Neurotrophin receptors are emerging targets in oncology, but their clinicopathologic significance in thyroid cancer is unclear. In this study, the neurotrophin tyrosine receptor kinase TrkA (also called NTRK1), the common neurotrophin receptor p75NTR, and the proneurotrophin receptor sortilin were analyzed with immunohistochemistry in a cohort of thyroid cancers ($n = 128$) and compared with adenomas and normal thyroid tissues ($n = 62$). TrkA was detected in 20% of thyroid cancers, compared with none of the benign samples ($P < 0.0007$). TrkA expression was independent of histologic subtypes but associated with lymph node metastasis ($P = 0.0148$), suggesting the involvement of TrkA in tumor invasiveness. Nerves in the tumor microenvironment were positive for TrkA. p75NTR was overexpressed in anaplastic thyroid cancers compared with papillary and follicular subtypes ($P < 0.0001$). Sortilin was overexpressed in thyroid cancers compared with benign thyroid tissues ($P < 0.0001$). Neurotrophin receptor expression was confirmed in a panel of thyroid cancer cell lines at the mRNA and protein levels.

Functional investigations using the anaplastic thyroid cancer cell line CAL-62 found that siRNA against TrkA, p75NTR, and sortilin decreased cell survival and cell migration through decreased SRC and ERK activation. Together, these data reveal TrkA, p75NTR, and sortilin as potential therapeutic targets in thyroid cancer. (Am J Pathol 2018, 188: 229–241; https://doi.org/10.1016/j.ajpath.2017.09.008)
found, suggesting a role in pathogenesis. However, the status of NGF/proNGF receptors in thyroid tissue is not clear. ProNGF binds to a complex between the membrane protein sortilin and the neurotrophin receptor p75<sub>NTR</sub>, initiating various signaling pathways, including NF-κB, RhoA, and JNK. In addition, proNGF has also been found to activate the neurotrophin tyrosine receptor kinase 1 (NTRK1 or TrkA). In thyroid cancer, the expression of sortilin has never been reported, and although p75<sub>NTR</sub> and TrkA are expressed, their clinicopathologic significance is not defined.

In this study, we found the increased expression of TrkA, p75<sub>NTR</sub>, and sortilin in a cohort of thyroid cancers compared with benign thyroid tissues (adenomas and normal thyroid tissues). Furthermore, in vitro experiments revealed that targeting these neurotrophin receptors resulted in a decreased growth and invasion of ATC cells, suggesting a potential utility as therapeutic targets.

**Materials and Methods**

**Thyroid Tissue Samples**

High-density tumor microarrays (TH801, TH641, TH8010) were obtained from US Biomax Inc. (Rockville, MD) and included 128 thyroid cancers (79 PTCs, 27 FTCs, 12 ATCs, 10 other subtypes), 6 adenomas, and 56 normal thyroid tissues. The other subtypes were follicular papillary carcinomas (n = 6), sarcomatoid carcinomas (n = 1), and MTCs (n = 3). Within the 27 cases of FTC, 3 cases were characterized as poorly differentiated, and 5 were characterized as moderately differentiated. The following information was available: patient age and sex, histologic subtype, tumor size, lymph node status, and stage. US Biomax Inc. quality controls are described as follows. Each single tissue spot on every array slide is individually examined by pathologists certified according to World Health Organization published standardizations of diagnosis, classification, and pathologic grade. Each specimen collected was consented to by both hospital and individual. Discrete legal consent was obtained, and the rights to hold research uses for any purpose or further commercialized uses were waived. The study was approved by the Human Research Ethics Committee of the University of Newcastle, Australia.

**Immunohistochemistry and Digital Quantification**

Immunohistochemistry and digital quantification of staining intensities were performed as previously described. Antibodies against TrkA (1/200 dilution, catalog number 2508; Cell Signaling Technology, Danvers, MA), p75<sub>NTR</sub> (1/400 dilution, catalog number 4201; Cell Signaling Technology), sortilin (0.8 μg/mL, catalog number ANT-009; Alomone Labs, Jerusalem, Israel), or PGP9.5 (1/200 dilution, catalog number ab15503; Abcam, Cambridge, United Kingdom) were applied. Negative controls with a rabbit monoclonal antibody IgG Isotype Control (DA1E, catalog number 3900; Cell Signaling Technology) were also performed (Supplemental Figure S1A). The specificity of the antibodies used for immunohistochemistry was assessed using Western blot analysis in a panel of thyroid tumor tissue samples (Supplemental Figure S1B). Thyroid tumor samples included one FTC and three PTCs. Specificity was also confirmed in the PC-12 cell line, which is known to express neurotrophin receptors (Supplemental Figure S1C). TrkA, p75<sub>NTR</sub>, and sortilin were all found to be expressed at their expected molecular weights (140 kDa for TrkA, 75 kDa for p75<sub>NTR</sub>, and 100 kDa for sortilin) in both the thyroid tumor tissue samples (Supplemental Figure S1B) and PC-12 cells (Supplemental Figure S1C). For quantification of p75<sub>NTR</sub> and sortilin staining intensity, pixel intensity values were used to determine the h-scores for each core (index calculated as the sum of 3 × percentage of pixels with strong staining + 2 × percentage of pixels with intermediate staining + 1 × percentage of pixels with weak staining). Staining intensities were categorized as negative (h-score < 25), low (h-score of 25 to 100), or high (h-score > 100). For TrkA, because of the limited proportion of cells that tested positive for TrkA, it was not possible to calculate a representative h-score; therefore, positivity versus negativity for TrkA staining was recorded. For statistical analyses, staining intensities for p75<sub>NTR</sub>, sortilin, and positivity or negativity for TrkA, were compared with clinicopathologic parameters: normal versus malignant, patient age and sex, histologic type, tumor size, lymph node status, stage, and grade. Simple unadjusted associations with pathologic variables were performed using a χ² test with SAS statistical software version 9.4 (SAS Institute, Cary, NC).

**Cell Culture**

Thyroid cancer cell lines CAL-62 (ATC), BCPAP (PTC), and ML-1 (MTC) were purchased from DSMZ (Braunschweig, Germany), which uses STR verification of cell line authenticity. The TPC-1 (PTC) cell line was obtained from Dr. Mareel’s laboratory (University of Gent, Gent, Belgium). The 8505c (ATC) cell line was a generous gift from Prof. Alfred Lam (Griffith University, Queensland, Australia). TPC-1 cell authenticity was validated using the GenePrint 10 System (catalog number B9510, Promega, Madison WI). PC-12 cells were obtained from Prof. Ralph A. Bradshaw (University of California, San Francisco, CA). All cell lines were maintained in RPMI-1640 with 10% (v/v) fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS) and 2 mmol/L l-glutamine in a humidified incubator at 37°C with 5% (v/v) CO₂. Routine Mycoplasma testing was performed using the MycoAlert Mycoplasma Detection Kit (catalog number LT07-118; Lonza, Basel, Switzerland). Cells were not maintained in culture for longer than 3 months to ensure passage number remained fit for purpose.
Preparation of Conditioned Media

Conditioned media was prepared as previously described. Briefly, $5 \times 10^6$ cells were seeded per T-75 cm$^2$ culture flask and grown in 10 mL of serum free media for 24 hours. The collected medium was centrifugated (800 × g for 5 minutes at 4°C) and the supernatant was concentrated and desalted using 10-kDa cut-off Amicon Ultra-15 filtration unit (catalog number UFC900324; Merck Millipore, Darmstadt, Germany) for 30 minutes (4000 unit (catalog number UFC900324; Merck Millipore). The recovered concentrate was stored at −80°C.

Protein Extraction and Western Blotting

Protein extraction from cell lines and Western blotting experiments were performed as previously described. For protein extraction from thyroid tumors, thyroid tumor tissue samples (obtained from the biobank of the Department of Endocrinology, John Hunter Hospital, Newcastle, Australia) were snap frozen with liquid nitrogen, crushed using a mortar and pestle, and transferred to individual 1.5 mL Precellys CK28 Lysing Tubes (catalog number KT03961-1-007.2; Bertin Technologies, Montigny-le-Bretonneux, France). Samples were lysed on ice using 1 mL of SDS extraction buffer (2% SDS, 1% IGEPAL, 0.5% sodium deoxycholate, 50 mmol/L Tris pH 7.5, 5 mmol/L EDTA). Complete mini-protease inhibitor cocktail tablets (catalog number 4906837001; Roche) were also used. Samples were homogenized in a Precellys 24 Homogenizer (Bertin Technologies) at 1500 × g (6 × 30 seconds intervals). Finally, samples were centrifuged at 16,000 × g for 15 minutes at 4°C, and the supernatant was extracted and stored at −80°C. Anti-TrkA (catalog number ANT018; Alomone Labs), anti-p75NTR (catalog number sc-6188; Santa Cruz Biotechnology, Dallas, TX), and anti-sortilin (catalog number ANT009; Alomone Labs) antibodies were used at a dilution of 1:500. Anti-proNGF (catalog number ab9040; Merck Millipore) and anti-NGF (catalog number sc-548; Santa Cruz Biotechnology) antibodies were used at a dilution of 1:200. β-Actin detection (1/5000 dilution, catalog number A2066; Sigma-Aldrich, St. Louis, MO) was used as the equal loading control. Antibodies from Cell Signaling Technology were used to assess cellular signaling pathways: anti–phospho-TrkA (anti–p-TrkA) (Tyr940, catalog number 9141), anti-Src (catalog number 2100), anti–phospho-Src (anti–p-Src) (Tyr416, catalog number 2101), anti–Erk1/2 (catalog number 9107), and anti–phospho–Erk1/2 (anti–p–Erk1/2) (Thr202/Tyr204, catalog number 4370).

Real-Time RT-PCR

Total RNA was isolated from thyroid cancer cell lines using the illustra RNAspin Mini RNA Isolation Kit (catalog number 25-0500-70; GE Healthcare Life Sciences, Little Chalfont, UK). Reverse transcription was performed with 1 µg of total RNA using the iScript cDNA Synthesis Kit (catalog number 1708890; Bio-Rad Laboratories Inc., Hercules, CA). Real-time PCR was performed using 2 µL of 1/10 cDNA using iTaq Universal SYBR Green Supermix (catalog number 172-5120; Bio-Rad Laboratories Inc.). The primers used were as follows: sortilin primers were Quantitect Primer Assay QT00073318 (Qiagen, Venlo, Netherlands); p75NTR primers were 5’-ACGG CTACTACCAGGATGAG-3’ (forward) and 5’-TGCCCTCG TCGGAATACGTG-3’ (reverse) (Sigma-Aldrich); TrkA primers were Quantitect Primer Assay QT00054110 (Qiagen); primers used for the reference gene GAPDH were 5’-CAC CAGGCGCTGCTTTTAACTCCTGTA-3’ (forward) and 5’-CCTTGACGGTGCCATGGAATTTG-3’ (reverse) (Sigma-Aldrich). PCR was performed in a ABI7500 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Wal- tham, MA) using the following conditions: 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 60 seconds followed by a continuous Melt curve from 65°C to 95°C. Data were analyzed using the ABI7500 Real-Time software version 2.3 (Applied Biosystems, Thermo Fisher Scientific). Relative expression was obtained using the 2$^{-ΔΔCt}$ method.

Transfection with siRNA

ATC cell lines CAL-62 and 8505c were transfected for 72 hours with 15 nmol/L of siRNA using lipofectamine RNAiMAX (catalog number 13778150; Life Technologies, Carlsbad, CA), according to the manufacturer’s recommendations. Cells were seeded in 6-well plates and transfected 24 hours later with siRNA against TrkA (siTrkA, CGAGAACCCCA CAUAACUCUCAGUGAT, catalog number SR303249; OriGene Technologies Inc., Rockville, MD), p75NTR (siP75NTR, GGAUUUGACUUCUGACUGACUG, catalog number SR303174; OriGene Technologies Inc.), and sortilin (siSort, CUCUGCUUAAACACACC[dt][dT]; Sigma-Aldrich) as well as a commercially available siRNA control sequence: MISSION siRNA Universal negative control 1 (catalog number SIC001; Sigma-Aldrich). The efficiency of TrkA, p75NTR, and sortilin knockdown was assessed using Western blot analysis at 24, 48, and 72 hours after transfection, using anti-TrkA (catalog number ANT018; Alomone Labs), anti-p75NTR (catalog number sc-6188; Santa Cruz Biotechnology), and anti-sortilin (catalog number ANT009; Alomone Labs) antibodies, respectively. β-Actin (catalog number A2066; Sigma-Aldrich) was used as an equal loading control.

Cell Growth and Apoptosis Assay

A total of $7 \times 10^5$ ATC cells (CAL-62 and 8505c) were seeded in 2 mL of culture medium (RPMI containing 10% FCS and 2 mmol/L L-glutamine) on 6-well plates and were allowed to adhere overnight in a humidified incubator at 37°C and 5% CO$_2$. Cells were transfected the following day with siTrkA, siP75NTR, siSort, a combination of the three
siRNAs (siCombo), or control siRNA (siCont). Images were obtained after 72 hours, and viable cell number was counted using Trypan Blue with a hemocytometer. For assessing apoptosis, 1 x 10^6 cells were incubated with 100 μL of Muse Annexin V and Dead Cell Reagent (catalog number MCH100105; Merck Millipore, Darmstadt, Germany) for 20 minutes at room temperature as per the manufacturer’s instructions. The Muse Cell Analyzer (Merck Millipore) was used to determine apoptosis, and the statistics obtained revealed the percentages of the cells represented by alive, apoptotic, and dead populations.

Cell Migration and Wound-Healing Assay
A total of 5 x 10^5 ATC cells (CAL-62 and 8505c) were seeded on 6-well plates in complete growth medium (RPMI containing 10% FCS and 2 mmol/L L-glutamine) and were allowed to adhere overnight in a humidified incubator at 37°C and 5% CO2. Cells were transfected the following day with siTrkA, siP75NTR, siSort, a combination of the three siRNAs (siCombo), or control siRNA (siCont). After 72 hours, during which the cells were allowed to grow to confluence, the cell monolayer was scratched with a 200-μL pipette tip, rinsed three times with phosphate-buffered saline to remove floating cellular debris, and replaced with media that contained 0.1% (v/v) FCS. The wound area that resulted from the scratch was monitored using the JuLI Stage automated cell imaging system (NanoEnTek Inc., Seoul, Korea). Images were taken automatically every 5 hours during a 20-hour postscratch period. Results are shown as the percentage reduction of the wound area, measured using ImageJ version 1.60_20 (NIH, Bethesda, MD; http://imagej.nih.gov/ij).

Cell Invasion Assay
Cell invasion assays were performed on CAL-62 and 8505c ATC cells using the QCM ECMatrix Cell Invasion Assay (catalog number ECM554; Merck Millipore), which is made up of 24-well plates and contains upper invasion chamber inserts with 8-μm pore size membranes. The extracellular matrix layer was rehydrated with 300 μL of prewarmed serum free media for 30 minutes at room temperature. Cells were loaded into the invasion chamber insert using 2 x 10^5 siRNA transfected cells (72 hours after transfection with siTrkA, siP75NTR, siSort, siCombo, and siCont.) in 250 μL of media that contained 0.1% (v/v) FCS. Five hundred microliters of media, in the presence of a chemoattractant (10% FCS), was added to the lower chamber. After a 20-hour incubation period, the upper invasion chamber inserts were rinsed with phosphate-buffered saline, and cells at the upper surface of the membranes were gently scraped and removed. Invading cells were dislodged by placing the invasion chambers in 225 μL of pre-warmed cell detachment buffer before being stained by CyQuant GR Dye (1:75 with 4x lysis buffer) for 15 minutes. Two hundred microliters of the mixture from each sample was transferred to a black 96-well plate (catalog number CLS3792-100; Sigma-Aldrich), and the fluorescence was recorded at 480/520 nm using a FLUOstar OPTIMA fluorescence plate reader (BMG Labtech, Durham, NC). Samples without cells but containing cell detachment buffer, lysis buffer, and CyQuant Dye were used as blanks, and background fluorescence was subtracted from all samples. The number of invading cells was determined by running a fluorescent cell dose standard curve.

Statistical Analysis for in Vitro Assays
For cell proliferation, apoptosis, and migration and invasion assays, each condition was performed at least in triplicate, and experiments were repeated on three separate occasions. Statistical analysis was conducted using the GraphPad Prism software version 6 (GraphPad Software Inc., La Jolla, CA). Cell proliferation, apoptosis, migration, and invasion assays

Figure 1  TrkA expression in thyroid cancers. A–F: TrkA immunostaining was performed on a cohort of thyroid cancers, adenomas, and normal thyroid tissues. TrkA is not observed in normal thyroid tissues (A) or adenomas (B). In contrast, TrkA staining is found in papillary thyroid carcinomas (C and D), follicular thyroid carcinoma (E), and anaplastic thyroid carcinoma (F) histologic subtypes. Only a small proportion of cancer cells are positive for TrkA. Recording of TrkA positivity is reported in Table 1. n = 128 thyroid cancers, 6 adenomas, and 56 normal thyroid tissues. Scale bars: 50 μm. Original magnification, ×400.
Results

The expression of TrkA, p75NTR, and sortilin was analyzed by immunohistochemistry in a series of 128 thyroid cancers of various histologic types, six adenomas, and 55 normal thyroid tissues. In addition, these neureotrophin receptors were detected in thyroid cancer cells in culture, and the functional impact of their targeting using siRNA was investigated.

Gene Versus Protein Expression

We initially started with data mining of gene expression, using cBioportal, of thyroid data sets in The Cancer Genome Atlas database. The results indicated that mRNA abundance in tumors does not reliably predict differences in protein levels. Therefore, we proceeded with analyzing protein expression.

Table 1. TrkA, p75NTR, and Sortilin Expression in Thyroid Cancers and Associations with Clinicopathologic Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal versus cancer</th>
<th>Cancer histologic subtype</th>
<th>Sex</th>
<th>Age, years</th>
<th>Tumor size (T)</th>
<th>Lymph node (N)</th>
<th>Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>TrkA intensity</td>
<td>Negative, Total (%)</td>
<td>Positive, Total (%)</td>
<td>P value</td>
<td>Negative, Total (%)</td>
<td>Positive, Total (%)</td>
<td>P value</td>
<td>Negative, Total (%)</td>
</tr>
<tr>
<td>Normal (n = 56)</td>
<td>56 (100) 0 (0)</td>
<td>6 (11) 49 (87) 1 (2)</td>
<td>&lt;0.0001</td>
<td>56 (100) 0 (0) 0 (0)</td>
<td>4 (67) 2 (33) 0 (0)</td>
<td>&lt;0.0001</td>
<td>48 (38) 79 (61) 1 (1)</td>
</tr>
<tr>
<td>Adenoma (n = 6)</td>
<td>6 (100) 0 (0)</td>
<td>0 (0) 6 (100) 0 (0)</td>
<td>&lt;0.0001</td>
<td>7 (26) 20 (74) 5 (19)</td>
<td>9 (75) 3 (25) 0 (0)</td>
<td>0.0415</td>
<td>6 (60) 4 (40) 0 (0)</td>
</tr>
<tr>
<td>Cancer (n = 128)</td>
<td>102 (80) 26 (20)</td>
<td>3 (2) 57 (45) 68 (53)</td>
<td>0.1020</td>
<td>26 (33) 52 (66) 1 (1)</td>
<td>9 (75) 3 (25) 0 (0)</td>
<td>0.8201</td>
<td></td>
</tr>
</tbody>
</table>

Immunohistochemical staining for each neurotrophin receptor was quantified. For TrkA, because only a small proportion of cancer cells were positive for TrkA, immunohistochemical staining in each sample was categorized only as negative versus positive. Representative images for TrkA staining are presented in Figure 1. For p75NTR and sortilin, immunohistochemical staining in each sample was digitally quantified and categorized as negative (h-score <25), low staining (h-score of 25 to 100), or high staining (h-score >100). Representative images for p75NTR and sortilin staining are presented in Figures 3 and 4, respectively. Statistically significant P values (P < 0.05, using the chi-square test) are shown in bold.
TrkA positivity was found in 20% of thyroid cancers compared with none of the benign samples \((P = 0.0007)\). An association was found between TrkA expression and lymph node metastases, with TrkA positivity observed in 41% of lymph node–positive cancers compared with only 13% of lymph node–negative cancers \((P = 0.0148)\). There was no association between TrkA expression and other clinicopathologic parameters (sex, age, tumor size, and stage).

Some staining for TrkA also appears to be in endothelial and other stromal cells. In other models, endothelial cells\(^{19}\) and fibroblasts\(^{20}\) have been reported to express TrkA; therefore, it is not unexpected to find some TrkA expression in stromal cells. Interestingly, TrkA expression was also detectable in nerves present in thyroid cancers (Figure 2). The presence of nerves in the tumor microenvironment was validated using the neuronal marker PGP9.5 (Figure 2, A and E). TrkA staining was observed in nerves (Figure 2, B and F), whereas p75\(^{NTR}\) (Figure 2C) and sortilin (Figure 2D) were not detected in serial tumor microarray cores.

### p75\(^{NTR}\) Expression in Thyroid Cancers

Expression of p75\(^{NTR}\) was detected in most benign thyroid tissues and cancers (Figure 3, A–F). Digital quantification of p75\(^{NTR}\) staining intensities (Figure 3G) indicated a median combined h-score of 57.86 for benign thyroid tissue compared with 102.5 for thyroid malignant tumors. Within cancer histologic subtypes, p75\(^{NTR}\) was expressed at higher levels in ATC, with a median h-score of 130.60 \((P < 0.0001)\) compared with 109.30 \((P < 0.0001)\) and 76.82 \((P = 0.0054)\) in PTC and FTC, respectively (Figure 3G). For analysis of the associations between p75\(^{NTR}\) expression and clinicopathologic parameters, p75\(^{NTR}\) staining intensities were categorized as negative \((h-score < 25)\), low \((h-score of 25 to 100)\), or high \((h-score > 100)\). The frequency distribution of staining intensities (Table 1) indicated that most normal tissues, adenomas, and cancers were positive for p75\(^{NTR}\), whereas only 0% to 2% of normal tissues and adenomas presented intense p75\(^{NTR}\) staining compared with 53% of cancers \((P < 0.0001)\).

By histologic subtypes, p75\(^{NTR}\) was expressed at higher levels in ATC, with high p75\(^{NTR}\) staining observed in 92% of ATC compared with 19% of FTC and 63% of PTC \((P < 0.0001)\). There was no association between p75\(^{NTR}\) expression and sex, age, tumor size, lymph node invasion, and stage (Table 1).

### Sortilin Expression in Thyroid Cancers

Sortilin was not detected in normal thyroid tissues (Figure 4A). In adenomas (Figure 4B), weak sortilin labeling was observed in epithelial cells of some tissue samples. Thyroid cancers were positive for sortilin, and the labeling was specifically observed in cancer cells (Figure 4, C–F). Digital quantification of staining intensities (Figure 4G) indicated that sortilin was higher in thyroid cancers compared with normal tissues and adenomas. Normal and adenoma thyroid tissues had a combined median h-score of 15.04 compared with 29.69 \((P < 0.0001)\) and 40.80 \((P < 0.0001)\) in the FTC and PTC cancer subtypes, respectively (Figure 4G). Sortilin expression in ATC yielded a median h-score of 24.11, which was not significantly different than benign thyroid tissues (normal and adenoma) (Figure 4G). For analysis of the association between sortilin expression and clinicopathologic parameters, sortilin staining intensities were categorized as negative \((h-score < 25)\), low \((h-score of 25 to 100)\), or high \((h-score > 100)\). The frequency distribution of staining intensities (Table 1) indicated that 62% of thyroid cancers were positive for sortilin compared with 33% of adenomas and 0% of normal tissues \((P < 0.0001)\). In terms of histologic subtypes, stronger sortilin staining was observed in FTC and PTC compared with ATC \((P = 0.0415)\). There was no association between sortilin expression and other clinicopathologic parameters (sex, age, tumor size, lymph node invasion, and stage).

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**Figure 2**  TrkA expression in nerves present in thyroid cancers. Immunohistochemical detection of the neuronal marker PGP9.5 was used to detect nerves in thyroid cancers, and the detection of TrkA, p75\(^{NTR}\), and sortilin was performed in serial sections. **A:** Nerves stained for the neuronal marker PGP9.5. **B–D:** Serial sections for the detection of TrkA (B), p75\(^{NTR}\) (C), and sortilin (D) in the nerve shown in A. **E:** Another nerve stained for PGP9.5. **F:** TrkA staining observed in a serial section of the nerve shown in E. Arrows indicate the location of nerves. Scale bars: 50 µm. Original magnification, ×400.
TrkA, p75NTR, and Sortilin Expression in Thyroid Cancer Cell Lines

Real-time RT-PCR and Western blot analysis were used to detect TrkA, p75NTR, and sortilin at the mRNA and protein levels in a series of thyroid cancer cell lines. The data (Figure 5) indicate that the three receptors are expressed in the four tested cancer cell lines (CAL-62, ML-1, BCPAP, and TPC-1). All three receptors were detected at their expected theoretical molecular mass: 140 kDa for TrkA, 75 kDa for p75NTR, and 100 kDa for sortilin. TrkA mRNA was present at higher levels in BCPAP, but again this difference

Figure 3  p75NTR expression in thyroid cancers. A–F: Immunohistochemical detection of p75NTR in thyroid cancers of various histologic types, adenomas, and normal thyroid tissues was performed. p75NTR was found in epithelial cells of normal tissue (A), adenomas (B), and thyroid cancers of various histologic subtypes: papillary thyroid carcinoma (PTC) (C and D), follicular thyroid carcinoma (FTC) (E), and anaplastic thyroid carcinoma (ATC) (F). Insets show higher magnification. G: p75NTR staining was quantified using the Halo image analysis platform; h-scores were calculated. p75NTR staining intensities are significantly higher for ATC (median h-score = 130.60), PTC (median h-score = 109.30), and FTC (median h-score = 76.82) compared with normal and adenoma thyroid tissues combined (median h-score = 57.86). Data are expressed as means (horizontal line in center of box), 25th and 75th percentiles (box limits), and interquartile ranges (whiskers) (G). n = 128 thyroid cancers, 6 adenomas, and 56 normal thyroid tissues. **P < 0.01, ****P < 0.0001 versus normal/adenoma tissue controls. Scale bars: 50 μm (A–F). Original magnification: ×50 (A–F, main images); ×400 (insets).

Figure 4  Sortilin expression in thyroid cancer versus normal thyroid tissue. A–F: Immunohistochemical detection of sortilin was performed on a cohort of thyroid cancers, adenomas, and normal thyroid tissues. Sortilin is not found in normal thyroid tissues (A) and in most adenomas (B). In contrast, sortilin staining is observed in cancer cells of papillary thyroid carcinoma (PTC) (C and D), follicular thyroid carcinoma (FTC) (E), and anaplastic thyroid carcinoma (ATC) (F). Insets show higher magnification. G: Sortilin staining was quantified using the Halo image analysis platform; h-scores were calculated. Sortilin staining intensities are significantly higher for PTC (median h-score = 29.69) and FTC (median h-score = 40.80) compared with normal and adenomas combined (median h-score = 15.04). Data are expressed as means (horizontal line in center of box), 25th and 75th percentiles (box limits), interquartile ranges (whiskers), and outliers (dots). n = 128 thyroid cancers, 6 adenomas, and 56 normal thyroid tissues. ****P < 0.0001 versus normal/adenoma tissue controls. Scale bars: 50 μm (A–F). Original magnification: ×50 (A–F, main images); ×400 (insets).
was not found at the protein level, and all cell lines had comparable levels of TrkA. For p75NTR, BCPAP cells expressed a higher level of mRNA than the other cell lines, but this difference was not found at the protein level, where p75NTR appears similar among cell lines. For sortilin, the mRNA and protein levels were similar among cell lines. The protein expression of NGF and its precursor proNGF was also examined in two ATC cell lines, CAL-62 and 8505c (Supplemental Figure S2). NGF and proNGF were detected in protein extracts from both cell lines. Furthermore, the conditioned culture media obtained from CAL-62 and 8505c cells revealed that proNGF was secreted by CAL-62 and 8505c cells, whereas NGF was secreted only by 8505c cells. The concomitant expression of TrkA, p75NTR, and sortilin with NGF and proNGF suggest the existence of an autocrine loop of stimulation in thyroid cancer cells.

Functional Impact of Targeting TrkA, p75NTR, and Sortilin

In vitro assays were performed on the highly aggressive CAL-62 (Figure 6). Cells were transfected with siRNA directed against TrkA, p75NTR, sortilin, and the impact on cell growth, apoptosis, migration, and invasion was assessed. The efficacy of the targeted siRNAs was determined using Western blot analysis at 24, 48, and 72 hours after transfection (Figure 6A). Sortilin protein expression was reduced after 24 hours and completely inhibited after 48 hours. However, maximal inhibition of TrkA and p75NTR expression was achieved 72 hours after transfection, indicating that it was necessary to evaluate the effects of the siRNAs after 72 hours. The combination of the three siRNA (siCombo) against TrkA, p75NTR, and sortilin similarly decreased the level of the three receptors 72 hours after transfection (Figure 6A).

Signaling Pathways

The effects of inhibiting TrkA, p75NTR, and sortilin on various tumorigenic and metastatic-related signaling pathways were explored using Western blot analysis (Figure 6A). p-TrkA was markedly decreased in response to siTrkA, siP75NTR, and siSort, suggesting that the activation of TrkA requires the presence of both p75NTR and sortilin. Total ERK1/2 and p-ERK1/2 were comparable for siCont, siP75NTR, and siSort; however, siTrkA resulted in a decreased level of p-ERK. Inhibition of TrkA, p75NTR, and sortilin all reduced the level of p-SRC. Targeting all three receptors simultaneously mirrored data obtained when targeting each receptor alone; however, the observed effect was not potentiated (Figure 6A).

Cell Growth and Apoptosis

CAL-62 cell growth was analyzed at 72 hours after transfection, and the effects of siTrkA, siP75NTR, siSort, and siCombo were compared with siCont-treated cells (Figure 6B). Treatment of cells with siTrkA, siP75NTR, p75NTR, and sortilin similarly decreased the level of the three receptors 72 hours after transfection (Figure 6B). Treatment of cells with siTrkA, siP75NTR,
siSort, and siCombo all resulted in a reduction in CAL-62 cell number compared with siCont treated cells ($P < 0.0001$). To further elucidate the effects of targeting TrkA, p75NTR, and sortilin, we determined the impact on apoptosis using Annexin V expression flow cytometry profiling (Figure 6C).

In comparison with siCont cells (7.5% apoptotic cells), the percentage of CAL-62 apoptotic cells increased after knockdown of TrkA (80.62%), p75NTR (63%), and sortilin (49.9%). Simultaneous knockdown of all three receptors resulted in 80.4% of cells entering apoptosis (Figure 6C).
Cell Migration and Invasion

Cellular migration was assessed using a scratch wound healing assay, in which a scratch was made to a cell monolayer to create a wound area. The rate of closure was monitored and quantitated (Figure 6D). Transfection with siTrkA \((P = 0.0002)\), siP75NTR \((P = 0.0019)\), siSort \((P < 0.0001)\), and siCombo \((P = 0.0002)\) all inhibited the migration of CAL-62 cells compared with siCont. The invasiveness of CAL-62 cells, in response to transfection with siRNA against TrkA, p75NTR, and sortilin or the combination (siCombo), was assessed using a Transwell assay (Figure 6E). Invasion of CAL-62 cells was significantly inhibited by siTrkA \((P = 0.0085)\), siSort \((P = 0.001)\), and siCombo \((P = 0.0021)\) compared with siCont cells. In contrast, knocking down p75NTR expression did not significantly alter the invasion of CAL-62 cells \((P = 0.1268)\).

The 8505c ATC cell line was also analyzed (Supplemental Figure S3C). The impact of siRNA against the three receptors, alone or in combination, was studied following the same experimental procedures as for CAL-62. Similar results were obtained with 8505c cells compared with the CAL-62 cells, with respect to the decreased cell growth (Supplemental Figure S3B), apoptosis (Supplemental Figure S3C), migration (Supplemental Figure S3D), and invasion (Supplemental Figure S3E). In terms of cell signaling, similar decreased level of p-TrkA, ERK1/2, and SRC were obtained using siCombo, but the individual siRNAs had a less clear impact on signaling pathways (Supplemental Figure S3A), probably indicating a synergic effect of inhibiting the three receptors simultaneously. Interestingly, 8505c cells did not express the membrane receptor p75NTR (Supplemental Figure S3A); thus, targeting this receptor with siRNA yielded similar results to those obtained with siCont cells. This finding highlights the specificity of the siRNA targeting in our experiment and points to a potential diversity of anaplastic cells in regard to neurotrophin receptor expression.

Discussion

New therapeutic strategies are required for treatment of thyroid cancers that do not respond to current treatment, in particular, ATC and metastatic differentiated thyroid cancer. We report that neurotrophin signaling may be an important component of tumor aggressiveness in ATC and differentiated thyroid carcinomas, which invites further examination as a potential drug target.

TrkA Expression and Targeting

This study found that TrkA protein expression is present in approximately 20% of thyroid cancers and is a marker of tumor aggressiveness, being associated with lymph node metastasis. Furthermore, targeting TrkA expression resulted in a decreased activation of SRC and ERK, ultimately resulting in decreased thyroid cancer cell growth and invasion. TrkA participates in the stimulation of cancer cell invasion in several cancers,\(^2\) including those of the breast,\(^21,22\) prostate,\(^23,24\) and pancreas,\(^23,28\) and is increasingly being considered as a therapeutic target in oncology. In breast cancer, TrkA activation participates in cancer cell invasion,\(^23\) and in lung cancer, Trk inhibitors are in clinical trials.\(^26\) The present data confirm the role of TrkA as an oncogenic protein and suggest a potential utility of targeting this pathway in thyroid cancer therapy.

In addition, TrkA expression was detected in the nerves present in the microenvironment of thyroid cancer. Although innervation of thyroid cancers has not been previously reported, using the neuronal marker PGP9.5, we found nerves in <5% of thyroid cancers (S.F., P.J., C.W.R., S.M.R.O., S.R., R.F.T., C.O., J.A., C.C.J., X.D.Z., M.M.W., H.H., unpublished data). This proportion of innervated tumors may be an underestimate because of the small sampling radius and depth of the tumor microarray cores. In developing neurons, TrkA activation results in the stimulation of various tyrosine kinase–induced signaling pathways, leading to neurite extension.\(^11\) The thyroid gland is principally innervated by the autonomic nervous system, with parasympathetic nerves from the vagus and sympathetic nerves distributed from the sympathetic trunk, entering the gland along blood vessels.\(^25\) Interestingly, the nerves observed in thyroid cancers were positive for TrkA but not for sortilin and p75NTR. Given that NGF\(^28\) and proNGF\(^10\) are expressed and released by thyroid cancer cells,\(^10\) it can be hypothesized that the activation of TrkA in nerves may lead to a stimulation of neurogenesis in the tumor microenvironment. NGF and TrkA are involved in perineural invasion in other cancers, as in pancreatic cancer,\(^29\) and TrkA may play a similar role in thyroid cancer. In any case, the nerve-cancer cell crosstalk is increasingly regarded as a promoter of cancer progression,\(^30,31\) and the expression of TrkA in nerves suggests a potential association with neural infiltration in thyroid cancer.

p75NTR Expression and Targeting

Although previous studies have found that p75NTR is expressed in PTC and is associated with the presence of BRAF (V600E) mutations,\(^12\) this study provides the first data that p75NTR is expressed in normal thyroid tissue, although at a significantly lower level than in thyroid cancers. In addition, we found for the first time the expression of p75NTR in FTC and ATC. p75NTR is the common receptor for all neurotrophins and proneurotrophins.\(^11\) It belongs to the tumor necrosis factor receptor gene family, and the proteins recruited by p75NTR signaling are the tumor necrosis factor receptor–associated factor proteins followed by the activation of the NF-κB transcription factor. In addition, p75NTR interacts with sortilin and TrkA and can modulate the tyrosine kinase activity of the latter on stimulation by neurotrophins.\(^11\) Presumably because of its wide

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range of interaction with various receptors and signaling, p75NTR exhibits various biological activities in neurons, including the stimulation of survival or apoptosis, differentiation, and neurite outgrowth. In cancer, the same variety of effects are observed. p75NTR acts as a tumor suppressor in gastric, bladder, and prostate cancers by blocking cell cycle progression and inducing apoptosis. In most other tumors, including melanoma, glioma, and breast cancer, p75NTR favors tumor development, in particular through the stimulation of the stem cell compartment. Interestingly, our data indicate that p75NTR is overexpressed in most ATC. ATC has a poor survival rate (approximately 5% after 5 years), and at this stage there is no targeted treatment. Therefore, our data warrant future functional exploration to determine the interest of p75NTR as a therapeutic target in thyroid cancer.

Sortilin Expression and Targeting

This study is the first report of sortilin expression in thyroid cancer. Sortilin is expressed in thyroid epithelial cells, where it contributes to the recycling of the thyroid hormone precursor thyroglobulin, but its protein expression in cancer. Sortilin is expressed in thyroid epithelial cells, initially described as neurotensin receptor 3, sortilin is more generally involved in sorting and trafficking of target proteins to distinct destinations. It is a common binding partner of tyrosine kinase receptors, G-protein-coupled receptors, and ion channels, for which it facilitates ligand-induced signaling. Sortilin has been identified as a co-receptor for proneurotrophins, including proNGF, in which it acts in a complex with the neurotrophin receptor p75NTR to induce neuron apoptosis. A few cancer cell lines express sortilin. In HT29 colon cancer cells, sortilin participates in the control of the growth-promoting activity induced by brain-derived growth factor through interacting with its tyrosine kinase receptor TrkB (NTRK2). In addition, sortilin mediates the release and transfer of exosomes in the A549 lung cancer cell line and regulates progranulin stimulatory activity of prostate cancer cells. In melanoma cell lines, sortilin is a co-receptor for proNGF and acts in cooperation with the neurotrophin receptor p75NTR to promote cancer cell invasion. In breast cancer, sortilin expression is increased in invasive carcinomas compared with healthy tissues, and sortilin expression in the primary tumor is also associated with lymph node invasion. In vitro, sortilin participates in the proinvasive effect of proNGF in breast cancer cells. In the present study, similar to breast cancer, sortilin expression was increased in thyroid cancer compared with normal tissues and adenomas. This increased level of sortilin protein in thyroid cancers, compared with benign thyroid tissues, suggested a potential role in the initiation and progression of the disease. Intriguingly, sortilin was expressed at higher levels in PTC and FTC compared with ATC. Sortilin is a ubiquitous receptor; its functions go beyond the sole control of cell growth and migration, and this probably accounts for the fact that its expression is not strictly related to tumor aggressiveness in thyroid cancer. However, siRNA-targeting sortilin resulted in an inhibition of anaplastic thyroid cancer cell growth, migration, and invasion. Although the molecular mechanism of sortilin activity and its direct interacting signaling partners remain to be fully elucidated in thyroid cancer, our data suggest that targeting sortilin is a potential therapeutic strategy in thyroid cancer.

Conclusion

The present study found an increased level of TrkA, p75NTR, and sortilin in thyroid cancers, which signals the potential value of these neurotrophin receptors as novel therapeutic targets. Further preclinical investigations in vivo are warranted to explore the therapeutic interest of targeting neurotrophin receptors in the different forms of thyroid cancers and particularly in ATCs, which are resistant to current treatment options. In addition, pain is a reported event in thyroid cancer, particularly in MTC and ATC. Neurotrophins and their receptors have been well characterized as important mediators of pain initiation and maintenance, and pharmacotherapies targeting the NGF/TrkA pathway are undergoing trials in the treatment of a variety of pain conditions, including cancer. Therefore, targeting neurotrophin receptors in ATC may potentially address the issue of pain, which also merits further attention.

Acknowledgments

We thank Sheridan Keene for excellent technical assistance and the Clinical Research Design, IT, and Statistical Support (CReDITSS) Unit of the University of Newcastle (Callaghan, New South Wales, Australia). The 8505c (ATC) cell line was a generous gift from Prof. Alfred Lam (Griffith University, Queensland, Australia).

H.H. conceived and supervised the study and drafted the manuscript; S.F., S.R., and C.C.J. prepared all figures and tables; C.W.R. and X.Z. provided images and tissue slide analyses and quantification; R.F.T.; S.M.R.O. and P.J. performed the quantification suppression; S.F. performed all cell culture experiments and analysis; J.A. and C.O. supervised statistical analyses; and all authors read and approved the final manuscript.

Supplemental Data

Supplemental material for this article can be found at https://doi.org/10.1016/j.ajpath.2017.09.008.
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