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Original Article

Neuron-derived neurotensin promotes pancreatic cancer invasiveness and gemcitabine resistance via the NTSR1/Akt pathway

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Abstract: Perineural invasion and neurogenesis are frequently observed in pancreatic ductal adenocarcinoma (PDAC) and link to poor outcome. However, how neural factors affect PDAC prognosis and the underlying mechanism as well as counteracting therapeutic are still unclear. *In silico* systematic analysis was performed with PROGgene to identify potential neural factor and its receptor in pancreatic cancer. *In vitro* assays including migration, invasion, 3D recruitment, and gemcitabine resistance were performed to study the effect of neuron-derived neurotensin (NTS) on pancreatic cancer behavior. Orthotopic animal study was used to validate the *in vitro* findings. Gene set enrichment analysis (GSEA) was performed to confirm the results from *in silico* to *in vivo*. Expression of NTS and its receptor 1 (NTSR1) predicted poor prognosis in PDAC. NTS synthetic peptide or neuron-derived condition medium promoted pancreatic cancer invasiveness and recruitment in 2D and 3D assays. NTS-induced effects depended on NTSR1 and PI3K activation. GDC-0941, a clinically approved PI3K inhibitor, counteracted NTS-induced effects *in vitro*. Inhibition of NTSR1 in pancreatic cancer cells resulted in decreased tumor dissemination and diminished PI3K activation *in vivo*. NTS boosted gemcitabine resistance via NTSR1 in pancreatic cancer. Our results suggest that neural cell-secreted NTS plays an important role in promoting PDAC.

Keywords: Neuron, neurotensin, pancreatic ductal adenocarcinoma, neurotensin receptor 1, Akt

Introduction

PDAC is one of the most lethal cancers in the world [1, 2], with a 5-year survival rate around 12% [2]. Targeting this cancer on both cancer cell and microenvironment simultaneously is important to improve treatment efficacy because stromal cells including cancer-associated fibroblasts, immune cells, and endothelial cells have been shown to secret soluble factors to enhance tumor growth, tissue desmoplasia and immune surveillance escape [3-5]. In addition to above cell types, neuronal cells play a key role in pancreatic tumorigenesis because these cells interact with pancreatic cancer cells to promote perineural invasion and neurogenesis [6-9]. Clinicopathological studies clearly demonstrated that perineural invasion and neurogenesis are frequently observed in PDAC and are associated with a poor prognosis [10, 11].

The underlying mechanisms by which neuronal cells stimulate growth and metastasis of PDAC are still under intensive investigation. Guo *et al.* demonstrated that neurotransmitter norepinephrine increased perineural invasion in PDAC via STAT3 signaling pathway [12]. Gil *et al.*

showed that glial cell-derived neurotrophic factor enhanced pancreatic cancer invasion via RET activation in a paracrine-dependent manner [13]. Recently, the substance P/neurokinin 1 receptor signaling axis has been shown to promote PDAC metastasis [14]. However, these studies did not address the importance of neuronal factors in PDAC prognosis. Whether neuronal factors may affect the response of PDAC cells to chemotherapeutic drugs is also unclear.

NTS is a neuron-derived factor that plays an important role in development and disease [15-19]. This 13-amino acid peptide was initially identified in bovine hypothalamus [20]. Subsequent studies demonstrated that NTS could modulate biological function of neuronal and gastrointestinal systems [21, 22]. Besides its normal physiological role, NTS also participates in the pathogenesis of neural diseases, such as Parkinson's disease [19] and various cancers [17, 18].

The biological effects of NTS are mediated by three cognate receptors on cell surface [15]. NTS receptor 1 (NTSR1) is a G-protein-coupled receptor which binds NTS with high affinity and is expressed in multiple human tissues. NTSR2, with a sequence homology around 65% to NTSR1, is a lower affinity receptor. NTSR3, also known as gp85 or sortilin, is a non-specific receptor which could interact with other proteins such as lipoprotein lipase, pro-neurotrophin and sphingolipid activator protein. NTSR3 may act as a co-receptor to modulate NTSR1 signaling [15, 23].

A previous study showed that NTSRs are expressed in 75% of PDAC tissues while they are not found in endocrine tumors [24]. By *in vitro* radiography, Korner *et al.* also detected the expression of NTSRs in primary PDAC and liver metastases [25]. NTS has been shown to stimulate proliferation and migration of pancreatic cancer cells [26-29]. However, most studies only used a single cell line and did not clarify the detailed mechanism.

Although the aforementioned studies suggested the involvement of NTS/NTSR in PDAC, several questions remain unresolved. First, what is the source of NTS produced in tumor microenvironment? Second, whether the expression of NTS or NTSRs have a prognostic value in PDAC? Third, what is the major pathway activated by NTS to accelerate tumor metastasis? Fourth, whether NTS/NTSR signaling modulates the sensitivity of pancreatic cancer cells to chemotherapeutic drugs?

In the present study we identified the prognosis importance of NTS as neural factor in PDAC via systematically bioinformatic analysis. We found that NTS is mainly expressed in neuronal cells while NTSR1 is mainly expressed in pancreatic cancer cell. Depletion of NTS in neuronal cells or knockdown of NTSR1 in pancreatic cancer cell decreased NTS-induced invasiveness of pancreatic cancer cells. Mechanistic study showed that the invasiveness-promoting activity is mediated by the NTSR1/Akt pathway and a PI3K inhibitor effectively counteracted NTS-induced invasiveness and gemcitabine resistance. Results of this study clarified the clinical significance of NTS/NTSR pathway in PDAC and provided mechanistic insight and therapeutic potential by targeting this axis.

Material and methods

PROGgene analysis

Prognosis prediction by NTS or NTSR1 in PDAC was analyzed with PROGgene [30].

Cell culture, treatment and reagents

Human pancreatic cancer cell lines MIA PaCa-2 and PANC-1 were cultured in DMEM (HyClone; Logan, UT). Mouse pancreatic cancer cell line KPC was cultured in RPMI (HyClone). Human neuroblastoma cell line SH-SY5Y was cultured in DMEM/F12 (HyClone). Mouse neuroblastoma cell line Neuro-2a was cultured in MEM (HyClone). Cell culture was performed as previously described [31]. NTS, retinoic acid, wortmannin, and gemcitabine were from Sigma (Darmstadt, Germany). GDC-0941 was from Selleck (Houston, TX).

Antibody against NTS, TUBB3, total Akt, or E-cadherin was from GeneTex (Hsinchu, Taiwan). Antibody against pan-cytokeratin was from Sakura (Torrance, CA). Antibody against phospho-Akt antibody was from Cell Signaling (Danvers, MA).

Table 1. RNA interference sequence

Name	Sequence
shLuc	GCGGTTGCCAAGAGGTTCCAT
sh-h-NTS-109	CACACTTATCTGTCTCTTCTA
sh-h-NTS-920	ATGGAAAGGAAGAAGTCATAA
sh-m-NTS-33	GCTTCCCATAAACTGCTAGTT
sh-m-NTS-36	GCCTTTCAACACTGGGAGATA
sh-h-NTSR1-6	GAACACCGACATCTACTCCAA
sh-h-NTSR1-7	CGTGCAGTGGTCATCGCCTTT
sh-m-NTSR1-3	GACGTGAACACTGACATTTAT
sh-m-NTSR1-4	CAAGGTCGTCATCCAGGTTAA

Table 2. Primer sequence

Name	Sequence
h-NTSR1-F	TACAACCTCGTCTCTGCCAA
h-NTSR1-R	CCGGGAGACACTAATGAGAA
h-GAPDH-F	AGAAGGCTGGGGCTCATTTG
h-GAPDH-R	AGGGGCCATCCACAGTCTTC
m-NTSR1-F	AAGTAGTGGCCCATCTAAGC
m-NTSR1-R	AATTTTGGGGCTTCTCTGGA
m-GAPDH-F	AGGTCGGTGTGAACGGATTTG
m-GAPDH-R	TGTAGACCATGTAGTTGAGGTCA

Cell proliferation assay

The indicated cells were seeded onto 96-well plates and treated with NTS at indicated concentrations. Afterward cells were stained with 1 mg/ml MTT (Sigma) for 3 h and dissolved with DMSO. Color densities were measured at 550 nm with FlexStation 3 (Thermo Fisher Scientific; Waltham, MA).

Colony formation assay

Colony formation assay was performed as previously described [31].

Migration and invasion assays

Migration and invasion assays were performed as previously described [32].

Conditioned medium preparation

Differentiated neuronal cells were seeded onto 10-cm dishes, and the medium was refreshed on the next day. Twenty-four hours after refreshment, conditioned medium was harvested and filtered through 0.45 μm filter (Pall; Washington, NY) to remove cell debris.

ELISA assay for the determination of NTS concentration

For standard curve preparation, recombinant NTS peptides (at the concentrations of 1.56-100 nM) in pH 9.6 medium were coated onto the wells of 96-well plates for 2 h at room temperature. NTS peptides in the conditioned media of undifferentiated and differentiated neuron cells were also coated by the same procedure. After coating, the wells were washed with PBS containing 0.1% Tween-20 (PBS-T) and blocked with 3% BSA (Sigma) in PBS for 1 h at room temperature. Anti-NTS antibody (GeneTex) was added and incubated for 1 h at room temperature. After washing, peroxidase-conjugated secondary antibody was added and the signal was developed using TMB (Sigma). The reaction was stopped by 2N H_2SO_4 and the optical density at 450 nm was measured by FlexStation 3.

RNA interference

RNAi was performed as previously described [32] and the target sequences were listed in **Table 1**.

RNA extraction and RT-PCR

RNA extraction and RT-PCR were performed as previously described [31]. Primer sequences were listed in **Table 2**.

Flow cytometry

Flow cytometry was performed as previously described [31, 32].

3D recruitment assay

3D recruitment assay was performed as previously described [33]. Briefly, neural cells were stained with green fluorescent dye DiO (Biotium; Fremont, CA) and pancreatic cancer cells were stained with red fluorescent dye DiI (Biotium) at the concentration of 5 μM in PBS for 20 min. Stained neural cells were suspended in 20 μl Matrigel (BD; Franklin Lakes, NJ) and seeded onto the center of 6-well. This mixture was allowed to solidify for 7 min, and medium containing stained pancreatic cancer cells was added. Cell recruitment in 3D was allowed for 24 h and the number of pancreatic cancer cells

Table 3. Neural factors predicted poor pancreatic cancer prognosis

Gene/dataset	GSE21501	TCGA-PAAD	GSE50827	GSE57495	GSE71729
AVP					1.4*
GNRH1				1.95*	1.48*
MLN					1.44*
NTS	1.35*				1.53***
PPY		1.04*			
PYY			1.64*		
SCT	1.6*				

*, P<0.05; ***, P<0.001.

migrating toward the Matrigel-embedded neural cells was analyzed under fluorescent microscope.

Immunofluorescence

Immunofluorescence on cell was performed as previously described [31, 32]. Mouse tumors were fixed with 10% formalin (Sigma) overnight at 4°C, dehydrated and embedded in paraffin. Tissue slide of 3 μm thickness was prepared. After rehydration and washing, slide was subjected to antigen retrieval with 1 mM EDTA (pH 9.0) by microwaving. After cooling down to room temperature, slide was blocked for peroxidase activity with 3% H_2O_2 (Sigma) for 10 min and non-specific protein binding with 3% BSA for 30 min both at room temperature. Primary antibody against NTS, TUBB3, or pan-cytokeratin was added onto slide and incubated overnight at 4°C. After washing, slide was stained with secondary antibody for 1 h and 5 μg/ml DAPI for 5 min at room temperature. After wash with ddH_2O slide was mounted with mounting gel (Agilent; Santa Clara, CA) and observed as above.

GSEA analysis

GSE71729 described above was utilized, and patients were divided into NTS-high and -low groups based on their NTS levels. GSEA analysis [34] was performed with datasets including KEGG and Reactome, and permutation number is 100. Enriched pathways, their normalized enrichment scores (NESs), and statistical significances were shown.

Orthotopic mouse model

Six to eight-week-old C57BL/6 were from National Laboratory Animal Center and anesthetized by isoflurane and injected with ketoprofen at the concentration of 1 mg/ml subcutaneously. Fur was removed by shaver and site of incision was cleaned with alcohol pad and povidone-iodine. A small incision was made on the skin and peritoneum of left lateral flank to exteriorize pancreas. 2×10^5 KPC cells of shLuc or shNTSR1 clone in 50 μl PBS were injected orthotopically via 29G insulin syringe into pancreas of mice [35, 36]. Bleeding was stopped by sterile cotton swab. The peritoneum was closed by sutures with No. 4 catgut (Unik; New Taipei City, Taiwan), and the skin was closed by clips. Mice were left on heated pad and monitored until recovery from anesthesia. Daily monitoring of wound-healing and food/water intake of the mice receiving surgery were executed. After 3 weeks, mice were sacrificed and primary/disseminated tumors were analyzed. Each group (shLuc or shNTSR1) has 4 mice. Animal study was approved by Institutional Animal Care and Use Committee (approval number 109029).

Apoptosis assay

Apoptosis assay was performed as previously described [31].

Statistical analysis

Statistical analysis was performed with GraphPad as previously described [31, 32].

Results

High expression of NTS and NTSR1 predicts poor prognosis in PDAC

To identify potential neural factors with prognostic significance in PDAC patients, we applied PROGgene analysis and found several genes including arginine vasopressin (AVP), gonadotropin releasing hormone 1 (GNRH1), motilin (MLN), neurotensin (NTS), pancreatic polypeptide (PPY), peptide YY (PYY), and secretin (SCT) predicted poor prognosis among 44 neuronal factors (**Table 3**). We focused on NTS because its upregulation was linked with poor patient's

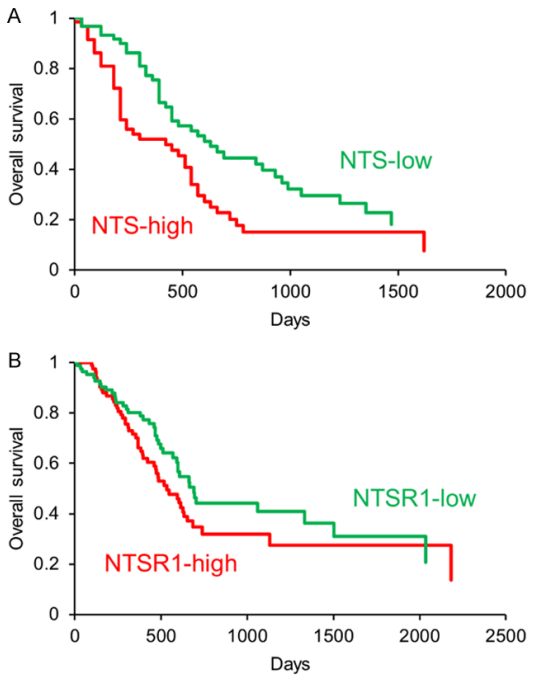

Figure 1. NTS and its receptor 1 predicted poor pancreatic cancer prognosis. 44 neural factors were analyzed for prognostic power in pancreatic cancer with PROGgene database, and the one for NTS was shown (A, GSE71729; HR=1.53; P=0.000437). NTSR1 was assayed similarly (B, TCGA-PAAD; HR=1.07; P=0.0469814).

outcome in multiple cohorts. As shown in **Figure 1A**, the survival of NTS-high PDAC patients was significantly worse than that of NTS-low patients (GSE71729; HR=1.53; P=0.000437). More importantly, high expression of NTSR1 also predicted shorter survival in PDAC patients, suggesting a critical role of NTS/NTSR1 signaling in this cancer (**Figure 1B**) (TCGA-PAAD; HR=1.07; P=0.0469814). In addition, levels of the other NTS receptors, NTSR2 and NTSR3, were not associated with PDAC outcome. These data suggested that the NTS/NTSR1 signaling axis may play a role in PDAC.

Neuron-derived NTS increases PDAC invasiveness

To elucidate the mechanism of NTS-induced tumor-promoting activity, we first tested the effect of synthetic NTS on human (MIA PaCa-2 and PANC-1) and mouse (KPC) pancreatic cancer cell lines. Our results showed that NTS did not significantly increase proliferation of PDAC cells (**Figure 2A**). In addition, colony-forming activity was only marginally enhanced by NTS, suggesting that NTS is not a potent growth-stimulating factor for PDAC cells (**Figure 2B**). On the contrary, NTS strongly increased migration and invasion of PDAC cells (**Figure 2C**, **2D**).

We applied flow cytometry to detect NTS expression in cell, and applied ELISA to detect NTS expression in conditioned medium. These neural cells expressed NTS and the level of NTS was further increased after differentiation. Treatment of conditioned media from differentiated SH-SY5Y and Neuro-2a cells potently enhanced migration and invasion of human and mouse PDAC cells, respectively (**Figure 3**).

To verify whether the effect of differentiated SH-SY5Y- or Neuro-2a-derived conditioned medium on the enhancement of PDAC invasiveness is mediated by NTS, we inhibited NTS expression by shRNA in differentiated SH-SY5Y and Neuro-2a cells (**Figure 4A-D**). Consistent with our hypothesis, differentiated SH-SY5Y-conditioned medium-induced migration and invasion in MIA PaCa-2 were significantly attenuated after NTS depletion (**Figure 4E**, **4G**). Inhibition of NTS in differentiated Neuro-2a cells was also achieved by shRNA (**Figure 4B**, **4D**). The stimulatory effects of differentiated Neuro-2a-conditioned medium on migration and invasion on KPC were also significantly attenuated by NTS depletion (**Figure 4F**, **4H**).

We next inhibited NTSR1 expression in MIA PaCa-2 and KPC (**Figure 5A**). Depletion of NTSR1 in MIA PaCa-2 cells reduced basal levels of migration and invasion (**Figure 5B**, **5C**). Moreover, NTS-increased migration and invasion were totally abolished in NTSR1-depleted PDAC (**Figure 5B**, **5C**). Reduction of NTS-induced migration and invasion in NTSR1-depleted KPC was also found (**Figure 5D**, **5E**). These results suggested that NTSR1 is the major receptor responsible for neuronal cell-promoted PDAC invasiveness.

Neuron-derived NTS promotes PDAC movement in 3D culture

Migration and invasion in 2D culture system sometimes may not precisely recapitulate the movement of cancer cells in 3D. We labeled neuron with green fluorescence and PDAC with

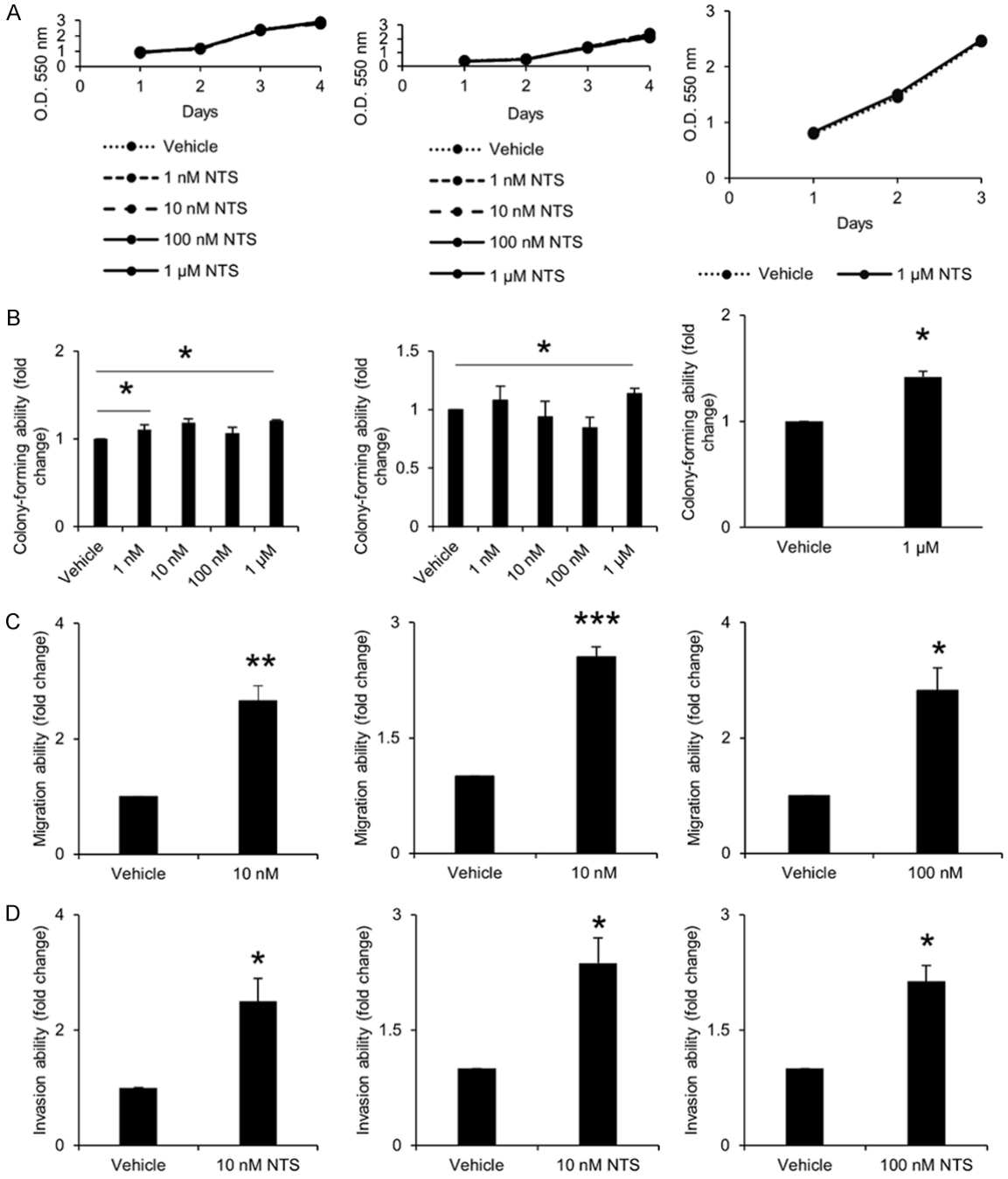

Figure 2. NTS increased pancreatic cancer invasiveness. Human pancreatic cancer cell lines MIA PaCa-2 (left column) and PANC-1 (middle column) as well as mouse pancreatic cancer cell line KPC (right column) were treated with various concentrations of NTS, and their effects on proliferation (A), colony formation (B), migration (C), and invasion (D) were assayed. *, P<0.05; **, P<0.01; ***, P<0.001.

red fluorescence respectively. Neuronal cells were suspended in Matrigel and dropped in the center of a dish. PDAC cells in culture medium were added into the plate and movement of PDAC cells toward neuronal cells was investigated. As in **Figure 6A**, the migration of MIA PaCa-2 cells toward NTS-depleted SH-SY5Y cells in 3D was decreased. Similarly, a significant reduction of Neuro-2a-induced 3D migration of KPC was found after NTS knockdown (**Figure 6B**). On the other hand, inhibition of NTSR1 expression in MIA PaCa-2 and KPC also

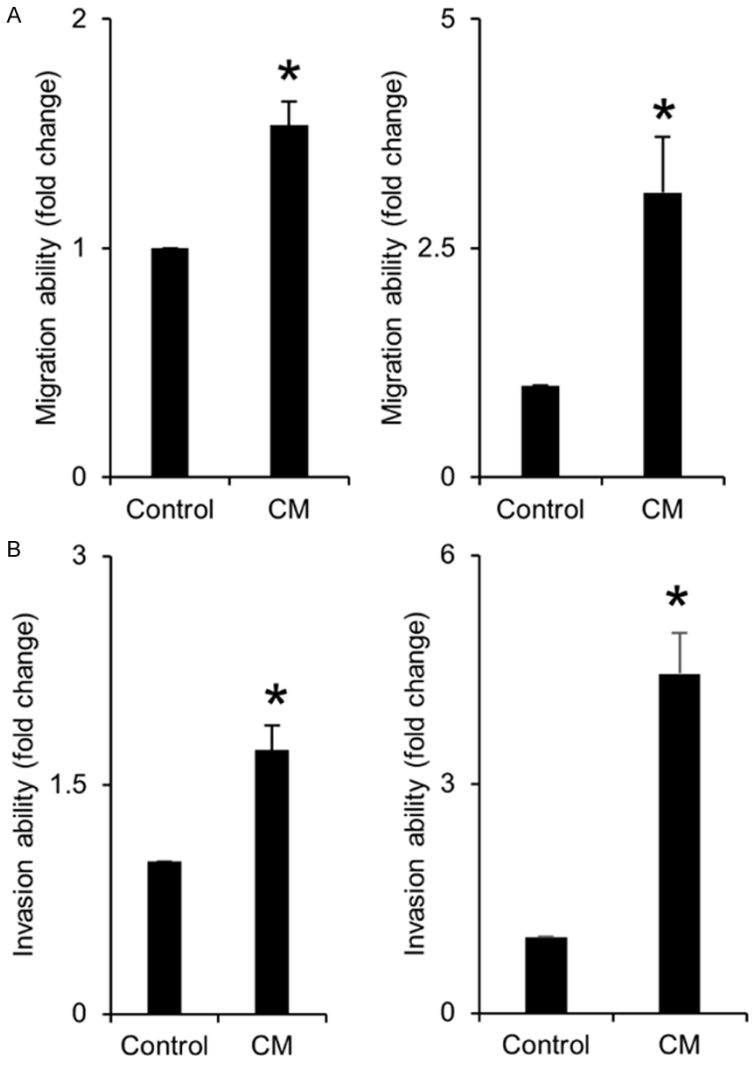

Figure 3. NTS from neural conditioned medium increased pancreatic cancer invasiveness. Conditioned medium of neural-like human SH-SY5Y cell line and mouse Neuro-2a cell line were treated onto MIA PaCa-2 (left column) and KPC (right column), respectively, and the effect on migration (A) and invasion (B) was assayed. *, P<0.05.

dramatically attenuated neuron-induced movement (**Figure 6C**, **6D**). These data further supported the importance of NTS/NTSR1 signaling in promoting PDAC invasiveness under 3D condition.

GSEA analysis reveals enriched cellular junction in NTS-low PDAC

To understand the mechanism of NTS in PDAC, we analyzed GSE71729 mentioned in **Figure 1**, and divided patients into NTS-high and -low groups based on their NTS levels. GSEA analysis revealed that cellular junction was enriched in NTS-low group, represented by the negative NESs in pathways REACTOME_TIGHT_JUNCTION_INTERACTIONS (NES=-2.18; P<0.001), KEGG_TIGHT_JUNCTION (NES=-1.88, P<0.001), and REACTOME_CELL_CELL_JUNCTION_ORGANIZATION (NES=-1.73, P<0.001) (**Figure 7**, Supplementary Figure 5). This bioinformatic analysis is in accordance with our observations that NTS increases PDAC migration, emphasizing the impact of NTS on PDAC motility.

PI3K inhibitor counteracts NTS-induced PDAC invasiveness

We next applied bioinformatic analysis with GEO database and Connectivity Map to predict therapeutics for NTS-high pancreatic cancer, and identified PI3K/Akt pathway as a potential candidate. As a G-protein coupled receptor [37], NTSR1 works as a high-affinity receptor for NTS and the interaction of these two causes a conformational change in NTSR1 [37], rendering signal molecules such as PI3K [38] to be activated as previously reviewed [17]. Indeed, NTS enhanced Akt activity in MIA PaCa-2 and KPC (**Figure 8A**). We detected Akt phosphorylation by immunofluorescence as we found direct fixation of cell at the end of NTS treatment best restrains the alteration in Akt phosphorylation as showed in **Figure 8A**. Treatment of PI3K inhibitors wortmannin (experimental compound) and GDC-0941 (drug under clinical trial) suppressed NTS-induced migration and invasion of MIA PaCa-2 (**Figure 8B**, **8C**). Moreover, these two inhibitors also inhibited 3D migration in MIA PaCa-2 (**Figure 8D**). Similar inhibitory effects on migration, invasion and 3D recruitment of KPC were also

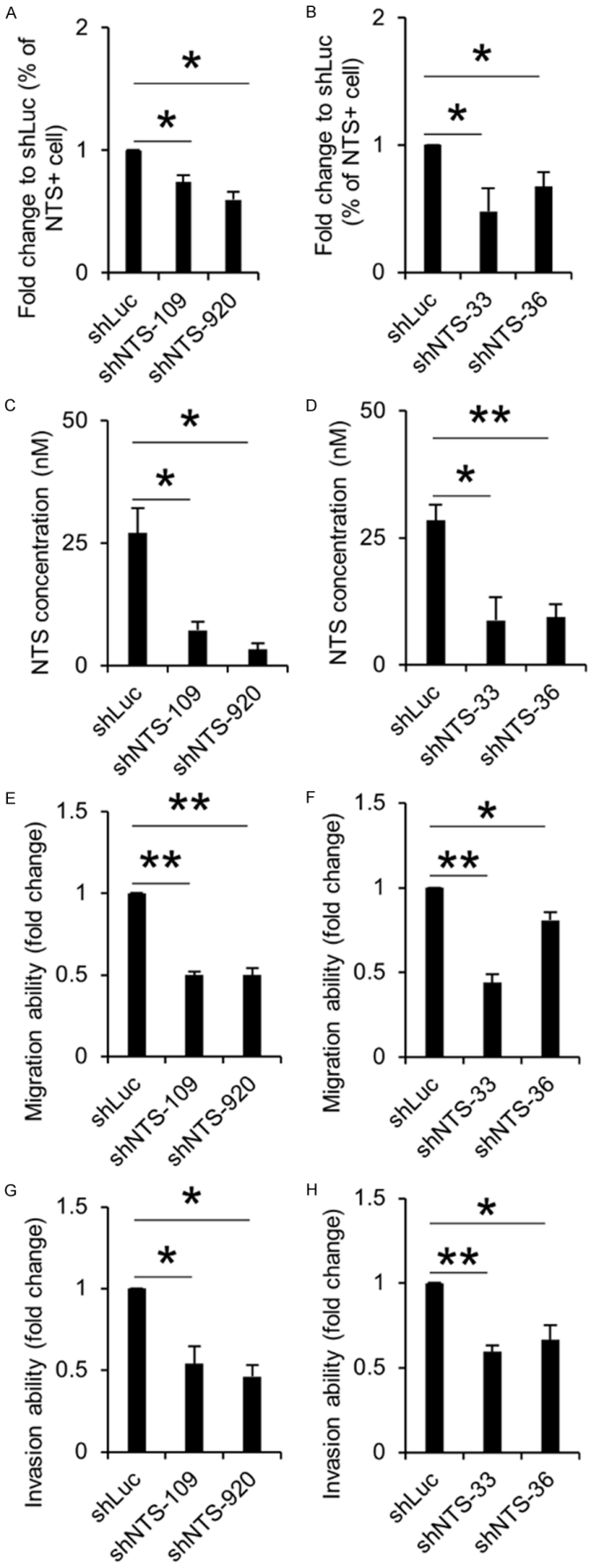

Figure 4. NTS knockdown in neuron decreased induction on pancreatic cancer invasiveness. Differentiated SH-SY5Y (A, C) or Neuro-2a (B, D) was transfected with shLuc or shNTS, and 48 h post transfection knockdown efficiency was assayed. Conditioned media from above clones were treated onto MIA PaCa-2 (E, G) or KPC (F, H), and the effect on migration (E, F) as well as invasion (G, H) was assayed. *, P<0.05; **, P<0.01.

observed (**Figure 8E-G**). These results suggested that PI3K/Akt axis is a downstream pathway to mediate NTSR1-induced PDAC invasiveness.

NTS promotes PDAC progression in vivo

We used orthotopic model to verify the importance of NTS/NTSR1 signaling in pancreatic tumorigenesis. As knockdown of NTS in neuron is less achievable and is beyond our main scope of pancreatic cancer at the present time, we applied the shLuc- or shNTSR1-transfected stable clones in KPC cell line for *in vivo* confirmation. 3 weeks post orthotopic inoculation, tumor growth and dissemination were examined. We found that NTSR1 knockdown marginally decreased the growth of primary tumors (**Figure 9A**). However, the number and size of disseminated tumors were obviously reduced (**Figure 9B**), confirming the effect of NTS in promoting PDAC progression. Immunofluorescent staining also demonstrated the decrease of NTSR1 and phospho-Akt in the tumors generated from shNTSR1-KPC (**Figure 9C**, **9D**). For enriched cellular junction in NTS-low PDAC, we also provided *in vivo* evidence that loss of NTS receptor 1 and related signaling led to increased E-cadherin expression in mouse tumors (Supplementary Figure 5).

The communication between pancreatic cancer cell and neural cell was reported in animal models of spontaneous one (**Figure 4B** of [39]) and orthotopic one (**Figure 2D** of [39]). Furthermore, this communication

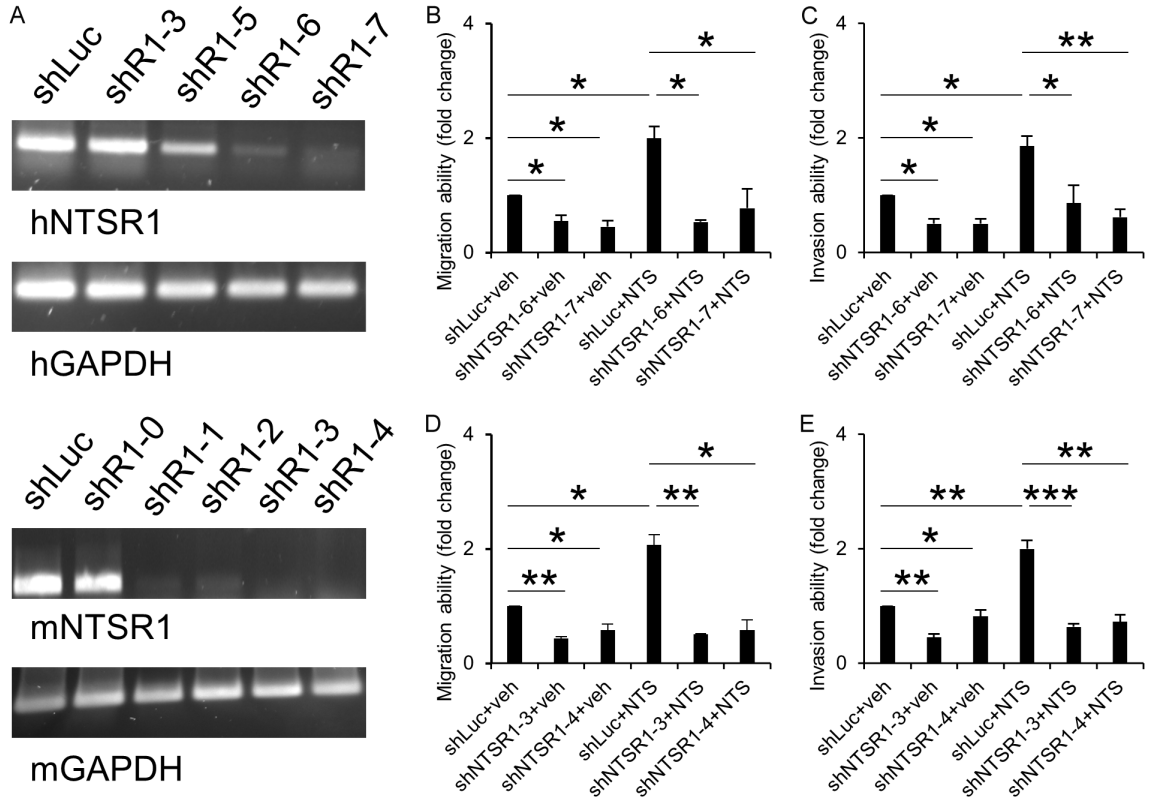

Figure 5. NTSR1 knockdown in pancreatic cancer decreased response toward NTS-affected motility. MIA PaCa-2 (A, upper) or KPC (A, lower) was transfected with shLuc or shNTSR1, and 48 h post transfection knockdown efficiency was assayed (A). Cells from above conditions were then assayed for NTS-affected migration (B, MIA PaCa-2; D, KPC) and invasion (C, MIA PaCa-2; E, KPC). *, P<0.05; **, P<0.01; ***, P<0.001.

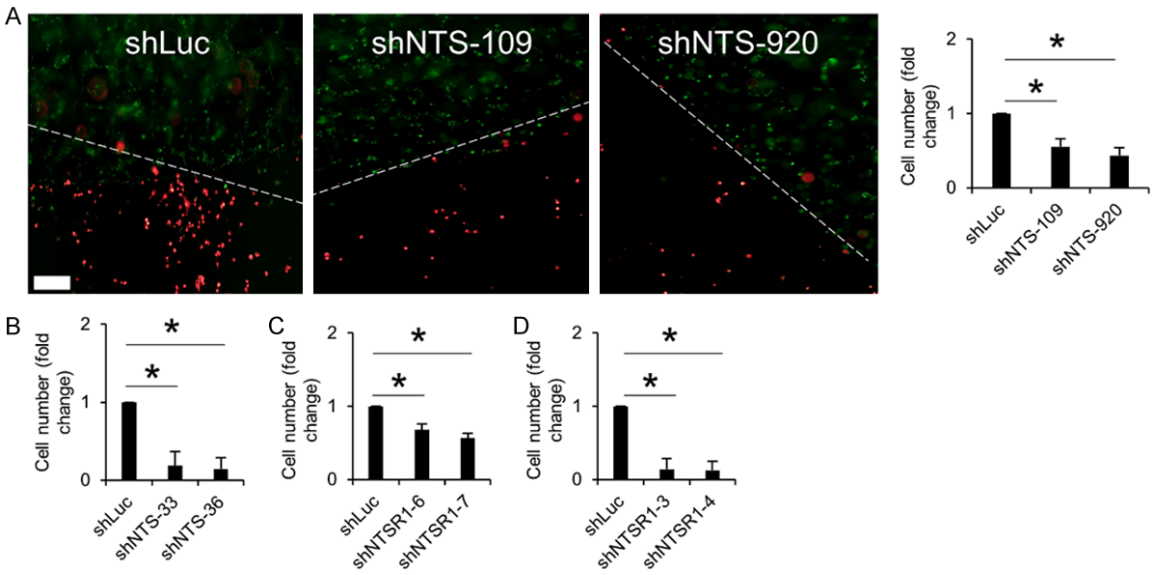

Figure 6. NTS knockdown in neuron and NTSR1 knockdown in PDAC decreased 3D pancreatic cancer motility. Differentiated SH-SY5Y or Neuro-2a was transfected with shLuc or shNTS, and 48 h post transfection cells were analyzed with 3D recruitment assay together with MIA PaCa-2 (A) and KPC (B), respectively. On the other hand, MIA PaCa-2 (C) or KPC (D) was transfected with shLuc or shNTSR1, and 48 h post transfection cells were analyzed with 3D recruitment assay together with differentiated SH-SY5Y and Neuro-2a, respectively. *, P<0.05. Scale bar, 50 μm. Cells with green fluorescence in Matrigel are DiO-stained differentiated SH-SY5Y. Cells with red fluorescence in medium are DiI-stained MIA PaCa-2.

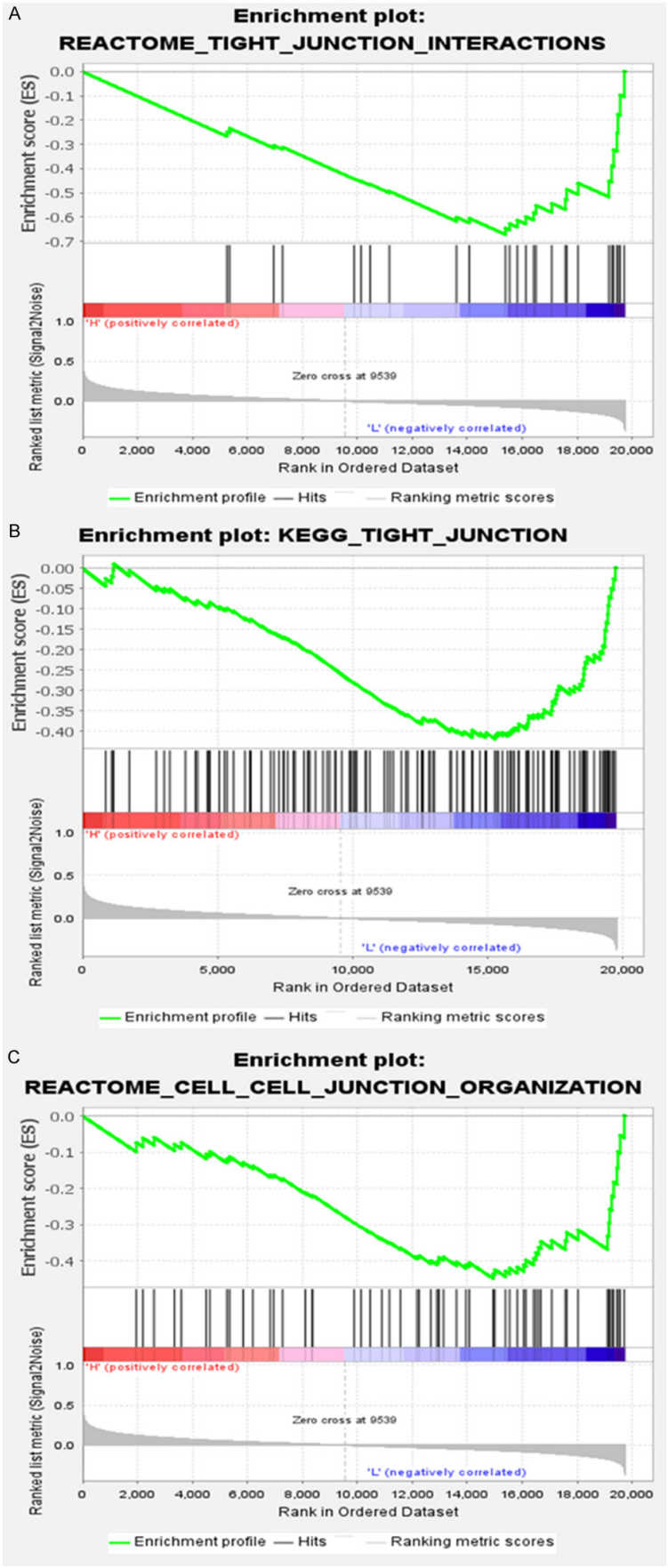

Figure 7. GSEA analysis revealed enriched cellular junction in NTS-low PDAC. GSE71729 as showed in **Figure 1** was analyzed for NTS expression in primary pancreatic cancer specimens, and NTS pattern grouping was determined by its mean expression value. These NTS-high or -low PDACs were then analyzed with GSEA, and enriched pathways in NTS-low group (A) REACTOME_TIGHT_JUNCTION_INTERACTIONS (NES=-2.18; P<0.001), (B) KEGG_TIGHT_JUNCTION (NES=-1.88, P<0.001), and (C) REACTOME_CELL_CELL_JUNCTION_ORGANIZATION (NES=-1.73, P<0.001) were shown.

could also be observed in clinical specimens of pancreatic cancer patients across America (**Figure 1A** of [40]) and Asia (**Figure 1A** of [39]; **Figure 1A** of [41]). In our orthotopic model, we analyzed whether NTSR1 on pancreatic cancer cell is involved in the neuron-PDAC communication. shNTSR1-KPC tumors indeed showed decreased interaction with neural cells, while shLuc-KPC tumors had more pancreatic cancer cells surrounding neural cells (**Figure 10**). As there is no obvious difference in neuron area of mouse tumors from shLuc-KPC or shNTSR1-KPC (**Figure 11**), the decreased communication between neuron and PDAC may be mainly resulted from NTSR1 loss on PDAC. This kind of neuron-PDAC interaction was reported frequently [39, 40, 42]. In quantitative result we also observed the decreased recruitment by neuron of pancreatic cancer cells in shNTSR1 group *in vivo* (**Figure 10**).

Inhibition of NTS/NTSR1 signaling sensitizes PDAC cells to gemcitabine

Gemcitabine is the first line chemotherapeutic drug for PDAC patients. Intrinsic or ac-

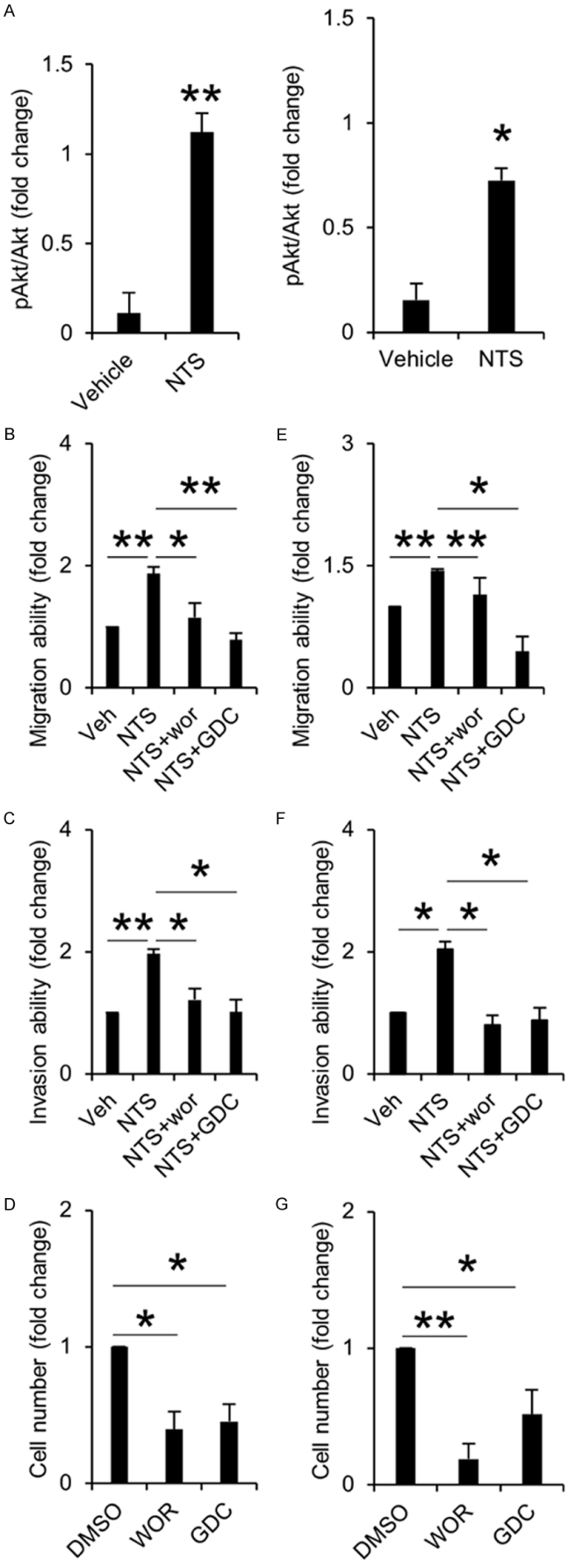

Figure 8. PI3K inhibitor decreased NTS-induced pancreatic cancer motility in 2D and 3D models. MIA PaCa-2 (A, left) and KPC (A, right) were treated with NTS with or without PI3K inhibitors wortmannin and GDC-0941, and the effect on Akt phosphorylation (A), migration (B, MIA PaCa-2; E, KPC), invasion (C, MIA PaCa-2; F, KPC), and 3D recruitment (D, MIA PaCa-2; G, KPC) was assayed. *, P<0.05; **, P<0.01.

quired resistance is the major cause leading to treatment failure. Whether NTS/NTSR1 signaling modulates the sensitivity of PDAC to chemotherapeutic drugs has not been reported. As shown in **Figure 12A**, gemcitabine (at 100 nM) significantly increased apoptosis in MIA PaCa-2, while NTS effectively countered gemcitabine-induced apoptosis. However, the protective effect of NTS was diminished when GDC-0941 was added, suggesting NTS acts via NTSR1/Akt signaling to enhance PDAC resistance to gemcitabine. To further confirm our hypothesis, we tested the effect of gemcitabine on NTSR1-depleted MIA PaCa-2. As in **Figure 12B**, depletion of NTSR1 in MIA PaCa-2 cells totally abolished NTS-induced gemcitabine resistance, confirming that NTS enhances resistance to gemcitabine via NTSR1. As clinical specimens with gemcitabine resistance characterization are yet to be publicly available, we searched NCBI gene expression omnibus database [43] for keyword “pancreatic cancer gemcitabine resistance” under the categories of “Homo Sapiens” and “tissue”. Among 16 datasets, GSE36563 is available for proper analysis as Van den Broeck *et al.* applied xenografts grown from human pancreatic tumors to analyze the side population (SP) having the properties of cancer stem cell and chemoresistance compared to those of the main population (MP) in bulky tumor [44]. From this microarray we indeed found side population, which is more resistant to gemcitabine according to the authors, also expressed more NTSR1 (Supplementary Figure 3). This analysis further strengthened our hypothesis on the impact of NTS-NTSR1 axis in pancreatic cancer drug resistance with clinical evidence.

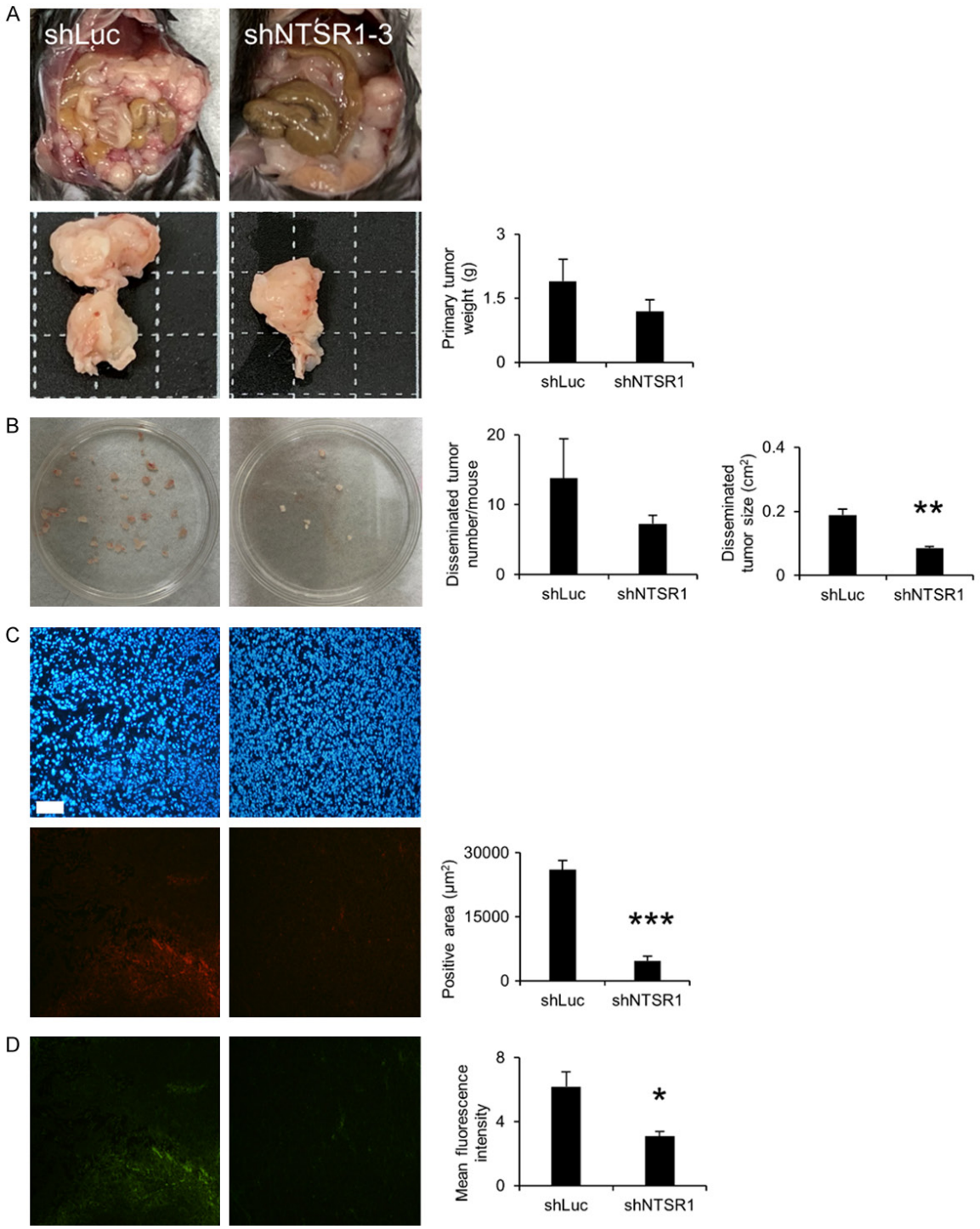

Figure 9. NTSR1 knockdown decreased tumor dissemination *in vivo*. 2×10^5 KPC cells of shLuc or shNTSR1 clones were injected orthotopically into the pancreas of C57BL/6. 3 weeks post inoculation mice were sacrificed, and tissues were harvested for analyses on primary (A) and disseminated (B) tumors, as well as expression of NTSR1 (C) and phospho-Akt (D). *, P<0.05; **, P<0.01; ***, P<0.001. Length of square in (A), 1 cm. Scale bar, 50 μm.

Discussion

In the present study, we answered several questions unresolved in previous studies. The first issue is the source of NTS in PDAC. When our study was ongoing, Takahashi *et al.* reported that NTSR1 signaling promotes the proliferation of PDAC through MAPK and NF-κB [45]. By

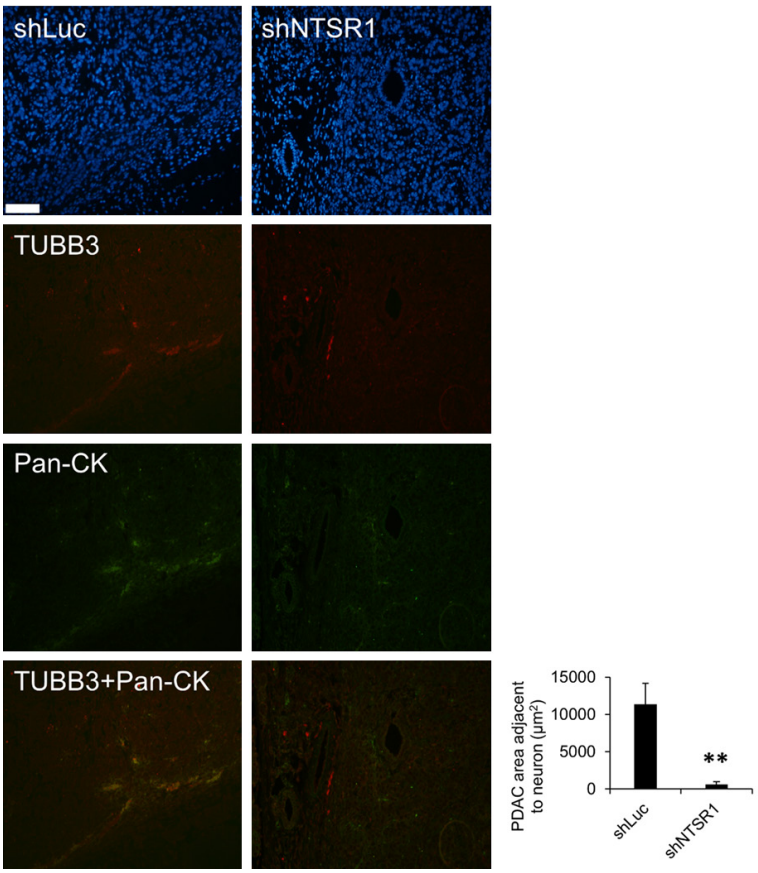

Figure 10. Effect of NTSR1 knockdown in KPC on interaction with neuron in orthotopic animal model. Pan-cytokeratin (Pan-CK)-positive pancreatic cancer and TUBB3-positive neuron were costained in mouse tumors of shLuc-KPC or shNTSR1-KPC, and area of PDAC surrounding neuron was analyzed. **, P<0.01. Scale bar, 50 μm.

using orthotopic model, they generated highly malignant PDAC sublines and tried to identify therapeutic targets by genomic screening. However, their results showed that NTSR1, but not NTS, is overexpressed in the sublines. We demonstrated that NTS is mainly produced by neuronal cells (**Figure 11**, Supplementary Figure 1) and acts via a paracrine mechanism to stimulate the invasiveness of PDAC. The importance of neural plasticity in cancer formation has been revisited recently [46-48]. As pancreas is an organ being innervated by different types of neurons, it is soaked in a microenvironment fulfilled with abundant neurotrophic factors, which help the initiation, promotion and progression of PDAC [46].

The second issue is the prognostic value of NTS/NTSR1 in PDAC. Although expression of NTSRs in PDAC have been reported [24, 25], its clinical significance is still unclear. Our results demonstrated that expression of NTS and NTSR1, but not NTSR2 and NTSR3, is associated with reduced PDAC patient survival.

The third issue is how NTS drives cancer invasiveness? Wang *et al.* demonstrated that NTS promoted IL8 expression and indirectly stimulated IL8-dependent migration in colon cancer, while curcumin treatment reversed these effects [49]. They also found that NTS increased activation of AP-1 and NF-κB, the upstream regulators of IL8 gene transcription [50], and these regulators may also contribute to NTS-induced migration. Akter *et al.* reported that NTS promoted migration and invasion of gastric cancer via MMP9 activation and this effect was mediated by multiple pathways including protein kinase C, MAPK and PI3K [51]. Servotte *et al.* found NTS only increased motility of glioma in laminin-coated substrate [52]. Under uncoated condition, NTS suppressed glioma migration via activation of Rac1 and Cdc42. Our results showed that NTS enhances motility of PDAC via NTSR1/Akt signaling axis. In addition, we confirmed our hypothesis in 2D and 3D systems, suggesting the importance of this pathway in NTS-promoted PDAC invasiveness. The contribution of NTS in pancreatic tumorigenesis may not be limited to cancer cells alone. Two previous studies demonstrated that NTS also enhanced motility and cytokine expression in macrophages [53, 54]. They showed that NTS increased intracellular calcium and JAK2-STAT1 activation when combined with other cytokines, resulting in enhanced migration of macrophages [53]. In addition, NTS induced IL1 expression and production in macrophages [54]. Besides macrophages, NTS also played an important role in the suppression of cytokine-triggered dendritic cell activation [55].

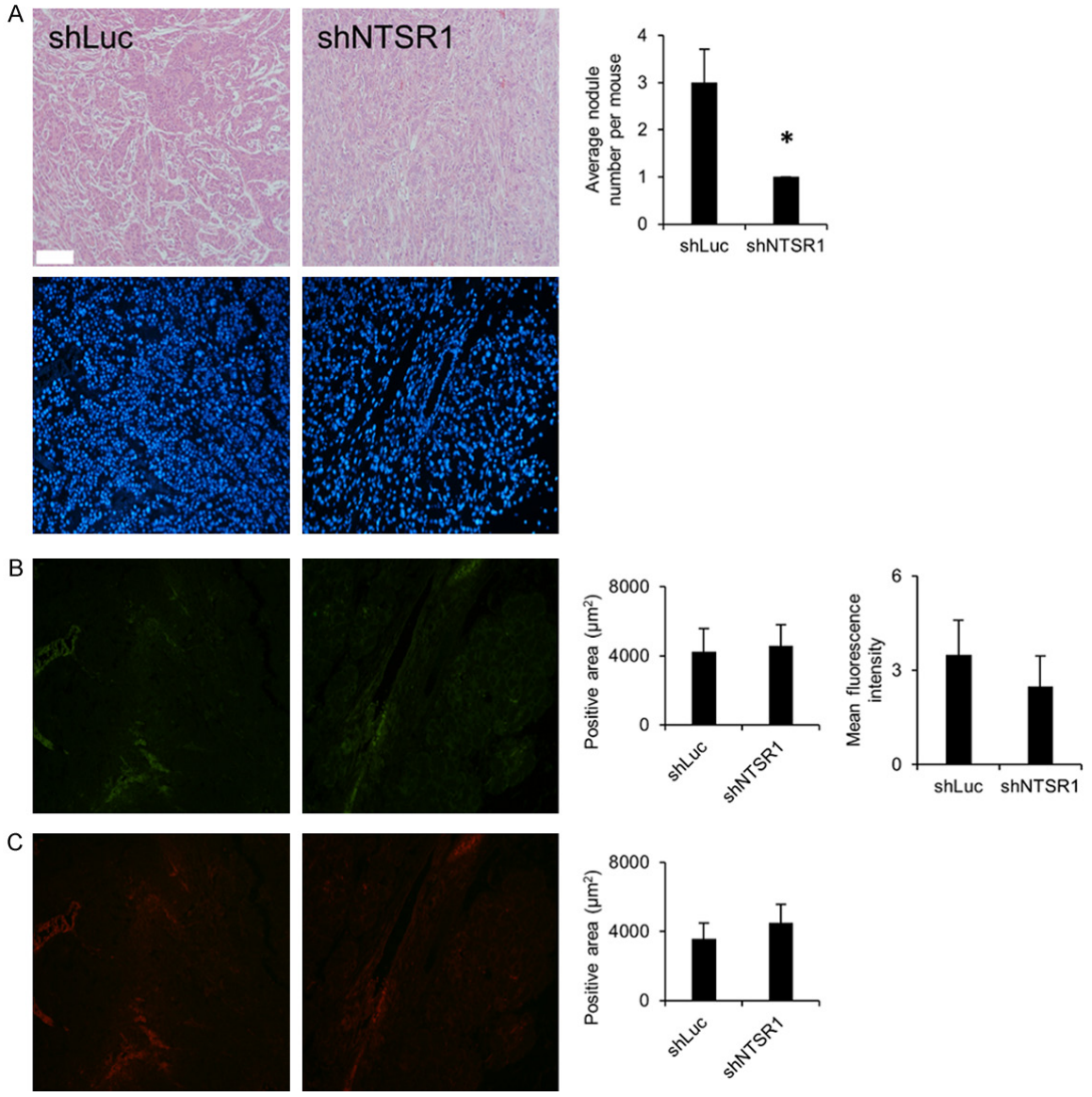

Figure 11. H&E stain and NTS stain on mouse primary tumors of shLuc-KPC and shNTSR1-KPC. (A) H&E stain and average nodule number per mouse were analyzed. NTS stain together with neural marker tubulin beta 3 class III (TUBB3) stain were performed on mouse primary tumors, and (B) NTS-positive area (middle) and intensity (right) as well as (C) TUBB3-positive area were analyzed. *, $P<0.05$. Scale bar, 50 μm.

Moreover, NTS increased taurocholate absorption and degranulation in mast cells [56, 57]. These studies suggested a complex role of NTS in the control of cancer cell behavior and the remodeling of tumor microenvironment.

We also provided the first evidence that NTS/NTSR1 signaling is involved in the regulation of gemcitabine resistance. Our results demonstrated that inhibition of NTSR1 or PI3K decreased the protective effect of NTS on gemcitabine-induced PDAC apoptosis. More than 80% of PDAC patients are diagnosed at late stages and could not undergo surgical resection due to local or distant metastasis. These patients mainly receive chemotherapy in clinical management. However, intrinsic or acquired resistance in PDAC cells frequently leads to treatment failure and early recurrence. Therefore, combinatory therapy by targeting different molecular pathways become the major strategy to enhance treatment efficacy. Wang

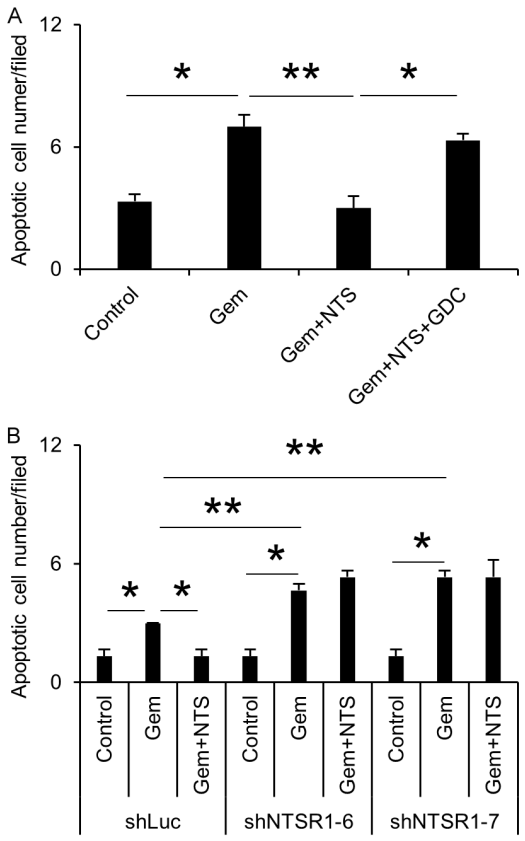

Figure 12. NTS increased pancreatic cancer gemcitabine resistance. Cells of indicated condition were stained with 50 μg/ml propidine iodine and cell apoptosis was analyzed under blockage of PI3K/Akt (A) or NTSR1 (B). *, P<0.05; **, P<0.01.

et al. showed that NTSR1 inhibitor SR48692 suppressed NTS-induced proliferation in pancreatic cancer [27]. Baum *et al.* demonstrated that another NTSR1 inhibitor ^{177}Lu-3BP-227 suppressed tumor growth in a small portion of patients with PDAC [58]. Isotope-conjugated NTS has also been used for tumor imaging and treatment. Kanellopoulos *et al.* showed that conjugation of technetium-99m with NTS analog yielded [99mTc]Tc-NT(7-13), which could be effectively taken by PDAC [59]. Visser *et al.* demonstrated 111In-labelled DTPA- and DOTA-conjugated NTS analogues could be useful for the detection and treatment of exocrine pancreatic cancer [60]. However, the effectiveness of NTS-derived molecules on cancer treatment has yet to be elucidated in clinical trials.

As in drug prediction database Connectivity Map wortmannin was predicted as the most potential therapeutic for NTS-high PDAC using the coexpression gene signature in GSE71729, we found in cell line and animal experiments phospho-Akt was affected once NTS-NTSR1 signaling was modulated. We checked that phospho-ERK was not significantly altered post NTS treatment in MIA PaCa-2 and KPC. The difference in signaling pathways post NTS treatment in PDAC may be resulted from differences in NTS treatment period and pancreatic cancer cell line used. For 100 nM NTS-induced activations of MAPK and NF-κB, Takahashi *et al.* showed that these were observed in Panc-1-3P cells (primary cell sublines established from orthotopic PANC-1 inoculation in nude mice for 3 repeats) under 5-minute NTS treatment [45]. Subsequently they found NTS receptor 1 overexpression in Panc-1 increased its growth in nude mice. We additionally identified neuron as NTS producer in pancreatic cancer microenvironment and the underlying mechanism for NTS-induced pancreatic cancer invasion, with PI3K activation being observed in both cell line and animal experiment for dual confirmation.

Based on analyses for expression and promoter in TCGA dataset in UALCAN database [61], NTSR1 is also detected in adjacent normal pancreas, but its expression is higher in pancreatic cancer (Supplementary Figure 2A). The NTSR1 promoter methylation is higher in tumor parts (Supplementary Figure 2B), indicating that promoter methylation may not be a direct cause of NTSR1 upregulation in pancreatic cancer. Transcription factor analysis in TF2DNA database [62] revealed that the transcription factor YY1 (Supplementary Table 1), affecting gene expression and epigenetic enzyme recruitment, is a potential transcription factor on the promotor of NTSR1. YY1, like NTSR1, is also increased in pancreatic cancer (Supplementary Figure 2C) and predicts its poor prognosis (Supplementary Figure 2D). These analyses revealed the potential of transcription factor YY1 as one of the mechanisms leading to NTSR1 upregulation in pancreatic cancer.

As disease-free survival curve in TCGA PAAD is yet to be publicly available, we assayed whether NTSR1 expression is associated with disease progression. After extractions of NTSR1 expression and clinical data for TCGA PAAD dataset from cBioPortal database, we found NTSR1 level indeed is higher in the tumors from patients with disease recurrence/progres-

sion than those from patients who are disease-free (Supplementary Figure 4).

While the importance of NTS and its receptor 1 were identified in pancreatic cancer in terms of prognosis and therapy, these characters were not observed in other gastrointestinal cancers such as those of liver, stomach, and colon in our subsequent bioinformatic analyses. Multiple reviews had suggested the expression alterations of NTS and its receptor 1 across cancer types [23, 63], but only some of them were prognostic. Takahashi *et al.* revealed the importance of NTSR1 in pancreatic cancer via TCGA analysis [45] as we found. Qiu *et al.* showed the importance of these factors in colon cancer via in-house dataset analysis [64]. Xiao *et al.* showed that the combination of NTS and interleukin 8 was a prognostic indicator in liver cancer via in-house dataset analysis [65]. Besides gastrointestinal cancers, NTSR1 was reported to be prognostic in cancers of brain [66], breast [67], endometrium [68], and lung [69]. These studies echoed the criticalness of NTSR1 in cancer formation of various origins, and we additionally emphasized the importance of its ligand NTS from the tumor microenvironment.

Collectively, our results reveal the importance of NTS/NTSR1 in promoting PDAC tumorigenesis. In addition, we elucidate the underlying mechanism by which NTS promotes invasiveness and drug resistance in pancreatic cancer cells, providing a new strategy for the treatment of PDAC by combining chemotherapeutic drugs and NTS pathway inhibitors.

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Disclosure of conflict of interest

None.

Abbreviations

NTS, neurotensin; PDAC, pancreatic ductal adenocarcinoma; NTSR1, neurotensin receptor 1.

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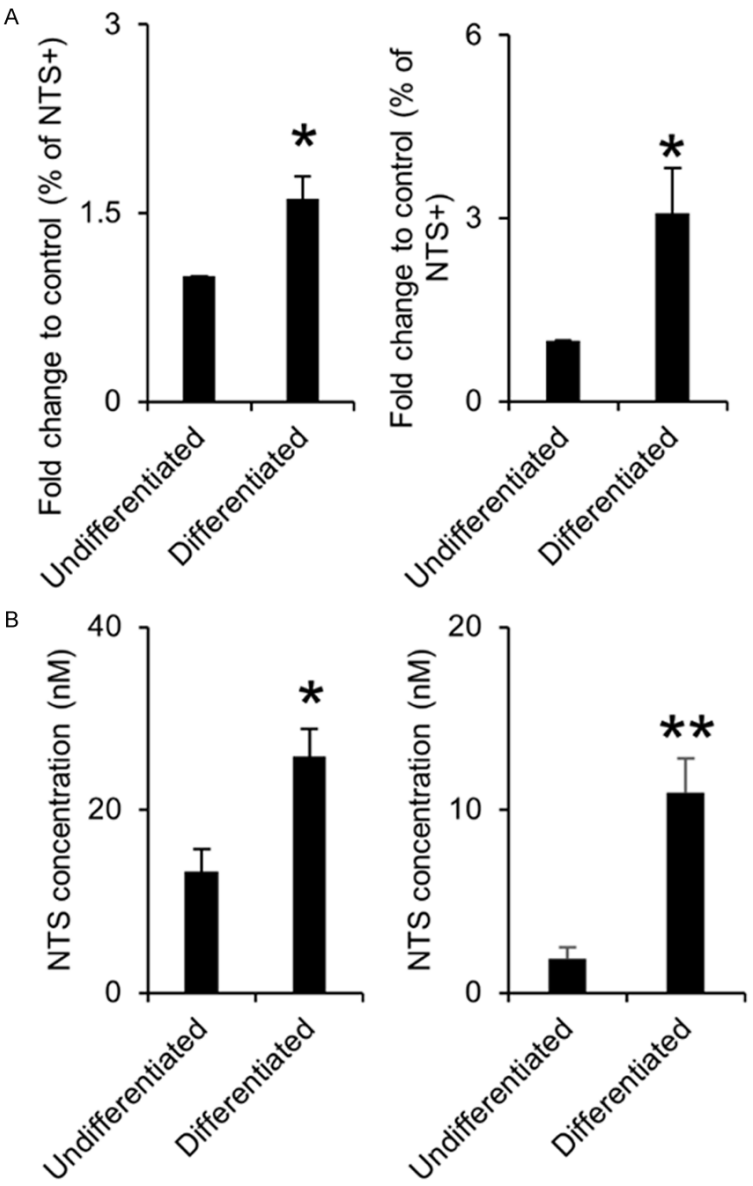
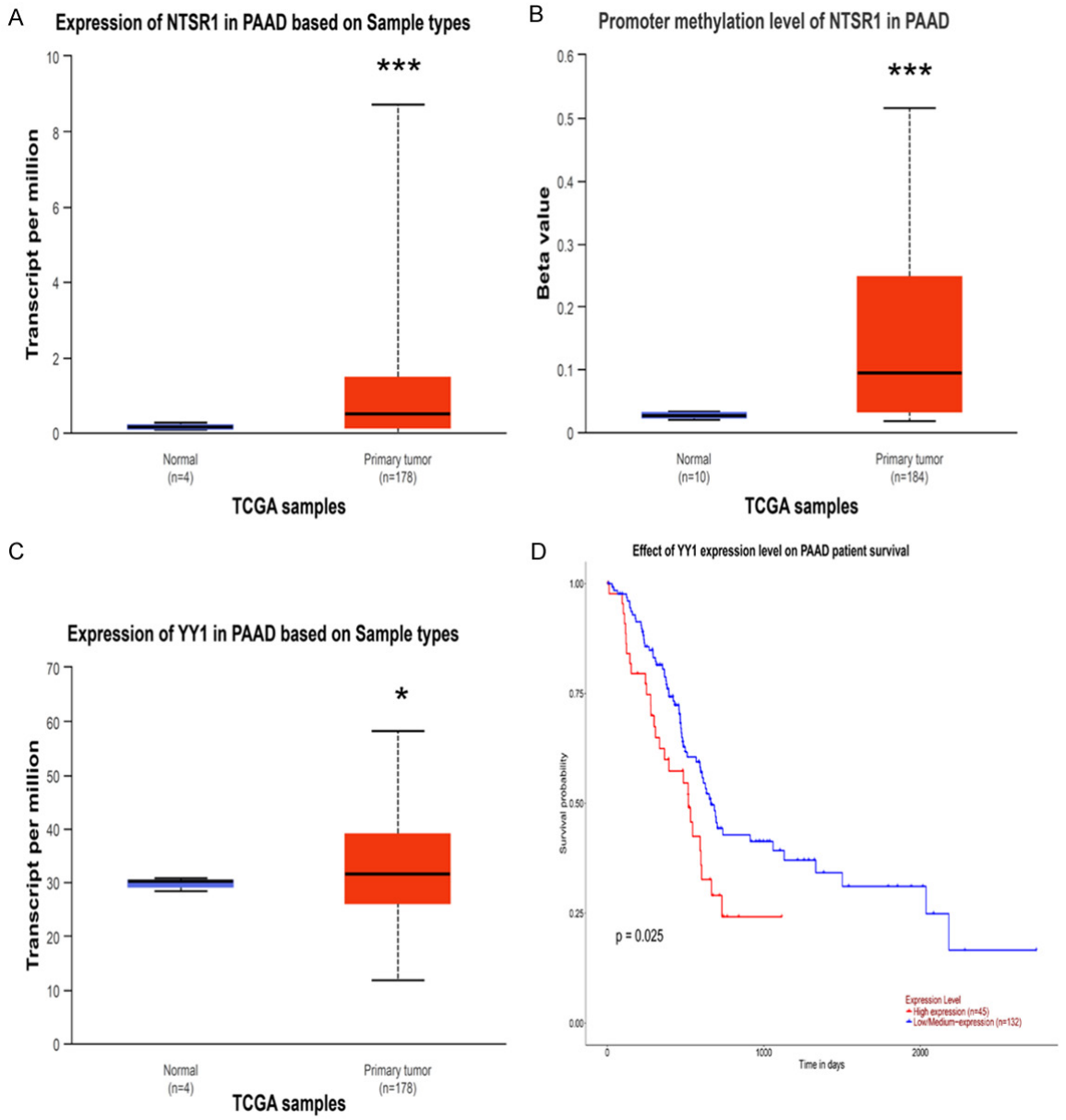
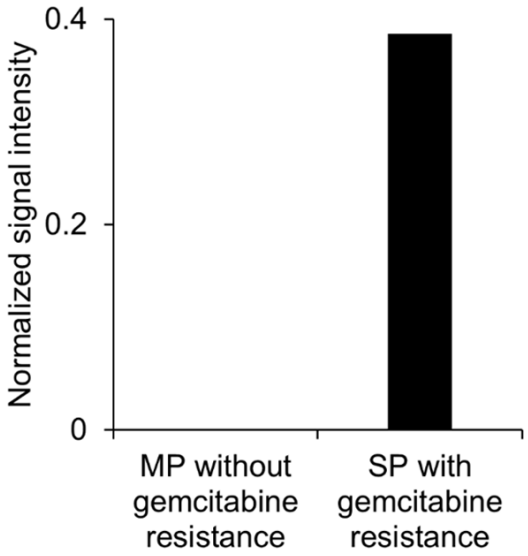


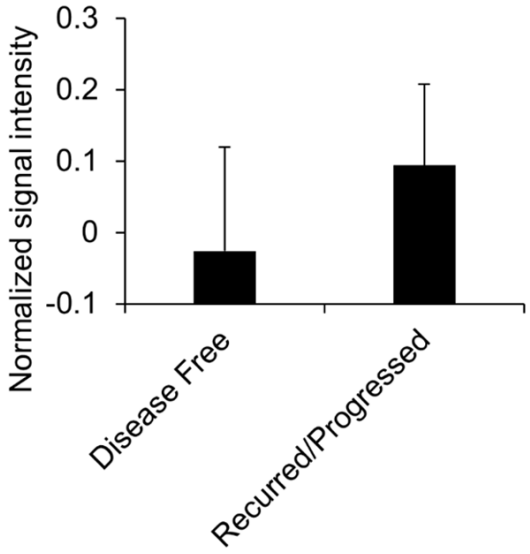
Supplementary Figure 1. Increased NTS expression after neural differentiation. SH-SY5Y (left column) and Neuro-2a (right column) with or without neural differentiation were compared for NTS expression with flow (A) and ELISA (B). *, P<0.05; **, P<0.01.



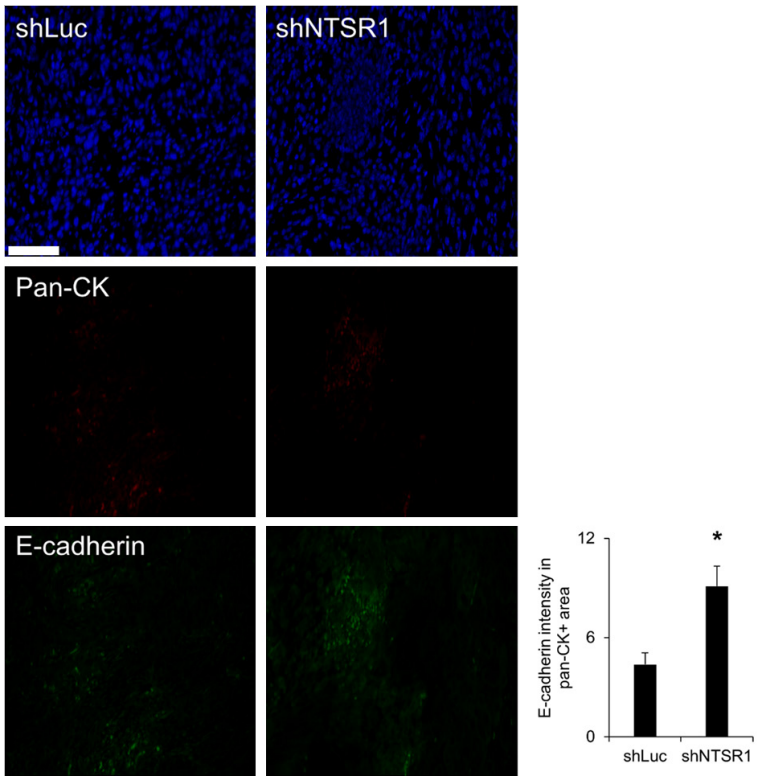
Supplementary Figure 2. The expression and associated phenotypes of NTSR1 and transcription factor YY1 in pancreatic cancer. The expression (A) and promoter methylation (B) of NTSR1 in TCGA PAAD dataset were analyzed with UALCAN database. As promoter hypomethylation was not observed in the analysis, we speculated specific transcription factors (Supplementary Table 1) might be involved in NTSR1 upregulation in pancreatic cancer. Screening through their expression alterations and prognosis predictions, transcriptional factor YY1 was found to be increased (C) and predicted poor prognosis (D) in pancreatic cancer. *, P<0.05; ***, P<0.001.



Supplementary Figure 3. NTSR1 expression is higher in gemcitabine-resistant pancreatic cancer. The potential involvement of NTSR1 in gemcitabine resistance in clinical specimen was identified in NCBI gene expression omnibus (GEO) database with the keyword "pancreatic cancer gemcitabine resistance" and categories "Homo Sapiens" as well as "tissue". GSE36563 was identified as this study applied xenografts grown from human pancreatic tumors to analyze the side population (SP) having the properties of cancer stem cell and chemoresistance compared to those of the main population (MP) in bulky tumor. Applying these criteria we found NTSR1 (probe A_24_P378806) was expressed at higher level in SP.



Supplementary Figure 4. NTSR1 expression is higher in recurred/progressed pancreatic cancer. The potential involvement of NTSR1 in disease recurrence/progression in clinical specimen was identified in TCGA PAAD dataset in cBioPortal database. Applying NTSR1 expression and clinical data we found NTSR1 was expressed at higher level in tumors from patients who are with recurred/progressed disease.



Supplementary Figure 5. E-cadherin expression is higher in shNTSR1-KPC tumors. E-cadherin expressions in KPC tumors of shLuc and shNTSR1 were compared. *, P<0.05. Scale bar, 200 μm.

Supplementary Table 1. Potential transcription factors for NTSR1 identified in TF2DNA database

Source dataset	Transcription factor
Kulakovskiy 2013	ELF2
Matys 2006	ZNF423
	YY1
Jolma 2013	TCF3
	KLF16
	ELF5
	ETV5
	GLI2
	ELK3
	HES5
TF2DNA	ZNF436
	ZBTB48
	ZNF222
	MZF1
	ADNP2
	KLF16
	ZNF25
	TLX1
	ZNF679
	ID4
	ZNF41
	ZNF420
	HINFP
	ZNF768
	REST
	TBR1
	TCF21
	ZNF273
	ZNF837