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The cytoskeleton-associated protein Destrin is involved in the perineural invasion of pancreatic cancer

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Meinen Eltern
Und meinem Ehemann
In Dankbarkeit

Zusammenfassung

Hintergrund/Zielsetzung: Die „perineurale Invasion“ ist ein wichtiges Merkmal des duktalen Adenokarzinoms des Pankreas (PDAC). Womöglich eröffnen sich neue therapeutische Optionen durch die Erforschung der molekularen Mechanismen, welche die malignen Zellen befähigen, infiltrierend in und entlang nervaler Strukturen zu wachsen. Kürzlich wurde ein neuartiges ex vivo Model etabliert, welches die perineurale Invasion im PDAC widerspiegelt [1]. Mit Hilfe dieses Models wurden Zellklone verschiedener humaner PDAC-Zelllinien isoliert, welche entweder ein hohes oder ein niedriges invasives Wachstum in neuralen Strukturen aufwiesen. Die vergleichende Analyse des Transkriptoms dieser Zellklone offenbarte, dass das Gen *DSTN* (*Destrin, actin depolymerizing factor*) in den hochinvasiv wachsenden Zellklonen signifikant überexprimiert war gegenüber den geringer invasiven Zellklonen. Die vorliegende Arbeit widmet sich der weiteren Erforschung dieses Genes und seiner zugehörigen Proteine, welche wichtige Regulatoren des Zytoskeletts sind. Destrin (DSTN) ist eingebunden in wichtige physiologische Prozesse wie Zytokinese, Zellmigration und gerichtetes Zellwachstum, aber auch in pathologische Prozesse wie Migration und Invasion von malignen Zellen.

Methoden und Ergebnisse: Die erhöhte Exprimierung von *DSTN* in den neural invasiven Pankreaszellklonen wurde mittels quantitativer *Realtime*-PCR und Westernblot-Analysen verifiziert. Veränderungen der Zellmorphologie der hochinvasiv wachsenden Pankreaszellklone konnten anhand von Immunfluoreszenzfärbungen demonstriert werden. Die zelluläre Exprimierung von DSTN wurde mittels zweier spezifischer siRNA Oligonukleotide in der Pankreaskrebszelllinie PANC-1 unterbunden und eine herabgesetzte Invasivität, Migrationsfähigkeit sowie Proliferation der so behandelten Zellen festgestellt.

Schlussfolgerung: DSTN ist in den isolierten neural stark invasiven Zellklonen überexprimiert. Die gesteigerte Exprimierung von DSTN steht im Zusammenhang mit der perineuralen Invasivität von Pankreaskrebszellen.

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1. Introduction

1.1. Pancreatic cancer

Pancreatic ductal adenocarcinoma (PDAC), usually referred to as pancreatic cancer, is known as one of the most aggressive human tumors leading to early local spread as well as lymph node, vascular and distant metastasis [40]. Invasion to intra- and extrapancreatic nerve structures is a further important key characteristic of this malignancy [11, 61]. Patients usually present symptoms at an advanced stage of the disease leading to late diagnosis [40]. Therefore, the majority of patients is diagnosed at a locally advanced or metastatic stage and only palliative approaches are available for these patients [80, 102]. Chemoresistance and poor response to radiation therapy limit the systematic approach to this disease and surgery remains the only potential curative treatment until now [99]. However, even the small share of operable patients who undergo surgery often suffer from early recurrence of the disease [88].

1.1.1. Epidemiology and Etiology

Pancreatic cancer is not one of the most common cancers, but it is one of the most lethal ones with a 5-year survival rate less than 5 % [26, 85]. In the year 2008, an estimated number of 277.000 new cases were diagnosed worldwide and nearly the same number of people, 266.000, were predicted to die from pancreatic cancer consistent with its poor

prognosis [33]. It ranks as the fourth leading cause among all estimated cancer deaths in the United States and European Union [66, 85].

Pancreatic cancer is more commonly diagnosed in developed countries [49]. Men are slightly more affected than women and most patients are diagnosed at ages above 60 years [85]. Major environmental risk factors are cigarette smoking, alcohol consumption leading to chronic pancreatitis and a high body mass index [78]. Smokers face a two times higher risk compared to non-smokers correlating with duration and numbers of cigarettes [19]. 5-10 % of all patients present with a family history of pancreatic cancer, these cases are thought to be caused by germ line disorders [63, 78]. However, most cases of pancreatic cancer occur sporadically. Somatic mutations, either occurring spontaneously or/and caused by environmental factors, are thought to play a major role in the development of sporadic cases [78]. Activating and inactivating mutations in oncogenes, tumor-suppressor genes and genome maintenance genes probably accumulate and lead to the carcinogenesis of pancreatic cancer [58].

1.1.2. Pathogenesis and Biology

Until now, the most common hypothesis about the origin of pancreatic cancer describes a malignant transformation of pancreatic ductal cells from premalignant lesions of pancreatic intraepithelial neoplasia (PanIN grades 1-3) to invasive cancer [46]. The

concept of this adenoma-carcinoma sequence with progressive development of epithelial dysplasia in line with increasing accumulation of genetic mutations has gained support by morphological studies, genetic analyses and animal models [47]. Activation of the HER-2/*neu* (human epidermal growth factor receptor 2) and *KRAS* (Kirsten rat sarcoma viral oncogene homolog) oncogenes, inactivation of *CDKN2A* (cyclin-dependent kinase inhibitor 2A) resulting in loss of p16 protein, *TP53* (tumor protein p53) and *DPC4* (deleted in pancreatic carcinoma, locus 4) are considered as being key in this context, as almost all patients with pancreatic cancer display one or more of these genetic defects. These genetic mutations have been identified as the sequence of genetic events that probably leads to the invasive transformation of pancreatic ductal cells. Interestingly, the mutation rate accelerates with the progress of the disease [32, 65]. Next to PanINs, two other potential premalignant lesions have been described deriving from ductal cells: intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs) [64].

The cell-of-origin of pancreatic cancer remains being discussed. Beside ductal cells, pancreatic cancer may possibly derive from other epithelial cells within the pancreas. A progression model originating in the centroacinar-acinar compartment developing PanIN lesions has been introduced and a cancer stem cell hypothesis as cell-of-origin is also discussed for pancreatic cancer [29, 54-55]. As 85% of all malignancies of the pancreas are classified as ductal adenocarcinomas, it will be a major step in the oncology of the pancreas to determine the cells of origin and pathogenesis of this malignant disease.

1.1.3. Clinical manifestations

The cancer-related symptoms depend on the location and stage of pancreatic cancer and become usually apparent after the tumor started to invade the local environment. Abdominal or mid-back pain, jaundice and weight loss are the main initial symptoms through which pancreatic cancer comes to clinical appearance [98].

Patients who present with painless jaundice as initial symptom mostly show better outcome as this symptom is often associated with resectable small tumors limited to the head of the pancreas. In contrast, painful jaundice or pain alone are often associated with a poor outcome as they are the leading symptoms in locally advanced or metastatic tumors frequently originating in the body or tail of the pancreas [50]. The jaundice may be accompanied by pruritus, pale stools and dark urine. Weight loss can be the result of maldigestion due to pancreatic duct obstruction, anorexia and cachexia as well as gastric-outlet obstruction with nausea and vomiting at further advanced stages. Patients may also develop dysglycaemia or diabetes mellitus and in some occasions pancreatitis and deep or superficial venous thrombosis [41, 58, 98]. Metastasis of pancreatic cancer has been shown to occur by continuous local spread, venous and lymphatic invasion as well as perineural invasion. Therefore, beside adjacent organs, the peritoneum, retroperitoneum and extra pancreatic nerve plexus as well as distant organs like liver and lung are common sites of metastasis [40].

1.1.4. Diagnosis, staging and treatment

The imaging procedure of choice is currently the multiphase, multi-slice helical computed tomography (CT) with intravenous contrast. With this procedure it is possible to predict resectability of the pancreatic tumor in 80-90% of cases [41]. Further evaluation and staging can be done by endoscopic ultrasound, endoscopic retrograde cholangiopancreatography, positron emission tomography-CT as well as chest imaging and measuring carbohydrate antigen 19-9 (CA 19-9) levels. Additional fine-needle aspiration guided by endoscopic/abdominal ultrasound or CT imaging is recommended to confirm the diagnosis or rule out benign diseases causing a pancreatic tumor mass. Laparoscopy or open laparotomy is reserved to patients who could not be clearly diagnosed or staged by the methods described [41, 58, 98].

In regard of the management of pancreatic cancer, patients are classified as suffering from resectable, unresectable, locally advanced disease or metastatic disease [58]. Pancreatic tumors with absence of distant metastasis or local invasion of surrounding organs and vessels are considered resectable. The standard operation procedure for tumors of the pancreas head is pancreaticoduodenectomy (Whipple-procedure) either done in the classical or in a pylorus-preserving way. Tumors of the pancreas body and tail are surgically resected by a distal subtotal pancreatectomy, which is usually combined with splenectomy. Additionally, due to the possibility to reconstruct tumor-involved veins,

tumors invading the portal vein, the superior mesenteric vein or the venous confluence are nowadays often eligible for resection [87]. Also local invasion of surrounding organs such as the colon does not always lead to unresectability and can sometimes be considered as marginally resectable. In studies, multivisceral resections reached the same 5-year survival rate as resections of tumors limited to the pancreas [84].

Although these major advancments have been made in pancreatic surgery, limitations to resection are reached in case of distant metastasis or if the pancreatic tumor involves the superior mesentery artery or celiac axis in the surroundings of the pancreas [89]. Unfortunately, the majority of patients present with such advanced stages of disease and therefore only 15-20% of patients can undergo surgery, the only potential cure for pancreatic cancer until now [58]. Patients with unresectable or metastatic pancreatic cancer may be treated with palliative approaches like chemotherapy and/or radiation as well as in some cases palliative surgery. Among these options, chemo radiation therapy was shown to be superior to radiation alone with gemcitabine being favored versus fluorouracil [20, 48, 91]. Despite these treatment options the median overall survival of palliative patients ranges from 9 to 10 months only [41].

Multimodal therapy strategies are not only applied for palliative patients but also for patients who have undergone surgery. Gemcitabine alone or fluorouracil and leucovorin

have been found to improve progression-free and overall survival [70-72]. In addition, a recent trial showed a trend towards improved overall survival for chemo radiation therapy with a combination of gemcitabine and fluorouracil for pancreas head tumors, yet these findings were not significant [39]. The discussion about adjuvant therapy in pancreatic cancer is still ongoing and there has no consensus been found yet. Nevertheless, the efforts made until now have not been in vain. By combining curative surgery with adjuvant chemotherapy, 5-year survival rates of 15-20% have been achieved and in case of a favorable node-negative status even 40% were reached [21].

Nevertheless, these survival rates may not be considered as sufficient, as almost all patients die at some point from metastasis and unrestrained tumor growth [58]. This aggressive nature of the tumor is one of the aspects that require further research. In addition to the ability of pancreatic cancer to metastasize early into lymph and blood vessels, the cancer also shows the ability to grow into, along and through the nerves of the pancreas and beyond, a process called perineural invasion. Perineural invasion is of particular importance in regard of the choice of secure resection margins. Therefore the concept and definition of complete resection (R0) in pancreatic cancer has changed over the recent years. It has been revealed that the rate of R1 resection is much higher than usually reported by a single center experience. Out of 111 macroscopically complete pancreatic head resections for PDAC, 76% were identified as R1 resections under the microscope [30]. In addition, the retroperitoneal margin was found to be the most

frequently invaded margin and, moreover, to be associated with a very poor prognosis [101]. These findings are of highest importance as curative resection has been identified as being a determining factor predicting the outcome in PDAC [99]. Therefore, perineural invasion is one of the aspects of the aggressive nature of pancreatic cancer and research of this aspect can help to find new therapeutic strategies to prevent or limit local invasion.

1.2. Perineural invasion in pancreatic cancer

The growth of cancer cells along nerve sheaths and their invasion into neural structures is referred to as perineural invasion (PNI) according to Batsakis' generally accepted definition of PNI from 1985 [12, 60]. PNI is a prominent and general feature of pancreatic cancer with a reported incidence of at least 70% up to 100% of all evaluated tumor specimens [11, 60]. This incidence of PNI is one of the highest amongst numerous tumor entities, which also show PNI [11]. It is an important pathologic feature of malignancies of the esophagus, stomach, billiary tract, colon and rectum, prostate, bladder, head and neck [16, 22, 28, 42, 60, 69, 83, 93, 97]. PNI has often been associated with decreased survival and poor prognosis of tumor patients [13, 17, 34, 38, 42, 52, 57, 59-60, 67, 75-76, 82, 90, 104].

In pancreatic cancer, PNI is an independent prognostic factor and probably the main mechanism by which pancreatic cancer cells invade into the retropancreatic space [37].

The tumor cell invasion into the retropancreatic nerve plexus has been stated as one of the determining factors influencing patient survival due to the high tumor recurrence rate after curative-intended surgery [61, 77]. Pancreatic cancer cells have not only been reported to grow continuously along the nerve but were also found to progress discontinuously [42, 51]. Thus, even if the margins have been classified as R0 after resection, pancreatic cancer cells might have remained and could lead to metastasis in noncancerous parts of the pancreas or in the retroperitoneum. Hence, PNI likely prevents curative resection and influences local recurrence after tumor resection [42, 109]. Additionally, it has also been demonstrated by Kayahara et al. that PNI may involve pancreatic cancer cell spreading into lymph node hili by continuous growth along perineural spaces [51]. These findings support a growing evidence that perineural invasion seems to be significantly associated with the high incidence of recurrence and therefore with the poor prognosis of pancreatic cancer [6, 25, 34, 61, 67, 74, 81, 92].

Although PNI seems to be an important factor not only for pancreatic cancer but also for many other malignancies, many of the processes behind PNI remain elusive. Regarding the prominent role of PNI in pancreatic cancer progression, a better understanding of these processes could lead to improved therapeutic approaches.

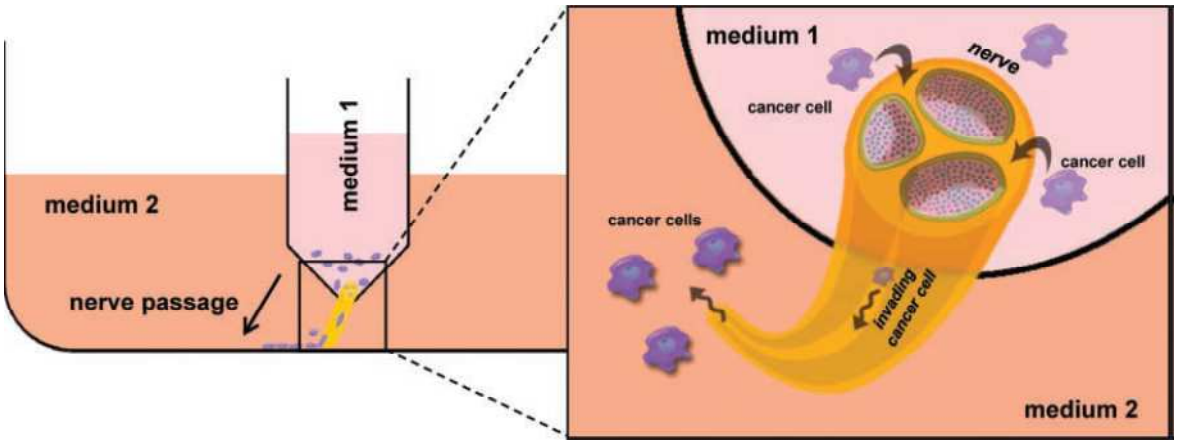
1.3. Perineural Invasion model

This study is based on the findings of the new ex vivo PNI model established by Abiatari et al. [1]. This model aims to provide insight into the molecular determinants of PNI in pancreatic cancer. For this purpose, it differentiates between highly nerve-invasive (NP3) and less nerve-invasive (NP0) human pancreatic cancer cell clones based on the difference in their ability to invade neural structures. A further examination of the cell clones' gene expression profiles allows for determining the genetic prerequisites potentially enabling pancreatic cancer cells to achieve a highly nerve-invasive geno- and phenotype.

The highly nerve-invasive pancreatic cancer cell clones were obtained from an ex vivo passage of human pancreatic cancer cells through surgically resected rat vagal nerves. The resected nerves were placed in a gap at the bottom of specifically designed nerve invasion chambers filled with a pancreatic cancer cell suspension (figure 1) [1]. The cancer cells placed at one end of the vagal nerves showed PNI and passed through the nerve. They were propagated after appearing in the culture plate in which the nerve invasion chamber has been placed. This procedure was conducted using the human pancreatic cancer cell lines PANC-1, COLO 375 and T3M4. By propagating the migrated cancer cells at three consecutive time-points, distinct cell clones were obtained differentiating in their invasiveness. The nerve-invasive potential of these clones was higher the faster they overcame the nerve passage. Therefore, out of the "wildtype" pancreatic cancer cells, which were labeled as none nerve-invasive (NP0), three d

pancreatic cancer cell clones with low, intermediate and high nerve-invasive potential were created (nerve invasion passages (NP) 1, 2 and 3).

In a subsequent step, genome-wide expression analyses were utilized to reveal the distinct transcriptome signature of the highly nerve-invasive (NP3) against none nerve-invasive pancreatic cancer cells (NP0). A consensus gene signature of the NP3 pancreatic cancer cell lines was identified (Figure 2, A) [1]. Further analysis of the consensus gene signature according to functional gene categories showed that gene ontology terms such as immune response, cell adhesion, cytoskeleton reorganization and cell motility were enriched in NP3 pancreatic cancer cells (Figure 2, B). The gene *DSTN* (*destrin)* was found to be up-regulated in the functional category of cytoskeleton reorganization and cell motility (Table 1). Thus, *DSTN* was demonstrated to be up-regulated in pancreatic cancer cells with a high capability for nerve invasion. Therefore *DSTN* was chosen to be further examined in this study.



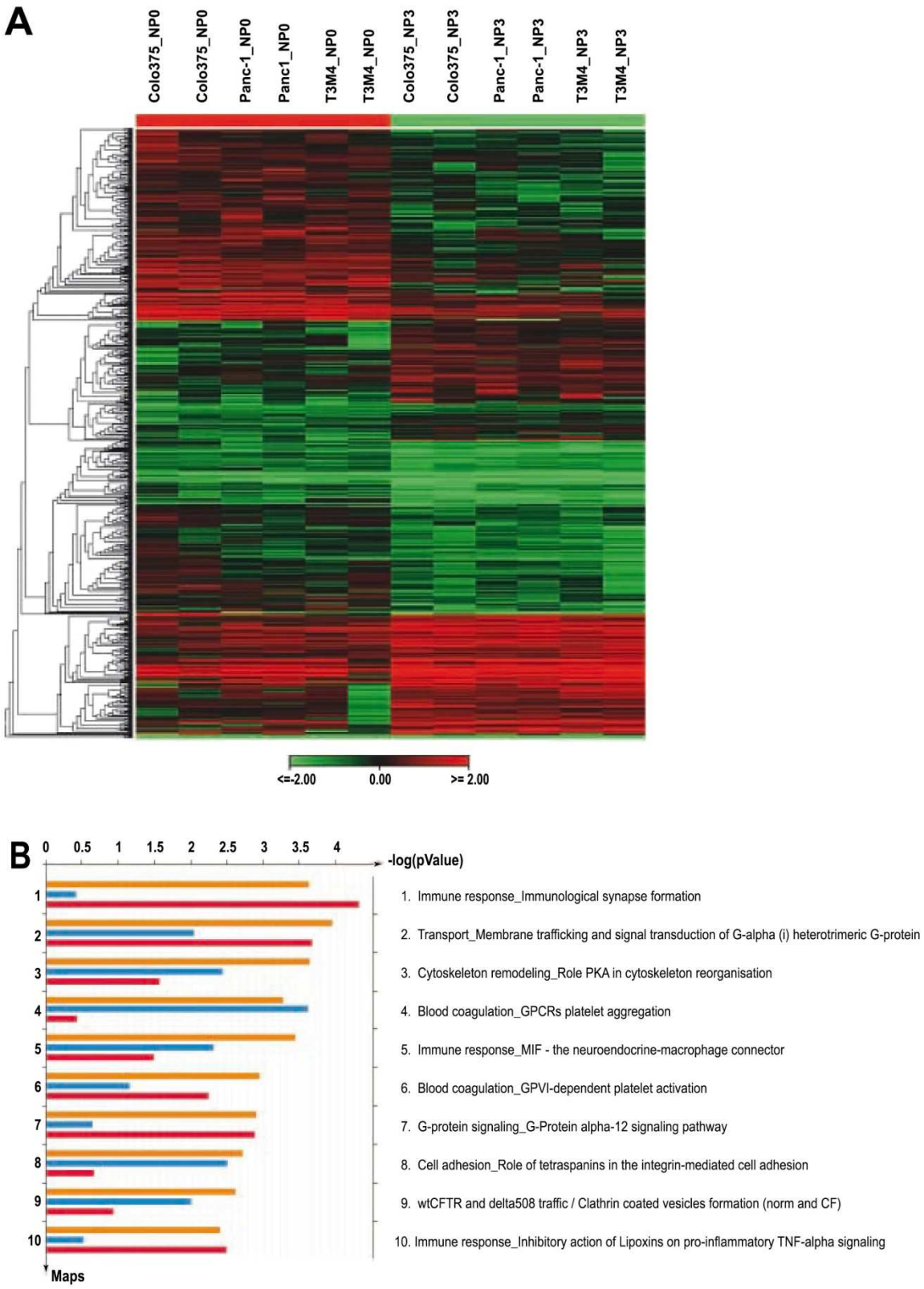
Abiatari et al., Molecular Cancer Therapies, 2009

Figure 1. Schematic demonstration of the ex vivo PNI model: the nerve invasion chambers filled with culture medium and a pancreatic cancer cell suspension (medium 1) were placed on tissue culture plates containing standard culture medium (medium 2). The different levels of the media prevented the leakage of pancreatic cancer cells from medium 1 to medium 2 without perineural passage [1].

Gene name	Gene description	Chromosome	RefSeq	Difference NP3-NP0
DSTN	Homo sapiens DSTN (actin depolymerizing factor)	20p12.1	NM_006870	4.03

Data was obtained by transcriptional analysis described in Abiatari et al., Molecular Cancer Therapies, 2009

Table 1 *DSTN* was found to be up-regulated in the functional category of cytoskleleton reorganization and cell motility, which was enriched in NP3 pancreatic cancer cells. The ifference in expression in NP3 compared to NP0 pancreatic cancer cells is presented as $\log_2$. [1].



Abiatari et al., Molecular Cancer Therapies, 2009

Figure 2. **(A**) The heat map representing the consensus transcriptional signature of highly invasive versus non-nerve-invasive pancreatic cancer cell clones. Altogether 680 transcripts were identified to be differentially regulated between NP3 and NP0 cell lines ($p<0.01$). Each row represents $\log_2$ expression ratios of an individual gene (pancreatic cell line versus reference RNA), and the columns indicate each respective sample. Expression ratios are colored according to the scale bar: green: down-regulation; red: up-regulation compared with universal reference RNA. (**B**) Functional categories of genes differentially regulated in PNI; Orange bars: all 680 genes differentially regulated; red bars: up-regulated genes and blue bars: down-regulated genes identified by consensus transcriptome analysis according to functional gene categories [1].

1.4. *DSTN* and its role in cytoskeleton reorganization

DSTN has been demonstrated to be up-regulated in the functional category of cytoskeleton reorganization and cell motility and was suggested to play a key role in the related processes.

1.4.1. Function of the cytoskeleton

The cytoskeleton enables cells to maintain shape, organize cellular components and execute cell division as well as to interact with the environment in a mechanical way and to perform coordinated movements [4]. Three protein filament types (intermediate filaments, actin filaments and microtubules) are the basal components of the cytoskeleton. They are subject to a constant dynamical turnover as the cytoskeleton is not merely a static framework, but rather a highly dynamic and continuously re-organized structure [3].

Actin filaments are flexible helical polymers consisting of the protein sub-unit actin and are organized in bundles and networks. The actin cytoskeleton is crucial for numerous cellular processes such as maintaining cell shape, cytokinesis, membrane trafficking, polarized cell growth and cell migration [8, 44]. The filaments are polarized structures with a fast-growing “plus” end and slow-growing “minus” end and are dynamically assembled and disassembled. This regulated dynamic turnover is essential for many of the functions of the actin cytoskeleton such as cell migration and is regulated by numerous actin

binding proteins (ABP). These proteins also control the filaments in many other ways for example by cross-linking, severing or bundling them. *DSTN* encodes for one of these actin binding proteins.

1.4.2. Cancer Cell Migration and the Cytoskeleton

There are different models describing cancer cell migration. Next to migrating collectively, independent migration of tumor cells has been observed in an amoeboid (lymphoma and small-cell lung carcinoma) or mesenchymal way [105]. The latter can be described as a periodical repetition of three essential steps: starting with the protrusion of pseudopodial structures such as lammellipodia or filopodia and the anchoring of these protrusions via adhesion molecules to the extracellular matrix (ECM) followed by contraction and translocation of the cell's body in the direction of the new sites of attachment [56]. Next to making and breaking of focal contacts, this process depends highly on the actin cytoskeleton. Filamentous actin is bundled to filopodia or is cross-linked in meshwork in lamellipodia. The rapid assembly and disassembly of the filaments enables the cell to create these highly motile and exploratory structures at its leading edge. Moreover, the locomotive force in the migrating cell is generally believed to be generated by the localized actin polymerization in lamellipodia (probably together with myosin motor activity) [35]. Recently, filopodia have been reported to be important not only for substrate sensing but also for formation of adhesions, actin bundles and hence force generation

[79]. Formation of actin stress fibers possibly helps to contract the cytoplasm and to drag the trailing edge of the migrating cell [18, 45]. Therefore, the cytoskeleton, especially its actin component, is essential for mesenchymal cancer cell migration.

1.4.3. DSTN is an important regulator of the actin cytoskeleton

DSTN encodes for DSTN (Destrin), also known as actin-depolymerizing factor (ADF), which belongs to the ADF/cofilin (AC) family of proteins. In eukaryotes these proteins are abundant and essential in almost every cell type [7]. In mammals, the protein family consists of three members: DSTN, Cofilin-1 (CFL1) and Cofilin-2 (CFL2). Cofilin-1 is the predominant form in non-muscle tissues and Cofilin-2 is found mainly in muscle tissues [62]. The AC proteins belong to the so-called actin binding proteins, which are involved in the modulation and precise regulation of the actin filament network [24, 27].

The rapid turnover of the actin filament network, termed actin dynamics, is essential for normal cells, e.g., for reaction to extracellular signals by moving and changing shape, and also for cancer cells enabling them to metastasize [24]. The AC family proteins are key regulators of this rapid turnover by enhancing it through de-polymerization and severing of the actin filaments. Although DSTN and Cofilin-1 are identical in about 70% of their amino-acid sequence and show similar functions in actin dynamics, they are involved in the actin turnover to a different extent [10, 15]. DSTN is the most potent turnover agent of actin as it is capable to sequester actin monomers more efficiently than Cofilin-1 [8, 44,

108]. Cofilin-1on the other hand is more efficient in severing and nucleating actin filaments [15, 108]. Actin dynamics and their regulating proteins are not only essential for the normal development and function of the cell, but also play a crucial role in tumor cell metastasis, due to their influence on adhesion-dependent growth, cell motility and cell division [9, 24, 105]. DSTN has been demonstrated to be up-regulated in diverse tumor diseases [23, 73]. In Isreco1 human colon cancer cells DSTN seemed to be needed for cancer cell migration and tissue invasion as its silencing by siRNA induced an adhesive cell morphology and significantly reduced tumor cell migration and invasiveness in the according assays [31].

2. Aim of this study

DSTN has been identified to be up-regulated in the functional category of cytoskeleton reorganization and cell motility. Cell motility is one of the gene ontology processes found to be enriched in highly nerve-invasive pancreatic cancer cell clones. The actin regulating protein DSTN is important not only for normal function of the cytoskeleton but also plays a crucial role during tumor cell metastasis. Based on these findings, we proposed the hypothesis that DSTN is over-expressed in NP3 pancreatic cancer cell clones and that this up-regulation is connected to the highly nerve-invasiveness of the NP3 cancer cell clones. Thus, DSTN may be generally connected to PNI.

To verify the hypothesis, different experiments were conducted. By using quantitative real-time polymerase chain reaction (QRT-PCR), the over-expression of *DSTN* was examined. Likewise the over-expression of its protein product DSTN was examined by using immunoblotting assays. Immunohistochemistry analysis was additionally performed to further investigate DSTN expression in normal pancreatic, chronic pancreatitis and pancreatic cancer tissue. Immunofluorescence analysis was carried out for investigation of changes on the cellular level. Furthermore, siRNA silencing of *DSTN* in PANC-1 pancreatic cancer cells was established for functional experiments.

3. Materials and methods

3.1. Materials

3.1.1. Laboratory equipment

Analytic balance	Sartorius, Göttingen
Balance	KERN, Balingen
Biophotometer	Eppendorf AG, Hamburg
Cell culture hood	Thermo Fisher SCIENTIFIC, Waltham, Massachusetts, USA
Centrifuge	Eppendorf AG
Heraeus Multifuge 3SR+	Thermo Fisher SCIENTIFIC
CO_2 incubator	SANYO, Moriguchi, Osaka, Japan
Electrophoresis/Electroblotting equipment/ power supply	Invitrogen™ BIOMETRA, Goettingen
Microscope	Leica Microsystems GmbH, Wetzlar Carl Zeiss, Jena Bresser, Rhede
pH-meter	BECKMAN COULTER, Brea, California, USA
QT-PCR: LightCycler Instrument LC480	Roche, Mannheim
Roller mixer	Progen Scientific, London, UK

Sterilgard Hood	Thermo SCIENTIFIC
Thermomixer	Eppendorf, Wesseling, Berzdorf
Tissue embedding machine	Leica, Bensheim
Tissue processor	Leica
Vortex	neoLAb, Heidelberg
Water bath	Lauda-Königshofen, Lauda
Water Distillator	Millipre, Schwallbach
X-ray film cassette	Eastman Kodak Company, Rochester, NY, USA

3.1.2. Consumables

Biocoat Matrigel Invasion Chamber	BD Bioscience, Heidelberg
Cell Scraper	SARSTEDT AG&Co., Nümbrecht
Cotton swabs	NOBA, Wetter
Coverslips	Marienfeld, Lauda-Königshofen
Disposable Scalpel	Feather, Fukushima, Japan
Filter (0.2 µm)	neoLab
Gel blotting Paper	Whatman, Sanford, ME, USA
Hyperfilm	Amersham, Buckinghamshire, UK

Nunc CryoTube	SIGMA-ALDRICH or Thermo SCIENTIFIC
PageRuler™ Prestained Protein Ladder	Fermentas, Burlington, Kanada
Parafilm	Pechiney plastic packaging, Chicago, USA
Pipet tips	Biozym, Oldendorf
Pure Nitrocellulose membrane (0.45 µM)	BIO-RAD, Hercules, CA, USA
Sterile serological pipette (2 ml; 5 ml; 10 ml; 50 ml)	CELLSTAR, greiner bio-one, Frickenhausen
Sterile needles (1 ml)	BD Medical, Franklin Lakes, USA
Syringe Injekt 40 solo	B. Braun, Melsungen
Tissue culture dishes (60x15 mm ; 100x20 mm)	CELLSTAR, greiner bio-one
Tissue culture Flasks (25 cm^2 ; 75 cm^2 ; 125 cm^2)	CELLSTAR, greiner bio-one
Tissue culture plates (6-well; 24-well; 96-well)	CELLSTAR, greiner bio-one
Tubes (15 ml; 50 ml)	CELLSTAR or BD Bioscience
Tubes (1.5 ml; 2.0 ml)	SARSTEDT
Tubes (Eppendorf® 1,5ml)	Eppendorf AG

3.1.3. Reagents

0.25% trypsin/EDTA	Invitrogen™
2-Mercaptoethanol	SIGMA-ALDRICH
3-(4,5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide	SIGMA-ALDRICH
Acetic acid	Merck Biosciences, Schwalbach
Acrylamide/Bis solution	Bio-RAD
Alexa Fluor® 488 phalloidin	Molecular probes, Inc. Eugene or Invitrogen™
Ammonium per sulfate (APS)	SIGMA-ALDRICH
Antibiotics: Penicillin, Streptomycin	Invitrogen™
Anti-fading mounting medium	Abcam, Cambridge, UK
Bicinchoninic Acid (BCA) protein assay kit	Thermo Fischer SCIENTIFIC
Bovine serum albumin (BSA)	Roth, Karlsruhe
Bromophenol blue	SIGMA-ALDRICH
Dimethyl sulfoxide	SIGMA-ALDRICH
Deoxyribonucleotide phosphate	Invitrogen™
DMEM	Invitrogen™
DNA SYBR Green I Master kit	Roche
Dulbecco's MEM	Invitrogen™

ECL detection reagent	Amersham
Envision antibody diluent	Dako GmbH, Hamburg
Ethanol	Roth
Ethidium bromide	Roth
Fetal calf serum	PAN Biotech, Aidenbach
Glycerol	Merck Biosciences
Glycine	Roche diagnostics, Mannheim
Haematoxylin	Merck Biosciences
HiPerFect transfection reagent	QIAGEN, Hilden
Histowax	Leica
Humidified chamber	TeleChem International Inc., USA
Hydrogen peroxide	Roth
Isopropanol	Roth
Laurylsulfate (SDS)	Roth
LightCycler 480 DNA SYBR Green I	Roche diagnostics, Mannheim
Liquid nitrogen	TMG Sol Group, Gersthofen
Liquid DAB & chromogen substrate	Dako GmbH, Hamburg
Methanol	Roth

3-(N-morpholino)propanesulfonic acid (MOPS)	Invitrogen™
DNA molecular weight marker	Fermentas, Life Sciences, Ontario, Canada
Disodium hydrogenphosphate	Merck Bioscience
Paraformaldehyde	Fischer, Kehl
Phosphate buffered saline (PBS) pH 7.4	Invitrogen™
PCR amplification kit	Roche diagnostics
Potassium chloride (KCl)	Merck Bioscience
Premount® (Mounting medium)	Fischer
Protease inhibitor cocktail	Roche diagnostics
Proteinase K	SIGMA-ALDRICH
QuantiTect Reverse transcription kit	QIAGEN
RNA-later	Ambion Europe Ltd., Huntington, Ambridgeshire, UK
RNAse-DNAse-free water	Invitrogen™
RNeasy Mini Kit	QIAGEN
Roticlear®	Roth
RPMI 1640 Medium	Invitrogen™
Sodium borate	Merck Biosciences

Sodium chloride (NaCl)	Merck Biosciences
Sodium citrate	Merck Biosciences
Sodium phosphate	Merck Biosciences
Tetramethylethylenediamine (TEMED)	SIGMA-ALDRICH
Toluidine blue	Merck Biosciences
Tris Base	Merck Biosciences
Triton-X-100	Merck Biosciences
Trypan blue solution	SIGMA-ALDRICH
Tween 20	Merck Biosciences

3.1.4. siRNA Molecules, Antibodies and negative controls

DSTN siRNAs siRNA#1 target sequence: TTGGTTGGAGATGTTGGTGTA siRNA#2 target sequence: TTAGGTGGATCCTTAATTGTA Negative control siRNA	QIAGEN
Rabbit polyclonal DSTN antibody (cat. #: ab11072)	Abcam
Rabbit polyclonal CFL1 antibody (D59)	Cell Signaling, Danvers, USA
Fluorescent-labelled secondary antibody Cy3	Invitrogen™
Alexa Fluor 488 goat anti-rabbit IgG (A-11008)	Invitrogen™

Rabbit IgG1 negative control	Dako GmbH
HRP Labelled Polymer Anti-Rabbit (K4003)	Dako GmbH
Rabbit GAPDH antibody (sc25778)	Santa Cruz Biotechnology, Santa Cruz, USA

3.1.5. Buffers and solutions

Immunohistochemistry	
10 x Tris buffered saline (TBS):	
Tris base NaCl pH to 7.4 with add H_2O to	12.1 g 85 g 5 M HCl 1000 ml
1 x TBS/0.1% BSA:	
10 x TBS BSA add H_2O to	100 ml 1 g 1000 ml
1 x TBS/0.1% BSA/0.05%Tween 20:	
1 x TBS/0.1% BSA Tween 20	1000ml 500 µl
1 x TBS/3% BSA	
1 x TBS BSA	100 ml 3g
Peroxidase Block:	
Methanol Hydrogen peroxide	900 µl 100 µl

Protein extraction and Western Blotting	
Cell lysis buffer **:**	
1M Tris-HCl (pH 7.5)	0.5 ml
0.5M EDTA (pH 8)	40 µl
5M NaCl	0.3 ml
20% SDS	500 µl
add H_2O to	9 ml
Protease inhibitor tablet	1
Sample buffer:	
1.25M Tris-HCL	100 µl
10% SDS	50 µl
87% Glycerol	95 µl
Mercaptoethanol	25 µl
Bromophenolblue	5 µl
Electrophoresis buffer:	
MOPS	209.2 g
Tris base	121.2 g
SDS	20 g
EDTA free acid	6 g
pH 7.7 with	5 M HCl
add H_2O to	1000 ml
Transfer buffer:	
Tris base	29.1 g
Glycine	14.7 g
SDS	0.1875 g
Methanol	1000 ml

add H_2O to	5000 ml
Blocking buffer:	
5 % dry milk powder in TBS/Tween20 (TTBS milk)	

3.2. Methods

3.2.1. Patient data and tissue collection

PDAC tissues were obtained from patients who have undergone pancreatic resections. Normal human pancreatic tissue samples were obtained through an organ donor program from previously healthy individuals. Written informed consent was obtained from all patients ahead of the date of operation. The use of tissue was approved by the local Ethics committees.

Samples were fixed in 4% paraformaldehyde solution for 24 h immediately after removal and then paraffin-embedded for further histological analysis. All embedded samples were confirmed histologically by light microscopy after preparation of a hematoxylin-eosin staining. A portion of each sample was preserved in RNAlater or was immediately flash frozen in liquid nitrogen and maintained at -80°C (storage in liquid nitrogen) until use for RNA or protein extraction in the future.

3.2.2. Cell Culture

Pancreatic cancer cell lines AsPC-1, BxPC-3, Capan-1, COLO 357, MIA PaCa-2, PANC-1, SU.86.86, and T3M4 were used. The cells were routinely grown in complete medium under sterile conditions in a humid chamber with 95% air atmosphere and 5% CO2 saturation at a steady temperature of 37°C. The culture medium RPMI-1640 was supplemented with 10% fetal calf serum, 100U/ml penicillin and 100µg/ml streptomycin for cell culture of AsPC-1, BxPC-3, COLO 357, SU.86.86, and T3M4. Likewise, the culture medium DMEM was complemented with 10% fetal bovine serum, 100U/ml penicillin and 100µg/ml streptomycin and used for the cell lines Capan-1, MIA PaCa-2 and PANC-1.

3.2.3. siRNA transfection

Small interfering RNA (siRNA) transfection was performed for transient silencing of protein expression. The experiment was carried out with two different molecules of validated siRNA for *DSTN* as well as negative control siRNA (all purchased from Qiagen; DSTN siRNA#1 target sequence: TTGGTTGGAGATGTTGGTGTA. DSTN siRNA#2 target sequence: TTAGGTGGATCCTTAATTGTA). In 6-well plates, $1.5x10^5$ cells per well were seeded and incubated for 24 hours. The cells were then transfected with 5 nM/well of specific DSTN siRNA#1 or #2 or negative control siRNA. Cells were transfected using HiPerFect (Qiagen) as transfection reagent. 74.8 µl of serum free medium was mixed with 1.2 µl of siRNA (specific or negative control) and 24 µl of HiPerFect transfection reagent

with a total of 100 µl transfection solution per well. The solutions were mixed thoroughly and after 10 minutes keeping at room temperature added drop-wise to the cells. The efficacy of the siRNA transfection was checked using immunoblotting analysis after 48, 72 and 96 h of transfection. Functional experiments were carried out after 96 h of transfection with the transfected cells being trypsinized and immediately being plated according to the experimental protocol (see also point 3.2.9-3.2.12). The purchased siRNAs and reagents were stored and prepared according to the manufacturer's protocol.

3.2.4. mRNA and cDNA preparation

Total cellular RNA isolation and RNA extraction from normal, CP and PDAC frozen tissues and pancreatic cancer cells was performed using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions: The frozen tissue was destructed and homogenized by using 600 µl RLT buffer and aspiration of the lysate about 20 times through a 22-gauge needle attached to a RNAse-free syringe. The homogenized lysate was centrifuged at full speed for 3 minutes and the supernatant collected in a fresh tube. Cells were collected and lysed by using 350 µl of RLT buffer per cell culture dish. Homogenization was completed by aspiration of the cell lysate through a 22-gauge needle about 20 times and transferred to a microcentrifuge tube. The following procedures were equally carried out for tissue or cell lysates: 70% Ethanol was added to lysates (1:1, v/v) and mixed thoroughly by pipetting. 700 µl of this mixture was added to a RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15 seconds at

≥8000×g. One by one, 700 µl of RW1 buffer, 500 µl of RPE Buffer and again 500 µl of RPE Buffer was added, centrifuged for 15 seconds at ≥8000×g (for 2 minutes after adding RPE Buffer the second time). The flow-through was discarded after every centrifugation step. Then, the RNeasy spin column was centrifuged at full speed for 1 minute after placing into a new collection tube. Afterwards, the RNeasy spin column was transferred again to a further collection tube, 30 µl of RNase-free water was added directly to the RNeasy spin column membrane, followed by centrifugation for 1 minute at ≥8000×g. The flow-through was added back to the spin column to repeat the last centrifugation step. The eluated RNA concentration was measured by using NanoPhotometer. The RNA was stored at -80°C or reverse transcription was performed immediately afterwards.

For reverse transcription in order to synthesize cDNA from total RNA the QuantiTect Reverse transcription kit was used according to the manufacturer's recommendations. The following steps were all carried out on ice with all components of the kit thawn on ice. According to RNA concentration measurement, a variable amount of RNA and RNase free water (with a total of 12 µl RNA+water mixture and a final concentration of 1 µg RNA) was mixed with 2 µl of gDNA Wipeout Buffer. The mixture was incubated using a heating block at 42°C for 2 minutes, then immediately put on ice again. In a following step, the Reverse transcription master mix was prepared: 1 µl Quantiscript Reverse Transcriptase + 4 µl Quantiscript RT Buffer + 1 µl RT Primer Mix. 6 µl of the master mix was added to the sample and incubated again at 42°C for 15 minutes, immediately put on ice and

directly incubated again at 95°C for 3 minutes. The resulting cDNA was diluted 1:5 with RNase-free water to a final concentration of 10 ng/µl and stored at -20°C.

3.2.5. Quantitative Real-Time Polymerase Chain Reaction

Real-Time PCR was performed with the LightCycler^{TM}480 using the DNA SYBR Green I Master kit. The samples were prepared in the following way: 2x SYBR Green master mix 10 µl + cDNA 5 µl (10 ng/µl) + PCR-Grade water 3 µl + primer mix (10nM) 2 µl. The primer sets were designed to amplify the region of *DSTN* (forward 5'-GGC CAG GGT CTC ACT GAG GGG-3', reverse 5'-CTC ACT GGC AGG TGC AGG GC-3') and *CFL1* (forward 5'-TAT GAG ACC AAG GAG AGC AAG-3', reverse 5'-CTT GAC CTC CTC GTA GCA GTT-3'). The annealing temperature (58°C) of the primer was optimized by normal PCR which amplified a single band of *DSTN* and *CFL1* at the predicted size. The calibration/ standard curves were performed by a serial gradient dilution of cDNA. Intercept as well as Ct values of each single QRT-PCR were determined by the LightCycler^{TM}480 software. The target concentration was expressed as a ratio relative to the expression of the reference human housekeeping gene (β-actin for DSTN and CFL1) in the same sample and normalized to the calibrator sample using the LightCyclerTM480 software release 1.5, version 1.05.0.39 (Roche). All experiments were repeated at least three times for reliable results.

3.2.6. Immunohistochemistry Analysis

On the first day, formalin-fixed and paraffin-embedded human pancreatic tissue sections (3-µm thick) were deparaffinized using Roticlear and gradually rehydrated in ethanol with progressively decreasing concentration (100%, 90%, 70% and 50% each for 3 minutes). After washing with tap water, antigen retrieval was performed by boiling the section in 10 mM citrate buffer (pH=6) for 10 minutes using a microwave oven. After cooling to room temperature (about 30 minutes), the sections were washed with deionized water for 2 minutes followed by washing with TBS/0.1%BSA for about 10 minutes. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in 30 % methanol. The sections were then washed with TBS/0.1%BSA and subsequently blocked with TBS/3%BSA for 30 min at room temperature before being exposed to primary antibody for overnight incubation at 4°C. The primary rabbit polyclonal DSTN antibody (Abcam; 1:1000 dilution) was used and rabbit IgG was used as a negative control. On the second day, tissue sections were first rinsed with TBS/0.1%BSA, before being washed with TBS/0.1%BSA/0.05%Tween20 and, subsequently, being washed with TBS/0.1%BSA again. As an following step, sections were incubated with the respective secondary anti-mouse or anti-rabbit antibody (EnVision+ System Labeled polymer HRP antibody) for 1 h at room temperature. All described incubation steps were performed using a wet chamber. After washing with TBS/0.1%BSA /0.05%Tween20 and TBS/0.1%BSA, tissue sections were subjected to 100 µl DAB chromogen substrate mixture and color reaction was stopped using running tap water. Counterstaining was performed with haematoxylin

and the sections were then washed and dehydrated using progressively increasing ethanol concentrations (50%, 70%, 90% and 100 % each for 3 minutes) followed by incubation with Roticlear 3x5 minutes. Finally mounting with xylene-based mounting medium was performed. Tissue sections were visualized using the Axioplan 2 imaging microscope. 2 independent researches carried out the evaluation of the sections.

3.2.7. Immunofluorescence

Cells were seeded in 8-well-chambers in complete medium and incubated overnight. Next, the cells were washed with PBS, fixed on slides with 1% formaldehyde/PBS for 15 min at room temperature and permeabilized with 0.2% Triton X-100/PBS for 10 min. Subsequently, after washing with PBS/0.1%BSA for 5 min, the cells on the slides were blocked with 3% BSA/PBS for 30 min. Subsequently, the slides were incubated overnight with the primary rabbit polyclonal DSTN antibody at 4°C (dilution 1:200). On the next day the slides were washed 3 times with PBS/0.05%Tween20 and incubated 1h with fluorescent-labelled secondary antibody Cy3 (red fluorescence, dilution 1:100) and Alexa Fluor® 488 phalloidin (green fluorescence, 1:250) in order to detect actin filament organization. Negative IgG controls were conducted accordingly. The slides were incubated with 4’,6 -diamidino-2-phenylindole (DAPI) for 2 min, before being rinsed with PBS and mounted with anti-fading medium. Finally, icroscopic analysis was performed using the Axioplan 2 imaging microscope and Leica fluorescent Microscope.

3.2.8. Immunoblotting Analysis

Protein extraction from cells

All following steps were conducted on ice and reagents were cooled on ice beforehand. Cells in dishes were washed two times with PBS after discarding the medium. Next, cell lysis buffer was added and the lysed cells collected using a cell scraper. The cell lysates were homogenized by passing through a syringe G27 needle 10 times. The cell homogenate was first incubated on ice for 10 min and then centrifuged at 13,200 rpm at 4°C for 10 minutes. The supernatant was immediately transferred to fresh tubes and aliquoted. The concentration of the extracted protein was determined using a BCA Protein Assay kit following the manufacturer's intructions. The sample aliquots were stored at -20ºC or used for Western blotting analysis immediately.

Western blotting analysis

20µg of total protein extract from cell lysates was loaded on a polyacrylamide gel (gradient 4-12%) after denaturation using 10% SDS-PAGE loading buffer. Prestained protein ladder was added in the first and last gel pocket for size reference. The protein samples were then separated by gel electrophoresis using Running Buffer and appropriate Western blotting equipment (120V/200mA). The electrophoresis was stopped after the prestained protein ladder reached the gel's lower edge. Secondly, the separated protein samples were eletrophoreticly transferred to nitrocellulose by using Blotting Buffer

and a appropriate blotting module (setup of “sandwich”: sponge-filter paper-gel-nitrocellulose transfer-membrane-filter paper; varying number of sponges to fill up blotting chamber; 30V/200mA, blotting time 70 minutes). The membrane was then blocked with 10 ml of 0.1% Tween20-TBS (T-TBS) with 5% milk for 60 min. The primary antibody was diluted in T-TBS/5% milk and the nitrocellulose membrane was incubated overnight at 4°C. The following antibodies were used: primary rabbit polyclonal DSTN antibody (Abcam; 1:1000 dilution), primary rabbit polyclonal CFL1 antibody (Cell Signaling; 1:1000 dilution) and rabbit GAPDH antibody (Santa Cruz Biotechnology; 1:5000 dilution). On the second day, the membrane was washed 2 times for 15 min with T-TBS and incubated with the secondary horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (1:3000 dilution) diluted in T-TBS milk for 60 min at room temperature. After 2 times washing with T-TBS for 15 min, enhanced chemoluminescence system (ECL) detection reagent was added to the membrane and signals were developed in a dark room using Hyperfilm®. The western blot films were then scanned and densitometry analysis was done using the ImageJ software.

3.2.9. Proliferation Assay

Cell proliferation was determined utsing the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) colorimetric growth assay. siRNA-transfected PANC-1 cells and control-transfected PANC-1 cells were plated in 96-well plates with 2,000 cells per well in complete medium and cultured for 24 hours under standard

conditions. The following day, cell growth was determined by adding MTT (50 µg/well) for 4h. Mitochondrial MTT was solubilized with acidic isopropanol and absorbance (= optical density) was measured at 570 nm wave length using an ELISA reader. The measured cell viability was set as day 0 (d0). The MTT assay was repeated as described after further 24h and 48h of cell incubation and cell viability therefore also determined at d1 and d2, respectively. The doubling time was calculated for the exponential growth phase. All experiments were performed in triplicates.

3.2.10. Matrigel Invasion Assay

Assays were done in 8 µm pore size BD Biosciences BioCoat Matrigel Invasion Chambers according to the manufacturer's instructions. Matrigel was first rehydrated with 500 µl warm serum-free DMEM medium for each filling of empty wells, followed by incubation under standard cell culture conditions for 2h. After discarding the medium, wells were refilled with 750 µl complete DMEM medium with 5% fetal bovine serum (FBS) and emptied chambers were carefully placed inside the wells. siRNA-transfected PANC-1 cells and control-transfected PANC-1 cells were immediately added to the chambers with 5×10^4 cells/ml (500 µl in total) and incubated for 24 h under standard conditions. On the next day, non-invading cells were removed from the upper surface of the chamber membrane and cells adhering to the lower surface were fixed with 70% ethanol and stained with Mayer's hematoxylin. The whole membrane was removed from the invasion

chamber, mounted, scanned and the invading cells were counted. The assay was performed in duplicates and repeated three times.

3.2.11. Wound Healing (Migration) Assays

siRNA-transfected and control-transfected PANC-1 cells were seeded in 6-well culture plates and incubated for 24 h under standard conditions. An artificial “wound” was created using a 10 µl pipette tip on confluent cell monolayers in the culture plates. Quantification of “wound” closure was carried out by counting the number of cells in the “wound” area after 8 h. The cell number was expressed as the average of three optical fields.

3.2.12. Adhesion assay

siRNA-transfected and control-transfected PANC-1 cells were plated in 96-well culture plates (5000 cells/well) with complete medium and incubated for 8h under standard conditions. The wells were then washed 3 times with PBS to remove cells not attached to the culture plate dish and filled with 200 µl of medium per well containing MTT solution (50 µg/well) for 4 h. The following solubilisation of reduced MTT was done with acidic isopropanol. The resulting absorbance (= optical density) was measured at 570 nm wave length using an ELISA reader. Values were normalized to control plates (incubated 5000 cells per well for 12 h and without the PBS washing step).

3.2.13. Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 5 software. Results were expressed as mean ± standard error of the mean (SEM). For statistical comparisons, the non-paired student t test was used unless indicated otherwise. Survival analysis was carried out using the Kaplan-Meier method for estimation of event rates and the log-rank test for survival comparisons between patient groups. Significance was defined as $p<0.05$.

4. Results

As described above, highly nerve-invasive clones (NP3) of pancreatic cancer cell lines were obtained by establishing a new perineural invasion model. The consensus transcriptome signature of these pancreatic tumor cells revealed a significant up-regulation of *DSTN* in the highly nerve-invasive pancreatic cancer cells [1]. As this gene is crucial for the dynamic regulation of the cytoskeleton and hence likely to be related to cell migration and invasion in NP3 pancreatic cancer cells, it has been specifically chosen to be investigated in more detail within this study.

4.1. DSTN and Cofilin-1 are endogenously expressed in human pancreatic cancer cell lines

The mRNA levels of *DSTN* and *Cofilin-1* were analyzed for several cultured pancreatic cancer cell lines (AsPC-1, BxPC-3, Capan-1, COLO 357, MIA-PaCa-2, PANC-1, SU.86.86 and T3M4), using quantitative RT-PCR method (Figure 6A, B). All tested pancreatic cancer cell lines displayed an endogenous expression of *DSTN* and *Cofilin-1* with COLO 357 pancreatic cancer cells demonstrating highest relative mRNA expression and SU.86.86 pancreatic cancer cells revealed lowest relative mRNA expression of *DSTN*. Correspondingly, for *Cofilin-1* the highest mRNA expression was demonstrated for the PANC-1 cancer cell line while the MIA PaCa-2 cell line displayed the lowest mRNA expression (Figure 6). For the experiments conducted as part of this study, the pancreatic

cancer cell lines PANC-1 and COLO 357 were selected. They both were utilized for the perineural invasion model experiments and either demonstrated the highest levels of mRNA for *DSTN* or *Cofilin-1* or showed an expression at the same level as the other pancreatic cancer cell lines tested.

4.2. DSTN is up-regulated whereas Cofilin-1 is down-regulated in highly nerve-invasive cells

The mRNA levels of both AC family genes were analyzed in greater detail by comparing NP3 to NP0 pancreatic cancer clones of the two selected pancreatic cancer cell lines PANC-1 and COLO 357 (Figure 6C). *DSTN* mRNA levels were increased in NP3 clones in contrast to the less nerve-invasive NP0 cells of both tested PANC-1 (158.5±32.2 fold, *p<0.05) and Colo357 (2.11±0.05 fold, *p<0.05) pancreatic cancer cell lines, confirming the previous results [1]. Interestingly, the *Cofilin-1* mRNA expression was reduced in the highly nerve-invasive clones (NP3) compared to NP0 cell clones in both pancreatic cancer cell lines PANC-1 (0.1±0.01 fold, *p<0.05) and COLO 357 (0.6±0.01 fold, *p<0.05). The increased *DSTN* mRNA expression and decreased *Cofilin-1* mRNA expression could be confirmed on the protein level using immunoblotting analysis (Figure 6D). DSTN up-regulation in highly nerve-invasive NP3 clones compared to NP0 clones was evident in PANC-1 (7.9±2.3 fold, *p<0.05) and COLO 357 (1.2±0.1 fold, p<0.05) cells. Furthermore, the down-regulation of Cofilin-1 could be demonstrated for the NP3

cell clones of PANC-1 pancreatic cancer cells (0.6±0.1 fold, *p<0.05) as well as for COLO 357 cells (0.7±0.1 fold, *p<0.05) when compared to the NP0 clones of these two cell lines.

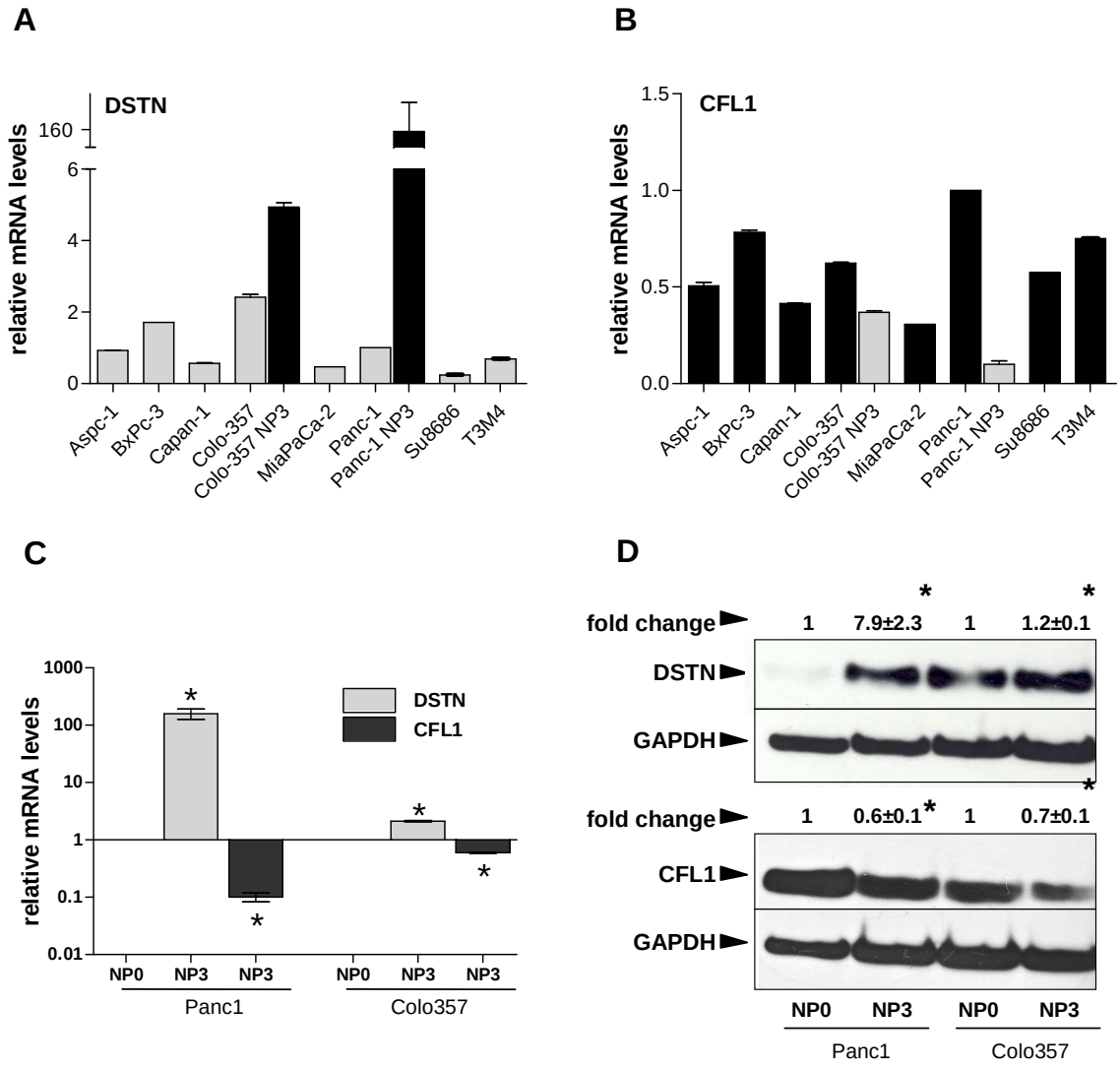


Figure 3. *DSTN* **(A)** and *Cofilin-1* **(B)** mRNA expression in cultured pancreatic cancer cell lines. Data is presented as mean (± SEM) from three independent experiments. Expression of DSTN and Cofilin-1 mRNA **(C)** and protein levels **(D)** in nerve passage clones NP0 and NP3 using QRT-PCR and immunoblot analysis as described in the Materials and Methods section. Data is presented as mean (± SEM) from three independent experiments. *p<0.05

4.3. Overexpression of DSTN is associated with poor prognosis

Gene expression of *DSTN* and *Cofilin-1* was analyzed in pancreatic bulk tissues of donor (n=10), chronic pancreatitis (n=10) and pancreatic cancer patients (n=20) using quantitative RT-PCR method (Figure 7A, B). This analysis revealed decreased mRNA expression of *DSTN* and *Cofilin-1* in pancreatic cancer bulk tissues compared to normal pancreas. Interestingly, the matching of the median *DSTN* mRNA expression level from pancreatic cancer bulk tissue with patient survival showed a tendency towards shorter survival of patients with a *DSTN* mRNA level above the median (number of analyzed samples for survival: 58) (Figure 7C). However, this difference did not reach statistical significance (p=0.23).

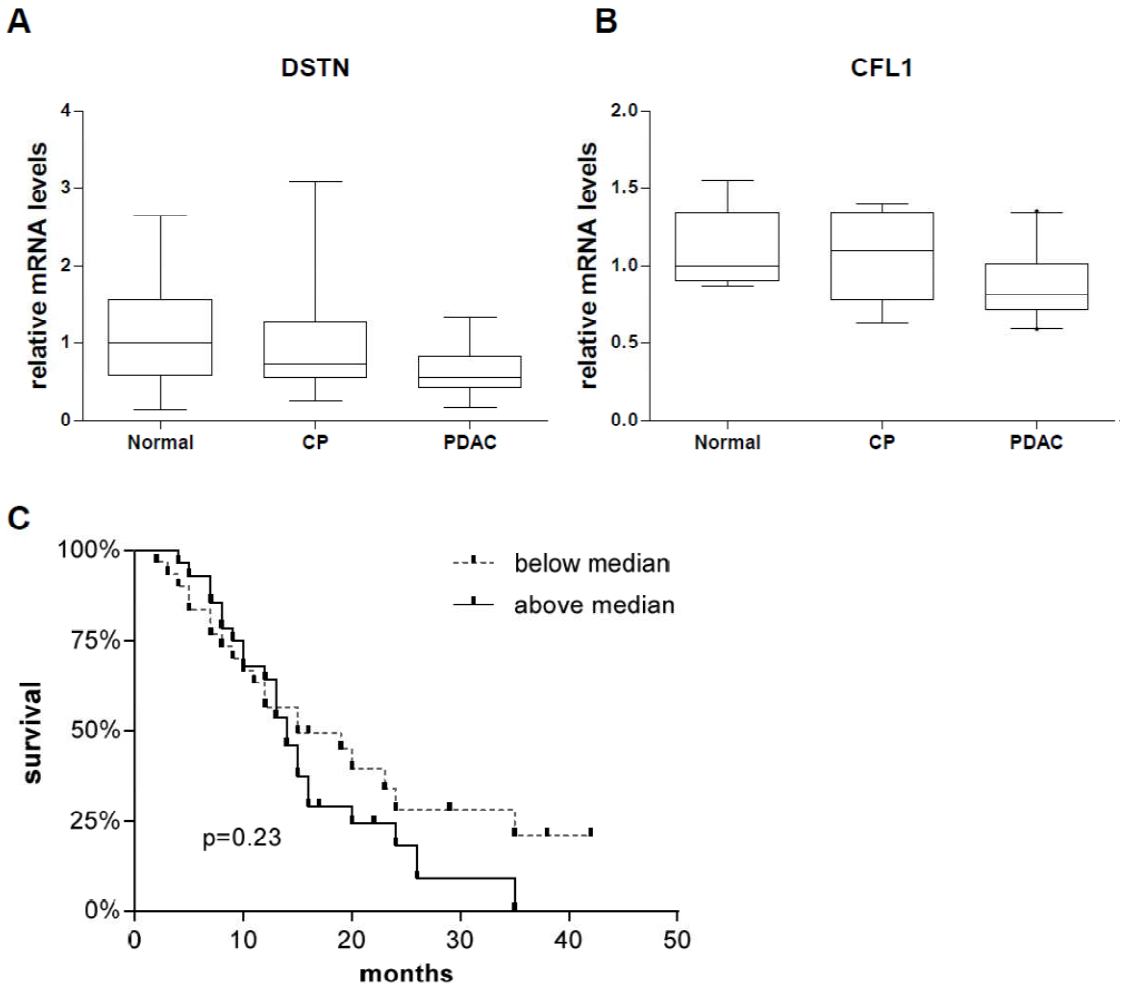


Figure 4. mRNA expression of *DSTN* **(A)** and *Cofilin-1* **(B)** in normal pancreatic, chronic pancreatitis and pancreatic cancer bulk tissues using QRT-PCR method, as described in the Materials and Methods section. Box and whisker plot with median expression level, interquartile range and 95% confidence interval. The median value of *DSTN* expression was taken as cut-off to define groups with high (straight line) and low (dashed line) *DSTN* mRNA levels. **(C)** The survival of patients in these groups was compared using the Kaplan-Meier analysis and the log-rank test.

4.4. DSTN is expressed in pancreatic acinar cells and pancreatic cancer cells

The localization of DSTN in pancreatic cancer tissue was investigated using immunohistochemistry staining (Figure 8). In normal pancreatic tissue DSTN exhibited a cytoplasmic expression pattern in acinar and ductal cells. The same expression pattern was present in islets and nerve cells in normal pancreatic tissue (Figure 8A, B). In pancreatic cancer tissue, the pancreatic cancer cells displayed overall strong DSTN expression with DSTN also being present in tubular complexes and hypertrophic nerves (Figure 8 C-F). The cytoskeletal-associated protein DSTN was also expressed in stromal cells of pancreatic cancer tissue (Figure 8 C-F). Moreover, DSTN was highly expressed in nerve-invasive pancreatic cancer cells (Figure 8G, H).

In conclusion, *DSTN* mRNA was up-regulated on the mRNA and protein level in highly nerve-invasive pancreatic cancer cell clones of the two selected pancreatic cancer cell lines. Although *Cofilin-1* was also endogenously expressed in several pancreatic cancer cell lines, it was found to be down-regulated on the mRNA and protein level in the NP3 pancreatic cancer cell clones in contrast to the closely related DSTN. Immunohistochemistry demonstrated DSTN localization in tubular complexes, nerves and stromal cells of pancreatic cancer tissues and in addition, high *DSTN* mRNA levels could be correlated with shorter survival.

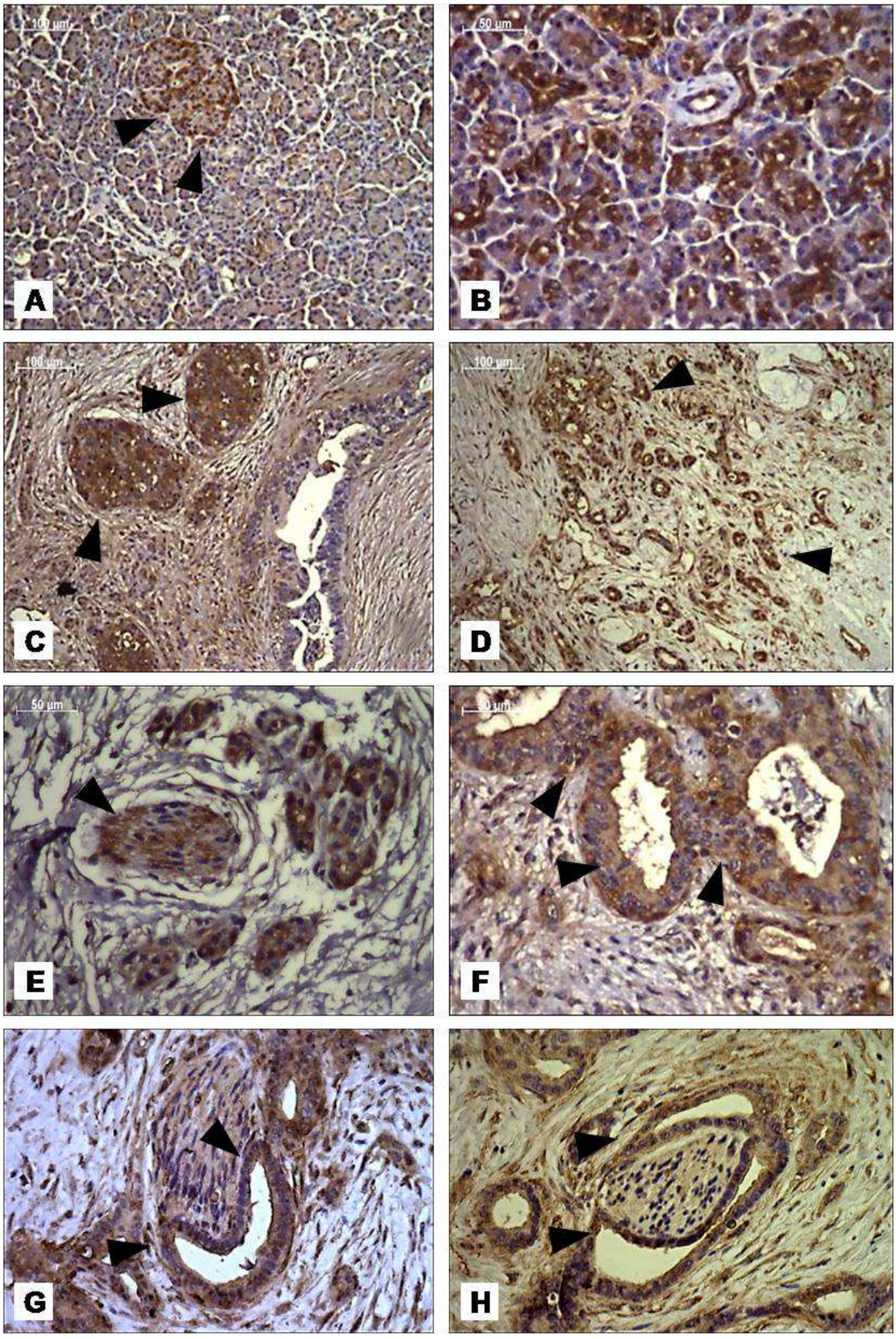

100 µm
A
50 µm
B
100 µm
C
100 µm
D
50 µm
E
F
G
H

Figure 5. Immunohistochemical analysis of normal pancreatic **(A, B)** and ductal adenocarcinoma **(C-H)** tissues using a specific DSTN antibody as described in the Materials and Methods section. DSTN is expressed in acinar cells and islets of normal pancreas tissues; arrowheads in **(A)** portray the islet region. DSTN is strongly expressed in cancer cells, nerves and tubular complexes **(C-F)** of pancreatic cancer tissues; arrowheads indicate islets **(C)**, tubular complexes **(D),** nerves **(E)**, ductal adenocarcinoma cells **(F)** and perineural invasive cancer cells **(G, H)**

As DSTN was found to be strongly expressed in NP3 pancreatic cancer cells and pancreatic cancer tissue, it has been chosen for conduction of further functional analyses. Down-regulation of Cofilin-1 indicated less relevance for carrying out the specific function of the AC family genes. Furthermore, functional experiments for DSTN described in the literature indicated this approach as being promising [31].

4.5. Down-regulation of DSTN leads to decreased proliferation and invasiveness of NP3 PANC-1 cells

To further explore the functional relevance of DSTN in pancreatic cancer cells, the endogenous expression levels of this protein were down-regulated using siRNA transient transfection. This assay was performed for NP3 PANC-1 pancreatic cancer cells, since this cell line showed a clearly higher expression of DSTN versus NP0 pancreatic cancer cells (Figure 9A, B). Two different siRNA molecules specific to *DSTN* mRNA were used for transfection of PANC-1 pancreatic cancer cells and their efficacy was confirmed on the protein level (Figure 9A). The maximum reduction of the DSTN protein expression was achieved 96 hours after transfection with a 0.2-fold down-regulation of DSTN using siRNA#1 and 0.3-fold down-regulation using siRNA#2 (each compared to control-transfected cells). The expression of the second AC family partner, Cofilin-1, was not significantly affected in the siRNA transfected PANC-1 cells within the 96 hours of monitoring (Figure 9A). The down-regulation of DSTN by using specific siRNAs in

PANC-1 NP3 pancreatic cancer cells resulted in reduced proliferation (for siRNA#1: 126.7±7.9% and for siRNA#2: 125.1±7.3%; at day 2 each) of these cells compared to control siRNA-transfected cells (198.7±28.6%; $p < 0.05$; Figure 9B).

The siRNA-transfected PANC-1 NP3 cells were tested for potential changes in basal invasive properties. Therefore, Matrigel invasion and wound healing assays were conducted. Compared to control cells, DSTN-silenced cells revealed a decreased invasiveness (68% for siRNA#1 and 61% for siRNA#2) as well as reduced migratory activity (70% for siRNA#1 and 68% for siRNA#1, $*p<0.05$; Figure 9C). In contrast to these findings, adhesion assays did not reveal a significant difference in adhesion capacity between DSTN-silenced and control-transfected cells (Figure 9C). Therefore, one can conclude that DSTN-silenced PANC-1 NP3 pancreatic cancer cells demonstrated reduced proliferation as well as decreased invasiveness and migratory activity whereas the adhesion capacity of these cells was not affected by down-regulation of DSTN.

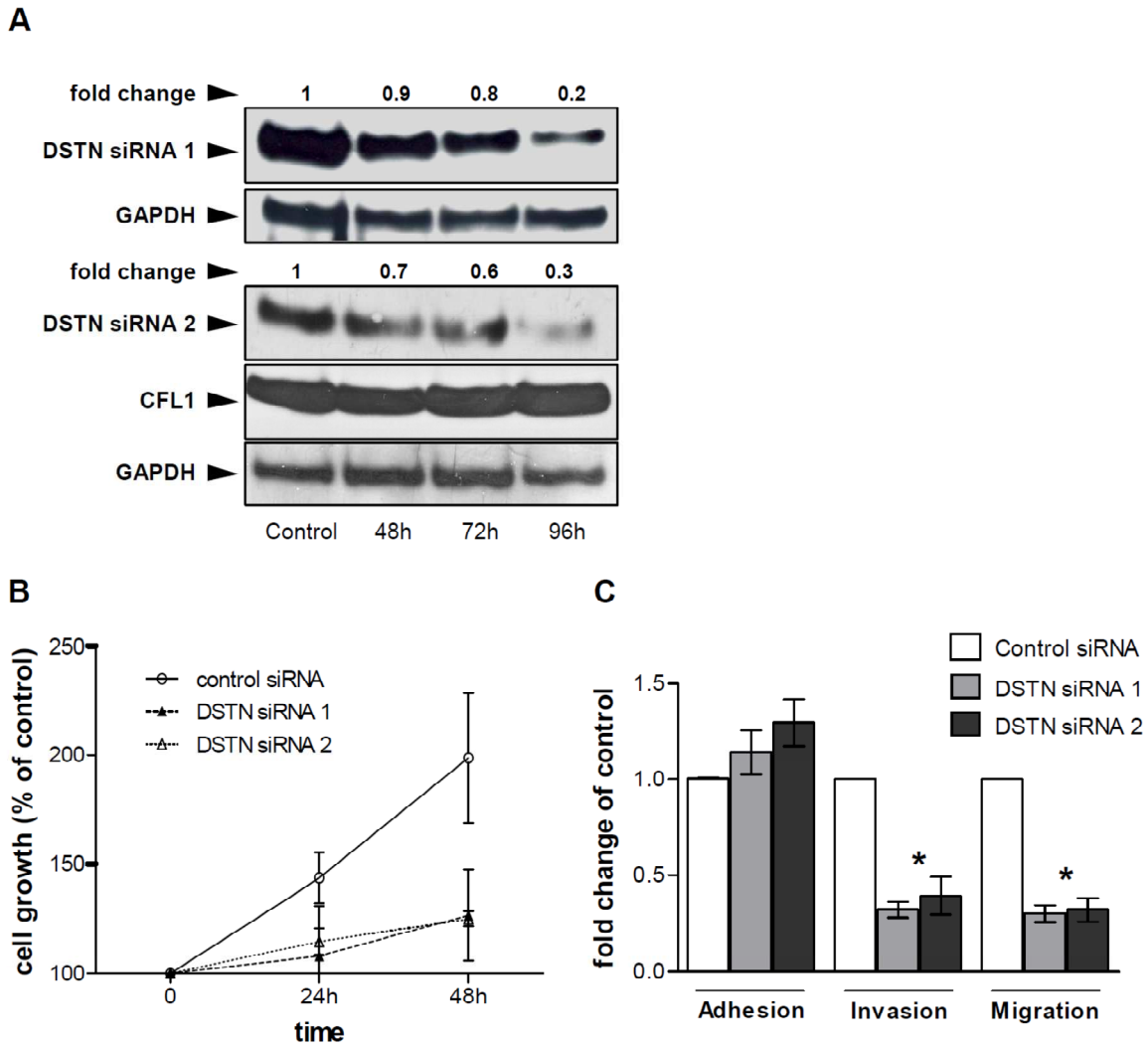


Figure 6. **(A)** DSTN and Cofilin-1 expression in NP3 PANC-1 cells after siRNA transfections using immunoblot and densitometric analysis as described in the Materials and Methods section. **(B)** MTT assay demonstrating proliferation of DSTN silenced NP3 PANC-1 cells. **(C)** Adhesion, invasion and migration capacities of DSTN siRNA-transfected NP3 PANC-1 cells compared to control-transfected cells as described in the Materials and Methods section. Data is presented as mean (± SEM) from three independent experiments. * $p<0.05$

4.6. DSTN-silenced NP3 cell clones present a disorganized actin cytoskeleton

For immunofluorescence analysis, a specific DSTN antibody as well as filamentous actin-specific phalloidin staining was used to determine differences in the morphology between NP0 and NP3 cells as well as in DSTN-silenced cells (Figure 10 A-D). In both control-transfected NP0 and NP3 cells, DSTN displayed a mainly nuclear and partly cytoplasmic expression pattern (Figure 10A, B). NP3 PANC-1 cells demonstrated solid organization of the actin cytoskeleton with dominant transverse stress fiber formation and well-defined adhesion junctions (Figure 10B). In comparison, NP0 PANC-1 cells displayed poorly organized cortical actin localization with strongly reduced cell-cell adhesion (Figure 10A). After DSTN siRNA transfection in NP3 PANC-1 cells the patterns of filamentous actin expression were remarkably changed. Moreover these cells demonstrated reduced transversal stress fibers and increased cortical localization of actin (Figure 10D). Interestingly, these changes in DSTN-silenced NP3 cells closely resembled the morphology of NP0 cells (Figure 10A). Indeed, down-regulation of DSTN expression induced a complete disorganization of the actin cytoskeleton in NP0 PANC-1 pancreatic cancer cells (Figure 10C). DSTN expression was not detectable in the DSTN-silenced cells (Figure 10C, D). Therefore, it can be summarized that PANC-1 NP3 cell clones with up-regulated DSTN levels displayed solid actin cytoskeleton organization as well as stress fiber formation whereas PANC-1 NP0 cell clones and moreover DSTN-silenced NP3 and NP0 clones showed poor or even complete disorganization of the actin cytoskeleton and correspondingly less transversal stress fiber formation.

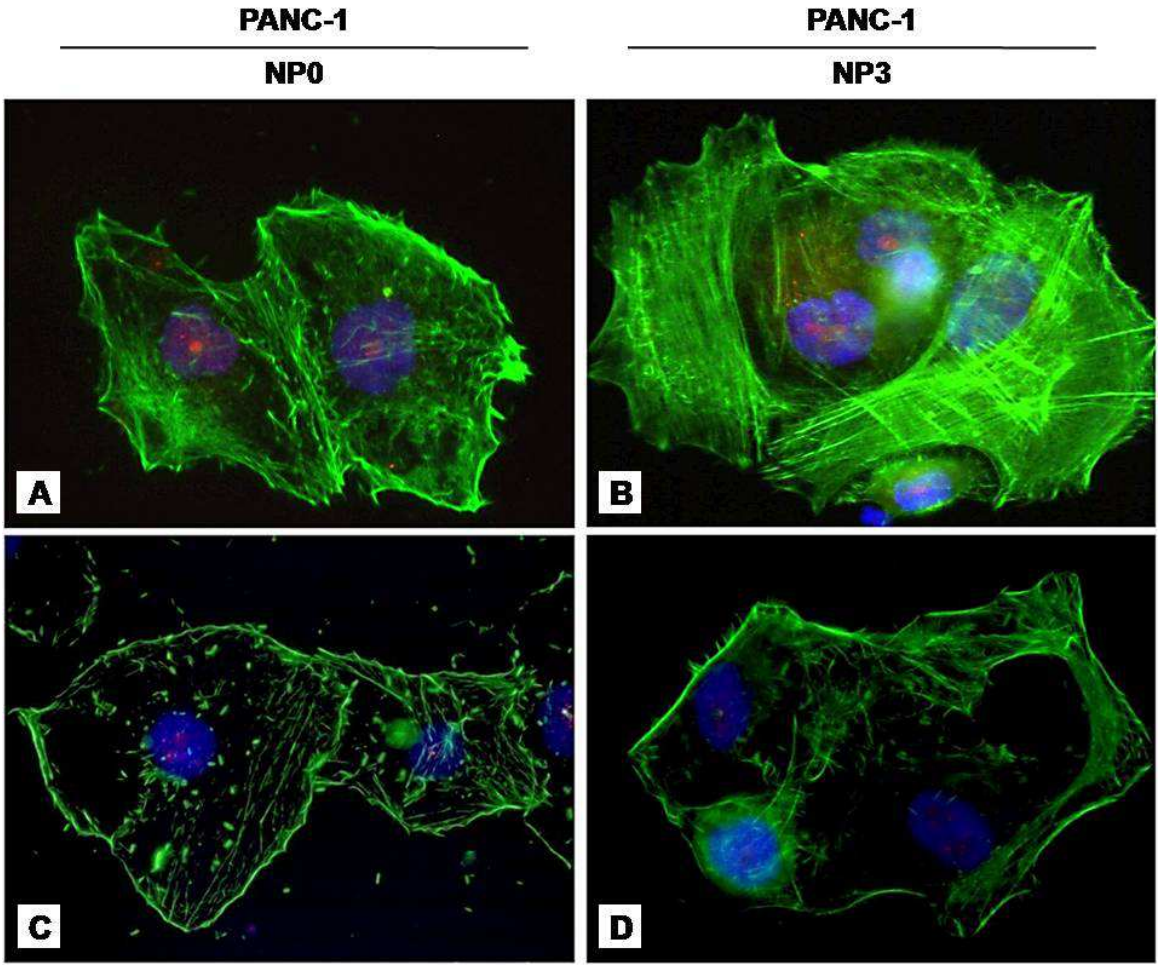


Figure 7. Analysis of DSTN and actin organization in NP0 and NP3 PANC-1 pancreatic cancer cells using immunofluorescence. Analysis of control-transfected **(A, B)** and DSTN siRNA-transfected **(C, D)** NP3 clones of PANC-1 pancreatic cancer cells as described in the Materials and Methods section. Red: DSTN; green: F-actin; blue: nuclear counterstaining.

5. Discussion

Reorganization of the actin cytoskeleton is crucial for the different modes of cancer cell migration. For example, single cancer cells are able to migrate in mesenchymal or amoeboid ways, and for both of these migrating modes actin dynamics are highly essential [36, 105]. It has been shown that manipulation of DSTN / Cofilin-1 activity and its regulators, affects the formation of protrusions (e.g. lamellipodia) and cell migration since they are involved in invadopodia formation [94, 103]. It is still unclear how the AC proteins exactly function in the mentioned processes and which circumstances lead to their different expression in cancer cells. It is not known whether DSTN and Cofilin-1 share the same functions or either act independently or in a mutual manner in the actin dynamics of cell migration and invasion. However, the expression of DSTN and Cofilin-1 is obviously differently regulated in perineural invasive pancreatic cancer cells. DSTN and Cofilin-1 are highly similar to each other but they display differences in local expression and show quantitatively different effects on actin dynamics [68, 95, 108]. The AC pathway appears to have a central role in the generation of free actin filament ends resulting in actin filament remodeling by polymerization and de-polymerization. This process is essential during chemotaxis, cell migration and invasion of tumor cells. Therefore, a balance of stimulatory and inhibitory parts of the pathway is critical for these processes [100]. It is known that knockdown of either DSTN or Cofilin-1 leads to defects in cell motility in mammalian (non-muscle) cells [44]. In DSTNcorn-1 mice (spontaneous mutant mice which lack DSTN), the phenotype is mainly restricted to the cornea, where DSTN

exists as the main AC family protein, suggesting a compensatory role of cofilins in other tissues [96]. It has been reported previously that DSTN/Cofilin isoforms are often co-expressed in the same cell types; nevertheless until now, the exact reasons for this co-expression have not been elucidated [94].

The recently published PNI genome-wide transcriptional analyses revealed expression changes of DSTN [1], which was verified by QRT-PCR and immunoblotting experiments as part of this study. Additionally, DSTN-depleted highly nerve-invasive cells were found to display a significantly altered phenotype, when using immunofluorescence staining. These results point towards a strong influence of DSTN expression on the actin cytoskeleton organization of these cells. Migration and invasion assays with DSTN knock out NP3 cells also showed significant functional changes in these cells. The surprising observation has been made that the invasive phenotype of PNI cells displayed a more organized actin cytoskeleton rather than the expected disorganized morphology. The reasons for this discrepancy are currently not known and have to be further investigated. It could be speculated, that DSTN has stronger actin filament de-polymerization activity than Cofilin-1 [95, 108].

Interestingly, DSTN is up-regulated in platinum-resistant ovarian cancer cells [106]. Cofilin-1 appears to be over-expressed in human chemoresistant pancreatic

adenocarcinoma [86]. Whether DSTN is involved in chemoresistance of pancreatic cancer is currently unknown and might be an interesting topic to investigate. Further studies have to be carried out to better understand this potential interaction.

Up-regulation of DSTN has also been observed in other tumor diseases, e.g., in primary anaplastic thyroid cancer and its derived cell lines as well as in malignant lung epithelial cells [23, 73]. Furthermore, DSTN is expressed in colon cancer cells. In the Isreco1 colon carcinoma cell line, DSTN and Cofilin-1 are expressed at a comparatively high level, but interestingly only DSTN appears to be required for cell migration and cell invasion [31]. In rat ascites hepatoma cells, simultaneous knockdown of DSTN and Cofilin-1 using specific siRNA decreases the motility and transcellular migration of these cells in two-dimensional culture. The knockdown of LIMK1, a kinase which inactivates the AC family of proteins through phosphorylation at Ser-3 [5, 107], led to the suppression of fibronectin-mediated cell attachments and focal adhesion formation [43]. In the approach described in this study, silencing of DSTN resulted in only slightly increased adhesion. Possibly, down-regulation of DSTN alone is not sufficient to increase adhesion in invasive pancreatic cancer cells and therefore depends on additional factors. It is unknown whether down-regulation of Cofilin-1 expression in PNI pancreatic cancer cells is directly induced or linked to the up-regulation of DSTN. However, our results indicate an involvement of the AC pathway in this phenomenon. DSTN has to be considered as one effector molecule in the actin dynamics - a complex process with precise spatial and

temporal regulation. Therefore, besides DSTN, the AC family of proteins and their direct or upstream regulators, such as LIMK1/2, SSH1-3 and rho family small GTPases, are most probably involved in migration and invasion of cancer cells [1, 14, 43, 94, 100, 105]. Targeting an effector at the end of a complex signaling pathway such as DSTN may therefore be of particular interest for developing successful therapeutic concepts.

6. Summary

Perineural invasion is a key feature of pancreatic cancer and influences disease recurrence and patient survival. Highly nerve-invasive and less invasive pancreatic cancer cell clones can be generated through the recently established ex vivo nerve invasion model. Genome-wide transcriptional analayses revealed the up-regulation of *DSTN* in the highly nerve-invasive pancreatic cancer cells [1].

With this study the increased expression of the cytoskleleton-associated protein DSTN in highly versus less nerve-invasive pancreatic cancer cells could be validated by using QRT-PCR, immunoblotting and immunohistochemistry analysis. Immunofluorescence analysis unveiled concomitant changes in cell morphology. After silencing of DSTN in Panc-1 pancreatic cancer cells using two specific siRNA oligonulceotides, these cancer cells showed reduced proliferation as well as decreased invasiveness and decreased migration [2, 53].

In conclusion, this study proofs that DSTN is up-regulated in highly nerve-invasive pancreatic cancer cells and that its expression is likely to be related to perineural invasion. Targeting this cytoskeleton-associated protein by further studys may reveal further insight in perineural invasion of pancreatic cancer and thus open a new ways for treatment of pancreatic cancer.

7. References

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8. Abbreviations

A	Ampere
ABP	Actin binding protein
AC	ADF/cofilin
ADF	Actin depolymerizing factor
APS	Ammonium per sulfate
BCA	Bicinchoninic Acid
BSA	Bovine serum albumin
CA 19-9	Carbohydrate antigen 19-9
cDNA	Complementary DNA
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CFL1	Cofilin-1
CFL2	Cofilin-2
CO2	Carbon dioxide
CP	Chronic pancreatitis
CT	Computed Tomography
°C	Degree Celsius
d	Day
DAB	3,3´- Diaminobenzidine
DAPI	4’,6-Diamidino-2-phenylindole
DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPC4	Deleted in pancreatic carcinoma, locus 4

DSTN	Destrin
ECL	Enhanced chemoilluminescence
ECM	Extracellular matrix
EDTA	Ethylenediamintetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
FCS	Fetal calf serum
g	Gram
G	Gauge
GAPDH	Glyceraldehyde 3-phospate dehydrogenase
h	Hour
HER-2/*neu*	Human epidermal growth factor receptor 2
HRPO	Horseradish peroxidase
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IPMN	Intraductal papillary mucinous neoplasm
kDa	Kilo Dalton
KCl	Potassium chloride
KRAS	Kirsten rat sarcoma viral oncogene homolog
M	Molar
MCN	Mucinous cystic neoplasm
mA	Milliampere
mg	Milligram
min	Minute

ml	Milliliter
µg	Microgram
µl	Microliter
µM	Micromole
µm	Micrometer
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide
NaCl	Sodium chloride
nM	Nanomolar
nm	Nanometer
NP0	Nerve invasion passage 0, none nerve-invasive
NP1-3	Nerve invasion passages 1-3, less-highly nerve-invasive
PANC-1	Human pancreatic carcinoma, epithelial-like cell line
PanIN	Pancreatic intraepithelial neoplasia
PBS	Phosphate buffered saline
PDAC	Pancreatic ductal adenocarcinoma
PNI	Perineural invasion
QRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute (medium)
RT	Room temperature
RtU	Ready to use
SDS	Sodium dodecyl sulfate

SEM	Standard error of the mean
siRNA	Small interfering RNA
SFM	Serum-free medium
TBS	Tris-buffered saline
T-TBS	Tween20-TBS
TEMED	Tetramethylethylenediamine
TP53	Tumor protein p53
Tris	Tris(hydroxmethyl)aminomethane
U	Units
V	Volts

9. Curriculum Vitae

Name	Theresa Bittner, née Klose
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Education

10/2004-11/2011	Study of human medicine at the Technische Universität München and Ludwigs-Maximilians-Universität, Munich
09/2007-07/2008	Exchange year at the faculty of medicine, Universidad Miguel Hernández, Elche (Spain)
1996-2004	A-Levels at Dr. Wilhelm André Gymnasium Chemnitz
1992-1996	Primary School in Chemnitz

Work and medical clerkship experience

Since 09/2012	Further specialization for internal medicine at the Zentralklinikum Augsburg
2010-2011	Clinical Internship Year
06-07/2011	Nephrology, Pulmology, Intensive Care at the New York Presbyterian Hospital, Cornell University, New York City
04-05/2011	Department of Cardiology (Intensive Care), Klinikum rechts der Isar, Munich
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