

Reactivation of the Silenced Thyroid Hormone Receptor β Gene Expression Delays Thyroid Tumor Progression

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That a knock-in mouse harboring a dominant-negative thyroid hormone receptor (TR)- β (*Thrb*) mutation develops metastatic thyroid cancer strongly suggests the involvement of TR β in carcinogenesis. Epigenetic silencing of the *THRB* gene is common in human cancers. The aim of the present study was to determine how DNA methylation affected the expression of the *THRB* gene in differentiated thyroid cancer (DTC) and how reexpression of the *THRB* gene attenuated the cancer phenotypes. We used methylation-specific PCR to examine the expression and promoter methylation of the *THRB* gene in DTC tissues. Thyroid cancer cells with hypermethylated *THRB* were treated with the demethylating agents 5'-aza-2'-deoxycytidine (5'-aza-CdR) and zebularine to evaluate their impact on the cancer cell phenotypes. *THRB* mRNA expression in DTC was 90% lower than in normal controls, and this decrease was associated with a higher tumor/lymph node staging. The promoter methylation level of the *THRB* gene had a significant negative correlation with the expression level of the *THRB* gene. Treatment of FTC-236 cells with 5'-aza-CdR or zebularine induced reexpression of the *THRB* gene and inhibited cell proliferation and migration. FTC-236 cells stably expressing TR β exhibited lower cell proliferation and migration through inhibition of β -catenin signaling pathways compared with FTC-236 without TR β . 5'-Aza-CdR also led to suppression of tumor growth in an *in vivo* xenograft model using FTC-236 cells consistent with the cell-based studies. These findings indicate that TR β is a tumor suppressor and could be tested as a potential therapeutic target. (*Endocrinology* 154: 25–35, 2013)

Thyroid hormone receptors (TRs) are members of the nuclear receptor superfamily that mediate the biological activities of the thyroid hormone (T_3) in development, growth, differentiation, and metabolism (1, 2). Two TR genes, *THRA* and *THRB*, encode four T_3 -binding receptor isoforms (TR α 1, TR β 1, TR β 2, and TR β 3). Previous studies have shown TR mutations are associated with human cancers, including hepatocellular carcinoma, renal cell carcinoma, breast cancer, and pituitary tumors (3–6). That a knock-in mouse model harboring a potent dominant-negative *THRB* mutation (denoted PV) spontaneously develops metastatic thyroid cancer indicates that the loss of normal functions of TR β by mutation contributes

to thyroid carcinogenesis (7). Silencing of the *THRB* expression by hypermethylation of the promoter region of the *THRB* gene is frequent in breast, lung, colon, acute lymphoblastic leukemia, and thyroid cancers (8–13). Recent studies have provided evidence that the expression of the *THRB* gene could also be repressed through a microRNA regulatory mechanism in papillary thyroid carcinoma (14). These findings raised the possibility that TR β could act as a tumor suppressor and potentially be a therapeutic target.

The incidence of thyroid cancer, the most common malignancy in the endocrine organs, has greatly increased in the past two decades around the world (15). Recent ad-

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Abbreviations: 5'-Aza-CdR, 5'-Aza-2'-deoxycytidine; DTC, differentiated thyroid cancer; FH, Flag-hemagglutinin; FTC, follicular thyroid carcinoma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PARP, poly-(adenosine-diphosphate ribose); p- β -catenin, phosphorylated β -catenin; TR, thyroid hormone receptor.

vances in the understanding of molecular genetics of thyroid cancer have revealed that molecular abnormalities accumulate by genetic and epigenetic mechanisms during thyroid carcinogenesis (16, 17). Aberrant DNA methylation of tumor-suppressor genes occurred in the early phases of tumorigenesis and plays an important role in cancer development and progression (17, 18). Several cell-based studies have shown beneficial effects by reactivating the expression of hypermethylated genes with the use of demethylating agents (19–22). These cell-based studies raised the possibility for a novel potential treatment modality for thyroid cancers. Although hypermethylation of the *THRB* gene was previously reported in differentiated thyroid cancer (DTC) (11), functional consequences of inactivation of the *THRB* gene by hypermethylation in thyroid cancer have not been elucidated.

In the present study, we first evaluated promoter methylation and the expression of the *THRB* gene in tissue specimens from patients with DTC and in several human thyroid cancer cell lines. We found a positive correlation between the extent of promoter hypermethylation of the *THRB* gene and the progression of DTC. When human thyroid cancer cell lines in which the *THRB* gene was silenced by hypermethylation were treated with demethylation agents such as 5'-aza-2'-deoxycytidine (5'-aza-CdR) and zebularine (a newer demethylating agent with more stable and less toxic properties), the expression of the *THRB* gene was reactivated concurrently with inhibition of cancer cell proliferation, migration, and tumor growth in xenograft model. Reexpression of *THRB* in thyroid cancer cell lines inhibited cell proliferation and migration through suppressing the activation of β -catenin signaling pathway. The present study demonstrated that TR β could act as a tumor suppressor in thyroid cancer and could be tested as a potential therapeutic target.

Materials and Methods

Human thyroid tissue specimens from patients with DTC and normal controls

Thyroid cancer tissue samples, patient demographics, and clinical and pathological information were collected after written informed consent under an Institutional Review Board-approved clinical protocol (NCI-09-C-0242 and NCT01005654). Samples were obtained at the time of thyroidectomy, snap frozen in liquid nitrogen, and stored at -80°C . The clinicopathological information of the patients with DTC is summarized in Supplemental Table 1 (published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>). We enrolled 17 patients with papillary thyroid carcinoma and four patients with follicular thyroid carcinoma (FTC); the mean age of these 21 patients (eight male and 13 female) was 50.2 yr. The mean maximal diameter of tumor was 3.6 cm; 12 of 21 patients (57%) were

classified in T1/T2 stage by the American Joint Committee on Cancer/International Union Against Cancer tumor-node-metastases classification staging classification (sixth edition). Cervical lymph node metastasis was present in 11 patients (52%), and distant metastasis was found in two patients (10%). In 11 patients undergoing thyroidectomy for benign disease, normal thyroid tissue was obtained from the contralateral thyroid lobe. Tissues with nodular hyperplasia and follicular adenoma were also obtained from patients for analysis.

Quantitative real-time RT-PCR

Total RNA from human thyroid tissues was extracted by TRIzol (Invitrogen, Carlsbad, CA), followed by ribonuclease-free deoxyribonuclease treatment and column purification (RNeasy Mini kit; QIAGEN, Valencia, CA). Total RNA from FTC-236 cells after treatment with 3 μM 5'-aza-CdR, 10 μM zebularine, or vehicle for 10 d was isolated using TRIzol as indicated by the manufacturer's protocol. For real-time RT-PCR, one-step RT-PCRs were performed with 100–200 ng total RNA using a QuantiTect SYBR green RT-PCR kit (QIAGEN) in a Roche LightCycler PCR instrument (Roche, Indianapolis, IN) according to the manufacturer's instructions. Specific primer sequences are listed in Supplemental Table 2. The reaction conditions were 50 $^{\circ}\text{C}$ for 20 min, 95 $^{\circ}\text{C}$ for 15 min, followed by 40–45 cycles of 94 $^{\circ}\text{C}$ for 15 sec, 59 $^{\circ}\text{C}$ for 25 sec, 72 $^{\circ}\text{C}$ for 25 sec, 65–95 $^{\circ}\text{C}$ with a heating rate of 0.1 $^{\circ}\text{C}/\text{sec}$, and finally a cooling step to 40 $^{\circ}\text{C}$.

DNA extraction and detection of DNA methylation

Genomic DNA from tissues and cells was isolated using a QIAamp DNA Micro kit (QIAGEN) according to the manufacturer's instructions. An isolated 100 ng of genomic DNA was bisulfite modified using a BisulFlash DNA modification kit (Epigentec, Brooklyn, NY) according to the manufacturer's instructions. Methylation-specific PCR was performed on 10 ng of bisulfite-treated DNA. The methylation-specific *THRB* primer sequences are listed in Supplemental Table 2. The PCR was performed for 40 cycles and the conditions were denaturation at 94 $^{\circ}\text{C}$ for 15 sec, annealing at 59 $^{\circ}\text{C}$ for 25 sec, and extension at 72 $^{\circ}\text{C}$ for 25 sec, followed by a final 5-min extension at 72 $^{\circ}\text{C}$. The PCR products were separated by electrophoresis onto a 2% Tris-acetate-ethylenediaminetetraacetic acid agarose gel, stained with ethidium bromide, and visualized under UV illumination. The relative ratios of methylated to unmethylated DNA were determined by measuring the intensities of the amplified bands visualized on DNA gel.

Western blot

Preparation of whole-cell lysates from human thyroid tissues or FTC-236 cells and nuclear extracts of FTC-236 cells was carried out as previously described (23). The protein samples (40 μg of cell lysates from human tissues and 20–30 μg of lysates from FTC-236 cells) were loaded and separated by SDS-PAGE. After electrophoresis, the protein was electrotransferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). Anti-TR β C4 antibodies were used for detection of TR β expression (24). The antibodies to phosphorylated β -catenin (p- β -catenin; 1:1000 dilution), β -catenin (1:1000 dilution), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000 dilution) were purchased from Cell Signaling

Technology (Danvers, MA). Antibody to cyclin D1 (1:500 dilution) was from Thermo Scientific (Waltham, MA), and antibody to α -tubulin (1:1000 dilution) was from Sigma-Aldrich (St. Louis, MO). Antibody to poly-(adenosine-diphosphate ribose) polymerase (PARP) (1:1000 dilution) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The blots were stripped with Re-Blot Plus (Chemicon, Temecula, CA) and reprobed with antibodies to GAPDH and PARP. Band intensities were quantified by using NIH ImageJ software version 1.44o (Wayne Rasband, National Institutes of Health, Bethesda, MD).

Cell lines and treatment with demethylating agents

FTC-133 and FTC-236 cells, kindly provided by Dr. Orlo H. Clark (UCSF Mount Zion Medical Center, San Francisco, CA), were derived from a primary human follicular thyroid carcinoma and neck lymph node metastases, respectively. These cells were maintained in DMEM/Ham's F12 (1:1) medium (Invitrogen) supplemented with 10% fetal bovine serum, 10 μ g/ml bovine insulin (Sigma-Aldrich), 1 mIU/ml bovine TSH (Sigma-Aldrich), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen). Culture media for other cell lines were described in Supplemental Table 3. The FTC-236 cell line was treated with 3 μ M 5'-aza-CdR and 10 μ M zebularine (Sigma-Aldrich). Media and reagents were changed every 3 d. Human *THRB* cDNA was cloned into pFH-IRESneo plasmid (a generous gift from R. G. Roeder, Rockefeller University, New York, NY) (25) to obtain Flag-hemagglutinin (FH)-tagged TR β cells. The preparation of FTC-236 cells stably expressing FH-TR β (FTC-236-TR β) and control cells (FTC-236-Neo) was similar to what was described for HeLa cells (26). FTC-236 cells were transfected with FH-TR β or the empty vector and selected with 200 μ g/ml G418 (Invitrogen). G418-resistant colonies expressing FH-tagged proteins were expanded for subsequent experiments. The expression of TR β protein was verified by Western blot analysis using anti-TR β C4 antibodies (24).

Cell proliferation assay

After 7 d of treatment with 3 μ M 5'-aza-CdR, 10 μ M zebularine, or vehicle, 5×10^4 cells were plated in six-well plates and cultured for 24 h. Nonattached cells were removed by gently washing twice with 2 ml PBS. After trypsin treatment and resuspension, the number of attached cells was counted every 24 h from d 1–6 by using a cell and particle counter (Beckmann Coulter, Indianapolis, IN).

Wound-healing assay

After 7 d of treatment with 5'-aza-CdR, zebularine, or vehicle, 1×10^6 cells were seeded in six-well plates. The wound was applied with a pipette tip on confluent cells, and plates were washed with PBS followed by the addition of complete culture medium. At regular intervals, a camera system with an inverted microscope visualized cell migration at $\times 100$ magnification. The migration rate was quantified by measuring the distance between the edges of wound, and the percentage of migration was determined as the ratio between migrated distance and initial distance of wound.

Immunohistochemistry of cultured FTC-236 cells

Cell pellets from 5×10^6 cells were fixed in 10% neutral-buffered formalin (Sigma-Aldrich) and subsequently embedded in paraffin. Five-micrometer-thick sections were prepared for staining. Immunohistochemistry was conducted as previously described (27). Rabbit anti-Ki-67 (1:300 dilution, NeoMarker; Thermo Scientific) was used for primary antibody.

In vivo mouse xenograft study

The National Cancer Institute Animal Care and Use Committee approved the protocols for animal care and handling in the present study. Four-week-old male athymic NCr-*nu/nu* mice were obtained from NCI-Frederick animal facility. FTC-236 cells (5×10^6 cells) in 200 μ l suspension mixture with Matrigel basement membrane matrix (BD Biosciences, San Jose, CA) were sc inoculated into the right flank of mice. Mice were randomly divided into two groups (six mice for each). Weekly 5'-aza-CdR (5 mg/kg) or vehicle treatment by ip injection started 2 d after cell inoculation. Tumor size was measured with calipers, and tumor volume was calculated as $(\text{length} \times \text{width}^2)/2$.

Statistics

Data are presented as mean \pm SE or as median/range. Student's *t* test or Mann-Whitney *U* test was used to compare continuous variables. Fisher's exact test was used for comparison of categorical variables. The pattern of relative expression of mRNA and relative methylation ratios in different groups were evaluated by examining the *P* value for trend via linear regression analysis. *P* values were two sided throughout, and *P* < 0.05 was considered statistically significant. Data were analyzed using SPSS statistics version 19.0 (SPSS Inc., Chicago, IL). GraphPad Prism version 4.0a (GraphPad Software, San Diego, CA) was used to draw graphs.

Results

The expression of the *THRB* gene is decreased in thyroid cancer and is inversely correlated with cancer progression

To understand the roles of the TR β in human DTC, we first examined the expression of the *THRB* mRNA levels in 21 DTC, eight nodular hyperplasia, eight follicular adenoma, and 11 normal thyroid tissues. Concurrently, we also determined the mRNA levels of a thyroid-specific transcription factor, the *PAX8* gene. The expression of the *PAX8* gene is known to be repressed in human thyroid cancers (28). Therefore, we used *PAX8* mRNA expression as a positive control and also for the validation of the thyroid cancer tissues. The *THRB* mRNA expression was 90% lower in cancer tissues than in normal controls (*P* = 0.0001, Fig. 1A). The *THRB* mRNA in nodular hyperplasia and follicular adenoma was higher than that in cancer tissues and was not significantly different from that in normal tissues (Fig. 1A). Consistent with reports by others (28), the expression of *PAX8* mRNA was decreased by

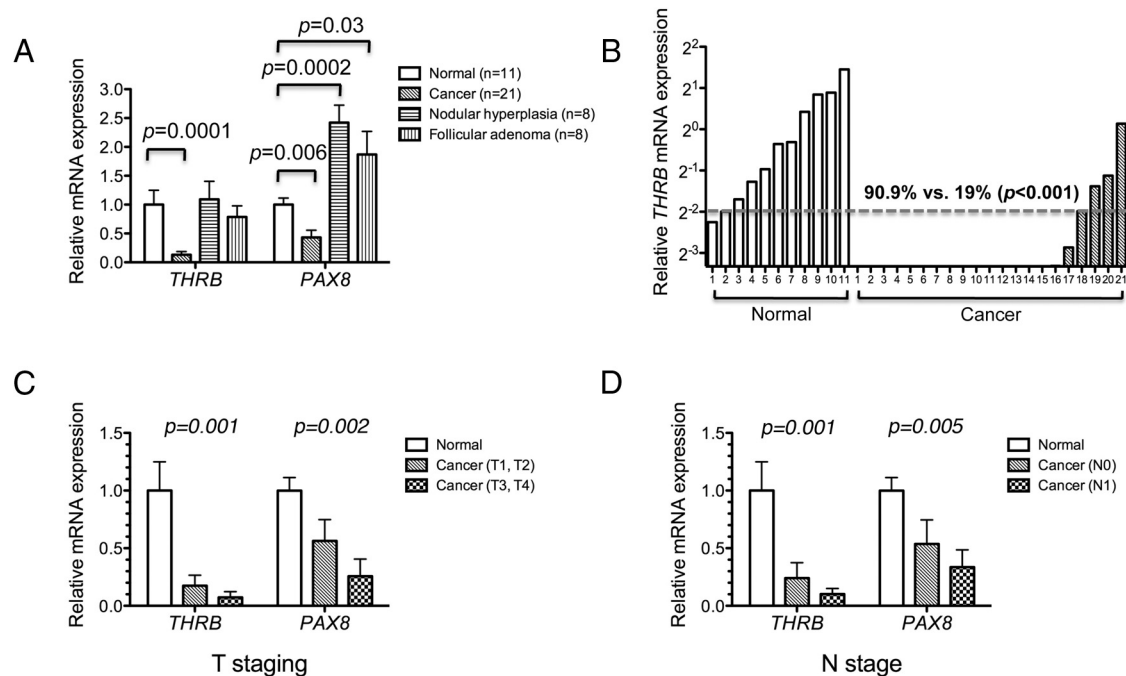


FIG. 1. Down-regulation of *THRB* gene in DTC tissues. A, Relative mRNA expression of *THRB* and *PAX8* in DTC (n = 21), nodular hyperplasia (n = 8), follicular adenoma (n = 8), and normal thyroid tissue (n = 11). The data are presented as mean \pm se and were analyzed by Student's *t* test. B, Relative *THRB* mRNA expression of individual tissue samples from DTC and normal thyroid determined by real-time RT-PCR assay. C, Relative *THRB* and *PAX8* mRNA expression according to lower (n = 12) and higher (n = 9) tumor (T) staging in DTC tissues. The relative expression of mRNA in different groups was evaluated by examining the *P* value for trend via linear regression analysis. D, Relative *THRB* and *PAX8* mRNA expression according to the absence (n = 10) or presence (n = 11) of lymph node (N) metastasis in DTC tissues. T staging and N staging were defined according to the sixth edition of the American Joint Committee on Cancer/International Union Against Cancer tumor-node-metastases (TNM) classification.

60% in cancer tissues as compared with normal controls ($P = 0.006$, Fig. 1A) but was higher than normal tissues in nodular hyperplasia and follicular adenoma ($P = 0.03$ and $P = 0.0002$, respectively). The extent of the *THRB* mRNA expression for each normal thyroid and cancer tissue is shown in Fig. 1B, indicating more normal tissues expressed *THRB* mRNA above one fourth of the mean (90.9%) than those of cancer tissues (19%; $P = 0.001$).

To correlate the clinical relevance of the *THRB* expression with the aggressiveness of DTC, we analyzed the *THRB* mRNA expression according to the subgroups of tumor (T) and lymph node (N) stage of cancer (see Supplemental Table 1). Figure 1C shows that the more advanced the thyroid cancer was, on the basis of T staging, the lower *THRB* and *PAX8* mRNAs were [normal (n = 11), T1/T2 cancer (n = 12); T3/T4 (n = 9)] according to trend analysis ($P = 0.001$ and $P = 0.002$, respectively). A similarly significant decreasing trend was detected when lymph node staging was used ($P = 0.001$; Fig. 1D). These data indicated that the expression of the *THRB* gene was lower in cancer tissue than in normal, and the decreased *THRB* expression was inversely correlated with the severity in the progression of DTC.

The expression of the *THRB* gene is repressed by hypermethylation of the *THRB* promoter in DTC

Epigenetic inactivation of the *THRB* gene through promoter hypermethylation has been reported in many human cancers (8–13). Using methylation-specific PCR, we examined the methylation status of the *THRB* promoter region in human thyroid tumors (n = 18). Figure 2A shows that the extent of *THRB* methylation was greater in thyroid cancer tissues than in normal controls. The ratio of methylated to unmethylated *THRB* (i.e. the relative *THRB* methylation ratio) was significantly increased by 4.6-fold in cancer tissues ($P = 0.01$, Fig. 2B; n = 18 for cancers and n = 7 for normal tissues). To understand the consequences of the repressed expression of the *THRB* gene by promoter methylation, we analyzed the relative *THRB* methylation ratios in normal and lower- and higher-stage cancers. As shown in Fig. 2C, the relative *THRB* methylation ratio was lower in normal tissues, higher in T1/T2 cancers and highest in T3/T4 cancer shown by T staging (P value by trend analysis = 0.012). The *THRB* methylation ratios of cancer categorized by lymph node staging also showed significant increasing trend comparing with normal tissues (P value by trend analysis = 0.016). The findings of significantly lower *THRB* meth-

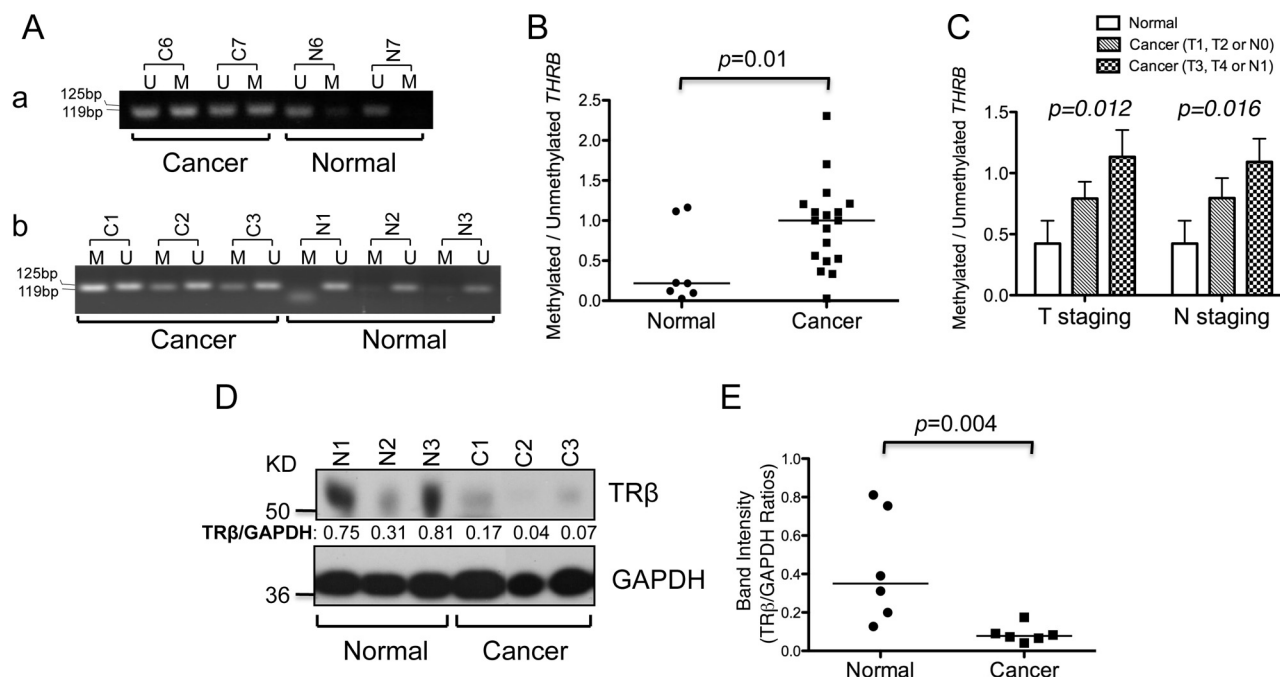


FIG. 2. *THRβ* is hypermethylated in DTC. A, Representative results of *THRβ* promoter methylation in cancer and normal thyroid tissue. The amounts of methylated (M) or unmethylated (U) *THRβ* promoter from bisulfite-treated genomic DNA of each tissue were determined by specific PCR analysis. B, The ratio of methylated to unmethylated *THRβ* promoter from normal (n = 7) and cancer tissues (n = 18). The data are presented as medians and analyzed by the Mann-Whitney U test. C, The relative *THRβ* methylation ratios of normal and DTC tissues according to lower (n = 10) and higher (n = 8) tumor (T) staging. The relative *THRβ* methylation ratios of normal and DTC tissues are shown according to lymph node (N) staging [the absence (n = 9) or presence (n = 9) of lymph node (N) metastasis]. The relative ratio of methylated and unmethylated *THRβ* in different groups was evaluated by examining the P value for trend via linear regression analysis. D, Representative results of Western blot analysis of TRβ and GAPDH as the loading control in normal and cancer tissues. E, Quantification of relative protein expression of TRβ after normalization via use of GAPDH as the loading control. The data are presented as medians and analyzed by the Mann-Whitney U test.

ylation ratios in normal tissues and higher ratios of *THRβ* methylation in aggressive disease strongly suggest that methylation of the promoter region is critical in regulating the expression of the *THRβ* gene in thyroid cancer.

We also examined the TRβ protein level by Western blot analysis in DTC tissue (n = 6) and normal controls (n = 6). Representative examples show that the TRβ protein abundance in DTC was decreased (Fig. 2D). The band intensities were quantified, and the data show that the TRβ protein levels were significantly lower (by 78%) in cancer tissues than in normal tissues ($P = 0.004$, Fig. 2E). These findings further support the notion that methylation of the *THRβ* promoter region inhibits *THRβ* expression in thyroid cancer.

The demethylating agents 5'-aza-CdR and zebularine induce the expression of the *THRβ* gene in human thyroid cancer cell lines

To elucidate the functional consequences of the silencing of the *THRβ* gene by hypermethylation, we turned to the use of human thyroid cancer cell lines. We tested five thyroid tumor cell lines and three breast cancer cell lines for detection of inactivation of the *THRβ* gene by hypermeth-

ylation. Figure 3A shows that the *THRβ* gene in T47D and MCF-7 breast cancer cells was not methylated, but it was methylated in MDA468 cells (lane 3), as previously reported (8). Increased methylation of the *THRβ* gene was detected in FTC-236 but not in four other thyroid cancer cell lines: SW1736, K-5, WRO, and FTC-133 cells (Fig. 3A, a and b). We therefore treated FTC-236 cells with the demethylating agents 5'-aza-CdR and zebularine to test whether they could demethylate the *THRβ* gene. Indeed, Fig. 3B shows that after treatment, the extent of methylation of the *THRβ* gene was decreased by 31% with 5'-aza-CdR and by 63% with zebularine (Fig. 3B, upper and lower panels). Importantly, the demethylation of the *THRβ* gene led to the increased expression of the *THRβ* mRNA by 5.6-fold ($P = 0.03$) and by 44-fold ($P < 0.001$) after treatment with 5'-aza-CdR and zebularine, respectively (Fig. 3Bb). We further showed that by Western blot analysis, TRβ protein was also detected by treatment of FTC-236 cells with 5'-aza-CdR or zebularine (Fig. 3C, upper panel). Therefore, these results indicate that the *THRβ* gene was silenced by hypermethylation and that demethylation resulted in the reexpression of the *THRβ* gene in FTC-236.

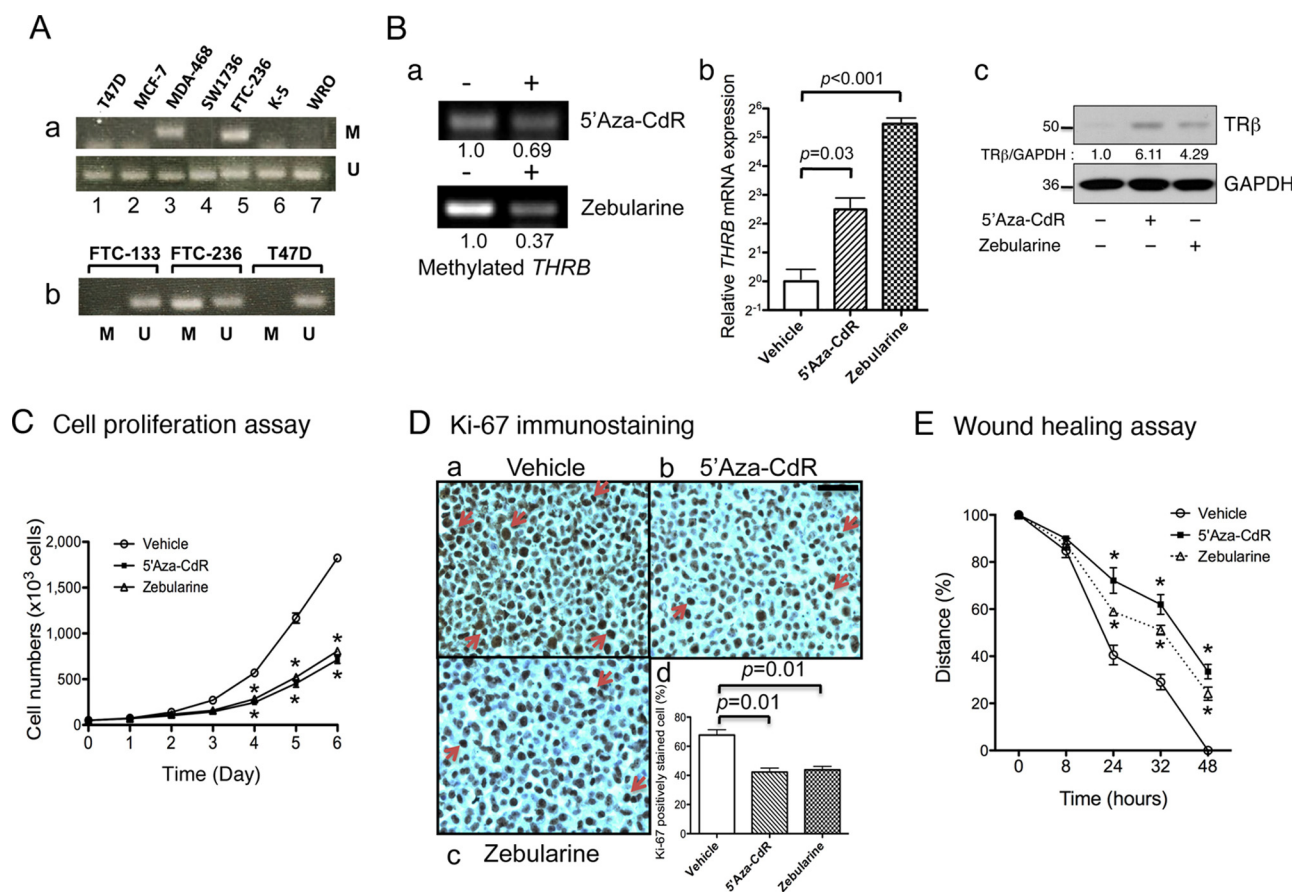


FIG. 3. Demethylation agents 5'-aza-CdR and zebularine increase *THRB* expression through reduced *THRB* promoter methylation. **A**, The amounts of methylated (M) or unmethylated (U) *THRB* promoter determined by methylation-specific PCR analysis in human breast cancer cells (T47D, MCF-7, MDA-468) and thyroid cancer cells (SW1736, FTC-236, K-5, WRO, and FTC-133). **Ba**, Methylation of *THRB* promoter was decreased after treatment with the demethylating agents, 5'-aza-CdR (upper panel) and zebularine (lower panel) in the FTC-236 human thyroid cancer cell line; **b**, relative mRNA expression of *THRB* was increased by treatment with the demethylating agents in FTC-236 cells (data presented as mean \pm SD and compared by Student's *t* test); **c**, protein abundance of TR β was increased by treatment with demethylating agents in FTC-236 cells as shown by Western blot analysis of total cell lysates with or without treatment with demethylating agents as marked (upper panel). GAPDH was used as the loading control (lower panel). **C**, Relative to vehicle treatment, cell proliferation was inhibited by treatment with the demethylating agents in FTC-236 cells (data presented as mean \pm SD). *, $P < 0.01$. **D**, Decreased Ki-67-positive cells after treatment with 5'-aza-CdR and zebularine in FTC-236 cells compared with vehicle treatment. Representative microphotographs of Ki-67 immunohistochemistry on paraffin-embedded cells after treatment with vehicle (a), 5'-aza-CdR (b), and zebularine (c). Cell proliferation index was determined by proportion of Ki-67-positive cells in FTC-236 cells (d). **E**, Relative to vehicle treatment, cell migration of FTC-236 was inhibited by treatment with demethylating agents, as determined by wound-healing assay (data presented as mean \pm SD). *, $P < 0.01$.

Demethylating agents inhibit cancer cell proliferation and migration through down-regulation of the β -catenin pathway in FTC-236 cells

The finding that the *THRB* gene in FTC-236 cells was reactivated by treatment with the demethylating agents 5'-aza-CdR and zebularine prompted us to evaluate whether the reexpressed TR β could lessen the cancer phenotype. As shown in Fig. 3C, FTC-236 cell proliferation was significantly reduced by treatment with 5'-aza-CdR or zebularine, beginning at d 3. On d 6, cell proliferation was 50% lower (Fig. 3C). Consistent with the decreased cell proliferation, Ki-67-positive cells were decreased by 37% ($P = 0.01$) and by 35% ($P = 0.01$) after treatment with 5'-aza-CdR and zebularine, respectively (Fig. 3D).

Cancer cell migration is an important process involving local cancer invasion to adjacent tissues, infiltration of the lymphatic system, and hematogenous metastasis. We therefore used a wound-healing assay to examine migration in cells treated with 5'-aza-CdR, zebularine, or vehicle (Fig. 3E). Significant decreases of cancer cell migration were observed after treatment with the demethylating agents (Fig. 3E). These data suggest that reduced methylation of the *THRB* gene by the demethylating agents 5'-aza-CdR and zebularine could be therapeutically beneficial in the treatment of thyroid cancer.

We further elucidated the molecular pathways affected by the treatment of FTC-236 cells with 5'-aza-CdR or zebularine. β -Catenin is a key regulator of the Wnt signaling pathway, which plays important roles in carcino-

genesis. Aberrant activation of β -catenin has been reported in many human cancers, including thyroid cancer (29, 30). Previously, we reported that β -catenin signaling is repressed in TR β -expressing cells but is activated in the thyroid tumors of *Thrb*^{PV/PV} mice (31). We therefore examined whether the β -catenin signaling pathway was affected after treatment of FTC-236 cells with demethylating agents. As shown in Fig. 4A, activation of the β -catenin signaling pathway was inhibited by treatment of 5'-aza-CdR or zebularine as evidenced by a decrease of p- β -catenin (serine 552) which is critical for β -catenin transcriptional activation (Fig. 4A, panel a). The quantification of the band intensities was shown in Fig. 4B (the first three bars). The attenuated β -catenin transcriptional activity was supported by reduced cyclin D1 (Fig. 4A, panel b), which is a direct target of β -catenin signaling. Figure 4B shows that the protein abundance was decreased by 50 and 35% after treatment with 5'-aza-CdR and zebularine, respectively (see the last three bars). Moreover, we found that nuclear β -catenin abundance was decreased by 72 or 78% after treatment with 5'-aza-CdR or zebularine, respectively (Fig. 4C, panel a). No changes in the β -catenin abundance in the cytoplasmic fraction were observed. However, a lower nuclear β -catenin abundance was consistent with the notion of the attenuation of β -catenin signaling. The marker protein for the nuclear fraction, PARP, was not affected and was used as loading controls (Fig. 4C, panel b). These findings identified the

down-regulation of β -catenin signaling as one of the pathways by which reexpression of TR β by treatment with 5'-aza-CdR and zebularine inhibit cancer cell proliferation. We also evaluated the protein abundance of p- β -catenin (serine 552) and cyclin D1 in cancer and normal thyroid tissue samples. Activation of the β -catenin signaling pathway was evident by higher p- β -catenin (serine 552) levels in human thyroid cancer tissues than in normal tissues (Fig. 4D, panel a). The protein abundance of cyclin D1 was also increased in human thyroid cancer samples as compared with normal controls (Fig. 4D, panel c). These findings provided additional support for the association of suppressed *THRB* expression with activation of β -catenin signaling pathway in human thyroid cancers.

TR β reexpression in FTC-236 cells reduces cancer cell proliferation and migration through down-regulation of the β -catenin pathway

To further support the idea that TR β acts as a tumor suppressor in thyroid carcinogenesis, we generated FTC-236 cells stably expressing TR β (TR β 9 and TR β 23) or only the neomycin gene as controls (Neo1 and Neo4). The expression of TR β at the protein level in FTC-236-TR β 9 and FTC-236-TR β 23 was confirmed by Western blot analysis (Fig. 5A, lanes c and d), whereas no TR β expression was observed in control Neo cells (Fig. 5, lanes a and b). The expression of TR β in FTC-236-TR β 9 and FTC-236-TR β 23 led to a decreased cell proliferation, as shown in

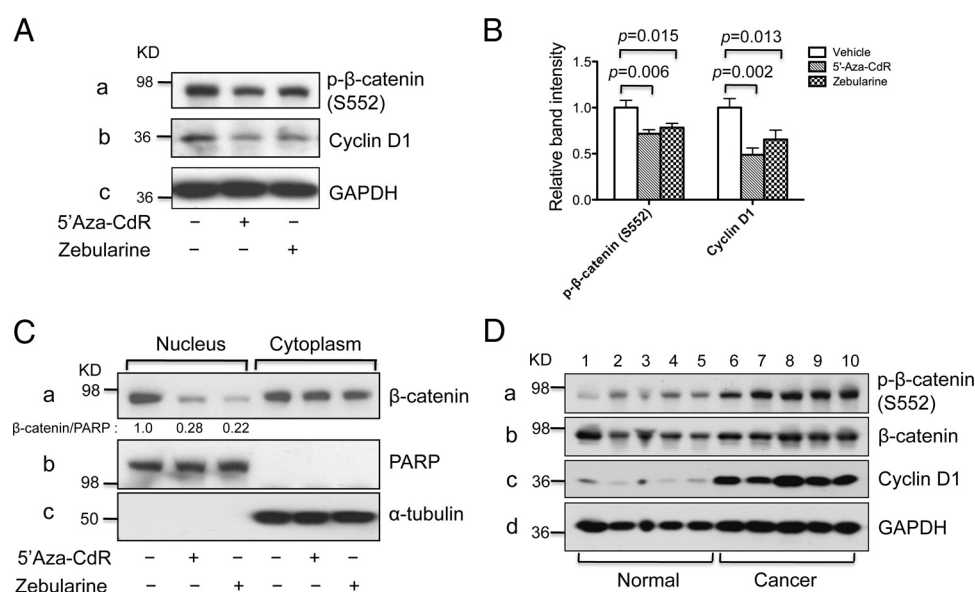


FIG. 4. Demethylating agents 5'-aza-CdR and zebularine decrease cell proliferation by attenuating β -catenin signaling in FTC-236. A, Western blot analysis of p- β -catenin (serine 552), cyclin D1, and GAPDH as the loading control in whole-cell lysates from FTC-236 cells after treatment with demethylating agents. B, Quantification of relative protein abundance of p- β -catenin (serine 552) and cyclin D1 after normalization by using GAPDH as a loading control. C, Western blot analysis of protein abundance of β -catenin in the nuclear and cytoplasmic cell fractions. PARP was used as loading control for nuclear fraction and α -tubulin was used as loading control for cytoplasmic fractions of FTC-236 cells. D, Western blot analysis of p- β -catenin (serine 552), total β -catenin, cyclin D1, and GAPDH as the loading control in human tissue lysates of normal thyroid and cancer samples.

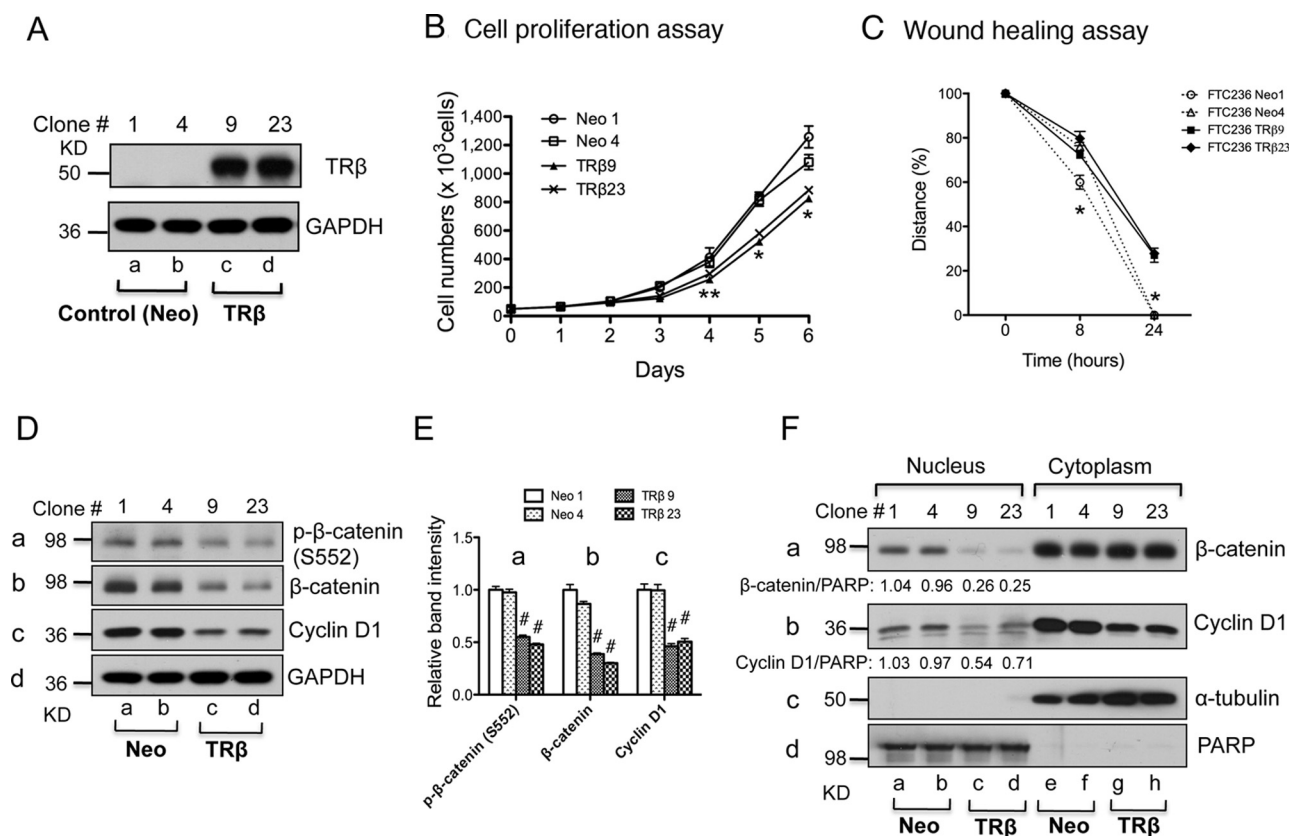


FIG. 5. Stable reexpression of TRβ in the FTC-236 thyroid cancer cells reduces cell proliferation and migration by attenuating β-catenin signaling. A, Western blot analysis of expression of TRβ in stable cells after transfection of control (Neo) or *THRB*-expressing vector in FTC-236 cells. B, Cell proliferation was lower in TRβ-expressing FTC-236 cells (TRβ9 and TRβ23) than in control FTC-236 cells (Neo1 and Neo4). Data are presented as mean ± SE. **, *P* < 0.05; *, *P* < 0.01. C, Wound-healing assay showed that reexpressing of the TRβ inhibited cell migration of FTC-236 cells. Data are presented as mean ± SE. *, *P* < 0.01. D, Western blot analysis of p-β-catenin (serine 552), total β-catenin, cyclin D1, and GAPDH in whole-cell lysates from TRβ-expressing FTC-236 cells and control FTC-236 cells. E, Quantification of relative protein abundance of p-β-catenin, total β-catenin, and cyclin D1 after normalization using GAPDH as a loading control. Data presented as mean ± SE. #, *P* < 0.001. F, Western blot analysis of protein abundance of β-catenin and cyclin D1 in the nuclear and cytoplasmic cell fractions. PARP was used as loading control for nuclear fraction, and α-tubulin was used as loading controls for cytoplasmic fraction from FTC-236 cells.

Fig. 5B. The effect of the expressed TRβ in FTC-236-TRβ9 and FTC-236-TRβ23 on cancer cell motility was evaluated by wound-healing assays. A decrease in cell motility was evident in that after 24 h, the cell migration was significantly impeded in FTC-236-TRβ9 and FTC-236-TRβ23 cells as compared with the control Neo cells (Fig. 5C). Activation of β-catenin signaling pathway was also inhibited in TRβ-expressing FTC-236 cells shown by decreased p-β-catenin (serine 552) and total β-catenin as compared with control Neo cells (Fig. 5D, panels a and b, respectively; see quantification of band intensities in Fig. 5E). The protein abundance of downstream target of β-catenin, cyclin D1, was also decreased by about 50% in TRβ-expressing FTC-236 cells as compared with control Neo cells (Fig. 5D, panel c; see quantification of band intensities in Fig. 5E). The nuclear abundance of β-catenin protein was clearly reduced, whereas no apparent changes in the cytoplasmic fraction were seen (Fig. 5F, panel a). Importantly, the protein abundance of a direct target of

β-catenin signaling, cyclin D1, was lower in both the nuclear fraction (Fig. 5F, panel b; lanes c and d vs. lanes a and b) as well as cytoplasmic fraction (Fig. 5F, panel b; lanes g and h vs. lanes e and f) than those in control Neo cells, providing direct evidence to indicate the attenuation of the β-catenin signaling. Taken together, these data indicate that TRβ could act as a tumor suppressor in thyroid carcinogenesis through down-regulation of the β-catenin pathway and that epigenetic modulation by reactivation of the silenced *THRB* gene expression could be a therapeutic target for treatment of thyroid cancer.

Demethylating agent 5'-aza-CdR inhibits growth of tumors induced by FTC-236 cancer cells in mouse xenograft models

To further validate our cell-based studies, we evaluated the effect of 5'-aza-CdR on *in vivo* mouse xenograft models. FTC-236 cells were sc inoculated into the right flanks of mice that were treated with or without 5'-aza-CdR

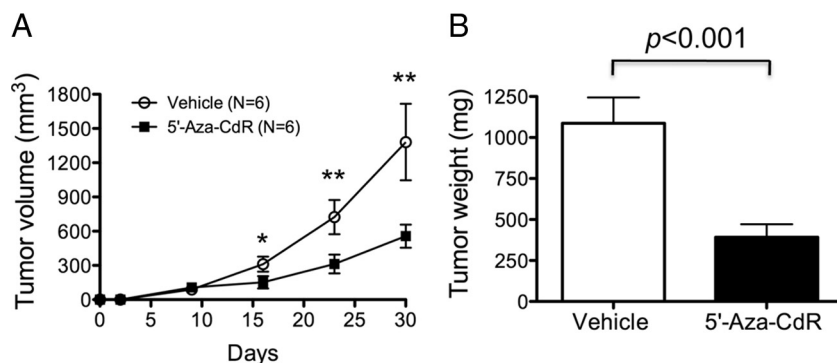


FIG. 6. Demethylating agent 5'-aza-CdR inhibits growth of tumors induced by FTC-236 cells in mouse xenograft models. A, Growth curves of FTC-236-induced tumors. FTC-236 cancer cells were sc inoculated into the right flank of athymic NCr-nu/nu mice. Mice were treated by weekly ip injection of 5'-aza-CdR (5 mg/kg; n = 6) or vehicle control (n = 6) for 30 d. Data are presented as mean \pm SE. *, $P < 0.01$; **, $P < 0.001$. B, Comparison of the weight of FTC-236 cell-induced tumors after treatment of 5'-aza-CdR (n = 6) or vehicle control (n = 6) at study endpoint. The data are represented as mean \pm SE. The differences are significant ($P < 0.001$).

(weekly 5 mg/kg body weight). As shown in Fig. 6A, tumor volume in mice treated with 5'-aza-CdR was significantly reduced as compared with vehicle-treated mice. The weights of FTC-236-induced tumors were measured at the end point (a month after cancer cell inoculation). As shown in Fig. 6B, tumor weight was significantly reduced ($\sim 75\%$) in the 5'-aza-CdR-treated group as compared with the vehicle-treated group (Fig. 6B). These findings are consistent with the cell-based studies in that demethylation of the *THRB* gene *in vivo* by 5'-aza-CdR led to suppression of tumor growth.

Discussion

The reduced expression resulting from hypermethylation, deletion, or truncation of the *THRB* gene in human cancers suggests that TR β could act as a tumor suppressor (8–13). A close association of somatic mutations of the *THRB* gene with several types of human cancer supports the hypothesis that loss of the normal suppressor functions of TR β could lead to increased cell proliferation and aberrant growth (3–6). The fact that mice deficient in total functional TRs (*Thra1^{-/-}Thrb^{-/-}* mice) or with a targeted homozygous mutation of the *Thrb* gene (*Thrb^{PV/PV}* mice) spontaneously develop metastatic thyroid carcinoma provides strong evidence of the critical roles of TRs in human cancer (7, 32).

In the present study, we first showed the correlation between reduced mRNA expression of the *THRB* gene and progression of DTC. We also found evidence showing that promoter methylation is one of the key regulatory mechanisms in the repression of *THRB* expression in DTC. Cell-based studies further showed that the demethy-

lating agents 5'-aza-CdR and zebularine induced *THRB* reexpression, leading to the inhibition of cancer cell proliferation and migration. Importantly, when TR β was stably expressed in FTC-236 cells, the cancer phenotype was markedly lessened. The cell-based findings were further validated by the observations of *in vivo* xenograft models. Thus, the present study supports the notion that TR β can act as a tumor suppressor in thyroid carcinogenesis.

DNA methylation plays critical roles in the regulation of normal physiology during embryogenesis and cell division, but this process is also involved in the initiation and progression of cancer as well as in genetic abnormalities. During the past two decades, numerous drugs

targeting DNA methylation have been developed to increase the efficacy and stability and to decrease the toxicity of cancer treatments (33). At present, 5'-aza-CdR (decitabine) is approved for the treatment of myelodysplastic syndromes (33). It is currently under investigation in a phase II clinical trial of metastatic, refractory DTC (NCT00085293). However, the toxicity and a short *in vivo* half-life (7–35 min) of 5'-aza-CdR could pose major obstacles to this drug's use in clinical settings (33, 35). Zebularine [1-(β D-ribofuranosyl)-1,2-dihydropyrimidin-2-1] is a new cytidine analog that forms a covalent complex with DNA methyltransferases, selectively inhibits human DNA methyltransferase 1 and has shown a preferential response in cancer cells (35, 36). However, little information is currently available for the effect of zebularine on thyroid cancer cells. In the present study, we showed that zebularine was as effective as 5'-aza-CdR in reactivation of the expression of the *THRB* gene, leading to decreasing cell proliferation and motility (Fig. 3). It also exhibited similar molecular actions in dampening the β -catenin signaling pathway to decrease cell proliferation. These preclinical data could be useful in paving the way for further investigations of the benefits of zebularine, a less toxic methylating agent, on the treatment of DTC.

5'-Aza-CdR has been used in the redifferentiation of thyroid carcinoma cell lines by reexpression of sodium iodide symporter, thyroid transcription factor-1, thyroglobulin, and retinoic acid receptor β 2 (21, 22). The present study shows that 5'-aza-CdR and a newer demethylating agent, zebularine, can reactivate the expression of the *THRB* gene. It is not clear whether different demethylating agents have different selectivity toward specific target genes or whether this class of demethylating agents has

general effects on most target genes. However, the present findings that reactivating the expression of the *THRB* gene by 5'-aza-CdR and zebularine lessened the cancer phenotype by decreasing cell proliferation and motility suggest that demethylating agents are promising candidates in the treatment of advanced DTC.

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