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BRAF V600E Maintains Proliferation, Transformation, and Tumorigenicity of BRAF-Mutant Papillary Thyroid Cancer Cells

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Context: Although the BRAF V600E mutant can initiate the formation of papillary thyroid cancer (PTC), it is unclear whether it is required to maintain cell proliferation, transformation, and tumor growth of *BRAF* mutation-harboring PTC.

Objective: The aim of the study was to investigate whether BRAF V600E is required for the proliferation, transformation, and tumorigenicity of *BRAF* mutation-harboring PTC cells.

Design: We addressed this issue using BRAF small interference RNA (siRNA) to transfect stably several *BRAF* mutation-harboring PTC cell lines, isolated clones with stable suppression of BRAF, and assessed their ability to proliferate, transform, and grow xenograft tumors in nude mice.

Results: PTC cell proliferation and transformation were suppressed in specific BRAF siRNA clones, but not in control scrambled siRNA clones. Specifically, taking the advantage of stable BRAF knockdown, we were able to show continued suppression of PTC cell proliferation and transformation, or anchorage-independent colony formation in soft agar, after long-term culture. Moreover, we also demonstrated that *in vivo* tumorigenicity and growth of tumors from the specific BRAF siRNA cell clones in nude mice were suppressed compared with control clones.

Conclusions: BRAF V600E is not only an initiator of PTC as demonstrated previously but is also a maintainer of proliferation, transformation, and tumorigenicity of PTC cells harboring *BRAF* mutation, and growth of tumors derived from such cells continues to depend on BRAF V600E. These results provide further support for potentially effective therapy targeted at BRAF for *BRAF* mutation-harboring PTC. **(*J Clin Endocrinol Metab* 92: 2264–2271, 2007)**

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B-RAF KINASE (BRAF) is a key component of the RET/PTC → Ras → Raf → MEK → MAPK/ERK signaling pathway (termed "MAPK pathway" hereafter) that transmits mitogenic signals from the cell membrane to the nucleus and promotes cell division and proliferation (1). Aberrant activation through genetic alterations, such as activating mutations of the gene for *BRAF*, plays an important role in tumorigenesis in human cancers (2). The T1799A transversion mutation of the *BRAF* gene, which causes a V600E amino acid substitution in the BRAF protein and constitutive activation of the kinase, is the most common *BRAF* mutation, accounting for more than 80% of all mutations of this gene (3). This mutation is commonly seen in human cancers, particularly in melanoma (2) and thyroid cancer (4–8). A mutual exclusivity among *BRAF* mutation, *Ras* mutation, and *RET/PTC* was reported in thyroid cancer (5, 7), supporting an independent oncogenic role of *BRAF* mutation through the MAPK pathway in thyroid cancer.

Papillary thyroid cancer (PTC) is the most common type of thyroid malignancy, accounting for more than 80% of all thyroid malignancies (9). Of various thyroid cancers, the T1799A *BRAF* mutation occurs virtually only in PTC, with a prevalence of 45% on average (10). Several large studies showed an association of *BRAF* mutation with aggressive characteristics of PTC, such as extrathyroidal invasion, lymph node metastasis, advanced tumor stage, and clinical recurrence (6, 11–13). Targeted expression of the V600E BRAF in the thyroid gland in transgenic mouse could induce the development of PTC with progression to undifferentiated thyroid cancer (14). These data demonstrated the ability of *BRAF* mutation to initiate PTC formation and progression. A recent study showed that conditional expression of the V600E BRAF mutant caused chromosomal instability (15), suggesting that secondary genetic alterations might play an important role in maintaining the malignancy and progression of PTC. Thus, it remains a question whether, after initiation and development of PTC, *BRAF* mutation is still necessary for the phenotypic maintenance of cell proliferation and transformed state, as well as tumor growth of PTC, and is, therefore, an effective therapeutic target for PTC harboring this mutation. It was recently proposed that further studies using molecular approaches, particularly stable transfection of specific small interference RNA (siRNA), are needed to assess this role of *BRAF* mutation (16). Therefore, we addressed this issue in the present study using a strategy employing stable transfection of BRAF-specific siRNA.

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Abbreviations: PTC, Papillary thyroid cancer; siRNA, small interference RNA.

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Materials and Methods

Cell culture

Human PTC-derived cell lines KAT5 and KAT10 were a kind gift from Dr. Kenneth B. Ain (University of Kentucky Medical Center, Lexington, KY) and NPA from Dr. Guy J. F. Juillard (University of California-Los Angeles School of Medicine, Los Angeles, CA). KAT5 and KAT10 cells harbor a heterozygous V600E BRAF mutation, and NPA cells harbor a homozygous mutation (4–6). These cell lines were routinely cultured at 37 C in RPMI 1640 medium with 10% calf serum and 5% carbon dioxide. For NPA cells, apo-transferrin (5 μg/ml), hydrocortisone (10 nM), insulin (10 μg/ml), and TSH (10 mIU/ml) were also supplemented to the medium.

siRNA plasmid construction and cell transfection

BRAF-specific siRNAs were designed using an established program of Imgenex Corp. (http://imgenex.com/sirna_tool.php; San Diego, CA). There were two siRNA target sequences, located from 313nt to 332nt and from 523nt to 542nt of the BRAF cDNA (the translation start site was set as +1), respectively, selected for BRAF RNAi: tacaccagcaagctagatgca (siRNA 313) and cctatcgttagagtcttcctg (siRNA 523). Two corresponding nonspecific siRNA duplex containing the same nucleotide composition but in random sequence (scrambled) were used as control: atagagcgatcacatacagcc (siRNA 313c) and ctattatagctctcgcgttcg (siRNA 523c). There were two complementary oligonucleotides, tcga-(target or control sense)-gagtcgtcg-(target or control antisense)-ttttt and ctagaaaaa-(target or control sense)-cgacgactc-(target or control antisense), synthesized for each target sequence and annealed *in vitro*. The annealed double-stranded oligonucleotides were cloned into the Sal I and Xbal I sites of siRNA expressing vector pMG800 (Imgenex Corp.). Cells were transfected using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For stable transfection, the transformants were selected in medium containing 0.4 mg/ml (for KAT5 and KAT10 cells) or 0.2 mg/ml (for NPA cells) G418 for 2–3 wk. Stable cell clones were confirmed by Western blotting analysis of BRAF protein and ERK phosphorylation. It should be noted that the NPA cells harbor homozygous *BRAF* mutation. Therefore, for this cell, even the BRAF siRNAs were not specifically designed for the V600E BRAF; it was the V600E BRAF that was targeted and knocked down. For KAT5 and KAT10 cells, both wild BRAF and the V600E BRAF were targeted.

Western blotting analysis

Cells were lysed in RIPA Buffer [150-mM NaCl, 10-mM Tris, pH 7.20, 1% sodium dodecyl sulfate, 1% Triton X-100, 1% deoxycholate, 5-mM EDTA, 2-mM NaF, 1-mM VaPO3, and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)]. Total cellular proteins were resolved on denaturing polyacrylamide gels, transferred to polyvinylidene fluoride membranes (Amersham Biosciences, Piscataway, NJ), and blotted with specific primary antibodies from Santa Cruz (Santa Cruz, CA), including anti-phospho-ERK (Sc-7383), anti-ERK1 (Sc-94), anti-BRAF (Sc-166), or from Cell Signaling (Beverly, MA), including anti-phospho-MEK1/2 (no. 9121) and anti-MEK1/2 (no. 9122). The antigen-antibody complexes were visualized using the HyGLO HRP detection kit (Denville Scientific, Inc., Metuchen, NJ) after further incubation with horseradish peroxidase-conjugated antimouse (Sc-2005; Santa Cruz) or antirabbit (Sc-2004; Santa Cruz) IgG antibodies.

Colony formation assay

For colony formation assay to examine cell growth in monolayer culture, cells (8×10^5/well) were transfected with plasmid in 6-well plate. After a 24-h culture, cells were stripped and inoculated into a 6-well plate (1×10^5 cells/well for KAT5 and 2×10^4 cells/well for KAT10). After an additional 24-h culture, G418 (0.4 mg/ml) was added for selection for 2 wk. Surviving colonies were counted after staining with crystal violet. For colony formation assay to examine anchorage-independent cell growth in soft agar, 4×10^3 cells/well (for NPA) or 2.5×10^3 cells/well (for KAT10) were plated in RPMI 1640 containing 10% fetal bovine serum, 0.2 mg/ml G418, and 0.33% agar in 6-well plates. After 3–4 wk of culture, colonies were photographed, and the colony number was counted under a microscope.

Cell proliferation assay

Cells (500/well) were seeded into a 96-well plate and cultured with 2.5% serum. MTT assay was performed daily over a 5-d time course to evaluate cell proliferation. The cell confluence was about 5–10% initially and reached 80–90% by d 5. At the end of the culture period, 10 μl of 5 mg/ml MTT (Sigma-Aldrich) was added to the culture. After incubation for 4 h, 100 μl of 10% sodium dodecyl sulfate solution was added, followed by incubation for another 12 h. The plates were then read on a microplate reader using a test wavelength of 570 nm and a reference wavelength of 670 nm. Four duplicates were performed to determine each data point. Cell proliferation rates among groups were analyzed by performing ANOVA followed by the Student-Newman-Keuls test as described previously (17, 18), and a P value < 0.05 was considered significant.

Xenograft tumor assay in nude mice

Cell clones stably transfected with control or specific BRAF siRNA were grown to approximately 80% confluence and harvested with 0.25% Trypsin/1 mM EDTA solution. Evenly suspended 2×10^6 cells in 100 μl of RPMI 1640 medium were injected sc into the flanks of nude mice (four for each group) at the age of about 5 wk (Harlan Sprague Dawley, Indianapolis, IN). Growth of tumors was assessed by measuring the size of the tumor on the skin surface twice a week and photographed periodically. The tumor volume is calculated by the formula "volume = $(\text{width})^2 \times \text{length}/2$," as described previously (19). At the end of 5 wk after the cell inoculation, mice were scarified, and the developed tumors were surgically removed, weighted, and photographed. For the assess-

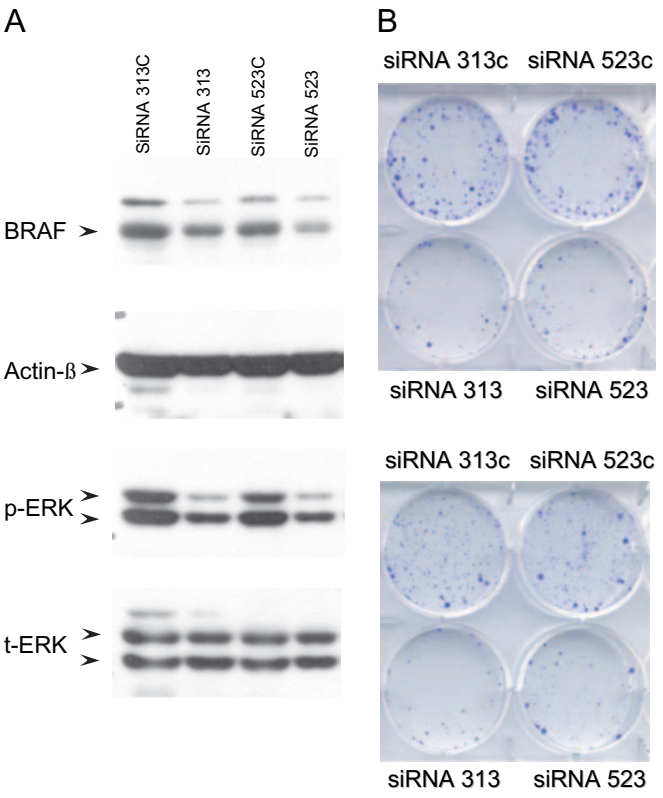

FIG. 1. Inhibition of V600E BRAF expression and cell growth by RNA interference. A, A representative result of Western blotting analysis of BRAF expression and ERK phosphorylation in KAT5 cells after transient transfection with siRNA vectors. B, A representative result of colony formation of KAT5 (*upper* panel) and KAT10 (*lower* panel) cells in monolayer culture after transfection of siRNA vectors followed by G418 selection for 2 wk. Cell colonies were stained with crystal violet. siRNA 313 and siRNA 523, BRAF-specific siRNA; si313c and si523c, nonspecific siRNA (with scrambled sequences); p-ERK, phosphorylated ERK; t-ERK, total ERK protein.

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ment of BRAF expression level in the tumors, 10-mg tumor tissue was weighted and lysed by homogenization for Western blotting analysis using the protocol described previously for Western blotting studies for cell lines.

Results

Inhibition of BRAF expression and cell proliferation by RNA interference

To knock down BRAF expression in human PTC cells harboring the T1799A *BRAF* mutation, we generated and tested a series of siRNAs targeting different sites of the human *BRAF* coding sequence. Of them, siRNA 313 and 523, which targeted respectively the sequences located from 313nt to 332nt and from 523nt to 542nt of the *BRAF* cDNA, were found to be most effective in knocking down BRAF protein and in suppressing ERK phosphorylation when transiently transfected into cells such as KAT5 cells, as shown in Fig. 1A. In contrast, no such effects were observed for the two controls siRNA 313c and 523c, which had the same nucleotide compositions as in siRNA 313 and siRNA 523, respectively, but in random sequence. We then tested whether the BRAF siRNA could inhibit proliferation/growth of thyroid cancer cells. Transfection of KAT5 and KAT10 cells with siRNA 313 or siRNA 523 expression vector, followed by 2 wk of culture in G418-containing media, resulted in significant inhibition of cell colony formation in monolayer culture (up to 60%) in both KAT5 and KAT10 cells when compared with control vectors expressing the scrambled siRNA (Fig. 1B).

Inhibition of cell proliferation by stable knockdown of BRAF

To study the consequence of durable suppression of aberrantly activated MAPK pathway signaling by BRAF V600E, we isolated siRNA tranfectant cell clones with stable BRAF knockdown from *BRAF* mutation-harboring PTC cell lines. There were three KAT10 cell clones transfected with siRNA 313, and three KAT10 and three NPA cell clones transfected with siRNA 523 identified in which BRAF expression was stably and effectively suppressed (up to >90%) (Fig. 2). Correspondingly, these cell clones lost most of the MAPK pathway signaling activity in terms of MEK and ERK phosphorylation. No apparent cell morphology changes were found in these cell clones with BRAF knockdown (data not shown). We subsequently investigated the effect of stable BRAF knockdown on proliferation of these specific siRNA clones by MTT assay. Detection of cell number was performed daily over a 5-d time course and cell proliferation curve was derived. As shown in Fig. 3, cell clones transfected with BRAF-specific siRNA had a significantly lower proliferation rate than the control cell clones transfected with scrambled siRNA in both KAT10 and NPA cells. On d 5, the total number of cells with BRAF knockdown was about three times less than that of the control cells ($P < 0.05$). Significant difference in cell number occurred on d 4 for KAT10 cell clones and on d 3 for NPA cell clones ($P < 0.05$). It is interesting to note that the effect of siRNA on proliferation become evident earlier for NPA cells than KAT10 cells. This might reflect the fact that

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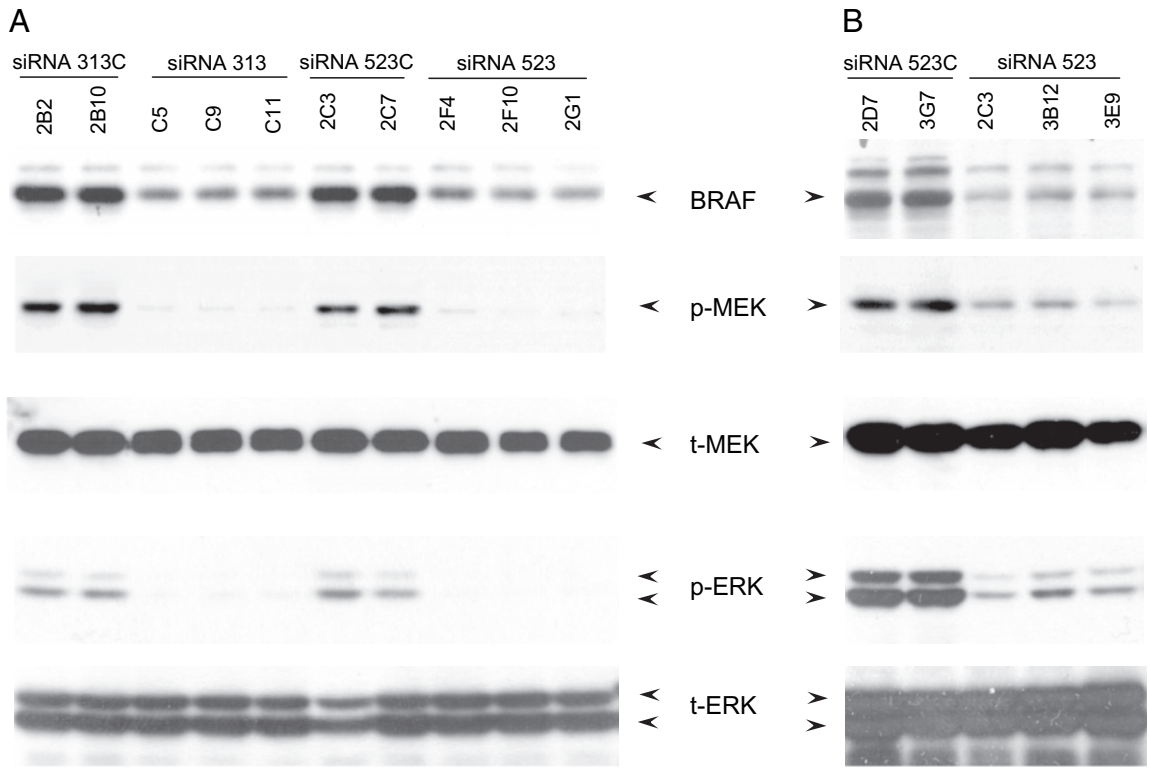

FIG. 2. Western blotting analysis of BRAF expression, MEK phosphorylation, and ERK phosphorylation in cell clones stably transfected with various siRNAs. A, Clones derived from KAT10 cells. B, Clones derived from NPA cells. Several different individual cell clones for each type of specific or control siRNA were selected and identified, all showing a dramatic decrease in BRAF protein, MEK phosphorylation, and ERK phosphorylation in specific siRNA clones compared with nonspecific siRNA clones. p-MEK, Phosphorylated MEK; t-MEK, total MEK protein.

NPA cells harbor a homozygous V600E BRAF mutation, whereas KAT10 cells harbor a heterozygous mutation, and, consequently, proliferation of the former relies on a stronger driving pressure from the MAPK pathway and may, therefore, be more susceptible to BRAF knockdown.

Inhibition of anchorage-independent cell growth by stable knockdown of BRAF

We also investigated the effect of stable BRAF knockdown on anchorage-independent cell growth in soft agar, an indication of cellular transformation. Figure 4 shows that although KAT10 cells with BRAF knockdown could still form colony in soft agar, the colony size and number were all much smaller than control cells transfected with scrambled siRNA. The difference in colony number between the specific siRNA and nonspecific control clones ranged from 2- to 8-fold. These results suggest that the activated mutant BRAF plays an important role in driving cell proliferation and growth, and in maintaining the malignant and transformed state of *BRAF* mutation-harboring PTC cells.

Inhibition of tumorigenicity and tumor growth of PTC cells harboring BRAF mutation in nude mice by stable knockdown of BRAF

To examine the effect of BRAF knockdown on tumorigenicity of BRAF mutation-harboring PTC cells, we established a xenograft tumor model using nude mice with sc injection of KAT10 cell clones transfected with specific BRAF siRNA (clone C9) or control scrambled siRNA (clone 2B2). Emerging tumors became visible in most mice in the control group, but not in the specific siRNA group within 1 wk after injection of cells. As shown in Fig. 5A, the tumor volume progressively grew in both groups of nude mice but with a slower pace in the specific siRNA group. At the end of wk 5 when the experiment was terminated, averaged volume and weight of the tumors in the two groups (each with n = 4) were 2.60 ± 0.48 cm^3 and 1.56 ± 0.29 g (for the control group) *vs.* 1.43 ± 2.52 cm^3 and 0.45 ± 0.11 g for the specific siRNA group, respectively (mean ± SD; P = 0.23 for tumor volume and P = 0.01 for tumor weight). As shown in Table 1, both tumor volume and weight were smaller in the mice in the specific siRNA group than in the control group, although the difference between the two groups of mice was statistically significant only for tumor weight. Figure 5B shows the tumor weight of each individual mouse in the two groups, more clearly illustrating the inhibition of the tumor by specific BRAF siRNA (P = 0.01). Figure 5C shows a representative tumor, the second smallest, from the control and specific siRNA groups before and after surgical removal, illustrating the inhibition of tumor growth by BRAF siRNA. It should be pointed out that, unlike the tumor weight measured after surgical removal that was accurate, the tumor volume estimated based on the dimensions of the tumor measured from the skin surface of the animal was likely not accurate. Therefore, the time courses of tumor growth shown in Fig. 5A might underestimate the difference in true tumor volumes between the two groups of mice.

We performed similar animal studies for NPA cells. Unfortunately, the NPA cells did not seem to be compatible with

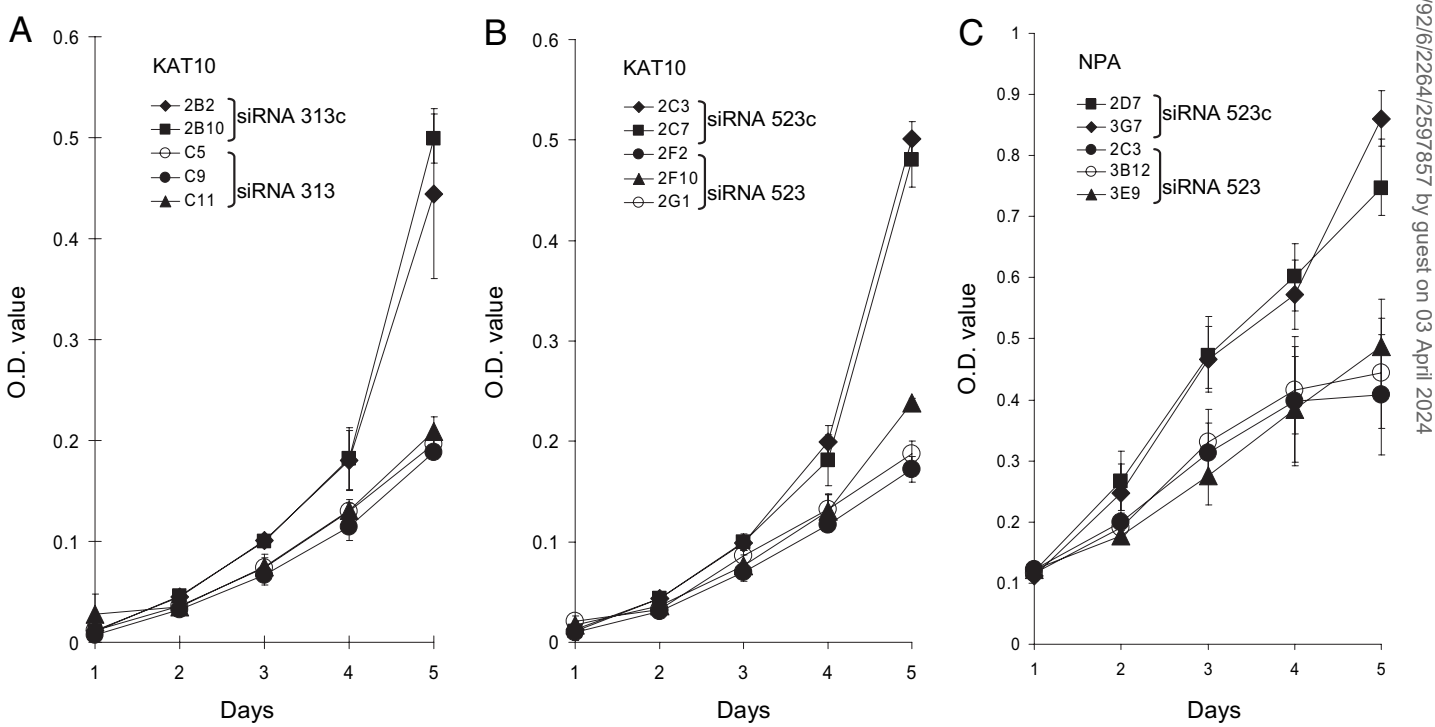

FIG. 3. Proliferation rate of the KAT10 and NPA cell clones stably transfected with various BRAF-specific or nonspecific scrambled siRNA. Cell proliferation rate was detected with MTT assay. The OD value was measured daily over a 5-d time course. Results are expressed as means ± SD (n = 3). A, KAT10 cells transfected with siRNA 313 or siRNA 313c. B, KAT10 cells transfected with siRNA 523 or siRNA 523c. C, NPA cells transfected with siRNA 523 or siRNA 523c. The types of various siRNA vectors are as defined in the legend to Fig. 1.

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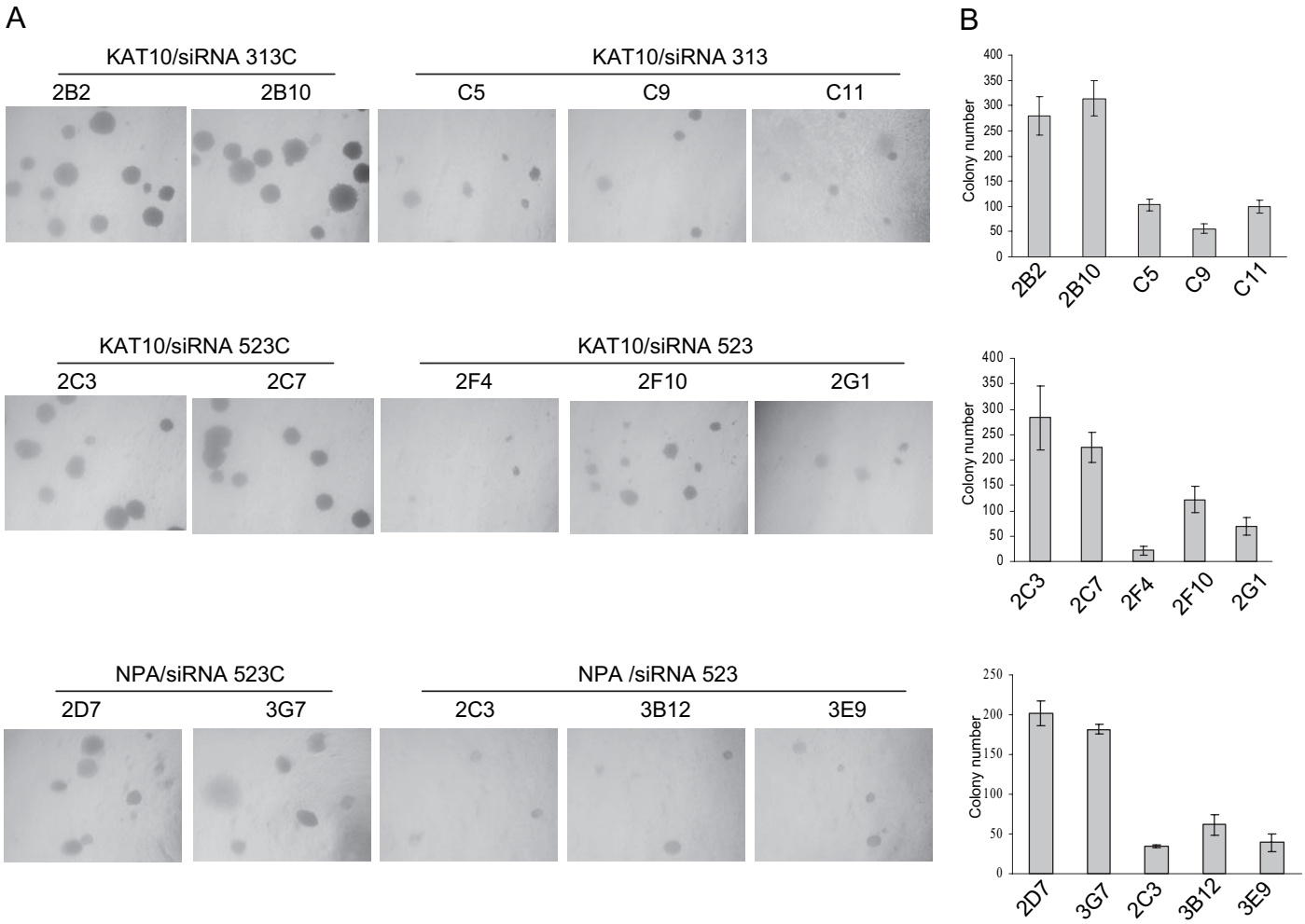

FIG. 4. Anchorage-independent growth of the KAT10 and NPA cell clones stably transfected with various BRAF-specific or nonspecific scrambled siRNA. After culturing in 0.33% soft agar for 3–4 wk, colonies were counted and photographed. A, Representative results of colony formation of different cell clones transfected with various siRNA vectors as indicated (magnification, ×40). B, Plots, corresponding to A, of the colony numbers from two experiments, with the average represented by the column and the range by the bar.

these nude mice because only a small tumor grew in two mice in the control group, and no tumors grew in the specific BRAF siRNA group (data not shown). However, this result itself seems to be consistent with the notion that BRAF knockdown made it even harder for NPA cells to grow into tumor in these mice.

We also examined the relationship between BRAF expression level and the weight of the final tumors. As shown in Fig. 6A, the BRAF level remained low in the tumors from the specific siRNA group compared with the control group, even after 5 wk of growth, corresponding to the smaller tumors in the siRNA group of mice (Table 1 and Fig. 5). Figure 6B shows a direct comparison of BRAF level with tumor weight, clearly illustrating the association of low BRAF level with small tumors in the specific BRAF siRNA group and the association of high BRAF level with large tumors in the control group. These results demonstrated that activated BRAF is important for the maintenance of tumorigenicity and tumor growth of PTC that harbor *BRAF* mutation.

Discussion

The ability of the V600E BRAF to initiate PTC was well demonstrated in a previous transgenic mouse model in which targeted expression of this mutant in thyroid gland induced PTC with poor histological differentiation and progression (14). These results recapitulated clinicopathological findings in thyroid cancer patients who showed a close association of *BRAF* mutation with increased aggressiveness and recurrence of PTC (6, 11–13). Although these animal and human studies support an important role of *BRAF* mutation in PTC tumorigenesis, including initiation of this cancer, they do not establish a necessary role of *BRAF* mutation in the maintenance of aberrant cell proliferation, transformed state, and progression of this cancer. In fact, it was recently shown that, unlike RET/PTC rearrangements, V600E BRAF caused chromosomal instability in thyroid cells (15), raising the possibility that secondary genetic alterations could play an important or even a primary role in maintaining the phenotypic aggressiveness and progression of PTC initiated by *BRAF* mutation. As Chiloeches and Marais (16) recently pointed out, whether the oncogenic *BRAF* mutation

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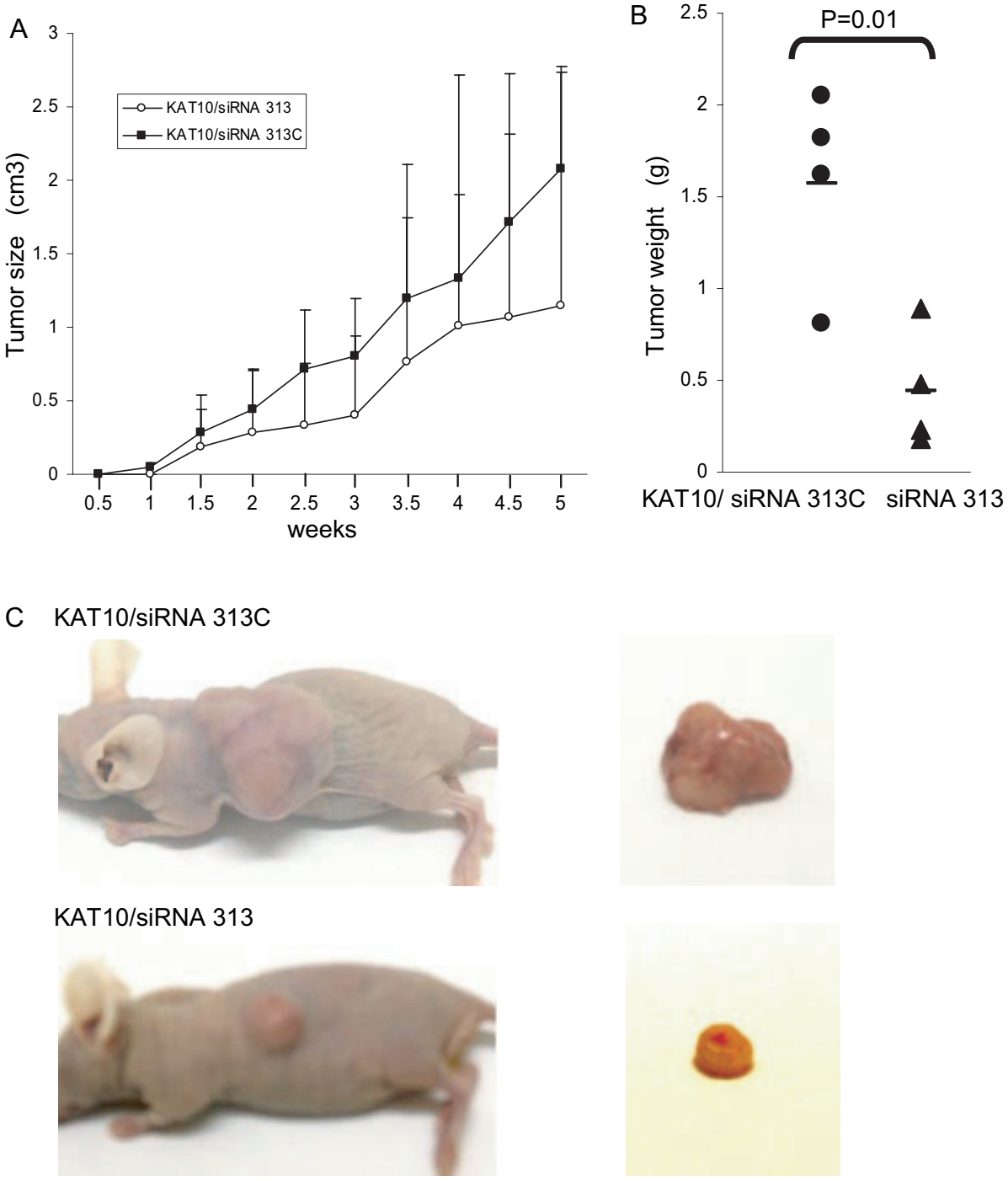

FIG. 5. Mouse xenograft assay of tumorigenicity of KAT10 cells with or without stable knockdown of BRAF. KAT10 cell clones stably transfected with control (clone 2B2) or BRAF siRNA (clone C9) were injected sc into the flanks of nude mice (n = 4 in each group), and the animals were subsequently monitored for tumor growth. A, Time course of tumor growth over a 5-wk period in mice injected with the indicated KAT10 cell clones. Tumor size was measured on the surface of the skin of the mouse, and tumor volume was calculated as described in *Materials and Methods*. Each time point represents the average ± SD of the values obtained from four mice in each group. B, Weight of individual tumors surgically removed from the animals in each group after their sacrifice. The average weight of the tumors from each group is indicated with a *short horizontal bar*. The *P* value was obtained by the independent samples *t* test. C, Representative tumors *in situ* and after surgical removal from mice. The second smallest tumor in each group of mice (or mouse no. 2 in each group in Table 1 and Fig. 6) is shown.

plays an important role in maintaining PTC remains an unsolved issue and requires further work to define. Two excellent recent studies showed that several Raf kinase-inhibiting compounds could suppress growth and proliferation of V600E mutation-harboring PTC cell lines (20, 21). However, concerns remain on the caveat of nonspecific actions of these compounds that may prevent one from clearly delineating a specific role of BRAF mutant in the phenotypic maintenance of PTC. Conse-

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TABLE 1. Tumor volume and weight 5 wk after cell injection in nude mice

Tumor	Cell clone	Nude mouse no.				Mean ± SD	*P* value
		1	2	3	4		
Volume (cm^3)	2B2 (control)	1.59	2.70	3.08	3.02	2.60 ± 0.48	0.23
	C9 (siRNA)	0.54	0.57	0.82	3.81	1.43 ± 2.52	
Weight (g)	2B2 (control)	0.81	1.62	1.82	2.05	1.56 ± 0.29	0.01
	C9 (siRNA)	0.18	0.23	0.48	0.89	0.45 ± 0.11	

quently, it has been suggested that the molecular approach, such as stable RNA interference, would be an ideal tool to assess this role of oncogenic BRAF (16).

The present study was conducted to address principally this issue by stably and specifically knocking down BRAF using specific siRNA expression vectors and examining its consequence to the behaviors of PTC cells that harbor the T1799A *BRAF* mutation. With this strategy we were able to knock down BRAF in several PTC-derived cell lines with a high efficacy of up to more than 90%. Remarkable suppression of cancer cell proliferation, colony formation in monolayer culture, and anchorage-independent cell growth in soft agar was all correspondingly achieved. These data were consistent with previous results achieved in melanoma cell lines (22, 23). A recent study by Sumimoto *et al.* (24) showed that simultaneous suppression of BRAF V600E and another oncoprotein Skp-2 effectively inhibited growth and invasiveness of melanoma cells. It would be interesting to see if this combined suppression of two major oncoproteins may cause even more significant inhibition of PTC cells. We also took a further step to examine the effect of stable knockdown of BRAF on tumorigenicity of *BRAF* mutation-harboring PTC cells in nude mice, and demonstrated the dependence of tumorigenicity and tumor growth on BRAF. This result is consistent with the strong association of BRAF V600E signaling with *in vivo* tumorigenicity of melanoma demonstrated using the inducible short-hairpin RNA technique for conditional knockdown of BRAF that resulted in tumor regression (25). Our data suggest that secondary oncogenic genetic alterations, if any, caused by *BRAF* mutation may be insufficient to overcome the effects of loss of BRAF V600E, and the oncogenic BRAF plays an important role in maintaining the transformed and progressive state of PTC cells. Therefore, V600E BRAF is not only an initiator of PTC as previously demonstrated but also a maintainer of PTC.

A recent study by Salvatore *et al.* (21) used transient transfection with synthetic siRNA oligos to study the role of *BRAF* mutation in PTC cell growth. Our study was different in that we used BRAF-specific siRNA expression vectors and were, therefore, able to establish cell clones with stable knockdown of BRAF. Although this technique

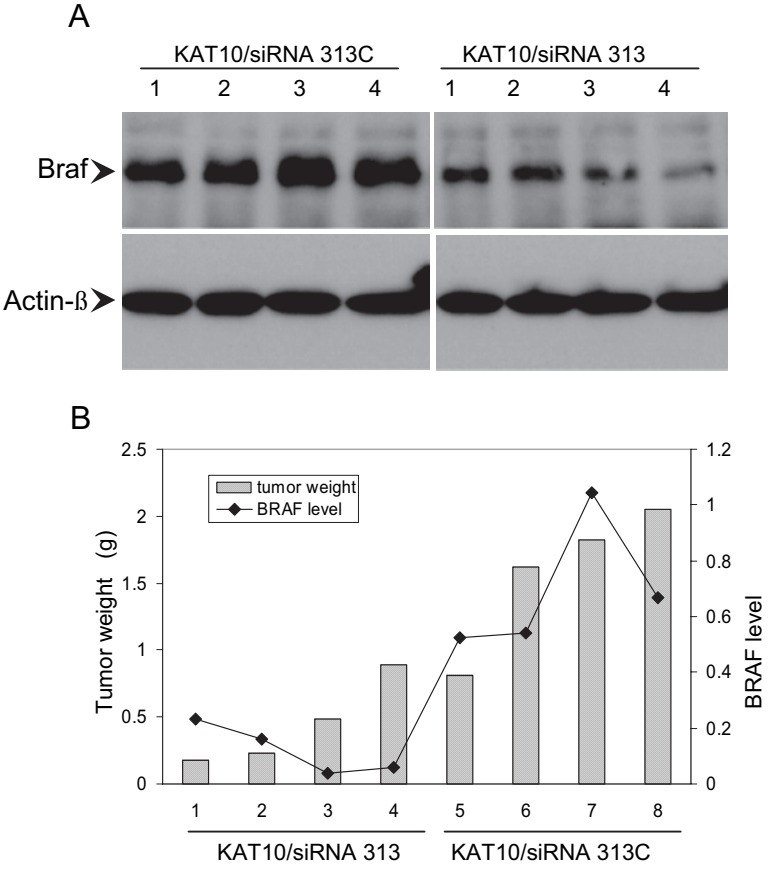

FIG. 6. Correlation between tumor weight and BRAF expression level in the tumor. A, Western blotting analysis of BRAF expression levels in the tumor tissues developed from KAT10 cells in nude mice. The β-actin was used to ensure the integrity of protein samples loaded to the gel. B, Comparison of BRAF level with tumor weight in each individual mouse. BRAF level is estimated by dividing the intensity of the BRAF band by that of β-actin obtained on densitometry of the gel in panel A. The data are plotted in the order of the smallest to the largest tumors in each group. Shown on the *left* and *right* y-axes are the tumor weights and BRAF levels, respectively.

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is more challenging and has not been previously used for *BRAF* mutation studies in thyroid cancer, with this approach, we were able to investigate the durability of the effect of BRAF knockdown on the behaviors of PTC cells that harbor *BRAF* mutation. In particular, we were able to investigate anchorage-independent cell growth, demonstrating for the first time that specific knockdown of BRAF could suppress transformation of *BRAF* mutation-harboring human PTC cells. With the stable transfectants, we were able to also show continued suppression of cell growth/proliferation and transformation by BRAF knockdown, even after a long-term culture of cells. Moreover, by taking this advantage of stable siRNA transfection, we were able to demonstrate suppression of *in vivo* tumorigenicity by sustained knockdown of BRAF.

In summary, with stable and specific siRNA knockdown of BRAF, we were able to investigate the durable effects of BRAF suppression on *BRAF* mutation-harboring PTC cell behaviors, including cell proliferation, transformation, and *in vivo* tumorigenicity. Our data support an important role of the V600E BRAF in the phenotypic maintenance of PTC in addition to its known role in the initiation of this cancer. This study also provides evidence demonstrating the reversibility of *BRAF* mutation-driven PTC cell proliferation and transformation, as well as tumorigenicity or tumor growth by specifically suppressing BRAF, confirming BRAF as an important therapeutic target for *BRAF* mutation-harboring PTC.

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