Aus der Chirurgischen Universitätsklinik Heidelberg
Klinik für Allgemein, Viszeral und Transplantationschirurgie
(Ärztlicher Direktor: Prof. Dr. med. Dr. h.c. mult. M.W. Büchler)

Simvastatin targets pancreatic cancer stem-like cells and sensitizes PDA cells to chemotherapeutic drugs via Sonic hedgehog signaling

Inauguraldissertation
zur Erlangung des Doctor scientiarum humanarum (Dr. sc. hum.)
an der
Medizinischen Fakultät Heidelberg
der
Ruprecht-Karls-Universität

vorgelegt von
Yefeng Yin
aus
Shanxi
Volksrepublik China
2017

Dekan: Prof. Dr. med. Wolfgang Herzog

Doktormutter: Prof. Dr. rer. nat. Ingrid Herr

DECLARATION BY THE CANDIDATE

I hereby declare that this thesis is my own work and effort. Where other sources of information have been used, they have been indicated or acknowledged.

Signature: _____

Date: _____

"Real knowledge, like everything else of value, is not to be obtained easily, it must be worked for, studied for, thought for, and more than all, must be prayed for."

– (Thomas Arnold)

To my Beloved

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. ABBREVIATIONS

ABBREVIATION	FULL NAME
5-FU	5-fluorouracil
ABC	ATP-binding cassette
AEC	Sodium POE 10 fatty alcohol ether carboxylate
ATCC	American type culture collection
AML	Acute myeloid leukemia
AKT	Protein kinase B
ALDH1	Aldehyde dehydrogenase 1
β-actin	Beta-actin
BCA	Bicinchoninic acid
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CAM	Chorioallantoic membrane
CSC	Cancer Stem-like Cell
CD133	Prominin-1
CD24	Cluster of differentiation 24
CD44	Cluster of differentiation 44
c-Met	Mesenchymal-epithelial transition factor and membrane receptor
CO	Control
CO_2	Carbon dioxide
Dapi	4'-6-diamidino-2-phenylindole
dH_2O	Distilled water
Dhh	Desert hedgehog
DNase	Deoxyribonuclease
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
dsDNA	Double-stranded DNA
DMEM	Dulbecco's modified Eagle's medium
EDD	Embryonic Development Day

EGF	Epidermal growth factor
EDTA	Ethylenediaminetetraacetate
EMT	Epithelial-mesenchymal-transition
FCS	Fetal calf serum
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEM	Gemcitabine
HCl	Hydrochloric acid
H&E	Hematoxylin and eosin
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
IF	Immunofluorescence
IgG	Immunoglobulin G
Hh	Hedgehog
IHC	Immunohistochemistry
Ihh	Indian hedgehog
mRNA	Messenger RNA
NaCl	Sodium chloride
NBT	Nitro blue tetrazolium
p53	Tumor protein p53
PDA	Pancreatic Ductal Adenocarcinoma
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
pH	Potential of hydrogen
Ptch	Patched
SCID	Severe combined immunodeficient
siRNA,	Small interfering RNA
Shh	Sonic hedgehog
siCO	Small interfering RNA Negative control
Smo	Smoothened
RT-PCR	Reverse transcription-polymerase chain reaction
RNA	Ribonucleic acid
RT	Room temperature

SD	Standard deviation
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline
UV	Ultraviolet
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

Units

°C	centigrade or degree celsius
cm	centimeter
g	gravitational acceleration ($9.81ms^{-2}$)
h	hours
KDa	kilo Dalton
L	liter
M	molar [mol/L]
mA	milliampere
min	minutes
mL	milliliter
mm	millimeter
mM	millimolar
µ	micro
µg	micrograms
µL	microliter
µm	micrometer
µM	micromolar
ng	nanogram
nM	nanomolar
rpm	rounds per minute
s	seconds
V	Volt

2. INTRODUCTION

2.1 Pancreatic cancer and therapy management

2.1.1 Epidemiology and demographics

As a malignant disease with a very bad prognosis, pancreatic cancer is rising to the third leading cause of cancer death among both men and women in the United States [1]. In 2012, around 16,700 patients were diagnosed with pancreatic cancer in Germany. According to a recent study, the major part of the new cases have been observed in Europe (30.7%), China (19.5%), and North America (14%) [2]. Besides, the incidence of pancreatic cancer is still expanding worldwide during the past years. It is assumed that pancreatic cancer would turn into the second leading lethal cancer after lung cancer in the US, surpassing colorectal, breast, and prostate cancer by 2030 [3]. Around the world, 338,000 new pancreatic cancer cases were diagnosed and 330,000 died of this disease approximately every year [2]. The high morbidity and mortality underlines the pressure demand of studying this disease. The risk factors for pancreatic ductal adenocarcinoma (PDA) include but are not limited to ethnicity and race [4], medical conditions [5], history of pancreatitis, obesity, helicobacter pylori infection, hepatitis B, human immunodeficiency virus (HIV), cirrhosis, hereditary [6], Lynch syndrome, Peutz-Jeghers syndrome, family history of pancreatic cancer, Li-Fraumeni syndrome, tobacco use, alcohol use, high fat and cholesterol diet [7]. Pain and weight loss are the most typical symptoms of pancreatic cancer, while the most common clinical sign is jaundice. One review has revealed the following frequency of signs and symptoms in patients with PDA, which is shown is Table 1 [8].

Table 1: Symptoms and signs in patients with PDA

Sign and symptom	Proportion	Sign and symptom	Proportion
Weakness/fatigue (asthenia)	86%	Weight loss	85%
Loss of appetite (anorexia)	85%	Dark urine	59%
Abdominal pain	79%	Jaundice	56%
Nausea	51%	Back pain	49%
Diarrhea	44%	Vomiting	33%

However, the giving signs and symptoms mentioned above are not special for pancreatic cancer diagnosis. That is the major reason why pancreatic cancer is mainly diagnosed in an advanced stage.

2.1.2 Pathology and staging

PDA is the most common type of pancreatic malignancies, attributing more than 85% in all types of pancreatic cancer [9]. Therefore, the name "Pancreatic Cancer" is regularly used to refer to PDA [10]. PDA is an epithelial tumor that emerges from pancreatic duct or ductal cells, from which the overwhelming majority of pancreatic neoplasms originate (Figure 1). The most common precursor lesion is PanIN, it was divided into three grades: PanIN-1, PanIN-2, and PanIN-3 according to the degree of epithelial atypia.

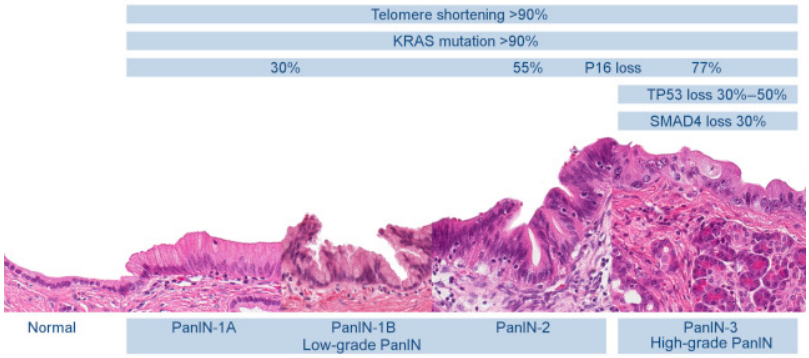

Figure 1: Majority of PDA cells originate from pancreatic duct or ductal cells (Adapted from [9])

An attempt of staging PDA should be made after a verified or highly suspected diagnosis of PDA. Based on the 7th edition of the American Joint Committee on

Table 2: American Joint Committee on Cancer staging system

	AJCC stage	Anatomic stage			Proportion	Median survival time
		T	N	M		
Early stage	Stage 0	Tis	N0	M0	10%	17-23 months
	Stage IA	T1	N0	M0		
	Stage IB	T2	N0	M0		
	Stage IIA	T3	N0	M0		
	Stage IIB	T1	N1	M0		
		T2	N1	M0		
		T3	N1	M0		
Advanced stage	Stage III	T4	Any N	M0	30%	8-14 months
	Stage IV	Any T	Any N	M1	60%	4-6 months

Primary Tumor (T); T0: No evidence of primary tumor; Tis: Carcinoma in situ; T1: Tumor limited to the pancreas, 2 cm or less in greatest dimension; T2: Tumor limited to the pancreas, more than 2 cm in greatest dimension; T3: Tumor extends beyond the pancreas but without involvement of the celiac axis or the superior mesenteric artery; T4: Tumor involves the celiac axis or the superior mesenteric artery**; Regional Lymph Nodes (N);** N0: No regional lymph node metastasis; N1: Regional lymph node metastasis**; Distant Metastasis (M)**; M0: No distant metastasis ;M1: Distant metastasis (Adapted from [10]**).**

Cancer (AJCC), the tumor-node-metastasis (TNM) staging system is most often used for staging pancreatic cancer, which is shown in Table 2 [10]. All the patients in stage IV and patients in stage III with tumor spread into surrounding tissues are defined as advanced stages. Patients in advanced stages, which account for about 60-90% among all PDA patients, indicate a poorer prognosis and less survival time.

2.1.3 Current therapy for pancreatic cancer

There are five types of standard treatments available for PDA patients: surgery, radiation therapy, chemotherapy, chemoradiation therapy and targeted therapy. In spite of developing studies of the pancreatic cancer biology, advancement in early diagnosis and the increase of pancreatic cancer centers, the origin of this disease is still not fully clear and the prognosis of pancreatic cancer remains extremely poor [11]. Even now, only complete surgical resection of the tumor is the definitive therapy for PDA. Nevertheless, the clinical outcome remains rather pessimistic, because only around 10-20% of the PDA cases have indication to undergo surgical resection [12]. To extend survival time or improve quality of life, chemotherapy is implied in the treatment of PDA in all cases [13, 14]. Gemcitabine is considered as the standard chemotherapy drug in the pancreatic cancer treatment [14]. After gemcitabine was first synthesized during the early 1980s it showed efficacy to kill leukemia cells in the preclinical studies [15]. In 1993, gemcitabine was first applied to treat pancreatic cancer [16]. Gemcitabine has been the cornerstone chemotherapeutic agent for pancreatic adenocarcinoma based on clinical benefit compared with 5-FU [17]. Since the approval of gemcitabine monotherapy as first-line treatment by the Food and Drug Administration (FDA) in 1996, gemcitabine and several combination therapies (for example: erlotinib, 5-FU and cisplatin) based on gemcitabine have been widely used in the treatment of many types of cancer, including breast cancer, lung cancer, ovarian cancer and pancreatic cancer [18-21]. Chemically gemcitabine is a nucleoside analog in which the hydrogen atoms on the 2' carbon of deoxycytidine are replaced by fluorine atoms (Figure 2).

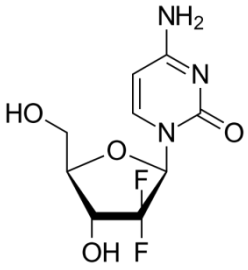

Figure 2: Molecular structure of gemcitabine
4-amino-1-(2-deoxy-2, 2-difluoro-β-D-erythro-entofuranosy l) pyrimidin-2(1H)-on. Fluorine atoms replace the hydrogen atoms on the 2' carbon of deoxycytidine.

The major mechanism of gemcitabine is the inhibition of DNA synthesis by incorporating of gemcitabine diphosphate (dFdCTP) into DNA. An additional mechanism of gemcitabine is self-potentiation by binding the active site of ribonucleotide reductase, decreasing activity and the synthesis of deoxy ribonucleotides, which is required in DNA replication and reparation [22]. In 1997, the results of a phase II trial were very encouraging. Compared to pancreatic cancer patients treated with 5-flurouracil (5-FU), patients treated with gemcitabine had a significantly better clinical response (from 4.8 to 23.8%) as well as a significant increase of median survival time (from 4.41 to 5.65 months) and survival rate (from 2 to 18%) [23]. In addition, patients tolerated the mild toxicity of gemcitabine well. From then on, more and more clinical and laboratory experiments demonstrated that gemcitabine was an effective chemotherapy drug for pancreatic cancer. Surprisingly, all efforts trying to improve the effects of gemcitabine in the clinic failed to show synergistic effects for example: gemcitabine plus erlotinib [24], 5-FU [25] or cisplatin [26]. Until now, gemcitabine is still considered as one of the standard and most important cytotoxic drugs in the treatment of advanced pancreatic cancer and widely used in the clinic. However, less than 25% of PDA patients benefit from gemcitabine treatment in total and the overall survival rate is still not improved remarkably. The current agents that are the standard of care in this setting are gemcitabine, or a combination of 5-FU and leucovorin. FOLFIRINOX (5-FU, leucovorin, irinotecan, oxaliplatin) illustrated a survival benefit for patients with metastatic or unresectable PDA despite its increased toxicity compared to gemcitabine alone [27]. Radiotherapy presents an alternative adjuvant treatment but the benefit of radiation in PDA is somewhat controversial because randomized trials

have suggested inconsistent outcome in locally advanced pancreatic cancer [13]. Therefore, new and better therapeutic options are required.

2.2 Cancer stem-like cells

Cancer stem-like cells (CSCs), also known as cancer-initiating cells or tumor-initiating cells [20] are defined as a group of cells within a tumor mass that possesses the capability of unlimited self-renewal and resistance to standard chemotherapy in both primary tumor and metastases and is able to reconstitute tumor growth *in vitro* and *in vivo* [28-32]. The stem-like characteristics of CSCs are sufficient to assimilate them into stem cells. Moreover, the discovered experimental and clinical features of metastatic cancer cells are very close to the established features of stem cells [21]. The fundamental hypothesis implies that CSCs drive metastasis and recurrence even when all signs of the cancer have been relieved by surgery or conventional chemotherapy (Figure 3). By targeting CSCs, cancer could regress due to differentiation and/or cell death [33].

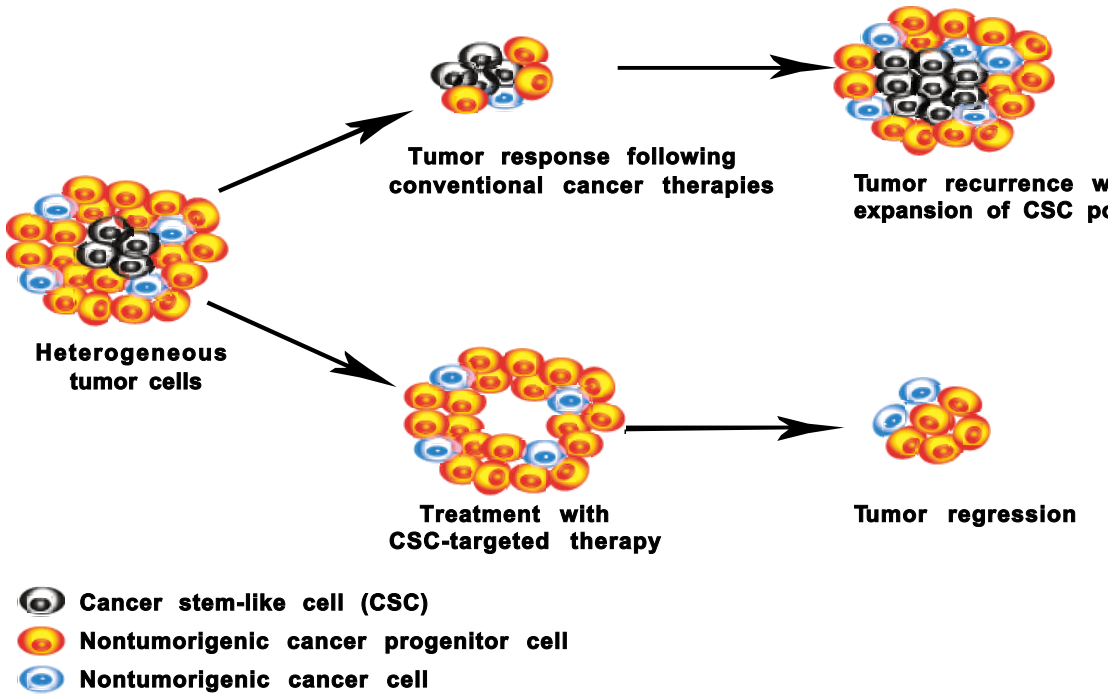

Figure 3: The cancer stem-like cell hypothesis

The cancer stem-like cell (CSC) hypothesis implies that CSC can generate a tumor, because this small group of cancer cells is capable of enormous proliferative potential and self-renewal properties. This theory suggests that targeting these subpopulations of cancer stem-like cells could destroy the tumor long-term without the necessity to fight the whole tumor (Modified from [33]).

In 1994, Lapidot and colleagues first identified CSCs in acute myelogenous leukemia (AML) by using cell-surface protein markers to identify a small group of stem-like

cells and detected that leukemic cell growth could be induced only by $CD34^+/CD38^-$ AML cells in SCID mice. These subpopulations of cells were also supported to have the capacities of self-renewal and differentiation in mouse models. In 2003, CSCs were first discovered in solid tumor by Al-Hajj and colleagues [34]. $ESA^+/CD44^+/CD24^-$ cells obtained from primary human breast cancer were identified as breast CSCs because these cells exhibited greater tumorigenicity than other cancer cells. Subsequently, various kinds of solid tumor CSCs were identified in brain, lung, colon, liver, prostate and ovarian cancers [35-39].

2.2.1 Pancreatic cancer stem-like cells

Increasing evidence has cropped up to prove the presence of CSCs in pancreatic cancer [40, 41]. In 2007, Li and colleagues were the first to identify $CD44^+/CD24^+/ESA^+$ cells as CSCs in pancreatic cancer [42]. These cells accounted for only less than 1% of total PDA cells and showed the stem cell properties of self-renewal and generating differentiated progeny. Hermann and colleagues proved that $CD133^+$ cells were highly resistant to standard chemotherapy and exclusively PDA tumorigenic [43]. Afterwards, various pancreatic cancer stem-like cell sub-population were identified using different methods. For instance, side population (SP) cells which can efflux Rhodamine 123 or Hoechst 33342 were found possessing features of cancer stem-like cells in pancreatic cancer [44]. $CD24^+/CD44^+$ [45] or c-Met^+ [46] or $Gdeg^{high}$ [41] were identified as the pancreatic cancer stem-like cell surface markers. Nanog and nuclear transcription proteins Oct4 were also proved to be associated with stemness of pancreatic cancer cells [47]. Cellular markers such as c-Met, ESA, EpCAM, ALDH1, nestin, CD24, CD44, CD133 and Lgr5 have been used to characterize pancreatic CSCs [41, 42, 48-50]. They possess several characteristics of carcinogenesis process such as self-renewal, proliferation, immortality and many signaling pathways. Signaling pathways like Sonic hedgehog, PTEN, Notch, PI3K/AKT and Wnt are altered by pancreatic cancer stem-like cells [51, 52]. The regulatory mechanisms triggering the CSCs are poorly defined for most types of cancer, it has been implicated that several key developmental signaling pathways related to normal stem and progenitor functions play important roles in CSCs regulation. In addition, sphere and colony formation assays are considered as partly mimic tumorigenicity *in vitro* [53].

2.3 Hedgehog signaling and pancreatic cancer

2.3.1 Introduction

In 1995, the Nobel Prize in Medicine was awarded for discoveries concerning "the genetic control of early embryonic development". Two Nobel laureates, Eric F. Wieschaus and C. Nüsslein-Volhard, isolated mutations in genes that control the segmentation pattern of Drosophila melanogaster embryos. Their mutagenesis screen discovered that loss of a gene function later to be called Hedgehog. There are three Hedgehog (Hh) genes named by the prefixes Indian (Ihh), Desert (Dhh) and Sonic (Shh) [54]. After translation as an around 46-kDa precursor, in addition to the removal of the signal peptide, Hh proteins are further processed by internal cleavage to generate two fragments. The 19 kDa N-terminal fragment (N-Hh) has all the known signaling activity of Hh. While the 25 kDa C-terminal fragment (C-Hh) undergo post-translational modifications by adding cholesterol at the C-terminus of N-Hh (N-Hh^{chol}) and engage in this processing reaction [55-63]

Canonical Hh pathway activation is originated by the binding of secreted Hedgehog molecules to a transmembrane receptor called Patched (Ptch), which relieves its inhibition on Smoothened (Smo), another transmembrane protein that is suppressed by the Ptch in the absence of Hh. Therefore, Smo operates its function only when Hh is present. Smo transduces the signal intracellularly through Gli zinc finger transcription factors. Consequently, activating Smo initiates an intracellular signaling cascade multiplying the formation of activated forms of Gli2 and Gli3, which directly induce Gli1. Three transcription factors composed Gli family and act as key mediators of Hh signaling in cancer by regulating the expression of target genes in which only Gli1 is a solely full-length transcriptional activator. Gli2 and Gli3 can be partly processed into truncated repressor forms [64, 65]. Finally, Suppressor of Fused (Sufu), is a key negative regulator of Hh signaling activity, controls the activation of the Gli transcription factors as a tumor suppressor gene. Sufu could prevent Hh pathway activation by directly binding the Gli transcription factors and inhibiting their translocation to the nucleus [66]. The binding of the Gli proteins with Sufu in the cytoplasm will promote processing and/or degradation of them and thus inhibit Hh signaling [67].(Figure 4).

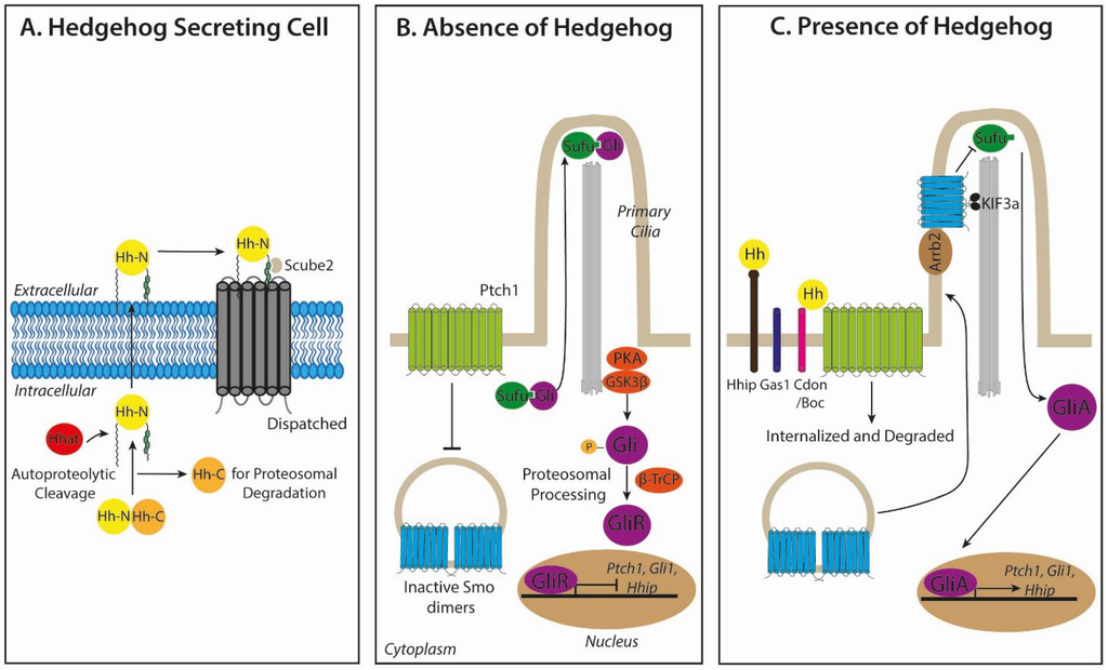

Figure 4: Mammalian Hedgehog (Hh) signaling

A. Hh ligand protein precursors are autoproteolytically cleaved to produce an N-terminal protein that goes through dual lipid modification, consisting of an N-terminus and a C-terminal cholesterol group, which advances the binding of Hh to sterol-rich membrane microdomains to restrict mobility. Then Dispatched in synergy with Scube2 mediates the release of active Hh ligand. **B**. In the absence of Hh, Ptch represses Smoothened (Smo), inhibiting its ciliary localization. In this case, Gli proteins are kept in a complex with Sufu at the ciliary tip. The recruitment of glycogen synthase kinase 3β (GSK3β), protein kinase A (PKA) and β-transducin repeat-containing protein (β-TrCP) to this complex lead to partial proteasomal degeneration to form Gli transcriptional repressors (GliR) that translocate to the nucleus and inhibit Gli target genes. **C**. In the presence of Hh, the inhibition of Smo is relieved by binding of Hh to its receptor Ptch1. Activation of Smo assists the release of Gli from Sufu, and then full-length Gli activators (GliA) transfer from plasmid to the nucleus and active Hh target genes (Adapted from Ref. [68]).

2.3.2 The Hedgehog signaling in pancreatic cancer

Hh signaling is an evolutionarily-conserved pathway essential for self-renewal and cell fate determination and a key pathway critical in embryonic development, stem cell biology and tissue homeostasis, cellular metabolism, synapse formation and nociception [69-73]. Sonic hedgehog signaling is conserved in vertebrates and highly active during mammalian development, but this signal usually shuts down after birth [74]. In recent years, aberrant activation of Shh signaling has been implicated in multiple aspects of cancer progression, from initiation to metastasis [64], including the maintenance of CSCs [68]. The dysregulation of Shh signaling is also known to drive several types of cancer, including basal cell carcinoma, breast, ovarian, lung, prostate, liver, gastrointestinal and bladder cancer [64, 65, 75-77]. Moreover, recent studies reveal that Hedgehog inhibition significantly prolonged survival in a mouse

model of pancreatic cancer [78] and inhibited pancreatic cancer invasion and metastatic spread [54, 78-83]. The precise mechanism of how Hh acts in pancreatic cancer is not completely clear yet may be caused by autocrine signaling, paracrine signaling or selective activation of cancer initiating stem cells. Additionally, mutant Kras activates Gli1 in pancreatic cancer [84-86], and Gli activity could be regulated by altering the activation of pathways which interact with Hedgehog signaling through intercellular cross talk, such as the mitogen-activated protein kinase RAF/MEK signaling pathway [54].

2.4 Cholesterol synthesis and metabolism in cancer

2.4.1 Normal cholesterol homeostasis

Cholesterol is mainly synthesized in the liver. Nevertheless, genome and/or transcriptome alterations cannot directly reflect cholesterol homeostasis, since lipids are not genetically encoded [87]. Cholesterol is transported to cells over the body through the bloodstream as a form of low-density lipoprotein (LDL)-bound [88, 89]. LDL is transported into cells by clathrin-mediated endocytosis and hydrolyzed to free cholesterol molecules after being transported to lysosomes through endocytic pathway. Afterwards, the free cholesterol molecules are transported to the cell membrane and cell membrane-bound organelles [87, 88, 90]. A tightly controlled protein network regulates cholesterol homeostasis, including its biosynthesis, import, export, metabolism and esterification [87]. Liver X receptors together with sterol regulatory element-binding protein transcription factor 2 (SREBF-2) play important roles in the regulation of cholesterol homeostasis [87, 90]. Endoplasmic reticulum (ER) cholesterol level plays a role as a sensor for intracellular cholesterol homeostasis. Sterol regulatory element binding protein 2 (SREBP-2) is translocated from ER to Golgi and then to the nucleus when ER cholesterol level decreased and this process results in transcription of genes involved in cholesterol synthesis, such as HMG-CoA. It was proved that SREBP-2 activity had a strong association with cell viability in human prostate tumor cells, on the other hand, cholesterol synthesis can also be turned off by increased intracellular cholesterol level, meanwhile assists cholesterol export via activation of LXR receptors by oxysterols [91].

2.4.2 Role of cholesterol metabolites in cancer development

The metabolic alteration from catabolic to anabolic metabolism has been considered as a classic hallmark of cancer [92]. With its complex biosynthesis and metabolism [89], cholesterol is not only important in the pathogenesis of cardiovascular diseases and diseases of brain vascular, but has been also involved in diabetes, dementias, and development of cancer types [90, 93, 94]. Cholesterol intake is indicated to increase the risk of breast cancer and induce tumor growth and metastasis in mouse breast cancer model by upregulating the level of oxysterol 27-hydroxycholesterol (27HC), which is a main cholesterol metabolite (Figure 5). Besides, cholesterol is the precursor of estrogen, and high blood level of estrogen is associated with an increased risk of breast cancer [96].

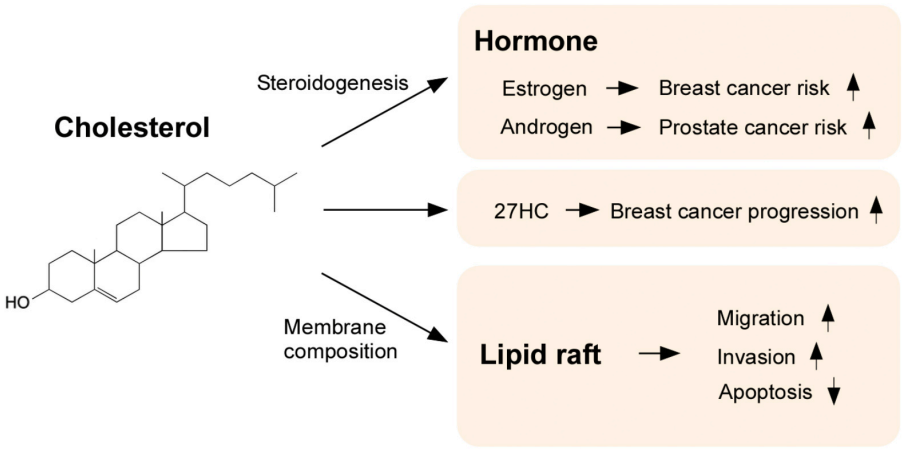

Figure 5: Potential signaling of cholesterol in cancer

Importantly, cholesterol is also the precursor of steroidogenesis, which can produce androgen, and the activation of androgen receptor by androgen results in prostate cancer cell proliferation and invasion [95, 96]. The mevalonate pathway is aberrant upregulated in prostate cancer cells, clinical and experimental evidence indicated a positive association between higher serum cholesterol level and higher risk for several cancer types [97-99], a 10 mg/dL increase in cholesterol contributed to a 9% rise in prostate cancer recurrence [99]. PDA cells are also highly dependent on cholesterol uptake through low-density lipoprotein receptor [100]. In PDA cells, reduction of cholesterol uptake or interruption of its distribution by blocking low-density lipoprotein receptor (LDLR), result in inhibition of cancer cell proliferation and ERK1/2 survival pathway. [101]. However, the role of cholesterol metabolites in carcinogenesis and tumor development needs expansion as well as the involvement

of different metabolites in various cancer types. Targeting the biosynthesis, transport, or metabolism of the cholesterol homeostasis pathways are options for controlling cancer development.

2.4.3 Targeting cholesterol synthesis in cancer

There are more than 15 proteins in cholesterol synthesis pathway, giving multiple potential targets to interrupt the cancer cellular pathway [88]. The proteins in the upstream process of cholesterol biosynthesis, such as mevalonic acid, farnesyl pyrophosphate and geranylgeranyl pyrophosphate, are essential for lipid modification and various cancer related signaling, including GPCR, PI3K, Akt, Ras and other GTPases signaling. HMG-CoA reductase is one of the most important key proteins in cholesterol synthesis, the chemotherapeutic potential of targeting these cholesterol synthesis genes has been extensively explored preclinically [102-104]. A recent example is that modulated cholesterol levels control cancer development, involving a short-term biomarker simvastatin, which reduces breast cancer recurrence by downgrading serum estrone sulfate levels [105]. However, long-term studies are needed to confirm this observation. Bisphosphonates and tocotrienols are examples of downstream inhibitors of the cholesterol synthesis pathway, which in preclinical studies suppress cultured cancer cells and tumor growth similar to the observation with statins [106, 107]. Thus, preclinical studies suggest that targeting the cholesterol synthesis pathways could be useful for controlling cancer.

2.4.4 Role of cholesterol in Hh signaling

HMG-CoA reductase controls the conversion of HMG-CoA to mevalonic acid, which is the precursor for the biosynthesis of several fundamental end-products including cholesterol, isoprenoids, dolichol, ubiquinone, and isopentenyladenine [108]. All of these end-products play crucial roles in promoting carcinogenesis. Statins are competitive inhibitors of HMG-CoA reductase widely used as cholesterol-lowering medications [109]. Cholesterol plays an important role in the Hh pathway because of its covalently modification with active Hh proteins, making this post-translational modification unique among all known proteins [56]. After synthesized as 49 kDa premature proteins, the precursors of Hh proteins firstly pass through a signal peptide cleavage after reaching the ER to create a C-terminal autoprocessing domain (Hh-C)

and an N-terminal signaling domain (Hh-N). The C-terminal has auotoproteolytic activity, which catalyzes an intramolecular splicing reaction by attaching a cholesterol moiety to the newly produced Hh-N C-terminus. The cholesterol-modified N-terminal domain further undergoes palmitoylation at the N-terminus producing a highly hydrophobic and activity protein [110]. Cholesterol is essential to maintain this cleavage process and the activity of Hh proteins, which play an important role in CSCs, suggesting that simvastatin may contribute to the elimination of pancreatic CSCs via Hedgehog pathway; however, to our knowledge, the mechanisms involved in the ant-proliferative and anti-CSC actions of simvastatin have not yet been fully studied. Hh requires cholesterol to enable active forms and function. Thus, lowering cholesterol levels by statin drugs such as simvastatin is a crucial and possible mechanism for inhibiting Hedgehog signaling and cancer.

2.5 The role of statins in cancer

2.5.1 Introduction of statin

The first statin is mevastatin, which was isolated by Akira Endo from the fungus Penicillium citrinium in 1976 [111]. In 1979, lovastatin was isolated from a strain of the fungus Aspergillus terreus by Hoffman and colleagues. During developing and researching lovastatin, simvastatin was synthesized from a fermentation product of A. terreus by scientists from Merck, which is a more efficient HMG-CoA inhibitor. [112]. There are currently seven types of statins approved by the FDA, namely pravastatin, fluvastatin, atorvastatin, lovastatin, rosuvastatin, pitavastatin and simvastatin (Figure 6). Notably, cerivastatin, which was approved in 1997, was withdrawn from the market worldwide in 2001 because the use of it was linked to rhabdomyolysis, which lead to kidney failure.

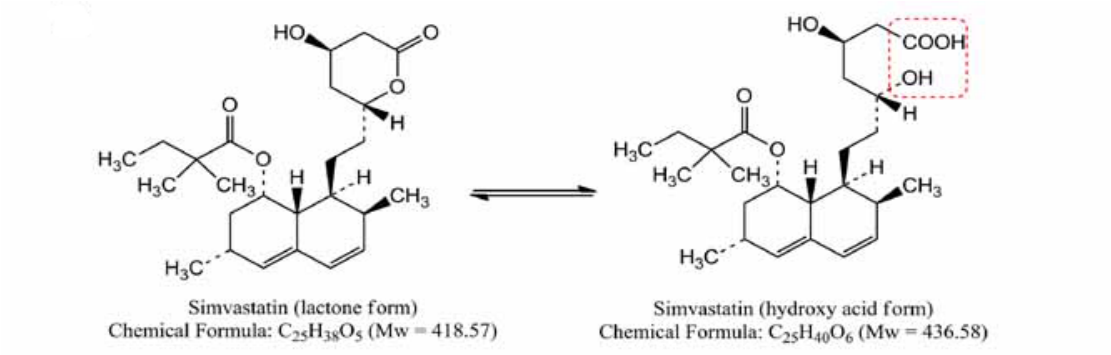

Figure 6: Structure of the lactone and hydroxyl acid form of simvastatin (Adapted from [113])

2.5.2 Effect of statins in cancer metabolism

As the rate-limiting enzyme, the inhibition of HMG-CoA reductase by statins prevents the transformation of HMG-CoA to mevalonate in the mevalonate pathway, and consequently prevents the formation of mevalonate and products derived from it. (Figure 7) [108].

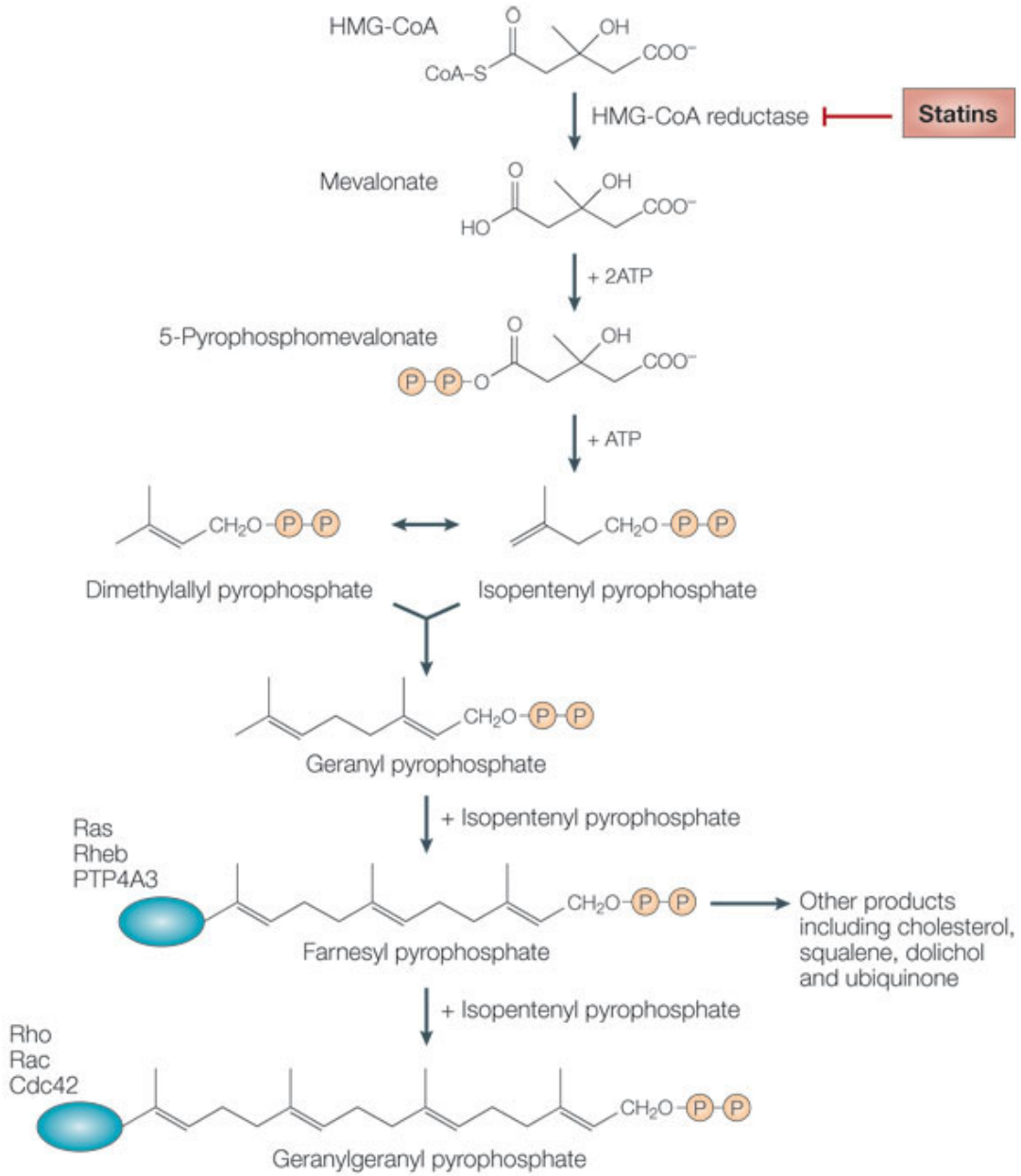

Figure 7: Statins inhibit the conversion of HMG-CoA to mevalonate

Statin's inhibition of mevalonate pathway decrease levels of both mevalonate and its downstream products (Adaped from Ref. [114]).

A lot of products from the mevalonate pathway are required for important cellular functions, including protein synthesis, membrane integrity, cell signaling, and cell cycle progression [108, 115]. Statin's interruptions of these processes in tumor cells may lead to control of tumor initiation, growth, and metastasis [115]. Inhibition of the mevalonate pathway by statins has the potential to decrease the cancer risk and prevent the recurrence of aggressive cancers. The localization and function of mevalonate downstream products are dependent on isoprenylation and the inhibition of downstream products may mediate the pleiotropic effects of statins, such as Ras, Rho, Rac and small GTP-binding proteins. It was found that statin showed antiproliferative, pro-apoptotic and anti-invasive effects in preclinical studies and animal models of different cancer types. Importantly, statins exhibited targeted action mainly in cancerous cell lines compared to non-malignant cells. Recent findings also indicated that statins had synergistic effects when they were used together with chemotherapeutic agent and radiotherapy. Same effect was also detected in chemotherapy-resistant tumors. These observed synergistic effects of statins to both chemotherapy and radiation might be mediated by arresting cell cycle progression at G0/G1 phase [116-119]. Likewise, population-based clinical studies also suggested that patients were benefited from statin treatment in many tumor types [120-126].

2.5.3 Anti-cancer efficacy of statins

In 1988, the association between cancer risk and statin use in animal model was firstly observed by MacDonald and his colleagues. They found that more lovastatin use was related to a higher incidence of pulmonary cancer [127]. Likewise, simvastatin induced follicular adenomas in rats [128]. However, in these studies statin-related cancer risk was limited too much higher dosages than that used in clinical treatment. Lots of studies *in vitro* found that statins had anti-carcinogenic activities. The promising antitumor effects of statins in preclinical studies have inspired investigations into their potential consequences as an antitumor agent in animal models. There are numerous studies about different satins in preclinical studies. Among all statins, simvastatin was suggested to exert the highest tumor-suppressive effects *in vitro* and *in vivo* [129, 130].

A mouse melanoma model proved that simvastatin inhibits tumor growth and metastasis. Furthermore, simvastatin was suggested to significantly improve the

survival rate in a mouse melanoma metastasis model by inhibiting Rho signaling pathways [131]. Borahay MA and his/her colleagues suggested that treatment with simvastatin (20 mg/kg/day) reduced expression of the proliferation marker Ki67 in xenograft tissue from uterine fibroid tumors [132]. Investigators suggested that simvastatin inhibits tumor growth at a high dosage (40 mg/kg/day) in breast cancer and implied that this effect may be related to simvastatin's inhibition of CSCs [133]. Similar result was found in another study, in which simvastatin reduced metastasis formation *in vivo* [134]. In the study of V. Fendrich and colleagues, simvastatin could significantly delay progression of pancreatic cancer in mice. These results indicate that administration simvastatin could be an effective strategy for the inhibition of cancer progression. The association between statins and cancer risk regarding different types of cancer *in vivo* was summarized in Table 3.

Table 3: The related anti-cancer effect of simvastatin *in vivo*

Effect	Statin	Tumor	Target	Ref
Inhibition of proliferation Anti-metastasis effect	Simvastatin Fluvastatin	Metastatic melanoma	Rho	[131]
Anti-metastasis effect	Simvastatin	Breast cancer	FOXO3a	[134]
Inhibition of proliferation Induction of apoptosis	Simvastatin	Uterine leiomyoma	Akt	[132]
Anti-invasion effect	Simvastatin	Pancreatic cancer	HMG-CoA	[135]
Inhibition of proliferation	Simvastatin	Breast cancer	CSCs	[133]

2.5.3 Clinical safety and efficacy of statins

Statins can have antitumor effects and synergize with certain chemotherapeutic agents to decrease the development of multidrug resistance [102, 136]. Several clinical trials have examined the potential chemo preventive and therapeutic efficacy of statins (Clinical trial identifier: NCT02534376, NCT02360618, NCT00584012, NCT01110785). Clinical studies involved in interaction between the risk of cancer or cancer-related mortality and statin use has been lasting for many years, however, only a few convincing evidences were found. Epidemiologic findings of the association between statin use, cancer risk and cancer prognosis have revealed different results. Many clinical studies indicated that statin use could prevent carcinogenesis and benefit the survival of cancer patients [121, 123-125]. Recently, association between

use of statin and liver cancer was also evaluated in a nested case-control study [137]. K.A. McGlynn and colleagues suggested that the use of statin was associated with an increased risk of liver cancer. However, more studies found that the statin use could decrease the risk of Hepatocellular carcinoma (HCC) [120] [137, 138]. Meta-analyses on randomized statin trials also suggested that the use of statins was associated with lower risk of primary liver cancer [122]. A retrospective cohort study showed a significant difference in survival between patients who received low-intensity doses of simvastatin and those who received moderate high-intensity doses. Statins are normally well tolerated despite the side effects after long-term administration, mainly myopathy, rhabdomyolysis and hepatotoxicity. The association between statin use and cancer risk and/or mortality obtained from clinical findings regarding different types of cancer is summarized in Table 4**.**

Table 4: Summary of published clinical studies using statins as anticancer therapy

Statin	Cancer	Influence on cancer risk, mortality and survival	Ref
S	Pancreatic cancer	No clinical benefit was found	[139]
A, C, F, Pr, R, S	Liver cancer	Reduce cancer risk	[137]
F, L, Pr, R, S	Liver cancer	Reduce cancer risk	[120]
A, F, Pr, R, S	Pancreatic cancer	Reduce cancer risk, improve survival	[121]
A, C, F, L, Pi, Pr, R, S	Liver cancer	Reduce cancer risk	[122]
A, F, L, Pr, R, S	Brain cancer	Reduce cancer risk	[123]
A, C, F, L, Pi, Pr, R, S	Colorectal cancer	Reduce cancer risk	[140]
S, L	Pancreatic cancer	Reduce cancer risk and mortality	[124]
All statins	Colorectal cancer	Reduce cancer risk	[125]
A, F, L, Pr, R, S	Liver cancer	No effect on cancer survival	[138]
A, F, L, Pr, R, S	Prostate Cancer	Reduced cancer aggressiveness	[126]
A, F, Pr, R, S	Esophageal cancer	Reduce cancer mortality	[141]
A, L, Pr, R, S	Breast cancer	No effect on cancer risk	[142]
A, F, L, Pr, R, S	Kidney cancer	Improve overall survival	[143]
A, C, F, L, Pr, R, S	Kidney cancer	No effect on cancer risk	[144]
S	Colorectal cancer	No effect on overall survival	[145]
A, S	Pancreatic cancer	Improve overall survival	[146]
A, F, L, Pr, R, S	Prostate cancer	Reduce cancer mortality	[147]
All statins	Prostate cancer	Reduce cancer risk and mortality	[148]
A, F, L, Pr, R, S	Pancreatic cancer	Reduce cancer risk	[149]
A, C, F, L, Pr, R, S	Endometrial Cancer	Improve disease-specific survival	[150]
A, F, L, Pr, R, S	Esophageal cancer	Reduce cancer risk	[151]
All statins	Prostate cancer	No effect on cancer risk	[152]
A, F, Pr, S	Prostate cancer	Improve disease-specific survival	[153]
A, C, F, Pr, R, S	Lung cancer	Reduce cancer risk and mortality	[154]
A, F, L, Pr, R, S	Pancreatic cancer	Improve median survival	[155]
A, F, L, Pr, R, S	Cholangiocarcinoma	Reduce cancer risk	[156]
A, F, Pr, S	Colorectal cancer	No effect on cancer survival.	[157]
A, F, L, Pr, R, S	Colorectal cancer	Improve cancer survival	[158]
A, F, Pr, R, S	Breast cancer	Reduce cancer mortality	[159]
A, C, F, L, Pr, R, S	Kidney cancer	No effect on cancer survival	[160]
A, F, L, Pr, R, S	Pancreatic cancer	Reduce cancer risk	[161]
All statins	Kidney cancer	Improve cancer survival	[162]
A, C, F, L, Pr, R, S	Glioblastoma	Improve cancer-specific survival	[163]
S, L	Lung cancer	Reduce cancer risk	[164]
A, C, F, L, Pr, R, S	Ovarian cancer	No effect on cancer risk	[165]
A, F, Pr, R, S	Pancreatic cancer	No effect on cancer risk	[166]
A, F, Pr, R, S	Colorectal cancer	Improve cancer survival	[167]
A, C, F, L, Pr, R, S	Breast cancer	Reduce cancer risk and mortality	[168]
A, F, L, Pi, Pr, R, S	Prostate cancer	Reduce cancer risk	[169]
A, C, F, Pr, R, S	Breast cancer	Reduce cancer mortality	[170]

Atorvastatin (A), Cerivastatin (C), Fluvastatin (F), Lovastatin (L), Pitavastatin (Pi), Pravastatin (Pr), Rosuvastatin (R) and Simvastatin (S).

2.5.4 Signaling by statins

The mevalonate pathway is also known as isoprenoid pathway, in which inhibition of HMG-CoA is a rate-limiting step. Isoprenoid is one of several products of the mevalonate pathway, which plays important role for various cellular functions. The inhibition of HMG-CoA by statins leads to reduce mevalonate levels and its downstream products, which in turn disturbs crucial cell functions including protein synthesis, cell signaling, cell cycle progression and membrane integrity. Therefore, tumor initiation, growth and metastasis might be inhibited by the effects of statins on these processes and subsequently on cancer cells (Figure 8) [171].

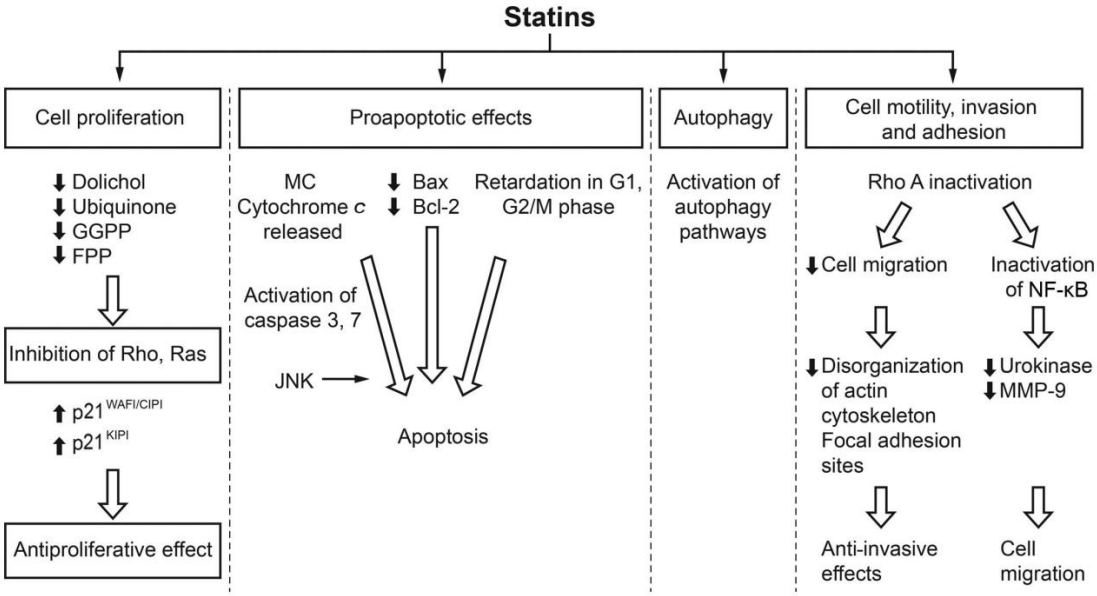

Figure 8: Pleiotropic effects of statins

Farnesyl pyrophosphate (FPP); geranylgeranyl pyrophosphate (GGPP); p21WAF1/CIP1 and p27KIP1, two cyclin-dependent kinase inhibitors; c-jun NH2-terminal kinase (JNK9); MC, mitochondria; MMP-9, matrix metalloproteinase-9 (MMP-9); nuclear factor κB (NF-κB) (Adapted from Ref. [171]).

2.5.5 Effect of simvastatin in pancreatic cancer

A recent study has precisely demonstrated that lipid metabolic pathways are disturbed in PDA and pancreatic cancer cells are highly dependent on cholesterol uptake [101]. A previous study suggested that simvastatin has a potential role for simvastatin-induced accumulation of cytosolic lipid droplets and upregulated genes involved in lipid metabolism in pancreatic cancer cells [172]. Notably, simvastatin showed stronger antitumor effects compared with other statins in several cancer cell lines *in vitro* [129, 173]. It has been also shown to significantly delay the progression

of PanIN lesions to PDA as well as to inhibit PDA growth in K-Ras mutant mice [135].

2.6 Aim of the study

According to the CSC theory solely the small subset of CSCs is held to be responsible for self-renewal, invasion, and metastasis. Recent clinical and epidemiological investigations demonstrate that daily intake of simvastatin not only reduces the risk of pancreatic cancer [121], but also inhibits metastasis and prolongs life of patients treated with simvastatin after diagnosed with prostate cancer [126], lung cancer [154] and renal cell carcinoma [143]. Aberrant activation of Shh signaling has been found in several types of cancer, including colon, breast, and pancreatic cancer. Moreover, Shh signaling plays an important role in carcinogenesis, differentiation, metastasis and resistance of chemotherapy and radiotherapy, indicating Shh pathway may be an intriguing therapeutic target focusing on pancreatic CSCs. **The hypothesis of this project is** that simvastatin targets pancreatic CSCs by inhibiting of Shh signaling. **The aim of this project is** to elucidate the effect of simvastatin to pancreatic CSCs with focus to self-renewal, metastasis, differentiation and chemotherapy resistance, and the underlying mechanisms. I studied the hypothesis of my thesis **1)** at the cellular level *in vitro*, **2)** in xenografts on fertilized chicken eggs *in vivo* and **3)** in human samples from well-characterized PDA patients *ex vivo*.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment

96 well plates (Cell star)	Greiner bio-one, Frickenhausen
Accu-jet pro	Brand, Wertheim
Analytical balance (Mettler P220)	Mettler Toledo, Gießen
BioStation IM-Q	Nikon, Düsseldorf
Blotting chamber	Starlab, Ahrensburg
Cell counter–Z^{TM} Series	Beckman Coulter, Indianapolis
Centrifuge–Capsulefuge PMC-060	Tomy, Fremont
Centrifuge–Varifuge 3.0R	Herraeus, Hanau
Cyrostat (CM 3050)	Leica, Wetzlar
DNA/RNA UV–cleaner box	Biosan, Riga
Electrophoresis power supply	Biotec Fischer, Reiskirchen
Electrophoresis unit	Bio-Rad, Munich
Fluorescence microscope	Leica, Wetzlar
Fluorometer	MS Laborgeräte
FLUOstar OPTIMA	BMG Labtech, Ortenberg
Ice machine (AF80)	Scotsman, Herborn
Incubator-Digital motor breeders	Siepmann, Herdecke
Inverted microscope–Eclipse TS100	Nikon, Düsseldorf
Lab frezeers (-20^{o}C, -80^{o}C)	Liebherr, Ludwigshafen
Laminar Flow Hood, HERA safe	Herraeus, Hanau
Light microscope	Leica, Wetzlar
Magnetic stirring hot plate	Heidolph, Schwabach
Micro Centrifuge–Biofuge 15R	Herraeus, Hanau
Mini Protean tetra system	Bio-rad, Munich
NanoDrop® Spectrophotometer	NanoDrop Tech, Wilmington
Neubauer hemacytometer	Brand, Wertheim
Odyssey® cLx imager	Li-cor®, Lincoln
pH-meter (pH 538)	WTW, Weilheim
Repeat pipettor, multistep	Eppendorf, Hamburg

Shaker–Unimax 2010	Heidolph, Schwabach
Spectrophotometer–Smart Spec 3000	Biorad, Munich
SPOT™ FLEX 15.2 64MP camera	Diagnostic Instruments, MI
Step One™ Real Time PCR system	Applied Biosystems, Nidderau
Thermal cyclers–C1000™	Bio-rad, Munich
Thermomixer	Eppendorf, Hamburg
Ultracentrifuge–L8-M	Beckman, Munich
Vortexer–REAX 2000	Heidolph, Schwabach
Water bath	Kottermann, Hänsingen
Xcell II™ Blot module	Life Technologies, Nidderau

3.1.2 Consumables

6, 12, 24, 48 and 96 well plates	Greiner, Frickenhausen
96 well white polystyrene plates	Sigma-Aldrich, Taufkirchen
96 well black polystyrene plates	Sigma-Aldrich, Taufkirchen
Cell culture dishes	TPP, Trasadingen
Cell culture flasks	TPP, Trasadingen
Cell culture plates	Nunc, Wiesbaden
Cell scraper	Greiner, Frickenhausen
Centrifuge tubes	TPP, Trasadingen
Chamber slides	Lab-Tek®, Naperville
Coverslips	Knittelgläser, Braunschweig
Cryotubes	Nunc, Wiesbaden
Dispenser tips	Nerbe, Winse
Disposable scalpels	Braun, Melsungen
Eppendorf tubes	Eppendorf
Eggs, fertilized, chicken	Geflügelzucht Hockenberger, Eppingen, Germany
Hemacytometer glasses	Fischer Scientific, Nidderau
Immobilon® transfer membrane	Millipore, Billerica
Microscope slides	Menzel, Braunschweig
Nitrocellulose membrane	Amersham, Piscataway
Parafilm M	Pechiney, Chicago

Pasteur pipettes	WTW, Weilheim
Pipette barrier tips	Starlab, Ahrensburg
Polypropylene tubes	Greiner, Frickenhausen
Sterile filters	Nalgene, Hamburg
Sterile Gloves	Hartmann, Heidenheim
Syringe	Hamilton, Bonaduz
Thermanox™ coverslips	Thermo Scientific, Schwerte
Tissue culture dishes	Greiner, Frickenhausen
Transwell® permeable support	Corning Inc., Acton
Weighing dishes	Neolab, Heidelberg

3.1.3 Media, supplements and reagents

Advanced DMEM/F12	Invitrogen, Karlsruhe
B27 supplement (50×)	Invitrogen, Karlsruhe
bFGF	Pepro Tech, Hamburg
Cell dissociation solution	Sigma-Aldrich, Taufkirchen
Dimethylsulfoxide (DMSO, >99%)	Applichem, Darmstadt
DMEM High Glucose	Thermo Scientific, Rochester
DNase/RNase free water	Sigma-Aldrich, St. Louis
Epidermal Growth Factor (rEGF)	R&D Systems, Wiesbaden
FCS	Sigma, Deisenhofen
Glutamax	Thermo Fischer, Waltham
HEPES	PAA, Cölbe
HEPES Buffer Solution	PAA, Posching
Human GAPDH primer	Qiagen, Hilden
Human Shh primer	Thermo Scientific, Rochester
Human Shh (GFP-tagged) plasmid	OriGene
Human Smo Primer	Thermo Scientific, Rochester
Insulin	Sigma-Aldrich, St. Louis
Lipofectamine 2000	Invitrogen, Dreieich
Lipofectamine RNAiMAX	Invitrogen, Dreieich
Matrigel	BD Biosciences, Heidelberg
OptiMEM®	Invitrogen, Karlsruhe

Shh siRNA Silencer®	Thermo Scientific, Rochester
SilencerTM Negative control No. 1 siRNA	Thermo Scientific, Rochester
Trypan blue	Biozol, Eching
Trypsin-EDTA	PAA, Cölbe
Water–Aqua ad injectabilia	Braun, Melsungen

3.1.4 Chemicals

Acetone	Sigma-Aldrich, St. Louis
AEC single solution	StemCell Tech., Köln
Alcian blue staining solution	Sigma-Aldrich, St. Louis
Avidin/Biotin Blocking Kit	Linaris, Werheim-Bettingen
Bovine Serum Albumin (BSA)	New England Biolabs, Frankfurt
DAPI	Sigma-Aldrich, Taufkirchen
EDTA	Sigma-Aldrich, St. Louis
Ethanol	Sigma-Aldrich, St. Louis
Fast BCIP/NBT tablet	Sigma-Aldrich, St. Louis
Fluoromount G	Biozol, Eching
Formalin	Carl Roth, Karlsruhe
Goat serum	Alexis, Grünberg
Isopropanol	Carl Roth, Karlsruhe
Milk powder	Carl Roth, Karlsruhe
MTT	Invitrogen, Karlsruhe
Narcoren®	IBF, Heidelberg
Oil Red O staining solution	Sigma-Aldrich, St. Louis
Paraformaldehyde (37%)	Merck, Darmstadt
PBS	Sigma-Aldrich, St. Louis
PMSF	Carl Roth, Karlsruhe
Primers	Biocat, Heidelberg
Protease inhibitor cocktail tablets	Roche, Mannheim
Protein ladder-PageRuler^{TM}	Fermentas, St. Leon-Rot
SDS pellets	Carl Roth, Karlsruhe
Simvastatin	Merck, Darmstadt
Sodium chloride	Carl Roth, Karlsruhe

Sodium citrate	Fischer, Schwerte
Sodium hydroxide	Carl Roth, Karlsruhe
TEMED	Merck, Darmstadt
Tris	Merck, Darmstadt
Tween-20	Sigma-Aldrich, St. Louis

3.1.5 Kits

Avidin Biotin Blocking Kit	Linaris, Wertheim-Bettingen
BCA™ protein assay kit	Thermo Fischer, Rockford
DNeasy® blood and tissue kit	Qiagen, Hilden
E.Z.N.A.® Tissue DNA kit	Omega Bio-tek, Norcross, USA
GeneChip® WT Plus Reagent kit	Affymetrix, Santa Clara, USA
GeneChip® Hybridization, Wash and Stain Kit	Affymetrix, Santa Clara, USA
High Capacity RNA-to-cDNA Kit	Thermo Fischer, Rockford
Plasmid mini and maxi preparation kits	Invitrogen, Karlsruhe
Proteinase K	Invitrogen, Karlsruhe
QIAquick® Gel Extract Kit	Qiagen, Hilden
RealTime-Glo™ MT Cell Viability Assay	Promega, Mannheim
RNeasy kit	Qiagen, Hilden
Site-Directed Mutagenesis Kit	Agilent, Santa Clara
Taqman®2× Universal PCR Master Mix, No AmpErase® UNG	Thermo Fischer, Rockford
Taqman® Endogenous Controls	Thermo Fischer, Rockford
TaqMan® Gene Expression Assay	Thermo Fischer, Rockford
Taqman® Reverse Transcription kit	Thermo Fischer, Rockford

3.1.6 Buffers and solutions

All buffers were prepared in distilled water unless otherwise specified.

10× Phosphate Buffered Saline (PBS) solution	2 g KCl 2 g KH_2PO_4 14.41 g $Na_2HPO_4 \times 2H_2O$ 80 g NaCl

	Made up to 1 L with ddH_2O
	pH 7.4
1.5 M Tris-HCl	36.32 g Tris
	200 mL ddH_2O
	pH 8.8
10% Sodium Dodecyl Sulfate (SDS)	10 g SDS
	100 mL ddH_2O
10× Triethanolamine Buffered Saline (TBS) solution	87.6 g NaCl (1.5 M)
	12.1 g Tris (100 mM)
	1 g NaN_3
	Made up to 1 L with ddH_2O
	pH 7.5
10× Western Blot Running buffer	144 g Glycine (1.92 M)
	10 g SDS (1%)
	30 g Tris (0.25 M)
	Made up to 1 L with ddH_2O
	pH 8.3
1 M Tris-HCl	24.2 g Tris
	200 mL ddH_2O
	pH 6.8
4× lämmli buffer	1 mL β-mercaptoethanol
	0.8g 8% SDS
	4 mL 40% glycerol
	2 mL 200 mM Tris-HCl (pH 6.8)
	Bromophenol blue
	Made up to 10 mL with ddH_2O
Chicken saline	7.2 g NaCl
	0.37 g KCl
	0.23 g $CaCl_2$
	Made up to 1 L with ddH_2O
LB media	10 mg/mL Bacto-tryptone 5 mg/ml yeast extract
	10 mg/ml NaCl

	2% w/v dextrose (pH 7.4)
LB/amp/X-Gal plates	LB media
	15 g/l agar
	75 µg/mL ampicillin
	100 µg/mL X-Gal
Membrane Extraction (ME) buffer	30 mM Tris
	150 mM NaCl
	0.5% Triton X-100
	0.5% Na-Desoxycholate
	1 µL/mL 1 M DTT
	10 µL/mL 100 mM PMSF
	1 µL/mL aproptinin (1 mg/mL)
Transfer buffer	2.930 g glycine (39 nM)
	5.810 g Tris (48 mM)
	0.375 g SDS (0.0375% w/v)
	200 mL methanol (20%)
	Made up to 1 L with ddH_2O
Tumorsphere medium	Advance DMEM/F12 medium
	B27 supplement (50×)
	20 ng/mL rEGF
	20 ng/mL bFGF
	5 µg/mL insulin
	5 mL Glutamax

3.1.7 Antibodies

Primary antibodies	
Anti-Shh, rabbit, polyclonal	Abcam, Cambridge
Anti-smoothed, rabbit, polyclonal	Abcam, Cambridge
Anti-Sufu, rabbit, monoclonal	Abcam, Cambridge
Anti-Ki67	Abcam, Cambridge
Anti-BMP4, rabbit, polyclonal	Abcam, Cambridge
Anti-Gli, mouse monoclonal	Cell Signaling, USA
Anti-Vimentin, mouse monoclonal	Abcam, Cambridge

Anti-CXCR4, rabbit polyclonal	Abcam, Cambridge
Anti-cMet, rabbit monoclonal	Cell Signaling, USA
Anti-β-actin, mouse monoclonal	Sigma-Aldrich, St. Louis
Secondary antibodies	
Anti-mouse-IgG, IRDye® 800CW Infrared Dye (Goat, anti-Mouse IgG, western blot dilution 1:5000)	LI-COR Biosciences GmbH, Bad Homburg, Germany
Anti-mouse-IgG, IRDye® 680RD Infrared Dye (Goat, anti-Mouse IgG, western blot dilution 1:5000)	LI-COR Biosciences GmbH, Bad Homburg, Germany
Anti-rabbit-IgG, IRDye® 680RD Infrared Dye (Goat, anti-Mouse IgG, western blot dilution 1:5000)	LI-COR Biosciences GmbH, Bad Homburg, Germany
Anti-rabbit-IgG, IRDye® 800CW Infrared Dye (Goat, anti-Mouse IgG, western blot dilution 1:5000)	LI-COR Biosciences GmbH, Bad Homburg, Germany
Anti-mouse-IgG, AlexaFluor 488	BD Pharmingen, Heidelberg
Anti-mouse-IgG, AlexaFluor 594	Molecular probes, Karlsruhe
Anti-rabbit-IgG, AlexaFluor 488	Molecular probes, Karlsruhe
Anti-rabbit-IgG, AlexaFluor 594	Molecular probes, Karlsruhe

3.1.8 Cell culture

The normal pancreatic duct fibroblast (CRL-4023), non-malignant human primary mesenchymal stromal cells (MSC) cell lines and the established human pancreatic cancer cell line BxPc-3，MIA-PaCa2, PANC1 were obtained from ATCC [174-177]. The primary cell line ASAN-PaCa was established and provided by N. Giese from European pancreas center [178]. Negative mycoplasma cultures were confirmed by monthly mycoplasma test.

3.1.9 Patient tissue

Surgical specimens were obtained from patients admitted to the Department of General, Visceral and Transplantation Surgery. The study was approved by the

ethical committee of the University of Heidelberg after receiving written informed consent from the patients. Clinical diagnoses were established by conventional clinical and histological criteria. All surgical resections were indicated by principles and practice of oncological therapy.

3.1.10 Preparation of stock solutions

Gemcitabine was purchased from the Pharmacy of the University Hospital Heidelberg supplied by Eli Lilly Company, with a concentration of 38 mg/ml. Gemcitabine stored at 4°C was freshly diluting aliquoted 100 µM stock in DMEM. According to average weight of chick embryo on embryonic development day (EDD) 15 and concentration for mouse model recently described [179], stock was diluted in chick saline to final concentration of 10 µM for intravascular injection. Simvastatin (≥95%) was bought from Merck and dissolved in ddH_2O (PH 7.0) with a stock concentration of 10 mM. The stock was divided into aliquots and stored in -20°C and were freshly diluted in culture medium to reach different concentrations according to the experiments purpose. Each stock was used only once immediately after thawing.

3.2 Methods

3.2.1 Cell culture

3.2.1.1 General cell culture

Cells were cultured in an incubator at 37°C, 5% CO_2. Media were supplemented, if not indicated differently, with 10% [v/v] FCS, 2 mM L-Glutamin, 10 mM HEPES (pH 7.4) and 2.5 µg/mL of Plasmocin. Media were pre-warmed at 37°C prior use. FCS was heat-inactivated for 30 min at 56°C. For determination of the number of viable cells, cell suspension was diluted 1:1 in Trypan blue solution (0.125%). Cells were counted using a Cell counter-Z^{TM} Series as recommended by the supplier.

3.2.1.2 Passaging of cells

When cells were 80-90% confluent in flasks, media were aspirated and cells were gently washed once with 5 mL 1× PBS. After aspirating PBS, 2 mL or 1 ml 1× Trypsin-EDTA was added to per T-150 flask and cells were incubated at 37°C and 5% CO_2 until more than 90% cells were observed detached from the flask. Trypsin was inactivated by 10 mL medium. Suspended cells were put in a 15 mL tube, and then

centrifuged at 1500 rpm for 5 min. The supernatant was removed and cells were resuspended with normal culture medium. After determination of the number of cells, cells were passaged as required ratio.

3.2.1.3 Thawing of cells

The cryotubes were put in the warm water bath at 37°C until a little piece of ice clot was still visible in the cryotube. Cell suspension was transferred to 10 mL of pre-warmed medium and centrifuged at 1500 rpm for 5 min. After removing supernatant, cells were carefully resuspended in appropriated fresh cell culture medium and transferred to a culture flask. In order to completely eradicate the toxicity of remained DMSO to cells, medium was normally changed after 24 h.

3.2.1.4 Freezing of cells

Cells in flasks were trypsinized after washed once with 5 mL 1× PBS. After quenched trypsin reaction with media, resuspended cells were centrifuged at 1500 rpm for 5 min, and then removed supernatant. Aliquots of 3×10^6 to 1×10^7 cells were well resuspended in 900 µL corresponding media with 10% FCS and 100 µL sterile DMSO in each labeled cryotube. Cryotubes were immediately put in an isopropanol-filled cryo-container to get a constant decrease in the temperature at a rate of 1 °C/min. Subsequently, cryotubes were transferred to the -80°C freezer. 24 h later cryotubes were transported to a freezer with a temperature of -140°C for long-term storage.

3.2.1.5 Preparation of chick eggs and transplantation of established cell lines

Fertilized eggs from highly genetically identical hybrid Lohman Brown (LB) chicks were obtained from a local ecological hatchery. All eggs are specific pathogen free animals and tested against infectious bronchitis, leucosis and mycoplasma. On EDD1, eggs were incubated in 37.8°C at a humidity of 45-55% in digital motor breeders Type 168/D. On EDD 4.5 ml of albumen was absorbed removed with a syringe, thus allowing detachment of the embryo. Afterward, a small window was cut into the eggshell, and the window was wrapped with tape. On EDD 9 small handmade rings from Thermanox™ coverslips were deposited on the chorioallantoic membrane (CAM), followed by adding 5×10^5 of MIA-PaCa2 cells in matrigel into the rings. For treatment, diluted gemcitabine, simvastatin or combination were intravenously injected in blood vessels of the CAM on EDD 12 and 15 respectively. Tumor growth

and tumor take rate were evaluated at EDD 18. All embryos that died before EDD 18 were excluded from further analyses. Tumor volumes were determined after resection of tumor xenografts by the following formula: Volum=4/3×π×r^3 (r=1/2×square root of diameter 1×diameter 2) [180]. Tumor take rate was calculated by the following method: N1×100/N2 (N1=number of embryos with tumor; N2=number of live embryos). Tumor tissue was kept frozen in dry ice and embedded in Tissue Tek O.C.T. compound for further analyses.

3.2.2 MTT assay

Measurement of cell viability and proliferation was detected by the MTT assay. Cells were seeded in 96-well flat bottom plates at a density of 3-5×10^4/mL, 100 µL per well. 24 h later cells were treated with corresponding drugs or transfected with siRNA or plasmid for different time points. At the end of each treatment time point, 10 µL of MTT stock solution (12 mM in PBS) per well was added. Subsequently, the 96-well plates were incubated for 4 h in an incubator at 37°C. After completely removing media, 200 µL pre-warmed DMSO was added. The plate was shaken in a shaker until all of the blue crystals were completely solubilized. Subsequently, the optical density of each well was detected at 560 nm with an ELISA reader.

3.2.3 Spheroid and colony formation assay

3.2.3.1 Spheroid formation assay

Cancer cells were cultured in human NeuroCult NS-A basal serum-free medium supplemented with 2 µg/mL Heparin, 20 ng/mL hEGF, 10 ng/mL hFGF-b and NeuroCult NS-A Proliferation Supplements. Low densities of cells (1.5×10^3 cells/mL) were seeded in 24-well ultra-low adhesion plates for spheroid formation, 1 mL per well. 24 h later cells were treated with simvastatin, gemcitabine or both agents together and incubated for 3 days. Then spheroids were photographed at day 5 and the percentage of cell spheroids was calculated. To evaluate potential for the secondary spheroids formation, equal numbers of cells were reseeded. Same manner of spheroid formation was quantified [181].

3.2.3.1 Colony formation assay

Briefly, cells were collected as described above and seeded in 6-well plates containing 1.0-2.0×10^5 cells/well, followed by treatment with different drugs or siRNA for 72 h. Subsequently, cells were collected again and re-plated at a density of 500-2000 cells per well in 6-well plates triplicates. After incubation for two weeks in incubator without changing medium, cells were fixed with 2 mL 3.7% paraformaldehyde (PFA) for 10 min followed by 2 mL 70% ethanol for another 10 min after wash once with 1× PBS. Then cells washed 3 times with water and stained with 0.05% Coomassie blue for 5 min. Subsequently, cells were washed with water and dried overnight. A colony was defined as a spot comprising more than 50 cells. The number of colonies was quantified under a dissecting microscope. The percentage of plating efficiency was calculated (plating efficiency of non-treated cultures=1) [176, 181]. To investigate potential for formation of the secondary colonies, cells were collected from the colonies above and equal numbers of cells were reseeded, colonies were calculated in the same manner.

3.2.4 Evaluation of differentiation potential

3.2.4.1 Evaluation of osteoblast differentiation

Cells were seeded at a density of 0.8-1.2×10^5 cells/well in a 6-well plate, 3 mL medium per well, and were treated with simvastatin for 72 h and the media were changed with 2 mL osteogenic differentiation media. The cells were grown for 10 days, with medium replacement triple a week. Osteogenic differentiation was measured by staining with SIGMAFASTTM BCIP®/NBT substrate according to the manufacturer's instructions. Images of staining cells were taken using a NikonEclipse TS100 microscope (200× magnification).

3.2.4.2 Evaluation of adipocyte differentiation

0.8-1.2×10^5 cells were seeded in a 6-well plate. After reaching 80-90% confluence, the cells were treated with simvastatin for 72 h and incubate in adipogenic differentiation medium for 12 days with changing the medium every 3 days. Cells were gently washed with PBS, fixed with methanol for 5-10 min and incubated in Oil Red O for 30 min at room temperature. Then the cells were washed with distilled water. Red staining cells were visualized using a Nikon Eclipse TS100 microscope.

3.2.5 Protein extraction and western blot analysis

3.2.5.1 Protein extraction

1.0-2.0×10^6 cells were seeded in 10 cm tissue culture plates and treated cells at and treated for 72 h. The protein samples were stayed on ice during the entire procedure to avoid degradation. All of cells were harvested by scraping and put into 15 mL tubes and then centrifuged at 1500 rpm for 5 min at 4°C. The pellet was resuspended in 1.0 mL cool 1× PBS and transferred into a 1.5 mL eppendorf tube. The samples were centrifuged at 2000 rpm for 5 min at 4°C and the supernatant was removed. The pellet was resuspended in 5× ME buffer containing complete protease inhibitor according to the pellet volume (ME buffer volume: pellet volume=4-5:1) and incubated on ice for 10 min after mix. After that, samples were centrifuged at 14,000 rpm for 20 min and then supernatant was transferred to a new 1.5 mL tube for determination of protein concentration. Subsequently, protein solutions were used for SDS-gel electrophoresis or were stored at -20°C.

3.2.5.2 Detection of protein concentration

For detection of total protein concentration, the Pierce BCA Protein Assay was used. Briefly, 5 µL of each standard and sample replicate are pipetted into each corresponding microplate well of a 96 well plate and then 200 µL of the working reagent (Kit BCA reagent A: B=50:1) were added to each well and incubated for 30 min at 37°C. After that, an ELISA-Reader well determined absorption in each. To determine the protein concentration of each unknown sample, a standard curve was prepared by plotting the absorption of each standard versus its concentration (µg/mL) and calculated the concentration of each detected samples according to the manufacturer's instructions.

3.2.5.3 SDS-polyacrylamid gel electrophoresis (SDS-PAGE)

Denatured proteins can be separated by polyacrylamid gel electrophoresis (PAGE) according to their molecular weight. The proteins are denatured and become negatively charged by SDS. The charge of the protein-SDS-complex is proportional to protein size and thus migration velocity through the gel is dependent on molecular weight. The SDS-gel consists of separating gel and stacking gel and the concentration

of the SDS-gel depends on the molecular weight of the desired protein (Table 5, Table 6).

Table 5: Composition of separation gel (10 ml)

Complements	6%	8%	10%	12%	15%
Protogel	2.0 mL	2.67 mL	3.33 mL	4.0 mL	5.0 mL
1.5M Tris pH 8.8	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL
10% SDS	100 µL	100 µL	100 µL	100 µL	100 µL
Water	4.79 mL	4.32 mL	3.46 mL	2.8 mL	1.8 mL
Glycerol	500 µL	500 µL	500 µL	500 µL	500 µL
10% APS	100 µL	100 µL	100 µL	100 µL	100 µL
TEMED	10 µL	10 µL	10 µL	10 µL	10 µL

Table 6: Composition of stacking gel (4 ml)

Components	4% gel
Protogel	520 µL
1.M Tris pH 6.8	500 µL
10% SDS	40 µL
Water	2.89 mL
10% APS	40 µL
TEMED	4 µL

After polymerization, gels were fixed in a gel chamber containing 1× running buffer. Protein solutions were diluted 1:3 with 4× loading buffer and boiled for 3 min at 99 °C to accelerate denaturation and binding of SDS to the proteins. The Loading buffer contains β-Mercaptoethanol, which can break disulphide bridges denatured proteins (polypeptides) become surrounded by negatively charged SDS-molecule micelles and migrate to the anode. Subsequently, cooked samples were incubated on ice for 5 min. After gel wells were rinsed several times by syringe to completely exclude the pieces of gels in wells, gels were loaded with 25-50 µg proteins and protein separation was performed at 80 V for 20 min followed by separation at 160 V. A prestained protein ladder was used as a marker for determination of protein size.

3.2.5.4 Electroblot

After electrophoresis, gels with separation proteins were electroblotted on a nitrocellulose membrane. The membrane was activated in 100% methanol for 15 s and rinsed under running water before use and then pre-soaked in transfer buffer for at least 10 min. Filter papers were also pre-soaked in transfer buffer before use. Three filter papers were placed on the bottom of the chamber followed orderly by the nitrocellulose membrane, gel, and another three filter papers. Then, the chamber was closed and the time and amperage of transfer were set based on the instructions of the manufacturer and the protein size.

3.2.5.5 Detection of proteins

To detect the proteins on the membranes, membranes were placed upside down in a blockholder and blocked for 3 min with 0.1% [w/v] milk powder in 0.1% TBS-Tweenat room temperature using a Millipore Snap ID. Subsequently, blocking solution was aspirated and membranes were incubated for 10 min at room temperature with 2 mL primary antibodies diluted in TBS-Tween (1:1000). After that, membranes were washed 3 times with 15 mL TBS-Tween and then incubated for another 30 min at room temperature with 2 mL secondary Infrared Dye conjugated second antibody diluted in TBS-Tween (1:5000). Then, membranes were washed with additional three times and put immediately onto the Odyssey® CLx Infrared Imaging System. Protein bands were measured and calculated with the correspond software.

3.2.6 Immunohistochemistry

Frozen slides (fixed with 4% PFA) were rehydrated in 1× TBS for 5 min and blocked with a blocking solution containing 20% goat serum and 4 drops/mL of avidin solution in 1× PBS with 0.2% Tween for 30 min. After blocking solution was removed, samples were incubated with 100 µl primary antibody solution in which primary antibody was diluted in 1× PBST including 4% goat serum and 4 drops/mL biotin solution (dilution 1:50 to 1:200) overnight at 4°C. At one slide of each group, omission of primary antibody was used as a negative control. After an overnight incubation, samples were washed twice in 1× PBS with 0.2% Tween for 5 min and once in 1× PBS for 5 min. Then, endogenous peroxidase was quenched by 3%

hydrogen peroxide in methanol for 10 min. Secondary antibody was diluted 1:200 in 1× PBS with 4% goat serum and 100 µl of secondary antibody solution was added to samples followed an incubation for 30 min at room temperature. Subsequently, samples were washed 2 times in 1× PBS with 0.2% Tween for 5 min and 1 time in 1× PBS for 5 min. After that, 100 µl ABC solutions (2.5 mL PBS + 1 drop avidin + 1 drop biotin) were pipetted to each sample and incubated for 30 min. After washing in 1× PBS 3 times for 5 min, AEC was added to each sample and incubated for 3-10 min until the color reaction was observed under the microscope and stopped when cells became brown or red. The reaction was stopped by washing with 1× PBS three times for 5 min. Subsequently, samples were counterstained for three min in haematoxylin followed by washing in tap water and distilled water (3×5 min). Finally, the samples were mounted in xylene and random regions of interest were chosen with the Leica DMRB microscope.

3.2.7 Immunofluorescence

Frozen tissue sections were fixed with 4% PFA for 10 min and were washed in 1× TBS for 5 min. Then samples were incubated with a blocking solution containing 20% goat serum in 1× PBS with 0.2% Tween for 30 min at room temperature to inhibit unspecific antibody binding. Subsequently, samples were incubated with 100 µl primary antibody solution in which primary antibody was diluted in 1× PBST including 4% goat serum overnight at 4°C, with omission of primary antibody served as a negative control. Then, samples were washed twice in 1× PBS with 0.2% Tween for 5 min and once in 1× PBS for 5 min and then incubated with Alexa Fluor 488-conjugated secondary antibody for 30 min in the dark. After being washed as described above, samples were incubated with 100 µl second primary antibody solution in which primary antibody was diluted in 1× PBST including 4% goat serum and nuclei were counterstained with DAPI (dilution 1:100). The DAPI containing solution centrifuged for 30 min at 14000 rpm before usage. Samples were washed as described above and then incubated with Alexa Fluor 594-conjugated for 30 min. After that, the samples were washed with 1× PBS once and dH_2O twice for 5 min. Finally, the samples were mounted with Fluoromount G and stored at -20°C in the dark. Randomly chosen fields were examined at 400× magnification using a Leica DMRB microscope. Images were captured using a SPOTTM FLEX 15.2 64Mp

shifting pixel digital color camera and analyzed with SPOT Basic/Advanced 4.6 software. The percentage of double-positive cells was calculated.

3.2.8 Haematoxylin and Eosin (H&E) Staining

Frozen tissue sections were fixed with 4% PFA for 10 min and were washed in 1× TBS for 5 min. Subsequently, samples were counterstained for 3-5 min in haematoxylin followed by washing in tap water until to get perfect blue nuclear staining. After that, samples were stained with Eosine for 3-5 min and then washed once with H_2O. Subsequently, samples were dipped in ethanol 70%, ethanol 80%, ethanol 90%, and ethanol 100% in sequence for 5 min, respectively, and held in xylene twice for 3 min. After dried completely, the samples were mounted and randomly fields were chosen with the Leica DMRB microscope.

3.2.9 Transplantation of tumor cells to fertilized chicken eggs

The chorioallantoic membrane (CAM) assay is an animal substitute model for cultivation of human tumors. 15 to 20 fertilized eggs were used for each study group. On day 1, eggs were washed with 70% ethanol and incubated in a special incubator at 37.8°C. On day 4, after 2-3 mL of albumen was removed with a syringe, a 2 cm diameter hole was penned on each egg using Leukosilk strips and curved scissors. The viability of the embryos was examined by observing a beating heart and clear blood vessels. Then, the holes on the eggs were covered with strips and put back in the incubator in a horizontal position. On day 8 of embryonic development, small handmade rings were placed on the CAM and 1×10^6 cancer cells mixed in matrigel were seeded in the middle of the ring surrounded by clear blood vessels. Tumor cells were only transplanted to eggs with viable embryos. After that, the egg shell was taped with a strip and replaced in the incubator for additional 10 days. On day 18, the tumor-take was performed and all embryos that died before day 18 were excluded from further analyses. To evaluate the tumors, tumor volume was determined (diameter 1 and 2) and calculated using the following formula: Volume=$4/3\times\pi\times r^3$ ($r=1/2\times\sqrt{}$ of diameter 1×diameter 2). Subsequently, the tumor tissues were fixed as soon as possible to achieve the best morphology for further investigations.

3.2.10 Isolation of genomic DNA and human Alu PCR amplification

Tissue DNA was extracted from fresh tissue obtained from the CAM adjacent the plastic ring using the E.Z.N.A.® Tissue DNA kit according to the manufacturer's instructions (Omega Bio-tek, Norcross, GA, USA). The PCR amplification was carried out with 0.5 µM of both forward and reverse primers using FastStart Taq DNA Polymerase, dNTPack according to the manufacturer's instructions (Roche, Mannheim, Germany). The total reaction volume was 50 µl, including 5 µl of synthesized cDNA or 200 ng genomic DNA. The following primers were used: Human Alu-sense - 5'-GTAAGAGTTCCGTAACAGGACAGCT-3'; Alu-antisense - 5'-CCCCACCCTAGGAGAACTTCTCTTT-3'. Chicken GAPDH Alu DNA were Alu-sense, 5'-GAG GAA AGG TCG CCT GGT GGA TCG-3', and Alu-antisense, 5'-GGT GAG GAC AAG CAG TGA GGA ACG-3'. The PCR conditions were 40 amplification cycles of 60 s at 94°C, 120 s at 58°C, and 120 s at 72°C. PCR products were analyzed on a 2% agarose gel.

3.2.11 RNA expression profiling and real time qualitative PCR

3.2.11.1 mRNA isolation and mRNA concentration measurement

Total RNA was isolated using the RNeasy Mini Kit from Qiagen and stored in -80°C or immediately used. One microliter of total mRNA was mounted onto NanoDrop 2000 Spectrophotometer for measurement.

3.2.11.2 mRNA expression profiling

mRNA expression profiling was done by Center of Medical Research (ZMF) Mannheim. Briefly, 500 ng mRNA was checked by quality control and the concentration was measured again, then gene expression profiling was performed using human HuGene-2_0-st-type array from Affymetrix. Biotinylated antisense cRNA was then prepared based on standard Affymetrix protocol with the GeneChip® WT Plus Reagent Kit and the GeneChip® Hybridization, Wash and Stain Kit. thereafter the hybridization on the chip was performed on a GeneChip Hybridization oven 640 and dyed in the GeneChip Fluidics Station 450, then scanned with a GeneChip Scanner 3000.

3.2.11.3 mRNA expression analysis

A Custom CDF Version 20 with ENTREZ based gene definitions was used to annotate the arrays [182]. The Raw fluorescence intensity values were normalized applying quantile normalization and RMA background correction. One-Way ANOVA was performed to identify differential expressed genes using a commercial software package SAS JMP10 Genomics, version 6, from SAS (SAS Institute, Cary, NC, USA). A false positive rate of a=0.05 with FDR correction was taken as the level of significance.

Gene Set Enrichment Analysis (GSEA) was used to evaluate whether defined lists of genes exhibit a statistically significant bias in their distribution within a ranked gene list using the software GSEA [183]. Pathways of different cell functions were obtained from public external databases (KEGG, http://www.genome.jp/kegg). The heat map was created by R software with the package "gplot".

3.2.11.4 Reverse transcription of mRNA to cDNA

mRNA was extracted as described above. A total of 1-100 ng mRNA was used for reverse transcription using the High Capacity RNA to c-DNA Kit. The reverse transcription reaction mix was prepared on ice according to Table 7. Then, the reverse transcription reaction was incubated for 60 min at 37°C and inactivated by heating to 95°C for 5 min and hold at 4°C. The cDNA was stocked in -80°C or diluted in 200 µL RNase-free water for immediate real-time PCR.

Table 7: Reverse-transcription reaction components

Component	Volume
2× RT (Reverse Transcription) Buffer	10 µL
20× RT Enzyme Mix	1 µL
Template RNA (50 ng)	9 µL
Total volume	20 uL

3.2.11.5 Real time quantitative PCR

Real time quantitative PCR was performed using Master mix without UNG. The reaction mix was prepared according to Table 8 and transferred to a 48-well plate.

Table 8: Reaction mix of Real Time PCR

Component	Volume
Primer Mix	1 µL
2× TaqMAN Gene Expression Master Mix	10 µL
Rnase-free water	5 µL
Template cDNA	4 µL
Total volume	20 uL

After the plate was tightly sealed with film, it was centrifuged for 3 min at 3000 g at room temperature to remove bubbles. Then the step one real time PCR was set up according to Table 9.

Table 9: Cycling conditions for real-time PCR

Step	Time	Temperature
PCR initial activation step	10 min	95°C
Denaturation	15 s	95°C
Annealing	1 min	60°C
Break	∞	4°C
Cycle number	40 cycles	

Human GAPDH from Qiagen was used as controls. The used primers were and Shh

3.2.12 Isolation of plasmid DNA

E.coli DH5α cells were used for plasmid cloning. Frozen DH5α cells were taken from the -80 °C freezer and thawed on ice for 5 min. 2 µg of plasmid DNA was added to 25 µl thawed DH5α cells. Lysogeny broth (LB, 900 µl) medium without antibiotics was added to the cells-plasmid mixture. The mixture was incubated in a 37°C shaking incubator at 250 rpm overnight to allow bacterial growth. The next day, 50 µl of bacterial cells were plated on the LB/antibiotic agar plates. Sterile pipette tips were used to create single colonies by running streaks in LB agar plates, Plates were placed upside down in incubator overnight at 37°C. After transformation, a single colony of bacteria was picked and inoculated in 4 ml liquid LB medium with antibiotic (Ampicillin, 100 µg/ml) overnight in a 37°C shaking incubator. After

recovering plasmid, the level of plasmid DNA was evaluated using plasmid mini-prep kit. 250 ml liquid LB medium mixed with 4 ml of prepared bacterial cells were inoculated overnight in a 37°C shaking incubator to amplify plasmid DNA for purification. DNA preparation was performed using a PureLink HiPure Plasmid Maxiprep Kit according to the manufacturer's recommendations (Invitrogen).

3.2.13 Transfection of siRNA and plasmid DNA

3.2.13.1 Transient transfection of plasmid DNA

For overexpression of Shh protein, cells were seeded on 6-well plates or 96-well plates in DMEM containing 10% FBS without antibiotics. After cells had been attached, human Shh plasmid was used for transfection with Lipofectamine 2000 according to the instructions manufacturer. Briefly, 4 µg of Shh plasmid and 10 µL of Lipofectamine 2000 were incubated separately in 250 µL of Opti-MEM I Reduced Serum Medium (Invitrogen). After 5 min of incubation at room temperature, the diluted plasmids and Lipofectamine 2000 were combined and incubated for an additional 20 min at room temperature to allow the formation of transfection complexes and then transferred to corresponding wells. Usually, cells under the transfection complexes were incubated at 37°C in a CO_2 incubator for 24 h, followed by changing with corresponding medium.

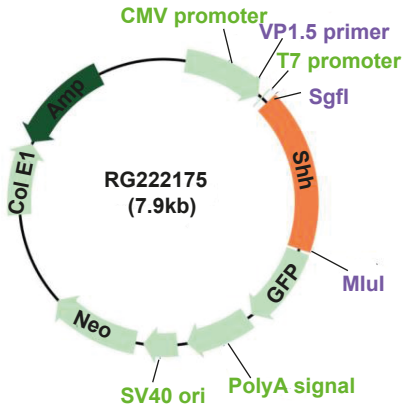

Figure 9: Plasmid map of human Sonic hedgehog

3.2.13.2 siRNA transient transfection

For knockdown of Shh protein expression, pooled siRNA duplexes were used, negative control used non-targeting siRNA. Lipofectamine 2000 from Invitrogen was

used to transient siRNA transfection according to the instructions of manufacturer. Transfected cells were cultured for 72 h at 37°C in a CO_2 incubator.

3.3 Statistical analysis

The IC50 doses and the combination index were calculated by the Chou-Talalay method [184, 185]. The data obtained in cell culture experiments are presented as mean ± SD for at least three independent experiments performed in triplicate. The statistical significance was evaluated by Student's *t*-test if data were normally distributed and groups had equal variance, otherwise, Mann-Whitney test was used. $P<0.05$ was considered statistically significant. One star represents $P<0.05$ and two stars represent $P<0.01$.

4. RESULTS

4.1 Simvastatin inhibits viability and enhances cytotoxicity of gemcitabine in PDA

To get knowledge about the chemosensitivity of pancreatic cancer cells, 2 highly aggressive (MIA-PaCa2, PANC-1) and 2 less aggressive (ASAN-PaCa, BxPc-3) human established PDA cell lines were treated with gemcitabine and the cell viability was measured by MTT. It was found that the aggressiveness corresponded to gemcitabine sensitivity (ASAN-PaCa＜BxPc-3＜MIA-PaCa2＜PANC-1) (Figure 10A). To evaluate the effect of simvastatin on proliferation，the cells were treated with simvastatin in concentrations found in plasma of patients, with typical plasma concentrations in the range of 1 nM to 2 mM [186, 187]. The results revealed the viability decreased in a time and dose dependent-manner especially after 48 h and 72 h treatment with increasing simvastatin concentration (Figure 10B). From the obtained data, the IC50 doses of simvastatin and gemcitabine was calculated with the Chou-Talalay method (Table 10).

Table 10: Simvastatin synergistically interacts with gemcitabine in pancreatic cancer cells

Cell line	SIM	GEM	SIM+GEM
	IC50 (μM)	IC50 (nM)	CI
ASAN-PaCa	25±2.2	10±2.8	0.75±0.07
BxPc-3	25±3.4	15±2.7	0.57±0.09
MIA-PaCa2	3±1.2	20±5.4	0.82±0.01
PANC-1	32±2.5	52±4.3	1.06±0.14

The combination effect of simvastatin and gemcitabine in ASAN-PaCa, BxPc-3, MIA-PaCa2 and PANC-1 cells.
The drug combination analysis was performed using the Chou-Talalay method. The graphs represent values of the combination index (CI) and they were generated automatically by using the CompuSyn software. Additive effects CI=1; Synergism CI<1; Antagonism CI>1; Measurement of viability of cells treated with simvastatin (SIM), gemcitabine (GEM) and combination (SIM+GEM) (refer to Table 10) for 72 h. Each value is represented as mean ± SD of three independent experiments.

The results show that MIA-PaCa2 cells are about 10 times more sensitive to simvastatin compared to the other cell lines, due to the very low IC50 dose of 3 μM; the same applies to gemcitabine-sensitivity (Figure 10A). Due to the obtained CI results there is a synergistic effect for combination treatment resulting in enhanced cytotoxicity of gemcitabine in ASAN-PaCa, BxPc-3 and MIA-PaCa2 (CI＜1＝ but not in the most aggressive PANC-1 cells (CI＞1). Additionally, simvastatin had an only marginal effect on the viability of non-malignant human primary mesenchymal

stromal cells (MSCs) or immortalized human pancreatic ductal (CRL-4023) cells after 72 h treatment with simvastatin (Figure 10C, D). The above results indicate that simvastatin not only inhibits viability but also may reverse the chemotherapy resistance of PDA *in vitro.*

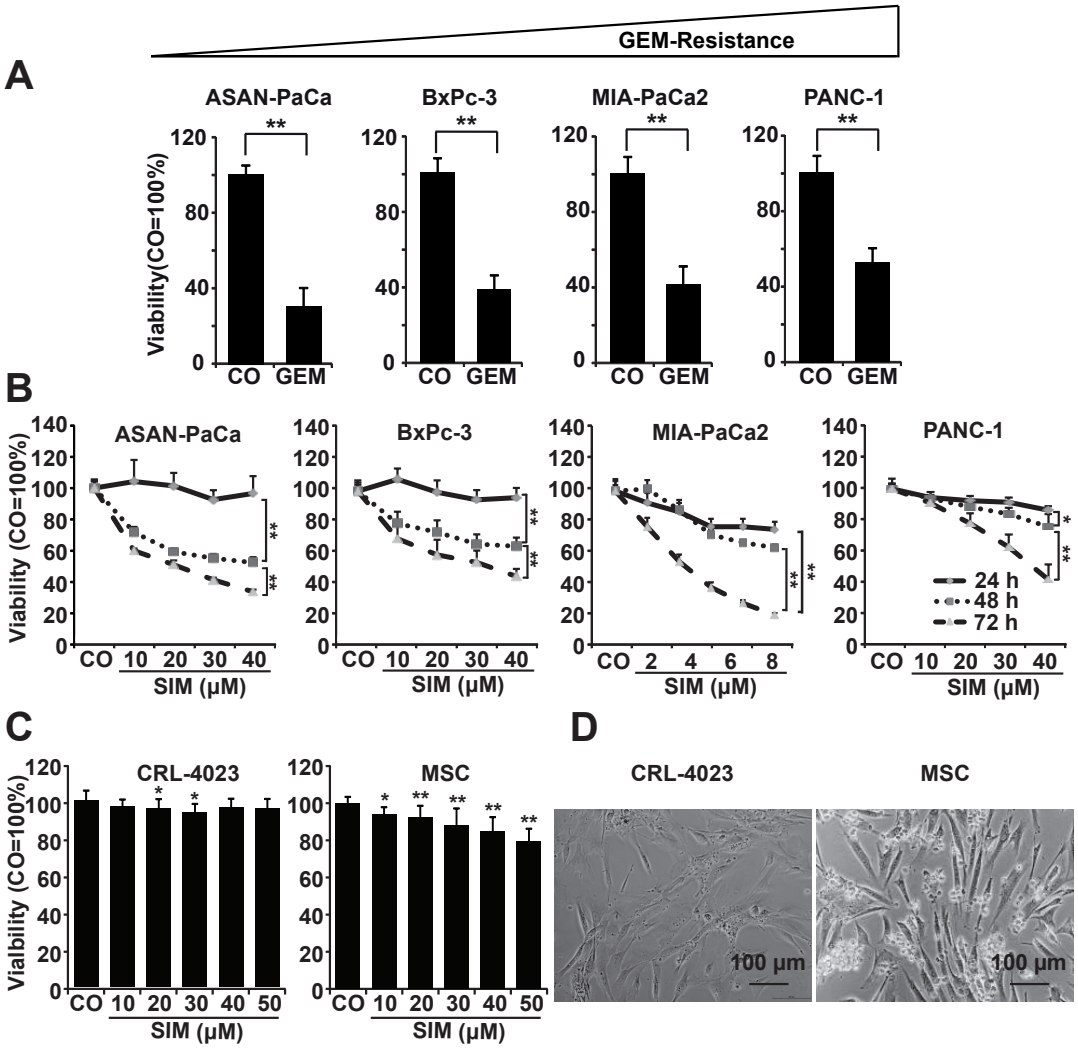

Figure 10: Simvastatin is selectively cytotoxic in pancreatic cancer cells

A. The human established PDA cell lines ASAN-PaCa, BxPc-3, MIA-PaCa2 and PANC-1 were left untreated (CO) or were treated with 50 nM gemcitabine (GEM), and the cellular viability was measured 72 h later with MTT assay. The gemcitabine resistance of the cells is indicated by a triangle above the diagrams. **B**. The human established PDA cell lines ASAN-PaCa, BxPc-3, MIA-PaCa2 and PANC-1 were left untreated (CO) or were treated with simvastatin at different time and concentrations as indicated, the cellular viability was evaluated with MTT assay. **C**. The non-malignant primary human cell lines CRL-4023 and MSCs were treated with different concentrations (10, 20, 30, 40, 50 μM) for 72 h. **D.** The morphology of CRL-4023 and MSCs after 72 h treatment with 50 μM simvastatin was documented by microscopy at 100× magnification. Representative pictures are shown, and the bar indicates 100 μm. (**P<0.01).

To further confirm synergistic effects of simvastatin to gemcitabine sensitivity, the cells were treated with IC50 doses of simvastatin or gemcitabine or combinations

thereof. As a result, the cell viability significantly declined to approximately 50% with single treatment after 72 h in all cell lines examined. Combination treatment significantly decreased cell viability compared to single treatment (Figure 11A). Similarly, morphological analysis showed that cells exposed to gemcitabine partly preserved the cell morphology of parental cells regardless of cytotoxicity of gemcitabine. However, simvastatin and combined with gemcitabine treated cells showed conspicuously difference in the cell morphology (Figure 11B). In short, treatment with simvastatin strongly enhanced the cytotoxicity of gemcitabine in all cells lines examined.

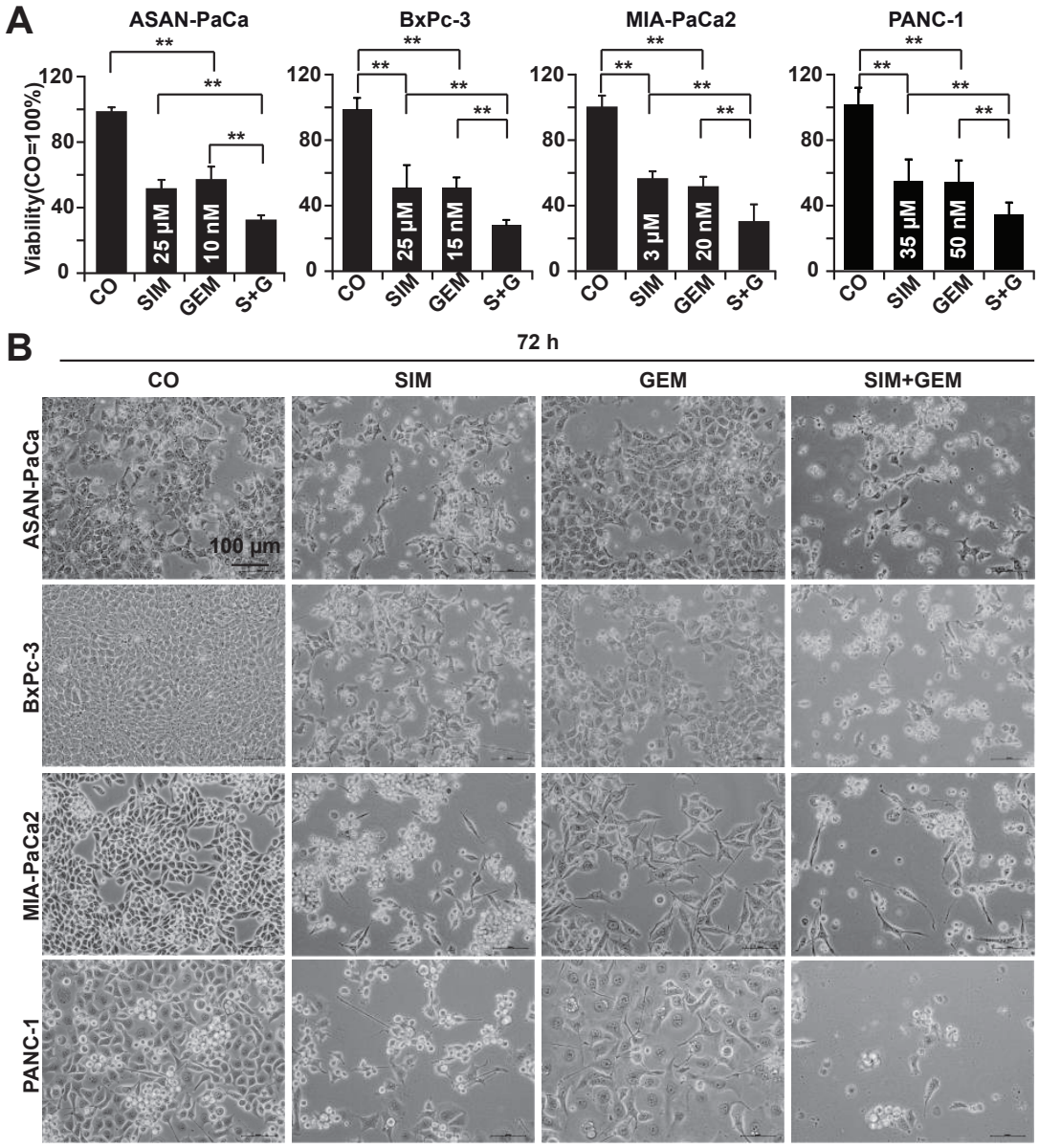

Figure 11: More aggressive pancreatic cancer cells response better to simvastatin

A. ASAN-PaCa, BxPc-3, MIA-PaCa2 and PANC-1 cells were treated as described above, and after 72 h cellular viability was measured by MTT assay. The cell viability was measured as described in Figure 10. **B**. The morphology of ASAN-PaCa, BxPc-3, MIA-PaCa2 and PANC-1 cells treated with simvastatin, gemcitabine or both together (SIM+GEM) after 72h or untreated (CO). Representative pictures are shown, and the bar indicates 100 μm. Data are presented as mean ± SD. (**P<0.01).

4.2 Simvastatin inhibits stemness and induces the differentiation potential of PDA *in vitro*

To study the influence of simvastatin on self-renewal capacity, ASAN-PaCa, BxPc-3, MIA-PaCa2 and PANC-1 cells were treated and then seeded for colony and spheroid formation. Whereas simvastatin and gemcitabine alone significantly reduced the colony and spheroid formation in both size and number, the combination of both substances had more pronounced effects on PDA cells (Figure 12, 13).

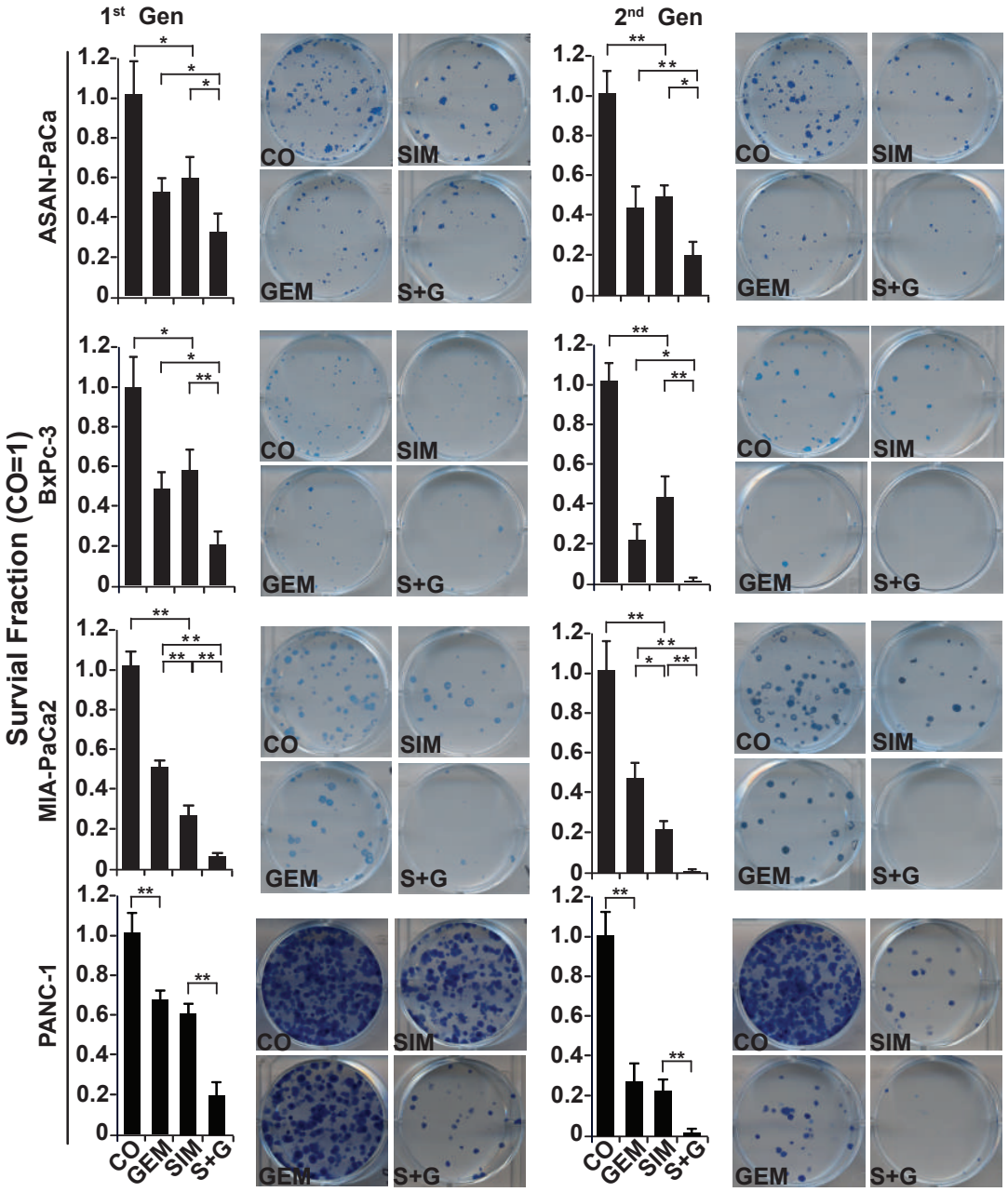

Figure 12: Simvastatin inhibits colony formation of pancreatic cancer cells

ASAN-PaCa, MIA-PaCa2, BxPc-3 and PANC-1 cells were treated as described in Figure 11, followed by re-plating of viable cells 72 h later at a low density (ASAN-PaCa: 1500 cells/well, MIA-PaCa2: 400 cells/well, BxPc-3: 1000 cells/well, PANC-1: 800 cells/well) in 6-well plates. After two weeks, colonies containing more than 50 cells were counted using a dissecting microscope. The number of surviving colonies in the control group was set to 1, and the survival fraction is presented on the left. After the first generation (1st Gen) of colony formation, the cells were harvested and treated as described above, and then treated cells were re-plated at the same density described in 6-well plates. After an additional 2 weeks, clonogenic survival was analyzed as described above. Representative photographs of fixed and the colonies of four Coomassie-stained cell lines are presented on the right of the quantification (2nd Gen). (**P<0.01).

To get information if the observed inhibition on colony and spheroid formation is maintained in a second passage after cell division, the survival colonies and spheroids

were harvested, and single, alive cells were isolated and seeded again for colony and spheroid formation. Also in this second-generation, and without additional simvastatin treatment, the spheroids and colonies were even stronger reduced than in the first-generation, suggesting that simvastatin indeed targeted CSCs *in vitro*.

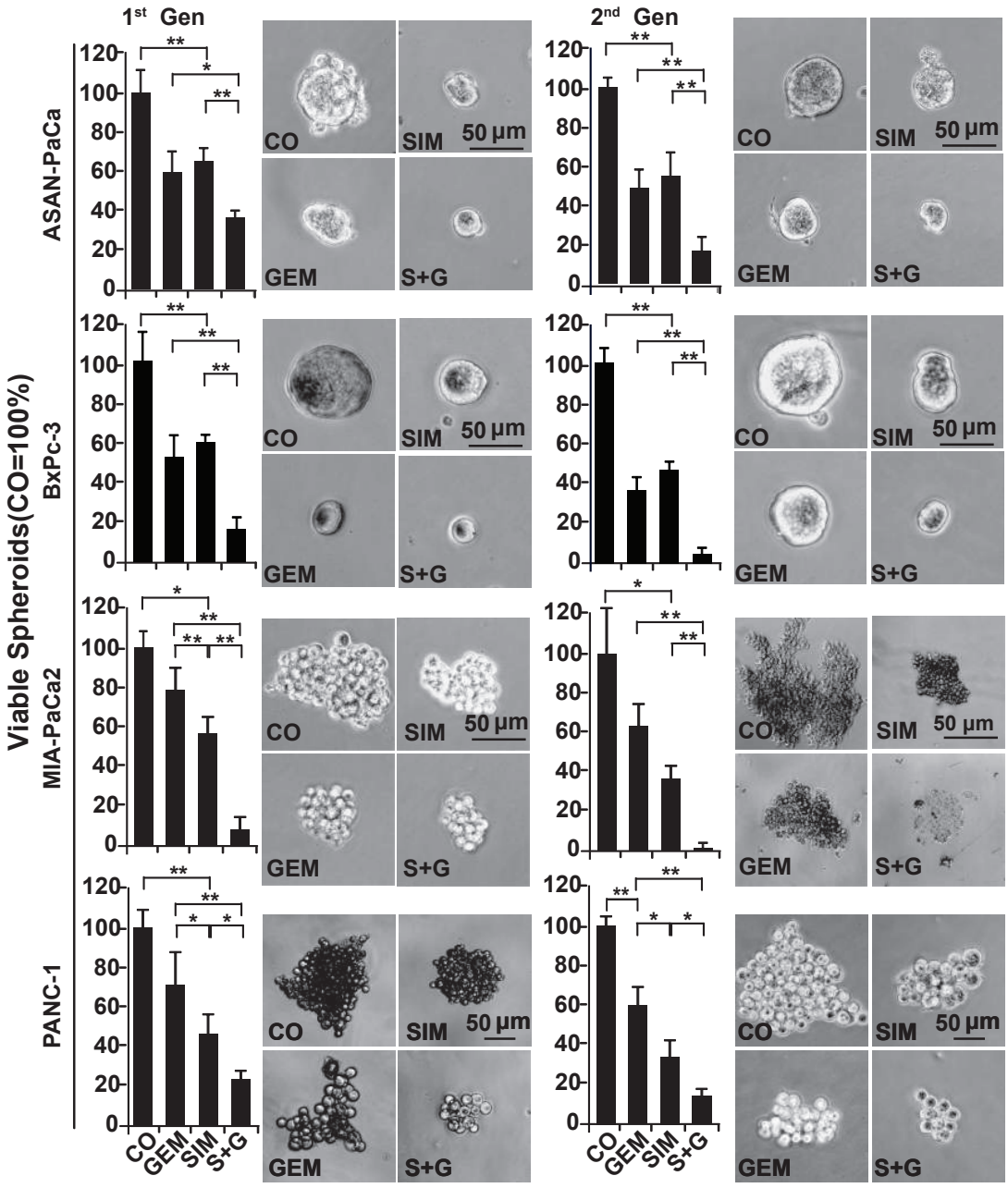

Figure 13: Simvastatin inhibits spheroid formation of pancreatic cancer cells

ASAN-PaCa, MIA-PaCa2, BxPc-3 and PANC-1 cells were seeded at a clonal density (1.0-2.0×10^3 cells/mL) in ultra-low attachment plates with serum-free but growth factor-containing medium for spheroid formation. The cells were treated as described in Figure 11, and 5 days later, the percentage of viable spheroids was determined (1st Gen). The quantity of spheroids in the control was set to 100%. Afterwards, the first generation spheroids were diluted to single cells, and equal quantity of live cells were re-plated at a concentration of 1.0-2.0×10^3 cells/mL. Upon spheroid formation 5 days later, the

cells were photographed at 100× magnification (on the right of the quantification) and quantified as described above (2nd Gen). The bar indicates 100 µm (**P<0.01).

To analyze the influence of simvastatin on the differentiation potential, cells were incubated with the IC50 concentration of simvastatin for 72 h. Then osteo-differentiation medium was added, to induce the osteoblastic differentiation. Ten days later, the cells were stained with BCIP/NBT to detect extracellular calcium deposits, which are secreted by mature osteoblasts. Such deposits were detected in all cells treated with simvastatin, but not in untreated cells (Figure 14A). Compared with non-simvastatin-treated control cells, the percentage of simvastatin-treated differentiated cells increased significantly to approximately 28% in ASAN-PaCa and BxPc-3 cells, followed by 23% in MIA-PaCa2 and 11% in PANC-1 cells (Figure 14B). To underline these results, the expression of bone morphogenetic protein 4 (BMP4) was evaluated, which is a marker for bone and cartilage development [188-191]. Western blot analysis shows that BMP4 is already expressed in untreated cells, however, after treatment with simvastatin for 72 h, the expression of BMP4 was increased significantly in all cell lines examined (Figure 14C). Simvastatin also induced adipocyte differentiation, but to a much lower extent (Figure 14D), as concluded from the absence of a significant red lipid droplet accumulation after simvastatin treatment. These results indicate that simvastatin induces osteoblastic differentiation.

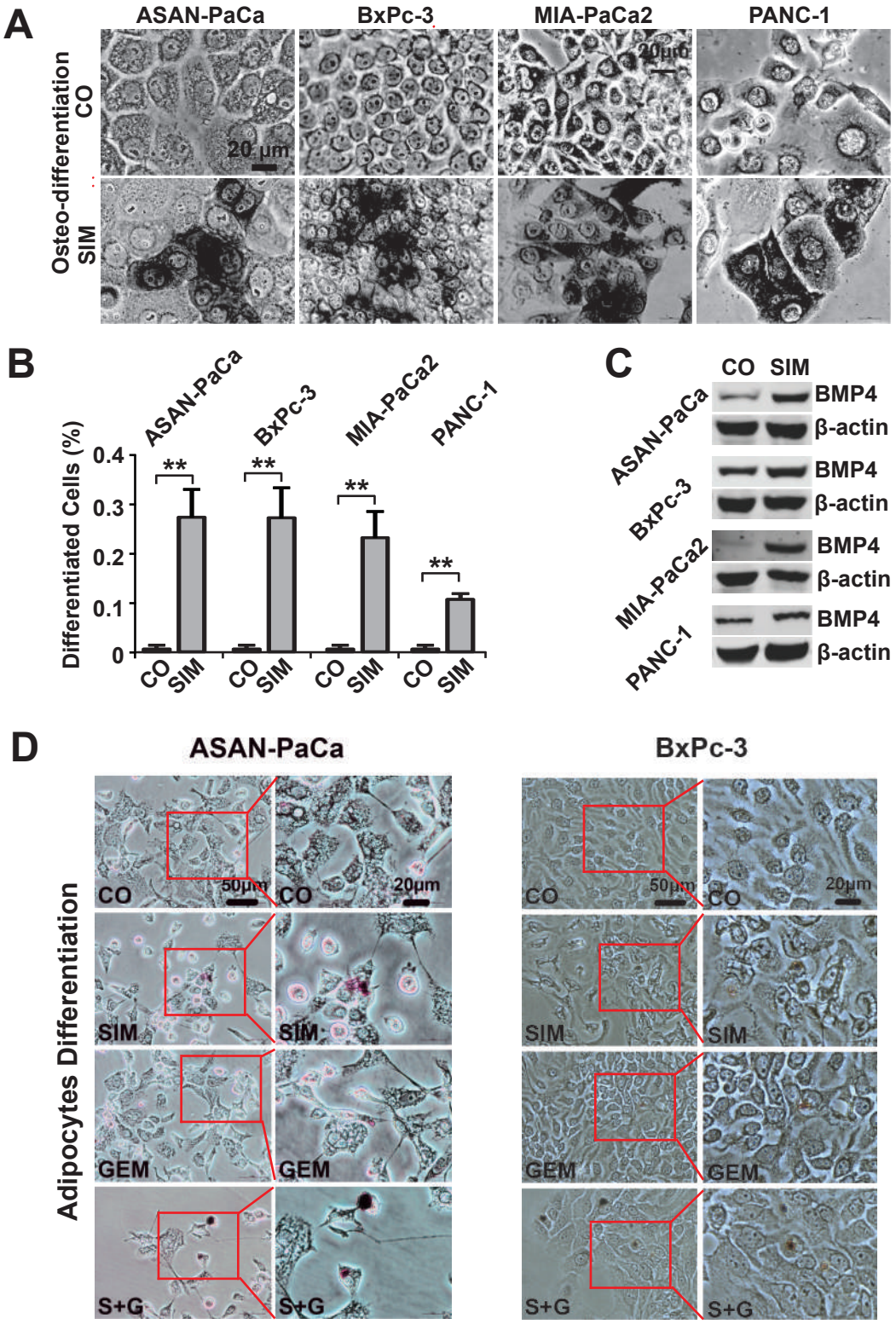

Figure 14: Simvastatin activates the differentiation potential

A. ASAN-PaCa, BxPc-3, MIA-PaCa2 and PANC-1 cells were treated with simvastatin (SIM) for 72 h or were untreated (CO) as described in Figure 11, and incubated in osteogenic medium for 10 days. Osteogenic differentiation was detected by BCIP®/NBT. Differentiated cells present in dark color. The bar indicates 20 µm. **B**. Quantitative analysis of stained areas was confirmed by counting the number of differentiated cells in 10 vision fields for each group and cell line, and the presentation of the obtained data as percentage of differentiated cells (**P<0.01). **C**. Three days after induction of

osteogenic differentiation, western blot analysis of BMP4 expression was performed. β-actin served as internal control. **D**. ASAN-PaCa and BxPc-3 cells were treated with or without simvastatin, lipid-droplets stained with Oil Red O. Differentiated cells present in red lipid droplet. The bar indicates 20 μm.

4.3 Simvastatin inhibits Hedgehog signaling

Because covalent modification of Hedgehog proteins with cholesterol mediates auto-cleavage and activation and Shh signaling has been implicated in tumorigenesis, I evaluated whether simvastatin may inhibit stemness by blocking Shh signaling in pancreatic cancer. To examine this hypothesis, the expression of the Hedgehog key members Shh, Smo, Gli1 and Sufu were detected before and after treatment of PDA cell lines with simvastatin and gemcitabine for 72 h followed by Western blot analysis. In all cell lines examined, simvastatin alone or combined with gemcitabine diminished the expression of Shh, Smo, and Gli1 and activated the expression of the Gli-inhibitor Sufu, whereas gemcitabine alone had no effect (Figure 15A). In contrast, gemcitabine alone did not modify the expression of these proteins significantly. To further examine the role of simvastatin in Hedgehog signaling, Shh was inhibited in PANC-1 cells by lipofection of specific Shh siRNA, whereas non-transfected cells or transfection of non-specific siRNA served as controls. First, the optimal siRNA concentration and time point after transfection were selected by time- and dose-response kinetics and the detection of Shh expression by Western blot analysis of transfected and untransfected cells. By this way, a dose of 75 nM and a time point of 3 days after transfection were identified as optimum (Figure 15B). These optimum siRNA conditions were then used to silence Shh expression in PANC-1 cells in the presence of the appropriate controls (Figure 15C). Interestingly, a knockdown of Gli1 could be achieved by the knockdown of Smo, suggesting cross-regulation. Furthermore, the silencing of Shh had a similar effect to the cells as simvastatin, because it reduced the viability in a time-dependent manner (Figure 15D) and inhibited the formation of colonies (Figure 15E). *Vice versa*, the overexpression of Shh by transfection of an expression vector for Shh cDNA, partially inhibited the tumor-preventive function of simvastatin (Figure 15F, G, H).

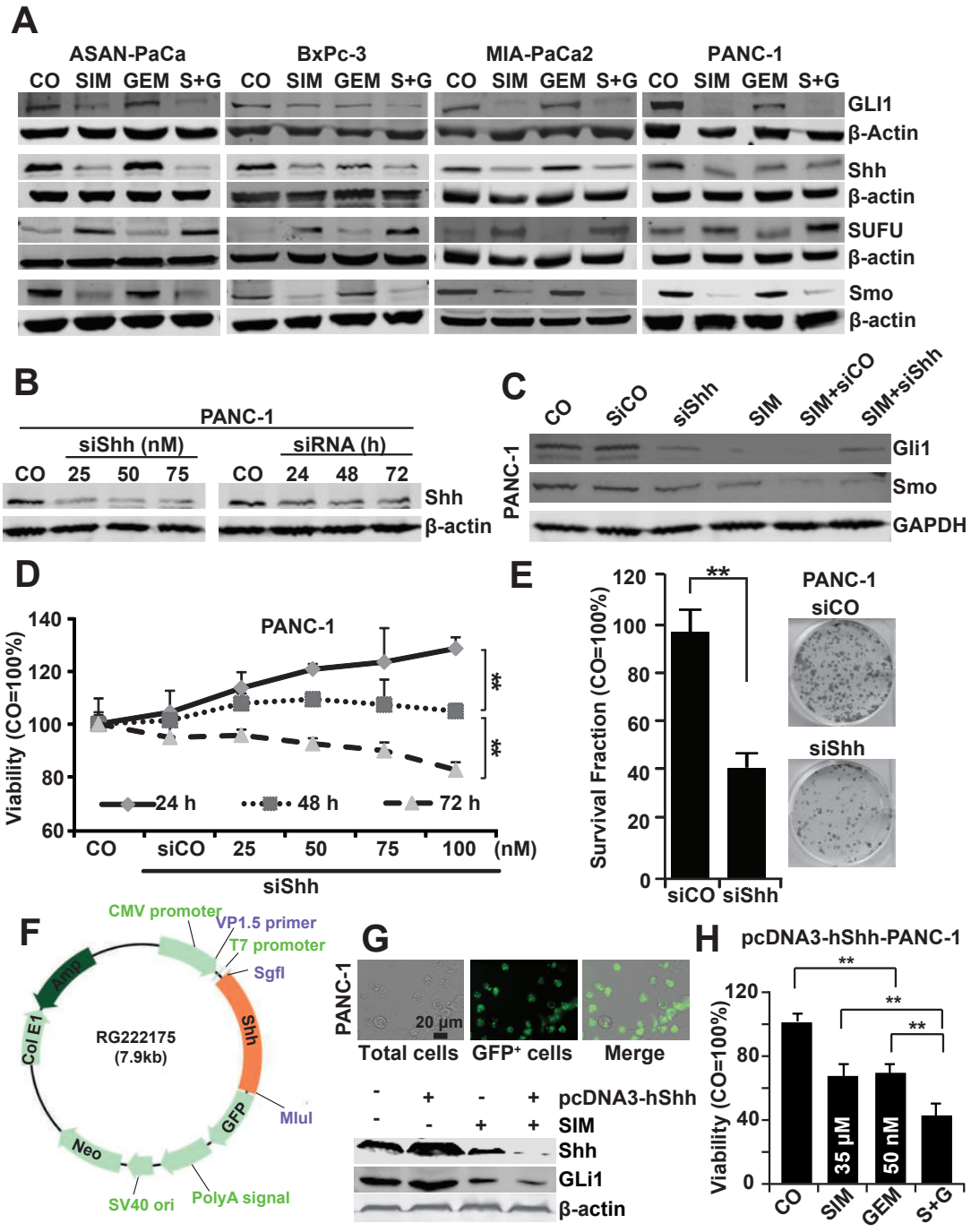

Figure 15: Simvastatin represses Shh signaling in pancreatic cancer cells

A. ASAN-PaCa, BxPc-3, MIA-PaCa2 and PANC-1were treated as described in Figure 11. After 72 h, the expression of Shh pathway related protein Gli1, Sonic Hedgehog (Shh), Smoothed (Smo) were measured by Western blot analysis in ASAN-PaCa, BxPc-3, MIA-PaCa2 and PANC-1 cell lines. β-actin served as a control. **B**. The efficiency of human Sonic hedgehog siRNA (siShh) against Shh was evaluated by western blot assay in PANC-1 cells at different time points (24, 48, 72 h) and concentrations (25, 50, 75 nM), where non-specific siRNA (siCO) with irrelevant sequence was used as reference (75 nM) and β-actin as internal control. **C**. PANC-1 cells were treated as indicated for 72 h, the effect of Shh siRNA on Gli1 and Smo were evaluated by Western blot. β-actin served as a control. Untreated (CO), non-specific siRNA (siCO), human Shh siRNA (siShh), simvastatin (SIM). **D**. Cell viability of PANC-1 cells after transfection of different concentrations (as indicated) of Shh siRNA for 24, 48 and 72 h. **E**. Survival fraction of PANC-1 cells after Shh siRNA knockdown with colony formation. non-specific siRNA (siCO), human Shh siRNA (siShh). **F.** The illustration shows

features of the Shh plasmid, which is derived from OriGene. **G**. PANC-1 cells were transfected with GFP-tagged plasmid of Shh, 48 h after transfection. G418 was added to the media at concentrations of 1 mg/ml. After 72 h growth in medium, efficiency of transient transfection was evaluated under inverted fluorescence. Bright-field and fluorescent images of GFP expression are taken at 72 h after selection. The bar indicates 20 μm. Cells transfected with Shh plasmid were treated as indicated for 72 h. Cells of all groups were harvested for Shh pathway related protein expression. Shh and Gli1 measurement expression by western blotting using β-actin served as an internal control. Human Shh plasmid (pcDNA3-hShh), simvastatin (SIM), Sonic hedgehog (Shh). **H**. Cell viability of Shh-transfected PANC-1 cells treated with simvastatin (SIM), gemcitabine (GEM) or both together (SIM+GEM) for 72 h was measured by MTT-assay, as described in Figure 11. The data are presented as mean ± SD. (**P<0.01).

To get knowledge which downstream signaling of progression markers may have been inhibited by simvastatin, to inhibit tumorigenicity, gene array analysis was performed. Whereas the expression of Shh genes, which was expected, was not significantly affected after simvastatin treatment (Figure 16A, B). Because the activation occurs on the posttranslational level, the expression of a broad panel of progression markers was inhibited (Figure 16C). Among them are CCNE, ZEB1, JAG2 and so on (Figure 16C).

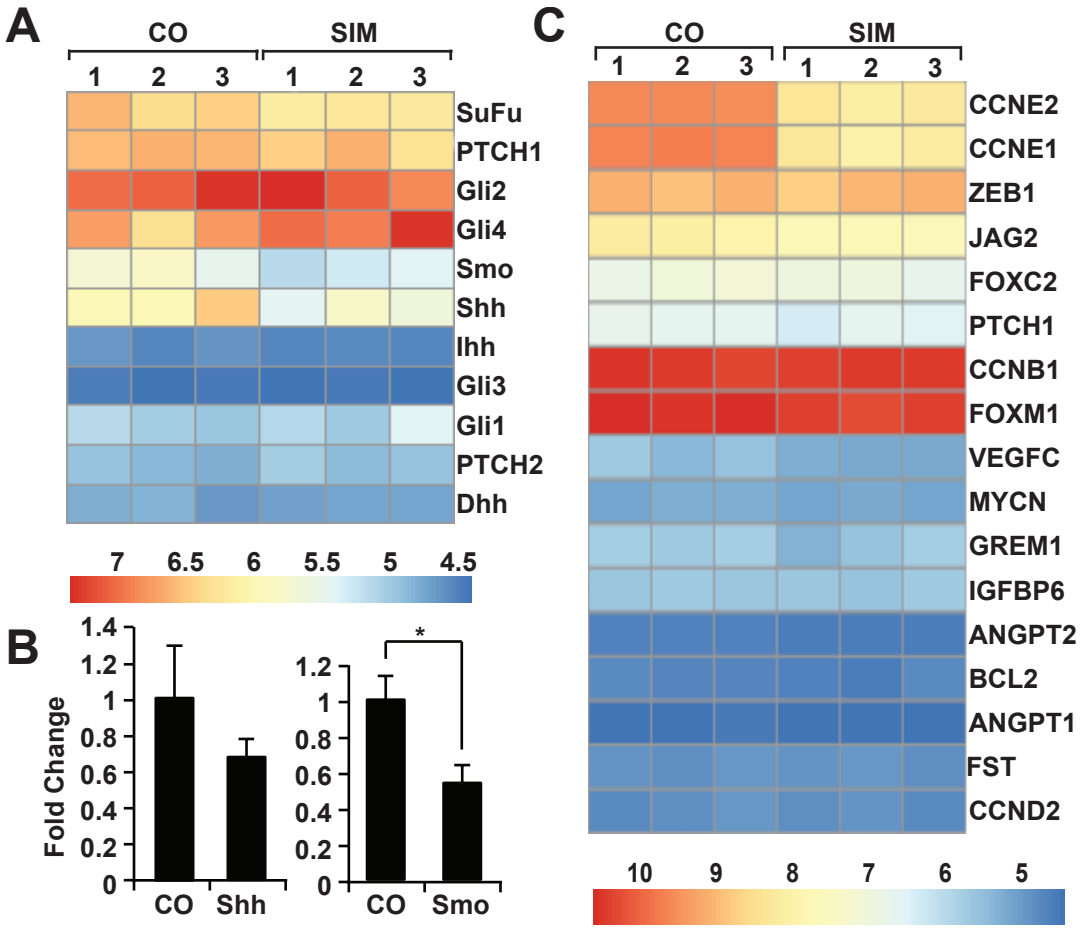

Figure 16: mRNA expression profiling of Shh signaling related genes in PANC-1 cells with or without simvastatin treatment

A. Heat map representation of the mRNA expression profiling showing selected genes within the Shh signaling pathway (annotated with KEGG pathway analysis) (*P<0.05). The red colors represent high expression and the blue colors indicated low expression within a scale from 4.5 to 7. **B**. Shh and Smo mRNA expression levels in PANC-1 cells 24h after treated with or without simvastatin (3 μM), as determined by qRT-PCR. The mean fold change of CO was set to 1. **C**. Heat map showed changes in the expression of Shh related progression markers in control (CO) and simvastatin (SIM) treatment groups. Shades of red and blue indicated high or low expression (see color scale). Experiments were performed in triplicate and data are shown as mean ± SD.

4.4 Simvastatin inhibits tumor growth, invasion and Hedgehog signaling *in vivo*

After establishing the functional effects of simvastatin *in vitro*, the results were further confirmed by using tumor xenotransplantation on fertilized chicken eggs. This *in vivo* system is naturally immunodeficient and enables the growth of well-perfused, tridimensional tumors with a pronounced tumor stroma, and with advantages to the mouse system regarding ethical reasons, bureaucracy and costs [192, 193]. The chick embryo needs 21 days breeding until hatching and xenografts do not grow before the blood vessels are well developed, so MIA-PaCa2 cells were transplanted at day 9 of development and injected the treatment or PBS in the control intravenously into chick blood vessels at days 12 and 15, until the tumor xenografts were harvested at day 18 of embryo development (Figure 17A). The presence of tumors on eggs was determined and is shown as percentage of tumor takes. In parallel, the size of the tumors was measured by calipers. The results reveal that all treatments (simvastatin, gemcitabine and both together) reduced the tumor take rate and the tumor size compared to the control xenografts (Figure 17B). The tumor take rate and the size of xenografts from chick embryos, which received double treatment were significantly smaller than after each single drug treatment. To study the effect of the treatment to tumor invasion chick tissues of the chorioallantoic membrane (CAM) adjacent to the tumor xenograft were resected and genomic DNA was extracted to perform a PCR with primers for detection of human Alu sequences. We found that 81% of CAM specimens from untreated chick embryos were Alu-positive, followed by 19%, 69% and 0% of CAM specimens derived from chick embryos treated with simvastatin, gemcitabine or a combination thereof, respectively (Figure 17C). An internal control PCR reaction was performed with the same tissue but with chicken GAPDH primers, resulting in the detection of the same band intensity in all probes, suggesting equal conditions. These results correlate to 43% of positive expression of the proliferation marker Ki67, which was decreased to 32% after each single treatment with a significant further reduction to around 22% upon double treatment, as measured by

immunohistochemistry staining followed by microscopy and evaluation of the number of positive cells (Figure 17D). Likewise, simvastatin, but not gemcitabine, reduced the expression of Shh, Smo and Gli1 to 23.6%, 21.9% and 21.3% compared to 36.6%, 70.3% and 69.5% of that in CO respectively. No further reduction was seen upon combination with gemcitabine. These results suggested that simvastatin inhibits tumor engraftment, tumor growth, invasion, and the expression of proliferation marker Ki67 and Shh signaling related proteins Shh, Smo and Gli1 *in vivo*.

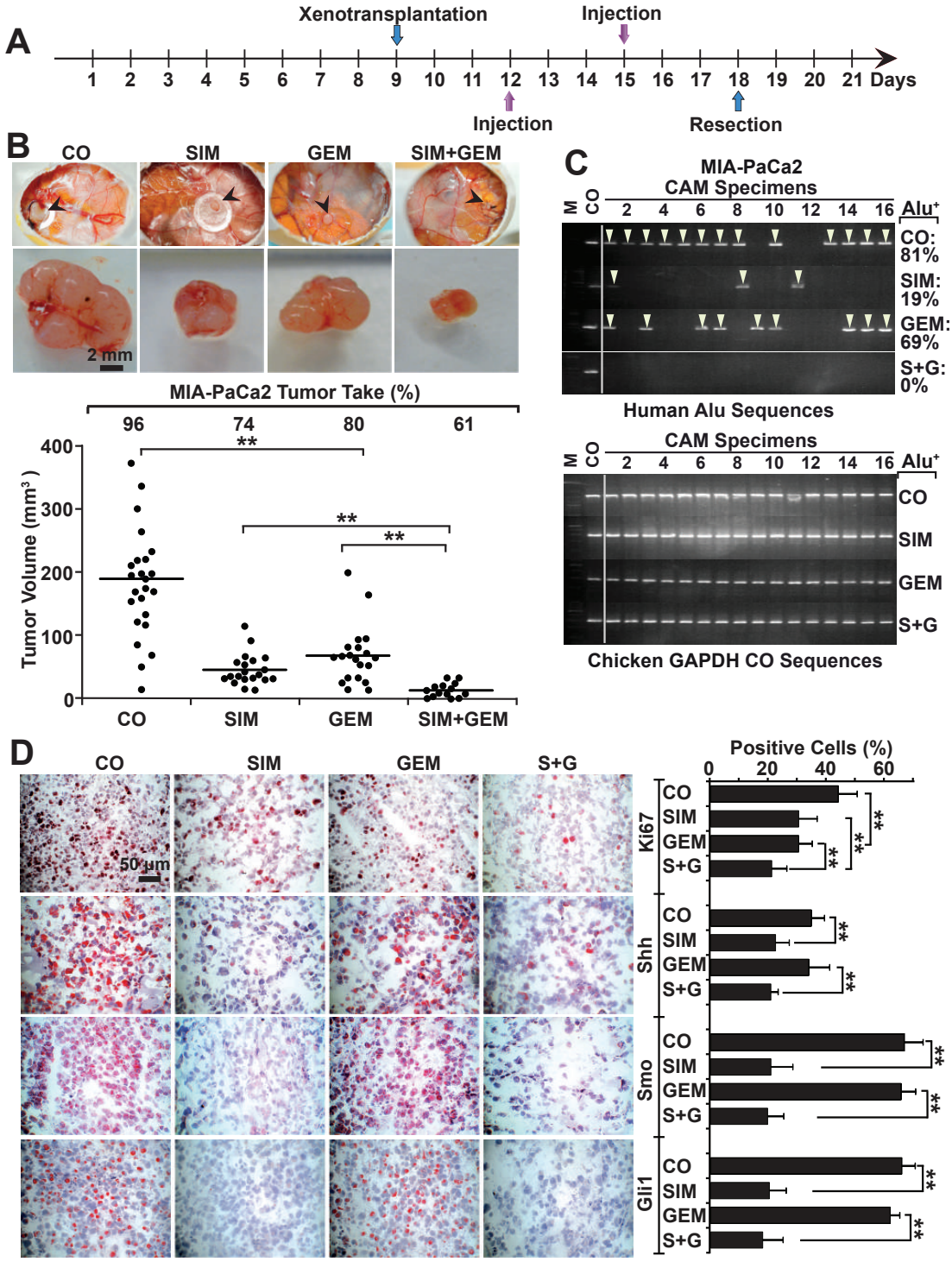

Figure 17: Simvastatin inhibits tumor growth and invasion in vivo and enhances gemcitabine efficacy

A. Experimental procedure: 5×10^5 MIA-PaCa2 cells were seeded onto the CAM of fertilized chick eggs at day 9 of embryonic development (25 eggs per group). At day 12 and 15, the CAM was injected with 20 µL of normal saline (CO), or 60 µg simvastatin in 20 µL of normal saline (SIM), or 75 µg gemcitabine in 20 µL of normal saline (GEM), or 60 µg simvastatin together with 75 µg gemcitabine in 20 µL of normal saline (SIM+GEM). The tumor xenografts were harvested at day 18. **B.** Representative photographs of a developed xenograft on the CAM (black arrow) and a resected xenograft tumor were shown below. The rate of engraftment (tumor takes) and the volumes of individual tumors (dots) and the means of each group (bar) are shown (**P<0.01). **C.** Genomic DNA was extracted from CAM tissue (n=16) directly adjacent to the tumor xenografts, and a PCR with

primers for human Alu sequences was performed. Genomic DNA in positive control (CO) was isolated from a tumor xenograft. The DNA marker is shown in the first line (Marker). **D.** Sections were prepared from the xenograft tumors and paraffin-embedded, and the expression of Ki67, Shh, Smo and Gli1 were examined by immunohistochemistry (left). The red or brown color represent positive signal. Representative images are shown. The scale bar indicates 50 μm. Quantification of Ki67, Shh, Gli1 and Sufu is shown on the right. The expression of positive cells was evaluated (right) by counting in 10 visual fields.

4.5 Statins inhibits Shh signaling in primary PDA patient tumors

To further examine the impact of statins in PDA patients, immunohistochemistry was performed and analyzed the Shh signaling related protein expression, Shh, Gli1 and Sufu, in patient tissue derived from normal pancreas (n=5) were analyzed, and PDA specimens derived from patients that had taken a statin such as simvastatin, atorvastatin and fluvastatin (n=34), or not (n=34) prior to resection. In normal pancreas, no Shh and Gli1 positive signal was found in the islets and ductal epithelium but evaluation of PDA tissue from patients without statin medication showed that the expression of Shh and Gli1 was 61% and 32% form specimens respectively, expression of Sufu was approximately 20% lower in PDA tissues compared with 25% Sufu positive in normal pancreas form specimens (Figure 18A, B). In contrast, expressions of Shh and Gli1 in tissue of patients with statin medication were almost absent. A significant higher Sufu positive signal was also detectable in PDA tissues from patients without statin medication (Figure 18A, B). Strong expression for c-Met, CXCR4 and vimentin in ductal cells and tumor stroma of patient tissue without simvastatin intake before surgery, whereas weakly positive staining for them in ductal cells and tumor stroma of PDA patient tissues with simvastatin intake (Figure 18C, D, Table 11, 12). These results confirm our *in vitro* and *in vivo* data and suggest that statin intake may prevent progression of PDA by inhibition of Hedgehog signaling. Basic on the results above, Shh signaling is aberrant high active in PDA cells, which has numerous and fundamental roles in proliferation, differentiation and migration in pancreatic cancer cells. After Shh is produced by PDA cells, covalent linkage of cholesterol to the Hedgehog proteins is required for their auto-cleavage, a necessary step in the processing and activation of the Shh. Inhibition of cholesterol biosynthesis mediated by simvastatin interrupts the post-translational modification of Shh and consequently prevents the inactive forms of Shh from binding to its receptor Patched, leading to inhibition of its downstream proteins Smo and nuclear transcription factor Gli1, which could enter the nucleus to active its target genes, such as Gli1, CD44 and CD133. Besides, simvastatin can also

impair Smo activation by directly reducing cholesterol level and induce expression of Gli1 inhibition protein Sufu in low-grade (Figure 18E).

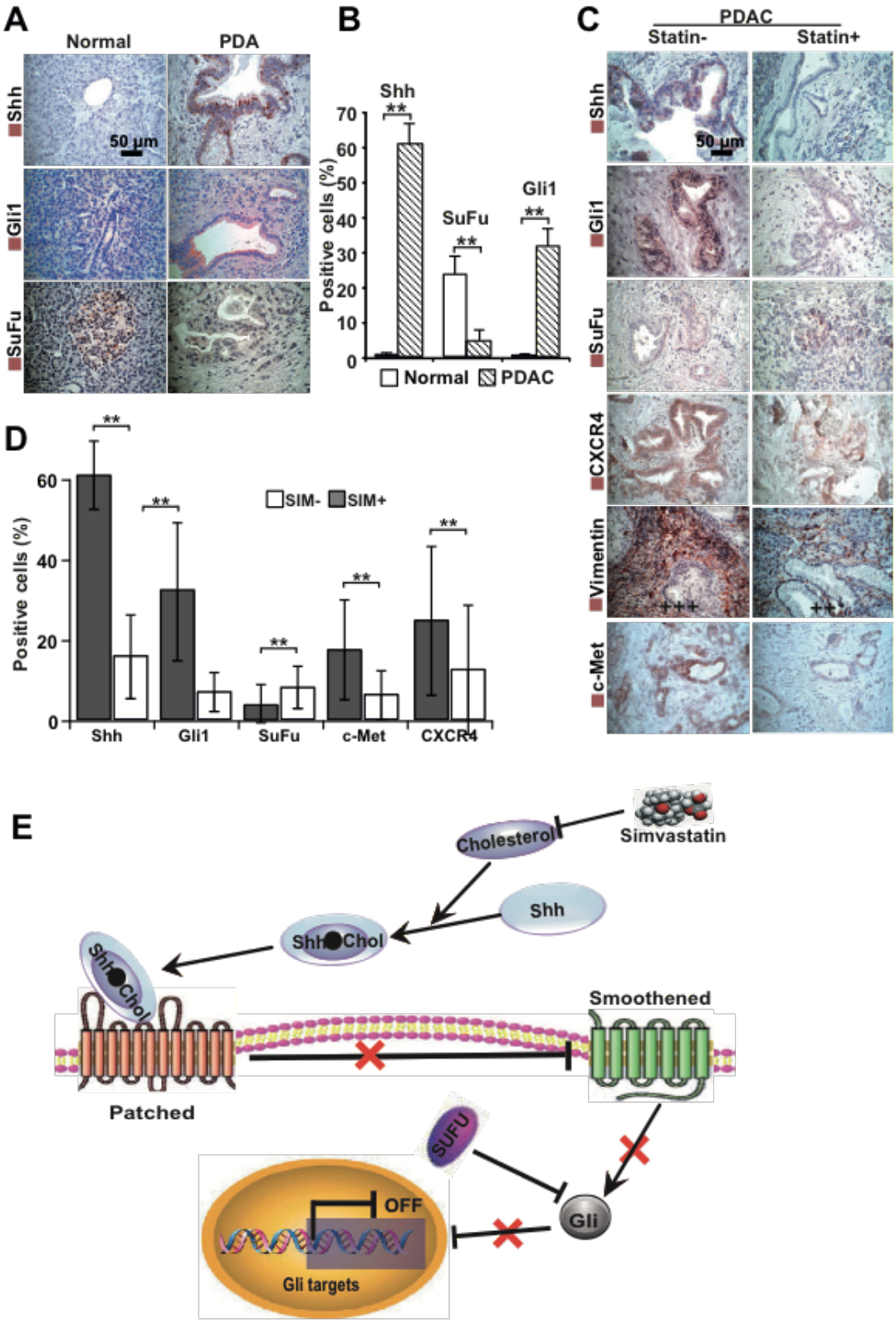

Figure 18: Shh signaling is inhibited in PDA specimens from patients with statin medication

A. Shh signaling related markers Shh, Gli1 and Sufu were detected by immunohistochemistry in normal pancreas (left) and PDA patients (right) specimens. Representative images are shown. The scale bar indicates 50 μm **B.** The numbers of dark brown cells in figure A were quantified in 10 visual fields, and the mean percentages ± SD are shown, **P<0.01. **C.** The patient tissue sections described were evaluated by immunohistochemistry for the expression of Shh, Gli1, Sufu and c-Met. Vimentin and CXCR4 in patient tissues were stained by immunohistochemistry. The scale bar indicates 50 μm. **D.** Quantification of Shh, Gli1, Sufu, c-Met, Vimentin and CXCR4 in figure C were determined by counting the number of dark brown cells in 10 visual fields, and the mean percentages ± SD are shown, **P<0.01. **E.** Role of simvastatin and cholesterol in the Shh signaling. Arrows suggest positive correlation, stopped lines is a sign of inhibition of the component and crosses indicate elimination of the inhibition of Smo and Gli1 when Shh presents. (**P<0.01).

Table 11: Protein expression in tissue of PDA patients without statin medication

	Patient ID.	Shh	Gli1	Sufu	Vim	CXCR4	c-Met
Controls without statin treatment	HD 5933-1B	54.6	34.7	1.23	+++	26.76	35.82
	HD 5956-1B	57.5	42.7	1.87	++	4.16	38.22
	HD 6026-1A	61.6	40.6	0	++	1.16	2.78
	HD 6076-1A	52.6	53.8	0.87	+++	14.3	0
	HD 6095-1A	64.5	38.8	0.78	+++	15.36	33.08
	HD 6102-1A	51.6	36.3	0.63	++	28.84	17.32
	HD 6111-1A	54.7	46	0.33	+++	9	20.58
	HD 6191-1A	52.7	54	0.28	++	5.02	0
	HD 5735-1A	53.8	2.16	0	+++	0	4.54
	HD 5835-1A	67.9	29.74	0	++	29.58	20.84
	HD 5897-1A	52.42	10.82	0	+++	17.82	40.5
	HD 5913-1B	51.4	9.6	0.36	++	0	0
	HD 5921-1B	69.9	52.78	9.98	+++	29.04	20.64
	HD 5934-1A	73.8	49.34	15.08	+++	20.72	45.68
	HD 6014-1A	45.45	0	6.96	++	18.56	12.4
	HD 6034-1A	61.78	44.5	3.42	+++	26.22	0
	HD 6040-1A	66.42	56.8	13.22	+++	42.22	32.48
	HD 6041-1A	69.26	11.98	9.64	+++	30.7	32.76
	HD 6053-1B	63.82	45.56	4.58	+++	14.52	17.24
	HD 6055-1C	69.56	47.42	2.03	+++	15.82	19.72
	HD 6065-1A	79.96	47.58	7.5	+++	0	67.2
	HD 6069-1A	63.7	57.24	5.06	+++	21.74	0
	HD 6070-1A	57.68	20.48	0	+++	25.52	30.16
	HD 6073-1A	44.25	24.86	0	+++	8.98	0
	HD 6074-1A	58.82	10.16	0	+++	25.52	29.36
	HD 6113-1A	56.58	17.76	7.62	+++	17.44	59.36
	HD 6121-1A	58.5	36.28	0	+++	4.96	15.96
	HD 6122-1A	71.18	7.34	8.36	+++	18.2	44.82
	HD 6127-1A	62.44	33.22	1.54	+++	27.6	38.82
	HD 6137-1A	72.583	23.88	9.28	+++	28.74	29.02
	HD 6138-1A	67.667	19.18	0.42	+++	0	49.48
	HD 6143-1A	60.2	20.3	14.36	+++	0	0
	HD 6144-1B	51.72	22.52	9.68	+++	44.46	37.76
	HD 6117-1A	Necrosis	Necrosis	Necrosis	Necrosis	Necrosis	Necrosis
Average		**60.62**	**31.77**	**4.09**	**+++**	**17.4**	**23.7**

The expression of Shh, Gli1, Sufu, Vimentin (Vim), CXCR4 and c-Met was determined by immunohistochemistry microscopy. The intensity expression and percentage of positive cells was determined by counting the number of differentiated cells in 10 vision fields for each group. Visual

judgment with Double-blind methods: extremely strong expression & very high percentage (≥75%) (+++), moderate expression & medium percentage (≤50%) (++)

Table 12: Protein expression in tissue of PDA patients with statin medication

Statin (mg/day)	Patient ID.	Shh	Gli1	Sufu	Vim	CXCR4	c-Met
Fluvastatin 80 mg	HD 4911-1A	0	0	6.04	++	2.16	0
Simvastatin 40 mg	HD 4921-1A	8.5	8.5	5.6	++	1.9	0
Simvastatin 40 mg	HD 4952-1A	3.1	3.1	4.5	++	0	0
Simvastatin 30 mg	HD 4953-1A	11.3	11.3	13	++	0	0
Simvastatin 20 mg	HD 5101-1A	12.8	12.8	12.7	+	Necrosis	0
Simvastatin 40 mg	HD 5393-1A	10.7	10.7	2.1	++	0	0
Simvastatin 40 mg	HD 5459-1A	12.2	12.2	10.9	+++	10.8	9.72
Simvastatin 40 mg	HD 5683-1A	1.7	1.7	7.5	++	0	4.94
Simvastatin 40 mg	HD 4907-1A	16.48	16.48	5.08	++	10.14	0
Simvastatin 40 mg	HD 4941-1A	14.56	14.56	0	++	4.02	11.06
Simvastatin 40 mg	HD 4990-1A	4.24	4.24	2.66	+++	0	3.96
Fluvastatin 20 mg	HD 5025-1A	0	0	5.1	+++	7.28	0
Simvastatin 20 mg	HD 5117-1B	13.82	13.82	5.56	+++	0.82	0
Simvastatin 20 mg	HD 5144-1A	21.24	21.24	4.46	+++	16.96	13.78
Pravastatin 20 mg	HD 5433-1A	13.86	13.86	11.74	+++	6.18	25.6
Simvastatin 5 mg	HD 5442-2A	17.08	17.08	19.82	+++	19.74	49.82
Simvastatin 40 mg	HD 5488-1A	26.48	26.48	15.34	++	15.66	0
Simvastatin 20 mg	HD 5511-1A	10.44	10.44	0	++	12.78	29.32
Fluvastatin 40 mg	HD 5530-1A	20.9	20.9	8.04	+++	9.4	34.64
Atorvastatin 40 mg	HD 5534-1A	17.06	17.06	7.04	+++	15.34	39.54
Atorvastatin 20 mg	HD 5657-4A	21.36	21.36	9.72	++	12.02	0
Simvastatin 10 mg	HD 5709-1A	18.58	18.58	5.1	++	0	0
Simvastatin 20 mg	HD 5752-1A	23.04	23.04	16.46	+++	4.34	50.34
Atorvastatin 10 mg	HD 5762-1A	1.3	1.3	5.02	++	0	0
Simvastatin 10 mg	HD 5777-1A	14.06	14.06	13.92	+++	8.06	5.8
Simvastatin 20 mg	HD 5858-1A	43.98	43.98	8.68	+++	2.08	18.18
Simvastatin 20 mg	HD 5853-1B	23.18	23.18	9.96	+++	13.46	42.28
Atorvastatin 10 mg	HD 5860-1A	33.9	33.9	1.35	Necrosis	0	22.9
Atorvastatin 20 mg	HD 5890-1A	3.38	3.38	2.28	+++	1.52	0
Simvastatin 20 mg	HD 5906-1A	36.62	36.62	13.62	+++	7.6	11.68
Simvastatin 20 mg	HD 5936-1A	9.66	9.66	9.54	+++	14	25.78
Simvastatin 20 mg	HD 5983-1A	15.32	15.32	16.9	+++	0	0
Atorvastatin 20 mg	HD 6051-1A	8.42	8.42	5.12	+++	0	0
Simvastatin 20 mg	HD 6018-1A	15.5	15.5	13.92	+++	12.18	21.18
Average		**14.85**	**6.75**	**8.20**	**++**	**6.3**	**12.9**

The expression of Shh, Gli1, Sufu, Vimentin (Vim), CXCR4 and c-Met was determined as described in Table 11

5. DISCUSSION AND CONCLUSION

5.1 Discussion

Pancreatic cancer is one of the worst prognoses of malignant diseases and the third leading cause of cancer death in the USA [1]. My experimental data suggest that statin medication may be helpful in prevention and treatment of this fatal disease. The clinical relevance of mechanistic studies on the incidence and progression of pancreatic cancer is unclear, because some studies suggest promotion, others the inhibition of tumor growth. Likewise, several clinical trials demonstrated that statin use was associated with a higher cancer risk [137]. For example, pravastatin was associated with an increase in breast cancer risk in one clinical trial [194], and total cancer and gastrointestinal cancer risk in another clinical trial [195], but pravastatin showed antitumor effect in an rat mammary gland carcinoma model. However, no other large randomized controlled trials of statins demonstrated an altered risk of cancer incidence [196-198]. Some authors emphasized that there was no evidence between the use of a statin and the development of colon, pancreatic or prostate cancer [139, 140, 152]. In the seven types of statins approved by the U.S. FDA, pravastatin is the most hydrophilic statin compared to the other statins, and differences in hydrophobicity may have clinical significance with respect to factors such as cancer risk. The different pharmacokinetic properties between hydrophilic and hydrophobic statins demonstrated differential outcomes on cancer risk [199, 200] and may explain the association between pravastatin use and high risk of cancer in two randomized controlled trials [194, 195]. Differences in hydrophobicity may be also the reason for inhibition of intracellular Ras protein translocation as observed for all statins except of pravastatin [129, 130, 201]. Contrary to concerns about the carcinogenicity of statins, growing clinical and epidemic evidence reveal that simvastatin in fact have a chemopreventive potential against multiple cancers [114], including colorectal, liver and prostate cancers [119, 202, 203], as well as pancreatic cancer [204]. These findings suggest that simvastatin may be a potential efficient agent to CSCs. This study provides evidence that simvastatin not only strongly inhibited the viability of pancreatic cancer cells but also sensitized established pancreatic CSCs to gemcitabine-mediated cytotoxicity by inhibiting cholesterol mediated Shh signaling pathway.

5.1.1 Simvastatin primarily targets highly aggressive cells and is well tolerated

To determine whether simvastatin primarily affects more aggressive cells, I used a set of established PDA cell lines with documented low, medium or high aggressiveness. My results showed that simvastatin mainly affected the more aggressive and gemcitabine-resistant cells but not the less-resistant or non-malignant cells. Similar results were found with several other cell lines of different tumor entities [205]. As most chemotherapeutic drugs have severe side effects, my observation of different sensitivity of non-malignant cells and cancer cells to simvastatin might have important effects on the option to use statins as co-treatment in cancer, similar to the already wide medication for treatment of hypercholesterolemia. To confirm my observation that simvastatin mainly attacks the highly aggressive pancreatic cancer cells, the effects of simvastatin on non-malignant pancreatic duct cells and mesenchymal stem cells was examined. I found that simvastatin exhibited slight cytotoxicity to these primary cells at a therapeutically active concentration of 50 μM *in vitro*. Likewise, simvastatin did not induce adverse side effects in chick embryos *in vivo*. Thus, simvastatin indeed may hold great potential benefits for both cancer prevention and treatment.

We proceeded to evaluate the effect of simvastatin on the therapeutic efficacy of gemcitabine. My results demonstrated that simvastatin significantly enhanced the ability of gemcitabine to reduce cell viability. A significant greater combined effect of simvastatin and gemcitabine combination compared to the single substances was also observed in PANC-1 cells, although it did not indicate synergism. This apparent contradiction can be explained by the T.C. Chou's theory, since synergism is determined by CI values instead of P values [184]. My *in vitro* data were further supported by my *in vivo* findings showing that simvastatin led to complete inhibition of tumor growth, engraftment and metastasis when administered in conjunction with gemcitabine.

5.1.2 Simvastatin inhibits CSCs self-renewal and induces differentiation in pancreatic cancer cells *in vitro*

We demonstrated that simvastatin strongly reduces the self-renewal potential of established and primary CSC cells by inhibition of colony and spheroid formation.

Similar results were recently found in breast cancer [133]. Since inhibition of cloning efficiency is generally considered the hallmark of differentiation, whether simvastatin induces osteogenic and adipocyte differentiation potential was further investigated in four established pancreatic cancer cell lines. It was found that simvastatin treatment activated the differentiation potential. These findings are consistent with recent studies, which reported that simvastatin induced osteogenic differentiation of Murine embryonic stem cells [188-190]. Similar results were also found in human periodontal ligament stem cells *in vitro* and *in vivo* [191].

I assume that these results may be partly due to the observed simvastatin-mediated inhibition of Sonic hedgehog activity, which is highly expressed in PDA aggressive CSC-like cells, which has been suggested that inhibition of Hedgehog signaling enhances delivery of chemotherapy in pancreatic cancer [56, 81, 206]. Indeed, almost all stages of PDA are associated with Hedgehog signaling [51], and activation of Shh and Smo is crucial for the development of pancreatitis [56, 207]. These events may be prevented by treatment with simvastatin, which thereby may reduce the risk of developing PDA or the progression of existing PDA.

5.1.3 Simvastatin impairs Hedgehog signaling

The mechanisms involved in the anti-proliferative and anti-CSC actions of simvastatin are not fully understood. Several lines of evidence indicate that an inhibitory effect on the Hedgehog signaling may be a major event in these antitumor actions [207-212]. The PDA cell lines have been reported to have high levels of Hh expression [213, 214]. Pancreatic adenocarcinoma cells can produce increasing amounts of Shh, and MIA-PaCa2 cell line showed higher Hh expression [215]. This is probably why simvastatin was maximally effective in MIA-PaCa2 cells, which is considered as one of the most malignant pancreatic cancer cell line.

Because of Hh signaling plays an important role of in cancer development, increasing studies are focus on safe and specific Hh inhibitors of clinical use [216]. By inhibiting HMG-CoA reductase activity, simvastatin is capable of blocking cholesterol synthesis, which was recognized essential in post-translational modification of Hedgehog proteins. Similar results were found in transgenic mouse models of the Smith−Lemli−Opitz syndrome, which revealed that cells defective in

cholesterol biosynthesis did not respond to Shh [217]. Moreover, cholesterol can directly or indirectly stimulate Smo activity [217, 218], a critical component of the Hh signal transduction. Contrarily, reduced cholesterol levels can also impair Smo activation [207]. *Vice versa*, high cholesterol levels are associated with accumulation of Smo on the plasma membrane, which is the first step to the activation of Smo [208]. Besides, some oxygenated derivatives of cholesterol can bind to Smo collaboratively with two well-known agonists, purmorphamine and SAG, which play key roles in Hh signaling [219]. In cancer treatment, Sufu is not usually targeted by medical compounds, however it plays an important role in clinical therapy since it is a tumor suppressor gene, mutations in Sufu could lead to the development of numerous types of cancer. In this study, my results show that the expression of Sufu is increased by simvastatin treatment. The reason of this alteration is not entirely clear. But the fact that Smo activates Gli transcription factors through the release of Gli from Sufu may explain this still unexplored mechanism [66].

5.1.4 Simvastatin has a preventive effect on the development of PDA.

I observed that simvastatin-treated PDA cells show an impaired potential for self-renewal due to the inhibition of spheroid and colony formation, and impaired tumor engraftment *in vivo*. These results are in accordance with recent data obtained in transgenic mouse models of pancreatic cancer, which demonstrated that daily i.p. injection of 10 mg/kg simvastatin significantly delayed the progression of mPanINs in LsL-KrasG12D; Pdx1-Cre mice [135]. The concentrations of simvastatin administered to mice may be relevant for humans, because simvastatin dose-dependently exerted a significant chemopreventive potential in lung cancer in COPD patients [220]. The daily intake of 30 mg statin was associated with a lower risk of cancer related death, especially among simvastatin users. After PDA diagnosis, the use of simvastatin indicated a longer survival among patients with non-metastatic PDA [121]. A recent clinical study also suggested that statins show a promising effect on "long-term response" to gemcitabine-erlotinib chemotherapy in unrespectable pancreatic cancer [221].

My data obtained with the chicken egg model adds important information to the increasing evidence that simvastatin indeed may be able to inhibit the growth of PDA in patients, as indicated in recently published clinical studies, which reported that

patients who had intake moderate-high-intensity doses of simvastatin have a significantly better survival compared to those who received low-intensity doses [124]. Statin use was associated with longer overall survival (OS) of PDA patients and significantly decreased the risk of pancreatic cancer and there was a significant dose-effect of statin use for the risk of pancreatic cancer [149, 161]. However, in contrast to my study, none of these studies addressed the efficacy of simvastatin toward CSC-like PDA cells by Hedgehog signaling.

My results obtained with PDA xenografts on chicken eggs, as well as my examinations of tissues from patients who had or had not taken simvastatin before surgery suggest that simvastatin strongly reduces the expression of Shh and Gli1. The effect of simvastatin on Hedgehog signaling in PDA has to my knowledge never been studied before. The obvious clinical relevance of my findings has recently been confirmed by several studies in colorectal cancer [222], prostate cancer [126], renal cell carcinoma [143] and lung cancer [154, 164]. These authors support my view and conclude that cancer patients may benefit from simvastatin medication.

5.2 Conclusion

My study demonstrates that simvastatin exhibits strong activity against pancreatic CSCs, which may be mediated by inhibition of the Shh signaling pathway. Importantly, simvastatin does not present obvious toxicity to non-malignant cells. Besides, the self-renewal of pancreatic CSCs is inhibited and the differentiation potential is activated *in vitro*. Tumor engraftment, growth, invasion and the activation of Shh signaling are inhibited *in vivo*. Immunohistochemistry of human pancreatic cancer tissue from patients that received a statin prior to surgery or not demonstrated that the activation of Shh signaling and progression markers was inhibited by statin medication. Therefore, I suggest simvastatin to be a promising adjuvant for prevention of PDA and to improve the efficacy of chemotherapy as a robust, cost-effective and well-tolerated drug.

6. SUMMARY

Pancreatic ductal adenocarcinoma (PDA) is a disease with an exceptionally poor prognosis, high therapy resistance and poor effective therapeutic options. Advances in therapeutic treatments are urgently required. Cancer stem-like cells (CSCs), capable of unlimited self-renewal, have been proposed as a mechanism for cancer growth, therapy resistance and metastasis, involving PDA. Besides a function in normal tissue development, Sonic hedgehog (Shh) is highly expressed at all stages of human PDA. Recent data demonstrate that the expression of Shh is highly upregulated in CSCs and regulates them. Simvastatin, which is widely prescribed as cholesterol-lowering drug, was shown to inhibit tumor growth, metastasis and cancer-specific mortality in some studies, but the available data are not consistent. Most importantly, **the hypothesis of my thesis, namely that simvastatin attacks pancreatic CSCs by inhibition of Sonic hedgehog signaling** was never examined before. In my thesis, I evaluated the effect of simvastatin on 3 established and 1 primary PDA cell lines, as wells as non-malignant pancreas cells and mesenchymal stromal cells from human bone marrow. Results from cell viability assays show that simvastatin significantly reduces viability even at low concentration in CSC-enriched cell lines. I observed synergetic effects on pancreatic cancer cells upon combination of simvastatin with gemcitabine *in vitro*. Colony and spheroid assays indicated that simvastatin inhibits self-renewal, with an even stronger effect upon combination with gemcitabine. In addition, simvastatin significantly induced the differentiation potential. My results obtained with pancreatic cancer xenografts transplanted on the CAM of fertilized chicken eggs show that simvastatin inhibits tumor growth and metastasis *in vivo*. Importantly，no pronounced toxic side effects of simvastatin to non-malignant cells or chick embryos occurred. My further experiments suggest that the underlying mechanism of simvastatin is associated with inhibition of cholesterol biosynthesis, which is essential for post-translational modification and thereby Sonic hedgehog activation. Simvastatin alone or combined with gemcitabine diminished the expression of Shh and related proteins Smo, Gli1 and activated the expression of the Gli-inhibitor Sufu. Likewise, the siRNA-mediated inhibition of Shh expression mimicked the simvastatin effect, whereas it´s overexpression prevented it. I confirmed these *in vitro* and *in vivo* findings in tissue of patients who did (n=34) or did not (n=34) receive simvastatin prior to surgery. Thus, the expression of Shh, its

downstream signaling protein Gli1, along with the levels of progression markers Vimentin, CXCR4 and c-Met were lower in PDA tissues from patients with statin medication. Therefore, I conclude that simvastatin, as a robust, cost-effective and well-tolerated drug for prevention of hypercholesterolemia, may also be suited to prevent PDA and to improve the efficacy of standard therapy in patients suffering from PDA.

7. REFERENCES

1. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer Statistics, 2017.* CA Cancer J Clin, 2017. **67**(1): p. 7-30.
2. *World Cancer Report 2014.* WHO, 2014.
3. Rahib, L., et al., *Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States.* Cancer Res, 2014. **74**(11): p. 2913-21.
4. Shavers, V.L., et al., *Racial/ethnic patterns of care for pancreatic cancer.* J Palliat Med, 2009. **12**(7): p. 623-30.
5. Berrington de Gonzalez, A., S. Sweetland, and E. Spencer, *A meta-analysis of obesity and the risk of pancreatic cancer.* Br J Cancer, 2003. **89**(3): p. 519-23.
6. Schenk, M., et al., *Familial risk of pancreatic cancer.* J Natl Cancer Inst, 2001. **93**(8): p. 640-4.
7. Yeo, T.P., *Demographics, Epidemiology, and Inheritance of Pancreatic Ductal Adenocarcinoma.* Seminars in Oncology, 2015. **42**(1): p. 8-18.
8. Porta, M., et al., *Exocrine pancreatic cancer: symptoms at presentation and their relation to tumour site and stage.* Clin Transl Oncol, 2005. **7**(5): p. 189-97.
9. Noe, M. and L.A. Brosens, *Pathology of Pancreatic Cancer Precursor Lesions.* Surg Pathol Clin, 2016. **9**(4): p. 561-580.
10. Edge, S.B. and C.C. Compton, *The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM.* Ann Surg Oncol, 2010. **17**(6): p. 1471-4.
11. Siegel, R., D. Naishadham, and A. Jemal, *Cancer statistics, 2013.* CA Cancer J Clin, 2013. **63**(1): p. 11-30.
12. Konstantinidis, I.T., et al., *Pancreatic Ductal Adenocarcinoma Is There a Survival Difference for R1 Resections Versus Locally Advanced Unresectable Tumors? What Is a 'True"R0 Resection?* Annals of Surgery, 2013. **257**(4): p. 731-736.
13. Wolfgang, C.L., et al., *Recent progress in pancreatic cancer.* Ca-a Cancer Journal for Clinicians, 2013. **63**(5): p. 318-348.
14. Vincent, A., et al., *Pancreatic cancer.* Lancet, 2011. **378**(9791): p. 607-20.
15. Hertel, L.W., et al., *Evaluation of the antitumor activity of gemcitabine (2',2'-difluoro-2'-deoxycytidine).* Cancer Res, 1990. **50**(14): p. 4417-22.
16. Schultz, R.M., et al., *Evaluation of new anticancer agents against the MIA PaCa-2 and PANC-1 human pancreatic carcinoma xenografts.* Oncol Res, 1993. **5**(6-7): p. 223-8.
17. Cid-Arregui, A. and V. Juarez, *Perspectives in the treatment of pancreatic adenocarcinoma.* World J Gastroenterol, 2015. **21**(31): p. 9297-316.
18. Moore, M., *Activity of gemcitabine in patients with advanced pancreatic carcinoma. A review.* Cancer, 1996. **78**(3 Suppl): p. 633-8.
19. Copley-Merriman, C., et al., *Economic value of gemcitabine compared to cisplatin and etoposide in non-small cell lung cancer.* Lung Cancer, 1996. **14**(1): p. 45-61.
20. Possinger, K., *Gemcitabine in advanced breast cancer.* Anticancer Drugs, 1995. **6 Suppl 6**: p. 55-9.
21. Lund, B. and J.P. Neijt, *Gemcitabine in cisplatin-resistant ovarian cancer.* Semin Oncol, 1996. **23**(5 Suppl 10): p. 72-6.

22. Gesto, D.S., et al., *Gemcitabine: a critical nucleoside for cancer therapy.* Curr Med Chem, 2012. **19**(7): p. 1076-87.
23. Burris, H.A., 3rd, et al., *Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial.* J Clin Oncol, 1997. **15**(6): p. 2403-13.
24. Moore, M.J., et al., *Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group.* J Clin Oncol, 2007. **25**(15): p. 1960-6.
25. Berlin, J.D., et al., *Phase III study of gemcitabine in combination with fluorouracil versus gemcitabine alone in patients with advanced pancreatic carcinoma: Eastern Cooperative Oncology Group Trial E2297.* J Clin Oncol, 2002. **20**(15): p. 3270-5.
26. Heinemann, V., et al., *Randomized phase III trial of gemcitabine plus cisplatin compared with gemcitabine alone in advanced pancreatic cancer.* J Clin Oncol, 2006. **24**(24): p. 3946-52.
27. Li, D. and E.M. O'Reilly, *Adjuvant and neoadjuvant systemic therapy for pancreas adenocarcinoma.* Semin Oncol, 2015. **42**(1): p. 134-43.
28. Fidler, I.J. and I.R. Hart, *The Development of Biological Diversity and Metastatic Potential in Malignant Neoplasms.* Oncodevelopmental Biology and Medicine, 1982. **4**(1-2): p. 161-176.
29. Heppner, G.H., *Tumor Heterogeneity.* Cancer Research, 1984. **44**(6): p. 2259-2265.
30. Penchev, V.R., et al., *Heterogeneity and targeting of pancreatic cancer stem cells.* Clin Cancer Res, 2012. **18**(16): p. 4277-84.
31. Magee, J.A., E. Piskounova, and S.J. Morrison, *Cancer stem cells: impact, heterogeneity, and uncertainty.* Cancer Cell, 2012. **21**(3): p. 283-96.
32. Li, Y., et al., *Pancreatic cancer stem cells: emerging target for designing novel therapy.* Cancer Lett, 2013. **338**(1): p. 94-100.
33. Das, S., M. Srikanth, and J.A. Kessler, *Cancer stem cells and glioma.* Nat Clin Pract Neurol, 2008. **4**(8): p. 427-35.
34. Al-Hajj, M., et al., *Prospective identification of tumorigenic breast cancer cells.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 3983-8.
35. Yang, Z.F., et al., *Significance of CD90+ cancer stem cells in human liver cancer.* Cancer Cell, 2008. **13**(2): p. 153-66.
36. Singh, S.K., et al., *Identification of a cancer stem cell in human brain tumors.* Cancer Res, 2003. **63**(18): p. 5821-8.
37. Dalerba, P., et al., *Phenotypic characterization of human colorectal cancer stem cells.* Proc Natl Acad Sci U S A, 2007. **104**(24): p. 10158-63.
38. Baba, T., et al., *Epigenetic regulation of CD133 and tumorigenicity of CD133+ ovarian cancer cells.* Oncogene, 2009. **28**(2): p. 209-18.
39. Collins, A.T., et al., *Prospective identification of tumorigenic prostate cancer stem cells.* Cancer Res, 2005. **65**(23): p. 10946-51.
40. Li, C.W., et al., *Identification of pancreatic cancer stem cells.* Cancer Research, 2007. **67**(3): p. 1030-1037.
41. Adikrisna, R., et al., *Identification of pancreatic cancer stem cells and selective toxicity of chemotherapeutic agents.* Gastroenterology, 2012. **143**(1): p. 234-45 e7.
42. Li, C., et al., *Identification of pancreatic cancer stem cells.* Cancer Res, 2007. **67**(3): p. 1030-7.

43. Hermann, P.C., et al., *Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer.* Cell Stem Cell, 2007. **1**(3): p. 313-323.
44. Yao, J., et al., *Side population in the pancreatic cancer cell lines SW1990 and CFPAC-1 is enriched with cancer stem-like cells.* Oncology Reports, 2010. **23**(5): p. 1375-1382.
45. Zhu, J., et al., *Identification of glycoprotein markers for pancreatic cancer CD24+CD44+ stem-like cells using nano-LC-MS/MS and tissue microarray.* J Proteome Res, 2012. **11**(4): p. 2272-81.
46. Li, C.W., et al., *c-Met Is a Marker of Pancreatic Cancer Stem Cells and Therapeutic Target.* Gastroenterology, 2011. **141**(6): p. 2218-U395.
47. Lu, Y.H., et al., *Knockdown of Oct4 and Nanog expression inhibits the stemness of pancreatic cancer cells.* Cancer Letters, 2013. **340**(1): p. 113-123.
48. Ohara, Y., et al., *Histological and prognostic importance of CD44(+) /CD24(+) /EpCAM(+) expression in clinical pancreatic cancer.* Cancer Sci, 2013. **104**(8): p. 1127-34.
49. Maeda, K., et al., *CD133 Modulate HIF-1alpha Expression under Hypoxia in EMT Phenotype Pancreatic Cancer Stem-Like Cells.* Int J Mol Sci, 2016. **17**(7).
50. Takao, S., Q. Ding, and S. Matsubara, *Pancreatic cancer stem cells: regulatory networks in the tumor microenvironment and targeted therapy.* J Hepatobiliary Pancreat Sci, 2012. **19**(6): p. 614-20.
51. Thayer, S.P., et al., *Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis.* Nature, 2003. **425**(6960): p. 851-6.
52. Zeng, G., et al., *Aberrant Wnt/beta-catenin signaling in pancreatic adenocarcinoma.* Neoplasia, 2006. **8**(4): p. 279-89.
53. Lee, C.J., J. Dosch, and D.M. Simeone, *Pancreatic cancer stem cells.* Journal of Clinical Oncology, 2008. **26**(17): p. 2806-2812.
54. Kelleher, F.C., *Hedgehog signaling and therapeutics in pancreatic cancer.* Carcinogenesis, 2011. **32**(4): p. 445-51.
55. Lee, Y., et al., *Loss of suppressor-of-fused function promotes tumorigenesis.* Oncogene, 2007. **26**(44): p. 6442-7.
56. Porter, J.A., K.E. Young, and P.A. Beachy, *Cholesterol modification of hedgehog signaling proteins in animal development.* Science, 1996. **274**(5285): p. 255-9.
57. Buglino, J.A. and M.D. Resh, *Hhat is a palmitoylacyltransferase with specificity for N-palmitoylation of Sonic Hedgehog.* J Biol Chem, 2008. **283**(32): p. 22076-88.
58. Lee, J.J., et al., *Autoproteolysis in hedgehog protein biogenesis.* Science, 1994. **266**(5190): p. 1528-37.
59. Bumcrot, D.A., R. Takada, and A.P. McMahon, *Proteolytic processing yields two secreted forms of sonic hedgehog.* Mol Cell Biol, 1995. **15**(4): p. 2294-303.
60. Roelink, H., et al., *Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis.* Cell, 1995. **81**(3): p. 445-55.
61. Briscoe, J., et al., *A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube.* Cell, 2000. **101**(4): p. 435-45.

62. Lewis, P.M., et al., *Cholesterol modification of sonic hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptc1.* Cell, 2001. **105**(5): p. 599-612.
63. Porter, J.A., et al., *The product of hedgehog autoproteolytic cleavage active in local and long-range signalling.* Nature, 1995. **374**(6520): p. 363-6.
64. Teglund, S. and R. Toftgard, *Hedgehog beyond medulloblastoma and basal cell carcinoma.* Biochim Biophys Acta, 2010. **1805**(2): p. 181-208.
65. Yang, L., et al., *Activation of the hedgehog-signaling pathway in human cancer and the clinical implications.* Oncogene, 2010. **29**(4): p. 469-81.
66. Kogerman, P., et al., *Mammalian suppressor-of-fused modulates nuclear-cytoplasmic shuttling of Gli-1.* Nat Cell Biol, 1999. **1**(5): p. 312-9.
67. Humke, E.W., et al., *The output of Hedgehog signaling is controlled by the dynamic association between Suppressor of Fused and the Gli proteins.* Genes Dev, 2010. **24**(7): p. 670-82.
68. Cochrane, C.R., et al., *Hedgehog Signaling in the Maintenance of Cancer Stem Cells.* Cancers (Basel), 2015. **7**(3): p. 1554-85.
69. Ingham, P.W. and A.P. McMahon, *Hedgehog signaling in animal development: paradigms and principles.* Genes Dev, 2001. **15**(23): p. 3059-87.
70. Briscoe, J. and P.P. Therond, *The mechanisms of Hedgehog signalling and its roles in development and disease.* Nat Rev Mol Cell Biol, 2013. **14**(7): p. 416-29.
71. Teperino, R., et al., *Hedgehog partial agonism drives Warburg-like metabolism in muscle and brown fat.* Cell, 2012. **151**(2): p. 414-26.
72. Courchet, J. and F. Polleux, *Sonic hedgehog, BOC, and synaptic development: new players for an old game.* Neuron, 2012. **73**(6): p. 1055-8.
73. Babcock, D.T., et al., *Hedgehog signaling regulates nociceptive sensitization.* Curr Biol, 2011. **21**(18): p. 1525-33.
74. Athar, M., et al., *Sonic hedgehog signaling in Basal cell nevus syndrome.* Cancer Res, 2014. **74**(18): p. 4967-75.
75. Pasca di Magliano, M. and M. Hebrok, *Hedgehog signalling in cancer formation and maintenance.* Nat Rev Cancer, 2003. **3**(12): p. 903-11.
76. Podlasek, C.A., et al., *Prostate development requires Sonic hedgehog expressed by the urogenital sinus epithelium.* Dev Biol, 1999. **209**(1): p. 28-39.
77. Szkandera, J., et al., *Hedgehog signaling pathway in ovarian cancer.* Int J Mol Sci, 2013. **14**(1): p. 1179-96.
78. Feldmann, G., et al., *Hedgehog inhibition prolongs survival in a genetically engineered mouse model of pancreatic cancer.* Gut, 2008. **57**(10): p. 1420-30.
79. Feldmann, G., et al., *Blockade of hedgehog signaling inhibits pancreatic cancer invasion and metastases: a new paradigm for combination therapy in solid cancers.* Cancer Res, 2007. **67**(5): p. 2187-96.
80. Rangarajan, P., et al., *Crocetinic acid inhibits hedgehog signaling to inhibit pancreatic cancer stem cells.* Oncotarget, 2015. **6**(29): p. 27661-73.
81. Olive, K.P., et al., *Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer.* Science, 2009. **324**(5933): p. 1457-61.
82. Hwang, R.F., et al., *Inhibition of the hedgehog pathway targets the tumor-associated stroma in pancreatic cancer.* Mol Cancer Res, 2012. **10**(9): p. 1147-57.

83. Rodova, M., et al., *Sonic hedgehog signaling inhibition provides opportunities for targeted therapy by sulforaphane in regulating pancreatic cancer stem cell self-renewal.* PLoS One, 2012. **7**(9): p. e46083.
84. Evangelista, M., H. Tian, and F.J. de Sauvage, *The hedgehog signaling pathway in cancer.* Clin Cancer Res, 2006. **12**(20 Pt 1): p. 5924-8.
85. Merchant, A.A. and W. Matsui, *Targeting Hedgehog--a cancer stem cell pathway.* Clin Cancer Res, 2010. **16**(12): p. 3130-40.
86. Ji, Z., et al., *Oncogenic KRAS activates hedgehog signaling pathway in pancreatic cancer cells.* J Biol Chem, 2007. **282**(19): p. 14048-55.
87. Ikonen, E., *Cellular cholesterol trafficking and compartmentalization.* Nat Rev Mol Cell Biol, 2008. **9**(2): p. 125-38.
88. Brown, M.S. and J.L. Goldstein, *A receptor-mediated pathway for cholesterol homeostasis.* Science, 1986. **232**(4746): p. 34-47.
89. Cherezov, V., et al., *High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor.* Science, 2007. **318**(5854): p. 1258-65.
90. Kuzu, O.F., M.A. Noory, and G.P. Robertson, *The Role of Cholesterol in Cancer.* Cancer Res, 2016. **76**(8): p. 2063-70.
91. Wang, Y., et al., *Regulation of cholesterologenesis by the oxysterol receptor, LXRalpha.* J Biol Chem, 2008. **283**(39): p. 26332-9.
92. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
93. Ikonen, E., *Mechanisms for cellular cholesterol transport: defects and human disease.* Physiol Rev, 2006. **86**(4): p. 1237-61.
94. Maxfield, F.R. and I. Tabas, *Role of cholesterol and lipid organization in disease.* Nature, 2005. **438**(7068): p. 612-21.
95. Heinlein, C.A. and C. Chang, *Androgen receptor in prostate cancer.* Endocr Rev, 2004. **25**(2): p. 276-308.
96. Feldman, B.J. and D. Feldman, *The development of androgen-independent prostate cancer.* Nat Rev Cancer, 2001. **1**(1): p. 34-45.
97. Shafique, K., et al., *Cholesterol and the risk of grade-specific prostate cancer incidence: evidence from two large prospective cohort studies with up to 37 years' follow up.* BMC Cancer, 2012. **12**: p. 25.
98. Pelton, K., M.R. Freeman, and K.R. Solomon, *Cholesterol and prostate cancer.* Curr Opin Pharmacol, 2012. **12**(6): p. 751-9.
99. Allott, E.H., et al., *Serum lipid profile and risk of prostate cancer recurrence: Results from the SEARCH database.* Cancer Epidemiol Biomarkers Prev, 2014. **23**(11): p. 2349-56.
100. Greenhill, C., *Pancreatic cancer: Disrupted lipid metabolic pathways in PDAC identified.* Nat Rev Gastroenterol Hepatol, 2015. **12**(4): p. 188.
101. Guillaumond, F., et al., *Cholesterol uptake disruption, in association with chemotherapy, is a promising combined metabolic therapy for pancreatic adenocarcinoma.* Proc Natl Acad Sci U S A, 2015. **112**(8): p. 2473-8.
102. Cruz, P.M., et al., *The role of cholesterol metabolism and cholesterol transport in carcinogenesis: a review of scientific findings, relevant to future cancer therapeutics.* Front Pharmacol, 2013. **4**: p. 119.
103. Solomon, K.R., et al., *Ezetimibe is an inhibitor of tumor angiogenesis.* Am J Pathol, 2009. **174**(3): p. 1017-26.

104. Ginestier, C., et al., *Mevalonate metabolism regulates Basal breast cancer stem cells and is a potential therapeutic target.* Stem Cells, 2012. **30**(7): p. 1327-37.
105. Higgins, M.J., et al., *A short-term biomarker modulation study of simvastatin in women at increased risk of a new breast cancer.* Breast Cancer Res Treat, 2012. **131**(3): p. 915-24.
106. Pavlakis, N., R. Schmidt, and M. Stockler, *Bisphosphonates for breast cancer.* Cochrane Database Syst Rev, 2005(3): p. CD003474.
107. Kuzu, O.F., et al., *Leelamine mediates cancer cell death through inhibition of intracellular cholesterol transport.* Mol Cancer Ther, 2014. **13**(7): p. 1690-703.
108. Wong, W.W., et al., *HMG-CoA reductase inhibitors and the malignant cell: the statin family of drugs as triggers of tumor-specific apoptosis.* Leukemia, 2002. **16**(4): p. 508-19.
109. Baigent, C., et al., *Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins.* Lancet, 2005. **366**(9493): p. 1267-78.
110. Pepinsky, R.B., et al., *Identification of a palmitic acid-modified form of human Sonic hedgehog.* J Biol Chem, 1998. **273**(22): p. 14037-45.
111. Liao, J.K. and U. Laufs, *Pleiotropic effects of statins.* Annu Rev Pharmacol Toxicol, 2005. **45**: p. 89-118.
112. Martinez, O.W.A.-M.J.J.D.S., *Case 10: Merck(A): Mevacor." In Allan Afuah. Innovation Management - Strategies, Implementation, and Profits*. 1998: Oxford University Press.
113. Simoes, S.M., et al., *Poloxamine-cyclodextrin-simvastatin supramolecular systems promote osteoblast differentiation of mesenchymal stem cells.* Macromol Biosci, 2013. **13**(6): p. 723-34.
114. Demierre, M.F., et al., *Statins and cancer prevention.* Nat Rev Cancer, 2005. **5**(12): p. 930-42.
115. Chan, K.K., A.M. Oza, and L.L. Siu, *The statins as anticancer agents.* Clin Cancer Res, 2003. **9**(1): p. 10-9.
116. Hoque, A., H. Chen, and X.C. Xu, *Statin induces apoptosis and cell growth arrest in prostate cancer cells.* Cancer Epidemiol Biomarkers Prev, 2008. **17**(1): p. 88-94.
117. Babcook, M.A., et al., *Combination simvastatin and metformin induces G1-phase cell cycle arrest and Ripk1- and Ripk3-dependent necrosis in C4-2B osseous metastatic castration-resistant prostate cancer cells.* Cell Death Dis, 2014. **5**: p. e1536.
118. Wang, G., et al., *Simvastatin induces cell cycle arrest and inhibits proliferation of bladder cancer cells via PPARgamma signalling pathway.* Sci Rep, 2016. **6**: p. 35783.
119. Wang, S.T., et al., *Simvastatin-induced cell cycle arrest through inhibition of STAT3/SKP2 axis and activation of AMPK to promote p27 and p21 accumulation in hepatocellular carcinoma cells.* Cell Death Dis, 2017. **8**(2): p. e2626.
120. McGlynn, K.A., et al., *Statin use and risk of hepatocellular carcinoma in a U.S. population.* Cancer Epidemiol, 2014. **38**(5): p. 523-7.
121. Lee, H.S., et al., *Statin Use and Its Impact on Survival in Pancreatic Cancer Patients.* Medicine (Baltimore), 2016. **95**(19): p. e3607.

122. Zhong, G.C., et al., *Meta-analysis of studies using statins as a reducer for primary liver cancer risk.* Sci Rep, 2016. **6**: p. 26256.
123. Chen, B.K., H.F. Chiu, and C.Y. Yang, *Statins are Associated With a Reduced Risk of Brain Cancer: A Population-Based Case-Control Study.* Medicine (Baltimore), 2016. **95**(17): p. e3392.
124. Wu, B.U., et al., *Impact of statin use on survival in patients undergoing resection for early-stage pancreatic cancer.* Am J Gastroenterol, 2015. **110**(8): p. 1233-9.
125. Ananthakrishnan, A.N., et al., *Statin Use Is Associated With Reduced Risk of Colorectal Cancer in Patients With Inflammatory Bowel Diseases.* Clin Gastroenterol Hepatol, 2016.
126. Allott, E.H., et al., *Statin Use and Prostate Cancer Aggressiveness: Results from the Population-Based North Carolina-Louisiana Prostate Cancer Project.* Cancer Epidemiol Biomarkers Prev, 2016. **25**(4): p. 670-7.
127. MacDonald, J.S., et al., *Preclinical evaluation of lovastatin.* Am J Cardiol, 1988. **62**(15): p. 16j-27j.
128. Smith, P.F., et al., *Studies on the mechanism of simvastatin-induced thyroid hypertrophy and follicular cell adenoma in the rat.* Toxicol Pathol, 1991. **19**(3): p. 197-205.
129. Gbelcova, H., et al., *Differences in antitumor effects of various statins on human pancreatic cancer.* Int J Cancer, 2008. **122**(6): p. 1214-21.
130. Newman, A., et al., *A comparison of the effect of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors simvastatin, lovastatin and pravastatin on leukaemic and normal bone marrow progenitors.* Leuk Lymphoma, 1997. **24**(5-6): p. 533-7.
131. Tsubaki, M., et al., *Statins improve survival by inhibiting spontaneous metastasis and tumor growth in a mouse melanoma model.* Am J Cancer Res, 2015. **5**(10): p. 3186-97.
132. Borahay, M.A., et al., *Novel effects of simvastatin on uterine fibroid tumors: in vitro and patient-derived xenograft mouse model study.* Am J Obstet Gynecol, 2015. **213**(2): p. 196 e1-8.
133. Renno, A.L., et al., *Decreased Expression of Stem Cell Markers by Simvastatin in 7,12-dimethylbenz(a)anthracene (DMBA)-induced Breast Cancer.* Toxicol Pathol, 2015. **43**(3): p. 400-10.
134. Wolfe, A.R., et al., *Simvastatin prevents triple-negative breast cancer metastasis in pre-clinical models through regulation of FOXO3a.* Breast Cancer Res Treat, 2015. **154**(3): p. 495-508.
135. Fendrich, V., et al., *Simvastatin delay progression of pancreatic intraepithelial neoplasia and cancer formation in a genetically engineered mouse model of pancreatic cancer.* Pancreatology, 2013. **13**(5): p. 502-7.
136. Warita, K., et al., *Statin-induced mevalonate pathway inhibition attenuates the growth of mesenchymal-like cancer cells that lack functional E-cadherin mediated cell cohesion.* Sci Rep, 2014. **4**: p. 7593.
137. McGlynn, K.A., et al., *Statin use and risk for primary liver cancer in the clinical practice research datalink.* J Natl Cancer Inst, 2015. **107**: p. djv009.
138. Jeon, C.Y., et al., *Statin Use and Survival with Early-Stage Hepatocellular Carcinoma.* Cancer Epidemiol Biomarkers Prev, 2016. **25**(4): p. 686-92.
139. Hong, J.Y., et al., *Randomized double-blinded, placebo-controlled phase II trial of simvastatin and gemcitabine in advanced pancreatic cancer patients.* Cancer Chemother Pharmacol, 2014. **73**(1): p. 125-30.

140. Mamtani, R., et al., *Disentangling the Association between Statins, Cholesterol, and Colorectal Cancer: A Nested Case-Control Study.* PLoS Med, 2016. **13**(4): p. e1002007.
141. Alexandre, L., et al., *Association Between Statin Use After Diagnosis of Esophageal Cancer and Survival: A Population-Based Cohort Study.* Gastroenterology, 2016. **150**(4): p. 854-865 e1.
142. Borgquist, S., et al., *Statin Use and Breast Cancer Risk in the Nurses' Health Study.* Cancer Epidemiol Biomarkers Prev, 2016. **25**(1): p. 201-6.
143. McKay, R.R., et al., *Statins and survival outcomes in patients with metastatic renal cell carcinoma.* Eur J Cancer, 2016. **52**: p. 155-62.
144. Pottegard, A., et al., *Long-term Use of Statins and Risk of Renal Cell Carcinoma: A Population-based Case-Control Study.* Eur Urol, 2015.
145. Lim, S.H., et al., *A randomised, double-blind, placebo-controlled multi-centre phase III trial of XELIRI/FOLFIRI plus simvastatin for patients with metastatic colorectal cancer.* Br J Cancer, 2015. **113**(10): p. 1421-6.
146. Kozak, M.M., et al., *Statin and Metformin Use Prolongs Survival in Patients With Resectable Pancreatic Cancer.* Pancreas, 2016. **45**(1): p. 64-70.
147. Sun, L.M., et al., *Statin Use Reduces Prostate Cancer All-Cause Mortality: A Nationwide Population-Based Cohort Study.* Medicine (Baltimore), 2015. **94**(39): p. e1644.
148. Jung, J., et al., *Effects of statin use on the response duration to androgen deprivation therapy in metastatic prostate cancer.* Korean J Urol, 2015. **56**(9): p. 630-6.
149. Chen, M.J., et al., *Statins and the risk of pancreatic cancer in Type 2 diabetic patients--A population-based cohort study.* Int J Cancer, 2016. **138**(3): p. 594-603.
150. Nevadunsky, N.S., et al., *Association Between Statin Use and Endometrial Cancer Survival.* Obstet Gynecol, 2015. **126**(1): p. 144-50.
151. Nguyen, T., et al., *Statin use reduces risk of esophageal adenocarcinoma in US veterans with Barrett's esophagus: a nested case-control study.* Gastroenterology, 2015. **149**(6): p. 1392-8.
152. Chan, J.M., et al., *Postdiagnostic Statin Use and the Risk of Lethal Prostate Cancer in the Health Professionals Follow-up Study.* Cancer Epidemiol Biomarkers Prev, 2015. **24**(10): p. 1638-40.
153. Harshman, L.C., et al., *Statin Use at the Time of Initiation of Androgen Deprivation Therapy and Time to Progression in Patients With Hormone-Sensitive Prostate Cancer.* JAMA Oncol, 2015. **1**(4): p. 495-504.
154. Cardwell, C.R., et al., *Statin use and survival from lung cancer: a population-based cohort study.* Cancer Epidemiol Biomarkers Prev, 2015. **24**(5): p. 833-41.
155. Jeon, C.Y., et al., *The association of statin use after cancer diagnosis with survival in pancreatic cancer patients: a SEER-medicare analysis.* PLoS One, 2015. **10**(4): p. e0121783.
156. Peng, Y.C., et al., *Statins are associated with a reduced risk of cholangiocarcinoma: a population-based case-control study.* Br J Clin Pharmacol, 2015. **80**(4): p. 755-61.
157. Hoffmeister, M., et al., *Statin use and survival after colorectal cancer: the importance of comprehensive confounder adjustment.* J Natl Cancer Inst, 2015. **107**(6): p. djv045.

158. Shao, Y.Y., et al., *Statin Use Is Associated With Improved Prognosis of Colorectal Cancer in Taiwan.* Clin Colorectal Cancer, 2015. **14**(3): p. 177-184 e4.
159. Desai, P., et al., *Statins and breast cancer stage and mortality in the Women's Health Initiative.* Cancer Causes Control, 2015. **26**(4): p. 529-39.
160. Viers, B.R., et al., *The association of statin therapy with clinicopathologic outcomes and survival among patients with localized renal cell carcinoma undergoing nephrectomy.* Urol Oncol, 2015. **33**(9): p. 388 e11-8.
161. Walker, E.J., et al., *Statin use and risk of pancreatic cancer: results from a large, clinic-based case-control study.* Cancer, 2015. **121**(8): p. 1287-94.
162. Kaffenberger, S.D., et al., *Statin use is associated with improved survival in patients undergoing surgery for renal cell carcinoma.* Urol Oncol, 2015. **33**(1): p. 21 e11-7.
163. Gaist, D., et al., *Statin use and survival following glioblastoma multiforme.* Cancer Epidemiol, 2014. **38**(6): p. 722-7.
164. Yang, T.Y., et al., *Correlation between use of simvastatin and lovastatin and female lung cancer risk: a nationwide case-control study.* Int J Clin Pract, 2015. **69**(5): p. 571-6.
165. Baandrup, L., et al., *Statin use and risk for ovarian cancer: a Danish nationwide case-control study.* Br J Cancer, 2015. **112**(1): p. 157-61.
166. Bradley, M.C., et al., *Statins and pancreatic cancer risk: a nested case-control study.* Cancer Causes Control, 2010. **21**(12): p. 2093-100.
167. Krens, L.L., et al., *Statin use is not associated with improved progression free survival in cetuximab treated KRAS mutant metastatic colorectal cancer patients: results from the CAIRO2 study.* PLoS One, 2014. **9**(11): p. e112201.
168. Murtola, T.J., et al., *Statin use and breast cancer survival: a nationwide cohort study from Finland.* PLoS One, 2014. **9**(10): p. e110231.
169. Song, C., et al., *Statin use after radical prostatectomy reduces biochemical recurrence in men with prostate cancer.* Prostate, 2015. **75**(2): p. 211-7.
170. Cardwell, C.R., et al., *Statin use after diagnosis of breast cancer and survival: a population-based cohort study.* Epidemiology, 2015. **26**(1): p. 68-78.
171. Altwairgi, A.K., *Statins are potential anticancerous agents (review).* Oncol Rep, 2015. **33**(3): p. 1019-39.
172. Gbelcova, H., et al., *The effect of simvastatin on lipid droplets accumulation in human embryonic kidney cells and pancreatic cancer cells.* Lipids Health Dis, 2013. **12**: p. 126.
173. Menter, D.G., et al., *Differential effects of pravastatin and simvastatin on the growth of tumor cells from different organ sites.* PLoS One, 2011. **6**(12): p. e28813.
174. Amponsah, P.S., et al., *microRNA-210 overexpression inhibits tumor growth and potentially reverses gemcitabine resistance in pancreatic cancer.* Cancer Lett, 2016. **388**: p. 107-117.
175. Zhang, Y., et al., *Aspirin counteracts cancer stem cell features, desmoplasia and gemcitabine resistance in pancreatic cancer.* Oncotarget, 2015. **6**: p. 9999-10015.
176. Kallifatidis, G., et al., *Sulforaphane targets pancreatic tumour-initiating cells by NF-kappaB-induced antiapoptotic signalling.* Gut, 2009. **58**(7): p. 949-63.
177. Kopp, E. and S. Ghosh, *Inhibition of NF-kappa B by sodium salicylate and aspirin.* Science, 1994. **265**(5174): p. 956-9.

178. Heller, A., et al., *Establishment and Characterization of a Novel Cell Line, ASAN-PaCa, Derived From Human Adenocarcinoma Arising in Intraductal Papillary Mucinous Neoplasm of the Pancreas.* Pancreas, 2016.
179. Zhang, Y., et al., *Aspirin counteracts cancer stem cell features, desmoplasia and gemcitabine resistance in pancreatic cancer.* Oncotarget, 2015. **6**(12): p. 9999-10015.
180. Balke, M., et al., *A short-term in vivo model for giant cell tumor of bone.* BMC Cancer, 2011. **11**: p. 241.
181. Kallifatidis, G., et al., *Sulforaphane increases drug-mediated cytotoxicity toward cancer stem-like cells of pancreas and prostate.* Mol Ther, 2011. **19**(1): p. 188-95.
182. Dai, M., et al., *Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data.* Nucleic Acids Res, 2005. **33**(20): p. e175.
183. Subramanian, A., et al., *Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles.* Proc Natl Acad Sci U S A, 2005. **102**(43): p. 15545-50.
184. Chou, T.C., *Drug combination studies and their synergy quantification using the Chou-Talalay method.* Cancer Res, 2010. **70**(2): p. 440-6.
185. Chou, T.C., *Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies.* Pharmacol Rev, 2006. **58**(3): p. 621-81.
186. Bjorkhem-Bergman, L., J.D. Lindh, and P. Bergman, *What is a relevant statin concentration in cell experiments claiming pleiotropic effects?* Br J Clin Pharmacol, 2011. **72**(1): p. 164-5.
187. Collins, R., et al., *Interpretation of the evidence for the efficacy and safety of statin therapy.* Lancet, 2016. **388**(10059): p. 2532-2561.
188. Qiao, L.J., K.L. Kang, and J.S. Heo, *Simvastatin promotes osteogenic differentiation of mouse embryonic stem cells via canonical Wnt/beta-catenin signaling.* Mol Cells, 2011. **32**(5): p. 437-44.
189. Pagkalos, J., et al., *Simvastatin induces osteogenic differentiation of murine embryonic stem cells.* J Bone Miner Res, 2010. **25**(11): p. 2470-8.
190. Yalom, A., et al., *In vitro osteoinductive effects of hydroxycholesterol on human adipose-derived stem cells are mediated through the hedgehog signaling pathway.* Plast Reconstr Surg, 2014. **134**(5): p. 960-8.
191. Zhao, B.J. and Y.H. Liu, *Simvastatin induces the osteogenic differentiation of human periodontal ligament stem cells.* Fundam Clin Pharmacol, 2014. **28**(5): p. 583-92.
192. Aleksandrowicz, E. and I. Herr, *Ethical euthanasia and short-term anesthesia of the chick embryo.* ALTEX, 2015. **32**(2): p. 143-7.
193. Fan, P., et al., *Continuous exposure of pancreatic cancer cells to dietary bioactive agents does not induce drug resistance unlike chemotherapy.* Cell Death Dis, 2016. **7**(6): p. e2246.
194. Sacks, F.M., et al., *The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators.* N Engl J Med, 1996. **335**(14): p. 1001-9.
195. Shepherd, J., et al., *Pravastatin in elderly individuals at risk of vascular disease (PROSPER): a randomised controlled trial.* Lancet, 2002. **360**(9346): p. 1623-30.

196. Downs, J.R., et al., *Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study.* Jama, 1998. **279**(20): p. 1615-22.
197. *West of Scotland Coronary Prevention Study: identification of high-risk groups and comparison with other cardiovascular intervention trials.* Lancet, 1996. **348**(9038): p. 1339-42.
198. *Major outcomes in moderately hypercholesterolemic, hypertensive patients randomized to pravastatin vs usual care: The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT-LLT).* Jama, 2002. **288**(23): p. 2998-3007.
199. Duncan, R.E., A. El-Sohemy, and M.C. Archer, *Statins and cancer development.* Cancer Epidemiol Biomarkers Prev, 2005. **14**(8): p. 1897-8.
200. Campbell, M.J., et al., *Breast cancer growth prevention by statins.* Cancer Res, 2006. **66**(17): p. 8707-14.
201. Sugiyama, M., et al., *Compactin and simvastatin, but not pravastatin, induce bone morphogenetic protein-2 in human osteosarcoma cells.* Biochem Biophys Res Commun, 2000. **271**(3): p. 688-92.
202. Li, G., et al., *Simvastatin inhibits tumor angiogenesis in HER2-overexpressing human colorectal cancer.* Biomed Pharmacother, 2017. **85**: p. 418-424.
203. Miyazawa, Y., et al., *Simvastatin Up-Regulates Annexin A10 That Can Inhibit the Proliferation, Migration, and Invasion in Androgen-Independent Human Prostate Cancer Cells.* Prostate, 2017. **77**(4): p. 337-349.
204. Huang, B.Z., et al., *Influence of Statins and Cholesterol on Mortality Among Patients With Pancreatic Cancer.* J Natl Cancer Inst, 2017. **109**(5).
205. Spampanato, C., et al., *Simvastatin inhibits cancer cell growth by inducing apoptosis correlated to activation of Bax and down-regulation of BCL-2 gene expression.* Int J Oncol, 2012. **40**(4): p. 935-41.
206. Rubin, L.L. and F.J. de Sauvage, *Targeting the Hedgehog pathway in cancer.* Nat Rev Drug Discov, 2006. **5**(12): p. 1026-33.
207. Blassberg, R., et al., *Reduced cholesterol levels impair Smoothened activation in Smith-Lemli-Opitz syndrome.* Hum Mol Genet, 2016. **25**(4): p. 693-705.
208. Riobo, N.A., *Cholesterol and its derivatives in Sonic Hedgehog signaling and cancer.* Curr Opin Pharmacol, 2012. **12**(6): p. 736-41.
209. Stottmann, R.W., et al., *Cholesterol metabolism is required for intracellular hedgehog signal transduction in vivo.* PLoS Genet, 2011. **7**(9): p. e1002224.
210. Jeong, J. and A.P. McMahon, *Cholesterol modification of Hedgehog family proteins.* J Clin Invest, 2002. **110**(5): p. 591-6.
211. Wendler, F., X. Franch-Marro, and J.P. Vincent, *How does cholesterol affect the way Hedgehog works?* Development, 2006. **133**(16): p. 3055-61.
212. Choudhry, Z., et al., *Sonic hedgehog signalling pathway: a complex network.* Ann Neurosci, 2014. **21**(1): p. 28-31.
213. Yauch, R.L., et al., *A paracrine requirement for hedgehog signalling in cancer.* Nature, 2008. **455**(7211): p. 406-10.
214. Berman, D.M., et al., *Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours.* Nature, 2003. **425**(6960): p. 846-51.
215. Damhofer, H., et al., *Assessment of the stromal contribution to Sonic Hedgehog-dependent pancreatic adenocarcinoma.* Mol Oncol, 2013. **7**(6): p. 1031-42.

216. Mas, C. and A. Ruiz i Altaba, *Small molecule modulation of HH-GLI signaling: current leads, trials and tribulations.* Biochem Pharmacol, 2010. **80**(5): p. 712-23.
217. Cooper, M.K., et al., *A defective response to Hedgehog signaling in disorders of cholesterol biosynthesis.* Nat Genet, 2003. **33**(4): p. 508-13.
218. Corcoran, R.B. and M.P. Scott, *Oxysterols stimulate Sonic hedgehog signal transduction and proliferation of medulloblastoma cells.* Proc Natl Acad Sci U S A, 2006. **103**(22): p. 8408-13.
219. Nachtergaele, S., et al., *Oxysterols are allosteric activators of the oncoprotein Smoothened.* Nat Chem Biol, 2012. **8**(2): p. 211-20.
220. Liu, J.C., et al., *Statins dose-dependently exert a chemopreventive effect against lung cancer in COPD patients: a population-based cohort study.* Oncotarget, 2016. **7**(37): p. 59618-59629.
221. Moon do, C., et al., *Concomitant Statin Use Has a Favorable Effect on Gemcitabine-Erlotinib Combination Chemotherapy for Advanced Pancreatic Cancer.* Yonsei Med J, 2016. **57**(5): p. 1124-30.
222. Ananthakrishnan, A.N., et al., *Statin Use Is Associated With Reduced Risk of Colorectal Cancer in Patients With Inflammatory Bowel Diseases.* Clin Gastroenterol Hepatol, 2016. **14**(7): p. 973-9.

8. OWN PUBLICATIONS

1. Pei Fan, Li Liu, **Yefeng Yin**, Zhefu Zhao, Yiyao Zhang, Prince S. Amponsah, Xi Xiao, Nathalie Bauer, Alia Abukiwan, Clifford C. Nwaeburu, Jury Gladkich, Chao Gao, Peter Schemmer, Wolfgang Gross, Ingrid Herr. MicoRNA-101-3p reverses gemcitabine resistance by inhibition of ribonucleotide reductase M1 in pancreatic cancer. Cancer Letters 2016.373(1):130-137.

2. Pan Fan, Yiyao Zhang, Li Liu, Zhefu Zhao, **Yefeng Yin**, Xi Xiao, Nathalie Bauer, Jury Gladkich, Jürgen Mattern, Chao Gao, Peter Schemmer, Wolfgang Gross, Ingrid Herr. Continuous exposure of pancreatic cancer cells to dietary bioactive agents does not induce drug resistance unlike chemotherapy. Cell Death Disease. 2016 Jun;7(6): e2246.

9. CURRICULUM VITAE

Family name:	Yin
Given name:	Yefeng
Gender:	Male
Date of Birth:	09th Oct, 1984
Place of Birth:	Shanxi, China
Marital status	Married
Father:	Lin Yin
Mother:	Youlan He

Education

Dec. 2014–present:	Molecular OncoSurgery, Department of General Surgery Heidelberg University Hospital, Heidelberg, Germany
Sep. 2010–Jun. 2013:	Surgery, Dalian University, Liaoning, China granted Master Degree
Sep. 2004–Jun. 2010:	Clinical medicine, Shanxi Datong University, Shanxi, China granted Bachelor Degree
Sep. 2001–Jul. 2004:	Shuochengqu No.2 Middle School, Shuozhou, Shanxi, China

Professional Experience

Jul. 2013–Oct. 2014:	Department of Orthopedics, Beijing Shijitan Hospital, Capital Medical University Affiliated Hospital, China

Languages

Chinese	Native language
English	Fluently
German	Basics

10. ACKNOWLEDGEMENT

This project would not have been possible without the kind help of individuals and organizations. I would like to extend my sincere gratitude to all of them.

Firstly, I want to express my deepest appreciation and most earnest gratitude to my doctoral advisor Professor Ingrid Herr for her constructive invaluable suggestions, generous help and for providing me with a precious opportunity to finish my thesis in her research group; I sincerely appreciate her continuous encouragement during my study. Her unrivaled scientific attitude and meticulous academic spirit inspired me deeply. I am extremely thankful for her guidance and critique in scientific writing and data preparation. This work would not have been possible without her consistent and selfless help.

Secondly, I am very grateful to express my sincere appreciation to my current and former colleagues. I want to thank Dr. Li Liu and Dr. Pei Fan for their constructive advice and hand-by-hand techniques training during my doctoral research.

I also would like to thank Jury Gladkich for assisting me with immunochemistry, and to Dr. Wolfgang Groß for performing statistical analysis, and to PD Dr. Svetlana Karakhanova, Dr. Michael Schäfer, Zhefu Zhao, Nathalie Bauer, Libo Yin, Clifford Nwaeburu, Alia Abukiwan, Christina Georgikou, Xi Xiao, Liping Bai, Sebastian Faus, Sonja Bauer, Katharina Piwowarczyk and Jale Ottinger for their constant support, brilliant comments and invaluable suggestions. I also owe a huge debt of gratitude to Ellen Watson, who contributed in many ways to my work and life.

Next I would like to express my sincere gratitude to Prof. Dr. Oliver Strobel for providing the patient tissue, to Dr. Torsten Hoppe-Tichy for providing the medical treatments, and to Prof. Norbert Gretz and his co-workers for their scientific and technical support.

Most importantly, I want to thank my beloved parents, Lin Yin and Youlan He. Without their love and constant support, particularly “in taking care of my newborn daughter”, my scientific education would not have been possible. Their belief in me was a driving force for me to reach my goals.

Most special acknowledgement goes to my amicable siblings, Caimei Yin, Caihua Yin and Pengqiang Yin, who assisted me in various ways.

My final and profound appreciation goes to my beautiful and virtuous wife, Xiulian Si, and my clever and lovely daughter, Siyuan Yin. They are my emotional anchor and the sources of strength against any hardships at any time of my doctoral studies. My wife unselfish love and permanent support kept my spirits up and made me concentrate on my studies. Thank you for all you have done for me. I would not have made it this far without your love, care and support. I love you all. I would also like to thank all my friends who supported me in writing and incented me to strive towards my goal.