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KRAS^{G12D}-mediated oncogenic transformation of thyroid follicular cells requires long-term TSH stimulation and is regulated by *SPRY1*

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KRAS^{G12D} can cause lung cancer rapidly, but is not sufficient to induce thyroid cancer. It is not clear whether long-term serum thyroid stimulating hormone (TSH) stimulation can promote *KRAS*^{G12D}-mediated thyroid follicular cell transformation. In the present study, we investigated the effect of long-term TSH stimulation in *KRAS*^{G12D} knock-in mice and the role of Sprouty1 (*SPRY1*) in *KRAS*^{G12D}-mediated signaling. We used TPO-*KRAS*^{G12D} mice for thyroid-specific expression of *KRAS*^{G12D} under the endogenous *KRAS* promoter. Twenty TPO-*KRAS*^{G12D} mice were given anti-thyroid drug propylthiouracil (PTU, 0.1% w/v) in drinking water to induce serum TSH and 20 mice were without PTU treatment. Equal number of wild-type littermates (TPO-*KRAS*^{WT}) was given the same treatment. The expression of *SPRY1*, a negative regulator of receptor tyrosine kinase (RTK) signaling, was analyzed in both *KRAS*^{G12D}-and *BRAF*^{V600E}-induced thyroid cancers. Without PTU treatment, only mild thyroid enlargement and hyperplasia were observed in TPO-*KRAS*^{G12D} mice. With PTU treatment, significant thyroid enlargement and hyperplasia occurred in both TPO-*KRAS*^{G12D} and TPO-*KRAS*^{WT} littermates. Thyroids from TPO-*KRAS*^{G12D} mice were six times larger than TPO-*KRAS*^{WT} littermates. Distinct thyroid histology was found between TPO-*KRAS*^{G12D} and TPO-*KRAS*^{WT} mice: thyroid from TPO-*KRAS*^{G12D} mice showed hyperplasia with well-maintained follicular architecture whereas in TPO-*KRAS*^{WT} mice this structure was replaced by papillary hyperplasia. Among 10 TPO-*KRAS*^{G12D} mice monitored for 14 months, two developed follicular thyroid cancer (FTC), one with pulmonary metastasis. Differential *SPRY1* expression was demonstrated: increased in FTC and reduced in papillary thyroid cancer (PTC). The increased *SPRY1* expression in FTC promoted TSH-RAS signaling through PI3K/AKT pathway whereas downregulation of *SPRY1* by *BRAF*^{V600E} in PTC resulted in both MAPK and PI3K/AKT activation. We conclude that chronic TSH stimulation can enhance *KRAS*^{G12D}-mediated oncogenesis, leading to FTC. *SPRY1* may function as a molecular switch to control MAPK signaling and its downregulation by *BRAF*^{V600E} favors PTC development.
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Thyroid cancer is the most common type of endocrine malignancies and its incidence is rising rapidly in recently years, especially among women.[1] Histologically, it can be classified into papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), and anaplastic thyroid cancer (ATC). PTC is the most common type of differentiated thyroid carcinomas, accounting for more than 80% of thyroid cancer, and FTC accounts for 15%.[2] The *BRAF*^{V600E} is the most common genetic alterations in PTC with overall rate of 44%,[3–5] and *RAS* (*HRAS*, *KRAS*, or *NRAS*) mutations are found in about 50% of FTC and can also been found in benign adenomas.[6,7]

In the earlier studies, several transgenic mouse models were created to study the molecular mechanisms of *RAS*-mediated transformation of thyroid follicular cells *in vivo*.[8–10] Both PTC and FTC were developed from overexpression of a mutant *RAS* gene under TG promoter, which may not reflect the activity of endogenous mutant *RAS* gene expressed at the

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physiological level. Overexpression of *KRAS* and *HRAS* could also downregulate expression in a dose-dependent manner of *NKX2-1*, *TG*, and *TPO* genes required for thyroid hormone synthesis.[11,12] Recent knock-in mouse studies have shown that endogenous expression of either $HRAS^{G12V}$ or $KRAS^{G12D}$ is not sufficient to induce thyroid dysfunction and cell transformation under their native promoters.[13,14]

It is known that TSH stimulates the growth or development of thyroid cancer and higher serum TSH is associated with both thyroid cancer incidence and recurrence.[15,16] It is not clear whether long-term TSH stimulation can induce $KRAS^{G12D}$-mediated thyroid follicular cell transformation. In the present study, we investigated the potential of long-term TSH stimulation on thyroid cancer development in $KRAS^{G12D}$ knock-in mice targeted to express its oncoprotein in thyroid at the physiological level. We also studied the role of SPRY1 in the regulation of $KRAS^{G12D}$- *vs* $BRAF^{V600E}$-mediated signaling.

MATERIALS AND METHODS

Experimental Animals

LSL-$KRAS^{G12D}$ (obtained from The Jackson Laboratory, ME, USA), LSL-$BRAF^{V600E}$, and TPO-Cre strains have been described previously.[17–19] LSL-$KRAS^{G12D}$ and LSL-$BRAF^{V600E}$ carry a latent mutant allele of *KRAS* and *BRAF*, respectively. Both LSL-$KRAS^{G12D}$ and LSL-$BRAF^{V600E}$ mice were kept as heterozygotes. LSL-$KRAS^{G12D}$ or LSL-$BRAF^{V600E}$ was crossed with TPO-Cre to generate TPO-$KRAS^{G12D}$ or TPO-$BRAF^{V600E}$ strain where $KRAS^{G12D}$ or $BRAF^{V600E}$ is conditionally expressed in thyroid follicular cells through Cre-mediated deletion of a floxed transcriptional stop sequence. The resulting TPO-$KRAS^{G12D}$ or TPO-$BRAF^{V600E}$ strain expressed mutant $KRAS^{G12D}$ or $BRAF^{V600E}$ transcripts at the physiological level under its endogenous promoter. The study was approved by the Animal Care and Usage Committee of the institution and conducted in compliance with the Public Health Service Guidelines for the Care and Use of Animals in Research.

Genotyping of TPO-$KRAS^{G12D}$ or TPO-$BRAF^{V600E}$ Strain

Genotyping of TPO-Cre mediated recombination of LSL-$KRAS^{G12D}$ or LSL-$BRAF^{V600E}$ targeted allele has been described previously.[17,19] Briefly, the following primers were used to detect *LSL-KRAS*^{G12D} recombination in mouse tissue: primer 1, 5′-GTCTTTCCCCAGCACAGTGC-3′, primer 2, CTCTTGCCTACGCCACCAGCTC-3′, and primer 3, 5′-AG CTAGCCACCATGGCTTGAGTAAGTCTGCA-3′. Primer 1+2 detects wild-type allele yielding a product of 622 bp. Primer 1+2 also detects Cre-recombined $KRAS^{G12D}$ allele (Lox-$KRAS^{G12D}$) yielding a product of 650 bp. This product is larger than the wild-type allele due to the presence of LoxP site that remains after Cre-mediated recombination. Primer 2 +3 detects the LSL-$KRAS^{G12D}$ allele yielding a product of 500 bp. Multiplex PCR containing three primers was used: 95 °C for 10 min followed by 35 cycles of amplification (95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min with final extension at 72 °C for 10 min). The following primers were used to detect LSL-$BRAF^{V600E}$ recombination: primer A, 5′-AGTCAATCATCCACAGAGACCT-3′, primer B: 5′-GCTTG GCTGGACGTAAACTC-3′, and primer C, 5′-GCCCAGG CTCTTTATGAGAA-3′. Primer A+C detects the wild-type allele yielding a product of 466 bp. Primer A+C also detects Cre-recombined allele (Lox-$BRAF^{V600E}$) yielding a product of 518 bp. Primer B+C detects the LSL-$BRAF^{V600E}$ allele yielding a product of 140 bp. The PCR conditions are at 95 °C for 5 min followed by 35 cycles of amplification (95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min with a final extension at 72 °C for 10 min).

Anti-Thyroid Drug Treatment

TPO-$KRAS^{G12D}$ mice (4–10 weeks of age) were divided into two groups and given 0.1% (w/v) anti-thyroid drug propylthiouracil (PTU, Sigma-Aldrich, MO) in drinking water *ad libitum*, changed once weekly, to induce serum TSH. One group ($n=10$) was observed for 8 months and the other group ($n=10$) was observed for 14 months. Twenty TPO-$KRAS^{G12D}$ mice were also divided into two groups (10 in each group) without PTU treatment, and observed for 8 and 14 months, respectively. Forty wild-type mice (TPO-$KRAS^{WT}$, 10 in each group) were given the same treatment as TPO-$KRAS^{G12D}$ mice.

Thyroid Hormone Measurements

Blood was collected by cardiac puncture. Serum TSH was measured using MILLIPLEX MAP Mouse Pituitary Magnetic Bead Panel following the manufacturer's instruction (EMD Millipore Corporation, Billerica, MA, USA). Serum total T4 was measured using MILLIPLEX MAP Steroid/Thyroid Hormone Magnetic Bead Panel (EMD Millipore Corporation).

Tumor Cell Culture

A PTC tumor from a 4-month-old TPO-$BRAF^{V600E}$ mouse was collected aseptically using blunt dissection and mechanically dissociated by mincing and passaged through a 40-μM mesh sterile screen, and suspended in DMEM/F12 growth medium (10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin). Cells were further dissociated by incubation in the growth medium containing 100 U/ml type I collagenase (Sigma-Aldrich) and 1.0 U/ml dispase I (Roche Diagnostics, Indianapolis, IN, USA) at 37 °C rocking water bath for 60 min. The cell suspension was washed twice and resuspended in 10 mm culture dish with DMEM/F12 growth medium containing 2 mU/ml bovine TSH (Sigma-Aldrich) to establish a BVE cell line ($BRAF^{V600E}$-induced tumor cell line). The genetic background was confirmed by genotyping.

Quantitative Real-Time Reverse Transcriptase-PCR Analysis for *SPRY1* Expression

Total RNA was isolated from thyroid of TPO-$KRAS^{WT}$mice treated with PTU, PTC of TPO-$BRAF^{V600E}$, or FTC of TPO-$KRAS^{G12D}$ mice by the quanidinium thiocyanate-phenol-chloroform method as described previously.[20] The integrity of

RNA was verified by denaturing gel electrophoresis. In all, 2 μg of each total RNA was reverse-transcribed to cDNA using the Promega RT system (Promega, Madison, WI, USA). LightCycler DNA Master SYBR Green 1 kit was used for quantitative real-time PCR analysis.[21] The cDNA mix was diluted 10-fold, and 2 μl of the dilution was used for real-time PCR. PCR primers for the 114-bp *SPRY1* cDNA fragment are 5′-GCGGAGGCCGAGGATTT-3′ (forward) and 5′-ATCAC CACTAGCGAAGTGTGGC-3′ (reverse). The forward primer spans the junction of exon 1 and exon 2 over 1.7 kb intron 1 so that contaminated genomic DNA will not be amplified. The *SPRY1* cDNA fragment was verified by DNA sequencing. The mRNA level of housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control, and a 155-bp PCR product was amplified using the following two primers: 5′-ATGTTCCAGTATGACTCCACT CACG-3′ (forward) and 5′-GAAGACACCAGTAGACT CCACGACA-3′ (reverse). The PCR conditions are 95 °C for 30 s followed by 30 cycles of amplification (95 °C for 10 s, 50 °C for 5 s, and 72 °C for 10 s). The resulting concentration of *SPRY1* PCR products was normalized by comparison with GAPDH and was used to determine the relative mRNA level of *SPRY1* in thyroid tumors.

Histology and Immunohistochemistry

Histology and immunohistochemical staining was described previously.[22] Briefly, 4-μm-thick formalin-fixed paraffin-embedded tissue sections were prepared and stained with hematoxylin and eosin or *SPRY1* (1:50 dilution, Abcam, Cambridge, MA, USA), p-ERK, or p-AKT antibody (1:50 dilution, Cell Signaling Technology, Danvers, MA, USA). Histological diagnosis was performed by a thyroid pathologist (MA) blinded to the genotype and the treatment status of the animal. DAKO LSAB+kit, HRP was used for immunostaining (DAKO, Carpinteria, CA, USA). The sections were counter-stained with Mayer's haematoxylin.

Regulation of TSH Signaling by SPRY1

Mouse SPRY1 cDNA in pCMV6 expression vector was obtained from OriGene (Rockville, MD, USA). BVE cell line was transfected with either pCMV6 vector or mSPRY1/pCMV6 using Lipofectamine (Invitrogen, CA). The culture medium was changed to DMEM/F12 growth medium 16 h after transfection, and the transfected cells were cultured for additional 48 h in the presence or absence of 10 mU/ml bTSH. The expression of SPRY1, phospho-ERK, and phospho-AKT was determined by western blot analysis.

Western Blot Analysis

BVE cell line was transfected with mouse SPRY1 cDNA under the control of CMV promoter (Origen). 40 μg of protein was loaded onto a 12% SDS-polyacrylamide gel. Proteins were transferred onto a PVDF membrane and subject to western blot analysis using anti-phospho-ERK 1/2, and phosphor-AKT antibody (1:1000, Cell Signaling Technology) or anti-*SPRY1* (1:1000, Abcam).

Bioinfomatic Anylysis of Human PTC Samples

The expression profiles of *SPRY1*, *SPRY2*, *SPRY3*, and *SPRY4* in the TCGA data set of 572 PTC samples[23] were analyzed using UCSC cancer genomics browser (https://genome-cancer.soe.ucsc.edu/proj/site/hgHeatmap/).

Statistical Analysis

Unpaired Student's *t*-test (two-tailed) was used. A *P*-value of 0.05 or less was considered as significant.

RESULTS

Without PTU treatment, only mild thyroid enlargement and hyperplasia were observed in TPO-$KRAS^{G12D}$ mice (Figure 1b). The thyroids were about two times larger as compared with those from the TPO-$KRAS^{WT}$ mice. Thyroid cancer development was not observed among 10 mice monitored for up to 14 months. With PTU treatment, significant thyroid enlargement with multinodular goiters and hyperplasia occurred in both TPO-$KRAS^{G12D}$ and TPO-$KRAS^{WT}$mice (Figure 2a). Massive thyroid enlargement was found in TPO-$KRAS^{G12D}$ mice: thyroids of TPO-$KRAS^{G12D}$ mice were about six times larger than TPO-$KRAS^{WT}$ mice (118.75 ± 13.15 mg *vs* 21.11 ± 6.33 mg, $P<0.001$, Figure 2b), indicating that TSH can cooperate with $KRAS^{G12D}$ to promote thyroid growth and hyperplasia. Interestingly, thyroid histology of TPO-$KRAS^{G12D}$ mice showed nodular hyperplasia with well-maintained follicular architecture whereas in TPO-$KRAS^{WT}$ mice such follicular architecture was replaced by papillary hyperplasia (Figure 2c). However, PTC was not diagnosed due to lack of characteristic nuclear features such as nuclear grooves, intranuclear cytoplasmic inclusion, fine chromatin texture, and nuclear envelope irregularity. As shown in Figure 2d, serum TSH was significantly elevated in PTU-treated mice as compared with the control ($24\,370.8 \pm 12\,942.5$ *vs* 426.4 ± 9.65 pg/ml, $P<0.01$). Serum T4 was significantly reduced in PTU-treated mice as compared with the control (5.21 ± 1.31 *vs* 22.68 ± 6.23 ng/ml, $P<0.05$). Among 10 TPO-$KRAS^{G12D}$ mice monitored for 14 months, two developed follicular thyroid cancer (FTC, Figure 3a) and one with pulmonary metastasis (Figure 3b). Since the cellular features were indistinguishable between follicular hyperplasia and cancer, the diagnosis of FTC was based on the invasion of surrounding tissues such as blood vessel and neck muscle (Figure 3a), and pulmonary metastasis (Figure 3b). Among 10 TPO-$KRAS^{WT}$ mice monitored for 14 months under PTU treatment, no tumor development was observed.

The Sprouty (*SPRY*) family of proteins is involved in the negative feedback regulation of growth factor-mediated MAPK activation.[24] To understand why TSH stimulation caused only FTC, but not PTC, we investigated *SPRY1* expression in thyroid tumors from TPO-$KRAS^{G12D}$ and TPO-$BRAF^{V600E}$ mice. *SPRY1* mRNA level was significantly higher

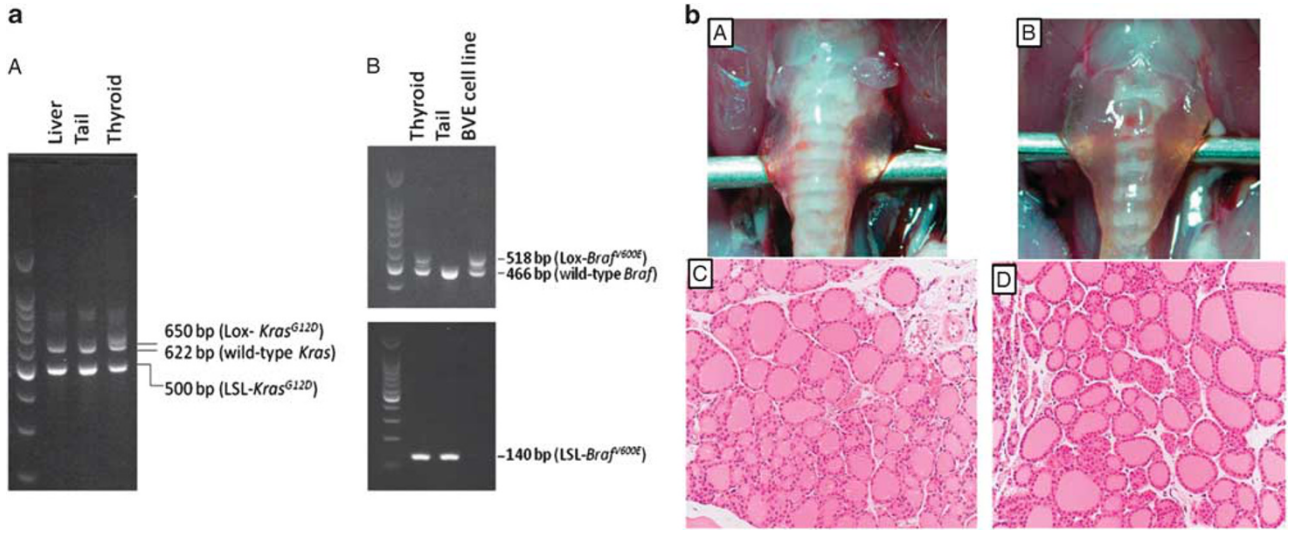

Figure 1 Activation of *KRAS*^{G12D} in thyroid induces mild thyroid enlargement and hyperplasia. (**a**) Genotyping of TPO-Cre mediated recombination of LSL-*KRAS* ^{G12D} (A) and LSL-*BRAF*^{V600E} (B). The PCR product was run on a 1.5% agarose gel. The activation of *KRAS*^{G12D} and *BRAF*^{V600E} occurred only in the thyroid as a result of Cre-mediated deletion of a floxed transcriptional stop sequence. (**b**) Mild thyroid enlargement and hyperplasia were observed in TPO-*KRAS*^{G12D} mice. Thyroids of a TPO-*KRAS*^{WT} mouse (A) and a TPO-*KRAS*^{G12D} mouse (B) at the age of 8 months were shown at the top panel. Histology of thyroids from the same TPO-*KRAS*^{WT} mice (C, ×20) and TPO-*KRAS*^{G12D} mice (D, ×20) was shown at the bottom panel.

in *KRAS*^{G12D}-induced FTC (9.23 ± 0.56) as compared with *KRAS*^{WT}control (1.03 ± 0.15, $P<0.01$, Figure 4a). SPRY1 expression was also increased in *KRAS*^{G12D} thyroid without PTU treatment (2.1 ± 0.2, $P<0.01$, Figure 4a), but the expression level was much lower than under PTU treatment, suggesting that the increased SPRY1 expression may be a compensatory mechanism to counter TSH-mediated growth stimulation. In contrast, *SPRY1* mRNA level was significantly lower in *BRAF*^{V600E}-induced PTC (0.31 ± 0.17) as compared with *BRAF*^{WT} control (1.00 ± 0.12, $P<0.05$, Figure 4b). The serum TSH was greater than 50 000 pg/ml (beyond the detection limit of the kit) and T4 was 3.87 ± 0.68 in TPO-*BRAF*^{V600E} mice ($n=6$) as compared with 445.2 ± 72.21 in TSH and 29.03 ± 3.62 in T4 from TPO-*BRAF*^{WT} mice ($n=6$), consistent with previous report that TPO-*BRAF*^{V600E} mice have severe hypothyroidism.[13] The increased *SPRY1* expression in FTC was confirmed by immunohistochemistry analysis. As shown in Figure 4c, increased *SPRY1* and p-AKT expression was observed in FTC (b and e) with normal p-ERK expression (h). In PTC, both p-ERK and p-AKT expression were increased (f and i). Although down-regulation of *SPRY1* expression in PTC was not clearly shown by immunohistochemistry (Figure 4cC), it was confirmed by western blot analysis, showing inverse correlation between *SPRY1* expression and p-ERK activation (Figure 4d).

To further investigate the role of SPRY1 in the regulation of TSH-mediated signaling, we used BVE cell line (derived from *BRAF*^{V600E}-induced PTC tumor) as a model, for it has low *SPRY1* expression. The cell line was transfected with mouse *SPRY1* cDNA and treated with or without TSH. As shown in Figure 5, *SPRY1* expression reduced p-ERK expression, and TSH stimulation only activated p-ATK when *SPRY1* expression was increased. When *SPRY1* expression was reduced, TSH stimulation increased both p-ERK and p-ATK expression (Figure 5). These data suggest that *SPRY1* may act as a molecular switch to control thyroid follicular cells to go through either MAP kinase (RAF-MEK-ERK) or PI3 kinase (PI3K/AKT) signaling pathway. When *SPRY1* is upregulated by *KRAS*^{G12D}, MAPK pathway is inhibited and only PI3K/AKT pathway is utilized, resulting in FTC. When *SPRY1* is downregulated by *BRAF*^{V600E}, thyroid follicular cells may go through both MAPK and PI3K/AKT pathways to initiate PTC.

Finally, we performed bioinformatic mining of the recent TCGA data set of 572 human PTC samples to compare *SPRY* gene (*SPRY1*, *SPRY2*, *SPRY3*, and *SPRY4*) expression between classic PTC (CPTC) and follicular variant PTC (FVPTC). FVPTC shares many molecular and pathological features with FTC. *RAS* mutation is frequently detected in FVPTC as compared with frequent *BRAF* mutation in CPTC. As shown in Figure 6, the *SPRY1* and *SPRY4* expression is reduced in normal thyroids. The expression of *SPRY2* and *SPRY3* tends to increase in normal thyroids and is variable in both CPTC and FVPTC. The *SPRY1* expression is also variable in CPTC and FVPTC. In CPTC, increased *SPRY1* expression is present in 36.6% (162/409) samples, decreased expression in 40.6% (166/409) and unchanged in 19.8% (81/409) samples. In FVPTC, 40.6% (43/106) samples have increased expression, 36.8% (39/106) have decreased expression, and 22.6% (24/106) unchanged. The expression of *SPRY4* is consistently increased in FVPTC: 66.1% (72/109) have increased expression and 13.8% (15/109) have decreased expression. These data suggest that the *SPRY* family of proteins is involved in the regulation of thyroid tumorigenesis. The exact role of each protein remains to be determined.

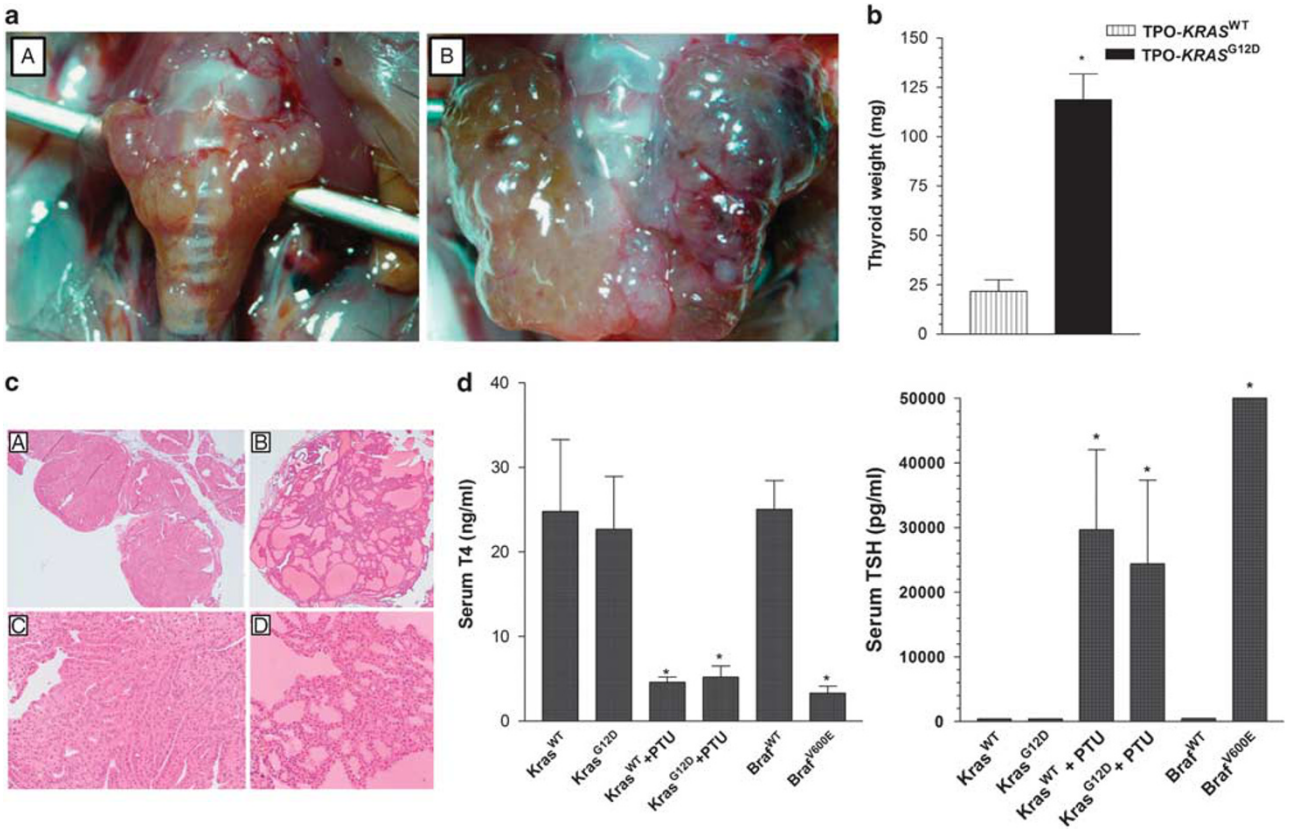

Figure 2 TSH cooperates with *KRAS*^{G12D} to induce massive thyroid hyperplasia. (**a**) Thyroids of a TPO-*KRAS*^{wt} mouse (A) and a TPO-*KRAS*^{G12D} mouse (B) at 16 months old were shown. The mice were treated with PTU in drinking water for 14 months. Goiters were present in both mice and significant thyroid enlargement was observed in TPO-*KRAS*^{G12D} mice. (**b**) Thyroid weight in PTU-treated mice. Thyroid was collected from 10 TPO-*KRAS*^{G12D} and 10 TPO-*KRAS*^{WT} mice treated with PTU for 14 months (at the end of experiment). Thyroid weight was measured and data were expressed as mean ± s.e.m., *$P<0.001$. (**c**) Histology of thyroids from the same TPO-*KRAS*^{WT} and TPO-*KRAS*^{G12D} mice was shown. Follicular architecture was replaced by papillary structure in hyperplastic goiters from a TPO-*KRAS*^{WT} mouse (A, ×4; C, ×20) whereas in the goiter of a TPO-*KRAS*^{G12D} mouse, the follicular architecture was intact (B, ×4, D, ×20). (**d**) Thyroid hormone levels in PTU-treated mice. Serum was collected from six TPO-*KRAS* mice treated with PTU for 1 month and from six mice of same age group without PTU treatment. The samples were assayed individually in triplicates. The data are expressed as mean ± s.e.m., *$P<0.05$.

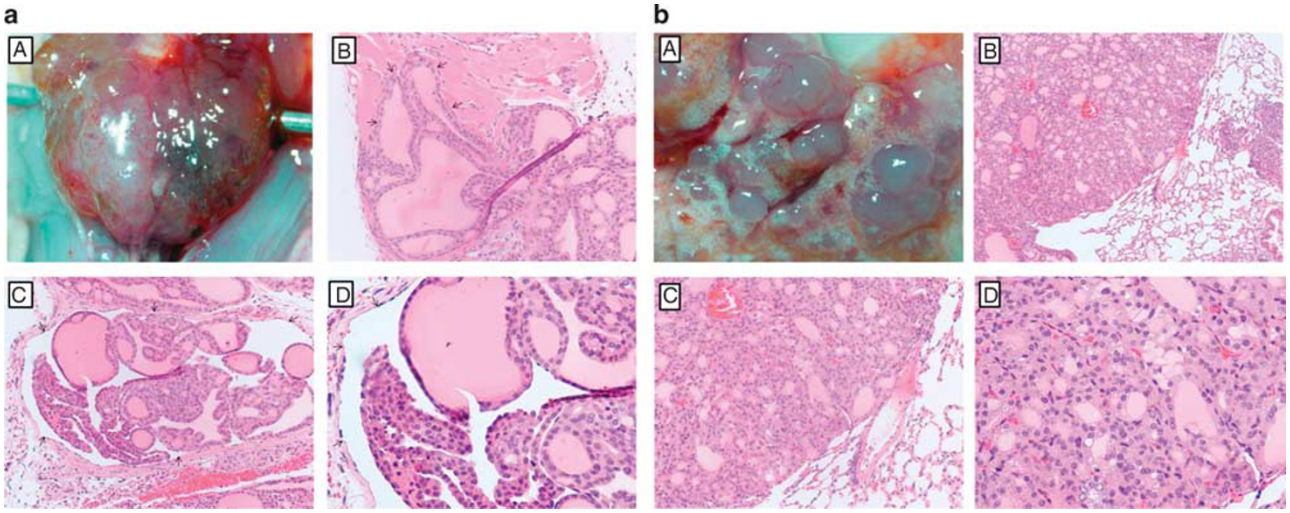

Figure 3 TSH cooperates with *KRAS*^{G12D} to induce transformation of thyroid follicular cells into FTC. (**a**) Thyroid of a TPO-*KRAS*^{G12D} mouse with FTC following 14-month PTU treatment (A). FTC with neck muscle invasion as indicated by arrows (B, ×10) and blood vessel invasion (indicated by arrows, C, ×20; D, ×40). (**b**) Pulmonary metastasis of FTC from the same mice. Multiple foci of metastasis can be seen from gross examination of the lung (A) and microscopic examination (B, ×10; C, ×20, D, ×40).

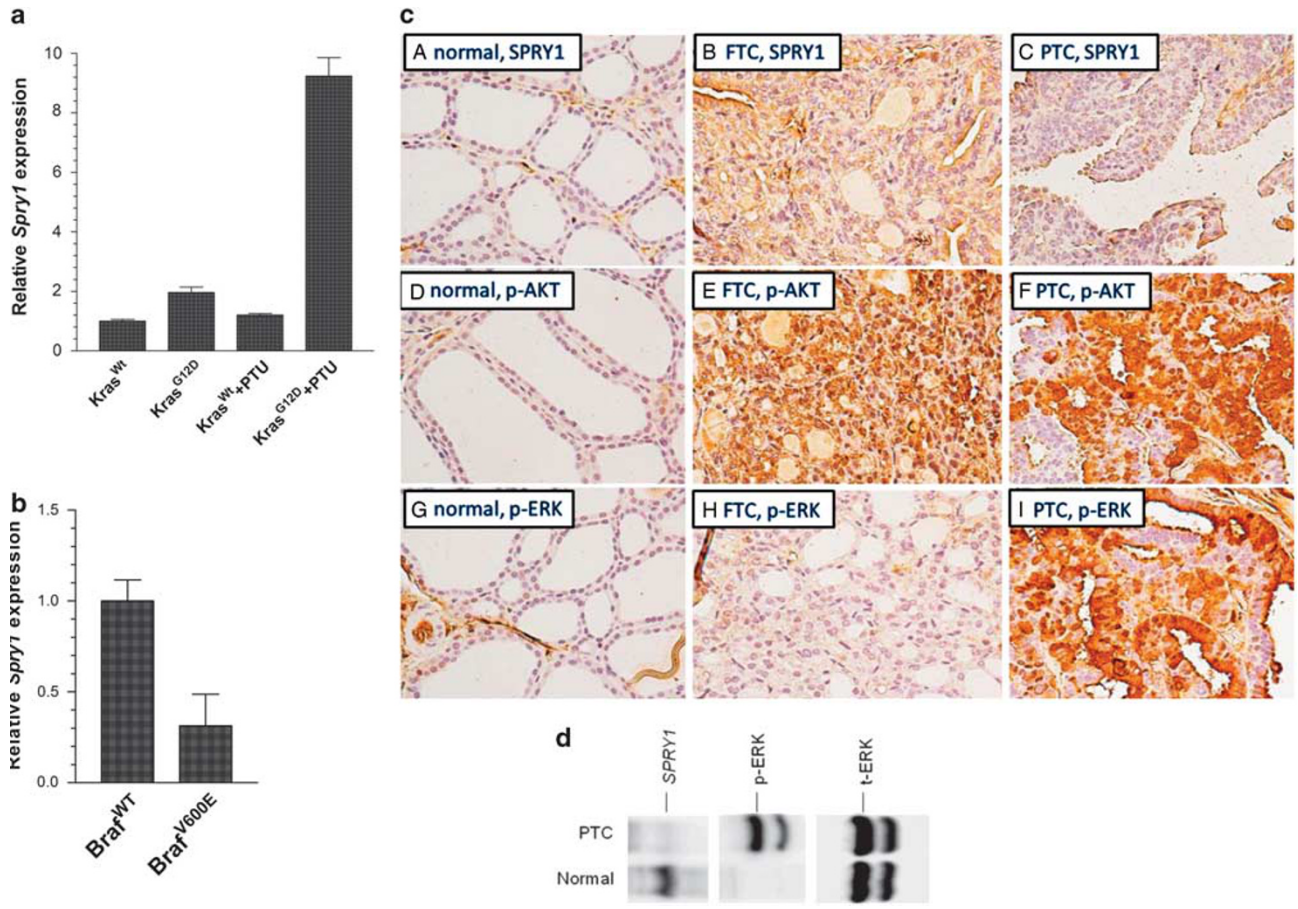

Figure 4 *SPRY1* expression in thyroid cancer. *SPRY1* mRNA from *KRAS*^{G12D}-induced FTC (**a**) and *BRAF*^{V600E}-induced PTC (**b**) was analyzed by qRT-PCR. RNA from TPO-*KRAS*^{WT} mouse thyroids and TPO-*BRAF*^{WT}mouse thyroids was used as a control. Data were expressed as mean ± s.e.m. of three separate experiments. (**c**) Immunohistochemistry staining of *SPRY1*, p-AKT, and p-ERK. Normal thyroid (A, D, and G, ×40), FTC from a TPO-*KRAS*^{G12D} mouse (B, E, and H, ×40), and PTC from a TPO-*BRAF*^{V600E} mouse were stained with *SPRY1* antibody (A, B, and C, 1:50 dilution), p-AKT antibody (D, E, and F, 1:50 dilution) and p-ERK antibody (G, H, and I, 1:50 dilution). Increased expression of *SPRY1* and p-AKT was seen in FTC. p-ERK was not activated in FTC whereas both p-AKT and p-ERK were activated in PTC. (**d**) Western blot analysis of *SPRY1* expression in a PTC tumor. Decreased *SPRY1* expression is observed in PTC as compared with normal thyroid.

DISCUSSION

In the present study, we have demonstrated that *KRAS*^{G12D} mutation alone is not sufficient to induce oncogenic transformation of thyroid follicular cells. With long-term TSH stimulation, *KRAS*^{G12D} can promote thyroid growth and hyperplasia, and eventually result in oncogenic transformation into follicular thyroid cancer. This demonstrates the significant oncogenic role of TSH in thyroid tumorigenesis: without TSH stimulation, thyroid cancer would not be able to develop or progress even in the presence of *KRAS*^{G12D} or *BRAF*^{V600E}.[25]

The animal model in the current study is very similar to congenital hypothyroidism in humans, which is caused by defects in thyroid hormone synthesis.[26] High levels of TSH are often present in patients with congenital hypothyroidism due to poor management or patients' non-compliance to treatment. We and others have reported thyroid cancer development as a result of long-term TSH stimulation.[27,28] Although both PTC and FTC were reported in those cases, *BRAF* mutation was found only in patients with PTC.[29] *RAS* mutations have been reported in both benign thyroid goiters and cancers,[30] indicating that additional factors are required to initiate malignant transformation. The current study has demonstrated that chronic TSH stimulation is one of the factors required for *RAS*-mediated carcinogenesis.

TSH stimulates growth and differentiation of thyroid follicular cells through its G protein-coupled receptor. TSH can activate both cAMP-protein kinase A (PKA) pathway for differentiation[31] and RAS-PI3K pathway for proliferation.[32,33] RAS allows growth promoting signals through both MAPK (RAF-MEK-ERK) and PIK3CA/AKT pathways. Previous studies have shown that cAMP can inhibit RAS-mediated signals to MAPK by blocking RAF-1 activation via RAP1.[34,35] Under these circumstances, PI3K signaling is the main mediator of RAS effects. RAP1 is a member of the RAS family of small G proteins that transmit signals from cAMP-

PKA pathway to MAPK pathway by interaction with BRAF.[36] Without BRAF, GTP-loaded RAP1 blocks RAS activation of RAF-1, thereby inhibiting MAPK signaling.[37] In a mouse model with constitutive RAP1 activation in thyroid, FTC was not developed until given 12 months goitrogen treatment, indicating that TSH signaling is required and is probably via RAS-PI3K pathway for FTC initiation.[38] In two different FTC mouse models, TSH signaling is constantly activated with simultaneous activation of either PI3K/AKT[39,40] or phospholipase C pathway.[41] FTC was developed in all mice with PI3K/AKT activation, suggesting that PI3K is an important mediator of RAS effects in TSH-induced FTC. FTC can develop rapidly without TSH signaling when both RAS and PI3K/AKT pathways are constitutively activated.[42] Although MAPK is activated in these mice,[42] PI3K signaling probably predominates over MAPK, resulting in FTC phenotype. Since serum TSH is very low in these mice, MAPK activation may reflect the loss of cAMP-mediated inhibition of RAS signals to MAPK.[43]

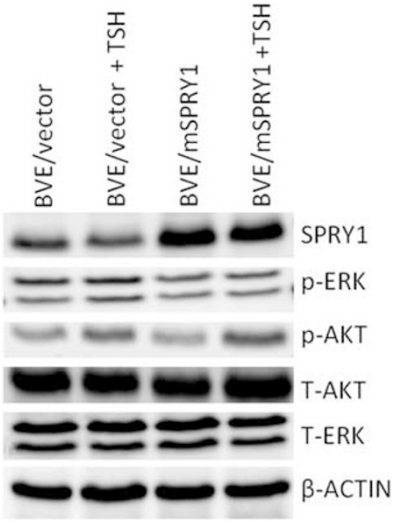

Figure 5 Regulation of TSH signaling by SPRY1. BVE cell line was transfected with mouse *SPRY1* cDNA and cultured in the presence or absence of 10 mU/ml bovine TSH for 48 h. The expression of SPRY1, phospho-ERK, and phospho-AKT was determined by western blot analysis. Reduction of p-ERK is observed by increased *SPRY1* expression, and TSH stimulation only activates p-ATK in the presence of increased *SPRY1* expression. TSH can increase both p-ERK and p-ATK expression when *SPRY1* expression is reduced.

TSH signaling promotes the tumorigenesis of PTC,[13] indicating a cross-talk between TSH-cAMP-PKA pathway and MAPK pathway.[37] As discussed above, RAP1 may be one of the molecular switches to control TSH-cAMP signaling through either RAS-PI3K to initiate FTC or RAS-MAPK to induce PTC. In the current study, we have identified that *SPRY1* is another molecular switch to regulate RAS-MAPK signaling. *SPRY1* has been reported as a candidate tumor-suppressor gene in medullary thyroid carcinoma[44] and its expression is decreased in human prostate cancer.[45] We also found reduced *SPRY1* expression in human PTC with $BRAF^{V600E}$ (data will be presented elsewhere). Concomitant

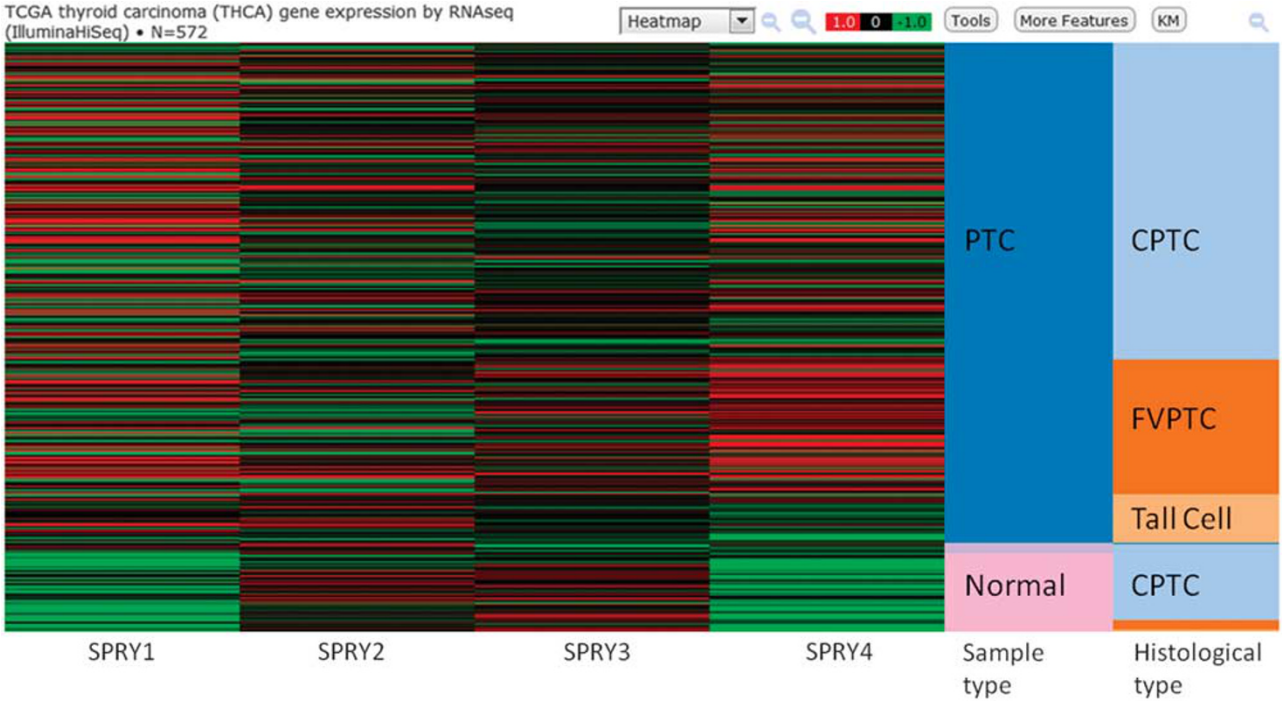

Figure 6 The gene expression profile of *SPRY* family of proteins in human thyroid cancer. The TCGA thyroid cancer gene expression data set of 572 thyroid cancer samples was used to examine gene expression profile among normal thyroid, PTC, classic PTC (CPTC), tall cell PTC, and follicular variant PTC (FVPTC). The gene expression profile is presented as a heatmap of red (upregulated), black (no change), and green (downregulated). The *SPRY1* and *SPRY4* expression is downregulated in normal thyroids. The expression of *SPRY4* is consistently increased in FVPTC.

loss of *SPRY1* and *SPRY2* results in hyperactive MAPK signaling and low-grade prostatic intraepithelial neoplasia.[46] However, when *SPRY1* and *SPRY2* loss-of-function occurs in the context of loss of one Pten allele, aberrant activation of AKT and invasive neoplasia occur, suggesting that Sprouty genes can negatively regulate the PI3K/AKT pathway as well.[46] Indeed, PI3K/AKT activation has been reported in PTC patients with $BRAF^{V600E}$.[47] Our data also demonstrate the activation of both MAPK and PI3K/AKT pathways in $BRAF^{V600E}$-induced PTC when *SPRY1* expression is reduced. In $KRAS^{G12D}$-induced lung cancer, *SPRY2* is upregulated via negative feedback loop to inhibit MAPK signaling.[48] However, we only found *SPRY1* upregulation in $KRAS^{G12D}$-induced FTC and *SPRY2* expression was not changed (data not shown). The differential *SPRY1* expression in TPO-$KRAS^{G12D}$ and TPO-$BRAF^{V600E}$ mice indicates that *SPRY1* can selectively regulate TSH-mediated RAS signaling pathways in thyroid: favoring MAPK pathway and PTC initiation when its expression is downregulated and PI3K/AKT pathway and FTC initiation when its expression is upregulated. It has been reported that supraphysiologic expression of a mutant RAS can result in PTC or mixed papillary–follicular features in early transgenic mice studies,[8,10] which appears to be contradictory to our hypothesis. However, overexpressed mutant RAS beyond physiological level may escape *SPRY1*-mediated feedback inhibition of MAPK signaling and induce PTC.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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