Expression and function of Neutral Endopeptidase/CD10 on pancreatic adenocarcinoma

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Thank God for all his blessings

My family for

continuous and unconditional support

Contents

Abbreviations

aa	Amino acid
A	Ampere
Abeta	Amyloid-beta peptide
AD	Alzheimer`s disease
AIDS	Acquired immune deficiency syndrome
Ala	Alanine
ANF	Atrial natriuretic factor
ANP	Atrial natriuretic peptide
Arg	Arginine
Asp	Aspartate
ATRA	All-trans retinoic acid
BA	Butyric acid
BK	Bradykinin
BLP	Bombesin like peptides
BRCA2	Breast cancer 2
bp	Base pair
BSA	Bovine serum albumine
CALLA	Common acute lymphocytic leukemia antigen
CD	Cluster of Differentiation
CDKN2A	Cyclin-dependent kinase inhibitor 2A
cDNA	Complementary deoxriboynucleic acid
Cdc42	Cell division cycle 42
CDS	Coding sequence
CGRP	Calcitonin gene-related peptide
COBRA	Combined of bisulfite and resection enzyme assay
CT	Threshold cycle
DEPC	Diethylpyrocarbonat
DMEM	Dulbecco’s minimum essential medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EC	Extra cellular
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis-(b-amino-ethyl ether) N,N,N´,N´-tetra-acetic acid

ET-1	Endothelin-1
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
fMLP	Formyl-metheonyl-leucyl-phenylalanine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Glu	Glutamate
GRP	Gene-related peptide
HBSS	Hank’s Balanced Salt Solution
hCG	Human chorionic gonadotropin
HDAC	Histone deacetylase
HEPES	N-[2-Hydroxyethyl]piperazine-N'-ethanesulfonic acid
His	Histidine
HIV 1	Human immunodeficiency virus 1
HNPCs	Human neuronal progenitor cells
IFN-β	Interferon-beta
IGF	Insulin like growth factor
IL-1β	Interleukin-1 beta
IL-6	Interleukin- 6
Ile	Isoleucine
kb	Kilo base
kDa	Kilo dalton
K-ras	Kirsten retrovirus-associated DNA sequences
Leu	Leucine
Lys	Lysine
mAb	Monoclonal antibody
Met	Methionine
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MSP	Methylation specific PCR
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NEP	Neutral endopeptidase
NKA	Neurokinin A
nm	Nanometer
NPY	Neuropeptide Y
NTC	No template control
OD	Optical density

PAK1	p21/Cdc42/Rac1-activated kinase 1
PAMP	Proadrenomedullin N-terminal 20 peptide
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with tween
PC	Pancreatic cancer
PCR	Polymerase chain reaction
PE	Phycoerythrin
Phe	Phenylalanine
PETs	Pancreatic endocrine adenocarcinomas
PI3K	Phosphoinositide-3-kinase
PI	Propidium iodide
PIN	Pancreatic intraepithelial neoplasia
PMN cells	Poly morpho nuclear cells
Pro	Proline
PVDF	Polyvinylidene difluoride
q	Long arm side of chromosome
Rac	Ras-related C3 botulinum toxin substrate
RhoA	Ras homolog gene family, member A
RNA	Ribonucleic acid
rNTP	Ribose nucleotide triphosphate
ROCK	Rho-associated, coiled-coil containing protein kinase
RhoGDIs	Rho Guanine nucleotide dissociation inhibitors
rpm	Rotations per minute
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMA	Smooth muscle actin
SP	Substance P
TBST	Tris buffered saline with tween
Thr	Threonine
Tm	Melting temperature
Trp	Tryptophan
Tsp-1	Thrombospondin-1
Tyr	Tyrosine
UICC	Union international contre le cancer
Val	Valine
VIP	Vasoactive intestinal peptide
VPA	Valproic acid

1 Introduction

1.1 Pancreas anatomy, histology and function

The pancreas a part of the digestive system is a long, flat gland (Figure 1), that is located deep in the abdomen sandwiched between the stomach and the spine.[1] It lies partially behind the stomach. The other part is nestled in the curve of the small intestine called the duodenum. Pancreas is described as having a head, body and tail.

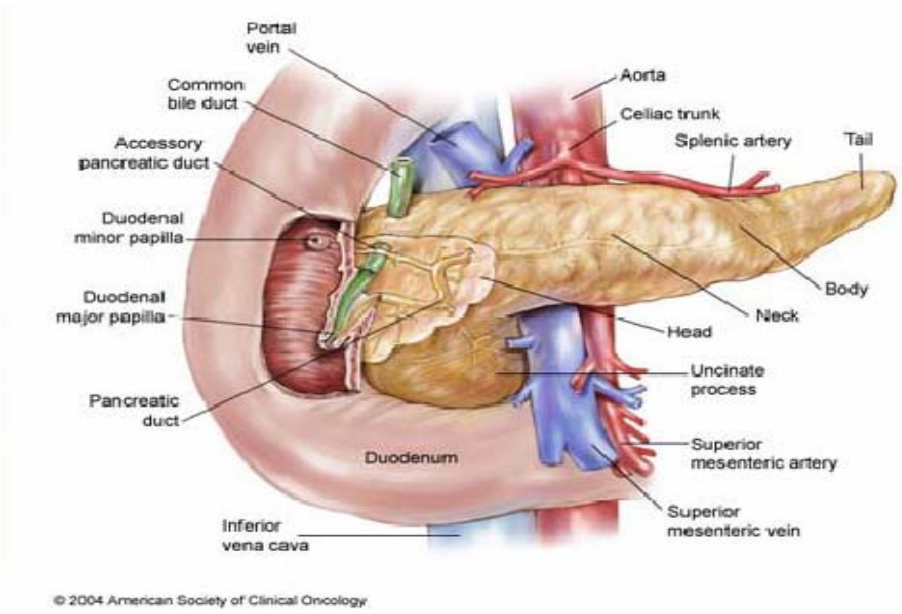


Fig. 1: Morphological structure and location of pancreas in the body

Histologically, the pancreatic parenchyma is divided into two components: (i) the exocrine portion, which is composed of ducts and acini and (ii) the endocrine component, (Figure 2) which is composed of hormone-secreting cells arranged in islets (islets of Langerhans). The pancreatic exocrine cells, the larger part (98-99 %), is composed of tubulo-acinar glands that drain, via a highly branched duct system, into the main pancreatic duct. This duct runs the whole length of the gland and opens into the duodenum through the ampulla of Vater. The acinar cells produce digestive enzymes and some duct lining cells yield a fluid rich in sodium and bicarbonate.[2] The enzymes are responsible for the breakdown of proteins mainly (trypsin, chymotrypsin, elastase and others), fats (lipase) and

carbohydrates (amylase). The endocrine cells are small clusters of cells (1-2 % of the adult pancreatic mass)[3] named the islets of Langerhans and some spread cells that occur single or in small groups.[4] The islets are scattered throughout the exocrine pancreas, although the islets are more numerous in the tail.[2] Endocrine cells are also found within the ductal system.[4] Human islet cells have been classified into four types based on their hormone production: A cells (glucagon); B cells (insulin); D cells (somatostatin); and PP cells (pancreatic polypeptide).[5,6] In the special islets of the pancreatic head the ratio of PP cells is increased even as the endocrine cells found outside the islets include both PP and D cells.[3,4] Two other cell types occur more rarely: D1 cells that secrete vasoactive intestinal peptide (VIP) and enterochromaffin cells that secrete serotonin.[6]

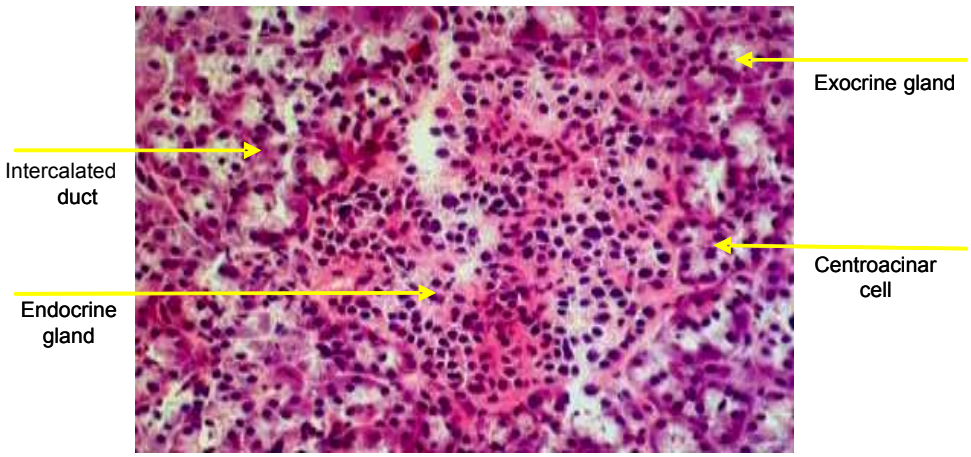


Fig. 2: Histological structure of pancreas (our slide)

1.2 Pancreas diseases

1.2.1 Pancreatitis

Pancreatitis is a rare disease in which the pancreas becomes inflamed. The mechanism that causes pancreatitis is not well known. It is thought that enzymes normally secreted by the pancreas in an inactive form become activated inside the pancreas and start to digest the pancreatic tissue. This process called auto digestion could lead to swelling, hemorrhage in the gland, serious tissue damage, infection and formation of cysts. Enzymes and bacterial toxins may then enter the bloodstream and seriously injure organs,

such as the heart, lungs and kidney. Most commonly caused by alcohol abuse or gallstones, the disease affects men more often than women. There are two forms of pancreatitis: acute and chronic. The acute form occurs suddenly and may result in life-threatening complications; nevertheless the majority of patients (80 %) recover completely. Chronic pancreatitis is a long-term inflammation of the pancreas, that is primarily marked by severe pain and loss of pancreatic function.[7]

1.2.2 Pancreatic cancer

Like all organs, the pancreas is made up of individual living cells. These cells are joined to form the tissues of the pancreas. There are several different types of cells in the pancreas to accomplish its functions as mentioned above. The cells divide rapidly while the pancreas is growing in the womb, childhood and through puberty. In adults, they only divide rarely to replace old, dying cells or injured ones. Cell division is normally under very tight control by the genetic material (genes) such as cyclines, p21, p53 etc, that control all mechanisms like proliferation, differentiation and other functions inside each cell. Pancreatic cancer begins in a single cell. A change occurs in the genes of this cell, which loses its controls to prevent haphazard division (Figure 3).

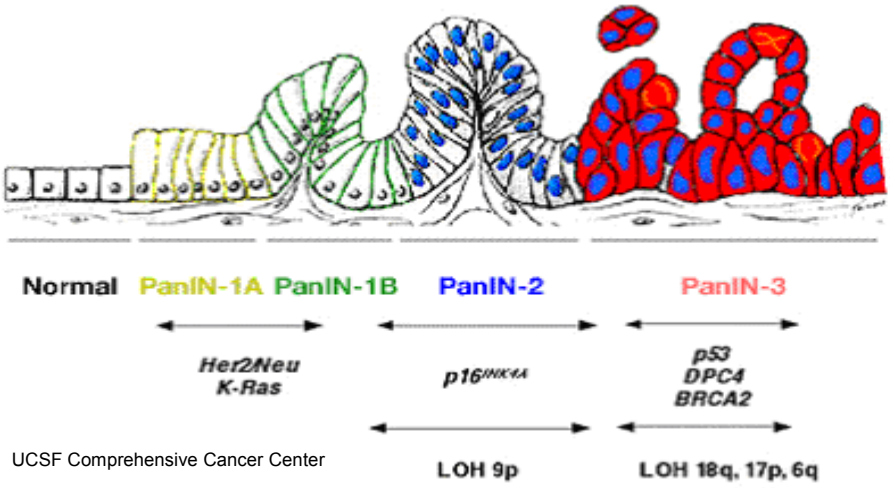


Fig. 3: Pancreatic cancer progression. Stages of pancreatic cancer progression from a normal polarized epithelial monolayer through hyperplasia (PanIN-1A), dysplasia (PanIN-IB/2), and ductal carcinoma in situ (PanIN-3).

The abnormal cell starts dividing rapidly, makes millions and billions of copies of it. Soon a clump of abnormal cells is produced, called adenocarcinoma. Adenocarcinoma simply means a swelling and is not necessarily malignant or cancerous. When the cells in the adenocarcinoma just grow in their local area and do not spread elsewhere, then the adenocarcinoma is benign. However, when the adenocarcinoma's cells, which are dividing out of control, gain the capacity to spread to distant body areas, this is a malignancy called cancer. Malignant adenocarcinomas can spread to any area of the body; the process of distant spread is called metastasis (Table 1). The cancer at first grows in its local area and interferes with the pancreas functions. When cancer metastasizes, it can grow in vital organs, cause symptoms there and eventually kill the patient.

1.2.3 Risk factors

Pancreatic cancer is the fourth most common cause of cancer death in Western society and is a leading cause of cancer death worldwide. Its incidence and mortality rates are almost identical. The 5-year survival rate is approximately 1-2 % and the median survival time after diagnosis is 4-6 months.

Pancreatic cancer is more common among males than females, with peak incidence occurring at age sixty.[8] The etiology of the disease remains unclear, but cigarette smoking, obesity and alcohol abuse have been related with an increased incidence of pancreatic cancer. Pancreatic cancer is more common among individuals with histories of the following conditions: cirrhosis (a chronic liver disease), chronic pancreatitis, diabetes and a history of surgery to the upper digestive tract.[8] Long term exposure to certain chemical carcinogens, such as dry cleaning chemicals, gasoline or metallurgic fumes, also appears to increase the risk of this cancer.[8] Inherited DNA changes that may increase a person's risk of developing cancer of the pancreas can also cause an increased risk for certain other cancers. Mutation in oncogene (K-ras in codon 12), tumor suppressor genes (CDKN2A/INK4A, TP53 and DPC4/SMAD4/MADH4) and caretaker genes (BRCA2) also have a higher rate of pancreatic cancer. Current studies on

pancreatic adenocarcinoma tissues and cell lines have shown that multiple subsets of genes undergo activation or inactivation during development and progression of disease.[9] Cancer of the endocrine pancreas includes a highly treatable and often curable collection of tumors. They are uncommon cancers with 200 to 1.000 new cases per year and occur in only 1.5 % of detailed autopsy series. About 95% of pancreatic cancers begin in the exocrine pancreas. Here in this study only exocrine pancreas cell lines will being investigated.

1.2.4 Cellular cancer classification of pancreatic adenocarcinoma

Cancer of the pancreas can be classified according to the UICC and TNM . Once cancer of the pancreas is found, more tests will be done to find out whether the cancer has spread from the pancreas to the tissues around it or to other parts of the body. This is called staging (Table 1).

Table 1: The UICC TNM classification for staging of cancers

	Involvement of lymph nodes N		
Primary adenocarcinoma T	**N0**	**regional node N1**	**distant nodes M**
T1 With in the pancreas 2 cm or smaller	Stage 1a	Stage 2b	Stage 4
T2 With in the pancreas larger than 2 cm	Stage 1b	Stage 2b	Stage 4
T3 Beyond the pancreas	Stage 2a	Stage 2b	Stage 4
T4 Invasion to the celiac artery or SMA*	Stage 3	Stage 3	Stage 4
Liver metastasis, lung metastasis, peritoneal dissemination[12] Stage 4:			

*Smooth muscle actins (SMA) are commonly used to elucidate mammary myoepithelial (ME) cells, whose presence or absence is a reliable criterion for differentiating in situ and invasive adenocarcinomas.

Cancers of the exocrine pancreas can be grouped according to where in the pancreas the cancer is or according to the type of cell the cancer has originated from.

- duct cell adenocarcinoma (90 % of all cases)
- acinar cell adenocarcinoma
- papillary adenocarcinoma
- adenosquamous adenocarcinoma

- undifferentiated adenocarcinoma
- mucinous adenocarcinoma
- giant cell adenocarcinoma
- mixed type
- small cell adenocarcinoma
- cyst adenocarcinoma
- unclassified
- pancreatoblastoma
- papillary-cystic neoplasm (this adenocarcinoma has lower malignant potential and may be cured with surgery alone)[10,11]

1.3 Ectoenzymes

Ectoenzymes are integral membrane proteins anchored by hydrophobic interactions with the lipids of the plasma membrane, that have their enzymatically active site outside the plasma membrane in the extracellular environment. Many ectoenzymes are type II integral membrane proteins with a short amino terminus in the cytosol or are glycosylphosphatidyl-inositol-linked molecules. But all other possible kinds of anchoring in cell membranes have been found. One of the first ectoenzymes to be identified was cholinesterase, which hydrolyses acetylcholine at neuronal synapses.[13] Ectoenzymes can be classified according to their enzymatic activities. Many of them are peptidases and proteinases, see below table 3. Other enzyme species include hydrolases and nucleotidases which hydrolyse extracellular nucleotides, NAD and NADP, or oxidases which oxidize various substrates (Table 2).[14]

Table 2: Ectoenzymes and substrates

Name	EC number	Catalytic activity	Substrates	Products
Nucleotidases and related enzymes				
CD39	EC 3.6.1.5	ATP diphosphohydrolase	ATP ATP ADP	AMP ADP AMP
CD73	EC 3.1.3.5	5.-Nucleotidase	AMP	Adenosine
ADP-ribosyl cyclases and ADP-ribosyltransferases				
CD38	EC 3.2.2.5	ADP-ribosyl cyclase	NAD(P)	cADPR(P) and nicotinamide
		NAD(P) hydrolase	NAD(P)	ADPR(P) and nicotinamide
		cADPR hydrolase	cADPR	ADPR
		Base-exchange catalyst	NAD(P)	NAAD(P)
CD157	EC 3.2.2.5	ADP-ribosyl cyclase	NAD(P)	cADPR(P) and nicotinamide
		NAD(P) hydrolase	NAD(P)	ADPR(P) and nicotinamide
		cADPR hydrolase	cADPR	ADPR
		Base-exchange catalyst	NAD(P)	NAAD(P)
ART2	EC 2.4.2.31	ADP-ribosyltransferase	NAD(P)	ADP-ribosylated proteins
Peptidases and proteases				
CD10	EC 3.4.24.11	Neutral endopeptidase	Peptides derivatives	Cleaved peptides
CD13	EC 3.4.11.2	Aminopeptidase N	Peptides derivatives	Cleaved peptide
CD26	EC 3.4.14.5	Dipeptidyl peptidase	X-Pro/Ala	Cleaved peptide
MT1-MMP	EC 3.4.24.80	Matrix metalloproteinase	Protein	Proteolytic fragments
CD156b	EC 3.4.24.86	Metalloproteinase	Protein	Proteolytic fragments
Oxidases				
VAP1	EC 1.4.3.6	Amine oxidase	Amine	Aldehyde, H_2O_2 and NH3
NADPH oxidase	EC 1.6.3.1	Oxidase	NADPH	Superoxide and H_2O_2

ADPR, ADP-ribose; ADPR(P), ADPR or ADPR phosphate; ART2, ADP-ribosyltransferase 2; cADPR, cyclic ADP-ribose; cADR(P), cADPR or cADPR phosphate; cAMP, cyclic AMP; H2O2, hydrogen peroxide; LPA, lyso-phosphatidic acid; MT1-MMP, membrane-type-1 matrix metalloproteinase; NAAD, nicotinic-acid-adenine dinucleotide; NAAD(P), NAAD or NAAD phosphate; NAD(P), NAD or NADP; NH3, ammonia; S1P, sphingosine 1-phosphate; VAP1, vascular adhesion protein 1; X, any amino acid.

Ectopeptidases may cleave regulatory peptides and peptide hormones at the adenocarcinoma cell surface, thereby modulating the biological effect of these peptides on adenocarcinoma cell proliferation, differentiation and also facilitating immune escape mechanisms.[15-17] Several ectopeptidases are also capable of transducing signals independently of their proteolytic activity, making them multifunctional membrane proteins. Ectopeptidases are present in a wide variety of tissues and cell types. They are classified according to the location of the cleavage site in the putative substrate (Table 3). Endopeptidases recognize specific amino acid in the middle of the peptide, whereas exopeptidases distinguish one or two terminal amino acids. Exopeptidases that attack peptides from the N-terminus (removing either single amino acids or a dipeptide) are

termed (dipeptidyl) aminopeptidases, whereas peptidases attacking the C-terminus are termed carboxypeptidases.[18]

Table 3: Ectopeptidases and substrates

Peptidase	Specificity*	Posssible natural substrates
Aminopeptidases		
APN	○ ÷ ● — = Ala, Leu	fMLP, opioid peptides, enkephalins
APA	○ ÷ ● — = Glu, Asp	Angiotensins
APP	● ÷ ○ — = Pro	BK, SP
DPP IV	● ÷ ○ — = Pro, Ala	SP, BK, NPY, chemokines (CXCL9, 10)
Carboxy peptidases		
CPN	— ● ÷ ○ = Arg, Lys	Anaphylatoxins
Endopeptidases		
NEP	—● ÷ ○ — ● — = Phe, Leu, Ile, Val, Thr, Trp, Ala	BK, SP, NKA, NPY, VIP, ANF, enkephalins ET-1, BLP, angiotensins
ACE	● — ● ÷ ○— ○ — = Relatively non-specific	Angiotensins, enkephalins, SP
ECE	— ○— ○ — ÷ ● — = Ile-Ile-Trp	Big ET-1

The peptidase cleaves peptides in which the open circle represents (one of) the mentioned amino acids. The closed circle can be any amino acid. The cleaved bond is represented by '÷'. Peptidases: APN, aminopeptidase N; APA, aminopeptidase A; APP, aminopeptidase P; DPP IV, dipeptidyl(amino)peptidase IV; CPN, carboxypeptidase N; NEP, neprilysin; ACE, angiotensin-converting enzyme; ECE, endothelin-converting enzyme. Substrates:fMLP, formyl-metheonyl-leucyl-phenylalanine; BK, bradykinin; SP, substance P; NKA, neurokinin A ; NPY, neuropeptide Y ; VIP, vasoactive intestinal peptide ; ANF, atrial natriuretic factor ; ET-1, endothelin-1 ; BLP, bombesin-like peptides.

1.3.1 Neutral endopeptidase

Membrane metalloendopeptidase EC 3.4.24.11 (Enkephalinase, neprilysin, neutral endopeptidase, NEP) is a mammalian type II integral membrane zinc-containing endopeptidase ectoenzyme. Immunophenotypically it is identified as the leukocyte cluster of differentiation CD10 or CALLA (common acute lymphoblastic leukemia antigen), which has specificity for cleaving oligopeptides (≤ 40 amino acids in length) at the amino side of hydrophobic amino acids (Val, Ile, Phe, Leu, Ala).[19]

1.3.1.1 Biochemical and molecular characteristics

Neutral endopeptidase was first characterized and isolated from rabbit kidney tubule brush borders. Cloning of the NEP/CD10 gene and subsequent cloning of the common acute lymphoblastic leukemia antigen (CALLA, CD10) showed that both sequences are identical.[20-22] NEP/CD10 is a glycoprotein with 750 amino acids.[20] It has a single 24 amino

acid hydrophobic segment that functions both as a transmembrane region and a signal peptide. The C-terminal 700 amino acids compose the extracellular domain which contains the pentapeptide consensus sequence (His-Glu-[Ile, Leu, Met]-X-His) of zinc-binding metalloproteases, whereas the 25 N-terminal amino acids form the cytoplasmic tail.

In most species, NEP/CD10 appears to exist as a non-covalently associated homodimer.[23] The crystal structure (Figure 4) of the extracellular domain complexed with phosphoramidon has recently been described.[24]

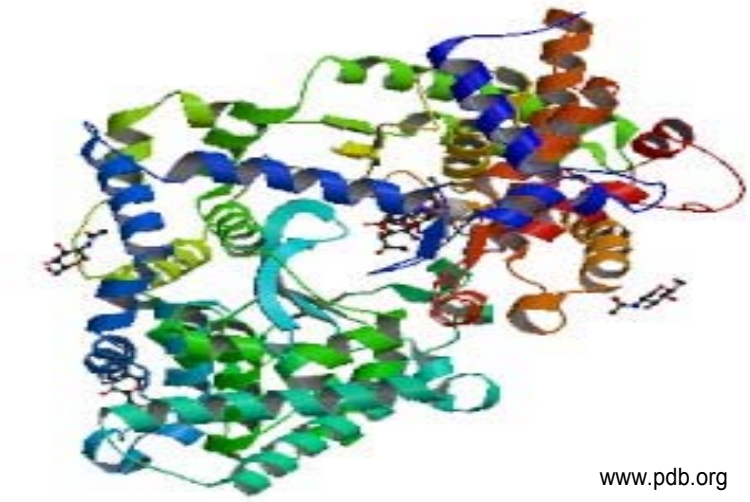


Fig. 4: Structure of human NEP/CD10 complexed with phosphoramidon enzyme inhibitor.

1.3.1.2 Gene and protein structure

NEP/CD10 gene maps to chromosomal region 3 (q25.1-q25.2), it spans more than 80 kb and is composed of 25 exons (Figure 5).[25,26] Exons 1, 1 bis and 2 encode 5' untranslated sequences; exon 3 encodes the initiation codon, the cytoplasmic and transmembrane domain; 20 short exons (exons 4-23) encode most of the extracellular region; and exon 24 encodes the C-terminal 32 amino acids of the protein and contains the entire 3' untranslated region.[18] Within exon 24 are five poly (A) signals. Alternative splicing of exon 1, exon 1bis, exon 2 (2a), or part of exon 2 (2b) to the common exon 3, resulting in four different transcripts, may be the origin of the tissue-specific or stage of development-

specific expression of NEP/CD10.[27] Indeed, two separate regulatory elements have been found in the NEP/CD10 promoter region and these elements may be regulated by the transcription factor CBF/ NF-Y in a tissue specific manner. A cDNA clone lacking the complete exon 16 has been isolated from human lung tissue.[28]

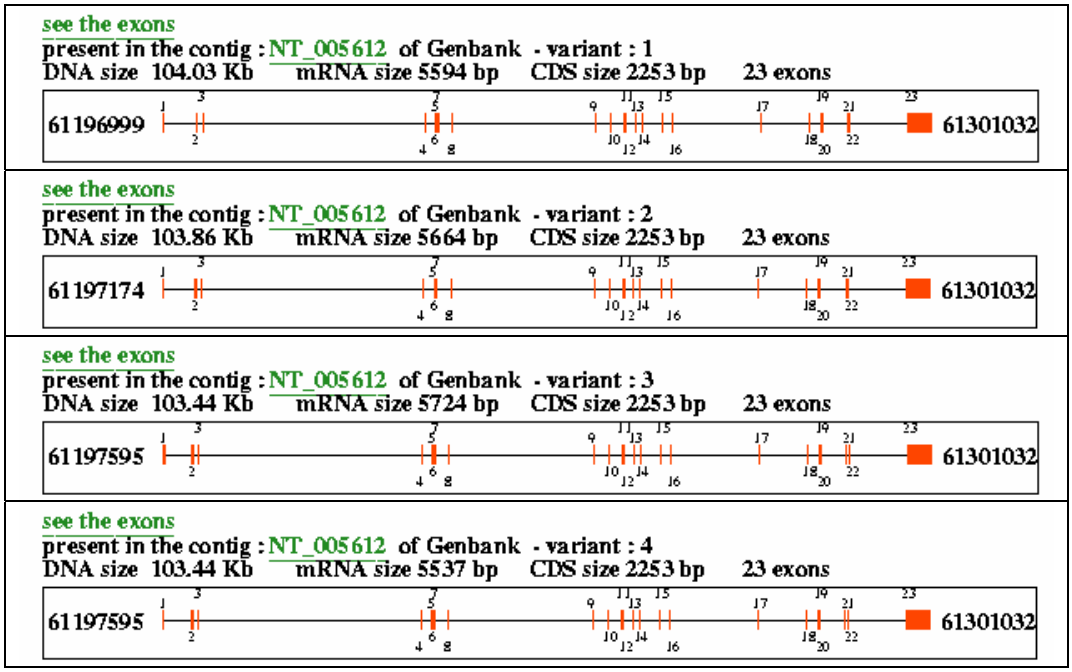


Fig. 5: Variant gene transcripts structure of NEP/CD10 confer four transcripts that present only one protein. Copied from gene data bank.

1.3.1.3 Enzymatic activity and biological function

NEP/CD10 is able to hydrolyse peptide bonds on the N-terminal site of hydrophobic amino acids, including Phe, Leu, Ile, Val, Tyr, Ala and Trp. However, sub-site interactions and conformational factors greatly influence the efficiency of hydrolysis. It hydrolyses a variety of physiologically active peptides including opioid peptides (Met- and Leu-enkephalin), substance P, atrial natriuretic factor (ANF), endothelin, neurotensin, oxytocin, bradykinin, angiotensin 1, 2, chemotactic peptide formyl-Met-Leu-Phe (f - MLP) , and bombesine like peptides.[19] Thus, one of the main roles of NEP/CD10 seems to be reducing the local concentration of biologically active peptides available for receptor binding and signal transduction. The ubiquitous occurrence of NEP/CD10 in mammalian organs renders possible a broad field of physiological functions as illustrated by more or less organ-

specific peptides proven to be cleft by the enzymes. In kidneys possible physiological substrates of NEP/CD10 are ANP, adrenomedullin and PAMP (see the list of abbreviations); in the brain, the substrates probably are enkephalins and oxytocin. Similarly, possible substrates in the lung are bombesin, BLP, GRP, neuromedin C, substance P and neurokinin A; in the cardiovascular system, angiotenisin II, bradykinin and CGRP; in the gut, VIP; on the neutrophil membrane, fMLP etc. Some substrates are not strictly tissue-specific, e.g. substance P. Preclinical and clinical trials explored possibilities of therapeutic application of the inhibitors of NEP/CD10, such as thiorphan in the management of pain, diarrhea, depression, arterial hypertension and asthma.[29-37] It has been reported that NEP/CD10 may also be able to hydrolyse certain larger substrates, including cytokines such as IL-1β and IL-6.[18] A role for NEP/CD10 in lymphoid development has been suggested by studies showing that inhibition of NEP/CD10 results in increased proliferation and maturation of B cells, both in vitro and in vivo.[38]

1.3.1.4 Distribution in tissues

The common acute lymphoblastic leukemia antigen (CALLA; CD10) was originally found on lymphoblasts from patients with pre-B type acute lymphoblastic leukemia.[39] Later it has been found to be widely associated with precursor B-cell acute lymphoblastic leukemia, T-cell acute lymphoblastic leukemia, as well as various types of lymphomas including follicular lymphomas and Burkitt lymphomas.[40-45]

Subsequently, immunohistochemical studies demonstrated the expression of this antigen on a variety of non hematopoietic normal and adenocarcinoma tissues, such as kidney, breast, fetal small intestine, fibroblasts, gliomas, melanomas, retinoblastomas, various mesenchymal adenocarcinomas, breast, colon adenocarcinoma cells, genitourinary tract, such as renal cell, transitional cell and prostate adenocarcinomas.[46-59] In the female genital tract, NEP/CD10 is a marker of trophoblastic adenocarcinomas and endometrial stromal neoplasms.[60] It is also expressed by serous ovarian adenocarcinomas.[60-62] In the gastrointestinal system, NEP/CD10 is expressed in hepatocellular adenocarcinomas.[63] It also has been detected in pancreatic ductal adenocarcinomas and is frequently observed

in solid pseudopapillary adenocarcinomas.[64-66] It has been reported to be positive in some pancreatic endocrine adenocarcinomas (PETs) in a small series.[64,67] This wide tissue distribution, suggests a crucial role in vivo of this antigen.

1.3.1.5 NEP/CD10 function in normal and carcinoma tissues

The various functions certainly depend on cell type or tissue origin. In muscles, NEP/CD10 may play an important role during muscle cell differentiation, possibly through the regulation, either directly or indirectly, of the insulin-like growth factor I driven myogenic program.[68] Furthermore, other results showed that elevated cardiac NEP/CD10 activity may increase the local degradation of bradykinin and natriuretic peptides promote to pressure loaded and failing human hearts.[69] Neutral endopeptidase may terminate the proinflammatory and mitogenic actions of neuropeptides in normal skin and wounds.[70] In addition it plays a specific role in promoting early T-cell development.[71] In human bronchi, it may regulate peptide-induced inflammation.[72] In human thymocytes, expression of functional NEP/CD10 suggests a role for this enzyme in the maturation of human thymocytes by hydrolysing thymopentin, a thymic peptide known to induce the maturation of prothymocytes into thymocytes.[73] NEP/CD10 of epithelial cells within human digestive tract (in the stomach, duodenum, small intestine, ascending, descending sigmoid colon and rectum) confirms the hypothesis of its participation in protein hydrolysis processes.[74] In the human respiratory mucosa, in addition to the modulating functions of NEP/CD10 on neuropeptide-mediated activities on vessels and glands, it is possible that in secreted form it plays a role in regulating mucosal responses to luminal neuropeptides or other as yet uncharacterized NEP/CD10 substrates.[75] In spleen, results suggest that NEP/CD10 inhibition promotes the reconstitution and maturation of splenic B-cells. Therefore, it may function to regulate B-cell ontogeny in vivo by hydrolyzing a peptide substrate that stimulates B-cell proliferation and or differentiation.[76]

In tumors, NEP/CD10 function seems to be also variable from one tissue to others. In stromal cells, it seems to promote invasion and metastasis of differentiated gastric carcinoma.[77] In malignant melanoma the expression is also associated with tumor

progression.[78] In epithelial ovarian carcinomas, it may play a role in the regulation of neoplastic transformation and tumor differentiation,[79] however other evidence suggests that NEP/CD10 functionally suppresses the progression of ovarian carcinoma.[80] In an integral part of colorectal carcinogenesis it seems to contribute to the invasion and thus probably facilitates metastasis.[81] In placenta, induction of choriocarcinoma cell differentiation is associated with an increase of NEP/CD10 expression at the cell surface, suggesting a role of this enzyme in regulating differentiated trophoblast functions such as human chronic gonadotrophin (hCG) secretion.[82] In brain, a partial reduction of extracellular amyloid-beta peptide (Abeta) levels by NEP/CD10 may facilitate development of human neuronal progenitor cells (HNPCs) in treatment of neurodegenerative diseases like Alzheimer's disease (AD).[83,84] Downregulation of NEP/CD10 in the progression of urothelial bladder carcinomas, probably facilitates invasion, especially into muscle.[85] In nasopharyngeal carcinoma it was revealed that NEP/CD10 expression by stromal cells in this malignancy plays an important role in tumor progression, particularly in older patients.[86] In cervical carcinoma cells results indicated that NEP/CD10 functions as a tumor-suppressor gene and its expression may have prognostic significance,[87] and in oral squamous cell carcinoma may have an important role in tumor invasion, probably facilitating the occurrence of metastases.[88]

1.4 NEP/CD10 expression in pancreatitis and carcinoma tissues

We investigated NEP/CD10 expression in pancreatitis and pancreatic tumor lesions.[89] Interestingly, there exists no significant difference in NEP/CD10 mRNA expression levels in pancreatic carcinoma compared to that of pancreatitis tissues (data not shown). Regarding protein expression, immunoreactivity for NEP/CD10 was detectable in a subset of pancreatic carcinomas exhibiting a membranous staining pattern in tumor cells (Figure 6). A positive staining was found in 6 of 24 pancreatic ductal adenocarcinomas (25 %). However, the heterogeneous NEP/CD10 expression pattern was not associated with tumor grading, staging and metastasis formation. In chronic pancreatitis 3 of 6 samples demonstrated a focal staining of residual ducts.

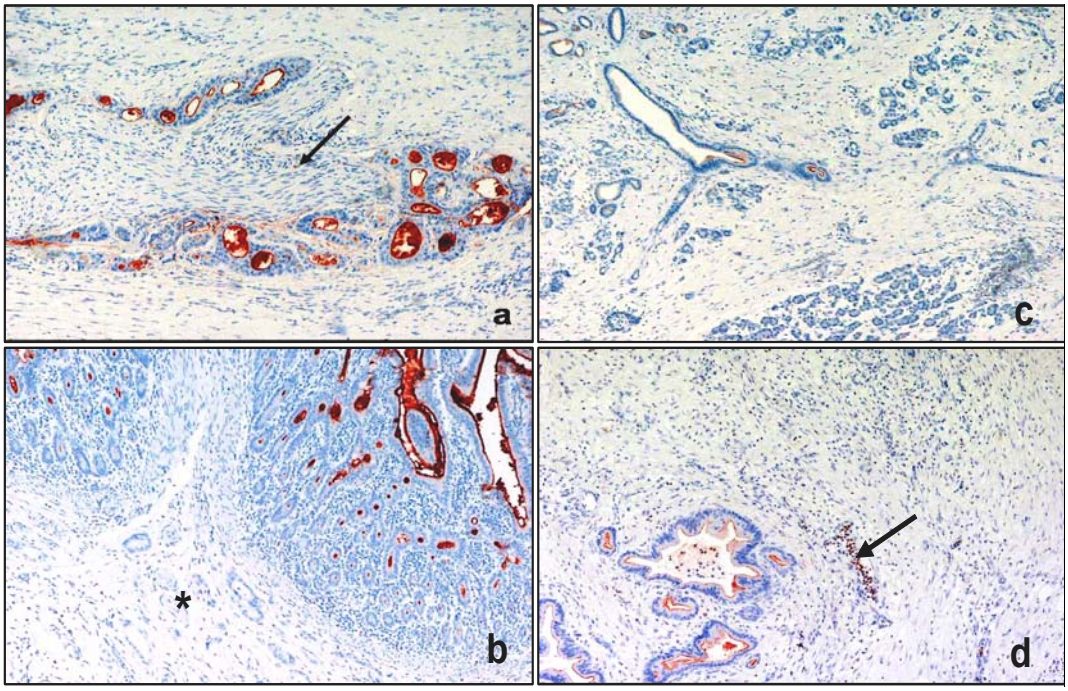


Fig.6: Immunohistochemical detection of NEP/CD10 expression in pancreatic adenocarcinomas as well as in chronic pancreatitis. Perineural (arrow indicating nerve) infiltrating ductal adenocarcinoma with membranous expression of NEP/CD10 (a). Ductal adenocarcinoma negative for NEP/CD10 (*), normal duodenal mucosa served as internal positive control (b). Chronic pancreatitis with focal expression of NEP/CD10 in residual ducts and intravascular neutrophils (arrow) (c, d). (Erhuma et. al.)[89]

1.5 Aim of the present investigation

Neutral endopeptidase is a membrane bound enzyme with various functions depending on cell type or tissue origin. Only limited information however exists about the NEP/CD10 expression in physiological and patho-physiological conditions of the pancreas.

In this study, the main purpose is in the following steps explained:

NEP/CD10 expression: investigation of the relevance of NEP/CD10 expression in pancreatic adenocarcinoma cell lines.

NEP/CD10 promoters methylation status: Examination of a subset of cases for evidence of two NEP/CD10 promoters methylation and correlateion the results with NEP/CD10 expression to determine the mechanism for the lack of NEP/CD10 expression in pancreatic adenocarcinoma cell lines.

NEP/CD10 regulation: Either hypermethylation of the 5' CpG island or deacetylation of chromatin results in a loss of NEP/CD10 expression in pancreatic adenocarcinoma cell lines. To differentiate between both mechanisms pancreatic carcinoma cell lines were exposed in separate to either the demethylating agent 5-aza-2'-deoxycytidine or acetylation agents, butyric acid and valproic acid.

Biological function of NEP/CD10: Investigation of the biological function of NEP/CD10 in pancreatic exocrine carcinoma using pancreatic carcinoma cell line as a model, transfected with pEGFP-C3 (Mock), NEP-pEGFP-C3 and mutated NEP^{H587E}-pEGFP-C3 in enzyme active site, as well as their effects on proliferation, motility and invasion via induction or repression of diverse proteins that play a crucial role in biological mechanisms.

Affecting of NEP/CD10 on signal cascades: Investigation of the influence of NEP/CD10 over expression on the proteins that play crucial roles in signal cascades that regulate proliferation, motility and invasion via stimulation through cell membrane receptors concluded our series of experiments.

2 Materials and Methods

2.1 Pancreatic adenocarcinoma cell lines and culture conditions

The pancreatic adenocarcinoma cell lines that were used in this study PA-TU-T, HUP-T4, HUP-T3, PA-TU-S, PA-TU-02, PANC-1, Capan-1, Capan-2 and MIA PaCa-2 were either obtained from the Deutsche Sammlung für Mikroorganismen und Zelllinien (DSMZ; Braunschweig, Germany) or the American Type Culture Collection (ATCC). Cells were cultured in a humidified atmosphere containing 5 % CO_2 at 37 °C. Their characteristics and growth conditions are listed in table 4. All media were supplemented with 1 % penicillin/streptomycin (Biochrom AG, Berlin, Germany). Cells were passaged every 4–7 days using trypsin-EDTA (Invitrogen, Karlsruhe, Germany) and $1x10^5$ cells were seeded onto 25 cm² tissue culture flasks (Greiner Bio-one, Frickenhausen, Germany) grown until confluence, then processed for mRNA or protein quantification respectively.

2.2 Plasmids

The following three plasmids were used to explore the biological function in the examined pancreatic adenocarcinoma cell line PANC-1, pEGFP-C3 (BD Biosciences Clontech, Heidelberg, Germany) was used to insert any desired investigated gene (Figure 7), NEP-pEGFP-C3, CDS of NEP/CD10 cloned inside pEGFP-C3 and mutated NEP^{H587E}-pEGFP-C3, that has a mutation in the enzyme active site of NEP/CD10 ($H^{587}E$) by substitution of histidine with aspartic acid.

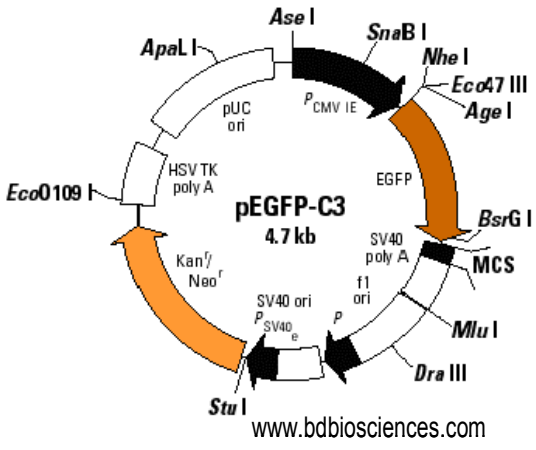


Fig. 7: Structure of the used pEGFP-C3 plasmid in our study

Table 4: Characteristics of pancreatic adenocarcinoma cell lines used in this study

Name and source	Abbrev.	Doubling time and morphology	Culture media	Characteristics	Metastasis	Differentiation status
PA-TU-8988 T DSMZ	PA-TU-T	about 22 h, adherent cells growing in monolayers	DMEM/F12 5 % FCS 5 % Horse serum 2 mM L-Glutamin	Liver metastases of a primary pancreatic adenocarcinima	no	well
HUP-T4 DSMZ	HUP-T4	about 38 h, epitheloid cells growing adherent in monolayers	MEM 10 % FCS 1 % non essential amino acides 1 % Na. pyruvate	Pancreas adenocarcinoma from malignant ascites	no	poor
HUP-T3 DSMZ	HUP-T3	about 38 h, epitheloid cells growing adherent in monolayers & clusters	MEM 10 % FCS 1 % non essential amino acides 1 % Na. pyruvate	Pancreas adenocarcinoma from malignant ascites	no	well
PA-TU-8988 S DSMZ	PA-TU-S	about 40-60h, adherent epitheloid cells growing in monolayers	DMEM/F12 5 % FCS 5 % Horse serum 2 mM L-Glutamin	Liver metastases of a primary pancreatic adenocarcinoma (sister of PATU-T)	yes, in particular lung	poor
PA-TU-8902 DSMZ	PA-TU-02	about 25-40 h, epithelial adherent cells growing in monolayers	DMEM/F12 5 % FCS 5 % Horse serum 2 mM L-Glutamin	Primary ductal pancreatic adenocarcinoma (grade II)	yes, in lung	poor
PANC-1 ATCC	PANC-1	about 52 h, adherent cells growing in monolayers	DMEM/F12 5 % FCS 5 % Horse serum 2 mM L-Glutamin	Liver metastases of a pancreatic ductal adenocarcinoma	yes	undifferentiated
Capan-1 DSMZ	Capan-1	about 50-100 h, adherent fibroblastic semi-confluent monolayer	RPMI 1640 15 % FCS	Liver metastases of a pancreatic ductal adenocarcinoma	yes	well
Capan-2 DSMZ	Capan-2	about 50-70 h, adherent epithelial-like cells growing in monolayer	RPMI 1640 15 % FCS	Pancreas adenocarcinoma	yes	well
MIAPaCa-2 ATCC	MIAPaCa-2	about 40 h, adherent cells growing in monolayers	DMEM/F12 1 % FCS 2.5 % Horse serum 1 % Na. pyruvate 4 mM L-Glutamin	Pancreatic adenocarcinoma	yes	well

2.3 RNA isolation and cDNA synthesis

Pancreatic adenocarcinoma cell lines, $1x10^5$ cells were seeded into 25 cm² tissue culture flasks (Greiner Bio-one) and were incubated to grow until confluence. Cells were lysed in 1 ml of TRIzol™ reagent (WKS, Frankfurt, Germany) and total cellular RNA was isolated from cells according to the manufacturers instructions developed by Chomczynski & Sacchi.[90] The concentration and purity of total cellular RNA was quantified by determination of optical density at 260, 280, 320 nm and integrity of the RNA was confirmed by denaturing agarose gel electrophoresis. cDNA was prepared as further described, 1 µg of RNA in total volume of 10 µl RNase free water, then incubated in trio-thermo-block (Biometra, Göttingen, Germany) at 70 °C for 3 min followed by addition of 15 µl of master mix to each sample 5 x reaction buffer 5 µl; 0.0 M DTT 2.5 µl; 12.5 mM dNTP-mix 1 µl; RNase free water 2.7 µl; 40U/µl RNasin 0.5 µl; 200U/µl superscript™ II RNase H-reverse transcriptase 0.3 µl; 100ng/µl random primer 3 µl (Invitrogen, Karlsruhe, Germany). The samples are incubated again in trio-thermo-block at 42 °C 45 min, 95 °C 3 min, 2 °C. The resulting complementary DNA (cDNA) was subjected either to conventional PCR amplification or Real Time-PCR.

2.4 NEP/CD10 standard preparation

To quantitate NEP/CD10, standard was prepared using SP6 promotor + NEP/CD10 sense primer: 5`-GATTTAGGTGACACTATAGAATACCTCCGAGAAAAGGTGGACAA-3` and antisense primer: 5' -TGAGTCCACCAGTCAACGAC-3` (Invitrogen). In the presence of cDNA of cell type that expresses the required gene, NEP/CD10 was amplified by thermo-cycler in a total volume of 50 µl containing: 1 µl of SP6-sense primer, 1 µl of antisense primer, 1.5 µl $MgCl_2$, 1 µl dNTP-mix, 40.2 RNase free water, 5 µl 10 X Taq buffer [500 mM KCl, 100 mM Tris-HCl, pH 8.3], 0.3 µl 1.25 U Taq Polymerase (Qiagen, Hilden, Germany). The tubes were incubated in thermo-cycler as follows, initial denaturation 95 °C, 5 min, cycle 1 which repeated 35 times with the following parameters: denaturation 95 °C, 30 s; annealing 60 °C, 30 s; extension 72 °C, 30 s, then followed by extension 72 °C, 10 min

and at the end 2 °C. The samples were loaded on gel electrophoresis. The bands were sliced, purified and eluted by using QIA quick Gel extraction kit (Qiagen).

RNA was transcribed in vitro using SP6 RNA polymerase (Roche, New Jersey, USA) in a total volume of 20 µl containing: 13 µl of eluted DNA, 2 µl of rNTP-mix, 2 µl of 10 x buffer, 1 µl of RNase-Inhibitor, 2 µl of SP6-RNA polymerase and incubated in thermocycler at 37 °C for 2 h. The transcription template was eliminated with 5 µl of DNase I (verified by PCR analysis) at 37 °C for 15 min and remaining RNA was precipitated using 2.5 µl of 9 M lithium chloride, 75 µl of cold absolute alcohol (-20 °C). The mixture was kept at – 70 °C for 30 min followed by centrifugation 14.000 rpm at 4 °C for 20 min. The supernatant was removed, 100 µl of 70 % ethanol was added to the tubes followed by centrifugation 14.000 rpm at 4 °C for 10 min. Finally the pellet was left for a few min to dry, then 11 µl of RNase free water was added to each tube. RNA standard was first quantified by measuring the absorbance at 260, 280, 320 nm and then stored at –20 °C until use. As described above the RNA standard 500 ng was co-reverse transcribed and amplified as cDNA in thermo-cycler. In the Real Time-PCR analysis, quantitative analysis of NEP/CD10 mRNAs was performed by incubating samples of cDNA with various concentration of cDNA standard each in separate tube as indicated in the legend.

2.5 Quantitative RT-PCR

The purpose of a PCR (Polymerase Chain Reaction) is to make a huge number of copies of a gene. There are three major steps in a PCR, which are repeated for 30 to 40 cycles. Quantitative PCR was performed in a RG2000 Cycler (LTF, Wasserburg, Germany) by using SYBR™ Green as double-strand DNA-specific binding dye and continuous fluorescence monitoring. Amplification was carried out in a total volume of 20 µl containing, 10 µl QuantiTect SYBR Green Master Mix, 8.5 µl RNase free water (Qiagen), 0.5 µl 10 pmole/µl of forward, revers primers (Table 5) and 1 µl of 1:10 diluted cDNA prepared as described above. The PCR reactions were applied as follows, initial denaturation 95 °C, 15 min, cycle 1 which repeated 40 times with the following parameters: denaturation 95 °C, 20 s; annealing 60 °C, 30 s; extension 72 °C, 30 s.

Fluorescence was acquired to cycling A on channel 1 (CH1). Melting curve analysis of amplification products was performed at the end of PCR reaction by cooling the samples to 60 °C, 5 s, then increasing the temperature 60-99 °C 1 s each temperature and acquired to melt A on CH1 using the Roter-Gene software 4.6. Control reactions for product identification consisted of: (i) Analyzing the melting points of the end product, (ii) use of NTC as negative control to monitor the purity of the reagent and primers. (iii) At the end 10 µl of each PCR product mixed with 2 µl loading buffer were visualized using agarose gel electrophoresis and ethidium bromide staining to determine the length of the PCR products.

Table 5: Number of primer sequences used in our study

Gene name	Primer sequence	Product size	Annealing temp.
CD10	F: 5'-CTCCGAGAAAAGGTGGACAA-3` R: 5`-TGA GTCCACCAGTCAACGAC-3`	251 bp	60 °C
S100A4	F: 5'-TCTCTCCTCAGCGCTTCTTC-3` R: 5`-GCTGTCCAAGTTGCTCATCA-3`	239 bp	60 °C
Rac 1	F: 5'-ACTGTCCCAACACTCCCATC-3` R: 5`-TCGCTTCGTCAAACACTGTC-3`	207 bp	60 °C
Rho A	F: 5'-AAGGACCAGTTCCCAGAGGT-3` R: 5`-ACTATCAGGGCTGTCGATGG-3`	195 bp	60 °C
Cdc42	F: 5'-TACTGCAGGGCAAGAGGATT-3` R: 5`-CCCAACAAGCAAGAAAGGAG-3`	172 bp	60 °C
LIMK 1	F: 5'-CAGGTGAGGTGATGGTGATG-3` R: 5`-GTCCTTGGCAAAGCTCACTC-3`	248 bp	60 °C

2.6 Flow cytometry

Flow cytometry is a method for analyzing the presence of antigens on cells by immunofluorescence. The method involves labeling of cells with a fluorescent antibody, then analyzing them using the flow cytometer. Fluorescence-activated cell sorting (FACS) was performed to quantify the expression levels of NEP/CD10 on the surface of pancreatic adenocarcinoma cell lines. The cells were incubated with phycoerythrin-conjugated anti-human mouse NEP/CD10 mAb IgG (H10a IgG1) (BD Biosciences Heidelberg, Germany) or iso IgG_2a k (BD, pharmingen) as control for 15 min, then fixed

with 2 % para-formaldehyde (Fluka, Germany) for 10 min, followed by washing twice with PBS (Applichem, Darmstadt, Germany). FACS data were acquired on a FACS calibur (Beckton Dickinson, Heidelberg, Germany), fluorescence data of 10.000 events were recorded and analyzed using CELL Quest software (Beckton Dickinson). Furthermore fluorescence data were expressed in (MFI) mean fluorescence intensity.

2.7 Genomic DNA extraction

Genomic DNA was isolated from pancreatic adenocarcinoma cell lines using DNeasy tissue kit (Qiagen) and its quality was assessed by spectrophotometry at 260 280, 320 nm and visualized by 1 % agarose gel electrophoresis with ethidium bromide staining.

2.7.1 Bisulfite modification of DNA

Modifying DNA using sodium bisulfite to convert unmethylated CpG site to UpG with out modifying methylated site, thus allowing their differentiation by methylation specific PCR (MSP), restriction digestion (COBRA), or sequencing after PCR amplification. From each pancreatic adenocarcinoma cell lines 2 µg DNA was denatured in a volume of 20 µl (18 µl, 2 µl 3 M NaOH, final concentration 0.3 M) (Carl Roth. Gmbh+Co) in water bath at 37 °C for 15 min. After denaturation, 12 µl of 0.1 M hydroquinone (Sigma) and 208 µl of 3.6 M sodium hydrogensulfite (Sigma) at pH 5 (400 µl of 3 M NaOH was added to sodium hydrogensulfite), both prepared freshly, then added and after mixing, the samples were incubated in a water bath at 55 °C for 16 h. Modified DNA was purified using wizard DNA purification resin (Promega) according to the manufacturer's protocol and eluted into 50 µl of water. Modification was completed with 5 µl NaOH (final concentration 0.3 M) treatment at water bath 37 °C for 15 min to remove the bisulfite product from the uracil ring, followed by 1 µl glycogen 2 µg/µl, 55 µl 7.5 M ammonium acetate and 250 µl absolute ethanol (Sigma). The samples were vigorously mixed, kept in –20 °C for 10 min, then centrifuged 13.000 rpm at RT for 10 min. The supernatants were removed, the pellets washed with 200 µl 75 % ethanol, followed by centrifugation 13.000 rpm at RT for 5 min. The supernatants were removed, DNA was dried, then suspended in 50 µl TE buffer and used immediately or stored at -20 °C.

2.7.2 NEP/CD10 MSP primer design

Methprimer program available in internet was used to obtain the area in untranscripted region of NEP/CD10 rich in CpG (Figure 8), that is indicator of promoter. The primers were designed in CpG island area. The following points were considered: (i) primers were designed to contain at least two times of CpG dinucleotides in their sequence. The primers contained CpG sites, they were designed to amplify methylated and unmethylated alleles equally (with a mixture of C or T used for the sense strand or a mixture of G or A used for antisense primers); (ii) primers were designed to contain a maximum number of thymidines converted from cytosines to avoid amplification of the no converted genomic sequence.

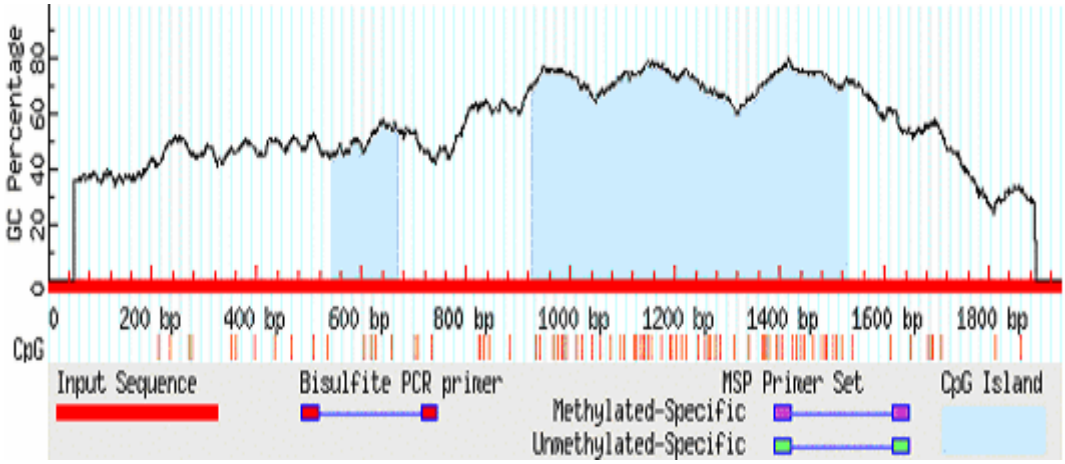


Fig. 8: NEP/CD10 promoter region: The untranscripted region in NEP/CD10 sequence where locate CpG island area in blue color that is an promoter indicator.

2.7.3 Methylation specific PCR assay

This assay is used to examine the methylation and unmethylated status of the gene promoters. Methylation Specific PCR (MSP) was carried out for the cell lines PA-TU-T, HUP-T4, HUP-T3, PA-TU-S, PA-TU-02, PANC-1, Capan-1, Capan-2, MIA PaCa-2 and FTC-238 that served as a positive control. Using 25 µl of reaction solution 4 x (two promoters to each methylated and unmethylated status) each sample (2.5 µl 10 x buffer, 2 µl 2.5 mM dNTP-mix, 4 µl 25 mM $MgCl_2$, 0.5 µl Taq gold (Roch), 3 µl of sodium bisulfite-treated DNA and 11 µl DNase free water), 1 µl methylated and unmethylated primers forward, reverse (Invitrogen) of NEP/CD10 promoter 1 and 2. The cycling conditions were

10 min at 95 °C, followed by 5 cycles of 94 °C for 30 s, 64 °C for 45 s and 72 °C for 45 s, then 35 cycles of 94 °C for 30 s, 62 °C for 45 s and 72 °C for 45 s with final extension elongated at 72 °C for 10 min, 4 °C. After amplification, PCR products were separated on 2 % agarose gels with ethidium bromide staining which gives a main product (Table 6).

Table 6: PCR primers of two NEP/CD10 promoters for MSP analysis

Primer pair	Methylated set (f, rev) Unmethylated set (f, rev)	Product size	Annealing temp.
NEP1 M	5`-GGGTCGGAGGGATGTTTAGGTGTTTC-3`	197 bp	62 °C
	5`-CTAAACCGACACATCCCGACCAATAAACG-3`		
UM	5`-GTGGGTTGGAGGGATGTTTAGGTGTTTT-3`	206 bp	62 °C
	5`-AAACTACTAAACCAACACATCCCAACCAATA-3`		
NEP2 M	5`-GCGGATGTACGGATTGAGAGGCG-3`	195 bp	62 °C
	5`-CCACGAACTCCCAACGCCCTAACG-3`		
UM	5`-TGGGTGGATGTATGGATTGAGAGGTG-3`	200 bp	62 °C
	5`-TCCCACAAACTCCCAACACCCTAACA-3`		

2.8 Cell culture stimulation

Cell lines, PA-TU-T, HUP-T4, PA-TU-S, MIA PaCa-2 and PANC-1 were grown in complete medium until sub confluence, followed by 24 h serum free medium interval before stimulation for 24, 48, 72 h with the following reagents: 0 (untreated); 5-aza-2-deoxycytidine (Sigma) 1 µM; sodium butyrate (Merck, Darmstadt, Germany) 2 mM BA; and valproic acid (Sigma) 2 mM VPA. The medium was changed every day and after the appropriate stimulation time cells were prepared for analysis of gene and protein expression.

2.9 Transformation

Competent cells E. coli DH5 α (Invitrogen) and the three vectors pEGFP-C3 (BD Biosciences Clontech), NEP-pEGFP-C3 and mutated NEP^{H587E}-pEGFP-C3, with mutation in enzyme active site, histidine was substituted with glutamic acid were used. The latter two were prepared by Jens Wulfänger and René Keil (Institute of Medical Immunology,

Martin Luther University). Plasmids were removed from -70 °C; thawed on ice. Three 1.5 ml tubes were labeled and placed on ice then competent cells were gently mixed and 40 µl was pipetted in each three labeled tubes followed by 250 ng from the plasmids pEGFP-C3, NEP-pEGFP-C3 and mutated NEP^{H587E}-pEGFP-C3 each in separate tube. The tubes were incubated on ice for 30 min followed by heat shock at 42 °C for 45 s, then placed on ice for 2 min; 500 µl SOC liquid medium (Invitrogen) was added to each tube and left on shaker at 37 °C for 1 h. At this point, dry LB-agar (life tech., Scotland) plates with 50 µg/ml kanamycin that were made earlier in fume hood for approximately 30 min or until (remove lid plate) condensation drops were dried. The incubated tubes were controlled when the medium turned turbid, under sterile hood 100 µl from each tube was pipetted independently in suitable labeled plate followed by spreading the transformed E. coli DH5 α bacteria over the plates with sterile glass rod. The plates were incubated upside down in incubator at 37 °C over night. Under a sterile hood three sterile flasks were filled with 100 ml LB (life tech., Scotland) and kanamycin 50 µg/ml, by stick wood one clone (transformed bacteria) from each plate was picked out and dropped in every flask in separate. The three flasks were kept on shaker at 37 °C over night. From each transformed bacteria in turbid flasks 850 µl was pipetted in separated labeled tube and 150 µl of glycerol (Merck, Germany) was added to each tube, mixed and kept in –20 °C as stock. The rest was transferred to 50 ml tube, each flask in two tubes. The tubes were centrifuged at 4000 rpm for 10 min, then the supernatant was removed and discarded from each tube. pEGFP-C3, NEP-pEGFP-C3 and mutated NEP^{H587E}-pEGFP-C3 were isolated by plasmid isolation kit under the instruction protocol (Qiagen). Integrity, purity of the plasmids were confirmed by agarose gel electrophoresis and the concentration of each plasmid type was quantified by determination of optical density at 260, 280, 320 nm.

2.10 Killing curve

Before transfection, a killing curve was performed with various concentrations of the antibiotic to determine the amount of antibiotic required to kill the cells after one or two weeks. Since pEGFP-C3 plasmid has a resistance gene sequence against Geneticin

(Invitrogen), PANC-1 $5x10^4$/well was seeded in 24 well plate and incubated to grow into standard DMEM/F12 medium at 37 °C, 5 % CO_2 in an incubator. After 48 h the medium was changed and new medium with different concentrations of 0, 250, 500, 1000, 1500 and 2000 µg/ml Geneticin (Invitrogen) was added to the cells, in duplicate wells. The cells were observed under microscope every 48 h, medium was aspirated out of the wells and new medium with the appropriate concentration of Geneticin was added. After two weeks, the Geneticin concentration 1.5 mg/ml which could kill PANC-1 cell line was used after transfection to select clones.

2.11 Transfection and clone selection

To obtain the NEP/CD10 function in pancreatic adenocarcinoma cancer, PANC-1 cell that has no endogenous NEP/CD10 expression was transfected with expression vectors for pEGFP-C3, NEP-pEGFP-C3 and mutated NEP^{H587E}-pEGFP-C3. In a six-well culture plate, approximately $1x10^5$ cells/well was seeded in 2 ml of DMEM/F12 free of antibiotic supplemented with 10 % FCS. The cells were cultured in a humidified atmosphere containing 5 % CO_2 at 37 °C in an incubator until the cells were 50-70 % confluent. The following solutions were prepared in four 1.5 ml labeled sterile tubes. For each transfection, in each three tubes in separate, 50 µl serum-free Opti-MEM (Invitrogen), 1 µg DNA (pEGFP-C3, NEP-pEGFP-C3, mutated NEP^{H587E}-pEGFP-C3 plasmids) and in the fourth tube 9 µl of Lipofectamine 2000 reagent 1 µg/µl (Invitrogen) was diluted into 150 µl of serum-free Opti-MEM (Invitrogen). For each tube with the different plasmids, 50 µl of Lipofectamine 2000-Opti-MEM complex was added. The tubes were gently mixed and incubated at room temperature to combine for 20 min. During this time the cells were washed with 2 ml of antibiotic-free medium followed with 1 ml of antibiotic-free DMEM/F12 supplement with 10 % FCS. Total 100 µl of pEGFP-C3, NEP-pEGFP-C3 and mutated NEP^{H587E}-pEGFP-C3–Lipofectamine 2000-Opti-MEM complex was added each in separate well. The transfected cells were incubated at 37 °C over night in an incubator. The medium was replaced with DMEM/F12 standard growth medium and the transfected cells were controlled under fluorescent microscope whether intense green fluorescence

colour could be seen, then incubated at 37 °C over night in an incubator. To select clones, medium was replaced with Geneticin (Invitrogen) 1.5 mg/ml DMEM/F12 medium. Every 48 h new Geneticin 1.5 mg/ml medium was added to the cells and checked under fluorescent microscope whether clones were formed. After two weeks, PANC-1-pEGFP-C3, –NEP-pEGFP-C3 and mutated- NEP^{H587E}-pEGFP-C3 clones (Table 7) were marked, picked out, then pipetted in 96 well plates, each clone in one well which has Geneticin (1.5 mg/ml) in DMEM/F12 standard growth medium. The plate was incubated at 37 °C in an incubator, every 48 h medium was replaced until confluent. The desired clones of cells were transferred to 24 well plate and so on, then sorted depending on intensity of green fluorescent protein using FACS instrument by PD Dr. med. Dagmar Riemann (Institute of Medical Immunology, Martin Luther University). The clones of cells were expanded, then evaluated for mRNA and protein expression by Real Time-PCR and flow cytometer respectively.

Table 7: Name and character of plasmid and clones used in the study

Name	Used name	NEP/CD10 expression	Enzyme activity
pEGFP-C3 (express GFP)	Mock1	no	no
p-C3 (express no GFP)	Mock2	no	no
NEP-pEGFP-C3	Wild type NEP CL1 and 2	yes	yes (high)
NEP^{H587E}-pEGFP-C3	Mutated mNEP CL1 and 2	yes	yes (low)

2.12 Freezing, thawing and adapting of transformed cells

2.12.1 Freezing cells

The selected Mock, NEP and mNEP clones that had low, middle and high expression of NEP/CD10, were cultured to proliferate in DMEM/F12 standard growth medium with 1.5 mg/ml Geneticin in a humidified atmosphere containing 5 % CO_2 at 37 °C in an incubator until the cells were confluent in 75 cm^2 cell culture flask. Every clone was prepared and collected in preserved solution contain 90 % FCS and 10 % DMSO, then divided in cryotubes (Greiner Bio-one) each tube containing 1 ml. The tubes were kept in

a slow-freezing container in –20 °C overnight, then transferred to liquid nitrogen tank for long preservation, using gloves and face protection.

2.12.2 Thawing cells

The desired clones suited for further investigation were thawed quickly using pre warmed DMEM/F12 standard growth medium 37 °C in water bath to prevent them from dying. Under sterile bank, the tube contents were transferred into 15 ml tube with regular medium, then centrifuged at 1500 rpm for 10 min. The cells were resuspended in DMEM/F12 standard growth medium with Geneticin 1.5 mg/ml, then transferred to cell culture plates to grow over night. The cells were observed under microscope, the medium was changed and cultured in an incubator to grow. Every 48 h medium was changed, the cells were split when they became confluent and $1x10^5$ cells were pipetted in culture plate with DMEM/F12 standard growth medium and Geneticin 1.5 mg/ml.

After three passages the clones were used to evaluate planned investigation.

2.13 Microarray gene expression analysis

Total cellular RNA of pancreatic adenocarcinoma cell lines PANC-1, Mock1 and NEP CL2 was lysed in 1 ml of TRIzol™ reagent (WKS) and total cellular RNA was isolated from cells according to the manufacturers instructions developed by Chomczynski & Sacchi.[90] The RNA integrity was confirmed by agarose-formaldehyde electrophoresis under denaturing conditions in 2.2 M formaldehyde (Fluka) according to Maniatis et al., using the MOPS buffer (Merck) system. Agarose is prepared by melting the required amount of agarose in DEPC water (Sigma), (40 % formaldehyde and 10 x MOPS were diluted in DEPC water to give 2.2 M formaldehyde and 1 x MOPS, respectively) then cooling to approximately 60 °C. Prior to loading, RNA samples were heated to 65 °C for approximately 10 min to denature any secondary structure, cooled on ice for 2 min and 2 µl of sterile loading buffer was added. Samples were loaded onto the gel, followed by electrophoreses. Visualization of RNA was achieved by illumination with UV light. Typical marker of RNA quality are 18S (~1900 bases) and 28S (~4800 bases) rRNA molecules. RNA samples 40 µg of PANC-1, Mock and NEP CL2 were sent to RZPD in Rostock for

application of Affymetrix chip array. Double stranded cDNA synthesis was performed using a superscript double-stranded cDNA synthesis kit. In vitro where transcription of biotin-labeled cRNA probes was done following the kit's instructions (Affymetrix). The cRNA probes were chemically fragmented using a fragmentation buffer (Affymetrix) and fragmented biotin-labeled cRNA was hybridized on Affymetrix human genome U133 2A chips. Genechip® Human Genome U133A 2A could analyses about 54.700 transcripts, this already containing 14.500 human characterized genes. On the used U133A 2A chips, every transcript is existing as set of 11 probe pairs, where by every probe pairs comprises one perfect-match and one miss-match. The perfect-match is the precise reverse complement of transcript sequence and the miss-match is the same oligonucleotide (25 mer) with bases substitution in the middle of transcript. Through comparison of hybridization signals between perfect-match and miss-match specific or not specific hybridization could be differentiated. The U133A 2A Chip of Mock was evaluated against PANC-1 and NEP CL2 to determine the discrepancy of gene expressions. The data of Affymetrix were changed to Excel data and the raw micro array data files were analyzed. For illustration the Affymetrix data, the signals were classified in present (P), absent (A) or marginal (M). The data were normalized using the robust multi-array average (RMA) algorithm. Gene expression ratios were calculated. Ratios greater than 2.0 were considered over expressed, otherwise they were considered as normal/under-expressed. The data were exploited using Eva program by Ivonne Pöschel (Institute of Medical Immunology, Martin Luther University).

2.14 Cell proliferation assay

Analysis of cell proliferation was performed on PANC-1, Mock1,2, wild type NEP1,2 and mutated NEP1,2 clones using a MTT assay, first described by Mosmann.[91] The product 3 -(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma) is a yellow water soluble tetrazolium dye that is reduced by living cells, but not by dead ones, to a purple formazan product that is insoluble in aqueous solutions. The cells were cultured 4 times into 96 wells plates $2x10^3$ cells/well in 200 µl of DMEM/F12 medium supplemented with

2 mM L-glutamine, 1 % penicillin/streptomycin, heat inactivated 5 % FCS, 5 % horse serum and allowed to attach for 24 h, followed by 72 h serum free medium to synchronize the cells, Geneticin 1.5 mg/ml medium was added to Mocks and clones. After three days starvation one plate was performed for MTT test as 0 h. The medium supplements with 5 % FCS was added to three well plates, Geneticin 1.5 mg/ml medium was added to Mocks and clones, every day medium was changed. At each time point 24, 48, 72 h MTT 5 mg/ml (Sigma) 20 µl was added to each well and the plates were incubated at 37 °C for 4 h to allow the conversion of MTT to formazan by mitochondrial dehydrogenase. The medium was then removed, formazan crystals were dissolved in 100 µl DMSO and gently mixed. Formazan quantification was performed measuring the optical density at 570 nm using 96 well multiscanner auto reader (TECAN, Austria GmbH). The proliferation rate was expressed as OD and comparing Mock, NEP and mNEP clones with PANC-1 cells.

2.15 NEP/CD10 indirect immunofluorescence

Because PANC-1 cell line and established clones need an adhesive surface to grow on, the glass slides were coated with poly-L-lysine hydrobromide (Sigma). In brief, the glass slides were immersed in 50 % H_2SO_4 for 1 h using a porcelain rack, then washed for 30 min in running tab water followed by rinse with dH_2O. The glass slides were immersed in 40 µg/ml of poly-L-lysine hydrobromide at RT for 1 h, then washed in running tap water for 1 h, followed by rinse 3 times 5 min each in dH_2O. The glass slides were dried on filter paper in dust-free area. The dried glass slides were arranged in porcelain rack and completely covered with foil aluminium, then sterilized in dry autoclave at 180 °C for 2 h. The confluent PANC-1, Mock, NEP and mNEP clones were detached from culture plates by incubating them in trypsin-EDTA (Invitrogen). Detached cells were resuspended in culture medium, then centrifuged. The pellets were resuspended again with new culture medium, counted under microscope using haemocytometer. Total number $2x10^4$ cells/ml of PANC-1, Mock, NEP and mNEP clones, 100 µl was dropped twice separately on each sterilized glass slide coated with poly-L-lysine hydrobromide in culture dishes, incubated in a humidified atmosphere containing 5 % CO_2 at 37 °C in an incubator up to cells were

semi confluent. The cells were rinsed with PBS once 5 min, followed by fixation with paraformaldhyde 2 % in PBS at RT for 15 min, followed by 3 times washing 5 min each in PBS. In every glass slide, the two areas with the fixed cells were closely circled with fatty pen (DAKO A/S), then 100 µl of 1:40 mouse anti-human NEP mAb (ALB_2, IgG_2a, immunotech, Hamburg, Germany) in one circle and 100 µl of 1:200 iso-IgG_2a kappa (Pharmingen) as control in the other spot. The glass slides were incubated in a wet chamber at RT for 40 min, then washed with PBS 4 times, 5 min each, followed by incubation with 50 µl of 1:100 diluted secondary antibody Cy3-conjugated anti-mouse IgG-$f(ab')_2$-fragment (Dianova, Hamburg, Germany) at RT for 40 min, protected from light. The cells were washed 4 times, 5 min each and 50 µl of 1:100 diluted 10 mg/ml Hoechst stain (Sigma) was added at RT for 1 min, protected from light, followed by washing 4 times for 5 min each. The Fluorescent mounting medium (DAKO cytomation, Carpinteria USA) was dispensed on the cells and the cover slide was applied on the glass slide. The expression of NEP/CD10 was photographed using fluorescence microscope.

2.16 Cell cycle analysis

The cell cycle analysis was performed as follows. In brief, $3x10^5$ of PANC-1, Mock, wild type NEP and mutated mNEP clones 4 times for each were seeded in small culture flasks parallel with proliferation test and incubated to grow into DMEM/F12 with heat inactivated 5 % FCS, 5 % horse serum and 2 mM L-glutamine at 37 °C, 5 % CO_2 in an incubator over night. For starvation of the cells, serum free-medium was added to PANC-1 cell line and in the same way, Geneticin in medium 1.5 mg/ml was also added to Mock, NEP and mNEP clones, then incubated in a humidified atmosphere containing 5 % CO_2 at 37 °C in incubator 72 h.

The cells of one flask from each PANC-1, Mock, wild type NEP and mutated mNEP clones as 0 h were prepared for cell cycle and the rest were cultured in 5 % FCS medium for PANC-1 and 5 % FCS medium with Geneticin 1.5 mg/ml for Mock, NEP and mNEP clones, then incubated in humidified atmosphere containing 5 % CO_2 at 37 °C in an incubator. Every day medium was changed and at target time point 24, 48 and 72 h the

cells of each flask were prepared for cell cycle analysis. The cells were detached from flasks by trypsin followed by washing twice with PBS, suspended in 150 µl 0.1 % glucose/PBS, then 1.5 ml of cold ethanol (-20 °C) as drops was added and gently mixed to fix the cells. The tubes were kept in refrigerator (4 °C) until performed for cell cycle analysis. The fixed cells of PANC-1, Mock, NEP and mNEP clones were centrifuged at 1500 rpm for 3 min. The ethanol was discharged from the tubes followed by washing twice with 1 ml PBS, then centrifuged at 1500 rpm for 3 min. PBS was discharged from the tubes, the pellet of each tube was suspended in mixture of RNase A 0.02 ml (10 mg/ml) (Sigma), in PBS and 0.3 ml (100 µg/ml) propidium iodide (Calbiochem, San Diego, CA), dissolved in PBS, then incubated at 37 °C for 30 min in water bath. The DNA content was then analyzed by a FACScan flow cytometer using the Cell Quest 3.1 software (Becton Dickinson) for acquisition and the Mod Fit LT 2.0 software (Verity, Topsham, ME) for analysis.

2.17 NEP/CD10 activity assay

Enzyme activity of NEP/CD10 was determined according to the method originally described by Mari et al and modified for microassay.[92,93] PA-TU-T, PA-TU-02, HUP-T4, PANC-1, Mock, wild type and mutated NEP clones, $2x10^5$ cells/50 µl PBS were incubated in PBS, with or without 10 µm thiorphan (Fluka), or 50 µM amastatin (Sigma) in separate for 20 min in 96 well microtiter plate (Greiner Bio-one) on shake instrument (Infors AG, Bottmingen, Germany) at 37 °C, then Suc-Ala-Ala-Phe-pNA substrate (Serva electrophoresis Gmbh) and aminopeptidase N prepared by PD Dr. med. Dagmar Riemann (Institute of Medical Immunology, Martin Luther University) were added in a volume of 200 µl (final concentration 1 mM, 0.5 U/ml, respectively) and incubated at 37 °C for 90 min. Optical density was measured at 405 nm using 96 well multiscanner auto reader (TECAN). NEP/CD10 activity was expressed as the amount of hydrolyzed substrate (µM/ min/$1x10^6$ cells).

2.18 Cell migration assays

Cell migration is a fundamental function of normal cellular processes, including embryonic development, angiogenesis, wound healing, immune response and inflammation. Microporous membrane inserts are widely used for cell migration and invasion assays. Migration experiments were performed using 10 mm tissue culture inserts with 8.0 µm polycarbonate membranes (Greiner Bio-one) as described elsewhere with minor modifications.[94] $3x10^5$ of PANC-1, Mock, wild type and mutated NEP clones were seeded twice in 6 well culture plates and incubated to grow into DMEM/F12 with heat inactivated 5 % FCS, 5 % horse serum and 2 mM L-glutamine at 37 °C, 5 % CO_2 in an incubator over night. The growing cells were cultured in serum-free media for 48 h. The cells were harvested, washed and resuspended in serum-free DMEM/F12. The cells were counted and 200 µl was added to the upper chambers at concentrations of $2x10^4$ cells/well. Media in lower chambers contained 1 % FCS. Cells were allowed to migrate at 37 °C for 18 h in a humidified atmosphere containing 5 % CO_2. Cells were washed with cold PBS, for 10 min, then fixed in 100 % methanol (Sigma), for 15 min followed by staining and visualized with toluidine blue (Merck, Darmstadt, Germany), 0.1 % in 2.5 % sodium acetate (Carl Roth, Karlsruhe, Germany). No migrated cells on upper side of the membrane were removed with a cotton swab. Migrated cells attached to the lower side of the membrane were enumerated using light microscope at 20 x magnification. Each data point represents the average cell number of five independent microscopic fields from each experiment. Statistical analyses were performed using an unpaired t-test.

2.19 Transfilter invasion assay

Invasion of adenocarcinoma cells into basement membrane has been used to characterize involvement of ECM receptors and matrix degrading enzymes which play roles in adenocarcinoma progression.

PANC-1, Mock, wild type and mutated NEP clones $3x10^5$ were seeded twice in 6 well culture plates and incubated to grow into DMEM/F12 with heat inactivated 5 % FCS, 5 % horse serum and 2 mM L-glutamine at 37 °C, 5 % CO_2 in an incubator over night. The

growing cells were cultured in serum-free media for 48 h. Cell culture inserts (Greiner Bio-one) containing trans well filters with 8 µm pores were precoated on upper surfaces with laminin 0.2 µg/ml (Sigma) and incubated under sterile bank at RT 2 h followed by 2 % BSA (Sigma) in PBS to block nonspecific binding for 1 h. During this time, the cells were harvested, washed and resuspended in serum-free DMEM/F12. The cells were counted, 200 µl of serum-free media containing a total of $1x10^5$ cells/ml of PANC-1, Mock, wild type and mutated NEP clones were placed in the upper compartment, media in lower chambers contained 1 % FCS and incubated at 37 °C for 16 h. Cells were washed with cold PBS for 10 min then fixed in 100 % methanol (Sigma) for 15 min followed by staining and visualized with toluidine blue (Merck) 0.1 % in 2.5 % sodium acetate (Carl Roth). The top surface of the filters were wiped to remove all adherent cells. Cells that had migrated to the lower surface of the filter were quantified by counting the number of cells in five fields of view per filter, using 20 x magnification.

2.20 Extraction of total protein from cells

Total protein extracts were prepared from cells PANC-1, Mock, wild type and mutated NEP clones using a protein lyses buffer 0.6 ml containing [20 mM HEPES pH 7.4, 1 % Trition X-100, 2 mM EGTA (Sigma), 10 % glycerol (Merck), 1 mM DTT (Carl Roth)] and protease inhibitors leupeptin (2 µg/ml) and pepstatin (1 µg/ml) (Roche) were added just prior to the addition of lyses buffer to the cells. The tubes were centrifuged at 14.000 rpm at 4 °C for 15 min. The supernatant which contains the protein was transferred in a new 1.5 ml tube. The protein concentrations in the extracts were quantified using the Bradford method (Bio-RAD laboratory Gmbh) according to the manufacturer's instructions. The protein samples were stored at –20 °C for later analysis.

2.21 Western Blot

Western blot is a method used in molecular biosciences to detect proteins of interest in a given sample from a mixture of a great number of proteins using specific labeling of the target proteins with antibodies. Western blotting can give information about the size of

target protein (with comparison to a size marker or ladder in kDa) and also give information on protein expression (with comparison to a control such as untreated sample or another cell type or tissue). An appropriate percentage of SDS-PAGE gel for protein of interest is used to obtain optimal separation. Typically 10 and 12 % resolving gel is used at the bottom with a pH 8.8 and a stacking gel (4-5 %) pH 6.8 is used to pack proteins together after loading was pipetted separately in a mini-Protean II device (Bio-Rad). From each extract of PANC-1, Mock, wild type and mutated NEP clones, 30 µg of total protein was mixed with loading buffer (0.04 % bromophenol blue) (3:1) proportionally and denatured at 95 °C for 5 min. The denatured proteins were loaded in slots of SDS-PAGE gel, then electrophoresis was performed at 20-40 mA for about 2 h at room temperature. Proteins are separated according to their mass into bands within each lane depending on molecular weight of proteins using separating buffer that contained [3 g Tris (Fluka), 14.4 g glycine (Serva electrophoresis), 600 mg SDS (Sigma)] in 1 liter dH_2O. After electrophoresis, separated proteins were transferred to presoaked 0.2 µm PVDF membranes (Amersham Biosciences, Freiburg, Germany) in methanol. The protein was blotted using transfer buffer that contained 9 g Tris-HCl, 42 g glycine and 600 ml methanol in 3 liter dH_2O, at 1 A, 6 °C for 2 h using wet mini-Transblot cell, according to manufacturer's instructions. After transfer, the separated proteins were visualized using ponceau stain (Sigma) to determine that proteins have migrated uniformly and evenly for a few seconds followed by washing with tape water to remove excess stain. Membranes were blocked in nonfat dry milk or bovine serum albumin dissolved in 1 x TBS-T (14 mM Tris, 154 mM NaCl, 0.1 % Tween-20, pH 7.5) or 1 x PBS-T (154 mM NaCl, 0.1 % Tween-20, pH 7.5) at RT for 1 h. Membranes were incubated with primary antibodies (table 7) at 4 °C over night. The membranes were washed in buffer depending on type of buffer used in blocking, three times, 10 min each. The antigen-antibody complexes were detected on membranes using the appropriate secondary antibodies conjugated to horseradish peroxidase (Promega, Madison, WI) at 1:20.000 dilution in buffer for 1-2 h at RT. The membranes were washed in buffer three times, 10 min each.

Table 8: Name of antibodies that were used in western blot analysis

Antibody	dilution	block buffer	second Ab.	MW
S100A4 (Dianova Gmbh)	1:500	PBS-T + 3 % BSA	goat anti rabbit	10 kDa
Rac 1/2/3 (Cell signaling Tech. Inc.)	1:500	TBS-T + 5 % N.F. milk	goat anti rabbit	18 kDa
Cdc 42 (Cell signaling Tech. Inc.)	1:500	TBS-T + 5 % N.F. milk	goat anti rabbit	18 kDa
Rho A (Cell signaling Tech. Inc.)	1:500	TBS-T + 5 % N.F. milk	goat anti rabbit	21 kDa
LIMK 1(Cell signaling Tech. Inc.)	1:500	TBS-T + 5 % N.F. milk	goat anti rabbit	60 kDa
Cofilin (Cell signaling Tech. Inc.)	1:500	TBS-T + 5 % N.F. milk	goat anti rabbit	18 kDa
phospho-Cofilin (Cell signalingTech. Inc.)	1:500	TBS-T + 5 % N.F. milk	goat anti rabbit	18 kDa
ß-Actin (Sigma)	1:10000	PBS-T + 3 % N.F. milk	goat anti mouse	45 kDa
Stathmin (Cell signaling Tech. Inc.)	1:1000	TBS-T + 5 % N.F. milk	goat anti rabbit	19 kDa
α-Tubuline (Invitrogen)	1:5000	PBS-T + 3 % BSA	goat anti mouse	55 kDa
acet.Tubuline (Sigma)	1:5000	PBS-T + 3 % BSA	goat anti mouse	55 kDa
GAPDH (Ambion)	1:10000	PBS-T + 3 % N.F. milk	goat anti mouse	36 kDa

2.21.1 Protein detection and results evaluation

Membranes were developed using ECL (Enhanced Chemiluminescence's) (Amersham Pharmacia Biotech). PVDF membrane was dipped in Pierce Western Blotting substrate Reagents for 1 min. Immunoreactivity was detected by exposing to X-ray film, amersham Hyper film^{TM} ECL (GE healthcare limited, Buckinghamshire, UK) in dark room for appropriate times to detect signal, then developed by immersion the film in developer for 1 min followed by washing step with tape water for few seconds in dark. The film was immersed in fixer solution (Amersham Pharmacia Biotech) for few minutes followed by washing step with tape water for few seconds in light, then left to dry. Numeric data were obtained by densitometry scanning analysis using Kodak Digital science 1D software (Kodak Digital Science Electrophoresis Documentation and Analysis System 120).

2.21.2 Membrane stripping

Membranes can be reused again to detect additional interesting protein. The membranes were immersed in stripping buffer pH 2.5 containing [15 g glycine, 0.5 ml tween-20 (Serva electrophoresis) in 1 liter dH_2O] to lose the antibody antigen immunoreactions followed by washing with TBS-T or PBS-T buffer depending on which antibody will being used next, three times 10 min each.

2.22 Statistical analysis

All experiments were reproduced at least three times with different passages of the pancreatic adenocarcinoma cell lines. The data of NEP/CD10 mRNA and protein expression levels are presented as mean and statistical analysis was performed using t-test. $P<0.05$ was considered statistically significant.

3 Results

3.1 Heterogeneous NEP/CD10 expression in different cell lines

3.1.1 NEP/CD10 mRNA expression

In vitro, the expression of NEP/CD10 was analyzed in 9 pancreatic adenocarcinoma cell lines. The NEP/CD10 mRNA expression of pancreatic adenocarcinoma cell lines was quantified in the presence of NEP/CD10 standard using a RG2000 cycler as described in methods (Figure 9). The pancreatic adenocarcinoma cell lines can be subdivided into four groups (Figure 10): one cell line with a high NEP/CD10 expression (PA-TU-T), one with intermediate expression (HUP-T4), one with a low expression (HUP-T3) and six cell lines (PA-TU-S, PA-TU-02, PANC-1, Capan-1, Capan-2 and MIA PaCa-2) where NEP/CD10 expression was not detected.

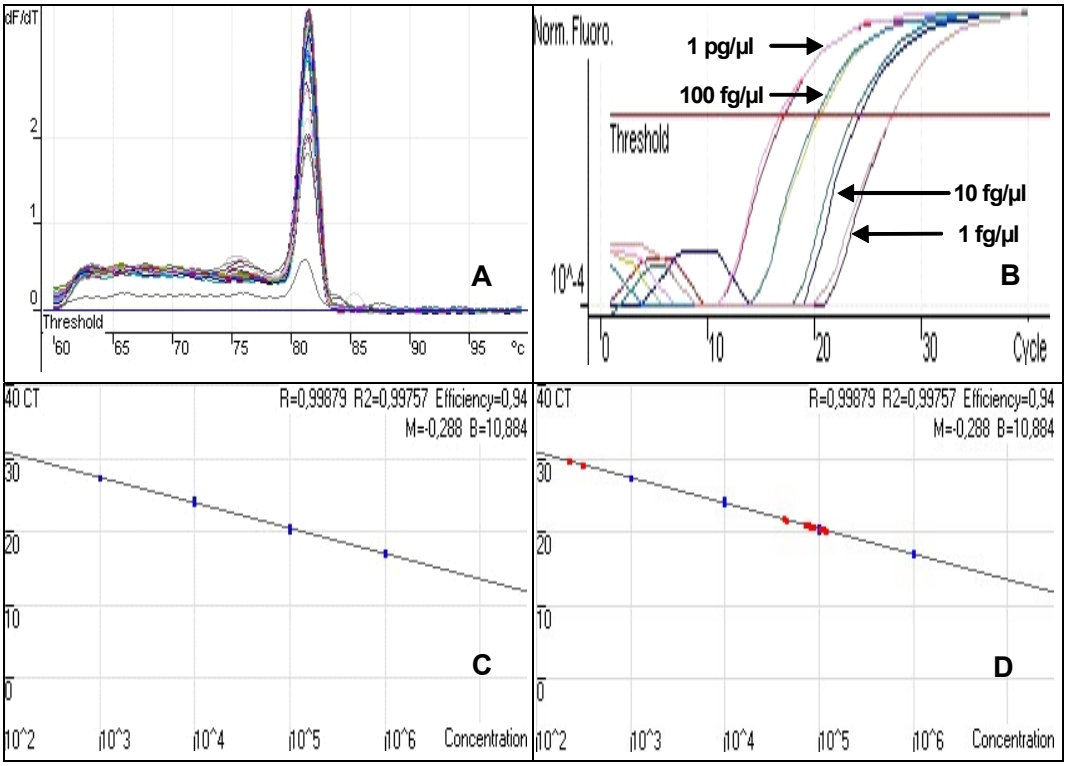


Fig. 9: Amplification plots of NEP/CD10 standard.

A: The melting curve analyses of the standard that is about 82ºC.
B: Shows PCR amplification of four serial dilutions of standard as a graph of log fluorescence intensity versus number of PCR cycles.
C: Shows the resulting standard curve as a graph of log concentration versus crossing point (1 fg, 10 fg, 100 fg, 1 pg).
D: Shows the resulting standard curve and samples as a graph of log concentration versus crossing point.

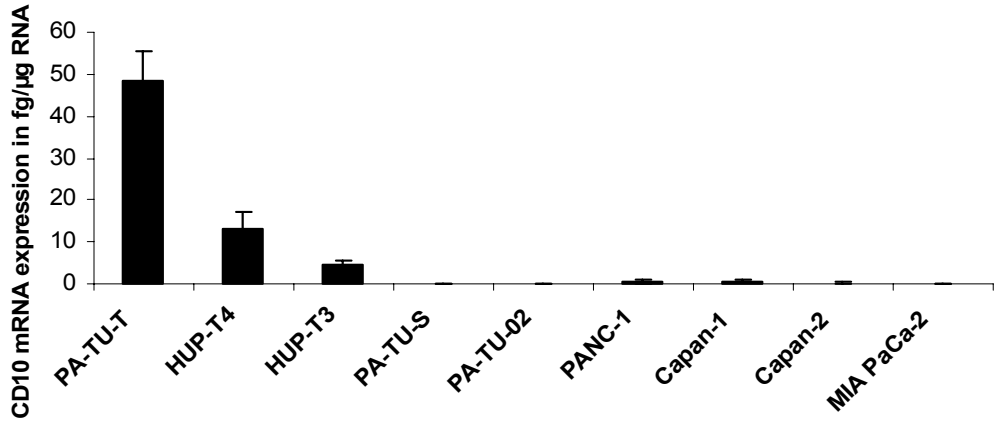


Fig. 10: Expression of NEP/CD10 mRNA. The RNA of examined pancreatic adenocarcinoma cell lines was isolated, cDNA was prepared as described in materials and methods . NEP/CD10 mRNA expression was quantified using real time-PCR.

3.1.2 NEP/CD10 protein expression

NEP/CD10 protein immunoreactive on the cell surface membranes of 9 pancreatic adenocarcinoma cell lines was measured by flow cytometry as described previously. NEP/CD10 protein expression was similar to the NEP/CD10 mRNA expression data (Figure 11). Regarding NEP/CD10 protein expression on the cell membranes of the cell lines, the tested pancreatic adenocarcinoma cell lines were subdivided into four groups: one cell line with a high NEP/CD10 expression (PA-TU-T), one with intermediate expression (HUP-T4), one with a low expression (HUP-T3) and six cell lines (PA-TU-S, PA-TU-02, PANC-1, CAPAN-1, CAPAN-2 and MIA PaCa-2) where NEP/CD10 expression was not detected.

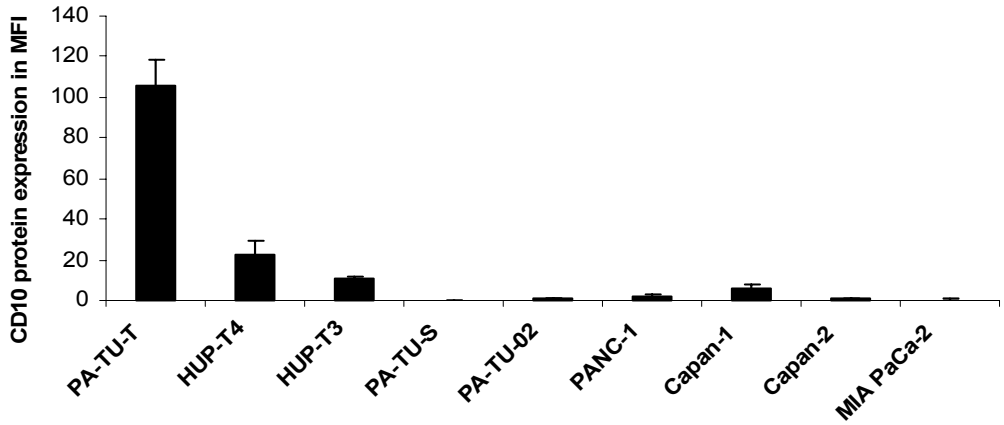


Fig. 11: NEP/CD10 protein expression. The pancreatic adenocarcinoma cell lines were reacted with anti-NEP/CD10 as described above and NEP/CD10 protein immunoreactive on the cell surface membranes of pancreatic adenocarcinoma cell lines was performed by flow cytometry.

3.1.3 NEP/CD10 enzyme activity

NEP/CD10 enzyme activity of pancreatic adenocarcinoma cell lines, PA-TU-T, PA-TU-02, HUP-T4 and PANC-1 was measured. From the result there is relation between protein expression and enzyme activity. PA-TU-T that expresses high NEP/CD10 protein also has high enzyme activity, HUP-T4 that expresses middle NEP/CD10 protein also has middle enzyme activity. Whereas on the other side both cell lines PA-TU-02, PANC-1 have neither NEP/CD10 protein expression nor enzyme activity (Figure 12). The NEP/CD10 enzyme activity in these cell lines was at the most inhibited by pretreatment with 10mM thiorphan a specific inhibitor of NEP/CD10. Furthermore 50mM amastatin, a specific inhibitor of CD13/APN also reduced enzyme activity of examined cell lines.

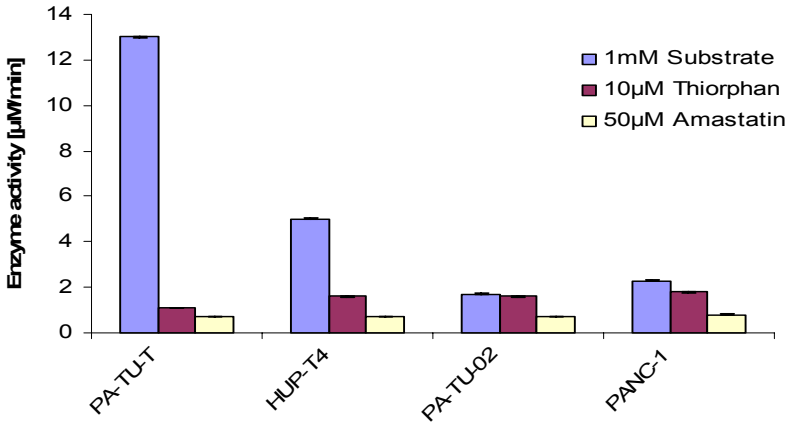


Fig. 12: NEP/CD10 enzyme activity. Characterization of the enzymatic activities responsible for the release of paranitroaniline from Suc-Ala-Ala-Phe-pNA. Total cell number of PA-TU-T, HUP-T4,PA-TU-02 and PANC-1 (2x10(5)/50 µl) were incubated at 37°C with 1 mM Suc-Ala-Ala-Phe-pNA as substrate in the presence of exogenously added aminopeptidase N. Enzymatic activity was determined in presence or absence of 10 µM thiorphan or 50 µM amastatine as described in Materials and methods.

3.2 NEP/CD10 promoters and methylation status

Methylation status of NEP/CD10 promoters in examined pancreatic adenocarcinoma cell lines was obtained using MSP method. The methylation status of the two promoters was variable in examined cell lines (Figure 13).

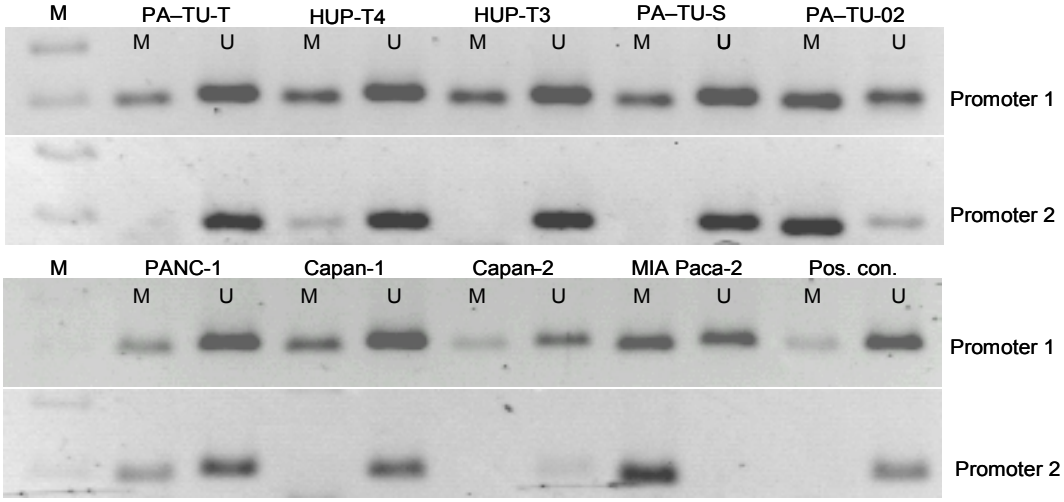


Fig.13: Methylation status of two NEP/CD10 promoters: DNA of examined pancreatic adenocarcinoma cell line was treated with sodium bisulfite and PCR was evaluated using MSP technique as described in materials and methods. M= methylated; U= unmethylated

As shown more detailed in table 9, promoter 1 was hemimethylated (one allel was methylated and the other not methylated) in all examined cell lines PA-TU-T, HUP-T4, HUP-T3, PA-TU-S, PATU-T-02, PANC-1, Capan-1, Capan-2 and MIA Paca-2, furthermore promoter 2 was hypermethylated (both allels are methylated) in MIA Paca-2, hemimethylated in HUP-T4, PATU-T-02 and PANC-1, others were unmethylated, in PA-TU-T, HUP-T3, PA-TU-S, Capan-1 and Capan-2.

Table 9:The distribution of NEP/CD10 promoters methylation

Cell lines	Promoter 1			Promoter 2		
	methylated	hemimethylated	unmethylated	methylated	hemimethylated	unmethylated
PA-TU-T						
HUP-T4						
HUP-T3						
PA-TU-S						
PA-TU-02						
PANC-1						
Capan-1						
Capan-2						
MIA Paca-2						

3.3 NEP/CD10 regulation on pancreatic carcinoma cell lines

3.3.1 Regulation of NEP/CD10 mRNA expression

The influence of either histone acetylation and promoter demethylation reagents on the NEP/CD10 mRNA expression of pancreatic adenocarcinoma cell lines was assessed by Real Time-PCR. NEP/CD10 mRNA expression of PA-TU-T and HUP-T4 cell lines was slightly up-regulated after stimulation with 1 µM 5-aza-2-deoxycytidine, while 2 mM butyric acid (BA) and valproic acid (VPA) induced more than 1.5 fold NEP/CD10 mRNA expression (Figure 14) ($P<0.05$). In contrast, the same reagents could not induce the NEP/CD10 mRNA expression in PA-TU-S, PA-TU-02, PANC-1 and MIA PaCa-2 cell lines (data not shown).

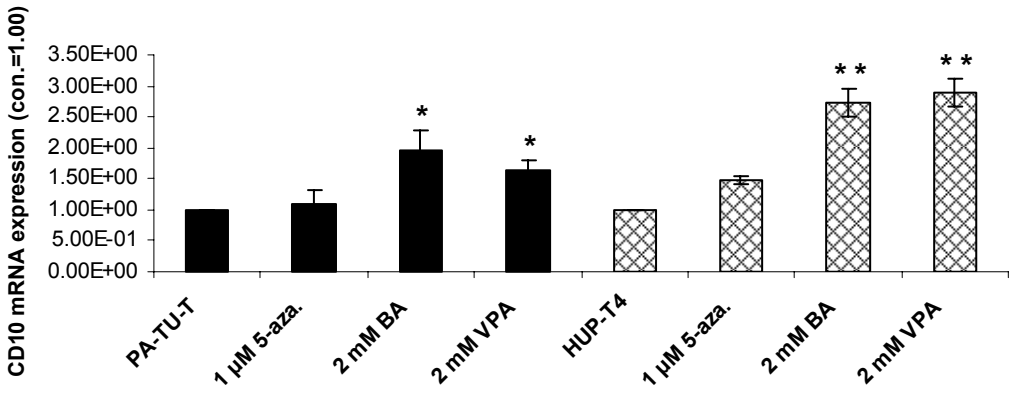


Fig. 14: NEP/CD10 mRNA expression: PA-TU-T and HUP-T4 were stimulated with 1 µM 5-aza-2-deoxycytidine (5-aza.), 2 mM butyric acid (BA) and 2 mM valproic acid (VPA) as described in materials and methods. RNA was isolated, then cDNA was transcribed and NEP/CD10 mRNA expression was quantified using real time-PCR.

3.3.2 Regulation of NEP/CD10 protein expression

The investigated cell lines were incubated with suitable concentrations of histone acetylation and promoter demethylation reagents. At the end of incubation time, cells were labelled with anti-CD10 antibody or with isotype control. Intensity of membrane NEP/CD10 expression was determined by means of flow cytometry. The NEP/CD10 protein expression was slightly up-regulated after stimulation with 1 µM 5-aza-2-deoxycytidine on PA-TU-T and HUP-T4 (Figure 15) cell lines, while 2 mM butyric acid (BA) and valproic

acid (VPA) induced more than 1.5 fold NEP/CD10 protein expression. In contrast, the same reagents could not induce the NEP/CD10 protein expression in PA-TU-02, PANC-1 PA-TU-S and MIA PaCa-2 cell lines (data not shown).

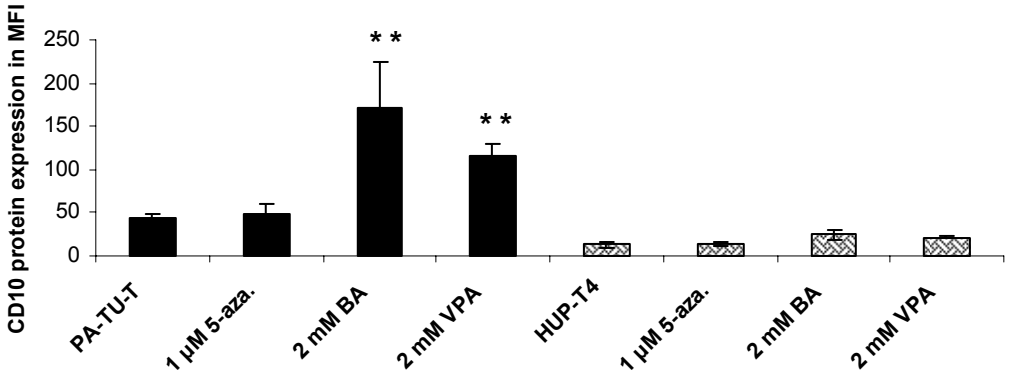


Fig. 15: **NEP/CD10 protein expression:** PA-TU-T and HUP-T4 were stimulated with 1 µM 5-aza-2-deoxycytidine (5-aza.), 2 mM butyric acid (BA) and 2 mM valproic acid (VPA) as described in materials and methods. Protein NEP/CD10 expression on cell membrane of cell lines was quantified using flow cytometry as MFI.

3.4 Transfection and NEP/CD10 expression on PANC-1 clones

3.4.1 NEP/CD10 mRNA over expression upon gene transfection

PANC-1 cell line was transfected with plasmids, pEGFP-C3 (Mock) alone or with insert encoding the CDS of wild type or mutated NEP. NEP/CD10 mRNA expression in PANC-1, Mock, NEP and mNEP clones was obtained using a RG2000 Cycler. NEP/CD10 mRNA expression in each NEP and mNEP clones was heterogeneous (Figure 16), that means in comparison to PANC-1, NEP/CD10 mRNA expression in wild type NEP clones was increased ~14 fold in NEP CL1 and ~64 fold in NEP CL2, whereas in mutated mNEP clones, NEP/CD10 mRNA expression was ~3 fold in mNEP CL1 and ~10 fold in mNEP CL2, statistically significant (< 0.05).

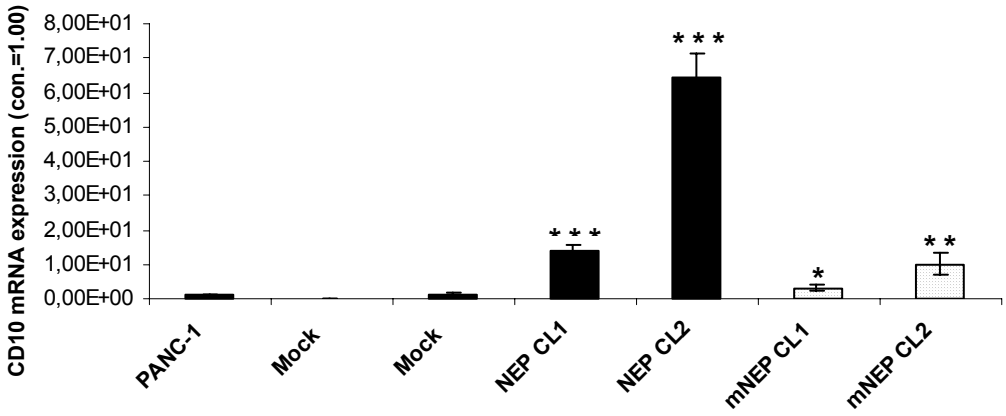


Fig.16: NEP/CD10 mRNA expression: Cellular RNA was isolated, then cDNA was transcribed, and NEP/CD10 mRNA expression in PANC-1, Mock, NEP and mNEP clones was quantified using Real Time-PCR.

3.4.2 NEP/CD10 protein over expression upon gene transfection

The NEP/CD10 protein expression on the cell surface membranes of PANC-1, Mock, wild type and mutated NEP clones was investigated using flow cytometry. As can be seen in figure 17, NEP/CD10 protein expression was heterogeneous in wild type NEP and mutated mNEP clones, that means in comparison to PANC-1, NEP/CD10 protein expression in wild type NEP clones was increased ~16 fold in NEP CL1 and ~66 fold in NEP CL2. Moreover in mutated mNEP clones, NEP/CD10 protein expression was augmented ~6 fold in mNEP CL1 and ~21 fold in mNEP CL2. Statistical significance was at the $P \leq 0.05$ level. This result is in good correlation to the mRNA expression of both the types of transfectants.

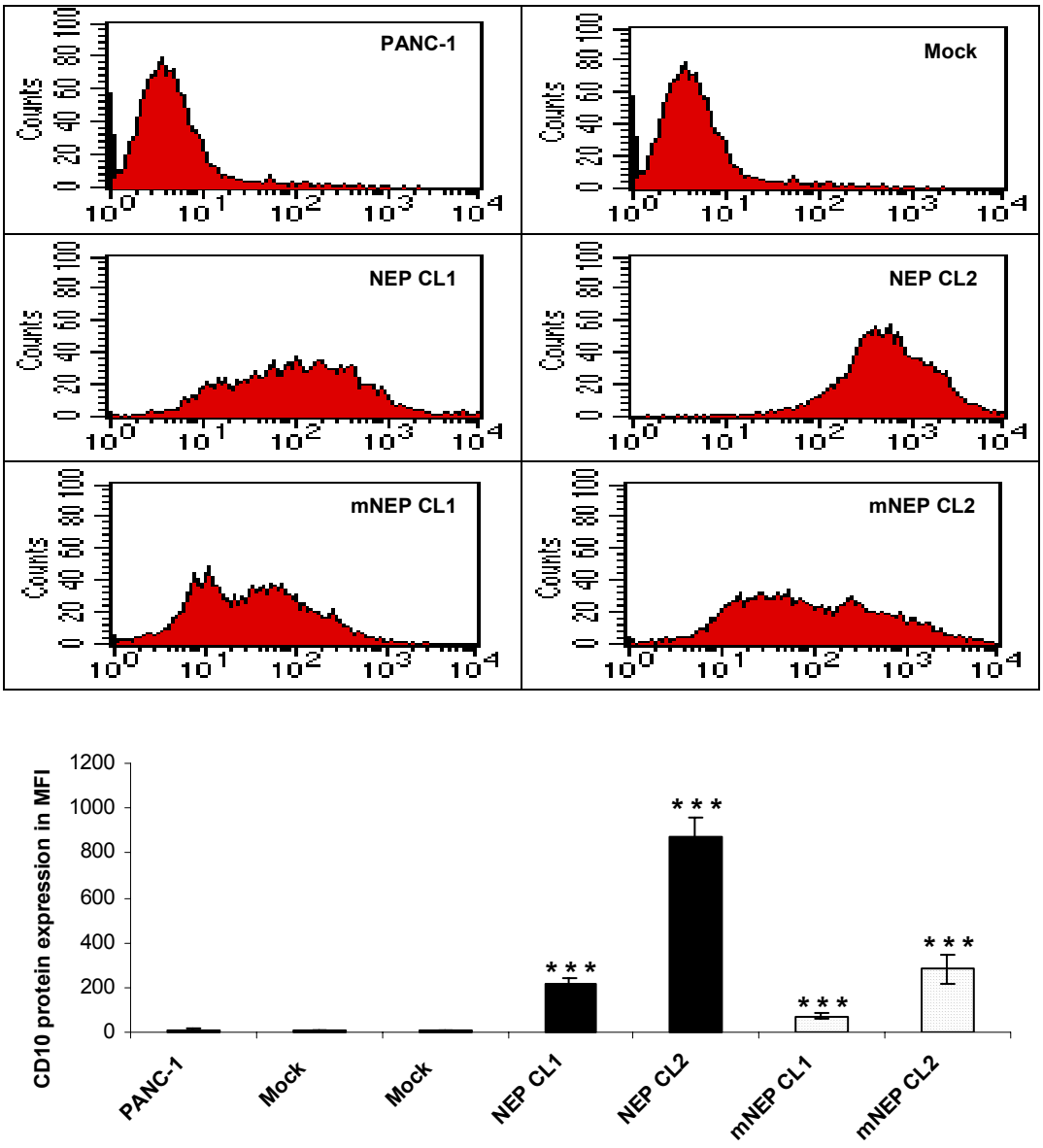


Fig. 17*:* The expression of NEP/CD10 protein: PANC-1, Mock, NEP and mNEP clones were immunoreacted with mab NEP/CD10 as described in materials and methods. NEP/CD10 protein expression on PANC-1, Mock, NEP and mNEP clones was quantified using flow cytometry (upper panel) and given in graphic as MFI (lower panel).

3.5 Immunofluorescence and localization of NEP/CD10

Localization pattern of the NEP/CD10 on pancreatic adenocarcinoma cell line PANC-1, Mock, wild type and mutated NEP clones was assessed. The immunoreaction distribution showed a general staining of whole plasma membrane as well as a positive immunoreaction of cytoplasm (Figure 18). Since pancreatic adenocarcinoma cell line PANC-1 was transfected with pEGFP-C3 plasmid, that express green fluorescence

protein in transfected cell line. The green fluorescent dye (Figure 18A) was distributed over all the investigated cells (Mock), on the other side, on NEP and mNEP clones, the green fluorescent dye was localized on the plasma membranes of the examined cells (Figure 18B). After staining PANC-1, Mock, wild type and mutated NEP clones with NEP/CD10 indirect PE-fluorescent Ab, no NEP/CD10 immunoreactive was observed on PANC-1 and Mock (negative data not shown), but on the other side on wild type NEP and mutated mNEP clones was found a strong immunoreaction on the plasma membrane and in the cytoplasm. NEP/CD10 was localized on the plasma membranes and distributed in the cytoplasm of the cells (Figure 18C). Since the CDS of NEP/CD10 was coupled with CDS of green fluorescence protein in NEP clones, two filters were used (Figure 18D) to confirm that NEP/CD10 antibody react specifically with NEP/CD10 protein, that coupled with green fluorescence protein.

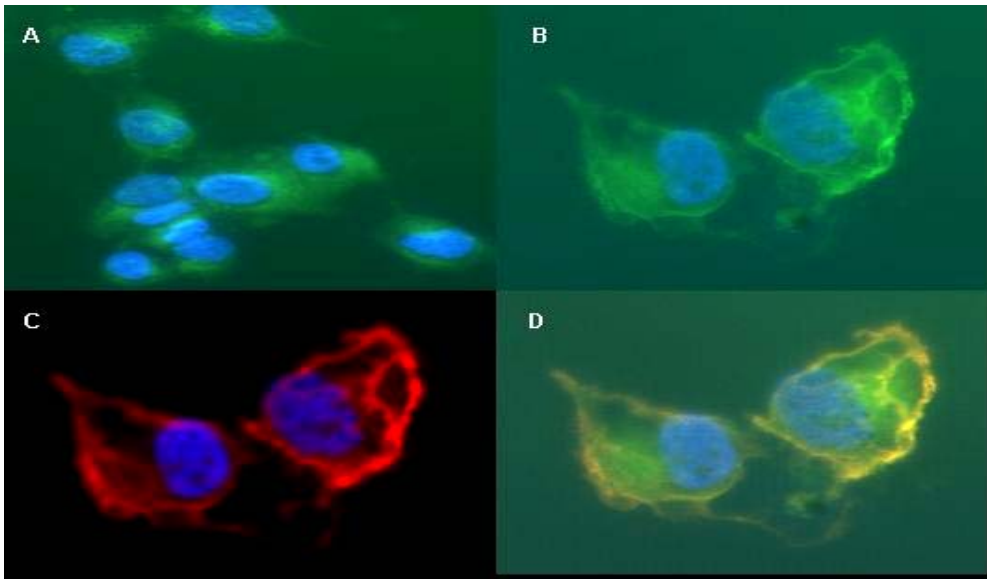


Fig. 18: Immunofluorescence localization of NEP/CD10: PANC-1, Mock, NEP and mNEP clones were cultured on glass slides, then immunoreacted with mab NEP/CD10 as described in materials and methods. NEP/CD10 localization on PANC-1, Mock, NEP and mNEP clones was photographed using fluorescent microscope. Mock shows green fluorescent dye distribution over all the cells (A). NEP and mNEP clones show green fluorescent dye localized on plasma membrane, and cytoplasm (B). NEP and mNEP clones show a strong immunoreactions on plasma membrane and cytoplasm (C). Two filters were used to confirm that immunoreactions are specific for NEP/CD10 coupled with green fluorescence protein (D).

3.6 NEP/CD10 enzyme activity assay

In order to find out whether NEP/CD10 protein expression was associated with enzyme activity, NEP/CD10 enzyme activity was examined by assessing the hydrolysis of succinyl-Ala-Ala-Phe-p-nitroanilide in total of $2x10^5$ cells of PANC-1, Mock, wild type NEP and mutated mNEP clones in absence or presence of 10 mM thiorphan or 50 mM amastatin for 90 min. At the end of incubation time, the enzyme activity of NEP/CD10 was measured using multi reader spectrophotometer suitable for 96 wells plate. As shown the high NEP/CD10 expressing NEP CL2 clone also exhibited a high level of enzyme activity (1000 %) comparing to PANC-1, whereas the moderate NEP/CD10 expressing NEP CL1 clone showed also moderate enzyme activity (800 %). Furthermore, mutated NEP clones, that have mutation in enzyme active site of NEP/CD10 had very low enzyme activity. These results demonstrated that the cell surface expression levels of NEP/CD10 were correlated with enzymatic activity. The NEP/CD10 activity in these cell lines was at the most inhibited by pretreatment with 10 mM thiorphan a specific inhibitor of NEP/CD10, the significant enzyme reduction was (91 %, 78 %) in wild type NEP CL1, NEP CL2, respectively. Furthermore 50 mM amastatin a specific inhibitor of CD13/APN also reduced enzyme activity (46 %, 39 %) in wild type NEP CL1, NEP CL2 respectively (Figure 19).

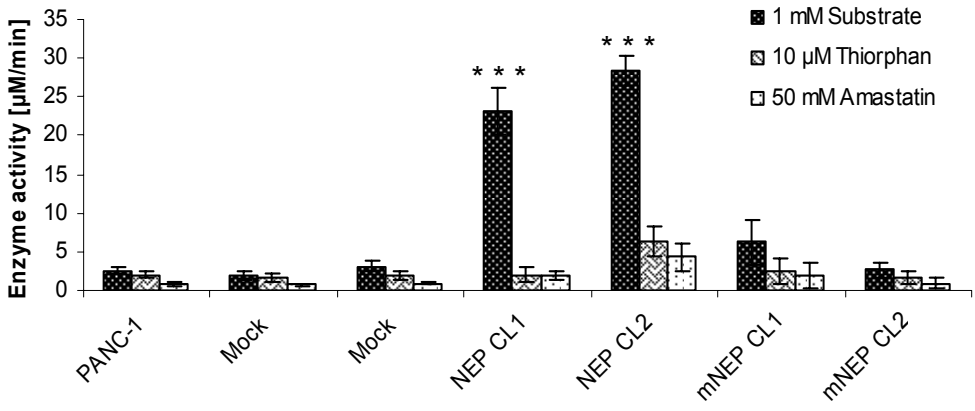


Fig. 19: Enzyme activity of NEP/CD10: Characterization of the enzymatic activities responsible for the release of paranitroaniline from Suc-Ala-Ala-Phe-pNA. Total cell number of PANC-1, Mock, NEP, and mNEP clones ($2x10^5$/50 µl) were incubated at 37 °C with 1 mM Suc-Ala-Ala-Phe-pNA as substrate in the presence of exogenously added aminopeptidase N. Enzymatic activity was determined in presence or absence of 10 µM thiorphan or 50 µM amastatin as described in materials and methods.

3.7 NEP/CD10 and cell lines proliferation assay

The proliferation rate and cell viability of PANC-1, Mock, wild type NEP and mutated mNEP clones were determined by MTT test every day (0, 24, 48, 72 h). Compared to PANC-1, a proliferation arrest effect was observed on either wild type NEP CL1 and 2 but only marginally on mutated mNEP CL1 and 2 clones. The reduced proliferate activity was readily apparent in NEP CL1 and 2, the reduction rate from 0-72 h was 24-28 % and 30-44 % respectively. This diminshed rate is the same at all time points (0-72 h). However, in mutated clones, mNEP CL1 and 2 the impaired proliferate activity was only marginally, the inhibition rate from 0-72 h was 0-19 % and 0-12 % respectively (Figure 20). The result was significant at all measuring time point 0-72 h ($p \leq 0.001$) of wild type NEP CL1 and 2.

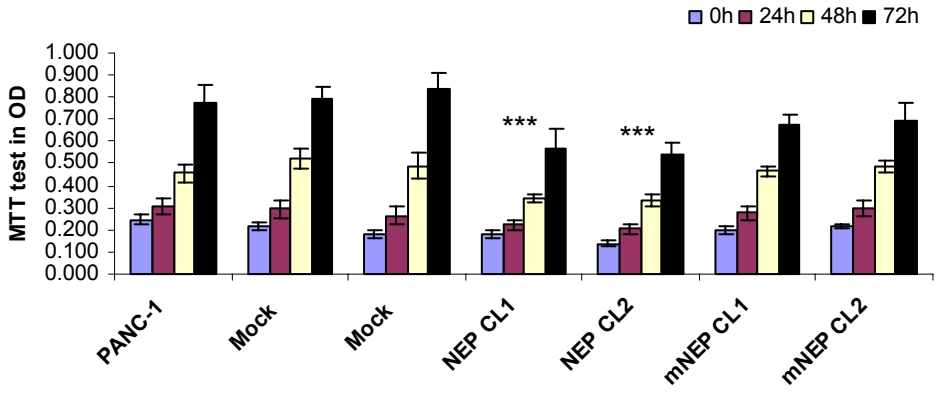


Fig. 20: Proliferation rate: Effect of NEP/CD10 on cell proliferation was examined using NEP/CD10 transfected PANC-1 adenocarcinoma cell line. The proliferation rate of PANC-1, Mock, NEP and mNEP clones was analyzed as described in Materials and Methods ($p \leq 0.001$).

3.8 Cell cycle analysis by flow cytometry

In the same time of measurement the cells proliferation rate, the cells were prepared for cell cycle to confirm proliferation rate result. The fixed cells were stained with PI, cell cycle phase was assayed by flow cytometry in parallel with proliferation analysis after synchronizing the cultured cells three days in serum free-medium as 0 h followed by

medium supplement with 5 % FCS for 24, 48 and 72 h. Histogram content of G0-G1, S- and G2/M phase distribution in PANC-1, Mock, NEP clones was presented (Figure 21A).

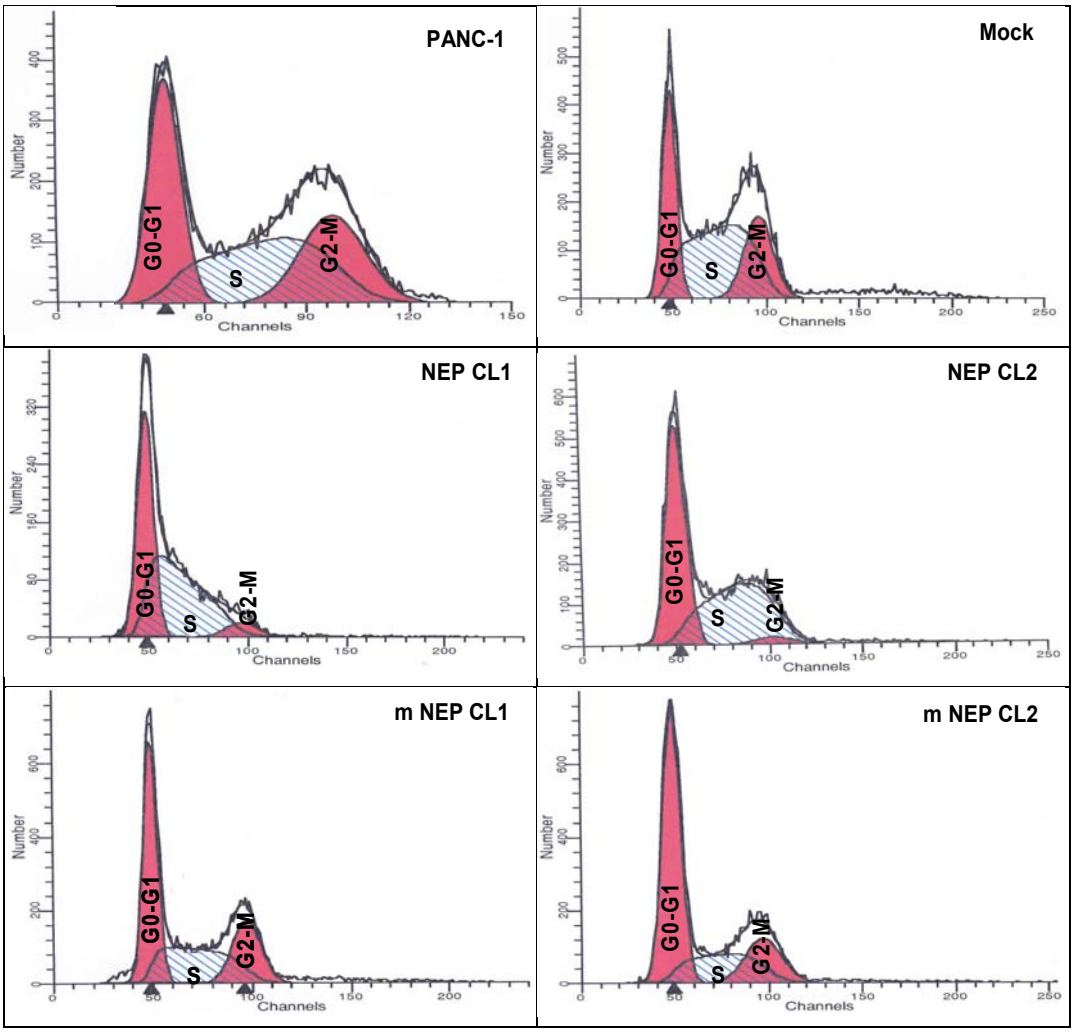


Fig. 21A: Cell cycle: Histogram content of G0-G1-, S- and G2/M phase distribution in PANC-1, Mock wild type and mutated NEP clones where the cells left three days in serum-free medium, followed by medium supplements with 5 % FCS for 24 h. Cell cycle of PANC-1 and clones was analyzed.

The mean of three times cell cycle results was obtained. In 0 h where the cells left to grow three days in serum free medium (Table 10), comparing to PANC-1 that has a markedly induced G2/M accumulation in the cells of 36.1 %, the rates of G2/M phase were 10.3 % in mock, 10.9 % in mock, 1.7 % in NEP CL1, 4.3 % in NEP CL2, 10.7 % in mNEP CL1 and 5.6 % in mNEP CL2 ($P<0.05$). The rate of S phase distribution was 13.4 % in PANC-1, 12.2 % in mock, 13.9 % in mock, 21.4 % in NEP CL1, 26.2 % in NEP CL2, 26.0 % in mNEP CL1 and 21.8 % in mNEP CL2. The rates of G0/G1 phase were 50.5 % in PANC-1,

77.5 % in mock, 75.2 % in mock, 76.9 % in NEP CL1, 69.5 % in NEP CL2, 63.6 % in mNEP CL1 and 72.6 % in mNEP CL2 (Figure 21B).

Table 10: Cell cycle distribution of PANC-1 and clones at 0h (after 3 days starvation).

0 h	PANC-1	Mock	Mock	NEP CL1	NEP CL2	mNEP CL1	mNEP CL2
G0-G1	50.5 ± 0.9	77.5 ± 1.6	75.2 ± 4.2	76.9± 1.7	69.5± 9.2	63.6 ± 1.4	72.6 ± 2.7
S	13.4 ± 1.7	12.2 ± 0.5	13.9 ± 3.0	21.4 ± 1.2	26.2± 4.9	26.0 ± 3.2	21.8 ± 3.4
G2-M	36.1± 2.6	10.3 ± 1.8	10.9 ± 1.2	1.7 ± 2.1	4.3± 4.7	10.4± 4.5	5.6± 6.1

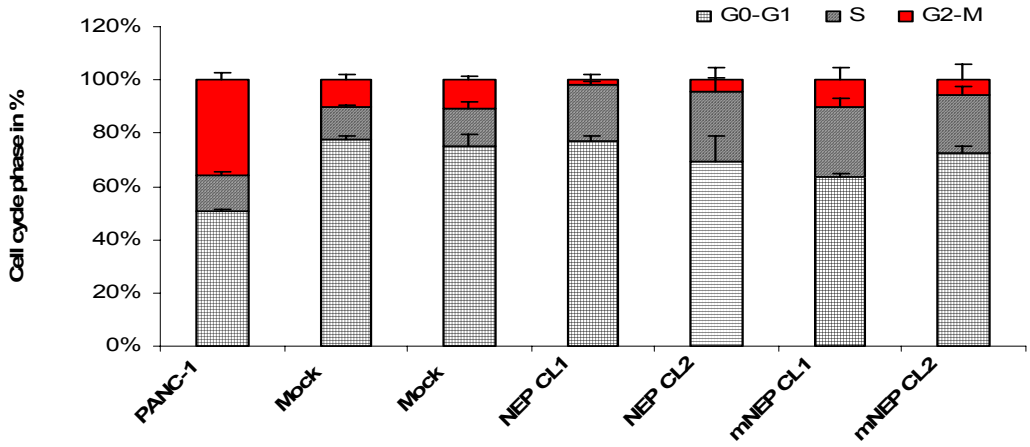


Fig. 21B: Cell cycle: The cells of PANC-1, Mock and clones were synchronized three days in serum free medium (0h), then the fixed cells were stained with PI. The cell cycle distribution (G0-G1-, S- and G2/M phase) was analyzed using flow cytometer, mean ± SD.

Furthermore, in 24 h where the cells left to grow one day in medium supplements with 5 % FCS (Table 11), comparing to PANC-1 G2/M accumulation in the cells were 30.3 %, while the rates of G2/M phase were 20.4 % in mock, 20.5 % in mock, 4.4 % in NEP CL1, 13.0 % in NEP CL2, 17.0 % in mNEP CL1 and 16.5 % in mNEP CL2 ($P<0.05$). Furthermore, the rate of S phase distribution was 35.9 % in PANC-1, 36.0 % in mock, 50.1 % in mock, 51.2 % in NEP CL1, 43.6 % in NEP CL2, 37.2 % in mNEP CL1 and 26.9 % in mNEP CL2. The rates of G0/G1 phase were 33.8 % in PANC-1, 43.6 % in mock, 29.4 % in mock, 44.4 % in NEP CL1, 43.4 % in NEP CL2, 45.8 % in mNEP CL1 and 56.6 % in mNEP CL2. The result demonstrated maximal G2/M accumulation in PANC-1, but in other side demonstrated minimal G2/M accumulation in NEP CL1,2 clones (Figure 21C).

Table 11: Cell cycle distribution of PANC-1 and clones at 24h (after 1 day 5% FCS).

24 h	PANC-1	Mock	Mock	NEP CL1	NEP CL2	mNEPCL1	mNEP CL2
G0-G1	33.8 ± 0.9	43.6 ± 6.0	29.4 ± 0.4	44.4± 1.7	43.4± 5.5	45.8± 4.0	56.6 ± 1.8
S	35.9 ± 7.6	36.0 ± 5.6	50.1 ± 7.7	51.2± 1.2	43.6± 3.6	37.2± 4.1	26.9 ± 8.6
G2-M	30.3 ± 7.4	20.4±11.4	20.5± 7.6	4.4 ± 2.1	13.0± 8.4	17.0± 7.3	16.5±10.0

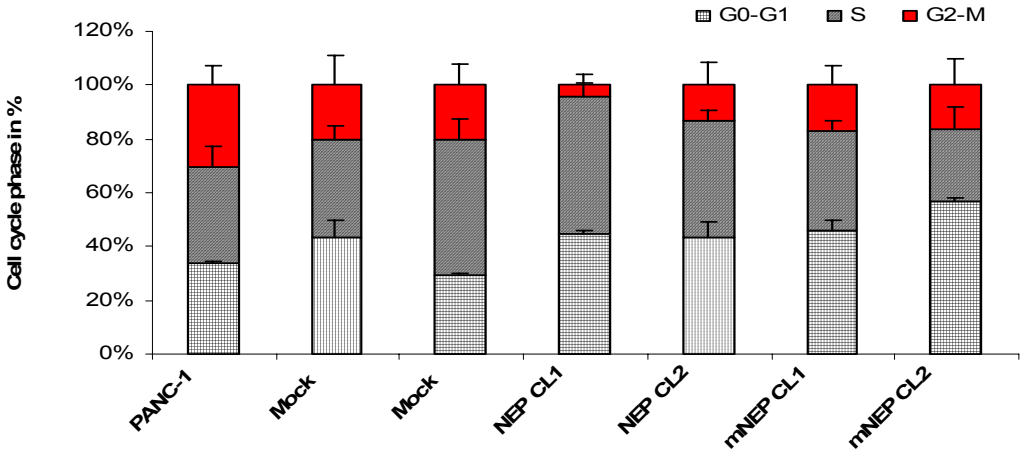


Fig. 21C: Cell cycle: The cells of PANC-1, Mock and clones were synchronized three days in serum with PI and the cell cycle distribution (G0-G1-, S- and G2/M phase) was analyzed using flow cytometer, mean ± SD.

3.9 NEP/CD10 expression and cell migration

The effect of NEP/CD10 expression on cell migration was analyzed using 10 mm cell culture inserts with 8.0 µm pores containing polycarbonate membranes. Over expression of NEP/CD10 decreased the migration rate of wild type NEP clones. The most reduced migration rate was found with NEP CL2 80 %, NEP CL1 60 % (P<0.01). Furthermore, on the other side the cell migration rate of mutated clones was slightly or not reduced: mNEP CL2, 30 %, but mNEP CL1 a reduced migration rate was not observed (Figure 22).

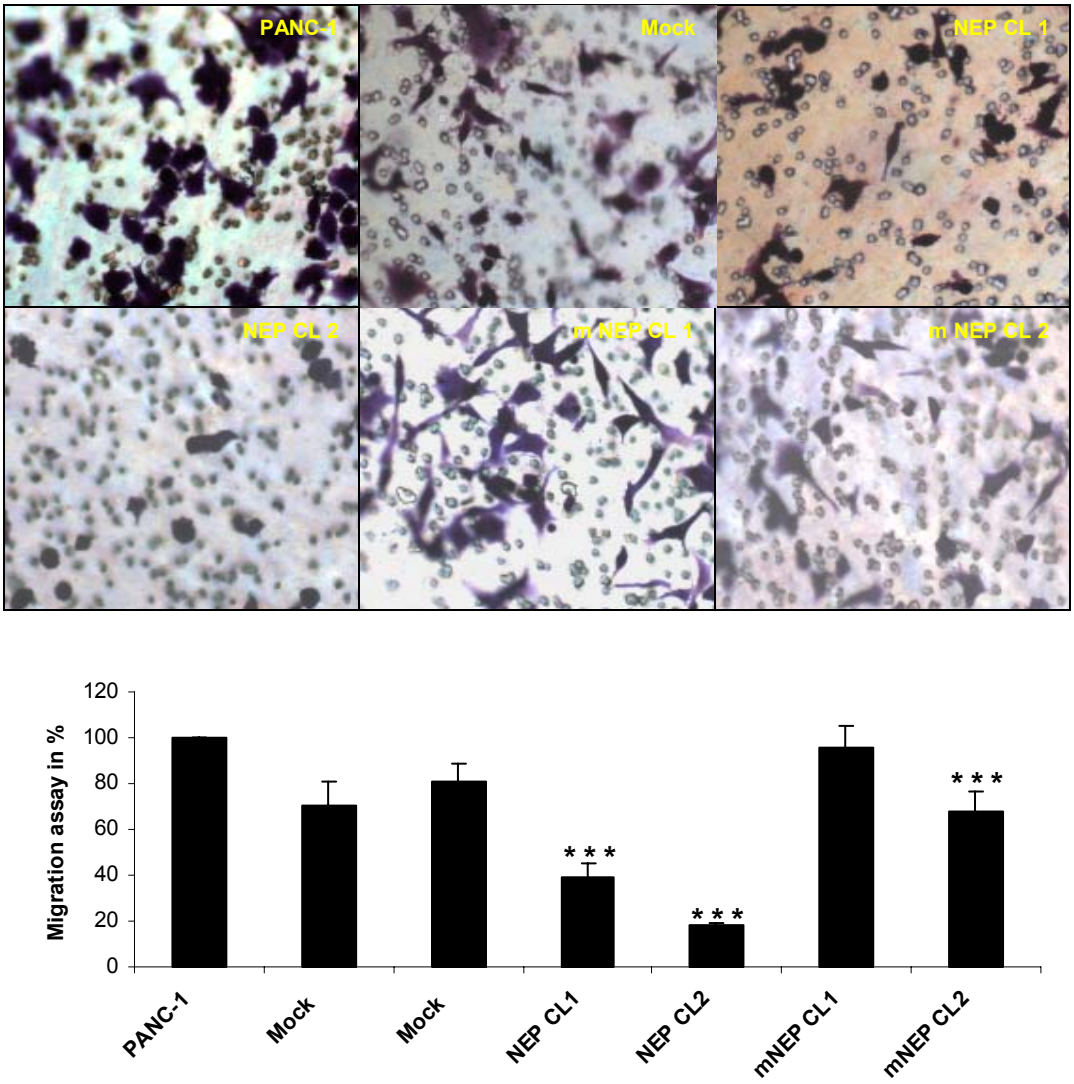


Fig. 22: The migration assay: Effect of NEP/CD10 on cell migratory potential was examined using NEP/CD10 transfected PANC-1 adenocarcinoma cell line. The cell migration of PANC-1, Mock, NEP and mNEP clones was photographed (upper panel) and analyzed in percentage as described in Materials and Methods (lower panel) (P < 0.01).

3.10 NEP/CD10 expression and cell invasion

The effect of NEP/CD10 expression on cell invasion was analyzed using 10 mm tissue culture inserts with 8.0 µm pores polycarbonate membranes coated with laminin. The invasion rate was decreased in over expressed NEP/CD10 clones (Figure 23) NEP CL1 and NEP CL2. The most reduced invasion rate was on NEP CL2 60 %, then NEP CL1 40 % ($P<0.01$). Furthermore, on the other side the cell invasion rate of mutated clones was slightly or not reduced. However, the reduced invasion rate was on mNEP CL2 20 %, while on mNEP CL1 the reduced invasion rate was not observed. These result are identical to the ones of the migration assay.

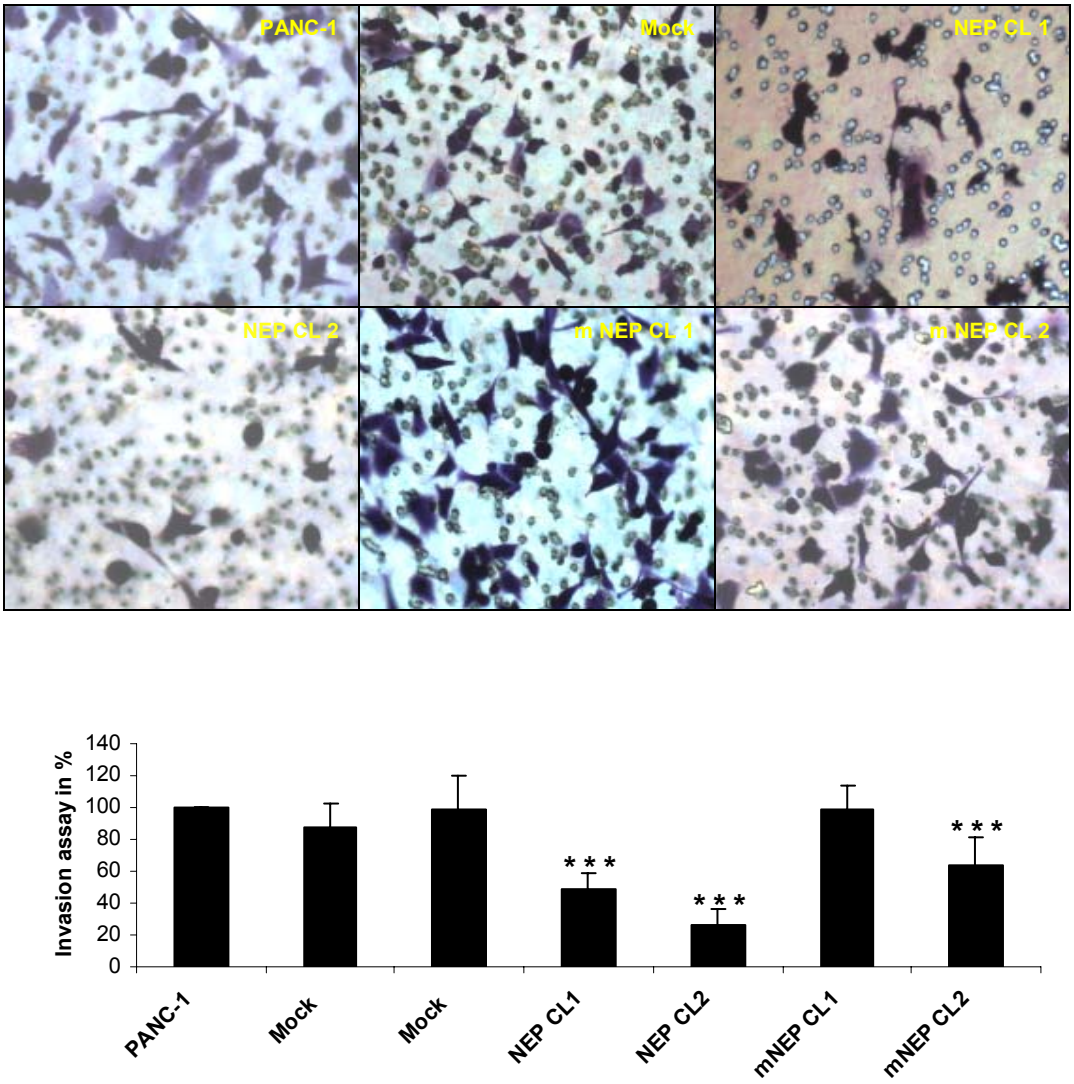
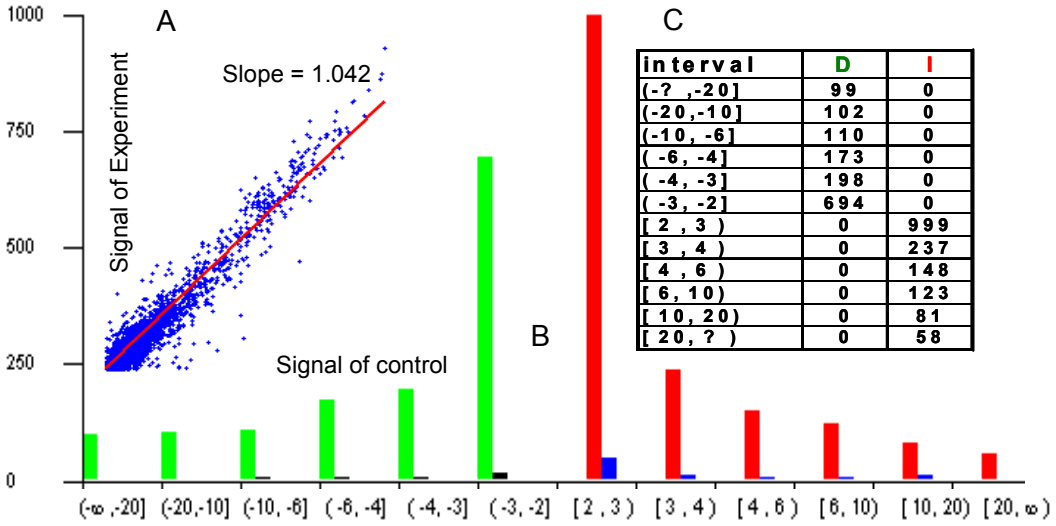


Fig. 23: The invasion assay: Effect of NEP/CD10 on cell invasion was examined using NEP/CD10 transfected PANC-1 adenocarcinoma cell line. The cells invasion of PANC-1, Mock, NEP and mNEP clones was photographed (upper panel) and analyzed in percentage as described in Materials and Methods (lower panel).

3.11 Microarray gene expression analysis

To obtain the number of up- and down-regulated genes related with biological cell mechanisms, as proliferation, adhesion, migration etc, the microarray data of PANC-1 and NEP CL2, were evaluated against Mock. The data of Affymetrix were transformed to Excel data. To illustrate the Affymetrix data, the signals were classified in present (P), absent (A) or marginal (M). The signal log ratios provide strong hybridization differences between the probes of both chips. The data were exploited using Eva program. The transcript profiles data shown in figure 24A represents the signal of NEP CL2 vs. Mock, where slope was (1.042). The graphic in figure 24B represents the number and interval of up-regulated genes given in red color, whereas the number and interval of down-regulated genes is shown in green color. The microarray data where ordered in a table as a general overview to represent the total number and interval of down- and up-regulated genes (Figure 24C).



interval	D	I
(-? ,-20]	99	0
(-20,-10]	102	0
(-10, -6]	110	0
(-6, -4]	173	0
(-4, -3]	198	0
(-3, -2]	694	0
[2 , 3)	0	999
[3 , 4)	0	237
[4 , 6)	0	148
[6, 10)	0	123
[10, 20)	0	81
[20, ?)	0	58

Fig. 24: Microarray data: NEP CL2 vs Mock that as signal in (A), number of decreased (D) in green clolour, and increased (I) genes in red colour as graphic in (B) and table where the total number of decreased (D) and increased (I) genes in interval (C).

3.11.1 Differential gene transcriptions

To verify the microarray data of NEP CL2 vs. Mock, a number of target up- and down-regulated genes were selected. These genes are related to essential biological mechanisms belonging to cell cycle regulation, motility, invasion etc. These data were classified in two tables, one of them (Table 12), includes the up regulated genes of NEP/CD10 (MME) that increased about 10 fold compared to Mock.

Table 12: Genes that positively correlate with NEP/CD10

Gene probe	Gene symbol	Increase fold	Gene name
203434_s_at	MME	9.96	membrane metallo-endopeptidase
200951_s_at	CCND2	2.62	cyclin D2
205411_at	STK4	2.88	serine threonine kinase 4
222595_s_at	DATF1	3.06	death associated transcription factor 1
203680_at	PRKAR2B	3.33	protein kinase, cAMP-dependent, regulatory, type II, beta
220334_at	RGS17	2.02	regulator of G-protein signalling 17
213418_at	HSP70B	2.21	heat shock 70 kDa protein 6
203896_s_at	PLCB4	2.29	phospholipase C, beta 4
203037_s_at	MTSS1	1.53	metastasis suppressor 1
202896_s_at	SIRPA	1.58	signal-regulatory protein alpha
201996_s_at	SPEN	1.58	transcriptional regulator
205376_at	INPP4B	1.60	inositol polyphosphate-4-phosphatase, type II
205659_at	HDAC9	1.71	histone deacetylase 9
204420_at	FOSL1	1.81	FOS-like antigen 1
209457_at	DUSP5	1.93	dual specificity phosphatase 5

The second table includes a number of down regulated genes that have also a relation with pivotal cell mechanisms like GTPase regulator, S100, Rho, Cdc42 and LIM, where all of them were down regulated compared to Mock (Table 13).

Table 13: Genes that negatively correlate with NEP/CD10

Gene probe	Gene symbol	decrease fold	Gene name
201508_at	IGFBP4	-4.89	insulin-like growth factor-binding protein 4
205069_s_at	FAK	-4.55	GTPase regulator associated with the focal adhesion kinase pp125
204351_at	S100P	-4.33	S100 calcium-binding protein P
204989_s_at	ITGB4	-3.17	integrin, beta 4
222853_at	FLRT3	-3.10	fibronectin leucine rich transmembrane protein 3
204731_at	TGFBR3	-2.97	transforming growth factor, beta receptor III
218693_at	tetraspan NET-7	-2.94	transmembrane 4 superfamily member
206544_x_at	SMARCA2	-2.88	SWISNF related, matrix associated, actin dependent regulator of chromatin, subfamily amember 2
223467_at	AGS1	-2.48	activator of G protein signaling
205880_at	PRKCM	-2.13	protein kinase C, mu
208510_s_at	PPARG	-2.13	peroxisome proliferative activated receptor, gamma
203256_at	CDH3	-2.08	cadherin 3, type 1, P-cadherin
201069_at	MMP2	-3.17	matrix metalloproteinase 2
201850_at	CAPG	-2.51	capping protein (actin filament), gelsolin-like
211559_s_at	CCNG2	-2.40	cyclin G2
201331_s_at	STAT6	-2.29	signal transducer and activator of transcription 6
203758_at	CTSO	-2.28	cathepsin O
202729_s_at	LTBP1	-2.07	latent transforming growth factor beta binding protein 1
203657_s_at	CTSF	-1.47	cathepsin F
227628_at	(GEF) 1	-1.22	Rho guanine nucleotide exchange factor
218062_x_at	CEP4	-1.09	Cdc42 effector protein 4; binder of Rho GTPases 4
224352_s_at	CFL	-0.31	cofilin isoform 1
200618_at	LASP1	-0.56	LIM and SH3 protein 1
204338_s_at	RGS4	-3.43	regulator of G-protein signaling 4
215111_s_at	TSC-22	-2.09	transforming growth factor beta-stimulated protein
200965_s_at	ABLIM	-1.15	actin binding LIM protein 1
209539_at	GEF	-1.37	RacCdc42 guanine exchange factor
209040_s_at	allele LMP7B	-1.45	proteasome subunit LMP7
208829_at	NGS-17	-0.97	tapasin

3.11.2 Validation of microarray data result

3.11.2.1 mRNA expression

To explore the microarray data of NEP CL2 vs. Mock, mRNA expression of S100A4, Rac1, Rho A, Cdc42 and LIMK1 in PANC-1, Mocks, wild type and mutated NEP clones was quantified using Real Time-PCR. Here, the result of S100A4 mRNA expression is presented in figure 25. Regarding to PANC-1, S100A4 mRNA expression was highly reduced in all, Mocks, wild type and mutated NEP clones. But on the other side, compared with Mock, S100A4 mRNA expression was also reduced in wild type and mutated NEP clones.

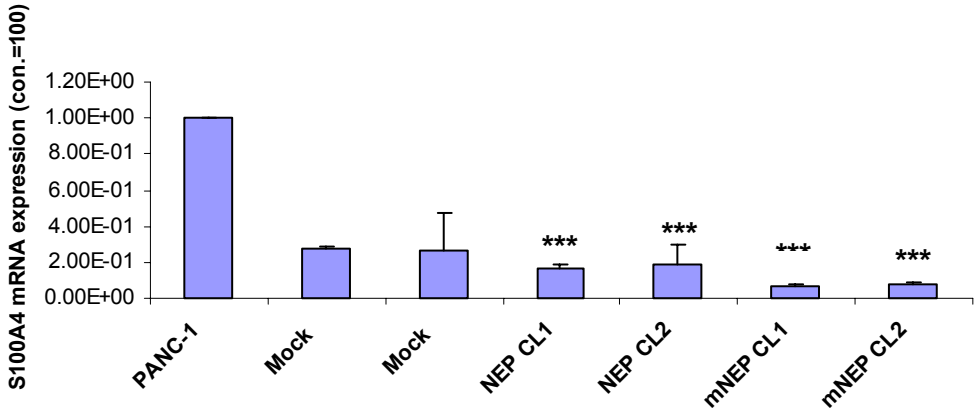


Fig. 25: S100A4 mRNA expression: Total RNA was isolated, then cDNA was transcribed and S100A4 mRNA expression in PANC-1, Mock, NEP and mNEP clones was quantified using Real Time-PCR. Con. is PANC-1.

3.11.2.2 Protein expression

To control the mRNA expression of genes related to pivotal cell mechanisms, protein expression in PANC-1, Mocks, wild type and mutated NEP clones was also investigated. Western blot was performed to target proteins that induce signal transduction where by promote proliferation, motility and invasion of the tumor cells like S100A4, Rac1/2/3, Cdc42, Rho A, LIMK1, Cofilin, phospho-Cofilin, Stathmin, α-Tubulin and acetylated-Tubulin. Generally comparing to PANC-1, all the examined GTPase proteins were clearly down regulated in wild type NEP CL1,2 clones (Figure 26).

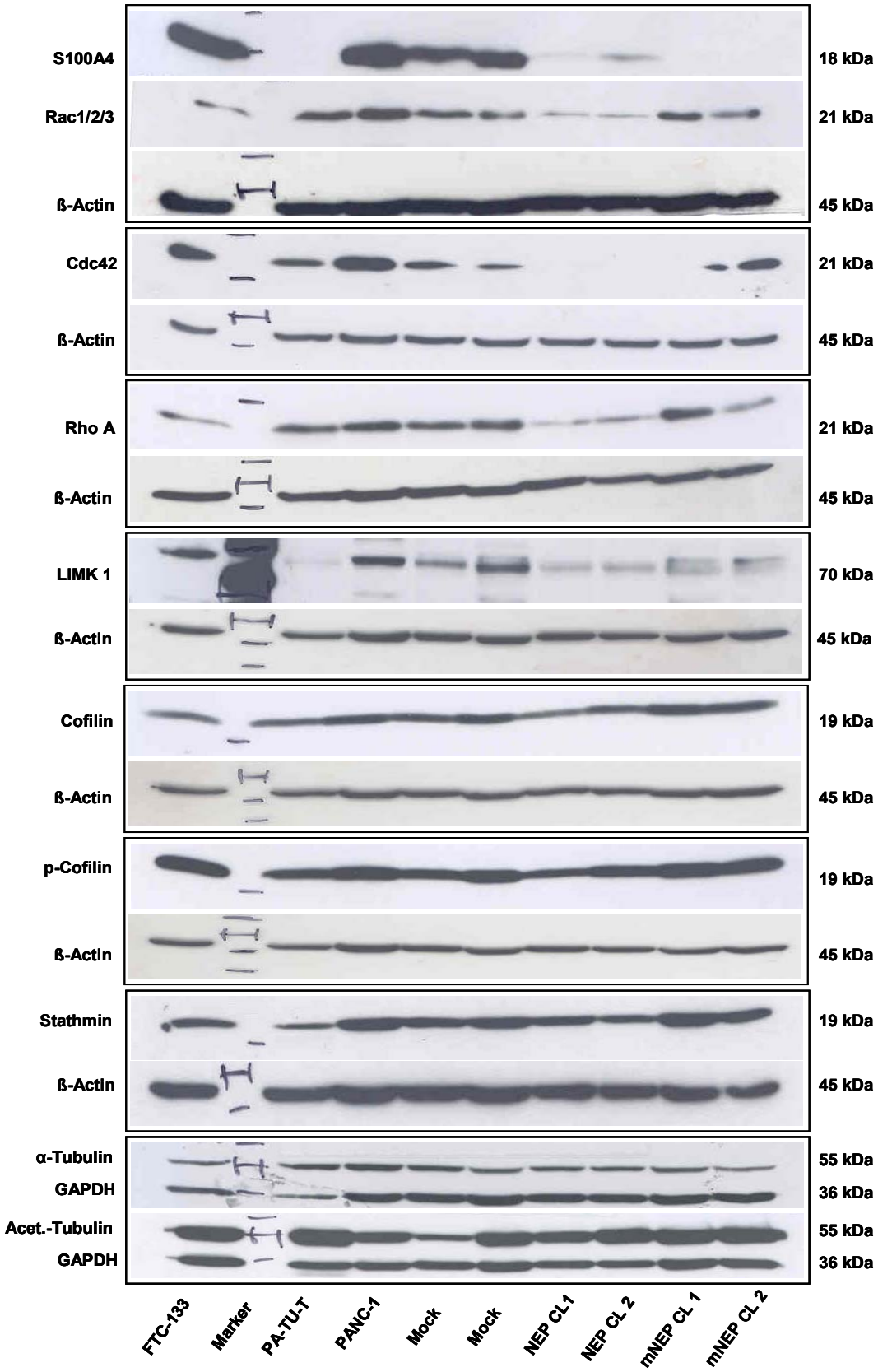


Fig.26: Western blot data Immunoblot analysis of different proteins expression in pancreatic adenocarcinoma PANC-1 cell line and clones. 30 µg of total cell lysate was electrophoresed on a 12% SDS polyacrylamide gel, then blotted on PVDF membranes. To each membrane desired antibody was added, then prepared to photograph as explained in materials and methods.

Furthermore, the examined proteins expression of S100A4, Rac1/2/3, Cdc42, Rho A, LIMK1, Cofilin, phospho-Cofilin, Stathmin, α-Tubulin and acetylated-Tubulin were semi quantified against PANC-1 and ß-Actin was used to normalize protein using Kodak Digital science 1D software (Kodak Digital Science Electrophoresis Documentation and Analysis System 120). The result was shown as relative expression in comparison to the PANC-1 (Table 14).

In fact, compared to PANC-1, protein expression of S100A4, Rac1/2/3, Cdc42, Rho A, LIMK1 Cofilin and phospho-Cofilin was highly reduced in wild type NEP CL1,2 clones as shown in table 14. Furthermore, the expression of mentioned protein was also reduced in mutated NEP CL1,2 clones, where the reduction level except for S100A4 was always in mutated NEP CL1,2 clones less than in wild type NEP CL1,2 clones. Stathmin protein expression was in general obviously effected in wild type NEP CL1,2 clones. Moreover, compared to PANC-1, protein expression of α-Tubulin was decreased in Mock, wild type and mutated NEP CL1,2 clones, but on the other hand protein expression of acetylated-Tubulin was up-regulated in wild type and in mutated NEP CL1,2 clones.

Table 14: Validation of western blot data using Kodak Digital science 1D software.

	PANC-1	Mock	Mock	NEP CL1	NEP CL2	mNEP CL1	mNEP CL2
S100A4	100	45	61	5	25	0	0
Rac1/2/3	100	72	54	16	14	62	43
Cdc42	100	46	31	0	0	19	74
Rho A	100	84	81	20	32	89	42
LIMK 1	100	60	108	26	37	45	47
Cofilin	100	88	108	60	88	110	82
p-cofilin	100	63	121	33	84	117	99
Stathmin	100	84	119	113	78	117	108
α-Ttubilin	100	81	70	76	74	79	40
acet.Tub.	100	62	147	118	142	112	125

4 Discussion

NEP/CD10 expression and promoter status in pancreatic cell lines:

This study investigated the expression and regulation of neutral endopeptidase in nine cell lines of pancreatic ductal adenocarcinoma. The results demonstrated heterogeneous NEP/CD10 expression levels in the different analysed pancreatic adenocarcinoma cells lines. Although the PA-TU-T and PA-TU-S cell lines derived from the same original tumor, they exhibit a different cell morphology, differentiation status, gene expression pattern and in vivo growth.[95] In this work could be shown, that PA-TU-T cells expressed high levels of NEP/CD10 mRNA and protein, whereas PA-TU-S expressed neither NEP/CD10 mRNA nor protein. HUP-T4-derived tumor as a well differentiated papillo tubular carcinoma [96] expressed moderate NEP/CD10 mRNA and protein levels. HUP-T3 cells showed low NEP/CD10 mRNA and protein expression. Expression of NEP/CD10 was not detected in the cell lines PA-TU-02, PANC-1, Capan-1, Capan-2 and MIA PaCa-2. NEP/CD10 enzyme activity was also correlated with NEP/CD10 protein expression using flow cytometry of examined pancreatic adenocarcinoma cell lines.

Although the different cell lines analyzed are of distinct differentiation status, [97-100] and also exhibit a distinct in vivo growth (Table 4), the heterogeneous NEP/CD10 expression pattern observed appears to be correlated with their biological behavior.

Heterogeneous expression of NEP/CD10 in different tumors suggests its transcriptional regulation. The NEP/CD10 mRNA expression can be regulated by two alternative promoters which control its tissue- and developmental stage-specific gene expression.[19,101] Both type 1 and 2 NEP/CD10 regulatory regions are characterized by the presence of multiple transcription initiation sites and the absence of a TATA box and consensus initiator elements. The type 2 promoter has functionally important transcription factor binding sites, one of which is identical to CCAAT-binding transcription factor/nuclear transcription factor Y (CBF/NFY), which might mediate tissue-specific expression of NEP/CD10 by alternative splicing.[102] Decreased NEP/CD10 expression in cancer can be

caused by hypermethylation of the NEP/CD10 promoter as shown e.g. in the human prostate cancer cell line PC3.[103] The NEP/CD10 promoters were examined using MSP method and as previously shown in results (Figure 13, Table 9). Promoter 1 in the examined pancreatic adenocarcinoma cell lines was hemimethylated whereas, the promoter 2 was variable i.e. in a number of examined cell lines it is methylated but in the others is not. From this results of examined cell lines it seems to be, there is not only promoter methylation that has influence on NEP/CD10 transcription but also other factors that also play together essential role in activation of NEP/CD10 promoter.

Regulation of NEP/CD10 expression in pancreatic adenocarcinoma cell lines:

To determine whether NEP/CD10 expression could be restored in vitro after epigenetic treatments, pancreatic adenocarcinoma cell lines PA-TU-T, PA-TU-S (unmethylated), HUP-T4, PA-TU-02 and PANC-1 (hemimethylated) and MIA PaCa-2 (methylated) were treated with 5-aza-2'-deoxycytidine demethylating agent. However, exposure of the cell lines to 5-aza-2'-deoxycytidine could not induce the transcription levels of NEP/CD10 in the non-expressing lines (see 3.3). Furthermore the pancreatic adenocarcinoma cell lines were exposed to both VPA and BA, members of the class of histone deacetylase inhibitors which are discussed as one of the most promising class of new anti-cancer drugs[48] separately or combined with 5-aza-2'-deoxycytidine. VPA induced an 1.5 fold higher NEP/CD10 expression in PATU-T and HUP-T4 cell lines whereas the effect of BA was 2 fold in PATU-T. Inhibitors of histone deacetylase induce hyperacetylation in chromatin usually resulting in activation of certain genes. In contrast, the same reagents alone or in combination could not induce the NEP/CD10 protein expression in PA-TU-S, PA-TU-02, PANC-1 and MIA PaCa-2 cell lines (data not shown). This result suggests that methylation alone is not likely to control NEP/CD10 expression in these cell lines and it seems that the methylation mechanism alone plays no crucial role in repression of NEP/CD10 promoters of pancreatic adenocarcinoma cell lines.

On the other hand a reacetylation could be required for the increase of NEP/CD10 expression in cell lines that already express NEP/CD10 but not for the other cell lines that

do not express NEP/CD10. From these data it seems that the promoters could be repressed with other biological mechanisms that work in correspondence with RNA polymerase to initiate the transcription progress. One of the factors that could inhibit the transcription status is mutation in promoter motifs, where transcription binding proteins bind to accelerate the transcription machinery of the gene or may be a lack of expression of one of transcription factors, that play vital role in transcription resulting in diminution of transcription level of the gene.

NEP/CD10 transfection in pancreatic adenocarcinoma PANC-1 cell line:

Gene delivery to eukaryotic cells by transfection is one of the fundamental techniques to study the regulation of gene expression and protein function.

Stable transfections that are experimentally useful are produced by introducing another gene that can give the cell a selection advantage, typically resistance to a particular toxin. After mitosis, the cells produced are exposed to the toxin. The transfected cells will survive, while most of the normal cells (non-transfected cells) will die. After many rounds of mitosis, only cells with the resistance and the desired genetic change will survive.

As a cell model to study NEP/CD10 function in pancreatic adenocarcinoma cells PANC-1 cells were used, a cell line of an aggressive pancreatic adenocarcinoma. It was transfected with empty plasmid alone or with NEP/CD10 insert, wild type and mutated in enzyme active site as a separate. Here to study the influence of NEP/CD10 enzyme activity on cell mechanisms regulation, mutation was obtained in enzyme active site of NEP/CD10 instead of using chemical inhibitors, like phosphoramidon or thiorphan that have cross reaction with other proteases that may promote to negative influence of cell progression.

Different clones were obtained and NEP/CD10 expression and enzyme activity of wild type and mutated NEP clones were examined. High to moderate NEP/CD10 mRNA, protein expression and enzyme activity were found in wild type NEP CL1,2, but in mutated mNEP CL1,2 moderate to high NEP/CD10 mRNA and protein expression and reduced enzyme activity.

NEP/CD10 over expression, proliferation and cell cycle phase:

Proliferation test was evaluated using MTT test to clarify the effect of NEP/CD10 over expression (clones) on the proliferation rate of pancreatic adenocarcinoma cell line and the role of this enzyme in regulation of proliferation rate of the examined cells in vitro. A reduced proliferation rate of wild type NEP CL1,2 cells was found after 72 h serum starvation condition (0 time). Subsequently, the lowest proliferation rate was demonstrated also in wild type NEP CL1,2 cells under 5 % serum conditions after 24 h from 72 h serum starvation condition (Figure 20). On the contrary, PANC-1, Mock- and mutated mNEP-transfected cells could proliferate in the serum starvation condition, also proliferation rate was increased under 5 % serum condition after 24 h from 72 h serum starvation condition. Apparently, a variety of mitogenic growth factors such as bioactive peptides or cytokines were released from tumor cells in an autocrine/paracrine fashion. However, these substances may be down regulated through enzymatic degradation by over expressing NEP/CD10 and this may lead to failure to proliferate in wild type NEP-transfected cells under serum-free conditions after 72 h.

In fact, this result suggests the involvement of NEP/CD10 via its enzyme activity in the regulation of proliferation rate through controlling the local concentrations of various bioactive peptides that bind to their receptors on cell membrane and result in inducing proliferation of the cells. This finding was in accordance with data of other malignancies such as splenic B cells, NSCLC, SCCa cell lines of the lung and cervical carcinoma CaSki cells indicating that NEP/CD10 expression was accompanied by inhibition of the proliferation. [38,104-106]

Furthermore, to confirm the proliferation rate result, in the same time the cell cycle phase distribution of PANC-1 and clones was analysed using flow cytometry. DNA histograms demonstrated presynthetic (G1), synthetic (S) and postsynthetic-mitotic (G2-M) populations. The cell cycle phases are calculated and the results are given as percentages of cells in the G1, S and G2-M phases. Interestingly, after 72 h of cells starvation (0 h), more than 30 % of PANC-1 cells entered G2-M phase and slightly less in

Mocks, but on the other hand more than 70 % of the cells of wild type NEP CL1,2 and mutated mNEP CL1,2 clones where in G0-G1 phase. In other words, the starvation condition had no influence on the proliferation rate of PANC-1 and Mock, while wild type NEP CL1,2 and mutated mNEP CL1,2 clones where affected. After 24 h from adding medium supplements with 5 % FCS, the proliferation rate was induced in PANC-1, Mock and mutated mNEP CL1,2 where 20-30 % of the cells entered the G2-M phase while wild type NEP CL1,2 clones where delayed and still in S phase. This result harmonized with the proliferation results, suggesting the involvement of enzyme activity of NEP/CD10 in controlling of cell cycle. This result was the same in osteoarthritic (OA) chondrocytes.[107]

NEP/CD10 over expression, migration and invasion:

The effects of NEP/CD10 over expression as wild type or mutated NEP clones on migration and invasion of pancreatic adenocarcinoma PANC-1 cell line were measured (Chapter 3.9, 3.10). The present study could demonstrate that the migratory and invasive potential of PANC-1 cells were markedly reduced by NEP transfection and that the migratory ability of NEP-over expressing PANC-1 cells was marginally reduced by mutation in NEP/CD10 enzyme active site. This result implies that increased NEP/CD10 expression might enzymatically, at least partially, contribute to a suppressive effect on migratory and invasive potentials caused by bioactive peptides in pancreatic adenocarcinoma cells. However, another mechanism independent on NEP/CD10 activity may exist in this tumor. Iwase et. al. showed that NEP/CD10 in prostate cancer mediated inhibition of cell adhesion and motility occurs in part through binding with ezrin/radixin/moesin proteins and that interaction between NEP/CD10 and ezrin/radixin/moesin proteins results from the potential of NEP/CD10 to recruit these proteins from CD44.[108] Similarly, in pancreatic adenocarcinoma NEP/CD10 may contribute to reduced invasive and migratory potential both via enzymatic activity and by a similar nonenzymatic mechanism. One could speculate that this interaction is inhibited by the mutation in active site of NEP/CD10, being the reason for the non-reduced migratory activity in mutated mNEP clones.

In prostate carcinoma, NEP/CD10 can regulate cell migration via mechanisms both dependent and independent on its catalytic function.[109] Furthermore, catalytically active NEP/CD10 inhibits neuropeptide-mediated activation of the insulin growth factor-1 and the resulting downstream activation of PI-3 kinase and Akt/PKB kinase.[16] Recently, in prostate cancer has been reported that NEP/CD10 and the PTEN tumor suppressor associate directly through electrostatic interactions.[110] In PMN cells, results demonstrate that inhibition of NEP/CD10 significantly reduced the concentration of fMLP needed for eliciting transmigration of PMN cells across intestinal epithelia.[111]

Considering the results of this study it seems that there is no one single effect or pathway mediating NEP/CD10 functions in all different cells/tissues expressing this ectoenzyme.

NEP/CD10 over expression and signal cascade pathways:

The identification of molecular pathways that contribute to tumor cells proliferation, their motility and invasion is essential for understanding how these activities are initiated in tumor cells and how the tumor microenvironment contributes to cell migration. Elucidation of mechanisms and molecules involved in most cell functions was the aim of the gene expression experiments with the transfectants over expressing NEP/CD10. Using microarray data, many target up- and down-regulated genes that related with pivotal mechanisms inside the cells were selected and classified in details as up regulated genes (Table 12) and down regulated genes (Table 13). Interesting target genes were confirmed with Real-Time PCR. While Proteins are the functional molecules which operate metabolic, developmental and regulatory pathways in a cell, tissue or an organism many target genes were verified using western blot. S100A4 is a member of the S100 family of calcium binding proteins and has been categorized as a metastasis-associated protein in breast cancer.[112] In addition to these data for breast cancer, an increase in S100A4 protein expression has been correlated with a worse prognosis for patients with colorectal, gallbladder, urinary bladder, esophageal, nonsmall-cell lung, gastric, medulloblastoma, pancreatic and hepatocellular cancers.[113-117] Studies to determine the mechanistic basis for S100A4 function have shown a potential role for S100A4 in several different facets of

tumor progression including motility, invasion and apoptosis.[118,119] Interestingly, these results could determine that PANC-1, as an aggressive pancreatic tumor cell line with high proliferation rate, motility and invasive potential in vitro, expresses very high S100A4 as compared to transfected PANC-1 cell line, wild type NEP CL1,2 and mutated mNEP CL1,2 (Chap. 3.11, Figure 25, 26) which lost S100A4 expression. Anyhow, one could found that in comparison to PANC-1, mutated mNEP CL1,2 express no S100A4 protein, while in wild type NEP CL1,2 the expression was negligible.

Rho GTPases mediate housekeeping aspects of cell biology including cell size, proliferation, apoptosis/survival, cytoarchitecture, cell polarity, cell adhesion and membrane trafficking.[120-123] They do so as signaling switches that regulate lipid metabolism, microtubules-, actin-based structures, epithelial cell-junctions, cell cycle, apoptosis regulatory proteins and transcription factors. There is abundant information regarding the expression levels of Rho GTPases, the Rho family (Rho A and Rho C), the Rac family (Rac1, Rac2 and Rac3) and the Cdc42 family in a large variety of human tumors compared to those of normal tissues.

Rho pathway is the downstream signaling cascade that is activated by PI3K and leads to ROCK stimulation, Myc phosphorylation and thrombospondin (TSP-1) repression. There is also evidence of the disbalance between some Rho family members and RhoGDIs and its correlation with invasion and metastasis in breast cancer.[124] Many data indicating that expression of Rho A correlates with tumor stage and aggressiveness have described in breast cancer, melanoma cells, colorectal carcinoma, lung carcinoma, testicular germ cell carcinoma and hepatocellular carcinoma cells.[125-132] In these data, one could found that Rho A expression was decreased in wild type NEP compared to PANC-1 and mutated NEP clones. This may indicate that a role of NEP/CD10 enzyme activity in regulation of Rho A expression via inactivation of bioactive peptides that induce signal cascades by activation transmembrane receptors upstream of Rho GTPase.

Furthermore, other data indicated a vital role of Rac1/2/3 over expression, upstream regulation of PAK1 in growth and metastasis of tumor cells, breast-, colorectal-, lung-

cancer, melanoma.[133-136] In these data one could found that PANC-1, lacking NEP/CD10 expression with enhanced proliferation rate and potential invasion in vitro, expresses high Rac1/2/3 expression, whereas wild type NEP clones with high NEP/CD10 and high enzyme activity having reduced proliferation rate, migration and invasion in vitro, showed a decline in Rac expression (Figure 26). More interestingly, mutated NEP clones that express NEP/CD10 but with a mutation in enzyme active site have unchanged proliferation rate, migration and invasion in vitro as compared to PANC-1 expressing high Rac compared with wild type NEP clones.

Cdc42 plays also a vital role in signal cascades that regulate tumor growth and invasiveness,[137-139] which is in accordance with a high expression of Cdc42 in this work in PANC-1 and in mutated mNEP clones but on the other hand there was no Cdc42 expression in wild type NEP clones (Figure 26).

Additionally, the downstream pathways of the corresponding GTPases that play pivotal roles in Microtubules, Actin organization and polarization with effects on cell cycle and motility of tumor cells via modulating intracellular signal transduction was also investigated as schematically indicated in figure 27.

Stathmin, as downstream signal of Cdc42 is highly expressed in a wide variety of cancers and its high level of expression seems to be necessary for the maintenance of transformed phenotype.[140-142] The levels of expression and phosphorylation of Stathmin are regulated in response to a variety of signals that affect the state of proliferation and/or differentiation of different cell types.[143-145]

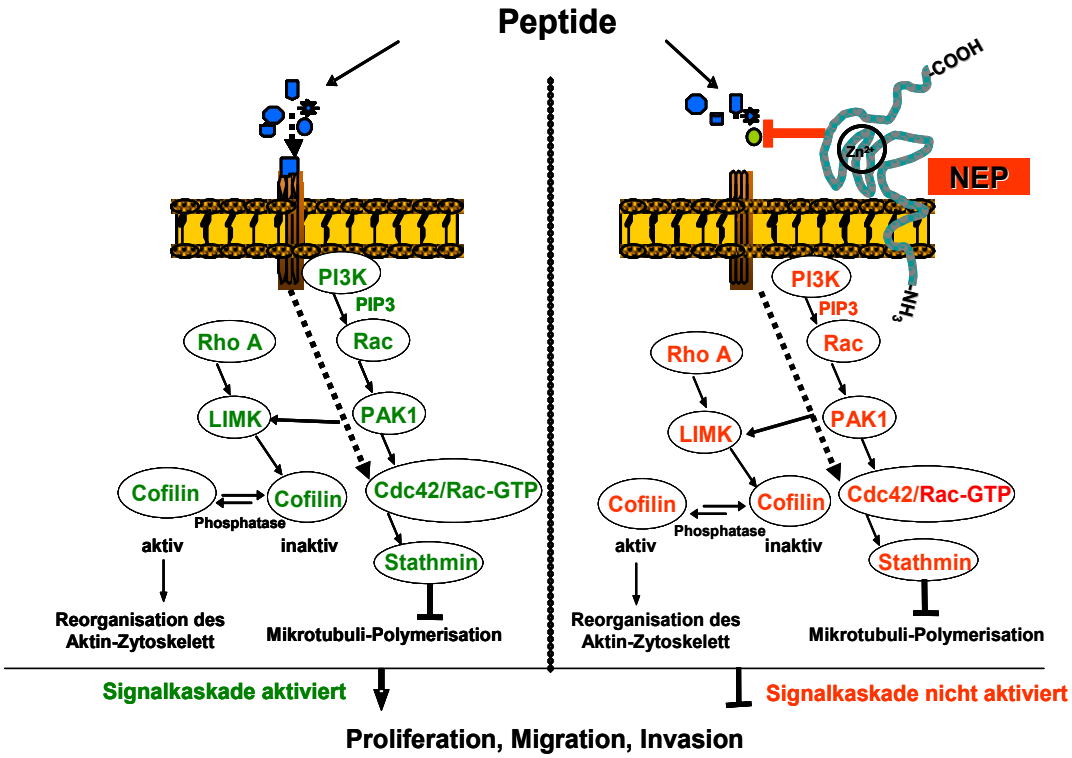


Fig. 27: NEP/CD10 and signal cascade In the left side, the down stream signal is induced by bioactive peptides (green color) and at the right side, the down stream signal is inhibited by NEP/CD10 expression (red color).

Stathmin was recently shown to increase the rate of transition of Tubulin polymers from growth to shrinkage known as Catastrophe.[146] Changes in the level of expression and the state of phosphorylation of Stathmin are important in the regulation of the dynamics of Microtubule polymerization, especially during cell cycle progression.[147]

Interestingly, in this study, wild type NEP clones that express high NEP/CD10 expression have a reduced Stathmin expression, while mutated NEP clones have high Stathmin expression as it was also found in PANC-1. We investigated also α-Tubulin and acetylated Tubulin, which are regulated by Stathmin. In general, PANC-1 cell line has low expression of acetylated Tubulin while wild type NEP and mutated NEP clones express highly acetylated Tubulin. When Tubulin is acetylated, it is no longer active resulting in negative influence on cell motility and invasion.

LIMK1 down stream of the Rho-GTPase family members, Rac, Rho and Cdc42, is a serine/threonine kinase that regulates actin polymerization by phosphorylating and inactivating its substrate, the Actin depolymerization factor, Cofilin.[148-152] Cofilin regulates

Actin dynamics by severing actin filaments and sequestering the Actin monomer from the pointed end of Actin filaments.[153] However, once phosphorylated at serine 3 by LIMK1, Cofilin can no longer bind to Actin, resulting in accumulation of Actin polymer.[154-156] In these data, LIMK1 expression was high in PANC-1 but reduced in all the transfectants, wild type NEP and mutated NEP clones. Despite phospho-Cofilin expression was high in PANC-1, mutated mNEP and low in wild type NEP clones, total Cofilin was also high in PANC-1, mutated mNEP and low in wild type NEP clones.

However, not only Microtubules and Actin expression but its polarization play a crucial role by affecting Microtubules and Actin cytoskeleton stabilization and destabilization of cells that accelerate contraction of the cells during migration. Finally, from these data one could demonstrate that in vitro, over expression of NEP/CD10 could decrease cell proliferation rate, motility and invasion in vitro by influencing Rho-GTPase signal cascade, Roh A, Rac, Cdc42, LIMK1, that regulate actin and Microtubules that play a vital role in multiple cellular processes such as mitosis, migration, protein, organelle transport. The influence of NEP/CD10 seems to be only partially dependent on the active site of the enzyme via degrading bioactive peptides binding to extracellular receptors and activating intracellular signal cascades.

Regarding to these data, NEP/CD10 seems to be a tumor suppressor gene that inhibits tumor progress, possibly it could be used as gene therapy for pancreatic tumor patients especially those with pancreatic tumor metastasis.

5 Conclusion

In this study to explain the cell biological role of NEP/CD10 in controlling pancreatic adenocarcinoma progression, PANC-1 cell line was used as models that derived from poorly differentiated pancreas carcinomas.

In the examined exocrine pancreatic carcinoma cell lines only the cell lines that have no ability to metastasis express NEP/CD10.

Demethylating reagent 5-aza-deoxy-2-citydine could not restore expression of NEP/CD10 in the examined pancreatic adenocarcinoma cell lines.

Acetylating reagents such as butyric acid and valproic acid could upregulate expression of NEP/CD10 in the cell line that already expresses NEP/CD10, but could not restore NEP/CD10 in the cell lines that do not express NEP/CD10.

PANC-1, an aggressive pancreatic adenocarcinoma cell line that has enhanced proliferation and invasive potential rate, shows after transfection with wild type NEP/CD10 gene that, proliferation and invasive potential rate was reduced in vitro. On the other hand after transfection with mutated NEP/CD10, the proliferation and invasive potential rate was marginally or not reduced in vitro.

NEP/CD10 over expression has indirect influence on upstream and down stream signal cascades that regulate proliferation and motility rate. This postulate as one of the possible mechanisms of NEP/CD10 over expression one that works via controlling bioactive peptides. Binding to their cell membrane receptors and initiation of signal cascades is partially dependent on enzyme active site as schematically indicated in figure 27.

Zusammenfassung:

In dieser Arbeit wurde die zellbiologische Rolle von NEP/CD10 beim exokrinen Pankreaskarzinom untersucht. Als Modelle wurden für diese Studie verschiedene Pankreaskarzinomzelllinien, insbesondere die Zelllinie PANC-1, welche aus einem undifferenzierten Pankreaskarzinom gewonnen wurde, verwendet. Die folgenden zellbiologischen Parameter wurden (mit an die Bedingungen der Pankreaskarzinom-Zelllinien adaptierten Methoden) quantitativ und qualitativ erfasst: mRNA Expression (Realtime-PCR), Protein Expression (mittels Durchflusszytometrie), Enzymaktivität (enzymatisch), Zellproliferation (MTT-Test), Zellmotilität (Migrationstest mit Transwell-kammern) und andere.

Die folgenden Punkte beinhalten die wesentlichen Ergebnisse:

Von allen untersuchten Pankreaskarzinomzelllinien zeigten nur die Zelllinien, welche nicht die Fähigkeit zur Metastasierung besitzen, die Expression von NEP/CD10.

Durch die Methylierung mittels 5-aza-deoxy-2-cytidin konnte die Expression von NEP/CD10 in den untersuchten Pankreaskarzinomzellen nicht induziert werden.

Reagenzien für die Azetylierung wie Butter– oder Valproinsäure konnten die Expression von NEP/CD10 in Pankreaskarzinomzellen, welche NEP/CD10 exprimieren, hoch regulieren. Auf der anderen Seite zeigte sich, dass die Expression von NEP/CD10 in Zelllinien, welche dieses Gen nicht exprimieren, nicht durch eine Azetylierung induziert werden konnte.

Die aggressive Pankreaskarzinomzelllinie PANC-1, welche ein erhöhtes Proliferations- und Invasionspotential besitz, zeigte in vitro nach Transfektion mit dem Wildtyp von NEP/CD10, ein erniedrigtes Proliferations- und Invasionsverhalten. Andererseits veränderte sich die Proliferations- und Invasionsrate nicht, wenn diese mit einer Mutante des NEP/CD10 Gens transfiziert wurde.

Aus den Daten lässt sich schließen, dass eine Überexpression von NEP/CD10 einen indirekten Einfluss auf die Signalkaskaden (up- und downstream) hat, welche für die Auslösung und Regulation der Proliferation und Motilität verantwortlich sind. Dabei könnte eine Überexpression von NEP/CD10 einen inhibierenden Einfluss in diesen Kaskaden durch den Abbau bioaktiver Peptide ausüben. Eine schematische Darstellung dieser Vorstellung ist in Abbildung 27 zu finden.

6 Prospective plane

Among the ideas for further experiments suggested by these results the following ones could be regarded:

NEP/CD10 promoter sequencing in pancreatic adenocarcinoma cell lines and tissues to obtain information whether there are mutations in promoter consensus motifs where transcription factors bind to accelerate transcription.

Investigation of transcription factors in pancreatic adenocarcinoma cell lines and tissues that accelerate gene transcription, whether they are expressed or not and mutated or not.

Use of nude mice xenograft to study NEP/CD10 function in vivo using PANC-1 that express no NEP/CD10, transfectants wild type and mutated NEP/CD10.

Transfection of NEP/CD10 in other cell lines that are not aggressive like, tumor grade I, or lymph node metastasis cell lines and study the gene function in vitro and in vivo to explore whether NEP/CD10 inhibits proliferation, invasion just in aggressive cell line or at all tumor grade.

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Zhouxun Chen, Rainer Finke, Karoline Rabara, **Erhuma Mabruk**, Oliver Gimm, Thomas Klonisch, Henning Dralle and Cuong Hoang-Vu (2007). Embryonic stem cell marker OCT4 is expressed in human thyroid neoplasia: new evidence supporting the thyroid fetal cell carcinogenesis hypothesis. Abstract in American Association for Cancer Research Annual Meeting;14-18 Los Angeles, CA, USA.

Eidesstattliche Erklärung

Hiermit erkläre ich, die vorliegende Arbeit selbstständig und ohne fremde Hilfe und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt zu haben. Die Stellen der Arbeit, die anderen Werken in Wort, Bild oder dem Sinn nach entnommen sind, wurden als Entlehnung kenntlich gemacht. Es wurde weder diese noch eine andere Arbeit zur Erlangung des Doktorgrades an einer anderen Einrichtung vorgelegt.

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