Expression of Neurotensin and NT1 Receptor in Human Breast Cancer: A Potential Role in Tumor Progression

Frédérique Souazé,[1] Sandra Dupouy,[1] Véronique Viardot-Foucault,[1,2] Erik Bruyneel,[3] Samir Attoub,[1] Christian Gespach,[1] Anne Gompel,[1,2] and Patricia Forgez[1]

[1]Institut National de la Sante et de la Recherche Medicale U673 and UPMC, Hôpital Saint-Antoine; [2]Unité de Gynécologie, AP-HP, Hôtel-Dieu de Paris, Paris, France and [3]Laboratory of Experimental Cancerology, Ghent University Hospital, Ghent, Belgium

Abstract

Emerging evidence supports neurotensin as a trophic and antiapoptotic factor, mediating its control via the high-affinity neurotensin receptor (NT1 receptor) in several human solid tumors. In a series of 51 patients with invasive ductal breast cancers, 34% of all tumors were positive for neurotensin and 91% positive for NT1 receptor. We found a coexpression of neurotensin and NT1 receptor in a large proportion (30%) of ductal breast tumors, suggesting a contribution of the neurotensinergic signaling cascade within breast cancer progression. Functionally expressed NT1 receptor, in the highly malignant MDA-MB-231 human breast cancer cell line, coordinated a series of transforming functions, including cellular migration, invasion, induction of the matrix metalloproteinase (MMP)-9 transcripts, and MMP-9 gelatinase activity. Disruption of NT1 receptor signaling by silencing RNA or use of a specific NT1 receptor antagonist, SR48692, caused the reversion of these transforming functions and tumor growth of MDA-MB-231 cells xenografted in nude mice. Our findings support the contribution of neurotensin in human breast cancer progression and point out the utility to develop therapeutic molecules targeting neurotensin or NT1 receptor signaling cascade. These strategies would increase the range of therapeutic approaches and be beneficial for specific patients. (Cancer Res 2006; 66(12): 6243-9)

Introduction

Breast cancer is the most frequent cause of cancer-related deaths among women in the western world (1). Today, early and systematic screening generates detection of early stages of breast cancer. In the last decade, improvement of adjuvant hormonal therapy and polychemotherapy has reduced the risk of recurrence and death from breast cancer (2, 3). Nonetheless, more accurate prognostic and predictive factors are needed to adjust the treatment and its aggressiveness.

For example, the overexpression of the HER-2/*neu* proto-oncogene was found to correlate with increased tumor aggressiveness, recurrence, and mortality in node-positive patients (4). The combination of chemotherapy and Herceptin, a blocker of the HER-2/*neu* growth factor receptor, provides benefits for metastatic breast cancer patients whose tumors overexpressed HER-2, suggesting that HER-2 signaling is critically involved in the carcinogenesis of the mammary gland (5). Several additional data and converging evidence support the concept that the HER family [epidermal growth factor (EGF) receptor (EGFR), HER-2, HER-3, and HER-4] and their ligands can contribute to breast cancer development and clinical course of the disease (6). Abnormal expression of HER members results in receptor hyperactivation due to abnormal transcriptional regulation ensuing in protein overexpression and stimulation enhancement by growth factors (7). Although the functionality of HER members, especially HER-2, has been extensively studied in breast cancer (6), the pathologic conditions resulting in HER member family activation is not fully understood. Emerging paradigms suggest that several growth-promoting peptides, such as angiotensin, endothelin, vasoactive intestinal peptide, bombesin, gastrin, and neurotensin, interact in a cross-talk with the EGFR signaling pathway (8, 9). Many of these peptide receptors, belonging to the G-protein-coupled receptor (GPCR) family, are often overexpressed in human solid tumors and activate several transforming functions, including cell proliferation, survival, invasion, tumor angiogenesis, and metastasis (10).

Several reports implicate neurotensin and its high-affinity neurotensin receptor (NT1 receptor) in several detrimental functions linked to the neoplastic progression, including proliferation of the pancreas, prostate, colon, and lung cancer cells (11), protection of breast cancer cells against apoptosis (12), and induction of the proinvasive potential of colon cancer cells (13). In colon and breast cancers, the NT1 receptor is a Wnt/β-catenin target gene (13).

The central and peripheral functions of neurotensin are mediated through its interaction with NT1, NT2, and NT3 receptors (14). NT1 and NT2 are GPCRs with high (subnanomolar) and low (nanomolar) affinity for neurotensin, respectively. *In vivo* and *in vitro* neurotensin effects are abolished by the specific antagonist of NT1 receptor SR48692, showing that NT1 receptor is the major mediator of these transforming actions (15).

Neurotensin is largely distributed along the gastrointestinal tract (16). Neurotensin is released into the blood circulation soon after a meal and persists for at least 10 hours, increasing its basal blood concentration from 10 to 20 up to 230 pmol/L (17). The physiologic functions of neurotensin include stimulation of pancreatic and biliary secretions, inhibition of small bowel and gastric motility, and facilitation of fatty acid translocation (18–20). In addition, neurotensin is a trophic factor on gastric antrum, small bowel, and colon tissue regeneration (11).

The potential malignant effect of neurotensin is further sustained by data showing increased NT1 receptor expression in human cancers versus the corresponding normal epithelium (21, 22). When NT1 receptor is challenged with neurotensin, phosphatidylinositols are hydrolyzed leading to Ca^{2+} mobilization, and extracellular signal-regulated kinase 1/2 (ERK1/2), Rho GTPases (RhoA,

Requests for reprints: Patricia Forgez, Institut National de la Sante et de la Recherche Medicale U673 and UPMC, Hôpital Saint-Antoine, Bâtiment Raoul Kourilsky, 184 rue du Faubourg St-Antoine, 75571 Paris Cedex 12, France. Phone: 33-1-49-28-46-69; Fax: 33-1-44-74-93-18; E-mail: forgez@st-antoine.inserm.fr.
©2006 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-06-0450

Downloaded from http://aacrjournals.org/cancerres/article-pdf/66/12/6243/2863610/6243.pdf by guest on 01 December 2023

Rac1, and Cdc42), and focal adhesion kinase (FAK) are activated, leading to immediate or delayed responses to neurotensin involving gene transcription activation, cell growth, death, or differentiation (23–25). More recently, it was shown that neurotensin via NT1 receptor transactivates EGFR by the shedding of transforming growth factor-α (TGF-α) in pancreas and heparin-binding EGF or amphiregulin in prostate cancer cells, both leading to ERK1/2 activation (26, 27).

In this article, we report on the coexpression of NT1 receptor and neurotensin in human breast ductal invasive adenocarcinomas. We showed the detrimental role of endogenous neurotensin and NT1 receptor activation on the tumor growth initiated by human breast cancer cells xenografted in nude mice. Neurotensin and NT1 receptor had a discernible malignant effect on human breast adenocarcinoma cell proliferation, migration, and invasion. This report is the first study to support the contribution of neurotensin and NT1 receptor in the progression of transformed human breast epithelial cells (HBEC) to malignancy.

Materials and Methods

Culture procedure. The human breast adenocarcinoma cell line, MDA-MB-231, was grown in DMEM (Invitrogen, Cergy Pontoise, France) supplemented with 10% FCS and 2 mmol/L glutamine. Normal HBECs were cultured as described previously in Gompel et al. (28). Briefly, breast tissue was obtained from 15 women undergoing reduction mammoplasty in ages between 15 and 25 years. Patients had no history of breast disease and pathologic studies revealed only normal breast tissue. Sampling of the tissues was done according to the French regulations on clinical experimentation. The tissue was digested with 0.15% collagenase and 0.05% hyaluronidase in Ham's F-10 (Invitrogen) and consecutively filtered through 300- and 150-μm sieves to retain undigested tissue and collected on 60-μm sieves. Cells were grown in Ham's F-10 with phenol red supplemented with 0.24% $NaHCO_3$, 1% penicillin-streptomycin, 5 ng/mL cortisol, 6.5 ng/mL T3, 10 ng/mL cholera toxin, 5 mg/mL transferrin, 5% compatible human serum, 0.12 units/mL insulin, and 10 ng/mL EGF in a humidified atmosphere of 5% CO_2, 95% air. Cells reached confluence after 15 to 20 days of primary culture.

Breast tumors. A total of 84 patients diagnosed for breast cancer and undergoing tumorectomy or mastectomy for complete resection of their primary tumors (Gynecology Department, Hôtel-Dieu Hospital, Paris, France) were studied. A series of 70 patients with invasive ductal breast cancers (IDC) with concomitant ductal carcinoma *in situ* (DCIS) were analyzed. The average age was 60.7 ± 12.1 years, with a mean tumor size of 2 ± 1.3 cm, and 28 patients exhibited invaded nodes (from a total of 67 cases studied). The malignancy of the infiltrating carcinomas was scored according to the Scarff-Bloom-Richardson (SBR) histoprognostic system (29); accordingly, 22, 26, and 22 patients were classified as grades 1, 2, and 3, respectively. Four DCIS and 10 invasive lobular breast carcinomas were also collected. Tumors were fixed in 10% formaldehyde and embedded in paraffin wax. Several sections (5 μm thick) were made for each case. The histologic diagnosis was routinely checked by microscopic examination of sections stained with H&E.

Immunohistochemistry. Immunostaining of NT1 receptor and neurotensin was carried out on deparaffinized sections using the avidin-biotin-peroxidase complex method. After inhibition of endogenous peroxidases with 3% hydrogen peroxide, slides were washed in TBS and incubated with 10% normal rabbit serum at room temperature for 30 minutes. NT1 receptor immunoreactivity was detected using a goat polyclonal antibody directed against the human COOH terminus of the receptor (1:100; C-20; Santa Cruz Biotechnology, Santa Cruz, CA). Neurotensin immunoreactivity was conducted using rabbit antibody directed against neurotensin (1:500; NA1230; Biomol International, Plymouth, PA) for 2 hours at room temperature in a humidified chamber. Neurotensin or NT1 receptor immunohistochemistry specificity was checked by omission of primary antibody and by displacement with neutralizing peptide (Santa Cruz Biotechnology) or neurotensin for 2 hours at room temperature. All slides were rinsed thrice with TBS; sections were incubated with biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. The antigen-antibody complex was revealed with avidin-biotin-peroxidase complex for 30 minutes according to the manufacturer's instructions for the Vectastain ABC kit (Vector Laboratories). Staining was done for 5 minutes with 3,3′-diaminobenzidine (Sigma-Aldrich, Lyon, France). All slides were counterstained with hematoxylin. A semiquantitative estimation of the number of positive cells was done by counting 1,000 reactive and nonreactive cells in 10 successive fields at the original ×250 magnification.

RNA extraction and reverse transcription-PCR. The protocols for total RNA extraction, reverse transcription, and PCR are documented by Souaze et al. (30). Reverse transcription was done on 2 μg total RNA using a specific NT1 receptor primer (5′-GCTGACGTAGAAGAG-3′) or 50 pmol oligo(dT) and oligo(dN). The PCR amplification was done on a 1:5 (v/v) of the reverse transcription reaction using 25 pmol of each primer 5′-CGTGGAGCTGTACAACTTCA-3′ and 5′-CAGCCAGCAGACCACAAAGG-3′ for NT1 receptor and 5′-TCCAATAGGTGATGTTGTCGT-3′ and 5′-TCCAA-TAGGTGATGTTGTCGT-3′ for matrix metalloproteinase (MMP)-9 and 1 unit Taq polymerase (Applied Biosystems, Courtaboeuf, France). The amplification profile consisted of denaturation at 94°C for 30 seconds, annealing at 57°C for 45 seconds, and extension at 72°C for 45 seconds. The PCR cycles were preceded by denaturation at 95°C for 5 minutes and were followed by a final extension at 72°C for 10 minutes. Amplification was done in a DNA thermal cycler 9700 (Perkin-Elmer Applied Biosystems, Courtaboeuf, France).

NT1 receptor small interfering RNA construction and transfection. Small interfering RNAs (siRNA) for human NT1 receptor (AAGAAGTTCAT-CAGCGCCATC) and scramble sequence (ATCGTCCGAACGTAAGTCAA) were prepared using pSilencer 3.1-H1 according the manufacturer's instruction (Ambion, Austin, TX). MDA-MB-231 cells were transfected using Lipofectamine reagent (Invitrogen). Stable transfectants were selected with hygromycin B (400 μg/mL) and colonies were screened using NT1 receptor reverse transcription-PCR (RT-PCR) and binding assay. Two MDA siNT1 receptor clones, MDA Si1 and Si2, and one pool of clones expressing the scramble sequence, MDA Scr, were chosen for further experiments.

Wound healing. For migration assays, MDA-MB-231 cells were plated in six-well dishes. Twenty-four hours after the cells reached confluence, scarification was done in a Moscona buffer with a pipette tip. Cells were maintained in culture in low-serum medium (0.1% FCS) and treated with 10^{-8} mol/L neurotensin agonist, JMV449, in the presence or absence of NT1 receptor antagonist, SR48692. Pictures were taken at the beginning of the treatment and 18 hours later.

Collagen invasion assays. The method was adapted from Bracke et al. (31). Petri dishes were filled with 2.5 mL neutralized type I collagen (0.18%; Upstate Biotechnology, Lake Placid, NY) and allowed to gel for 4 hours at 37°C and 5% CO_2. MDA-MB-231 cells were harvested and isolated using Moscona buffer and trypsin/EDTA, and 0.2 × 10^6 cells were seeded on top of the gel collagen. Dispersed cells were cultured for 24 hours at 37°C in the presence or absence of indicated effectors. Counting was done under code: invasive and superficial cells were counted in 12 fields of 0.157 mm^2. The invasion index was calculated as the percentage of cells invading the gel divided by the total number of cells.

Gelatin zymography. Metalloproteinase activity in supernatants of MDA-MB-231 cells cultured in serum-free medium and conditioned or not conditioned for 24 hours with neurotensin agonist, JMV449, and was tested by zymography. Supernatants were desalted on G25 Sephadex column and then concentrated by lyophilization. Total protein (50 μg) was analyzed under nonreducing conditions on 8% polyacrylamide gels containing 0.1% gelatin. For development, gels were washed twice in 2.5% Triton X-100, incubated overnight at 37°C in 50 mmol/L Tris-HCl (pH 7.6), 5 mmol/L $CaCl_2$, 0.02 mg/mL NaN_3, and stained with Coomassie blue.

Animals. Xenografts were initiated by s.c. injection of 3 × 10^6 MDA-MB-231 wild-type (MDA wt), their silenced NT1 receptor counterparts (MDA Si1 or Si2), or the control MDA scramble cells (MDA Scr). Cells were resuspended in 100 μL PBS and 100 μL Matrigel. Three days after injection, the mice were randomly divided in two groups of 10 animals each and

Downloaded from http://aacrjournals.org/cancerres/article-pdf/66/12/6243/2863610/6243.pdf by guest on 01 December 2023

received the following treatments as i.p. injection: control, vehicle solution; NT1 receptor antagonist, SR48692 (1 mg/kg) resuspended daily in 0.5% Tween 20 in 9‰ NaCl. The treatment was continued for 27 days. The tumor volume was calculated using the formula: length × width^2 × 0.4. After the treatment period, mice were sacrificed and the tumors were dissected and weighed.

Statistical analysis. The biochemical data were compared by Student's *t* test or ANOVA followed by Neuman-Keuls' test.

Results

NT1 receptor is not expressed in normal human epithelial cells and breast mammary tissue. We first studied NT1 receptor mRNA expression in normal HBECs. As shown in Fig. 1*A*, the NT1 receptor is very poorly expressed or absent in normal cells (*lanes 2-6*) in comparison with MCF-7 cells (*lane 1*). NT1 receptor expression was also studied in 10 additional samples of HBEC (data not shown). In these patients, NT1 receptor expression was either feeble or absent. As confirmation of this result, immunohistochemistry in normal tissue exhibited no staining in the epithelial and in the myoepithelial cells of ducts or in the lobular cells (Fig. 1*B*).

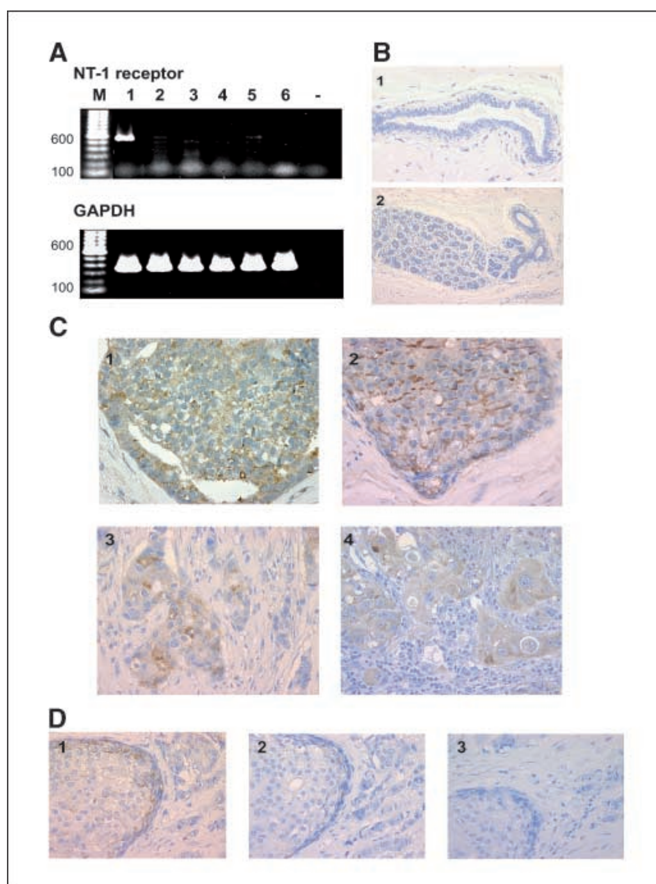

Figure 1. NT1 receptor expression in normal human breast cells, normal human breast tissues, and human breast carcinomas. *A,* 1 μg total RNA from HBEC and MCF-7 cells was reverse transcribed. A PCR experiment was done using specific primers for NT1 receptor and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). *Top,* NT1 receptor amplicons from MCF-7 cells (*lane 1*) and HBEC (*lanes 2-6*); *bottom,* GAPDH amplicons from the same samples. For both gels, *lane* - corresponds to PCR from reverse transcription without mRNA. *B,* normal duct (*1*) and lobule (*2*) from reduction mammoplasty samples exposed to NT1 receptor antibody at 1:100 dilution. *C,* typical staining of DCIS or IDCs exposed to 1:100 of COOH-terminal NT1 receptor antibody: DCIS (*1*), ductal component of SBR histologic grade 2 (*2*), and invasive component of SBR histologic grade 2 (*3*) or grade 3 (*4*). *D,* IDCs exposed to NT1 receptor antibody at 1:200 dilution (*1*) after preincubation with the antigen peptide for 2 hours at 1:20 (*2*) or without primary antibody (*3*). Original magnification, ×200 (*B*) and ×400 (*C* and *D*).

NT1 receptor is expressed in human breast cancers. NT1 receptor expression was studied in patients diagnosed for lobular carcinoma, DCIS, or IDC. Globally, NT1 receptor immunoreactivity was observed in the ductal or invasive compartment or both and with variable intensity. In DCIS (Fig. 1*C, 1*) or in the ductal component of IDC with SBR histologic grade 2 (Fig. 1*C, 2*), the staining of the cancer cells was granular and located at the cell surface. Interestingly, a polarized cell staining was often observed as shown in Fig. 1*C, 2*. Concerning the invasive component, the staining was mainly restricted to the cytoplasm. As shown in Fig. 1*C, 3* and *4*, no major difference could be noticed within the SBR histologic grades. We confirmed the specificity of the COOH-terminal antibody directed against human NT1 receptor; the labeling observed when incubated with NT1 receptor was totally suppressed by a preincubation with the antigen peptide (Fig. 1*D, 2*) or by omission of the primary antibody (Fig. 1*D, 3*).

Among the breast carcinomas studied, the majority exhibited a high proportion of NT1 receptor–positive cells (50-100%). Nonetheless, patients were considered positive with ≥10% positive cells. According to this assessment, we observed that 9 of 10 invasive lobular breast cancers, 4 of 4 DCIS, and 64 of 70 (91%) of the patients with IDCs were positive. Interestingly, NT1 receptor expression was not homogeneous in the ductal or the invasive compartment and varied according to the SBR grade. We observed that in 59% of patients with histologic grade 1 NT1 receptor reactivity was restricted to the intraductal compartment compared with only 4% for grade 3. In sharp contrast, the NT1 receptor–positive patients in the invasive component were predominantly in the histologic grade 3 (90%), with only 36% in grade 1.

Neurotensin is expressed in human breast carcinomas. Neurotensin expression was studied by immunohistochemistry on 50 patients previously studied for NT1 receptor expression to establish the potential NT1 receptor activation via neurotensin. As shown in Fig. 2, neurotensin labeling was detected in the IDCs. The labeling was cytoplasmic in both ductal and invasive compartments. Neurotensin reactivity was either absent or intense in both compartments (Fig. 2, *1* and *2*). Neurotensin antibody specificity was assessed by antibody preincubation with neurotensin as well as by the omission of primary antibody; in both cases, no labeling was observed (Fig. 2, *3* and *4*). Among the 50 patients, 44 (88%) were NT1 receptor positive, 17 (34%) neurotensin positive, and 15 (30%) positive for both NT1 receptor and neurotensin.

Tumor growth in the human breast cancer cell line MDA-MB-231 is affected by NT1 receptor expression. We next investigated the expression of NT1 receptor in the highly tumorigenic and metastatic breast cancer cell line MDA-MB-231. A specific PCR amplicon with a size of 590 bp, corresponding to the NT1 receptor, was detected (Fig. 3*A*). To establish the contribution of NT1 receptor on tumor breast progression, expression vectors encoding siRNA to silence NT1 receptor gene expression were stably introduced into parental MDA-MB-231 cells (MDA-wt). As expected, NT1 receptor transcripts were depleted in the two clones Si1 and Si2 (Fig. 3*A*). As a control, we verified that the NT1 receptor amplicon was unchanged in MDA-MB-231 cells stably transfected by the expression vector encoding the scrambled NT1 receptor silencing RNA sequence (Scr). MDA wt and Scr cells bound 2.89 ± 0.4 and 2.98 ± 0.3 fmol [^{125}I]neurotensin/mg crude

Downloaded from http://aacrjournals.org/cancerres/article-pdf/66/12/6243/2863610/6243.pdf by guest on 01 December 2023

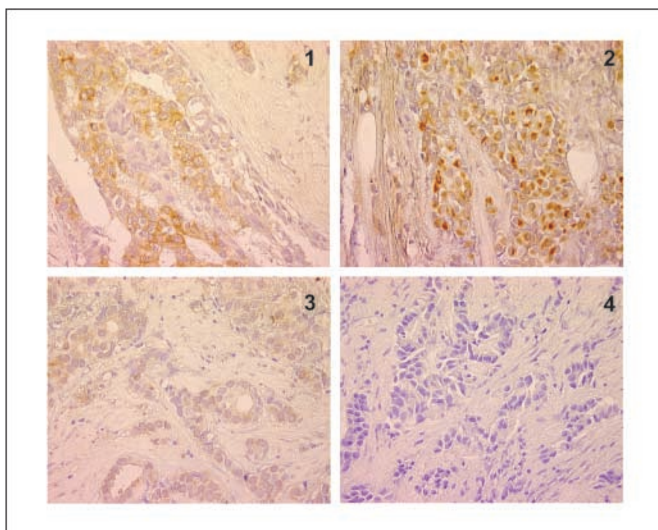

Figure 2. Neurotensin is expressed in human breast carcinomas. Typical staining of IDCs exposed to 1:500 of neurotensin antibody: ductal (*1*) and invasive (*2*) component of SBR histologic grade 2. IDCs exposed to neurotensin antibody at 1:500 dilution after preincubation with 10^{-8} mol/L neurotensin (*3*) or without primary antibody (*4*). Original magnification, ×400.

membrane protein, respectively, whereas MDA Si1 and Si2 cells did not bind [^{125}I]neurotensin.

Using these cellular models, we examined the effect of NT1 receptor depletion on the tumorigenic potential of MDA-MB-231 cells xenografted in nude mice. Silenced, scrambled, and parental cells were injected s.c. in the right flank of nude mice, and the tumor size was measured 5 weeks after inoculation. As shown in Fig. 3*B*, depletion of NT1 receptor in MDA Si1 and Si2 cells is accompanied by a significant 40% and 70% fold decrease in tumor volume, 25% and 70% fold decrease in the final tumor weight, and 35% and 80% lengthening of tumor doubling time compared with MDA wt cells. The strength of the two siRNAs (Fig. 3*A*) correlated with their ability to inhibit tumor growth in MDA cells, with Si2 being the more affected clone.

This result substantiates NT1 receptor involvement in promoting breast tumor growth. To validate the *in vivo* effect, xenografted nude mice with MDA wt cells were administrated with daily doses of the specific NT1 receptor antagonist, SR48692 (15), for 27 days. Five days after inoculation, the initial breast tumor volume was the same in control and treated groups (191 ± 10 and 192 ± 3.4 mm^3, respectively). As shown in Fig. 3*C*, SR48692 reduced by 25% and 30% the volume and weight of the MDA-MB-231 breast tumor xenografts and, accordingly, lengthened the tumor doubling time by 35%.

Neurotensin-induced motility and cellular invasion of breast cancer cell lines through NT1 receptor activation. Because active migration and invasive growth of transformed cells are characteristics of cancer progression, we examined the effect of neurotensin and NT1 receptor in the wound closure and collagen type I invasion assays using MDA wt and Si1 and Si2 cells. As shown in Fig. 4, the weakly degradable neurotensin agonist, JMV449, remarkably increased the migration and wound healing of parental MDA-MB-231 cell layers. The residual gap size after 18 hours was smaller in cells treated with JMV449 compared with control cells (35% of the original open gap in the treated cells versus 50% in control cells). The specific NT1 receptor antagonist, SR48692, completely inhibited the agonist-induced wound healing effect in MDA wt (Fig. 4). The Scr cells exhibited the same response as the parental cells (data not shown). In contrast, wound repair was not accelerated in the presence of JMV449 for the silenced cell lines. Compared with the original gap, after 18 hours, the MDA Si1 residual gap was 64% for treated cells and 67% for nontreated control cells, respectively (Fig. 4). The residual gap sizes after 18 hours were similar for control and treated MDA Si2 cells (42% and 38%; data not shown).

As shown in Fig. 5*A* (and *inset*), neurotensin and JMV449 were both found to promote collagen I invasiveness in MDA wt cells. The induced proinvasive effects were dose dependent and abolished by cotreatment with the specific antagonist, SR48692. Neurotensin proinvasive effects were abolished in the MDA Si1 and MDA Si2 cell lines (Fig. 5*B*), whereas silenced cells remained responsive to TGF-α. As control, MDA Scr cells were shown to be responsive to neurotensin, JMV449, and TGF-α (Fig. 5*B*).

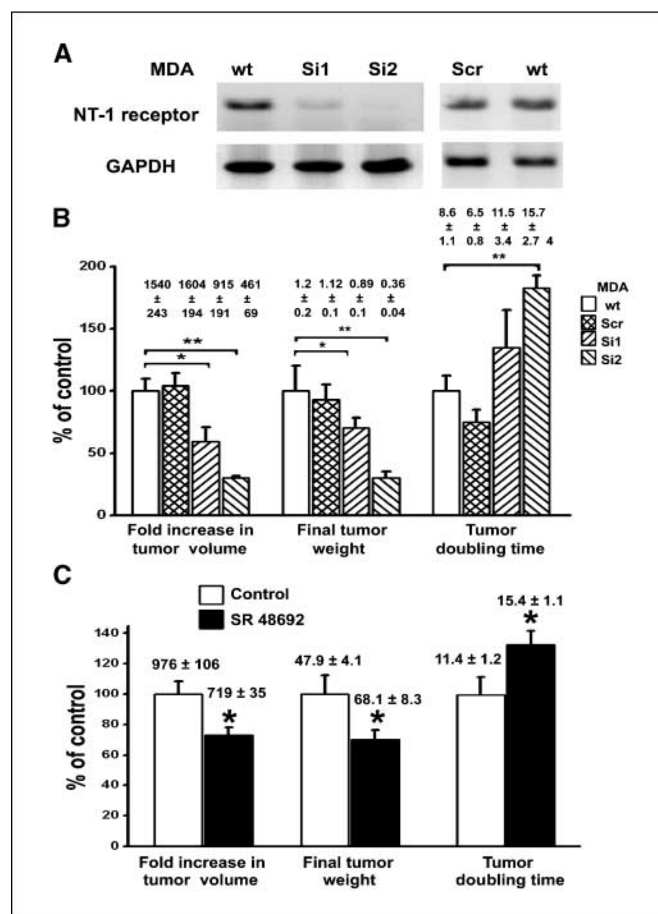

Figure 3. NT1 receptor depletion and pharmacologic blockade by SR48692 reduced the tumorigenic potential of MDA-MB-231 cells xenografted in nude mice. *A,* 1 μg total RNA from MDA wt, MDA Si1 or Si2 for NT1 receptor, or scramble (Scr) was reverse transcribed. A PCR experiment was done using specific primers for NT1 receptor and GAPDH. Amplicons were electrophoresed on agarose gel and stained with ethidium bromide. *B,* nude mice were xenografted with 3 × 10^6 MDA wt, Scr, Si1, or Si2 cells. Five weeks after inoculation, the mice were killed and tumor size and weight were measured. Representative of three independent experiments. *Columns,* mean percentage of values (g, mm^3, or days) from MDA wt cells; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$, ANOVA and Student's-Neuman-Keuls' test. *C,* MDA wt cells were xenografted in nude nice and treated or not with 1 mg/kg SR48692 for 27 days. Representative of two experiments. *Columns,* mean (in g, mm^3, or days); *bars,* SE. *, $P < 0.05$, Students' *t* test.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/66/12/6243/2863610/6243.pdf by guest on 01 December 2023

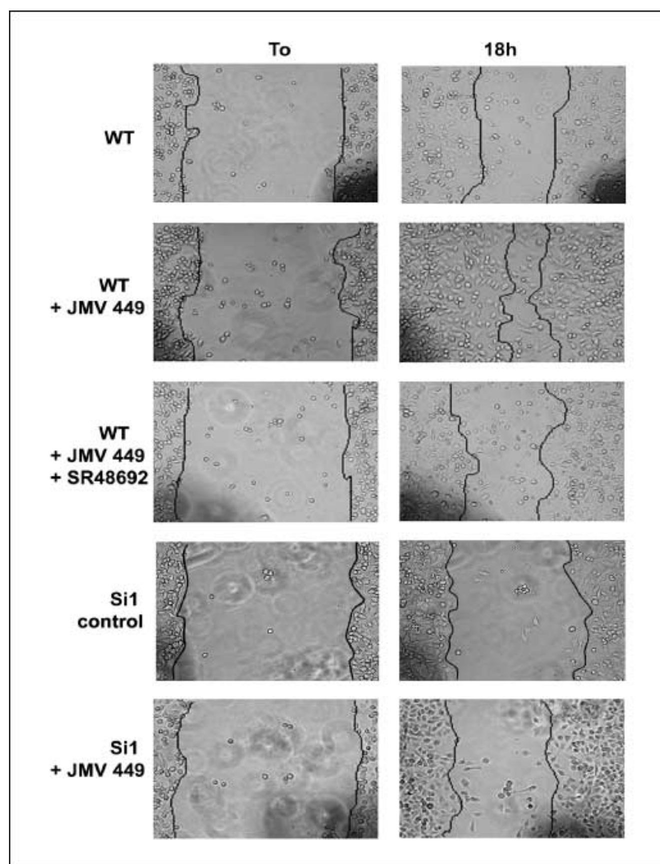

Figure 4. Neurotensin agonist promotes cellular migration of MDA-MB-231 cells. The wound repair process was studied in MDA wt and Si1 cells. Cell monolayers were incubated in the presence of 0.5% serum. Initial gap (*To*) and remaining gap after 18-hour incubation (*18h*) at 37°C. *Top,* control MDA wt cells treated with JMV449 (10^{-8} mol/L) alone or combined with SR48692 (10^{-6} mol/L); *bottom,* MDA Si1 control and treated with JMV449. Representative of two to three independent experiments.

Activation of cellular invasion in MDA-MB-231 cells treated by JMV449 was blocked by selective pharmacologic inhibitors, including the mitogen-activated protein kinase (MAPK) activation blockers, PD098059 and SB203580, and by drugs targeting phospholipase C (U73122) and protein kinase C (PKC; Gö6976 and GF109). The two latter indicated the participation of the Gαq/Gβγ and PKC signaling pathways in this cellular response. Equally interesting, the proinvasive activity of the NT1 receptor remained in the presence of pharmacologic inhibitors for phosphatidylinositol 3-kinase (PI3K; wortmannin), Rho GTPases and Rho kinase (C3T and Y27632), and Gαo/i subunits (pertussis toxin) and protein kinase A (KT5720), two signaling elements involved in the cyclic AMP (cAMP) pathways.

Neurotensin-induced cell invasion requires MMP-9 activation. Tumor cell invasion through matrix and tissue barriers requires the combined effects of increased cell motility and regulated proteolytic degradation of the matrix. In breast tumors, the MMP-9 has been detected in vascular pericytes, cancer cells, and tumor stromal cells (32, 33). Elevated levels of the MMP-9 in tumor tissue have been generally correlated with cancer cell invasion and metastasis (34). To show the requirement for MMP-9 activation in neurotensin induced invasion, we preincubated MDA-MB-231 cells with a specific MMP-9 inhibitor for 30 minutes (35). The invasive activity induced by the NT1 receptor agonist JMV449 was selectively abolished because these breast cancer cells remained sensitive to the EGFR agonist TGF-α (Fig. 6*A*). We further confirmed that neurotensin induced MMP-9 expression by raising the levels of MMP-9 mRNA (Fig. 6*B*). As a consequence, the MMP-9 gelatinase activity was elevated as visualized by zymography in MDA-MB-231 cells (Fig. 6*C*).

Discussion

Because GPCRs can be exposed to an excess of locally produced or circulating agonist, increasing evidence argues for a direct correlation between aberrant GPCR signaling and cancer progression (9). The current study documents the overexpression of NT1 receptor in most human ductal breast cancers and the frequent concomitant expression of its specific ligand. We also confirm the participation of neurotensin in several deleterious characteristics associated with breast cancer progression, including cellular migration, invasion, and tumor growth. The tumorigenic potential observed here on human breast cancer tissue and cells was shown to be reversible by silencing the NT1 receptor by RNA interference or by NT1 receptor pharmacologic blockade. Building on previous reports showing the neurotensin trophic and survival function of neurotensin in breast cancer cells, the present results strengthen the argument for the involvement of neurotensin in breast cancer growth and progression (12).

Cancer progression involves a sequential series of critical genetic and molecular alterations inducing the deregulation of cell proliferation, adhesion, migration, and invasion and leading to the lethality associated with metastatic spread of malignant tumors. It has been shown previously that NT1 receptor gene is a target of the Wnt/APC oncogenic pathways connected with the β-catenin/Tcf transcriptional complex, known to activate genes involved in cell proliferation and transformation (13). Because an exact correlation was found between NT1 receptor expression and β-catenin cytoplasmic or nuclear localization in colonic adenomas, we concluded that NT1 receptor overexpression in cancer is an early event in colonic cell transformation (13).

Wnt genes are frequently up-regulated in breast cancer and are associated with mammary tumorigenesis (36). We observed previously that Wnt factors activate NT1 receptor gene expression in normal human epithelial breast cells (13), indicating a possible up-regulation of NT1 receptor in breast cancer. In the present study, we observed NT1 receptor expression mainly located in the ductal compartment within histologic grade 1, reinforcing the concept that NT1 receptor expression is an early event of breast epithelial cell transformation. In addition, we also detected intracellular NT1 receptor localization as well as neurotensin labeling predominantly in the invasive compartment in histologic grades 2 and 3, suggesting that intensive internalization follows receptor activation by intense and sustained ligand exposure. This event is associated with a chronic self-activation loop between neurotensin and the NT1 receptor and is a mechanism driving constitutive activation of the MAPK signaling pathways coupled with cell division (37). These critical agonist exposure conditions would seem to occur in breast cancer patients expressing both ligand and NT1 receptor and consequently enable the potential action of neurotensin/NT1 receptor in human breast cancer.

The molecular mechanisms involved in neurotensin gene up-regulation observed in human colon tumors (38), carcinoid tumors

Downloaded from http://aacrjournals.org/cancerres/article-pdf/66/12/6243/2863610/6243.pdf by guest on 01 December 2023

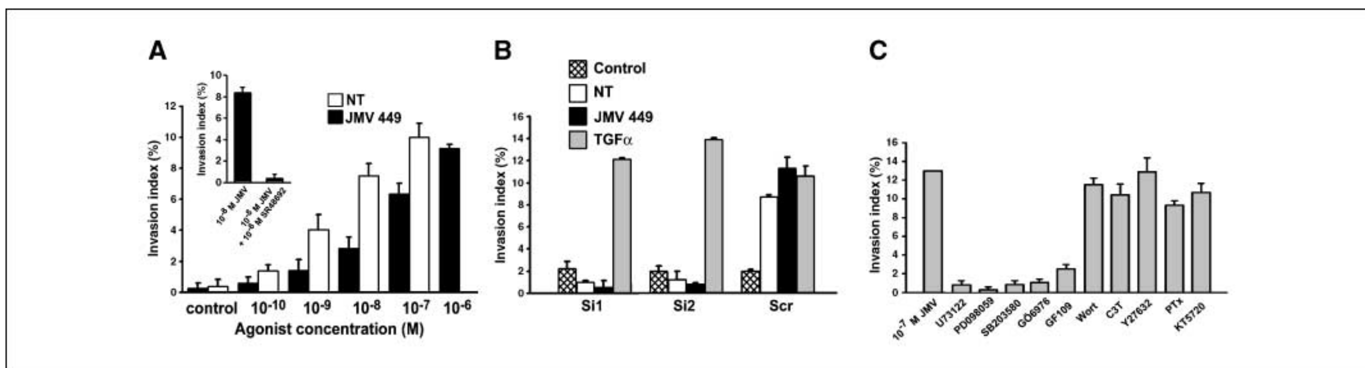

Figure 5. Neurotensin agonist promotes cellular invasion of MDA-MB-231 cells. *A,* neurotensin or JMV449 dose-dependent induction of cellular invasion in type I collagen by MDA wt cells. *Inset,* neurotensin (10^{-8} mol/L) induced cellular invasion blockade by SR48692 (10^{-6} mol/L). *B,* neurotensin or JMV449 (10^{-7} mol/L) induced invasion in type I collagen was compared in MDA wt, S1, S2, or Scr cells. TGF-α (10 ng/mL) was used as positive control. *C,* JMV449 (10^{-7} mol/L) induced cellular invasion blockade by phospholipase C inhibitor, U73122 (10^{-5} mol/L); MAPK inhibitors, PD098059 (5×10^{-5} mol/L) and SB203580 (10^{-5} mol/L); PKC inhibitors, Gö6976 (10^{-5} mol/L) and GF109 (10^{-6} mol/L); PI3K inhibitor, wortmannin (*Wort*; 10^{-5} mol/L); Rho GTPase inhibitor, C3T (5 μg/mL); ROCK inhibitor, Y27632 (10^{-5} mol/L); Gi component of adenylate cyclase inhibitor, pertussis toxin (*PTx*; 200 ng/mL); and cAMP-dependent protein kinase (PKA) inhibitor, KT5720 (10^{-5} mol/L).

(39), and pancreatic tumors (40) are not fully clarified. Evidence shows that neurotensin gene hypomethylation (41), along with the activation of transcription factors activator protein-1 (AP-1) and cAMP-responsive element-binding protein and the oncogenic signaling factors RAS and Src, are potential mechanisms leading to neurotensin overexpression and secretion during cancer progression (42). It is noteworthy that estrogens can also activate neurotensin expression in the brain through the cAMP cascade and through an additional, nonconventional, steroid receptor pathway (43). A future challenge will be to evaluate the possible participation of estrogens in neurotensin expression in breast cancer.

No clear correlation has been described today between circulating neurotensin and the stages of pancreas, prostate, or medullar thyroid tumors (44) probably due to its high degradability and rapid clearance by the liver. Nevertheless, neurotensin was found to be released from gut and pancreas cancers and has been identified as a gene associated with enhanced metastasis in a lung carcinoma cell line (40, 45). Additionally, it has been shown that the NT1 receptor antagonist SR48692 inhibited the growth of human colon and lung cancer cells xenografted in mice (46). In this report, we further establish the contribution of endogenous or tumoral neurotensin within the breast cancer progression by showing the reversibility of its tumorigenic effects through silencing RNA and direct pharmacologic blockades. Nevertheless, direct determination of the local neurotensin concentration, at the tumor vicinity, would provide the clearest evaluation of neurotensin secretions by the tumor and its sequential placement within cancer progression.

Neurotensin signaling-associated effectors, such as nuclear factor-κB, ERK1/2, AP-1, Ras, Src, Rho family protein, and FAK, substantiate the potential neurotensin/NT1 receptor oncogenic role (23–25, 47, 48). These signaling factors are correlated with cell and tissue growth, cell death, and differentiation. Here, we illustrated the proinvasive activity of neurotensin and the NT1 receptor in MDA-MB-231 cells mediated by MMP-9. Interestingly, expression of MMP-9 has been also associated with high potential of metastasis in several human carcinomas, including breast carcinomas. MMP-9 immunohistochemistry revealed MMP-9 in malignant and stromal cells, predicting a poor survival index when expressed in stromal cells within a hormone-responsive tumor, whereas it has a favorable survival index when expressed in carcinoma cells (49).

This article is the first report on neurotensin/NT1 receptor in human breast cancer and brings new data on neurotensin malignant effects in breast cancer cells, suggesting that NT1 receptor may not only be a marker but also participates as part of the process of breast cancer progression. Further analysis will

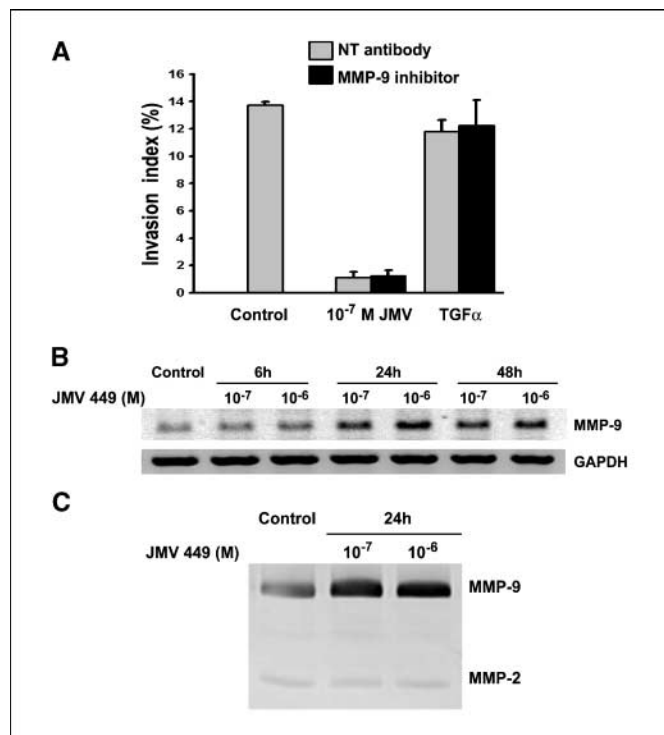

Figure 6. Neurotensin agonist activates MMP-9 expression and activity in MDA-MB-231 cells. *A,* neurotensin agonist (10^{-8} mol/L) induced cellular invasion blockade induced by MMP-9-specific inhibitor I (25×10^{-9} mol/L). *B,* accumulation of endogenous MMP-9 transcripts detected by RT-PCR following treatment of MDA wt cells with JMV449 (10^{-7} or 10^{-6} mol/L) for 6, 24, and 48 hours. Representative of one of three independent experiments. *C,* gelatinolytic activities assessed by zymography on lyophilized medium of MDA wt cells treated or not with JMV449 (10^{-7} or 10^{-6} mol/L) for 24 hours. Representative of one of three independent experiments.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/66/12/6243/2863610/6243.pdf by guest on 01 December 2023

detail the position and the participation of neurotensin in breast tumorigenesis, particularly the signaling, its partner (HER, EGF-like ligands, interleukin, etc.), and its place in the clinical course. The data described here should aid in the search for neurotensinergic-blocking agents to be joined to the adjuvant antihormonal or the chemotherapy treatments for breast cancer.

Acknowledgments

Received 2/7/2006; revised 3/31/2006; accepted 4/18/2006.

Grant support: Institut National de la Sante et de la Recherche Medicale, Association pour la Recherche sur le Cancer grant 3543, Ligue national contre le cancer grant 75/00-RS/40 France, and Belgian Federation against Cancer Research Belgium.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. D. Gully (Sanofi-Synthelabo) for providing the NT1 receptor antagonist (SR48692), Dr. D. Pélaprat (U 773 EP 11 Hôpital Bichat) and Dr. N. Insdorf for very useful discussions, Isabelle Renault (Hôpital Saint-Antoine) for assistance with the animals, and Dr. D. Hugol (Laboratoire d'Anatomie Pathologique, Hôtel-Dieu de Paris) for reviewing the pathology of breast cancer biopsies.

References

1. Althuis MD, Dozier JM, Anderson WF, Devesa SS, Brinton LA. Global trends in breast cancer incidence and mortality 1973-1997. Int J Epidemiol 2005;34:405–12.

2. Early Breast Cancer Trialists' Collaborative Group. Polychemotherapy for early breast cancer: an overview of the randomised trials. Lancet 1998;352:930–42.

3. Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet 1998;351:1451–67.

4. Borg A, Tandon AK, Sigurdsson H, et al. HER-2/*neu* amplification predicts poor survival in node-positive breast cancer. Cancer Res 1990;50:4332–7.

5. Yeon CH, Pegram MD. Anti-erbB-2 antibody trastuzumab in the treatment of HER2-amplified breast cancer. Invest New Drugs 2005;23:391–409.

6. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2001;2:127–37.

7. LeBedis C, Chen K, Fallavollita L, Boutros T, Brodt P. Peripheral lymph node stromal cells can promote growth and tumorigenicity of breast carcinoma cells through the release of IGF-I and EGF. Int J Cancer 2002;100:2–8.

8. Pierce KL, Luttrell LM, Lefkowitz RJ. New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. Oncogene 2001;20:1532–9.

9. Marinissen MJ, Gutkind JS. G-protein-coupled receptors and signaling networks: emerging paradigms. Trends Pharmacol Sci 2001;22:368–76.

10. Rozengurt E. Neuropeptides as growth factors for normal and cancerous cells. Trends Endocrinol Metab 2002;13:128–34.

11. Thomas RP, Hellmich MR, Townsend CM, Jr., Evers BM. Role of gastrointestinal hormones in the proliferation of normal and neoplastic tissues. Endocr Rev 2003;24:571–99.

12. Somai S, Gompel A, Rostene W, Forgez P. Neurotensin counteracts apoptosis in breast cancer cells. Biochem Biophys Res Commun 2002;295:482–8.

13. Souaze F, Viardot-Foucault V, Roullet N, et al. Neurotensin receptor 1 gene activation by the Tcf/β-catenin pathway is an early event in human colonic adenomas. Carcinogenesis 2006;27:708–16.

14. Tyler-McMahon BM, Boules M, Richelson E. Neurotensin: peptide for the next millennium. Regul Pept 2000;93:125–36.

15. Gully D, Canton M, Boigegrain R, et al. Biochemical and pharmacological profile of a potent and selective nonpeptide antagonist of the neurotensin receptor. Proc Natl Acad Sci U S A 1993;90:65–9.

16. Reinecke M. Neurotensin. Immunohistochemical localization in central and peripheral nervous system and in endocrine cells and its functional role as neurotransmitter and endocrine hormone. Prog Histochem Cytochem 1985;16:1–172.

17. Gullo L, Pezzilli R, Tomassetti P, de Giorgio R. Plasma cholecystokinin and neurotensin after an ordinary meal in humans. A prolonged time study717. Gastroenterol Clin Biol 1998;22:25–8.

18. Baca I, Feurle GE, Schwab A, Mittmann U, Knauf W, Lehnert T. Effect of neurotensin on exocrine pancreatic secretion in dogs. Digestion 1982;23:174–83.

19. Andersson S, Rosell S, Hjelmquist U, Chang D, Folkers K. Inhibition of gastric and intestinal motor activity in dogs by (Gln4) neurotensin. Acta Physiol Scand 1977;100:231–5.

20. Armstrong MJ, Parker MC, Ferris CF, Leeman SE. Neurotensin stimulates [3H]oleic acid translocation across rat small intestine. Am J Physiol 1986;251:G823–9.

21. Elek J, Pinzon W, Park KH, Narayanan R. Relevant genomics of neurotensin receptor in cancer. Anticancer Res 2000;20:53–8.

22. Wang L, Friess H, Zhu Z, et al. Neurotensin receptor-1 mRNA analysis in normal pancreas and pancreatic disease. Clin Cancer Res 2000;6:566–71.

23. Zhao D, Kuhnt-Moore S, Zeng H, Wu JS, Moyer MP, Pothoulakis C. Neurotensin stimulates IL-8 expression in human colonic epithelial cells through Rho GTPase-mediated NF-κB pathways. Am J Physiol Cell Physiol 2003;284:C1397–404.

24. Leyton J, Garcia-Marin L, Jensen RT, Moody TW. Neurotensin causes tyrosine phosphorylation of focal adhesion kinase in lung cancer cells. Eur J Pharmacol 2002;442:179–86.

25. Ehlers RA, Zhang Y, Hellmich MR, Evers BM. Neurotensin-mediated activation of MAPK pathways and AP-1 binding in the human pancreatic cancer cell line, MIA PaCa-2. Biochem Biophys Res Commun 2000;269:704–8.

26. Hassan S, Dobner PR, Carraway RE. Involvement of MAP-kinase, PI3-kinase and EGF-receptor in the stimulatory effect of Neurotensin on DNA synthesis in PC3 cells. Regul Pept 2004;120:155–66.

27. Zhao D, Zhan Y, Koon HW, et al. Metalloproteinase-dependent transforming growth factor-α release mediates neurotensin-stimulated MAP kinase activation in human colonic epithelial cells. J Biol Chem 2004;279:43547–54.

28. Gompel A, Malet C, Spritzer P, Lalardrie JP, Kuttenn F, Mauvais-Jarvis P. Progestin effect on cell proliferation and 17β-hydroxysteroid dehydrogenase activity in normal human breast cells in culture. J Clin Endocrinol Metab 1986;63:1174–80.

29. Bloom HJG, Richardson WW. Histological grading and prognosis in breast cancer. Br J Cancer 1957;3:359–77.

30. Souaze F, Rostene W, Forgez P. Neurotensin agonist induces differential regulation of neurotensin receptor mRNA. Identification of distinct transcriptional and post-transcriptional mechanisms. J Biol Chem 1997;272:10087–94.

31. Bracke M, Boterberg T, Bruyneel E, Mareel M. Collagen invasion assay. In: Cancer invasion and metastasis. Totowa (NJ): Humana Press; 2000. p. 81–9.

32. Nielsen BS, Sehested M, Kjeldsen L, Borregaard N, Rygaard J, Dano K. Expression of matrix metalloprotease-9 in vascular pericytes in human breast cancer. Lab Invest 1997;77:345–55.

33. Scorilas A, Karameris A, Arnogiannaki N, et al. Overexpression of matrix-metalloproteinase-9 in human breast cancer: a potential favourable indicator in node-negative patients. Br J Cancer 2001;84:1488–96.

34. Nabeshima K, Inoue T, Shimao Y, Sameshima T. Matrix metalloproteinases in tumor invasion: role for cell migration. Pathol Int 2002;52:255–64.

35. Levin JI, Chen JM, Du MT, et al. The discovery of anthranilic acid-based MMP inhibitors. Part 3. Incorporation of basic amines. Bioorg Med Chem Lett 2001;11:2975–8.

36. Howe LR, Brown AM. Wnt signaling and breast cancer. Cancer Biol Ther 2004;3:36–41.

37. Toy-Miou-Leong M, Cortes CL, Beaudet A, Rostene W, Forgez P. Receptor trafficking via the perinuclear recycling compartment accompanied by cell division is necessary for permanent neurotensin cell sensitization and leads to chronic mitogen-activated protein kinase activation. J Biol Chem 2004;279:12636–46.

38. Evers BM, Zhou Z, Dohlen V, Rajaraman S, Thompson JC, Townsend CM, Jr. Fetal and neoplastic expression of the neurotensin gene in the human colon. Ann Surg 1996;223:464–70.

39. Calhoun K, Toth-Fejel S, Cheek J, Pommier R. Serum peptide profiles in patients with carcinoid tumors. Am J Surg 2003;186:28–31.

40. Theodorsson-Norheim E, Oberg K, Rosell S, Bostrom H. Neurotensinlike immunoreactivity in plasma and tumor tissue from patients with endocrine tumors of the pancreas and gut. Gastroenterology 1983;85:881–9.

41. Dong Z, Wang X, Evers BM. Site-specific DNA methylation contributes to neurotensin/neuromedin N expression in colon cancers. Am J Physiol Gastrointest Liver Physiol 2000;279:G1139–47.

42. Evers BM. Endocrine gene neurotensin: molecular mechanisms and a model of intestinal differentiation. World J Surg 2002;26:799–805.

43. Watters JJ, Dorsa DM. Transcriptional effects of estrogen on neuronal neurotensin gene expression involve cAMP/protein kinase A-dependent signaling mechanisms. J Neurosci 1998;18:6672–80.

44. Goettler DM, Levin L, Chey WY. Postprandial levels of prolactin and gut hormones in breast cancer patients: association with stage of disease, but not dietary fat. J Natl Cancer Inst 1990;82:22–9.

45. de Lange R, Dimoudis N, Weidle UH. Identification of genes associated with enhanced metastasis of a large cell lung carcinoma cell line. Anticancer Res 2003;23:187–94.

46. Moody TW, Chiles J, Casibang M, Moody E, Chan D, Davis TP. SR48692 is a neurotensin receptor antagonist which inhibits the growth of small cell lung cancer cells. Peptides 2001;22:109–15.

47. Zhao D, Keates AC, Kuhnt-Moore S, Moyer MP, Kelly CP, Pothoulakis C. Signal transduction pathways mediating neurotensin-stimulated interleukin-8 expression in human colonocytes. J Biol Chem 2001;276:44464–71.

48. Banker NA, Hellmich MR, Kim HJ, Townsend CM, Jr., Evers BM. Src-mediated activation of the human neurotensin/neuromedin N promoter. Surgery 1997;122:180–5.

49. Pellikainen JM, Ropponen KM, Kataja VV, Kellokoski JK, Eskelinen MJ, Kosma VM. Expression of matrix metalloproteinase (MMP)-2 and MMP-9 in breast cancer with a special reference to activator protein-2, HER2, and prognosis. Clin Cancer Res 2004;10:7621–8.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/66/12/6243/2863610/6243.pdf by guest on 01 December 2023