RET receptor expression and interaction with TRK receptors in neuroblastomas

LAURA H. TETRI[1], VENKATADRI KOLLA[1], REBECCA L. GOLDEN[1], RADHIKA IYER[1], JAMIE L. CROUCHER[1], JEE-HYE CHOI[1], SUZANNE P. MACFARLAND[1], KOUMUDI NARAPARAJU[1], PENG GUAN[1], FERRO NGUYEN[1], KRUTIKA S. GAONKAR[2,3], PICHAI RAMAN[2,3] and GARRETT M. BRODEUR[1]

[1]Division of Oncology, Department of Pediatrics, The Children's Hospital of Philadelphia and The University of Pennsylvania; [2]Department of Biomedical and Health Informatics, [3]Center for Data-Driven Discovery in Biomedicine, Children's Hospital of Philadelphia, Philadelphia, PA 19104-4302, USA

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Abstract. Neuroblastomas (NBs) have heterogeneous clinical behavior, from spontaneous regression or differentiation to relentless progression. Evidence from our laboratory and others suggests that neurotrophin receptors contribute to these disparate behaviors. Previously, the role of TRK receptors in NB pathogenesis was investigated. In the present study, the expression of RET and its co-receptors in a panel of NB cell lines was investigated and responses to cognate ligands GDNF, NRTN, and ARTN with GFRα1-3 co-receptor expression, respectively were found to be correlated. RET expression was high in NBLS, moderate in SY5Y, low/absent in NBEBc1 and NLF cells. All cell lines expressed at least one of GFRα co-receptors. In addition, NBLS, SY5Y, NBEBc1 and NLF cells showed different morphological changes in response to ligands. As expected, activation of RET/GFRα3 by ARTN resulted in RET phosphorylation. Interestingly, activation of TrkA by its cognate ligand NGF resulted in RET phosphorylation at Y905, Y1015, and Y1062, and this was inhibited in a dose-dependent manner by the TRK inhibitor (CEP-701). Conversely, RET activation by ARTN in NBLS cells led to phosphorylation of TrkA. This suggests a physical association between RET and TRK proteins, and cross-talk between these two receptor pathways. Finally, RET, GFR and TRK expression in primary tumors was investigated and a significant association between RET, its co-receptors and TRK expression was demonstrated. Thus, the present data support a complex model of interacting neurotrophin receptor pathways in the regulation of cell growth and differentiation in NBs.

Correspondence to: Dr Garrett M. Brodeur, Department of Pediatrics, The Children's Hospital of Philadelphia and The University of Pennsylvania, 3501 Civic Center Blvd., Philadelphia, PA 19104-4302, USA
E-mail: brodeur@email.chop.edu

Abbreviations: RT-PCR, reverse transcriptase polymerase chain reaction; GDNF, glial cell line-derived neurotrophic factor; NRTN, neurturin; ARTN, artemin; PSPN, persephin; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; RTK, receptor tyrosine kinase

Key words: neuroblastoma, RET, glial cell line-derived neurotrophic factor, neurturin, artemin, neurotrophic receptor tyrosine kinase 1, neurotrophic receptor tyrosine kinase 2, nerve growth factor, brain-derived neurotrophic factor

Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor of childhood, accounting for 8% of childhood cancers and 15% of childhood cancer deaths. It arises from sympathoadrenal precursors and generally occurs in the adrenal medulla or along the sympathetic chain. NBs demonstrate clinical heterogeneity, from spontaneous regression to relentless progression (1,2). We and others have identified different patterns of genetic change that underlie these disparate clinical behaviors (3,4), and that receptor tyrosine kinase (RTK) expression likely contributes to this (5,6). Several RTKs have been implicated in the pathogenesis or clinical behavior of NBs including TRK genes, ALK and RET. The TRK family of neurotrophin receptors plays critical roles in the development and maintenance of the central and peripheral nervous system (5). Neurotrophic receptor tyrosine kinase 1 (NTRK1, also known as TrkA), neurotrophic receptor tyrosine kinase 2 (NTRK2, also known as TrkB) and neurotrophic receptor tyrosine kinase 3 (NTRK3, also known as TrkC) are the cognate receptors for nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3), respectively. TrkB also binds to NT4, and all three can be activated by NT3. TrkA (and TrkC) are important for the development of sensory and sympathetic neurons, whereas TrkB is important for motoneuron development (7).

RET is an RTK that is expressed in many NB tumors and tumor-derived cell lines (8-10). RET is essential for the migration, growth, axon formation, and differentiation of normal sympathetic neurons during development (11-13). RET is one of few RTKs that requires a co-receptor for ligand binding, rather than direct ligand binding (14). Most studies of RET in NB have not investigated co-receptor expression, or the correlation between co-receptor expression and ligand-specific activation. The four RET ligands are glial-cell line derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN), and persephin (PSPN). They associate with GDNF co-receptors GFRα1-GFRα4, respectively. A ligand homodimer must bind to a co-receptor (GFRα1-GFRα4) to induce co-receptor homodimerization (15). The ligand/co-receptor complex then recruits RET into lipid rafts, leading to homodimerization and autophosphorylation of the RET tyrosine kinase domain (11,16-18).

RET phosphorylation leads to the activation of specific post-receptor signaling pathways. Of the 16 RET autophosphorylation sites, the three most important for signaling are Y905, Y1015 and Y1062 (11,17,18). RET is further regulated by posttranslational modifications and exists in the cell in three forms: Unglycosylated (~120 kDa), partially glycosylated (~150 kDa)-present only at the endoplasmic reticulum, and fully glycosylated (~170 kDa)-present on the cell surface (11). Besides differences in glycosylation, RET also exists as two main transcriptional isoforms-RET9 and RET51-which have different signaling properties (19,20).

RET is expressed in most NB tumors and cell lines, although it has not been associated with patient outcome (8-10,19,21-23). Thus far, no mutations or genomic alterations have been identified leading to activation (or inactivation) of RET in NB (22-25). Higher RET mRNA and protein expression levels have been associated with differentiation of NB cells (9,26-33), but other studies have suggested that RET may play a role in proliferation or metastasis in NBs (31,34-36). The pattern of TRK family gene expression clearly contributes to the survival, growth, and differentiation of NBs, and it has been suggested that RET can cooperate with TrkA and B to cause neuronal differentiation in response to ligands or to 13-cis retinoic acid (37,38). However, the direct or indirect interaction of RET with other receptors has not been fully explored.

Here, the expression of the RET and its co-receptors was investigated, as well as TRKs in a panel of NB cell lines and the correlation of their responses to GDNF, NRTN, and ARTN with GFRα1-3 expression, respectively, was investigated. It was also demonstrated TRK activation by ARTN, and RET activation by NGF, suggesting a physical association between RET and TrkA receptors. Finally, the expression of RET, GFRs and TRKs in publicly available databases of NB mRNA expression was examined, and significant associations were validated in additional databases. The present data provide insights into the complex interactions of these two receptor pathways in neuroblastoma that may contribute to NB pathogenesis or differentiation.

Materials and methods

Cell lines, culture conditions. All cell lines used in this study were maintained in our lab or obtained from American Type Culture Collection. The NLF line was first isolated at Washington University (St. Louis, MO, USA) in the 1970's by Dr Milton Goldstein. Both TrkA and TrkB clones were developed in Dr Brodeur's laboratory. Cell lines were grown in RPMI-1640 medium containing 10% fetal bovine serum, 500 U/l penicillin and 500 μg/l streptomycin, 2 mM L-glutamine and 25 mg/l gentamicin (all from Gibco; Thermo Fisher Scientific, Inc.). Cells were grown at 37˚C in a humidified incubator with 95% air and 5% CO_2. TRK-null SH-SY5Y cells were stably transfected with TrkA (SY5Y-TrkA, clone P23A) or TrkB (SY5Y-TrkB, clone BR6) (39-52). Transfected cells were maintained in media containing 0.3 mg/ml G418 sulfate (Corning Inc.). Other NB lines used in this study were NBLS, NBEBc1 and NLF. Cells were harvested using 0.2% tetrasodium EDTA in phosphate buffered saline. We tested these cell lines for endotoxins, mycoplasma, bacterial and viral contamination, as well as genetic identity validation by multiplex PCR techniques. These tests are performed annually at our facility.

Prior to treatment with ligand or drug, cells were serum-starved in RPMI-1640 with 1% fetal bovine serum (Life Technologies; Thermo Fisher Scientific, Inc.) to minimize serum factor signaling. Cells were treated with lestaurtinib (CEP-701, Cephalon/TEVA Pharmaceutical Industries), a TRK-selective inhibitor, at the indicated concentration for one hour before ligand treatment. Cells were treated with 50 ng/ml of GDNF, NRTN, and ARTN (R&D Systems, Inc.), or 100 ng/ml NGF and BDNF (PeproTech, Inc.) in RPMI-1640 with 1% fetal bovine serum for 15 min before cell lysis. Control cells received either the same volume of PBS or no treatment. For longer treatments to determine effects on morphology, cells remained in the drug and ligand-containing media for 24 h, 3 days or 6 days.

Antibodies and other reagents. Antibodies targeting phosphorylated (p) RET-Y905 (cat. no. 3221), TRK-Y490 (cat. no. 9141) and RET (cat. no. 14556) were obtained from Cell Signaling Technology, Inc. Antibodies targeting pRET-Y1015 (cat. no. ab74154) and pRET-Y1062 (cat. no. ab51103) were purchased from Abcam. GFRα1-3 (cat. nos. MAB7141, MAB6131 and MAB6701, respectively) antibodies were from R&D systems, Inc. RET (isoforms 51, cat. no. sc-1290) and pan-TRK (cat. no. sc-11) antibodies were obtained from Santa Cruz Biotechnology, Inc. NGF and BDNF (50 ng/ml final working concentrations) were obtained from PeproTech, Inc.

Whole cell extracts. To prepare whole cell extracts, cells were lysed and protein collected using cell lysis buffer (Cell Signaling) with 500 ml of buffer concentrate, 50 μl of 100 mM PMSF in ethanol, 750 μl of Protease inhibitor from cOmplete™, Mini Protease Inhibitor Cocktail Tablets (Roche Diagnostics) and 3.75 ml of distilled water.

RET inhibition studies. NBLS cells were induced with RET ligand ARTN (50 ng/ml) and inhibited using increasing concentrations of CEP-701 (10, 50, 100, 200 and 400 nM). Whole cell extracts were prepared as aforementioned, and used for western blot analysis.

Western blot analysis. Whole cell extracts (100 μg) were subjected to polyacrylamide gel electrophoresis

(4-12% SDS-PAGE), using NuPAGE Bis-Tris gels with MOPS-SDS Running Buffer (Invitrogen; Thermo Fisher Scientific, Inc.). Proteins were transferred to nitrocellulose membranes (GE Healthcare Life Sciences). Membranes were blocked with 5% non-fat milk in PBS-Tween-20, and incubated with primary antibodies (1:1,000) overnight at 4˚C, either in 5% non-fat milk or 1% BSA (for phosphorylated-specific antibodies). After 3-4 washes with PBS-Tween-20, the membranes were incubated with secondary antibodies (1:3,000) in similar buffers at room temperature for 1 h. Blots were washed four time with PBS-Tween-20, developed using chemiluminescence reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and signals were detected using autoradiography.

RNA isolation and reverse-transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from cell pellets using the RNeasy Mini kit (Qiagen, Inc.) and quantified using Nanodrop spectrometer (Thermo Fisher Scientific, Inc.). cDNA was prepared from total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed using an ABI 7900HT Fast Real-Time PCR system with TaqMan Gene Expression Assays (Applied Biosystems; Thermo Fisher Scientific, Inc.) for RET (RET: Hs01120032_m1), GFRα1-3 (GFRα1: Hs00237133_m1; GFRα2: Hs00394700_m1; GFRα3: Hs00181751) and GAPDH (GAPDH: Hs99999905_m1). All samples were run in triplicate, and each experiment was conducted at least three times. Values were calculated as relative rates from a standard curve, and GAPDH was used as an internal control.

Phase contrast microscopy. Cell morphology was assessed using phase-contrast microscopy, and captured images at 20x magnification (Leica Microsystems Inc.). For longer treatments to examine the effects of ligand on cell morphology, the media was changed every 3 days, and fresh ligand was added. Various NB cell lines were plated in either 6-well or 10 cm culture dishes, and cells were treated with GDNF, ARTN or NRTN. Ligand-induced cells were assessed for neuronal differentiation by changes in cell shape, and by measuring neurite outgrowth. Cells were considered differentiated when they had three or more times the size of undifferentiated cells, with development of euchromatin and prominent nucleoli. Neurite outgrowth was also assessed by counting the number of cells that had neurites extending more than the length in longest diameter of the cell, as assessed by ocular micrometer measurement.

Statistical analysis. For analysis of gene expression, statistical analyses were performed using GraphPad Prism and the Prism-ANOVA method. Each experiment was performed at least three times, and triplicate readings were used and reported for all P-values. Data are expressed as the standard error of the mean. Values are the mean of triplicate readings from three or more independent experiments and $P<0.05$ was considered to indicate a statistically significant difference.

Relative expression of RET and its co-receptors was assessed by western blotting and was scored qualitatively from '+++' for the highly RET positive NBLS line to '-' for the NLF line. A similar approach was used for GDNFα1-3 expression and for RET phosphorylation. Morphologic changes, such as cell body enlargement, flattening and neurite outgrowth, were also quantitated on a '-' to '+++' scale, where '-' indicated no change, '+' indicated a modest change, '++' indicated a moderate change, and '+++' indicated a dramatic change, based on direct inspection of phase-contrast images.

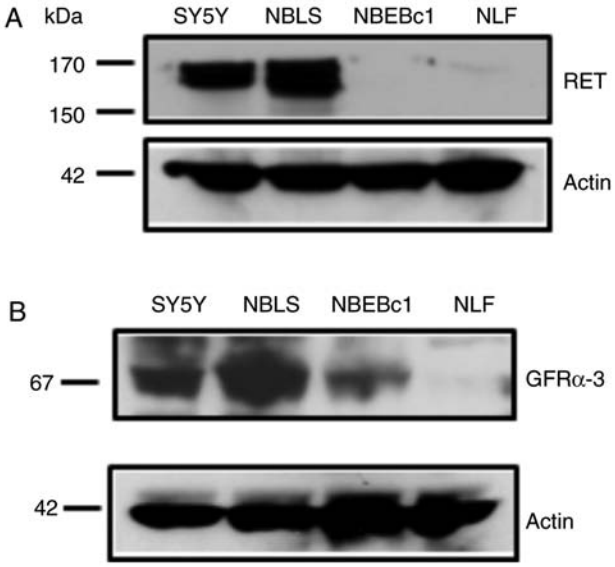

Figure 1. RET expression in NB cell lines. (A) Whole cell extracts from SY5Y, NBLS, NBEBc1 and NLF cell lines were subjected to western blotting. SY5Y and NBLS cells show the highest levels of RET expression. (B) Expression of co-receptor GFRα3 is high in NBLS, moderate in SY5Y, low in NBEBc1 and absent in NLF cells.

Data from publicly available NB microarray expression and RNA-seq studies were analyzed for the expression of RET with its co-receptors GFRα1, -2, -3, and -4, as well as with NTRK1 and -2, to determine if there were significant associations. The ggplot2 package (version 2.21; https://mran.microsoft.com/snapshot/2017-04-11/web/packages/ggplot2/index.html) was used to generate scatter plots comparing RET with GFRα1, 2, 3, 4 and NTRK1, 2 from the TARGET NB RNA-sequencing data (53). Pearson's correlation analysis was used to estimate correlation between RET and the expression of the GFRα1 receptors. Meta-analysis was performed using the meta package (54). Both fixed and random effects model for the analysis and forest plots were generated to summarize the data. All data was analyzed using the R statistical (version 3.43) language and framework.

Results

Expression of RET and its co-receptors in NB cell lines. In order to determine RET expression levels in a panel of NB cell lines (SY5Y, NBLS NBEBc1 and NLF), western blot analysis using whole cell extracts was performed. RET was detected at moderate to high levels in both the NBLS and SY5Y lines, but it was low/absent in NBEBc1 and NLF cells (Fig. 1A). Next, the expression of endogenous RET co-receptor expression was assessed. GFRα3 was expressed at various levels, ranging from low to high in this panel of NB cell lines, and high expression of GFRα3 was seen in NBLS, with lower levels detected in NBEBc1 (Fig. 1B). In addition, we analyzed mRNA

Table I. RET and RET co-receptor expression in NB cell lines.

Ligand treatment	Characteristics	NBLS	SY5Y	NB-EBc1	NLF
No treatment	RET expression[a]	+++	++	+/-	-
No treatment	GFRα1 expression	++	-	+	+
No treatment	GFRα2 expression	+	-	+	++
No treatment	GFRα3 expression	+++	+++	+	+
GDNF treatment	RET phosphorylation	++	+	+	-
	Morphologic changes[b]	+	++	+	-
NRTN treatment	RET phosphorylation	++	-	+	-
	Morphologic changes	++	+	+	-
ARTN treatment	RET phosphorylation	+++	-	+	-
	Morphologic changes	++	+	+	-

[a]Relative expression of RET and its co-receptors was assessed by western blotting and was scored qualitatively from '+++' for the highly RET positive NBLS line to '-' for the NLF line. A similar approach was used for GDNFα1-3 expression and for RET phosphorylation. [b]Morphologic changes, such as cell body enlargement, flattening and neurite outgrowth, were also quantitated on a '-' to '+++' scale.

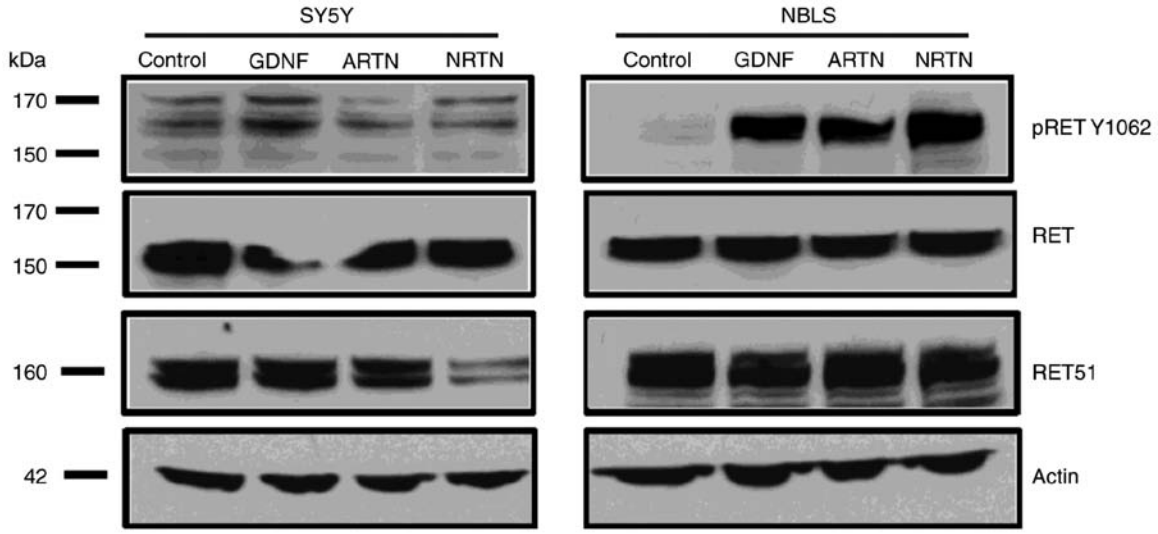

Figure 2. RET phosphorylation in response to ligand. Cell extracts from SY5Y and NBLS were analyzed by western blotting. Phospho-RET is induced by exposure to all three ligands, GDNF, NRTN and ARTN, in NBLS. SY5Y shows modest induction of phosphor-RET by GDNF. NB, neuroblastoma; GDNF, glial cell line-derived neurotrophic factor; ARTN, artemin; NRTN, neurturin.

expression of RET and its co-receptors GFRα1-3 in these cell lines by quantitative RT-PCR. The results correlating RET and co-receptor expression are summarized in Table I. GFRα4 and PSPN expression levels were negative in all cell lines and were excluded from further analysis.

Expression of phospho-RET in NB cell lines. To further investigate whether ligand exposure leads to RET phosphorylation, SY5Y and NBLS cells, which expressed both RET and GFRα3 (Fig. 1), were treated with 50 ng/ml of ligands GDNF, NRTN and ARTN for 15 min. Whole cell extracts were prepared and western blotting was performed. RET expression in response to ligands was analyzed using a RET-Y1062 antibody. All ligands readily induced phosphorylation of RET in NBLS cells, but there was only a modest increase in phosphorylation of RET in respond to GDNF in SY5Y (Fig. 2). Phosphorylated RET in response to various ligands in different cell lines is shown in Table I. These results confirm that RET activation occurs in NB cell lines by phosphorylation in response to ligand in the presence of their cognate co-receptors.

Morphological changes of NB cell lines in response to RET ligands. Morphological differences in NB cell lines in response to ligands were assessed. SY5Y, NLF, NBLS, and NB-EBc1 cells were exposed to GDNF, ARTN, or NRTN (50 ng/ml) or no ligand, and the cells were assessed qualitatively by phase contrast microscopy at 0, 24 h, 3 days, and 6 days. SY5Y cells showed morphologic differentiation and neurite outgrowth in response to GDNF and NRTN, but not ARTN after 6 days of exposure (Fig. 3). NLF cells showed a flattened shape and neurite outgrowth compared to control cells in response to ligand (Fig. 3). NBLS and SY5Y had the highest levels of endogenous RET expression, and showed morphologic

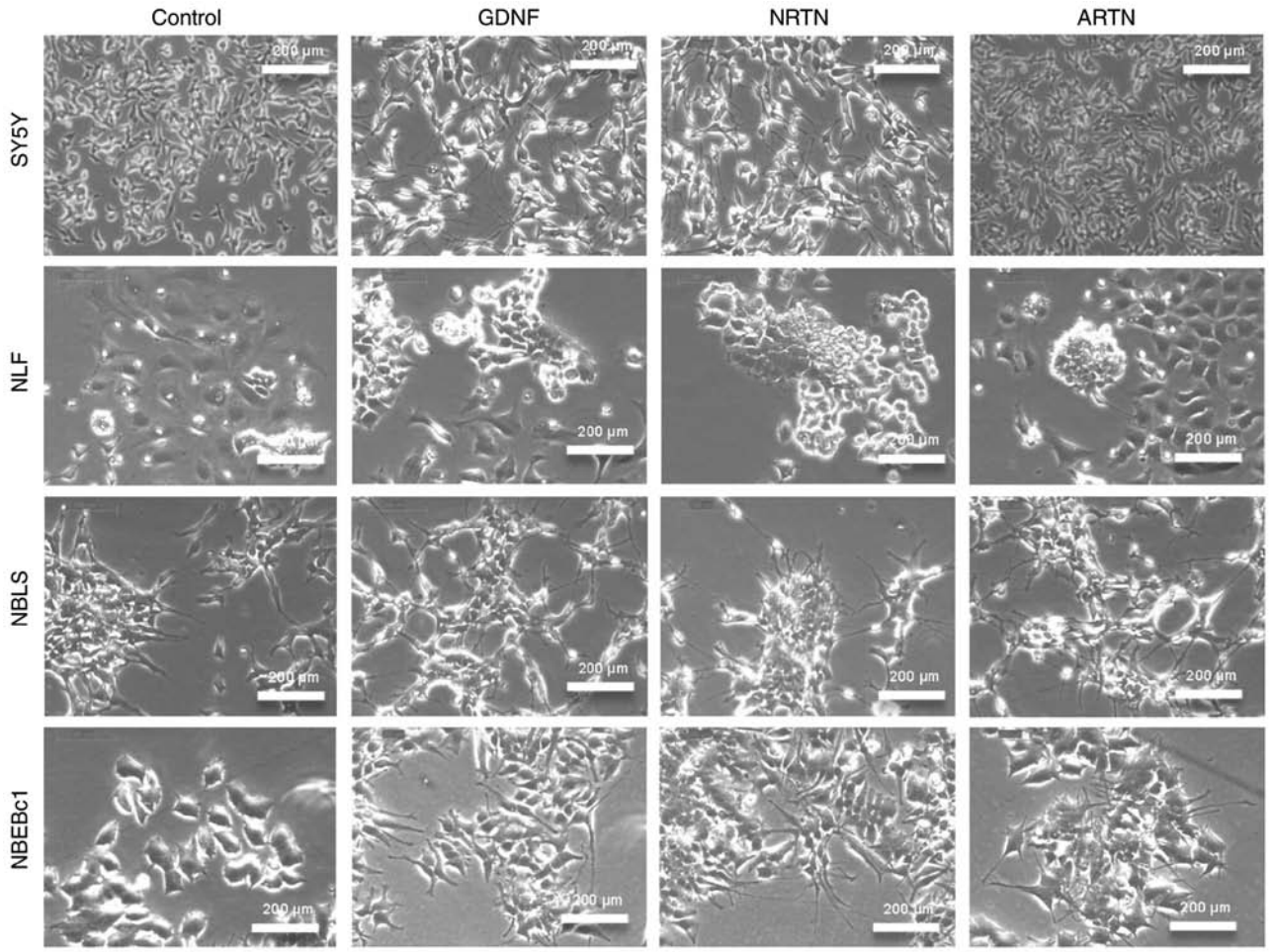

Figure 3. Representative phase contrast images depicting morphology change in response to GDNF, NTRN and ARTN ligands (6 days) in SY5Y, NLF, NBLS and NBEBc1 NB cell lines. Cells respond to ligand activation and show morphological changes, such as cell flattening, cell enlargement and neurite outgrowth, which is most pronounced in NBLS and SY5Y, modest in NBEBc1 and not observed in NLF. NB, neuroblastoma; GDNF, glial cell line-derived neurotrophic factor; ARTN, artemin; NRTN, neurturin.

changes characteristic of differentiation (Fig. 3 and Table I). Neurite outgrowth was observed in NBLS treated with GDNF, NRTN and ARTN, and by 6 days the most dramatic morphologic changes were seen in NRTN- and ARTN-treated cells. NB-EBc1 cells treated with NRTN and ARTN had short neurites at 3 days, and longer, more complex neurites in 6 days. The morphology of the NBLS and NB-EBc1 without ligand exposure did not change at 6 days. These results suggest that there was clear morphological differentiation upon ligand induction in cell lines expressing RET and the corresponding co-receptor.

Effect of CEP-701 on RET expression. Since differential expression of RET and TRK in SY5Y and NBLS cells was observed, the effect of TRK inhibition with CEP-701 was examined. SY5Y is RET positive and TRK null; and NBLS expresses both RET and TRK. These cells were treated with 100 nM CEP-701 for 1 h before adding ARTN (50 ng/ml). To examine possible TRK or RET inhibition by CEP-701, western blot analysis with phospho-specific antibodies was performed. Results indicated that CEP-701 significantly inhibited both TRK and RET activation, and there was reduced RET phosphorylation at all three residues (Y905, Y1062 and Y490) in NBLS and SY5Y cells (Fig. 4A). Interestingly, ARTN treatment activated TRK at tyrosine 490 in NBLS cells, and this was completely inhibited by CEP-701 (Fig. 4A). However, there was no effect of RET ligands on NLF, which is had no RET expression (Fig. 1).

In order to determine the most effective dose of CEP-701 against both RET and TRK, we treated NBLS cells with CEP-701 at 0, 10, 50, 100, 200 and 400 nM concentrations, and performed western blot analysis. Reduced phosphorylation at all three tyrosine residues was observed in a dose dependent manner. CEP-701 inhibited TRK at lower concentrations compared with those required for RET inhibition. RET phosphorylation was almost completely inhibited at 200 nM, whereas TRK was completely inhibited at 50 nM (Fig. 4B). These results suggest that RET expression is inhibited by CEP-701, presumably through inhibition of TRK activation, and TRK activation by ARTN requires RET activation.

RET and TRK receptor interactions. Since we observed TrkA phosphorylation upon exposure of NBLS cells to ARTN, which was inhibited by CEP-701 (Fig. 4A), if RET could be activated by exposure of TRK-expressing cells to their cognate ligands

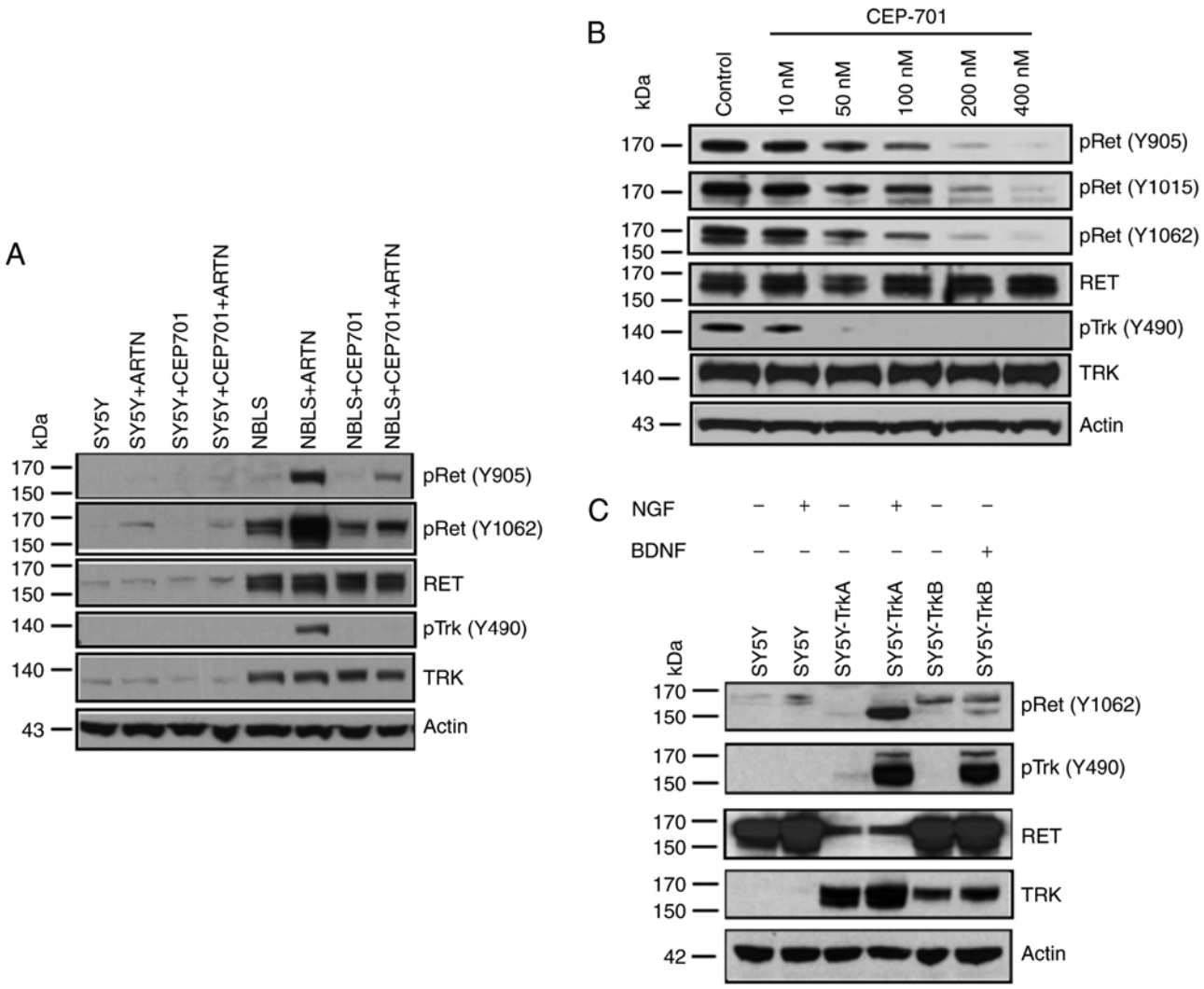

Figure 4. Effect of RET/TRK inhibition by lestaurtinib (CEP701). (A) Inhibition of RET expression by CEP701. SY5Y and NBLS cells were treated with 100 nM CEP701 and/or ARTN, and analyzed for the expression of RET, phospho-RET, TRK, and phospho-TRK. SY5Y cells showed modest induction of phospho-RET by ARTN compared with untreated cells, but the effect of CEP701 could not be assessed due to the lack of TRK expression. NBLS cells showed strong RET activation and TRK activation, and this activation was inhibited by CEP701. (B) Dose dependent inhibition of RET expression by CEP701. NBLS cells were induced with the RET ligand ARTN and inhibited using increasing concentrations of CEP701. Dose dependent inhibition of RET (and TrkA) was observed upon CEP701 exposure. (C) RET phosphorylation in response to TRK ligands. SY5Y (TRK-null), SY5Y-TrkA and SY5Y-TrkB cells were exposed to their cognate ligands (NGF for TrkA, BDNF for TrkB), and phospho-RET, total RET, phospho-TRK, and total TRK expression was measured. RET is phosphorylated strongly by NGF exposure to SY5Y-TrkA, and modestly by BDNF exposure to SY5Y-TrkB.

was investigated next. There was no effect on RET by adding NGF to TRK-null SY5Y cells (Fig. 4C). However, even though RET expression was lower in SY5Y-TrkA cells compared with NBLS cells, it was strongly phosphorylated by adding NGF, and phosphorylated less by adding BDNF to SY5Y-TrkB cells. RET phosphorylation occurs in response to TrkA activation by NGF, and TrkA is phosphorylated in response to RET activation by ARTN, which suggests that RET and TrkA (and possibly TrkB) physically interact and can induce reciprocal activation in response to ligand activation, but this needs to be investigated further.

Association of RET expression with its co-receptors and with TRK genes. Next, mRNA expression of *RET* with its co-receptors and with *TRK* genes (*NTRK1*, *NTRK2*) in a large series of primary NBs was assessed. Data from 249 primary NBs from the NB TARGET Initiative (ocg.cancer.gov/programs/target/projects/neuroblastoma) was analyzed (Fig. 5). There was no correlation between *RET* expression and *GFRα1*, but there was a significant positive correlation between *RET* and *GFRα2*, and a significant negative correlation between *RET* and *GFRα3*; *GFRα4* was expressed at very low levels in all tumors. Interestingly, there was an inverse correlation between *RET* and *NTRK1* expression, but positive correlation between RET and NTRK2. *MYCN* amplification status did not correlate with GFR co-receptors, but *NTRK1* expression was significantly lower in tumors with *MYCN* amplification. These correlations were validated in additional expression datasets (2,579 cases total), and the same correlations were seen in all datasets (Fig. 6). In addition, age vs candidate Log2 FPKM gene expression was compared in patients <18 months and >18 months and identified RET, GFR α2 and NTRK1 expressions as significant (Fig. S1).

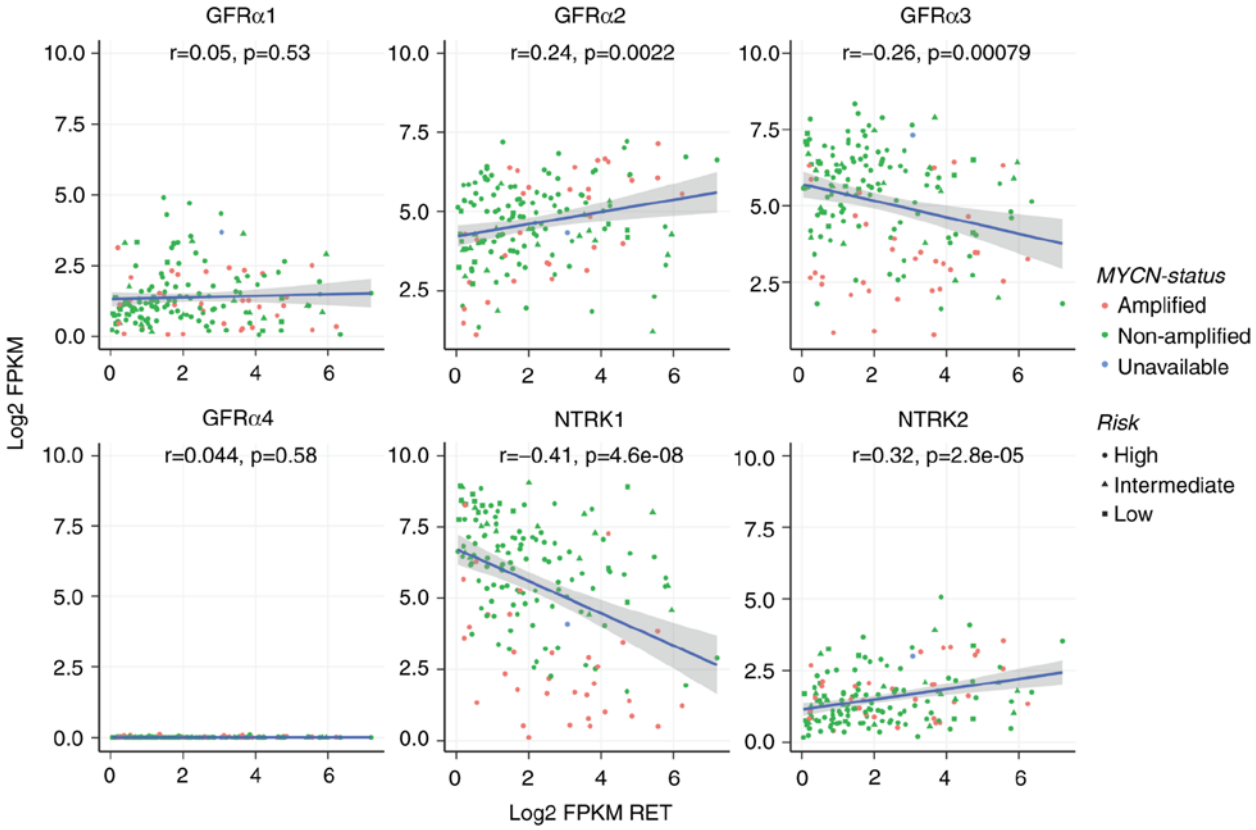

Figure 5. Correlation between RET expression with RET co-receptor and TRK genes. Scatter plot of the Log2 FPKM mRNA expression of RET vs. GFRα1, -α2, -α3, and -α4, as well as NTRK1 and NTRK2 for all neuroblastoma samples with RNA sequencing data. Data from 249 primary NBs from the NB TARGET Project (ocg.cancer.gov/programs/target/projects/neuroblastoma) were included in the analysis. NTRK, neurotrophic tyrosine receptor kinase.

Discussion

In the present study, it was demonstrated that RET and its co-receptors are expressed in some but not all NB cell lines. It was also demonstrated activation of RET with GDNF, NRTN and/or ARTN in the RET positive cell lines examined. RET activation by cognate ligands demonstrates intact RET function in NB cell lines that is correlated with expression of the respective GFR co-receptor.

Cell lines with the highest RET expression also had the highest GFRα3 expression. NLF, a RET null line, had very low GFRα3 expression and yet was unresponsive to ARTN. This suggests that ARTN activation of RET is primarily through GFRα3 and requires RET expression in NB. Baloh *et al* (55) first identified ARTN as a survival factor for sympathetic, sensory and mid-brain neurons. Shortly after this, Masure *et al* (56) described ARTN under a different name, enovin or neublastin, as inducing neurite outgrowth in SY5Y cells and rescuing them from the toxic effects of taxol. In the present study, morphologic evidence of neuronal differentiation as well as significant neurite outgrowth was noticed in SY5Y and NBLS when treated with NRTN and ARTN. Several reports have also suggested a role of GDNF, GFRα1 and RET in NB differentiation (9,26-33). Others have shown that RET is essential for retinoic acid induced differentiation (9,27,32). Finally, Hishiki *et al* (57) suggested a role for NRTN and GDNF in neurological differentiation.

The intensity of phosphorylation of RET by different ligands correlated well with co-receptor mRNA and protein expression in the present study. In particular, the expression of GFRα3 and RET activation by ARTN were quite consistent with what was expected. Studies by others have shown that GFRα1 forms a physical complex with RET upon GDNF stimulation by recruiting RET into lipid rafts (58-61). Based on sequence and functional homology between GFRα1 and GFRα3 and what is known about the mechanism of interaction between GFRα1 and RET, it could be hypothesized that GFRα3 also recruits RET into lipid rafts in a similar manner. PSPN and GFRα4 were not examined in detail, as their mRNA and protein expression were very low/absent in the NB lines, and the literature suggests that PSPN does not play an important role in the sympathetic nervous system (55).

Previous studies have shown RET activation with TRK ligands (37,38), but there are no published studies that show TRK activation with RET ligands. The present study demonstrated that treatment of NBLS, a TRK and RET positive cell line, with the RET ligand ARTN resulted in phosphorylation and activation of TRK, as well as RET. However, RET is not activated by NGF in the TRK-null SY5Y line, suggesting TrkA expression is required. Inhibition of both RET and TRK activation in the presence of a TRK inhibitor CEP-701. Was shown. Two other groups have suggested that RET and TRK crosstalk is essential for NB differentiation (37,38). Further studies into RET and TRK interactions may provide a more

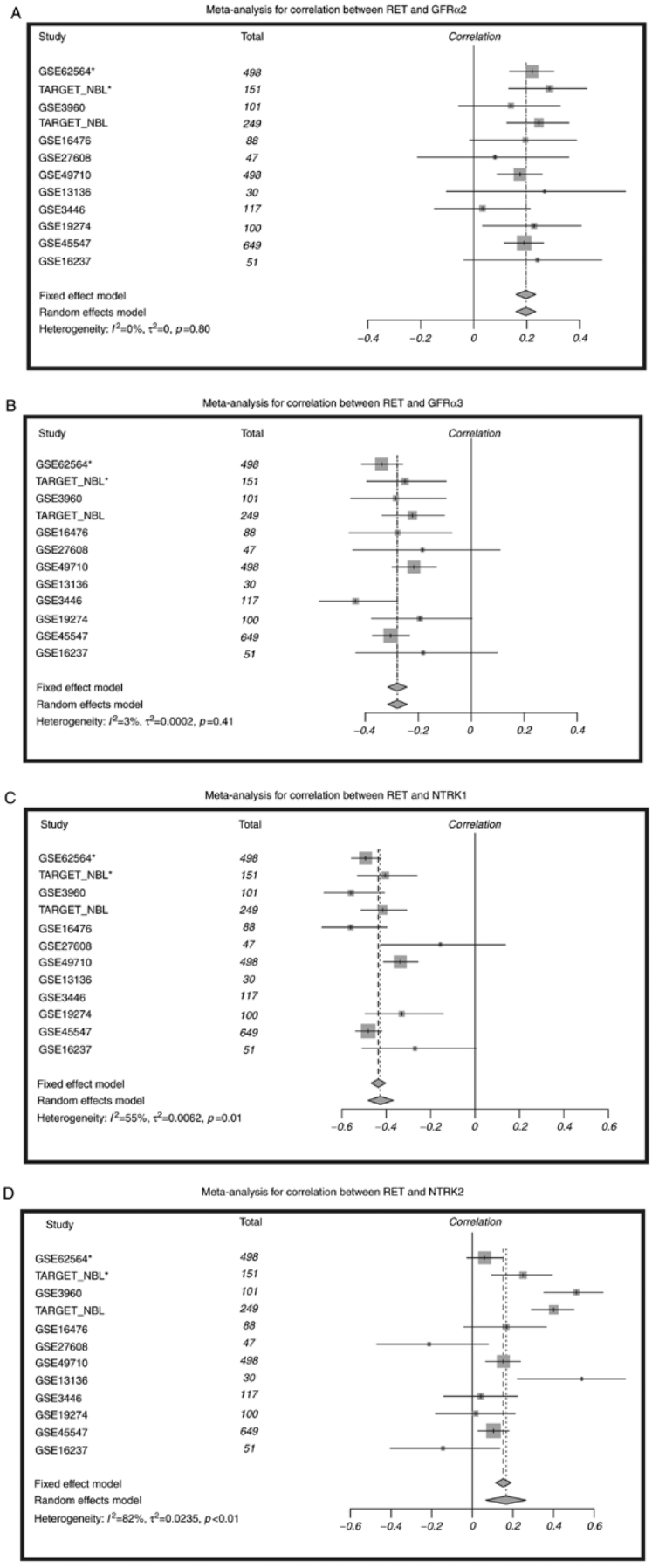

Figure 6. Correlation analysis between RET, GFR and TRK gene expression by meta-analysis. Forest plots displaying the meta-analysis of estimated Pearson's correlation of (A) RET with GFRα2; (B) RET with GFRα3; (C) RET with NTRK1 and (D) RET with NTRK2, from 12 publicly available NB microarray expression and RNA-sequencing studies. Studies with an asterisk represent RNA sequencing data. The remaining studies represent gene expression microarray data. NTRK, neurotrophic tyrosine receptor kinase.

insight into the nature of the interaction between RET and TRK pathways.

In conclusion, the expression and function of RET and its co-receptors in NB cell lines was investigated. RET activation may play a role in differentiation of NB cell lines, and uninhibited RET and TRK activation are essential for survival and growth of NB cells *in vitro*. It was shown that TRK activation by ARTN may occur through a RET-dependent mechanism, and RET activation by TrkA in response to NGF. It has been suggested that RET and TrkB expression are both required for differentiation induced by retinoic acid, a compound known to induce differentiation in NB (37). The same study showed that RET can be activated by BDNF (37). NGF, the ligand associated with TrkA, has been shown to induce RET phosphorylation and the activation of RET and TrkA can also induce NB differentiation (38,62). Understanding the importance of RET and its interactions with TRK for differentiation and survival of NB may provide new therapeutic avenues involving this complex signaling pathway.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request. The transfected lines are available from Kerafast (https://www.kerafast.com/contactus). Data from 249 primary NBs from the NB TARGET Project (ocg.cancer.gov/programs/target/projects/neuroblastoma) were included in the analysis.

Authors' contributions

LHT, RLG, RI, JLC, JHC, KN, PG and FN performed the experiments. KSG, PR performed the statistical analysis. LHT, VK, JLC, SPM and GMB designed the present study and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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