Role of MEN2A-Derived RET in Maintenance and Proliferation of Medullary Thyroid Carcinoma

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Background: Dominant-activating mutations in the RET protooncogene, a receptor tyrosine kinase, have been identified as a cause of medullary thyroid carcinoma. Such oncogenic RET mutations induce its ligand-independent constitutive trans-autophosphorylation. We investigated the role of endogenous oncogenic RET autophosphorylation in maintaining the neoplastic phenotype in medullary thyroid carcinoma cells and orthotopic medullary thyroid carcinomas in RET transgenic mice. Methods: We constructed adenoviral vectors expressing a dominant-negative truncated form of RET, termed RETΔTK, and analyzed its effect on cell viability, apoptosis, and proliferation of TT medullary thyroid carcinoma cells. We investigated the effect of RETΔTK on downstream signaling by assessing alterations in phosphorylation or in gene expression. The effect of RETΔTK in primary medullary thyroid carcinomas in transgenic mice was assessed by monitoring tumor growth. All statistical tests were two-sided. Results: Cell viability was reduced. Phosphorylation of Akt and extracellular signal-regulated kinase (ERK), components of downstream signal transduction pathways, was abolished, and cell cycle progression was reduced. Expression of cell cycle regulator cyclin D1 was reduced, and expression of cell cycle regulators p21CIP1/WAF1 and p27KIP1 was increased. Apoptosis was stimulated and concurrently the expression of BCL-2 decreased. All in vitro experiments compared TT cells expressing RETΔTK with untreated control cells or control vector-treated cells. Furthermore, 2 weeks after injecting adenovirus-carrying RETΔTK into thyroid glands of transgenic mice with orthotopic medullary thyroid carcinoma, tumors were statistically significantly smaller than their initial size in mice treated with RETΔTK (43.6%, 95% confidence interval [CI] = 30.7% to 56.5%; P = .010; two-sided unpaired Student’s t test), whereas tumors in mice treated with a control vector were larger than their initial size (139.8%, 95% CI = 120.3% to 159.3%; P < .001). Conclusion: Selective disruption of oncogenic RET signaling in medullary thyroid carcinoma in vitro and in vivo is associated with loss of the neoplastic phenotype of medullary thyroid carcinoma and should be investigated further as the basis for new therapeutic approaches for this disease. [J Natl Cancer Inst 2004;96:1231–9]

Thyroid malignancies occur with an incidence of three per 100,000 people in Europe and the United States. Medullary thyroid carcinoma accounts for 5%–10% of such thyroid carcinomas, and it originates from the parafollicular C cells, whose main function is to produce and secrete calcitonin. About 1000 people are diagnosed with medullary thyroid carcinoma each year in the United States alone, and the 10-year survival rate of patients with medullary thyroid carcinoma is estimated at 60%–70% (1,2). The current treatment is characterized by surgical removal of neoplastic tissue. However, radiotherapy and chemotherapy have produced inconsistent results, indicating the need for alternative therapies (3).

Dominant activating mutations in the RET protooncogene have been identified as the key cause for the development of medullary thyroid carcinoma; these mutations confer dominant ligand-independent constitutive trans-autophosphorylation activity on the mutant RET protein (4,5). The RET gene encodes a receptor tyrosine kinase that binds neurotrophic factors of the glial cell line–derived neurotrophic factor family and is involved in the development of the kidney and in the survival of various neurons of the enteric nervous system (6,7).

Medullary thyroid carcinoma occurs in sporadic (approximately 75%) or hereditary (approximately 25%) forms. Hereditary medullary thyroid carcinoma is inherited in an autosomal dominant manner with the following three clinical manifestations: multiple endocrine neoplasia types 2A (MEN2A) and 2B (MEN2B) and familial medullary thyroid carcinoma. The MEN2A subtype accounts for approximately 90% of all inherited medullary thyroid carcinomas; most mutations affect one of the cysteine residues in the cysteine-rich domain (i.e., 609, 611, 618, 620, or 634). These mutations and those identified in familial medullary thyroid carcinoma lead to the formation of permanent receptor dimers with constitutive autophosphorylation activity. The active dimers stimulate downstream signal transduction pathways and thus promote cell transformation. The most aggressive disease is associated with the MEN2B subtype; mutations identified with this subtype appear to induce a conformational change in the tyrosine kinase domain of RET that alters substrate specificity (8–10).

Oncogenic RET proteins activate a complex network of signal transduction pathways that contributes to cellular transformation (11,12), including the Raf/Mek/extracellular signal-regulated kinase (ERK) cascade, which can regulate cell proliferation (13), and the phosphatidylinositol 3-kinase (PI3K)/Akt signal transduction pathway, which regulates cellular survival (14). Activation of the PI3K/Akt pathway is required for oncogenic RET-mediated transformation (15).

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To investigate the mechanism used by the most common oncogenic RET protein in medullary thyroid carcinoma type MEN2A cells to maintain neoplastic properties, we constructed RET\textsuperscript{3TK}, a dominant-negative truncated RET protein that lacks the entire cytoplasmic tyrosine kinase domain, to inhibit intrinsic oncogenic RET autophosphorylation. Baldassarre et al. (16) reported that a related dominant negative RET mutant efficiently inhibits glial cell line–derived neurotrophic factor–mediated RET activation and the activity of MEN2A-derived RET by dimerizing with endogenous RET to form an inactive dimer that lacks trans-autophosphorylation activity. We used adenoviral vectors to insert the RET\textsuperscript{3TK} gene into cells and tumors and investigated which signal transduction pathways are altered by the inhibition of oncogenic RET activity with RET\textsuperscript{3TK} in vivo and in vitro.

**Materials and Methods**

**Cell Lines**

The human medullary thyroid carcinoma cell line TT (product CRL-1803, American Type Culture Collection, Manassas, VA) was derived from a patient with aggressive medullary thyroid carcinoma and carries a frequent RET mutation (C634W) identified in patients with MEN2A disease (17). TT cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum. All medium contained 2 mM L-glutamine, penicillin at 100 U/mL, and streptomycin at 100 U/mL.

**Construction of Adenoviral Vectors**

Adenoviral vectors expressing truncated RET were constructed by first using the polymerase chain reaction (PCR) to obtain a cDNA fragment corresponding to amino acids 1–718 of wild-type RET with the primer pair 5′-CCGGCAAGGCGACGTCCGGTGCC-3′/H11032 and 5′-GGCGGGA AGCTTTAGAGGCTAGCATAATCAGGAACATCATACGAGCTTTAGAGGCTAGCATAATCAGGAACATCATACGGAATTC CACTTGGATCCTCCAGGATCTTGAAG-3′. These primers gave rise to the RET cDNA lacking the complete intracellular region with the tyrosine kinase domain but including an in-frame hemagglutinin tag and followed by a stop codon (Fig. 1, A). This cDNA fragment—designated RET\textsuperscript{3TK}—was then cloned into pShuttleCMV via HindIII restriction endonuclease cleavage sites that have been added to the primer sequences to generate an adenoviral vector expressing RET\textsuperscript{3TK} (Ad.RET\textsuperscript{3TK}) under control of the cytomegalovirus (CMV) promoter (Fig. 1, B). We used the AdEasy System (18) as previously described (19) for this cloning. Adenoviral vectors expressing green fluorescent protein (GFP; designated Ad.GFP) or wild-type human p53 (designated Ad.p53), used as control vectors, have also been described (20). Adenovirus maintenance, propagation, and titration in 293 cells were described previously by Pützer et al. (21).

**Western Blot Analysis**

To prepare whole cell extracts, cells were lysed in ice-cold RIPA buffer (150 mM NaCl, 10 mM Tris–HCl [pH 7.2], 0.1% sodium dodecyl sulfate, 0.1% Triton X-100, 1% deoxycholate, 5 mM EDTA, supplemented with the protease inhibitor mixture Complete Mini [Roche, Mannheim, Germany]) as previously described (19). In brief, proteins in whole-cell extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subsequently transferred to nitrocellulose membranes (Amersham Pharmacia, Braunschweig, Germany) with the Trans Blot SD semi-dry transfer system (Bio-Rad, München, Germany). Membranes were subsequently probed, according to the manufacturer’s guidelines, with antibodies against hemagglutinin (product sc-805), RET\textsuperscript{3TK} (product sc-805), RET\textsuperscript{3TK} (product sc-1290), RET H-300 (product sc-13104), β-actin (product sc-1616), or phosphorylated RET [Tyr-1062] (product sc-20252) from Santa Cruz Biotechnology, Heidelberg, Germany; with antibodies against Akt (product 9272), phosphorylated Akt (product 9271), p44/42 mitogen-activated protein kinase (i.e., ERK1/2; product 9102), phosphorylated p44/42 mitogen-activated protein kinase (product 9101), cleaved caspase 3 (product 9661), or cleaved poly(ADP-ribose) polymerase (product 9541) from Cell Signaling Technologies, Frankfurt, Germany; or with antibodies against BCL-2 (product OP60-100UG) from Calbiochem, Baden.
Soden, Germany. Primary antibodies were detected with appropriate secondary antibody–horseradish peroxidase conjugates (Amersham Pharmacia).

**Semi quantitative Reverse Transcription–PCR**

Total RNA, prepared with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol, was transcribed into cDNAs by use of oligodeoxynucleotide primers (Applied Biosystems, Darmstadt, Germany) and an Omniscript Reverse Transcription Kit (Qiagen). To obtain a semiquantitative result within the linear range, PCR was performed with the minimum number of cycles necessary to acquire a clear signal. Precise PCR conditions and primer sequences are shown in Table 1. PCR products were stained with SYBR Gold nucleic acid gel stain (Molecular Probes, Leiden, Netherlands) and quantitated in relative software units using a Bio-Imaging-Analyzer (Fuji, Düsseldorf, Germany) with the TINA program, version 2.09. All mRNA levels were normalized to the mRNA level of the ribosomal S9 gene.

**5-Bromo-2’-Deoxyuridine (BrdU) Incorporation Assay and Determination of Sub-G1 DNA Content**

To determine the cell cycle distribution, cells were treated with adenoviral vectors as indicated. Cells in S phase were labeled with BrdU at a final concentration of 10 μM for 1 hour by use of the FLUOS In Situ Cell Proliferation Kit (Roche), according to the manufacturer’s protocol. BrdU-labeled cells were measured by flow cytometry with a FACSVantage flow cytometer (BD Biosciences Immunocytometry Systems, Heidelberg, Germany).

For flow cytometry analysis to detect the population of cells with a sub-G1 DNA content, cells were harvested 72 hours after infection and fixed in 70% ethanol, and DNA was stained with propidium iodide. Flow cytometry analysis was carried out in a FACSVantage flow cytometer using CellQuest Software.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide (MTT) Assay**

Cells were placed in 96-well plates and allowed to adhere for 48 hours before infection. Cell viability in triplicate wells was determined 2 days, 4 days, and 6 days after infection by use of the CellTiter 96 AQFluor One Solution Cell Proliferation Assay (Promega, Mannheim, Germany), an MTT-based assay, as described by the manufacturer.

**Animal Experiments**

Transgenic mice expressing the germline MEN2A-derived mutant RET (C634R) have been described previously (22). Because medullary thyroid carcinoma cells produce and secrete calcitonin, serum calcitonin levels can be used to indicate tumor progression. We measured serum calcitonin with the Calcitonin Chemiluminescence Assay Kit (Nichols Institute Diagnostics, Bad Vilbel, Germany) according to the manufacturer’s protocol.

Transgenic mice were infected with adenoviral vectors as follows: Mice were fully anesthetized with ketamine (100 mg/kg of body weight) and xylazine (2 mg/kg). Thyroid glands were surgically exposed, and their volumes were measured in two perpendicular diameters with calipers. A total of 1 × 10⁸ plaque-forming units of adenoviral vectors (5 × 10⁸ plaque-forming units per thyroid lobe) were injected into multiple sites on different sides of each thyroid gland to ensure uniform distribution, and the incision was sutured. We treated a total of 13 animals with Ad.RETΔTK and another 12 with Ad.GFP. Fourteen days later the animals were killed, and the thyroid glands were exposed and measured with calipers. Tumor volumes were calculated from the longest diameter and average width by assuming a prolate spheroid shape (23). Previous comparison of thyroid and tumor volumes revealed these volumes were statistically significantly correlated with each other, thus, we used thyroid volume as an indication of tumor size (24). Tumor tissue was fixed in phosphate-buffered formalin, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin for histologic examination. Phosphorylated ERK and phosphorylated Akt proteins in tumor sections were immunohistochemically labeled by use of antibodies specific for phosphorylated p44/42 mitogen-activated protein kinase (Thr-202/Tyr-204; product 9101), phosphorylated Akt (Ser-473; product 9277), unphosphorylated Akt (product 9272), and unphosphorylated p44/42 mitogen-activated protein kinase (ERK1/2; product 9102) (all from Cell Signaling Technologies). All animal experiments were approved by and carried out according to the guidelines set forth by the Animal Research Ethics Board of the University of Essen, Essen, Germany.

**Statistical Analysis**

Variations in phosphorylation and/or expression levels of various cellular proteins, BrdU incorporation, DNA content in the sub-G1 population of cells, and tumor size were compared with an unpaired Student’s t test. All statistical tests were two-sided.

**RESULTS**

**RETΔTK Protein Expression**

To confirm that the adenoviral vector Ad.RETΔTK can express RETΔTK, we infected RET-negative H1299 cells with Ad.RETΔTK vectors, and 48 hours later, we measured the expression of RETΔTK protein in these cells or in uninfected
cells by use of western blot analysis. As shown in Fig. 1, C, RET\(^\Delta\)TK protein was expressed in a viral load-dependent manner, as detected by use of antibodies against either the hemagglutinin tag attached to the recombinant protein or the extracellular RET epitope by the use of the RET antibody H-300, which recognizes an extracellular epitope. The truncated RET proteins were identified in bands of 135 and 115 kd, corresponding to RET\(^\Delta\)TK proteins located at the cell surface and the endoplasmic reticulum, respectively. RET proteins are generally detected as fully glycosylated proteins located in the cell membrane and immature glycosylated proteins present in the endoplasmic reticulum (6).

**RET Autophosphorylation and Cell Viability**

To determine whether the expression of RET\(^\Delta\)TK interferes with RET-mediated signal transduction, we used the TT cell line, a well-established in vitro model for medullary thyroid carcinoma. Expression of RET\(^\Delta\)TK in TT cells decreased RET-mediated trans-autophosphorylation 1 and 2 days after transduction (Fig. 2), as measured by the phosphorylation of tyrosine residue 1062, a multifunctional docking site for numerous downstream effector proteins (16). The level of total endogenous RET protein remained constant, indicating that RET\(^\Delta\)TK interfered with endogenous RET-mediated trans-autophosphorylation but not with RET expression. Because oncogenic RET stimulates cell proliferation and survival (16,17), we next investigated whether the expression of RET\(^\Delta\)TK and the correspondingly reduced endogenous RET trans-autophosphorylation altered TT cell viability. We infected TT cells with adenoviral vectors expressing GFP (Ad.GFP), wild-type p53 (Ad.p53), or RET\(^\Delta\)TK (Ad.RET\(^\Delta\)TK) and assayed cell viability over a 6-day period. Infection with Ad.GFP had no effect on cell viability, whereas decreased cell survival was detected after infection with Ad.p53 (survival rate of 39.8% after 6 days) or with Ad.RET\(^\Delta\)TK (survival rates of 74.7% after 4 days and 45.9% after 6 days) compared with the viability of untreated control TT cells (Fig. 3, A). To investigate whether RET\(^\Delta\)TK alone inhibited cell viability, we infected RET-negative H1299 cells with the same vectors used in TT cells above and investigated the viability of these cells. Infection with Ad.p53 decreased the viability of H1299 cells (survival rates of 28.3% after 4 and 27.4% after 6 days; Fig. 3, B) compared with that of untreated control H1299 cells, but infection with Ad.GFP or with Ad.RET\(^\Delta\)TK did not alter the viability of H1299 cells. Thus, RET\(^\Delta\)TK appears to act by specifically blocking the action of oncogenic RET.

**Phosphorylation of Downstream Effectors ERK1/2 and Akt**

We further characterized the mechanism of the loss of cell viability by determining whether transformation-relevant signal transduction pathways were inhibited. TT cells were infected with Ad.RET\(^\Delta\)TK or with Ad.GFP vectors (as a control to exclude virus-related effects), and then the phosphorylation status of ERK1/2 and Akt was assessed. ERK1/2 and Akt are kinases with major roles in neoplastic transformation because they are part of two important signal transduction pathways activated by oncogenic RET (13–15); both kinases are activated by phosphorylation. Infection with the control Ad.GFP virus vector did not alter the endogenous constitutive phosphorylation of ERK1/2 or Akt, whereas 2 days after infection with Ad.RET\(^\Delta\)TK vectors, phosphorylation of ERK1/2 was substantially decreased, and 2 days after such an infection, phosphorylation of Akt was not detected (Fig. 4). Thus, expression of RET\(^\Delta\)TK inhibits the phosphorylation of both ERK1/2 and Akt in TT cells.

**Regulation of Cell Proliferation and Survival**

To investigate how reduced levels of phosphorylated ERK and Akt may contribute to the loss of TT cell viability, we analyzed the regulation of cell proliferation and apoptosis by these two pathways and determined whether changes in the expression of various factors regulated by activated ERK and/or Akt were associated with reduced cell viability. Because Akt and ERK participate in the control of cell proliferation (25–27), we first assessed the fraction of actively proliferating cells by determining whether transformation-relevant signal transduction pathways were inhibited. TT cells were infected with Ad.RET\(^\Delta\)TK or Ad.GFP. The percentage of TT cells in S phase after the expression of RET\(^\Delta\)TK was statistically significantly reduced (3.9%, 95% CI = 2.3% to 5.5%) compared with that of untreated TT cells (10.2%; difference = 6.3%, 95% CI = 7.9% to 12.5%; P = .039) or that of TT cells infected with
To investigate the effects of RET\textsuperscript{ΔTK} \textit{in vivo}, we used a medullary thyroid carcinoma model, the RET C634R transgenic mouse that develops orthotopic bilateral medullary thyroid carcinoma with almost complete penetrance at 6–8 months of age (22,24). Because medullary thyroid carcinomas secrete calcitonin, we monitored serum calcitonin levels between 6 and 12 months after birth to determine when the tumors developed. After calcitonin levels increased, we confirmed the presence of tumors immunohistochemically (data not shown). After serum calcitonin levels increased to more than 1000 pg/mL, usually after 10–12 months, we exposed the thyroid glands of 13 mice to calcitonin (30), 48 and 72 hours after infection with Ad.RET\textsuperscript{ΔTK} but not after infection with Ad.GFP (Fig. 5, C). Thus, inhibition of oncogenic RET activity with RET\textsuperscript{ΔTK} inhibits cell proliferation by increasing the expression of genes for the cyclin-dependent kinase inhibitors p21\textsuperscript{CIP1/WAF1} and p27\textsuperscript{KIP1} as well as by decreasing the expression of cyclin D1 and induces apoptosis by suppressing the activity of antiapoptotic proteins.

Treatment of Medullary Thyroid Carcinoma \textit{In Vivo}

Ad.GFP (9.4%; difference = 5.5%, 95% CI = 8.0% to 10.8%; P = .004) (Fig. 5, A, upper panel). Second, we investigated whether oncogenic RET influences cell cycle progression by activating ERK and/or Akt, by measuring changes in the expression of the cell cycle regulators cyclin D1, p21\textsuperscript{CIP1/WAF1}, and p27\textsuperscript{KIP1}. Thirty-six hours after treatment with Ad.RET\textsuperscript{ΔTK}, when endogenous RET trans-autophosphorylation was inhibited, expression of mRNAs for the cyclin-dependent kinase inhibitors p21\textsuperscript{CIP1/WAF1} and p27\textsuperscript{KIP1} increased, and expression of cyclin D1 mRNA decreased substantially, indicating that oncogenic RET enhances cell cycle progression in medullary thyroid carcinoma cells. Because Akt enhances cell survival and inhibits apoptosis (25,26), we investigated whether oncogenic RET inhibited apoptosis by a pathway involving Akt phosphorylation and BCL-2, an antiapoptotic protein that is regulated by Akt (28,29), as well as other factors. We found reduced levels of BCL-2 protein 48 hours after infection with Ad.RET\textsuperscript{ΔTK} (Fig. 5, C), indicating that oncogenic RET may suppress the induction of apoptosis by activating pro-survival pathways. To test this possibility, we explored whether the RET\textsuperscript{ΔTK}-mediated decreased cell viability is accompanied by the induction of apoptosis by determining the percentage of apoptotic TT cells (i.e., the population of cells with a sub-G₁ DNA content) 72 hours after infection. Cultures infected with Ad.RET\textsuperscript{ΔTK} contained more apoptotic cells (18.1%, 95% CI = 12.4% to 23.8%) than uninfected cultures (5.3%, 95% CI = 4.1% to 6.5%; P = .006) or cultures infected with Ad.GFP (mock infected) (8.5%, 95% CI = 8.4% to 8.6%; P = .04) (Fig. 5, A, bottom panel). We also detected cleaved products of procaspase 3 and poly(ADP-ribose) polymerase, which are also indicative of apoptosis (30), 48 and 72 hours after infection with Ad.RET\textsuperscript{ΔTK} but not after infection with Ad.GFP (Fig. 5, C). Thus, inhibition of oncogenic RET activity with RET\textsuperscript{ΔTK} inhibits cell proliferation by increasing the expression of genes for the cyclin-dependent kinase inhibitors p21\textsuperscript{CIP1/WAF1} and p27\textsuperscript{KIP1} as well as by decreasing the expression of cyclin D1 and induces apoptosis by suppressing the activity of antiapoptotic proteins.

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In medullary thyroid carcinoma, activated oncogenic RET plays a major role in cellular transformation (8–10). In this study, we disrupted signaling by oncogenic RET to investigate the mechanisms used by oncogenic RET to contribute to tumor development and to begin a foundation for an alternative therapeutic approach to medullary thyroid carcinoma. We used a dominant negative truncated RET mutant, designated RET$^{\Delta TK}$, to block oncogenic RET activity in TT cells, a line derived from a patient with medullary thyroid carcinoma. Our results and those from Baldassarre et al. (16) indicate that co-expression of RET$^{\Delta TK}$ is capable of blocking endogenous RET trans-autophosphorylation, most likely by forming an inactive dimer with oncogenic RET, because RET$^{\Delta TK}$ lacks the entire cytoplasmic tyrosine kinase domain, which is required to bind and activate signaling adapter molecules when phosphorylated (11) thereby ablating RET trans-autophosphorylation activity. The constitutive tyrosine kinase activity of oncogenic RET appears to be a prerequisite for cellular transformation, at least in the MEN2A type of medullary thyroid carcinoma, in which mutations in the cysteine-rich region of RET lead to the constitutive formation of active dimers. We found that disrupting oncogenic RET activity decreased cell viability and cell proliferation, indicating that oncogenic RET stimulates proliferation and blocks apoptosis. Results of previous reports (13–15) indicate that the PI3K/Akt and Raf/ERK signaling pathways contribute to RET-mediated transformation. Our results also indicate that both pathways are involved, because the activities of Akt and ERK1/2 are eliminated after the expression of the dominant negative RET inhibitor RET$^{\Delta TK}$. Both pathways regulate proliferation and/or survival by different but overlapping mechanisms (25,27).

Consistent with another report (31), we detected elevated transcription of cyclin D1 mRNA in cells expressing oncogenic RET. Cyclin D1 mRNA is tightly regulated by the Raf/Mek/ERK cascade through phosphorylation of the API1 transcription factor complex (32). In addition, the PI3K/Akt pathway has also been implicated in the regulation of cyclin D1 by sustaining protein stability with a mechanism involving interference with...
Fig. 6. Antitumor activity of the adenoviral vector Ad.RET<sup></sup>TK against primary medullary thyroid carcinomas in RET C634R transgenic mice. A) Tumor volumes were assessed on the day of virus injection and 14 days after treatment. The difference in the relative increase of the size of Ad.GFP- versus Ad.RET<sup></sup>TK-treated tumors at day 14 was statistically significant (P < 0.01). Tumor volume before treatment was statistically significantly larger than that after treatment with Ad.RET<sup></sup>TK (Ad.RET<sup></sup>TK [day 0] versus Ad.RET<sup></sup>TK [day 14]; P = 0.010). B) Total and phosphorylated (phospho-) extracellular signal-regulated kinase 1/2 (ERK1/2) (44/42 kd) and Akt (62 kd) levels in Ad.GFP- and Ad.RET<sup></sup>TK-treated tumors 14 days after injection. Left: Whole-cell protein extracts were prepared from tumor tissue and analyzed by western blot. Right: Immunohistochemical staining of paraffin-embedded tissue for phosphorylated Akt and phosphorylated ERK after RET<sup></sup>TK treatment. Micrographs show the cytoplasmic, nuclear, and membrane localization of phosphorylated Akt, and the nuclear localization of phosphorylated ERK1/2 in tumors treated with Ad.GFP or Ad.RET<sup></sup>TK. Scale bar = 100 µm. Arrowheads indicate phosphorylated Akt and ERK1/2 proteins in tumor tissue.

protein degradation (25). Furthermore, we observed increased expression of mRNAs for cyclin-dependent kinase inhibitors p21<sup>CIP1/WAF1</sup> and p27<sup>KIP1</sup> after oncogenic RET activity was inhibited by RET<sup></sup>TK. Because Akt inhibits the expression of p21<sup>CIP1/WAF1</sup> and p27<sup>KIP1</sup> by phosphorylating the transcription factor AFX/FKHR (25,33), oncogenic RET may also contribute to enhanced cell cycle progression, possibly by inhibiting the expression of p21<sup>CIP1/WAF1</sup> and p27<sup>KIP1</sup> mRNA through the PI3K/Akt pathway. However, because RET, ERK, and Akt have many downstream targets, other targets could also be responsible for the observed decrease in cell number in TT cells infected with Ad.RET<sup></sup>TK. In addition to the Raf/ERK and PI3K/Akt cascades, RET activates a complex network of signal transduction pathways, including the c-Jun amino-terminal kinase pathway, the p38 mitogen-activated protein kinase pathway, the extracellular signal-regulated kinase 5 pathway, or the signal transducer and activator of transcription 3 pathway (11,34). Akt can also modulate cell cycle progression via many other mechanisms, including the regulation of proliferating-cell nuclear antigen or phosphorylation of glycogen synthase kinase 3β that leads to inhibition of cyclin D1 protein degradation (35,36). The variety of pathways potentially regulated by RET and their contribution to cancer have still to be discovered in detail.

After oncogenic RET activity was disrupted, cells actively underwent apoptosis, indicating that oncogenic RET appears to inhibit apoptosis. Other oncogenic tyrosine kinases, including MET, BCR-ABL, HER-2/neu, or RON, have antiapoptotic activity that is involved in cellular transformation. This activity has also been attributed to enhanced PI3K/Akt signaling, although Raf/ERK activation may be involved as well (37–40). Thus, stimulation of antiapoptotic signaling might be a common mechanism used by oncogenic tyrosine kinases in the multistage process of tumorigenesis.

Because BCL-2 plays a major role in medullary thyroid carcinoma progression and because the level of BCL-2 protein expression is generally elevated in medullary thyroid carcinomas (41), we examined the expression of BCL-2 and found that inhibition of oncogenic RET activity decreased the level of BCL-2 protein. BCL-2 strongly inhibits apoptosis by maintaining mitochondrial membrane integrity and thus preventing caspase activation (42). Therefore, our results provide an explanation of how oncogenic RET stimulates cell survival. BCL-2 expression, among other mechanisms, is tightly regulated by nuclear factor-κB transcription factors (28), which in turn are stimulated by enhanced Akt activity by the phosphorylation of IκB kinase (29). Thus, stimulation of Akt may be one mechanism that links oncogenic RET activity with pro-survival signaling mediated by enhanced BCL-2 expression.

Our results suggest that orthotopic medullary thyroid carcinomas in transgenic mice also develop a state of dependence on signals mediated by activated oncogenic RET. Injection of RET<sup></sup>TK into primary tumors led to a statistically significantly decreased tumor volume after 2 weeks compared with that in mice injected with the appropriate control vectors. Our results indicate that inhibition of oncogenic RET activity might be a viable target for the development of new treatments for medullary thyroid carcinomas. Results of previous studies have demonstrated that oncogenic RET activity can be nonspecifically blocked by small-molecule compounds (43,44) or the expression of tyrosine phosphatases (45). In this study, we describe pheno-
oncogenic RET may be the most promising target. For medullary thyroid carcinoma therapy, we propose that disrupting these results alone could have important implications for future pathways. Oncogenic RET increases antiapoptotic signaling by autophosphorylation with RET/H9004.

Repression of mRNA of the cyclin-dependent kinase inhibitors p21CIP1/WAF1 and the extracellular signal-regulated kinase 1/2 and cyclin D1 mRNA induction is accompanied by enhanced S-phase progression via constitutive activation and enhanced proliferation triggered by a MEN2A-derived RET oncogene. RET is activated by increased S-phase progression via constitutive activation of the extracellular signal-regulated kinase 1/2 and cyclin D1 mRNA induction. Repression of mRNA of the cyclin-dependent kinase inhibitors p21CIP1/WAF1 and p27KIP1 also confers to elevated cell cycle progression. Oncogenic RET directly stimulates protein expression of the antiapoptotic factor BCL-2 via a pathway involving Akt phosphorylation thereby suppressing apoptosis.

typic changes induced by specifically inhibiting RET trans-autophosphorylation with RET-TK.

In summary, oncogenic RET increases cell proliferation by regulating the expression of cyclin D1, p21CIP1/WAF1, and p27KIP1 mRNAs through the PI3K/Akt and Raf/ERK signaling pathways. Oncogenic RET increases antiapoptotic signaling by increasing the expression of BCL-2 (Fig. 7). Although each of these results alone could have important implications for future medullary thyroid carcinoma therapy, we propose that disrupting oncogenic RET may be the most promising target.

REFERENCES


NOTES

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