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# Neurotensin is a versatile modulator of *in vitro* human Pancreatic Ductal Adenocarcinoma Cell (PDAC) migration

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**Abstract.** *Background*: While the neurotensin (NT) roles in pancreatic cancer growth are well documented, its effects on pancreatic cancer cell migration have not been described. *Methods:* The NT-induced effects on the migration process of human pancreatic ductal adenocarcinoma cells (PDACs) were characterized by means of various assays including computer-assisted videomicroscopy, fluorescence microscopy, ELISA-based, small GTPase pull-down and phosphorylation assays. *Results*: The NT-induced modifications on *in vitro* PDACs migration largely depended on the extra-cellular matrix environment and cell propensity to migrate collectively or individually. While NT significantly reduced the level of migration of collectively migrating PDACs on vitronectin, it significantly increased the level of individually migrating PDACs. These effects were mainly mediated through the sortilin/NTR3 receptor. Neurotensin both induced altered expression of $\alpha$V and $\beta$5 integrin subunits in PDACs cultured on vitronectin resulting in modified adhesion abilities, and caused modifications to the organization of the actin cytoskeleton through the NT-mediated activation of small Rho GTPases. While the NT effects on individually migrating PDACs were mediated at least through the EGFR/ERK signaling pathways, those on collectively migrating PDACs appeared highly dependent on the PI 3-kinase pathway. *Conclusion*: This study strongly suggests the involvement of neurotensin in the modulation of human PDAC migration.

Keywords: Neurotensin, pancreatic cancer, cell migration, integrins, sortilin/NTR3

## 1. Introduction

Pancreatic adenocarcinomas are one of the most common causes of death from cancer in the United States [18]. The overall cumulative 5-year survival rates are below 1% because most pancreatic ductal adenocarcinoma (PDA) patients are diagnosed at an advanced stage and the disease has a definite leaning towards undetected metastases [18]. The processes of pancreatic cancer initiation, progression and metastasis are still not fully understood [18] but a striking feature of pancreatic cancer development involves the presence of neurotensin (NT) receptors (NTRs) in a large majority of PDACs whereas endocrine pancreatic cancers, chronic pancreatitis and normal pancreatic acini, ducts and islets do not express NTRs [38,46]. It is thus presumed that NT and its receptors are involved in PDA initiation and/or development. At least three subtypes of NTRs have been cloned (NTR1, NTR2 and sortilin/NTR3 from the Vps10p-domain receptor family, and also SorLA). The high-affinity NTR1 (also known as NTS1) and the low affinity NTR2 (also known as NTS2) belong to the seven transmembrane domain/G protein-coupled receptor (GPCR) family [20,38,46]. NT also binds to some members of the Vps10p-domain receptor family such as the gp95/sortilin receptor (also designated as sortilin/NTR3). This is a single transmembrane domain sorting recep-

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tor predominantly associated with vesicular organelles and the Golgi apparatus [20,37]. NT which is a tridecapeptide originally isolated from bovine hypothalami has a wide range of biological activities when acting on the cardiovascular, gastrointestinal, reproductive and central nervous systems (for reviews see [5,39,44,46]). In the gastrointestinal tract NT has trophic effects on the large and small intestine, the pancreas, and the stomach; NT inhibits small intestine and gastric motility and stimulates colonic motor activity [5,39,44,46]. NT is also a proinflammatory neuropeptide in colonic inflammation [5] and regulates the growth levels of a large majority of cancers [4,5,29,39,44,46], including those of the pancreas [16,17,40]. While NT-mediated effects on cell migration features have already been reported for human microglia [30], cutaneous T cell lymphoma cells [27] and astrocytoma cells [42], nothing has been published to date, at least to the best of our knowledge, on the potential role of NT in pancreatic cancer cell migration. This topic is thus the subject of the present study.

Cell migration at the single cell level is a very complex biological process of coordinated steps, and a clear distinction has to be drawn between so-called collective cell – as opposed to single cell-migration [7,28]. An additional level of complexity appears with respect to the characterization of cell migration features in pancreatic cancers, i.e. the cross-talk between the extra-cellular matrix (ECM) and pancreatic cancer cells. Indeed, alterations to the ECM composition are a key feature of ductal adenocarcinomas of the pancreas, which differ from other solid tumors in their enormous production and deposition of ECM around the tumor cells [24,25]. In addition to mesenchymal cells, the epithelial tumor cells themselves contribute to the production and assembly of ECM proteins [24,25]. Of these proteins vitronectin is a major ECM component synthesized and secreted by PDACs [24,25]. All these biological features have been taken into account in our assessment of the characterization of NT-mediated effects on human pancreas cancer cell migration.

## 2. Materials and methods

### 2.1. Human pancreatic ductal adenocarcinoma cell lines

All the cell lines used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA), i.e. the Bx-PC3 (CRL-1687), the Capan-1 (HTB-79), the CF-PAC (CRL-1918) and the Capan-2 (HTB-80) models. The pancreatic origin of these cells was confirmed by means of standard immunochemical markers, i.e. CA19.9 (carbohydrate antigen 19.9) positivity, CK7 (cytokeratin-7) positivity and CK20 (cytokeratin-20) negativity (data not shown).

### 2.2. Compounds

Neurotensin, levocabastine (a NTR2 antagonist used at a final concentration of 1 $\mu$M) and human laminin, fibronectin and collagen-I were purchased from Sigma-Aldrich (Bornem, Belgium), while human vitronectin and tenascin came from Biognost (Heule, Belgium). Phallacidin, ionomycin and Fura-2-AM were obtained from Invitrogen (Merelbeke, Belgium). Sortilin/NTR3 antagonist pro-peptide resulting from the furin cleavage of the precursor form of sortilin/NTR3 [36] was synthesized by Eurogentec (Liège, Belgium) and was used at a final concentration of 1 $\mu$M, as already used in [30]. The NTR1 antagonist SR48692 was a gift from Sanofi-Synthelabo (Toulouse, France) and was used at a final concentration of 1 $\mu$M. Two EGFR inhibitors, i.e. AG-1468 and PD-153035 were obtained from Calbiochem (VWR, Leuven, Belgium). The PI 3-kinase inhibitor LY-294002 was obtained from Sigma-Aldrich (Bornem, Belgium) and was used at a final concentration of 50 $\mu$M.

### 2.3. Determination of expression of the ECM components and integrins in PDACs

The fluorescent detection of the ECM components and the integrin expression in PDACs cultured on glass cover slips was performed by means of computer-assisted fluorescence microscopy, as previously described [2]. The anti-integrin $\alpha$V and $\beta$3 antibodies were purchased from Becton Dickinson Biosciences (Erembodegem, Belgium) while the other antibodies (anti-integrin $\beta$1, anti-integrin $\beta$5, anti-vitronectin, anti-collagen-I, anti-tenascin, anti-laminin and anti-fibronectin) came from Santa Cruz Biotechnology (Tebu-Bio, Boechout, Belgium). Immunocytochemical expression of integrin subunits was evaluated in Bx-PC3 and Capan-2 cells in the absence and presence of NT (10 nM).

### 2.4. Western blotting

Cell extracts were prepared by the lysis of subconfluent PDACs directly in the boiling lysis buffer (10 mM Tris pH 7.4, 1 mM $Na_3O_4V$, 1% SDS, pH 7.4). 40 $\mu$g of extracted proteins (evaluated by the BCA protein assay; Pierce, Perbio Science, Erembodegem, Belgium) were loaded onto a denaturating polyacrylamide gel. Western blotting was carried out as detailed previously [31]. The primary antibodies: anti-NTR2 (1/50) and anti-NTR1 (1/20) were provided by respectively: AbCam, Cambridge, UK and Santa Cruz, Tebu-Bio, Boechout, Belgium. The antiserum against the sortilin/NTR3 was generated as detailed previously [36,37]. The secondary antibodies were obtained from Pierce (PerbioScience, Erembodegem, Belgium) and AbCam (Cambridge, UK). Western blots were developed using the Pierce Supersignal Chemiluminescence system (PerbioScience, Erembodegem, Belgium).

### 2.5. Small GTPases activation assays

The activation of small GTPases (Rho, Rac1, cdc42) immediately following 15 min of 10 nM neurotensin treatment of PDACs seeded onto vitronectin-coated culture flasks was evaluated by pull-down-based assays (EZ-detect activation kits, Pierce, Erembodegem, Belgium) following the manufacturer's instructions.

### 2.6. Determination of EGFR phosphorylation levels and levels of total and phospho-p85 PI3K, FAK, Src and ERK expression

PDACs were incubated in the presence and absence of NT (10 nM). Each experimental condition was evaluated in triplicate. Thereafter the determination of EGFR tyrosine phosphorylation levels was performed using DuoSet®IC Human Phospho-EGFR ELISA (R&D Systems, Abingdon, UK) according to the instructions provided and using 100 $\mu$g of protein extract for the assay. The levels of p85 PI3K, FAK, Src and ERK expression were determined using the Fast Activated Cell-based ELISA (FACE™) Kit (Active Motif, Rixensart, Belgium).

### 2.7. Real-time RT-PCR

The procedures used for total RNA isolation, quality control and standard and real-time RT-PCR were identical to those published previously [21]. Real-time PCR reactions were performed with 20 ng of purified cDNA in a LightCycler thermocycler instrument (Roche Diagnostics) using LC-Fastart DNA Master SYBR Green 1 (Roche Diagnostics). After amplification, data analysis was carried out by means of the "Fit points" algorithm of the Lightcycler quantification software. A standard curve enabled the quantification of the samples to be effected.

The primers employed included:

- Human Neurotensin Receptor type 1 (NTR1): external forward: 5′-TTAAGAAGGTCGCCTAA GC-3′ and reverse: 5′-AAGTGCAGCGAATA GGC-3′; internal forward: 5′-GCCTAAGAGAA GACAGTCC-3′ and reverse: 5′-TCGACTCTCA TTTCTCAGAC-3′.
- Human Neurotensin Receptor type 2 (NTR2): external forward: 5′-CTGTACAATTTCTACCACT ACTTC-3′ and reverse: 5′-ACTTTAGTCTCAG GCAACAC-3′; internal forward: 5′-GGACCTG AATGTAATGCAAG-3′ and reverse: 5′-GCAAC ACTAAGAGATGGGT-3′.
- Human Neurotensin Receptor type 3 (sortilin/NTR3): external forward: 5′-GGCTACGCAAGT CATCC-3′ and reverse: 5′-GCTATTCCAAGAG GTCCTC-3′; internal forward: 5′-AGAATGGTC GAGACTATGTTG-3′ and reverse:5′-TTTTCCG GACTCAAAAAGTT-3′.

### 2.8. *In vitro* adhesion assay

PDACs were seeded onto vitronectin-coated (0.1 $\mu$g/cm$^2$) 30 mm-cell culture dishes 24 h before the adhesion assay. The cells were treated with 10 nM NT for 4 hours and were then gently scraped using non-enzymatic methods in order to maintain the cell organization. The cells were then seeded onto $\alpha$V$\beta$5-mediated cell adhesion kit microplates (Chemicon, Biognost, Heule, Belgium) and incubated for 6 h (without any addition of NT). A monoclonal anti-$\alpha$V$\beta$5 antibody was immobilized onto a goat anti-mouse antibody coated microplate and used to capture cells expressing the $\alpha$V$\beta$5 integrin on their surfaces. The adherent cells were subsequently fixed and stained. The relative cell attachment was determined using absorbance readings. Each experimental condition was evaluated in triplicate.

### 2.9. *In vitro motility assay*

Human PDAC motility was defined using a device previously described [2,6] that quantifies the trajectories of living cells maintained in culture. The greatest linear distance covered by each cell was calculated on the basis of these trajectories. This distance, normalized for the observation time for the cell analyzed, defined the Maximum Relative Distance from the point of Origin, i.e. the MRDO quantitative variable [2,6]. PDACs cultured on vitronectin-coated (0.1 $\mu$g/cm$^2$) culture dishes were exposed to NT treatment at 0.1 and 10 nM. The data presented relate to the 25% of the most motile cells analyzed for each of the 4 human pancreas cancer cell lines under study. All the experiments were performed over 8 h and one image was recorded every 4 minutes. Since the analyses were conducted in sextuplicate, between 142 and 241 cells were analyzed for each experimental condition.

### 2.10. *Characterization of the organization of the actin cytoskeleton*

Fluorescent phallacidin conjugated with Alexa Fluor® 488 fluorochrome (Molecular Probes Inc., Invitrogen, Merelbeke, Belgium) was used to label the fibrillar actin, and Alexa Fluor® 594-conjugated DNAseI (Molecular Probes Inc) used to stain the globular actin. Prior to fluorescence staining, PDA cells were cultured on vitronectin-coated glass cover slips for 2 h in the absence of (controls) or presence of 10 nM NT as previously described [22,31].

### 2.11. *Intracellular calcium measurements*

Cells were cultured (30–50% confluence) on glass coverslips to quantify intra-cellular calcium levels ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ measurements were performed using Fura-2-AM as previously described [8].

### 2.12. *Data analyses*

Statistical comparisons of more than 3 independent groups of data were made using the Kruskal–Wallis test (a non-parametric one-way analysis of variance). In cases where this test revealed some significant differences or where only two groups were involved, applying the Mann–Whitney test compared pairs of groups. All the statistical analyses were performed using Statistica (Statsoft, Tulsa, Oklahoma).

## 3. Results

### 3.1. *Differential effects induced by NT on collectively and individually migrating human PDACs*

As illustrated in Figs 1A–D, Capan-1 (Fig. 1A) and Capan-2 (Fig. 1B) cells migrate collectively in contrast to Bx-PC3 (Fig. 1C) and CF-PAC (Fig. 1D) cells which migrate individually. By analyzing the type of components secreted by these cell lines, it was observed that the collectively migrating cells (Capan-1 and -2) secreted more vitronectin than those migrating individually (Bx-PC3 and CF-PAC; data not shown). NT was also found to significantly ($p < 0.01$ to $p < 0.001$) decrease motility in the collectively migrating cell populations cultured on vitronectin, but significantly ($p < 0.01$ to $p < 0.001$) increased it in individually migrating cells (Fig. 1E). These marked NT-induced dual effects observed with cells cultured on vitronectin were not seen when PDACs were cultured on other substrates (such as collagen-I, fibronectin, laminin and tenascin) on which lesser NT-induced effects were observed (data not shown).

### 3.2. *Attempts to identify which types of NTRs could be involved in the NT-mediated effects on human PDACs migration*

The presence of NTR1, NTR2 and sortilin/NTR3 were evidenced at both the mRNA (Fig. 1F) and protein (data not shown) level in the four human PDA cell lines under study. Given the similarities in the motility and NTRs profiles of Capan-1 versus Capan-2 and Bx-PC3 versus CF-PAC (Figs 1E–F), further investigations were confined to 10 nM NT-induced effects on the Capan-2 and Bx-PC3 cell lines alone. Using these two models it was observed that while the NTR1 antagonist SR48692 (SR: at 1 $\mu$M) and the NTR2 antagonist levocabastine (LV: at 1 $\mu$M) did not modify the NT-induced effects on cell motility ($p > 0.05$), the sortilin/NTR3 antagonist pro-peptide (PP: at 1 $\mu$M) did so in a statistically significant manner ($p < 0.01$; Fig. 1G). Furthermore, while modification of $[Ca^{2+}]_i$ is a usual event encountered in the case of NTR1-mediated effects on cells (although the functionality of NTR1 cannot be completely assessed by $[Ca^{2+}]_i$ measurements) [9,11], it was observed that NT induced no modification in $[Ca^{2+}]_i$ in human PDACs (data not shown).

All these data suggest that NTR3 mediates the NT-induced effects on PDAC migration rather than NTR1 or 2.

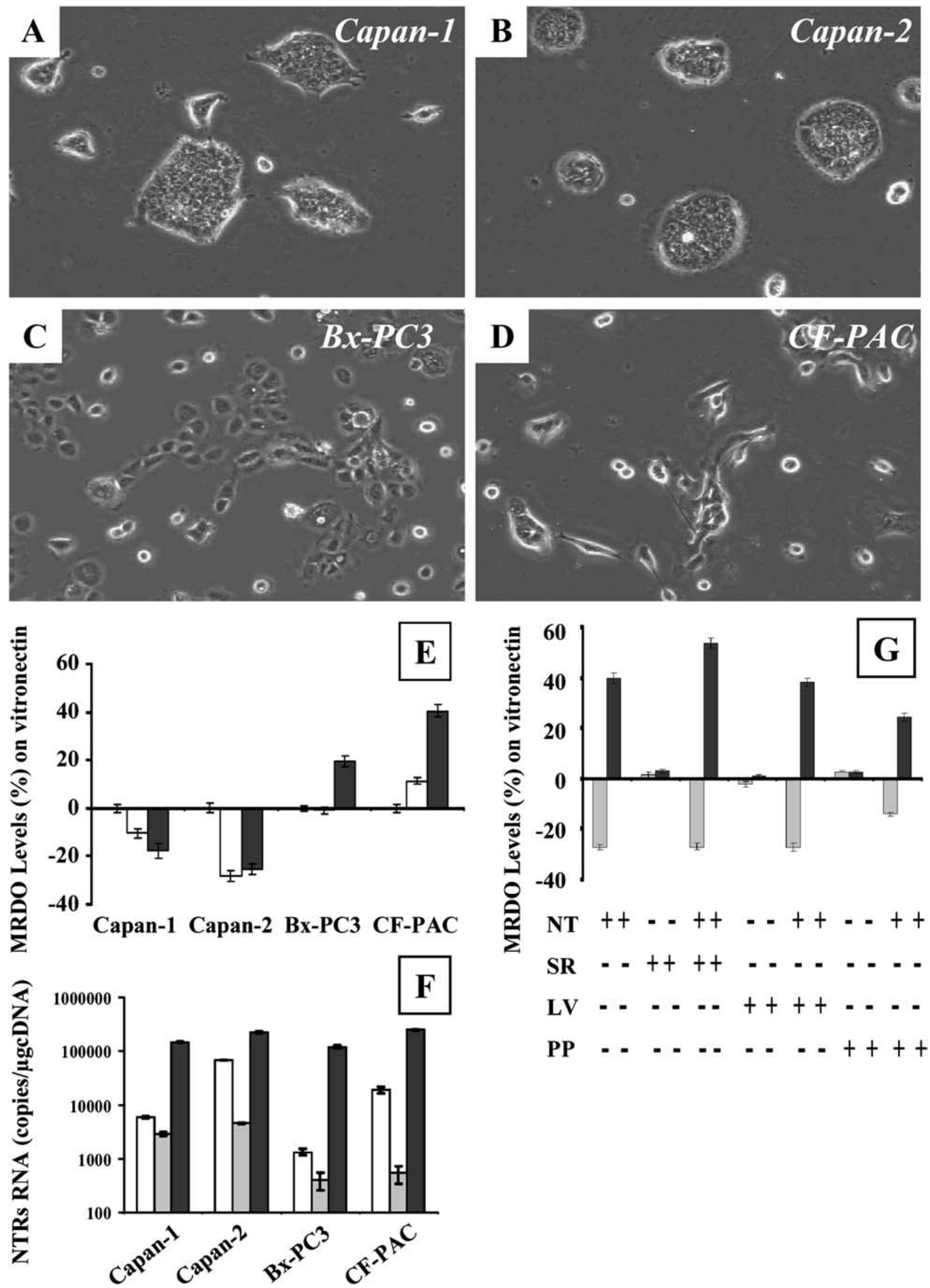


Fig. 1. (A–D) Illustrations of the different migratory behavior patterns encountered with the four human PDAC lines studied: collective in the case of Capan-1 (A) and Capan-2 (B) *versus* individual in the case of Bx-PC3 (C) and CF-PAC (D). (E) The effects on the motility (MRDO variable) of the PDACs cultured on vitronectin (0.1 $\mu$g/cm$^2$) due to NT treatment at 0.1 nM (open bars) and 10 nM (black bars). The data are reported as means $\pm$ SEM expressed in percentages of variation as compared to control (arbitrarily set at 0%). (F) Evaluation by means of the real-time RT-PCR of the mRNA expression of NTR1 (open bars), NTR2 (gray bars) and sortilin/NTR3 (black bars) in four human PDAC cell lines (logarithmic scale). (G) Similarly to Fig. 1E, the 10 nM NT-induced effects on Capan-2 (gray bars) and Bx-PC3 (black bars) cell motility observed on a vitronectin coating without pre-treatment, or after pre-treatment with the NTR1 antagonist (SR48692, indicated as SR; 1 $\mu$M), the NTR2 antagonist (levocabastine, indicated as LV; 1 $\mu$M) and the sortilin/NTR3 antagonist pro-propeptide (indicated as PP; 1 $\mu$M).

### 3.3. Modifications to the expression of $\alpha$V and $\beta$5 integrins parallel the NT-induced modifications to human PDAC cell adhesion and migration on vitronectin

Expression levels of certain integrins involved in vitronectin-mediated adhesion and their variation in response to NT (10 nM) have been investigated. While there was no evidence of any NT-induced modification to $\beta$1 and $\beta$8 expression (data not shown), significant NT-induced changes ($p < 0.01$ to $p < 0.001$) were observed with respect to the levels of $\alpha$V, $\beta$3 and $\beta$5 subunit expression. While dual effects were observed in the case of the $\beta$5 subunit between the collectively (Capan-2) and the individually migrating (Bx-PC3) cells (Fig. 2A: gray and black bars respectively), an increase in expression was observed in the case of the $\alpha$V (Fig. 2A) and $\beta$3 subunits (data not shown) in the two cell types. Evaluation of $\alpha$V$\beta$5-mediated adhesion abilities by means of dynamic adhesion assay also demonstrated NT-induced dual effects on the two investigated cell types (Fig. 2A).

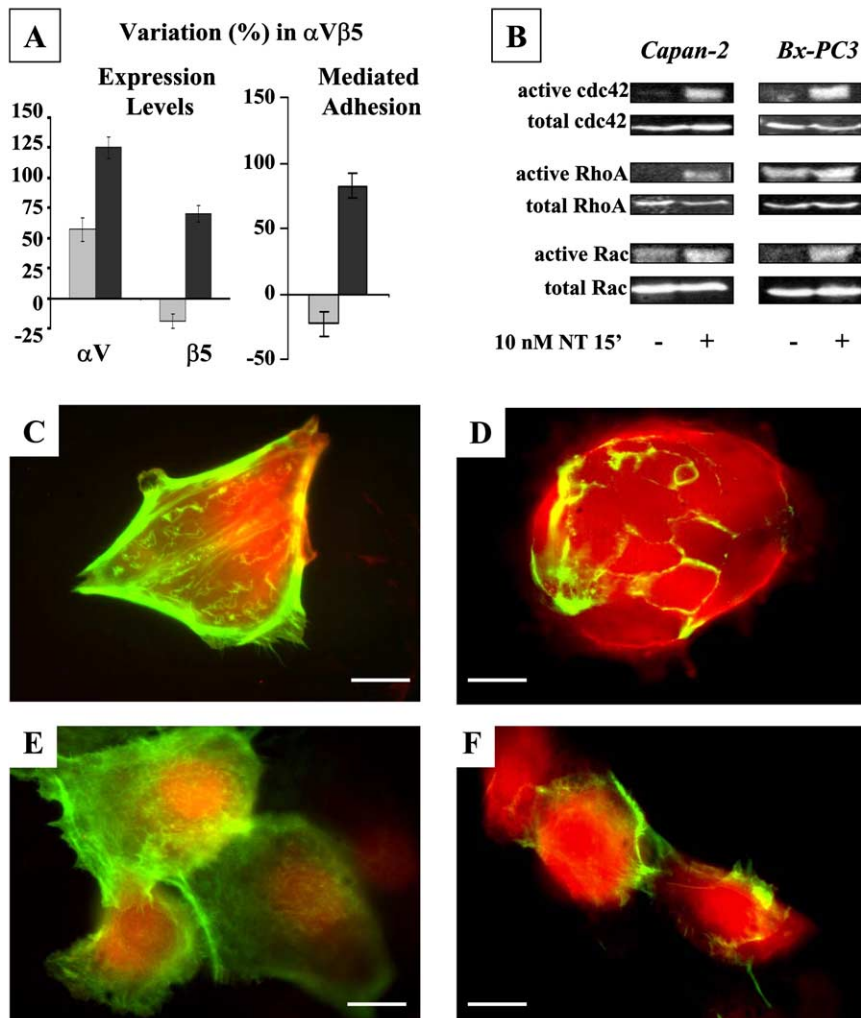


Fig. 2. (A) Variations in immunocytochemical expression of the $\alpha$V and $\beta$5 integrin subunits and $\alpha$V$\beta$5-mediated adhesion in the collectively migrating Capan-2 (gray bars) and the individually migrating Bx-PC3 (black bars) cells due to 10 nM NT. The data are reported as means $\pm$ SEM expressed in percentages as compared to control (set at 0%). (B) The expression and activation of small GTPases (Rho, Rac1, cdc42) in untreated cells and cells treated with 10 nM neurotensin for 15 min and visualized by western-blotting after pull-down assays. (C–F) Illustration of the fibrillary (green fluorescence) and globular (red fluorescence) actin patterns in untreated (C, E) and in 10 nM NT-treated (D, F) Capan-2 (C, D) and Bx-PC3 (E, F) cells cultured on vitronectin-coated slides. Scale bar: 50 $\mu$m.

### *3.4. NT modifies the organization of the actin cytoskeleton in human PDACs and affects the activation levels of Cdc42, Rho and Rac1*

Figures 2C–F illustrate the distribution of fibrillary (green fluorescence) as opposed to globular (red fluorescence) actin in untreated (C, E) and 10 nM NT-treated (D, F) Capan-2 (C, D) and Bx-PC3 (E, F) cells. While F-actin is located at the edge of collectively migrating PDAC colonies (Fig. 2C), individually migrating PDACs show high levels of F-actin located in each cell (Fig. 2E). In both cell types, NT induces a clear decrease in the fibrillarly actin (Figs 2D, 2F). In view of the differential NT-induced effects on cell motility (Fig. 1E) it thus appears that an impairment of the highly organized cortical actin sub-cellular structures of collectively migrating PDACs should decrease the cells' migratory ability. In contrast, the transformation of the rigid actin cytoskeletons of individually migrating PDACs into a more flexible structure should enhance their migratory abilities.

In parallel to the actions on the actin cytoskeleton, Fig. 2B evidences various NT-induced modifications to the activation levels of cdc42, Rho and Rac1, with some differences between individually and collectively migrating cells. Without modifying its total level of expression, 10 nM NT activated Rac1 in individually migrating Bx-PC3 cells (in which basal levels of active Rac1 are low) while it seemed to have no effect on it in collectively migrating Capan-2 cells (in which the basal levels of Rac1 activation are high). The opposite was evidenced in the case of Rho, which was activated by 10 nM NT in Capan-2 cells and left unchanged in Bx-PC3 ones. In contrast, 10 nM NT stimulated cdc42 activation in both cell lines (without modifying the total level of expression).

### *3.5. Partial characterization of the signaling pathways activated by neurotensin with respect to its effects on human PDAC migration*

In view of the data reported in the literature [1, 19,34] the involvement of the EGFR/ERK and the PI3-K signaling pathways in the NT-induced effects on PDACs were investigated. The data in Fig. 3A show that 10 nM NT slightly (20–30% on average)

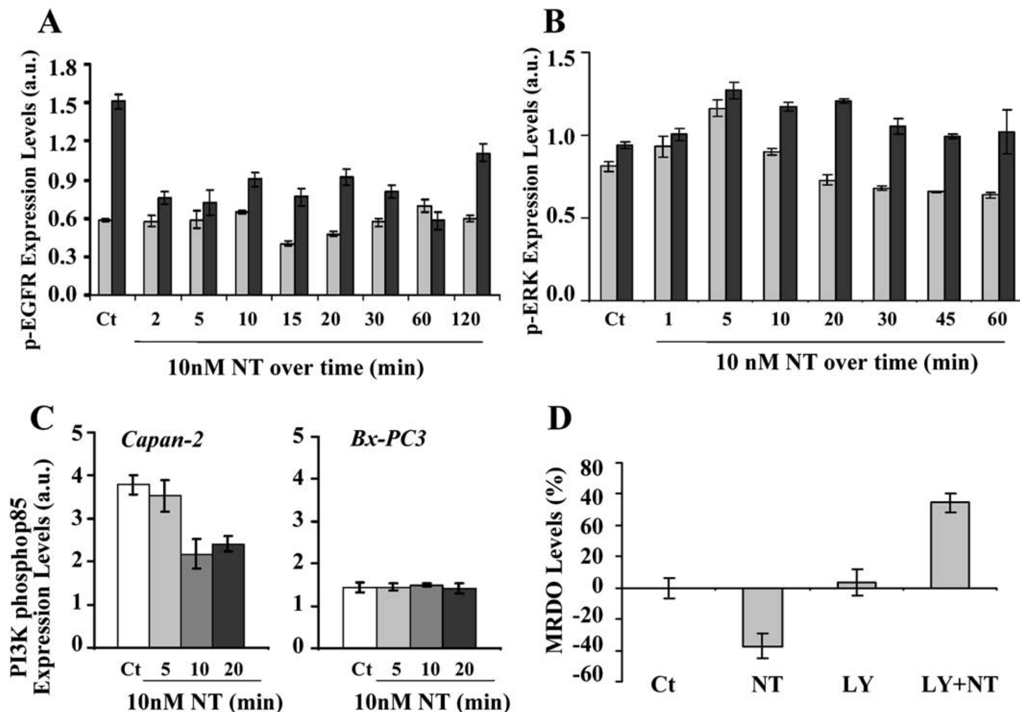


Fig. 3. Characterization of the signaling pathways affected by 10 nM NT in PDACs cultured on vitronectin. Evaluation of the NT effects on the EGFR (A) and ERK (B) phosphorylation levels in Capan-2 and Bx-PC3 (gray and black bars respectively). (C) The NT effects on the levels of expression of phosphorylated p85 PI 3-kinase. (D) The NT effects on the motility levels (expressed with control taken as reference) of Capan-2 cells cultured on vitronectin in the presence and absence of a PI3-K inhibitor (LY-294002; 50 $\mu$M). All the data are expressed as means $\pm$ SEM.

and only transiently decreased the EGFR phosphorylation levels in the Capan-2 cells, but did so durably and in a more pronounced manner (43–52%) in the Bx-PC3 cells. However, the phospho-EGFR inhibitors AG-1468 and PD-153035 did not significantly modify the NT-induced effects on the motility of these two cell lines (data not shown). Additionally, 10 nM NT did not significantly modify the Src phosphorylation levels in either the Capan-2 or the Bx-PC3 cells (data not shown). Figure 3B shows that while 10 nM NT slightly (30% to 40% on average) and only transiently increased the ERK phosphorylation levels in the Capan-2 cells (gray bars), it did so in a more prolonged manner in the Bx-PC3 cells (black bars). Indeed in the case of Capan-2 cells ERK phosphorylation levels actually declined significantly at later time points, whereas the two cell types exhibited similar basal ERK phosphorylation levels (data not shown). Figure 3C shows that 10 nM NT clearly decreased the phosphorylation levels of the regulatory p85 PI 3-kinase subunit in the collectively migrating Capan-2 cells, but not in the Bx-PC3 individually migrating ones. Furthermore, a PI 3-kinase inhibitor (LY-294002) completely reversed the NT-induced effects on Capan-2 cell migration (Fig. 3D). This suggests that NT may also be able to activate another pathway that results in the enhanced migration of Capan-2 cells in the absence of PI 3-kinase activation. As NT induces sortilin/NTR3 internalization, LY-294002 could disable sortilin/NTR3 recycling which depends on the PI 3-kinase pathway in particular [32] and could thus allow the activation of another pathway mediated by another receptor. Taken together, all these data suggest that NT modifies migration in collectively migrating Capan-2 cells independently (or only in a weakly dependent manner) of the EGFR/ERK signaling pathways, but in a highly dependent manner with respect to the PI 3-kinase pathway. A different situation seems to occur with respect to individually migrating Bx-PC3 cells, in which NT elicits clear modifications to EGFR/ERK phosphorylation levels, but not of PI 3-kinases.

## 4. Discussion

PDACs can adopt two different migration modes: collective- *versus* single-cell migration. Collective-cell movement could be a primary mechanism for invasion and metastasis by highly differentiated tumors, while single-cell movement could represent the typical invasion pattern of undifferentiated tumors [7,12]. As emphasized by Hegerfeldt et al. [12], single-cell and collective-cell migration strategies are both dependent on front-rear asymmetry driven by a dynamic leading edge and the coordinated detachment of the trailing edge. In single-cell migration forefront force generation is provided by adhesion receptors of the integrin family interacting with extracellular matrix components, a process which supports the concept of haptokinetic adhesion-dependent migration [7,12]. Collective-cell movement requires that several cells grouped together by adhesive cell–cell contacts generate front-rear asymmetry via unipolar ruffling in the cells at the leading edge, while those at the trailing edge remain largely non-motile [7,12,28]. As is also illustrated by our results, the organization of the actin cytoskeleton plays a number of key roles in these two cell migration patterns.

The data from the present study evidence that neurotensin is able to affect PDAC migration at different levels. The NT-induced effects on PDAC migration are clearly modulated by the molecular nature of the ECM and by the fact that the PDACs migrate individually as opposed to collectively. The essential NT-induced effects are observed on vitronectin and are schematically summarized in Fig. 4. As mentioned in the Introduction, NT binds to different NTR receptors with different levels of affinity. Furthermore, NT is able to bind directly to and to activate the bradykinin B2 receptor [35] and to inhibit the $Na^+/K^+$-ATPase activity [26]. Although significant amounts of NTR1, NTR2 and sortilin/NTR3 were evidenced in the different human PDA cell lines, further observations strongly suggested that NT acts mainly on cell migration modulators in PDACs through sortilin/NTR3. In contrast, data in the literature point to the direct involvement of NTR1 in the regulation of the growth of PDACs [16,17,40]. It should also be noted that upon sortilin/NTR3 engagement by neurotensin the NT/receptor complex is internalized and recycled intracellularly. This makes intracellular neurotensin signaling possible. Sortilin/NTR3 is also present on the surfaces of certain cancer cells and could be released to form a soluble protein able to bind exogenous NT [33].

As represented in Fig. 4, several reports [1,19,34] emphasize the interconnections and cross-talk of different signaling networks involving the molecular actors investigated in the present study. As reviewed by Iijima et al. [15], PI 3-kinase is emerging as a key regulator of cell polarity – an essential event involved in collective and individual cell migration, as is pointed

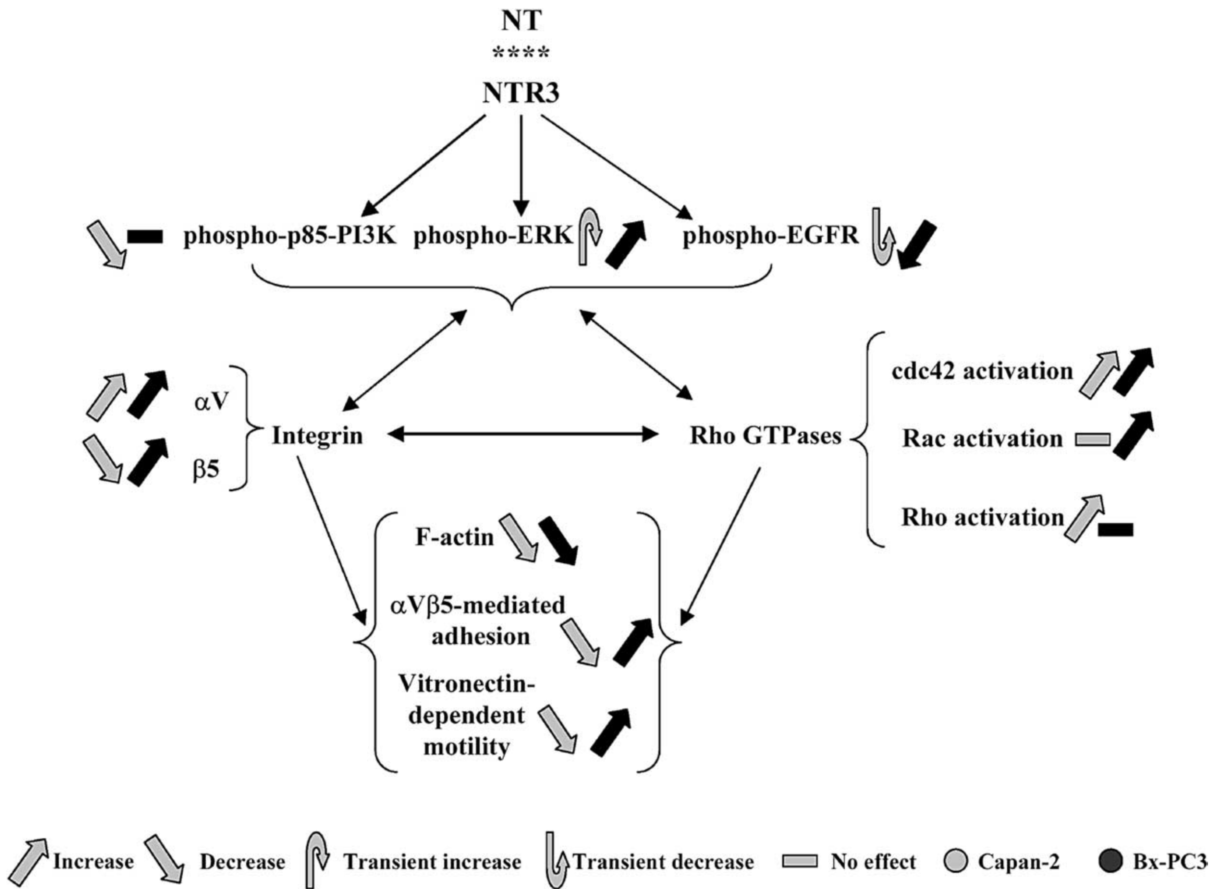


Fig. 4. Hypothetical model explaining the NT-induced effects on PDAC cell migration suggested by our experimental data.

out above. To date, PI 3-kinase has also been shown to activate predominantly Rac1 and cdc42 [43,45]. Many chemokines and growth factor receptors (including EGFR) engage Rac, cdc42 and/or RhoA by interfering with the PI 3-kinase pathway [3,43]. In addition, the overexpression of RhoA-GTP induces the activation of EGFR, the dephosphorylation of focal adhesion kinase and increased motility in breast cancer cells [3]. The Ras-ERK pathway is regulated by integrin $\alpha V\beta 5$ with consequences for cell proliferation, migration and angiogenesis [13]. More generally, integrins are known to play important regulatory roles in cell adhesion and migration [14]. In particular, integrins are able to activate both Rho and Rac [10]. GTPase cross-talk in migration is however complex. For example, activated Rac has been reported as decreasing or increasing Rho activity in different cell types [10,41], with possible negative feedback loops [23,47].

To investigate the NT-sortilin/NTR-3 effects on the aforementioned signaling pathways, a pro-peptide was used that is obtained from the furin cleavage of the precursor form of sortilin/NTR3. This pro-peptide which is a selective ligand of the soluble form of sortilin/NTR3 [30,36] that has been shown to antagonize the exogenous effects of NT on C13NJ microglial cell migration [30]. In agreement with our data, these NT-induced effects (at concentrations ranging between 1 and 10 nM) comprise a profound modification of the F-actin cytoskeleton and the stimulation of the motility of these individually migrating microglial cells [30]. Sortilin/NTR3 activation in the case of these microglial cells leads to the phosphorylation of ERKs [30], a feature also observed here with respect to the PDACs (Fig. 4). The same authors also evidence a PI 3-kinase-mediated upregulation of Akt phosphorylation [30], a fact which was not observed in the present study (data not shown). However, a decrease in PI 3-kinase phosphorylation was observed in collectively Capan-2 migrating cells (Fig. 4) without any effect on the Akt phosphorylation status (data not shown). Investigation

of other pathways, in particular those involving Src, urokinase-type plasminogen activator / focal adhesion kinase (uPA/FAK), failed to reveal evidence of any NT-mediated effects on these pathways in the PDACs under study (data not shown).

Acting mainly through the sortilin/NTR3 receptor, NT significantly reduced the level of migration in collectively migrating PDACs on vitronectin, while it significantly increased migration levels in individually migrating cells. As hypothesized in Fig. 4, our data suggest that these effects could be due to the NT-induced modifications to integrin subunit expression (resulting in altered adhesion abilities) and the Rho-GTPase activation levels with consequences for the organization of the actin cytosketelon. More particularly, in collectively migrating PDACs, NT-induced effects seem to be mediated through integrin $\beta$5 down-regulation, the deactivation of the PI 3-kinase pathway, and Rho activation. In contrast, in individually migrating PDACs the NT-induced effects seem to be mediated through integrin $\beta$5 up-regulation, the activation of both the EGFR/ERK pathway and Rac1. Related NT-induced effects were recently observed in the human U373 glioblastoma cell line [42]. The motility on laminin (one of the main ECM components of glioma) of these individually migrating cells was up-regulated by 10 nM NT-treatment, with this effect mediated through cdc42 and Rac1 activation. The versatility of the observed NT effects could, at least partly, shed new light on the reasons of failure of the NT receptor-based therapies in the case of PDAC.

In order to clarify the roles played by NT in pancreatic cancer as compared to the normal situation the present study could be interestingly completed by an analysis of the behavior of normal pancreatic cell lines under a NT treatment.

In conclusion, we are the first to show that neurotensin is able to modify the migration features of human pancretic ductal adenocarcinoma cells *in vitro*. These neurotensin-induced effects are mainly mediated through the sortilin/NTR3 receptor and are markedly influenced by whether cells migrate either individually or collectively.

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## References

[1] M. Baumgart, E. Heinmoller, O. Horstmann, H. Becker and B.M. Ghadimi, The genetic basis of sporadic pancreatic cancer, *Cell. Oncol.* **27** (2005), 3–13.

[2] N. Belot, S. Rorive, I. Doyen, F. Lefranc, E. Bruyneel, R. Dedecker, S. Micik, J. Brotchi, C. Decaestecker, I. Salmon, R. Kiss and I. Camby, Molecular characterization of cell substratum attachments in human glial tumors relate to prognostic features, *Glia* **36** (2001), 375–390.

[3] M. Caceres, J. Guerrero and J. Martinez, Overexpression of RhoA-GTP induces activation of the Epidermal Growth Factor Receptor, dephosphorylation of focal adhesion kinase and increased motility in breast cancer cells, *Exp. Cell. Res.* **309** (2005), 229–238.

[4] I. Camby, I. Salmon, E. Bourdel, N. Nagy, A. Danguy, J. Brotchi, J.L. Pasteels, J. Martinez and R. Kiss, Neurotensin-mediated effects on astrocytic tumor cell proliferation, *Neuropeptides* **30** (1996), 133–139.

[5] I. Castagliuolo, C.C. Wang, L. Valenick, A. Pasha, S. Nikulasson, R.E. Carraway and C. Pothoulakis, Neurotensin is a proinflammatory neuropeptides in colonic inflammation, *J. Clin. Invest.* **103** (1999), 843–849.

[6] O. Debeir, P. Van Ham, R. Kiss and C. Decaestecker, Tracking of migrating cells under phase-contrast video microscopy with combined mean-shift processes, *IEEE Trans. Med. Imaging* **24** (2005), 697–711.

[7] P. Friedl and K. Wolf, Tumour–cell invasion and migration: diversity and escape mechanisms, *Nat. Rev. Cancer* **3** (2003), 362–374.

[8] P. Gailly and M. Colson-Van Schoor, Involvement of trp-2 protein in store operated influx of calcium in fibroblasts, *Cell. Calcium* **30** (2001), 157–165.

[9] P. Gailly, E. Hermans and J.M. Gillis, Role of [$Ca^{2+}$]i in "$Ca^{2+}$ stores depletion-$Ca^{2+}$ entry coupling" in fibroblasts expressing the rat neurotensin receptor, *J. Physiol.* **491** (1996), 635–646.

[10] F.G. Giancotti and E. Ruoslahti, Integrin signaling, *Science* **285** (1999), 1028–1032.

[11] D. Gully, M. Canton, R. Boigegrain, F. Jeanjean, J.C. Molimard, M. Poncelet, C. Gueudet, M. Heaulme, R. Leyris, A. Brouard et al., Biochemical and pharmacological profile of a potent and selective nonpeptide antagonist of the neurotensin receptor, *Proc. Natl. Acad. Sci. USA* **90** (1993), 65–69.

[12] Y. Hegerfeldt, M. Tusch, E.B. Bröcker and P. Friedl, Collective cell movement in primary melanoma explants: Plasticity of cell-cell interaction, $\beta$1-integrin function, and migration strategies, *Cancer Res.* **62** (2002), 2125–2130.

[13] J.D. Hood, R. Frausto, W.B. Kiosses, M.A. Schwartz and D.A. Cheresh, Differential alphaV integrin-mediated Ras-ERK signaling during two pathways of angiogenesis, *J. Cell. Biol.* **162** (2003), 933–943.

[14] A. Huttenlocher, R.R. Sandborg and A.F. Horwitz, Adhesion in cell migration, *Curr. Opin. Cell. Biol.* **7** (1995), 697–706.

[15] M. Iijima, Y.E. Huang and P. Devreotes, Temporal and spatial regulation of chemotaxis, *Dev. Cell.* **3** (2002), 469–478.

[16] J. Ishizuka, C.M. Townsend Jr. and J.C. Thompson, Neurotensin regulates growth of human pancreatic cancer, *Ann. Surg.* **217** (1993), 439–445.

[17] K. Iwase, B.M. Evers, M.R. Hellmich, H.J. Kim, S. Higashide, D. Gully, J.C. Thompson and C.M. Townsend Jr., Inhibition of neurotensin-induced pancreatic carcinoma growth by a nonpeptide neurotensin receptor antagonist, SR48692, *Cancer* **79** (1997), 1787–1793.

[18] S. Keleg, P. Büchler, R. Ludwig, M.W. Büchler and H. Friess, Invasion and metastasis in pancreatic cancer, *Mol. Cancer* **2** (2003), 1–7.

[19] K. Kisfalvi, S. Guha and E. Rozengurt, Neurotensin and EGF induce synergistic stimulation of DNA synthesis by increasing the duration of ERK signaling in ductal pancreatic cancer cells, *J. Cell. Physiol.* **202** (2005), 880–890.

[20] P. Kitabgi, Targeting neurotensin receptors with agonists and antagonists for therapeutic purposes, *Curr. Opin. Drug Discov. Devel.* **5** (2002), 764–776.

[21] F. Lefranc, C. Decaestecker, J. Brotchi, C.W. Heizmann, O. Dewitte, R. Kiss and T. Mijatovic, Co-expression/co-location of S100 proteins (S100B, S100A1 and S100A2) and protein kinase C (PKC-beta, -eta and -zeta) in a rat model of cerebral basilar artery vasospasm, *Neuropathol. Appl. Neurobiol.* **31** (2005), 649–660.

[22] F. Lefranc, T. Mijatovic, V. Mathieu, S. Rorive, C. Decaestecker, O. Debeir, J. Brotchi, P. Van Ham, I. Salmon and R. Kiss, Characterization of gastrin-induced proangiogenic effects *in vivo* in orthotopic U373 experimental human glioblastomas and *in vitro* in human umbilical vein endothelial cells, *Clin. Cancer Res.* **10** (2004), 8250–8265.

[23] Z. Li, C.D. Aizenman and H.T. Cline, Regulation of rho GTPases by crosstalk and neuronal activity *in vivo*, *Neuron* **33** (2002), 741–750.

[24] S. Linder, E. Castanos-Velez, A. von Rosen and P. Biberfeld, Immunohistochemical expression of extracellular matrix proteins and adhesion molecules in pancreatic carcinoma, *Hepatogastroenterology* **48** (2001), 1321–1327.

[25] M. Löhr, B. Trautmann, M. Gottler, S. Peters, I. Zauner, B. Maillet and G. Kloppel, Human ductal adenocarcinomas of the pancreas express extracellular matrix proteins, *Br. J. Cancer* **69** (1994), 144–151.

[26] M.G. Lopez Ordieres and G. Rodriguez de Lores Arnaiz, Neurotensin inhibits neuronal $Na^{+}$, $K^{+}$-ATPase activity through high affinity peptide receptor, *Peptides* **21** (2000), 571–576.

[27] M. Magazin, E. Poszepczynska-Guigne, M. Bagot, L. Boumsell, C. Pruvost, P. Chalon, J.M. Culouscou, P. Ferrara and A. Bensussan, Sezary syndrome cells unlike normal circulating T lymphocytes fail to migrate following engagement of NTR1 receptor, *J. Invest. Dermatol.* **122** (2004), 111–118.

[28] M. Mareel and A. Leroy, Clinical, cellular, and molecular aspects of cancer invasion, *Physiol. Rev.* **83** (2003), 337–376.

[29] S. Martin, V. Navarro, J.P. Vincent and J. Mazella, Neurotensin receptor-1 and -3 complex modulates the cellular signaling of neurotensin in the HT29 cell line, *Gastroenterology* **123** (2002), 1135–1143.

[30] S. Martin, J.P. Vincent and J. Mazella, Involvement of the neurotensin receptor-3 in the neurotensin-induced migration of human microglia, *J. Neurosci.* **23** (2003), 1198–1205.

[31] V. Mathieu, T. Mijatovic, M. Van Damme and R. Kiss, Gastrin exerts pleiotropic effects on human melanoma cell biology, *Neoplasia* **7** (2005), 930–943.

[32] A. Morinville, S. Martin, M. Lavallee, J.P. Vincent, A. Beaudet and J. Mazella, Internalization and trafficking of neurotensin via NTS3 receptors in HT29 cells, *Int. J. Biochem. Cell. Biol.* **36** (2004), 2153–2168.

[33] V. Navarro, J.P. Vincent and J. Mazella, Shedding of the luminal domain of the neurotensin receptor-3/sortilin in the HT29 cell line, *Biochem. Biophys. Res. Commun.* **298** (2002), 760–764.

[34] S.S. Ng, M.S. Tsao, S. Chow and D.W. Hedley, Inhibition of phosphatidylinositide 3-kinase enhances gemcitabine-induced apoptosis in human pancreatic cancer cells, *Cancer Res.* **60** (2000), 5451–5455.

[35] T.J. Park and K.T. Kim, Activation of B2 bradykinin receptors by neurotensin, *Cell. Signal.* **15** (2003), 519–527.

[36] C.M. Petersen, M.S. Nielsen, C. Jacobsen, J. Tauris, L. Jacobsen, J. Gliemann, S.K. Moestrup and P. Madsen, Propeptide cleavage conditions sortilin/neurotensin receptor-3 for ligand binding, *EMBO J.* **18** (1999), 595–604.

[37] C.M. Petersen, M.S. Nielsen, A. Nykjaer, L. Jacobsen, N. Tommerup, H.H. Rasmussen, H. Roigaard, J. Gliemann, P. Madsen and S.K. Moestrup, Molecular identification of a novel candidate sorting receptor purified from human brain by receptor-associated protein affinity chromatography, *J. Biol. Chem.* **272** (1997), 3599–3605.

[38] J.C. Reubi, B. Waser, H. Friess, M. Buchler and J. Laissue, Neurotensin receptors: a new marker for human ductal pancreatic adenocarcinoma, *Gut* **42** (1998), 546–550.

[39] J.C. Reubi, Peptide receptors as molecular targets for cancer diagnosis and therapy, *Endocr. Rev.* **24** (2003), 389–427.

[40] N.M. Ryder, S. Guha, O.J. Hines, H.A. Reber and E. Rozengurt G, protein-coupled receptor signaling in human ductal pancreatic cancer cells: neurotensin responsiveness and mitogenic stimulation, *J. Cell. Physiol.* **186** (2001), 53–64.

[41] E.E. Sander, J.P. ten Klooster, S. van Delft, R.A. van der Kammen and J.G. Collard, Rac downregulates Rho activity: reciprocal balance between both GTPases determines cellular morphology and migratory behavior, *J. Cell. Biol.* **147** (1999), 1009–1022.

[42] S. Servotte, I. Camby, O. Debeir, C. Deroanne, C.A. Lambert, C.M. Lapière, B. Nusgens and C. Decaestecker, The in vitro influences of neurotensin on the motility characteristics of human U373 glioblastoma cells, *Neuropathol. Appl. Neurobiol.* **32** (2006), 575–584.

[43] S. Srinivasan, F. Wang, S. Glavas, A. Ott, F. Hofmann, K. Aktories, D. Kalman and H.R. Bourne, Rac and Cdc42 play distinct roles in regulating PI(3,4,5)P3 and polarity during neutrophil chemotaxis, *J. Cell. Biol.* **160** (2003), 375–385.

[44] R.P. Thomas, M.R. Hellmich, C.M. Townsend and B.M. Evers, Role of gastrointestinal hormones in the proliferation of normal and neoplastic tissues, *Endocr. Rev.* **24** (2003), 571–599.

[45] F. Wang, P. Herzmark, O.D. Weiner, S. Srinivasan, G. Servant and H.R. Bourne, Lipid products of PI(3)Ks maintain persistent cell polarity and directed motility in neutrophils, *Nat. Cell. Biol.* **4** (2002), 513–518.

[46] J. Wang, H. Friess, Z. Zhu, H. Graber, A. Zimmermann, M. Korc, J.C. Reubi and M.W. Buchler, Neurotensin receptor-1 mRNA analysis in normal pancreas and pancreatic disease, *Clin. Cancer. Res.* **6** (2000), 566–571.

[47] G.C. Zondag, E.E. Evers, J.P. ten Klooster, L. Janssen, R.A. van der Kammen and J.G. Collard, Oncogenic Ras downregulates Rac activity, which leads to increased Rho activity and epithelial-mesenchymal transition, *J. Cell. Biol.* **149** (2000), 775–782.