Inhibition of Tumorigenesis by the Thyroid Hormone Receptor β in Xenograft Models

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Background: Previous studies showed a close association between several types of human cancers and somatic mutations of thyroid hormone receptor β (TR β) and reduced expression of TR β due to epigenetic inactivation and/or deletion of the *THRB* gene. These observations suggest that TR β could act as a tumor suppressor in carcinogenesis. However, the mechanisms by which TR β could function to inhibit tumorigenesis are less well understood.

Methods: We used the human follicular thyroid cancer cell lines (FTC-133 and FTC-236 cells) to elucidate how functional expression of the *THRB* gene could affect tumorigenesis. We stably expressed the *THRB* gene in FTC cells and evaluated the effects of the expressed TR β on cancer cell proliferation, migration, and tumor growth in cell-based studies and xenograft models.

Results: Expression of TR β in FTC-133 cells, as compared with control FTC cells without TR β , reduced cancer cell proliferation and impeded migration of tumor cells through inhibition of the AKT-mTOR-p70 S6K pathway. TR β expression in FTC-133 and FTC-236 led to less tumor growth in xenograft models. Importantly, new vessel formation was significantly suppressed in tumors induced by FTC cells expressing TR β compared with control FTC cells without TR β . The decrease in vessel formation was mediated by the downregulation of vascular endothelial growth factor in FTC cells expressing TR β .

Conclusions: These findings indicate that TR β acts as a tumor suppressor through downregulation of the AKT-mTOR-p70 S6K pathway and decreased vascular endothelial growth factor expression in FTC cells. The present results raise the possibility that TR β could be considered as a potential therapeutic target for thyroid cancer.

Introduction

THYROID HORMONE RECEPTORS (TRs) are ligand-dependent transcription factors that mediate the biological actions of the thyroid hormone (triiodothyronine, T₃) in development, growth, and differentiation and maintenance of metabolic homeostasis (1,2). Two human TR genes, *THRA* and *THRB*, located on different chromosomes, encode three major T₃-binding TR isoforms. Although there has been significant progress in the understanding of molecular mechanisms by which TRs act to maintain normal physiological cellular functions, their role in human carcinogenesis is less well understood. Evidence suggesting that TRs could be involved in human carcinogenesis came from the discoveries of mutated TRs in human hepatocellular carcinoma, renal clear cell carcinoma, breast cancer, pituitary tumors, and thyroid cancer (3–10). The loss of normal expression of the *THRB* gene located on chromosome 3p due to truncation or deletion was

also observed in many malignancies, including lung, melanoma, breast, head and neck, renal cell, uterine cervical, ovarian, and testicular tumors (10–13). Decreased *THRB* expression by promoter hypermethylation has been reported in human breast cancer, lung cancer, and thyroid carcinoma (14–16). These findings raise the possibility that TR β could act as a tumor suppressor in human cancers.

To test the hypothesis that the loss of normal functions of TR β contributes to thyroid cancer development and progression, several genetic engineered mice have been developed (10). The *Thrb*^{PV/PV} mouse, harboring a knock-in dominant negative mutation, known as PV, in the *Thrb* gene locus, spontaneously develops metastatic follicular thyroid carcinoma (FTC) similar to human FTC (17). As *Thrb*^{PV/PV} mice age, pathological changes in thyroid glands progress from hyperplasia to capsular invasion, vascular invasion, anaplasia, and distant metastasis (18). Moreover, another mouse model with the loss of total functional TRs (*Thra1*^{-/-}

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Thrb^{-/-} mice) also leads to spontaneously developed FTC with a similar pathological progression (19). These findings indicate that the loss of normal functional TR β by deletion or mutation contributes to thyroid carcinogenesis in mice. However, the functional consequences and the molecular actions of the re-expression of the silenced *THRB* gene in human thyroid cancer cells have not been well studied.

In the present study, we adopted the gain-of-function approach by expression of the *THRB* gene in human FTC cells, FTC-133 and FTC-236. These two cell lines were derived from the same patient with FTC, but were from the primary thyroid lesion and a neck lymph node metastasis, respectively (20). We stably expressed the *THRB* gene in these two cell lines and evaluated the effects of the expression of TR β on cell proliferation and migration and tumor development in xenograft models. We found inhibition of cancer cell proliferation and impediment of migration by the expression of the *THRB* gene in FTC cells. These inhibitory responses were mediated by downregulation of the AKT-mTOR-p70 S6K signaling pathway due to the expression of TR β . Moreover, in xenograft models, the expression of TR β in FTC-133 and FTC-236 significantly reduced tumor growth and inhibited generation of new blood vessels in tumors. Further studies indicated that the decreased angiogenesis was mediated by downregulation of vascular endothelial growth factor (VEGF) by the expression TR β in FTC cells. Thus, TR β could act as a tumor suppressor in thyroid carcinomas by modulation of cancer angiogenesis.

Materials and Methods

Cell lines

FTC-133 and FTC-236 cells (provided by Orlo H. Clark, MD, San Francisco) were obtained from a primary human FTC and a metastatic lymph node, respectively. These cells were maintained in Dulbecco's modified Eagle's medium/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 thyrotropin (Sigma Aldrich), 100 units/mL penicillin, and 100 μ g/mL streptomycin (Invitrogen), as previously described (21). The human *THRB* cDNA encoding TR β 1 subtype was cloned into pFH-IRESneo plasmid (a generous gift from R.G. Roeder, Rockefeller University, New York, NY) (22) to obtain Flag-hemagglutinin-tagged (FH)-TR β 1 cells. The preparation of FTC cells stably expressing FH-TR β 1 and control cells harboring the empty vector was similar to steps described for HeLa cells (23). The FTC-133 and FTC-236 cells expressing FH-TR β 1 were designated FTC-TR β and the control cells expressing the Neo only were designated FTC-Neo cells. Cells were selected with 200 μ g/mL G418 (Invitrogen) after transfection with expression plasmids encoding TR β 1 or the Neo. G418-resistant colonies expressing FH-tagged proteins were expanded for subsequent experiments. The expression of TR β 1 protein was verified by Western blot analysis using anti-TR β 1 C4 antibodies (24).

Cell proliferation assay and wound healing assay

Cell proliferation assay was done as previously described (21). Briefly, 5×10^4 cells were cultured for 24 hours in a 6-well plate, and nonattached cells were removed by gently washing twice with 2 mL of phosphate-buffered saline. The number of

attached cells was counted every 24 hours by using a cell and particle counter (Beckmann Coulter). To evaluate the effect of T3 on cell proliferation, cells were cultured in the medium containing 10% thyroid hormone-deficient bovine serum (Td medium) for 24 hours. Cells were re-seeded in 6-well plates at a density of 5×10^4 cells/well in Td medium (day 0). Cells were further cultured in the medium with T3 (100 nM) or without T3, and cells were counted each day for 6 days.

Wound healing assay was carried out as previously described with some modifications (21). The wound was applied with a pipette tip on the confluent cells, and nonattached cells were removed by washing with phosphate-buffered saline followed by addition of a complete culture medium. We visualized cell migration with an inverted microscope at $\times 100$ magnification at baseline, 8 hours, and 24 hours. The cell migration rate was quantified by measuring the distance between the edges of the wound, and the percentage of migration was determined as the ratio between migrated distance and initial distance of the wound.

To evaluate the T3 effect, a similar protocol was used, except that cells were seeded at 1,000,000 per well of a 6-well plate in Td medium for 24 hours. Cells were further cultured in the medium with T3 (100 nM) or without T3, and the wound healing assay was performed as described above.

Western blot analysis

Preparation of whole-cell lysates from FTC-133 and FTC-236 cells was carried out as previously described (25). The protein samples (30 μ g of lysates from FTC-133 and FTC-236 cells) were loaded and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, the protein was electrotransferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). Anti-TR β C4 antibodies were used for detection of TR β expression. The antibodies against phosphorylated (p-)retinoblastoma (Rb) (1:500 dilution), total Rb (1:250 dilution; C-15, Cat# SC-50; Santa Cruz), p-AKT (1:1000 dilution), total AKT (1:1000 dilution), p-p70 S6K (1:1000 dilution), total p70 S6K (1:1000 dilution), p-eIF4B (1:1000 dilution), total eIF4B (1:1000 dilution), p-GSK3 β (1:1000 dilution), total GSK3 β (1:1000 dilution), p-4EBP1 (1:1000 dilution), total 4EBP1 (1:1000 dilution), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000 dilution) were purchased from Cell Signaling Technology. Antibodies against cyclin B1 (1:200 dilution), cyclin D1 (1:300 dilution), cyclin E (1:200 dilution), and VEGF (1:200 dilution) were purchased from Santa Cruz Biotechnology. The blots were stripped with Re-Blot Plus (Chemicon) and re-probed with antibodies against GAPDH and poly(ADP-ribose) polymerase. Band intensities were quantified by using NIH Image software (ImageJ 1.44o; Wayne Rasband, NIH, Bethesda, MD).

In vivo mouse xenograft study

The National Cancer Institute Animal Care and Use Committee approved the protocols for animal care and handling in this study. Four-week-old male athymic NCr-nu/nu mice were obtained from the NCI-Frederick animal facility. FTC-133 cells or FTC-236 cells (5×10^6 cells) in 200 μ L of suspension mixture with Matrigel (BD Biosciences) were subcutaneously inoculated into the right flank of mice, similar to our previous report (21). Mice were randomly divided into each group (six

mice per group). Tumor size was measured with calipers, and tumor volume (V) was calculated as $(\text{length} \times \text{width}^2)/2$. Tumor weight was determined after dissection of tumor tissues from euthanized mice at the study endpoint.

Histopathological analysis

Tissues from xenografted tumor were fixed in 10% neutral buffered formalin (Sigma-Aldrich) and subsequently embedded in paraffin. Five-micrometer-thick sections were prepared and stained with hematoxylin and eosin. Immunohistochemistry was performed as previously described with some modification using Vectastain ABC kit (Vector Laboratories) (26). A rabbit anti-CD31 antibody (1:50 dilution; Abcam) was used as primary antibody.

Statistical analysis

Data are presented as mean $\pm$ SE or as median/range. One-way analysis of variance with Tukey's *post-hoc* test was used to compare continuous variables between groups. Two-way analysis of variance with Bonferroni's *post-hoc* test was used to compare cell proliferation and cell migration according to the time and groups. p -Values were two-sided throughout, and $p < 0.05$ was considered statistically significant. Data were analyzed using GraphPad Prism 4.0a (GraphPad Software).

Results

TR β expression in FTC-133 cells inhibits cancer cell proliferation and migration

To elucidate the functional consequences of re-expression of TR β in thyroid cancer cells, we generated FTC-133 cells stably expressing TR β (TR β 8 and TR β 13) or only the neomycin gene as controls (Neo3 and Neo5). The protein abundance of TR β in FTC-133-TR β 8 and FTC-133-TR β 13 was confirmed by Western blot analysis (Fig. 1A), whereas no TR β was detected in control Neo cells (Fig. 1A). When culturing in the medium containing 10% regular fetal bovine serum, cancer cell proliferation was clearly less in FTC-133 cells expressing TR β (TR β 8 and TR β 13) than in control FTC-133 cells ($p < 0.001$ and $p < 0.001$, respectively; Neo3 and Neo5; Fig. 1B-i). No significant differences in proliferation rates between two TR β clones (TR β 8 and TR β 13) were observed (Fig. 1B-i). We further evaluated the effect of T3 on the cell proliferation of TR β 8 cells and Neo cells by first culturing cells in the medium containing Td serum followed by culturing the cells in the Td medium with or without T3. As shown in Figure 1B-ii, no effect of T3 was observed on the proliferation of Neo cells that did not express TR β . In contrast, T3 further potentiated the inhibitory effect on the proliferation of FTC133 cells expressing TR β .

The effect of TR β expression in FTC-133 cells on cancer cell motility was evaluated by wound healing assays. When the wound healing assays were conducted in cells cultured in the medium containing 10% regular fetal bovine serum, the cell migration was markedly impeded in FTC-133 cells expressing TR β (TR β 8 and TR β 13 clones) compared with the control Neo FTC-133 cells (Fig. 1C-i). Quantitative analysis showed that the migration was significantly inhibited ($p < 0.001$ and $p < 0.001$, respectively (Fig. 1C-ii). We further evaluated the

effect of T3 on migration of TR β 8 cells and Neo cells by first culturing cells in the medium containing Td serum followed by culturing the cells in the Td medium with or without T3. As shown in Figure 1D-i, no significant effect of T3 was observed on the proliferation of Neo cells. In contrast, T3 further impeded the migration of FTC133 cells expressing TR β (Fig. 1D-i). Quantitative analysis showed that the migration was further impeded by T3 in FTC133 cells expressing TR β ($p < 0.001$ and $p < 0.001$, respectively (Fig. 1D-ii).

We further examined the abundance and activity of key regulators involved in cell cycle progression in thyroid cancer cells. The level of p-Rb represents the G1-S cell cycle progression because unphosphorylated Rb acts as a negative regulator of the G1-S phase. Western blot analysis shows that the protein abundance of p-Rb (S780) was clearly less in FTC-133 cells expressing TR β (TR β 8 and TR β 13) than in control FTC-133 cells (Fig. 2A-i) without apparent changes in the protein abundance of total Rb (Fig. 2A-ii). Figure 2A also shows that the protein levels of cyclin E, cyclin B1, and cyclin D1 were lower in FTC-133 cells expressing TR β than in control FTC-133 cells (Fig. 2A-iii-v). The band intensities were quantified, and the results are shown in Figure 2B, indicating the ~50% decrease in p-Rb/total Rb ratios (data group 1) and 75%, 80%, and 25% reduction in the protein abundance of cyclin E (data group 2), cyclin B1 (data group 3), and cyclin D1 (data group 4), respectively. These findings suggested that TR β expression could suppress thyroid cancer cell proliferation by delaying cell cycle progression.

AKT is a central player in the signal transduction pathways activated in response to many growth factors, hormones, cytokines, and nutrients. Via several downstream phosphorylation cascades, it controls a myriad of cellular functions, including proliferation and survival, metabolism, angiogenesis, and motility. Studies indicate that aberrant AKT activation is common in a variety of human cancers, including thyroid cancer (27,28). Our previous studies revealed that increased AKT activities contribute to thyroid carcinogenesis in the *Thrb*^{PV/PV} thyroid cancer mouse model (29). Therefore, we evaluated whether AKT activity was altered via TR β re-expression by focusing on AKT-mTOR- p70 S6K signaling in FTC-133 thyroid cancer cells. As shown in Figure 2C-i, the protein level of p-AKT was decreased in TR β -expressing FTC-133 cells (TR β 8 and TR β 13) compared with control FTC-133 cells (Fig. 2C-i). No apparent changes in the total AKT level were observed (Fig. 2C-ii). The level of p-p70 S6K (T421/S424), the downstream target of AKT-mTOR signaling, was lower in FTC-133 cells expressing TR β than in control FTC-133 cells (Fig. 2C-iii). Consistent with these findings, the protein abundance of p-eIF4B (S422), the downstream target of p70 S6K, was also lower in TR β -expressing FTC-133 cells than in control FTC-133 cells (Fig. 2C-v). Even though there was no significant change in the protein abundance of p-GSK3 β and total GSK3 β (Fig. 2C-vii,viii), the protein abundance of p-4EBP1 (T70) was significantly decreased in FTC-133 cells expressing TR β without changes in total 4EBP1 protein levels (Fig. 2C-ix,x). Figure 2D shows the relative ratios of p-AKT/total AKT, p-p70 S6K/total p70 S6K, p-eIF4B/total eIF4B, p-GSK3 β /total GSK3 β , and p-4EBP1/total 4EBP1. These ratios were obtained after normalization of the band intensities against GAPDH signals shown in Figure 2C. These ratios indicated significant attenuation of the AKT-p70 S6K-p-4EBP1 pathway by re-expression of TR β in FTC-133 cells.

FIG. 1. Stable expression of TR β in FTC-133 thyroid cancer cells reduces cell proliferation and migration. **(A)** Western blot analysis of expression of TR β in stable cells after transfection of control (Neo) or *THR β* -expressing vector in FTC-133 cells. **(B-i)** Cell proliferation was lower in TR β -expressing FTC-133 cells (TR β 8 and TR β 13) than in control FTC-133 cells (Neo3 and Neo5). Data are presented as mean $\pm$ SE, and analysis between each stable clone was done by two-way ANOVA with Bonferroni's *post-hoc* test. **(B-ii)** Effect of T3 on the proliferation of Neo and FTC-133 cells expressing TR β . The proliferation assay was carried out as described in **Materials and Methods**. Data are presented as mean $\pm$ SE, and analysis between each stable clone was done by using two-way ANOVA with Bonferroni's *post-hoc* test. **(C-i)** Representative cell pictures of wound healing assay in TR β -expressing FTC-133 cells (TR β 8 and TR β 13) and in control FTC-133 cells (Neo3 and Neo5). **(C-ii)** Cell migration was inhibited in TR β -expressing FTC-133 cells (TR β 8 and TR β 13) compared with control FTC-133 cells (Neo3 and Neo5) in wound healing assay. **(D-i)** Representative cell pictures of wound healing assay in TR β -expressing FTC-133 cells (TR β 8) and in control FTC-133 cells (Neo3 cells) in the presence or absence of T3 as marked. **(D-ii)** Cell migration of FTC-133 cells expressing TR β was further impeded by T3. Data are presented as mean $\pm$ SE, and analysis between each stable clone was done by two-way ANOVA with Bonferroni's *post-hoc* test. ANOVA, analysis of variance; NS, not significant; T3, triiodothyronine; TR β , thyroid hormone receptor β .

TR β expression inhibits growth of tumors induced by FTC-133 and FTC-236 cells in mouse xenograft models

To further validate the findings of the cell-based studies described above, we evaluated the effects of the expression of TR β on tumorigenesis using *in vivo* mouse xenograft models. FTC-133 cells expressing TR β (TR β 8 and TR β 13) and control

cells (Neo3 and Neo5) were subcutaneously inoculated into the right flanks of athymic nude mice. As shown in Figure 3A, the volume of tumors induced by FTC-133-TR β 8 and FTC-133-TR β 13 was significantly less than that of tumors induced by control FTC-133 (Neo3 and Neo5) cells ($p < 0.001$ and $p < 0.001$, respectively). The weights of FTC-133-induced tumors were measured at the endpoint (4 weeks after cancer cell inoculation). The weight of tumors induced by FTC-133-TR β 8

FIG. 2. Stable expression of TR β in FTC-133 thyroid cancer cells attenuates the phosphoinositide 3 kinase (PI3K)-AKT signaling pathway. **(A)** Western blot analysis of phosphorylated (p-) Rb (S780), total Rb, cyclin E, cyclin B1, and cyclin D1, and GAPDH as a loading control in whole cell lysates from TR β -expressing FTC 133 cells (TR β 8 and TR β 13) and control FTC-133 cells (Neo3 and Neo5). **(B)** Quantification of relative protein abundance of p-Rb/total Rb, cyclin E, cyclin B1, and cyclin D1 after normalization using GAPDH as a loading control. Data are presented as mean $\pm$ SE and analyzed by one-way ANOVA with Tukey's *post-hoc* test. **(C)** Western blot analysis of (i) p-AKT (S473), (ii) total AKT, (iii) p-p70 S6K, (iv) total p70 S6K, (v) p-eIF4B (S422), (vi) total eIF4B, (vii) p-GSK3 β (S9), (viii) total GSK3 β , (ix) p-4EBP1 (T70), and (x) total 4EBP1, with (xi) GAPDH as a loading control in whole cell lysates from TR β -expressing FTC-133 cells and control FTC-133 cells. **(D)** Quantification of the relative ratio of p-AKT to total AKT and p-total protein to total protein abundance in p70 S6K, ERK, eIF4B, GSK3 β , and 4EBP1 after normalization using GAPDH as a loading control. Data are presented as mean $\pm$ SE and analyzed by one-way ANOVA with Tukey's *post-hoc* test. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

and FTC-133-TR β 13 was also significantly less than that of control tumors ($p < 0.001$ and $p < 0.001$, respectively; Fig. 3B).

We also inoculated FTC-236 cells in athymic nude mice and evaluated the effects of the expression of TR β tumor growth. FTC-236 cells were derived from neck lymph node metastases of the same patient from whom FTC-133 cells were derived using the primary tumor (20). Previous studies showed that FTC-236 cells had a more invasive phenotype and were less responsive to thyrotropin than FTC-133 cells (20,30). In our previous study, we showed that the *THRB* gene was inactivated by promoter hypermethylation in FTC-236 cells, and re-activation of the *THRB* gene led to a less aggressive phenotype in an *in vitro* study (21). We, therefore, examined the *in vivo* effect of TR β expression in FTC-236 cells to confirm the effect of TR β expression and to ascertain whether common signaling pathways inhibited tumor growth. Figure 3C shows that, indeed, the volume of tumors induced by FTC-236 cells expressing TR β (FTC-236-TR β 9 and FTC-236-TR β 23) was also significantly lower than that of control FTC-236-Neo4-induced tumors ($p < 0.001$ and $p < 0.001$, respectively). At the study endpoint, the weight of tumors induced by FTC-236 cells expressing in TR β was significantly lower than that of FTC-236-Neo4 ($p < 0.001$ and $p < 0.001$, respectively; Fig. 3D). These findings confirmed the tumor-suppressing effect of TR β

in vivo, not only in FTC-133, but also in FTC-236 thyroid cancer cells.

TR β expression reduces tumor growth of thyroid cancer cells by inhibiting new vessel formation

To understand how TR β expression inhibits *in vivo* tumor growth, we evaluated the pathohistological features of tumors induced by FTC-133 cells without the expression of TR β (Fig. 4A-i) or with it (Fig. 4A-ii). Hematoxylin and eosin-stained cells revealed the presence of undifferentiated cancer cells with brisk mitotic activity and with minimal apoptosis (Fig. 4A-i, ii). There was no major apparent histological difference in tumor cells induced by FTC-133 cells with or without TR β . However, it is important to note that the number of vascular structures clearly visible in tumors induced by control FTC-133 cells (indicated by arrows in Fig. 4A-i) was decreased in tumors induced by TR β -expressing FTC-133 cells (Fig. 4A-ii).

New vessel formation is known to be a critical factor for tumor growth, and an antiangiogenesis approach has been proposed as an important cancer therapy (31). Therefore, we evaluated the extent of new vessel formation in FTC-133 cell-induced tumors by immunostaining the endothelial marker,

FIG. 3. TR β expression inhibits growth of tumors induced by FTC-133 cells and FTC-236 cells in mouse xenograft models. **(A)** Growth curves of FTC-133-induced tumors in mouse xenograft. FTC-133 cancer cells were subcutaneously inoculated into the right flank of athymic NCr-nu/nu mice ($n=6$ in each group). Data are presented as mean $\pm$ SE. The difference between tumor growth in TR β -expressing cells (TR β 8 and TR β 13) and control cells (Neo3 and Neo5) was analyzed by two-way ANOVA with Bonferroni's *post-hoc* test. **(B)** Comparison of the weights of FTC-133-induced tumors ($n=6$ in each group). Data are presented as mean $\pm$ SE. The difference in tumor weight between each of the groups was analyzed by one-way ANOVA with Tukey's *post-hoc* test. **(C)** Growth curves of FTC-236-induced tumors in mouse xenograft. FTC-236 cancer cells were subcutaneously inoculated into the right flank of athymic NCr-nu/nu mice ($n=6$ in each group). Data are presented as mean $\pm$ SE. The difference between tumor growth in TR β -expressing cells (TR β 9 and TR β 23) and control cells (Neo4) was analyzed by two-way ANOVA with Bonferroni's *post-hoc* test. **(D)** Comparison of the weight of FTC-236-induced tumors ($n=6$ in each group). Data are presented as mean $\pm$ SE. The difference in tumor weight between each of the groups was analyzed by one-way ANOVA with Tukey's *post-hoc* test.

CD31. The expression of CD31 in TR β -expressing FTC-133 cell-induced tumors (indicated by arrows in Fig. 4D-i, ii and Fig. 4E-i, ii [high magnification] and in Fig. 4D-iii, iv and Fig. 4E-iii, iv [low magnification]) was clearly less than with control FTC-133 tumors (indicated by arrows in Fig. 4B-i, ii and Fig. 4C-i, ii [high magnification] and in Fig. 4B-iii, iv and Fig. 4C-iii, iv [low magnification]). The negative controls are shown in Figure 4F for the control Neo cells and FTC-133 cells expressing TR β 8 (Fig. 4F-i and F-ii, respectively). These findings suggested that TR β expression reduces tumor growth in FTC-133 thyroid cancer cells by inhibiting new vessel formation.

TR β expression inhibits expression of VEGF in FTC-133 and FTC-236 cells

VEGF is a main regulator of tumor-induced angiogenesis and acts on vascular endothelial cells to induce vascular permeability and to reprogram gene expression, leading to endothelial cell proliferation and migration *in vitro*, and to generation of new blood vessels *in vivo* (31–33). Therefore, we

examined the level of VEGF in TR β -expressing cells and control cells. As shown in Figure 5A, the protein abundance of VEGF was lower in FTC-133-TR β 8 and FTC-133-TR β 13 cells than in control FTC-133 cells (Neo3 and Neo5). The quantification of the band intensities shows that the protein abundance of VEGF was decreased by about 50% in TR β -expressing FTC-133 cells (Fig. 5B). Consistently, we also found that the protein abundance of VEGF was lower in FTC-236-TR β 9 and FTC-236-TR β 23 cells than in control FTC-236 cells (Neo1 and Neo4; Fig. 5C). The quantification of the band intensities is shown in Figure 5D. These findings revealed the downregulation of VEGF expression by TR β to be an important mechanism by which TR β expression in thyroid cancer cells inhibits new vessel formation.

Discussion

Previous reports indicating a close association of reduced expression and somatic mutations of the *THRB* gene with several human cancers support the hypothesis that TR β could

FIG. 4. TR β expression inhibits neovascularization of tumors induced by FTC-133 cells in a mouse xenograft model. (A) Representative microphotography of H&E staining on tumors induced by TR β -expressing FTC-133 cells (ii) and control FTC-133 cells (i). The number of vascular structures (arrows) in tumors was lower in TR β -expressing FTC-133 tumors (D, E) than in control FTC-133 tumors (B, C). Representative CD31 IHC staining of tumors induced by control FTC-133 Neo cells (B and C for clones Neo3 and 5, respectively; each duplicates). Representative CD31 IHC staining of tumors induced by TR β -expressing FTC-133 cells (D and E for clones TR β 8 and TR β 13, respectively; each duplicate). Panels with high magnification ($\times 400$; i, ii) and medium magnification ($\times 100$; iii, iv) are marked. Negative controls (F-i, F-ii) shown for Neo3 and TR β 8, respectively, are as marked. H&E, hematoxylin and eosin; IHC, immunohistochemical. Color images available online at www.liebertpub.com/thy

act as a tumor suppressor (3–10). In line with these studies, we have recently shown a significant correlation of reduced *THRB* gene expression with more advanced cancer stage in human differentiated thyroid cancers (21). Direct *in vivo* evidence to support the tumor suppressor role of TR β in thyroid

cancer came from the observations that mice deficient of all functional TRs (*Thra*^{-/-}*Thrb*^{-/-} mice) or with a targeted homozygous mutation of the *Thrb* gene (*Thrb*^{PV/PV} mice) spontaneously develop metastatic thyroid cancer similar to human cancer (17,19).

FIG. 5. TR β expression decreases protein abundance of VEGF in FTC-133 and FTC-236 thyroid cancer cells. **(A)** Western blot analysis of VEGF and GAPDH as a loading control in whole cell lysates from TR β -expressing FTC-133 cells (TR β 8 and TR β 13) and control FTC-133 cells (Neo3 and Neo5). **(B)** Quantification of relative protein abundance of VEGF in FTC-133 cells after normalization using GAPDH as a loading control. Data are presented as mean $\pm$ SE and analyzed by one-way ANOVA with Tukey's *post-hoc* test. **(C)** Western blot analysis of VEGF and GAPDH as a loading control in whole cell lysates from TR β -expressing FTC-236 cells (TR β 9 and TR β 23) and control FTC-236 cells (Neo1 and Neo4). **(D)** Quantification of relative protein abundance of VEGF in FTC-236 cells after normalization using GAPDH as a loading control. Data are presented as mean $\pm$ SE and analyzed by one-way ANOVA with Tukey's *post-hoc* test. VEGF, vascular endothelial growth factor.

In the present study, we adopted the gain-of-function approach by expression of TR β in human thyroid cancer cells. The expression of TR β in FTC cells led to less tumor cell proliferation and impeded the migration of cancer cells. Moreover, in addition to these cell-based results, we showed that the growth of tumor induced by TR β -expressing FTC cells was inhibited in *in vivo* xenograft models. Importantly, we found that new vessel formation, critical for carcinogenesis and cancer progression, was suppressed in tumors induced by TR β -expressing FTC cells. The expression of a critical growth factor that stimulates angiogenesis, VEGF, was suppressed in TR β -expressing FTC cells. Therefore, the present study provides additional evidence to show that TR β acts as a tumor suppressor in thyroid carcinogenesis.

Tumor angiogenesis is an essential process for blood supply to support tumor growth and metastasis (34). To obtain this blood supply, tumor cells can tilt the balance toward stimulatory angiogenic factors to drive vascular growth by attracting and activating cells from within the microenvironment of the tumor (34). VEGF is one of the essential angiogenic factors needed to induce activation of endothelial cells, and its expression is commonly elevated in most types of human cancer cells (35). Recently, many kinds of anti-angiogenic agents targeting VEGF signaling pathways have

been developed and are in clinical use. In the present study, we found that tumor growth induced by TR β -expressing FTC cells was decreased through inhibition of tumor angiogenesis. The expression of endothelial marker CD31 was clearly reduced in tumors induced by TR β -expressing FTC cells (Fig. 4D, E). Currently, the molecular mechanisms by which TRs act to affect tumor angiogenesis are not known. In models of thyroid hormone-induced cardiac hypertrophy, thyroid hormone could enhance angiogenesis by engaging in crosstalk with the VEGF signaling, and a derivative of thyroxine, tetraiodothyroacetic acid (tetrac), was shown to inhibit vascular budding in response to VEGF (35). The present findings that the expression of VEGF was decreased by expression of TR β in FTC cells are consistent with the observations of reduced new vessel formation in xenografted tumors. Clearly, further investigation is needed to elucidate the role of TR β in tumor angiogenesis.

It was reported that FTC-133 cells have hemizygous *PTEN* allele deletion and a splice variant IVS4-19G $\rightarrow$ A in the remaining allele (36). The decreased *PTEN* mRNA and protein levels result in the overactivation of the AKT-mTOR-p70S6K signaling pathway in FTC-133 cells. The present studies showed that the overactivated AKT-mTOR-p70S6K pathway was attenuated by TR β expressed in FTC-133 cells (Fig. 2). Aberrant activation of the AKT-mTOR-p70S6K pathways, as well as activated VEGF/receptors signaling, has been described in both thyroid cancer mouse models of *Thra1*^{-/-} *Thrb*^{PV/PV} mice and *Thrb*^{PV/PV} mice (19,29). Our previous studies also showed that treatment of *Thrb*^{PV/PV} mice with a PI3K inhibitor, LY294002, effectively delays thyroid tumor progression and inhibits distant metastasis (37). We also reported that another mouse model of follicular thyroid cancer, *Thrb*^{PV/PV} *Pten*^{+/-} mice, displayed overactivation of the AKT-mTOR-p70S6K signaling pathway (38). Treatment of *Thrb*^{PV/PV} *Pten*^{+/-} mice with a specific mTOR inhibitor (RAD001) reduced thyroid tumor growth (39). The findings from these mouse models showed that the loss of normal functions of wild-type TR β due to gene knockout (*Thra1*^{-/-} *Thrb*^{-/-} mice) or mutations (*Thrb*^{PV/PV} mice and its derived strain *Thrb*^{PV/PV} *Pten*^{+/-} mice) promotes thyroid carcinogenesis. In line with the findings from animal models, the present study shows that re-gaining of TR β functions inhibits tumor development in xenograft models, at least in part, via attenuation of the AKT-mTOR-p70S6K signaling pathway. Interestingly, TR β , when overexpressed in FTC-236 cells, acts to attenuate β -catenin signaling to decrease cell proliferation (21). However, in contrast to FTC-236 cells, we did not find that the re-expressed TR β in FTC-133 cells affected the β -catenin signaling (data not shown). These observations suggested that the tumor suppressor actions of TR β differ in FTC-133 and FTC-236 cells. FTC-133 cells were derived from the primary lesions, whereas FTC-236 cells were derived from a lymph node metastasis from the same patient (20). Thus, these two cells are known to have distinct phenotypes (30,40). It is reasonable to expect that the signaling pathways affected by TR β would be different due to different genetic alterations in these two thyroid cancer cell lines.

In summary, TR β expression in FTC cells decreased cancer cell proliferation, impeded tumor cell migration, and inhibited tumor growth *in vivo* through downregulation of the AKT-mTOR-p70 S6P signaling pathway and suppression of VEGF expression. These findings indicate that TR β could act

as a tumor suppressor in thyroid cancer by modulation of cancer angiogenesis.

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Author Disclosure Statement

The authors have nothing to disclose.

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