Suppression of BRAF/MEK/MAP Kinase Pathway Restores Expression of Iodide-Metabolizing Genes in Thyroid Cells Expressing the V600E BRAF Mutant

Dingxie Liu, Shuying Hu, Peng Hou, David Jiang, Stephen Condouris, and Mingzhao Xing

Abstract

Purpose: The V600E BRAF mutant plays an important role in the pathogenesis of papillary thyroid cancer (PTC) and is associated with loss of expression of thyroid iodide-metabolizing genes. This study was done to investigate the restorability of expression of these genes by suppressing the BRAF/extracellular signal-regulated kinase (MEK)/mitogen-activated protein (MAP) kinase pathway in V600E BRAF – harboring thyroid cells and to explore the mechanisms involved.

Experimental Design: We used inducible expression of V600E BRAF, small interfering RNA transfection, and MEK-specific inhibitor to alter the MAP kinase pathway activities and subsequently examined the changes in expression, promoter activities, and methylation status of thyroid genes.

Results: MEK inhibitor U0126 or cessation of V600E BRAF expression in PCCL3 cells restored expression of thyroid genes silenced by induced expression of V600E BRAF. U0126 also restored the expression of these genes in V600E BRAF – harboring PTC-derived NPA cells. Knockdown of BRAF by specific small interfering RNA restored expression of some of these genes in NPA cells. Luciferase reporter assay using thyroid-stimulating hormone receptor gene as a model showed that the promoter activity was modulated by the MAP kinase pathway. Promoter methylation in association with DNA methyltransferase expression played a role in gene silencing by MAP kinase pathway in NPA cells.

Conclusions: We showed the restorability of expression of thyroid iodide-metabolizing genes silenced by V600E BRAF, and linked this process to gene methylation in PTC cells. The results provide clinical implications that therapeutic targeting at the BRAF/MEK/MAP kinase pathway may be a good approach in restoring thyroid gene expression for effective radioiodine therapy for BRAF mutation-harboring PTC.

Papillary thyroid cancer (PTC) accounts for 80% of all thyroid cancers and is the most common endocrine malignancy, with a currently rapidly increasing incidence (1, 2). Following thyroidectomy, radioiodine ablation is the mainstay of medical treatment for PTC, but patients may fail it when the cancer has lost radioiodine avidity, a primary cause for thyroid cancer – related morbidity and mortality (3, 4). This radioiodine treatment takes advantage of the unique function of thyroid cells to uptake, concentrate, and organify iodide, a substrate normally used for synthesis of thyroid hormones. Several thyroid-specific protein molecules play a key role in this iodide-metabolizing process, including thyroid stimulating hormone receptor (TSHR), sodium iodide symporter (NIS), thyroglobulin (Tg), thyroperoxidase (TPO), and the thyroid gene transcription factors TTF-1 and Pax-8 (5). Loss of expression of the genes for these molecules is common in thyroid cancer and is a sufficient cause for the loss of radioiodine avidity and failure of radioiodine therapy in this cancer (6–10). Thus, a novel approach leading to restoration of expression of these thyroid iodide-metabolizing genes would provide great hope for those patients with thyroid cancer that is currently incurable.

A somatic oncogenic mutation, the T1799A transversion mutation of the BRAF gene, has been recently found in PTC, with a high prevalence of 44% on average (11). This mutation causes a V600E amino acid substitution and constitutive activation of the BRAF protein kinase, which is oncogenic through activation of the RAS/RAF/extracellular signal-regulated kinase (ERK) kinase (MEK)/mitogen-activated protein (MAP) kinase pathway. The V600E BRAF mutant plays an important role in PTC tumorigenesis (11) and is associated with aggressive pathologic and clinical outcomes (12–15). BRAF mutation is also associated with silencing of various thyroid iodide-metabolizing genes (16–19) and loss of...
radioiodine avidity (14). In the present study, we investigated the restorability of expression of these genes by suppression of the BRAF/MEK/MEK1 kinase pathway and the molecular mechanism involved and obtained results with important therapeutic implications for PTC.

**Materials and Methods**

**Cell culture.** The human PTC-derived NPA cell line was a gift from Dr. Guy J.F. Juillard (University of California-Los Angeles School of Medicine, Los Angeles, CA). NPA cells were routinely maintained in a humidified incubator with 5% CO₂ at 37°C. RPMI 1640 (Invitrogen, Carlsbad, CA) was used as culture medium and was supplemented with 10% heat-inactivated calf serum, 10 μg/mL apotransferrin, 10 mM hydrocortisone, 10 μg/mL insulin, 10 mM thrytropin, and, in each 500 mL medium, 77 mg sodium pyruvate (Invitrogen, Santa Ana, CA), 750 mg sodium bicarbonate (Invitrogen), 7 mL of 100 × MEM nonessential amino acid (Invitrogen), 5 mL of 100 × antimycotic solution (Omega Scientific, Tarzana, CA), and 1 mL of 50 mg/mL gentamicin (Invitrogen). For some experiments, NPA cells were treated with 10 μM/L U0126 for 5 days with medium and agents changed and replenished daily before total protein and RNA extraction for Western blotting and reverse transcription-PCR (RT-PCR) analysis, respectively. Differentiated rat thyroid cell line PCCL3 with inductive expression of V600E BRAF was a gift from Dr. James A. Fagin (University of Cincinnati College of Medicine, Cincinnati, OH). Cells were cultured as described previously (17). Doxycycline (Sigma, St. Louis, MO) at 1 μg/mL was used to induce V600E BRAF expression for the indicated durations, and U0126 (Sigma) at 10 μM/L was included in some experiments as indicated.

**RNA extraction and RT-PCR analysis.** Total cellular RNA was isolated using TRIzol regent according to the instructions of the manufacturer (Invitrogen). Normal control human thyroid RNA samples were purchased from Stratagene (La Jolla, CA). The reverse transcription synthesis of DNA was conducted with the SuperScript First-Strand Synthesis kit according to the instructions of the manufacturer (Invitrogen). RT-PCR of the gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was simultaneously conducted as quality control. PCR was done with Platinum Taq DNA Polymerase (Invitrogen). 1.5 mM/L MgCl₂, 0.2 mM/L deoxyribonucleotide triphosphate, and 0.33 μM/L of each primer. The primers of the genes analyzed are listed in Table 1.

**Small interfering RNA plasmids and transfection.** A specific small interfering RNA (siRNA) target sequence, located from 523 to 542 nucleotides (nt) of the BRAF cDNA (the translation start site was set as +1), was selected for BRAF RNA interference: 5'-CCTATCGTATACAGTGGAC-3'. A nonspecific siRNA (siRNA 523c) containing the same nucleotide composition but in scrambled sequence was used as the control: 5'-CTATATTACGCTTCGGGCTCG-3'. Two complementary oligonucleotides, 5'-TCGA-(target or control sense)-GAGTCGGCG-(target or control antisense)-TTTTT-3' and 5'-CTAGAAAAA-(target or control sense)-CGACGACT-(target or control antisense)-3', were synthesized and annealed in vitro. The annealed double-stranded oligonucleotides were cloned into the Sall and XhoI sites of siRNA-expressing vector pMg800 (Imgenex, San Diego, CA). Cells were transfected using LipofectAMINE (Invitrogen) according to the manufacturer's protocol. After 48 h of transfection, the transfected cells were selected in a medium containing 0.2 mg/mL G418 for 2 to 3 weeks. Stable cell clones were isolated and confirmed by Western blotting analysis of BRAF protein and ERK phosphorylation.

**Western blotting analysis.** Cells were lysed in radioimmunoprecipitation assay buffer containing 150 mM/L NaCl, 10 mM/L Tris (pH 7.20), 1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM/L EDTA, 2 mM/L NaF, 1 mM/L NaPO₄, and protease inhibitor cocktail (Sigma). Total cellular proteins were resolved on denaturing polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ), and blotted with different primary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA), including anti–phospho-ERK, anti-ERK1, anti-BRAF, and anti-actin. The antigen-antibody complexes were visualized using horseradish peroxidase–conjugated anti-mouse (Santa Cruz Biotechnology) or anti-rabbit (Santa Cruz Biotechnology) IgG antibodies and HyGLO horseradish peroxidase detection kit (Denville Scientific, Metuchen, NJ).

**Construction and transfection of luciferase reporter plasmids and luciferase detection.** Luciferase-expressing vectors pTSHR-Luc and pACTB-RL were generated by inserting TSHR and β-actin promoter fragments into the multiple cloning sites of plasmids PGL2-enhancer and PRL-null (Promega, San Luis Obispo, CA), respectively. Both the TSHR and β-actin promoter fragments were obtained by PCR amplification of human genomic DNA as template. The primers for TSHR promoter amplification were 5'-CCCAACAAAGAGCAGACGACGAC-3' (forward) and 5'-CACCGGGATCCGGGCTTTATT-3' (reverse), which amplified a 3.9 kb DNA fragment spanning −3,827 to +152 nt (numbered from the transcription start site, which is 156 nt upstream of the translation start site of TSHR). The primers for β-actin promoter were 5'-CATTTACTACCGCGCGTCCCGA-3' (forward) and 5'-TCCGATTCCCCGCCAACATTACGAGACG-3' (reverse), which amplified a 1.8-kb DNA fragment spanning −1,390 to +373 nt (numbered from the transcription start site, which is 569 nt upstream of the translation start site of β-actin). For TSHR promoter activity analysis, PCCL3 or NPA cells were cotransfected with pTSHR-luc and pACTB-RL plasmids, the latter being used to normalize cell number and transfection efficiency. Five hours after transfection with luciferase reporter plasmids, PCCL3 cells were treated with 1 μM/mL doxycycline for 67 h to induce V600E BRAF expression. Forty-eight hours after

### Table 1. Primer sequences used in RT-PCR analysis

<table>
<thead>
<tr>
<th>Genes</th>
<th>Species</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product length (bp)</th>
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<td>TSHR</td>
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<tr>
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<td>GCCAGTGGACTCCACGAGC</td>
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transfection of NPA cells with luciferase reporter plasmids, cells were treated with 10 μmol/L U0126 for an additional 24 h. Cell lysates were then prepared after a total of 72 h of transfection, and firefly and Renilla luciferase activities in 5 μL of lysate were measured with the Dual Luciferase Reagent (Promega).

**Sodium bisulfite treatment of genomic DNA and gene methylation analysis using real-time quantitative methylation-specific PCR.** DNA bisulfite treatment was done as described previously (20, 21). Briefly, 2 μg of DNA were denatured by treatment with 0.2 mol/L NaOH in 20 μL H2O at 50°C for 20 min and subsequently incubated for 3 h at 70°C in 500 μL of a freshly prepared solution containing 3 mol/L sodium bisulfite and 10 mmol/L hydroquinone. Treated DNA was purified using a Wizard DNA Clean-Up System (Promega), and eluted into H2O, followed by precipitation with ethanol, vacuum drying, and resuspension in H2O. Bisulfite-modified DNA was used as the template for fluorescence-based quantitative methylation-specific PCR (QMSP; Taqman) as previously described (20). The relative level of methylation was normalized using the ratio obtained from the values of the gene of interest over the values of the internal reference gene (β-actin). Fluorogenic QMSP assays were carried out in a reaction volume of 20 μL on a 384-well plate in an Applied Biosystems 7900 Sequence Detector (Perkin-Elmer, Foster City, CA). Normal leukocyte DNA after in vitro methylation with Sss I methylase (New England Biolabs, Beverly, MA) was used as a positive control. Each plate contained multiple water blanks, negative unmethylated controls, duplicate patient samples, and serial dilutions of positive methylated control to construct the calibration curve. The primers and probes for TSHR and β-actin used were the same as described previously (21). Conventional MSP was used to detect TSHR gene promoter methylation in rat PCCl3 cells using a previously described protocol (21) and the following primers: 5’-TGTTGTTGTGTGGTGTCCG-3’ and 5’-TTTACCGATATAACGACCG-3’ for amplification of methylated DNA, and 5’-TTTTGGTGTTGTGTGTGTGTTGTTTG-3’ and 5’-CCCTTACACATATA-CACACCCA-3’ for unmethylated DNA.

The results shown in each figure are representative of two to four similar experiments.

**Results**

**Restoration of iodide-metabolizing genes in V600E BRAF–harboring rat thyroid cells by suppression of BRAF/MEK/MAP kinase pathway.** To test our hypothesis that BRAF mutant–induced silencing of thyroid genes is reversible, we first used a rat thyroid cell line, PCCl3, with inducible expression of V600E BRAF (17). Consistent with previous findings (17), doxycycline-induced conditional expression of the V600E BRAF for 6 days down-regulated the expression of TSHR, NIS, Tg, and PAX-8 genes in this cell (Fig. 1A and B). We additionally found down-regulation of the TTF-1 gene by V600E BRAF expression. Expression of the TPO gene was not affected. To take a further step toward testing our hypothesis, we tested the effect of a MEK-specific inhibitor, U0126 (20), and showed that treatment of cells with this inhibitor could restore the expression of all the genes down-regulated by acute induction of V600E BRAF (Fig. 1A and B). Removal of doxycycline to shut off V600E BRAF expression correspondingly restored the expression of thyroid genes (Fig. 1C). The induction of V600E BRAF and the effect of U0126 were respectively correlated with the activation and blockade of the BRAF/MEK/MAP kinase signaling (Fig. 1D).

BRAF V600E can cause chromosomal instability (17) and possible secondary genetic alterations, which may lead to permanent and potentially irreversible biological consequences to the cell. It is conceivable that some of these biological consequences of BRAF mutation in PTC are results of the long-term effect of this mutation. We therefore rationalized that reversibility of thyroid gene silencing induced by acute induction of the V600E BRAF might not necessarily apply to naturally existing PTC cells that persistently express V600E BRAF.

Consequently, we investigated whether expression of thyroid genes could still be restored when silenced after a long-term induction of V600E BRAF. As shown in Fig. 1A and B, after 30 days of persistent induction of the V600E BRAF, a more profound suppression of gene expression, particularly TSHR and NIS genes, was observed, perhaps reflecting different biological consequences of short- and long-term effect of V600E BRAF. Importantly, these silenced thyroid genes could still be restored by U0126 treatment after the long-term induction of V600E BRAF. In fact, restoration of the long-suppressed expression interestingly showed an “overshot” for the TSHR and NIS genes after U0126 treatment of cells (Fig. 1B). These results also showed that among the thyroid genes examined, expression of the TSHR and NIS genes was most susceptible to the influence of V600E BRAF mutant and was also most readily restorable.

**Restoration of iodide-metabolizing genes in V600E BRAF–harboring human PTC cells by suppression of BRAF/MEK/MAP kinase pathway.** To better assess the reversibility of silencing of thyroid genes induced by BRAF mutation in a condition that more closely mimicked human PTC cells than rat thyroid cells, we next tested the effect of U0126 on NPA cell, a human PTC–derived cell line that harbored homozygous BRAF mutation with strong activation of the BRAF/MEK/MAP kinase pathway (12). As shown in Fig. 2A, expression of the TSHR and NIS was naturally silenced in this BRAF mutation–harboring cell and was restored after treatment of the cell with U0126. A low-level expression of Tg was naturally present in this cell, which was significantly enhanced by treatment of the cell with U0126 (Fig. 2A). This U0126 treatment was correlated with suppression of the BRAF/MEK/MAP kinase signaling with inhibition of ERK phosphorylation (Fig. 2B). We were not able to achieve restoration of the TPO, TTF-1, and PAX-8 genes under these conditions in NPA cells (data not shown).

We took a further step to assess the restorability of thyroid gene expression by specific knockdown of BRAF using the siRNA approach. After transfection with a plasmid expressing specific BRAF siRNA followed by G418 selection, three NPA cell clones were obtained in which the BRAF expression was stably knocked down (Fig. 3A). Two control clones stably transfected with nonspecific siRNA containing scrambled nucleotides showed no suppression of BRAF protein. RT-PCR was done for thyroid gene expression analysis and showed that expression of the TSHR gene was restored by specific knockdown of BRAF (Fig. 3B). In contrast, no reexpression of the TSHR gene was seen in the clones transfected with nonspecific siRNA (Fig. 3B). Unlike the effects of U0126 on thyroid gene expression in NPA cells (Fig. 2), no reexpression of NIS and Tg occurred in these specific siRNA clones (data not shown). However, we did observe reexpression of Tg in pooled cells transiently transfected with siRNA before individual clone isolation (Fig. 3C), suggesting that the Tg gene was reexpressed in a subpopulation of cells contained in the cell pool. Expression of other thyroid genes was not restored with this siRNA approach. One possible explanation for the different outcomes achieved in gene expression with the MEK inhibitor and with the siRNA approach is that MEK inhibitor was able to almost completely

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U0126 restored the expression of iodide-metabolizing thyroid genes that were down-regulated by conditional expression of V600E BRAF. A. RT-PCR analysis of expression of the indicated thyroid-specific genes. Total RNA was isolated from PCCL3 cells without or with V600E BRAF induction by continuous treatment of cells without or with doxycycline (−DOX and +DOX, respectively) for 6 or 30 d. During the last 4 d of doxycycline induction, cells were additionally treated with 10 μmol/L U0126 before RNA isolation. RT-PCR of β-actin was used as quality control.

B. Quantitative illustration of expression of thyroid genes. Densitometry was done to measure the density of the corresponding electrophoresis bands of the RT-PCR products shown in (A).

C. reexpression of V600E BRAF silenced thyroid genes after withdrawal of doxycycline in PCCL3 cells. Shown in the upper portion of the panel is the RT-PCR analysis of the TSHR and NIS genes before and after DOX induction of V600E BRAF (for 6 d), and 3, 5, and 7 d after removal of DOX from the culture medium after a 6-d treatment. RT-PCR of β-actin was used as quality control. Shown in the lower portion of the panel is a quantitative illustration of expression of the indicated thyroid genes using densitometry to measure the density of the electrophoresis bands of the corresponding RT-PCR products shown in the upper portion of the panel. D. BRAF/MEK/MAP kinase pathway activity analysis in PCCL3 cells. The treatment conditions of cells were identical to those in (A) before extraction of total cellular proteins. The MAP kinase signaling pathway activity was reflected by ERK phosphorylation level detected by Western blotting of cellular proteins using specific anti–phosphorylated ERK (p-ERK) antibodies. Total ERK (t-ERK) was used for quantity control of proteins.
block the BRAF/MEK/MAP kinase pathway and eliminate ERK phosphorylation (Fig. 2B), whereas the siRNA used could only knockdown BRAF and did not affect other forms of Raf kinases, leaving significant residual ERK phosphorylation (Fig. 3A). Involvement of other molecular abnormalities in addition to aberrant activation of the BRAF/MEK/MAP kinase pathway is also possible in the silencing of different thyroid genes in PTC-derived NPA cells. This hypothesis is consistent with the fact that U0126 treatment could restore the expression of Pax-8 and TTF-1 genes silenced by induction of V600E BRAF in well-differentiated PCCL3 thyroid cells (Fig. 1) but not in NPA cells. The effective restoration of the TSHR gene expression by the siRNA transfection was consistent with the data in Figs. 1 and 2 showing that TSHR gene was most sensitive to the influence of BRAF/MEK/MAP kinase pathway.

**Modulation of the TSHR gene promoter activity by BRAF/MEK/MAP kinase pathway.** We rationalized that the effect of V600E BRAF mutant on thyroid gene expression was through affecting the gene promoter activity. We therefore next used TSHR as a model to study its promoter activity under various activity states of the BRAF/MEK/MAP kinase pathway. To this end, we constructed an expression vector in which the TSHR promoter drove firefly luciferase expression and used it to transfect NPA cells and V600E BRAF-expressible PCCL3 cells. As shown in Fig. 4B, the activity of exogenously introduced human TSHR promoter in PCCL3 cells was diminished upon induced expression of the V600E BRAF mutant. The exogenously introduced TSHR promoter activity was enhanced by treatment with U0126 in NPA cells. These results suggest that inhibition of thyroid gene expression by BRAF/MEK/MAP kinase pathway and restoration of the expression were mediated through modulation of the gene promoter activities.

**Link between TSHR promoter methylation and BRAF/MEK/MAP kinase pathway in PTC cells.** To explore further the molecular mechanism involved in the modulation of thyroid gene expression and promoter activity by BRAF/MEK/MAP kinase pathway, we again used TSHR gene as a model to examine the relationship between its promoter methylation and BRAF/MEK/MAP kinase pathway signaling. As shown in Fig. 5A, we first showed that in NPA cells, the TSHR promoter was methylated, which silenced the gene, and demethylation by treatment of cells with 5-aza-2′-deoxycytidine could restore the expression of TSHR. These results were consistent with the finding of TSHR gene methylation in the regulation of its expression in other PTC-derived cell lines (21). We next examined the relationship of TSHR promoter methylation with the BRAF/MEK/MAP kinase pathway. As shown in Fig. 5B, methylation of the TSHR promoter was decreased by treatment of NPA cells with U0126 in a time- and concentration-dependent manner. To specifically look at the role of V600E BRAF, we examined the effect of specific knockdown of V600E BRAF on TSHR promoter methylation by siRNA in the NPA cell line.
cell that naturally harbored homozygous Braf mutation. As shown in Fig. 5C, the Tshr promoter methylation level was significantly lower in cell clones stably transfected with Braf siRNA than cell clones transfected with nonspecific siRNA. Interestingly, no methylation of Tshr promoter was found in PCCL3 cells upon induced expression of the V600E Braf (Fig. 5D) although expression of Tshr gene was diminished under these conditions (Fig. 1). Unlike NPA cells in which treatment with the demethylating agent 5-aza-2'-deoxycytidine could restore expression of Tshr (Fig. 5A), treatment with 5-aza-2'-deoxycytidine had no effect on V600E Braf–suppressed expression of the Tshr gene in PCCL3 cells (data not shown). Thus, acute silencing of thyroid genes by aberrant activation of MAP kinase pathway did not seem to involve gene methylation in PCCL3 cells.

It was reported that activated MAP kinase pathway upregulated expression of DNA methyltransferase (DNMT; ref. 22). As gene methylation was involved in MAP kinase pathway–induced silencing of thyroid genes in NPA cells, we investigated whether alteration in DNMT expression could be a mechanism in these cells. We therefore examined the expression levels of DNMT genes, including DNMT1, DNMT3a, and DNMT3b, in these cells. As shown in Fig. 6, U0126 treatment and Braf siRNA transfection diminished the expression of these DNMT genes to various extents, with DNMT1 and DNMT3b being affected more significantly and consistently. Expression of all of the three DNMT genes was up-regulated in the V600E Braf–harboring NPA cells compared with normal thyroid tissues (Fig. 6A and B). It is also worth noting that serum starvation in day 3 lowered the expression of DNMT1 and DNMT3b even in the control nonspecific siRNA-transfected cells in comparison with day 1 (starting day; Fig. 6C and D), suggesting that removal of growth factors in the culture medium that stimulated MAP kinase pathway had a negative effect on the expression of these two DNMT enzymes. A small decrease in Tshr methylation was correspondingly seen also in the nonspecific siRNA-transfected NPA cells after 3 days of serum starvation (Fig. 5C).

**Discussion**

Although thyroid cancer is usually associated with a relatively indolent course and good prognosis, many patients become incurable with significant morbidity and mortality (3, 4). As the incidence of thyroid cancers is currently rapidly increasing, the number of patients with increased morbidity and mortality is also expected to be increasing (1, 2). A major cause for these patients’ poor outcome is their failure to respond to radioiodine ablation therapy after surgical thyroidectomy. This failure is due to the loss of radioiodine avidity of the cancer, which is, in turn, due to aberrant silencing of iodide-metabolizing genes in thyroid cancer cells (6–10). In PTC, silencing of these genes was found to be particularly associated with the V600E Braf (16–19). It is not known whether such V600E Braf–induced gene silencing is reversible by altering the BRAF/MEK/MAP kinase pathway signaling, a potentially novel and effective therapeutic approach for PTC. The molecular mechanism involved in this V600E Braf–induced silencing of thyroid genes is also unclear.

In the present study, we showed the restorability of the expression of several key thyroid iodide-metabolizing genes by suppressing BRAF/MEK/MAP kinase pathway in thyroid cells expressing the V600E Braf mutant. Using Tshr gene as a model, we showed that the effect of the BRAF/MEK/MAP kinase pathway on thyroid gene expression occurred through alteration of gene promoter activity, which may involve methylation. In NPA cells, methylation-mediated alteration of Tshr gene expression in relation to BRAF/MEK/MAP kinase pathway echoed an alteration in the expression of DNMTs, particularly DNMT1 and DNMT3b. DNMT1 and DNMT3b account for nearly all the DNMT activities and cooperatively maintain DNA methylation and gene silencing in human cells. As gene methylation was involved in MAP kinase pathway–induced silencing of thyroid genes in NPA cells, we investigated whether alteration in DNMT expression could be a mechanism in these cells. We therefore examined the expression levels of DNMT genes, including DNMT1, DNMT3a, and DNMT3b, in these cells. As shown in Fig. 6, U0126 treatment and Braf siRNA transfection diminished the expression of these DNMT genes to various extents, with DNMT1 and DNMT3b being affected more significantly and consistently. Expression of all of the three DNMT genes was up-regulated in the V600E Braf–harboring NPA cells compared with normal thyroid tissues (Fig. 6A and B). It is also worth noting that serum starvation in day 3 lowered the expression of DNMT1 and DNMT3b even in the control nonspecific siRNA-transfected cells in comparison with day 1 (starting day; Fig. 6C and D), suggesting that removal of growth factors in the culture medium that stimulated MAP kinase pathway had a negative effect on the expression of these two DNMT enzymes. A small decrease in Tshr methylation was correspondingly seen also in the nonspecific siRNA-transfected NPA cells after 3 days of serum starvation (Fig. 5C).

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![Diagram](https://example.com/diagram.png)
cancer cells (23). Overexpression of these two DNMTs and associated aberrant gene methylation are commonly seen in human cancers (24–27). Consistent with the data in the present study, MAP kinase pathway was previously reported to cause DNMT expression and associated methylation and silencing of certain genes, such as T lymphocyte genes in lupus (22) and the tumor-suppressor gene Par-4 in transformed epithelial cells (28). Thus, promoter methylation may be a common mechanism in mediating gene silencing induced by aberrant activation of the MAP kinase pathway. Interestingly, in MAP kinase pathway–promoted TSHR gene silencing, methylation involvement was cell selective; it occurred in PTC-derived NPA cells naturally expressing V600E BRAF, but not in differentiated PCCL3 thyroid cells acutely expressing the V600E BRAF. The latter presumably involves alternative mechanisms. Involvement of promoter methylation and silencing of thyroid genes in NPA cells may reflect a result of long-term effect of V600E BRAF on epigenetic programming in these PTC-derived cells. The finding of the link of gene methylation to V600E BRAF–induced silencing of TSHR represents an interesting further step from our previous studies showing promoter methylation as a mechanism in silencing of this gene in thyroid cancer (21). These data on the relationship among TSHR gene expression, methylation, and BRAF/MEK/MAP kinase pathway signaling are also consistent with previous findings in human PTC tissues of the association of BRAF mutation with aberrant methylation of SLC5A8 (16, 29), a putative iodide transporter gene in the apical membrane of follicular thyroid cells (30, 31).

Regardless of the specific molecular mechanisms involved, the restorability of the expression of thyroid genes by suppression of V600E BRAF–activated BRAF/MEK/MAP kinase pathway has important clinical implications. It suggests that clinical use of specific inhibitors to suppress the BRAF/MEK/MAP kinase pathway in conjunction with radioiodine therapy may improve the treatment efficiency for BRAF mutation–harboring PTC, particularly in those with impaired or lost radioiodine avidity. This novel therapeutic approach to thyroid cancer is attractive and practically possible given the availability of inhibitors of the RAF/MEK/MAP kinase pathway that were recently shown to inhibit the growth of BRAF mutation–harboring human cancer cells, including those derived from human PTC (32, 33). It is worth noting that among the thyroid genes examined in the present study, the TSHR gene was most easily restorable. TSHR plays a central role in up-regulation of other thyroid genes (5). Therefore, induced expression of TSHR could facilitate

**Fig. 5.** Relationship between expression and methylation of TSHR gene and BRAF/MEK/MAP kinase pathway in NPA cells. A, reexpression of TSHR gene (left) and corresponding demethylation of TSHR promoter (right) in NPA cells treated with 5 μmol/L demethylating agent 5-aza-2’-deoxycytidine (5-Aza-dC) for 24 h. Expression of TSHR was analyzed by RT-PCR, and methylation was analyzed by QMSP as described in Materials and Methods. B, demethylation of TSHR promoter in NPA cells by treatment with U0126 in a time-dependent (left) and concentration-dependent (right) manner. U0126 (10 μmol/L) was used in the time course study; in the concentration-response study, cells were treated with the indicated concentrations of U0126 for 5 d. Methylation was analyzed by QMSP. C, demethylation of TSHR promoter in NPA cells by stable V600E BRAF knockdown. 2D7, cell clone stably transfected with nonspecific control siRNA. 2C3 and 3B12, cell clones stably transfected with specific BRAF siRNA. A clear difference in TSHR promoter methylation in these clones was seen after 3 d of serum starvation. Methylation was analyzed by QMSP. D, methylation analysis by conventional MSP of TSHR promoter in PCCL3 cells with expression of V600E BRAF induced for 6 or 30 d as indicated. M, methylated; U, unmethylated. The genomic DNA isolated from PCCL3 and treated with SsII methylase was used as positive control for MSP assay.
expression of other iodide-metabolizing genes in thyroid cancer. In recent years, human recombinant thyroid stimulating hormone is increasingly used to facilitate radioiodine management of patients with thyroid cancer (34). The readily restorable TSHR expression in PTC cells suggests that use of BRAF/MEK/MAP kinase inhibitors in conjunction with radioiodine ablation therapy may be particularly effective for thyroid cancer when recombinant thyroid stimulating hormone is used.

In summary, using several unique molecular techniques, we showed the restorability, by suppression of the BRAF/MEK/MAP kinase pathway, of the expression of thyroid iodide-metabolizing genes silenced by V600E BRAF. Using the TSHR gene as a model, we also showed that modulation of gene promoter activity, through methylation, is a mechanism for thyroid gene silencing by the V600E BRAF–activated BRAF/MEK/MAP kinase pathway in PTC-derived cells. These data provide important therapeutic implications for BRAF mutation–harboring PTC.

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Fig. 6. Suppression of expression of DNMT by inhibition of BRAF/MEK/MAP kinase pathway in NPA cells. A, RT-PCR analysis of expression of the three DNMT genes in NPA cells treated with or without 10 μmol/L U0126 for 5 d. B, densitometric presentation of the results in (A), as described in Fig. 1B. C, RT-PCR analysis of expression of DNMT genes in NPA cells stably transfected with BRAF siRNA. RT-PCR of β-actin was used as quality control. D, densitometric presentation of the results in (C). Cell clones are as defined in Fig. 3 legend.

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