Conditional Activation of RET/PTC3 and BRAFV600E in Thyroid Cells Is Associated with Gene Expression Profiles that Predict a Preferential Role of BRAF in Extracellular Matrix Remodeling

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Abstract

Papillary thyroid cancers (PTC) are associated with nonoverlapping mutations of genes coding for mitogen-activated protein kinase signaling effectors (i.e., the TK receptors *RET* or *NTRK* and the signaling proteins *RAS* and *BRAF*). We examined the pattern of gene expression after activation of these oncoproteins in thyroid PCCL3 cells, with the goal of identifying pathways or gene subsets that may account for the phenotypic differences observed in human cancers. We hybridized cDNA from cells treated with or without doxycycline to induce expression of $BRAF^{V600E}$, RET/PTC3, or RET/PTC3 with small interfering RNA–mediated knockdown of BRAF, respectively, to slides arrayed with a rat 70-mer oligonucleotide library consisting of 27,342 oligos. Among the RET/PTC3-induced genes, 2,552 did not require BRAF as they were similarly regulated by RET/PTC3 with or without BRAF knockdown and not by expression of $BRAF^{V600E}$. Immune response and IFN-related genes were highly represented in this group. About 24% of RET/PTC3-regulated genes were BRAF dependent, as they were similarly modified by RET/PTC3 and $BRAF^{V600E}$ but not in cells expressing RET/PTC3 with knockdown of BRAF. A gene cluster coding for components of the mitochondrial electron transport chain pathway was down-regulated in this group, potentially altering regulation of cell viability. Metalloproteinases were also preferentially induced by BRAF, particularly matrix metalloproteinase 3 (MMP3), MMP9, and MMP13. Accordingly, conditional expression of BRAF was associated with markedly increased invasion into Matrigel compared with cells expressing RET/PTC3. The preferential induction of MMPs by BRAF could explain in part the more invasive behavior of thyroid cancers with *BRAF* mutations. (Cancer Res 2006; 66(13): 6521-9)

Introduction

Papillary thyroid cancers (PTC) are the most common type of thyroid malignancy. They are associated with characteristic oncogenic mutations with clearly shown roles in the early stages of tumor development. Notable among them are the tyrosine kinase receptor oncogenes *RET/PTC* and *NTRK*, which arise by intrachromosomal inversions or translocations, leading to expression of chimeric proteins, and constitutive activation of their respective tyrosine kinases (1). Recently, an activating mutation of *BRAF* was found to be the most common oncogene thus far identified in sporadic forms of the disease, with an overall prevalence of ~40% (reviewed in ref. 2). The mutation is most commonly a thymine-to-adenine transversion at position 1799, previously designated as 1796, leading to a valine-to-glutamate substitution at residue 600 (V600E), with other point mutations being comparatively rare. An additional mechanism of BRAF activation in PTC is through an intrachromosomal rearrangement, leading to the formation of the *AKAP9-BRAF* fusion gene, coding for an oncoprotein with a constitutively activated BRAF kinase (3). In addition, activating mutations of the three *RAS* genes are also found in a smaller subset of PTCs, particularly follicular variant forms of these cancers (4).

Several groups have examined PTCs for mutations of all or most of the known PTC oncogenes [i.e., *RET/PTC, NTRK, NRAS, HRAS, KRAS*, and *BRAF*, all of which activate the mitogen-activated protein kinase (MAPK) signaling pathway (5–7)]. The mutations are mutually exclusive, providing genetic evidence for a requirement of mutation of MAPK signaling components for transformation to PTC. Thus, inappropriate activation of any of these effectors is sufficient to evoke the phenotype, and additional mutation of any of the others would not confer further clonal advantage and thus not be perpetuated. This is consistent with evidence that RET/PTC-induced dedifferentiation (8) and thyroid-stimulating hormone (TSH)–independent growth (9) require activation of the MAPK pathway in thyroid cell lines. Moreover, several lines of evidence indicate that deregulated MAPK activity in PTC occurs early in tumorigenesis and may be important for tumor maintenance.

PTCs with *RET/PTC, RAS*, or *BRAF* mutations have distinct pathologic characteristics and clinical behavior. Thus, follicular variant PTCs, which are less likely to develop lymph node metastases but have a greater predisposition to metastasize to lung, are associated with mutations of *RAS* or with PAX8-peroxisome proliferator-activated receptor γ recombinations (10). By contrast, PTCs with *BRAF* mutations present more often with extrathyroidal invasion and at a more advanced stage and may be more prone to progress to undifferentiated thyroid cancer (11, 12). Thus, despite the fact that all these oncoproteins share the common property of signaling via MAP/extracellular signal-regulated kinase (ERK) kinase (MEK) and ERK, they have unique phenotypic features that likely arise as a consequence of activation of unique patterns of gene expression. This has recently been shown in human tissue samples. Giordano et al. generated expression profiles of 51 PTCs that had been genotyped for *RET/PTC, RAS*, and *BRAF* mutations and found that the expression data

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separated the tumors into three distinct groups corresponding to their mutational status (13). Mellilo et al. examined the transcriptional profile of mass transfected populations of clonal rat thyroid PCCL3 cells stably expressing RET/PTC, oncogenic RAS, or BRAF (14). A large fraction of the genes activated by RET/PTC3 were also activated by RAS or BRAF, consistent with the primary role of this pathway in transformation. This was confirmed for a subset of genes by use of a RNA interference (RNAi) for BRAF, or by MEK inhibitors. Moreover, they showed a critical role of this pathway in stimulating cell motility, mediated in part by induction of the chemokines CXCL1 and CXCL10.

The purpose of this study was to compare the expression profile induced by expression of RET/PTC3 and BRAF in thyroid PCCL3 cells using oligonucleotide microarrays, with the goal of identifying functional categories of genes that may help explain some of the common features of these cancers, as well as those that make them distinct from each other. This is important because PTCs with *BRAF* and *RET/PTC* mutations seem to have distinct biological behavior. As the activation of these oncogenes is believed to take place early in the course of tumorigenesis, we determined the transcriptional profile at early time points after oncoprotein expression *in vitro*, which allowed for a rigorous comparison between cells subjected to similar conditions. These experiments allowed us to define functional gene clusters that were preferentially activated by RET, and conversely, others that were induced preferentially by BRAF. Additional confirmation on the role of the BRAF in RET/PTC-mediated gene expression was obtained through the use of cell lines with RNAi-mediated deletion of this kinase. Notable among the genes regulated primarily by oncogenic BRAF were the matrix metalloproteases (MMP), a major group of enzymes that regulate cell-matrix composition through cleavage of extracellular matrix components. Enhanced production of MMPs in the tumor environment, either by stromal or tumor cells, is believed to be an important determinant of tumor invasion (15). Accordingly, we show that PCCL3 thyroid cells conditionally expressing oncogenic BRAF show much greater MMP expression, enzymatic activity, and cell invasiveness than cells expressing RET/PTC3, which is consistent with the biological behavior of human PTC with these mutations.

Materials and Methods

Cell lines and transfections. Rat thyroid PCCL3 cells were maintained in H4 medium consisting of Coon's medium/F-12 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 apotransferrin, 10 nmol/L hydrocortisone, and penicillin/streptomycin. PTC3-5 and PC-BRAF^{V600E} cells were derived from PCCL3 cells to obtain doxycycline-inducible expression of RET/PTC3 or BRAF^{V600E}, respectively, as previously described (16, 17). PTC3-BRAF^{RNAi} cells were obtained by stable expression of a BRAF RNAi in PTC3-5 cells as previously described (18).

Oligonucleotide microarray. PTC3-5, PC-BRAF^{V600E}, or PTC3-BRAF^{RNAi} cells were grown to full confluency. TSH was then removed for 2 days, after which cells were treated with doxycycline for 0, 1, 2, or 4 days to induce expression of the respective oncoprotein. At each time point, samples derived from three independent cell preparations were used for RNA extraction with TRIzol (Invitrogen, San Diego, CA) and purification with an RNAeasy kit (Qiagen, Alameda, CA). The rat 70-mer oligonucleotide library version 3.0 consists of 27,342 optimized oligos (Qiagen) and was arrayed and printed as previously outlined. The complete gene lists can be viewed at http://microarray.uc.edu. Fluorescence-labeled cDNAs were synthesized from total RNA using an indirect amino allyl labeling method via an oligo(dT)-primed, reverse transcriptase reaction. The cDNAs were labeled with monofunctional reactive cyanine-3 and cyanine-5 dyes (Cy3 and Cy5; Amersham, Piscataway, NJ). Pairwise hybridizations were done between labeled cDNAs corresponding to unstimulated versus doxycycline-treated cells for each of the cell lines and time points. In addition, to increase the statistical power of the experiment, paired hybridizations were done to compare expression between cell lines at the same time points before or after oncoprotein activation. Details of hybridization and washing conditions can be found in the indicated web site. Imaging and data generation were carried out using a GenePix 4000A and GenePix 4000B (Axon Instruments, Union City, CA) and associated software from Axon Instruments, Inc. (Foster City, CA). The microarray slides were scanned with dual lasers with wavelength frequencies to excite Cy3 and Cy5 fluorescence emittance. Images were captured in JPEG and TIFF files, and DNA spots were captured by the adaptive circle segmentation method. Information extraction for a given spot is 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. The Cy3 and Cy5 fluorescence signal intensities were normalized. Data normalization was done in two steps for each microarray separately (19–21). First, background-adjusted intensities were log transformed, and the differences (R) and averages (A) of log-transformed values were calculated as $R = \log_2(X_1) - \log_2(X_2)$ and $A = [\log_2(X_1) + \log_2(X_2)] / 2$, where X_1 and X_2 denote the Cy5 and Cy3 intensities after subtracting local backgrounds, respectively. Second, data centering was done by fitting the array-specific local regression model of R as a function of A. The difference between the observed log-ratio and the corresponding fitted value represented the normalized log-transformed gene expression ratio. The statistical analysis was done for each gene separately by fitting a mixed-effects linear model (22). Assumptions about model variables are the same as described in ref. (23), with array effects assumed to be random and treatment and dye effects assumed to be fixed. Statistical significance of differential expression was assessed by calculating Ps and adjusting for multiple hypotheses testing by calculating false discovery rates (24). Estimates of fold change were also calculated. Data normalization and statistical analyses were done using SAS statistical software package (SAS Institute, Inc., Cary, NC). Gene annotation was supplemented with human and mouse homologues for unknown oligos. As mentioned, the identification of differentially expressed genes was augmented by clustering expression profiles across different experimental comparisons. At each time point, duplicate samples derived from three independent cell preparations were used. In our experience and that of others, the fidelity of such an approach dispenses with the need for subsequent validation steps by real-time PCR. Clusters analysis was done using the Bayesian infinite mixture models as implemented in the *GIMM* software (http://eh3.uc.edu/gimm).

Real-time reverse transcription-PCR. Total RNA (2 μg) was reverse transcribed with SuperScript III (Invitrogen) in the presence of random hexamers. Real-time PCR was done using QuantiTect SYBR Green PCR kit (Qiagen) in a LightCycler instrument (Cepheid, Sunnyvale, CA). The cycle threshold value, which was determined using the second derivative, was used to calculate the normalized expression of the indicated genes using Q-Gene Software, using *β-actin* as a reference gene. The following primer pairs were used: β-actin, 5′-CTGAACCCTAAGGCCAACCGTG-3′ and 5′-GGCATACAGGGACAGCACAGCC-3′; MMP2, 5′-TGACGATGAGCTGTGGACTC-3′ and 5′-CTGCTGTATTCCCGACCATT-3′; MMP3, 5′-GCTCATCCTACCCATTGCAT-3′ and 5′-GCTTCCCTGTCATCTTCAGC-3′; MMP9, 5′-CCCTGCGTATTTCCATTCAT-3′ and 5′-AGTTGCCCCCAGTTACAGTG-3′; MMP13, 5′-GAGGTGAAAAGGCTCAGTGC-3′ and 5′-ATGAGGCGGGGATAGTCTTT-3′, MT1-MMP, 5′-TCATGGCTCCCTTTTACCAG-3′ and 5′-CTTTGTGGGTGACCCTGACT-3′; tissue inhibitor of metalloproteinase 2 (TIMP2), 5′-GCATCACCCAGAAGAAGAGC-3′ and 5′-TGATGCAGGCAAAGAACTTG-3′.

Western blotting. Cells were lysed in a buffer containing 20 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 150 mmol/L NaCl, 0.5% Triton X-100, 50 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate, 2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, and protease inhibitor cocktail (Sigma, St. Louis, MO). Serum-free medium was collected from the dish, spun down to remove the dead cells, and concentrated 20 times using Centricon Concentrator 10 (Amicon, Inc.,

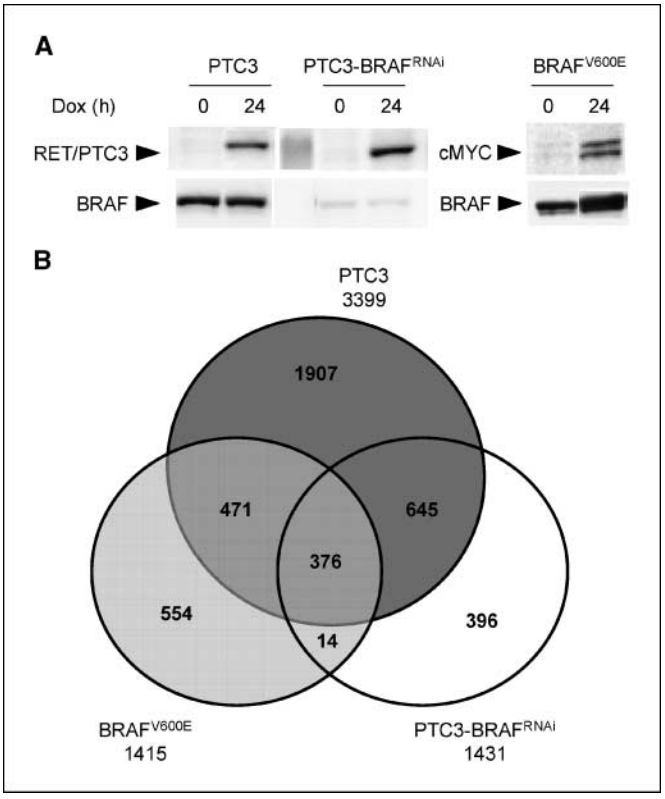

Figure 1. Conditional expression of RET/PTC3 and BRAF^{V600E} in PCCL3 cells. *A,* Western blots of the indicated cell lines incubated with or without doxycycline for 24 hours. PTC3-5 and PTC3-BRAF^{RNAi} cells show doxycycline-induced expression of RET/PTC3. *Bottom,* immunoreactive BRAF, which is markedly down-regulated in cells stably expressing BRAF^{RNAi}, as described (18). *Right,* doxycycline-inducible expression of oncogenic myc-tagged BRAF^{V600E} in PC-BRAF^{V600E} cells. *B,* Venn diagram showing the transcripts either up-regulated or down-regulated in at least one of the time points after treatment with doxycycline of the three cell lines. Overlapping regions indicate transcripts that were significantly regulated in the same direction, using a false discovery rate of 0.05 as a cutoff.

Billerica, MA). Fetal bovine serum was used as a positive control for MMP2 and MMP9. After measurement of protein concentration using a Micro Bicinchoninic Acid Protein Assay Reagent (Pierce, Rockford, IL), 50 μg of the cell lysate or 30 μL of the concentrated medium were separated by SDS-PAGE and blotted onto a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The following primary antibodies were used: anti-MMP2-AB809, anti-MMP3-MAB3306, anti-MMP9-AB19016, anti MMP13-MAB13426 (Chemicon International, Temecula, CA), anti-ERK K-23 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-RET (provided by Yuri Nikiforov, University of Cincinnati, Cincinnati, OH), anti-BRAF F-7 (Santa Cruz Biotechnology), anti-myc 9E10 (Oncogene, Boston, MA). The antigen-antibody complexes were visualized using horseradish peroxidase–conjugated anti-mouse or rabbit IgG antibody (Santa Cruz Biotechnology) and enhanced chemiluminescence system (Amersham Biosciences).

Gelatin and casein zymography. Confluent PTC3-5 and BRAF^{V600E} cells were treated with or without doxycycline for 2 or 4 days in the absence of TSH. Cells were maintained in serum-free media for the final 24 hours of incubation. Cell-conditioned medium was harvested on ice, centrifuged at 3,000 rpm to remove dead cells and debris, and concentrated 20 times using Centricon Concentrator 10 (Amicon). Thirty microliters of concentrated media were loaded onto 7.5% or 10% polyacrylamide gels containing 1 mg/mL of gelatin (Sigma G8150) or β-casein (Sigma C6905), respectively. The following positive controls were used: fetal bovine serum for MMP2 and MMP9 in the gelatin gel and human MMP3 (Chemicon CC1035) in the casein gel. After electrophoresis, the gels were washed for 30 minutes in 2.5% Triton X-100 and 30 minutes in developing buffer [50 mmol/L Tris, 0.2 mol/L NaCl, 5 mmol/L $CaCl_2$, 0.02% Brij 35 (pH 7.4)] at room temperature. Fresh developing buffer was added, and the gel was incubated for 18 hours at 37°C. The gels were stained with 0.2% Coomassie blue R-250 in 30% methanol and 10 % acetic acid for 3 hours and then destained with 45% methanol and 10% acetic acid. The substrate degrading enzymes were identified as clear bands in the blue background. The gels were photographed by Nikon coolpix 5700 and converted to grayscale.

Cell motility assay. The cell motility experiment, also known as *in vitro* wounding assay, assesses the rate of repopulation after disruption of a cell monolayer. Ninety percent confluent PTC3-5 and BRAF^{V600E} cells were treated with or without doxycycline for 2 days in absence of TSH. The respective monolayers were disrupted by scratching with a pipetman equipped with a 10-μL tip. Repopulation of the cell-free area was examined under an inverted microscope after 0, 12, 22, and 38 hours. In this time frame, cell division is negligible in a TSH-free medium. After 22 hours, cells that entered the gap were counted in two scratches over the entire length of the dish. When grown in H3 medium, RET/PTC3 and BRAF did not increase cell proliferation over this time period.

Matrigel invasion assay. Invasion was examined in Transwell cell culture chambers using polycarbonate membranes with pores of 8 μm (Costar, Cambridge, MA). Both sides of the Transwell membrane were coated with rat collagen I, and Matrigel was placed as a barrier (60 μg/Transwell) on the upper side. Cells that had been cultured for 4 days in the presence or absence of doxycycline were harvested using trypsin/EDTA and suspended in medium with or without doxycycline in the absence of TSH. Cells (2.5×10^4) were placed in the upper chamber. The lower chamber contained 0.6 mL of medium containing the indicated concentrations of SDF-1 as a chemoattractant. This chemokine has been previously shown to function as a chemoattractant for PCCL3 cells chronically expressing RET/PTC1 (25). Cells were allowed to invade for 24 hours at 37°C and 5% CO_2. Nonmigrated cells on the upper chamber were removed with a cotton swab; the filters were fixed in 4% paraformaldehyde and stained with crystal violet. The total number of cells migrated to the lower surface was counted

Table 1. Immune response genes up-regulated ≥4-fold change at day 4 of doxycycline treatment in PTC3 or BRAF^{V600E} cells

Genes	PTC3	BRAF^{V600E}	PTC3-BRAF^{RNAi}
BRAF independent			
CXCL11 (*IP9*)	61.62	1.54	37.38
Best 5	36.33	2.74	34.14
CD74	26.62	3.89	6.7
CCL4 (*MIP1β*)	23.98	3.31	27.77
SLAMF1 (*IPO3*)	16.59	1.76	18.18
IFIT4	16.14	1.06	6.77
GBP2	15.91	2.05	8.46
BF	15.18	1.42	10.74
Mx2	13.55	1.45	10.72
IFI27	11.03	3.01	9.19
CXCL10 (*IP10*)	10.54	1.92	6.26
MOB5	9.66	1.79	4.71
IFIT2	7.84	1.45	3.89
IRF7	7.57	−1.43	4.26
IGTP	5.4	1.19	3.62
RT1.Db	5.35	1.37	3.81
SOCS1	4.31	−1.17	3.57
IRF1	4.03	1.91	2.87
BRAF dependent			
CCL7 (*MCP3*)	9.52	39.55	6.12
CCL2 (*MCP1*)	31.8	39.29	7.73
GM-CSF	1.28	12.11	2.17
CCR1	−1.03	5.77	−1.14

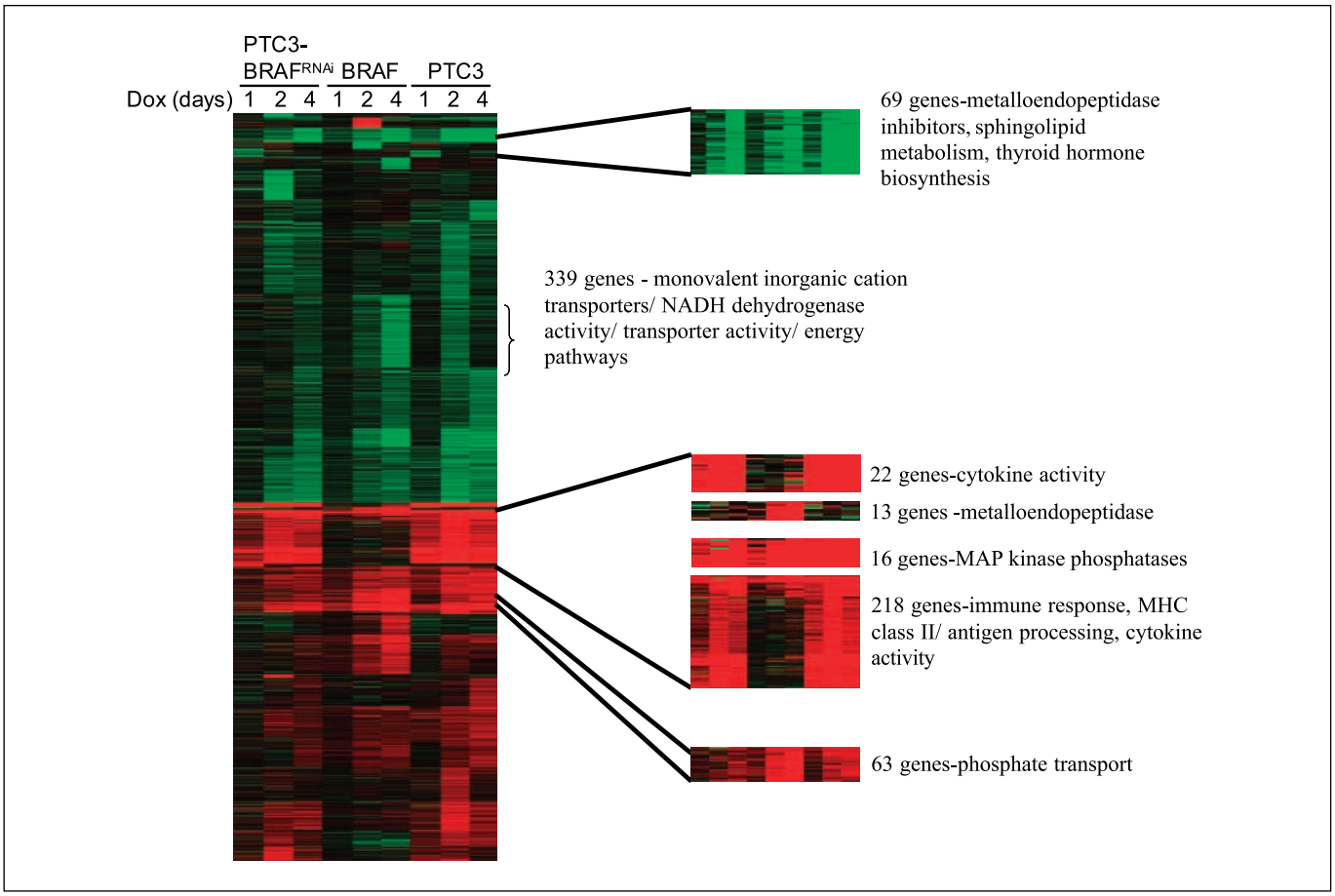

Figure 2. Clusters of coexpressed genes were created from the significantly changed transcripts using advanced Bayesian modeling. Columns indicate expression ratios versus the respective control at 1, 2, and 4 days after doxycycline treatment of PTC3-BRAF^{RNAi}, PC-BRAF^{V600E}, and PTC3-5 cells, respectively. A subset of functional clusters is highlighted (see text for discussion). The entire data set is publicly available as a supplement at http://eh3.uc.edu.

under bright-field microscope. In the experiments assessing the involvement of matrix metalloproteinases, equimolar concentrations of the indicated MMP inhibitors were maintained in both chambers. GM6001 (Calbiochem, La Jolla, CA), a potent broad spectrum MMP inhibitor, was compared with a structurally modified negative control. Invasion was also measured in the presence of the MMP13-specific inhibitor CL-82198 (Calbiochem). Compounds were dissolved in DMSO and diluted so that the final concentration of vehicle was <1% in the assay.

Results

Gene expression profile after acute activation of RET/PTC3, BRAF, or RET/PTC3 with a knockdown of BRAF in thyroid PCCL3 cells. PTC3-5, PTC3-BRAF^{RNAi}, and PC-BRAF^{V600E} cells were treated with doxycycline for 1, 2, or 4 days to induce expression of RET/PTC3 or BRAF^{V600E}. The adequacy of expression of the respective oncoproteins was confirmed in a protein aliquot by Western blotting before generating cDNA to probe the microarrays (Fig. 1*A*). The actual RNA samples used for microarray hybridization were also checked by Northern blotting (data not shown). Care was also taken to approximate the expression of RET/PTC3 in the cell lines without or with a knockdown of BRAF. We have recently shown that knockdown of BRAF, but not CRAF, fully abrogates ERK activation by RET/PTC3 (18).

Altogether, RET/PTC3 regulated the expression of 3,399 genes, whereas BRAF^{V600E} had effects on a more discrete population of transcripts (1,415; Fig. 1*B*). Of these, 847 were coregulated by both stimuli. In turn, 471 of these were not regulated in PTC3-BRAF^{RNAi} cells, showing that they required BRAF for the RET/PTC3-mediated effect. The latter were therefore considered to be highly enriched in MAPK-dependent genes. By contrast, 645 genes were regulated by RET/PTC3, regardless of whether the wild-type BRAF protein was expressed or not, and were not induced by oncogenic BRAF^{V600E}. This subset is highly enriched for genes regulated by pathways that do not require MAPK activation.

Table 2. Fold changes of MMPs and related genes at day 4 of doxycycline treatment

Gene name	PTC3	BRAF^{V600E}	PTC3-BRAF^{RNAi}
MMP3	45.94	97.40	31.62
MMP13	1.33	39.64	1.10
MMP9	−1.14	4.41	1.08
MMP2	−1.03	−1.59	−1.09
MT-MMP1	−1.59	−1.84	−1.26
TIMP2	−4.10	−2.63	−1.79
TIMP3	−3.76	−2.46	−2.21
UPAR	16.14	11.16	7.11

Identification of functional categories activated by RET/PTC3 and BRAF by unsupervised cluster analysis and pathway analysis. Clusters of coexpressed genes were created from the significantly changed transcripts using advanced Bayesian modeling, which allows for assessment of the statistical significance of identified expression patterns. Functional tests were done against the biological process and molecular function branches of Gene Ontology using the EASE program. Fisher's exact probability, using a multiple testing adjustment, was calculated for each gene category (26) for several clusters. Among the down-regulated genes, there is a group that is clearly BRAF dependent, as this cluster is down-regulated more intensely by $BRAF^{V600E}$ than by RET/PTC3, and the inhibitory effects of the latter are blunted by BRAF knockdown (Fig. 1*B*). Components of the mitochondrial respiratory chain (MRC) pathway were highly represented ($P = 9.60 \times 10^{-21}$) in this cluster. The MRC consists of five enzyme complexes, with the NADH ubiquinone oxidoreductase subunits lying within complex I. Eight of 42 genes in this complex were down-regulated primarily via BRAF activation. The magnitude of down-regulation was on average ~2-fold.

As previously reported, the most highly represented cluster up-regulated by RET/PTC3 consisted of genes involved in the immune response (*P*s for the various clusters ranged from 1.7×10^{-3} to 3.1×10^{-15}). A small subset of these required signaling primarily via BRAF, as their induction was blunted in PTC3-$BRAF^{RNAi}$4-8 cells, and they were induced more robustly by $BRAF^{V600E}$. Notably, those showing BRAF dependency are primarily related to genes regulating innate immune responses, such as granulocyte macrophage colony-stimulating factor (GM-CSF), the macrophage chemokines MCP-1 and MCP-3, and the chemokine receptor CCR1. By contrast, the large majority of genes in the immune response cluster did not require the presence of BRAF for activation by RET/PTC3 and consisted primarily of genes involved in antigen presentation ($P = 6.13 \times 10^{-7}$) and in the IFN signaling pathway (*P*s for the various clusters ranged from 9.3×10^{-8} to 3.1×10^{-15}, depending on the cluster area; Table 1; Fig. 2).

A cluster of MAPK phosphatases was regulated robustly by both $BRAF^{V600E}$ and RET/PTC3. The two prototypic ERK phosphatases (MKP-3 and DUSP5) were markedly induced by both oncoproteins, which was confirmed by reverse transcription-PCR (RT-PCR) and Northern blotting (data not shown).

Among the functional categories that showed preferential regulation by $BRAF^{V600E}$, metalloendopeptidases were highly represented. As shown in Table 2, MMP3, MMP9, and MMP13 were markedly induced by expression of oncogenic BRAF. A number of other genes involved in matrix remodeling showed a similar pattern of expression. As invasiveness is a recognized feature of human PTC with BRAF mutation, and of transgenic mice overexpressing $BRAF^{V600E}$ in the thyroid, we focused in greater detail on this family of matrix-degrading enzymes, as will be described later in Results.

Manual enrichment of unsupervised clusters. A key strength of unsupervised clustering in combination with pathway analysis is that it provides an unbiased analysis of the possible functional

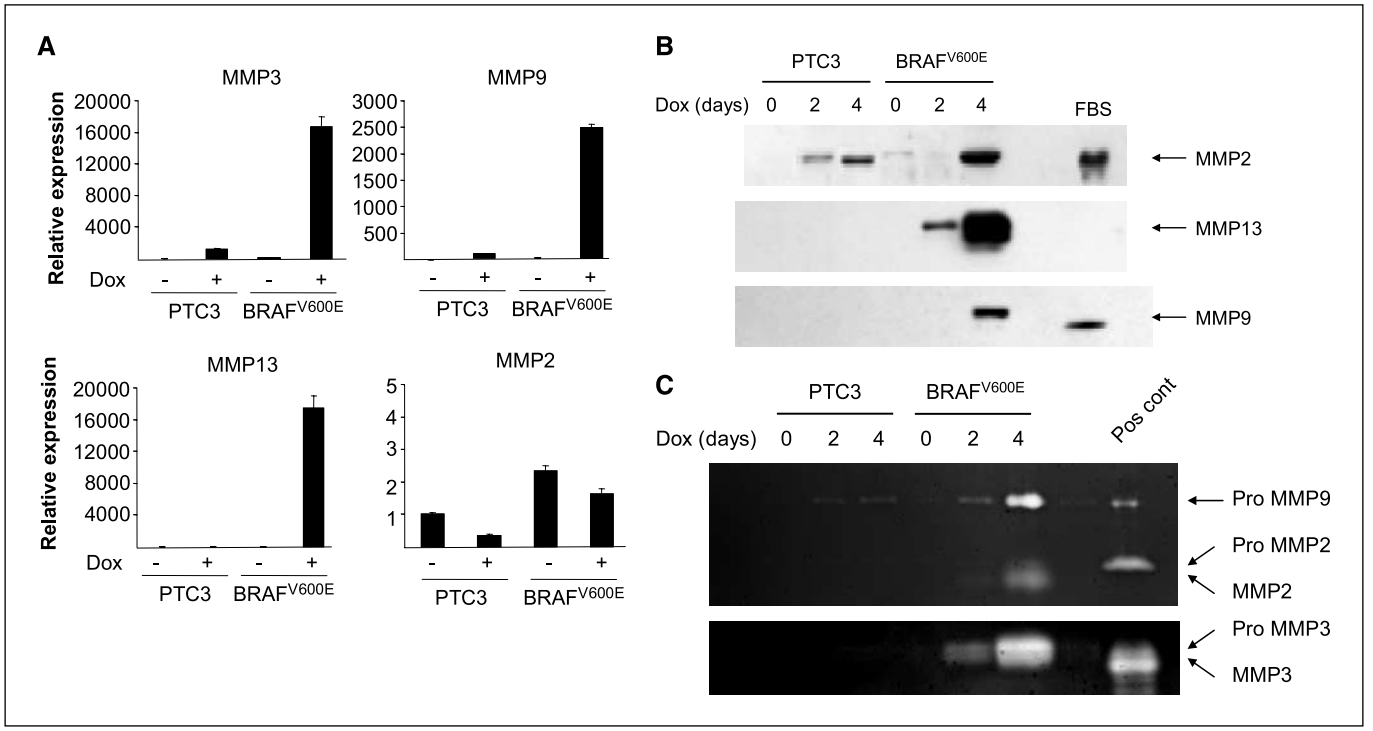

Figure 3. Effect of RET/PTC3 and $BRAF^{V600E}$ activation on MMPs and related genes. *A,* abundance of the indicated MMP mRNAs determined by RT-PCR in the indicated cell lines treated with or without 1 μg/mL doxycycline for 4 days in the absence of TSH. Represented relative to expression in PTC3-5 cells in the absence of doxycycline. *Columns,* mean of triplicate samples; *bars,* SE. Representative experiment that was replicated once. *B,* Western blots of conditioned media of the indicated cells treated with or without doxycycline for the indicated time in the absence of TSH. Fetal bovine serum (*FBS*) was removed from the media for the final 24 hours in each sample. Thirty microliters of 20× concentrates were size separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the indicated antibodies. Fetal bovine serum was used as a positive control for MMP2 and MMP9, respectively. Gels show representative examples of two to three independent experiments. *C,* gelatin (*top*) and casein (*bottom*) zymograms of 30-μL aliquots of concentrates of conditioned media from the indicated cell lines. Arrows point to MMP9 (92 kDa), pro-MMP2 (72 kDa), and MMP2 (66 kDa) in the gelatin zymogram and to pro-MMP3 (59 kDa) and MMP3 (50 kDa) in the casein zymogram. Pro-MMP9, pro-MMP2, and pro-MMP3 have enzymatic activity because of activation by denaturation with SDS present in the sample-loading buffer.

consequences of a pathologic or physiologic perturbation. The technique has limitations due in part to the continually evolving knowledge of gene function. We therefore examined the gene lists using a candidate approach based on the investigators understanding of gene function to enrich the information provided in the initial analysis.

Manual enrichment of the clusters showed complex regulation by BRAF of genes whose function either amplifies or dampens signaling along the MAPK pathway. The ephrin receptor EphA2 and its ligand ephrin A1 were induced 4.4- and 1.9-fold by BRAF, respectively. EphA2 is a tyrosine kinase receptor that is transcriptionally activated by the Ras/Raf/MAPK pathway (27). Ligand-stimulated EphA2 inhibits growth factor–induced activation of Ras, hence creating a negative feedback loop that regulates Ras activity. The mitogen-induced Mig-6 protein down-regulates epidermal growth factor receptor (EGFR)–mediated activation of ERK2 and was also induced by BRAF (3.2-fold) and by RET/PTC3 in a BRAF-dependent manner. Thus, like the MAPK phosphatases DUSP5 and MKP3, activation of EphA2 and Mig-6 would be predicted to result in down-regulation of BRAF-induced ERK activation. By contrast, transforming growth factor α, a ligand for EGFR, was induced by 3.4-fold by BRAF.

Among genes that could participate in innate immunity, *CCL15* (*MIP1γ*), a chemoattractant for T cells and monocytes, did not score in that category by unsupervised clustering but was also induced by 2.1-fold by BRAF.

Some structural and adhesion genes that could play a role in matrix remodeling and invasiveness, such as tissue factor, lipocalin 2, syndecan 1, nidogen 2, integrin α_3, integrin α_2, collagen α1 XII chain, and cathepsin D, were also not in the cluster significant for structural function and cell adhesion but were also up-regulated via BRAF (by 8-, 7.3-, 3.1-, 2.6-, 3.5-, 3.6-, 2.5-, and 2.3-fold, respectively).

As predicted based on prior studies in thyroid cell lines, thyroglobulin, type I deiodinase, and the transcriptional factor TTF-2 were down regulated by both RET-PTC3 and BRAF.

Preferential regulation of MMPs by BRAF^{V600E}. Unsupervised clustering of genes involved in matrix remodeling ($P = 2.03 \times 10^{-3}$) is shown in Table 2. We confirmed BRAF-dependent induction of MMP3, MMP9, and MMP13 mRNA by RT-PCR (Fig. 3*A*). Secretion of MMP13 and MMP9 was also shown by Western blotting (Fig. 3*B*). By contrast, expression of MMP2 mRNA (Fig. 3*A*) and protein (data not shown) was not induced by either RET/PTC or BRAF in cell lysates. However, MMP2 secretion was markedly increased, consistent with post-translational regulation via BRAF (Fig. 3*B*). Gelatin zymography of conditioned media showed a preferential BRAF^{V600E}-induced increase of MMP9, pro-MMP2, and MMP2, whereas casein gel zymograms showed BRAF-mediated stimulation of secretion of pro-MMP3 and MMP3 (Fig. 3*C*). The expression of TIMP3 and UPAR were confirmed by RT-PCR (data not shown) and were consistent with the data shown in Table 2.

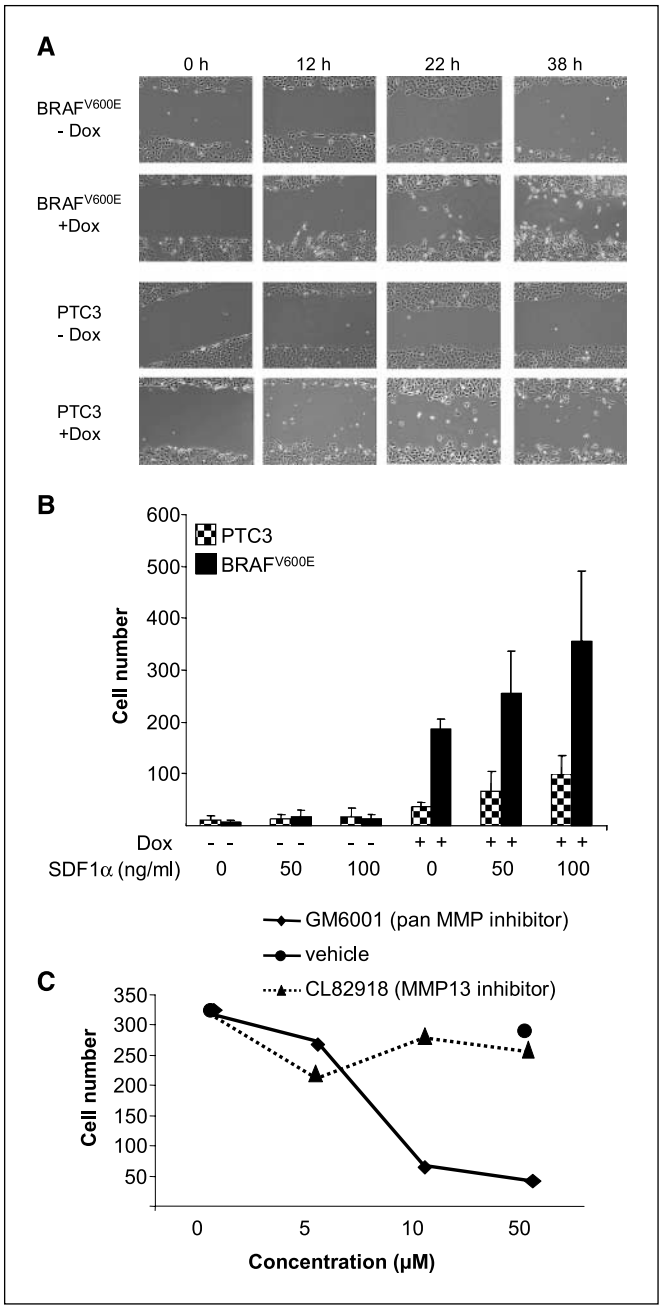

Figure 4. *A,* BRAF^{V600E} and RET/PTC3 induce similar degrees of cell migration in PCCL3 cells. Time course of repopulation of a wound-induced gap in confluent monolayers of the indicated cell lines after treatment with or without doxycycline for 48 hours in the absence of TSH. Repopulation of the cell-free area was examined under an inverted microscope. Cells were counted in two scratches over the entire length of a P-100 Petri dish at 22 hours in two independent experiments and were not statistically different. *B,* preferential stimulation of cell invasion by doxycycline-induced expression of BRAF^{V600E}. Invasion was examined in Transwell cell culture chambers using polycarbonate membranes with pores of 8 μm (Costar). Both sides of the Transwell membrane were coated with rat collagen I, and Matrigel was placed as a barrier (60 μg/Traswell) on the upper side. Cells that had been cultured for 4 days in the presence or absence of doxycycline were harvested using trypsin/EDTA and suspended in H3 medium with or without doxycycline. Cells (2.5×10^4) were placed in the upper chamber. The lower chamber contained 0.6 mL of H3 medium containing the indicated concentrations of SDF-1 as a chemoattractant. The cells were allowed to invade for 24 hours at 37°C and 5% CO_2. Nonmigrated cells on the upper chamber were removed with a cotton swab; the filters were fixed in 4% paraformaldehyde and stained with crystal violet. The total number of cells migrated to the lower surface was counted under a brightfield microscope. *Columns,* mean of a representative experiment with three replicates; *bars,* SE. Statistical analysis is described in the text. *C,* BRAF-induced invasion into Matrigel is inhibited by a pan-MMP antagonist but not by an inhibitor of MMP13. Matrigel invasion assays were done on PC-BRAF^{V600E} cells as described in (*B*), except that the indicated concentrations of the pan-MMP inhibitor GM6001, a structurally modified negative control, or the MMP13-specific inhibitor CL-82198 were added before layering them on the Transwell chambers.

Matrigel invasion is preferentially stimulated by doxycycline-induced expression of BRAF^{V600E}. Next, we did functional assays to determine the consequences of RET/PTC3 and BRAF^{V600E} expression on migration and cell invasion. To examine cell motility, we counted the number of cells repopulating a scraped gap of the monolayer after *in vitro* wounding at timed intervals following doxycycline-induced expression of either RET/PTC3 or BRAF^{V600E}. Both oncoproteins induced similar rates of repopulation after 2 days of doxycycline treatment (Fig. 4*A*). The capacity to invade into Matrigel was examined in BRAF^{V600E} and PTC3-5 cells 4 days after treatment with doxycycline using different concentrations of SDF-1 as a chemoattractant. Expression of BRAF^{V600E} induced Matrigel invasion by 35-fold in the absence of SDF-1 ($P < 0.005$), whereas RET/PTC3 evoked only a 3.7-fold increase, which was of borderline statistical significance ($P = 0.07$; Fig. 4*B*). The difference between BRAF- and RET/PTC3-induced migration was clear ($P = 0.008$). In the presence of SDF-1, similar trends were apparent, as shown in Fig. 4*B*. Invasion into Matrigel was inhibited in a concentration-dependent manner by the pan-MMP inhibitor GM6001, whereas the MMP13-specific inhibitor CL82918 was without effect (Fig. 4*C*).

Discussion

PTCs are associated with non-overlapping mutations of at least six different genes whose products signal along the MAPK kinase pathway. At least two of these oncogenes (*RET/PTC* gene recombinations and *BRAF* mutations) are likely early events in transformation of follicular thyroid cells. To explore the early consequences after activation of these oncoproteins, we did gene expression profiles of rat thyroid PCCL3 cells acutely expressing BRAF^{V600E} or RET/PTC3. We previously determined that RET/PTC3-mediated activation of ERK phosphorylation requires BRAF (18). Thus, to more accurately identify MAPK-dependent genes, we also profiled the expression signature after RET/PTC3 activation in cells with concomitant small interfering RNA (siRNA)–dependent knockdown of BRAF.

The number of genes modulated by RET/PTC3 is more than twice that activated by BRAF. This is not an unexpected finding as RET/PTC is upstream in the pathway and can signal through many other effectors besides MAPK. About 25% of genes were coregulated by RET/PTC3 and BRAF, consistent with MAPK dependency. When a stricter criterion is applied by including only genes no longer regulated by RET/PTC3 in the absence of BRAF, only 14% remain. Melillo et al. (14) did a microarray study in PCCL3 cells chronically expressing RET/PTC3, oncogenic RAS, BRAF, or the RET/PTC mutants Y1062F and Y1015F. They found a much higher proportion of RET/PTC3-regulated genes to be MAPK dependent, as 48% of the up-regulated and 36% of the down-regulated genes were coregulated by BRAF, and 90% of these required the integrity of RET-Y1062. The models differ significantly in that Melillo examined PCCL3 cells that stably and chronically expressed the indicated oncoproteins and grew in a TSH-independent manner. Acquisition of TSH independence does not result directly from expression of either RET/PTC (17) or BRAF (16), indicating that additional changes may have taken place during selection of the cells. By contrast, the genes we identified represent those activated shortly after oncoprotein expression. RET-Y1062 is a docking site for Src homology and collagen, and its substitution efficiently inactivates Ras recruitment and Raf/MAPK activation. However, this same residue also interacts with Enigma and FRS2, potentially evoking a more profound disruption of signaling, and may delete regulation of both MAPK-regulated genes as well as others that are not controlled through this pathway. By contrast, the knockdown of BRAF, while resulting in nearly complete abrogation of RET/PTC-induced ERK1/2 phosphorylation, may still conceivably allow some MAPK-regulated genes to be modulated, perhaps through residual BRAF expression due to incomplete knockdown efficiency. Nevertheless, those genes that fulfill all three criteria (i.e., regulation by RET/PTC3, by BRAF^{V600E}, and reversal by BRAF knockdown) are very likely to require MAPK and provide a good data set to generate hypotheses on possible functional consequences of constitutive MAPK activation in thyroid cells.

RET/PTC regulated functional clusters not requiring BRAF. Most of the up-regulated genes that did not require BRAF were involved in the acquired immune response, and many are known to belong to the IFN-regulated pathway. These findings confirm our previous report in which we examined a single time point after activation of RET/PTC3 on a more limited microarray of 4,273 known genes (29). Although the present study significantly expanded this functional cluster and showed that MAPK is not required for expression of the majority of these gene products, we will not further dwell on this observation. The likely mediators of this expression cluster are signal transducers and activators of transcription 1 (STAT1) and STAT3, based on preliminary data from our lab.[4]

BRAF-dependent genes. The potential functional consequences of the modest down-regulation of MRC complex I genes via BRAF are unknown. Inhibition of MRC complex I has been shown to have opposite effects on cell survival in different studies. Thus, rotenone, a selective MRC complex I inhibitor, and tumor necrosis factor induce mitochondrial membrane permeability transition, cytochrome *c* release, and apoptosis in the leukemia cell line ML-1 (30). By contrast, the predominance of the literature indicates that inhibition of complex I activity protects cells from apoptosis (31). Mutations of MRC complex I genes were recently found in medullary thyroid cancer specimens (32), although their significance remains to be established.

Whereas genes involved in adaptive immunity for the most part did not require BRAF for expression, a significant subset of genes involved in innate immune responses was markedly induced via BRAF. Among them are the monocyte-macrophage chemoattractants MCP1, MCP3, GM-CSF, and CCL15. Russell et al. previously showed that MCP-1 and GM-CSF expression in PCCL3 cells is activated by RET/PTC3, and that this requires RET kinase activity (33). Tumor-infiltrating macrophages are found in about 70% of human PTCs (28). Macrophage infiltration has also been described in thyroid glands from transgenic mice with thyroid cell–specific overexpression of RET/PTC (34) and BRAF (35). In the latter, significant macrophage infiltration was present in at least 90% of Tg-BRAF mice. The role of chronically activated innate immune cells in cancer development has been studied extensively. Because of their ability to produce numerous cytokines, MMPs, reactive oxygen species, and other mediators, they can have key effects on cancer progression (reviewed in ref. 36). This can take place through direct effects on the cancer cell, for instance, by promotion of DNA damage or by paracrine production of growth factors. Tumor-associated macrophages also alter the tumor microenvironment and can promote matrix remodeling and angiogenesis.

[4] L. Zhang et al., unpublished.

$CD11b^+$ macrophages have also been shown to physically contribute to lymphangiogenesis under pathologic conditions, specifically in a model of corneal inflammation (37). As PTCs with BRAF mutation have a higher prevalence of lymphatic metastases, it is possible that BRAF-mediated recruitment of monocyte/macrophages to the tumor microenvironment may contribute to lymphatic spread.

MMPs are zinc-dependent endopeptidases that degrade components of the extracellular matrix and basement membrane. MMPs are synthesized as latent zymogens. The production of active enzymes requires the proteolytic removal of the prodomain. They are subdivided into collagenases, stromelysins, gelatinases, and membrane-type MMP (MT-MMP) based on their substrate preference. The ability of cancer cells to degrade extracellular matrix components is required for invasion of surrounding tissues, entry into, and exit from blood/lymphatic vessels and to metastasize to distant organs. MMPs are good candidates for this property because they can degrade all components of the extracellular matrix. In addition they are up-regulated in virtually all types of cancer and many cancer cell lines. Overexpression of MMPs is associated with cell invasion and poor prognosis in cancer (reviewed in ref. 15). MMP expression has been examined in human thyroid cancer tissues, cell lines, and in serum from patients with the disease. Expression of MMP2, MMP9, TIMP1, and TIMP2 has been found to be elevated in PTCs compared with normal thyroid tissues (38, 39). By contrast, human thyroid cancer cell lines showed a much broader profile of MMP overexpression, as MMP1, MMP2, MMP9, MMP11, and MMP13 were higher in thyroid cancer cell lines than in normal thyrocytes (40). Increased serum levels of MMP2 (41) and MMP9 (42) have been reported in patients with PTC. The association of MMP expression with mutational status of human PTC has not been investigated. In this study, we have shown the preferential induction of MMP3 and MMP9 and the exclusive induction of MMP13 by $BRAF^{V600E}$ compared with RET/PTC3 in PCCL3 cells. These effects of oncogenic BRAF manifested in greater invasiveness compared with PTC3-5 cells, which was abrogated by coincubation with a broad-spectrum MMP inhibitor.

As the induction of MMP13 occurred exclusively in response to BRAF, we examined its contribution to BRAF-induced invasion of PCCL3 cells into Matrigel. Incubation with the specific MMP13 inhibitor CL82198 showed that this metalloproteinase is not required for invasiveness in this model. As MMP13 is critical for endochondral ossification (43), it is tempting to speculate that expression of this protease may favor colonization of bone by metastasizing thyroid cancer.

The expression of MMPs is transcriptionally regulated by cytokines and growth factors. MMP1, MMP3, MMP7, MMP9, MMP10, MMP12, and MMP13 contain activator protein (AP-1) binding sites in their respective proximal promoters (reviewed in ref. 44). Both BRAF and RET/PTC signal through MAPK, which could explain their shared ability to induce MMP expression. The reason for the differences in MMP expression between PCBRAF^{V600E} and PTC3-5 cells is not known but may relate in part to the intensity of ERK activation, which is much higher after conditional expression of oncogenic BRAF than of RET/PTC3 (data not shown). Several other transcription factors play important roles in modulating expression of each of the MMP genes, including *ETS*, *NF-κB*, and *STAT*, among others (44). IFN-γ-mediated activation of STAT1 is associated with inhibition of MMP13 expression in transformed human keratinocytes (40). RET/PTC3 induces marked STAT1 and STAT3 phosphorylation (45, 46), whereas $BRAF^{V600E}$ does not,[5] which may contribute to the fact that overexpression of MMP13 is specific to BRAF.

MMP2 and MT1-MMP mRNA levels were not induced by either RET/PTC3 or $BRAF^{V600E}$, which is consistent with the fact that neither have AP-1 sites in their promoter regions. Interestingly, we did observe increased secretion and activation of pro-MMP2 after oncoprotein expression. Activation of MMP2 requires the MMP inhibitor TIMP2 and MT1-MMP, which form a ternary complex at the cell surface that activates MMP2 by cleavage (47). This process is dependent on the concentration of MT1-MMP, although the role of TIMP2 is more complex. TIMP2 is necessary; yet, high concentrations can also block MMP2 activation (47). We found a modest inhibition of MT1-MMP and TIMP2 in BRAF and PTC3-5 cells (confirmed by RT-PCR; data not shown). Inhibition of TIMP2 could explain in part the MMP2 activation in our system. The induction of MMP2 secretion we observed in the absence of transcriptional regulation could also be a MAPK-dependent phenomenon, as MMP2 secretion is a PKC- and ERK-dependent process in human ciliary muscle cells (48).

Despite the vast amount of preclinical data implicating MMP expression with cancer progression, the clinical trials using MMP inhibitors in cancer patients were disappointing. The stage and type of cancer selected and the lack of a reliable marker for assessment of MMP inhibitory activity are some weaknesses of the studies. Moreover, the MMP substrates are complex and diverse, leading to effects that are unrelated to degradation of the extracellular matrix, such as stimulation of proliferation or induction of apoptosis (reviewed in ref. 15). It is therefore premature to dismiss the significance of MMP overexpression in cancer based on the failure of the clinical trials.

Although our focus was to study the role of oncoproteins signaling via MAPK on gene expression, it should be noted that disruption of signaling along the phosphatidylinositol 3-kinase (PI3K) pathway also has a prominent role in thyroid cancer pathogenesis. Loss of expression of PTEN (49) and activating mutations of RAS result in AKT activation, primarily in follicular neoplasms. Moreover, somatic mutations of *PIK3CA* (50), the gene encoding for catalytic subunit of PI3K, are found in 23% of anaplastic carcinomas. Moreover, AKT activation has also been implicated in thyroid cancer invasiveness (51).

In conclusion, our study shows a fairly clear demarcation between some of the functional gene programs activated by RET/PTC3 and BRAF in thyroid cells. RET/PTC3 induces expression of a remarkably rich array of genes involved in acquired immunity, whereas BRAF evokes expression of genes that promote activation of an innate immune reaction. Moreover, although both genes induce expression of genes involved in matrix remodeling, BRAF does so more robustly, which could account in part for the greater predisposition of PTC with BRAF mutation to invade surrounding tissues. These observations need to be tested and verified in appropriately genotyped human thyroid cancer specimens.

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[5] L. Zhang, unpublished.

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