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Neurotensin Receptor 3/Sortilin Contributes to Tumorigenesis of Neuroendocrine Tumors Through Augmentation of Cell Adhesion and Migration[1]

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Abstract

Neurotensin (NTS), a 13–amino acid peptide which is distributed predominantly along gastrointestinal tract, has multiple physiologic and pathologic functions, and its effects are mediated by three distinct NTS receptors (NTSRs). Overexpression and activation of NTS signaling components, especially NTS and/or NTSR1, are closely linked with cancer progression and metastasis in various types of cancers including neuroendocrine tumors (NETs). Although deregulation of NTSR3/sortilin has been implicated in a variety of human diseases, the expression and role of NTSR3/sortilin in NETs have not been elucidated. In this study, we investigated the expression and oncogenic effect of NTSR3/sortilin in NETs. Increased protein levels of NTSR3/sortilin were noted in the majority of human clinical NETs ($n = 21$) by immunohistochemical analyses compared with normal tissues ($n = 12$). Expression of NTS and NTSR3/sortilin was also noted in all tested NET cell lines. In addition, small interfering RNA–mediated knockdown of NTSR3/sortilin decreased cell number without alteration of cell cycle progression and apoptosis induction in NET cell lines BON and QGP-1. Moreover, silencing of NTSR3/sortilin significantly suppressed cell adhesion and cell migration with inhibition of focal adhesion kinase and Src phosphorylation in the NET cells. Our results demonstrate increased expression of NTSR3/sortilin in NET patient tissues and a critical role of NTSR3/sortilin on NET cell adhesion and migration suggesting that NTSR3/sortilin contributes to NET tumorigenesis.

Neoplasia (2018) 20, 175–181

Introduction

Neuroendocrine tumors (NETs) are rare and slow-growing tumors, but recent studies indicate that they are increasing in incidence [1,2]. NETs originate in the diffuse endocrine system and are capable of producing and secreting bioreactive peptides, neuroamines, and hormones [2,3]. Some of the vasoactive substances (e.g., serotonin) are associated with specific clinical syndromes characterized by flushing, diarrhea, and heart disease and are used as diagnostic biomarkers or therapeutic targets for NETs [2,3]. Several peptide receptors are also expressed in many cancers and appear promising as a diagnostic marker as well as a potential therapeutic target [4,5].

Neurotensin (NTS), a tridecapeptide, has multiple physiological functions including proliferation of normal intestinal mucosa [6,7]. Furthermore, NTS modulates intestinal inflammation and augments the growth of many cancers [6,8]. The physiopathological effects of NTS are triggered by interaction with three distinct receptors: two G protein–coupled receptors, high-affinity NTSR1 and low-affinity NTSR2, and a single transmembrane domain receptor, NTR3/sortilin [8]. In particular, NTSR1, which is highly expressed in a variety of solid tumors, mediates the effects of NTS on cell proliferation, survival, migration, and invasion through multiple signaling pathways such as PKC and MAPK activation [8–10].

NTSR3/sortilin, which is also a member of the Vps10p sorting receptor family, is expressed in a wide range of cell types and tissues [11–14]. It plays important roles in various biological processes such

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as the transport of a variety of intracellular proteins and works as the co-receptor for the precursor of nerve growth factor (pro-NGF) and the 75-kDa neurotrophin receptor (p75NTR), or a regulator of atherosclerosis [11,13]. Deregulation of NTSR3/sortilin function has been implicated in a variety of human diseases including cardiovascular disease and cancer [11–13]. Elevated expression NTSR3/sortilin is also found in some types of human cancer cells and tissues including brain, breast, and prostate, which suggests a role for this protein in tumorigenesis [13,15–18].

Recently, we found that NTS stimulates the growth of NET cells as a target of the Wnt/β-catenin pathway [19]. In addition, we showed increased expression of NTSR1 in NETs and oncogenic functions of NTSR1 such as cell growth, attachment, migration, and invasion in NET cell lines [9]. Although consistent expression of NTSR3/sortilin is noted in NET cells, expression of NTSR3/sortilin in patient tissues and its roles in tumorigenesis have not been clearly elucidated in NETs. In our present study, we analyzed the expression of NTSR3/sortilin in clinical NET tissues and cell lines. Moreover, we delineated the tumorigenic functions of NTSR3/sortilin on NET cell adhesion and migration. Based on our findings, notably increased expression in NET patient tissues and promotion of cell adhesion and migration, NTSR3/sortilin appears to contribute to NET tumorigenesis and may possibly serve as a therapeutic target.

Materials and Methods

Immunohistochemistry

The slides, which were used in previous studies [9,20], were provided by the University of Kentucky Department of Pathology and the Markey Cancer Center Biospecimen Procurement and Translational Pathology Shared Resource Facility. Immunostaining was carried out as described previously [9,20] with an antibody against NTSR3/sortilin purchased from Alomone Labs (Jerusalem, Israel).

Cell Culture and siRNA Transfections

Four human NET cell lines, BON, QGP-1, NCI-H727, and UMC-11, were used as described previously [9,19,20]. The cell lines were authenticated by STR profiling in March and May 2016 by Genetica DNA Laboratories (LabCorp Specialty Testing Group, Burlington, NC). Transfection with nontargeting control and SMARTPool NTSR3/sortilin siRNA (Dharmacon, Lafayette, CO) was performed as previously described [9].

RNA Isolation and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA isolation and qRT-PCR were carried out as described previously [9]. Expression levels of NTS and NTSR3/sortilin were normalized to GAPDH.

Western Blot Analysis

Western blotting was carried out as described previously [9]. The antibodies for proliferating cell nuclear antigen (PCNA) and β-actin were obtained from Santa Cruz Biotechnology (Dallas, TX) and Sigma-Aldrich (St. Louis, MO), respectively. The antibodies for poly (ADP-ribose) polymerase (PARP), focal adhesion kinase (FAK), phospho-FAK (Tyr397), Src, and phospho-Src (Tyr416) were from Cell Signaling (Danvers, MA).

Cell Counting and Apoptosis Analysis

Cell counting and apoptosis analyses were performed as described previously [9,20].

Cell Cycle Analysis by Flow Cytometry

Transfected NET cells were collected by trypsinization, washed, fixed with 70% ice-cold ethanol, and stored at −20°C until analysis by flow cytometry. The fixed cells were washed, resuspended in FxCycle Propidium iodide/RNase staining solution (Life Technologies, Carlsbad, CA), and incubated for 15 to 30 minutes in the dark. The UK Flow Cytometry Service Facility conducted cell cycle analysis using the Becton-Dickinson FACSort flow cytometer (BD Biosciences, NJ). The cell cycle data were analyzed using CellQuest program (BD Biosciences) to determine the percentage of cells at different stages of the cycle (G1, S, and G2/M).

Adhesion Assay

A cell adhesion assay was performed to assess cell binding ability to direct or the extracellular matrix (ECM)–coated culture plate as described previously [9]. Briefly, equal numbers of detached NET cells were plated to each uncoated well or type I collagen–coated well of 48-well plates and incubated for 6 hours or 30 minutes, respectively.

Transwell Migration Assay

A Boyden chamber migration assay carried out with control and NTSR3/sortilin knockdown NET cells as described previously [9]. The chambers were incubated at 37°C for 24 or 48 hours, respectively, and the cells were fixed with methanol and stained with 0.5% crystal violet in 20% methanol.

Statistical Analysis

Descriptive statistics for immunoreactivity score, mRNA levels, cell counts, and apoptosis were calculated according to experimental groups. Correlations between NTSR1 versus NTSR3/sortilin were assessed using Spearman's correlation coefficient. Pairwise comparisons were performed using two-sample t test and multiple group comparisons using analysis of variance with adjustment for multiple testing based on Holm's P value adjustment method.

Results

Expression Analysis of NTSR3/Sortilin in NET Tissues and Cell Lines

Although a number of studies, including those from our laboratory, have demonstrated increased expression of NTS and NTSR1 in many tumor types including NETs [8–10], the expression of NTSR3/sortilin has not been well studied in NETs. To assess NTSR3/sortilin expression, immunohistochemistry was performed using clinical NET patient samples (13 gastrointestinal [GI], 6 lung and 2 thymus tissues) which were analyzed for β-catenin and NTSR1 in our previous reports [9,20]. Statistical comparisons of immunoreactivity scores between normal (5 GI, 5 lung, and 2 thymus tissues) versus NETs showed significantly increased expression of NTSR3/sortilin in 9 GI and in all lung and thymus NET samples (Figure 1***A***). An analysis of the possible relationship between NTSR1 and NTSR3/sortilin expression was also carried out due to high expression levels of these receptors in the NET samples. There was little to no correlation in the NET samples (Spearman correlation = − 0.0075, P = .975; data not shown). In addition, predominant cytoplasmic staining of NTSR3/sortilin was observed in NET tissues, suggesting localization in intracellular organelles, such as the Golgi body and endoplasmic reticulum (Figure 1***B***).

In addition, to evaluate quantitative expression of NTS and NTSR3/sortilin, endogenous levels of mRNA and protein were also checked by qRT-PCR and Western blotting, respectively. All tested

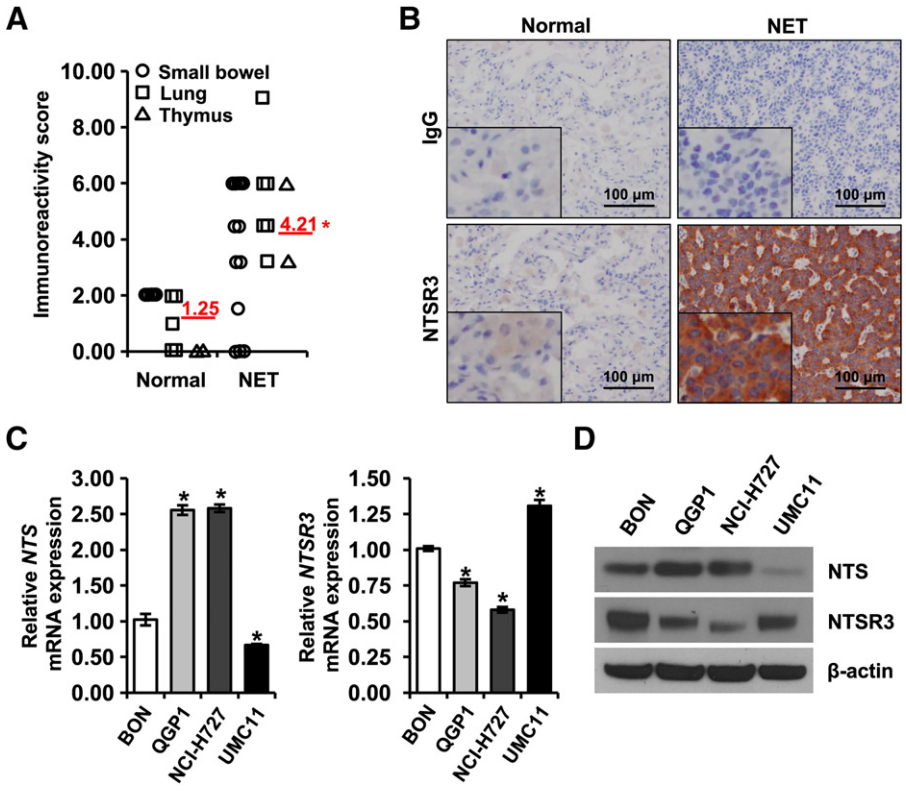

Figure 1. Expression analysis of NTSR3/sortilin in clinical NET tissues and four NET cell lines. (A) Human normal (nonneoplastic, $n = 12$) and NET ($n = 21$) tissue sections were stained with anti-NTSR3/sortilin antibody. Immunoreactivity scores were determined by multiplication of the values for staining intensity (0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining) and for percentage of positive staining cells (0, no positive; 1, 0%-10% positive; 2, 11%-50% positive; 3, 51%-100% positive); $*P < .05$ versus normal tissue. (B) Representative images are shown for IgG (upper) or NTSR3/sortilin (bottom) staining in normal and lung NET tissues. (C) Relative expression of mRNA levels for *NTS* (left) and NTSR3/sortilin (right) in NET cells was assessed by qRT-PCR ($*P < .05$ versus BON). (D) Analysis of protein expression for NTS, NTSR3/sortilin, and β-actin in NET cells was performed by Western blot analysis. β-Actin was used as an internal control for protein loading.

NET cell lines demonstrated varying levels of mRNA expression for NTS and NTSR3/sortilin by qRT-PCR analysis (Figure 1***C***). By comparison with respective NET cells, higher expression levels of NTS were noted in QGP-1 and NCI-H727 cells, whereas increased expression of NTSR3/sortilin was detected in BON and UMC-11 compared with QGP-1 and NCI-H727 cells (Figure 1***C***). Consistent with mRNA expression levels, the protein expression of NTS and NTSR3/sortilin was confirmed in the four human NET cells by Western blotting (Figure 1***D***). Overall, all NET cell lines expressed NTS and NTSR3/sortilin proteins which closely approximated the mRNA expression levels of the corresponding genes.

The Effect of NTSR3/Sortilin Knockdown on NET Cell Number

Recently, we have shown that inhibition of NTS or NTSR1 suppressed tumorigenic functions in NET cells [9,19]. To elucidate the potential role of NTSR3/sortilin in NET cells, we used small interfering RNA (siRNA) against NTSR3/sortilin in BON and QGP-1 cells and determined the effect on cell number by direct cell counting. Knockdown of NTSR3/sortilin decreased BON and QGP-1 cell numbers at 48 and 96 hours compared with cells transfected with nontargeting control siRNA (Figure 2***A***). Furthermore, we determined the levels of PCNA and PARP cleavage, which were used as markers for cell proliferation [21] and apoptosis [22], respectively, since change in cell number may be related to a decrease in cell cycle progression and/or induction of apoptosis. NTSR3/sortilin silencing did not change the level of PCNA and induce cleaved PARP in either BON or QGP-1 cells as noted by Western blot analysis (Figure 2***A***).

As a next step, we directly measured cell cycle progression and apoptosis in the cells under basal culture conditions. Flow cytometric analysis using propidium iodide staining demonstrated no effect on the cell cycle profile in either control or NTSR3/sortilin-transfected BON and QGP-1 cells (Figure 2***B***). In addition, no significant change in apoptosis was noted in BON cells transfected with NTSR3/sortilin siRNA as determined by the Cell Death Detection ELISA (Figure 2**C**, *left*). In contrast, NTSR3/sortilin knockdown QGP-1 cells demonstrated significantly decreased induction of apoptosis, which may be due to decreased cell number (Figure 2**C**, *right*). Collectively, these findings suggest that knockdown of NTSR3/sortilin significantly reduced the number of NET cells independent of a decrease in cell proliferation and induction of cell death.

The Effect of NTSR3/Sortilin Silencing on NET Cell Adhesion

To analyze whether NTSR3/sortilin knockdown alters cell adhesion which can influence cell number change, we conducted two separate adhesion assays, which determine binding ability of NET cells to uncoated culture plates or plates coated with type I collagen. As shown in Figure 3, the direct binding of NTSR3/sortilin knockdown BON (Figure 3***A***) and QGP-1 (Figure 3***B***) cells to uncoated cell plates was

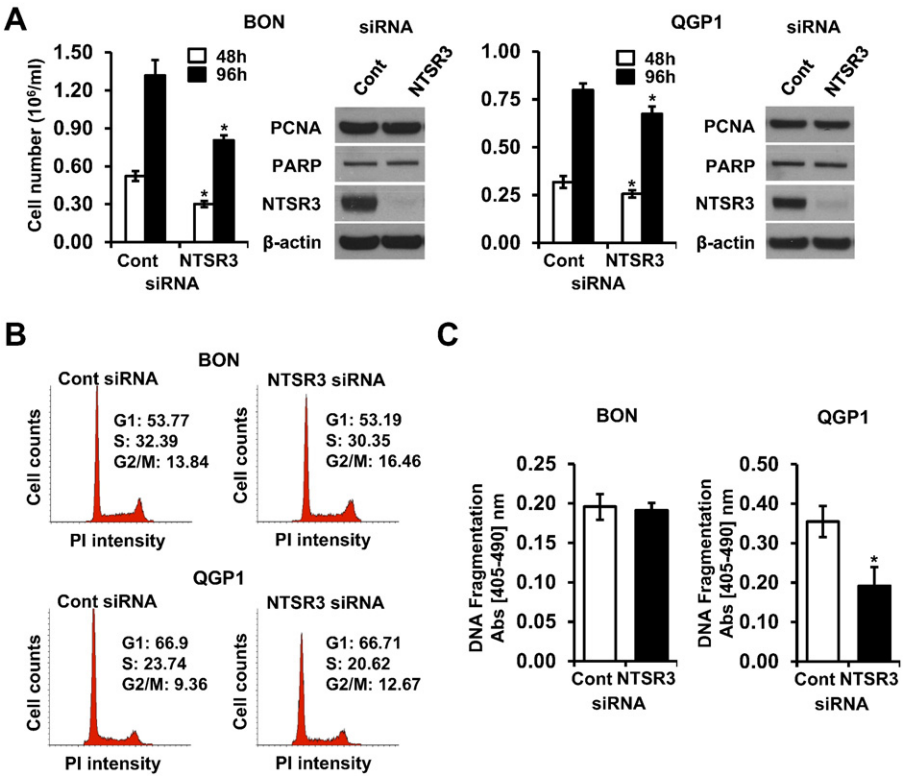

Figure 2. The effect of NTSR3/sortilin knockdown on proliferation and survival of NET cells. (A) Equal numbers of BON and QGP-1 cells transfected with siRNA against control or NTSR3/sortilin were plated in 24-well plates. Cell numbers were counted in triplicate after 48- and 96-hour incubation using a cell counter (left; $*P < .05$ versus control siRNA). Expression levels of PCNA, a marker for proliferation, or PARP, a marker for apoptosis, and NTSR3/sortilin were measured by Western blotting using siRNA-transfected NET cells (right). (B) Flow cytometry analysis with siRNA-transfected BON and QGP-1 cells. The percentage of cells in G1, S, and G2/M phases is shown. (C) Apoptosis assays were performed in quadruplicate using Cell Death Detection ELISA^{plus} (Roche, Indianapolis, IN).

significantly decreased. Moreover, knockdown of NTSR3/sortilin in BON (Figure 3***C***) and QGP-1 (Figure 3***D***) cells also demonstrated lower numbers of attached cells on type I collagen–coated plates compared with control cells. These data indicate that knockdown of NTSR3/sortilin decreases adhesion of NET cells.

The Effect of NTSR3/Sortilin on NET Cell Migration

To further delineate the role of NTSR3/sortilin on cell migration, we examined the effect of NTSR3/sortilin knockdown using a Boyden chamber migration assay with type I collagen–coated Transwells. NTSR3/sortilin silencing decreased the migratory potential of BON cells at 24 (Figure 4***A***) and 48 (Figure 4***B***) hours, respectively. Additionally, the migration of QGP-1 cells was also significantly suppressed by NTSR3/sortilin knockdown at 24 (Figure 4***C***) and 48 (Figure 4***D***) hours.

Moreover, we have verified the activation levels of cell migratory-related signaling pathways such as FAK and Src, which facilitate cell movement, cell cycle progression, and cell survival in many cancer cells [23–25]. In accordance with cell adhesion and migration, phosphorylation of FAK and Src was dramatically reduced by knockdown of NTSR3/sortilin in BON and QGP-1 cells (Figure 5). Taken together, these findings suggest that NTSR3/sortilin knockdown inhibits cell migration of NET cells, in part, through a decrease of FAK and Src activation.

Discussion

It is becoming increasingly evident that NTS signaling components, in particular, NTS and/or NTSR1, are overexpressed in various types of cancers and that activation of this signaling is associated with cancer progression and metastases [8–10]. We also found that NTS [19] and NTSR1 [9] play a role in tumorigenic function such as cell proliferation, anchorage-independent growth, cell migration, and invasion in NETs. Recently, it has also been reported that increased expression level of NTSR3/sortilin is found in some human cancer cells and tissues [13,15–18]. Notably, elevated protein level of NTSR3/sortilin was noted in invasive breast ductal carcinomas and was closely associated lymph node invasion [17]. In addition, the level of NTSR3/sortilin in high-grade gliomas and aggressive prostate cancers was higher than those in normal and low-grade (or indolent) tumor tissues [16,18]. Higher expression of NTSR3/sortilin, as well as the ectopic expression of NTS, was also examined in pituitary adenomas [15]. In our current study, we found that NTSR3/sortilin protein is strongly expressed in a majority of NET clinical samples and that their staining was intensely noted in the cytoplasm of NET cells. Along with breast cancers, gliomas, and pituitary adenomas, increased level of NTSR3/sortilin is correlated with tumor occurrence, indicating that this protein may be involved in tumorigenic characteristics and aggressiveness of several cancers including NETs. Together, strong staining of NTSR3/sortilin in the cytoplasm is in

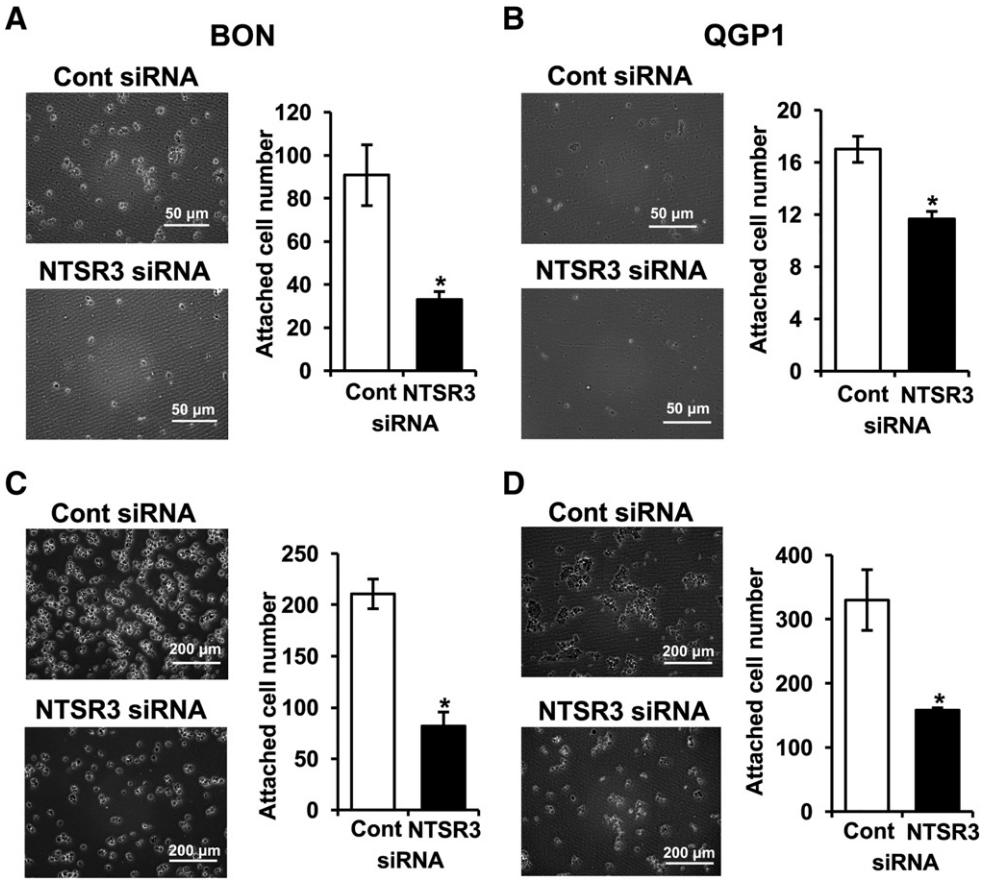

Figure 3. The influence of NTSR3/sortilin knockdown on NET cell adhesion. The same number of siRNA-transfected BON (A, C) and QGP-1 (B, D) cells was added directly onto cell culture plates for 6 hours (A, B) or type I collagen–coated plates for 30 minutes (C, D). The attached cells were fixed and then stained with crystal violet. Microscopic examination of the attached BON and QGP-1 cells in 48-well plates (left). The number of attached cells was counted in triplicate, and the mean values were determined (right; *P, < .05 versus control siRNA).

accordance with previous reports [26,27] that 5% to 10% of NTSR3/sortilin is detected at the cell surface and NTSR3/sortilin is mainly associated with the Golgi apparatus, vesicular organelles, and saccules of endoplasmic reticulum, suggesting the function of NTSR3/sortilin in intracellular sorting processes.

It has been reported that NTS stimulated cell proliferation of stable NTSR3/sortilin CHO and immortalized human microglia-SV40 cells through NTSR3/sortilin [28,29]. In addition, NTSR3/sortilin leads to NTS-induced migration in human microglial cells and human pancreatic ductal adenocarcinoma [30–32]. In addition to functioning as a receptor for NTS, NTSR3/sortilin has multiple other functions and regulates numerous biological processes. NTSR3/sortilin has multiple binding partners such as a lipoprotein lipase, proneurotrophins (e.g., pro-NGF and precursor of brain-derived neurotrophic factor), and the p75NTR as a receptor or co-receptor. Recently, it was reported that NTSR3/sortilin is involved in promotion of cell migration and invasion by pro-NGF in melanoma and breast cancer cell lines with p75NTR and tropomyosin (or tyrosine) receptor kinase A, respectively [33,34]. In addition, Roselli and colleagues [17] showed that knockdown of NTSR3/sortilin decreased cell number by reducing cell adhesion without affecting cell proliferation and survival of breast cancer cells. Furthermore, they found that NTSR3/sortilin silencing inhibited cell migration and invasion only in highly invasive and triple-negative MDA-MB-231 cells [17]. Similarly, our data showed that knockdown of NTSR3/sortilin decreased the adhesion of NET cells without significant change of cell growth or cell death. Moreover, NTSR3/sortilin knockdown reduced cell migration in both BON and QGP-1 cells.

It is well established that activation of NTSR1 is involved in cell proliferation, adhesion, migration, and invasion similar to the effect of NTSR3/sortilin in this study [8–10]. Moreover, in our previous study, increased expression of NTSR1 was assessed in the same clinical NET samples [9]. Additionally, with NTS treatment, NTSR1-NTSR3/sortilin can interact to form a complex and become internalized [8]. Based on this relationship, we determined whether there was a correlation between NTSR1 and NTSR3/sortilin expression and found no correlation in the expression in the NET samples. Furthermore, the silencing effect of NTSR3/sortilin in BON and QGP-1 cells on cell adhesion and migration is identical despite the distinction of NTSR1 expression (moderate and no NTSR1 transcripts in BON and QGP-1 cells, respectively). In line with the above evidence, our findings suggest that NTSR3/sortilin may be involved in NET cell adhesion and migration that is unrelated to NTSR1.

Nonreceptor tyrosine kinases FAK and Src have various cellular functions, and their activation promotes cell motility, cell cycle progression, and cell survival [23–25]. Numerous studies have shown

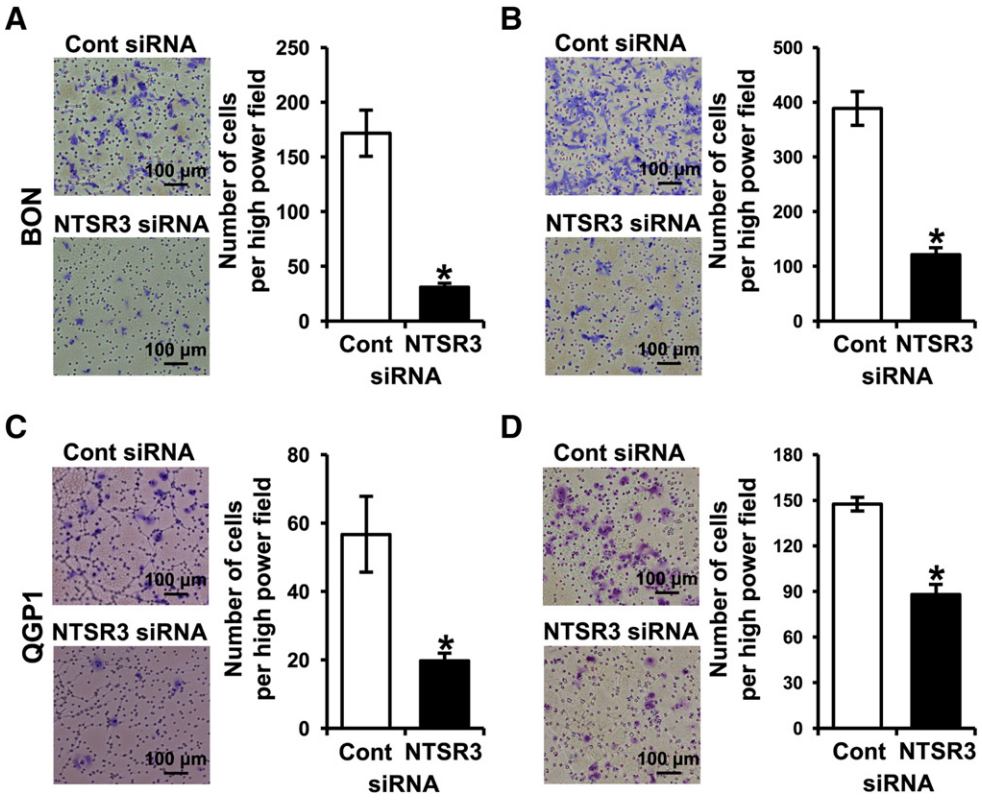

Figure 4. The effect of NTSR3/sortilin silencing on migration of NET cells. Migration analyses using Boyden chambers were performed using control and NTSR3/sortilin knockdown BON cells for 24 (A) and 48 (B) hours. Phase-contrast microscopic images (left) and quantification of migrated cells observed in four different fields using an inverted microscope (right) are shown (*$P < .05$ versus control). Using control and NTSR3/sortilin knockdown QGP-1 cells, migration assays were also performed at 24 (C) and 48 (D) hours.

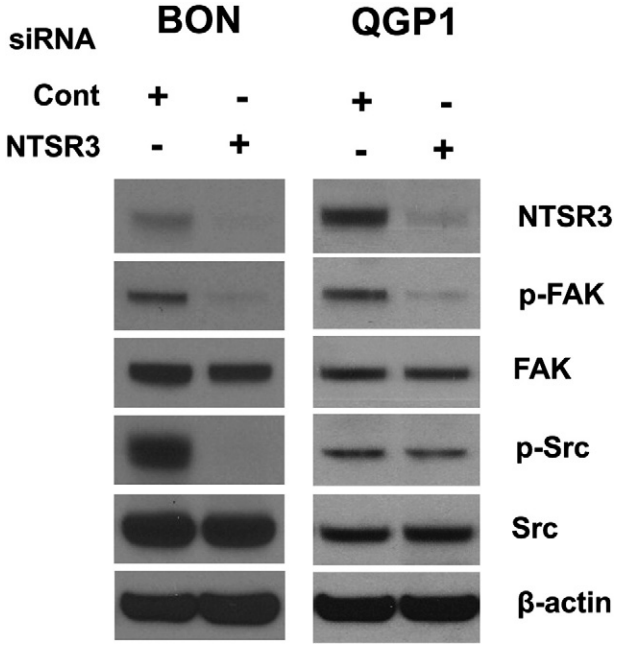

Figure 5. Regulation of cell adhesion and motility modulating proteins by NTSR3/sortilin knockdown in NET cells. BON (left) and QGP-1 (right) cells were transfected with siRNA directed to NTSR3/sortilin or control. Protein was extracted, and the cell lysates were analyzed by Western blotting using antibodies against NTSR3/sortilin, FAK, phospho-FAK (Tyr397), Src, phospho-Src (Tyr416), or β-actin.

that FAK and Src kinases are activated in tumor cells and that this activation facilitates tumor growth, invasion, and metastasis [23–25]. Recently, it has been shown that the soluble form of NTSR3/sortilin leads to an increase of FAK- and Src-dependent activation of the PI3 kinase pathway in the HT29 human colon cancer cells line [35]. Decreased phosphorylation of FAK and Src by NTSR3/sortilin knockdown was also found in breast cancer cells [17]. Consistent with these findings, we also observed decreased activation of FAK and Src with inhibited expression of NTSR3/sortilin, suggesting that the inhibitory effect of NTSR3/sortilin on cell adhesion and migration may be mediated by FAK and Src.

As noted above, NTSR3/sortilin functions as a (co)receptor for multiple factors including NTS and pro-NGF. In our study, we determined the effect of NTSR3/sortilin by gene knockdown alone without treatment of NTS or proneurotrophin. Although further work is needed to elucidate the detailed molecular mechanisms for these effects and precise binding partners, our current data suggest that NTSR3/sortilin contributes to tumorigenesis of NETs.

Conclusions

In summary, our data demonstrate that NTSR3/sortilin is significantly overexpressed in human NET patient tissues. We have also shown that inhibition of NTSR3/sortilin suppressed NET cell adhesion and migration using BON and QGP-1 cells. Our findings suggest a potential role for NTSR3/sortilin in NET tumorigenesis and a basis to explore NTSR3/sortilin as a novel prognostic marker and/or therapeutic target of NETs.

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