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Neurons chemoattract cancer cells via MCP-1-CCR4-induced paxillin phosphorylation and activation in the cancer cell cytoskeleton

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1.0 Introduction

1.1 Pancreatic cancer

Pancreatic cancer is one of the most aggressive and lethal malignancies with a high rate of mortality. It is the 4th cause of cancer-related death in both the United States and Europe (Malvezzi et al. 2013, Von Hoff et al. 2013), and the overall 5-year survival is as low as 8% (Siegel et al. 2017, Siegel et al. 2018). It is reported that by 2030, pancreatic cancer will become the second leading cause of cancer-related death (Rahib et al. 2014). GLOBOCA estimated that there will be 458,918 new cases (2.5% of all sites) diagnosed with pancreatic cancer worldwide in 2018, and 432,242 deaths (4.5% of all sites), almost as many as new cases (Bray et al. 2018). Despite great improvements have been achieved in diagnosis, chemotherapy and perioperative care, the 5-year survival rate only increased from 3.4% to 7% over the past 20 years in the palliative setting. Even after the radical operation, the 5-year survival is still as low as 25% because of local recurrence or distant metastasis. Under this situation, new strategies are urgent for understanding this disease.

The initiating event of pancreatic cancer in the majority of patients is the activating mutation of the KRAS oncogene (Roe et al. 2017). The oncogenic KRAS ($Kras_{G12D}$ mutations) leads to sustained proliferation, survival and cancerous development due to continuous signaling (Collins et al. 2012). Subsequently, pancreatic intraepithelial neoplasia (PanINs), which is the pre-malignant/pre-invasive lesion in PDAC, arises in the intralobular ducts (Roe et al. 2017). Loss of tumor suppressor genes, including TP53, p16, DPC4, BRCA2, and MKK4, results in uncontrollable cell proliferation and drives the progression of PanINs to PDAC at the primary site (Hruban et al. 1998, Kim et al. 2006, Makohon-Moore et al. 2016). TP53 is one of the most important tumor suppressor genes and most frequently mutated genes in pancreatic

cancer. TP53 gene can mediate proliferation, apoptosis (el-Deiry et al. 1994) and cell cycle (el-Deiry et al. 1993). The heterozygous inactivation of TP53 was observed in 90% of pancreatic carcinomas (Hruban et al. 1998), and the homozygous inactivation combined with the mutation was observed in 50-75% of pancreatic cancer samples (Redston et al. 1994).

Symptoms of pancreatic ductal adenocarcinoma (PDAC), which account for more than 90% of all pancreatic malignancies (Tempero et al. 2017), comprise pain, weight loss, jaundice, pruritus, diabetes, ascites, nausea, vomiting. However, it occurs in a stealth mode and no warning signs have been detected in the early stage. Weight loss is the most common symptom in pancreatic cancer. It happens in as high as 92% of pancreatic head cancer, and almost 100% of body and tail cancer (Rabow et al. 2017). Abdominal or back pain is another common symptom, which can be classified as both neuropathic and nociceptive, thus “mixed type” pain (Rabow et al. 2017).

Pancreatic cancer is primarily diagnosed by imaging, including ultrasound, multidetector-row CT (MDCT), MRI, endoscopic ultrasound (EUS), endoscopic retrograde cholangiopancreatography (ERCP). MDCT has almost the same sensitivity and specificity with MRI for pancreatic cancer (Park et al. 2009). The serum tumor biomarkers are also important indicators. The diagnostic biomarkers for pancreatic cancer include carbohydrate antigen 19-9 (CA 19-9), carbohydrate antigen125, carcinoembryonic antigen (CEA), and laminin γC (LAMC2).

To date, the therapeutic strategies for pancreatic cancer contain surgical resection, adjuvant chemotherapy, chemoradiation therapy, systemic chemotherapy, and palliative care. Surgical resection remains the only potentially curative option and few other effective therapies exist for prolonging the overall survival. Unfortunately, as the majority patients are asymptomatic until the PDAC progresses to a late stage, up to 80%-85% of patients are not

eligible for surgical resection (Ryan et al. 2014) because of the local infiltration or distant metastasis. Depending on the location of the resectable tumors, the surgical methods are divided into pancreaticoduodenectomy (Whipple procedure), distal pancreatectomy, and total pancreatectomy (Kamisawa et al. 2016). The purpose of curative surgery is to achieve R0 resection, as R1 resection is positively correlated with impaired survival (Demir et al. 2018). To increase the resectability rates, neoadjuvant therapy has been increasingly investigated in recent years. Our previous study demonstrated that response to neoadjuvant therapy is positively correlated with a decreased tumor stage, increased rates of R0 resection, and less neural invasion (Schorn et al. 2017).

1.2 Neural invasion in pancreatic cancer

As for the neuropathic pain, an important mechanism seems to be the infiltration of the intrapancreatic and extrapancreatic nerves by pancreatic cancer cells (Dobosz et al. 2016), which occurs in up to 100% of the patients with true pancreatic ductal adenocarcinoma (Bapat et al. 2011, Demir et al. 2015). Besides, neural invasion is also associated with local recurrence and metastasis (Bapat et al. 2011), and has been identified as an important prognostic factor for pancreatic cancer (Schorn et al. 2017). Therefore, understanding the mechanisms of the neural invasion is indispensable in pancreatic cancer.

Current insights indicate that several signaling molecules from nerves or/and from pancreatic cancer cells and the interactions between nerves and tumor cells are essential for neural invasion (Bapat et al. 2011). The molecules contain a variety of neurotrophic factors (such as the neurotrophin family, glial cell line-derived neurotrophic factor family) (Zhu et al. 1999, Schneider et al. 2001), cytokines (such as Transforming growth factor alpha) (Bockman

et al. 1994), as well as chemokines (such as CX3CL1) (Marchesi et al. 2008, Marchesi et al. 2010).

Neurotrophic factors are important in controlling the survival, growth, and differentiation of cancer and nerve cells (Liebig et al. 2009, Imoto et al. 2013). Neurotrophin family is composed of 4 members: Nerve growth factor (NGF), brain-derived neurotrophic factor (BDGF), neurotrophin-3 and neurotrophin-4 (Bapat et al. 2011). NGF family can interact with two types of receptors, tropomyosin-receptor kinase (Trks, the high-affinity receptor) and p75 neurotrophin receptor ($p75_{LNTR}$, the low-affinity receptor) (Lee et al. 2001, Nykjaer et al. 2004). Each neurotrophin has a specific Trk receptor: 1) NGF binds with high affinity to TrkA and at low affinity to $p75_{NTR}$ (Kaplan et al. 1991); 2) Brain-derived neurotrophic factor and neurotrophin-4 bind with TrkB (Klein et al. 1991, Soppet et al. 1991, Squinto et al. 1991); 3) Neurotrophin-3 binds with TrkC (Lamballe et al. 1991). Interestingly, the binding of nerve growth factor and TrkA is increased in the presence of $p75_{LNTR}$ (Esposito et al. 2001). The expressions of nerve growth factor, Trks and $p75_{LNTR}$ are strikingly up-regulated in pancreatic cancer cells (Miknyoczki et al. 1999, Zhu et al. 1999, Schneider et al. 2001) and positively associated with the poor prognosis and the severity of neural invasion (Wang et al. 2009). Interaction of nerve growth factor with TrkA can activate the ERM1/2 signaling pathway and increase the expression of matrix metalloproteinase-2 (MMP-2) (Okada et al. 2004). Specifically, the binding of nerve growth factor with TrkA contributes toward the proliferation and neural invasion in pancreatic cancer cells (Ketterer et al. 2003). Brain-derived neurotrophic factor is also over-expressed in pancreatic cancer, which can stimulate the proliferation and invasive capacity of cancer cells (Kowalski et al. 2002, Zhu et al. 2002).

The glial cell line-derived neurotrophic factor family, which contains 4 members [glial cell line-derived neurotrophic factor family (GDNF), artemin (ARTN), neurturin (NRTN) and

persephin (PSPN)], also plays an important role in the neural invasion (Ceyhan et al. 2008). The GDNF receptor family consists of 4 members: GFRα1 (binding to GDNF), GFRα2 (binding to NRTN), GFRα3 (binding to ARTN), and GFRα4 (binding to PSPN). GDNF promotes tumor cells invasiveness by modulating matrix metalloproteinase-9 (MMP-9) through several different signaling pathways in pancreatic cancer (Okada et al. 2003). The expression of artemin is up-regulated in human pancreatic specimens and closely correlated with pancreatic cancer cells neural invasion capacity (Ceyhan et al. 2006, Ceyhan et al. 2010, Demir et al. 2010). The expression of neurturin and its receptor GFRα2 in pancreatic cancer is overall up-regulated (Wang et al. 2014). The neurturin/GFRα2 axis promotes sustained proliferation and neuron-directed migration in pancreatic cancer (Wang et al. 2014).

The chemokine CX3CL1 and its receptor CX3CR1 are also involved in neural invasion. Human pancreatic cancer cell lines strongly express CX3CR1 and also in pancreatic cancer patients, the high expression of CX3CR1 is positively correlated with neural invasion and early recurrence (Marchesi et al. 2008). CX3CL1 can influence cell adhesive properties which subsequently affect the neural invasion (Imai et al. 1997, Fong et al. 1998). Moreover, CX3CL1/CX3XR1 signaling also contributes to apoptosis resistance and mediates proliferation through AKT/NF-κB/p65 signaling pathway in pancreatic cancer cells (Wang et al. 2017).

C-X-C motif chemokine 12 (CXCL12) has been demonstrated to be ubiquitously expressed in many cancers (Schrader et al. 2002). It is important in mediating proliferation and metastasis in pancreatic cancer cells (Sun et al. 2010, Roy et al. 2014). CXCR4 and CXCR7 are two proved receptors of CXCL12. The CXCL12/CXCR4 axis mediates cancer cells migration and angiogenesis, thereby influencing progression (Koshiba et al. 2000, Marechal et al. 2010). In our previous study, we also demonstrated that CXCL12/CXCR4/CXCR7 axis can regulate the chemoattraction of human Schwann cells to pancreatic cancer cells, and subsequently influence neural invasion (Demir et al. 2017).

Pancreatic cancer cells have a strong tendency to invade to nerves. Neural invasion is strongly associated with the generation of pain in pancreatic cancer (Ceyhan et al. 2010, Demir et al. 2010). Abdominal or back pain sensation is a representative feature of pancreatic adenocarcinoma. During the neural invasion (NI), the features of intrapancreatic nerves are increased in size (neural hypertrophy) and density (Friess et al. 2002, Ceyhan et al. 2009, Demir et al. 2011, Demir et al. 2012), which may result from the interactions of pancreatic cancer cells and nerves. In the interaction, the sheaths of nerves are damaged by tumor cells, remaining the nerves assailable to be stimulated by infiltrated inflammatory cells, extracellular matrix, as well as cancer cells (Ceyhan et al. 2008, di Mola et al. 2008). Another mechanism of pain generation is vascularization in pancreatic cancer. A possible explanation is that the molecular factors, including vascular endothelial growth factor, artemin, can stimulate the formation of the vessel which also promote the growth of nerve fibers (Lindsay et al. 2005, di Mola et al. 2008).

The severity of the neural invasion is an independent prognostic factor regarding the overall survival for many solid tumors (Ceyhan et al. 2009, Schorn et al. 2017), including pancreatic cancer (Friess et al. 2002, Ceyhan et al. 2009, Demir et al. 2011, Demir et al. 2012), prostate cancer (Feng et al. 2011), head and neck cancer (Johnston et al. 2012). Neural invasion is correlated with local recurrence and neuropathic pain sensation (Demir et al. 2015). The presence of abdominal or back pain reduces the quality of life of pancreatic cancer patients. Neural invasion-targeting therapeutic strategies are a potential and convincing approach for pancreatic cancer patients since it would not only prevent the invasion and progression of cancer, but also relieve the pain from the neural invasion and improve the quality of life. Nevertheless, an effective targeted treatment for neural invasion is still not available (Scanlon et al. 2015). The NGF/TrkA pathway is a potential strategy, which

intervenes in the interaction between NGF and TrkA by using NGF-neutralizing antibody (Hefti et al. 2006, Abdiche et al. 2008, Watson et al. 2008, Wood 2010).

1.3 Cytoskeleton and cytoskeletal proteins

The cytoskeleton is composed of 3 elements: actin, microtubules and intermediate filaments (Dogterom et al. 2019). In normal cells, a fairly large number of core biological cellular activities lean on the cytoskeleton and its components (Dogterom et al. 2019). The crosstalk of actin and microtubules is essential for regulating cell migration (Huda et al. 2012). Microtubules play vital roles in the mediation of cell proliferation, migration, invasion, vesicular transport, and mitosis (Jordan et al. 2004). The coordination and remodeling of actin are essential for cell motility, cell differentiation, and cell division (Gunning et al. 2008). The activities of cytoskeleton can be sorted by intermediate filaments through conveying information between the cell surface and cytoplasmic interior (Chang et al. 2004).

On the other hand, the reorganization of the cytoskeleton and the formation of membrane protrusions and the abnormal expression of related proteins are vital for the acquirement of migratory and chemoresistance properties of the cancer cells (Yilmaz et al. 2009, Fife et al. 2014). The metastasis of cancer cells occurs over several multifaceted steps. First, cancer cells detach and escape from the primary tumor. Then, cancer cells invade into and spread along either the lymphatics or vessels. After extravasation, the secondary tumor is developed at the new site or organ (Steeg 2006). An important alteration within migrating cells is the dynamic reorganization of the cytoskeleton, which also plays an important role in the epithelial-mesenchymal transition (EMT) process (Thiery et al. 2009). In the context of the metastatic and EMT processes, the orchestrated cytoskeleton and its components are critical for maintaining the cell structure (Fife et al. 2014).

The Rho GTPases act as cytoskeletal regulators during cancer cell migration. The Rho GTPase family is comprised of several subfamilies, including Rac subfamily, Cdc42 subfamily, RhoA subfamily, and other Rho GTPases (Ridley 2006). The Rho GTPase family affects cell migration through regulating the cytoskeleton by interacting with various effectors (Jaffe et al. 2005). For example, the components Rho, Rac, and Cdc42 emerge as regulators in the assembly and the organization of the actin cytoskeleton in different kinds of cells, including fibroblasts, epithelial cells and mast cells (Hall 1998). Rac stimulates the formation of lamellipodia by reorganizing the actin cytoskeleton. Cdc42 also can promote the formation of filopodia, thereby initiating and controlling the migration directed by chemokines (Hall 2012). Moreover, high expression of Rho GTPase in several tumors is positively associated with increased metastasis and poor prognosis (Karlsson et al. 2009).

Focal adhesion kinase (FAK), a tyrosine kinase, affects the migration of tumor cells by sensing the mechanical forces and consequently regulates the reorganization of cytoskeleton during tumor cell migration (Huang et al. 1999). During the processes of tumor cell migration or adhesion, the activity of FAK is associated with changes in the stabilization of actin filaments (Lim et al. 2008). Moreover, the high expression of FAK is also correlated with increased metastasis and poor prognosis (de Vicente et al. 2013).

The typical migratory phenotypes of cancer cell are characterized by the formation of migratory protrusions, including filopodia, lamellipodia, and invadopodia (Alblazi et al. 2015). Filopodia are narrow, dynamic and actin-rich structures that direct the migration of cancer cells. The formation of filopodia is mediated by Cdc42, a component of the Rho family small G protein, via a series of proteins, including fascin, diaphanous, and Mena/VASP (Machesky 2008). Fascin, an actin-bundling protein, has the ability to encourage the growth of filopodia (Svitkina et al. 2003). Diaphanous is involved in the interactions with microtubules (Faix et al. 2006). Mena/VASP, composed of the membrane-targeting module and the actin-interacting module,

is able to enforce the extension of the long parallel actin bundles and plays an important role in the formation of filopodia (Applewhite et al. 2007). Filopodia are vital for cell motility (Stevenson et al. 2012) and cell adhesion (Vasioukhin et al. 2000). During the process of cancer cell invasion and migration, the transport of signal molecules between cells and filopodia contributes to the formation of the intercellular junctions (Vasioukhin et al. 2000).

Lamellipodia are also observed in migrating cells in the edge of the migrating side and sustain the migratory “drive”. Lamellipodia are among the most prominent cytoskeletal alterations that play clear and established roles in driving cell locomotion (Johnson et al. 2015). Similar with filopodia, the formation of lamellipodia is signalled by another component of small G protein of the Rho family, the Rac protein (Machesky 2008).

Moreover, the formation of lamellipodia and filopodia is also controlled by cytokines, including epidermal growth factor (EGF) (Yasui et al. 2017), transforming growth factor-β (TGF-β) (Feng et al. 2013), tumor necrosis factor (TNF) (Marivin et al. 2014), hepatocyte growth factor (HGF) (Yasui et al. 2017). However, the significance of filopodia and lamellipodia during neural invasion in pancreatic cancer remains unclear.

1.4 Paxillin

Actin-bundling-proteins, like paxillin (Lopez-Colome et al. 2017), play essential roles in the formation of migratory protrusions (Salvi et al. 2017). Paxillin is a major component of focal adhesions (FAs) with a multifunctional focal adhesion adapter protein, which is vital in the conversion of signals from extracellular into endocellular.

Paxillin protein, which is 64.5 kDa in molecular weight and contains 591 amino acids, is encoded by the paxillin gene. The activation of paxillin is stimulated by phosphorylation.

Various stimuli can stimulate the phosphorylation of paxillin, including growth factors, as well as integrin-dependent adhesion to the extracellular matrix (ECM). In addition to the major phosphorylation sites tyrosines 31 and tyrosines 118, paxillin also has other sites, such as Tyr40, Tyr88, Ser85, Ser126, Ser130 (Webb et al. 2005). After phosphorylation of paxillin by integrin ligation via Src kinase at Tyr118, the cellular proliferation state and the reorganization of the cytoskeleton are provoked via mitogen-activated protein kinase (MAPK) (Brown et al. 2004) signaling pathway.

Paxillin is critical for cancer development and metastasis (Lopez-Colome et al. 2017). Changes in the expression of paxillin are linked with cancer cell progression and invasion, including osteosarcoma (Azuma et al. 2005), lung carcinomas (Mackinnon et al. 2011), colorectal cancer (Cui et al. 2006), breast tumors (Farmer et al. 2005, Finak et al. 2008), prostate tissue (Mestayer et al. 2003, Miyoshi et al. 2003).

Reorganization of focal adhesions via phosphorylation of paxillin by FAK and Src is essential for the promotion of cancer cell migration, which is considered as an indicator of metastasis (Pribic et al. 2012, Devreotes et al. 2015). Phosphorylation of paxillin and FAK are requested in the insulin-like growth factor (IGF)-induced cell motility (Leventhal et al. 1997). Interactions between FAK and paxillin result in the initiation of migration signaling (Wu et al. 2013). Vinculin emerges as a modulator in FAK-paxillin interactions, and thereby controls cell survival and motility by regulating ERK pathway (Subauste et al. 2004). In pancreatic cancer cells, loss of paxillin is strongly correlated with inhibition of migratory ability (Burdyga et al. 2013). Overexpression of paxillin is positively associated with poor survival and increased migration and invasion ability (Sun et al. 2017).

2.0 Aim of the present study

Neural invasion (NI) is the leading cause of local recurrence and neuropathic pain sensation in many solid cancers including pancreatic ductal adenocarcinoma and colorectal cancer. The alterations that occur in the cytoskeleton and the abnormal expression of cytoskeletal proteins play an important role during cancer cell migration and neural invasion. Therefore, dissecting what happens specifically to the cytoskeleton and cytoskeletal-related proteins, such as paxillin, during neural invasion is vital for facilitating the development of novel therapeutics targeting pancreatic cancer and neural invasion, which may improve patient survival and, especially, quality-of-life. However, the alterations of the cytoskeleton and the "cytoskeletal" mechanisms underlying the impacts of cytoskeletal-related proteins on the neural invasion of pancreatic cancer cells have, however, never been analyzed.

Therefore, the first aim of the presented study was to illuminate what happens specifically to the cytoskeleton and to elucidate the alterations of the migratory protrusions, including filopodia and lamellipodia. For this purpose, a three-dimensional (3D) migration assay was used and the morphology of pancreatic cancer cells in the migration front was compared with that of cells in the back front. Furthermore, pancreatic cancer cells were stained for phalloidin, and the formation of the migratory protrusions was quantified with the FiloQuant® software and compared between the pancreatic cancer cells in the migration front and the back front.

The second aim of the study was to elucidate the effect of cytoskeletal related protein on the neural invasion of pancreatic cancer cells. Since paxillin phosphorylation plays an important role in the alteration of cytoskeleton. Hereby, the expression of phospho-paxillin of pancreatic cancer cells in the neural invasion front was characterized and compared to that in the back front in the 3D migration assay.

The third aim of the study was to investigate the cytokines which possibly be involved in the stimulation of paxillin phosphorylation during the neural invasion of pancreatic cancer cells. For this purpose, pancreatic cancer cells and DRG neurons were either cultured alone or cultured together. After cultivation, the supernatant was collected, and the concentration of related cytokines was measured by sandwich ELISA assay.

The fourth aim of the study was to ascertain the mechanism of how the cytokines affect the neural invasion of pancreatic cancer cells. For this purpose, pancreatic cancer cells were exposed to cytokines or receptor antagonists. Afterwards, the alteration of the neuron-directed migration ability was determined by 3D migration assay.

3.0 Materials and Methods

3.1 Materials

3.1.1 List of the antibodies

Primary antibodies

Antibody	Catalogue number	Application	Source
Rabbit anti-phospho-paxillin (Y118) Ab	2541	WB, IHC	Cell Signaling Technology
Rabbit anti-paxillin Ab	12065S	WB	Cell Signaling Technology
Rabbit anti-phospho-Src (Y416) Ab	2101S	WB	Cell Signaling Technology
Rabbit anti-Src Ab	2102S	WB	Cell Signaling Technology
Rabbit anti-phospho-FAK (Y397) Ab	8556S	WB	Cell Signaling Technology
Rabbit anti-FAK (D507U) Ab	71433S	WB	Cell Signaling Technology
Rabbit anti-phospho-ERK1/2 (T202/Y204) Ab	4370S	WB	Cell Signaling Technology
Rabbit anti-ERK1/2 (137F5) Ab	4695s	WB	Cell Signaling Technology
Rabbit anti-PGP9.5 Ab	Z5116	IF	Dako Deutschland GmbH
Mouse anti-S100 Ab	MAB079-1	IHC	EMD Millipore
Mouse anti-Cytokeratin Pan Ab	Ab17154	IF	Abcam
Rat anti-CK19 Ab	-	IF	Developmental Studies Hybridoma Bank
Mouse anti-GAPDH Ab	SC-32233	WB	Santa Cruz Biotechenology
Phalloidin-TRITC	P1951	IF	Sigma-Aldrich Chemie GmbH

Secondary antibodies

Antibody	Catalogue number	Application	Source
Alexa Fluor goat anti-mouse IgG 488/594	1834337/1830459	IF	Thermo Fisher Scientific
Alexa Fluor goat anti-rabbit IgG 488/594	1885240/1851471	IF	Thermo Fisher Scientific
Goat HRP-Labelled Polymer Anti-Mouse Ab	K4000	IHC	Dako Deutschland GmbH
Goat HRP-Labelled Polymer Anti-Rabbit Ab	K4003	IHC	Dako Deutschland GmbH
ECL anti-mouse IgG Horseradish peroxidase lined antibody	NA931V	WB	GE Healthcare Life Sciences
ECL anti-rabbit IgG Horseradish peroxidase lined antibody	NA9340V	WB	GE Healthcare Life Sciences

3.1.2 Chemicals and Reagents

Chemical/Reagent	Product number	Source
Albumin Fraction V (BSA)	T844.3	Carl Roth GmbH
Ammonium persulfate (APS)	9592.1	Carl Roth GmbH
B-27 Supplement (50x)	1116531	Gibco
BCA protein assay	23225	Thermo Fisher Scientific
Citric acid (Monohydrate)	3958.4	Carl Roth GmbH
Collegenase type 2	LS004176	Worthington Biochemicals
Dulbecco's Phosphate Buffered Saline	D8537	Sigma
ECL Plus Western Blotting substrate	32132	Thermo Fisher Scientific
EGF	17005042	Invitrogen

Ethanol 70%	7078027	Otto Fischer GmbH
Ethanol 96%	7138032	Otto Fischer GmbH
Ethanol absolute	7127114	Otto Fischer GmbH
Ethanol absolute	64-17-5	Merck KGaA
Extracellular matrix (ECM) gel	E1270	Sigma-Aldrich Chemie GmbH
Fetal Bovine Serum	F7524	Sigma-Aldrich Chemie GmbH
Fluorescence Mounting Medium	S3023	Dako Deutschland GmbH
formaldehyde		Carl Roth GmbH
Glycin	3908.3	Carl Roth GmbH
Hank's BSS	H15-010	PAA
HEPES solution	H0887	Sigma-Aldrich Chemie GmbH
hydrogen peroxide 30 %	9681.1	Carl Roth GmbH
Keratinocyte SFM	17005042	Invitrogen
L-Glutamine solution	G7513	Sigma-Aldrich Chemie GmbH
Methanol	4627.5	Carl Roth GmbH
Milk	T145.3	Carl Roth GmbH
Minimum Essential medium Eagle (MEM) media	M2279	Sigma-Aldrich Chemie GmbH
Natriumchlorid (NaCl)	3957.2	Carl Roth GmbH
Neuralbasal medium	21103	Gibco
Normal goat serum	50062Z	Life technologies
PBS Dulbecco	L182-50	Biochrom GmbH
Penicillin-Streptomycin	P0781	Sigma-Aldrich Chemie GmbH
phosphatase inhibitor	4906837001	Sigma-Aldrich Chemie GmbH
protease inhibitor	4693159001	Sigma-Aldrich Chemie GmbH

RIPA buffer	R0278	Sigma-Aldrich Chemie GmbH
Roticlear	A538.1	Carl Roth GmbH
Rotiphorese Gel 30	3029.1	Carl Roth GmbH
RPMI-1640 Medium	R8758	Sigma-Aldrich Chemie GmbH
SDS, ultra-pure	2326.2	Carl Roth GmbH
SuperSignal West Pico PLUS Chemiluminescent Substrate	34577	Thermo Fisher Scientific
TEMED	2367.3	Carl Roth GmbH
Transfer Membrane 0.2 um	ISEQ00010	Merck Millipore
Tris base	T1503	Sigma-Aldrich Chemie GmbH
Tris-HCl	T3253	Sigma-Aldrich Chemie GmbH
Triton X 100	3051.2	Carl Roth GmbH
Tween 20	9127.2	Carl Roth GmbH
Typsin-EDTA solution	T3924	Sigma-Aldrich Chemie GmbH
VectaMount permanent mounting medium	H-5000	Vector
ε-aminocaproic acid	7260	Sigma-Aldrich Chemie GmbH

3.1.3 Buffers and Solutions

Immunohistochemistry

10x Tris Buffered Saline (TBS)

Tris base	24.2 g
NaCl	85 g
Distilled Water	800 mL
Adjust pH to 7.4 with	5 M HCl

Constant volume with distilled water to	1000 mL

20x Citrate buffer

Citric acid (Monohydrate)	21 g
Distilled Water	300 mL
Adjust pH to 7.4 with	5 M NaOH
Constant volume with distilled water to	500 mL

Washing Buffer (1x TBST)

10x TBS	100 mL
Tween 20	1 mL
Constant volume with distilled water to	1000 mL

Washing Buffer (1x PBST)

PBS	9.55 g
Tween 20	1 mL
Constant volume with distilled water to	1000 mL

Western Blotting

Electrophoresis buffer (10x)

Tris base	30.3 g
Glycin	144 g
SDS	10g
Constant volume with distilled water to	1000 mL

Lower Tris buffer (4x)

Tris-HCl	181.5 g
SDS	4 g

Check pH 8.8	
Constant volume with distilled water to	1000 mL

Upper Tris buffer (4x)

Tris-HCl	60.5 g
SDS	4 g
Check pH 6.8	
Constant volume with distilled water to	1000 mL

10% APS

APS	10 g
Constant volume with distilled water to	100 mL

Anode buffer I

Tris base	36.3 g
Methanol	200 mL
Constant volume with distilled water to	1000 mL

Anode buffer II

Tris base	3.03 g
Methanol	200 mL
Constant volume with distilled water to	1000 mL

Cathode buffer

Tris base	3.03 g
Methanol	200 mL
ε-aminocaproic acid	5.24 g
Constant volume with distilled water to	1000 mL

Washing Buffer (1x TBST)

10x TBS	100 mL
Tween 20	1 mL
Constant volume with distilled water to	1000 mL

Blocking Buffer (1x TBST)

Dry milk or BSA	0.5 g
Washing buffer	10 mL

3.1.4 Kits

SCF ELISA Kit	Sigma-Aldrich Chemie GmbH
IGF ELISA Kit	Sigma-Aldrich Chemie GmbH
PDGF ELISA Kit	Sigma-Aldrich Chemie GmbH
EGF ELISA Kit	Sigma-Aldrich Chemie GmbH
MCP-1/CCL2 ELISA Kit	Sigma-Aldrich Chemie GmbH
HGF ELISA Kit	Sigma-Aldrich Chemie GmbH
VEGF ELISA Kit	Sigma-Aldrich Chemie GmbH
NGF ELISA Kit	Sigma-Aldrich Chemie GmbH

3.1.5 Laboratory equipment

Accumax	Sigma-Aldrich Chemie GmbH
Balance	SCAL TEC SBC 52
centrifuge	Eppendorf
Freezer -20℃	

Freezer -80℃	Heraeus
Microscopes	Olympus IX50 inverse microscope and Zeiss Axioplan 2
PH-meter	BECKMAN (Washington, DC, USA)
Power supple	Bio RAD MODLL 200/2.0
Refrigerator 4℃	
Vortex Mixer	
Water bath	Lauda ecoline RE 104, MEDAX
Tissue embedding machine	Leica
Tissue processor	Leica
Shaker	IKA-Shaker MTS 4
Glass coverslips	Plano
Magnetic mixer	IKA-COMBIMAG RET
Photometer	Thermo-Labsystem Opsys MR
Stereomicroscope	Zeiss Stemi 2000
Imaging software	Olympus analysis software and Zeiss KS300 program
microtome	Leica JUNG RM2055
X-ray films	Hyperfilm, Amersham Bioscience

3.2 Methods

3.2.1 Cell culture

Pancreatic cancer cell lines AsPC-1, BxPC-3, Capan-1, Colo-357, MIA PaCa-2, Panc-1, SU.86.86 were purchased from American Type culture collection (ATCC) and the pancreatic cancer cell line T3M4 was a kind gift from Dr. Metzgar (Durham. North Carolina). All cancer cell lines were cultured according to the supplier's recommendations at 37℃, 5% CO_2. The human immortalized pancreatic epithelial cell line HPDE was a kind gift from Professor M.S. Tsao of the Ontario Cancer Institute (Toronto, Canada) (Furukawa et al. 1996, Ouyang et al. 2000) and was cultured with Keratinocyte SFM, +EGF + bovine pituitary extract supplemented with 1% Penicillin/Streptomycin.

3.2.2 Isolation of dorsal root ganglia

The primary dorsal root ganglia (DRG) neurons were isolated from the lumbar spinal region of newborn C57BL/6 mouse (2-day to 2-week old) after anterior laminectomy, as described previously (Ceyhan et al. 2008). The neurons were kept in MEM on ice, and the roots were removed under microscopy. After collection, the DRG neurons were incubated in 900 µL Hanks' BSS and 100 µL collagenase type II for 40 minutes in incubator for digesting the DRG capsules, easing the subsequent dissociation of neurons.

3.2.3 Three-dimensional (3D) migration assay

The 3D migration assay and digital time-lapse microscopy were used to determine the affinity and the neuron-directed migration of cancer cells to DRG neurons. 10_5 SU.86.86

pancreatic cancer cells and DRG neurons were respectively suspended in an extracellular matrix (ECM) gel droplet, placed at a distance of 1 millimeter. To generate a chemoattractive gradient, an ECM or fibronectin “bridge” was applied between the suspensions. An additional cell-free ECM gel droplet was placed at the other side and connected to the cancer cell suspension so that unspecific cellular migration could be excluded. After polymerization of the suspensions and bridges in the incubator, neurobasal medium supplemented with 100U/mL penicillin and 100 µg/mL streptomycin, 2% B-27, 0.5mM L-Glutamine and 10% FCS were applied to the assay. After 48 hours incubation, the migratory behavior of cancer cells was recorded by digital time-lapse microscopy. To analyze the migratory behavior of the cancer cells, several morphometric parameters were applied, including *velocity* (cancer cells migrate to neuronal structures), the *Euclidean distance*, and *forward migration index* (FMI).

3.2.4 DRG neurons supernatant treatment

After collection, the DRG neurons were seeded in 24-well plates precoated with 4 µg /mm^2 poly-D-lysine hydrobromide, supplied with 500 µL neurobasal medium supplemented with 100U/mL penicillin and 100 µg/mL streptomycin, 2% B-27, 0.5mM L-Glutamine and 10% FCS. The medium was changed to serum-free medium 24 hours later, and the supernatant was collected after an additional 48 hours and kept at -80°C. SU.86.86 and T3M4 pancreatic cancer cells were seeded in 6-well-plate and serum-starved overnight and then treated with control medium or DRG neurons supernatant for 15 or 25 minutes.

3.2.5 Western blotting

Cells were washed with PBS for 3 times and total protein were extracted with lysis buffer which contain protease inhibitor and phosphatase inhibitor. Protein concentration was measured by BCA protein assay kit. After measurement, 20 μg of protein was diluted into same volume and boiled for 5 minutes, and cooled on ice. Proteins were electrophoretically fractionated in 10% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. After blocked with 5% BSA, the membrane was incubated with primary antibody at 4℃ overnight. After incubation, the membrane was washed with washing buffer for 3 times and incubated with ECL IgG horsereddish peroxidase.coupled secondary antibody for 1 hour at room temperature. Afterwards, antibody detection was performed with ECL Plus Western Blotting substrate. After scanning of the films, the density of the target bands was measured by ImageJ.

3.2.6 Immunohistochemistry, Immunofluorescence

3 μm sections were prepared from paraffin-embedded tissues. After deparaffinization in Roticlear® and rehydrated in ethanol, the sections were boiled with citrate buffer for the retrieval of the antigen for 15 minutes and blocked with normal goat serum to exclude the non-specific binding for 1 hour at room temperature. Then, the sections were incubated with the primary antibody at 4℃ overnight. After incubation, the sections were washed with washing buffer for 3 times and incubated with Dako Envision+ system-HRP secondary antibody for 1 hour at room temperature. Then, Dako Liquid DAB+ Substrate Chromogen System was used for the color reaction. Afterwards, the sections were then dehydrated with ethanol and Roticlear® successively.

For immunofluorescence, after incubation with the primary antibody and wash with washing buffer, the sections were incubated with Alexa Fluor goat IgG secondary antibody.

3.2.7 Immunocytochemistry

For immunocytochemistry, the cultured cells were fixed with 4% formaldehyde for 15 minutes and blocked with normal goat serum for 1 hour at room temperature. After incubation with primary antibody overnight, the cells were washed and incubated with Alexa Fluor goat IgG secondary antibody and Phalloidin-TRITC for 1 hour at room temperature. After washing again with washing buffer, the slides were mounted with Fluorescence Mounting Medium.

3.2.8 ELISA

10_5 SU.86.86/T3M4 pancreatic cancer cells or/and DRG neurons were seeded in 2 cm dish and cultured with the completed medium in 37℃, 5% CO_2. After 24 hours incubation, 4 mL serum-free medium was added to control group, cancer cell mono-culture group, DRG neuron mono-culture, co-culture group, and 2 mL serum-free medium was added to the mixed groups (cancer cells supernatant mixed with DRG neurons supernatant after cultivation). After 48 hours cultivation, the supernatant was collected and kept at -80°C. The concentrations of each cytokine, including MCP-1/CCL2, IGF-1, VEGF, EGF, PDGF, HGF, SCF and NGF, in each group were measured by sandwich ELISA assay.

100 µl of standard and samples from each group were added into appropriate wells and incubated with gentle shaking at 4°C overnight. After incubation, the solution in appropriate wells was discarded carefully. After washing with wash solution, 100 µl of prepared detection antibody was added into each well and incubated for 1 hour at room temperature with gentle shaking. After washing, 100 µl of Streptavidin solution was added into each well and incubated

for 45 minutes at room temperature with gentle shaking. After washing again, 100 µl of TMB One-Step Substrate Reagent was added into each well and incubated for 30 minutes at room temperature with gentle shaking. After incubation, 50 µl of Stop Solution was added into each well. The absorbance was measured at 450 nm immediately.

3.2.9 Statistical analysis

Statistical analysis was performed by the GraphPad Prism 8 Software. The data was presented with mean± SEM from three independent experiments. *T*-test was used for determining statistically significant and an effect was considered statistically significant at a *p* value ≤ 0.05.

4.0 Results

4.1 Pancreatic cancer cells show affinity to DRG neurons

To evaluate the affinity of pancreatic cancer cells to neurons, we used our recently demonstrated three-dimensional (3D) migration assay, in which the pancreatic cancer cell line SU.86.86 and DRG neurons were suspended in an ECM gel to simulate the in vivo circumstance (Demir et al. 2014). Here, cancer cells were confronted with mouse DRG neurons on one side and empty ECM gel on the other side to excluded the unspecific interaction (*Fig. 1a*).

The affinity of pancreatic cancer cells to DRG neurons is best demonstrated by the differences between the migration front FMI and the back front FMI. Comparative quantification of the neuron-directed cancer cell migration showed that the cancer cells revealed a highly targeted migration toward mouse DRG neurons at the migration front, which obviously surpassed the FMI values in the back front (0.32 versus 0.16) (*Fig. 1b*).

When the cancer cells in the migration front were compared with that in the back front, it is apparent that pancreatic cancer cells migrated much faster toward DRG neurons, as evidenced by their higher velocity values to DRGs (0.09 μm/min versus 0.05 μm/min) (*Fig. 1c*).

Comparing the euclidean distance between two groups, neuron facing cancer cells in the migration-front covered prominently longer distance than those in back front (42.21μm versus 24.57μm) (*Fig. 1d*). These results indicated that pancreatic cancer cells have strong affinity to neurons.

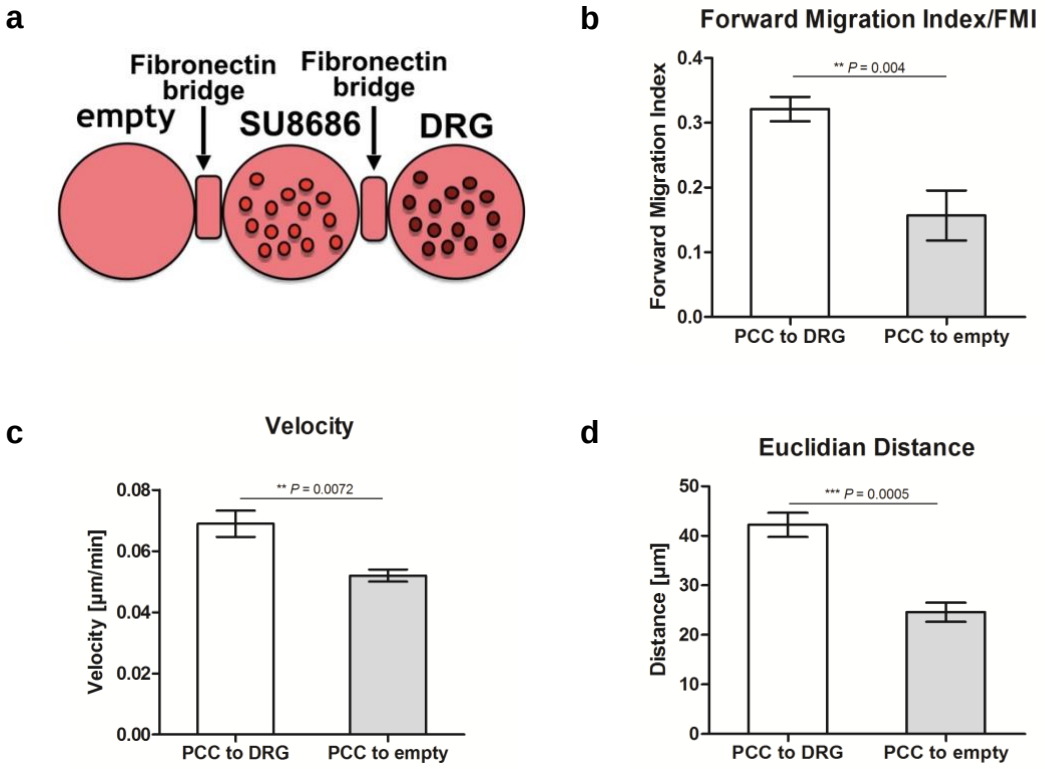


Figure 1. Chemoattraction of DRG to pancreatic cancer cells. (a) Schematic illustration of 3D-migration-assay. (b, c, d) Quantification of pancreatic cancer cell FMI, velocity and Euclidian distance toward DRG in comparison to the control extracellular matrix gel suspension. The results showed that the cancer cells' FMI, velocity and Euclidian distance in the migration front were significantly increased. All data are expressed as mean ± SEM from triplicate independent experiments. *P* value was determined by unpaired *t*-test.

4.2 Pancreatic cancer cells exhibit a more "polygonal shape" during neural invasion.

The reorganization of the cytoskeleton is essential for the acquirement of migratory and invasive properties of pancreatic cancer cell. To better understand the alteration of the cytoskeleton in cancer cells during the neural invasion, cancer cells were immunostained with Phalloidin-TRITC and DAPI. Here, we demonstrated, that in the migration front, the number of the migrating cells was dramatically increased *(Fig. 2a)*.

Interestingly, the neuron-facing cancer cells in the migration front acquired a typical morphological change in their cytoskeleton. In particular, phalloidin staining showed that pancreatic cancer cells without the stimulation of neurons revealed a relatively round shape, whereas cells migrating towards DRG exhibited a more “polygonal shape” and a rather spindle-like morphology with a consequent increase of cell volume *(Fig. 2b)*. In addition, the actin filaments in the neuron-facing pancreatic cancer cell seemed to align along the macroaxis of the cancer cells.

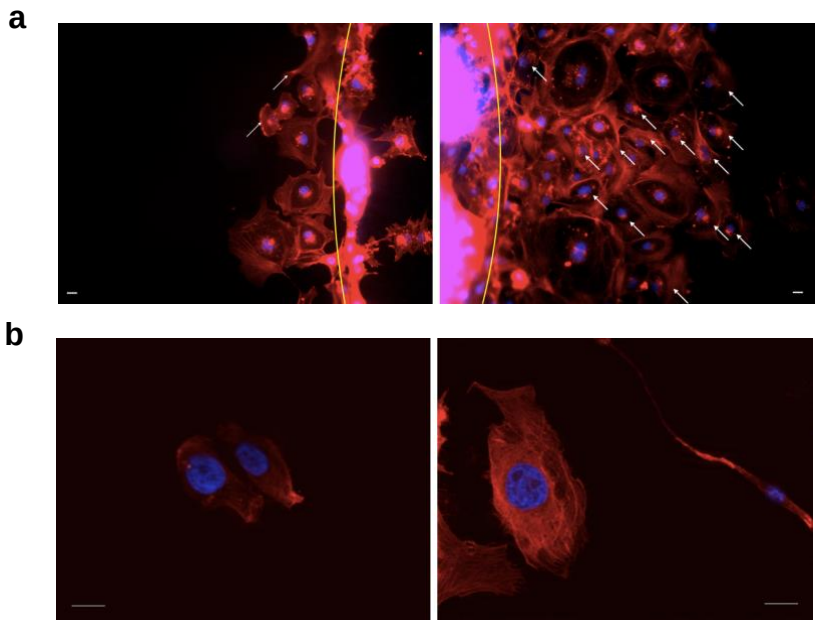
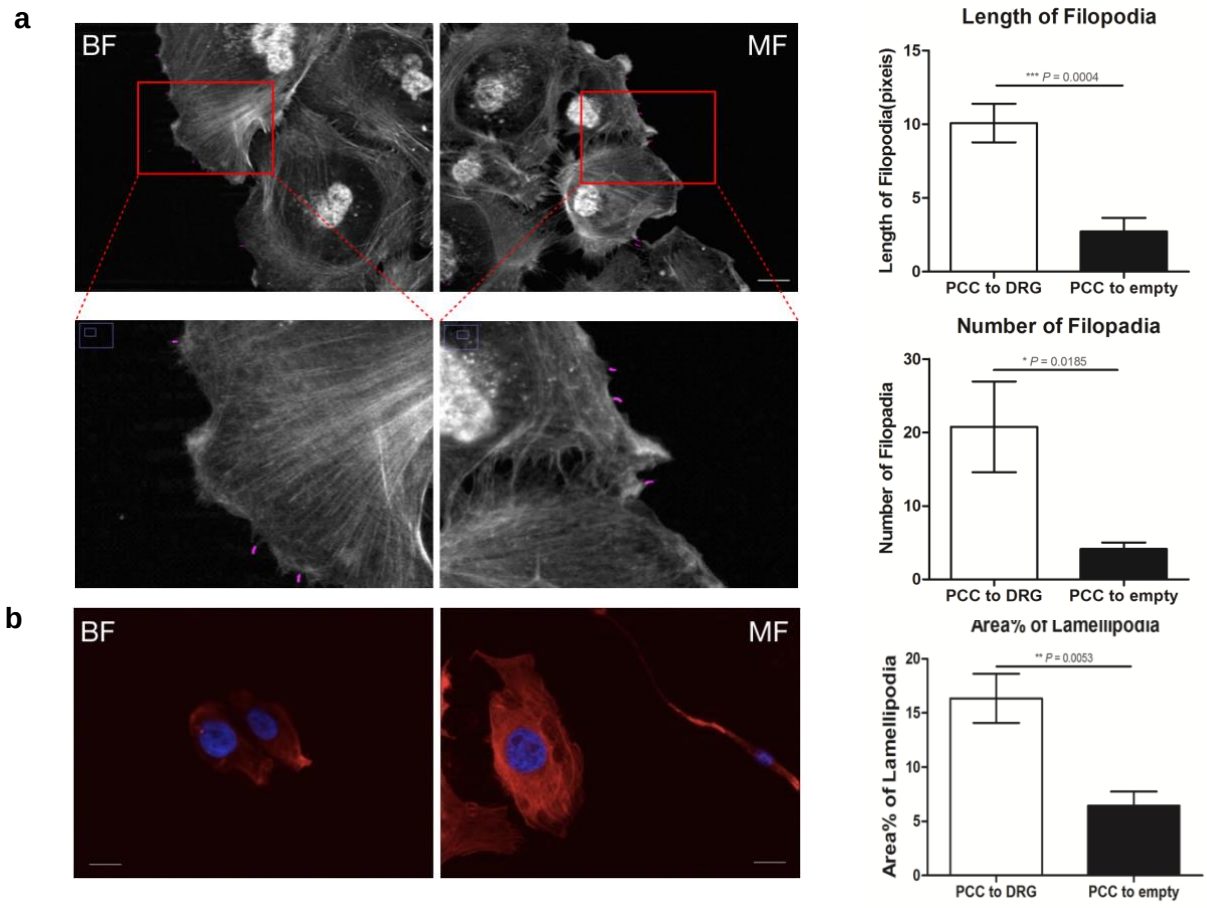


Figure 2. The alterations of cytoskeleton during the neural invasion. (a) Pancreatic cancer cells were simultaneously confronted with DRG neurons on one side and ECM gel on the other side in a 3D-migration assay. More migrating cells (white arrows) were observed in migration-front in （right） comparison to back-front (left). (b) Cell cytoskeleton changes of cancer cells. The neuron-facing tumor cells acquired a typical morphological change of their cytoskeleton. Cancer cells (images to the right) migrating towards DRG exhibited a more “polygonal shape” with a consequent increase of their cell volume. (Scale bar: 20μm).

4.3 The formation of migratory protrusions is significantly increased during the neural invasion of pancreatic cancer cells.

Next, to further investigate the impact of cytoskeletal reorganization on the neural invasion, we determined the formation of migratory protrusions in nerve-infiltrating pancreatic cancer cells. For this purpose, we applied Apotome-supported digital tome-lapse microscopy after immunostaining in the 3D migration assay. Further on, the density and the length of cancer cell filopodia were quantified with the FiloQuant® software (Jacquemet et al. 2017). Intriguingly, cancer cells in the migration front exhibited a prominently increase in the density and length of filopodia (*Fig. 3a*). Additionally, the number and the area of the lamellipodia was also increased in the neuron-facing cancer cells (*Fig. 3b*). These findings confirmed the reorganization of the cytoskeleton and formation of microfilaments specifically in neuron-targeting cancer cells.



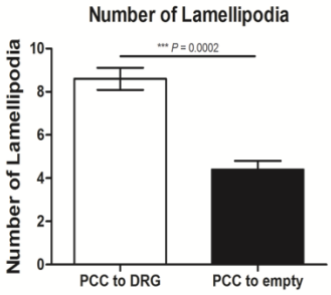
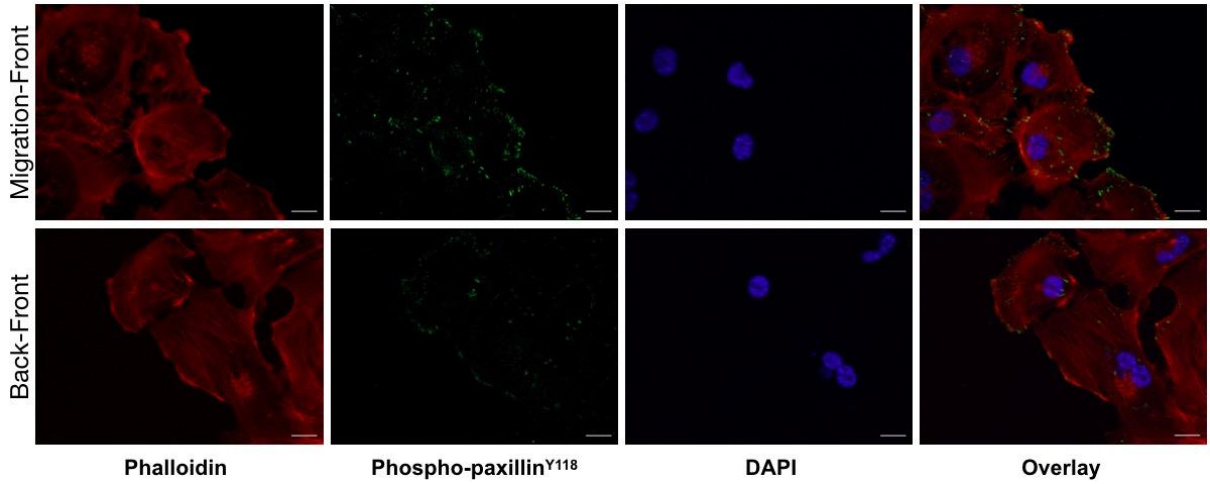


Figure 3. The formation of filopodia and lamellipodia during the neural invasion. (A) After 3D-migration assay, the density and length of cancer cell filopodia were quantified with the FiloQuant® software. Filopodia density was quantified and compared between cells. The density and length of filopodia were increased in the migration front (MF). (B) The number and the area of lamellipodia was also increased in the migration front (white arrows). Data are expressed as mean ± SEM from triplicate independent experiments. *P* value was determined by unpaired *t*-test. (Scale bar: 20μm).

4.4 Pancreatic cancer cells specifically amplify phospho-paxillin in their lamellipodia during neural invasion.

One of the major aims of this study was to investigate the impact of cytoskeleton-related proteins on neural invasion in pancreatic cancer. Paxillin activation is strongly associated with the cytoskeletal reorganization. For this purpose, the expression of phospho-paxillin was determined by double immunolabeling with Phalloidin-TRITC and phospho-paxillin after 3D migration assay. The phospho-paxillin positive dots were counted and compared between the migration front and back front.

Here, phospho-paxillin-positive dots were seen to be abundant in the neuron-facing pancreatic cancer cells, as opposed to the cells in the back front. By comparison with cancer cells in the back front, there was an evident increase in the amount of phospho-paxillin-positive dots in the neuron-facing cells, demonstrating that DRG neurons could stimulate the phosphorylation of paxillin *(Fig. 4)*.



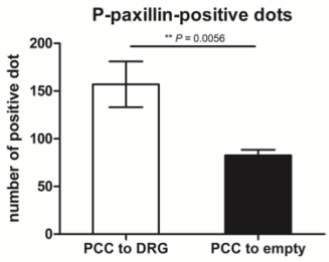


Figure 4. Phospho-paxillin expression in 3D migration. Pancreatic cancer cells in migration-front (upper panel) and back-front (lower panel) were immunofluorescently stained with phalloidin and phospho-paxillin after 3D-migration assay. The filopodia exhibited a specific enrichment of phospho-paxillin in the migration front, when compared to the back front. Data are expressed as mean ± SEM from triplicate independent experiments. *P* value was determined by unpaired *t*-test. (Scale bar: 20µm).

4.5 The expression of phospho-paxillin is upregulated in PCC after treatment with DRGs supernatants

The up-regulation of phospho-paxillin in the neuron-facing cells in 3D migration assay prompted us to further assess the role of DRG neurons in neural invasion. For this purpose, SU.86.86 and T3M4 human pancreatic cancer cells were treated with DRG neurons supernatants for different time intervals. The amount of phospho-paxillin was measured by western blotting. DRG neurons were supplied with serum-free medium and the supernatant was collected after 48 hours. Then, cancer cells were treated with DRG neurons supernatant or control medium for 15 or 25 minutes. Western blotting results revealed that the phosphorylation of paxillin was amplified after treatment with DRG neuron supernatants *(Fig.*

5). Hence, it was conceivable that DRG neurons can secrete cytokines which can induce the phosphorylation of paxillin.

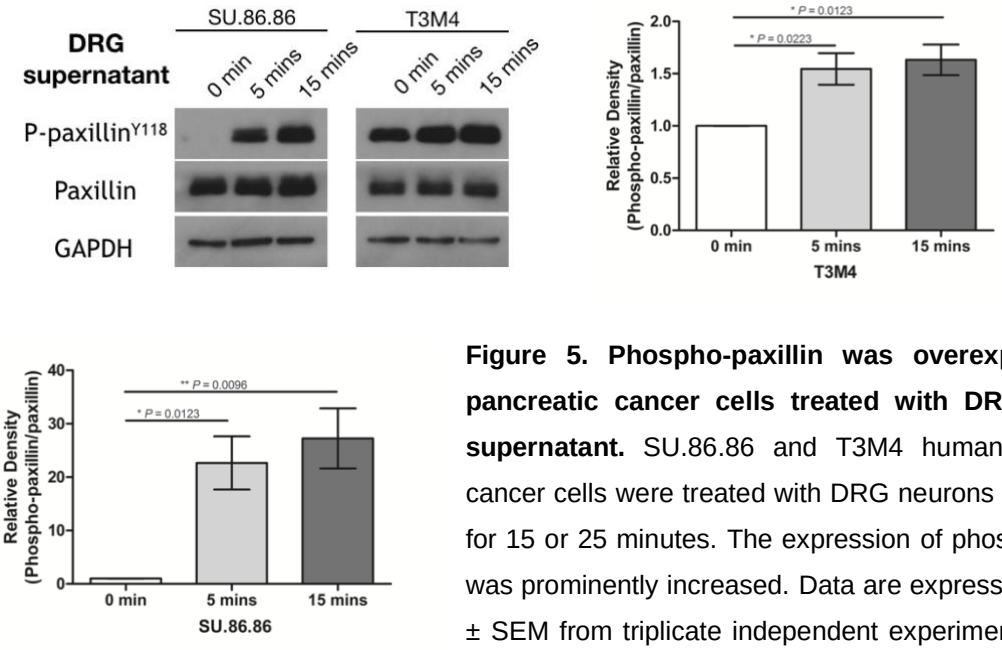


Figure 5. Phospho-paxillin was overexpressed in pancreatic cancer cells treated with DRG neurons supernatant. SU.86.86 and T3M4 human pancreatic cancer cells were treated with DRG neurons supernatant for 15 or 25 minutes. The expression of phospho-paxillin was prominently increased. Data are expressed as mean ± SEM from triplicate independent experiments. *P* value was determined by unpaired *t*-test.

4.6 The expression of phospho-paxillin downstream targets in pancreatic cancer cell lines is prominently up-regulated

In the next step, to further investigate the potential role of paxillin phosphorylation in pancreatic cancer cells, we determined the expression of phospho-paxillin, phospho-FAK, phospho-Src and phospho-ERK1/2 content of human PDAC cell lines.

We detected that phospho-paxillin, phospho-FAK, phospho-Src and phospho-ERK1/2 and phospho-FAK proteins were elevated in all different PDAC cell lines, including AsPC-1 (AsP), BxPC-3 (BxP), Capan-1 (Cap), Colo-357 (Col), MIA PaCa-2 (MIA), Panc-1 (Pan), SU.86.86 (SU) and T3M4 (T3), which exhibited much higher levels that the immortalized pancreatic epithelial cell line HPDE (HP) *(Fig. 6)*. These findings imply that the expression of phospo-paxillin, phospho-Src and phospho-FAK is abnormally up-regulated in cancer cells.

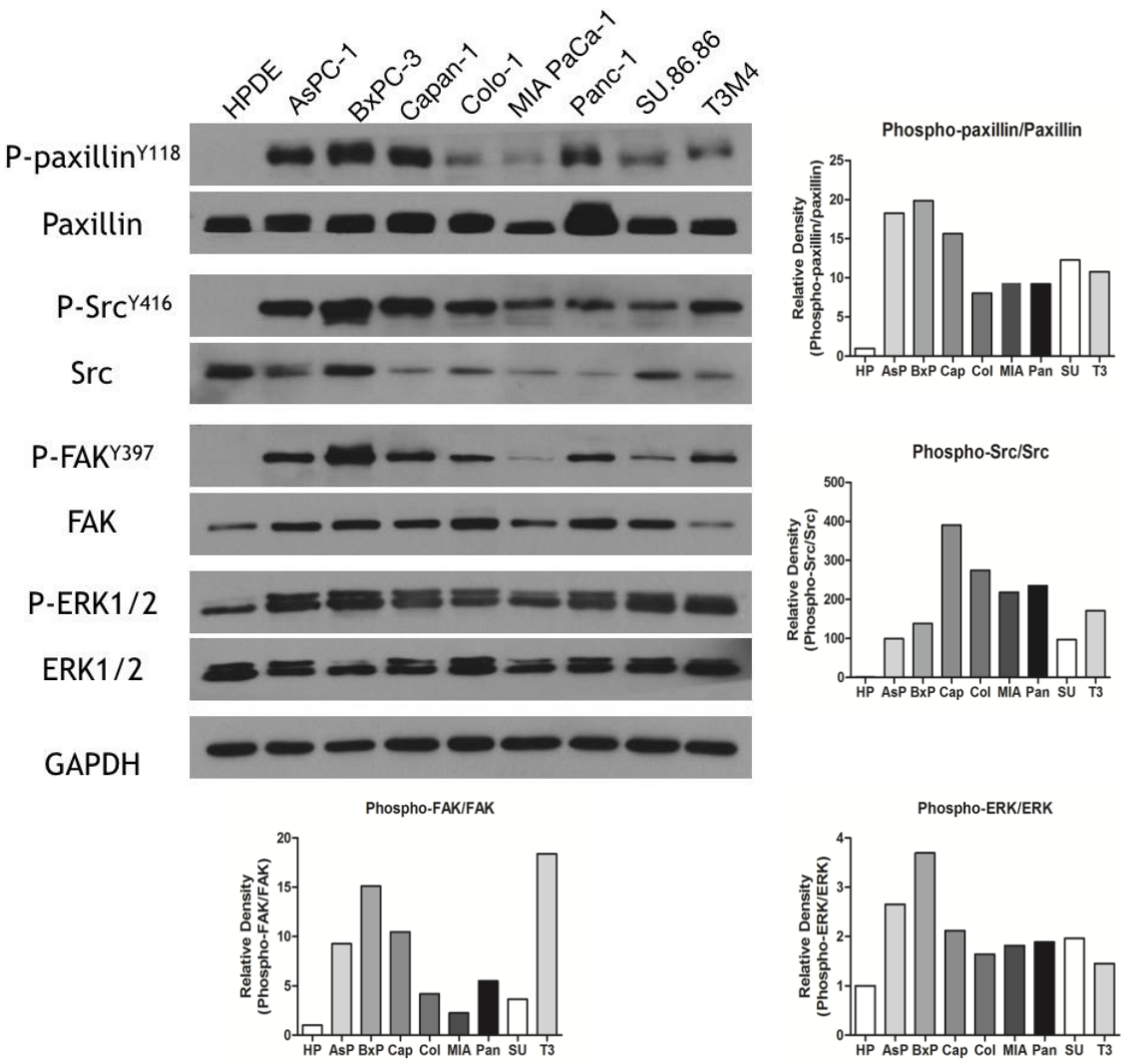


Figure 6. The expression of phospho-paxillin, phospho-FAK, phospho-Src and phospho-ERK in different cell lines was prominently up-regulated. The expressions of phospho-paxillin, phospho-FAK, phospho-Src and phospho-ERK in different pancreatic cancer cell lines were increased in comparison to HPDE.

4.7 Paxillin is increasingly phosphorylated in cancer cells around nerves in the cancer tissue.

Because of the strong affinity of cancer cells to DRG neurons, we next investigated the expression of phospho-paxillin around neurons in pancreatic cancer tissues using two consecutive 3-um-thick section obtained from PDAC patients. The first slide was

immunostained against anti-PGP 9.5/anti-Cytokeratin Pan for locating the neural invasion regions. The second slide was immunostained against anti-Phospho-paxillin/anti-Cytokeratin Pan for detecting the expression of phospho-paxillin. The phospho-paxillin content of cancer cells around nerves was compared to cancer cells that were in no contact with nerves. In accordance with the in vitro results, we frequently detected a relatively stronger presence of phospho-paxillin in cancer cells invading nerves *(Fig. 7a)*.

The genetically engineered mouse models (GEMMs) provided a unique opportunity to investigate the characteristic features of the tumor and improved our understanding of neural invasion. $Pdx1_{cre/+}$;LSL-KrasG12D/+;$Tp53_{fl/+}$ (KPC) mouse tumor model was reported to harbor neural invasion (Na'ara et al. 2018). Consistent with our findings in human PDAC tissue, the phospho-paxillin content was significantly increased in cancer cells that were in the vicinity of nerves, when compared to cancer cells away from nerves *(Fig. 7b)*

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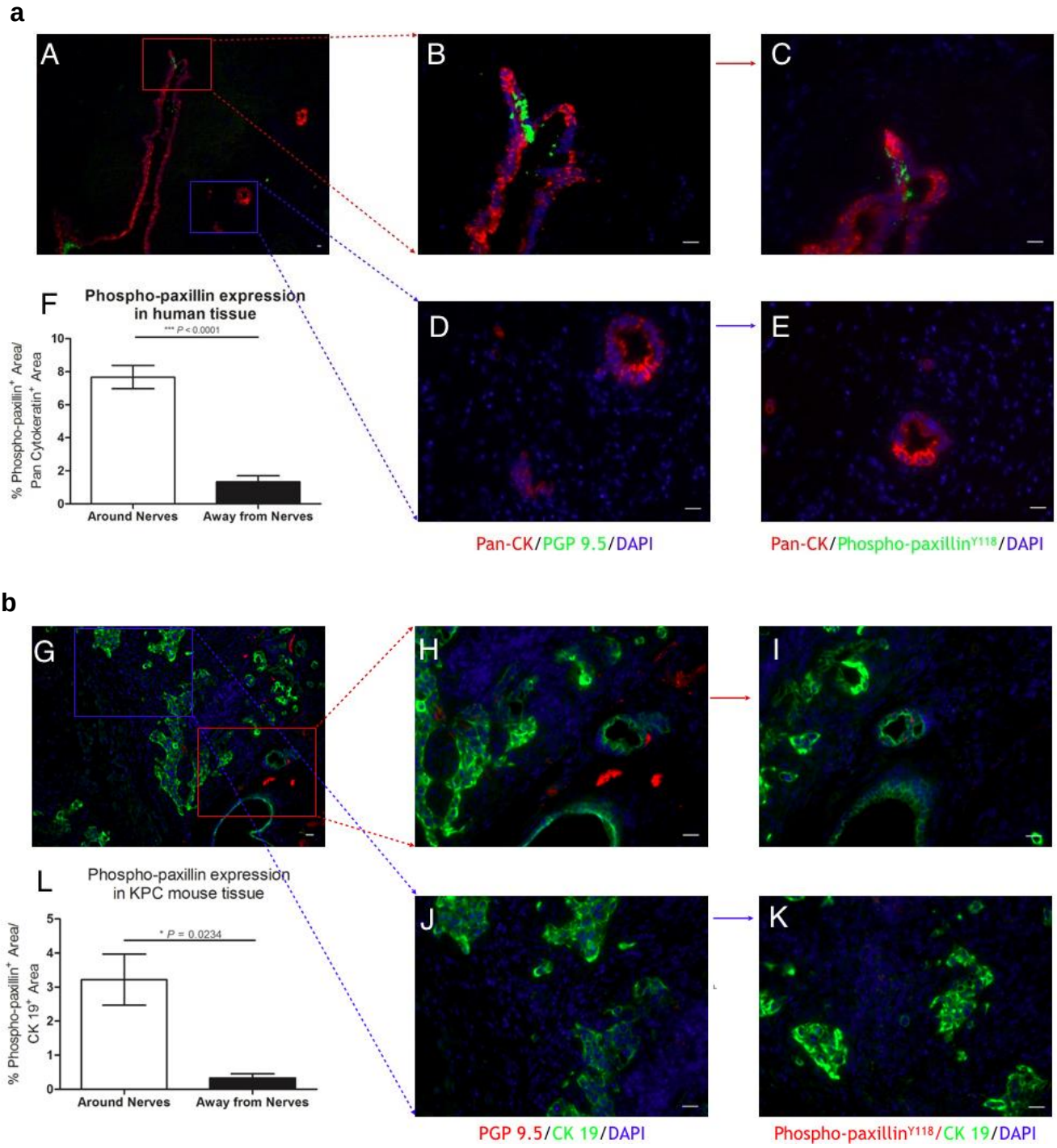


Figure 7. Phospho-paxillin is also prominently expressed around nerves in the primary PDAC tissue. Two consecutive 3-um-thick sections obtained from PDAC patients (A) or KPC mice (B) were immunostained against cancer cell marker pan-cytokeratin (A, red), or against phospho-paxillin (A, green), or against the pan-neural marker PGP9.5 (B, red). The phospho-paxillin content of cancer cells around nerves was compared to cancer cells that were in no contact with nerves. The expression phospho-paxillin of was increased in tumor cells around nerves when compared to tumor cells away from nerves. Data are expressed as mean ± SEM from triplicate independent experiments. *P* value was determined by *t*-test. (Scale bar: 20μm).

4.8 MCP-1 is prominently enriched in the co-culture supernatants of cancer cell and DRG neurons

The up-regulated expression of phospho-paxillin in the pancreatic cancer cells treated with DRGs supernatant motivated us to further investigate the stimuli for paxillin phosphorylation during neural invasion. The phosphorylation of paxillin can be triggered by a series of cytokines, including Monocyte chemoattractant protein 1 (MCP-1, also known as CCL2) (Yamasaki et al. 2001), insulin-like growth factor I (IGF-I) (Butler et al. 1997), vascular endothelial growth factor (VEGF) (Abedi et al. 1997), epidermal growth factor (EGF) (Tapia et al. 1999), platelet-derived growth factor (PDGF) (Rankin et al. 1996), hepatocyte growth factor (HGF) (Herrera 1998), stem cell factor (SCF) (Scott et al. 1996), and nerve growth factor (NGF) (Melamed et al. 1995). To investigate the roles of these cytokines in neural invasion, DRG neurons or cancer cells were seeded either alone or co-culture with each other and the levels of these cytokines in the supernatants were assayed by sandwich ELISA. 5 groups were measured, including control group, cancer cell monoculture group, DRG neuron monoculture group, co-culture group and mixed group. In the mixed group, cancer cell supernatant was mixed with DRG neuron supernatant after cultivation to compare the alteration in a more intuitively way. Intriguingly, when SU.86.86 cancer cells were monocultured, we did not observe any significant variation in the MCP-1/CCL2 secretion compared to control group *(Fig. 8a)*. Meanwhile, when T3M4 cancer cells were monocultured, the MCP-1/CCL2 levels in the supernatant was slightly increased *(Fig. 8a)*. Specifically, when DRG neurons were monocultured, the secretion was mildly increased *(Fig. 8a, 8b)*. However, the secretion level of MCP-1/CCL2 was dramatically increased when DRG neurons were co-cultured together with either SU.86.86 or T3M4 PDAC cancer cells *(Fig. 8a, 8b)*, which implied that MCP-1/CCL2 plays a critical role in neural invasion.

Furthermore, the expression of VEGF *(Fig. 8b)* and PDGF *(Fig. 8c)* was apparently enhanced when DRG neurons or cancer cells monocultured, whereas the expression level was not observed to significantly change in the co-culture situation. On the other hand, as for IGF *(Fig. 8e)*, EGF *(Fig. 8f)*, and NGF *(Fig. 8g)*, there was no obvious alteration between the control group and monoculture group. Besides, compared to monoculture, co-culture of DRG neurons and cancer cells did also not influence the expression of IGF, EGF, and NGF. Concerning SCF *(Fig. 8d)*. In fact, although the expression was increased in the cancer cell supernatant, the expression was attenuated when co-cultured with DRG neurons. As for HGF *(Fig. 8h)*, neither DRG neurons nor cancer cells altered the expression of HGF. Moreover, the expression was even hampered in the co-culture condition.

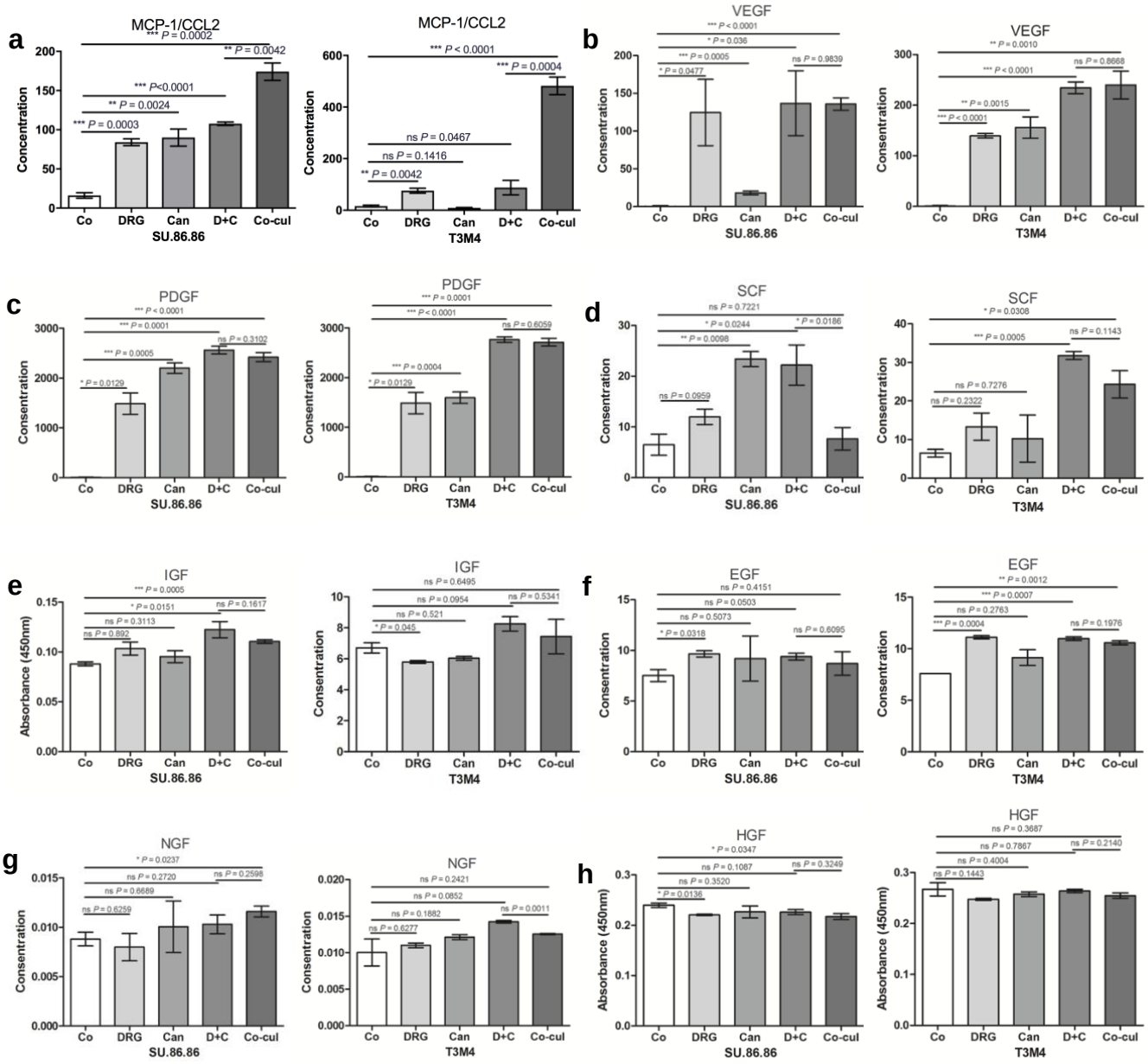


Figure 8. MCP-1 is dramatically elevated in the co-culture of cancer cell and DRG neurons supernatant. (a) The expression level of MCP-1 was dramatically increased when DRG neurons were mixed together with cancer cells. (b, c) The expression of VEGF and PDGF was enhanced when DRG neurons or cancer cells monocultured, whereas the expression level was not significantly changed in the co-culture situation. (d) The expression of SCF was slightly attenuated in the co-culture group. (e, f, g) The secretion of IGF, EGF and NGF showed no obvious alteration between control group and monoculture group. Besides, compared to monoculture, co-culture of DRG neurons and cancer cells did not influence their expression. (h) Neither DRG neurons nor cancer cells altered the expression of HGF. Data are expressed as mean ± SEM from triplicate independent experiments. *P* value was determined by *t*-test. (Co: control supernatant. DRG: DRG supernatant. Can: Cancer supernatant. D+C: DRG supernatant + cancer supernatant. Co-cul: Co-culture supernatant.)

4.9 The expression of MCP-1 is significantly upregulated in neural invasion in pancreatic cancer patients

To find out the potential role of MCP-1/CCL2 during neural invasion, we investigated the expression of MCP-1/CCL2 in human pancreatic cancer tissue by immunohistochemistry. Two consecutive 3-um-thick section were used. The first slide was immunostained with anti-S100 for labeling the nerves and the second slide was immunostained with anti-MCP-1. Nerves were separated into NI group and non-NI group depending on the presence of the NI. The immunoreactivity of MCP-1 differed considerably between NI group and non-NI group. Figure 9 shows that the expression of MCP-1 in non-NI group was quite low in nerves and cancer cells, whereas in the NI group, the expression was prominently up-regulated. Interestingly, in the NI-group, the expression of MCP-1 in pancreatic cancer cells was also increased compared with non-NI group.

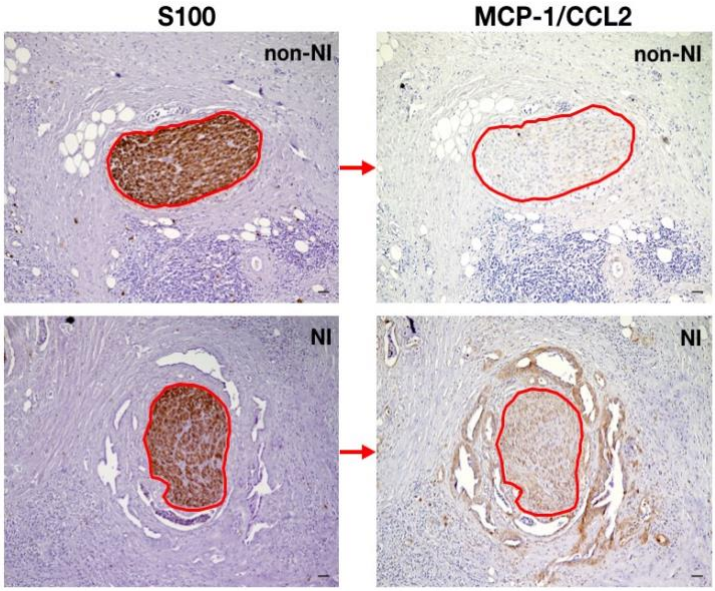


Figure 9. The expression of MCP-1 was significantly overexpressed at sites of neural invasion in pancreatic cancer. The expression of MCP-1 in non-NI group was quite low, whereas in the NI group, the expression was prominently up-regulated. (Scale bar: 50μm)

4.10 MCP-1/CCL2 induces the phosphorylation of paxillin and enhances the neuron-directed migration ability in vitro

Due to the important role of MCP-1/CCL2 in promoting neural invasion in pancreatic cancer cells, we sought to evaluate if MCP-1/CCL2 can stimulate paxillin phosphorylation. Strikingly, figure 10a shows that 100 ng MCP-1/CCL2 in the SU.86.86 cancer cell supernatant significantly enhanced phosphorylation of paxillin at 5, 15 and 25 minutes by 172%, 186%, and 150%, respectively. Similarly, 100 ng MCP-1/CCL2 addition to T3M4 cancer cells also increased the phosphorylation of paxillin by 130%, 181%, and 133%, respectively. Subsequently, cancer cells were lysed15 minutes after the treatment of MCP-1/CCL2. Figure 10b shows that 15 minutes after addition of 0 ng, 10 ng, 50 ng and 100ng MCP-1/CCL2 to SU.86.86 and T3M4 pancreatic cancer cell, phosphorylation of paxillin was significantly increased in a dose-dependent manner.

In additional 3D migration assays, DRG neurons were simultaneously confronted with cancer cells treated with vehicle and with cancer cells treated with MCP-1/CCL2 protein or the CCR4 antagonist C 021 dihydrochloride *(Fig 10c)*. After 48 hours incubation, the neuron-targeted migration of cancer cells was recorded via digital time-lapse microscopy. Here, we observed significantly amplified neuron-directed migration ability of pancreatic cancer cells upon treatment with MCP-1CCL/2, as demonstrated by the increase in their FMI (0.33 versus 0.25) *(Fig 10d)*. Moreover, the migration of stimulated pancreatic cancer cells was characterized by a rather longer Euclidian distance (46.71 µm versus 41.61 µm) and faster velocity (0.10 µm/min versus 0.08 µm/min) than those of vehicle-treated cancer cells *(Fig 10d)*.

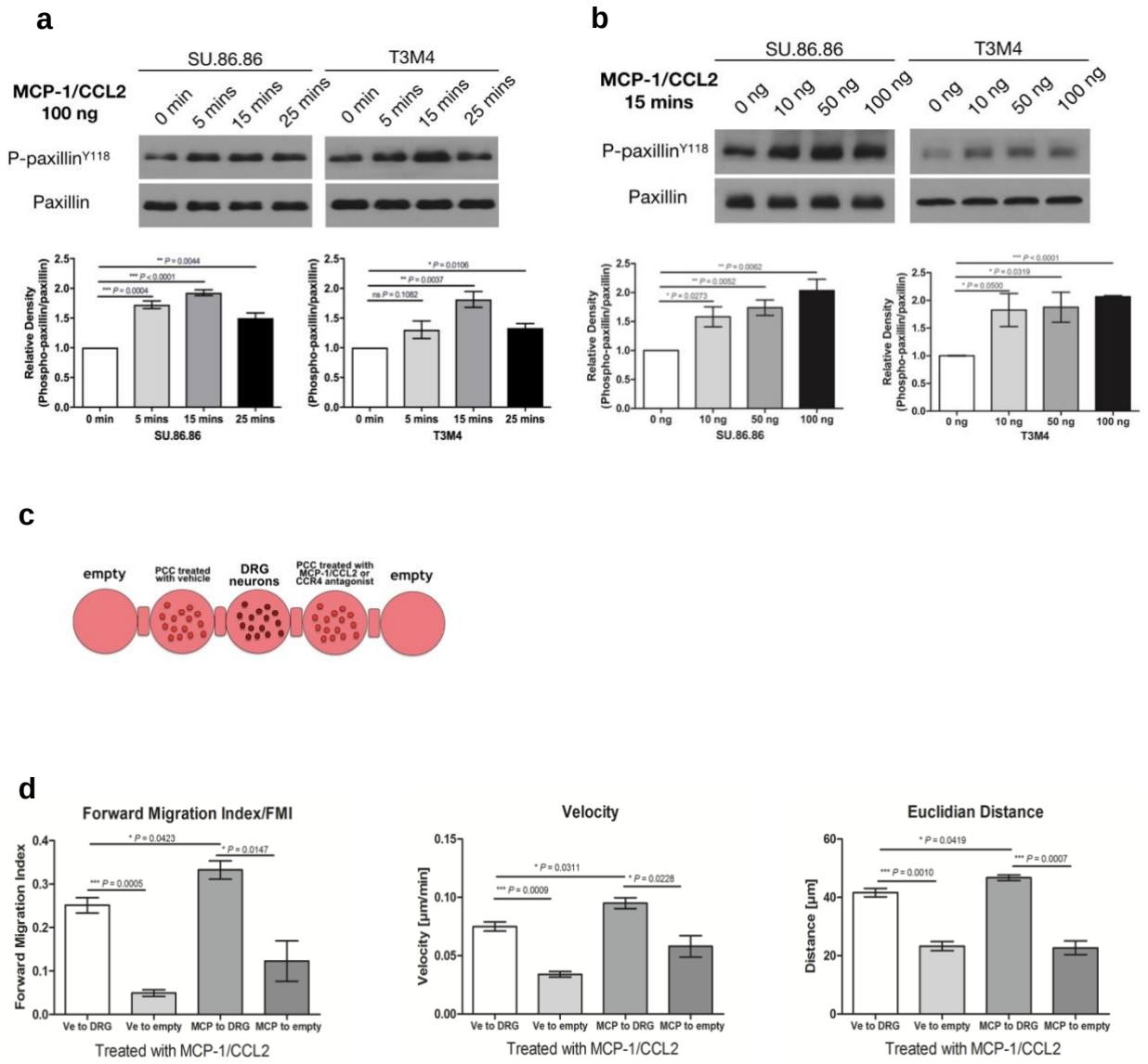


Figure 10. MCP-1/CCL2 induces the phosphorylation of paxillin and enhances the neuron-directed migration ability in vitro. (a) When SU.86.86 and T3M4 pancreatic cancer cells were treated with 100 ng MCP-1/CCL2 for different times, the expressions of paxillin were significantly enhanced. (b) 15 minutes after addition of 0 ng, 10 ng, 50 ng and 100ng MCP-1/CCL2 to SU.86.86 and T3M4 pancreatic cancer cell culture medium, phosphorylation of paxillin was significantly increased in a dose-dependent manner. (c) Schematic illustration of 3D migration assay. (d) The FMI, velocity and Euclidian distance of pancreatic cancer cells treated with MCP-1 were prominently greater than that of pancreatic cancer cells treated with vehicle. Data are expressed as mean ± SEM from triplicate independent experiments. *P* value was determined by *t*-test.

4.11 CCR4 antagonist reduced the phosphorylation of paxillin and diminished the neuron-directed migration ability of cancer cells in vitro

To further ascertain the potential role of phospho-paxillin in cancer cell migration to neurons in more detail, SU.86.86 and T3M4 pancreatic cancer cells were treated with the antagonist of MCP-1/CCL2 receptor CCR4 (C 021 dihydrochloride) and receptor CCR2 (RS 504393). The amount of phospho-paxillin was identified by western blotting. After treatment with the RS 504393, we did not observed alteration in the expression of phospho-paxiilin (*Fig. 11a*). However, figure 11b shows that treatment with C 021 led to a dose-dependent attenuation of paxillin phosphorylation. Quantification of the band density suggested that 0.14 μM C 021 dihydrochloride significantly lowered the phosphorylation of paxillin at 5, 15, and 25 minutes in both SU.86.86 and T3M4 cells.

Meanwhile, when CCR4 was blocked by the specific antagonist, decreased migration ability towards DRG neurons was observed. As shown in figure 11c, the FMI value of C 021-treated cancer cells was diminished in comparison to the vehicle-treated cancer cells (0.17 versus 0.24). In line with this, after treatment of C 021 dihydrochloride, the velocity of pancreatic cancer cells toward DRG neurons was prominently decelerated (0.05 μm/min versus 0.07 μm/min) and the Euclidian distance was also reduced (31.03 μm versus 40.12 μm), implying an essential role for the MCP-1/CCL2-CCR4 cytokine-cytokine receptor in the neural invasion in pancreatic cancer.

4.12 Phosphorylation of paxillin mediates cancer cells migration to neurons via Src/paxillin/ERK signaling pathway

ERK1/2 plays an essential role in promoting migration ability in cancer cells, including breast cancer (Wang et al. 2018), neuroblastoma (Yeh et al. 2016), gastric cancer (Hou et al. 2016), thyroid cancer (Ren et al. 2017), as well as pancreatic cancer (Ono et al. 2014). For

this purpose, we sought to investigate whether paxillin mediates ERK1/2 phosphorylation during neural invasion. SU.86.86 and T3M4 pancreatic cancer cells were treated with MCP-1/CCL2 or the CCR4 antagonist, and the expressions of phospho-Src and phospho-ERK1/2 were checked via western blotting. Here, we detected that the protein levels of phospho-Src and phospho-ERK1/2 were both up-regulated when treated with MCP-1/CCL2 *(Fig. 12a)*, whereas the amounts were down-regulated after the treatment of CCR4 antagonist *(Fig. 12b)*. Meanwhile, consistent with MCP-1/CCL2, after treatment with DRG supernatant, we observed up-regulation of phospho-Src and phospho-ERK1/2 in both SU.86.86 and T3M4 pancreatic cancer cells *(Fig. 12c)*. Our findings illuminated that Src and ERK1/2 phosphorylation is closely linked to migration in pancreatic cancer cells. However, the expression of phospho-FAK did not change after treatment of MCP-1/CCL2 or with CCR4 antagonist *(Fig. 12d)*.

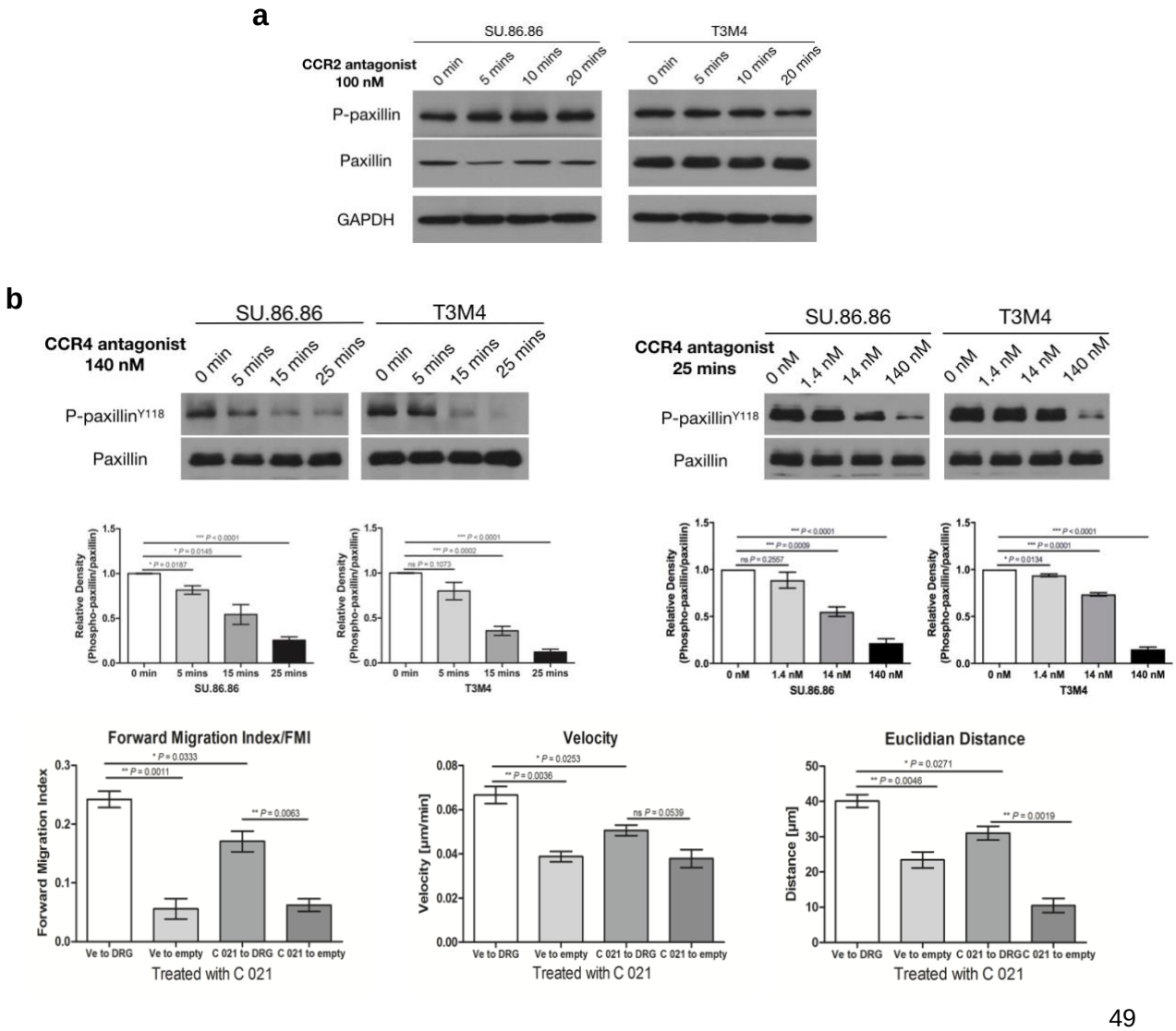
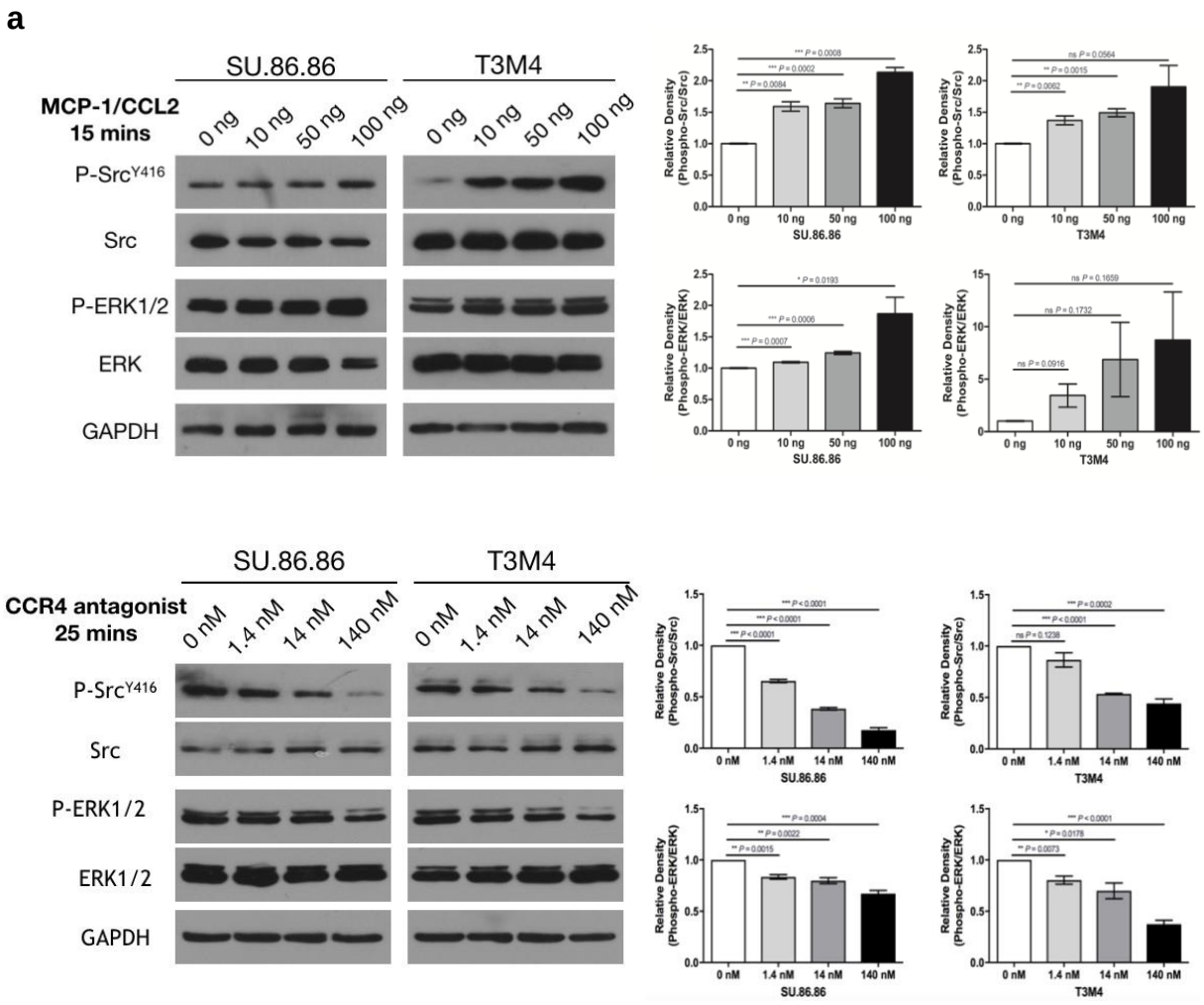


Figure 11. CCR4 antagonist reduced the phosphorylation of paxillin and discourages the neuron-directed migration ability in vitro. (a) after the treatment of the CCR4 antagonist C 021 dihydrochloride (C 021), the expression of phospho-paxillin in SU.86.86 and T3M4 pancreatic cancer cells were decreased in a dose-dependent manner. (b) The FMI, velocity and Euclidian distance of pancreatic cancer cells treated with C 021 were less than that of pancreatic cancer cells treated with vehicle. Data are expressed as mean ± SEM from triplicate independent experiments. *P* value was determined by *t*-test.



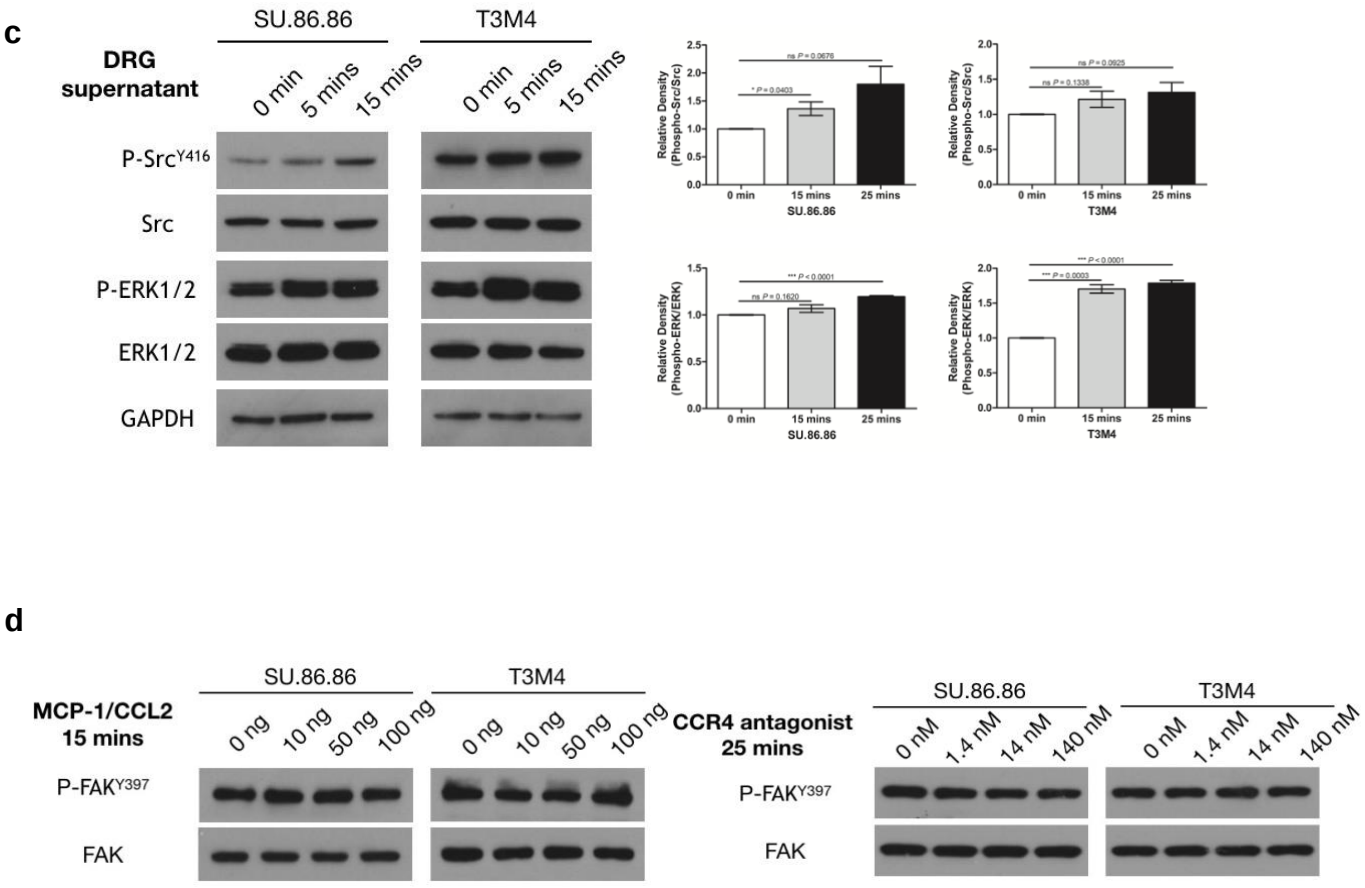


Figure 12. Paxillin mediates cancer cells migration ability via Src/paxillin/ERK signaling pathway. (a) 15 minutes after addition of 0 ng, 10 ng, 50 ng and 100ng MCP-1/CCL2 to SU.86.86 and T3M4 pancreatic cancer cells, phospho-Src and phospho-ERK1/2 were significantly increased. (b) After the treatment with the CCR4 antagonist (C 021 dihydrochloride), the amounts of phospho-Src and phospho-ERK1/2 in SU.86.86 and T3M4 pancreatic cancer cells were decreased. (c) After treatment with DRG supernatants, the phospho-Src and phospho-ERK1/2 expression was prominently enhanced in PDAC cells. (d) The expression of phospho-FAK did not change after treatment with MCP-1/CCL2 or with CCR4 antagonist. Data are expressed as mean ± SEM from triplicate independent experiments. *P* value was determined by *t*-test.

4.13 Dephosphorylation of ERK1/2 decreases the neuron-directed migration ability of pancreatic cancer cells

As we found a close correlation between the phospho-paxillin and phospho-ERK1/2 in mediating the neuron-directed migration of cancer cells, in the next step, we suppressed the phospho-ERK1/2 by the ERK1/2 phosphorylation inhibitor AZD8330. SU.86.86 and T3M4 pancreatic cancer cells were treated with different concentrations of the inhibitor for 1 hour and the expression of phospho-ERK1/2 was subsequently determined by western blotting. The results showed that ERK1/2 phosphorylation inhibitor AZD8330 markedly induced the dephosphorylation of ERK1/2 in both SU.86.86 and T3M4 pancreatic cancer cells. Quantification of the band density revealed that the treatment with AZD8330 for 1 hour decreased the amounts of phospho-ERK1/2 at 0.004 nM and 0.04 nM by 5.3%, 2.5% in SU.86.86 and 2.0%, 1.9% in T3M4 cells, respectively (*Fig. 13*).

In the next step, to further assess the role of phospho-ERK1/2 in the neural invasion of pancreatic cancer cells, the 3D migration assay was utilized. DRG neurons were simultaneously confronted with cancer cells treated with vehicle and with cancer cells treated with 0.04 nM ERK1/2 phosphorylation inhibitor AZD8330. Here, we observed significantly decreased migration ability. As shown in figure 13b, the FMI value of inhibitor-treated pancreatic cancer cells was attenuated in comparison with vehicle-treated cancer cells. In accordance with FMI, the cancer cells also decelerated after the treatment of ERK1/2 inhibitor.

Moreover, we also observed that the inhibitor-treated pancreatic cancer cells covered a shorter Euclidian distance.

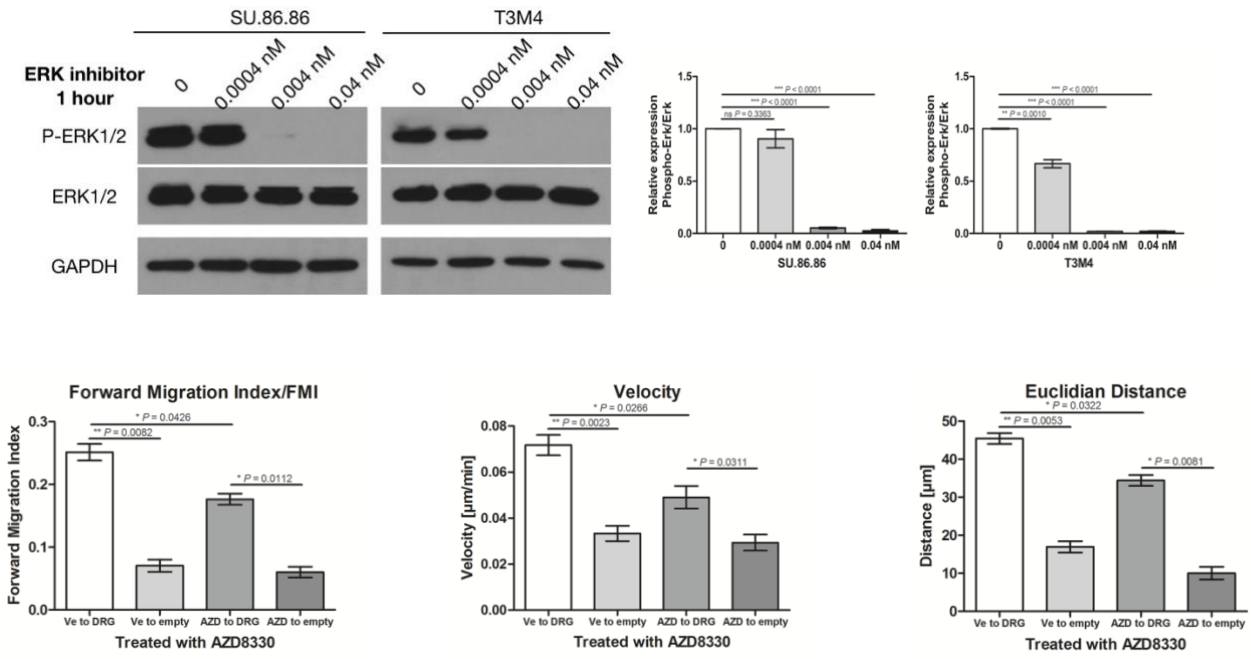


Figure 13. ERK1/2 phosphorylation inhibitor discourages the neuron-directed migration ability in vitro. (a) After treatment with the ERK1/2 phosphorylation inhibitor AZD8330, the expression of phospho-ERK1/2 in SU.86.86 and T3M4 pancreatic cancer cells were decreased in a dose-dependent manner. (b) The FMI, velocity and Euclidian distance of pancreatic cancer cells treated with AZD8330 were less than that of pancreatic cancer cells treated with vehicle. Data are expressed as mean ± SEM from triplicate independent experiments. *P* value was determined by *t*-test.

5.0 Discussion

The present study aimed to illustrate the cytoskeletal changes, and the role of paxillin phosphorylation in neural invasion in pancreatic cancer. In this study, we demonstrate that, during neural invasion, the cellular conformation of pancreatic cancer cells is altered, and the microfilament formation is increased in the cytoskeleton. Moreover, the formation of migratory protrusions, including filopodia and lamellipodia, is markedly increased. More importantly, we also detected that neurons can secrete cytokine MCP-1/CCL2, which amplifies the paxillin phosphorylation in the filopodia via MCP-1/CCR4/Src/ERK pathway. Activation or inhibition of MCP-1/CCL2 by MCP-1/CCL2 protein or CCR4 receptor antagonist could mediate the phosphorylation of paxillin and influence the neuron-directed migration of pancreatic cancer cells. We furthermore demonstrate that the inhibition of ERK1/2 phosphorylation suppresses the neural invasion. Collectively, our findings clarify that paxillin phosphorylation is involved in tumor progression and neural invasion, which makes paxillin a potentially novel important therapeutic target.

Previous studies showed that alterations of the cytoskeleton are continually detected particularly in cancer cells, from which cancer cells acquire specific characteristic features, including invasion, migration, uncontrolled proliferation, adhesion, and death evasion (Sherr 1996, Hanahan et al. 2011). It is also reported that the cytoskeleton is an important target of chemotherapy due to its established role in proliferation and survival (Zhou et al. 2005). Rearrangement of the cytoskeleton contributes to migration and invasion of several solid tumors, including pancreatic cancer (Pandey et al. 2015, Taniuchi et al. 2018, Taniuchi et al. 2018), lung cancer (Zhao et al. 2015), breast cancer (Wicki et al. 2006, Meng et al. 2014), epithelial ovarian cancer (Tang et al. 2015), glioblastoma (Memmel et al. 2017). Alteration of actin structures has been proven to contribute to pancreatic cancer metastasis (Pandey et al.

2015). High expression of WAVE2, a member of the actin cytoskeletal regulatory protein, augmented motility and invasiveness of pancreatic cancer cells by inducing the establishment of cell protrusions. More importantly, over-expression of WAVE2 is positively correlated with impaired prognosis and survival in pancreatic cancer patient (Taniuchi et al. 2018). Similar results were observed in BCL7B, a member of the B-cell CLL/lymphoma 7 families, which can influence the formation of membrane protrusions in pancreatic cancer cells via dephosphorylation of CREB. Overexpression of BCL7B in pancreatic cancer cells resulted in amplified migratory and invasive capacity, and strongly associated with diminished overall survival (Taniuchi et al. 2018). Consistent with pancreatic cancer, in the study by Wicki et al. (Wicki et al. 2006), the investigators also found that rearrangement of the actin cytoskeleton in lung cancer was positively correlated with the formation of filopodia, cancer cells' migratory and invasive ability. In epithelial ovarian cancer cells, the microtubule-associated protein 1 light chain 3B (LC3B) mediated tumor cell migration and invasion by cytoskeletal rearrangement. Down-regulation of light chain 3B by siRNA or specific inhibitor suppressed tumor cell migratory and invasive ability, whereas elevated expression of light chain 3B promoted cell migration and invasion (Tang et al. 2015). These studies revealed the importance of cytoskeletal properties on a variety of cancer cells, as well as on prognosis. Yet the roles of the cytoskeleton in neural invasion still remain unclear.

Consistent with these studies, in our study, we found that during neural invasion of pancreatic cancer cells, the cells altered their cytoskeleton. Pancreatic cancer cells exhibited a more "polygonal shape" with a consequent increase in their cell volume. More importantly, we observed that the formation of migratory protrusions, including filopodia and lamellipodia, was significantly increased, which contributed to the motility of pancreatic cancer cells in neural invasion. Previous studies have also proven that the formation of filopodia in the pancreatic cancer cell line PANC-1 increased the cell motility (Bao et al. 2015). These observations

provided important insights into the effects of the cytoskeletal rearrangement in pancreatic cancer cells migrating toward neurons.

Paxillin is one of the main components of focal adhesions, and it is involved in the transduction of signals, regulation of cell morphology, and control of cell spread and migration by interaction with a multitude of structural proteins (Huang et al. 2004). Recent studies have shown that phosphorylation of paxillin by FAK or Src at Tyr118 and Ser178 is necessary for the stimulation of the cancer cell migration. It is reported that paxillin phosphorylation was related with cell adhesion, epithelial-mesenchymal transition, and cell cycle via WNT5A/JNK pathway in pancreatic cancer cells (Wei et al. 2013). Phosphorylation of paxillin by gastrin promoted cancer cell migration and invasion. Furthermore, phosphorylation of paxillin regulated the formation of focal adhesions and cell polarization in pancreatic cancer cells (Mu et al. 2018). More importantly, paxillin was reported to be a potential target in pancreatic cancer (Kanteti et al. 2016).

Changes in paxillin protein expression were shown to be involved in the acceleration of several other cancers, including breast carcinoma (Madan et al. 2006), lung cancer (Jagadeeswaran et al. 2008), hepatocellular carcinoma (Li et al. 2005), melanoma (Velasco-Velazquez et al. 2008). Moreover, overexpression of paxillin augmented migration and invasiveness and induced metastasis in colorectal cancer (Jun et al. 2011). A previous study demonstrated that the down-regulation of paxillin protein levels was associated with suppression of cell migration proliferation, migration and invasion in colorectal cancer (Qin et al. 2015). Chen et al. also demonstrated phosphorylation events of paxillin were vital for cancer cell migration in breast cancer (Chen et al. 2012). In lung carcinoma, the expression of paxillin was positively correlated with higher stage and metastasis. Furthermore, suppression of paxillin expression by siRNA decreased the cell viability in lung cancer cell lines, whereas promotion of paxillin expression increased tumor growth and invasiveness *in vivo*

(Jagadeeswaran et al. 2008). In melanoma cells, the reduction of paxillin expression weakened tumor cell adhesion and motility (Velasco-Velazquez et al. 2008).

In this study, we demonstrated that during the neural invasion, the expression of phospho-paxillin was amplified particularly in the lamellipodia and filopodia of pancreatic cancer cells. In line with this, by suppression or stimulation of phospho-paxillin, we further demonstrated that the neuron-directed migration ability of cancer cells was significantly altered. In accordance with this, paxillin phosphorylation is linked with cancer cells' migration and plays a critical role in mediating neural invasion of pancreatic cancer cells.

Based on our findings, the phospho-paxillin expression was markedly enhanced in neural invasion. Along with this line, we identified cytokines involved in stimulation of phospho-paxillin by performing ELISA assays in the supernatants of cancer cells, DRG neurons, or cancer cell-neuron co-culture. Initially, we did not observe the obvious variations of MCP-1/CCL2 levels in the monoculture supernatant of the pancreatic cancer cell line SU.86.86, but the level was slightly increased in the supernatant when DRG neurons or the pancreatic cancer cell line T3M4 were cultured alone, indicating that the differential expression of MCP-1/CCL2 might be attributed to DRG neurons in our experiments. Surprisingly, we detected highly enhanced secretion of MCP-1/CCL2 in co-culture supernatants, which suggested that the interaction between neurons and pancreatic cancer cells is critical during neural invasion and MCP-1/CCL2 is vital in this interaction. MCP-1/CCL2 has been demonstrated to be one of the potent chemokines mediating the migration of monocytes/macrophages, natural killer cells, and T cells (Bose et al. 2013). In breast cancer, the high levels of MCP-1/CCL2 were significantly associated with early recurrence and played a vital role in the meditation of angiogenesis (Ueno et al. 2000). Moreover, the MCP-1/CCL2 expression level was significantly different between the various breast cancer genotypes, and breast cancer patients with low expression of MCP-1/CCL2 trended to have better outcomes (Wang et al. 2015). In bladder cancer, MCP-

1/CCL2 mediated the migration and invasiveness of bladder cancer cells. Inhibition of MCP-1/CCL2 decreased the motility in bladder cancer cells (Chiu et al. 2012). Similarly, MCP-1/CCL2 secreted by hepatic myofibroblasts could also increase the migratory and invasive ability of human hepatoma cells (Dagouassat et al. 2010).

In this line, it is conceivable that cancer-infiltrated nerves are injured or stimulated and consequently secrete MCP-1/CCL2, which causes a vicious circle by aggravating neural invasion. Similar results were also found in prostate cancer (He et al. 2015). In the studies by He et al., they also demonstrated that DRG neurons could express MCP-1/CCL2, and it was a leading candidate in mediating nerve-derived migration capacity of prostate cancer cells. Moreover, neural invasion was significantly hampered in the migration assay by using DRG neurons isolated from MCP-1/CCL2$^{-/-}$ mouse (He et al. 2015). It is noted that the MCP-1/CCL2 was deregulated after nerve injury (Kim et al. 2011). Overall, MCP-1/CCL2 may, therefore, be a crucial factor for neural invasion of pancreatic cancer.

It was noted that paxillin phosphorylation stimulates cancer cells' migration and invasiveness. Nevertheless, little is known about the exact pathogenesis. Some reports have linked paxillin phosphorylation with activation of the MAPK pathway (Monami et al. 2006, Zhu et al. 2007, Lee et al. 2012, Montone et al. 2018). It has been reported that in bladder cancer cells, the migration and invasiveness were enhanced by the growth factor proepithelin through the phosphorylation of ERK1/2 via paxillin/ERK signaling pathway, whereas reduction of endogenous paxillin withdrew the proepithelin-mediated migration ability (Monami et al. 2006). In hepatocellular carcinoma, Sorafenib combined with vitamin K1 significantly inhibited the hepatocellular carcinoma cell migration through decreased ERK1/2 phosphorylation (D'Alessandro et al. 2018). Furthermore, inhibition of the platelet-derived growth factor receptor resulted in an obvious suppression of migration and proliferation in glioma cells via regulation of the FAK/paxillinERK signaling pathway (Singh et al. 2018).

Meantime, several studies have also reported that MCP-1/CCL2 is tightly correlated with MAPK pathway (Fang et al. 2012, Li et al. 2012, Wang et al. 2015). In human breast cancer, CC-chemokine receptor-like protein 2 (CCRL2) was hampered in highly invasive cancer cells, while blocking MCP-1/CCL2-induced phosphorylation of p38 MAPK by CCRL2 suppressed invasion and growth of cancer cells in vivo and in vitro. It was also reported that MCP-1/CCL2 mediated EMT and migration via ERK pathway (Li et al. 2017). Similarly, MCP-1/CCL2 can augment the proliferation and invasiveness in a dose- and time-dependent manner in endometrial stromal cells, whereas the anti-MCP-1/CCL2 neutralizing antibody can block proliferation and invasiveness (Li et al. 2012). In the present study, we demonstrated that after treatment with MCP-1/CCL2, the expression of phospho-Src and phospho-ERK1/2 was increased in both SU86.86 and T3M4 pancreatic cancer cells. Meanwhile, after treatment with the receptor CCR4 antagonist, the expression of phospho-Src and phospho-ERK1/2 was decreased. Consistent with this, the expression of phospho-ERK1/2 was decreased after treatment with the CCR4 receptor antagonist in Merkel cell carcinoma (Rasheed et al. 2018). Altogether, paxillin-mediated neuron-directed migration in pancreatic cancer is herewith shown to be associated with the Src/ERK1/2 pathway.

6.0 Summary and Conclusion

In conclusion, this study investigated the alterations of the cytoskeleton and the modification of related proteins during neural invasion of pancreatic cancer cells. Here, we demonstrated that the cytoskeletal rearrangements are important phenomena in pancreatic cancer and can accelerate neural invasion and migration of pancreatic cancer cells. Moreover, we also found that the expression of phospho-paxillin was specifically up-regulated in lamellipodia during neural invasion. Furthermore, this study illuminated one of the key cytokines, i.e. MCP-1/CCL2, to be crucial for neural invasion. In fact, MCP-1/CCL2 can mediate the upregulation of phospho-paxillin and contribute to neural invasion via the Src/ERK1/2 MAPK pathway. Understanding the mechanisms of neural invasion and the interaction between cancer cells and neurons are important for facilitating the development of innovative treatments for cancer as well as for neural invasion. Overall, our findings elucidate a novel and potential strategy for targeted therapeutics against neural invasion, which merits further study.

7.0 Literature

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