RET/PTC-induced gene expression in thyroid PCCL3 cells reveals early activation of genes involved in regulation of the immune response

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Abstract

RET/PTC rearrangements represent key genetic events involved in papillary thyroid carcinoma (PTC) initiation. The aim of the present study was to identify the early changes in gene expression induced by RET/PTC in thyroid cells. For this purpose, microarray analysis was conducted on PCCL3 cells conditionally expressing the RET/PTC3 oncogene. Gene expression profiling 48 h after activation of RET/PTC3 identified a statistically significant modification of expression of 270 genes. Quantitative PCR confirmation of 20 of these demonstrated 90% accuracy of the microarray. Functional clustering of genes with greater than or less than 1.75-fold expression change (86 genes) revealed RET/PTC3-induced regulation of genes with key functions in apoptosis (Ripk3, Tdga), cell–cell signaling (Cdh6, Fn1), cell cycle (Il24), immune and inflammation response (Cxcl10, Scya2, Il6, Gbp2, Oas1, Tap1, RT1Aw2, C2ta, Irf1, Lmp2, Psme2, Prkr), metabolism (Aldob, Ptges, Nd2, Gss, Gstt1), signal transduction (Socs3, Nf1, Jak2, Cpg21, Dusp6, Socs1, Stat1, Stat3, Cish) and transcription (Nr4a1, Junb, Hfh1, Runx1, Foxe1). Genes coding for proteins involved in the immune response and in intracellular signal transduction pathways activated by cytokines and chemokines were strongly represented, indicating a critical role of RET/PTC3 in the early modulation of the immune response.

Endocrine-Related Cancer (2005) **12** 319–334

Introduction

Rearrangements of the proto-oncogene *RET* resulting in its constitutive activation are believed to play a causative role in the pathogenesis of a significant proportion of papillary carcinomas of the thyroid (PTC) (Santoro *et al.* 2002). The *RET* gene encodes a transmembrane tyrosine kinase (TK) receptor whose expression and function is normally restricted to a subset of cells derived from the neural crest. In thyroid follicular cells, RET activation occurs through chromosomal recombination resulting in illegitimate expression of a fusion protein consisting of the intracellular TK domain of RET coupled to the N-terminal fragment of a heterologous gene. The promoter region of the partner gene drives constitutive expression of the chimeric gene. There are at least ten different types of *RET/PTC* that differ according to the 5′ partner gene involved in the rearrangement. *RET/PTC1* and *RET/PTC3* are the most common types, accounting for more than 90% of all the rearrangements. The resulting fusion proteins dimerize in a ligand-independent manner and constitutively activate the TK function of RET (Durick *et al.* 1995, Tong *et al.* 1997). Several signaling pathways activated by RET/PTC have been identified. Among these, autophosphorylation of tyrosine residues Y905, Y1015, Y1062 of the full length of RET, which form docking sites for Grb7/Grb10, PLCγ and Shc/Snt(Frs2)/Enigma respectively (Borrello *et al.* 1996, Arighi *et al.* 1997, Durick

DOI:10.1677/erc.1.00947
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et al. 1998), are required for its oncogenic function *in vitro*.

RET rearrangements are found in 5–40% of PTCs in the adult population (Santoro *et al.* 2002). They are significantly more prevalent in pediatric PTCs, and in cancers from children exposed to radiation after the Chernobyl nuclear accident (Fugazzola *et al.* 1995, Bongarzone *et al.* 1996, Nikiforov *et al.* 1997). There is compelling evidence pointing to *RET*/*PTC* as a key first step in thyroid cancer pathogenesis (Ito *et al.* 1993, Viglietto *et al.* 1995, Jhiang *et al.* 1996, Santoro *et al.* 1996, Mizuno *et al.* 1997, Powell *et al.* 1998, Sugg *et al.* 1998, Nikiforov *et al.* 1999, Nikiforova *et al.* 2000). Moreover, recent molecular genetic studies show the consistent occurrence of mutations of genes coding for effectors signaling along the MAP kinase (MAPK) pathway in PTCs, namely rearrangements of the receptor TKs *RET* or *TRK*, as well as point mutations of the intracellular signaling proteins *B-RAF* and *RAS*. Interestingly, there is little or no overlap between these genetic events in the same cancer, pointing to the requirement for activation of MAPK for thyroid cell transformation to PTC.

Relatively little is known about the events that follow *RET*/*PTC* activation and that may be associated with PTC progression. Acute *RET*/*PTC* activation stimulates both DNA synthesis and apoptosis in thyroid cells, is associated with loss of thyroid-specific differentiation, and interferes with thyrotropin (TSH) receptor-mediated intracellular signaling at various levels (Wang *et al.* 2003). Moreover, it triggers a rapid and vigorous induction of microsomal prostaglandin E synthase-1 (mPGES-1) and cyclooxygenase (COX)-2 expression and stimulation of PGE_2 biosynthesis (Puxeddu *et al.* 2003). Very recently, thyroid cells engineered to express *RET*/*PTC3* have also been shown to produce high amounts of monocyte chemoattractant protein 1 (MCP1) and granulocyte-macrophage colony stimulating factor (GM-CSF) (Russell *et al.* 2003) and to express osteopontin and its receptor CD44, all of which have been implicated in the determination of the mitogenic and invasive phenotype of RET/PTC-transformed thyroid cells (Castellone *et al.* 2004*a*,*b*). Taken together, these data suggest that expression of RET/PTC in normal follicular cells induces significant phenotypic changes oriented towards neoplastic transformation.

The RET/PTC oncoproteins may be directly involved in inducing inflammatory responses in thyroid tissues. This hypothesis is supported by the observation that RET/PTC3-expressing thyrocytes express high levels of proinflammatory cytokines such as MCP1 and GM-CSF (Russell *et al.* 2003), the appearance of inflammatory cells in thyroid glands from RET/PTC1 and RET/PTC3 transgenic mice (Jhiang *et al.* 1996, Santoro *et al.* 1996, Powell *et al.* 1998), the rejection of transplanted RET/PTC3-expressing thyroid tumors in syngeneic mice (Powell *et al.* 2001) and the demonstration of immunogenicity of the RET/PTC3 protein (Powell *et al.* 2003). This has led to speculation that RET/PTC may account for the chronic inflammatory infiltrates and foci of thyroiditis observed in 10–30% of tumor specimens of patients with PTC (Kebebew *et al.* 2001). Some authors report the detection of RET/PTC in non-neoplastic Hashimoto's thyroiditis (Wirtschafter *et al.* 1997), although this has not been confirmed by others (Nikiforov 2002). RET has recently been shown to regulate the activation and transcriptional activity of Stat1 and Stat3 (Schuringa *et al.* 2001, Hwang *et al.* 2003, Hwang *et al.* 2004). Stats are a family of latent transcription factors that are activated in response to many cytokines and growth factors and play critical roles in the regulation of the immune response and cellular growth. RET/PTC expression induces the phosphorylation of Stat1 at Y701, resulting in the induction of expression of interferon (IFN)-responsive genes (Hwang *et al.* 2004). Moreover, MEN2A-RET (Schuringa *et al.* 2001), a constitutive active mutant of RET causing multiple endocrine neoplasia type 2A, as well as RET/PTC (Hwang *et al.* 2003), associates with Stat3 and induce its phosphorylation and transcriptional activity (Schuringa *et al.* 2001). The evidence that oncogenic RET mutants regulate Stat3 is of interest because of its role in tumorigenesis. Stat3 is up-regulated in many human cancer types, protects from apoptosis and promotes cell proliferation in several cell types. In addition, Stat3 induces the expression of genes involved in survival and proliferation (reviewed in Pedranzini *et al.* 2004). Moreover, Stat3 is required for development of skin tumors in the two-step model of chemically induced skin carcinogenesis (Chan *et al.* 2004). Stat3 has also been shown to inhibit the immune response elicited by growing and invading tumor cells either by suppressing release of proinflammatory stimuli or by repressing the capacity of dendritic cells to sense tissue damage and the presence of inflammation (Wang *et al.* 2004). By contrast, Stat1 mediates primarily proinflammatory signals in part by increasing transcription from type IV IFN-γ-responsive promoters of class II transactivator genes (Hwang *et al.* 2004).

Here we report the results of gene expression profiling experiments performed in PCCL3 cells induced to conditionally express RET/PTC3. Two hundred and seventy genes displayed a statistically significant

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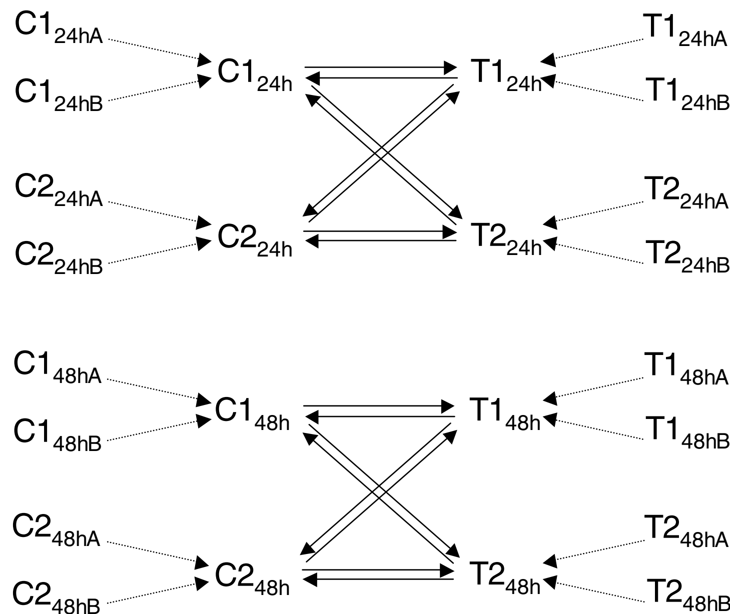

Figure 1 Design of the microarray experiment. Microarray slides were co-hybridized (→) with cDNA from PTC3-5 cells treated with (T) or without (C) dox for 24 or 48 h. Each RNA sample contained the pooled (arrows with dotted lines) RNA from two plates ($_A$ and $_B$). For each time point, cDNA from two independent experiments was tested (1 and 2). Cross-co-hybridization (arrows at 45 degrees) between treated and control samples from two independent experiments was also performed. Every co-hybridization was repeated a second time switching the Cy3 and Cy5 dyes (←). Altogether, for each time point eight independent hybridizations were performed.

RET/PTC3-induced modification of expression. As expected, a subset of these genes represented possible mediators of RET/PTC-dependent thyrocyte transformation and others grouped into a variety of functional classes. A particularly striking subset of genes (14 out of 86 with greater than or less than 1.75-fold change) belongs to a cluster involved in the regulation of the immune response.

Experimental procedures

Cell lines and reagents

PCCL3 cells, a clonal rat thyroid line requiring TSH for growth, were maintained in H4 complete medium consisting of Coon's medium/F12 high zinc supplemented with 5% fetal bovine serum, 0.3 mg/ml L-glutamine, 1 mIU/ml TSH, 10 µg/ml insulin, 5 µg/ml apo-transferrin, 10 nM hydrocortisone and penicillin/streptomycin. H3 complete medium was identical to H4 complete medium but without addition of TSH. The PTC3-5 line was derived from PCCL3 cells to obtain doxycycline (dox)-inducible expression of RET/PTC3, as described elsewhere (Wang *et al.* 2003).

RNA isolation

PTC3-5 cells were grown in 10 cm dishes until almost confluent and then preincubated with H3 medium for 3 days before addition of dox. After incubation with or without 1 µg/ml dox for the indicated time, total RNA was isolated using TRIzol reagent (Invitrogen) (1 ml for a 10 cm dish) following the manufacturer's recommended protocol. The RNA was further purified using RNeasy Mini Kit (Qiagen) following the accompanying RNA Cleanup protocol.

Microarray analysis

Experimental design is depicted in Fig. 1. Microarray slides were spotted with a combination of a commercial rat 70-mer oligonucleotide library as well as purified PCR products of a cDNA subtractive suppression hybridization library generated from PTC3-5 cells with or without expression of RET/PTC3. The rat 70-mer oligonucleotide library, representing 4273 known genes, was obtained from Qiagen-Operon (Alameda, CA, USA). The PTC3-5 substractive suppression hybridization library was generated in our laboratory and included 364 known sequences. The construction

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of this library and the purification of the library PCR products for custom microarray generation has been described elsewhere (Puxeddu *et al.* 2003). The oligonucleotides and the PCR products were spotted in duplicate at 22 °C and 65% relative humidity using a high-speed robotic machine (OmniGrid model; GeneMachines, San Carlos, CA, USA) with Stealth pins (SMP 3 pins) from Telechem (Sunnyvale, CA, USA) on a mounted 48-pin head. Spot volumes were 0.5 nl and spot diameters were 75–85 µm. The 70-mer oligonucleotides and the PCR products were suspended in 3 × SSC at 30 mM and 0.2–1 µg/µl respectively, and printed on aminosilane-coated UltraGAPS slides (Corning, Acton, MA, USA). The DNAs were crosslinked to the slide substrate by exposure to 600 mJ of ultraviolet light. For the hybridization step (DeRisi *et al.* 1996), the slides were placed in a hybridization station (Genomic Solutions, Inc., Ann Arbor, MI, USA) using Microarray Hybridization Buffer #1 from Ambion (Austin, TX, USA). Details of the hybridization protocol can be found at http://microarray.uc.edu.

The oligonucleotides on the microarray slides were co-hybridized with cDNA from PTC3-5 cells treated with or without dox for 24 or 48 h. Each RNA sample contained the pooled RNA from two plates. For each time point, cDNA from two independent experiments was tested. Cross-co-hybridization between treated and control samples from two independent experiments was also performed. Every co-hybridization was repeated a second time switching the Cyanine-3 and Cyanine-5 dyes (Cy3 and Cy5) (Amersham). Altogether, for each time point, eight independent hybridizations were performed (Fig. 1).

Fluorescence-labeled cDNAs were synthesized from 20 µg total RNA using an indirect amino allyl labeling method (DeRisi *et al.* 1996). The cDNA was synthesized by an oligo(dT)-primed, reverse transcriptase reaction, and the cDNA labeled with monofunctional reactive Cy3 and Cy5 dyes. Details can be found at http://microarray.uc.edu. Imaging and data generation were carried out using a GenePix 4000A and GenePix 4000B and associated software from Axon Instruments, Inc. (Foster City, CA, USA). The microarray slides were scanned with dual lasers with wavelength frequencies to excite Cy3 and Cy5 fluorescence emission. Images were captured in JPEG and TIFF files, and DNA spots captured by the adaptive circle segmentation method. Information extraction for a given spot was based on the median value for the signal pixels minus the median value for the background pixels to produce a gene set data file for all the DNA spots.

Data analysis and normalization

The data representing raw spot intensities generated by the GenePix software were analyzed to identify differentially expressed genes and characterize gene expression profiles under different experimental conditions. Data normalization was performed separately in three steps for each microarray. First, channel-specific local background intensities were subtracted from the median intensity of each channel (Cy3 and Cy5). Secondly, background-adjusted intensities were log-transformed and the differences (R) and averages (A) of log-transformed values were calculated as $R = \log_2(X1) - \log_2(X2)$ and $A = [\log_2(X1) + \log_2(X2)]/2$, where X1 and X2 denote the Cy5 and Cy3 intensities of each spot after subtracting local backgrounds respectively. Thirdly, data centering was performed by fitting the array-specific local regression model of R as a function of A (Yang *et al.* 2002). The difference between the observed log-ratio and the corresponding fitted value represented the normalized log-transformed gene expression ratio. Normalized log-intensities for the two channels were then calculated by adding a half of the normalized ratio to A for the Cy5 channel and subtracting half of the normalized ratio from A for the Cy3 channel.

Identification of differentially expressed genes

The statistical analysis was performed for each gene separately by fitting the following mixed effects linear model (Wolfinger *et al.* 2001): $Y_{ijkl} = \mu + A_i + S_j + C_k + C * A_{ik} + R_{l(i)+}\varepsilon_{ijkl}$, where Y_{ijkl} corresponds to the normalized log-intensity on the ith array ($i = 1, \ldots, 16$), with the jth treatment-time combination ($j = 1, \ldots, 4$ for control 24 and 48 h, and treated 24 and 48 h respectively), labeled with the kth dye ($k = 1$ for Cy5, and 2 for Cy3), and on the lth replicate spot of the ith array ($l(i) = 1,2$). μ is the overall mean log-intensity, A_i is the effect of the ith array, S_j is the effect of the jth treatment time and C_k is the effect of the kth dye. Assumptions about model parameters were the same as described (Wolfinger *et al.* 2001), with array and replicate spot effects assumed to be random, and treatment and dye effects assumed to be fixed. Statistical significance of the differential expression between treatment and control at 24 and 48 h, after adjusting for the additional effects, was assessed by calculating *P*-values for corresponding linear contrasts. Multiple hypothesis testing adjustment was performed by calculating false discovery rates (Hochberg & Benjamini 1990, Benjamini *et al.* 2001). Data normalization and statistical analyses were performed using SAS statistical software package (SAS Institute, Inc., Cary, NC, USA).

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Quantitative RT-PCR

Confirmation of the microarray results was carried out by quantitative real-time PCR (QPCR) analysis. Cells were harvested at the indicated time and RNA isolated using TRIreagent as directed by the manufacturer (Molecular Research Center, Inc., Cincinnati, OH, USA). One microgram of total RNA was reverse transcribed in a 20 µl reaction containing 200 U SuperScript III reverse transcriptase (Invitrogen), 50 ng random primers, 500 µM dNTP mix, 5 mM dithiothreitol and 40 U RNaseOUT (Invitrogen) for 60 min at 50 °C, followed by a 15 min heat inactivation at 70 °C. Twenty-five nanograms of the obtained cDNA were then used as a template in a PCR reaction containing 200 nM of the primer pairs for amplification of either β-actin or the indicated gene product (Table 1). PCR amplifications were performed using the Platinum SYBR Green qPCR SuperMix UDG as directed by the manufacturer (Invitrogen). The amplification conditions were optimized for the PerkinElmer Life Sciences ABI 5700 instrument (Applied Biosystems, Foster City, CA, USA) and assays were run in triplicate under the following conditions: Platinum Taq polymerase activation, 50 °C for 2 min followed by 95 °C for 2 min; PCR, 50 cycles of 95 °C, 15 s and 60 °C, 30 s. PCR products were demonstrated to be a single PCR product by melting curve and electrophoretic analysis. To avoid potential problems from contaminating genomic DNA, PCR primers were designed to span introns whose location was determined by blasting each rat cDNA sequence of interest against the NCBI rat genome database. This was further verified by the absence of signal when reactions were performed without reverse transcriptase. The cycle threshold (CT) value coupled with individualized amplification efficiencies for each primer set was used to calculate the normalized expression of the indicated gene mRNA using the *Q-Gene* program (Muller *et al.* 2002).

Table 1 Primers used for the real-time quantitative PCRs

	Primers sequences (5′ – 3′)
β-Actin	TCATCACTATCGGCAATGAG CTTTACGGATGTCAACGTCA
STAT-1	TGAAGCTGAGACTGTTGGTG GAACTCAGCTGCCAAACTTC
BTG3	TAGCAGGGCTCTTGATAAGG CATTGGAAGAGGTGGGAATA
TMOD1	TGGATGAGCTAGACCCTGAT CTCGTTTCTCCCCTGTGTAG
IL-6	CAGGGAGATCTTGGAAATGA AACTCCAGAAGACCAGAGCA
PSMB9	GGACGCAGCTTATAAACCAG TGGTGACCAGGTAGATGACA
RTN4	CCAGGCTATCCAGAAATCAG CCGCCTCAGTTCTTTTATTG
CEACAM 1	TGAGAAGCTCCAGACATCCT CTTTGACTGTGGTGTTGGTG
IRF1	GACGGACTGAGCAGCTCTAC CTGCATATGCCACTCAGAGA
PRKR	GGTACTGGTTTCGGTGCTAA ATAGACGAGCTGCTGGAAAA
PSME2	AAGGTGCTGGAGAGAGTGAA TCCATTACATGGGTGTCCTT
C2TA	GAGAACAAGATCGGGGACAA GCTCAGCCTTAGGAGGGACT
TAA1	TCCCAACTCCACTCATTTCC TCCGATGATCAGGACACAAA
OGFR	GCCGCGCTTCCACAATC GGGCCCCCAGACAAACTC
PNLIPRP1	CCGGGACTTTGTGGCTTGTAACC ATCGGCATAGTGACCCATCTG
CHRNB1	CATCGAGTCTCTCCGTGTCA TGCAATTCTGCCAGTCAAAG
S100A10	TTGACAAAGGAGGACCTGAGA CCCCGCCACTAGTGATAGAA
PER3	TGGCAGTGTTTTCGTTTCTG CTGCAGAAAAAGGGCTTCAC
Scya2	ATGCAGTTAATGCCCCACTC TTCCTTATTGGGGTCAGCAC
GBP2	CTCGACTGTGCATCAGGAAA TAGGTCTGCACCAGGCTCTT
TAP1	TTCATGATTTGGGGGTCATT GCCTAAACTTCTGGGCCTCT

The sense primer is listed first and the antisense primer second.

Functional clustering

Differentially expressed genes were clustered in 12 functional categories based on the ‘Biological Process’ principle of the GeneOntology Consortium. The categories included: *Apoptosis*, *Cell–cell signaling*, *Cell cycle*, *Cell motility*, *Cell organization and biogenesis*, *Developmental process*, *Immune and inflammation response*, *Metabolism*, *Signal transduction*, *Transcription and translation*, *Transport* and *Unknown*. Functional definition of the genes was based on the information obtained through the Gene Link at the NCBI website (http://www.ncbi.nlm.nih.gov), containing GeneOntology and RefSeq annotations, the Rat Genome Database link (http://rgd.mcw.edu) and specific literature references. In the case of insufficient information for the rat gene, the human homologue was searched through the Gene Link at the NCBI website (http://www.ncbi.nlm.nih.gov) and specific information obtained through the analysis of GeneOntology and RefSeq annotations, OMIM (Online Mendelian Inheritance in Man) links and specific literature references.

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Table 2 Results of the real-time quantitative PCR (QPCR) confirmation of 20 genes of the microarray and comparison of the QPCR results with the array results

		QPCR results		Array results	
Clone ID	**Description**	**Fold**	***P*-value**	**Fold**	***P*-value**
AF251305	MHC class II transactivator (C2ta)	19.21	8.44E – 05	2.17	1.65E – 03
L12025	Tumor-associated antigen 1 (Taa1)	16.06	3.07E – 12	2.04	7.80E – 05
AF156878	Opioid growth factor receptor (Ogfr)	2.54	2.62E – 07	2.01	1.94E – 03
X61925	Pancreatic lipase related protein 1 (Pnliprp1)	>7.0	*	2.02	9.13E – 05
X74833	Cholinergic receptor, nicotinic, beta polypeptide 1 (Chrnb1)	1.56	5.84E – 05	– 2.05	1.36E – 03
AF465254	Calpactin I light chain (P10 protein) (S100a10)	3.03	3.95E – 05	1.93	1.96E – 03
AF311875	Period homologue 3 (*Drosophila*) (Per3)	– 2.89	5.28E – 07	– 2.32	4.54E – 03
AF205604	Signal transducer and activator of transcription 1 (Stat1)	6.06	7.89E – 04	1.98	6.73E – 05
AF087037	B-cell translocation gene 3 (Btg3)	2.13	6.76E – 05	2.04	3.72E – 07
U59241	Tropomodulin 1 (Tmod1)	– 2.35	8.68E – 11	1.94	4.63E – 06
M26744	IL-6 (I16)	39.2	3.34E – 03	9.88	9.00E – 05
D10757	Proteasome subunit, beta type, 9 (Psmb9 alias Lmp2)	2.98	4.38E – 02	2.05	6.35E – 05
AF132046	Nogo-A (Rtn4)	1.50	2.19E – 03	2.05	5.04E – 05
AJ277105	Carcinoembryonic antigen-related cell adhesion molecule 1 (Ceacam1)	– 2.09	2.48E – 06	– 2.0	5.15E – 05
M34253	Interferon regulatory factor 1 (Irf1)	5.37	3.39E – 04	2.10	1.35E – 05
L29281	Protein kinase, interferon-inducible double-stranded RNA-dependent (Prkr)	3.41	7.91E – 04	1.94	1.68E – 05
D45250	Protease 28 subunit, beta (Psme2)	2.42	4.70E – 05	1.98	6.73E – 05
AF058786	Small inducible cytokine A2 (Scya2)	3030	5.89E – 03	10.10	7.04E – 04
M80367	Guanylate binding protein 2, interferon-inducible (Gbp2)	22.8	5.81E – 03	5.97	3.84E – 07
X57523	Transporter 1, ABC (ATP binding cassette) (Tap1)	20.2	5.66E – 06	4.12	1.41E – 05

*In the control sample pancreatic lipase related protein 1 was not detectable at 40 cycles. Therefore 40 cycles was chosen as the CT value.

'Humanization' of the gene list and comparison with data obtained in human PTCs

In order to compare our gene list with the microarray data obtained by Huang *et al.* (2001) in human PTCs (http://thinker.med.ohio-state.edu), we applied the following strategy. First, we identified genes common to our gene list and to the HG-U95A Affymetrix oligonucleotide array used in the human tissue analysis. This task was accomplished by matching all the obtainable GenBank accession numbers of the human homologues and/or the gene symbols (obtained through Gene Link, the Homologene database (Wheeler *et al.* 2004) and the Nucleotide database at the NCBI website (http://www.ncbi.nlm.nih.gov)) with the gene list of the HG-U95A Affymetrix oligonucleotide array. In this way a list of genes in common between our genome and the genes analyzed in PTCs was obtained.

Results and discussion

Microarray analysis and QPCR validation

Analysis of the microarray results showed a statistically significant RET/PTC3-induced modification of expression of 270 genes after 48 h of activation of the oncoprotein. Of these, 172 genes were up-regulated with a fold-increase ranging from 1.18 to 17.95 and 98 down-regulated with a fold decrease ranging from –1.16 to –2.32. There were 82 genes showing statistically significant regulation at 24 h (68 up-regulated and 14 down-regulated). With the exception of Hyou1 (24 h –2.48, 48 h 1.32), all showed consistent regulation at the two time points, in most cases with a peak of expression modification at 48 h. To further validate the microarray results, QPCR confirmation was performed for 20 genes, 12 of which were characterized by a 1.9- to 2.1-fold increase, four by a –2- to –2.32-fold decrease, and four others by a >4-fold change at 48 h (Table 2). Eighteen genes out of 20 (90%) showed a consistent change between the microarray and the QPCR. In 15 of these, the change detected by QPCR was significantly greater. This indicates that the rigorous statistical criteria applied to the microarray data succeeded in minimizing false positive results. This is consistent with previous studies describing the validation of this technique (Guo *et al.* 2004, Sartor *et al.* 2004). However, based on previous experiments conducted in this cell line by our group, we are aware of several genes present on the microarray chip and

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known to be markedly up-regulated by RET/PTC that did not score (e.g. COX-2, vascular endothelial growth factor (VEGF), PGE_2 receptor 2 (EP2), EP4) (Puxeddu *et al.* 2003). Therefore, it is apparent that by minimizing Type I error some true positives were missed. The strict dependence of gene expression modifications on RET/PTC3 activation, and not on antibiotic treatment, is supported by our previous studies in which we found no effects of dox on phenotype and expression of thyroid-specific genes in the rtTA control cell lines (Puxeddu *et al.* 2003, Wang *et al.* 2003). However, we did not formally perform this control in this microarray model. As the 48 h gene list included a larger number of genes, it was chosen for further investigation.

Functional clustering

For the purpose of functional clustering, we further restricted the gene list by using a cut-off value of 1.75-fold (Table 3). This yielded 77 genes showing up-regulation and 9 down-regulation. The genes were clustered in 12 categories, namely *Apoptosis*, *Cell–cell signaling*, *Cell cycle*, *Cell motility*, *Cell organization and biogenesis*, *Developmental processes*, *Immune and inflammation response*, *Metabolism*, *Signal transduction*, *Transcription and translation*, *Transport* and *Unknown*.

Genes involved in the regulation of cell growth and apoptosis

Acute RET/PTC activation stimulates both DNA synthesis and apoptosis in thyroid cells (Wang *et al.* 2003). Paradoxically, very few genes belonged specifically to the *Apoptosis* (two) and *Cell cycle* (four) categories. However, at least three of these are key regulators of these biological processes. Ripk3 has been implicated in the activation of caspases 2, -3, -7 and -10, in HeLa cells, implying that it might enhance the dominant FADD/caspase-8 pathway triggered by tumor necrosis factor-α or those of other death receptors, such as DR5 and DR6 (Kasof *et al.* 2000). Similarly, Tdga, cloned by differential display in conditionally immortalized rat hippocampal and septal cells undergoing cell death following differentiation with several factors, has been shown to trigger apoptosis when expressed in the hippocampal H19-7 cell line (Gomes *et al.* 1999). Moreover, a reduction of expression of its human homologue, PHLDA1, was observed in primary and metastatic melanomas compared with normal melanocytic nevi and its ectopic expression was associated with increased basal apoptosis in several human melanoma cell lines (Neef *et al.* 2002).

Recently, a functional role of interleukin (IL)-24 in autocrine regulation of growth and survival of RET/PTC3-expressing thyroid cells has been identified (Shinohara & Rothstein 2004). However, loss of IL-24 expression in advanced cancers seems to indicate that its induction by oncogenes may support tumor growth only at the early stages of cancer development (Shinohara & Rothstein 2004). Moreover, ectopic expression of its human homologue MDA7 in H0-1 human melanoma cells suppressed growth (Jiang *et al.* 1995). Similarly, infection of human breast cancer cells with a recombinant adenovirus expressing MDA7 inhibited growth and induced apoptosis, independently of the p53 status, with an increase in Bax protein and in the ratio of Bax to Bcl-2 (Su *et al.* 1998). Thus, the precise role of IL-24 in mediating RET/PTC3-induced oncogenic properties is still unresolved. Nr4a1, although included in the *Transcription and translation* category because it codes for a nuclear orphan receptor, deserves to be mentioned because of its role in regulation of cell death. Indeed, its human homologue, also known as Nur77, has been shown to bind Bcl-2 at the mitocondrial level and to trigger a conformational change of Bcl-2 that exposes its BH3 domain, resulting in conversion of Bcl-2 from a cell protector to a cell killer (Lin *et al.* 2004). The remaining three genes in the *Cell cycle* category, namely Emp1, Btg3 and S100a10, code for negative regulators of cell proliferation, and their possible role in mediating RET/PTC biological effects is unclear. Up-regulation of several genes in the *Cell–cell signaling*, *Signal transduction*, *Transcription and translation* and eventually also in the *Immune and inflammatory response* categories may participate in the regulation of cell growth. Besides being implicated in cell–cell adhesion and cell migration, fibronectin (Fn1, *Cell–cell signaling*), has been shown to activate survival and mitogenic signals, including the MEK-ERK pathway that is particularly critical for thyroid cell transformation (Gu *et al.* 2002). Similarly, up-regulation of Stat3 expression (*Signal transduction*) and activation of Stat3 pathways exert tumorigenic functions through the induction of cell cycle genes such as cyclin D1 and cMyc and antiapoptotic genes such as Bcl-xl (Bromberg 2002). Indeed, Stat3 has been found to be overexpressed in human PTC (Trovato *et al.* 2003). Moreover, RET/PTC1 has been shown to activate Stat3 phosphorylation in thyroid cells (Schuringa *et al.* 2001, Pedranzini *et al.* 2004). Ran (*Signal transduction*) is a small G-protein that functions in several critical processes in eukaryotic cells including nuclear transport, nuclear envelope formation and spindle formation, the last two processes being a critical component

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Table 3 Functional clustering of the genes regulated by RET/PTC3 in PCCL3 cells

		Fold change
Apoptosis		
AF036537	Receptor-interacting serine-threonine kinase 3 (Ripk3)	3.16
AF192802	T-cell death associated gene (Tdag)	2.52
Cell–cell signaling		
D25290	Cadherin 6 (Cdh6)	3.03
U61261	Laminin 5 alpha 3 (Lama3)	2.75
X01032	Cholecystokinin (Cck)	2.64
X15906	Fibronectin 1 (Fn1)	2.56
M25297	Natriuretic peptide precursor type B (Nppb)	2.49
AF189709	Collagen type XVIII, alpha 1 chain (Col18a1)	2.31
Cell cycle		
Z54212	Epithelial membrane protein 1 (Emp1)	7.06
AF269251	IL-24 (I124)	5.88
AF087037	B-cell translocation gene 3 (Btg3)	2.04
AF465254	Calpactin I light chain (P10 protein) (S100a10)	1.93
Cell motility		
AF132046	Nogo-A (Rtn4)	1.85
AF083269	Actin-related protein complex 1b (Arpc1b)	1.78
Cell organization and biogenesis		
U59241	Tropomodulin 1 (Tmod1)	1.94
AF306457	RAN, member RAS oncogene family (Ran)	1.76
AF028784	Glial fibrillary acidic protein (Gfap)	2.94
Developmental processes		
Y07704	Best5 (Best5)	17.95
AF304446	Adamts1 (Adamst1)	9.71
AK034239	Semaphorin 3C (Sema3c)	1.88
AF016296	Neuropilin (VEGF 165 receptor) (Nrp)	1.79
Immune and inflammation response		
U22520	Interferon-induced protein 10 (Cxcl10)	11.90
AF058786	Small inducible cytokine A2 (Scya2)	10.10
M26744	IL-6 (I16)	7.64
M80367	Guanylate binding protein 2, interferon-inducible (Gbp2)	5.97
Z18877	25 oligoadenylate synthetase (Oas1)	4.20
X57523	Transporter 1, ABC (ATP binding cassette) (Tap1)	4.12
L40365	mRNA for MHC class I antigen, EU allele (RT1Aw2)	2.77
AF251305	MHC class II transactivator (C2ta)	2.17
M34253	Interferon regulatory factor 1 (Irf1)	2.10
D10757	Proteasome subunit, beta type, 9 (Psmb9 alias Lmp2)	2.05
D45250	Protease 28 subunit, beta (Psme2)	1.98
L29281	Protein kinase, interferon-inducible double stranded RNA dependent (Prkr)	1.94
AJ277105	Carcinoembryonic antigen-related cell adhesion molecule 1 (Ceacam1)	−2.02
X78985	CD5 antigen (Cd5)	−1.82
Metabolism		
M10149	Aldolase B (Aldob)	10.73
AF162130	MAGUK p55 subfamily member 6 (Mpp6)	10.67
AF280967	Prostaglandin E synthase 1 (Ptges)	7.29
AJ278701	Branched chain aminotransferase 1, cytosolic (Bcat1)	5.27
M58364	GTP cyclohydrolase 1 (Gch1)	4.84
AJ428514	NADH dehydrogenase subunit 2 (Nd2)	4.65
U76997	Aminopeptidase Vp165 (Vp165)	4.04
L38615	Glutathione synthetase (Gss)	2.98
U18942	Adenosine deaminase, RNA-specific (Adar)	2.40
X61925	Pancreatic lipase related protein 1 (Pnliprp1)	2.02
AF010306	Cathepsin K (Ctsk)	1.79
X67654	Glutathione S-transferase 1 (theta) (Gstt1)	1.75
M88344	Cystathionine beta synthase (Cbs)	−1.97

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Table 3 *continued*

		Fold change
Signal transduction		
AJ249240	Suppressor of cytokine signaling 3 (Socs3)	5.14
Y18812	Glutamate receptor, metabotropic 1 (Grm1)	4.14
D45201	Neurofibromatosis type 1 (Nf1)	2.59
L13041	Calcitonin receptor (Calcr)	2.46
AJ000557	Janus kinase 2 (Jak2)	2.40
AF013144	MAP-kinase phosphatase (Cpg21)	2.32
U42627	Dual specificity phosphatase 6 (Dusp6)	2.24
AJ243123	Suppressor of cytokine signaling 1 (Socs1)	2.09
AF156878	Opioid growth factor receptor (Ogfr)	2.01
AF205604	Signal transducer and activator of transcription 1 (Stat1)	1.98
L06441	Decidual prolactin-related protein (Dtprp)	1.85
U40652	Protein tyrosine phosphatase, receptor type, N (Ptprn)	1.81
X91810	Signal transducer and activator of transcription 3 (Stat3)	1.80
AF065161	Cytokine inducible SH2-containing protein (Cish)	1.79
M22924	Membrane-spanning 4-domains, subfamily A, member 2 (Ms4a2)	1.75
L27081	Melanocortin 5 receptor (Mc5r)	1.75
X74833	Cholinergic receptor, nicotinic, beta polypeptide 1 (Chrnb1)	−2.05
Transcription and translation		
U17254	Immediate early gene transcription factor NGFI-B (Nr4a1)	2.92
M29039	Jun B proto-oncogene (Junb)	2.90
L13201	HNF-3/forkhead homolog-1 (Hfh1)	2.88
L35271	Runt-related transcription factor 1 (Runx1)	2.75
AB025017	TIS11 (Zpf36)	1.77
Y11321	Forkhead box E1 (thyroid transcription factor 2) (Foxe1)	−1.78
Transport		
X60729	beta-hO-r gene for beta-globin chain (Betahor)	10.07
AB002801	Cyclic nucleotide gated channel alpha 3 (Cnga3)	3.61
AF144082	Aquaporin 4 (Aqp4)	2.91
M85299	Solute carrier family 9, member 1 (S1c9a1)	2.84
X98399	Solute carrier family 14, member 1 (S1c14a1)	2.19
BC003281	B aggressive lymphoma (Ba1)	1.86
AJ003065	Potassium inwardly-rectifying channel J14 (Kcnj14)	1.76
Unknown		
U07619	Coagulation factor 3 (F3)	3.37
L12025	Tumor-associated antigen 1 (Taa1)	2.04
AF065438	Peptidylprolyl isomerase C-associated protein (Ppicap)	1.79
BC005419	Interferon-γ induced GTPase (Gtpi-pending)	1.75
AF311875	Period homologue 3 (*Drosophila*) (Per3)	−2.32
M18839	T-cell receptor active beta-chain V-region (V-beta10-J-beta2.5) (M18839)	−2.01
AF034900	Olfactory receptor-like protein (SCR D-7)	−2.00
AF054270	Prolactin-inducible protein (Pip)	−1.85

of cell cycle progression (Li & Zheng 2004). The Runx1/Aml1 gene (*Transcription and translation*) is one of the most frequently mutated genes associated with human acute leukemia and encodes the DNA-binding subunit of the heterodimeric transcriptional factor complex that also includes core-binding factor (Ito 2004). Its function is not completely understood, but is believed to be related to lymphohematopoiesis. Finally, Santoro *et al.* (Castellone 2004*b*) have shown that Cxcl10 and Scya2 (*Immune and inflammation*) are expressed in PCCL3 cells after chronic activation of RET/PTC and that chemokines, such as Cxcl10, may have a role in the promotion of cell proliferation and invasiveness.

RET/PTC induces expression of genes involved in the glycolytic cascade (such as Aldob and Nd2), amino acid metabolism (such as Bcat1, Vp165, Ctsk, Cbs) and lipid metabolism (such as Pnliprp1). This would be expected to increase metabolic activity consistent with the requirements for cell proliferation. The

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oncoprotein also induced genes involved in the protection of cells from oxidative damage by free radicals and detoxification of xenobiotics (such as Gss and Gstt1), and in water and solute transport (such as Aqp 4, Slc9a1, Slc14a1, Bal, Kcnj14).

RET/PTC was found to induce microsomal PGE synthase 1 (Ptges), a key enzyme in PGE_2 biosynthesis. Besides mPGES1, RET/PTC also induces expression of COX-2, resulting in marked stimulation of PGE_2 (Puxeddu *et al.* 2003). PGE_2 in turn may play a role in autocrine tumor growth regulation, besides having immunomodulatory properties and serving as a promoter of angiogenesis (Murakami & Kudo 2004). Several studies have demonstrated increased COX-2 immunostaining in human thyroid adenomas and carcinomas (Cornetta *et al.* 2002, Specht *et al.* 2002). To the best of our knowledge there are no published clinical trials examining the effects of COX antagonists on thyroid tumor formation or progression. A recent report described inhibition of growth of the PTC cell line TPC-1 expressing RET/PTC1 by the specific COX-2 inhibitor NS-398 (Kajita *et al.* 2005).

Genes involved in metastasis and angiogenesis

Some of the genes included in the *Cell–cell signaling*, *Cell motility*, *Developmental processes* and in the *Metabolism* categories, may participate in the acquisition of invasiveness, metastatic potential and angiogenesis. Cadherin (Cdh6) and fibronectin (Fn1) (*Cell–cell signaling category*) have been implicated in the activation of cell motility and metastasis (Shimoyama *et al.* 1995, Clark *et al.* 2000). Moreover, Arpc1b (*Cell motility category*) may directly mediate modifications of the cytoskeleton required for cell motility (Welch *et al.* 1997). As mentioned above, RET/PTC induces expression of rate-limiting enzymes involved in PG biosynthesis, which could be significant in promotion of angiogenesis via PGE_2. In addition, VEGF is also up-regulated by RET/PTC, although this gene product did not score in the microarray. By contrast, Adamts1 (Vazquez *et al.* 1999) (*Developmental processes*) and a product of Col18a1 catabolism, endostatin (O'Reilly *et al.* 1997), exert anti-angiogenetic functions. The balance between the production of angiogenetic and of angiostatic factors may be important for the ordered development of new vessels in the developing tumoral lesion. This phenomenon may help explain the rarity of progression of microcarcinomas to clinically significant forms of the disease, with the latter being characterized by the capacity to actively promote capillary development (Folkman & Kalluri 2004).

Genes involved in signal transduction

The *Signal transduction category* included 16 up-regulated genes and one down-regulated gene. Three genes up-regulated by RET/PTC3, namely Cpg21, Dusp6 and Nf1 are negative regulators of the RAS–BRAF–MAPK signal transduction pathway, known to be activated by RET/PTC3. Cpg21, which is homologous to the human dual-specificity phosphatase DUSP5, inactivates ERK-1 (Ishibashi *et al.* 1994), whereas DUSP6 inactivates ERK-2 (Muda *et al.* 1996), while Nf1, the product of the neurofibromatosis tumor suppressor gene, is a negative regulator of RAS intracellular signaling (Reed & Gutmann 2001). The induction of expression of these three genes may reflect a compensatory mechanism aimed to terminate signaling through the MAPK pathway to maintain cellular homeostasis. The *Signal transduction category* includes also Socs3, Jak2, Socs1, Stat1, Ptprn, Stat3 and Cish, all of which regulate pathways activated by the cell surface cytokine receptor superfamily (Balkwill 2004, Howard & Galligan 2004). As discussed in the following sections, these genes may drive signal transduction pathways involved in the regulation of the immune response.

Genes involved in loss of differentiation

Sustained expression of RET/PTC1 (De Vita *et al.* 1998) or acute expression of RET/PTC1 and RET/PTC3 (Wang *et al.* 2003) in PCCL3 cells is associated with loss of thyroid-specific differentiated properties, manifesting as impaired expression of TSH receptor (TSHR), thyroid peroxidase, thyroglobulin (Tg) and the sodium iodide symporter. This is due in part to loss of expression or activity of the thyroid transcription factors Pax8 and TTF1 (De Vita *et al.* 1998) or with interference of TSHR-mediated intracellular signaling at sites distal to TSHR (Wang *et al.* 2003). This present study was not designed to look at the impact on thyroid differentiation, because cells were not maintained in the presence of TSH. Despite this, expression of Foxe1 (also known as Ttf2) (Zannini *et al.* 1997), a forkhead domain transcription factor thought to play a role in the early stages of thyroid cell differentiation, was decreased by RET/PTC3.

Genes involved in the regulation of the immune response

Twelve up-regulated and two down-regulated genes belonged to the *Immune and inflammation response*

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category. Three of these code for chemokines or cytokines (Cxcl10, Scya2, IL-6) potentially involved in the modulation of the immune response (Houshmand & Zlotnik 2003). Three others code for signaling proteins (Gbp2, C2ta, Irf1) that together with seven others previously mentioned in the *Signal transduction category* (Socs3, Jak2, Socs1, Stat1, Ptprn, Stat3, Cish) are known to mediate intracellular transduction of cytokines (Samuel 2001, Houshmand & Zlotnik 2003, van den Elsen *et al.* 2004). Four genes are known to be involved in the regulation of antigen presentation to cytotoxic T cells, whose expression is typically IFN-dependent (Tap1, RT1Aw2, Lmp2, Psme2) (Marincola *et al.* 2000, 2003). Two additional IFN-regulated genes, Oas1 and Prkr, code for molecules typically expressed during viral infection and involved in its elimination. Although Adar has similar activity, because of its broader spectrum of action it is listed in the *Metabolism category* (Samuel 2001). Finally, two adhesion molecules, Ceacam1 and Cd5, were down-regulated. Ceacam1, also known as CD66a, has been shown to protect melanoma cells from natural killer (NK) cell cytotoxicity (Markel *et al.* 2002). Conceivably, CD66a down-regulation may increase thyroid cell susceptibility to NK lysis. Cd5 is instead generally expressed on the surface of lymphocytes and is considered as an inhibitor of antigen receptor-mediated signals in T cells. The explanation for its expression in PCCL3 cells and its down-regulation after RET/PTC3 activation is not clear.

In summary, the genes specifically involved in the regulation of the immune response represented 16.3% (14 of 86) of the entire list considered for the functional clustering. Moreover, the proportion increases if genes with broader function are included, as described above (22 of 86, 25.6%). The strong representation of immune and inflammation response-related genes after RET/PTC activation points to a critical role of this oncoprotein in the early modulation of the immune response. Immune surveillance systems may operate at early stages of tumorigenesis, possibly activated by the formation of neoantigens or by perturbation of the antigen density resulting from genetic alterations and major genetic instability (Marincola *et al.* 2000). Our data raise the possibility that activation of RET/PTC induces immunogenicity of the tumor cell through up-regulation of the MHC class I antigen-presenting machinery and by down-regulation of molecules involved in protection from NK cytotoxicity.

Many of the genes of this category (Cxcl10, Gbp2, C2ta, Tap1, RT1Aw2, Lmp2, Psme2, Oas1, Prkr), and some included in other functional subgroups (Best5, Gtpi-pending, Adar), are typically induced by IFN-α/-β and/or -γ. Paradoxically, IFN-α, -β and -γ mRNA was not significantly up-regulated by RET/PTC3 in the microarray. Thus, IFN-independent upstream effectors may be involved in those events. Indeed, there is recent evidence that RET/PTC interacts with and constitutively activates Stat1 through mechanisms that do not require Jak or Src (Hwang *et al.* 2004). The induction and activation of Stat1 may account for the up-regulation of so many IFN-responsive genes, such as Irf1 and C2ta, previously described as RET/PTC targets (Hwang *et al.* 2004), most of which are involved in activation of the immune response. However, RET/PTC also activates Stat3 (Hwang *et al.* 2003), which may exert primarily inhibitory effects on the immune response. Indeed, Stat3 activation was shown to inhibit the immune response elicited by tumor cells by suppressing release of proinflammatory mediators or by repressing the functional maturation of dendritic cells (Wang *et al.* 2004). We show that RET/PTC3 up-regulates Stat3 expression, which may amplify the effects following RET-mediated phosphorylation of Stat3. Moreover, Stat3 may also be further activated in a paracrine fashion through IL-6, which is also up-regulated by RET/PTC.

Ligand activation of c-RET, as well as oncogenic forms of RET, can activate nuclear factor-κB (NF-κB) (Visconti *et al.* 1997, Hayashi *et al.* 2000, Ludwig *et al.* 2001). NF-κB in turn promotes the transcription of many proinflammatory cytokines and in our model may account for the up-regulation of Scya2 (Russell *et al.* 2003) and IL-6 (Kikumori *et al.* 1998). Scya-2, also known as MCP1, IL-6 and CXCL10 (also known as IP10), may represent key regulators of chemo-attraction of inflammatory cells and lymphocytes at the tumor formation site. Interestingly, NF-κB and proinflammatory mediators induced by NF-κB activation, produced either by cells of the immune system or by transformed cells, were recently shown to mediate tumor promotion in two mouse models of inflammation-associated cancers (Greten *et al.* 2004, Pikarsky *et al.* 2004).

In conclusion, RET/PTC3 may potentially increase tumor cell immunogenicity through the regulation of IFN-responsive genes, such as those involved in MHC class I antigen-presentation to cytotoxic T cells (Tap1, RT1Aw2, Lmp2, Psme2), eventually increasing susceptibility to NK lysis, and through the up-regulation of cytokines/chemokines involved in the recruitment and activation of cells of the immune system. RET/PTC3 also activates Stat3, which may promote

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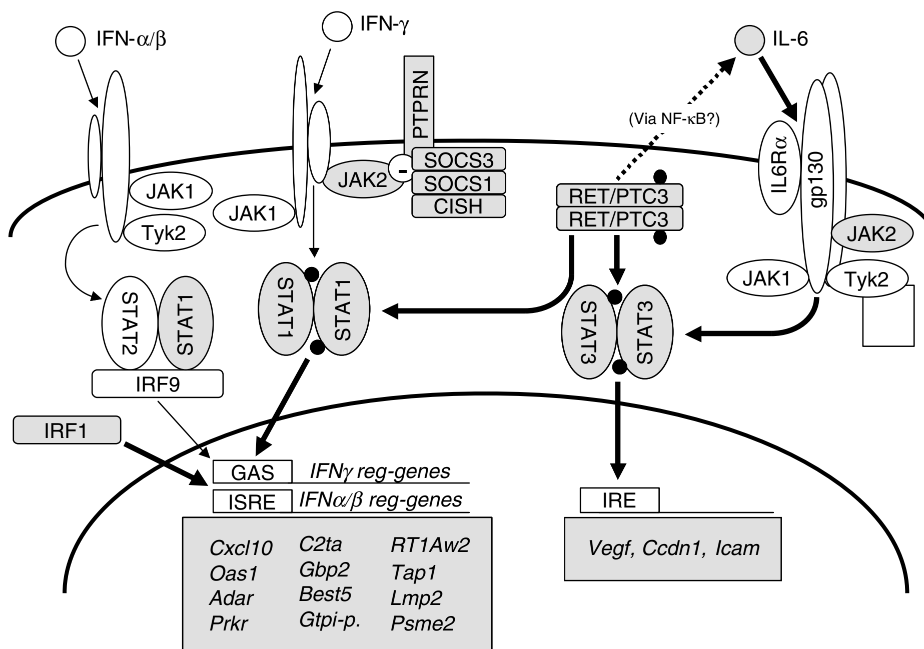

Figure 2 Schematic map of the relationships between RET/PTC3 and IFN- and cytokine-activated signal transduction pathways according to the microarray results and to the literature. Up-regulated genes are shaded, whereas black dots indicate activation by phosphorylation. RET/PTC3 induces the expression of Stat1 and Stat3, involved respectively in the regulation of IFN and cytokine (e.g. IL-6) signal transduction pathways. RET/PTC3 also activates both transcription factors through direct phosphorylation of critical tyrosine residues (Hwang *et al.* 2003, Hwang *et al.* 2004) and through increased IL-6 expression, possibly via NF-κB (Visconti *et al.* 1997, Ludwig *et al.* 2001), stimulates Stat3 activation. In addition RET/PTC3 induces the expression of additional components of these two pathways, such as Jak2, Socs1 and 3, Cish and Ptprn. RET/PTC3-induced activation of Stat1 results in the up-regulation of IFN-responsive genes, whereas RET/PTC1 or RET/PTC3-induced activation of Stat3 results in the up-regulation of Vegf, Cyclin D1 and Icam (Hwang *et al.* 2003).

Table 4 Results of the comparison of the expression profile of PCCL3 cells expressing RET/PTC3 with the expression profiles of papillary thyroid carcinomas reported by Huang *et al.* (2001)

Rat clone ID	Human clone ID	Description	Fold change		
			Rat	Human	*P*-value
M80367	M55543	Guanylate binding protein 2, interferon-inducible (Gbp2)	6.0	2.1	0.19
D25290	D31784	Cadherin 6 (Cdh6)	3.0	1.5	0.35
L38615	U34683	Glutathione synthetase (Gss)	3.0	1.9	0.47
L35271	D43969	Runt-related transcription factor 1 (Runx1)	2.8	7.9	0.01
X15906	X02761	Fibronectin 1 (FN1)	2.6	10.2	0.00
AF013144	U15932	MAP-kinase phosphatase (Cpg21)/Dual specificity phosphatase 5 (DUSP5)	2.3	5.3	0.02
U42627	AB013382	Dual specificity phosphatase 6 (DUSP6)	2.2	4.7	0.00
AF087037	D64110	B-cell translocation gene 3 (BTG3)	2.0	1.5	0.02
AF205604	M97935	Signal transducer and activator of transcription 1 (Stat1)	2.0	1.9	0.11
AJ277105	X16354	Carcinoembryonic antigen-related cell adhesion molecule 1 (Ceacam1)	−2.0	−1.6	0.65

immune evasion by blocking production and responsiveness to inflammatory signals arising from multiple components of the immune system (Wang *et al.* 2004) (Fig. 2). How the combined effects of Stat1 and Stat3 may be contributing to the ultimate immune response remains speculative.

Comparison of the expression profile of PCCL3 cells expressing RET/PTC3 with the expression profiles of PTCs

The PCCL3 cell line is a well-differentiated clonal line derived from rat thyroid. Previous studies have

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demonstrated shared properties with normal thyroid follicular cells (e.g. dependence on TSH for growth, capacity to uptake iodine and secrete Tg, necessity of multiple oncogenic events to induce transformation) (Fusco *et al.* 1987). Thus, it represents a convenient *in vitro* model to study aspects of thyroid cell pathophysiology. Comparison of the expression profile of PCCL3 cells expressing RET/PTC3 with the expression profile of PTCs reported by Huang *et al.* (2001) yielded a handful of genes that are commonly regulated (Table 4). However, these tumors were not genotyped for mutations of *RET/PTC*, *RAS* or *BRAF*, so it is not possible to determine from this limited set of tumors which of the genes acutely regulated by RET/PTC in thyroid cells *in vitro* are similarly modulated in human cancers. Moreover, the statistical criteria used for analysis of the microarray data from the human samples were less stringent, and the actual number of genes differentially regulated in the PTC samples much lower. Nevertheless, these types of comparisons may in the future yield important insights into the pathogenesis of PTCs, by linking the genes expressed in cancer tissues to specific oncogenic events.

Acknowledgements

This work was supported in part by NIH grant CA50706 (J A F). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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