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Neuromedin U is overexpressed in pancreatic cancer and increases invasiveness via the hepatocyte growth factor c-Met pathway

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ABSTRACT

Neuromedin U (NmU) is a bioactive peptide, ubiquitously expressed in the gastrointestinal tract. Here, we analyzed the role of NmU in pancreatic ductal adenocarcinoma (PDAC) pathogenesis. NmU and NmU receptor-2 mRNA were significantly overexpressed in PDAC and in metastatic tissues. NmU and NmU receptor-2 were localized predominantly in cancer cells. NmU serum levels decreased after tumor resection. Although NmU exerted no effects on cancer cell proliferation, it induced c-Met and a trend towards increased invasiveness as well as an increased hepatocyte growth factor (HGF)-mediated scattering. Thus, NmU may be involved in the HGF-c-Met paracrine loop regulating cell migration, invasiveness and dissemination of PDAC.

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1. Introduction

Neuromedin U (NmU) is a highly conserved bioactive peptide first isolated from the spinal cord in 1985 and named for its ability to cause smooth muscle contraction in the uterus [1,2]. Since then, various studies have been performed to localize NmU and to identify its possible physiological roles. NmU mRNA is widely expressed throughout the rat and human intestinal tracts, with the most pronounced levels found in the duodenum and jejunum, smaller amounts in the ileum and colon, and low expression in the esophagus, stomach and pancreas [3,4]. In the intestinal tract, it is primarily localized in nerve axons and nerve bodies as well as in epithelial cells of the mucosa [5–7].

There are two cognate G protein coupled receptors for NmU—termed NmUR1 and NmUR2 [8]—and these have a distinct distributional pattern. Whereas NmUR1 is mainly expressed in peripheral tissues and blood cells, NmUR2 is predominantly expressed in the central nervous system (CNS) [8]. Low NmUR1 and NmUR2 mRNA levels have been described in the gastrointestinal tract, liver, pancreas, genitourinary tract, and some endocrine glands [9,10]. Functionally, activation of NmU receptors leads to intracellular signal transduction via calcium mobilization, phosphoinositide signaling, and the inhibition of forskolin-stimulated accumulation of cAMP [11,12].

Although the precise physiological role of NmU has not been completely elucidated, different effects have been described in experimental settings. When locally administered, it leads to smooth muscle contraction and thus regulates of intestinal motility [13] and regional blood flow [14,15]. Intracerebral administration of NmU stimulates the hypophysis–pituitary–adrenal axis via CRH secretion; in rats it reduces food intake and increases grooming behaviour, body temperature, heart frequency and systemic blood pressure [16–18]. There is experimental

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evidence that NmU plays a modulatory role in pain perception which is sustained by the expression of NmUR2 in the dorsal horn of the spinal cord [19].

Until now, there are only a few reports addressing the role of NmU in cancer. A recent study demonstrated highly increased NmU levels in blood samples of acute myeloid leukemia patients and showed a cell growth-enhancing effect of NmU peptide in myb-deficient cells deprived of endogenous NmU. The authors concluded that NmU has an oncogenic effect via its cognate receptor NmUR1 [20]. Furthermore, NmU was found to be significantly overexpressed in human non-small-cell lung cancers and ovarian cancer cell lines compared to immortalized non-cancerous ovarian cell lines [21,22]. In contrast, downregulation of NmU mRNA has been observed in microdissected tissue samples from oral carcinomas [23]. In esophagus squamous cell carcinoma cell lines with epigenetic silencing of the NmU gene, growth inhibitory effects of exogenous NmU have been demonstrated, suggesting a tumor-suppressive function of NmU [24].

Hepatocyte growth factor (HGF), which was found to be identical to the scatter factor (SF), was originally identified as a mitogen of hepatocytes [25–27]. It is predominantly expressed in cells of mesenchymal origin, and its tyrosine kinase receptor c-Met is expressed by epithelial and endothelial cells [28,29]. Activation of c-Met by HGF can induce a variety of cellular responses, including proliferation, motility (scattering), morphology changes and invasion. In addition, it plays an important role in many developmental processes [28,29].

The c-Met oncogene is genetically altered or overexpressed in many human cancers. A variety of c-Met mutations have been well described in multiple solid tumors and some hematological malignancies. For example, a Met kinase-activating fusion protein has been found in hereditary and sporadic papillary renal carcinomas [30–32]. Whereas c-Met can be mutated in other tumor types, this is rare in PDAC, but overexpression is frequently found in ductal adenocarcinoma of the pancreas (78% by immunohistochemistry) [33,34].

Here, we demonstrate specific upregulation of NmU and its receptor NmUR2 in human pancreatic cancer tissues, as well as an NmU-mediated increase in HGF-induced scattering which implicates NmU in pancreatic cancer metastasis.

2. Materials and methods

2.1. Patients and tissue sampling

Tissue samples were obtained from patients undergoing surgery for pancreatic cancer or chronic pancreatitis at the Department of General Surgery, University of Heidelberg, Germany, and at the Inselspital, Bern, Switzerland. Tissues were either snap-frozen in liquid nitrogen (protein extraction), stored in RNA-later (Ambion, Huntingdon, UK; mRNA isolation) or formalin-fixed and paraffin-embedded (for immunohistochemical analysis). Histological examination was carried out by an experienced pathologist. Normal pancreatic tissue was obtained through an organ donor program whenever there was no suitable recipient for transplantation. The use of tissue for this study was approved by the local Ethics committees and written informed consent was obtained from the patients prior to the operation.

2.2. Cell culture

Eight pancreatic cancer cell lines—ASPC1, BxPc3, Capan1, Colo357, MiaPaca2, SU86.86, Panc1 and T3M4—were cultured on 100 mm dishes (Becton Dickinson Labware Europe, Le Port de Claix, France; B&D) in RPMI 1640 medium (Gibco, Paisley, UK) with fetal calf serum (FCS; 20% for ASPC1 and Capan1, 10% for the other cell lines; Pan Biotech GmbH, Aldenbach, Germany) and 1% penicillin/streptomycin (Gibco) at 37 °C 5% CO_2.

2.3. Quantitative RT-PCR

mRNA extraction, cDNA synthesis and quantitative RT-PCR were performed as described previously (specimens used: normal *n* = 18; CP *n* = 20; PDAC *n* = 31; normal lymph nodes *n* = 4; metastatic lymph nodes *n* = 11; normal liver *n* = 9; liver metastasis *n* = 11) [35,36]. Primers for NmU, NmUR1, NmUR2 and c-Met were obtained from Search LC (Heidelberg, Germany). QRT-PCR of microdissected cells was performed as described previously [37].

2.4. Immunohistochemistry

Immunohistochemical analysis was performed as previously described in detail [35,38]. Rabbit anti-NmU, rabbit anti-NmUR1 and rabbit anti-NmUR2 (Alpha Diagnostics International, San Diego, CA, USA) were diluted at 1:100, 1:50 and 1:200, respectively. The slides were subsequently analyzed by two independent researchers, and representative pictures were taken.

2.5. ELISA

An NmU ELISA kit (Peninsula Laboratories, San Carlos, CA, USA) was used according to the manufacturer's instructions on sera and urine from pancreatic cancer patients, chronic pancreatitis patients or healthy donors (Serum: healthy donors *n* = 10; CP *n* = 10; postoperative CP *n* = 5; PDAC *n* = 15; postoperative PDAC *n* = 15. Urine: healthy donors *n* = 5; CP *n* = 5; PDAC *n* = 10). Samples were taken at standardized time points before and after resection.

2.6. Western blot

Protein expression patterns of NmUR1 and NmUR2 in pancreatic cancer cell lines were analyzed using standard Western blot protocols, as described previously [35,39]. NmUR1 and NmUR2 antibodies (Alpha Diagnostics International, San Diego, CA, USA) were diluted 1:750. Equal loading was confirmed with an anti-gamma-tubulin antibody (at a dilution of 1:3000, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). To analyze levels of total Met following treatment with NmU-25, Su86.86 pancreatic cancer cells (low endogenous NmU but high NmUR2 expression)

were incubated with 1 μM of NmU-25 for 3, 6, 12, 24 and 48 h. Protein lysates were prepared and were then analyzed for total Met expression using immunoblotting.

2.7. Proliferation assays

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assays were performed as described before [37]. Cells were incubated with 0.1 nM–1 μM human NmU-25 (Bachem, Bubendorf, Schweiz) or PBS for 24, 48 and 72 h, respectively.

2.8. Microarray of NmU-stimulated pancreatic cancer cells

The cell line SU86.86 was stimulated with 1 μM NmU-25 (Bachem, Bubendorf, Switzerland), and RNA was extracted at the time points 0, 0.5, 6 and 24 h. RNA was then subjected to microarray as described below. The experiment was repeated three times, and only consistent results in all three experiments were regarded as significant.

Approximately 3800 human complementary cDNAs representing genes that are of relevance to pancreatic cancer were selected [40]. PCR amplification was carried out in 96-well microtiter plates with amino-modified M13 forward primer d(GTTTTCCCAGTCACGACGTTG) and amino-modified reverse primer d(AGCGGATAACAATTTCACA CAGG). The PCR products were arrayed onto slides with an epoxy surface (Epoxy Slides, Quantifoil Micro Tools GmbH, Jena, Germany) with a spotter from Engineering Services Inc. (Virtek's Arrayer System, BioRad, Munich, Germany) or a MicroGrid II arrayer (BioRobotics, Cambridge, UK) using SMP3 pins (TeleChem International Inc., Sunnyvale, CA, USA). Subsequently, the slides were treated according to the manufacturer's instructions.

Sample preparation, hybridization and detection were performed as described in detail previously [40]. Data quality assessment, normalization and correspondence cluster analysis were performed with the MIAME-compatible analysis and data warehouse software M-CHiPS, which currently holds data of more than 8500 experiments (www.mchips.org) [41,42]. The signal intensities were normalized and significance levels were assessed by the highly stringent "min–max separation" criterion, which is calculated by taking the minimum distance between all data points of two conditions [43].

Cluster analyses were performed using correspondence analysis. Correspondence analysis is an explorative computational method for the investigation of associations between variables, such as genes and hybridizations, in a multi-dimensional space. Much like principle component analysis, it displays a low dimensional projection of this data. In contrast, however, it does so simultaneously for two variables, thus revealing associations between them. In the analysis of array-based transcript data, the display of genes and experiments has proven very valuable for biological data interpretation [44] (www.mchips.org).

2.9. Scatter assay

Cancer cell lines were seeded on six-well plates (B&D) at 300 cells per well and were cultured for 5–10 days until cell clones of twenty or more cells could be distinguished. Medium was changed before a 2-h incubation with 1 μM NmU-25. Subsequently, a pretreated and a control well were stimulated with rHGF (R&D Systems, Minneapolis, MN, USA) at 20 nM. After 36 h, the supernatants were discarded and the cells were dried and stained with undiluted May-Grunwald solution (Merck, Darmstadt, Germany) and counterstained with Giemsa (Merck) at a dilution of 1:20. Representative pictures were taken and evaluated by a researcher who was blinded to the experimental setup.

2.10. In vitro invasion assays

Assays were performed in BD Biocoat Matrigel Invasion Chambers with 8 μm pore size (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions [45]. Matrigel was rehydrated with 500 μl serum-free cell culture medium and was incubated at 37 °C, 5% CO2 atmosphere for 2 h. 1.25×10^4 cells (Su86.86) were seeded into the upper chamber and were pre-incubated with or without NuM-25 (1 μM) for 1 h, followed by stimulation with recombinant HGF (1 ng/ml) for 24 h. Cells without pre-incubation with NmU-25 and without stimulation by rHGF were regarded as controls. Cells adhering to the lower surface of the membrane were fixed with 75% methanol mixed with 25% acetone and were stained with 1% toluidine blue. The whole membrane was scanned and the invaded cells were counted. The assays were repeated three times. The invasion index is expressed as the ratio of the invasion of the treated versus the control cells.

2.11. Statistical analysis

For statistical analyses, the GraphPad Prism 4 Software (GraphPad, San Diego, CA, USA) was used. Analysis of variance (ANOVA) was performed for comparisons of different groups, followed by a post hoc Bonferroni's multiple comparison test (the extreme outliers of NmU and NmUR2 expression in the CP group were excluded for the analysis). A paired t-test was used for comparisons of pre- and post-operative NmU serum levels. The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. Overexpression of NmU and NmUR2 in pancreatic cancer tissues

To determine the mRNA expression of NmU, we performed quantitative RT-PCR in the normal pancreas (NP), chronic pancreatitis (CP) and pancreatic ductal adenocarcinoma (PDAC) as well as in normal and metastatic tissues from lymph nodes and liver. In NP and CP, NmU expression was near the detection limit, whereas in PDAC we found significantly increased NmU mRNA levels (Fig. 1A; NP versus PDAC: $p < 0.0001$ and CP versus PDAC: $p < 0.01$). In liver metastasis, there was also an upregulation of NmU mRNA levels (Fig. 1A; normal liver versus liver metastasis: $p = 0.0065$), while metastatic lymph nodes expressed NmU in 45% of the cases (Fig. 1A). NmU receptor-1 (NmUR1) mRNA was expressed at relatively low levels in all examined human pancreatic tissues, with no significant differences between normal controls, CP and PDAC tissues (Fig. 1B). In metastasis tissues, NmUR1 mRNA levels were lower than in the corresponding normal tissues (normal versus metastatic lymph nodes: $p = 0.0061$; normal liver versus liver metastasis: 3.6-fold reduction, not significant; Fig. 1B). In contrast, NmU receptor-2 (NmUR2) mRNA was

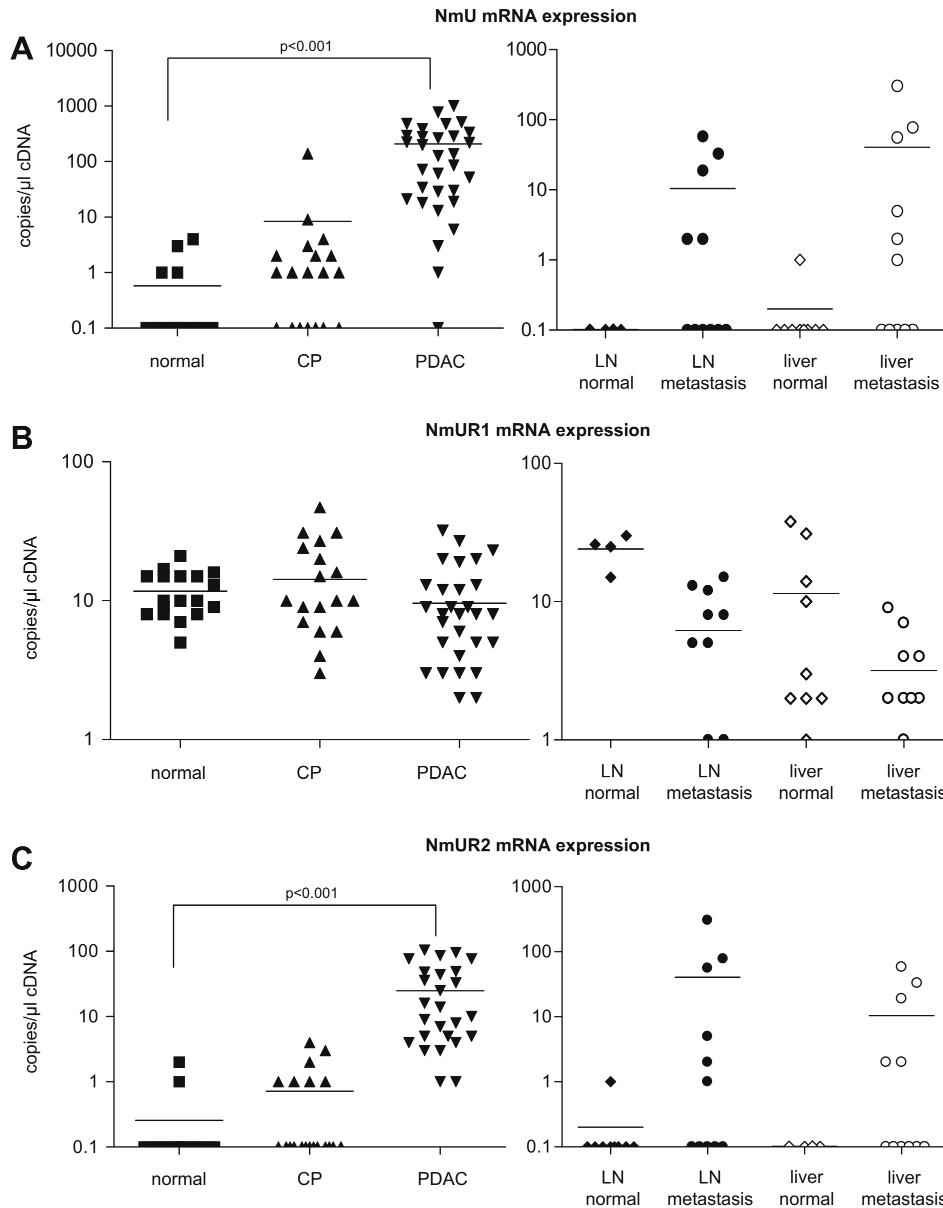

Fig. 1. mRNA expression of neuromedin U (NmU) (A), neuromedin U receptor-1 (NmUR1) (B), and neuromedin U receptor-2 (NmUR2) (C) in human specimens from the normal pancreas, chronic pancreatitis (CP) and pancreatic ductal adenocarcinoma (PDAC). NmU and NmUR2 are expressed significantly higher in PDAC compared to the normal pancreas (A and C left side). NmU is also significantly increased in liver metastasis compared to normal liver (A right side). NmUR1 mRNA expression is low in all analyzed tissues (B). NmUR2 is expressed only in some metastasis tissues, although there is trend towards increased NmUR2 expression levels in lymph node and liver metastases (C right side).

found to be overexpressed in pancreatic cancer samples, with a 149-fold increase compared to normal controls ($p < 0.0001$; Fig. 1C). While in a few metastasis samples there was NmUR2 upregulation, this was not consistently observed and was therefore not statistically significant (normal lymph nodes versus metastasis: $p = 0.214$; normal liver versus liver metastasis: $p = 0.069$).

To analyze NmU and NmUR1/2 protein levels and to locate their expression patterns, immunohistochemistry and immunoblot analyses were performed. In the normal pancreas, no NmU or NmUR2 immunoreactivity was observed (Fig. 2A and D). In pancreatic cancer, there was weak immunostaining for NmU in the cytoplasm of cancer cells (Fig. 2B) and moderate to strong immunoreactivity in enlarged intrapancreatic nerves (Fig. 2C, "N" = nerve). Possibly due to low mRNA expression levels, NmUR1 was not detectable by immunohistochemistry or Western blot on the protein level (data not shown). Enlarged pancreatic nerves (Fig. 2E, arrow) and pancreatic cancer cells (Fig. 2E and F) were strongly immunoreactive for NmUR2, which was mainly distributed in the cytoplasm and the cell membrane. Furthermore, single cancer cells also showed nuclear staining for NmUR2 (Fig. 2F, arrows).

3.2. NmU expression in microdissected pancreatic cancer cells of human tissues

Microdissected ductal cells as well as normal pancreatic cancer tissues exhibited only low levels of NmU mRNA (Fig. 2G). Benign ductal cells in chronic pancreatitis tissues exhibited slightly elevated levels. However, in microdissected ductal adenocarcinoma cells, NmU mRNA expression was significantly higher than in bulk normal pancreas tissues, in normal

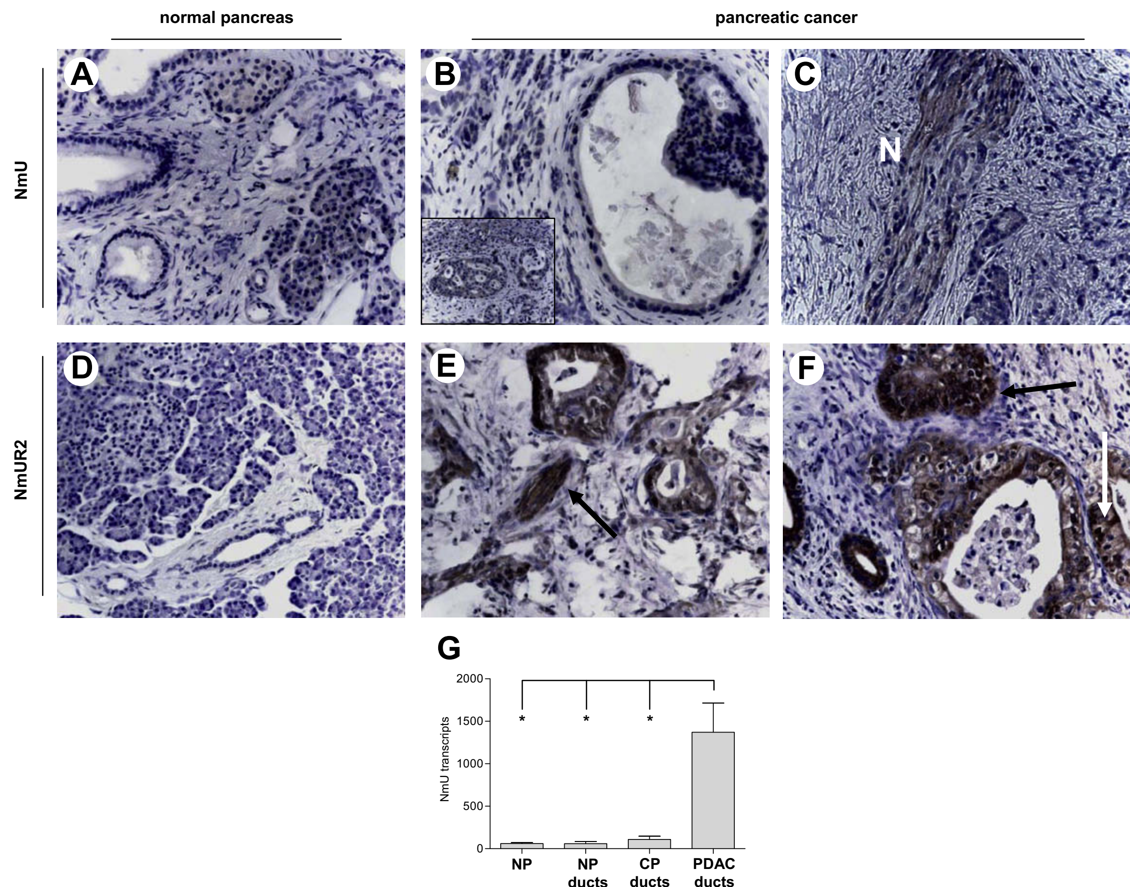

Fig. 2. Immunohistochemistry of NmU and NmUR2 in the normal pancreas and in PDAC. No immunoreactivity for NmU and NmUR2 was observed in the normal pancreas (A and D). In pancreatic cancer, cytoplasmic NmU immunoreactivity was seen in cancer cells and enlarged nerves (B and C). NmUR2 immunoreactivity was found in enlarged nerves (E, arrow) and in cancer cells with a cytoplasmic and membranous staining pattern (F, arrows). N = nerve. (G) Laser-capture microdissection followed by QRT-PCR of NmU. NP: bulk (whole) pancreatic tissue ($n = 3$); NP ducts: microdissected normal pancreatic ducts ($n = 3$); CP ducts: microdissected ducts from CP tissues ($n = 5$); PDAC ducts: microdissected ducts from pancreatic cancer tissues ($n = 6$). $^{*}p < 0.05$ as assessed by ANOVA followed by Bonferroni's multiple comparison test.

pancreatic ducts, and in chronic pancreatitis ducts (all $p < 0.05$, as assessed by ANOVA followed by Bonferroni's multiple comparison test; Fig. 2G).

3.3. Differential expression of NmU, NmUR1 and NmUR2 in pancreatic cancer cell lines

To assess the NmU and NmU receptor status of eight pancreatic cancer cells lines (ASPC1, BxPC3, Capan1, Colo357, MiaPaca2, Panc1, SU86.86 and T3M4), quantitative RT-PCR was performed. While NmU and NmUR2 were expressed at different levels in these cell lines (Fig. 3A and C), NmUR1 expression levels were below 5 copies/μl of cDNA, suggesting that NmUR1 is not transcribed in pancreatic cancer cell lines (Fig. 3B). The mRNA expression profiles of NmUR2 (Fig. 3C) in the various cancer cell lines were reflected on the protein level, with ASPC1, Capan1, Colo357 and SU86.86 showing a specific protein band in the immunoblot analysis (Fig. 3D; NmUR2-positive cell lines marked bold).

3.4. NmU serum levels decrease following pancreatic cancer resection

To evaluate the potential of NmU to serve as a disease marker, we examined NmU protein levels in serum and urine from healthy donors and from CP and PDAC patients. Serum analyses revealed no differences in NmU levels between these groups (controls mean 2.402 ng/ml; CP mean 3.017 ng/ml; PDAC mean 1.900 ng/ml; Fig. 4A). In urine samples, NmU levels were generally lower than in sera, but there were also no differences when comparing normal controls, CP and PDAC patients (controls mean 0.246 ng/ml; CP mean 0.293 ng/ml; PDAC mean 0.270 ng/ml; Fig. 4B). In a next step, we assessed whether surgical resections influence NmU serum levels. Although there was a trend toward reduced NmU serum levels after pancreatic head resection for chronic pancreatitis, statistical significance was not reached (Fig. 4C; $p = 0.15$). However, 5 days after pancreas resection for pancreatic cancer, NmU levels decreased significantly (Fig. 4D; $p = 0.022$).

3.5. NmU does not influence cell proliferation

In order to evaluate the mitogenic activity of NmU, we cultured four pancreatic cancer cell lines following treatment with NmU. Capan1, SU86.86, MiaPaca2 and Panc1 were treated with increasing doses from 0.1 nM to 1 μM NmU for 72 h, and in a second set of experiments with 1 μM NmU for 24, 48 and 72 h. None of the cell lines responded significantly to NmU incubation as measured by MTT proliferation assays (data not shown). We chose NmU concentrations between 0.1 and 1 μM NmU since concentrations of 0.5 and 1 μM NmU have been reported to promote growth of other mammalian cells (COS-7).

3.6. Neuromedin U induces c-Met expression

To identify target genes of NmU in SU86.86 pancreatic cancer cells, we performed microarray analysis (Table 1) followed by QRT-PCR of

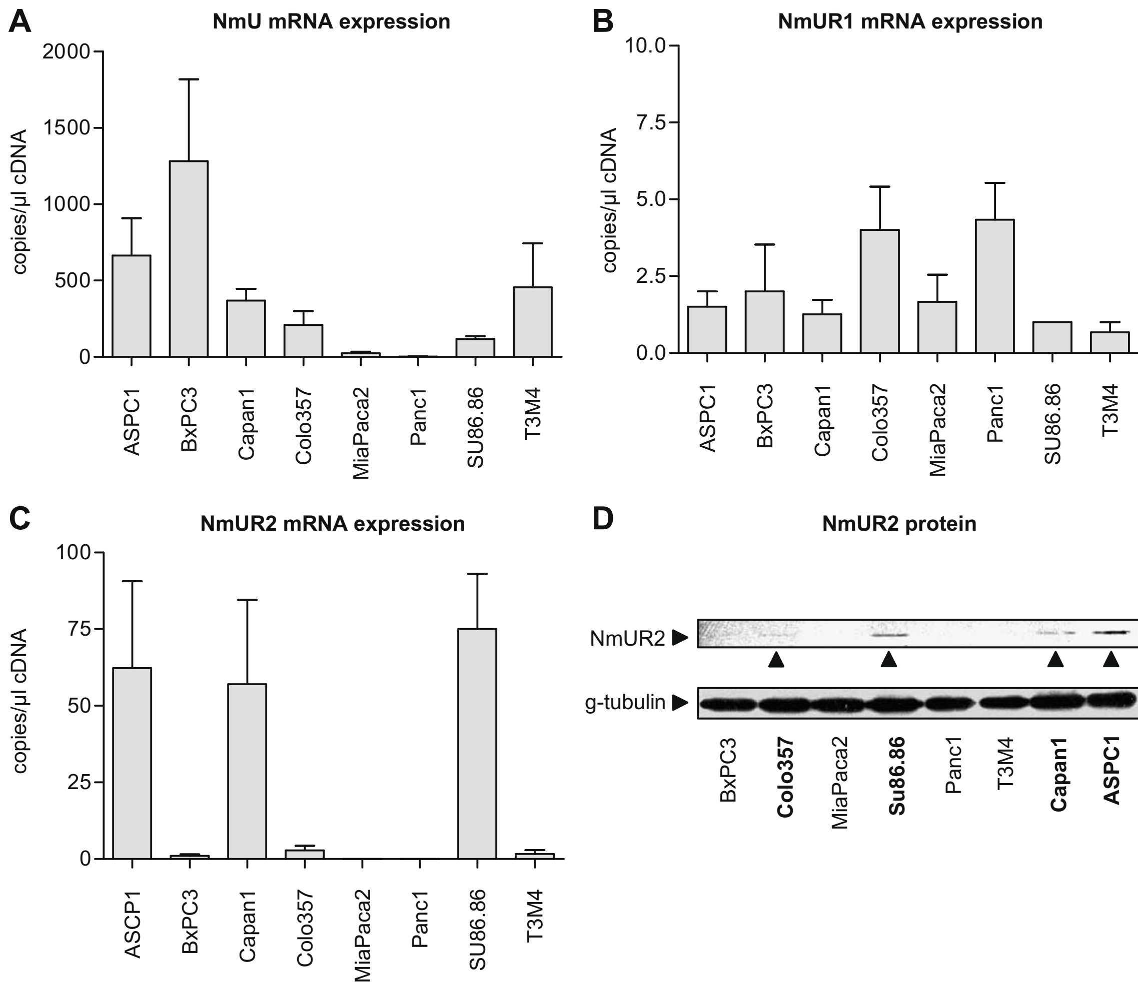

Fig. 3. NmU (A), NmUR1 (B) and NmUR2 (C) mRNA expression in pancreatic cancer cell lines. NmU and NmUR2 mRNA are expressed at different levels. NmUR1 mRNA expression is near the detection limit in all analyzed pancreatic cancer cell lines. In concordance with the QRT-PCR findings (C), ASPC1, Capan1, Colo357 and SU86.86 exhibit NmUR2 protein expression (D).

several potential target genes after a 24-h treatment with 1 µM recombinant NmU. This analysis revealed a 2.4-fold upregulation of the expression of the oncogene c-Met (Table 1). c-Met induction was also shown to occur following NmU treatment in BxPC3 (1.5-fold upregulation) and Capan1 (3-fold upregulation) pancreatic cancer cell lines. Immunoblot analysis of total Met following stimulation of Su86.86 pancreatic cancer cells with NmU (1 µM) demonstrated a time-dependent increase in total Met expression upon incubation of the cells with NmU (Fig. 4E; densitometry of three independent experiments; normalized to the respective GAPDH expression; immunoblotting shows one representative experiment).

3.7. Neuromedin U increases HGF-mediated scattering in pancreatic cancer cell lines

To investigate the biological significance of c-Met induction following NmU incubation, we performed scatter assays with hepatocyte growth factor (HGF) alone and in combination with NmU. Untreated cancer cell colonies (Fig. 5A and E) and NmU-treated cells (Fig. 5B and F) were not scattered, with all tested cell lines (BxPC3 and SU86.86) showing scattering of the cell colonies upon HGF stimulation (Fig. 5C and G). This effect was significantly increased through a 2-h pre-incubation with NmU (Fig. 5D and H). These results were confirmed in Capan1 and ASPC1 cancer cell lines (data not shown).

3.8. Neuromedin U increases HGF-mediated invasion in pancreatic cancer cell lines

In order to further elucidate the effects of NmU and NmU/HGF on pancreatic cancer cell motility, in vitro invasion assays were performed. In line with the results obtained by the scattering assays, incubation of Su86.86 cancer cells with a combination of NmU and HGF increased their invasiveness more than two times (Fig. 5I).

4. Discussion

The role of neuromedin U in cancer is not yet fully defined. Downregulation in oral epithelial cancer, in head and neck squamous cell carcinoma (HNSCC) and in esophageal squamous cell cancer (ESCC) as well as promoter hypermethylation in ESCC and HNSCC led to speculation that neuromedin U might act as a tumor suppressor gene [23,24,46]. On the other hand, NmU was found to be overexpressed in ovarian cancer cells, in non-small-cell lung cancers and in myb-deficient acute myeloid leukemia (AML) cells [20]. Additionally, NmU is also regulated by

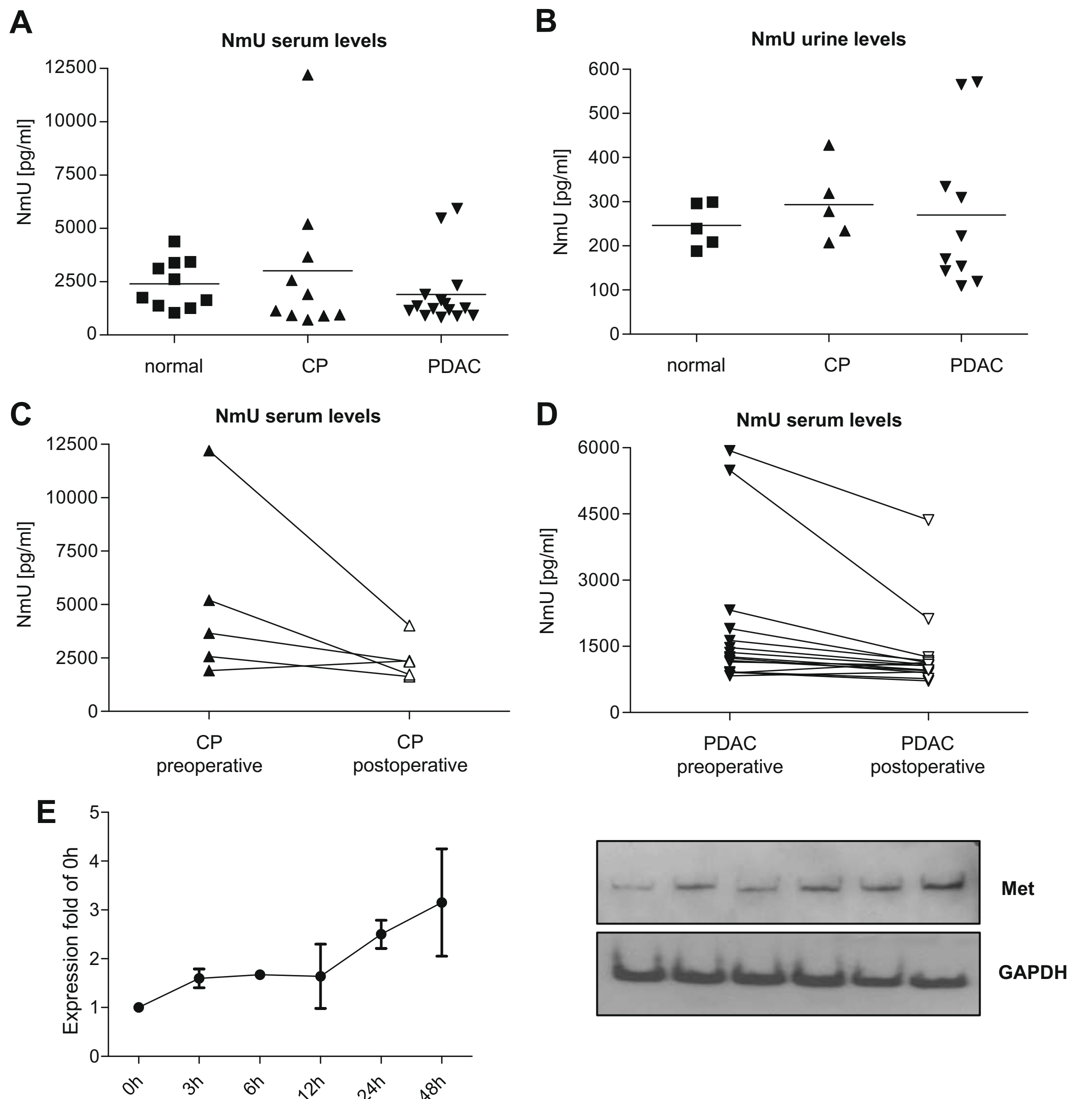

Fig. 4. Neuromedin U (NmU) peptide levels in serum (A, C, D) and urine (B) as determined by ELISA. No significant differences were found comparing healthy donors (normal), chronic pancreatitis patients (CP) and patients suffering from pancreatic ductal adenocarcinoma (PDAC). The decrease of NmU serum levels one week after major pancreatic resection was significant in pancreatic cancer patients (D; $p = 0.022$) but not in chronic pancreatitis patients (C). Total Met expression following stimulation of Su86.86 pancreatic cancer cells with Nmu (E; 1 μM; densitometry of three independent experiments; normalized to the respective GAPDH expression; immunoblotting shows one representative experiment).

the metastasis suppressor RhoGDI2 in lung cancer, supporting its role as a tumor promoter [47]. Functionally, neuromedin U exerted pro-proliferative effects which were mediated mainly through the neuromedin U receptor-1 [20–22].

In this study, we demonstrate that neuromedin U and its receptor NmUR2 are overexpressed in human pancreatic cancers compared with normal pancreas. In pancreatic cancer cell lines, expression of NmU was found to be highly variable. The differences in the expression of NmU among the various cell lines could be attributed to the varying expression of c-myb or RhoGDI2 which were shown to regulate expression of NmU. In addition, hypermethylation of the NmU promoter region might also be responsible for the low expression of NmU in several tested cell lines. Interestingly, less differentiated cell lines such as Panc1 (grade2) and MiaPaCa2 (grade3) express NmU at lower levels than the more differentiated cell line Capan1 (grade1). However, the in vivo relevance of such a difference remains unclear.

We could also show that NmU and its receptor NmUR2 are overexpressed in lymph node and liver metastases of pancreatic cancer. Localization studies revealed distinct patterns of NmU and NmUR2 distribution mainly in cancer cells. These data suggest that NmU and the NmU receptor-2 may play a specific role in pancreatic cancer pathobiology.

With regard to the NmU receptor-1 (NmUR1), there were only very low expression levels in all examined pancreatic samples. In lymph node metastasis, NmUR1 was

Table 1
Differentially regulated genes in SU86.86 following NmU stimulation (microarray).

Gene	Upregulation (*x*-fold)	Gene	Upregulation (*x*-fold)
LCN2 (lipocalin 2)	6.99	mdm2 (Sequence 1)	2.57
MAC30 (meningeoma-associated protein 30)	3.75	UBE2B (ubiquitin-conjugating enzyme 2b)	2.51
LHCGR (luteinizing hormone receptor)	3.51	IMAGp998G10824Q2	2.48
Keratin 19	3.38	FPGH	2.47
PXN paxilin	3.34	c-Met	2.41
TFPI2 (tissue factor pathway inhibitor)	3.29	TNFSF10 (TNF-ligand superfamily 10)	2.39
IRAK1 (interleukin-1-receptor-associated kinase	3.19	IMAGp998L19659Q2	2.33
ULK1 (UNC51-like kinase 1)	3.19	CNK (cytokine inducible kinase)	2.32
GPR2/G (G-protein coupled receptor-2)	2.98	Mucin3	2.31
PHKB (phosphorylase-kinase beta subunit)	2.95	NAIP/occluding	2.31
GTF2H1 (general transcription factor 2H1)	2.85	PIK3C2B (phosphatidylinositol3 kinase 2B)	2.31
TS (thymidilate synthase)	2.81	IMAGp998D087297Q2	2.29
Adenosine A	2.79	Rho C (ras-homolog gene family member C)	2.25
TRAF5 (TNFR-associated factor 5)	2.74	mdm2 (Sequence 2)	2.21
TNFRSF10B (TNF receptor superfamily 10b)	2.71	RFC replication factor 3	2.08
ITGAL (antigen CD11A)	2.63	cyclin D2	2
IEX-1(=DIF2) (immediate early response 3)	2.58		

SU86.86 pancreatic cancer cell lines were treated with 1 μM NmU for 24 h. The relative mRNA expression according to the DNA array is shown as the fold change compared to untreated normal control SU86.86 cells.

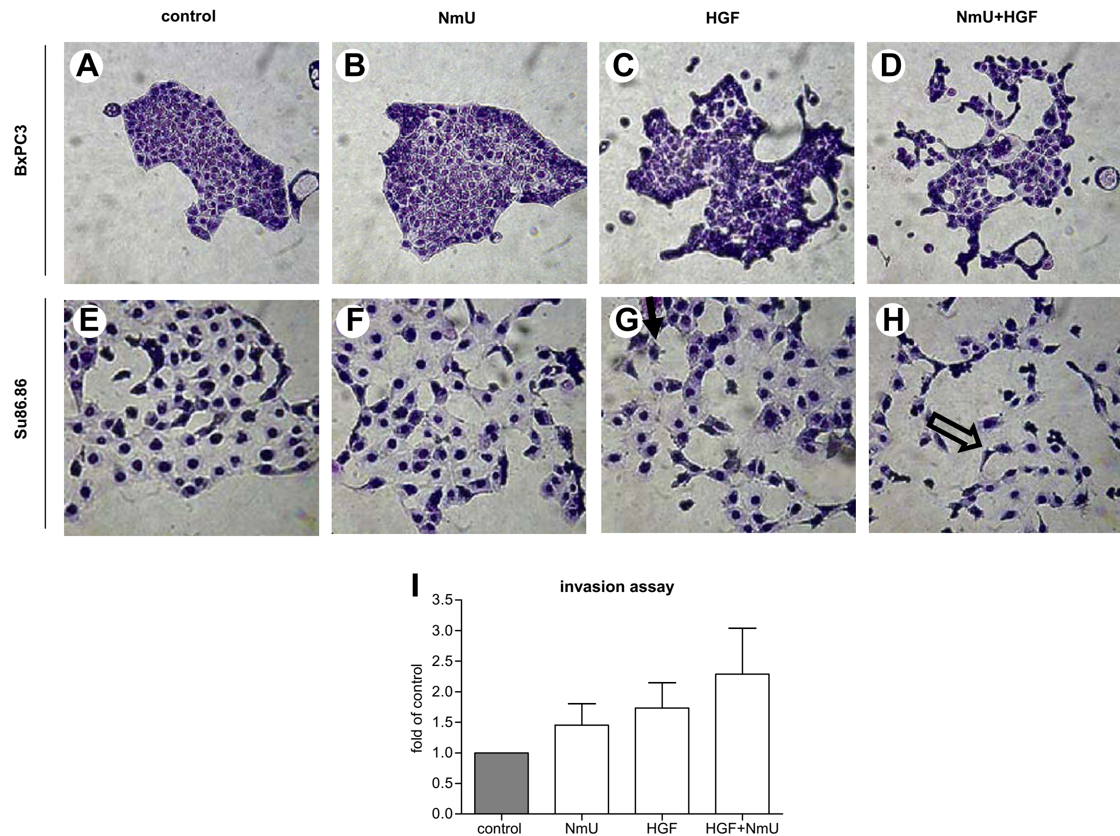

Fig. 5. Scatter assay with BxPC3 (A–D) and SU86.86 (E–H) human pancreatic cancer cell lines. Cells were grown until colonies were formed (controls without treatment A and E). Treatment with 1 μM NmU alone had no effect (B, F). The visible scattering after treatment with HGF (20 nM) (C, G) was remarkably increased when there was treatment with HGF and NmU simultaneously (D, H). The black arrow indicates a podocyte-forming cell; the open arrow points to a sickle-shaped cell with a prolate nucleus. Invasion assays (I) demonstrate increased invasiveness of Su86.86 cancer cells following incubation with a combination of NmU and HGF.

expressed at even lower levels than in normal lymph nodes, pointing towards the considerable transcription rate of NmUR1 in lymphatic tissue, which may be diluted by infiltrating tumor cells with low NmUR1 mRNA expression levels. It is known that NmU stimulates mast cells and eosinophils and promotes IL-6 production of macrophages

via NmUR1 [48–50]. Thus, NmU-negative carcinomas with lymph node metastasis may be a result of an anti-metastatic effect mediated by the immune system against NmU-positive cancer cells.

For the first time, we could demonstrate measurable amounts of NmU in serum and urine by ELISA. Most likely due to NmU production in multiple tissues, there was no difference between healthy donors, chronic pancreatitis patients and PDAC patients before surgery. However, after surgery, the NmU serum levels dropped significantly in the cancer group but not in the chronic pancreatitis group with a comparable extent of surgery. Therefore, serum NmU may be derived from the pancreatic cancer itself, while the general tendency for lower NmU values in both groups may be explained by diluting effects of infusions and blood transfusions. Furthermore, total protein levels are usually decreased after major surgical procedures. Interestingly, three patients (1 CP and 2 PDAC) who suffered from postoperative gut-motility dysfunctions (gastric emptying, intestinal motility) had increased NmU serum levels after surgery. Since it has been shown that NmU is a potent prokinetic factor in gastrointestinal motility [51], it may be possible that NmU production is upregulated following postoperative gut-motility dysfunction.

To evaluate the pathobiology of the NmU/NmUR2 system in the carcinogenesis of PDAC, we first analyzed effects of exogenous NmU on cancer cell proliferation. In our study, NmU had no effects on growth in cell culture experiments. However, a recent study demonstrating neuromedin U overexpression in human samples of non-small-cell lung cancers revealed pro-proliferative effects of NmU at high concentrations (up to 15 μM) [22]. Since NSCLC tissue samples and cells did not express NmUR1 or NmUR2, two novel receptors were identified which bind to NmU (growth hormone secretagogue receptor-1b and neurotensin receptor-1) and which were overexpressed in NSCLC. However, the NmU concentrations used for binding to these receptors were much higher (micromolar range) than those used in studies with the cognate neuromedin U receptors (nanomolar range) [11].

As NmU did not alter pancreatic cancer cell growth, an indirect approach was chosen to identify downstream targets which were then used to define potential effects of NmU on pancreatic cancer cells. Screening experiments with RT-PCR analysis revealed an upregulation of c-Met in pancreatic cancer cell lines upon treatment with neuromedin U. In line with these results, we showed that total Met protein was also upregulated following incubation with NmU. These data support the notion that the NmU-Met axis seems to be involved in pancreatic cancer invasiveness and potentially also metastasis.

Activation of c-Met by its ligand HGF stimulates cancer cell invasion and mobility and enhances metastasis. In the current study, rHGF alone had a scattering effect on pancreatic cancer cells which was further enhanced when the cells were co-stimulated with NmU and HGF. It has recently been reported that NmU-transfected COS-7 cells were significantly more invasive than the control cells [22]. Furthermore, NmU expression is regulated by the metastasis suppressor RhoGDI2, and bladder cancer cells expressing high NmU levels showed enhanced anchorage-independent growth in vitro and more frequent tumor formation and lung metastases in nude mice xenografts [47]. In line with the results of our in vitro studies, these observations suggest that overexpression of NmU and the NmU receptor-2 induces increased invasiveness and an enhanced metastatic potential in pancreatic cancer.

Conflict of interest statement

None of the authors has a financial or other interest with regard to the submitted manuscript that might be construed as a conflict of interest.

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