Differential roles of RET isoforms in medullary and papillary thyroid carcinomas

Eric Y Lian^{1,2}, Sarah M Maritan^{1,2}, Jessica G Cockburn^{1,2}, Katayoon Kasaian³, Mathieu J F Crup^{1,2}, David Hurlbut², Steven J M Jones^{3,4}, Sam M Wiseman⁵ and Lois M Mulligan^{1,2}

¹Division of Cancer Biology and Genetics, Cancer Research Institute, Queen's University, Kingston, Ontario, Canada

²Department of Pathology & Molecular Medicine, Queen's University, Kingston, Ontario, Canada

³Michael Smith Genome Sciences Centre, British Columbia Cancer Research Centre, Vancouver, British Columbia, Canada

⁴Department of Medical Genetics, University of British Columbia, British Columbia Cancer Research Centre, Vancouver, British Columbia, Canada

⁵Department of Surgery, St Paul's Hospital & University of British Columbia, Vancouver, British Columbia, Canada

Correspondence should be addressed to L M Mulligan

Email
mulligal@queensu.ca

Abstract

The RET receptor tyrosine kinase mediates cell proliferation, survival and migration in embryogenesis and is implicated in the transformation and tumour progression in multiple cancers. RET is frequently mutated and constitutively activated in familial and sporadic thyroid carcinomas. As a result of alternative splicing, RET is expressed as two protein isoforms, RET9 and RET51, which differ in their unique C-terminal amino acids. These isoforms have distinct intracellular trafficking and associated signalling complexes, but functional differences are not well defined. We used shRNA-mediated knockdown (KD) of individual RET isoforms or of total RET to evaluate their functional contributions in thyroid carcinoma cells. We showed that RET is required for cell survival in medullary (MTC) but not papillary thyroid carcinoma (PTC) cells. In PTC cells, RET depletion reduced cell migration and induced a flattened epithelial-like morphology. RET KD decreased the expression of mesenchymal markers and matrix metalloproteinases and reduced anoikis resistance and invasive potential. Further, we showed that RET51 depletion had significantly greater effects on each of these processes than RET9 depletion in both MTC and PTC cells. Finally, we showed that expression of RET, particularly RET51, was correlated with malignancy in a panel of human thyroid tumour tissues. Together, our data show that RET expression promotes a more mesenchymal phenotype with reduced cell–cell adhesion and increased invasiveness in PTC cell models, but is more important for tumour cell survival, proliferation and anoikis resistance in MTC models. Our data suggest that the RET51 isoform plays a more prominent role in mediating these processes compared to RET9.

Key Words

- ▶ RET isoforms
- ▶ thyroid carcinoma
- ▶ epithelial–mesenchymal transition
- ▶ migration
- ▶ invasion

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Introduction

The RET receptor tyrosine kinase is required for the development of multiple human tissues (Mulligan 2014). Under normal conditions, RET is activated by binding a glial cell line-derived neurotrophic factor (GDNF) family ligand and a cell surface co-receptor (GFR α) (Airaksinen & Saarma 2002). However, RET can also act as an oncogene and is implicated in development and progression of multiple human cancers, most notably carcinomas of the thyroid (Prescott & Zeiger 2015, Romei et al. 2016). Constitutive activation of RET by specific point mutations gives rise to medullary thyroid carcinoma (MTC), a tumour of thyroid C-cells, as part of the familial cancer syndrome multiple endocrine neoplasia 2 (MEN2), and also occur in sporadic forms of MTC (Mulligan 2014). In contrast, in the follicular cell tumour papillary thyroid carcinoma (PTC), rearrangements of the *RET* gene result in a constitutively active RET kinase fusion protein (RET/PTC) localised in the cytosol (Prescott & Zeiger 2015, Romei et al. 2016). Although RET mutations are thought to act as initiating drivers in MTC, the contributions of RET/PTC to PTC tumourigenesis are less clear (Prescott & Zeiger 2015, Wells et al. 2015). Recent studies have also identified rare RET fusion oncoproteins in other cancers, including lung adenocarcinomas, chronic myelomonocytic leukaemia (CMML) and Spitzoid tumours (Mulligan 2014, Wiesner et al. 2014) suggesting broader implications of RET rearrangements than those previously recognised.

RET is expressed as two conserved protein isoforms, RET9 and RET51, generated by alternative splicing of 3' exons (Supplementary Fig. 1, see section on supplementary data given at the end of this article), which share the first 1063 residues but have 9 or 51 unique C-terminal amino acids, respectively (Mulligan 2014). RET isoforms are generally co-expressed but have distinct molecular and functional properties. RET9 and RET51 differ in membrane localisation and in their intracellular trafficking upon activation (Richardson et al. 2012, Crupi et al. 2015) and each recruits distinct signalling complexes, leading to different target gene expression patterns (D'Alessio et al. 1995, Rossel et al. 1997, Ishiguro et al. 1999, Tsui-Pierchala et al. 2002, Hickey et al. 2009). RET9 has been shown to have a more prominent role in the development of kidney and neural cell types (de Graaff et al. 2001). Constitutively active RET9 and RET51 mutants found in human thyroid carcinomas have different transforming potentials and abilities to promote cell scattering *in vitro* (Rossel et al. 1997, Ishiguro et al. 1999, Degl'Innocenti et al. 2004). Although RET9 is generally more highly expressed,

RET51 is significantly more effective at inducing cell proliferation and anchorage-independent growth *in vitro* (Pasini et al. 1997, Rossel et al. 1997, Iwashita et al. 1999, Le Hir et al. 2000). Despite these functional differences, the contributions of endogenous RET9 and RET51 isoforms to human thyroid tumourigenesis and to tumour metastasis or invasive spread have not been well characterised.

Here, we investigated the roles of total RET and each individual RET isoform in MTC and PTC models using shRNA-mediated knockdowns (KD). We showed that RET is required for the survival of MTC cells harbouring an activating RET MEN2 mutation. In PTC cells expressing a constitutively active RET/PTC1 fusion protein, RET is not essential for cell survival but promotes changes in cell morphology and transcriptional pattern, and contributes to cell migration and invasion, consistent with a change to a more mesenchymal growth pattern. Interestingly, our data show that RET51 has greater effects than RET9 on each of these processes. Finally, we evaluated the expression of individual RET isoforms *in situ* by immunohistochemistry (IHC) in benign and malignant human thyroid tissues. Together, we show that RET acts primarily as a survival factor in MTC but has roles in establishing and maintaining a more mesenchymal phenotype in PTC cells. Further, our data suggest a more prominent role for RET51 in promoting these events in both MTC and PTC.

Materials and methods

Adherent cell culture

The PTC cell line TPC-1 (obtained from Dr Sylvia Asa) and MTC cell line TT (ATCC) were cultured in DMEM or F-12K medium, respectively, supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich). RET KD cell lines were maintained in medium supplemented with 1 μ g/mL puromycin.

Antibodies

For western blotting, RET was detected using isoform-specific antibodies for RET9 (C19G) and RET51 (C20) or an antibody that detects both isoforms (C19) (Santa Cruz Biotechnology). The anti- γ -tubulin antibody was obtained from Sigma-Aldrich. For immunofluorescence, primary antibodies to total RET (C19), E-cadherin (C36, BD Biosciences, San Jose, CA, USA) and N-cadherin (C32, BD Biosciences) and Alexa Fluor-conjugated

secondary antibodies (Thermo Fisher Scientific) were used. IHC detection of RET expression was performed using isoform-specific RET9 (C19G) and RET51 (EPR2871, Abcam) antibodies.

shRNAs for RET knockdown

shRNA sequences and target site locations for constructs targeting all RET isoforms and RET51 (GE Healthcare) or RET9-specific shRNAs (Sigma-Aldrich) are shown in [Supplementary Fig. 1](#). Polyclonal shRNA KD or empty vector control (MockKD) cell lines were generated by lentiviral transduction, as previously described ([Crupi et al. 2015](#)). Four to 10 independent shRNA constructs were evaluated for each target and construct combinations yielding >85% KD were used ([Supplementary Fig. 1](#)). Transduced cells were selected in 1 µg/mL puromycin for a minimum of 7 days to generate RET9 KD (RET51only, expressing only RET51), RET51 KD (RET9only, expressing only RET9) or total RET KD (noRET) cell lines. Low passage (<5) KD cells were used for all assays.

Protein isolation and western blotting

Protein isolation and immunoblotting were performed as previously described ([Gujral et al. 2008](#), [Crupi et al. 2015](#)). Protein concentrations were determined using the Pierce BCA Protein Assay (Thermo Fisher Scientific). Proteins were separated on SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. Blots were probed with indicated primary (1:1000 dilution) and appropriate HRP-linked secondary antibodies (1:3000 dilution): anti-mouse, anti-rabbit (Cell Signaling) or anti-goat (Santa Cruz Biotechnology). Immunoreactive bands were visualised by enhanced chemiluminescence (Perkin Elmer).

Cell proliferation, migration and chemotaxis assays

Cell proliferation was evaluated by MTT reduction assay, as previously described ([Gujral et al. 2008](#)). Briefly, 1×10^4 cells/well were seeded into 96-well plates and allowed to proliferate for 72 h. MTT (Thermo Fisher Scientific) was added to a final concentration of 450 µg/mL for 24 h. One volume acidified 20% SDS was added, and cells were incubated for 4 h with shaking. Formazan product was quantified by absorbance at 570 nm and normalised to control wells containing only MTT, medium and acidified SDS.

Collective cell migration was evaluated in wound healing assays. Briefly, 3.5×10^5 TPC-1 or 1×10^6 TT cells/well were grown in reduced (0.1%) FBS medium to form a confluent monolayer before wounding with a silicone pick. Cell monolayers were washed, and wound edges were allowed to adhere before capturing baseline images (time=0 h). Images of cell migration into the wound were captured after 18 h. Wound width measured at 10 points along the wound was averaged. A minimum of three experimental replicates were performed, each including three technical replicates.

Real-time tracking of individual migrating cells was performed at low (10% confluence) and high (30% confluence) cell densities in 0.1% FBS medium. Cells were allowed to adhere for 4 h, and then images were captured every 45 min over 20 h using an Incucyte ZOOM system equipped with a 4× objective lens (Essen BioScience, Ann Arbor, MI, USA). Images were exported as multidimensional TIFFs and aligned using Metamorph software (Molecular Devices, Sunnyvale, CA, USA). ImageJ software (Freeware, NIH) and the MTrack2 plugin (<http://valelab.ucsf.edu/~nsturman/ijplugins/MTrack2.html>) were used to threshold and assign X–Y coordinates to cells at each time point. DiPer software ([Gorelik & Gautreau 2014](#)) was used to determine mean squared displacement (MSD) over time for a minimum of 100 cells/condition.

Gelatin zymography

Matrix metalloproteinase (MMP) secretion was assessed by gelatin zymography. Briefly, conditioned medium, prepared by incubating serum-free medium with a confluent cell monolayer, was separated by SDS-PAGE on gels containing 1–2 mg/mL gelatin. Gels were incubated for 18 h at 37°C in zymography buffer (1% Triton X-100, 50 mM Tris–Cl (pH 8.0), 5 mM CaCl₂ and 0.02% Brij-35 (Bioshop Canada, Burlington, ON, Canada)). Proteolytic degradation of gelatin was visualised by Brilliant Blue G-250 (Thermo Fisher Scientific) staining and quantified by densitometry using ImageJ software.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. qRT-PCR was performed using a QuantiTect SYBR Green RT-PCR kit (Qiagen) with a BioRad iCycler iQ system. A minimum of three technical replicates, performed in triplicate ($n \geq 9$), were averaged for each

RNA sample. Primers for PCR amplification were *CDH1* (forward 5'-TGGAGGAATTCTTGCTTTGC-3'; reverse 5'-CGTACATGTCAGCCAGCTTC-3') (Aigner et al. 2007); *CDH2* (forward 5'-TGACCAGCCTCCAACTGGTATCTT-3'; reverse 5'-CCCTCAAATGAAACCGGGCTATCT-3'); *SNAIL* (forward 5'-TTCTCTAGGCCCTGGCTGC-3'; reverse 5'-TACTTCTTGACATCTGAGTGGGTCTG-3') (Fenouille et al. 2012); *MMP2* (forward 5'-CCAAGTGGGACAAGAACCAGATCA-3'; reverse 5'-AGACTTGGAAGGCACGAGCAAA-3'); *MMP9* (forward 5'-TGCCACTTCCCCTTCATCTTCG-3'; reverse 5'-AAACTGGCAGGGTTCCCATCA-3') (Fenouille et al. 2012); *TWIST1* (forward 5'-GGCTCAGCTACGCCTTCTC-3'; reverse 5'-TCCTTCTCTGGAAACAATGACA-3') (Sung et al. 2011); *ZEB1* (forward 5'-GCACCTGAAGAGGACCAGAG-3'; reverse 5'-TGCATCTGGTGTCCATTTT-3') (Drake et al. 2009), and *GUSB* (forward 5'-GAAAATACGTGGTTGGAGAGC-3'; reverse 5'-AAGGAACGCTGCACTTTTGTG-3') was used as a reference gene (Hickey et al. 2009).

Immunofluorescence

Cells seeded onto poly-L-lysine-coated glass coverslips were fixed in 2.5% neutral-buffered formalin for 15 min at 25°C, permeabilised with 0.1% Triton X-100 in PBS for 7 min and blocked in 3% BSA for 30 min. Fixed cells were incubated with the indicated primary antibodies (1:100 dilution) for 30 min at 37°C, and with appropriate Alexa Fluor secondary antibodies (1:200 dilution) and Hoechst 33342 nuclear stain (1:2000 dilution; Thermo Fisher Scientific) for 20 min at 37°C. A Quorum Wave FX spinning disc confocal microscope system (Quorum Technologies, Guelph, ON, Canada) with a 60× objective lens (Olympus Canada) and Metamorph software were used to acquire 0.2 µm optical sections. ImageJ software was used to normalise channel intensities and generate maximum-intensity Z-projections.

Contact inhibition and anchorage-independent growth assays

To assess contact inhibition of growth, 1×10^5 TPC-1 or 5×10^5 TT cells were seeded into 6-well plates and cell numbers/well ($n \geq 3$) were counted daily to generate growth curves. To assess anchorage-independent growth, 1×10^3 cells/well were seeded into 96-well U-bottom cell-repellent plates (Greiner Bio-One, Frickenhausen, DE, Germany) in medium with 5% FBS and 30 mg/mL

methylcellulose (Sigma-Aldrich) and allowed to proliferate for 2 weeks in suspension. A minimum spheroid size threshold of 30 µm for TPC-1 and 15 µm for TT cells was used. Spheroid size and number were quantified for a minimum of 12 wells per condition.

Spheroid generation and invasion assays

Cells were seeded into U-bottomed cell-repellent plates with low-density (3 mg/mL) methylcellulose and incubated overnight to generate a single spheroid/well. These pre-formed spheroids were collected and suspended in 2.5 mg/mL collagen (Corning), overlaid with medium, and invasion into the surrounding matrix was quantified.

Statistical analyses

Biological assays were repeated a minimum of three times, with a minimum of three technical replicates each. Statistical significance was assessed using one-way ANOVA. Mean and s.e.m. were calculated for each experimental condition, and significance between conditions was determined using a two-tailed Student's *t*-test for $\alpha = 0.025$.

Thyroid tissue microarray construction, IHC and scoring

Thyroid tissue microarrays (TMA) were constructed as previously described from formalin-fixed paraffin-embedded archival patient specimens (Parker et al. 2002, Wiseman et al. 2008a,b) consisting of 215 benign thyroid lesions (Benign pathology), 289 malignant thyroid carcinoma specimens (Malignant pathology) and 39 lymph node metastases (3 MTC; 36 PTC). Clinical and pathologic characteristics are summarised in Table 1. RET mutation status was not available for our patient cohort. Briefly, areas of interest in each donor block were identified and two tissue core samples extracted using a tissue microarrayer with a 0.6 mm diameter cylinder (Beecher Instruments, Silver Spring, MD, USA) and transferred to a recipient paraffin block for construction of a 2-fold redundant TMA. This study was approved by our institutional Research Ethics Boards (University of British Columbia; Queen's University). Serial 4 µm paraffin sections from TMA blocks were transferred onto glass slides for immunostaining (Queen's Laboratory for Molecular Pathology, Kingston, ON, Canada). Staining was optimised using RET isoform-specific positive and negative controls (Supplementary Fig. 2) and a commercially available TMA (UNC241, US Biomax, Rockville, MD, USA) containing cores from a wide variety

Table 1 Clinical and pathologic characteristics of entire study cohort (504 patients) and cancer patient subset (289 patients).

Patient set/clinical variable	Categories	Frequency
Benign pathology (n=215)	Follicular adenoma	71
	Goiter	110
	Hashimoto's thyroiditis	9
	Hurthle cell adenoma	25
Malignant pathology (n=289)	FTC	20
	PTC	220
	MTC	12
	ATC	37
Benign patient cohort (n=215)		
	Sex	
	Male	45
Age	Female	170
	<45	104
	≥45	111
PTC patient cohort (n=220)		
	Sex	
	Male	51
Age	Female	169
	<45	109
	≥45	111
Extrathyroidal extension	Yes	63
	No	157
Tumour size (cm)	<2	101
	2–4	90
	>4	23
Vascular invasion present	Yes	23
	No	197
Tumour multifocality present	Yes	85
	No	135
Distant metastases present	Yes	3
	No	217
Complete resection	Yes	191
	No	29
T score	T1	76
	T2	63
	T3	29
	T4	40
	Unknown	12
N score	N0	114
	N1	70
	Unknown	36
M score	M0	140
	M1	3
	Unknown	77
AMES risk group ^a	Low risk	110
	High risk	33
	Unknown	77
Patient status	No recurrence	158
	Dead	6
	Local recurrence	6
	Unknown	50

^aAMES: age, metastases, extent and size.

ATC, anaplastic thyroid carcinoma; FTC, follicular thyroid carcinoma; MTC, medullary thyroid carcinoma; PTC, papillary thyroid carcinoma.

of tissue was used as an additional tissue immunostaining control. High-resolution scans were acquired using the Aperio Scanscope CS system (Leica Biosystems), and Aperio Spectrum software (Pham *et al.* 2007, Farris *et al.* 2009) was used to annotate regions of interest within each core. Briefly, nests of tumour cells within each core were manually selected for scoring, with care taken to exclude areas of high background and other immunohistochemical staining artefacts. The annotated regions of each core were then processed using nuclei detection algorithms in HALO (Indica Labs, Corrales, NM, USA) to generate segmentation masks, which consisted of the surrounding cytoplasm and membranes. A cytoplasmic+membranous staining intensity scoring algorithm was optimised in HALO and independently validated by a pathologist (DH). This scoring algorithm was then applied to the segmentation masks to generate staining intensity scores for the annotated regions of each core. Staining intensity was quantified using a continuous 0 (no stain) to 100 (most intense) scale. Descriptive statistics were performed to examine RET9 and RET51 expression. One-way ANOVA was used to determine statistical differences between mean RET9 and RET51 expression in tumour subtypes. A Tukey's *post hoc* analysis was used for multiple comparison of subtypes. Pearson correlations were calculated to determine the significant associations between RET9 and RET51 expression in various thyroid subtypes.

The correlation of clinical and pathologic characteristics with RET isoform expression was assessed using contingency table statistics (Pearson χ^2 or the Fisher exact test) and scripts written in the R programming language (version 2.4.1, R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria). Analyses were performed with categorical scorings of RET staining, grouped as either 'negative/weak' (negative, HALO score $50 \leq$) or 'moderate/strong' (positive, HALO score >50). All tests were 2-tailed, and a *P* value of less than 0.05 was considered statistically significant.

Results

RET has distinct roles in PTC and MTC cells

We evaluated the contributions of RET and each of its isoforms individually in two well-characterised human thyroid carcinoma cell models. The MTC cell line TT expresses both wild-type RET and a mutant MEN2-RET with an activating point mutation (C634W), whereas the PTC cell line TPC-1 expresses a cytosolic activated RET/PTC1 protein in which RET kinase sequences are

Figure 1

RET isoform knockdown alters cell morphology and growth of thyroid carcinoma cell lines. (A) RET isoform expression in the PTC cell line TPC-1 and MTC cell line TT was quantified by real-time PCR using isoform-specific primers. Expression was normalized to the housekeeping gene *GUSB*. (B) Expression of total RET or individual RET isoform proteins in TPC-1 and TT cells. Whole cell lysates of MockKD and knockdown (KD) cell lines depleted for RET51 (RET9only), RET9 (RET51only) or total-RET KD (noRET) were immunoblotted using antibodies specific for each RET isoform or a panRET antibody (total RET) that detects both isoforms, as indicated. Protein was not obtained from noRET TT cells (not shown) as these cells were not viable and did not proliferate sufficiently for analysis. In TT cells, full-length RET appears as a doublet of immature and fully glycosylated forms. The chimeric RET/PTC1 in TPC-1 appears as a single band representing cytosolic protein. Tubulin serves as a loading control. (C) RET KD induces isoform-specific changes in TPC-1 and TT cell morphology. Phase contrast images (40×) showing morphology of the indicated RET KD cells. Scale bar = 100 μm. (D) Growth of TPC-1 and TT RET KD cells. Cells were transduced with lentiviral shRNA constructs (day 0) and growth was monitored over time as indicated. Representative images show cell and colony morphology at indicated times. (E) Quantification of proliferation of RET KD cells. Growth of indicated cells was measured by MTT assay after 72-h incubation. Data are shown as mean ± S.E.M. of three experiments, each with a minimum of three technical replicates, normalized to MockKD. In TPC-1 cells, RET51only, RET9only and noRET cell lines all proliferated significantly less than MockKD (* $P < 2.4 \times 10^{-9}$) and were each significantly different from each other (RET51only vs RET9only † $P = 4.4 \times 10^{-9}$; RET9only vs noRET ‡ $P = 3.0 \times 10^{-9}$). RET51only and RET9only TT cells proliferated less than MockKD cells (* $P < 3.0 \times 10^{-5}$) but were not significantly different from each other ($P = 0.20$).

fused with N-terminal sequences of CCDC6 (Romei *et al.* 2016). RET9 and RET51 isoforms are co-expressed in both cell lines (Fig. 1A and B) and RET-activating mutations have similar distribution in each isoform. Using shRNAs (Supplementary Fig. 1), we generated polyclonal single-isoform KD cell lines depleted for RET51 (RET9only) or RET9 (RET51only) and total RET KD (noRET) in both TPC-1 and TT cells. Knockdown validation by western blotting showed >86% reductions in target proteins (Fig. 1B). noRET TT cells had reduced viability and stained with Annexin V and propidium iodide (not shown) suggesting apoptotic cell death. A noRET TT cell line could not be established in culture (Fig. 1D and E), indicating that RET expression is required for TT cell survival. Notably, using an antibody that recognises both RET isoforms (total RET, Fig. 1B), we showed that single-isoform RET KD TT cells had similar total quantities of RET remaining, suggesting that RET9 and RET51 were expressed at similar levels in parental TT cells. In contrast, total remaining RET expression in RET51only TPC-1 cells was much less than in RET9only TPC-1 cells, consistent with relatively lower levels of RET51 than RET9 expression in parental cells.

MockKD TPC-1 cells were spindle-shaped with multiple long protrusions and formed loose colonies of highly motile cells that dispersed readily (Fig. 1C and D; Supplementary videos). RET51only TPC-1 cells were more flattened and epithelial-like, with fewer protrusions but had similar colony morphology (Fig. 1C and D; Supplementary videos). RET9only and noRET TPC-1 cells were increasingly epithelial-like, with few short protrusions, and formed colonies of tightly packed cells with little cell dispersal (Supplementary videos). RET KD TT cell lines were similar to corresponding TPC-1 RET KD cells, with flattened morphologies and reduced cell protrusions (Fig. 1C and D). We assessed proliferation of our RET KD cell lines in MTT assays. Proliferation of all TPC-1 RET KDs was reduced, but long-term viability was minimally affected, consistent with pro-proliferative but not survival roles for RET in these cells. We also noted significant differences in relative proliferation between TPC-1 KD cell lines, with MockKD > RET51only > RET9only > noRET (Fig. 1E). Knockdown of each individual RET isoform in TT cells reduced proliferation to a similar extent compared to MockKD controls (Fig. 1E). noRET TT cells did not proliferate.

RET promotes individual and collective cell motility in TPC-1 cells

As TPC-1 and TT cells express constitutively active RET mutants, these cells did not respond to exogenous RET ligands in chemotaxis assays (Supplementary Fig. 3A).

Thus, we evaluated non-directional RET-mediated cell motility in RETKD cells grown at low and high cell density by real-time tracking of individual cell movement and mean squared displacement (MSD) analyses to quantify the area that cells explored over time. TPC-1 cells expressing any RET form were significantly more motile and had significantly greater MSD (dispersal) than noRET TPC-1 cells grown at low density, suggesting that RET contributes to this process (Fig. 2A). At higher confluence, RET9only TPC-1 cells were also significantly less motile, with MSD intermediate to noRET and MockKD TPC-1 cells (Fig. 2B). In contrast, RET51only cells showed similar motility and MSD to MockKD TPC-1 cells under all conditions. TT cell motility was minimal under all conditions (Supplementary Fig. 3B).

In time lapse images (Supplementary videos), motile RET9only and noRET TPC-1 cells came in contact and remained associated in clusters, whereas RET51only and MockKD cells continued to move individually, suggesting differences in cell–cell adhesion that could contribute to isoform-specific differences in collective cell migration. Thus, we assessed collective movement of RET KD cells in wound healing assays. Consistent with our previous findings, RET9only and noRET TPC-1 cells had significantly reduced ability to move into a wound scratched in a cell monolayer, whereas RET51only and MockKD TPC-1 cells were not significantly different (Fig. 2C). TT cell movement into a scratch wound was not detected (Supplementary Fig. 3C). Together, we showed that RET promotes both individual TPC-1 cell movement and collective cell migration but not TT cell motility and that the RET51 isoform is more effective at promoting TPC-1 movement than RET9. Further, the phenotypic changes in cell and colony morphologies in response to RET knockdown indicate that RET depletion leads to increased TPC-1 cell contact and enhanced cell–cell adhesiveness, consistent with a more epithelial-like phenotype suggesting that RET, and particularly RET51, promotes more mesenchymal phenotypes in parental TPC-1 cells.

RET depletion promotes mesenchymal phenotypes in TPC-1 cells

The more migratory phenotype of transformed cells is frequently associated with changes in morphology and transcriptional profile as they undergo epithelial to mesenchymal transition (EMT) (Lamouille *et al.* 2014). The increased cell–cell adhesiveness and reduced motility of RET KD TPC-1 cells suggested that RET may sustain

Figure 2

RET knockdown induces isoform-specific changes in TPC-1 cell motility. Mean squared displacement (MSD) analysis of indicated RET KD TPC-1 cells seeded at (A) low or (B) high densities. Displacement from initial position was tracked at 45-min intervals over 20h for a minimum of 100 individual cells/indicated condition. MSD for all tracked cells $\pm$ S.E.M. is plotted for each time point (left). Individual paths taken by 30 randomly selected cells are shown for each condition (right). MSD of noRET cells was reduced relative to MockKD at both low and high cell densities ($P=2.1 \times 10^{-6}$ and 5.6×10^{-7} , respectively). RET9only MSD was significantly lower compared to MockKD at high cell densities ($P=1.2 \times 10^{-2}$) but not at low cell density ($P=0.42$). (C) Representative phase-contrast images of the indicated TPC-1 cells migrating into a scratched wound in a confluent cell monolayer. Remaining wound area at the indicated time is highlighted by hatched lines. Wound closure was quantified after 18h (right). RET9only and noRET TPC-1 cell wound closure was significantly reduced relative to MockKD control ($*P=6.3 \times 10^{-3}$ and 3.7×10^{-5} , respectively).

this mesenchymal phenotype in TPC-1 and that loss of RET allows cells to revert to epithelial-like phenotypes. To evaluate this, we assessed the expression of a panel of epithelial and mesenchymal markers in RET KD cells. In TPC-1 RET KD cells, expression of the EMT transcription factors *ZEB1* and *TWIST1* was reduced relative to MockKD cells (Fig. 3A). Decreased expression was most pronounced in RET9only and noRET TPC-1 cells, whereas RET51only TPC-1 cells retained notably higher expression, consistent with their more mesenchymal phenotype. *ZEB1* was also reduced to similar extents in RET9only and RET51only TT cells, but *TWIST1* was undetectable in all TT RETKD cell lines (Fig. 3B).

As *ZEB1* and *TWIST1* negatively regulate the expression of the epithelial cell adhesion molecule E-cadherin, we assessed the expression of E-cadherin and the mesenchymal cell adhesion molecule N-cadherin. E-cadherin (*CDH1*) gene expression was below detectable levels in all TT and RET-expressing TPC-1 cells and rose only minimally above background in noRET TPC-1 cells (Fig. 3A). N-cadherin (*CDH2*) expression was decreased in RETKD TPC-1 cells such that MockKD > RET51only > RET9only and noRET (Fig. 3A), consistent with the relative reductions in cell motility and

increases in cell–cell adhesiveness we observed in these cells. *CDH2* expression was significantly reduced only in RET9only TT cells (Fig. 3B), consistent with the more mesenchymal phenotype of these cells. We evaluated the changes in E-cadherin and N-cadherin protein expression in RET KD TPC-1 cells by immunofluorescence. MockKD TPC-1 cells had strong N-cadherin staining at the cell membrane but no detectable membranous E-cadherin (Fig. 3C and D). RET9only and RET51only TPC-1 cells had reduced cell membrane N-cadherin and no membranous E-cadherin expression, whereas noRET TPC-1 cells had reduced N-cadherin staining but showed an increase in membrane-associated E-cadherin (Fig. 3C and D), suggesting that RET KD led to a shift away from mesenchymal marker expression but was not alone sufficient to promote a corresponding large increase in the expression of epithelial markers.

EMT is associated with increased expression and secretion of matrix metalloproteinases (MMPs), especially MMP2 and MMP9, which degrade the extracellular matrix and facilitate invasion. MMP9 was not detectable in TT cells and low levels of MMP2 transcript and protein were detected only in MockKD and RET51only TT cells (Fig. 4A and B) suggesting RET9 does not efficiently

Figure 3

RET knockdown alters the expression of epithelial and mesenchymal markers in TPC-1 and TT cells. (A and B) Expression of epithelial and mesenchymal marker transcripts quantified by qRT-PCR in the indicated RET KD (A) TPC-1 and (B) TT cells. Expression was normalized to expression of *GUSB* and expressed as fold change (2^{-ddCt}) relative to MockKD. (A) RET KD in TPC-1 cells did not affect expression of *SNAI1*. However, expression of mesenchymal marker *ZEB1* was reduced in all RET KDs ($*P < 0.043$) with RET9only ($*P = 0.001$) and noRET ($*P = 0.006$) cells showing significantly greater reductions than RET51only cells. Expression of *TWIST1* was reduced in RET9only and noRET cells ($*P < 0.0023$). *CDH1* was below detection threshold in all cell lines except noRET. *CDH2* expression was reduced by RET KD ($*P < 0.023$), with expression in RET9only and noRET cells significantly decreased relative to either MockKD or RET51only cells ($*P < 7.6 \times 10^{-6}$). (B) RET KD in TT cells did not affect *SNAI1* expression, whereas *ZEB1* expression was significantly reduced in both RET9only and RET51only cells ($*P < 3.3 \times 10^{-6}$). *TWIST1* and *CDH1* expression was undetectable in all conditions, whereas *CDH2* expression was significantly reduced only in RET9only cells ($*P = 7.1 \times 10^{-5}$). (C and D) Immunofluorescence localization of (C) E-cadherin (red), RET (green) and (D) N-cadherin (green) in TPC-1 cells. (C) Arrows indicate accumulations of membranous E-cadherin in noRET cells. (D) Concentrations of N-cadherin (arrows) are visible at cell-cell contacts in MockKD, RET51only and RET9only cells but not in noRET cells. Nuclei are stained with Hoescht.

promote MMP2 expression. In contrast, MockKD TPC-1 cells expressed high levels of both *MMP2* and *MMP9* transcripts. RET KD reduced *MMP2* transcripts, such that MockKD > RET51only > RET9only and noRET (Fig. 4A). *MMP9* expression was similar in RET51only and MockKD TPC-1 cells but was significantly diminished in RET9only and noRET TPC-1 cells. Pro-MMP9 protein was reduced in conditioned medium from all RET KD TPC-1 cell lines such that MockKD > RET51only > RET9only and noRET cells. Pro-MMP2 secretion by RET9only and noRET TPC-1 cells was reduced compared to both MockKD and RET51only TPC-1 cells (Fig. 4B and C). Further, levels of activated MMP2, generated by cleavage of the pro-peptide, were significantly reduced in conditioned medium from RET9only and noRET compared to either MockKD or RET51only TPC-1 cells (Fig. 4B and C). Active MMP9 was detectable in conditioned medium after longer incubations (72h) but did not vary between KD cell lines (not shown). Thus, in TPC-1 cells, RET51 not only promotes the expression of both *MMP2* and *MMP9* but

also significantly enhances the activation of *MMP2*, and RET51 plays greater roles than RET9 in these processes.

RET abrogates contact inhibition of growth and promotes adhesion-free cell survival and invasion

EMT reduces cell contact inhibition of growth that would normally limit proliferation and enhances anoikis resistance and migratory or invasive potential of tumour cells (Huang et al. 2013, Tsai & Yang 2013). We evaluated the changes in proliferation of RET KD cells over time as they were grown to confluence (Fig. 5A). Proliferation of all TPC-1 KD cell lines slowed abruptly as they reached confluence (~3–4 days in culture). RET9only and noRET TPC-1 cells formed epithelial-like monolayers with minimal growth after reaching confluence, whereas RET51only and MockKD TPC-1 cells continued to proliferate slowly, forming multicellular foci, consistent with reduced contact inhibition of proliferation (Fig. 5A). TT cells continued to proliferate for more than 60 days

Figure 4

RET knockdown reduces matrix metalloproteinase expression and secretion. (A) Quantification of *MMP9* and *MMP2* transcripts by qRT-PCR in the indicated RET KD TPC-1 (left) and TT (right) cells. Expression was normalized to *GUSB* mRNA expression level and expressed as fold change ($2^{-\Delta\Delta Ct}$) relative to MockKD. *MMP9* expression was significantly reduced in RET9only and noRET TPC-1 cells compared to MockKD ($*P < 5.2 \times 10^{-6}$) and RET51only TPC-1 ($*P < 9.5 \times 10^{-12}$). *MMP2* expression was reduced in all TPC-1 RET KD cells compared to MockKD ($*P < 6.0 \times 10^{-4}$) and in RET9only and noRET cells compared to RET51only TPC-1 ($*P < 5.3 \times 10^{-3}$). *MMP9* expression was not detected in TT cells, but *MMP2* expression was significantly reduced in RET9only TT cells relative to either MockKD or RET51only TT cells ($*P < 1.2 \times 10^{-9}$). (B) Representative images showing secretion of MMPs by RETKD TPC-1 and TT cells detected using gelatin zymography. Immature, secreted pro-MMP9 and pro-MMP2 are indicated by black arrowheads. The mature active MMP2 is indicated with a gray arrowhead. 1% FBS served as a positive control for enzymatic activity (gelatin degradation). (C) Bar graphs show the intensity of MMP bands in TPC-1 samples as shown in (B) quantified by densitometry and expressed relative to the corresponding pro-MMP band in the FBS control sample. Secretion of pro-MMP9 (left) was significantly reduced in all KD TPC-1 cell lines compared to MockKD cells ($*P < 2.5 \times 10^{-3}$) and reduced in RET9only and noRET cells relative to RET51only cells ($*P < 5.8 \times 10^{-3}$). Secretion of pro-MMP2 (centre) was significantly reduced in RET9only and noRET TPC-1 cells compared to MockKD ($*P < 1.7 \times 10^{-5}$) and RET51only ($*P < 3.3 \times 10^{-4}$) cells. Secretion of mature active MMP2 (right) by RET9only and noRET TPC-1 cells was significantly lower than MockKD ($*P < 5.6 \times 10^{-4}$) or RET51only ($*P < 5.5 \times 10^{-4}$) cells.

and formed dense multicellular foci that did not exhibit contact inhibition of growth. Over extended periods, growth of MockKD > RET51only >> RET9only TT cells.

We developed 3D spheroid models of RET KD TPC-1 and TT cell growth for evaluation of anchorage-independent growth and anoikis resistance by suspending cells in an inert non-proteinaceous methylcellulose matrix that does not support cell invasion (Maritan et al. 2016). After 2 weeks in culture, MockKD TPC-1 and TT cells formed large spheroids, whereas RET51only cells formed fewer, smaller spheroids (Fig. 5B). RET9only TPC-1 and TT cells formed fewer and smaller spheroids than corresponding MockKD or RET51only cells. noRET TPC-1 cells were not able to grow in the methylcellulose matrix (Fig. 5B). Together, these data suggest that RET is critical for 3D spheroid formation and anoikis resistance of both TPC-1 and TT cells.

To evaluate the invasive potential of RET KD cells, spheroids were pre-formed by incubating cells (1000 cells/spheroid) in methylcellulose overnight, and then transferred to collagen matrix and cell invasion into the surrounding matrix monitored. MockKD TPC-1 (Fig. 6A) and TT (Fig. 6B) cells invaded more and farther than all RET KD cells. RET51only cell invasion was also significantly greater than RET9only for both TT and TPC-1 cells. Invasion of noRET TPC-1 cells was minimal. Together, our data indicate that RET confers anoikis resistance and promotes cell invasiveness in both TPC-1 and TT cells. Further, the RET51 isoform promotes anchorage-independent growth and invasion into surrounding matrix more effectively than RET9, suggesting that it could play a greater role in these aggressive phenotypes in thyroid cancers *in vivo*.

Figure 5

RET knockdown restores TPC-1 contact inhibition of growth and reduces anchorage-independent growth of TPC-1 and TT cells. (A) Evaluation of contact inhibition of growth in RET KD cell lines. TPC-1 cells (left) and TT cells (right) were seeded at 1×10^5 cells/well and counted over indicated days to generate growth curves. Representative images of super-confluent layers of TPC-1 cells (day 7) and TT cells (day 30) are shown. (B) RET KD reduces anchorage-independent growth. TPC-1 cells (left) and TT cells (right) embedded in 30 mg/mL methylcellulose after 2-week incubation. In both TPC-1 and TT cells, RET51only and RET9only cells formed significantly fewer ($*P < 1.2 \times 10^{-8}$) and smaller ($*P < 1 \times 10^{-4}$) spheroids relative to MockKD cells (lower panels). RET9only cells of either cell line also formed significantly fewer spheroids than RET51only cells ($^{\dagger}P < 1.9 \times 10^{-9}$) but were significantly different in size only for TT cells ($^{\dagger}P = 9.6 \times 10^{-4}$). noRET TPC-1 cells did not form spheroids.

Expression of RET isoforms in human thyroid tissues

We evaluated RET9 and RET51 expression by IHC on a TMA consisting of human thyroid tissue specimens including

benign and malignant thyroid lesions and lymph node metastases (Fig. 7A and Table 1). RET expression varied significantly between pathological thyroid specimens

Figure 6

RET knockdown reduces cell invasion. (A and B) RET KD in both TPC-1 and TT cells reduces invasion into collagen matrix. Pre-formed spheroids of 1×10^3 TPC-1 or TT cells were embedded in 2.5 mg/mL collagen matrix and allowed to invade for the indicated times. (A) RET51only TPC-1 cells show a modest reduction in invasiveness relative to MockKD, whereas RET9only and noRET cells have dramatically reduced invasive potential and invade a significantly shorter distance away from the spheroid than either MockKD or RET51only ($*P < 8.2 \times 10^{-9}$). noRET cells travel significantly shorter distances relative to RET9only ($^{\dagger}P = 4.5 \times 10^{-5}$). (B) RET51only TT cells display a modest reduction in invasiveness relative to MockKD, whereas RET9only cells invade significantly shorter distances relative to both MockKD and RET51only cells ($*P < 1.9 \times 10^{-2}$).

($P < 0.0001$) (Fig. 7B). Mean RET51 expression was significantly higher in PTC and MTC compared to benign, FTC and ATC tissues ($^{\dagger}P < 0.001$), whereas RET9 expression was significantly higher only in PTC compared to benign specimens ($*P < 0.001$) and was not significantly different from other thyroid cancer types. Further, in PTC, RET51 expression was significantly higher than RET9 ($^{\dagger}P = 0.003$). RET9 and RET51 expression was positively correlated in primary tumours when all histological types were considered ($R = 0.457$, $P < 0.0001$).

Only our PTC primary tumour cohort was sufficiently large for the evaluation of RET expression relative to clinical and pathologic characteristics (Table 2). In these specimens, expression of RET9 and RET51 was correlated with increased tumour size (T score). RET51 but not RET9 was associated with AMES (age, metastasis, extent, size) high-risk group, whereas RET9 was associated with lower incidence of lymph node metastasis.

When expression in lymph node metastasis specimens from patients with MTC or PTC were considered, RET9 and RET51 expression was significantly correlated for PTC but not MTC metastases. Interestingly, RET51 expression in PTC and MTC lymph node metastases was significantly greater than that in benign specimens ($P < 0.0001$), whereas the increase in RET9 expression was not as significant ($P = 0.04$). Although the sample size was small, RET9 and RET51 expression appeared overall higher in MTC lymph node metastases when compared to primary tumours.

Discussion

RET has established oncogenic roles in thyroid tumourigenesis, and mounting evidence indicates it also

contributes to metastatic spread of many tumour types (Mulligan 2014). Constitutive activation of the full-length RET receptor occurs in familial and sporadic MTC, whereas PTCs harbour chimeric active RET/PTC kinases in the cytosol. Here, we demonstrate that RET promotes proliferation and contributes to anoikis resistance of the MTC cell line TT and that RET depletion blocks survival and leads to apoptotic cell death (Fig. 8). These data are consistent with the effects of the active RET mutants found in MEN2 patients, which promote hyperplastic growth of thyroid C-cells, the precursor lesion of MTC (Wells et al. 2015). Interestingly, RET does not play a prominent role in TT cell motility or invasion, consistent with the low levels of RET-associated MMP secretion, suggesting other genetic events also contribute to these progressional events in MTC.

In contrast, cytosolic RET/PTC1 in the PTC cell line TPC-1 is dispensable for survival but promotes individual and collective cell motility and invasiveness (Fig. 8). Upon shRNA RET KD, we saw changes in TPC-1 morphology and growth pattern reflective of a more epithelial-like phenotype including increased cell-to-cell adhesiveness and decreases in motility, anoikis resistance and invasiveness into collagen matrix (Fig. 8). RET KD TPC-1 had reduced expression of mesenchymal transcription factors (ZEB1 and TWIST1), adhesion molecules (N-cadherin) and matrix metalloproteinases (MMP2 and MMP9), but the corresponding increases in membranous expression of the epithelial adhesion molecule E-cadherin were minimal. These findings suggest that RET contributes to a more mesenchymal phenotype in TPC-1 cells and that RET KD impairs or prevents this transition but that loss of RET does not enhance the re-expression of epithelial markers to the same extent, possibly due to additional

Figure 7

Immunostaining of thyroid tissues for RET isoform expression. (A) Representative IHC staining of the indicated thyroid tissues for RET9 or RET51. Insets show low magnification image of the corresponding TMA tissue core. (B) RET9 and RET51 expression in benign and malignant thyroid tissues. Staining intensity scores for RET9 and RET51 are shown for each tissue subtype (bottom) and represented as box plots (top). Whiskers represent the data within the 1.5 interquartile range, whereas boxes represent the first (25%) and third quartiles (75%) as well as the median (50%). Outliers are represented by data points (dots) beyond the interquartile range. Mean RET9 staining intensity was significantly greater only in PTCs relative to benign tissues ($*P < 0.001$), while mean RET51 staining intensity was significantly greater in both PTC and MTCs relative to all other subtypes ($^{\dagger}P < 0.001$), RET51 staining was significantly stronger than RET9 only in PTCs ($^{\dagger}P = 0.003$).

progressional events in this established tumour cell line. Our data are consistent with reports of low levels of E-cadherin expression in PTCs *in vivo* and corresponding high levels of mesenchymal transcription factors that downregulate E-cadherin expression (Hardy *et al.* 2007, Vasko *et al.* 2007, Wang *et al.* 2014, Lv *et al.* 2016). In particular, the mesenchymal transcription factor TWIST1 (Wang *et al.* 2013, Lv *et al.* 2016) has been correlated with

lymph node metastasis in PTC, suggesting that increased expression of mesenchymal markers contributes to PTC invasiveness and metastasis. Consistent with this, kinase fusion oncogenes have recently been associated with more extensive lymphatic and lymphovascular invasion in paediatric PTC, suggesting a role for RET/PTC in the tumour dissemination process (Adeniran *et al.* 2006, Cancer Genome Atlas Research Network 2014, Prasad *et al.* 2016).

Table 2 RET isoform expression relative to clinical and pathologic characteristics for PTC cohort (220 patients).

PTC patient cohort study (n = 220)	Test	RET9	RET51
Sex	Pearson χ^2	1.00	0.67
Age	Pearson χ^2	0.35	0.15
Extrathyroidal extension	Pearson χ^2	0.13	0.30
Vascular invasion	Fisher's exact	0.42	0.72
Tumour multifocality	Pearson χ^2	0.53	0.87
Distant metastases	Fisher's exact	0.57	1.00
T score	Pearson χ^2	0.01	0.09
N score	Pearson χ^2	0.02	0.62
M score	Fisher's exact	0.56	1.00
AMES risk group	Pearson χ^2	0.83	0.04

Together, these data suggest a role for RET in regulating the mesenchymal gene expression profile and promoting a motile, invasive cell phenotype in PTC. These results are consistent with RET's roles in other tumours, including carcinomas of the breast and pancreas, where it has been linked to tumour invasion and metastasis (Kang *et al.* 2009,

Gil *et al.* 2010, Mulligan 2014). Recent identification of RET/PTC-like rearrangements in lung adenocarcinoma, CML and Spitzoid tumours suggest that RET fusion proteins could also be an important contributor to more advanced disease in other cancer models (Mulligan 2014, Wiesner *et al.* 2014, Tsai *et al.* 2015).

RET is expressed as two major protein isoforms that differ in their protein interactions and downstream signalling (Tsui-Pierchala *et al.* 2002, Richardson *et al.* 2012, Crupi *et al.* 2015). These isoforms have established roles in normal development (de Graaff *et al.* 2001, Heanue & Pachnis 2008); however, the contributions of individual RET isoforms to tumourigenesis and metastasis remain poorly understood. Previous studies using RET overexpression models have shown that RET51 has greater transforming potential, compared to RET9 (Rossel *et al.* 1997, Ishiguro *et al.* 1999, Iwashita *et al.* 1999). Here, we used isoform-specific KD of individual endogenous RET isoforms to investigate their contributions to

Figure 8 RET functions in thyroid carcinoma cells. Localization and functions of RET9 and RET51 in MTC (left) and PTC (right) cells. Full-length RET9 (green) and RET51 (yellow) are shown at the cell membrane, whereas RET/PTC RET9 (green/purple) and RET51 (yellow/purple) localize in the cytosol. RET-associated processes common to MTC and PTC are indicated in green. RET-mediated processes occurring primarily in MTC (red) or in PTC (purple) are indicated. Arrow width is indicative of relative contributions of each RET isoform to the indicated cellular process.

RET-mediated cell phenotypes. Both RET9 and RET51 were able to contribute to RET functions in TPC-1 and TT cells; however, cells expressing RET51 alone were more similar to the parental or MockKD control cells, suggesting that RET51 was more effective in sustaining normal RET functions than RET9. In TT cells, RET51 enhanced proliferation and anoikis resistance compared to RET9. RET51 had greater abilities to promote TPC-1 cell motility, including individual and collective cell migration and was associated with maintenance of a more mesenchymal cell phenotype than RET9. RET51only TPC-1 cells were also more resistant to anoikis and had greater ability to invade into surrounding matrix when grown in 3D culture. RET9only TPC-1 cells were more similar to the total RET KD cells (noRET), with flattened epithelial-like morphology, more pronounced cell-to-cell adhesiveness and relatively reduced expression of mesenchymal markers and metalloproteinases and had significantly less ability to degrade or invade extracellular matrix. Notably, RET51 expression is much lower than RET9 in parental TPC-1 cells; thus, our RET51only cells retained less total RET than the RET9only cells, indicating that differences in RET-mediated processes were not due to overall fewer RET receptors in RET9only cells. Together, these data suggest that RET9 alone is not sufficient to maintain full RET-mediated cellular functions in TPC-1 and TT cells (Fig. 8).

Full-length RET is expressed at relatively low levels in adult thyroid and in some benign thyroid lesions including Hashimoto's thyroiditis and has been detected at lower levels in other follicular cell-derived lesions (Bunone et al. 2000, Fluge et al. 2001, Barroeta et al. 2006, Kang et al. 2007). In our TMA studies, both mean RET9 and RET51 expression were significantly higher in PTC compared to all benign lesions. Although RET mutation data were not available for our patient cohort, RET/PTC rearrangements are rare in most populations (Romei et al. 2016). Thus, the RET staining observed is unlikely to be exclusively due to RET fusion proteins and may reflect the expression of the wild-type *RET* allele in these tumours. Previous RT-PCR and IHC studies using several different RET antibodies have suggested that full-length RET may also be expressed in some PTC (Bunone et al. 2000, Fluge et al. 2001, Inaba et al. 2003, Kang et al. 2007, Zhu et al. 2010) and that RET could have some roles in normal follicle formation (Reynolds et al. 2001, Inaba et al. 2003), although this has yet to be confirmed *in vivo*.

High levels of RET expression characterise MTC and have been associated with more aggressive disease in PTC (Fluge et al. 2006, Mulligan 2014, Prasad et al. 2016, Romei et al. 2016). However, previous studies

have not evaluated the expression of individual RET isoforms in thyroid lesions. We showed that expression of both RET9 and RET51 correlated with malignancy in thyroid tissues, but RET51 expression was more strongly associated with the malignant phenotype, consistent with our data showing that RET51 promotes anoikis resistance, mesenchymal marker expression and more motile invasive phenotypes *in vitro*. Taken together, our data are consistent with a greater contribution of RET51 or coexpression of both isoforms, to promotion of thyroid metastatic processes. Future studies examining the relative expression of RET51 and RET9 in primary tumours may prove useful in determining patients with a heightened risk of tumour spread.

In summary, RET is an important contributor to tumour growth and spread in thyroid carcinoma models. Our data highlight the differences between RET function in PTC and MTC that have relevance for the distinct clinical pictures of these diseases. Although MTC and PTC have been reported to co-occur in rare cases (Kim et al. 2010, Shah et al. 2015), full-length RET mutant forms are relatively poorly transforming in thyroid follicular cells and thus PTC is not a common occurrence in MEN2 patients (Melillo et al. 2004). Previous studies have shown that RET/PTC proteins differ in expression level, subcellular trafficking, stability and downregulation compared to full-length RET forms (Richardson et al. 2009), which may contribute in part to the differences in cell phenotypes we observe *in vitro* and *in vivo*. In the MTC cell line TT, RET acts as a driver of tumour cell proliferation and anoikis resistance. However, in the PTC cell line TPC-1, RET promotes a more migratory, matrix invasive, mesenchymal phenotype. In each case, our data show that RET51 plays greater roles than RET9 in these processes, which may be important to their individual contributions to RET-associated tumour growth and invasive spread *in vivo*.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-16-0393>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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