Postnatal Expression of BRAF^{V600E} Does Not Induce Thyroid Cancer in Mouse Models of Thyroid Papillary Carcinoma

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The mutant BRAF (BRAF^{V600E}) is the most common genetic alteration in papillary thyroid carcinomas (PTCs). The oncogenicity of this mutation has been shown by some genetically engineered mouse models. However, in these mice, BRAF^{V600E} is expressed in all the thyroid cells from the fetal periods, and suppresses thyroid function, thereby leading to TSH elevation, which by itself promotes thyroid tumorigenesis. To overcome these problems, we exploited 2 different approaches, both of which allowed temporally and spatially restricted expression of BRAF^{V600E} in the thyroid glands. First, we generated conditional transgenic mice harboring the *lox*P-neo^{R}-*lox*P-BRAF^{V600E}-internal ribosome entry site-green fluorescent protein sequence [*Tg(LNL-BRAF^{V600E})*]. The double transgenic mice (*LNL-BRAF^{V600E};TPO-Cre*) were derived from a high expressor line of *Tg(LNL-BRAF^{V600E})* mice and *TPO-Cre* mice; the latter expresses Cre DNA recombinase under the control of thyroid-specific thyroid peroxidase (TPO) promoter and developed PTC-like lesions in early life under normal serum TSH levels due to mosaic recombination. In contrast, injection of adenovirus expressing Cre under the control of another thyroid-specific thyroglobulin (Tg) promoter (Ad-TgP-Cre) into the thyroids of *LNL-BRAF^{V600E}* mice did not induce tumor formation despite detection of BRAF^{V600E} and pERK in a small fraction of thyroid cells. Second, postnatal expression of BRAF^{V600E} in a small number of thyroid cells was also achieved by injecting the lentivirus expressing *lox*P-green fluorescent protein-*lox*P-BRAF^{V600E} into the thyroids of *TPO-Cre* mice; however, no tumor development was again observed. These results suggest that BRAF^{V600E} does not appear to induce PTC-like lesions when expressed in a fraction of thyroid cells postnatally under normal TSH concentrations. **(*Endocrinology* 154: 4423–4430, 2013)**

Thyroid carcinomas are the most common endocrine malignancies with their incidence rapidly increasing in recent years (1). Most of them are made up of differentiated thyroid carcinomas, such as papillary and follicular types. The mutant v-RAF murine sarcoma viral oncogene homolog B (BRAF^{V600E} in which valine at position 600 is changed to glutamic acid) is the most frequently detected in sporadic papillary thyroid carcinomas (PTCs), followed by rearranged during transfection (RET)-PTC and rat sarcoma virus oncogene (RAS) mutations (2). Although it is now clear that these mutants enhance the ERK/MAPK signaling; whether BRAF^{V600E} by itself is sufficient or it

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Abbreviations: CMV, cytomegalovirus; EGFP, enhanced GFP; GFP, green fluorescent protein; IRES, internal ribosome entry site; MMI, methimazole; NLS, nuclear localization signal; PTC, papillary thyroid carcinoma; RAS, rat sarcoma virus oncogene; RET, rearranged during transfection; Tg, thyroglobulin; TgP, Tg promoter; TPO, thyroid peroxidase.

needs the aid of mutation(s) in other gene(s) (eg, tumor suppressor genes), to induce PTC remains unknown.

In in vitro experiments, conditional BRAF^{V600E} expression by Tet-ON system has failed to transform rat differentiated thyroid $PCCl_3$ cells and induced apoptosis in parallel with increased DNA synthesis, dedifferentiation, and chromosomal instability (3). Induction of oncogene-induced senescence, not oncogenic transformation, has also been demonstrated in thyroid cells in primary culture transduced with BRAF^{V600E} (4). Conversely, however, transgenic mice specifically expressing BRAF^{V600E} in the thyroid follicular epithelial cells by using thyroid-specific thyroglobulin (Tg) promoter (*Tg-BRAF^{V600E}*) have developed PTC early in life (5). The essentially same results have also been observed in a recent study by the same group (6) with *LSL-BRAF^{V600E};TPO-Cre* mice, in which BRAF^{V600E} could be specifically knocked into the thyroid cells (7, 8). Although in vivo experiments with mice are generally more meaningful than in vitro cell culture experiments, these mice have serious intrinsic problems. For example, 1) BRAF^{V600E} is expressed in all the thyroid follicular epithelial cells from the fetal periods, which indicates that these 2 mice are rather models of familial (not sporadic) thyroid cancer (although the timing of BRAF activation may not be the same between these models and familial thyroid cancer with BRAF^{V600E} if present); 2) BRAF^{V600E} inhibits thyroid function, thereby leading to TSH elevation that by itself induces thyroid diffuse enlargement and sometimes promotes tumorigenesis (2); and 3), in the former mice, Tg promoter activity may be changed (presumably silenced) as a dedifferentiation of the thyroid cells by BRAF^{V600E} proceeds.

To overcome these problems in these mouse models of BRAF^{V600E}-induced PTC, we here exploited 2 novel experimental approaches. Our results demonstrate that the postnatal expression of BRAF^{V600E} in a part of the thyroid cells is not able to induce thyroid cancer under normal TSH concentrations. Thus the timing of BRAF activation appears to be a determining factor in thyroid cell transformation under the physiological TSH condition as recently speculated (6).

Materials and Methods

Mice used

The conditional transgenic BRAF^{V600E} mice were constructed as follows. The cDNA fragment encoding BRAF^{V600E} (9) was ligated into pIRES2-AcGFP1 (TaKaRa-Clontech) to create the plasmid pBRAF^{V600E}-IRES2-AcGFP1, where internal ribosome entry site (IRES) and green fluorescent protein (GFP) indicate internal ribosomal entry site and green fluorescent protein, respectively. The DNA fragment containing BRAF^{V600E}-IRES2-AcGFP1 was then released and ligated into pCALNL5 (clone 1862, RIKEN BioResource Center) yielding pCALNL-BRAF^{V600E}-IRES2-AcGFP1. This plasmid has the neomycin-resistant (neo^R) gene flanked by two *lox*P sites under the CMV early enhancer/chicken β actin promoter (CAGp), followed by BRAF^{V600E}-IRES2-AcGFP1 sequence. pCALNL-BRAF^{V600E}-IRES2-AcGFP1 was linealized and injected into fertilized B6C3F1 mouse eggs, which were implanted into pseudopregnant female mice. The 12 pups were confirmed by PCR (see below) to have integrated the transgene, of which 5 proved to express neomycin-resistant genes by culturing tail fibroblasts with neomycin. Lines were then generated from founder animals by crossing them with wild-type B6C3F1 mice. BRAF^{V600E} expression in the thyroid glands was confirmed by Western blotting after crossing with thyroid peroxidase (TPO)-Cre mice (see below). Finally, 2 lines, designated *Tg(LNL-BRAF^{V600E})#302MM* (low expressor) and *#213MM* (high expressor), were established.

TPO-Cre mice that express Cre DNA recombinase under the control of thyroid peroxidase (TPO) gene promoter were previously generated (8).

All mice were kept in a specific pathogen-free facility. Animal care and all experimental procedures were performed in accordance with the Guideline for Animal Experimentation of Nagasaki University with approval of the Institutional Animal Care and Use Committee.

PCR for genotyping

Genotyping of offspring was performed by PCR with tail tip DNA (REDExtract-N-Amp Tissue PCR KIT; Sigma Chemical Co) that was amplified with the appropriate primer pairs. The primers used to detect the BRAF^{V600E} transgene in *Tg(LNL-BRAF^{V600E})* were 5′-tat ttg gtt tag agt ttg gca aca-3′ (forward) and 5′-att tca cac agg aaa cag cta tga-3′ (reverse), yielding a 335-bp PCR product. Those for *TPO-Cre* mice were 5′-tgc cac gac caa gtg aca gca atg-3′ (forward) and 5′-aga gac gga aat cca tcg ctc g-3′ (reverse). Thermocycling conditions consisted of 40 cycles of 30 seconds at 94°C, 30 seconds at 55–57.5°C, and 30 seconds at 72°C.

PCR analysis for Cre-mediated DNA recombination

Genomic DNA was extracted from the thyroid tissues using DNeasy Blood & Tissue Kit (QIAGEN). Selected regions of the target genes were amplified and analyzed by PCR. The following primers (Figure 1) were used to detect recombination Primer A (forward): 5′-ata ttg ctg aag agc ttg gcg gcg a-3′; primer B (reverse): 5′-acc gct cag cgc cgc cat ctt ata a-3′; primer C (forward): 5′-ctc tag agc ctc tgc taa cca tgt t-3′. Primers A and B detect the LNL-BRAF^{V600E} allele yielding a product of 550 bp. Primers B and C detect the Cre-recombined BRAF allele yielding a product of 229 bp.

Western blotting

The thyroid tissues were placed in lysis buffer (20 mmol/L Tris-HCl [pH 7.5], 1 mmol/L EDTA, 0.5% Triton X-100, 150 mmol/L NaCl, and protease inhibitor cocktail) and homogenized using a homogenizer. Protein lysates were centrifuged, supernatant was collected, and protein concentration was determined using Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc).

Protein lysates were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with antibody to BRAF (Santa Cruz Biotechnology) and horseradish peroxidase ABC method (Vectastatin Universal ABC kit; Vector Laboratories).

Construction of adenovirus expressing Cre recombinase under the control of Tg promoter (Ad-TgP Cre)

The DNA fragment for nuclear localization signal (NLS)-Cre DNA recombinase from pxCANCre (clone 1675; RIKEN) was ligated into the adenovirus shuttle vector pHMCMV6 (10) to generate pHMCMV6-NLS-Cre. CMV promoter in pHMCMV6-NLS-Cre was replaced with the bovine Tg promoter (TgP) (a generous gift from Vassart G, IRIBHM), yielding pHMCMV6-TgP-NLS-Cre. The DNA fragment containing TgP-NLS-Cre was ligated into the adenovirus vector pAdHM15 (11). The resultant pAdHM15-TgP-Cre was linealized and transfected into 293 cells with PolyFect (QIAGEN), yielding Ad-TgP-Cre. The virus was propagated in 293 cells and purified by CsCl density-gradient centrifugation, and viral particle concentration was determined by measuring the absorbance at 260 nm (3.5×10^{12} virus particles/mL).

Construction of lentivirus expressing BRAF^{V600E} or lacZ

PCR-amplified cDNAs for BRAF^{V600E} or lacZ were subcloned into pLenti6/V5-D-TOPO (Invitrogen) according to the manufacturer's instruction, and then the floxed enhanced GFP (EGFP) DNA (PCR amplified from pEGFP-N1 [TaKaRa-Clontech]) was ligated between CMV promoter and BRAF^{V600E}/lacZ. The resultant plasmids, pLEL-BRAF^{V600E}-V5 and pLEL-LacZ-V5, have the CMV promoter followed by the floxed EGFP and BRAF^{V600E}/lacZ C-terminally fused to V5 epitope. For lentivirus production, 293FT cells (Invitrogen) were transfected with the pLEL-BRAF^{V600E}-V5 or pLEL-lacZ-V5 plasmid together with ViraPower Packaging Mix (Invitrogen) containing pLP1, pLP2, and pLP/VSVG using Lipofectamine 2000 (Invitrogen). Lentiviruses were collected 48 hours after transfection and concentrated by ultracentrifugation, yielding 6×10^7 cfu/mL viral stock.

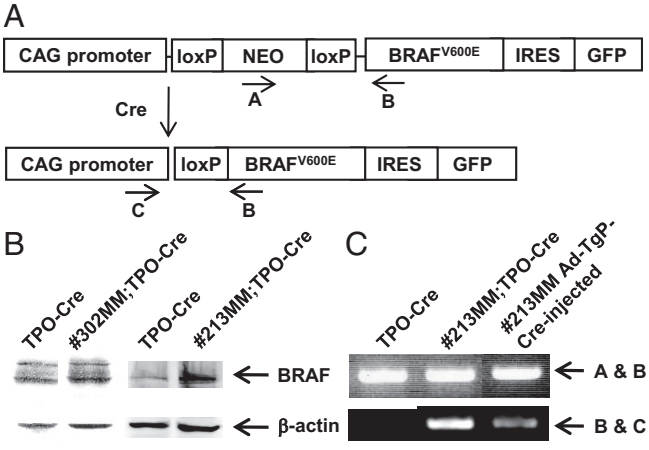

Figure 1. The Transgene BARF^{V600E} expression and Cre-mediated DNA recombination in *Tg(LNL-BRAFV^{600E})* mice.A, The structure and Cre-mediated recombination of the transgene in *Tg(LNL-BRAFV^{600E})*. Primers A, B, and C were used for PCR analysis of Cre-mediated DNA recombination. B, Western blot for BRAF expression. Total cell lysates were extracted from the thyroids of *TPO-Cre*, *#302MM:TPO-Cre* and *#213:TPO-Cre* mice (female, 4–8 weeks of age) and subjected to Western blot as described in *Materials and Methods*. C, PCR analysis of recombination. Genomic DNA was extracted from the thyroids of *TPO-Cre*, *#213MM:TPO-Cre* and *#213MM* injected with Ad-TgP-Cre (8 weeks old), and subjected to PCR analysis of Cre-mediated DNA recombination as described in *Materials and Methods*. Primers A, B, and C are shown in panel A.

Experimental design

Mice (4 weeks old) were anesthetized with isoflurane using Anesthetizer (Muromachi). An approximate 1-cm long midline incision was made on the anterior neck under sterile conditions. The underlying submandibular salivary glands were separated to both sides to visualize laryngotrachea and strap muscles. The strap muscles were then cut to expose the thyroid lobes (12). One microliter containing approximately 3.5×10^9 adenovirus particles or 6×10^4 cfu lentivirus was injected to the left lobes using a 25-μL Hamilton microsyringe tipped with a 30 G needle. Subsequently the submandibular glands were returned to their normal position, and the skin incision was closed using Reflex Skin Closure System (CellPoint Scientific).

Hypothyroidism was induced by administration of 0.5% sodium perchlorate and 0.05% methimazole (MMI) (both from Sigma) given in the drinking water (13) to 2-week pregnant mothers and the pups.

At the indicated time points, mice were anesthetized by ip injection of sodium pentobarbitone. Sera were collected via pericardial tap and the animals were euthanized by cervical dislocation. Thyroids were removed and either flash-frozen in liquid nitrogen or fixed in 10% neutral-buffered formalin.

Hematoxylin and eosin staining and immunohistochemistry of the thyroid glands

Thyroid tissues fixed in formalin were embedded in paraffin. Sections (5μm thick) were prepared and stained with hematoxylin and eosin or immunostained with rabbit anti-phospho-p44/42 MAPK (ERK1/2) (Cell Signaling Technology; a dilution of 1:200), rabbit anti-Ki67 (Abcam; a dilution of 1:100), or anti-V5 antibody (PM003, Medical and Biological Laboratories; a dilution of 1:100) and the colors were developed with Vectastatin Elite ABC kit (Vector Laboratories) without additional antigen retrieval.

Real-time PCR

Thyroid lobes were surgically removed and immediately placed in liquid nitrogen. RNA was isolated using ISOGEN (Nippon Gene) and 0.5 μg RNA was reverse transcribed with SuperScript III (Invitrogen) in the presence of random hexamers to generate cDNA. Quantitative RT-PCR was done using SYBR Premix Ex Taq (Takara) and primer pairs for β-actin (ctg aac cct aag gcc aac cgt g and ggc ata cag gga cag cac agc c); Tg (tgt ccc acc aag tgt gaa aa and cca agg aaa gct tgt tca gc); and sodium iodine symporter (gct cag tct cgc tca aaa cc and cgt gtg aca ggc cac ata ac). The cycle threshold values were determined using Thermal Cycler Dice Real-Time System (Takara) and used to calculate the relative expression levels of the target genes normalized against β-actin.

Serum TSH and T_4 measurements

Serum-free T_4 concentrations were measured with a RIA kit (DPC free T_4 kit; Diagnostic Products). The normal range was defined as the mean + 3 SD of control mice. Serum TSH was measured with a specific mouse TSH RIA as previously described (14) except that mouse TSH reference (AFP9090D) was used instead of mouse TSH/LH reference (AFP51718MP). The normal range was defined as the mean ± 3 SD of control untreated mice.

Statistical analyses

All data were analyzed by either Student's *t* test or χ^2 test. A $P < .05$ was considered statistically significant.

Results

The first approach with Tg(LNL-BRAF^{V600E}) transgenic mice and *Ad-TgP-Cre*

Tg(LNL-BRAF^{V600E}), transgenic mice we generated here, harbor a transgene of CAGp-*lox*P-neo^R-*lox*P-BRAF^{V600E}-IRES2-AcGFP1 sequence. In these mice, BRAF^{V600E} expression is normally suppressed by the presence of the floxed neo^R but can be induced when this gene is rearranged by Cre DNA recombinase (Figure 1A). Two lines, #*302MM* and #*213MM*, were selected by their lower and higher, respectively, expression levels of BRAF^{V600E} protein in Western blotting (Figure 1B) and used the subsequent experiments.

To confirm the structural and functional integrity of these transgenic mice, mice were first crossed with *TPO-Cre* mice, which express Cre specifically in the thyroid follicular epithelial cells (8). DNA recombination by Cre in *LNL-BRAF^{V600E};TPO-Cre* mice was confirmed by PCR using genomic DNA extracted from the thyroids, showing the presence of the recombined gene (B and C in Figure 1C) in addition to the intact transgene (A and B in Figure 1C). The growth rates were indistinguishable among *LNL-BRAF^{V600E}*, *TPO-Cre*, and *LNL-BRAF^{V600E};TPO-Cre* mice (data not shown). However, the thyroid glands were markedly enlarged throughout the experiments in both lines of *LNL-BRAF^{V600E};TPO-Cre* mice (Figure 2, A and C). However, thyroid function assessed by serum T_4 and TSH at 4 weeks of age was normal in both lines (Figure 3, A and B), consistent with mosaic pattern of recombination sparing normal tissues (see below). Expression of thyroid-specific genes, Tg and sodium iodine symporter, was decreased in both sexes of the higher expressor #*213MM* and only male of the lower expressor #302MM (Figure 3C).

In thyroid histology, all #*302MM;TPO-Cre* mice examined (16 mice, 8 males and 8 females) showed marked proliferation of epithelial cells in the interfollicular areas at 4 weeks of age, which mostly disappeared, however, at 26 and 52 weeks (Figure 4A and data not shown). These results suggest temporal epithelial proliferation induced by lower expression levels of BRAF^{V600E}, which is consistent with immunohistochemical data of Ki67 staining (a cell proliferation marker), which was positive only early in life and then became negative (Figure 4D). Presumably due to this nonsustained cell proliferation, tumor formation was not observed for up to 52 weeks.

Treatment of these mice with sodium perchlorate and MMI induced further enlargement of the thyroid glands (thus a difference in thyroid weights between *TPO-Cre* mice and #*302MM;TPO-Cre* mice was no longer observed) (Figure 2B), increased TSH, decreased T_4 (Figure 3A) and epithelial cell proliferation with invasion to muscles, parathyroids and/or lymphatics, and occasional tumor formation (Figure 4B and data not shown) in 31% (5/16) #*302MM;TPO-Cre* mice at 52 weeks of age. However, these ex-

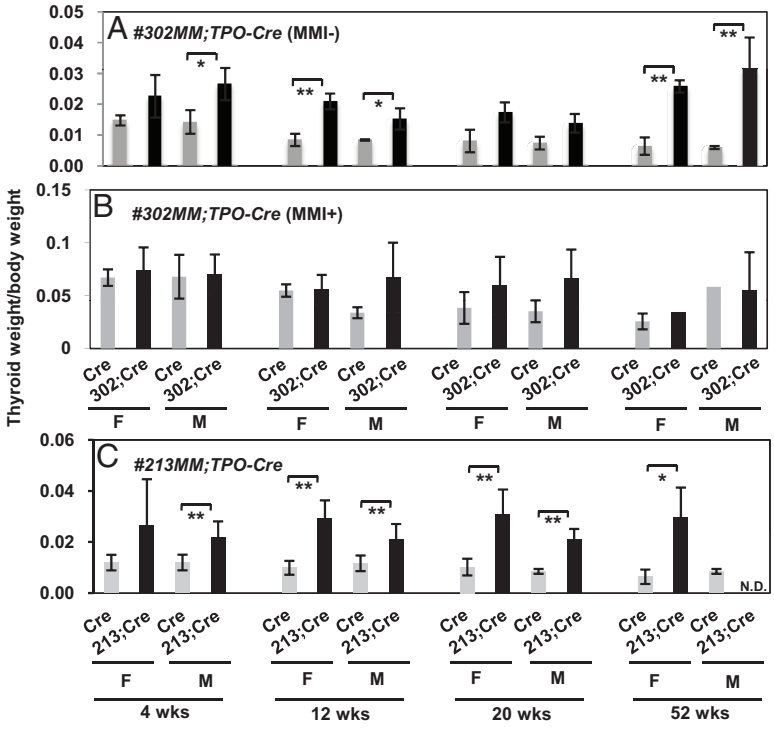

Figure 2. Thyroid weights/body weights of *Tg (LNL-BRAF^{V600E});TPO-Cre* mice with/without MMI/perchlorate. Thyroid weights/body weights were regularly measured in *TPO-Cre, #302MM;TPO-Cre* with/without MMI/perchlorate (A and B) and #*213MM;TPO-Cre* (C). Data are means ± SD (n = 3–4). *, $P < .01$; **, $P < .05$. F, female; M, male.

trathyroidal invasions, usually considered as a sign of cancer in humans, were also observed in 25% (2/8, $P > .05$) sodium perchlorate/MMI-treated *TPO-Cre* mice (Figure 4B), which were therefore considered as the effect of long-term elevation of TSH.

In contrast, all *#213MM;TPO-Cre* mice readily showed sustained interfollicular epithelial proliferation and many foci of hyperplastic epithelial cells at 4 weeks of age, of which 63% (10/16; 8 males and 8 females) developed PTC-like lesions at 52 weeks with 30% (3/10) tumors showing occasional invasion into muscles (Figure 4C). Overexpression of BRAF and pERK was confirmed by immunohistochemistry in 5 randomly selected specimens (Figure 5). Of interest, these changes were observed in a patchy pattern, indicating a mosaic Cre-mediated recombination. Also, unlike *#302MM;TPO-Cre* mice, Ki67 staining was continuously positive in *#213MM;TPO-Cre* mice during the experimental periods (Figure 4D).

These data indicate that the expression levels of $BRAF^{V600E}$ in the *#213MM;TPO-Cre* line are sufficient for thyroid cancer induction on a single cell basis. Thus, *#213MM* mice (4 weeks of age) were used for intathyroidal injection of Ad-TgP-Cre. We incorporated Tg promoter in this adenovirus vector to precisely target the expression of Cre to the thyroid follicular epithelial cells. Intrathyroidal injection of 3.5×10^9 particles of Ad-TgP-Cre clearly induced Cre-mediated recombination detected by PCR (Figure 1C) and expression of pERK by immunohistochemistry in 4 randomly selected tissues 4 and 52 weeks after injection (Figure 5), although the number of pERK-positive cells was somewhat decreased in the latter. Following the injection, mice were humanely destroyed 4, 26, and 52 weeks later (14–16 mice [6–8 males and 6–8 females] at each time point). No tumor formation was however observed (Supplemental Figure 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

The second approach with TPO-Cre mice and lentivirus *LEL-BRAF^{V600E}-V5*

LEL-$BRAF^{V600E}$-*V5* and *LEL-LacZ-V5*, the lentiviruses we constructed, harbor *CMVp-lox*P-EGFP-*lox*P-$BRAF^{V600E}$/LacZ-V5 sequences. $BRAF^{V600E}$/LacZ are expressed only when the viruses are infected into the thyroid cells of *TPO-Cre* mice (Supplemental Figure 2A). Indeed, V5 expression, a surrogate marker for $BRAF^{V600E}$/LacZ expression, was observed exclusively in thyroid follicular epithelial cells of *TPO-Cre* mice in which the virus was injected into their thyroids (Supplemental Figure 2B). Following the injection of LEL-$BRAF^{V600E}$-V5 (6×10^4 cfu), mice were humanely destroyed 12, 24, and 48 weeks later (16 mice at each time point; 8 males and 8 females). Again, no tumor formation was observed (Supplemental Figure 2C).

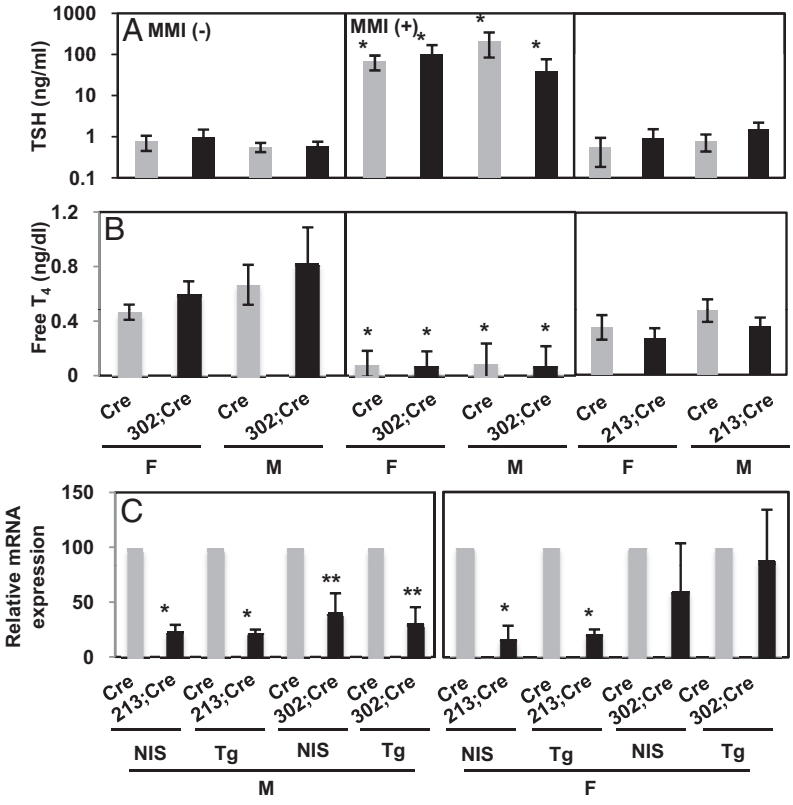

Figure 3. TSH, free T_4 (A and B) and real-time RT-PCR analysis of thyroid-specific genes (sodium iodine symporter [NIS] and Tg). A and B, TSH and free T_4concentrations were measured by RIA as described in *Materials and Methods* in *TPO-Cre* and *#302MM;TPO-Cre* with/without MMI/perchlorate and *#213MM;TPO-Cre* mice at 4 weeks of age. C, NIS and Tg mRNA levels in the thyroids. Total RNA was extracted from the thyroids of *TPO-Cre*, *#302MM;TPO-Cre*, and *#213MM;TPO-Cre* mice and subjected to real-time PCR for NIS and Tg expression as described in *Materials and Methods*. Data are means ± SD (n = 5 in panels A and B; n = 3 in C). *, $P < .01$; and **, $P < .05$ vs controls. F, female; M, male.

Discussion

We here used 2 novel approaches to clarify whether or not $BRAF^{V600E}$ by itself is sufficient for PTC induction in mice. Both approaches, enabling temporally and spatially restricted expression of $BRAF^{V600E}$ in the thyroid glands, can recapitulate the key pathological conditions of human sporadic PTC that usually arise from

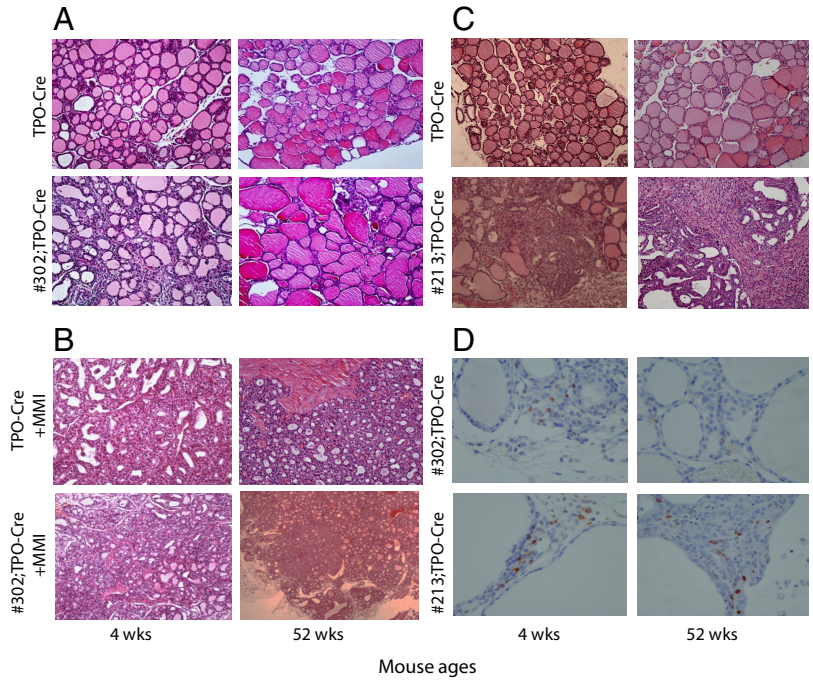

Figure 4. Thyroid histology of *TPO-Cre*, *#302MM;TPO-Cre* mice with/without MMI treatment and *#213MM;TPO-Cre* mice. A–C, Hematoxylin and eosin staining. D, Ki67 staining. Representative data at 12th and 52nd weeks were shown.

a single cell or a small number of cells postnatally under physiological serum TSH concentrations. Detections of BRAF^{V600E} overexpression and pERK by immunohistochemistry and/or Cre-mediated DNA recombination by PCR in the thyroid glands all confirmed the validity of our approaches. Although it has been reported that the effects of oncogenic BRAF is expression level dependent with the lower expression promoting cell quiescence/cell cycle arrest/senescence (15), development of PTC-like lesions in *#213MM;TPC-Cre* mice indicates that expression levels of BRAF^{V600E} following Cre-mediated recombination are high enough for inducing carcinoma on a single-cell basis at least in the first approach. However, our 2 approaches did not lead to cancer formation. The essentially same results have been reported with RET/PTC, another common mutation in PTC. Thus, the transgenic mice in which RET/PTC expression is derived from Tg promoter (like *Tg-BRAF^{V600E}*) developed PTC (16, 17), but another transgenic mouse line that expresses RET/PTC postnatally by using Tet-ON system failed to do so (18).

What are the reasons for the inconsistent data between our present experiments and the others (5, 6)? The key issues that concerned us about the previous reports showing induction of PTC by BRAF^{V600E} are 1) expression of BRAF^{V600E} in the entire thyroid glands, 2) its expression from the prenatal periods, and 3) elevated TSH.

Regarding the first issue, as mentioned above, BRAF^{V600E} induction of transformation occurs in a single cell or a small number of cells within an epithelial sheet in human sporadic PTC. Of interest, transformed cells in the initial phase of carcinogenesis have recently been experimentally demonstrated to be cleared out by the surrounding normal cells, although they grow normally when cultured by themselves (the cell competition theory) (19, 20). However, the detection of pERK-positive cells, ie, BRAF^{V600E}-expressing cells, 52 weeks after adenovirus injection excludes this possibility, although their number appeared to be lower than that at 4 weeks. Yet the possibility cannot be completely excluded that the surrounding normal cells inhibit the growth of oncogene-expressing cells (21).

Turning to the second issue, the different property and proliferation status of thyroid cells between the prenatal and postnatal (particularly postpubertal) periods may explain the distinct predisposition of BRAF^{V600E} to induce cancer in these 2 periods. Thyroid cells in the fetal periods are immature and proliferate well, whereas they are mature and quiescent after puberty. The difficulties in inducing cancer by introducing oncogenes by themselves into quiescent normal cells have been shown by various mouse cancer models using the combination of genetically engineered mice generated with Cre-*lox*P technology and viral vectors. For example, using adenovirus expressing Cre, proliferating tracheal and colon epithelial cells have shown to transform into hyperplasia, benign tumors, and, in some

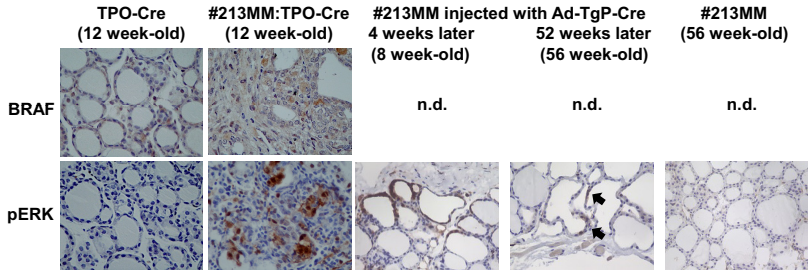

Figure 5. Immunohistochemical analysis of BRAF and pERK expression in the thyroids of control *TPO-Cre* (12 weeks of age), *#213MM;TPO-Cre* (12 weeks), and *#213MM* mice injected with Ad-TgP-Cre (8 and 56 weeks) and control *#213MM* (56 weeks). n.d., not determined. The arrows indicate the pERK-positive areas.

cases, malignant tumors in *LSL-Kras*^{G12D}, *LSL-BRAF*^{V600E} and *APC*^{580S} mice (22–24), whereas quiescent muscles have failed to do so in *LSL-Kras*^{G12D} mice unless both alleles of p53 gene were deleted (25). Retroviral or lentiviral mediated expression of the mutant molecules in MAPK kinase/ERK/MAPK signaling pathway (K-Ras^{G12D}, ΔRaf1–22W, and Braf^{V600E}) or H-ras^{V12D} specifically in poorly proliferating neural stem/progenitor cells has also failed to induce brain tumors unless combined with Akt activation or deletion of the Ink4a/Arf locus (25–28). Thus introduction of an oncogene alone into dividing cells (including tracheal and colon epithelial cells) can lead to transformation but that into non or poorly dividing cells (muscle and brain as well as thyroid cells) does not.

Furthermore, importantly, it has been shown, using *K-Ras*$^{+/LSLG12Vgeo}$*;Elas-tTa;tetO-Cre* mice, that expression of K-Ras^{G12V} in embryonic cells of pancreatic acinar/centroacinar lineage results in intraepithelial neoplasia and invasive ductal adenocarcinoma, but that adult mice are refractory to K-Ras^{G12V} induction of tumorigenesis (29).

When considering the difference(s) between fetal and adult thyroids, we should also take into account involvement of normal stem/progenitor cells for thyroid carcinogenesis. Assuming that aberrant activation of BRAF in normal stem/progenitor cells is a prerequisite for thyroid carcinogenesis, mosaic DNA recombination in *#213MM; TPO-Cre* mice and almost complete recombination in *LSL-BRAF*^{V600E}*;TPO-Cre* mice may well explain the lower penetrance of tumor formation in the former compared with the latter. Furthermore, Because fetal thyroids likely contain more stem/progenitor cells than adult thyroids, the possibility of induction of BRAF^{V600E} expression in such cells by intrathyroidal adenovirus injection is fairly low.

It should be noted here that attempts to generate new mouse models in which BRAF^{V600E} can be induced in the postnatal periods have recently been reported (30, 31). In the first paper, in theoretical, their mice, *Thyro::CreER*^{T2}*; BRAF*^{CA}, specifically express BRAF^{V600E} only when tamoxifen is administered. However, untreated, 1-month-old *Thyro::CreER*^{T2}*;BRAF*^{CA} mice displayed increased thyroid volumes, indicating leaky expression of CreER^{T2} (thereby BRAF^{V600E}) without tamoxifen from the fetal periods (30). In the second one, *Tg-rtTA/tetO-BRAF*^{V600E} mice, upon doxycycline administration, postnatally expressed the mutant BRAF and developed thyroid tumors, although extremely high TSH levels was also induced concomitantly; these high levels must play a serious role in tumorigenesis because the tumors underwent involution with doxycycline withdrawal (31).

The third issue is TSH elevation. As shown in the present study, it is well known that antithyroid drug-induced TSH elevation leads to development of thyroid cancer, albeit at low penetrance, in rodents (2). Indeed TSH was markedly elevated in all the mouse models of thyroid cancer so far mentioned (5, 6, 30, 31), because BRAF^{V600E} impairs thyroid function. Although development of PTC in *LSL-BRAF*^{V600E}*;TPO-Cre;TSHR* knockout (KO) mice, albeit less aggressive and latency being longer, suggests that the dispensable role for TSH signaling in development of PTC by prenatal expression of BRAF^{V600E} (6), it is possible that postnatal expression of BRAF^{V600E} may be insufficient for PTC induction in a normal TSH condition.

In melanoma, which harbors BRAF^{V600E} mutation at high frequency, the oncogenicity of BRAF^{V600E} itself for melanoma development is controversial. *Tyr::CreER/Braf*^{CA} mice, in which skin-specific expression of BRAF^{V600E} can be induced postnatally by administration of tamoxifen, have developed benign melanocytic hyperplasia that failed to progress to melanoma, unless combined with PTEN silencing (22). In contrast, the essentially same mice (*Tyr::CreERT2/LSL-Braf*^{V600E}) have been shown to develop melanoma (32).

Finally, our results suggest that BRAF^{V600E} itself may not be sufficient for PTC development in our experimental approaches, which however does not necessarily mean that BRAF^{V600E} is not the driver mutation, and rather suggests that additional genetic and/or epigenetic changes may be required to induce full transformation. A recent study has shown that deletions, mutations, and epigenetic inactivation through aberrant methylation of the PTEN gene exist in thyroid tumors, which activate the phosphatidylinositol 3-kinase-AKT-mmmalian target of rapamycin signaling pathway (33). Also the mutations have recently been identified in AKT1 gene (34). Therefore, future studies with *#213MM* with additional gene mutation(s) will definitely be necessary to confirm the role of BRAF^{V600E} for PTC formation.

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