (<0.2%) (Data were not shown). Fluorescent staining supported this observation showing a stronger staining of ABCG2 and CD44 in OE19-SP as compared to OE19-non-SP cells (Figure IV.26). β-catenin had an accumulated nuclear localization in OE19-SP cells; whereas OE19-non-SP showed more cytomembraneous staining which indicated that Wnt signaling is activated in SP cells (Figure IV.26).

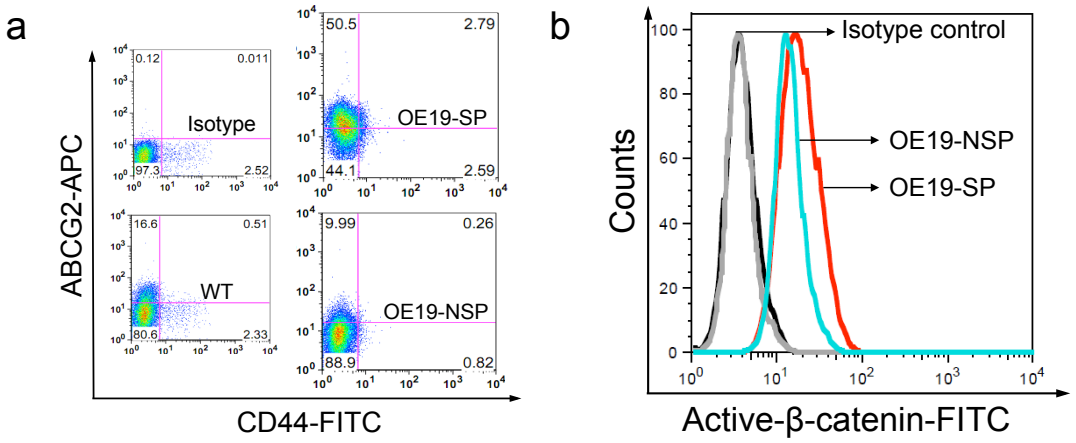

Figure IV.25 Side population cells express cancer stem cells markers. a) Representative flow cytometric analysis of ABCG2 and CD44 in OE19 cells and sorted SP and non-SP subpopulations showed an enrichment of ABCG2 and CD44 positive cells in SP cells. b) Sorted SP cells were detected with 24.4% active-β-catenin positive cells, as compared to 6.6% active-β- catenin positive cells in non-SP cells.

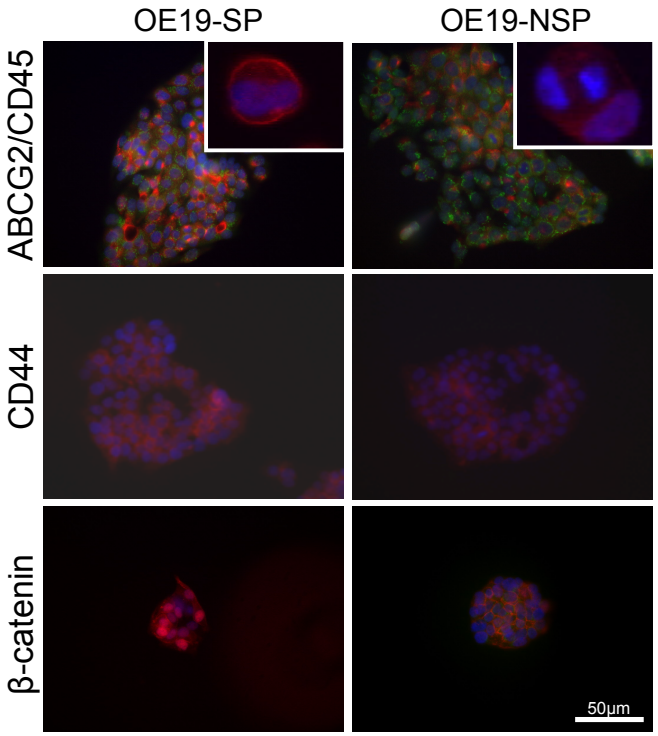

Figure IV.26 Side population cells express cancer stem cells markers. ABCG2 (red), CD44 (red), and CD45 (green) were localized on cell membrane, whereas β-catenin (red) was localized in the nucleus in SP cells as compared to a cytomembraneous staining in non-SP cells (400× magnification; scale bar=50 μm).

4.2.2 Stem like side population and chemoresistance of esophageal cancer cell lines

4.2.2.1 Enrichment of side population cells based on acquired resistance to 5-FU or cisplatin

To study the potential association between chemotherapy resistance and enrichment of SP cells in the PT1590 and LN1590 cell lines, which lacked an initial stable SP subpopulation, each cell line was cultured in medium with increasing concentrations of 5-FU and cisplatin starting from 0.1 µg/ml up to 5 µg/ml for more than 10 months. Stable chemotherapy resistant cell lines PT1590/5-FU_{res}, PT1590/$CDDP_{res}$ as well as LN1590/5-FU_{res} and LN1590/$CDDP_{res}$ were established with significantly higher IC_{50} values (Table IV.5). Protein levels of thymidylate synthase were highly elevated in PT1590/5-FU_{res} and LN1590/5-FU_{res} as compared to the sensitive cells (Figure IV.27a). Accordingly, ERCC1 was markedly higher expressed in PT1590/$CDDP_{res}$ and LN1590/$CDDP_{res}$ using FACS analysis as compared to the respective sensitive cell lines (Figure IV.27b).

Table IV.5 IC_{50} values of both sensitive and resistant PT1590 and LN1590 cells.

IC_{50} ± s.d. µg/ml	PT1590		LN1590	
	Parental	Resistant	Parental	Resistant
5-FU	5.3 ± 3.3	80.1 ± 2.9	1.2 ± 0.5	68.2 ± 4.8
Cisplatin	1.4 ± 0.7	9.1 ± 0.7	1.4 ± 0.6	7.2 ± 0.6

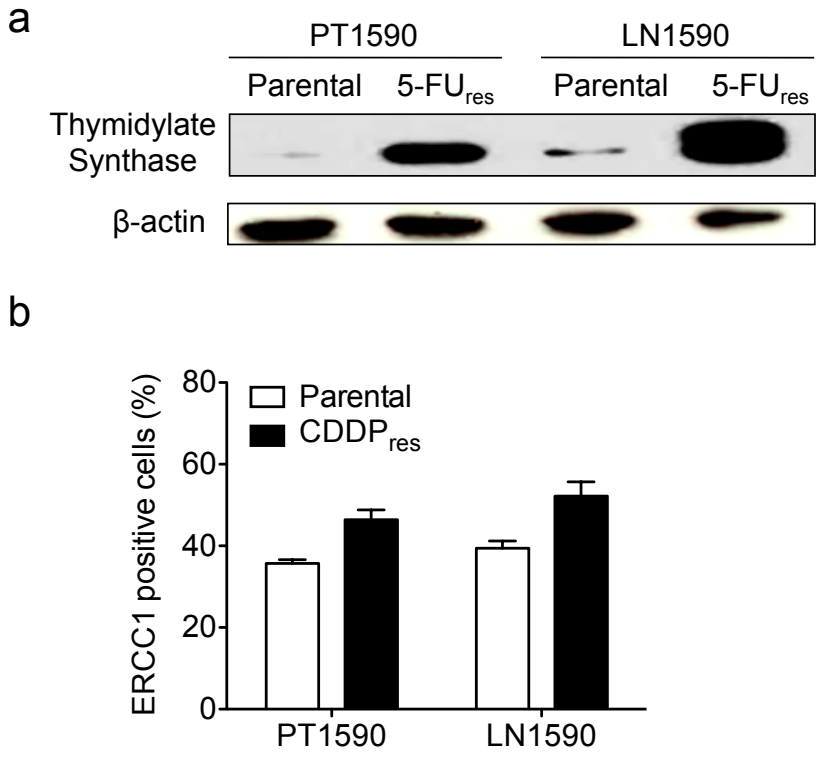

Figure IV.27 Selection of 5-FU and cisplatin resistant cells in PT1590 and LN1590. a) Western blot analysis indicates that resistance to 5-FU is attributed to elevation of thymidylate synthase activity. b) Cisplatin resistant marker-ERCC1 was detected by flow cytometry and has relatively higher expression in cisplatin induced chemotherapy resistant cells (PT1590 vs. PT1590/CDDP$_{res}$: 35.7% ± 0.9 vs. 46.4% ± 2.4; LN1590 vs. LN1590/CDDP$_{res}$: 39.4% ± 1.8 vs. 52.2% ± 3.5, *$p<0.05$).

4.2.2.2 Enrichment of side population cells based on acquired resistance to 5-FU or cisplatin

The SP fraction was significantly enriched in both 5-FU and cisplatin resistant cell lines, raising from 0.01% to 0.23% and 0.85% in PT1590/5-FU$_{res}$ and PT1590/CDDP$_{res}$ cells, and from 0.04% to 2.80% and 1.06% in LN1590/5-FU$_{res}$ and LN1590/CDDP$_{res}$ cells (Figure IV.28a and b). With increasing duration of chemotherapy the SP subpopulation was step wisely expanded. For example, PT1590/CDDP$_{res}$ showed approximately 0.1% of SP cells after 2 months continuous treatment with cisplatin, 0.7% after 4 months and 1.3% of SP cells after 8 months of chemotherapy (Figure IV.28c).

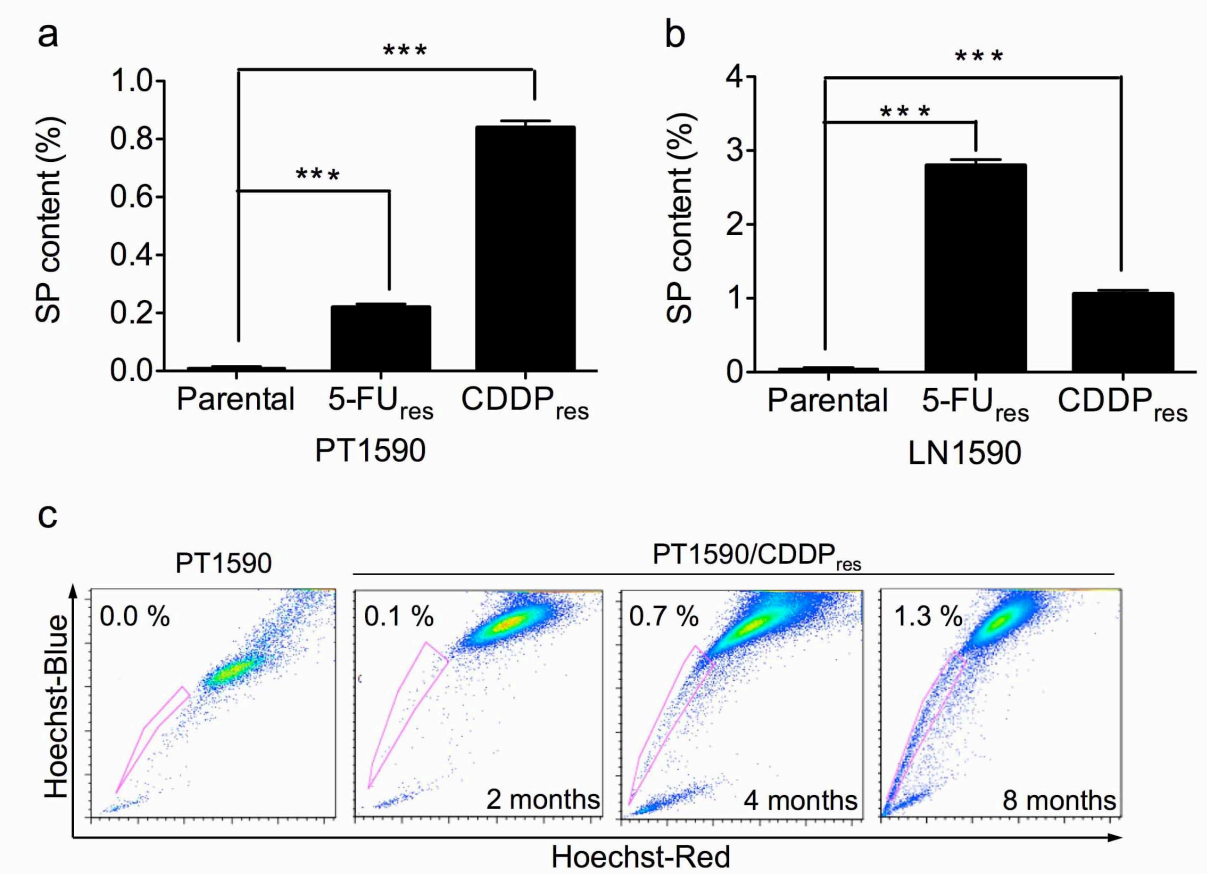

Figure IV.28 Side population increased after chemotherapy selection in both PT1590 and LN1590 cell lines. a) SP amount in PT1590 increased from 0.01% ± 0.01 to 0.22% ± 0.02 and 0.84% ± 0.04 in 5-FU and cisplatin resistant cells, ***p<0.0001. b) SP amount in LN1590 increased from 0.04% ± 0.02 to 2.8% ± 0.08 and 1.06% ± 0.05 in 5-FU and cisplatin resistant cells, ***p<0.0001. c) With increasing duration of chemotherapy, the SP subpopulation is growing from 0.01% up to 0.1%, 0.7%, and 1.3% following 2, 4, and 8 months of therapy, respectively.

4.2.3 5-FU induced chemoresistance is associated with EMT

4.2.3.1 Chemoresiatance and EMT in OE19

OE19 cells were then continuously cultivated over a 6-12 month period in the presence of 1-20µg/ml 5-FU. The surviving cells (OE19/5-FU$_{res}$) resembled spindle–shaped mesenchymal-like cells that grew differently as compared to sensitive cells, which were packed in tissue culture (Figure IV.29). OE19/5-FU$_{res}$ cells demonstrated a significantly higher IC$_{50}$ value of 191.9 ± 3.1µg/ml compared to sensitive OE19 cells with an IC$_{50}$ value of 26.8 ± 0.2 µg/ml after 5-FU application for 48 hours (p=0.00017). Since we observed a different morphology of OE19/5-FU$_{res}$ cells (with a mesenchymal-like phenotype), Vimentin, which is a type III

intermediate filament (IF) protein expressed in mesenchymal cells, was determined in both cell lines by immunofluorescent staining and western blot analysis (Figure IV.30). The protein level of Vimentin was found to be elevated in OE19/5-FU_{res} cells and predominantly expressed at the cell surface. Furthermore, ABCG2/BCRP1, a calcium-sensitive cell surface protein excluding the Hoechst dye, conferring resistance to several chemotherapeutic agents as previously reported, was significantly elevated in OE19/5-FU_{res} cells with 4.97 ± 1.5 fold positive ABCG2 cells compared to sensitive OE19 cells (p=0.0105) (Figure IV.31). Immunofluorescent staining for ABCG2 displayed a cytomembraneous expression in OE19/5-FU_{res} cells (Figure IV.31). In concordance with recent studies describing the presence of the ABCG2 transporter highly correlates with the SP phenotype, we could demonstrate a significant enhancement of SP cells from 2.18% ± 0.25 in sensitive OE19 cells compared to 19.74% ± 4.06 in OE19/5-FU_{res} cells (p=0.026) (Figure IV.32).

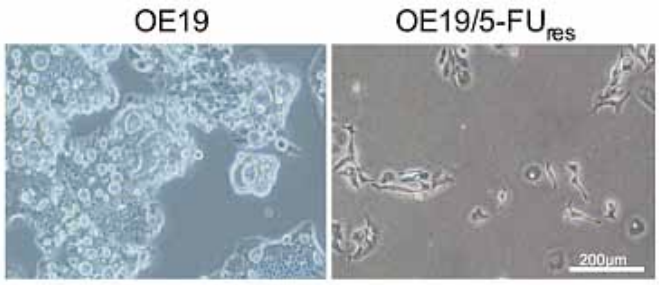

Figure IV.29 Morphology of sensitive OE19 and OE19/5-FU_{res} cells. (100× magnification; scale bar=200 µm).

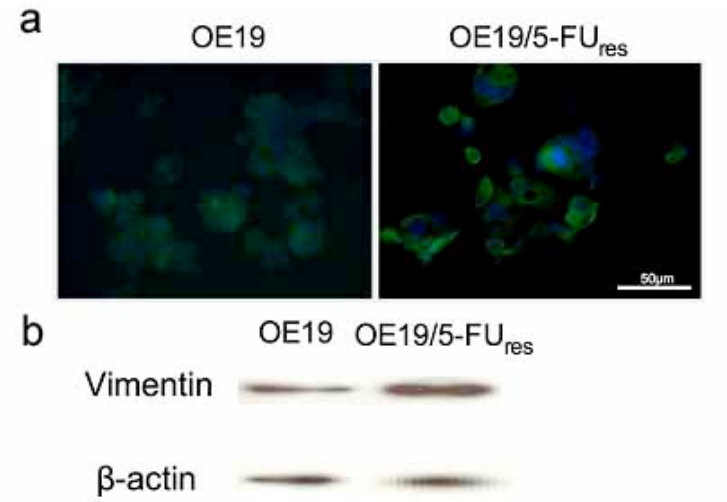

Figure IV.30 Establishment of OE19/5-FU_{res} cell lines: association to a mesenchymal like phenotype. a) The mesenchymal marker Vimentin was expressed on the cell surface of OE19/5-FU_{res} cells showing a mesenchymal-like phenotype. b) The protein level of Vimentin

was elevated in OE19/5-FU$_{res}$ cells. (400× magnification; scale bar=50 µm).

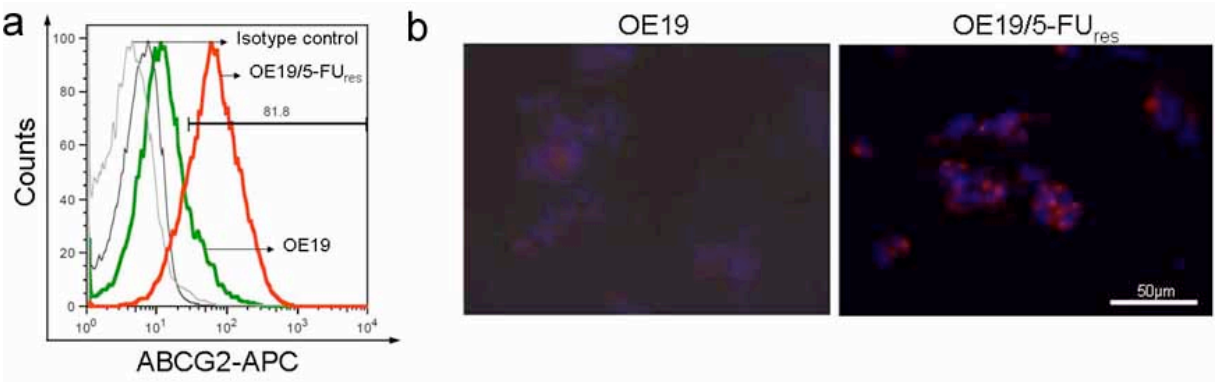

Figure IV.31 ABCG2 up regulated in OE19/5-FU$_{res}$. a) ABCG2 positive cells were significantly higher in OE19/5FU$_{res}$ cells (red line) as compared to sensitive OE19 cells (green line). b) Immunofluorescent staining for ABCG2 demonstrated a markedly stronger cytomembraneous expression in OE19/5FU$_{res}$ cells. (400× magnification; scale bar=50 µm).

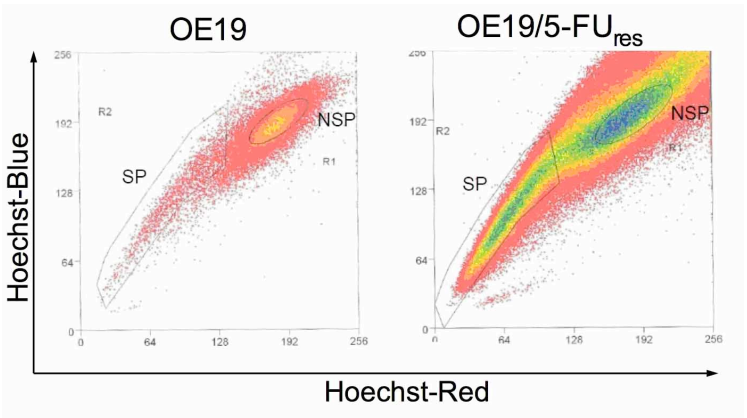

Figure IV.32 Side population increased significantly in OE19 in response to 5-FU. The percentage of SP cells was 2.4% compared to 16.9% in OE19 and OE19/5FU$_{res}$ cells, respectively.

4.2.3.2 EMT is associated to chemotherapy resistance in side population cells

Finally, we investigated whether SP cell induced chemotherapy resistance is associated with epithelial-mesenchymal transition. A commercial EMT PCR array (The Human Epithelial to Mesenchymal Transition RT² Profiler™ PCR Array) was used to profile the expression of 84 key genes that either change their expression during EMT, or regulate alterations of EMT associated gene expression. SP cells isolated from OE19 and OE19/5-FU$_{res}$ were analyzed as

described in materials and methods. The mRNA expression of SLUG (SNAI2), CALD1, WNT11, MSN, ZEB1, SERPINE1, VCAN, COL3A1, ERBB3, TMEFF1, TCF4, ITGA5, TIMP1, GSK3B, ITGAV, BMP1, MMP9, COL5A2, FOXC2, MMP3, NOTCH1, VIMENTIM, MAP1B, FN1, DSC2 and COL1A2 was found to be significantly up-regulated in SP cells from OE19/5-FU_{res} cells (>4 fold changes) when compared to expression in the SP subpopulation of sensitive OE19 cells. In contrast, a decrease of TSPAN13 and IL1RN mRNA expression in OE19/5-FU_{res}-SP cells was found. Key factors of the Wnt, Notch, and TGF-β/BMP signaling pathway such as WNT11, NOTCH1, and BMP1 were also differentially expressed in OE19/5-FU_{res}-SP cells as compared to the SP subpopulation of sensitive OE19 cells (Table IV.6).

Table IV.6 Side population cells regulate EMT associated genes during chemoresistance.

Gene Symbol	Gene Description	Fold Regulation	Status
SNAI2	Snail homolog 2	34.98	Up
CALD1	Caldesmon 1	17.65	Up
WNT11	Wingless-type MMTV integration site family, member 11	16.00	Up
MSN	Moesin	8.74	Up
ZEB1	Zinc finger E-box binding homeobox 1	8.51	Up
SERPINE1	Serpin peptidase inhibitor, clade E, member 1	4.44	Up
VCAN	Versican	4.44	Up
COL3A1	Collagen, type III, alpha 1	4.32	Up
ERBB3	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3	4.08	Up
JAG1	Jagged 1	4.06	Up
TMEFF1	Transmembrane protein with EGF-like and two follistatin-like domains 1	4.06	Up
TCF4	Transcription factor 4	4.04	Up
ITGA5	Integrin, alpha 5	4.04	Up
TIMP1	TIMP metallopeptidase inhibitor 1	4.04	Up
GSK3B	Glycogen synthase kinase 3 beta	4.03	Up
ITGAV	Integrin, alpha V	4.03	Up
BMP1	Bone morphogenetic protein 1	4.02	Up
MMP9	Matrix metallopeptidase 9	4.02	Up
COL5A2	Collagen, type V, alpha 2	4.01	Up
FOXC2	Forkhead box C2	4.01	Up
MMP3	Matrix metallopeptidase 3	4.00	Up
NOTCH1	Notch 1	4.00	Up
VIM	Vimentin	3.99	Up
MAP1B	Microtubule-associated protein 1B	3.98	Up
FN1	Fibronectin 1	3.97	Up
DSC2	Desmocollin 2	3.97	Up
COL1A2	Collagen, type I, alpha 2	3.77	Up
TSPAN13	Tetraspanin 13	0.5	Down
IL1RN	Interleukin 1 receptor antagonist	0.25	Down

4.2.4 Cancer stem cells targeted therapy via Wnt signaling pathway

4.2.4.1 Aspirin inhibits SP in esophageal cancer cell lines

As detailed earlier, Wnt signaling is a key pathway linked to the regulation of CSCs. CSCs contribute to drug resistance and are enriched during the process of chemoresistance. Recent

reports indicate that the emergence of CSCs occurs in part, as a result of EMT, and accumulating evidence suggests that EMT of tumor cells not only causes increased metastasis, but also contributes to drug resistance. Aspirin has been described as Wnt signaling inhibitor. Therefore, suppression Wnt pathway activated cancer stem cells or stem like subpopulations by aspirin might provide an easy and effective therapeutic strategy safely.

We investigated potential aspirin effects on side population cells in esophageal cell lines directly. Treatment with 5 mM aspirin has been previously reported to induce apoptosis in CRC cells (Din et al 2004). This concentration of aspirin tested at 24h, 48h and 72h in OE19 was shown to lead to a reduction in SP cell fraction from the percentage of 19.7 ± 1.0 to 6.9 ± 1.0, 3.4 ± 0.9 to 1.8 ± 1.4, respectively (Figure IV.33).

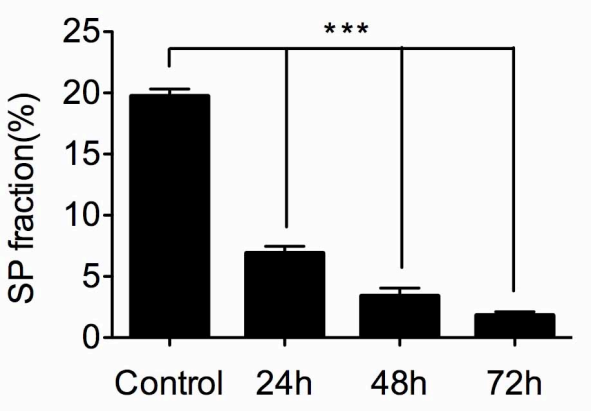

Figure IV.33 Aspirin decreased the proportion of side population cells in OE19. Cells after 5 mM aspirin treatment were collected, counted and further stained with Hoechst 33342 at 2.6 μg/ml per 10^6 living cells. 72h incubation generated a SP reduction of 90.8%, ***p<0.0001.

4.2.4.2 Aspirin inhibits cell proliferation, especially on SP subpopulations in esophageal cancer cell line

The effect of aspirin treatment on OE19, sorted OE19-SP, OE19-NSP cell proliferation was determined using the MTT assay. Aspirin concentrations lower than 1mM aspirin did not affect OE19 cell proliferation. 5 mM aspirin inhibited cell proliferation and showed cell viability as high as 107.8%, 89.3%, 72.2% and 72.3% on 6h, 24h, 48h and 72h, while 91.5%, 57.3%, 29.5% and 21.1% at 10mM and 63.2%, 36.6%, 11.0% and 0.2% (Figure IV.34). This indicated the cytoxicity of aspirin on esophageal cell line is time and dose dependent.

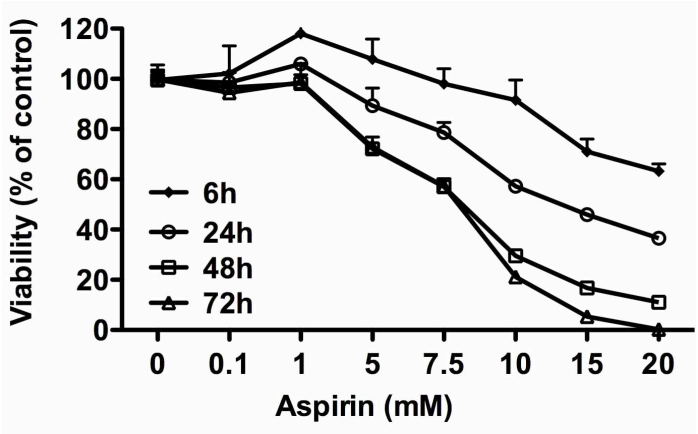

Figure IV.34 Aspirin affecting cell proliferation is time and dose dependent. There is no significant difference between 48h and 72h treatment at the concentration lower than 10 mM.

Aspirin had a significant stronger effect on SP cell proliferation as compared with the NSP cells. Cell viability of SP is percentage of 85.5 ± 0.7, 61.5 ± 2.7, and 51.4 ± 2.7 as compared to 92.3 ± 4.8, 82.9 ± 10.5 and 59.2 ± 5.3 of NSP under the treatment of aspirin at 2.5 mM, 5 mM and 10 mM (Figure IV.35).

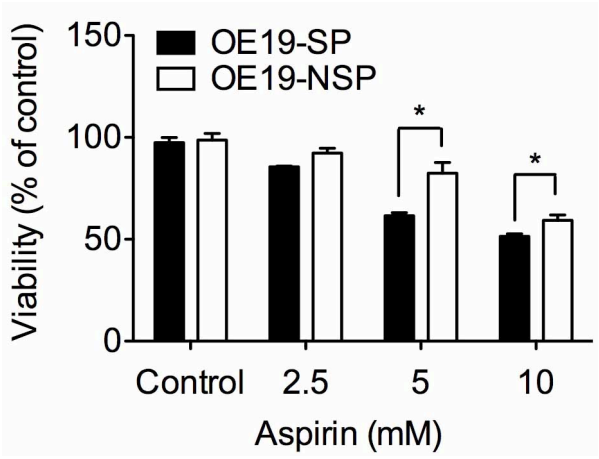

Figure IV.35 Aspirin decreased SP cell proliferation more than NSP. SP cells are more sensitive than NSP with aspirin 24h therapy and display a biggest difference at 5 mM, *p<0.05.

4.2.4.3 Aspirin inhibit 5-FU enriched side population cells in esophageal cancer cell lines

OE19 and OE19/5-FU_{res} were treated with 5mM aspirin for 48h and further detected the SP proportion by Hoechst 33342 staining. OE19/5-FU_{res} has as high as 40.7% of SP, which can

be decreased significantly to lower than 1% of SP. Representative results are shown (Figure IV.36).

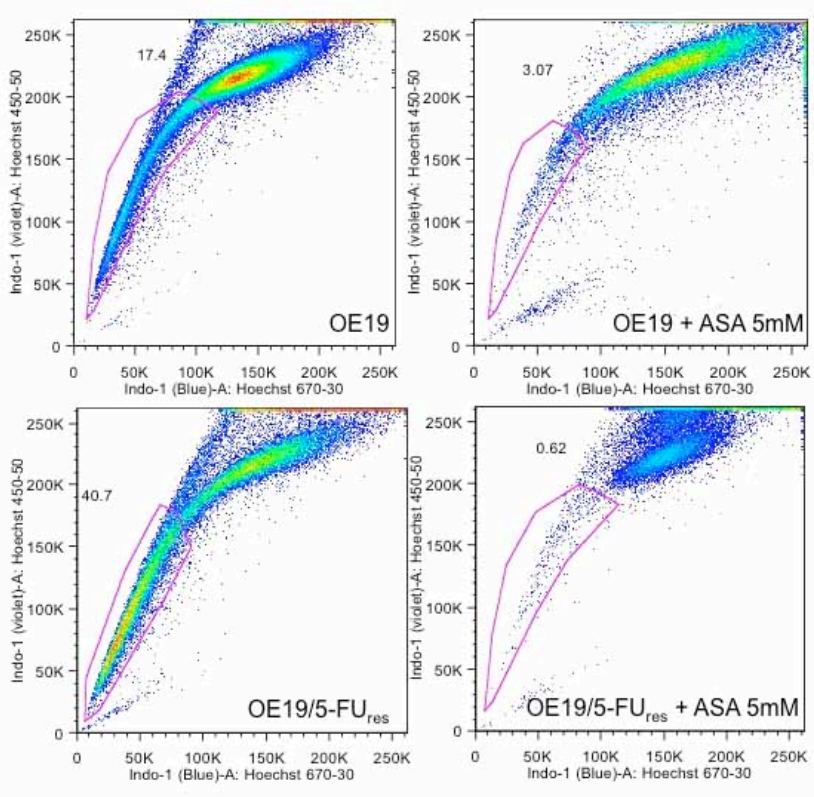

Figure IV.36 Aspirin decreased chemoresistant SP of OE19 5-FU resistant cell line. Both parental OE19 and OE19/5-FU$_{res}$ cells after 48h 5 mM aspirin treatment were collected, counted and further stained with Hoechst 33342 at 2.6 μg/ml per 10^6 living cells. 5-FU resistant cells enriched SP and are able to target by aspirin.

4.2.4.4 Aspirin may target the Wnt signaling pathway

Our previous data showed an activation of Wnt/β-catenin signaling in side population and chemoresistant cell lines. We analyzed the dysregulation of this pathway by using Wnt signaling target array. OE19/5-FU$_{res}$ was incubated with or without 5mM aspirin for 48h and further isolated RNA to detect the gene expression on mRNA level. After normalization with housekeeping genes (ACTB B2M, GAPDH, HPRT1 and RPLPO), the relative expression of 84 key Wnt target genes was analyzed (Table IV.7). SOX2, BMP2, MMP7, NRCAM and NTRK2 are dramatically down regulated after aspirin treatment (> 4 fold change); in addition COX2, BIRC5, LEF1, DAB2, CCND1 and CD44 were also decreased over 2 fold change.

Table IV.7 Aspirin inhibits Wnt target genes associated with 5-FU induced chemoresistance.

Gene symbol	Gene description	Fold regulation	Status
SOX2	SRY (sex determining region Y)-box 2	-9.22	Down
BMP4	Bone morphogenetic protein 4	-9.17	Down
MMP7	Matrix metallopeptidase 7 (matrilysin, uterine)	-4.63	Down
NRCAM	Neuronal cell adhesion molecule	-4.61	Down
NTRK2	Neurotrophic tyrosine kinase, receptor, type 2	-4.61	Down
PTGS2(COX-2)	Prostaglandin-endoperoxide synthase 2	-2.32	Down
BIRC5	Baculoviral IAP repeat containing 5	-2.31	Down
LEF1	Lymphoid enhancer-binding factor 1	-2.30	Down
DAB2	Disabled homolog 2, mitogen-responsive phosphoprotein	-2.30	Down
CCND1	Cyclin D1	-2.28	Down
CD44	CD44 molecule (Indian blood group)	-2.28	Down

V. DISCUSSION

5.1 Pancreatic cancer

Today gemcitabine is still the standard chemotherapy for patients with locally and/or systemic advanced pancreatic cancer. Although clinical trials showed that gemcitabine based single or combined chemotherapeutic treatments improved overall and progression free survival in patients with advanced pancreatic cancer, still there exist no convincing predictive marker for therapy response and patients´ selection (Conroy et al 2011). In particular, gemcitabine induced resistance and systemic toxicities leads to limitations regarding its therapeutic application. Emerging evidence supports the notion that cancer stem cells (CSC) contribute to chemotherapy resistance and tumor progression. Preclinical research results support the fact that chemotherapy and/or radiation therapy enrich the proportion of CSCs within different cancer cell lines *in vitro*. For pancreatic cancer Du et al. found that chemo- and radiation resistant pancreatic cancer cells express high levels of pancreatic CSCs markers (CD24 and CD133) (Du et al 2011). Side population cells are also considered to possess stem cell characteristics in many tumors. In our previous studies we identified side population cells as a cellular subpopulation of metastatic pancreatic cancer cells demonstrating the ability to self-renew *in vitro* with a tumorigenic and metastatic phenotype *in vivo* (data unpublished). Our *in vivo* results repeatedly revealed the tumor initiating capacity of pancreatic cancer SP cells since there was no significant difference in tumor weight of primary tumors generated from $1x10^6$ L3.6pl$_{Gres}$ whole population cell compared to $1x10^5$ L3.6pl$_{Gres}$-SP cells whereas primary pancreatic tumors generated from $1x10^5$ L3.6pl$_{Gres}$-NSP cells were significantly smaller (Figure IV.12). In addition, the metastatic activity of $1x10^5$ L3.6pl$_{Gres}$-SP cells was equivalent to $1x10^6$ L3.6pl$_{Gres}$ whole population cells (Table IV.4). Others recently described stem-like properties including self-renewal ability and chemoresistance in BxPC-3-LN highly lymphatic metastatic pancreatic cancer cells. BxPC-3-LN cells also expressed higher levels of sonic hedgehog and cancer stem cell surface markers (CD133 and CXCR4) compared to the parental BxPC-3 cells (Luo et al 2013).

We further demonstrated a significant enrichment of side population cells in L3.6pl pancreatic cancer cells after long-term treatment with increasing concentrations of gemcitabine (Figure IV.2). Side population cells are able to efflux chemotherapeutic drugs, which might be one explanation of their resistance against anti-cancer therapy (Donnenberg and Donnenberg 2005, Zhou et al 2001).

MiRNAs are a class of conserved small non-coding RNAs that regulate gene expression by either repressing the translation or causing degradation of multiple-target mRNAs, so that miRNA dysregulation results in profound cellular consequences because individual miRNAs can bind to and regulate multiple mRNAs (Ambros 2004, Gregory et al 2005). In normal cells multiple miRNAs are responsible for the maintenance of cell homeostasis. Aberrant expression of miRNAs has been reported in many cancer related biological processes including angiogenesis, metastasis, as well as chemoresistance, EMT, and apoptosis (Esquela-Kerscher and Slack 2006, Kasinski and Slack 2011, Kuehbacher et al 2008, Nicoloso et al 2009, Urbich et al 2008).

In this study, we focused on miR-21 and miR-221 as the most strongly differentially expressed miRNAs in pancreatic adenocarcinoma (Lee et al 2007, Moriyama et al 2009, Park et al 2009). A significant up regulation of both miRNAs was found in pancreatic cancer SP as compared to NSP cells (Table IV.1Figure IV.1).

MiR-21 increases tumor cell proliferation, migration, and invasion through targeting a series of tumor suppressor genes including programmed cell death 4 (PDCD4), phosphatase and tensin homolog deleted on chromosome ten (PTEN), tumor suppressor gene tropomyosin 1 (TPM1) and maspin. MiR-21 can also target the inhibitors of matrix metalloproteinases (TIMPs) and RECK (reversion-inducing cysteine-rich protein with Kazal motifs), resulting in increased expression of matrix metalloproteinases (MMPs). The miRNAs also regulate multiple cellular pathways including the PI3K-protein kinase B (AKT) pathway and mitogen-activated protein kinase (MAPK)/extracellular signal regulated kinase1/2 (ERK1/2) pathway (Krichevsky and Gabriely 2009). In addition, Huang et al. showed that miR-21 expression is increased via the MAPK pathway upon stimulation with HER2/neu (Huang et al 2009b). Recently, reports have shown that down regulation of miR-21 inhibits the EGFR pathway and suppresses the growth of human glioblastoma cells independent of PTEN status (Zhou et al 2010). Correlations between the overexpression of miR-21 and resistance to anticancer agents have been reported (Blower et al 2008). Specifically, inhibition of miR-21 led to increased sensitivity to gemcitabine in PDAC and cholangiocarcinoma cells (Meng et al 2006, Moriyama et al 2009, Park et al 2009). In colorectal cancer, miR-21 did not affect gemcitabine- induced apoptosis in colon cancer cells (Wang et al 2009), whereas is did appear to induced resistance to 5-fluorouracil by down regulating human DNA MutS homolog 2 (hMSH2) (Valeri et al 2010). As one of the most abundant and easily detectable miRNAs, several studies have evaluated the potential application of miR-21 as a diagnostic or

prognostic biomarker. The expression of miR-21 has been correlated with clinical stage, lymph node, and distant metastasis as well as poor prognosis in glioma, colon, breast, tongue and esophageal squamous cell cancers (Kimura et al 2010, Li et al 2009a, Schetter et al 2009, Yan et al 2008). High miR-21 expression was associated with more aggressive pancreatic endocrine tumors, characterized by increased tumor cell proliferation and liver metastasis (Roldo et al 2006).

MiR-221 plays important role in the process of tumor progression by targeting cell cycle regulators such as p27/Kip1 and CDKN1C/p57. In addition, it can regulate cell apoptosis by inhibiting PUMA and pro-apoptotic protein Bim and modulatong temozolomide response via a DNA repair enzyme MGMT (Fornari et al 2008, Galardi et al 2007, Garofalo et al 2012). In addition, Di Leva et al discovered a regulating stem-loop within miR-221 and estrogen receptor -α (ER-α) (Di Leva et al 2010). They found that miR-221 inhibited the expression of ER-α and ER-α enabled to repress miR-221 transcriptional activation. Multiple oncogenic signaling pathways (PI3K/AKT, Wnt, ERK, and MAPK signaling pathways) are linked to miR-221 through its target genes. These include pathways associated with cell proliferation, differentiation, migration and invasion (Garofalo et al 2012). In addition to its role of miR-221 in cancer development, miR-221 may also play a role in the potential response during therapy. For example, miR-221 has a prominent in the acquisition of anti-ER reagent (such as tamoxifen) and fulvestrant resistance in breast cancer (Rao et al 2011). Pogribny and colleagues identified miR-221 up regulated in cisplatin resistance (Pogribny et al 2010). Another study found miR-221 could regulate radiosensitivity by directly modulating of PTEN expression (Chun-Zhi et al 2010). Antagomirs (ASOs) have been designed against miRNAs to specifically inhibit their expression. Since we identified up regulated expression of specific miRNAs in pancreatic cancer stem-like SP compared to NSP cells the aim of this study was to evaluate the therapeutic potential of specific miRNA antisense oligonucleotides (ASOs) to antagonize SP cell associated stem-like biological functions and thereby to consecutively restore chemosensitivity in pancreatic cancer. In L3.6pl whole cell population we observed that ASOs against miR-21 and miR-221 indeed influenced cell proliferation, apoptosis, and chemotherapy sensitivity (Figure IV.6; Figure IV.7; Figure IV.8; Figure IV.9). The results were comparable to the results of Park et al (Park et al 2009). We further confirmed a significant overexpression of both miRNAs following gemcitabine induced chemotherapy resistance in L3.6pl$_{Gres}$ pancreatic cancer cells (Figure IV.3). Li et al. reported an overexpression of other miRNAs such as miR-200 and let-7 in gemcitabine resistant pancreatic cancer cells (Li et al 2009b). The results shown here indicate that the proportion of

stem-like side population cells was enriched in gemcitabine resistant pancreatic cancer cells (Figure IV.2). Notably, combined ASOs therapy against miR-21 and -221 led to a significant reduction of stem like side population and consecutively to a reconstitution of sensitivity to either 5-FU or gemcitabine (Figure IV.9). . Kasinski and Slack postulated that a combinatorial use of ASOs against different miRNAs could decrease the chance of mutation-induced resistance (Kasinski and Slack 2011).

The metastatic capacity of pancreatic cancer SP cells has been linked to activation of EMT (Kabashima et al 2009). Growing evidence demonstrates a potential role of miR-21 in regulating embryonic stem cell pathways and biological functions of CSCs especially EMT (Bao et al 2011). Recently, Han et al. elucidated a reversal of EMT in a breast CSC model by antagomir-induced inhibition of miR-21 (Han et al 2012a, Han et al 2012b). ASOs therapy against miR-21 or miR-221 resulted in a significantly reduced transmigration of L3.6pl *in vitro* (Figure IV.8). ASOs therapy substantially lowered the incidence of liver metastases *in vivo*. Intriguingly, combined ASOs therapy led to complete abolishment of detectable metastatic lesions.

Anticancer miRNA-based therapy has the theoretical advantage of targeting multiple biological cancer related pathways. *In vitro* inhibition of miR-21 and -221 in the unselected L3.6pl whole cell population affected a series of regulated genes such as IRAK3, C5ORF41, KLF12, and MAPK10 (Figure IV.10) involved in transcription activity and interacting with important cancer associated biological functions such as proliferation, migration, and apoptosis. IRAK3 functions as a key inhibitor of TLR2/NFkB-mediated chronic inflammation that is negatively associated with oxidative stress, and obesity-related insulin resistance and metabolic syndrome (Hulsmans et al 2012). MAPK10 may function as a tumor-suppressor gene by regulating apoptosis (Ying et al 2006). Recently, miR-21 is further identified as both a target and a regulator of ERK/NF-κB and JNK/c-Jun and the feedback regulations of miR-21 and MAPKs via Pdcd4 and Spry1 are involved in arsenite-induced malignant transformation of HELF cells (Shen et al 2013). These co-targeted genes by both miR-21 and miR-221 and co-regulating pathways such as PI3K/AKT, Wnt, ERK, and MAPK signaling pathways and auto-regulatory loops mediated by miR-21 (Garofalo et al 2012, Pan et al 2010), indicating a potential cross talk between both miRNAs and may help to explain the phenomena that inhibition of miR-21 together with miR-221 had a more than additive effect on miR-221 expression.

The direct transfection of antagomirs or negative all stars control oligo into freshly isolated

side population cells with a transfection efficiency about 85%-95%. Cell viability after transfection just before orthotopic tumor cell injection was higher than 90%.

Histomorphological and immunohistochemical tissue analysis of primary pancreatic tumors following orthotopic injection of ASOs transfected L3.6pl$_{Gres}$-SP cells showed a higher level of apoptotic cells, and reduced proliferative tumor cells coincident with a decrease in MVD, in particular following transfection with anti-miR-221 confirming the anti-angiogenic effect of miR-221 inhibition for pancreatic cancer.

To demonstrate the clinical relevance of miRNAs for pancreatic cancer, we further analyzed the expression of miR-21 and miR-221 in human pancreatic cancer and adjacent normal tissue samples. Both miRNAs were significantly overexpressed in tumor as compared to the corresponding adjacent normal pancreatic tissue (Figure IV.11). Aberration of miR-155 expression has already been observed in PanIN-2, miR-21 abnormalities in PanIN-3 lesions during the multistep progression towards pancreatic cancer (Ryu et al 2011). Aberrant miRNA expression may provide diagnostic biomarkers for pancreatic adenocarcinoma in the future.

In summary, both microRNAs are up regulated in pancreatic cancer stem-like SP cells and contribute to important biological functions of cancer progression including proliferation, apoptosis, migration, and chemotherapy resistance. ASOs therapy against miR-21 and -221 reduced the proportion of SP cells and effectively suppressed L3.6pl$_{Gres}$-SP induced primary pancreatic tumor growth and metastasis *in vivo*. Our data suggest that inhibition of miR-21 and -221 as future therapeutic strategy for pancreatic cancer is particularly suitable to target stem-like subpopulations and further address both miRNAs specific biological function to promote tumor progression (Figure V.1).

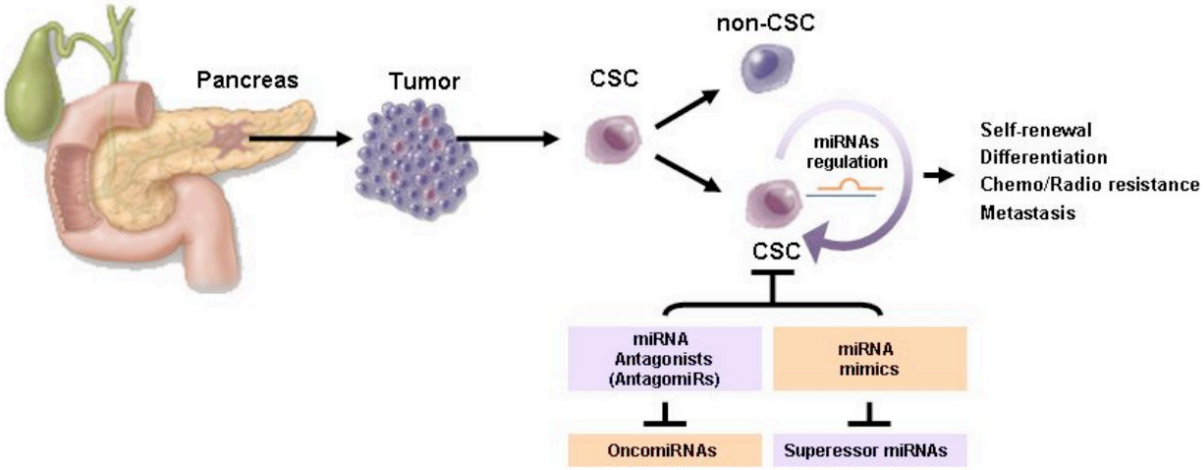

Figure V.1 Systematic study design of pancreatic CSC targeted therapy. Cancer stem cells or stem cell like subpopulation cells play important roles in self-renewal, differentiation, chemo or radiotherapy induced resistance and metastasis. These cells are regulated by some miRNAs, strategic replacement these tumor suppressor miRNAs or targeting oncogenic miRNAs are able to diminish CSCs, and sensitizing tumors to chemotherapy, decreasing metastasis and leading to systemic CSC targeted therapy.

5.2 Esophageal cancer

More than 90% of esophageal cancers are either squamous cell carcinoma or adenocarcinoma (Daly et al 2000). The incidence of esophageal adenocarcinoma located in the distal esophagus and gastroesophageal junction is rising and often associated with a history of gastroesophageal reflux disease and Barrett's esophagus (Enzinger and Mayer 2003). More than two third of patients present with unresectable or metastatic disease at the time of diagnosis, most patients with localized disease will develop metastases despite therapy (Enzinger and Mayer 2003, Parkin et al 1999). Traditional chemotherapy is based on cisplatin and 5-fluorouracil, nowadays, a combination of both chemotherapeutic agents with epirubicin or taxane constitutes the most effective treatment option (Cunningham et al 2008, Thallinger et al 2011), however, development of resistance to chemotherapy remains a major challenge. An understanding of the molecular mechanisms of resistance primarily to 5-FU and cisplatin is important to effectively treat esophageal cancer in the future.

Cancer stem cells are often resistant against chemotherapeutic agents either based on clonal selection of drug resistant cancer cells with high tumorigenic potential with respect to lung cancer (Levina et al 2008), or caused by elevation of drug transporters that enable the ejection of chemotherapeutic agents such as 5-FU and irinotecan (Chikazawa et al 2010, Yu et al 2007). In the present study we analyzed the function of SP cells for chemotherapy resistance and metastasis in esophageal cancer. One key feature to characterize SP cells as stem-cell like cells is higher resistance to chemotherapy than the bulk of cancerous cells (resistance to apoptosis, expression of certain pumps, including ABCC1, ABCG2 and MDR1, which are the principal mediators of multidrug-resistance, quiescent in the absence of specific stimulation from the microenvironment). The SP assay constitutes a highly valuable primary purification strategy for isolating potential stem/progenitor cells from various tissues or cell lines, particularly in the absence of specific cell surface markers (Moserle et al 2010). The results presented suggest that SP cells in esophageal cancer indeed mirror the proposed biology of cancer stem cells.

Using Hoechst 33342 dye we detected by FACS analysis different proportions of side population cells in 4 esophageal AC and one SCC cell line varying from 0 to nearly 20% (Dean et al 2005, Wu and Alman 2008). Side population cells are generally identified based on their drug/dye efflux efficiency maintained by the breast cancer resistant protein 1 Brcp1/ABCG2, a member of ATP-binding cassette (ABC) family (Zhou et al 2001), which has been associated with multidrug resistance in diverse malignancies (Donnenberg and

Donnenberg 2005). Our results reproducibly demonstrated that OE19 cells contained a larger proportion of SP cells displaying intrinsically resistance towards 5-FU (Figure IV.32) and cisplatin (data not shown). Shi et al. found SP cells with high resistance to 5-FU, mitomycin, and cisplatin in hepatocellular carcinoma cell lines with different metastatic potential including HCCLM3, MHCC97H, MHCC97L, and Hep3B cells (Shi et al 2008), while Haraguchi et al. evaluated the same tendency of SP cells in Huh7 response to Doxorubicin, 5-FU, and gemcitabine (Haraguchi et al 2006b). Previous studies have shown the existence of even radio-resistant stem like cells in esophageal cancer. Che et al. developed a radio-resistant subtype - Eca109R50Gy cells - which show some properties of CSCs (Che et al 2011), while Zhang et al. found an enrichment of side population cells in radio-resistant esophageal cancer cell lines following fractionated irradiation with high expression of stem cell markers such as β-catenin, Oct3/4, and β1-integrin (Zhang et al 2008b).

To support the hypothesis that the existence of SP cells contributes to chemotherapy resistance, we developed 5-FU and cisplatin resistant variants of different cell lines. The corresponding resistant cells displayed a significantly higher IC_{50} value for 5-FU and cisplatin cytotoxicity (Table IV.5) and substantially elevated resistant markers such as thymidylate synthase and ERCC1 (Figure IV.27). By stepwise elevation of the respective 5-FU and cisplatin concentration a SP subpopulation was generated in SP negative PT1590 and LN1590 cell lines. SP fractions increased with the duration of chemotherapy (Figure IV.28). OE19-SP cells showed a higher expression of ABCG2 and CD44 than that seen in OE19-NSP cells (Figure IV.26). The importance of ABCG2 for chemotherapy resistance was first demonstrated in stem cells derived from ABCG2-deficient mice that were more sensitive to the ABCG2 substrate mitoxantrone (Zhou et al 2002).

Conflicting data has been reported supporting the existence of common CSC surface markers on esophageal cancer cells. CD44 is a transmembrane glycoprotein widely used as a surface marker for cancer stem cells (Jaggupilli and Elkord 2012). $CD44^+/CD24^-$ cells were about 10–60 fold more resistant to chemotherapy in relation to corresponding non-$CD44^+/CD24^-$ cells in breast cancer (Gong et al 2010). The expression of CD44 has been found to correlate with a poor prognosis in esophageal SCC according to Chai (Chai et al 2007) and Takayama (Takayama et al 2003). Other stem cell associated tumor cell subpopulations also contribute to chemotherapy resistance e.g. $CD133^+$ cells are enriched both after cisplatin exposure in lung cancer and following gemcitabine therapy in pancreatic cancer. $CD133^+$ cells in glioblastoma, lung cancer, and hematopoietic malignancies have been linked to poor

prognosis (Mizrak et al 2008). However, increasing evidence suggests that CD133 may not be suitable as ideal cancer stem cell marker in HCC and esophageal cancer cell lines or tissues (Ma et al 2007). Furthermore, signal transduction pathways such as PI3K/Akt/mTOR and Wnt have been generally associated to cancer stem cell survival (Li et al 2003, Martelli et al 2010). Interestingly, these pathways are often aberrantly regulated in esophageal cancer (Hildebrandt et al 2009, Hsu et al 2008).

Self-renewal and the potential of differentiation are key properties of cancer stem cells and manifest the fundamental aspects of tumorigenicity. The SP cells described here were able to reproduce both SP and NSP cells with an enrichment of the SP fraction after re-sorting compared to the whole cell population (Figure IV.21). SP cells displayed tumorigenic capacity both *in vitro* and *in vivo* in colony formation assay, soft agar assays, and in a subcutaneous *in vivo* model of esophageal cancer (Figure IV.19; Figure IV.20; Figure IV.22). These results are in accordance to previous discoveries found in hepatocellular and brain cancer (Haraguchi et al 2006a, Singh et al 2004).

The metastatic potential of SP cells has been controversial. Chua et al. described SP cells of the U87MG glioma cell line that were significantly more invasive than corresponding NSP cells (Chua et al 2008), whereas others found that SP cells of glioma or human hepatocellular cancer cell lines exhibited a slower migration rate in monolayers as well as Boyden chamber migration assays (Weber et al 2010). Interestingly, emerging evidence suggests an association between chemotherapy resistance and epithelial mesenchymal transition (EMT) in cancer (Rosano et al 2011). EMT and the reciprocal mesenchymal to epithelial transition (MET) are linked to tumor metastasis, stem cell differentiation, and development. Brabletz et al. identified cells at the invasive front of tumors, a subtype of malignant cells displaying stem cell like characteristics that acquired the ability of metastasis through EMT (Brabletz et al 2005). During EMT, epithelial cells lose their apical and basolateral polarities, break their intercellular tight junctions, and degrade basement membrane extracellular matrix components to become migratory mensenchymal cells. In human esophageal cancer, changes in the expression of EMT key regulators such as Snail, Slug, and Twist play an important role in tumorigenesis and progression, and are significantly higher expressed in advanced stages and metastatic lesions (Kuo et al 2011, Lee et al 2012, Zhang et al 2011). Tomizawa et al. presented some EMT and cancer stem cell markers in specimens of early esophageal adenocarcinoma in Barrett´s esophagus at the invading edges of the tumor which abundantly express Snail, Slug, and Twist, suggesting that early stage cancers predominantly constitute

cells with metastatic potential (Tomizawa et al 2012).

We observed a mesenchymal like phenotype together with Vimentin overexpression and an increasing SP cell fractions during the generation of resistant OE19/5-FU_{res} *in vitro* (Figure IV.30). To evaluate whether SP cell related chemotherapy resistance leads to induction of EMT processes in esophageal cancer, we analyzed the expression of EMT associated genes in SP cells from both resistant OE19/5-FU_{res} and sensitive OE19 cells via a high through-put PCR array. Zhang et al. have shown the relevant role of Slug expression in apoptosis, invasion, and metastasis of human esophageal adenocarcinoma cells and its relationship to E-cadherin and BCL-2 expression *in vitro* and *in vivo* (Zhang et al 2011). Here Slug (Snail2) was significantly up regulated in OE19/5-FU_{res} SP cells. The EMT activator ZEB1 is linked to tumor progression towards metastasis (Peinado et al 2007). Ohashi et al. found that ZEB1 and ZEB2 are associated to TGF-β-mediated EMT in cells with EGFR overexpression during esophageal carcinogenesis (Ohashi et al 2010). Recently, the ZEB/miR-200 feedback loop has been identified as the molecular motor for cellular plasticity in developmental processes and for cancer progression towards metastasis (Burk et al 2008, Wellner et al 2009). In resistant OE19/5-FU_{res} cells the SP subpopulation showed 8.5 fold up-regulation of ZEB1 as compared to the SP subpopulation of sensitive OE19 cells. Interestingly, IL1RN, the interleukin 1 receptor antagonist, a negative regulator of heterotypic cell-cell adhesion was 4 fold down-regulated in the SP subpopulation of OE19/5-FU_{res} cells. In addition, genes regulating the Wnt pathway such as GSK3B and WNT11 were significantly higher expressed in the SP subpopulation of OE19/5-FU_{res} as compared to SP cells of sensitive OE19. Activated β-catenin has been already demonstrated in the SP as compared to the corresponding NSP fraction of sensitive OE19 cells (Figure IV.26). These findings suggest that the application of chemotherapy may lead to propagation of stem cell like subpopulations of esophageal cancer cells that are not only therapy resistant but also potentially metastatic due to activated EMT related pathways.

Loss of the β-catenin/E-cadherin interaction in immortalized breast epithelium is associated with both the epithelial-mesenchymal transition and a CSC-like phenotype (Gupta et al 2009). The authors found that $CD44^{+}CD24^{-/low}$ cells displayed an EMT phenotype as characterized by the loss of E-cadherin and gain of Vimentin expression. Although activation of the Wnt signaling cascade was not formally shown, the dramatic increase in the $CD44^{high}$ $CD24^{low}$ population may mark activated Wnt signaling. Therefore, it is interesting to speculate that Wnt inhibitors that influence epithelial-mesenchymal transition, may help overcome CSC

enhanced drug resistance, and metastatic capacity in a variety of malignancies (Takahashi-Yanaga and Kahn 2010).

A number of existing drugs and natural compounds have been identified as inhibitors and/or modulators of Wnt/β-catenin signaling pathway (Takahashi-Yanaga and Kahn 2010). Aspirin is cheap and easy to handle in clinical application, especially, daily use of aspirin has already been considered as an effective cancer prevention strategy. Aspirin or other NASIDs affect cell proliferation, angiogenesis and metastasis and induced cell apoptosis in colorectal cancer (Ricchi et al 1997, Yao et al 2005). COX-2 elevation was observed in cancer cells and correlated with increased prostaglandin PGE2 production (Yoshida et al 2003). PEG2 can efficiently prevent β-catenin degradation by interfering with both GSK3-β and Axin2 function. In an esophageal cancer model, Navtej S et al has shown that either selective or non-selective COX-2 inhibitors can inhibit inflammation, COX-2 activity, and the development of adenocarcinoma induced by reflux (Buttar et al 2002). COX-2 contributes to P-glycoprotein-mediated multidrug resistance via JNK pathway (Sui et al 2011), which suggested that modulation of COX-2 might regulate SP cells associated chemoresistance. In our study, under aspirin treatment, we firstly observed a significant inhibition of SP cells that also show comparable sensitive to aspirin (Figure IV.35). The reduction was strengthened in SP enriched chemoresistant cells that could be associated with decreased COX-2 mRNA expression. The cell adhesion protein family member-CD44 regulates growth, survival, differentiation and migration and is thereby prone to be involved in tumor progression and metastasis. Furthermore, CD44 was identified as CSC marker in various cancer, for instance, homing of leukemia cancer stem cells is dependent on CD44 (Jin et al 2006). CD44 expression is up regulated in esophageal epithelial cells undergoing EMT (Le Bras et al 2011) and associated with acquirement of 5-FU resistance in ESCC cell lines (Zhao et al 2011). We found higher CD44 expression in OE19-SP cells than OE19-NSP cells, and could be down regulated by aspirin in resistant cells (Table IV.7). Other Wnt target genes, growth factors such as BMP4 is important in cell development and differentiation, for example, enquired for $CD133^+$ CSCs maintenance (Zhang et al 2012). Cell cycle related genes-SOX2, CCND1, COX-2 and adhesion or migration genes MMP7, NRCAM were all down regulated under aspirin therapy.

However, it is known that aspirin and other NSAIDs may cause gastrointestinal bleeding and heartburn (Huang et al 2010, McQuaid and Laine 2006) and it is possible that patients with early symptoms of esophageal, gastric, and other digestive tract neoplasms may selectively

avoid using it. There are also some reports suggesting a cytoprotective effect of aspirin, suggesting a co-treatment with aspirin might limit the outcome of anticancer therapy (di Palma et al 2006). Aspirin function involved in COX2 signaling and crosstalk with Wnt/β-catenin, mTOR, AMPK, PI3K/AKT, which influenced numerous biological activities (Markowitz 2007), therefore is still complicated to clarify its therapeutic effect in cancer. The safety use of aspirin as adjuvant therapy for cancer patients is necessary to be further developed.

Taken together, our data provides evidence that 1) SP cells represent a stem cell like subpopulation in human esophageal cancer *in vivo* and *in vitro*, 2) SP cell biology may contribute to intrinsic and acquired chemotherapy resistance to 5-FU and cisplatin, and 3) SP cells mediated chemotherapy resistance was associated with changes in EMT regulation in esophageal cancer. 4) Aspirin might target SP cells directly by down regulating Wnt/β-catenin signaling.

The results presented suggest that long term application of chemotherapy may create acquired resistance in originally chemotherapy sensitive esophageal cancers by enrichment of SP related cells and thereby promote disease progression towards distant metastasis. Targeting SP cell mediated EMT activation may represent an efficient additional cancer therapy to overcome traditional chemotherapy resistance but also prevent disease progression towards distant metastases by eradicating the SP subpopulation (Figure V.2). Our results indicate in particular the application of Wnt inhibitors to target resistant side populations in esophageal cancer.

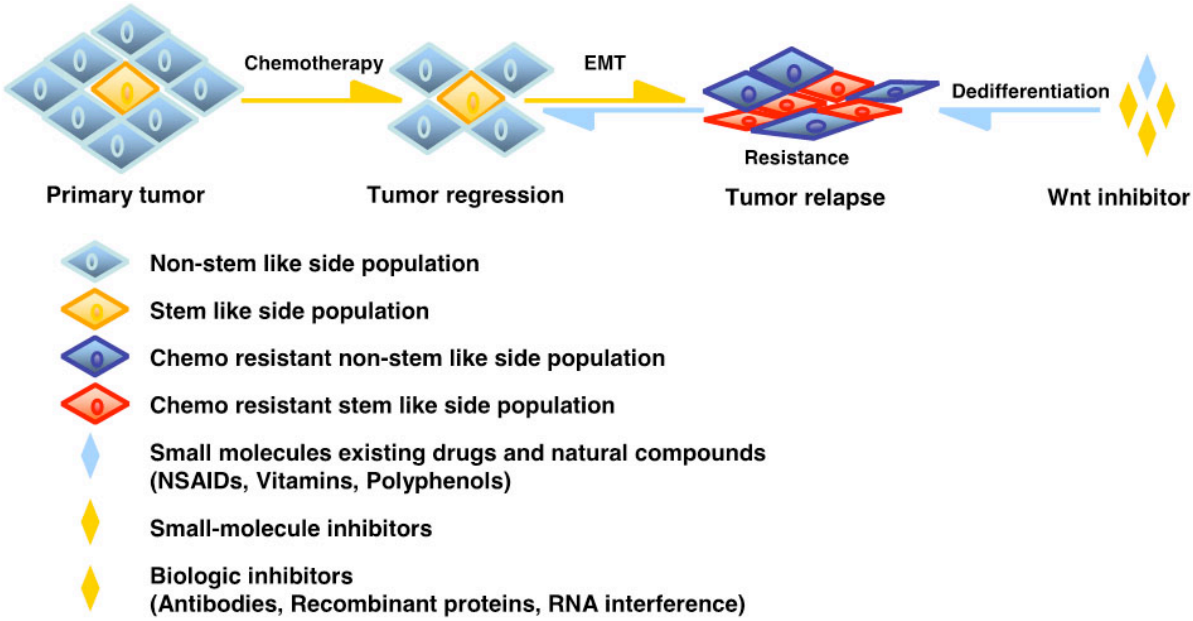

Figure V.2 The involvement of CSC, EMT and Wnt signaling in tumor progression. Traditional chemotherapy regresses the tumor size by targeting and killing rapidly dividing differentiated tumor cells, which constitute the bulk of the tumor, but fail to eradicate CSC populations. These subpopulation cells are thought to play a critical role in drug resistance and are associated with the acquisition of the EMT-like phenotype. Recent evidence correlated the interaction of Wnt/β-catenin signaling with both EMT and CSC-like phenotype. A number of existing drugs and natural compounds have been identified as inhibitors and/or modulators of Wnt signaling pathway, which might provide us a simple and safety strategy to overcome chemoresistance by eradicating CSC and reversing the EMT phenotype.

VI. SUMMARY

- Earlier studies from our group had identified SP cells from the highly metastatic pancreatic cancer cell line L3.6pl showed stem cell-like properties. This subpopulation was able to induce fast and aggressive tumor formation in nude mice. Gene expression analysis demonstrated a significant difference in microRNA expression of miR-21 and miR-221 between SP and non-SP (NSP) subtypes. Knock down of these miRNAs was achieved using selective antagomir transfection, which resulted in a significantly reduced SP cell fraction with decreased downstream target gene regulation. The treated cells showed reduced L3.6pl cell proliferation, invasion, and chemoresistance against gemcitabine and 5-Fluorouracil. SP cells from gemcitabine resistant L3.6pl cells ($L3.6pl_{Gres}$-SP) following miRNA-21 and/or -221 antagomir transfections were then orthotopically implanted in nude mice. Combined antagomir therapy significantly inhibited primary tumor growth and metastasis as compared to single antagomir treatment. These findings suggest that the inhibitions of miR-21 and miR-221 appear particularly suitable for the targeting of stem cell-like subpopulations of pancreatic tumors.

- In the second part of this study, the biology of SP subpopulations in esophageal cancer cell lines and their relation to chemotherapy resistance and metastasis was characterized. SP subpopulations were detected in five esophageal cancer cell lines OE19, OE21, OE33, PT1590, and LN1590. Chemotherapy-resistant cell lines were developed after long-term exposure to 5-FU and cisplatin and validated by analysis of their expression of the resistance markers thymidylate synthase and ERCC1. While neither LN1590 nor PT1590 cell lines showed detectable SP cells, OE19, OE21, and OE33 cells were found to contain varying levels of SP cells. With increasing duration of 5-FU or cisplatin therapy SP subpopulations were found to outgrow in the PT1590 and LN1590 lines. OE19-SP cells displayed significantly higher tumorigenicity than OE19-NSP cells following subcutaneous tumor cell injection *in vivo*. Cancer stem cells have been proposed to alter EMT status. The SP fraction of OE19/5- FU_{res} showed a dramatic up-regulation of EMT-related genes as compared to the SP fraction of OE19.

Our results provide evidence that SP cells exhibit stem cell-like properties and are associated to chemotherapy resistance. Long-term chemotherapy effectively selects for the outgrowth of SP cells, which then show an altered EMT gene profile, as compared to the parental cell line. These results suggest that cells similar to these subpopulations may be the source of systemic disease relapse. Targeting the EMT associated genes in SP cells in esophageal cancer via modulation of Wnt signaling may show efficacy in improving sensitivity of the tumors to standard chemotherapy regimens.

VII. ZUSAMMENFASSUNG

- In eigenen Vorarbeiten haben wir in der hoch-metastatischen humanen Pankreaskarzinom-Zelllinie L3.6pl die sogenannten ‘side population’ (SP) als Zellsubpopulation identifiziert, die Stammzelleigenschaften hat. Diese Tumorzellsubpopulation führte zu aggressivem Tumorwachstum im orthotopen Nacktmausmodel. Genexpressions-Analysen zeigten einen signifikanten Unterschied in der microRNA-Expression insbesondere von miR-21 und miR-221 zwischen SP und Non-SP-Zellen. Selektiver knock-down über Antagomir-Transfektion führte zu einer signifikanten Abnahme der SP-Subpopulation mit Abnahme der downstream induzierten Genexpression. Die transfizierten L3.6pl-Zellen zeigten eine reduzierte Proliferationsrate, Invasionsfähigkeit sowie Resistenz gegen Chemotherapie wie Gemcitabine und 5-FU. Gemcitabine resistente L3.6pl-SP-Zellen ($L3.6pl_{Gres}$-SP) wurden dann nach Transfektion mit Antagomirs gegen miR-21 und/oder miR-221 orthotop ins Pankreas von Nacktmäusen injiziert. Die kombinierte Antagomir-Transfektion führte zur signifikanten Inhibition des Primärtumorwachstums und der Metastasierung im Vergleich zur jeweiligen einzelnen Antagomir-Transfektion. Diese Ergebnisse lassen vermuten, dass die Inhibition von miR-21 und miR-221 beim Pankreaskarzinom sich insbesondere als gezielte Therapie für Stammzell-ähnliche Tumorzellsubpopulationen eignet.

- Der zweite Teil unserer Studien befasste sich mit der Biologie der SP Subpopulationen in Ösophaaguskarzinomzellinien und deren Verbindung zu Chemotherapieresistenz und Metastasierung. SP subpopulationen wurden in 5 verrschiedenen Ösophaguskarzinomzellinien (OE19, OE21, OE33, PT1590 und LN1590) untersucht. Des Weiteren wurden chemotherapie-resistente Zellinien durch langzeitige Behandlung mit 5-FU und Cisplatin generiert. Zur Validierung der Resistenzentwicklung wurden die entsprechenden Resistenzmarker Thymidylatsynthase sowie ERCC1 analysiert. Während weder LN1590 noch PT1590 Zellen primär nachweisbare SP Subpopulationen aufwiesen, enthielten OE19, OE21 und OE33 Zellen unterschiedliche prozentuale Anteile an SP Subpopulationen. Mit zunehmender Behandlungszeit durch 5-FU und Cisplatin ließ sich sogar eine langsam ansteigende Anzahl an SP Zellen in den beiden Zellinien PT1590 und LN1590

nachweisen. OE19-SP Zellen zeigten eine signifikant höhere Tumorigenität als OE19-NSP Zellen nach subkutaner Tumorzellinjektion. Des Weiteren hat sich herausgestellt, dass Tumorstammzellen den EMT Status verändern. Die SP Subpopulation von OE19/5- FU_{res} Zellen zeigte eine dramatische Hochregulierung EMT-relevanter Genen verglichen mit der SP Subpopulation von OE19 Zellen.

Unsere Ergebnisse zeigen, dass SP Zellen Stammzelleigenschaften haben und mit Chemotherapie-Resistenz vergesellschaftet sind. Langzeitgabe von Chemotherapie führt eindeutig zur Anreicherung von SP Zellen, die veränderte EMT-Genprofile im Vergleich zu SP Zellen aus parentalen Zellinien haben. Unsere Ergebnisse lassen die Vermutung zu, dass diese Subpopulationen möglicherweise die Quelle für Rezidiventwicklung sind. Durch gezielte Therapie der hochregulierten EMT-Gene in Ösophaguskarzinom-SP Zellen zum Beispiel durch Modulation des Wnt-Signaltransduktionsweges könnte die Sensitivität gegenüber Standardchemotherapie möglicherweise wiederhergestellt werden.

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ABBREVIATION

5-FU, fluorouracil

ABC, avitin biotin complex

Balb/C nu/nu, bagg-albino/c nude/nude

CD, cluster of differentiation

CDDP, cisplatin

CDK, cyclin-dependent kinase

cDNA, complementary desoxyriboic acid

CFU, colony-forming units

CT, cycle threshold

CSCs, cancer stem cells

CXCR, alpha chemokine receptor

DAPI, 4', 6-Diamidin-2-phenyl-Indol

DMEM, dulbecco's modified eagle medium

DMSO, dimethyl sulfoxide

DNA, desoxyribonucleic acid

FACS, fluorescence activated cell scan

FCS, fetal calf serum

EAC, esophageal adenocarcinoma

ELISA, enzyme-linked immunosorbent assay

EMT, epithelial to mesenchymal transitions

ESCC, esophageal squamous cell carcinoma

HE, hematoxylin and eosin

HLA-DR, human leukocyte antigens-DR (major histocompatibility complex, MHC class II)

HRP, horseradish peroxidase

hTERT, human telomerase reverse transcriptase

IFN-β, beta-Interferon

IL, interleukin

MHC, major histocompatibility complex

miRNA, micro ribonucleic acid

mRNA, messenger ribonucleic acid

MVD, microvascular density

NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells

PanIN, pancreatic intraepithelial neoplasia

PCR, polymerase chain reaction

PECAM-1, platelet-endothelial-cell-adhesion-molecule-1 (CD31)

RNA, ribonuecleic acid

RT-PCR, reverse transcriptase polymerase chain reaction

s.c., subcutaneous

SP, side population

TGF-β, transforming growth factor beta

TNF-α, tumor necrosis factor-alpha

SDF, stroma derived factor

Shh, sonic hedgehog

TNF, tumor necrosis factor

VEGF, vascular endothelial growth factor

TABLE OF FIGURES AND TABLES

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   06-10. April, 2013, Washington DC, US. Poster presentation

2. 2nd International Meeting on Molecular-Based Treatment of GI Cancer

   01-02. March, 2013, Göttingen, Germany. Poster presentation

3. 16th. Chirurgische Forschungstag

   04-06. October, 2012, Regensburg, Germany. Oral presentation

4. DGAV-Jahrestagung - Viszeralmedizin 2012

   09-22. September, 2012, Hamburg, Germany. Oral presentation

5. 47th Congress of the European Society for Surgical Research

   06-09. June, 2012, Lille, France. Oral presentation

6. 3rd EUNE Gastric Cancer International Workshop

   08-09. June, 2012, Köln, Germany. Poster presentation

7. 129th. Kongress der Deutschen Gesellschaft für Chirurgie

   22-25. April, 2012, Berlin, Germany. Oral presentation

8. The International Liver Congress 2012

   16-18. April, 2012, Bacerlona, Spain. Poster presentation

9. 30th. Deutscher Krebskongress 2012

   22-25. February, 2012, Berlin, Germany. Poster presentation

10. 15th. Chirurgische Forschungstage

    22-24. September, 2011, Dresden, Germany. Oral presentation

11. 14th. Chirurgische Forschungstage

23-25. September, 2010, Rostock, Germany. Poster presentation

12. 2008 International HongKong-Shanghai Liver Disease Congress

12-15. June, 2008, Hongkong, P.R. China. Poster presentation

Educational meetings and academic communications

1. Doktorandenkolloqium & Dienstagskolloqium in Surgery Department, LMU

   29. March, 2011, Munich, Germany. Oral presentation

2. Doktorandenkolloqium & Dienstagskolloqium in Surgery Department, LMU

   11. October, 2011, Munich, Germany. Oral presentation

3. Journal Club/Methodenseminar in Surgery Department, LMU

   30. January, 2010, Munich, Germany. Oral presentation

4. Academic visit in Zhongshan hospital with Prof. Jauch, Prof. Stiegler and Prof. Bruns, Shanghai, China

   08. March, 2011, Shanghai, China. Oral presentation

5. Doktorarbeitstag Medizin, LMU

   14. May, 2011, Munich, Germany. Poster presentation

6. CSC-LMU program auditing seminar, LMU

   23-24. May, 2011, Munich, Germany. Oral presentation

Publications

1. **Zhao Y**, Bao Q, Schwarz B, Zhao L, Mysliwietz J, Ellwart J, Renner A, Hirner H, Niess H, Camaj P, Angele M, Gros S, Izbicki J, Jauch KW, Nelson PJ, Bruns CJ.. Stem cell like side populations in esophageal cancer: a source of chemotherapy resistance and metastases. **Stem Cells Dev**. 2013 Sep 10. Epub ahead of print.

2. Bao Q*, **Zhao Y***, Niess H, Conrad C, Schwarz B, Jauch KW, Huss R, Nelson PJ, Bruns CJ. Mesenchymal stem cell-based tumor-targeted gene therapy in gastrointestinal cancer. **Stem Cells Dev**. 2012 Sep 1;21 (13):2355-63. (* Co-first authorship)

3. 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. Epub ahead of print. German.

4. Bao Q, Niess H, Djafarzadeh R, **Zhao Y**, Schwarz B, Angele MK, Jauch KW, Nelson PJ, Bruns CJ. Recombinant TIMP-1-GPI inhibits growth of fibrosarcoma and enhances tumor sensitivity to doxorubicin. Target Oncol. 2013 Aug 10. Epub ahead of print

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Manuscripts in preparation

1. **Y Zhao**, P Camaj, L Zhao, I Ischenko, Q Bao, B Schwarz, H Nieß, Y Wang, K-W Jauch, P J. Nelson, J W. Ellwart, C J. Bruns. Antisense inhibition of microRNA-21 and -221 in tumor-initiating stem-like cells modulates biological functions of pancreatic cancer including tumorigenesis, metastasis, and chemoresistance. *Submitted.*

Patent

Haplotypes and reconstruction of OPN in assay and application of HCC prediction

Qiong Zhu Dong, Lun Xiu Qin, Hu Liang Jia, **Yue Zhao**, Zhao Wei Yan, Xiao Fei Zhang.

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