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

Running title: BRAF$^{V600E}$ in thyroid cancer

Mika Shimamura[#], Mami Nakahara[#], Florence Orim[#], Tomomi Kurashige, Norisato Mitsutake, Masahiro Nakashima, Shinji Kondo, Masanobu Yamada, Ryo Taguchi, Shioko Kimura, Yuji Nagayama

Departments of Molecular Medicine (*M.S., Mam.N., T. K., Y.N.*), Radiation Medical Sciences (*F.O., N.M.*) and Tumor and Diagnostic Pathology (*M.N.*), Atomic Bomb Disease Institute, Nagasaki University, Nagasaki, Japan; Department of Clinical Pharmacology (*S.K.*), Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; Department of Medicine and Molecular Science (*M.Y., R.T.*), Graduate School of Medicine, Gunma University, Maebashi, Japan; Laboratory of Metabolism (*S.K.*), National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

#, These authors equally contributed.

Correspondence and reprint request: Yuji Nagayama, M.D., Department of Molecular Medicine, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523 Japan (TEL) 81+95-819-7173 (FAX) 81+95-819-7175 (E-MAIL) nagayama@nagasaki-u.ac.jp

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**Abstract**

The mutant BRAF ($BRAF^{V600E}$) is the most common genetic alteration in papillary thyroid carcinomas (PTC). 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 elavation, which by itself promotes thyroid tumorigenesis. To overcome these problems, we exploited two different approaches both of which allowed temporally and spatially restricted expression of $BRAF^{V600E}$ in the thyroid glands. Firstly, we generated conditional transgenic mice harboring the *lox*P-$neo^R$-*lox*P-$BRAF^{V600E}$-IRES-GFP sequence [*Tg(LNL-*$BRAF^{V600E}$*)*]. The double transgenic mice (*LNL-*$BRAF^{V600E}$*;TPO-Cre*) 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, 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. Secondly, postnatal expression of $BRAF^{V600E}$ in a small number of thyroid cells was also achieved by injecting the lentivirus expressing *lox*P-GFP-*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. (242 words)

**Introduction**

Thyroid carcinomas are the most common endocrine malignancies with their incidence rapidly increasing in recent years (1). The majority of them are comprised 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 (PTC), 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 extracellular signal-regulated kinase (ERK)/mitogen activated protein (MAP) kinase signaling, whether $BRAF^{V600E}$ by itself is sufficient or it needs the aid of mutation(s) in other gene(s) (*e.g.*, 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, (i) $BRAF^{V600E}$ is expressed in all the thyroid follicular epithelial cells from the fetal periods, which indicates that these two 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), (ii) $BRAF^{V600E}$ inhibits

thyroid function, thereby leading to TSH elevation that by itself induces thyroid diffuse enlargement and sometimes promotes tumorigenesis (2), and (iii), 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 two 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, Tokyo, Japan) to create the plasmid pBRAF$^{V600E}$-IRES2-AcGFP1, where IRES and 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, Tsukuba, Japan) 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 beta 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 twelve pups were confirmed by polymerase chain reaction (PCR) (see below) to have integrated the transgene, of which five 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 TPO-Cre mice (see below). Finally, two 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., St. Louis, MO) 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 sec at 94 C, 30 sec at 55-57.5 C, and 30 sec at 72 C.

**PCR analysis for Cre-mediated DNA recombination**

Genomic DNA was extracted from the thyroid tissues using DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA). Selected regions of the target genes were amplified and analyzed by PCR. The following primers were used to detect recombination (Fig. 1). 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 & B detect the LNL-$BRAF^{V600E}$ allele yielding a product of 550 bp. Primers B & 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 collected, and protein concentration determined using Bio Rad Protein Assay (Bio-Rad, Inc. CA, USA). Protein lysates were subjected to SDS-PAGE, transferred to PVDF membranes and probed with antibody to BRAF (Santa Cruz, Santa Cruz, CA) and horse radish peroxidase ABC method (Vectastatin Universal ABC kit, Vector Laboratories, Burlingame, CA).

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

The DNA fragment for 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, Bruxelles, Belgium), 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 determined by measuring the absorbance at 260 nm ($3.5 \times 10^{12}$ 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, Carlsbad, CA) according to the manufacture's instruction, and then the floxed 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 hrs after transfection and concentrated by ultracentrifugation, yielding 6 x $10^7$ cfu/ml viral stock.

**Experimental design**

Mice (4 week-old) were anesthetized with isoflurane using Anesthetizer (Muromachi, Tokyo, Japan). 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 μl containing approximately 3.5 x $10^9$ adenovirus particles or 6 x $10^4$ cfu lentivirus were injected to the left lobes using a 25 μL Hamilton micro-syringe 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 Scietific, Gaitherburg, MD).

Hypothyroidism was induced by administration of 0.5% sodium perchlorate and 0.05% methimazole (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 intraperitoneal injection of sodium pentobarbitone. Sera were collected via pericardial tap and the animal euthanized by cervical dislocation. Thyroids were removed and either flash-frozen in liquid nitrogen or fixed in 10% neutral-buffered formalin.

**H & E staining and immunohistochemistry of the thyroid glands**

Thyroid tissues fixed in formalin were embedded in paraffin. Five-micrometer-thick sections were prepared and stained with H&E or immunostained with rabbit anti-phospho-p44/42 MAPK (ERK1/2) (Cell Signaling Technology; a dilution of 1:200), rabbit anti-Ki67 (Abcam, Cambridge, MA; a dilution of 1:100) or anti-V5 antibody (PM003, MBL; a dilution of 1:100) and the colors 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, Tokyo, Japan) and 0.5 μg RNA was reverse-transcribed with SuperScript III (Invitrogen, Carlsbad, CA) in the presence of random hexamers to generate cDNA. Quantitative reverse transcription-PCR (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); sodium iodine symporter (NIS) (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, Los Angeles, CA). The normal range was defined as the mean $\pm$ 3 S.D. 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 $\pm$ 3 S.D. of control untreated mice.

**Statistical analyses**

All data were analyzed by either Student's *t*-test or chi-square test. A *p*-value of less than 0.05 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 (Fig. 1 A). Two lines, *#302MM* and *#213MM*, were selected by their lower and higher, respectively, expression levels of BRAF$^{V600E}$ protein in western blotting (Fig. 1 B) 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 & C in Fig. 1 C) in addition to the intact transgene (A & B in Fig. 1 C). 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 (Fig. 2 A and C). However, thyroid function assessed by serum $T_4$ and TSH at four weeks of age was normal in both lines (Fig. 3 A and B), consistent with mosaic pattern of recombination sparing normal tissues (see below). Expression of thyroid-specific genes, Tg and NIS, was decreased in both sexes of the higher expressor *#213MM* and only male of the lower expressor #302MM (Fig. 3 C).

In thyroid histology, all *#302MM;TPO-Cre* mice examined (16 mice, eight males and eight

females) showed marked proliferation of epithelial cells in the inter-follicular areas at 4 weeks of age, which however mostly disappeared at 26 and 52 weeks (Fig. 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 (Fig.4 D). Presumably due to this non-sustained 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) (Fig. 2B), increased TSH, decreased $T_4$ (Fig. 3A) and epithelial cell proliferation with invasion to muscles, parathyroids and/or lymphatics, and occasional tumor formation (Fig. 4B and data not shown) in 31 % (5/16) *#302MM;TPO-Cre* mice at 52 weeks of age. However, these extrathyroidal invasion, usually considered as a sign of cancer in humans, were also observed in 25 % (2/8, $p>0.05$) sodium perchlorate/MMI-treated *TPO-Cre* mice (Fig. 4B), which were therefore considered as the effect of long-term elevation of TSH.

In contrast, all *#213MM;TPO-Cre* mice readily showed sustained inter-follicular epithelial proliferation and many foci of hyperplastic epithelial cells at 4 weeks of age, of which 63 % (10/16, eight males and eight females) developed PTC-like lesions at 52 weeks with 30 % (3/10) tumors showing occasional invasion into muscles (Fig. 4C). Overexpression of BRAF and pERK was confirmed by immunohistochemistry in five randomly selected specimens (Fig. 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 (Fig.4 D).

These data indicate that the expression levels of $BRAF^{V600E}$ in *#213MM;TPO-Cre* line are sufficient for thyroid cancer induction on a single cell basis. Thus, *#213MM* mice (four week-old) 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 x $10^9$ particles of Ad-TgP-Cre clearly induced Cre-mediated recombination detected by PCR (Fig. 1C) and expression of pERK by immunohistochemistry in four randomly selected tissues four and 52 weeks after injection (Fig. 5), although the number of pERK-positive cells was somewhat decreased in the latter. Following the injection, mice were sacrificed 4, 26 and 52 weeks later (14-16 mice (six-eight males and six-eight females) at each time point). No tumor formation was however observed (supplement Fig. 1).

***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 (supplement Fig. 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 (supplement Fig. 2B). Following the injection of LEL-BRAF$^{V600E}$-V5 (6 x $10^4$ cfu), mice were sacrificed 12, 24 and 48 weeks later (16 mice at each time point, eight males and eight females). Again, no tumor formation was observed (supplement Fig. 2C).

**Discussions**

We here used two 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 which usually arise from 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 two 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 we concerned about the previous reports showing induction of PTC by $BRAF^{V600E}$ are (i) expression of $BRAF^{V600E}$ in the entire thyroid glands, (ii) its expression from the prenatal periods and (iii) 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, that is, $BRAF^{V600E}$-expressing cells, 52 weeks after adenovirus injection excludes this possibility, although their number appeared to be lower than that at four 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 post pubertal) periods may explain the distinct predisposition of $BRAF^{V600E}$ to induce cancer in these two periods. Thyroid cells in the fetal periods are immature and proliferate well, while 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 cases malignant tumors in *LSL-Kras*$^{G12D}$, *LSL-BRAF*$^{V600E}$ and *APC*$^{580S}$ mice (22-24), while quiescent muscle 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 MEK/ERK/MAK 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 account of 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 than the latter. Furthermore, since 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, one 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 (dox) administration, postnatally expressed the mutant BRAF and developed thyroid tumors, although concomitantly extremely high TSH levels was also induced, which must play a serious role for tumorigenesis because the tumors underwent involution with dox 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 ofBRAF$^{V600E}$ 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 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 PI3K-AKT-mTOR 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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**Figure Legends**

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 four to eight week-old), and subjected to western blot as described in the 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 (eight week-old), and subjected to PCR analysis of Cre-mediated DNA recombination as described in the Materials and Methods. Primers A,B and C were shown in (A).

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 $\pm$ S.D. (n =3-4). *, $p<0.01$; **, $p<0.05$.

Figure 3. TSH, free $T_4$ (A and B) and real-time RT-PCR analysis of thyroid-specific genes (NIS and Tg) . (A and B) TSH and free $T_4$concentrations were measured by RIA as described in the 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 the Materials and Methods. Data are means $\pm$ S.D. (n =5 in A and B, n = 3 in C). *, $p<0.01$ and **, $p<0.05$ *versus* controls.

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

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), *#213MM* mice injected with Ad-TgP-Cre (8 and 56 weeks) and control #213MM (56 weeks). The arrows indicate the pERK-positive areas.

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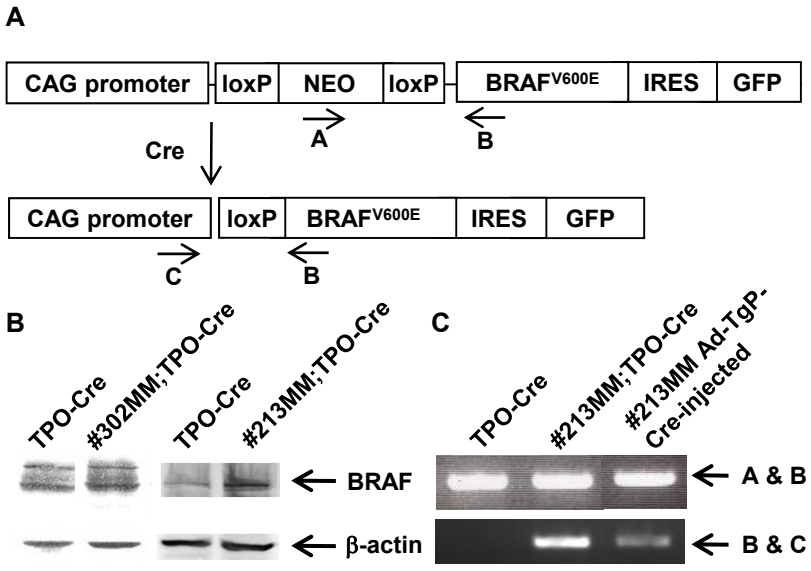
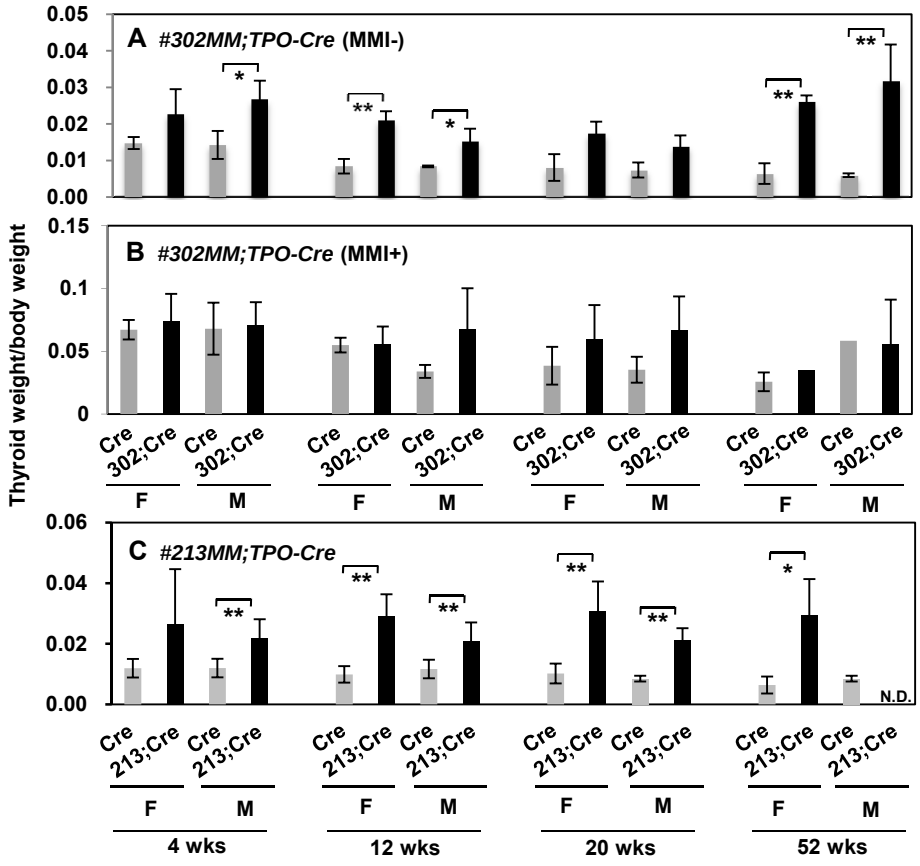



**Shimamura et al. Fig. 1**

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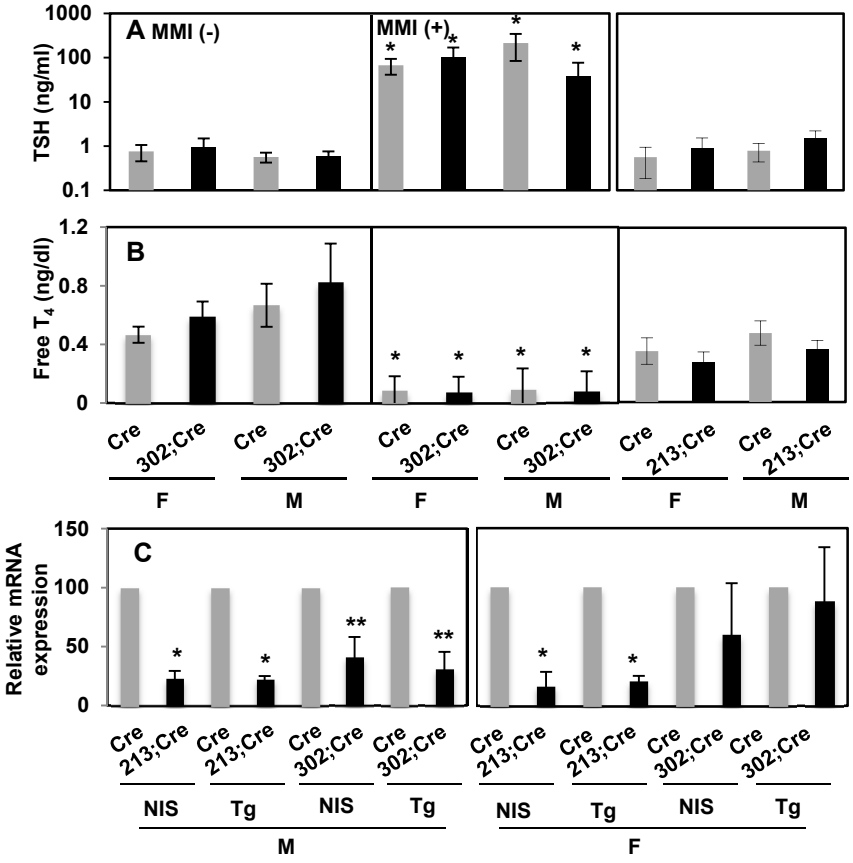


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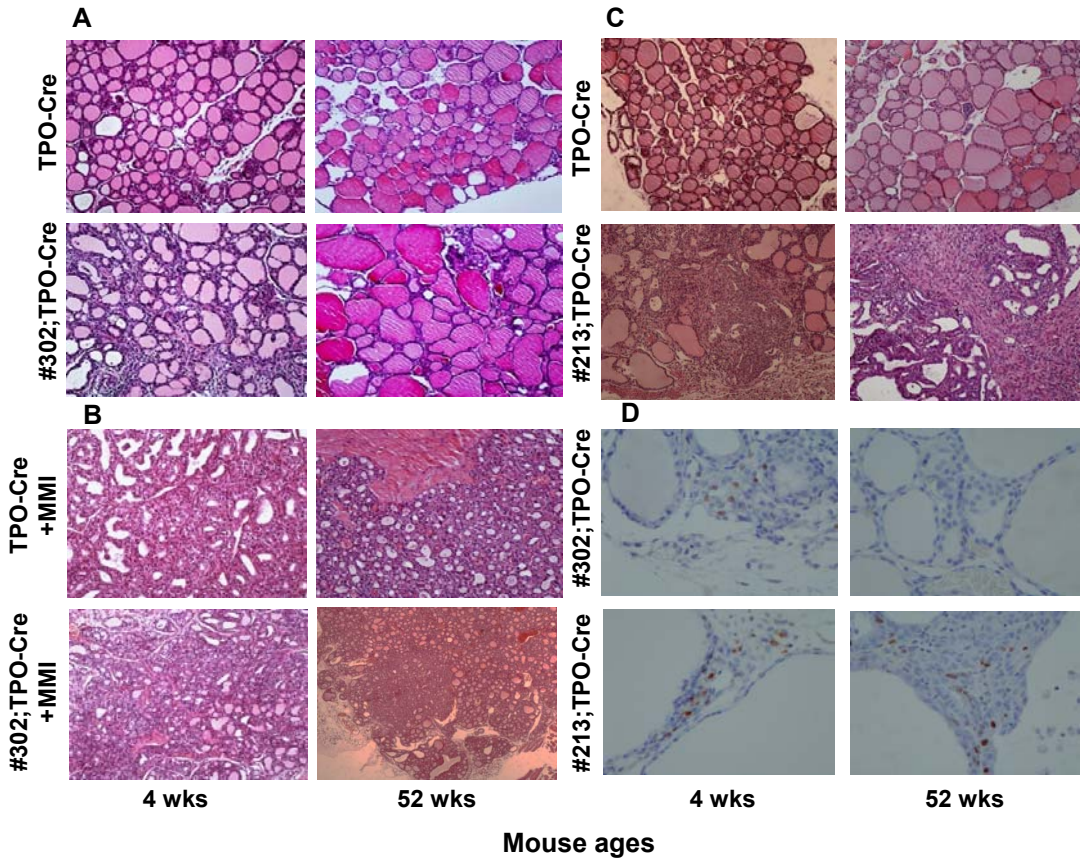


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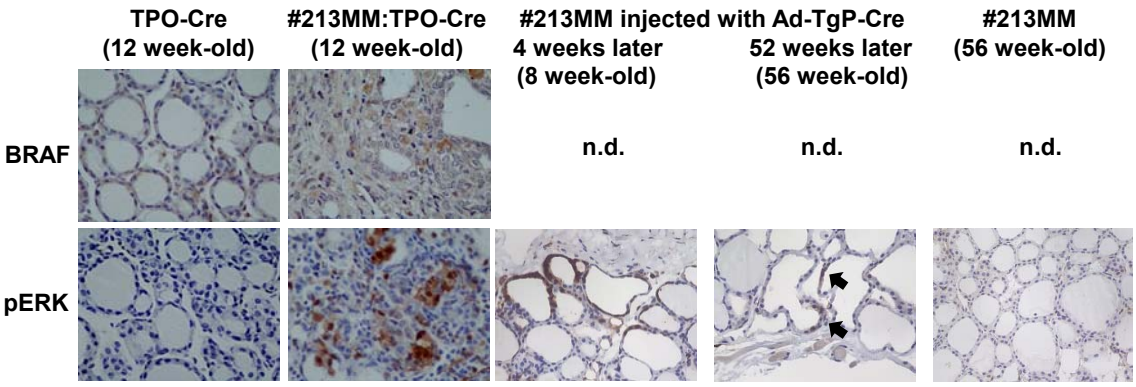


**Shimamura et al. Fig. 5**