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Mutant Thyroid Hormone Receptor β Represses the Expression and Transcriptional Activity of Peroxisome Proliferator-activated Receptor γ during Thyroid Carcinogenesis

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ABSTRACT

The molecular genetics underlying thyroid carcinogenesis is not clear. Recent identification of a *PAX8-peroxisome proliferator-activated receptor γ* (*PPARγ*) fusion gene in human thyroid follicular carcinoma suggests a tumor suppressor role of PPARγ in thyroid carcinogenesis. Mice harboring a knockin mutant *thyroid hormone β receptor* (*TRβPV*) spontaneously develop thyroid follicular carcinoma through pathological progression of hyperplasia, capsular invasion, vascular invasion, anaplasia, and eventually, distant organ metastasis. This mutant mouse ($TR\beta^{PV/PV}$ mouse) provides an unusual opportunity to ascertain the role of PPARγ in thyroid carcinogenesis. Here, we show that the expression of *PPARγ* mRNA was repressed in the thyroid gland of mutant mice during carcinogenesis. In addition, TRβPV acted to abolish the ligand (troglitazone)-mediated transcriptional activity of PPARγ. These results indicate that repression of PPARγ expression and its transcriptional activity are associated with thyroid carcinogenesis and raise the possibility that PPARγ could be tested as a therapeutic target in thyroid follicular carcinoma.

INTRODUCTION

Thyroid cancers in humans consist of an array of several different histological and biological types (papillary, follicular, medullary, clear cell, anaplastic, Hurthle cell, and others; Ref. 1), but the majority of clinically important human thyroid cancers are of the papillary and follicular types. The molecular genetic events underlying these thyroid carcinomas are not clearly understood. Several genes, however, have been identified to be involved in the development of papillary thyroid carcinoma. Rearrangements of the *RET tyrosine kinase receptor gene* (*RET/PTCs*) are found in 2.6–34% of papillary carcinomas in the adult population (2). Overexpression of RET/PTC1 (3, 4) or RET/PTC3 (5) in thyroid cells of transgenic mice results in tumors with histological and cytological characteristics similar to those of human papillary carcinoma, providing evidence for the involvement of RET/PTCs in the initiation of papillary carcinoma.

Accumulated evidence indicates that follicular carcinomas arise through an oncogenic pathway distinct from that of papillary carcinoma, probably from the point of clonal initiation (2). The major differences in the molecular genetics between these two types of carcinomas are a higher prevalence of activating mutations of all three *RAS* genes and a greater disposition to develop DNA copy abnormalities (2, 6). Recently, however, Kroll *et al.* (7) reported the identification of a chromosomal rearrangement t(2:3)(q13;p25), yielding a *PAX8-PPARγ1*[2] fusion gene in 5 of 8 human follicular carcinomas but not in 10 papillary carcinomas. This unique genetic rearrangement in follicular carcinoma was further confirmed by subsequent analyses using a larger number of samples (8). When fused to PAX8, PPARγ1 not only loses its capability to stimulate thiazolidinedione-induced transcription but also acts to inhibit PPARγ1 transcriptional activity (7). However, how the loss of PPARγ1 transcriptional activity impacts the normal functions of thyroid follicular cells is unclear.

We have recently created a mutant mouse by targeting a mutation (*PV*) to the *TRβ* gene locus (*TRβPV* mice; Ref. 9). *TRβPV* was derived from a patient (PV) with RTH (10). RTH patients manifest the symptoms of dysfunction of the pituitary-thyroid axis with high circulating levels of thyroid-stimulating hormone in the face of high circulating levels of thyroid hormones (T3 and T4; Ref. 10). There is only one reported homozygous RTH patient who died at an early age (11). Patient PV has one mutant *TRβ* gene allele and manifests severe RTH characterized by attention-deficit hyperactivity disorder, short stature, low weight, goiter, and tachycardia (12). *PV* has a unique mutation in exon 10, a C-insertion at codon 448, which produces a frameshift of the COOH-terminal 14 amino acids of TRβ1. PV has lost T3 binding completely and exhibits potent dominant negative activity (13).

Remarkably, as $TR\beta^{PV/PV}$ mice aged, they spontaneously developed thyroid carcinoma (14). Histological evaluation of thyroids of 5–14-month-old mice showed capsular invasion (91%), vascular invasion (74%), anaplasia (35%), and metastasis to the lung and heart (30%). Thus, as previously reported, the $TR\beta^{PV/PV}$ mouse is a unique mouse model of human thyroid carcinoma (14). Additional analyses in the present study indicate that the thyroid carcinoma was of the follicular type. The availability of a mouse model of thyroid follicular carcinoma provides an unusual opportunity to ask the question whether the loss of ligand-dependent PPARγ transcriptional activity is associated with thyroid follicular carcinoma. Here, we show that during thyroid carcinogenesis, the expression of *PPARγ* mRNA became repressed. Moreover, troglitazone-activated PPARγ transcriptional activity was repressed by mutant PV. These findings suggest a critical role of PPARγ in the development of thyroid follicular carcinoma.

MATERIALS AND METHODS

Mouse Strains. The animal protocol used in the present study has been approved by the National Cancer Institute Animal Care and Use Committee. The mice harboring the *TRβPV* gene were created by introducing the *PV* mutation onto the *TRβ gene* locus via homologous recombination as described previously (9). Genotyping was carried out using RT-PCR as described previously (9). The wild-type littermates were used as controls.

Northern Blot Analysis. Total RNA was isolated from thyroids using Trizol Reagent (Invitrogen, Carlsbad, CA). Total RNA (5 μg) was used for Northern blot analysis. The probes were cDNA for *TRβ1* or *PPARγ*, labeled

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[2] The abbreviations used are: PPARγ, peroxisome proliferator activated receptor γ; EMSA, electrophoretic mobility gel shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LpL, lipoprotein lipase; NIS, sodium iodide symporter; PPRE, peroxisome proliferator-activated receptor response element; RTH, thyroid hormone resistance syndrome; RT-PCR, reverse transcription-PCR; RXR, the retinoid X receptor; Tg, thyroglobulin; TRβ, thyroid hormone β receptor.

with [α-^{32}P]dCTP using a random primer hexamer protocol. For normalization, the blots were stripped and rehybridized with a [α-^{32}P]dCTP-labeled *GAPDH* cDNA. After quantification by NIH image 1.61, the intensities of the mRNA bands were normalized against the intensities of *GAPDH* mRNA.

Determination of the Expression of *PV* Mutant RNA in Tissues by RT-PCR. RT-PCR was carried out using total RNA (3 μg) as a template and using ploy (dT) as a primer for cDNA synthesis by SuperScript II reverse transcriptase (Invitrogen). The DNA fragments for the wild-type *TRβ* or mutant *PV* were amplified in the presence of 5′-primer (primer N), 5′-ATGGGGAAATGGCAGTGACACGAG and 3′-primer (primer C), 5′-TGGGAGCTGGTGATGACTTCGTGC using Tag DNA polymerase (Takara, Madison, WI). The mutant *PV* sequence contained a *Bam*HI site that was not present in the mouse endogenous *TRβ* gene. PCR products were digested with *Bam*HI to yield two 380- and 309-bp fragments for mutant *PV*, as analyzed by gel electrophoresis.

Quantitative Real-Time RT-PCR. LightCycler-RNA Amplification kit Sybr Green I was used according to the manufacturer's protocols (Roche, Mannheim, Germany). A typical reaction mixture contained 5.2 μl of H_2O, 2.4 μl of $MgCl_2$ stock solution, 4 μl of LightCycler-RT-PCR Reaction Mix Sybr, 2 μl of resolution solution, 0.4 μl of LightCycler-RT-PCR Enzyme Mix, 2.5 μl of forward primer (2 μM), 2.5 μl of reverse primer (2 μM), and 1 μl of total RNA (200 ng). The cycles were: 55°C for 30 min; 95°C for 30 s; 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s; and 65°C to ~95°C with a heating rate of 0.1°C/sec and cooling step to 40°C. The primers used are as follows: *PPARγ*, forward primer 5′-TCTGGCCCACCAACTTCGGA-3′, reverse primer 5′-CTTCACAAGCATGAACTCCA-3′; *LpL*, forward primer 5′-TGCCATGACAAGTCTCTGAAG-3′, reverse primer 5′-ATGGGCCATTAGATTCCTCA-3′; and *GAPDH*, forward primer 5′-CCCTTCATTGACCTCAACTACAT-3′, reverse primer 5′-ACAATGCCAAAGTTGTCATGGAT-3′.

Preparation of Primary Mouse-cultured Thyroid Cells. Mouse primary thyrocytes were prepared with modifications from Jeker *et al.* (15). Briefly, pieces of thyroid lobes were washed by HBSS and digested with type 2 collagenase (0.2% in HBSS containing 1% BSA, 3 mM $CaCl_2$, and 50 ng/ml gentamicin) at 37°C for 30 min. After digestion, cells were collected by centrifuged for 3 min at 500 × *g*, which were subsequently resuspended in 1 ml of 6H culture medium (F-12 medium with 5% calf serum, 10 μg/ml insulin, 1 nM hydrocortisone, 2 ng/ml glycyl-histidyl-L-lysine acetate, 5 μg/ml transferrin, 10 ng/ml somatostatin, and 1 mU/ml thyroid-stimulating hormone). The medium was changed every third day.

Transfection. Mouse primary thyroid cultured cells prepared as shown above or PC cells (16, 17) were transfected with 1 μg of reporter plasmid (pPPRE-TK-Luc) and 100 to ~300 ng of expression vector for *TRβ1* (pCLC51), *PV* (pCLC51PV), or *PPARγ1* (pSG5-mPPARγ1) using FuGENE6 (Roche) according to the manufacturer's protocols. Five h after transfection, cells were cultured 6H medium containing 5% calf serum or serum deficient in thyroid hormone (Td serum). After 24 h, 100 nM T3 or 20 μM Troglitazone were added and incubated for an additional 24 h. Cells were lysed, and the luciferase activity was determined. The values were normalized against the protein concentrations that were determined by the BCA protein assay kit (Pierce, Rockford, IL).

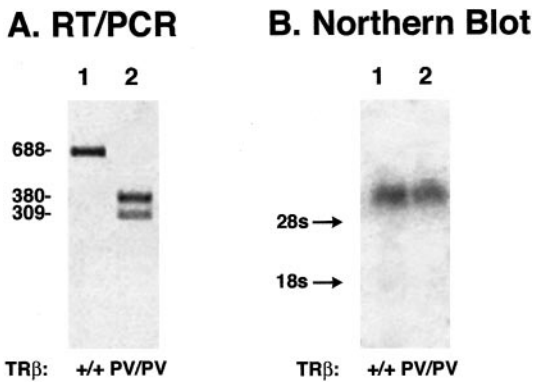

Fig. 1. Expression of PV mRNA in the thyroid gland. *A*, RT-PCR was carried out as described in "Materials and Methods." A 689- or 688-bp cDNA fragment was obtained from total RNA of $TR\beta^{PV/PV}$ and wild-type mice, respectively. However, only the cDNA from the mutant *TRβ* gene could be restricted with *Bam*HI to yield two 380- and 309-bp fragments (*Lane 2*). *B*, Northern blot analysis. Total RNA of the thyroid from wide-type thyroid (*Lane 1*) and mutant mice (*Lane 2*) was used in the Northern blot analysis.

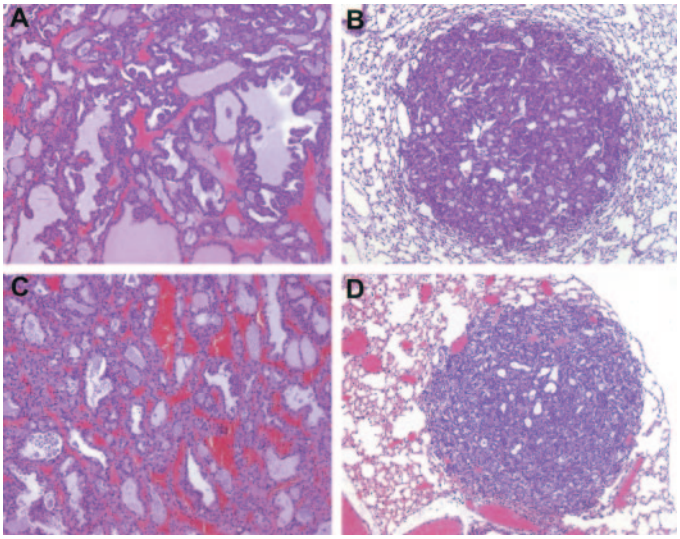

Fig. 2. Histological appearance of the hyperplastic thyroid and lung metastases in the same $TR\beta^{PV/PV}$ mice. H&E stains of paraffin sections from the thyroids (*A* and *C*) and lung metastases (*B* and *D*) from two $TR\beta^{PV/PV}$ mice (mouse no. 1: *A* and *B*; mouse no. 2: *C* and *D*). Note the extensive papillary hyperplasia in the thyroids but the clear follicular morphology of the metastatic carcinoma foci in the lungs. No local lymphoid node metastatic lesions were found in any of the $TR\beta^{PV/PV}$ mice. This morphology and metastatic pattern is consistent with follicular, rather than papillary, carcinoma of thyroid epithelial origin.

EMSA. The double-stranded oligonucleotide containing the PPRE (PPRE-5′, GAACGTGACCTTTGTCCTGGTCCCCTTTGCT and PPRE-3′, GGGACCAGGACAAAGGTCACGTTCGGGAAAGG) was labeled with [^{32}P]dCTP similarly as described by Zhu *et al.* (18). PPARγ1, TRβ1, and PV were synthesized *in vitro* by using the TNT-quick-coupled transcription/translation system (Promega, Madison, WI). About 0.2 ng of probe (3–5 × 10^4 cpm) were incubated with *in vitro* translated PPARγ1, TRβ1, or PV with or without +RXRβ (2 μl) in the binding buffer for 30 min at room temperature. DNA bound complexes were resolved on a 5.2% polyacrylamide gel. After electrophoresis for 2.5 h at 250 V, the DNA bound complexes were detected by autoradiography.

Histological and Immunohistochemical Methods. Tissues (thyroid and lung) were removed from mice and fixed in formaldehyde followed by paraffin embedding. For histology, sections were stained with H&E for microscopic examination. For immunohistochemistry, sections prepared from these paraffin blocks were deparaffinized, then treated with 0.3% hydrogen peroxide for 10 min at room temperature, followed by treatment with Antigen Unmasking Solution (Vector Labs, Burlingame, CA) at 97°C for 1 h. The sections were then blocked in 10% normal goat serum in PBS, followed by incubation in primary antibodies (rabbit anti-Tg antibody, 1:1000 in 1%BSA-PBS or rabbit anti-NIS antibody, 1:1000; Ref. 19) at 4°C overnight. The rabbit anti-Tg antibody was a generous gift from Dr. Roberto Dilauro, and the rabbit anti-NIS antibody was a generous gift from Dr. Nancy Carrasco, Albert Einstein College of Medicine. After the primary antibody step, the sections were washed and incubated in affinity-purified goat antirabbit IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Groove, PA) at 25 μg/ml in BSA-PBS for 30 min at room temperature. The sections were then routinely processed using diaminobenzidine-peroxide substrate solution and counterstained with hematoxylin. Images were captured using a Zeiss Axioplan 2 microscope equipped with an Axiocam camera and assembled using Adobe PhotoShop (version 7.0).

RESULTS

***$TR\beta^{PV/PV}$* Mice Develop Thyroid Follicular Carcinoma.** To confirm the expression of the *PV* gene in the thyroids of $TR\beta^{PV/PV}$ mice, RT-PCR was used to assess the expression of the *PV* mutant allele at the RNA level. Primers flanking the mutated exon 10 were

used (9), and the resultant cDNA was digested with *Bam*HI (Fig. 1*A*). The cDNA derived from the mutant allele yielded two fragments with sizes 380- and 309-bp (Fig. 1*A*, *Lane 2*), whereas only a 688-bp fragment was obtained from the wild-type mRNA (Fig. 1*A*, *Lane 1*). Fig. 1*B* shows that by Northern blot analysis, a ~6.1-kb band with similar intensity was detected for *TRβ* (Fig. 1*B*, *Lane 1*) and *PV* (Fig. 1*B*, *Lane 2*) mRNA, additionally confirming the expression of the PV mRNA in the thyroid of $TR\beta^{PV/PV}$ mice.

As $TR\beta^{PV/PV}$ mice aged, the thyroid glands became enlarged beginning at ~2 months of age, as reported previously (14). Histologically, these glands show extensive hyperplasia in a papillary pattern but none of the nuclear changes associated with papillary carcinoma. Some of these glands also develop foci of spindle cell anaplasia. At >10 months of age, some of these mice develop pulmonary metastases, the morphology of which are mostly in a pattern consistent with follicular carcinoma of the thyroid, as shown in Fig. 2. No local lymph node metastases were detected in these mice. The only other site of metastasis detected in these mice was the rare presence of metastatic lesions on the surface of the endocardium in the heart. These metastatic patterns, vascular rather than lymphatic, are consistent with human thyroid follicular carcinoma rather than papillary thyroid carcinoma.

We additionally evaluated whether the metastatic lesions still retain thyroid differentiation markers such as NIS (20) and Tg. For positive controls, Fig. 3, *A* and *B*, shows the immunostaining of the normal thyroid follicular cells in the wild-type mice with anti-NIS antibodies (Fig. 3*A*), or anti-Tg antibodies (Fig. 3*B*). As expected, NIS was expressed in the basolateral plasma membrane of the follicular epithelial cells (19, 20). Tg was detected in the lumen of the follicles and on the apical surface of the follicular cells. Fig. 3*C* shows that in the thyroid of $TR\beta^{PV/PV}$ mice, NIS was found in the plasma membrane of the hyperplastic follicular epithelial cells. Tg was detected in the hyperplastic follicular cells (Fig. 3*D*). Fig. 3*E* shows that in the metastatic lesions in the lung, NIS was detected in the membranes of the neoplastic cells (Fig. 3*F*). Tg was detected in the neoplastic cells but not in the adjacent normal lung parenchyma. These data additionally confirm the thyroid origin of the metastatic lesions shown in Figs. 2 and 3. The morphological features and the metastatic patterns indicate that the type of thyroid cancer detected in the $TR\beta^{PV/PV}$ mice is follicular carcinoma.

Repression of the Expression of *PPARγ* during Thyroid Carcinogenesis. Kroll *et al.* (7) and Nikiforova *et al.* (8) reported that PAX8-PPARγ fusion protein was detected mainly in human follicular carcinomas, rarely in follicular adenomas, but not in other thyroid carcinomas. Although it is not clear how the expression of the PAX8-PPARγ fusion protein leads to follicular carcinoma, it was shown that PAX8-PPARγ failed to respond to ligand-dependent transcriptional activity of PPARγ (7). The findings that $TR\beta^{PV/PV}$ mice developed thyroid follicular carcinoma prompted us to examine the expression of the *PPARγ* gene expression in the thyroid of $TR\beta^{PV/PV}$ mice. We first examined the expression of *PPARγ* mRNA in the thyroid of $TR\beta^{PV/PV}$ mice at the age of 5 months by Northern blot analysis (Fig. 4*A*).

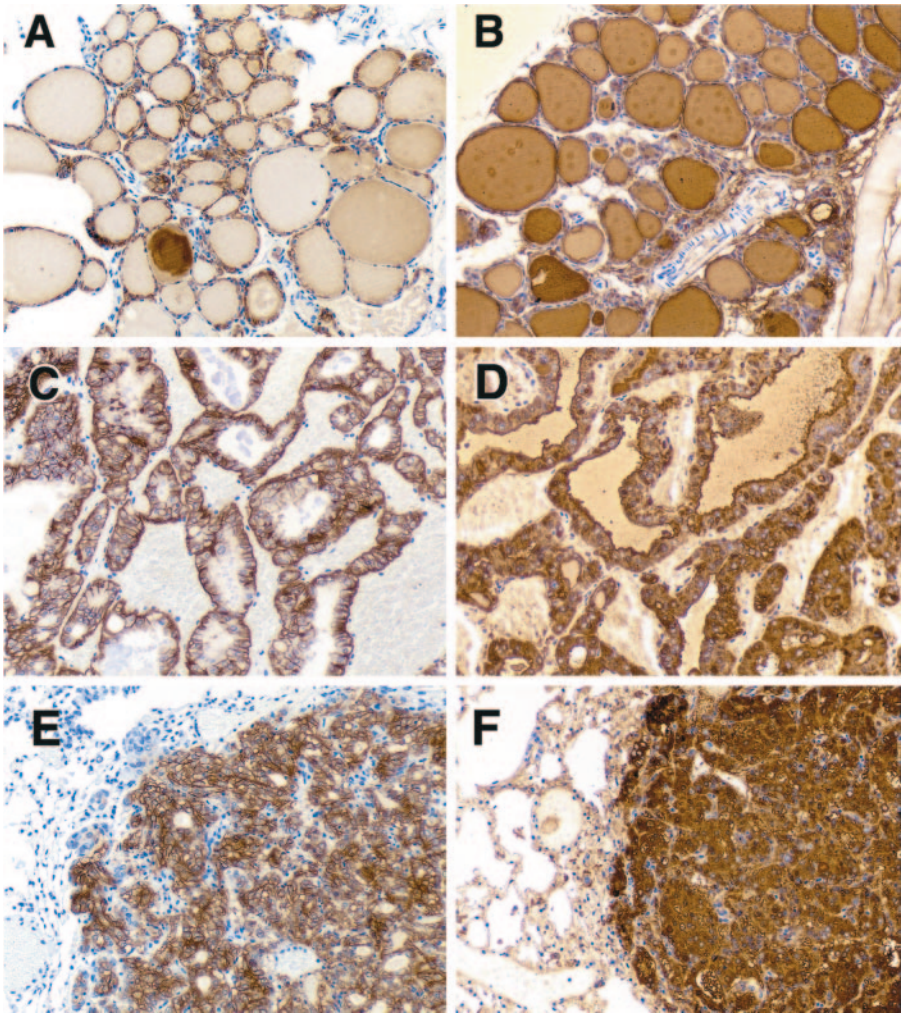

Fig. 3. Immunohistochemical demonstration of the thyroid origin of the metastatic lesions found in $TR\beta^{PV/PV}$ mice. Thyroids (*A* and *B*, wild-type; *C* and *D*, $TR\beta^{PV/PV}$ mice) or lung metastases (*E* and *F*, $TR\beta^{PV/PV}$ mice) were fixed and embedded in paraffin. Sections from these tissues were labeled with antibodies to Tg or the NIS (as markers for thyroid differentiation) using immunohistochemistry. Note that the NIS pattern in normal thyroid is that of a polarized plasma membrane distribution, a pattern also seen in the hyperplastic thyroid and the carcinoma metastasis in the lung of a $TR\beta^{PV/PV}$ mouse. Anti-Tg antibody shows a colloidal and an apical plasma membrane in normal thyroid epithelium, as well as a similar pattern in both the hyperplastic thyroid and the metastatic carcinoma lesions in the lung of the $TR\beta^{PV/PV}$ mice. These data indicate the preservation of thyroid epithelial differentiation in the metastatic lesions seen in these mice, supporting the interpretation that this neoplastic process is consistent with metastatic follicular carcinoma of the thyroid.

Clearly, the expression of *PPARγ* mRNA was lower than that of the wild-type siblings. After quantification and normalization against *GAPDH*, Fig. 4*B* shows that the expression of *PPARγ* mRNA was repressed in the thyroid of $TR\beta^{PV/PV}$ mice by 50% as compared with the wild-type siblings.

$TR\beta^{PV/PV}$ mice spontaneously develop thyroid carcinoma through different pathological changes from hyperplasia, capsular invasion, vascular invasion, anaplasia to distant organ metastasis (14). Hyperplasia of the thyroid was observed beginning at 2–3 months of age, capsular invasion at 4–5 months of age, vascular invasion and anaplasia beginning at 5–7 months of age, and most of the distant organ metastasis occurs after 9 months of age (14). We, therefore, ascertained the temporal profiles in the expression of *PPARγ* mRNA during carcinogenesis by comparing pairs of age-matched wild-type and of $TR\beta^{PV/PV}$ mice at the ages of 4 months (Fig. 5*A*, *bars a* and *b*), 6 months (Fig. 5*A*, *bars c* and *d*), and 12 months (Fig. 5*A*, *bars e* and *f*). The expression of *PPARγ* mRNA in the wild-type mice (Fig. 5*A*, *bars a*, *c*, and *e*, respectively) was increased 1.6- and 1.4-fold at the ages of 6 and 12 months, respectively, as compared with that at 4 months of age. Compared with the wild-type mice, however, the expression of *PPARγ* mRNA was repressed in $TR\beta^{PV/PV}$ mice at each time point (Fig. 5*A*, compare *bars b* with *a*, bar *d* with *c*, and bar *f* with *e*). The relative ratios were graphed in Fig. 5*B*, showing that the expression of *PPARγ* mRNA became repressed (~50–60%) during the time when the thyroid is undergoing carcinogenesis (14).

Mutant PV Represses the Transcriptional Activity of PPARγ. Previously, Kroll *et al.* (7) showed that the fusion of PAX8 to the NH_2 terminus of PPARγ inactivates the ligand-dependent transcriptional activity of PPARγ, suggesting that the loss of its transcriptional activity could play a role in the development of follicular carcinoma. We therefore tested the hypothesis that in addition to the repression in the expression of *PPARγ* mRNA, PV could also act to interfere with

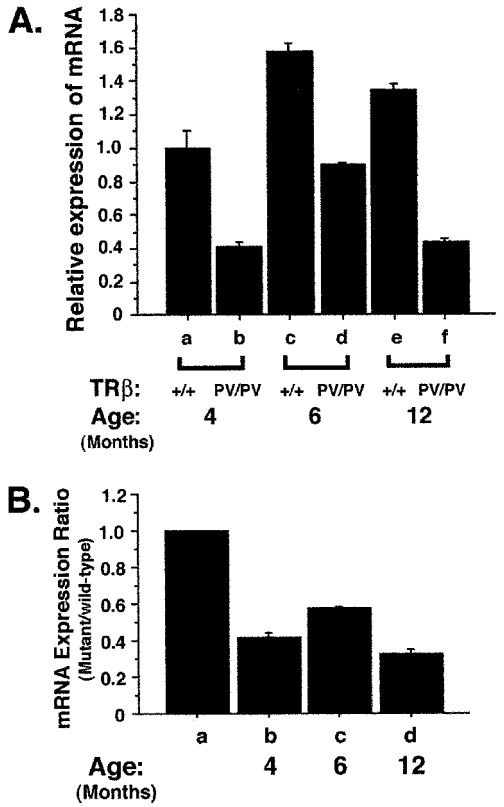

Fig. 5. Comparison of *PPARγ* mRNA between wild-type and $TR\beta^{PV/PV}$ mice at different ages by real-time RT-PCR. *A*, relative expression levels of *PPARγ* in the thyroid glands were determined in wild-type and $TR\beta^{PV/PV}$ mice using age-matched mice as marked. Relative quantification was determined as described in "Materials and Methods." *B*, the ratios of expression of *PPARγ* mRNA in $TR\beta^{PV/PV}$ and wild-type mice at the ages of 4, 6, and 12 months. The data are expressed as mean ± SD ($n = 4$).

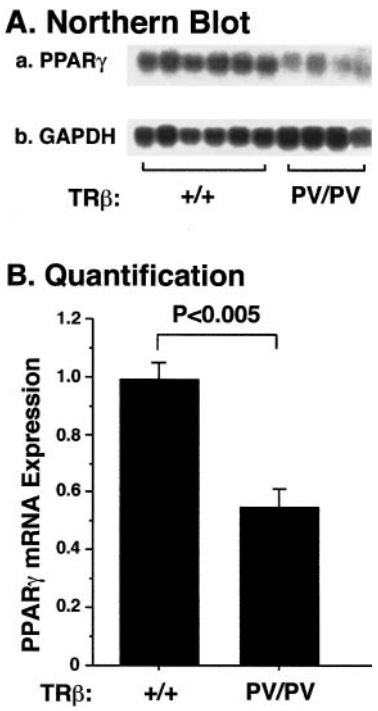

Fig. 4. Analysis of the expression of *PPARγ* mRNA by Northern blotting. *A*, comparison of the mRNA expression of *PPARγ* (*a*) and *GAPDH* (control) (*b*) in thyroids of wild-type ($n = 6$) and $TR\beta^{PV/PV}$ mice ($n = 4$) at the age of 5 months. *B*, quantitative comparison in the expression of *PPARγ* mRNA between wild-type and $TR\beta^{PV/PV}$ mice. The data are expressed as mean ± SE. Differences between two groups were examined for statistical significance using Student's *t* test. $P < 0.005$ is statistically significant.

the ligand-dependent transcriptional activity of PPARγ in the thyroid of $TR\beta^{PV/PV}$ mice. The luciferase reporter containing PPARγ response element (AGGTACXAGGTCA; DR1) was cotransfected with or without *TRβ1* or *PV* into cultured thyroid PC cells (Fig. 6*A*). Fig. 6*A*, *bars 1–4*, show that the basal activities were not significantly affected by the presence or absence of ligands (T3 or troglitazone), indicating the absence of a role of PPARγ in normal nontransfected cells. Cotransfection of *TRβ1* in the absence of T3 led to 50% repression of the basal activity (Fig. 6*A*, *bars 5* and *6*) but was derepressed by the presence of T3 (Fig. 6*A*, *bars 7* and *8*). However, the extent of repression and derepression was not affected by the ligand of the PPARγ, troglitazone (Fig. 6*A*, *bars 5–8*). Cotransfection of PV only led to 50% repression whether T3 was present (Fig. 6*A*, *bars 11* and *12*) or not (Fig. 6*A*, *bars 9* and *10*) as PV does not bind T3. The transcription of the cotransfected PPARγ was activated by troglitazone (Fig 6*A*, *bars 14* and *16*) but not by T3 (Fig. 6*A*, *bars 13* and *15*).

The complex ligand-dependent and -independent interaction of PPARγ with TRβ1 is illustrated in bars Fig. 6*A*, *bars 17–20*. Cotransfection of both *PPARγ* and *TRβ1* in the absence of both ligands led to repression (Fig. 6*A*, *bar 17*), but this repression was derepressed by the presence of troglitazone (Fig. 6*A*, *bar 18*) or T3 (Fig. 6*A*, *bar 19*). In the presence of both ligands (T3 and troglitazone) and both receptors, a synergistic 2.5-fold activation was observed (Fig. 6*A*, *bar 20*). A markedly different picture emerged when *PV* and *PPARγ* were cotransfected into PC cells (Fig. 6*A*, *bars 21–24*). Fig. 6*A*, *bar 22*, shows that troglitazone derepressed the basal activity, whereas T3 failed to do so (Fig. 6*A*, *bar 23*). Significantly, PV abolished the troglitazone-dependent activation of PPARγ transcriptional activity

(Fig. 6*A*, *bar 24*). These results clearly demonstrate the cross-signaling between the wild-type TRβ1 and PPARγ pathways. More importantly, this cross-signaling could be blocked by the dominant negative action of PV. Thus, these data provide a functional link of PV action to the transcriptional activity of PPARγ.

We further demonstrated the repression of the transcriptional activity of PPARγ by PV in primary thyrocytes of wild-type mice. As shown in Fig. 6*B*, *bar 2*, cotransfection of PPRE-containing reporter with *PPARγ* led to 3.5-fold activation of the transcriptional activity. Consistent with the results shown in the cultured thyrocytes, the transfected PV abolished the troglitazone-induced transcriptional activity to the basal level (Fig. 6*B*, *bar 3*). These findings additionally support the notion that the expression of PV in the thyroid of $TR\beta^{PV/PV}$ mice repressed the transcriptional activity of PPARγ.

Mutant PV Binds to PPRE. It is known that PV binds to thyroid hormone response elements with the half-site binding motifs in three different arrangements (palindromic, inverted repeats, and direct repeats separated by four nucleotides; Refs. 21, 22). Similar to TRβ1, PV binds to these thyroid hormone response elements as a homodimer and as a heterodimer with the RXR. The results described above in Fig. 6 suggested that TRβ1 as well as PV could bind to PPRE. We, therefore, evaluated the binding of TRβ1 and PV to PPRE (DR1) by EMSA. Consistent with other studies (23), binding of PPARγ to PPRE as homodimers was too weak to be detected by EMSA (Fig. 7, *Lane 2*). However, PPARγ bound to PPRE-DR1 as heterodimers with RXR (Fig. 7, *Lane 3*). Similarly, neither TRβ1 nor PV bound to PPRE as homodimers, but TRβ1 and PV bound to PPRE each as heterodimers with RXR, albeit weaker than that of PPARγ/RXR heterodimers (Fig. 7, compare *Lanes 5* or *7* with *Lane 3*). These results indicate that PV could compete with TRβ1 or PPARγ for binding to PPRE as PV/RXR heterodimers on the PPARγ target genes.

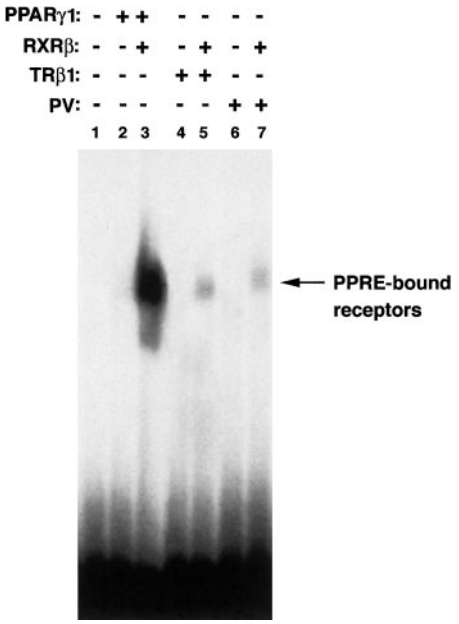

Fig. 7. Binding of PPARγ1, TRβ1, or PV to PPRE by EMSA. Lysates containing *in vitro* translated PPARγ1, TRβ1, or PV proteins (2 μl) in the presence or absence RXRβ (2 μl) were incubated with [^{32}P]-labeled PPRE and analyzed by gel retardation as described in "Materials and Methods." Volumes of lysate were kept constant by the addition of unprogrammed lysate as needed. The lanes are marked.

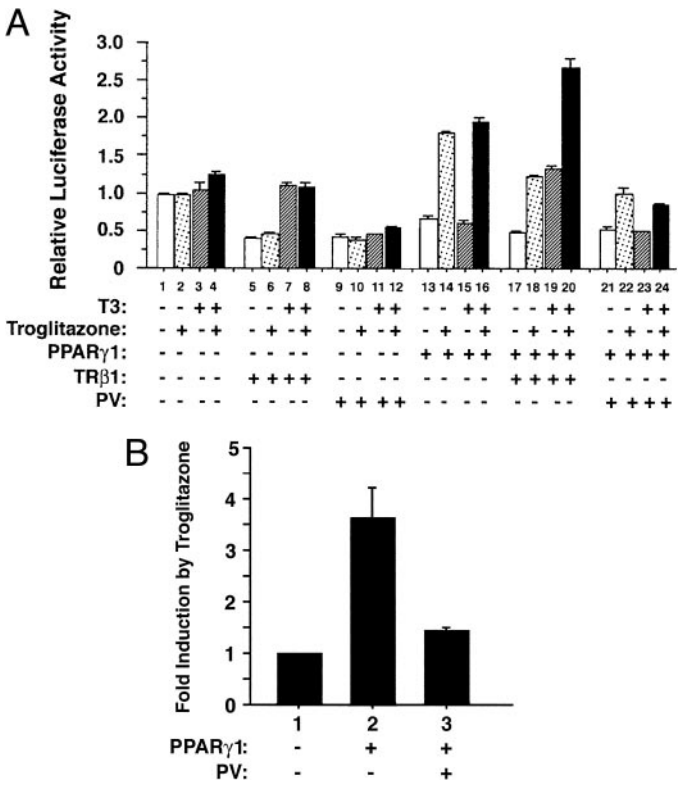

Fig. 6. Inhibition of the ligand-dependent transcriptional activity of PPARγ by PV. *A*, PC cells were cotransfected with 1 μg of the reporter plasmid (pPPRE-TK-Luc) and various cDNA expression vectors [empty vector, pCLC 51 for *TRβ1* (0.3 μg), pCLC51PV for *PV* (0.3 μg), or pSG%-mPPARγ1 for *PPARγ1* (0.1 μg)], as indicated. Cells were treated with either DMSO as vehicle or troglitazone (100 nM) in the absence or presence of T3 (100 nM), as marked. Data were normalized against the protein concentration in the lysates. Relative luciferase activity was calculated and shown as fold induction relative to the luciferase activity of PPRE in the cells treated by DMSO in the absence of T3, defined as 100. The data are expressed as mean ± SD ($n = 3$). *B*, primary thyroid cells were isolated from adult wild-type mice. cDNA expression vectors of *PPARγ1* (0.1 μg) or *PV* (0.3 μg) were cotransfected into thyrocytes as marked according to "Materials and Methods." The data are expressed as mean ± SD ($n = 3$).

Repression of the Expression of *LpL* in the Thyroid of $TR\beta^{PV/PV}$ Mice during Carcinogenesis. To address the question as to whether the repression in the mRNA expression and transcriptional activity of PPARγ by PV shown above is functionally relevant, we evaluated the expression of a known PPARγ downstream direct target gene, *LpL*. LpL is the primary enzyme responsible for conversion of lipoprotein triglycerides into free fatty acids and monoglycerides (24). A typical PPRE, −169 *TGCCCTTCCCCC* −157 (DR1), was identified in the promoter of the *LpL* gene (23). Furthermore, the transcriptional activation of the *LpL* gene by thiazolidinediones was shown mediated by PPAR/RXR heterodimers (23). We, therefore, compared the expression of LpL in the thyroids of $TR\beta^{PV/PV}$ mice and their wild-type siblings at the time the expression of *PPARγ* was repressed (Fig. 5). Fig. 8*A* shows that the expression of *LpL* was not significantly altered as the wild-type mice aged (from 4 to 12 months; Fig. 8*A*, *bars a*, *c*, and *e*). However, the expression of *LpL* was repressed 60, 90, and 90% in $TR\beta^{PV/PV}$ mice at the ages of 4, 6, and 12 months, respectively (Fig. 8*B*). The repression of the expression of a PPARγ direct downstream target gene supports the notion that PV-induced repression in the expression, and the transcriptional activity of PPARγ is functionally significant. Importantly, these data indicate that during carcinogenesis, transcriptional activity of PPARγ became repressed.

DISCUSSION

Little is known about the molecular genetics in the pathogenesis of thyroid follicular carcinoma. The identification of the *PAX8-PPARγ* fusion gene in thyroid follicular carcinoma ushers in a new paradigm to study the molecular genetic events underscoring the development

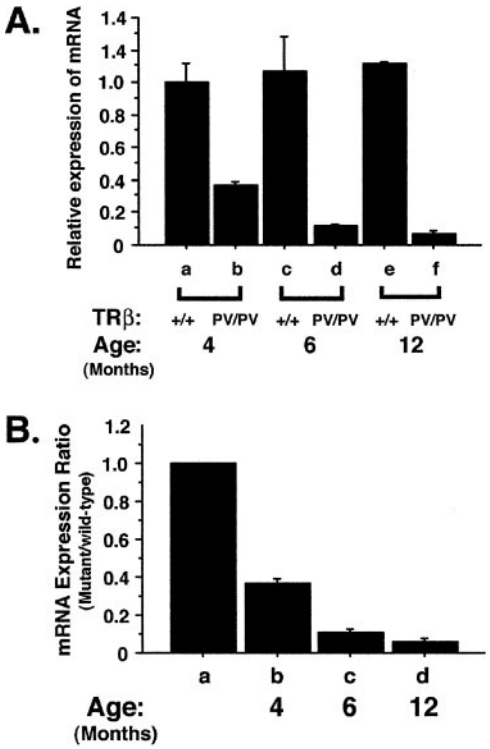

Fig. 8. Comparison of expression of *LpL* mRNA in the thyroids of wild-type and $TR\beta^{PV/PV}$ mice at different ages by real-time PCR. *A*, relative expression levels of *LpL* mRNA in the thyroid glands were determined using age-matched wild-type and mutant mice at the ages of 4, 6, and 12 months as marked. *B*, the ratios of expression of *LpL* mRNA in $TR\beta^{PV/PV}$ and wild-type mice at the ages of 4, 6, and 12 months. The data are expressed as mean ± SD ($n = 4$).

of follicular carcinoma. At present, how the rearranged product, *PAX8-PPARγ* fusion gene, is involved in tumorigenesis is unclear. It is known, however, that the fusion of PPARγ1 to PAX8 inactivates the ligand-transcriptional activity of PPARγ1, suggesting that the loss of the ligand-transcriptional activity of PPARγ1 could contribute to the tumorigenesis. The availability of $TR\beta^{PV/PV}$ mice with follicular thyroid carcinoma provides an unusual opportunity to test this hypothesis. We found that, indeed, during carcinogenesis and progression in the thyroids of $TR\beta^{PV/PV}$ mice, the expression of *PPARγ* mRNA became repressed. Importantly, PV was further shown to inhibit the ligand-dependent transcriptional of PPARγ. These dual actions of PV keep PPARγ repressed both on its expression and activity. These findings suggest a critical role of PPARγ in maintaining the normal phenotype of the thyroid.

A close association of somatic mutations of *TRβ* with several human cancers has been reported (25–27). In these studies, how *TRβ* mutants could be involved in the carcinogenesis *in vivo* has not been addressed. PV has been shown to act dominant negatively to interfere with the transcriptional activity of TRβ *in vitro* and *in vivo*, resulting in abnormal expression patterns of T3 target genes (9, 13, 28). The present study shows TRβ1 bound to PPRE, albeit weaker than PPARγ. However, in the presence of both T3 and troglitazone, a synergistic PPRE-mediated transactivation activity was detected (Fig. 6*A*), suggesting that TRβ1 could function to enhance the transcriptional activities of PPARγ *in vivo*. Similar to TRβ1, PV also bound to PPRE, but because PV cannot bind T3, PV acts to interfere with the enhancing functions of TRβ1 on PPARγ. It is possible that for some PPARγ target genes, the enhancing action of T3-bound TRβ1 is obligatory for their functions. For these genes, the dominant negative action of PV acts to obliterate their functions, leading to deleterious consequences.

Increasing evidence supports the belief that tumorigenesis occurs as a result of accumulative abnormal genetic events (29). Cross-signaling of these genetic pathways makes dissecting the genetic events underlying carcinogenesis a challenge (30). In many cases, where the abnormal genes are identified, little is known about how the interplay of their molecular pathways contributes to tumorigenesis. The present study highlights how the mutation of a nuclear transcription factor could silence the activity of another nuclear transcription factor, leading to pathogenic consequences.

Emerging evidence suggests that the loss of PPARγ expression could be an important risk factor in the development of carcinoma. Recent animal studies have shown that reduced expression of the *PPARγ* gene enhances carcinogenesis; $PPAR\gamma^{+/-}$ mice are at markedly enhanced risk for azoxymethane-induced colon carcinogenesis (31). Furthermore, Akiyama *et al.* (32) also showed that $PPAR\gamma^{+/-}$ mice were more susceptible than wild-type controls to the development of 7,12-dimethylbenz(*a*)anthracene-induced skin papillomas, mammary tumors, and ovarian tumors, suggesting that PPARγ might have a protective role against tumor development.

It is unclear how the loss of the *PPARγ* gene and/or the repression of ligand-dependent transcriptional activity of PPARγ are involved in thyroid carcinogenesis. The findings that its downstream direct target gene, *LpL*, was concurrently repressed indicate that the repression of PPARγ led to functional consequences. Therefore, PPARγ could act via downstream pathways to inhibit the proliferation of cell growth and to induce apoptosis. The loss of these activities of PPARγ results in uncontrolled cell growth. This notion is supported by recent studies showing that PPARγ agonists and PPARγ overexpression leads to a drastic reduction of cell growth and an increase in apoptotic cell death of PPARγ overexpressing thyroid carcinoma cells (33, 34). These human thyroid carcinoma cells express PPARγ (33, 34). In addition, troglitazone was found to significantly inhibit tumor growth and prevent distant metastasis of tumors induced by human papillary thyroid cancer BHP18–21 cells in nude mice *in vivo* (34). The genes and signaling pathways affected by PPARγ and its ligands that lead to growth inhibition and apoptosis await future studies. However, these studies raise the possibility that PPARγ could be an important potential therapeutic target and $TR\beta^{PV/PV}$ mice could be used to test PPARγ ligands as chemopreventive agents in thyroid follicular carcinoma.

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